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Accurate determination of sesame allergens in processed food by LC-MS/MS based on isotope-labelled derived peptides as internal standard

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ABSTRACT: As one of the eight major allergens recognized by the World Health Organization and the Food and Agriculture Organization of the United Nations, it is crucial to determine sesame allergens in food. Employing liquid chromatography-tandem mass spectrometry (LC-MS/MS), which exhibits exceptional precision and sensitivity, enables the identification of sesame allergen proteins. In this investigation, we established and validated a methodology for quantifying sesame allergen protein (Ses i 1-7) in processed foods utilizing LC-MS/MS. The key innovation lies in screening sesame allergen characteristic peptides with processing robustness and using isotope labeled derived peptides as internal standards to correct trypsin digestion efficiency, significantly improving quantitative accuracy. The quantitative method showed excellent sensitivity (limit of detection: 1.0-28 µg/g; limit of quantitation: 2.9-56 µg/g), accuracy (recovery rate: 90%-110%), and repeatability (intra/inter-day precision: 1.06%-7.59%/2.68%-6.98%). In addition, this method has been successfully employed in the examination of commercial food. The quantitative method established in this study is beneficial for the management of sesame allergens in processed foods.

Keywords: Sesame allergen; Quantitation; Processed food; Derived peptides; LC-MS/MS

1. Introduction

Sesame is a globally cultivated crop, according to the FAOSTAT database, the annual production of sesame seeds can reach millions of tons (Pi et al., 2022). Sesame is rich in material composition, providing lipids, protein, carbohydrates, minerals, and polyphenols (Mujtaba et al., 2020). Because of its pleasant aroma when heated, favorable sensory characteristics, and significant nutritional benefits, sesame is extensively utilized in the food industry (Torricelli et al., 2020). However, on 10 May 2021, the ad hoc Joint FAO/WHO Expert Consultation on Risk Assessment of Food Allergens identified sesame as a new member of the "Big Eight" replacing soybean, based on epidemiological, potency, and allergic reaction severity (Zeng et al., 2024). Cases of sesame allergy have been reported in several countries, including the United Kingdom and the United States (Nemni et al., 2021). It may cause adverse reactions in 0.1%–0.2% of the general population, and the incidence rate is increasing year by year (Protudjer & Abrams, 2019). Individuals with sesame allergies may

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experience a range of symptoms, from mild reactions such as hives and dizziness to severe and potentially life-threatening reactions (Touma et al., 2021). To protect individuals with the sesame allergy, many countries have established allergen regulations (Regulation (EU) No 1169/2011 of the European Parliament and of the Council, and the Food Allergen Labeling and Consumer Protection Act of 2004 in the United States, etc.) mandating that explicit disclosure of sesame allergen detail on the food packaging (Linghu, Wang, & Lu, 2022; Zeng et al., 2024).

The most reliable approach to safeguarding sesame-allergic individuals is to strictly avoid sesame consumption, which requires strict and accurate food labeling as well as accurate and reliable testing methods. At present, sesame detection is predominantly achieved through immunological methods focused on specific proteins, molecular biology methods relying on the amplification of nucleic acid fragments, and mass spectrometry (Koppelman et al., 2015; López-Calleja et al., 2017; Villa et al., 2023; Waiblinger et al., 2014). Immunological methods rely on the specific antibodies to identify the target allergen, which may sometimes lead to false positive/negative results (Gavage et al., 2020). Molecular biology methods are commonly used for qualitative detection of allergen species, but cannot accurately quantify allergen proteins. (Sena-Torralba et al., 2020; Tuzimski & Petruczynnik, 2020). With the progress in technological development, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been gradually utilized for detecting food allergens (Boo et al., 2018; Fan et al., 2023). Although the technique requires the use of expensive instrumentation and the implementation of lengthy pre-treatment procedures, it is capable of the detection of multiple allergens with high specificity and sensitivity (Stella, 2020).

Two main LC-MS/MS quantitative studies have been conducted on sesame-allergenic proteins: one only detects sesame allergenic protein Ses i 6, and the other can simultaneously identify seven sesame allergens (Huschek et al., 2016; Ma et al., 2020). However, these existing methods have two major limitations: (1) they used raw sesame for peptide selection without considering processing robustness, and (2) they employed conventional isotope-labeled peptides that cannot correct for trypsin digestion variability. The alteration of the secondary structure of sesame allergen protein during processing may affect the reaction intensity of hydrolyzed peptides, thereby reducing the accuracy of the results (Achouri & Boye, 2013; Marzano et al., 2020). As highlighted in recent research on food allergen analysis (Zhang et al., 2022), the two critical aspects require improvement: the selection of processing-resistant signature peptides and the development of internal standards that account for enzymatic digestion variability. To overcome these limitations, the use of isotope-labeled derived peptides has been proposed as an innovative internal standard strategy. These peptides, designed by extending characteristic peptides with trypsin cleavage sites at both termini, undergo co-digestion with sample proteins, effectively correcting for variations in trypsin efficiency (Chen et al., 2015). This approach has been successfully applied in quantifying bovine lactoferrin, where it demonstrated excellent repeatability (RSD < 6.4%) by compensating for trypsin-induced deviations (Zhang et al., 2014). Therefore, the derived peptides could be designed as internal standards according to the quantitative peptides of seven

sesame allergen proteins to establish a precise quantitative method, this will help future food allergen labels to indicate the allergen protein content.

This study focused on developing and validating a LC-MS/MS method for the simultaneous quantification of seven sesame-allergenic proteins (Ses i 1 to Ses i 7) in processed foods. To address the limitations of existing approaches, we introduced two key innovations: first, we systematically screened for characteristic peptides with processing robustness using twelve laboratory-processed sesame matrices subjected to various processing techniques (including stir-frying, frying, baking, steaming, boiling, and grinding); second, we designed and implemented isotope-labeled derived peptides as internal standards that undergo co-digestion with sample proteins, thereby correcting for losses in trypsin digestion efficiency and achieving more accurate quantification. The established method demonstrates accuracy and precision that meet the performance standards of the AOAC Allergen Analysis (SMPR® 2016.002).

2. Materials and methods

2.1 Reagents and Chemicals

Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, $\geq 99\%$), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), ammonium bicarbonate (NH_4HCO_3 , $\geq 98\%$), urea ($\geq 98\%$), thiourea ($\geq 98\%$), iodoacetamide (IAA, $\geq 98\%$), and dithiothreitol (DTT, $\geq 98\%$) were acquired from Sigma-Aldrich Chemical (St. Louis, MO, USA). Water (MS grade), acetonitrile (ACN, MS grade), formic acid (FA, MS grade), and hexane were obtained from ANPEL Laboratory Technologies (Shanghai, China). The Qubit™ Protein Assay Kit and trypsin protease (MS Grade) were provided by ThermoFisher Scientific (Waltham, MA, USA). 0.22 μm polyethersulfone (PES) membrane (Merck Millipore, USA) and 5 kDa ultra-filtration centrifuge tubes (Sartorius, Göttingen, Germany) were also employed. Peptide standards were custom-synthesized and supplied by MOTIF BIOTECH., Ltd. (Suzhou, China). Commercial food products for method validation were obtained from a supermarket (Beijing, China).

2.2 Preparation of test materials

Processed sesame matrices were prepared under laboratory conditions. White sesame and black sesame are often processed into various foods through different processing techniques. Therefore, all test materials were produced in duplicate, with one using white sesame and the other using black sesame, aiming to comprehensively cover sesame processed products. As shown in Figure 1, sesame products involving six processing techniques were produced in the laboratory as test materials. After cooling at room temperature, they were ground under liquid nitrogen and stored in the refrigerator. As shown in Table S1, fifteen different varieties of sesame and fifteen common food allergens purchased in the local market (Beijing, China) were applied to verify the specificity of sesame allergen peptides screened.

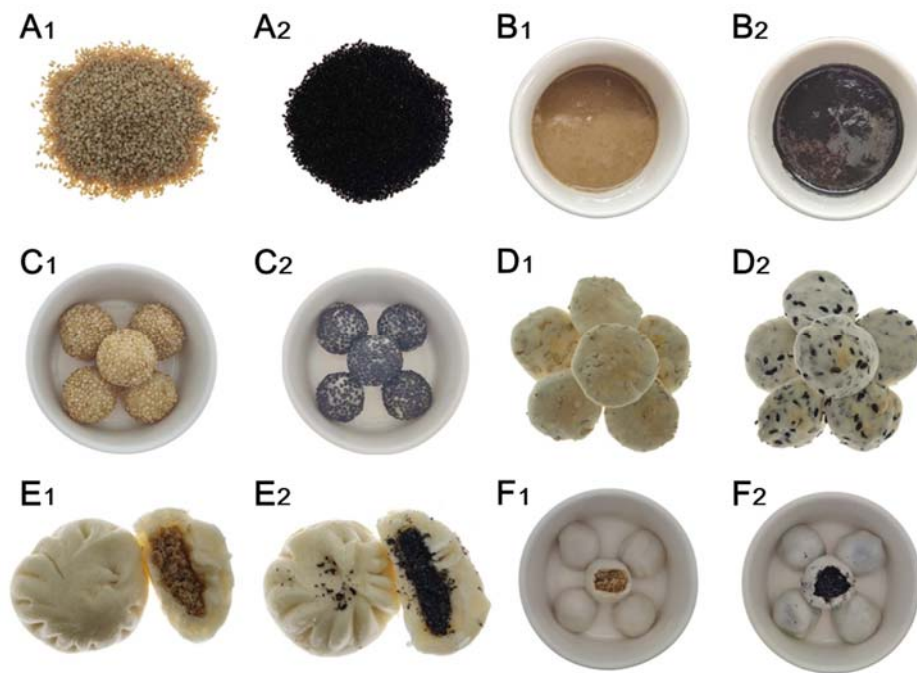


Figure. 1 Processed sesame matrices produced in the laboratory. Twelve sesame matrices prepared in the laboratory have undergone different processing techniques, including stir-frying, grinding, deep frying, baking, steaming, and boiling (A-F). All materials were produced in duplicate (1: using white sesame; 2: using black sesame).

2.2.1 Production of the stir-fried sesame matrix

Raw sesame in batches of 100 g was washed with water and stir-fried (around 150 °C) for 10 minutes. After cooling at room temperature, sesame seeds were ground and homogenized by a Tissue Lyser (Qiagen, Hilden, Germany) at maximum speed for 30 seconds, vacuum-packed, and stored in the dark at 4 °C.

2.2.2 Production of the fried sesame matrix

100 g raw sesame, 500 g glutinous rice flour, and warm water were mixed to form the dough. The dough was evenly divided into 10 g each, fried at 120 °C for 15 minutes, and then fried at 160 °C for 1 minute. After cooling, the product was ground, vacuum packed, and stored in the dark at 4 °C.

2.2.3 Production of the baking sesame matrix

Mix 100 g of raw sesame seeds, 500 g of flour, and warm water to form a dough. Divide the dough evenly into 10 g portions and place them in an oven (oven model K38FK613, Supor, China) to bake at 180 °C for 20 minutes. Spread the product evenly in a single layer to ensure even baking.

2.2.4 Production of the boiled sesame matrix

500 g of glutinous rice flour and cold water were mixed into dough. The dough was divided into 10 g and pressed into pancakes. Sesame powder was added to make glue pudding. The glue pudding was boiled in water for 10 minutes.

2.2.5 Production of the steamed sesame matrix

500 g of flour, 5 g of yeast powder, and warm water were mixed to form dough and fermented for 1 hour. The fermented dough was evenly divided into 10 g each, and sesame powder was added to make sesame stuffed buns, which were steamed for 15 minutes.

2.2.6 Production of the grinding sesame matrix

500 g of raw sesame were stir-fried for about 10 minutes, mixed with 50 g of sesame oil, and ground using a high speed blender (L12-P726, Joyoung, China) for 5 minutes to make sesame paste. Sesame paste was vacuum-packed and kept at 4 °C.

2.3 Sample preparation

Sesame products were thoroughly ground and then continuously stirred for 20 min in hexane (1:5, w/v) to remove fat, followed by centrifugation (2200 g, 10 min, 4 °C, repeated three times). The defatting step using hexane was essential for high-fat matrices like sesame paste to improve protein extraction efficiency and reduce matrix interference in LC-MS/MS analysis. The defatted sesame powder was dried completely in a chemical hood and then stored at -80 °C. A 1 g sample of defatted flour was mixed with 10 mL of 50 mmol/L Tris-HCl (7 mol/L urea, 2 mol/L thiourea, 25 mmol/L DTT, 4% CHAPS, pH 8.5) by shaking (25 °C, 20 min), and subsequently sonicated on ice for 20 min. After centrifugation (8800 g, 20 min, 4 °C), the supernatants were gathered to filtered through 0.22 µm PES membranes, and then quantified protein content by Bradford method.

To lessen matrix interference, ultrafiltration membranes were employed to facilitate trypsin digestion. The solution containing 200 µg of protein was diluted with the same extraction buffer (50 mmol/L Tris-HCl with 7 mol/L urea, 2 mol/L thiourea, 25 mmol/L DTT, and 4% CHAPS, pH 8.5) to 50 µL. Next, proteins were mixed with 10 µL DTT solution (120 mmol/L DTT, 8 mol/L urea, and 0.1 mol/L Tris-HCl, pH 8.5) for reduction (1 h, 37 °C). And they were alkylated by adding 10 µL IAA solution (600 mmol/L IAA, 8 mol/L urea, and 0.1 mol/L Tris-HCl, pH 8.5) for 15 min in the dark. The solution was transferred to the 5 kDa centrifuge tube and centrifuged (15000 g, 4 °C, 20 min). All samples were mixed with 100 µL of 50 mmol/L NH_4HCO_3 solution and centrifuged. The waste solution was removed, followed by washing the tube. Subsequently, 100 µL of 50 mmol/L NH_4HCO_3 was added and mixed thoroughly. Enzymatic digestion was then carried out by introducing trypsin at a 1:50 mass ratio in a final volume of approximately 220 µL and incubating (8 h, 37 °C). After centrifugation (15000 g, 20 min), 100 µL of 25 mmol/L NH_4HCO_3 solution was added, and the mixture was centrifuged once more. The resulting peptide solution was spin-dried under vacuum (Concentrator Plus, Eppendorf, Germany) and dissolved in 100 µL buffer (0.1% FA, 2% CAN, 98% water). After centrifugation (15000 g, 5 min, 4 °C), the peptide solution was transferred to LC injection vials.

2.4 HPLC-Q-TOF-MS/MS analysis and protein identification

The peptide solution (5 μ L for injection) was separated using NanoLC-Ultra 2D plus (Eksigent Technologies, Dublin, USA) fitted with an Xbridge Peptide BEH C18 column (4.6 mm $\times$ 150 mm, 3.5 μ m, 300 Å, Waters). And eluted peptides were analyzed in positive ion detection mode with the Triple TOF 6600 System (AB SCIEX, Foster City, USA). The separation was performed at a flow rate of 0.25 mL/min while maintaining column temperature at 40 °C. The accumulation time of precursor ion scanning was 250 ms, and the charge range was between 2+ and 5+ charge states. 20 s and 2.0 s were used as the dynamic exclusion time and total cycle time, respectively. The composition of the mobile phase, gradient elution program, and other parameters of mass spectrometry were set according to our previous research (Li et al., 2025).

The data was processed using ProteinPilot Software v.5.0 (AB SCIEX) with the default settings. The Sesame Protein Database was imported from the UniProt Knowledgebase (UniProtKB) (<http://www.uniprot.org>) (Date of access: August 12, 2023). The ALLERGEN NOMENCLATURE website (<https://www.allergen.org/>) was visited to obtain information on sesame allergen proteins (Ses i 1- Ses i 7). The accessions of them were Q9AUD1, Q9XHP1, Q9AUD0, Q9FUJ9, Q9XHP2, Q9XHP0, and Q9AUD2 in order.

2.5 Determination of signature and quantitative peptides

Referring to the previous research of our team (Li et al., 2025), 12 kinds of processed sesame substrates produced in the laboratory were used as test materials for mass spectrometry analysis to identify the characteristic peptides of sesame allergens. In brief, the key selection criteria were as follows: Firstly, peptides with identification reliability higher than 95%, mass-to-charge ratio less than 1250, and without variable post-translational modification sites were initially retained. Crucially, to experimentally evaluate "processing robustness," these pre-selected peptides were further required to be consistently identified across all twelve laboratory-processed samples (covering six processing techniques in duplicate). Then, the specificity of the preselected peptide was evaluated and verified by the Basic Local Alignment Search Tool (BLAST) and positive/negative samples (Table S1). Finally, the pre-selected peptides that can stably exist after processing stability verification are used as characteristic peptides for Ses i 1-Ses i 7.

The quantitative peptides for Ses i 1-Ses i 7 were identified from the characteristic peptides through targeted analysis. The final quantitative peptides for Ses i 1–Ses i 7 were selected from the characteristic peptides through a structured, multi-criteria decision process. Candidate peptides were evaluated based on the following objective metrics: (1) signal intensity ($S/N > 50$); (2) linearity ($R^2 > 0.99$ across 0–400 μ g protein); (3) sensitivity (lowest LOD/LOQ); (4) chromatographic performance (symmetric peak shape, stable retention time); and (5) repeatability (intra-day CV $< 10\%$). The peptide with the highest composite score across these criteria was chosen as the quantitative peptide for each allergen. To assess the dose-response relationship, sesame crude protein spanning a concentration range of 0-400 μ g was analyzed for the linearity between

characteristic peptide signal intensity and protein content. For each sesame allergen protein, the quantitative peptides were identified from the characteristic peptides through targeted analysis based on optimal linearity ($R^2 > 0.99$), highest signal response intensity, and stable retention time.

2.6 HPLC- Q-TRAP analysis

HPLC-Q-TRAP 5500 (AB SCIEX, USA) was used to develop the Multiple Reaction Monitoring (MRM) method. The samples (10 μ L) were injected into the HPLC system (LC-20AD XR, SHIMADZU, Japan), and it was separated by the XBridge peptide BEH C18 LC column (4.6 mm $\times$ 150 mm, Waters, Milford, MA, USA) with a 3.5 μ m particle size and a 300 Å pore size. Data were acquired in positive ions utilizing the Q-TRAP 5500 equipped with a Turbo V electrospray ionization (ESI) source. According to the research published (Li et al., 2025), the parameters of chromatography and mass spectrometry were set. Optimize the MRM method by determining the optimal collision energy (CE) and de-clustering potential (DP). For each transition, 11 DP values (20-130 V) and 11 CE values (initial value ± 10 V) were systematically tested at intervals of 10 V and 2 V, respectively. The optimal CE and DP values were selected based on the highest transition response intensity.

2.7 Preparation of calibration curves

Standard quantitative peptides and isotope-labeled [^{13}C , ^{15}N] peptides (purity $> 99\%$) were custom synthesized by MOTIF BIOTECH., Ltd. (Suzhou, China). To correct for variations in trypsin digestion efficiency, derived peptides were designed as internal standards by extending 6-10 amino acids at both termini of each quantitative peptide, ensuring the inclusion of tryptic cleavage sites (K/R) while avoiding unstable terminal residues (M/Q). Complete sequences are provided in Table S3. Calibration curves were prepared using the stable isotope dilution method. Specifically, the standard solution of the native (unlabeled) quantitative peptide was diluted to a series of concentrations (0.4–2000.0 fmol/ μ L). A fixed volume of each of these native peptide solutions was then spiked with a fixed volume of the corresponding isotope-labeled derived peptide internal standard solution (at a constant concentration of 80 fmol/ μ L). The peak area of each peptide was integrated after analysis. The calibration curve was generated by performing a linear regression analysis of the ratio of the native peptide peak area to the labeled peptide peak area against the concentration of the native peptide. The resulting calibration curve was used to determine the molar amount (fmol) of the quantitative peptide in the processed sample extracts. This value was then converted to the concentration of the intact allergen protein in the original food sample ($\mu\text{g/g}$) using the molecular weight of the specific allergen protein and accounting for all sample preparation factors (e.g., sample weight, extraction volume, and dilution factors).

2.8 Method validation

Method validation mainly included the evaluation of sensitivity, accuracy, and precision. The goodness of fit for linearity was validated using the Shapiro-Wilk test (residual normality) and a lack-of-fit F-test. Method accuracy (recovery) was statistically evaluated with a one-sample t-test against 100%. Detection limit (LOD) and quantification limit (LOQ) were determined using concentration levels where signal-to-noise ratios reached minimum thresholds of 3 and 10, correspondingly. Different levels (low, medium, and high concentrations) of quality control samples (QCS) were used to calculate the accuracy of the method. QCS were prepared by spiking known amounts of sesame allergen peptides at low, medium, and high concentrations into the sesame-free β -casein solution with extraction buffer (50 mmol/L Tris-HCl with 7 mol/L urea, 2 mol/L thiourea, 25 mmol/L DTT, and 4% CHAPS, pH 8.5). Mass spectrometry analysis was performed on protein enzymatically hydrolyzed peptides from samples prepared in the laboratory containing a certain amount of sesame, with six repetitions per day and a total of 30 repetitions for five days, to evaluate the precision of the method.

A sesame-free β -casein solution prepared in the extraction buffer was initially employed as a simplified blank matrix for QCS to assess fundamental performance parameters under minimized matrix interference. However, to evaluate accuracy under more realistic conditions, a complex surrogate food matrix (prepared from wheat flour, eggs, butter, and sugar) was also used. Some processed food models containing wheat flour, sugar, butter, eggs, and seven sesame allergen proteins were prepared by different processing technologies. The processed food without sesame allergen protein was used as the blank matrix. All products are dried and ground to form a uniform powder. The processed food containing protein was diluted with a blank sample so that the content of each of the seven sesame allergen proteins was 20 ppm (based on weight). Then all samples were pretreated according to the above steps. Isotope labeled derived peptides were mixed with the crude protein extract and digested by trypsin together with the protein in the sample.

3. Results and discussion

3.1 Determination of signature peptides

Signature peptide is critical in developing food allergen detection methods utilizing LC-MS/MS, as it can directly make sure the specificity and sensitivity of the method (Marzano et al., 2020; Yang et al., 2024). Based on the screening process of characteristic peptides in our team's previous research (Li et al., 2025), ultimately nine peptides were chosen as pre-selected signature peptides of Ses i 1-Ses i 7. There were significant differences in the transition response intensity of the pre-selected peptides of Ses i 1-Ses i 7 (Figure 2(A)), which may be explained that the different contents and extraction efficiencies of these allergen proteins. In order to improve the sensitivity of the detection method, the ion conversion response intensity of these preselected peptides is greater than 10^5 .

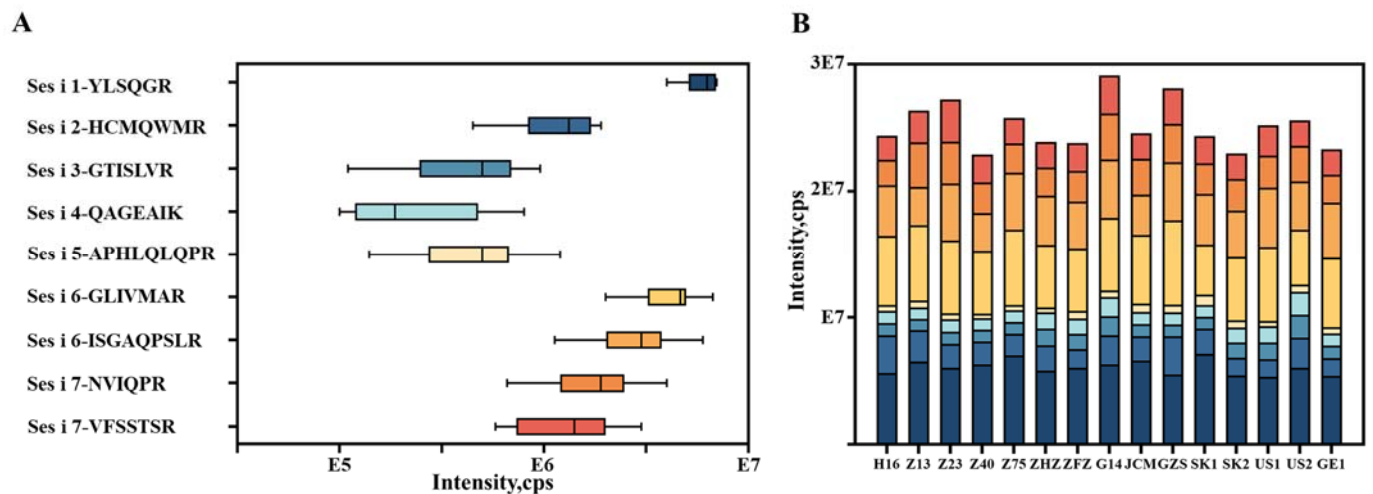


Figure 2 Response intensity of nine characteristic peptides from seven sesame allergen proteins detected by LC-MS/MS. (A) Twelve simulated processed products prepared in the laboratory; (B) Fifteen different varieties of sesame.

Sesame is often mixed with various nuts and grains to make all kinds of foods. Research has shown that sesame may share production lines with grains and nuts during the production process, causing unintentional cross-contamination (Meima et al., 2021). These food allergens may contain the same amino acid sequence, such as the sequence of 2S albumin from peanuts and sesame, which showed certain similarity (29%-60%) (Dreskin et al., 2021). Therefore, the selected characteristic peptides must ensure their specificity. Not only was the BLAST algorithm in the UniProt database used, but also some common food allergens were as negative samples for specific validation of nine pre-selected peptides. The results indicate that these pre-selected peptides do not exist in other species. The results in Figure 2 (B) indicated that proteins of different varieties of sesame could be hydrolyzed to produce nine pre-selected peptides. These nine pre-selected peptides are specific and could be used as characteristic peptides of Ses i 1-Ses i 7 for detection. These nine peptides are consistent with the results of the characteristic peptides screened from sesame commercial products as samples (Li et al., 2025). This may be because both commercial products and processed products prepared in the laboratory have undergone similar processing technology. These nine characteristic peptides are robust to processing and can exist stably in different kinds of processed products. The response intensity differences observed in Figure 2A reflect how different processing techniques distinctly affect protein extractability and digestibility. The consistently higher intensities in dry-heat processed samples (stir-fried, baked) compared to boiled samples suggest that thermal processing methods differentially impact protein structure and accessibility. Ses i 6 and Ses i 7 maintained high intensities in all processes, while Ses i 1 and Ses i 3 decreased by 40%-60% after boiling/steaming, indicating higher sensitivity to moist-heat treatments. Meanwhile, the consistent detection of all nine peptides across fifteen sesame varieties (Figure 2B) confirms their reliability as universal biomarkers, while the observed intensity variations reveal intrinsic differences in allergen composition among varieties, with important implications for varietal-specific allergen risk assessment.

Using sesame matrices prepared in the laboratory through stir-frying, frying, boiling, steaming, grinding, and baking as the test material, nine peptides that are stable and specific were selected as the biomarker characteristic peptides for seven sesame allergen proteins. Defatting proved particularly beneficial for high-fat samples, enhancing protein extraction and reducing ion suppression, consistent with established allergen analysis protocols (Chen et al., 2022). During mass spectrometry analysis, peptide fragments are induced to dissociate by collision, producing a large number of small bits, including y-ions and b-ions. Research has shown that when determining the MRM transition of characteristic peptides, the precursor ion of the first quadrupole should preferably be the y-ion (Ma et al., 2020). In this study, three to four transitions were selected for each signature peptide to ensure the sensitivity and reproducibility of the measurement results. Among them, the transition with the strongest response intensity is used for quantification. Table S2 displays all the information about the amino acid sequences, precursors, and fragment ions of nine signature peptides, laying the foundation for the development of subsequent quantitative methods.

3.2 Determination of quantitative peptides

3.2.1 Linear analysis of signature peptides

The method of quantifying proteins based on LC-MS/MS targets the hydrolyzed characteristic peptide of the protein, which is the quantitative peptide. Therefore, the peak area of the quantitative peptide needs to accurately reflect protein content. A high response intensity of quantitative peptide transitions is beneficial for sensitivity of detection. By analyzing the linear correlation between the peak area of nine characteristic peptides and the sesame protein content, the peptides with better linearity and response intensity were determined as the quantitative peptides for sesame allergen proteins. The results (Figure S2) indicated that there was a good linear relationship ($R^2 > 0.99$) between protein content and peak areas of nine characteristic peptides. According to the response intensity, the quantitative peptides were determined (Table 1). It is noteworthy that some selected quantitative peptides contain methionine (M) or cysteine (C) residues, which can be susceptible to oxidation or modification. These peptides were ultimately chosen because they demonstrated superior processing robustness and specificity over alternative sequences. Furthermore, the use of isotope-labeled internal standards with identical sequences ensures that any variability in sample preparation affects both the native and labeled peptides equally, thereby correcting for potential response variations and ensuring quantification accuracy.

The linear range of quantitative peptide determination is 0.4-2000 fmol/ μ L, with 80 fmol/ μ L isotope labeled peptide spiked. Linear analysis was performed on the concentration and peak area ratio (quantitative peptide/isotope labeled peptide). The results (Figure S3) showed that the peak area ratio and concentration of the seven quantitative peptide segments were linear ($R^2 > 0.99$), with a relative standard deviation (RSD) below 15%. The quantitative peptide content can be assessed through the peak area ratio measurements, and

then the content of the sesame allergen proteins in the sample can be calculated according to the molar equivalent relationship between the quantitative peptide and the target protein.

Table 1 Peptides with process robustness of seven sesame allergen proteins.

Allergen	Signature peptides	Quantitative peptides	Derivative peptides
Ses i 1	YLSQGR	YLSQGR	QFRSCQRYLSQGREVLEMSTGN
Ses i 2	HC[CAM]MQWMR	HC[CAM]MQWMR	ESRQCQMRHC[CAM]MQWMRSMRGQYE
Ses i 3	GTISLVR	GTISLVR	FVAKGRGTISLVRQDRRESLN
Ses i 4	QAGEAIK	QAGEAIK	YVGEKTKQAGEAIKSTAKEGGREG
Ses i 5	APHLQLQPR	APHLQLQPR	GQQQQTRAPHLQLQPRAQRVVKAA
Ses i 6	GLIVMARISGAQPSLR	GLIVMAR	SEEEERGLIVMARERMTF
Ses i 7	NVIQPRVFSSTSRR	NVIQPR	AGVAAVRNVIQPRGLLLPHYN

3.2.2 Design of derived peptides

To correct for variations in trypsin digestion efficiency, isotope-labeled derived peptides were designed and used as internal standards, as detailed in the Methods section (2.7). The key advantage of these derived internal standards is that they are co-digested with the sample proteins, thereby accounting for losses during the enzymatic step. The sequences of the final derived peptides selected for each allergen are provided in Table S3.

3.3 Method validation and performance

In order to assess the sensitivity of the established method, the linear range, LOD, and LOQ were determined. The quantitative standard curve demonstrated that the seven quantitative peptides could be quantified at concentrations as low as 0.4 fmol/ μ L and as high as 2000 fmol/ μ L. The LOD was defined as the minimal peptide concentration generating an S/N of 3:1, and the LOQ was the peptide concentration corresponding to an S/N of 10:1. The sensitivity of the method was evaluated. As illustrated in Table 2, the calculated LOD and LOQ for the seven sesame allergenic proteins in the food matrix ranged from 1.0 to 28 μ g/g and 2.9 to 56 μ g/g, respectively. The established detection method exhibits high sensitivity, with LOQ values for the specific allergen proteins in the low ppm range, which is notable for the LC-MS/MS analysis of allergenic proteins in processed foods. The linearity of all calibration curves was statistically confirmed (lack-of-fit test, $P > 0.05$). The recovery rates (90%–110%) showed no significant difference from 100% (one-sample t-test, $P > 0.05$).

Table 2 The linear analysis of calibration curves and sensitivity verification of methods.

Allergen	Quantitative peptides	Linear equations	R ²	LOD (μg/g)	LOQ (μg/g)
Ses i 1	YLSQGR	Y=0.01259X+0.07767	0.9983	3.6	7.2
Ses i 2	HC[CAM]MQWMR	Y=0.009947X-0.08511	0.9984	28	56
Ses i 3	GTISLVR	Y=0.01449X+0.1436	0.9981	2.3	4.5
Ses i 4	QAGEAIK	Y=0.008423X+0.03235	0.9982	5.1	11.9
Ses i 5	APHLQLQPR	Y=0.01202X-0.09736	0.9982	1.2	3.6
Ses i 6	GLIVMAR	Y=0.01439X+0.09747	0.9993	1.0	3.1
Ses i 7	NVIQPR	Y=0.01121X-0.02039	0.9988	1.7	2.9

The β -casein solution (sesame-free) was employed as a simplified matrix for preliminary quality control samples (QCS) to assess fundamental method performance under minimized matrix interference. The accuracy of the established method was evaluated by analyzing the recoveries of the QCS at three different concentrations of the sesame allergen peptides spiked (corresponding to 2 LOQ, 10 LOQ, and 50 LOQ). As illustrated in Table 3, the recoveries of QCS exhibited a range of 90% to 110%, indicating good accuracy under these controlled conditions.

Table 3 Recovery rates of quantitative methods for seven sesame allergen proteins.

Allergen	Quantitative peptides	Recovery rates (%)		
		2 LOQ	10 LOQ	50 LOQ
Ses i 1	YLSQGR	96.89	98.83	98.58
Ses i 2	HC[CAM]MQWMR	104.42	97.49	97.12
Ses i 3	GTISLVR	96.83	97.45	97.63
Ses i 4	QAGEAIK	96.24	98.12	96.90
Ses i 5	APHLQLQPR	104.37	96.11	98.09
Ses i 6	GLIVMAR	106.80	104.50	98.49
Ses i 7	NVIQPR	93.92	96.60	97.71

The repeatability of the method was evaluated by measuring the intra- and inter-day precision. Trypsin-digested peptides derived from specific protein concentrations were analyzed six times to assess the intra-day reproducibility. To assess inter-day reproducibility, five consecutive working days were selected for LC-MS/MS measurements. The findings revealed that the relative standard deviation (RSD) of each data set was below 10%, indicating high reproducibility and confirming that the established method is reliable and reproducible (Table 4).

Table 4 Summary of intra/inter-day validation of method precision.

Allergen	Relative standard deviation (%)					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1-5
Ses i 1	3.62	2.75	2.60	2.95	3.60	3.06
Ses i 2	3.31	2.01	2.98	1.21	1.06	2.68
Ses i 3	2.29	2.38	2.82	3.48	3.28	3.01
Ses i 4	3.91	4.11	4.85	5.95	5.53	5.15
Ses i 5	6.10	5.62	4.94	4.28	6.24	6.31
Ses i 6	4.69	5.02	6.19	6.43	5.99	6.98
Ses i 7	6.07	5.85	7.59	5.15	2.99	5.37

To more rigorously evaluate the accuracy and practical applicability of the established method in a realistic food matrix, simulated processed products containing wheat flour, eggs, butter, sugar, and a certain amount of seven sesame allergen proteins were prepared in the laboratory. The matrix containing seven allergen proteins was diluted with the blank matrix (the products without allergen proteins), resulting in a sesame allergen protein content of 20 ppm. Protein extraction, trypsin digestion, and mass spectrometry were performed on the simulated processed products. Isotope labeled derived peptides and quantitative peptides (non-derived) were used as internal standards, respectively. Critically, when using the complex food matrix, the recovery rates improved substantially with derived peptide internal standards (80%-105%; Table 5) compared to non-derived peptides (74%-90%). The assumption of identical digestion efficiency between derived peptides and native proteins, while fundamental to the method, is supported by the consistent high recoveries (80%-105%) across all seven allergens in complex matrices. This demonstrates effective correction of digestion variability, consistent with established proteomic methodologies. These results demonstrate that the quantitative method using derived peptides as internal standards provides accurate quantification even in challenging, food-relevant matrices, confirming its practical utility for processed food analysis.

Table 5 The results of the recovery rate of seven allergen proteins in the processed products containing 20 ppm sesame allergen protein prepared in the laboratory.

Internal Standard	Ses i 1	Ses i 2	Ses i 3	Ses i 4	Ses i 5	Ses i 6	Ses i 7
Non-derived Peptides	74.36%	87.77%	79.26%	77.60%	84.96%	80.85%	81.96%
Derived Peptides	81.78%	103.17%	87.81%	102.47%	85.70%	94.96%	90.78%

The enhanced recovery rates (80%–105%) using derived peptides across all seven structurally diverse allergens provide strong functional validation that their digestion efficiency closely matches that of the native proteins. This is because any significant mismatch would result in inconsistent corrections and variable recoveries, which was not observed. This approach of using extended peptide sequences to better mimic proteolytic processing is supported by principles in quantitative proteomics, where co-digested isotope-labeled standards are established to account for hydrolysis variability (Zhang et al., 2014).

3.4 Application of the developed method to detect sesame allergens in foods

This study identified the qualitative and quantitative peptides of Ses i 1- Ses i 7. These peptides existed in different varieties of sesame, although the protein composition of different varieties of sesame was different. To assess the practicability of the method, twelve commercial food products were obtained from markets (Beijing, China) as samples, of which six goods indicated the presence of sesame. After protein extraction, isotopically labeled derived peptides were added and digested with trypsin along with the sample protein. According to Table S3, the scheduled MRM method was established to perform mass spectrometry analysis on the samples and determine seven sesame allergen proteins.

The results (Table 6) showed that seven sesame allergen proteins were detected in all six samples, indicating the presence of sesame components. However, there were significant differences in the protein

concentration among different samples. In some samples ($n = 5$), the concentration of sesame allergen protein exceeded the linear range of the calibration curve, and the concentration of sesame allergen protein could only be accurately determined after dilution. Among the six samples that did not indicate the presence of sesame, seven sesame allergen proteins were detected in only one sample. This finding has important public health implications. For individuals with sesame allergy, even trace amounts of the allergen can trigger severe reactions. The detection of sesame in a product not declared on the label strongly suggests unintended cross-contamination, potentially occurring through shared production lines, equipment, or during the sourcing of raw materials (Meima et al., 2021). Such incidents underscore the limitations of relying solely on precautionary allergen labeling (e.g., "may contain traces") and highlight the very real risk of accidental exposure for allergic consumers. This finding also has significant regulatory implications, demonstrating the method's capability to identify non-compliant products and protect allergic consumers. It is worth noting that the concentration of the seven sesame allergen proteins varied significantly among different commercial products (Table 6). While the use of different sesame varieties is one plausible explanation, other critical factors related to food manufacturing contribute to these variations. The degree of processing (e.g., thermal treatment intensity) can alter protein extractability and digestibility, thereby affecting the quantified levels. Furthermore, the non-homogeneous distribution of sesame ingredients in complex food matrices can lead to significant sampling variability. Therefore, the observed variations likely result from a combination of the intrinsic protein profile, processing-induced modifications, and product heterogeneity. Analysis revealed a consistent allergen profile across commercial and laboratory samples, dominated by Ses i 6 and Ses i 7 (60%-75% of total content). This stable fingerprint validates our method's reliability for sesame identification in diverse food products.

Table 6 The content of seven sesame allergens in different commercial food products. Among them, six commercial products were clearly labeled with sesame in the ingredient list, and the other six products were labeled as also producing productions containing sesame on this production line.

Commercial food	Allergen labeling information	Sesame allergen protein content($\mu\text{g/g}$)						
		Ses i 1	Ses i 2	Ses i 3	Ses i 4	Ses i 5	Ses i 6	Ses i 7
Cookie-1	The ingredient list clearly indicates the presence of sesame.	14	139	237	80	40	1299	597
Dipping sauce-1		83	512	1002	292	132	4915	2199
Cake-1		39	227	394	130	72	2030	921
Candy-1		70	411	825	245	113	4004	1826
Bread-1		88	558	1069	322	146	5315	2324
Cake-2		76	463	932	280	125	4535	2003
Crisps	The product line also process productions containing sesame.	ND	ND	ND	ND	ND	ND	ND
Cookie-2		ND	ND	ND	ND	ND	ND	ND
Bread-2		10	67	117	42	21	613	308
Vegetable protein milk		ND	ND	ND	ND	ND	ND	ND
Candy-2		ND	ND	ND	ND	ND	ND	ND
Dipping sauce-2		ND	ND	ND	ND	ND	ND	ND

ND denotes not detected.

Although the purchased commercial food products have undergone multiple processing techniques, seven sesame allergen protein contents have also been successfully detected. This indicates that the biomarker peptides of seven sesame allergen proteins identified in this study are robust to processing, and the method is suitable for the detection of processed products. This research result provides an effective method and meaningful references for accurately quantifying sesame allergenic proteins in various processed foods.

The quantitative results for commercial samples must be considered in the context of potential variations in the relative abundance of individual allergen proteins across different sesame varieties. In the absence of a Certified Reference Material (CRM) for sesame allergens, which precludes absolute quantification, our method is designed to provide the most reliable assessment possible. The strategy of quantifying multiple major allergens (Ses i 1-7) collectively mitigates the risk of underestimation that could occur if relying on a single, variable protein marker. While the absolute concentration of a specific allergen might vary by cultivar, the sum of the concentrations of these seven key allergens provides a robust and comprehensive indicator for the total allergenic protein content from sesame in a food matrix. This approach offers a reliable and consistent measure for detecting and quantifying sesame, which is the primary objective for food safety control and labeling compliance.

4. Conclusion

In this study, we established and validated an LC-MS/MS method for accurately quantifying seven sesame allergen proteins (Ses i 1- Ses i 7) in processed foods. To overcome key limitations of existing methods, we implemented two major innovations: screening for processing-robust signature peptides using diverse laboratory-processed matrices, and most importantly, employing isotope-labeled derived peptides as internal standards to correct for trypsin digestion efficiency. The method demonstrated excellent sensitivity (LOQ: 2.9-56 µg/g), accuracy (recovery: 90%-110%). At three different distinct spiked levels of sesame-free food additives, the intra-day and inter-day precision were 1.06%-7.59% and 2.68-6.98%, respectively.

Compared to existing LC-MS/MS methods for sesame allergens (Huschek et al., 2016; Ma et al., 2020), the present method offers distinct advantages: (1) It simultaneously quantifies all seven major sesame allergens (Ses i 1-7) with greater comprehensiveness; (2) The use of processing-robust signature peptides, screened from a wide array of laboratory-processed matrices, ensures higher reliability for analyzing real-world food products; (3) Most importantly, the innovative adoption of isotope-labeled derived peptides as internal standards effectively corrects for trypsin digestion variability, leading to superior quantitative accuracy (recovery rates of 90%-110%) and precision (RSDs < 7.59%) over previously reported values. In addition, this method can also be successfully applied to the detection of commercial food products. This study has broad potential in accurately quantifying food allergens other than sesame. Recent advances in food allergen research indicate that future work should explore the stability of allergens under various processing conditions and support the development of standardized reference methods (Chen et al., 2024). Our method

provides both a practical tool for allergen management and a validated framework for improving the accuracy of food safety analysis.

CRedit authorship contribution statement

Jing Li: Investigation, Formal analysis, Data curation, Writing-original draft. Wenhan Kang: Methodology, Software, Writing-review & editing. Yang Wan: Conceptualization, Software, Writing-review & editing. Jiukai Zhang: Investigation, Project administration, Writing-review & editing. Yiqiang Ge: Resources, Supervision, Project administration, Funding acquisition. Ning Yu: Investigation, Writing-review & editing, Formal analysis. Ying Chen: Resources, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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