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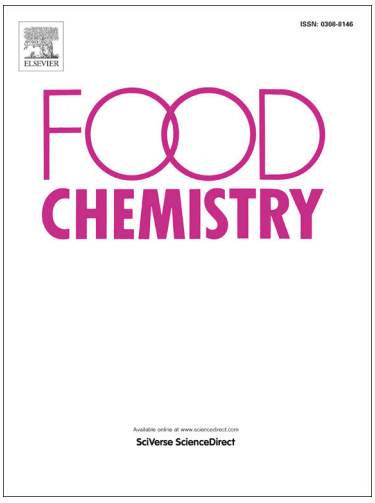

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An integrated calibration strategy for the development and validation of an LC-MS/MS method for accurate quantification of egg allergens (Gal d 1-6) in foods

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Abstract: Accurate determination of egg allergens in food is vital for allergen management and labeling. However, quantifying egg allergens with mass spectrometry poses challenges and lacks validation methods. Here, we developed and validated an LC-MS/MS method for quantifying egg allergens (Gal d 1-6) in foods. Sample extraction, enzymatic digestion, purification, proteins/peptides selection, and calibration curves were optimized. VMVLC[+57]NR (Gal d 1) and GTDVQAWIR (Gal d 5) exhibited outstanding sensitivity and stability, serving as quantitation markers for egg white and yolk. Using a matrix-matched calibration curve with allergen ingredients as calibrants and labeled peptides as standards, we achieved highly accurate quantitation. Validation involved spiking egg protein into egg-free foods, showing excellent sensitivity (LOQ: 1-5 mg/kg), accuracy (62.4%-88.5%), and reproducibility (intra-/inter-day precision: 3.5%-14.2%/8.2%-14.6%). Additionally, we successfully applied this method to commercial food analysis. These findings demonstrate optimal allergen selection, peptides, and calibration strategy are crucial parameters for food allergen quantification via MS-based methods.

Key words: food allergen, allergen quantification, validation methods, LC-MS, calibration curves

1. Introduction

Food allergy is a significant public health and food safety concern, generating global attention, particularly in developed nations (Sampath et al. 2021; Yuan and Capucilli 2023). Epidemiological data indicates that approximately 8% of children and 5% of adults suffer from food allergies, with a notable increase in prevalence over the past decades (Editorial 2021; Muraro et al. 2022; Yuan and Capucilli 2023). Unfortunately, there is currently no specialized therapy available for this condition. Allergy sufferers must strictly avoid allergenic foods as the only effective measure (Sampath et al. 2021). To protect consumers, major food allergens should be clearly labeled when present in foods, as practiced in the USA, Canada, European Union, China, and other regions (Ebisawa et al. 2020; Holzhauser et al. 2020; Sena-Torralba et al. 2020). Eggs, rich in proteins, fats, and vitamins, are commonly consumed in daily diets. However, eggs are a leading cause of food allergies, particularly among children (Dona and Suphioglu 2020). Additionally, eggs and their derivatives are widely used in the food industry, making complete elimination from the diet challenging. Although egg allergies tend to naturally resolve with the maturation of the immune system, especially when eggs are consumed in highly processed forms, it can still be severe and life-threatening in some patients (Dona and Suphioglu 2020). Therefore, the risk of food allergy triggered by accidental exposure to eggs should not be underestimated (Dona and Suphioglu 2020). Thus, it is critically important to establish a robust, efficient, and sensitive analytical method for the quantitative determination of egg allergens in foods (Sena-Torralba et al. 2020; Tuzimski and Petruczynik 2020).

Liquid chromatography tandem mass spectrometry (LC-MS) offers exceptional advantages such as high sensitivity, specificity, and high throughput, making it the preferred tool for allergen detection

(Monaci et al. 2018; Thomas et al. 2022). In practice, allergenic proteins are extracted from foods, followed by reduction, alkylation, and enzymatic digestion using trypsin to generate peptides (Croote and Quake 2016; Monaci et al. 2018). Signature peptides are selected and then analyzed and quantified using LC-MS. Another method for allergenic protein quantitation relies on antibodies and is available as commercial kits. However, the antibody-based method may exhibit limited sensitivity for foods subjected to thermal processing and can yield false-positive results due to antibody cross-reactivity (Tuzimski and Petruczynik 2020). In contrast, the LC-MS based method overcomes these limitations by relying on the detection of allergen-specific peptides, ensuring high specificity (Marzano et al. 2020; Sena-Torralba et al. 2020).

Over the past decade, several LC-MS based methods have been developed for the detection of up to four egg allergens, such as Gal d 1-4 (Downs et al. 2022; Gavage et al. 2019; Kyohei Kiyota 2021; Pilolli et al. 2017; Sufang Fan 2023). Ovalbumin (Gal d 2), comprising approximately 54% of egg white protein, is the most abundant protein and the primary target in reported LC-MS methods, as more than 94% of egg-allergic patients are sensitized to Gal d 2 (Jolivet et al. 2006). However, Gal d 2 is not the ideal protein for accurate egg allergen quantitation, as it is prone to degradation during food processing. In fact, a total of 10 egg allergens (Gal d 1-10) have been identified according to the Allergen Nomenclature Subcommittee of the World Health Organization and the International Union of Immunological Societies (WHO/IUIS) official allergen database. Gal d 1-4 are the main allergenic proteins in eggs, primarily found in the egg whites, while Gal d 5-6 are mainly present in the yolk (Amo et al. 2010; Gavage et al. 2019). Recently, Gal d 7-10, primarily found in chicken meat, have also been identified as allergens (Klug et al. 2020). Although Gal d 7-10 can be detected in eggs, they are present in trace amounts. Eggs, egg whites, and egg yolks are all crucial ingredients in food

processing, and one or more of these allergenic components are commonly observed in processed foods (Dona and Suphioglu 2020). For instance, Gal d 4 (lysozyme) is widely used as a food additive due to its antimicrobial activities (Downs et al. 2022). Focusing only on Gal d 1-4 in egg whites neglects the risk posed by allergens in egg yolk to allergic patients. However, reported detection methods have mainly concentrated on Gal d 1-4 in egg whites, with little emphasis on Gal d 5-6 and other allergens in egg yolks (Gavage et al. 2020; Ogura et al. 2019; Pilolli et al. 2021; Planque et al. 2019; Stella et al. 2020). Furthermore, protein extraction and digestion efficiencies for different types of egg allergens vary, even when subjected to the same food processing, resulting in significant differences in measured values using MS-based methods (Johnson and Downs 2019). Analyzing multiple allergens can effectively reduce the risk of false negatives or underestimation of food allergens. The MS-based method, with its excellent multiple quantitative detection capabilities using multiple reaction monitoring (MRM), can meet the requirements for multiple allergen quantification in foods. Thus, the development of an efficient analytical method for the simultaneous determination of egg allergens (Gal d 1-9) originating from egg whites and yolks in foods using LC-MS is urgently needed.

The process of quantifying food allergens using LC-MS generally involves four stages: protein and signature peptide selection, peptide specificity verification, quantitative LC-MS method development, and method validation (Downs and Johnson 2018; Johnson and Downs 2019; New et al. 2020). Method validation is a crucial step in the development of LC-MS based quantitative methods (Gavage et al. 2023; Johnson and Downs 2019; New et al. 2020). However, it has been rarely adopted in reported LC-MS methods for detecting egg allergens due to the lack of established guidelines for MS-based quantification of allergens in foods, hampering harmonization among different analytical

laboratories. Recent global recognition highlights the indispensability of harmonization and transparency during the development of MS-based methods (Gavage et al. 2023; Johnson and Downs 2019; New et al. 2020). This study aims to develop and validate an LC-MS based method for the simultaneous quantification of major egg allergens, namely Gal d 1-4 (from egg white) and Gal d 5-6 (from egg yolk), following the latest WHO/IUIS allergen database. The accuracy and precision of the developed method should meet the performance requirements outlined in the AOAC (Association of Analytical Communities) for allergen analysis (SMPRVR 2016.002). Additionally, the quantification limits align with the action levels specified in the Food Industry Guide to the VITALVR Program Version 3.0 and the proposed FAO/WHO threshold levels for egg allergens in foods (Holzhauser et al. 2020; Johnson and Downs 2019).

2. Materials and methods

2.1. Chemicals and reagents

Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, ≥99%), dithiotheritol (DTT, ≥98%), 2-iodoacetamide (IAA, ≥98%), ammonium bicarbonate (NH_4HCO_3, ≥98%), urea (≥98%), and hydrochloric acid (HCl, 37 %) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Sequencing-grade modified trypsin (100 μg, 4000 units/mg) was purchased by Promega GmbH (Mannheim, Germany). The HPLC grade methanol, acetonitrile, and formic acid were from Thermo Fisher Scientific (Waltham, USA). Additionally, the solid phase extraction cartridge (SPE) C18 spin columns were also obtained from Thermo Fisher Scientific (Waltham, USA). One kilogram of fresh eggs was purchased from the local stores in Beijing, China. Ultra-pure water generated from Milli-Q system for the experiments.

2.2. Peptide standards

A total of six signature peptides originating from Gal d 1-6 was selected in this study, in which VMVLC[+57]NR (Gal d 1, Ovomucoid), HIATNAVLFFGR (Gal d 2, Ovalbumin), YFGYTGALR (Gal d 3, Ovotransferrin), GTDVQAWIR (Gal d 4, Lysozyme), APFSEVSK (Gal d 5, Serum albumin), ATAVSLLEWQR (Gal d 6, YGP42) and their corresponding stable isotope-labeled internal standard (IS) peptides (R^{*}-$^{13}C_6{}^{15}N_4$, K^{*}-$^{13}C_6{}^{15}N_2$) were synthesized by China Peptides Co., Ltd. (Shanghai, China). The purity was >98% for the synthetic peptides and the ISs, which has been confirmed by HPLC. The standards stock solutions (1 mg/mL) and working solutions (1 μg/mL) for each peptides and the corresponding IS was dissolved in 50% acetonitrile, which was stored at -20 ºC and 4 ºC, respectively. All the working solutions were freshly prepared and used within two weeks.

2.3. Protein extraction and digestion, and purification of peptides.

Sample preparation protocol was adopted from a previously reported method with some modifications (New et al. 2020). Soft boiled whole egg, egg white, egg yolk were heated at 65 ºC for 15 min, which were used as the reference materials (allergen ingredients) for allergen quantification. Additionally, the protein concentration for the selected allergen ingredients was measured by the enhanced Bicinchoninic Acid (BCA) Protein Assay Kit. The commercial egg-free cakes and cookie are purchased from local stores in Beijing, China, which are served as blank matrix. These blank samples were homogenized twice before the spiking of allergen ingredients. Generally, three major steps are involved in samples preparation, namely the extraction of proteins from food matrices; protein reduction, alkylation and trypsin digestion; and the purification of peptides using solid phase extraction (SPE) column. Two grams of each sample was spiked with 20 mL protein extraction buffer

containing 2 M urea and 100 mM Tris buffer and subjected for 180 min of shaking. Subsequently, the samples were centrifuged at 5000 *g* for 10 min, after which 10 mL of the supernatant was transferred to a new falcon tube. Next, the proteins was reduced and alkylated in 100 mM dithiothreitol under 70 ºC for 30 min and 100 mM indole-3-acetic acid in the dark for 30 min, respectively. Trypsin solution (50:1 substrate to enzyme) was added for overnight digestion at 37 ºC. Then, 100 μL formic acid was added to stop trypsin digestion. Additionally, the stable isotope-labeled IS peptides was spiked in the mixtures at this step. Afterwards, the digested mixtures were loaded onto C18 SPE cartridges that were preconditioned with 3 mL of methanol followed by 3 mL of water for peptide purification. The loaded samples were washed with 3 mL of water and finally eluted into new 10 mL centrifuge tubes with 3 mL of methanol. The elution was then dried under a gentle nitrogen stream, and the dry residue was reconstituted with 200 μL 0.1% formic acid before the injection by LC-MS analysis.

2.4. LC-MS methods

The global proteomics data for whole egg, egg white, and egg yolk was performed on UHPLC-Q Orbitrap mass spectrometer equipped with heated-electrospray ionization (HESI) (Q Exactive Plus, Thermo Fisher Scientific). Peptide was separated with a Hypersil GOLD C18 column (100 mm × 2.1 mm, 1.9 μm, Thermo Fisher Scientific) using the Dionex UltiMate 3000 liquid chromatography system. A gradient elution with the flow rate at 0.3 mL/min was applied as follows: 0-1.0 min, 5% B; 1.0-2.0 min, 15% B; 2.0-13.0 min, 40% B; 13.0-14.0 min, 95% B; 14.0-17.0 min, 95% B; 17.0-17.2 min, 5% B; and 17.2-20.0 min, 5% B (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile). The injection volume was set as 5.0 μL. The major HESI parameters were set as electrospray voltage: 3.25 kV for positive mode, source temperature: 350 ºC, ion transfer capillary temperature: 320 ºC, and sheath gas flow rate: 40 arbitrary. Full mass-ddMS2 mode was used for the

identification of peptides in which a full MS scan was acquired first following with the five most abundant peaks for further MS2 analysis. For MS scan, mass range was 300-1200 Da with the resolution of 70000 at m/z 200; and the AGC target and IT were 3e6 and 250 ms, respectively. While for ddMS2, top 5 intense ions were selected within 2 Da isolation window for further fragment under a normalized collision energy of 25, and resolution was 15000 at m/z 200. The AGC target and IT were 1e5 and 80 ms, respectively. Additionally, the dynamic exclusion time for ddMS2 was set as 8 s. During the development of UHPLC-Q Orbitrap, we fully referred to the reported methods with slight parameter adjustments (Chen et al. 2020; Van et al. 2020). Acquisition and analysis of the data were performed using TraceFinder 3.0, and the raw MS data would be imported to PEAKS Studio software for the identification of peptides and proteins by matching with the online library of NCBI and UniProt.

The quantitation of targeted peptides was performed on the LC-MS/MS system (Agilent 6495). Chromatography was conducted using the same column and mobile phases, while the gradient elution was different as follows: 0.0-0.5 min, 5% B; 0.5-3.0 min, 5% B to 25% B; 3.0-7.0min, 25% B to 45% B; 7.0-7.5 min, 45% B to 100% B and maintained for 1.5 min; 9.0-10.0 min, 5% B. The total running time was 10.0 min with a flow rate of 0.3 mL/min. In addition, the important MS/MS parameters were set as follow: electrospray capillary is 4.0 kV for positive mode; the sheath gas temperature and flow were 400 ^{o}C and 12 L/min, respectively. Selected reaction monitoring (SRM) transitions were adopted in MS/MS method for each peptide, and the detail information about precursor and daughter ions, collision voltages were available in **Table 1**.

2.5. Preparation of calibration curves

The synthetic target peptides and allergen ingredients coupled with the ISs were adopted for the

preparation of external calibration. In this study, a total of four different calibration strategies (Cal. A, B, C and D) were prepared and the overview for them is indicated in **Table S1** (supplementary material). In short, the synthetic peptides were used in Cal. A, while allergen ingredients were adopted in other calibration Cal. B, C and D. Meanwhile, SIL peptides were used in Cal. A, B and D, and match matrix was employed in calibrations Cal. A, B and C, while the similar matrix were used in calibration Cal. D. The Cal. A was prepared by spiking synthetic peptides at 0, 0.1, 0.5, 1, 5, 20, and 100 μg/kg in the matched matrix. For Cal. B, C and D, allergen ingredient was spiked at 0, 1, 5, 10, 30, 100, 200, and 500 mg/kg in the matched and similar matrix. Taking into consider that the preparation of individual calibration samples at different concentration is quite labor-intensive; the dilution strategy was used in this study. Additionally, the commercial egg-free cakes and cookie were served as the blank matrix.

2.6. Method validation

The linearity, sensitivity, accuracy, precision (intra-day variability) and reproducibility (inter-day variability) were evaluated for method validation (Xiong et al. 2021; New et al. 2020). In brief, allergen ingredients with different concentration was spiked into the egg-free cakes and chicken-free sausage, which was used for the validation study. The limit of detection (LOD) and limit of quantification (LOQ) for each egg allergen was assessed according to the signal-to-noise (S/N) >3 and >10, respectively. Additionally, recovery and precision parameters were calculated by spiking allergen ingredients with three different concentrations (10, 20 and 100 mg/kg) in the blank samples. The percent recovery for each egg allergen was calculated by the measure values / the spiked concentration * 100%, which was obtained by six replicates (n=6). The intra-day and inter-day coefficients of variation (CVs) were evaluated by analysis of eighteen replicates from three batches for each spiked level on the same day

(n=18) and on three different days (n=18), respectively.

3. Results and Discussion

3.1. Overall Research Approach

The objective of this study is to identify and selected the optimal allergenic protein and its peptide present in eggs for precise and sensitive quantification of egg allergens in food using LC-MS. A comprehensive research strategy, along with the principal technical methodologies employed, is depicted in **Figure 1**. Despite the considerable amount of published papers in the literature on LC-MS/MS detection techniques for egg allergens, there is still a dearth of validated quantitative analytical methods (Gavage et al. 2020; Gavage et al. 2019; Kyohei Kiyota 2021; Ogura et al. 2019; Pilolli et al. 2021; Van Vlierberghe et al. 2020). Given the ubiquitous usage of eggs in the food industry, either independently or in specified egg white and yolk proportions, it is imperative to select quantitative peptide from both components to achieve accurate quantification of egg allergens in food products (Sufang Fan 2023). To address this, in this study, the proteomic approach combining LC-MS was employed to analyze the protein composition of eggs, egg white, and egg yolk. Subsequently, the acquired mass spectrometry data were subjected to analysis using the proteomic software Peaks, in conjunction with the Uniprot online database, for peptide identification and protein attribution. While our investigation identified and characterized over 400 distinct protein types from eggs, particular emphasis was placed on allergenic proteins (Gal d 1-9). Although Gal d 10 is also considered an allergenic protein, it was excluded due to the unavailability of its amino acid sequence within the database. **Table S2** (supplementary material) presents pertinent information regarding Gal d 1-9, alongside the quantity of uniquely identified peptide segments. To ensure the accuracy and robustness

of the quantitative methodology, further refinement of the identified peptide segments was essential to select optimal candidates for LC-MS method development, followed by a systematic methodological evaluation.

3.2. Selection of Quantitative Peptides

Peptide selection is a crucial and intricate step in the development of LC-MS quantitative methods for food allergens, as it directly impacts the specificity and sensitivity of the method (Croote and Quake 2016; Downs and Johnson 2018; Johnson and Downs 2019; Marzano et al. 2020). The selection process follows a set of principles that include the following: 1. ensuring specificity by representing unique peptide of the target protein; 2. prioritizing peptides with strong mass spectrometry signal intensity; 3. excluding peptides with missed cleavage sites (K and R); 4. considering peptides with good thermal stability and resistance to degradation; 5. opting for peptides with a length of 6-20 amino acids; 6. avoiding peptides with easily modifiable amino acids (M, W, Q, N, E, etc.). Based on these principles, we selected 2-3 peptides for each of Gal d 1-9, as presented in **Table S3** (supplementary material). Blast analysis using the Uniprot database was conducted to ensure the specificity of these peptides. **Table S3** provides comprehensive information on the amino acid sequences, accurate mass-to-charge ratios of precursor and fragment ions, and retention times of the selected peptides. Moreover, the major b and y fragment ions of these peptides MS/MS spectra were assigned, serving as a foundation for the subsequent development of the MRM method.

Egg white and egg yolk contribute to 60% and 40% of the edible protein in eggs, with significantly different protein compositions (Dona and Suphioglu 2020). Major proteins in egg white include ovalbumin (Gal d 2, 54%), ovotransferrin (Gal d 3, 13%), ovomucoid (Gal d 1, 11%), and lysozyme C (Gal d 4, 3.5%). Conversely, egg yolk consists of low-density lipoprotein (65%), high-density

lipoprotein (16%), serum albumin phosvitin (Gal d 5, 10%), and a minor component called vitellogenin I (Gal d 6). Consequently, substantial variations exist in the peak areas of Gal d 1-9 peptides between whole egg, egg white, and egg yolk. The findings of this study validate this observation, as shown in **Figure 2(A)**, which illustrates the peak areas and coefficients of variation of Gal d 1-9 peptides in whole egg, egg white, and egg yolk. Peptide peak areas are primarily influenced by ionization efficiency and concentration, rendering accurate representation of protein concentrations challenging. Nonetheless, peptides with high mass spectrometry signal response are preferable for allergen quantification methods (Johnson and Downs 2019; Ogura et al. 2019). **Figure 2(A)** demonstrates that the peak areas of Gal d 1-6 peptides are 2-4 orders of magnitude higher than those of Gal d 7-9, mainly due to the trace amounts of Gal d 7-9 in eggs. The peak areas of Gal d 1-4 peptides in egg white exhibit strong signal values and high S/N ratio, making them suitable for quantitative analysis of allergens in egg white. Additionally, higher peak areas of Gal d 1-4 peptides are observed in egg yolk, primarily because of the presence of a small amount of egg white in separated egg yolk. However, compared to egg white, the signal values of these peptides in egg yolk are lower by approximately one order of magnitude. Notably, Gal d 5 and 6 peptides display larger peak areas in egg yolk, with signal values approximately 1-2 orders of magnitude higher than those in egg white, as Gal d 5 and 6 are primarily present in egg yolk, making them suitable for quantifying proteins in single egg yolks.

Thermal processing is a common practice in the production of food products derived from eggs, which can lead to the degradation of certain allergenic proteins (Shimin Chen 2022). Therefore, it is crucial to select proteins and peptides with thermal stability during method development to ensure accurate quantification. In this study, we investigated the degradation of Gal d 1-6 in egg liquid after subjecting it to heating at temperatures of 70°C, 100°C, and 130°C in an oil bath for 10 and 20 minutes.

Detailed relative peak area values of Gal d 1-6 peptides under different heating conditions are presented in **Figure 2(B)**. Overall, all peptides exhibited varying degrees of reduction in peak area after thermal processing of eggs, particularly under high temperature and long-duration heating conditions, where degradation was more pronounced. Interestingly, two peptides derived from Gal d 1, specifically VMVLC[+57]NR, demonstrated the highest thermal stability, retaining 92% relative abundance even after heating at 130°C for 20 minutes, indicating their suitability as quantitative peptides. Previous studies have also recognized Gal d 1 as the most stable protein in eggs, resistant to heat and enzymatic digestion, but quantitative investigations of egg allergens have predominantly focused on Gal d 2 and 4 (Gavage et al. 2019; Kyohei Kiyota 2021; Sufang Fan 2023).

Among the reported peptides for Gal d 2, HIATNAVLFFGR and GGLEPINFQTAADQAR are the most commonly adopted ones (Gavage et al. 2019; Kyohei Kiyota 2021; New et al. 2020; Ogura et al. 2019). However, their sensitivity is 3 to 6 times lower than Gal d 1. Peptides derived from Gal d 3 (SAGWNIPIGTLLHR and YFGYTGALR) and Gal d 4 (GTDVQAWIR and FESNFNTQATNR) exhibited an improved sensitivity in comparison to Gal d 2. Additionally, for Gal d 5 and 6 in egg yolk, the peptide APFSEVSK derived from Gal d 5 displayed relatively good thermal stability, making it suitable for quantifying proteins in egg yolk. Based on the aforementioned analysis, Gal d 1-6 were ultimately selected for detecting egg allergens in food, with one peptide chosen for quantitative analysis and another for qualitative analysis for each allergen. Furthermore, stable isotope-labeled peptides were employed to correct for matrix effects and instrumental analysis fluctuations in the selection of quantitative peptides. **Table 1** presents detailed MRM parameter settings for the 12 peptides and 6 SIL-IS peptides. The selection of fragment ion primarily followed these principles: 1) choosing fragment ions with *m/z* values greater than the precursor ions; 2) prioritizing fragment ions

with strong mass spectrometry signals; 3) minimizing interference signals and achieving a high signal-to-noise ratio; 4) preferentially selecting y ions. **Figure S1** (supplementary material) displays the MS/MS spectra of the six quantification peptides and the chromatograms of the six quantitation peptides.

3.3. Development and Optimization of Sample Preprocessing Methods

In general, sample preprocessing for allergen detection in food involves several key steps, including sampling, protein extraction, denaturation, protein reduction, alkylation, enzymatic digestion, and SPE purification of digested peptides (Croote et al. 2019; Johnson and Downs 2019; Marzano et al. 2020). Additionally, for samples with high concentrations of other matrix constituents (fats, salts, and sugars), defatting and ultrafiltration treatments are necessary. It is important to recognize that allergenic contamination in food is typically unevenly distributed, often occurring in hotspots, which distinguishes it from pesticide and veterinary drug residues. To address this, we meticulously ground 200-500 g of each test sample to ensure the uniform distribution of allergens within the food matrix.

Protein extraction is the most critical step in sample preprocessing, with the aim of transferring food allergens from the sample to the solution, rather than extracting all proteins present (Johnson and Downs 2019). Due to the diversity of food types and processing methods, there is currently no standardized protein extraction method. The commonly employed approach involves using a 40-200 mM Tris-HCl solution for protein extraction. However, considering that heat processing can induce cross-linking between allergens and other proteins, hindering the effective extraction of allergens using Tris-HCl alone, we supplemented the extraction solution with 2M urea. This addition disrupted the protein cross-linking, facilitating their solubilization. Experimental results validated the efficacy of this strategy, as the inclusion of urea enhanced the extraction efficiency of egg allergens in food from

30% to 70%. Although the denaturing agent SDS can promote protein dissolution, its significant impact on subsequent enzymatic digestion and LC-MS/MS analysis and therefore it was excluded in our study.

Fats, sugars, and salts are major constituents of food, and their removal during preprocessing minimizes interference with subsequent enzymatic digestion. Conventionally, defatting and removal of small molecular substances like salts and sugars are achieved through n-hexane extraction and ultrafiltration. However, adopting these steps would introduce additional complexity and labor intensity to the preprocessing protocol. Remarkably, our study demonstrated that omitting these two steps still met the requirements for sensitivity and stability. Consequently, we excluded them from our preprocessing methodology.

Protein reduction, alkylation, and enzymatic digestion are the most consistent steps in allergen detection using LC-MS (Downs and Johnson 2018; Marzano et al. 2020). These steps are primarily derived from common sample preparation procedures in proteomics research, warranting only minor modifications in our study. In proteomics, the enzymatic digestion system is typically designed with a volume of 500 μL, accounting for the limited availability and cost of samples. Conversely, in food analysis, sample accessibility is not a limitation. Therefore, in our study, we adopted a larger volume of 2 mL for protein solution during reduction, alkylation, and enzymatic digestion, resulting in a total volume of 4 mL for the digestion system. While this larger volume entails increased reagent and trypsin consumption, it enhances the robustness of quantitative results and reduces reliance on the skills of laboratory personnel.

The SPE step offers purification, enrichment, and desalting of peptide solutions following enzymatic digestion, ultimately enhancing sensitivity in mass spectrometry analysis. When selecting

the appropriate SPE cartridge, we initially tested commonly used small-sized SPE cartridges (10 mg/1 mL) employed in proteomics research. However, we observed that some peptides were not adequately retained, possibly due to the large volume of the digestion solution during loading or the high total amount of digested peptides. Consequently, we opted for a SPE cartridge with a higher C18 packing capacity (60 mg/3 mL). Our results demonstrated excellent purification effects, surpassing a tenfold increase in sensitivity compared to the method without SPE treatment.

3.4. Calibration Curve Preparation Strategies and Selection

In the field of quantitative analysis of allergens, the focus is primarily on determining the total protein content of allergenic food rather than specific proteins (Cubero-Leon et al. 2023; Holzhauser et al. 2020). This approach differs significantly from targeted proteomics analysis. However, the direct analysis of allergens using LC-MS presents challenges due to their larger molecular weights. Prior to LC-MS analysis, allergens must undergo enzymatic digestion to form peptide segments. As standardized quantitative analysis methods for allergens using LC-MS are yet to be established, there is a wide diversity in the selection of calibration curve preparation strategies (Johnson and Downs 2019; Xiong et al. 2021).

In this study, three commonly employed strategies were investigated: (1) external calibration, (2) internal calibration, and (3) external calibration with internal standards (ISs). The External calibration involves diluting and enzymatically digesting allergens with known concentrations to prepare calibration curves, while the iInternal calibration utilizes a heavy-labeled peptide of known concentration to calculate the molar concentration of a light-labeled peptide segment, enabling the determination of total protein content through subsequent calculations. To accurately quantify egg allergens in food, the current study systematically explores four types of matrix-matched calibration

curves: Cal. A, synthetic peptide calibrants and ISs; Cal. B, allergen ingredient calibrants and ISs; Cal. C, allergen ingredient calibrants without ISs; and Cal. D, allergen ingredient calibrants and ISs under different matrices.

Figure 3(A) and **3(B)** depict the distribution of measured values and actual values of allergens using different calibration curves at low and high concentrations. From **Figure 3(A)**, it is evident that Cal. B yields the most accurate quantitative results, followed by Cal. D and C, while Cal. A performs the poorest. The primary distinction between Cal. A and the others lies in the choice of calibrants, as it employs synthetic peptides instead of allergen ingredients. Given that the method's target is the total protein content of allergenic food, Cal. A requires conversion factors to translate measured peptide concentrations into total protein concentrations. This approach poses practical challenges due to the need to consider the target protein's molecular weight and proportion in total protein, leading to inherent limitations. Nevertheless, Cal. A remains the most widely used strategy for LC-MS quantification of allergens (Gavage et al. 2020; Johnson and Downs 2019; Pilolli et al. 2021; Planque et al. 2019). However, Cal. A exhibits significantly lower recovery rates (ranging from 10.6% to 25.1%) compared to the actual added values. The low recovery rate is primarily attributed to the use of synthetic peptides as calibrants, which cannot compensate for allergen losses during sample extraction and enzymatic digestion. In reality, protein extraction typically yields only 50-70% of the target protein, and enzymatic digestion efficiency is also not 100%. These losses become more pronounced in highly processed foods, making it challenging to accurately quantify egg allergen content in cakes and biscuits using Cal. A.

Cal. B, C and D exhibit recovery rates ranging from 61.9% to 88.5% for the three tested sample concentrations, with Cal. B demonstrating the best results. The reason for this improvement is that all

three calibration curves employ allergen ingredients as calibrants, enabling determination of allergen concentrations in samples based on external calibration curves without the need for conversion factors (Xiong et al. 2021). Ideally, 5-6 different concentrations of allergen ingredients should be added to blank matrices, subjected to the same thermal processing steps as the test samples, and processed to obtain matrix-matched calibration curve solutions. However, the complexity and diversity of food matrices and processing methods present significant practical challenges for this approach. Consequently, the current study adds a high concentration of allergen ingredients to a similar blank matrix, applies similar thermal processing steps, and selects the resulting blank sample solution for dilution to prepare six different concentrations for calibration curve points. Our study also attempts to spiked allergen ingredients with different concentrations to blank matrices, but the resulting calibration curves exhibit poor performance, with an r2 value often below 0.90, especially for low-concentration additions where signal detection becomes challenging. This aligns with previous research by Chen et al., which suggests difficulties in achieving a uniform distribution of allergens in low-concentration samples (Chen et al. 2021).

The use of stable isotope internal standards enhances the accuracy and robustness of the analysis method. Current options for internal standards primarily include isotopically labeled peptides and proteins. Although SILIC proteins can compensate for pre-processing steps and instrument analysis effects, making them ideal internal standards, their high cost and complex preparation methods limit their practical application (Johnson and Downs 2019; Xiong et al. 2021). Conversely, isotopically labeled peptides are more affordable and easier to synthesize, enabling wider use. However, they only correct matrix effects in mass spectrometry analysis and errors between different instruments, making it challenging to compensate for errors in protein extraction and enzymatic digestion. Nonetheless, the

study results indicate that the addition of ISs significantly improves the recovery rate and accuracy of the method, even when the tested food matrix undergoes certain variations. Cal. C without ISs exhibits poor r2 values and quantification errors significantly greater than those of Cal. B (**Figure 3(C)** and **(D)**), primarily due to the excellent performance of ISs in correcting matrix effects and instrument analysis fluctuations (Xiong et al. 2021). The research findings clearly demonstrate that allergen ingredient calibrants and ISs are the optimal strategy for preparing calibration curves in the LC-MS quantification of egg allergens, and this approach can also be applied to the quantitative analysis of other food allergens.

3.5. Method validation and performance

3.5.1. Selectivity, limits of detection (LOD), quantification (LOQ) and linearity

In this study, we conducted an evaluation of selectivity, LOD, LOQ, and linearity. Ten common allergenic foods and three food ingredients, namely milk, soybean, peanuts, sesame, fish, shrimp, walnuts, hazelnuts, cashews, almonds, wheat, rice, and corn were test to assess the specificity of the developed methods. As a result, no interference signals was observed during the LC-MS analysis, indicating the specificity of the selected peptide sequences found exclusively in egg proteins. Additionally, the selected peptides were well blast using the Uniprot online database. As depicted in **Table 2**, LODs and LOQs were determined based on signal-to-noise ratios of equal to or greater than 3 and 10, respectively.

Although each peptide corresponds to a specific protein, our target analyte for allergen quantification analysis was the total egg protein. Given the prevalent use of egg whites and yolks alone or in combination in the food industry, we separately investigated the performance of LC-MS quantitative analysis for total egg protein in samples containing whole eggs, egg whites, and egg yolks.

Gal d 1-6 peptides were utilized for quantifying total egg protein in each of these components. Due to variations in mass spectrometry signal intensities among different peptide segments, distinct LODs and LOQs were obtained. Detailed information regarding the LODs and LOQs of Gal d 1-6 for quantifying total egg protein in baked goods can be found in **Table 2**.

Our results revealed that the peptide VMVLC[+57]NR, derived from Gal d 1, exhibited the highest sensitivity with an LOQ of 1 mg/kg for egg whites and egg total protein. Similarly, the peptides APFSEVSK and ATAVSLLEWQR, derived from Gal d 5 and 6 in egg yolks, respectively, demonstrated excellent performance with an LOQ of 5 mg/kg. To establish calibration curves for egg total protein, we employed allergen ingredient calibrants and internal standards at seven concentration levels (1, 5, 10, 30, 100, 200, 500 mg/kg) in blank cakes and cookies. These calibration standards met the ±15% criterion, and their correlation coefficients (r2 values) exceeded 0.99, thereby validating the suitability of the matrix-matched calibration curve.

3.5.2. Method accuracy and precision

The accuracy and precision was evaluated by incorporating samples with three different concentrations (5, 20, and 100 mg/kg) of whole eggs, egg whites, and egg yolks into blank cake and cookie matrices, followed by baking and other thermal processing steps. Average recoveries were calculated based on six replicates at each spiked level analyzed by LC-MS (n = 6). Additionally, the relative standard deviations within a batch (RSDv) and between three different days (RSDV) were obtained from eighteen replicates for each spiked level analyzed by LC-MS (n = 3). As presented in **Table 3**, the mean recovery values (n = 6) ranged from 62.4% to 88.5% across the three spiked levels. The RSDv and RSDV values were 3.5-14.2% and 8.2-14.6%, respectively. Notably, VMVLC[+57]NR, derived from Gal d 1, exhibited the highest accuracy and precision, followed by Gal d 2 and 4. On the

other hand, Gal d 3, 5, and 6 displayed inferior performance due to the superior thermal stability of Gal d 1, which is less susceptible to degradation during food processing.

The validation data obtained in this study demonstrate that our developed method meets the performance requirements (SMPRVR 2016.002) for allergen analysis, as specified by the AOAC (New et al. 2020). Furthermore, the quantification limits of our method align with the action levels outlined in the Food Industry Guide to the Voluntary Incidental Trace Allergen Labelling (VITALVR) Program Version 3.0 for egg allergens in foods (Holzhauser et al. 2020). Despite the existence of numerous methods for egg allergen detection, systematic evaluations of these methods remain relatively scarce. The results of our study highlight that the accuracy and precision of the method can be enhanced through the selection of appropriate calibration curves and sample pretreatment methods, offering valuable references for the development of LC-MS quantitative analysis methods targeting other food allergens.

3.6. The application of the developed method to investigate the egg allergen in foods

To evaluate the practicality of the developed method, 26 samples of cake and cookie were purchased from different local market (Beijing, China), in which 11 samples explicitly indicating the presence of egg ingredients. Following homogenization and pre-processing, the samples underwent injection analysis utilizing the developed LC-MS method. Results showed that eggs were detected in all 26 samples (100%). However, there were substantial variations in the concentration of total egg protein among the different samples. In 69% samples (n=18), the concentration exceeded the LOD values, and accurate measurement of egg protein concentration was only achievable after dilution. The protein concentration in the remaining 8 samples ranged from 4.5 to 263.2 mg/kg. Given that these products lacked egg allergen labeling, it is hypothesized that the presence of egg proteins in these

samples may originate from food ingredient contamination or cross-contamination during the production processes.

Significantly, there were notable disparities in the quantitative results of total egg protein based on Gal d 1 and 5 in 14 samples. In nine samples, the concentration measured with Gal d 1 was more than 6.4-10.8 times higher than that obtained with Gal d 5, while in the other five samples, the opposite trend emerged, with the concentration based on Gal d 5 being 5.2-8.7 times higher than that based on Gal d 1. This discrepancy likely arises from contaminated eggs in these samples originating from egg whites or egg yolks rather than whole eggs. Solely relying on Gal d 1 for quantitative analysis would yield considerable deviations from the actual results, leading to overestimation or underestimation of the total egg protein content in samples contaminated with egg whites or egg yolks. In such cases, suitable correction factors must be employed to adjust the measurements of Gal d 1 derived from egg whites and Gal d 5 derived from egg yolks.

In general, egg whites and egg yolks constitute approximately 60% and 40% of the total egg protein. Thus, when significant discrepancies arise in the quantitative results based on Gal d 1 and 5, it implies the likelihood of separate utilization of egg whites and egg yolks during food processing. In such instances, the quantitative result of Gal d 1 can be multiplied by 60% and combined with the quantitative result of Gal d 5 multiplied by 40% to determine the total protein content accurately. This approach enables the precise determination of the total egg protein content in the samples.

In addition to eggs, commonly consumed food items such as milk, soy, peanuts, and wheat often incorporate processed protein components as ingredients to enhance flavor and physical properties. Quantitative analysis relying on a single allergenic protein may result in either an underestimation or an overestimation of the concentration of allergenic proteins present in these foods.

In light of this, the present shows a reliable approach for the precise measurement of total allergenic protein content by analyzing multiple allergenic proteins and incorporating appropriate correction factors. The findings of our work offer an effective method and a valuable reference for the accurate quantification of total allergenic protein in various food products.

4. Conclusion

In this study, we developed and validated an LC-MS/MS method for accurately quantifying egg allergens (Gal d 1-6) in foods. The field of MS-based methods for food allergen detection lacks standardization and expert consensus, posing significant challenges to precise allergen quantitation. While several mass spectrometry-based allergen detection methods have been reported, most lack systematic methodological validation. To address this gap, we carefully selected appropriate reference materials, pre-processing methods, quantitation peptides, and LC-MS detection techniques, and rigorously validated our developed method. Our investigation involved the comparison of four different calibration curves, revealing that the matrix-matched calibration curve employing allergen ingredients as calibrants and stable isotope-labeled peptides as internal standards yielded the most accurate quantitative results. Additionally, we identified VMVLC[+57]NR (Gal d 1) as the optimal quantitation peptide for its outstanding sensitivity and stability, even in processed foods, making it an ideal choice for quantifying whole egg and egg white allergens. Furthermore, we discovered GTDVQAWIR (Gal d 5) as a suitable quantitation peptide specifically for egg yolk allergen quantification. The limits of quantitation ranged from 1-5 mg/kg, while the accuracy fell between 62.4% and 88.5%. The inter-day and intra-day precision values were 3.5-14.2% and 8.2-14.6%, respectively, across three distinct spiked levels in egg-free food. These results underscore the high sensitivity,

accuracy, and reproducibility of our proposed method. Moreover, the proposed method was successfully applied to analyze 26 commercial cake and cookies. These findings demonstrate the suitability of LC-MS as an effective tool for quantifying egg allergens in food, serving as a valuable reference for the future development of mass spectrometry analysis methods targeting other food allergens.

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CRediT authorship contribution statement

Shupeng Yang: Conceptualization, data curation, methodology, investigation, writing the original draft, supervision, conceptualization and funding acquisition. **Jingjing Chen**: data curation, methodology, validation, formal analysis. **Mohamed F. Abdallah**: writing and revising the draft. **Haopeng Lin**, **Peijie Yang**, **Jianxun Li**, **Rong Zhang**, **Qianqian Li** and **Peng Lu:** data curation, formal analysis, writing-review & editing. **Shuyan Liu** and **Yi, Li:** Supervision and 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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Table S1-S3 and Figure S2 were available in Supplementary Material.

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Figure caption:

Figure 1 Graphical overview about the optimization of mass spectrometry based method for protein allergen detection and quantitation.

Figure 2 Egg allergen (Gal d 1-9) abundance distributions using the top 3 most abundant peptides (MS1 peak area) via LC-Q/Orbitrap. (A) The abundance of targeted peptide for the major egg allergies originated from the raw whole egg, egg white, and egg yolk. (B) Change of composition of the major egg allergies under different roasting conditions.

Figure 3 Different calibration curves can significantly affect the accuracy and robustness of food allergen quantification results. The calculated egg total protein accuracy based on four different calibration curves by spiked low (10 mg/kg, A) and high concentration (100 mg/kg, B) whole egg, egg white and egg yolk in egg-free cake. Cal. A, synthetic peptide calibrants and ISs; Cal. B, allergen ingredient calibrants and ISs, Cal. C, allergen ingredient calibrants without ISs, and Cal. D, allergen ingredient calibrants and ISs under different matrices. In addition, the quantitation peptides performance in serial dilutions of egg protein by spiked whole egg, egg white and egg yolk in egg-free cake following the dilution of (A) digests and (B) extracts one by one. The VMVLC[+57]NR (Gal d 1) was served as the quantitation of egg allergies in whole egg and egg white, while GTDVQAWIR was used for egg yolk.

Table 1 The optimized MRM parameters for target light and heavy labeled ($K_{-}{}^{13}C_6{}^{15}N_2$ or $R_{-}{}^{13}C_6{}^{15}N_4$) peptides originated from egg allergen proteins (Gal d 1-6) using LC-MS/MS

Allergen	Peptide sequence	RT (min)	Prec Ion (*m/z*)	Charge state	Prod Ion (*m/z*)	CE (V)	Dwell Time (ms)	Prod Ion ratio
Gal d 1	**VMVLC[+57]NR**[a]	3.56	446.2	2	**661.4 (y5)**[b]	12	20	-
					562.3 (y4)	18	20	2.0
					792.3 (y6)	18	20	10.9
	VMVLC[+57]NR*	3.54	451.2	2	**671.4 (y5)**[b]	12	20	
					572.3 (y4)	18	20	2.1
					802.3 (y6)	18	20	11.2
	DVLVC[+57]NK	2.89	424.2	2	**633.4 (y5)**[b]	14	20	-
					520.3 (y4)	18	20	2.2
					421.3 (y3)	19	20	2.9
Gal d 2	**HIATNAVLFFGR**	4.55	673.3		**1095.6 (y10)**[b]	28	20	-
					1024.3 (y9)	20	20	5.2
					1208.7 (y11)	30	20	3.9
	HIATNAVLFFGR*	4.55	675.3		**1100.6 (y10)**[b]	28	20	-
					1029.3 (y9)	20	20	5.1
					1213.7 (y11)	30	20	4.0
	GGLEPINFQTAADQAR	4.09	844.4	2	**1331.7 (y12)**[b]	36	20	-
					1121.6 (y10)	32	20	1.4
					860.5 (y8)	35	20	1.5
Gal d 3	**YFGYTGALR**	3.86	524.3	2	**737.5 (y7)**[b]	28	20	-
					517.4 (y5)	20	20	6.4
					884.5 (y8)	20	20	9.2
	YFGYTGALR*	3.86	529.3	2	**747.5 (y7)**[b]	28	20	-
					527.4 (y5)	20	20	6.2
					894.5 (y8)	20	20	9.5
	SAGWNIPIGTLLHR	4.02	768.1	2	**906.7 (y8)**[b]	32	20	-
					1133.7 (y10)	34	20	1.1
					1019.7 (y9)	32	20	3.2
Gal d 4	**GTDVQAWIR**	3.92	523.3	2	**545.4 (y4)**[b]	20	20	-
					673.3 (y5)	20	20	1.2
					887.5 (y7)	24	20	3.0
	GTDVQAWIR*	3.92	528.3	2	**683.3 (y5)**[b]	20	20	-
					550.4 (y4)	20	20	1.4
					892.5 (y7)	24	20	3.2
	FESNFNTQATNR	3.26	714.9	2	1152.6 (y10)	28	20	-
					804.4 (y7)	30	20	1.1
					461.4 (y4)	30	20	1.3
Gal d 5	**APFSEVSK**	3.16	432.8	2	**696.4 (y6)**[b]	19	20	-
					549.3 (y5)	21	20	1.1
					793.5 (y7)	19	20	4.1
	APFSEVSK*	3.16	436.8	2	**704.4 (y6)**[b]	19	20	-
					557.3 (y5)	21	20	1.2
					801.5 (y7)	19	20	4.4
	LLINLIK	5.08	413.8	2	**600.4 (y5)**[b]	15	20	-
					713.5 (y6)	15	20	4.6
					487.3 (y4)	15	20	10.8
Gal d 6	**ATAVSLLEWQR**	4.74	637.4	2	**931.4 (y7)**[b]	28	20	-
					731.5 (y5)	20	20	1.2
					618.3 (y4)	18	20	1.4
	ATAVSLLEWQR*	4.74	642.4	2	**941.4 (y7)**[b]	28	20	-
					741.5 (y5)	20	20	1.1
					628.3 (y4)	18	20	1.6
	FIITTR	3.77	375.5	2	**490.4 (y4)**[b]	14	20	-
					377.3 (y3)	19	20	5.5
					603.6 (y5)	19	20	12.4

Note: RT, retention time; Prec Ion, precursor ion; Prod Ion, product ion; CE, collision energy; Prod Ion ratio, the peak area ratio between the quantitation and confirmation ion transition; a, quantitation peptide; b, Quantitation ion; *, the heavy-labeled peptides served as the SIL-IS.

Table 2 Summary of the linearity range, correlation coefficient (r^2), LOD and LOQ for the total egg proteins base on Gal d 1-6 using LC-MS/MS.

Food	Spiked protein	Selectivity and linearity	Gal d 1	Gal d 2	Gal d 3	Gal d 4	Gal d 5	Gal d 6
Cake	Whole egg	Linearity range (mg/kg)	1-500	3-500	5-500	5-500	5-500	5-500
		r2	0.998	0.992	0.995	0.994	0.997	0.991
		LOD (mg/kg)	0.3	1	2	2	2	2
		LOQ (mg/kg)	1	3	5	5	5	5
	Egg white	Linearity range (mg/kg)	1-500	3-500	5-500	5-500	-	-
		r2	0.997	0.995	0.992	0.993	-	-
		LOD (mg/kg)	0.3	1	2	2	-	-
		LOQ (mg/kg)	1	3	5	5	-	-
	Egg yolk	Linearity range (mg/kg)	-	-	-	-	5-500	5-500
		r2	-	-	-	-	0.995	0.992
		LOD (mg/kg)	-	-	-	-	2	2
		LOQ (mg/kg)	-	-	-	-	5	5
Biscuit	Whole egg	Linearity range (mg/kg)	2-500	5-500	5-500	5-500	5-500	5-500
		r2	0.997	0.991	0.993	0.994	0.996	0.992
		LOD (mg/kg)	0.5	2	2	2	2	2
		LOQ (mg/kg)	2	5	5	5	5	5
	Egg white	Linearity range (mg/kg)	2-500	5-500	5-500	5-500	-	-
		r2	0.999	0.993	0.991	0.995	-	-
		LOD (mg/kg)	0.5	2	2	2	-	-
		LOQ (mg/kg)	2	5	5	5	-	-
	Egg yolk	Linearity range (mg/kg)	-	-	-	-	5-500	5-500
		r2	-	-	-	-	0.997	0.995
		LOD (mg/kg)	-	-	-	-	2	2
		LOQ (mg/kg)	-	-	-	-	5	5

Note: In this study, the target substance for quantification was the total protein of eggs, egg white or egg yolk in foods, rather than a specific protein. The allergen ingredient calibrants and ISs was adopted during the quantitative analysis using LC-MS/MS. In addition, the curve was generated by utilizing least square regression analysis with a linear model while incorporating a 1/x^2 weighting. Do not force the regression through zero.

Table 3 Overview of the validation parameters of accuracy and precision for the quantitation of egg allergens in foods using the developed LC-MS/MS method.

Food	Spiked protein	Allergen	Low spiked level (5 mg/kg)			Medium spiked level (20 mg/kg)			High spiked level (100 mg/kg)		
			R_A (%)	RSDv (%)	RSDV (%)	R_A (%)	RSDv (%)	RSDV (%)	R_A (%)	RSDv (%)	RSDV (%)
Cake	Whole egg	Gal d 1	72.5	11.7	13.3	77.2	9.4	9.9	85.6	5.3	8.2
		Gal d 2	70.3	10.5	11.9	68.4	7.8	12.6	74.3	8.4	11.5
		Gal d 3	68.3	8.7	12.4	72.6	11.5	11.9	73.8	10.2	11.7
		Gal d 4	66.7	9.8	11.4	70.4	8.3	9.5	72.0	7.5	9.3
		Gal d 5	69.6	11.3	13.5	66.3	9.5	12.3	68.5	7.3	10.8
		Gal d 6	65.2	12.5	14.8	68.7	7.4	10.3	67.9	8.3	9.6
	Egg white	Gal d 1	71.7	9.2	12.4	78.3	8.2	10.6	88.5	3.6	9.7
		Gal d 2	73.8	11.6	13.5	75.7	10.2	11.8	82.9	7.1	8.4
		Gal d 3	64.6	14.2	14.6	69.4	9.3	12.2	75.6	6.9	12.5
		Gal d 4	67.2	11.8	12.2	73.3	10.7	13.5	80.2	3.5	11.7
	Egg yolk	Gal d 5	63.8	9.7	12.4	74.8	6.4	10.2	78.8	7.3	9.6
		Gal d 6	67.5	11.6	13.5	72.7	10.6	12.8	81.4	8.7	10.8
Biscuit	Whole egg	Gal d 1	71.4	12.3	12.9	80.7	10.3	11.6	87.1	8.6	10.4
		Gal d 2	67.3	9.6	10.2	74.8	11.1	13.8	81.3	7.7	11.8
		Gal d 3	62.8	12.4	14.1	69.4	8.5	12.5	77.2	5.5	9.2
		Gal d 4	66.5	10.2	13.6	73.7	11.3	12.8	74.1	8.4	11.3
		Gal d 5	62.7	13.3	13.8	69.4	9.5	9.8	70.7	8.3	9.5
		Gal d 6	60.8	13.7	14.1	70.4	11.2	13.6	69.7	5.3	11.4
	Egg white	Gal d 1	72.1	11.6	11.9	75.3	6.7	11.2	84.9	8.6	9.1
		Gal d 2	67.8	9.8	12.6	73.6	8.9	10.6	80.3	10.1	12.6
		Gal d 3	62.4	9.5	10.2	66.7	10.5	11.2	73.1	9.6	10.8
		Gal d 4	64.7	8.5	12.1	72.9	8.9	12.7	77.8	7.5	9.5
	Egg yolk	Gal d 5	67.4	11.3	14.2	72.5	12.3	13.6	75.7	9.2	10.7
		Gal d 6	63.7	8.5	10.8	69.5	7.3	9.9	80.8	7.4	9.3

Note：The total proteins of whole egg, egg white and egg yolk were spiked into egg-free cakes or biscuit at three different concentrations, respectively. Additionally, the prepared samples should be made by mimicking the thermal processing of food. R_A refers to the average recovery, which was determined by analyzing samples at various spiked levels six times (n=6); RSDv represents the relative standard deviation for within-day precision, where samples at each spiked level were analyzed three times within a single day (n=18); Similarly, RSDV represents the relative standard deviation for between-day precision, involving the analysis of samples three times over three consecutive days (n=18).

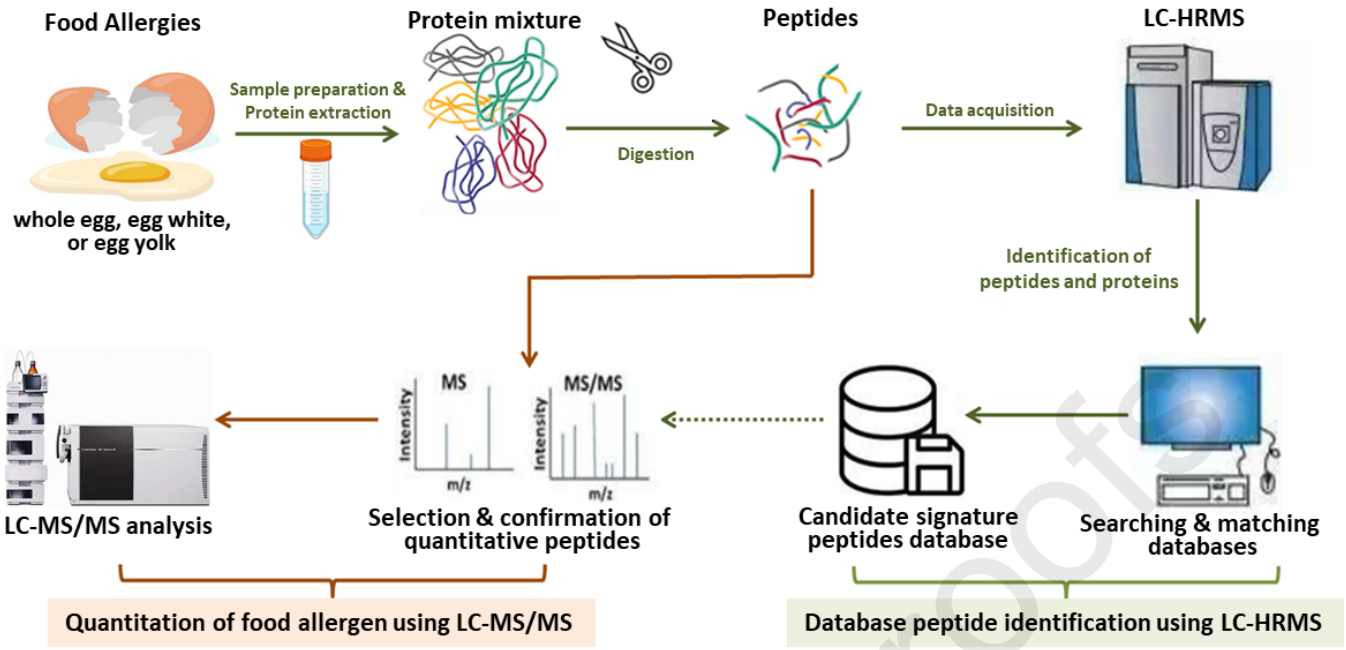

Figure 1

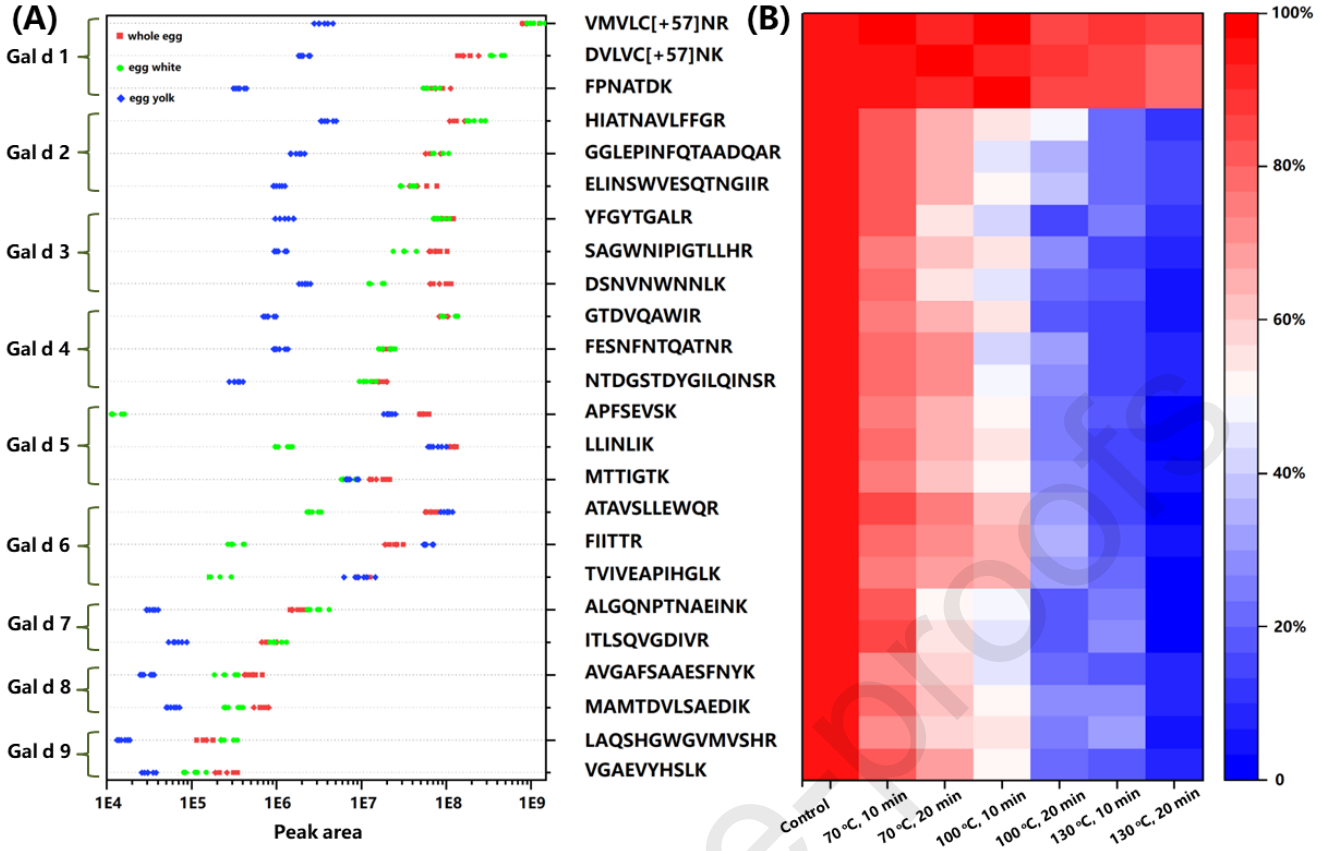

Figure 2

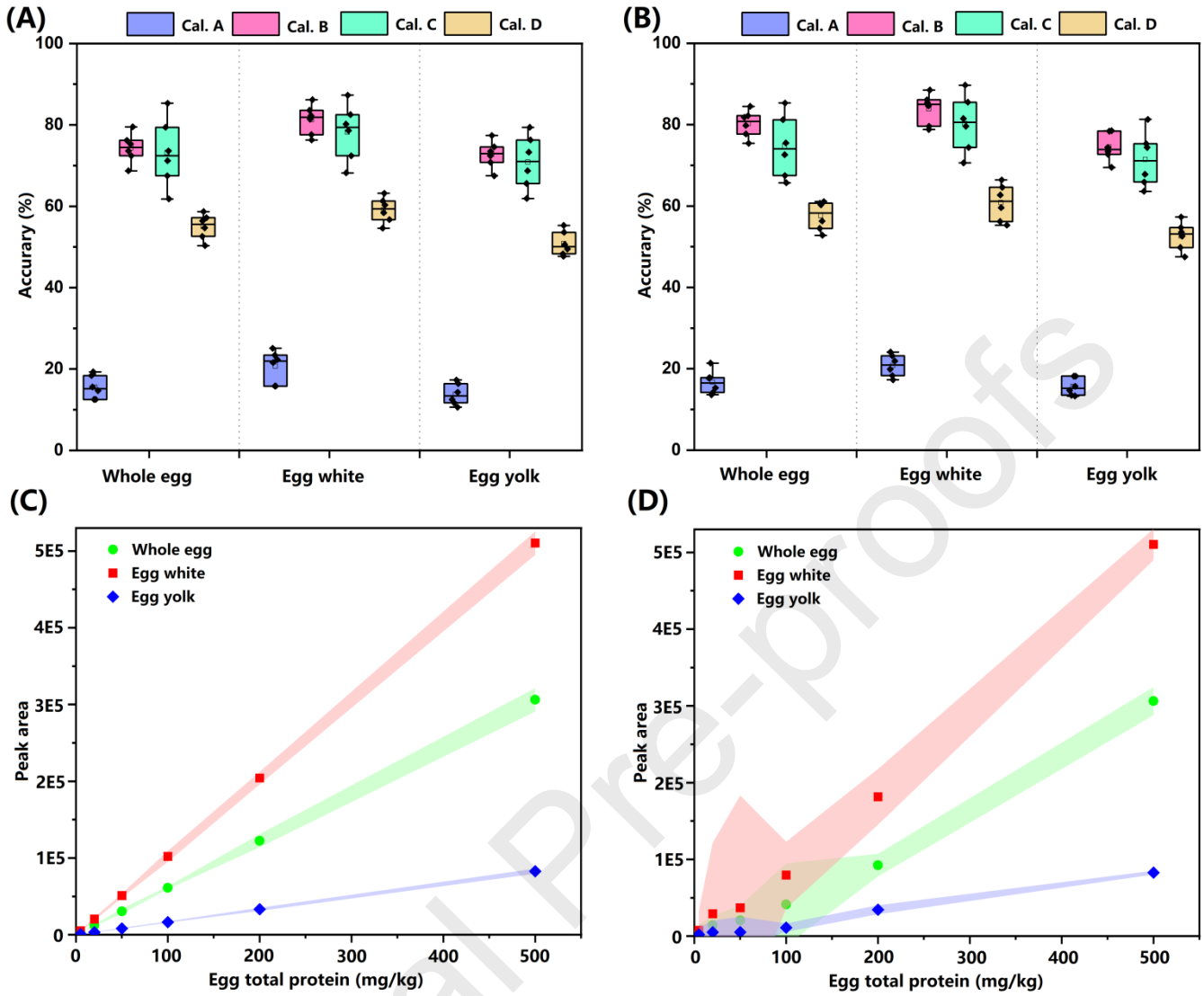

Figure 3

Highlights

- A LC-MS/MS method was developed and validated for the quantification of egg allergens (Gal d 1-6) in foods.
- VMVLC[+57]NR (Gal d 1) and GTDVQAWIR (Gal d 5) were employed for analyzing egg allergens using LC-MS/MS.
- Best practice: employing a matrix-matched calibration curve with allergen ingredients and internal standards for precise food allergen quantification.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Shupeng Yang: Conceptualization, data curation, methodology, investigation, writing the original draft, supervision, conceptualization and funding acquisition. **Jingjing Chen**: data curation, methodology, validation, formal analysis. **Mohamed F. Abdallah**: writing and revising the draft. **Haopeng Lin**, **Peijie Yang**, **Jianxun Li**, **Rong Zhang**, **Qianqian Li** and **Peng Lu:** data curation, formal analysis, writing-review & editing. **Shuyan Liu** and **Yi, Li:** Supervision and funding acquisition.