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Screening of Characteristic Peptide Biomarkers for Edible Locust Allergens by LC-MS/MS

Yijun Pan
Chinese Academy of Quality and Inspection & Testing
Wenhan Kang
Chinese Academy of Quality and Inspection & Testing
Yang Wan
Chinese Academy of Quality and Inspection & Testing
Jiukai Zhang
Chinese Academy of Quality and Inspection & Testing
Xuguang Qiao
Shandong Agricultural University
Ning Yu
Chinese Academy of Quality and Inspection & Testing
Ying Chen
chenyingcaiq@163.com
Chinese Academy of Quality and Inspection & Testing

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Abstract

Driven by the concept of a comprehensive food perspective, edible insect proteins such as locust protein have attracted considerable attention. However, the allergic reactions they trigger have become a critical obstacle to industrialization, highlighting the need for accurate detection methods for locust allergenic proteins. In this study, the East Asian locust (Locusta orientalis) was used as the research material. After protein extraction and enzymatic hydrolysis, five optimal characteristic allergenic peptides were identified based on evaluations of specificity, stability, and N-terminal composition. Meanwhile, Skyline software was applied to optimize MRM detection parameters, laying a foundation for the sensitive and accurate detection of locust allergens using liquid chromatography-mass spectrometry (LC-MS). The identified characteristic peptides can serve as core biomarkers for the quantitative detection of locust allergens in food. This work supports the development of food safety standards and the standardization of allergen labeling, thereby protecting the health and safety of allergic consumers.

1 Introduction

Amid the widespread adoption of the Great Food Concept, new resource food and alternative protein development has become a major research focus (Suh SM et al. 2024; Tata A et al.2022). Edible insects, boasting rich nutritious and controllable in safety, have emerged as a new food source. Insects typically contain 35% to 60% protein, and the Food and Agriculture Organization of the United Nations (FAO) has designated locusts, crickets, and other such insects as key candidates for future food resources (Hasnan FFB et al. 2023; Van Huis A et al.2013). Edible insect consumption has a long history in Africa, Asia, and Latin America: Africans commonly eat shea caterpillars, while Asian nations including Thailand and China have a custom of consuming crickets and silkworm pupae, and Latin America has maintained a tradition of eating locusts since Aztec civilization, a practice that endures to this day (Abril S et al. 2022; Yeh CH et al.2020). However, as a widely consumed insect species, the edible safety of locusts (Locusta migratoria) faces challenges, and the allergenic risk cannot be ignored. Consumption may cause symptoms such as vomiting and diarrhea in allergic individuals, and even induce systemic allergic reactions in severe cases (Scala E et al. 2025; Kim SY et al. 2023). Furthermore, processed locust products have gained widespread market access, yet consumers' poor awareness of insect allergenicity and the lack of relevant regulatory standards have led to a significant underestimation of such risks (Marien A et al. 2025; Aguilar-Toalá JE et al. 2022). Moreover, edible insects, crustaceans such as shrimp and crabs, and dust mites all belong to the phylum Arthropoda, and their allergen molecules contain conserved sequences that are prone to inducing cross-allergic reactions (De Gier S and Verhoeckx K 2018). Identified locust allergens include tropomyosin, arginine kinase, enolase, hexamerin-like protein, and glyceraldehyde-3-phosphate dehydrogenase, establishing an accurate and reliable method for detecting locust allergens is crucial (Gonzalez-Perez R et al. 2025; Phiriyangkul P et al. 2015; Wang Y et al. 2022; Barre A et al. 2021).

Currently, the detection of insect allergens primarily relies on technologies including immunology, molecular biology, and biosensors, yet these traditional approaches suffer from distinct limitations. For instance, enzyme-linked immunosorbent assay (ELISA), an immunological method, is susceptible to interference from antibody cross-reactivity. When crustacean allergen-targeted ELISA is applied to detect samples such as crickets, obvious antibody cross-reactivity occurs in the assay and leads to false-positive results, as both insects and crustaceans belong to the phylum Arthropoda and their allergens share conserved sequences (Awogbindin IO et al. 2023). Polymerase Chain Reaction (PCR) is designed for species detection rather than allergenic protein analysis, and it may produce false-negative results due to DNA degradation. In addition, biosensor technology features simple operation and rapid detection, but it is mostly still in the laboratory research stage, and its stability and repeatability in complex sample matrices need to be further optimized (Emilia M et al. 2025). Among the diverse array of allergen detection approaches, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as an increasingly prevalent gold standard technique in this field, owing to its high specificity and capacity for simultaneous multi-analyte detection.

In recent years, the application of mass spectrometry in insect allergen detection has been extensively explored. For example, Suh SM et al. performed a comparative analysis of LC-MS/MS and real-time fluorescence quantitative PCR for

identifying silkworm allergens, demonstrating that the former reaches a detection sensitivity as low as 0.0005% (2024). Despite these advances, mass spectrometry-based research targeting locust allergens remains in its nascent stage. Current investigations into edible insects are predominantly confined to the determination of basic nutritional constituents, such as crude protein and crude fat, while protein-focused analyses have long stagnated at the level of crude profiling, lacking a standardized workflow for the screening of allergen-specific characteristic peptides (Arp CG and Pasini G 2024; Malla N et al. 2023). To date, only a handful of international research teams have attempted to quantify insect allergens via mass spectrometry, and none have yet succeeded in establishing universally accepted standardized methodologies (Spiric J et al. 2023).

To address the aforementioned industrial bottlenecks, this study developed a precise screening method for locust allergen characteristic peptides by focusing on the identification of locust-specific qualitative and quantitative signature peptides. The experiment used Locusta migratoria manilensis as the test material and screened out characteristic peptide fragments that remained stable after processing by simulating the frying process. After protein extraction and tryptic hydrolysis for peptide fragment preparation, UHPLC-Q-TOF MS was applied to detect and analyze the samples. Following the screening of allergen characteristic peptides with robust processing stability, the present research further optimized core mass spectral parameters such as collision energy and declustering potential. The characteristic peptide markers obtained through this optimized strategy realize the accurate and highly sensitive quantitative detection of locust-derived allergens in processed food matrices. This established detection method not only provides a traceable technical means for regulatory agencies to implement effective allergen risk control in the food industry, but also furnishes essential technical backing for the safe production and standardized industrial development of edible insect-derived food products.

2 Materials and methods

2.1 Reagents and chemicals

The experimental apparatus included 10 μL-1000 μL adjustable pipettes; a vacuum rotary evaporator; a 5910 Ri refrigerated centrifuge (Eppendorf AG, Hamburg, Germany); an ME403E electronic balance (Mettler Toledo Instruments (Shanghai) Co., Ltd., Shanghai, China); brown Eppendorf (EP) tubes (Aisijin Biotechnology (Hangzhou) Co., Ltd., Hangzhou, China); a PowerPac™ Basic Gel Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA); a K38FK613 drying oven (Supor Co., Ltd., China); an HNDSY800 water bath shaker (Wiggens GmbH, Berlin, Germany); a WNB7 7 L electric constant-temperature water bath (Memmert GmbH, Schwabach, Germany); an L12-P726 household blender (Joyoung Electric Co., Ltd., Jinan, China); a Triple-TOF® 6600 high-resolution mass spectrometer (AB Sciex LLC, Framingham, MA, USA); a Nexera X2 high-performance liquid chromatograph (HPLC, Shimadzu Corporation, Kyoto, Japan); and an XBridge® Peptide BEH C18 column (4.6 mm × 150 mm, 3.5 μm particle size, 300 Å pore size, Waters Corporation, Milford, MA, USA).

Ultrapure aqueous solution was sourced from Guangzhou Watsons Food and Beverage Co., Ltd., located in Guangzhou, China. Ammonium bicarbonate (ABC, purity ≥ 98%), urea (purity ≥ 98%), thiourea, sodium hydroxide, sodium phosphate, dithiothreitol (DTT, purity ≥ 98%) and iodoacetamide (IAA, purity ≥ 98%) were all acquired from Sigma-Aldrich Co. LLC in St. Louis, Missouri, USA. Acetonitrile (ACN, MS grade), formic acid (FA, MS grade), acetone, acetic acid, trypsin of chromatography grade, trypsin of MS grade as well as the Qubit™ Protein Assay Kit were procured from Thermo Fisher Scientific Inc. based in Waltham, Massachusetts, USA. Tris-HCl buffer at a concentration of 1.5 mol/L (pH 8.8), Tris-HCl buffer at 1.0 mol/L (pH 6.8), 30% acrylamide solution, ammonium persulfate, protein standard marker and tetramethylethylenediamine (TEMED) were commercial products of Bio-Rad Laboratories, Inc. in Hercules, California, USA. Coomassie Brilliant Blue R-250 was supplied by Shanghai Yuanye Biotechnology Co., Ltd. in Shanghai, China. Hydrophilic filter membranes with a pore size of 0.22 μm were bought from Merck Millipore Ltd. in Darmstadt, Germany, while ultrafiltration centrifuge tubes with a molecular weight cut-off of 5 kDa were obtained from Sartorius AG in Göttingen, Germany.

2.2 Insects and other samples

As depicted in Fig. 1, the East Asian migratory locusts (Locusta migratoria manilensis) utilized in this study were sourced from the Huize Xiaoshan Grasshopper Breeding Base. To verify the specificity of the candidate protein-derived peptides, seven additional samples of commonly consumed edible insects and crustaceans were procured from a local market in Beijing, China. These samples comprised bee larvae, silkworm pupae, cicadas, bamboo worms, mealworms, tiger prawns, and freshwater crabs.

The pretreatment procedures for locust samples are as follows: live locusts were initially frozen at -80°C in an ultra-low temperature refrigerator, subsequently rinsed and blotted dry to remove surface moisture, and then placed in a drying oven until a constant weight was achieved. The dried locust samples were placed in a liquid nitrogen environment and fully ground into powder using a tissue lyser (Qiagen, Hilden, Germany), followed by sieving through an 80-mesh sieve to obtain homogeneous fine locust powder. Acetone solution was added at a solid-liquid ratio of 1:2 (g/mL, locust fine powder: acetone) for defatting treatment. Following 48 hours of defatting, centrifugation was conducted at 12000 r/min for 10 minutes; the supernatant was discarded, and this defatting process was repeated three times to guarantee thorough defatting. Subsequently, the treated locust powder was naturally dried in a fume hood and stored in a -80°C refrigerator for later use.

2.3 Protein extraction

The protein extraction and quantification operations were as follows: 2.5 g of defatted locust powder was weighed, and 25 mL of extraction buffer (containing 7 mol/L urea, 2 mol/L thiourea, pH 9.0) was added. Vortex oscillation was performed for 1 min to fully disperse the powder; after ultrasonic treatment in an ice bath for 30 min, the mixture was placed at -4°C for extraction for 60 min. After the extraction process, the sample was subjected to centrifugation at 4°C and 12000 r/min for 20 minutes; the resulting supernatant was collected and filtered through a 0.22 μm PES filter membrane to prepare the crude protein extract of locusts. The concentration of the extracted protein was determined quantitatively using the Qubit™ Protein Assay Kit, which yielded standardized samples for the subsequent experimental assays.

To separate and analyze the crude locust protein extract, we further performed SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The specific operations were as follows: Gels were cast following Bio-Rad's recommended formulation, with the stacking gel containing a final 5% acrylamide concentration and the separating gel 12%. Quantified protein extracts were diluted to a concentration of 1 mg/mL, combined with 2× loading buffer at a 1:1 v/v ratio, and heat-denatured for 10 min in a 95°C water bath. 10–20 μL of the denatured sample was loaded, and the electrophoresis program was initiated at 4°C: first, electrophoresis was conducted at a constant voltage of 80 V for approximately 20 min; after the protein sample entered the separating gel, the voltage was adjusted to 120 V and constant voltage electrophoresis was continued for approximately 90 min. Electrophoresis ceased when the bromophenol blue indicator reached the bottom of the separating gel. After completing electrophoresis, the gel was placed into Coomassie Brilliant Blue R250 stain and stained at 25°C for 2 h under isothermal conditions; following stain removal, a decolorizing solution (10% acetic acid: 25% methanol: 65% ultrapure water, volume ratio) was applied for the decolorization process. The decolorizing solution was refreshed several times during this period until the gel background was completely colorless and distinct protein bands were visible. The gel was ultimately scanned with a Bio-Rad gel imager to conduct subsequent image analysis.

2.4 Enzymatic digestion

Based on the quantified protein concentration, 200 μL of the crude protein extract was aliquoted into a brown Eppendorf (EP) tube. A volume of 10 μL of 120 mmol/L dithiothreitol (DTT) solution was added to the tube, and the mixture was vortexed thoroughly prior to incubation at 37°C for 1 h to facilitate protein reduction. Subsequently, 10 μL of 600 mmol/L iodoacetamide (IAA) solution was incorporated into the tube, followed by incubation at room temperature in the dark for 15 min to induce protein alkylation. The resultant mixture was transferred to a 5 kDa ultrafiltration centrifugal tube, and 100 μL of 50 mmol/L ammonium bicarbonate (ABC) solution was added; the tube was then centrifuged at 12,000 rpm for 15 min for membrane washing. This washing-centrifugation step was repeated three times until no residual liquid was visible on the ultrafiltration membrane. The waste filtrate in the collection tube was discarded, and the tube was rinsed with ultrapure

water. Subsequently, 100 μL of 50 mmol/L ABC solution and 4 μL of 1 μg/μL trypsin solution were added to the ultrafiltration membrane; the mixture was vortexed to ensure complete homogeneity and then incubated in a 37°C water bath for 10 h to allow for enzymatic digestion.

After proteolytic digestion, the ultrafiltration centrifugal tube was centrifuged at 12,000 rpm for 20 min to allow the digested solution to filter through to the tube's collection bottom. Next, 100 μL of 25 mmol/L ammonium bicarbonate (ABC) buffer was added to the tube, followed by an additional centrifugation step at the same rotational speed for 15 min. This washing procedure was repeated three times, and the peptide-containing filtrate was collected to recover small peptide fragments. The ultrafiltration membrane was then removed, and the collection tube containing the filtrate was transferred to a vacuum rotary evaporator for lyophilization via rotary evaporation. During the drying process, 100 μL of MS-grade water was added to the tube; after complete drying, this rehydration-drying cycle was repeated three times to ensure the thorough removal of residual ammonium bicarbonate. Finally, 100 μL of mobile phase A (composed of 98% ultrapure water, 2% acetonitrile, and 0.1% formic acid, v/v) was added to the dried peptide mixture, and the solution was vortexed vigorously to achieve complete reconstitution. The reconstituted solution was centrifuged at 4°C at 10,000 rpm for 10 minutes, after which 80 μL of the obtained supernatant was carefully pipetted into a liquid chromatography vial and preserved at 4°C for subsequent mass spectrometry analysis.

2.5 HPLC-Q-TOF-MS/MS method development

Peptide fractionation was conducted on an Xbridge Peptide BEH C18 column (4.6 mm × 150 mm, 3.5 μm particle size, 300 Å pore size, Waters Corporation). The separation system was composed of a NanoLC-Ultra 2D plus integrated with a NanoFlex system (Eksigent Technologies, Dublin, USA), and the column temperature was kept constant at 40°C during the entire process.

Eluent composition was set as follows: Mobile phase A consisted of 2% acetonitrile (ACN) and 98% ultrapure water, while mobile phase B was prepared with 98% ACN, 2% water and 0.1% formic acid (FA). The gradient elution procedure was configured as below: 0.0 min, 5% mobile phase B (v/v); 0.5 min, elevated to 8% mobile phase B (v/v); 0.6 min, further increased to 12% mobile phase B (v/v); 25 min, raised to 30% mobile phase B (v/v); 30 min, up to 35% mobile phase B (v/v); 30.5 min, increased to 80% mobile phase B (v/v); 38 min, held constant at 80% mobile phase B (v/v); 38.5 min, reduced to 5% mobile phase B (v/v); 50 min, maintained at 5% mobile phase B (v/v) until the end of the program.

Mass spectrometry analysis was performed using a Triple Time-of-Flight Mass Spectrometer (Triple TOF 6600) system (AB SCIEX, Foster City, USA) equipped with a Nanospray III ion source. Relevant parameters were set as follows: ion spray voltage 2.5 kV, nebulizer gas pressure 6 PSI, curtain gas pressure 30 PSI. Data acquisition employed data-dependent acquisition (DDA) mode. The primary mass spectrum (MS) scan range was 350–1500 m/z, with a precursor ion scan accumulation time of 250 ms. Dynamic exclusion time was set to 20 s, total cycle time to 2.0 s, and collision energy (CE) was enabled.

Secondary mass spectrometry (MS/MS) detection parameters: Product ion scan range set to 100–1500 m/z; DDA trigger threshold set to 120 cps; Precursor ion charge range restricted to 2 + to 5+.

2.6 Analysis and Selection of Target Peptides

2.6.1 Target Peptide Identification

From the UniProt Knowledge Base (UniProt KB), we retrieved five locust allergen-associated proteins: tropomyosin, arginine kinase, enolase, hexamerin-like protein 2 and glyceraldehyde-3-phosphate dehydrogenase. The corresponding amino acid sequences were downloaded as FASTA files and then uploaded to ProteinPilot Software v.5.0 to conduct subsequent analyses.

Based on the respective UniProt accession numbers corresponding to each target protein, namely A6M9J4 (arginine kinase), H8YU84 (glyceraldehyde-3-phosphate dehydrogenase), E0WBM7 (hexamerin-like protein 2), G9C5D8 (enolase), and P31816 (tropomyosin), their FASTA-format sequence files were downloaded separately and imported into ProteinPilot Software v.5.0 to facilitate database construction and peptide matching analyses.

ProteinPilot software was used to analyze the peptides produced by protein hydrolysis, with the software parameters set as follows: Sample type: Identification; Digestion enzyme: Trypsin; Search effort: Rapid ID; ID focus: Biological modifications; Fixed modification: Carbamidomethylation (C); Cysteine alkylation reagent: Iodoacetamide (IAA); Variable modifications: Not specified; Precursor ion tolerance: ± 0.05 Da; MS/MS fragment ion tolerance: 0.03 Da; Maximum allowed missed cleavages: 2. After importing the mass spectrometry data files, the target proteins and their corresponding hydrolysates (peptides) were identified successfully.

2.6.2 Selection of Signature Peptides

On the results interface of ProteinPilot Software v.5.0, peptides with a reliability score below 95% were first excluded from subsequent analysis. Only those peptides with an amino acid residue length of 7 to 24 (i.e., more than 6 and fewer than 25 residues) were retained, which served to ensure the specificity of the target peptides. Furthermore, detection sensitivity is closely correlated with ion transition response intensity. To facilitate subsequent method development for the Q-Trap mass spectrometry system, the mass-to-charge ratio (m/z) of the candidate peptides was further constrained to a value below 1250. Following completion of the above screening criteria, the resultant peptide sequences were subjected to specificity analysis using BLAST software. To verify the processing stability of the screened peptides, they were subjected to deep-frying treatments at three temperatures (120°C, 150°C, and 180°C) with processing durations set at 1, 2, 3, 4, 5, 6, 7, and 8 min, respectively. After the fried samples were cooled to room temperature, they were subjected to sample pretreatment following the protocols described above.

2.7 Establishment of the MRM method

A multiple reaction monitoring (MRM) analytical method was established using high-performance liquid chromatography coupled with quadrupole-tandem mass spectrometry (HPLC-Q-TRAP 5500). Sample separation was conducted on an LC-20 AD XR HPLC system (Shimadzu Corporation, Kyoto, Japan) with an injection volume of 10 μL. The chromatographic separation was performed using a Waters XBridge® Peptide BEH C18 column (4.6 mm × 150 mm, 3.5 μm particle size, 300 Å pore size; Waters Corporation, Milford, MA, USA). The column temperature was maintained at a constant 40°C throughout the analysis, and the mobile phase flow rate was set at 0.4 mL/min.

The mobile phase system composition is as follows: Mobile phase A consists of a 2% acetonitrile (ACN)-98% aqueous solution containing 0.1% formic acid (FA); Mobile phase B consists of a 98% acetonitrile-2% aqueous solution containing 0.1% formic acid. The gradient elution program was set as follows: 0.1-1.0 min, mobile phase B volume fraction maintained at 3%; 1.0–10.0 min, mobile phase B volume fraction increased linearly from 3% to 30%; 10.0–13.0 min, mobile phase B volume fraction increased linearly from 30% to 55%; 13.0-13.1 min: mobile phase B volume percentage rapidly increased to 80%; 13.1–16.0 min: mobile phase B volume percentage maintained at 80%; 16.0-16.1 min: mobile phase B volume percentage decreased to 3%; 16.1–20.0 min: mobile phase B volume percentage maintained at 3%.

Mass spectrometric analysis was conducted using an AB SCIEX Q-TRAP 5500 mass spectrometer (AB SCIEX, USA) equipped with a Turbo V electrospray ionisation (ESI) source. Data acquisition was performed entirely in positive ion scanning mode. The core operational parameters for the ion source were configured as follows: ion source heating temperature regulated at 500°C, spray ionisation voltage set to 4500 V, primary nebuliser gas pressure at 35 psi, secondary nebuliser gas pressure at 65 psi, and curtain gas pressure adjusted to 50 psi. Mass spectrometry detection employed a pre-set multiple reaction monitoring (MRM) scanning mode in positive ion mode. The detection time window was set to 120 seconds, with each mass spectrometry scan duration controlled at 3 seconds.

2.8 Optimization of MRM method parameters

Skyline v.2.5 software was utilized to perform MRM method optimization, enabling the determination of initial DP and collision energy (CE) parameters for peptide fragments. The collision energy optimization gradient was set at CE ± 2, CE ± 4, CE ± 6, CE ± 8, and CE ± 10. Declustering potential was adjusted across 11 gradient levels (20–130 V) at 10 V intervals according to the initial DP value, and the optimal DP and CE parameters for each ion pair identified via this optimization strategy effectively improved the ion transition response intensity of the target peptides.

2.9 Data analysis

High-resolution mass spectrometry and computer technology were applied to acquire raw data that included mass-to-charge ratio (m/z), retention time (RT), and response intensity for trypsin-digested peptides via IDA, with the data subsequently compiled into WIFF files. All experiments were performed in triplicate, and the obtained results were expressed as mean ± standard deviation (SD). Data processing was conducted with IBM SPSS Statistics 21 software; in the figures, different lowercase letters denote statistically significant differences at the level of $P < 0.05$. All figures and tables in this study were constructed using Origin 2021.

3 Results and discussion

3.1 Protein Extraction and SDS-PAGE Analysis of Locust

Locusts are characterized by high protein and fat contents: common species such as Schistocerca gregaria and Locusta migratoria have a crude protein content ranging from 16.6% to 77.3%, with the maximum crude fat content reaching 54.9% (Ahmed I and İnal F 2025). Building on previous research, this study achieved efficient extraction of allergenic proteins through an optimized process combining defatting pretreatment and denaturant solution extraction. The results demonstrated that with a mixed solution of 7 mol·L^{-1} urea and 2 mol·L^{-1} thiourea (pH = 9) serving as the extraction solvent, under the parameters of a solid-to-liquid ratio (mass/volume) of 1:10 (g·mL^{-1}) and ultrasonic-assisted extraction for 30 min, the extraction concentration of allergenic proteins from Locusta migratoria manilensis exceeded 30 mg·mL^{-1}.The mechanism underlying this efficient extraction lies in the synergistic effect of urea and thiourea in disrupting protein spatial structure and improving the yield of hydrophobic proteins, the enhanced protein solubility in an alkaline environment, and the accelerated protein release facilitated by ultrasonic treatment (Li H et al. 2025; Takeya K et al. 2018; Momen S et al. 2021; Dagher SM et al. 2000; Qi L et al. 2025; Rahman MM et al. 2021). Verification via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that the crude extract prepared by the optimal extraction system, after gradient dilution to 1 ~ 5 mg·mL^{-1} and loading of 10 μL for detection, could be separated into approximately 30 bands, including 10 major bands with the highest protein abundance at around 75 kDa (Figure A1). The allergen-related characteristic bands were distributed in the molecular weight ranges of 30 ~ 44 kDa, 47 ~ 50 kDa, 55 ~ 60 kDa, and 80 ~ 130 kDa, which were confirmed to be accurate and reliable through calibration. Additionally, the crude extract exhibited good protein integrity without significant degradation, and 4 mg·mL^{-1} was determined as the optimal loading concentration for SDS-PAGE analysis.

3.2 Screening and Identification of Signature Peptide Biomarkers

3.2.1 Selection of Allergen Proteins and Characteristic Peptides

The precise screening of target proteins constitutes a prerequisite for identifying allergen-derived characteristic peptide biomarkers in edible locusts. First, the UniProt database was utilized to retrieve and download protein-related data annotated as locust allergens from the locust-specific sequence repository. The screening criteria for candidate protein information were defined as follows: proteins must have explicit species origin information, complete amino acid sequences, accurate residue counts, sequence coverage exceeding 90%, and no significant deletions in functional domains. These strict criteria were implemented to ensure the reliability of subsequent peptide prediction analyses. After downloading and saving the qualified protein sequences in FASTA format, the dataset was imported into Protein Pilot v.5.0 software to construct a customized search database. Using this in-house database as the search reference, the raw data

acquired via high-resolution mass spectrometry detection were subjected to database searching for protein identification. This process yielded peptide sequence information corresponding to the proteins in the sample and enabled the preliminary screening of peptides with high matching scores and robust signal intensities.

Following completion of the mass spectrometry analysis, the resultant data were compared against the constructed search library. Specifically, the identification report generated by Protein Pilot software was accessed to extract detailed peptide information from the sample proteolytic hydrolysates. Peptides satisfying the following criteria were further screened as pre-selected characteristic peptides: high mass spectrometry response intensity; amino acid residue length ranging from 6 to 25; mass-to-charge ratio (m/z) below 1250; a confidence level of over 95% for peptide identification; absence of missed cleavage sites; no non-enzymatic cleavage events; and no variable post-translational modifications.

Trypsin was designated as the specific protease for the gel-based digestion of edible locust allergen proteins, primarily owing to its distinctive enzymatic characteristics and well-established advantages in proteomics research. First, trypsin demonstrates stringent substrate specificity, precisely cleaving the carboxyl-terminal peptide bonds of lysine (Lys, K) and arginine (Arg, R) residues with an extremely low non-specific cleavage rate (Olsen JV et al. 2004; Schilling O et al. 2018). This ensures the homogeneity and predictability of the enzymatically generated peptides, thereby laying a robust foundation for the accurate qualitative matching of downstream mass spectrometry data. Second, trypsin exhibits superior stability and high catalytic activity under the standard digestion condition of 37°C, enabling the efficient hydrolysis of denatured locust allergen proteins into peptides with a molecular weight suitable for mass spectrometric analysis (Kiser JZ et al. 2009; Nickerson JL and Doucette AA 2018). This attribute not only enhances the ionization efficiency of peptides in the mass spectrometry ion source but also facilitates the preliminary screening of pre-selected characteristic peptides derived from allergen proteins, thus significantly improving the sensitivity and accuracy of subsequent mass spectrometry detection.

Due to insufficient sequence specificity and weak signal response in myosin and arginine kinase, preliminary screening and review ultimately identified three candidate locust allergen proteins in this study. These primarily include Glyceraldehyde 3-phosphate dehydrogenase (H8YU84), Hexamerin-like protein 2 (E0WBM7), and Enolase (G9C5D8).

Among locust allergen proteins, candidate characteristic peptide segments capable of characterizing each protein were screened using Skyline software, primarily derived from enolase, hexameric-like protein 2, and glyceraldehyde-3-phosphate dehydrogenase. To ensure the accuracy of qualitative detection, restrictions were imposed on the amino acids composing the peptide segments. Characteristic peptides should exclude variable modification amino acids such as methionine and N-terminal glutamine. During actual detection, locust content in processed locust products is reflected by the mass spectrometry response intensity of characteristic peptides. Therefore, ideally, these characteristic peptides should exhibit only one specific mass. The molecular weight and mass-to-charge ratio of modified and unmodified peptides differ, which does not affect targeted qualitative detection. However, the unstable occurrence of modification may impact quantitative accuracy. Among the peptides derived from Hexamerin-like protein 2 (E0WBM7), most sequences contain methionine and have glutamine (Q) at the N-terminus, making them prone to modification. Some peptides exhibit low ion response intensity and suffer from insufficient sequence specificity. Although several peptides fall within the 6–25 amino acid range, they contain unstable residues and are unsuitable as signature peptides. Four peptides were selected as candidates after screening. The peptide corresponding to Glyceraldehyde 3-phosphate dehydrogenase (H8YU84) lacks methionine and does not have glutamine at the N-terminus, with one peptide included as a candidate. For Enolase (G9C5D8), most peptides (e.g., SQWLSMEK) contained methionine (Met) or unstable amino acid residues. After screening, only DALALISEAIEK and LAALYTEFIK met the criteria, thus selected as preliminary candidate peptides for Enolase. After preliminary screening, the sequence lengths of the seven peptides were all within the range of 10–21 amino acids. Among them, LAALYTEFIK contained 10 amino acids; ADGDSLVVNGQK and DALALISEAIEK contained 12 amino acids each; DGIDYGYLAGYNYEK is a peptide consisting of 16 amino acid residues; AVDYNHPVLVGYYPELR comprises 17 amino acid residues; NPILEHGDLHAAGFPFDR has 18 amino acid residues; and VVEFEFDVPNAHFDETFVVHR contains 21 amino acid residues. This length range can not only avoid the problems of easy degradation and insufficient specificity associated with short peptides, but also circumvent the defects of low enzymatic hydrolysis efficiency and poor ionization performance in mass

spectrometry detection observed in long peptides. After identification and alignment using Protein Pilot software, none of these seven peptides exhibited protein modifications or trypsin missed cleavages, and the credibility of their identification all reached 99%. In summary, a total of seven candidate specific peptides corresponding to three allergy-related proteins of locusts were obtained through screening (Table 1), which laid a foundation for the subsequent verification of characteristic peptides of locust allergens.

Table 1
Table of candidate specific peptide information

No.	Allergen	UniProt Accession Number	Candidate signature peptides
1	Hexamerin-like protein 2	E0WBM7	NPILEHGDLHAAGFPFDR
2	Hexamerin-like protein 2	E0WBM7	VVEFEFDVPNAHFDETFVVHR
3	Hexamerin-like protein 2	E0WBM7	AVDYNHPVLVGYYPELR
4	Hexamerin-like protein 2	E0WBM7	DGIDYGYLAGYNYEK
5	Enolase	G9C5D8	DALALISEAIEK
6	Enolase	G9C5D8	LAALYTEFIK
7	Glyceraldehyde-3-phosphate dehydrogenase	H8YU84	ADGDSLVVNGQK

3.2.2 Specific validation of peptides

Among protein detection methodologies relying on mass spectrometry, multiple reaction monitoring (MRM) is universally recognized as the most sensitive analytical technique. This method enables targeted detection of specific ion pairs and has been extensively applied in the field of targeted protein detection in food (Wissing J et al. 2007). Given the incompleteness of species-specific protein databases, candidate specific peptide segments identified through software screening require further validation of their specificity via MRM to mitigate potential impacts from incomplete database coverage. Based on peptide ion pair information and retention times, false-positive mass spectrometry peaks can be eliminated to confirm peptide specificity. Specifically, these target peptides are detectable only in locusts, with no corresponding signals observed in other edible insects or crustaceans. Based on the theoretical information of the screened candidate peptides, ion pair data were generated using Skyline software. To improve the reliability of peptide detection, the top four fragment ions with the highest response intensities were selected for each peptide. The mass spectrometer employed has a maximum detectable m/z value of 1250, thus all precursor ions and their matching product ions exceeding this cut-off were eliminated. If any of the initially selected top four product ions were removed due to this m/z limitation, the vacant positions were sequentially filled with subsequent product ions ranked by their response intensities to ensure a total of four qualified ions per peptide. Detailed ion pair information for each peptide is provided in Table 2.

The extracted ion chromatograms (EICs) of the seven candidate peptides in negative control samples are presented in Fig. 2. Visual inspection of the chromatograms indicated that the target peptides exhibited notably low abundance across all negative control samples: the peak ion response intensity of these peptides was approximately 3000, with the lowest response (around 1000) observed in the cicada pupa samples. This low signal intensity was far below the characteristic response threshold of peptides in positive locust samples, which was fully consistent with the preset screening criteria for candidate specific peptides. It indicated that these peptides would not bind non-specifically to proteins in other closely related edible insects and crustaceans, effectively eliminating the false positive risk caused by cross-reactivity. Based on the above experimental results, these 7 candidate characteristic peptides passed the specificity verification. They exhibited both structural stability and species specificity, and were suitable for the qualitative detection of target locust allergens without interference from matrices of related species. These peptides provide a core basis for the subsequent establishment of an accurate LC-MS/MS-based detection method for locust allergens.

Table 2
Table of specific peptide information

No.	Allergen	Precursor Ion	Product Ion				Candidate signature peptides
1	Hexamerin-like protein 2	669.334	880.431	809.394	738.357	534.267	NPILEHGDLHAAGFPFDR
2	Hexamerin-like protein 2	845.076	1149.569	1002.5	965.461	887.473	VVEFEFDVPNAHFDETFVVHR
3	Hexamerin-like protein 2	669.011	514.298	897.446	677.362	840.425	AVDYNHPVLVGYYPELR
4	Hexamerin-like protein 2	870.891	957.468	1177.552	1120.531	897.399	DGIDYGYLAGYNYEK
5	Enolase	636.856	676.351	789.435	902.519	973.556	DALALISEAIEK
6	Enolase	584.834	984.540	913.503	637.356	1055.577	LAALYTEFIK
7	Glyceraldehyde-3-phosphate dehydrogenase	601.804	844.489	757.457	754.373	1016.537	ADGDSLVVNGQK

Note

A: Bee Pupae; B: Yellow Mealworms; C: Silkworm Pupae; D: Bamboo Caterpillars; E: Cicada Nymphs; F: Litopenaeus vannamei; G: Flower Crabs

3.2.3 Stability verification of peptides

As an edible insect, locusts are typically subjected to thermal processing methods such as deep-frying prior to consumption. High temperatures alter the spatial conformation of allergenic proteins and disrupt the integrity of peptide bonds, resulting in the degradation or destabilization of specific peptide segments (Zhao J et al. 2022). To evaluate the thermal stability of preselected characteristic peptides, locust samples were baked at gradient temperatures of 120℃, 150℃, and 180℃ for durations ranging from 1 to 8 minutes. The candidate peptides were next subjected to multiple reaction monitoring (MRM) analysis, with comparisons made of the changes in mass spectrometry response intensity for seven preselected peptides before and after heat treatment (Fig. 3). The experimental results showed that there was no significant difference in the response intensity of ADGDSLVVNGQK, a hydrolytic peptide of glyceraldehyde-3-phosphate dehydrogenase, before and after heat treatment. This result may be closely related to the amino acid composition and spatial structure of the peptide: the ratio of hydrophobic amino acids to hydrophilic amino acids in its sequence is balanced, and there are no fragile peptide bonds that are easily broken at high temperatures (Kurokawa M et al. 2023). It is not prone to conformational changes or peptide bond cleavage under high temperatures, thus maintaining a stable mass spectrometric response and good stability, and can be used as a characteristic peptide for the locust allergen glyceraldehyde-3-phosphate dehydrogenase. For some peptides, such as DALALISEAIEK, a hydrolytic peptide of enolase, the response intensity only slightly decreased after heating at 150°C for 8 minutes. The reason may be that the local conformation underwent reversible changes under high temperatures, but its core peptide bond structure remained intact and could still be accurately identified by mass spectrometry. This indicates that it has good tolerance to moderate-intensity heat treatment, is suitable for most common locust food processing scenarios, meets the screening requirements for characteristic peptides, and can be included in the candidate range. In contrast, the hexamerin-like protein 2-derived peptide VVEFEFDVPNAHFDETFVVHR exhibited weak response signals after frying and a marked decrease in intensity after baking at 180℃ for 8 minutes, indicating poor processing stability and leading to its exclusion. Similarly, another peptide from hexamerin-like protein 2, DGIDYGYLAGYNYEK, consistently showed weak signals throughout the stability validation and was also discarded. The instability of VVEFEFDVPNAHFDETFVVHR may be related to its long sequence length. Long peptides are more prone to imbalance in intramolecular forces under high-temperature conditions, and their peptide chain backbones

have a larger exposed area, making them more susceptible to oxidation, degradation, and other reactions, which leads to the destruction of peptide integrity (Stevens CA et al. 2017). In contrast, the weak response of DGIDYGYLAGYNYEK may originate from the oxidizable groups in its amino acid composition; these groups are prone to oxidative modification at high temperatures, which affects the efficiency of mass spectrometry ionization and thus results in a weak response signal that cannot meet the detection sensitivity requirements for characteristic peptides. In summary, among the 7 preselected characteristic peptides, LAALYTEFIK and ADGDSLVVNGQK showed the highest response intensities. Except for the two excluded peptides mentioned above, the remaining 5 preselected characteristic peptides all exhibited ideal processing stability.

3.3 Optimization of mass spectrometry parameter

Collision energy (CE) and declustering potential (DP) are critical mass spectrometric parameters required to develop a pre-specified MRM detection method. The response intensity of fragment ions produced during peptide fragmentation varies with collision energy (CE). The influence of CE on fragment ions differs not only between peptides but also among different cleavage sites within the same peptide. As a key parameter governing further fragmentation of parent ions, CE directly determines both the efficiency of parent ion fragmentation and the mass of resulting product ions [28]. By regulating the kinetic energy transfer between parent ions and neutral gas molecules (e.g., argon, nitrogen) in the collision cell, CE affects the accumulation of internal energy in ions and the extent of chemical bond cleavage (Li Y et al. 2024). Declustering potential (DP) shows a positive correlation with the energy transferred to ions. An appropriate DP provides moderate energy to ions, disrupting interactions between ions and solvent molecules. This effectively suppresses cluster ion formation and prevents adsorption of solvent molecules, thereby enhancing the purity and transfer efficiency of target ions and establishing a foundation for subsequent detection. However, excessively high DP values may lead to unintended fragmentation of peptide segments (Pino LK et al. 2020). To establish stable and reproducible detection conditions, collision energy and declustering potential were optimized for 20 ion pairs to determine the optimal CE and DP values for each.

To acquire the initial declustering potential (DP) and collision energy (CE) values, the five signature peptides of locusts were imported into Skyline software, which generated the initial CE values following analysis. Afterwards, the CE values were adjusted at a step size of 2 V over five gradient intervals and set as CE ± 10, CE ± 8, CE ± 6, CE ± 4 and CE ± 2, respectively. In the same manner, the initial DP values were obtained via software analysis and then further optimized within the range of 20 V to 140 V at a step size of 10 V, resulting in a total of 11 DP gradient values. Ultimately, based on the response signal intensity of each ion pair to corresponding characteristic peptide segments, the optimal dissociation potential (DP) and collision energy (CE) parameters for each ion pair were determined.

Based on the liquid chromatography-mass spectrometry (LC-MS) analysis, the peak areas reflecting response intensities were calculated for each optimized CE and DP value across different ion pairs. For example, considering the characteristic peptide NPILEHGDLHAAGFPFDR from hexamerin-like protein 2 with the ion pair 669.334/809.394 (Fig. 4), the optimal CE value was determined as 34.1. Similarly, for the enolase-derived peptide DALALISEAIEK with the ion pair 636.856/789.435 (Fig. 4), the optimal DP value was identified as 110 based on comparison of peak area and corresponding response intensity. Through this experiment, the optimal CE and DP values were established for a total of 20 ion pairs corresponding to five characteristic peptides (Table 3).

Table 3
Optimal CE and DP Values of Candidate Ion Pairs for Specific Peptides of Different Locust Allergen Proteins

No.	Allergen	Peptides	Precursor ion (m/z)	Fragment ions (m/z)	Fragment type	DP (V)	CE (V)	Rt (min)
1	Hexamerin-like protein 2	NPILEHGDLHAAGFPFDR	669.334	880.441	y8	130	32.1	13.21
				809.414	y7	140	34.1	
				738.357	y6	130	30.1	
				534.287	y4	140	34.1	
2	Hexamerin-like protein 2	AVDYNHPVLVGYYPELR	669.011	514.268	y4	130	24.1	13.76
				897.427	y7	140	26.1	
				677.322	y5	130	22.1	
				840.385	y6	130	22.1	
3	Enolase	DALALISEAIEK	636.856	676.321	y6	110	24.2	15.75
				789.405	y7	110	24.2	
				902.509	y8	100	28.2	
				973.516	y9	130	22.2	
4	Enolase	LAALYTEFIK	584.834	984.510	y8	140	21.7	14.95
				913.473	y7	130	21.7	
				637.336	y5	130	23.7	
				1055.547	y9	100	21.7	
5	Glyceraldehyde 3-phosphate dehydrogenase	ADGDSLVVNGQK	601.804	844.469	y8	110	24.5	10.04
				757.437	y7	120	24.5	
				757.353	b8	120	24.5	
				1016.517	y10	120	24.5	

4 Conclusions

As one of the common foodborne allergens, locusts are prone to causing allergic reactions after consumption, posing potential threats to the health of allergic populations. Therefore, establishing a reliable and sensitive method for detecting locust allergens in food is of great practical significance. This study used the Oriental migratory locust (Locusta migratoria manilensis) as the test material and adopted characteristic peptide screening technology, aiming to screen processing-stable characteristic peptides of locust allergens and lay a foundation for constructing a high-sensitivity detection method based on tandem mass spectrometry.In accordance with the screening criteria for amino acid composition, species specificity, and processing stability, five processing-stable characteristic peptides were obtained from three major locust allergenic proteins, namely hexamerin-like protein 2, glyceraldehyde-3-phosphate dehydrogenase, and enolase. Furthermore, 20 groups of qualitative ion pairs suitable for the accurate identification of these three allergenic proteins were screened out, including 8 pairs corresponding to hexamerin-like protein 2, 4 pairs to glyceraldehyde-3-phosphate dehydrogenase, and 8 pairs to enolase. The confirmation of these qualitative ion pairs further ensures the specificity and precision of the established detection method.Notably, significant differences were observed in the high-temperature tolerance of hydrolytic peptides derived from different allergenic proteins, which are closely related to the structural characteristics of the proteins

themselves and the sequence features of the peptides. In subsequent studies, further in-depth exploration of the key amino acid sites in peptide sequences that affect thermal stability can be conducted, providing a more accurate theoretical basis for the precise screening of characteristic peptides.In addition, to further improve the detection response intensity, this study systematically optimized the mass spectrometry parameters associated with the ion pairs of the five characteristic peptides. By determining the stable retention time of each peptide, the declustering potential (DP) and collision energy (CE) under electrospray ionization were adjusted stepwise, and the optimal conditions for the efficient collision-induced dissociation of parent ions into high-response fragment ions were obtained. These optimizations significantly enhanced the response intensity of the target ion pairs.In the establishment of liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection methods, the use of characteristic peptides with high response intensity is crucial for achieving a lower limit of detection. The five characteristic peptides identified in this study not only have good processing stability but also exhibit excellent ion transition efficiency. Accordingly, these peptides can be used to establish a precise LC-MS/MS method for the detection of locust allergenic proteins in food matrices, providing reliable technical support for the risk monitoring and control of food allergens.

Declarations

Author Contribution

Conceptualization, Ying Chen and Ning Yu; investigation, Yijun Pan; writing—original draft preparation, Yijun Pan; writing—review and editing, Ning Yu, Wenhan Kang, Yang Wan, Jiukai Zhang, Xuguang Qiao; all authors have read and agreed to the published version of the manuscript.

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval

This article does not contain any studies with humans or animals.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures



Figure 1

Migratory Locust in East Asia

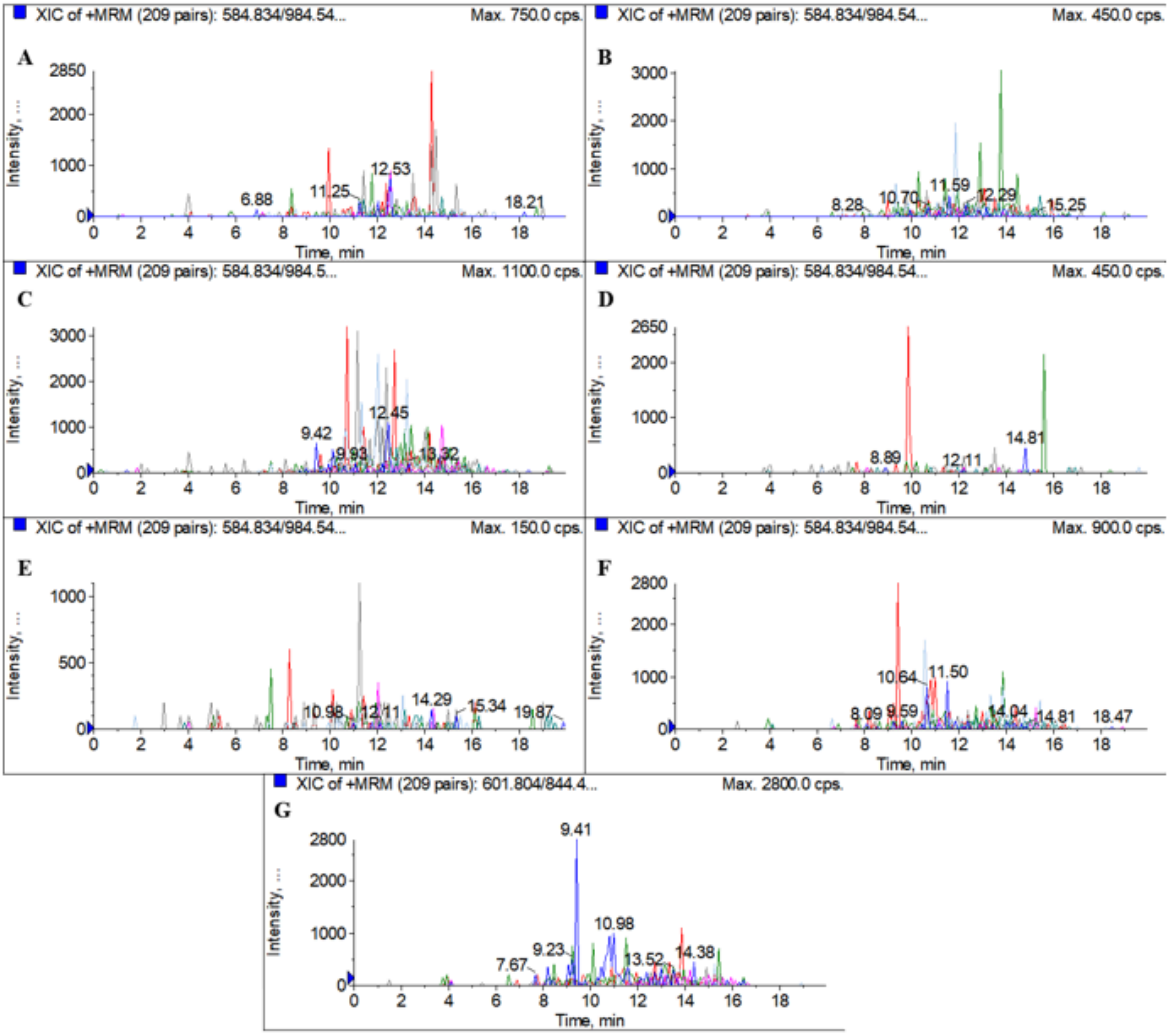


Figure 2

Extracted Ion Chromatogram of Candidate Signature Peptides in Negative Samples

Note: A: Bee Pupae; B: Yellow Mealworms; C: Silkworm Pupae; D: Bamboo Caterpillars; E: Cicada Nymphs; F: Litopenaeus vannamei; G: Flower Crabs

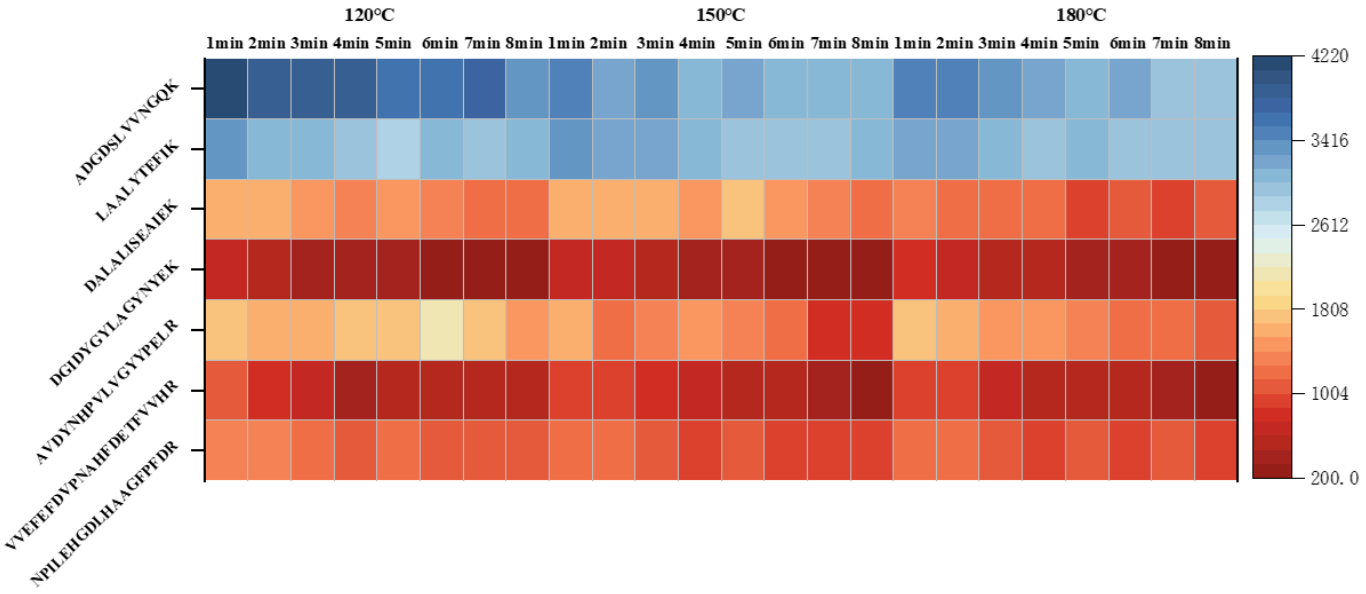


Figure 3

The Effect of Processing Techniques on the Stability of Seven Preselected Characteristic Peptides from Locust Allergens

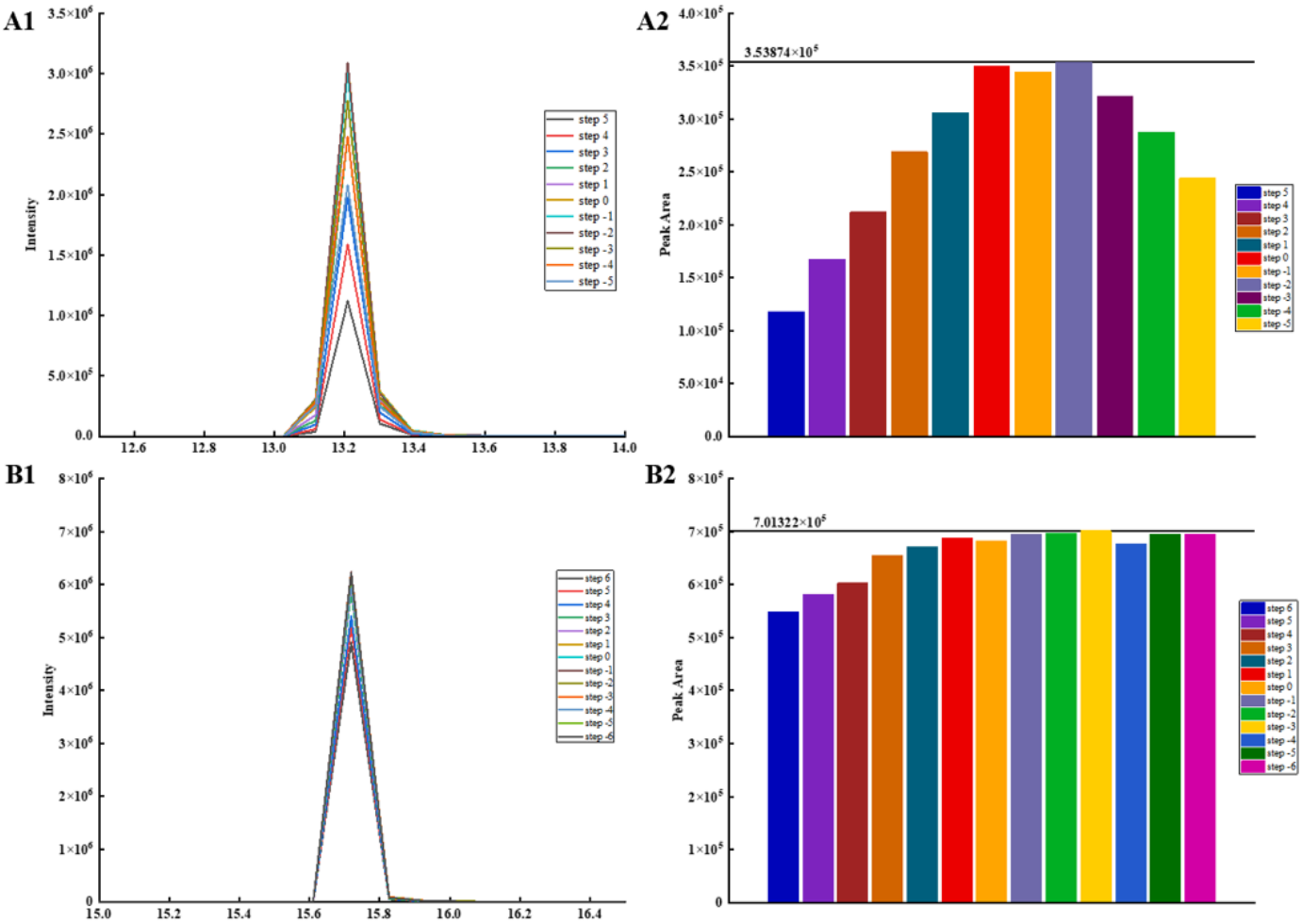


Figure 4

Optimisation of mass spectrometry parameters for characteristic peptides of locust allergen proteins. Optimisation of the CE value for the ion pair 669.334/809.394 of NPILEHGDLHAAGFPFDR; optimisation of the DP value for the ion pair 636.856/789.435 of DALALISEAIEK

Supplementary Files

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- Appendix.docx