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Running title: Quantification of Crustacean Tropomyosin in Foods using HPLC-MS/MS

Quantification of Crustacean Tropomyosin in Foods using High-Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) method

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Abstract

BACKGROUND: Allergic reactions to crustacean products have been increased owing to the rising consumption. Tropomyosin (TM) is the main crustacean allergen with a coiled-coil structure, which showed stability to various food processing methods. Crustacean processed products have been used in several food products, thereby causing greater difficulties in detecting TM in these products. We aimed to develop an assay based on High-Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) for the accurate and reproducible quantification of crustacean TM in foods.

RESULTS: Three peptides of IQLLEEDLER, LAEASQAADSEER, and IVELEEEELR were selected as peptide markers, and the peptide of IVELEEEELR was selected as the quantitative marker. Extraction conditions and enzymatic digestion conditions were completely optimized. The extraction solution of Tris-HCl buffer (50 mM, pH=7.4) containing 1 mol·L⁻¹ KCl and the enzymatic treatment at 1:15 ratio (enzyme/protein, m/m) for 13 h showed excellent efficiency. The method exhibited a good linear relationship with the qualified coefficient of determination ($R^2=0.9994$) in the wide range of 1 to 1000 $\mu\text{g}\cdot\text{L}^{-1}$. The accuracy was validated based on spiked recovery at three spiking levels (12.5, 25.0, and 50.0 $\mu\text{g}\cdot\text{kg}^{-1}$, TM/matrix) in the blank matrices including chicken sausages, beef balls, and egg-milk biscuits. The recoveries ranged from 91% to 109% with qualified relative standard deviations (RSDs) < 15% with the limit of quantification (LOQ) of 1.6 $\text{mg}\cdot\text{kg}^{-1}$ (TM/matrix).

CONCLUSION: The new approach can be used for the qualitative and quantitative detection of crustacean TM in various food matrices.

Keywords: allergen; tropomyosin; HPLC-MS/MS; crustacean; peptides

INTRODUCTION

Crustacean includes a range of species such as shrimps, prawns, crabs, and lobsters, which are among the highly consumed crustaceans. Crustacean allergy is one of the most life-threatening food allergies that affects 0.2% of people worldwide, among them crab and shrimp are the species causing the most frequent reactions.^{1,2} Tropomyosin (TM) is the major allergen identified in crustaceans having two identical subunits with molecular masses ranging from 35 kDa to 38 kDa. TM showed stability to various food processing methods, such as high-temperature processing, pasteurization, and grinding.³⁻⁶ Crustacean products pose serious concerns for patients allergic to TM.^{7,8} However, the performance of TM detecting methods would be influenced by food processing operations. Therefore, a simple, sensitive, and accurate method was intended to develop that can identify and quantify these processed TM in food products.

Allergen-containing foods may not have an allergy precautionary label that indicating the possible presence of the undeclared allergen due to analytical challenges.⁹ Enzyme-linked immunosorbent assay (ELISA) and real-time quantitative polymerase chain reaction (RT-qPCR) method have been widely used to quantify allergens in food matrices.¹⁰⁻¹⁴ ELISA method is based on the principle of specific binding of antibodies and allergens and is the most commonly used method in routine laboratories. However, this method may give false positive or negative results due to the changes in epitopes upon binding to the antibodies during the food processing.¹⁰⁻¹³ Polymerase chain reaction (PCR) cannot directly detect allergenic proteins and differentiate the source of allergens (i.e., DNA from chicken and egg are the

same).¹⁴ The development of high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) methods have obvious advantages of excellent accuracy, sensitivity, specificity, and reproducibility. The proteomics analysis of allergens has been found useful for allergen detection in processed food products.¹⁵⁻²²

Bottom-up analysis by HPLC-MS/MS has been widely investigated to detect multiple allergens in foods, such as milk,¹⁵ eggs,¹⁶ fish,¹⁷ crustacean,^{18,19} nuts,²⁰ wheat, peanuts,²¹ and soybeans.²² Allergens in food samples are extracted and tryptic digested to peptides. The proper peptides will be selected as surrogates of proteins of interest. LC-MS/MS analysis for the detection of TM in crustaceans has been probed by Nagai et al.¹⁹ Six peptides of ADTLEQQNK, IQLLEEDLER, LAEASQAADER, SLSDEER, FLAEEADR, and IVELEELR were selected for detection, where FLAEEADR was used for quantification only depending on the intensity. This approach could discriminate the TM from different species among fish, crustaceans, mollusks, and insects, thus avoid false positive which is superior to ELISA in this regard. However, it is not enough to choose the quantitative peptide just on the basis of its sensitivity. The extraction and digestion protocols of TM are necessary to be optimized the validation of established LC-MS/MS needs to be conducted especially to apply in various food matrices.

In this study, a stable HPLC-MS/MS method for the quantification of crustacean TM in foods has been developed. Novel marker peptides were selected combined with bioinformatics software simulation, high-resolution mass spectrometry (HRMS) identification, and basic local alignment search tool (BLAST) analysis. The extraction and tryptic digestion were optimized

for the best performance of LC-MS/MS. The established method was completely validated via linearity, LOQ, accuracy, and application in other food matrices.

MATERIAL AND METHODS

Reagents, standards, and samples

Acetonitrile (LC-MS grade) and methanol (LC-MS grade) were purchased from Merck (Darmstadt, Germany). Formic acid (FA), ammonium bicarbonate (NH_4HCO_3), and Tris were purchased from Sigma Aldrich (St. Louis, USA). Potassium chloride (KCl) was purchased from MACKLIN (Shanghai, China). Sequencing grade modified trypsin was purchased from Roche Diagnostics (Indianapolis, USA) and Strata-X SPE column (30 mg/1 mL, 30 μm) was purchased from Phenomenex (Aschaffenburg, Germany).

The peptide markers (95% purity) were chemically synthesized by Sangon Biotech (Shanghai, China). Stock peptides solutions were prepared on a weight basis in formic acid-water (1:100, v/v) at $1\text{ mg}\cdot\text{mL}^{-1}$ and stored at $-20\text{ }^\circ\text{C}$ for future usage.

Penaeus vannamei was purchased from the local market of Qingdao, China. Prawn crackers, shrimp flakes, spicy shrimp biscuits, shrimp balls, salmon sausages, beef balls, chicken sausages, egg-milk biscuits, and fish balls were purchased from the local supermarket (Qingdao, China).

Purification of TM and determination of the purity of TM by SDS-PAGE

TM was extracted and purified from *Penaeus vannamei* according to the method described earlier.²³ Briefly, an acetone powder of myofibrillar proteins was prepared from shrimp muscle

and re-suspended in the Tris-HCl buffer. After isoelectric precipitation (pH 4.5) and salt extraction (50% ammonium sulfate saturation), TM precipitate was obtained from myofibrillar proteins solution. Dialysis membrane (Solarbio, China) was used to dialyze the aqueous solution of TM to remove the salt ions for 24 hours, and the dialysate water was changed every 6 hours. Then the dialyzed TM solution was freeze-dried into powder and stored at -20 °C before use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli et al.²⁴ Samples were separated by 15% gel using SDS-PAGE Gel Kit (Solarbio, China) and stained with Coomassie brilliant blue G250 (Solarbio, China).

Screening of peptide markers segments by LC-HRMS

Pretreatment

10 μL of purified TM working solution ($1\text{ mg}\cdot\text{mL}^{-1}$ in water) and 20 μL of sequencing grade modified trypsin solution ($0.1\text{ mg}\cdot\text{mL}^{-1}$ in 50 mM ammonium bicarbonate) were added in 1.5 mL microtubes with 168 μL of water. Samples were incubated in a water bath at 37 °C for at least 16 h to allow complete digestion. The proteolysis reaction was terminated by adding 2 μL of FA directly used for LC-HRMS.

LC-HRMS analysis

Chromatographic separation was achieved with a Dionex UltiMate 3000 UHPLC+ system (Thermo Scientific, San Jose, CA, USA) equipped with an Agilent Poroshell 120 EC-C18 analytical column (100 mm $\times$ 3 mm, 2.7 μm). The mobile phase for the ESI+ detection consisted of 0.1% (v/v) FA in water (solvent A) and 0.1% (v/v) FA in acetonitrile (solvent B).

The following gradient was used: 0 min, 3% B; 15 min, 30% B; 18 min, 100% B; 22 min, 100% B; 22.1, min 3% B; 25 min, 3% B. The LC flow rate was 0.3 mL·min⁻¹, the column temperature was set at 35 °C and the injection volume was 5 µL.

Mass spectrometry analysis was carried out using a Q Exactive Focus spectrometer (Thermo Scientific, San Jose, CA, USA) and the instrument was operated using heated electrospray ionization (HESI) for positive ionization of the compounds. The aux gas heater temperature was 300 °C, the capillary temperature was 320 °C, and the electrospray voltage for positive mode was set at 3500 V. The data acquired by full MS scan mode with a resolving power of 70000 full widths at half maximum (FWHM, m/z 200), the scan range was set 400–1400 m/z for positive modes. The dd-MS² confirmation mode used a resolving power of 17500 FWHM (m/z 200) and the isolation window was 2.0 m/z with an AGC target of 2e5 and the Max IT of 50 ms. The collision energy of quadrupole ranged between 10 and 30 eV.

Data processing

Data were processed with MaxQuant software.²⁵ The amino acid sequence of *Penaeus vannamei* TM (Accession number: B4YAH6, <http://www.uniprot.org>, accessed on 2020 December 22) was used as the database; the digestion mode was trypsin; the trypsin missed cleavage was zero, and the peptide length was between 8 and 25 amino acids.

Detection of TM in food sample using HPLC-TQMS

Extraction

The food samples were crushed in a homogenizer, and then 1.0 g was weighed into a 50 mL centrifuge tube. Next, 10 mL of protein extraction solution (Tris-HCl buffer (50 mM, pH=7.4)

with 1 mol·L⁻¹ KCl) were added. Tubes were placed into the HS501 vibration shaker (IKA, Germany) and shook for 30 min, then centrifuged for 30 min at 20700×g and 4 °C using a CR21G high-speed refrigerated centrifuge (Hitachi, Japan). To purify thermostable TM, 5 mL of the supernatant was transferred into a 15 mL centrifuge tube then heated at 100 °C in a water bath for 5 min. Subsequently, extracts were centrifuged at 20700×g and 4 °C for 30 min, and the supernatant was used for tryptic digestion. Protein concentrations were measured by the BCA protein assay kit (Solarbio, USA), with three replicates at each level.

Tryptic digestion

Trypsin was used to digest the aliquot of the extract of samples referring to the literature.²⁶ Sample extract (100 µL) and water (900 µL) were added into the 2 mL tube and digested with a certain amount of sequencing grade modified trypsin (1 mg·mL⁻¹ in 50 mM NH₄HCO₃) with 1:15 of enzyme/protein ratio (m/m). The proteins were digested to peptides at 37 °C and 13h. At last, the proteolysis reaction was terminated by adding 10 µL of formic acid (FA).

The digested samples were loaded on a Strata-X SPE column which had previously been activated and equilibrated with 1 mL methanol (MeOH) and 1 mL of 1% FA. Next, the sample was washed with 1 mL of 5% MeOH/1% FA and eluted with 1 mL of 90% MeOH/10% FA in proper order. The eluent was collected in a 1.5 mL tube, dried by nitrogen (40 °C), and resuspended in 500 µL of 0.1% FA.

Liquid chromatography-tandem quadrupole mass spectrometer (LC-MS/MS) analysis

For the LC analysis, an Agilent 1200 HPLC system (Agilent, USA) with a binary pump, an autosampler, column, and solvents were consistent with LC-HRMS. The flow was set at 0.3

mL·min⁻¹, and the following gradient was used: 0 min 10% B, 4 min 25% B, 10 min 30% B, 10.1 min 100% B, 12 min 100% B, 14 min 10% B, and 17 min 10% B.

For the mass spectrometric analysis, a 6430 QQQ MS/MS system (Agilent Technologies, Palo Alto, CA, USA) equipped with an electrospray ionization (ESI) source was used. Peptides were detected in positive ion mode using the Multiple Reaction Monitoring (MRM) mode. The MS conditions were as follows: drying gas temperature 300 °C, drying gas flow 12.0 L·min⁻¹, nebulizing pressure 40 psi, and capillary voltage 3500 V for positive mode. Nitrogen was served as the nebulizer and collision gas. MassHunter Data Acquisition Qualitative Analysis and Quantitative Analysis software (Agilent Technologies, Palo Alto, CA, v.B.03) were used for method development and data acquisition.

Data processing

TM concentration ($C_{(TM)}$) was calculated by quantitative peptide concentration ($C_{(IVE\ Peptide)}$) in enzymatic hydrolysate as equation (1), and this equation was used to sample analysis and recovery tests.

$$C_{(TM)} = D \times C_{(IVE\ peptide)} \times \frac{M_{(TM)}}{M_{(IVE\ peptide)}} \times V \div m \quad (1)$$

In the equation, D stood for multiples of sample extract diluted during enzymatic hydrolysis and purification, and $D=5$. $M_{(TM)}$ stood for the relative molecular mass of TM, $M_{(TM)}$ of *Penaeus vannamei* is 36000 g·mol⁻¹. $M_{(IVE\ Peptide)}$ stood for the relative molecular mass of quantitative peptide, and $M_{(IVE\ Peptide)}= 1129.3$ g·mol⁻¹. V stood for the volume of protein extraction solution, and $V=10$ mL. m stood for the mass of the sample for extracting, and $m=1$

g. So, for *Penaeus vannamei* TM, $C_{(TM)}=1.5939C_{(IVE \text{ peptide})}$. The higher concentration of IVELEEELR (peak), the higher content of TM.

RESULTS AND DISCUSSION

Selection of marker peptides

We firstly selected peptide marker candidates via a bioinformatics approach. To select TM peptides markers that could detect all crustacean TMs correctly, a sequence alignment of TMs from 35 crustaceans (shrimp, squilla, crawfish, lobster, and crab) (Table 1) that were available in the UniProtKB database was conducted using DNAMAN software. Results indicated that the most conserved region is the amino acid sequence between the residues 80 to 215, with a homology of up to 98.52% across species.²⁷ A highly conserved sequence provides a theoretical basis for the simultaneous detection of TM from different crustaceans in various food products. According to the rules, the proper peptide markers should meet the following requirements: 8 to 25 amino acids, no internal arginine or lysine residues, no posttranslational modifications, hydrophobicity, species specialty, and high signal intensity.^{21,22} Peptides of IQLLEEDLER, LAEASQAADER, FLAEEADR, IVELEEEL, and SLEVSEEK were found after the in-silico digestion of the most conserved region using the Skyline v 2.6 (Fig.1).

To compare the score and intensity of peptides, the *Penaeus vannamei* TM was purified, tryptic digested, and analyzed by LC-HRMS. The higher the score and intensity, the more identified ion fragments of peptides.²⁸ For protein quantification, at least three peptides are supposed to be used, where the most sensitive peptide is selected as the quantitative peptide, others are qualitative peptides. As shown in Table 2, the intensities of IVELEEELR,

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QLEEDLER, LAESQAADER were higher than others, where the peptide of IVEEEELR was most sensitive. Thus, IVEEEELR was suggested to be used for TM quantification, others used for qualitative detection. Nagai et al.¹⁹ choose peptides of ADLEQQNK, QLEEDLER, LAESQAADER, SLEDER, FLAEEADR, and IVEEEELR as the specific peptides and FLAEEADR was used as the quantitative (target) peptide because of the higher intensity. However, the intensity of IVEEEELR is much higher than the peptide of FLAEEADR in our study (Table 2).

For the selection of the marker peptides, the uniqueness and mass abundance of peptides should be considered simultaneously.²⁹ To determine the unique feature of the three marker peptides, they were analyzed by the Basic Local Alignment Search Tool (BLAST) in the UniProtKB database. The results showed that these peptides existed not only in crustaceans, but also in insects, arachnoidea, and mollusks. Insects and arachnoidea are not used for food purposes, therefore, mollusks were given priority consideration for the cross-reaction. The search results of the three marker peptides in cephalopod and mollusks were shown in Table 3. From the results, peptides of IVEEEELR and LAESQAADER were unique for the detection of crustacean in foods, and could further discriminate most of Mollusca. Besides, the established method also could be used in TM detection of insects, arachnoidea, and mollusks.

Optimization of the extraction conditions

The TM to be detected in the actual sample was existed in the form of crustacean muscle and very homologous across different crustaceans. Therefore, the extraction and decontamination pretreatment protocols were optimized with *Penaeus vannamei* as a reference substance.

Extraction of TM from the muscle of *Penaeus vannamei* using protein extraction solution (PES) was optimized. The composition of different PES was indicated as PES1 to PES7, and the concentration of extracted proteins was calculated by BCA kit. As shown in Table 4, the protein concentrations using PES1 and PES3 were much higher than the results of PES2 and PES4. To enhance the extraction efficiency, we increased the concentration of potassium chloride (KCl) in PES5, PES6, PES7. Results showed KCl plays a key role in protein extraction since protein solubility in salt solutions is the main function of the interaction between salt and protein. KCl as a salting-out electrolyte can hydrate proteins, thus, the magnitude of the salt and protein interaction can be expanded by an increase in the ionic strength of the solution.³⁰ Even though the PES7 could extract more proteins, there is a minor distinction with PES6. Considering the cost-effectiveness, the solution contains 20 mM Tris-HCL with 1M KCL (PES6) was selected as the best extraction buffer.

Optimization of the tryptic digestion conditions

Few reports optimized the tryptic digestion of TM when detected using LC-MS/MS. The degree of digestion of proteins will seriously impair the accuracy of LC-MRM-MS/MS.^{31,32} The purified *Penaeus vannamei* TM with 97% purity was used for the enzymatic optimization (Fig.2). Enzyme-to-substrate ratio and enzymatic hydrolysis time are the key factors for tryptic digestion. The extent of digestion was assessed by quantifying the released marker peptides using LC-MS/MS with three replicates at each level.

The enzyme-to-substrate ratio is the primary indicator of complete digestion, commonly used from 1:1 to 1:100.³³⁻³⁵ In the present study, enzyme-to-protein ratios (m/m) including 1:30,

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1:15, 1:10, 2:15, 1:6, 1:5, and 1:3 were analyzed with the incubation time of 16 h (Fig. 2A). When the ratio is 1:30, the enzymatic rates of LAEASQAADER, IVELEEELR, and IQLLEEDLER were 96%, 108%, and 27%, respectively. With the increase of enzyme/protein ratio, IVELEEELR relative response didn't show significant change, whereas IQLLEEDLER relative response increased gradually. The peptide of LAEASQAADER seemed more susceptible to trypsin and decreased gradually. The enzyme-to-protein ratio of 1:15 was adopted in our assay by comprehensively considering the variation trend of three peptides. Furtherly, the incubation times at 1 h, 3 h, 5 h, 7 h, 9 h, 11 h, 13 h, and 16 h were optimized with the enzyme-to-protein of 1:15, as shown in Fig. 2B. Three peptides simultaneously reached enzymatic equilibrium at the time of 13 h. Taken together, the tryptic digestion protocol for TM was conducted by employing 1:15 of the enzyme-to-protein ratio and a 13 h incubation time.

Besides, even though peptides of LAEASQAADER and IVELEEELR reached a higher level of enzymatic hydrolysis (close to 100%), significantly, IVELEEELR was more tolerant of the changes of enzyme-to-protein ratios than LAEASQAADER. Therefore, IVELEEELR was the best choice of the quantitative peptide, and LAEASQAADER and IQLLEEDLER could be used as qualitative peptides.

Validation by HPLC-MS/MS method

The parameters including linearity, accuracy, precision, and sensitivity of the established method were validated by detecting quantitative peptide of IVELEEELR. The retention time and MS/MS parameters were summarized in Table 5. The linearity of peptides was evaluated

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at a wide concentrations range: 1, 5, 10, 20, 50, 100, 200, 400, and 1000 $\mu\text{g}\cdot\text{L}^{-1}$. The equation of IVELEEEELR linearity of HPLC-MS/MS response (y) with IVELEEEELR concentration (x) was $y=1.1586x+425.3614$ with a 0.9994 coefficient of determination. The accuracy of this method was validated by detecting recovery after spiking tropomyosin into the blank matrices including chicken sausages, beef balls, and egg-milk biscuits. As shown in Table 6, the mean recoveries of tropomyosin with 12.5, 25.0, and 50.0 $\mu\text{g}\cdot\text{kg}^{-1}$ spiked levels ranged from 91% to 109%, with RSD values below 15%. The results indicated that the method we established has good accuracy and precision, which could meet the requirements of TM quantification in various food matrices. Furthermore, the loss rate of three proposed peptides exhibited a minor variation range from 7%-11% indicated that the matrix interference is weak to TM detection from the above-described foodstuffs. The sensitivity was assessed via measuring LOQ based on a signal-to-noise (S/N) ratio of 10:1. The LOQ of this method was 1.6 $\text{mg}\cdot\text{kg}^{-1}$ (TM/matrix). The sensitivity of the LC-MS/MS method is based on the quality of the mass spectrometer and the removal rate of impurities.

Application in commercial food products

The proposed method was applied to detect TM in common commercial food products. As shown in Table 7, there are no TM peptides in chicken sausage, beef balls, and egg-milk biscuits which were used as blank matrices. The TM content of *Penaeus vannamei* was 5251.4 $\mu\text{g}\cdot\text{g}^{-1}$, and the chromatogram and mass spectrum are shown in Fig. 3A. The concentrations of TM in prawn crackers, spicy shrimp biscuits, and shrimp balls were 6.7, 1.7, 1566.8 $\mu\text{g}\cdot\text{g}^{-1}$, respectively. Allergens were not detected in salmon sausages and the food production line

might contain crustaceans. However, the peptide of IQLLEEDLER was detected in the sample of fishballs with a minor peak, while peptides of LAEASQAADSEER and IVELEEEELR were not found (Fig. 3B). The fishballs may be contaminated by squid and/or cuttlefish not crustacean TM. The LC-MS/MS method was further demonstrated to have the advantage of distinguishing the TM from different species. In summary, the present method could be used to quantify TM in different food matrices.

CONCLUSIONS

A simple, accurate, and precise HPLC-MS/MS method was developed to directly quantify the major crustacean allergen-tropomyosin in foodstuffs. The novel peptide of IVELEEEELR was selected as the quantitative peptide marker due to the higher intensity and stabler state. The extraction and enzymatic treatment were completely optimized to assure the TM had been completely extracted and tryptic digested properly. Then the method was validated at sensitivity, accuracy, precision, and application. In conclusion, this method could be successfully applied in the qualitative and quantitative determination of crustacean TM in various foodstuffs.

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CONFLICTS OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

REFERENCES

1. Khan MU, Ahmed I, Lin H, Li Z, Costa J, Mafra I, Chen Y and Wu YN, Potential efficacy of processing technologies for mitigating crustacean allergenicity. *Crit Rev Food Sci Nutr* **59**:2807–2830 (2019).
2. Sicherer SH, Epidemiology of food allergy. *J Allergy Clin Immunol* **127**:594–602 (2011).
3. Lopata A and Lehrer L, Chemical and biological properties of food allergens. In: J. Jedrychowski J, Wichers HJ Ed. Seafood allergen overview: focus on crustacean. Boca Raton: Crc Press/ Taylor and Francis Group. p. 233–257 (2010).
4. Li Z, Lin H, Cao L and Jameel K, Effect of high intensity ultrasound on the allergenicity of shrimp. *J Zhejiang Univ-Sci* **7**:251-256 (2006).
5. Ahmed I, Lin H, Xu L, Li S, Costa J, Mafra I, Chen G, Gao X and Li Z, Immunomodulatory effect of laccase/caffeic acid and transglutaminase in alleviating shrimp tropomyosin (Met e 1) allergenicity. *J Agric Food Chem* **68**:7765–7778 (2020).
6. Ahmed I, Lv L, Lin H, Li Z, Ma J, Chen G, Sun L and Xu L, Effect of tyrosinase-aided crosslinking on the IgE binding potential and conformational structure of shrimp (*Metapenaeus ensis*) tropomyosin. *Food Chem* **248**:287–295 (2018).
7. Liu G, Cheng H, Nesbit JB, Su W, Cao M and Maleki SJ, Effects of boiling on the IgE-binding properties of tropomyosin of shrimp (*Litopenaeus vannamei*). *J Food Sci* **75**:1-5 (2010).

8. Nakamura A, Sasaki F, Watanabe K, Ojima T, Ahn D and Saeki H, Changes in Allergenicity and Digestibility of Squid Tropomyosin during the Maillard Reaction with Ribose. *J Agric Food Chem* **54**:9529-9534 (2006).
9. Watanabe H, Saita K, Akaboshi C, Ohsawa N, Hashiguchi S and Miyazawa M, Detection of allergenic substances (shrimp, crab) in processed seafood. *Food Hyg Saf Sci* **55**:41-54 (2014).
10. Fu T and Maks N, Impact of thermal processing on ELISA detection of peanut allergens. *J Agric Food Chem* **61**:5649-5658 (2013).
11. Parker CH, Khuda SE, Pereira M, Ross MM, Fu TJ, Fan X, Wu Y, Williams KM, Devries JW, Pulvermacher B, Bedford B, Zhang X and Jackson LS, Multi-allergen quantitation and the impact of thermal treatment in industry-processed baked goods by ELISA and liquid chromatography-tandem mass spectrometry. *J Agric Food Chem* **63**:10669-10680 (2015).
12. Poms R, Agazzi ME, Bau A, Brohee M, Capelletti C, Norgaard JV and Anklam E, Inter-laboratory validation study of five commercial ELISA test kits for the determination of peanut proteins in biscuits and dark chocolate. *Food Addit Contam Part A-Chem Anal Control Expo Risk Assess* **22**:104-112 (2005).
13. Verhoeckx K, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S, Herouetguicheney C, Holzhauser T, Shimojo R, Van Der Bolt N, Wichers HJ and Kimber I, Food processing and allergenicity. *Food Chem Toxicol* **80**:223-240 (2015).
14. Eischeid AC, Kim B and Kasko SM, Two quantitative real-time PCR Assays for the detection of penaeid shrimp and blue crab, crustacean shellfish allergens. *J Agric Food*

Chem **61**:5669-5674 (2013).

15. Yang W, Liqing W, Fei D, Bin Y, Yi Y and Jing W, Development of an SI-traceable HPLC–isotope dilution mass spectrometry method to quantify β -lactoglobulin in milk powders. *J Agric Food Chem* **62**:3073-3080 (2014).
16. Monaci L, Losito I, De Angelis E, Pilolli R and Visconti A, Multi-allergen quantification of fining-related egg and milk proteins in white wines by high-resolution mass spectrometry. *Rapid Commun Mass Spectrom* **27**:2009-2018 (2013).
17. Carrera M, Canas B and Gallardo JM, Rapid direct detection of the major fish allergen, parvalbumin, by selected MS/MS ion monitoring mass spectrometry. *J Proteomics* **75**:3211-3220 (2012).
18. Korte R, Monneuse JM, Gemrot E, Metton I, Humpf HU and Brockmeyer J, New high-performance liquid chromatography coupled mass spectrometry method for the detection of lobster and shrimp allergens in food samples via multiple reaction monitoring and multiple reaction monitoring cubed. *J Agric Food Chem* **64**:6219-6227 (2016).
19. Nagai H, Minatani T and Goto K, Development of a method for crustacean allergens using liquid chromatography/tandem mass spectrometry. *J AOAC Inter* **98**:1355-1365 (2015).
20. Korte R and Brockmeyer J, MRM³-based LC-MS multi-method for the detection and quantification of nut allergens. *Anal Bioanal Chem* **408**:7845-7855 (2016).
21. Huschek G, Bonick J, Lowenstein Y, Sievers S and Rawel HM, Quantification of allergenic plant traces in baked products by targeted proteomics using isotope marked peptides. *LWT - Food Sci Technol* **74**:286-293 (2016).

22. Hoffmann B, Münch S, Schwägele F, Neusüß C and Jira W, A sensitive HPLC-MS/MS screening method for the simultaneous detection of lupine, pea, and soy proteins in meat products. *Food Contr* **71**:200-209 (2017).
23. Huang M and Ochiai Y, Fish fast skeletal muscle tropomyosins show species-specific thermal stability. *Compar Biochem Physiol B* **141**:461-471 (2005).
24. Laemmli UK, Cleavage of structural protein during the assembly of the head of bacterio phase T4. *Nature* **227**:265-275 (1970).
25. Cox J and Mann M, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnol* **26**:1367-1372 (2008).
26. Montowska M and Pospiech E, Species-specific expression of various proteins in meat tissue: Proteomic analysis of raw and cooked meat and meat products made from beef, pork and selected poultry species. *Food Chem* **136**:1461-1469 (2013).
27. Zhang Y, Matsuo H and Morita E, Cross-reactivity among shrimp, crab and scallops in a patient with a seafood allergy. *J Dermatol* **33**:174-177 (2006).
28. Olsen JV and Mann M, Improved peptide identification in proteomics by two consecutive stages of mass spectrometric fragmentation. *PNAS* **101**:13417-13422 (2004).
29. Johnson PE, Baumgartner S, Aldick T, Bessant C, Giosafatto V, Heick J, Mamone G, O'Connor G, Poms R, Popping B, Reuter A, Ulberth F, Watson A, Monaci L and Mills ENC, Current perspectives and recommendations for the development of mass spectrometry methods for the determination of allergens in foods. *J AOAC Int* **94**:1026-

1033 (2011).

30. Oomah BD, Mazza G and Cui W, Optimization of protein extraction from flaxseed meal.

Food Res Int **27**:355-361 (1994).

31. Burkhardt JM, Schumbrutzki C, Wortelkamp S, Sickmann A and Zahedi RP, Systematic and quantitative comparison of digest efficiency and specificity reveals the impact of trypsin quality on MS-based proteomics. *J Proteomics* **75**:1454-1462 (2012).

32. Norrgran J, Williams TL, Woolfitt AR, Solano MI, Pirkle JL and Barr JR, Optimization of digestion parameters for protein quantification. *Anal Biochem* **393**:48-55 (2009).

33. Sun L, Lin H, Li Z, Sun W, Wang J, Wu H, Ge M, Ahmed I and Pavase TR, Development of a method for the quantification of fish major allergen parvalbumin in food matrix via liquid chromatography-tandem mass spectrometry with multiple reaction monitoring. *Food Chem* **276**:358-365 (2019).

34. Smith BJ, Protein Sequencing Protocols. *Methods in Molecular Biology*TM 211 (2003).

35. Suder P, Bierczynska A, Konig S and Silberring J, Acid-labile surfactant assists in-solution digestion of proteins resistant to enzymatic attack. *Rapid Commun Mass Spectrom* **18**:822–824 (2004).

TABLES

Table 1. The 35 species of crustacean TMs (16 species of shrimp TMs, 3 types of squilla TMs, 2 types of crawfish TMs, 4 kinds of lobster TMs and 10 kinds of crab TMs) were analyzed for sequence alignment.

Crustacean	Scientific name	TM in UniProKB
Shrimp	<i>Penaeus monodon</i> , <i>Marsupenaeus japonicus</i> , <i>Pandalus</i>	A1KYZ2, A2V731, A2V732,
	<i>eous</i> , <i>Euphausia superba</i> , <i>Euphausia pacifica</i> , <i>Penaeus</i>	A7VKE0, A7VKE1, B4YAH6,
	<i>vannamei</i> , <i>Fenneropenaeus chinensis</i> , <i>Macrobrachium</i>	D2KMW0, D3XNR9, D3XNS0,
	<i>rosenbergii</i> , <i>Fenneropenaeus merguiensis</i> , <i>Crangon</i>	D7F1J4, E5BBS3, M4M2H6,
	<i>crangon</i> , <i>Pandalus borealis</i> , <i>Melicertus latisulcatus</i> ,	Q25456, Q3Y8M6, W5RFX7,
	<i>Metapenaeus ensis</i> , <i>Farfantepenaeus aztecus</i> , <i>Thenus</i>	W6EKR0
	<i>orientalis</i> , <i>Macrobrachium nipponense</i>	
Squilla	<i>Squilla aculeate</i> , <i>Squilla oratoria</i> , <i>Oratosquilla oratoria</i>	A5D6I3, A5JVN3, A9CSJ9
Crawfish	<i>Procambarus clarkii</i> , <i>Cherax destructor</i>	C0LU07, W5RFX0
Lobster	<i>Jasus edwardsii</i> , <i>Jasus lalandii</i> , <i>Homarus americanus</i> ,	W5RFZ5, K9UTQ8, O44119,
	<i>Panulirus stimpsoni</i>	O61379
Crab	<i>Scylla olivacea</i> , <i>Portunus sanguinolentus</i> , <i>Paralithodes</i>	A1KYZ3, A1YYV6, A2V733,
	<i>camtschaticus</i> , <i>Chionoecetes opilio</i> , <i>Eriocheir sinensis</i> ,	A2V735, A2V736, A4URH3,
	<i>Eriocheir sinensis</i> , <i>Scylla serrata</i> , <i>Portunus</i>	A7L5V2, A7L5V3 M1H607,
	<i>trituberculatus</i> , <i>Portunus pelagicus</i> , <i>Charybdis feriata</i>	Q9N2R3

Table 2. Marker peptides screening with LC-HRMS.

Amino acids	Peptides sequence	Score	Intensity
190-198	IVELEEEELR	147	9.04×10^8
92-101	IQLLEEDLER	211	4.77×10^8
113-125	LAEASQAADER	281	3.52×10^8
153-160	FLAEEADR	136	3.26×10^8
206-213	SLEVSEELR	106	2.85×10^8

Table 3. The three marker peptides cross-reaction with proteins from mollusca by BLAST.

Peptide	Mollusca
IQLLEEDLER	<i>Biomphalaria glabrata</i> , <i>Helix aspersa</i> , <i>Pinctada fucata</i> , <i>Sinonovacula constricta</i> , <i>Sepioteuthis lessoniana</i> , <i>Ommastrephes bartramii</i> , <i>Sepia esculenta</i> , <i>Todarodes pacificus</i> , <i>Octopus vulgaris</i> , <i>Neptunea polycostata</i> , <i>Arion vulgaris</i> , <i>Heterololigo bleekeri</i> , <i>Sepia officinalis</i> , <i>Octopus bimaculoides</i>
LAEASQAADER	<i>Sinonovacula constricta</i>
IVELEEEELR	<i>Sinonovacula constricta</i>

Table 4. The extraction solution (PES) was optimized on the basis of the concentration (TPC)

of total proteins.

PES	Tris-HCL (pH 7.4)	DTT	KCL	TPC (mg/mL)
1	20 mM	0.1 mM	0.15 M	1.40±6%
2	20 mM	0.1 mM		1.10±2%
3	20 mM		0.15 M	1.50±4%
4	20 mM			1.13±5%
5	20 mM		0.5M	1.49±5%
6	20 mM		1M	1.63±3%
7	20 mM		1.5M	1.64±5%

Table 5. Retention time and MS/MS conditions for marker peptides.

Peptide	RT (min)	Precursor ion	Mr (g/mol)	Fragment (V)	Quantification transition	Identification transition	CE (eV)	Ion ratio (%)
LAE	2.12	688.8	1376.4	90	688.8>777.4	688.8>706.4	15, 15	88.1
IVE	6.10	565.3	1129.3	90	565.3>917.4	565.3>788.4	10, 15	53.8
IQL	6.56	629.4	1257.4	120	629.4>1016.4	629.4>903.4	15, 15	69.3

Table 6. Determination of TM recovery in chicken sausages, fish balls, and egg-milk biscuits by LC-MS/MS.

	TM concentration	IVE in enzymatic	Concentration of TM ($\mu\text{g/g}$)	Mean recovery, % (RSD, %) (n=6)
	addition ($\mu\text{g/g}$)	hydrolysate ($\mu\text{g/L}$)		
Chicken Sausages	12.5	7.3	11.6	93(8)
	25.0	14.8	23.6	94(3)
	50.0	28.5	45.5	91(1)
Beef balls	12.5	8.6	13.7	109(4)
	25.0	17.3	27.5	110(1)
	50.0	29.0	46.2	92(2)
Egg milk biscuits	12.5	8.2	13.09	105(2)
	25.0	17.3	27.6	110(1)
	50.0	30.4	48.4	97(15)

Table 7. The detection of crustacean TM in market samples.

Market samples	Ingredient	Detection	TM ($\mu\text{g/g}$)
<i>Penaeus vannamei</i>	<i>Penaeus vannamei</i>	Yes	5251.4
prawn crackers	shrimp	Yes	6.7
spicy shrimp biscuits	shrimp	Yes	1.7
Shrimp balls	shrimp	Yes	1566.8
salmon sausages	salmon	No	
beef balls	no allergens	No	
chicken sausages	no allergens	No	
egg and milk biscuits	no allergens	No	

Figure Legends

Figure 1. Simulated enzymatic hydrolysis of the residues 80 to 215 of crustacean's TM. The bold peptides were considered as marker peptides.

Figure 2. The optimization of enzyme-to-protein ratios (A) and enzymolysis times (B) on digestion efficiency.

Figure 3. The total ion Chromatogram of target peptides from *Penaeus vannamei* (A) and fishballs (B).

Figure 1.

NAEGEVAALNR	R	IQLLEEDLER	SEER	LNTATTK	LAEASQAADER	MR
80-90	91	92-101	102-105	106-112	113-125	126-127
K	VLENR	SLSDEER	MDALENQLK	EAR	FLAEEADR	K
128-129	133	134-140	141-149	150-152	153-160	161
LAMVEADLER	AEER	AETGESK	IVELEELR	VVGNNLK	SLEVSEEK	AN
169-178	179-182	183-188	189	190-198	199-205	206-213
						214-215

Figure 2.

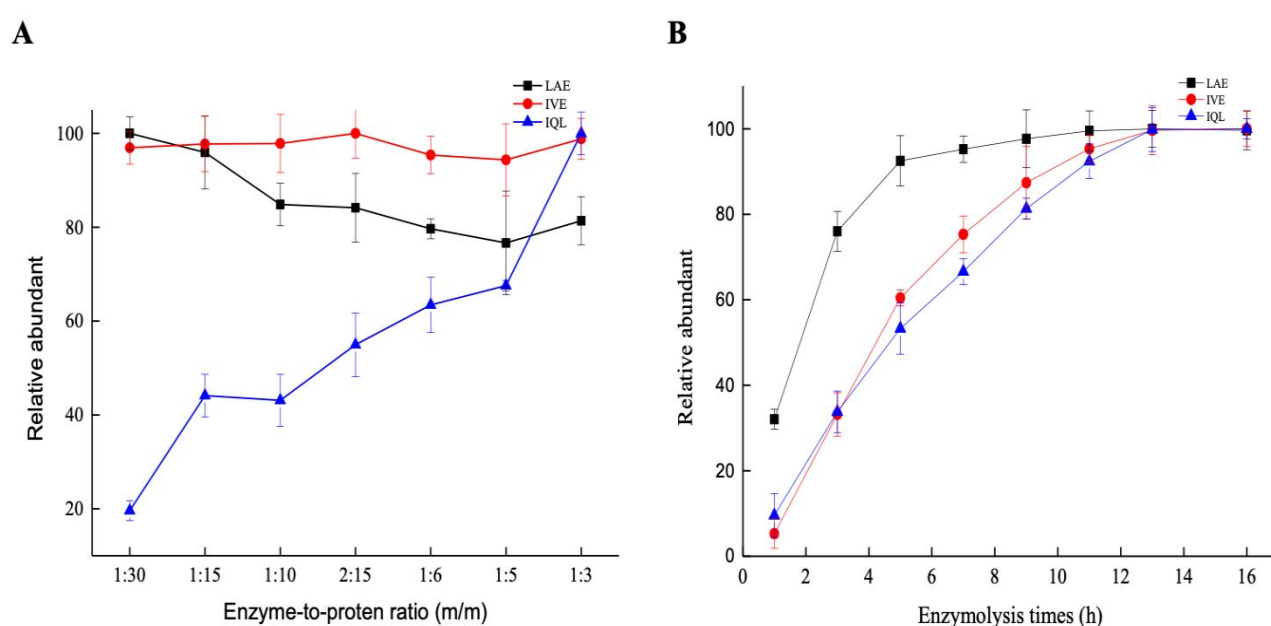


Figure 3.

