

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

Lirui Sun^a, Hong Lin^a, Zhenxing Li^{a,*}, Weihong Sun^b, Jianhua Wang^c, Haiyan Wu^b, Minmin Ge^c, Ishfaq Ahmed^a, Tushar Ramesh Pavase^a

^a College of Food Science and Engineering, Ocean University of China, No. 5, Yushan Road, Qingdao, Shandong Province 266003, People's Republic of China

^b Key Laboratory of Testing and Evaluation for Aquatic Product Safety and Quality, Ministry of Agriculture; Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, People's Republic of China

^c Technical Center of Inspection and Quarantine, Shandong Entry-Exit Inspection and Quarantine Bureau 266002, People's Republic of China

ARTICLE INFO

Chemical compounds studied in this article:

Tris (PubChem CID:6503)
Glycine (PubChem CID:750)
Dithiothreitol (PubChem CID:446094)
Iodoacetamide (PubChem CID: 3727)
Ammonium bicarbonate (PubChem CID:14013)
Formic acid (PubChem CID:284)
Acetonitrile (PubChem CID:6342)
Trypsin (PubChem CID:33613)

Keywords:

Allergen
Parvalbumin
Liquid chromatography
Mass spectrometry
Quantification

ABSTRACT

The availability of analytical methods for quantification of allergens is crucial for the correct assessment and labeling of products in order to protect allergic consumers. For this purpose, a simple, sensitive and accurate technique was developed based on liquid chromatography-tandem mass spectrometry and multiple reaction monitoring mass spectrometry (LC-MRM-MS/MS). The proposed method uses a simple purification with heat and a completely optimized tryptic digestion. This method has been validated according to the requirements defined by ICH (Q2 [R1]), having a linear range from 0.10 to 1179.36 nM with $r > 0.999$. The parvalbumin beta in flounder (*Paralichthys olivaceus*) has been quantified at a low level down to 0.10 µg/g with satisfactory precision (RSD < 18.35%) and accuracy (< 13.3%). The new approach was successfully applied for the determination of parvalbumin beta in the other food matrices.

1. Introduction

Currently, food allergies have become one of the main concerns in the food safety sector. For sensitized individuals, a small amount of allergens could cause allergy symptoms ranging from minor (e.g. dermatitis, nausea, vomiting...) to severe (e.g. anaphylactic shock). However, there is no effective measure but to assure the total exclusion of allergens from the diet of sensitive individuals.

To guarantee the security of the allergenic customers, numerous regulations regarding food allergenic components have been enforced (Commission directive 2000/13/EC, 2000; Commission Directive 2007/68/EC, 2007; Regulation (EU) No 1169/2011, 2011; US Food and Drug Administration, Food Allergen Labeling and Consumer Protection

Act, 2004). In the European Union (EU), the labeling legislation entails a mandatory declaration of the existence of 14 main allergenic foods including fish and its products in the labels (Regulation (EU) No 1169/2011, 2011). Usually, the warning applies in ambiguous and inconsistent manners (e.g. “may contain, be free of...”), and lack the sufficient interpretation of precautionary labels, thereby misleading the customers. Sometimes, the ignorance and negligence of allergenic peoples also lead to severe allergic reactions (Ballmer-Weber et al., 2015). The new terminology “reference doses” of allergenic foods, a basis for the definition of impending threshold values, has been brought up in recent years (Ballmer-Weber et al., 2015; Heinonen, 2014; Taylor et al., 2014). The reference dose of fish protein was reported to be 27.3 mg of protein, likely provoking allergic reactions in 10% of the

Abbreviations: PV, Parvalbumin; HPLC-MS, High performance liquid chromatography-tandem mass spectrometry; MRM, Multiple reaction monitoring; VITAL, Voluntary Incidental Trace Allergen Labeling; ELISA, Enzyme-linked immunosorbent assay; RT-qPCR, Real-time quantitative polymerase chain reaction; RSD, Relative standard deviation; LOD, Limit of detection; LOQ, Limit of quantification; BCA, Bicinchonininc acid; CE, Collision energy; DP, Decluster potential

* Corresponding author.

E-mail address: lizhenxing@ouc.edu.cn (Z. Li).

<https://doi.org/10.1016/j.foodchem.2018.10.014>

Received 16 April 2018; Received in revised form 11 September 2018; Accepted 2 October 2018

Available online 03 October 2018

0308-8146/ © 2018 Published by Elsevier Ltd.

sensitized individuals (Ballmer-Weber et al., 2015). As a consequence, considering the regulations and allergen threshold levels discussed above, it is necessary to establish accurate, sensitive and simple methods to detect traces of allergenic substances in the food and its products.

To date, enzyme-linked immunosorbent assay (ELISA) and real-time quantitative polymerase chain reaction (RT-qPCR) are widely used in routine laboratories to detect allergens in food (Jayasena et al., 2015; Khuda et al., 2012; Sathe, Teuber, & Roux, 2005; Verhoeckx et al., 2015). The ELISA method, which is based on the interaction of an allergen with its antibody, is the most commonly used method because of its sensitivity, operability and potential for standardization (Parker et al., 2015). But this method has significant drawbacks in terms of reproducibility and specificity. The qualification results from the same sample are not identical between commercial ELISA kit from different manufacturers, especially for the analysis of thermally processed foods (Poms et al., 2005). The interference of matrix components target allergen's epitopes which may be modified to be not detected during food processing, resulting in false-negative results (Fu & Maks, 2013; Jayasena et al., 2015; Khuda et al., 2012; Sathe et al., 2005; Verhoeckx et al., 2015). The RT-qPCR method is established using the specific DNA sequence of the allergenic protein as the biomarker (Eischeid, Kim, & Kasko, 2013). With regards to the characteristics of DNA (i.e., heat stable, specificity etc.), this method is less affected by heat processing and has a low chance of cross-reactions than protein-based method. Yet DNA will be separated and degraded during food production, as a result, might perform the false-negative results (Holzhauser & Röder, 2015).

Due to the limitations of the above mentioned two methods, high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) method has been developed for allergenic protein analysis in foodstuffs (Carrera, Canas, & Gallardo, 2012; Downs, Baumert, Taylor, & Mills, 2016; Koeberl, Clarke, & Lopata, 2014; Korte & Brockmeyer, 2016; Planque et al., 2016; Sayers et al., 2018). The combination of the effective separation techniques (e.g. nano-LC, HPLC, UHPLC) with stable monitoring techniques (e.g. Q-TOF, PRM, SRM, MRM, MS3) advances this method better in terms of sensitivity and specificity for the detection of multiple food allergens simultaneously both in raw and processed samples. The main steps involved in LC-MS/MS method includes signature peptide markers selection, minimization of interference from matrix components, optimization of mass spectrometric and chromatographic performances, and validation of method in terms of reproducibility, accuracy, sensitivity, linearity and quantification (Koeberl et al., 2014). The labeled or unlabeled peptides were frequently used as the chemical substitutes to quantify allergen peptide markers (Posada-Ayala et al., 2015; Ippoushi, Sasanuma, Oike, Kabori, & Maeda-Yamamoto, 2016). The mass spectrometry method based on multiple reaction monitoring mode (MRM) focused on proteotypic peptides, are more widely used (Korte et al., 2016; Lin, Chen, Zhang, Li, & Fu, 2018). Although several studies had been validated in terms of the limit of quantification (LOQ), limit of detection (LOD), signal-to-noise (S/N) and linearity curve, there are few methods systematically validated following the guidelines (Mindaye, Spiric, David, Rabin, & Slater, 2017; ICH.Q2 [R1], 2005; Commission Decision of 2002/657/EC, 2002).

Fish allergy is an important concern in the seafood processing environment. Numerous fish allergens including parvalbumin, enolase, aldolase and vitellogenin have been reported, and parvalbumins beta (β -PVs) are identified as the major allergens (Kuehn, Swoboda, Arumugam, Hilger, & Hentges, 2014). β -PVs are EF-hand calcium-binding globular protein having a molecular mass weight of 10–12 kDa, an acidic pI (3–5) and play a role of muscle relaxation. Due to this structure, especially the Ca^{2+} binding site, β -PVs are resistant to tryptic digestion and heat treatment (Swoboda et al., 2002). Both of the two characteristics increase the difficulty to quantify β -PVs using HPLC-MS/MS. Carrera et al. (2012) reported a rapid and direct HPLC-SMIM-MS/

MS method for the qualitative detection of β -PVs in foodstuffs. This method allows the analysis of β -PVs in less than 2 h by ultrasound-assisted trypsin digestion, however, the selected peptides were not proved to be used in quantitative analysis and lacked the validation procedures.

The purpose of the present research was to establish a stable HPLC-MS/MS method for the quantitative analysis of β -PV in flounder (*Paralichthys olivaceus*). The method, using mass spectrometry with MRM, suits the routine laboratory equipment involved in food analysis. The established method was completely validated via a set of parameters in accordance with the guidelines of ICH Q2 (R1), including linearity, LOD, LOQ, precision and accuracy. Additionally, the validated method was employed for determining the presence of allergens in different matrices acquired from local supermarkets.

2. Materials and methods

2.1. Chemicals and reagents

Tris, glycine, dithiothreitol (DTT), ammonium bicarbonate (NH_4HCO_3), iodoacetamide (IAA) and BCA protein quantitation Kit were procured from Sigma-Aldrich (St. Louis, MO, USA). Formic acid and acetonitrile (ACN) of HPLC grade were obtained from Merck (Darmstadt, Germany). RapiGest SF acid labile surfactant (ALS) and sequencing grade trypsin were purchased from Waters Corporation (Manchester, UK) and Roche Diagnostics (Indianapolis, USA), respectively. Water was obtained from a Milli-Q Gradient A 10 system (Millipore, Bedford, MA, USA). DTT, IAA, RapiGest SF and trypsin were dissolved in 50 mM ammonium bicarbonate solution.

The reference peptides including ALTDATK, SDFIEDELK and LFLQNFASAR (95% standard) were chemically synthesized by Sangon Biotech (Shanghai, China). Stock solutions (10.0 mM) were prepared in methanol/water (50/50, v/v) and diluted to 10.0 mL, then stored at -70°C in dark for a maximum of 3 months. Working standard solutions at different levels were prepared for the calibration curves by diluting the stock solutions with the digests of turbot muscle matrix, and were kept at 4°C in the dark and found to be stable for 48 h.

The recombinant β -PV (Gene Bank number: BAF98925.1) was expressed and purified from *Escherichia coli* in Sangon Biotech (Shanghai, China). Finally, 13.0 mg of recombinant β -PV was obtained with the purity of over 95%.

2.2. Preparation of fishes and matrices

Flounder (*Paralichthys olivaceus*), turbot (*Scophthalmus maximus*), brown-marbled grouper (*Epinephelus fuscoguttatus*), small yellow croaker (*Pseudosciaena polyactis*) and silver carp (*Hypophthalmichthys molitrix*) were obtained from local supermarkets. Food matrices including pork, shrimp, chicken muscle and beef were purchased from local supermarkets. To validate the method, incurred matrices were prepared by the addition of 1.0 g of the minced muscle of flounder to 9.0 g non-contaminated minced matrices with homogenization.

2.3. Sample extraction

The white muscle of flounder was homogenized using a homogenizer. 1.0 g of muscle was transferred into a 10.0 mL polypropylene-based centrifuge tube and extracted with 4.0 mL of 0.1 M Tris, 0.5 mM glycine and 1.0 mM DTT, then homogenized for 2.0 min using an Ultra Turrax T-18D (Ika, Staufen, Germany) at 8000 rpm/min. After centrifugation for 20.0 min at 4°C and $12000\times g$, the supernatant was pipetted into a new 10 mL polypropylene centrifuge tube. Then, the extracts were heated for purification in a water bath at 90°C for 3.0 min. After centrifugation for 20 min at 4°C and $12000\times g$, the concentration of supernatants was measured by the bicinchoninic acid (BCA) kit (Sigma-Chemical Co., USA) (Smith et al., 1985). The other fish muscles

were prepared using the same protocol.

2.4. Sample digestion

A 10.0 μ L aliquot of supernatant was diluted in 90.0 μ L of 0.1% RapiGest SF. After vortexed for 10 s, 10.0 μ L of 100.0 mM IAA was mixed with alkylate proteins, followed by incubation for 45.0 min at 37 °C in the dark. A 10.0 h trypsin digestion was performed (protein: trypsin ratio 1:10) at 37 °C. Formic acid (1.0%) was added to stop the digestion and remove RapiGest SF incorporation with centrifugation (12000 $\times$ g, 10.0 min). At last, the volume was adjusted to 200.0 μ L with the initial mobile phase. Recombinant β -PV (1.3 mg/mL) was digested using the same protocol as a sample.

2.5. LC-MS/MS method development

Data were attained on a QTRAP 5500 LC – MS/MS system (AB Sciex Corp., Framingham, MA, USA) coupled with Essentia LC-16 HPLC (SHIMADZU Corp., Japanese). The chromatographic separation was achieved using Waters BEH C18 column (100 mm $\times$ 2.1 mm; i.d. 2.4 μ m) at 40 °C. Ten microliters of sample were injected with the help of auto-sampler. A gradient elution was performed at a flow rate of 0.3 mL/min with a mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), such that at time 0 min it was 3% B; at 8.0 min it was 95% B; at 9.0 min it was 95% B; at 9.1 min it was 3% B; and at 11.0 min it was 3% B.

The mass spectrometer was equipped with an ESI source operating in positive MRM and MS3 mode, respectively. The data was acquired by Analyst version 1.6.2 software (AB Sciex Pte. Ltd., Concord, Ontario, Canada). A turning for MRM and MS3 parameters was performed by directly infusing 100.0 μ M of working solution with an electronically controlled integrated syringe followed by the tuned program. The parameters of MS3 refer to the reported methods of Korte and Brockmeyer (2016). The following parameters were used: ionSpray voltage, 4.5 kV; temperature, 450 °C. The pressure of an auxiliary gas (nitrogen), spray gas (nitrogen), collision gas and curtain gas was set at 45, 50, 8 and 30 psi, respectively. Miscellaneous optimized settings and retention times for each peptide are presented in Table 1.

2.6. Quality parameters

The proposed LC-MS/MS approach for quantitative analysis of fish allergen was assessed via a set of parameters to conform the recommendations as defined by ICH protocols (ICH.Q2 [R1], 2005). The sensitivity of the developed method was validated by LOD and LOQ. The LOD, defined as the lowest concentrations of analyte where it could be detected, was measured based on a signal-to-noise (S/N) ratio of

between 3:1 and 2:1. While, LOQ was the minimum concentration at which the analyte can be quantified, and the distinctive signal-to-noise ratio is 10:1. The precisions, including inter-day and intra-day precision, were assessed as the relative SD (RSD) by analyzing peptides from three independently spiked turbot muscle at 20.0, 50.0 and 100.0 μ g/g, respectively. The intra-day precision was evaluated from RSD of three samples in one day. The inter-day precision was evaluated from RSD of the concentration of the three independent samples over three days. The accuracy of the proposed method was tested by correlating the concentration of recombinant β -PV quantified by LC-MS/MS with the results of BCA protein quantitation kit (1.3 mg/mL). The spiked turbot muscle was extracted and digested in multiple replicates, then quantified using the optimized LC-MS/MS method.

3. Results and discussion

3.1. Selection of peptides

The candidate peptides selection was performed based on the amino acid sequence composition (8 to 25 amino acids, exclude cysteine and methionine), tryptic cleavage efficiency and reproducibility (no internal arginine or lysine residues), physicochemical properties (post-translational modifications and hydrophobicity) and characteristic of chromatogram (high signal intensities) (Mallick et al., 2007). Moreover, the exogenous and endogenous interferences in complex foodstuffs can also influence food allergen detection, then, at least 3 or 4 peptides of each allergen were needed to ensure the specificity of the LC-MS/MS method.

The range of amino acid identity among fish β -parvalbumins was reported at 46–100% with an average of 72% (Swoboda et al., 2002). Therefore, unlike other allergens, detection of different fish parvalbumins has to choose numerous peptides as the biomarkers. Carrera et al. (2012) selected at least 16 peptides for detecting most fish β -PVs. However, these peptides could not be used for quantification of β -PV extracted from flounder. The most conserved region of amino acid sequence from 163 teleostei β -PVs was identified among the residues 46 to 77 (Carrera et al., 2012). This region encompasses one helix-loop-helix motif and Ca^{2+} -binding loop which are the characteristic sequence of fish β -PVs (Swoboda et al., 2002). Thus, the biomarkers were ideally selected from this region.

In this study, the sequence of β -PV of flounder (*Paralichthys olivaceus*) (accession number: A9ZTF2) was searched in the UniProt KB database (<http://www.uniprot.org>, accessed on 2018 April 22). An in silico digestion of the target peptide sequence was performed using the software Skyline v 2.6. Four potential candidate peptides were predicted with the ionization parameters of MRM (Table 1). To further validate these peptides, a 100.0 μ g/mL recombinant β -PV solution was

Table 1
MRM and MS3 parameters of peptides for the identification of β -PV by LC-MS/MS.

Sequence	Position	Precursor ion (charge state) (m/z)	Production (fragment)	Collision Energy (ev)	Declustering Potential (ev)	Transitions (MS3) (m/z)	Excitation Energy	Retention time (min)
ALTDAETK	76–83	424.6 ⁺⁺	664.3 (y6 ⁺) [*] 563.2 (y5 ⁺) 448.2 (y4 ⁺) 875.5 (y7 ⁺) 762.3 (y6 ⁺) [*] 633.3 (y5 ⁺) 880.4 (y8 ⁺) 752.3 (y7 ⁺) [*] 638.3 (y6 ⁺) 788.4 (y7 ⁺) 717.4 (y6 ⁺) 618.3 (y5 ⁺)	28 27 26 22 23 19 18 16 15	100	N	N	2.68 $\pm$ 0.01
SDFIEEDELK	55–64	612.9 ⁺⁺			90	612 ⁺⁺ $\rightarrow$ 762.3 ⁺ $\rightarrow$ 744.2 ⁺	0.35	3.63 $\pm$ 0.01
LFLQNFSASAR	65–75	627.4 ⁺⁺			100	627 ⁺⁺ $\rightarrow$ 753.2 ⁺ $\rightarrow$ 735.2 ⁺	0.43	4.06 $\pm$ 0.02
AAFAVIDQDK	45–54	539.3 ⁺⁺		-	-	-	-	-

^{*}Ion for quantification.

^NNo data were detected.

Table 2

The matching scores, intensities and charges of the completely digested peptides of recombinant flounder β -parvalbumin.

Peptide Sequence	Position	Score	Intensity	Charges
AAFAVIDQDK	46–55	166.5	4.251E + 10	2
SDFIEDELK	56–65	98.2	6.436E + 09	2
LFLQNFSASAR	66–76	177.4	6.711E + 10	2
ALTDAETK	77–88	261.4	1.49E + 11	2
IGVDEFAAMVK	98–108	93.6	6.3E + 10	2

digested using trypsin then detected by tandem mass spectrometry in Q Exactive™ Plus (ThermoFisher, USA). The Data were processed using Proteome Discoverer 1.3 against UniProt KB database (<http://www.uniprot.org>, accessed on 2018 April 22). All of the mentioned peptides were detected with high matching scores and intensities (Table 2). After a Basic Local Alignment Search Tool (BLAST) analysis of the retained peptide sequences, the peptide of SDFIEDELK was found as the unique in the β -parvalbumin sequence of flounder (Table S1). Besides, the other peptides were also searched in different fishes. Therefore they could be used as the biomarkers for the detection of other fish parvalbumins. The peptide of AAFAVIDQDK was excluded because of a relatively low homology. Finally, the peptides of ALTDAETK, SDFIEDELK and LFLQNFSASAR were selected for the LC-MS/MS analysis. The intensity of the peptide of SDFIEDELK was found to be the highest than the others in same concentration during standard work solution (Fig. 1). However, it was lowest when detected β -PV extracted from flounder because of the low digestion efficiency (Fig. 2). Finally, ALTDAETK was selected as the quantitative peptide for determining β -PVs of flounder in present assay due to the highest sensitivity.

3.2. Optimization of chromatography and mass spectrometry

All the purified extracts were digested using trypsin, and separated by RP-HPLC, subsequently measured by ESI-MS/MS operated with MRM scan mode to detect the selected peptides derived from samples.

The HPLC parameters including column, mobile phase and elution program were optimized for a good separation of each peptide prior to MS detection. An excellent separation of peptides with high reproducibility was achieved via this method (Fig. 1). The retention times of the

peptides ALTDAETK, SDFIEDELK and LFLQNFSASAR were 2.68 ± 0.07 , 3.63 ± 0.01 and 4.06 ± 0.02 , respectively.

The optimization of mass spectrometry revolves around ionization and ion transmission conditions were performed by infusing 100.0 μ M synthetic peptides. The MS parameters including mass fragments and peptides were set according to the results of in silico digested by Skyline. The ESI source and the positive ion mode were designated for quantitative assessment owing to the amidogen in the chemical structures. Three peptides and their relevant MRM fragments were identified and optimized according to the signal quality and strength. We determined that the charge state for all precursor ions was only +2 (Fig. S1). At least two y-ion transitions (product ions) of each precursor ion were usually selected for confirmation of each peptide and the most abundant was utilized for quantitative purposes (Table 1). Subsequently, the collision energy (CE) and declustering potential (DP) of each transition were optimized. Table 1 shows the target peptides together with the MRM fragments and optimized MS/MS parameters in the final LC-MS/MS assay.

Recently, Korte and Brockmeyer (2016) had reported the multiple reaction monitoring cubed (MRM3) technology in the QTRAP systems that could significantly reduce the matrix interference, increase the specificity and obtain high abundant signals in quantitative confirmation of lobster, shrimp and nut allergens. In the present study, we applied MRM3 mode according to the reported approach. The Q3 was set to a unit resolution and used in LIT mode. MRM3 parameters were also turned with synthetic peptides, aided by syringe pump injection. The CE and DP of each peptide were set as the same as the optimized value in MRM mode. The excitation energy (AF2) was further optimized by taking into account the signal intensity (Table 1). Finally, just two MS3 transitions were selected, namely $627^{++} \rightarrow 753.2^{+} \rightarrow 735.2^{+}$ (LFLQNFSASAR) and $612^{++} \rightarrow 762.3^{+} \rightarrow 744.2^{+}$ (SDFIEDELK) (Fig. S2). For evaluating this method, we detected these two different concentrations of peptides by MRM and MRM3 mode, respectively. As seen in Fig. 3, the signal intensities of two peptides detected with MRM3 mode were far lower than the levels in MRM mode. Although the MRM3 method has the advantage of specificity and anti-interference, it's unavailable to every food allergen because of the low signal sensitivity.

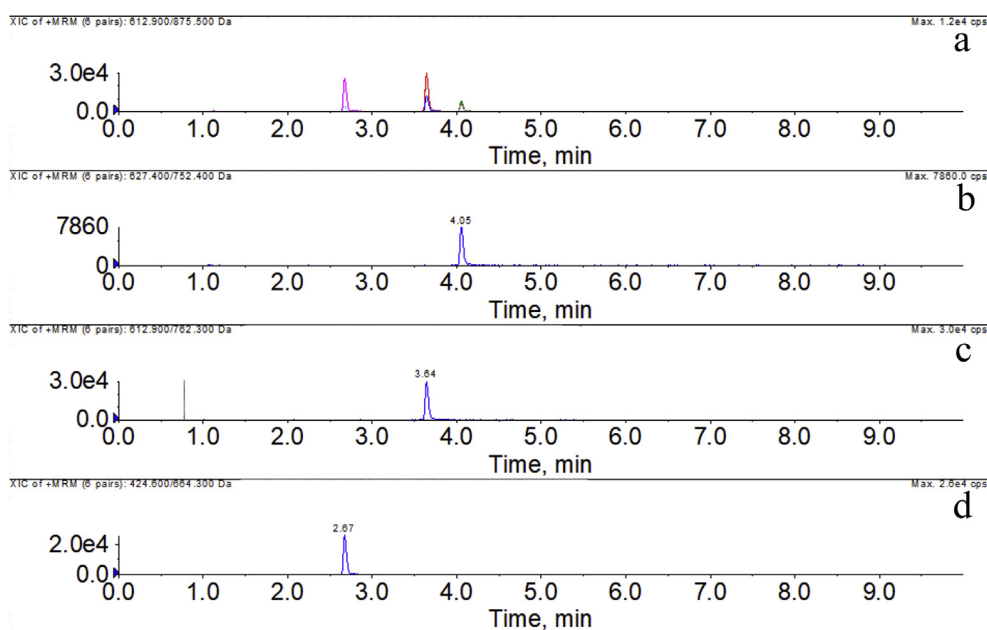


Fig. 1. Total ion chromatogram (a) and extracted ion chromatogram (b, c, d) of target peptides by analyzing work solution (concentration = 5 nM). b, c and d are the peptides of LFLQNFSASAR ($627.4^{++} \rightarrow 753.2^{+}$), SDFIEDELK ($612.9^{++} \rightarrow 762.3^{+}$) and ALTDAETK ($424.6^{++} \rightarrow 664.3^{+}$), respectively.

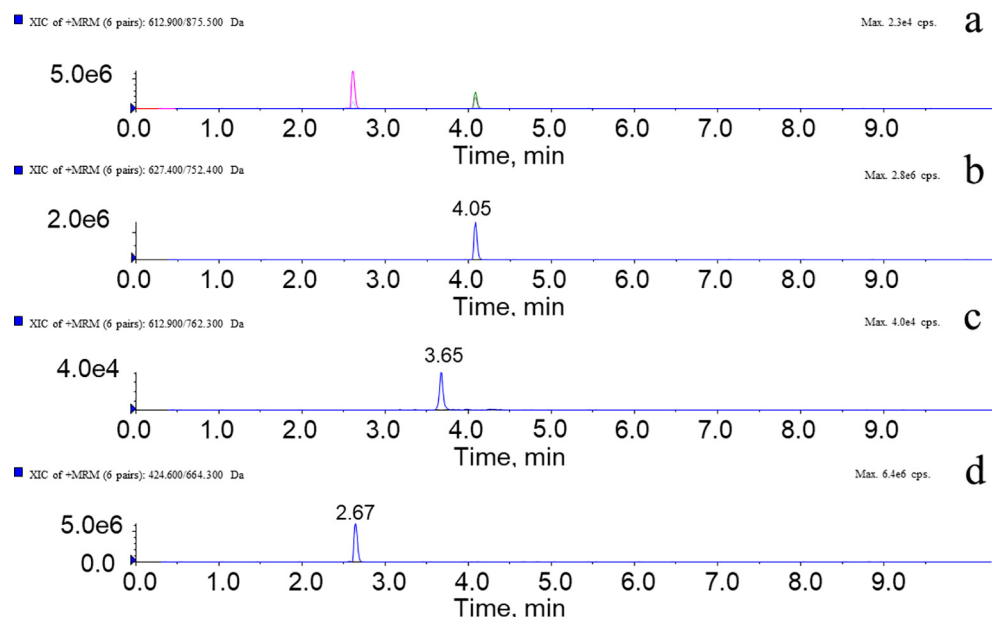


Fig. 2. Total ion chromatogram (a) and extracted ion chromatogram (b, c, d) of target peptides by analyzing muscle of flounder (*Paralichthys olivaceus*) with the concentration of 2686.3 nM. b, c and d are the peptides of LFLQNFASAR (627.4⁺⁺ → 753.2⁺), SDFIEDELK (612.9⁺⁺ → 762.3⁺) and ALTDATK (424.6⁺⁺ → 664.3⁺), respectively.

3.3. Digestion conditions optimization

For the complete digestion of allergens, parameters including selection of denaturants, amount of RapiGest SF, time of incubation and enzyme to substrate ratio should be optimized according to the maximum peptide yield. Several helices in β -PVs form a cavity in which special amino acid sequences (SDFIEDELK) are buried. This structure may contribute to its resistance to trypsin (Ikura, 1996; Swoboda et al., 2002). Therefore, β -PVs were supposed to be pretreated with denaturants such as SDS, urea, guanidine and RapiGest SF (an acid-labile

surfactant) prior to digestion (Burkhart, Schumbrutski, Wortelkamp, Sickmann, & Zahedi, 2012; Norrgran et al., 2009; Suder, Bierczynska, König, & Silberring, 2004). Also, the denaturants should be removed prior to MS analysis that will adversely affect the efficiency of trypsin and response of MS. RapiGest SF was adopted because it helps to increase the proteins more soluble and more susceptible to tryptic digestion. Besides, it also has the advantage of easy removal by acidification, as it is acid labile and degraded into components which has no influence on the MS analysis at pH < 2.0 (Suder et al., 2004). Therefore, RapiGest SF was selected as the ideal protein denaturant in tryptic

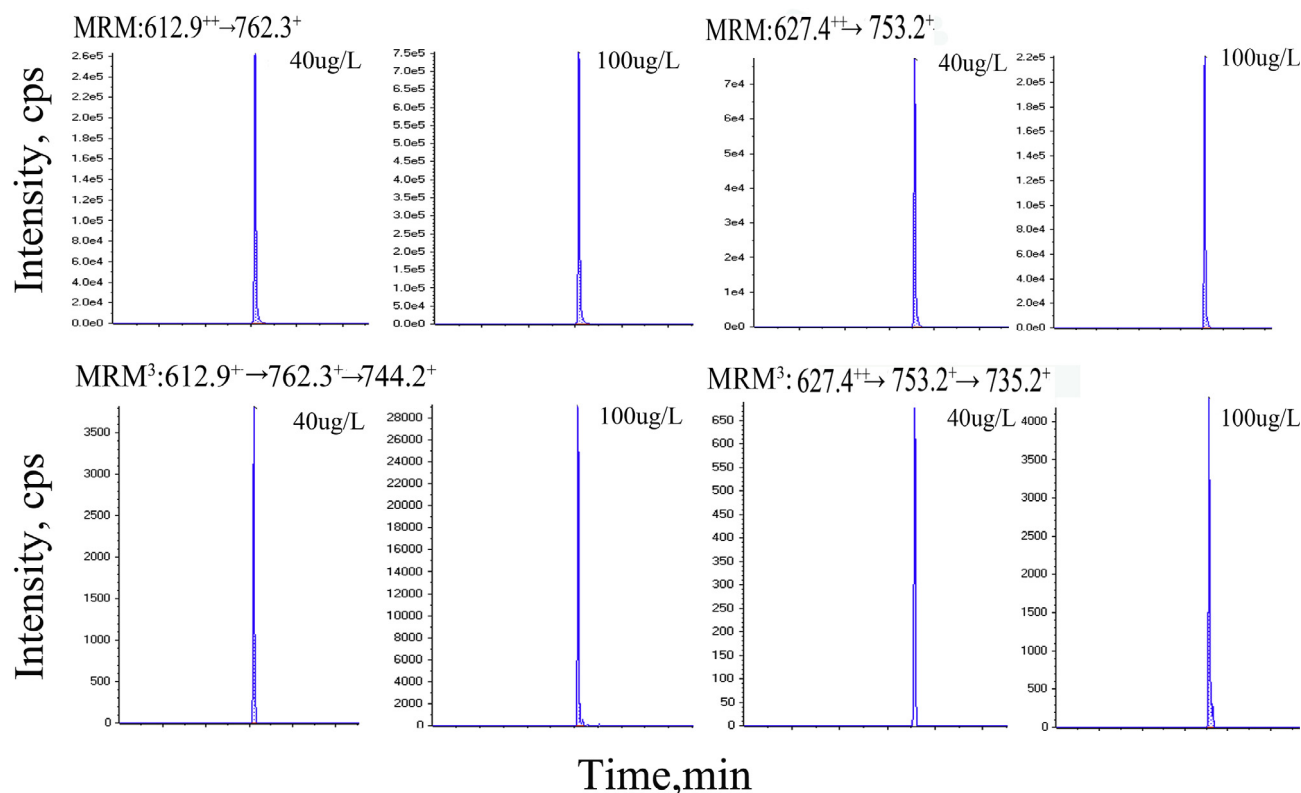


Fig. 3. The comparison of signal intensity of peptides by analyzing work solution with two different concentrations (40 µg/L and 100 µg/L) in MRM and MRM3 mode.

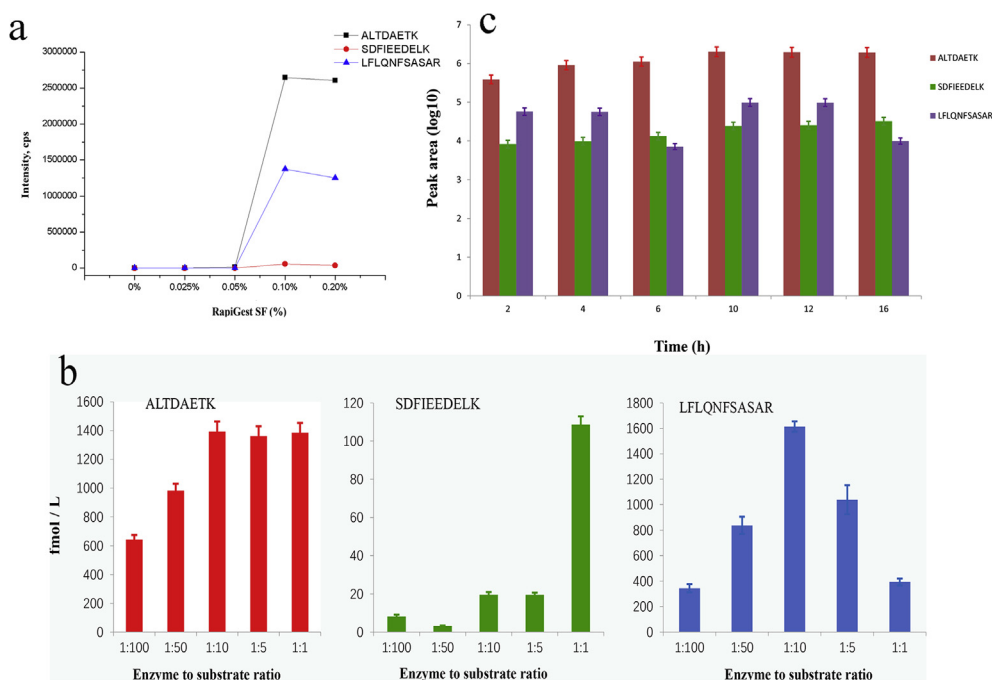


Fig. 4. The optimization of the concentration of RapiGest SF (a), enzyme to substrate ratio (b) and the time of incubation (c) on digestion efficiency. The error bars represent standard deviation.

digestions. In addition, the effect of concentrations of RapiGest SF at five levels: 0%, 0.025%, 0.05%, 0.1% and 0.2% were also explored. Fig. 4a shows that digestion was not efficient without the use of RapiGest SF. A significant difference was observed between 0.025% and 0.2% RapiGest SF with an earlier increasing and later decreasing trend. This might be because of the high concentration (above 0.2%) of RapiGest SF has negatively influenced the activity of trypsin (Norrgran et al., 2009).

Enzyme-to-substrate ratio was also one of the important indicators of complete digestion. Most digestion methods typically use ratios of 1:100 to 1:10 (Smith, 2003; Suder et al., 2004). We evaluated this factor by adding trypsin to the extrats of flounder at ratios of 1:100, 1:50, 1:10, 1:5 and 1:1, as shown in Fig. 4b. Three peptides have the different optimum enzyme-to-substrate ratios. The maximum digestion efficiency for the peptide of SDFIEDELK was found at the high enzyme-to-substrate ratio of 1:1 due to the buried structure. Comparatively, the peptides of LFLQNFSASAR and ALTAETK were more susceptible to trypsin and decreased at concentrations above the ratio of 1:10. Therefore, the ratio of 1:10 was selected in our assay. The time of incubation was also investigated from 2.0 to 16.0 h. A 10.0 h digestion duration was adapted for the assay based on the highest peptide yields (Fig. 4c). Taken together, the digestion protocol for β -PVs was adopted by employing 0.1% RapiGest SF, a high level of trypsin, and a 10.0 h digestion time.

3.4. Evaluation of the performance of LC-MS/MS method

The performance of this method was validated from the parameters including linearity, sensitivity, precision and accuracy according to ICH protocols (ICH.Q2 [R1], 2005). Linearity was replicated over three days using fresh standard peptides with a wide concentration range (0.07–1179.36 nM). Calibrations were constructed from log-transformed data because of the wide concentration range. The method exhibited a good linear relationship with the qualified correlation coefficients ($r > 0.999$). Sensitivity was assessed by LOQ and LOD calculated by S/N ratio. The results were listed in Table 3, showing that the β -PV could be detected in fish at $< 0.1 \mu\text{g/g}$, lower than the prior reports by ELISA (Shibahara, Uesaka, Wang, Yamada, & Shiomi, 2013).

Precession was evaluated using repeated quantification of recombinant β -PV which was spiked into the muscle of turbot with different concentrations. The muscle of turbot was selected as the blank samples because the turbot is same with flounder belonging to the flatfish family Bothidae and was also verified excluding the selected peptides (Fig. S3). The LC-MS/MS method was precise with the RSD of inter-day and intra-day for all peptides ($< 18.35\%$). Quantification accuracy was assessed by comparing the quantitative results of recombinant parvalbumin (purity $> 95\%$) in turbot muscle using the optimized LC-MS/MS method with BCA Protein Quantitation Kit. The concentration of recombinant parvalbumin was calculated at 1.3 mg/mL by BSA. Table 3 shows the accuracy of the LC-MS/MS assay within 13.3% of the target quantity. The validated parameters confirm the LC-MS/MS method could meet the requirements for quantitative detection of β -PV in fish (ICH.Q2 [R1], 2005).

To assess the matrix effect, the peptide stock solutions were diluted to three levels with the digests of the turbot muscle (20 nM, 50 nM and 100 nM) and analyzed by LC-MS/MS. Comparing with the peak area of peptides in methanol/water (50/50, v/v) with the same concentrations. The matrix interference was considered significant when the peak areas differed by $> 20\%$. The matrix effect was not the same with all peptides from the same allergen. The differences of three peptides of ALTAETK, SDFIEDELK and LFLQNFSASAR were between 9.8% and 17.3%, 10.5%–14.5% and 10.9%–18.3%, respectively. The low degree of interference confirms that the matrix has a very minor effect on the peptides quantification.

3.5. Analysis in other matrices

For the purpose of evaluating the applicability of this method in other matrices, we detected three peptides from the incurred matrices. The incurred matrices were prepared by adding 1.0 g muscle of fish to the 9.0 g minced matrices (pork, shrimp, chicken muscle and beef) which were obtained from local supermarkets. All of the peptides were detected with the acceptable inter-day RSDs ($< 10\%$) (Fig. S4). In addition, the method was successfully applied to detect other fish parvalbumins (Fig. S5). The peptide of ALTAETK was detected in brown-marbled grouper (*Epinephelus fuscoguttatus*), small yellow croaker

Table 3

Results of linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy for target peptides.

Peptide	Linearity (nM)	LOD ^d (μg/g)	LOQ ^e (μg/g)	Conc (μg/g)	Inter-day Precision (RSD ^f , %)	Intra-day Precision (RSD ^f , %)
ALTDAETK	$Y^a = 0.9738X^b + 3.0751$ $r^c = 0.9997$ Range: 0.10–1179.36 nM	0.03	0.10	20.0 50.0 100.0	8.79 2.39 6.06	4.23 4.54 4.74
SDFIEDELK	$Y = 0.9992X + 5.1533$ $r^c = 0.9998$ Range: 0.07–816.80 nM	0.02	0.07	20.0 50.0 100.0	9.85 9.89 18.35	16.09 7.01 4.78
LFLQNFSASAR	$Y = 1.0184X + 3.2069$ $r^c = 0.9999$ Range: 0.12–797.81 nM	0.04	0.12	20.0 50.0 100.0	12.66 13.82 10.23	2.77 3.51 3.91
Accuracy	Replicate 1 (mg/mL)		Replicate 2 (mg/mL)		Replicate 3 (mg/mL)	
Analyzing by LC-MRM MS	1.365		1.127		1.128	
% difference	5.0%		13.3%		13.2%	

^a log₁₀(x).^b log₁₀(y).^c Correlation coefficient.^d Limit of detection (signal-to-noise ratio of 3/1).^e Limit of quantification (signal-to-noise ratio of 10/1).^f Relative standard deviation (ratio between standard deviation and mean found concentration).

(*Pseudosciaena polyactis*) and silver carp (*Hypophthalmichthys molitrix*) with high concentration, respectively. Therefore, the present method could be used to detect β-PVs in different food matrices. Moreover, the sample pre-preparation methods should be optimized based on the matrix and the appropriate internal standards (labeled peptide or unlabeled peptide) were recommended when quantified β-PVs in complex matrices.

4. Conclusions

A simple, sensitive and precise method was established for determining β-PVs by analyzing special peptides using HPLC-MS/MS. This buffer solution with DTT as an extraction solvent and the simple heating protocol as the clean-up approach were selected. The parameters of tryptic digestion and spectrometry were completely optimized. Then the proposed method was completely validated in accordance with the ICH protocols. In addition, the method was successfully applied to analyze other fish parvalbumins and β-PV in typical food matrices. What should be done in the future with this new method is following: to detect parvalbumin in complex matrices, to analyze the variation rules of parvalbumin during food processing, to verify the species identification, and to assess the allergenicity of parvalbumin coupled with other models.

Acknowledgement

The authors would like to express their sincere thanks to the Fundamental Research Funds for the Central Universities (201762004) and National Natural Science Foundation of China (31871719) for supporting the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2018.10.014>.

References

- Ballmer-Weber, B. K., Fernandez-Rivas, M., Beyer, K., Defernez, M., Sperrin, M., Mackie, A. R., ... Mills, E. N. (2015). How much is too much? Threshold dose distributions for 5 food allergens. *The Journal of Allergy and Clinical Immunology*, 135(4), 964–971.
- Burkhardt, J. M., Schumbrutzki, C., Wortelkamp, S., Sickmann, A., & Zahedi, R. P. (2012). Systematic and quantitative comparison of digest efficiency and specificity reveals the impact of trypsin quality on MS-based proteomics. *Journal of Proteomics*, 75(4), 1454–1462.
- Carrera, M., Canas, B., & Gallardo, J. M. (2012). Rapid direct detection of the major fish allergen, parvalbumin, by selected MS/MS ion monitoring mass spectrometry. *Journal of Proteomics*, 75(11), 3211–3220.

- Commission directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs, OJ L109, pp. 29–42.
- Commission Decision (2002). Commission Decision of 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC), Official Journal of European Communities, L221, pp. 8–36.
- Commission Directive 2007/68/EC of 27 November 2007 amending Annex IIIa to Directive 2000/13/EC of the European Parliament and of the Council as regards certain food ingredients, OJ L 310, p. 11–14.
- Downs, M. L., Baumert, J. L., Taylor, S. L., & Mills, E. N. (2016). Mass spectrometric analysis of allergens in roasted walnuts. *Journal of Proteomics*, 142, 62–69.
- Eischeid, A. C., Kim, B. H., & Kasko, S. M. (2013). Two quantitative real-time PCR assays for the detection of penaeid shrimp and blue crab, crustacean shellfish allergens. *Journal of Agriculture and Food Chemistry*, 61(24), 5669–5674.
- Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, OJ L 304, 22. 11. 2011, p. 18–63.
- FDA (2004). *Food Allergen Labeling and Consumer Protection Act*. Silver Spring: US Food and Drug Administration.
- Fu, T. J., & Maks, N. (2013). Impact of thermal processing on ELISA detection of peanut allergens. *Journal of Agriculture and Food Chemistry*, 61(24), 5649–5658.
- Heinonen, I. M. (2014). Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes. *Efsa Journal*, 12(11), n/a-n/a.
- Holzhauser, T., & Röder, M. (2015). Polymerase chain reaction (PCR) methods for detecting allergens in foods. *Handbook of Food Allergen Detection and Control* (pp. 245–263). UK: Wood head Publishing Limited.
- ICH. Q2(R1) Validation of Analytical Procedures: Text and Methodology. 2005. [http://academy.gmp-compliance.org/guidemgr/files/Q2\(R1\).PDF](http://academy.gmp-compliance.org/guidemgr/files/Q2(R1).PDF). Accessed 18. 04. 12.
- Ikura, M. (1996). Calcium binding and conformational response in EF-hand proteins. *Trends in Biochemical Sciences*, 21(1), 14–17.
- Ippoushi, K., Sasanuma, M., Oike, H., Kobori, M., & Maeda-Yamamoto, M. (2016). Absolute quantification of pru av 2 in sweet cherry fruit by liquid chromatography/tandem mass spectrometry with the use of a stable isotope-labelled peptide. *Food Chemistry*, 204, 129–134.
- Jayasena, S., Smits, M., Fiechter, D., de Jong, A., Nordlee, J., Baumert, J., ... Koppelman, S. J. (2015). Comparison of six commercial ELISA kits for their specificity and sensitivity in detecting different major peanut allergens. *Journal of Agriculture and Food Chemistry*, 63(6), 1849–1855.
- Khuda, S., Slate, A., Pereira, M., Al-Taher, F., Jackson, L., Diaz-Amigo, C., ... Williams, K. M. (2012). Effect of processing on recovery and variability associated with immunochemical analytical methods for multiple allergens in a single matrix: Sugar cookies. *Journal of Agriculture and Food Chemistry*, 60(17), 4195–4203.
- Koeberl, M., Clarke, D., & Lopata, A. L. (2014). Next generation of food allergen quantification using mass spectrometric systems. *Journal of Proteome Research*, 13(8), 3499–3509.
- Korte, R., & Brockmeyer, J. (2016). MRM(3)-based LC-MS multi-method for the detection and quantification of nut allergens. *Analytical and Bioanalytical Chemistry*, 408(27), 7845–7855.
- Korte, R., Monneuse, J. M., Gemrot, E., Metton, I., Humpf, H. U., & Brockmeyer, J. (2016). 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. *Journal of Agriculture and Food Chemistry*, 64(31), 6219–6227.
- Kuehn, A., Swoboda, I., Arumugam, K., Hilger, C., & Hentges, F. (2014). Fish allergens at a glance: Variable allergenicity of parvalbumins, the major fish allergens. *Frontiers in Immunology*, 5, 179.
- Lin, N., Chen, S., Zhang, H., Li, J., & Fu, L. (2018). Quantification of major royal jelly protein 1 in fresh royal jelly by ultra-performance liquid chromatography-tandem mass spectrometry. *Journal of Agriculture and Food Chemistry*, 66(5), 1270–1278.

- Mallick, P., Schirle, M., Chen, S. S., Flory, M. R., Lee, H., Martin, D., ... Werner, T. (2007). Computational prediction of proteotypic peptides for quantitative proteomics. *Nature Biotechnology*, 25(1), 125–131.
- Mindaye, S. T., Spiric, J., David, N. A., Rabin, R. L., & Slater, J. E. (2017). Accurate quantification of 5 German cockroach (GCr) allergens in complex extracts using multiple reaction monitoring mass spectrometry (MRM MS). *Clinical and Experimental Allergy*, 47(12), 1661–1670.
- Norrgran, J., Williams, T. L., Woolfitt, A. R., Solano, M. I., Pirkle, J. L., & Barr, J. R. (2009). Optimization of digestion parameters for protein quantification. *Analytical Biochemistry*, 393(1), 48–55.
- Parker, C. H., Khuda, S. E., Pereira, M., Ross, M. M., Fu, T. J., Fan, X., ... Jackson, L. S. (2015). Multi-allergen quantitation and the impact of thermal treatment in industry-processed baked goods by ELISA and liquid chromatography-tandem mass spectrometry. *Journal of Agriculture and Food Chemistry*, 63(49), 10669–10680.
- Planque, M., Arnould, T., Dieu, M., Delahaut, P., Renard, P., & Gillard, N. (2016). Advances in ultra-high performance liquid chromatography coupled to tandem mass spectrometry for sensitive detection of several food allergens in complex and processed foodstuffs. *Journal of Chromatography A*, 1464, 115–123.
- Poms, R. E., Agazzi, M. E., Bau, A., Brohee, M., Capelletti, C., Norgaard, J. V., & Anklam, E. (2005). Inter-laboratory validation study of five commercial ELISA test kits for the determination of peanut proteins in biscuits and dark chocolate. *Food Additives & Contaminants*, 22(2), 104–112.
- Posada-Ayala, M., Alvarez-Llamas, G., Maroto, A. S., Maes, X., Munoz-Garcia, E., Villalba, M., ... Cuesta-Herranz, J. (2015). Novel liquid chromatography–mass spectrometry method for sensitive determination of the mustard allergen Sin a 1 in food. *Food Chemistry*, 183, 58–63.
- Sathe, S. K., Teuber, S. S., & Roux, K. H. (2005). Effects of food processing on the stability of food allergens. *Biotechnology Advances*, 23(6), 423–429.
- Sayers, R. L., Gethings, L. A., Lee, V., Balasundaram, A., Johnson, P. E., Marsh, J. A., ... Mills, E. N. C. (2018). Microfluidic separation coupled to mass spectrometry for quantification of peanut allergens in a complex food matrix. *Journal of Proteome Research*, 17(1), 647–655.
- Shibahara, Y., Uesaka, Y., Wang, J., Yamada, S., & Shiomi, K. (2013). A sensitive enzyme-linked immunosorbent assay for the determination of fish protein in processed foods. *Food Chemistry*, 136(2), 675–681.
- Smith, B. J. (2003). Protein Sequencing Protocols. *Methods in Molecular Biology*[™], 211.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., ... Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, 150(1), 76–85.
- Suder, P., Bierczynska, A., Konig, S., & Silberring, J. (2004). Acid-labile surfactant assists in-solution digestion of proteins resistant to enzymatic attack. *Rapid Communications in Mass Spectrometry*, 18(7), 822–824.
- Swoboda, I., Bugajska-Schretter, A., Verdino, P., Keller, W., Sperr, W. R., Valent, P., ... Spitzauer, S. (2002). Recombinant carp parvalbumin, the major cross-reactive fish allergen: a tool for diagnosis and therapy of fish allergy. *The Journal of Immunology*, 168(9), 4576–4584.
- Taylor, S. L., Baumert, J. L., Kruizinga, A. G., Remington, B. C., Crevel, R. W., Brooke-Taylor, S., ... Houben, G. (2014). Establishment of Reference Doses for residues of allergenic foods: Report of the VITAL Expert Panel. *Food and Chemical Toxicology*, 63, 9–17.
- Verhoeckx, K. C., Vissers, Y. M., Baumert, J. L., Faludi, R., Feys, M., Flanagan, S., ... Kimber, I. (2015). Food processing and allergenicity. *Food and Chemical Toxicology*, 80, 223–240.

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

Lirui Sun^a, Hong Lin^a, Zhenxing Li^{a*}, Weihong Sun^b, Jianhua Wang^c, Haiyan Wu^b, Minmin Ge^c, Ishfaq Ahmed^a, Tushar Ramesh Pavase^a

^a*College of Food Science and Engineering, Ocean University of China, No. 5, Yushan Road, Qingdao, Shandong Province 266003, People's Republic of China*

^b*Key Laboratory of Testing and Evaluation for Aquatic Product Safety and Quality, Ministry of Agriculture; Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, People's Republic of China*

^c*Technical Center of Inspection and Quarantine, Shandong Entry-Exit Inspection and Quarantine Bureau 266002, People's Republic of China*

Corresponding Author

*Institution: College of Food Science and Engineering, Ocean University of China, No. 5, Yushan Road, Qingdao, Shandong Province 266003, People's Republic of China;

E-mail address: lizhenxing@ouc.edu.cn.

Table S1. The BLAST results of each peptide with 100% identities against UniProt KB database

Peptide sequence	Identities	Protein name	Source
SDFIEEDELK	10/10 100%	Parvalbumin beta	<i>Paralichthys olivaceus</i>
AAFAVIDQDK	10/10 100%	Parvalbumin beta	<i>Paralichthys olivaceus</i>
		Parvalbumin alpha	<i>Cyprinus carpio</i>
LFLQNFSASAR	11/11 100%	Parvalbumin beta	<i>Paralichthys olivaceus</i>
		Parvalbumin beta	<i>Xenopus laevis</i>
		Parvalbumin beta-2	<i>Gadus chalcogrammus</i>
		Parvalbumin beta 2	<i>Salmo salar</i>
		Parvalbumin beta 2	<i>Oncorhynchus mykiss</i>
ALTDAETK	8/8 100%	Parvalbumin beta	<i>Paralichthys olivaceus</i>
		Parvalbumin beta	<i>Gadus morhua callarias</i>
		Parvalbumin-2	<i>Danio rerio</i>
		Parvalbumin beta	<i>Merluccius bilinearis</i>
		Parvalbumin alpha	<i>Cyprinus carpio</i>
		Parvalbumin beta	<i>Merlangius merlangus</i>
		Parvalbumin beta 2	<i>Salmo salar</i>
		Parvalbumin beta 2	<i>Merluccius australis</i>
		Parvalbumin beta 2	<i>polylepis</i>
		Parvalbumin beta 2	<i>Merluccius polli</i>
		Parvalbumin beta 2	<i>Merluccius productus</i>
		Parvalbumin beta 3	<i>Merluccius bilinearis</i>
		Parvalbumin beta 2	<i>Oncorhynchus mykiss</i>
		Parvalbumin beta	<i>Squalius cephalus</i>
		Parvalbumin beta 1	<i>Macruronus magellanicus</i>
		Parvalbumin beta 3	<i>Merluccius gayi</i>
		Parvalbumin beta	<i>Scomber japonicus</i>
		Parvalbumin beta-2	<i>Gadus chalcogrammus</i>

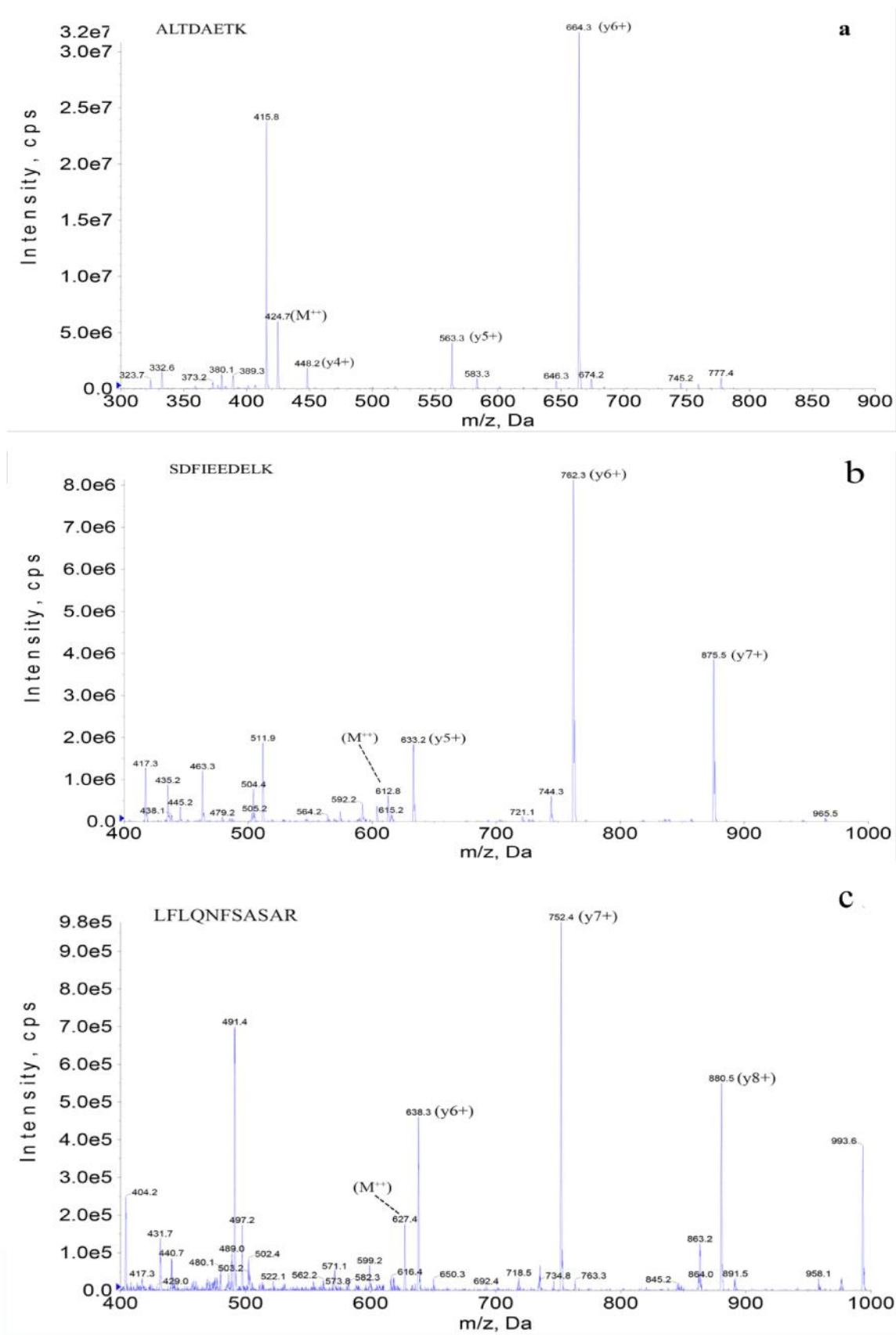


Figure S1. The ESI (+) MS/MS spectra of peptides of ALTDAETK (a), SDFIEEDELK (b), and LFLQNFSASAR (c) by analyzing work solution (100 nM) in MRM mode.

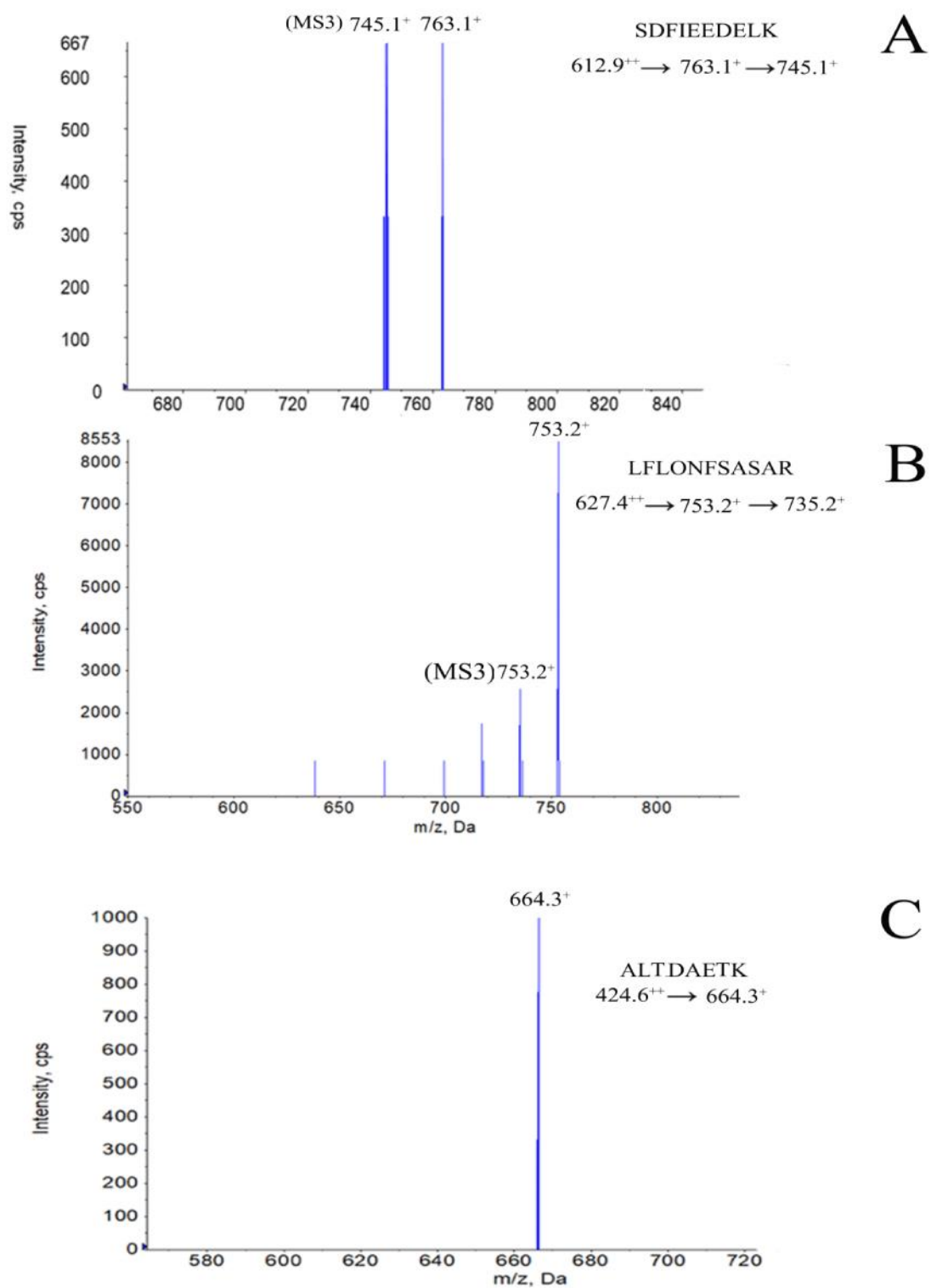


Figure S2. The ESI (+) MS3 spectra of peptides of SDFIEEDELK (A), LFLQNFSASAR (B) and ALTDAETK(C) by analyzing work solution (100 nM) in MRM3 mode. The selected MS3 transitions were $612.9^{++} \rightarrow 762.3^{+} \rightarrow 744.2^{+}$ (SDFIEEDELK) and $627.4^{++} \rightarrow 753.2^{+} \rightarrow 735.2^{+}$ (LFLQNFSASAR), respectively.

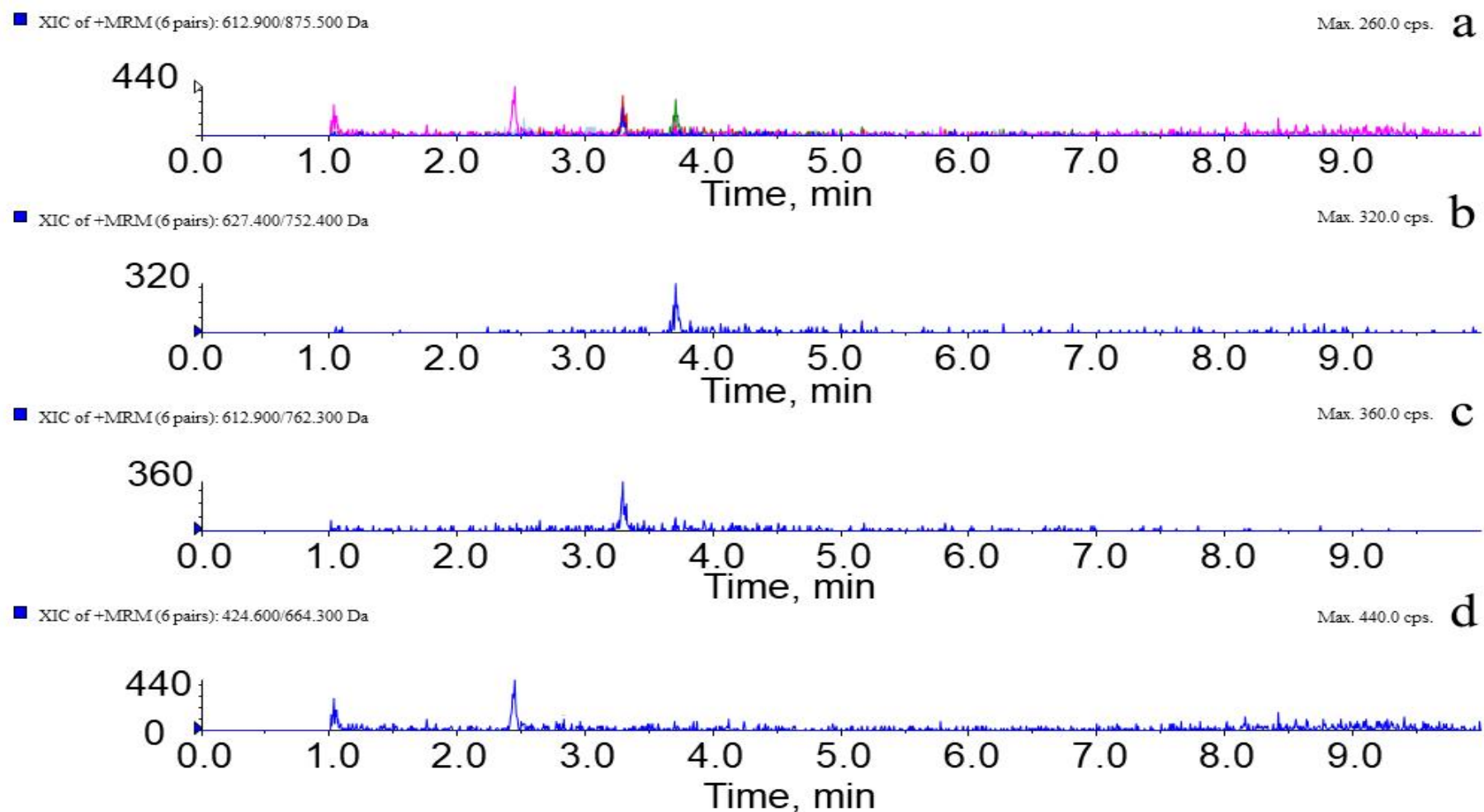


Figure S3. Total ion chromatogram (a) and extracted ion chromatogram (b, c, d) of target peptides by analyzing target peptides in turbot muscle (*Scophthalmus maximus*).

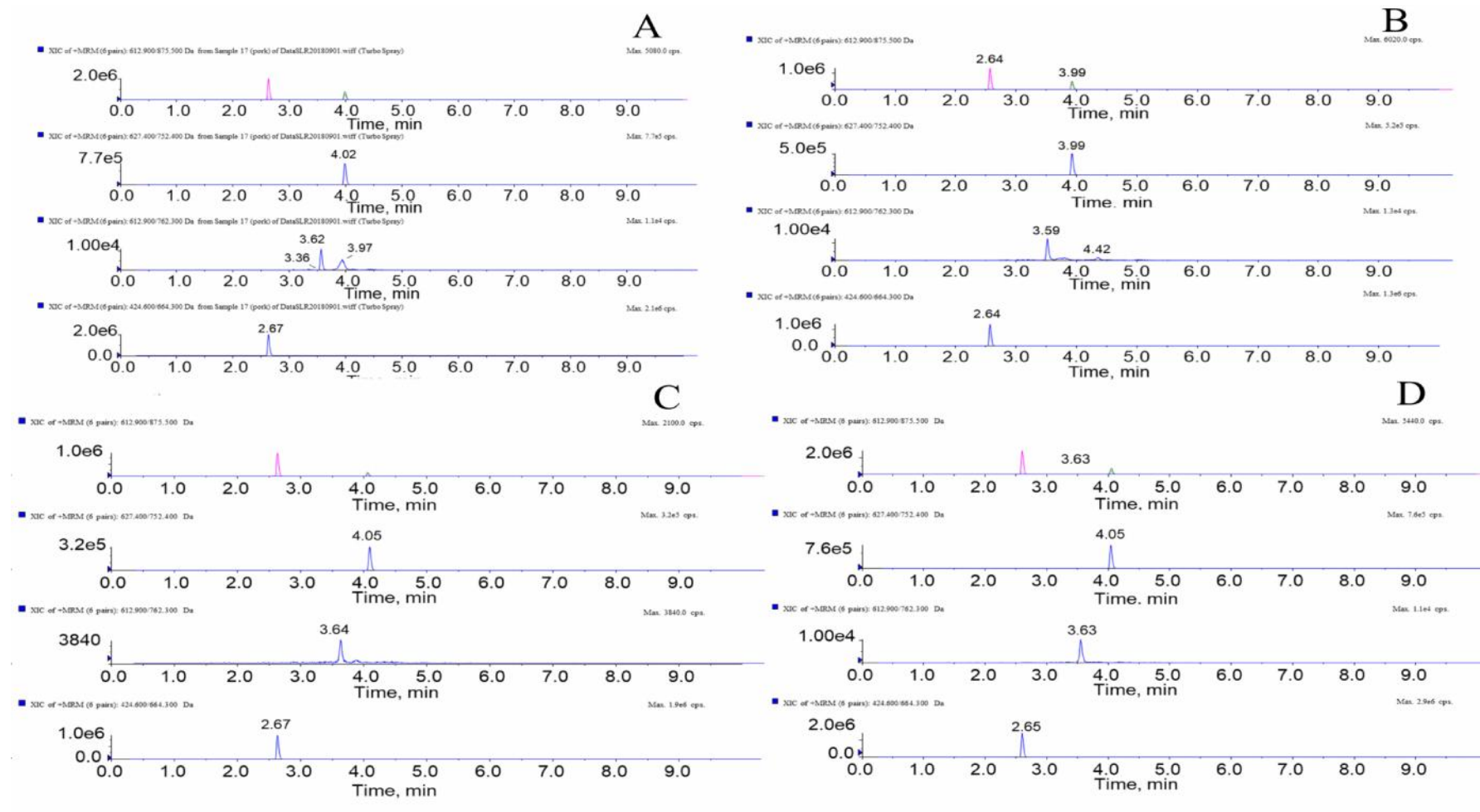
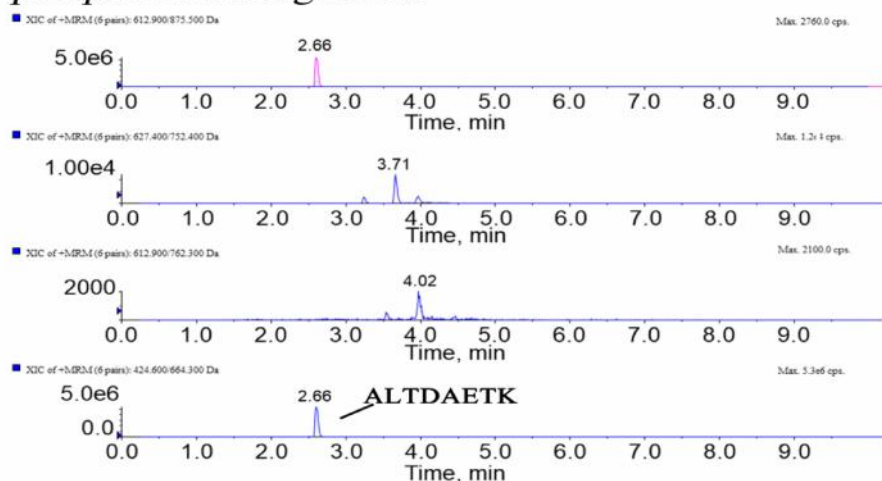


Figure S4. Total ion chromatogram and extracted ion chromatogram of target peptides by analyzing β -PV in pork (A), shrimp (B), chicken muscle (C) and beef (D) with the concentration of 193.37nM, 119.1nM, 140.5nM and 213.9nM, respectively.

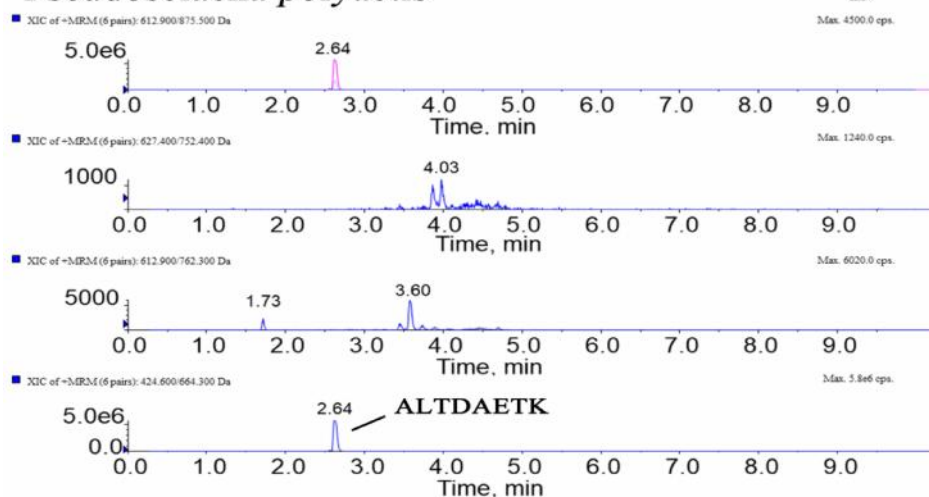
Epinephelus fuscoguttatus

A



Pseudosciaena polyactis

B



Hypophthalmichthys molitrix

C

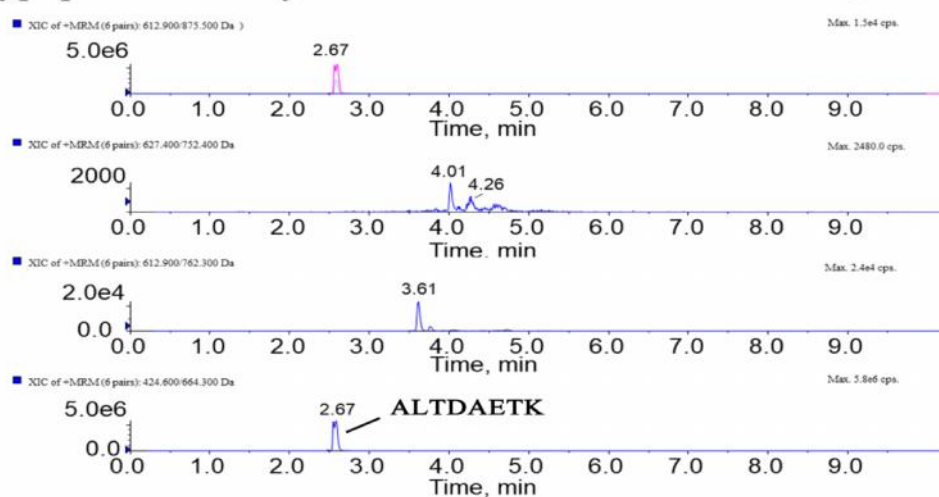


Figure S5. Total ion chromatogram and extracted ion chromatogram of target peptides by analyzing parvalbumin of brown-marbled grouper (*Epinephelus fuscoguttatus*), small yellow croaker (*Pseudosciaena polyactis*) and silver carp (*Hypophthalmichthys molitrix*) with the concentration of 1994.1nM, 1573.4nM and 2265.5nM, respectively.