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Rapid Direct Detection of the Major Fish Allergen, Parvalbumin, by Selected MS/MS Ion Monitoring Mass Spectrometry

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ABSTRACT

Parvalbumins beta (β -PRVBs) are considered the major fish allergens. A new strategy for the rapid and direct detection of these allergens in any foodstuff is presented in this work. The proposed methodology is based on the purification of β -PRVBs by treatment with heat, the use of accelerated in-solution trypsin digestion under an ultrasonic field provided by High-Intensity Focused Ultrasound (HIFU) and the monitoring of only nineteen β -PRVB peptide biomarkers by Selected MS/MS Ion Monitoring (SMIM) in a linear ion trap (LIT) mass spectrometer. The present strategy allows the direct detection of the presence of fish β -PRVBs in any food product in less than 2 hours. This new affordable assay would be very useful for the sanitary and inspect authorities to protect sensitive consumers from allergic reactions and to guarantee their safety.

Keywords: allergen, proteomics, Selected MS/MS Ion Monitoring (SMIM), High Intensity Focused Ultrasound (HIFU), mass spectrometry, parvalbumin, PRVB, fish, food, marine.

INTRODUCTION

Fish is one of the most frequent causes of immunoglobulin E (IgE)-mediated food allergy. In the general population, its prevalence has been estimated to be around 0.2-0.6% [1]. The symptoms of this type of allergy (type-I) appear within 60 minutes of exposure and include acute and generalized urticaria, nausea, vomiting, abdominal cramps, diarrhoea, wheezing and asthma [2]. In the most severe cases, anaphylaxis shocks can potentially be life threatening [3]. The only proven and effective treatment is to conduct a diet free of fish and their derivatives. However, new developments in the characterization of epitopes on fish allergens are becoming the target for the development of novel diagnostic tools and specific immunotherapies [4-6]. Fish-sensitive patients are commonly allergic to numerous fish species [7]. In fact the clinical cross-reactivity is strongest for taxonomically closely related species, due to homologies in the sequence of the major allergen [8]. Fish species that cause allergy can be classified based on cluster analysis of immunoglobulin reactivities in mainly two groups, (a) salmon and mackerel and (b) cod and tuna [9].

Parvalbumins beta (β -PRVBs), which are found in high amounts in the sarcoplasmic fraction of white muscle of fishes, are considered as the major fish allergens [10-13]. They have a molecular weight around 10-12 kDa, an acidic pI (3.0-5.0) and three EF-hand motifs, two of them with high affinity by Ca^{2+} . The allergenic properties of these proteins are related with their resistance to certain gastrointestinal enzymes and their heat resistance [14].

The first identified and purified fish allergen corresponded to the β -PRVB of the cod *Gadus callarias*, also known as allergen M or Gad c1 [10]. Recently, results of the extensive *de novo* mass spectrometry sequencing have allowed obtaining the sequence of 41 new β -PRVB isoforms from Merlucciidae family [15]. Thus, a total of 163 β -PRVB sequences for all Teleostei group are available in the UniProtKB database (November 2011).

To guarantee the security to the consumers, a number of regulations in terms of food allergy have been implemented (Directive 2007/68/EC) [16]. In the European Union, these regulations compel the producers to label the fourteen food allergens, including fish and products thereof, when these have been intentionally introduced in the foodstuffs. However, some products on the market could contain traces of allergens due to cross-contaminations during the food manufacturing processes. As consequence, accurate, sensitive and fast detection methods that permit the direct recognition of allergens in food samples are highly recommendable.

At present the methods more used for the direct detection of β -PRVBs in the food products are the immunological methods [17]. Several polyclonal and monoclonal antibodies have been development [18-20]. However, the limiting factors of these techniques are the availability of an universal antibody or a combination of antibodies that covers all β -PRVBs isoforms, combined with the cross-reaction problems and the alteration of antibody binding in whose foodstuffs that were subjected to heating or technological food processing. DNA-based methods have been also developed [21, 22]. However the presence of fish DNA in a food product does not guarantee the presence of the allergen. Therefore, the development of an alternative and direct fast method that presents high reproducibility, sensitivity and specificity is necessary.

Given the limitations of the methods described above, a mass spectrometry-driven detection could provide a good alternative tool. Systematic analysis using high-resolution separation techniques in combination with mass spectrometry (MS) is used to detect and identify several allergenic proteins in the foodstuffs [23, 24]. Targeted-mass spectrometry approaches based on selective reaction monitoring (SRM) focused on specific peptides from allergen/s that result from a tryptic digest, are the specific monitoring methods more widely

used [24-26]. However, the application to the direct detection of allergenic fish proteins has not been explored.

The optimization for a definite SRM assay is also a time-consuming procedure and in general terms complete MS/MS spectra are not registered. The MS/MS spectrum of a peptide is of paramount importance to confirm the structure of the compound detected. Selected MS/MS Ion Monitoring (SMIM) in a linear ion trap (LIT) mass spectrometer is a monitoring-scanning mode that at the same time allows obtaining complete structural information about the peptide fragmented [27]. In this operating mode, the MS analyzer is programmed to perform continuous MS/MS scans on one or more selected precursor peptide ions along the whole chromatographic run or during a scheduled narrow retention time window. MS/MS spectra are recorded and virtual transitions for all different fragment ions can be plotted. This method has the capability to obtain high confident MS/MS spectra due to the average of individual spectra. The utility of this operating monitoring mode has been demonstrated in several previously published studies [15, 27-30].

Once SMIM targeted-approach has been optimized, the sample preparation continues being one the most time-consuming steps in any bottom-up proteomics workflow. In order to simplify this step, if a minor number of different proteins are the targets of the analysis, a lower risk of false positives and a minor time of SMIM analysis will be necessary. Fast and easy protein fractionation or purification steps conducted prior to LC-MS analysis, makes the analysis simpler and faster [31]. In addition, procedures to enhance the protease activity, such as the application of microwaves [32], high pressure [33], or the energy produced by ultrasound [34], can accelerate the time consuming trypsin digestion. The application of only 1-2 minutes of High Intensity Focused Ultrasound (HIFU) to in-solution tryptic digestions has been reported to achieve an efficiency and reproducibility similar to that obtained by traditional overnight protocols [34-36].

Therefore, in this study we propose a new strategy for the fast direct detection of the fish β -PRVBs based on: (a) the purification of β -PRVBs by heat treatment (Time: 45 min), (b) their accelerated tryptic digestion using HIFU (Time: 2 min) and (c) the monitoring of several common β -PRVBs peptide biomarkers (nineteen) by SMIM in a LIT mass spectrometer (Time: 60 min). Each step was individually adjusted to minimize the time of analysis. With this strategy, the direct detection of fish β -PRVBs in any food products, including processed and precooked can be achieved in less than 2 h.

MATERIALS AND METHODS

1. Reference species and commercial foodstuffs

A total of 16 different raw fish species and 6 commercial sea-foodstuffs were employed in this study (Table 1). These were purchased from local markets and were selected in order to include the most commonly consumed fish species in Europe including the main fish species that cause allergy in children [9]. The reference species were identified by a marine expert biologist and by genetic identification in the Food Biochemistry laboratory from the Marine Research Institute (Vigo, Pontevedra, Spain) with the fished Kit (Bionostra SL, Madrid, Spain). To validate the method against other species, 6 non-fish food species were also included. Thus, all the 28 different samples were analyzed in duplicate.

2. Parvalbumin purification

Sarcoplasmic protein extraction was carried out by homogenizing 5 g of white muscle in 10 mL of 10 mM Tris-HCl pH 7.2, supplemented with 5 mM PMFS, during 30 s in an Ultra-Turrax device (IKA-Werke, Staufen, Germany) [37]. The sarcoplasmic proteins extracts were then centrifuged at 40000 g for 20 min at 4 °C (J221-M centrifuge; Beckman, Palo Alto, CA). β -PRVBs were purified by taking advantage of their thermostability, heating the sarcoplasmic extracts at 70 °C for 5 min [15]. After centrifugation at 40000 g for 20 min (J221-M centrifuge, Beckman, Palo Alto, CA), supernatants composed mainly by β -PRVBs were quantified by the bicinchoninic acid (BCA) method (Sigma-Chemical Co., USA).

3. Parvalbumin digestion using HIFU

PRVBs supernatants were subjected to HIFU-assisted trypsin digestion as previously described [34]. A total of 20 µg of heated extract were subjected to in-solution digestion with 1 µg trypsin without adding urea, DTT or iodoacetamide (Promega, Madison, WI, USA) applying simultaneously HIFU. A high-intensity ultrasonic probe of 1 mm probe tip (Dr. Heilscher, Teltow, Germany) was set to 50% of amplitude and was used to perform the ultrafast digestion for 1 min. Another 1 µg of trypsin was added again to the sample and the HIFU application was repeated for 1 min.

4. LC-MS/MS analysis.

Peptide digests were acidified and analyzed by LC-ESI-IT-MS/MS using a Agilent 1100 microflow system (Agilent Technologies) coupled to an LTQ LIT mass spectrometer (Thermo Fisher, San Jose, CA). The peptide separation (1 µg) was performed on a RP column (75 µm x 15 cm) packed in house with C₁₈ resin (Magic C₁₈ AQ 5 µm; Michrom BioResources, Auburn, CA) using 0.15% formic acid in Milli-Q-water and 98% ACN and 0.15% formic acid as mobile phases A and B, respectively. A 60 min linear gradient from 5 to 40% B, at a flow rate of 1.2 µL/min was used. For ionization, 1.95 kV of spray voltage and 230°C of capillary temperature were used. Peptides were analyzed in positive mode from 400 to 1600 amu (three microscans), followed by four data-dependent MS/MS scans (three microscans), using an isolation width of 3 amu and a normalized collision energy of 35%. Fragmented masses were set in dynamic exclusion for 3 min after the second fragmentation event, and singly charged ions were excluded from MS/MS analysis.

5. Selected MS/MS Ion Monitoring (SMIM)

SMIM analysis was performed using a Agilent 1100 microflow system (Agilent Technologies) coupled to an LTQ LIT mass spectrometer (Thermo Fisher, San Jose, CA), as described previously [27] with minor modifications. The peptide separation (1 μ g) was performed on an RP column (75 μ m x 15 cm) packed in house with C₁₈ resin (Magic C₁₈ AQ 5 μ m; Michrom BioResources, Auburn, CA) using 0.15% formic acid in Milli-Q-water and 98% ACN and 0.15% formic acid as mobile phases A and B, respectively. A 45 min linear gradient from 5 to 40% B, at a flow rate of 1.2 μ L/min was used. For ionization, 1.95 kV of spray voltage and 230°C of capillary temperature were used. Peptides were detected in positive ion mode using the SMIM mode [27]. For this method, the MS instrument was programmed to perform continuous MS/MS scans (3 μ scans) of doubly-charged precursor ions from all candidate peptide biomarkers along the complete chromatographic separation. Normalized collision energy was set to 35% and a 1 amu mass window was used to fragment selected parent ions.

6. Mass spectrometry data processing

MS/MS spectra were searched using SEQUEST (Bioworks 3.1 package, Thermo Fisher), against the Teleostei UniProt/TrEMBL database (release 2010_12; 158,545 entries), which also included their respective decoy sequences. The following constraints were used for the searches: semi-tryptic cleavage with up to two missed cleavage sites and tolerances 1.2 Da for precursor ions and 0.5 Da for MS/MS fragments ions. The variable modifications allowed were methionine oxidation (Mox), carbamidomethylation of Cys (C*) and acetylation of the N-terminus of the protein (N-Acyl). The database search results were subjected to statistical analysis with the PeptideProphet algorithm (v.4.4) [38]. The FDR was kept below 1%.

Virtual chromatograms traces were plotted and optimized using QualBrowser software (Thermo Fisher) to show the selected transitions for each parent ion. MS/MS spectra collected from the SMIM mode were used to validate the peptide identities using the search engine SEQUEST as is described before.

RESULTS AND DISCUSSION

1. Strategy for the rapid and direct detection of the major fish allergen (β -PRVB)

The strategy for the rapid and direct detection of the major fish allergen (β -PRVB) in any foodstuff is summarized in Figure 1. This strategy integrates three main steps: (a) purification of β -PRVBs by a short heat treatment followed by centrifugation (time 45 min), (b) in-solution trypsin digestion accelerated using HIFU (time 2 min) and (c) monitoring of 19 β -PRVB peptide biomarkers by SMIM using a LIT mass spectrometer (time 60 min). With this strategy, the presence of the major fish allergen, β -PRVBs, can be detected in any seafood product, including processed and precooked, in less than 2 h. The detailed results for each step and the validation of this new direct monitoring strategy using commercial products are shown in the following sections.

2. Parvalbumin purification and enzymatic digestion accelerated by HIFU

β -PRVBs were purified from the sarcoplasmic extracts taking advantage of their thermostability [15]. Figures S-1a to S-1k in the Supplemental Data 1 (Supporting Information), show a summary of the protein composition in the extracts for each species before and after the treatment with heat (70°C for 5 min). Complete list of proteins and peptides for both samples identified by LC-MS/MS and Sequest search are present in the Supplemental Data 2 (original) and 3 (heated). For the majority of species tested and taking

into account the availability of entries of β -PRVBs sequences in the UniProtKB database, after treatment with heat the majority of identified peptides corresponded to β -PRVBs (30~90%). These results demonstrated that the treatment with heat is a simple, fast and effective procedure to purify and enrich the samples in β -PRVBs.

Once purified the β -PRVBs, accelerated tryptic digestions using HIFU were compared with conventional overnight procedures. According to previous publications [34] the results showed a successful and reproducible in-solution HIFU digestion equivalent to the conventional overnight incubation methods (data not shown) [34]. Moreover, the absence of urea in the buffer prevented undesired peptide side reactions, such as carbamylation of N-termini and Lys residues, which may occur when HIFU is applied in presence of urea [33, 39].

The combination of a fast and easy protein purification procedure (Time: 45 min) with the use of HIFU for the protein digestion (Time: 2 min), considerably simplified and reduced the time needed for the sample preparation, reflected in the overall time needed for monitoring.

3. Selection of β -PRVBs peptide biomarkers

The next step in the proposed strategy was to select the smaller number of β -PRVB peptides which can function as biomarkers. The results of the sequences alignment by ClustalW of 163 teleostei β -PRVBs available in the UniProtKB database, identified the most conserved region, corresponding to the sequence between the residues 46 to 77 (Figure 2). This region contains one helix-loop-helix (EF-hand) motif (CD domain) [40], functional for chelating Ca^{2+} , which is composed by a central 12-residue Ca^{2+} -binding loop flanking with two α -helix positioned roughly perpendicular to each other. The Ca^{2+} ion is coordinated by residues located in position (x, y, z, -x, -y, -z) related to the binding loop [41]. Thus, in this

CD domain, all residues localized in the Ca^{2+} -binding positions were conserved (x: Asp53, y: Asp55, z: Ser57, -x: Phe59, -y: Glu61, -z: Glu64) (Figure 2).

To determine the expected peptides in this conservative region, an *in silico* tryptic digestion using PeptideMass [42] was done on of 163 β -PRVB sequences. The results showed a total of 79 different fully tryptic peptides. Among them and according to specificity criteria by BLAST were selected only 17 as peptide biomarkers to cover the identification of all 163 β -PRVB sequences. Twelve of them were present only in β -PRVBs from fishes (B3, B5, B7, B9, B10, B11, B12, B13, B14, B15, B16, B17) and the remaining five (B1, B2, B4, B6, B8) shared sequences with β -PRVBs of others organisms such as different species of frogs, monotremes, lizards and birds. Consequently, an extra peptide biomarker (B18), specific for poultry species, was added in order to avoid cross-reactivity problems in food samples. Therefore, a total of 19 different peptide biomarkers were selected for this purpose (Table 2).

4. Rapid detection of β -PRVBs using SMIM

For each of the reference species (Table 1), β -PRVBs peptide pools obtained from the accelerated tryptic digestions were subjected to SMIM analysis in a LIT mass spectrometer focusing the MS/MS events on the corresponding precursor ions for the 19 peptides selected (Figure 3). The selected m/z value for each of precursor ion corresponded to the predominant charge state, which was +2 for all of them (Table 2). Figure S-3 in the Supplemental Data 1 details the MS/MS spectra for each peptide. Once MS/MS spectra were recorded, structural characterization was carried out and virtual chromatograms for all the different fragment ions could be obtained. For each of the peptide markers, mass transitions were noted according to the criteria of sensitivity and selectivity. As the peptide mixture used is not too complex, selectivity was not a matter of concern and the transitions chosen in every case was in accordance with the maximum intensity of the fragments, which mostly corresponded to y-

ions. Therefore, the combination of highly sensitive transitions (precursor $m/z \rightarrow$ fragment m/z) (Table 2), together with the use of simple peptide mixtures (coming mainly from β -PRVBs), make possible the representation of specific transitions with a high signal-to-noise (S/N) ratio. Tracing these transitions for each peptide biomarker described in the Table 2, it was possible to detect unequivocally the presence of β -PRVBs in all reference raw species in less than 2h (Figure 3).

To corroborate the method against other species, 6 non-fish species were also subjected to analysis (Figure 4). Due to cross-reactivity problems with the peptide biomarker (B2; SGFIEEEELK) in poultry species, an extra biomarker specific only for poultry species (B18; AVGAFSAAESFNYK) was included (Figure 4). In addition, an extra peptide biomarker specific only for frog species (B19; IGVEEFQALVK) was also included to discriminate the frog species (Figure 4).

Finally, in order to validate this new strategy in commercial sea-foodstuffs, six commercial seafood products were analysed. Table 1 summarizes the products that were tested, which had been previously subjected to one or more processing treatments, even precooked. Results for the detection of β -PRVBs in commercial samples using the designed strategy are shown in the Figure 5. In all the samples, included precooked products, the presence of β -PRVBs was detected. Thus, the heat resistance properties of these allergens, allows the possibility of their fast and direct detection in different type of products, including battered precooked.

CONCLUSIONS

A new strategy for a rapid and direct detection of the major fish allergen, β -PRVB, in any foodstuff is described. The principle it is based on the use of a fast purification step of the β -PRVBs by treatment with heat, the acceleration of in-solution protein digestion by HIFU, and the monitoring of 19 peptide biomarkers by SMIM in a linear ion trap mass spectrometer. The methodology reported here allows the direct detection of presence of fish β -PRVBs in any food product, in less than 2 hours. To our knowledge, this is the fastest method to achieve the direct detection of these allergens.

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FIGURE CAPTIONS

Figure 1: Analytical scheme for the fast detection of the fish β -PRVBs proposed in this work.

Figure 2: Highly conserved region of the teleostei β -PRVBs sequence between residues 46-77.

Figure 3: SMIM traces for each of the reference fish species, plotting the corresponding canonical transition for each peptide biomarker derived from β -PRVBs tryptic digestion.

Figure 4: SMIM traces for each of the reference non-fish species, plotting the corresponding canonical transition for each peptide biomarker derived from β -PRVBs tryptic digestion.

Figure 5: SMIM traces for each of the commercial foodstuffs, plotting the corresponding canonical transition for each peptide biomarker derived from β -PRVBs tryptic digestion.

Table 1. Reference species and commercial foodstuffs considered in the study.

Reference fish species

Sample	Species (Order)	Common name	β -PRVBs in UniProKB or NCBI
S1	<i>Brama brama</i> (Perciformes)	Ray's bream	---
S2	<i>Diplodus sargus</i> (Perciformes)	White seabream	---
S3	<i>Gadus morhua</i> (Gadiformes)	Cod	Q90YK9, A5I874, Q90YL0, A5I873
S4	<i>Genypterus blacodes</i> (Ophidiiformes)	Pink cusk-eel	---
S5	<i>Lepidorhombus boscii</i> (Pleuronectiformes)	Four-spot megrim	---
S6	<i>Lophius piscatorius</i> (Lophiiformes)	Angler	---
S7	<i>Merluccius australis australis</i> (Gadiformes)	Austral hake	P86745, P86747, P86748, P86746
S8	<i>Merluccius paradoxus</i> (Gadiformes)	Deep-cape hake	P86756, P86757, P86755
S9	<i>Pagellus bogaraveo</i> (Perciformes)	Common seabream	---
S10	<i>Salmo salar</i> (Salmoniformes)	Salmon	Q91482, B5DH15, E0WD98, Q91483, E0WD99
S11	<i>Scomber japonicus</i> (Perciformes)	Club mackerel	P59747
S12	<i>Solea solea</i> (Pleuronectiformes)	Common sole	---
S13	<i>Sparus aurata</i> (Perciformes)	Gilthead seabream	D0VB96, Q4Qy67
S14	<i>Thunnus albacares</i> (Perciformes)	Yellowfin tuna	C6GKU3
S15	<i>Trachurus trachurus</i> (Perciformes)	Horse mackerel	---
S16	<i>Xiphias gladius</i> (Perciformes)	Swordfish	B9W4C2

Non-fish species

Sample	Species	Common name	β -PRVBs in UniProKB or NCBI
S17	<i>Gallus gallus</i> (muscle leg)	Common chicken	P19753, P43305

S18	<i>Meleagris gallopavo</i> (muscle leg)	Common turkey	XP_ 03210583
S19	<i>Bos taurus</i>	Beef	---
S20	<i>Sus scrofa</i>	Pork	---
S21	<i>Penaeus vannamei</i>	Shrimp	---
S22	<i>Rana spp.</i>	Frog	P02617, P84536, Q802R7, Q8JIT9, Q8JIU1
Commercial sea-foodstuffs			
Sample	Species		
S23	Dried salted fillets of cod (Gadiformes)		
S24	Frozen surimi		
S25	Baby food with vegetables and angler (Lophiiformes)		
S26	Baby food with peas, rice and hake (Gadiformes)		
S27	Baby food with vegetables and hake (Gadiformes)		
S28	Baby food with béchamel sauce and common sole (Pleuronectiformes)		

Table 2. Peptide biomarkers for the detection of the β -PRVBs.

Biomarker code	Peptide Sequence	SMIM Transition m/z precursor ion (z) $\rightarrow$ m/z fragment ion	163 PRVB	Cross-reaction with PRVBs from other organisms by BLAST	Retention time (min)
B1	SGFIEEDELK	583.78 $\rightarrow$ 762.35 (y''_6^+)	68	<i>Gallus gallus</i> , <i>Meleagris gallopavo</i> , <i>Taeniopygia guttata</i> , <i>Ornithorhynchus anaticus</i> , <i>Anolis carolinensis</i> , <i>Xenopus laevis</i> , <i>Xenopus tropicalis</i>	22.10
B2	SGFIEEEELK	590.79 $\rightarrow$ 776.36 (y''_6^+)	23	<i>Gallus gallus</i> , <i>Meleagris gallopavo</i> , <i>Taeniopygia guttata</i> , <i>Xenopus laevis</i> , <i>Xenopus tropicalis</i> , <i>Rana catesbeiana</i> , <i>Cavia porcellus</i> , <i>Triakis semifasciata</i>	22.06
B3	SDFVEEDELK	605.77 $\rightarrow$ 861.42 (y''_7^+)	18	----	21.08
B4	LFLQNFSAGAR	612.33 $\rightarrow$ 963.50 (y''_9^+)	48	<i>Xenopus laevis</i> , <i>Xenopus tropicalis</i> , <i>Latimeria chalumnae</i>	26.25
B5	LFLQTFSAGAR	605.83 $\rightarrow$ 709.36 (y''_7^+)	12	----	26.90
B6	LFLQNFSASAR	627.33 $\rightarrow$ 752.37 (y''_7^+)	20	<i>Gallus gallus</i> , <i>Xenopus laevis</i> , <i>Xenopus tropicalis</i>	27.68
B7	VIDQDASGFIEVEELK	896.45 $\rightarrow$ 1336.66 (y''_{12}^+)	8	----	20.78
B8	SGYIEEEELK	598.78 $\rightarrow$ 889.45 (y''_7^+)	3	<i>Latimeria chalumnae</i>	26.36
B9	FFAIIDQDHSGFIEEEELK	1134.04 $\rightarrow$ 1675.74 (y''_{14}^+)	10	----	31.46
B10	LFLQNFAAGAR	604.44 $\rightarrow$ 706.36 (y''_7^+)	3	----	23.25
B11	LFLQNFCPK	555.29 $\rightarrow$ 736.34 (y''_6^+)	9	----	23.41
B12	AFAIIDQDNSGFIEEDELK	1077.51 $\rightarrow$ 1638.71 (y''_{14}^+)	3	----	28.54
B13	LFLQTFGAGAR	590.82 $\rightarrow$ 679.35 (y''_7^+)	1	----	30.42
B14	EGFIEEDELK	604.78 $\rightarrow$ 875.44 (y''_7^+)	1	----	nd
B15	AFAIIDQDNSGFIEEEELK	1084.52 $\rightarrow$ 1537.70 (y''_{12}^+)	2	----	31.70
B16	AFAIIDQDISGFIEEEELK	1084.04 $\rightarrow$ 1408.68 (y''_{12}^+)	1	----	nd
B17	AFHLLDADNSGFIEEEELK	1089.02 $\rightarrow$ 1821.87 (y''_{16}^+)	1	----	nd
B18	AVGAFSAAESFNKY	731.35 $\rightarrow$ 1163.54 (y''_{10}^+)	----	<i>Gallus gallus</i> , <i>Meleagris gallopavo</i> , <i>Taeniopygia guttata</i>	31.06
B19	IGVEEFQALVK	616.85 $\rightarrow$ 963.51 (y''_8^+)	----	<i>Rana temporaria</i> , <i>Rana esculenta</i> , <i>Rana catesbeiana</i> , <i>Xenopus laevis</i> , <i>Limnonectes macrodon</i>	25.80

nd (not determined)

Figure 1

a) Time: 45 min

SAMPLE PROBLEM

SARCOPLASMIC
PROTEIN
EXTRACTION

PURIFICATION OF THERMOSTABLE
PROTEINS (**PARVALBUMINS**)

70°C for 5min

b) Time: 2 min

ACCELERATED TRYPTIC DIGESTION BY **HIFU**

c) Time: 60 min

MONITORING OF **19** PRVB PEPTIDE BIOMARKERS BY **SMIM**

FISH ALLERGEN (PRVB) DETECTION

Total time: <2h (107 min)

Figure 2

Figure 3

Figure 4

Figure 5

Graphical abstract

Highlights

The present paper allows the direct detection of the presence of fish β -PRVBs (the major fish allergens) in any food product in less than 2 hours. The proposed methodology is based on the purification of β -PRVBs by treatment with heat, the use of accelerated in-solution trypsin digestion under an ultrasonic field provided by High-Intensity Focused Ultrasound (HIFU) and the monitoring of only nineteen β -PRVB peptide biomarkers by Selected MS/MS Ion Monitoring (SMIM) in a linear ion trap (LIT) mass spectrometer. This new affordable assay would be very useful for the sanitary and inspect authorities to protect sensitive consumers from allergic reactions and to guarantee their safety.