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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.



## 1. 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, nauseas, vomiting, abdominal cramps, diarrhoea, wheezing and asthma [2]. In the most severe cases, anaphylaxis shocks can potentially 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 p*I* (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

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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.

## 2. Materials and Methods

### 2.1. *Reference species and commercial foodstuffs*

A total of 16 different raw fish species and 6 commercial seafoodstuffs 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 fishID 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.

### 2.2. *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).