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Fish Authentication by MALDI-TOF Mass Spectrometry

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Recent EU directives and regulations for quality control and authentication of food products have prompted the development of new methods for large-scale tests to ensure the protection of consumers. In view of this, an innovative method based on MALDI-TOF mass spectrometry has been developed and successfully applied to fish authentication. Highly specific mass spectrometric profiles from 25 different fish species were obtained. Signals generated from proteins with molecular weights of about 11 kDa have been selected as specific biomarkers for unambiguous discrimination. This method is also suitable for verifying commercial product authenticity and to rapidly discriminate species subjected to fraudulent substitutions, such as those belonging to Gadidae and Pleuronectiformes. For example, biomarkers for fillets of sole (*m/z* 11975.21), European plaice (*m/z* 11351.73, 11763.63) and Greenland halibut (*m/z* 11432.38) were defined. Structural characterization by mass spectrometry of several proteins generating biomarker signals allowed us to identify them as parvalbumins, known to be among the major fish allergens.

KEYWORDS: Fish authentication; MALDI-TOF-MS; molecular profiling; fish frauds

INTRODUCTION

Consumers are increasingly aware of the beneficial effects of fishery products on human health. These are likely due to the high content of polyunsaturated fatty acids belonging to the ω-3 series which have been proven to help prevent cancer and cardiovascular and inflammatory diseases (*1*). The rise in the consumption of fishery products is mainly due to these scientific findings. As a result, the market has seen an increase in the sale of fish captured in Asian and African seas and of a wider range of processed fish-based foods.

The assessment of fish authentication and origin is an important requisite to ensure adequate quality and safety controls, especially in terms of consumer protection. Moreover, fish authentication obtained by innovative methodologies is key to detecting possible falsification, deceptive practices, and fraudulent substitution of commercially valuable fish species with inferior ones (*2, 3*). In fact, identifying morphological characteristics, such as the head, fins, skin, or bones, are lost during processing, and, in addition, a growing number of species are used for transformed products mostly because of globalization and freer markets. Since 2002, European Union directives and regulations regarding fishery and aquaculture products have established that the species, its geographical origin, and production method (wild or cultivated) must be labeled (Council Regulation (EC) No.104/2000 and 2065/2001 of the European Parliament) in order to guarantee market transparency. Moreover, the European Food Safety Authority has established a comprehensive system of traceability for food (including fishery and aquaculture products) and feed businesses to ensure food safety at all stages (EC regulation no. 178/2002 of the European Parliament reviewed in ref *4*).

In this context, methods based on DNA analysis, such as RAPD-PCR, RFLP, and SSCP (*5−7*) or protein analysis, including SDS-PAGE, IEF (*8, 9*), and immunological techniques (*10*), have been developed (*11*). Furthermore, proteomics has been applied to identify fish species (*12−15*) and to characterize protein alterations due to postmortem changes or processing conditions (*16*). However, the need for rapid screening of large numbers of samples calls for research activity aimed to develop high-tech approaches with minimal time consumption, low costs, high accuracy, and sensitivity and that could successfully complement methods already in use.

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Table 1. List of the Analyzed Fish Species

order	family	genus	species	common name[a]
Perciformes	Serranidae	*Dicentrarchus*	*Dicentrarchus labrax*	seabass
		Epinephelus	*Epinephelus marginatus*	dusky grouper
	Sparidae	*Sparus*	*Sparus auratus*	seabream
		Pagellus	*Pagellus acarne*	axillary seabream
		Pagellus	*Pagellus erythrinus*	common pandora
		Diplodus	*Diplodus sargus*	white seabream
	Centracantidae	*Spicara*	*Spicara maena*	blotched picarel
	Mullidae	*Mullus*	*Mullus barbatus*	red mullet
	Uranoscopidae	*Uranoscopus*	*Uranoscopus scaber*	Atlantic stargazer
	Percidae	*Perca*	*Perca fluviatilis*	European perch
	Triglidae	*Aspitriglia*	*Aspitrigla cuculus*	East Atlantic red gurnard
	Cichlidae	*Tilapiini*	*Tilapiine cichlids*	tilapias
Gadiformes	Gadidae	*Gadus*	*Gadus morhua*	Atlantic cod
		Merluccius	*Merluccius merluccius*	European hake
			Merluccius capensis	shallow-water cape hake
			Merluccius hubbsi	Argentine hake
			Merluccius paradoxus	deep-water cape hake
		Trisopterus	*Trisopterus minutus minutus*	poor cod
		Molva	*Molva elongata*	Mediterranean ling
		Phycis	*Phycis blennioides*	greater forkbeard
		Micromesistius	*Micromesistius poutassou*	blue whiting
Pleuronectiformes	Bothidae	*Arnoglassus*	*Arnoglossus laterna*	scaldfish
	Pleuronectidae	*Reinhardtius*	*Reinhardtius hippoglossoides*	Greenland halibut[b]
		Pleuronectes	*Pleuronectes platessa*	European plaice
	Soleidae	*Solea*	*Solea solea*	common sole

[a] From FishBase (www.fishbase.org). [b] Analysis has been carried out on fillets.

Recently "molecular profiling" strategies based on matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) have been thoroughly applied to biomarker discovery in life science (*17*), as well as to food science (*18*) and bacteria identification (*19*). In this study, we applied the molecular profiling approach to fish authentication. MALDI-TOF-MS analysis of protein extracts from muscle tissue yielded molecular profiles exhibiting a pattern of a few highly intense signals that could be considered species-specific biomarkers. The developed method allowed for discrimination among different species, even those phylogenetically closely related, and the authentication of commercial products. Peptide mass fingerprint (PMF) strategy (*20*) and tandem mass spectrometric experiments led to the identification of proteins, giving rise to some of those biomarkers such as parvalbumins, proteins which are particularly abundant in fish muscle tissue and responsible for fish allergy (*21*).

MATERIALS AND METHODS

Cytochrome C, α-cyano-4-hydroxycynnamic acid (CHAC), dithiothreitol, angiotensin III, adrenocorticotropic hormone (clip 18−39) (ACTH), trypsin, and iodacetamide were purchased from Sigma (St. Louis, MO). Trifluoroacetic acid (TFA) and all the used organic solvents were of the highest purity available from Carlo Erba (Milan, Italy). C_{18} ZipTip pipette tips were from Millipore (Billerica, MA). *Gadus morhua*, *Merluccius* species, *Trisopterus minutus minutus*, *Molva elongata*, *Phycis blennoides*, and *Micromesistius poutassou* were provided by colleagues at the Department of Biological Science, Division of Zoology, University of Naples "Federico II", Naples, Italy, who also identified, by means of morphological criteria, all the other fish species analyzed (**Table 1**) purchased from the local market (*22, 23*).

The composition of commercial products analyzed (fillets and fishsticks) was taken from the product labels. All samples were stored at −20 °C until use.

Extraction of Muscle Tissue Proteins. Samples of fish muscle were cut in small pieces, and 0.1−0.2 g aliquots were extracted in 0.5 mL of 0.1% TFA; samples were centrifuged at 13000 rpm for 10 min, and supernatants were used for the following analyses. Experiments were carried out on five different individuals, and three protein extracts were obtained for each individual. Protein concentration was measured by Bradford assay (*24*) (1.5−2.0 μg/μL for all the analyzed samples). Samples of *Sparus auratus, Solea solea*, *Merluccius merluccius*, *Molva elongata*, *Phycis blennoides*, *Micromesistius poutassou* were also treated at 100 °C for 15 min before protein extraction.

MALDI-TOF-MS Analysis. One microliter of protein extract was deposited onto a MALDI target plate, mixed with 1 μL of matrix (10 mg/mL CHAC, 50% acetonitrile (ACN) in 0.1% TFA containing 1 pmol/μL of cytochrome C as internal standard) and dried under ambient conditions. All mass spectra were acquired on a MALDI-TOF mass spectrometer Voyager DE^{PRO} (Applied Biosystems, Foster City, CA), operating in linear, delay extraction, and positive-ion mode. The laser intensity (N_2, 337 nm) was set just above the ion generation threshold. The low mass gate was set to *m/z* 1990, the delay time was 500 ns, the accelerating voltage was 25000 V, and the grid voltage and guide wire were set to 95% and 0.1% of the accelerating voltage, respectively. Mass spectra were acquired by accumulating 100 laser shots in a *m/z* range 2000−15000 and calibrated using as internal standards the doubly and singly charged ions originated from cytochrome C (*m/z* 6181.05 and 12361.10, respectively). All *m/z* values were reported as average.

Mass spectra were run in triplicate on each protein extract so that for each species 45 mass spectra were acquired; arithmetic means and standard deviations for the *m/z* values were calculated. The accuracy of mass measurements was such that standard deviations were lower than 2 Da (**Table 2**).

Structural Analyses of Proteins. Protein extracts from *Trisopterus minutus minutus*, *Merluccius merluccius*, and *Sparus auratus* were analyzed by RP-HPLC on a Vydac C18 column (25 × 0.46 cm, 5 μm) using a Waters HPLC instrument (Waters Milford, MA) and an elution system consisting of 0.1% TFA (solvent A) and 95% ACN in 0.07% TFA (solvent B). Separation was achieved by means of a linear gradient from 35% to 65% solvent B over 30 min. Fractions were analyzed by MALDI-TOF-MS, and those containing proteins generating biomarkers were dried in a Speed-Vac centrifuge. Protein digestion, reduction and alkylation, desalting of the obtained tryptic peptide mixture, and MALDI-TOF-MS analyses were carried out as already described (*25*).

Protein Identification. Identification of proteins giving rise to biomarkers was achieved by PMF strategy using the MASCOT search engine (http://www.matrixscience.com) for searching against the NCBInr database. Parameters for database searches were as follows: no taxonomic category was specified, trypsin as enzyme, carbamidom-

Table 2. MALDI-TOF-MS Analysis of the Different Fish Species: The Biomarker Pattern Is Reported

analyzed species	$m/z \pm$ SD [a]	analyzed species	$m/z \pm$ SD [a]
Dicentrarchus labrax	11404.49 ± 0.97	*Gadus morhua*	11366.46 ± 0.88
	11495.56 ± 1.14		11463.68 ± 0.76
Epinephelus marginatus	11606.77 ± 0.22	*Merluccius merluccius*	8432.86 ± 0.80
	11645.19 ± 0.68		11338.59 ± 0.94
			11361.27 ± 1.29
			11388.58 ± 1.30
Sparus auratus	11441.99 ± 1.00	*Merluccius capensis*	8432.93 ± 1.25
	11370.28 ± 1.23		11337.95 ± 1.47
			11360.84 ± 1.39
			11387.81 ± 1.74
Pagellus acarne	11563.26 ± 0.85	*Merluccius hubbsi*	8436.57 ± 1.56
	11407.36 ± 0.72		11338.68 ± 1.28
			11362.18 ± 1.25
			11386.91 ± 1.24
Pagellus erythrinus	11606.18 ± 0.97	*Merluccius paradoxus*	8476.48 ± 0.91
	11585.81 ± 0.72		11338.63 ± 0.72
	11429.89 ± 0.66		11387.36 ± 1.30
Diplodus sargus	11445.73 ± 0.89	*Trisopterus minutus minutus*	11351.36 ± 0.60
	11301.48 ± 0.78		11310.70 ± 1.52
Spicara maena	11507.34 ± 0.17	*Molva elongata*	11550.99 ± 0.79
	11374.41 ± 0.75		
	11247.55 ± 1.18		
Mullus barbatus	11383.54 ± 1.20	*Phycis blennoides*	11552.78 ± 0.55
	11544.64 ± 1.04		11446.69 ± 0.78
Uranoscopus scaber	11731.26 ± 1.30	*Micromesistius poutassou*	11448.28 ± 0.37
	12078.70 ± 1.23		11350.48 ± 0.41
	11874.14 ± 1.78		
Perca fluviatilis	11434.85 ± 0.95	*Arnoglossus laterna*	11550.12 ± 1.50
	11402.86 ± 1.02		11478.29 ± 1.29
			11783.03 ± 1.28
Aspitrigla cuculus	11639.45 ± 0.61	*Reinhardtiius hippoglossoides*	11433.51 ± 1.30
	11519.68 ± 0.64		
	11195.20 ± 0.97		
Tilapiine cichlids	11382.01 ± 0.51	*Pleuronectes platessa*	11764.14 ± 0.84
	11559.88 ± 0.86		11351.65 ± 0.63
		Solea solea	11975.58 ± 0.80

[a] The average values are reported as calculated from the 45 mass spectra acquired for each species.

ethyl as fixed modification for cysteine residues, up to two missed cleavages, and 30 ppm as mass tolerance for the monoisotopic peptide masses.

Tandem Mass Spectrometric Experiments. MS/MS experiments were carried out on an orthogonal hybrid quadrupole time-of-flight mass spectrometer (QStar/Pulsar, Applied Biosystems) equipped with a static nanoelectrospray ionization (nano-ESI) source (Proxeon Biosystems A/S, Odense, Denmark) as already described (*26*).

RESULTS AND DISCUSSION

The present study aimed to develop an innovative strategy based on MALDI-TOF-MS for fish authentication and for rapid detection of fraudulent substitutions. The applicability of the method was ascertained by analyzing protein extracts from 25 different fish species, selected among largely consumed products, either of high commercial value or commonly involved in frauds (**Table 1**).

Protein extracts from muscle tissues have been directly analyzed by MALDI-TOF-MS. Mass spectra acquired in the m/z range 2000−60000 revealed the presence of a few strong signals in a well-limited m/z range of 2000−15000; therefore, all the reported data refer to this m/z range. The extracted sarcoplasmic protein mixture was quite complex, as clearly demonstrated by SDS-PAGE and HPLC analyses of samples from *Dicentrarchus labrax* (seabass) and *Sparus auratus* (seabream), reported as an example in Supporting Information (Figures S-1 to S-3). Nevertheless, the corresponding mass spectra were extremely simple and could be considered as highly specific molecular profiles suitable for fish authentication. In fact, the signal pattern generated from proteins with molecular weights of about 11 kDa was specific of each analyzed species, and these signals could be selected as species-specific biomarkers for an unambiguous discrimination (**Table 2**). Mass spectra obtained from each analyzed species are reported in Supporting Information (Figures S-4 to S-21).

For example, *Dicentrarchus labrax* and *Sparus auratus,* two species widely consumed and of high commercial value, were easily discriminated. In fact, the most intense signals in the mass spectrum of *Dicentrarchus labrax* were at m/z 11403.70 and 11495.33, whereas for *Sparus auratus* two strong peaks were detected at m/z 11441.24 and 11370.03 (**Figure 1**).

Similarly, two very closely phylogenetically related species of the *Pagellus* genus (*Pagellus acarne* and *Pagellus erythrinus*) were discriminated by peaks at m/z 11563.26 and 11407.36 present in the mass spectrum of *Pagellus acarne* and absent in that of *Pagellus erythrinus,* which showed instead three biomarkers at m/z 11606.18, 11585.81, and 11429.89 (Figures S-5, S-6 in Supporting Information).

It is worth noting that the proposed method assures a rapid authentication of widely consumed species within the Gadidae family, such as *Merluccius* species, *Gadus morhua*, *Trisopterus minutus minutus*, *Phycis blennoides*, *Molva elongata*, and *Micromesistius poutassou*. In particular, the commercially valuable *Gadus morhua* (biomarkers at m/z 11366.46, 11463.68) or *Merluccius* species (genus-specific biomarkers at approximately m/z 11338, 11387), used for many transformed products, can be rapidly discriminated from *Molva elongata* (biomarker at m/z 11550.99) and *Phycis blennoides* (biomarkers at m/z

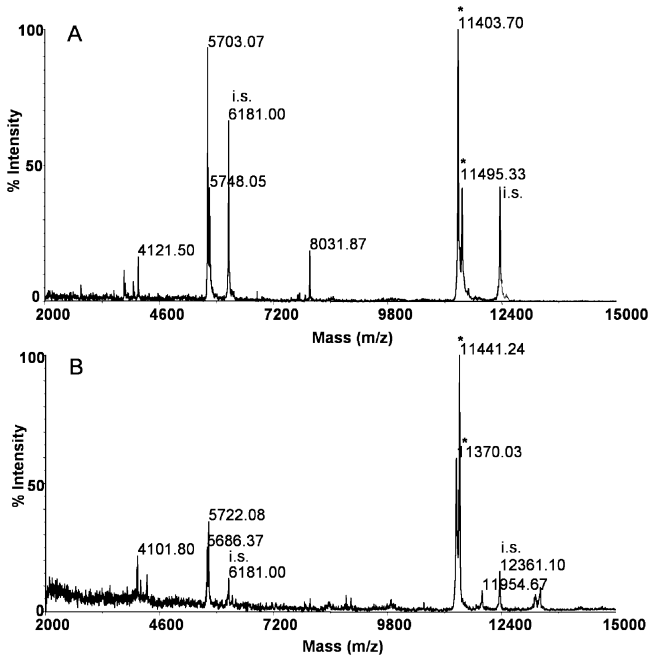

Figure 1. MALDI-TOF mass spectra obtained from the analysis of (A) *Dicentrarchus labrax* (seabass) and (B) *Sparus auratus* (seabream). Biomarker pattern is indicated with asterisks.

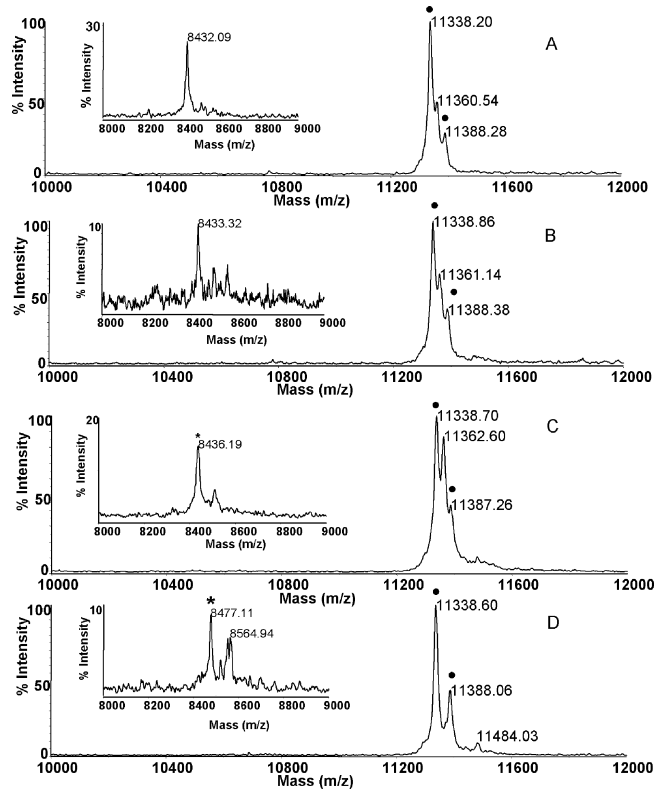

Figure 2. MALDI-TOF mass spectra of different *Merluccius* species: (A) *Merluccius merluccius*; (B) *Merluccius capensis*; (C) *Merluccius hubbsi*; (D) *Merluccius paradoxus*. Genus-specific biomarkers are indicated with dots; biomarkers for *Merluccius hubbsi* and *Merluccius paradoxus* are indicated with asterisks.

11552.78 and 11446.69), two species having less commercial value and often used in the substitution of fresh products. Similarly, *Trisopterus minutus minutus* (biomarkers at *m/z* 11351.36 and 11310.70) and *Micromesistius poutassou* (biomarkers at *m/z* 11448.28 and 11350.48) being smaller in size and commonly used for fraudulent substitutions of transformed products (e.g., canned fish), can be identified by a specific signal pattern (**Figure 2** and Figures S-14 to S-18 in Supporting Information). As already reported, these species can be discriminated at molecular level by more laborious techniques such as PCR-RFLP (*27, 28*), SSCP analysis (*29*), or IEF analysis (*30*). This confirms the reliability and the usefulness of the present methodology.

To assess method specificity, experiments were performed on four different *Merluccius* species (*Merluccius merluccius, Merluccius capensis*, *Merluccius hubbsi*, and *Merluccius paradoxus*). The mass spectra of all of them shared the most intense signal at approximately *m/z* 11338 and a minor peak at approximately *m/z* 11387 (**Figure 2**). By allowing the rapid discrimination of *Merluccius*, these two genus-specific biomarkers can have the utmost importance in the development of quality control tests for commercial products labeled as *Merluccius*.

Mass spectra of *Merluccius merluccius, Merluccius capensis*, and *Merluccius hubbsi* also showed a strong signal at approximately *m/z* 11362 that was not present for *Merluccius paradoxus*. Moreover, in the mass spectra of *Merluccius merluccius* and *Merluccius capensis*, a signal at approximately *m/z* 8432 was present, while the mass spectra of *Merluccius hubbsi* and *Merluccius paradoxus* showed a signal at *m/z* 8436.19 and one at *m/z* 8477.11, respectively. Those patterns allowed a strict discrimination among the analyzed species (**Figure 2**).

Merluccidae are phylogenetically divided in two major classes, the American one including both Eastern Pacific and Western Atlantic species (*Merluccius bilinearis*, *Merluccius albidus*, *Merluccius hubbsi*, *Merluccius australis*, *Merluccius productus*, *Merluccius angustimanus*, *Merluccius gayi*), while the Euro-African species include the Eastern Atlantic hakes (*Merluccius merluccius*, *Merluccius senegalensis*, *Merluccius polli*, *Merluccius capensis*, *Merluccius paradoxus*). The biomarker patterns obtained are strongly related to the phylogeny of *Merluccius* genus. In fact, genetic data reported a wide gap between the two Euro-African lineages of hakes, e.g. *Merluccius paradoxus* and *Merluccius polli* and the group including *Merluccius merluccius*, *Merluccius capensis*, and *Merluccius senegalensis* (*31*).

Interestingly, proteins generating biomarkers exhibited a remarkable stability; in fact, mass spectra obtained from heat-treated samples of *Sparus auratus*, *Merluccius merluccius*, *Molva elongata*, *Phycis blennoides*, *Micromesistius poutassou*, and *Solea solea* (Figures S-24 to S-29 in Supporting Information) showed the same biomarker pattern of the untreated samples (**Figures 1**, **2** and Figures S-16 to S-18, S-21 in Supporting Information), suggesting the feasibility of the method for cooked products.

The present method could also be applied to verify commercial product authenticity, as demonstrated by experiments carried out on cod or sole fillets and fishsticks. Mass spectra of cod fishsticks and fillets not only showed the two *Merluccius* genus specific biomarkers but also identified fishsticks as *Merluccius capensis* and fillets as *Merluccius hubbsi,* in agreement with the labels (Figures S-22, S-23 in Supporting Information). Furthermore, the more valuable *Solea* species have often been subjected to fraudulent substitution (*30*). Worth noting, we were able to differentiate among fillets of sole (*Solea solea*; *m/z* 11975.21), European plaice (*Pleuronectes platessa*; *m/z* 11351.73, 11763.63), and Greenland halibut *(Reinhardtius hippoglossoides*; *m/z* 11432.38) on the basis of the specific signal patterns (**Figure 3**).

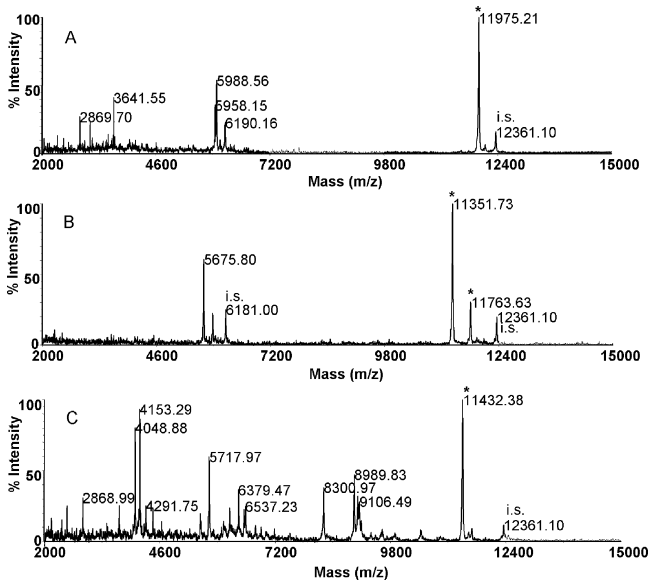

Figure 3. MALDI-TOF mass spectra obtained from fillets of (A) sole (*Solea solea*); (B) European plaice (*Pleuronectes platessa*); (C) Greenland halibut (*Reinhardtius hippoglossoides*). Biomarker pattern is indicated with asterisks.

It is important to note that this new method requires neither preliminary information of the sample under investigation nor preliminary identification of the proteins generating biomarkers. However, our data indicate that several biomarkers originated from parvalbumins, calcium-binding proteins with molecular weights in the 10000−12000 Da range, relatively abundant in muscle tissues and known as the major allergy-eliciting proteins (*21*).

In fact, the *Gadus morhua* biomarkers at *m/z* 11463.68 and 11366.46 corresponded to two isoforms of parvalbumin beta (accession numbers gi|32363376 (Allergen Gad m 1) and gi|14531014, respectively), lacking the Met residue in position 1 and having the N-terminal residue acetylated. These modifications already annotated in protein databases for the Allergen Gad m 1 could only be inferred for the other isoform (gi|14531014) on the basis of the measured molecular weight.

The *Merluccius* genus-specific biomarker at *m/z* 11338 matched the molecular weight of the parvalbumin beta isoform from *Merluccius merluccius* (accession number gi|131116) having the same modifications. The protein corresponding to the *Merluccius* genus-specific biomarker at *m/z* 11387, purified by HPLC (Figure S-30 in Supporting Information), was identified by PMF strategy as parvalbumin beta from *Merluccius bilinearis* (accession number gi|3024436, molecular weight 11310 Da, Mascot score 57, protein coverage 27%). Protein identification was confirmed by tandem mass spectrometric experiments. Up to now, only a limited number of parvalbumin sequences from fish species have been annotated in protein databases. Therefore, PMF strategy could lead to the identification of parvalbumins from species different from those under investigation which share a high sequence identity. In fact, the protein generating the *Trisopterus minutus minutus* biomarker (*m/z* 11351.36) was identified by PMF as parvalbumin beta from *Gadus morhua* (accession number gi|14531014, Mascot score 85, coverage 37%) with a molecular weight of 11454.0 Da, in disagreement with the *m/z* value of the *Trisopterus minutus minutus* biomarker. Tandem mass spectrometric experiments defined the differences in the primary structure of the *Trisopterus minutus minutus* protein, listed here: (i) deletion of the N-terminal Met; (ii) Asn26 instead of Ser; (iii) Ala37 instead of Ser; (iv) Ala38 instead of Gly; (v) Ser71 instead of Lys. Furthermore, we can infer that the N-terminal residue of the characterized protein was acetylated.

Finally, *Sparus auratus* biomarker at *m/z* 11370.28 was identified by PMF as parvalbumin from *Evynnis japonica* (accession number gi|165905305, Mascot score 66, coverage 37%), a fish species belonging to the same order and family of *Sparus auratus.*

Because of their high interspecies variability and thermostability, as demonstrated also by our results, parvalbumins have already been proposed as biomarkers for fish authentication by IEF and proteomics in fresh or processed seafood products (*14, 32, 33*). Interestingly, these proteins also exhibited an extremely high ionization efficiency under the MALDI-TOF experimental conditions since sarcoplasmic protein extracts gave rise to mass spectra with only a few highly intense signals referable to parvalbumins. Moreover, because of this peculiar feature, we can hypothesize that good quality mass spectra could be obtained also from processed samples, where protein denaturation and precipitation can significantly reduce parvalbumin concentration. This may ensure the overall applicability of the method for fish authentication.

Methods based on molecular biology or protein analysis may demand some previous knowledge of the sample under investigation and, more importantly, can be slow and laborious with results possibly dependent on experimental variables. However, the developed approach is straightforward, with minimal time consumption and low costs (a few minutes are necessary for sample preparation and mass spectrum acquisition) and, at the same time, is highly accurate and sensitive without requiring any previous information of the species under analysis.

The construction of a molecular profile database, currently underway, supported by bioinformatics, could integrate the method here proposed, thereby providing a high-tech tool in large scale screening for control and authentication of fishery products.

ABBREVIATIONS USED

ACN, acetonitrile; ACTH adrenocorticotropic hormone (clip 18−39); CHAC, α-cyano-4-hydroxycynnamic acid; IEF, isoelettric focusing; i.s., internal standard; HPLC, high performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; MW, molecular weight; *m/z*, mass to charge ratio; PMF, peptide mass fingerprint; RAPD-PCR, random amplification of polymorphic DNA-polymerase chain reaction; RFLP, restriction fragment length polymorphism; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SSCP, single strand conformation polymorphism; TFA, trifluoroacetic acid.

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Supporting Information Available: SDS-PAGE and HPLC profiles of protein extracts of *Dicentrarchus labrax* and *Sparus auratus*, HPLC chromatogram of *Merluccius merluccius*, MALDI-TOF mass spectra of all the fish species, commercial products, and heat-treated samples analyzed in this study and not reported in the manuscript's figures.This material is available free of charge via the Internet at http://pubs.acs.org.

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FISH AUTHENTICATION BY MALDI-TOF MASS SPECTROMETRY

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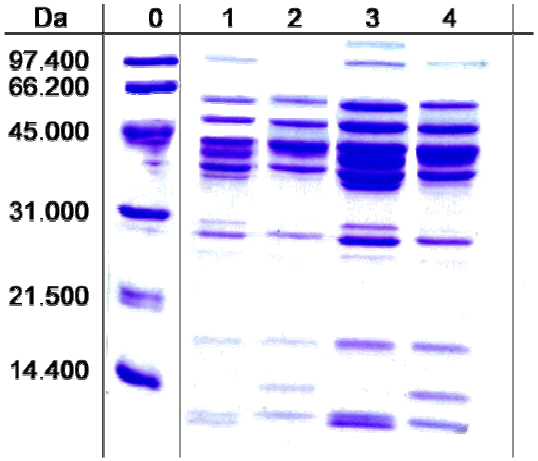
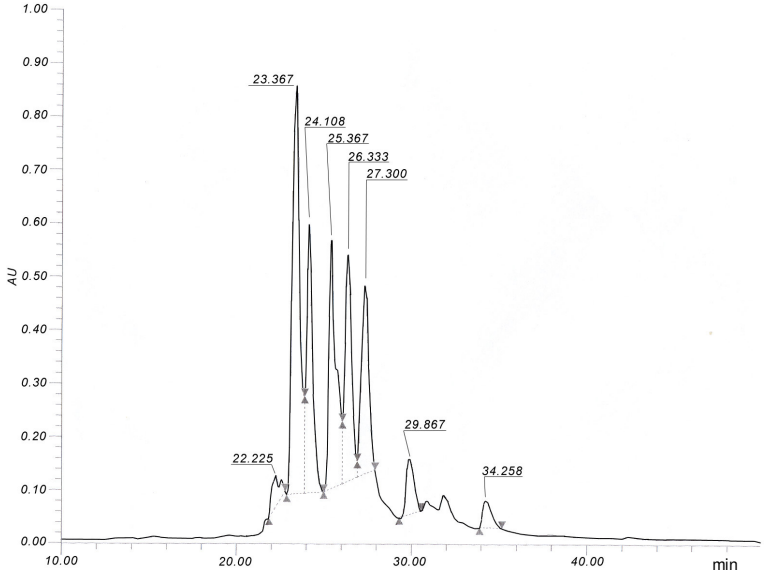
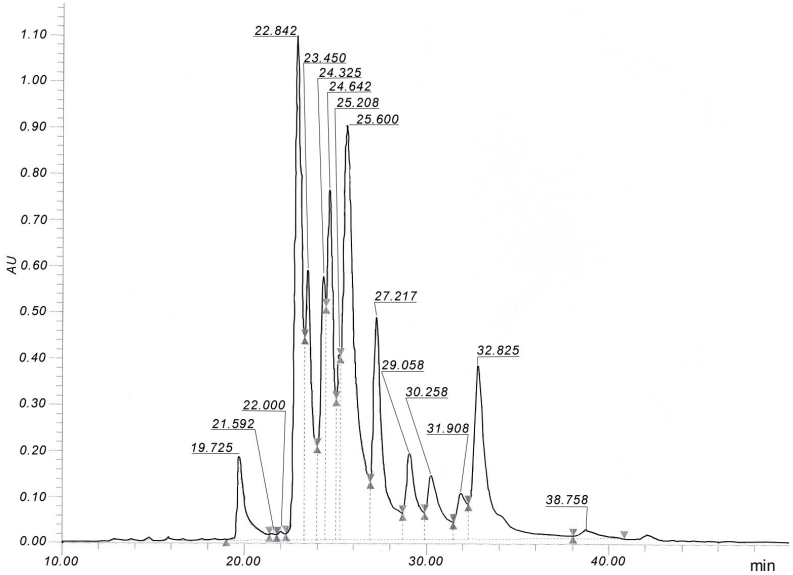
Supporting Information Available: SDS-PAGE and HPLC profiles of protein extracts of *Dicentrarchus labrax* and *Sparus auratus*, HPLC chromatogram of *Merluccius merluccius*, MALDI-TOF mass spectra of all the fish species, commercial products and heat treated samples analyzed in this study and not reported in the manuscript's figures.



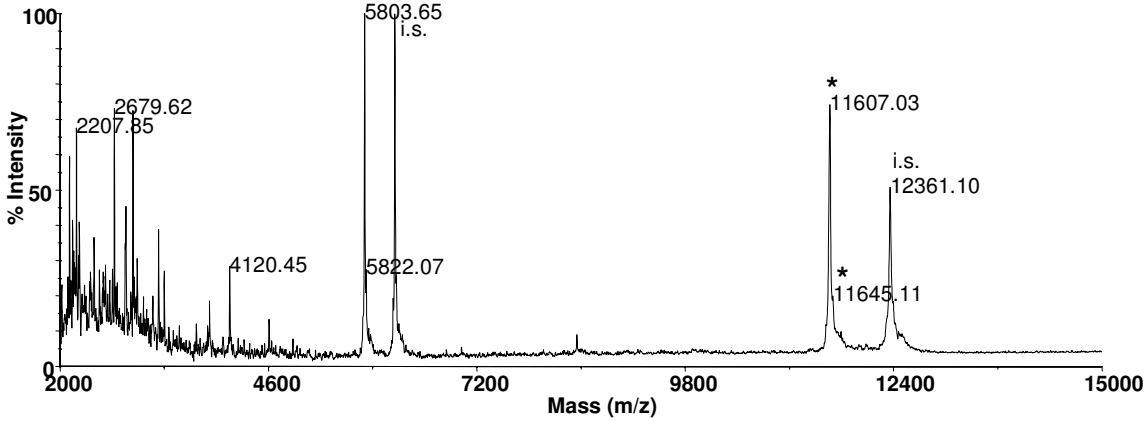
S-1: SDS-PAGE analysis of protein extracts of *Sparus auratus* (lane 1=15 µg, lane 3 =40 µg) and *Dicentrarchus labrax* (lane 2=15 µg lane 4=40 µg). Lane 0=MW standard



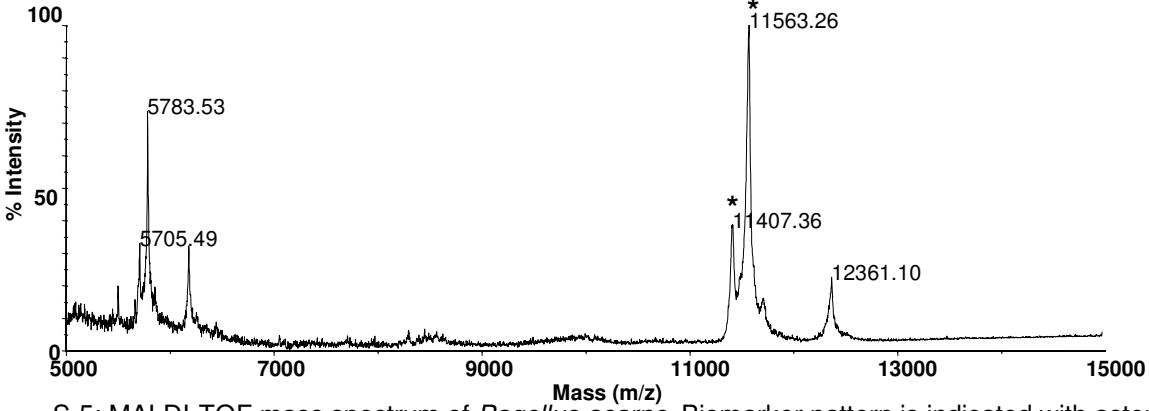
S-2: HPLC analysis of protein extracts of *Dicentrarchus labrax*



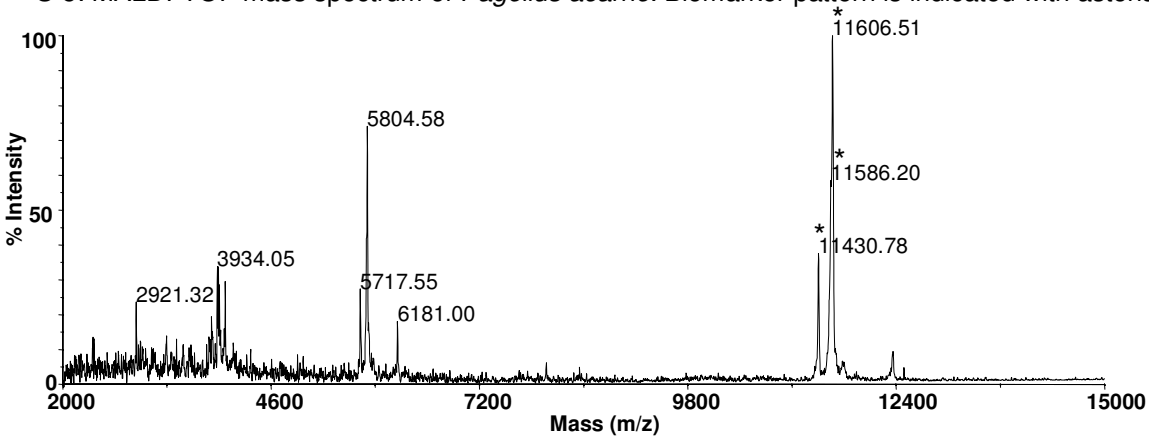
S-3: HPLC analysis of protein extracts of *Sparus auratus*



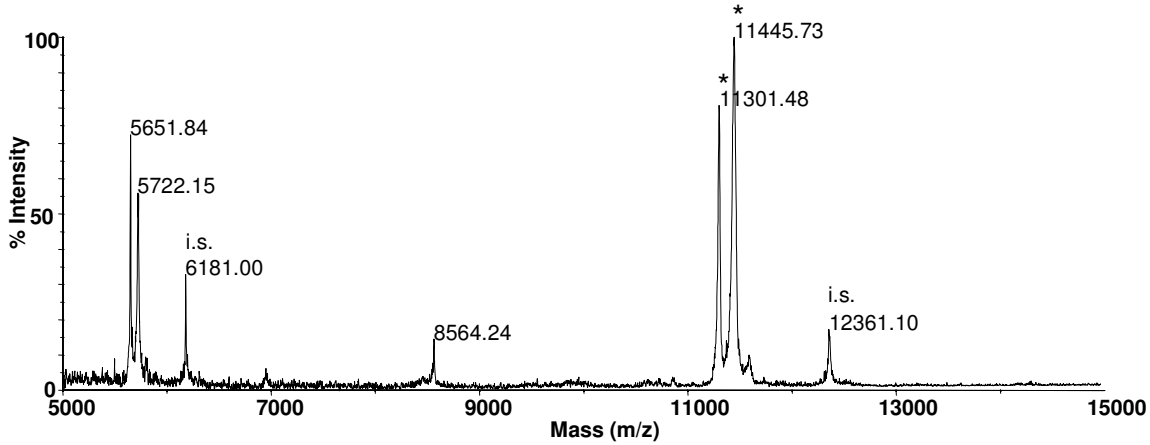
S-4: MALDI-TOF mass spectrum of *Epinephelus marginatus.* Biomarker pattern is indicated with asterisks



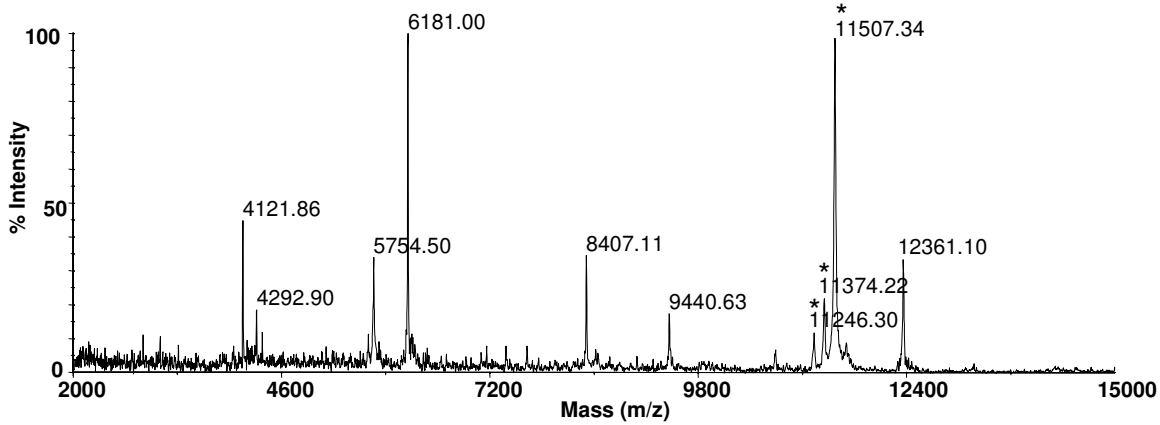
S-5: MALDI-TOF mass spectrum of *Pagellus acarne.* Biomarker pattern is indicated with asterisks



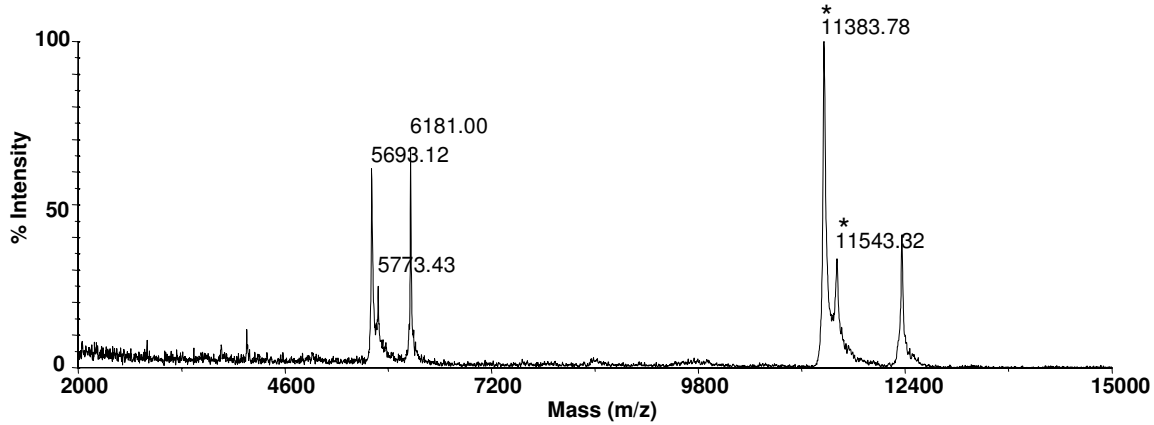
S-6: MALDI-TOF mass spectrum of *Pagellus erythrinus.* Biomarker pattern is indicated with asterisks



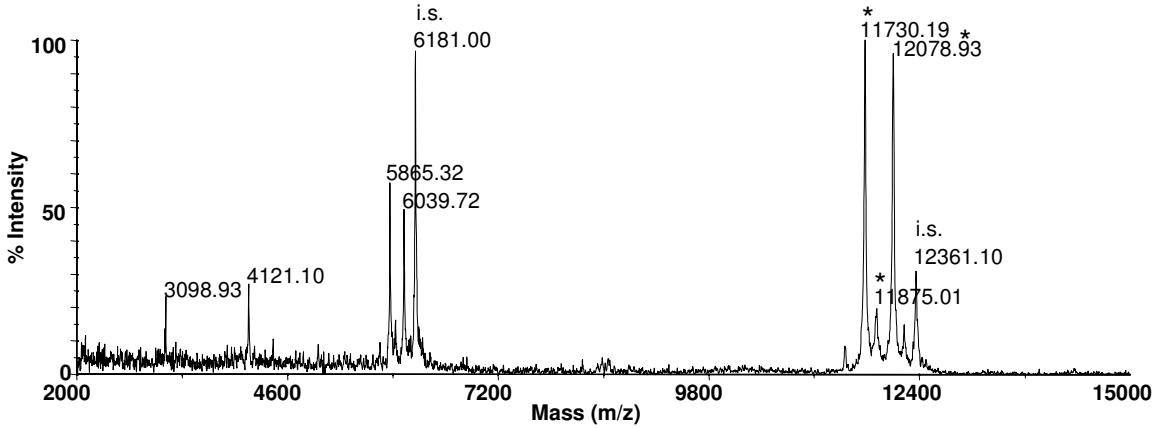
S-7: MALDI-TOF mass spectrum of *Diplodus sargus.* Biomarker pattern is indicated with asterisks



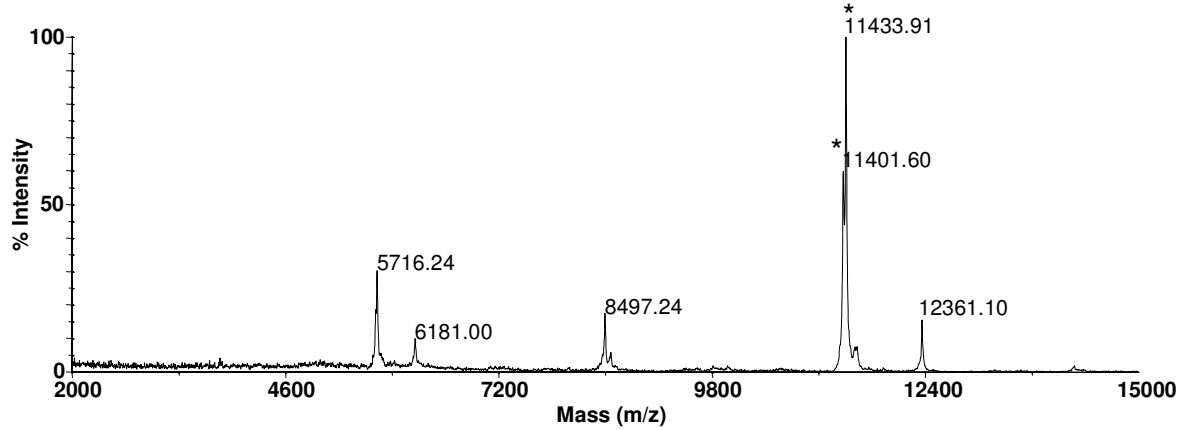
S-8: MALDI-TOF mass spectrum of *Spicara maena*. Biomarker pattern is indicated with asterisks



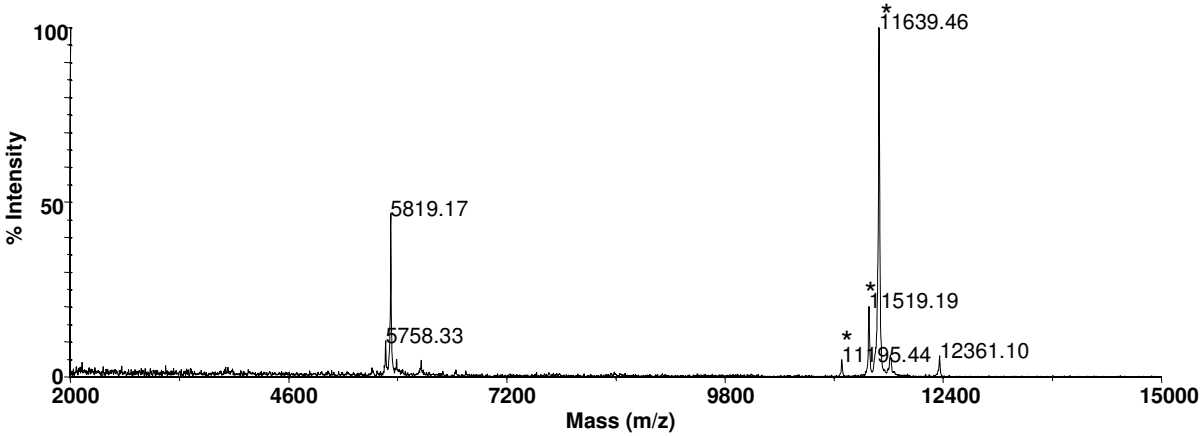
S-9: MALDI-TOF mass spectrum of *Mullus barbatus.* Biomarker pattern is indicated with asterisks



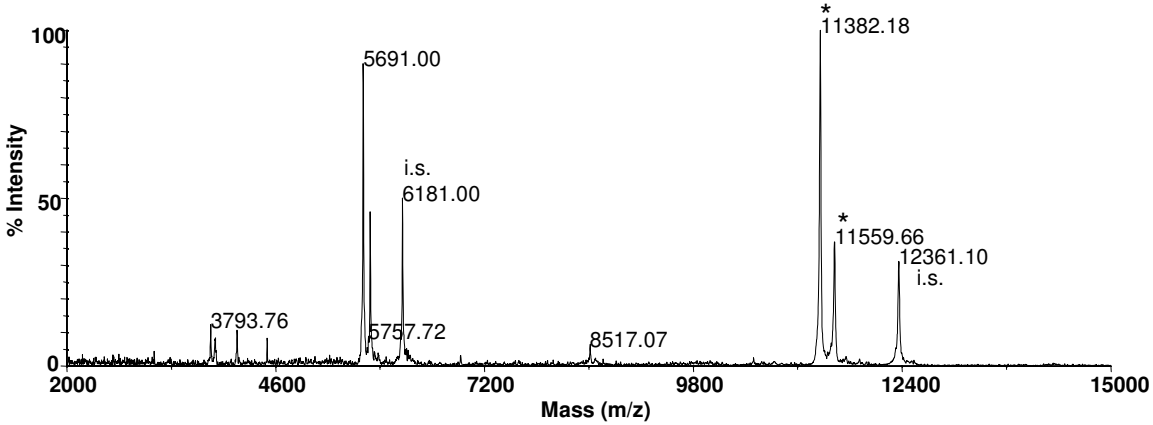
S-10: MALDI-TOF mass spectrum of *Uranoscopus scaber.* Biomarker pattern is indicated with asterisks



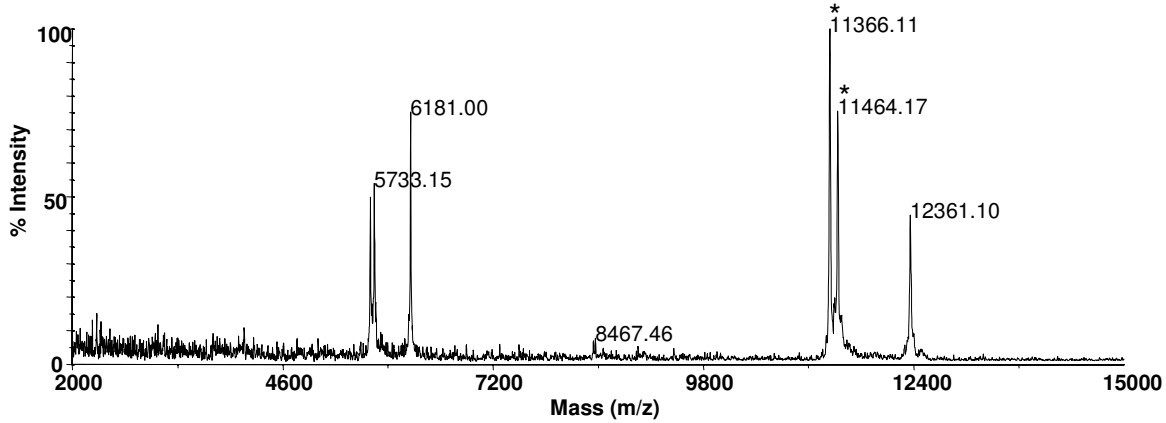
S-11: MALDI-TOF mass spectrum of *Perca fluviatilis.* Biomarker pattern is indicated with asterisks



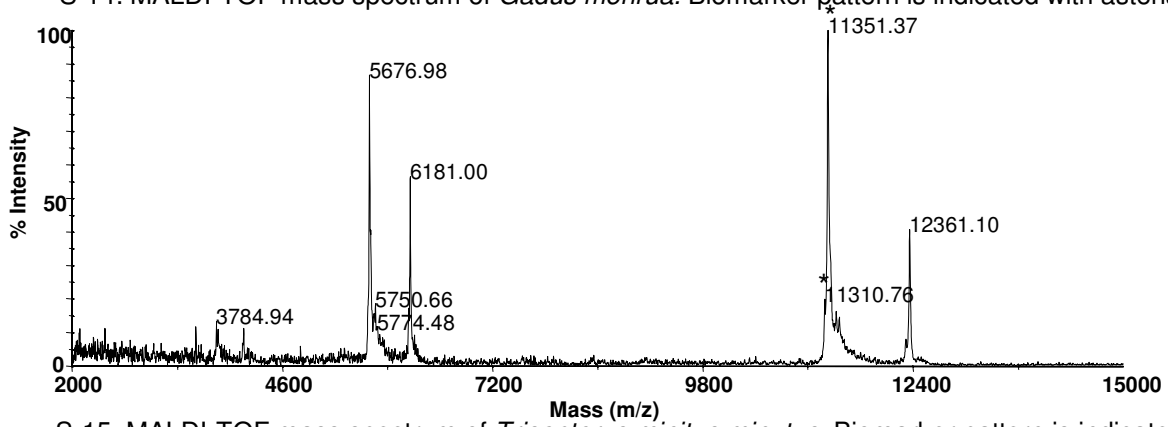
S-12: MALDI-TOF mass spectrum of *Aspitrigla cuculus.* Biomarker pattern is indicated with asterisks



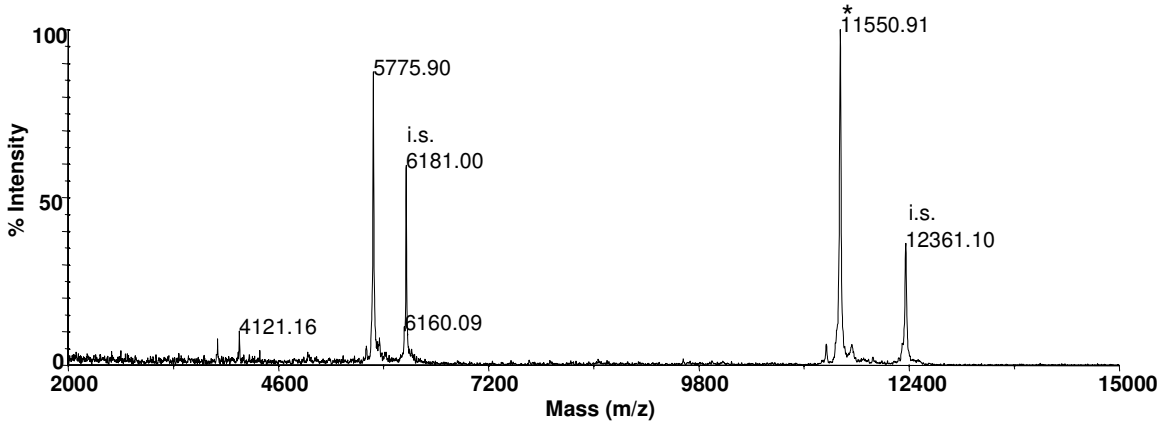
S-13: MALDI-TOF mass spectrum of *Tilapiine cichlids.* Biomarker pattern is indicated with asterisks



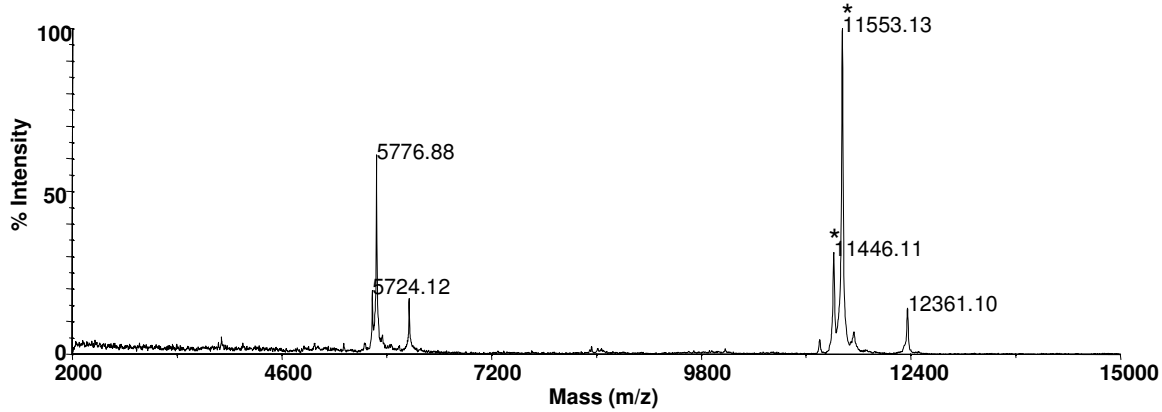
S-14: MALDI-TOF mass spectrum of *Gadus mohrua.* Biomarker pattern is indicated with asterisks



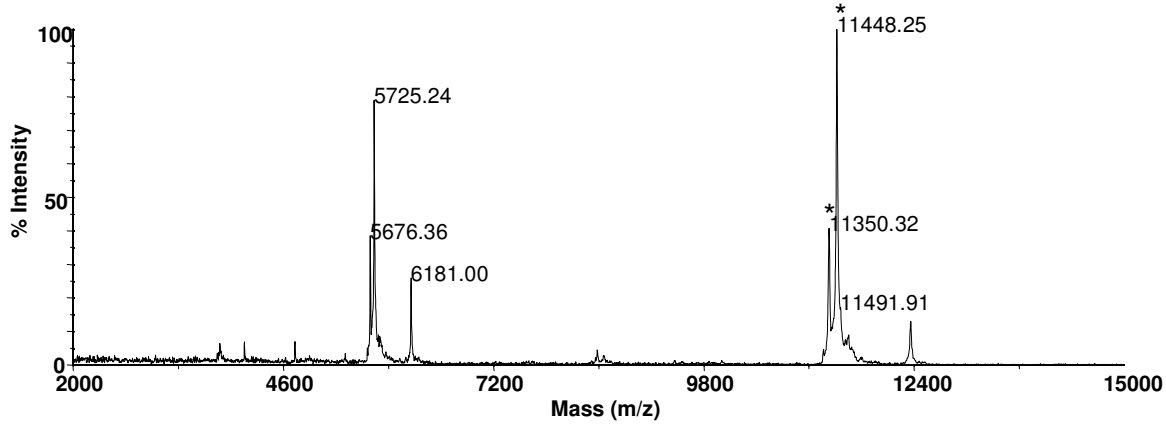
S-15: MALDI-TOF mass spectrum of *Trisopterus minitus minutus.* Biomarker pattern is indicated with asterisks



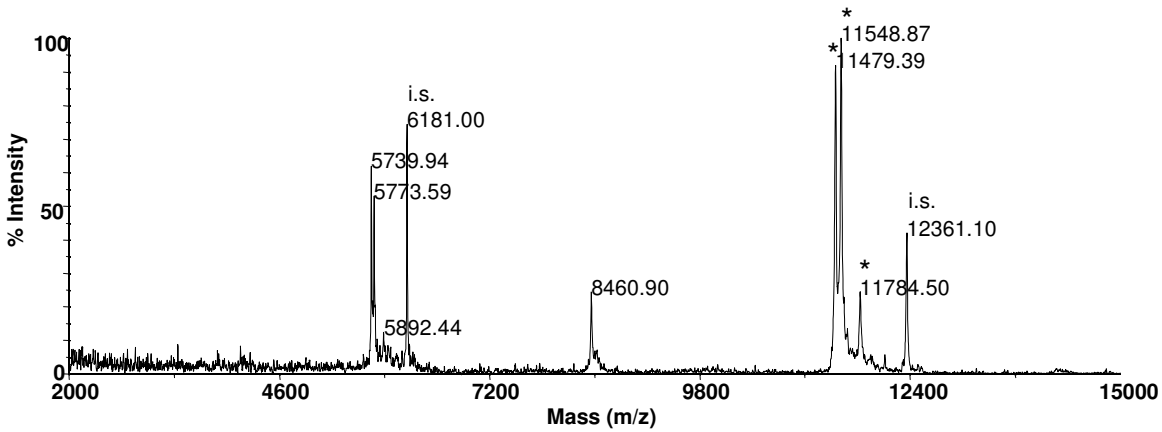
S-16: MALDI-TOF mass spectrum of *Molva elongata.* Biomarker pattern is indicated with asterisks



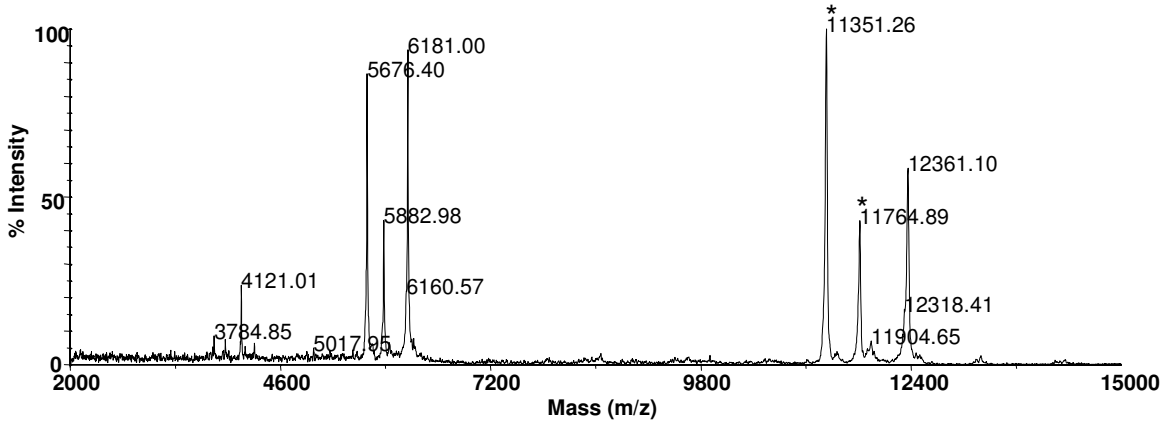
S-17: MALDI-TOF mass spectrum of *Phycis blennoides.* Biomarker pattern is indicated with asterisks



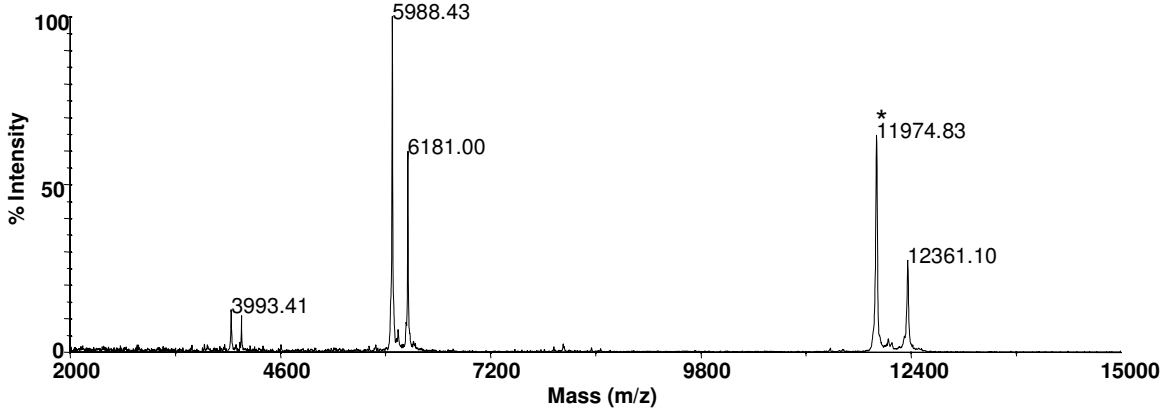
S-18: MALDI-TOF mass spectrum of *Micromesistius poutassou.* Biomarker pattern is indicated with asterisks



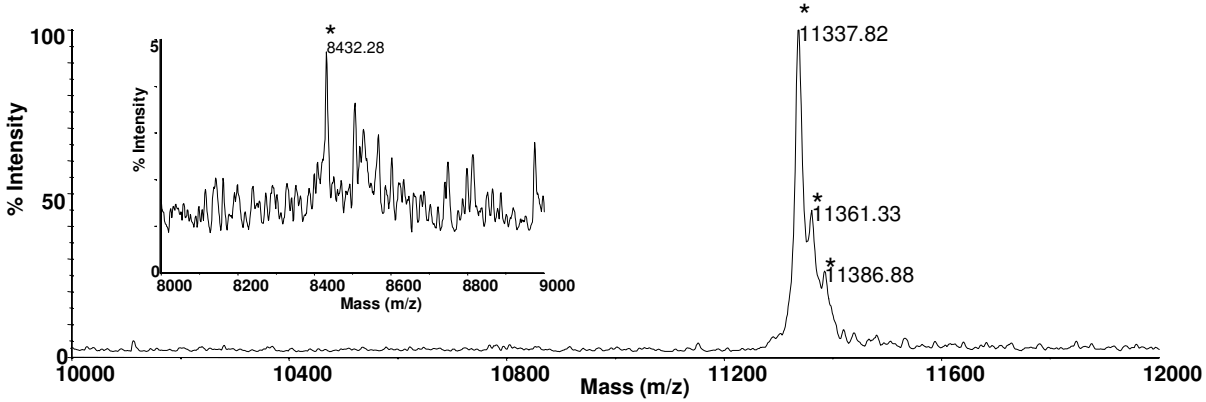
S-19: MALDI-TOF mass spectrum of *Arnoglossus lanterna.* Biomarker pattern is indicated with asterisks



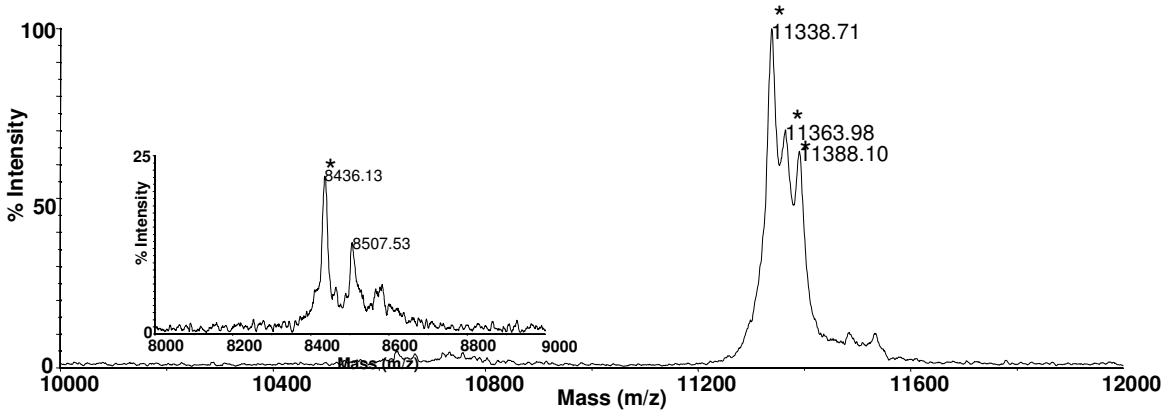
S-20: MALDI-TOF mass spectrum of *Pleuronectes platessa.* Biomarker pattern is indicated with asterisks



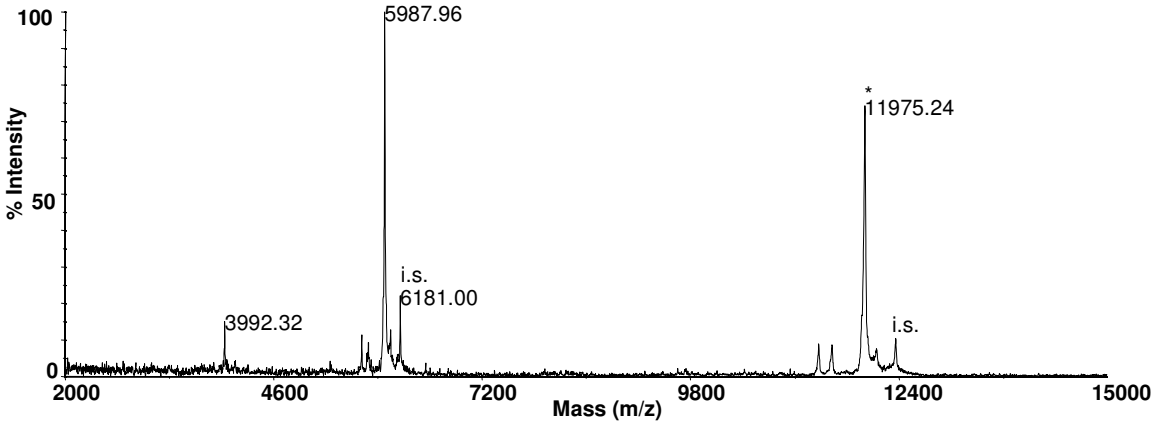
S-21: MALDI-TOF mass spectrum of *Sole solea.* Biomarker pattern is indicated with asterisks



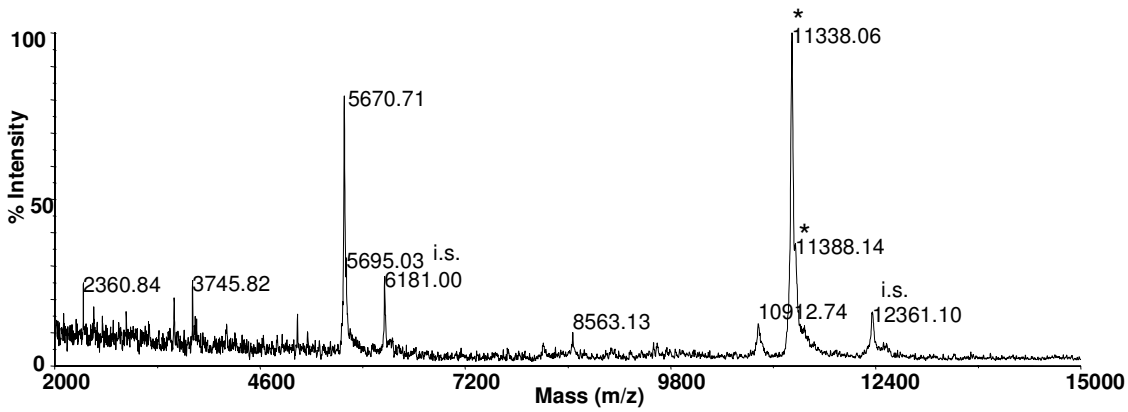
S-22: MALDI-TOF mass spectrum of cod fishstick. Biomarker pattern is indicated with asterisks



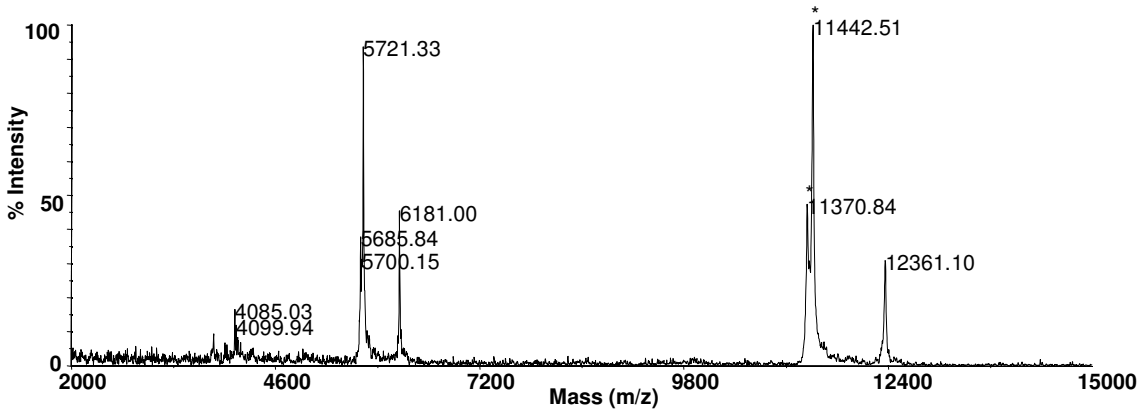
S-23: MALDI-TOF mass spectrum of cod fillet. Biomarker pattern is indicated with asterisks



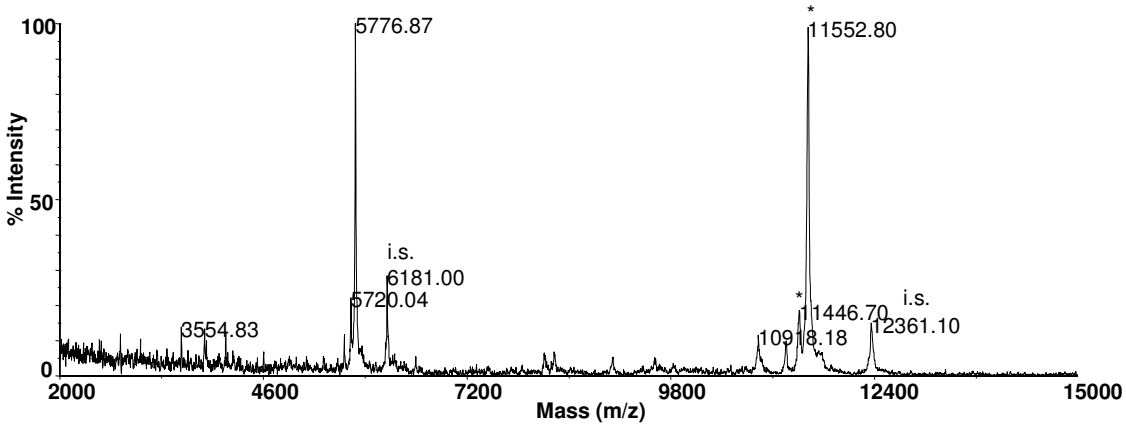
S-24: MALDI-TOF mass spectrum of *Solea solea* after treatment at 100℃. Biomarker pattern is indicated with asterisks



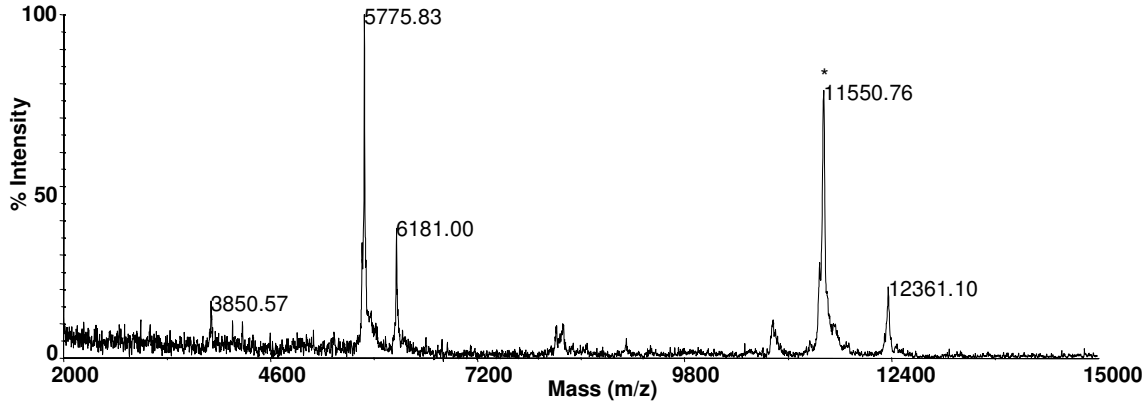
S-25: MALDI-TOF mass spectrum of *Merluccius merluccius* after treatment at 100℃. Biomarker pattern is indicated with asterisks



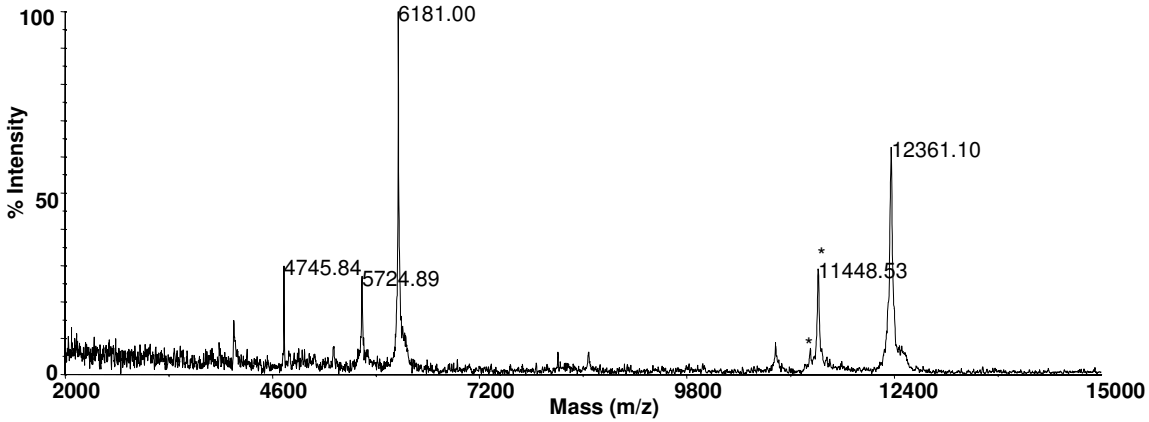
S-26: MALDI-TOF mass spectrum of *Sparus auratus* after treatment at 100℃. Biomarker pattern is indicated with asterisks



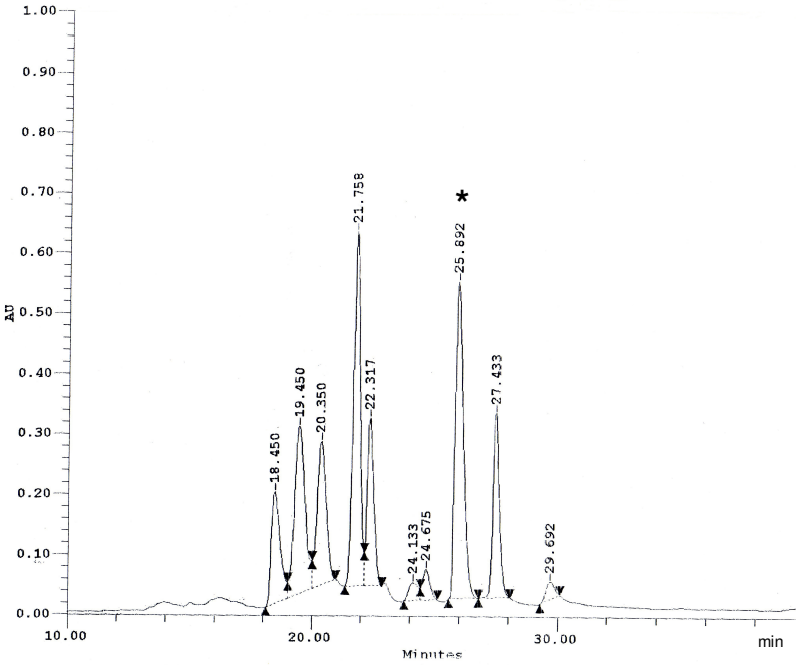
S-27: MALDI-TOF mass spectrum of *Phycis blennoides* after treatment at 100℃. Biomarker pattern is indicated with asterisks



S-28: MALDI-TOF mass spectrum of *Molva elongata* after treatment at 100℃. Biomarker pattern is indicated with asterisks



S-29: MALDI-TOF *mass spectrum of Micromesistius poutassou* after treatment at 100℃. Biomarker pattern is indicated with asterisks



S-30: HPLC analysis of protein extracts of *Merluccius merluccius.* Fraction containing the biomarker at m/z 11387 is indicated with an asterisk