# Food Authentication by MALDI MS: MALDI-TOF MS Analysis of Fish Species

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**Abstract** Consumer demand for healthy and well-sourced food has been growing in recent times. In particular, demand for fish is constantly increasing due to the awareness of beneficial effects of fishery products on human health. Furthermore, the opening of new markets and the use of a larger number of fish species are strong and timely reminders for the urgent need to guarantee safety, traceability, and authenticity of seafood. Recent European Union directives and regulations for quality control of food products have prompted the development of new methods for large-scale tests to ensure consumer protection. MALDI-TOF MS has provided a significant contribution to food science, proving to be a key tool in the analysis of several food matrices, including fish, especially in studies aimed to assess food quality, safety, and authenticity.

This chapter is focused on an innovative molecular profiling strategy based on MALDI-TOF MS analysis of sarcoplasmic protein extracts from fish muscle, successfully applied to fish authentication. The described method allows to rapidly discriminate different fish species, to verify commercial product authenticity and to detect fraudulent substitutions.

## 1 Introduction

Consumption of fish products that enhances the intake of long chain and shorter chain omega 3 fatty acids can positively affect human health, in particular contributing to the prevention of cancer and cardiovascular events (Hooper et al. 2006). Therefore, in the last decades, consumer demands for seafood has rapidly increased. To meet these demands, the market has enlarged fish species assortment including fish captured in Asian and African seas and using a growing number of species to produce transformed fish-based foods. Unfortunately, as a consequence, globalization and freer markets have favored a seafood mislabeling phenomenon that is

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associated with potential health risks and, at the same time, illegal economic gains. These issues emphasize the need to determine fish authenticity and origin in order to guarantee proper quality and safety controls and to protect consumers. Reliable quality control methods are crucial to detect deceptive practices of seafood substitution that occurs when one species of fish, crustacean or shellfish is sold as another species (Arvanitoyannis et al. 2005b; Herrero 2008).

Conventional identification methods rely on the analysis of anatomical and morphological characteristics, such as the head, fins, skin, or bones, which are lost during processing, thus making any identification impossible. In agreement with European Union directives and regulations on fishery and aquaculture products, the species, geographical origin, and production method (wild or cultivated) must be provided in fish labeling (Council Regulation (EC) No.104/2000 and 2065/2001 of the European Parliament) to ensure market transparency. Furthermore, the European Food Safety Authority provided complete procedures for the traceability of food (including fishery and aquaculture products) and feed businesses to guarantee food safety at all stages (EC regulation no. 178/2002 of the European Parliament reviewed in Arvanitoyannis et al. 2005a).

Classical methodologies for fish authentication are based on the analysis of protein extracts by electrophoretic, chromatographic, and immunological methods. The isoelectric focusing (IEF) analysis of the sarcoplasmic proteins has been applied to fish and shrimp authentication (Etienne et al. 2000; Piñeiro et al. 2000; Rehbein et al. 2000; Ortea et al. 2010) and has been adopted by the Association of Official Analytical Chemistry as the validated method for species identification purposes (Helrich 1990). DNA-based procedures (mainly DNA sequencing of the cytochrome-b gene) are also routinely used for the authentication of fish species, as they present a number of advantages over protein-based methods, in particular for the analysis of highly processed samples (Rasmussen and Morrissey 2009; Carrera et al. 2013a). However, a fundamental drawback in DNA-based methods is the difficulty to standardize protocols and techniques. This is mandatory to rule out inconsistencies in results from different laboratories that could have regulatory or legal implications and to obtain a rigorous standard operating procedure (SOP) applicable across different countries (Griffiths et al. 2014).

The need for rapid screening of a large number of samples requires the development of high-tech approaches with minimal time consumption, low costs, and high reliability, which can successfully complement or substitute methods already in use.

In the last decades, the introduction of omics platforms has significantly contributed to research activities in food science, cumulating in the term ‘foodomics’ being coined in 2009 to indicate ‘a new discipline that studies food and nutrition domains through the application of advanced omics technologies to improve consumer’s well-being, health, and confidence’ (Cifuentes 2009; Herrero et al. 2010). Analytical methodologies based on mass spectrometry (MS) play a central role in foodomics. In particular, proteomics has been used to investigate several aspects of food quality and safety, including, traceability, authenticity, absence of contaminating, and/or adulterating agents and impact of the processing/storage methods (Herrero et al. 2012).

## 2 Applications

Recently, 'molecular profiling' strategies based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS have emerged as a general tool for the discovery of biomarkers that are potentially useful as indicators of authenticity for several food matrices (Cozzolino et al. 2001; Wang et al. 2009; Nunes-Miranda et al. 2012; Ciarmiello et al. 2014). A similar strategy has also recently been applied to the identification of shrimp at the species level (Salla and Murray 2013).

In this chapter, we describe an innovative molecular profiling approach based on MALDI-TOF MS analysis of sarcoplasmic protein extracts from fish muscle, developed by our group and successfully applied to the authentication of fish species (Mazzeo et al. 2008). The main strengths of this approach are the straightforward sample preparation protocol and the low demands on time and cost for the analysis (only a few minutes are necessary for sample preparation and mass spectra acquisition). It does not require any preliminary information on the sample under investigation or the identification of the biomarker-generating proteins. The methodology is highly accurate and sensitive, and due to the high unambiguity of mass spectrometric results, fish identification can be achieved with high confidence. Therefore, this strategy holds the potential to become a reliable first-line authenticity test for fish.

Our previously published data and the data presented here demonstrate that MALDI-TOF MS can be employed as a powerful tool in fish authentication. The presented methodology can be upgraded by exploiting the outstanding performance of modern MALDI-TOF instruments that provide high accuracy and resolution in molecular mass measurements, improving the definition of the biomarker pattern, and thus increasing confidence and reliability in the identification of unknown samples. Furthermore, these instruments assure faster analysis, perform completely automated data acquisition and processing and are specifically designed to be user-friendly, so that highly specialized operators would not be required to carry out mass spectrometric analyses.

As a matter of fact, these innovations and the development of specific software have paved the way to the introduction of MALDI-TOF MS-based methodologies for bacterial identification as routine tests in clinical microbiological laboratories (see Seng et al. 2009). Similarly, we can foresee that the creation of a specific database containing mass spectra and/or reference peak lists of a growing number of fish species, as well as ad hoc bioinformatics tools for database querying will prompt the application of this analytical approach as a routine method in fish and other food products authentication. In addition, as such database can be continuously updated by the operator, this method will be quite flexible and easily adaptable to specific analytical needs of the market. As proof of concept, a commercial MALDI mass spectral fingerprint matching software has been applied for the first time in food science to the discrimination at species level of 72 shrimp samples from the market (Salla and Murray 2013).

In conclusion, these studies strongly suggest that in the near future analytical strategies based on MALDI-TOF MS will play a key role in the assessment of food quality and safety and are going to represent robust tools suitable to be integrated in or substitute current official screening methods.

## 3 Materials and Protocols

The robustness of the method has been assessed by analyzing protein extracts from 40 different fish species, selected from widely consumed products of high commercial value or commonly involved in frauds. The presented data were obtained from fish species belonging to seven different Orders and twenty Families, representing one of the most comprehensive repertoires of species analyzed in fish authentication studies (Table 1), including species previously reported in a similar study (Mazzeo et al. 2008). The general workflow employed in our lab is outlined in Fig. 1.

**Table 1** List of the analyzed fish species[a]

| Order | Family | Genus | Species | Common name |
|---|---|---|---|---|
| Perciformes | Serranidae | *Dicentrarchus* | *Dicentrarchus labrax* | Seabass |
| | | *Epinephelus* | *Epinephelus marginatus* | Dusky grouper |
| | Sparidae | *Sparus* | *Sparus auratus* | Seabream |
| | | *Pagellus* | *Pagellus acarne* | Axillary seabream |
| | | | *Pagellus erythrinus* | Common pandora |
| | | *Diplodus* | *Diplodus sargus* | White seabream |
| | | | *Diplodus vulgaris* | Common two-banded seabream |
| | | | *Diplodus puntazzo* | Sharpsnout seabream |
| | | | *Diplodus annularis* | Annular seabream |
| | | *Dentex* | *Dentex dentex* | Common dentex |
| | | *Pagrus* | *Pagrus pagrus* | Red porgy |
| | 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 |
| | Scombridae | *Auxis* | *Auxis thazard* | Frigate tuna |
| | | *Sarda* | *Sarda sarda* | Atlantic bonito |
| | | *Scomber* | *Scomber scombrus* | Atlantic mackerel |
| | Coryphaenidae | *Coryphaena* | *Coryphaena hippurus* | Common dolphinfish |
| | Carangidae | *Trachurus* | *Trachurus trachurus* | Atlantic horse mackerel |

(continued)

**Table 1** (continued)

| Order | Family | Genus | Species | Common name |
|---|---|---|---|---|
| Gadiformes | Gadidae | *Gadus* | *Gadus morhua* | Atlantic cod |
| | | *Merluccius* | *Merluccius capensis* | Shallow-water cape hake |
| | | | *Merluccius merluccius* | European hake |
| | | | *Merluccius hubbsi* | Argentine hake |
| | | | *Merluccius paradoxus* | Deep-water cape hake |
| | | *Trisopterus* | *Trisopterus minutus minutus* | Poor cod |
| | | *Micromesistius* | Micromesistius poutassou | Blue whiting |
| | | *Molva* | *Molva elongata* | Mediterranean ling |
| | | *Phycis* | *Phycis blennioides* | Greater forkbeard |
| Pleuronectiformes | Bothidae | *Arnoglassus* | *Arnoglossus laterna* | Scaldfish |
| | Pleuronectidae | *Reinhardtius* | *Reinhardtius hippoglossoides* | Greenland halibut |
| | | *Pleuronectes* | *Pleuronectes platessa* | European plaice |
| | Soleidae | *Solea* | *Solea solea* | Common sole |
| Lophiiformes | Lophiidae | *Lophius* | *Lophius piscatorius* | Angler |
| Salmoniformes | Salmonidae | *Salmo* | *Salmo salar* | Atlantic salmon |
| Clupeiformes | Engraulidae | *Engraulis* | *Engraulis encrasicolus* | European anchovy |
| | Clupeidae | *Sardina* | *Sardina pilchardus* | European pilchard |
| Siluriformes | Pangasiidae | *Pangasius* | *Pangasius pangasius* | Striped catfish |

[a]Adapted with permission from Mazzeo et al. (2008) J Agric Food Chem 56:11071-11076. Copyright (2014) American Chemical Society

## *3.1 Extraction of Sarcoplasmic Proteins from Muscle Tissue*

The protocol can be applied to the analysis of fresh and frozen fish samples. One gram of white tissue muscle is taken from three individuals of each species without damaging any organ in order to avoid any contamination and stored at −20 °C. Sarcoplasmic protein extraction is carried out by vortexing 0.1–0.2 g fish muscle in 100–200 μL of 0.1% trifluoroacetic acid (TFA) for 1 min. Protein extracts are then centrifuged at 13,000 rpm for 5 min and the recovered supernatants are diluted 1:10 in 0.1% TFA and immediately analyzed by MALDI-TOF MS. For fish

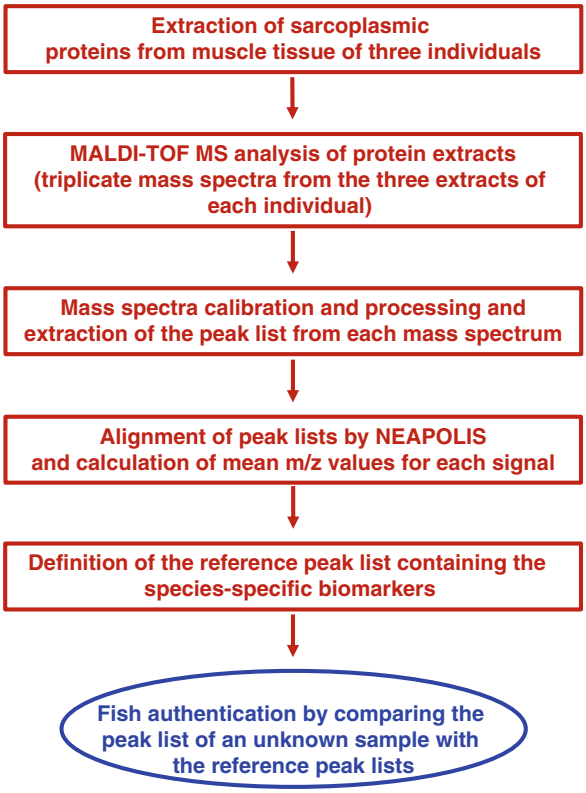


**Fig. 1** Analytical scheme of the described authentication method

species particularly rich in fats, sample extracts are subjected to a defatting step with chloroform, i.e., using 0.1% TFA/chloroform (1:4; v/v) that improves mass spectra quality.

## *3.2 MALDI-TOF MS Analysis*

MALDI-TOF MS analyses are carried out using as matrix solution a solution of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile (ACN)/0.1% TFA (10 mg/mL) that contains 1 pmol/μL of cytochrome C as internal standard. One microliter of the analyte extract is mixed with 1 μL of matrix solution and deposited onto a MALDI target plate so that co-crystallization occurs under ambient conditions. In some cases, mass spectra quality is improved by adding 1 μL of matrix solution directly on the crystallized samples and analyses are carried out after subsequent recrystallization. In our experiments, mass spectra are acquired on a Voyager-DE PRO MALDI-TOF mass spectrometer (AB-SCIEX, Foster City, CA, USA), operating in linear, positive-ion mode with delayed extraction, using a pulsed

nitrogen laser (337 nm; 3 ns). Parameters for data acquisition are the following: laser intensity set just above the ion generation threshold, low mass gate at 1990, delay time at 500 ns, accelerating voltages at 25,000 V, grid voltage and guide wire voltage at 95% and 0.1% of the accelerating voltage, respectively.

Mass spectra are typically acquired by accumulating spectra obtained from 100 laser shots in the *m*/*z* range of 2000–15,000. Internal calibration is performed using the doubly and singly charged ions of cytochrome C (*m*/*z* 6181.05 and 12,361.10, respectively). All *m*/*z* values are recorded as average values.

Reference molecular profiles are constructed from triplicate MALDI-TOF MS analysis of analyte extracts from three individuals for each species.

### *3.3 Data Analysis*

Mass spectra are processed applying baseline subtraction and smoothing algorithms and transformed into a list containing the *m*/*z* values of signals present in the *m*/*z* range of 8000–15,000 with an ion signal intensity of >10%. In our lab, DataExplorer 5.1 software (AB-SCIEX) is used for these data processing steps. For each species, the peak lists obtained from the nine processed mass spectra are aligned along the *m*/*z* axis using the NEAPOLIS software (www.bioinformatics.org/bioinfo-af-cnr/NEAPOLIS) (Mangerini et al. 2011) which calculates the mean *m*/*z* value for each signal. The threshold mass tolerance value for the alignment is fixed to 500 ppm, so that, among the aligned signals, the minimum and maximum *m*/*z* values differ by <500 ppm and the standard deviations of mass measurement are <3 Da. The mean *m*/*z* values of signals present in all replicate mass spectra are included in the reference peak list for each analyzed species. A direct comparison of the reference peak lists shows that the pattern of signals included in individual reference peak lists is unique for each analyzed species and therefore unequivocally identifies that species. This pattern, containing as few as one to four signals, is considered the species-specific biomarker pattern and can be used to discriminate fish species unambiguously. Table 2 details the reference peak lists for the 40 fish species studied in our lab.

To identify an unknown sample, its peak list is obtained following the previously described method and compared to all the reference peak lists in the pre-recorded database. A positive match is obtained if the peak list of the unknown sample completely matches one database reference peak list (Table 2, Fig. 1).

### *3.4 General Remarks and Examples*

MALDI-TOF MS analyses of sarcoplasmic protein extracts yield mass spectra characterized by a pattern of a few highly intense signals, mainly in the *m*/*z* range of about 11,000–12,000, that can be considered as species-specific biomarkers. These specific molecular profiles are suitable for fish authentication and allow the differentiation of

**Table 2** Biomarker patterns useful for discriminating fish species, as obtained by MALDI-TOF MS analysis[a]

| Scientific name | Common name | Biomarker pattern[b] | | SD | |
|---|---|---|---|---|---|
| *Dicentrarchus labrax* | Seabass | 8032.0 | ± | 0.8 | |
| | | 11,404.7 | ± | 1.0 | M*[c] |
| | | 11,495.9 | ± | 1.1 | |
| *Epinephelus marginatus* | Dusky grouper | 11,606.8 | ± | 0.2 | M* |
| *Sparus auratus* | Seabream | 11,370.5 | ± | 1.1 | |
| | | 11,442.3 | ± | 0.9 | M* |
| *Pagellus acarne* | Axillary seabream | 11,407.4 | ± | 0.7 | |
| | | 11,563.3 | ± | 0.8 | M* |
| *Pagellus erythrinus* | Common pandora | 11,429.9 | ± | 0.7 | |
| | | 11,588.2 | ± | 2.3 | |
| | | 11,606.4 | ± | 0.9 | M* |
| *Diplodus sargus sargus* | White seabream | 11,301.5 | ± | 1.4 | |
| | | 11,456.7 | ± | 1.3 | M* |
| *Diplodus vulgaris* | Common two-banded seabream | 11,343.4 | ± | 1.0 | |
| | | 11,457.5 | ± | 0.8 | M* |
| *Diplodus puntazzo* | Sharpsnout seabream | 11,380.7 | ± | 1.7 | |
| | | 11,457.8 | ± | 1.5 | M* |
| *Diplodus annularis* | Annular seabream | 11,270.7 | ± | 1.2 | |
| | | 11,461.5 | ± | 1.5 | M* |
| | | 11,488.2 | ± | 1.2 | |
| *Dentex dentex* | Common dentex | 11,519.8 | ± | 1.0 | M* |
| | | 11,567.4 | ± | 1.0 | |
| *Pagrus pagrus* | Red porgy | 11,489.8 | ± | 2.2 | |
| | | 11,582.2 | ± | 1.4 | M* |
| *Spicara maena* | Blotched picarel | 11,376.2 | ± | 2.5 | |
| | | 11,507.9 | ± | 1.2 | M* |
| *Mullus barbatus* | Red mullet | 11,383.5 | ± | 2.1 | M* |
| | | 11,544.9 | ± | 2.0 | |
| *Uranoscopus scaber* | Atlantic stargazer | 11,731.1 | ± | 1.0 | M* |
| | | 12,078.9 | ± | 1.0 | |
| *Perca fluviatilis* | European perch | 11,403.0 | ± | 1.1 | |
| | | 11,434.6 | ± | 0.8 | M* |
| *Aspritrigla cuculus* | East Atlantic red gurnard | 11,519.7 | ± | 0.6 | |
| | | 11,639.4 | ± | 0.6 | M* |
| *Tilapiine cichids* | Tilapias | 11,382.0 | ± | 0.5 | M* |
| | | 11,559.9 | ± | 0.9 | |
| *Auxis thazard* | Frigate tuna | 11,377.1 | ± | 0.8 | M* |
| | | 11,419.5 | ± | 0.7 | M* |
| *Sarda sarda* | Atlantic bonito | 11,459.3 | ± | 0.4 | |
| *Scomber scombrus* | Atlantic mackerel | 11,456.2 | ± | 0.5 | M* |
| | | 11,468.1 | ± | 0.6 | M* |
| *Coryphaena hippurus* | Common dolphinfish | 11,452.6 | ± | 0.2 | |
| | | 11,638.3 | ± | 0.1 | M* |

(continued)

**Table 2** (continued)

| Scientific name | Common name | Biomarker pattern[b] | | SD | |
|---|---|---|---|---|---|
| *Trachurus trachurus* | Atlantic horse mackerel | 11,295.1 | ± | 1.7 | |
| | | 11,483.1 | ± | 1.5 | M* |
| *Gadus morhua* | Atlantic cod | 11,366.6 | ± | 0.5 | M* |
| | | 11,464.0 | ± | 0.6 | |
| *Merluccius capensis* | Shallow-water cape hake | 8432.7 | ± | 0.9 | |
| | | 11,338.4 | ± | 0.5 | M* |
| | | 11,361.4 | ± | 0.6 | |
| | | 11,387.9 | ± | 0.8 | |
| *Merluccius merluccius* | European hake | 8432.3 | ± | 0.5 | |
| | | 11,338.6 | ± | 0.4 | M* |
| | | 11,388.2 | ± | 0.3 | |
| | | 11,361.0 | ± | 0.8 | |
| *Merluccius hubbsi* | Argentine hake | 8437.5 | ± | 1.2 | |
| | | 11,339.3 | ± | 0.3 | M* |
| | | 11,362.7 | ± | 0.9 | |
| | | 11,387.4 | ± | 1.1 | |
| *Merluccius paradoxus* | Deep-water cape hake | 8476.9 | ± | 0.5 | |
| | | 11,339.1 | ± | 0.5 | M* |
| | | 11,389.2 | ± | 0.7 | |
| *Trisopterus minutus minutus* | Poor cod | 11,310.7 | ± | 1.5 | |
| | | 11,351.3 | ± | 0.6 | M* |
| *Micromesistius poutassou* | Blue whiting | 11,350.8 | ± | 0.7 | |
| | | 11,448.3 | ± | 0.4 | M* |
| *Molva elongata* | Mediterranean ling | 11,550.7 | ± | 0.6 | |
| *Phycis blennoides* | Greater forkbeard | 11,447.4 | ± | 0.8 | |
| | | 11,553.3 | ± | 0.6 | M* |
| *Arnoglossus lanterna* | Scaldfish | 11,478.7 | ± | 1.8 | |
| | | 11,548.2 | ± | 2.0 | M* |
| | | 11,783.0 | ± | 1.3 | |
| *Reinhardtius hippooglossoides* | Greenland halibut | 11,433.8 | ± | 1.2 | |
| *Pleuronectes platessa* | European plaice | 11,351.6 | ± | 0.6 | M* |
| | | 11,764.1 | ± | 0.8 | |
| *Solea solea* | Common sole | 11,976.3 | ± | 1.4 | |
| *Lophius piscatorius* | Angler | 11,522.6 | ± | 0.6 | M* |
| | | 11,588.9 | ± | 0.7 | |
| *Salmo salar* | Atlantic salmon | 11,295.7 | ± | 0.9 | |
| | | 11,825.3 | ± | 0.9 | M* |
| *Engraulis encrasicolus* | European anchovy | 11,537.2 | ± | 0.3 | |
| *Sardina pilchardus* | European pilchard | 11,360.2 | ± | 0.2 | M* |
| | | 11,731.4 | ± | 0.4 | M* |
| *Pangasius pangasius* | Striped catfish | 11,555.1 | ± | 0.8 | M* |
| | | 12,075.8 | ± | 2.4 | |

[a]Adapted with permission from Mazzeo et al. (2008) J Agric Food Chem 56:11071-11076. Copyright (2014) American Chemical Society

[b]Mean *m*/*z* values of signals present in the replicate mass spectra are reported. Average *m*/*z* values are recorded in the mass spectra

[c]M* indicates the most intense signal in MALDI-TOF mass spectra

fish species, even phylogenetically closely related, as well as the authentication of commercial products. In a few cases, the identification is achieved by taking into account also signals present in a broader *m*/*z* range. The fish species analyzed up to now and their species-specific biomarker pattern are reported in Table 2. The potential of the method is well demonstrated in the following examples.

The mass spectrum of *Dicentrarchus labrax* (seabass) obtained from applying the above method exhibits three intense signals at *m*/*z* 8031.9, 11,403.7, and 11,495.3, whereas for *Sparus auratus* (seabream), two strong peaks were detected at *m*/*z* 11,441.2 and 11,370.0 (Fig. 2). Therefore, these two species that are widely consumed and of high commercial value are easily discriminated.

The method specificity assures the discrimination of very closely related species as shown by the analysis of four different species belonging to the *Diplodus* genus. Mass spectra of *Diplodus sargus sargus* (white seabream), *Diplodus vulgaris* (common two-banded seabream) and *Diplodus puntazzo* (sharpsnout seabream) share a major peak at *m*/*z* 11,457 while minor intense peaks can be detected at *m*/*z* 11,301.5

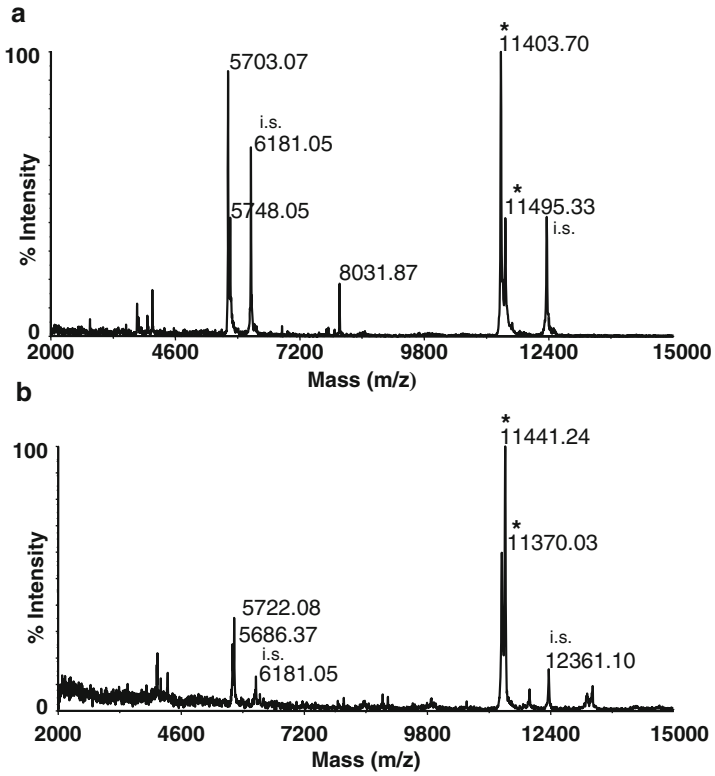


**Fig. 2** MALDI-TOF mass spectra obtained from the analysis of *Dicentrarchus labrax* (seabass) (**a**) and *Sparus auratus* (seabream) (**b**). Signals selected as biomarkers are indicated with *asterisks*. Adapted with permission from Mazzeo et al. (2008) J Agric Food Chem 56:11071-11076. Copyright (2014) American Chemical Society

for *Diplodus sargus sargus*, *m*/*z* 11,343.4 for *Diplodus vulgaris*, and *m*/*z* 11,380.7 for *Diplodus puntazzo*. The molecular profile of *Diplodus annularis* is quite different showing two signals with almost the same intensities at *m*/*z* 11,461.5 and 11,488.2 and a minor one at *m*/*z* 11,270.7 Therefore a species-specific biomarker pattern can be defined for the *Diplodus* species (Table 2).

Similarly, two phylogenetically related species of the *Pagellus* genus can be discriminated by peaks at *m*/*z* 11,563.3 and 11,407.4 present in the mass spectrum of *Pagellus acarne* (axillary seabream) and absent in that of *Pagellus erythrinus* (common pandora), which is characterized instead by three signals at *m*/*z* 11,606.4, 11,588.2, and 11,429.9.

The proposed method also allows a rapid authentication of widely consumed species within the *Gadidae* family, such as *Merluccius* species, *Gadus morhua* (atlantic cod), *Trisopterus minutus minutus* (poor cod), *Phycis blennoides* (greater forkbeard), *Molva elongata* (mediterranean ling), and *Micromesistius poutassou* (blue whiting) (Table 2).

It is noteworthy that this method takes advantage of a fast extraction protocol and of some important technical features of MALDI-TOF MS, such as sensitivity of analysis and tolerance to contaminants. In fact, the obtained protein extracts could be directly analyzed without any prior purification and/or concentration step. Moreover, although the analyzed protein mixtures are quite complex, MALDI-TOF mass spectra show a few strong signals. Therefore, the biomarker pattern is quite simple and fish identification can be easily and rapidly achieved.

### 3.4.1 Detection of Frauds

The ability of this approach to verify the authenticity of fish and commercial products (such as fillets and fishsticks) is demonstrated by the following exemplary case studies.

A common fraudulent practice is the substitution of the high value species *Dentex dentex* (common dentex), generally present in the market as fillets, with the low-cost species *Pagrus pagrus* (red porgy). Figure 3 shows the mass spectral profiles of the two species, characterized by the presence of two intense signals at *m*/*z* 11,519.9 and 11,567.2 for *Dentex dentex* and at *m*/*z* 11,488.0 and 11,581.5 for *Pagrus pagrus*. These signals immediately and unambiguously allow to discriminate the two species.

*Pangasius pangasius* (pangas catfish) and *Tilapiine cichids* (tilapias) fillets are often mislabeled and sold as fillets of a vast number of fish products, including cod fillets. MALDI-TOF MS analysis of pangas catfish led to the identification of a very intense signal at *m*/*z* 11,555.1 and a minor one at *m*/*z* 12,075.8 as species-specific biomarkers, while the signals at *m*/*z* 11,382.0 and 11,559.9 were characteristic of tilapias. Therefore, the straightforward discrimination of these fish species from more valuable ones, such as species belonging to Gadiformes, could be achieved (see biomarker pattern in Table 2).

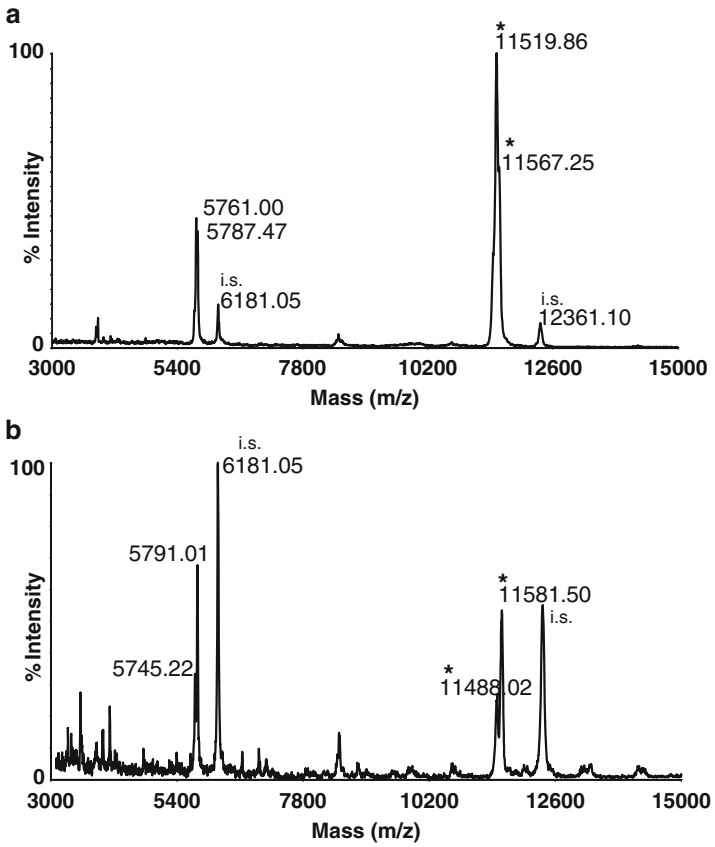


**Fig. 3** MALDI-TOF mass spectra obtained from the analysis of *Dentex dentex* (common dentex) (**a**) and *Pagrus pagrus* (red porgy) (**b**). Signals selected as biomarkers are indicated with *asterisks*

Similarly, it was possible to differentiate among fillets of sole (*Solea solea*; biomarker at *m*/*z* 11,976.3), European plaice (*Pleuronectes platessa*; biomarkers at *m*/*z* 11,351.6 and 11,764.1), and Greenland halibut (*Reinhardtius hippoglossoides*; biomarker at *m*/*z* 11,433.8) based on the specific signal patterns.

Furthermore, the developed method permits to verify the correctness of what is declared on labels as demonstrated by the mass spectra of some commercial cod fishsticks, which were identical to those obtained from *Merluccius capensis*, and thus in agreement with the label (Mazzeo et al. 2008).

It is worth stressing that the presented method can be applied to the analysis of cooked products as mass spectra obtained from some heat-treated samples (*Sparus auratus*, *Merluccius merluccius*, *Molva elongata*, *Phycis blennoides*, *Micromesistius poutassou*, and *Solea solea*) show the same biomarker patterns as the untreated samples (Mazzeo et al. 2008). The method can also be applied to the analysis of processed products such as homogenized baby foods.

### 3.4.2 Parvalbumins as Species-Specific Biomarkers for Fish Authentication

Earlier analyses based on tandem mass spectrometric experiments determined the primary structure of a few protein isoforms (such as that from *Trisopterus minutus minutus*) present in the fish muscle extracts and demonstrated that biomarker signals in the *m*/*z* range of 11,000-12,000 originate from parvalbumins (Mazzeo et al. 2008). Parvalbumins are calcium-binding proteins with molecular weights in the 11–12 kDa range, relatively abundant in muscle tissues and known as the major allergy-eliciting proteins in fish.

These proteins can be regarded as the most suitable biomarkers for fish species authentication due to the interspecies variability of their sequences, their high concentration in the fish muscle, and solubility in aqueous buffers that make the extraction protocol extremely fast and easy. Moreover, parvalbumins exhibit a high ionization efficiency in MALDI-TOF MS analysis so that, regardless of the complexity of the analyzed sarcoplasmic extracts, the obtained mass spectra predominantly show signals originating from these proteins (Mazzeo et al. 2008; Carrera et al. 2013b). The structural stability of parvalbumins even under harsh conditions such as heat is paramount to utilize these biomarkers also for the authentication of fish species sold as thermally processed products (Elsayed and Bennich 1975; Kawai et al. 1992; Carrera et al. 2010).

The interspecies variability of parvalbumin sequences is fundamental for the discrimination of different fish species as assessed by earlier proteomic analyses performed on species belonging to the Merlucciidae family (Piñeiro et al. 2001). Proteomic studies integrating two-dimensional electrophoresis (2-DE) with MALDI-TOF MS peptide mass mapping for protein identification allowed the characterization of the 2-DE parvalbumin-specific pattern and the definition of a set of specific tryptic peptides suitable for the identification of nine hake species (Carrera et al. 2006). More recently, exploiting the improved performance of new instruments such as Fourier-transform ion-cyclotron resonance (FTICR) mass spectrometers and linear ion trap (LIT) mass spectrometers, innovative strategies for the extensive characterization of parvalbumins have been proposed. These studies led to the de novo sequencing of 25 isoforms from all commercial species of the Merlucciidae family and to the rapid and direct detection of the presence of fish allergens in all of the investigated food products (Carrera et al. 2010; Carrera et al. 2012).

These latest results provide the structural evidence and demonstrate the potential of parvalbumins as suitable biomarkers for fish authentication and their importance in MALDI-TOF molecular profiling strategies such as the one described in this chapter.

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