Chapter 12
Fish Authentication

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12.1 Introduction

The intake of fish species by consumers is increasing due to the strong evidence of their positive benefits in human health. These benefits are mainly due to their high content of polyunsaturated ω-3 fatty acids proven to help in the prevention and treatment of cardiovascular, neurological, and inflammatory diseases (Hooper et al. 2006). In addition, fish are also excellent sources of micronutrients, including various vitamins (A, D, E, B1, B6, and B12) and minerals (Fe, I, P, Na, Ca, and K), and an affordable source of high-quality animal protein. A portion of 150 g of fish provides about 50–60% of the daily protein requirements for an adult (Piggot 1976). In 2007, the average annual per capita fish supply in developing countries was 9.0–15.1 kg (FAO 2010).

Attributable to this high demand, the fishery market is showing a dramatic growth in sales, producing an overexploitation of resources and the search for new or alternative fish species, which may be hazardous in an increasingly globalized market environment. Figure 12.1a,b show, respectively, the dominant species in marine fishery catches and the main seafood groups dedicated to human consumption (FAO, SOFIA 2010). Nowadays, fish can be produced in one country, processed in a second, and consumed in a third. The process of globalization has created substantial opportunities, but hand in hand with inherent risks. A very common fraudulent

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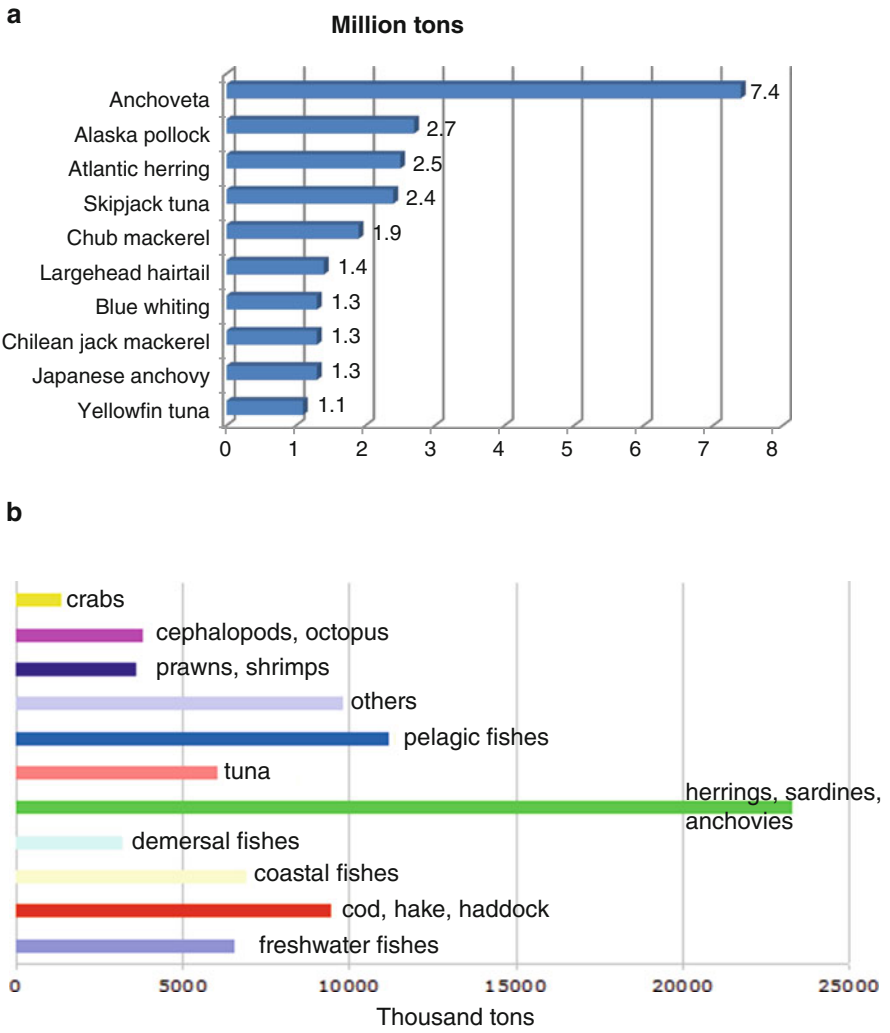

Fig. 12.1 Marine capture fisheries production: top ten species in 2008 (**a**), and the main seafood groups dedicated for human consumption (**b**) (FAO 2010)

practice is species substitution, which can be done unintentionally, but more frequently, looking for tax evasion, to launder illegally caught fish, or to sell low-priced fish species in a fraudulent substitution of more valuable higher-priced species. In addition, potential human health risks may appear as the fraudulent species can be harmful and may aggravate symptoms in sensitive human allergic patients. In fact, fish are one of the foods with more prevalence of food allergies (Sicherer and Sampson 2010).

Nowadays, consumers demand clear and reliable information about the species they eat, therefore the establishment of new standardized labeling and normative and inspection control regulations is growing continuously. The use of acceptable market names is essential for the identification of seafood because of the exceedingly great number and variety of species. Furthermore, in some cases and depending on the country, different genera share the same generic commercial name. This is the case, for example, of the generic denomination of "hake," used for commercialized species belonging to the *Merluccius* genera in Argentina, Spain, Chile, Australia, the United States, and the United Kingdom; nevertheless, other disparate species belonging to the genera *Dissostichus*, *Rexea*, or *Urophycis* are sold in Argentina, the United States, and the United Kingdom, respectively, under the same denomination of "hake." The need of common and acceptable market names for the seafood sold in interstate commerce and the need to assist manufacturers in labeling seafood products led to the publication in 1993 of *The Seafood List* by the U.S. Food and Drug Administration (U.S. FDA) to provide a source of names of several seafood species and reduce confusion within producers and consumers (Guidance for Industry 2012).

In the European Union, the need for labeling regulations has led to the Council Regulation (EC) No 104/2000 on the common organization of the markets in fishery and aquaculture products. This regulation advises that fish should be correctly labeled indicating: (1) the commercial designation of the species, (2) the production method (caught or farmed), and (3) the catch area. For this purpose, the member states draw up and publish a list of the commercial designations accepted in their territory indicating the scientific name for each species and the name in the language or languages of the member state. The indication of the catch area mentioned above is normalized in the Council Regulation (EC) No 2065/2001 in the annex following the FAO Yearbook (FAO yearbook 2000). Regulation (EC) No 104/2000 also indicates the importance of labeling seafood products with their scientific name to ensure traceability. These requirements have been implemented in each of the European states, such as Spain, where several regulations have been promulgated to assure the correct labeling and identification of seafood products (Royal Decree 1380/2002; Royal Decree 121/2004; Royal Decree 1702/2004).

To comply with all these regulations, accurate, sensitive, and fast detection methods that permit the direct authentication of fish in any food product are highly recommended. Conventional identification of unprocessed fish is done by examination of their anatomical and morphological features. However, even for marine expert biologists this is a difficult task in the case of very closely related fish species that coexist in the same catch area. This is the case, for example, of Cape hakes, *Merluccius capensis* and *Merluccius paradoxus*, two different species belonging to the Merlucciidae family with similar morphological features that overlap their geographical distribution on the South African coast. Due to this overlapping distribution, the species are caught and managed jointly and no distinction is made in stock management. In addition, and due to the development of the fishing industry, seafood products can be processed (beheaded, eviscerated, skinned, filleted, smoked, cooked,

or canned), often making the identification of their external anatomical or morphological features impossible.

For all these reasons, the use of molecular tools is therefore a suitable strategy to circumvent such problems. Although over the last two decades several DNA and protein molecular markers have been developed, recent successes of proteomics methodologies make them a promising strategy for fish authentication purposes. In light of this, a comprehensive overview of the state of the art and the future of proteomics approaches for fish species authentication is given in this chapter.

12.2 Traditional Molecular Strategies Used to Assess Fish Authenticity

12.2.1 Classical Protein-Based Methods Used for Fish Authenticity

Methodologies based on the detection of biomarker proteins representative of a particular species, using mainly electrophoretic or immunological assays, have been extensively exploited for the authentication of fish species.

In this respect, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of myofibrillar and sarcoplasmic proteins has been used for the identification of commercial fish species in both raw and processed products (Scobbie and Mackie 1988; Piñeiro et al. 1999a; Martinez et al. 2001). Using specific enzymatic staining procedures, Keenan and Saklee in 1985 published a collection of the most common enzyme patterns by SDS-PAGE for 164 different fish species. SDS-PAGE has also been used to identify those species or products subject to some thermal processing, except sterilization (Piñeiro et al. 1999a; Martinez et al. 2001).

Isoelectric focusing (IEF) is the most commonly used protein-based technique for species identification (Piñeiro et al. 2000; Tepedino et al. 2001). In fact, IEF was adopted by the Association of Official Analytical Chemists as the official validated method for species identification purposes (AOAC 1990) and the U.S. FDA offers an Internet library of IEF patterns of sarcoplasmic proteins from different fish (Regulatory Fish Encyclopedia, FDA). Urea IEF gels have been applied for discrimination of unrelated fish species in products that have been heat treated (Mackie et al. 2000; Rehbein et al. 2000). Parvalbumins are the sarcoplasmic proteins that show higher interspecific variability by IEF (Rehbein et al. 2000). Due to their thermal stability, these proteins can also be used by native IEF for the discrimination of cooked products, in addition to raw species (Carrera et al. 2010). In addition, the experiences accumulated in our lab using IEF on narrow strips of pH 4–6.5 have demonstrated the suitability of parvalbumin isoforms as species-defining markers for all commercial fish species belonging to the Merlucciidae family (Piñeiro et al. 1998; Carrera et al. 2006; Carrera 2008).

Several reports concerning the application, with authentication purposes, of the two-dimensional gel electrophoresis (2DE) to the characterization of fish proteins have been published (Piñeiro et al. 1998; Martinez and Jakobsen Friis 2004; Carrera et al. 2006). Interestingly, the application of 2DE has allowed the detection of potential species-specific proteins for the discrimination of closely related fish species, such as hake (Piñeiro et al. 1998, 2001; Carrera et al. 2006), puffer fish (Chen et al. 2004), commercial flat fish (Piñeiro et al. 1999b), and perch species (Berrini et al. 2006). 2DE database servers for different organisms are available on the Internet (World-2DPAGE List, http://world-2dpage.expasy.org/list/; Appel et al. 1996); although up to date, for Teleostei species, only one 2DE image for rainbow trout is included in the FishProm database from Aberdeen University (http://www.abdn.ac.uk/fishprom/).

The detection of potential species-specific proteins by 2DE can be further investigated by other complementary techniques such as mass spectrometry (MS). Thus, following a classical bottom-up proteomics approach, consisting of 2DE, tryptic in-gel digestion, and MS/MS analysis, representative spots or clusters of spots for the Merlucciidae family, corresponding to nucleoside-diphosphate kinase proteins (NDK) (Carrera et al. 2007), to aldolase proteins (Carrera et al. 2009), and to parvalbumin (PRVB) isoforms, were identified, characterized, and de novo sequenced (Carrera et al. 2006, 2010). Immunological methods, based on the specificity of the antigen–antibody (Ag–Ab) reaction, are particularly attractive because they combine specificity, sensitivity, and simplicity. The first efforts to produce Abs to identify fish species were described by Mairs and Sindermann in 1962, who prepared polyclonal Abs (pAbs) to discriminate clupeid species. Furthermore, pAbs against certain sarcoplasmic proteins have been developed for the discrimination of species such as sardines, salmon, trout, halibut, haddock, grouper, and Nile perch (Domínguez et al. 1997; Céspedes et al. 1999a; Asensio et al. 2003a). The production of pAbs is simple and economical. However, it presents a number of drawbacks: (1) low specificity, resulting in cross-reactivity problems, (2) limited amount of Ab, and (3) requiring continuous immunizations of new animals, leading to the existence of nonreproducible batches. These problems can be solved with the generation of monoclonal Abs (mAbs). These Abs are selected by their ability to discriminate species with high specificity and absence of cross-reactivity against other species. Also, the production of mAbs after obtaining the hybridoma is unlimited. Several mAbs have been generated against species of red snapper (Huang et al. 1995), grouper (Asensio et al. 2003b), and catfish (McNulty and Klesius 2005; Gajewski, et al. 2009). The main immunological techniques that have been employed using pAbs or mAbs are the immunodiffusion (Domínguez et al. 1997), ELISA (Huang et al. 1995; Céspedes et al. 1999a; Asensio et al. 2003a, b; McNulty and Klesius 2005) and Western blot (Zhang and Rasco 1996; Domínguez et al. 1997). Immunoassays are advantageous in that, once developed, they are easy to use, having high sensitivity and throughput, thus allowing the process of a high number of samples in a short time. However, this technology is expensive and time consuming, and is not completely exempt from potential cross-reactivity. Apart from this, immunoassay can display important limitations in the analysis of processed food because processing

can greatly affect the tertiary structure of the protein and thus negatively affect recognition of the target protein by the antibody.

General limitations of these classical protein-based methods, such as being very laborious and time-consuming methodologies, may be solved with the introduction of alternative methods based on DNA amplification and hybridization.

12.2.2 Common DNA-Based Methods Used for Fish Authenticity

Analytical methods based on nucleic acid detection, mainly DNA, have achieved an outstanding position in the authentication of fishery products in recent decades (Sotelo and Pérez-Martín 2007; Rasmussen and Morrissey 2009). Compared with the above-mentioned methodologies, DNA techniques have considerably higher discriminating power as they are based on identification at the sequence level of specific DNA fragments that are unique for a particular species. Together with their sensitivity, this is why DNA-based procedures have become popular methods for unambiguous identification of fish species even for closely related species.

The analysis of specific genetic sequences can be applied to both fresh and processed products, thanks to the stable nature of the DNA molecule, its ubiquitous character, and high content in diriment information, which is not affected by variations of expression. Nuclear genes such as the 5S ribosomal DNA, 5S ribosomal RNA, internal transcribed spacer 1 (ITS1), and certain microsatellite loci have been considered for the study of phylogenetic relationships among fish (Céspedes et al. 1999b; Castillo et al. 2003; Asensio et al. 2004; Pérez and García-Vázquez 2004). Among the DNA targets, mitochondrial DNA (mtDNA) is generally preferred because of its maternal inheritance, a relatively fast evolutionary rate, and the lack of intermolecular genetic recombination. The most commonly used mtDNA markers include the cytochrome b gene (Rehbein et al. 1997; Sotelo et al. 2001; Calo-Mata et al. 2003; Chapela et al. 2007), the mtDNA control region (Quinteiro et al. 2001), and the 12S rRNA region (Comesaña et al. 2003; Zhang et al. 2006). Today, most DNA-based methods for species identification in foods consist of the highly specific amplification of one or more DNA fragments by means of polymerase chain reaction (PCR). This technique presents high potential due to its simplicity, sensibility, and specificity. In this sense, several methods have been developed in order to perform polymorphism searches such as the restriction fragment length polymorphism (PCR-RFLP) used for the identification of different species of salmon, gadoids, flatfish, and hake (Russell et al. 2000; Quinteiro et al. 2001; Sotelo et al. 2001; Pérez et al. 2004; Aranishi et al. 2005). Other techniques include the amplified fragment length polymorphism (PCR-AFLP) (Maldini et al. 2006); the single-stranded conformational polymorphism (PCR-SSCP) for the identification of tuna, salmon, flatfish, Nile perch, and hake (Colombo et al. 2005; Chapela et al. 2007); the random amplified polymorphic DNA (RAPD) for the discrimination of

Tilapia species, Nile perch, and grouper (Partis and Wells 1996; Asensio et al. 2002); and multiplex PCR for grouper fillets (Trotta et al. 2005). In addition, some examples of sequencing techniques, such as forensically informative nucleotide sequencing (PCR-FINS) have been used to identify anchovies, sardines, and hake species (Jérôme et al. 2003; Santaclara et al. 2006). The latest and more fashionable methods for fish species identification are the approaches based on real-time PCR (Sánchez et al. 2009), microarrays (Kochzius et al. 2008), and lab-on-a-chip systems (Chen et al. 2011) for their potential to identify and quantify seafood species on a large scale.

Despite all these advantages, DNA-based procedures are not exempt from some important limitations that are especially important in the case of processed foods. During the processing of fish products, disruption of the cellular integrity can occur, causing the release of hydrolytic enzymes. Together with this, heat treatment and an acid environment can negatively affect DNA integrity, reducing the length of fragments to be amplified and consequently increasing the chances of having nonspecific identifications. Another important limitation is the complexity of foods, which can yield important matrix effects that negatively affect the accuracy and robustness of results. This complicates the development of standardized protocols for DNA extraction, thus being necessary to optimize them for each particular situation to ensure that enough DNA is obtained for the analysis and inhibitors of the reduced or eliminated PCR.

Although over the last two decades, several DNA and protein molecular markers have been developed, proteomics methodologies are emerging as a promising strategy for fish authentication.

12.3 Proteomics Technologies for Fish Authentication

As a discipline, proteomics is defined as the large-scale analysis of proteins in a particular biological system at a particular time (Pandey and Mann 2000). Recent successes illustrate the role of mass spectrometry, mainly matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) and electrospray-ion trap (ESI-IT) mass spectrometry, as an indispensable tool for proteomics studies (Aebersold and Mann 2003). But the history of proteomics began in the 1970s with the development of 2DE, which provided the first method for displaying hundreds or thousands of proteins on a single gel (Klose 1975; O'Farrell 1975). Nowadays, bioinformatics treatment of the data has increased the scale of proteomics tools, representing a powerful strategy for a high-throughput protein and peptide identification and quantification.

Proteomics methodologies have been used for the identification of some seafood species such as mussels (López et al. 2002) and shrimp (Ortea et al. 2009), but their application on Teleostei species authentication is still scarce. An idea of the current limited impact of proteomics in any type of research involving the Teleostei group can be obtained if one considers that protein databases include 239,454 amino acid

sequences from Teleostei, whereas there are more than 1,118,730 amino acid sequences reported for the Tetrapoda group (UniProtKB, February, 2012) (Fig. 12.2).

Figure 12.3 shows the proteomics pipeline and tools that are currently being used at our laboratory for fish authentication. Two consecutive phases, the discovery phase (Carrera et al. 2006, 2010) and the target-driven phase (Carrera et al. 2011) are described in detail in the following sections using as an example the identification of all the commercial fish species belonging to the Merlucciidae family.

12.3.1 Discovery Phase

In this phase, to identify new potential peptide biomarkers for the identification of fish species (i.e., Merlucciidae species), organisms whose genomes remain unsequenced, we use a classical bottom-up proteomics approach (Fig. 12.3).

Thus, potential specific proteins, according to 2DE analysis, are selected, subjected to tryptic digestion, and the recovered peptides then ionized and analyzed by means of MS. As mentioned above, different spots or clusters of spots corresponding to parvalbumin (PRVB) isoforms (11.20–11.55 kDa and p*I* 3.75–4.57 units) (Piñeiro et al. 2001; Carrera et al. 2006, 2010), nucleoside-diphosphate kinase proteins (NDK) (16.80–18.60 kDa and p*I*5.04–5.47 units) (Piñeiro et al. 2001; Carrera et al. 2007), and aldolase proteins (42–43 kDa and p*I* 6.5 units) (Carrera et al. 2009), showed noticeable qualitative interspecific differences by 2DE, and were further investigated by MS.

The capacity of the peptide mass fingerprinting (PMF) methodology by MALDI-TOF MS was ascertained by analyzing 10 closely related commercial species of the Merlucciidae family (Carrera et al. 2006). MALDI-TOF mass fingerprints of the sarcoplasmic protein PRVB defined a set of molecular fish authentication markers, relying on the presence or absence of species-specific peptide masses, providing: (1) the selective differentiation between the genus *Merluccius* and *Macruronus*; (2) the classification of the hake species in two groups according to their geographic procedence, American or Euro-African hake; and (3) the unequivocal identification of several hake species, *M. bilinearis*, *M. australis polylepsis*, *M. australis australis*, *M. productus*, *M. paradoxus,* and *M. polli*, whereas the rest of the hake species can be grouped in two clusters, comprising *M. hubbsi* and *M. gayi* in one and *M. merluccius* and *M. capensis* in the other. Due to PRVB interspecific variability and high concentration in the muscle from fish, we can forecast that this protein can be used as a good biomarker for fish species identification. The selection of PRVB as a target protein has additional importance inasmuch as it is a protein that presents a high thermostability (Kawai et al. 1992; Elsayed and Bennich 1975; Carrera et al. 2010). For that, the monitoring of peptide masses ensures an overall applicability of the method for fish authentication in both fresh and processed seafood products. A similar approach was further successfully applied for the identification of 25 different fish species (Mazzeo et al. 2008). The authors, using the same strategy previously reported for our group, characterized specific PRVB peptide masses capable of

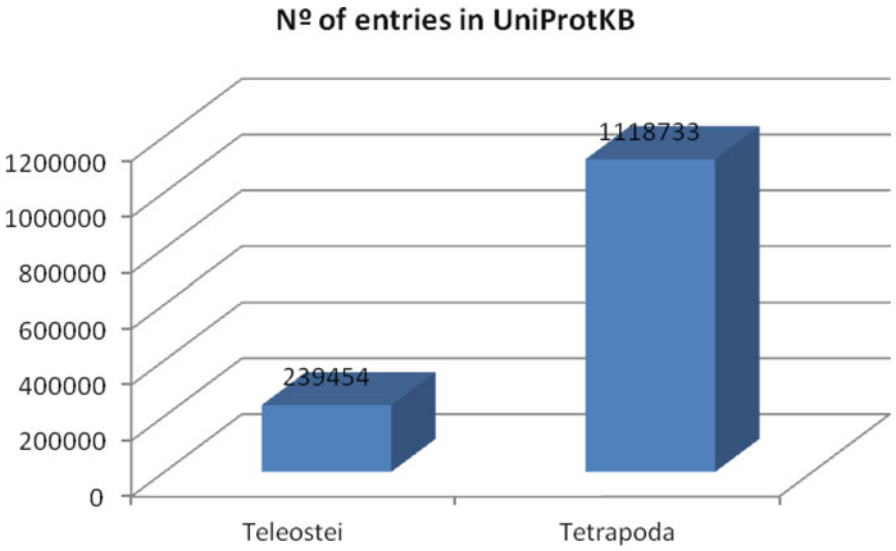

Fig. 12.2 Protein sequences annotated in UniProtKB database for Teleostei and tetrapods

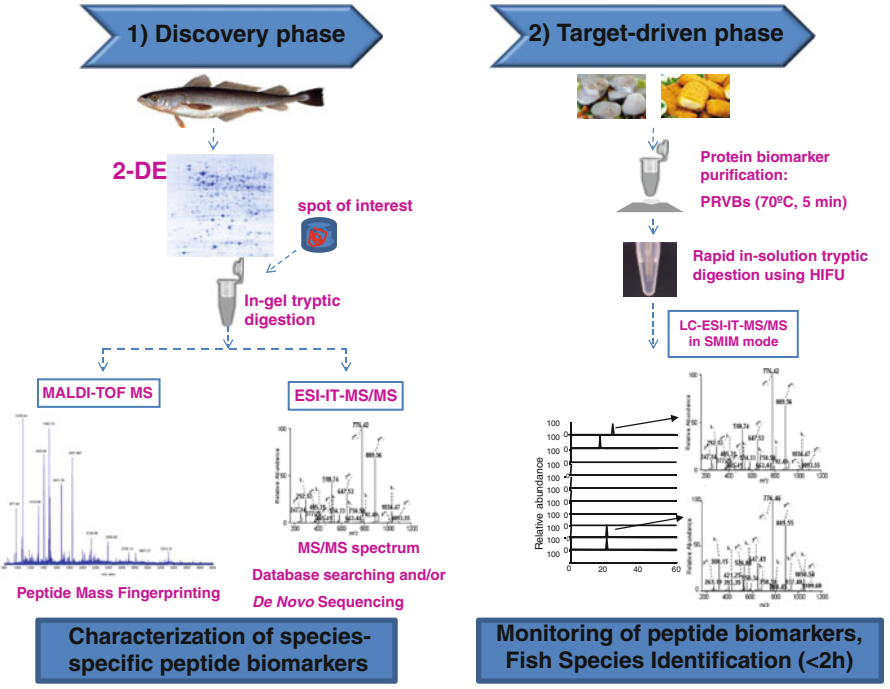

Fig. 12.3 Proteomics pipeline considered for the identification, characterization and detection of species-specific peptide biomarkers with fish authentication purposes

differentiation by MALDI-TOF MS a large number of fish species belonging to three different orders (Perciformes, Gadiformes, Pleuronectiformes).

In a subsequent study (Carrera et al. 2010), we proposed a novel strategy for the extensive characterization of all the PRVBs isoforms in all the commercial species from the Merlucciidae family (the previous 10 species and *M. senegalensis*). This strategy is based on the integration of a classical bottom-up proteomics approach with accurate M_r determination by Fourier-transform ion-cyclotron resonance (FTICR)-MS of intact proteins and selected tandem mass spectrometry (MS/MS) ion monitoring (SMIM) of peptide mass gaps. For each PRVB, mass spectra obtained by LC-ESI-IT-MS/MS from two digests (trypsin, Glu-C) were followed by database searching using Sequest (Eng et al. 1994) and *de novo* sequenced manually with the help of two programs, PEAKS (Ma et al. 2003) and DeNovoX (Thermo Electron Co.) (Scigelova et al. 2007). The deduced peptide sequences were arranged and the theoretical M_r for the resulting sequences was calculated. Experimental M_r for each PRVB was measured with high mass accuracy by FTICR-MS (0.05–4.47 ppm). The masses of several missing peptide gaps were estimated by comparing the theoretical and experimental M_r, and the MS/MS spectra corresponding to these ions were obtained by LC-ESI-IT-MS/MS in the SMIM scanning mode. Finally, all peptide sequences were combined to generate the final protein sequences. This approach allowed the complete *de novo* MS-sequencing of 25 new PRVB isoforms. This study constitutes the report accounting for the higher number of new proteins completely sequenced making use of MS-based techniques only.

Several species-specific peptide biomarkers were selected to effectively identify all the species from the Merlucciidae family. PRVBs peptide sequences with high interspecific variability, obtained after the extensive *de novo* sequencing of PRVBs previously published (Carrera et al. 2010), were used for this purpose. Eleven tryptic peptides were selected on the basis of the information that their combined presence or absence could be used to confidently identify all of the species under study. A flow diagram for the unambiguous systematic discrimination was also achieved (Carrera et al. 2011). According to this scheme, the presence/absence of several peptide biomarkers achieves: (1) identification if any member from the Merlucciidae family is present in the sample, (2) discrimination between the genera *Merluccius*, (3) classification of hake species into two groups according to their geographic distribution: American hake or Euro-African hake, and (4) finally the combination of the presence/absence of eight other peptide biomarkers allows the unambiguous identification of any specific species from the Merlucciidae family.

The selection of PRVB as the protein biomarker is particularly interesting because it is considered the major fish allergen (Elsayed and Bennich 1975), and therefore an analysis targeting this protein would have a double application, both for species identification and food safety purposes.

Moreover, particular attention was also focused on the characterization and identification by the MS-based biomarker discovery of several other peptide markers to discriminate all the commercial species belonging to the Merlucciidae family. In fact, using the same classical bottom-up proteomics approach, the characterization

by *de novo* peptide sequencing of the different nucleoside diphosphate kinase (NDK) (Carrera et al. 2007) and aldolase proteins (Carrera et al. 2009), allowed the characterization of additional species-specific peptides that also can be used for fish authentication purposes.

These peptide biomarkers could be used to develop easy-to-use kits based on antibodies or monitored by MS using an innovative strategy that is described in the next phase of our pipeline.

12.3.2 Target-Driven Phase

In the second phase of the proteomics pipeline used in our laboratory for fish authentication purposes (Fig. 12.3), we developed a new targeted MS-based strategy for the fast monitoring of the species-specific peptide biomarkers found in the discovery phase (Carrera et al. 2011).

The performance of this target-driven method was established for the unequivocal identification of all commercial fish species belonging to the Merlucciidae family. It was based on (1) the purification of PRVBs by heat treatment (time 45 min), (2) their accelerated tryptic digestion using high-intensity focused ultrasound (HIFU; time 2 min), and (3) the monitoring of 11 PRVB peptide biomarkers by selected tandem mass spectrometry ion monitoring in a linear ion trap (LIT) mass spectrometer (time 60 min). Each step was individually adjusted to minimize analysis time. Thus, PRVBs, considered as the best protein biomarker for fish authentication of Merluciidae species, were purified from the sarcoplasmic extracts, taking advantage of their thermostability (Kawai et al. 1992). After treatment with heat (70ºC, 5 min), the majority of identified peptides corresponded to PRVBs (77.87%). These results demonstrated that the treatment with heat is a simple, fast, and effective procedure to purify and enrich the samples in only PRVBs. Purified PRVBs were digested with trypsin using a fast procedure by HIFU. Accelerated HIFU-tryptic digestions produced results comparable to those obtained by the conventional overnight incubation methods. Thus, the combination of a fast and easy protein purification procedure (time 45 min) with the use of HIFU for protein digestion (time 2 min) considerably simplified and reduced the time needed for sample preparation, reflected in the overall time needed for monitoring. Then, a particular combination of only 11 peptides (Table 12.1), resulting from the HIFU-assisted tryptic digestion of the thermostable proteins PRVBs, were subjected to SMIM analysis in an LIT mass spectrometer focusing the MS/MS events on the corresponding precursor ions. Once MS/MS spectra were recorded, virtual chromatograms for all the different fragments could be obtained. Tracing the highly sensitive transitions (precursor $m/z \rightarrow$ fragment m/z) for each peptide biomarker is possible for the unequivocal identification of all Merlucciidae species (Fig. 12.4). Also, the use of the SMIM mode for scanning gives the possibility of obtaining full MS/MS information necessary for the validation of the peptide biomarker sequence.

Table 12.1 Peptide biomarkers and specific transitions for the identification of all the species from the Merlucciidae family (Reprinted with permission from Carrera M, Cañas B, López-Ferrer D, Piñeiro C, Vázquez J, Gallardo JM. Fast monitoring of species-specific peptide biomarkers using high-intensity-focused-ultrasound-assisted tryptic digestion and selected MS/MS ion monitoring. Anal. Chem. 2011, 83, 5688-5695. Copyright 2011 American Chemical Society)

Biomarker code	Parvalbumin (PRVB) Peptide Sequence	SMIM Transition *m/z* precursor ion (*z*) → *m/z* fragment ion	Retention time (min)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	Cross-reaction with proteins from other organisms by BLAST
F-MER604	LFLQVFSAGAR	604.84 (2+)→948.52 (y''^{+}_{9})	23.00	■	■	■	■	■	■	■	■	■	■	■	■	-
G-MER517	VFGIIDQDK	517.78 (2+)→ 788.41 (y''^{+}_{7})	20.00	■	■	■	■	■	■	■	■	■	■	■	□	-
G-MAC524	VFAIIDQDK	524.79 (2+)→ 802.43 (y''^{+}_{7})	20.00	□	□	□	□	□	□	□	□	□	□	□	■	*Xenopus laevis, Xenopus tropicalis*
S-MER967	AGDSDGDGAIGVDEFAVLVK	967.97 (2+)→ 1189.68 (y''^{+}_{11})	21.50	□	□	□	□	□	■	■	■	■	■	■	□	-
S-MER794	(N-Ac)A FSGILADADIAAALK	794.93 (2+)→ 1187.59 (b_{12})	25.00	□	□	□	□	□	□	□	□	□	□	■	□	-
S-MER612	IGVDEFTAMLK	612.32 (2+)→ 954.46 (y''^{+}_{8})	22.50	□	□	□	□	□	□	□	□	□	■	□	□	-
S-MER736	AEGTFTHGEFFTK	736.35 (2+)→ 966.46 (y''^{+}_{8})	23.00	□	□	□	□	□	□	□	■	■	□	□	□	-
S-MER721	AEGTFTHGVFFTK	721.36 (2+)→ 936.49 (y''^{+}_{8})	23.50	□	□	□	□	□	□	□	□	■	□	□	□	-
S-MER590	IGVDEFAAMVK	590.31 (2+)→ 910.43 (y''^{+}_{8})	22.50	■	□	□	■	■	■	■	■	■	□	■	□	*Trachurus japonicus and others*[a]
S-MER973	AGDSDGDGAIGVDEWAALVK	973.46 (2+)→ 1087.57 (y''^{+}_{10})	23.00	□	□	□	□	■	□	□	□	□	□	□	□	-
S-MER987	AGDSDGDGAIGVDEWAVLVK	987.48 (2+)→ 1115.61 (y''^{+}_{10})	22.50	□	□	□	■	□	□	□	□	□	□	□	□	*Gadus morhua*

m/z (mass/charge).

(■) Denotes the presence of a peptide biomarker and (□) the absence.

S1 (*Merluccius merluccius*), S2 (*M.capensis*), S3 (*M. senegalensis*), S4 (*M. polli*), S5 (*M. paradoxus*), S6 (*M. hubbsi*), S7 (*M. gayi*), S8 (*M. australis polylepis*), S9 (*M. australis australis*), S10 (*M. productus*), S11(*M. bilinearis*), S12(*Macruronusspp.*).

([a]) *Sparus aurata, Fundulus grandis, Fundulus similis, Fundulus heteroclitus, Hypophthalmichthys nobilis, Paralichthys olivaceus, Theragra chalcogramma.*

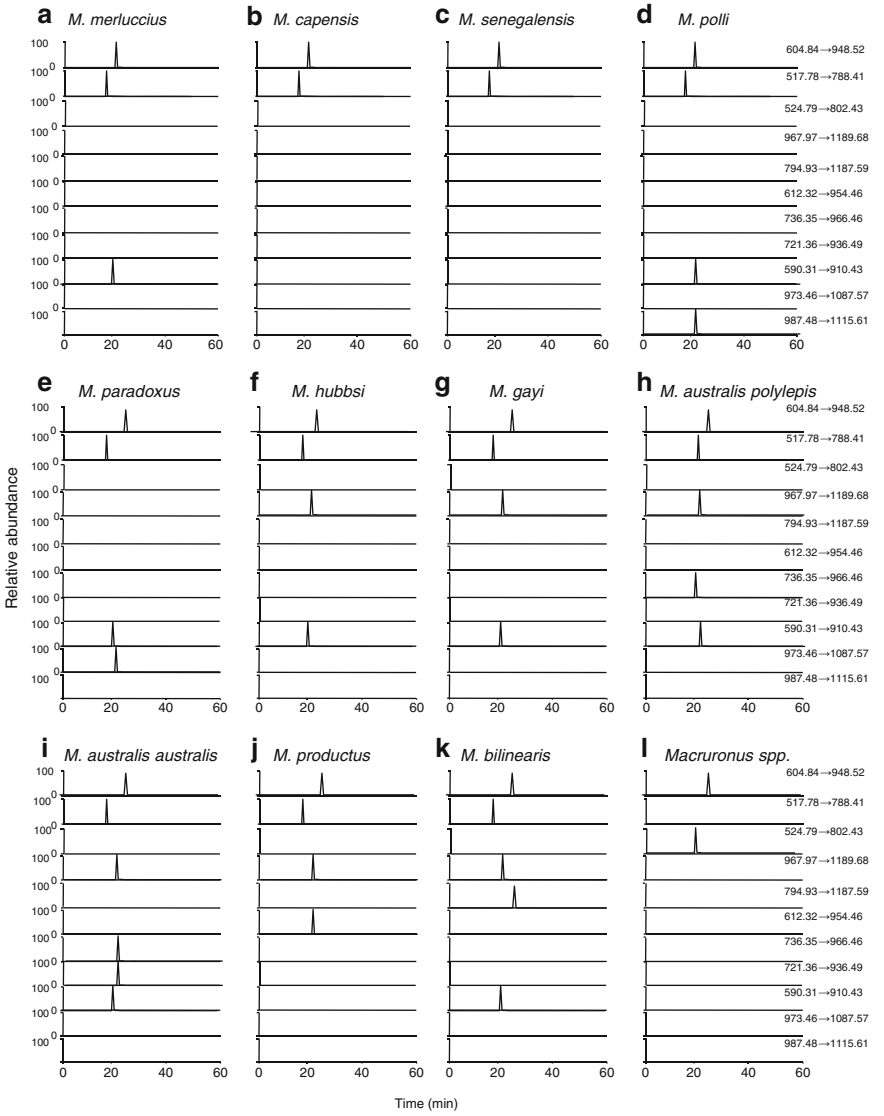

Fig. 12.4 Reference SMIM traces for each Merlucciidae species, plotting the corresponding canonical transition for each PRVB tryptic peptide biomarker (Reprinted with permission from Carrera M, Cañas B, López-Ferrer D, Piñeiro C, Vázquez J, Gallardo JM. Fast monitoring of species-specific peptide biomarkers using high-intensity-focused-ultrasound-assisted tryptic digestion and selected MS/MS ion monitoring. Anal. Chem. 2011, 83, 5688-5695. Copyright 2011 American Chemical Society)

An additional validation step using commercial fish products successfully demonstrated the applicability of this new targeted strategy for the fast detection of mislabeling practices in both fresh and processed fish products.

With this new strategy, we demonstrated that all relevant fish species belonging to the Merlucciidae family present in any seafood product can be unequivocally identified in less than 2 h.

This workflow constitutes the fastest method for peptide biomarker monitoring and its application to food quality control provides authorities with a rapid and effective method for food authentication and traceability to guarantee quality and safety to consumers.

12.4 Concluding Remarks and Future Outlook

As we discussed in this chapter the potentiality of the proteomics pipeline developed in our laboratory for fish species identification is noticeable. The two consecutive steps (discovery phase and target-driven phase) allow the identification and characterization of species-specific peptides that can be monitored by MS allowing the unequivocal and fast identification of fish species in any seafood product. Currently this pipeline is also being successfully applied in our laboratory for the identification of other species belonging to the order Decapoda (Ortea et al. 2009) and to develop a rapid and reliable method for bacterial identification in foodstuffs (Böhme et al. 2011). Nevertheless, we consider that its application field is not restricted to food authentication purposes and that may offer new opportunities to the food science sector such as the detection of allergens, the characterization of bioactive peptides, the study of the effects caused by processing and storing on food proteins, and so on. In addition, we consider that the procedures and results obtained using this proteomics pipeline may be stored in a centralized and web-accessible open source that supports their dissemination and their potential applicability to further food science projects.

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