Chapter 10
Evaluation of Fish Quality and Safety by Proteomics Techniques

Carmen Piñeiro and Iciar Martinez

10.1 Introduction

Food quality is a complex concept. In marketing and economics literature, there are two main approaches to define food quality (Grunert 2005): the holistic approach, which includes within the concept of food quality "all the desirable characteristics a product is perceived to have," and the excellence approach, which views food quality as referring only to characteristics that pertain to a higher, more restrictive, or "superior" specification of the product. The holistic approach leaves wide scope for interpretation: quality can mean conforming to standards (including standards pertaining to the environment, local specialities, organic production, ethics, and even taste and smell) and it can refer to subjectively perceived quality attributes. Quality is also a factor that involves the entire production process, from raw materials, processing, and packaging up to consumption of the product.

The terms "food safety," "food security," and "food quality" should not be confused. Food safety refers to all those hazards, whether chronic or acute, that may make food injurious to the health of the consumer. Food safety is not negotiable: all food items must be safe. Food security is defined by Pinstrup-Andersen (2009) as "a situation that exists when all people, at all times, have physical,

C. Piñeiro
Institute for Marine Research (IIM), Spanish Council for Scientific Research (CSIC), C/Eduardo Cabello 6, Vigo E-36208, Spain

I. Martinez (✉)
Deptarment of Zoology and Cellular Biology, University of the Basque Country, Leioa E-48940, Spain

IKERBASQUE, Basque Foundation for Science, Bilbao E-48011, Spain
e-mail: iciar.martinez@ehu.es

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social, and economic access to sufficient, safe, and nutritious food that meets their dietary needs and food preferences for an active and healthy life." Food quality, as mentioned above, refers to those attributes that influence a product's value to the consumer.

According to the plain English definitions used in ISO 9000, 9001, and 9004: "The quality of something can be determined by comparing a set of inherent characteristics with a set of requirements. If those inherent characteristics meet all requirements, high or excellent quality is achieved. If those characteristics do not meet all requirements, a low or poor level of quality is achieved." According to this definition, the concept of quality is subjective and relative to how well the product fulfills the customer's expectations. Its limits are usually set by what the customer will be willing to pay and in the case of fish products, it entails aspects related to the species, size, sex, and condition, geographic origin, chemical composition (its content in vitamins, minerals, bioactive compounds, protein, and fat as well as its digestibility and desirable fatty acid composition), freshness, production method (wild, intensively or organically farmed), type of processing if applicable (frozen vs. not frozen, canned, smoked, salted, etc.), and the resulting sensory aspects (taste, texture, color, smell).

In general, however, the term "seafood quality" also includes the product's safety (Yaktine et al. 2008) and it is important to emphasize that safety and quality management of seafood may be complex due to the very large number of edible species; the fact that even farmed species are not truly domesticated (they have been commercially farmed for only a few generations); the greater variability in their environment, accessibility, and biodiversity; the difficulty in controlling their environmental conditions (temperature, pH, salinity); and the potential presence of toxic compounds in the environment.

Proteins play a crucial role in almost every biological process. In addition to providing structural support, they are responsible for an ample variety of physiological functions, including catalysis, defense, transport, and sensing, in all living systems. Whereas the genome of an organism is more or less constant and specific, its proteome (i.e., the entire complement of proteins expressed by the genome) is highly dynamic. The type and amount of proteins expressed vary not only between different types of cells in the same organism but also in a given cell type as a response to a wide diversity of stimuli and environmental factors.

The formidable capability in post-genomic analyses built up over the past few years, including the use of DNA microarrays and proteomics, underpins the ongoing research strategy. One single gene can produce a variable number of proteins whose function may be modulated by further post-translational modifications. Given that one of the major objectives of proteomics is to quantify protein levels and their dynamic changes (Hocquette et al. 2005), this approach can be used, when applied to food matrices, to improve our knowledge of the interrelationship between the changes induced during the production and processing of seafood on the protein map and the quality and safety of the product, thus opening the possibility of using proteomic techniques as robust tools to monitor the state of seafood products in a given step within the production chain.

Only complete knowledge of the exact chemical composition of a food item allows the correct evaluation of its nutritional, toxicological, and technological properties. Novel analytical technologies, including proteomics, will generate comprehensive databases of the protein composition of food items. These databases should include particularly interesting minor components that may have been overlooked by conventional methods of protein analysis, as well as changes in the protein composition originating as a consequence of applying different production and processing methods. Furthermore, proteome analysis adds the intriguing perspective of achieving a systematic overview of the metabolic routes within defined cells or tissues of food components (Pischetsrieder and Baeuerlein 2009), providing valuable information about the cellular processes that have occurred in the organism prior to slaughtering, including stress responses, inflammation, defense, apoptosis, and immunomodulation. This adds the possibility of identifying biomarkers to indicate the exposure of the organism to stresses and contaminants, in addition to helping map the chemical composition and properties of the seafood. As a consequence, proteomic analyses of seafood have the potential to tremendously increase our knowledge of the composition, safety, and effect of the production and processing methods of the raw materials and, therefore, on the impact of these variables on seafood product quality.

Further nonenzymatic post-translational protein modifications (nePTM) occur frequently during food processing and storage, rendering the food proteome even more complex. nePTMs are mostly caused by oxidation and by nonenzymatic reactions of sugars with amino acid side chains (Maillard reaction or glycation). Additionally, other nePTMs have been reported, such as condensation, elimination, or hydrolysis of side chains or breakdown of the peptide backbone. nePTMs also have significant consequences regarding the technological, nutritional, and toxicological properties of processed food and proteomic techniques have been applied to the systematic study of the formation of nePTMs in processed food items, many of which are largely unknown (Pischetsrieder and Baeuerlein 2009). The challenges of nePTM analysis are their low abundance compared to the unmodified or PTM-modified side chains and their high heterogeneity. Knowledge of the chemical structure and binding sites of nePTMs, however, is crucial to evaluate their effects on food safety and quality. The analysis of the proteome, optimally combined with the metabolome, promises to yield a systematic overview of the changes in food that are caused by changes in production parameters. More important, the recently proposed FOODOMICS approach (Herrero et al. 2012) intends to use proteome research to actually understand the molecular processes that link production parameters to food quality. Currently, proteomics is built upon the foundations of genomics, in such way that a lack of genomic information on a particular species can substantially limit success in protein identification (Graham et al. 2005) and, although sequence data from marine fish and shellfish species have significantly increased in the last decade, they are still scarce (Piñeiro et al. 2003; Forné et al. 2010). Japanese pufferfish (*Takifugu rubripes*), zebra fish (*Danio rerio*), and the estuarine fish *Fundulus heteroclitus* are the first three bony fish whose complete genome sequences became available. According to the NCBI protein database there were over 2.6

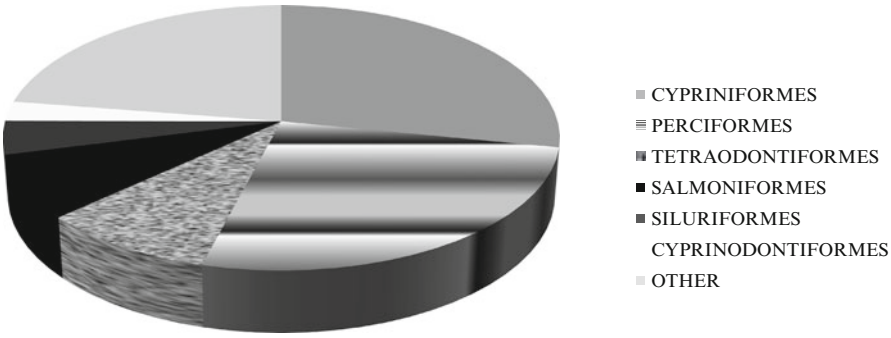

Fig. 10.1 Euteleostei orders with the highest number of protein sequences (Source NCBI protein data base)

million proteins sequenced for the subphylum Vertebrata at the beginning of 2012 of which about 14% belong to bony fish. Figure 10.1 shows the Teleostei orders with a larger number of known DNA sequences and in Table 10.1 the species of the same taxonomic class with a larger number of protein sequences available in the NCBI database are listed.

Almost 40% of the fish proteomic papers deal with issues related to aquaculture. The studies carried out thus far have clearly demonstrated the potential of proteomics to identify physiologically relevant molecules and mechanisms, biomarkers for seafood management and fish welfare, and to evaluate the impact of environmental pollution. In addition, some studies combining proteomics with genomic, metabolomic, and functional approaches provided a wider vision of the physiological functions of interest and pointed out the direction for future research (Forné et al. 2010). Interestingly, these authors stated that the reasons why proteomic techniques have not been applied to resolve problems in fishery product technology remain the same already mentioned 10 years ago by Piñeiro et al. (2003), namely that seafood proteins easily become insoluble and/or aggregate, displaying high molecular weights in addition to exhibiting a large number of isoforms and weak ionization levels.

10.2 Mapping Fish Quality by Proteomics Techniques

Research on seafood quality by means of proteomics is a complicated task: the term "seafood" refers to a large number of different species that may present tissue-, developmental stage-, and temperature-acclimation-dependent polymorphisms (Martinez et al. 1991; Watabe et al. 1992) and on the other hand, processing will further alter the protein components, thus increasing the number of spots and protein sequences with potential diagnostic value for quality determination.

The introduction of consumer perception and marketing studies enlarges the list of quality definitions previously mentioned. Santesmases (2004) distinguished between objective and subjective quality and Verdú (2003) referred to subjective or

Table 10.1 *Euteleosteii* species with the highest number of protein sequences. In *brackets* the number of sequenced proteins at the beginning of 2012 according to NCBI protein database

71,000-1,500 sequences	1,500-500 sequences	500-400 sequences	400-350 sequences	350-315 sequences
Danio rerio (70,887)	*Kryptolebias marmoratus(1,389)*	*Rutilus rutilus(515)*	*Oncorhynchus kisutch(394)*	*Pomatoschistus minutus(346)*
Tetraodon nigroviridis(28.512)	*Anoplopoma fimbria(1,280)*	*Eleutheronema tetradactylum(497)*	*Oreochromis mossambicus(389)*	*Larimichthys crocea(345)*
Oreochromis niloticus(24,076)	*Paralichthys olivaceus(1,048)*	*Oncorhynchus clarkii(495)*	*Etheostoma rufilineatum(383)*	*Gadus macrocephalus(345)*
Salmo salar(17,341)	*Larimichthys polyactis(1,031)*	*Alosa fallax(478)*	*Conger myriaster(381)*	*Mugil cephalus(336)*
Oncorhynchus mykiss(6,735)	*Epinephelus coioides(992)*	*Typhlichthys subterraneus(459)*	*Anguilla japonica(378)*	*Hypophthalmichthys nobilis(336)*
Ictalurus punctatus(4,386)	*Scleropages formosus(883)*	*Scophthalmus maximus(449)*	*Barbatula barbatula(373)*	*Epinephelus bruneus(334)*
Takifugu rubripes(2,702)	*Ctenopharyngodon idella(856)*	*Acanthemblemaria spinosa(448)*	*Rhinichthys osculus(370)*	*Denariusa bandata(331)*
Oryzias latipes(2,638)	*Oncorhynchus tshawytscha(754)*	*Acanthemblemaria aspera(436)*	*Atherina boyeri(366)*	*Anguilla anguilla(331)*
Gadus morhua(2,245)	*Ictalurus furcatus(744)*	*Gadus chalcogrammus(430)*	*Siniperca chuatsi(360)*	*Megalobrama amblycephala(329)*
Dicentrarchus labrax(,2201)	*Salvelinus alpinus(681)*	*Etheostoma simoterum(426)*	*Solea solea(358)*	*Ctenogobiops feroculus(323)*
Cyprinus carpio(1,921)	*Salmo trutta(628)*	*Oncorhynchus keta(422)*	*Perca flavescens(356)*	*Galaxias sp. 'southern'(322)*
Esox lucius(1,743)	*Sparus aurata(603)*	*Pseudorasbora parva(422)*	*Oncorhynchus kisutch(394)*	*Squalius pyrenaicus(320)*
Carassius auratus(1,539)	*Fundulus heteroclitus(570)*	*Oncorhynchus masou(414)*	*Oreochromis mossambicus(389)*	*Brevoortia tyrannus(320)*
Gasterosteus aculeatus(1,503)	*Poecilia reticulata(529)*	*Galaxias gollumoides(410)*	*Etheostoma rufilineatum(383)*	*Salmoniformes sp.(316)*
Osmerus mordax(,1493)	*Cynoglossus semilaevis(519)*	*Misgurnus anguillicaudatus(400)*	*Opsariichthys bidens(354)*	*Etheostoma caeruleum(315)*

Other taxa about 168,000

perceived quality. In the present work, we address the application of proteomics to the examination of the quality aspects inherent to the organism and already present before its harvesting and those aspects acquired or modified after its harvesting.

Quality aspects inherent to the organism would include its species, production method (wild, farmed), and food safety aspects related to exposure to environmental contaminants. Quality aspects acquired or modified after harvesting would include post-mortem changes and sensory attributes, the effect of processing and storage (cold storage, freezing, smoking), and food safety aspects related to post-harvest contaminations, mainly those of microbial origin.

10.2.1 Pre-mortem Quality and Safety Aspects of Seafood

10.2.1.1 Species and Size

Species and size are two main parameters determining the quality and price of seafood. There is an entire chapter of this book on the issue of species identification, accordingly we only mention here that in the 2DE analysis of muscle extracts, the patterns of myosin light chains (Ochiai et al. 1990; Martinez et al. 1990) and of sarcoplasmic proteins (Piñeiro et al. 1998, 2001) are always species-specific. However, given that myosin light chains and sarcoplasmic proteins are relatively abundant in the white muscle, it would be highly desirable to develop a method that, bypassing the 2DE step, would allow a direct sequencing and identification of the sample.

10.2.1.2 Production Method, Crowding, and Feeds

A second perceived quality factor is the production method, that is, whether the fish is farmed or wild, a subject which is also comprehensively dealt in another chapter of this book. Monti and co-workers (2005) showed differences between the peptide profiles of wild and farmed sea bass, especially those of some glycolytic enzymes and the parvalbumin fraction, supporting the belief that farming practices affect muscle composition (Monti et al. 2005; Eriksson and Fenyö 2005). These results were further confirmed in cod by 2DE analysis: farmed cod muscle seemed to display a different protein expression and/or a different post-mortem degradation pattern than wild cod, which was attributed to stress during cultivation, differences in post-mortem muscle conditions (e.g., pH), and/or to qualitative and quantitative differences in the expression or regulation of proteases with a role in post-mortem muscle tenderization (Martinez et al. 2007).

The muscle proteome of wild and farmed gilthead sea bream was mapped by Addis et al. (2010) who found, as expected, that the protein expression pattern in muscle was more stable than in liver (which is a more dynamic tissue) and, more interestingly, that the protein expression profiles of muscle tissue of wild and

maricultured gilthead sea bream of commercial size were comparable, indicating that offshore farming in floating cages favors proper muscle tissue development and the production of high-quality fish.

Animal welfare is a highly relevant topic involving breeding and slaughtering. Farmed rainbow trout subjected to intense pre-slaughter activity displayed differences on the levels of proteins involved in energy-producing pathways and of structural proteins. Desmin in particular showed consistently lower levels in the muscle of stressed fish (Morzel et al. 2006). Regardless of whether the reduced relative amount of desmin is due to proteolysis, denaturation, or a combination of both processes, it is bound to have a negative effect on the integrity and texture of the trout muscle inasmuch as it is one of the main constituents of the cytoskeleton in muscle cells.

Veiseth-Kent et al. (2010) showed that pre-slaughter crowding in Atlantic salmon induced classic signs of both primary and secondary stress responses. It was accompanied by alterations in the amounts of 27 proteins in muscle and of 17 proteins in blood plasma, all of them involved in secondary and tertiary stress responses, including altered energy metabolism, osmotic regulation, and immune function. The main changes in the muscle of crowded salmon seemed to be increased proteolysis and/or dissociation of structural proteins (actin, myosin heavy and light chains, tropomyosin) and increased levels of enzymes involved in anaerobic energy production (creatine kinase, enolase, phosphoglycerate kinase). Apolipoprotein A-I decreased in blood plasma and the angiotensinogen complement component C3 increased. These results indicate that short-term exposure to crowding may induce changes in the immune system of salmon and explain the mechanisms causing an accelerated muscle pH decline and rigor mortis contraction.

Ochiai (2010) addressed a serious quality problem, known as "burnt meat," in the highly appreciated flesh of bluefin tuna by 2DE. He showed a high degree of protein aggregation and decomposition and mainly the absence of creatine kinase in the portion of burn muscle, in both farmed and wild specimens. "Burnt" meat lacks the characteristic bright red meat color, it has a more watery, softer texture, and it is often seen in tuna and mackerel under stress conditions when fish are caught during the spawning period in summer (Yamashita 2010). Yamashita and co-workers (2010) attributed this oxidative stress to selenium deficiency and hypoxia and indicated the relevance of the recently discovered selenoneine (with a high antioxidant ability to bind to heme proteins and protecting them from iron auto-oxidation; see Yamashita and Yamashita 2010) to prevent this quality flaw.

The amount and composition of the diet are main determinants of seafood quality. The traditional source of protein and fat in the manufacture of fish feeds has been pelagic fish stocks. Nowadays, the fact that most of these are overexploited together with ethical issues regarding the use of high-value lipid and protein sources for animal feeds rather than human food, has forced feed producers to look for alternative and more acceptable sources of nutrients most of which are of vegetable origin. Given the relevance of the liver to the general fish metabolism, most of the studies on the effect of alternative feed ingredients on fish growth have targeted this organ instead of the edible muscle tissue.

Martin et al. (2003) published a very interesting proteomics work on the effect of using soy as a protein source on the 2DE protein profile of rainbow trout liver. Fish fed diets with higher levels of soy meal displayed higher protein consumption and protein synthesis rates, increased ammonia excretion, increased activities of hepatic glutamate dehydrogenase and aspartate amino transferase, and lower efficiency of retention of synthesized protein (Martin et al. 2003). Of the approximately 800 liver protein spots whose expression pattern was examined, 33 were found to be differentially expressed according to the feed. Two structural proteins, keratin II and β-tubulin, were downregulated in fish fed the high-soy diet, suggesting an increased requirement for energy metabolism and therefore lower energy available to synthesize structural proteins in these fish. Several heat shock proteins, including at least two chaperones (HSP70 and HSP78) were also downregulated in fish fed high-soy diets. Additional differences seemed to indicate an immune response and increased emphasis on catabolism relative to anabolism in the fish fed the high-soy diet. Interestingly, a hepatic selenium-binding protein identified as a potential biomarker in this work has been previously found to increase in the presence of aryl hydrocarbons (Ishida et al. 2002). As already mentioned above, Se-containing proteins seem to be of relevance to fight oxidative stress. The authors attributed the cause for the observed altered metabolism to the copurification of antinutritional factors from soy, such as phytoestrogens, antigenic agents together with the soy meal. On the other hand, there were no apparent differences between the liver proteomes of salmon fed GM versus non-GM soy-based diets (Nini et al. 2010).

10.2.1.3 Seafood Safety: Biomarkers of Exposure to Environmental Contaminants

Classical analytical techniques used to detect many of the relevant environmental contaminants, such as polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCBs), dioxins, heavy metals, and their species (methyl mercury, arsenicum, cadmium, etc.) are time consuming, expensive, and require specialized equipment and trained personnel. Food safety and environmental monitoring are currently targeting the identification of biomarkers in order to map the exposure to contaminants and the effect that such exposure has at the molecular, tissue, and organism levels. Identification of biomarkers is obviously of high value not only to food safety but also to environmental monitoring, as indicated by Miracle and Ankley (2005) in their review about the applications of -omics techniques in the field of ecotoxicogenomics.

The effect on the proteome of juvenile Atlantic cod plasma to the exposure to crude North Sea oil spiked with alkyl phenols and polycyclic aromatic hydrocarbons was investigated by Bohne-Kjersem et al. (2009) who identified 137 proteins that were differentially expressed depending on the levels of crude oil used. The most noticeable changes, many of which occurred even at low levels, indicated alterations of fibrinolysis and the complement cascade, the immune system, fertility-linked proteins, bone resorption, fatty acid metabolism, increased oxidative

stress, impaired cell mobility, and increased levels of proteins associated with apoptosis. The following proteins were identified as potential biomarkers: alpha enolase, plasminogen, alpha-1-antitrypsin, alpha-2-macroglobulin, alpha-2- antiplasmin, prothrombin, pentraxin, tropomyosin, serotransferrin, hemopexin, Fetuin B, apolipoprotein B, and NTPase. Not surprisingly, many of the responses seemed to be linked to each other, which made the authors suggest that the use of an array of these biomarker candidates would give a better indication of adverse effects induced in the fish by oil and/or produced water compared to single biomarkers alone (Bohne-Kjersem et al. 2009).

The same research group (Meier et al. 2010) examined the response in embryos, larvae, and juvenile fish to the complex chemical mixture found in real produce water containing dispersed oil, metals, alkylphenols (APs), polycyclic aromatic hydrocarbons (PAHs), and other chemicals. In general, APs bioconcentrate in fish tissue in a dose and developmental stage-dependent manner during PW exposure and cod exposed to 1% produce water (but not to either 0.1% or 0.01%) had significantly higher levels of the biomarkers vitellogenin and CYP1A in plasma and liver, respectively (Meier et al. 2010). However, continuous exposure from egg to fry in cod to different levels of North Sea-produced water, induced large changes in the proteome also at the lower levels of 0.01% and 0.1% produce water (Bohne-Kjersem et al. 2010): the expression and modifications of myosin heavy chain were affected and those of alpha-actin, alpha-actinin, keratin K8b (S2), and cytokeratin 4 (Krt4), Hsc71 (a heat shock protein belonging to the Hsp70 family) were downregulated. In muscle, exposure changed the levels of myosin, alpha-actin, and alpha-actinin suggesting an impact of produced water on the fast skeletal muscle development, essential for somatic growth, potentially impairing general growth and development of cod fry. Furthermore, the downregulation of keratin suggests an effect on tissue integrity. In summary, myosin heavy chain, fast skeletal muscle alpha-actin, Hsc71, alpha-actinin, ATP synthase, and keratin were identified as potential biomarker candidates of the effects of produced water and 17 β-oestradiol on cod fry. These responses reflect a potential impairment of the general growth and development of cod fry with the consequent value as indicators for lowered muscle quality and exposure to safety risks. These results are of obvious interest and should be followed to document the effect of environmental contaminants on market-size fish muscle protein makeup and properties.

Surface-enhanced laser desorption/ionization (SELDI) proteomics together with genomics (heterologous cDNA arrays) were used to investigate the integrated response of rainbow trout gills to sublethal concentrations of zinc for up to 6 days' exposure (Hogstrand et al. 2002): seven proteins were unique to zinc exposure whereas four others were suppressed. A spot that remained unidentified, but suspected to be metallothionein (a low molecular weight, metal-binding peptide inducible upon exposure to metals) was upregulated. Other changes seemed to lead to promoting glycolysis and stimulating the cellular production of energy. The proteomic studies also showed an activation of an inflammatory response during zinc exposure, thus confirming the postulated immunomodulatory role for zinc, which apparently protects rainbow trout against cadmium and mercury-induced immunotoxicity (Sanchez-Dardon et al. 1999).

The effect of exposure to increasing levels of cadmium on the proteome of bastard halibut's brain was examined by Zhu et al. (2006). Among the 24 proteins identified on a 2-D-PAGE gel, 9 demonstrated a synchronous response to acute cadmium exposure, suggesting that they might represent a biomarker profile. The changes affected both cytoplasmic and mitochondrial proteins; thus creatine kinase, transcriptional regulator, and endoglucanase were upregulated and actin 1, a putative xylose repressor, transferrin, and a dehydrogenase were downregulated. The authors propose an important role for transferrine in this system as a biomarker and a key molecule in cadmium detoxification.

In summary, exposure to environmental contaminants and toxic metals induces changes in the proteome of the fish tissues examined consistent with altered metabolism, increased glycolysis and oxidative stress, possible degradation of structural proteins (myosin, actin, desmin, keratins), and upregulation of proteins involved in detoxification mechanisms (transferring, HSPs, metallothioneins). All these changes have a negative effect on the quality of the fish (mostly shown by the degradation of structural proteins) and have a clear value as seafood quality and safety indicators.

10.2.2 Quality Aspects Acquired or Modified After Harvesting

The level of denaturation of proteins in seafood has been reported to be closely related to the sensory and technological properties of the respective products (Piñeiro et al. 2003). Post-mortem changes and spoilage are the result of mainly three basic mechanisms: enzymatic autolysis, oxidation, and microbial growth. Low-temperature storage and the addition of chemicals are the methods most commonly used by the industry today to control the levels of water activity and enzymatic and oxidative reactions in order to delay product deterioration and microbial growth (Ghaly et al. 2010).

The electrophoretic and mass spectroscopic techniques used to investigate the mechanisms of post-translational protein modifications have proven useful to follow up protein modifications during food production and storage and to elucidate the relationship between the nature and structure of proteins (i.e., protein size, amino acid composition, and sequence) and their functionality (Piñeiro et al. 2003). It has become clear that protein functionality exerts a significant effect on the physical and sensory properties of seafood, highlighting the suitability of investigating relevant proteome changes in seafood products for which two-dimensional polyacrylamide gel electrophoresis (2DE-PAGE) is still the most popular technique (Piñeiro et al. 2003).

10.2.2.1 Post-mortem Changes and Sensory Attributes

Washing, bleeding, and eviscerating (in the case of fish), temperature, and degree of handling are the most relevant factors influencing the quality of seafood

(Terlouw et al. 2008; Borderías and Sánchez-Alonso 2011). The initial biochemical changes that take place in the edible tissues are due to the enzymatic breakdown of major molecules and the level of post-mortem autolysis in the muscle (with the corresponding degradation of extra- and intracellular structural proteins and variation in the composition of peptides, amino acids, and small water molecules) is a major determinant of the texture, flavor, and odor of the final products (FAO 2005). During the last few years, proteomics have been used to further understand these processes, especially in meat, where the identification of naturally generated small peptides derived from myofibrillar proteins such as myosin light chain 1, titin, and actin, have expanded the knowledge of the different proteases that influence food properties (Bendixen et al. 2011).

As far as we are aware, proteomics methodologies were initially applied to assess how variations in processing conditions during the manufacture of surimi made from pre-rigor and post-rigor cod affected the composition of the final product (Martinez et al. 1992). The results indicated an increase in protein degradation with decreasing freshness and upon the addition of Ca^{2+} salts to post-rigor, but not to pre-rigor cod, valuable information to have in order to optimize the processing parameters according to the quality of the raw material and the desired texture of the final products.

Verrez-Bagnis et al. (2001) identified a 16 kDa protein whose disappearance from the proteome of sea bass muscle with increasing post-mortem storage time indicated its potential as a biomarker for freshness in that species. In cod, Kjærsgård and Jessen (2003) identified significant changes in 11 protein spots of the partial muscle proteome (p*I* 3.5–8.0, MW 13–35 kDa): for 8 of the 9 spots whose intensity increased during the first 8 days of ice storage the increase was already significant within the first 2 h post mortem, and 2 other spots whose intensity decreased only displayed significant changes after 8 days, indicating that different biochemical processes are involved in the post-mortem changes of cod muscle.

Several gel electrophoresis and mass spectrometric studies of post-mortem changes in sea bass muscle stored at 18°C and 1°C for 5 days showed a temperature-dependent increase in proteolysis that mainly affects the myosin heavy chain and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Terova et al. 2011). Interestingly the same study shows a rapid and significant decrease in the abundance of nucleoside diphosphate kinase B and phosphoglycerate mutase 2 that appears to be temperature-independent (Terova et al. 2011). A similar preliminary study of post-mortem deterioration in ice-stored sea bream by Schiavone et al. (2008) showed that α-actin and tropomyosin were among the most stable proteins during 6 days of storage, whereas myosin light 3 (MLC3) and major histocompatibility complex Class II beta 1 proteins increased and Sec 13-like and parvalbumin significantly decreased. The relevance of these results must await the understanding of the causes for these changes. For example, an increase in the intensity of the MLC3 spot would agree with an increase in the liberation of myosin from the rigor actomyosin complex and subsequent degradation of the myosin heavy chain, which will increase the level of free MLCs. Rigor actomyosin and the intact myosin molecule are difficult to solubilize and hardly enter the 2-D gels, but MLCs are easily solubilized and visualized. These results confirm the higher susceptibility of myosin heavy chain and

the stability of the light chains to proteolytic degradation shown by Martinez (1992) in frozen stored extracts of isolated actomyosin.

One of the characteristics of the proteome is that it is dynamic and one must understand the causes for the changes before these methods may be fully useful to the aquaculture and fisheries industries. The above-mentioned study by Terova et al. (2011) showed a decrease in the intensity of the parvalbumin spot upon post-mortem storage. Taking into account that parvalbumin is one of the major fish allergens, this result may confirm a previous work by Dory et al. (1998) who showed that both the number of IgE-reactive bands and the intensity of the reaction in cod extracts was greater if the fish had been stored for several days, than if the extracts had been obtained from post-rigor fish immediately after rigor mortis resolution, suggesting not a loss of allergens but their aggregation and increased allergenicity. This stresses the need to understand the reasons for the variation in intensity of the protein spots: although the formation of aggregates of parvalbumins will also induce the loss of the characteristic low molecular mass spot of these proteins, the implications for the quality and safety of the fish muscle would be opposite if the diminishing of this spot were due to the disappearance of the allergen or to its aggregation and consequent increase in potential allergenicity.

Post-mortem pH is a highly relevant variable that would affect the quality and composition of fish as well as its proteome and yet it has been largely ignored in proteome studies of seafood. It is known that post-mortem pH varies depending, for example, on the condition of the fish and the degree of stress prior to death (Roth et al. 2012). Interestingly, the only work examining the effect of the pH on post-mortem proteolytic activities showed the great impact of this variable (Wang et al. 2011). Although that work did not use proteomics, it clearly demonstrated that different proteases are active depending on the post-mortem pH, a fact illustrated by the different pattern of degradation of the myosin heavy chain. This means that the post-mortem proteome for each species will vary according to the final post-rigor pH value and that the post-mortem pH value will have more relevance on the proteome than the storage temperature, because although the changes are usually temperature-dependent in that increased storage temperature accelerates the changes, variations in the pH value will cause the appearance of different spots (i.e., originate a different proteome), due to the preferential action of different proteases.

10.2.2.2 Processing and Storage

There is a scarcity of proteomic studies addressing the modifications suffered by seafood proteins during their processing and storage, and most of them refer to chilled and frozen species of commercial value such as pufferfish, Atlantic cod, Atlantic salmon, rainbow trout, and skipjack tuna muscle. Thus, Lu and coworkers (2010) identified 21 proteins in the skeletal muscle of Japanese pufferfish using 2DE and MALDI-TOF/TOF MS with different and well-known cellular functions, and Gebriel et al. (2010) used 1D-PAGE, nanoliquid chromatography peptide

fractionation, and LTQ-MS in order to achieve an Atlantic cod muscle proteome catalogue.

Specific protein modifications during processing and storage as well as the effect of lactic fermentation with *Lactobacillus* starters in Atlantic salmon muscle were studied using 2DE (Morzel et al. 2000). Their results showed that the main quantitative protein changes were due to endogenous enzymes and affected the acidic range of proteins, whereas alkaline proteins and tropomyosin were more susceptible to microbial proteases.

The production of low molecular weight peptides (<5 kDa) in post-mortem rainbow trout muscle stored in ice and their stability during cooking were studied by Bauchart et al. (2007). Post-mortem proteolysis in muscle is an important factor affecting fish texture, and low molecular weight peptides affect the taste of the products. The main peptides in trout muscle were anserine and glutathione whose concentration was almost unaffected by the 7 days of ice storage and vacuum cooking for 5 min at 70°C. MS analysis revealed the highly reproducible appearance of a limited number of not yet characterized small peptides following the treatment.

Kjærsgård and coworkers (2006a) described changes in the proteome of farmed Atlantic cod muscle stored under different cold and frozen storage temperatures using 2DE and ESI-MS/MS. Their results showed the main changes in myosin light chain, triose-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, aldolase A, two α-actin fragments, and nuclease diphosphate kinase B, that seemed to keep a closer relationship to the length of freezing storage than to the storage temperature.

The same research group (Kjærsgård et al. 2006b) documented how freezing storage induced an increase in the level of protein oxidation in rainbow trout muscle. These authors identified several carbonylated proteins by LC-MS/MS, including nucleoside diphosphate kinase, adenylate kinase, pyruvate kinase, actin, creatine kinase, tropomyosin, myosin light chains 1 and 2, and myosin heavy chain, and showed a reduced solubility of nucleoside diphosphate kinase in fish stored at −20°C for 2 years compared to fish stored at −80°C. The authors observed that some proteins seemed to be more susceptible to oxidation than others, and attributed these differences to their abundance, cellular localization, amino acid sequence, or biochemical function, confirming earlier findings by the same research group (Kjærsgård and Jessen 2004).

Kinoshita and coworkers (2007) identified several spots by 2DE and MALDI-TOF/TOF that corresponded to oxidized peptides in brine-frozen bonito (*Katsuwonus pelamis*) muscle defrosted and stored at 15°C for 4 days. Sequencing of five of the oxidized spots led to the identification of four as: enolase 3, aldolase, aldolase A, and L-lactate dehydrogenase A chain.

Finally, because an important source of free radicals taking part in protein oxidation is the Fenton reaction, dependent on ferrous ions present in the tissue, Pazos et al. (2011) investigated the susceptibility of sarcoplasmic and myofibrillar cod muscle proteins to in vitro catalyzed oxidation, in an attempt to identify candidates that might play a major role in the deterioration of fish quality. After 2DE separation, selected proteins whose carbonyl groups had been labeled by

fluorescein-5-thiosemicarbazide (FTSC) were identified by MALDI-TOF/TOF mass spectrometry. The most vulnerable proteins to ferrous-catalyzed oxidation seemed to be lactate dehydrogenase, triosephosphate isomerase, creatine kinase, enolase, glyceraldehyde 3-phosphate dehydrogenase, nucleoside diphosphate kinase B (NDK), and phosphoglycerate mutase. Different isoforms of the last three proteins showed different susceptibilities to metal-catalyzed oxidation, indicating that post-translational modifications may change the resistance of proteins to oxidative damage. In addition, the Fe (II)/ascorbate treatment significantly increased carbonylation of mainly actin and myosin, some of whose degradation products exhibited increased carbonylation levels.

10.2.2.3 Post-harvest Food Safety: Microbial and Other Contaminations

Microbial growth is a major cause of fish spoilage resulting in the production of biogenic amines such as putrescine, histamine, and cadaverine, organic acids, sulphides, alcohols, aldehydes, and ketones with unpleasant and unacceptable off-flavors (Ghaly et al. 2010). Fresh fish stored at ambient temperature are usually spoiled by Gram-negative, fermentative bacteria, whereas chilled fish spoil under the action of psychrotolerant Gram-negative bacteria, such as *Pseudomonas* spp. and *Shewanella* spp. (Gram and Huss 2000).

Proteomics allow addressing the molecular processes underlying the physiological behavior of pathogens in food matrices, a major challenge in food microbiology. For that, it is necessary to identify both the micro-organisms present in the food and the food components that are relevant in determining the microbial stability of such foods. The latter range from small molecules (including taste compounds and food preservatives) to the macro-ingredients such as proteins, carbohydrate polymers, and fats (Havelaar et al. 2010).

Since the first bacterial genome of *Haemophilus influenza* was described by Fleischmann et al. in 1995, top-down or bottom-up proteomics approaches have allowed the fast and sensitive characterization of individual micro-organisms in mixtures (Welker 2011). Nowadays, genome sequences are available for many food-borne micro-organisms (Abee et al. 2004) as evidenced by the number of strains that are currently sequenced as reviewed by O'Flaherty and Klaenhammer (2011).

There are many studies from the early years of proteomics applying these tools to bacterial classification and to the study of common pathogenic seafood bacteria such as *Listeria, Campilobacter,* or *Staphylococcus* (Piñeiro et al. 2010; Forné et al. 2010) both under refrigeration and after high-pressure preservation procedures (Cacace et al. 2010; Bièche et al. 2011).

However, proteomics have only recently been applied to the characterization of how processing may affect the virulence of microbial pathogens: Guilbaud et al. (2008) showed that liquid smoke affected the proteomic pattern of *Listeria monocytogenes,* decreased its growth and survival, and inhibited its hemolytic potential without affecting the *hly* gene expression. These results help us understand why in spite of the apparently high prevalence of *L. monocytogenes*, particularly in lightly preserved

smoked seafood, these products are rarely linked to listeriosis and support the attenuated infectious potential of *L. monocytogenes* strains isolated from the smoked salmon industry indicated by Norton et al. (2001) and Gudmundsdóttir et al. (2006).

Hazen et al. (2009) used whole-cell MALDI-TOF MS analysis to identify species and strains of *Vibrio parahaemolyticus* isolated from different geographical locations and at different times. *V. parahaemolyticus* is the leading causative agent of bacterial seafood-borne gastroenteritis in the United States, and this technique permitted the fast identification of the pathogen and its clear differentiation from the closely related *V. alginolyticus*, *V. harveyi*, and *V. campbellii.*

Böhme et al. (2010a, b) used a special extraction protocol from intact microbial cells together with MALDI-TOF-MS techniques to identify the main 26 species of seafood spoilage and pathogenic Gram-negative bacteria, including *Aeromonas hydrophila, Acinetobacter baumanii, Pseudomonas* spp., and *Enterobacter* spp. They also constructed a reference library containing the spectral fingerprints of 32 Gram-positive reference strains, including *Bacillus* spp., *Listeria* spp., *Clostridium* spp., *Staphylococcus* spp., and *Carnobacterium* spp. (Böhme et al. 2011a) and identified a variety of seafood pathogens such as *Stenotrophomonas maltophilia*, *Proteus vulgaris*, *Pseudomonas syringae*, *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Bacillus cereus*, *Bacillus subtilis,* and *Serratia marcescens*, in iced and vacuum-packed mild heat-treated seafoods (Böhme et al. 2011b). The same group has recently published the first report on the identification of *Streptococcus parauberis* in seafood in general and in vacuum-packed food products in particular using the same methodology (Fernández-No et al. 2012).

Mass spectrometry (MS) is an instrumental technique that permits the identification of all types of compounds in food toxicology including seafood (Malik et al. 2010). Fernandez-No and coworkers (2011) used MS to isolate and identify the main histamine-producing bacteria from farmed turbot and blackspot sea bream and histamine production was measured by HPLC. The study revealed *Pseudomonas fragi* and *Pseudomonas syringae* to be the major histamine-forming bacteria present in farmed turbot and blackspot sea bream, respectively. Self and coworkers (2011) developed a method for the extraction of agmatine, cadaverine, histamine, phenyethylamine, putrescine, tryptamine, tyramine, and urocanic acid from canned tuna and frozen tuna loin matrices by matrix solid-phase dispersion, followed by separation and quantification of these compounds by ultrahigh-performance hydrophilic interaction chromatography (UHPLC-HILIC) with orbitrap mass spectrometric detection. This streamlined approach eliminates the need for derivatization, which has been the traditional option for liquid or gas chromatographic analysis for many of these compounds.

10.3 Challenges

The relevance of high-throughput proteomic approaches is to increase insight and understanding of how the physiology of the organism, breeding, harvesting, storage, and processing methods affect the quality and safety of ready-to eat

seafood. Such understanding can then be used to optimize the entire production chain from the water to the table: from selection and breeding to processing and cooking.

There are, however, still some pitfalls associated with the techniques themselves and on how to deal best with the tremendous amount of data they generate (Pedreschi et al. 2010). Some of the limitations to the application of proteomics technologies derive from the instability of proteomes, the large number of proteins, and possible post-translational modifications, the limited detection of low abundance and highly acidic or basic proteins by 2-D gel electrophoresis, limited reproducibility, and the fact that not all proteins in a sample can be identified (Van Vliet 2011). Thus there is an urgent demand to develop sophisticated, robust, and fast analytical methodologies, capable of identifying in parallel hundreds of seafood proteins, with a variety of post-translational modifications at different expression levels (Zhang et al. 2008).

According to recent publications, the laser capture microdissection approach has been successfully applied in fish immune response studies (Prunet et al. 2012), but the use of this technique often implies the recovery of only small amounts of biological material for further proteomics analysis. Small sample size makes it difficult to detect differentially expressed proteins. Perhaps the introduction of lab-on-a-chip devices for sample processing where protein separation and trypsin digestion can take place, and directly coupling these chips to mass spectrometers, could be a good solution for the near future but it is currently a challenge (Forné et al. 2010).

Optimally analytical proteomic techniques should be combined with other "omic" approaches, particularly transcriptomic and metabolomic information together with the indispensable help of bioinformatic tools (Cifuentes et al. 2011) to map and improve seafood production, quality, and safety.

On the other hand, the high biodiversity and shortage of sequences for the large number of organisms used as seafood makes it difficult to undertake -omics strategies in the short term. There is a need to begin the compilation of useful data obtained after fully controlling all the significant variables involved, starting with the selection of relevant species on which to perform the studies under strictly controlled conditions (environmental conditions, nutrition, age, sex, stressors, contaminants, etc.). However, the results from descriptive punctual analyses without knowledge of the organism's prior history would be of only limited value and likely difficult to reproduce.

Given that most current fish farming practices seek to improve flesh quality together with the notion that white muscle is the main product for the fishing industry, a compilation of data about white muscle structure, function, and ontogeny followed by an account of the changes induced by the environment, feed, contaminants, stressors, swimming behavior, and performance related to the use of white muscle during growth from larva to adult, would be essential to fully understand the results of proteomic and other -omic studies (Videler 2011) and to devise strategies to optimize seafood breeding, harvesting, and processing.

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