

Omics approaches in fish quality and safety

Procedimentos ômicos aplicados em qualidade e segurança de pescado

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Abstract

Omics is a new technology that uses genomics, proteomics, and metabolomics to investigate metabolites from foods. The global demand for fish has shown a progressive increase because it is a significant source of high quality protein, polyunsaturated fatty acids, especially omega-3, and essential minerals. However, there are barriers in the fishery production chain such as lack of standardization, knowledge, and technology transfer to industry. Moreover, fish effective monitoring is difficult due to restricted quality parameters and analytical methods determined by current Brazilian legislation. This review details the limiting chemical parameters and recent advances in analytical procedures for fish quality determination. To improve fish quality monitoring, total volatile basic nitrogen (TVB-N), trimethylamine (TMA), ammonia, pH, and biogenic amines values should be revised and established by fish category and/or type of fish product. On the other hand, protein carbonyl concentration, free fatty acids (FFAs), peroxide values (POV), and thiobarbituric acid reactive substances (TBARS) should be included in the national legislation. Simultaneously, the official authorities should take into account effective, practical, and low cost analytical methodologies, which lead to faster results in order to facilitate and enhance the quality control of the products from the fish production chain, ensuring the consumer's health. Moreover, analytical techniques for the identification of fish species must be introduced in the Brazilian legislation in order to avoid illegal substitutions and negative impacts to consumers.

Keywords: Advanced analytical methods. Fish production chain. Legislation. Quality monitoring.

Resumo

Os procedimentos ômicos são uma nova tecnologia que utiliza a genômica, proteômica e metabolômica para avaliar metabólitos dos alimentos. A demanda mundial de pescado tem aumentado progressivamente devido à elevada qualidade de proteínas, minerais e ácidos graxos poli-insaturados, especialmente ômega-3. Todavia, a cadeia produtiva aquícola apresenta limitações como falta de padronização, ausência de conhecimento e transferência de tecnologia para as indústrias. Além disso, torna-se difícil garantir um monitoramento efetivo do pescado em decorrência das limitações dos parâmetros de qualidade atuais e dos métodos analíticos estabelecidos pela legislação nacional. O presente trabalho analisa os fatores limitantes relacionados aos parâmetros químicos, bem como os avanços recentes nos procedimentos analíticos, para determinação da qualidade do pescado. Levando-se em consideração a melhoria no controle de qualidade dessa matriz, os parâmetros de bases voláteis totais (BVT), trimetilamina (TMA), amônia, pH e aminas biogênicas deveriam ser revisados e estabelecidos por categorias de pescado e/ou por tipo de produto à base de pescado. Em contrapartida, parâmetros relacionados à concentração de carbonilas, ácidos graxos livres (AGLs), índice de peróxidos (IP) e malonaldeído (MDA) poderiam ser inseridos na legislação nacional. Simultaneamente, as autoridades oficiais devem levar em consideração metodologias analíticas que apresentem efetividade, praticidade, baixo custo e rapidez, facilitando e aprimorando o controle de qualidade de produtos de pescado e garantindo a saúde dos consumidores. Além disso, técnicas analíticas para identificação das espécies de peixes devem ser incluídas na legislação brasileira visando evitar substituições ilegais e impactos negativos aos consumidores.

Palavras-chave: Cadeia produtiva de pescado. Legislação. Metodologias analíticas sofisticadas. Monitoramento da qualidade.

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Introduction

Worldwide capture fisheries' production remains stable whereas aquaculture has increased to supply the growing demand for nutritive foods (FAO, 2012). Brazilian contribution to the world's fish production has risen steadily (BRASIL, 2012). Fish is marketed throughout the world mostly as fillets and frozen whole for direct consumption (FAO, 2012). However, the number of fish products on the market has increased because consumers currently prefer ready-to-eat (RTE) fish products that require less preparation time (BRUNSDØ et al., 2009).

Fish and fish products represent a valuable source of nutrients for balanced nutrition and good health. The protein fraction is composed by a wide variety of essential amino acids that act on decreasing anxiety and artery spasm risk as well as enhancing the immune system (CHENG et al., 2008; UDDIN et al., 2010). The lipid fraction is especially related to eicosapentaenoic and docosahexaenoic acids, which reduce health risk factors including various types of cancer (HOOPER et al., 2006; CALDER; YAGOOB, 2009). Nevertheless, these nutritive compounds contribute to fish perishability and during the degradation process they result in substances that are harmful for human health, which emphasizes the need for monitoring (JUNEJA; SOFOS, 2010; MUÑOZ-ATIENZA et al., 2011).

Brazilian legislation is lacking effective monitoring parameters for fish and fish products. Therefore, there is a need for changes in the official parameters and for more advanced analytical methodologies indicated in some studies (MONTEIRO et al., 2012; RODRIGUES et al., 2012; CARNEIRO et al., 2013; CUNHA et al., 2013; RODRIGUES et al., 2013; ANDRADE et al., 2014; BORGES et al., 2014; MONTEIRO et al., 2014; PALMEIRA et al., 2014).

Moreover, the growing consumption and demand for certain fish species lead to cases of economic fraud (MARTINEZ et al., 2005). In Brazil, fish species are identified by their morphological characteristics, which

are absent in processed foods, and thus fraud detection is impaired. Therefore, studies on more accurate analytical methods for species authentication are necessary (WOOLFE; PRIMROSE, 2004; GIL, 2007; RASMUSSEN; MORRISSEY, 2008).

In this context, this review focuses on the obstacles to fish quality monitoring in Brazil as limiting chemical parameters and traditional analytical methods established by the official legislation in order to encourage the application of omics approaches to improve the fish production chain.

Parameters of metabolites from protein degradation

Total volatile basic nitrogen (TVB-N) expresses the amount of ammonia and primary, secondary, and tertiary amines as monomethylamine (MMA), dimethylamine (DMA), and trimethylamine (TMA), respectively. These compounds are related to bacterial or enzymatic degradation and used as spoilage indicators (XU et al., 2009).

Ammonia is formed mainly through the oxidative deamination of protein components from fish muscle and by bacterial deamination of amino acids during storage (CONTRERAS-GUZMÁN, 2002). Therefore, ammonia is present even in very fresh fish representing the most abundant volatile base at the beginning of the degradative process (ÖZOGUL; ÖZOGUL, 2000) and can markedly increase mainly after seven days of storage (CONTRERAS-GUZMÁN, 2002). Therefore, the national legislation could include a quantitative method for measuring this metabolite.

Nessler's test is a method often used for ammonia determination in fish. It is based on the reaction between potassium tetraiodomercurate and ammonia resulting in a colorimetric complex (reddish orange) (BARTELS, 1971). However, this analysis is subjective and can lead to ambiguous results. Monteiro et al. (2012) observed consistent results for qualitative ammonia by using Nessler's reagent in tilapia fillets stored at $0 \pm 1^\circ\text{C}$. On the other hand, Rodrigues et al. (2013) only found correlation between ammonia and sensory deterioration in rainbow trout after the 11th day of storage at $0 \pm 1^\circ\text{C}$. In this context, it is important to study more accurate methodologies and to encourage the official authorities to establish a quantitative limit for this metabolite.

Spectrophotometry is an alternative method for the determination of ammonia concentration, but there is lack

of information about quantitative ammonia measurements in fish. Few studies have been carried out in the past and were mainly related to blood and water (MCCULLOUGH, 1967; SVEHLA, 1996). However, the adaptation of McCullough's (1967) colorimetric method is possible by using the Nessler reaction and a spectrophotometer. In brief, 1 g of sample was added to 10 mL of Milli-Q water, homogenized for 30 s in a tube, and let to stand. After decanting, 1 mL of the supernatant was removed and

2mL of Nessler's reagent was added. Next, the tube was homogenized for 30s and the measurement was performed immediately on a SmartSpec™ Plus spectrophotometer (Bio-Rad Laboratories, São Paulo, Brazil) at a wavelength of 425 nm.

A calibration curve was drawn after reading seven different concentrations (1, 2.5, 5, 7.5, 10, 15 and 20 µg NH₃/g) of a standard solution of ammonium sulphate (2 µg/mL) (Table 1).

Table 1 – Different concentrations of standard solution of ammonium sulfate (2 µg/mL)

	Blank	St. I	St. II	St. III	St. IV	St. V	St. VI	St. VII
Water	2 mL	1.9 mL	1.75 mL	1.5 mL	1.25 mL	1 mL	0.5 mL	-
St. solution	-	0.1 mL	0.25 mL	0.50 mL	0.75 mL	1 mL	1.5 mL	2 mL

St. solution = standard solution of ammonium sulphate

Each concentration was added to a 10mL tube containing 1mL of tungstate. One mL of N sulphuric acid was added from a quick delivery pipette and the contents of the tube were mixed thoroughly. The tube was centrifuged at 503 g for 10 min and the protein-free supernatant removed; 0.5 mL of clear supernatant was pipetted into a 10 mL tube and 2 mL of Nessler's reagent was added. The tube was homogenized for 30 s and the measurement was performed immediately on a SmartSpec™ Plus spectrophotometer (Bio-Rad Laboratories, São Paulo, Brazil) at a wavelength of 425 nm. The regression equation and regression coefficient ($R^2 = 0.995$) were calculated from the linear calibration curve previously prepared, and the results were expressed in µg NH₃/g.

TMA is a product of bacterial degradation of trimethylamine oxide (TMAO), which is naturally present in live marine fishes with an osmoregulation function. Generally, the muscle tissue from marine fish species presents between 1% and 5% TMA, which is practically absent in freshwater species (CHUNG; CHAN, 2009).

Therefore, TVB-N values slowly increase during storage in fishes that do not contain TMAO or where spoilage is due to a non-TMAO reducing flora, probably as the result of the deamination of amino acids (HUSS, 1995). In addition, Contreras-Guzmán (2002) affirm that TMAO degradation results, especially, in TMA at refrigerated storage, whereas in DMA and MMA under frozen storage. The Brazilian legislation defines the allowed limits for TVB-N and TMA

as below 30 mg TVB-N/100g, except for elasmobranchs, and 4 mg TMA/100 g, respectively; no limit is determined for freshwater fish or by type of fish product taking into account the process (BRAZIL; BRAZIL, 2017). Soccol et al. (2005); Monteiro et al. (2012), and Rodrigues et al. (2013) argue that TVB-N and TMA values should not be considered good quality indicators for fish. Therefore, they suggest that the interpretation of these results must be performed in association with other parameters such as sensory proprieties (CASTRO et al., 2006) as well as other BAs, such as cadaverine and putrescine (SHI et al., 2012).

The Brazilian authorities must be encouraged to establish the limits for different fish categories, similarly to the European legislation (EUROPEAN COMMISSION, 1995). TVB-N and TMA quantifications are performed according to Conway micro-diffusion method, as stated in the manual of Siang and Kim (1992). When compared to other simple methodologies (semi micro steam distillation and flow injection gas diffusion), Conway procedure represents a viable alternative to laboratory routine (ÖZOGUL; ÖZOGUL, 2000).

More advanced methods were studied in the past, such as gas chromatography, high performance liquid chromatography, capillary electrophoresis with laser-induced fluorescence detection, and capillary electrophoresis with indirect UV-detection (DABEK-ZLOTORZYNSKA; MARUSZAK, 1998; OETJEN; KARL, 1999; TIMM; JØRGENSEN, 2002). Although these methods present higher

sensitivity and specificity, they depend on the solubility and volatility of the compounds, and require expensive materials and apparatuses (DABEK-ZLOTORZYNSKA; MARUSZAK, 1998).

Biogenic amines (BAs) are basic nitrogenous compounds with low molecular weight (HALÁSZ et al., 1994). The main route of biogenic amine formation occurs by exogenous decarboxylase enzymes (produced by microorganisms), which remove the α -carboxyl group from the structure of a specific amino acid resulting in the corresponding amine (SHALABY, 1996). Low levels of BAs in fish products do not present a serious risk to human health because human intestine has amine oxidases that rapidly detoxify these compounds. Nevertheless, the ingestion of high levels of BAs may result in severe toxicological symptoms (MOHAMED et al., 2010; MUÑOZ-ATIENZA et al., 2011) including headache, urticaria, edema, nausea, vomiting, diarrhea, abdominal pain, cardiac palpitations, and even death (SHALABY, 1996; JUNEJA; SOFOS, 2010; MUÑOZ-ATIENZA et al., 2011). In addition, BAs may react with nitrates resulting in nitrosamines, which are carcinogenic compounds (JUNEJA; SOFOS, 2010).

The Brazilian legislation recommends 10 mg/100 g as the maximum level of histamine in muscle of fish species from *Scombridae*, *Scombresocidae*, *Clupeidae*, and *Coryphaenidae* families (BRASIL, 1997), which present high levels of histidine in their natural composition. In this case, the legislation draws attention for fishes that have greater possibility of histamine formation, including thermally processed products, because these substances are heat stable (cooking or prolonged exposure to heat does not eliminate the toxin) (GONZAGA et al., 2009). However, there is no limit for other biogenic amines (BRASIL, 1997) such as tyramine (considered as toxic as histamine), putrescine, and cadaverine (which potentiates the effects of histamine and tyramine), which are also thermostable (ALVAREZ; MORENO-ARRIBAS, 2014). Moreover, no limit is established for different products subjected to other processes (e.g. salting) and to other fish categories (freshwater fish) (BRASIL, 1997). Koral et al. (2013) observed that BA levels vary depending on the product type and salting methods. Pombo et al. (2009) reported that most fish cured by salting and fermentation presented histamine levels above the limits authorized by Brazilian regulations. In addition, the BAs formed in food directly depend on the amino acid composition of fish muscle and on the ingredients used in product formulation. Rodrigues et al. (2013) reported that putrescine and cadaverine might be considered suitable

indicators of the degradation process of rainbow trout meat. Palmeira et al. (2014) affirmed that BAs might be considered a promising quality parameter in meatball-type products prepared from rainbow trout waste, mainly in those with soy protein addition, which have precursor amino acids (e.g. arginine, lysine, tyrosine).

Regarding the analytical methods, until recently, the Brazilian legislation allowed only the fluorometric method for BA determination (BRASIL, 1997). Currently, High-Performance Liquid Chromatography (HPLC) was included as official methodology for BA determination in fish and derived products (BRASIL, 2011). The HPLC methodology was validated by Lázaro et al. (2013), and several researchers confirmed that biogenic amines could be considered a good quality indicator for fish and fish products (CUNHA et al., 2013; RODRIGUES et al., 2013; PALMEIRA et al., 2014). This was a positive advance in national regulations, however, HPLC is time consuming, expensive, and requires experienced personnel (FUCHS et al., 2011). In this context, the legislation should also include simpler and faster analytical methods taking into account the laboratorial routine and the reality of fishery industries (demand for quality control of fresh and frozen fishes, as well as canned fish, mainly tuna and sardine).

The potential of hydrogen (pH) is an important indicator of fish quality (OKEYO et al., 2009). Bacterial and enzymatic activity changes the pH value, which is measured by the concentration of free hydrogen (OGAWA; MAIA, 1999). An increase in pH values during the storage period may be attributed to the accumulation of alkaline compounds (e.g., ammonia and trimethylamine), which affect fish quality and consumer acceptance (MONTEIRO et al., 2012; RODRIGUES et al., 2012; RODRIGUES et al., 2013). However, catching, processing, and storing methods directly influence the pH values (MORZEL et al., 2003; ESAIASSEN et al., 2004; LOPES et al., 2004; AURSAND et al., 2009; REBOUÇAS et al., 2012; CARNEIRO et al., 2013).

Monteiro et al. (2012) found pH 6.35 and 6.92 in tilapia fillets stored at $0 \pm 1^\circ\text{C}$, on days 0 and 7, respectively. Rodrigues et al. (2013) evaluated rainbow trout stored at $0 \pm 1^\circ\text{C}$ and observed pH values of 6.47 on the first five days of storage, whereas 6.77 between days 10 and 15. Monteiro et al. (2010) observed pH 5.7 and 5.3 in whole and eviscerated tuna stored at $0 \pm 1^\circ\text{C}$, respectively. Rodrigues et al. (2012) found different pH values for tuna sashimi (5.55) and salmon sashimi (5.95) on day 0 at refrigerated storage (4°C). However, the national legislation does not establish pH

parameters for categories of fish and fish products, only that pH values must be below 7.0 (BRASIL, 1952). Therefore, pH analysis is generally used as secondary information. In general, the pH value is interpreted in association with other quality parameters or compared with similar studies described in the literature (OKEYO et al., 2009; RODRIGUES et al., 2012). This fact should be reassessed by the national rules mainly because there are studies that confirm the pH parameter as a good indicator of quality loss in fish, and because the analysis is an easy and fast procedure. Generally, there are two main methods for pH determination (colorimetric or electrometric); the second is more commonly used because it allows a direct, simple, and accurate measurement. For this analysis, a homogenized solution of 10g of muscle sample in 100mL of distilled water is prepared and the measure is performed using a digital pH meter equipped with an electrode (BRASIL, 2008).

Another important parameter is related to protein oxidation, which is currently one of the most novel approaches within the Food Chemistry field (LUND et al., 2011b). The oxidation of food proteins can occur by reactive oxygen species (ROS) from mitochondria (aerobic metabolism) or from external sources (e.g. X-ray, UV irradiation, and chemical compounds) (VALENTINE, 2007). In addition, ROS can oxidize DNA, carbohydrates, and unsaturated fatty acids, which can accelerate protein oxidation (FOYER; NOCTOR, 2003). Therefore, this deteriorative process can occur during processing and storage, negatively affecting the sensory properties (flavor, tenderness, juiciness, and color) (XIONG, 2000) especially in fish muscle that is more prone to lipid oxidation due to high amount of polyunsaturated fatty acids (PUFAs) (FOYER; NOCTOR, 2003). Moreover, oxidized proteins are associated to a variety of age-related diseases (e.g. Alzheimer) and vascular dementia (REDDY et al., 2009; TAGUCHI, 2009). Among the markers of protein oxidation, protein carbonylation is the most employed (VALENTINE, 2007). The protein carbonyls are formed directly by three routes: oxidation of the side chains of several amino acids, conjugation with lipid peroxidation byproducts (free radicals, hydroperoxides, and aldehydes), and with reactive carbonyl derivatives from the reaction with reducing carbohydrates (RICHTER et al., 2005; VALENTINE, 2007).

There are some analytical methodologies to identify specific protein carbonyls in fish muscle such as the techniques based on avidin/biotin affinity by mass spectrometry (MIRZAEI; REGNIER, 2005), as well

as derivatization with 2,4-dinitrophenylhydrazine (DNPH) labeling of protein carbonyls with 2-DE, nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS) (KJAERSGARD et al., 2006), fluorescein-5-thiosemicarbazide (FTSC) associated to two-dimensional gel electrophoresis (2-DE), and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) mass spectrometry (PAZOS et al., 2011). However, the reaction of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazones (DNP) with spectrophotometer reading at 370nm is a routine method for assessing protein carbonyl concentration. Although the colorimetric DNPH assay does not allow the individual identification of the oxidized proteins, it is a simple and effective method taking into account its cost and the time it consumes (LEVINE et al., 1990; NIKOO et al., 2015). In addition, there is no protein loss during protein transfer from the gel electrophoresis to the membrane (MIRZAEI; REGNIER, 2005).

Passi et al. (2005) verified a range of protein carbonyls (0.15 to 0.42nmol/mg protein) in the muscle of *Pagellus erythrinus* during seven days of storage in ice; these authors observed a significant increase after the 3rd day of storage. Tuckey, Forster, and Gieseg (2009) verified that protein carbonyl concentrations increased 73% in Chinook salmon (*Oncorhynchus tshawytscha*) fillets during storage at 15°C. According to Baron (2014), protein oxidation induces quality losses and leads to a negative impact on protein functionality, fish muscle texture, and food nutritional value.

Therefore, the national legislation should include protein carbonyl concentration as a parameter for fish quality monitoring, considering that changes are undesirable in foods and for human health.

Parameters of metabolites from lipid degradation

The lipid fraction of fish is rich in essential fatty acids, which generate positive effects in human health. The two main polyunsaturated fatty acids (PUFAs) in fish are eicosapentaenoic acid (EPA; C20:5n3) and docosahexaenoic acid (DHA; C22:6n3), which act in the prevention of human coronary artery disease, improvement of retina and brain development, and have anti-inflammatory and anti-carcinogenic effects (STEVANATO et al., 2010; LINSEISEN et al., 2011; LUND et al., 2011a; TACON;

METIAN, 2013). However, PUFAs are compounds with high amount of unsaturated bonds, which are chemically unstable and facilitate the occurrence of both hydrolytic and oxidative rancidity (MAPIYE et al., 2012). These are the two main types of decomposition of lipids and lead to undesirable effects on the sensory properties of foods (volatile aromatic compounds), and negative effects on human health (e.g., malondialdehyde) (CHAIJAN, 2008; ZAKI et al., 2014).

The hydrolytic rancidity is caused by a hydrolysis reaction of triglycerides generating glycerol and free fatty acids (FFAs), which leads to rancid odor and taste. The decomposition process is mostly attributed to lipase enzymes that can be endogenous or from bacterial action. The hydrolysis reaction can be catalyzed by light exposure and processing conditions such as high temperature and milling. Moreover, the presence of FFAs accelerates the oxidative rancidity (MCCLEMENTS; DECKER, 2008).

Lipid oxidation is caused by the interaction of unsaturated lipids with oxygen, leading to the formation of peroxide, hydroperoxide, and secondary compounds (aldehydes, ketones, carbonyls, alcohols, and acids), which negatively impact flavor and human health (CHAIJAN, 2008; MCCLEMENTS; DECKER, 2008; ZAKI et al., 2014). Zaki et al. (2014) concluded that an increase in malondialdehyde (MDA) decreases the paraoxonase (PON1) level leading to a greater risk of dyslipidemia, insulin resistance, and high blood pressure, which are considered important components in the pathogenesis of the metabolic syndrome, especially in obese adolescents. In addition, aldehydes have been associated with atherosclerosis, putative mutagens, and cancer formation (DUTHIE et al., 2013). The oxidative rancidity can start by an energy source (heat, light, high-energy radiation or metal ions) with free radical production, lipoxygenase action, and photosensitive compounds (e.g., riboflavin, chlorophyll, myoglobin, erythrosine, and heavy metal ions), which are excited to a high-energy state by absorbing light (ALLEN, 1994).

Regarding the analytical methodologies, the methods commonly used are simple, inexpensive, effective, and do not require experienced personnel. FFA quantification is based on lipid extraction with an organic solvent (ether) and anhydrous sodium sulphate without heat processing. Then, the lipid extracted (5 g) is dissolved with alcohol-ether solution (1:2, v/v) and a pH indicator (phenolphthalein solution) is added. The titration is

performed with sodium hydroxide solution (0.1 M or 0.01 M) until pink coloration. The results are expressed in percentage. The measurement of peroxide values (POV) also requires previous lipid extraction, which is performed as described for FFA quantification. Next, the lipid extracted (5 g) is dissolved with acetic acid-chloroform solution (3:2, v/v) and saturated potassium iodide solution is added. The mixture must be left to stand for 1min and distilled water is added. After that, the titration is performed with sodium thiosulphate solution (0.01 N) until the yellow color disappears. Starch indicator solution is added and the titration is continued until disappearance of the blue color derived from the iodine. The results are expressed in mEq/kg. Peroxide determination is important to verify the beginning of oxidative rancidity (BRASIL, 2008).

To evaluate the final products of lipid oxidation, one of the most common methods is the quantification of MDA, which is considered the main secondary compound from lipid decomposition. In this assay, there are several analytic methods that basically differ in relation to the extraction method (by distillation or acid addition) and use of heating or not. However, all methodologies cited are based on the reaction of one molecule of MDA with two molecules of 2-thiobarbituric acid (TBA) to form a stable pink chromophore which are called thiobarbituric acid reactive substance (TBARS) test and the results are expressed in mg of MDA per 1,000g of sample (TARLADGIS et al., 1960; YIN et al., 1993; KARLSDOTTIR et al., 2014a).

There are no limits established by legislation for any of the compounds of lipid decomposition in fish or in their derived products. However, several studies confirmed the increase of FFAs, peroxide, and MDA during storage in fresh and frozen fishes of different species (MONTEIRO et al., 2012; INDERGARD et al., 2014; KARLSDOTTIR et al., 2014a, 2014b; QIU et al., 2014), as well as in fish products (STEVANATO et al., 2010; NIELSEN; JACOBSEN, 2013; PALMEIRA et al., 2014; NIKOO et al., 2015; OLIVEIRA et al., 2015). This means that hydrolytic and oxidative rancidity can occur even in low-fat fishes, depending on the fatty acid composition, and during refrigerated and frozen storage, which are the most common conservation methods used for fishes.

Therefore, FFAs, peroxide, and MDA parameters could be used as quality loss indicators for fish and derived products, indicating possible failures during the

production steps and preventing health risks associated to fish ingestion.

Species authentication

In Brazil, the substitution of species of high value for less valuable ones is often found (CARVALHO et al., 2011) mainly due to growing fish demand and fish consumption (MARTINEZ; JAMES; LOREAL, 2005). According to the Brazilian legislation, fish species identification is based on the morphological characteristics, which complicates fraud detection in processed foods such as frozen fillets and precooked seafood due to the absence of external features (GIL, 2007; MAFRA et al., 2008).

Assays to verify the species authenticity is important for economic, safety, health, and legal reasons, including the labeling regulations, which require that the species be accurately conveyed to the consumer (ONG et al., 2007). Fernández et al. (2002), Horstkotte and Rehbein (2003) and Ataman et al. (2006) have reported analytical methods for fish species identification such as electrophoretic techniques (e.g. isoelectric focusing or SDS-PAGE), chromatographic techniques, and immunological techniques such as immunodiffusion and ELISA. In general, the authentication of fish species can be achieved using proteins or DNA sequences as species-specific markers. The methods based on the protein sequences provide accurate results, but they are not suitable for laboratory routine due to the loss of biological activity after animal death and heat lability. In addition, the presence and characteristics of protein molecules depend on the cellular types. Moreover, these methods are time consuming, difficult, complex, expensive, and their specificity is limited, being inadequate to discriminate between species that have similar phylogenetic relation (ABDEL-RAHMAN et al., 2009).

Therefore, to prevent the detrimental effects on both the industry and the consumer, sensitive analytical methods must be developed for fish species authentication. DNA molecules are present in all types of tissue and they vary greatly with the genetic code. Therefore, analytical procedures related to the determination of DNA sequences may be considered a promising method for fish species identification, mainly in processed matrices (LOCKLEY; BARDSLEY, 2000). Currently, fish species identification can be performed by molecular techniques based on using PCR to amplify the specific fragments of the DNA that are of interest. Next, the amplicons (products of amplification)

can be analyzed by a variety of methods such as PCR (Polymerase chain reaction) sequencing, multiplex PCR, PCR-RFLP (Restriction fragment length polymorphism), PCR-SSCP (Single-strand conformation polymorphism), RAPD (Random Amplified Polymorphic DNA), and real time PCR (BAJZÍK et al., 2010). These methods have been developed and could be applied as alternatives to the official identification of species to enforce labeling regulations (RASMUSSEN; MORRISSEY, 2008; HERRERO et al., 2011, 2012; LOBO et al., 2014).

Generally, in PCR sequencing fragments from ribosomal DNA (alpha-actine) and mitochondrial DNA sequences (cytochrome b) are sequenced for the identification of several fish species. Although sequencing produces a great amount of information, it is time consuming and requires high technology (JÉRÔME et al., 2003; PEPE et al., 2005). Nevertheless, prior sequence knowledge allowed designing species-specific primers even with minor differences (polymorphisms) within the same species. On the PCR-specific primers, the amplification of one fragment occurs using one pair of primers on each reaction. In special conditions, species-specific primers generate a product with determined size in the presence of DNA of a given species and the identification is confirmed by electrophoresis in agarose gel from appropriately sized amplicon (HSIEH et al., 2004).

On the other hand, multiple primers are used in the multiplex-PCR, which leads to the simultaneous amplification of many fragments and, consequently, amplicons of varied sizes and specific to different DNA sequences are produced (LOBO et al., 2014). This assay can be interesting when the desired information is obtained because there is higher saving of time and reagents than species-specific PCR primers. Nevertheless, as multiple genes are placed at once, similar bands can impair fragment discrimination on gel electrophoresis (RASMUSSEN; MORRISSEY, 2008; DURAND et al., 2010). In addition, species identification can be performed by Real-time PCR, which is used to amplify and simultaneously detect or quantify a fragment of DNA molecule. For this quantification, non-specific fluorescent dyes and sequence-specific DNA probes with fluorescent compounds are more commonly used due to their capacity of binding with any double-stranded DNA and detecting their complementary sequence, respectively (DALMASSO et al., 2007). In these cases, electrophoresis needs not be used (MARMIROLI; PEANO; MAESTRI, 2003).

PCR-RFLP is based on DNA digestion by a restriction enzyme leading to the separation of DNA fragments, which are discriminated by their lengths on gel electrophoresis. A single primer pair can produce a fragment that can be used for the identification of multiple species (CHEN et al., 2008). This method has been used for the identification of some fish species of the Cyprinidae family (CHEN et al., 2012), fishes belonging to the genus *Epinephelus* (SUMATHI et al., 2015), and fish species in the Engraulidae family (CHAIRI; REBORDINOS, 2014). Moreover, when compared to conventional DNA sequencing and nucleotide sequence analysis, PCR-RFLP is a rapid and cheap method for species identification (CHEN et al., 2012). However, an incomplete digestion can difficult the restriction sites, the DNA fragment size can be decreased by heat processing, and additives added (spices and sauces) on manufactured fish products might lead to an inhibitory effect on the PCR (ZHANG et al., 2006). Nevertheless, this can be solved by obtaining PCR of the fishes destined for processing, and checking the compliance with labelling from those samplings.

PCR-SSCP analysis is a molecular technique that does not require prior knowledge of DNA sequence. This method is very sensitive for detecting a single base exchange or a few differences between sequences of short DNA fragments (from 100 to 400 bp), which are separated by their different conformations (HAYASHI, 1996). Different sequences migrate unevenly on gel electrophoresis due to distinct molecular weight and shape. This fact occurs, mainly, because single-strand DNA fragment conformation (secondary structure) depends on the nucleotide sequence (primary structure), and the mobility of the single-strand DNA fragment is affected by minor changes in the sequence, resulting in distinct banding patterns. PCR-SSCP is used primarily to detect known or novel polymorphisms and mutations in genes (PETERS et al., 2000).

PCR-RAPD consists in PCR amplification of random segments of DNA using single primers of arbitrary nucleotide sequence, which are purchased from commercial companies. It is expected that the fragments of DNA generated are consistent with the primer, DNA, and conditions used. The choice of the primer is random and the PCR is performed using a large template of genomic DNA, expecting that the fragments will amplify. The amplification products are separated on agarose gel in the presence of ethidium bromide and visualized under ultraviolet light. The information generated can range from differentiation at the individual to species level (WILLIAMS et al., 1990).

The PCR-RAPD technique has been used to develop markers for sex differentiation in many fish species such as in Nile tilapia (*Oreochromis niloticus*) (BARDAKCI, 2000), African catfish (*Clarias gariepinus*) (KOVÁCS et al., 2000), and blowfish (*Tetradon nigroviridis*) (LI et al., 2002). This method is simple and quick, only a small amount of DNA is needed, it targets many sequences in the DNA of the sample allowing simultaneous comparison, and, most importantly, no prior knowledge of the genetic make-up of the fish is required (WILLIAMS et al., 1990). However, it is not practical for species identification in products containing mixtures of species, and is not adequate for the analysis of degraded material as those subjected to severe heat treatment (autoclaved products) (MARTÍNEZ; MALMHEDEN YMAN, 1998). Currently, PCR-RAPD has been described as a potential molecular technique for the evaluation of fish genotoxicity caused by heavy metals (SALEM et al., 2014), molecular characterization, and genotoxic study of *Dicentrarchus labrax* embryonic cells (DLEC) (ROCCO et al., 2014), as well as genetic knowledge (specification of populations) of marine fish commercially important such as saithe (*Pollachius virens*) (BEHRMANN et al., 2015).

Regarding fish species identification, the main challenge is to develop and validate a molecular method that be accurate, specific, sensitive, and applicable to detect adulterations and misbranding of a variety of fish and fish products. Moreover, it needs to identify simultaneous species with relatively low cost and duration even in processed foods containing highly degraded material. It is important to introduce a molecular technique as official methodology for fraud monitoring and ensure the quality control of fishery industries (KITPIPIT et al., 2014; ALI et al., 2015).

Conclusions

Based on the negative effects on fish sensory properties and consumer's health, the limits of total volatile basic nitrogen (TVB-N), trimethylamine (TMA), ammonia, pH, and biogenic amines should be established by fish categories and/or type of fish product. Moreover, official parameters must be added for metabolites of protein oxidation (carbonyls levels) and lipid degradation, such as free fatty acids (FFAs), peroxide values (POV), and malondialdehyde (MDA). Furthermore, the Brazilian legislation needs to consider analytical methods (effective, practical, low-cost, and fast) that facilitate the routine analyses and allow a

dynamic quality control of fish and fish derived products, resulting in enhanced monitoring and lower risk to human health. Regarding illegal substitutions, the national legislation should include analytical methodologies for the identification of each fish in order to facilitate fraud detection and ensure that the consumer's rights be respected.

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