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New methods to determine fish freshness in research and industry

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SUMMARY – In this paper a description of the new trends in fish quality determinations is described. First, the quality definition is discussed, considered as a broad concept, addressing all the features of the fish appreciated by a potential consumer. Fresh fish is a product with a very short commercial life, and a high variability. In fact, the number of marketable species is enormous. Those characteristics make very difficult to apply the same systems used in other foods. New economic and fast determinations are necessary to develop applying these on three premises: (i) consideration of fish autolysis in the freshness determination; (ii) development and study of forward markers of fish spoilage; and (iii) a double requirement in the development of these forward markers – rapidity and reliability. Different recommended methods and analysis are discussed such as: *rigor mortis*, objective methods (amine concentration, nucleotides, protein modifications and lipid oxidation), rapid microbiological determinations and enzymatic and histological analysis. As conclusions, to consider the different methods described as reference protocols, it will be necessary to develop specialised laboratories in fish quality determination, distributing applications on the basis of concrete needs and monitoring of the quality system.

Key words: Fish quality, fish freshness determination.

RESUME – *"Nouvelles méthodes pour déterminer la fraîcheur du poisson dans le domaine de la recherche et de l'industrie". Cet article présente une description des nouvelles tendances pour la détermination de la qualité du poisson. En premier lieu, la définition de la qualité est discutée, considérée comme un concept très vaste, en examinant toutes les caractéristiques du poisson perçues par un consommateur potentiel. Le poisson frais est un produit qui a une vie commerciale très courte, et une forte variabilité. En fait, le nombre d'espèces commercialisables est immense. Ces caractéristiques font qu'il est très difficile d'appliquer les mêmes systèmes qui sont utilisés pour d'autres aliments. Il est nécessaire de mettre au point de nouvelles déterminations rapides et économiques en les appliquant sur trois bases : (i) considération de l'autolyse du poisson pour la détermination de la fraîcheur ; (ii) développement et étude de marqueurs précoces de l'altération du poisson ; et (iii) une double exigence concernant le développement de ces marqueurs précoces : rapidité et fiabilité. Différentes méthodes et analyses recommandées sont discutées comme :* rigor mortis*, méthodes objectives (concentration en amines, nucléotides, modifications des protéines et oxydation des lipides), déterminations microbiologiques rapides et analyses enzymatiques et histologiques. En conclusion, pour la prise en compte des différentes méthodes décrites comme protocoles de référence, il sera nécessaire de développer des laboratoires spécialisés dans la détermination de la qualité du poisson, et de distribuer les applications sur la base des besoins concrets et du suivi du système de qualité.*

Mots-clés : *Qualité du poisson, détermination de la fraîcheur du poisson.*

Introduction

For the determination of fish quality is essential, first, consider what is our concept of this word. In general, we may accept in general a broad concept, considering all the features of the fish appreciated positively by the consumer conscious or unconsciously. What means this? In our language, that it will be very difficult to determine on a specific manner, the exact fish quality. Over the years, different studies has been developed to determine the quality of meat, milk and vegetable foods, and different techniques has been recommended to assess their quality and freshness features. However, fish has been studied from a different perspective. Fresh fish is a product with a very low commercial life, and a high variability. In fact, the number of marketable species is enormous. Those characteristics make very difficult to apply the same systems used in other foods. Furthermore, the chemical methods developed are expensive and time-consuming. For these raisons they cannot be used in a product that need economic and fast determinations. Our objective of study the fish quality is based on three premises: (i) consideration of fish autolysis in the freshness determination to very fresh fish and frozen

fish; (ii) research of forward markers of fish spoilage; and (iii) a double requirement in the development of these forward markers – rapidity and reliability.

Freshness is a property of fish that has a considerable influence on its quality. It is the most important single criterion for judging the quality of the majority of fish products. Loss of freshness followed by spoilage is a complex combination of microbiological, chemical and physical processes (Pedrosa-Menabrito and Regenstein, 1990). The early spoilage detection of fish is a problem not resolved nowadays. It is accepted that the organoleptic evaluation of fish quality is the most economic and rapid technique. However, this is a subjective method. To obtain comparative and representative results, analysis could be performed by trained personnel. Then is essential to obtain objective techniques, such as chemical analysis. Comparative studies to establish correlations between both parameters are necessary. Several chemical parameters have been proposed to establish a freshness scale. These techniques are: volatile organic acids' determination, fat changes, valuation of indole, eschatole.

The main objective is monitoring the fish quality. Reliable and objective criteria and methods are necessary, to confirm incipient spoilage and to have the opportunity to brake it. A reliable detection of the raw material quality could let: (i) the best product to the consumers; (ii) assess a better management of fresh stocks; and (iii) reduce the level of returned products.

Recommended analysis

Rigor mortis

It is a parameter very influenced by the temperature and the time. Fish quality is a very used word, but without a concrete and viable application to all kind of fish species. The most interesting parameter associated with fish quality is the *rigor mortis*. It is presented when the muscular ATP stores are exhausted, and it indicates the better quality of fish, their better freshness, and assures a very good nutritional composition. Any parameter referred to maintenance of *rigor mortis* will assure freshness of fresh fish.

Objectives methods

Amines concentration

Analysed individually or associated in equations. These equations pretend to relate natural amines with autolitic and bacterial-formed amines. The result wants to obtain a prediction of the freshness of the fish. Nowadays, is preferred to use methods such as Total Volatile Basic Nitrogen determination (TVBN) (Billon *et al*., 1979; Civera *et al*., 1995) and trimethylamine (TMA) (Civera *et al*., 1995). The Directive 91/493/EEC, which establishes the sanitary practices to be adopted during the handling and marketing of seafood, mentions TMA and TVBN determination as additional investigations besides the organoleptic tests. However, these techniques let to appreciate, a *de facto* situation. Under a value, product could be consumed, over this value consumption is not possible. Furthermore, TVBN is not reliable in all fish species, and TMA and TVBN contents, are not sensitive indexes of freshness because of their high variability. Then, these tests are usually reserved for fish near the limit of acceptance (Malle and Tao, 1987; Malle and Poumeyrol, 1989; Civera *et al*., 1995).

Amines concentration (Valle *et al*. 1996):

$$AI = \frac{\frac{NH_4^+}{1{,}000} + put + cad + his + ltyr}{\frac{NH_4^+}{1{,}000} + put + cad + his + met + spd + spm}$$

By the other hand biogenic amines could be used such a quality index (Karmas and Mietz, 1978; Moret *et al*., 1992). On this way, the development of enzymatic methods to determine histamine and the diamines putrescine and cadaverine followed during storage time, may contribute to study the evolution of those amines during the spoilage of different fish species (Rodríguez-Jerez *et al*., 1994a).

To valuate the fish spoilage, several works have been carried out, oriented to quantify biogenic amines by different reasons. Biogenic amines formation is due to a decarboxylation of aminoacids by bacterial enzymes. Bacterial growth is a determinant factor on fish spoilage, and asses the level increases of biogenic amines (Rodríguez-Jerez *et al*., 1994a). The most important microbial species responsible of fish spoilage springs from: environment, gut, gill and skin. This microflora is formed by Gram negative bacteria. The study of bigenic amines, let a taxonomic approach of these bacteria (Busse and Auling, 1988; Auling *et al*., 1991), suggesting an advanced bacterial contamination (Gouygou *et al*., 1989). Ingestion of foods with an elevated level of some biogenic amines, determine the development of a food ill. Histamine ingestion upper to 100 mg/100 g of flesh, could involve a histamine food poisoning (Edmunds and Eitenmiller, 1975). In 1991, The EU published the Directive 91/493, pointing out that histamine level in scombrid fish could be lower than 10 mg/100 g. Amines could be toxic by reaction with nitrites from salted products, due to a nitrosamines formation (Gennaro *et al*., 1988). Since hypoxanthine peaked at ten days and declined at 15 days of storage, and diamines did not develop until eight days after storage. The combination of these two tests could reflect the freshness and incipient spoilage of fish (Pedrosa-Menabrito and Regenstein, 1990).

Quantification of biogenic amines, will be revised as a function of different parameters: solvent used in the extraction, extraction time, optimization of the time of extraction and stability of fluorescent compounds. HPLC techniques should be used. However, an initial study about the quantification methodologies should be performed and also it is necessary to determine the ideal relation between matrix and eluent. Especial attention will be taken to nucleotid extraction. The idea is to use the same matrix HPLC than is used for amines quantification (Valle *et al*., 1996), considering different points:

(i) Amines extraction: this technique need a previous extraction of the molecules to quantify. A destruction of cellular structures and a protein precipitation by alcohols or acids. The results of an acid extraction with both perchloric or trichloroacetic acids or with methanol are as for cheese samples (Zee *et al*., 1985). In fish flesh a previous confirmation is necessary, and also an extraction with cholorhydric acid is much more difficult (Lin and Lai, 1980; Huy and Taylor, 1983; Moret *et al*., 1992). Then, the amines could be recovered into an organic solvent or into a solvent mixture with different polarities. At acid pH, amines are dissolved in the hydrophilic phase. However, at alcaline pH, amines change their polarity. A migration to lipophile phase is observed. An evaporation of organic phase could let a concentration of amines. For these reasons, an extraction with organic solvents and at alkaline pH will be used to facilitate the concentration and to minimize the wastage (Rosier and Van Peteghem, 1988; Lebiedziñska *et al*., 1991).

(ii) Amines derivation: acid halogenures as benzoyl chloride (Yen and Hsieh, 1991), dansyl chloride (Mietz and Karmas, 1977; Chiavari *et al*., 1989) or dabsyl chloride (Lin and Lai, 1980) may be used. Their optimal activity is between pH 8 and 10. Produced compounds are relatively stable but unspecific. Derived compounds with specific fluorogen reagents as orthophtalaldehyde (OPT), could be used too (Walters, 1984; Yamanaka, 1990). These compounds are more specific but with a lower stability.

(iii) Detection: different techniques has been used to detect biogenic amines. Chromatography and especially HPLC allow a high specificity in the amines quantification, because is possible to leave the biogenic amines with a big sensibility. Furthermore, HPLC can be used with halogens and fluorogen products.

Nucleotides

In parallel, to the previous methodology, it is necessary to develop a rapid technique to study the degradation of nucleotides. A cellular structure disruption and a protein precipitation with acids is necessary. Perchloric or trichloroacetic acids could be used. Then, a neutralization with NaOH or KOH is necessary to adjust the pH between 6.4-7.8. To achieve nucleotides detection, a column of ionic interchange or reverse phase will be used (Hu *et al*., 1991). Results with that technique is the same that using a biosensor specific method (Luong *et al*., 1991). The interest to apply HPLC in front to biosensors is because biosensors loose their effectiveness after 40 analysis (Mulchandani *et al*., 1989; Lee *et al*., 1992).

The aim of this method is to simulate the ATP spoilage, on a natural point of view. Their aim is to simulate the normal formation and resolution of *rigor mortis*. It is preferable to consider more specific

molecules, directly related with transformation processes on the fish flesh as the degradation study of ATP metabolism, and particularly the hypoxanthine (Pedrosa-Menabrito and Regenstein, 1990; Luong *et al*., 1992; Lee y coll., 1992). Hypoxanthine, and derived nucleotides of enzymic breakdown of ATP (K value), are a normal constituents of fish flesh, although it is present in very low concentration in the living animal. These nucleotides are better spoilage indicators that TVBN and TMA.

They are applicable to a wider range of species and products, and are not stopped with the freezing. It can be said that if little or not hypoxanthine is present, the fish is fresh (Jacober and Rand, 1982; Pedrosa-Menabrito and Regenstein, 1990). Hypoxanthine being one of the best indicators of fish ageing and damage, lead to the development of a patented test strip by Jahns *et al*. (1976), for the determination of hypoxanthine concentration in fish tissues. However, it is difficult to readily differentiate the colour changes on the strip. This test is not being used commercially.

The K value is a measure of the percentage of the total adenosine-derived nucleotides pool, and their use will determine the incipient freshness of fish. A second step, to develop is the study of nucleotid degradation. Especially, inosine and hypoxantine levels could be studied. These parameters are particularly interesting, because the autolysis evaluation is permitted before the bacterial growth start.

K Value (Valle *et al*., 1996):

$$K = \frac{\text{Inosine + hypoxanthine}}{\text{Inosine + hypoxanthine + IMP}}$$

ATP disappearance is in a direct relation with fish freshness (Fujii *et al*., 1973; Mulchandani *et al*., 1990; Lee *et al*., 1992). ATP level at the fish flesh depend on capture conditions, time after catching, sexual maturity and storage conditions (Luong *et al*., 1991). After the fish dead, a rapid decrease of ATP level is observed. That situation is derived of a dysfunction of glycogenolysis (enhanced by Ca^{++}); an increase of phosphocreatine; the arrest of Lohman reaction (ATP regeneration by action of ADP and Ca^{++}) and the stimulation of the miosine-ATPase by Ca^{++}. On this moment, a junction between the actin and myosin is observed. When the ATP concentration reached 0%, the *rigor mortis* is established (Burt, 1977; Jouve *et al*., 1991). ADP and AMP levels decrease quickly too. Then an accumulation of inosine and hypoxanthine is detected (Williams *et al*., 1991).

To quantify the K value, different enzymes will be used. The first step is the isolation and purification of an enzyme (nucleoside oxidase) produced by a microorganism (*Pseudomonas maltophilia*) (Isono *et al*., 1988, 1989a,b). Then with the enzymes nucleoside phosphorylase and xanthine oxydase (commercialized by Boehringer Manheim, Co.), the total nucleotides will be quantified, using colourimetric methods in the different points of the enzymic chain (Isono, 1990).

Protein modifications

Is more applicable to processed fishes. Proteins are not very spoiled in fresh fish, and consequently it will be very difficult to obtain significance parameters using this methodology. If amines are used, is necessary remember that these compounds are formed after a bacterial decarboxylation of aminoacids. A previous proteolysis is necessary. Although this parameter could be used when a significant level of microorganisms is detected, then spoilage is already started.

Lipid oxidation

Lipid spoilage is due to the unsaturated fatty acids. These are very sensible to oxygen, and then, to the autooxidation. Evaluation of fat oxidation may have a well correlation with organoleptic features.

Rapid microbiological determinations

Microbiological determinations in fish need at least 48 hours to obtain reliable results. All the parameters, which capacity to obtains results in less than 24 hours, will help in freshness determination or evaluation of fish. Fish spoilage start when it is captured. After catching, fish is in contact with a different microflora, and normally, it is stored at ice temperature (0°C). These conditions merely slow down microbial activity, delaying but not inhibiting, the spoilage of fish.

Recently, some papers have appointed the necessity to use different bacterial groups than traditional total count used in other food products. Consequently H_2S^+ bacteria has been recommended, and in particular *Shewanella putrefaciens* specie. These bacteria are more sensitive to additional NaCl in the medium. Valle *et al*. (1998) have demonstrated that the better conditions to perform a representative bacterial total count in fish are presence of oxygen, TSA medium with a 1.5% NaCl, 20°C, during 2-4 days. However, This king of analysis require the development of rapid methods, because 2-4 days are not reliable to a very perishable product as fish.

Enzymatic and histological analysis

An important quantity of enzymatic and histological analysis has been recommended to evaluate quality of fish. Only few of them are really useful. Our work could be differentiate and select them.

To apply these new methods, it will be important to calibrate our product, according with the better system, and to assure the better quality, in relation with their evolution and time. To assure this objective, some points should be considered: (i) development of specialised laboratories; (ii) distribution of application on the basis of concrete needs; and (iii) monitorization of the quality system.

An initial method has been published by Lerke *et al*. (1983) and latter modified by Lopez-Sabater *et al*. (1993). That technique is based on the action, at pH 6.8, of the diamine oxidase enzyme over the histamine present in the sample. This enzyme catalyses the breakdown of histamine. As product of enzymic activity, imidazole acetaldehyde, ammonia and hydrogen peroxide are formed (Fig. 1). A second enzyme (peroxidase), in presence of a chromogen (LCV) in reduced form (colourless), caused its oxidation into crystal violet (coloured form). After incubation, lectures are performed at 596 nm, after a incubation of 2 hours at 37°C. This method was performed in an incubator and on these conditions a variation in the optimum wavelength was observed (Rodríguez-Jerez *et al*., 1994c). A modification of the enzymic method was recommended, incubating at 37°C during 15 minutes and measuring the optical density at 580 nm. With these conditions a linearity was observed between 1 and 25 mg of histamine per kg of flesh.

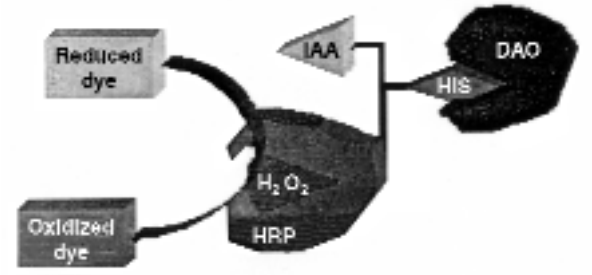

Fig. 1. Enzymic method.

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