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Biochemical changes and quality loss during chilled storage of farmed turbot (*Psetta maxima*)

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Abstract

Changes in three of the major biochemical components – nucleotides, lipids and proteins – related to quality loss in farmed turbot, were determined during 29 days of iced storage; results were complemented with sensory analysis. Nucleotide degradation, as estimated by the *K* value, underwent a gradual increase until day 19, in agreement with the loss of freshness observed for the sensory scores (high quality: days 0–2; good quality: days 3–14; fair quality: days 15–19). After day 19, the fish was judged unacceptable and the *K* value did not show differences until the end of storage. Lipid hydrolysis and oxidation occurred at slow rates, free fatty acid contents and the peroxide value being below 20.0 g kg^{-1} lipids and 4.00 meq active oxygen kg^{-1} lipids, respectively, during the whole storage. The content of fluorescent compounds did not increase significantly until day 19, when a sharp increase was detected. The electrophoretic protein profiles of turbot muscle did not point to any major protein degradation event or any significant change in protein during storage. However, a new band, corresponding to 22 kDa, could be observed at day 2 in the low-ionic strength buffer extract, whose concentration seemed to increase at days 9 and 14 and was present until the end of the chilled storage. The results obtained in this work indicate slow and gradual biochemical changes and long shelf life and good quality times (19 and 14 days, respectively) for iced turbot; these long times would be very profitable when turbot commercialisation is carried out in places distant from production farms.

Keywords: Turbot; Aquaculture; Chilling; Nucleotides; Lipids; Proteins; Quality

1. Introduction

Seafood products have attracted considerable attention as a source of high amounts of important nutritional components to the human diet (Ackman, 1989; Piclet, 1987). However, in recent years the fishing sector has suffered from dwindling stocks of traditional species as a result of dramatic changes in their availability. This has prompted fish technologists and the fish trade to pay more attention to aquaculture techniques as a source of fish and other seafood products (FAO, 2000; Josupeit, Lem, & Lupin, 2001).

Assurance of both the quality and safety of seafood will be a major challenge faced by humankind in this new century. In this sense, wild and farmed fish species are known to deteriorate after death due to the action of different mechanisms (Hsieh & Kinsella, 1989; Pigott & Tucker, 1987). During fish chilled storage, biochemical changes are known to take place, such as changes in the protein and lipid fractions and the formation of amines (volatile and biogenic) and hypoxanthine. As a consequence of these events, a deterioration in sensory quality, a loss of nutritional value, and negative modifications of the physical properties of fish muscle have been reported (Bennour, El Marrakchi, Bouchriti, Hamama, & El Ouadaa, 1991; Nunes, Batista, & Morâo de Campos, 1992; Olafsdóttir et al., 1997).

Turbot (*Psetta maxima*, also known as *Scophthalmus maximus*) is a flat fish species of high commercial value found in Northern waters and widely appreciated for its firm, white, and flavourful flesh. In recent years, the

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increasing production of this species as an aquaculture product has made it more available to consumers, when the wild product is increasingly less consumed because of its low availability and high cost. Extensive work has been carried out on the effects of diet on turbot growth (Cáceres-Martínez, Cadena-Roa, & Métailler, 1984; Danielssen & Hjertnes, 1993; Regost et al., 2001) and on the development of tools for the identification of turbot with respect to other fish species (Etienne et al., 2000; Prost, Serot, & Demaimay, 1998). However, research concerning the quality changes that might occur during *post-mortem* storage only includes high pressure (Chevalier, LeBail, & Ghoul, 2001) and thermal (Madeira & Penfield, 1985) treatment, such that – to date – the mechanisms of damage taking place in farmed turbot during chilled storage remain relatively unknown.

In the light of this situation, in the present work, we were prompted to investigate the biochemical changes involved in the loss of quality undergone by farmed turbot during chilled storage. To this end, the changes in the most relevant biochemical components – lipids, proteins and nucleotides – were evaluated during a long storage period (29 days) in ice and complemented by sensory analysis.

2. Materials and methods

2.1. Raw material, sampling and processing

Two-year old farmed turbot (*P. maxima*) specimens were obtained from Stolt Sea Farm, S.A. (Carnota, Galicia, Spain). Fish specimens were sacrificed in a water-ice mixture and then kept in ice for 10 h until they arrived at our laboratory. The length of the fish was in the 39–46 cm range, while the width was in the 29–35 cm range; the weight range was 1.600–1.900 g. All the fish specimens were stored on ice in an isothermal room at 4 °C. Samples were taken for analysis on days 0, 2, 5, 9, 14, 19, 22, 26 and 29. Three different batches were laid down and studied separately along the whole experiment. Once whole fish had been subjected to sensory analysis, the white muscle was separated and homogenised to obtain extracts for analyses.

2.2. Sensory analysis

This was conducted by a taste panel consisting of five experienced judges, based on traditional guidelines concerning fresh and chilled fish (DOCE, 1989). Four categories were ranked: highest quality (E), good quality (A), fair quality (B) and unacceptable quality (C). Sensory assessment of the fish included the following parameters: skin, external odour, gills, consistency and flesh odour.

2.3. Chemical composition

The water content was determined by weight difference of the homogenised muscle (1–2 g) before and after heating at 105 °C for 24 h. The results were calculated as g water kg^{-1} muscle.

The lipid fraction was extracted by the Bligh and Dyer (1959) method. Quantification results was calculated as g lipids kg^{-1} wet muscle.

Two different protein extraction procedures were considered in this work. Preparation of urea-soluble protein extracts was carried out in extraction buffer A [8 M urea + 4% (w/v) CHAPS (3,3-chloramidopropyl-dimethylammonio-1-propanesulphate) + 40 mM Tris]. Sarcoplasmic protein extracts were prepared in a low-ionic-strength buffer – extraction buffer B [10 mM Tris–HCl, pH 7.2, +50 mM pentamethyl sulphonic acid]. In both cases, 500 mg of muscle were homogenised for 60 s in 4 ml of the required buffer solution, as previously described (Piñeiro et al., 1999). Then, all extracts were centrifuged at 12,500 rpm for 15 min, in a JA20.1 rotor (J221-M centrifuge, Beckman-Coulter, London, UK) at 20 °C, and the supernatants were recovered. Extracts prepared in buffer A were kept for 30 min at room temperature before spinning. All extracts were maintained at −80 °C until analysis.

Protein concentrations in the extracts were determined as follows: extracts prepared in buffer A were subjected to the PlusOne 2-D Quant kit (Amersham Biosciences, Uppsala, Sweden) while the protein concentration in extracts prepared in buffer B were determined by means of the protein microassay method (Bio-Rad Laboratories Inc., Hercules, CA, USA). In both cases, a standard curve, constructed for bovine serum albumin, was used as reference and results were expressed as g kg^{-1} muscle.

2.4. Nucleotide analysis

Nucleotide extracts were prepared according to the method of Ryder (1985) and stored at −30 °C until analysis.

Nucleotide analysis was performed by HPLC, using a Beckman device provided with the programmable solvent module 126 (Beckman), and the scanning detector module 167 (Beckman) connected to the System Gold software, version 8.1 (Beckman). Separations were achieved on a reverse-phase Spherisorb ODS-2 C_{18} 250 × 4.60 mm column (Waters, Milford, MA), with an internal particle diameter of 5 μm. The composition of the mobile phase was as follows: solvent A was composed of 0.04 M KH_2PO_4 + 0.006 M K_2HPO_4, pH 7; solvent B was acetonitrile. Solvents were filtered through a 0.45 μm aqueous filter before use. Separations were carried out using a continuous gradient elution with solvent A and solvent B. Table 1 summarises the tech-

Table 1
Technical conditions employed for the HPLC analysis of nucleotide degradation

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (ml min^{-1})
0	100	0	1.6
4	80	20	1.6
8	70	30	1.8
10	100	0	1.6
15	100	0	1.6

nical conditions used in the chromatographic analysis. The eluent was monitored at 254 nm and the running time was 10 min. Standard curves for adenosine 5′-triphosphate (ATP) and each compound involved in its degradation pathway, adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine 5′-monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx), were constructed in the 0–1 mM range. All nucleotide standards were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). *K* values were calculated according to the following concentration ratio: K value $= 100 \times (\mathrm{Hx} + \mathrm{Ino})/(\mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP} + \mathrm{IMP} + \mathrm{Ino} + \mathrm{Hx})$.

2.5. Analysis of lipid damage

The free fatty acid (FFA) content was determined by the method of Lowry and Tinsley (1976), based on complex formation with cupric acetate-pyridine. Results are expressed as g FFA kg^{-1} lipids.

The peroxide value (PV) – expressed as meq active oxygen kg^{-1} lipids – was determined according to the ferric thiocyanate method (Chapman & Mckay, 1949).

The formation of interaction compounds was investigated by means of fluorescent properties. To do so, fluorescence formation (Perkin–Elmer LS 3B) at 393/463 and 327/415 nm was studied as previously described (Aubourg, Medina, & Gallardo, 1998a). Relative fluorescence (RF) was calculated as follows: $\mathrm{RF} = F/F_{\mathrm{st}}$, where F is the fluorescence measured at each excitation/emission maximum, and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 μg ml^{-1} of 0.05 M H_2SO_4) at the corresponding wavelength. The fluorescence ratio (FR) was calculated as the ratio between both RF values: $\mathrm{FR} = \mathrm{RF}_{393/463\,\mathrm{nm}}/\mathrm{RF}_{327/415\,\mathrm{nm}}$. The FR value was analysed in the aqueous phase resulting from the lipid extraction (Bligh & Dyer, 1959).

2.6. Analysis of proteins by electrophoresis

Electrophoretic analysis was initially carried out using both homemade vertical and commercial horizontal SDS–PAGE gels. Because of their higher resolution and the reproducibility of their results, commercial precast polyacrylamide $245 \times 110 \times 1$ mm gels (Excel-Gel SDS Homogeneous 15%, Amersham Biosciences) for horizontal electrophoresis were selected. Anode and cathode buffer strips (Amersham Biosciences) were also employed. Electrophoretic studies were performed on a Multiphor II electrophoresis system (Amersham Biosciences) provided with a MultiTemp III refrigerated circulator bath (Amersham Biosciences). Running conditions were as follows: 1000 V/40 mA/40 W for 165 min. Once the bromophenol blue had reached the anode, the gels were fixed and stained using a standard silver staining protocol (Amersham Biosciences). A low molecular weight protein standard (94–14 kDa) from Amersham Biosciences was employed as reference. This protein standard mixture was comprised of the following proteins (MW values are indicated in brackets): phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The mobilities of protein bands were determined using the Whole Band Analyser Software (BioImage Systems Corporation, MI, USA) and the corresponding MWs were calculated by comparison with the size of the protein standards.

2.7. Statistical analysis

Data from the different biochemical measurements were subjected to one-way analysis of variance ($p < 0.05$); comparison of means was performed using a least-squares difference (LSD) method (Statsoft, 1994). Linear correlations between chilled time and biochemical parameters were studied; non-linear fittings (logarithmic and quadratic) were also evaluated and their results are expressed when equal or superior to the linear ones.

3. Results and discussion

3.1. Sensory analysis

Turbot specimens maintained high and good quality (categories E and A, respectively) during the first 14 days of chilled storage (Table 2). Afterwards, the quality decreased and by day 20 the fish specimens were no longer acceptable. The main concern relating to the loss of sensory quality was the segregation of mucus from the skin, which led to a very unpleasant odour. In general terms, both the aspect of the skin and the external odour proved to be the limiting factors of sensory quality, whereas the other three features examined – gills, consistency, and flesh odour – were still acceptable up to day 29 of chilled storage.

The results of the sensory analysis obtained for turbot are consistent with the shelf life times obtained for other medium-sized fish species, such as albacore and hake

Table 2
Sensory acceptance[a] of chilled turbot

Storage time (days)	Sensory acceptance
0	E
2	E
5	A
9	A
14	A
19	B
22	C
26	C
29	C

[a] Freshness categories: E (excellent), A (good), B (fair) and C (unacceptable).

(Pérez-Villarreal & Pozo, 1990; Ruiz-Capillas & Moral, 2001) and this shelf life is far longer than those of smaller fish species, such as sardine and horse mackerel (Aubourg, 2001; Nunes et al., 1992).

3.2. Chemical composition

The water content of turbot muscle ranged between 760 and 800 g kg^{-1} while the lipid content was in the 8.0–12.0 g kg^{-1} range. The differences in both components may be explained in terms of individual variations in the fish rather than being due to chilled storage. Comparison of the present results with those obtained in previous research reveals, first, a higher water content for turbot than for fattier fish species (Aubourg, Sotelo, & Pérez-Martín, 1998b) but, second, a lower water content than other leaner fish species (Aubourg & Medina, 1999; Aubourg et al., 1998a). These results correlate well with the expected inverse ratio between water and lipid matter, as previously described (Piclet, 1987).

Fig. 1 summarises the results of protein extractability in turbot muscle during the storage period. As expected, in general terms the urea-containing extraction buffer A afforded extracts with higher protein concentrations than their counterparts extracted with the low-ionic-strength buffer B. Protein extractability remained high when the urea buffer was used, even after advanced storage periods; a significant increase was observed on day 22 (Fig. 1). Once diluted, the protein extracts were studied by horizontal electrophoresis, this allowing the observation of clear and almost equivalent protein profiles, regardless of the extraction buffer considered. A decreasing trend with chilling time was observed for sarcoplasmic protein content, leading to a good correlation value ($r^2 = -0.97$; Table 3). In the case of urea-soluble protein, an interesting correlation value was also obtained ($r^2 = 0.94$).

From this part of the study, it was concluded that, since the protein concentrations in extracts prepared in buffer B were more reproducible and less affected by storage time than the extracts prepared in buffer A, the sarcoplasmic fraction should be selected for evaluating the protein changes in turbot muscle during chilled storage.

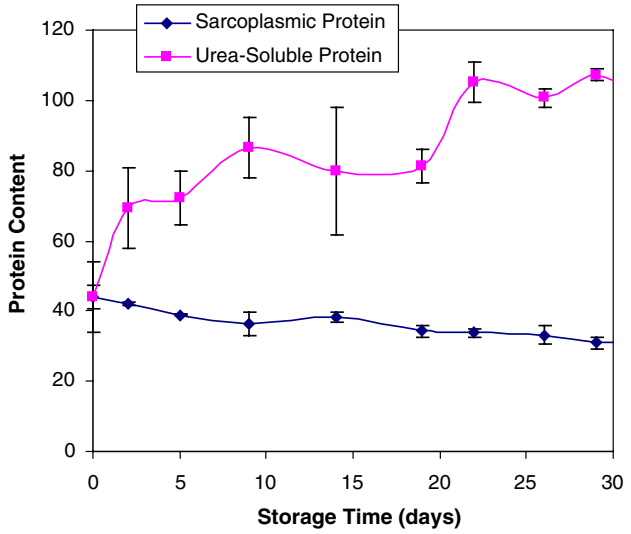

Fig. 1. Evolution of protein (g kg^{-1} muscle) extractability from turbot muscle during chilled storage.

Table 3
Linear correlations between chilled storage time and different biochemical determinations

Biochemical index	Correlation value[a]
Sarcoplasmic protein	−0.95
	(−0.97)
Urea-soluble protein	0.90
	(0.94)
K value	0.91
	(0.96)
Free fatty acids	0.96
Peroxide value	0.74
	(0.83)
Fluorescence ratio	0.87

[a] Non-linear fittings (logarithmic) are expressed in brackets when equal to or superior to the linear ones.

3.3. Nucleotide analysis

Nucleotide degradation in turbot muscle during storage was studied on the basis of the *K* value (Fig. 2). A gradual increase in this index was observed in the 0–19 days period; the muscle obtained in this period was judged by the sensory analysis as eatable. The *K* value reflected the loss of freshness (Table 2) of chilled turbot during the 0–19 days period. After day 19, no changes were observed for the *K* value. During the complete storage period, a good correlation (Table 3) was obtained with the chilling time ($r^2 = 0.96$, logarithmic fitting).

According to previous research (Olafsdóttir et al., 1997; Ryder, Buisson, Scott, & Fletcher, 1984), the IMP

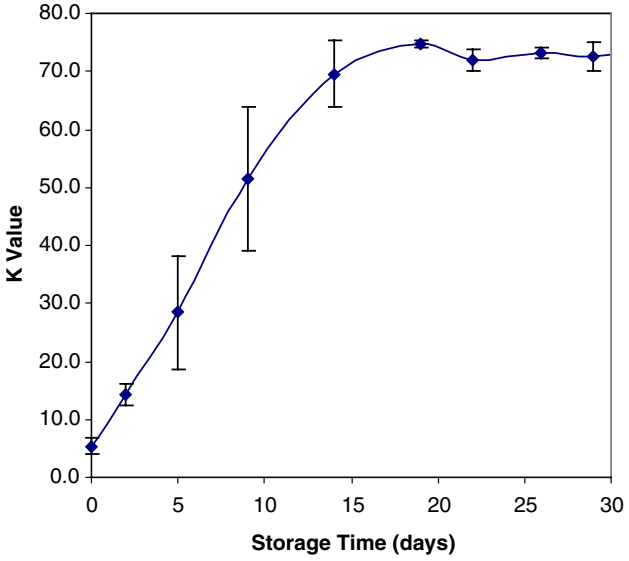

Fig. 2. Evolution of K value in farmed turbot muscle during chilled storage. Results are expressed as the ratio of nucleotide concentrations described in the text.

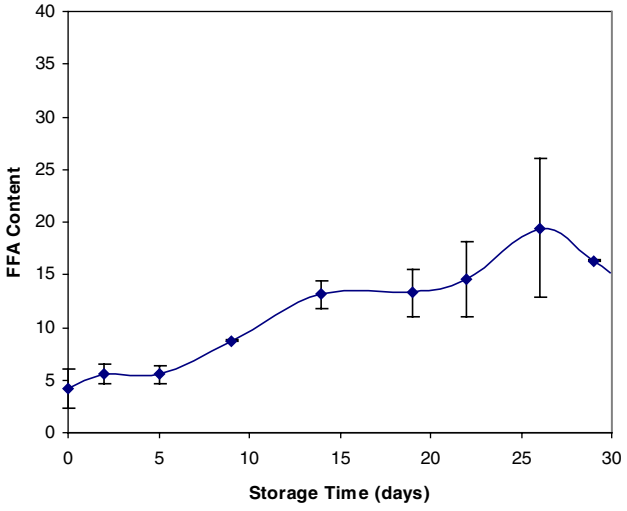

Fig. 3. Evolution of FFAs content in farmed turbot muscle during chilled storage. Results are expressed as g FFA kg^{-1} lipids.

concentration is the determinant in the K value, such that the K value did not increase any more because the IMP was practically non-existent. As regards its K value, the evolution of turbot muscle with time was similar to that of the sardine (Gómez, Cañada, & Moral, 1990) and black skipjack (Mazorra-Manzano, Pacheco-Aguilar, Díaz-Rojas, & Lugo-Sánchez, 2000). However, other fish species, such as mackerel (Ryder et al., 1984) and seabream (Huidobro, Mendes, & Nunes, 2001), show slower increases in their K values.

3.4. Analysis of lipid damage

Lipid hydrolysis occurred during chilled storage (Fig. 3). In comparison with the initial material, a significant increase was observed on day 9 and then on day 14. After this, few differences in FFA concentrations were observed, the highest mean value being at day 26 (19.4 g kg^{-1} lipids). A good correlation value with chilling time was obtained ($r^2 = 0.96$; Table 3). With respect to other previously studied chilled fish species, such as sardine (Aubourg, Sotelo, & Gallardo, 1997), horse mackerel (Aubourg, 2001), and blue whiting (Aubourg et al., 1998a), the initial FFA content was lower, and lipid hydrolysis developed at a slower rate in the fish species studied here.

Peroxide formation in chilled turbot, proved to be very slow during chilled storage, as can be observed in Fig. 4. Thus, the concentration of peroxides was in all cases below 4.00 meq kg^{-1} lipids; a fair correlation with time was obtained ($r^2 = 0.83$; Table 3). This finding also suggests that lipid oxidation mechanisms may occur at a lower rate in turbot than in other fish species, such as

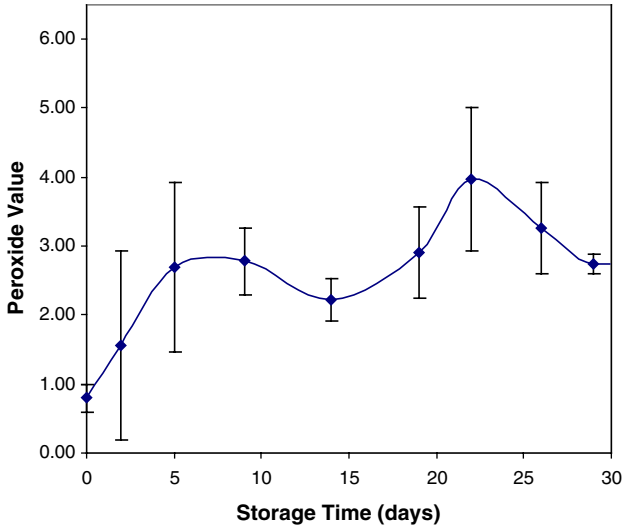

Fig. 4. Evolution of peroxide value in farmed turbot muscle during chilled storage. Results are expressed as meq active oxygen kg^{-1} lipids.

blue whiting (Aubourg et al., 1998a) or herring (Undeland, Hall, & Lingnert, 1999).

The investigation of the formation of fluorescent compounds in turbot during storage revealed no significant differences until day 14 (Fig. 5). After this time, a sharp increase was detected, the FR value rising from 1.4 to 6.2, which was followed by no significant variations. A fair correlation with the chilling time was obtained ($r^2 = 0.87$; Table 3).

3.5. Protein electrophoretic analysis

Fig. 6 compiles the results obtained in the electrophoretic analysis of sarcoplasmic protein extracts during storage. In general terms, no major change in the

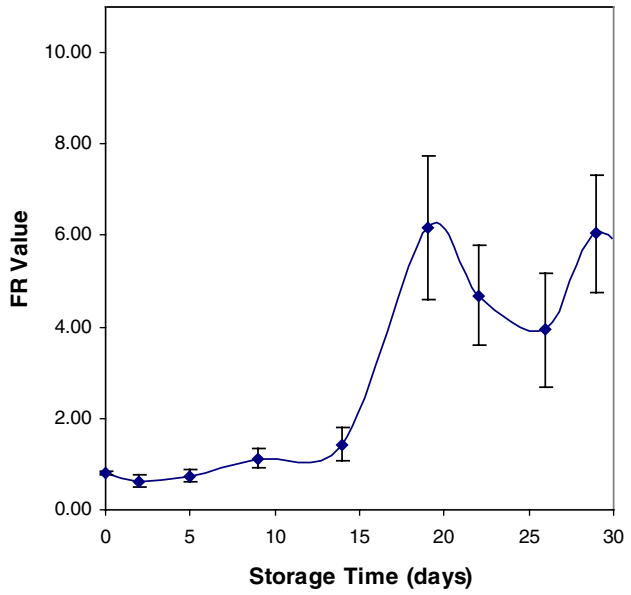

Fig. 5. Evolution of interaction compound formation in farmed turbot muscle during chilled storage. The FR value was calculated as described in the text.

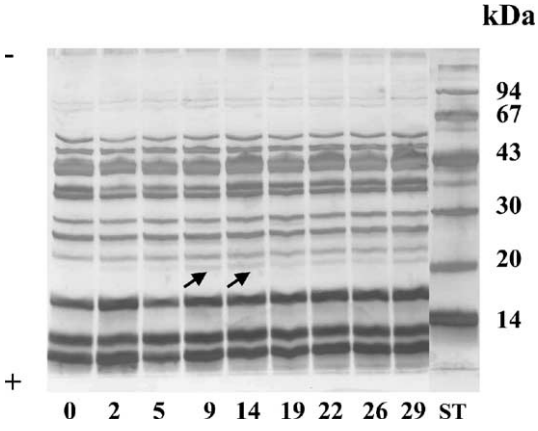

Fig. 6. Electrophoretic profiles of the proteins extracted from farmed turbot muscle with buffer B during chilled storage. The numbers bellow the lanes indicate the days of chilled storage. ST: MW protein standard. The cathodic (−) and anodic (+) sides are indicated. Arrows indicate the new appeared band.

protein profiles was observed, even after advanced chilled storage. However, minor changes, such as the appearance of a protein band of ca. 22 kDa was observed from day 2 of chilled storage. The concentration of this polypeptide seemed to increase at days 9 and 14 and was present until the end of the chilled storage. It should be stressed that this polypeptide had not been observed in the initial protein extract on day 0, even after using the extremely sensitive silver staining protocol.

Several works addressing the application of electrophoretic tools for monitoring the changes that occur in seafood proteins as a consequence of *post-mortem* changes and different storage methods have been undertaken (Jessen, 1996; Martinez, Ofstad, & Olsen, 1990; Papa, Alvarez, Vérrez-Bagnis, Fleurence, & Benyamin, 1996; Papa et al., 1997; Vérrez-Bagnis, Ladrat, Morzel, Noël, & Fleurence, 2001; Vérrez-Bagnis, Noël, Sautereau, & Fleurence, 1999). Thus, Kjærsgård and Jessen (2002) employed electrophoretic tools to follow up the protein changes occurring in iced cod. More recently, other authors have investigated the *post-mortem* protein changes occurring in sea bass by analysing electrophoretic profiles (Vérrez-Bagnis et al., 2001). These authors reported very slight changes in the high-molecular-mass protein fraction (>100 kDa) in total protein extracts, and a more interesting change in the concentration of a 16 kDa protein from a protein extract prepared in a low-ionic-strength buffer. In our study, the latter type of extract was also found to afford differences in the concentration of the 22 kDa polypeptide. Other authors have also reported that changes in cytoskeletal proteins from fish may affect proteins of very high MW, such as nebulin and titin, with MWs considerably above 600 kDa and sometimes even above 2500 kDa, although such changes would be difficult to appraise with conventional electrophoretic tools due to the high MW of such proteins (Estrade, Vignon, Rock, & Monin, 1993; Vérrez-Bagnis et al., 2001). Likewise, other works focussing on the investigation of *post-mortem* protein degradation in porcine and bovine muscle have also failed to detect changes in structural proteins, such as titin, nebulin, desmin, filamin and vinculin, probably because these proteins would be cleaved by complex and non-specific mechanisms, resulting in a complex electrophoretic pattern, characterised by the presence of small amounts of a high number of polypeptides (Estrade et al., 1993; Görg et al., 2000; Lametsch & Bendixen, 2001). By contrast, a study describing the proteolytic breakdown of low MW-metabolic proteins, such as glycogen phosphorylase, creatine kinase, pyruvate kinase and myokinase, among others, belonging to the sarcoplasmic protein fraction, and underlining their potential use as markers of proteolytic activity, has been recently reported (Lametsch & Bendixen, 2001).

In summary, and also according to the microbiological parameters evaluated for the same species (Rodríguez, Barros-Velázquez, Ojea, Piñeiro, & Aubourg, 2003), farmed turbot has shown slow and gradual biochemical changes and long shelf life and good quality times (19 and 14 days, respectively) that should be very profitable when commercialisation is carried out in places distant from production farms. Nucleotide determination proved to be a valuable tool for measuring the loss of freshness during the eatable time range (days 0–19). FFAs and peroxide formation were particularly low. Most biochemical indices showed very good correlation values with chilled time ($r^2 = 0.94$–0.97), except for lipid oxidation parameters ($r^2 = 0.83$–0.87). Moreover, although the electrophoretic protein

profiles of turbot remained unchanged during chilled storage, the appearance of a 22 kDa polypeptide, whose nature, level of expression and composition are currently being investigated at our laboratory, may be of applied interest for monitoring the degradation mechanisms that occur in the muscle of farmed turbot.

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