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Full Length Research Paper

Biochemical and textural properties of frozen stored (-22˚C) gilthead seabream (*Sparus aurata*) fillets

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Skinned, vacuum packed post-rigor gilthead seabream (Sparus aurata) fillets were stored frozen at -22°C for up to 340 days. Sampling was carried out on fresh fillets at days 34, 91, 183, 266 and 340 of frozen storage. Tests related to muscle integrity (activity of α-glucosidase and the protein content of centrifugal tissue fluids), myofibrillar protein denaturation (Ca^{2+}- and Mg^{2+}-ATPase activities in actomyosin extracts) and lipid degradation products (free fatty acids, peroxide values and thiobarbituric reactive substances) showed that storage time affected the integrity of muscles, and caused structural changes to myosin (or 'actomyosin') and hydrolysis and oxidation of lipids. A slight decrease in salt soluble proteins was observed after 266 days of frozen storage suggesting that storage time hardly affected the formation of aggregates. The water holding capacity of the stored frozen fillets decreased with storage time and was associated with the damage in muscle structures (protein content in centrifugal tissue fluids), denaturation of myofibrillar proteins and lipid degradation products (free fatty acids and peroxide value). The firmness and toughness of the frozen fillets, as measured by the Warner-Bratzler shear knife, increased slightly with storage time. The changes in toughness were associated with the state of myofibrillar proteins and changes in water holding capacity of the stored frozen fillets.

Key words: Gilthead seabream, fillets, frozen storage, quality.

INTRODUCTION

Frozen storage is the only large scale preservation method that facilitates exports and minimizes variations in supply of raw fish (Persson and Londahl, 1993). Frozen storage is, therefore, an important method for processing of fish. However, when seafoods are frozen and stored in frozen state they inevitably lose quality (Mackie, 1993). Loss in quality of frozen stored fish is mainly due to changes in muscle integrity, proteins and lipids (Shenouda, 1980).

Formation of inter-cellular ice and accretion of intra-cellular ice, that is, re-crystallization of ice, in a rapidly frozen muscle are almost inevitable during storage at commercially storage temperatures (Hamm, 1986). Re-crystallization of ice reduces the number of free water molecules in frozen stored fish muscle, which may cause changes in structural elements and mechanical damage to cell membranes (Huber et al., 1979). Therefore, frozen storage may cause lysis of organelles, such as mitochondria and lysosomes, and disintegration of membranes, resulting in a loose, disorganised fish muscle structure (Karvinen et al., 1982). The damage of fish muscle during frozen storage can be studied by the activities of enzymes in muscle tissue fluids, enzymes that in fresh tissue are retained in sub-cellular organelles. The enzyme α-glucosidase is not specifically associated with any particular sub-cellular organelle, but the activity of this enzyme under acid assay conditions is regarded as evidence of lysosomal activity, that is, cell damage (Barrett and Heath, 1977). The activity of α-glucosidase in fluids obtained by centrifugation, 'centrifugal tissue fluids', and the protein content of the fluids have been found to correlate with the cellular damage caused by the length of time a frozen fish remains in frozen storage

Abbreviations: TBARS, Thiobarbituric acid reactive substances; **CTF**, centrifugal tissue fluid; **AG**, α-glucosidase; **SH**, sulfhydryl groups; **EGTA, N,N,N´,N´** -tetraacetic acid; **TCA**, trichloroacetic acid.

(Nilsson and Ekstrand, 1995; Benjakul et al., 2003).

The alterations in texture and water holding capacity of frozen fish muscle after thawing are the most obvious effects of frozen storage, which have been associated with changes in myofibrillar proteins (Haard, 1992). The changes in myofibrillar proteins can be detected in the form of reduced solubility and extractability in saline and other extracting solutions, and also reductions in myosin and actomyosin ATP-ase activities, sulfhydryl groups, apparent viscosity, gel-forming ability e.t.c (Shenouda, 1980)

Cellular disintegration during frozen storage can cause hydrolysis of lipids to free fatty acids (Shewfelt, 1981). Accumulation of free fatty acids in frozen fish is related to some extent with lack of acceptability, because free fatty acids are known to undergo oxidation and cause texture deterioration by interacting with proteins (Mackie, 1993).

The changes in fish muscle fibres, proteins, lipids and textural properties during frozen storage have been studied for several decades because of their economic importance (Shenouda, 1980; Shewfelt, 1981; Haard, 1992; Mackie, 1993). However, these changes can be influenced by various factors, for instance fish species, freezing and storage conditions (Haard, 1992). Consequently, information about the freeze-induced alterations and stability in storage of species coming either from fisheries or aquaculture is relevant for commercially important species.

Gilthead seabream (*Sparus aurata*) is one of the most important fish species farmed in the Mediterranean region. It is much prized as a food and is usually marketed fresh on ice. The gilthead seabream aquaculture industry has grown strongly in the last decade, from 33,000 tonnes in 1996 to 86,700 tonnes in 2006 (FAO: www. fao.org; accessed in June 2007), with Greece producing 50% of the global production of this species (FAO: www. globefish.org; accessed in June 2007). Nevertheless, the increased supply of gilthead seabream caused prices to decline by more than two-thirds between 1990 and 2002 (FAO: www. fao.org; accessed in June 2007). In certain periods of the year (from June to January), there is plentiful supply of fresh fish in market, which causes a further decline of the prices (FAO: www. globefish.org; accessed in June 2007). In order, therefore to regulate the market, there is a need to look for new ways of processing gilthead seabream. Freezing of fish is an important consideration in this regard. Some recent studies have shown that frozen storage can be a commercial alternative for marketing of whole gilthead seabream (Pastor et al., 1999; Tejada et al., 2003; Huidobro and Tejada, 2004). However, there is hardly any information available about the effects of the length of time in frozen storage on the quality of gilthead seabream fillets. It would be extremely useful to inform industry how gilthead seabream fillets perform in frozen storage, since individually frozen fish fillets present simplicity in use and thereby they are preferred by consumers compared to whole frozen fish.

Therefore, the present study, aimed to investigate the effects of the length of time in frozen storage on the quality of gilthead seabream fillets in regard to the integrity of muscle structure, myofibrillar protein denaturation and aggregation, lipid degradation and texture changes.

MATERIALS AND METHODS

Gilthead seabream fillets processing and storage

A total number of 30 gilthead seabreams (*Sparus aurata*; average weight and length 568 ± 38 g and 32.1 ± 0.89 cm (average ± S.D.), respectively) were obtained from a commercial cage culture unit located in Western Greece. Fish were fasted for two days prior to harvesting and were slaughtered by immersion in ice-cold water (hypothermia). They were packed into an insulated polystyrene container with flaked ice and delivered to the Technological Educational Institute of Messolonghi on the same day of their harvesting. At the laboratory, the whole fish were packed individually in polyethylene bags and buried in ice in a polystyrene container provided with holes for drainage. The container was then stored in a refrigerator chamber for three days from harvesting for resolution of rigor mortis. The ice was renewed daily. At the end of storage period, the fish were filleted and skinned by hand. Subsequently, each fillet was weighed, placed between two aluminium foil sheets in a polyethylene food bag and vacuum packed. No washing was applied prior to freezing in order to avoid any variability due to absorbed or melted water. The mean weight of the skinned fillets was 86 ± 10 g (average ± S.D.).

A total number of twenty five pairs of matched fillets were frozen in lots of five fillets in a -80°C ultra-freezer until the thermal centre of the fillets reached -20°C, for 28 min. During freezing the temperature of the thermal centre of one fillet was recorded as described previously (Makri et al., 2007). Immediately after freezing, the fillets were stored in a freezer cabinet with working temperature -22°C for 340 days. The temperature in frozen storage was monitored every hour with a K type thermocouple and a recording thermometer. Sampling was carried out on fresh fillets and at days 34, 91, 183, 266, 340 of frozen storage. On each sampling day, five matched pairs of fillets (ten fillets) were thawed in a refrigerator chamber at 4 to 7°C overnight (12 h). The thawed fillets were re-weighed for thaw loss determinations and divided into two groups of five fillets. In each group, there were fillets coming from five different fish. The fillets of the first group were sliced in three equal sized portions as follows: a) the anterior portion, which was used for extraction of lipids and thiobarbituric acid reactive substances (TBARS) determinations b) the middle portion, which was used for salt soluble protein determinations and extraction of actomyosin, and related measurements. The anterior and the middle portions of the fillets were wrapped in aluminium foil, vacuum packed in polyethylene bags and stored at -80°C until they were thawed (20 min at 25°C) for analysis. The third portion (c) was the tail portion, which was used immediately after thawing of the fillets for the preparation of centrifugal tissue fluid (CTF). The CTF were stored at -80°C until analysis.

The fillets of the second group were stored whole at -80°C until they were used for textural determinations.

All chemical and biochemical analyses were performed using the white muscle of the fillets. The results were the means of five independent determinations.

Proximate analyses

Proximate analyses were performed using the white muscle of the fresh fillets. Water content was measured following the method of (AOAC, 1997). The ash content was obtained by heating the residue from the moisture determination in a furnace at 550°C for

24 h. Crude protein was analyzed by the Kjeldahl method (AOAC, 1997). Crude lipid was measured by the method of (Bligh and Dyer, 1959)

Determinations of the muscle damage parameters

Centrifugal tissue fluids (CTF) were prepared according to the method of Nilsson and Ekstrand (1993) using 5 to 7 g of chopped muscle. The CTF was determined by weighing the sample before and after centrifugation. The results were expressed as g of fluid released per kg of tissue.

The protein content in the centrifugal tissue fluid was measured by the bicinchoninic acid (BCA) procedure (Sigma procedure no. TPRO-562). Bovine serum albumin was used as standard. The results were expressed as g of protein in CTF released per kg of tissue.

α-Glucosidase (AG; enzyme class EC 3.2.1.20) was measured spectrophotometrically using p-nitrophenyl-α-glucopyranoside as substrate. The assay were performed according to Nilsson and Ekstrand (1993) and activity was calculated according to Benjakul and Bauer (2000). Results were expressed as milliunits per g of tissue.

Total and surface (reactive) sulfhydryl determinations in atomyosin

The determination of total and surface (reactive) sulfhydryl (SH) contents in actomyosin was carried out according to the titration method of Ramirez et al. (2000). Actomyosin was prepared according to the method of MacDonald and Lanier (1994) using 4 g of chopped muscle. The numbers of SH groups in actomyosin were calculated using a molar extinction coefficient of 13600 M^{-1} $cm^{-1}$and expressed as SH moles per 5 x 10^5 g protein (Ellman, 1959).

ATPase activities in actomyosin extracts

ATPase activities in actomyosin extracts were carried out at 0.06 M KCl and pH 7.0 (25 mM Tris maleate) according to Herrera (1993). The substrate for the reactions was 1 mM ATP. The remaining conditions were: 5 mM calcium chloride and 0.5 mg protein/ml for Ca^{2+}-ATPase; 1 mM magnesium chloride and 0.2 mg protein/ml for Mg^{2+}-ATPase. 1 mM magnesium chloride, 0.5 mM N,N,N´,N´ -tetraacetic acid (EGTA) and 0.7 mg protein/ml for Mg^{2+}-EGTA-ATPase. Samples were incubated for 3 min at 25℃ and the reaction was stopped by adding 5 ml of 15 % (w/v) chilled trichloroacetic acid (TCA) solution to 10 ml of the reaction mixture. The reaction mixtures were centrifuged at 28,000 g for 20 min at 4℃. The amount of inorganic phosphorus liberated was determined according to Fiske and Subbarow (1925). Blank measurements were conducted by adding the chilled TCA solution prior to the addition of ATP. Specific activity was expressed as µmol inorganic phosphate (P_i) released per mg protein per minute.

Salt-soluble protein

The extracts for the salt-soluble protein determinations were prepared according to Shiku et al. (2004) using 2 g of chopped muscle and 40 ml of 0.6 M KCl (pH 7.0). Protein measurements in extracts and muscles were conducted by the Kjeldahl method (AOAC, 1997). The results were expressed as the ratio of salt soluble protein to total protein.

Lipid damage measurements

Total free fatty acid (FFA) determinations were performed on the extracted crude lipids according to Lowry and Tinsley (1976). Results were expressed as g of FFA per kg of lipid.

The estimation of peroxides(peroxide value; PV) in gilthead seabream crude lipids was determined by the ferric thiocyanate method described by Chapman and Mackay (1949). Results were expressed as meq oxygen per kg of lipid.

The thiobarbituric acid reactive substances (TBARS, mg malondialdehhyde per kg of sample) was determined according to Lemon (1975).

Determinations of the water holding capacity parameters

Thawing weight losses were determined by weighing gilthead seabream fillets before freezing and after thawing. Thawing loss was expressed as gram of weight loss per kg of initial weights.

The CTFs prepared as is described previously were taken as measurements of expressible fluids of gilthead seabream fillets (Trout, 1988). Results were expressed as gram of fluid released per kg of gilthead seabream fillets prior to centrifugation.

Texture determination as measured by the texture analyzing system

Shear force measurements were performed on fresh and stored frozen fillets according to Morkore and Einen (2003). Three cylinders of standard diameter (15 mm) were excised from the middle part of the fillets close to the lateral line by using a cork borer. The height of the cylinders was arranged from 10 to 12 mm by using scissors. The individual cylinders were wrapped in aluminum foil and kept in a refrigerator chamber until measurement. All measurements were performed within half an hour from the preparation of the cylinders. During measurements, the core temperature of the cylinders was 6.2 ± 1.2℃.

The shear forces of the cylinders were measured using a Texture Analyzer TA-XTi plus (Stable Micro Systems Ltd., Surry, U.K.) with a V- shaped Warner - Bratzler shear knife attached to a 5 kg load cell.

Individual cylinders were inserted through the triangular opening of the blade and placed on the instrument's heavy duty platform in such a position that the muscle fibres were parallel to blade penetration.

The knife approached the sample at a speed of 2 mm s^{-1} and cut it with a speed of 2 mm s^{-1} to a distance of 30 mm. Then the knife returned to the start position at a speed of 10 mm s^{-1}. The trigger force, that is, the force required for the texture analyzer to initiate capturing data, was 0.20 N. The force-time graphs were recorded by a computer and analyzed using the Texture Exponent software (version 32, Stable Micro Systems Ltd., Surry, U.K.).

Parameters recorded from the force–time graphs were the maximum force and the area under the time-force curve.

Statistical analyses

One-way analyses of variance (ANOVA) or 't-test' with Welch's correction (Zar, 1984) were performed to test for the effects of the length of time of storage at -22℃ on chemical, biochemical, physical and textural parameters measured.

Spearman's rank correlation coefficients (r_s) were calculated in order to examine the possibility of linear relationships between the parameters. Data were subjected to non-parametric correlation analyses in order to avoid possible unjustified assumptions on the distributions of parameters as being normally distributed (Zar, 1984) Simple linear regressions were performed when required.

ANOVAs showing significant differences were followed by a Tukey honestly significant difference. Significance was accepted when $P<0.05$ (Zar, 1984).

Table 1. Effect of length of time of storage at -22˚C on protein content in centrifugal tissue fluids and α-glucosidase activities*.

Storage time (days)	Protein content (g kg^{-1})	AG activity (mU g^{-1})
0	14.7 ± 0.67 d	0.421 ± 0.049 d
34	23.3 ± 0.69 bc	1.292 ± 0.151 c
91	21.5 ± 0.65 c	1.547 ± 0.198 bc
183	24.6 ± 0.46 bc	2.228 ± 0.157 a
266	28.1 ± 0.91 a	2.048 ± 0.190 ab
340	25.0 ± 0.56 b	1.804 ± 0.112 abc

Means ± S.E.M., n = 5.
Different letters (a, b, c, d) in the same column denote a significant difference (P<0.05).
The '0' storage time represents fresh gilthead seabream fillets.

RESULTS AND DISCUSSION

Proximate composition

The mean values of water, crude protein, crude lipid and ash of fresh fillets were 730.8 ± 5.79, 228.0 ± 3.76, 27.2 ± 2.91 and 15.4 ± 0.53 g kg^{-1} (means ± S.D.), respectively. Kyrana et al. (1997) report mean values of water, crude protein, crude lipid and ash of fresh whole skinless gilthead seabream fillets, coming from diverse Greek fish farm units, from 703 to 753.3, 219 to 233, 32.6 to 73.8 and 13.0 to 14.8 g kg^{-1} of tissue, respectively. Therefore, the results of the water content, crude protein and ash content of fresh gilthead seabream fillets of the present study are similar to those reported by Kyrana et al. (1997). The discrepancy in the lipid content of fresh fillets between the study by Kyrana et al. (1997) and the present one may be related to the differences in samples that were analyzed, that is, white muscle in the present study as opposed to whole muscle in their study. It is known that the white muscle of fish contains less lipids than the red muscle (Shewfelt, 1981).

Effect of storage time on muscle integrity

The protein content in centrifugal tissue fluids from frozen fillets after 34 and 340 days in frozen storage was 1.6 and 1.7 times compared to the values of fresh fillets, respectively. Maximum values of protein content in centrifugal tissue fluids from frozen fillets were found after 266 days in frozen storage, which were 1.9 times compared to the respective values of fresh samples (Table 1). In addition, from previous experiments of the effects of freezing times on the quality parameters of gilthead seabream fillets, the weight of proteins in centrifugal tissue fluids from fillets frozen at a freezing time of 27.2 min and immediately thawed increased about 1.2 times compared to the respective values of fresh samples (Makri et al., 2007). Therefore, the results of the present study may indicate the release of intra-cellular fluids and/or the presence of cell fragments in exudates of frozen gilthead seabream fillets as consequence of the length of time of storage at -22°C (Love, 1966).

AG activities in the frozen gilthead seabream fillets increased 3.1 and 4.3 fold compared to the activities of fresh samples after 34 and 340 days in frozen storage, respectively (Table 1). Maximum AG activities in the muscle of frozen gilthead seabream fillets were observed after 183 days of frozen storage, and these were 5.3 and 1.7 more compared to the activities in fresh samples and those in the stored frozen fillets for 34 days at -22ºC, respectively (Table 1). Also, a previous study suggested that the activity of the AG enzyme in the frozen gilthead seabream fillets at a freezing time of 27.2 min and immediately thawed increased by about 3.2 fold compared to the activity of the fresh fillets (Makri et al., 2007). Thus, the results of the present study and those by Makri et al. (2007) may indicate the release of the enzyme from lysosomes into the gilthead seabream muscles as consequence of the length of time the frozen fillets remained in storage at -22ºC. In addition, the changes in the release of the AG enzyme from lysosomes found after 34 days of frozen storage of fillets may have resulted from the freezing process itself. After 183 days of storage of frozen fillets, a decrease in AG activities were observed up to 340 days of storage (Table 1). This result implies the denaturation of the enzyme during prolonged storage of frozen fillets (Benjakul et al., 2003).

Altogether, the results of this section may suggest that the length of time of storage at -22°C affected the integrity of cell-membrane structures of stored frozen gilthead seabream fillets. Formation of inter-cellular ice and accretion of intra-cellular ice during prolonged storage at -22°C, that is, after 183 days (almost 6 months), of the frozen fillets could have damaged the cell structures by denaturating the cellular wall proteins due to concentration of salts and/or by disrupting the cells due to enlargement of intra-cellular ice crystals (Love, 1966).

Table 2. Effect of length of time of storage at -22°C on total and surface (reactive) sulfhydryl groups (SH moles/5 x 10^5 g actomyosin) in actomyosin from frozen gilthead seabream fillets*.

Storage time (days)	Total sulfhydryl Groups	Surface (reactive) sulfhyhdryl groups
0	37.9 ± 0.35 a	32.8 ± 0.33 a
34	34.9 ± 1.08 bc	31.0 ± 1.33 ab
91	33.9 ± 0.30 c	30.1 ± 0.35 b
183	34.8 ± 0.81 bc	30.9 ± 0.32 b
266	35.9 ± 0.39 b	32.2 ± 0.30 a
340	36.0 ± 1.63 abc	31.8 ± 1.28 ab

*Means ± SEM, n = 5.
Different letters (a, b, c) in the same column denote a significant difference (P<0.05).
The '0' storage time presents fresh gilthead seabream fillets.

Effects of storage time on total and surface (reactive) SH groups

The results of the present study showed that the content of total and surface (reactive) SH groups in actomyosin extracts from stored frozen gilthead seabream fillets decreased during the first 91 and 183 days of storage respectively; thereafter, an increasing trend was observed towards the end of storage period in the number of total and surface (reactive) sulfhydryl groups (Table 2).

Suarez et al. (2002) found a significant decrease in total sulfhydryl groups from frozen sardines stored for 4 months (almost 120 days) at -20°C followed by an increase during the next two months (almost 60 days) of storage. In contrast to these findings, Ramirez et al. (2000) showed that total and surface (reactive) sulfhydryl groups of frozen tilapia actomyosin in 0.6 M KCl decreased during storage for 10 days at -20°C. Benjakul et al. (2003) found a continuous decrease in total sulfhydryl groups with concomitant increase in disulfide bond formation in frozen lizardfish, croaker, threadfin bream and bigeye snapper during storage for 24 weeks (almost 168 days) at -18°C. Therefore, the findings of the present study are partially in agreement with those recorded in literature for the changes in sulfhydryl groups in actomyosin extracts from different fish and fishery products. This may be related to differences in species studied and to the length of time of storage applied, that is, almost one year in the present study as opposed to maximum half year in the studies by Ramirez et al. (2000) and Benjakul et al. (2003).

During frozen storage of fish, sulfhydryl groups of myosin (or 'actomyosin') molecules are prone to oxidation and are involved in sulfhydryl-disulfide inter-change reactions contributing to denaturation and formation of large molecular weight aggregates (Buttkus, 1971). In addition, conformational changes take place in proteins during frozen storage and cause continuous exposure and burial of sulfhydryl groups (Herrera and Mackie, 2004). Accordingly, in the gilthead seabream fillets stored frozen for 91 to 183 days, oxidation of SH groups may have been faster than protein unfolding which leads to exposure of natively buried SH groups, so that the number of surface (reactive) SH groups decreased with a concomitant decrease in the number of total SH groups in actomyosin extracts from stored frozen fillets. Similar suggestions are reported by Herrera and Mackie (2004) after studying the effects of storage time and cryoprotectants on frozen trout actomyosin in 0.6 M KCl.

Total sulfhydryl groups in actomyosin from stored frozen gilthead seabream fillets were expected to decrease further towards the end of storage period, when a further reduction in Ca^{2+}-ATPase activities and a decrease in salt soluble proteins were observed in extracts from the stored frozen gilthead seabream fillets (see the following sections). However, aggregation and denaturation of proteins due to disulfide bond formation may take place without a net change in the total sulfhydryl groups, by sulfhydryl – disulfide exchange reactions, as Buttkus (1970) showed for stored frozen myosin from trout. Additionally, Badii and Howell (2001) have indicated that non-covalent hydrogen bonding, electrostatic and hydrophobic interactions contributed to a higher extent than disulfide bonds in aggregation of myofibrillar proteins from frozen cod and haddock fillets stored for 65 weeks (almost 455 days) at -30°C. In addition, surface (reactive) SHs in actomyosin extracts of frozen gilthead seabream fillets increased after 266 days of storage probably showing unfolding of the protein molecule (Ko et al., 2006). By means of surface hydrophobicity, Benjakul et al. (2003) showed that storage for 24 weeks (168 days) at -18°C caused significant unfolding of actomyosin extracted from frozen threadfin bream and bigeye snapper. It is, thus, likely that in the present study unfolding of myosin molecules during prolonged storage of frozen seabream fillets took place. This unfolding would favour the formation of inter-molecular secondary forces, which would contribute to aggregation of myofibrillar proteins to a higher extent than the disulfide bonds do.

Therefore, the decrease in sulfhydryl groups of actomyosin may indicate that disulfide bonds were formed during early periods of frozen storage of gilthead seabream fillets. These bonds could have been developed further during the subsequent periods of storage without a decrease in the total sulfhydryl groups by sulfhydryl-disulfide exchange reactions contributing with secondary forces to formation of aggregates and loss in salt soluble proteins.

Effects of storage time on ATPase activities in actomyosin extracts

Ca^{2+} and Mg^{2+}-ATPase of actomyosin extracts from gilthead seabream fillets decreased during the 340 days of frozen storage (P<0.01; Table 3). Changes in Ca^{2+}-ATPase activities have been recorded in a number of fish and fishery products. This is the case with stored frozen

Table 3. Effect of length of time of storage at -22°C on ATPase activities (µmoles pi/ mg protein/min) of actomyosin from frozen gilthead seabream fillets*.

Storage time (days)	Ca^{2+}-ATPase	Mg^{2+}-ATPase	Mg^{2+}-EGTA-ATPase
0	0.204 ± 0.014 a	0.166 ± 0.018 a	0.030 ± 0.001 a
34	0.165 ± 0.007 ab	0.157 ± 0.021 ab	0.042 ± 0.005 a
91	0.143 ± 0.007 bc	0.105 ± 0.011 bc	0.032 ± 0.002 a
183	0.143 ± 0.006 bc	0.105 ± 0.004 bc	0.031 ± 0.001 a
266	0.117 ± 0.008 cd	0.102 ± 0.008 bc	0.033 ± 0.003 a
340	0.106 ± 0.012 d	0.097 ± 0.014 c	0.031 ± 0.003 a

Means ± SEM, n = 5.
Different letters (a, b, c, d) in the same column denote a significant difference (P<0.05). The '0' storage time presents fresh gilthead seabream fillets.

mackerel and Atlantic mackerel (Saeed and Howell, 2002), croacker, lizardfish, threadfish and bigeye snapper (Benjakul et al., 2003). Decreases in Ca^{2+}-ATPase and Mg^{2+}-ATPase activities were observed in frozen trout actomyosin stored at -20°C for 8 weeks (56 days) (Herrera and Mackie, 2004). Leelapongwattana et al. (2005) found a continuous decrease in Ca^{2+}-ATPase activities in actomyosin extracted from whole and fillets of frozen lizardfish stored at -20°C for 24 weeks (6 months). Jarsa et al. (2006) showed that Ca^{2+}-ATPase activities decreased in frozen carp stored for 24 months (almost 720 days) at -20 and -30°C and the degree of changes was dependent on the storage temperature. Paredi et al. (2006) found a continuous decrease in Ca^{2+} and Mg^{2+}-ATPase activities extracted from frozen male and female squids during storage for 9 months (almost 270 days) at -30°C. Pastor et al. (1999) found that Ca^{2+}-ATPase activities in actomyosin extracted from frozen gutted gilthead seabreams stored at -20°C for 10 months (almost 300 days) did not change with storage time; however when un-gutted frozen gilthead seabreams were kept at -20°C for 10 months a continuous decrease in Ca^{2+}-ATPase activities in actomyosin extracts was observed.

Therefore, the results of the present study and those of Pastor et al. (1999) imply that pre-freezing treatments may affect the integrity and functionality of myosin (or 'actomyosin') from stored frozen gilthead seabream. In general, filleting operations can affect the quality of frozen fish through the activation of enzymic systems of fillets as compared to those of the whole fish (Ciarlo et al., 1985; Simeonidou et al., 1997).

In the present study, the decrease in ATPase activities in actomyosin from stored frozen gilthead seabream fillets suggests denaturation of myosin or ('actomyosin'), especially in the head region, which contains the active site of the enzyme and the actin interaction site. The denaturation of myosin (or 'actomyosin') might have been caused by the increased concentration of salts in the unfrozen phase of fillets leading to unfolding of protein (Benjakul et al., 2003) and/or aggregation of myosin's subunits (Shenouda, 1980). Furthermore, the decreases in ATPase activities in actomyosin extracts from frozen fillets stored for 34 and 91 days of storage were somewhat coincidental with the decrease in total and surface sulfhydryl groups. This may imply that oxidation of sulfhydryls, located in the head region of myosin molecule, contributed to loss in ATPase activities during at least the first periods of storage of frozen gilthead seabream fillets. Similar suggestions are recorded for other stored frozen fish species and products (Jiang et al., 1988ab; Ramirez et al., 2000; Benjakul et al., 2003; 2005). In addition, hydrophobic effects of free fatty acids on proteins and interaction of oxidized lipids with functional groups of proteins could have contributed to denaturation of myosin (or 'actomyosin') of stored frozen gilthead seabream fillets (Shenouda, 1980; Mackie, 1993). These assumptions are supported by the negative and significant relationships between Ca^{2+}-ATPase activities and free fatty acids (r_s = -0.812, P<0.01) peroxide values (r_s = -0.560, P<0.01) and TBARS (r_s = -0.569, P<0.01) found in the present study.

No significant changes in Mg^{2+}-EGTA-ATPase activities in actomyosin extracts from fresh and stored frozen fillets were observed (P>0.05; Table 3). This may imply that frozen storage did not induce changes in the troponin-tropomyosin complex in the stored frozen gilthead seabream fillets. By means of Mg^{2+}-EGTA-ATPase activities, Benjakul et al. (2003) showed that storage for up to 6 months (almost 180 days) at -18°C induced changes in the troponin-tropomyosin complex of several tropical fish species, and the degree of changes depended on the species. However, the changes in myofibrillar ATPase activities during frozen storage of fish may vary depending on the species (Nambudiri and Gopakumar, 1992). Thus, the discrepancy between the study by Benjakul et al. (2003) and the present one may be related mainly to differences in the species examined.

Therefore, frozen storage may have resulted in denaturation of myosin (or 'actomyosin') of gilthead seabream fillets, as shown by the decrease in Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in actomyosin extracts, but it hardly affected the tropomyosin-troponin complex.

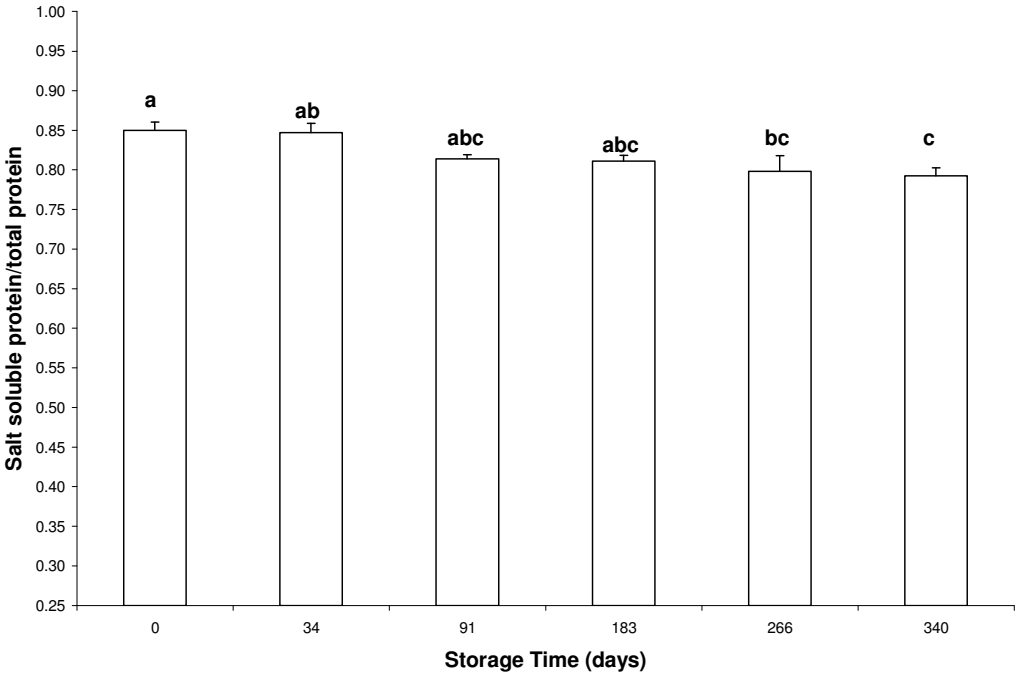

Figure 1. Effect of length of time of storage at -22°C on the ratio of salt soluble/total protein. Means ± SEM, n = 5. Groups with different letters (a, b, c) are significantly different (P<0.05). The '0' storage time presents fresh gilthead seabream fillets

Effect of storage time on salt- soluble proteins

The solubility of proteins in salt solutions from the frozen gilthead seabream fillets was initially high and remained almost stable until 183 days of storage; after 266 days (almost 8.5 months) of frozen storage a small but significant decrease in salt soluble proteins was observed with respect to fresh fillets (P<0.05; Figure 1). Several investigations have reported a reduction in solubility of proteins in saline solutions during storage of frozen fish. This is the case with cod and haddock (Badii and Howell, 2001; 2002), sardines (Suarez et al., 2002), Atlantic mackerel (Saeed and Howell, 2002), lizardfish (Leelapongwattana et al., 2005) and common carp (Ganesh et al., 2006). In addition, Huidobro and Tejada (2004) showed that the salt soluble proteins from frozen whole and gutted gilthead seabreams were significantly reduced after 349 days of storage at -20°C. The results of these investigations and those of the present study may imply that prolonged storage of the frozen gilthead seabream fillets caused aggregation of the myofibrillar proteins. However, the values of salt soluble proteins observed in the present study were within the same range as those reported by Tejada et al. (2003) and Huidobro and Tejada (2004) for the whole stored frozen seabream, and higher than those reported for all the other mentioned stored frozen fish.

Gilthead seabream is a fish species which does not produce formaldehyde during frozen storage (Tejada et al., 2003). Therefore, the aggregation of salt soluble proteins of stored frozen gilthead seabream fillets could have been caused mainly by ice crystals and/or interactions of proteins with lipid degradation products (Shenouda, 1980; Mackie, 1993). Accretion of intra- or inter-cellular ice and/or formation and accretion of inter-cellular ice may have caused an increase in the ionic strength of the liquid phase of stored frozen samples, and water migration from myofibrillar spaces of frozen gilthead seabream fillets. These changes may have resulted in disruption and/ or weakening of the forces that stabilise the native structure of proteins with concomitant unfolding of the molecules. Unfolded molecules of myofibrillar proteins could have then cross-linked with other proteins by secondary interactions and disulfide bridges to form insoluble protein-protein aggregates (Badii and Howell, 2001; Herrera and Mackie, 2004). These assumptions are supported by the changes in surface (reactive) sulfhydryl groups in actomyosin extracts from the frozen fillets particularly after 266 days of storage (discussed in the next section), and by the positive and significant relationship between the salt soluble proteins and surface (reactive) sulfhydryl groups found in the present study (r_s = 0.562, P<0.01).

The changes in salt soluble proteins from the stored frozen gilthead seabream fillets were to some extent coincidental with the changes in free fatty acids (r_s = -0.686, P<0.01), peroxide values (r_s = -0.518, P<0.01) and TBARS (r_s = -0.479, P<0.01) of the frozen fillets. Several

Table 4. Effect of length of time of storage at -22°C on lipid damage parameters*.

Storage time (days)	Total lipid content (g kg^{-1}tissue)	Free fatty acids (FFA) (g kg^{-1}lipid)	Peroxide value (PV) (meq 0_2 kg^{-1}lipid)	Thiobarbituric reactive substances (TBARS) (mg kg^{-1}tissue)
0	27.24 ± 1.30 a	6.36 ± 0.21 c	2.26 ± 0.25 b	0.092 ± 0.013 c
34	51.29 ± 6.24 b	7.72 ± 0.77 c	2.19 ± 0.22 b	0.165 ± 0.011 b
91	55.01 ± 5.36 b	12.97 ± 1.01b	2.34 ± 0.11 b	0.205 ± 0.027 ab
183	62.59 ± 1.31 b	13.79 ± 0.61b	4.25 ± 0.19 a	0.198 ± 0.034 a
266	64.30 ± 10.89 b	18.05 ± 1.93 b	4.05 ± 0.54 a	0.236 ± 0.026 a
340	46.49 ± 10.69b	25.35 ± 1.97 a	5.47 ± 0.50 a	0.159 ± 0.013 b

*Means ± SEM, n = 5.
Different letters (a, b, c) in the same column denote a significant difference (P<0.05).
The '0' storage time presents fresh gilthead seabream fillets.

Table 5. Effect of the length of time of storage at -22°C on water holding parameters of frozen gilthead seabream fillets*.

Storage time (days)	Thawing weight losses	Centrifugal tissue fluids	Total weight losses
0		121.0 ± 5.46 c	121.0 ± 5.46 c
34	19.7 ± 1.14 a	182.7 ± 4.54 b	202.4 ± 4.62 b
91	17.4 ± 1.93 a	173.8 ± 2.49 b	191.2 ± 3.34 b
183	25.2 ± 2.53 a	183.8 ± 2.92 b	209.0 ± 4.43 b
266	20.9 ± 2.40 a	207.5 ± 4.19 a	228.5 ± 2.93 a
340	21.9 ± 3.86 a	204.4 ± 2.73 a	226.5 ± 5.52 a

Means ± S.E.M, n = 5.
Different letters (a, b, c) in the same column denote a significant difference (P<0.05).
The '0' storage time represents fresh gilthead seabream fillets.

studies have suggested a relationship between aggregation of myofibrillar proteins and lipid degradation products in stored frozen fish and fishery products, including frozen minces of sardines stored for 150 days at -20ºC (Verma et al., 1995), frozen sardines stored for 12 weeks (almost 84 days) at -20ºC (Sarma et al., 2000), frozen minces of carp stored for 180 days at -18ºC (Siddaiah et al., 2001), frozen Atlantic mackerel stored for 12 months (almost 360 days) at -20 and -30°C (Saeed and Howell, 2002) and several tropical frozen fish stored for 6 months (i.e. almost 180 days) at -18°C (Benjakul et al., 2005). Altogether these results may imply that aggregation of salt soluble proteins from gilthead seabream fillets could have been caused, in part at least, by the interaction of myofibrillar proteins with free fatty acids and lipid oxidation products.

It can, therefore, be concluded that long term storage of gilthead seabream fillets at -22°C caused a small but significant decrease in the salt soluble proteins.

Effects of storage time on lipid damage parameters

The results of the present study show that the weight of total lipid extracted from the stored frozen fillets was significantly higher than that from fresh fillets (P<0.05; Table 4). Roldan et al. (2005), using a methanol-chloroform method to extract lipids from pre-spawned muscle of hake, recorded an increase of about 90% in total lipid extracted from muscles after freezing and attributed this change to denaturation of myofibrillar proteins, and to formation of exudates which caused dehydration of frozen muscle. These observations and the fact that the gilthead seabreams used in the present study were in pre-spawned stage (harvested in July) might explain the mentioned differences. Thereafter, no significant changes (P>0.05; Table 5) were observed in total lipid content of frozen gilthead seabreams stored for up to 340 days, in agreement with the findings of Aubourg et al. (1999) and Roldan et al. (2005) for stored frozen hake. As it will be shown later in this study, during frozen storage of gilthead seabream fillets, hydrolysis and oxidation of lipids occurred. The products of degradation of lipids might have interacted with protein molecules of stored frozen fillets, promoting denaturation of proteins (Shenouda, 1980; Mackie, 1993). Because of this, some decrease in total lipids extracted from frozen gilthead seabream fillets might be expected. However, free fatty acids bonded to myofibrillar proteins during frozen storage of gilthead seabream fillets can also be extracted

with the methanol-chloroform system, as Roldan et al. (2005) reported for the stored frozen hake fillets.

The mean value of free fatty acids from fresh fillets was 6.36 g per kg of extracted lipid, and this is similar to those recorded for fatty fish (horse mackerel and mackerel) (Aubourg et al., 2004; Stodolnik et al., 2005), but lower than those of lean fish species (hake and blue whiting) (Aubourg et al., 1999; Aubourg, 1999).

As is shown in Table 4, hydrolysis of lipids in frozen gilthead seabream fillets increased gradually during storage, so that a good linear correlation of free fatty acids with time was observed (R^2 = 0.840, P<0.0001). Similar observations have been recorded for other species. This is the case for whole hake, horse mackerel and mackerel stored frozen for 12 months (almost 360 days) at -20°C (Koning and Mol, 1991; Aubourg et al., 2004; Aubourg et al., 2005; Stodolnik et al., 2005). In addition, Sequeira-Munoz et al. (2005) showed an increase in the content of free fatty acids of lipids extracted from frozen carp fillets stored at -20°C for 75 days. Aranda et al. (2006), also, found that free fatty acids increased linearly with the length of time of storage at -18°C of frozen jack mackerel stored for 120 days (almost 4 months). These results may imply that lipolytic enzymes were active in the white muscle of frozen gilthead seabream throughout the storage period of 340 days.

The results of the present study show that peroxides in lipids extracted from fillets stored frozen for up to 91 days were similar. After that time, an almost progressive increase was observed till the end of storage (Table 4). As a consequence, a fairly good linear correlation of peroxide values with storage time was obtained (R^2 = 0.720, P<0.01). Similar results are recorded by Aubourg et al. (2004) and Aubourg et al. (2005) for frozen horse mackerel and mackerel fillets stored at -20°C for 12 months (almost 360 days), respectively. However, Aubourg et al. (2004) recorded a rate of increase in peroxide values much higher than that of the present study. This discrepancy may be related to differences in the species examined, for example, differences in the composition of free fatty acids (Nawar, 1985). Also, harvesting season may influence, also, the hydrolysis and oxidation of fish lipids, as Aubourg et al. (2005) showed for mackerel harvested at different seasons of the year and stored frozen at -20°C for one year. In addition, exclusion of oxygen could have reduced the rate of auto-oxidation of free fatty acids to peroxides in the vacuum packaged frozen gilthead seabream fillets (Brewer and Harbers, 1991).

Considering the changes in TBARS during storage of frozen gilthead seabream fillets, an increase until 266 days was observed (Table 4). After 340 days of frozen storage, a significant decrease in TBARS values was observed. This can be explained by the fact that thiobarbituric reactive substances are prone to interact with biological constituents present in fish muscle, leading to decrease in the TBARS detection in spite of the increasing fish damage (Aubourg, 1993). As a consequence, a significant but small linear correlation between TBARS and the length of time the frozen gilthead fillets remained stored at -22°C was found (r_s = 0.484, P<0.01). This result may imply that the TBARS index is not a reliable indicator for assessing the quality loss of the stored frozen seabream fillets, in agreement with the findings of other studies (Simeonidou et al., 1997; Aubourg et al., 1999 ; Aubourg, 1999; Ben-Gigirery et al., 1999; Namulema et al., 1999; Aubourg et al., 2004; Stodolnik et al., 2005).

Effects of storage time on water holding parameters

From the results of the present study, thawing losses of stored frozen gilthead seabream fillets were not affected by the length of time the fillets remained frozen in frozen storage (P>0.05; Table 5). In contrast, centrifugal tissue fluids and total weight losses due to thawing and centrifugation showed a significant increase after 266 days (almost 8.5 months) of frozen storage compared to those of fresh samples and stored frozen samples from 34 to 183 days (P<0.05; Table 5). Since an increase in the values of the expressible fluids indicates a decrease in the water holding capacity of a stored frozen muscle (Trout, 1988), the mentioned results suggest that the length of time of storage at -22ºC decreased the water holding capacity of the stored frozen gilthead seabream fillets. In addition, after 34 days of frozen storage total weight losses were about 67% more than those of fresh samples. From previous experiments of the effects of freezing times on the quality parameters of gilthead seabream fillets, the total weight losses of the frozen fillets at a freezing time of 27.2 min and immediately thawed were 46% more than those of fresh samples (Makri et al., 2007). Therefore, the changes in the water holding capacity parameters of the frozen fillets stored for 34 days at -22°C might have resulted from the freezing process and the length of time these fillets remained in cold storage. However, it seems that the freezing process itself exerted a more profound effect on the water holding capacity of the frozen fillets stored for 34 days, than did the storage time.

The water holding capacity has been found by other researchers to decrease in stored frozen fish. Simeonidou et al. (1997) showed that the expressible fluids from frozen whole and fillets of horse mackerel and Mediterranean hake stored at -18°C for 12 months (almost 360 days) were about 115% more than those of fresh samples. Benjakul et al. (2003) showed that expressible fluids increased, while the water holding capacity decreased, in several frozen tropical fish stored at -18°C for six months and the rate of the mentioned changes was dependent on the species. According, also, to the study of Benjakul et al. (2003), expressible fluids of the frozen fish at the end of the storage period were 215 to 537% more than those of fresh samples. Natseba et al. (2005) showed that expressible moisture increased, while

the water holding capacity decreased, in frozen Nile perch stored at -20°C for five months (almost 150 days). Ozbay et al. (2006) found an almost linear increase in expressible fluids, i.e. decrease in water holding capacity, of frozen fillets of salmon stored for 10 months (almost 300 days) at -20°C. Also, the values of the expressible fluids increased by about 7-fold in frozen salmon fillets stored for 10 months compared to those of fresh samples. Huidobro and Tejada (2004) found that the water holding capacity decreased slightly in frozen whole and gutted gilthead seabream stored at -20°C for almost one year (for 349 days).

Therefore, the results of the present study are in agreement with these other studies. However, the changes in the water holding parameters of the stored frozen gilthead seabream fillets were much less than those observed by Simeonidou et al. (1997), Benjakul et al. (2003) and Ozbay et al. (2006) for several other commercial important fish species.

Total weight losses were significantly correlated with the protein content in centrifugal tissue fluids (r_s = 0.850, P<0.01), Ca^{2+}-ATPase activities (r_s = 0.638, P<0.01), free fatty acids (r_s = 0.594, P<0.01) and peroxide values (r_s = 0.529, P<0.01)

These observations may suggest that the water holding capacity of stored frozen gilthead seabream fillets was affected by the damage in cell structures, as measured by the protein content in centrifugal tissue fluids of stored frozen samples, and the denaturation of myofibrillar proteins, as measured by the Ca^{2+}-ATPase activities in actomyosin extracts of stored frozen gilthead seabream. Lipid degradation products, that is, free fatty acids and peroxides, could have an indirect effect on the water holding capacity of stored frozen fillets through their interactions with myofibrillar proteins (Shenouda, 1980; Mackie, 1993) As a result; water could be released from gilthead seabream muscles easily, particularly when the storage time increased. Similar suggestions are reported by Benjakul et al. (2003) for several tropical species.

Effects of storage time on instrumental texture measurements

The results of the present study show that peak shear forces (firmness values) obtained from the fresh fillets were significantly higher than those of frozen fillets stored for up to 91 days. Similarly, toughness values from the fresh fillets were significantly higher than those of frozen fillets stored for up to 183 days (Figures 2 and 3). These results suggest that storage of fillets for up to 91 days at -22°C caused softening (decrease in firmness) and tenderness of raw fillets. Sigurgisladottir et al. (2000) showed that the firmness (peak shear force) of thawed fillets of salmon stored frozen for one month at -20°C was lower than that of fresh fillets. Carbonell et al. (2003) found that the instrumental firmness (or hardness) and chewiness values of frozen whole gilthead seabream stored for two months at -20°C were lower than those of fresh fish. As the results of the present study show, frozen storage caused changes in the muscle integrity and denaturation and aggregation of myofibrillar proteins of gilthead seabream fillets. As was shown earlier in the present study, the most profound changes in the muscle integrity indices were found during the first 34 days of storage and these were attributed mostly to the freezing process. In addition, the changes in the myofibrillar proteins of the stored frozen fillets were more profound at the end than at beginning of the storage period, as indicated by the changes in the values of salt soluble proteins of the present study. It is, therefore, presumed that at the beginning of the storage period the tissue-softening factors (release of proteolytic enzymes from lysosomes) overcame the tissue-hardening factors (denaturation and aggregation of myofibrillar proteins) with concomitant softening of the muscle of raw stored frozen fillets with respect to the fresh fillets.

Evaluation of the texture of stored frozen cod fillets with a texture analysing system showed that increased length of time at -10°C caused hardening (firming) of the fillets (Badii and Howell, 2002). Ozbay et al. (2006) showed that shear forces of cooked salmon fillets increased almost linearly with storage time when the frozen fillets were stored at -20°C for one year. Dorado-Rodelo et al. (2007) found variable shear forces from raw frozen fillets of spotted rose snapper stored at -20°C for 120 days. Pastor et al. (1999) found no changes in shear strengths of whole and gutted gilthead seabreams stored frozen at -20°C for 10 months (almost 300 days). Huidobro and Tejada (2004) recorded a small but significant increase in the shear forces of cooked minced samples from frozen whole gilthead seabreams after 349 days in storage at -20°C. Therefore, the results of the present study with stored frozen gilthead seabream fillets are in agreement with most of these other studies

From the results of the present study, there was a tendency of the peak shear forces (firmness values) and toughness values of the stored frozen fillets to increase with the storage time (Figures 2 and 3).

Negative linear correlations were found between the values of toughness of the stored frozen gilthead seabream fillets and those of Ca^{2+}-ATPase activities (r_s = -0.594, P<0.05). In contrast, the values of toughness were correlated positively with those of free fatty acids (r_s = 0.722, P<0.05), peroxide values (r_s = 0.827, P<0.01) and water holding capacity (r_s = 0.749, P<0.001). In addition, values of firmness were correlated positively with those of free fatty (r_s = 0.578, P<0.05) and peroxide values (r_s = 0.763, P<0.01). These results suggest that the development of toughness and hardness (increase in firmness) in raw stored frozen fillets was, in part at least, due to denaturation and of myofibrillar proteins and to changes in water holding capacity of muscles. They may imply, also, that lipid degradation products contributed to changes in texture of stored frozen raw samples, possibly through their interaction with myofibrillar proteins, as was

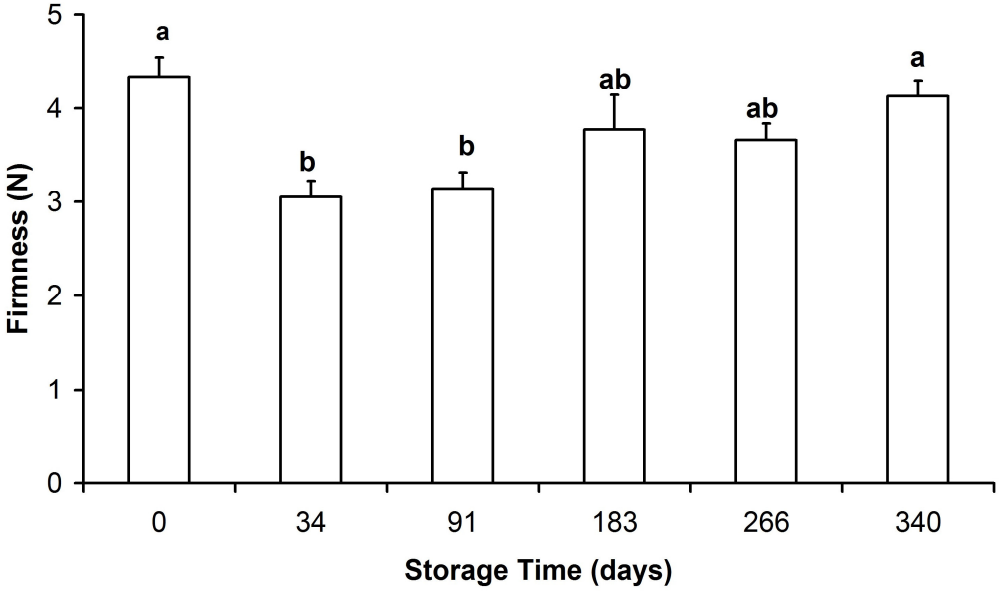

Figure 2. Effect of length of time of storage at -22°C on instrumental firmness of gilthead seabream fillets. Means ± SEM, n = 5. Groups with different letters (a, b) are significantly different (P<0.05). The '0' storage time presents fresh gilthead seabream fillets.

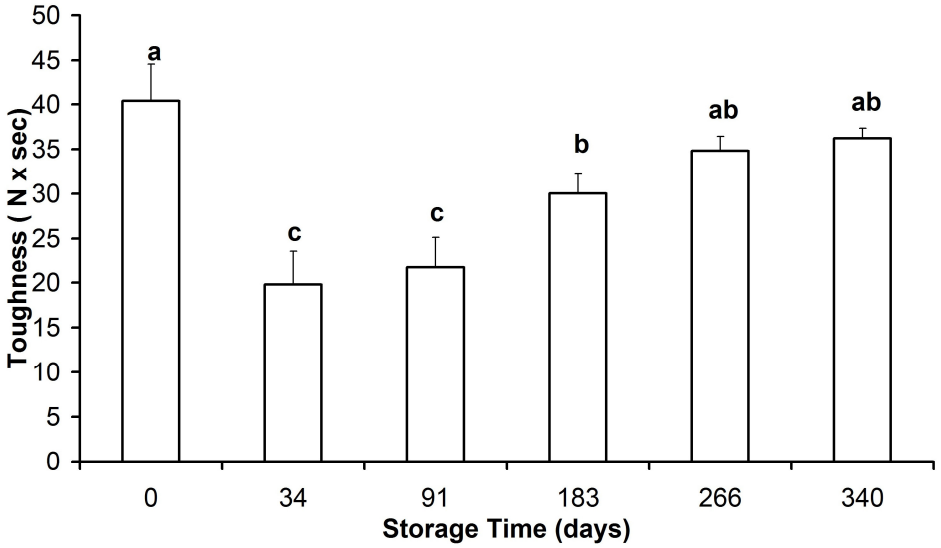

Figure 3. Effect of length of time of storage at -22°C on instrumental toughness of gilthead seabream fillets. * Means ± SEM, n = 5. Groups with different letters (a, b, c) are significantly different (P<0.05). The '0' storage time presents fresh gilthead seabream fillets.

suggested earlier in the present study.

Conclusions

The results of the present study indicate that the length of time of storage at -22ºC affected the integrity of muscles, reduced the water holding capacity, caused aggregation of myofibrillar proteins and denaturation of myosin (or 'actomyosin'), degradation of lipids, and affected the texture of gilthead seabream fillets as measured by the texture analyzing system. However, most of these changes were less profound than those recorded in literature for other stored frozen fish.

Therefore, gilthead seabream fillets were quite stable in frozen storage, and this suggests that gilthead seabream could be marketed in the form of frozen fillets. However, stored frozen gilthead seabream fillets may be more vulnerable to changes than whole gilthead seabreams as regards texture and integrity of myosins (or 'actomyosins') (Pastor et al., 1999; Huidobro and Tejada, 2004).

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