EVALUATION OF THE QUALITY OF INDIVIDUAL QUICK FROZEN FISH PRODUCTS

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To my family

Abstract

In the present study the effects of characteristic freezing times and storage time at -22°C on the quality of the adductor muscle of post-rigor scallops (*Pecten maximus)* and gilthead seabream fillets (*Sparus aurata*) were studied in regard to the integrity of muscle structure, myofibrillar protein denaturation and aggregation, lipid degradation, texture and sensory changes. This information would be useful for achieving optimal conditions for freezing these species and assessing their quality during frozen storage for commercial purposes.

Scallop muscles and gilthead seabream fillets were frozen individually with characteristic freezing times that can be met in commercial practice of freezing seafoods. After freezing, the samples were thawed and their quality was evaluated. Fresh samples were analyzed as controls. Intermediate characteristic freezing times (i.e. 89 and 49 minutes for scallop muscles and 74 minutes for gilthead seabream fillets) caused more damage to cell structure of both species than the shorter and longer characteristic freezing times tested. Short characteristic freezing times (i.e. 19 minutes for scallop muscles, and 2 and 18 minutes for gilthead seabream fillets) reduced the thawing losses of both species compared to the longer characteristic freezing times (i.e. 235 to 1000 minutes for scallop muscles, and 640 minutes for gilthead seabream fillets) tested. Freezing at short characteristic freezing times produced raw fillets similar in texture to the fresh fillets. Therefore, short characteristic freezing times (equal to or less than 19 minutes) are beneficial for freezing both species.

Scallop muscles and gilthead seabream fillets were kept frozen for up to 301 and 340 days, respectively. Sampling was carried out at regular intervals on fresh and stored frozen samples. Storage time affected the integrity of intra-cellular organelles, reduced the water holding capacity, caused

structural changes to myofibrillar proteins and affected the sensory attributes of both species. Frozen scallop muscles were in acceptable eating condition after a storage period of ten months, with most of the changes in bio-chemical and physical properties being pronounced after three months of storage. Based on the changes in taste scores versus storage time, it was assessed that the practical storage life of frozen gilthead seabream fillets was circa 5 to 6 months

Ca^{2+}-ATPase activities for scallop muscles and a linear model that combines free fatty acids, peroxide values and protein content in centrifugal tissue fluids for gilthead seabream fillets, may be reliable methods for industry to use for assessing their quality during long term storage at -22°C.

Key words: King scallops, gilthead seabream fillets, freezing, frozen storage, quality

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CONTENTS

Introduction

Seafoods are highly perishable commodities that, unless appropriately frozen and stored, will deteriorate rapidly owing to the growth of spoilage micro-organisms and bio-chemical/chemical reactions in the dead muscle. Deterioration is reflected in gradual development of undesirable flavours, softening of the flesh and eventually substantial losses of fluid containing protein and fat (Food and Agricultural Organisation (FAO), 1977).

By reducing the temperature of seafoods to a point at which almost all the water in them has frozen, the activity of micro-organisms is inhibited and the biochemical reactions are slowed down. As a result, freezing of seafoods can result in little change in their quality, and potentially offers the consumers products very similar in quality to fresh ones (FAO, 1977). Thus, the need for freezing arises when preservation of seafoods by other means, such as chilling with ice, is unsuitable for the period of storage time involved.

It is, also, important to underline that freezing is the only large-scale preservation method to bridge seasons as well as variations in supply and demand of raw fish and shellfish. Freezing, also, makes it possible to move large quantities of fish and shellfish over geographical distances (i.e. facilitates exports; Persson and Londahl, 1993).

As a result of these advantages, the frozen seafood industry grew substantially in industrialized countries from 1,992,825 metric tons in 1961 to 6,428,632 metric tons in 2004 (FAO: http://faostat.fao.org; accessed in May 2006)

However, despite the growth in the frozen seafood industry over the last decades, a slowdown and even a decline is now being experienced in industrialized countries (Table 1-1). This is due to high demand for

seafoods, which led to over -fishing of most important commercial finfish species such as cod, haddock and turbot. It is widely acknowledged that exploitation of other natural seafood stocks and developments in aquaculture will play an increasingly important role in supply of fish and shellfish to seafood freezing industry. Thailand, for instance, produced 83,823 tonnes of farmed shrimps in 1990, from which 77,600 tons were exported as frozen products (Morrison, 1993).

Table 1-1 Frozen seafood output from industrialized countries*

Year	Frozen seafood output (Metric tonnes)
1961	1,992,825
1971	3,541,432
1981	5,242,481
1991	6,780,513
2001	6,316,116
2002	6,397,855
2003	6,512,701
2004	6,428,632

Table note: * From: FAO (http://faostat.fao.org; accessed in May 2006)

Freezing and frozen storage, therefore, form an important section of the seafood processing industry. Nevertheless, these processes may cause physical, chemical and bio-chemical changes in frozen seafood muscle which eventually influence the quality of frozen seafood. The physical, chemical, bio-chemical and sensory properties of frozen seafoods have been studied for several decades because of their economic importance. However, these changes can be influenced by various factors, for instance species, freezing, and storage conditions (Haard, 1992). In addition, thawing methods were found to play an important role in the bio-chemical and sensory attributes of stored frozen seafoods (Nilsson and Ekstrand, 1994; Nilsson and Ekstrand, 1995; Srinivasan *et al.*, 1997). 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.

King scallop (*Pecten maximus*), a bivalve mollusc, is widely distributed in northwest Europe (Brand, 1991). It is much prized as food, and a number of commercial fisheries exist around U.K. Figure 1-1, below, shows the production of King scallops and other scallop species in U.K. for the years 1995 to 2005 (FAO: http://faostat.fao.org; accessed in September 2007). Apart from the years 1999 and 2000, the production of King scallops increased substantially from 9,400 tonnes in 1995 to 17,806 in 2005, with Scotland to take about half the total U.K. production of King scallops. Also, the production of King scallops accounted for 31% to 81% of the total production of scallop species in U.K. in years 2001 to 2005.

Figure 1-1. Global production of King scallop and other scallop species in U.K*.

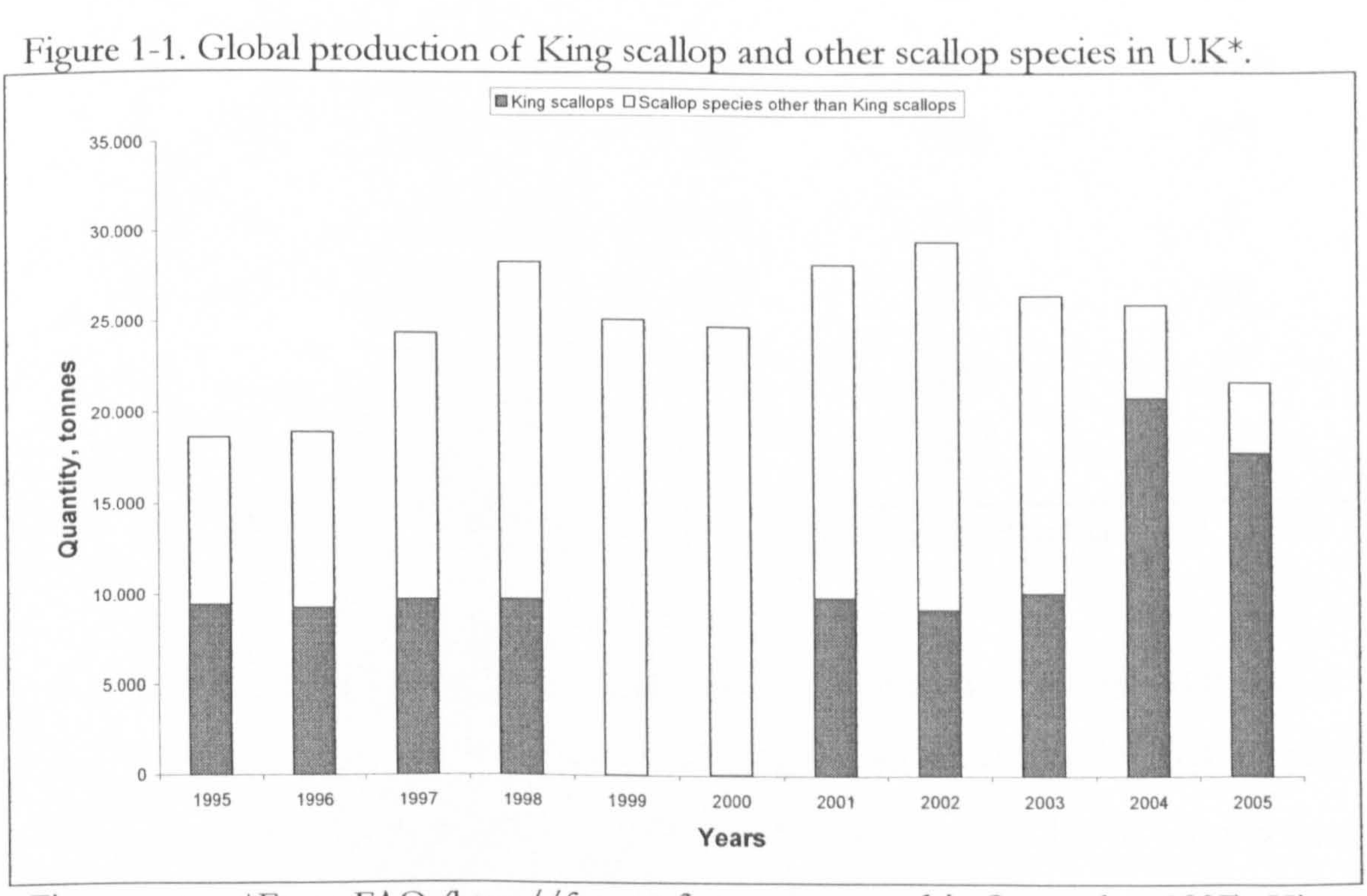


Figure notes: *From FAO (http://faostat.fao.org; accessed in September 2007). King scallops production for years 1999 and 2000 were equal to 27 and 41 tonnes, respectively.

The adductor muscle, the major edible part of King scallop, is offered to U.K. consumers mainly as fresh product (Hardy and Smith, 1986).

However, the U.K. exports of scallops are based mostly on frozen products. The total production of frozen scallop muscles in U.K. showed an increasing trend, from 2,378 tonnes in 1997 to 5,350 tonnes in 2004, which accounted for 10% and 20% of the total production of scallops in the respective years (FAO: http://faostat.fao.org; accessed in September 2007; Table 1-2).

Table 1-2. Production, exports and imports of frozen and exports of fresh muscles of scallop species, in tonnes*.

Years	Production of frozen scallop muscles	Exports of frozen scallop muscles	Imports of frozen scallop muscles	Exports of fresh scallop muscles
1997	2.378	2.378	1	156
1998	2518	2518	21	.12
1999	3.174	3.174	60	1363
2000	4092	4092	100	545
2001	3.338	3.338	112	3
2002	3.220	3.220	247	19
2003	2.789	2.789	259	21
2004	5.350	3.652	274	1

Table note:* From FAO (http://faostat.fao.org; accessed in September 2007)

Freezing, therefore, forms an important section of scallops industry in U.K. There are some reports in the literature on freezing and cold storage of scallop species (Dyer and Hiltz, 1974; Aurell *et al*, 1976; Chung and Merritt 1991ab; Kawashima and Yamanaka 1992; Kawashima and Yamanaka 1995ab; Kawashima and Yamanaka, 1996) but there is a lack of information on the quality of frozen King scallop adductor muscle. Therefore, the freeze-induced alterations and stability in storage of King

scallop adductor muscle need to be investigated.

By contrast, gilthead seabream (*Sparus aurata*) is one of the most important fish species farmed in Mediterranean, including Greece. It is much prized as 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 being the main producer of this species (FAO: www. globefish.org; accessed in June 2007; Table 1-3, bellow).

Table 1-3 Word production of farmed gilthead seabream (*Sparus aurara*), in 1000 tonnes

Countries	Years					
	2001	2002	2003	2004	2005	2006
Greece	40.9	38.1	38.6	49.0	50.0	49.0
Turkey	14.0	12.4	17.5	24.0	15.5	-
Spain	11.5	12.4	13.7	13.5	15.6	21.1
Italy	10.5	8.0	12.0	8.5	7.8	8.8
Egypt	3.4	4.1	3.8	-	-	-
Israel	2.7	2.6	2.5	-	-	-
Portugal	2	2.1	1.5	2.5	3.5	2.5
France	2	1.8	1.8	1.3	1.9	1.9
Others	4.7	5.2	6.2	1.5	1.5	3.4
Total	91.7	86.5	97.6	100.3	91.1	86.7

Table note: From FAO: www. globefish.org; accessed in September 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, also, periods of the year, when new generations of gilthead seabream reach the marketable size (i.e. 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). There is, therefore, a need to look for ways of processing gilthead seabream in order to regulate the market and have stocks for more even commercialisation. Freezing of fish is an important consideration in this regard. The demand for research on this subject comes from the gilthead seabream farming and processing industries, and 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 freezing process and 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 on freezing and in frozen storage, since individually frozen fish fillets present simplicity in use and thereby they are preferred by consumers compared to whole frozen fish (Botta, 1995). Therefore, the freeze-induced alterations and stability in storage of gilthead seabream fillets need to be investigated.

For all these reasons, the present study, which was carried out in Aberdeen, U.K., and in Greece, aimed to investigate the effects of different freezing treatments and the length of time in cold storage on the quality of adductor muscle scallops (*Pecten maximus*) and gilthead seabream (*Sparus aurata*) fillets. The information obtained would be useful for achieving optimal conditions for freezing these species and assessing their quality during storage for commercial purposes.

CHAPTER 1 Literature review

1.1. General aspects of freezing and cold store

In this section, basic aspects of freezing and cold storage of frozen seafood are discussed. This will give useful information for the subsequent discussion of the alterations of seafood muscle on freezing and during frozen storage.

1.1.1 Definitions and explanations

1.1.1.1. Seafood freezing

Seafood freezing is a processing method by which 'almost all the water in a seafood product has solidified by extracting heat and reducing temperature' (Whittle and Howgate, 2002).

1.1.1.2. Freezable and non-freezable water in (muscle) products

Since the main effect of freezing is to remove most of the water from a (muscle) product and convert it into ice, it is important to define the types of water that exist in the muscle. Fennema (1985) summarized the different categories of water as follows:

- 'Constitutional water', immobile, located in interstitial regions or form integral part of the protein as chemical hydrates.
- 'Vicinal water', located at hydrophilic sites and micro-capillaries constituting the first hydration layer.
- 'Multilayer water', located at other sites of the first hydration layer, as well as comprising the following ones.
- 'Bulk-phase water', similar to normal or dilute salt solution water, it is held in muscle by capillary forces.

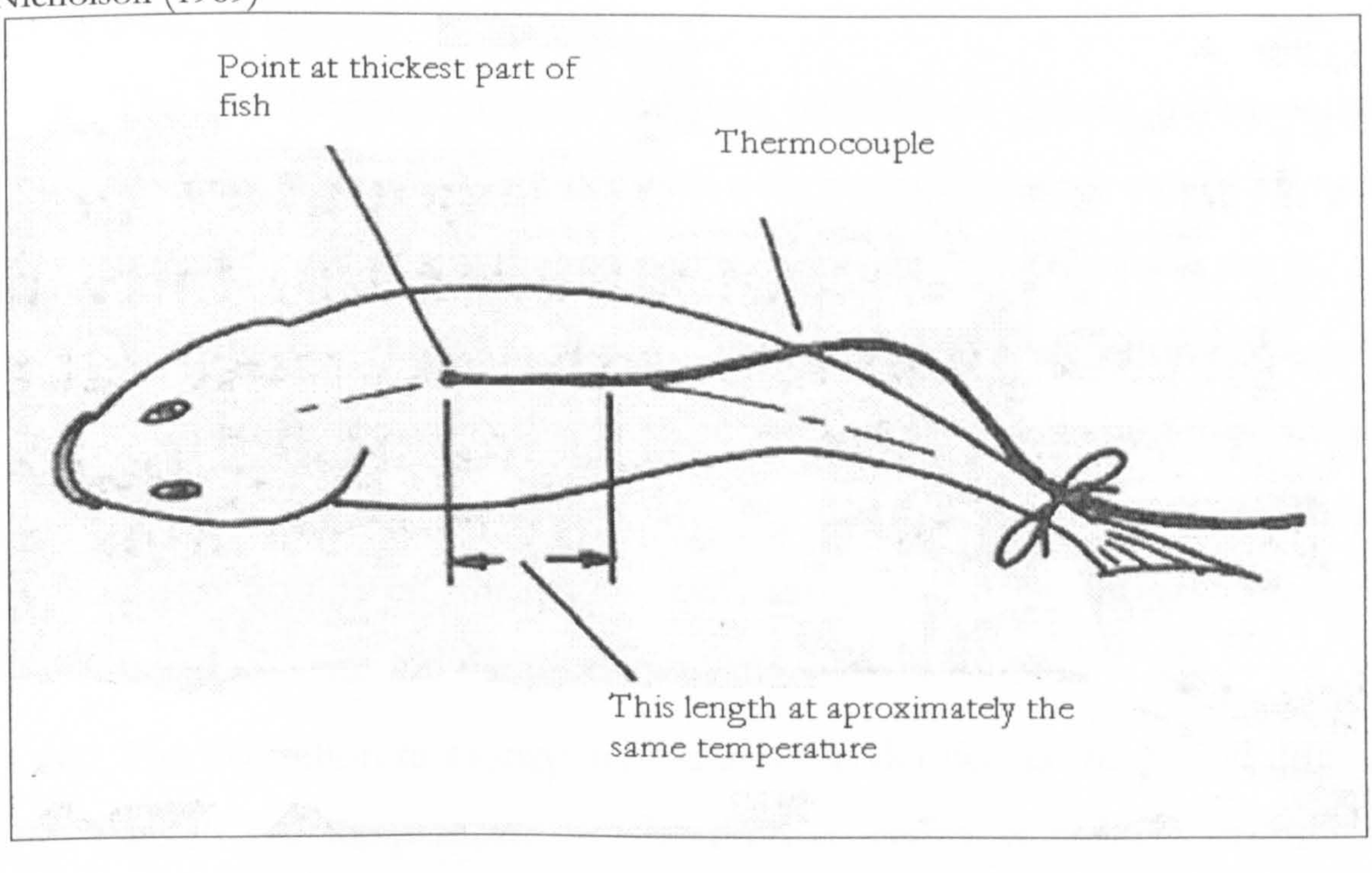
The same author defined 'bound water' or 'non-freezable' water as that fraction of the water in the muscle that does not freeze at -40°C. It has reduced mobility and comprises, according to previous classification, the

constitutional, vicinal and part of the multilayer water fractions. The rest of the water in muscle, i.e. the 'bulk-phase water' and a part of the 'multilayer water', is defined as the 'freezable water'.

1.1.1.3. The freezing process

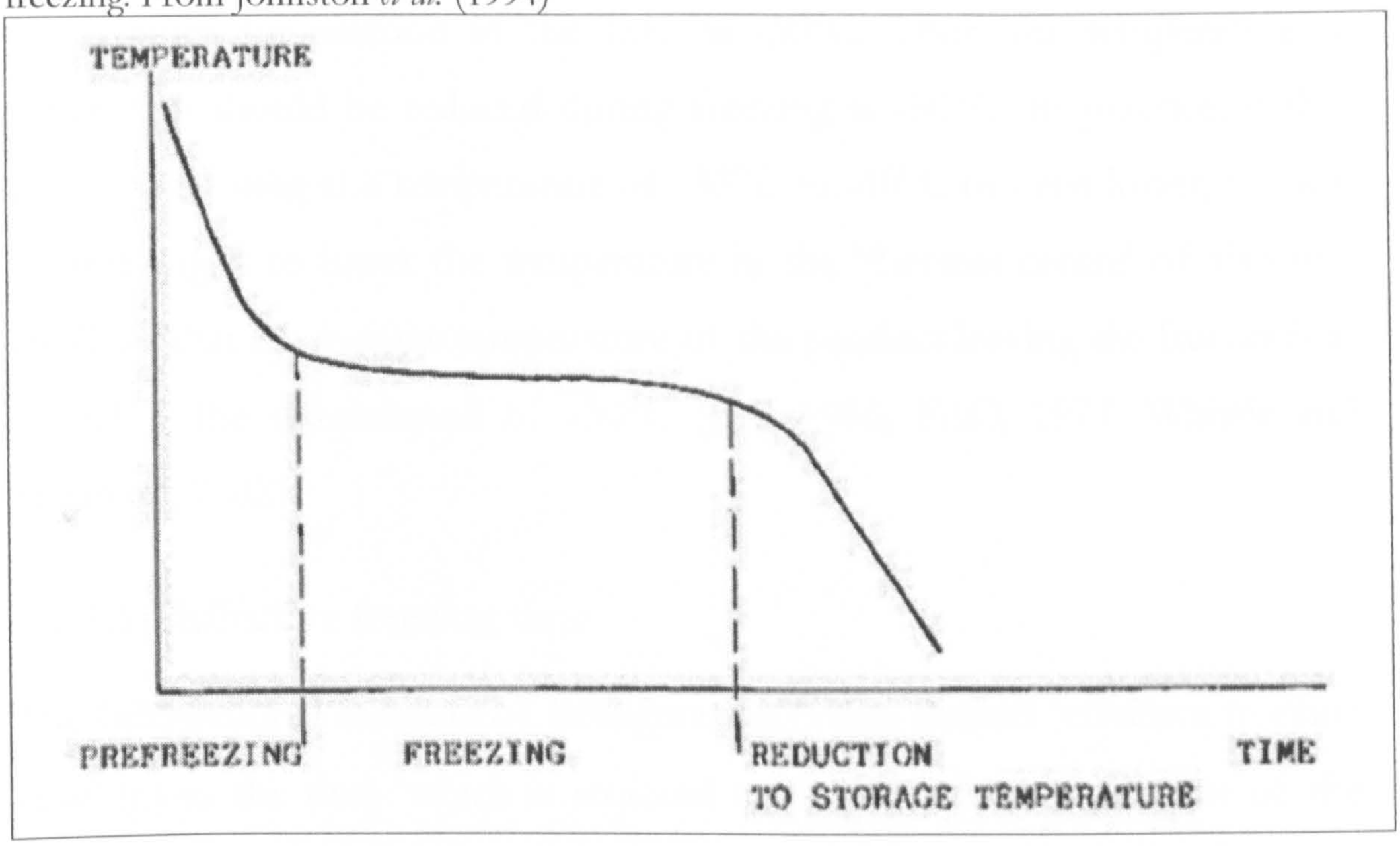
'The point in a product that has the highest temperature at the end of the freezing process' is defined as 'thermal centre' of the product (International Institute of Refrigeration (IIR), 1986). The 'thermal center' of a single fish is located in the centre of its thickest part (Nicholson, 1969). During freezing the temperature at the thermal centre of a fish is measured by using a special type of thermometer known as thermocouple (Nicholson, 1969). Figure 1.1.1.3-1 shows the correct method of locating thermocouples during freezing of a single

Figure 1.1.1.3-1 Correct method of locating thermocouples for freezing. From Nicholson (1969)



The time-temperature profile at the 'thermal centre' of a product during the freezing process is presented in Figure 1.1.1.3-2 (Johnston *et al.*, 1994).

Figure 1.1.1.3- 2. Time- Temperature profile at the 'thermal centre' of a product during freezing. From Johnston *et al.* (1994)



According to the International Institute of Refrigeration (1986), three stages are defined, as follows:

- The 'pre-freezing' stage is the time period that elapses between the moment at which a product with a high temperature is subjected to a freezing process and the moment at which ice crystallization starts
- The 'freezing stage' (or zone of maximal ice crystallization or period of thermal arrest) is the time period during which the temperature remains almost stationary at the thermal centre due to heat abstraction from the product. During this stage the majority of freezable water of the product changes phase into ice.
- The 'reduction to storage temperature' is defined as the period during which the temperature of the thermal centre is reduced from the temperature of the freezing stage to the storage temperature.

1.1.1.4. Storage temperature of seafood products

The storage temperature of frozen seafood products depends on species,

type of product and intended time of storage. The recommended storage temperature for seafood in the U.K. is -30°C. Thus, the temperature to which fish should be reduced during freezing is -30°C. In practice, with a freezer operating at a temperature of -35°C to -40°C or even lower, the aim of freezing is to lower the temperature in the 'thermal centre' of fish to -20°C, so that the average temperature of the product leaving the freezer is at or below the temperature of -30°C (IIR, 1986; FAO, 1977; Whittle and Howgate, 2002).

1.1.1.5. Effective freezing time

The International Institute of Refrigeration (1986) defines 'effective freezing time' (t_e) as the time 'which is required to decrease the temperature of the thermal centre of a product from the start of the 'pre-freezing stage' to the intended storage temperature'. The freezing time of a seafood product depends mainly on:

- the type of freezer
- the temperature of freezer
- air speed in an air-blast freeze
- product temperature
- product thickness
- product shape
- product contact area and density
- product packaging
- species of seafood (Nicholson, 1969).

The 'effective freezing time' (or 'freezing time') of a given seafood product can be measured during freezing or calculated by means of mathematical formulae (FAO, 1977). It can be used in designing freezers, freezing plants and estimating loading capacity of freezers.

1.1.1.6. Freezing rate

Freezing rate (°C/h) in a given point of a product is defined by the International Institute of Refrigeration (1986) as the difference between the initial and final temperature divided by the effective freezing time. It can also be evaluated by the speed (cm/h) of movement of the ice front through a product.

1.1.1.7. Characteristic freezing time

The definition of 'characteristic freezing time' is based on the time necessary for a given point of a product to pass through the temperature range of the 'freezing stage'. In order to define this stage, two temperatures need to be specified, i.e. the temperature at which the 'freezing stage' starts and the temperature at which most of the freezable water has been converted into ice. Bevilaqua *et al.* (1979) defined a constant temperature of -1°C as the temperature where the freezing of meat starts (i.e. the highest temperature at which ice crystals have a stable existence in a food material), and -7°C as the temperature where 80% of the total freezable water is converted into ice. These authors defined the time that corresponds to the 'freezing stage' as the time in minutes necessary for a given point of a piece of meat (e.g. thermal centre) to pass from -1 to -7°C, and they named this time the 'characteristic freezing time' (t_c). This time is important in studies dealing with the effects of freezing on quality of frozen (meat) products, since this mainly determines the size and location of ice crystals (Love, 1955; Bevilaqua *et al.*, 1979;Bevilaqua and Zaritzky, 1980; Añòn and Calvelo, 1980). It is widely accepted that the size and location of ice crystals formed during freezing and storage are the main causes for the deterioration of frozen (meat) products, for instance through the distortion and destruction of fibres, desiccation of cells, and exposure of the cell interiors to high concentrations of electrolytes (Shenouda, 1980).

1.1.1.8. Quick freezing

There is no widely accepted definition of quick freezing (sometimes called rapid freezing). Most International Standards and legislations from different countries (including the U.K.), define quick-frozen seafood products as those that undergo a freezing process whereby the "freezing stage" is crossed quickly and also the temperature at the end of the freezing period has been reduced to the recommended storage temperature (Codex Standard 1, 190: 1995; Jukes, 1997)

With respect to freezing rates, the International Institute of Refrigeration (1972) defines quick frozen (meat) products as those that have undergone a freezing process with freezing rates (as rate of movement of ice front, cm/h) ranging from 0.5 to 3 cm/h.

Therefore, 'quick freezing' is a general term for a freezing process that can be realized using different industrial freezing methods, and which depends mainly on the type of product (Garthwaite, 1997).

1.1.1.9. Some other terms of describing the freezing process

- 'Deep freezing' is used imprecisely in the context of quick freezing, to describe freezing of seafood to a temperature of -18°C or below (IIR, 1986; Whittle and Howgate, 2002).
- 'Bulk freezing' refers to 'freezing loose unpackaged products or to freezing large quantities of seafood for storage prior to further processing' (Whittle and Howgate, 2002).
- 'Slow freezing' describes a freezing process that is 'undesirably long, as for instance freezing of seafood products by blowing cold air in a cold store'. It is sometimes referred to as 'sharp freezing' (Whittle and Howgate, 2002).

1.1.1.10. Individual quick freezing (IQF)

'Individual quick freezing (IQF) is the quick freezing of seafood items in such a manner that each unit of product remains separate when frozen, in contrast to a frozen block of product (e.g. laminated blocks)' (Whittle and Howgate, 2002). Individually quick frozen (IQF) fillets, shrimp and mollusks are typical frozen products of this type. Cryogenic freezing is mainly used for small IQF products, such as shrimps and small fillets, which have comparatively short freezing times (Jul, 1984; IIR, 1986). Air blast freezers are the most frequently used freezers for production of IQF seafood products.

1.1.2 Ice formation during freezing of seafood products

On freezing, ice crystals are formed in seafood tissue. Their size, shape and location, inter- or intra-cellular, all depend on (characteristic) freezing time (t_c) and whether or not the flesh is frozen pre- or post-rigor. The subject has been reviewed by Love (1966).

At long characteristic freezing times (e.g. equal or more than 100 minutes for post-rigor cod fillets) a few crystallization centers are formed at first inter-cellularly. The freezing of this portion of water increases the concentration of dissolved solids in the remaining water, which in turn increases the osmotic pressure of the inter-cellular liquid. In order to equalize the concentration of solids in the liquid between inside and outside the fibres, water diffuses from the fibres into inter-cellular spaces. This water freezes by 'sticking' to crystals already formed causing them to grow. When the meat temperature decreases to the point that allows formation of intra-cellular crystallization centers, this does not occur. This is due to the fact that, from a thermodynamic viewpoint, the growth of existing crystals is more probable than the process of forming additional centers of crystallization.

If a similar specimen is frozen more rapidly, the heat removal is sufficient to

initiate formation of ice crystals both inter- and intra-cellularly at the same time, but since there is not enough time for the water to leave fibres it freezes mainly intra-cellularly. In this case the intra-cellular ice is in form of a large ice column occupying the whole cell, roughly synchronized with inter-cellular ice. If still more rapid freezing is used, the internal ice phase becomes discontinued and is broken laterally and longitudinally forming short ice spears. At short freezing times (less than 20 minutes for post-rigor cod) small ice crystals are distributed fairly evenly throughout the cell.

In contrast to post-rigor muscles, freezing of pre-rigor muscle at any freezing time results in intra-cellular ice formation (Love and Haraldsson, 1961).

Another important effect of freezing, which is connected with the size and location of ice crystals, and the final freezing temperature of a muscle tissue, is the concentration of solutes within the remaining unfrozen aqueous phase in the muscle cell. Freezing removes most of the water from proteins of muscle tissue to form ice, but a significant amount of the cellular water remains unfrozen ('bound water'). This allows marked differences in concentration of solutes throughout the frozen system, resulting in substantial changes in several properties of the unfrozen water phase of the frozen tissue, including pH, ionic strength, viscosity, oxidation – reduction potential (Mackie, 1993;Sahagian and Goff, 1996). The amount of the unfrozen water in a frozen tissue is related to the temperature at which the tissue is frozen. By -30°C about 90 % of the water is removed to form ice in frozen haddock, and as temperature is reduced, the proportion of ice to unfrozen water increases slowly (Reidel 1956 cited in Mackie, 1993).

It is important, also, to mention that storage of frozen muscle at temperatures above its eutectic point (i.e. the temperature at which the muscle is solidified completely; Love, 1966), which is usually between -40 to

-60°C (Hamm, 1986), changes the number, size, shape and location of ice crystal formed on freezing ('ice re-crystallization'; Sahagian and Goff, 1996). Formation of inter-cellular ice and accretion of intra-cellular ice in a rapidly frozen muscle tissue are almost inevitable at commercially used storage temperatures (Hamm, 1986).

The aforementioned changes, may lead to secondary effects (e.g. changes in micro-structure of fibres and denaturation and aggregation of proteins), which have the potential to result in a loss of quality of frozen seafood products.

1.1.3 Freezing methods

In commercial practice, freezing of seafoods is carried out in batch or continuous freezers. In batch freezers the product remains stationary, i.e. the freezer is loaded completely at the start of freezing and emptied when freezing is completed. In a continuous freezer the fish is moved continuously placed on trucks (truck freezer), or on a conveyor belt (belt freezer). There is in use a wide range of freezing equipments, which can be classified in three categories:

- Air blast freezing equipments, by passing a continuous stream of cold air over the product
- Plate or contact freezing equipments, by placing the product in direct contact with hollow metal freezer plates, through which cold fluid is passed
- Spray or immersion freezing equipments, by placing the product in direct contact with a refrigerant fluid (Whittle and Howgate, 2002).

Air blast and plate or contact freezing equipments are called mechanical freezers (George, 1993).

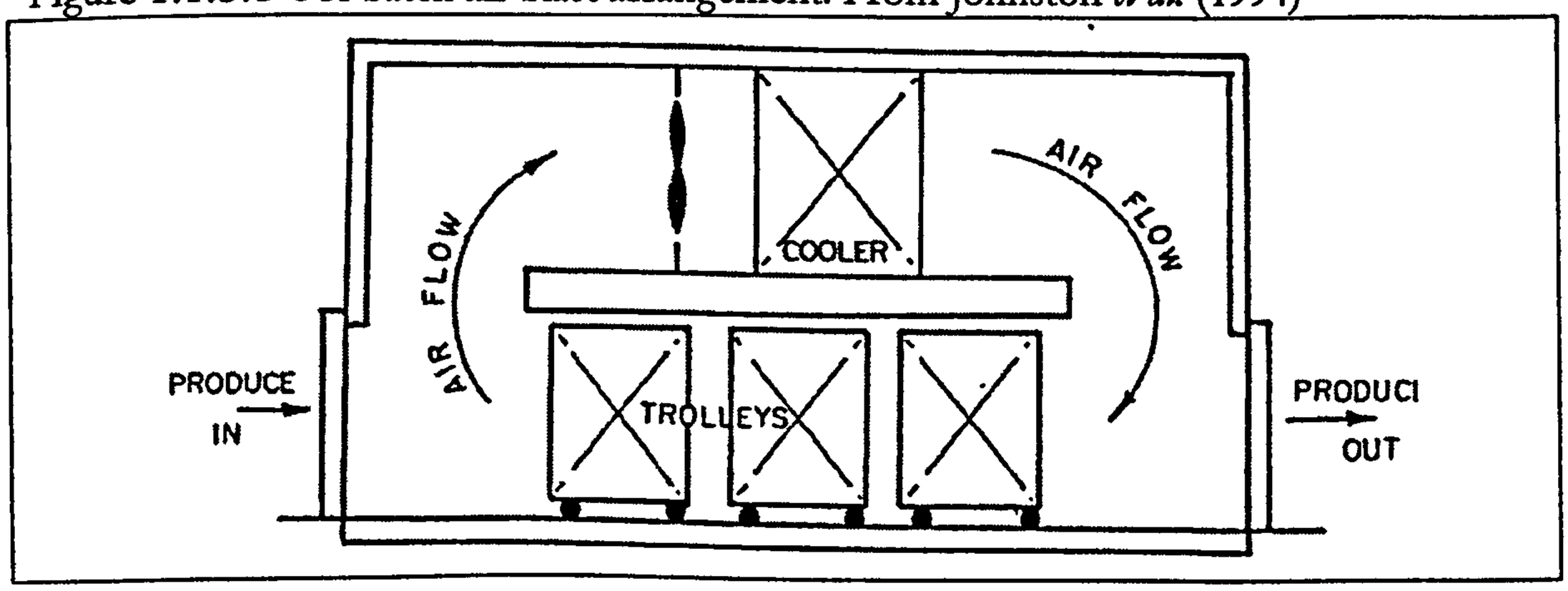
1.1.3.1. Air blast freezers

They can be operated on either a batch or a continuous basis. In good commercial practice, air blast freezing is carried out with air at -35°C (or as low as -60°C in some designs) and air velocity of 3 to 25 m/s. Uniform freezing is achieved only if the temperature and air velocity over the product is constant (Whittle and Howgate, 2002). Air blast freezers have lower freezing rates compared to freezing in liquefied gasses (Table 1.1.3.4-2; IIR, 1986). Prolonged and high air velocity may dehydrate the surface of a product and cause severe freezer burn (Whittle and Howgate, 2002).
There are many different designs of air blast freezers both for batch and continuous operation.

a) Batch air blast freezers

Batch air freezers use pallets, trolleys or shelf arrangements for loading the product. The freezer is fully loaded, and when freezing is complete, the freezer is emptied and reloaded for a further batch freeze. A batch freezer arrangement is shown in Figure 1.1.3.1-1, below. In this model, the trolleys are loaded from the side of the freezer and the air flows across the three trolleys in line.

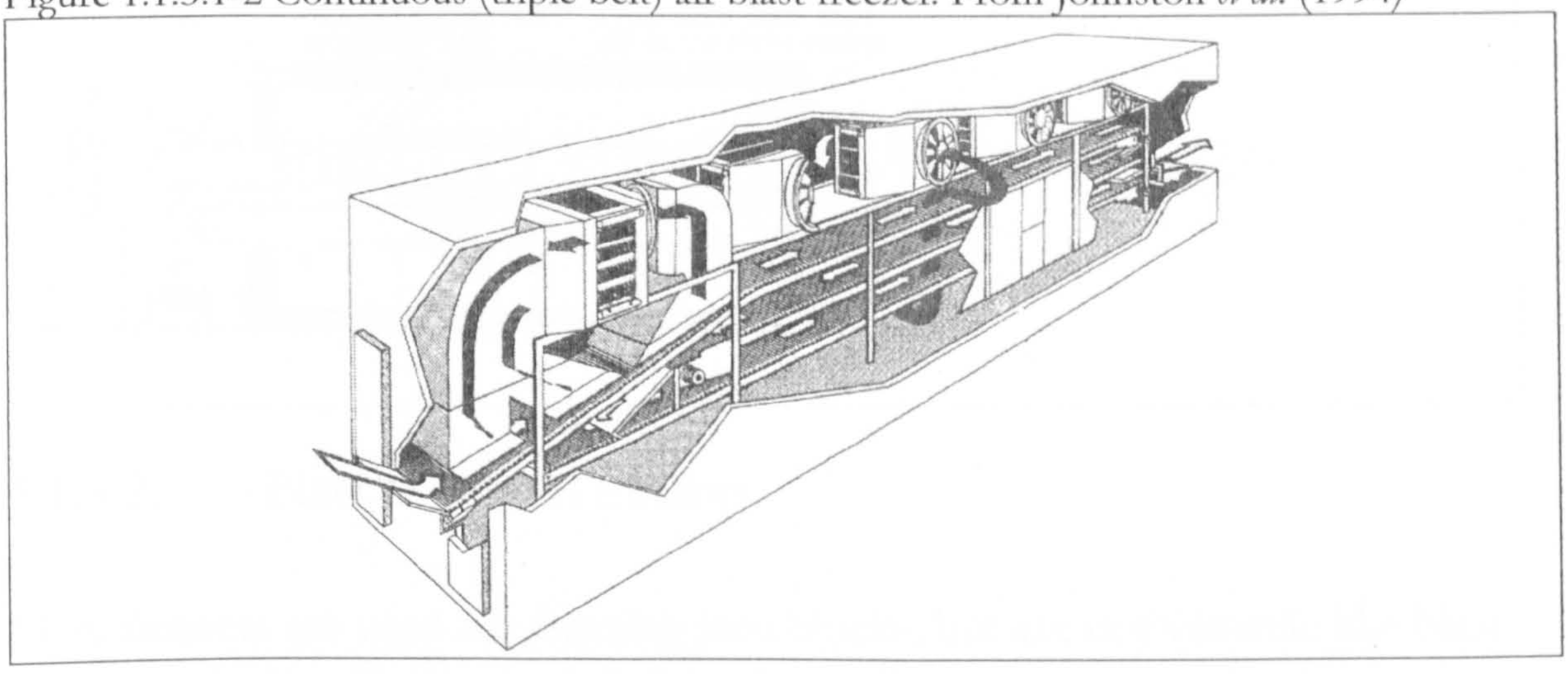
Figure 1.1.3.1-1 A batch air-blast arrangement. From Johnston *et al.* (1994)



b) Continuous air blast freezers

In continuous air blast freezers, the fish are conveyed through the freezer (on trucks or trolleys or they may be loaded on a continuously moving belt or conveyor) usually entering at one end and leaving at the other (Figure 1.1.3.1-2).

Figure 1.1.3.1-2 Continuous (triple belt) air blast freezer. From Johnston *et al.* (1994)



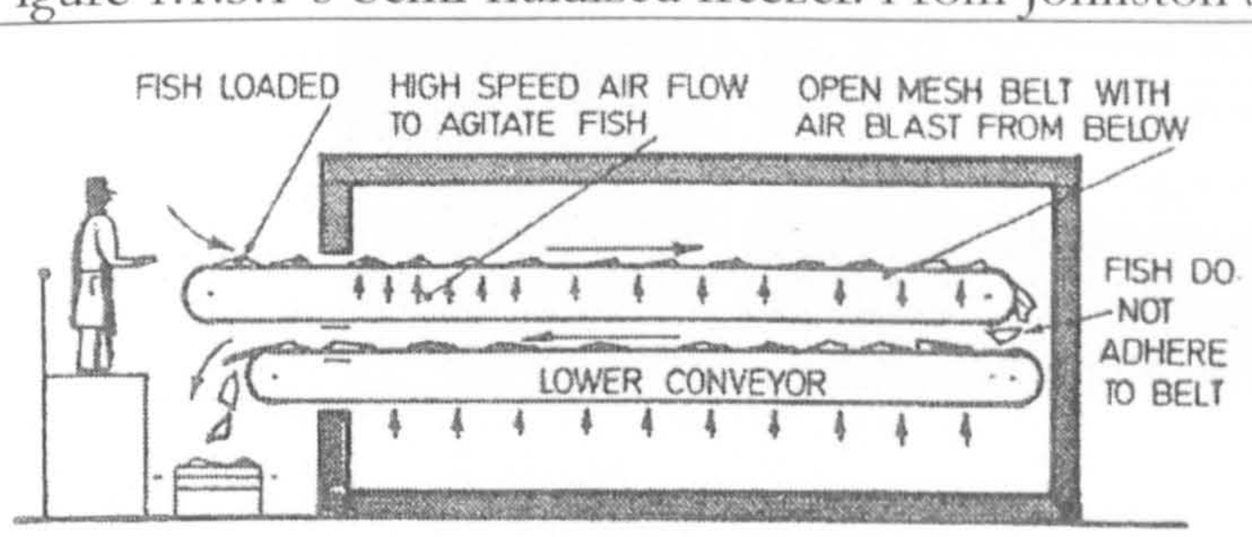
Continuous air blast freezers using belts or conveyors for moving the product through the freezer can only be used if the product can be frozen quickly. It is unlikely that a product with a freezing time of more than 30 minutes would be suitable for this freezer. The reason for the limitation on freezing time is that the freezer will become too long and cumbersome if a long freezing time is required (FAO 1977).

c) Fluidized and semi-fluidized freezers

One type of air blast freezer fluidizes the product with a strong blast of air from below. This type of freezer has been used successfully for such products as garden peas. This method of freezing has not a wide application for seafood products; small cooked and shelled shrimp is one of the few products that have been successfully frozen by this method.

A modified fluidized freezer, which may be termed a semi-fluidized freezer, has also been used for seafood freezing (Figure 1.1.3.1-2). At the beginning of freezing, a conventional conveyor is used. Sufficient air is blown from below the belt to agitate the product and ensure that individual portions of seafoods remain separate.

Figure 1.1.3.1-3 Semi-fluidized freezer. From Johnston *et al.* (1994)



1.1.3.2. Plate or contact freezers

Plate freezers are used for freezing into blocks, but are not versatile like blast freezers. They are of horizontal (see Figure 1.1.3.2-1, below) or vertical type (see Figure1.1.3.2-2, below) according to the arrangement of the plate (Whittle and Howgate, 2002). Hydraulic systems move the plates to create more compact product spacing, thereby giving higher density. Freezer operating temperatures is usually -40°C. Plate freezers are suitable for freezing deformable, uniform shaped foods, but not suitable for irregular shaped or non-deformable foods. If the thickness of a product is small, freezing rates can be increased to those found in blast freezers. Plate freezing is usually a batch type operation and hence involves unproductive periods and additional labour (FAO 1977)

Figure 1.1.3.2-1 Horizontal plate freezer. From Johnston *et al.*(1994)

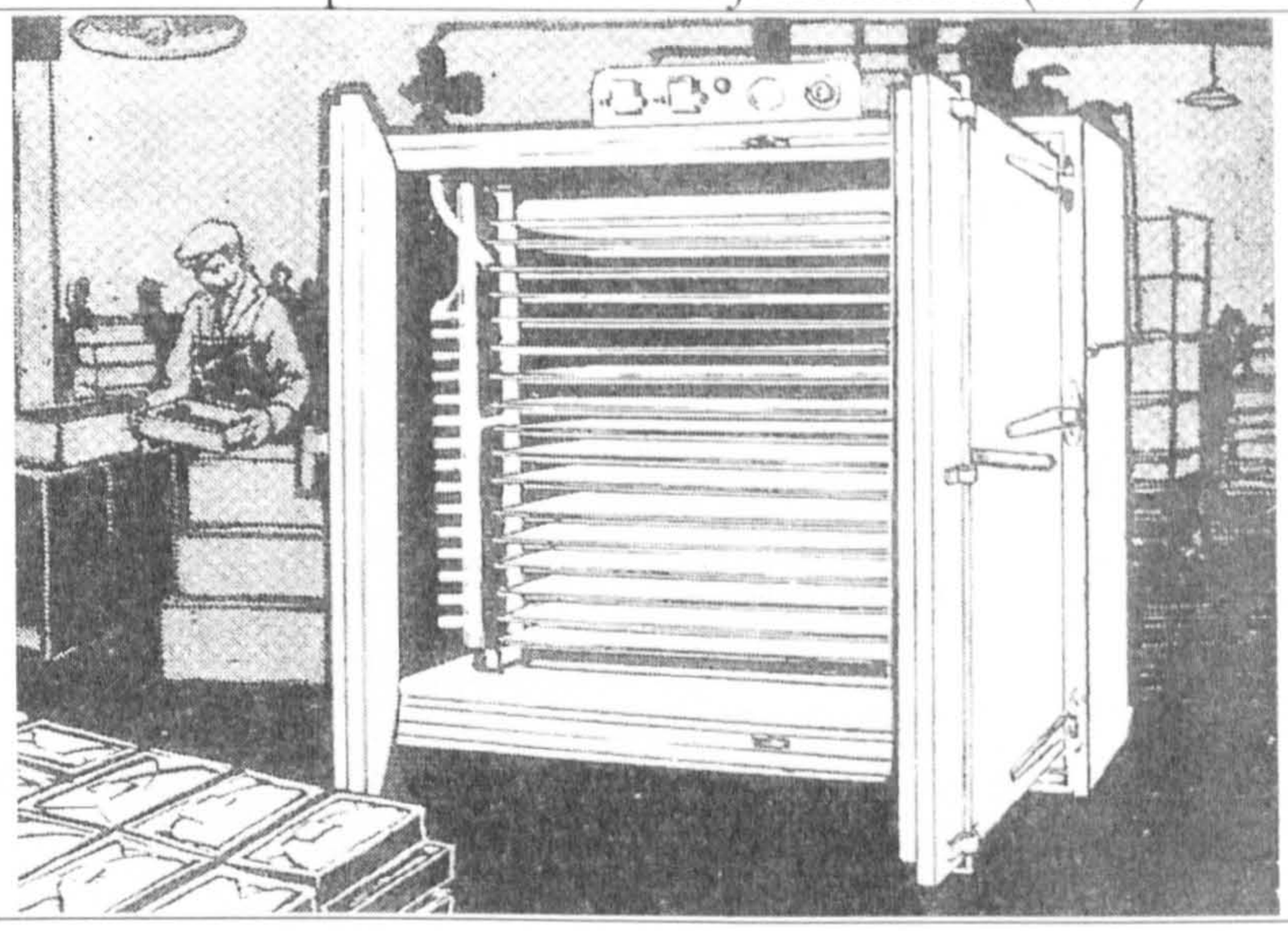
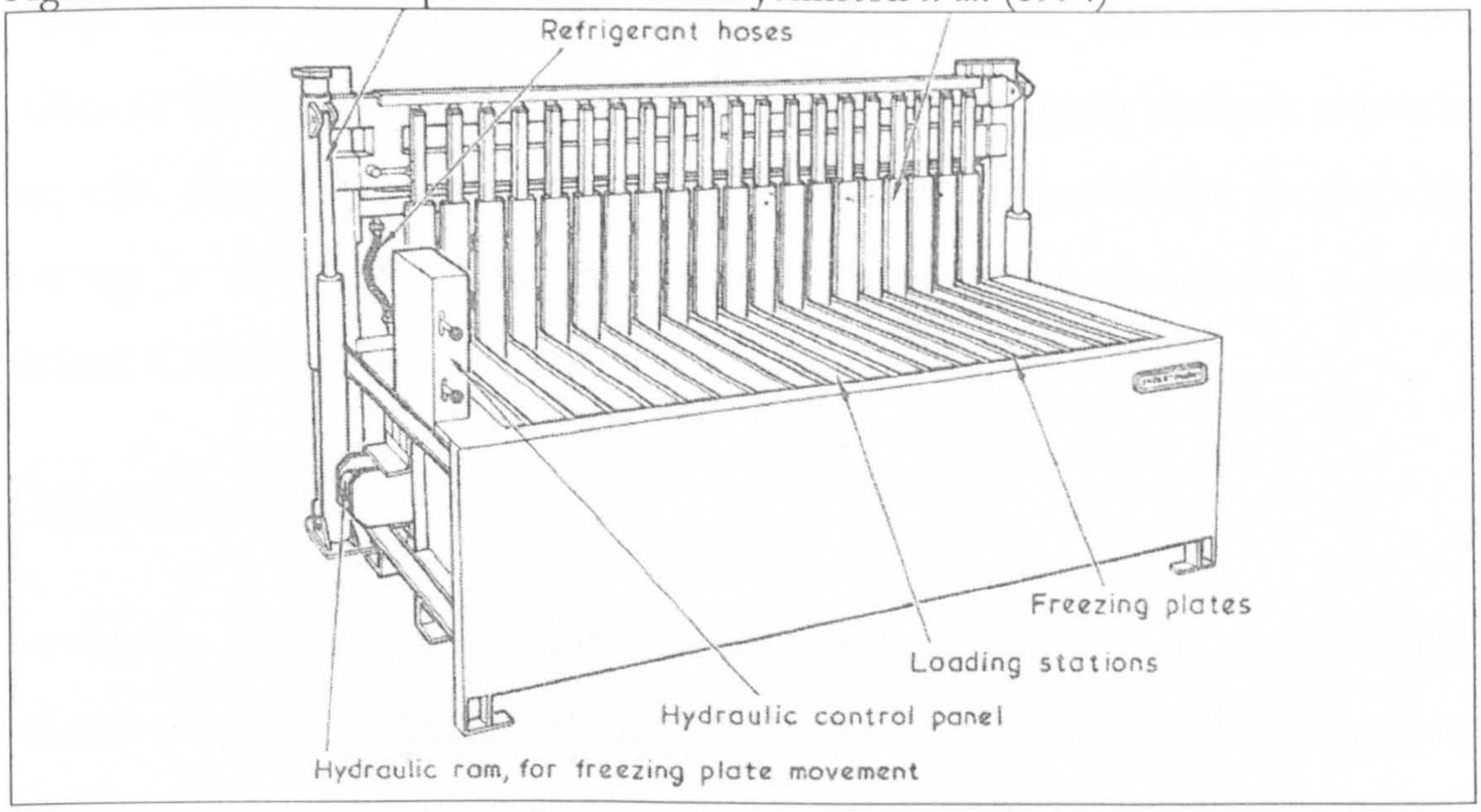


Figure 1.1.3.2-2 Vertical plate freezer. From Johnston *et al.* (1994)



1.1.3.3. Spray or Immersion freezers

These freezers find limited application in the freezing industry because they are suitable only for specialized products. Examples are:

- Immersion freezers.
- Cryogenic freezers which include:
- liquid nitrogen freezers (LNF)
- liquid freezant freezers (LFF)
- carbon dioxide (CO_2) freezers

a) Immersion freezers

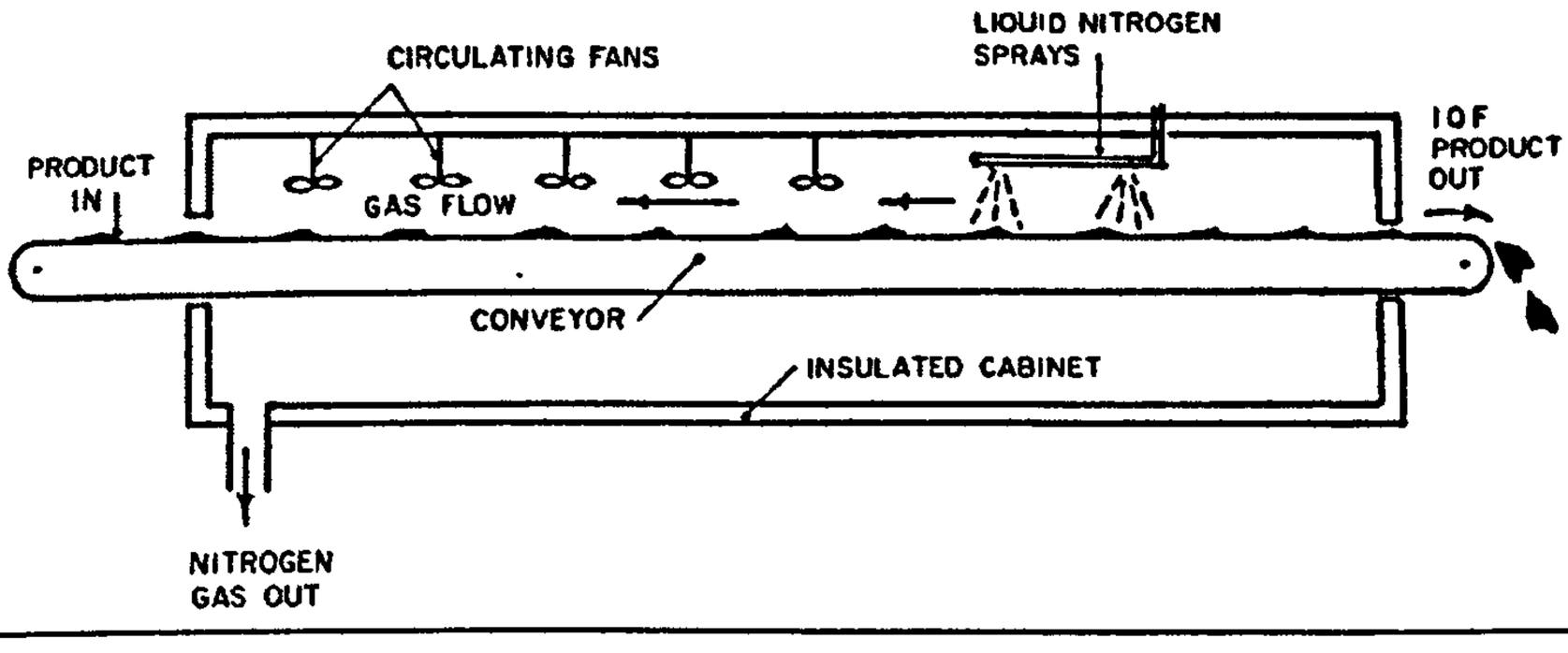
Freezing by immersion in refrigerated brine is most commonly used for the preservation of large fish such as tuna, which are intended for reprocessing into canned foods. With this method, it is important that the freezing medium should not impart any objectionable odours or flavours to the product or affect its quality in any other way. When using sodium chloride brine, care should be taken to minimize salt penetration of the product by removing it from the brine as soon as freezing is completed (Codex Standard CAS/RCP 16:1978).

b) Cryogenic freezers

In cryogenic freezing, a very rapid freezing rate is achieved by exposing the products either unpacked or thinly packed to an extremely cold refrigerant (i.e. boiling nitrogen, purified CCl_2F_2, subliming carbon dioxide) with low boiling point. In this method the refrigerant is sprayed on the product and heat removal is accomplished during a change of state, liquid to vapour or solid to gaseous, by the refrigerant (Figure 1.1.3.3, below). Cryogenic freezing is suitable only for high value products of prawns and lobsters, either whole or tails, because the process is more expensive but faster than

other traditional quick methods (FAO, 1977).

Figure 1.1.3.3 Liquid nitrogen freezer. From Johnston *et al.* (1994)



1.1.3.4. Freezing times and rates of freezing methods

The design of a freezer and calculating the capacity of a freezing plant depend on the type, quantity and freezing time of a particular seafood product to be frozen. The longer the freezing time, the bigger the freezer has to be for a given output (Johnston *et al.*, 1994).The freezing time of a new product in a particular freezer should always be measured. However, freezing times observed for other products, such as those presented in Table 1.1.3.4-1, will provide the designers and operators of the freezing plants some idea of what to expect.

Table 1.1.3.4-1 Freezing times for seafood products. From Johnston *et al.* (1994).

Product	Freezing Method	Product initial Temperature (°C)	Operating temperature (°C)	Freezing time Hour	Min
Whole cod, block 100mm thick	Vertical plate	5	-40	3	20
Single tuna, 90 Kg	Air blast	20 to -45°C at centre	-50 to -60	26	00
Single tuna, 50 Kg	Sodium chloride immersion	20 to-18°C at centre	-12 to -15	72	..00
Cod fillets, laminated block 57 mm thick in waxed carton	Horizontal plate	6	-40	1	20
Whole lobster 500g	Liquid nitrogen spray	8	-80	0	12
Scampi meats 18 mm thick	Air blast 3m/s	5	-35	0	26
Shrimp meats	Liquid nitrogen	6	-80	0	05
Packaged fillets 50 mm thick	Sharp freezer	8	-12 to -30	15	00
Packaged fillets 50mm thick	Air-blast 2.5 to 5 m/s	5	-35	5	15

Where several seafood products are to be frozen, the freezer should be designed to accommodate the required load of the product with the longest freezing time; the freezing rate for that freezer is then fixed. Table 1.1.3.4-2 present typical freezing rates that can be encountered in commercial freezers for seafood products (International Institute of Refrigeration, 1986)

Table 1.1.3.4-2 Typical freezing rates for seafood products

Freezing method	Freezing rate (cm/h)
Bulk freezing in a batch air blast freezer room	0.1
Quick freezing in a tunnel air blast freezer	0.3 to 0.5
Plate freezing	1.2 to 2.5
Quick freezing in a continuous air blast freezer	1.5 to 3.0
Freezing in liquefied gases	3.0 to 10.0

1.1.4 Cold storage

The objective of cold storage is to maintain the initial 'freshness' quality of the stored frozen seafood. The frozen product should be immediately transferred to the cold store, preferably to -30°C, but definitely not above -20°C. To avoid dehydration of the stored frozen seafood products, glazing or wrapping should be applied to frozen seafood products prior to storage, unless they have been packaged before freezing. The practical storage life of seafood products can be extended by reducing the cold storage temperature, as is shown in Table 1.1.4 (Johnston *et al.*, 1994), below.

Table 1.1.4 Storage life of frozen seafoods. From Johnston *et al.* (1994)

Fish product	Storage life, months		
	-18°C	-25°C	-30°C
Fatty fish	4	8	12
Lean fish	8	18	24
Flat fish	9	18	24
Lobster, crabs	6	12	15
Shrimp	6	12	12

Fluctuating temperatures and the presence of temperature gradients in cold storage rooms reduce the storage life of fish (Johnston *et al.*, 1994).

1.15 Dehydration changes of frozen and stored frozen seafood products

An unpacked frozen product loses a portion of its water content, which may vary from 1 to 2% or more (IIR, 1986).Weight loss due to dehydration depends on the:

- type of freezer
- freezing time
- type of product
- shape and size of product
- air velocity
- freezer operating conditions (FAO, 1977).

In cryogenic type freezers, which give very short freezing times, and in contact freezers, weight loss is low. The latter mainly occurs in air blast and other freezers, which use gases such as nitrogen and carbon dioxide in direct contact with the product (FAO, 1977). Some weight losses as a function of products and freezing methods are given in Table 1.1.5 (FAO, 1977).

Table 1.1.5 Weight loss from fish during freezing

Product	Method of freezing	Percentage of weight loss
IQF* shrimp	Air blast	2 to 2.5
IQF haddock	Air blast	1.2
IQF haddock	Carbon dioxide freezer	0.6
IQF products	Liquid nitrogen freezer	0.3 to 0.8
Tray of fillets	Air blast	1.0
Large fish or blocks	Air blast	0.5
Blocks of fish	Contact freezer (metal to fish contact)	0
Cartons of Fish	Contact freezer	0.5 within pack

Table note: *IQF= Individually Quick Frozen.

Weight loss is smaller when the freezing process is faster, provided that the other factors are constant. This is due to the fact that faster rates of freezing decrease the temperature of the product quickly to a value where the rate of moisture evaporation or sublimation is low (IIR, 1986).

Dehydration during frozen storage is a more serious problem because of the duration of storage usually involved. This is undesirable for reasons other than the most obvious one that the product will lose weight. Most important is that drying accelerates the alteration of proteins and oxidation of lipids. Frozen fish that have suffered severe drying in cold store, develop a chalky white, dry and wrinkled appearance on the surface associated with a tough texture that is characteristic of the condition known as 'freezer burn'. The surface of the thawed product may have a similar dry wrinkled look and in exceptionally severe cases, the flesh beneath can become spongy and very

light (FAO, 1977). Dehydration during storage can be minimized and/or avoided by tight-fitting water vapour proof packaging. In cases where frozen foods are stored unpacked or are protected by materials with water vapour permeability, dehydration will be kept to a minimum only by maintaining a low, uniform and steady temperature at cold storage (IIR, 1986).

1.2. Quality of frozen seafood

Quality as related to a seafood product is 'the aggregate of the properties that influence the acceptability of the product for the consumer' (Whittle and Howgate, 2002) and involves safety, nutritional value, 'freshness', availability, integrity and convenience (Botta, 1995).

Seafood safety is probably the most important category of seafood quality. In broad sense, this means that a seafood product is free of any harmful chemical or microbial contaminant at the time of its consumption. However, consumers or users are generally not capable of determining the safety quality of a particular seafood item and, thus, they are based on the seafood industry's declaration that the particular seafood product being purchased is indeed safe.

The determination of nutritional value of a seafood product is often complicated and/or expensive, and it cannot normally be readily determined by the buyer or user. Therefore, the importance of safety and nutritional value in determining if a particular seafood item is purchased or used is not great (Botta, 1995).

'Freshness' quality is defined by Botta (1995) as 'the degree of excellence to which a seafood meets the characteristics concerning appearance, flavour, odour, and /or texture that the buyer, user, and regulatory agency normally associates with a particular seafood when it is caught at the best time of the year, caught in the best location, caught by the best method, and handled and/or processed in the best manner.' The degree to which the 'freshness' quality of a seafood product meets the consumers' expectations concerning 'freshness' quality will greatly affect the acceptability of that product by the consumers (Botta, 1995). Thus the evaluation of 'freshness' quality of seafoods is of prime importance for the seafood industry. Sensory evaluation of 'freshness' quality of seafood products is the most direct method for determining their acceptability by consumers. However, these

methods may be subjective or need highly trained assessors and a quite large quantity of sample to be destroyed. Thus, sensory assessment of 'freshness' quality of seafoods is quite expensive and difficult to be organized. Consequently, seafood industry needs simple, quick, objective, and if possible non destructible methods to evaluate 'freshness' quality of seafoods. Several of these methods are discussed in section 1.4 of this Chapter.

Seafood products must present integrity and convenience. A seafood product must be what the supplier claims it is (in terms of net weight, count, species, ingredients, origin, etc.) and similar to what it was the previous time(s) it was purchased or consumed, in order to be acceptable. Seafood products are preferred by the consumers when they present simplicity in use and cooking (e.g. IQF fish fillets) (Botta, 1995). Therefore, whenever a seafood product is available for purchasing, 'freshness' quality, integrity and convenience are the controlling factors that determine if that seafood will be purchased more than once.

With respect to storage life of frozen seafood, the International Institute of Refrigeration (1986) has two definitions:

- High Quality Life (HQL) is 'the minimum time during storage at which a change can be detected in a sensory difference test'
- Practical Storage Life (PQL) is 'the period of frozen storage after freezing of a high quality product during which the organoleptic quality remains suitable for consumption or for the process intended.'

The alterations in 'freshness' quality of stored frozen seafood products are related to physical, chemical and biochemical reactions that take place in the frozen muscle.

1.3. Changes in seafood muscle caused by freezing and cold storage

A considerable amount of information is available on the physical, chemical and biochemical changes, which take place due to freezing and storage of seafood products (reviewed by Love, 1966; Mills, 1975; Jul, 1984; Haard, 1992; Mackie, 1993; Sikorski and Kolakowska, 1994). The most important of these changes are discussed below.

1.3.1. Changes in the integrity of seafood muscles caused by freezing and cold storage.

Formation of ice crystals during freezing and re-crystallization of ice during frozen storage reduces the number of free water molecules, which causes changes in structural elements. Ice crystals may, also, grow under certain conditions and cause mechanical damage to cell membranes (Huber *et al.*, 1979). Therefore, freezing, frozen storage and thawing 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). These changes have been considered to be the result of either one or both of the following: (a) denaturating of cellular wall proteins by the concentration of salts, or (b) disruption of cellular membranes by the formation ice crystals (Love, 1966).

Love (1955; 1958 ab) and Love and Karsti (1958) studied the effects of ice crystals formation on tissue damage of post-rigor cod fillets and found that small intra-cellular ice crystals caused little change in cells, whereas large inter-cellular ice crystals distorted and shrunk the fibres. Large intra-cellular ice crystals disrupted the cells. Similar results were obtained for chicken breasts (Grigler and Dawson, 1968) and beef muscles (Bevilaqua *et al.*, 1979; Añòn and Calvelo, 1980; Grujic *et al.*, 1993).

The alterations in muscle integrity, which are caused by different freezing

treatments and during frozen storage, influence the quality of the frozen seafood products. For instance, the enzymes that are released from intra-cellular organelles of a frozen muscle tissue may be more active than in the bound state (Hultin, 1985). Thus, the release of dehydrogenases from mitochondria might influence the redox potential of tissues, and the release of lipases from lysosomes may cause more rapid breakdown of lipids (Shewfelt, 1981; Civera *et al.*, 1996). The mechanical damage of sarcollemas and/or the allocation of the intra-cellular water to inter-cellular spaces of frozen seafood products may result in high drip losses during thawing. This has several economic disadvantages e.g. loss of weight, an unpleasant appearance; and the wet surface promotes bacterial spoilage. There is also a loss of valuable nutrients that are dissolved in the exudates, such as proteins, vitamins and minerals, as well as a loss of flavour components (Hamm, 1986).

1.3.2 Changes in proteins of seafood muscles caused by freezing and cold storage.

Undesirable textural changes, e.g. development of toughness, dryness and loss in tenderness, are a major consideration in judging the quality of frozen seafood products. In addition, several seafoods do not have a strong flavour and, therefore, texture becomes very important for their acceptance by consumers (Hyldig and Nielsen, 2001).

Also, irreversible changes in muscle proteins may cause undesirable alterations in colour and appearance (Shenouda, 1980; Mackie, 1993).

In the following sections, basic aspects of protein denaturation in the frozen seafood muscle are discussed.

1.3.2.1. Definition of protein denaturation

The native structure of proteins is a consequence of the balance between a series of different intra-molecular forces (i.e. Van der Waals, electrostatic interactions, hydrogen bonds, hydrophobic interactions and disulphide bonds) and the loss of conformational entropy (Howell, 1992). The native conformation of proteins is marginally stable and therefore any stressing effect can cause its denaturation (Cheftel *et al.*, 1985).

Protein denaturation is 'any modification in conformation (secondary, tertiary or quaternary) not accompanied by the rupture of peptide bonds involved in prime structure' (Cheftel *et al.*, 1985). Denaturation of proteins is an elaborate phenomenon during which new conformations appear, although often intermediary and short-lived Denaturation of proteins involves unfolding of the compact tertiary and quaternary protein chain structure by breaking the covalent (disulphide) and non-covalent (electrostatic, hydrogen and hydrophobic) bonds that stabilize the native conformation (Howell, 1992).

It has been postulated that, depending on the extent of denaturation, protein in the form of an extended hydrated random polypeptide coil can be involved in secondary interactions with the formation of intra-/inter-molecular covalent cross-links. Furthermore, aggregation between native protein molecules may also occur, leading to loss of extractability and alterations of the properties of the proteins (Sikorski and Kolakowska, 1994).

Denaturation may be reversible or irreversible. When disulfide cross-links contribute to the conformation of the protein and if these are broken, denaturation is often irreversible. The sensitivity of a protein to denaturation is related to the readiness with which a denaturating agent breaks the interactions or linkages that stabilize the protein's secondary, tertiary, or quaternary structures (Cheftel *et al.*, 1985).

1.3.2.2. Factors causing protein denaturation during freezing and frozen storage.

According to (Shenouda, 1980) the factors causing protein denaturation in the frozen fish muscle are related to:

a) changes in fish moisture

b) changes in fish lipids, and

c) the activity of a specific enzyme (trimethylamine oxidase (TMAOase)

a) Moisture as a factor in the denaturation of fish protein

Changes in the moisture phase during freezing and frozen storage of fish create an environment that is conductive to protein denaturation. The effects of these changes can be classified to three major patterns:

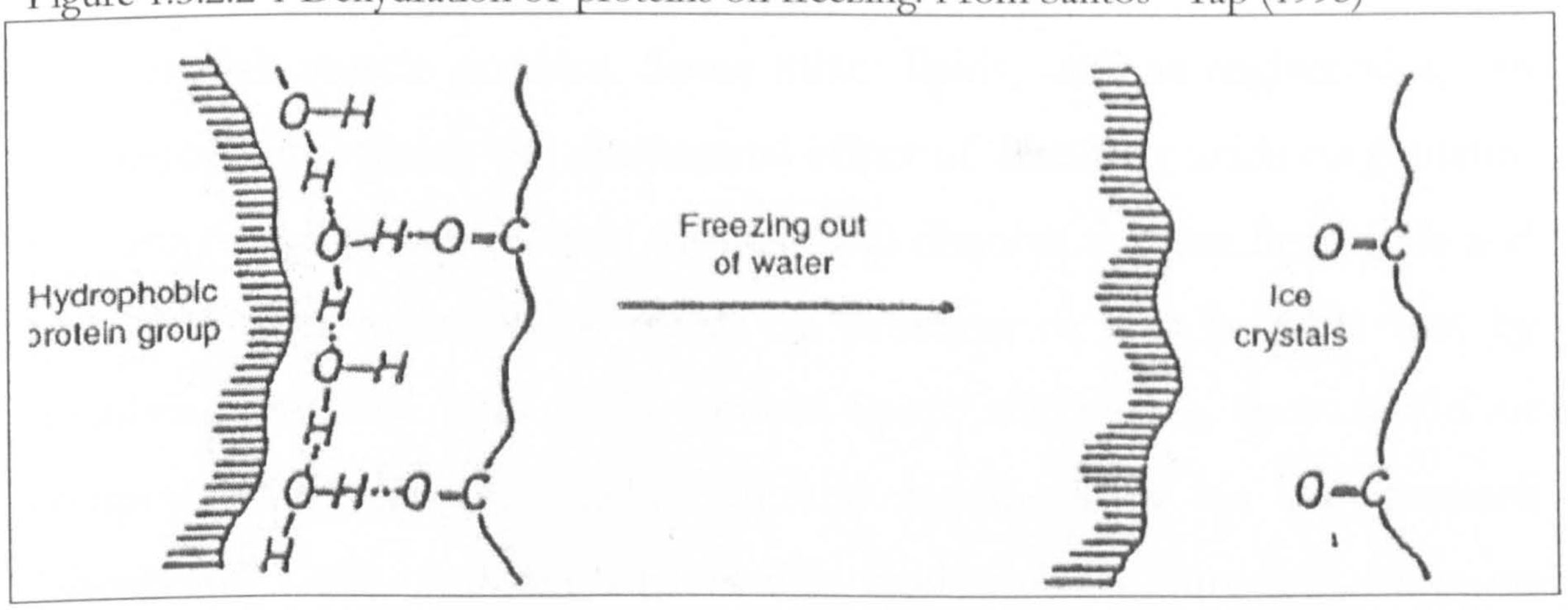
- damage due to formation of ice crystals,
- damage due to dehydration, and
- damage due to an increase in salt concentration.

Ice crystals formation and accretion due to freezing and prolonged storage of frozen seafood muscle may decrease the centre-to-centre distance between the thick filaments of the sarcomere (Liljemark, 1969). The reduction of the distance between filaments favours the formation of cross-linkages between molecules and stiffens the frozen fish fibres. Such inter-molecular cross-linkages result in aggregation, which leads to the formation of high molecular weight polymers and subsequent loss of extractability and alterations of the properties of proteins (Matsumoto, 1979).

The dehydration theory suggests that protein denaturation can be caused by freezing out of water. The conformation of most native proteins has hydrophobic side chains buried inside the molecule, some of them being exposed at the molecular surface (Figure 1.3.2.2-1, below). It has been suggested that surrounding water molecules arrange themselves around these exposed hydrophobic side chain groups (Lewin, 1974 cited in Santos-

Yap, 1995), thereby forming a network of hydrogen bonds which contributes to the stability of the three-dimensional protein structure (Figure 1.3.2.2-1, below). As water molecules freeze out, they migrate to form ice crystals, which result in the disruption of the hydrogen bonding system that stabilizes the protein structure. As the freezing process continues, the hydrophobic and hydrophilic regions of the protein molecules become exposed to a new environment, which may allow the formation of molecular cross-linkages (Matsumoto, 1979) either within the same protein molecule, causing deformation of their three-dimensional structure, or between two adjacent molecules, leading to protein-protein interaction.

Figure 1.3.2.2-1 Dehydration of proteins on freezing. From Santos –Yap (1995)



Freezing does concentrate solids, including mineral salts and small organic molecules, within the remaining unfrozen aqueous phase in the cell. Theoretically, the effect of salt concentration on protein denaturation, aggregation, or dissociation could be based on the effect of salts on the secondary forces (ionic, van der Waals, hydrogen, and hydrophobic), which help to stabilize the tertiary and quaternary configuration of protein macromolecules. The stability of ionic bonds, as well as other secondary forces in protein molecules, is dependant on the dielectric constant, the pH, and the ionic strength of the media. Thus, an increase in salt ions may cause

competition with the existing electrostatic bonds in proteins and break down some of them. It may also disturb other secondary forces; and although the net result cannot be precisely predicted, it will be a mixture of dissociational, aggregational, and conformational changes (Shenouda, 1980)

b) Factors related to fish lipids

The effects of lipids and their degradation products can be considered from three aspects:

- whole lipids
- free fatty acids, and
- products of oxidation.

There are conflicting views in the literature on the effects that whole lipids have on fish muscle proteins. Some intact lipids, such as triglycerides, can counteract or diminish the detrimental effect of free fatty acids on proteins. It is assumed that neutral lipid droplets will dissolve the free fatty acids and neutralize their hydrophobic effect on proteins; or it is possible that by dissolving the free fatty acids, neutral lipids will reduce their action or compete with free fatty acids for the binding sites on the proteins (Shenouda, 1980). Another view is that lipids exert a damaging effect on proteins. The destruction of membranes by ice crystals liberates lipids and proteins from their natural compartments, leading to formation of new lipoprotein complexes. Since these complexes are dissimilar to natural ones, this may affect the texture of fish muscle (Shenouda, 1980; Mackie, 1993).

The result of the presence of free fatty acids in frozen fish tissue, as it was postulated by Sikorski and Kolakowska (1994), is that the free fatty acids attach themselves (either hydrophobically or hydrophilically) to an appropriate site on the protein surface. Consequently, they can create more hydrophobic regions in place of polar or charged groups and surround the protein surface with a more hydrophobic microenvironment. The end result

is a decrease in protein solubility in aqueous buffers, or further inter-molecular linkages extensive enough to decrease extractability (Shenouda, 1980).

Several reports indicate that the unstable free radicals, formed during autoxidation of free fatty acids, attack the protein molecules, leading to the formation of protein free radicals (e.g. Saeed and Howell, 2002; Figure 1.3.2.2-2).

Figure 1.3.2.2-2 Potential reactions of proteins with radical and their oxidation products. From Santos-Yap (1995)

LH + R □ LOO + LOOH + RH + S

PH +LOO + S □ P + LH + LOOH + POO + PHS + PHSPH

P+ P □ PP

P + POO □POOP

P + L □PL

POO + L □ POOL

POO + LH □ POOL + H + POOH + L

PH + S □ PHSP

Figure notes: LH= intact free fatty acid, R= lipid radical, L00= peroxide radical, L00H= hydroperoxide, S= lipid scission product, PH= protein, P= protein free radical

These protein free radicals may cross link with other proteins to form protein-protein aggregates (PP) and with lipids to form protein–lipid aggregates (PL). Such free radicals may also initiate other reactions, as shown in Figure 1.3.2.2-3 (Santos-Yap, 1995).

Another possible mechanism for reaction between oxidized lipids and proteins occur through stable oxidation products of lipids such as malondialdehyde, butyaldehyde, propanal, and hexanal, which covalently react with specific functional groups on protein side chains, including the –SH group of cysteine, the amino group of lysine, and the N-terminal amino

group of aspartic acid, tyrosine, methionine, and arginine, leading to the formation of stable protein-lipid aggregates (Haard, 1992; Santos-Yap, 1995; Saeed and Howell, 2002). Such interactions also increase the hydrophobicity of proteins, making them less water soluble (Shenouda, 1980). Also a stronger hydrophobic interaction may occur between individual filaments, entangling them into aggregates (Buttkus, 1970).

c) Enzymatic activity of trimethylamine oxidase (TMAOase)

The enzyme trimethylamine oxidase (TMAOase) exists in a limited group of fish, particularly in gadoid fish, and is capable of activating, *in vivo* and *in vitro,* the degradation of trimethylamine oxide (TMAO) to dimethylamine (DMA) and formaldehyde (FrHO) at freezing temperatures. Formaldehyde, formed by this reaction, is very reactive and capable of interaction with many functional groups of proteins including intra- and inter-molecular cross-links. Thus, it was presumed that the rapid textural deterioration of frozen stored fish, which contain TMAOase, was due to production of formaldehyde from the above- mention reaction (Amano and Yamad, 1965 cited in Shenouda 1980). However, other findings throw doubt on the role of formaldehyde as a methylene cross-linking agent acting during frozen storage of cod and similar species (Badii and Howell, 2002).

1.3.3 Changes in lipids of seafood muscles caused by freezing and cold storage

The lipids of seafood are highly unsaturated in nature and thus are susceptible to oxidation during post mortem storage. Production of off-flavour compounds by lipid oxidation constitutes the primary decrease in quality of stored frozen seafood products. In addition, lowered nutritional value, changed texture and colour may also result from lipid oxidation (Shenouda, 1980; Undeland, 1997).

Total lipid content is highly variable among and within different fish species. Lean fish such as cod and haddock contain less than 2% lipid, whereas in fatty species, such as mackerel and herring, values of lipids range from 2% up to 30%. Seafood lipids can roughly be divided in two main groups, i.e. neutral and polar lipids. The former consists of triglycerides (TG), which occur as large droplets within the adipose tissue or as smaller droplets within the muscle cells or inter-cellular space. The content of triglycerides correlates positively to the total fat content of the fish and type of tissue, thus it varies widely due to species, season, diet, geographic origin etc. The polar lipids consist mainly of phospholipids (PL) and are important constituents of membranes. Therefore, the total amount of PL is relatively constant among different fish, and also, is independent of environmental factors (Undeland, 1997).

Degradation of lipids in frozen seafood muscle falls into two broad categories:

- hydrolysis of lipids, and
- oxidation of lipids.

These categories of degradation of lipids are discussed below.

1.3.3.1 Hydrolysis of lipids in frozen seafood muscle

In frozen seafood muscle, phospholipids and triglycerides are hydrolyzed by phospholipases and lipases, respectively (Shewfelt, 1981; Nawar, 1985). Fish lipases and phospholipases retain much of their activity in frozen fish and some may be activated (Sikorski and Kolakowska, 2000).

Hydrolysis of phospholipids in fish muscle may occur at a faster rate at temperatures below the freezing point than at temperatures above it. This appears to be due to cellular disruption rather than to dehydration or concentration effects caused by freezing and during frozen storage (Haard, 1992). Freeze activation of phopholipase A has been suggested by Olley and Lovern (1960). In addition, lysosomal lipases in fish muscle can be activated by temperature fluctuation and slow freezing, as it was shown for trout muscle by Geromel and Motgomery (1980) (cited in (Shewfelt, 1981).

The lipolytic activity of fish tissue appears to be species dependent (Shewfelt, 1981). Studies on lipid damage during frozen storage have been focused mostly on fatty species, e.g. horse mackerel (Aubourg *et al.*, 2004), mackerel (Stodolnik, 2005) and farmed steelhead (Ozbay, 2006). Concerning the origin of free fatty acids during frozen storage, some authors have reported a decrease in phospholipid content in lean fish as the only source of free fatty acids (e.g. Haardy *et al.*, 1979 cited in Shewfelt, 1981). Other investigators suggested that in hake species stored at -18°C, the free fatty acids were produced by both neutral lipids and phospholipids (Koning *et al.*, 1987; Koning and Mol, 1990). However, Rordan *et al.* (2005) found that the gonadal condition of hake influenced the lypolytic activity of muscle during freezing and frozen storage. Lipolysis occurs principally on phospholipids in frozen stored fillets from pre-spawned hake, whereas the triacylglycerols were the main substrates hydrolyzed during the frozen storage of fillets from post-spawned hake.

The role of lipolysis in deterioration of frozen seafood quality remains

uncertain despite a general correlation between free fatty acid production and quality deterioration. Available information suggests that triglyceride hydrolysis stimulate lipid oxidation whereas phospholipids hydrolysis retards lipid oxidation (Shewfelt, 1981).

1.3.3.2 Oxidation of lipids in frozen seafood muscle

Peroxides are the main initial products of lipids' oxidation and their measurement is an useful indicator of uptake of oxygen in the early stages of oxidation of lipids (Perez-Villarreal and Howgate, 1991). Hultin (1988) stated that lipid oxidation occurs via a free radical process as described below, where RH represents a fatty acid:

INITIATION	$RH + O_2 \rightarrow$ free radicals ($R^{\cdot}$, $ROO^{\cdot}$)
PROPAGATION	$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$
	$ROO^{\cdot} + RH \rightarrow ROOH + H^{\cdot}$
	$ROOH \rightarrow RO^{\cdot} + {\cdot}OH$
TERMINATION	$R^{\cdot} + ROO^{\cdot} \rightarrow ROOR$
	$R^{\cdot} + R^{\cdot} \rightarrow RR$
	$2ROO^{\cdot} \rightarrow ROOR + O_2$

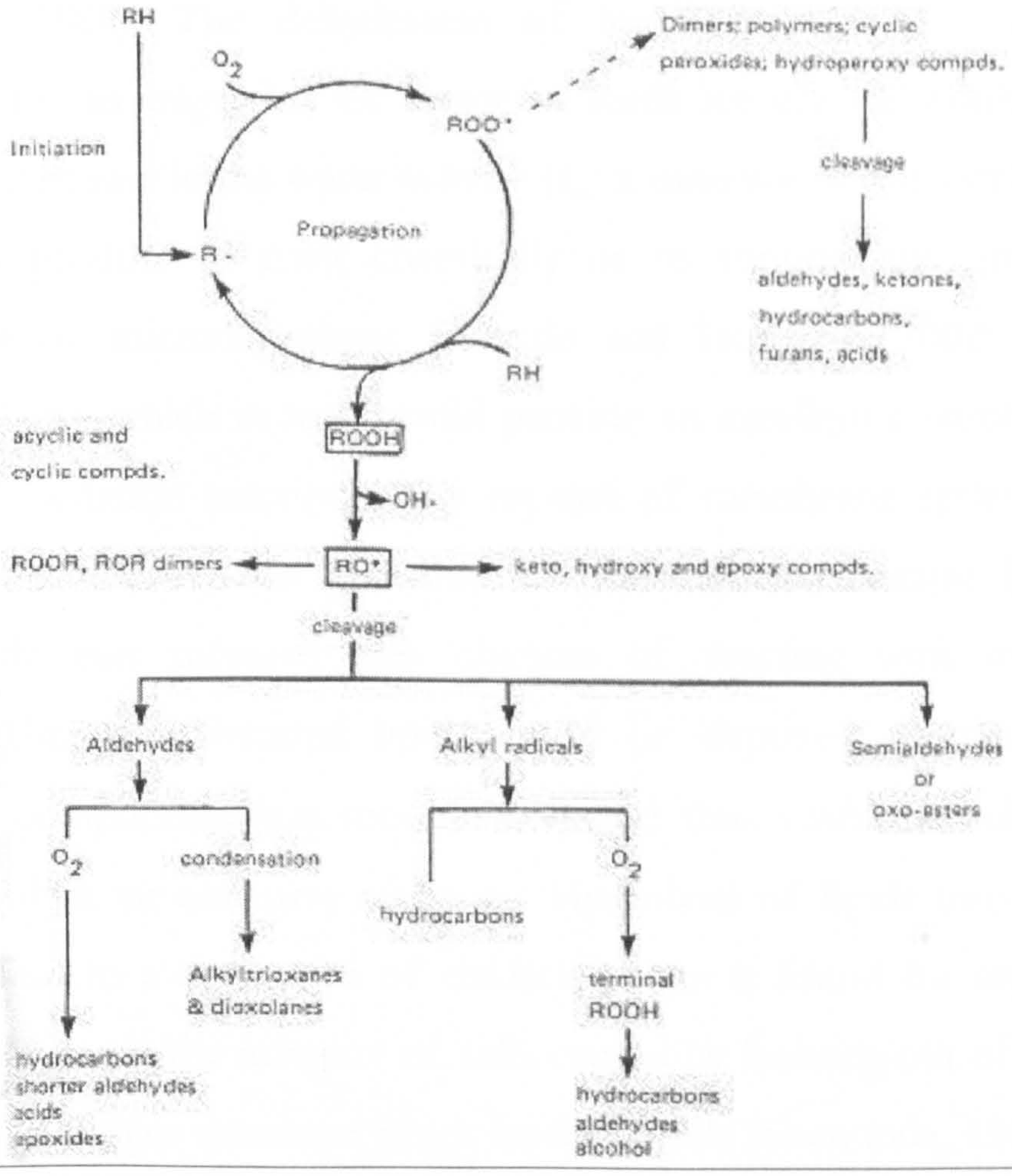
Three distinct types of initiation reactions have been identified. The process may initiate by extraction of hydrogen from a fatty acid. This type of initiation, which is usually called 'true autoxidation', can be brought about by any substance having substantial energy to abstract hydrogen from a methylene group of a free fatty acid. Another type of initiation of the reactions involves the addition of singlet oxygen over the double bond of fatty acid. In fish tissue, possible sensitizers of ground oxygen to form singlet oxygen are flavins, retinals or porphyrins. A third type of initiation is the addition of oxygen to a fatty acid molecule catalyzed by enzymes, e.g. lipoxygenases. Thus, the two later types of initiation of the reactions include

the direct formation of peroxy-radicals (ROO·), whereas in the first one, initiation proceeds through production of a free alkyl-radical (R·), which then rapidly reacts with oxygen giving rise to peroxy-radicals (ROO·). This step, together with subsequent reaction between peroxy-radicals (ROO·) and a new fatty acid (RH), is referred as 'propagation' process. The result of the 'propagation' process is the production of free alkyl-, alkoxy- and peroxy-radicals (R·, RO·, ROO·). After the production of a large number of free radicals, two free radicals may combine to terminate the 'propagation' process (termination stage).

Peroxides are tasteless and odourless and are generally referred to as primary oxidation products. They enter into numerous and complex breakdown and interaction mechanisms responsible for the production of myriad compounds of various molecular weights. A general scheme summarizing the overall picture of lipid autoxidation is given in Figure 1.3.3.2, below.

Through scission reactions, peroxides can be cleaved into various secondary products like aldehydes, ketones and alcohols. In fish, many of the unsaturated aldehyldes and ketones formed have very low odour thresholds, but are responsible for fishy, oily or rancid flavour. Some of the secondary products can react further, e.g. with compounds containing free amino groups, yielding tertiary products such as Schiff's bases. The latter are proposed as being precursors for compounds that are responsible for yellow-brownish discoloration of fish tissue (Nawar, 1985; Undeland, 1997).

Figure 1.3.3.2 Generalized scheme for autoxidation of lipids. From Nawar (1985)

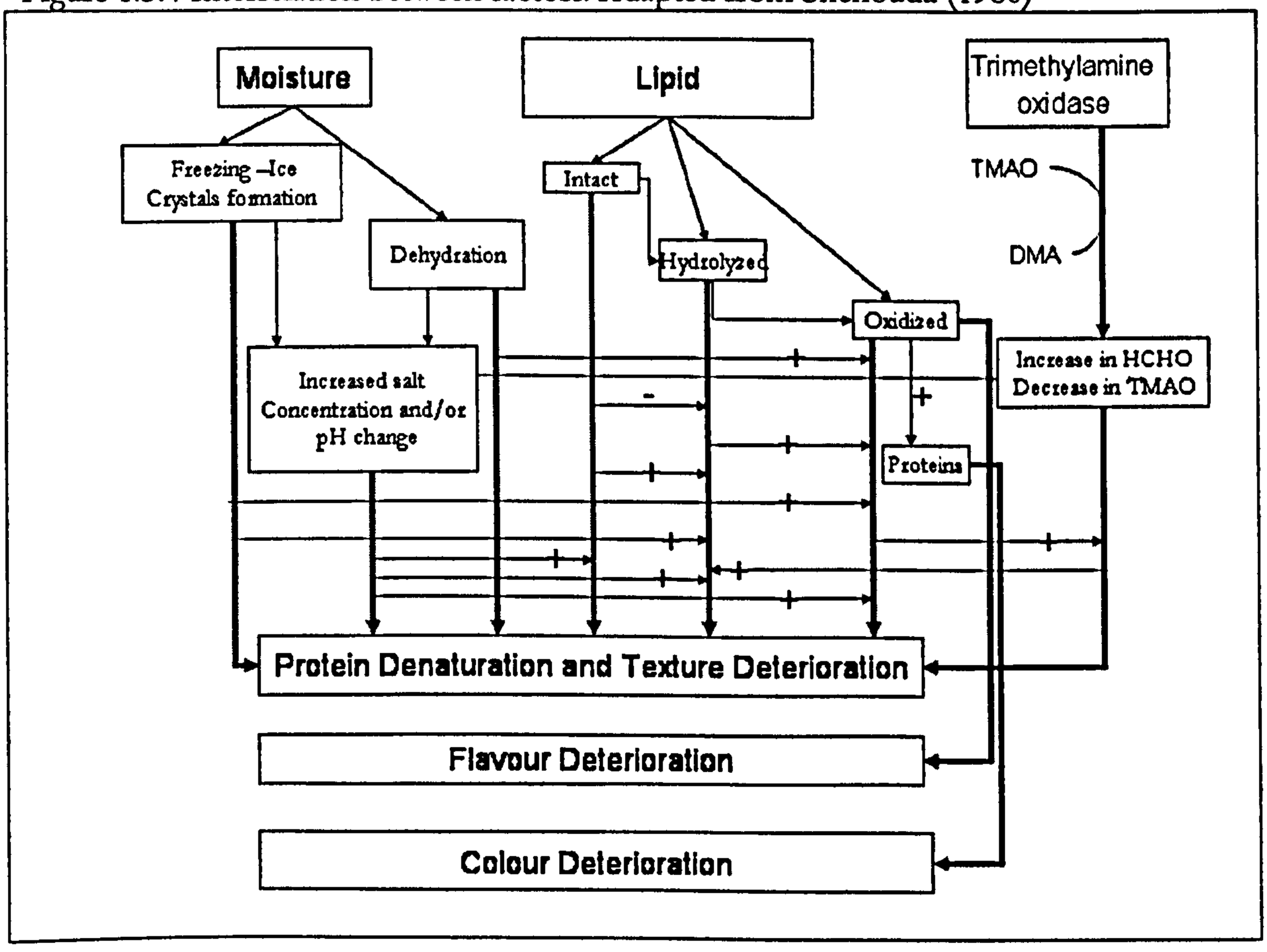


1.3.4. Interrelation between factors

In previous sections, direct effects of factors related to changes in muscle integrity, proteins denaturation and degradation of lipids on several sensory attributes (e.g. texture, flavour and colour) caused by freezing and during storage of seafood products were discussed. Figure 1.3.4 shows these effects by vertical arrows. However, the above mentioned factors may interact (horizontal arrows in Figure 1.3.4) and eventually influence sensory characteristics indirectly. This means that the increase in concentration of a product of a reaction could have a positive effect (accelerate, catalyze, or trigger) or a negative effect (inhibit or decrease) on the rate of other reactions. In addition, for instance, to the direct effect of salt concentration (as a consequence of freezing) on protein denaturation (texture deterioration), high salt concentrations and activation of lipases may

stimulate hydrolysis of lipids and accelerate the liberation of free fatty acids (Shenouda, 1980). The dehydration of localized areas or sub-cellular structures due to migration of water to form ice crystals could cause a substantial decrease in the water activity (a_w; a measure of the availability of water in a product to react chemically or to support the growth and metabolism of microorganisms (Whittle and Howgate, 2002) of these confined places, which in turn would provide an excellent environment for faster lipid oxidation reactions. The rupture of membrane systems by the formation of ice crystals probably liberates the membrane lipids and consequently may increase their chances of reacting with myofibrillar proteins. Also, the liberated lipids could be deprived of their natural protection compounds (e.g. tocopherols) and thus could be subjected to faster hydrolytic or oxidative reactions. Hydrolysis of lipids into free fatty acids will lead to a faster rate of oxidation than is found for intact lipids. Overall, changes in the integrity of cells caused by freezing out of the water in cells could trigger reactions which involve lipids (Shenouda, 1980). These reactions may lead to accumulation of secondary oxidation products of lipids, which could influence directly the texture and flavour or which could interact with proteins and thereby cause off colours. On the other hand, dispersion of some deposited fats may help to dissolve the free fatty acids and thus reduce their damaging action on proteins.

Figure 1.3.4 Interrelation between factors. Adapted from Shenouda (1980)



TMAO has shown a synergistic effect on the activity of γ-tocopherols in inhibition of lipid oxidation. Thus a depletion of TMAO, due to the activity of the TMAOase, will indirectly accelerate the autoxidation reactions of lipids. Oxidized lipids could furnish the necessary reduced media for maximum activity of TMAOase. This in turn could increase the concentration of formaldehyde, which was found to accelerate the hydrolysis of some fish lipids (Shenouda, 1980).

Therefore, different interrelated secondary reactions in the frozen tissue may alter (decrease or increase) the concentration of certain substances (e.g. free fatty acids, oxidized lipids' products), which may directly or indirectly affect certain sensory attributes of stored frozen seafood products.

In summary, freezing and frozen storage are believed to furnish favourable conditions for alterations in muscle integrity, proteins and lipids. Knowledge about these changes is important if deterioration of frozen seafood quality

is to be more fully understood and avoided.

1.4. Methods used to detect changes in frozen fish

To quantify the undesirable changes in 'freshness' quality of frozen seafood, scientists have tried to correlate them with various analytical parameters. Methods included in this review are limited to the most common of those that have been applied to measuring changes in fibres, proteins, lipids and sensory attributes during frozen storage of seafood products.

1.4.1 Methods related to the integrity of fish muscle

Light, transmission electron or scanning electron microscopic techniques have been used to show differences in size and location of ice crystals and changes in structure of muscle fibres of meat products on freezing and during frozen storage of seafoods (including Love,1955; Love, 1958ab; Love and Karsti, 1958; Pan and Yeh, 1993; Chen and Pan, 1997; Chevalier *et al.*, 2000; Sigurgisladottir *et al.*, 2000; Chevalier *et al.*, 2001). A drawback of these techniques is that the fixing techniques of frozen tissues may create artifacts, which can alter the ultra-structure images or mask the micro-changes between different treatments (Shenouda, 1980).

The freeze-damage of fish muscle can be studied also by the activities of enzymes in muscle tissue fluids, enzymes that in fresh tissue are retained in sub-cellular organelles. The enzymes α-glucosidase and β- N- acetyl-glucosaminidase are not specifically associated with any particular sub-cellular organelle, but the activity of these enzymes under acid assay conditions is regarded as evidence of lysosomal activity i.e. cell damage (Barrett and Heath, 1977).

The activities of α-glucosidase and β- N- acetyl-glucosaminidase in fluids obtained by centrifugation i.e. 'centrifugal tissue fluids', and the volume (or weight) and protein content of fluids, have been found to correlate with the cellular damage caused by different freezing and thawing treatments, and during cold storage of several frozen fish species (Rehbein *et al.*, 1978;

Nilsson and Ekstrand, 1993; Nilsson and Ekstrand, 1995ab; Benjakul and Bauer, 2000; Rehbein, 2000; Benjakul and Bauer, 2001); Dulfos *et al.*, 2002; Benjakul *et al.*, 2003).

The mitochondrial enzyme β-hydroxy-acyl-coenzyme A -dehydrogenase (HADH) is tightly bound to the inner mitochondrial membrane and is involved in the oxidation of fatty acids in cells (Gottesman and Hamm, 1983). HADH activity in exudates, which can be revealed by adding to them aceto-acetyl-coenzyme A, is a measure of the damage caused in mitochonria by various freezing and thawing treatments of meat products (Hamm and Gottesmann, 1982; Gottesman and Hamm, 1983). The HADH activity in extracts of fish and shellfish has mostly been found to increase due to freezing (Gartia de Fernando *et al.*, 1992; Hoz *et al.*, 1992; Hoz *et al.*, 1993; Fernandez *et al.*, 1999; Dulfos *et al.*, 2002) and for certain species, a trend to increase in HADH activity with decreasing freezing temperatures has been recorded (Gartia de Fernando *et al.*, 1992; Pavlov *et al.*, 1994).

1.4.2 Methods related to changes in proteins

Methods used to study the changes that occur in seafood proteins during frozen storage are presented in the Table 1.4.2., below. The most common methods are discussed in the following sections.

1.4.2.1. Protein solubility or extractability methods

Preliminary work by Reay (1933, 1934 cited in Mills, 1975) and Reay and Kuchel (1936 cited in Mills, 1975) showed that a decrease in the amount of soluble proteins in neutral salt solution occurs during storage of frozen fish. Later, it was shown that the mentioned decline was due to a decrease of the myofibrillar fraction of total proteins of frozen fish muscle (Dyer and Dingle 1961 cited in Mackie, 1993). Thus, by determining the salt soluble protein of a muscle tissue, one can estimate the denaturation of myofibrillar proteins.

A main drawback of these tests is that the extracting conditions are not standardized. For instance, the ionic strength, pH, the type of salt, the buffering capacity of the extracting solution may vary. Dissimilarities also exist in the ratio of muscle to solution, and in the duration and speed of the mincer. All these variables are rarely duplicated in the literature, in spite of their importance for determining the degree of solubilizing of proteins (Shenouda, 1980).

Salt soluble proteins have been found to decrease during storage in a number of frozen fish species, i.e. in sardines (Suarez *et al.*, 2002), in cod and haddock fillets (Badii and Howell, 2001,2002), in Atlantic mackerel (Saeed and Howell, 2002), in several tropical fish(Benjakul *et al.*, 2005), in whole and fillets of lizardfish (Leelapongwattana *et al.*, 2005), in common carp (Ganesh *et al.*, 2006), in squids (Paredi *et al.*, 2006) and in whole and gutted gilthead seabream (Huidobro and Tejada, 2004). Several of these studies have shown a clear relationship between the decrease in the salt-soluble proteins and the

increase in the toughness in frozen fish products.

Table 1.4.2 Methods used to detect or monitor changes or deterioration in fish protein during frozen storage. From Shenouda (1980).

1. Extractability of fish proteins
    - Total extractable proteins
    - Protein groups: myofibrilllar, sarcoplasmic, actomyosin
    - Protein species: myosin, actin, tropomyosin, etc.
2. Protein solubility in
    - Aqueous buffers
    - Detergents
    - Proteolytic enzymes
    - Tissues and texture
    - Drip-thaw
    - Water-holding properties
    - Objective textural measurements
4. Ultra-structure features
    - Light microscopy, scanning electron microscopy, transmission electron microscopy
5. Extracted proteins
    - Viscosity, molecular weight, specific volume
    - Functional groups: available lysine, reactable -SH
    - Spectrometric analysis: UV, ORD, NMR, IR, x-ray patterns
    - Mobility and fractionation under external forces:
        a)Ultracentrifuge sedimentation pattern
        b) Electrophoretic pattern
        c) Isoelectric focusing pattern
        d) Chromatographic separation (ion exchange, molecular sieves, adsorption systems)
6.. Enzymatic activity
    - ATPase, aldolase trimethylamine oxides, malic enzyme, glycerophosphate dehydrogenase
7. Formation of low-molecular-weight degradation products:
    - Lipid hydrolysis: FFA
    - Lipid oxidation: ketones, aldehydes, peroxides, free radicals, TBARS
    - TMAO hydrolysis: formaldehyde, dimethylamine

1.4.2.2. Tissue properties and texture

In this group of methods intact fish muscle tissues are used. It includes tests related to thawing losses and other water holding properties of fish muscle, and objective measurements of texture by using instruments capable to stimulate mouth characteristics, as for instance hardness, chewiness and cohesiveness.

a) Thawing losses and water holding properties

'The water holding capacity of meat is its ability to bind the natural water content and the capacity of myofibrils to retain additional water' (Hamm, 1986).

Hamm (1986) proposed the following terminology for measurements of the water holding capacity depending on the phenomenon investigated:

- *Drip loss*: Formation of exudates from meat or meat systems (except thawing loss) without application of external forces.
- *Thawing loss*: Formation of exudates from meat systems after freezing, storage and thawing without application of external forces
- *Cooking loss*: Release of fluid after heating of meat or meat systems either without or with application of external forces (i.e centrifugation or pressing).
- *Expressible fluid*: release of fluid from unheated meat or meat systems (also after freezing and thawing) during application of external forces such as pressing (e.g. filter paper press method), centrifugation methods, or suction methods (e.g. capillary method).

Measuring the volume (or weight) of thawing loss and determining the changes in the water holding capacities of fish are among the simple methods to determine the capacity of fish muscle proteins to reabsorb the water of melted ice crystals during thawing. These changes are attributed to the surface de-hydration of myofibrillar proteins and/or, to a lesser extent,

to physical damage in the cells or cell membranes (Shenouda, 1980). The water holding property of meat and fish in terms of thawing loss, expressible fluid and cooking loss, has often been found to decrease following the process of freezing and thawing (without freezer storage; Añòn and Calvelo, 1980; Petrovic *et al.*, 1993; Benjakul and Bauer, 2000). These properties may be influenced by the rate of freezing (Añòn and Calvelo, 1980; Petrovic *et al.*, 1993); and/or by the water holding properties of the meat product (e.g. pH) prior to freezing (Hamm, 1975). Several investigations have shown that the quantities of exudates, in terms of thawing losses, expressible fluids and cooking losses, are influenced from the time a fish product is kept in the frozen state (Ciarlo *et al.*, 1985; Castro *et al.*, 1996; Simeonidou *et al.*, 1997; Chevalier *et al.*, 2001; Suarez *et al.*, 2002; Benjakul *et al.*, 2003; Huidobro and Tejada, 2004; Natseba *et al.*, 2005; Ozbay *et al.*, 2006; Dorado-Rodelo *et al.*, In Press).

b) Objective measurements of texture

Texture describes the structure of a tissue (Jellinek, 1993) and encompasses 'all the mechanical, geometrical and surface attributes of a product perceptible by means of mechanical, tactile and, whereas appropriate, visual and auditory receptors' (ISO 5492:1992).

Texture of seafoods is an important quality attribute that depends on several parameters, both intrinsic and extrinsic (Barroso *et al.*, 1998; Mackie, 1993). The predominant methods used to quantify textural attributes of frozen seafoods are sensory and instrumental methods. Objective evaluations of texture by sensory methods usually require highly trained panels and are time consuming (Hyldig and Nielsen, 2001). Hence instruments have been designed to stimulate the mouth-feel characteristics of a meat product, e.g. the cutting effect, hardness and tenderness (Botta, 1995; Barroso *et al.*, 1998; Hyldig and Nielsen, 2001).

The most common types of measurements of seafood texture by instruments are based on rheological principles:

- shear resistance,
- puncture, and
- compression

The most commonly used shearing devices are the 'Kramer shear-compression cell' and the 'Warner-Bralzer shear cell'. The 'Kramer shear-compression cell' consists of an upper part with blades that penetrate a lower part, which is a metal case with slots. Foods flow through the slots in the cell when force is applied, thus undergoing a process of extrusion combined with shearing and compression. The 'Warner-Bralzer shear cell' consists of a triangular blade that penetrates a device with slot. The seafood sample is cut guillotine-style and is subjected to a complex combination of strain, compression and shearing (Barroso *et al.*, 1998). Shear resistance of seafoods has been mainly studied during frozen storage. It has been found to increase in minced muscle of red hake and European hake, chopped fillets of European hake, cod fillets, and sticks and blocks of red hake fillets (reviewed by Barroso *et al.*, 1998). For some frozen species small changes in shear resistance may be found, as is the case of whole farmed gilthead seabream frozen and stored at -20°C for nine (Pastor *et al.*, 1999) or twelve months (Huidobro and Tejada, 2004).

The puncture test consists of measuring the force required to push a plunger into a food sample, which is thus subjected to a combination of compression and shearing. This method has been used mainly in assessing changes in texture of seafoods during storage in ice rather than in frozen storage (Barroso *et al.*, 1998; Hyldig and Nielsen, 2001)

Compression tests can include one or two successive compressions. Those with two successive compressions result in curves from which several texture parameters can be obtained directly or indirectly (e.g. Hyldig and

Nielsen, 2001). Compression tests showed that intensively farmed gilthead seabreams stored frozen for one month at -30°C were softer than the extensively farmed frozen stored gilthead seabreams (Orban *et al.*, 1997). Evaluation of the texture of stored frozen cod fillets with compression tests showed that increased length of time at -10°C caused hardening of the fillets (Badii and Howell, 2002).

1.4.2.3. Enzymatic activities

The special textural attributes of seafoods are due to functional properties of the myofibrillar proteins and to their structural configurations which constitute the contractile elements of the muscle fibres (i.e. myofibrils; Mackie, 1993).

Myofibrillar proteins, may suffer a number of functional changes on freezing and during storage which damage the structure of myofibrils and eventually the texture of frozen seafoods. Functional changes of myofibrillar proteins can serve to evaluate the textural alterations in frozen seafoods provided that their change with time of frozen storage has a trend which is easy to follow (e.g. linear; Careche, 1977).

Myosin, the major myofibrillar protein, is an ATPase. Changes in ATPase activities of myosin on freezing and cold storage indicate 'denaturation' in the sense used by protein chemists, i.e. a change in the conformation of polypeptide chain (Love, 1966). ATPase activity of pure myosin is stimulated by Ca^{2+} and is inhibited by Mg^{2+} ions.

When myosin is extracted from fish muscle, the extract contains myosin and other myofibrillar proteins (actin, tropomyosin, troponin e.t.c). This extract is called actomyosin and maintains myosin's enzymatic properties. There are three different types of ion-activated ATPases activities with actomyosin function:

- Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities.

- Ca^{2+}-ATPase activity, and
- Mg^{2+}-EGTA-ATPase activity

In contrast to pure myosin, actomyosin may be activated by Mg^{2+} and Ca^{2+}ions, due to the presence of actin in the actomyosin complex. Thus, Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities of actomyosin from fish extracts are related to the affinity of myosin to bind with actin in actomyosin complex. Ca^{2+}-ATPase activity of actomyosin is an indicator of the changes in myosin's head which contains the active site of the enzyme complex. Thus, changes in Mg^{2+}-ATPase, Mg^{2+}-Ca^{2+}-ATPase and Ca^{2+}-ATPase activities of actomyosin extracts on freezing and during frozen storage could imply a change in myosin's head, which contains both the active site of the enzyme and the actin interaction site (Wagner and Añòn, 1985). The tropomyosin -troponins complex is necessary for control by Ca^{2+} ions of actin-myosin interaction of vertebrate muscle (Huxley 1972 cited in Roura and Crupkin (1995). Thus, Mg^{2+}-ATPase activity of actomyosin without Ca^{2+}ions (i.e. Mg^{2+}-EGTA-ATPase activity) is connected to the functionality of the troponin- tropomyosin complex in actomyosin extract (Benjakul *et al.*, 1997). From Mg^{2+}-Ca^{2+}-ATPase and Mg^{2+}-EGTA-ATPase activities, Ca^{2+} sensitivity of actomyosin extract can be calculated. Ca^{2+} sensitivity is an indicator of Ca^{2+} regulation by myofibrillar proteins in actomyosin extracts. Therefore, the extent of denaturation of myofibrillar proteins can be indirectly measured by ATPase activities of actomyosin extracts. This makes ATPase activities useful for quality assessment of frozen seafood muscle.

It is well established from studies on fish and fish prducts, i.e. in mackerel and amberfish (Jiang *et al.*, 1985), in actomyosin from milk fish (Jiang *et al.*, 1988 b), in Alaska Pollock (Scott *et al.*, 1988), in mullet, pearlspot, milkfish and tilapia (Nambudiri and Gopakumar, 1992), in myctophid species (Seo *et al.*, 1997), in Atlantic mackerel (Saeed and Howell, 2002), in croaker, lizardfish, threadfish bream and bigeye snapper (Benjakul *et al.*, 2003), in

actomyosin from trout (Herrera and Mackie, 2004), in whole and fillets of lizardfish (Leelapongwattana *et al.*, 2005) in carp (Jarsa *et al.*, 2006), in squid (Paredi *et al.*, 2006) and in whole gilthead bream (Tejada *et al.*, 2003), that a loss in ATPase activities occurs following frozen storage. These changes, however, can vary depending on species, physiological state, type of muscle and *post-mortem* treatments of fish (Shenouda, 1980). In contrast, the effects of the freezing process itself on ATPase activities of actomyosin extracts from different species of fish seem to be contradictory. Several studies have shown that the freezing process itself produces no drastic changes (Reid *et al.*, 1986; Tejada *et al,* 2003), whereas others have shown significant reductions in the activity of Ca^{2+}ATPase activity of actomyosin extracts (e.g. Benjakul and Bauer, 2000).

1.4.2.4. Tests on extracted proteins

The physical and chemical properties of proteins extracted from frozen fish attempt to give information on the changes that have occurred in fish proteins at the molecular level. The emulsifying capacity, viscosity and the gel-forming properties show the general condition of fish proteins. Other tests on extracted proteins may show the changes of susceptible functional groups and consequently they can be used to reveal the existence of protein cross-linking or to predict deformation and explain aggregation phenomena (Shenouda, 1980).

Sulfhydryl groups may oxidize to disulfide bonds during denaturation of proteins. Thus the changes in total and surface (reactive) sulfhydryl groups of extracted fish actomyosin can be used to explain denaturation and aggregation phenomena due to freezing and in stored frozen fish (Shenouda, 1980). In early investigations, no significant changes in the number of sulfhydryl groups in muscles of stored frozen fish could be evidenced (Connell, 1968). However, cross – linking of proteins due to disulfide bond

formation may take place without a significant net change in the total sulfhydryl groups by sulfhydryl – disulfide exchange reactions (Buttkus, 1970). Furthermore, the results of several more recent studies showed significant decrease in total sulfhydryl content of actomyosin (or myosin) extracted from frozen fish. In most of these studies the decrease of the total and/or surface (reactive) sulfhydryl groups was correlated to the changes of ATPase activities and Ca^{2+}sensitivity of fish actomyosin extracts (Jiang *et al.*, 1988ab; Benjakul and Bauer, 2000;Ramirez *et al.*, 2000; Benjakul *et al.*, 2003; Herrera and Mackie, 2004).

A number of other techniques are included in this group of tests, which are used to explore the changes in shape, size, charge, weight and tertiary structure of fish protein molecules during frozen storage (Table 1.4.2, number 5).

1.4.2.5. Measurement of low-molecular-weight-degradation products

These tests give an indirect indication of certain reactions that ultimately lead to protein denaturation (see Table 1.4.2, number 7). As for instance, the enzymatic breakdown of trimethylamine oxide in certain fish species (e.g. cod) into formaldehyde has been used to evaluate the quality of the stored frozen fish. In this group of tests, certain methods related to degradation of lipids are included, which are discussed in the following section.

1.4.3 Methods related to lipid degradation

The most common tests, which are used to quantify chemically the degree of degradation of lipids, are the free fatty acids content, the peroxide value and thiobarbituric acid reactive substances (TBARS), which are discussed in the following section

1.4.3.1. Free Fatty Acid content

A number of studies have shown an increase in free fatty acid formation during frozen storage of seafoods. This is the case with mullet (Deng, 1978), whole fish and fillets of horse mackerel, hake (Koning and Mol, 1991; Simeonidou *et al.*, 1997; Aubourg *et al.*, 1999; Aubourg *et al.*, 2004), sardine mince (Verma *et al.*, 1995; Sarma *et al.*, 2000; Serdaroglu and Felekoglu, 2005), blue whiting (Aubourg, 1999), silver carp mince (Siddaiah *et al.*, 2001), mackerel (Stodolnik *et al.*, 2005) and Nile perch (Namulema *et al.*, 1999; Natseba *et al.*, 2005). Changes in free fatty acids have been shown to correlate with time of storage in several fish species (including hake Koning and Mol, 1991; Aubourg *et al.*, 1999), sardines (Verma *et al.*, 1995), blue whiting (Aubourg, 1999), mackerel (Stodolnik *et al.*, 2005), carp fillets (Sequeira-Munoz *et al.*, 2005) and jack mackerel (Aranda *et al.*, 2006) and deteriorations in sensory attributes (including hake (Koning and Mol, 1991); sardine mince (Verma *et al.*, 1995), silver carp mince(Siddaiah *et al.*, 2001) and mackerel (Stodolnik *et al.*, 2005). Altogether these findings imply that the detection of free fatty acids, at least in several species, could be used as an estimate of changes in the quality of stored frozen fish.

1.4.3.2. Peroxide Value

Peroxides can be measured by techniques based on their ability to liberate iodine from potassium iodide, or to oxidize ferrous to ferric ions. Their content is usually expressed in terms of millequivalents of oxygen per

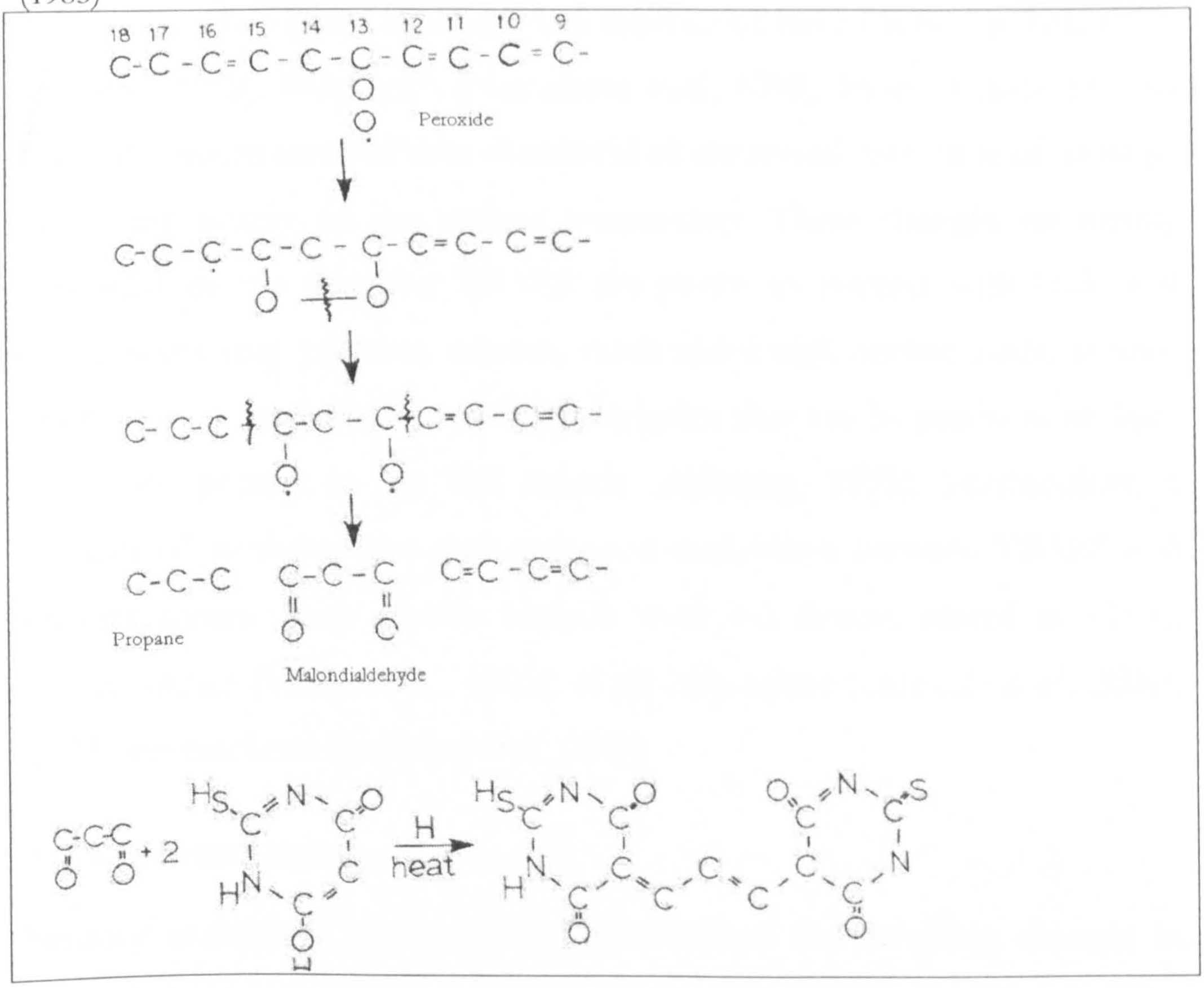
kilogram of fat (the 'peroxide value'; (Nawar, 1985).

The changes in peroxide value during storage were studied in numerous frozen fish species and products, i.e. mullet (Deng, 1978), European hake (Perez-Villarreal and Howgate, 1991), horse mackerel and Mediterranean hake (Simeonidou *et al.*, 1997; Stodolnik *et al.*, 2005), blue whiting (Aubourg, 1999), albacore tuna (Ben-Gigirery *et al.*, 1999), sardine mince (Verma *et al.*, 1995; Serdaroglu and Felekoglu, 2005), sardines (Sarma *et al.*, 2000) and silver carp mince (Siddaiah *et al.*, 2001). In most cases the peroxide value reached a maximum and then decreased or leveled off. These temporal changes in peroxide values were attributed to the decomposition or interaction of peroxides with other constitutes of frozen muscle. Thus, different authors concluded that the determination of peroxide value cannot always provide an accurate method for quality assessment. However several studies have shown that the peroxide value correlated well with the time of storage and/or with the changes in texture, flavour/ odour, appearance and acceptability of frozen stored fish and fish products (including European hake (Perez-Villarreal and Howgate, 1991), silver carp mince (Siddaiah *et al.*, 2001), sardine (Verma *et al.*, 1995), horse mackerel (Stodolnik *et al.*, 2005).

1.4.3.3. Thiobarbituric acid reactive substances (TBARS) test

This is one of the most widely used tests for evaluating the extent of lipid oxidation in biological food materials. Oxidation products of unsaturated systems produce colour reactions with thiobarbituric acid. It is believed that the chromagen results from condensation of two molecules of thiobarbituric acid with one molecule of malondialdehyde, which is a product of lipid oxidation and is considered the major thiobarbituric reactive substance (Figure 1.4.3.3; Nawar, 1985; Hoyland and Taylor, 1991).

Figure 1.4.3.3 Speculative mechanism for malondialdehyde formation. From Nawar (1985)



However malondialdehyde is not always found in all oxidized systems. Many alkanals, alkenals, and 2,4-dienals produce a yellow pigment (λmax=450nm) in conjunction with thiobarbituric acid , but only dienals produce a red pigment (λmax=530nm). (Nawar, 1985). Thus, the TBARS test not only measures malondialdehyde content, but also a wide range of lipid metabolites produced during processing and storage of foods (Hoyland and Taylor, 1991). Although interfering substances for this assay have been reported, the method is still used as a general index of oxidation of lipids, with results referred to as TBA-reactive substances (TBARS; Hoyland and Taylor, 1991; Botsoglou et al., 1994; Draper et al., 1993).

Changes in TBARS during cold storage have been studied in mullet (Deng, 1978), chub mackerel and smooth hound (Vareltzis *et al.*, 1988), horse

mackerel, hake (Simeonidou *et al.*, 1997), albacore tuna (Ben-Gigirery *et al.*, 1999), blue whiting and light and dark muscles of hake (Aubourg *et al.*, 1999; Aubourg, 1999), Nile perch (Namulema *et al.*, 1999). In most cases TBARS reached a maximum and then fluctuated or decreased with time of storage, depending mostly on the storage temperature. These changes are mainly explained by the fact that TBARS are prone to interact with biological constituents (e.g. proteins, amines, nucleosides and nucleic acids, amino-containing phospholipids, or other aldehydes that are by-products of lipid oxidation) present in the fish muscle (Aubourg, 1993). Furthermore, a number of workers have reported good correlation between TBARS and sensory scores. Such reports include work on frozen, stored at -20°C, sardine mince (Verma *et al.*, 1995), silver carp mince (Siddaiah *et al.*, 2001), and horse mackerel (Aubourg *et al.*, 2004).

1.4.4. Sensory tests

Sensory evaluation is the most direct method for detecting changes in flavour, odour, texture and appearance in frozen (meat) products. Most standards or specifications of frozen meats include assessment by tasting and the applicability of chemical or instrumental methods to assessing changes is usually judged by their performance against sensory assessment (Mills, 1975).

According to ISO 6658:1985, sensory tests of food products are divided into three groups:

a) difference tests used to determine whether a sensory difference exists between two products', which are divided to:

- paired comparison tests,
- triangular tests,
- duo-trio tests,
- two-out-of five tests, and

- 'A'- 'not A' tests

b) tests using scales and categories, to estimate the order or size of differences or the categories or classes to which samples should be allocated', which are divided to:

- ranking tests,
- classification tests,
- rating tests,
- scoring tests, and
- grating tests

c) analytical or descriptive tests, used to identify attributes present in a sample; the tests may also be quantitative, which are divided to:

- simple descriptive tests, and
- quantitative descriptive and sensory profiling tests

Although difference tests are simple, they require a large number of trained (selected) assessors or untrained assessors depending on the specific test applied. Thus, whenever a large number of samples is to be assessed, these tests are uneconomical. In addition difference tests, do not reveal the specific attributes that provoke the sensory quality changes of the evaluated foods (Carbonell *et al.*, 2003).

The specific level of any particular sensory attribute of a product can be identified by using scales and /or descriptive tests. Descriptive analysis of a food product, which is performed by a group of expert or selected assessors, is very sophisticated and extremely demanding (ISO 6658:1985; Botta, 1995). In contrast, scales can be used with panellists with a minimum of training and require much less time compared to descriptive tests (Botta, 1995).

Most of the studies on the effects of fast and moderate freezing rates on sensory attributes of seafood have failed to show any freezing rate effect (Aurell *et al.*, 1976). Freezing rates higher than 0.5 cm/h do not appear to

influence the sensory quality of frozen fish with the exception of some shellfish, e.g. individually frozen shrimp and mussels, which seem to benefit from rapid freezing (e.g. cryogenic freezing; Jul, 1984).

Changes in the sensory attributes of fish have been widely studied mainly during frozen storage. Thus, changes in sensory attributes during frozen storage have been recorded for Patagonian hake (Ciarlo *et al.*, 1985), for mackerel and smooth hound (Vareltzis *et al.*, 1988), for post-rigor scallop meats (Chung and Merritt, 1991), for horse mackerel and Mediterranean hake (Simeonidou *et al.*, 1997), for Nile perch (Namulema *et al.*, 1999), for guitarfish (Yilmaz and Akpinar, 2003) and whole gilthead seabream (Pastor *et al.*, 1999; Huidobro and Tejada, 2004) .Most of these studies showed that the length of time of cold storage deteriorates the sensory quality of frozen seafoods. However, these changes are mainly dependant on species, pre-freezing treatment, and time and temperature of frozen storage.

1.5. Objectives of the present study

In commercial seafood industries, seafoods are frozen at a range of freezing times (rates) that depends mostly on the type of seafood, the type of freezer and freezer's operating conditions. Based on the work reported in the literature, freezing of seafoods at different freezing times may furnish favorable conditions for alterations in muscle structure, muscle proteins and lipids, and textural properties in general. These changes are related to alterations in quality of frozen seafoods and may affect their market. Therefore, knowledge of optimal conditions with respect to freezing rates for freezing commercially important seafoods is relevant to the fish industry. This information can be obtained by experimental studies on changes in the chemical, bio-chemical, physical and sensory properties of seafoods frozen at different freezing times.

A considerable amount of literature information has shown that when seafoods are frozen and stored in frozen state they inevitably lose quality. Loss in quality of stored frozen seafoods is mainly due to changes in muscle integrity, proteins and lipids. Such changes usually determine the practical storage life of frozen seafoods and are very much dependent on species and the length of time a frozen seafood remains in frozen state. The literature describes a wide variety of methods that have been applied to measuring quality in stored frozen seafoods. For commercial applications sensory methods may be sufficient to evaluate the quality of stored frozen seafoods. However, sensory methods are time consuming, expensive and can be performed only by trained assessors. Thus, industry needs standardized chemical and physical tests that can be used to predict changes in sensory properties of stored frozen fish. Such information can be obtained by relationships between various tests and sensory parameters of stored frozen fish in experimental studies. Consequently, information about the changes in chemical, bio-chemical, physical and sensory properties of species coming

either from fisheries or aquaculture is relevant to seafood industry for commercially important species.

Thus, the objectives of the present study, which was carried out in Scotland and Greece, were:

- to investigate the effects of freezing times (rates) on the quality parameters of King scallop adductor muscle and gilthead seabream fillets
- to ascertain the effects on the quality of frozen King scallop adductor muscle and gilthead seabream fillets during cold storage;
- to compare the results obtained from the two different species of seafood.

The effects of freezing times (rates) and time of frozen storage on the quality of King scallop adductor muscle and gilthead seabream fillets were studied in regard to the integrity of muscle structure, myofibrillar protein denaturation and aggregation, lipid degradation, texture and sensory changes.

CHAPTER 2 Materials and Methods

2.1. The bio-chemical, physical and sensory properties of the adductor muscle of scallops (Pecten maximus) frozen at different freezing times and during storage at -22°C

2.1.1. Chemicals

Acetoacetyl Coenzyme A, calcium chloride, adenosine 5- triphoshate (disodium salt; ATP), β-nicotinamide adenine dinucleotide reduced form (pre-weighed vials) bicinchoninic acid protein and inorganic phosphorous determination kits were purchased from Sigma-Aldrich, U.K.

2.1.2. Instruments and equipment

In the present study the following instruments and equipments were used:

- Deep freezer (Forma, model 786, Life Sciences International, Basingstoke, Hampshire, U.K.) with working temperature – 80 °C.
- Domestic freezer cabinet (Bosch, Germany) with working temperatures –20°C and –35 °C.
- Thermocouples T type, 0.5 mm diameter (Comark Instruments, U.K.)
- Recording thermometer (Comark 8600 Tempscan, Comark Instruments, U.K.) and PC output.
- Food vacuum packaging machine (Multivac A300 Packaging Machine, Germany).
- Temperature data logger (Micro - Log 1.1, Fourier Systems, Israel).
- Steven's Texture Analyzer.
- Ultra – Turax homogenizer (IKA, T-25 basic, Germany) with the S25 NK-19 G dispersing tool.
- Double beam UV-visible recording spectrophotometer (Perkin-

Elmer, Lambda 2, USA).

2.1.3. Raw material

2.1.3.1. Origin of raw material

Whole scallops (*Pecten maximus*), from the Orkney fishing area, were purchased from the Aberdeen fish market. They were hand-picked by divers and maintained alive for 24 to 48 hours in seawater holding tanks in Orkney until dispatched. They were transported to the fish market in Aberdeen within 12 to 14 hours after their removal from the holding tanks. The whole live scallops were delivered to the laboratory and packed in crushed ice on the same day as their arrival at the fish market

2.1.3.2. Treatment of raw material prior to freezing

At the laboratory, the scallops were shucked and the striated part of the adductor muscle (from now on named 'scallop muscle') was separated from all other tissues (Figure 2.1.3.2).

Figure 2.1.3.2 Scallop with right shell valve and mantle lobe removed to show viscera

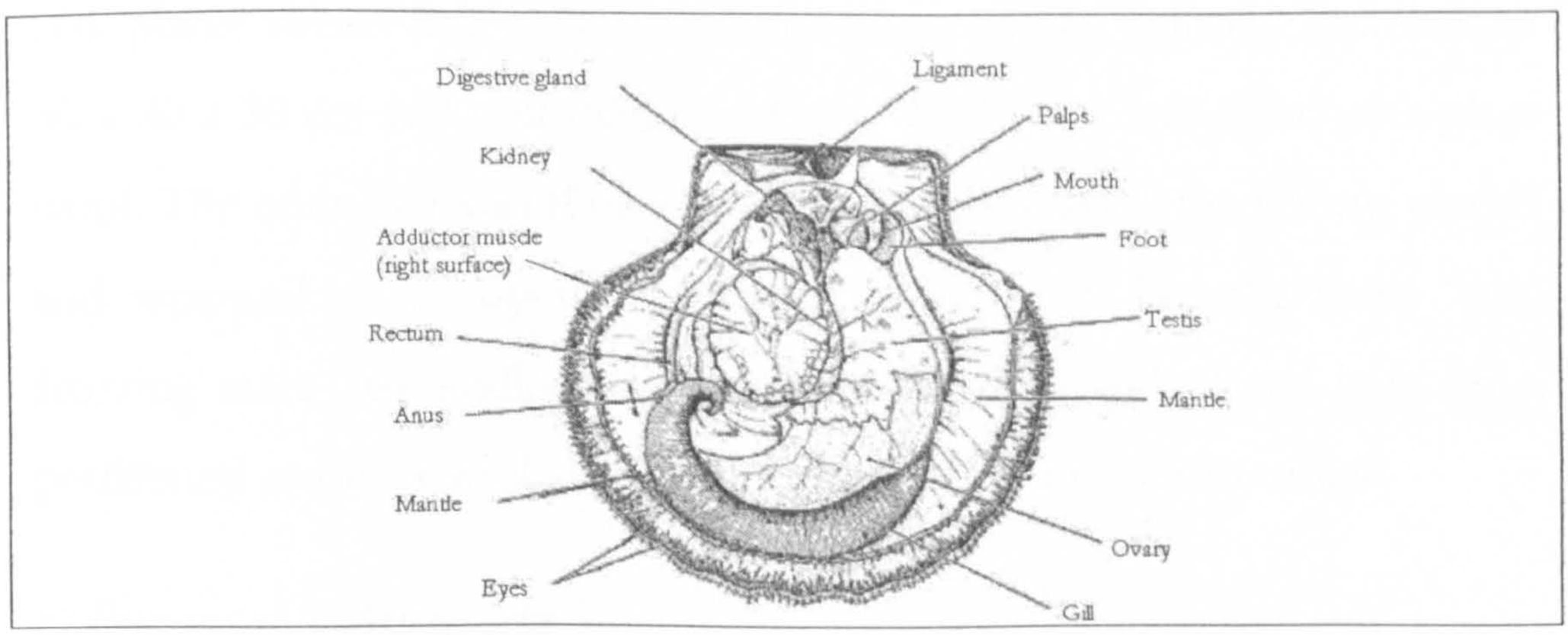


Figure note: From Mason (1983)

In order to ensure that the scallop muscles were in post rigor state, they were stored in glass jars without any washing, buried in crushed ice and stored in a chill room at 2°C to 4°C for 3 days. Post-rigor muscles were

required in order to ensure that any difference in instrumental texture measurements would indeed result from differences in freezing and freezing rates, and were not due to the development of rigor in the raw scallop muscles prior to freezing (see Appendix 1). The mean weight of muscles was 35 ± 5.6 g (mean ±S.D.).

2.1.4. Experimental setup

2.1.4.1. Freezing times experiments

a) Preparation of scallop muscles for freezing

In order to create a range of freezing rates, the scallop muscles were individually frozen at -20°C, -35°C, and -80°C insulated in a polystyrene box (from now on named 'slow' freezing rates) and on a perforated metal sheet (from now on named 'fast' freezing rates), without any wrapping. Additionally, any washing or glazing was not applied, which avoided any variability attributed to absorbed or melted water. For the 'slow' freezing rates five scallop muscles were weighed and placed between two cellophane sheets in a polystyrene container having external dimensions 30 x 30 x 30 cm and wall thickness 6 cm, which was half-filled with glass wool. The container was then filled up with glass wool; the lid was placed and wrapped at the opening with packaging tape. For the three 'fast' freezing rates, five scallop muscles were weighed and placed bare on a perforated stainless steel sheet with hexagonal 7 mm perforations.

b) Freezing of scallop muscles

The experiments were performed in triplicate from December 2000 until the end of February 2001. The duration of each replicate was 3 weeks. The first week of each replicate, the scallop muscles were frozen 'slow' and 'fast' at -20°C, the second week at -80°C, and the third week at -35°C.

Each week 15 scallop muscles were handled as described in section 2.1.3.2. Each day of freezing, the muscles were divided into 3 batches, each consisting of 5 scallop muscles. One batch was immediately analysed as unfrozen controls. The two other batches were weighed and frozen at the same temperature, either 'slow' for 36 hours or 'fast' for 24 hours. Immediately after freezing, the scallop muscles were weighed and then thawed, as is described in section 2.1.5. The freezing facilities are described in section 2.1.2

During freezing, the temperature of the centre of two 'slow' frozen scallop muscles and two 'fast' frozen scallop muscles, was monitored using T type thermocouples and a recording thermometer (see section 2.1.2).

2.1.4.2. Frozen storage experiments

a) Freezing and storing of scallop muscles

The effect of storage time on the quality of scallop muscles was investigated by freezing the scallop muscles unwrapped at -80 °C. Post-rigor scallop muscles were frozen and stored in December 2000, January, February, March and April 2001. Each month, three lots of ten scallop muscles were delivered at the laboratory in three successive weeks. Once the scallop muscles were in post-rigor stage, they were divided into two batches of five scallop muscles, and one batch was analyzed immediately as the unfrozen control and the other was frozen at -80°C for 24 hours. Immediately after freezing, the scallop muscles were weighed and placed in 30 x 19 cm polyethylene food bags (Fishpack LD, U.K.) that had, according to the supplier, 90 μm thicknesses and water vapour transmission rate of 1g/m^2/day at 23°C. The bags were vacuum-packed (see section 2.1.2) at a gauge reading of –1000 mbar and a setting of sealing temperature and time at 4 and 7 respectively. They were then

stored in a domestic freezer cabinet at -22°C. The temperature in cold storage was recorded every hour by using a temperature data logger (see section 2.1.2).The fluctuation of temperature in the freezer cabinet during storage of frozen scallop muscles is presented in Appendix 2.

b) Sampling of stored frozen scallop muscles

The stored frozen scallop muscles were analyzed for physical, biochemical and sensory properties after 28, 91, 154, 210 and 301 days in cold storage. The analyses of scallop muscles started in May 2001 with scallop muscles stored in April 2001, and ended in October 2001 with scallop muscles stored in December 2000. In each sampling period, fifteen scallop muscles were analyzed in three successive weeks. Each week, five scallop muscles were thawed and handled as described in section 2.1.5 and then subjected to the analyses that are described in section 2.1.7.

2.1.5. Thawing and handling of frozen scallop muscles

The frozen scallop muscles were weighed and then allowed to thaw in a chill room at 4°C overnight (12 hours). The thawed scallop muscles were re-weighed for thawing loss determinations and subsequently sensory, expressible fluids and instrumental texture determinations were performed (see sections2.1.7.3, 2.1.7.6 and 2.1.7.7).

The remaining tissue from those determinations and a slice of approximate weight of 2 g, which was removed from the right surface of each scallop muscle (Figure 2.1.5, below), were used for the biochemical and chemical assays.

Figure 2.1.5 Intact scallop shell *

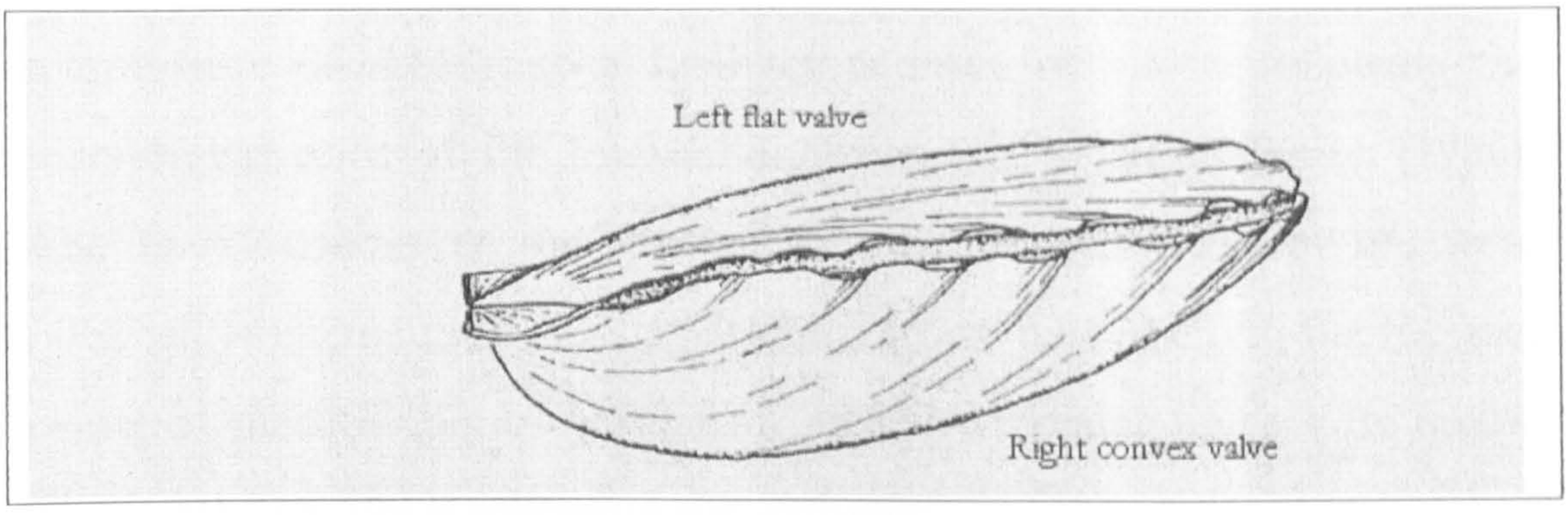


Figure notes: *From Mason (1983). The right and left surface of the adductor muscle correspond to right convex and left flat valves of the intact shell, respectively.

The tissue and the slices of scallop muscles were wrapped with aluminium foil then sealed in polyethylene bags and kept in chilled storage. The next day, the slices were used for the preparation of filtrates for the determination of β-hydroxy-acyl-coenzyme-A dehydrogenase (HADH) activity and the remaining tissues from the five scallop muscles were pooled first and then minced in a domestic mincer, having 0.5 cm drum holes, pre-chilled at 4°C. The minced scallop muscles were then immediately used for the determination of the water content and preparation of extracts. The extracts were stored in an -80°C freezer until analysis.

2.1.6. Freezing times and rates determinations

All temperature measurements were done with T type thermocouples and a recording thermometer (see section 2.1.2). The thermocouples were placed at the centre of the thickest part of the scallop muscles which was taken as the maximum distance between the right and the left surface of the muscles, and was measured by using a vernier instrument. The thermocouple was inserted at the centre of the thickest part from the lateral surface of the scallop muscle.

The freezing time (t_e) was calculated as the time (minutes) required to

decrease the temperature of the thermal centre from an average initial temperature of 4±1°C to a final temperature of -20°C following the recommendations of the International Institute of Refrigeration (1986). The freezing rates at the thermal centre, expressed as cm h^{-1}, were obtained from the ratios of the distance from the surface to the thermal centre of the scallop muscles and the effective freezing times, t_e (in hours; Chen and Pan, 1995) following the formula:

Freezing rate (cm h^{-1}) = Half thickness of scallop muscle (cm) x t_e^{-1}

The characteristic freezing time (t_c) was calculated according to Bevilaqua *et al.* (1979) as the time (in minutes) for which the thermal centre of scallop muscles was in the temperature range of maximal ice crystallization i.e. from -1 to -7°C.

2.1.7. Methods used to measure changes in fresh and frozen/ thawed scallop muscles

2.1.7.1. Determination of β-hydroxy-acyl-coenzyme-A dehydrogenase (HADH) activity.

The slices, prepared as described in section 2.1.5, were immersed in two volumes of 0.1 M phosphate buffer, pH 6.0, at room temperature for 15 min. The extract obtained was filtered through Whatman paper no 2. The filtrate was assayed for HADH activity according to Garcia de Fernando *et al.* (1992). To a methacrylate disposable semimicro spectrophotometric cell (light pathway 1 cm and capacity 1.5 ml) the following were added:

- 34 µl of extract
- 70 µl of EDTA (34.4 mM)
- 880 µl phosphate buffer (0.1M, pH 6).

The mix was maintained at room temperature for 3 min and then 20 µl of NADH (1.5mM) and 20 µl of aceto-acetyl coenzyme A were added. The

cell contents were mixed to start the reaction and the absorbancies at 340nm (against air in reference cell) were recorded every 0.5 min up to 3 min.

The HADH activity was determined using the following formula:

$$\text{HADH activity (mU g}^{-1}) = 4.781 \times \Delta A \times \Delta t^{-1} \times V \times W^{-1} \times 1000$$

Where:

- ΔA was the decrease in absorbance after 3 min of reaction
- Δt was the reaction time
- V was the volume of the extract in ml
- W was the weight of the sample in g

Three determinations per extract were performed.

2.1.7.2. Determination of weight losses due to freezing

Weight losses due to freezing were established by weighing the scallop muscles before and after freezing (Aurell *et al.*, 1976). The weight losses were expressed as g per kg of weight before freezing. The following formula was used:

$$\text{Weight losses (g kg}^{-1}) = (W_0 - W_1) \times W_0^{-1} \times 1000$$

Where W_o and W_1 were the weight in g of scallop muscles before and after freezing, respectively.

2.1.7.3. Determination of water holding capacity parameters

a) Thawing losses

The method described by Chung and Merritt (1991a) was followed. Each frozen scallop muscle was weighed and placed on a wire gauge set on top of a plastic cup. The whole apparatus was enclosed in a plastic bag to

prevent evaporation and kept at + 4°C in a refrigerator for 12 hours. Thawed scallop muscles were removed from wire gauges and re-weighed. The thawing loss was calculated from the weight difference between the initial weight of the frozen and the final weight of the thawed scallop muscle. The following formula was used:

$$\text{Thawing losses (g kg}^{-1}) = (W_2\text{-}W_3) \times W_2^{-1} \times 1000$$

Where W_2 and W_3 were the weight in g of scallop muscles after freezing, and thawing, respectively.

b) Expressible fluids

Two cylindrical portions of each scallop muscle, 4 mm in thickness and 20 mm in diameter, were excised from the left surface of scallop muscles by means of a ring having 4mm thickness and 20mm diameter. Each cylinder was weighed accurately with a Mettler analytical balance and placed on a double thickness filter paper Whatman No 1(diameter 42.5 mm). It was then covered with another double thickness filter paper and the pack was subjected to a 1,000 g force for 1 min using a Steven's Texture Analyzer fitted with a cylindrical flat probe (50 mm diameter and 20 mm height). The cylinders were re-weighed and the expressible drip was expressed as g per kg of weight of frozen/thawed scallop muscles, following the formula:

$$\text{Expressible fluids (g kg}^{-1}) = (W_4\text{-}W_5) \times W_4^{-1} \times 1000$$

W_4 and W_5 were the weights in g of cylinders before and after pressing, respectively.

The above-mentioned force was chosen since it would cause the least possible damage to the cylinders (Chung and Merritt, 1991a). Two measurements per scallop muscle per treatment were taken.

2.1.7.4. Determination of Ca^{2+}-ATPase activity in actomyosin extracts

a) Extract preparation

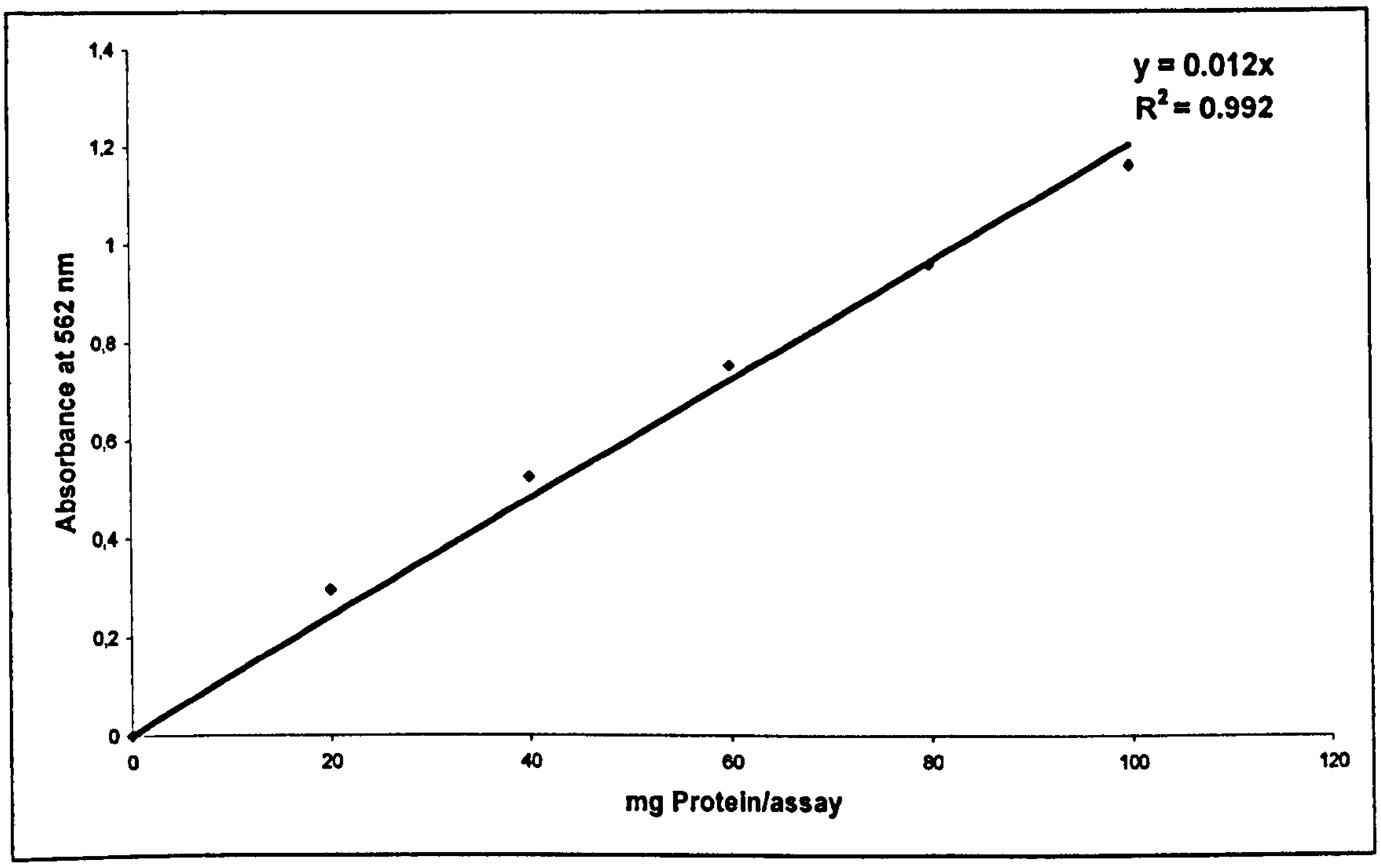
A portion (5 g) of the scallop muscles' mince was washed with 25 ml of ice-cold de-ionised water for 15 minutes and drained through a chilled Buchner No 3 funnel under vacuum. This step was needed to deplete the mince of sarcoplasmic proteins. It was repeated twice more.

A volume of 20 ml of iced-cold 5% (w/v) NaCl (pH=7) was added to the washed mince. The slurry was allowed to stand at 0 to 4°C for an extraction period of one hour and subsequently was centrifuged for 30 min at 5,000 g at 4°C. The supernatant solution was used for protein content and Ca^{2+}-ATPase activity measurements.

b) Measurement of protein concentration

The protein concentration was determined by the bicinchoninic acid (BCA) procedure (Sigma Procedure TPRO-562, BCA-1, Sigma Biochemicals Co., St. Louis, Mo., U.S.A). A 2 ml portion of BCA reagent (one part of C-2284 solution and 50 parts of B-9643 solution) was added to 0.1 ml of the unknown protein solution. This mixture was incubated at 37°C for 30 min and then allowed to cool at room temperature. The absorbance of the mixture was read at 562 nm. Triplicate assays were carried out. A calibration line was made using a standard solution of bovine serum albumin (P-014, Sigma Biochemicals). This is shown in Figure 2.1.7.4-1, below.

Figure 2.1.7.4-1 Protein calibration line



c) Assay for Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity was determined following the method described by Carvajal *et al.* (1999).

To 100 µl of salt extract the following were added:

- 50 µl of 0.5 M Tris-maleate (pH=7)
- 50 µl of 0.1M calcium chloride
- 750 µl de-ionized water and
- 50 µml 20mM ATP solution (pH=7)

The mixture was incubated for 3 minutes at 25°C and the reaction was stopped by adding 0.5 ml of chilled 15% (w/v) of trichloroacetic acid solution (TCA). The mixture was then centrifuged at 10,000 g for 30 min and the supernatant solution was used for inorganic phosphorus measurements. Sample blanks were prepared in the same manner except that the acid was added before ATP.

d) Inorganic phosphorus measurements

The inorganic phosphorus content of the TCA extracts was measured according to the Sigma procedure No 670. The supernatant fluid was allowed to react with ammonium molybdate in an acid solution to form phosphomolybdate. A mixture of sodium bisulphite, sodium sulphite and 1-amino-2-naphthol-4-sulphonic acid (Fiske and Subbarrow solution) reduced the phosphomolybdate to form a phosphomolybdenum blue complex. The intensity of the colour was measured at 660 nm.

The procedure was as follows:

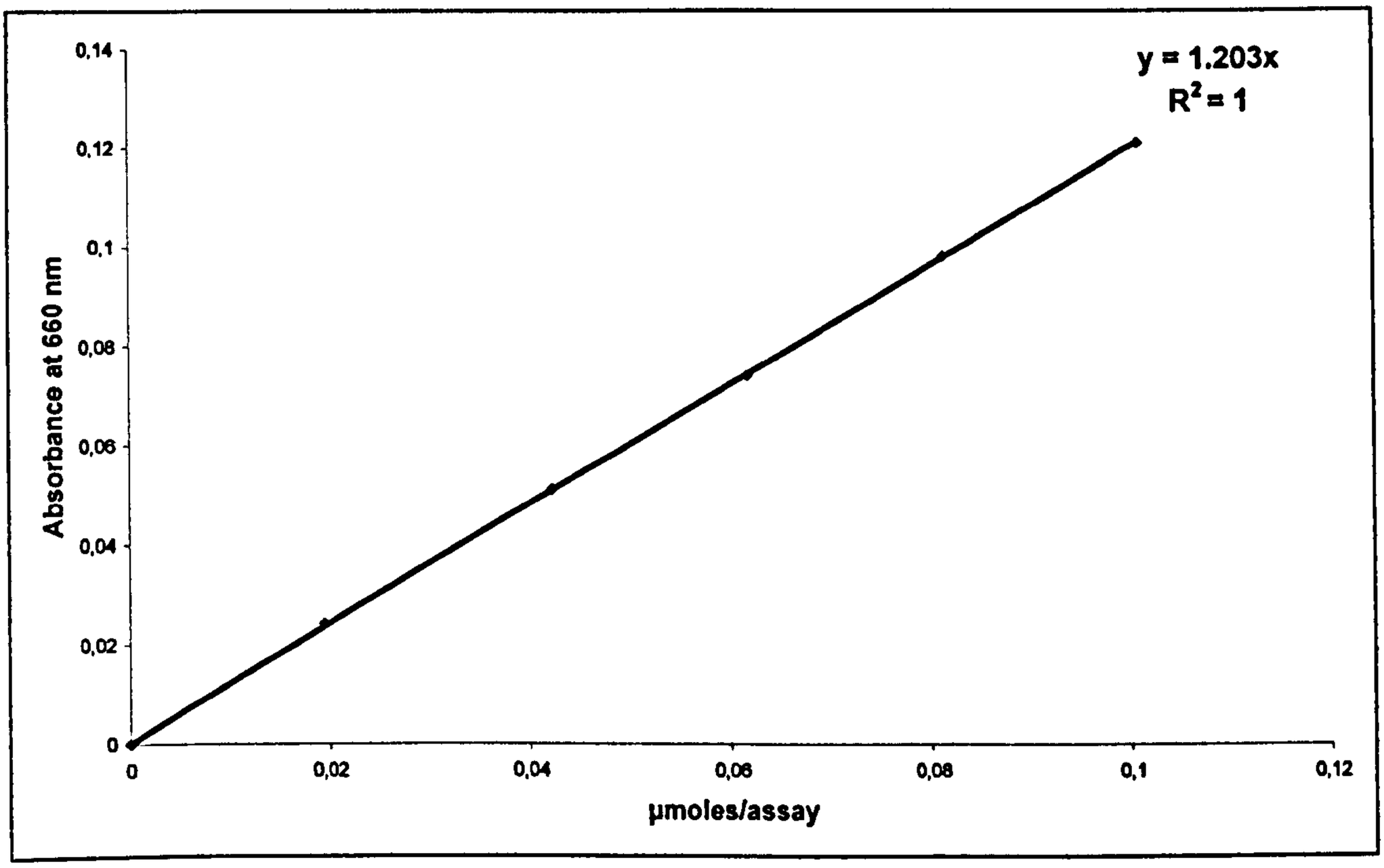
To 1 ml of TCA extract the following were added:

- 1.5 ml of distilled water
- 0.5 ml of acid molybdate solution
- 0.125 ml of Fiske and Subbarrow solution

The solution was mixed by inversion of the tube, which was then allowed to stand for 10 minutes at room temperature for colour development. Then the absorbance was read at 660 nm against a blank prepared by the same manner as the samples, except that the supernatant was replaced by 15% (w/v) TCA.

The concentration of inorganic phosphorus was determined from a calibration curve prepared using the phosphorus standard solution (Figure 2.1.7.4-2, below).

Figure 2.1.7.4-2 Inorganic phosphorus calibration line



One extract per treatment was prepared and four measurements per extract were carried out. The Ca^{2+}-ATPase total activity was defined as micromoles of inorganic phosphorus liberated per min per mg of protein (µmoles P_i/ mg protein/ min) at 25°C.

2.1.7.5. Thiobarbituric acid reactive substances (TBARS) determination

a) Water content determination

Water content was determined according to the method of AOAC (1997). A portion (2 - 3 g) of the scallop muscles' mince was accurately weighed on a pre-dried and weighed metal dish that was subsequently placed into an oven at 105°C for 24 hours. Then the dish was transferred to a desiccator and allowed to cool for one hour and then was re-weighed. The water content was determined from the weight difference of the sample before and after drying and the results were expressed as g of water per 100g of the scallop muscle.

The following formula was used:

$$\text{Water content, per cent} = (W_8 - W_9) \times W_8^{-1} \times 100$$

W_8 and W_9 were the weights in g of the scallop muscles' mince before and after drying respectively. Three measurements were performed on each pooled mince of scallop muscles and freezing procedure.

b) Extract preparation

A portion (5 g) of the scallop muscles' mince was homogenized with 20 ml chilled 7.5% (w/v) trichloroacetic acid (TCA) for 0.5 minutes and filtered using Whatman N°1 filter paper (Vyncke, 1970).

c) TBARS reaction

The method described by Vyncke (1970) was followed with the following slight modifications.

A volume of thiobarbituric acid reagent (0.5 ml of 0.02 M 2-thiobarbituric acid in distilled water) was mixed with 0.5 ml of filtrate in a 2-ml Eppendorf tube, which was then tightly capped and placed for 40 min in a hot water bath (70°C). The absorbance was read at 538 nm after cooling under running tap water for 10 minutes. Turbid samples were centrifuged at 10,000 g for 15 minutes at 4°C, and the supernatants were removed for measurement of absorbance at 538 nm.

c) Calculations

The content of malondialdehyde (MDA) in the filtrate, expressed as mg MDA, was determined from a calibration curve (Figure 2.1.7.5, below) using 1,1,3,3 tetraethoxypropane as standard.

The concentration of MDA in the scallop muscles, expressed as mg MDA per kg of scallop muscle, was calculated from the following type:

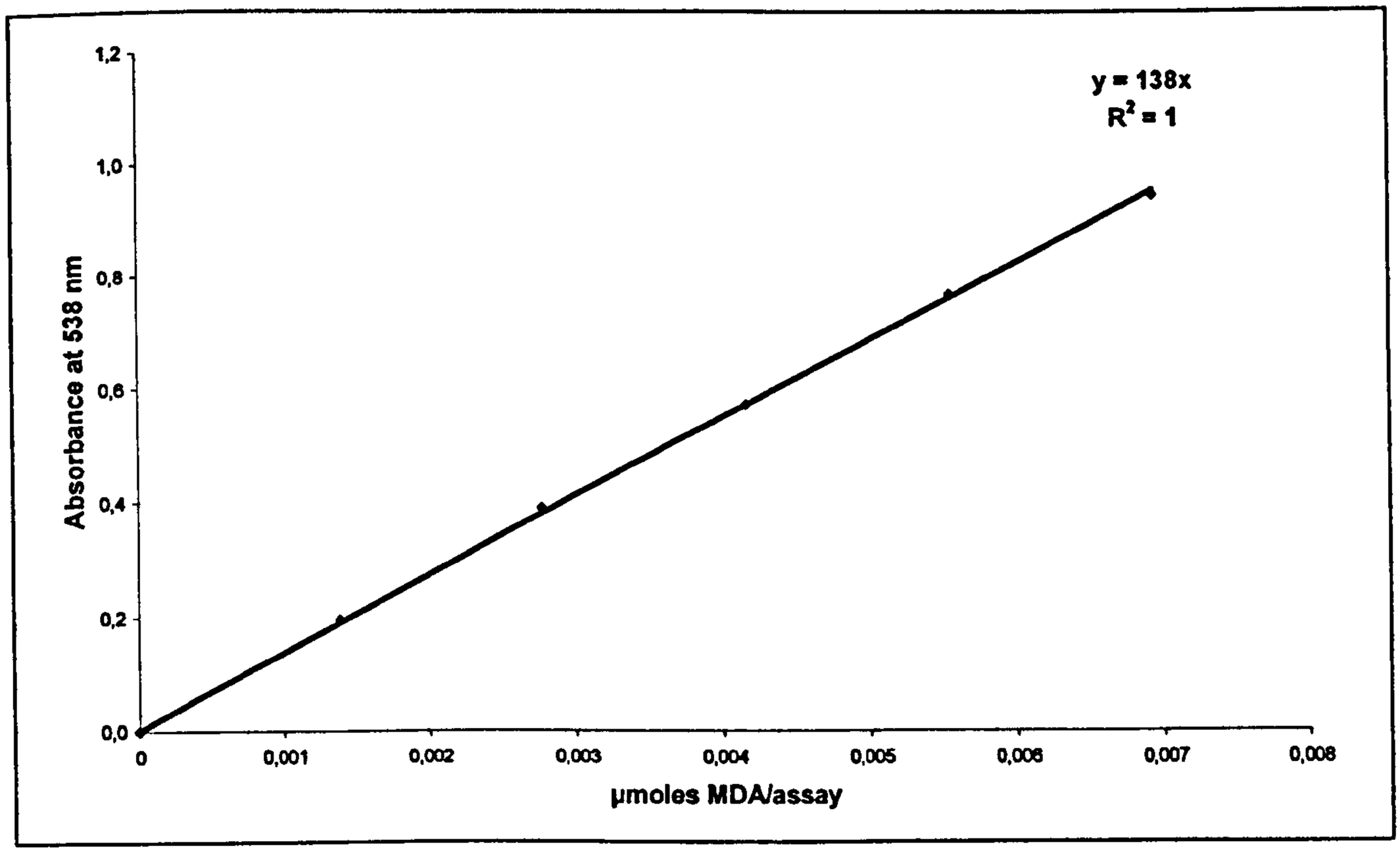
$$TBARS\ (mg\ MDA\ kg^{-1}) = A \times \alpha^{-1} \times [(WC_s \times W_s) + V] \times 0.5^{-1} \times W_s^{-1} \times 72$$

Where:

- A was the absorbance of the reaction mixture
- α was the slope of the calibration line (Figure 2.1.7.5)
- WC_s was the water content of the scallop muscle, per cent
- W_s was the weight of the scallop muscle sample in g, i.e. 5 g
- V was the volume of the extracting solution in ml
- 72 is the molecular weight of MDA

Four determinations per extract and treatment were performed.

Figure 2.1.7.5 TBARS calibration line



2.1.7.6. Texture determination as measured by the texture analyzing system

Texture measurements were performed according to Chung and Merritt, (1991b). Peak shear force measurements were performed with a Steven's texture analyzer at a crosshead speed of 50 mm per min. Shear strengths were measured with a V-shaped Warner-Bratzler shear probe mounted on the Steven's load cell.

From the central part of each scallop muscle two cylinder portions (10 mm in diameter and 10 to 15 mm long) were excised longitudinal to muscle fibres using a cork borer. Individual cylinders were weighed (Ohaus, Scout electronic balance, USA) and then inserted through the triangular opening of the blade and placed on the load cell in such a position that the scallop muscle fibres were at right angles to the blade penetration. The peak shear force, expressed in gram-force (g*), required to cut the cylinder into two pieces was read from the control panel of the analyzer. The shear peak force was adjusted to units of g*per g of scallop muscle cylinder to take into account of variations in weight of scallop muscles cylinders (Chung and Merritt, 1991b).

2.1.7.7. Sensory assessments

a) Sensory assessments for the freezing times (rates) experiments

Scallop muscles from each freezing treatment were assessed against fresh post-rigor scallop muscles using triangular comparisons following the methods described by Jellinek (1993) and Botta (1995).

A portion (4 -5 g) of muscle was removed from the anterior part of each scallop muscle longitudinal to muscle fibres. It was steam-cooked for 10 minutes using a domestic steam cooker. After cooking, the portions of scallop muscles were placed on wire gauze supported on a plastic cup; the whole apparatus was covered with a polyethylene bag and left at room

temperature to cool for 30 minutes. The portions of scallop muscles were then used for triangular tests. Three coded samples, two fresh and one frozen, were simultaneously presented to five assessors. After tasting, the assessors were asked to indicate in a questionnaire (Appendix N° 3) which portion of scallop muscle was (a) the different (b) the preferable and (c) presented differences in textural properties. Fifteen assessments in three sessions per freezing rate were carried out. The number of correct judgments (i.e. these that correctly distinguished the frozen from the fresh samples) was compared to an appropriate Table of minimum number of correct judgments to achieve a specified probability level. The differences between the fresh controls and frozen scallop muscles were significant when the number of correct judgments corresponded to the probability level (*P*) of 0.05. To determine which of the two scallop muscles (fresh or frozen) was preferred or presented textural differences, the results from the correct judgments were compared to a paired-tests Table containing the minimum number of judgments to establish differences at different levels. The differences were significant when the number of judgments corresponded to the probability level (*P*) 0.05.

b) Sensory assessments for the storage experiments

The stored frozen scallop muscles were evaluated, by the assessors who participated in the triangular evaluations, for flavour, texture and overall acceptability. The assessors were not specifically trained for these evaluations. The definitions of the sensory attributes were discussed in a session before the first trial (Table 2.1.7.7, below).

Figure 2.1.7.7 Flavour and texture definitions*

Flavour Definitions	Texture Definitions
Sweet = The basic sensation of which the taste of sucrose is typical	Tender = Low level of chewiness (e.g. young peas)
Sour (sharp) = The taste sensation caused by acids (e.g. vinegar or lemon)	Tough = High level of chewiness (e.g. old cow meat, bacon rind)
Rancid = Tasting like stale fat or linseed oil, pungent, acrid, bitter	Succulent = High level of moisture (e.g. water chestnut, oysters) Dry = Absence of moisture (e.g. cream crackers)

Table note: *According to ISO 5492:1992

The fifteen frozen scallop muscles of each storage period were evaluated in three sessions held in three consecutive weeks. In each session, every assessor was given two coded cooked portions of scallop muscles, one fresh and one frozen/thawed. The portions of scallop muscles were prepared as described in section 2.1.7.7-a. After tasting, panellists were asked to indicate in a score sheet their assessments for the sensory attributes (Table 2.1.7.7). Flavour and overall acceptability were scored on a five-point scale, and texture on a six-point scale.

The scoring system for flavour was as follow:

5= normal sweet taste

4= no sweet flavour, neutral

3= slight sour or rancid flavour

2= moderately strong sour and rancid flavour

1= very unpleasant putrid or rancid flavour

The degree of liking (overall acceptability) was rated using a five point hedonic scale Botta (1995). The scale was as follows:

5= like very much

4=like

3=neither like or dislike

2=dislike

1= dislike very much.

For texture rating, the scoring system, described by Koning and Mol (1991) with slight modifications, was used. The scoring system was as follow:

5=Tender, succulent, normal texture

4= Slightly tough and dry

3= Tough, dry but edible

2= Very tough and dry

1= Stringy, unable to swallow

0= Very stringy, completely inedible.

2.1.8. Statistical analyses

Statistical analyses were performed with Minitab13 for Windows (Minitab Inc., 2000). The assumption of normality of distributions was tested with the Kolmogorov-Smirnov test and that of homogeneity of variances with the Bartlett's test. Data were log – transformed when necessary.

For the freezing rates experiments, one way analysis of variance (ANOVA) was performed to test for a) differences between the fresh experimental groups and b) freezing time (t_f) effects on each parameter measured.

Data of the fresh experimental groups from the frozen storage experiments were first subjected to ANOVA for determination of possible session effects. Subsequently, the combined data from fresh experimental groups and those of the five storage periods were subjected

to ANOVA to test for the effects of storage time on each parameter measured.

The sensory results on flavour, texture and overall acceptability required analysis by non-parametric statistical methods since scoring was limited to discrete values. Thus, ANOVA based on Kruskal-Wallis method (described in Zar, 1984) was used to examine the effects of storage time on each sensory attribute.

Data from the cold storage experiments was, also, subjected to a linear regression in order to examine the relation between the bio-chemical/ chemical parameters and sensory properties. Linear regressions between the bio-chemical/ chemical parameters and sensory properties were required, since linear models are the most useful methods for assessing the changes in quality of stored frozen seafoods for commercial purposes.

The parametric and non parametric ANOVAs showing significant differences were followed by a Tukey HSD and Dunn post-hock test respectively (described in Zar, 1984). In all statistical analyses significance was accepted when $P<0.05$ (Zar, 1984).

2.2. The bio-chemical, physical and sensory properties of gilthead seabream (Sparus aurata) fillets frozen at different freezing times and during storage at -22°C

2.2.1. Chemicals

p-nitrophenyl-α-glucopyranoside, *p*-nitrophenyl-*N*-*β*-D-glucose amide, acetoacetyl coenzyme A, calcium chloride, magnesium chloride, ethyleneglycol bis (β-aminoethyl ether) N,N,N', N' -tetraacetic acid (EGTA), 5,5' dithiobis 2- nitrobenzoic acid (DTNB), urea and adenosine 5- triphoshate (disodium salt; ATP), β-nicotinamide adenine dinucleotide reduced form (pre-weighed vials) and oleic acid (cis-9-octadecenoic acid) were purchased from Sigma-Aldrich, Germany

2.2.2. Instruments and equipment

In the present study the following instruments were used:

- Food vacuum packaging machine (VM-T12 AGK, Germany).
- Deep freezer (Heto, Denmark) with working temperature -80°C.
- Domestic freezer cabinet (Zanussi, model ZV231 MR, Germany) with working temperature -20°C.
- Thermocouples K type, 0.5 mm diameter (Comark Instruments, U.K).
- Recording thermometer (Comark KM1242, Comark Instruments, UK).
- Laboratory oven (WTB Binder, Germany)
- Furnace (1400 model, Thermolyne, Grmany
- Digestion heating block (DH6, Velp Scientifica, Italy)
- Steam distillation unit (Vapodest 20, Gerhardt, Germany)

- Automatic titrator (TitroMatic 1S, Chrison, Spain)
- Heating rotary evaporator (Rotavapor R-114 Büchi, Switzerland)
- Centrifuge (3K 18,Sigma, Germany)
- Ultra-Turrax homogenizer (T25 basic, IKA Labortechnik, Germany)
- Closed dispersing tool (S 25 KR-25 G, IKA Labortechnik, Germany)
- Omni –mixer homogenizer (model 17106, Omni International , USA) fitted with a dispersing tool
- Spectrophotometer Pharmacia Biotech, Novaspec II, U.K
- Vortex agitator (IKA, Yellowline TTS, IKA Works, Inc., USA)
- Texture analyzer TA-XT plus and a Warner – Bratzler blade (type HDP/BS; Stable Micro- Systems Ltd., Surrey , U.K)

2.2.3. Raw material

2.2.3.1. Origin of raw material

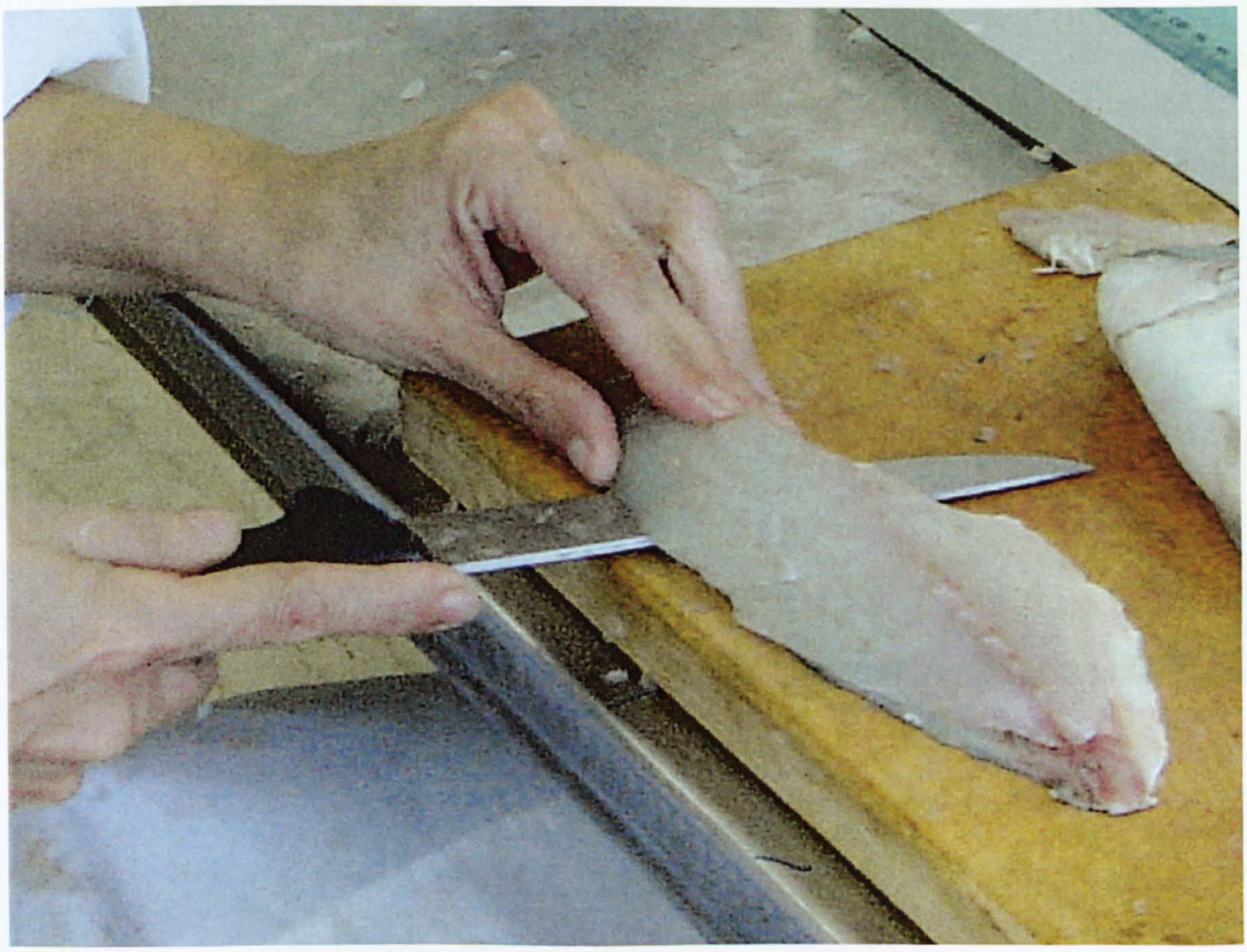
Gilthead sea bream (*Sparus aurata*), used in this study, were produced in a commercial cage culture unit located in Western Greece (TASTY FISH, S.A, Echinades Islands, Ionian Sea). Fish were fasted for two days prior to harvesting and were slaughtered by immersion in ice cold water (hypothermia). They were packed into an insulated polystyrene container with flaked ice and delivered to the Technological Institute of Messolonghi on the same day of their harvesting. At the laboratory, the fish were treated as is described in the following paragraph.

2.2.3.2. Treatment of raw material prior to freezing

Fish fillets were frozen in pre- or post-rigor state. Freezing of fillets in rigor state affects negatively the collagen structure and the general texture

after thawing, and directly after thawing the fillets pass through a sort of rigor (Nilsson and Ekstrand, 1993). Freezing of pre-rigor fillets is carried out mostly on sea-vessels and in some inland fisheries, since it requires the filleting process to take place shortly after harvesting of fish (Connell, 1975). Moreover, it is an accepted practice that fish are best processed once rigor mortis has resolved from 3 to 5 days after post-mortem storage in ice (Einen *et al.*, 2002). For these reasons, and in order to ensure that the fish had passed rigor mortis prior to filleting, 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. Ice was renewed daily. After the ice storage period was completed, the fish were filleted and skinned by hand (Figure 2.2.3.2-1).

Figure 2.2.3.2-1. Filleting and skinning of gilthead seabream fillets.



Subsequently, each fillet was weighed and placed between two aluminium

foil sheets in a 20 x 10 cm polyethylene food bag that had, according to the supplier, 90 μm thickness and water vapour transmission rate of 1g/m^2/day at 23°C (Fishpack LD, U.K). The food bags were vacuum-packed (see section 2.2.2, Figure 2.2.3.2-2) at a setting of 'sealing temperature' and vacuum at 7. Fillets were vacuum packed in order to avoid dehydration during freezing in air-freezing methods and to prevent contamination of the fillets with methanol in the methanol freezing method. Additionally, washing prior to freezing was not applied, which avoided any variability attributed to absorbed or melted water.

Figure 2.2.3.2-2 Vacuum packaging of the fillets

2.2.4. Experimental setup

2.2.4.1. Freezing times experiments

A total number of twenty fish (average weight and length 412 g and 28.2 cm, respectively) were obtained in two batches of ten fish on 17/06/2004

and 1/07/2004. Once the fish were in post-rigor condition (i.e. on 20/06/2004 and 04/07/2004 for the first and second batch respectively), they were filleted, skinned, weighed and vacuum packed; they were then divided into five groups. In each group there were fillets coming from four different fish. The mean weight of the skinned fillets was 63.4 ± 6 g (average ± S.D.). The fillets from the first group were analyzed as unfrozen controls and the others were frozen until the thermal centre reached -20°C as follows:

- In methanol pre-cooled for 24 hours at -80°C in a deep freezer. During freezing the tray containing the methanol was kept in the deep freezer.
- In a deep freezer with working temperature of -80°C. During freezing the fillets were placed on a stainless steel tray.
- In a freezer with working temperature of -20°C. During freezing the fillets were placed on a perforated stainless steel sheet.
- In a freezer at -20°C. The samples were insulated in a polystyrene container that was filled with glass wool and had external dimensions 40 x 30 x 16 cm and wall thickness 2 cm.

During freezing, the temperature of the centre of a fillet was monitored using a K type thermocouple and a recording thermometer (see section 2.2.2). Gilthead seabream fillets frozen in methanol were frozen individually and the temperature of the centre of each sample was recorded. Immediately after freezing, the vacuum packed fillets were allowed to thaw in a refrigerator chamber at 4 to 7°C overnight (12 hours). The fresh fillets from the first batch were handled as is described in section 2.2.5 whereas those of the second batch were stored at - 80°C until they were used for sensory evaluations (see section 2.2.7.13). The mean weight of the skinned fillets was 86 ± 10 g (average ± S.D.).

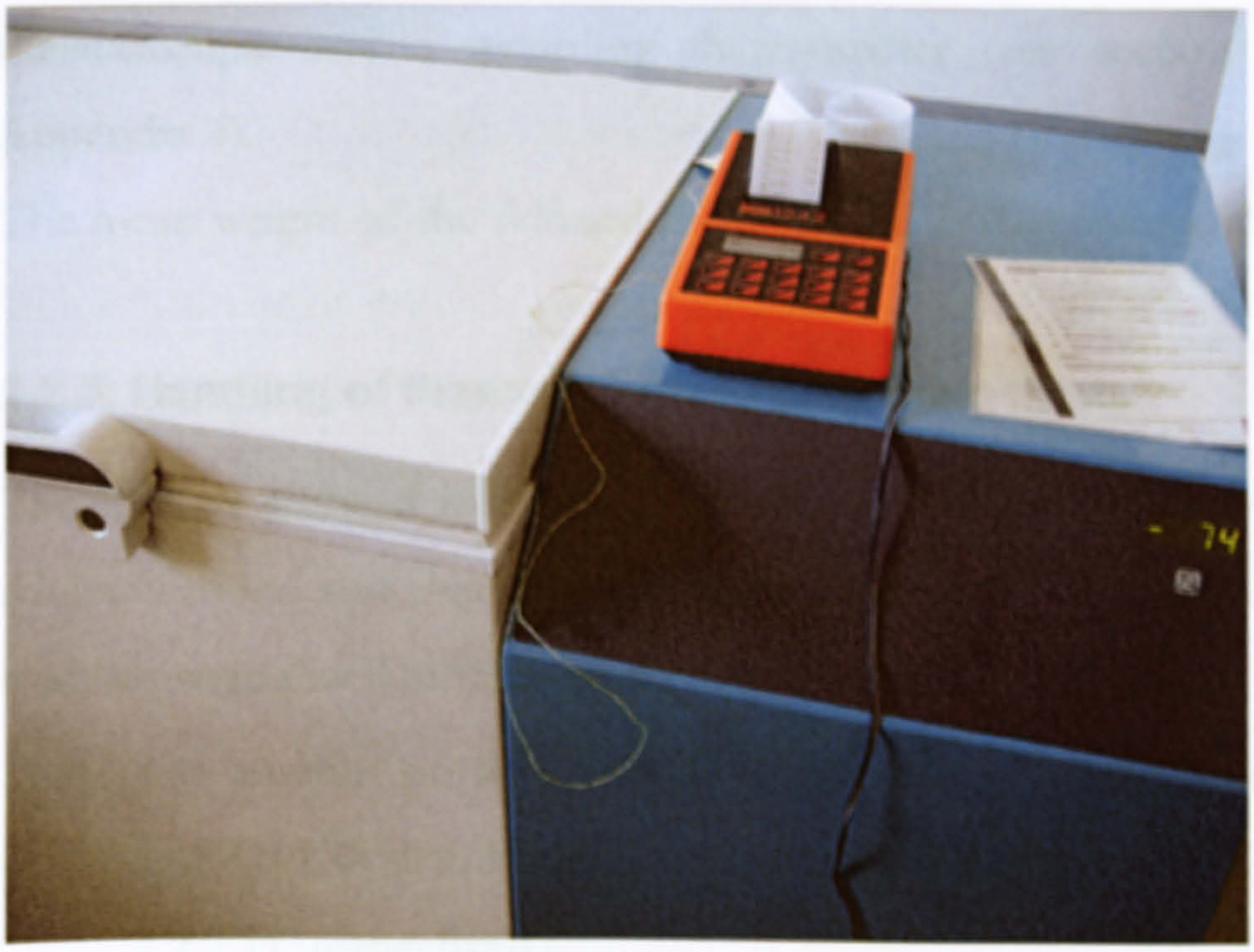
A total number of fifteen gilthead seabream were obtained on 9/02/2007

for determination of instrumental texture of fillets frozen at different characteristic freezing times (t_c values). Once the fish were in post-rigor state, they were filleted, skinned and vacuum packed; they were then divided into five groups. In each group there were six fillets, coming from three fish. The mean weight of the skinned fillets was 72.5 g ± 12.6 g. The fillets of the first group were analyzed for instrumental texture determinations immediately after filleting, and the others were frozen as is described earlier in this section. Immediately after freezing the fillets were thawed for 12 hours and their texture was determined by the texture analyzing system (see section 2.2.7.12). The results were the means of six determinations.

2.2.4.2. Frozen storage experiments

Thirty fish of mean weight 568 ± 38 g and length 32.1 ± 0.89 cm (average ± S.D.) were obtained on 11/07/2005. Once the fish were in post-rigor condition (i.e. on 14/07/2005), they were filleted, skinned, weighed and vacuum packed in polyethylene food bags. Subsequently, a total number of 50 fillets were frozen in lots of five fillets in the -80°C freezer (see section 3.1.2) until the thermal centre of the fillets reached -20°C. During freezing the fillets were placed on a stainless steel tray and the temperature of the thermal centre of one fillet was recorded using a K type thermocouple and a recording thermometer (see section 2.2.2 and Figure 2.2.4.2, below).

Figure 2.2.4.2. Temperature recording during freezing of the fillets



Immediately after freezing, the fillets were stored in a freezer cabinet with working temperature -22°C for up 340 days. The temperature in cold storage was monitored every hour with a K type thermocouple and a recording thermometer (see section 2.2.2). The fluctuation of temperature in the freezer cabinet during storage of frozen gilthead seabream fillets is shown in Appendix 4. 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, i.e. ten fillets, were thawed in a refrigerator chamber at 4 to 7°C overnight (12 hours) and handled as is described in the section 2.2.5. Five fillets were used for chemical and biochemical analyses and five for the sensory evaluations.

For the evaluations of texture by the texture analyzing system, ten fish were handled and frozen on 21/07/2005 as described earlier in this section. In addition ten fillets were preserved at -80°C as controls for the sensory and instrumental texture measurements. The results were the means of four determinations.

The temperature in cold storage was monitored every hour using a K type thermocouple and a recording thermometer (see section 2.1.2 and Appendix 4).

The mean weight of the skinned fillets was 86 ± 10 g (average± S.D.).

2.2.5. Handling of thawed gilthead seabream fillets

The thawed gilthead seabream fillets were re-weighed for thawing loss determinations and then the fillets used for chemical and bio-chemical analyses were sliced in three equal sized portions as follows:

- The anterior portion, which was used for the extraction of lipids and TBARS determinations (as described in sections 2.2.7.1-d and 2.2.7.11, respectively)
- The middle portion, which was used for the water content and salt soluble protein determinations, and the extraction of actomyosin, and the related measurements (as described in sections 2.2.7.1-a, and 2.2.7.5 to 2.2.7.8).
- The tail portion, which was used immediately after thawing of the fillets for the preparation of centrifugal tissue fluid and the extract for β-hydroxy-acyl-coenzyme-A dehydrogenase (HADH) assay (as described in sections .2.2.7.2 and 2.2.7.3)

The anterior and the middle portions of the fillets were wrapped in aluminium foil, vacuum packed in polyethylene bags and re-frozen with the centrifugal tissue fluids and HADH extracts at -80°C until they were semi-thawed (20 minutes at 25°C) for analysis.

The temperature of -80°C was used to preserve the portions of the fillets for protein and lipid extractions since it has been shown that the protein and lipid damage indices in frozen fish were not affected within the first month of storage at -80°C (Aubourg *et al*, 2004).

The anterior and the middle parts of each stored frozen fillet were

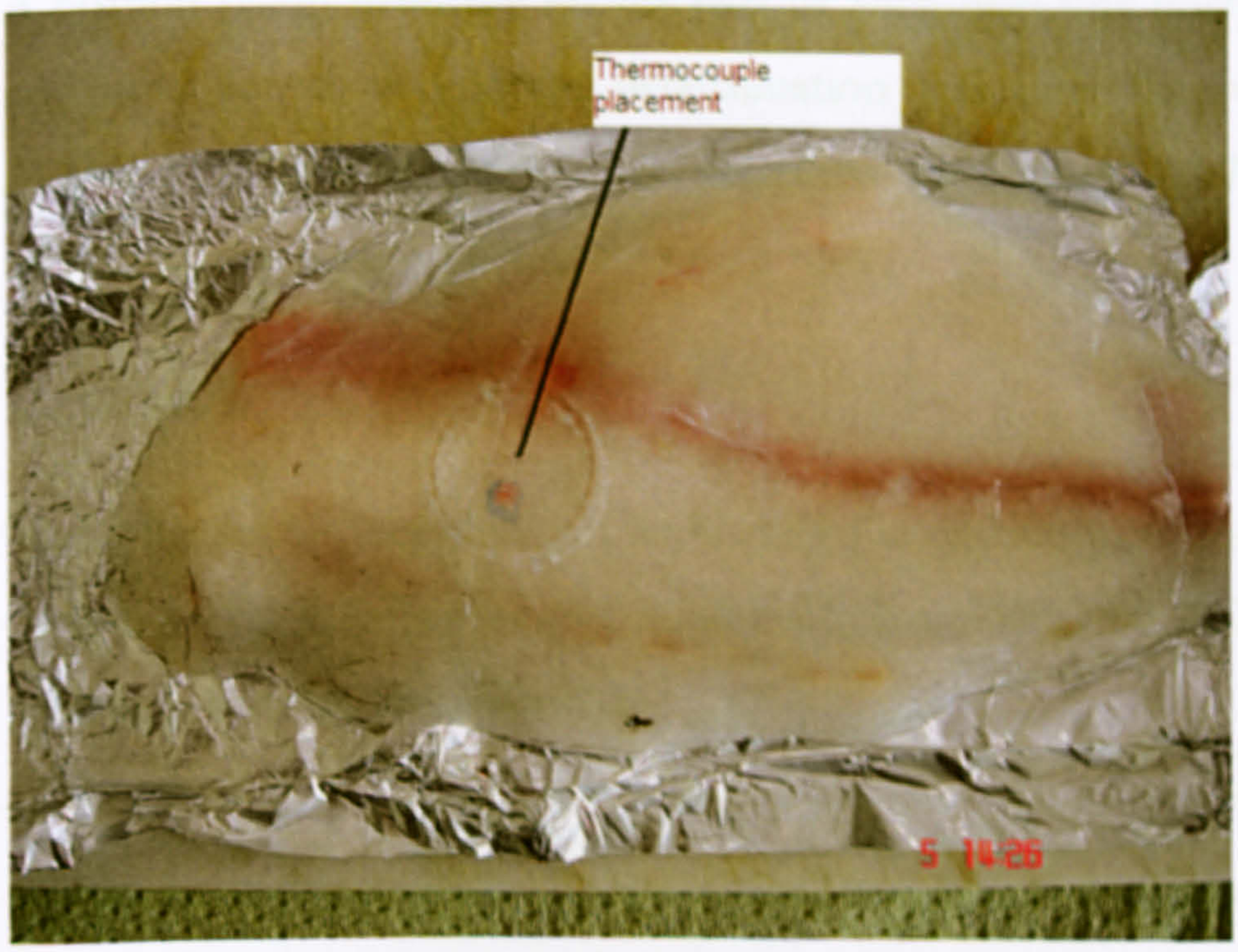
preserved separately at -80°C for taste and chewiness evaluations, respectively.

All chemical bio-chemical and sensory analyses were performed using the white muscle of the gilthead seabream fillets, and were completed within one month after thawing. The results of the freezing times experiments were the means of four independent determinations and those of the frozen storage experiments were the means of five independent determinations.

2.2.6. Freezing times and rates determinations

All temperature measurements were done with K type thermocouples and a recording thermometer (see section 2.2.2). The height of the thickest part of the fillet, i.e. under the dorsal fin and just above the lateral line, was measured by using a vernier instrument and the thermocouple was placed in the centre of that part (Figure 2.2.6, below).

Figure 2.2.6 Position on the fillet for the placement of the thermocouple



The freezing time (t_f) was calculated as the time (minutes) required to

decrease the temperature of the thermal centre from an average initial temperature of 5±1°C to a final temperature of -20°C following the recommendations of the International Institute of Refrigeration (1986). The freezing rates at the thermal centre, expressed as cm/h, were obtained from the ratio of the distance from the surface to the thermal centre of the fillets and the freezing times (in hours; (Chen and Pan, 1995). The characteristic freezing time (t_c) was calculated according to Bevilaqua *et al.* (1979) as the time (in minutes) for which the thermal centre of the fillet was in the temperature range of maximal ice crystallization i.e. from -1 to -7°C (see section 2.1.6).

2.2.7. Methods used to measure changes in frozen / thawed samples

2.2.7.1. Proximate analysis

a) Moisture content

A portion (1-2g) from the middle part of each fillet was finely chopped and used for water content determination following the method of AOAC (1997). The method is described in section 2.1.7.5-a. Two measurements were performed on each fillet.

b) Ash content

The ash content was obtained by heating the residue from the moisture determination in a furnace (see section 2.2.2) at 550°C for 24 hours. The following formula was followed:

$$\text{Ash content (g kg}^{-1}) = (W_{c1} - W_{c2}) \times W_s^{-1} \times 1000$$

Where:

- W_{c1} was the weight in g of the empty pre-dried container

- W_{c2} was the weight in g of the container after heating at 550°C and cooling in a dessicator at room temperature
- W_s was the weight of sample prior to drying for moisture determination

c) Crude protein content

Crude protein content of the gilthead seabream fillets was measured by the Kjeldahl method (AOAC 1997) as follows:

A portion (1 g) of white muscle excised from the middle part of the fillet was accurately weighed on 7 cm nitrogen-free filter paper (Whatman 541) and transferred to a 250 ml digestion tube. One Kjeldahl catalyst tablet, 15 ml of concentrated nitrogen-free H_2SO_4 and 3 ml of 35 % (w/v) H_2O_2 were then added. Subsequently, the tube was placed in a digestion block (see section 2.2.2) preheated at 410 °C and the mixture was digested until it became clear (30 minutes digestion time approximately).

The tube was then removed from the digestion block and cooled to room temperature. A volume of 50 ml of distilled water was then added and the tube was attached to a steam distillation unit (see section 2.2.2). A 250 ml receiving flask containing 25 ml of 4 % (w/v) H_3BO_3 solution was placed on the receiving platform. A volume of 75 ml of 32 % (w/v) NaOH solution was then dispensed from the steam distillation unit to the digestion tube and distillation was then performed until a volume of 100 ml of extract was collected (3 minutes distillation time). The absorbing solution (i.e. the solution containing the distillate and the boric acid) was titrated with 0.1 M HCl standard solution using an automatic titrator (see section 2.2.2). A reagent blank was prepared and titrated similarly.

The crude protein of the fillets, expressed as g per kg of fillets' weight, was calculated as follows:

$$\text{Total Protein (g kg}^{-1}) = (V_A - V_B) \times 14.01 \times M \times 6.25 \times W^{-1}$$

Where:

- V_A and V_B were the volumes in ml of standard acid required for sample and blank respectively
- 14.01 was the equivalent weight of nitrogen
- M was the molarity of the standard HCl acid, i.e. 0.1 M
- W was the weight of the fillet muscle used for the analyses in g

Triplicate measurements were performed on each fillet.

d) Crude lipid content

Crude lipid content was determined according to Bligh and Dyer (1959) as follows:

A portion (10g) of white muscle, taken from the anterior portion of the fillet, was chopped and accurately weighed into a 250 ml homogenizing flask. Sufficient water (about 8 ml) was then added to adjust the total moisture content, as near as possible, to 16 ml, together with 40 ml methanol and 20 ml chloroform. The mixture was blended for 2 minutes, holding the flask in an ice-water mixture. After adding further 20 ml chloroform, the mixture was homogenized for one minute. After addition of 20 ml water, a final 30 seconds homogenization was carried out. Then, the homogenate was filtered through a glass fibre filter paper (Whatman N° 4) on a Buchner funnel with slight suction. The filtrate was transferred to a 250ml separating funnel and allowed to settle down into a top aqueous and bottom chloroform layer. The latter was transferred into a pre-dried and weighed 100 ml round bottom flask and the chloroform was removed on a heated rotary evaporator (see section 2.2.2). Traces of chloroform were removed by blowing nitrogen into the flask and then the flask was re-weighed.

The lipid content of the muscle, expressed as g of extracted lipid per kg of muscle was determined from the following formula:

$$\text{Crude lipid content (g kg}^{-1}) = (W_{f2} - W_{f1}) \times 1000 \times Ws^{-1}$$

Where:

- W_{f1} was the weight in g of the pre-dried flask
- W_{f2} was the weight in g of the flask with the extracted lipid
- W_s was the weight of the fillet muscle used for the analyses in g

The extracted lipid was transferred with chloroform into an Eppendorf tube. After de-aeration by bubbling a stream of nitrogen, the extracted lipids were stored at -80°C until taken for free fatty acids and peroxide value determinations (see sections 2.2.7.9 and 2.2.7.10, respectively).

2.2.7.2. Determination of β–hydroxy-acyl-coenzyme-A dehydrogenase (HADH) activity

Two slices of approximate 2 g weight were removed from the tail portion of each fillet and used for the preparation of the extract for HADH assays according to Garcia de Fernando *et al*, (1992). The methods are described in section 2.1.7.1. One extract was prepared from each fillet and three measurements were performed on each extract.

2.2.7.3. Determination of the activities of the enzymes *α*-glucosidase and *β*-*N*-acetyl-glucosaminidase

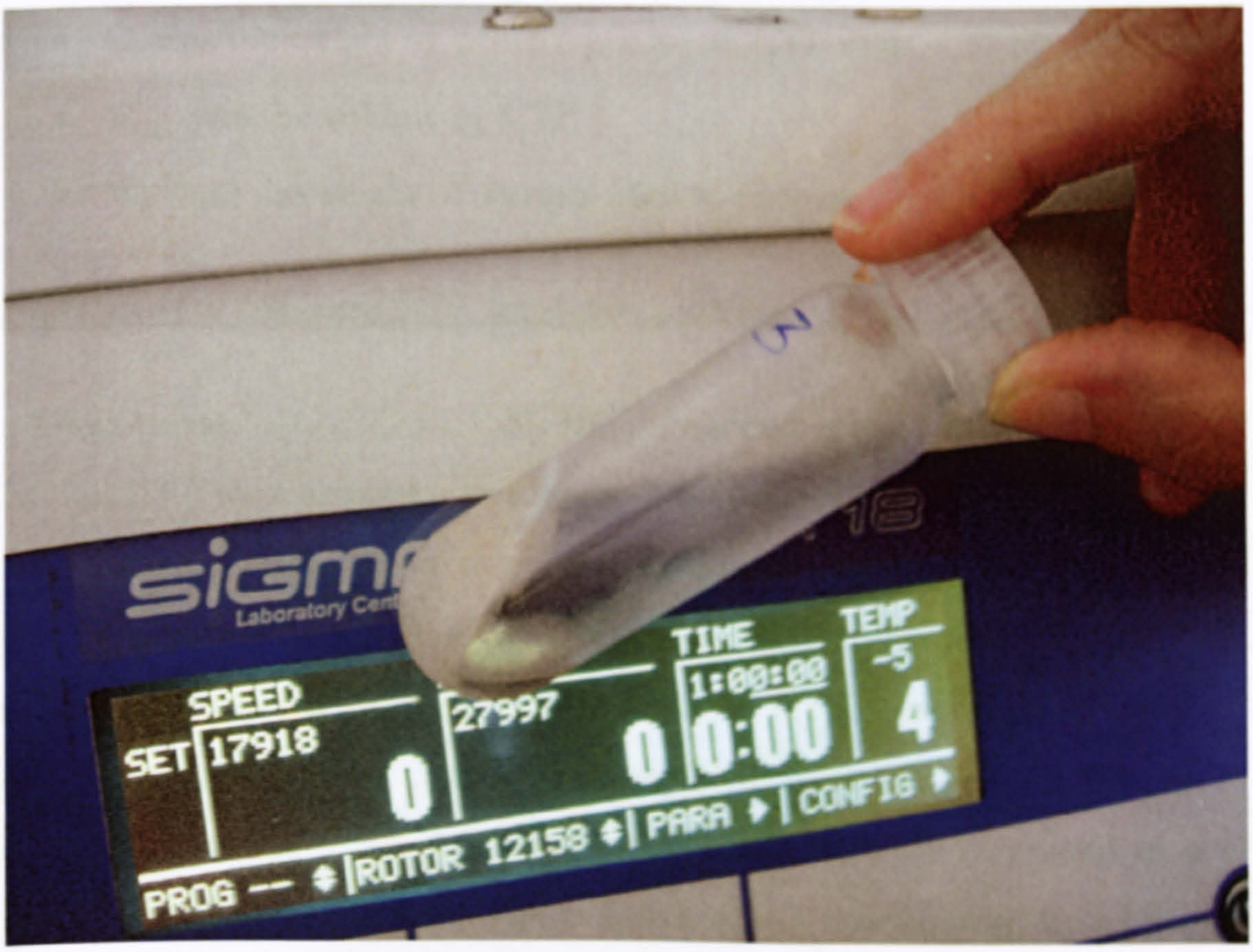
a) Centrifugal tissue fluids preparation

Centrifugal tissue fluids (CTF) were prepared according to Nilsson and Ekstrand (1993).

A sample of white muscle (5 to 7g), taken from tail portion of the fillet, was chopped into small pieces and centrifuged at 4°C at 28,000 g for 60 minutes (see section 2.2.2; Figure 2.2.7.3, below). The fluids that leaked

out from the tissues were collected using a Pasteur pipette. The centrifugal tissue fluids were determined by weighing the samples before and after centrifugation. The volumes of the fluids were estimated by assuming that one gram of fluids' weight corresponded to one ml of fluids' volume.

Figure 2.2.7.3 Preparation of centrifugal tissue fluids.



The fluids were stored in an -80°C freezer until analysis. The frozen samples of fluids were thawed in ice cold water and were centrifuged at 28,000 g at +4°C for 5 minutes to obtain clear supernatants. The supernatants were subjected to the determinations of protein, α-glucosidase and β- N- acetyl-glucosaminidase activity.

b) Protein content released in the centrifugal tissue fluids

The protein content in the centrifugal tissue fluid was measured by the bicinchoninic acid (BCA) procedure, described in section 2.1.7.4-b.

Results were expressed as grams of protein released in centrifugal tissue fluids per kg of gilthead seabream fillets.

The following formula was used:

$$\text{Protein released in CTF (g kg}^{-1}) = C_p \times V_{CTF} \times W_s^{-1} \times 1000$$

Where:

- C_p was the weight of protein per ml of CTF in g
- V_{CTF} was the volume of CTF
- W_s^{-1} was the weight of sample used to prepare the CTF in g

c) α-Glucosidase (AG) and β- N- acetyl-glucosaminidase activity (NAG) assay.

α-Glucosidase (AG; EC 3.2.1.20) and *β-N*-acetyl-glucosaminidase (NAG; EC 3.2.1.30) were assayed according to Nilsson and Ekstrand (1993).

For the AG assay, the activity was measured using *p*-nitrophenyl-α-glucopyranoside as substrate. The reaction mixture contained 0.3 ml of 0.1M sodium citrate buffer (pH=4), 0.2ml of 0.1M NaCl and 0.8 ml of centrifugal tissue fluid diluted with distilled water to a total protein content of about 5 mg. The reaction mixture was pre-incubated in a water-bath at 37°C for 10 minutes. The reaction was initiated by adding 0.2 ml of 4.2 mM *p*-nitrophenyl-α-glucopyranoside solution. After 60 minutes at 37°C the reaction was terminated by adding 1ml of 0.2M KOH with vigorous stirring. The absorbance was measured at 405 nm using a spectrophotometer (see section 2.2.2). Negative controls were prepared in the same manner except that the substrate was incubated separately from the reaction mixture. The substrate and the reaction mixture were combined after the addition of the stopping reagent. Blank measurements were performed using distilled water instead of centrifugal tissue fluid. Their respective absorbancies were subtracted from the values obtained from the samples.

NAG activity was determined using *p*-nitrophenyl-*N*-*β*-D-glucose amide as substate. The reaction mixture contained 0.3 ml of 0.1M sodium citrate buffer (pH=4.5), 0.2 ml of 0.6 M KCl and 0.5 ml of centrifugal tissue fluid diluted with distilled water to a total protein content of about 2 mg. The reaction mixture was pre-incubated in a water bath at 37°C for 10 minutes. The reaction was initiated by adding 0.2 ml of 1.0 mM *p*-nitrophenyl-*N*-*β*-D-glucose amide solution. After 30 minutes at 37°C the reaction was terminated by adding 1 ml of 0.3 M KOH with vigorous stirring on a Vortex mixer (see section 2.2.2). Blank and negative controls were run as described above. The absorbance was measured at 405 nm. The amount of *p*-nitrophenol released was calculated using a molar extinction coefficient of 19500 M^{-1} cm^{-1}. One unit of enzyme was defined as the activity that released 1 µmol of *p*-nitrophenol per minute (Benjakul and Bauer, 2000). Results were expressed as mU per gram of tissue using the following formulae:

$$AG\ activity\ (mU\ g^{-1}) = 1.28 \times \Delta A \times V_{CTF} \times V_s^{-1} \times W_s^{-1}$$

$$NAG\ activity\ (mU\ g^{-1}) = 2.05 * \Delta A * V_{CTF} * V_s^{-1} \times W_s^{-1}$$

Where:

- ΔA was the decrease in the absorbance after the reaction time
- V_{CTF} was the volume of CTF in ml
- V_s was the volume of CTF in the reaction mixture in ml
- W_s was the weight of the fillet muscle used for the preparation of CTFs in g.

Three determinations were performed for each sample of centrifugal tissue fluid.

2.2.7.4. Determinations of the water holding capacity parameters

a) Thawing weight losses

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 following formula was used:

$$\text{Thawing losses (g kg}^{-1}) = (W_1 - W_2) \times W_1^{-1} \times 1000$$

Where W_1 and W_2 were the weight in g of gilthead seabream fillets before freezing and after thawing, respectively.

b) Centrifugal tissue fluids (expressible fluids) determinations

The press method, which was used in experiments with scallop muscles to measure expressible fluids (see section 2.1.7.3-b), requires an extremely homogenous sample of small size (0.5 to 2 g; Trout, 1988). This was difficult to be obtained from the gilthead seabream fillets because of the heterogeneity of the fillets. Thus, the centrifugal tissue fluids (CTFs), prepared as is described in section 2.2.7.3-a, 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 using the following formula:

$$\text{Centrifugal tissue fluids (g kg}^{-1}) = (W_3 - W_4) \times W_4^{-1} \times 1000$$

W_3 and W_4 were the weights in g of fillets samples before and after centrifugation, respectively.

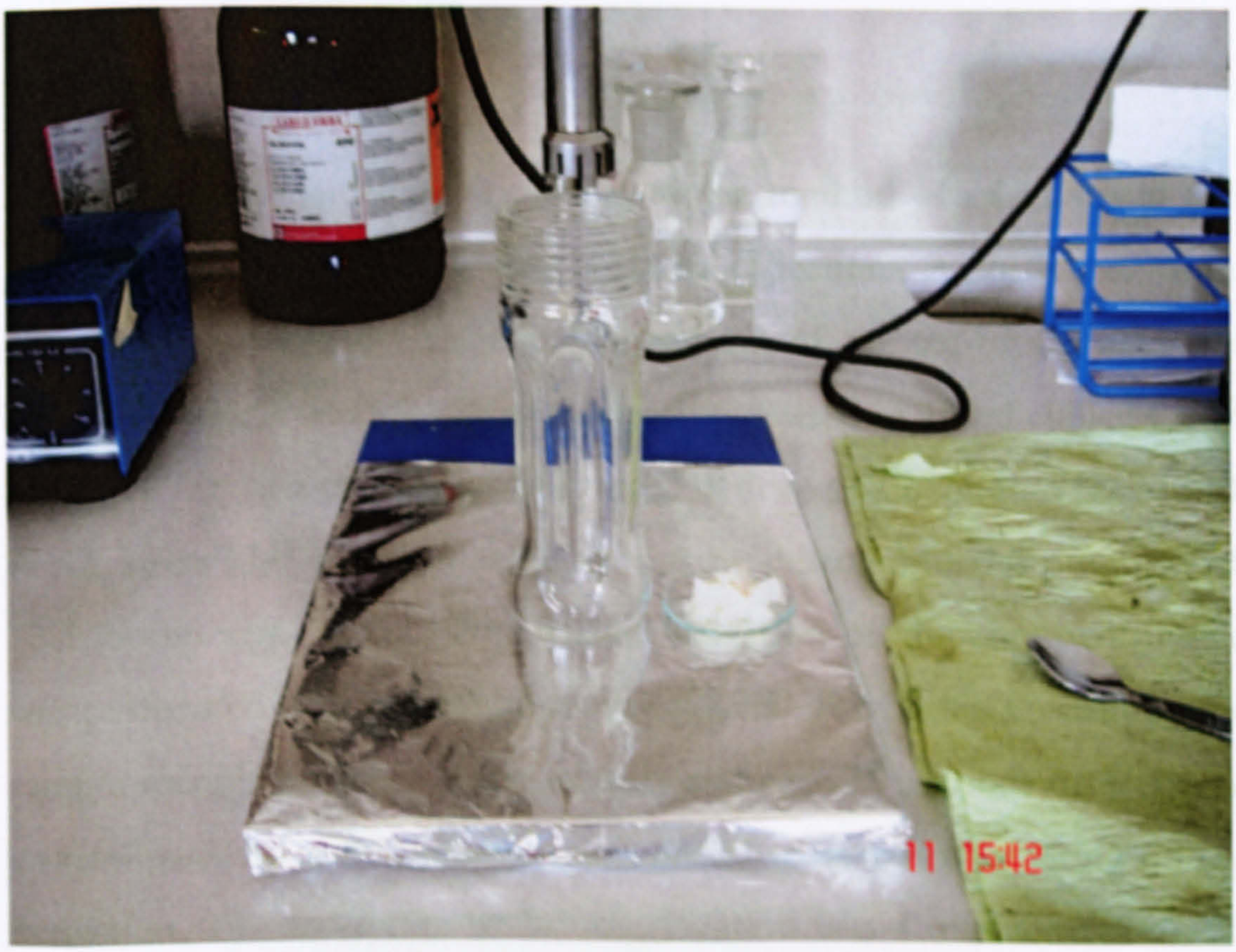
2.2.7.5. Salt soluble proteins

Extracts were prepared according to Shiku *et al.* (2004). A portion of white muscle (2 g) was excised from the middle part of the sea-bream fillet and chopped using a scalpel. It was then homogenized in 40 ml chilled 0.6 M KCl, pH 7.0 for 1 minute.

Homogenization of the muscle was performed using an Ultra-Turrax homogenizer (see section 2.2.2), a closed dispersing tool (see section 2.2.2) and a beaker that had a special design to minimize formation of vortex and frothing during homogenization (Bioblock Scientific, France; Figure 2.2.7.5).

The beaker containing the sample was placed in ice-water mixture in order to avoid overheating during blending. The homogenate was then centrifuged at 3,900 g for 20 minutes at 4°C. A 10 ml volume of the supernatant was used for protein determination using the Kjeldahl technique (section 2.2.7.1-c; AOAC, 1997).

Figure 2.2.7.5. The beaker and the dispersing tool for the extraction of proteins.



The salt soluble protein per kg of fillet was determined as follows:

$$\text{Salt soluble protein (g kg}^{-1}) = (V_A - V_B) \times 14.01 \times M \times (40 + MC) \times 6.25 \times W^{-1}$$

Where:

- V_A and V_B were the volumes in ml of standard acid required for sample and blank respectively
- 14.01 is the atomic weight of nitrogen
- M was the molarity of the standard HCl acid, i.e. 0.1M
- W was the weight of fillet sample in g
- MC was the moisture content of the fillet sample

The results were expressed as the ratio of salt soluble protein to total protein.

One extract was prepared from each fillet and triplicate measurements of protein were performed on each extract.

2.2.7.6. Extractability of actomyosin

a) Actomyosin preparation

Actomyosin was prepared according to the method of MacDonald and Lanier (1994) with the following slight modifications. A portion of white muscle (4g) excised from the middle portion of the sea-bream fillet was chopped using a scalpel. It was then homogenized in 40 ml chilled 0.6 M KCl, pH 7.0. Actomyosin was extracted by using KCl solution instead of NaCl, since Mg^{2+}-ATPase activities can be detected in the presence of KCl (Sompongse *et al*, 1996). Homogenization of the chopped muscle was performed as described in section 2.2.7.5. The beaker containing the sample was placed in ice water mixture and each 30 seconds of blending was followed by a 30 seconds rest interval to avoid overheating during extraction. The total time of homogenization was 4 minutes i.e. 8 x 30

seconds. The homogenate was kept in an ice-water mixture for an extraction period of 30 minutes prior to centrifugation at 5000 g for 30 minutes at 4°C. The supernatant was mixed with three volumes of chilled distilled water to precipitate actomyosin overnight at 4°C, which was then collected by centrifuging at 5000 g for 20 minutes at 4°C. The residue was then re-dissolved in an equal volume of chilled 1.2 M KCl solution (pH 7.0), and stirred gently at 4°C for 30 minutes in a refrigerator using a magnetic stirrer. Un-dissolved material was removed by centrifuging at 5000 g for 20 minutes at 4 °C. During the analysis, the actomyosin was kept in an ice-water mixture.

The mentioned method was used to prepare actomyosins from gilthead seabream fillets instead of that described in section 2.1.7.4 (experiments with scallop muscles), since it allows the implementation of analyses on the extracted actomyosins more than ATPases activities (described below).

b) Extractability of actomyosin determination

A 5 ml volume of actomyosin was diluted with an equal volume of 0.6M KCl, pH 7.0, and the determination of protein content of the diluted actomyosin was conducted using the BCA method (see section 2.1.7.4-b). Results were expressed as mg of actomyosin per gram of tissue following the formula:

$$\text{Extractability of actomyosin (mg g}^{-1}) = C \times \{V + (W_s \times M)\} \times W_s^{-1}$$

Where:

- C was the concentration of the protein of the diluted actomyosin in mg ml^{-1}
- V was the volume of the extracting solution in ml
- W_s was the weight of the sample in g

- M was the moisture content of the sample in %
- Triplicate determinations were performed per extracted actomyosin.

2.2.7.7. Sulfhydryl content in actomyosin extracts

The determination of sulfhydryl groups (-SH) in actomyosin was carried out according to the titration method of Ramirez *et al.* (2000). 5,5' dithiobis 2- nitrobenzoic acid (DTNB) was employed as titrating agent. The total number of -SH groups was evaluated by the addition of 150 µl actomyosin solution (2mg/ml) to 1.350 ml chilled solution of KH_2PO_4-K_2HPO_4, 6mM ethylenediaminetetraacetic acid (EDTA), 0.6 M KCl, and 8 M urea, pH 7.5. Samples were homogenized in a Vortex agitator (see section 3.1.2) at 2500 rpm for 30 seconds. Subsequently, the samples were mixed with 100 µl of 10 mM DTNB reagent and incubated at 25°C in a water bath for 45 minutes. Samples were centrifuged for 10 minutes at 28000 g at 25°C before reading absorbance of the supernatant at 412 nm. The total number of reactive –SH groups was determined similarly, but in the absence of urea.

A reagent blank prepared in the absence of actomyosin and a sample blank prepared in the absence of DTNB, but with actomyosin added, was used in all analyses. Their respective absorbancies were subtracted from the values obtained for the titration of the actomyosin solution.

The numbers of –SH groups in actomyosin were calculated using a molar extinction coefficient of 13600 M^{-1} cm^{-1}(Ellman, 1959) and expressed as SH moles/ 5 x 10^5 g actomyosin. The following formula was followed:

$$\text{SH moles/ } 5 \times 10^5 \text{ g actomyosin} = (A \times \varepsilon^{-1} \times V_f) \times (V_{AM} \times C_{AM})^{-1} \times (5 \times 10^5)^{-1}$$

Where:

- A was the net absorbance of the reaction mixture

- ε was the molar extinction coefficient of 13600 M^{-1} cm^{-1}
- V_r was the volume of the reaction mixture (i.e. 1.6 ml)
- V_{AM} the volume of actomyosin in the reaction mixture equal to 0.15ml
- C_{AM} the concentration of actomyosin in mg ml^{-1}
- Triplicate determinations were performed per extracted actomyosin.

2.2.7.8. ATPase activities in actomyosin extracts.

ATPase activity assays were carried out at 0.06 M KCl and pH 7.0 (25mM Tris maleate) according to Kawashima *et al.* (1973) and Herrera (1993). The substrate for the reactions was 1 mM ATP. The remaining conditions were:

- 5mM calcium chloride and 0.5 mg protein/ml for Ca^{2+}-ATPase
- 1mM magnesium chloride and 0.2 mg protein/ml for Mg^{2+}-ATPase
- 5mM calcium chloride, 1mM magnesium chloride and 0.2 mg protein/ml for Mg^{2+}-Ca^{2+}-ATPase
- 1mM magnesium chloride, 0.5 mM ethyleneglycol bis (β-aminoethyl ether) N,N,N', N' -tetraacetic acid (EGTA) and 0.7 mg protein/ml for the Mg^{2+}–EGTA-ATPase.

Samples were incubated for 3 minutes at 25°C in a water bath and the reaction was stopped by adding 5ml of a chilled 15 % (w/v) TCA solution to 10 ml of the reaction mixture. Subsequently, the reaction mixtures were centrifuged at 28000 g for 20 minutes at 4°C. The amount of the inorganic phosphorus (P) liberated was determined according to Fiske and Subbarow (1925) (described in paragraph 2.1.7.4-d). Blank measurements were conducted by adding the chilled TCA solution prior to the addition of ATP. Duplicate ATPase activities measurements were

performed on each extracted actomyosin.

Ca^{2+} sensitivity was calculated according to Benjakul *et al*, 1997 as follows:

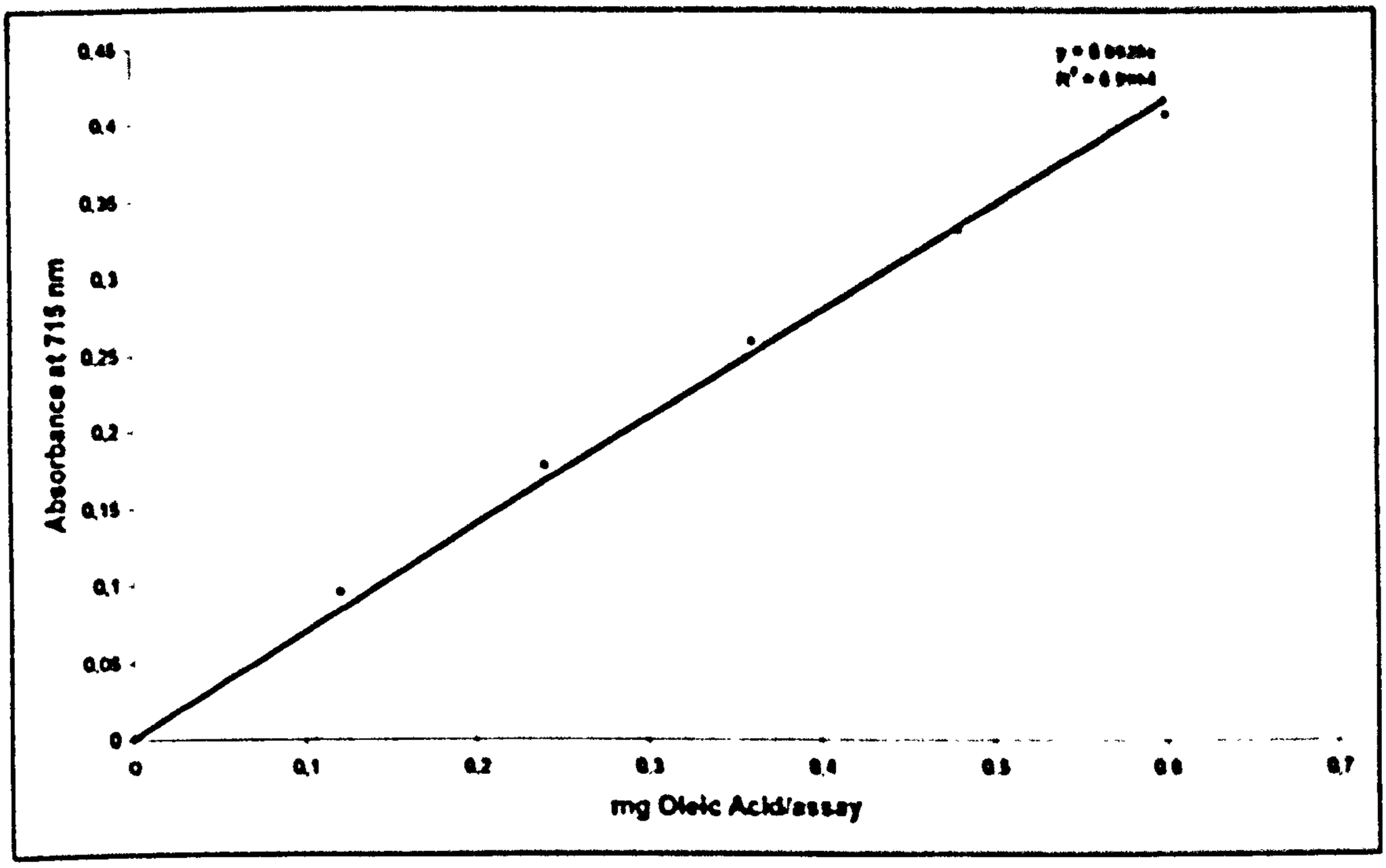
$$Ca^{2+}\ \text{sensitivity} = \{1-(Mg^{2+}\text{-EGTA-ATPase activity})/(Mg^{2+}\text{-}Ca^{2+}\text{-ATPase activity})\} \times 100$$

2.2.7.9. Determination of Free Fatty Acid (FFA) content in lipid extracted from the muscle

Total free fatty acids content in lipids extracted from the gilthead seabream muscle was determined according to Lowry and Tinsley (1976) with slight modifications. The extraction of lipids from the seabream muscle is described in section 2.2.7.1-d. A portion of the extracted lipid (0.1 to 0.08g) was accurately weighed in a 5 ml volumetric flask. A volume of 2ml benzene was added and the flask was swirled to dissolve the lipid. Benzene was then added to final volume of 5 ml. Subsequently, 1.25 ml of the benzene-lipid solution was transferred to a 2 ml Eppendorf tube and 0.25 ml of 5 % (w/v) cupric acetate-pyridine reagent (pH=6.0) was added. The biphasic system was homogenized using a Vortex mixer at 2500 rpm for 2 minutes. After centrifugation at 2300 g, the absorbance of the supernatant was read at 715 nm.

The concentration of free fatty acids (FFA) in the extracted lipids, expressed as g FFA per kg of lipid, was determined from a calibration curve (Figure 2.2.7.9) that was prepared using oleic acid as standard.

Figure 2.2.7.9 Calibration line for free fatty acids determination



For the determination of free fatty acids, the following formula was followed

$$\text{Free fatty acids (g kg}^{-1}) = A \times \alpha^{-1} \times W_s^{-1} \times 40$$

Where :

- A was the absorbance
- α was the slope of the calibration curve
- W_s was the weight of the lipid in g

Three FFA determinations per extracted lipid sample were performed.

2.2.7.10. Determination of Peroxide Value (P.V)

The estimation of peroxides in gilthead seabream lipids was determined by the ferric thiocyanate method described by Chapman and Mackay (1949) as follows:

a) Reagents

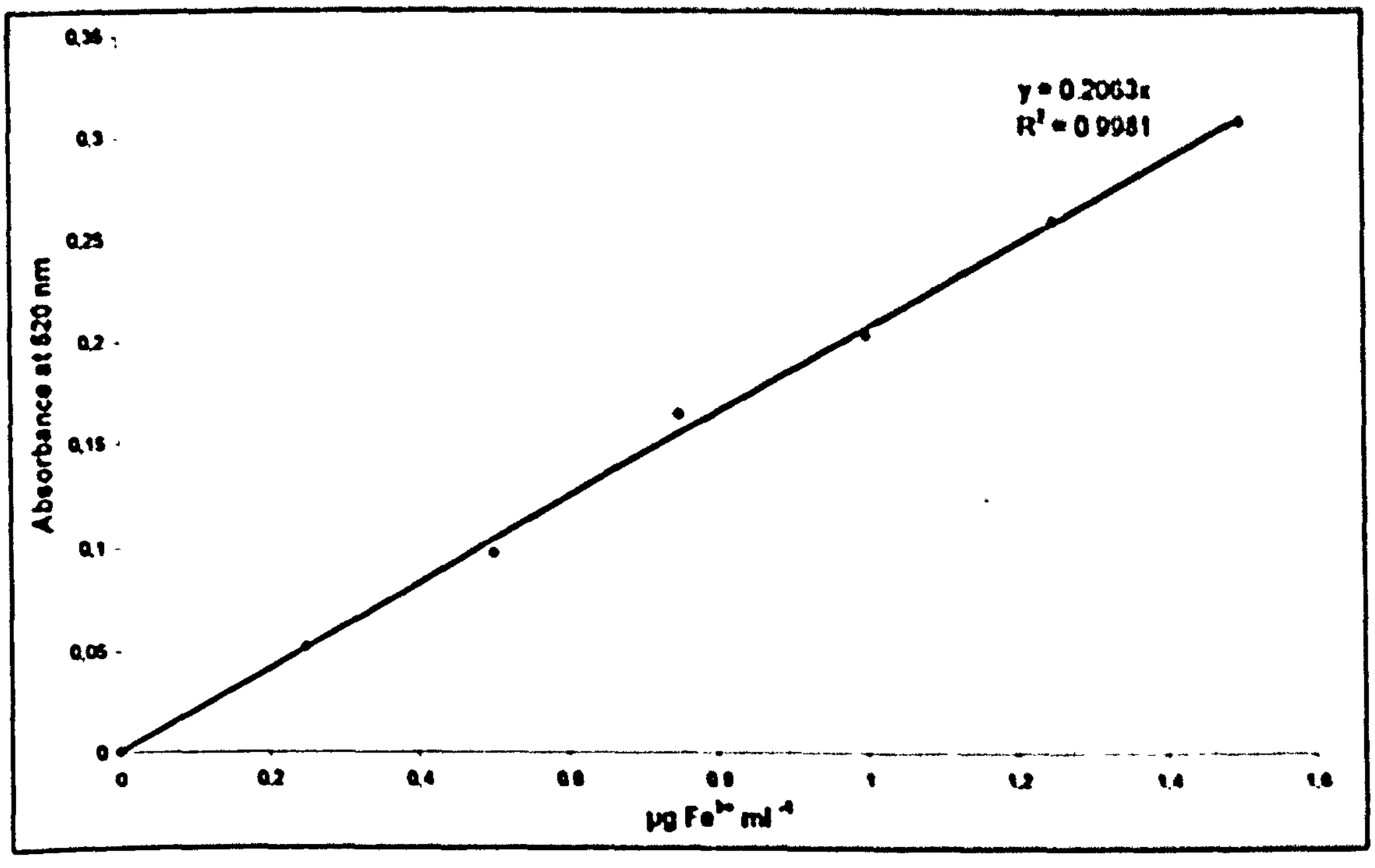
- Benzene – methanol solvent. The solvent was a mixture of 70 volumes of benzene and 30 volumes of absolute methanol.
- Ferrous chloride solution. A volume (5 ml) of a 0.8 % (w/v) aqueous solution of $BaCl_2 \bullet 2H_20$ was added slowly with stirring to 5 ml of 1 % (w/v) aqueous solution of $FeS0_4 \bullet 7H_20$ and 0.2 ml of 10 M HCl was then added. The precipitate of barium sulphate was allowed to settle, and the clear solution was used for PVs determinations.
- Ammonium thiocyanate solution. This solution was prepared by dissolving 30 g of ammonium thiocyanate in water to a volume of 100 ml.
- Ferric iron solution. This solution was prepared by diluting 0.1ml of 0.55 % (w/v) $FeCl_3 \bullet 4H_20$ in benzene – methanol with 9.9 ml of benzene methanol solvent. This solution, was used for the preparation of the calibration line (Figure 2.2.7.10, below).

b) Procedure

A portion of the extracted lipid (0.1 to 0.08g) was accurately weighed in a 5 ml volumetric flask. A volume of 2ml benzene – methanol solvent was added and the flask was swirled to dissolve the lipid. Benzene – methanol solvent was then added to final volume of 5 ml and then 1 ml of this solution was diluted to 10 ml with the same solvent. Subsequently, 1.98 ml of the diluted benzene – methanol - lipid solution was transferred to a 2 ml Eppendorf tube and 10 µl of ammonium thiocyanate and 10 µl of ferrous chloride were then added. The tube was shaken and the placed in a water bath at 50°C for two minutes. The tube was then cooled under running water and readings of the absorbencies were taken at 520 nm

(Figure 2.2.7.10, below). A blank containing all reagents except the lipid and subjected to the same treatment as the other tubes was included.

Figure 2.2.7.10 Calibration line for peroxide value determinations



c) Calculations

The peroxide value was calculated as follows:

$$P.V.\ (meq\ oxygen\ kg^{-1}\ lipid) = A \times \alpha^{-1} \times 50 \times W_s^{-1} \times 55.84^{-1}$$

Where:

- A was the absorbance
- α was the slope of the calibration curve
- 50 was the dilution factor
- W_s was the weight of sample in g
- 55.84 is the atomic weight of ferrous ion.

2.2.7.11. Thiobarbituric acid reactive substances (TBARS) determination

Extracts were prepared following the method described by Lemon (1975)

with the following slight modifications. A portion (5 g) of finely chopped white muscle, coming from the anterior part of the fillet, was homogenized with 10 ml of chilled 7.5 % (w/v) TCA solution, which contained 0.1 % (w/v) propyl-gallate as antioxidant, for 1 minute. Homogenization of the muscle was performed using an Omni homogenizer (see section 2.2.2) in a centrifuge tube. Then, the homogenate was centrifuged for 20 minutes at 5000 g at 25°C and the supernatant was filtered through a glass fibre filter paper (GF/B, 25 mm diameter, Whatman). Then, a 0.5 ml of the clear supernatant was used for the TBARS reaction described in section 2.1.7.5-b. The concentration of malondialdehyde (MDA) in the sample was expressed as mg MDA per kg of muscle following the calculation presented in section 2.1.7.5-c.

2.2.7.12. 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). The fillets for these measurements were preserved whole at -80°C and allowed to semi-thaw for 20 minutes at 25°C prior to preparing the test samples. The test samples were cylinders of standard diameter (15mm), which were excised from the middle part of the fillets close to the lateral line using a cork borer. (Figure 2.2.7.12-1, below).

Figure 2.2.7.12-1 Preparation of cylinders for texture determinations

The middle part of the fillets was selected for the preparation of the samples because it has approximately uniform height, i.e. from 12 to 14 mm. Since the precision and accuracy of the results of the shear tests depend on the size of the samples, the height of the cylinders were all arranged to 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 °C.

The shear forces of the cylinders were measured using a texture analyzer with a V- shaped Warner - Bratzler shear knife attached to a 5 kg load cell (see section 2.2.2, Figure 2.2.7.12-2 below).

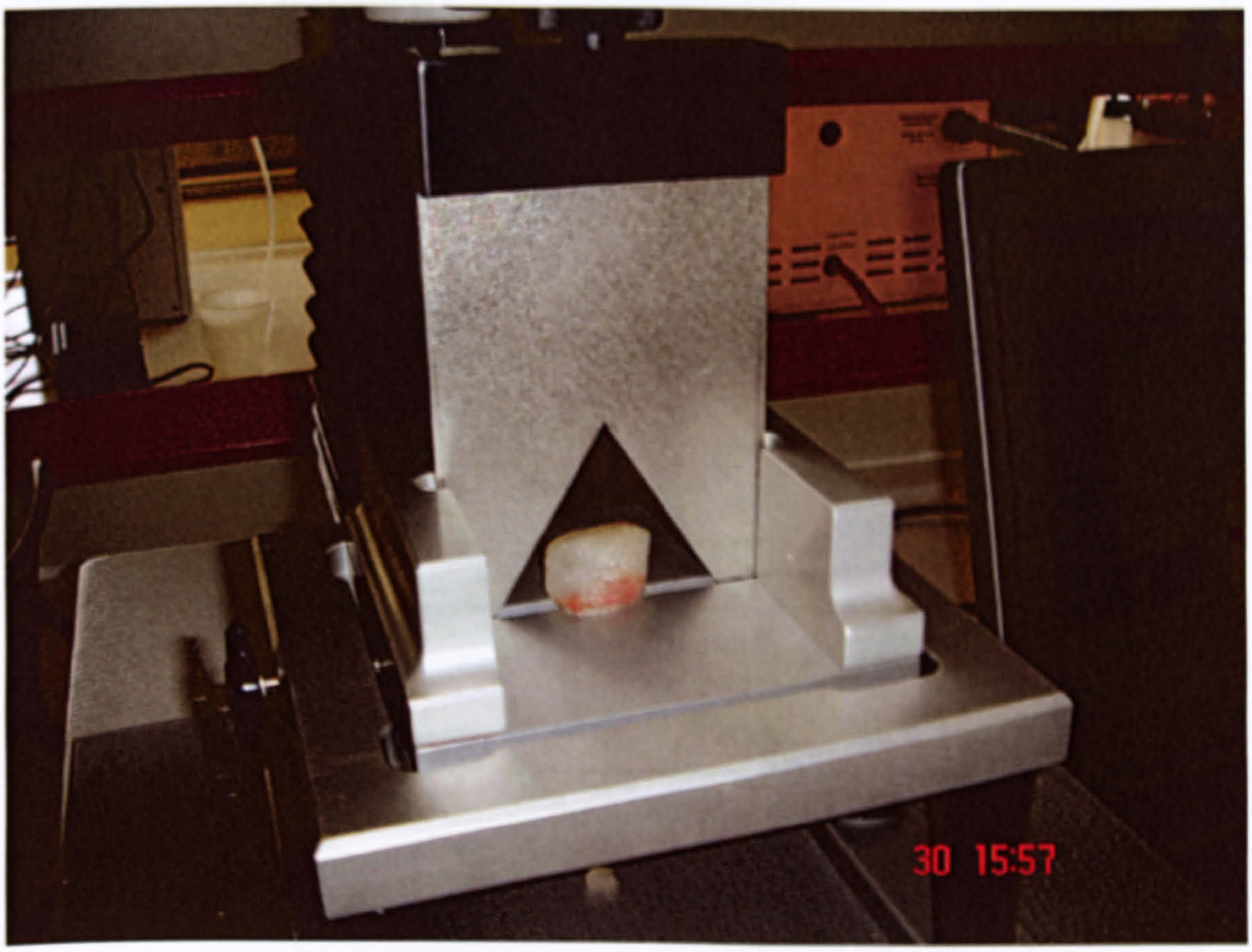
Figure 2.2.7.12-2 The texture analyzing system.

Calibration of height

Calibration of force

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 (Figure 2.2.7.12-3, below).

Figure 2.2.7.12-3 The Warner – Bratzler shear knife and location of the sample during measurements



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

Parameters recorded from the force – time graphs were the maximum force (F_{max}) and the area under the time-force curve (A_{max}) (Figure 2.2.7.12-4, below).

Figure 2.2.7.12-4 The force-time curve

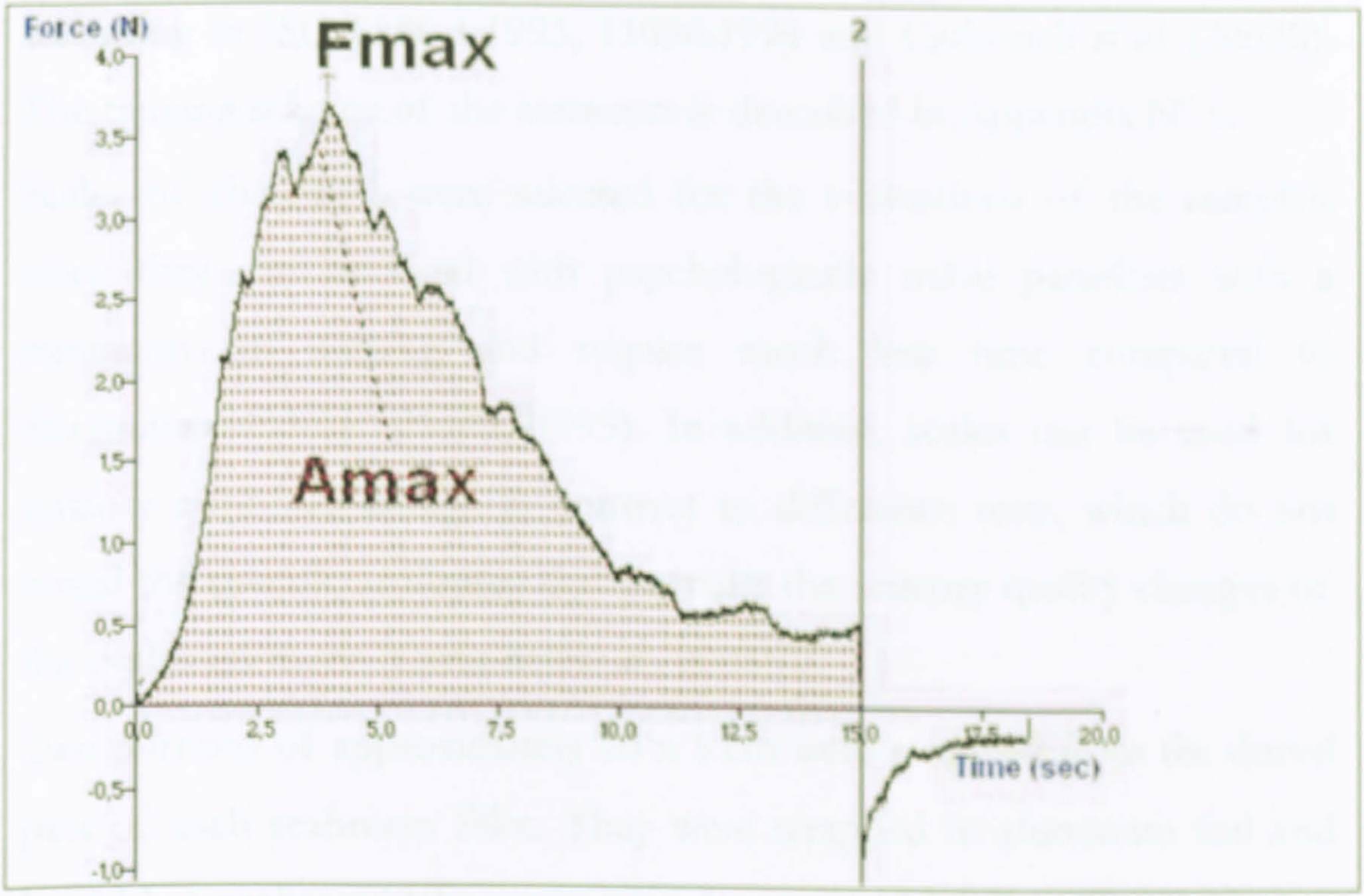


Figure notes: Fmax and Amax denote the firmness and the toughness of the samples, respectively.

The maximum force denotes the point at which the sample fills completely the triangular cavity of the blade and cuts through the sample surface. After this point shearing continues throughout the whole sample until the blade has passed through the base plate slot. According to the manufacturer's recommendations, the F_{max} and A_{max} represent the firmness and the toughness of the sample respectively (Stable Micro Systems Ltd). Triplicate measurements were taken per fillet.

2.2.7.13. Sensory assessments

a) Sensory assessments for the freezing times experiments

Gilthead seabeam fresh fillets and fillets frozen at t_c of 18, 74 and 640 minutes were evaluated for the chewiness textural attribute (ISO 11036:1994). Gilthead seabream fillets frozen at t_c of 2 minutes were not evaluated since these fillets were frozen in methanol. The evaluations

were performed by four assessors, who were trained in applying scales according to ISO 8586-1:1993, 11036:1994 and Carbonell *et al.* (2003b). The training scheme of the assessors is described in Appendix N° 5.

Scales of chewiness were selected for the evaluations of the samples, since they can be used with psychologically naïve panellists with a minimum of training and require much less time compared to discriminative tests (Botta, 1995). In addition, scales can be used for sensory attributes ratings in contrast to difference tests, which do not reveal the specific attributes that provoke the sensory quality changes of the evaluated foods (Carbonell *et al*, 2003b).

Two portions of approximately 10 x 3 cm were removed from the dorsal part of each seabream fillet. They were wrapped in aluminum foil and baked in a preheated laboratory oven (see section 3.1.2) at 200°C. During baking, the temperature of the thermal centre of each sample was monitored using the equipment described in paragraph 2.2.2. Once the centre temperature of the samples reached 70°C, they were transferred to a desiccator and allowed to cool for one hour. Then the portions were sliced into smaller samples of 1.5 to 1.7g, and served to the assessors wrapped in aluminum foil and coded with a three-digit random number. Each assessor was given two or three samples of each fillet. At each session, one fresh seabream fillet and three from different frozen groups were evaluated simultaneously. For the evaluations, a 6-point semi-unstructured 15 cm line scale, with the reference standard products anchored at certain intervals, was used (see Appendix N° 6). After tasting, each assessor was asked to place a vertical line on the scale that best indicated his/her rating of chewiness of each sample.

b) Sensory assessments for the cold storage experiments

The fresh and stored frozen fillets were evaluated for chewiness and

acceptability of taste by the four assessors, who participated in the sensory evaluations of the freezing times experiment. The middle and the anterior portion of each fillet were used for assessing chewiness and taste respectively in two separate sessions per storage period. The procedures used to cook the muscle portions and evaluate the chewiness texture attribute are described in the section 3.1.7.13-a. The cooked portion of the anterior part of each fillet was sliced into 2.5 to 3 g samples, and served to the assessors wrapped in aluminum foil and coded with a three-digit random number. Each assessor was given one sample of each fillet. At the beginning of each session, a control sample (i.e. fresh sample preserved at -80°C) was evaluated by each assessor and the individual evaluations were discussed. After tasting, the assessors were asked to indicate in a nine point hedonic scale (9 like extremely, 1 dislike extremely; see Appendix 7) their degree of liking of taste (Botta 1995). In addition, the assessors were asked to write in the score sheet their perceptions of taste of the evaluated samples. All evaluations were performed at ambient temperature (23 to 25°C).

2.2.8. Statistical analyses

Statistical analyses of data were performed with Minitab14 for Windows (Minitab Inc., 2002). The assumption of normality of distributions was tested with the Anderson-Darling test and that of homogeneity of variances with the Bartlett's test. Data were log – transformed when necessary.

One-way analyses of variance (ANOVA) were performed to test for freezing time effects on physical, chemical and biochemical parameters measured.

One-way analyses of variance or Kruskal-Wallis analyses or 't-test' with Welch's correction (described in Zar, 1984) were performed to test for the

effects of the length of time of storage at -22°C on physical, chemical, biochemical and sensory (taste) parameters measured.

Data from the cold storage experiments were subjected to non-parametric correlations in order to examine the possibility of linear relationships between the bio-chemical/ chemical parameters, physical and sensory properties. Simple or multiple linear regressions were performed when required.

General linear (GL) modelling (samples and assessors) was applied to the scores from chewiness analyses to test for effects of freezing times and length of time of storage at -22°C on seabream samples and assessor-sample interactions, and also to test for differences between assessors.

The parametric and non parametric ANOVAs showing significant differences were followed by a Tukey HSD and Dunn post-hock test respectively. Significance was accepted when $P<0.05$ (Zar, 1984).

CHAPTER 3 The bio-chemical, physical and sensory properties of the adductor muscle of scallops (*Pecten maximus*) frozen at different freezing times and during storage at -22°C. Results - Discussion - Conclusions

3.1. Results

3.1.1. Freezing times (rates) experiments: The bio-chemical, physical and sensory properties of the adductor muscle of scallops (*Pecten maximus*) frozen at different freezing times

3.1.1.1. The freezing processes

The freezing temperatures, the freezing times, the corresponding freezing rates and the characteristic freezing times are presented in Table 3.1.1.1.The freezing times (t_f values) ranged from 2,153 to 28 minutes and the freezing rates from 0.04 to 3.17 cm/h. The characteristic freezing times (t_c values), i.e. the time periods the scallop muscles were at -1 to -7°C, ranged from 19 to 1000 minutes.

Table 3.1.1.1 Experimental conditions for freezing the scallop muscles *.

Freezing Temperature (°C, ±S.D.)	Freezing Environment	Effective Freezing Time (t_f, min)**	Freezing Rate (r_f, cm/h)***	Characteristic Freezing Time (t_c, min)****
-20±1	B.S	182±21*****	0.49±0.050	89±11
	I.S	2153±93	0.04±0.001	1000±148
-35±1	B.S	77±10	1.28±0.170	49±10
	I.S	976±98	0.09±0.070	555±120
-80±2	B.S	28±3	3.17±0.290	19±3
	I.S	417±53	0.21±0.030	235±57

Table notes:
BS= scallop muscles frozen bare (i.e. 'fast' frozen). IS= scallop muscles frozen insulated in containers (i.e. 'slow' frozen)
* Mean height of the thickest part was 29.9 mm
** From an initial temperature 4°C to reach central temperature -20°C (International Institute of Refrigeration 1986)
***Freezing rate was calculated by dividing the half-thickness of the thickest part of the scallop muscles by the freezing time (Chen and Pan 1995)
****From -1°C to reach central temperature -7°C (Bevilaqua *et al.* 1979)
***** Values are means ± S.D., n=6.

Figures 3.1.1.1-1, 2 and 3 show the Time-Temperature profiles during freezing of scallop muscles at -20°C, -35°C and -80°C. 'Fast' freezing at -20°C, -35°C and -80°C reduced the characteristic freezing times (t_c values) by 11.2, 11.3 and 12.4 times, respectively, compared to 'slow' freezing at the same respective final freezing temperature.

Figure 3.1.1.1-1 Time –Temperature profile during freezing of scallop muscles at -20°C.

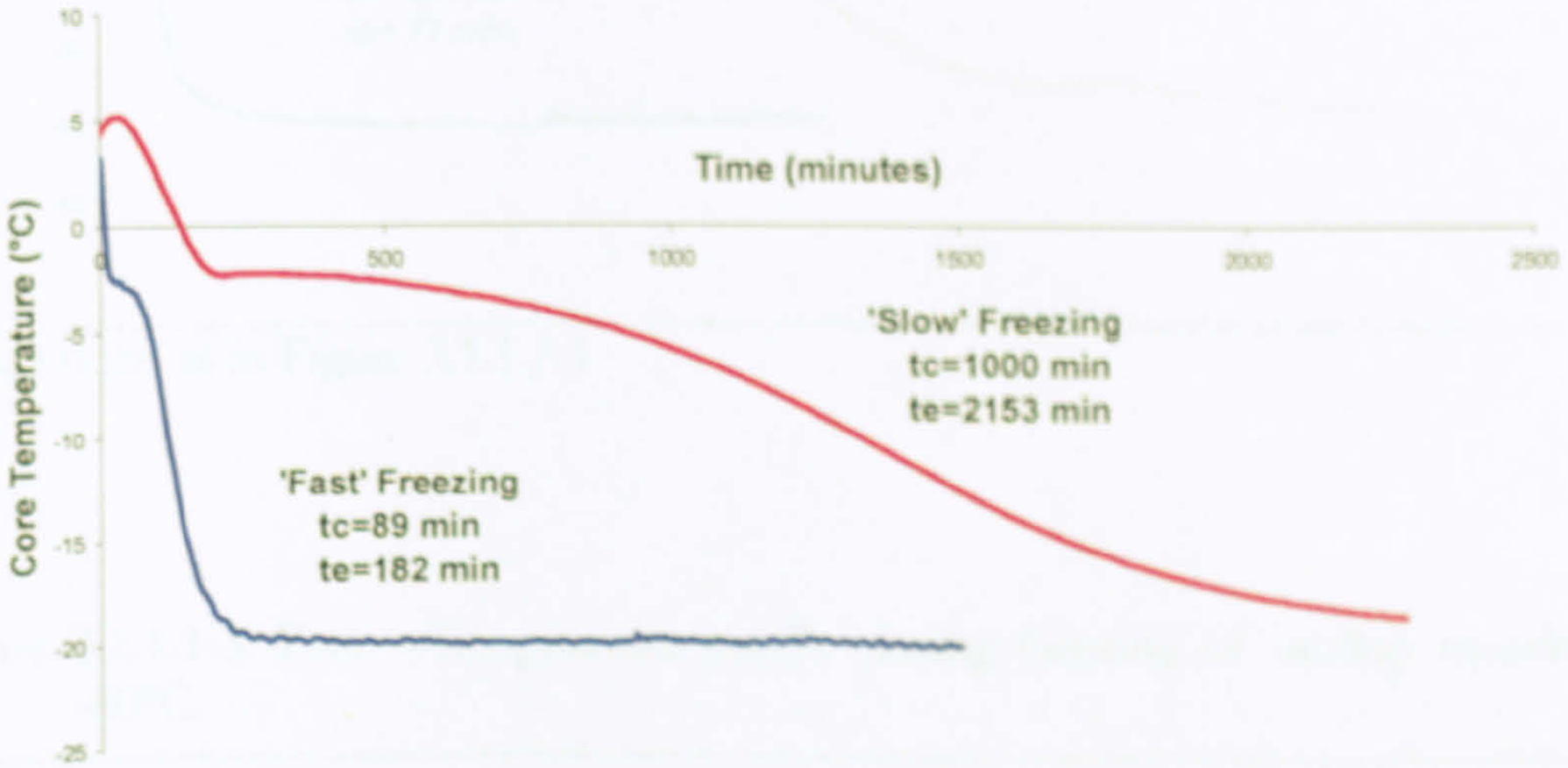


Figure notes: 'Fast' freezing = scallop muscles frozen bare, 'Slow' freezing = scallop muscles frozen insulated in containers, tc= characteristic freezing time, te=freezing time.

Figure 3.1.1.1-2 Time –Temperature profile during freezing of scallop muscles at -35°C.

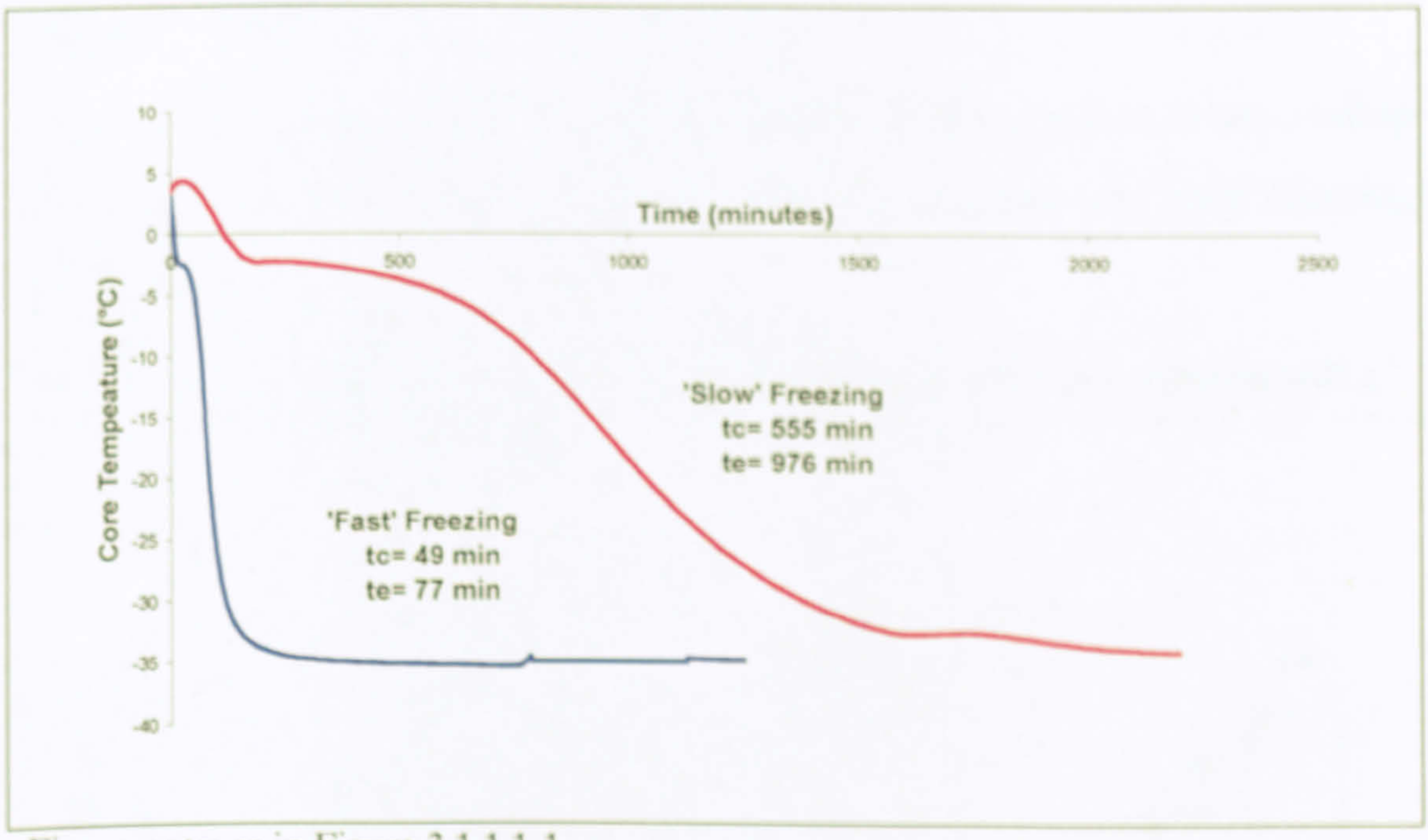


Figure note: as in Figure 3.1.1.1-1

Figure 3.1.1.1-3 Time –Temperature profile during freezing of scallop muscles at -80°C.

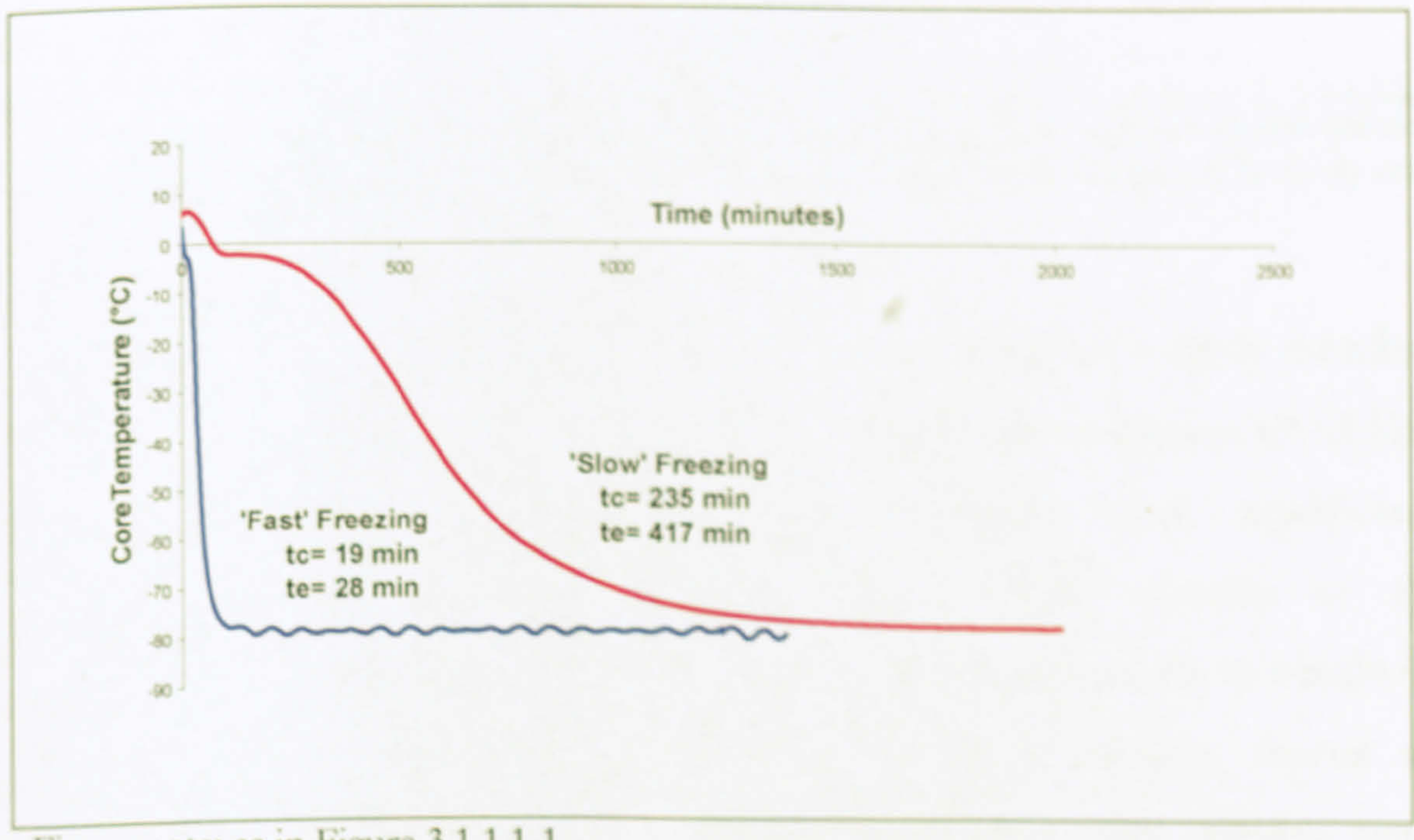


Figure note: as in Figure 3.1.1.1-1

3.1.1.2. Changes in β-hydroxy-acyl-coenzyme-A dehydrogenase (HADH) activities of scallop muscles frozen at different characteristic freezing times.

Figure 3.1.1.2 shows the HADH activities in the filtrates from scallop muscles frozen at different freezing times (t_c values) and final freezing temperatures.

Figure 3.1.1.2 β-hydroxy-acyl-coenzyme-A dehydrogenase (HADH) activities, mU g^{-1}

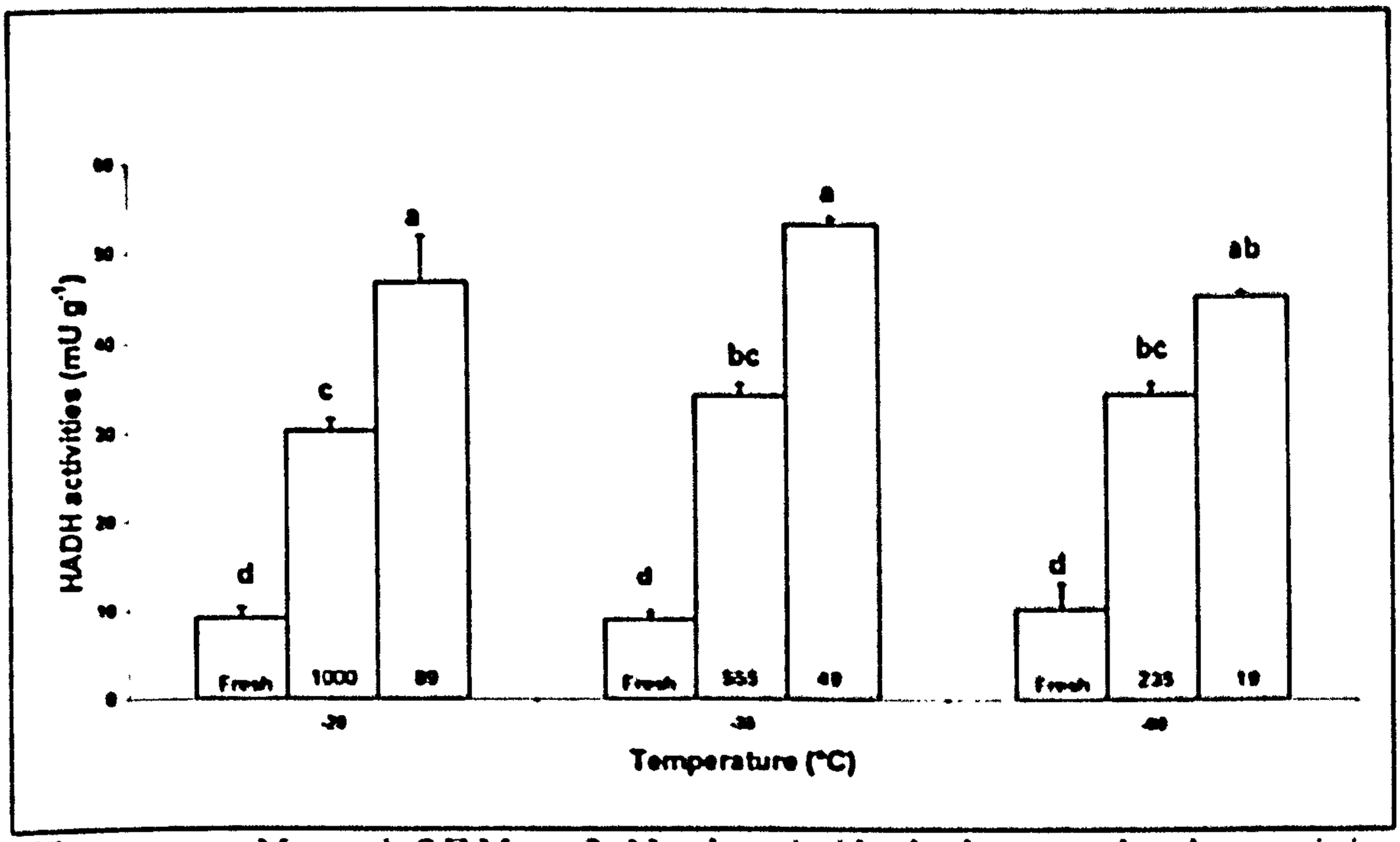


Figure notes: Means ± S.E.M, n=3. Numbers inside the bars are the characteristic freezing times (tc values) in minutes. Groups with different letters (a, b, c, d) are significantly different ($P<0.05$)

Mean values of HADH in filtrates from fresh groups of scallop muscles ranged from 9 to 10 mU g^{-1} and were not significantly different ($P>0.05$). Differences between the experimental groups were significant ($P<0.0001$). HADH activities of frozen scallop muscles at all characteristic freezing times were significantly higher than those obtained from fresh scallop muscles. Activities of scallop muscles frozen at characteristic freezing times (t_c values) from 1000 to 235 minutes were lower compared with those at t_c values from 89 to 19 minutes. Activities of scallop muscles frozen at a characteristic freezing time (t_c) of 1000 minutes were the lowest and significantly different from those at freezing

times of 89, 49 and 19 minutes.

3.1.1.3. Changes in weight losses in scallop muscles due to freezing in bare conditions or insulated in containers

Weight losses due to freezing are shown in Table 3.1.1.3.

Table 3.1.1.3 Weight losses due to freezing.

Scallop muscles frozen in the container		Scallop muscles frozen bare	
Characteristic freezing time (t_c, min)	Weight losses ($g\ kg^{-1}$)	Characteristic freezing time (t_c, min)	Weight losses ($g\ kg^{-1}$)
1000	13.8±0.9	89	39.4±1.1a
555	13.5±0.5	49	18.2±0.7b
235	14.0±0.5	19	11.1±0.3c

Table notes: t_c= characteristic freezing time, minutes. Values (means ± SEM) in columns, n=9. Values in the same row without letters are not significantly different ($P>0.05$).

Significant differences were found only between the bare frozen scallop muscles ($P<0.0001$) with the lowest weight loss values found at a characteristic freezing time (t_c value) of 19 minutes.

3.1.1.4. Changes in water holding capacity parameters of scallop muscles frozen at different characteristic freezing times

a) Thawing weight losses

Figure 3.1.1.4-1 shows the changes in thawing weight losses of scallop muscles frozen at different characteristic freezing times (t_c values).

Figure 3.1.1.4-1 Thawing weight losses, g kg^{-1}

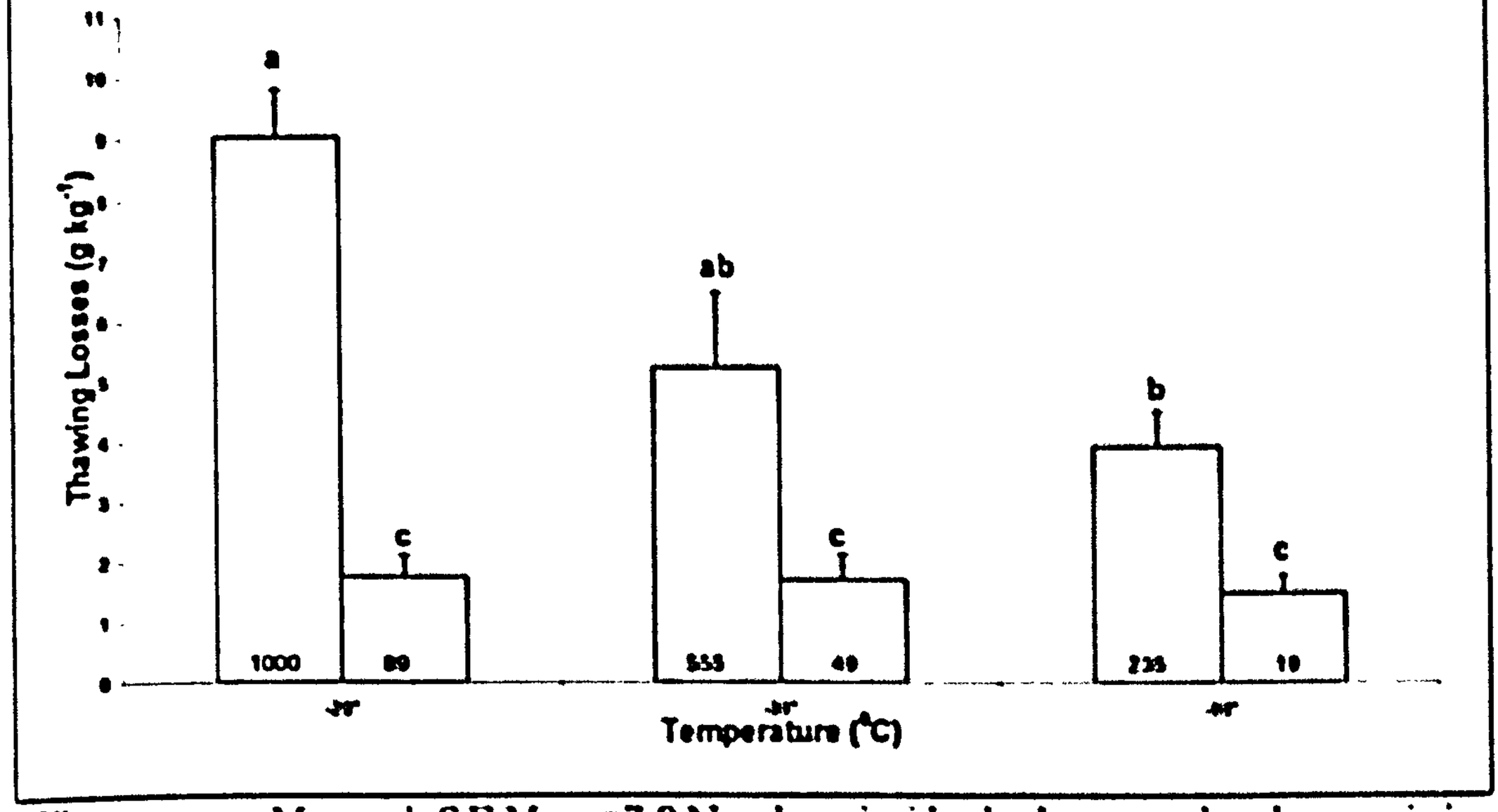


Figure notes: Means ± S.E.M., n=7-9.Numbers inside the bars are the characteristic freezing times (t_c values) in minutes. Groups with different letters (a, b, c) are significantly different ($P<0.05$)

Thawing weight losses obtained from scallop muscles frozen at a characteristic freezing time (t_c) of 1000 minutes were significantly more than those obtained from scallop muscles frozen at characteristic freezing times (t_c values) between 235 and 19 minutes ($P<0.05$).Thawing weight losses obtained from scallop muscles frozen at a characteristic freezing time (t_c) of 19 minutes were significantly less than thawing weight losses obtained from scallop muscles frozen at characteristic freezing times (t_c values) of 555 and 235 minutes ($P<0.01$).

b) Expressible fluids

Mean values of expressible fluid from fresh groups of scallop muscles ranged from 34.5 to 34.9 g kg^{-1} and were not significantly different ($P>0.05$, Figure 3.1.1.4-2).

Figure 3.1.1.4-2 Expressible fluids, g kg^{-1}

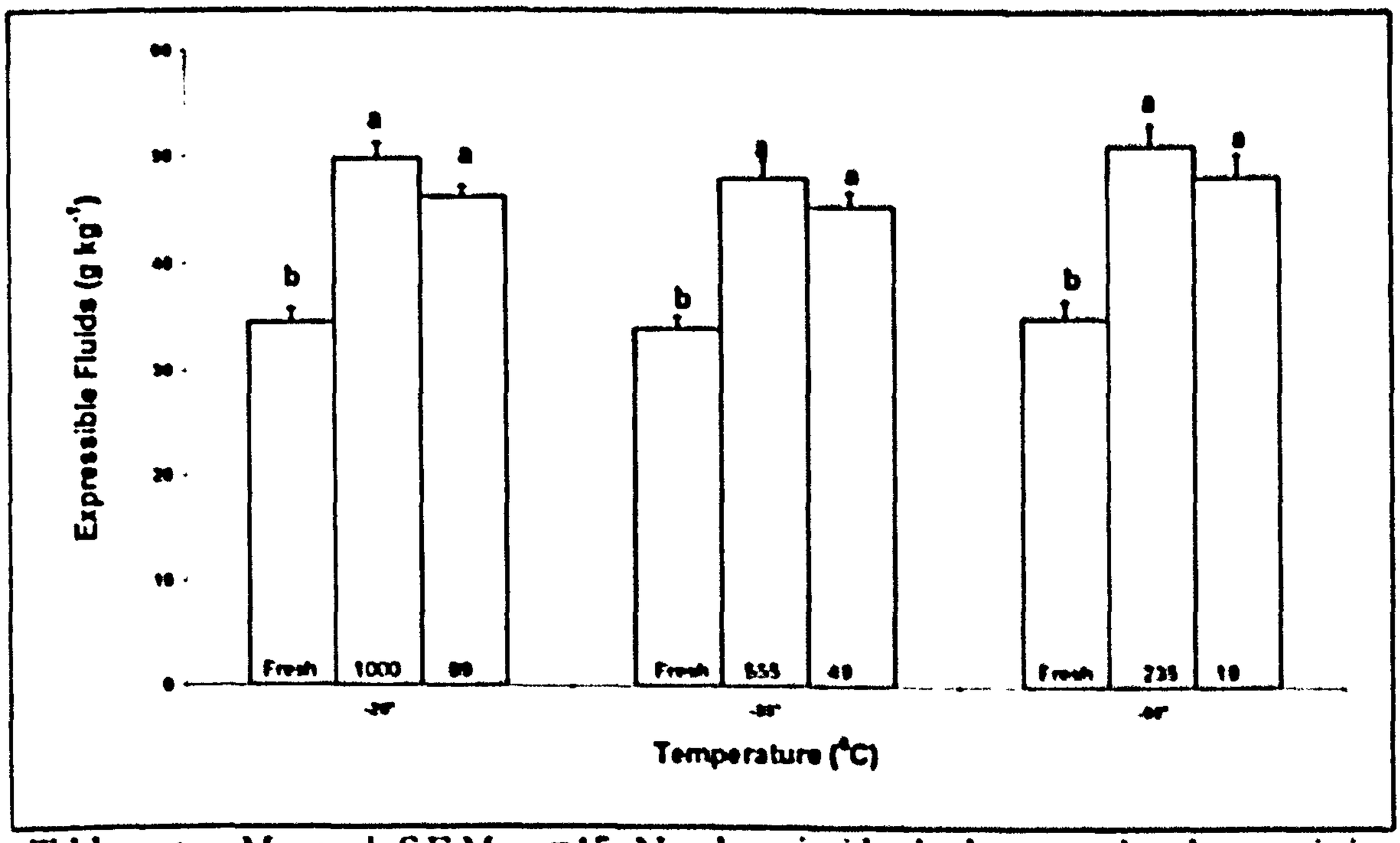


Table notes: Means ± S.E.M., n=15. Numbers inside the bars are the characteristic freezing times (t_c values) in minutes. Groups with different letters (a, b) are significantly different ($P<0.05$).

ANOVA showed significant differences between fresh and frozen scallop muscles ($P<0.0001$), but not between the frozen groups of scallop muscles themselves.

c) Total weight losses

Figure 3.1.1.4-3 shows the changes of total weight losses due to thawing weight losses and expressible fluids of scallop muscles frozen at different characteristic freezing times (t_c values). This parameter was obtained by adding the thawing weight losses and expressible fluids of scallop muscles. It was significantly higher in frozen than in fresh scallop muscles ($P<0.0001$). Differences between the frozen groups themselves were not observed ($P>0.05$).

Figure 3.1.1.4-3 Total weight losses, g kg^{-1}

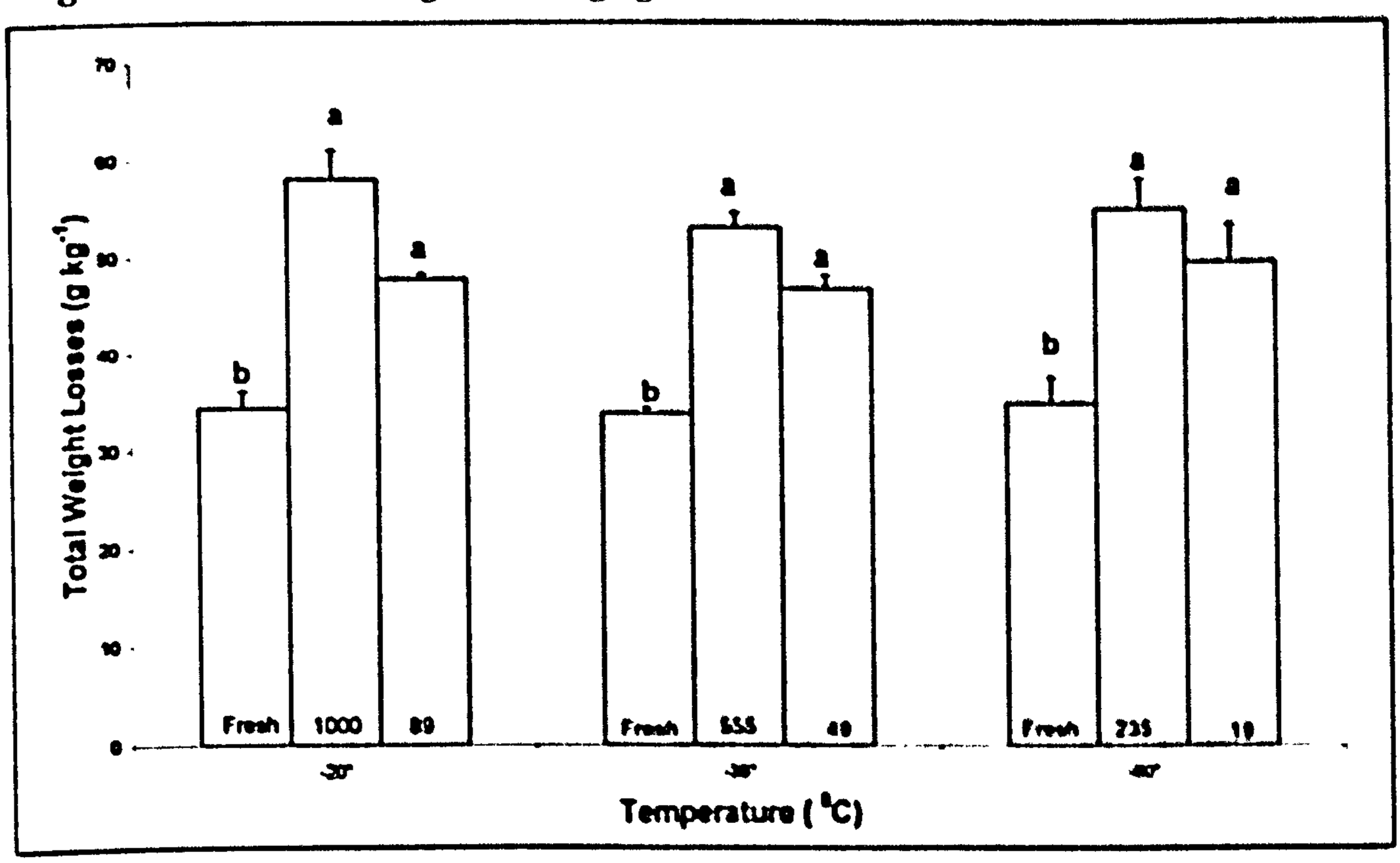


Figure notes: Means ±S.E.M, n=3. Numbers inside the bars are the characteristic freezing times (t_c values) in minutes. Groups with different letters (a, b) are significantly different ($P<0.05$).

3.1.1.5. Changes in Ca^{2+}-ATPase activities of scallop muscles frozen at different characteristic freezing times.

Figure 3.1.1.5 shows the changes in Ca^{2+}-ATPase activities of scallop muscles frozen at different characteristic freezing times. Mean values of Ca^{2+}-ATPase activities in actomyosin extracts from fresh groups of

scallop muscles ranged from 0.340 to 0.377 μmoles Pi/mg protein/min and were not significantly different (P>0.05). Also, ANOVA showed no significant differences in mean values of Ca^{2+}-ATPase activity in actomyosin extracts from scallop muscles frozen at different freezing times (P>0.05).

Figure 3.1.1.5 Ca^{2+}-ATPase activities, μmoles Pi/mg protein/min

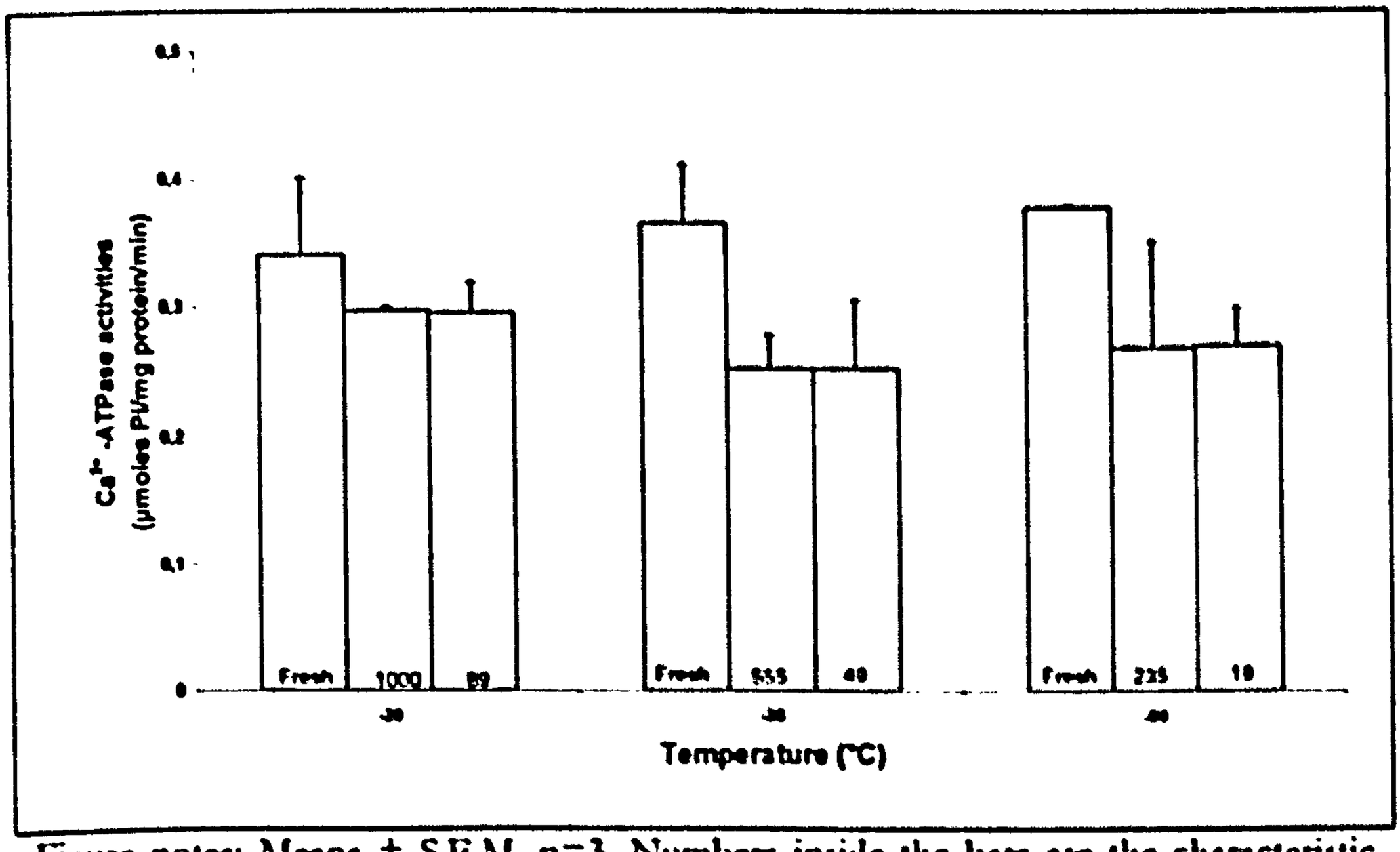


Figure notes: Means ± S.E.M, n=3. Numbers inside the bars are the characteristic freezing times (tc values) in minutes. The differences between the groups were not significant (P>0.05)

However, the mean value of Ca^{2+}-ATPase activity from all fresh groups of scallop muscles was 0.358±0.029 μmoles Pi/mg protein/min and significantly higher than those of 'fast' (i.e. characteristic freezing times from 19 to 89 minutes 0.251±0.024 μmoles Pi/mg protein/min) and 'slow' (i.e. freezing times from 235 to 1000 minutes 0.250±0.028 μmoles Pi/mg protein/min) frozen scallop muscles (P<0.05).

3.1.1.6. Changes in texture of scallop muscles frozen at different characteristic freezing times as measured by the texture analyzing system

Figure 3.1.1.6 shows the changes in peak shear forces of scallop muscles frozen at different characteristic freezing times (t_c values).

Figure 3.1.1.6 Peak shear forces, $g^* g^{-1}$

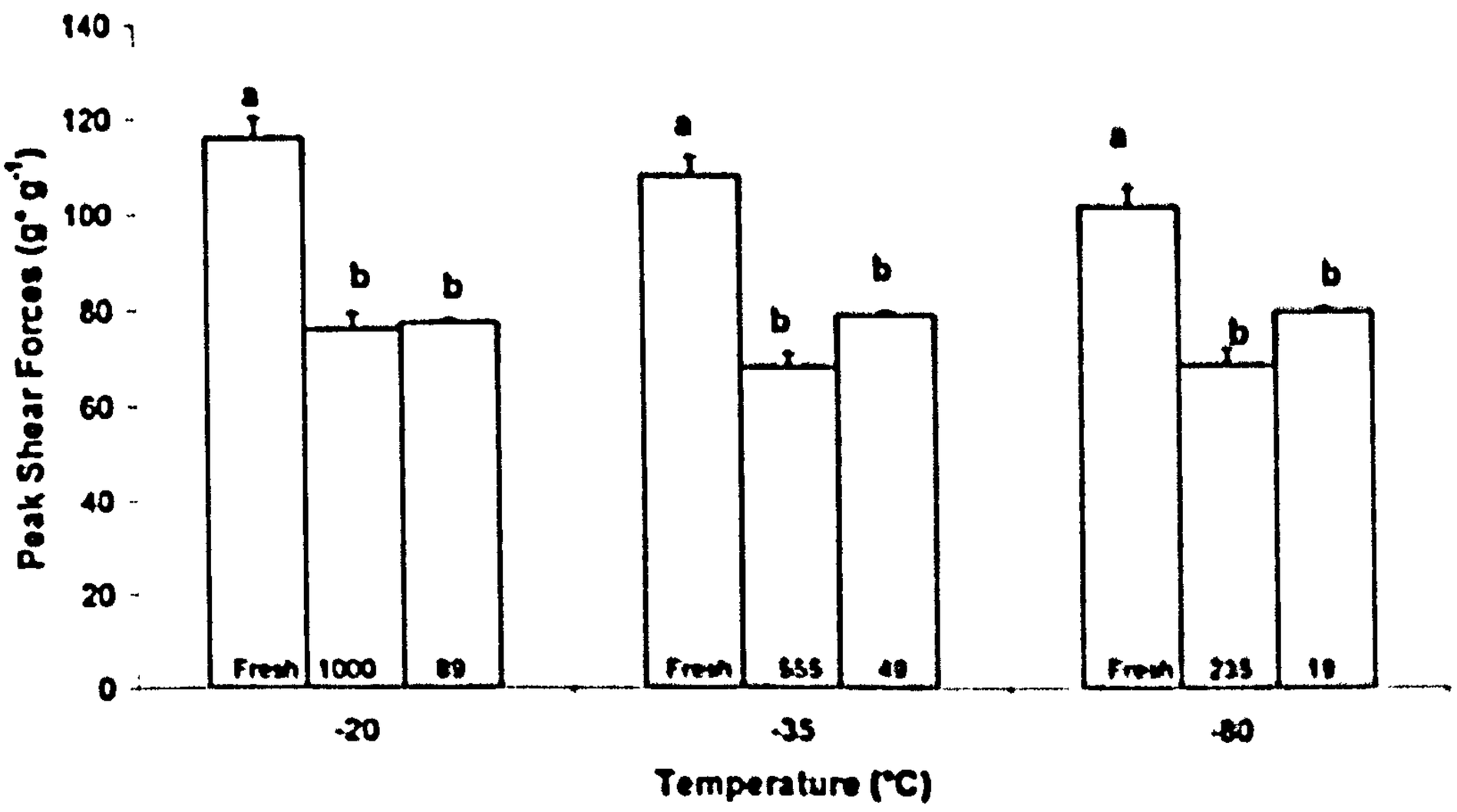


Table notes: Means ± S.E.M., n=15. Numbers inside the bars are the freezing times (tc values) in minutes. Groups with different letters (a, b) are significantly different ($P<0.05$)

Mean values of peak shear force from fresh experimental groups of scallop muscles ranged from 116 to 101 $g^* g^{-1}$ and were not significantly different ($P>0.05$). Peak shear forces obtained from the fresh scallop muscles were always significantly higher than those of frozen scallop muscles ($P<0.0001$). Differences between the frozen groups themselves were not observed ($P>0.05$).

3.1.1.7. Changes in sensory attributes

Table 3.1.1.7 shows the results of triangular comparisons between fresh and frozen scallop muscles

Table 3.1.1.7 Results of the triangle comparisons between fresh and frozen scallop muscles.

t_c (min)	Total number of evaluations*	Correct number of evaluations	Incorrect number of evaluations	More chewy		More juicy		More firm		Preferred	
				Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh
19	15	7	8	-	-	-	-	-	-	-	-
49	15	9 x	6	3	6	2	7	6	3	5	4
89	15	7	8	-	-	-	-	-	-	-	-
235	15	8	7	-	-	-	-	-	-	-	-
555	15	7	8	-	-	-	-	-	-	-	-
1000	15	10 xx	5	9 x	1	3	7	5	5	3	7

Table notes: *Frozen scallop muscles were evaluated by 5 assessors in three sessions per treatment. tc= characteristic freezing time. Numbers followed by x were significant correct at 5% level. Numbers followed by xx were significant correct at 1% level.

Significant sensory differences were found only when fresh scallop muscles were assessed against scallop muscles frozen at characteristic freezing times (t_c values) of 49 and 1000 minutes. Significant differences in texture (i.e. chewiness) were observed only in comparisons between the fresh scallop muscles and those frozen at a characteristic freezing time (t_c) of 1000 minutes

3.1.2. Storage experiments: The bio-chemical, physical and sensory properties of the frozen (-80°C) adductor muscle of scallops (*Pecten maximus*) stored at -22°C

3.1.2.1. Changes in β-hydroxy-acyl-coenzyme-A (HADH) activities of stored (-22°C) frozen scallop muscles.

Figure 3.1.2.1 shows the release of HADH in filtrates from stored frozen scallop muscles.

Figure 3.1.2.1 β-hydroxy-acyl-coenzyme-A (HADH) activities, mU g^{-1}

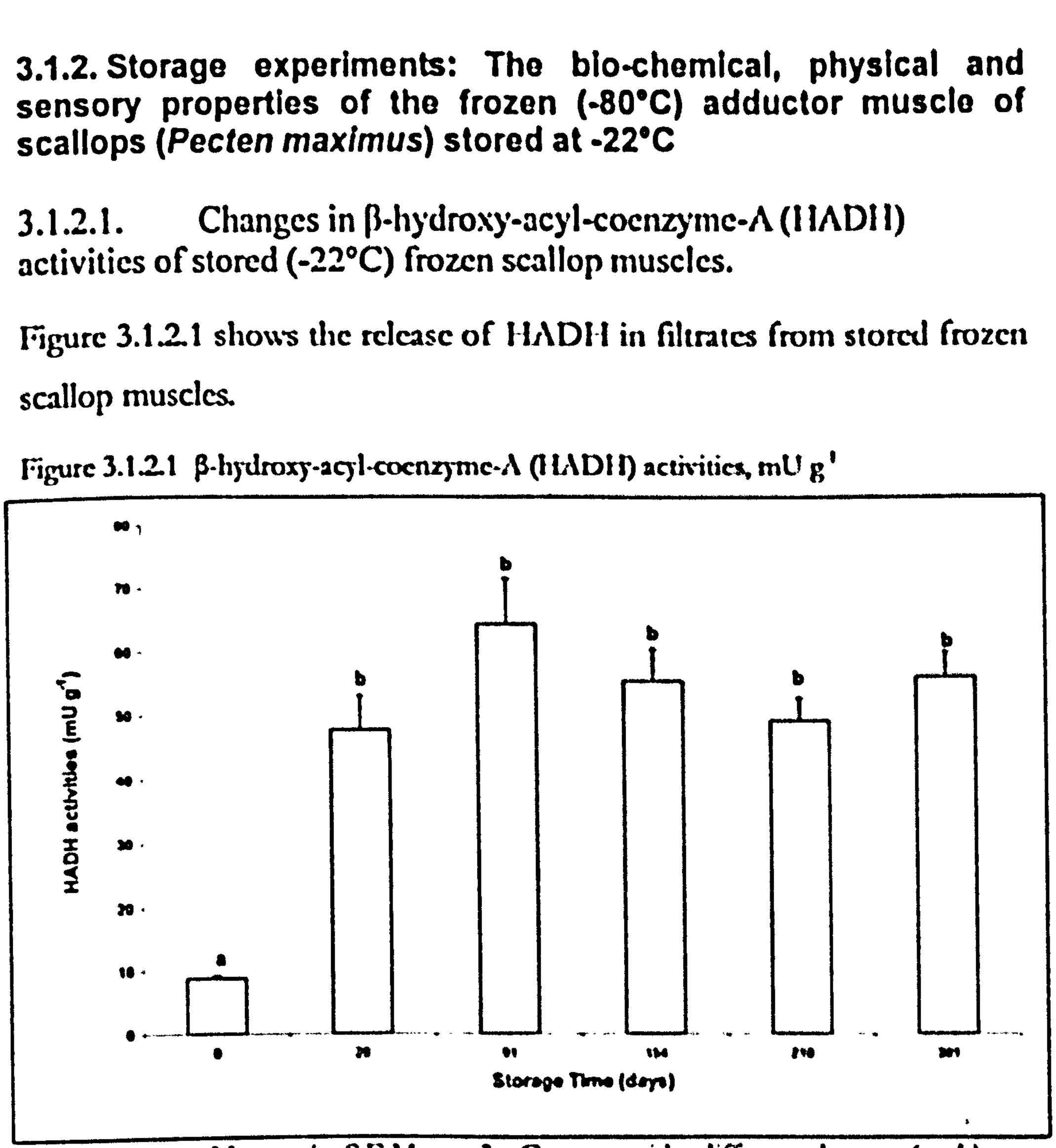


Figure notes: Means ± S.E.M, n=3. Groups with different letters (a, b) are significantly different ($P<0.05$). The '0' storage time represents fresh scallop muscles.

Mean values of HADH activities in filtrates from fresh groups of scallop

muscles ranged from 8 to 11 mU g^{-1} and were not significantly different ($P>0.05$). Significant differences were found only between the fresh and frozen experimental groups ($P<0.0001$; Figure 3.1.2.1), but not between the frozen scallop muscles stored for different times.

3.1.2.2. Changes in water holding capacity parameters of stored (-22°C) frozen scallop muscles.

a) Thawing weight losses

Thawing weight losses obtained from scallop muscles stored for 28 days were significantly lower than thawing weight losses obtained from scallop muscles stored for 91, 154, 210 and 301 days ($P<0.05$; Figure 3.1.2.2-1).

Figure 3.1.2.2-1Thawing weight losses, g kg^{-1}

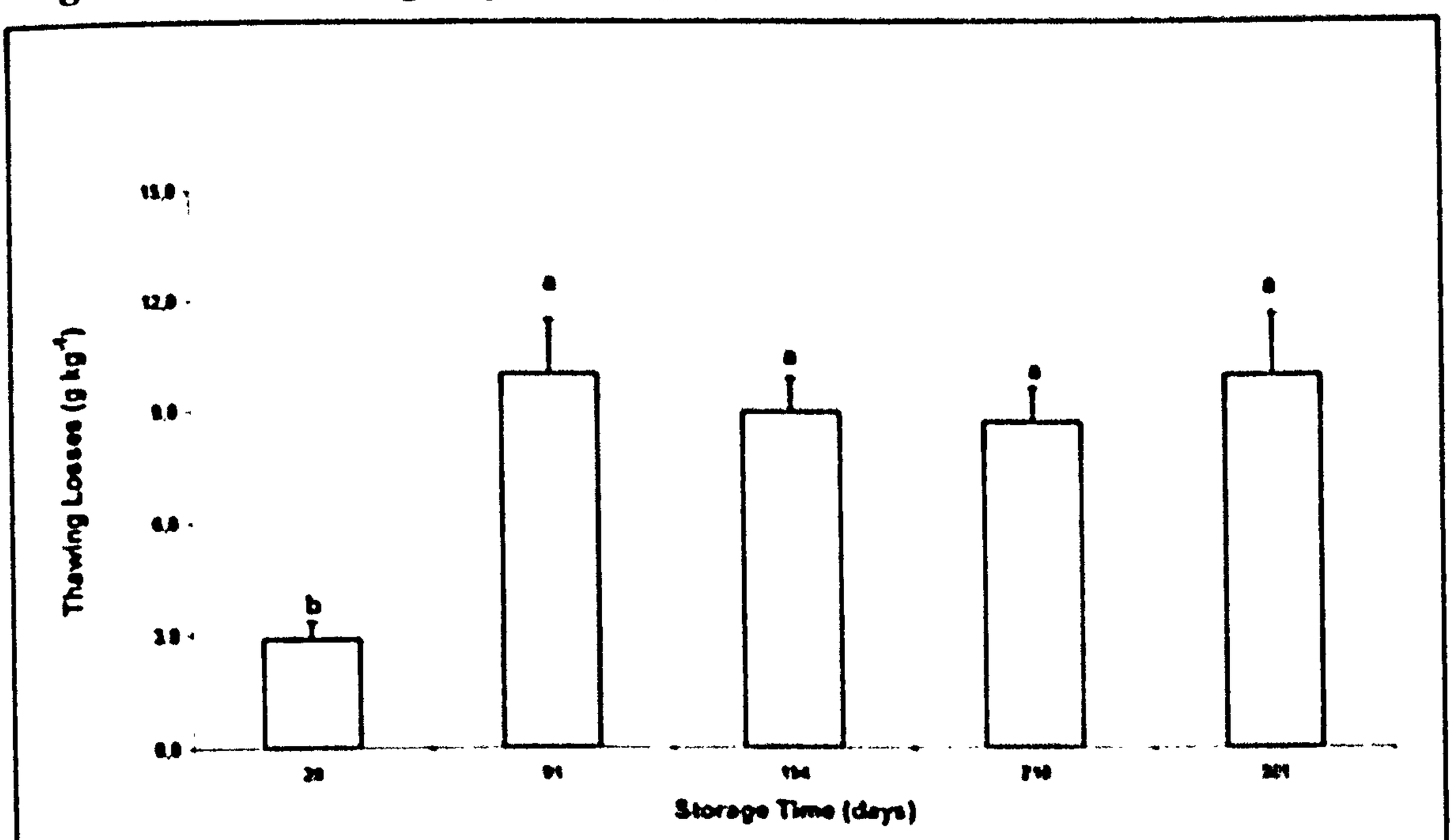


Figure notes: Means ± SEM, n=15. Groups with different letters (a, b) are significantly different ($P<0.05$).

Variable thawing loss values were observed at storage times between 91-301 days. But these were not significantly different from one another ($P>0.05$).

b) Expressible fluids

Mean values of expressible fluid from fresh groups of scallop muscles ranged from 28.1 to 34.1 g kg^{-1} and were not significant ($P>0.05$). Figure 3.1.2.2-2 shows the expressible fluid values from the fresh and stored frozen scallop muscles.

Figure 3.1.2.2-2 Expressible fluids, g kg^{-1}.

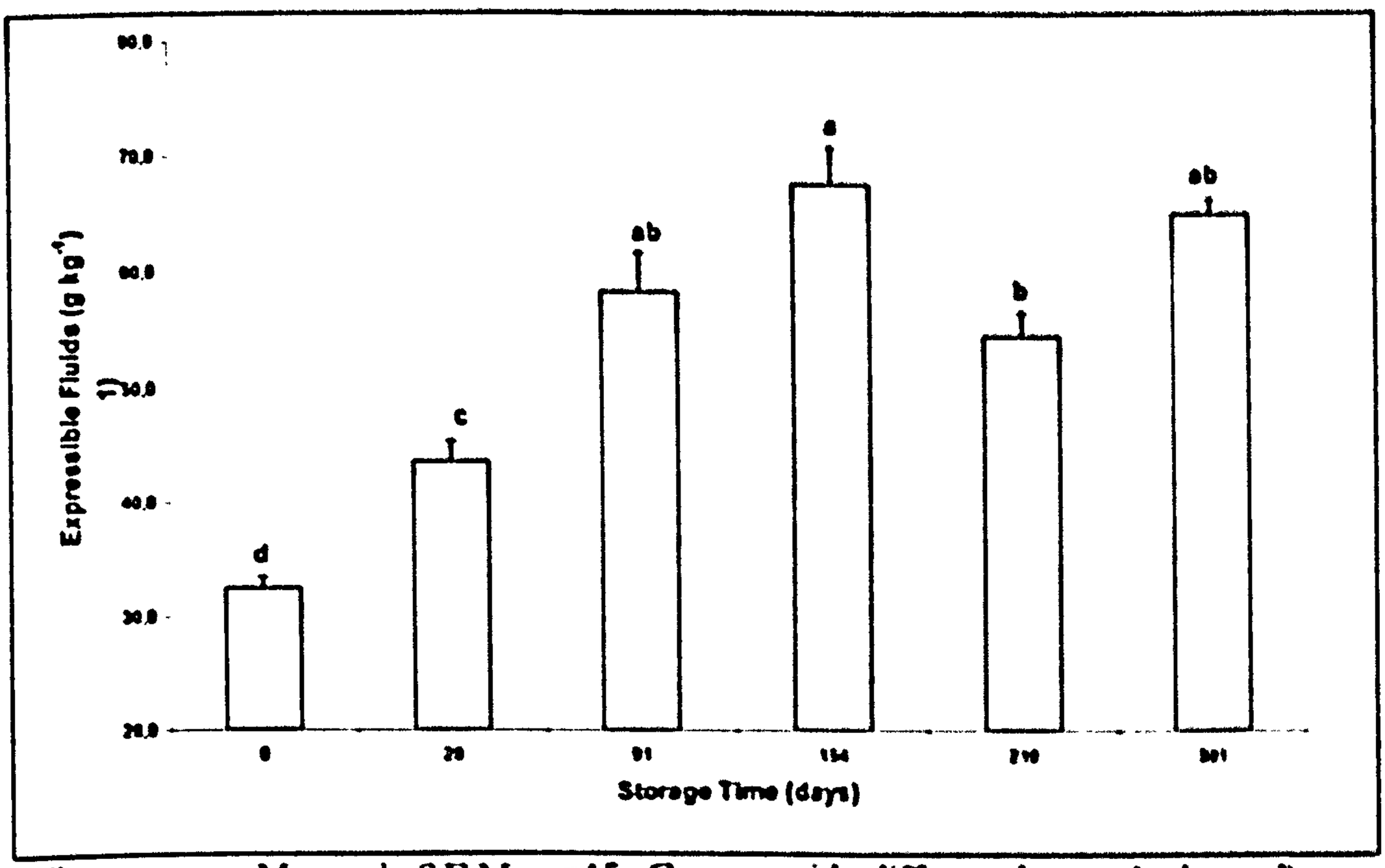


Figure notes: Means ± S.E.M, n=15. Groups with different letters (a, b, c, d) are significantly different ($P<0.05$). The '0' storage time represents fresh scallop muscles.

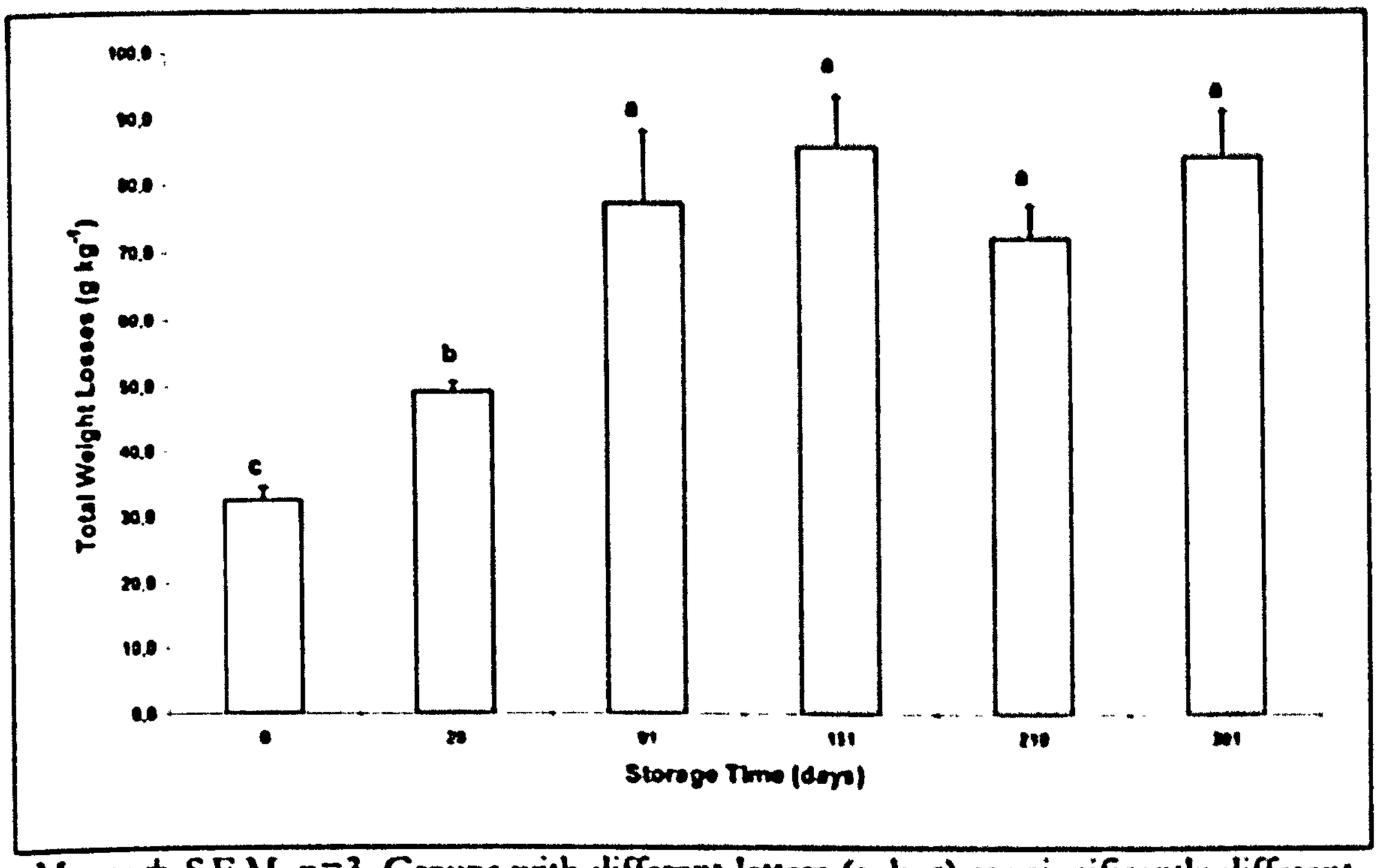
Expressible fluids from fresh scallop muscles were significantly less compared to those obtained from frozen scallop muscles in all storage periods ($P<0.05$). The mean expressible fluid values of frozen scallop muscles that were stored for 28 days were significantly lower than those obtained from scallop muscles stored for 91 to 301 days ($P<0.05$).Variable expressible fluid values were observed at storage times between 91 to 301 days. Expressible fluid values from frozen scallop muscles stored for 210 days were significantly lower than those of scallop muscles stored for 154 days ($P<0.05$), but not from those stored for 91 and 301 days ($P>0.05$). Significant differences in expressible fluid values

between scallop muscles stored frozen for 91, 154 and 301 days were not observed ($P>0.05$).

c) Total weight losses

Total weight losses of thawing weight losses and expressible fluids of fresh and stored frozen scallop muscles are presented in Figure 3.1.2.2-3.

Figure 3.1.2.2-3 Total weight losses, g kg^{-1}



Means ± S.E.M, n=3. Groups with different letters (a, b, c) are significantly different ($P<0.05$). The '0' storage time represents fresh scallop muscles.

Total weight losses were significantly higher in frozen stored than in fresh scallop muscles ($P< 0.05$). Total weight losses of frozen scallop muscles that were stored for 28 days were significantly lower than those obtained from scallop muscles stored for 91 to 301 days ($P<0.05$). Variable total weight losses were observed at storage times between 91 to 301 days, but these were not significantly different from one another.

3.1.2.3. Changes in Ca^{2+}-ATPase activities of stored (-22°C) frozen scallop muscles

Ca^{2+}-ATPase activities mean values in actomyosin extracts from fresh groups of scallop muscles ranged from 0.425 to 0.327 µmoles Pi/mg protein/min and were not significantly different ($P>0.05$). Figure 3.1.2.3 shows the Ca^{2+}-ATPase activities of fresh and stored frozen scallop muscles.

Figure 3.1.2.3 Ca^{2+}-ATPase activities, µmoles Pi/mg protein/min

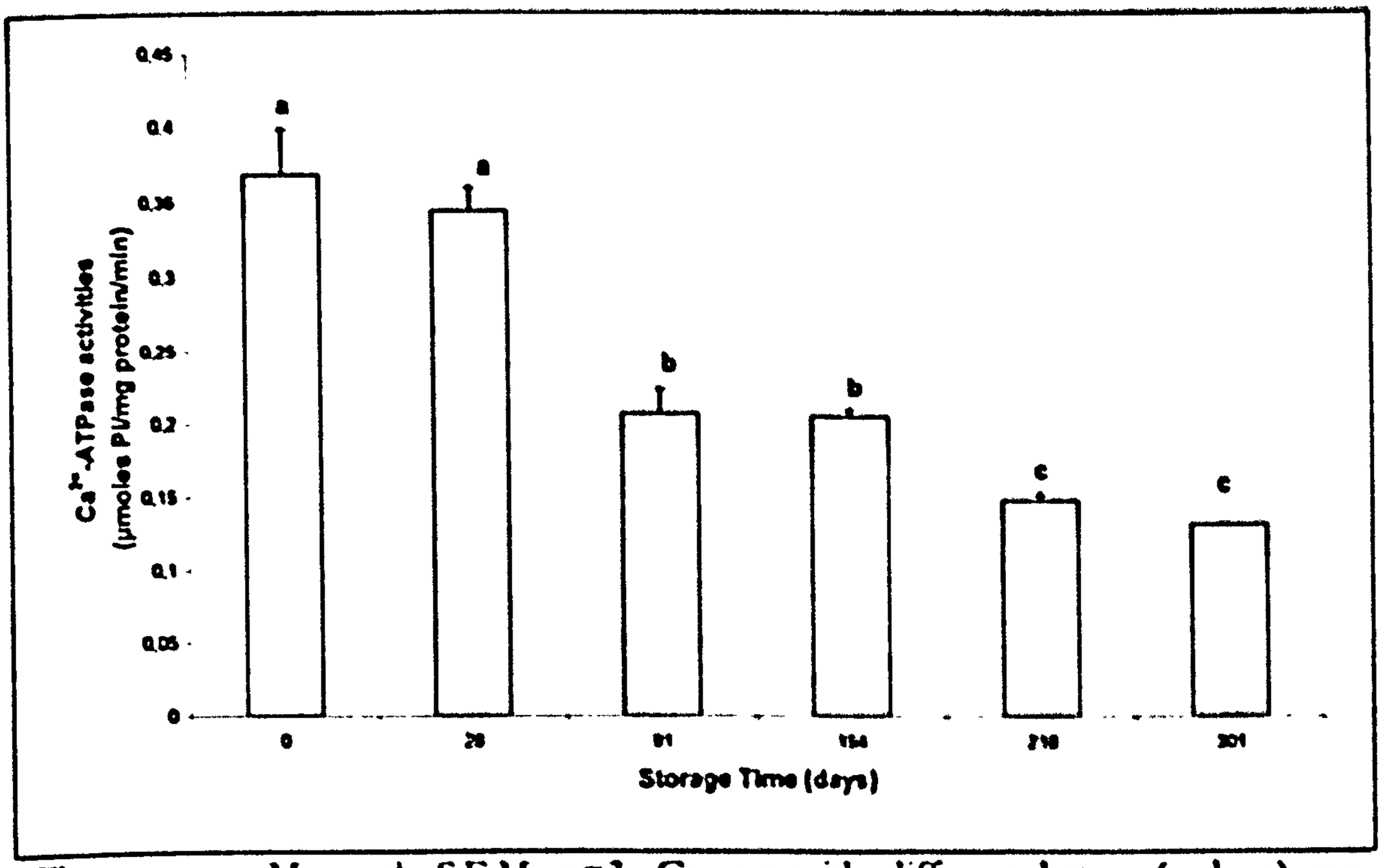


Figure notes: Means ± S.E.M, n=3. Groups with different letters (a, b, c) are significantly different ($P<0.05$). The '0' storage time represents fresh scallop muscles.

Differences between the experimental groups of scallop muscles were highly significant ($P<0.0001$). Differences in Ca^{2+}-ATPase activities between fresh and frozen scallop muscles were significant at storage periods from 91 to 301 days, but not at 28 days. Actomyosin extracts from scallop muscles stored for 91 and 154 days had mean Ca^{2+}-ATPase activities significantly lower compared to those of scallop muscles stored for 28 days. Mean Ca^{2+}-ATPase activities in actomyosin extracts from scallop muscles stored for 210 and 301 days were significantly lower than

those stored for 28 to 154 days, but not from each other.

3.1.2.4. Changes in thiobarbituric reactive substances (TBARS) of stored (-22°C) frozen scallop muscles

Figure 3.1.2.4 shows the TBARS values of stored (-22°C) frozen scallop muscles compared to fresh scallop muscles.

Figure 3.1.2.4 Thiobarbituric reactive substances (TBARS), mg MDA kg^{-1}

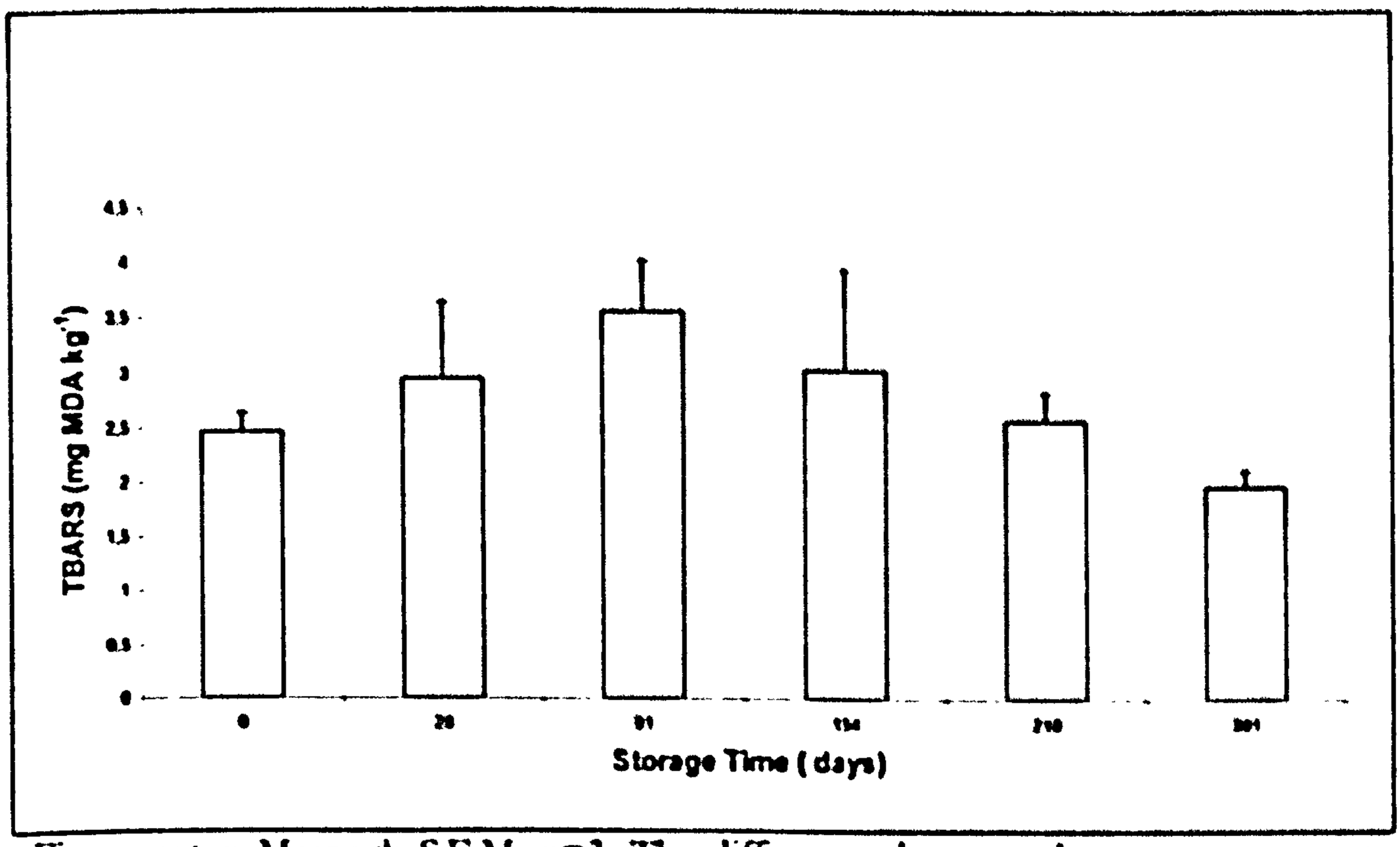


Figure notes: Means ± S.E.M, n=3. The differences between the groups were not significant (P>0.05). The '0' storage time represents fresh scallop muscles.

Mean values of TBARS from fresh groups of scallop muscles ranged from 2.96 to 2.20 mg MDA kg^{-1} of scallop muscles and were not significantly different ($P>0.05$). Also, no significant differences in TBARS values were found between the stored frozen groups of scallop muscles ($P>0.05$).

3.1.2.5. Changes in texture of stored (-22°C) frozen scallop muscles as measured by the texture analyzing system

Mean values of peak shear force of fresh groups of scallop muscles ranged from 101 to 126 $g^* g^{-1}$ and were not significantly different from each other ($P > 0.05$).

Figure 3.1.2.5 shows the peak shear values of fresh and stored (-22°C) frozen scallop muscles.

Figure 3.1.2.5 Peak shear forces, $g^* g^{-1}$

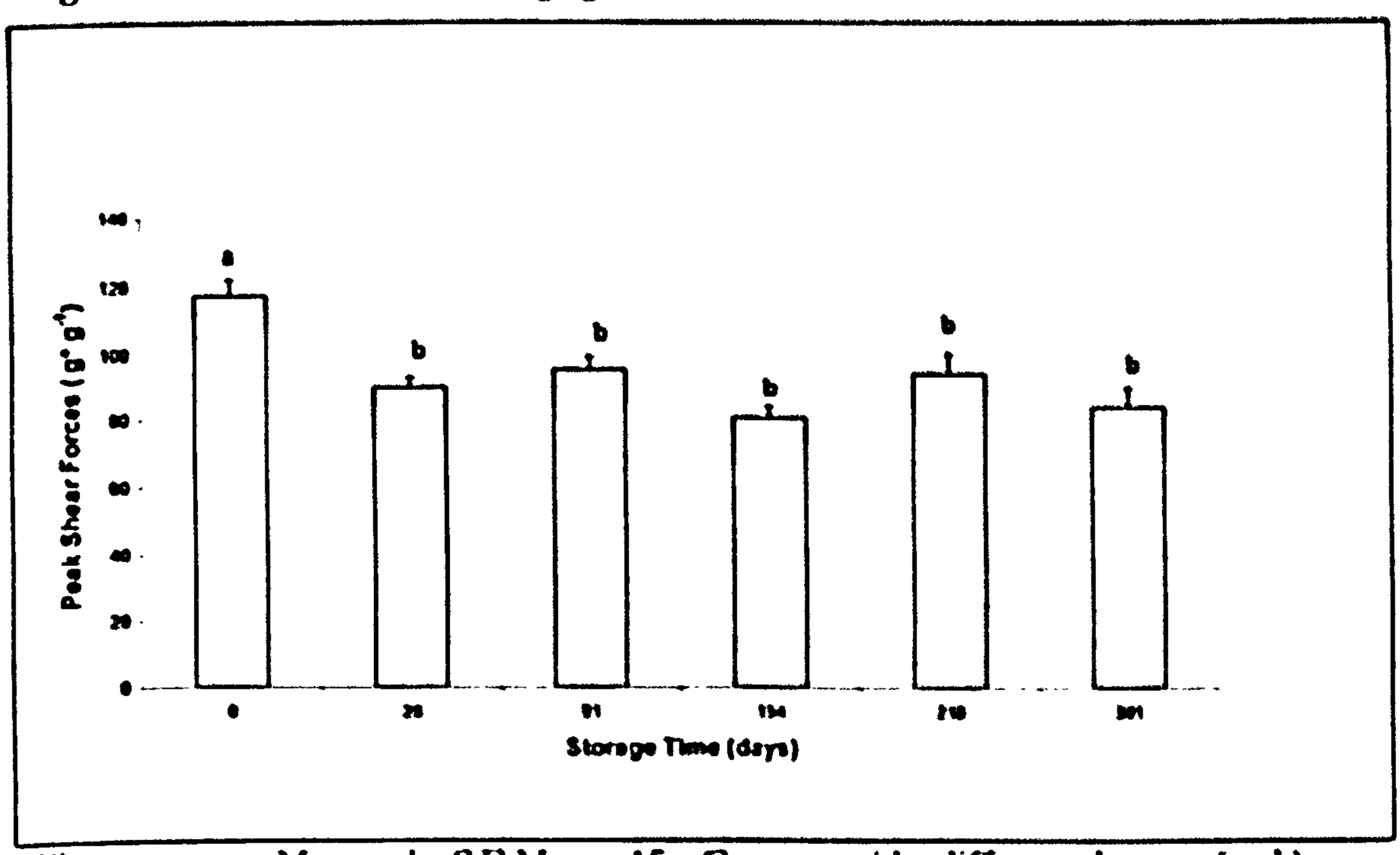


Figure notes: Means ± SEM, n=15. Groups with different letters (a, b) are significantly different ($P<0.05$). The '0' storage time represents fresh scallop muscles.

Differences in the values of peak shear forces between the fresh and frozen scallop muscles were significant at all storage period ($P<0.01$), but differences between the frozen groups themselves were not observed ($P>0.05$).

3.1.2.6. Changes in sensory attributes of frozen scallop muscles during storage (-22°C).

Figures 3.1.2.6-1, 2, and 3 show the changes in sensory scores of flavour, texture and acceptability of stored frozen scallop muscles up to 301 days at -22°C. The '0' storage time represents fresh scallop muscles.

Figure 3.1.2.6-1 Flavour sensory scores.

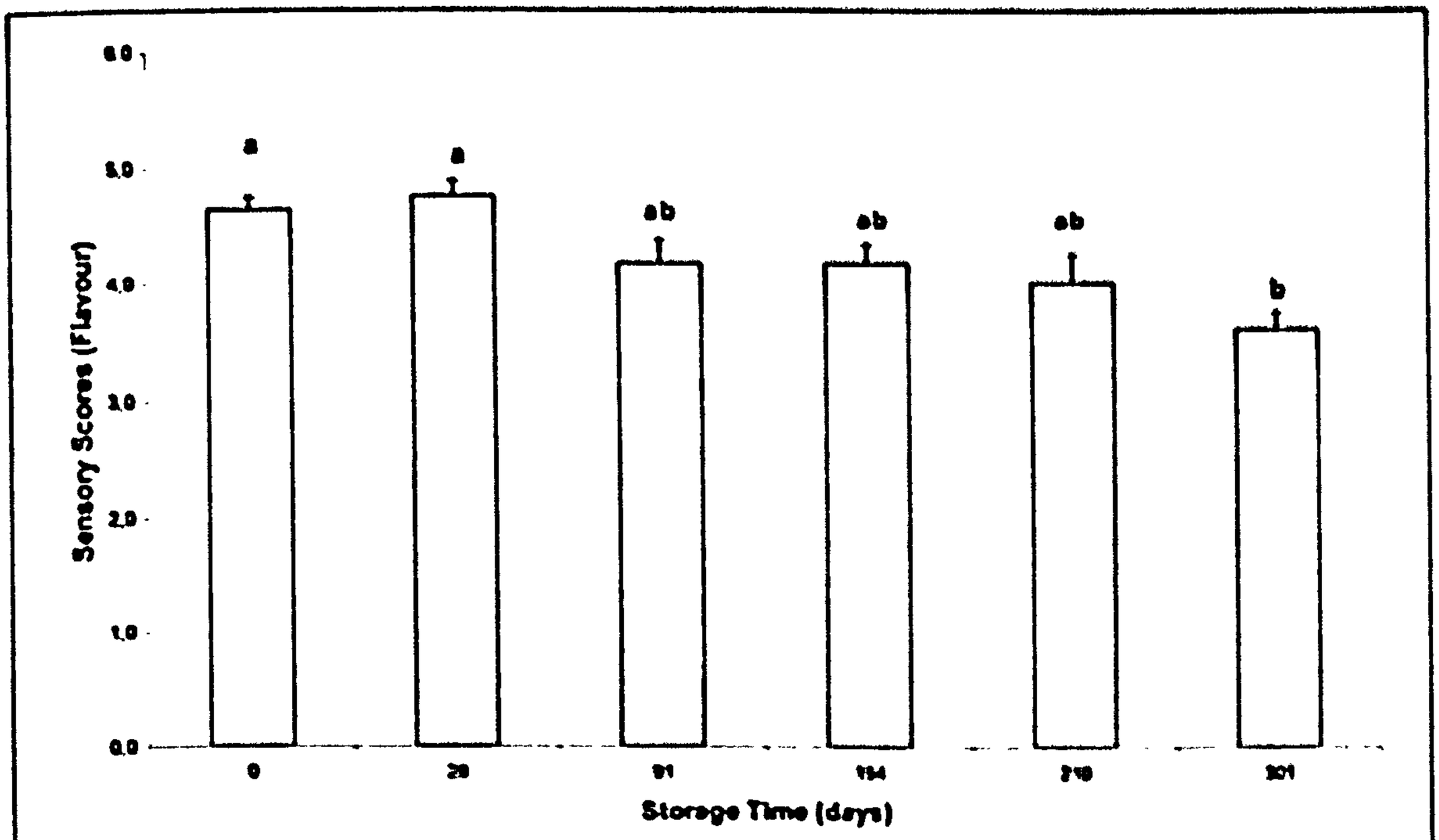


Figure notes: Means ± S.E.M, n=15. Groups with different letters (a, b) are significantly different ($P<0.05$). The '0' storage time represents fresh scallop muscles.

Figure 3.1.2.6-2 Texture sensory scores.

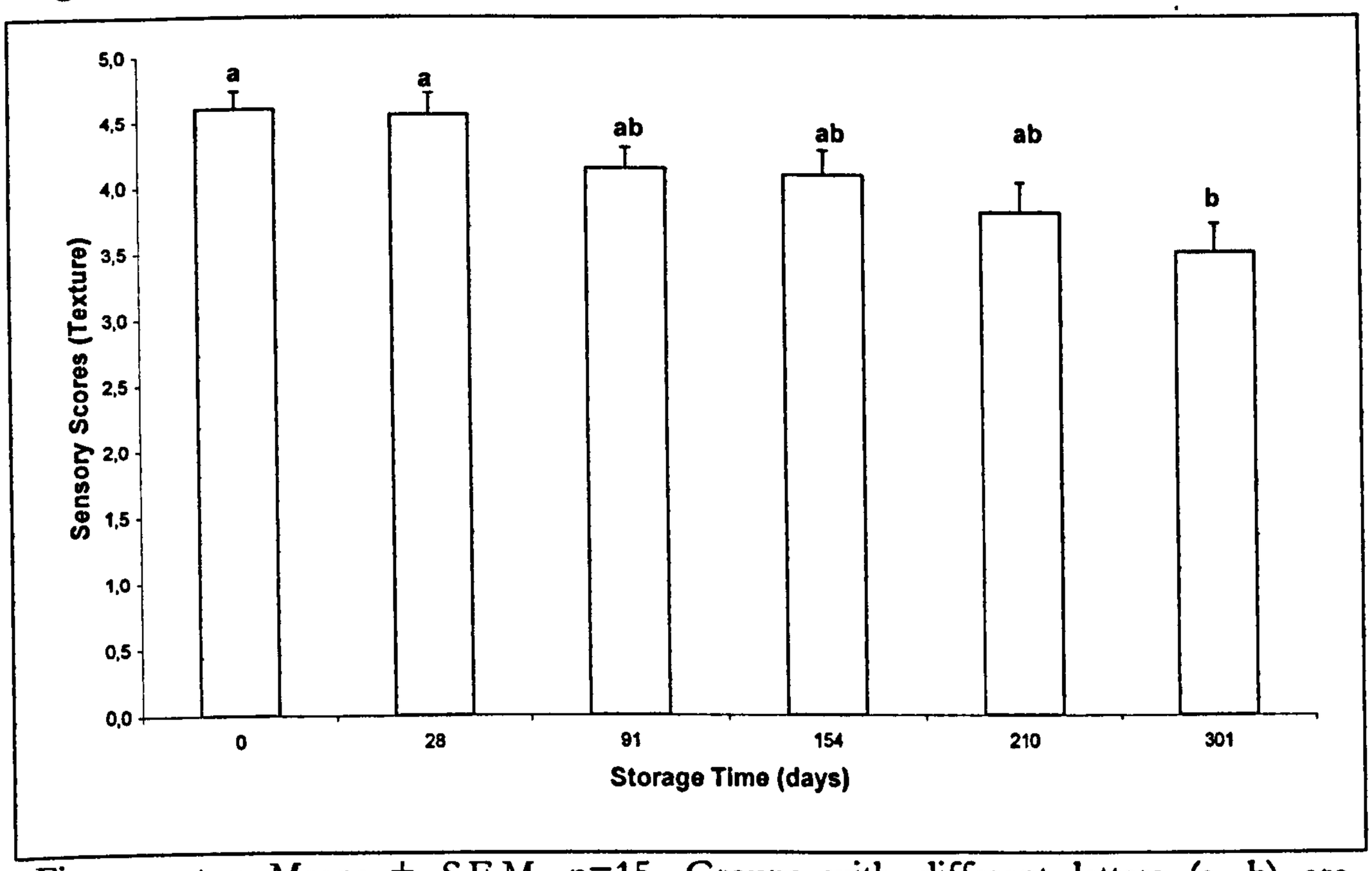


Figure notes: Means ± S.E.M, n=15. Groups with different letters (a, b) are significantly different (P<0.05). The '0' storage time represents fresh scallop muscles.

Figure 3.1.2.6-3 Acceptability sensory scores.

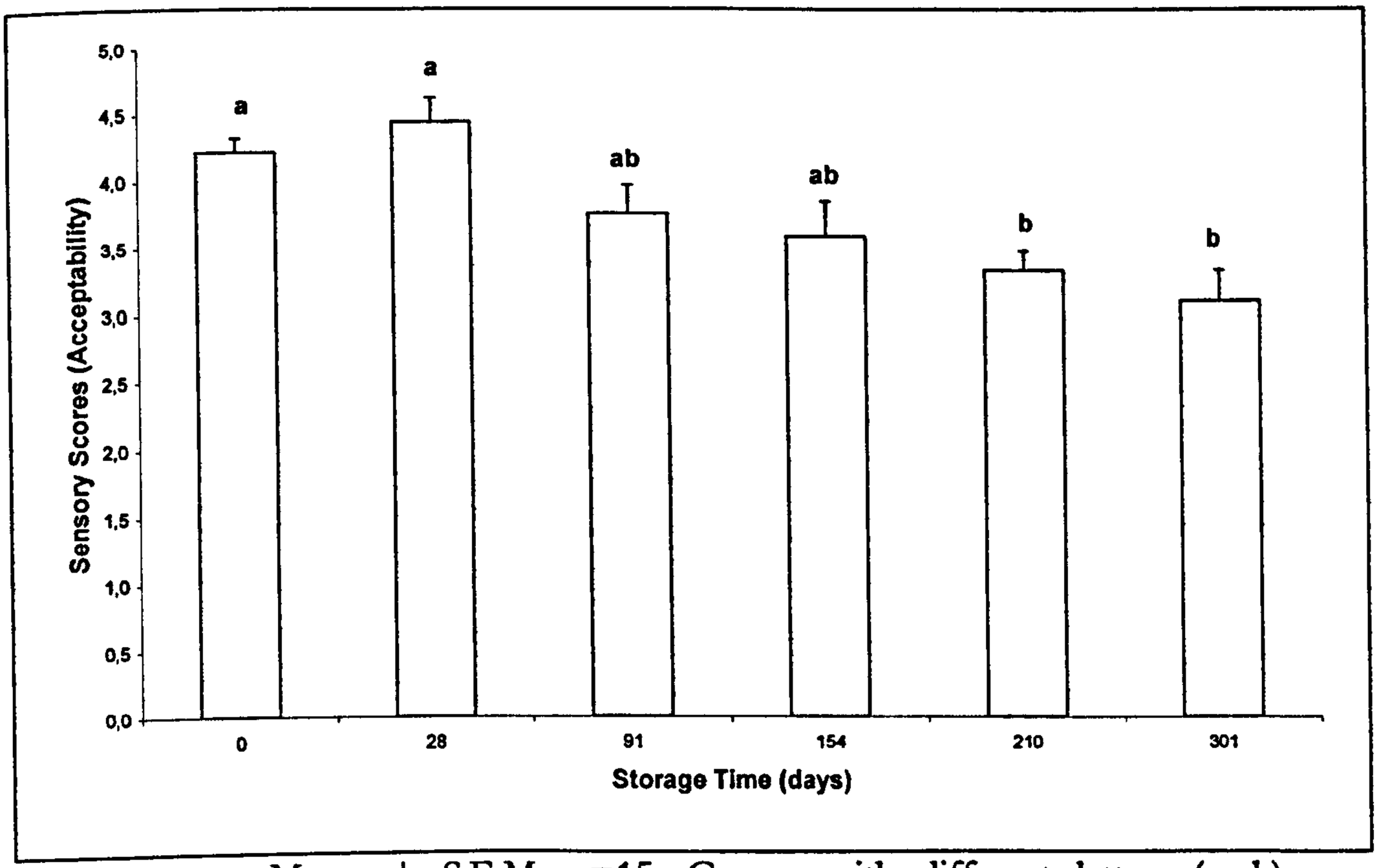


Figure notes: Means ± S.E.M, n=15. Groups with different letters (a, b) are significantly different (*P*<0.05). The '0' storage time represents fresh scallop muscles.

Flavour and texture scores of fresh and frozen scallop muscles stored for 28 days were significantly higher than those of scallop muscles stored for

301 days ($P<0.05$, Figure 3.1.2.6-1,-2), but not of those stored between 91 and 210 days. Acceptability scores of fresh and frozen scallop muscles stored for 28 days were significantly higher compared to those of scallop muscles stored for 210 and 301 days ($P<0.05$, Figure 3.1.2.6-3), but not of those stored for 91 and 154 days. Differences in flavour, texture and acceptability scores of scallop muscles stored between 91 and 301 days were not observed.

3.1.2.7. Correlations between parameters studied

Table 3.1.2.7 and Figure 3.1.2.7, below, show the coefficients of determinations (R^2) and the linear regressions between the different parameters.

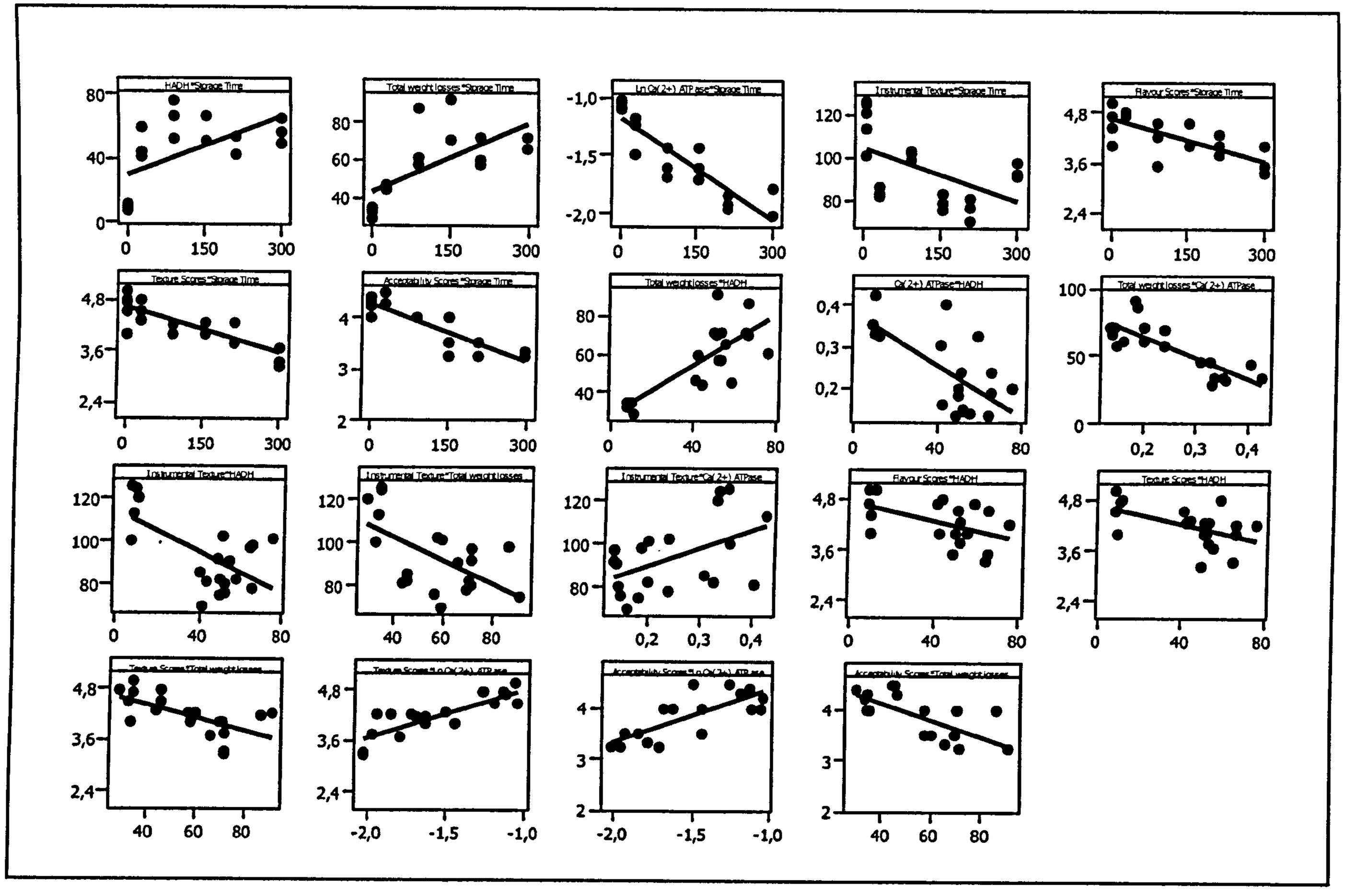
The deterioration in flavour of stored frozen seafoods is due primarily to lipid oxidation and to other biochemical reactions, such as lipid hydrolysis, proteolysis and nucleotide catabolism (Haard, 1992). Also, changes in the integrity of muscle cells could activate reactions, which involve lipids, and thereby they may cause deteriorations in flavour of stored frozen seafoods (see section 1.3.4). Thus, sensory scores of flavour were correlated with the values of TBARS and HADH activities of stored frozen scallop muscles and not with the parameters related to protein and texture deterioration (i.e. total weight losses, Ca^{2+}-ATPase activities and instrumental texture measurements)

Table 3.1.2.7 Coefficients of determination (R^2) from linear regression between different parameters

Parameter compared	R^2
HADH activities vs. Storage Time	0.363**
Total weight losses vs. Storage Time	0.570**
ln Ca^{2+}-ATPase activities vs. Storage Time	0.903**
TBARS vs. Storage Time	0.059
Instrumental Texture vs. Storage Time	0.270*
Flavour sensory scores vs. Storage Time	0.545**
Texture sensory scores vs. Storage Time	0.684**
Acceptability sensory scores vs. Storage Time	0.763**
Total weight losses vs. HADH activities	0.690**
Ca^{2+}-ATPase vs. HADH activities	0.481**
TBARS vs. HADH activities	0.014
Total weight losses vs. Ca^{2+}-ATPase	0.755**
Total weight losses vs. TBARS	0.080
Ca^{2+}-ATPase vs. TBARS	0.000
Instrumental Texture vs. HADH activities	0.400**
Instrumental Texture vs. Total weight losses	0.350**
Instrumental Texture vs. Ca^{2+}-ATPase	0.244*
Instrumental Texture vs. TBARS	0.000
Flavour sensory scores vs. HADH activities	0.242*
Flavour sensory scores vs. TBARS	0.010
Sensory texture scores vs. HADH	0.279*
Sensory texture scores vs. Total weight losses	0.389**
Sensory texture scores vs. ln Ca^{+2}ATPase	0.632**
Sensory texture scores vs. TBARS	0.060
Sensory texture scores vs. Instrumental Texture	0.126
Acceptability sensory scores vs. ln Ca^{+2}ATPase	0.588**
Acceptability sensory scores vs. HADH	0.198
Acceptability sensory scores vs. Total weight losses	0.494**
Acceptability sensory scores vs. TBARS	0.102
Acceptability sensory scores vs. Instrumental Texture	0.185

Table notes: * Significant at 5 % level. ** Significant at 1% level. Degrees of freedom =19.

Figure 3.1.2.7 Scatter plots and regression lines between various parameters



From the data in Table 3.1.2.7 and the scatter plots in Figure 3.1.2.7, significant linear correlations were observed only between:

- Ca^{2+}-ATPase and storage time,
- texture sensory scores and storage time,
- acceptability sensory scores and storage time,
- total weight losses and Ca^{2+}-ATPase activities, and
- sensory texture and Ca^{2+}-ATPase activities.

3.2. Discussion and conclusions

3.2.1. Freezing times (rates) experiments: The bio-chemical, physical and sensory properties of the adductor muscle of scallops (*Pecten maximus*) frozen at different freezing times.

3.2.1.1. The freezing processes

It is commercial practice to freeze scallop muscles individually in air blast freezing systems (Mason, 1983; Hardy and Smith, 1986). Plate freezers are normally designated for production of frozen foodstuffs into blocks, but they can, also, be used for freezing of individual particles (Aurell *et al.*, 1976). Typical freezing rates that can be met by freezing of seafood by such freezing systems range from 0.3 to 3 cm/h. Freezing rates of 0.2 to 0.1 cm/h can be met by bulk freezing of seafood in batch air blast–rooms (International Institute of Refrigeration, 1986). Although quick freezing of scallop muscles is a common commercial practice, slow freezing of scallop muscles can occur in badly designed and operated freezers. Therefore, the experimental conditions of freezing scallop muscles in the present study produced freezing times and rates (Table 3.1.1.1) that can be met in commercial practice of freezing scallop muscles.

3.2.1.2. Changes in the release of β-hydroxy-acyl-coenzyme-A dehydrogenase (HADH) activity of scallop muscles frozen at different characteristic freezing times.

In fresh tissue the enzyme HADH is retained in mitochondria. However, HADH activities were found in the filtrates of fresh scallop muscles (Figure 3.1.1.2). These results suggest that mitochondria were damaged at the surfaces where the scallop muscles were cut, and thus a certain amount of the enzyme had leaked from damaged mitochondria into the muscle (Gottesman and Hamm, 1983). Moreover, autolysis of the scallop

muscles could have caused disruption of some mitochondria, since the fresh scallop muscles were stored in ice for three days prior to analysis (Hoz *et al.*, 1992; Hoz *et al.*, 1993; Pavlov *et al.*, 1994).

The activities of HADH in frozen and thawed scallop muscles were significantly higher than those in fresh scallop muscles (Figure 3.1.1.2). HADH activities have been reported to have increased due to freezing in trout (Garcia de Fernando *et al.*, 1992; Hoz *et al.*, 1992), kuruma prawn (Hoz *et al.*, 1993), sole, salmon, prawn, Norwegian lobster (Fernández *et al.*, 1999), plaice, whiting and mackerel (Dulfos *et al.*, 2002). These findings and the results of the present study indicate that the freeze-thaw process itself affects the integrity of mitochondria of scallop muscles. The freeze damage of mitochondria may be due to dehydration of the mitochondrial inner membrane and/or mechanical disruption of mitochondrial membranes by ice crystals (Hamm and Gottesmann, 1982). The results of the present study show that the activities of HADH in frozen scallop muscles at times (t_c values) from 1000 to 235 minutes were significantly less than those in scallop muscles frozen at times from 89 to 19 minutes. It has been reported by Love (1966) that freezing times of about 100 minutes marked the changeover from intra- to inter-cellular ice formation in post-rigor cod. In addition, this process of ice formation was accompanied by a migration of water from the muscle fibers into inter-cellular spaces causing dehydration of cod muscle cells Thus, it is suggested that freezing of scallop muscles at times from 1000 to 235 minutes should result in inter-cellular ice formation and hence the freeze damage of mitochondria could be due to dehydration of mitochondrial membranes. Freezing of scallop muscles at times (t_c values) from 89 to 19 minutes should, mainly, result in intra-cellular ice formation (Love, 1955). Thus, the additional damage of mitochondrial membranes observed at times from 89 to 19 minutes compared to that of the times from 1000 to

235 minutes could be due to mechanical damage and dehydration of mitochondrial membranes caused by ice crystals formation in the vicinity of, or even inside, the mitochondria. Similar observations and results have been reported by previous workers for bovine muscle (Hamm and Gottesmann, 1982), trout (Garcia de Fernando *et al.*, 1992), squid and hake (Pavlov *et al.*, 1994). However, the characteristic freezing time of 19 minutes seems to cause less release of the HADH enzyme from mitochodria into scallop muscles than the characteristic freezing times of 35 and 89 minutes (Figure 3.1.1.2). This observation implies that freezing of scallop muscles at the characteristic freezing time of 19 minutes might have caused less damage to intra-cellular organelles than the longer times of 35 and 89 minutes. This suggestion may be attributed to the smaller and less destructive intra-cellular ice crystals, which would have formed in the scallop muscles frozen at the short characteristic freezing time of 19 minutes compared to the larger and more destructive intra-cellular ice crystals formed in scallop muscles frozen at the longer times (t_c values) of 35 and 89 minutes (Love 1955; see also section 4.2.1.2).

Overall, the results show that the freezing process itself clearly affected the integrity of scallop muscles' mitochondria. Moreover, freezing of scallop muscles at times (t_c values) from 1000 to 235 minutes caused less damage to mitochondria than the shorter times tested.

3.2.1.3. Changes in weight losses due to freezing in bare conditions or insulated in containers.

The results of the present study show that an increase in freezing times (t_c values) caused an increase in weight loss during freezing of the unpacked scallop muscles (Table 3.1.1.3). Similar results for other food products have been reported by other workers. Aurell *et al.* (1976) evaluated the effects of freezing by liquid freon freezant (freezing times from 50 seconds to 3 minutes) and air blast (freezing times from 6 to 22

minutes) on weight losses of lemon sole, shrimps and scallop muscles. According to the results of that study, weight losses of all products were less by freezing in liquid freon freezant than by air blast. Nusbaum *et al.* (1983), investigating the effects of four freezing times, i.e. 6, 30 80 and 100 minutes, on weight losses during freezing of unpacked beef patties, showed that short freezing times caused less weight losses of the patties compared to longer times. Petrovic *et al.* (1993) showed that an increase in freezing rate caused a decrease in weight loss during freezing of unpacked beef steaks. Altogether, these findings suggest that freezing times affected the weight losses during freezing of the unpacked scallop muscles, with the freezing time of 19 minutes showing the lowest losses. This can be due to the fact that freezing of scallop muscles over a time of 19 minutes decreased the surface temperature of the scallop muscles quickly to a value where the rate of moisture evaporation or sublimation was lower than the evaporation values at the other freezing times (International Institute of Refrigeration, 1986).

3.2.1.4. Changes in water holding parameters of scallop muscles frozen at different characteristic times

Thawing weight losses of scallop muscles frozen at times from 1000 to 235 minutes were higher than those of scallop muscles frozen at t_c values from 19 to 89 minutes (Figure 3.1.1.4-1) Several other studies have reported increases in thawing weight losses of meat products with increases in freezing times. Thawing weight losses of poultry meat frozen in containers at -18°C (slow freezing) were greater than those obtained by freezing in a methanol-dry ice mixture (-80°C, fast freezing; Khan and Berg, 1967). It has been reported that the drip losses after thawing of scallop muscles were higher when using an air blast freezer (freezing time 15 minutes) compared to a liquid freon freezant (freezing time 3 minutes; Aurell *et al*, 1976). Vacuum packaged beef cuts which were frozen in an

air-blast at -30°C (fast freezing, freezing times 2 -4h), showed less thawing losses than those frozen in containers in still air at -18°C (slow freezing, freezing time 16 to 24 h; Khan and Lentz, 1977). Similarly, an increase in freezing rate (from 0.22 to 5.66 cm/h) caused a decrease in weight loss during thawing of beef steaks (Petrovic *et al.*, 1993). Whiting fillets frozen at a freezing rate of 0.77°C/min showed less thawing losses than those frozen at a rate of 0.14°C/min (Chevalier *et al.*, 1999).

The differences in thawing weight losses of meat products frozen at different freezing times have been attributed mainly to changes in myofibrillar proteins and/or to distortion and destruction of muscle fibres by ice-crystals. However, as will be shown in the following section, the freezing times tested in the present study did not seem to cause an irreversible denaturation of myofibrillar proteins of 'fast' and 'slow' frozen scallop muscles (in terms of Ca^{2+}-ATPase activities of actomyosin), possibly because no storage time was included in the present study. Thus, the differences in the amount of exudates released from scallop muscles during thawing might be due to differences in the size and location of ice crystals and to differences in the mechanical damage of muscle fibres Scallop muscles frozen at the 'slow' characteristic freezing times (t_c values) of 1000, 555 and 235 minutes, would have inter-cellular ice that led to internal water diffusing out of the cells, leaving little ice in the cells. It is, therefore, suggested that when the slow frozen scallop muscles were thawed, much thawing drip exuded, presumably because a quantity of the inter-cellular water formed by melting of the inter-cellular ice in the inter-cellular spaces was free to escape from inter-cellular spaces before it could be reabsorbed by the fibres. Scallop muscles frozen at a 'fast' characteristic freezing time (t_c) of 19 minutes would have intra-cellular ice crystals. These ice crystals would be small in size since it took only 28 minutes to freeze the scallop muscles at -20°C (

effective freezing time (t_e value) of 28 minutes; Table 3.1.1.1) Thus, it is likely that when these scallop muscles were thawed the water being already *in situ* did, not to any appreciable extent escape as drip. The scallop muscles frozen at the characteristic freezing times (t_c values) of 49 and 89 minutes might have frozen fast enough to form intra-cellular ice, but these scallop muscles continued to freeze at a much slower rate such that the intra-cellular ice might have time (effective freezing times (t_e values) of 77 and 189 minutes respectively; Table 3.1.1.1) to grow to disruptive size and rupture the cells before the scallop muscles reached at -20°C. As a consequence of such cell damage, more drip loss during thawing of the scallop muscles frozen at the characteristic times of 49 and 89 minutes compared to those frozen at 19 minutes would be expected. However, this was not the case; thawing weight losses from scallop muscles frozen at a characteristic freezing times of 19 minutes were equal to 14.9 g kg^{-1} of tissue and slightly less than those of scallop muscles frozen at characteristic freezing times of 49 (i.e. 17 g kg^{-1} of scallop tissue) and 89 minutes (i.e. 17.6 g kg^{-1} of scallop tissue) (Figure 3.1.1.4-1). This might have been due to the higher dehydration losses the scallop muscles suffered during freezing at the characteristic freezing times of 49 and 89 minutes compared to those of 19 minutes (Table 3.1.1.3). Thus, a part of the water that could have been released during thawing of scallop muscles frozen at characteristic freezing times of 49 and 89 minutes might have been evaporated during freezing. These suggestions are in agreement with the statement made by Hamm (1986) that there is a direct relation between the freezing time, the movement of the water within muscle and the weight loss during thawing of a meat product. It must be mentioned, also, that the total weight losses due to freezing and thawing of scallop muscles frozen at a characteristic freezing of 19 minutes (i.e. 26 g kg^{-1} of scallop muscle) were less compared to

those of scallop muscles frozen at characteristic freezing times of 49 minutes (i.e. 35.2 g kg^{-1} of scallop muscle) and 89 minutes (i.e. 60 g kg^{-1} of scallop muscle), respectively.

In contrast to thawing weight losses, the values of the expressible fluids were similar for all frozen groups of scallop muscles and significantly higher compared to those of fresh scallop muscles (Figure 3.1.1.4-2). In the present study, expressible fluid measurements were taken by pressing the scallop muscles after thawing, i.e. after the scallop muscles had released part of the unbound or loosely bound water. These measurements can be considered as indicators of the state of the remaining quantity of loosely bound water in thawed scallop muscles, as reported by Petrovic *et al.* (1993) for frozen beef. Since water holding capacity of a meat product is indicative of the water loosely bound to protein (Trout, 1988), the total losses during thawing and pressing were taken as a measure of the water holding capacity (WHC) of the fresh and frozen scallop muscles. Total weight losses (WHC) were significantly higher in frozen than in fresh scallop muscles (Figure 3.1.1.4-3), but differences between the frozen groups themselves were not observed. Since changes in WHC are very sensitive indicators of the changes in the charges and structure of myofibrillar proteins (Mackie, 1993), the results suggest that the freezing process itself, but not the freezing times, affected the properties of myofibrillar proteins of scallop muscles. This suggestion was further investigated by analyses related to myofibrillar protein denaturation.

3.2.1.5. Changes in Ca^{2+}-ATPase activities of scallop muscles frozen at different characteristic freezing times

In order to examine the possibility of denaturation of myofibrillar proteins, Ca^{2+}-ATPase activities in actomyosin extracts from fresh and frozen groups of scallop muscles were measured. Although, the results

showed that this parameter did not change significantly when all the experimental groups of scallop muscles were compared (Figure 3.1.1.5), the mean value of Ca^{2+}-ATPase activities from all fresh groups of scallop muscles was significantly higher ($P<0.05$) compared to the mean values from all 'slow' (freezing times from 235 to 1000 minutes) and 'fast' (freezing times from 89 to 19 minutes) frozen scallop muscles (see section 3.1.1.5). Since Ca^{2+}-ATPase activity can be used as an indicator for the integrity of myosin molecules, the decrease of Ca^{2+}-ATPase activity due to freezing could indicate a change on the myosin head, which contains the active site of the enzyme. This change could be a result of partial tertiary structural change (unfolding) of myosin due to a weakening of intra-molecular hydrophobic bonds. This could be a result of local increase of ionic strength as a consequence of freezing and water migration from the myofibrillar space or dehydration of myofibrillar proteins (Benjakul and Bauer, 2000). Similar suggestions were reported by Wagner and Añòn (1985) after studying the effects of freezing and freezing times on the denaturation of myofibrillar proteins of beef muscle.

There was not any clear effect of the freezing times on myofibrillar proteins (in terms of Ca^{2+}-ATPase activity) of scallop muscles. Although more research is needed to confirm this, it may be related to the very short time that the scallop muscles remained to frozen state, i.e. 24 hours for the 'fast' frozen scallop muscles and 36 hours for the 'slow' frozen scallop muscles. During freezing of fish muscle, part of intra-cellular water freezes out but a considerable portion of it remains unfrozen. Consequently, the concentration of solutes in the unfrozen phase increases considerably. An increase in solute concentration may affect protein properties. At high ionic strength solutions, as might be the case of 'slow' frozen scallop muscles, myosin molecules undergo a rapid, but

reversible, dissociation into heavy cores and light polypeptide chains. Extended storage of myosin in those solutions could cause irreversible aggregation between myosin's subunits (Shenouda, 1980). Several previous studies have shown no changes in myofibrillar proteins of meat products frozen at different freezing times. Reid *et al.* (1986) found no differences in Ca^{2+}-ATPase activities between fast (2 hours) and slowly (24 hours) frozen Pacific rockfish immediately after freezing. Srinivasan *et al.* (1997b) showed no significant changes in myosin of freshwater prawns after blast (fast) and still (slow) freezing. Sakata *et al.* (1995) found no changes in myofibrillar proteins and water holding capacity of fast (still air at -80°C) and slow (still air at -20°C) frozen pork. Ngapo *et al.* (1999) showed that freezing times from 12 minutes to more than 900 minutes did not cause protein denaturation of small pork samples immediately after freezing, but the thawing losses from samples frozen at freezing times of 240 and 900 minutes were significantly more than those of samples frozen from 12 to 120 minutes.

Therefore, the results of this section suggest that there was not any clear effect of freezing times on Ca^{2+}-ATPase activities of myofibrillar proteins of scallop muscles, but that the freezing process itself might have caused changes in myofibrillar proteins of scallop muscles (in terms of Ca^{2+}-ATPase activities).

3.2.1.6. Changes in texture of scallop muscles frozen at different characteristic freezing times as measured by the texture analyzing system.

The peak shear forces obtained from the fresh scallop muscles were significantly higher than those of frozen scallop muscles (Figure 3.1.1.6). This means that the freezing process itself caused softening of raw scallop muscles. Freezing and thawing cause lysis of lysosomes and release into sarcoplasm of proteases, which cause breakdown of muscle

proteins and consequent tissue-softening (Pan and Yeh, 1993; Civera *et al.*, 1996). Therefore, it is likely that the freezing process itself causes release of proteolytic enzymes from lysosomes of scallop muscles with concomitant softening of their tissue. Although significant differences between the frozen experimental groups of scallop muscles were not observed, scallop muscles frozen at freezing times (t_c values) from 19 to 89 minutes showed slightly higher peak shear forces compared to those frozen from 235 to 1000 minutes. Nilsson and Ekstrand (1993) showed that short freezing times released less lysosomal enzymes in trout muscle compared to longer ones. Hence, the freezing times from 19 to 89 minutes might have caused less damage to lysosomes, and therefore less softening, than the times from 235 to 1000 minutes.

Therefore, the freezing process itself appeared to cause softening of scallop muscles.

3.2.1.7. Changes in sensory attributes

The results from the sensory assessments of the present study may suggest that scallop muscles frozen at a freezing time (t_c value) of 1000 minutes were less tender than the fresh ones. However, differences in the textural attributes between the fresh scallop muscles and the other frozen groups of scallop muscles were not observed (Table 3.1.1.7). Other reported studies on the effects of short and moderate freezing times on sensory attributes of frozen seafoods have failed to show any freezing time effect. This was the case with scallop muscles frozen at times from 3 to 45 minutes, lemon sole frozen at times from 4 to 41 minutes, shrimps frozen at times from 50 seconds to 30 minutes (Aurell *et al.*, 1976). In addition, Desrosier and Tressler (1977) reported that a freezing time of several hours to as long as 26 hours did not significantly influence the quality of the frozen fish as judged by organoleptic examination. Moreover, Love (1966) stated that the differences, which may exist in the

sensory quality of foodstuffs frozen at different rates and thawed after freezing, i.e. without cold-storage, are not great enough to be obvious to many of the investigators.

Therefore, the results of the present study suggest that freezing times (t_c values) up to 555 minutes may not influence the sensory quality of frozen scallop muscles. Differences may exist in the sensory quality of frozen groups of scallop muscles, but these were not large enough to be obvious to the panel of this study.

3.2.1.8. Conclusions

In summarizing the main findings and discussion of the effects of freezing times on quality parameters of scallop muscles, it can be said that:

- there was a clear effect of freezing times only on the integrity of intra-cellular organelles (mitochondria) and weight losses due to freezing and thawing of scallop muscles,
- the longer freezing times tested (i.e. from 235 to 1000 minutes) caused less injury to mitochondria and more thawing losses than the shorter times tested,
- the short freezing time of 19 minutes reduced the total weight losses due to freezing and thawing more than the times of 49 and 89 minutes. It appeared, also, to cause less damage to intra-cellular organelles (mitochondria) than the freezing times of 49 and 89 minutes;
- triangle sensory comparisons between fresh and frozen scallop muscles suggested that characteristic freezing times up to 555 minutes may not influence the sensory quality of frozen scallop muscles muscles.

Therefore, post rigor scallop muscles can be frozen at a variety of freezing times and rates that can be met in most common commercial freezing methods (e.g. air blast freezing systems and plate freezers; see section 1.1.3) without considerable change in their sensory quality.

However, freezing of scallop muscles at the short freezing time of 19 minutes reduced freezing and thawing weight losses compared to longer times tested. From the work reported in the literature, increased weight losses on freezing and thawing of frozen seafoods may influence adversely their quality and consequently their market. It is suggested, therefore, that short characteristic freezing times, such as that of 19 minutes (i.e. effective freezing time of 28 minutes and freezing rate of 3.17 cm/h), may be beneficial for freezing post-rigor scallop muscles compared to longer characteristic freezing times tested. Freezing rates of 3.17 cm/h can be obtained by freezing scallop muscles in continuous air-blast freezers (see section 1.1.3.1-c and 1.1.3.4)

3.2.2. Storage experiments: The bio-chemical, physical and sensory properties of frozen adductor muscle of scallops (*Pecten maximus*) stored at -22°C.

3.2.2.1. Changes in the release of β-hydroxy-acyl-coenzyme-A dehydrogenase (HADH) activity of stored (-22°C) frozen scallop muscles

From the results of the present study, an increase in HADH activity in scallop muscles was observed in the first 91 days of storage at -22°C, followed by a slight decrease up to 301 days (Figure 3.1.2.1). HADH activities in the scallop muscles of frozen scallop muscles stored frozen for 91 days were 7.3 times the HADH activities of fresh muscles. In addition, the HADH activities in the scallop muscles, frozen at a t_c value of 19 minutes and thawed after 36 hours in frozen stage, increased about 4.5 times the activity of the fresh scallop muscles (see section 3.1.1.2).

Thus, a part of the release of HADH in frozen scallop muscles, stored for 91 days at -22°C, may be related to the time the scallop muscles remained in frozen storage and not only to the freezing process. By means of lysosomal marker enzymes, Benjakul *et al.* (2003) showed that storage over 24 weeks at -18 °C caused disintegration of membrane structures of several tropical fish. The storage of a rapidly frozen muscle at temperatures above its eutectic temperature goes together with the growth of intra-cellular ice, and formation and accretion of inter-cellular ice (see section 1.1.2). Therefore, the additional release of the HADH enzyme, observed in scallop muscles stored for 91 days at -22 °C, may be due to further mechanical damage of mitochondrial membranes by the enlargement of intra-cellular ice and/or dehydration of membranes by the formation and accretion of inter-cellular ice (Hamm and Gottesmann, 1982). Moreover, the decrease in HADH activity in scallop muscles after 91 days of storage may be due to the denaturation of the released enzyme during prolonged storage. Similar results and suggestions were reported by Benjakul *et al.* (2003) after studying the effects of prolonged cold storage at -18°C on membrane integrity of croaker and lizardfish as determined by marker lysosomal enzymes.

Overall, the time in cold storage at -22 °C may affect the integrity of intra-cellular (mitochondria) organelles of scallop muscles via ice re-crystallization, it may also affect the activity of released mitochondrial enzymes.

3.2.2.2. Changes in water holding capacity parameters of stored (-22°C) frozen scallop muscles

Thawing losses, expressible fluids and total weight losses due to thawing and pressing of frozen stored scallop muscles showed a pronounced increase during the first 91 days of storage at -22°C. From that point until 301 days of storage, there were no significant changes in thawing and

total weight losses of frozen stored scallop muscles (Figures 3.1.2.2-1, 2 and 3). Several investigations have shown that the quantities of exudates are influenced from the time a meat product is kept in the frozen state. This was the case with frozen stored Patagonian hake fillets (Ciarlo *et al.*, 1985), ground pork (Brewer and Harbers, 1991), sardine mince gels (Martí de Castro *et al.*, 1996), whole and fillets of horse mackerel and Mediterrancan hake (Simeonidou *et al.*, 1997), turbot (Chevalier *et al.*, 2001), round sardines (Suarez *et al.*, 2002), whole and gutted seabream (Huidobro and Tejada, 2004) and Nile perch (Namulema *et al.*, 1999; Natseba *et al.*, 2005).

Total weight losses and Ca^{2+}-ATPase activities in actomyosin extracts from frozen stored scallop muscles were related by the following regression equation:

$$\text{Total weight losses} = 87.16 - 135.8 \times Ca^{2+}\text{-ATPase}$$

$$(R^2 = 0.757,\ T_{slope} = -7.06,\ P < 0.05)$$

The coefficient of determination (R^2) would indicate a good correlation between these two parameters. Moreover, the above-mentioned equation would indicate that a decrease in Ca^{2+}-ATPase would correspond to an increase in total weight losses, i.e. decrease in the water holding capacity of frozen stored scallop muscles. This observation, therefore, suggests that the water holding capacity of frozen stored scallop muscles was possibly affected by the denaturation of myofibrillar proteins, as measured by the Ca^{2+}-ATPase activities in actomyosin extracts. Similar suggestions were reported by Benjakul *et al.* (2003) for several tropical fish species.

3.2.2.3. Changes in Ca^{2+}-ATPase activities of stored (-22°C) frozen scallop muscles

Ca^{2+}-ATPaseactivity in actomyosin extracts from frozen stored frozen scallop muscles decreased throughout the 301 days of frozen storage at -

22°C. The marked decrease was observed within the first 91 days of frozen storage (Figure 3.1.2.3). The decrease in Ca^{2+}-ATPase activities in actomyosin extracts during extended frozen storage of scallop muscles could indicate conformational changes (unfolding) and aggregation of the head region of myosin (or 'actomyosin'), which contains the active site of the enzyme. Re-arrangements of protein via protein – protein interactions might have contributed also to the loss in ATPase activity. These changes in myosin (or 'actomyosin') might have been caused by the increased salt concentration in the unfrozen phase of scallop muscles as a consequence of ice re-crystallization. Similar suggestions were reported by Benjakul *et al.* (2005) after studding the effects of frozen storage on Ca^{2+}-ATPaseactivity in actomyosins extracted from several tropical fish at -18°C for up to six months.

Decrease in Ca^{2+}-ATPaseactivities in mackerel and amberfish muscle was observed during storage at -10 and -40 °C for up to 6 months (Jiang *et al.*, 1985). Ca^{2+}-ATPase in Alaska Pollock decreased during frozen storage at -29 °C for 9 months (Scott *et al.*, 1988). Ca^{2+}-ATPase in myctophid species decreased during frozen storage, and the degree of decrease depended on the fish species (Seo *et al.*, 1997). Ca^{2+}-ATPase activities decreased in croaker, lizardfish, threadfish bream and bigeye snapper during storage at -18 °C for 24 weeks (Benjakul *et al.*, 2003). Tejada *et al.* (2003) showed that the Ca^{2+}-ATPase activities decreased in the muscles of whole frozen stored gilthead seabream and hake after one year at -20 °C.

The results of the present study with stored frozen scallop muscles are, therefore, in agreement with these other studies.

The natural logarithm of Ca^{2+}-ATPase activities in actomyosin extracts from stored frozen scallop muscles showed a good correlation with storage time (R^2= 0.903 P<0.01; Table 3.1.2.7). This may suggest that

Ca^{2+}-ATPase activities may be a reliable index for assessing the quality loss of stored frozen scallop muscles.

3.2.2.4. Changes in thiobarbituric acid reactive substances (TBARS) of stored (-22°C) frozen scallop muscles

From the results of the present study, TBARS did not change significantly during storage of frozen scallop muscles at -22°C. However, TBARS reached a maximum up to 91 days of storage and then decreased (Figure 3.1.2.4). Changes in TBARS during cold storage have been studied in chub mackerel and smooth hound (Vareltzis *et al.*, 1988), horse mackerel and hake (Simeonidou *et al.*, 1997), blue whiting and light and dark muscles of hake (Aubourg, 1999; Aubourg *et al.*, 1999), albacore tuna (Ben-Gigirery *et al.*, 1999) and Nile perch (Namulema *et al.*, 1999). In most of these cases TBARS reached a maximum and then fluctuated or decreased. TBARS is a measure of malondialdehyde, which is an end-product of lipid oxidation. The decrease of malondialdehyde in frozen stored fish muscle was attributed to interactions of malondialdehyde with amines, nucleosides, nucleic acids, amino-containing phospholipids, proteins or other by-products of lipid oxidation (Aubourg, 1993).

3.2.2.5. Changes in texture of stored (-22°C) frozen scallop muscles as measured by the texture analyzing system

From the results of the present study, there was no sign of a tendency for peak shear values of frozen scallop muscles to change in up to 301 days of storage at -22°C (Figure 3.1.2.5). Similarly it has been reported that shear strength values from frozen stored gilthead seabream did not change in up to 9 months of storage at -20°C (Pastor *et al.*, 1999). Tissue toughening is common to many low-fat fish species stored at subzero temperatures. This is the case with minced European hake (Careche and Tejada, 1991), cod and haddock (Badii and Howell, 2002). The changes in

the texture of fish muscle during frozen storage have been associated with the denaturation and aggregation of myofibrillar proteins, among other causes (Haard, 1992; Mackie, 1993). The composition of scallop muscles is somewhat similar to white flesh of less fatty fish (Webb *et al.*, 1969).

Moreover, the results of the present study show a significant effect of storage time on denaturation of actomyosin extracted from frozen stored scallop muscles (section 3.2.2.3). Thus, scallop muscles were expected to toughen with time in frozen storage. However, factors related to tissue softening, e.g. release of proteases from lysosomes and cell disruption by ice crystals formation, may also exist in frozen and thawed seafoods and counteract the tissue-toughening factors (Srinivasan *et al.*, 1997a). Thus, it is likely that factors which caused the two opposing effects, i.e. tissue-softening and tissue-toughening, might both have been active in frozen stored scallop muscles; because of their counteraction, individual effects on textural changes in stored frozen scallop muscles up to 301 days at -22°C might have been diminished.

3.2.2.6. Changes in sensory attributes

According to the ratings of the sensory panel, the fresh scallop muscles had a sweet to neutral taste and tender to slightly tough texture (ratings between 5 and 4). The frozen scallop muscles stored for 301 days had neutral to slightly sour and rancid taste and slightly tough to tough and dry texture (ratings between 4 and 3; Figures 3.1.2.6-1, 2). The overall acceptability ratings of frozen scallop muscles stored at -22°C decreased significantly by 210 days (Figure 3.1.2.6-3), but the mean acceptability score was more than 3 (i.e. 'neither like or dislike') after 301 days of storage indicating that the scallop muscles remained acceptable at the end of the storage period. Changes in flavour, texture and acceptance of stored frozen seafoods have been recorded by other workers. These

changes are mainly dependant on species, pre-freezing treatment, and time and temperature of frozen storage (Mackie, 1993). Thus, Ciarlo *et al.* (1985) showed that frozen fillets of Patagonian hake remained acceptable after 12 months in storage at -20 and -30 °C. Similarly, Vareltzis *et al.* (1988) found no changes in flavour, texture and overall acceptability of chub mackerel and smooth hound stored at -22 °C for 8 weeks. Simeonidou *et al.* (1997) found that the taste and texture of horse mackerel and Mediterranean hake were reduced during 360 days of storage at -18 °C, but these attributes were still at an acceptable level at the end of the storage period. Namulema *et al.* (1999) showed that the texture, taste and overall acceptability of stored frozen Nile perch at -27 °C for 10 weeks were similar to fresh fish. Yilmaz and Akpinar (2003) showed, also, that the stored frozen fillets of guitarfish at -18 °C were acceptable after 6 months of storage. In addition, post-rigor scallop muscles, frozen within 6 days after shucking, were acceptable after 6 months at -30 °C (Chung and Merritt, 1991a).

The results of the present study are in agreement with these other studies and suggest that there was a loss in flavour, texture and overall acceptability of stored frozen scallop muscles during 301 days storage at -22 °C, but that these products were in acceptable condition at the end of the storage period.

3.2.2.7. Correlations between parameters studied

From the results of the present study, a poor but significant correlation of flavour scores of frozen scallop muscles and HADH activities was found (R^2=0.242, P<0.05; Table 3.1.2.7). This suggests that the HADH test may be a poor indicator for assessing the changes in flavour of stored frozen scallop muscles. This suggestion may be attributed to denaturation of the enzyme during prolonged storage of scallop muscles (see section 3.2.2.1).

The correlation of flavour scores of frozen scallop muscles and TBARS values proved insignificant (R^2=0.01, P>0.05; Table 3.1.2.7). Therefore, it is suggested that the TBARS test may be a poor indicator of changes in flavour of stored frozen scallop muscles. This suggestion may be attributed to the fact that the malondialdehyde interacted with other biological constituents in the scallop muscle, and thus its concentration did not correlate with the changes in taste of stored frozen scallop muscles. Similar results and observations were reported by Ciarlo *et al.* (1985) for stored frozen Patagonian hake.

Organoleptic texture deterioration in stored frozen seafoods is associated primarily with the denaturation and aggregation of myofibrillar proteins (Haard, 1992; Mackie, 1993). Thus, the sensory texture scores (T.S.) obtained from the stored frozen scallop muscles were correlated with the Ca^{2+}-ATPase activities in the actomyosin extracts:

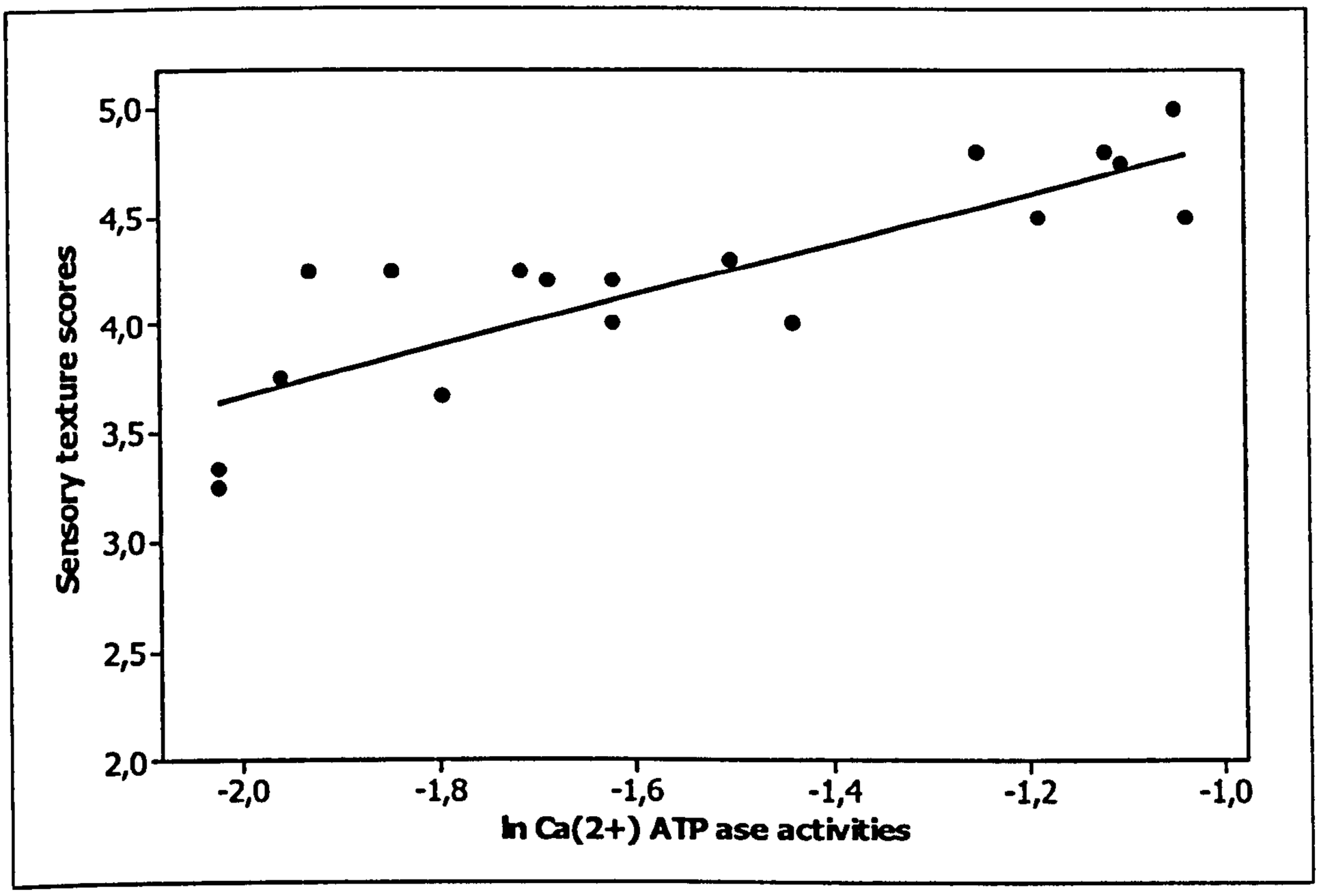
$$T.S.= 5.713 +1.004 \times \ln Ca^{2+}\text{-ATPase}$$

$$(R^2=0.632, T_{slope}=5.40, P<0.0001)$$

The coefficient of determination (R^2) of this equation implies a linear relationship between sensory texture scores and the natural logarithm of Ca^{2+}-ATPase activities in actomyosin extracts from stored frozen scallop muscles

The mentioned equation is shown in Figure 3.2.2.7, below:

Figure 3.2.2.7 Regression of sensory texture scores on Ca^{2+}-ATPase activities from stored frozen scallop muscles.



Significant correlations between parameters related to changes in myofibrillar proteins and sensory texture are recorded in the literature for stored frozen fish products. This is the case with stored frozen Patagonian hake at -20 and -30 °C, (Ciarlo *et al.*, 1985), stored frozen fillets and minces of hake at -18 °C (Koning and Mol, 1991) and stored frozen minced sardines at -18°C (Verma *et al.*, 1995). Thus, it is suggested that Ca^{2+}-ATPase activities may be used in assessing sensory texture during storage of frozen scallop muscles. This means that if a texture rating between 4.5 to 4 is arbitrarily selected as the borderline of the normal texture of stored frozen scallop muscles (i.e. a texture similar to that of fresh scallop muscles), it can be calculated from the mentioned equation that this corresponds to Ca^{2+}-ATPase activities of 0.298 ± 0.037 and 0.182 ± 0.025 μmoles Pi/mg protein/min (means ± 95 % confidence intervals), respectively. Above the value of 0.298 ± 0.037 μmoles Pi/mg protein/min, the quality of texture of frozen scallop muscles would be normal. Below the value of 182 ± 0.025 μmoles of Pi/ mg protein/min,

the texture of stored frozen scallop muscles will be inferior to that of fresh scallop muscles (i.e. the normal texture). Between these values no consistent suggestions can be made on the texture of frozen scallop muscles.

3.2.2.8. Conclusions

The results of the present study indicate that the length of time of storage at -22 °C affected the integrity of intra-cellular (mitochondria) organelles, reduced the water holding capacity, caused denaturation of myosin (or 'actomyosin') and affected the sensory attributes (flavour, texture and overall acceptability) of the frozen scallop muscles. Most of these changes in scallop muscles were more pronounced after 91 days of storage at -22°C. It can, therefore, be concluded that although the frozen scallop muscles were in acceptable condition up to 301 days (i.e. almost 10 months), holding of these products for up to three months (i.e. 91 days) at -22 °C may prevent the negative changes in muscle structure, water holding capacity, myofibrillar proteins and sensory quality which occur with longer storage.

Among the different indices checked, Ca^{2+}-ATPase activities in actomyosin extracts may be useful for assessing the quality loss of the scallop muscles stored frozen at -22°C, since a good linear correlation was obtained with time and sensory scores of texture.

CHAPTER 4. The biochemical, physical and sensory properties of the gilthead seabream (*Sparus aurata*) fillets frozen at different freezing times and during storage at -22°C. Results–Discussion-Conclusions

4.1. Results

4.1.1. Freezing times (rates) experiments: The bio-chemical, physical and sensory properties of the gilthead seabream (*Sparus aurata*) fillets frozen at different freezing times (rates)

4.1.1.1. The freezing processes

The freezing times, the corresponding freezing rates and the characteristic freezing times are presented in Table 4.1.1.1 and Figures 4.1.1.1-1 and 2.

Table 4.1.1.1 Experimental conditions for freezing fillets of gilthead seabream*

Freezing Temperature (°C)	*Freezing Environment*	*Freezing Time ** (t_f, min)*	*Freezing Rate *** (r_f, cm/h)*	*Characteristic Freezing Time **** (t_c, min)*
-80	Methanol	4*****	8.34	2
	Still Air	27	1.35	18
-20	Still Air	154	0.20	74
	Insulated in container	1175	0.03	640

Table notes:
* Mean height of the thickest part of fillets was 12 mm.
** From an initial temperature 5°C to reach central temperature -20°C (International Institute of Refrigeration, 1986)
*** Freezing rate was calculated by dividing the half-thickness of the thickest part of the fillet by freezing time (Chen and Pan, 1995)
**** From -1°C to reach central temperature -7°C (Bevilaqua *et al.*, 1979)
***** Mean values, n=2 to 8.

The freezing times (t_f) ranged from 4 to 1,175 minutes and the freezing rates from 8.34 to 0.03 cm/h. The characteristic freezing times (t_c values), i.e. the time periods the fillets were at -1 to -7°C, ranged from 2 to 640 minutes.

Figure 4.1.1.1-1 Time-Temperature profiles during freezing of seabream fillets in methanol and still air at -80°C

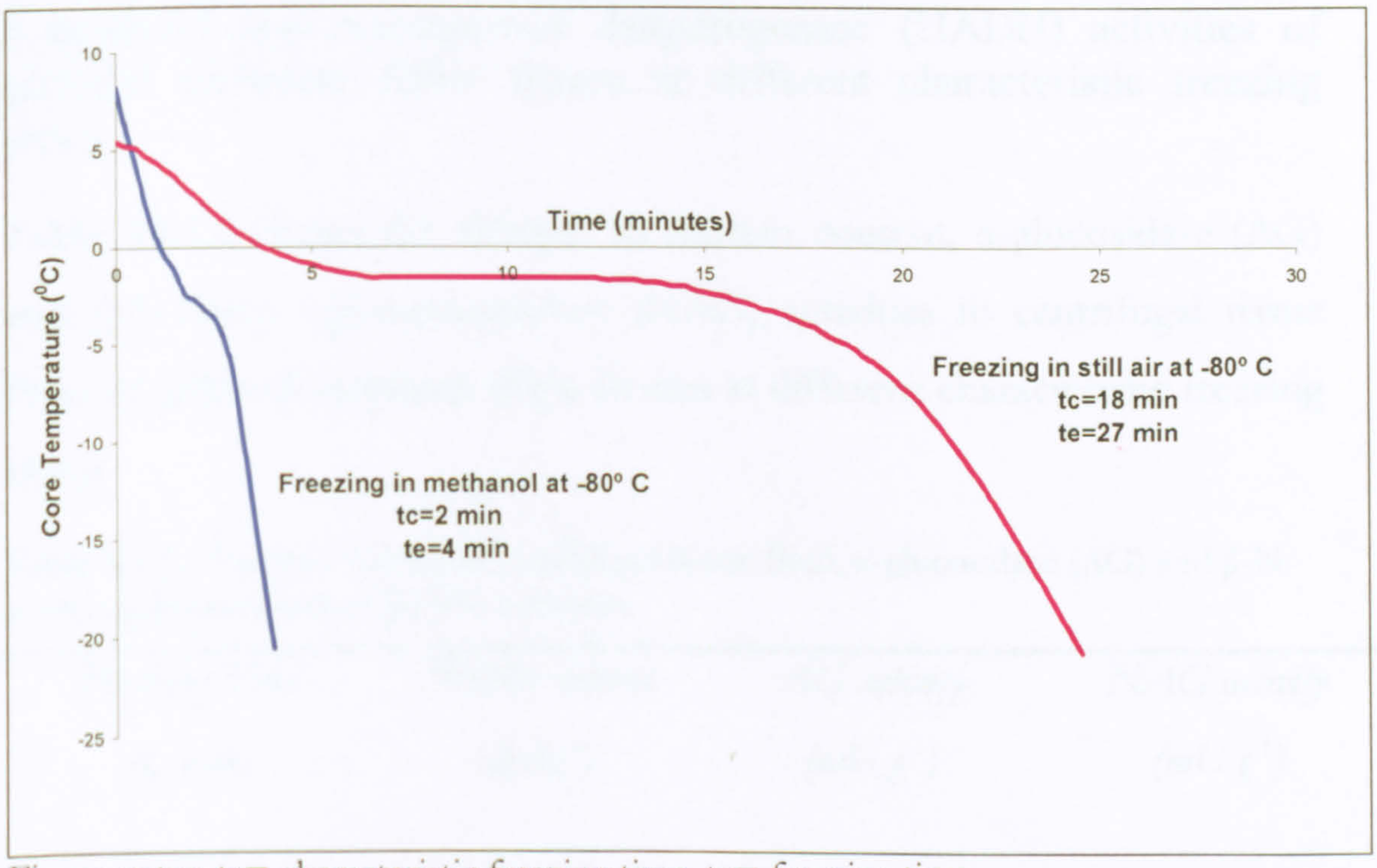


Figure notes: tc= characteristic freezing time, te= freezing time

Figure 4.1.1.1-2.Time-Temperature profiles during freezing of seabream fillets at -20°C

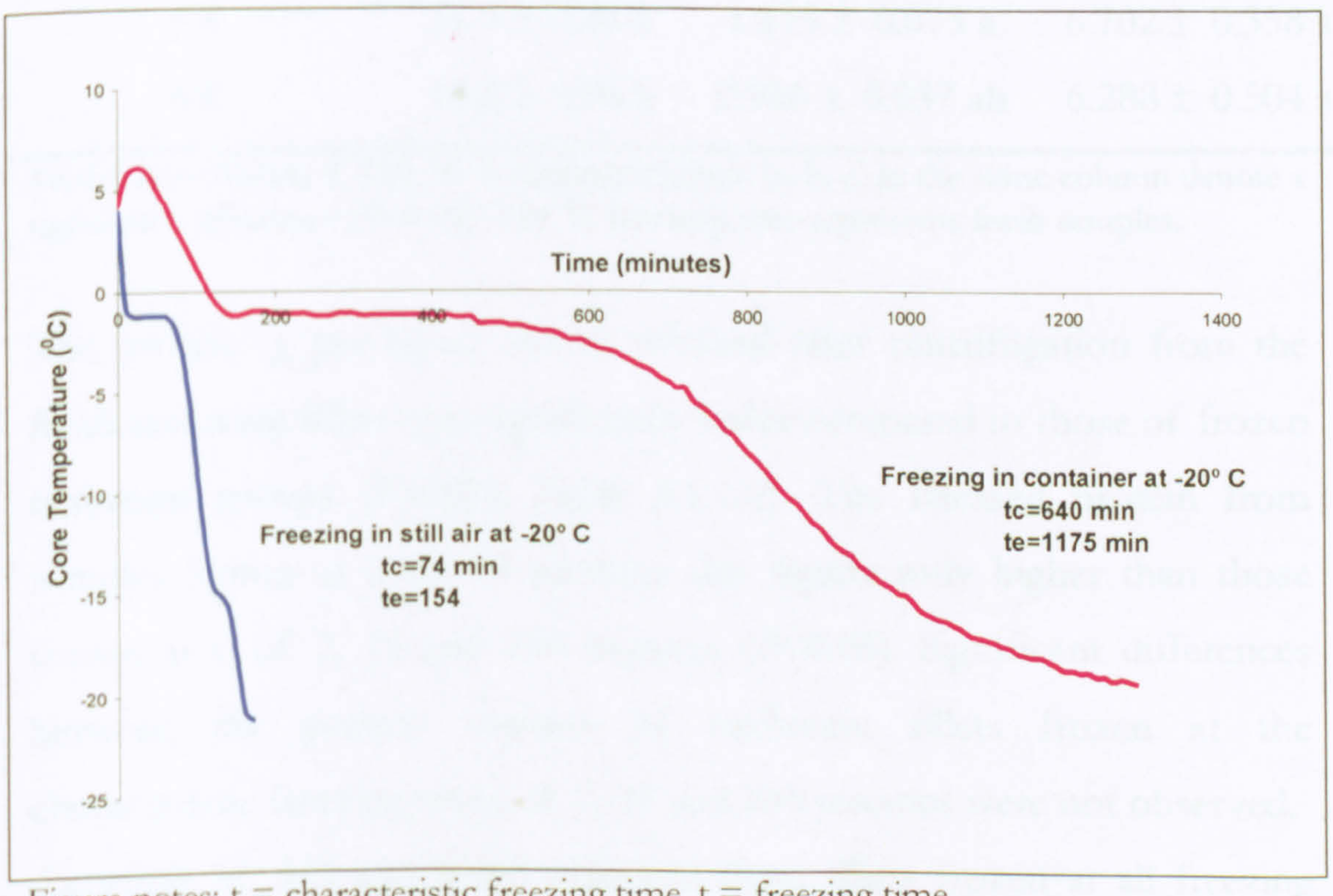


Figure notes: t_c= characteristic freezing time, t_e= freezing time

4.1.1.2. Changes in protein content of centrifugal tissue fluids (CTFs), α-glucosidase (AG), β-N-acetyl –glucosaminidase (NAG) and β–hydroxyl–acyl-coenzyme–A dehydrogenase (HADH) activities of gilthead seabream fillets frozen at different characteristic freezing times

Table 4.1.1.2 shows the changes in protein content, α-glucosidase (AG) and β-N-acetyl –glucosaminidase (NAG) activities in centrifugal tissue fluid of gilthead seabream fillets frozen at different characteristic freezing times

Table 4.1.1.2 Protein content in centrifugal tissue fluid, α-glucosidase (AG) and β-N-acetyl –glucosaminidase (NAG) activities.

Freezing Time (t_c, *min*)	*Protein content* ($g\ kg^{-1}$)	*AG activity* ($mU\ g^{-1}$)	*NAG activity* ($mU\ g^{-1}$)
0	13.8± 2.02 c	0.328 ± 0.021 c	2.128 ± 0.172 b
2	17.9 ± 1.47 b	0.906 ± 0.059 b	6.232 ± 0.390 a
18	17.1 ± 2.16 b	1.047 ± 0.050 ab	6.180 ± 0.414 a
74	21.5 ± 2.41 a	1.115 ± 0.073 a	6.702 ± 0.358 a
640	18.2 ± 0.86 b	0.916 ± 0.037 ab	6.288 ± 0.504 a

Table notes: Means ± S.D., n=4. Different letters (a, b, c) in the same column denote a significant difference ($P<0.05$). The '0' freezing time represents fresh samples.

The protein (g per kg of tissue) released after centrifugation from the fresh seabream fillets was significantly lower compared to those of frozen seabream groups ($P<0.05$; Table 4.1.1.2). The released protein from samples frozen at t_c of 74 minutes was significantly higher than those frozen at t_c of 2, 18 and 640 minutes ($P<0.05$). Significant differences between the protein content of seabream fillets frozen at the characteristic freezing times of 2, 18 and 640 minutes were not observed. Activities of AG and NAG obtained from fillets frozen at all freezing times were significantly higher compared to those of fresh fillets

(P<0.0001; Table 4.1.1.2). Activities of AG from samples frozen at t_c of 74 minutes were significantly higher than the activities of samples frozen at t_c of 2 minutes (P<0.05), but not from those frozen at t_c of 18 and 640 minutes. Differences in NAG activities between the frozen groups themselves were not observed.

Figure 4.1.1.2 shows the effects of freezing times on HADH activities of fresh and frozen seabream fillets.

Figure 4.1.1.2 β-hydroxy-acyl-coenzyme-A dehydrogenase (HADH) activities, mU g^{-1}

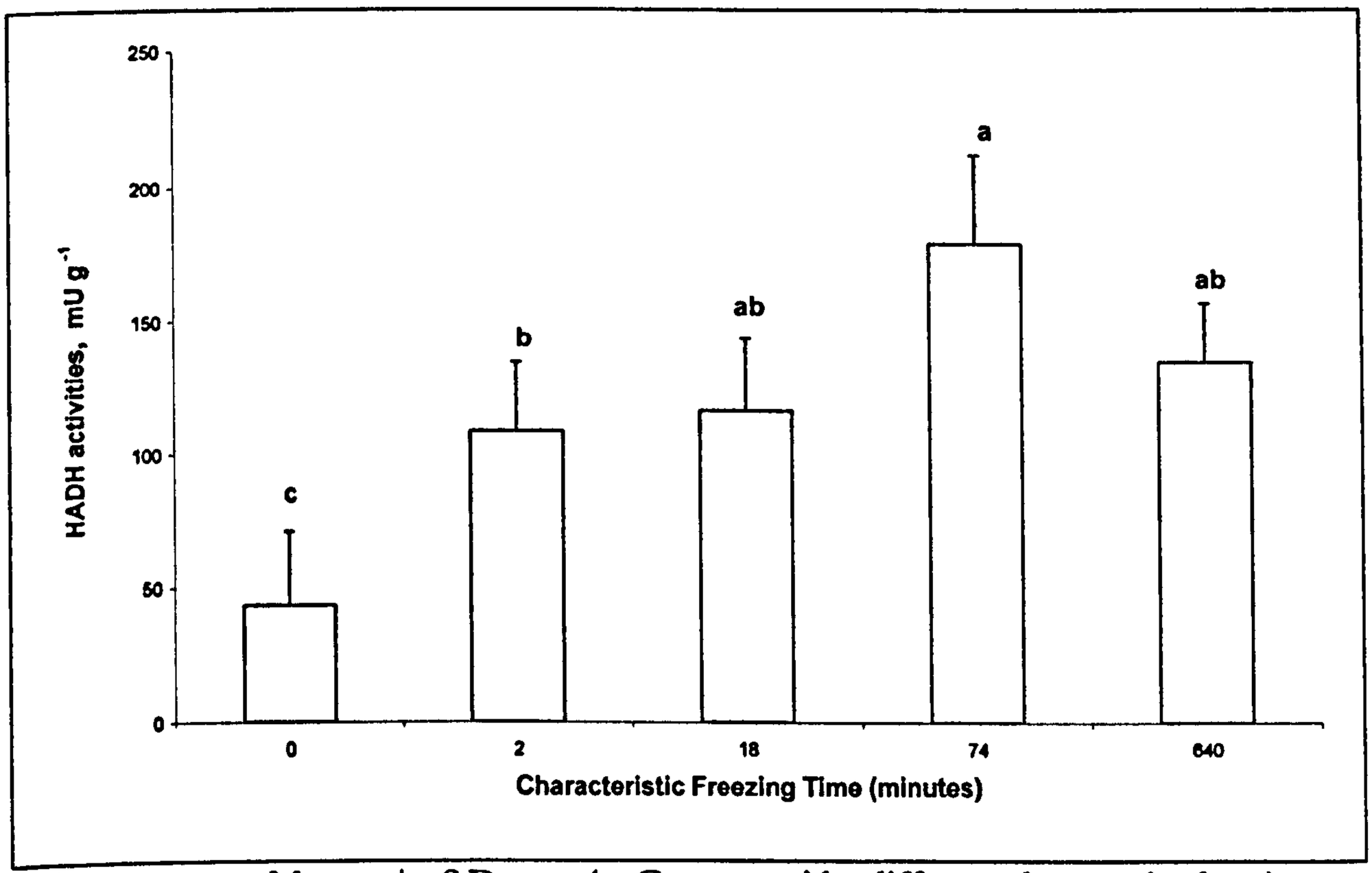


Figure notes: Means ± S.D., n=4. Groups with different letters (a, b, c) are significantly different (P<0.05). The '0' freezing time represents fresh samples.

Differences between the experimental groups were highly significant (P<0.0001). HADH activities of frozen samples at all freezing times were significantly higher from the activities of fresh samples (P<0.001). Activities of samples frozen at t_c of 74 minutes were significantly higher than the activities of samples frozen at t_c of 2 minutes (P<0.05), but not from those frozen at t_c of 18 and 640 minutes. Significant differences between the seabream fillets frozen at the characteristic freezing times of 2, 18 and 640 minutes were not observed.

4.1.1.3. Changes in the water holding parameters of gilthead seabream fillets frozen at different characteristic freezing times

Freezing times influenced significantly the thawing losses of gilthead seabream fillets ($P<0.01$; Table 4.1.1.3). Thawing losses obtained from fillets frozen at freezing time of 640 minutes were significantly higher than those frozen at t_c values of 2 and 18 minutes. Significant differences between the other experimental groups were not observed ($P> 0.05$).

Table 4.1.1.3. Thawing weight losses, centrifugal tissue fluids and total weight losses, g kg^{-1} of gilthead seabream tissue. *

Freezing Time (t_c, min)	*Thawing Weight Losses*	*Centrifugal Tissue Fluids*	*Total Weight Losses*
0	-	121.1 ± 18.55 b	121.1 ± 18.55 b
2	21.6 ± 4.35 b	157.1 ± 10.62 a	178.7 ± 9.10 a
18	21.7± 9.28 b	152.4 ± 22.68 a	174.0 ± 29.52 a
74	33.4 ± 13.54 ab	168.3 ± 9.32 a	201.7 ± 21.76a
640	59.4± 8.66 a	137.8 ± 12.21ab	197.2 ± 8.75 a

Table notes: Means ± S.D., n=4.Different letters (a, b) in the same column denote a significant difference ($P<0.05$). The '0' freezing time represents fresh samples.

Fresh seabream fillets showed significantly lower losses due to centrifugation than those frozen at freezing times of 2 , 18 and 74 minutes, but not from those frozen at tc of 640 minutes ($P=0.001$; Table 4.1.1.3). Significant differences between the frozen groups themselves were not observed.

Total weight losses were obtained by adding weight losses during thawing and centrifugation. This parameter was taken as a measure of the water holding capacity of the fillets (see section 4.2.1.3), and was significantly higher in frozen than in fresh seabream fillets ($P<0.0001$; Table 4.1.1.3). Significant differences between the frozen groups themselves were not observed ($P>0.05$).

4.1.1.4. Changes in salt soluble proteins and extractability of actomyosin from gilthead seabream fillets frozen at different characteristic freezing times

Figure 4.1.1.4-1 shows the changes in salt soluble proteins and from the gilthead seabream fillets frozen at different characteristic freezing times.

Figure 4.1.1.4-1 Salt soluble proteins, ratio of salt soluble protein/total protein.

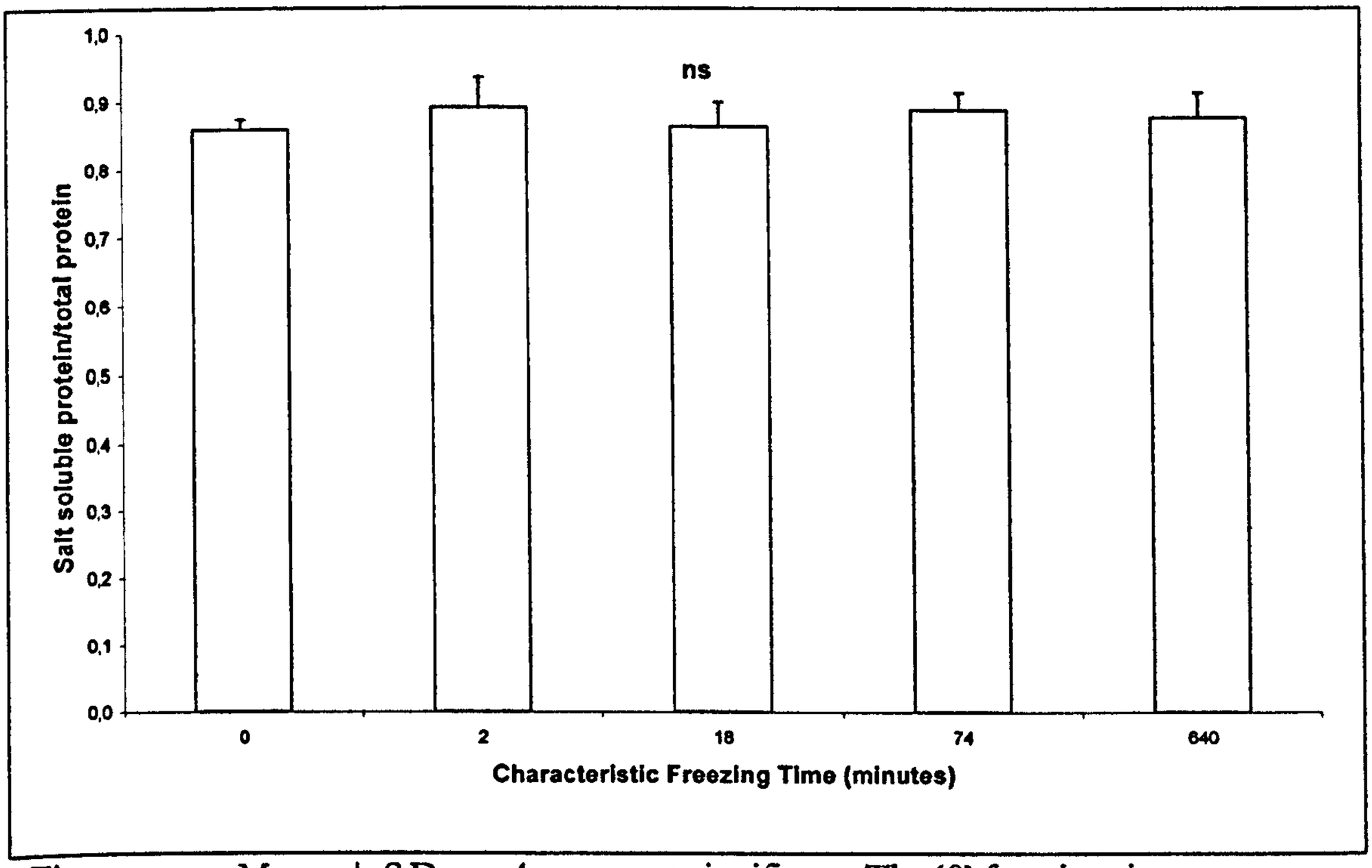


Figure notes: Means ± S.D., n=4, ns = not significant. The '0' freezing time represents fresh samples.

The results of the present study showed no significant differences between the fresh and frozen gilthead seabream fillets in regard to salt soluble protein ($P>0.05$; Figure 4.1.1.4-1)

Figure 4.1.1.4-2 shows the changes in the extractability of actomyosin from gilthead seabream fillets frozen at different characteristic freezing times.

Figure 4.1.1.4-2 Extractability of actomyosin, g g^{-1} of gilthead seabream tissue.

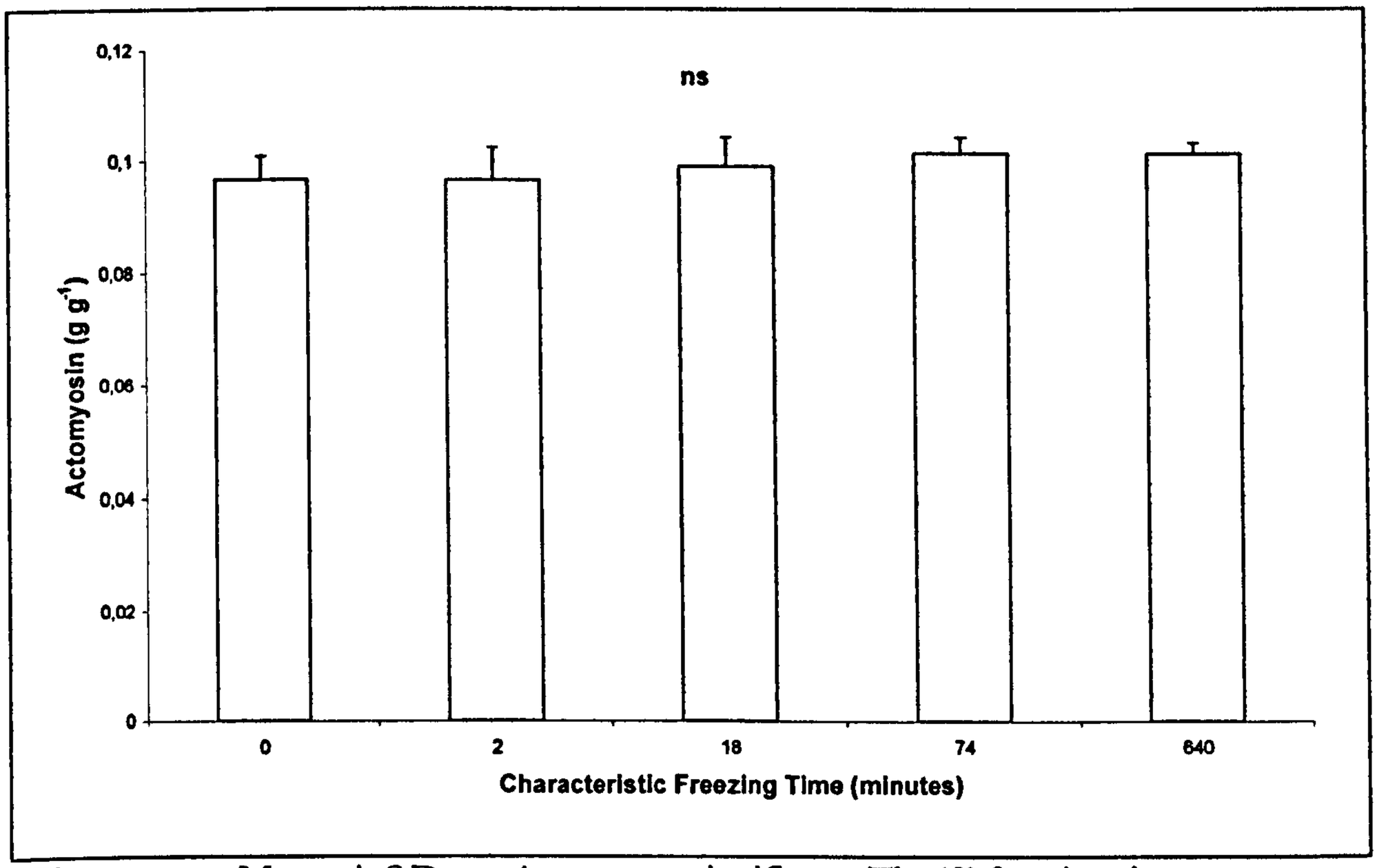


Figure notes: Means ± S.D., n=4, ns = not significant. The '0' freezing time represents fresh samples.

ANOVA showed that the differences between the experimental groups were not significant in regard to the extractability of actomyosin from fresh gilthead seabream fillets and frozen at different characteristic freezing times ($P>0.05$; Figure 4.1.1.4-2).

4.1.1.5. Changes in total and surface (reactive) sulfhydryl (SH) groups in actomyosin extracts from gilthead seabream fillets frozen at different characteristic freezing times

ANOVA analysis showed no significant effects of freezing times on total and surface (i.e. reactive) SH groups of actomyosin extracted from fresh and frozen gilthead seabream fillets ($P>0.05$; Figure 4.1.1.5).

Figure 4.1.1.5 Total and surface (reactive) sulfhydryl groups, SH moles/ 5 x 10^5g actomyosin (AM).

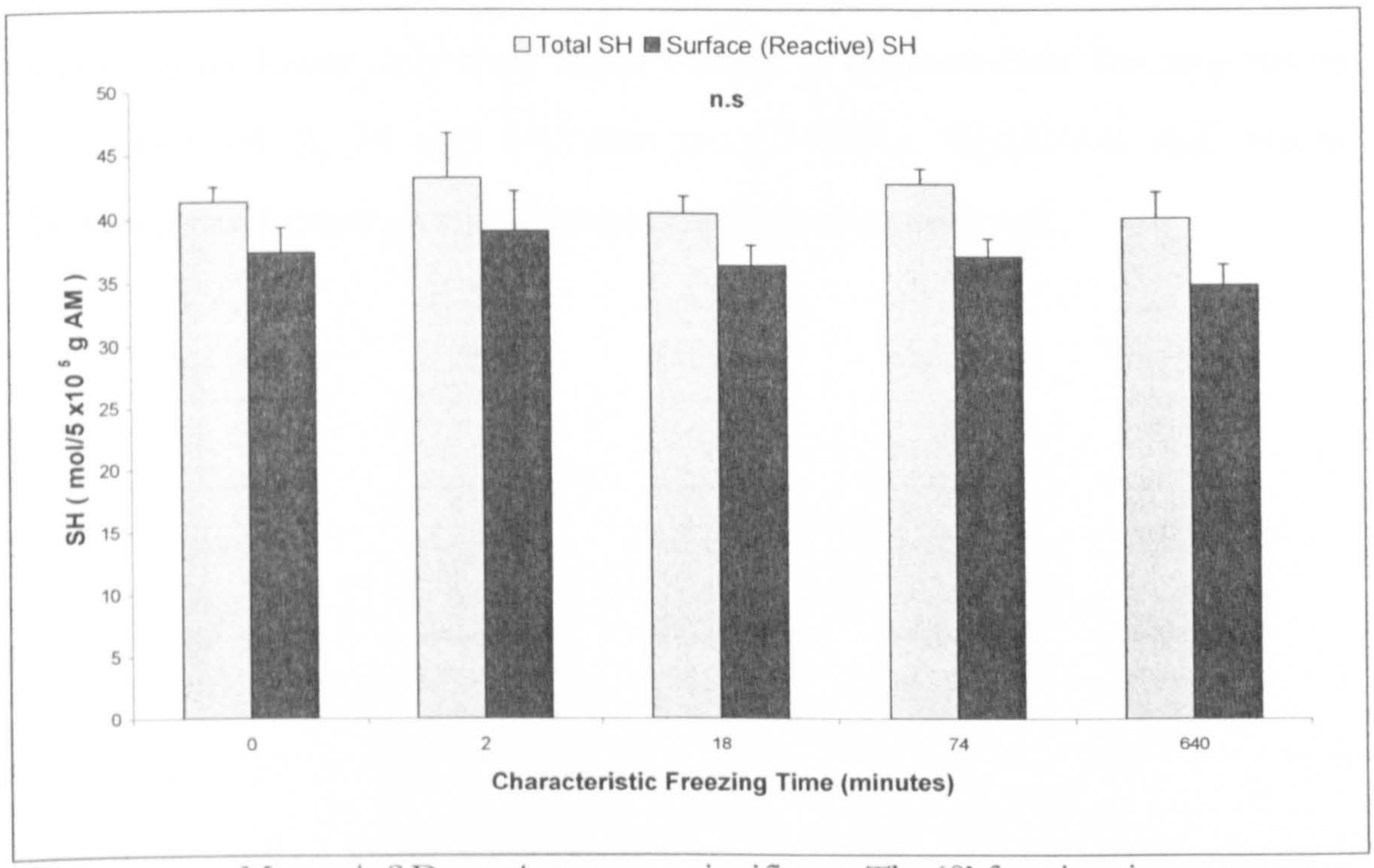


Figure notes: Means ± S.D., n=4, ns = not significant. The '0' freezing time represents fresh samples.

4.1.1.6. Changes in ATPase activities of actomyosin extracts from gilthead seabream fillets frozen at different characteristic freezing times

Table 4.1.1.6 shows the effects of freezing times on the ATPase activities of actomyosin from seabream fillets. Ca^{2+}-ATPase, Mg^{2+}-Ca^{2+}-ATPase and Mg^{2+}-ATPase activities of actomyosin from fresh fillets were significantly higher compared with those of the frozen seabream groups ($P<0.05$). Mg^{2+}-EGTA-ATPase activities from fresh samples were significantly lower only than those frozen at characteristic freezing times (t_c values) of 2, 74 and 640 minutes ($P<0.01$). Significant differences between the frozen groups themselves were not observed.

Table 4.1.1.6 ATPase activities of actomyosin, μmoles Pi/mg protein/min.

Freezing Time (t)	*Ca^{2+}-ATPase*	*Mg^{2+}-Ca^{2+}-ATPase*	*Mg^{2+}-ATPase*	*Mg^{2+}-EGTA-ATPase*
0	0.292± 0.013a	0.307± 0.036a	0.380± 0.089a	0.023± 0.005b
2	0.266± 0.007b	0.207± 0.021b	0.147± 0.013b	0.034± 0.004a
18	0.259± 0.004b	0.179± 0.052b	0.158± 0.025b	0.029± 0.000ab
74	0.265± 0.006b	0.141± 0.012b	0.146± 0.024b	0.034± 0.003a
640	0.266± 0.010b	0.154± 0.009b	0.165± 0.040b	0.033± 0.004a

Table notes: Means ± S.D., n=4.Different letters (a, b) in the same column denote a significant difference ($P<0.05$). The '0' freezing time represents fresh sample

4.1.1.7. Changes in Ca^{2+} sensitivity of actomyosin from gilthead seabream fillets frozen at different characteristic freezing times

Figure 4.1.1.7 shows the changes in Ca^{2+} sensitivity in actomyosin extracts from fresh and frozen gilthead seabream fillets at different freezing times (t_c values) gilthead seabream fillets.

Figure 4.1.1.7 Ca^{2+} sensitivity of gilthead seabream actomyosin, (%).

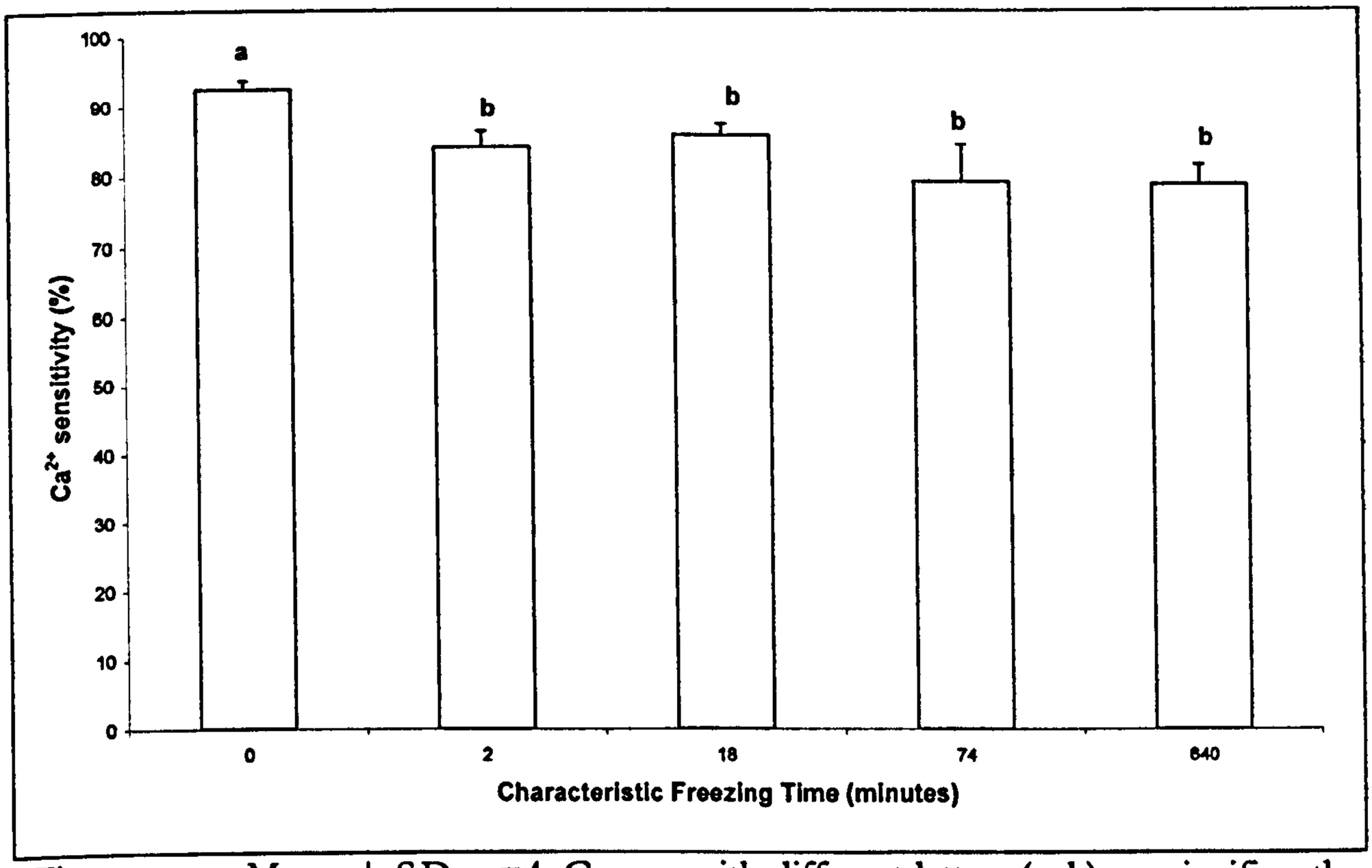


Figure notes: Means ± S.D., n=4. Groups with different letters (a, b) are significantly different ($P<0.05$). The '0' freezing time represents fresh samples.

Ca^{2+}sensitivity of actomyosin from fresh fillets was significantly higher compared to that of frozen fillets at all characteristic freezing times ($P<0.0001$; Figure 4.1.1.7). Differences between the frozen groups themselves were not observed ($P>0.05$).

4.1.1.8. Changes in total free fatty acids (FFA) in extracted lipids from gilthead seabream fillets frozen at different characteristic freezing times

Figure 4.1.1.8 shows the effects of characteristic freezing times on the concentration of total free fatty acids in lipids extracted from the fresh and frozen seabream fillets.

Figure 4.1.1.8 Total free fatty acids (FFA), g kg^{-1} of lipid.

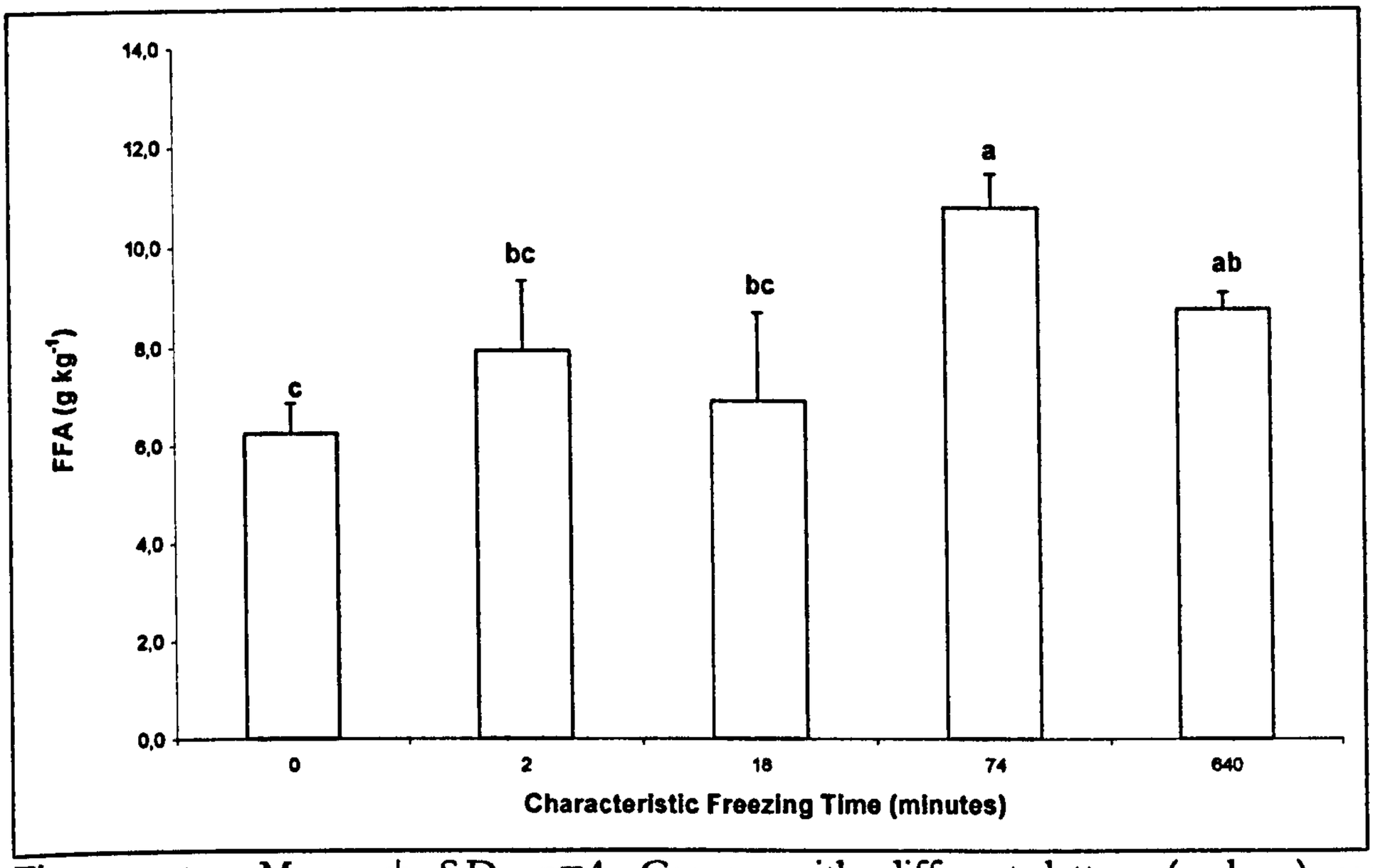


Figure notes: Means ± S.D., n=4. Groups with different letters (a, b, c) are significantly different ($P<0.05$). The '0' freezing time represents fresh samples.

Differences between the experimental groups were highly significant ($P<0.0001$). The concentrations of FFA from fresh seabream fillets were significantly lower than those frozen at t_c of 74 and 640 minutes, but not than those frozen at t_c of 2 and 18 minutes. Free fatty acids values in lipids from samples frozen at t_c of 74 minutes were significantly higher only than those frozen at t_c of 2 and 18 minutes.

4.1.1.9. Changes in the texture of gilthead seabream fillets as measured by the texture analyzing system.

Table 4.1.1.9 shows the changes in firmness and toughness values of gilthead seabream fillets frozen at different characteristic freezing times as measured by the texture analyzing system:

Table 4.1.1.9 Instrumental firmness and toughness values.

Freezing time (t_c, *min*)	*Firmness* (*N*)	*Toughness* (*N* × *sec*)
0	4.87 ± 0.296 b	35.10 ± 2.779 b
2	4.77 ± 0.537 b	35.86 ± 2.457 b
18	4.48 ± 0.399 bc	33.83 ± 3.113 bc
74	3.74 ± 0.203 c	29.14 ± 1.977 c
640	6.28 ± 0.804 a	43.14 ± 4.19 a

Table notes: Means ± S.D., n=6. Different letters (a, b, c) in the same column denote a significant difference ($P<0.05$). The '0' freezing time represents fresh samples.

The differences between the experimental groups of fillets were significant for both parameters ($P<0.0001$). Firmness and toughness values of samples frozen at a characteristic freezing time of 640 minutes were significantly higher than those of fresh and frozen fillets at characteristic freezing times of 2, 18 and 74 minutes. Firmness and toughness values of fillets frozen at a characteristic freezing time of 74 minutes were significantly less than those of fillets fresh and those frozen at characteristic freezing times of 2 and 640 minutes, but not than those frozen at a characteristic freezing time of 18 minutes. Differences in firmness and toughness values of fresh fillets and those frozen at characteristic freezing times of 2 and 18 minutes were not observed.

4.1.1.10. Changes in chewiness of gilthead seabream fillets frozen at different characteristic freezing times

Assessors' mean chewiness score of fresh seabream fillets was 5.3 and was significantly lower than those of frozen groups ($P<0.0001$; Figure 4.1.1.10). Differences between the frozen groups themselves were not observed. Moreover, general linear modeling did not reveal any significant effect of assessor and/or assessor -sample interaction ($P>0.05$). The background of these analyses is described in details in Appendices 1 and 2.

Figure 4.1.1.10 Assessors' chewiness scores of assessors.

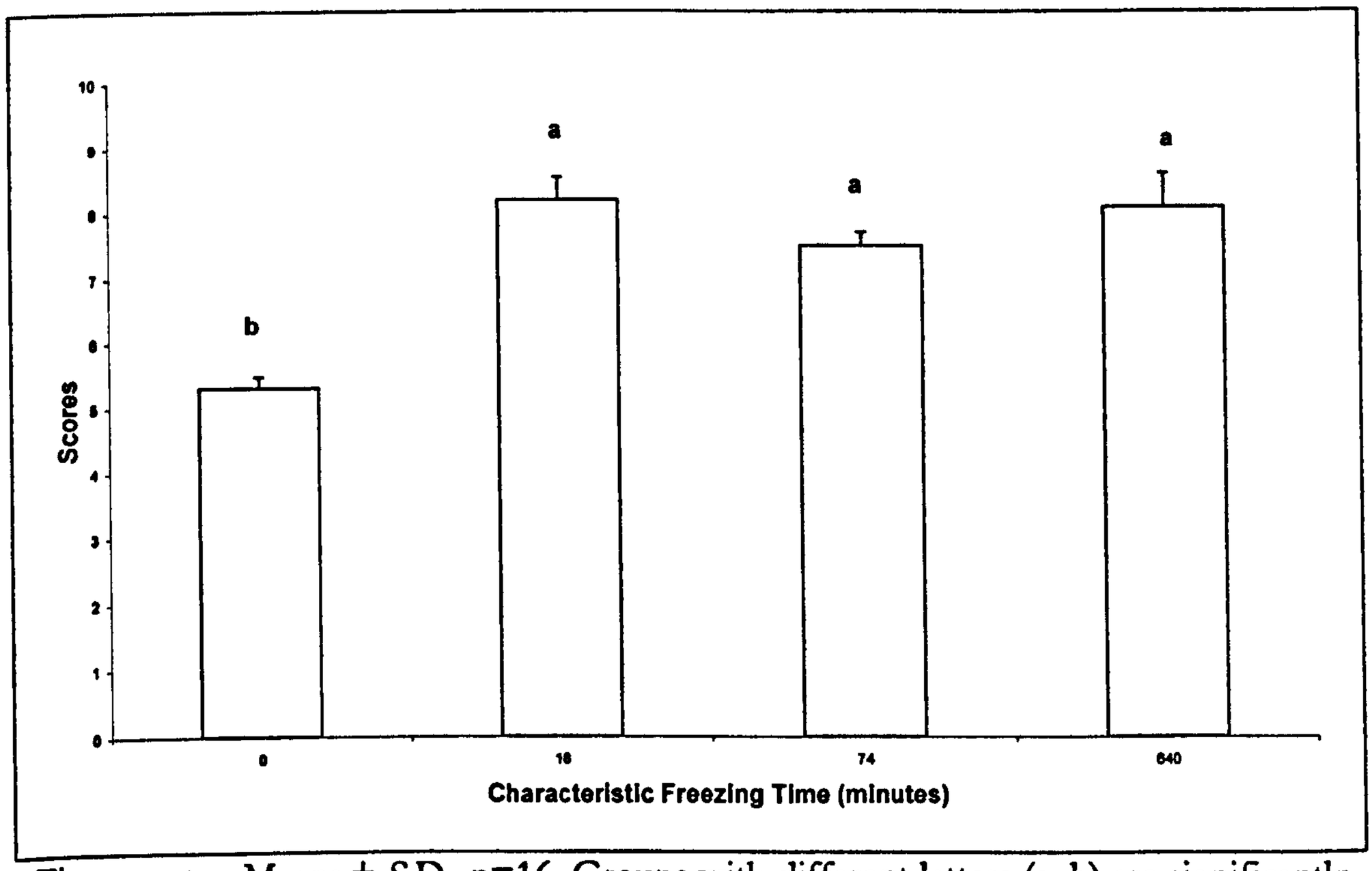


Figure notes: Means ± S.D., n=16. Groups with different letters (a, b) are significantly different ($P<0.05$). The '0' freezing time represents fresh samples.

4.1.2. Storage experiments: The bio-chemical, physical and sensory properties of the stored (-22°C) frozen fillets of gilthead seabream (*Sparus aurata*)

4.1.2.1. Proximate composition of fresh gilthead seabream fillets

Table 4.1.2.1 presents the proximate composition of fresh gilthead seabream fillets. Analyses were performed on the white muscle of fresh fillets.

Table 4.1.2.1 Proximate composition of fresh gilthead seabream fillets, g kg^{-1} of gilthead seabream tissue.

Water	*Ash*	*Crude Protein*	*Crude Lipid*
730.8 ± 5.79	15.4± 0.53	228.0± 3.76	27.2 ± 2.91

Table notes: Means ± S.D., n=5.

4.1.2.2. Changes in protein content of centrifugal tissue fluids (CTFs), α-glucosidase (AG), β-N-acetyl –glucosaminidase (NAG) and β–hydroxy–acyl-coenzyme–A dehydrogenase (HADH) activities of stored (-22°C) frozen gilthead seabream fillets

The protein released in the centrifugal tissue fluids (CTF) from the fresh gilthead seabream fillets was significantly less than that of stored frozen seabream fillets ($P<0.0001$; Table 4.1.2.2). The mean value of protein released in CTF from fillets stored for 266 days was significantly higher compared to the other stored frozen fillets. Variable values of protein content in the centrifugal tissue fluids were observed at storage times between 34 to 183 days and 340 days.

Table 4.1.2.2 Protein content in centrifugal tissue fluid, α-glucosidase (AG) and β-N-acetyl –glucosaminidase (NAG) activities.

Storage Time (days)	*Protein content ($g\ kg^{-1}$)*	*AG activity ($mU\ g^{-1}$)*	*NAG activity ($mU\ g^{-1}$)*
0	14.7 ± 0.67 d	0.421 ± 0.049 d	0.740 ± 0.092 b
34	23.3 ± 0.69 bc	1.292 ± 0.151 c	5.301 ± 0.151 a
91	21.5 ± 0.65 c	1.547 ± 0.198 bc	4.492 ± 0.416 a
183	24.6 ± 0.46 bc	2.228 ± 0.157 a	5.431 ± 0.581 a
266	28.1 ± 0.91 a	2.048 ± 0.190 ab	5.634 ± 0.503 a
340	25.0 ± 0.56 b	1.804 ± 0.112 abc	5.839 ± 0.582 a

Table notes: 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.

Significant differences were found in AG and NAG activities between the fresh and frozen experimental groups ($P<0.0001$; Table 4.1.2.2). Activities of AG from fillets stored for 183 days were significantly higher than the activities of fillets stored for 34 and 91 days ($P<0.05$), but not than those of fillets stored for and 266 and 340 days. Variable values in AG activities were observed at storage times between 34, 91 and 340 days. Significant differences in NAG activities between the frozen groups themselves were not observed ($P>0.05$).

Figure 4.1.2.2 shows the changes in HADH activities in filtrates from fresh and stored frozen gilthead seabream fillets.

Figure 4.1.2.2 β-hydroxy-acyl-coenzyme-A dehydrogenase (HADH) activities, mU g^{-1}.

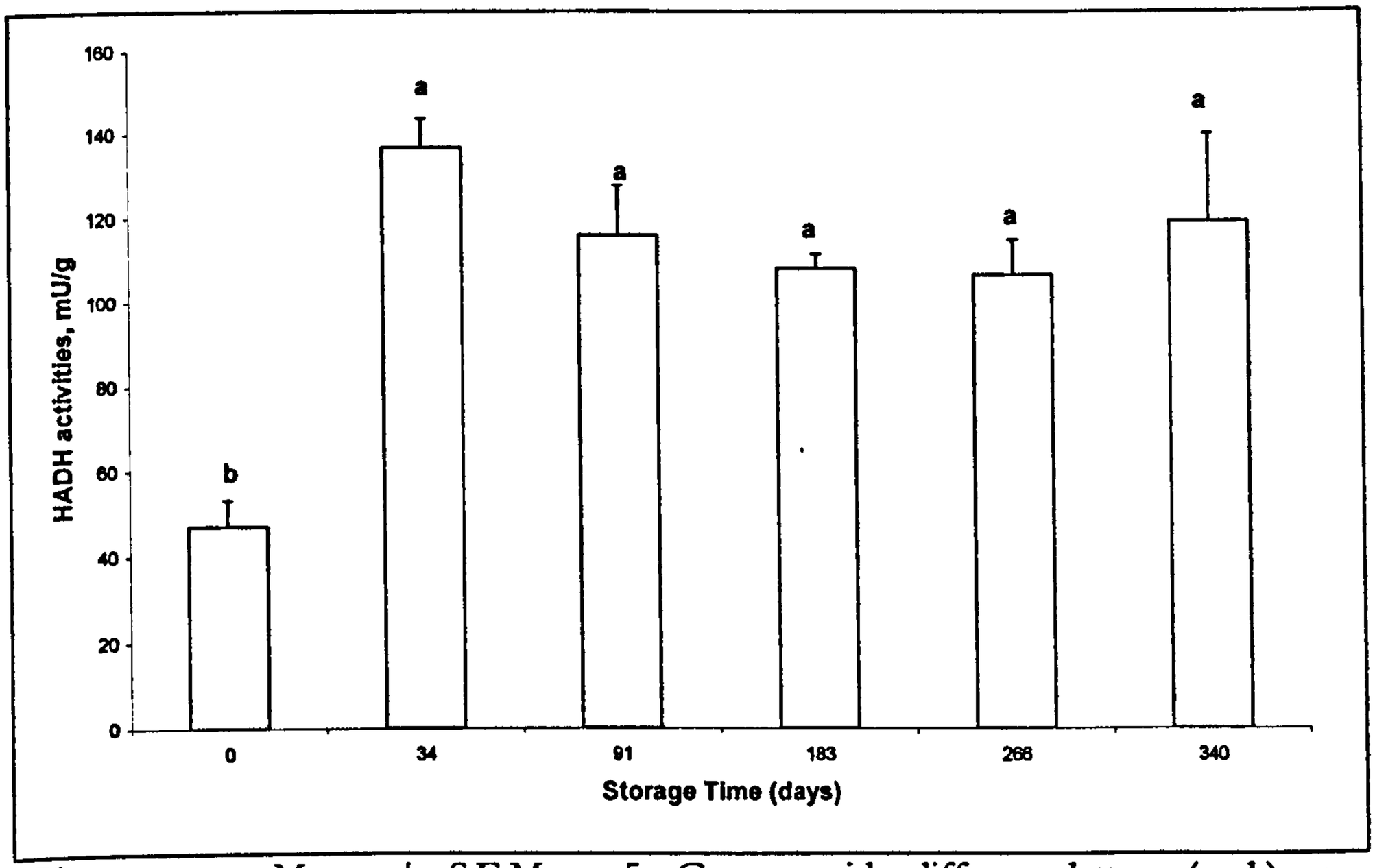


Figure notes: Means ± S.E.M, n=5. Groups with different letters (a, b) are significantly different ($P<0.05$). The '0' storage time represents fresh gilthead seabream fillets.

HADH activities in filtrates from stored frozen samples were significantly higher than those of fresh samples ($P<0.0001$), but differences between the frozen samples themselves were not observed ($P>0.05$).

4.1.2.3. Changes in the water holding capacity parameters of stored (-22°C) frozen gilthead seabream fillets

Figure 4.1.2.3 shows the changes in thawing weight losses, centrifugal tissue fluids and total weight losses due to thawing and centrifugation during storage at -22°C of frozen gilthead seabream fillets.

Table 4.1.2.3 Thawing weight losses, centrifugal tissue fluids and total weight losses, g kg^{-1} of gilthead seabream tissue.

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

Table notes: 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.

ANOVA showed no significant differences between the frozen experimental groups for thawing losses ($P>0.05$; Table 4.1.2.3). Centrifugal tissue fluid measurements and total weight losses due to thawing and centrifugation of fresh fillets were significantly less than those obtained from the frozen experimental groups ($P<0.0001$). The centrifugal tissue fluid values and total weight losses of frozen fillets that were stored from 34 to 183 days were significantly lower than those obtained from samples stored for 266 and 340 days ($P<0.05$; Table 4.1.2.3), but differences were not observed between the samples stored from 43 to 183 days.

4.1.2.4. Changes in salt soluble protein and extractability of actomyosin from stored (-22°C) frozen gilthead seabream fillets.

Figure 4.1.2.4-1 shows the changes in salt soluble proteins of stored frozen gilthead seabrem fillets.

Figure 4.1.2.4-1. Salt soluble proteins, ratio of salt soluble to total protein.

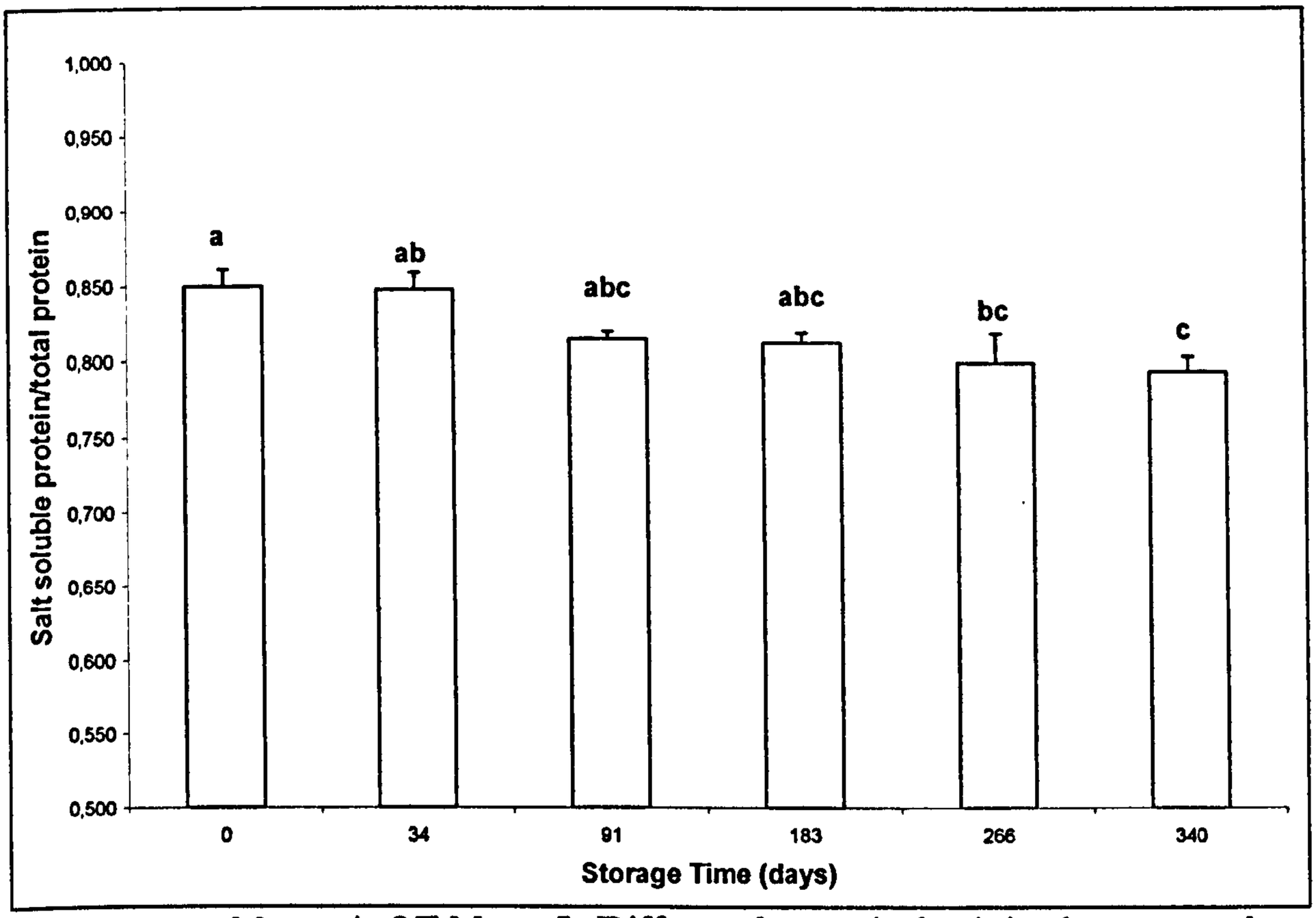


Figure notes: Means ± S.E.M, n=5. Different letters (a, b, c) in the same column denote a significant difference ($P<0.05$). The '0' storage time represents fresh gilthead seabream fillets.

ANOVA showed that salt soluble proteins extracted from the fresh samples were significantly different only than those of samples stored for 266 and 341 days ($P<0.01$; Figure 4.1.2.4-1). Salt soluble proteins extracted from gilthead seabream fillets stored for 340 days were significantly lower than those of samples stored from 34 days, but not from those stored for 91 to 266 days ($P<0.05$). Differences in salt soluble values from samples stored frozen from 34 to 266 days were not observed ($P>0.05$).

Figure 4.1.2.4-2 shows the effects of storage time at -22°C on extractability of actomyosin from fresh and stored frozen gilthead seabream fillets. The differences between the experimental groups were not significant for this parameter ($P>0.05$).

Figure 4.1.2.4-2 Extractability of actomyosin, g g-1 of gilthead seabream tissue.

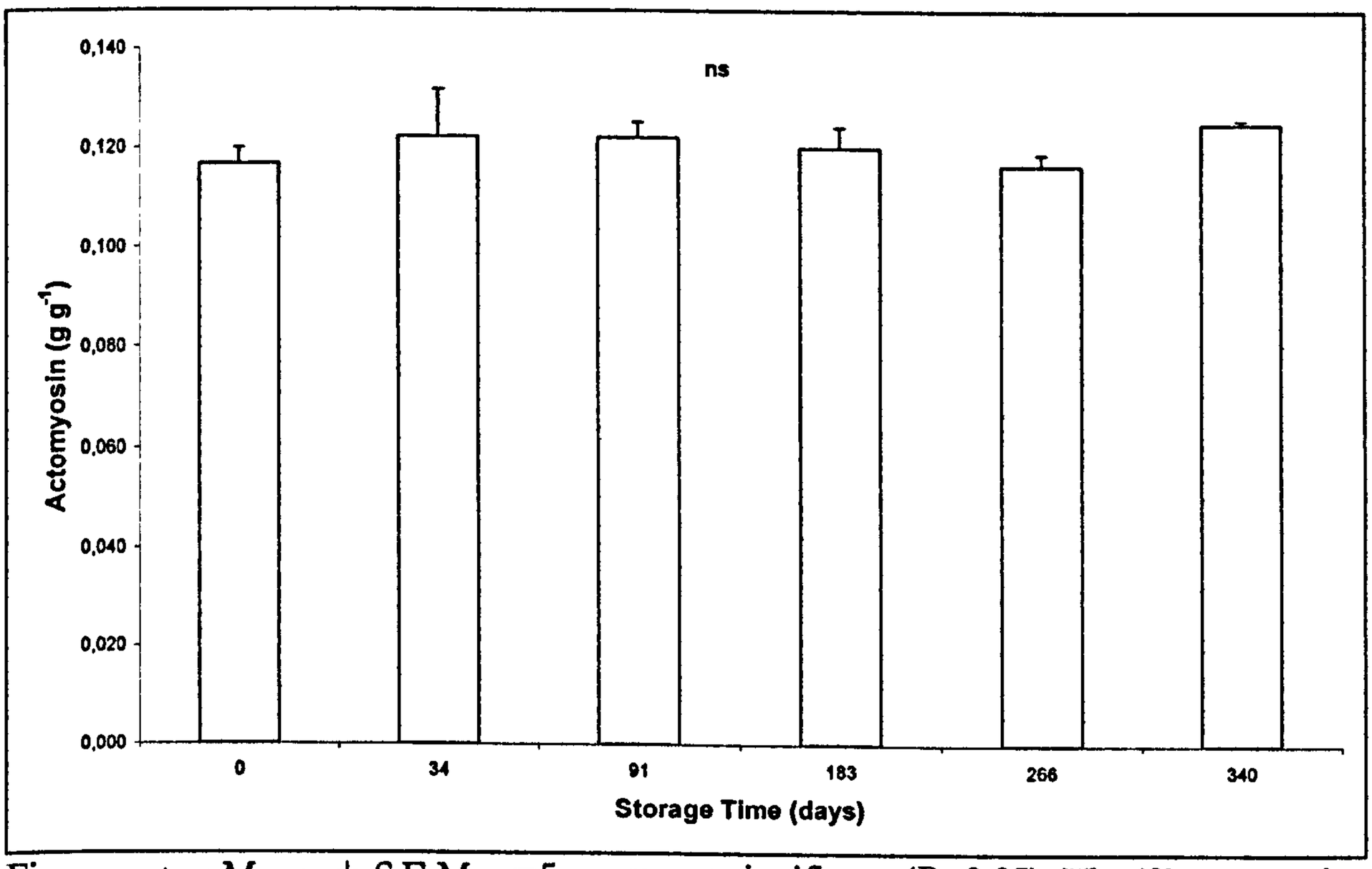


Figure notes: Means ± S.E.M, n=5. ns = not significant ($P>0.05$). The '0' storage time represents fresh gilthead seabream fillets.

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4.1.2.5. Changes in total and surface (reactive) sulfhydryl groups of stored (-22°C) frozen gilthead seabream fillets

Table 4.1.2.5 shows the changes in total and surface (reactive) sulfhydryl groups in actomyosin extracts from fresh and stored frozen fillets.

Table 4.1.2.5 Total and surface (reactive) sulfhydryl groups, SH moles/ 5 x 105g actomyosin.

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

Table notes: 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.

Total sulfhydryl groups of actomyosin from the fresh gilthead fillets were significantly higher compared to those from the fillets stored from 34 to 266 days ($P<0.01$), but not from those stored for 340 days ($P>0.05$). Total sulfhydryl groups of actomyosin extracted from samples stored for 91 days were significantly lower compared to those from samples stored for 266 days ($P<0.01$), but not from those stored for 34, 183 and 340 days ($P>0.05$). Significant differences between the total number of sulfhydryl groups of actomyosin extracted from samples stored for 34, 183, 266 and 340 days were not found ($P>0.05$).

Data of surface (reactive) sulfhydryl groups were analyzed by the t-test assuming not equal variances of distributions, since the Bartlett's test showed that the variances of surface (reactive) sulfhydryl distributions

after log-transformation were not equal ($P<0.05$).

Surface (reactive) sulfhydryl groups of actomyosin extracted from the fillets stored for 91 and 183 days were significantly lower compared to those from fresh fillets and those stored frozen for 266 ($P<0.05$), but not from those stored for 34 and 340 days ($P>0.05$). Significant differences between the number of surface (reactive) sulfhydryl groups of actomyosin extracted from fresh fillets and those stored frozen for 34, 266 and 340 days were not found ($P>0.05$).

4.1.2.6. Changes in ATPase activities in actomyosin extracts from stored (-22°C) frozen gilthead seabream fillets

Table 4.1.2.6 shows the effects of storage time at -22°C on the ATPase activities of actomyosin from fresh and stored frozen gilthead seabream fillets.

Table 4.1.2.6 ATPase activities of actomyosin, μmoles P_i/mg protein/min.

Storage Time (days)	*Ca^{2+}-ATPase*	*Mg^{2+}-Ca^{2+}-ATPase*	*Mg^{2+}-ATPase*	*Mg^{2+}-EGTA-ATPase*
0	0.204 ± 0.014 a	0.178 ± 0.007 a	0.166 ± 0.018 a	0.030 ± 0.001 a
34	0.165 ± 0.007 ab	0.111 ± 0.015 b	0.157 ± 0.021 ab	0.042 ± 0.005 a
91	0.143 ± 0.007 bc	0.093 ± 0.009 b	0.105 ± 0.011 bc	0.032 ± 0.002 a
183	0.143 ± 0.006 bc	0.095 ± 0.005 b	0.105 ± 0.004 bc	0.031 ± 0.001 a
266	0.117 ± 0.008 cd	0.093 ± 0.009 b	0.102 ± 0.008 bc	0.033 ± 0.003 a
340	0.106 ± 0.012 d	0.091 ± 0.012 b	0.097 ± 0.014 c	0.031 ± 0.003 a

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. The '0' storage time represents fresh gilthead seabream fillets.

Ca^{2+}-ATPase activities of actomyosin from fresh samples were significantly higher compared with those of the frozen samples stored from 91 to 340 days ($P<0.01$), but not with those stored for 34 days ($P>0.05$). Ca^{2+}-ATPase activities of actomyosin from samples stored for 34 days were significantly higher than those of the samples stored for 266 and 340 days ($P<0.05$), but not than those stored for 91 and 183 days ($P>0.05$). Ca^{2+}-ATPase activities of actomyosin from samples stored for 340 days were significantly lower than those of samples stored from 34 to 183 days ($P<0.05$), but not than those stored for 266 days ($P>0.05$). Significant differences in Ca^{2+}-ATPase activities of actomyosin from samples stored from 91 to 266 days were not found ($P>0.05$).

Mg^{2+}-Ca^{2+}-ATPase activities of actomyosin from fresh samples were significantly higher compared with those of the stored frozen seabream fillets ($P<0.01$), but significant differences between the stored frozen groups themselves were not observed ($P>0.05$).

Mg^{2+}-ATPase activities of actomyosin from fresh samples were significantly higher compared with those of the frozen samples stored from 91 to 340 days ($P<0.01$), but not with those stored for 34 days ($P>0.05$). Mg^{2+}-ATPase activities of actomyosin from samples stored for 34 days were significantly higher than those stored for 340 days ($P<0.05$), but not than those stored for 91 to 266 days ($P>0.05$). Significant differences in Mg^{2+}-ATPase activities of actomyosin from samples stored from 91 to 266 days were not found ($P>0.05$).

Significant differences in Mg^{2+}-EGTA-ATPase activities of actomyosin between the experimental groups of seabream were not found ($P>0.05$).

4.1.2.7. Changes in Ca^{2+} sensitivity of actomyosin from stored (-22°C) frozen gilthead seabream fillets

Figure 4.1.2.7 shows the effects of the length of time of storage at -22°C on Ca^{2+} sensitivity of actomyosin extracted from the fresh and frozen gilthead seabream fillets.

Figure 4.1.2.7 Ca^{2+} sensitivity of gilthead seabream actomyosin, (%).

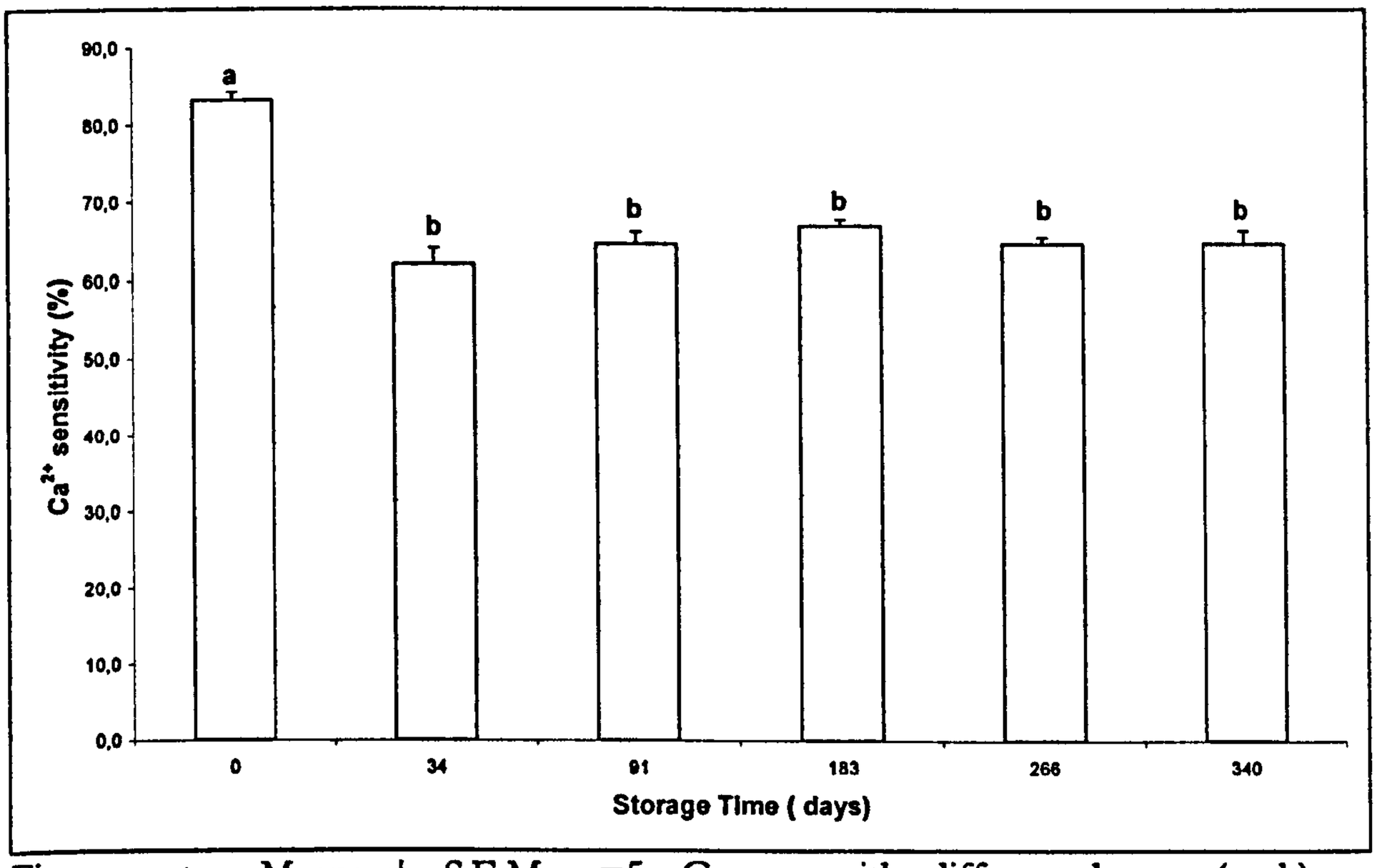


Figure notes: Means ± S.E.M, n=5. Groups with different letters (a, b) are significantly different ($P<0.05$). The '0' storage time represents fresh gilthead seabream fillets.

Ca^{2+} sensitivity of actomyosin from fresh fillets was significantly higher compared to that of frozen fillets at all storage periods ($P<0.001$; Figure 4.1.2.7). Differences between the frozen groups themselves were not observed ($P>0.05$).

4.1.2.8. Changes in lipids extracted from stored (-22°C) frozen gilthead seabream fillets

a) Total lipid content

The total lipid content of fresh and stored frozen fillets is shown in Table 4.1.2.8. The Kruskal-Wallis test for comparison of the medians of the distributions of the total lipid contents from the experimental groups showed that the differences were significant ($P<0.05$). Total lipid content from fresh fillets was significantly higher compared to that of frozen fillets at all storage periods ($P<0.05$). Differences between the frozen groups themselves were not observed ($P>0.05$).

b) Total Free Fatty acids (FFA)

Table 4.1.2.8 shows the effects of storage time at -22°C on the concentration of total free fatty acids (FFA) in lipids extracted from the fresh and frozen gilthead seabream fillets. Differences between the experimental groups were significant ($P<0.0001$). The concentrations of FFA from fresh gilthead seabream fillets and those of stored frozen for 34 days were significantly lower compared with those of the fillets stored from 91 to 340 days ($P<0.001$). Free fatty acids values in lipids from samples stored for 340 days were significantly higher than those stored from 91 to 266 days ($P<0.05$). Differences in FFA values in lipids from fillets stored from 91 to 266 days were not observed ($P>0.05$).

Table 4.1.2.8 Total lipid content, total free fatty acids (FFA), peroxide value (PV) and thiobarbituric reactive substances (TBARS).

Storage Time (days)	*Total Lipid Content (g kg^{-1} tissue)*	*Free Fatty Acids (FFA) (g kg^{-1} lipid)*	*Peroxide Value (PV) (meq O_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

Table notes: 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.

c) Peroxide Value (PV)

As shown in Table 4.1.2.8, the concentration of peroxides (PV values) in lipids from fresh gilthead seabream fillets and those of stored frozen for 34 and 91 days were significantly lower compared with those of the fillets stored from 183 to 266 days ($P<0.0001$), but not between themselves. Differences in PV values in lipids from fillets stored from 183 to 340 days were not observed ($P>0.05$).

d) Thiobarbituric reactive substances (TBARS)

The changes in TBARS in fresh and stored frozen fillets are shown in Table 4.1.2.8.

Since the Bartlett's test showed that the variances of TBARS distributions after log-transformation were not equal ($P<0.05$), data were analyzed by the t-test assuming not equal variances of TBARS distributions. TBARS values of fresh samples were significantly less compared with those of stored frozen gilthead seabream fillets ($P<0.001$). TBARS values of stored frozen fillets for 34 days were significantly less than those of the fillets stored for 183 and 266 days ($P<0.05$), but not of those stored for 91 and 340 days. Significant differences in TBARS values were found between fillets stored for 340 days and those stored for 183 and 266 days ($P<0.05$), but not with those stored for 91 days ($P>0.05$). Differences in TBARS values between the fillets stored from 91 to 266 days were not found ($P>0.05$).

4.1.2.9. Changes in texture of stored (-22°C) frozen gilthead seabream fillets as measured by the texture analyzing system

Figure 4.1.2.9-1 shows the changes in firmness of stored frozen gilthead seabream fillets as measured by the texture analyzing system.

Figure 4.1.2.9-1 Instrumental firmness values, N

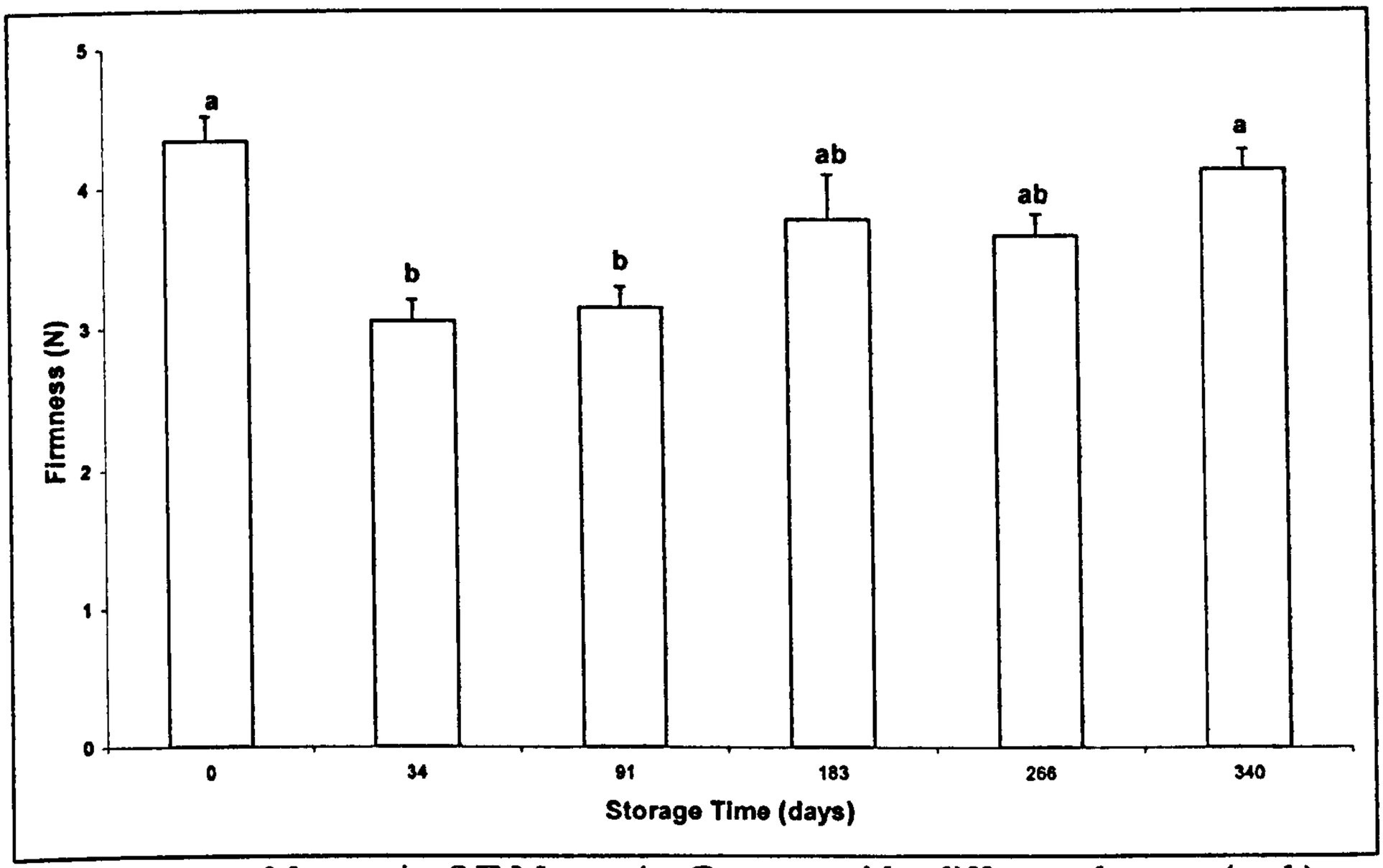


Figure notes: Means ± S.E.M, n=4. Groups with different letters (a, b) are significantly different ($P<0.05$). The '0' storage time represents fresh gilthead seabream fillets.

ANOVA showed significant differences in firmness values between the experimental groups ($P<0.01$). Firmness values of samples stored frozen for 34 and 91 days were similar, and significantly less than those of fresh fillets and those stored frozen from 183 to 340 days ($P<0.05$). Differences between the fresh fillets and those stored frozen from 183 to 340 days were not observed ($P>0.05$).

As shown in Figure 4.1.2.9-2, the toughness of frozen fillets stored for 34 and 91 days were similar, and significantly less than those obtained from the other experimental groups of fillets ($P<0.0001$). Toughness values of stored frozen fillets for 183 days were significantly less than those of fresh fillets, but not than those stored frozen for 266 and 340 days ($P>0.05$). Significant differences in toughness values between the fresh fillets and those frozen for 266 and 340 days were not found ($P>0.05$).

Figure 4.1.2.9-2 Instrumental toughness values, N x sec

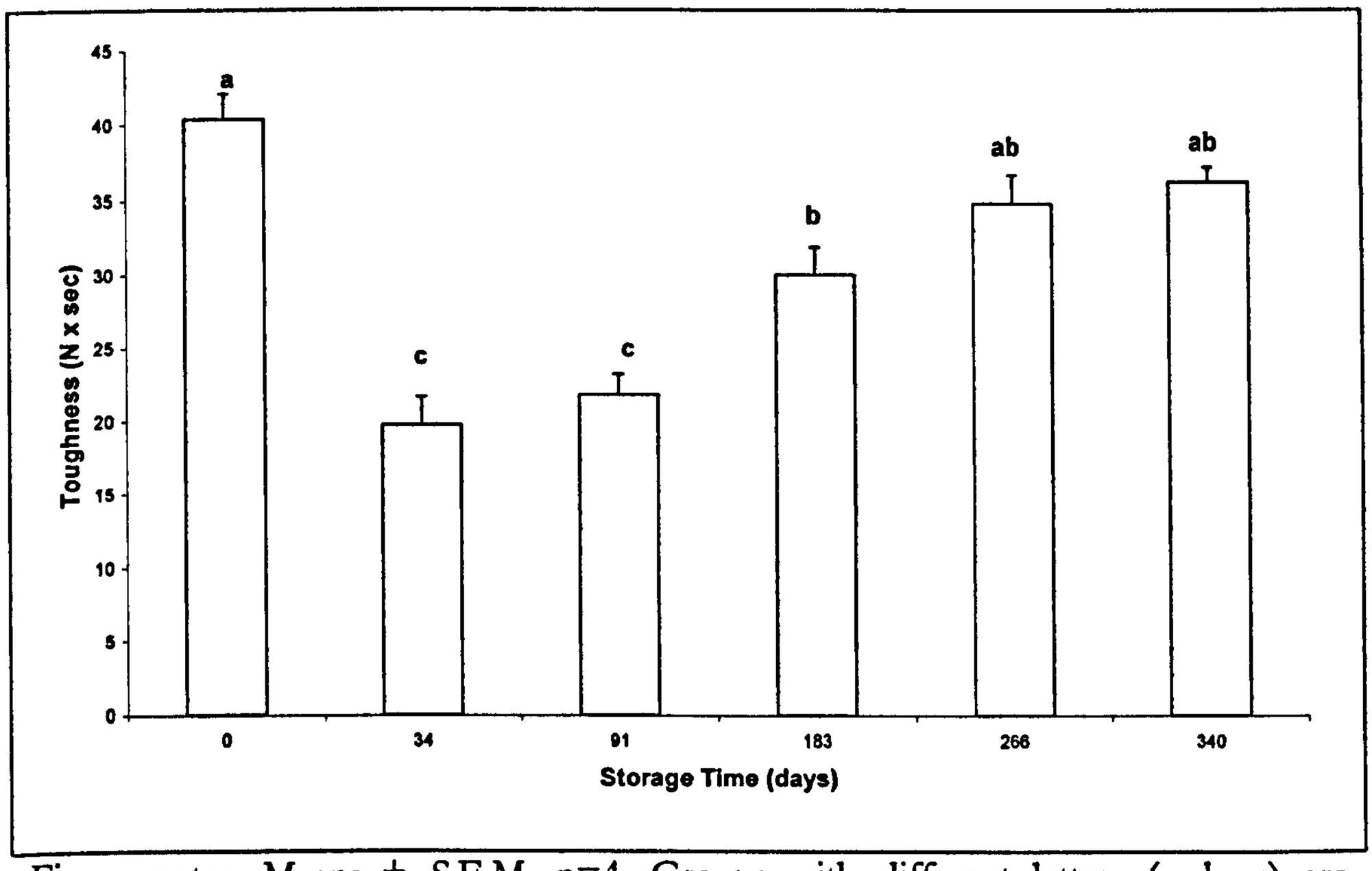


Figure notes: Means ± S.E.M, n=4. Groups with different letters (a, b, c) are significantly different ($P<0.05$). The '0' storage time represents fresh gilthead seabream fillets.

4.1.2.10. Changes in sensory attributes of stored frozen (-22°C) gilthead seabream fillets

a) Changes in chewiness

As shown in Figure 4.1.2.10-1, the assessors rated the stored frozen fillets significantly more chewy than the fresh fillets ($P<0.0001$). Samples stored frozen from 91 to 340 days were rated as significantly more chewy than those stored frozen for 34 days ($P<0.0001$), but there was not a significant difference between themselves ($P>0.05$).

Table 4.1.2.10-1 Assessors' chewiness scores.

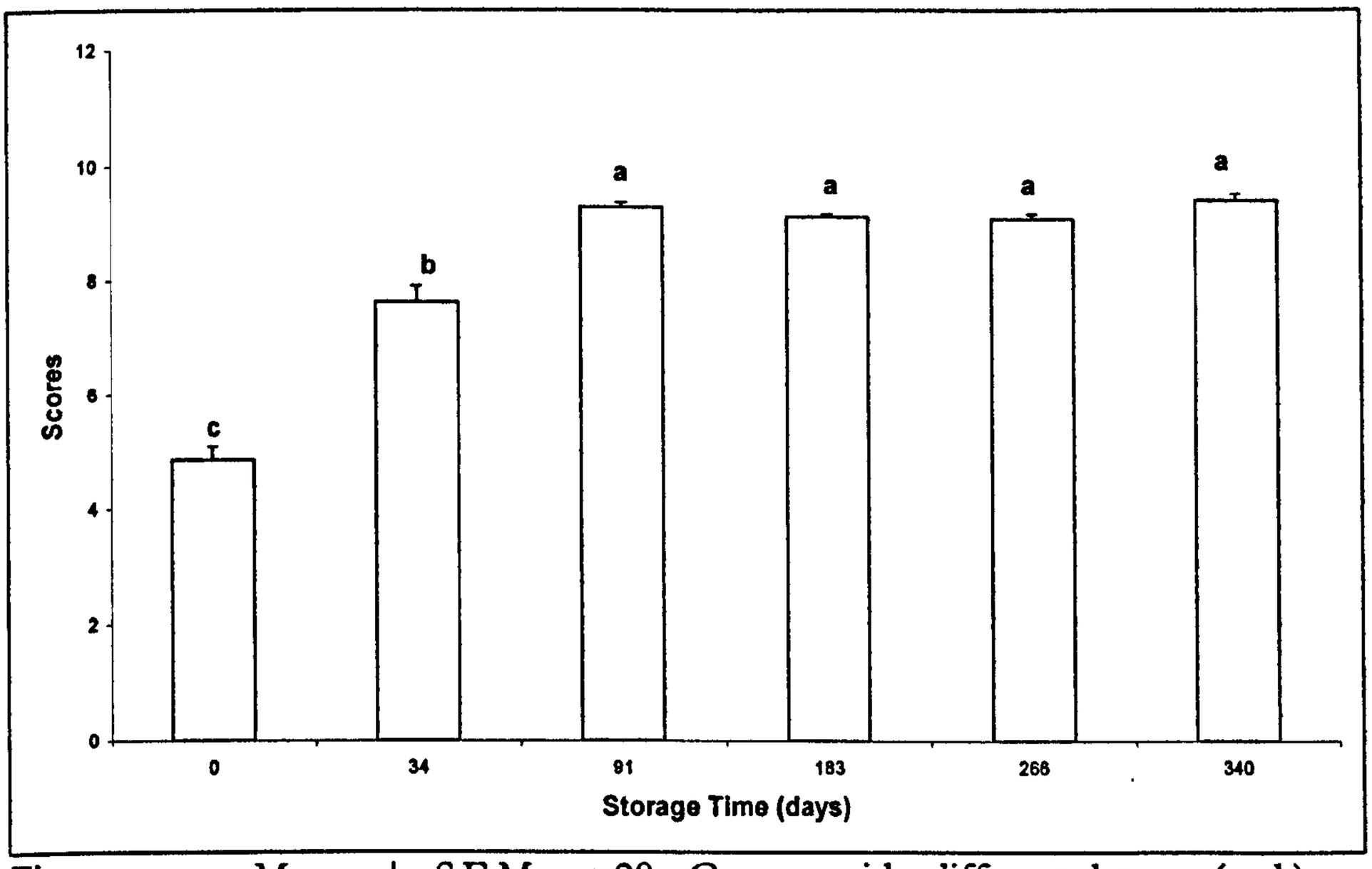


Figure notes: Means ± S.E.M, n=20. Groups with different letters (a, b) are significantly different ($P<0.05$).

b) Changes in acceptability scores of taste

Assessors' acceptability scores of taste are shown in Figure 4.1.2.10-2. Acceptability scores of taste recorded for fresh samples and those for fillets stored frozen for 34 days were significantly higher than those recorded for fillets stored from 91-340 days, but were similar to each other. Taste scores recorded for samples stored for 91 and 183 days were

similar and significantly higher than those for samples stored for 266 and 340 days ($P<0.05$). Taste scores recorded for samples stored for 266 days were significantly higher than those for samples stored for 340 days ($P<0.05$). There was a general decline of acceptability of taste with increasing length of storage time of frozen fillets.

Figure 4.1.2.10 -2 Assessors' acceptability of taste.

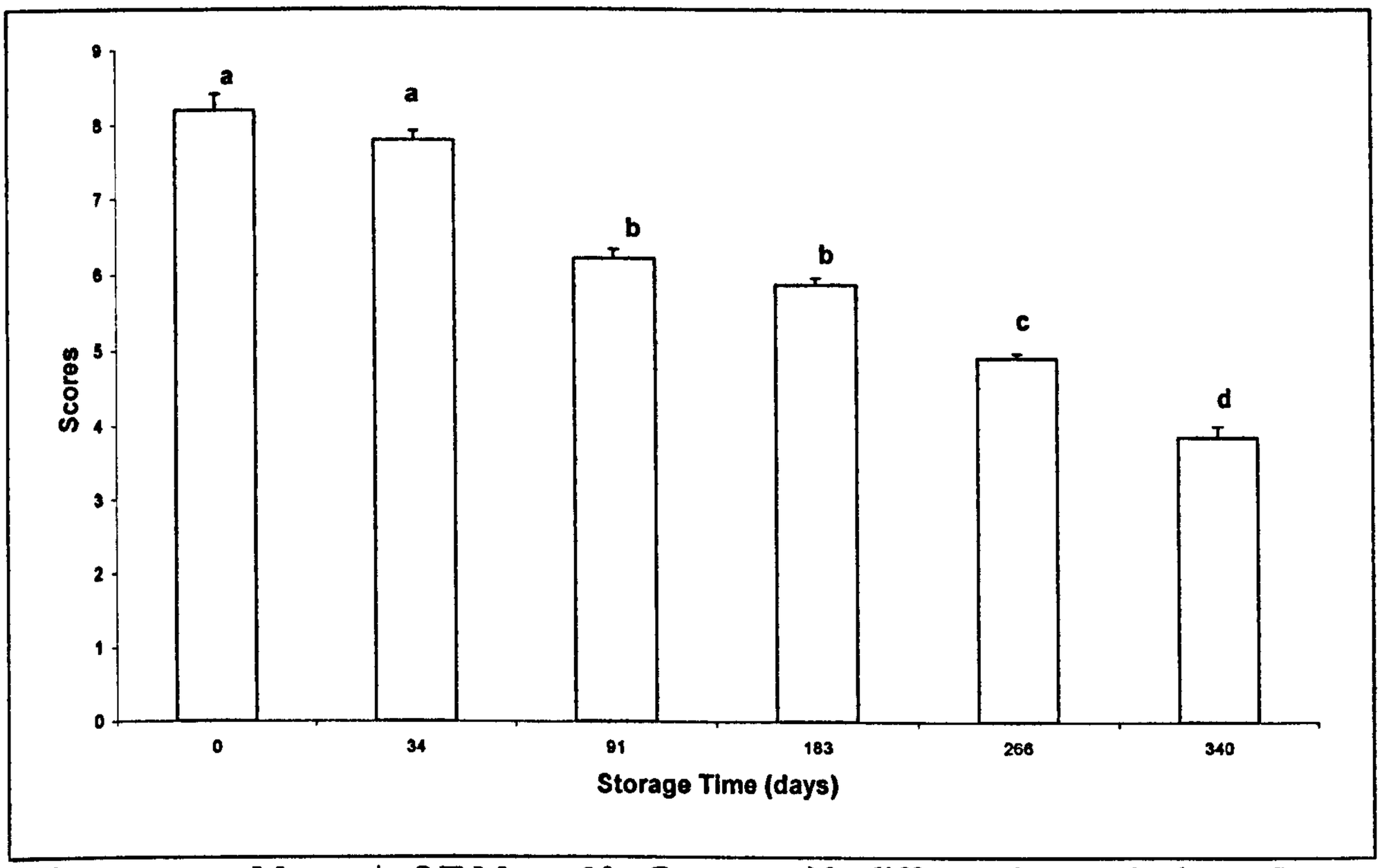


Figure notes: Means ± S.E.M, n=20. Groups with different letters (a, b, c, d) are significantly different ($P<0.05$). The '0' storage time represents fresh gilthead seabream fillets.

c) Changes in appearance

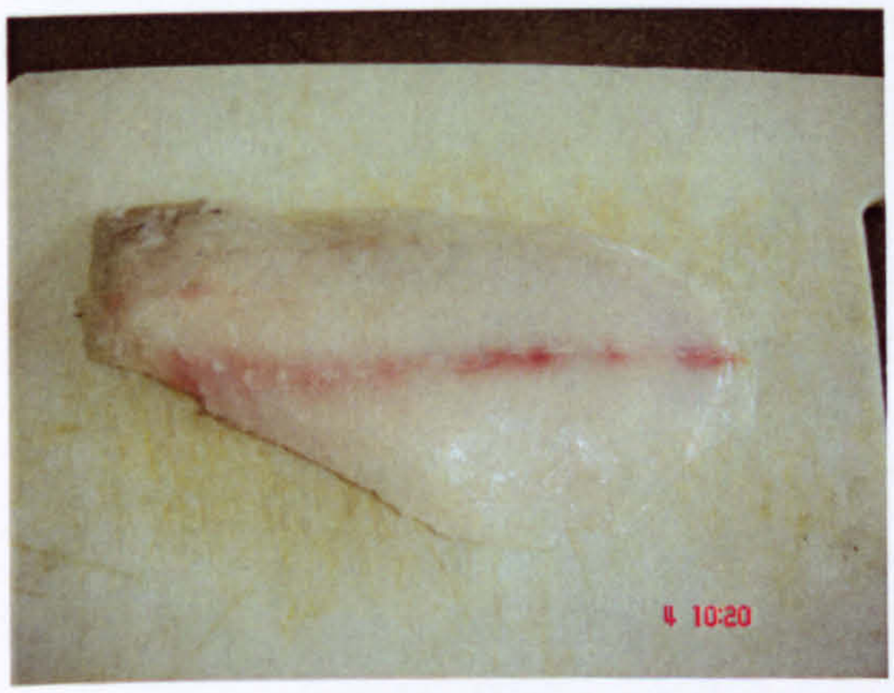
With respect to the appearance of the stored frozen fillets, gaping or dehydration was not observed. However, some fillets stored frozen for 286 and 340 days had a slight yellow or brown colour, probably due to oxidation of lipids and/ or interaction of lipids' degradation products and proteins (Figure 4.1.2.10-3, below ; see section 1.3.4)

Figure 4.1.2.10-3 Appearance of the stored frozen seabream fillets

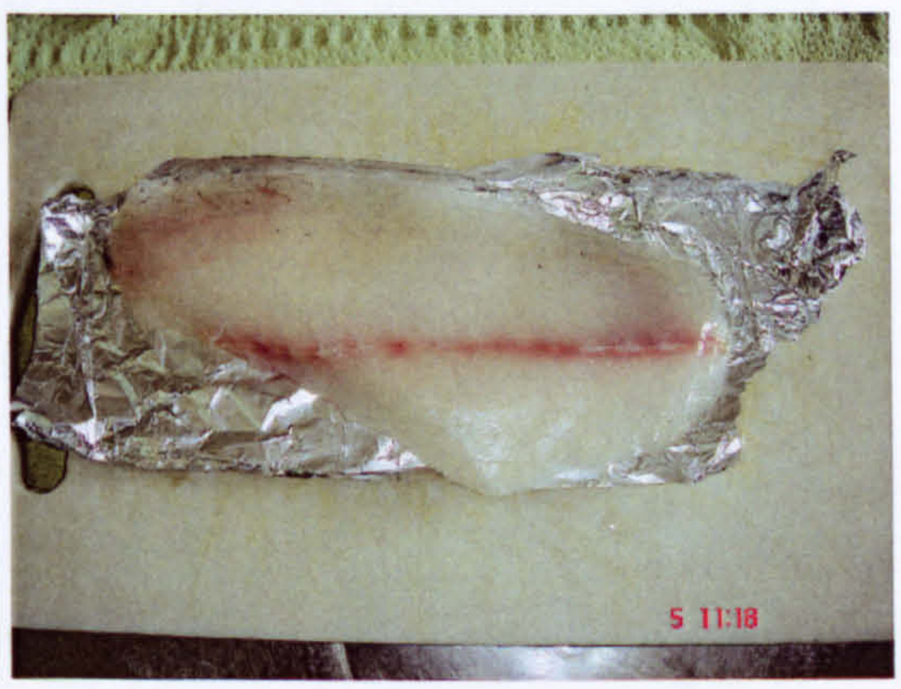
Fresh fillet

Stored frozen fillet for 34 days

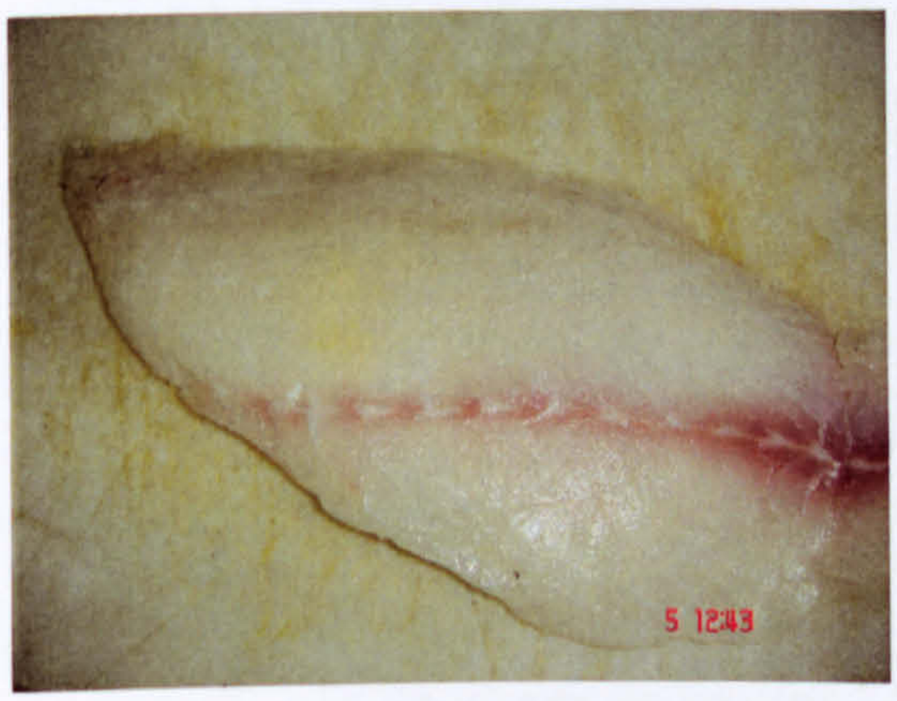
Stored frozen fillet for 91 days



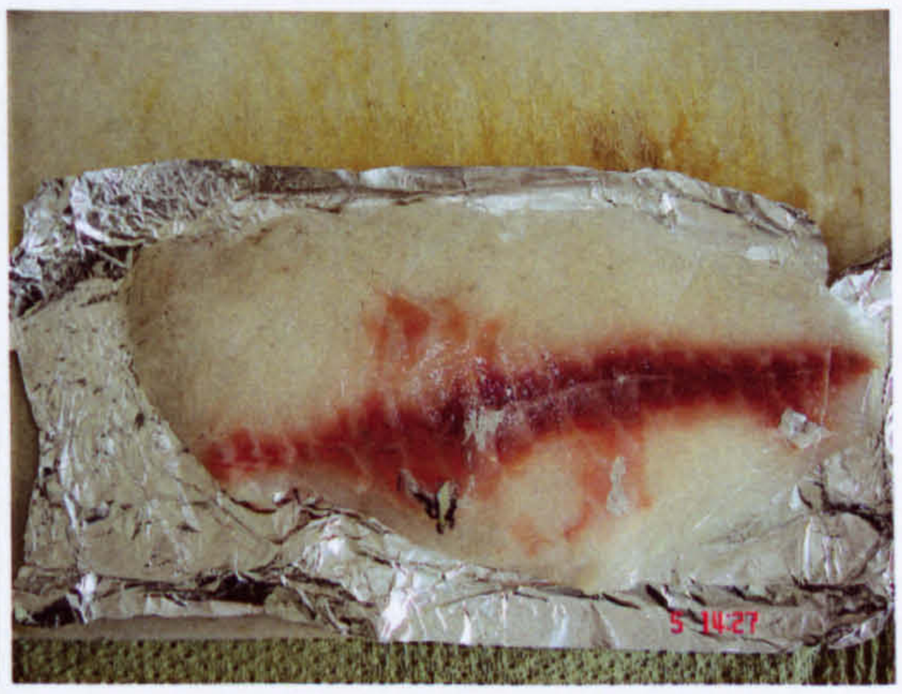
Stored frozen fillet for 186 days



Stored frozen fillet for 286 days



Stored frozen fillet for 340 days



4.1.2.11. Correlations between the parameters

Data from the cold storage experiments 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). For this, pair-wise scatter graphs were plotted and the Spearman's rank correlation coefficients (r_s) were calculated between the parameters.

Figure 4.1.2.11 and Table 4.1.2.11-1 show the scatter plot matrix and the Spearman's rank correlation coefficients (r_s) between the storage time, the physical, chemical, bio-chemical and sensory parameters of the stored frozen gilthead seabream fillets, respectively.

Figure 4.1.2.11 Scatter plot matrix between the parameter

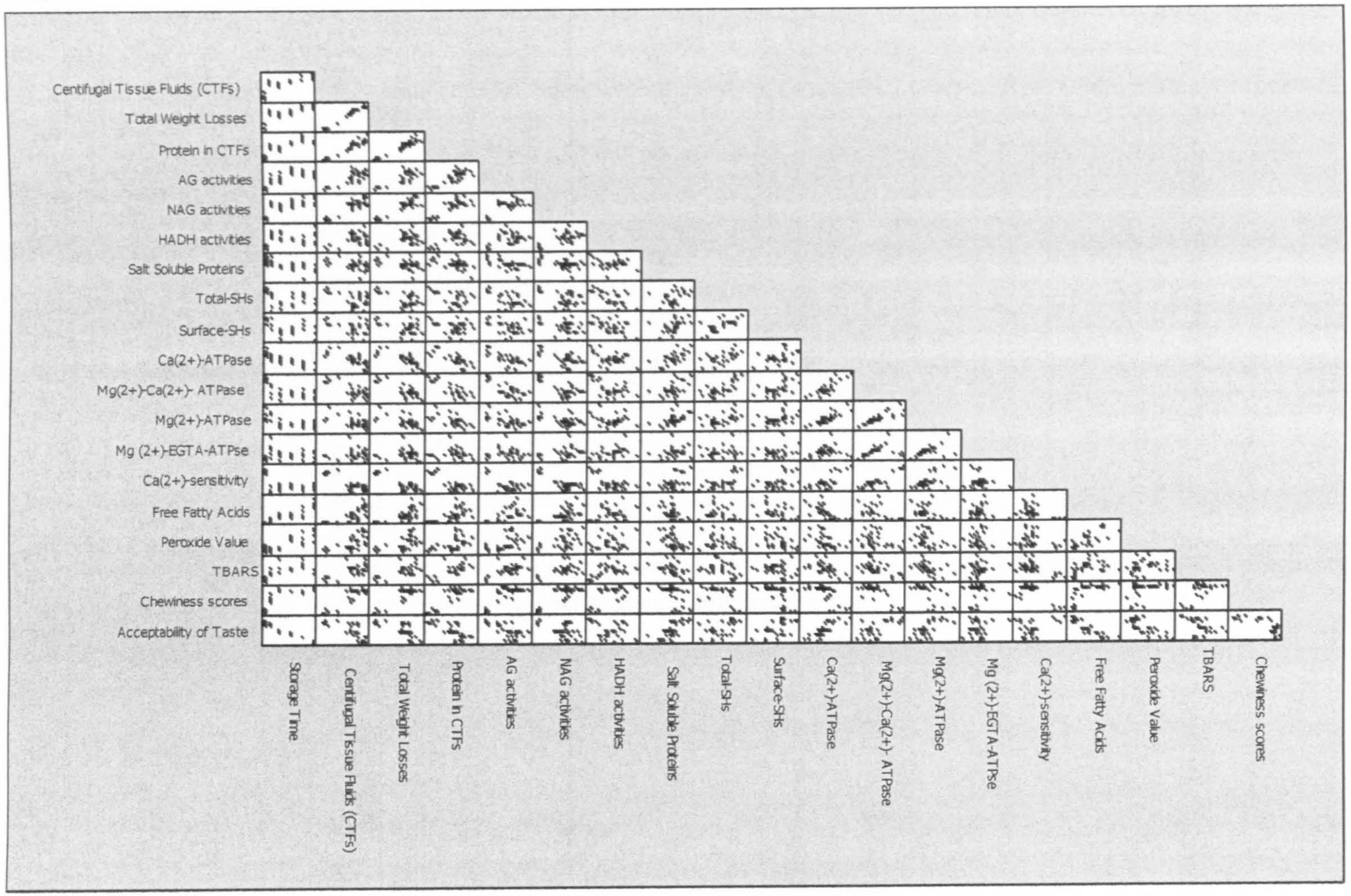


Table 4.1.2.11-1 Spearman's correlation coefficients between the parameters of the stored frozen gilthead seabream fillets

Parameters	Storage Time	Centrifugal Tissue Fluids (CTFs)	Total Weight Losses	Protein weight in CTFs	AG activities	NAG activities	HADH activities	Salt Soluble Proteins	Total-SHs	Surface-SHs	Ca(2+)-ATPase	Mg(2+)-Ca(2+)-ATPase	Mg(2+)-ATPase	Mg (2+)-EGTA-ATPse	Ca(2+)-sensitivity	Free Fatty Acids	Peroxide Value	TBARS	Chewiness scores	Acceptability of Taste
Storage Time	1																			
Centifugal Tissue Fluids (CTFs)	*0.694***	*1*																		
Total Weight Losses	*0.726***	*0.951***	*1*																	
Protein weight in CTFs	*0.576***	*0.802***	*0.850***	*1*																
AG activities	*0 489**	*0.189*	*0.311*	*0.416*	*1*															
NAG activities	*0.216*	*0.192*	*0.295*	*0.200*	*0.103*	*1*														
HADH activities	*-0.380*	*-0 215*	*-0.229*	*-0.346*	*-0.332*	*0.188*	*1*													
Salt Soluble Proteins	- 0.682**	*-0 045*	*-0.086*	*-0.181*	*-0.261*	*-0.089*	*0.377*	1												
Total-SHs	-0.385*	*0.217*	*0.241*	*-0.187*	*-0.087*	*0.159*	*-0.317*	0.301	1											
Surface-SHs	-0 503**	*0.178*	*0.137*	*0 196*	*-0.092*	*0.003*	*-0.257*	0.562**	0 691**	1										
Ca(2+)-ATPase	-0.871**	*-0.590***	*-0.638***	*-0.597***	*-0.477**	*-0 047*	*0.417*	0.639**	0.516**	0.455*	1									
Mg(2+)-Ca(2+)- ATPase	-0.546**	*-0.124*	*-0.206*	*-0.441*	*-0.411**	*0.150*	*0.273*	0.513**	0 653**	0.525**	0.775**	1								
Mg(2+)-ATPase	-0.632**	*-0.246*	*-0.344*	*-0.473**	*-0.400*	*0.136*	*0.419*	0.513**	0.497**	0.492**	0.831**	0.940**	1							
Mg (2+)-EGTA-ATPse	0.030	*-0.128*	*-0.204*	*-0.495**	*-0.461**	*0.193*	*0.332*	0.135	0.074	0.149	0.226	0.459*	0.491**	1						
Ca(2+)-sensitivity	-0.309	*-0.022*	*-0 048*	*-0.086*	*0.156*	*-0.010*	*-0.164*	0.270	0.510**	0.302	0.531**	0.703**	0.551**	-0.019	1					
Free Fatty Acids	**0.914****	*0.534**	*0.594***	*0.444**	*0.433**	*0.352*	*-0.344*	-0.686**	-0.354	-0.507**	-0.812**	-0.502**	-0.568**	0.053	-0.283	1				
Peroxide Value	0 817**	*0.516**	*0.529***	*0.478**	*0.434**	*0.218*	*-0.315*	-0.518**	-0.222	-0.455*	-0.560**	-0.304	-0.359	-0.027	-0.020	0.713**	1			
TBARS	0.484**	*0.085*	*0.153*	*0.314*	*0.385*	*0.115*	*-0.430*	-0.479**	-0.264	-0.120	-0.569**	-0.562**	-0.550**	-0.013	-0.464**	0.482**	0.269	1		
Chewiness scores	0.705**	*0.175*	*0.210*	*0.098*	*0.231*	*0.004*	*0.070*	0.270	-0.603**	-0.740**	0.531**	0.703**	0.551**	-0.019	0.581*	-0.283	0 020	-0.464*	1	
Acceptability of Taste	**-0.970****	*-0 670***	*-0.709***	*-0.569***	*-0.511**	*-0 225*	*0.392*	-0 657**	0 374*	0 484**	-0 890**	0.538**	-0.646**	0 031	0 282	-0.906**	-0 757**	0.474**	-0 749**	1

Table notes: * Significant at level 5%. **Significant at level 1%. Numbers followed by no * are not significant, n=30. For numbers in italic form, n=25. Numbers in bold imply a strong relationship between the parameters.

As shown in Figure 4.1.2.11, certain scatter plots (e.g. between total weight losses and ATPase activities) showed two distinct groups of observations, one of which corresponded to the fresh samples and the other to frozen samples. In order, therefore, to explore whereas a correlation existed between these parameters due to the length of time of storage of the frozen fillets, the Spearman's rank correlation coefficients (r_s) were determined taking into account the values of stored frozen samples. These are represented in Table 4.2.2.11-1 with numbers in italic form.

The scatter plots and the Spearman's rank correlation coefficients (r_s) suggested good relationships of storage time with:

- Ca^{2+}-ATPase activities,
- free fatty acids,
- peroxide values, and
- acceptability of taste.

Also, chewiness scores were significantly correlated with Mg^{2+}-Ca^{2+}-ATPases activities, and acceptability of taste with:

- free fatty acids, and
- peroxide values.

Since non-sensory parameters which show linear correlations with storage time and sensory parameters are useful methods for assessing the quality of stored frozen seafoods, linear regressions were performed between the mentioned parameters. The coefficients of determinations (R^2) from the these regressions are presented in the Table 4.1.2.11-2, below.

Table 4.1.2.11-2 Coefficients of determinations (R^2) from linear regressions between different non-sensory parameters of stored frozen gilthead seabream fillets with storage time and quality parameters.

Parameter compared	R^2
Ca^{2+}-ATPase activities vs. storage time	0.669
Free fatty acids vs. storage time	0.840
Peroxide value vs. storage time	0.720
Acceptability of taste vs. storage time	0.946
Chewiness scores vs. Mg^{2+}-Ca^{2+}-ATPase activities	0.715
Acceptability of taste vs. free fatty acids	0.847
Acceptability of taste vs. peroxide value	0.614

Table notes: R^2 values were significant at levels less than 0.01%.

From the data in Table 4.1.2.11-2, the best linear relationships were between:

- free fatty acids vs. storage time,
- acceptability of taste vs. storage time, and
- acceptability of taste vs. free fatty acids.

As shown in Figure 4.1.2.11 and Table 4.1.2.11-1, total weight losses of stored frozen gilthead seabream fillets were significantly correlated with:

- centrifugal tissue fluids,
- protein content in centrifugal tissue fluids,
- Ca^{2+}-ATPases activities,
- free fatty acids, and
- peroxide values.

The relationship between centrifugal tissue fluid and total weight losses would have arisen by the integration of centrifugal tissue fluids measurements into the calculations of total weight losses (see section 2.2.7.4). The relationships between total weight losses and the other mentioned parameters are discussed in section 4.2.2.3.

Salt soluble proteins showed positive and significant relationship with surface sulfhydryl groups and negative relationships with free fatty acids and peroxide values (discussed in section 4.2.2.4). Ca^{2+}-ATPase activities of actomyosin extracts had negative and significant relationships with free fatty acids, peroxide values and TBARS of gilthead seabream fillets (discussed in section 4.2.2.6).

The mean values of firmness and toughness values of stored frozen gilthead seabream fillets, as measured by the texture analyzing system, were correlated with the mean values of the physical, chemical, bio-chemical parameters and chewiness scores. This was performed because the measurements of instrumental texture and those of physical, chemical, bio-chemical and sensory properties were performed on fillets coming from different fish.

Table 4.1.2.11-3 shows the Spearman's correlation coefficients (r_s) between the instrumental firmness and toughness and different quality parameters of the stored frozen fillets.

Table 4.1.2.11-3 Spearman's correlation coefficients (r_s) between the instrumental firmness and toughness and quality parameters of stored frozen gilthead seabream fillets.

Parameters	*Firmness*	*Toughness*
Storage time	0.675**	0.881**
Centrifugal tissue fluids (CTFs)	0.721	0.875
Total weight losses	0.793	0.914*
Protein in CTFs	0.581	0.791
AG activities	0.711	0.747
NAG activities	0.761	0.770
HADH activities	-0.412	-0.650
Salt soluble proteins	-0.813	-0.883*
Total shulfhydryl groups	-0.036	0.224
Surface (reactive) shulfhydryl groups	-0.401	-0.186
Ca^{2+}-ATPase	-0.836	-0.918*
Mg^{2+}-Ca^{2+}- ATPase	-0.653	-0.688
Mg^{2+}-ATPase	-0.687	-0.721
Mg^{2+}-EGTA-ATPse	-0.684	-0.680
Ca^{2+}sensitivity	0.580	0.502
Free Fatty Acids	0.886*	0.888*
Peroxide value	0.999**	0.943**
TBARS	-0.041	0.256
Chewiness scores	0.643	0.643

Table notes: * Significant at level 5%. ** Significant at level 1%. Values followed by no * are not significant. n=5, n=20.

Significant positive correlations were observed between the instrumental firmness values and storage time, free fatty acids and peroxide values. Instrumental toughness values were correlated positively with storage time, total weight losses, free fatty acids and peroxide values, and

negatively with salt soluble proteins and Ca^{2+}-ATPase activities. These relationships are discussed in section 4.2.2.9.

4.2. Discussion and conclusions

4.2.1. Freezing times (rates) experiments: The bio-chemical, physical and sensory properties of the gilthead seabream (*Sparus* aurata) fillets frozen at different freezing times

4.2.1.1. The freezing processes

In the commercial fish industries, fish products are frozen mostly at rates of 3 to 10 cm/h in liquefied gases or 0.3 to 3 cm/h in mechanical freezers. Freezing rates of around 0.2 cm/hr can be obtained by bulk freezing of fish in batch air-blast rooms (International Institute of Refrigeration, 1986). Although rapid and quick freezing is a common commercial practice for freezing fish, slow freezing has been recorded in badly designed and operated freezers. Therefore, the experimental conditions for freezing of gilthead seabream fillets in the present study produced freezing times and freezing rates that are representative of commercial practice of fish freezing (Table 4.1.1.1).

4.2.1.2. Changes in protein content of centrifugal tissue fluids (CTFs), α-glucosidase (AG), β-N-acetyl –glucosaminidase (NAG) and β–hydroxy–acyl-coenzyme–A dehydrogenase (HADH) activities of gilthead seabream fillets frozen at different characteristic freezing times

In fresh tissue AG, NAG and HADH enzymes are retained in the intracellular organelles. However, AG, NAG and HADH activities were found in the water soluble extracts of fresh gilthead seabream fillets (Table 4.1.1.2 and Figure 4.1.1.2). These results suggest that lysosomes and mitochondria were damaged at the surfaces where the gilthead seabream samples were cut, and thus a certain amount of the enzymes had leaked from damaged organelles into the muscle (Gottesman and Hamm, 1983). Moreover, autolysis of the gilthead seabream muscle could have caused disruption of some lysosomes and mitochondria, since the

fresh fish were stored in ice for three days prior to analysis (Rehbein *et al.*, 1978; Nilsson and Ekstrand, 1993).

The weight of, and the protein content in centrifugal tissue fluids (CTFs) and the activities of the enzymes AG, NAG and HADH in frozen and thawed gilthead seabream fillets were significantly higher than those in fresh fillets ($P<0.05$; Tables 4.1.1.2, 4.1.1.3; Figure 4.1.1.2). Similar results for other fish species and shellfish have been reported by other workers (Rehbein *et al.*, 1978; Nilsson and Ekstrand, 1993, 1994; Dulfos *et al.*, 2002). Altogether these findings indicate that the freeze-thaw process itself affects the integrity of cells of gilthead seabream fillets. The freeze damage of cells can be due to denaturation of cellular wall proteins by the concentration of salts and/ or disruption of cells by ice crystals (Love, 1966).

From gilthead seabream fillets frozen at the characteristic freezing time (t_c) of 74 minutes, the protein content of CTF was significantly higher than that of the other times ($P<0.05$; Table 4.1.1.2). This could be the result of either release of intra-cellular fluids, which accounts for most of the protein present in the exudates, or by the presence of cell fragments, as the result of ice particles breaking the cell walls (Grigler and Dawson, 1968). The activities of AG and HADH enzymes from the fillets frozen at the characteristic freezing time (t_c) of 74 minutes were higher compared to those of the other times ($P<0.05$; Table 4.1.1.2 and Figure 4.1.1.2). These results suggest that freezing of gilthead seabream fillets at the characteristic freezing time (t_c) of 74 minutes caused more damage to cells than freezing at the other times. Love (1955) showed that freezing of post rigor fillets at times from 80 to 120 min resulted in higher amounts of intra-cellular material (specifically DNA) in exudates than freezing at shorter or longer times. He suggested that the cell damage of cod fillets frozen at those times was due to the rupture of sarcollemas by large intra-

cellular masses of ice. Therefore, the gilthead seabream fillets frozen at a time (t_c) of 74 minutes might have frozen fast enough to form intra-cellular ice, but as the fillets continued to freeze at a much slower rate such that the intra-cellular ice might have had time (154 min) to grow to disruptive size and rupture the cells before the fillets reached -20°C.

In the present work, data from CTF, AG, NAG and HADH analyses did not produce evidence of differences in cell-damage of gilthead seabream fillets frozen at 2, 18 and 640 minutes ($P>0.05$; Tables 4.1.1.2, 4.1.1.3; Figure 4.1.1.2). Love (1955) showed that post-rigor cod fillets, frozen at times (times to cool the centre of the fillets from 0 to -5°C) of less than 20 minutes, contained small intra-cellular ice crystals, which were distributed fairly evenly throughout the cells, with little change seen in cell structure. In addition, Love (1958a) showed that freezing cod fillets at very long freezing times (more than 500 minutes) resulted in large inter-cellular ice masses. Those fillets showed less cell damage than fillets frozen at intermediate rates. In the present study, the gilthead seabream fillets frozen at the fast freezing times (t_c values) of 2 and 18 minutes would probably have had intra-cellular ice crystals. These ice crystals would be small in size since it took only 4 and 27 minutes to freeze the fillets. Consequently, any small ice crystals formed as a result of fast freezing would cause little damage in the cell structure of the fillets. On the other hand, fillets frozen at the very slow freezing time (t_c) of the 640 minutes, would probably have had large inter-cellular ice crystals that led to internal water diffusing out of the cells, leaving little ice in the cells. Therefore, the very slow freezing process would prevent the formation of large destructive intra-cellular ice crystals. As the results show, the cell damage in the fillets frozen at characteristic freezing time (t_c) of 640 minutes did not seem to be different from the damage of fillets frozen at times of 2 and 18 minutes.

Overall, these results show that the freezing process itself clearly affected the integrity of gilthead seabream muscle cells. Moreover, freezing of gilthead seabream fillets at the characteristic freezing time (t_c) of 74 minutes caused more damage to cells than the other freezing times, shorter or longer.

4.2.1.3. Changes in water holding parameters of gilthead seabream fillets frozen at different characteristic freezing times

Thawing weight losses of fillets frozen at a characteristic freezing time (t_c) of 640 minutes were higher than those of fillets frozen at the other freezing times and significantly more compared to those of fillets frozen at t_c values of 2 and 18 minutes ($P<0.01$; Table 4.1.1.3). Several other studies have reported increases in thawing losses of meat products with increases in characteristic freezing times (Khan 1964; Aurell *et al.*, 1976; Petrovic *et al.*, 1993; Chevalier *et al.*, 1999). These differences are attributed to changes in mainly myofibrillar proteins and/ or to distortion and destruction of muscle fibres by ice-crystals (Añón and Calvelo, 1980; Wagner and Añón 1985; Petrovic *et al.*, 1993). As will be shown in the following sections, the freezing times tested did not seem to cause an irreversible denaturation of myofibrillar proteins (in terms of changes in salt soluble proteins, sulfhydryl groups, ATPase activities and Ca^{2+} sensitivities of actomyosin), possibly because no storage time was included in the present study. Thus, the differences in the amount of exudates released from the fillets during thawing might be due to differences in the size and location of ice crystals and to differences in the mechanical damage of muscle fibres, as discussed earlier in this work (see section 4.2.1.2). It is, therefore, likely that the inter-cellular water, formed by melting of the large ice crystals in the inter-cellular spaces of slowly frozen fillets, was not as well reabsorbed by the muscle cells as the water formed by melting of the intra-cellular ice crystals in the fillets

frozen at the shorter freezing times i.e. faster freezing rates. It was presumed earlier in this study that large intra-cellular ice crystals might have ruptured the cells of the fillets with a freezing time of 74 minutes. This and the size of ice crystals as well, could be the reason why there was more drip loss during thawing of these fillets compared to those frozen at times of 2 and 18 minutes. Similar suggestions have been reported by other authors (Bevilaqua *et al.*, 1979; Añón and Calvelo, 1980; Petrovic *et al.*, 1993).

In contrast to thawing weight losses, centrifugal tissue fluids (CTFs) obtained from gilthead seabream fillets frozen at characteristic freezing time (t_c) of 640 minutes were less compared to those of the other freezing times (Table 4.1.1.3). Several other investigations have shown that slow freezing results in more thawing losses than quick freezing and in less expressible fluid in frozen meat products (e.g. Khan and Lentz, 1977). In the present study, centrifugal fluid measurements were taken after thawing, i.e. after the fillets had released part of the unbound or loosely bound water. These measurements can be considered as indicators of the state of the remaining quantity of loosely bound water in thawed fillets, as reported by Petrovic *et al.*, (1993) for frozen beef. Therefore, the low CTF value of the fillets frozen at a characteristic freezing time (t_c) of 640 minutes is probably due to the losses that these fillets suffered during thawing. Since water holding capacity of a meat product has been suggested as being indicative of the water loosely bound to protein (Trout, 1988), in the present study the total losses during thawing and centrifugation can be taken as a measure of the water holding capacity (WHC) of the fresh and frozen gilthead seabream fillets (Ciarlo *et al.*, 1985). The total weight losses were significantly higher for frozen than for fresh seabream fillets ($P<0.0001$; Table 4.1.1.3), but differences between the frozen groups themselves were not observed. Since changes in WHC

are very sensitive indicators of the changes in the charges and structure of myofibrillar proteins (Mackie, 1993), the results suggest that the freezing process itself may have affected the properties of myofibrillar proteins of seabream fillets. This suggestion was further investigated by analyses related to myofibrillar protein denaturation and aggregation.

4.2.1.4. Changes in salt soluble proteins and extractability of actomyosin of gilthead seabream fillets frozen at different characteristic freezing times

The results of the present study showed no significant differences between the fresh and frozen gilthead seabream fillets in regard to salt soluble protein and extractability of actomyosin ($P>0.05$; Figures 4.1.1.4-1 and 2). This is in agreement with Wagner and Añón (1985) who found no significant differences in salt soluble proteins of beef frozen at different freezing times. Pan and Yeh (1993) investigating the effects of freezing methods on the solubility of myofibrillar proteins of grass shrimp, showed that there were no significant differences immediately after freezing between shrimps frozen at rates from 3.41 to 16.1 cm/h. Also, Tejada *et al.* (2003) showed that there were no significant differences in the salt soluble proteins and extractability of actomyosin between gilthead seabreams fresh and frozen at -20°C when fish were thawed immediately after freezing. Altogether, these findings suggest that the freezing process itself and the tested freezing times did not cause aggregation of myofibrillar proteins of gilthead seabream fillets if they were thawed immediately after freezing. This suggestion could be associated with the very short time the fillets remained in the frozen state. Love (1958c) and Love and Ironside (1958) found that there were no consistent differences in salt soluble protein of cod fillets frozen at different times and thawed immediately after freezing; however, these workers found that when cod fillets were stored for one year at -29 °C, a

temperature at which the pattern of ice crystals maintained intact, differences in soluble protein were observed that related to the original rates of freezing.

4.2.1.5. Changes in total and surface (reactive) sulfhydryl (SH) groups in actomyosin extracts from gilthead seabream fillets frozen at different characteristic freezing times

The results of the present study showed that the content of SH groups (total and surface) in actomyosin solutions extracted from the fresh and frozen gilthead seabream fillets were similar ($P>0.05$; Figure 4.1.1.5). Considering the data of the salt soluble protein, this result seems to support the previous suggestion in that aggregation of myofibrillar proteins might not have occurred in frozen seabream fillets. It is, also, therefore, likely that oxidation of sulfhydryl groups was not involved in denaturation of gilthead seabream actomyosin extracted from frozen samples.

4.2.1.6. Changes in ATPase activities of actomyosin extracts from gilthead seabream fillets frozen at different characteristic freezing times

Ca^{2+}-ATPase, Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities of actomyosin from seabream fillets frozen at all freezing times were significantly lower than those of fresh samples ($P<0.05$; Table 4.1.1.6). Ca^{2+}-ATPase activity can be used as an indicator for the integrity of myosin molecules, whereas Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities indicate the integrity of actin-myosin complex in the presence of endogenous and exogenous Ca^{2+} ions (Roura and Crupkin, 1995; Benjakul *et al.*, 1997). From the results of the present study the decrease of Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities, after the freeze-thaw process, could indicate a decrease of myosin affinity for actin and/ or an alteration of the enzyme properties of actomyosin (or myosin). On the

other hand, the decrease of Ca^{2+}-ATPase activity could indicate a change in free myosin structure.

The decrease in ATPase activities might imply a change on the myosin head, which contains both the active site of the enzyme and the actin interaction site. Loss of enzymatic activity of fish actomyosin due to freezing may be due to tertiary structural changes caused by ice crystallization and/ or to changes in the SH groups (Benjakul and Bauer, 2000). In the present study, since the total and surface SH contents in actomyosin from fresh and frozen gilthead seabream fillets were similar, the loss of ATPase activities could be a result of partial tertiary structural change (unfolding) of myosin due to a weakening of intra-molecular hydrophobic bonds. This could be a result of local increase of ionic strength as a consequence of freezing and water migration from the myofibrillar space, or dehydration of myofibrillar proteins (as suggested by Benjakul and Bauer, 2000). In the present study, if there was unfolding, this did not induce protein aggregation as indicated by the fact that the amount of salt soluble protein did not change by freezing. Similar results and suggestions were reported by Wagner and Añòn (1985) after studying the effects of freezing times on the denaturation of myofibrillar proteins of beef muscle.

In contrast, Mg^{2+}–EGTA-ATPase activities of actomyosin extracted from frozen samples were significantly higher compared to those of fresh fillets ($P<0.01$; Table 4.1.1.6).This increase coincided with the loss of Ca^{2+} sensitivity (Figure 4.1.1.7). This result is similar to the previously reported change in Mg^{2+} –EGTA-ATPase activity of actomyosin from cod subjected to freeze-thaw cycles (Benjakul and Bauer, 2000). Mg^{2+}-EGTA-ATPase activity is indicative of the integrity of the troponin-tropomyosin complex in meat samples and was reported to increase by treatment with lysosomal proteases (Quali and Valin, 1981). Therefore, in

the present study it may be that lysosomal proteases contributed to the changes in Mg^{2+}-EGTA-ATPase activity observed in gilthead seabream fillets due to freezing and thawing; this would be in accordance with the increase in AG and NAG activities, which were used as indicators of cell disintegration.

4.2.1.7. Changes in Ca^{2+}sensitivity of actomyosin from gilthead seabream fillets frozen at different characteristic freezing times

The Ca^{2+} sensitivity of actomyosin from fresh seabream fillets was significantly higher than from frozen samples ($P<0.0001$; Figure 4.1.1.7). The Ca^{2+}sensitivity of myofibrillar proteins has been attributed to the activity of native tropomyosin (Ebashi *et al.*, 1968). This loss of Ca^{2+} sensitivity is considered to be the result of degradation of myofibrils, due to hydrolysis by proteases (Okitani *et al.*, 1980), but it may also be due to the modification of actin-myosin interaction (Benjakul and Bauer, 2000). The decrease in Ca^{2+} sensitivity of seabream actomyosin caused by the freezing process itself could, therefore, be the result of both proteolysis and the modification of actin-myosin interaction by unfolding of myosin.

4.2.1.8. Changes in total free fatty acids (FFA) in extracted lipids from seabream fillets frozen at different characteristic freezing times

Freezing of seabream fillets at a characteristic freezing time (t_c) of 74 minutes resulted in higher amounts of free fatty acids (FFA) compared to fresh fillets and those frozen at t_c values of 2 and 18 minutes ($P<0.0001$; Figure 4.1.1.8). Hanaoka and Toyomizu (1979; cited in Shewfelt, 1981) showed that in carp muscle hydrolysis of phospholipids was faster at temperatures below the freezing point. They suggested this to be the effect of cellular disintegration rather than dehydration or concentration of solutes in frozen cells. Also, triglycerides are hydrolyzed to FFA by lipases, some of which are thought to be lysosomal (Shewfelt, 1981).

Therefore, in the present study, the maximal concentration of FFA in lipids extracted from the fillets frozen at a characteristic freezing time (t_c) of 74 minutes may be due to the maximal disintegration of cells and lysosomes being observed in that freezing time. The disintegration of cells could have caused increased levels of phospholipids from the breakdown of membranes, whereas the disruption of lysosomes could have caused additional release of lipases in the tissue at the other times. These assumptions are in agreement with decreased muscle integrity shown by the increased levels of released protein, AG and NAG activities found in seabream fillets frozen at a characteristic freezing time (t_c) of 74 minutes compared to those in the fillets frozen at the other freezing times.

4.2.1.9. Changes in texture of gilthead seabream fillets frozen at different characteristic freezing times as measured by the texture analyzing system.

Freezing of gilthead seabream fillets at different freezing times influenced the texture of raw fillets as measured by the texture analyzing system used in the present study (Table 4.1.1.9). Freezing at short characteristic freezing times (i.e. 2 and 18 minutes) produced raw fillets similar in texture (i.e. firmness and toughness values) to fresh fillets. Freezing of fillets at a t_c value of 2 minutes (or at a t_e value equal to 4 minutes; see section 4.1.1.1) produced fillets even closer to the texture of fresh samples compared to fillets frozen at a freezing time (t_c) of 18 minutes.

The texture of raw frozen and thawed seafoods is the result of factors, which cause two opposing effects, i.e., tissue softening (e.g. due to release of protease from lysosomes on freezing and thawing and mechanical damage of muscle cells by ice crystals formed on freezing) and tissue hardening (e.g. due to fibre shrinkage on freezing, weight loss on thawing and denaturation and cross-linking of proteins; Srinivasan *et al.*, 1997).

As the results of the present study show, the freezing process itself caused considerable damage to fibres, decreased the water holding parameters and caused structural changes to myofibrillar proteins of gilthead seabream fillets (see sections 4.2.1.2, 4.2.1.3 and 4.2.1.6). Therefore, the tissue-softening and hardening factors were both active in the frozen fillets and their mutual action would have determined the texture.

It was shown earlier in the present study that freezing of gilthead seabream fillets at a characteristic freezing time of 640 minutes did not produce evidence of differences in cell-damage compared to fillets frozen at t_c values of 2 and 18 minutes (see section 4.2.1.2). However, thawing losses of fillets frozen at a freezing time (t_c) of 640 minutes were bigger than those of fillets frozen at the other freezing times (see section 4.2.1.3). It is, therefore, likely that the tissue-hardening factors (e.g. thawing weight losses) overcame the tissue-softening factors (e.g. release of proteolytic enzymes from lysosomes) with concomitant hardening of gilthead fillets frozen at a freezing time (t_c) of 640 minutes. In contrast, the tissue-softening factors were apparently prevalent in fillets frozen at a characteristic freezing time of 74 minutes. This assumption is similar to the previously reported change in muscle integrity found in gilthead seabream fillets frozen at a characteristic freezing time (t_c) of 74 minutes (see section 4.2.1.2). Therefore, it is suggested that the release of proteolytic enzymes from lysosomes and the disruption of cells by ice crystals could have caused softening of the fillets frozen at a characteristic freezing time of 74 minutes (Pan and Yeh, 1993; Civera *et al.*, 1996). The texture of fillets frozen at t_c values of 2 and 18 minutes were similar to fresh samples possibly because the tissue-hardening factors counteracted the tissue-softening factors and their individual effects on texture might have been diminished. Boonsumrej *et al.* (2007) showed, also, that freezing

of tiger shrimp at a freezing time (t_e value) of 214 seconds (i.e. almost 3.5 minutes) had similar firmness values (i.e. shear forces) to fresh samples. Therefore, freezing of gilthead seabream fillets at different freezing times influenced the texture of raw fillets as measured by the texture analyzing system used in the present study.

4.2.1.10. Changes in chewiness (tenderness/toughness) of gilthead seabream fillets frozen at different characteristic freezing times

Tenderness of fish is highly related to the state of the myofibrillar proteins and changes in the water holding capacity of fish muscles (Mackie, 1993). The results of the present study showed that cooked frozen seabream fillets were perceived as being chewier than the fresh ones ($P<0.0001$; Figure 4.1.1.10), in agreement with the findings of (Carbonell *et al.*, 2003a) for whole cultured gilthead seabream. Since chewiness scores of a meat product are related inversely to tenderness (ISO 5492:1992) the results suggest that the frozen seabream fillets were less tender than the fresh ones. However, differences in chewiness (or tenderness) were not observed between the fillets frozen at t_c values of 18, 74 and 640 minutes. These suggestions are in agreement with the changes in myofibrillar proteins and water holding capacities found in this study.

4.2.1.11. Conclusions

The results of the present study show that the freezing process itself caused considerable damage to muscle fibres, reduced the water holding capacity of the fillets, caused structural changes to myofibrillar proteins (in terms of ATPase activities) and produced chewier cooked fillets. Freezing times hardly affected the water holding capacity of the fillets, the properties of myofibrillar proteins and the sensory attributes of cooked fillets. However, there was a clear effect of freezing times on thawing

losses, damage of muscle fibres, hydrolysis of lipids and texture of raw fillets as measured by a texture analyzing system. In particular:

- freezing of fillets at the long characteristic freezing time of 640 minutes increased the hydrolysis of lipids compared to fresh fillets, gave greater thawing losses, and produced harder and tougher raw fillets compared to fresh fillets and those frozen at the other freezing times;
- freezing of fillets at the characteristic freezing time of 74 minutes caused more damage to muscle fibres, increased hydrolysis of lipids, and produced softer raw fillets compared to fresh fillets and those frozen at the shorter freezing times;
- freezing of fillets at the short freezing times of 2 and 18 minutes hardly affected the hydrolysis of lipids with respect to fresh samples and reduced thawing losses compared to the longer times tested and produced raw fillets similar in texture to that of the fresh fillets. Freezing of fillets at the very short freezing time of 2 minutes produced fillets even closer to the texture of fresh samples, compared to fillets frozen at a freezing time of 18 minutes.

From the work reported in the literature, increased weight losses on thawing of frozen seafoods and deteriorations in their texture influence adversely their quality and consequently their market. In addition, release of enzymes from intra-cellular organelles, hydrolysis and oxidation of lipids may affect the texture, flavour and appearance of fish in subsequent storage (see sections 1.3.1, 1.3.2.2 and 1.3.3).

Based on the mentioned findings and suggestions, the following conclusions can be drawn:

1. short characteristic freezing times, such as that of 2 and 18 minutes (i.e. freezing rates of 8.34 and 1.35 cm/h, respectively), are

beneficial for freezing post-rigor gilthead seabream fillets compared to times of 74 and 640 minutes (i.e. freezing rates of 0.2 and 0.03 cm/h). Freezing rates of 8.34 cm/h (or effective freezing time (t_e) equal to 4 minutes) and 1.35 cm/h (or effective freezing time (t_e) equal to 27 minutes) can be obtained by freezing gilthead seabream fillets in liquefied gases (cryogenic freezers, see sections 1.1.3.3-b and 1.1.3.4) and mechanical freezers (e.g. continuous air-blast freezers, see sections 1.1.3.1-b and 1.1.3.4), respectively;

2. for seafood markets in which the texture of raw thawed fillets is an important quality attribute (e.g. markets of frozen/thawed seafood products), use of cryogenic freezers may be beneficial for freezing gilthead seabream fillets, because they give faster freezing rates than mechanical freezers.

4.2.2. Storage experiments: The bio-chemical, physical and sensory properties of stored (-22°C) frozen fillets of gilthead seabream (*Sparus aurata*)

4.2.2.1. Proximate composition of fresh gilthead seabream fillets.

The mean values of water, crude protein, crude lipid and ash of fresh gilthead seabream samples 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 (Table 4.1.2.1). 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 gilthead seabream fillets between the study by Kyrana *et al.* (1997) and the present one may be related to the differences in samples that were analyzed, i.e. white dorsal 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 and more water than the red muscle (Shewfelt 1981).

4.2.2.2. Changes in protein content of centrifugal tissue fluids (CTFs), α-glucosidase (AG), β-N-acetyl –glucosaminidase (NAG) and β–hydroxy–acyl-coenzyme–A dehydrogenase (HADH) activities of stored (-22°C) frozen gilthead seabream fillets

The protein content in centrifugal tissue fluids from frozen gilthead seabream fillets after 34 and 340 days in cold storage was 1.6 and 1.7 times compared to the values of fresh samples, respectively. Maximum values of protein content in centrifugal tissue fluids from frozen gilthead seabream fillets were found after 266 days in cold storage, which were 1.9 times compared to the respective values of fresh samples (Table 4.1.2.2). In addition, from the 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 t_c value of 18 minutes and immediately thawed increased about 1.2 times compared to the respective values of fresh samples (see sections 4.1.1.2 and 4.2.1.2). These results 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 cold storage, respectively (Table 4.1.2.2). Maximum AG activities in the muscle of frozen gilthead seabream fillets were observed after 183 days of cold 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 -22°C, respectively (Table 4.1.2.2). Also, the activity of the AG enzyme in the frozen gilthead seabream fillets at a t_c value of 18 minutes and immediately thawed increased by about 3.2-fold compared to the activity of the fresh samples (Table 4.1.2.2). These results 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 -22°C. In addition, the changes in the release of the AG enzyme from lysosomes found after 34 days of cold 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 4.1.2.2). This result implies the denaturation of the enzyme during prolonged storage of frozen fillets (Benjakul *et al.*, 2003).

In contrast to AG activities, NAG activities in the stored frozen gilthead seabream fillets showed a small tendency to increase during storage up to 340 days, but significant differences between the stored frozen groups of fillets were not observed (Table 4.1.2.2).

Nilsson and Ekstrand, (1995) showed that storage for 18 months (i.e. almost 540 days) at -18°C and -40°C had not a major significant effect on NAG activities of frozen trout. In contrast, Benjakul *et al.* (2003) showed that AG and NAG activities increased in frozen croaker, lizardfish, threadfin bream and big-eye snapper over storage for 24 weeks (i.e. 168 days) at -18°C, and that the degree of changes was dependent upon the species.

Therefore, the results of the present study and those of Nilsson and Ekstrand (1995) and Benjakul *et al.* (2003) may indicate differences in stability of fish tissues during frozen storage as well as differences in enzyme activities in the organelles among fish species.

Activities of HADH enzyme of frozen groups of fillets were similar to each other regardless of storage time, and on average were 2.5 times the activities of fresh samples (Figure 4.1.2.2). Hoz *et al.* (1993) showed that HADH activities of frozen kuruma prawns stored for one and six months at -18°C were similar to each other. Pavlov *et al.* (1994) showed that HADH activities of frozen squid, mackerel, seabream (*Pagellus centrodontus*), sole and hake were not affected over 6 months (i.e. almost 180 days) of storage at -18°C. Therefore, the results of the present study with respect to the changes in HADH activities in frozen gilthead seabream stored for almost twelve months (i.e. 340 days) at -22°C are in agreement with those by Hoz *et al.* (1993) and Pavlov *et al.* (1994).

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. Also, they imply that the protein content in centrifugal tissue fluids and the marker enzyme AG may be more sensitive indicators in assessing the changes of cell-membrane structures of the stored at -22°C frozen gilthead seabream fillets rather than the marker enzymes NAG and HADH. This suggestion is supported

by the positive and significant correlations which were found in the present study between the storage time and protein weight in centrifugal tissue fluids (r_s=0.576 P<0.01; Table 4.1.2.11-1) and AG activities in stored frozen fillets (r_s=0.489 P<0.01; Table 4.1.2.11-1). These relationships are shown in the Figure 4.2.2.2, below.

Figure 4.2.2.2 Scatter plots and regression lines of protein weight in centrifugal tissue fluids (CTFs) and α-glucosidae (AG) activities versus storage time

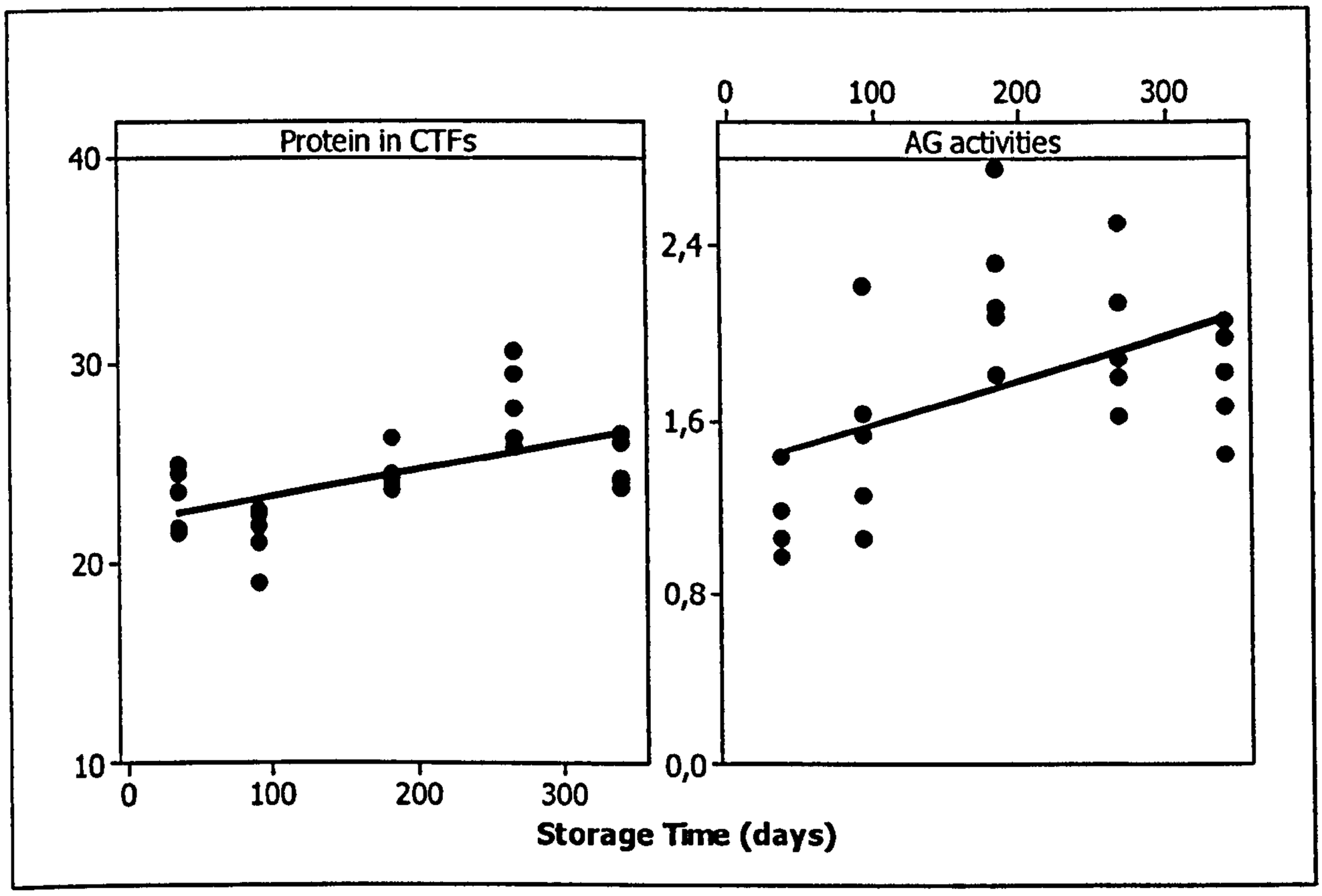


Figure note: The '0' storage time represents the fresh samples.

It was assumed earlier in this Chapter (see section 4.2.1.2) that freezing of seabream fillets at a t_c value of 18 minutes would result in small intra-cellular ice crystals. However, ice re-crystallization in the stored frozen seabream fillets should have occurred at the storage temperature of -22°C (see section 1.1.2). Therefore, formation of inter-cellular ice and accretion of intra-cellular ice during prolonged storage at -22°C, i.e. 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).

4.2.2.3. Changes in water holding parameters of stored (-22°C) frozen gilthead seabream fillets

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 cold storage ($P>0.05$; Table 4.1.2.3). In contrast, centrifugal tissue fluids and total weight losses due to thawing and centrifugation showed a significant increase after 266 days (i.e. 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 4.1.2.3). In addition, total weight losses of frozen gilthead seabream fillets stored for 340 days at -22°C were 87% more than those of fresh samples. 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 -22°C 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 the experiments of the effects of freezing times on the quality parameters of gilthead seabream fillets, the total weight losses in the frozen fillets at a t_c value of 18 minutes and immediately thawed were 46 % more than those of fresh samples. Therefore, the changes in the water holding capacity 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 samples 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 and fishery products. Total weight losses

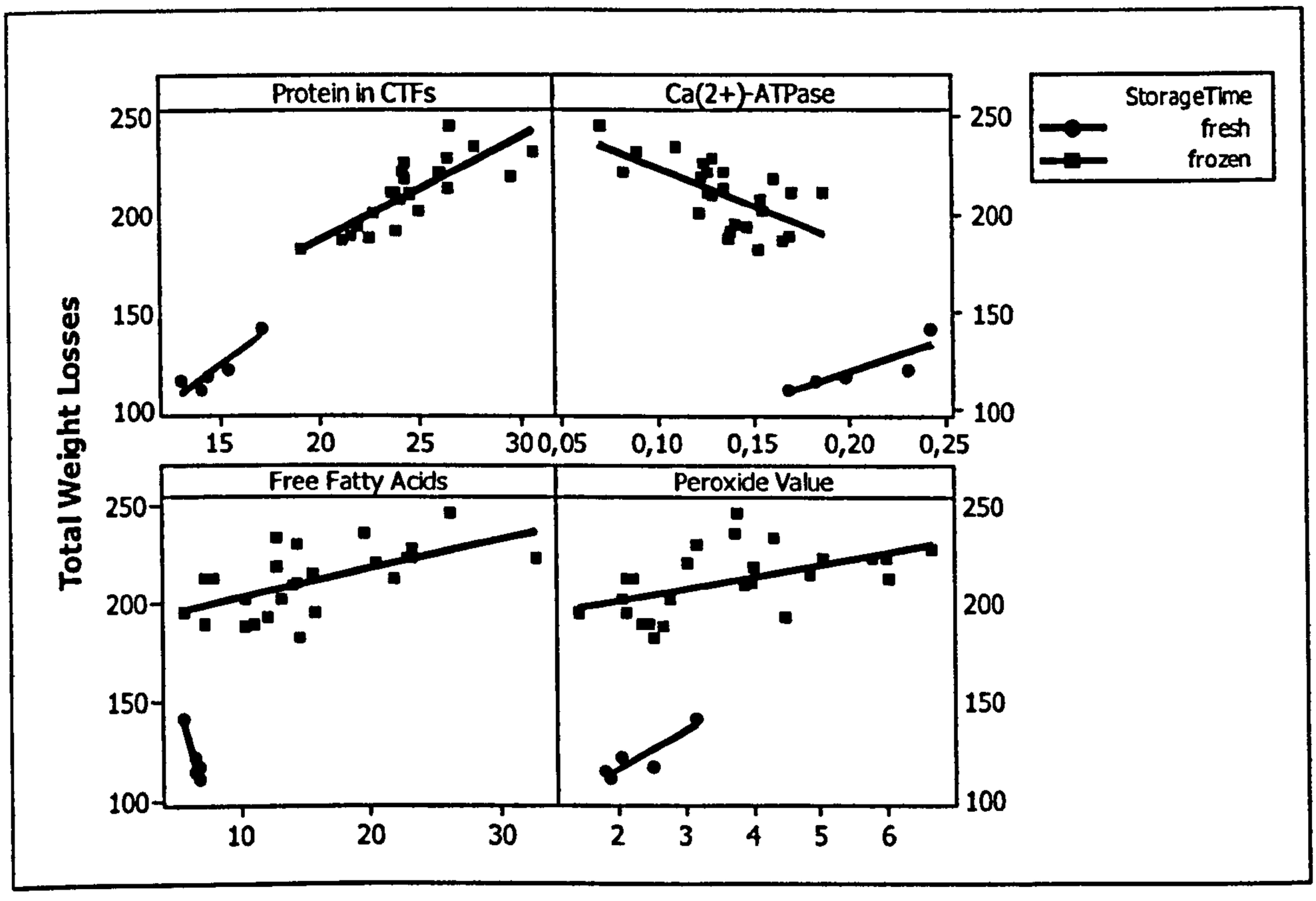
due to thawing and centrifugation increased, while the water holding capacity decreased, in Patagonian hake fillets and mince stored frozen at -20°C and -30°C for 10 months (i.e. almost 300 days; Ciarlo *et al.*, 1985). Martí de Castro *et al.* (1996) showed that the water holding capacity decreased in sardine gels stored frozen at -18°C and -12°C for 180 days (i.e. almost 6 months). 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 (i.e. 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 were 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 (i.e. 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 (i.e. almost 300 days) at -20°C. Also, the expressible fluids values increased by about 7-fold in frozen salmon fillets stored for 10 months compared to those of fresh samples. Dorado-Rodelo *et al.* (In Press) showed that the water holding capacity of frozen spotted rose snapper fillets decreased after 120 days (i.e. almost 4 months) of storage at -20°C. 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 (i.e. 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.

As shown in Table 4.1.2.11-1 and Figure 4.2.2.3, 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)

Figure 4.2.2.3 Scatter plots and regression lines of total weight losses versus protein content in centrifugal tissue fluids (CTFs), Ca^{2+}ATPase activities, free fatty acids and peroxide value



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, i.e. 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 (see sections 1.3.2.2 and 1.3.4) 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.

4.2.2.4. Changes in salt soluble proteins and extractability of actomyosin from stored (-22°C) frozen gilthead seabream fillets

The solubility of proteins in salt solutions from the frozen gilthead seabream fillets was initially high and remained almost stable until the 183 days of storage; after 266 days (i.e. 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 4.1.2.4-1). Several investigations have reported a reduction in solubility of proteins in saline solutions during storage of frozen fish. Badii and Howell (2001, 2002) recorded a significant decrease in protein solubility of frozen cod and haddock fillets with time of storage at -10°C and -30°C for 65 weeks (i.e. almost 14 months). Suarez *et al.* (2002) showed that salt soluble proteins from frozen sardines were decreased during storage at -18°C for six months. Similarly, Saeed and Howell (2002) showed a decrease in salt soluble proteins of frozen Atlantic mackerel stored for 12 months at -20°C and -30°C. Leelapongwattana *et al.* (2005) found a continuous decrease in salt soluble proteins extracted from whole, and fillets of, frozen lizardfish stored at -20°C during 24 weeks (i.e. 6 months). Ganesh *et al.* (2006) showed that salt soluble proteins extracted from frozen common carp decreased during storage at -18°C for 180 days (i.e. almost 6 months). Paredi *et al.* (2006) investigating the effects of the length of time of storage on the myofibrillar proteins of frozen male and female squids found a continuous decrease in salt soluble proteins during storage at -30°C for 9 months. Benjakul *et al.* (2005) recorded a continuous decrease in solubility of proteins of stored frozen tropical fish for 24 weeks (i.e. 6

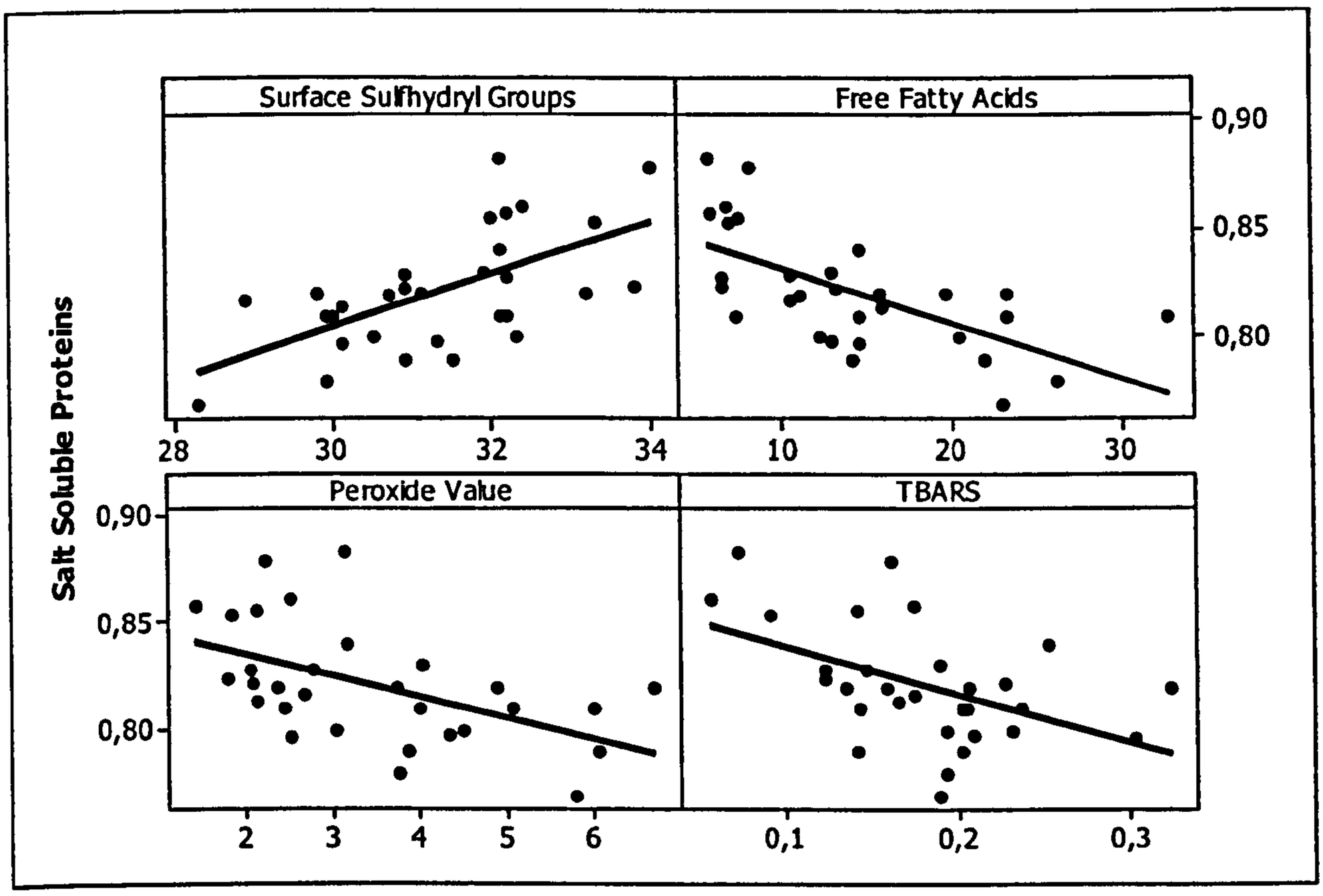
months) at -18°C. Huidobro and Tejada (2004) showed that the salt soluble proteins from frozen whole and gutted gilthead seabreams were significantly reduced after 349 days of storage at -20°C. The results of these investigations and those of the present study may imply that prolonged storage of the 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 seafoods.

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 (see section 1.3.2.2).

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 (Tejada *et al.*, 1996; 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, 4.2.2.5), 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; Table 4.1.2.11-1). This relationship is presented in the Figure 4.2.2.4, below.

Figure 4.2.2.4 Scatter plots and regression lines of salt soluble proteins versus surface sulfhydryl groups, free fatty acids, peroxide value and thiobarbituric reactive substances (TBARS).



As shown in Figure 4.2.2.4, the changes in salt soluble proteins from the stored frozen fillets were to some extent coincidental with the changes in free fatty acids (r_s = -0.686, P< 0.01; Table 4.1.2.11-1), peroxide values (r_s = -0.518, P< 0.01; Table 4.1.2.11-1) and TBARS (r_s = -0.479, P< 0.01; Table 4.1.2.11-1) of the frozen fillets. Several 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 -20° (Verma *et al.*, 1995), frozen sardines stored for 12 weeks (i.e. almost 84 days) at -20°C (Sarma *et al.*, 2000), frozen minces of carp stored for 180 days at -18°C (Siddaiah *et al.*, 2001), frozen Atlantic mackerel stored for 12 months (i.e. 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. Possible mechanisms of protein aggregation by free fatty acids and lipid oxidation products are discussed in the section 1.3.2.2.

Actomyosin extracted from the stored frozen gilthead seabream fillets remained almost stable through the storage period (Figure 4.1.2.4-2). After 340 days of storage, the weight of actomyosin extracted from the frozen fillets (i.e. 0.125 ± 0.009 g g^{-1} of gilthead seabream tissue) was similar to that extracted from the fresh fillets (0.117 ± 0.003 g g^{-1} of gilthead seabream tissue). Similar results have been recorded for frozen whole gilthead seabream stored for one year at -20°C (Tejada *et al.*, 2003). It can, therefore, be concluded that long term storage of gilthead seabream fillets at -22°C hardly affected the extractability of actomyosin, but it did cause a small and significant decrease in the salt soluble proteins.

4.2.2.5. Changes in total and surface (reactive) sulfhydryl (SH) groups in actomyosin extracts from stored (-22°C) frozen gilthead seabream fillets

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 4.1.2.5). Suarez *et al.* (2002) found a significant decrease in total sulfhydryl groups from frozen sardines stored for 4

months (i.e. almost 120 days) at -20°C followed by an increase during the next two months (i.e. almost 60 days) of storage. In contrast to these findings, Jiang *et al.* (1988a) indicated that total and surface (reactive) sulfhydryl groups of frozen milkfish actomyosin in 0.6 M KCl decreased during storage for 10 weeks (i.e. almost 70 days) at -20°C. 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 (i.e. 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, i.e. almost one year in the present study as opposed to maximum half year in the studies by Jiang *et al.* (1988a), 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 0.6M 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 decrease in salt soluble proteins and a further reduction in Ca^{2+}-ATPase activities were observed in extracts from the stored frozen gilthead seabream fillets (see sections 4.2.2.4 and 4.2.2.6). 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 (i.e. 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 (i.e.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.

4.2.2.6. Changes in ATPase activities of actomyosin extracts from stored (-22°C) frozen gilthead seabream fillets

Ca^{2+}-ATPase, Mg^{2+}-Ca^{2+}-ATPase and Mg^{2+}-ATPase of actomyosin extracts from gilthead seabream fillets decreased during the 340 days of frozen storage ($P<0.01$; Table 4.1.2.6). Changes in Ca^{2+}-ATPase activities have been recorded in a number of fish and fishery products. This is the case with frozen stored mackerel and amberfish (Jiang *et al.* 1985), Alaska Pollock (Scott *et al.* 1988), myctophid species (Seo *et al.* 1997), Atlantic mackerel (Saeed and Howell, 2002), croacker, lizardfish, threadfish and bigeye snapper (Benjakul *et al.*, 2003), and these are discussed in section 3.2.2.3 of the present study. Also, Ca^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities decreased in frozen actomyosin from milk fish stored for 8 weeks (i.e. 56 days) at -20°C and -35°C (Jiang *et al.*, 1988b). Decreases in Ca^{2+}-ATPase and Mg^{2+}-ATPase activities were observed in frozen trout actomyosin stored at -20°C for 8 weeks (i.e. 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 (i.e. 6 months). Jarsa *et al.* (2006) showed that Ca^{2+}-ATPase activities decreased in frozen carp stored for 24 months (i.e. almost 720 days) at -20°C 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+}-ATPase and Mg^{2+}-ATPase activities extracted from frozen male and female squids during storage for 9

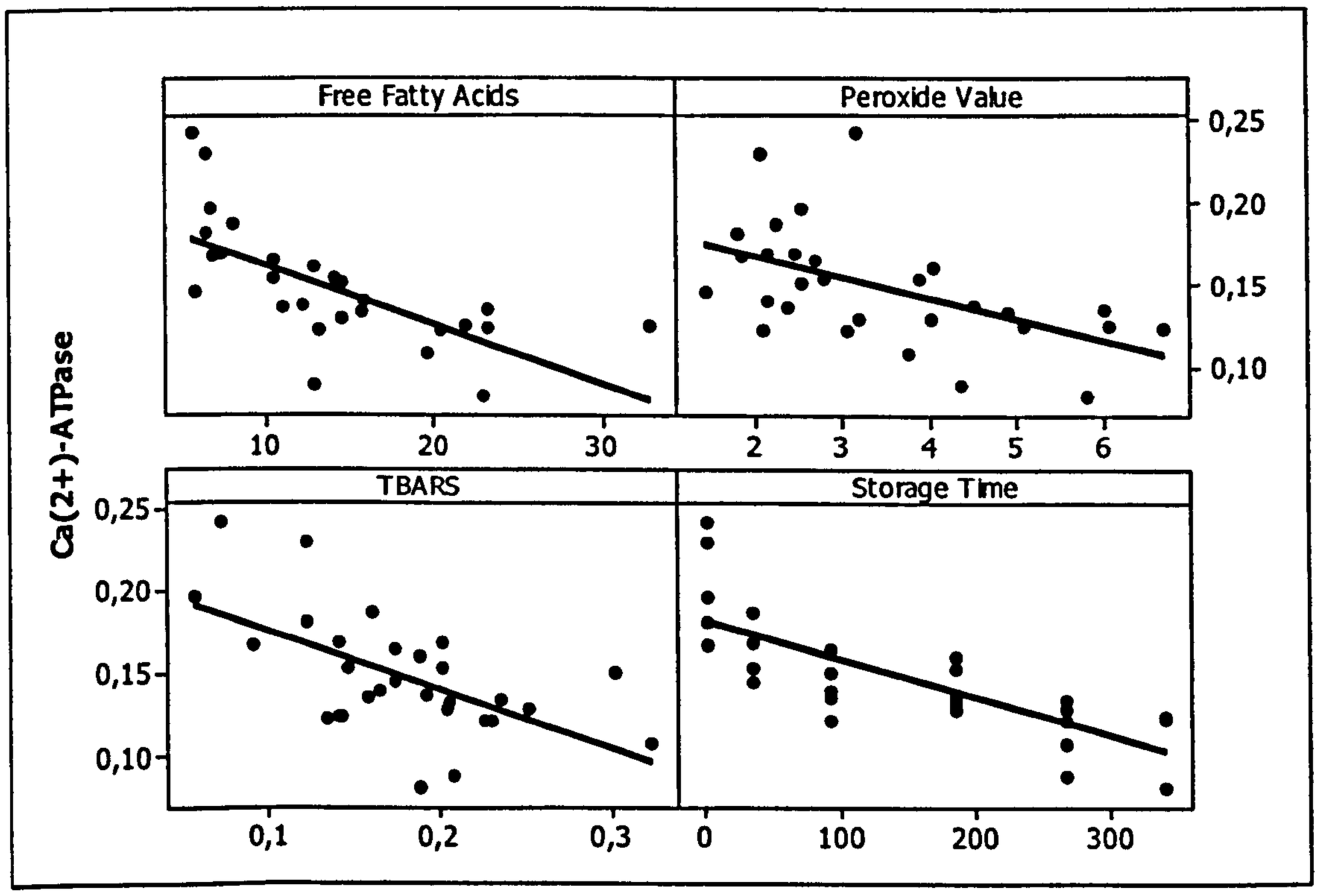
months (i.e. almost 270 days) at -30°C. Pastor *et al.* (1999) investigating the effects of pre-freezing treatments on quality parameters of the frozen gilthead seabream found no changes in Ca^{2+}-ATPase activities from gutted gilthead seabreams stored at -20°C for 10 months (i.e. almost 300 days), but a continuous decrease in Ca^{2+}-ATPase activities was observed in the un-gutted stored frozen fish. 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 *etal.*, 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 (see sections 1.3.2.2-a and 4.2.2.5). Furthermore, the decreases in ATPase activities of 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 (Table 4.1.2.11-1). 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; Benjakul *et al.*, 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 (see section 1.3.2.2-b). 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 (Table 4.1.2.11-1 and Figure 4.2.2.6, below.).

Figure 4.2.2.6 Scatter plots and regression lines of Ca^{2+}-ATPase activities versus free fatty acids, peroxide value, thiobarbituric reactive substances (TBARS) and storage time



Ca^{2+}-ATPase activities in actomyosin extracts from stored frozen fillets showed a good negative correlation with storage time (r_s= -0.871, P<0.01; Table 4.1.2.11-1 and Figure 4.2.2.6) indicating that Ca^{2+}-ATPase activities may be a reliable index for assessing the quality loss of stored frozen gilthead seabream fillets.

No significant changes in Mg^{2+}-EGTA-ATPase activities in actomyosin extracts from fresh and stored frozen fillets were observed (P>0.05; Table

4.1.2.6). 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 (i.e. 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, Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities in actomyosin extracts, but it hardly affected the tropomyosin-troponin complex.

4.2.2.7. Changes in Ca^{2+}sensitivity of actomyosin from stored (-22°C) frozen gilthead seabream fillets

Ca^{2+}sensitivities of actomyosin from stored frozen gilthead seabream fillets were similar and significantly lower only than those of fresh fillets (Figure 4.1.2.7). The changes in Ca^{2+}sensitivity were in accordance with the changes in Mg^{2+}-Ca^{2+}-ATPase activities (see section 4.2.2.6). Losses in Ca^{2+}sensitivity of myofibrillar proteins of stored frozen fish have been attributed to denaturation of troponin-tropomyosin complex (Benjakul *et al.*, 2003) and/ or modification of actin-myosin interaction caused by oxidation of sulfhydryl groups in myosin molecules (Jiang *et al.*, 1988b). From the results of the present study, changes in Ca^{2+}sensitivity of actomyosin from fresh and stored frozen fillets could be due to modification of actin-myosin interaction, as indicated by the decrease in

Mg^{2+}-Ca^{2+}-ATPase activities. This modification could have been caused by the oxidation of sulfhydryls in myosin molecules, as indicated by the decrease in total and surface (reactive) sulfydryls of actomyosin within the first month of storage of frozen fillets (see section 4.2.2.5).

4.2.2.8. Changes in lipids extracted from stored (-22°C) frozen gilthead seabream fillets

a) Total lipid

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 4.1.2.8). By using a methanol-chloroform method to extract lipids from pre-spawned muscle of hake, Roldan *et al.* (2005) 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 4.1.2.8) 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. During frozen storage of gilthead seabream fillets, hydrolysis and oxidation of lipids occurred (see sections 4.2.2.8-b and c). The products of degradation of lipids might have interacted with protein molecules of stored frozen fillets, promoting denaturation of proteins (see sections 4.2.2.4 and 4.2.2.6). 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.

b) Lipid hydrolysis

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 4.1.2.8 and Figure 4.2.2.8-1, 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; Table 4.1.2.11-2).

Figure 4.2.2.8-2 Scatter plot and regression line of free fatty acids versus storage time

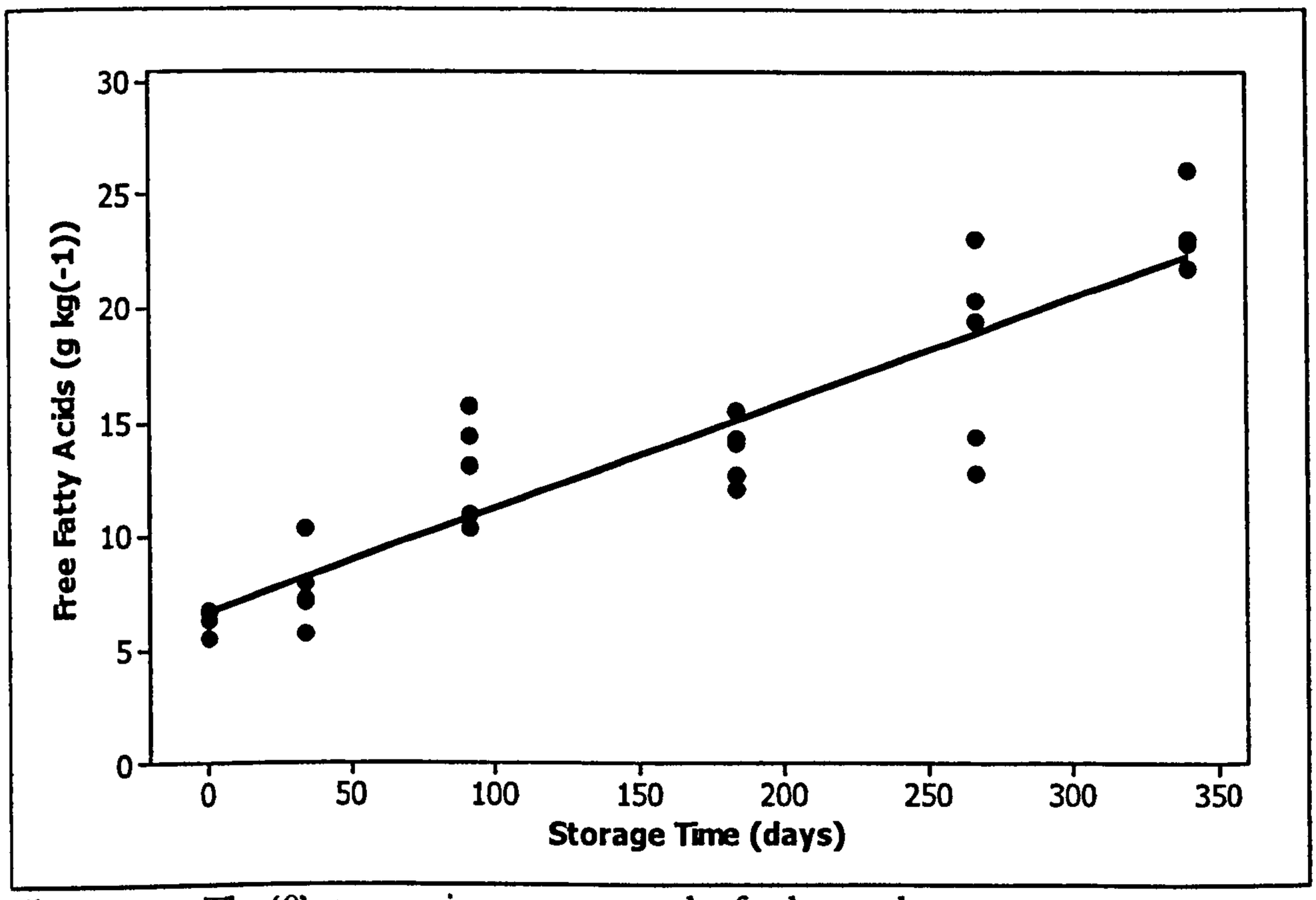


Figure note: The '0' storage time represents the fresh samples.

Similar observations have been recorded for other species. This is the

case for whole hake, horse mackerel and mackerel stored frozen for 12 months (i.e. almost 360 days) at -20°C (Koning and Mol, 1991; Aubourg *et al.*, 1999; 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 (i.e. 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.

c) Lipid oxidation

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 4.1.2.8). As a consequence, a fairly good correlation of peroxide values with storage time was obtained (r_s=0.817, P<0.01; Table 4.1.2.11-1; Figure 4.2.2.8-2). 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 (i.e. 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 e.g. 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).

Figure 4.2.2.8-3 Scatter plots and regression lines of peroxide value and TBARS versus storage time

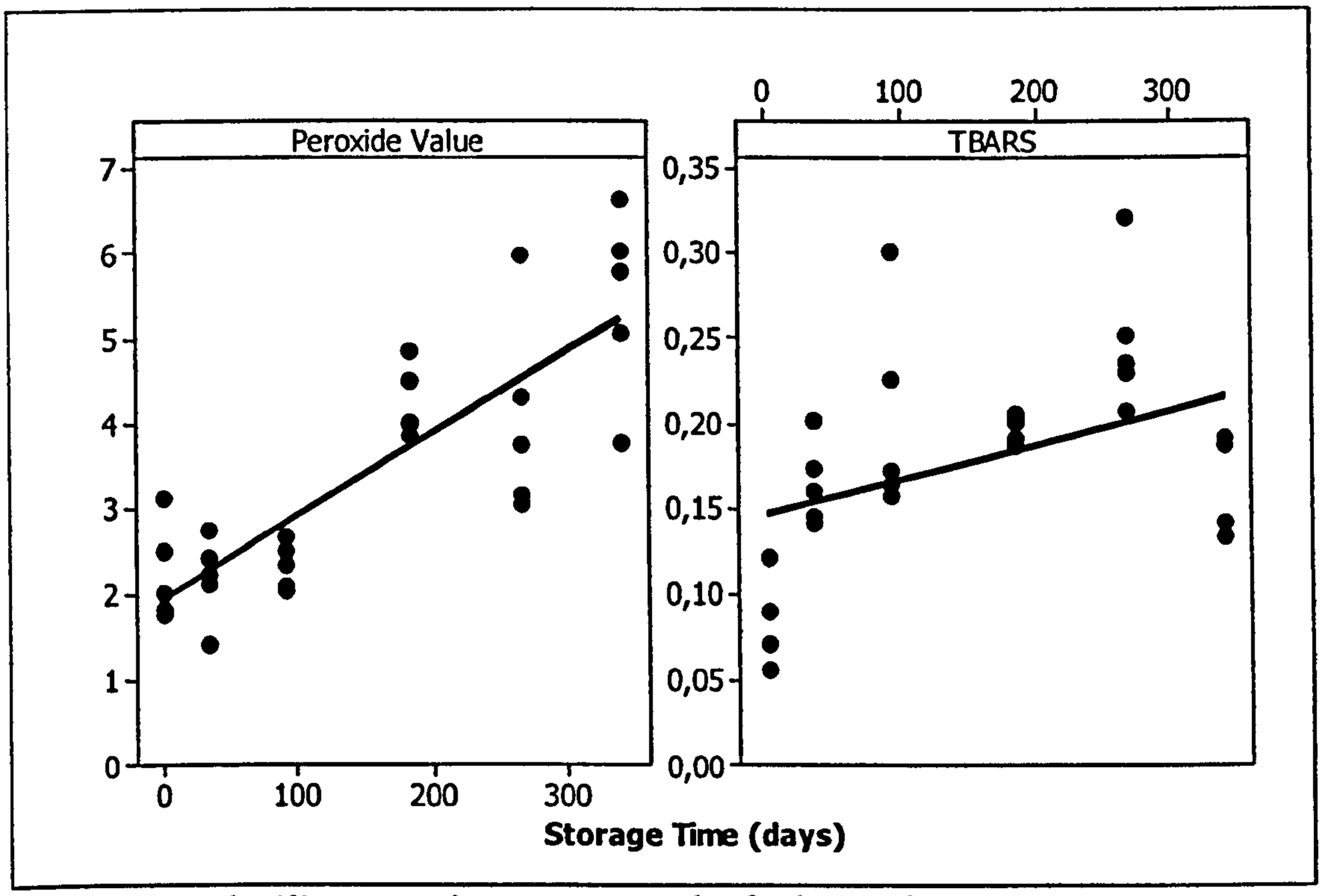


Figure note: The '0' storage time represents the fresh samples.

Considering the changes in thiobarbituric reactive substances (TBARS) during storage of frozen gilthead seabream fillets, a progressive increase until 266 days was observed (Table 4.2.2.8.). 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 (see section 1.4.3.3). 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; Table 4.1.2.11-1; Figure 4.2.2.8-2, above). 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).

Therefore, from the indices, used to monitor the degradation of lipids, free fatty acid and peroxide values may be reliable indicators for assessing the quality loss in stored frozen gilthead seabream. Correlations of these parameters with sensory scores are discussed in section 4.2.2.11.

4.2.2.9. Changes in texture of stored (-22°C) frozen gilthead seabream fillets as measured by the texture analyzing system

The results of the present study show that peak shear forces (i.e. 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 4.1.2.9-1 and 2). These results suggest that storage of fillets for up to 91 days at -22°C caused softening (i.e. decrease in firmness) and tenderness of raw fillets. Sigurgisladottir *et al.* (2000) showed that the firmness (i.e. 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.* (2003a) 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, freezing and frozen storage caused changes in the muscle integrity and denaturation and aggregation of myofibrillar proteins of gilthead seabream fillets (see sections 4.2.2.2, 4.2.2.3 and 4.2.2.6). However, 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 (see section 4.2.2.2). In addition, the changes in the myofibrillar proteins of the stored frozen fillets were higher at the end of the storage period, as indicated by the changes in the values of salt soluble proteins (see section 4.2.2.4). It is,

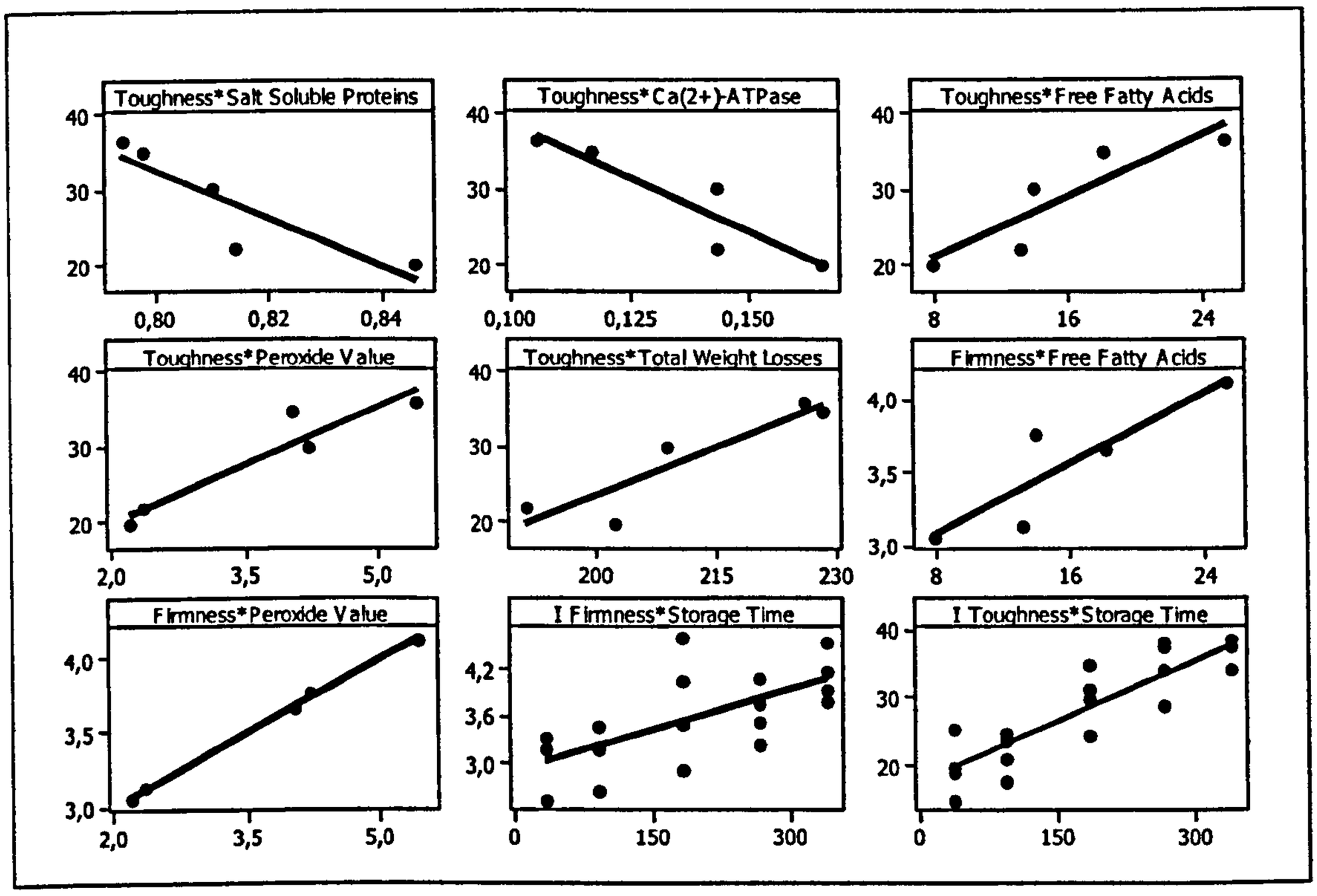
therefore, presumed that at the beginning of the storage period the tissue-softening factors overcame the tissue-hardening factors with concomitant softening of the muscle of raw stored frozen fillets with respect to the fresh fillets.

From the results of the present study, there was a tendency of the peak shear forces (i.e. firmness values) and toughness values of the stored frozen fillets to increase with the storage time (Figures 4.1.2.9-1 and 2). Several other studies have recorded changes in texture as measured by different texture analyzing systems of stored frozen seafoods. 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 (i.e. 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.* (In Press) 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 (i.e. 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.

As shown in Table 4.1.2.11-2 and Figure 4.2.2.9, negative linear correlations were found between the mean values of toughness of the stored frozen gilthead seabream fillets and those of salt soluble proteins (r_s=-0.883 P<0.05) and Ca^{2+}-ATPase activities (r_s=-0.918, P<0.05). In contrast, the mean values of toughness were correlated positively with those of free fatty acids (r_s=0.888, P<0.05) and peroxide values (r_s=0.943

$P<0.01$). In addition, mean values of firmness were correlated positively with those of free fatty (r_s=0.886, $P<0.05$) and peroxide values (r_s= 0.999 $P<0.01$). These results suggest that the development of toughness and hardness (i.e. increase in firmness) in raw stored frozen fillets was, in part at least, due to denaturation and aggregation of myofibrillar proteins. 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 suggested in section 4.2.2.6.

Figure 4.2.2.9 Scatter plots of instrumental toughness and firmness with quality parameters of stored frozen gilthead seabream fillets and storage time.



As shown in the Figure 4.2.2.9 and in Table 4.1.2.11-2, a positive linear relationship between instrumental toughness and total weight losses of stored frozen fillets was found (r_s=0.914, $P<0.05$). Ozbay *et al.* (2006) showed, also, that expressible fluids from frozen fillets of salmon stored at -20°C for 10 months were positively correlated with the texture of cooked samples as measured by a Kramer-shear cell.

Altogether these results, therefore, suggest that the tenderness of stored

frozen fillets was related to the state of the myofibrillar proteins and changes in water holding capacity of gilthead seabream muscles (Shenouda 1980; Mackie 1993).

Toughness values obtained from stored frozen gilthead seabream fillets were correlated linearly with the storage time (r_s=0.881, P<0.01; Table 4.1.2.11-2; Figure 4.2.2.9). This result may imply that toughness as measured by the texture analyzing system and the Warner-Braltzer knife may be a reliable index in predicting changes in texture of stored frozen gilthead seabream fillets.

Therefore, the length of time of storage at -22°C affected the texture of gilthead seabream fillets as measured by the texture analyzing system used in the present study.

4.2.2.10. Changes in sensory attributes of stored (-22°C) frozen gilthead seabream fillets

From the results of the present study, stored frozen fillets were chewier, i.e. more tough, than the fresh samples (Figure 4.1.2.10-1) in agreement with the findings of Carbonell *et al.* (2003a) for whole gilthead seabream stored frozen for two months at -20°C. This result is the opposite of the findings of toughness measurements by the texture analyzing system (see section 4.2.2.9). This may be due to the fact that the mechanical response of the fresh tissue to freezing might be very different from the effect of cooking on frozen tissue as detected in the mouth, as Carbonell *et al.* (2003a) reports the whole frozen gilthead seabream. According to the ratings of the sensory panel, toughness scores of samples stored frozen from 91 to 340 days were similar and significantly higher than those of samples stored frozen for 34 days (Figure 4.1.2.10-1). Similar results are reported for frozen fillets of horse mackerel and Mediterranean hake stored for 360 days at -18°C (Simeonidou *et al.*, 1997). Thus, the length of time of storage at -22°C affected the tenderness of frozen gilthead

seabream fillets (r_s= 0.705; P<0.01; Table 4.1.2.11-1), and this change was consistent with Mg^{2+}-Ca^{2+}-ATPase activities in actomyosin extracts from stored frozen fillets (r_s=-0.703 ,P<0.01; Table 4.1.2.11-1).

The sensory scores of acceptability of taste of stored frozen fillets reduced gradually with increased time of storage (Figure 4.1.2.10-2). Thus, a good linear correlation of sensory scores of acceptability of taste with storage time was observed (R^2= 0.946, P<0.0001; Table 4.1.2.11-2). This relationship and the linear equation between the mentioned parameters are presented in the Figure 4.2.2.10, below

Figure 4.2.2.10-1 Regression of scores of taste from stored frozen gilthead seabream fillets on storage time

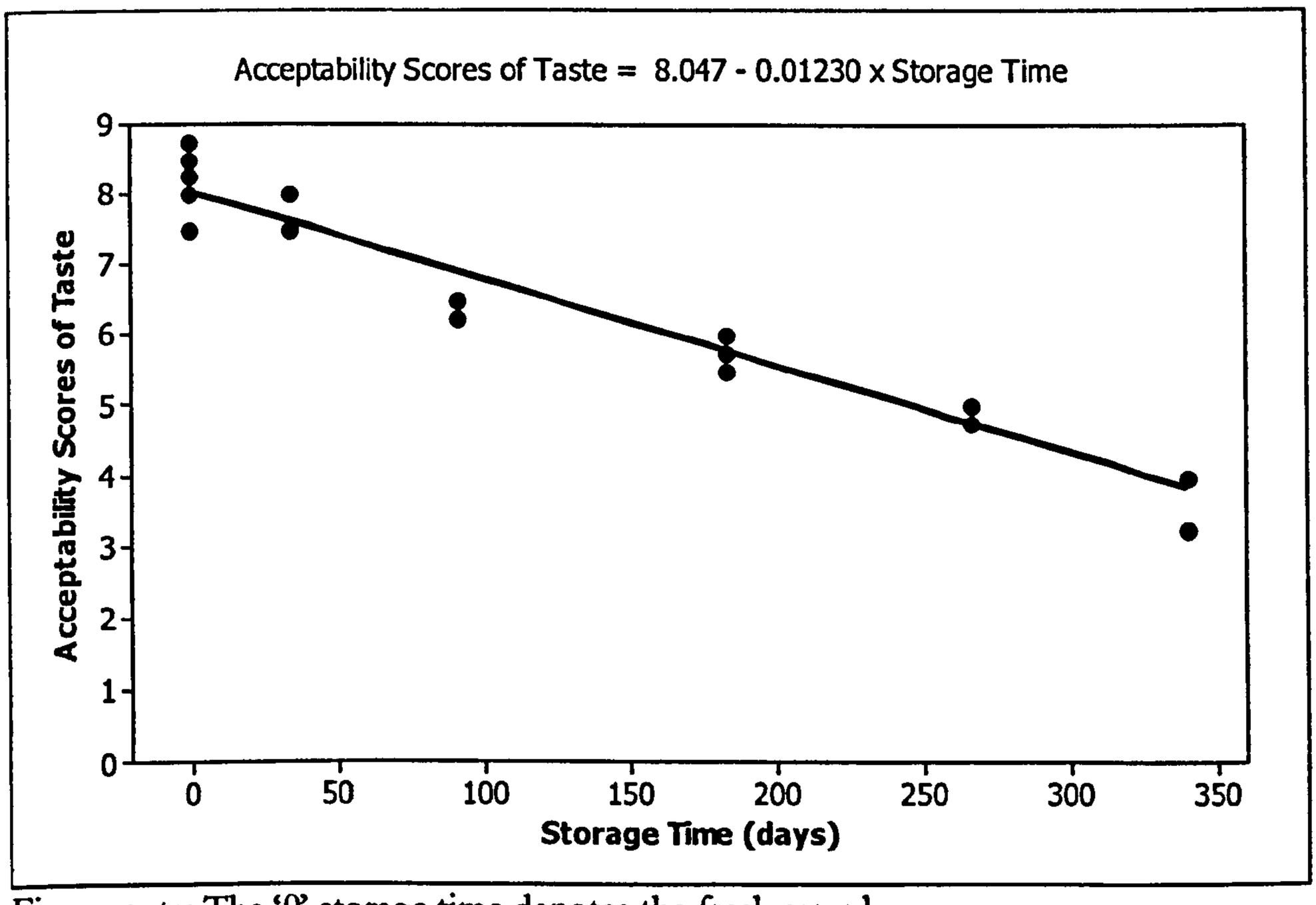


Figure note: The '0' storage time denotes the fresh samples.

Based on the linear equation between the taste scores and storage time and by using 6 (i.e. 'like slightly') as a rejection threshold for taste, it is assessed that the frozen fillets would be acceptable up to 166 days (circa 5 to 6 months) of storage. This storage time is higher than that reported for frozen sardines stored at -18°C (i.e. 4 months; Suarez *et al.*, 2002) and frozen mackerel fillets stored at -20°C (i.e. 3 months; Aubourg *et al.*,

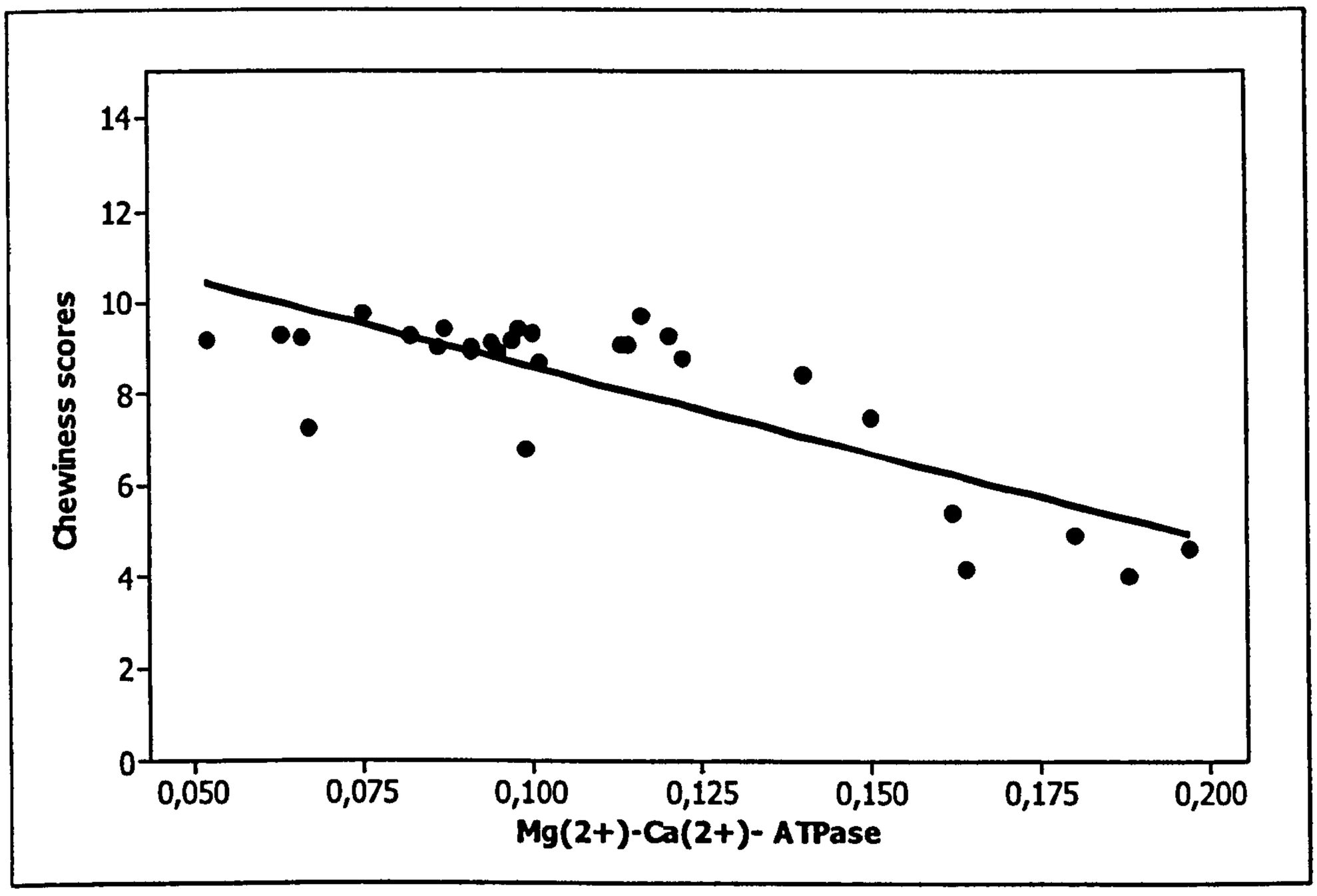
2005), but less than that reported for lean fish (see Table 1.1.4). Furthermore, it is suggested that the high quality life (HQL; see section 1.2) of stored frozen gilthead seabream fillets would be between 34 and 91 days, since significant differences in taste scores between the fresh and stored frozen fillets were detected after 91 of storage (see section 4.1.2.10).

Huidobro and Tejada (2004) investigating the effects of pre-freezing treatments on the sensory attributes of frozen whole gilthead seabream, found a small increase in sensory texture (i.e. development of toughness) after 349 days of storage at -20°C. However, flavour scores of cooked fish changed profoundly during storage, and by day 268 the fish were close to rejection. The results, therefore, of the present study are in basic agreement with those by Huidobro and Tejada (2004), in that the main sensory changes in the stored frozen gilthead fillets were in taste rather than in texture (i.e. development in toughness).

4.2.2.11. Correlations between parameters studied with sensory evaluations

From the scatter plots and the Spearman's correlation coefficients between the chewiness scores and the quality parameters of stored frozen fillets, the best linear correlation was found between the sensory chewiness scores and Mg^{2+}- Ca^{2+}-ATPase activities (Figure 4.1.2.11-1 and Table 4.1.2.11-1). This is shown in the Figure 4.2.2.11-1, below.

Figure 4.2.2.11-1 Scatter plot of chewiness scores and Ma^{2+}-Ca^{2+}-ATPas activities of stored frozen gilthead seabream fillets



Several other studies have shown significant correlations between parameters related to changes in myofibrillar proteins and sensory texture, and these are discussed in the section 3.2.2.7 of the present study.

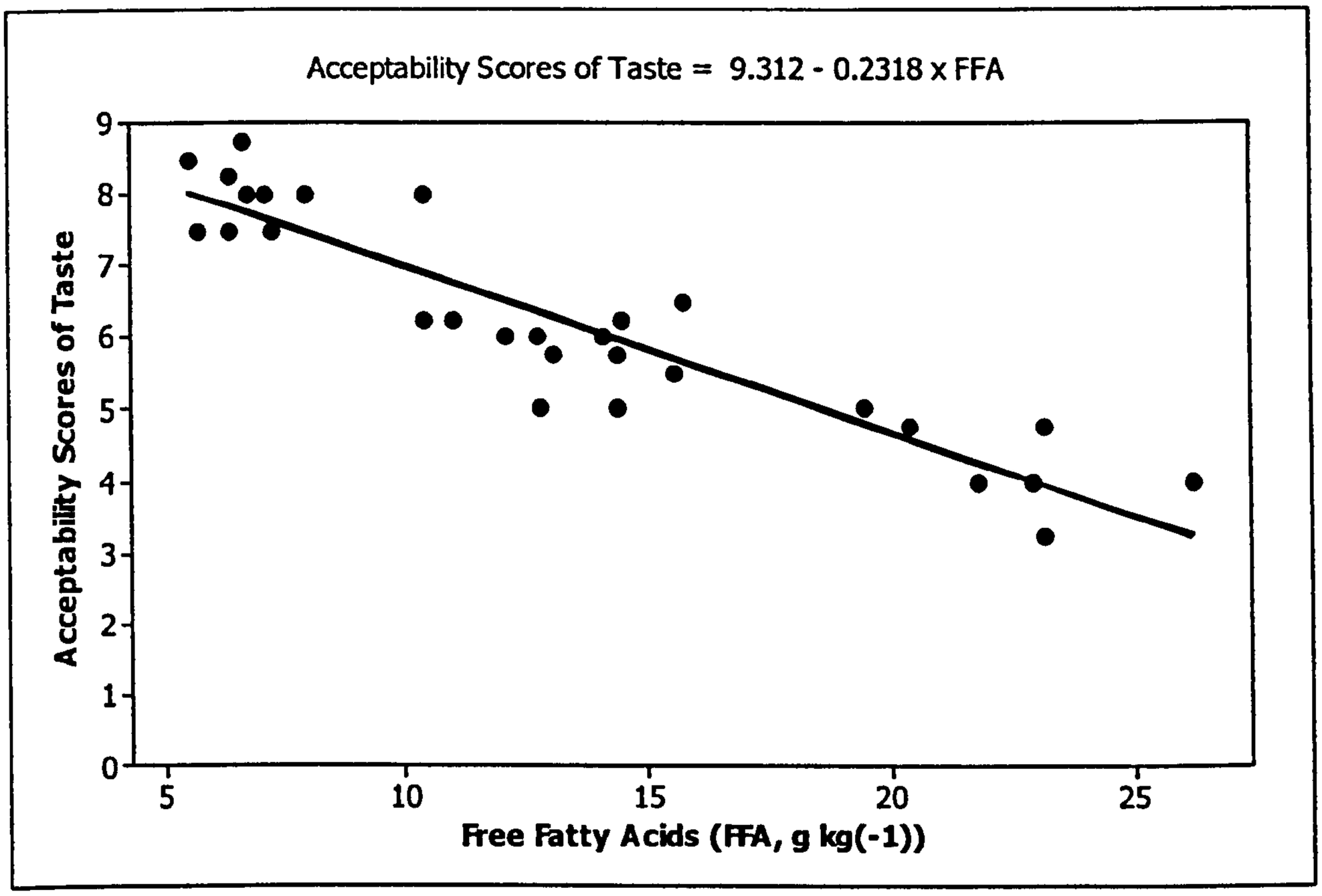
The results of the present study show a good linear correlation between the acceptability of taste and the content of free fatty acids in the lipids extracted from the stored frozen gilthead seabream fillets:

$$\text{Scores of Taste} = 9.312 - 0.2318 \times \text{FFA}$$

(R^2=0. 847, T=-12.21, P< 0.0001; Table 4.1.2.11-2)

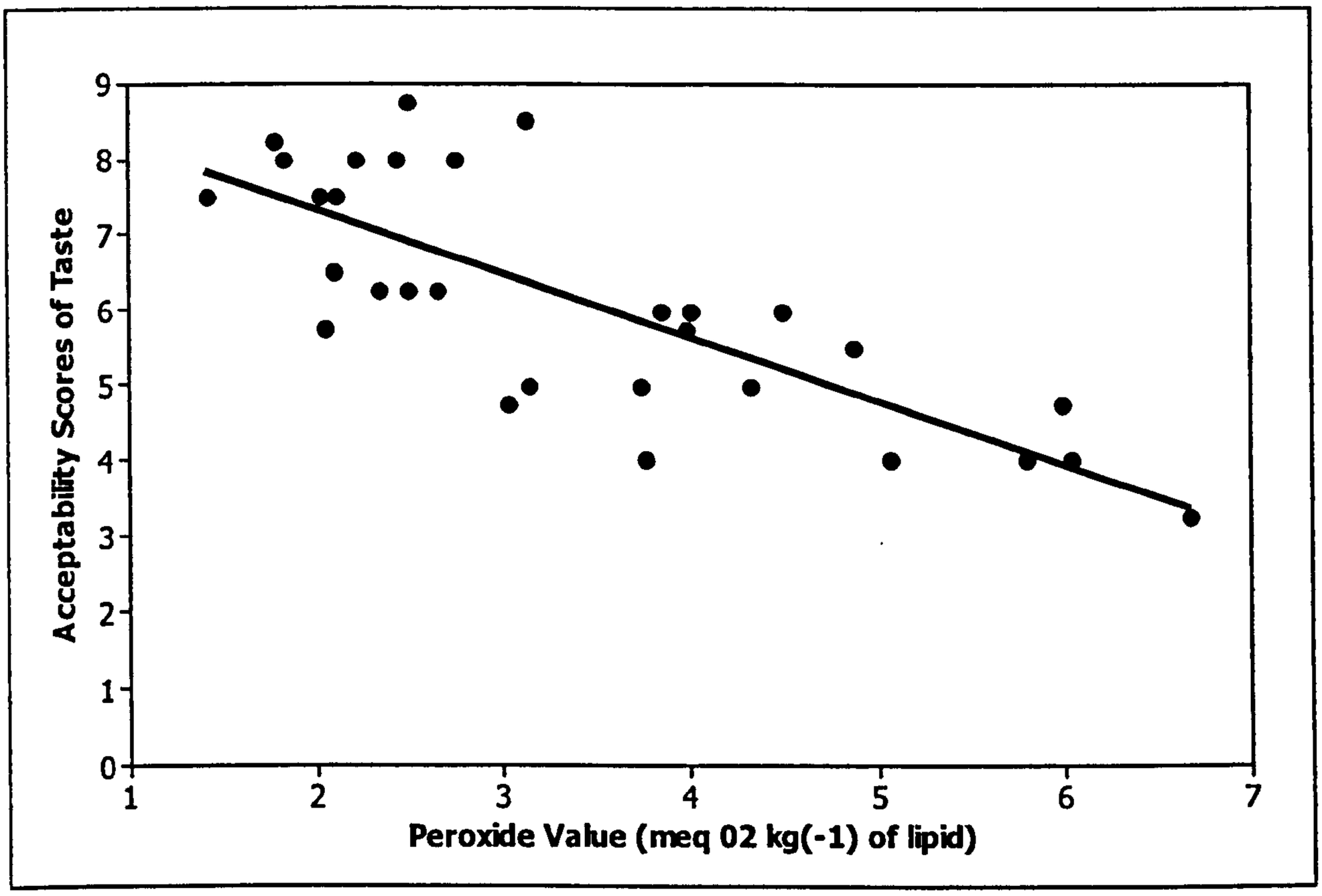
The linear regression line of scores of taste versus free fatty acids from the stored frozen gilthead seabream fillets is presented in the Figure 4.2.2.11-2, below.

Figure 4.2.2.11-2 Regression of scores of taste on free fatty acids from stored frozen gilthead seabream fillets.



Also, the sensory scores of taste were significantly correlated with peroxide values in lipids extracted from gilthead seabream fillets. However, the Spearman's coefficient of these two parameters implies a rather poor linear relationship (r_s=-0.757, P<0.05; Table 4.1.2.11-1). The relationship between the acceptability scores of taste and peroxide values of stored frozen fillets is presented in the Figure 4.2.2.11-3, below.

Figure 4.2.2.11-3 Scatter plot and regression line of acceptability scores of taste and peroxide values of lipids from stored frozen gilthead seabream fillets.



These results are in agreement with the findings for frozen hake (Koning and Mol, 1991), sardine mince (Verma *et al.*, 1995), silver carp mince (Siddaiah *et al.*, 2001) and mackerel (Stodolnik *et al.*, 2005), and imply that the total content of free fatty acids in lipids from stored frozen gilthead seabream fillets may be a reliable index to assess quality loss in frozen gilthead seabream fillets.

4.2.2.12. Prediction of frozen storage time of gilthead seabream fillets by non-sensory methods

Regression models with storage time as dependent variable and some non-sensory parameters as independent variables have been proposed as methods that can be used by frozen seafood industries to evaluate the quality of their batches of frozen seafoods. Evaluation of the quality of frozen batches of seafoods helps industries with the management of their stocks (e.g. control of sales) and provides information that may concern their customers (e.g. classification of frozen seafoods in quality

categories) (Herrero and Careche, 2006).

In order to explore whether storage time of frozen gilthead seabream fillets could be predicted by non sensory methods, stepwise multiple linear regression analyses were performed with storage time as dependent variable and the chemical and bio-chemical parameters as independent parameters. In the mentioned analyses data of individual fillets were used. The results suggest that the storage time of frozen fillets can be predicted by linear equations with one up to four parameters, i.e. free fatty acids (FFAs), peroxide values (PVs), Ca^{2+}-ATPase activities (CAA) and protein content in centrifugal tissue fluids (PrCTF), as follows:

Predicted storage time (PST, days) = -44.16+14.6 x FFAs (1)

PST (days) = -89.64+9.4 x FFAs+34.1xPVs (2)

PST (days) = 126.93+6.2 x FFAs+33.4 x PVs-1223xCAA (3)

PST (days)=-88.33+6.8 x FFAs+29.9 x PVs-832 xCAA+6.8 x PrCTF (4)

The statistics of the mentioned equations from individual fish and mean values from each storage period are shown in Tables 4.2.2.12-1 and 2, respectively.

Table 4.2.2.12-1 Statistics of the regression models of storage time versus chemical and bio-chemical parameters of stored frozen gilthead seabream fillets. Data from individual fish

Equation	*1*	*2*	*3*	*4*
Constant	*-46.16*	*-89.64*	*126.93*	*-88.33*
Free Fatty Acids	**14.6**	**9.4**	**6.2**	**6.8**
T-Value	8.69	5.37	4.28	5.27
P-Value	0.000	0.000	0.000	0.000
Peroxide Value		**34.1**	**33.4**	**29.9**
T-Value		4.29	5.82	5.75
P-Value		0.000	0.000	0.000
Ca(2+)-ATPase			**-1223**	**-832**
T-Value			-4.38	-2.91
P-Value			0.000	0.009
Protein in CTFs				**6.8**
T-Value				2.62
P-Value				0.017
S	54.1	40.0	28.9	25.3
R^2	78.22	88.66	94.36	95.92
R^2(adj)	77.19	87.53	93.46	95.01
R^2 (predicted)	72.26	83.81	92.13	93.69

Table notes: Numbers in italic and bold forms are the constants and the coefficients of the linear regressions, respectively. S values are the typical error of estimations in days.

Linear regression models, which show small estimation errors, high predictive abilities and use simple methods as independent parameters may be the models of choice for the prediction of storage time of frozen fish (Herrero and Careche, 2006).

As the results of the present study show, prediction of storage time of frozen gilthead seabream fillets by equations that combine one or two measurements techniques (i.e. free fatty acids and peroxide values; equations 1 and 2) show low predictive abilities (72.26% and 83.81 %; Table 4.1.2.112-1) and high estimation errors (54 and 40 days; equations 1 and 2 in Table 4.1.2.112-1). By contrast, prediction of storage time of frozen gilthead seabream fillets by equations that combine three or four measurement techniques (i.e. free fatty acids, peroxide values, Ca^{2+}-ATPase activities and protein content in CTFs; equations 3 and 4) show high predictive abilities (92.13 % and 93.69%; Table 4.1.2.112-1) and

small estimation errors (29 and 25 days; equations three and four in Table 4.2.2.12-1). However, prediction of storage time by the mentioned equations requires the determination of Ca^{2+}-ATPase activities in actomyosin extracts from the frozen fillets, which is a rather complicated and time consuming method, and hence these models may not be of commercial importance. By removing, therefore, the data of ATPase activities from the dataset of independent variables, the results from stepwise multiple linear regression analyses (Table 4.2.2.12-2) suggested that the storage time of frozen gilthead seabream fillets can be predicted by an equation with FFA, PVs and PrCTFs as independent variables, as follows:

PST (days)=319.9+8.7xFFA+28.2xPVs+10.8xPrCTF.....(5)

This model shows high predictive ability (90.9%) and quite small estimation error (29.8 days; equation 5 in Table 4.2.2.12-2).

Table 4.2.2.12-2 .Results from stepwise multiple linear regression analyses of storage time versus chemical and bio-chemical parameters excluding ATPases data. Data from individual fish.

Equation	*1*	*2*	*5*
Constant	*-46.16*	*-89.64*	*-319.90*
Free Fatty Acids	**14.6**	**9.4**	**8.7**
T-Value	8.69	5.37	6.61
P-Value	0.000	0.000	0.000
Peroxide Value		**34.1**	**28.2**
T-Value		4.29	4.63
P-Value		0.000	0.000
Protein in CTFs			**10.8**
T-Value			4.11
P-Value			0.001
S	54.1	40.0	29.8
R^2	78.22	88.66	94.00
R^2(adj)	77.19	87.53	93.05
R^2(predicted)	72.26	83.81	90.93

Table notes: As in Table 4.2.2.12-1

Figure 4.2.2.12-1, below, presents the predicted storage time values from equation 5 versus the actual storage time, showing a good linear

relationship.

Figure 4.2.2.12-1 Predicted values of storage time versus actual storage time from frozen gilthead seabream fillets stored for 340 days at -22°C

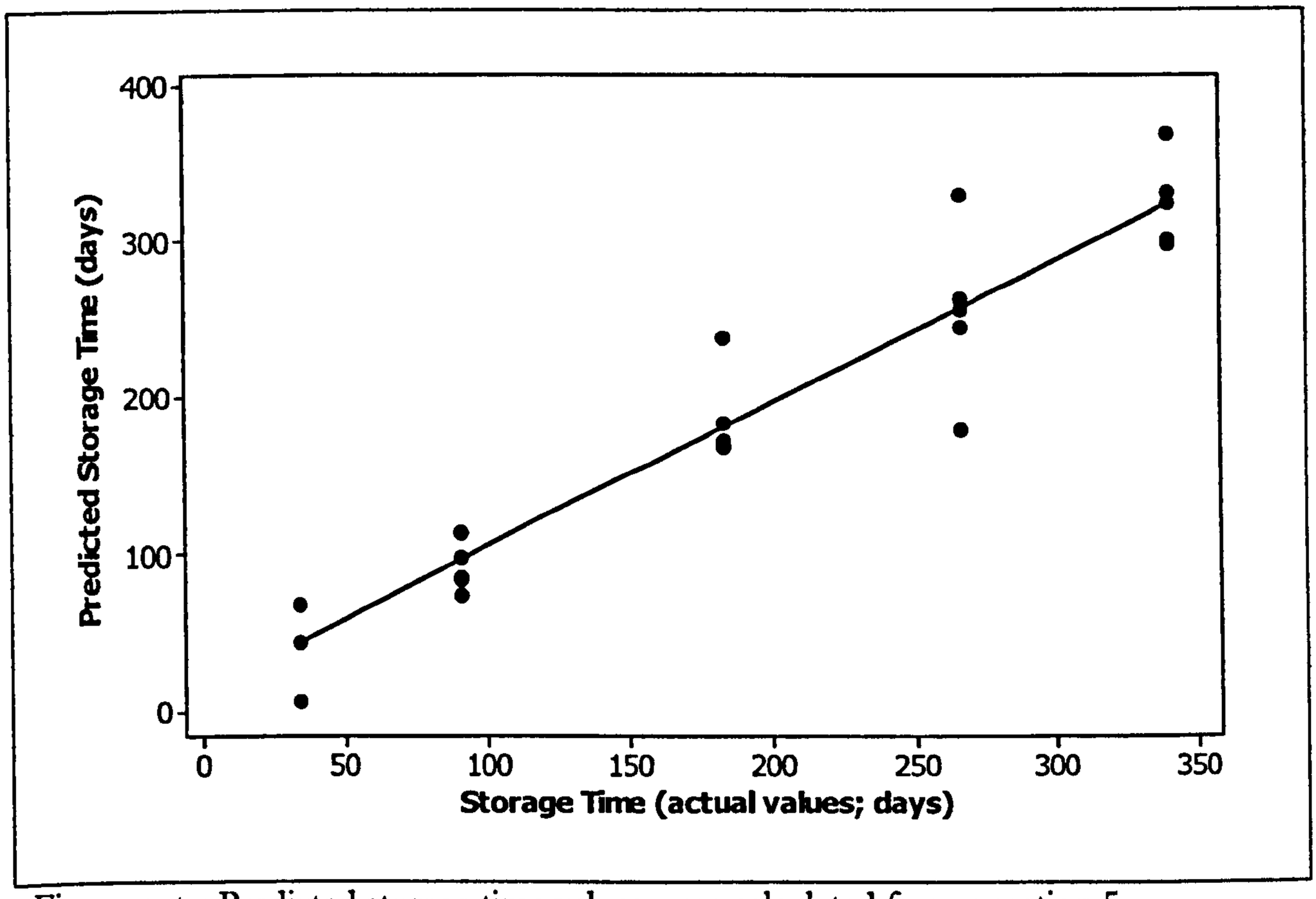


Figure note: Predicted storage time values were calculated from equation 5.

When the mentioned regression modeling applied to the mean data values of free fatty acid, peroxides and protein content in centrifugal tissue fluids from each storage period, the predictive ability of the model increased to 99.5 % and the typical error of estimation decreased to almost 6 days (Table 4.2.2.12-3; Figure 4.2.2.12-2)

Table 4.2.2.12-3 Statistics of the regression models of storage time versus chemical and bio-chemical parameters of stored frozen gilthead seabream fillets. Data from mean values

Statistics	*Equation 1*	*Equation 2*	*Equation 3*	*Equation 4*	*Equation 5*
S (days)	32	23.7	18.9	9.8	5.8
R^2 (%)	92.2	96.5	98	98.8	99.8
R^2 (adjusted, %)	89.6	95.3	97.3	98.4	99.7
R^2 (predicted, %)	77.0	89.0	93.7	97.1	99.5

Table notes: S values are the typical error of estimations in days

Figure 4.2.2.12-2. Predicted values versus storage time for mean data values of free fatty acids and peroxide values from each storage period

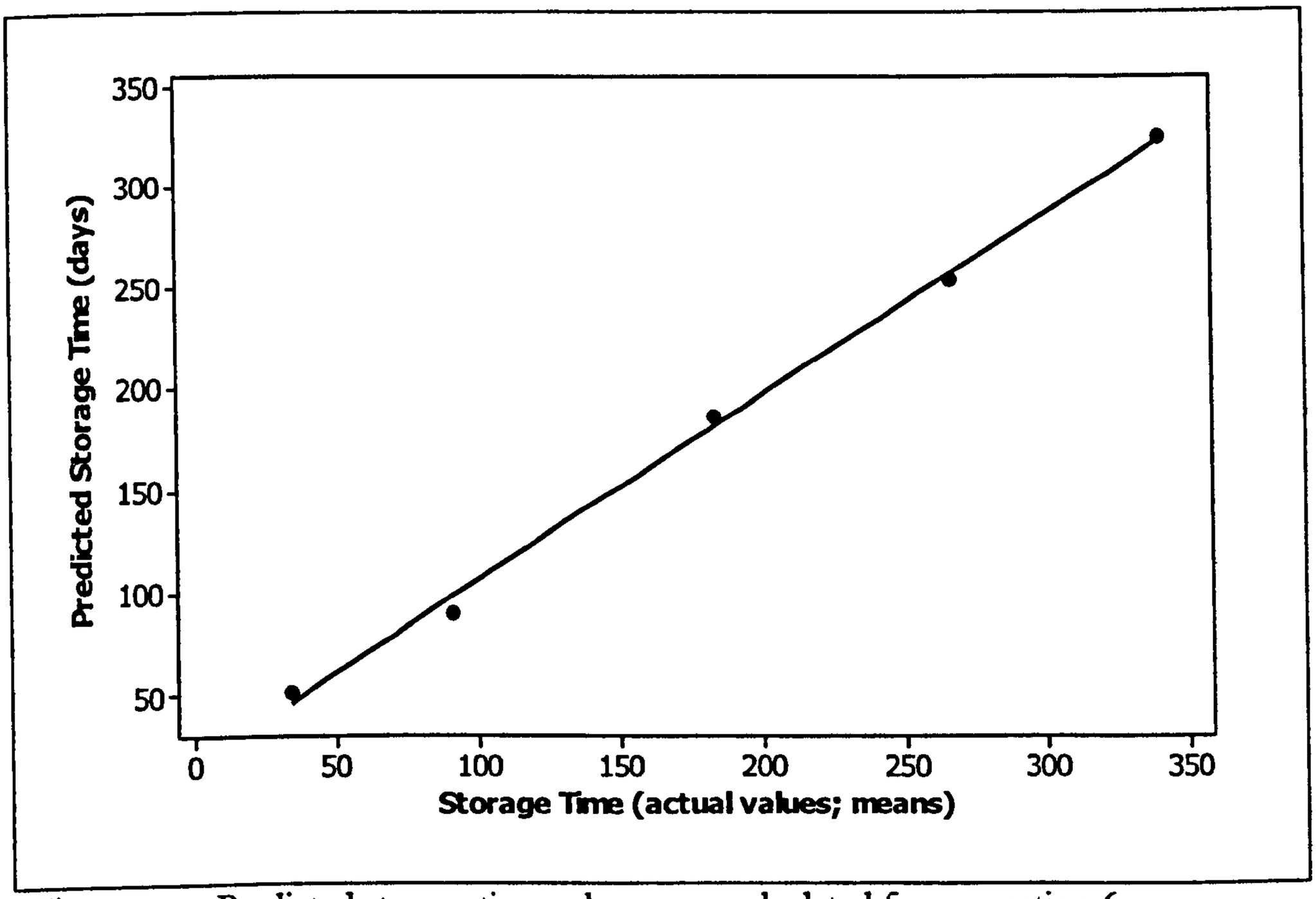


Figure note: Predicted storage time values were calculated from equation 6.

Taking into account that free fatty acids, peroxide values and protein content in CTFs were correlated also significantly with the sensory scores of acceptability of taste (Table 4.1.2.11-1 and section 4.2.2.11), it can be concluded that the storage time of gilthead seabream fillets may be

predicted by a linear model with free fatty acids (FFAs), peroxide values (PVs) and protein content in centrifugal tissue fluids (PrCTF) as independent variables, as follows:

Predicted storage time (days) =

-319.9+8.7x FFAs (means)+28.2 x PVs (means)+10.8PrCTF (means) (6)

Such a model may be of commercial relevance, since it shows a linear trend with storage time (Figure 4.2.2.12-2), has very high predictive ability (99.5%), gives very small estimation error (approximately 6 days) and involves relatively simple and fast measurement techniques (free fatty acids, peroxide values and protein content in centrifugal tissue fluids).

Simple linear regression analysis with the scores of taste acceptability as dependent variable and the values of predicted storage time, calculated from equation 6, as independent variable suggested that the quality (in terms of taste acceptability) of frozen gilthead seabream fillets stored at -22°C can be predicted by the model:

Quality= 8.027-0.0127 x Predicted Storage Time (7).

(S=0.47, R^2= 88.9, R^2 (adj) =88.4%, T(slope)=-13.56, *P*(slope)<0.0001)

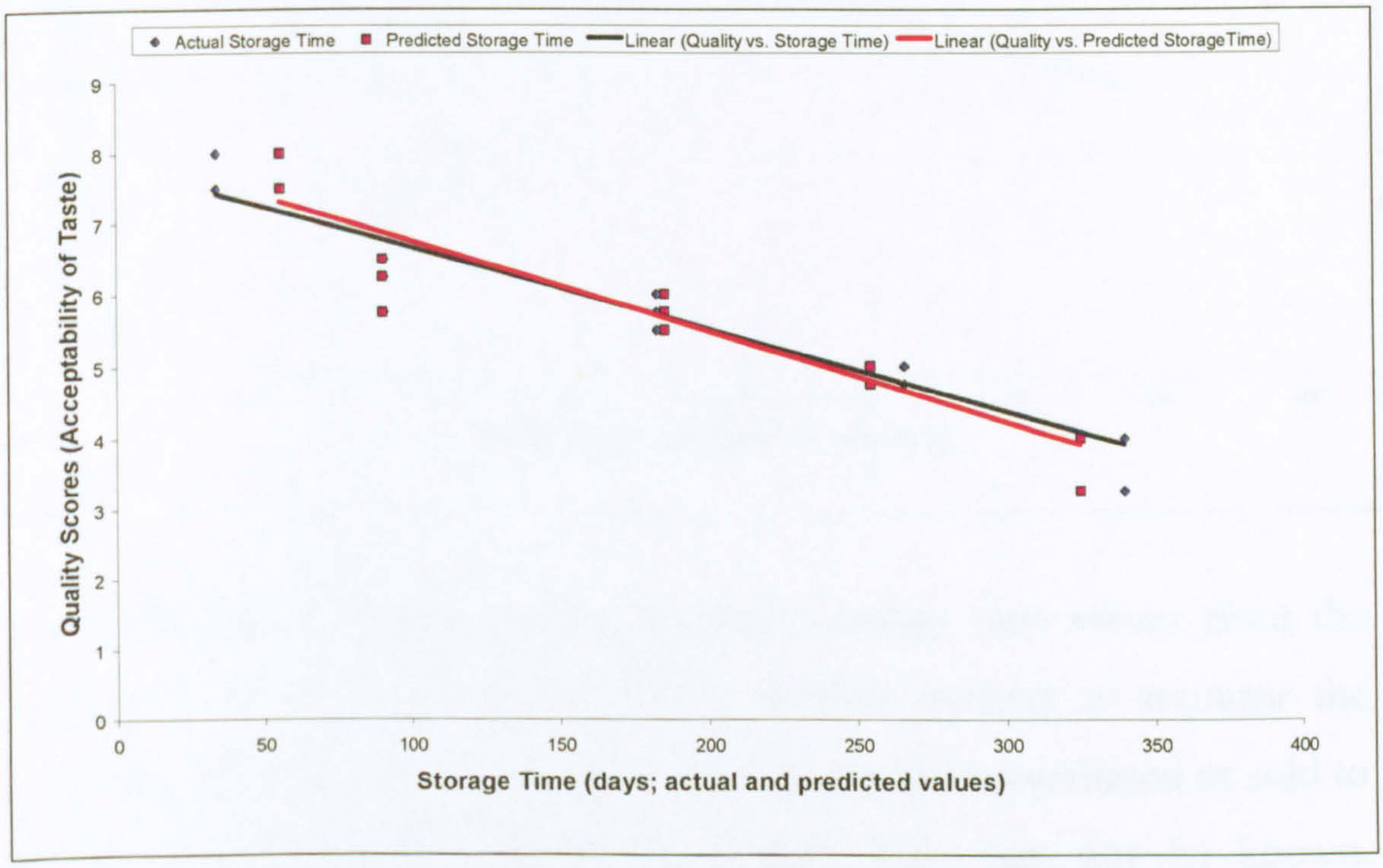
Also, simple linear regression of scores of taste acceptability as dependent variable and the values of storage time from the frozen groups of gilthead seabream fillets suggested that the quality of seabream fillets can be predicted by the model:

Quality= 7.82-0.0115 x Storage Time (8).

(S=0.41, R^2= 91.4, R^2 (adj) =91.1%, T(slope)=-15.66 *P*(slope)<0.0001)

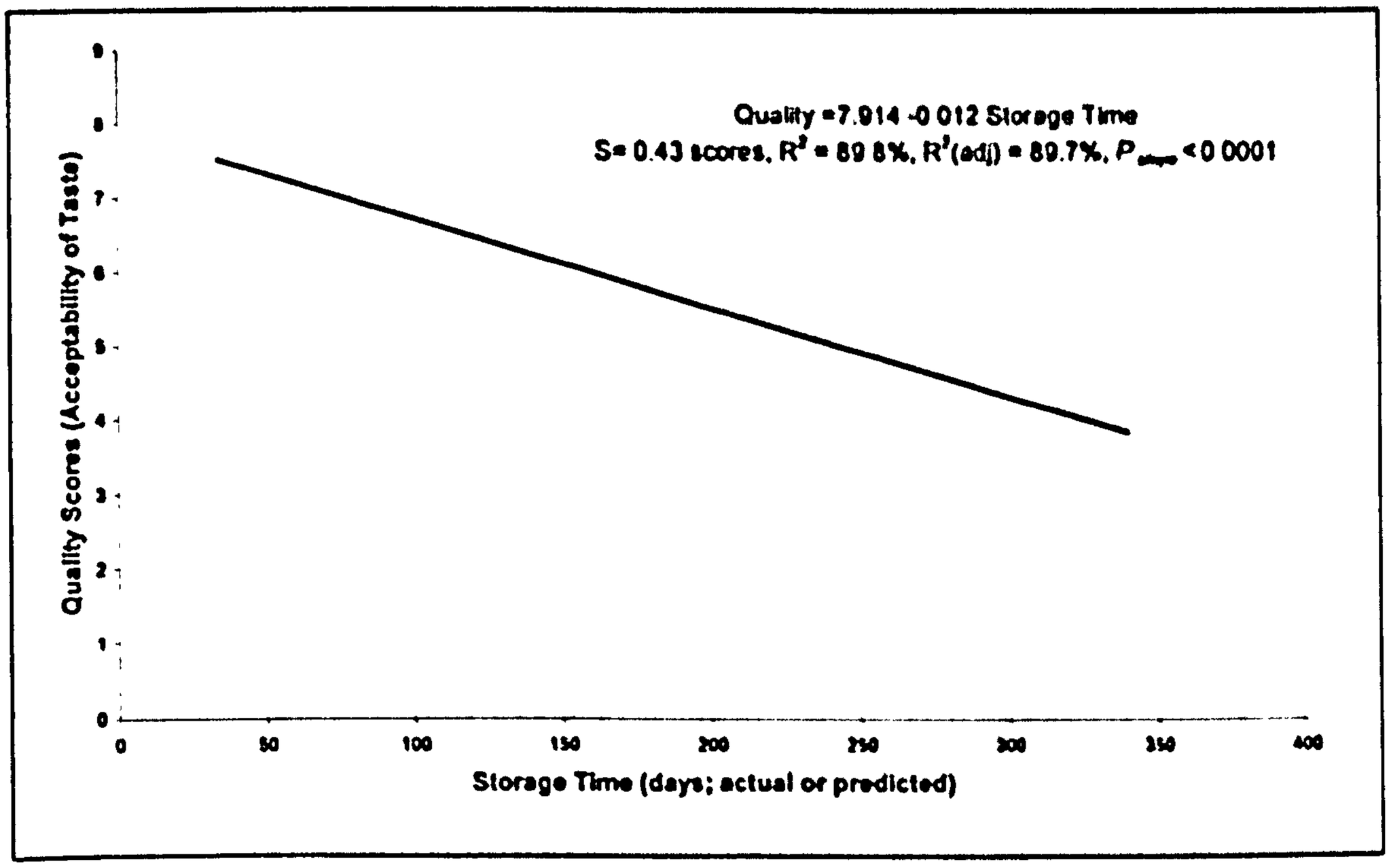
The regression lines of equations 7 and 8 are shown in Figure 4.2.2.12-3, below. The slopes and the elevations of these lines were compared by using the Student's t test according to Zar (1984), and the results suggested no significant differences for both parameters ($P>0.05$).

Figure 4.2.2.12-3 Regression lines of quality (acceptability of taste) vs. storage time (actual values) and predicted storage time



Therefore, the quality of frozen gilthead seabream fillets could be predicted by a regression line common for the actual and predicted storage time values (Zar, 1984). The regression coefficient and the intercept of this line were calculated according to Zar (1984), and the line is presented in Figure 4.2.2.12-4, below

Figure 4.2.2.12-4. Prediction of quality of stored frozen gilthead seabream fillets from the actual and predicted storage time values.



The mentioned model and the predicted storage time values from the model 6 can be used by the frozen seafood industry to monitor the quality of batches of frozen gilthead seabream fillets purchased or sold to external suppliers, for which the storage time may not be known. However, in most cases frozen seafood industries have storage time records of their batches. In those cases the predicted values of storage time can be used to check deviations from the expected quality, which is determined from the actual storage time of frozen batches and the model presented in the Figure 4.2.2.12-4. Such information can be used by frozen seafood industries to determine the actual length of time the batches of frozen fillets can be stored at -22°C, permitting a better control of their market.

Therefore, the proposed model for prediction of storage time could be a useful quality control method for assessing the quality of stored frozen gilthead seabream fillets for commercial purposes, which requires customer satisfaction.

4.2.2.13. Conclusions

The results of the present study indicate that the length of time of storage at -22 °C 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 the literature for other stored frozen fish.

Storage time of frozen gilthead seabream fillets influenced the tenderness and taste of the cooked fillets, however only the scores of taste acceptability correlated (inversely) with storage time. Based on the linear regression equation between the taste scores and storage time, it was assessed that the fillets would be acceptable up to 166 days at -22 °C. This time was higher than the practical storage life given in the literature for other frozen fatty fish stored at almost the same temperature with the fillets of the present study. The high quality life of the stored frozen fillets was assessed as being between 34 to 91 days, since significant differences in scores of taste acceptability between the fresh and the stored frozen fillets were found after 91 days of frozen storage.

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).

A linear model that combines free fatty acids, peroxide values and protein content in centrifugal tissue fluids for gilthead seabream fillets, may be a reliable method for industry to use for assessing the quality of frozen

gilthead seabream fillets during long term storage at -22°C.

CHAPTER 5. Comparison of the quality parameters obtained from frozen adductor muscles of scallops (*Pecten maximus*) and gilthead seabream *(Sparus aurata*) fillets – Conclusions - Suggestions for further studies.

5.1. The effects of freezing times (or rates) on quality parameters of scallop adductor muscles and gilthead seabream fillets

Figure 5.1-1 shows the mean HADH activities in scallop muscles and gilthead seabream fillets frozen at different characteristic freezing times.

Figure Σφάλμα! Δεν υπάρχει κείμενο καθορισμένου στυλ στο έγγραφο.-1 β-hydroxyl-acyl- coenzyme-A dehydrogenase (HADH) activities, mU g^{-1}

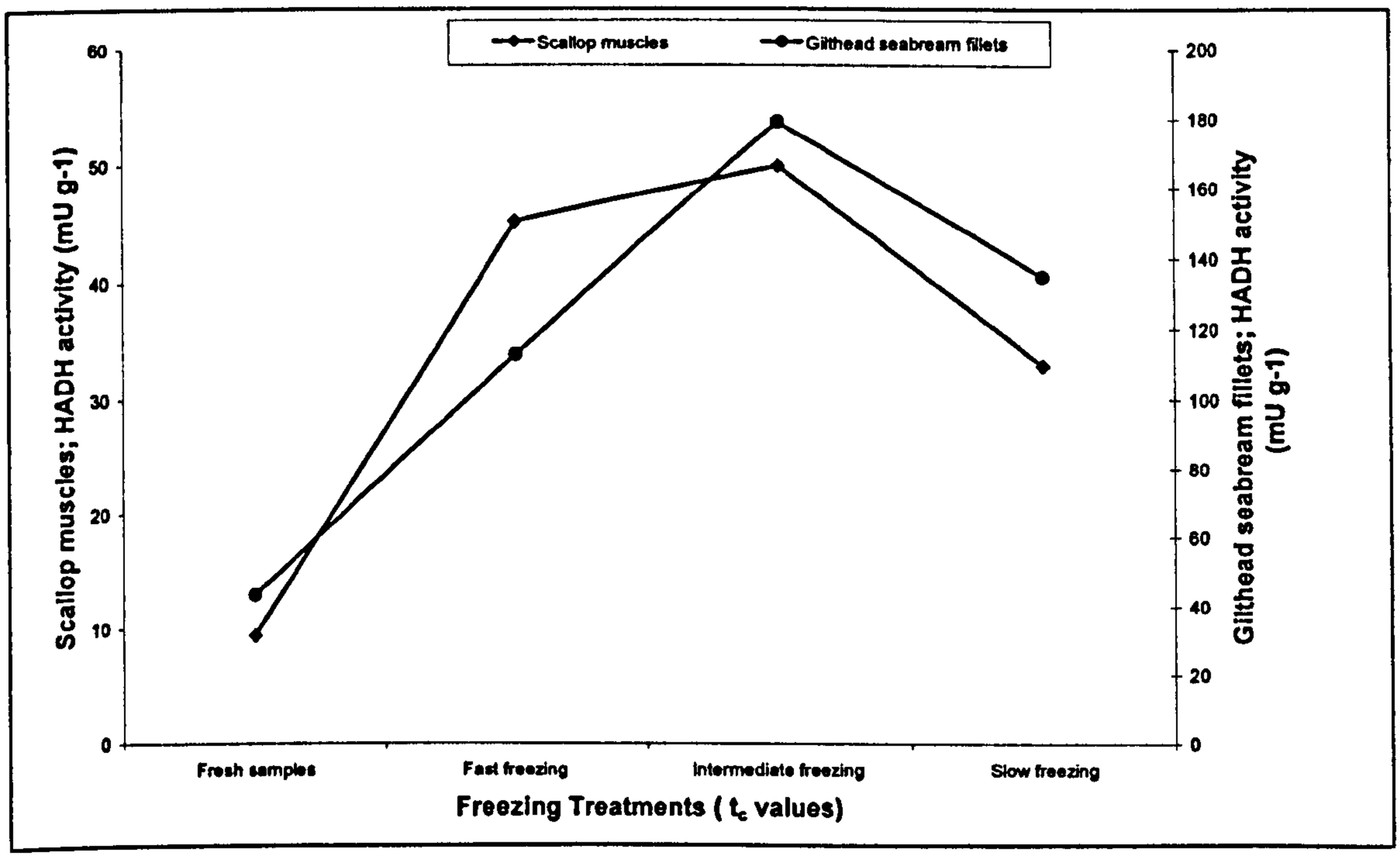


Figure notes: t_c= characteristic freezing times. Fast freezing = 19 minutes for scallop muscles; average values of gilthead seabream fillets frozen 2 and 18 minutes (t_c values). Intermediate freezing = average values of scallop muscles frozen at 89 and 45 minutes; 74 minutes for gilthead seabream fillets (t_c values). Slow freezing = average values of scallop muscles frozen at 235 to 1000 minutes; 640 minutes for gilthead seabream fillets (t_c values).

Mean HADH activities in the fresh scallop muscles and gilthead seabream fillets were 9 and 43 mU per gram of scallop muscle, respectively. On average, the mean values for HADH activities from the thawed groups of scallop muscles and gilthead seabream fillets were 43 and 142 mU per gram of muscle, respectively. Thus, the activity of the enzyme increased on thawing 4.5 and 3.3 -fold in scallop muscles and gilthead seabream fillets, respectively. Dulfos *et al.* (2002) found that the activity of HADH increased on thawing about three, eight and two -fold in whiting, place

and mackerel, respectively. Therefore, the results of the study by Dulfos *et al.* (2002) and those of the present study suggest that the degree of damage in intra-cellular organelles (mitochondria) due to freezing may be species dependent. Biological factors such as age, spawning, food supply and water temperature, or conditions of harvesting, processing and storage might also contribute to mentioned differences (Benjakul and Bauer, 2000), but these have not been considered in the present study.

The intermediate characteristic freezing times (i.e. 89 and 49 minutes for scallop muscles and 74 minutes for gilthead seabream fillets) caused higher release of the enzyme HADH in the muscles of both species than the shorter or longer characteristic freezing times tested (Figure 5.1-1). These results suggest that freezing of both species at intermediate freezing times may cause more damage to intra-cellular organelles (mitochondria) than the other freezing times. This can be due to mechanical damage and dehydration of mitochondrial membranes caused by large intra-cellular ice crystals, which might have been formed in the vicinity of or even inside the mitochondria of scallop muscles and gilthead seabream fillets during freezing at the mentioned characteristic freezing times (Hamm and Gottesmann, 1982; see sections 3.2.1.2 and 4.2.1.2).

Freezing of scallop muscles and gilthead seabream fillets at 'slow' characteristic freezing times (i.e. from 235 to 1000 minutes for scallop muscles, and 640 minutes for gilthead seabream fillets), caused higher thawing losses than those of samples of both species frozen at fast and intermediate characteristic freezing times (i.e. 19 to 89 minutes for scallop muscles, and 2 to 74 minutes for gilthead seabream fillets). These differences were mainly attributed to distortion and destruction of fibres of tested species by ice crystals rather than to denaturation of myofibrillar proteins (see sections 3.2.1.4 and 4.2.1.3).

As shown in Figure 5.1-2, thawing losses of gilthead seabream fillets were greater in gilthead seabream fillets than in scallop muscles. This may be attributed to the higher surface to mass ratio of the fillets compared to that of scallop muscles (Ramsbottom and Koonz 1939; cited in Añón and Calvelo, 1980).

Figure 5.1-2 Thawing weight losses, g kg^{-1}

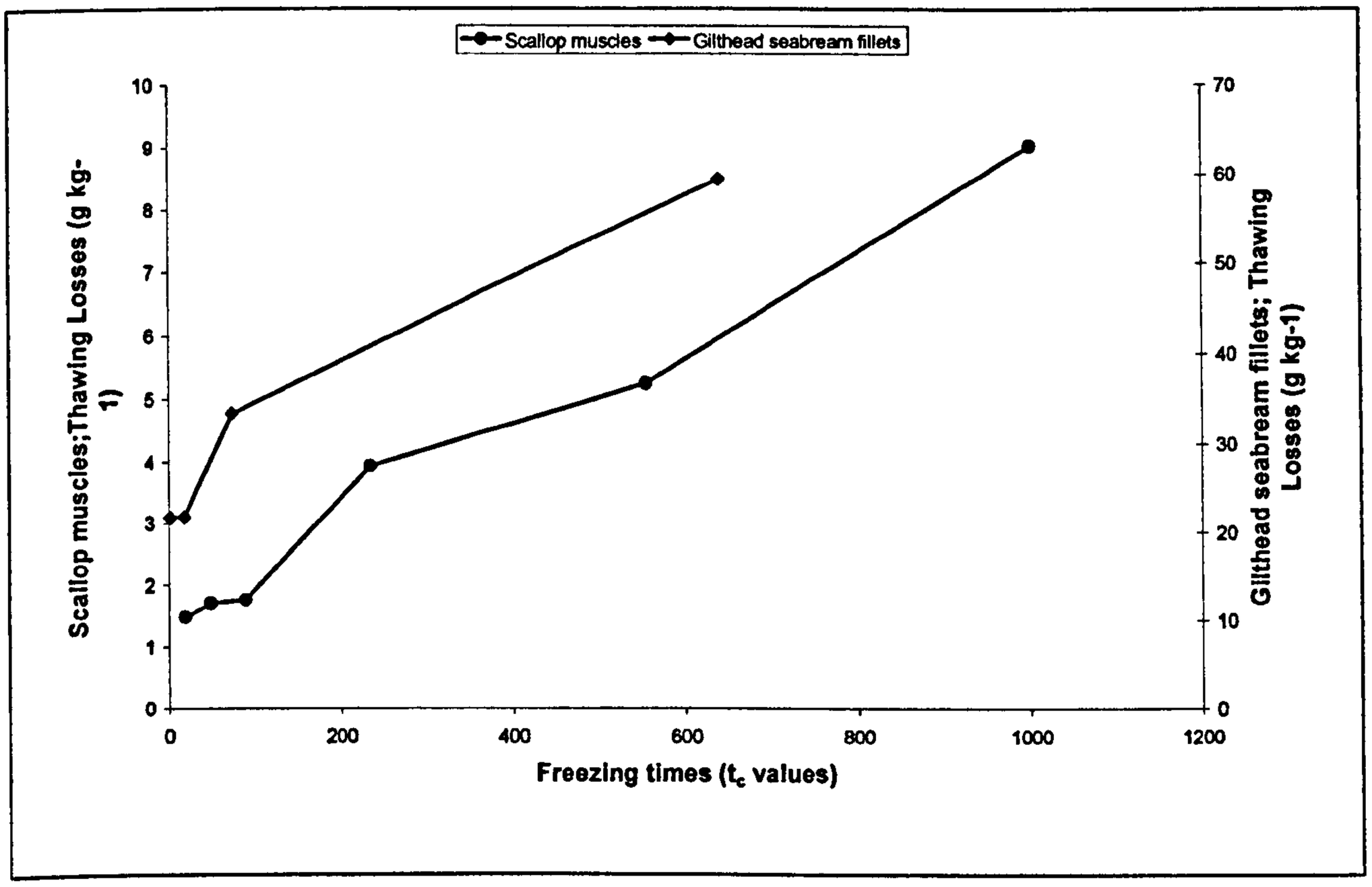


Figure 5.1-3 shows the total weight losses of expressible fluids and thawing losses from scallop muscles and gilthead seabream fillets frozen at different characteristic freezing times.

Figure 5.1-3 Total weight losses, frozen: fresh.

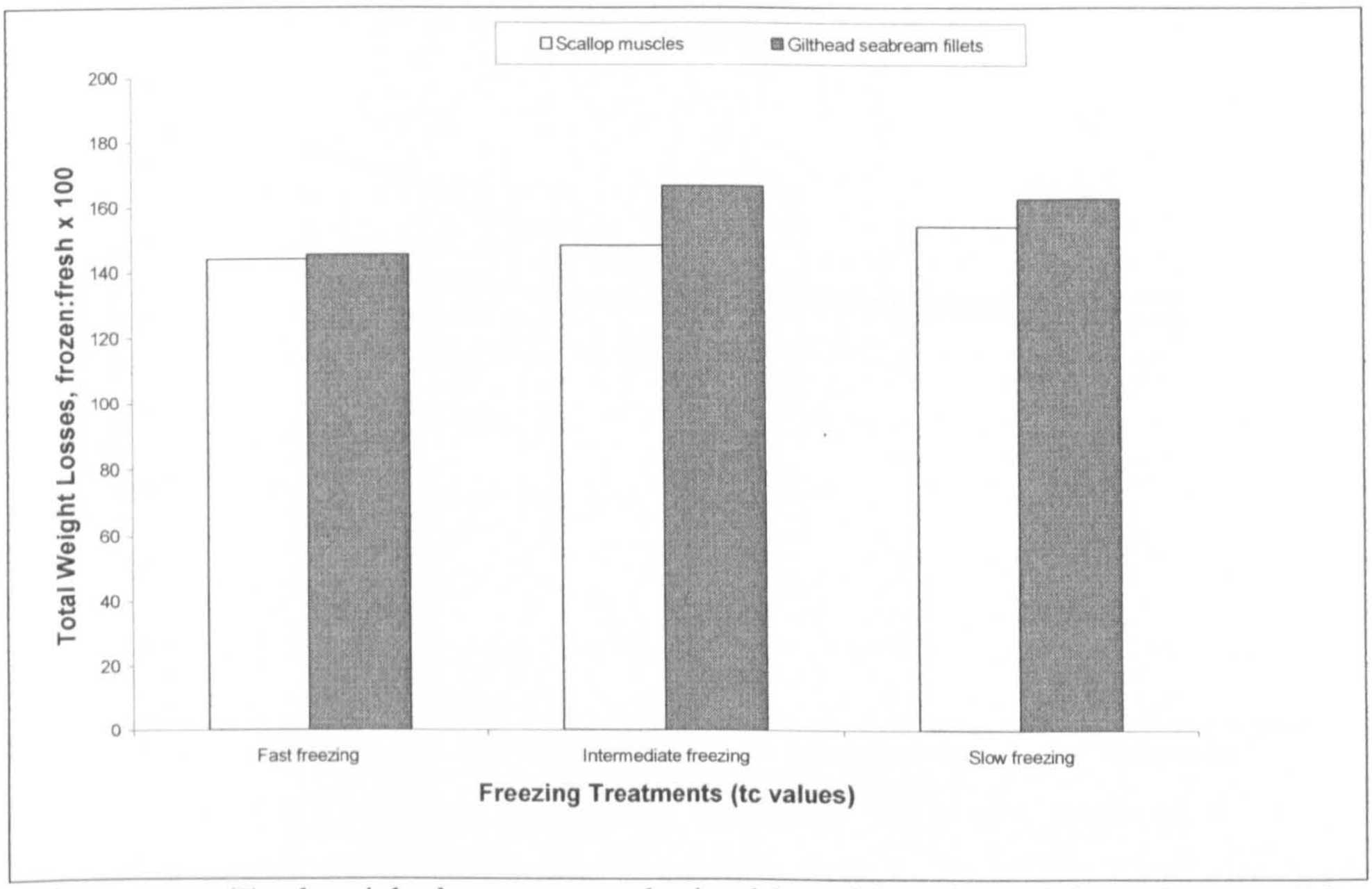


Figure note: Total weight losses were obtained by adding the weights of expressible fluids and thawing losses.

Expressible fluids values and total weight losses were hardly affected by the characteristic freezing times at which the scallop muscles and gilthead seabream fillets were frozen. However, the mentioned parameters were significantly affected by the freezing process itself for both species (see sections 3.2.1.4 and 4.2.1.3). These results may suggest that the freezing process itself affected the properties of myofibrillar proteins, which is consistent with the changes in Ca^{2+}-ATPase activities observed for actomyosin extracts from both species (see sections 3.2.1.5 and 4.2.1.6).

Figure 5.1-4 shows the mean Ca^{2+}-ATPase activities in actomyosin extracts from scallop muscles and gilthead seabream fillets frozen at different characteristic freezing times.

Figure 5.1-4 Ca^{2+}-ATPase activities, μmoles P_i/mg protein/min

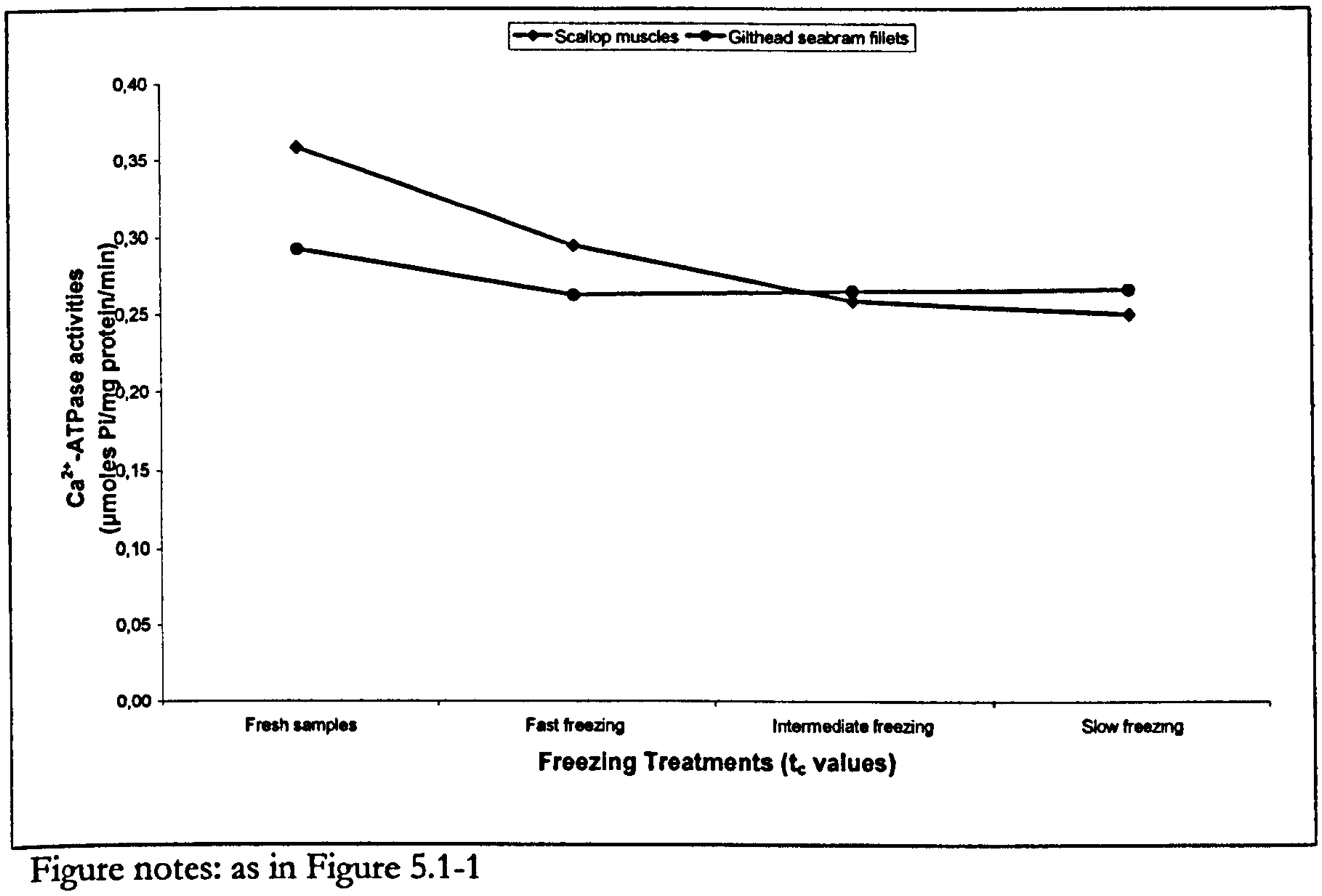


Figure notes: as in Figure 5.1-1

Mean values of Ca^{2+}-ATPase activities in actomyosin extracts from fresh scallop muscles and gilthead seabream fillets were 0.358 and 0.292 μmoles P_i/ mg protein /min, respectively. This difference may be attributed to differences in the species examined and/or most probably to the different methods used in the present study to extract actomyosins from the two species, i.e. by simple immersion of washed minced scallop muscle in a solution of sodium chloride as opposed to homogenizing chopped gilthead seabream samples with a solution of potassium chloride (see sections 2.1.7.4 and 2.2.7.6). Kawashima *et al.* (1973) showed that Ca^{2+}-ATPase specific activities of actomyosins extracted from surimi without homogenization were greater compared to the activities of actomyosins extracted by homogenization of samples for diverse times.

Freezing of scallop muscles and gilthead seabream fillets at different freezing times hardly affected Ca^{2+}-ATPase activities of actomyosin extracts from frozen scallop muscles and gilthead seabream fillets, probably due to the short time the samples of both species remained in the frozen stage before the activities were measured (see sections 3.2.1.5 and 4.2.1.6). Ca^{2+}-ATPase activities from all frozen groups of scallops were on average 27 per cent less compared to the activities of fresh samples, whereas Ca^{2+}-ATPase activities from all frozen groups of fillets were on average 10 per cent less than those of fresh fillets. These results indicate that the freezing process itself caused structural changes in myosin (or 'actomyosin') molecules of both species, but that the myosin (or 'actomyosin') molecules from gilthead seabream fillets were more stable during freezing than those from scallop muscles. This may be related to adaptation of gilthead seabream proteins to a seawater environment with higher and more variable temperature than the lower and more stable temperature experienced by scallops (Ochiai and Chow, 2000).

Figure 5.1-5 shows the peak shear forces (ratio of frozen to fresh) of scallop muscles and gilthead seabream fillets frozen at different characteristic freezing times.

Figure 5.1-5 Peak shear forces, frozen: fresh

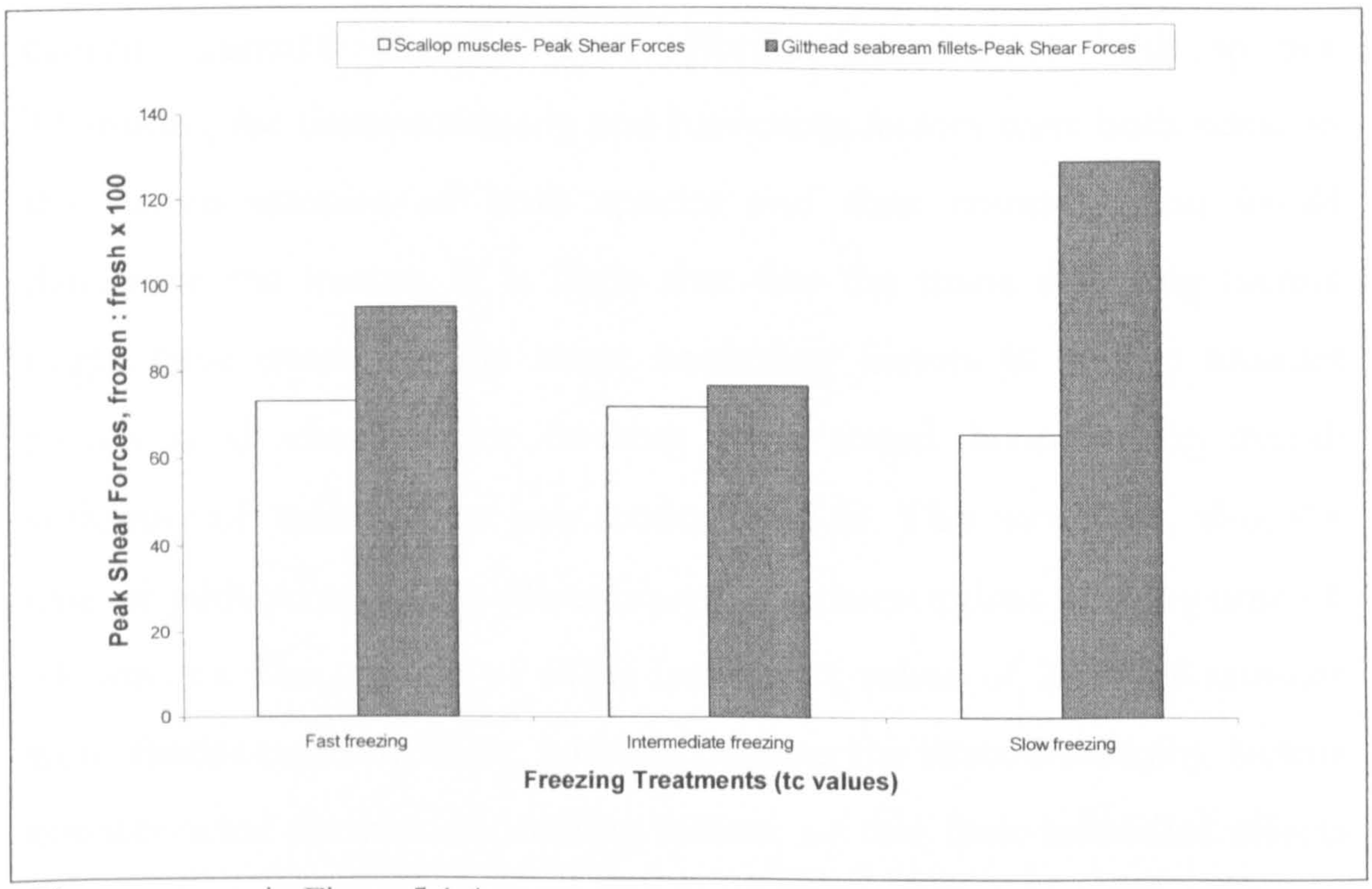


Figure note: as in Figure 5.1-1.

Freezing of scallop muscles at all characteristic freezing times tested produced softer (i.e. less firm) raw samples compared to fresh scallop muscles. In contrast, the peak shear forces (i.e. firmness values) of fillets frozen at t_c values of 2 and 18 minutes ('Fast freezing' in the Figure 5.1-5, above) were similar to those of fresh samples. Freezing of gilthead seabream fillets at a characteristic freezing time of 74 minutes ('Intermediate freezing' in the Figure 5.1-5 above) produced fillets significantly softer than the fresh fillets and those frozen at all the other characteristic freezing times. Freezing of gilthead seabream fillets at a characteristic freezing time of 640 minutes ('Slow freezing' in the Figure 5.1-5, above) produced fillets harder (i.e. more firm) than all the other experimental groups of gilthead seabream fillets and scallop muscles. Altogether, these results suggest that the characteristic freezing times

tested influenced the texture (i.e. firmness values) of gilthead seabream fillets, but not of scallop muscles.

As the results of the present study show, the freezing process itself caused damage to fibres, decreased the water holding parameters and caused structural changes to myofibrillar proteins of both species. Therefore, the tissue-softening and hardening factors were both active in the frozen samples of both species and their counter-action would determine the texture. It is likely that that the tissue softening factors might have overcome the tissue hardening factors in scallop muscles frozen at all characteristic freezing times tested, hence giving overall softening of their tissues (see section 3.2.1.6). This would be, also, the case of gilthead seabream fillets frozen at a characteristic freezing time of 74 minutes. The textures of fillets frozen at t_c values of 2 and 18 minutes were similar to fresh fillets, possibly because the tissue-hardening factors counter-acted the tissue-softening factors, so that their individual effects on texture might have been diminished (see section 4.2.1.9). By contrast, the tissue-hardening factors (e.g. thawing weight losses) would overcome the tissue-softening (e.g. release of proteolytic enzymes from lysosomes) causing hardening of the fillets frozen at a longer freezing time (t_c value) of 640 minutes, compared to the samples of the other experimental groups of both species. This suggestion is supported by the higher thawing weight losses of the gilthead seabream fillets frozen at a t_c value of 640 minutes compared to the samples of the other experimental groups of both species (Figure 5.1-2).

Freezing of gilthead seabream fillets at all characteristic freezing times tested produced chewier, i.e. less tender, cooked fillets compared to fresh fillets (see sections 4.1.1.10 and 4.2.1.10). However, scallop muscles were perceived as less tender than the fresh scallop muscles only when the fresh samples were compared with samples frozen at a slow characteristic

freezing time of 1000 minutes (section 3.1.1.7 and 3.2.1.7).

These results suggest that if the freezing process had caused changes in the sensory texture of scallop muscles, these might have been less pronounced than those of gilthead seabream fillets. It is known that freezing and thawing causes shrinkage, i.e. reduction of the cross sectional area of fish muscle fibres (Pan and Yeh, 1993; Chen and Pan, 1997; Sigurgisladottir *et al.*, 2000). Also, the effect of freezing and frozen storage on shrinkage of fibres is dependent on the fibre cross sectional area of the fresh fish material, i.e. fish with bigger cross sectional area shrink on freezing and frozen storage to a higher extent than fish that have smaller fibre cross sectional area (Sigurgisladottir *et al.*, 2000). In addition, Hurling *et al.* (1996) found a high correlation between cross sectional area and sensory perception of texture (firmness) in seven fish species. The cross sectional area of the muscle fibres of different scallop species is about 1 by 10 microns (Findlay and Stanley, 1984), which is much less than those recorded for different finfish muscle fibres (i.e. 100 to 150 by 10 microns; Hatae *et al.*, 1984). Thus, on freezing, the cross sectional area of gilthead seabream fibres might have been decreased to a greater extent than those of scallop muscles. Consequently, it is likely that the process of freezing and thawing would alter the sensory texture more profoundly in gilthead seabream fillets than in scallop muscles.

5.2. The effects of storage time at -22°C on quality parameters of scallop adductor muscles and gilthead seabream fillets

The activities of the β-hydroxy-acyl-coenzyme-A dehydrogenase (HADH) enzyme in the frozen scallop muscles and gilthead seabream fillets were used as markers of the integrity of intra-cellular organelles (mitochondria) during frozen storage of both mentioned species.

Figure Σφάλμα! Δεν υπάρχει κείμενο καθορισμένου στυλ στο έγγραφο.-1 β-hydroxy-acyl-coenzyme-A dehydrogenase activities, frozen :fresh

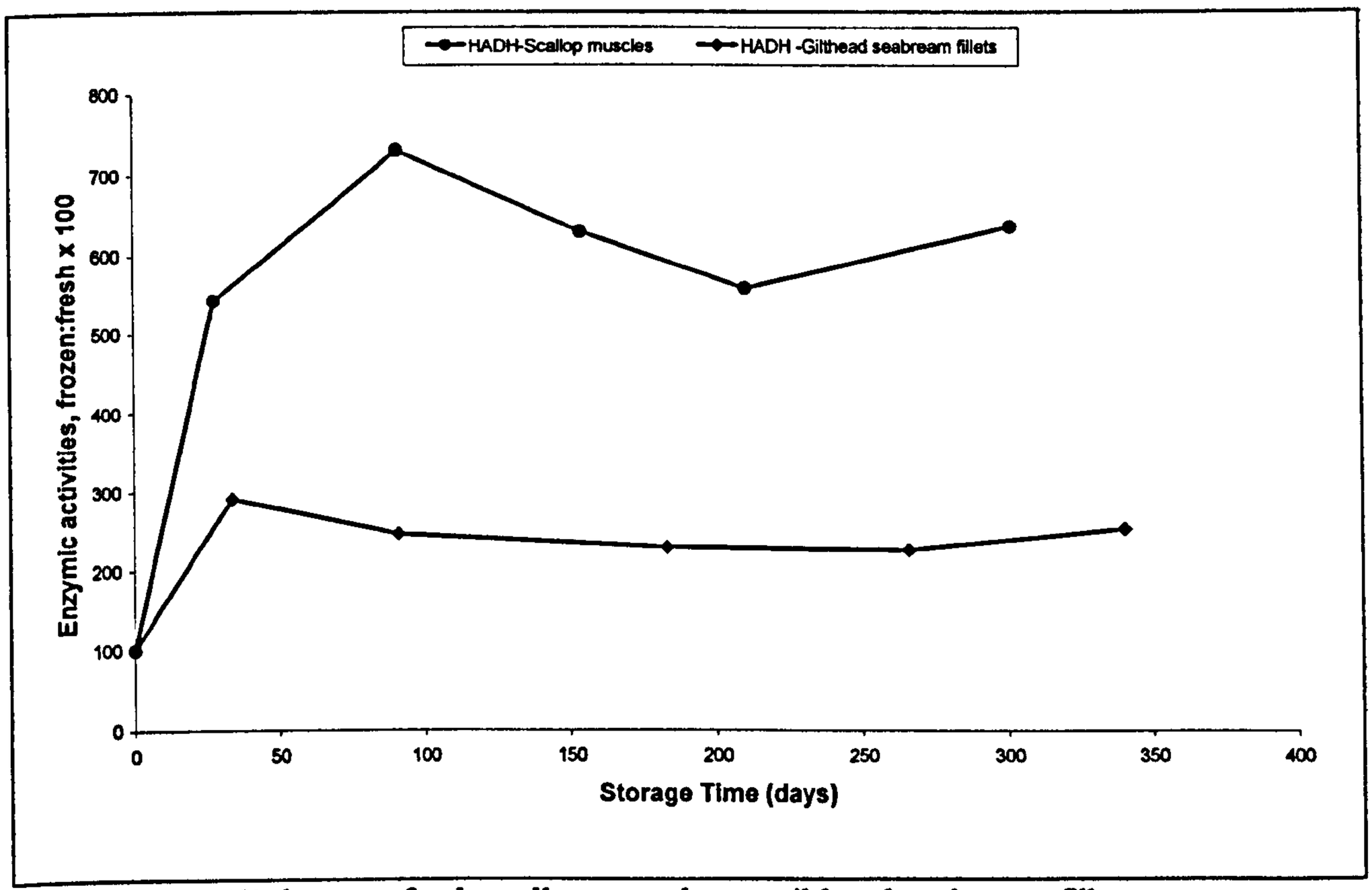


Figure note: '0' denotes fresh scallop muscles or gilthead seabream fillets

As is shown in Figure 5.2-1, the HADH activities in frozen scallop muscles increased 5.4 and 6.3 -fold compared to the activities of fresh samples after 28 and 301 days in cold storage, respectively. Maximum HADH activities in the muscles of frozen scallop muscles were observed after 91 days of storage (see section 3.2.2.1). In contrast, the HADH activities in stored frozen gilthead seabream fillets were similar to each other regardless of storage time (see section 4.2.2.2). These results suggest that the marker enzyme HADH may be a sensitive indicator in

assessing the changes in the integrity of intra-cellular organelles (mitochondria) of stored frozen scallop muscle, but not of stored frozen gilthead seabream fillets

The effects of the length of time of storage at -22°C on cell or tissue integrity of frozen gilthead seabream fillets were studied also by the marker lysosomal enzymes α-glucosidase (AG) and β-N-acetyl – glucosaminidase (NAG) (Figure 5.2-2, below). The results suggested that the enzyme AG may be a more sensitive indicator than the enzyme NAG in assessing the mentioned changes in stored frozen gilthead seabream fillets (discussed in section 4.2.2.2).

Figure 5.2-2 Enzymic activities of stored frozen gilthead seabream, frozen: fresh

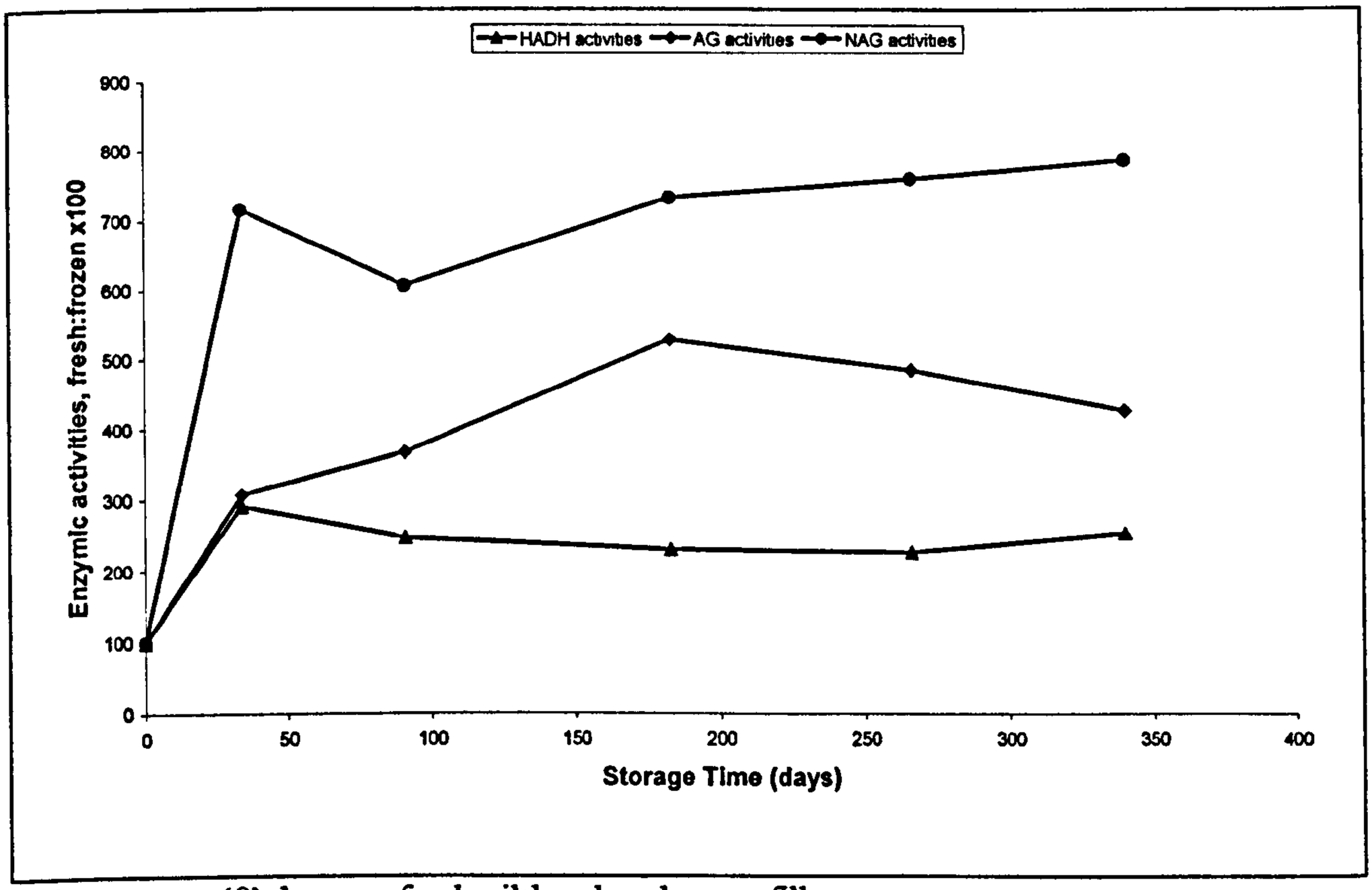


Figure note: '0' denotes fresh gilthead seabream fillets.

The differences in the activities of the aforementioned enzymes may indicate the differences in stability of scallop muscles and gilthead seabream fillets during storage at -22°C. For both species, the cell and tissue damage during cold storage was mainly attributed to ice crystals growth as well as the increased salt concentration in the unfrozen phase

(see sections 3.2.2.1and 4.2.2.2).

Total weight losses due to thawing and expressible fluids were taken as indices of the water holding capacities of stored frozen scallop muscles and gilthead seabream fillets, and are shown in Figure 5.2-3.

Figure 5.2-3 Total weight losses, frozen: fresh

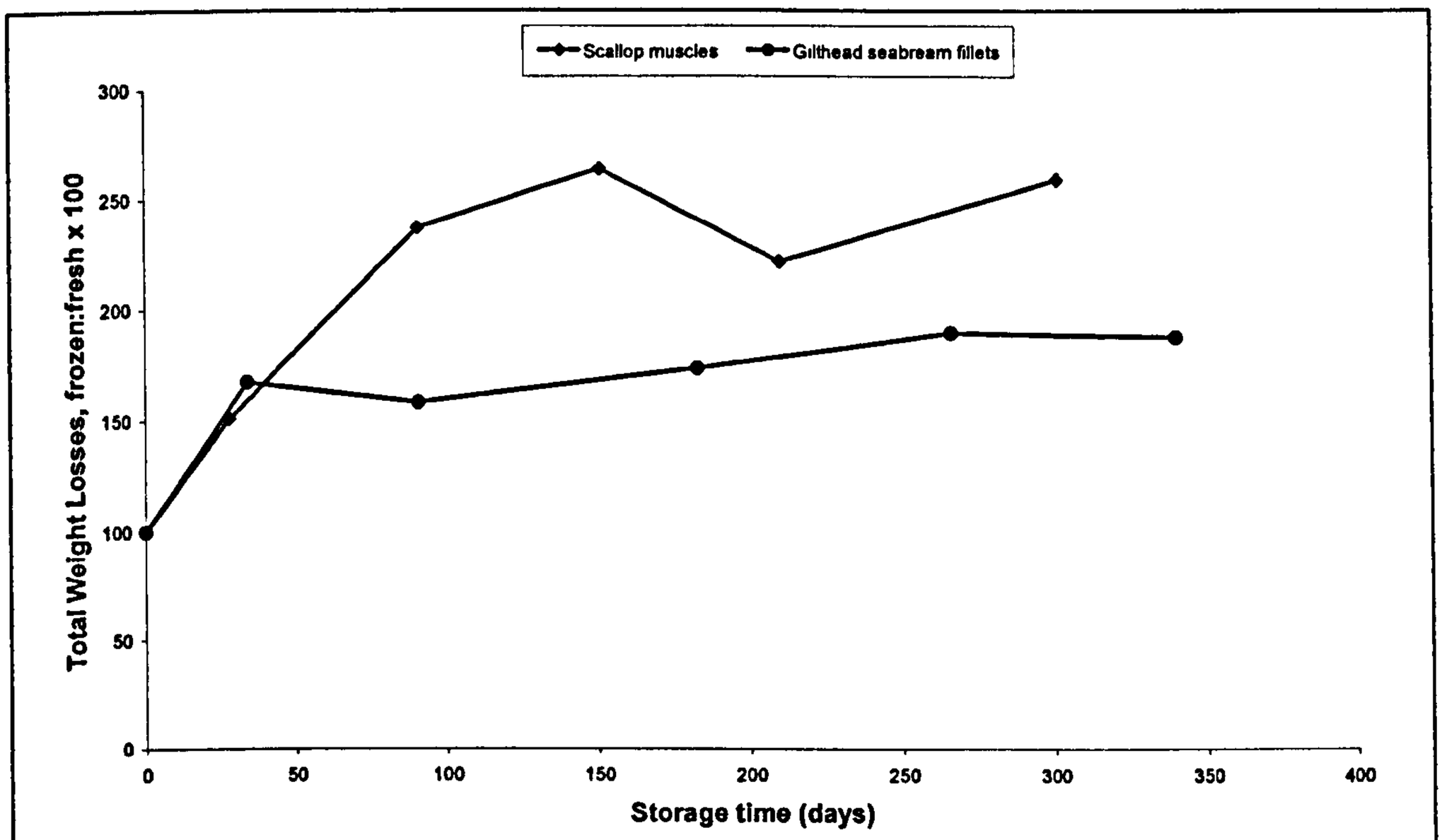


Figure note: '0' denotes fresh scallop muscles or gilthead seabream fillets.

Total weight losses of frozen scallop muscles stored for 301 days at -22°C were increased by 159 % compared with those of fresh samples, whereas for the frozen gilthead seabream fillets stored for 340 days at -22°C total weight losses were 87% more than those of fresh samples. Therefore, the length of time of storage at -22°C reduced the water holding capacity of both species; however these changes were greater in scallop muscles than gilthead seabream fillets, in agreement with the changes in Ca^{2+}-ATPase activities (Figure 5.2-4, below).

Ca^{2+}-ATPase activities of actomyosin extracts from both species decreased throughout the frozen storage. As Figure 5.2-4 shows, Ca^{2+}-ATPase activities from scallop muscles had a greater decreasing rate

compared to that of Ca^{2+}-ATPases activities from gilthead seabream fillets.

Figure 5.2-4 Ca^{2+}-ATPase activities, frozen: fresh

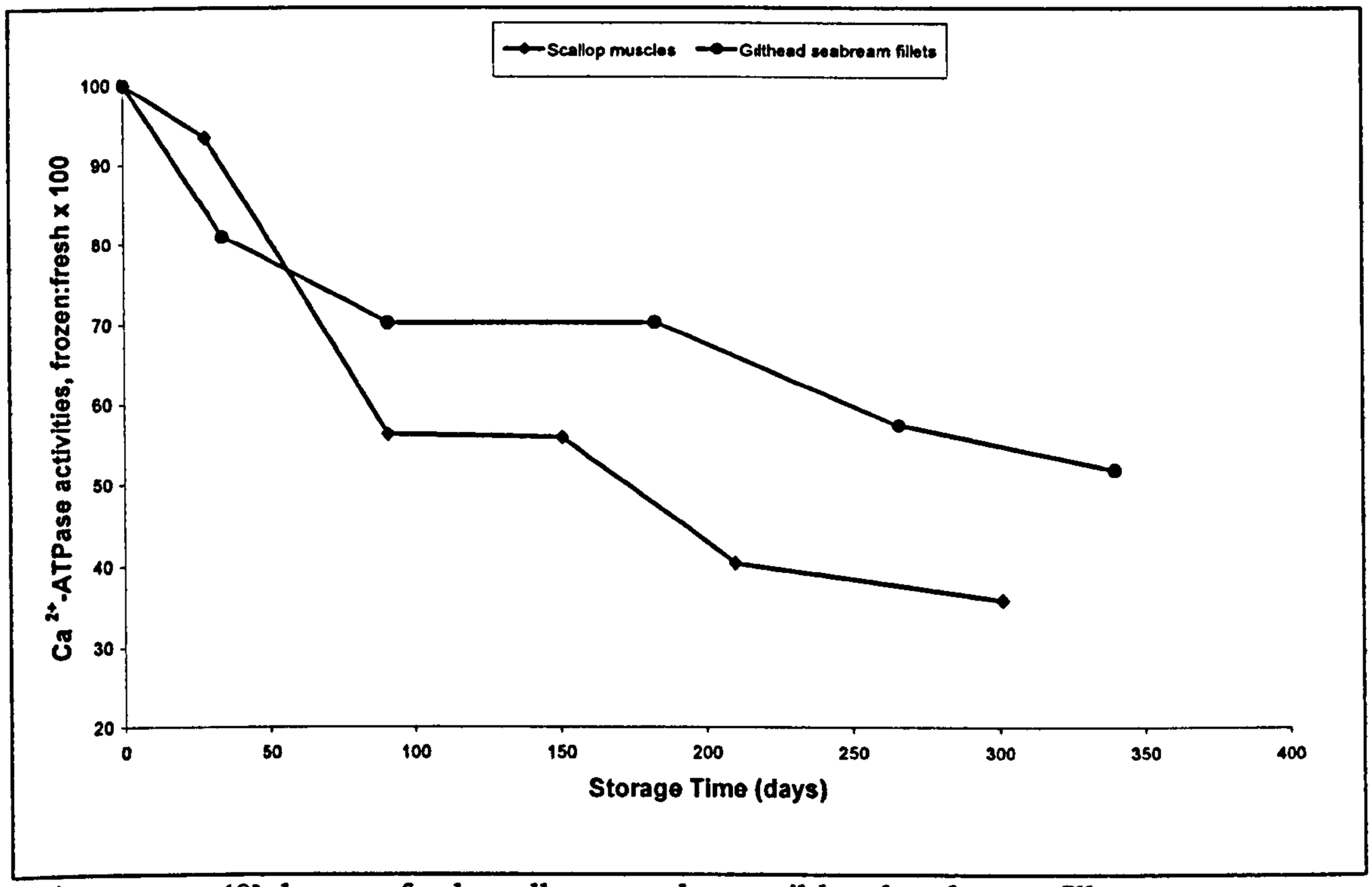


Figure note: '0' denotes fresh scallop muscles or gilthead seabream fillets.

Ca^{2+}-ATPase activities from frozen scallop muscles stored for 301 days at -22°C were decreased by 64 % compared to those of fresh samples. After 340 days of frozen storage, Ca^{2+}-ATPase activities had decreased by 48 % compared to those of fresh gilthead seabream fillets. These results suggest that the myosins (or 'actomyosins') from gilthead seabream fillets were more stable during frozen storage than those from scallop muscles. Stability of fish myosin during frozen storage is dependent on the temperature of their inhabiting waters (Ochiai and Chow, 2000). Changes in the thermal characteristics of myosin subunits during frozen storage suggest that myosin subunits of cold-water fish deteriorate more rapidly than myosin subunits of tropical fish (Howell *et al.*, 1991). Seo *et al.* (1997) showed that ATPase activities from vertically migratory myctophid fish were more stable than from the non-migratory myctophid fish during storage at -15° for one month. These authors attributed the mentioned

findings to the adaptation of myofibrillar proteins of the migratory species to the changes in seawater temperatures between the surface and deep layers. Scallops used in the present study were obtained from a fishing area, i.e. the Orkney fishing area of Scotland, with lower and possibly more stable seawater temperature than that of the Ionion Sea, in which the fish farm unit of gilthead seabream used in the present study was located. Therefore, the higher stability of myosins (or 'actomyosins') from gilthead seabream fillets during frozen storage compared to those of scallop muscles might be related to adaptation of the proteins of the gilthead seabream to higher and more variable seawater temperatures than the lower temperatures experienced by the scallops.

As is shown in Figure 5.2-5, thiobarbituric acid reactive substances (TBARS) in stored frozen scallop muscles and gilthead seabream fillets reached a maximum and then decreased with time of cold storage, in agreement with the findings of other studies (see section 1.4.3.3). This was attributed to the interactions of TBARS with other biological constituents present in the fish muscle (see sections 3.2.2.4 and 4.2.2.8-c).

Figure 5.2-5 Thiobarbituric acid reactive substances (TBARS), frozen: fresh

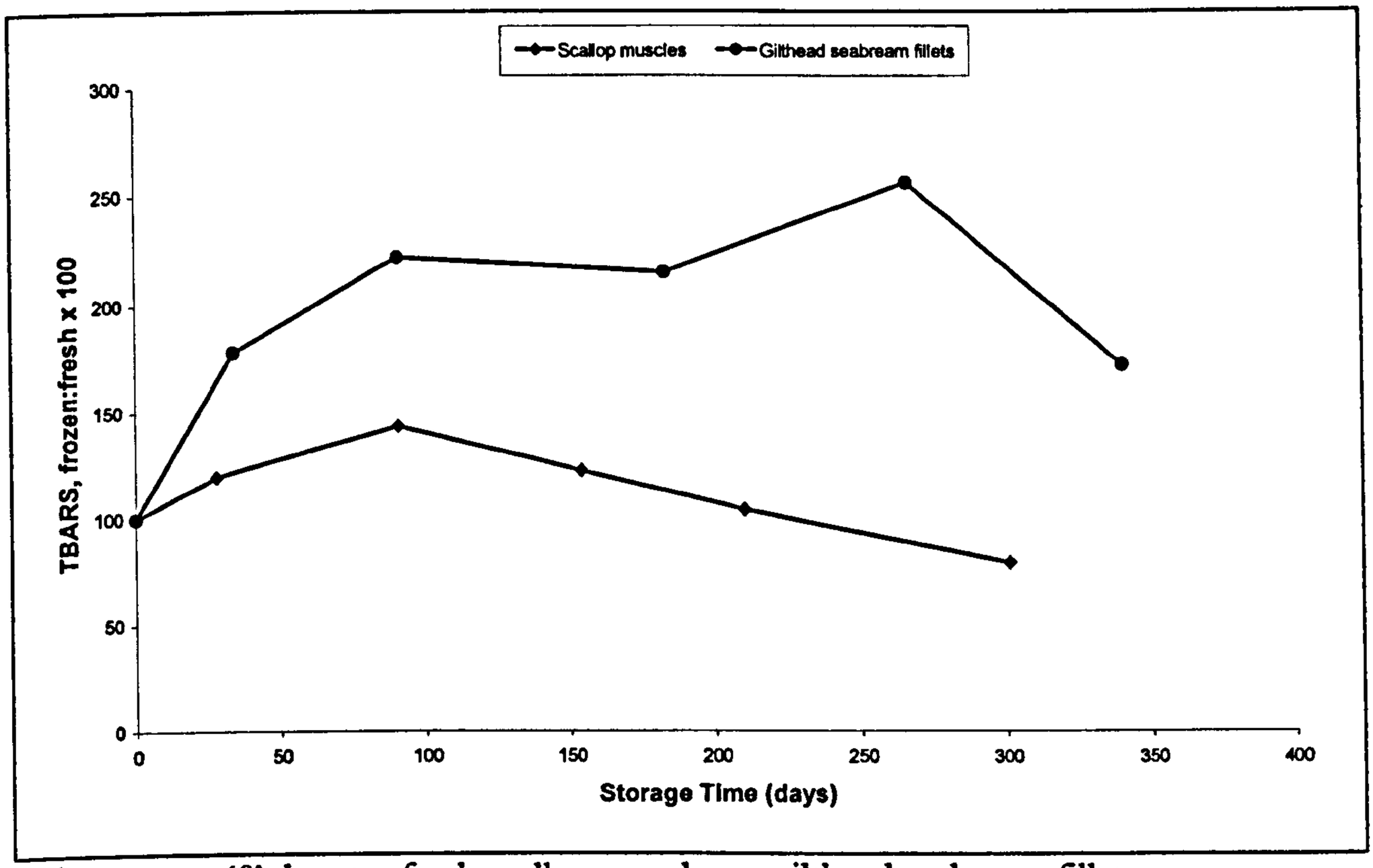


Figure note: '0' denotes fresh scallop muscles or gilthead seabream fillets.

However, the rate of changes in TBARS in stored frozen scallop muscles and gilthead seabream fillets was different. TBARS values reached a maximum after 91 and 210 days of storage in scallop muscles and gilthead seabream fillets, respectively. By those times, TBARS in scallop muscles and gilthead seabreams fillets were 44 % and 121 % more than the TBARS values of fresh samples, respectively. These differences may suggest that the rate of oxidation of lipids was higher in gilthead seabream fillets than in scallop muscles and/ or that the rate of interactions of TBARS with other biological constituents was higher in scallop muscles than in gilthead seabream fillets. Differences in total lipid

content and composition of polyunsaturated fatty acids could have contributed to different rates of lipid oxidation in stored frozen scallop muscles and gilthead seabream fillets, in agreement with the suggestions of Benjakul *et al.* (2005) for the frozen croaker and lizardfish stored for 24 weeks at -18°C.

As is shown in Figure 5.2-6 the peak shear forces of fresh scallop muscles and gilthead seabream fillets were always higher than those of stored frozen fillets.

Figure 5.2-6 Peak shear forces, frozen: fresh

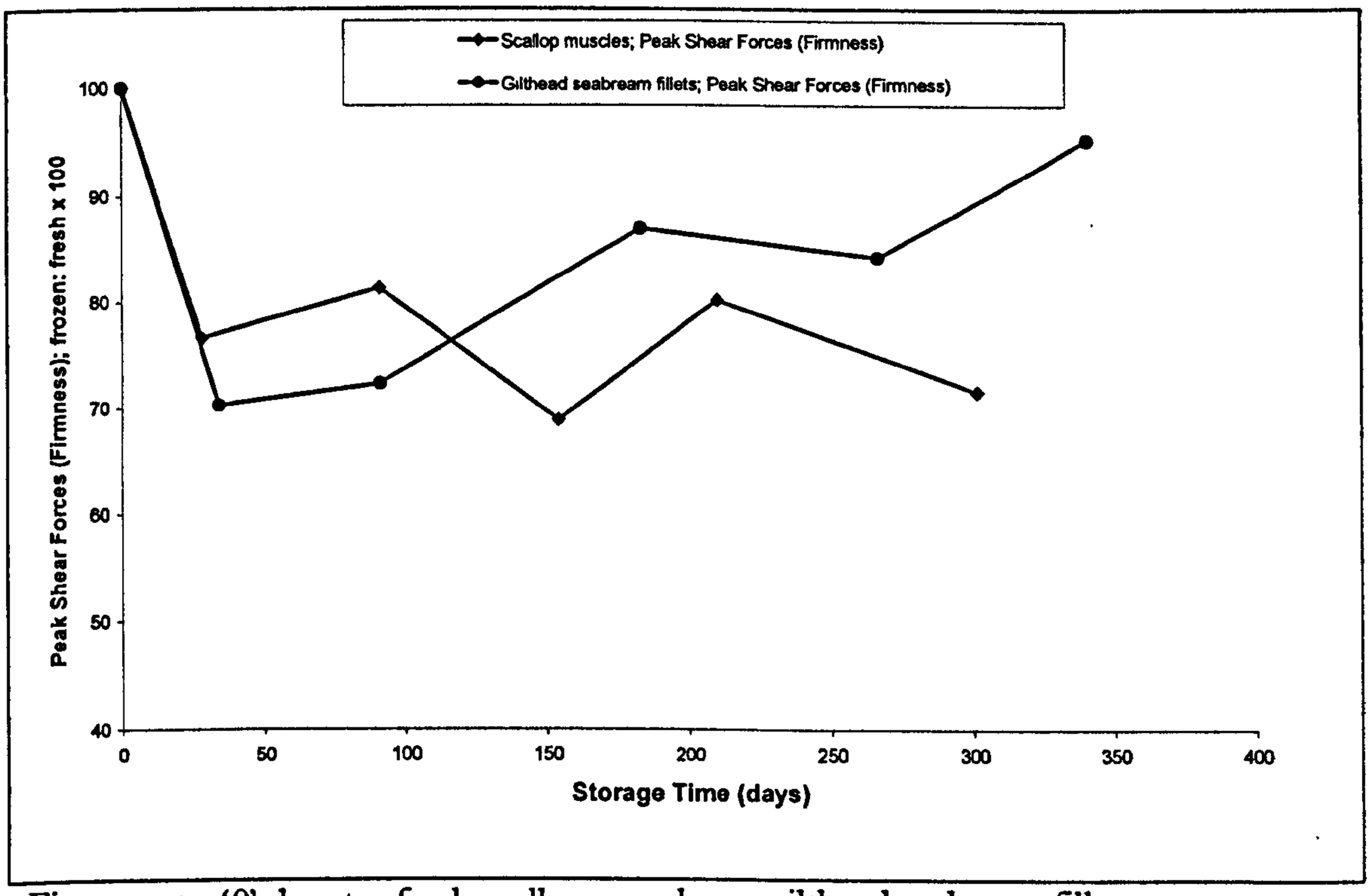


Figure note: '0' denotes fresh scallop muscles or gilthead seabream fillets.

There was no sign of a tendency for peak shear forces of scallop muscles to change in up to 301 days of storage at -22°C. In contrast, an increasing trend of peak shear forces values were obtained in the frozen gilthead seabream fillets with storage time. These results suggest that the length of time of storage at -22°C increased the firmness (or hardness) of raw frozen gilthead seabream fillets, but not that of frozen scallop muscles. As was shown earlier in this section, Ca^{2+}-ATPase activities of

actomyosins from stored frozen scallop muscles had a greater decreasing rate compared to those of stored frozen gilthead seabream fillets. This may imply a higher rate of denaturation of myofibrillar proteins in scallop muscles than in gilthead seabream fillets with storage time at -22°C.

Since the changes in the texture of fish muscle during frozen storage have been associated mainly with the denaturation and aggregation of myofibrillar proteins, it was expected that the peak shear forces of frozen scallop muscles would increase, and more so, than those of gilthead seabream fillets. This contradiction may be related to the differences in the content of connective tissue of scallop and gilthead seabream muscles. Fish skeletal muscle contains 3 to 10% connective tissue as opposed to 0.6% in scallop muscle (Findlay and Stanley, 1984;Mackie, 1993). Montero and Borderias (1990) suggested that collagens, which represent the main proteins of the connective tissue (Mackie, 1993), undergo denaturation on frozen storage of hake, and this is believed to contribute to an increase in the overall hardness of the stored frozen hake muscle. Thus, it is presumed that the mentioned differences in the peak shear forces between scallop muscles and gilthead seabream fillets resulted, in part at least, from the different content of collagens in their muscles.

The results of the present study show that the length of time of storage at -22°C affected the sensory texture (chewiness) and taste (flavour) of the frozen scallop muscles and gilthead seabream fillets, in agreement with the findings with other studies (see sections 3.2.2.6 and 4.2.2.10).

Figure 5.2-7 Scores of texture

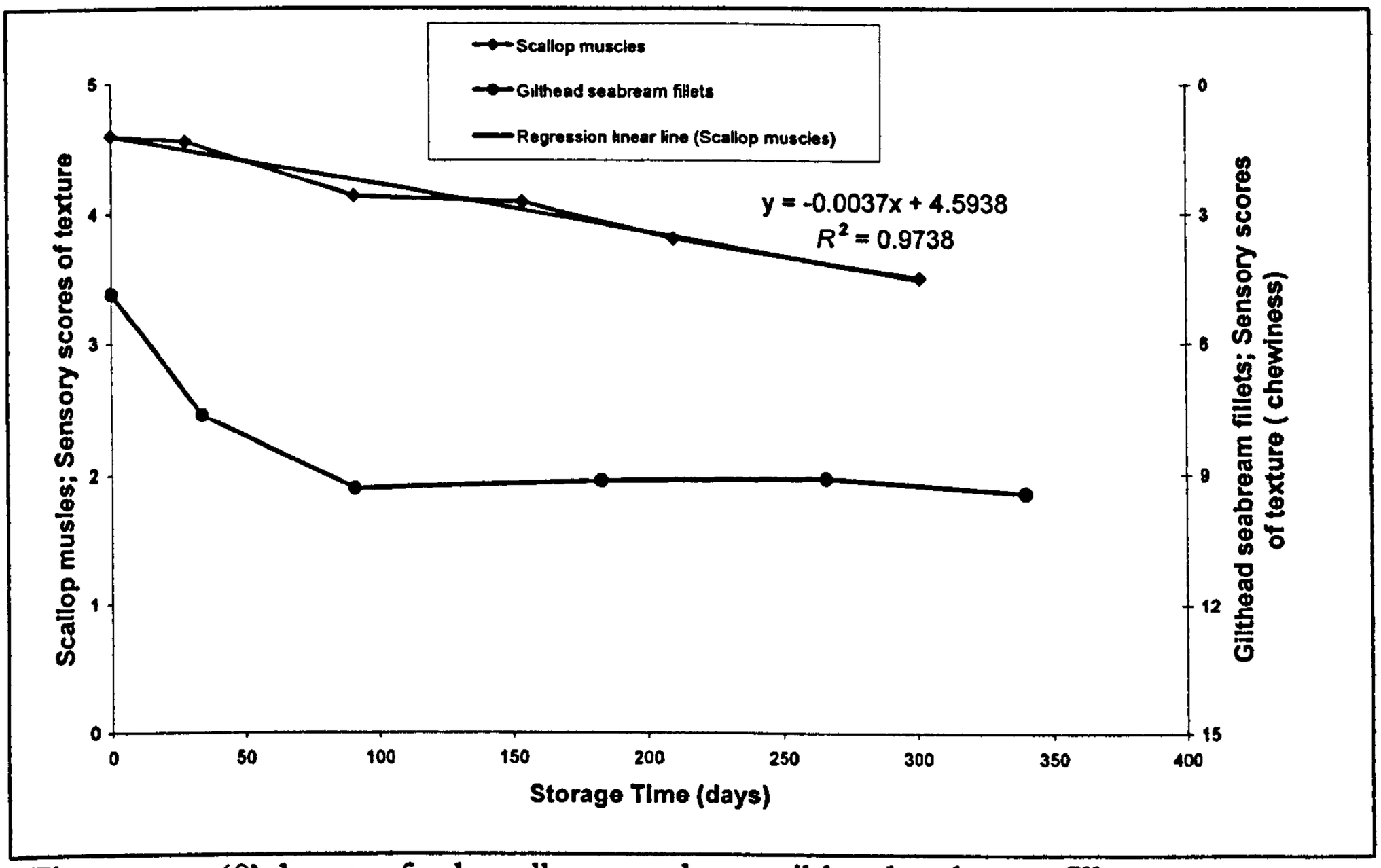


Figure note: '0' denotes fresh scallop muscles or gilthead seabream fillets.

As it can be seen from Figure 5.2-7, the sensory scores of texture of stored frozen scallop muscles decreased slightly and linearly with storage time. Thus, in stored frozen scallop muscles, the texture scores at the end of storage decreased by about 20% compared to the texture scores of fresh samples. In contrast, gilthead seabream fillets showed a greater change in the first 91 days of storage than in the remaining months of storage. By 91 days chewiness scores (or toughness scores) were very significantly more than the chewiness scores of fresh samples. These results suggest that gilthead seabream fillets were more vulnerable than scallop muscles to texture deterioration during frozen storage at -22°C. This suggestion is contradictory to the mentioned changes in water holding capacities and Ca^{2+}-ATPase activities. Thus, differences in other

parameters might have caused the dissimilarities in texture deterioration between the species tested. Differences in the degree of shrinkage of muscle fibres and the content of connective tissue may be some of the factors related to the mentioned differences in texture between the species tested (Hyldig and Nielsen, 2001).

As it can be seen from Figure 5.2-8, the scores of taste of frozen scallop muscles and gilthead seabream fillets decreased linearly with storage time. However, the rate of changes in taste was greater in gilthead seabream fillets than in scallop muscles. This difference might be explained by the higher rate of lipid oxidation observed in gilthead seabream fillets compared to scallop muscles (discussed earlier in the present section).

Figure 5.2-8 Scores of taste

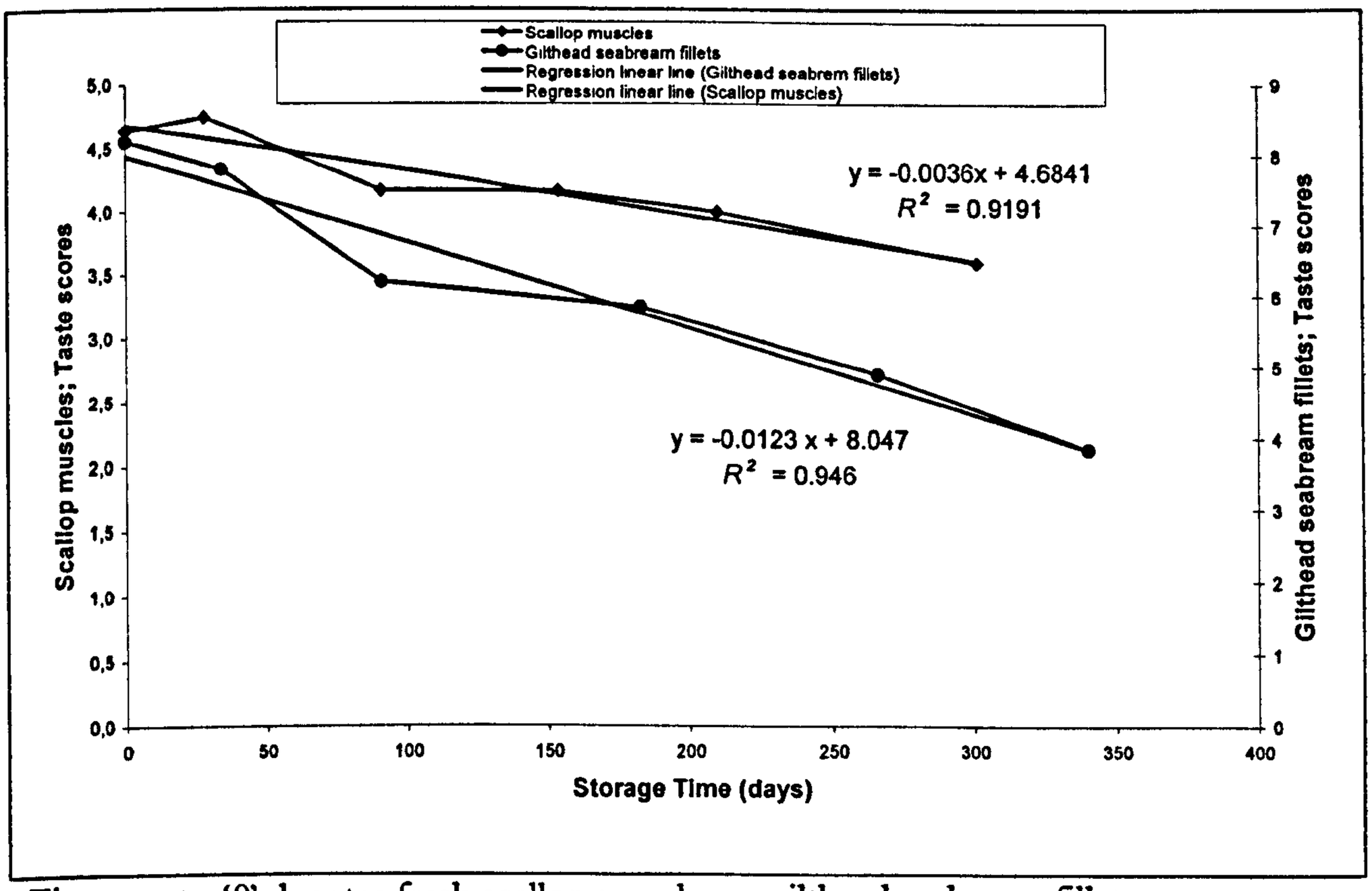


Figure note: '0' denotes fresh scallop muscles or gilthead seabream fillets.

5.3. Conclusions

The main findings from the comparison of the quality parameters obtained from frozen scallop muscles and gilthead seabream fillets are summarized as follows:

a) Effects of freezing at different freezing times:

- the freezing process itself caused the main differences in the bio-chemical and physical properties of scallop muscles and gilthead seabream fillets frozen at different characteristic freezing times;
- short characteristic freezing times (i.e. 19 minutes for scallop muscles, and 2 and 18 minutes for gilthead seabream fillets) caused less weight losses due to freezing and thawing of scallop muscles, and reduced the thawing losses of gilthead seabream fillets, compared to the longer characteristic freezing times tested; hence short characteristic freezing times may be economically advantageous in commercial freezing of both species;
- intermediate characteristic freezing times (i.e. 89 and 49 minutes for scallop muscles and 74 minutes for gilthead seabream fillets) seemed to cause more damage to intra-cellular organelles (mitochondria) of both species than the shorter and longer characteristic freezing times tested;
- there was a clear effect of characteristic freezing times on the instrumental texture of raw gilthead seabream fillets, but not of scallop muscles. Freezing at short characteristic freezing times (i.e. 2 and 18 minutes) produced raw fillets similar in texture to the fresh fillets.

Increased weight losses on freezing and thawing, release of enzymes from intra-cellular organelles, increased levels of lipid hydrolysis and deterioration in the texture of frozen seafoods may affect adversely their

quality and consequently their market.

Therefore, for all these reasons, short characteristic freezing times (equal to or less than 19 minutes) are beneficial for freezing post-rigor scallop muscles and gilthead seabream fillets for commercial purposes.

b) Effects of the length of time of storage

The length of time of storage at -22°C of frozen scallop muscles and gilthead seabream fillets affected their quality parameters as follows:

- there were changes in the integrity of the muscle tissues of both species (in terms of HADH activities for scallop muscles and AG activities and protein content of centrifugal tissue fluids for gilthead seabream fillets). These changes were less profound compared to those caused by the freezing process for both species.
- water holding parameters were reduced and there were structural changes (in terms of changes in ATPase activities) of myosins (or 'actomyosins') of both species. These changes were more profound in scallop muscles than in gilthead seabream fillets;
- there was oxidation of lipids (in terms of changes in TBARS) of both species, but the rate of oxidation of lipids seemed to be higher in gilthead seabream fillets than in scallop muscles;
- there was an increase in the peak shear forces (i.e. firmness values) of frozen gilthead seabream fillets, but not of scallop muscles;
- the sensory texture of scallop muscles and the chewiness (or tenderness) of gilthead seabream fillets were affected, but the rate of changes was bigger in gilthead seabream fillets than in scallop muscles;
- the taste of both species was affected, but these changes were more profound in gilthead seabream fillets than in scallop muscles.

Also, the results of the effects of storage time on the quality parameters of scallop muscles and gilthead seabream fillets showed that:

- frozen scallop muscles were in acceptable condition after a storage period of 301 days, with most of the changes in bio-chemical and physical properties being pronounced after 91 days of storage at -22°C;
- frozen gilthead seabream fillets were in acceptable condition up to 166 days of storage. Also, the high quality life of stored frozen gilthead seabream fillets would appear to be between 34 and 91 days (i.e. up to three months), since significant differences in scores of the acceptability of taste between the fresh and stored frozen fillets were found after 91 days of frozen storage.

Therefore, the length of time of storage at -22°C affected the quality parameters of stored frozen scallop muscles and gilthead seabream fillets. Gilthead seabream fillets appear to be generally more vulnerable to changes on storage than scallop muscles, except as regards water holding capacity parameters and Ca^{2+}-ATPase activities of myosins (or 'actomyosins') extracts. Based on sensory changes, and hence on customer acceptability, the frozen scallop muscles seemed to be more stable in storage at -22°C than the frozen gilthead seabream fillets. Keeping both seafoods frozen for up to three months at -22°C may prevent negative changes in quality parameters, which occur with longer storage.

The bio-chemical and physical parameters used in the present study to monitor the changes in the quality of stored frozen scallop muscles and gilthead seabream fillets, were related with the storage time and sensory parameters as follows:

- Ca^{2+}-ATPase activities of frozen scallop muscles showed good linear correlations with storage time and had satisfactory time-independent correlations with the scores of sensory texture over the 301 days of storage period.
- Ca^{2+}-ATPase activities, free fatty acids, peroxide values and instrumental toughness measurements of gilthead seabream fillets showed good and significant linear correlations with storage time However, Ca^{2+}-ATPase activities and instrumental toughness measurements showed poor relationships with the sensory scores of texture. The correlations of free fatty acids and peroxide values with the scores of acceptability of taste were significant, but only free fatty acids showed a strong linear correlation with the scores of acceptability of taste over the 340 days of storage period.
- Mg^{2+}-Ca^{2+}-ATPase activities of gilthead seabream fillets showed a poor linear correlation with storage time, but a satisfactory correlation with the sensory scores of chewiness.
- Thiobarbituric reactive substances (TBARS) values and activities of the enzyme β-hydroxy-acyl-coenzyme A (HADH) showed poor correlations with storage time and sensory attributes for both species.
- Salt soluble proteins, sulfhydryl (total and reactive) groups, Mg^{2+}-ATPase and Mg^{2+}-EGTA-ATPase activities, Ca^{2+}sensitivity values, and α-glucosidase (AG) and β-N-acetyl –glucosaminidase (NAG) activities of gilthead seabram fillets showed poor and/ or insignificant correlations with the storage time and sensory attributes.

The commercial significance of these results is that non-sensory parameters which show satisfactory linear correlations with storage time

and sensory parameters may be useful methods for assessing the quality of stored frozen seafoods for commercial purposes, which requires customer satisfaction.

Therefore, Ca^{2+}-ATPase activities for scallop muscles, and free fatty acids for gilthead seabream fillets, may be reliable methods for industry to use for assessing their quality during long term storage at -22°C.

Stepwise multiple linear regression analyses of the data from stored frozen gilthead seabream fillets with storage time as dependent variable and the chemical and bio-chemical parameters as independent parameters suggested that the quality (in terms of predicted storage time) of frozen gilthead seabream fillets stored at -22°C may be evaluated by a linear model that combines three relatively simple measurement techniques, i.e. determination of free fatty acids, peroxide values and protein content in centrifugal tissue fluids. This would be useful for the frozen seafood industry.

However, it must be taken into account that the changes in quality parameters with the length of time of storage of frozen seafoods are influenced by factors related to place and season of harvesting, pre-freezing and freezing conditions and storage temperature (Haard, 1992). Therefore, the effects of the mentioned factors on the quality parameters of the stored frozen gilthead seabream fillets have to be considered in the development of a robust model that could be used by industry as quality control tool.

As a starting point, the results of the present study suggest that methods related to the integrity of muscle structure and lipid degradation products may serve as tools for the frozen seafood industry to obtain predictive information about the changes in the quality of stored frozen gilthead seabream fillets.

5.4. Suggestions for further studies

Based on the results of this study, it is suggested to investigate the following:

- the effects on the quality of scallop muscles frozen at freezing times (rates) that are representatives for freezing seafoods in cryogenic freezers,
- the effects on structure and ultra-structure of scallop muscles and gilthead seabream fillets frozen at different freezing times (rates) in order to elucidate the changes in certain quality parameters (e.g. texture), which occurred on freezing in the muscles of both species,
- the effects on the quality of frozen scallop muscles stored for more than ten months at -22°C, including measurements of free fatty acids and peroxide values,
- the effects on sensory and instrumental texture profiles (i.e. textural attributes of hardness, springiness, juiciness, chewiness, greasiness) of frozen gilthead seabream fillets stored at a set temperature (e.g. -22°C) in order to examine the possibility of predicting sensory texture from instrumental measurements,
- the possibility of increasing the shelf-life of stored frozen gilthead seabream fillets by using anti-oxidant substances,
- the effects on the quality of scallop muscles and gilthead seabream fillets as related to freezing rates, thawing methods (e.g. refrigeration versus tap water or microwave) and length of time of storage at a set temperature (e.g. -22°);
- the possibility of predicting storage time of stored frozen scallop muscles and gilthead seabream fillets from non-sensory quality parameters by varying place and time of catch, pre-freezing and freezing conditions, and storage temperature.

Appendices

Appendix 1: Development of rigor mortis in raw scallop muscles

The development of rigor mortis in the scallop muscles was monitored up to 4 days in chilled storage. It was measured with a Warner-Bratzler shear triangular blade fitted in a Steven's Texture analyzer at a cross-head speed of 50 mm min^{-1} as described in section 2.1.7.6.

Twenty-five scallop muscles prepared as described in section 2.1.3.2 were divided into five lots. One lot was used after shucking for peak shear force measurements (firmness) and the other four lots were stored in glass jars, buried in crushed ice and stored in a chill room at 2°C to 4°C. These lots were analysed for peak shear force measurements after one, two, three and four days in chilled storage. The results are shown in Table 1.1.

Table 1.1 Peak shear forces of scallop muscles during ice storage, g* g^{-1}

Days in ice	*Peak shear forces*
0	161±33 a
1	112±27 b
2	119±25 b
3	88±24 bc
4	78±13 c

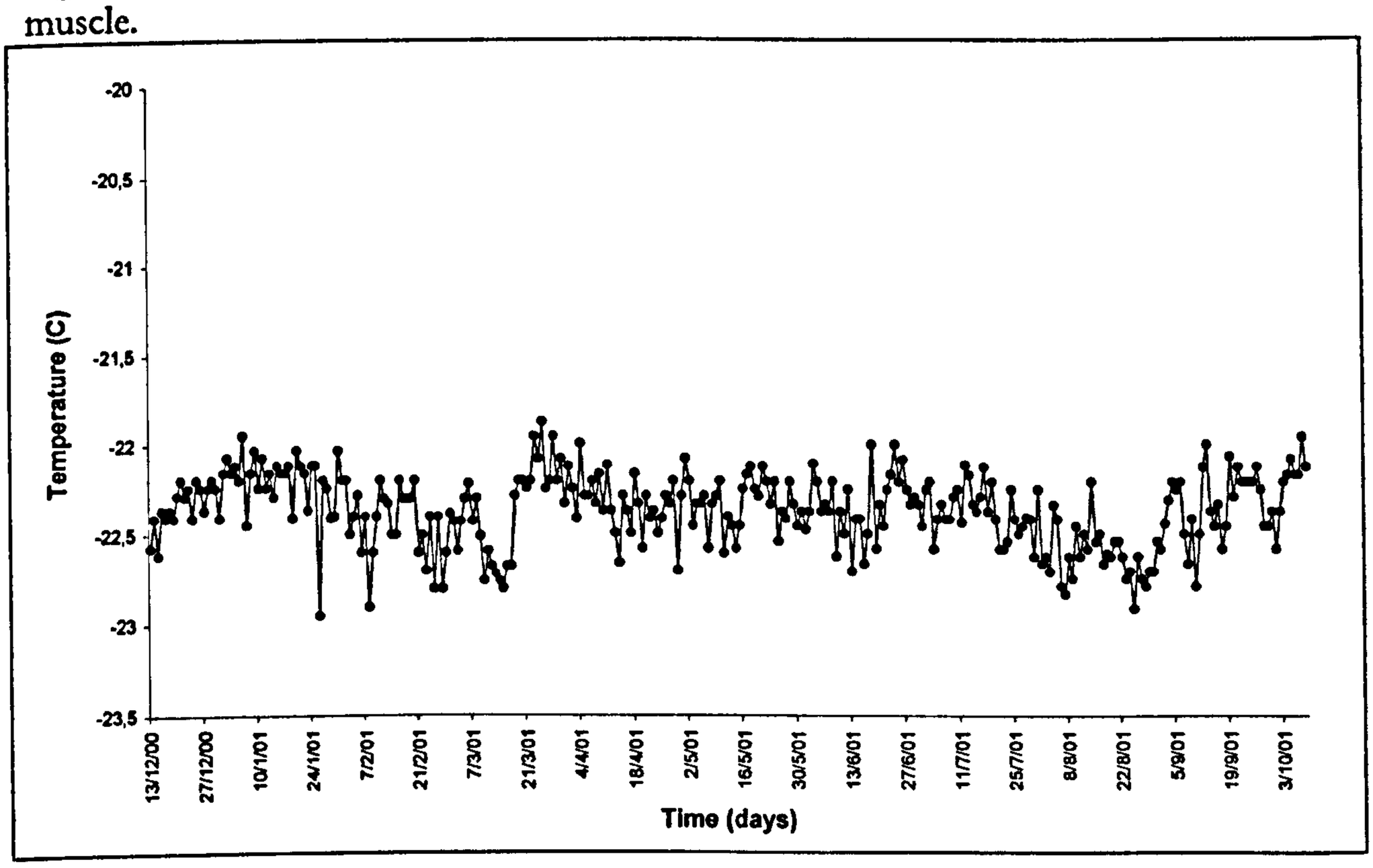
Table notes: means ± S.D., n=5. Numbers followed by different letter are significantly different ($P < 0.05$).

The mean peak shear force (g* g^{-1}) of the scallop muscles after shucking (day 0) was 161±33 (mean ± S.D.) and significantly higher ($P<0.05$) than the mean peak shear force of the scallop muscles stored in ice for one, two, three and four days.

Appendix 2: Temperature fluctuation in the freezer cabinet during storage of frozen scallop muscles

Figure 2.1 shows the daily temperatures in the freezer cabinet during storage of frozen scallop muscles. The minimum and maximum recorded temperatures in the freezer cabinet were -23°C and -21.9°C, respectively. The mean temperature in the freezer cabinet throughout the storage period was -22.4°C.

Figure 2.1 Daily temperatures in the freezer cabinet during storage of frozen scallop muscle.



Appendix 3: Scoring sheet for the triangular evaluations

Test Product: Frozen Scallop muscles (*Pecten maximus*)

Name……..

Test subject N°…..

You will receive a set of three samples. Two samples are identical and the other is different. You have the following tasks:

1) Please circle the number of odd sample

   Sample numbers

   52 54 56

2) Please circle which sample you prefer the:

   Single sample Duplicate sample

3) Which sample is more chewy, the:

   Single sample Duplicate sample

4) Which sample is more juicy, the:

   Single sample Duplicate sample

5) Which sample is more firm, the:

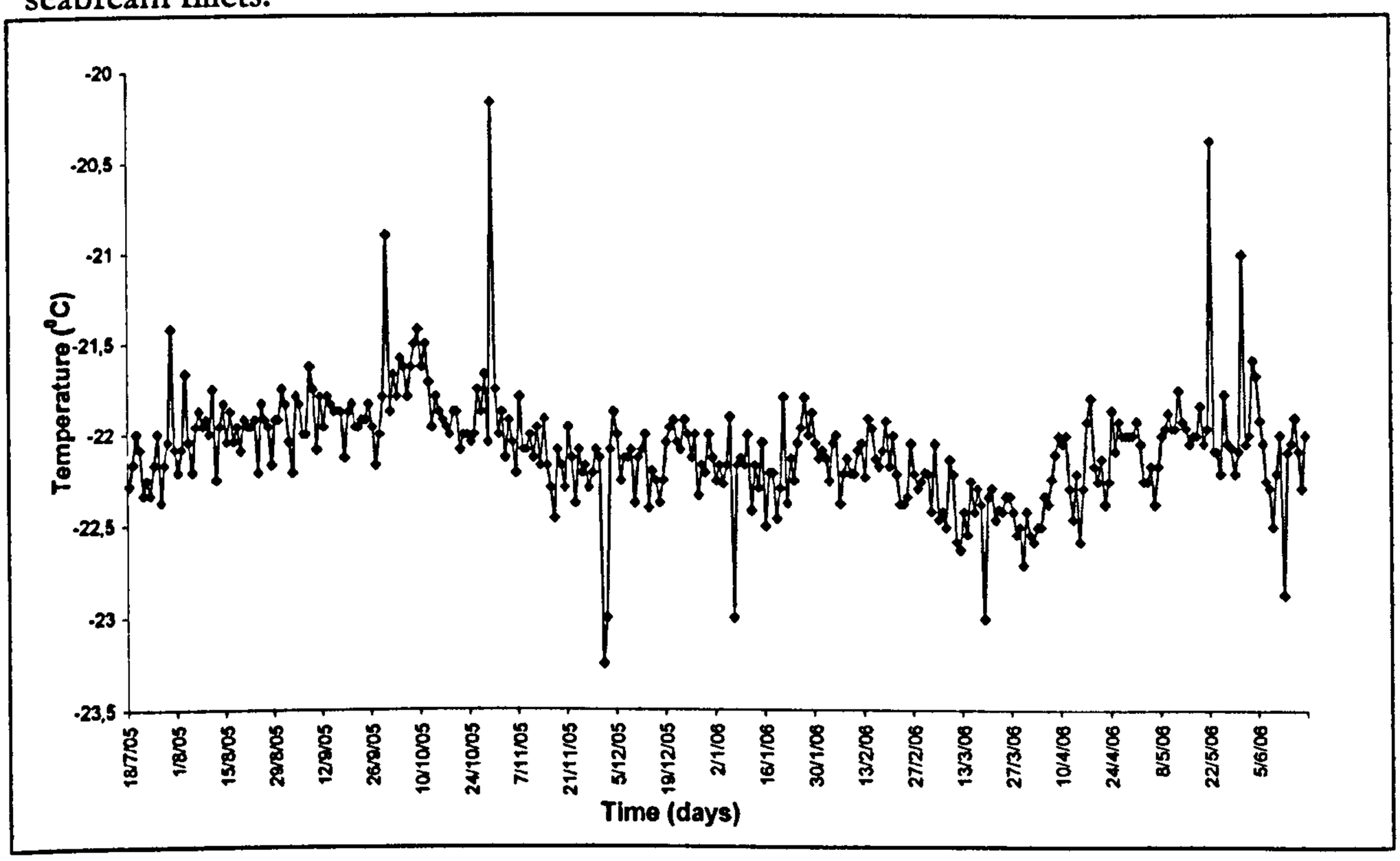
   Single sample Duplicate sample

THANK YOU

Appendix 4: Temperature fluctuation in the freezer cabinet during storage of frozen gilthead seabream fillets.

The daily temperatures in the freezer cabinet during storage of frozen gilthead seabream fillets are shown in Figure 4.1. The minimum and maximum recorded temperatures in the freezer cabinet were -23.3 °C and -20.2°C, respectively. The mean temperature in the freezer cabinet throughout the storage period was -22.1°C.

Figure 4.1 Daily temperatures in the freezer cabinet during storage of frozen gilthead seabream fillets.



Appendix 5: Training and selection of assessors for the chewiness evaluations of the gilthead seabream experiments

5.1 Selection of assessors

Six assessors (four women and two men) were selected among the staff and students of the Technological Educational Institute of Messolonghi (TEI). The selection of the assessors was based on behaviour criteria, i.e. on their availability, interest and liking of the product.

5.2 Materials and methods

5.2.1 Materials

- The reference products, used in the preliminary sessions, were purchased from a local supermarket.
- Gilthead seabreams (mean weight 410g) for the training and performance sessions were obtained from a local fish farm unit and delivered to the laboratory within one day of harvesting.
- For the training sessions, a total number of 8 fish were used. For the resolution of rigor mortis, the fish were kept for three days in ice from the time of harvest. They were then filleted, skinned, and vacuum packed in polyethylene bags and kept in a – 80°C freezer until used. The temperature of -80°C was used to preserve the fillets because it has been shown that that temperature preserves the quality of fish for one month at least (Aubourg *et al.*, 2004)
- For the performance sessions, a total number 12 fish were used. Two fish were immediately sampled, while the rest were repacked with ice into a polystyrene container provided with holes for drainage. The container was then stored in a refrigerator chamber

for a period of 13 days from the time of harvest. Ice was renewed as required. Samplings for sensory analyses took place on days 1, 3, 5, 8, 11 and 13. Each day of sampling, two fish were retrieved from the container and filleted, skinned, and vacuum packed in polyethylene bags and kept in a - 80°C freezer until used.

5.2.2 Sample preparation and cooking method

The reference products, used in the preliminary sessions, were prepared as is described in Table 5.5-1

The frozen fillets of gilthead seabream were allowed to semi-thaw at 25°C for 20 minutes. Two portions of 5 x 3 cm were cut from the anterior-middle part of each fillet. They were wrapped in aluminum foil and cooked in an oven preheated at 200°C until the temperature of the thermal centre reached 70°C. The portions were allowed to attain room temperature in a desiccator and then sliced into pieces of 1.5 to 1.7 g (from now on named 'seabream samples'). The 'seabream samples' were served at room temperature to the assessors wrapped in aluminium foil and coded with a three-digit random number. Each assessor received two to three 'seabream samples' from each fillet in each session.

5.3 Sessions

A total of 23 sessions (8 preliminary, 7 training and 8 for the evaluation of the performance of the panel) were performed over three months. The sessions (1/2 –1 hour each) were held in the morning (11.00 to 13.00) twice per week.

The first three preliminary sessions were aimed to:

- explain to the assessors the definition of chewiness (Table 5.3-1),
- select the reference products (Table 5.5-1),

- determine the evaluation procedures of the reference products and seabream samples (ISO 11036:1994; Table 5.3.-1), and
- define the final score sheet for the evaluation of the gilthead seabream samples.

Gum drops were used as a reference food in those three preliminary sessions.

Table 5.3-1. Definition and method of evaluation for the chewiness textural attribute

Sensory definition[1]	*Technique*[1]	*Manner of consumption*[2]
Mechanical textural attribute related to cohesiveness and to the length of time or number of chews required to masticate a solid product into a state ready for swallowing.	Place sample in the mouth and manipulate at one chew per second at a force equal to that required to penetrate a gum drop in ½ second, and evaluate the number of chews required to reduce to a state ready for swallowing.	Place the sample whole in mouth. Manipulate the sample with molars only. Reduce the sample as particles suspended in saliva before swallowing.

Table notes: [1] According to ISO 11036:1994 ; [2] Established by the panel of assessors.

On the following four sessions the assessors studied the chewiness attribute of reference products, most of which represented points on the reference scale of chewiness developed by Szczesniak *et. al.*, 1963 (cited in ISO 11036:1994). Peanuts chews and short textured sugar–based gums from the Szczesniak's scale were not included, since the assessors considered that seabream samples would not be chewier than the steak's samples. Moreover, they included 'cream cheese' as a low intensity reference product because they alleged that fresh 'seabream samples' could be less chewy than rye bread, i.e. the low intensity standard reference product in Szczesniak's scale. In each session, three samples of each reference product (Table 5.5-1) were evaluated. The assessors were requested to record the mean number of chews required to break down

and swallow the reference products. At the last preliminary session, the assessors tasted a variety of other foods including 'seabream samples', and then discussed their evaluations of 'seabream samples'. A structured scale with 6 categories which would correspond to the reference products, and an unstructured line scale were considered. Usually, a 10 or 15 cm unstructured line uses words to describe only the two different extremes of the attribute being assessed (e.g. very tender and very tough) anchored at or near the opposite ends of a horizontal line. Such scales produce scores of 0 to 100 or 0 to 150 and do not have the coarseness of a structured scale with several different categories. However, a drawback of using unstructured line scales is the inconsistency of the panellists during evaluations. This is due to the fact that it is more difficult for the panellists to remember a position on a line scale than to remember a number (Botta, 1995). To avoid these inconveniences and partially counteract the dispersion of the results, the assessors selected as their score sheet a 6-point semi-unstructured line scale, with the reference standard products anchored at certain intervals. This is shown in Table 5.3-2, below, and Appendix 6.

Table 5.3-2. Correspondence between intervals in structured scale and ratings in the semi-unstructured line scale (15cm) for chewiness textural attribute

Semi-unstructured line scale (15cm)	*Standard rating scale*	*Standard food*	*Mean Chews*[3]
0	1	Cream cheese[1]	6
2.5	2	Rye bread[2]	11
5.5	3	Frankfurter sausage[2]	17
9.0	4	Gum drop[2]	24
12.0	5	Starch-based gum drop[2]	30
15.0	6	Steak[2]	36

Table notes: [1] Standard food selected by the panel in the preliminary sessions, [2]Standard foods selected from the chewiness standard scale of Szczesniak *et.. al.*, 1963 (ISO 11036:1994), [3]Mean numbers of chews before swallowing established by the panel in the preliminary sessions.

Seven training sessions were carried out using fresh seabream samples. Samples were evaluated according to the procedures described above (Table 5.3-1) and using the score sheet established in the preliminary session (see Appendix 6).

The evaluation of the performance of the assessors was aimed to select the final panel for the gilthead seabream evaluations in the main experiments. In such evaluations, the assessors have to assess in more than one session six different actual samples that are of good quality. For this purpose an ice-storage experiment was performed. Fish were stored in ice for 13 days, since the upper limit of freshness of seabream stored in ice is about 14 days, as was shown by Kyrana *et al.* (1997) by using chemical, microbiological and sensory methods. Moreover, it was expected to find perceptible differences in the chewiness textural attribute between fish stored in ice for different time-periods (Huidobro 2000). For panel performance evaluation, eight sessions were performed. In each session three fillets, coming from three different batches (days in ice), were evaluated.

5.4 Statistical analyses

Standard error of the average scores of chewieness from the four preliminary and the seven training sessions was considered as an index of variability among assessors' scores (Carbonell *et al.*, 2003). ANOVA was used to analyze the data obtained by each assessor from panel performance sessions. General linear modelling (sample and assessor) was applied to the combined data from panel performance sessions to test for effects of samples, assessors, and sample-assessors interactions (ISO 8586-1:1993). Significance was accepted when $P<0.05$ (Zar, 1984)

5.5 Results and selection of the assessors

Table 5.5-1 shows the reference products used in this study, the panel performance and rating of the reference products. The mean standard error for the tasted products from the four performed sessions is shown in Table 5.5-1 and Figure 5.5-1.

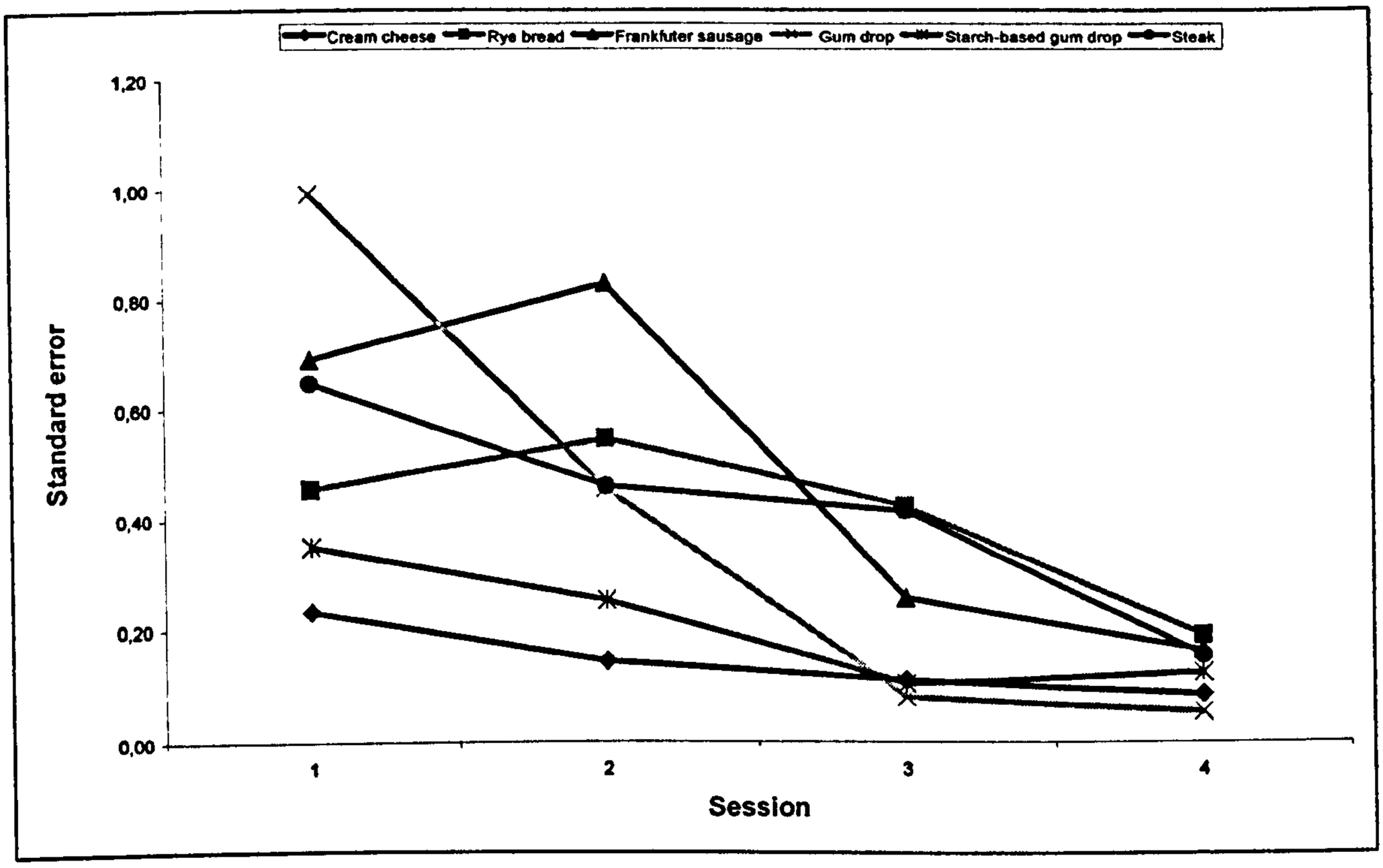
Table 5.5-1. Assessors' scoring and rating of the reference products

Reference product	Session	Assessors						Mean	SD	S.E	Overall mean (b)
		1	2	3	4	5	6				
Cream cheese	1	5,3 (a)	6,3	6	6	7	5,9	6,2	0,45	0,18	6
	2	6,0	6	6,3	6	7	6	6,2	0,40	0,16	
Sample size: 1,25 cm	3	6,3	6,7	6,7	6	6,3	6,3	6,4	0,27	0,11	
Tasted at room temperature	4	6,9	6,3	6,3	6,3	6,3	6,5	6,4	0,24	0,10	
Rye bread	1	11,3	8,8	11,4	11,0	12,6	10	10,9	1,30	0,53	11
Fresh, centre cut	2	10	10,6	9,8	8	12,3	12	10,5	1,58	0,64	
Sample size. 1,25 cm	3	12,7	12,7	12,5	13	12	9,7	12,1	1,22	0,50	
Tasted at room temperature	4	11	11	11	10	10	11	10,7	0,52	0,21	
Frankfuter sausage	1	18,3	16,2	18,4	13,3	18	18	17,0	2,00	0,82	17
Large, uncooked, skinless	2	17,5	14,2	20,1	15,4	17,3	20	17,4	2,38	0,97	
Sample size: 1,25 cm	3	17,3	17	18,5	16,3	17,7	17	17,3	0,75	0,30	
Tasted at room temperature	4	17	16	17	17,3	18	17	17,0	0,64	0,26	
Gum drop	1	20,1	20,8	26,8	24	19	22,7	22,2	2,87	1,17	24
	2	26,5	25,6	26,3	23	25	25,0	25,2	1,26	0,52	
Sample size: 1 piece	3	25,3	24,7	25	24,7	25	24,9	24,9	0,23	0,09	
Tasted at room temperature	4	25,0	24,7	24,7	25	25	24,9	24,9	0,15	0,06	
Starch-based gum drop	1	30,7	32	31	29	30,3	30,6	30,6	0,98	0,40	30
	2	31	29,7	29,5	31,3	30,7	30,3	30,4	0,72	0,29	
Sample size: 1 piece	3	30,0	30,6	30,7	30	30,3	30,4	30,3	0,29	0,12	
Tasted at room temperature	4	29,7	30,3	30,6	30	30,6	30,4	30,3	0,35	0,14	
Steak	1	36,0	38	32,5	37,0	35,3	35,7	35,8	1,87	0,76	36
Cooked in microwave 3min	2	34,3	33,7	36,3	36,7	36,7	35,9	35,6	1,29	0,53	
Sample size: 1,25 cm	3	36,3	35,7	33,7	33,7	36,3	34,9	35,1	1,20	0,49	
Tasted at room temperature	4	36,7	35	36	37	36,3	36,1	36,2	0,69	0,28	

Table notes: (a) mean number of chews before swallowing, (b) overall mean of chews before swallowing.

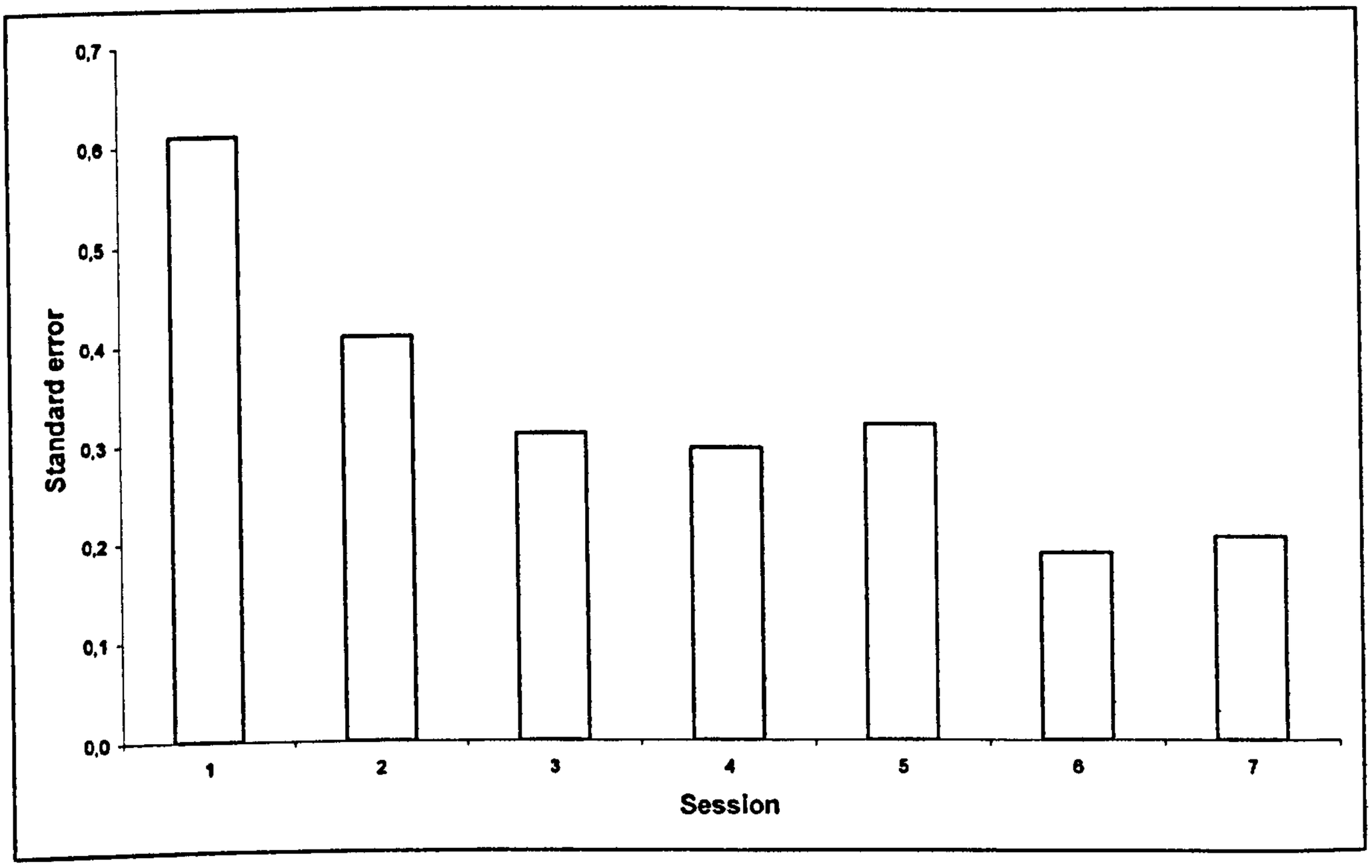
The mean standard error can be considered as an index of discordance among assessors in the evaluation of the intensity of an attribute (Carbonell *et al.*, 2003). This index decreased from a maximum value 1.17 in the first session for the gum drops to less than 0.3 for all products in the 4th session. This suggested that by the forth session the assessors had reached unanimity for the chewiness attribute of the tested reference products.

Figure 5.5-1. Variation of standard error chewiness attribute men values along preliminary sessions



The evolution along the training sessions of the standard error of mean cheweiness values is shown in Figure 5.5-2, below.

Figure 5.5-2. Variation of standard error of chewiness mean values along training sessions



The standard error of the mean which was calculated from the chewiness values of the assessors per session, decreased from a maximum value 0.61 in the first session to 0.21 in the 7th session (Figure 5.5-2). This suggested that the training led to an acceptable level of concordance for the panel of assessors with a final mean standard error of 0.2.

The assessment by each assessor of four seabream fillets from six batches of fish stored in ice for different lengths of time gave the results shown in Table 5.5-2 (individual scores and means).

Table 5.5.-2. Assessor's scores. Individual scores and means

Batches (Days in ice)	Assessor's scores												Mean
	Assessor 1		Assessor 2		Assessor 3		Assessor 4		Assessor 5		Assessor 6		
	Score	Mean	Score	Mean	Score	Mean	Score	Mean	Score	Mean	Score	Mean	
1	5.7		4.5		5.3		6.9		7.2		5.6		
	5.8		5.5		4.9		7.2		7.6		6.5		
	6.0		5.8		5.1		6.4		7.8		6.0		
	5.8	5.83	5.2	5.25	4.8	5.03	9.5	7.5	7.8	7.6	5.6	5.93	6.19
3	5.0		7.0		6.2		6.8		7.9		4.5		
	4.7		4 6		4.2		6.6		7.6		5.5		
	5.0		5.5		5.5		8.2		7.8		6.0		
	6.3	5.25	5.0	5.53	5.0	5.23	7.8	7.4	8.1	7.9	5.5	5.38	6.10
5	7.8		8.1		7.0		11.0		11.0		11.0		
	9.4		9.8		10.5		10.5		10.5		7.0		
	8.3		9.2		8.5		8.0		8.0		11.0		
	7.2	8.18	7.3	8.60	8.1	8.53	6.5	9.0	6.5	9.0	9.5	9.63	8.82
8	9.8		9.7		10.1		7.5		7.5		11.0		
	10.1		10.2		9.2		10.5		10.5		10.5		
	9.6		9.6		9.8		11.0		11.0		9.0		
	10.2	9.93	10.4	9.98	10.4	9.88	9.0	9.5	9.0	9.5	9.0	9.88	9.78
11	9.0		10.0		10.0		12.0		11.8		9.5		
	9.5		10.6		10.6		12.0		7.5		9.5		
	9.5		9.8		11.0		9.5		9.5		10.0		
	9.0	9.25	10.5	10.23	10.5	10.53	8.0	10.4	8.0	9.2	9.5	9.63	9.87
13	8.4		8.6		9.5		11.4		10.0		8.0		
	9.6		9.3		9.3		11.3		11.0		8.5		
	7.3		9.5		8.9		10.2		8.5		9.0		
	6.8	8.03	8.0	8.85	8.2	8.98	10.8	10.9	7.0	9.1	11.0	9.13	9.17
Mean	7.74		8.07		8.03		9.11		8.71		8.26		8.32

General linear modeling (GLM; Zar, 1984) of the combined data showed that samples' and assessors' variation was significant (Table 5.5-3).

Table 5.5-3. Analysis of variance. Combined data

Source of variation	Degree of freedom v	Sum of Squares, *SS*	Mean squares, *MS*	*F*
Between assessors	v=5	359.482	71.896	57.81 a
Between samples	v=5	30.31	6.062	4.87 a
Interaction	v=25	53.109	2.124	1.71 a
Residual	v=108	134.325	1.244	
Total	v= 143	577.226		

Table notes: a= significant at level P=0.05. Interaction = Interaction sample x assessor. F= group MS/residual MS.

These results indicate that the assessors as a panel differentiated successfully the samples, but one or more assessors gave scores consistently higher or lower than the others. Moreover, the interaction of sample x assessor was significant (Table 5.5-3). This indicates that two or more assessors had a different perception of the dissimilarities between two or more samples or they were in disagreement with the others about the ranking of the samples (ISO 8586-1:1993).

Table 5.5-4 shows the analysis of variance of scores from each assessor.

Table 5.5-4 Analysis of variance. Data not combined.

Source of variation	Degrees of freedom v	Assessor											
		1		2		3		4		5		6	
		MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Between samples	v=5	13.75	26.59 a	18.87	33.04 a	22 13	36.33 a	8 6	3.77a	2.47	1.07b	16.69	14.31 a
Residual	v=18	0.517		0.571		0.609		2.28		2.32		1.17	
	Residual standard deviation	0.719		0.756		0.78		1.51		1.523		1.08	

Table notes: as in Table 5.5-3. MS=Mean squares.

It can be seen that assessor 4 had high residual standard deviation, but significant variation between the samples. Assessor 5 had high residual standard deviation and insignificant variation between the samples. Assessors 1, 2, 3 and 6 had low residual standard deviations and statistically significant variation between samples. Assessors with high residual standard deviation and /or insignificant variation between samples should be considered for rejection from the panels of assessors (ISO 8586-1:1993).

By subtracting from the combined data the scores of the assessors 4 and 5, GL modeling (Table 5.5-5) revealed a significant effect only of samples.

Table 5.5-5 Analysis of variance – Combined data from assessors 1, 2, 3 and 6.

Source of variation	Degree of freedom v	Sum of Squares, *SS*	Mean squares, *MS*	*F*
Between assessors	v=5	364.406	69.281	96.78 a
Between samples	v=3	3.284	1.095	0.214 b
Interaction	v=15	10.823	0.722	0.457b
Residual	72	51.542	0.716	
Total	93	412.055		

Table notes: as in Table 5.5-3

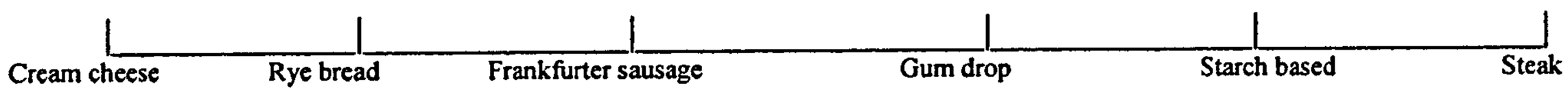
This implies that assessors 1, 2, 3, and 6 as a panel differentiated successfully the samples, they were in agreement about the ranking of samples and consistent in their scoring (ISO 8586-1:1993). Therefore, these four assessors from the initial panel were selected for the further evaluations of sea bream samples.

Appendix 6: Score sheet and instructions to the assessors.

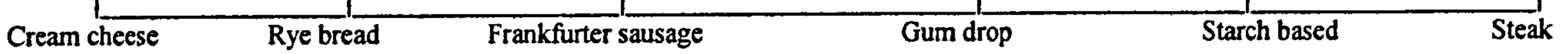
PANELIST: DATE:

Please consider the attached instructions and then evaluate the coded samples in the order, top to bottom, presented on the evaluation form. While evaluating chewiness of each sample, please place a vertical line on the location, of the line scale, that best indicates your rating of chewiness of each coded sample. Once a coded sample has been assessed, please rinse your mouth with water before you assess next coded sample.

CODE.....

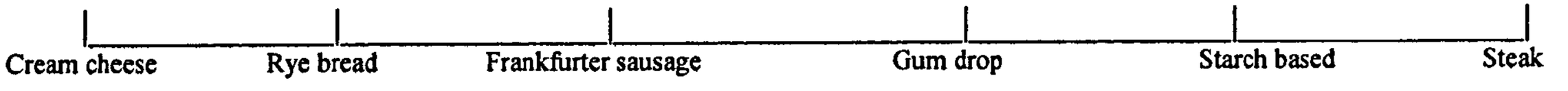


CODE.....



CODE.....

CODE.....



Instructions

Place the sample whole in mouth. Manipulate the sample at one chew per second with molars only. Evaluate the number of chews required to reduce the sample to a state ready for swallowing. Swallow the sample as particles suspended in saliva.

Before scoring, please consider the following table, which gives the correspondence between the intervals in the line scale, the standards foods and mean chews.

Correspondence between intervals in structured scale and ratings in the semi-unstructured line scale for chewiness textural attribute

Semi-unstructured line scale (15cm)	*Standard rating scale*	*Standard food*	*Mean Chews*
0	1	Cream cheese	6
2.5	2	Rye bread	11
5.5	3	Frankfurter sausage	17
9.0	4	Gum drop	24
12.0	5	Starch-based gum drop	30
15.0	6	Steak	36

Appendix 7: Score sheet for the acceptability of taste evaluations.

PANELIST: DATE:

Evaluate the coded samples in the order (left to right) presented on the evaluated form. While assessing each sample, check the statement that best describes your feelings about the taste of the sample being assessed. Once a coded sample has been assessed, please rinse your mouth with water before you assess next coded sample.

CODE_____	CODE_____	CODE_____
__Like extremely	__Like extremely	__Like extremely
__Like very much	__Like very much	__Like very much
__Like moderately	__Like moderately	__Like moderately
__Like slightly	__Like slightly	__Like slightly
__Neither like nor dislike	__Neither like nor dislike	__Neither like nor dislike
__Dislike slightly	__Dislike slightly	__Dislike slightly
__Dislike moderately	__Dislike moderately	__Dislike moderately
__Dislike very much	__Dislike very much	__Dislike very much
_ Dislike extremely	__Dislike extremely	__Dislike extremely

CODE_____	CODE_____
__Like extremely	__Like extremely
__Like very much	__Like very much
__Like moderately	__Like moderately
__Like slightly	__Like slightly
__Neither like nor dislike	__Neither like nor dislike
__Dislike slightly	__Dislike slightly
__Dislike moderately	__Dislike moderately
__Dislike very much	__Dislike very much
__Dislike extremely	__Dislike extremely

Remarks

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THE BIO-CHEMICAL AND SENSORY PROPERTIES OF GILTHEAD SEABREAM (*Sparus aurata*) FROZEN AT DIFFERENT CHARACTERISTIC FREEZING TIMES

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ABSTRACT

Skinned, vacuum packed, post-rigor gilthead seabream fillets were frozen individually with characteristic freezing times of 2, 18, 74 and 640 minutes (times to cool the thermal centre of the fillets from -1 to -7°C). Immediately after freezing, the fillets were thawed and their quality was evaluated with tests related to muscle integrity, myofibrillar protein denaturation and aggregation, lipid degradation and changes in tenderness. The muscle integrity indices (activities of α- glucosidase, β-N-acetyl-glucosaminidase and β –hydroxy-acyl-coenzyme-A dehydrogenase and the amount and protein content of centrifugal tissue fluids) showed that the freezing process itself clearly affected the integrity of muscles. Freezing of fillets with characteristic freezing times of 74 minutes caused more damage to muscles and hydrolysis of lipids than the other freezing times. ATPase activities and Ca^{2+} sensitivity of actomyosin extracts suggested that the freezing process itself, but not the freezing times, caused structural damage to myofibrillar proteins. No difference in the levels of salt soluble proteins and sulfhydryl contents in actomyosin extracts were found between the fresh and frozen fillets. Sensory evaluations showed that the cooked frozen fillets were less tender than the cooked fresh ones.

Keywords: freezing times; gilthead seabream; fillets; enzymes; actomyosin

INTRODUCTION

Freezing and frozen storage are important methods for fish preservation. However, these processes may cause structural and bio-chemical changes in frozen fish muscle that can affect certain sensory attributes of the product.

Formation of ice crystals during freezing reduces the number of free water molecules, which causes changes in structural elements. Ice crystals may, also, grow under certain conditions and cause mechanical damage of cell membranes (Huber *et al.* 1979). Therefore, freezing and thawing 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 freeze-damage of fish muscle can be studied by the activities of enzymes in muscle tissue fluids, enzymes that in fresh tissue are retained in sub-cellular organelles. The enzymes β-N acetyl-glucosaminidase and α-glucosidase are not specifically associated with any particular sub-cellular organelle, but the activity of these enzymes under acid assay conditions is regarded as evidence of lysosomal activity i.e. cell damage (Barrett and Heath 1977).The activities of β-N acetyl-glucosaminidase and α-glucosidase in fluids obtained by centrifugation i.e. 'centrifugal tissue fluids', and the volume and protein content of the fluids have been found to correlate with the cellular damage caused by different freezing and thawing treatments in several fish species (Nilsson and Ekstrand 1993, 1994; Benjakul and Bauer 2000). Moreover, the activity of the mitochondrial enzyme β –hydroxy-acyl-coenzyme-A dehydrogenase has been regarded as a measure of the damage caused in mitochondria by various freezing and thawing treatments of meat products (Gottesman and Hamm 1983).

The decrease in the water holding capacity of fish muscle after thawing is one of the most obvious effects of freezing. Such a decrease is associated with the fact that during freezing water-protein associations are replaced by protein-protein associations or other interactions (Hamm 1975). The alterations in texture and water holding capacity of fish muscle during freezing and cold storage have been associated with changes in myofibrillar proteins (Haard 1992). Such changes 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 etc (Shenouda 1980).

Cellular disintegration during freezing 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 sensory properties during freezing and 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.

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ORIGINAL

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. Some recent studies have shown, also, that frozen storage can be a commercial alternative for marketing of whole gilthead seabream (Tejada *et al.* 2003; Huidobro and Tejada 2004). However, there is hardly any information available about the effects of the freezing process on the integrity, physicochemical and sensory properties of gilthead seabream fillets. This would be useful for achieving optimal conditions for freezing fillets of this species for commercial purposes.

Therefore, in the present study the effects of freezing times on the quality of gilthead seabream fillets were studied in regard to the integrity of muscle structure, myofibrillar protein denaturation and aggregation, lipid degradation and sensory changes.

MATERIALS AND METHODS

Chemicals

p-nitrophenyl-α-glucopyranoside, *p*-nitrophenyl-*N*-β-D-glucose amide, acetoacetyl Coenzyme A, calcium chloride, magnesium chloride, ethyleneglycol bis (β-aminoethyl ether) N,N,N′,N′ -tetraacetic acid (EGTA), 5,5′ dithiobis 2- nitrobenzoic acid (DTNB), urea and adenosine 5- triphoshate (disodium salt; ATP) and β-nicotinamide adenine dinucleotide reduced form (pre-weighed vials) were purchased from Sigma-Aldrich, Germany.

Raw material origin

A total number of twenty gilthead sea breams (*Sparus aurata*; average weight and length 412 g and 282 mm, respectively) were obtained in two batches of ten fish. The fish were produced in 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 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 63.4 + 6 g (average+ S.D.).

Freezing, thawing and handling of thawed samples

The fillets were divided into five groups. In each group there were fillets coming from four different fish. The fillets from the first group were analyzed as unfrozen controls and the others were frozen until the thermal centre reached -20°C as follows: a) in methanol pre-cooled at -80°C, b) in a -80°C ultra-freezer, c) in a -20°C freezer, as still sharp freezing and d) in a -20°C freezer insulated in a polystyrene container. Immediately after freezing, the vacuum packed fillets were allowed to thaw in a refrigerator chamber at 4 to 7°C overnight (12 hours). The thawed samples were re-weighed for thaw loss determinations and then the fillets of the first batch of ten fish were sliced in three equal sized portions as follows: a) the anterior portion, which was used for extraction of lipids, 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 minutes 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) and the extract for β –hydroxy-acyl-coenzyme-A dehydrogenase (HADH) assay. The CTF and the extracts for HADH determination were stored at -80° C until analysis.

The thawed fillets of the second batch were stored whole at - 80°C until they were used for sensory evaluations.

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

Freezing times and rates determinations

All temperature measurements were done with K type thermocouples (0.5 mm, Comark Instruments, U.K) and a recording thermometer (Comark KM1242, Comark Instruments, UK). The height of the thickest part of the fillet i.e. under the dorsal fin and just above the lateral line, was measured by using a vernier and the thermocouple was placed in the centre of that part

The freezing time (t_e) was calculated as the time (minutes) required to decrease the temperature of the thermal centre from an average initial temperature of 5±1°C to a final temperature of -20°C following the recommendations of the International Institute of Refrigeration (1986). The freezing rates at the thermal centre, expressed as cm/h, were obtained from the ratios of the distance from the surface to the thermal centre of the fillets and the freezing times (in hours; Chen and Pan 1995). The characteristic freezing time (t_c) was calculated according to Bevilaqua *et al.* (1979) as the time (in minutes) for which the thermal centre of the fillet was in the temperature range of maximal ice crystallization i.e. from -1 to -7°C.

Thawing losses determinations

Free thaw drip was determined by weighing samples before freezing and after thawing. The results were expressed as g of weight loss per kg of weight prior to freezing.

Centrifugal tissue fluids determinations

Centrifugal tissue fluids 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.

Determination of the protein content in the centrifugal tissue fluids

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 and β-N-acetyl –glucosaminidase activity assay

α-Glucosidase (AG; EC 3.2.1.20) and *β-N*-acetyl-glucosaminidase (NAG; EC 3.2.1.30) were measured spectrophotometrically using *p*-nitrophenyl-α-glucopyranoside and *p*-nitrophenyl-*N*-β-D-glucose amide as substrates respectively. The assays were performed according to Nilsson and Ekstrand (1993) and activities were calculated according to Benjakul and Bauer (2000). Results were expressed as mU per g of tissue.

β–Hydroxy-acyl-coenzyme-A dehydrogenase activity assay

Two slices of seabream muscle of approximately 2 g each were used for the preparation of the filtrate for the HADH (EC 1.2.35) assays according to Fernadez *et al.* (1999). The HADH released in the filtrate was assayed according to Fernadez *et al.* (1999). Results were expressed as mU per g of tissue

Salt soluble protein

Extracts 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 1984).The results were expressed as the ratio of salt soluble protein to total protein.

Total and surface (reactive) sulfhydryl determinations in actomyosin

Actomyosin was prepared according to the method of MackDonald and Lanier (1994) using 4 g of chopped muscle. The determination of sulfhydryl (SH) contents in actomyosin was carried out according to the titration method of Ramirez *et al.* (2000). 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 of actomyosin

ATPase activity assays were carried out at 0.06 M KCl and pH 7.0 (25mM Tris maleate) according to Herrera (1993). The substrate for the reactions was 1 mM ATP. The remaining conditions were: 5mM calcium chloride and 0.5 mg protein/ml for Ca^{2+}-ATPase; 1mM magnesium chloride and 0.2 mg protein/ml for Mg^{2+}-ATPase; 5mM calcium chloride, 1mM magnesium chloride and 0.2 mg protein/ml for Mg^{2+}-Ca^{2+}-ATPase; 1mM magnesium chloride, 0.5 Mm EGTA and 0.7 mg protein/ml for Mg^{2+}-EGTA-ATPase. Samples were incubated for 3 minutes at 25°C and the reaction was stopped by adding 5ml of a chilled 15 % (w/v) trichloroacetic acid (TCA) solution to 10 ml of the reaction mixture. The reaction mixtures were centrifuged at 28,000 g for 20 minutes at 4°C. 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.
Ca^{2+} sensitivity was calculated according to Benjakul *et al.* (1977) as follows:

$$Ca^{2+}\ \text{sensitivity} = \left(1 - \frac{Mg(2+) - EGTA - ATPase\ activity}{Mg(2+) - Ca(2+) - ATPase\ activity}\right) \times 100$$

Determination of free fatty acids

The extraction of lipids was performed according to the method of Bligh and Dyer (1959) using 10g of seabream tissue. Total free fatty acid (FFA) determinations were performed according to Lowry and Tinsley (1976). The concentration of free fatty acids in the extracted lipids was expressed as g of FFA per kg of lipid.

Sensory evaluations

Fresh gilthead seabream fillets and those frozen by the air-freezing methods were evaluated for the chewiness textural attribute (ISO 11036:1994). The evaluations were performed by four assessors trained in applying scales with reference food products according to ISO 8586-1:1993. For the sensory evaluation of gilthead seabream fillets, two portions of approximately 10 x 3 cm were removed from the dorsal part of each fillet. They were wrapped in aluminum foil and baked in a preheated laboratory oven at 200°C. During baking, the temperature of the thermal centre of each sample

was monitored using a recording thermometer. Once the centre temperature of the samples reached 70°C, they were transferred to a desiccator and allowed to cool for one hour. The portions were then sliced into test portions of 1.5 to 1.7g, and served to the assessors wrapped in aluminium foil and coded with a three-digit random number. Each assessor was given two to three test portions of each fillet. At each session, one fresh seabream fillet and three from different frozen groups were evaluated. Gilthead seabream fillets were evaluated in four sessions. For the evaluations, a 6-point semi-unstructured 15 cm line-scale, with reference standard products anchored at certain intervals, was used (Table 1). After tasting, each assessor was asked to place a vertical line on the scale that best indicated his/her rating of chewiness of each sample.

TABLE 1.
CORRESPONDENCE BETWEEN INTERVALS IN STRUCTURED SCALE AND RATINGS IN THE SEMI-UNSTRUCTURED LINE SCALE FOR CHEWINESS TEXTURAL ATTRIBUTE

Semi-unstructured line scale (15cm)	*Standard rating scale*	*Standard food*	*Mean Chews****
0	1	Cream cheese*	6
2.5	2	Rye bread**	11
5.5	3	Frankfurter sausage**	17
9.0	4	Gum drop**	24
12.0	5	Starch-based gum drop**	30
15.0	6	Steak**	36

* Standard food selected by the panel in the preliminary sessions.
** Standard foods selected from a chewiness standard scale (ISO 11036:1994).
*** Mean numbers of chews before swallowing established by the panel in preliminary sessions.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to test for freezing time effects on physical, chemical and biochemical parameters measured. General linear (GL) modelling (samples and assessors) was applied to the scores from sensory analyses to test for effects of freezing times on seabream samples, assessors and assessor-sample interactions. All ANOVAs showing significant differences were followed by a Tukey HSD post-hock test. Significance was accepted when $P<0.05$.

RESULTS AND DISCUSSION

Freezing times and rates

In the commercial fish industries, fish products are frozen mostly at rates of 3 to 10 cm/h in liquefied gases or 0.3 to 3 cm/hr in mechanical freezers. Freezing rates of around 0.2 cm/hr can be obtained by bulk freezing of fish in batch air-blast rooms (International Institute of Refrigeration 1986) Although rapid and quick freezing is a common commercial practice for freezing fish, slow freezing has been recorded in badly designed and operated freezers. Therefore, the experimental conditions for freezing of gilthead seabream fillets of the present study produced freezing times and freezing rates (Table 2) that are representative of commercial practice of fish freezing.

TABLE 2.
EXPERIMENTAL CONDITIONS FOR FREEZING FILLETS OF GILTHEAD SEABREAM*

Freezing Temperature (°C)	*Freezing Environment*	*Freezing Time*** (t_e, min)	*Freezing Rate**** (r_e, cm/h)	*Characteristic Freezing Time***** (t_c, min)
-80	Methanol	4******	8.43	2
	Still Air	27.2	1.35	18
-20	Still Air	154	0.20	74
	Insulated in container	1175	0.03	640

* Mean height of the thickest part of fillets was 12 mm.
** From an initial temperature 5°C to reach central temperature -20°C (International Institute of Refrigeration 1986)
*** Freezing rate was calculated by dividing the half-thickness of the thickest part of the fillet by freezing time (Chen and Pan 1995)
**** From -1°C to reach central temperature -7°C (Bevilaqua *et al.* 1979)
***** Mean values, n=2 to 8.

Effect of freezing times on muscle integrity

In fresh tissue AG, NAG and HADH enzymes are retained in the intracellular organelles. However, AG, NAG and HADH activities were found in the extracts of fresh gilthead seabream fillets (Table 3 and Figure 1). These results suggest that lysosomes and mitochondria were damaged at the surfaces where the gilthead seabream samples were cut, and thus a certain amount of the enzymes had leaked from damaged organelles into the muscle (Gottesman and Hamm 1983). Moreover, autolysis of the gilthead seabream muscle could have caused disruption of some lysosomes and mitochondria,

since the fresh fish were stored in ice for three days prior to analysis (Rehbein *et al.* 1978; Nilsson and Ekstrand 1993)

The amount and the protein content in centrifugal tissue fluids (CTFs) and the activities of the enzymes AG, NAG and HADH in frozen and thawed gilthead seabream fillets were significantly higher than those in fresh fillets ($P<0.05$; Tables 3, 4; Figure 1). Similar results for other fish species and shellfish have been reported by other workers (Rehbein *et al.* 1978; Nilsson and Ekstrand 1993, 1994; Dulfos *et al.* 2002). Altogether these findings indicate that the freeze-thaw process itself affects the integrity of cells of gilthead seabream fillets. The freeze damage of cells can be due to denaturation of cellular wall proteins by the concentration of salts and/ or disruption of cells by ice crystals (Love 1996).

The protein content of CTF from gilthead seabream fillets frozen at the characteristic freezing time (t_c) of 74 minutes was significantly higher than that of the other times ($P<0.05$; Table 3). This could be the result of either release of intra-cellular fluids, which accounts for most of the protein present in the exudates, or by the presence of cell fragments, as the result of ice particles breaking the cell walls (Grigler and Dawson 1968). The activities of AG and HADH enzymes from the fillets frozen at the characteristic freezing time (t_c) of 74 minutes were higher compared to those of the other times ($P<0.05$; Table 4 and Figure 1). These results suggest that freezing of gilthead seabream fillets at the characteristic freezing time (t_c) of 74 minutes caused more damage to cells than freezing at the other times. Love (1955) showed that freezing of post rigor fillets at times from 80 to 120 min resulted in higher amounts of intra-cellular material (specifically DNA) in exudates than freezing at shorter or longer times. He suggested that the cell damage of cod fillets frozen at those times was due to the rupture of sarcollemas by large intra-cellular masses of ice. Therefore, the gilthead seabream fillets frozen at a time (t_c) of 74 minutes might have frozen fast enough to form intra-cellular ice, but the fillets continued to freeze at a much slower rate such that the intra-cellular ice might have time (154 min) to grow to disruptive size and rupture the cells before the fillets reached at -20°C.

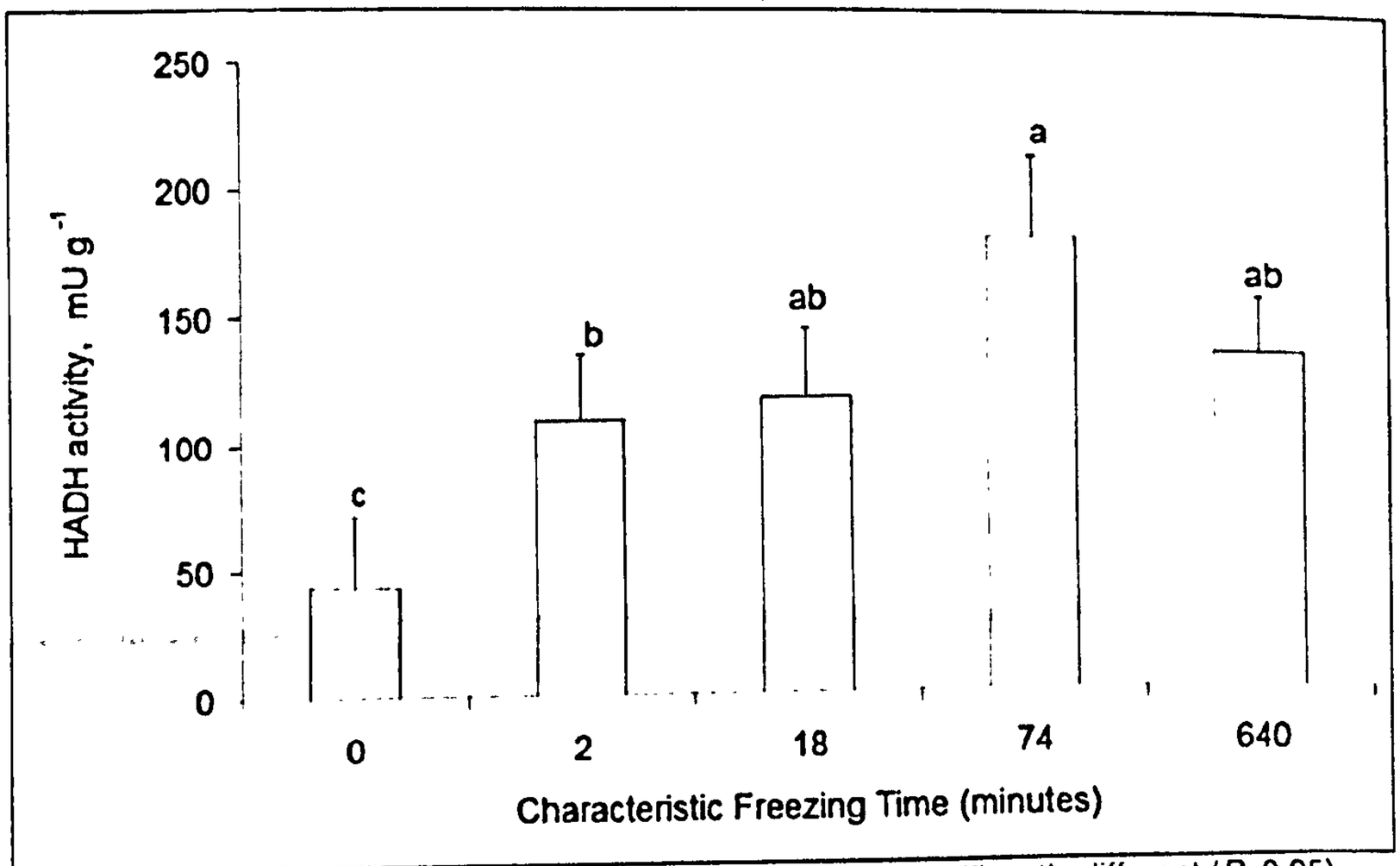
TABLE 3.
THE EFFECT OF CHARACTERISTIC FREEZING TIMES (t_c) ON PROTEIN CONTENT IN CENTRIFUGAL TISSUE FLUID, α-GLUCOSIDASE (AG) AND β-N-ACETYL –GLUCOSAMINIDASE (NAG) ACTIVITIES*

Freezing Time (t_c, min)	*Protein content ($g\ kg^{-1}$)*	*AG activity ($mU\ g^{-1}$)*	*NAG activity ($mU\ g^{-1}$)*
0	13.8± 2.02 c	0.328 ± 0.021 c	2.128 ± 0.172 b
2	17.9 ± 1.47 b	0.906 ± 0.059 b	6.232 ± 0.390 a
18	17.1 ± 2.16 b	1.047 ± 0.050 ab	6.180 ± 0.414 a
74	21.5 ± 2.41 a	1.115 ± 0.073 a	6.702 ± 0.358 a
640	18.2 ± 0.86 b	0.916 ± 0.037 ab	6.288 ± 0.504 a

*Means ± S.D, n=4.Different letters (a, b, c) in the same column denote a significant difference ($P<0.05$).

In the present work, data from CTF, AG, NAG and HADH did not produce evidence of differences in cell-damage of gilthead seabream fillets frozen at 2, 18 and 640 minutes (P>0.05; Tables 3 and 4; Figure 1). Love (1955) showed that post-rigor cod fillets, frozen at times (times to cool the centre of the fillets from 0 to -5°C) of less than 20 minutes, contained small intra-cellular ice crystals, which were distributed fairly evenly throughout the cells, and exhibited little change in cell structure. In addition, Love (1958) showed that freezing cod fillets at very long freezing times (more than 500 minutes) resulted in large inter-cellular ice masses.

FIGURE 1.
THE EFFECT OF CHARACTERISTIC FREEZING TIMES (t_c) ON HADH ACTIVITY*



*Means ± S.D, n=4. Groups with different letters (a, b, c) are significantly different (P<0.05).

Those fillets showed less cell damage than fillets frozen at intermediate rates. In the present study, the gilthead seabream fillets frozen at the fast freezing times (t_c values) of 2 and 18 minutes would have intra-cellular ice crystals. These ice crystals would be small in size since it took only 4 and 27 minutes to freeze the fillets at -20°C. Consequently, any small ice crystals formed as a result of fast freezing would cause little damage in the cell structure of the fillets. On the other hand, fillets frozen at the very slow freezing time (t_c) of the 640 minutes, would have inter-cellular ice that led to internal water diffusing out of the cells, leaving little ice in the cells. Therefore, the very slow freezing process would prevent the formation of large destructive intra-cellular ice crystals. As the result shows, the cell damage in the fillets frozen at characteristic freezing time (t_c) of 640 minutes did not seem to be different from the damage of fillets frozen at times of 2 and 18 minutes.

Overall, these results show that the freezing process itself clearly affected the integrity of gilthead seabream muscle cells. Moreover, freezing of gilthead seabream fillets at the characteristic freezing time (t_c) of 74 minutes caused more damage to cells than the other freezing times, shorter or longer.

Effects of freezing times on thaw losses, centrifugal tissue fluids and total weight losses

Thaw losses of fillets frozen at a characteristic freezing time (t_c) of 640 minutes were higher than those of fillets frozen at the other freezing times and significantly more compared to those of fillets frozen at t_c values of 2 and 18 minutes (P<0.01; Table 4). Several other studies have reported increases in thaw losses of meat products with characteristic freezing times (Aurell *et al. 1976;* Khan and Lentz 1977; Petrovic *et al.* 1993; Chevalier *et al.* 1999). These differences are attributed to changes in mainly myofibrillar proteins and / or to distortion and destruction of muscle fibres by ice-crystals (Añòn and Calvelo 1980; Wagner and Añòn 1985; Petrovic *et al.* 1993). As will be shown in the following sections, the freezing times tested did not seem to cause an irreversible denaturation of myofibrillar proteins (in terms of changes in salt soluble proteins, sulfhydryl groups, ATPase activities and Ca^{2+} sensitivities of actomyosin), possibly because no storage time was included in the present study. Thus, the differences in the amount of exudates released from the fillets during thawing might be due to differences in the size and location of ice crystals and to differences in the mechanical damage of muscle fibres, as discussed earlier in this work. It is, therefore, likely that the inter-cellular water, formed by melting of the large ice crystals in the inter-cellular spaces of slowly frozen fillets, was not as well reabsorbed by the muscle cells as the water formed by melting of the intra-cellular ice crystals in the fillets frozen at the other freezing times. It was presumed earlier in this study that large intra-cellular ice crystals might have ruptured the cells of the 74 minutes fillets. As a consequence of such cell damage and the size of ice crystals as well, there was more drip loss during thawing of the fillets frozen at that time compared to those frozen at 2 and 18 minutes. Similar suggestions are reported by other authors (Bevilaqua *et al.* 1979; Añòn and Calvelo 1980; Petrovic *et al.* 1993).

TABLE 4.
THE EFFECT OF CHARACTERISTIC FREEZING TIMES (t_c) ON THAWING LOSSES, CENTRIFUGAL TISSUE FLUIDS AND TOTAL WEIGHT LOSSES, g kg^{-1} *

Freezing Time (t_c, min)	*Thawing Losses*	*Centrifugal Tissue Fluids*	*Total Weight Losses*
0	-	121.1 ± 18.55 b	121.1 ± 18.55 b
2	21.6 ± 4.35 b	157.1 ± 10.62 a	178.7 ± 9.10 a
18	21.7± 9.28 b	152.4 ± 22.68 a	174.0 ± 29.52 a
74	33.4 ± 13.54 ab	168.3 ± 9.32 a	201.7 ± 21.76a
640	59.4± 8.66 a	137.8 ± 12.21ab	197.2 ± 8.75 a

*Means ± S.D, n=4.Different letters (a, b) in the same column denote a significant difference (P<0.05)

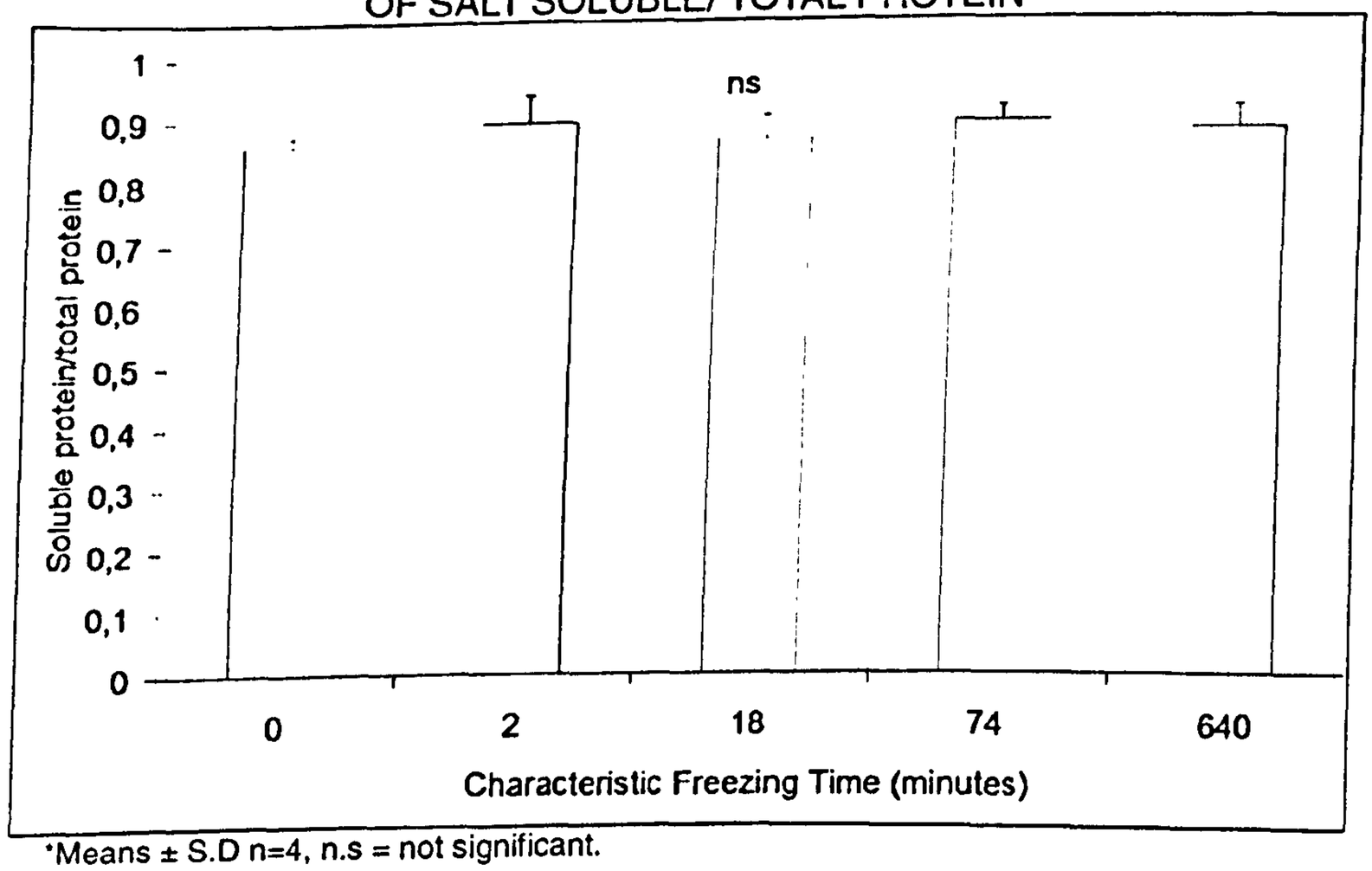
In contrast to thaw losses, centrifugal tissue fluids (CTFs) obtained from gilthead seabream fillets frozen at characteristic freezing time (t_c) of 640

minutes were less compared to those of the other freezing times (Table 4). Several other investigations have shown that slow freezing results in more thawing losses than quick freezing and in less expressible fluid in frozen meat products (e.g. Khan and Lentz 1977). In the present study centrifugal fluid measurements were taken after thawing, i.e. after the fillets had released part of the unbound or loosely bound water. These measurements can be considered as indicators of the state of the remaining quantity of loosely bound water in thawed fillets, as reported by Petrovic *et al.* (1993) for frozen beef. Therefore, the low CTF value of the fillets frozen at a characteristic freezing time (t_c) of 640 minutes is probably due to the losses that these fillets suffered during thawing. Since water holing capacity of a meat product is indicative of the loosely bound water to protein (Trout 1988), the total losses during thawing and centrifugation were taken as a measure of the water holding capacity (WHC) of the fresh and frozen gilthead seabream fillets. The total weight losses were significantly higher in frozen than in fresh seabream fillets ($P<0.05$; Table 4), but differences between the frozen groups themselves were not observed. Since changes in WHC are very sensitive indicators of the changes in the charges and structure of myofibrillar proteins (Mackie 1993), the results suggest that the freezing process itself, but not the freezing times, affected the properties of myofibrillar proteins of seabream fillets. This suggestion was further investigated by analyses related to myofibrillar protein denaturation and aggregation.

Salt soluble proteins

The results of the present study showed no significant differences between the fresh and frozen gilthead seabream fillets in regard to salt soluble protein ($P>0.05$; Figure 2). This is in agreement with Wagner and Añòn (1985) who found no significant differences in salt soluble proteins of beef frozen at different freezing times. Pan and Yeh (1993) investigating the effects of freezing methods on the solubility of myofibrillar proteins of grass shrimp, showed that there were no significant differences immediately after freezing between shrimps frozen at rates from 3.41 to 16.1 cm/h. Also, Tejada *et al.* (2003) showed that there were no significant differences in the salt soluble proteins between gilthead seabreams fresh and frozen at -20°C when fish were thawed immediately after freezing. Altogether, these findings suggest that the freezing process itself and the tested freezing times did not cause aggregation of myofibrillar proteins of gilthead seabream fillets immediately after freezing. This suggestion could be associated with the very short time the fillets remained in the frozen state. Love (1958) and Love and Ironside (1958) found that there were no consistent differences in salt soluble protein of cod fillets frozen at different times and thawed immediately after freezing; however, when cod fillets were kept for one year at -29 °C, a temperature at which the pattern of ice crystals maintained intact, differences in soluble protein were observed according to the original rates of freezing.

FIGURE 2
THE EFFECT OF CHARACTERISTIC FREEZING TIMES (t_c) ON THE RATIO OF SALT SOLUBLE/ TOTAL PROTEIN*

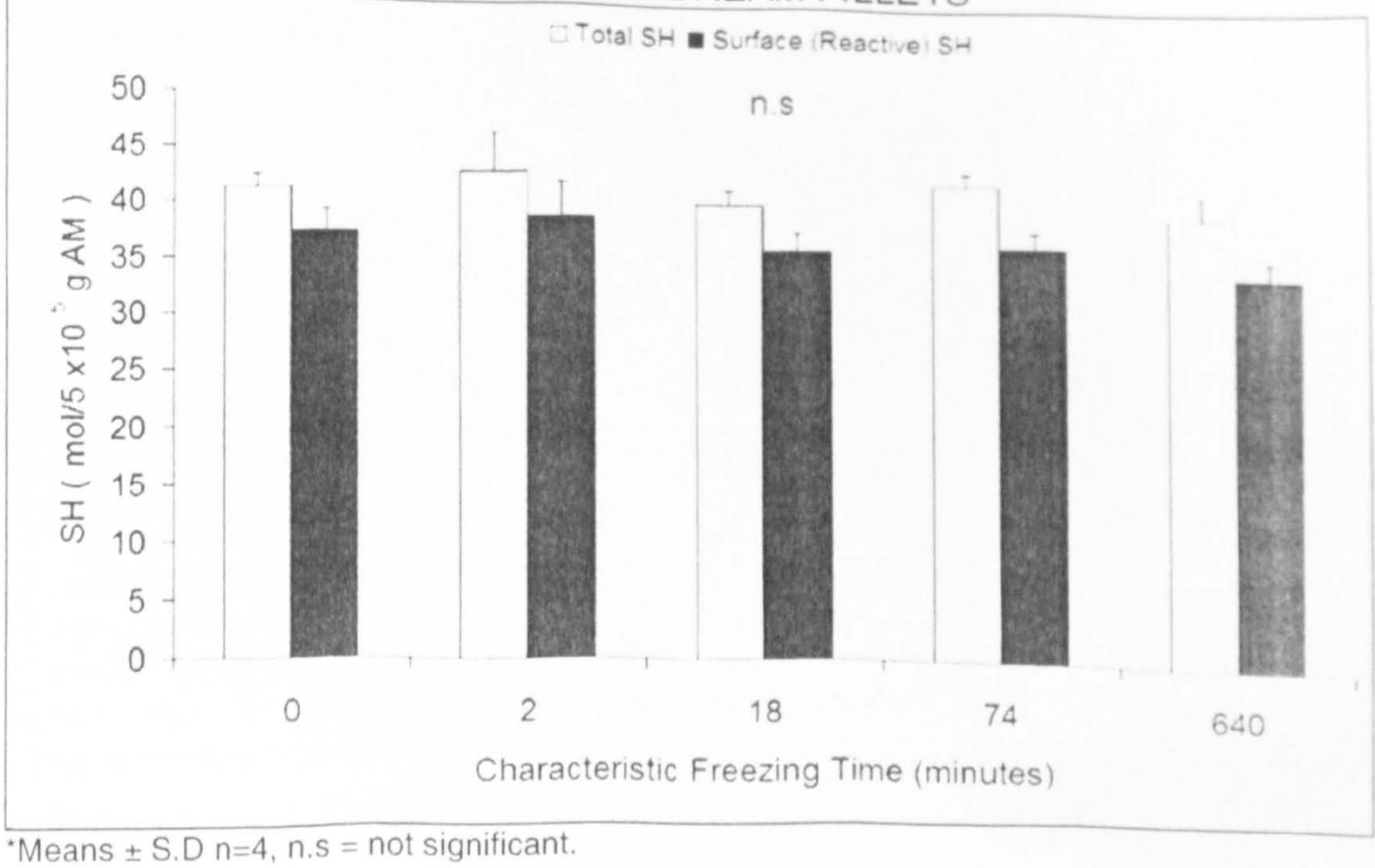


*Means ± S.D n=4, n.s = not significant.

Total and surface (reactive) sulfhydryl (SH) groups

The results of the present study showed that the content of SHs in actomyosin solutions extracted from the fresh and frozen fillets were similar ($P>0.05$; Figure 3). Considering the data of the salt soluble protein, this result seems to support the previous suggestion in this that aggregation might not have occurred in frozen seabream fillets. It is, also, likely that oxidation of sulfhydryl groups was not involved in denaturation of gilthead seabream' actomyosin extracted from frozen samples.

FIGURE 3.
THE EFFECT OF CHARACTERISTIC FREEZING TIMES (t_C) ON TOTAL AND SURFACE (REACTIVE) SH GROUPS OF ACTOMYOSIN (AM) FROM GILTHEAD SEABREAM FILLETS*



*Means ± S.D n=4, n.s = not significant.

ATPases in actomyosin extracts

Ca^{2+}-ATPase, Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities of actomyosin from seabream fillets frozen at all freezing times were significantly lower than those of fresh samples (P <0.05; Table 5). Ca^{2+}–ATPase activity can be used as an indicator for the integrity of myosin molecules, whereas Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities indicate the integrity of actin-myosin complex in the presence of endogenous and exogenous Ca^{2+} ions (Benjakul *et al.* 1977; Roura and Crupkin 1995).

From the results of the present study the decrease of Mg^{2+}-ATPase and Mg^{2+}-Ca^{2+}-ATPase activities, after the freeze-thaw process, could indicate a decrease of myosin affinity for actin and/ or an alteration of the enzyme properties of actomyosin (or myosin). On the other hand, the decrease of Ca^{2+}-ATPase activity could indicate a change in free myosin structure. The decrease in ATPase activities might imply a change on the myosin head, which contains both the active site of the enzyme and the actin interaction site. Suzuki (1967) and Hatano (1968) postulated that during frozen storage the loss of enzymatic activity of fish actomyosin was due to tertiary structural changes caused by ice crystallization. However, Buttkus (1971) and Hamada *et al.* (1977) concluded that the decrease in Ca^{2+}-ATPase of fish myosin was strongly related to changes in the SH groups. In the present study, since the total and surface SH contents in actomyosin from fresh and frozen gilthead

seabream fillets were similar, the loss of ATPase activities could be a result of partial tertiary structural change (unfolding) of myosin due to a weakening of intra-molecular hydrophobic bonds. This could be a result of local increase of ionic strength as a consequence of freezing and water migration from the myofibrillar space or dehydration of myofibrillar proteins (as suggested by Benjakul and Bauer 2000). If there was unfolding, this did not induce protein aggregation as indicated by the fact that the amount of salt soluble protein did not change by freezing. Similar results and suggestions were reported by Wagner and Anõn (1985) after studying the effects of freezing times on the denaturation of myofibrillar proteins of beef muscle.

TABLE 5.
THE EFFECT OF CHARACTERISTIC FREEZING TIMES (t_c) ON ATPASE ACTIVITIES OF ACTOMYOSIN FROM SEABREAM, μmolesP$_i$/min/mg protein*

Freezing Time	*Ca^{2+} ATPase*	*Mg^{2+}-Ca^{2+} ATPase*	*Mg^{2+} ATPase*	*Mg^{2+}-EGTA ATPase*
0	0.292±0.013a	0.307±0.036a	0.380±0.089a	0.023±0.005b
2	0.266±0.007b	0.207±0.021b	0.147±0.013b	0.034±0.004a
18	0.259±0.004b	0.179±0.052b	0.158±0.025b	0.029±0.000ab
74	0.265±0.006b	0.141±0.012b	0.146±0.024b	0.034±0.003a
640	0.266±0.010b	0.154±0.009b	0.165±0.040b	0.033±0.004a

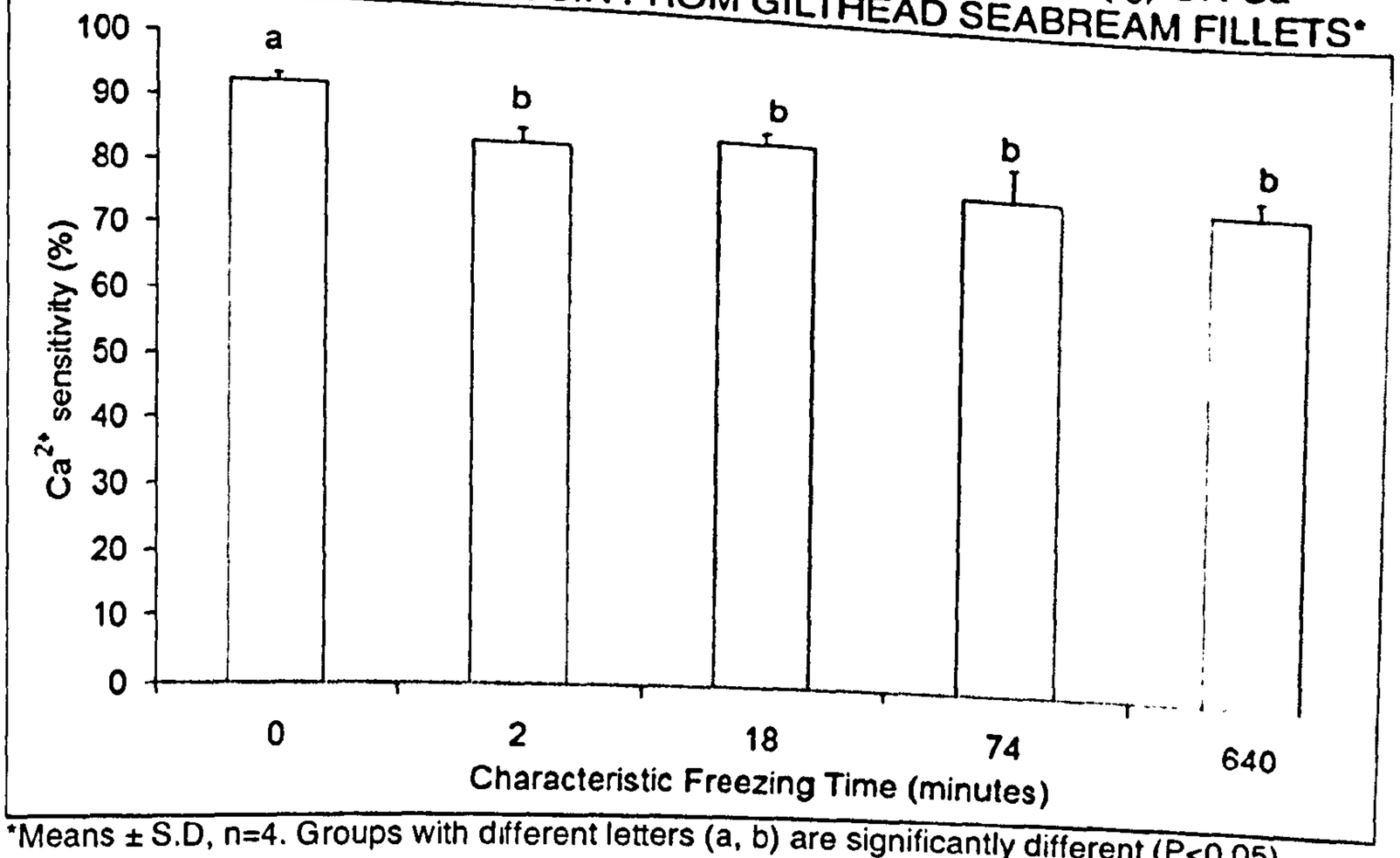
*Means ± S.D n=4.Different letters (a, b) in the same column denote a significant difference (P<0.05)

In contrast, Mg^{2+}–EGTA-ATPase activities of actomyosin extracted from frozen samples were significantly higher compared to those of fresh fillets (P<0.05; Table 5).This increase coincided with the loss of Ca^{2+} sensitivity (Figure 4). This result is similar to the previously reported change in Mg^{2+} – EGTA-ATPase activity of actomyosin from cod subjected to freeze-thaw cycles (Benjakul and Bauer 2000). Mg^{2+}-EGTA-ATPase activity is indicative of the integrity of the troponin- tropomyosin complex in meat samples and was reported to increase by treatment with lysosomal proteases (Quali and Valin 1981). Therefore, in the present study it may be that lysosomal proteases contributed to the changes in Mg^{2+}-EGTA-ATPase activity observed in gilthead seabream fillets due to freezing and thawing; this would be in accordance with the increase in AG and NAG activities, which were used as indicators of cell disintegration.

Ca^{2+} Sensitivity of actomyosin

The Ca^{2+} sensitivity of actomyosin from fresh seabream fillets was significantly higher than from frozen samples (P<0.05; Figure 4).

FIGURE 4.
THE EFFECT OF CHARACTERISTIC FREEZING TIMES (t_c) ON Ca^{2+} SENSITIVITY OF ACTOMYOSIN FROM GILTHEAD SEABREAM FILLETS*



*Means ± S.D, n=4. Groups with different letters (a, b) are significantly different ($P<0.05$).

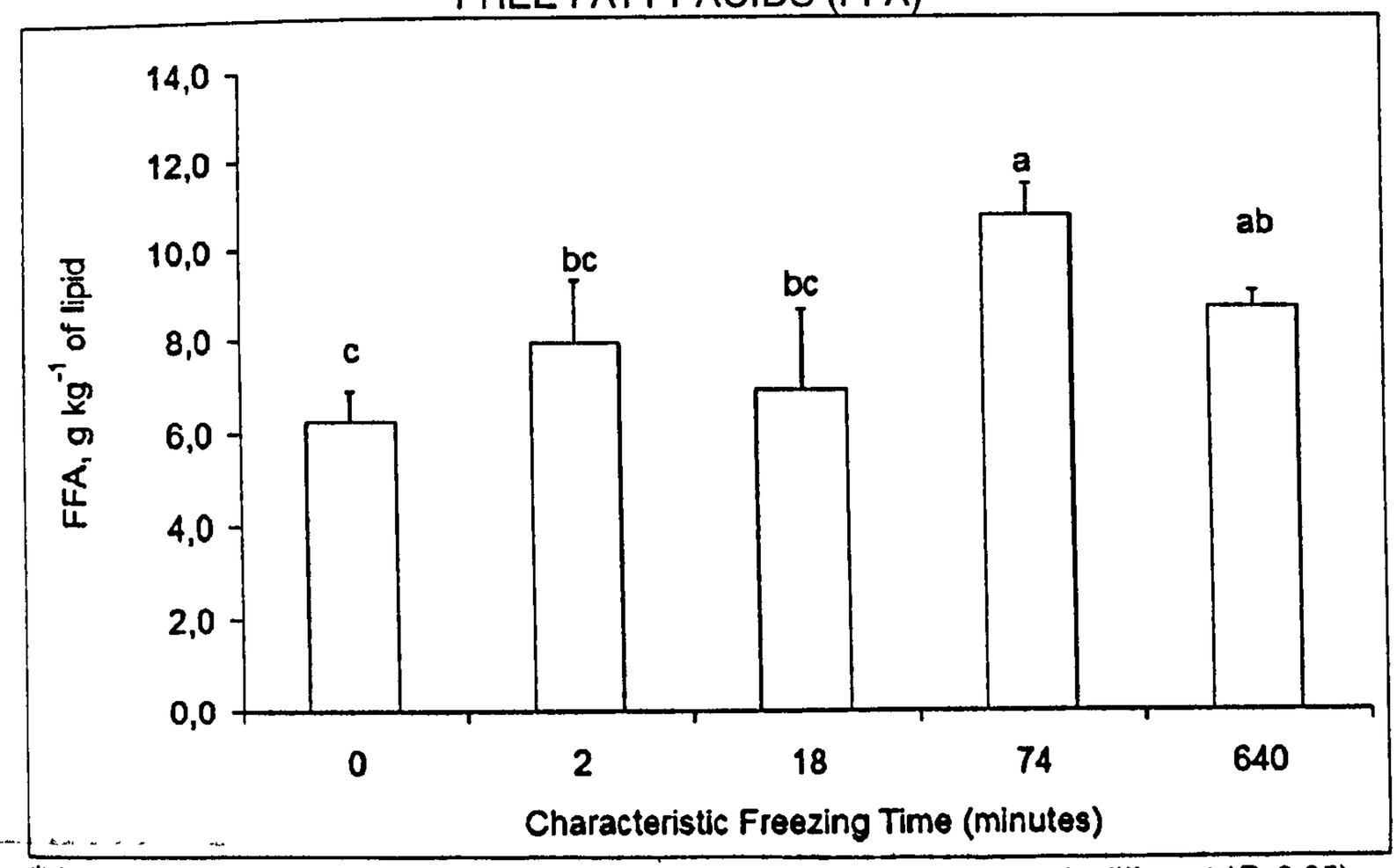
The Ca^{2+}sensitivity of myofibrillar proteins has been attributed to the activity of native tropomyosin (Ebashi *et al.* 1968). This loss of Ca^{2+} sensitivity is considered to be the result of filamentation of myofibrils, due to hydrolysis by proteases (Okitani *et al.* 1980) but, it may also be due to the modification of actin-myosin interaction (Benjakul and Bauer 2000). The decrease in Ca^{2+} sensitivity of seabream actomyosin caused by the freezing process itself could, therefore, be the result of both proteolysis and the modification of actin-myosin interaction by unfolding of myosin.

Effect of freezing times on total free fatty acids in extracted lipids from seabream fillet.

Freezing of seabream fillets at a characteristic freezing time (t_c) of 74 minutes resulted in higher amounts of free fatty acids (FFA) ($P<0.05$; Figure 5). Hanaoka and Toyomizu (1979) showed that in carp muscle hydrolysis of phospholipids was faster at temperatures below the freezing point. They suggested this to be the effect of cellular disintegration rather than dehydration or concentration of solutes in frozen cells. Also, triglycerides are hydrolyzed to FFA by lipases, some of which are thought to be lysosomal (Shewfelt 1981). Therefore, in the present study, the maximal concentration of FFA in lipids extracted from the fillets frozen at a characteristic freezing time (t_c) of 74 minutes may be due to the maximal disintegration of cells and lysosomes being observed in that freezing time. The disintegration of cells could have caused increased levels of phospholipids from the breakdown of membranes, whereas the disruption of lysosomes could have caused additional release of lipases in the tissue at the other times. These assumptions are in agreement

with decreased muscle integrity, increased levels of released protein, AG and NAG activities being found in seabream fillets frozen at a characteristic freezing time (t_c) of 74 minutes compared to those in the fillets frozen at the other freezing times.

FIGURE 5.
THE EFFECT OF CHARACTERISTIC FREEZING TIMES (t_c) ON TOTAL FREE FATTY ACIDS (FFA)*

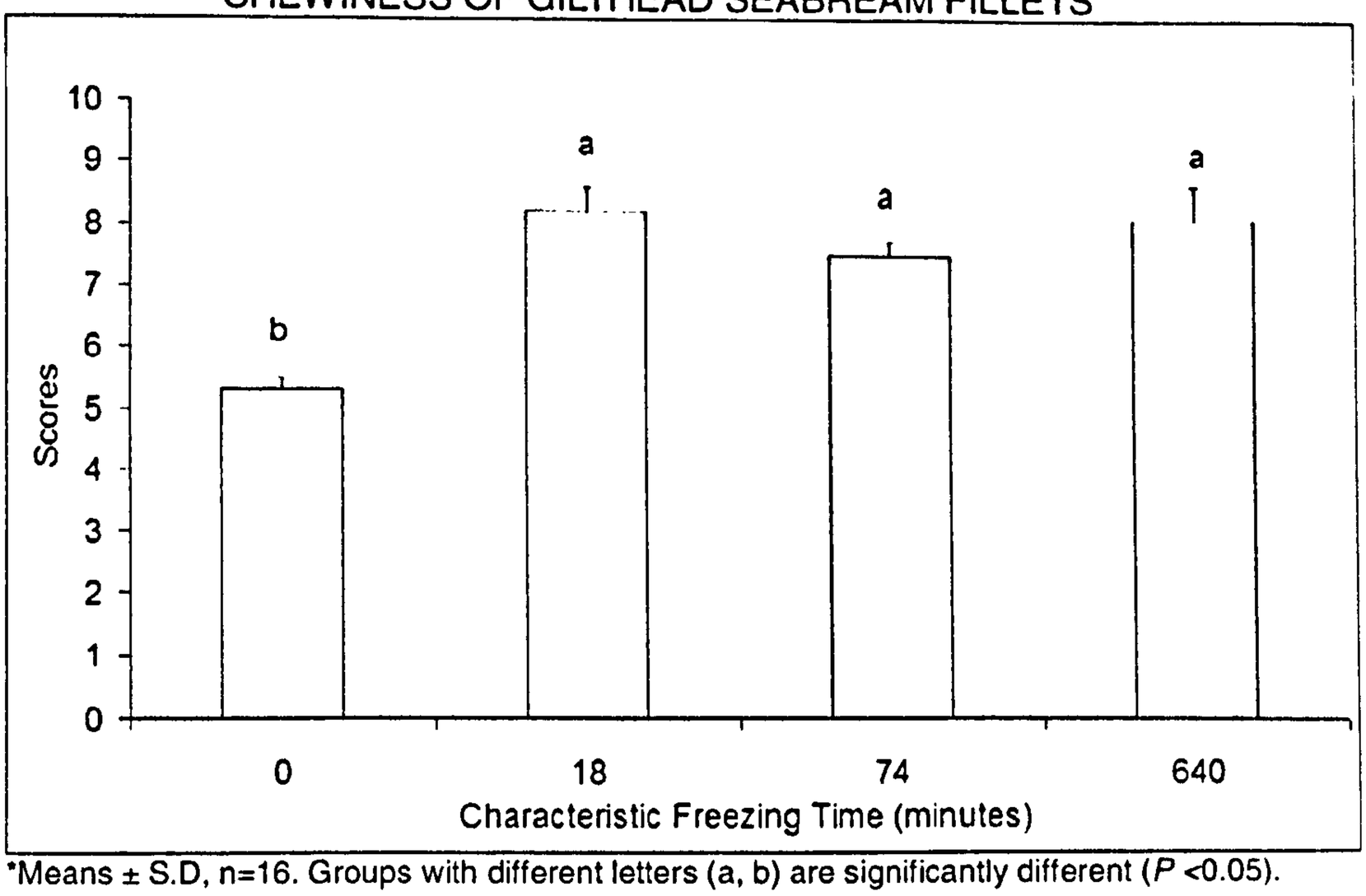


* Means ± S.D, n=4. Groups with different letters (a, b, c) are significantly different (P<0.05).

Effect of freezing times on sensorial attribute

Tenderness of fish is highly related to the state of the myofibrillar proteins and changes in the water holding capacity of fish muscles (Mackie 1993). The results of the present study showed that cooked frozen seabream fillets were perceived as being chewier than the fresh ones (P<0.05; Figure 6), in agreement with the findings of Carbonell *et al.* (2003) for whole cultured gilthead seabream. Since chewiness scores of a meat product are related inversely to tenderness (ISO 5492:1992) the results suggest that the frozen seabream fillets were less tender than the fresh ones. However, differences in tenderness were not observed between the fillets frozen at t_c values of 18, 74 and 640 minutes. These suggestions are in agreement with the changes in myofibrillar proteins and water holding capacities found in this study.

FIGURE 6.
THE EFFECT OF CHARACTERISTIC FREEZING TIMES (t_c) ON CHEWINESS OF GILTHEAD SEABREAM FILLETS*



*Means ± S.D, n=16. Groups with different letters (a, b) are significantly different (P <0.05).

Conclusions

The results of the present study showed that freezing of gilthead seabream fillets at a characteristic freezing time (t_c) of 74 minutes caused more damage to fibres and hydrolysis of lipids than the other tested freezing times. The release of enzymes from intracellular organelles, hydrolysis and oxidation in lipids may affect the texture and flavour of fish in subsequent storage (Mackie 1993; Civera *et al.* 1996). Since long freezing times, such as that of 640 minutes, are hardly encountered in good commercial practice for freezing seafood, it can be concluded that short freezing times, such as that of 2 and 18 minutes, could be beneficial for freezing post-rigor fillets of gilthead seabream, compared to a time of 74 minutes. However, it must be pointed out that for a meaningful assessment of the frozen gilthead seabream fillets to be sold on a commercial scale, a subsequent frozen storage study is very important. The bio-chemical, physical and sensory properties of gilthead seabream fillets during cold storage are being investigated and will be the subject of a future communication.

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