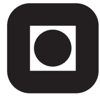

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Protein oxidation in Atlantic mackerel (Scomber scombrus) during chilled and frozen storage

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Summary

There are numerous health benefits associated with the consumption of fatty fishes such as mackerel. They provide important nutrients linked to their lipid, proteins, vitamins and minerals. As well as being a rich source of omega-3 fatty acids, fatty fish is an excellent protein source. Fish provides proteins of high biological value which contains essential amino acids with high digestibility. There are many steps during pre-handling, handling, storage and processing which may affect lipids, proteins and vitamins of fatty fishes leading to the reducing nutritional values. To maximize the health benefits of fatty fish, it is therefore important to know how these steps will affect the different components of fish and how these compounds can be retained during different processing steps.

The main aim of this work has been to study the oxidation of sarcoplasmic and myofibrillar proteins of white, black and whole mackerel muscle during chilled (+4°C) and frozen storage (-30°C). The samples were either chilled stored for 4 and 9 days or frozen stored for 1, 7 and 12 months. The contents of protein carbonyl and total thiol were used to measure the extent of protein oxidation during fish storage. Protein carbonyl was measured by the use of a conventional DNPH-based and an ELISA method. The second aim was to evaluate the effects of protein oxidation on water holding capacity and protein solubility. The lipid oxidation during chilled and frozen storage of the same samples as used in the present work, have been evaluated in a master project and the results was used to consider the relationship between lipid and protein oxidation.

The conventional DNPH-based method was found not to be a useful method for measuring the content of protein carbonyls in fatty fish like mackerel due to the incomplete resolubilization of the protein pellet in guanidine hydrochloride.

Carbonyl contents of sarcoplasmic and myofibrillar protein of all muscle types were relatively stable during chilled and 1 month frozen storage of samples. This could implicate that protein carbonylation was not developed in samples during refrigerated and 1 month frozen storage. However, the total thiol group generally decreased in myofibrillar proteins during chilled and 1 month frozen storage, which could be an implication of protein thiol group oxidation in those samples. WHC was relatively stable during chilled and 1 month

frozen storage. Sarcoplasmic protein solubility was also stable during this period of storage while the myofibrillar protein solubility generally decreased.

Frozen storage for 7 and 12 months significantly influenced the oxidative stability of sarcoplasmic and myofibrillar proteins of all mackerel muscle types. Sarcoplasmic and myofibrillar protein carbonyl contents increased significantly during frozen storage at -30°C for 7 and 12 months compared to the chilled and 1month frozen storage. At the same time, the total thiol group contents decreased significantly. These results show that duration of frozen storage has a strong impact on protein oxidation in mackerel.
Increased protein carbonyl contents and decreased thiol group contents in 7 and 12 months frozen samples occurred concomitantly with a loss of WHC and protein solubility. It could be concluded that protein oxidation had a significant effects on the loss of WHC and protein solubility.

Myofibrillar proteins had significantly higher carbonyl content, lower total thiol group content and lower protein solubility compared to the sarcoplasmic proteins in both chilled and frozen stored samples. This may implicate the higher stability of sarcoplasmic proteins with regard to protein oxidation and freeze denaturation compared to myofibrillar proteins.

While the black and mixed muscle had a significantly higher sarcoplasmic and myofibrillar carbonyl contents compared to the white muscle, no clear relationship between muscle types and loss of thiol groups, WHC and protein solubility was observed.

The results from the lipid oxidation study on the same mackerel samples show decreased level of TBARs after 7 months frozen storage. The increased carbonyl contents and decreased thiol group contents at the same time may indicate a correlation between protein and lipid oxidation progress in mackerel.

Preface

First of all, I would like to express my special thanks to my supervisor Turid Rustad, Professor at the Biotechnology department of NTNU, for her supervision, guidance in scientific writing and support throughout the work on this thesis.

I wish to thank all my friends in Trondheim for their supports, kindness and friendship throughout these three years. My warm thanks go to my friend Maryam, for her invaluable supports and her comments on my thesis draft.

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Abbreviations

Protein Oxidation	Pox
Reactive Oxygen Species	ROS
Poly unsaturated fatty acids	PUFA
Monounsaturated fatty acid	MUFA
Eicosapentaenoic acid	EPA
Docosahexaenoic acid	DHA
Myoglobin	Mb
Hemoglobin	Hb
Adenosine diphosphate	ADP
Adenosine triphosphate	ATP
low-density lipoprotein	LDL
Water holding capacity	WHC
hypochlorous acid	HOCL
4-hydroxy-2nonenal	HNE
metal ion-catalyzed oxidation	MCO
α-aminoadipic semialdehyde	AAS
γ-glutamic semialdehyde	GGS
cysteine	RSH
reactive nitrogen species	RNS
sulfenic acid	RSOH
Enzyme-Linked Immunosorbent Assay	ELISA
Dinitrophenylhydrazine	DNPH
2, 4-dinitrophenyl hydrazone	DNP
trichloroacetic acid	TCA
5,5'- Dithiobis-(2-nitrobenzoic acid)	DTNB
2-nitro-5-thiobenzoic acid	TNB
Myofibrillar protein	MP
hydrochloric acid	HCl
malondialdehyde	MDA
sulfinic acid	$CysSO_2H$

sulfonic acid	$CysSO_3H$
sulfenic acid	$CysSOH$

1 Introduction

1.1 Background

Fish is a vital source of food. Fatty fish including pelagic species like herring and mackerel represent a valuable source of nutrients of fundamental important in a healthy diet. A significant proportion of pelagic fish is reduced, or dehydrated, to make fish meal and oil which are used in manufactured animal feeds [1]. However, in many countries small pelagic fish are important parts of the human diet. It is traditionally a source of cheap protein in some countries in Africa and Asia. Herring and mackerel have a long tradition for being used for human consumption in Norway and Iceland [2]. The consumption of these fatty fishes provides numerous important nutrients linked to their lipids, proteins and micronutrients such as vitamins and minerals. The lipids are rich in omega-3 fatty acids with documented positive health effects. Fish is also an excellent protein source due to the high quality proteins which contains essential amino acids with high digestibility. Fatty fish are highly susceptible to deterioration in storage due to their high contents of unsaturated fatty acids [3].There are many steps during pre-handling, handling, storage and processing which may affect lipids, proteins and vitamins of fatty fish leading to reduction of nutritional values. To maintain the health benefits of pelagic fish it is therefore important to know how these steps will affect the different components of fish and how these compounds can be retained during different processing steps. In addition, it is highly important to have good sensory quality and consumer acceptability for the end products. This thesis is a part of ProhealthPelagic project, funded by the Norwegian Research Council. The project aims at investigation of potential changes in health promoting compounds present in raw herring and mackerel throughout processing operations like salting, marinating and canning. The project work is led by SINTEF Fisheries and Aquaculture with NTNU, DTU and Chalmers as research partners. In addition, several industry partners are participating. The aim of this thesis has been to investigate protein oxidation in mackerel during chilled and frozen storage. Lipid oxidation was investigated on the same mackerel samples in a master thesis done as a part of ProhealthPelagic project.

Over the past decades, there has been an increasing interest among food scientists on the occurrence and impact of protein oxidation (Pox) in food systems, following many years of medical research on the possible essential role of the Pox in the variety of human diseases [4] [5] [6] [7]. The oxidative degradation of food components like proteins and lipids is one of the major causes of chemical, physical and biological deterioration. Lipid oxidation in food has been studied in depth for many years. In contrast, protein oxidation has been ignored for decades perhaps because of the high complexity of the food protein oxidation mechanisms, the lack of the methods for investigation of Pox in food systems and the fact that protein oxidation products may not be involved in the changes immediately perceived by human sense [8] [9]. One of the first studies of food protein oxidation was the finding that turkey white muscle myofibrillar proteins was susceptible to oxidative reactions [10]. Since then, numerous studies implicated that proteins from muscle foods are susceptible to protein oxidation during post-mortem, processing and storage [11] [12] [13] [14] [15] [16] [17] [18] [19] [20] [21]. Fish may be more susceptible to protein oxidation compared to the land animals due to the high level of poly unsaturated fatty acids (PUFA). However the researches conducted on the fish protein oxidation are few. Storage such as chilling and freezing have been found to induce lipid oxidation in fish and proteins are also expected to be oxidatively modified [22]. Studies shows that protein oxidation products was developed during frozen storage of rainbow trout [22] and chilled storage of mince and washed mince horse mackerel (*Trachurus trachurus*) [23] and mince mackerel (*Scomber scombrus*) [24].

Pox in muscle foods could be induced either directly by reactive oxygen species (ROS) or indirectly by secondary byproducts of oxidative stress [25].

The interaction between free radicals and proteins and peptides results in alterations in backbone and the side chains of amino acids. Proteins are the most abundant component of muscle and play an essential role in the technological, nutritional and sensory aspect of muscle foods [26]. Pox could result in biochemical and structural changes in proteins including destruction of amino acids, loss of protein functionality, loss of enzyme activity and impaired digestibility. These modifications might have serious consequences on protein functionality and muscle quality including alteration in protein solubility, water holding capacity, viscosity and emulsification. However, the precise mechanisms of potential implication of Pox on muscle food quality have not been identified yet [27] [28]. Recent findings have hypothesis the potential negative effects of Pox not only on the sensory or technological attributes of the muscle, but also on nutritional aspects and consumer health

[29] [27]. Although interesting advances have recently been achieved in the field of protein oxidation in muscle foods, more studies are needed to understand the mechanism and the impact of oxidized proteins on the quality attributes of muscle and consumer health.

1.2 The aim of this thesis

The main aim of this work has been to study the oxidation of sarcoplasmic and myofibrillar proteins of white, black and whole Atlantic mackerel (*Scomber scombrus*) muscle during chilled (+4ºC) and frozen (-30ºC) storage. Protein oxidation was evaluated by measuring carbonyl and thiol content of the muscle at each sampling point. The second aim was to evaluate the effects of protein oxidation on the quality of fish. The effects of protein oxidation on quality attributes of muscle was assessed by the determining the solubility of salt and water soluble proteins and water holding capacity of mackerel fillet.

1.3 Atlantic mackerel (*Scomber scombrus*)

Atlantic mackerel (*Scomber scombrus*) is a fatty fish belonging to the pelagic and fast swimming species (Figure 1.1). It can be found in the Northeast Atlantic from the Northwestern part of Africa north to the Barents Sea, and westwards in the Norwegian Sea to Iceland. Mackerel mainly feeds on zooplankton, molluscs and small fishes like herring. They live in deeper water over winter but move closer to shore in spring [30]. The spawning begins in February and last till June depending on the area where the fish live. Mackerel is sold as fresh and frozen fillets and whole fish. There is a wide range of well-known mackerel products including hot smoked, cold smoked and pepper smoked mackerel. Canned mackerel is also a famous mackerel product. Mackerel is one of the main capture species in Norway. It is caught in the North Sea by purse seiners or trawlers from September to December. Bulk processors mainly produce whole frozen, gutted frozen or frozen fillets, and export as semi products to the end producers mainly located in other countries. During the first half of 2014 Norwegian exports of frozen mackerel were 70,700 tons [31]. A few processors in Norway produce end-products of mackerel including mackerel in tomato sauce. Canned mackerel is manufactured from fresh or frozen fish. The manufacturing steps involves thawing of frozen mackerel, cutting, filling of mackerel and tomato sauce in the box, sealing hermetically and processing by heat to achieve commercial sterility and prevent spoilage. Canned mackerel in tomato sauce is

frequently eaten processed seafood in Norway. It is a popular food on breakfast and lunch.

Figure 1-1 Atlantic mackerel (Scomber scombrus)

1.4 Fish muscle

1.4.1 Chemical composition of fish muscle

Fish consists mainly of water, protein, lipid and to a lesser extent of carbohydrate. Fish is also a rich source of vitamin A, D and the B vitamins like B6 and B12. Iodine and selenium are important dietary minerals of fish. Iron and zinc are found in moderate amounts in most fishes [32].

The chemical composition of fish vary greatly depending on species, and within a species depends on age, sex, environment, feeding and season. In the same fish the composition of muscle varies depending on specific muscle type. Fish species living at different temperatures have different biochemical composition. The variation in the chemical composition of fish is also closely related to season. Pre-slaughter starvation and handling stress are also found to have significant effects on the chemical composition of fish muscle [33]. In addition, the physiological state and sexual changes of fish such as spawning would affect the chemical composition of fish. Wild and farmed fish have different chemical compositions. Wild fish usually contains less fat than farmed fish [34]. Table 1.1 gives an overview of the chemical composition of muscle in Atlantic mackerel.

Table 1-1 % of water, lipid and protein of fish fillet of Atlantic mackerel(*Scomber scombrus*) [35]

Alantic mackerel (*Scomber scombrus*)	**Fish (fillet)**
Water %	60-74
Lipid %	1-23.5
Protein %	16-20

Water in muscle

Water is the main constituent of fish flesh. The water content in fish muscle is typically in the range of 66-81 % [36]. In fish muscle there is an inverse relationship between water and fat content, which together make up about 80 % of the muscle [37]. The amount of water and its distribution in muscle foods are important properties influencing quality, shelf life and processing. The water can be divided into three main categories, based on the degree of freedom. A small part of the water is free water which is held in the muscle with weak surface forces. Another fraction of water is bound water which is exist in the vicinity of non-aqueous constituents like proteins and has reduced mobility [38]. This water is strongly bound to proteins, very resistant to freezing and only makes up less than 10% of the total water in muscle [39]. The third and major fraction of water is termed entrapped, immobilized or bulk water. This water is not directly bound to the proteins but is held within the structure of muscle either by steric effects and/or by attraction to the bound water. The entrapped water is the water that is affected by the rigor process and the processing like freezing, heating and salting. It can be removed from the muscle during rigor as drip loss, and can be easily converted to ice upon freezing [39].

The majority of water in muscle; about 85% , is held between the myofibrillar protein network (intra-myofibrillar) and 15% is located outside the myofibrillar network (extra-myofibrillar) [40] .

Water in the intra-myofibrillar space is located:

• In the myofibrils in the space between the thick and thin filaments by capillary forces [41]

Water in the extra-myofibrillar space is located:

•In the sarcoplasm in the space between myofibrils (inter-myofibrillar)

• Between muscle fibers and in the inter-fascicular space (inter-fascicular) [42]

• Between the muscle bundles

Lipids in muscle

The lipids are one of the most important nutritional components of the fish but at the same time they contribute to many unwanted changes in fatty specie [43]. Lipid and fatty acid composition vary greatly between species and within the same species. There is usually noticeable seasonal variation in the fat content of fatty fishe. Atlantic mackerel can have less than 4 % fat during late spring after spawning while it can have about 24% fat during late November due to the active feeding [44]. Descending order for the fat content in different part of mackerel and sardines are reported as follows: skin, viscera, dark muscle and white muscle [45]. In mackerel half of the fat is found under the skin in contact with the dark muscle and 30% is localized in the white muscle [46]. Phospholipids and triglycerides are the major classes of lipids in fish. Triglycerides are the main form of storage fat and would be present as either large droplets within the adipose tissue or as smaller droplets between or within muscle cells [47]. Phospholipids are one of the main building blocks of muscle cell membrane bilayers. Fish is a rich source of long-chain polyunsaturated fatty acids (PUFAs) in particular eicosapentaenoic (EPA, 20:5 -3) and docosahexaenoic acids (DHA, 22:6 -3) which are found in higher level in the membrane phospholipids than in storage triglycerides [48]. Dark muscle fishes have higher level of PUFAs compared to white flesh species.

Proteins in muscle

Fish is one of the most valuable sources of proteins. The content of the 8 essential amino acids is high and balanced in fish. Proteins make up about 15 - 20% of the weight of muscle in different species. The amount of protein is more constant compared to fat.

The proteins in fish muscle can be divided into three groups: (1) Structural proteins or myofibrillar proteins, (2) Sarcoplasmic proteins, and (3) Stroma or connective tissue proteins. Structural or myofibrillar proteins are myosin, actin, actomysin, and troponin which constitute about 40 – 80% of the total crude protein in fish. Sarcoplasmic proteins are myoalbumin, globulin and enzymes, account for the approximately 30% of total muscle proteins. The rest of the muscle proteins are classified as connective tissue or stromal proteins, consist mainly of collagenous material [49].

- **Structural (myofibrillar) proteins**

Fish flesh is formed by two fillets that go from the head to the tail on each side of the body. The fish muscles are separated by thin connective tissue membranes called myocommata, into bundle of short fiber called myotome (Figure 1.2). Each myotome is composed of numerous cells, called muscle fiber or muscle cell. The muscle fiber consists of all the components of a cell and also a number of myofibrils. The cells are surrounded by a connective tissue called the sarcolemma which contains thin collagenous fibrils. The myofibrils are constructed of contractile units, sarcomeres, which are made up of thick filaments of myosin and thin filaments of actin and are bordered by z- lines [50]. Structural or myofibrillar proteins make up more than 60% of fish muscle. It includes mainly myosin (55-60%) and actin (15-30%). Tropomyosin, and troponins C, I, and T are also included in the myofibrillar proteins [51]. These proteins can be extracted from the fish with neutral salt solutions of ionic strength larger than 0.4. They are also called salt soluble proteins. Myosin contains two heavy chains associated with two pairs of light chains ranging from 16 to 28 kD [37]. Fish myosin isoforms differ mainly in the content and position of specific amino acid residues such as Pro and Gly [52]. Different fish species have different myosin stability based on their habitat temperature [34]. Actin is a globular protein of about 40 kD. This protein is the major component of thin filaments. Unlike myosin, actin is one of the most highly conserved proteins in the animal kingdom [34]. Figure 1.3 gives an overview of different parts of a skeletal muscle fiber.

Myofibrillar proteins are mainly responsible for the characteristic texture and structure of fish products such as tenderness, as well as functional properties of muscle proteins such as water holding capacity, solubility, gel forming ability and lipid emulsifying properties [32] [50]. They undergo changes during rigor mortis and processes such as freezing and heating.

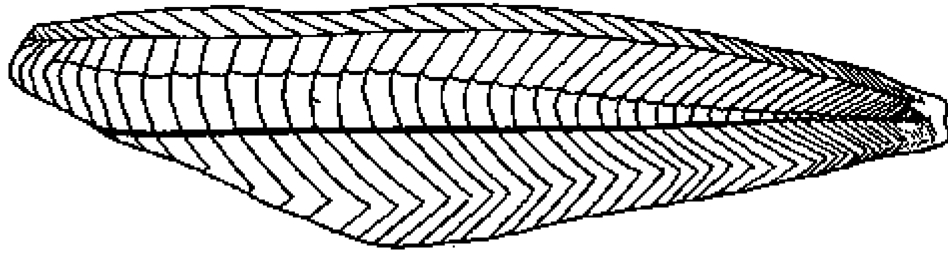


Figure 1-2 The metameric structure of fish muscleThe longitudinal sections represent the arrangement of sheets on connective tissue in the muscle [53]

- **Sarcoplasmic proteins**

The term sarcoplasmic proteins usually refers to the proteins of the sarcoplasm, the components of the extracellular fluid, and the proteins contained in the small particles of sarcoplasm [51]. These proteins are soluble in water or salt solutions of low ionic strength. Pelagic fish muscle has higher sarcoplasmic protein compared to demersal fish [50]. The sarcoplasmic proteins contain generally low molecular weight compounds including albumins, myoglobin, and hemoglobin, enzymes of the glycolytic pathway, hydrolytic enzymes of the lysosomes and various proteases [37]. In the sarcoplasmic fraction there are also the enzymes catalyzing the degradation of nitrogenous compounds. Fish albumin proteins are composed of over 100 various proteins, most of them having enzymatic activity [50]. Sarcoplasmic proteins can contribute in the quality of fish through the effects on the color, taste, texture or nutritional value of fish. Proteases hydrolyze the muscle proteins after animal death and resolve the rigor mortis contraction. They play an important role in the quality of fish flesh during postmortem period [54].

Myoglobin (Mb) and hemoglobin (Hb) are responsible for fish color. Hb is lost during the bleeding of fish and contributes less than Mb to the appearance and color of the muscle [55]. Myoglobin is the primary oxygen carrier of muscle tissue while hemoglobin is an oxygen carrier protein in the red blood cells. The color of dark muscle flesh is defined by the relative amount of oxymyoglobin, deoxymyoglobin and metmyoglobin. Deoxymyoglobin or reduced form of myoglobin is the dark red pigment of deep muscle.

Myoglobin concentration in the muscle depends on species and the age of the animal, muscle type, muscular activity, oxygen availability, type of feed as well as the way the muscle is treated [56]. Myoglobin and hemoglobin of fish are localized mainly in the red or dark

muscle which is used frequently or continuously for swimming and so need more oxygen supply.

- **Connective tissue (stroma) proteins**

Connective tissue holds the muscle fiber together and binds the muscle to the skeleton [57]. There are three types of connective tissue in muscle: epimysium, perimysium and endomysium. Epimysium is a thick sheath of connective tissue which surrounds the entire muscle, perimysium is a thin layer that groups muscle fibers into bundles and endomysium is a fine layer that ensheaths each individual myocyte [58]. Stroma proteins are insoluble in water or neutral salt solutions.

Collagen makes up approximately 90 % of fish connective tissue proteins and the rest is elastin [59]. Collagen presents in the skin, tail, swim bladder, myocommata, etc. The content and composition of collagen contributes greatly to the texture of fish muscle [34]. Aggregation of collagen during long term frozen storage could contribute to the hardening of fish flesh [60]. Thickness and stability of collagen fibril in fish muscle is affected by the proportion of these two types of collagens.

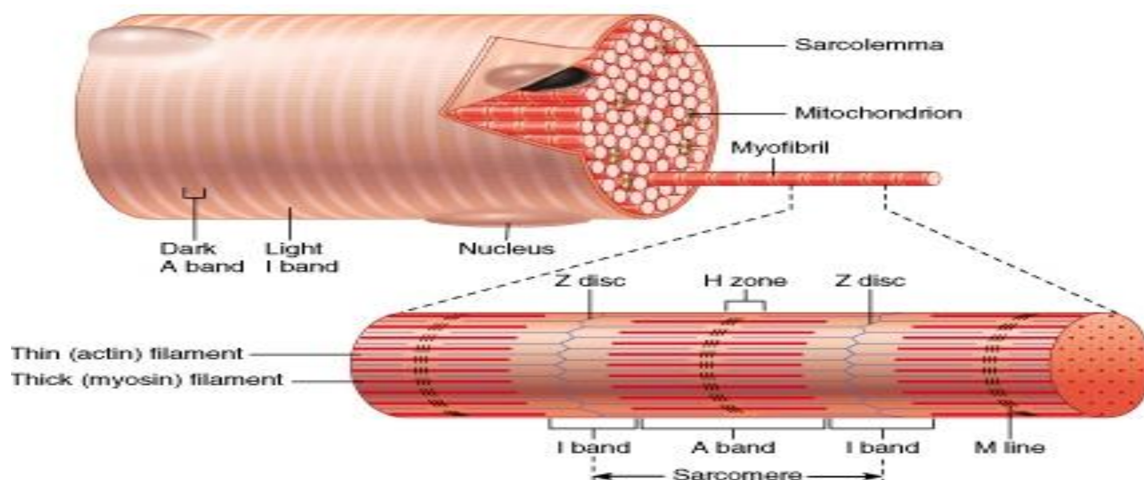


Figure 1-3 illustration of muscle structure [61]

1.4.2 Dark (red) and white muscle fibers

Fish contains two major fiber types classified by their color: (1) white muscle fiber and (2) red or black muscle fiber. These fibers are arranged in separate and distinct anatomical regions. The bulk of the muscle often consists of white fibers, while the dark fibers are located along the side of the body under the skin. The proportion of dark to light muscle varies according to the species. In pelagic fish like mackerel and herring, up to 48% of the body weight is made up of dark muscle [62], while the amount of dark muscle in demersal fishes is very small [63]. The white fibers are used for high-speed, short-time swimming using anaerobic metabolic pathways to provide energy; while the red muscle fibers are employed for continuous swimming motion such as migration using aerobic metabolic pathways to provide sustained energy [37]. Dark fibers have greater quantities of mitochondria and less sarcoplasmic reticulum than white fibers. These fibers also contain higher content of lipid (2 to 5 times), B vitamins, glycogen, and nucleic acids than white muscles [50]. In addition, dark muscle contains higher amount of myoglobin and more abundant vascular supply compared to the white muscle. The content of enzymes also vary in both muscle types, dark muscle is richer in the enzymes of citric acid cycle, while white muscles have higher ATPase activity [64]. Figure 1.4 shows a schematic representation of the distribution of fiber types in mackerel muscle.

Figure 1-4 Dark muscle regions in mackerel[51]

1.5 Freezing of fish

Freezing is one of the most important preservation methods for fish. According to FAO, it represents the main method of processing fish for human consumption. It accounted for 54% of total processed fish for human consumption and 25% of total fish production in 2012 [65] . Fish is mainly composed of water; normally 60-80% depending on the species. The freezing process converts most of this water into ice. During freezing, water crystallizes to form ice crystals. Frozen storage is used to retard undesirable physical and chemical deterioration in fish. However, during frozen storage several chemical and physical reactions can occur between different fish components. In this section, thermodynamics of the freezing process and the impact of freezing on the quality attribute of fish is described.

1.5.1 Thermodynamics of the freezing process

During the first stage of freezing, the temperature falls rapidly below the freezing point of the product. Due to the dissolved substances in the moisture within the muscle, this initial freezing point is lower than initial freezing point of the pure water (0℃). During the second stage of freezing the water is initially changing to ice and there is a little change in temperature due to the high amount of heat required to remove from flesh to change the phase. As a portion of the water in the muscle is crystallized, the remaining solution becomes more concentrated. This reduces the freezing point of the unfrozen portion of the muscle. As the temperature drops, ice crystal formation increases the concentration of the solutes in solution and depresses the freezing point. In the third stage of freezing the temperature again begin to fall rapidly to create the desired ice content in the food. At the end of this stage the food temperature tends to the temperature of the heat transfer medium.

1.5.2 The impact of freezing on quality attribute of fish

Freezing is related to the quality deterioration in foods. Formation of ice crystals may result in textural changes and disruption of cell components that result in the release of chemically reactive components such as enzymes and pro-oxidants. Furthermore, as the

water is converted to ice, the remaining solution will be more concentrated. This increased concentration of solutes may cause changes in muscle in two ways: (1) it may cause a change in pH of muscle. The acid-base equilibrium (pH) is important in the stability of substances in muscle cells. Upon crystallization, salts and other components which are slightly soluble such as phosphate will precipitate. This can result in a severe change in pH (up to 2 pH units). This change often affects the physiochemical properties of muscle.

(2) Although, lowering the temperature slows down the rate of physiochemical reactions, at the same time potential reactants such as enzymes and substrates become more concentrated in the unfrozen fraction and the rate of physiochemical reactions may accelerate [66].

The most common physiochemical changes that can occur during freezing and frozen storage are dehydration, changes in lipids, flavor deterioration, degradation of pigments and vitamins, enzymatic browning, and protein denaturation. Since this master is focused on the changes in proteins during frozen storage of mackerel, these changes are explained in detail.

- **Changes in proteins during frozen storage**

Proteins may undergo physical and chemical changes during frozen storage. Protein denaturation and aggregation are the major changes occurring in proteins during frozen storage. These two phenomena may change the texture and functional properties of proteins. Denaturation can be defined as the changes in protein structure due to the disruption of the chemical bonds and by secondary interactions with other constituents of muscle [67]. Ice crystal growth during freezing could physically rupture the membrane and change the structure of muscle cells. Both volume changes in muscle fibers resulting from freezing and formation of large ice crystals in extracellular location can result in mechanical damage to the cells. At a slow freezing rate, the exterior fluid of cells cools more rapidly than the interior fluid. As crystallization proceeds, extracellular concentrated salts create an osmotic pressure gradient across the cell membrane. As a result, flowing of intracellular moisture outward will lead to dehydration and an increase to the ionic strength of the cells. Freezing out of the water onto the existing extracellular ice crystals will cause them to grow, thus distorting and

damaging the membrane and proteins. Temperature fluctuation during frozen storage also could change the structure of ice crystals leading to the cellular damage of fish or structural proteins. Chemical damage to the proteins arises from changes in concentration of solutes, dehydration and reaction of proteins with intact lipids and oxidized lipids. The increased concentration of mineral salts and soluble organic substances in the unfrozen fraction will change the pH and ionic strength of the unfrozen matrix and cause conformational changes in proteins. Changes in pH alters the electrostatic interactions between charged amino acids and cause the breakdown of some electrostatic bonds [68]. The effect of salt concentration on protein denaturation, aggregation or dissociation could be related to the effect of salts on the secondary forces (ionic, van der Waals, hydrogen and hydrophobic forces), which help to stabilize the tertiary structure of protein [68].

Interaction of proteins with intact, oxidized and hydrolyzed lipids also leads to the conformational changes in proteins. Contradictory results are found with regard to the effect of intact lipids on proteins [69]. On one hand, they seem to protect proteins, on the other, they form lipoprotein complexes with proteins which could affect protein properties [70]. The protective effect of intact lipids is credited to the neutral lipid fractions such as triglycerides. These lipids could diminish the effects of the free fatty acids on proteins [71]. The detrimental effect of intact lipids on protein is due to the interaction between liberated lipids and proteins from breakdown in membranes or deformation of other micro organelles. The resulting lipid-protein complexes are dissimilar to natural lipoprotein complexes and will affect the textural quality of the muscle tissue. [71].

- **Changes in WHC during frozen storage**

Water holding capacity is usually defined as the ability of muscle to retain water under external forces. This is frequently expressed as drip loss, expressible water, cook loss, and cooling loss depending upon the stage during processing in which it was measured [72]. Water holding capacity is one of the most important quality characteristics of muscle foods. Impaired water holding capacity costs the meat industry millions of dollars annually [38]. This is also the case for the fish industry. Once, the fish is caught the amount of water and location can change depending on a number of pre and post mortem factors. Genetic, diet and

stress before or through the slaughter are the factors which could affect the WHC. Further processing such as freezing, salting and smoking will also influence water holding capacity of fish. Freezing is a dehydration process in which water is removed from its original location and collected in the form of ice crystals [73]. This water may or may not be reabsorbed into its original place within the muscle microstructure. In addition to the physiological and biochemical status of the muscle and the intrinsic water binding strength prior to freezing, the factors that determine thawing drip loss include the extent of protein denaturation during freezing, the size and location of ice crystals, the rate of thawing, and the rate and extent of water reabsorption [73].

- **Changes in protein solubility during frozen storage**

Protein solubility or extractability, usually in salt containing solutions, is widely used as an indicator of myofibrillar protein denaturation during frozen storage [74]. Formation of protein-protein bonds due to the denaturation of myofibrillar proteins leads to the decrease in extractability in salt solutions [75]. Disulfide bond formation during frozen storage was suggested as a contributing factor to loss of protein extractability of halibut mince [76]. Intra- and inter-molecular disulfide bonds in proteins are formed during the oxidation of sulfhydryl containing amino acids such as cysteine. Hydrogen and hydrophobic interactions during frozen storage also has been known to cause the aggregation during frozen storage. Side-by-side aggregation of myofibrillar proteins followed by rupturing of different bonds in the native conformation of proteins, especially myosin, leads to the formation of intermolecular cross-linkages [77]. Such intermolecular cross linkages result in aggregation which leads to decreased protein solubility [78]. Protein-lipid complexes and the lipid oxidation products has well known to produce protein aggregates which decrease solubility [79].

1.6 Chilling of fish

Fish is a very rapid perishable food because of the high contents of unsaturated fatty acids. Therefore, it should be chilled as soon as possible after cath. The aim of chilling is to prolong the shelf-life of fish by slowing the action of enzymes and bacteria, and the physical

and chemical changes which can affect quality. Chilled foods are typically stored at temperature close to their freezing point, ranging from + 8℃ to − 1℃ [80] . The most common chilling method for fish is by the use of ice. Other methods are chilled water, ice slurries, refrigerated sea water (RSW) and cold air storage. As the refrigerated sea water was used to transport the mackerel to land and cold air storage at + 4℃ was used as a cold storage method during this master project, these two methods will be shortly described here.

1.6.1 Refrigerated sea water

Refrigerated sea water system have been used for salmon, sardine, halibut, menhaden, shrimp, mackerel, herring, blue whiting and many other species [81]. In this system the sea water is cooled to below 0℃ by mechanical refrigeration or by the addition of ice [82]. The basic components of mechanical refrigeration system are a heat exchanger to remove heat from seawater, a mechanical refrigerator to discharge heat from the system and a circulatory system to transport the refrigerant between the heat exchanger and the refrigerator.

1.6.2 Cold air storage

Fish is normally stored in chill rooms at 0-4℃ prior to processing [83]. The maximum storage life of fresh fish is 10 to 15 days depending on species. Temperatures below 0℃ in cold room should be avoided to prevent the formation of ice crystals. Also the humidity of the air should be over 90% and the air velocity should be low to minimize dehydration [83].

1.6.3 Effects of chilling on fish

Lipid oxidation is the main chemical reaction resulting in quality deterioration of fatty fish during chilling [80]. The extent of lipid oxidation varies depending on the fish species. Fatty fishes are more susceptible to lipid oxidation during chilled storage. The products of lipid oxidation reactions have been associated with changes in proteins of fish muscle, affecting protein solubility and causing adverse textural changes in muscle. Lipid

oxidation products also contribute to the development of off flavor and odor. Discoloration due to myoglobin oxidation is also observed during refrigerated storage of fish.

1.7 Protein oxidation

In this section the protein oxidation is discussed in relation to the mechanism, markers for evaluation of protein oxidation, the methods used for assessing protein oxidation, and the implication of Pox on fish.

1.7.1 Mechanism of protein oxidation

Protein oxidation in muscle foods could be induced either directly by reactive oxygen species (ROS) or indirectly by secondary byproducts of oxidative stress [25]. Many free radicals such as superoxide (O2 •−), hydroperoxyl (HO2 •) and hydroxyl (HO•) radicals and non-radical species (hydrogen peroxide, singlet oxygen, peroxynitrite, hypochlorous acid, ozone, hypervalent myoglobin species and reduced transition metal) have been identified as possible precursors of oxidation in muscle proteins [29].These reactive species are usually formed in living cells either via metabolic processes or as a response to external stimuli such as stress, irradiation and pollution [84]. Living cells could deal with ROS through enzymatic and antioxidant defense system. After animal death, both the metabolism and enzymatic systems are impaired, resulting in an imbalance between the production of ROS and the antioxidant defense system and as a consequence accumulation of ROS and protein and lipid oxidation products [9]. Natural components of muscle tissue such as transition metals, heme pigments, unsaturated lipids and oxidative enzymes are potential catalysts for the formation of ROS and therefore, play an important role in the initiation of muscle protein oxidation [27]. In a complex matrix such as muscle, there may be a link between protein and lipid oxidation [85] [86] [15] [87] [88] . The interaction between free radicals and proteins and peptides results in alterations in backbone and the side chains of amino acids. These oxidative modifications include formation of cross linked protein derivatives, alteration of side chains amino acids and oxidative cleavage of peptide chains. The specific pathway and chemical nature of final oxidation products depends on the target, the oxidizing system and the intensity of oxidation conditions [89]. The most remarkable and measurable changes of protein oxidative modifications in muscle food system consists of generation of protein carbonyls, loss of thiol groups and formation of protein-cross links and aggregates [29]. In

this part first the pro-oxidants presented in fish muscle will be considered and then the oxidation of protein backbone and side chain is discussed in detail.

1.7.2 Pro-oxidants in fish muscle

There are many compounds that are naturally present in fish muscle and can serve as pro-oxidants by contributing in the oxidation processes [90]. They include transition metals, heme proteins, reducing agents, peroxidases, and lipoxygenases [91]. Transition metals have been reported as one of the major pro-oxidants of muscle foods [92]. The main transition metal in seafood is iron, but copper is also present. Iron is either associated with heme pigments or as non-heme iron complexed with proteins or low molecular weight metabolites. Reducing components of the tissue such as ascorbate can form active ferrous iron from inactive ferric iron [91]. This reactive iron species could contribute to the formation of reactive radical species such as hydroxyl radical ($^{\cdot}OH$). Peroxidases are the enzymes which are naturally present in muscle and can act as an electron donor and catalyze the formation of reactive radicals. Lipoxygenases are a family of iron containing enzymes which catalyze the oxidation of poly unsaturated fatty acids (PUFA) to form fatty acid hydroperoxides. As it will be discussed later in this chapter, the lipid hydroperoxides could induce protein oxidation. Heme proteins such as hemoglobin and myoglobin also play a key role in protein oxidation. The mechanism is explained later in this chapter.

1.7.3 Oxidation of protein backbone

As shown in Figure 1.5 oxidative attack of the polypeptide backbone are induced by radical oxidants by the $^{\cdot}OH$-dependent abstraction of the α-hydrogen atom of an amino acid residue to form a carbon-centered protein radical ($p^{\cdot}$)(Figure 1.5, Reaction a). The initial $p^{\cdot}$ will react with O_2 to produce an alkylperoxyl radical intermediate (Reaction b), which can give rise to the alkylperoxide (Reaction d), followed by formation of an alkoxyl radical (Reaction e). Alkoxyl radical could convert to a hydroxyl protein derivative (Reaction g). Fe^{2+} and Cu^{+} can also catalyze many steps of this pathway.

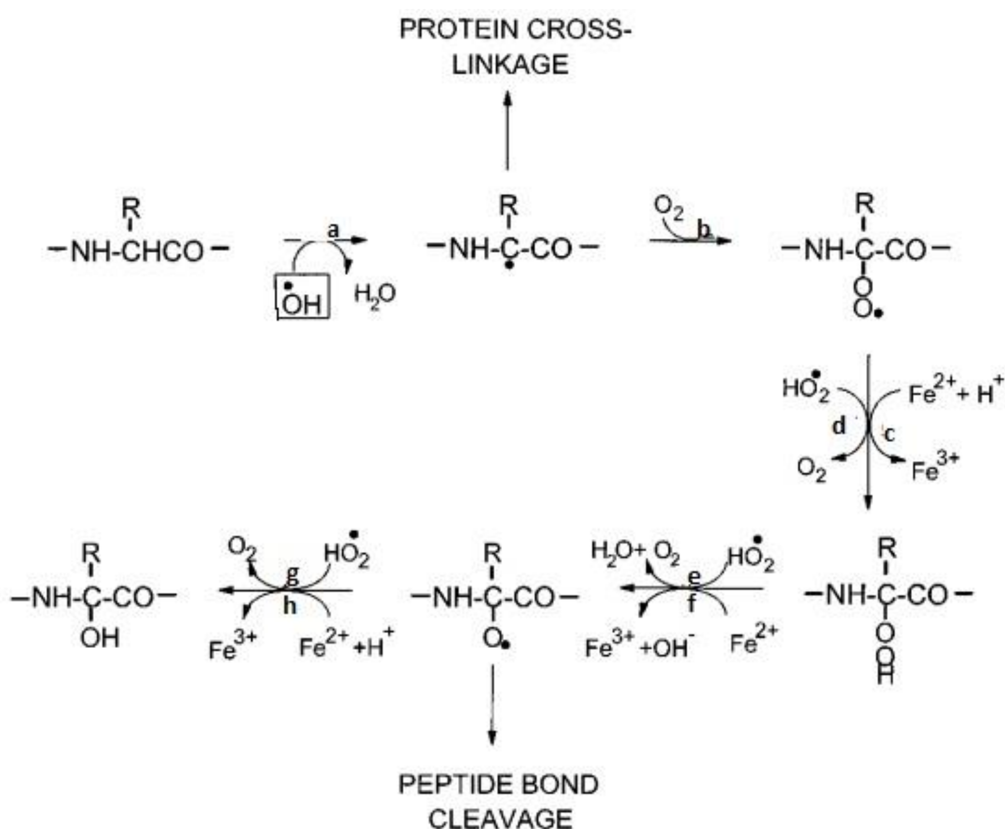
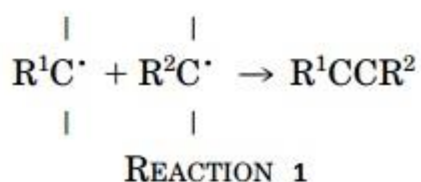


Figure 1-5 Free radical mediated oxidation of the protein backbone [93]

In the absence of O_2 the carbon-centered radical may react with another carbon-centered radical to form a protein-protein cross-linked derivative (Reaction 1).



Furthermore, alkoxyl radicals (Figure 1.5, Reaction e and f) could undergo β-scission or fragmentation reactions by cleavage of the peptide bonds. This results in the formation of carbonyl groups and acyl radicals [93]. The process of generation of carbonyl derivatives is discussed in detail further in this section. In addition, protein fragmentation can occur as a

result of ROS attack of glutamyl, aspartyl and prolyl side chains [94]. Additionally, reaction of hypochlorous acid (HOCL) with proteins can result in backbone cleavage [95]. This process may lead to formation of nitrogen-centered radicals.

1.7.4 Oxidation of amino acid side chains

All amino acid residues are susceptible to oxidation by $^{\cdot}OH$. However, cysteine and methionine are the most susceptible to oxidative modifications by all forms of ROS due to their sulfur atoms [93]. Under even mild conditions cysteine residues can convert to thiyl radicals. These thiyl radicals could either react with other thiol/thiolate to form disulfide, or react with O_2 to generate thiyl peroxyl radicals ($RSOOa^{\cdot}$). The thiol group oxidation is discussed in detail further in this section. Furthermore, cysteine oxidation can result in the formation of sulfenic acid (CysSOH), sulfinic acid ($CysSO_2H$), and sulfonic acid ($CysSO_3H$) [96]. These species are unstable and can form oxyacids by hydrolysis reactions or disulfide bonds by reacting with another thiol group [97] [98]. Methionine residues can be easily oxidized by various kinds of oxidants to form methionine sulfoxide (MeSOX) residues. For aromatic amino acids, including histidine, phenylalanine, tryptophan and tyrosine the major reaction is the addition to aromatic side chains [98]. Tyrosine can be converted to dityrosines by free radicals, peroxynitrite, lipid hydroperoxides and UV and γ-irradiation [99]. These dityrosines may lead to the formation of protein cross-linkages.

1.7.5 Markers for evaluation of protein oxidation

Formation of carbonyl groups, loss of thiol and formation of protein dityrosine are the most common markers of protein oxidation in foods [9]. Since the protein carbonylation and loss in thiol groups are used as protein oxidation markers during this master thesis, these two processes are discussed in detail below. Formation of protein carbonyl could occur in fish during frozen storage. Baron et al. found a significant increase in carbonyl content upon storage of rainbow trout at -20°C up to 13 months [100]. Thiol group oxidation is also reported during storage of fish. Eymard et al. found a significant decrease in thiol group of horse mackerel mince stored for up to 96 h at 5°C [23].

- ## Formation of carbonyl derivatives

Carbonylation is an irreversible and non-enzymatic modification of proteins that involves the formation of carbonyl residues (CO) induced by oxidative stress and other mechanisms [93]. Carbonyls (aldehydes and ketones) can be formed in proteins through four major pathways, namely, (i) direct oxidation of amino acid side chains, (ii) oxidative cleavage of the peptide backbone via the α-amidation pathway or via oxidation of glutamyl side chains, (iii) reacting with reducing sugars by non-enzymatic glycation, (iv) Covalent binding to non-protein carbonyl components such as 4-hydroxy-2nonenal (HNE) and malondialdehyde [8] (Figure 1.6). Among the four pathways, the direct oxidation of arginine, lysine, proline, and threonine residues has been highlighted as the main pathway for protein carbonylation [101] [6]. In addition, Estevez demonstrated that it is the only mechanism that has proved to produce carbonyls from meat proteins [8] and this could also be the case about fish proteins. The formation of carbonyl derivatives from lysine, threonine, arginine and proline side- chains is usually via metal ion-catalyzed oxidation (MCO) systems [102]. None heme iron and other transition metal ions can catalyze protein oxidation in the presence of H_2O_2 in muscle tissue through the mechanism called Fenton reaction or mixed function oxidation [103]. In fact, the combination of transition metals with H_2O_2 results in a highly effective pro-oxidant system for generating carbonyl group from proteins. Amino acid residues situated at metal binding sites are sensitive to MCO by a site specific mechanism. MCO systems catalyze the reduction of Fe (III) to Fe (II) and of O_2 to H_2O_2. These Pro-oxidants react at metal-binding sites on the protein to produce active oxygen species which attack the side chains of amino acid residues. Among other modifications, carbonyl derivatives of some amino acid residues are formed [6]. According to MCO mechanism, reduced forms of transition metals would reduce H_2O_2 to form a reactive intermediate (Hydroxyl radical; $^{\cdot}OH$) via the Fenton reaction (reaction 2) in the proximity of susceptible amino acid side chain [8].

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + HO^- + {}^{\cdot}OH \quad (2)$$

According to scientific evidence two oxidation states of iron (Fe^{2+} / Fe^{3+}) and copper (Cu^+ /Cu^{2+}) are able to promote in vitro formation of carbonyl groups [104]. The reduced forms act as electron donors and the oxidized forms act as electron acceptors. For instance, Fe^{3+} could produce $^{\cdot}HO_2$ radicals from H_2O_2 (reaction 3).

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + {}^{\cdot}HO_2 + {}^{\cdot}H^{+} \quad (3)$$

Furthermore, the reduced forms of transition metals could also reduce molecular oxygen to form super oxide anion radical (reaction 4), which undergoes successive reactions to form hydrogen peroxide (reaction 5) and hydroxyl radical (reaction 6), which could contribute to the formation of carbonyl groups from amino acid side chains.

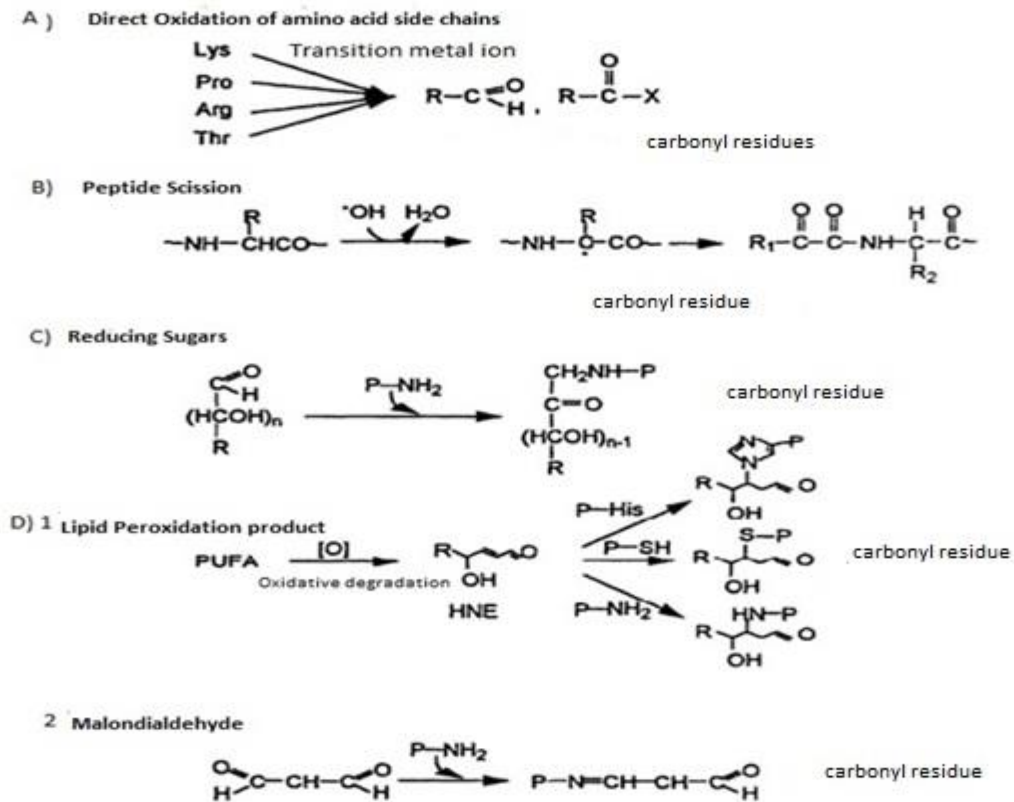


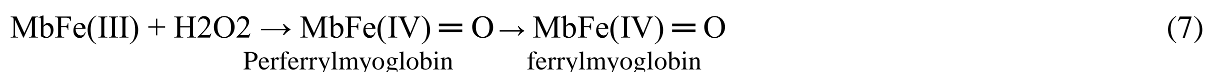
Figure 1-6 Mechanisms involved in the formation of protein carbonyls A) Metal catalyzed oxidation of basic amino acid side chains. B) Peptide backbone cleavage or peptide scission. C) Reaction with reducing sugars. D) Binding to non-protein carbonyl components such as 1. Lipid peroxidation products such as 4-hydroxy-2-nonenal(HNE) and 2. Malondialdehyde [27]

$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + {}^{\cdot}O_2^{-} \quad (4)$$

$$2O_2^{\cdot -} + 2H^{+} \rightarrow H_2O_2 + O_2 \quad (5)$$

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + HO^{-} + HO^{\cdot} \quad (6)$$

As a consequence of MCO, threonine is converted into α-amino-3-keto butyric acid, lysine into α-aminoadipic semialdehyde (AAS), and arginine and proline into γ-glutamic semialdehyde (GGS). AAS and GGS were proposed as biomarkers of oxidative damage to proteins by Daneshvar and coworkers [105]. These biomarkers were found to account for up to 70% of the total protein carbonyls produced in meat products [8]. Reaction of susceptible amino acid side chains with ˙OH radical which is produced by MCO could yield AAS and GGS. In this pathway, the side chain of amino acids is oxidatively deaminated in the presence of transition metals. The reactive species would abstract a hydrogen atom of amino acid residues, leading to the formation of carbon-centered radical. Transition metals may accept an electron of the carbon radical to form an imino group which is hydrolyzed to yield aldehyde moiety [104]. The MCO system is reported as a pathway for carbonylation of muscle food proteins [8]. Decker et al. reported that the combination of transition metals such as Fe^{3+} / Cu^{2+} with ascorbic acid was effective at inducing the formation of carbonyls from oxidized Turkey myofibrillar proteins [10]. The results indicate that transition metals could generate reactive radicals from O_2 or H_2O_2 even in the absence of ascorbic acid. Beside transition metals other natural components of muscle such as myoglobin (Mb), have been known to induce protein carbonylation [8]. Heme proteins such as hemoglobin (Hb) and myoglobin (Mb) are present in fish muscle tissue. Hb is usually present in fish light muscle in small quantities whereas Mb is predominant in dark muscle [9]. A study performed on mackerel reported that myoglobin and hemoglobin make up about 44% and 56% of the total heme protein of dark muscle in bled samples, respectively [106]. In the presence of H_2O_2, MbFe(III) form hypervalent species such as ferrylmyoglobin (MbFe (IV) $=$ O) (reaction 7) which have been found to initiate protein and lipid oxidation [107]. Also, some studies have shown that MbFe(IV) $=$ O is able to transfer its protein radical to other proteins and hence generate reactive secondary protein radicals with extremely long half-lives [108] [109]. Promeyrat et al. recently found that myoglobin is a good predictive marker of carbonyl formation in meat systems which highlight its role in protein carbonylation [110]. They proposed that the release of iron from iron carrying proteins due to the pH decrease after death, leads to the formation of free iron or catalytic iron. This free iron can react with hydrogen peroxide to give hydroxyl radical (˙OH) which is capable of the initiating protein oxidation.



Besides the presence of transition metals and myoglobin, the oxidation of proteins is affected by the lipid-derived reactive oxygen species. The impact of lipid oxidation products in protein oxidation is discussed in more detail in section 1.7.6. In addition to lipid oxidation products, the oxidation of amino acids and proteins are affected by the numerous environmental factors such as pH, temperature, water activity and the presence of other promoters and / or inhibitors such as phenolic compounds [111] [112] [27]. Light and irradiation are also capable of initiate protein oxidation but the knowledge of the pathway is limited [113] [13].

Reactivity of protein carbonyls

In general, carbonyl moieties from biomolecules could participate in multiple reactions [114]. Until recently, the lack of knowledge on the definite chemical nature of food protein carbonyls hindered the investigation of the involvement of such unknown components in further reactions [8]. Xiong hypothesized the interaction of protein-bound carbonyls with free amines leading to produce cross-links between polypeptides [115]. Later, Stadtman and Levine and Akagawa et al. found the likely contribution of protein carbonyl residues in condensation reaction with amino groups from neighboring amino acid side chains to form cross-links through Schiff-base formation [101] [104]. Estevez et al. and Estevez and Heinonen reported a decrease of AAS and GGS of MP followed by their increase as a result of the in vitro MCO of MP [116] [88]. The aldehyde moiety from specific protein carbonyls can contribute in several reactions including [8]:

(1) Oxidizing into a carboxylic acid. Under intense oxidative condition, the oxidation of AAS will lead to the formation of a stable end product, α-aminoadipic acid (AAA). Recent researches have confirmed the formation of AAA in MP oxidized in vitro with a model system(Fe^{3+}, H_2O_2,ascorbate) [8]. Sell et al. recently reported that AAA may be a more reliable marker of protein oxidation than its carbonyl precursor [117].
(2) Forming an aldol condensation product by the reaction with an aldehyde moiety from another protein-bound carbonyl residue. Several meat researchers have investigate the formation of carbonyl-amine condensation in MP [112] [27] [118]. The condensation reactions could result in the formation of cross-linked proteins and polypeptides.

(3) Forming covalent bond via Schiff base formation with an amino group from neighboring protein-bound amino acid mainly lysine. Schiff base can be formed from the reaction between an aliphatic or aromatic amine and a carbonyl moiety by a nucleophilic addition to form an imine. This reaction could lead to the formation of cross-links between peptide chains.

(4) Forming a Strecker aldehyde via Strecker-type degradation –oxidative deamination and decarboxylation of the amino acids by the reaction with an α-amino group from a free amino acid. The formation of Strecker aldehydes is usually ascribed to the oxidative deamination and decarboxylation of the free amino acids in the presence of α-dicarbonyl compounds formed in the Maillard reaction and/or from lipid oxidation such as alkadienals and ketodienes [119]. A recent study reported the protein degradation products, free amino acids and free oxidized amino acids as a sources of Strecker aldehydes in the absence of reducing sugars and oxidizing lipids [120].

- **Loss of thiol group**

The thiol group of cysteine (RSH) and methionine is highly susceptible to oxidation in the present of different kinds of reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydrogen peroxide (H_2O_2) and nitrogen dioxide (NO_2), which are formed in cells and accumulated in muscle post-mortem. The initial oxidation products of cysteine is sulfenic acid (RSOH) (reaction 8). Sulfenic acids may be stabilized by the protein microenvironment or serve as a central intermediate to other reversible and largely irreversible species. Condensation with the tripeptide glutathione (γ-L-Glu-L-Cys-Gly, GSH) or with a protein thiol results in glutathione–protein mixed disulfides or intramolecular and intermolecular protein disulfides cross-links(RSSR) (reaction 9). Alternatively, sulfenic acid can be oxidized to form sulfinic acid and, under more severe oxidizing conditions, to sulfonic acid [121]. Figure 1.7 shows a schematic pathway of the protein thiol group oxidation and reactions.

$RSH + H_2O_2 \rightarrow RSOH + H_2O$ (8)

$RSOH + RSH \rightarrow RSSR + H_2O$ (9)

In more complex reactions thiyl radicals are formed which may, in turn, form disulfide cross- links (reaction 10).

$RS^{\cdot} + RS^{-} \rightarrow (RSSR)^{\cdot -} \xrightarrow{O_2 \;\rightarrow\; O_2^{\cdot -}} RSSR$ (10)

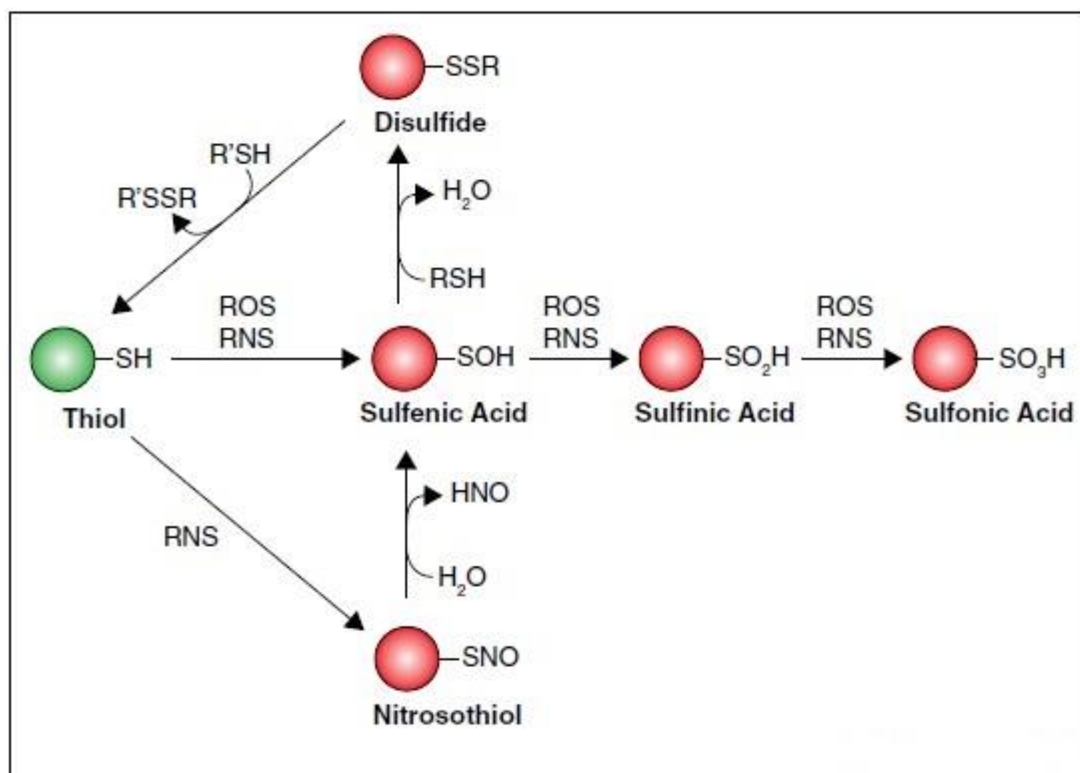


Figure 1-7 The pathway of protein thiol group oxidationProtein thiols react with ROS and RNS. The initial oxidation product of this reaction is sulfenic acid. This transient modification may be stabilized or condense with a second cysteine resulting in formation of intramolecular or intermolecular protein disulfides. Alternatively, sulfenic acid may be oxidized to sulfinic acid and under severe oxidizing conditions, sulfonic acid. Reaction of thiols with RNS also generates nitrosothiols [121].

Myosin has about 42 sulfhydryl groups and no disulfide bond, while actin has around 12 sulfhydryl groups. Many of the sulfhydryl in myosin and some of those in actin are accessible to the pro-oxidants presented in the muscle after the animal death [122]. Therefore, it is not surprising that oxidized myofibrils showed changes in sulfhydryl and disulfide bonds. Methionine residues can also be easily oxidized to methionine sulfoxide.

1.7.6 Role of the oxidizing lipids on protein oxidation

Lipid-derived peroxidation products, such as radicals, lipid hydroperoxides and reactive aldehyde derivatives could also initiate modifying of proteins both *in vivo* and *in vitro* [123]. Several authors reported the coupling of lipid and protein oxidation process in a model

system [16, 112, 123, 124]. Theoretically, the oxidative reactions could be transferred either way between lipids and proteins [29], However, the lipid oxidation onset would take place faster than protein oxidation [125] [124] [126] [86], and therefore it is more likely that lipid-derived radicals induce Pox than the other way around. In contrast, Liu and Xiong have reported that Pox was minimally affected by lipid peroxidation [127]. ROS derived from the first stages of lipid oxidation such as $^{\cdot}OH$, $^{\cdot}O_2$, and $^{\cdot}ROO$ can abstract hydrogen from susceptible amino acids, leading to a radical mediated chain reaction similar to that of lipid oxidation [102]. The reaction of the primary lipid peroxidation products, lipid hydroperoxides (LOOH), with proteins is not well characterized. This could be due to the low stability of LOOH, which decomposes rapidly to aldehydic products [128] [129]. End-products of lipid peroxidation, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal are very reactive and could induce protein damage. MDA could react with lysine residues of proteins to form carbonyl derivatives. α ,β- unsaturated aldehydes such as 4-hydroxy-2-nonenal could undergo Michael addition reaction with amino group of lysine residues, the thiol group of cysteine residues and imidazole group of histidine residues [130] [131] [132] [133] [134]. Hene et al. reported fatty acid dependent generation of carbonyl derivatives and loss of lysine residues in bovine serum albumin (BSA) in the presence of ascorbate and Fe^{3+} ions in a model oxidation system [123]. They demonstrated that these modifications are dependent on the degree of unsaturation of the fatty acid and that polyunsaturated fatty acid alkoxyl radicals produced during degradation of lipid hydroperoxides are likely involved in the formation of protein carbonyl derivatives. Park and Xiong found that the incubation of MP with linoleic acid and lipooxidase leads to formation of carbonyl compounds [16]. In addition, The reaction between lipid derived carbonyls and amino groups from proteins can lead to the formation of Polymerized forms of Schiff bases [124]. It is noteworthy that MP possesses antioxidant potential and can protect themselves and other food components, such as lipids from oxidative reactions [125] [135]. It could be attributed to the oxidation of sulfur-containing amino acids in MP which could act as electron scavengers and endogenous protection mechanism against ROS [136]. In the other hand, some proteins in muscle foods such as myoglobin could act as pro-oxidant that induce and accelerate lipid oxidation [137].

1.7.7 Methods for measuring protein oxidation

There are various types of protein oxidation products. Hence, there are different methods for detection and quantification of these modifications. The most commonly measured products

of protein oxidation are contents of protein carbonyls and thiol groups. In this section the methods used for quantification of protein oxidation during work on this master thesis are discussed and the advantages and disadvantages of these methods are mentioned.

Methods for detection of protein carbonyl groups

Protein carbonyls are widely determined as a measure of protein oxidation because they are chemically stable, which is useful for their detection [138]. Many assays are available for detection of protein carbonyls. These methods include DNPH-based spectrophotometric method (conventional), DNPH-based Enzyme-Linked Immunosorbent Assay (ELISA), Tritiated sodium borohydride method, Gel electrophoresis based detection of carbonyls, DNPH dot blot method, Fluorophores with carbonyl reactive groups, GC and HPLC detection of carbonyls, Mass spectrometry and Novel carbonyl-reactive isobaric labels for quantitative analysis of protein-bound carbonyls [139]. Since the conventional DNPH-based spectrophotometric and ELISA methods are used in this master thesis, these two methods are investigated here (the protocols used are described in chapter 2).

- ### DNPH-based spectrophotometric method (conventional)

The DNPH method is the most widely used method for protein carbonyl determination which was established by Fields and Dixon in 1971 [140]. The principle of the method is derivatization of carbonyl groups (ketones and aldehydes) using 2, 4 Dinitrophenylhydrazine (DNPH), which is normally prepared in hydrochloric acid (HCl) with a paired control sample derivatization in acid alone. The DNPH reagent reacts with the ketone and aldehyde functional groups and form 2, 4-dinitrophenyl (DNP) hydrazone which displays a maximum absorbance peak at around 370 nm (Figure 1.8). Excess DNPH is required for derivatization of all protein carbonyl groups in the sample, but as the unbound DNPH absorbs at the same wavelength as the protein- bound DNPH, it is necessary to remove the unbound DNPH after the derivatization step by precipitating out the protein with trichloroacetic acid (TCA) and washing the sample extensively with organic solvents such as ethanol/ethylacetate.

After the washing steps the protein pellet is dissolved in guanidine hydrochloride. Quantitation of protein carbonyls after derivatization is determined by measuring absorbance at 370 nm and calculating hydrazone concentration using the molar extinction coefficient of 22 000 M^{-1} cm^{-1} per mg of protein for dinitrophenyl hydrazone.

The original method was developed for analyzing the oxidative stress in biological samples and has subsequently been employed with some modifications by food scientists [8]. These modifications include using high ionic strength buffers to facilitate the extract of particular food proteins such as MP and treating the samples with a hydrochloric acid-acetone solution in order to remove interfering chromophore substances such as hemoglobin, myoglobin and retinoids [28].

Conventional DNPH-based assay is a simple, convenient and cheap method and provides useful and meaningful information of total carbonyls present in sample. However, several drawbacks have been reported by researchers. Carbonyl moieties can be present in proteins due to mechanisms that do not involve the oxidation of amino acids [141]. This could result in the overestimation of protein carbonyls. In addition, the extensive washing steps to remove unbound DNPH results in a loss of protein (approximately 10-15%) which in turn results in the underestimation of carbonyl contents [139]. Incomplete re-solubilization of the protein in guanidine is also reported as a problem encountered in the conventional method and may result in underestimation of the protein carbonyl content and turbidity in the solution due to incomplete solubilization in detergent which can interfere with spectrophotometric analysis [139]. Besides, the method lacks the specificity to determine the specific nature of oxidation products. Meat scientists often complain of the difficulty encountered to obtain reliable and consistent results [8]. Cao and Cutler have reported that DNPH method is unsuitable for measuring the actual level of protein in animal tissue because of presenting artifacts such as nucleic acids and chromophores [142]. Additionally, some oxidative reactions in proteins might not lead to the generation of carbonyl compounds and DNPH method would underestimate the overall oxidative damage to proteins. Hence, other methods should be used as complementary techniques together with the DNPH method (loss of sulfhydryl groups, formation of cross-links, tryptophan oxidation etc.) to have a reliable overall picture on the extent of the oxidative damage [8].

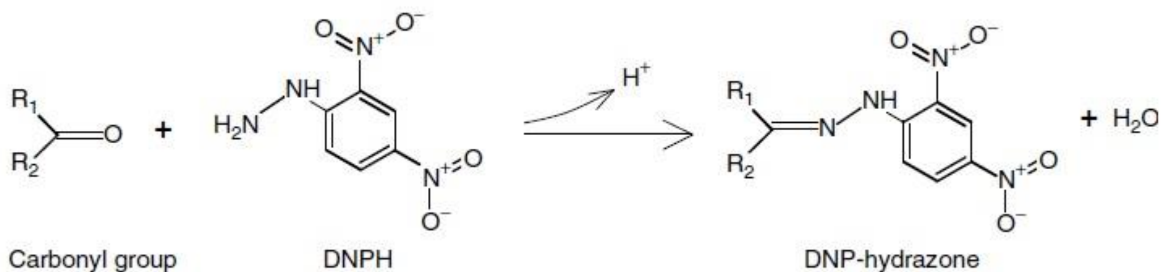


Figure 1-8 The reaction between 2,4-dinitrophenylhydrazine and protein carbonyl groups to form a hydrazone which may be determined spectrophotometrically at 370 nm [28].

- ## DNPH-based Enzyme-Linked Immunosorbent Assay (ELISA)

The Protein Carbonyl ELISA was first developed by Buss and co-workers [143]. The method has been modified by Alamdari et al. to increase the sensitivity for analysis of samples with low protein concentration [144]. ELISA is a plate based assay technique which is typically performed in a 96-well polystyrene plate. The principle of protein carbonyl determination by immunoassay is based on the detecting DNPH using anti DNPH antibody. Nowadays, a variety of ready- to- use ELISA kits are commercially available for detecting of carbonyl groups. There are different types of ELISA assays including: direct, indirect, competitive and sandwich ELISA. Since an indirect ELISA method was used in this master work, this method is considered in detail.

Indirect ELISA is a two-step assay which involves two binding processes of a primary antibody and labeled secondary antibody. The procedure consists of three major steps; immobilization of sample on the ELISA plate, DNPH derivatization, and antibody-based detection. Protein samples of specified protein concentration are first allowed to adsorb to wells of a 96-well plate and then react with DNPH. Different washing steps are used to remove unbound DNPH. Blocking solution is added to block remaining protein bonding sites. Then primary antibody is added followed by adding secondary anti body, which recognizes and binds to the primary anti body. Then the substrate solution is added and converted to a detectable and colored product and the absorbance is read in a plate reader spectrophotometer. Figure 1.9 shows a simple scheme of the indirect ELISA procedures.

The ELISA test has important advantages. It is a rapid, easy and sensitive method. A producer claimed that their protein carbonyl ELISA kit is 5-10 times more sensitive than the conventional DNPH method [145]. In addition, it requires only microgram amounts of sample. However it has some drawbacks. It is an expensive method and, like the spectrophotometric assay, does not provide any information on the extent of oxidative damage to a particular protein in a complex mixture [138]. Some studies reported that the ELISA method is very sensitive for analysis of purified proteins but not for complex mixtures [142] [146]. The commercially available ELISA standards are not standardized according a common reference and therefore the concentration of carbonyl estimated in identical samples may vary depending on the kit used [147]. Rogowska-Wrzesinska et al. attributed the differences between different DNPH-ELISA assays to the preferential adsorption of certain pools of protein carbonyls to the plate, selective reactions of certain types of adsorbed DNPH-reacted proteins with primary and secondary antibodies and difficulties in removing unreacted DNPH [147].

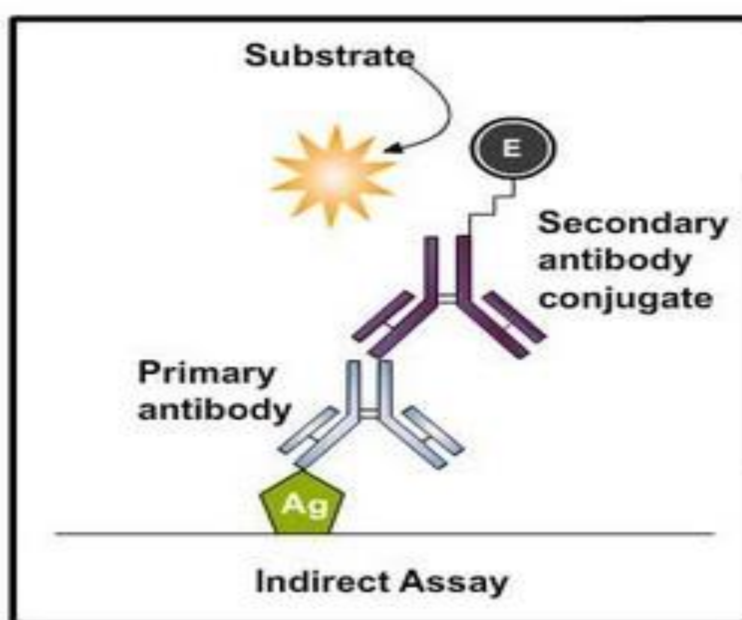


Figure 1-9 A simple scheme of the indirect ELISA procedure.The antigen (Ag) of interest is immobilized by direct adsorption to the assay plate. Detection of the antigen (carbonyl group) is performed using a matched set of unlabeled primary antibody and conjugated secondary anti body [148].

- **Detection of Thiol groups**

The content of thiol groups in muscle food does not seem to be affected by post-mortem aging and is a good marker of protein oxidation during storage of muscle foods [29]. The measurement of the loss of thiol groups in protein from muscle foods is often based on use of 5,5'- Dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) which is the most common reagent for the quantification of thiols [29]. The method is described in chapter 2.
It is a relatively easy method to measure the contents of total thiol in the water soluble and MP fraction of muscle foods. However, the reaction of DTNB with protein thiols is hindered by its high polarity and negative charges [149]. In addition, DTNB assay is suitable for nanomolar ranges of thiol and this can be a problem when measuring thiol concentrations in dilute protein solutions [150].

1.7.8 Effect of protein oxidation on fish quality

Proteins are the major components of muscle tissue and therefore modification of the native structure and/or integrity of muscle proteins are known to affect muscle food quality including texture, aroma, water holding capacity, color and functionality [8].

As already mentioned Pox causes physico-chemical modifications in proteins including amino acid destruction, changes in protein solubility and functionality, loss of enzymes activity and impaired protein digestibility [27]. Considering these severe modifications, it could be hypothesized that oxidation-induced changes in fish proteins could also affect fish quality. As the water holding capacity and protein solubility are considered in this master thesis, the impact of Pox on these two properties are considered in this part.

MP are the most abundant muscle proteins and the specific amino acid composition and primary sequence of amino acids of these proteins largely influence their native structure which in turn determines their functionality [8]. Therefore, the distribution and characteristics of the amino acid side chains play a relevant role in their structure. In muscle, the polar (hydrophilic) residues of amino acids are exposed to the water phase, while the non-polar (hydrophobic) groups are occluded in the molecule. So, the interaction between the polar groups of amino acids and water is essential for their water-related functionality such as water holding capacity [38]. These polar groups are also more accessible to pro-oxidants present in muscle, and hence more susceptible to

oxidative reactions. Protein carbonylation may lead to the loss of amino groups which in turn results in the alteration of the distribution of the electrical charges and the overall arrangement of MP. An intensive protein oxidation modification could even result in the changes of isoelectric points of proteins. As the net charge of protein approaches zero, the positive and negative groups within the protein are attracted to each other and result in the reduction in the amount of water that can be held by that protein. In addition, repulsion of the structures within the myofibril is reduced and the enlarged extracellular space between adjacent muscle fibers in oxidized muscle could result in the decreased water holding capacity [38]. Additionally, formation of cross-linked oxidation products such as dityrosine and disulfide in oxidized myofibril are also reported to reduce the water holding capacity of muscle foods [151].

The decreased solubility (extractability) of myofibrillar and sarcoplasmic proteins due to protein oxidation are reported by the different researchers [152] [10] [13] [153]. JARENBÄCK and LILJEMARK found significant decrease in myofibrillar protein solubility of cod muscle upon oxidation in a model system [154].

The changes in the intermolecular and intramolecular interaction within a protein due to the protein oxidation, lead to the conformational changes of protein. These changes can increase the surface hydrophobicity of the protein due to the changes in tertiary structure. In addition, formation of dimer, timer, and polymer and other inter and intra cross-linkages due to protein oxidation can lead to the protein aggregation. Severe protein oxidation can lead to the protein denaturation and precipitation, which is associated with decreased protein solubility [98].

2 Material and Methods

2.1 Experimental design

Mackerel (*Scomber scombrus*) was caught in the North Sea on February 2nd, 2013. The fish was stored in chilled seawater at approximately 2 ºC and transported to land. They arrived on land on the 5th of February. Mackerel was filleted by hand on day three after catch and was packed in 10 kg blocks, a total of 15 units (Figure 2.1). Six units were put on ice at +4 ºC and nine units were frozen at -20ºC and transported to SINTEF. At SINTEF, the ice stored samples were stored at +4ºC for four and nine days. Then, they were separated into white, black and whole muscle. The whole muscle contains black, white and skin (mixed muscle). The samples were minced, vacuum packed and stored in a -80ºC freezer until analyzed. Frozen samples were stored in a -30ºC freezer for one, seven and twelve months. At each sampling point the blocks were thawed and separated into white, black and mixed muscle. Then, they were minced, vacuum packed and transferred to a -80 ºC freezer until analyzed. After each storage period the samples were thawed in a +4 ºC cold room overnight and the following analyses were carried out: The content of Carbonyl and thiol groups, solubility of salt and water soluble proteins, water-holding capacity and water content. Figure 2.2 gives an overview of sample preparation and the experimental set-up.

Figure 2-1 Mackerel fillets (left) and 10 kg blocks of mackerel (right). Photo was taken by Revilija Mozuraityte at SINTEF.

2.2 Chemicals

All chemicals used during this work were of analytical grade. The chemicals used in this master work and the producers are listed in table 2.1.

Table 2-1 List of Chemicals used

Chemical	Producer	Chemical	Producer
Trisma base	MERK	Ethanol	VWR
EDTA	MERK	Sodium chloride	Chiron AS
Sodium dodecyl sulfate(SDS)	VWR international AS	Potassium chloride	MERK
Butyl hydroxyl toluene(BHT)	Sigma	DTNB	Sigma
Chloroform	VWR	Urea	VWR
Methanol	VWR	$Na_2Hpo_{4.2H2O}$	MERK
Trichloroacetic Acid(TCA)	MERK	$NaH_2PO_{4.H2O}$	MERK
Acetone	Sigma	EDTA	MERK
Hydrochloric Acid	MERK	Brilliant Blue G-250	Chiron
2,4-Dinitrophenylhydrazine (DNPH)	VWR	BSA	Sigma
Potassium dihydrogen phosphate	MERK	NaOH	VWR
Ethylacetate	Apotekproduksjon As		
Guanidine hydrochloride	Sigma		

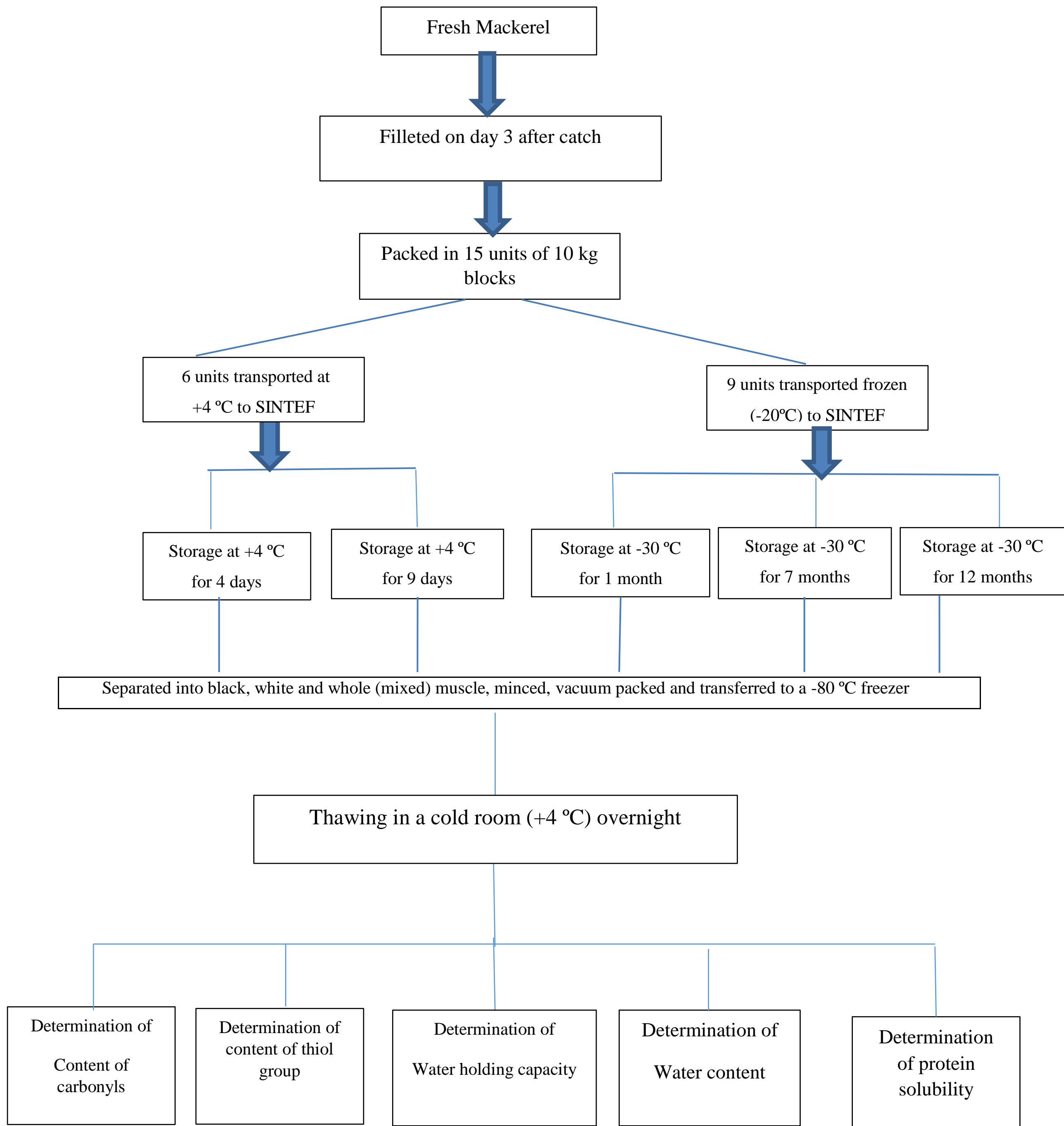


Figure 2-2 Flow chart of sample preparation and experimental design

2.3 Methods

2.3.1 Protein solubility

Protein solubility was determined by extraction of water and salt soluble proteins as described by Anderson and Ravesi and Licciardello et al [155] [156]. About 4 grams of fish was homogenized with an Ultra Turrax (IKA®, T 25 Germany) with 80 ml of 0.05 M phosphate buffer (0.05 M KH_2PO_4, 0.029 M NaOH, pH7) for 30 seconds and centrifuged at 8000 xg for 20 minutes at 4°C (Sorvall ® Ultraspeed centrifuge, RC5C). To obtain the water soluble fraction the supernatant was filtered through glass wool and the volume was made up to 100 ml with phosphate buffer. To extract the salt soluble fraction the precipitate was homogenized with 80 ml 0.05 M phosphate buffer with 0.6 M KCl and centrifuged at 8000 xg for 20 minutes at 4°C. The supernatant was filtered and the volume was adjusted to 100 ml with phosphate buffer containing KCl. Protein measurement was carried out using the BioRad method as described in section 2.3.2.

2.3.2 Spectrophotometric determination of protein concentration (Bio-Rad method)

Concentration of water and salt soluble proteins were measured using the method described by Bradford [157]. The Bradford or Bio-Rad Protein Assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The method is based on the binding of protein to the Coomassie Brilliant Blue G-250 dye in an acidic environment. The maximum absorbance of an acidic solution of Coomassie® Brilliant Blue G-250 shifts from 465 nm to 595 nm, when the color binds to protein. Water and salt soluble proteins were extracted as described in section 2.3.1. A blank was prepared using 0.05 M phosphate buffer instead of sample in water soluble proteins and phosphate buffer with KCl in salt soluble proteins.

A series of standards were prepared with different concentrations of Bovine Serum Albumin (BSA). 0.2, 0.4, 0.6, 0.8 and 1 mg/ml dilutions were made by diluting the Standard Protein Stock with distilled water.

Dye reagent was diluted with distilled water (1:5) and 5 ml was added to 100µl of standards, suitably diluted samples and blanks and mixed well with a whirl mixer (Ika (R),MS_2

Minishaker). Absorbance was read at 595 nm after 5 minutes incubation at room temperature with a UV/visible spectrophotometer (Pharmacia biotech, Ultrospec 2000). The samples and standards were run in triplicate. Protein concentration in samples was calculated using equation of the standard curve line in Excel program. Results were expressed as mg of protein in ml of extract and percentage of protein in gram wet weight of fish.

2.3.3 Protein oxidation

Protein oxidation was evaluated by measuring the total carbonyl and total thiol content. Protein carbonylation was determined by a conventional DNPH method and an enzyme linked Immunosorbent Assay (ELISA). Thiol content was measured by Ellman's reagent. The methods are described in section 2.3.3.1 and 2.3.3.2 for protein carbonyl and thiol group contents, respectively.

Protein carbonyls

- **Conventional DNPH based spectrophotometric method**

Protein carbonyls were measured by derivatization with 2,4-dinitrophenylhydrazine (DNPH) according to the method described by Levine et al. [158] and Reznick and Packer [159] with some modifications [160] [161] as described below. The method is based on the derivatization of the carbonyl groups with 2, 4-dinitrophenylhydrazine (DNPH), leading to formation of 2, 4-dinitrophenyl hydrazone (DNP) products. The DNP conjugated products can be detected with a spectrophotometer at 370nm (Pharmacia biotech, Ultrospec 2000).

1 gram of thawed minced sample was homogenized with an Ultra Turrax (IKA®, T 10, Germany) in 10 ml of tris buffer (50 mM trisma base, 1 mM EDTA, 1 % Sodium dodecyl sulfate, pH 7.4) and 100 µl butyl hydroxyl toluene (BHT) 0.01% as an antioxidant, for 1 minute. This is the total homogenate fraction. 3 portions of 500 µl homogenate fraction were pipetted out into eppendorf tubes and the rest was centrifuged (Sorvall ® Ultraspeed centrifuge, RC5C) for 10 minutes at 17,300 xg and 4°C to extract the water soluble fractions. 3 portions of 500 µl water soluble fraction were pipetted out into eppendorf tubes. Then, 100 µl of chloroform/methanol (2:1) mixture was mixed with 500 µl of homogenate and water soluble fraction to remove lipid contaminants. Furthermore, 200 µl of the homogenate fraction and 500 µl of the water soluble fraction were precipitated with 50 µL saturated

Trichloroacetic acid (TCA) in three parallels and one blank. Blanks were made using tris buffer instead of sample for both homogenate and water soluble fractions.

The samples were mixed with a whirl mixer for 10 seconds (Ika [(R)],MS_2 Minishaker,Germany) and centrifuged for 4 minutes at 13,400 xg and 4℃ (Eppendorf 5415R). The supernatant was decanted and 1 ml Aceton/HCl (3:100, v/v) was added to protein pellets to remove chromophores and help to denature protein, and centrifuged for 5 min at 13,400 xg (Eppendorf 5415R). Further, the protein pellets were washed with 1 ml tris buffer and reprecipitated by adding 50 µl saturated TCA.

The pellets and blanks were incubated in dark with 500 µl of 10 mM DNPH in 2 M HCl for one hour. The derivatization reaction of protein carbonyls with DNPH leads to formation of 2,4-dinitrophenyl hydrazone (DNP) products with a yellow to red- color. Subsequently 50 µl of saturated TCA was added to the samples and blanks and mixed with a whirl mixer for 10 seconds followed by centrifugation at 13,400 xg for 4 minutes at 4℃.

Pellets were washed 5 times with 1 ml ethanol/ethylacetate 1:1 (v/v) to remove excess DNPH followed by mixing on a whirl mixer for 10 seconds and centrifugation at 13,400 xg for 4 minutes at 4℃ (Eppendorf 5415R) after each washing step. The pellets were dissolved in 1 ml 5 M guanidine hydrochloride in 20 mM potassium dihydrogenphosphate (pH 2.3). The samples and blanks were incubated at + 4ºC in a cold room overnight. Finally, the samples and blanks were heated in a 37°C water bath (Heto-Holten) for 30 minutes. The samples were mixed with a whirl mixer and centrifuged at 13,400 xg for 10 minutes at 20ºC. Carbonyl content was determined by measuring the absorbance with a UV/visible spectrophotometer (Pharmacia biotech, Ultrospec 2000) at 370nm. Protein concentration was measured by a Biorad method as described in section 2.3.2. A molar absorption coefficient of 22, 000 M^{-1} $cm^{-1}$was used for calculation of protein carbonyls. Carbonyl content was expressed as nanomole of carbonyl per milligram of protein and was calculated from equation 2-1. Carbonyl content was also calculated in µmol of carbonyl per gram wet weight of fish from equation 2-2.

$$\textbf{concentration of carbonyl}\left(\frac{\mathbf{M}}{\mathbf{L}}\right)=\frac{\textbf{ABS at 370nm}\times\textbf{total volume in cuvette(ml)}}{\varepsilon\times\textbf{cuvette width}\times\textbf{sample volume in cuvette(ml)}}$$

$$\mathbf{2-1}\ \textbf{concentration of carbonyl group}\left(\frac{\textbf{nmol carbonyl}}{\textbf{mg protein}}\right)=\frac{\textbf{carbonyl content (M carbonyl per L)}\times\mathbf{10^6}}{\textbf{Protein concentration (mg protein per ml)}}$$

$$\mathbf{2-2}\ \boldsymbol{concentration\ of\ carbonyl\ group}\ \left(\frac{\mu\boldsymbol{mol\ carbonyl}}{\boldsymbol{gr\ wet\ weight}}\right)$$

$$=\frac{\boldsymbol{carbonyl\ content\ (nmol\ per\ \ mg\ protein)}\times\boldsymbol{protein\ concentration\ (mg\ per\ ml)}\times\boldsymbol{volume\ of\ extracted\ prtein(ml)}}{\boldsymbol{Weight\ of\ sample(gr)}\times\mathbf{1000}}$$

ABS= Absorbance

Cuvette width = 1 cm

$\varepsilon =$ Molar absorption coefficient of 2,4-dinitrophenyl hydrazone (22,000 M^{-1} cm^{-1})

Sample volume in cuvette or volume of extracted protein = 0.2 ml for homogenate fraction and 0.5 ml for water soluble fraction

Total volume in cuvette = 1ml

Enzyme-linked immunosorbent (ELISA) assay

- ## Enzyme-linked immunosorbent (ELISA) assay

The method was developed by Buss and coworkers [143] . It is based upon derivatization of carbonyl groups with DNPH and probing of protein-bound DNP with an anti-DNP antibody. Indirect ELISA kit, STA-310 OxiSelect™, was purchased from CELL BIOLABS, INC. Company. The procedure is as follows:

Bovine Serum Albumin (BSA) standard preparation

10µg/ml of reduced and oxidized BSA were freshly prepared by diluting 1 mg/ml oxidized or reduced BSA standards in 1X phosphate buffer saline (PBS) (137 mM NaCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4, 2.7 mM KCl, pH 7.4). A series of carbonyl BSA standards were prepared by mixing the oxidized and reduced BSA in the proper ratios according to table 2.2.

Table 2-2preparation of BSA standard Protein carbonyl standard was made by different concentration of reduced and oxidized BSA to get specified concentration of protein carbonyls (adapted from ELISA protocol Cell Biolabs,INC)

Standard tubes	10 µg/ml oxidized BSA (µl)	10 µg/ml reduced BSA (µl)	Protein carbonyl nmol/mg
1	400	0	7.5
2	320	80	6.0
3	240	160	4.5
4	160	240	3.0
5	80	320	1.5
6	40	360	0.75
7	20	380	0.375
8	0	400	0

Sample preparation

The water and salt soluble proteins were extracted by the method described in section 2.3.1. Protein content of water and salt soluble extracts was determined after suitable dilution by BioRad method using BSA as standard (see section 2.3.2). The protein concentration was adjusted to 10µg/ml with 1XPBS. Salt soluble protein which contains high concentrations of protein carbonyl needed further dilution with 10µg/ml reduced BSA to keep the samples in the range of standard curve.

ELISA Assay protocol

100 µl of the 10µg/ml prepared BSA standards and protein samples were added to the 96-well protein binding plate. Standards and samples were run in duplicates. The wells containing standards and samples were incubated at 4℃ overnight for 14 hours. The proteins adsorbs to the surface of the well plate due to hydrophobic interaction between proteins and the plastic well plate.

The wells were washed 3 times with 250 µl 1X PBS per well. After the last wash, wells were tapped on an absorbent pad or paper towel to remove excess 1XPBS. Then, 100µl of 0.04 mg/ml DNPH (Freshly prepared by diluting 1 mg/ml DNPH stock solution in DNPH diluent) were added to the wells and incubated at room temperature in the dark for 45 minutes. The derivatization reaction leads to the formation of dinitrophenyl hydrazone products.

Further, the wells were washed 5 times with 250µl of 1X PBS/Ethanol (1:1, v/v) with incubation on an orbital shaker (Heidolph unimax 1010, MERCK eurolab) for 5 minutes after each washing steps. After the last wash the wells were tapped on an absorbent pad or paper towel to remove excess washing solution, followed by washing 2 times with 250µl of 1XPBS. Then 200 µl of blocking solution 5% (prepared by dissolving 5 gr of blocking reagent in 100 ml of 1XPBS) was added to each well and incubated for one and half hour at room temperature on an orbital shaker. The blocking solution blocks the unbound proteins and prevents them from binding to antibody in the next step. The wells were washed 3 times with 250 µl of 1X washing buffer (Prepared by diluting 10X wash buffer with deionized water) to remove excess blocking reagent followed by tapping wells on an absorbent pad or paper towel after the last wash.

100 µl of the anti-DNP antibody (prepared by diluting anti- DNPH antibody with blocking solution 5%, 1:1000) was added to wells following incubation for 1 hour at room temperature on an orbital shaker. The anti-DNP antibody acts as a detecting antibody, specifically recognizing and bounding to the DNP-carbonyl group hydrazones. The wells were washed 3 times with 250 µl of 1X wash buffer to remove unbound antibody (Prepared by diluting 10X wash buffer with deionized water). Then, 100 µl of the HRP conjugated secondary antibody (1:1000 dilution, prepared by diluting secondary antibody with blocking solution 5%) was added to all wells and incubated for 1 hour at room temperature on an orbital shaker followed by washing 5 times with 250 µl of 1X wash buffer to remove unbound secondary antibody. Secondary antibody is a horseradish peroxidase -conjugated detection antibody and binds to the primary antibody.

The substrate solution was warmed to room temperature and 100 µl was added to each well. The plates were incubated at room temperature on an orbital shaker for an appropriate time to get a gradient of blue color, with a bright blue color in the highest concentration of oxidized BSA standard and a very faint color in the lowest concentration of oxidized BSA standard (Figure 2.3). The enzyme reaction was stopped by adding 100 µl of stop solution to each well. The blue color will change to yellow after adding stop solution. The absorbance was read immediately at 450 nm in a plate reader spectrophotometer (TECAN, Infinite M200 PRO, Austria) using fully reduced BSA standard as absorbance blank. Carbonyl concentration of samples was calculated from the standard curve. Results are expressed as nmol of total carbonyl per milligram of protein and µmol of carbonyl per gram wet weight of fish.

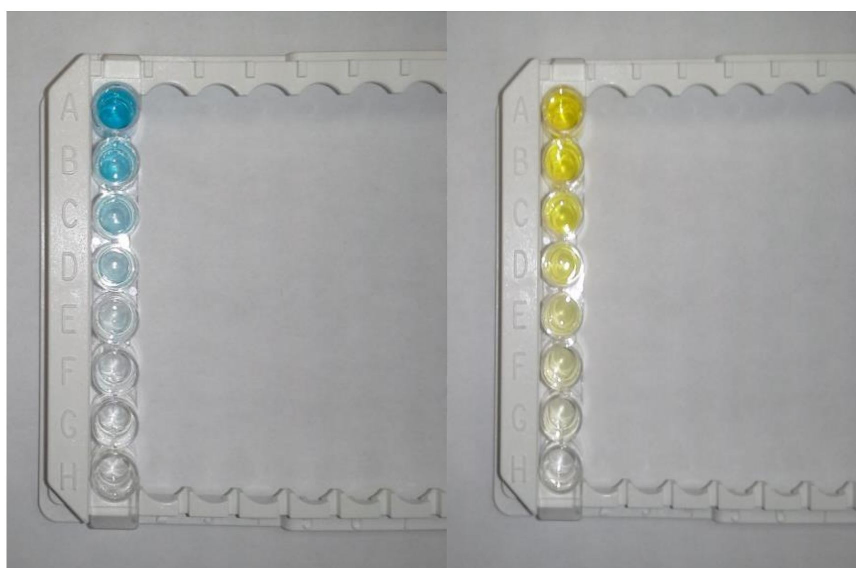


Figure 2-3 The gradient of color in standard samples in ELISA well platesThe left one is a developed blue color in standard samples after adding substrate solution and the right one is a developed yellow color after adding stop solution to standard samples.

Determination of total thiol group content

Total thiol concentration was determined spectrophotometrically after derivatization by Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB [162] [163]. DTNB reacts with the thiols and yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB). Since this is a stoichiometric reaction with the ratio of 1 TNB for 1 protein thiol, the formation of TNB can be used to determine the number of thiols.

The water and salt soluble proteins were extracted as described in part 2.3.1. A blank was made using 100 µl phosphate buffer instead of sample for water soluble proteins and 100 µl phosphate buffer with KCl for salt soluble proteins. 800µl of 8 M urea in the reaction buffer ((0.1 M Na phosphate buffer (77.4 ml of 1M Na_2HPO_4 and 22.6 ml 1 M NaH_2PO_4), 1mM EDTA, pH 7.4)), and 100 µl DTNB was added to 100 µl of water and salt soluble samples and blanks. Urea acts as a denaturant to denature proteins and make the thiols accessible to react with DTNB.

The samples and blanks were mixed with a whirl mixer (Ika [(R)], MS2 Minishaker) and then incubated at room temperature for 30 minutes. Further, they were centrifuged for 3 minutes

at 13,400 xg at room temperature (Eppendorf 5415R). The absorbance was measured at 412 nm (Pharmacia biotech, Ultrospec 2000) with the blank as reference and the thiol content was calculated using a molar extinction coefficient of 14,290 M^{-1} cm^{-1} from the equation 2-3. Results were expressed as nmol of total thiol per milligram of protein. The results was also calculated as µmol of thiol per gram wet weight of fish according to equation 2-4 .Water and salt soluble protein concentration was measured as described in section 2.3.2.

$$\text{Concentration of thiol}(M/L) = \frac{\text{absorbance of TNB at 412nm} \times \text{total volume of substrate in cuvette(ml)}}{\text{cuvette width or length of light path} \times \text{extinction coefficient for TNB} \times \text{sample volume in cuvette(ml)}}$$

$$2-3\ \textit{Concentration of thiol}\ \left(\frac{nMol\ thiol}{mg\ protein}\right) = \frac{\text{Concentration of thiol}\left(\frac{M}{L}\right) \times 10^6}{\text{Protein concentration of sample (mg/ml)}}$$

$$2-4\quad \text{Concentration of thiol}\ \left(\frac{\mu\text{Mol thiol}}{\text{gr wet weight}}\right) = \frac{\text{Concentration of thiol}\left(\frac{nMol}{mg}\right) \times \text{protein concentration}\left(\frac{mg}{ml}\right) \times \text{volume of extracted protein} \times 10^3}{\text{Weight of sample (gr)}}$$

Total volume of substrate in cuvette = 1ml

Volume of sample in cuvette =0.1 ml

Cuvette width = 1cm

Extinction coefficient for TNB = 14,290 M^{-1} cm^{-1}

Volume of extracted protein= 100 ml

2.3.4 Water content

Water content was determined based on an oven drying method. 2 grams of thawed minced sample were weighed into a crucible and dried in an oven (Termaks) at 105 °C for 24 hours and the water content was measured as the loss in sample weight after drying divided by the original sample weight and expressed in percentage according to equation 2-5.

$$2-5\ \%\ \text{water content} = \frac{\text{weight of sample holder and sample befor drying} - \text{weight of sample holder and sample after drying}}{\text{weight of sample}} \times 100$$

2.3.5 Water holding capacity

Water holding capacity (WHC) was determined on minced muscle by low speed centrifugation as described by Eide et al. [164]. A centrifugal force of 210 g was used instead of 1500g. About 2 gr of fish mince were weighed into cylindrical tubes with a nylon membrane. The tubes were then placed in centrifuge tubes containing glass marbles to provide filtration space. The tubes were centrifuged (Sigma-202, West Germany) for 5 minutes at 210 g and 4°C. The test was done in four replicates and WHC was calculated according to equation 2-6. Water content was determined from equation 2.5. % of weight loss was measured according to equation 2-7.

$$2-6\ \textbf{WHC}\ (\%) = \frac{\textbf{water content} - \textbf{weight loss}}{\textbf{Water content}} \times \textbf{100}$$

$$2-7\ \textbf{Weight loss}\ (\%) = \frac{\textbf{weight of sample holder and sample before centrifugation} - \textbf{weight of sample holder and sample after centrifugation}}{\textbf{weight of sample}} \times \textbf{100}$$

2.4 Statistical analysis

Statistical analysis and data processing were performed using Microsoft Excel 2010. All experiments were performed in duplicate or greater and results are expressed as means ± SD. One way analysis of variance (ANOVA) was used to determine significant differences in the same group or between groups. Whenever the ANOVA test was found to be significant, a one-tailed Student's t test was used to determine significant differences between 2 sets of data. To establish a relationship between protein oxidation products and functional properties of muscle (water holding capacity and protein solubility), Pearson correlations were calculated. Differences were considered to be significant at a critical p value of <0.05.

3 Results and Discussion

3.1 Water content

Monitoring the changes in water content of fish muscle during chilled and frozen storage is important because water plays a significant role in physical and biochemical changes in muscle. Changes in water content and distribution of water in muscle lead to changes in the physicochemical properties of muscle. Analyses for determination of water content were performed in four replicates and results are expressed as gram of water per 100 gram of sample. The results are shown in Figure 3.1.

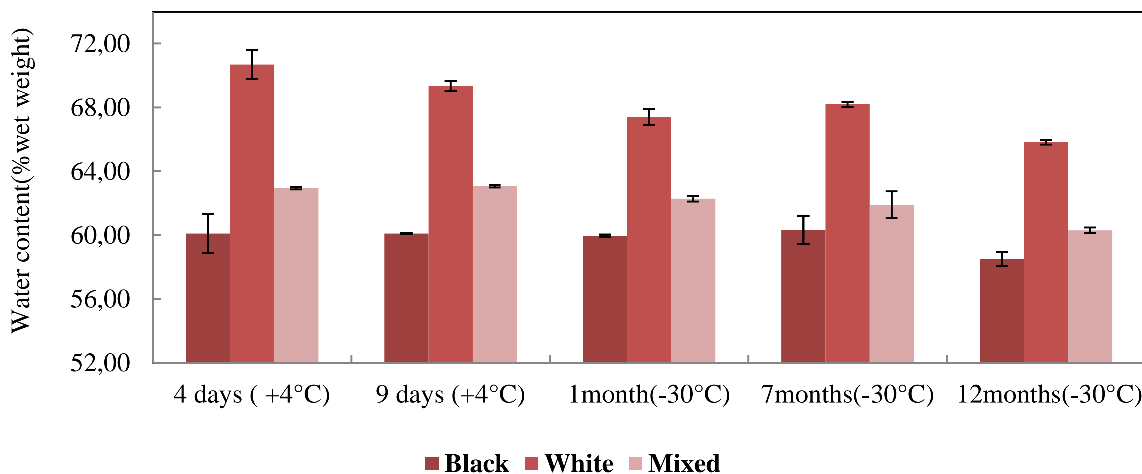


Figure 3-1 Water content (% of wet weight)of black, white and mixed mackerel muscle stored for 4 and 9 days at +4°C and 1, 7 and 12 months at -30°C. Error bars indicate standard deviation(n=4).

As expected, white muscle had a significantly higher water content compared to both black and mixed muscles at all storage times ($p < 0.05$).

While the water content of black and mixed muscle was relatively constant during chilled and frozen storage for 1 and 7 months, water content of white muscle significantly decreased during storage.

Water content of all muscle types was significantly lower in frozen samples stored for 12 months compared to frozen samples stored for 1 and 7 months ($p < 0.05$).

The higher water content of white muscle compared to black and mixed muscle could be explained by the higher fat content of black and mixed muscle and the inverse relationship between the content of moisture and fat in muscle. The results are in agreement with the results of Chaijan and coworkers who reported that the moisture contents of white muscle was generally higher than in dark muscle in sardine and mackerel [165].

The relatively constant water content of black and mixed muscle compared to white muscle during refrigerated and frozen storage for 1 and 7 months could be attributed to the higher fat content of black and mixed muscle. Meat with lower fat cover lost more moisture during frozen storage compared to the meat with higher fat cover [166]. Fat could act as an insulator to prevent water from escaping from the intracellular space and consequently prevent water loss from the surface of tissue.

Significantly lower water content of 12 months frozen stored samples compared to 1 and 7 months frozen stored samples could be due to the loss of moisture during storage through sublimation, evaporation and exudation. Evaporation losses depend on the freezing condition such as temperature and storage time and fish characteristics such as size of pieces. Sublimation losses depend on the storage temperature, presence or absence of packaging and fluctuation in the temperature during storage period [167].

3.2 Water holding capacity

Water holding capacity (WHC) is a very important quality characteristic of muscle foods as it affects both the economics of their production and their quality. The analyses of water holding capacity were performed in four replicates and results are expressed as retained water in % of original water in the sample. The results are shown in Figure 3.2.

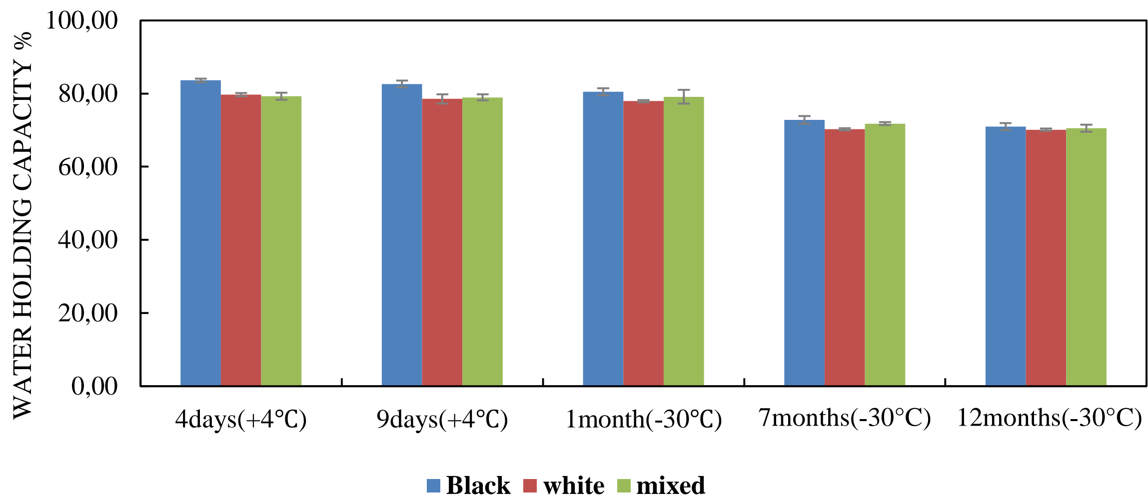


Figure 3-2 Water holding capacity (% of original water)of black, white and mixed mackerel stored for 4 and 9 days at +4°C and 1, 7 and 12 months at -30°C. Error bars indicate standard deviation(n=4).

While dark muscle had a significantly higher water holding capacity compared to white muscle in the samples stored at 4°C for 4 and 9 days and -30°C for 1 and 7 months ($p<0.05$), no significant difference was observed in water holding capacity of dark, white and mixed muscle stored at -30°C for 12 months.

Water holding capacity was relatively stable during refrigerated storage of samples for 4 and 9 days. Similar results were reported by Mørkøre et al who found no significant differences in liquid leakage and consequently water holding capacity during storage of rainbow trout at 4°C for 7 days [168].

Water holding capacity of all muscle types decreased significantly during frozen storage for 7 and 12 months compared to 1 month frozen samples and chilled samples ($p<0.05$).

The higher water holding capacity of dark muscle compared to white muscle in refrigerated and 1 and 7 months frozen samples could be attributed to the lower water content of dark muscle compared to white muscle. Higher water content in a sample may provide more free water which readily could move out from the tissue during storage and processing. Qiao et al. found a negative correlation between water holding capacity and water content in broiler breast fillets [169].

The lower water holding capacity of 7 and 12 months frozen stored samples compared to chilled samples could be due to the formation of ice crystals and increasing concentration of solutes and enzymes in unfrozen solutions. This may result in the structural damage of

membrane as well as protein denaturation. Denaturation of proteins may decrease the ability of myofibrillar proteins to retain and bind water [67] .
The majority of water (85%) in muscle is located within the myofibrils in the spaces between the thick (myosin) and thin (actin) filaments and 15% is located outside the myofibrillar network in the sarcoplasm, between muscle fiber in the inter-fascicular space, between the muscle fasciculi and between the muscle cells [170] [42]. Hence, any changes in muscle proteins, especially in myofibrillar proteins may have an impact on the drip loss from muscle and consequently on water holding capacity. Changes in content, distribution and mobility of water begin after the death of animal. Solidification of water is the most obvious change that occurs during freezing, in which the water is removed from its original location within the tissue [171]. If fish has been stored at low, stable temperature, most of the water will be retained in myofibrillar network and the changes in water holding capacity will be small. However, freezing is often associated with the loss of water holding capacity in the muscle.
Temperature fluctuation during frozen storage leads to recrystallization of ice crystals which in turn could lead to increased drip loss and decreased water holding capacity [172].

Freezing rate could also be a factor influencing water holding capacity of frozen fish. At a slow freezing rate, formation of large ice crystals leads to protein denaturation, tissue damage and leakage of various organelles. As a result, water could be released from muscle more easily, particularly during long periods of frozen storage. On the other hand, at a high freezing rate, small ice crystals form inside the cells. Therefore, the freeze-induced denaturation of proteins decreases [173].

The significantly lower water holding capacity of all muscle types stored at -30℃ for 7 and 12 months compared to those stored at the same temperature for 1 month could be explained by the increasing extent of protein alteration with storage time, resulting in changes in muscle network.

The observed loss in water holding capacity after 7 and 12 months frozen storage could also be due to protein oxidation. In postmortem muscle, protein oxidation is possibly involved in mediating the conversion of muscle to meat, and thus, regulating the meat quality including water holding capacity [174] [98]. During frozen storage of muscle foods, proteins may undergo oxidative reactions [27] [175] [176] [177]. The results from the determination of protein oxidation in this work, confirm that the carbonyl content of myofibrillar and sarcoplasmic proteins significantly increased during frozen storage of mackerel for 7 and 12

months and at the same time thiol content decreased (see section 3.4.1 and 3.4.2 for myofibrillar and sarcoplasmic protein carbonyl content and thiol group content, respectively). There was a significant correlation between the formation of carbonyls in sarcoplasmic and myofibrillar proteins and loss of WHC during storage of Atlantic mackerel ($r = -0.44$ and -0.89, $p<0.05$, for sarcoplasmic and myofibrillar proteins, respectively). Also, a significant correlation was found between loss of thiol group and WHC during storage ($r = +0.89$ and $r = +0.75$, $p<0.05$, for sarcoplasmic and myofibrillar proteins, respectively).

Protein oxidation and protein cross linkage could result in the loss of water holding capacity during storage of muscle foods [38] [29] [27] [178] [10]. Bertram et al. reported the reduced water holding capacity of porcine myofibrillar protein upon protein oxidation in a model system with H_2O_2 [151]. Decreased WHC in cod muscle upon frozen storage for 18 months at -20°C was found together with an increase in carbonyl content [179]. The decreased water holding capacity could be explained by the modifications in the morphological properties of muscle proteins resulting from oxidative reactions during frozen storage. Liu et al. ascribed the loss of water holding capacity of muscle proteins upon oxidation to the enlargement of extracellular space between adjacent fibers due to fiber shrinkage [180]. These authors claimed that increasing the hydrophobic and covalent interactions between myofilaments would lead to increased association of myofilaments and decreased water retention in muscle.

Loss of water holding capacity could be due to the chemical modifications of the amino acid side chains and protein peptide bonds that are triggered during muscle oxidation. These could lead to the modification of charge distribution and the isoelectric point of myofibrillar proteins, which in turn lead to the loss of interactions between polar groups from myofibrillar proteins and water molecules, and decrease the ability of muscle proteins to bind and hold water [38] [181] [178].

In addition, protein oxidation has been reported to trigger protein denaturation and insolubilization due to the chemical and structural changes which have negative effects on water holding capacity.

Also, oxidative modifications lead to polymerization and massive aggregation of the proteins [27]. As a result, the loss of the protein-protein interactions that is favored for functional properties of muscle would reasonably lead to an alteration in water holding capacity. Estevez et al. found that the loss of water holding capacity of porcine muscle stored at -18°C for 12 weeks occurred simultaneously with intense protein oxidation in the muscle [178].

However, there is a lack of knowledge relating the effects of protein oxidation during frozen storage on the quality parameters of muscle foods, especially in fish.

These results are in contrast with the results of Burgaard and Jørgensen who found no significant changes in water holding capacity of rainbow trout stored at -30°C to -80°C for 18 months [182]. This could be due to the differences between Atlantic mackerel and rainbow trout. Furthermore, the fat content of Atlantic mackerel which was used in the present work is higher than in the rainbow trout (16.7 ± 1.1 % compared to 8.2±1.3 %). The higher fat content in fish may lead to more extensive lipid oxidation during frozen storage. Lipid hydroperoxide and secondary lipid oxidation products may react with muscle proteins which may in turn lead to modification of the proteins [183] [184]. Lipid hydroperoxide – protein interactions may result in formation of protein-protein and protein-lipid cross linkages. The secondary products arising from hydroperoxide degradation also readily damage protein and amino acids due to the formation of covalent bonds with amino acids [185]. Damaged proteins may cause poor quality attributes in muscle and loss of water holding capacity.

3.3 Protein solubility (extractability)

Solubility properties of proteins are correlated with other functional properties. Protein solubility measurements were used to determine the effects of storage on protein extractability of mackerel muscle. Analyses were performed in triplicate and the results are presented in Figure 3.3 and 3.4 for sarcoplasmic (water soluble) and myofibrillar (salt soluble) proteins, respectively. Results are expressed as percentage (%) of protein in wet weight of mackerel. The protein concentration in extract was also calculated in milligram of protein per milliliter of extract and the results are presented in table 3.1 for sarcoplasmic and myofibrillar proteins.

3.3.1 Sarcoplasmic (water soluble) proteins

Figure 3.3 shows the solubility of sarcoplasmic proteins during chilled and frozen storage of mackerel.

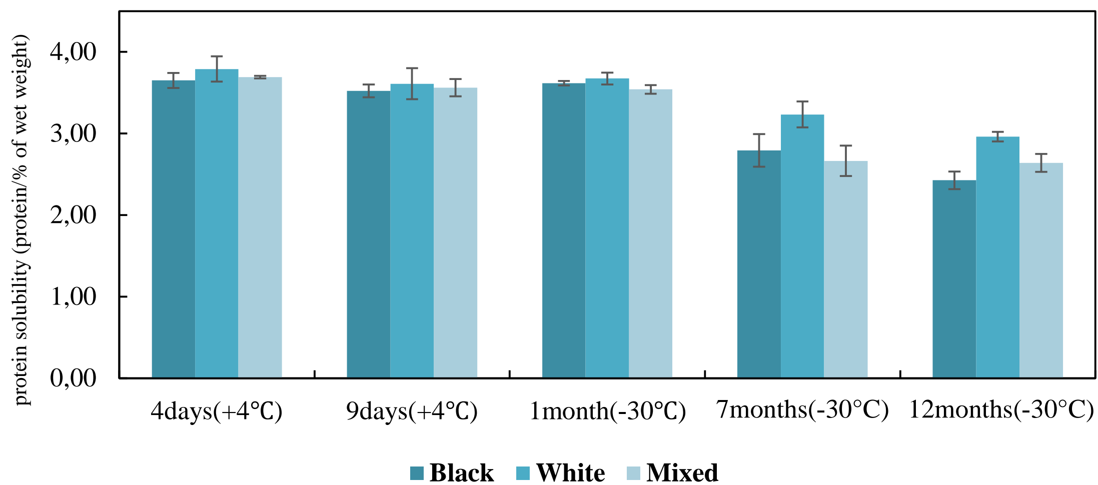


Figure 3-3 Sarcoplasmic protein solubility(protein in % of wet weight) of black, white and mixed mackerel muscle stored for 4 and 9 days at 4°C and 1, 7 and 12 months at -30°C. Error bars indicate standard deviation (n=3).

White muscle had a significantly higher sarcoplasmic protein solubility compared to black and mixed muscle in frozen samples stored for 7 and 12 months ($p < 0.05$).

Sarcoplasmic protein solubility of all muscle types was significantly lower in 7 and 12 months frozen samples compared to the chilled and 1 month frozen sample ($p < 0.05$).

Sarcoplasmic protein solubility decreased significantly during frozen storage ($p < 0.05$) with the exception of mixed muscle in 12 months frozen samples which was approximately the same as for mixed muscle in 7 months frozen samples.

3.3.2 Myofibrillar (Salt soluble) proteins

Figure 3.4 shows the solubility of myofibrillar proteins during chilled and frozen storage of mackerel.

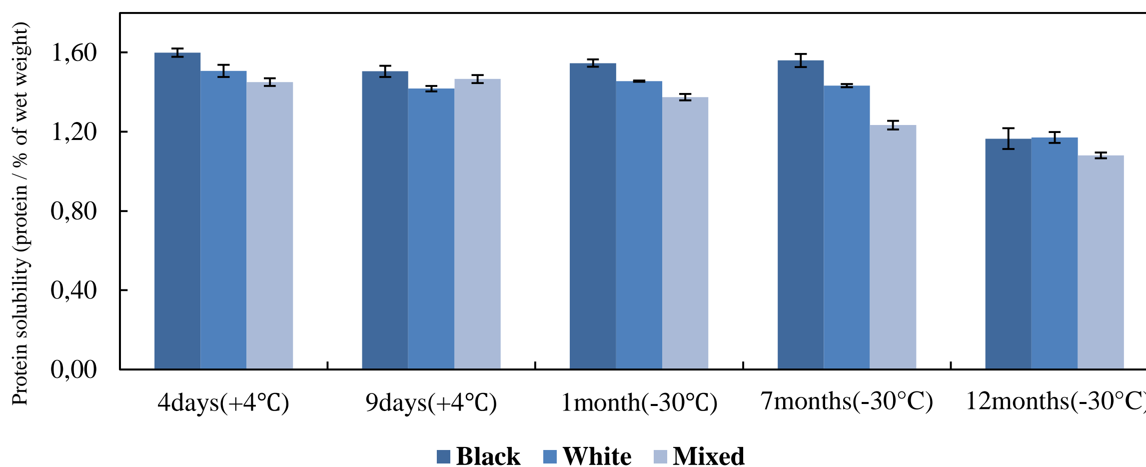


Figure 3-4 Myofibrillar protein solubility(protein in % of wet weight) of black, white and mixed mackerel muscle stored for 4 and 9 days at 4°C and 1, 7 and 12 months at -30°C. Error bars indicate standard deviation (n=3).

Black muscle had a significantly higher myofibrillar protein solubility compared to white and mixed muscle in samples stored at 4°C for 4 and 9 days and samples stored at -30°C for 1 and 7 months ($p < 0.05$).

Myofibrillar protein solubility of black and white muscle decreased significantly during chilled storage of samples ($p < 0.05$).

Myofibrillar protein solubility of all muscle types was significantly lower in frozen samples compared to 4 days chilled samples ($p < 0.05$) except for black and white muscles stored for 1 and 7 months which were approximately the same as for 4 days chilled muscle. Myofibrillar protein solubility significantly decreased during frozen storage except for black and white muscles stored for 7 months at -30°C which was approximately the same as black and white muscle of 1 month frozen samples ($p < 0.05$).

Comparing the results from sarcoplasmic and myofibrillar protein solubility in the present work reveals that sarcoplasmic proteins had a significant higher protein solubility compared to myofibrillar proteins, through the whole storage period. Initially, myofibrillar proteins constitute higher fraction in muscle compared to sarcoplasmic proteins (60-65% vs 20-35% respectively). However, sarcoplasmic proteins have been reported to be more stable than myofibrillar protein during storage of fish [78] [186] [171]. Hence, it seems reasonable to expect higher sarcoplasmic protein solubility than myofibrillar protein solubility during

chilled and frozen storage of mackerel in the present work. However, Hultmann and Rustad found higher protein solubility in myofibrillar proteins compared to the sarcoplasmic proteins in iced stored Atlantic salmon [187]. Chomnawang et al, also reported higher protein solubility in myofibrillar protein compared to sarcoplasmic protein during 15 days storage of hybrid catfish fillets at 4ºC [188].

Significantly higher myofibrillar protein solubility of black muscle compared to white muscle could be due to the higher fat content of black muscle compared to white muscle. Fat could act as a protective layer for proteins to protect them from denaturation especially during frozen storage. However these results are in contrast with the results of Mohan et al. who found higher myofibrillar protein solubility in white muscle compared to black muscle in fresh mackerel [63].

The significantly lower solubility of sarcoplasmic proteins in 7 and 12 months frozen samples and the myofibrillar proteins in some of the frozen samples compared to the chilled samples indicates that freezing reduced the solubility of both myofibrillar and sarcoplasmic proteins. There are many reasons reported for this solubility decrease.

First, freezing may result in denaturation and aggregation of proteins resulting in reduced protein solubility. As water molecules migrate to form ice crystals, the organized H-bonding system that stabilize the protein structure, especially the myofibrillar protein structure, would be disrupted. As a result, the hydrophobic and hydrophilic regions of protein molecules will be exposed to a new environment, which may result in formation of intermolecular cross-linkages within the same protein molecule or disulfide and non-disulfide covalent bonds between two adjacent protein molecules [77] [189] [70]. These secondary interactions between protein molecules will result in formation of insoluble protein-protein aggregates [190] [191] [192]. In addition, increased concentration of dissolved solutes in the unfrozen water phase, will result in changes in ionic strength and possibly pH, leading to denaturation of proteins [68] [193]. Several studies have reported a reduction in protein solubility during frozen storage of fish [194] [195] [196] [197] [198] [199].

Aggregation and denaturation of proteins resulting from interaction between lipid free radicals and proteins is also reported as a cause of decreased protein solubility. In addition, free fatty acids derived from lipid hydrolysis can interact with proteins creating more hydrophobic regions resulting in a decrease in protein solubility [68].

Furthermore, frozen storage has been found to have a significant effect on protein oxidation and, in turn, on functional properties of protein including protein solubility [8] [200] . Protein oxidation could result in the formation of cross-linkages between polypeptides and proteins, which can further lead to protein aggregation which is associated with decreased protein solubility [98] [201]. As can be seen from the protein carbonylation results in section 3.4.1 and protein thiol content in section 3.4.2, carbonyl content of frozen samples increased significantly compared to the chilled samples and the thiol group decreased at the same time. These results show that protein oxidation increased during frozen storage of mackerel. There was a significant negative correlation between myofibrillar protein carbonylation and protein solubility ($r = -0.77$, $p<0.05$) and a significant positive correlation between myofibrillar protein solubility and protein thiol content ($r = 0.94$, $p<0.05$) during frozen storage of mackerel at -30°C. Protein oxidation will result in increased protein carbonyl residues which in turn may result in protein aggregation and decreased protein solubility. At the same time, protein oxidation will decrease the sulfhydryl (–SH) groups contents to form intra- and inter-molecular disulfide bonds which result in decreased protein solubility. Decker et al. reported decreased myofibrillar protein solubility with increasing carbonyl content in a model oxidant system [10]. Rowe et al. found a decrease in sarcoplasmic protein solubility of beef muscle stored for 14 days at 4℃ with increasing carbonyl contents in an oxidation model system [13].

Table 3-1 Protein solubility (mg of protein/ml of extract) of sarcoplasmic and myofibrillar protein of black, white and mixed mackerel muscle stored for 4 and 9 days at 4°C (chilled) and 1,7 and 12 months at -30°C (Frozen).

Muscle type	Storage time				
	Chilled 4 days	Chilled 9days	Frozen 1month	Frozen 7 months	Frozen 12 months
Black(sarcoplasmic protein)	1.49±0.37	1.49±0.033	1.47±0.012	1.14±0.081	1.00±0.044
Black(Myofibrillar protein)	0.65±0.009	0.64±0.012	0.63±0.007	0.63±0.013	0.47±0.021
White(sarcoplasmic protein)	1.55±0.063	1.53±0.081	1.51±0.030	1.35±0.067	1.20±0.024
White(Myofibrillar protein)	0.61±0.013	0.60±0.006	0.60±0.001	0.58±0.003	0.048±0.012
Mixed(sarcoplasmic protein)	1.53±0.006	1.47±0.044	1.50±0.023	1.10±0.076	1.06±0.044
Mixed(Myofibrillar protein)	0.60±0.008	0.60±0.009	0.58±0.007	0.52±0.002	0.47±0.006

Table 3.1 presents the protein solubility given in milligram of sarcoplasmic and myofibrillar protein per milliliter of extract. As expected myofibrillar and sarcoplasmic protein solubility follow the same trend according in percentage of protein solubility in gram wet weight of mackerel and milligram of protein per milliliter of extract.

3.4 Protein oxidation

As already stated, the main aim of the work presented in this master thesis was to investigate the oxidation of myofibrillar and sarcoplasmic proteins during storage of mackerel muscle at +4℃ for 4 and 9 days and at -30℃ for 1, 7 and 12 months. Protein oxidation in muscle foods may cause changes in physiochemical properties of proteins including solubility, water holding capacity, texture- forming ability and susceptibility to proteolysis [27]. Carbonyl and thiol group contents were measured as protein oxidation biomarkers and the results are presented in section 3.4.1 and 3.4.2, respectively.

3.4.1 Carbonyl content

Direct oxidation of susceptible amino acid side chains such as lysine, threonine, arginine and proline, is the main route for protein carbonylation in meat systems [116] [88]. In order to measure protein carbonyls, a classical DNPH spectrophotometric method developed by Levin et al. and Reznick and Packer was first applied [158] [202]. A difficulty was encountered to redissolve the protein pellet precipitated with saturated TCA. This was more problematic in myofibrillar proteins from black muscles. It was assumed that the difficulty in redissolving the protein pellet arose from the high fat content of mackerel and the low incubation temperature of samples with 5 M guanidine hydrochloride. Hence the carbonyl protocol assay was modified by employing three additional steps. The method is briefly explained in chapter 2. The three added steps are as follows:

1- Although lipids were separated from the phase containing proteins, as a pad typically floating on the top layer, the extract may still contain lipids. Hence, 100 µl chloroform/methanol (2:1) was mixed well with homogenate and water soluble fraction after extraction of proteins according to a protocol developed by Wang et al. [160]. The chloroform/methanol mixture could remove the lipid contaminants from

the protein extracts. Resolubilisation of protein extracts containing lipid contaminants is difficult, since protein-lipid complexes are stable due to van der Waals forces between non polar amino acid residues in protein and lipid chains [203].

2- Washing protein pellet with 1 ml Aceton/HCl (3:100, V/V) after the first protein precipitation with saturated TCA, according to a modified carbonyl assay described by Fagan [161]. Aceton/HCl will not only remove chromophores especially from water soluble fraction proteins, but also possibly denature proteins and thus help further protein solubilization with guanidine hydrochloride. Chromophores, such as the heme containing proteins, myoglobin and hemoglobin, present in the water soluble protein fractions were found to strongly absorb at the wavelength used for the quantitation of the carbonyl hydrazine and interfere with the carbonyl assay. Furthermore, acetone will also remove excess salt from protein solutions [204] [205].

3- Heating the undissolved pellet in a 37°C water bath for 30 minutes after incubating with 5 M guanidine hydrochloride and 20mM potassium dihydrogen phosphate (pH 2.3) at 4°C overnight, to speed up the solubilization process.

In spite of these attempts the pellet was not dissolved completely. One explanation could be the formation of covalent bonds such as disulfide bridges resulting in the insolubilization of the pellet in guanidine hydrochloride. Disulfide bonds usually forms by the oxidation of cysteine sulfhydryl groups. Friedli reported that some proteins may aggregate upon denaturation with guanidine hydrochloride. This is probably due to the formation of disulphide bridges between sulphydryl groups made accessible by the unfolding of the polypeptide chains [206]. The author claimed that the addition of iodoacetate may inhibit such reactions.

Incomplete resolubilization of the protein in guanidine have also been reported by other researchers [207] [147] [208] [209]. Resolubilization of TCA precipitated proteins is even more problematic [210] [211] [212].

Finally, an indirect ELISA method was applied to measure carbonyl content as described in chapter 2. Although some difficulties were encountered in starting up the method due to the high level of protein carbonyls in the myofibrillar (salt soluble) proteins, reliable results were finally produced. Analyses were performed in duplicate and the results are expressed as nanomoles carbonyl per milligram of protein. The results are presented in Figure 3.5 and 3.6 for sarcoplasmic (water soluble) and myofibrillar (salt soluble) proteins, respectively.

Carbonyl content was also calculated in µmol of carbonyl per gram wet weight of mackerel and the results are presented in table 3.2 for sarcoplasmic and myofibrillar proteins.

Sarcoplasmic (water soluble) proteins

Figure 3.5 shows the carbonyl content of sarcoplasmic proteins.

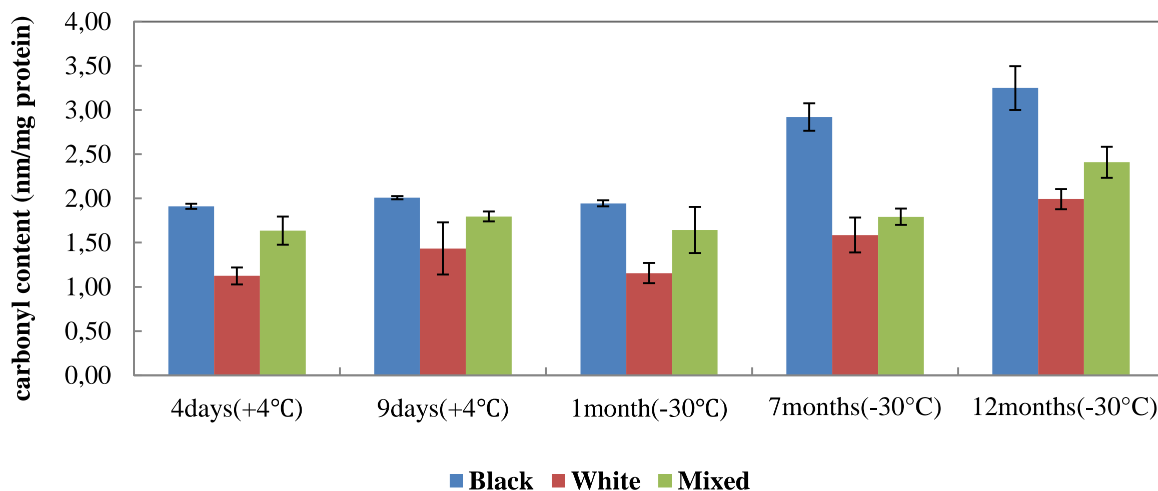


Figure 3-5 Sarcoplasmic protein carbonyl content(nmol carbonyl/mg protein) of black, white and mixed mackerel muscle stored for 4 and 9 days at 4°C and 1, 7 and 12 months at -30°C. Error bars indicate standard deviation (n=2).

Black and mixed muscle had a significantly higher sarcoplasmic protein carbonyl content compared to white muscle at all storage times except for the mixed muscle stored for 7 months at -30°C which was approximately the same as for white muscle ($p<0.05$).
Sarcoplasmic protein carbonyl content was slightly higher in all muscle types stored at 4°C for 9 days compared to the samples stored at -30°C for 1 month but the differences were not significant.
Sarcoplasmic protein carbonyl content was significantly higher in all muscle types stored for 7 and 12 months at -30°C compared to the chilled and 1 month frozen samples.

Myofibrillar (salt soluble) proteins

Figure 3.6 shows the carbonyl content of myofibrillar proteins.

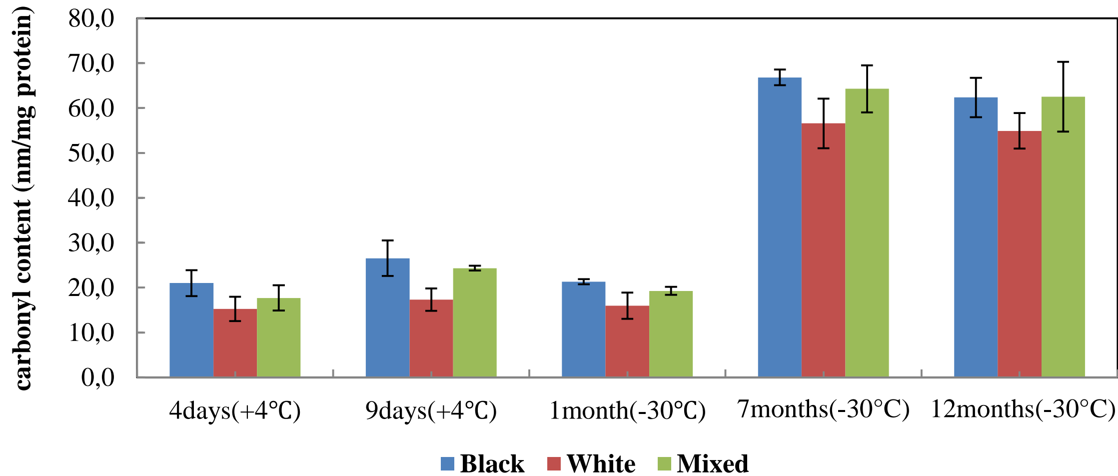


Figure 3-6 Myofibrillar carbonyl content(nmol carbonyl/mg protein) of black, white and mixed mackerel muscle stored for 4 and 9 days at 4°C and 1, 7 and 12 months at -30° C. Error bars indicate standard deviation (n=2).

Black and mixed muscle had a significantly higher myofibrillar protein carbonyl content compared to white muscle at all storage times ($P< 0.05$).

Myofibrillar protein carbonyl content of all muscle types was significantly higher in samples stored for 7 and 12 months at -30° C compared to the samples stored at the same temperature for 1 month and chilled samples ($P< 0.05$).

Myofibrillar protein carbonyl content was slightly lower in frozen samples stored for 12 months compared to frozen samples stored for 7 months but the differences were not significant.

The results presented in Figure 3.5 and 3.6 indicate that myofibrillar proteins had significantly higher carbonyl content compared to sarcoplasmic proteins ($p<0.05$). Carbonyl content was almost tenfold higher in myofibrillar protein compared to sarcoplasmic protein in chill stored samples and 1 month frozen stored samples. Carbonyl content of myofibrillar proteins in samples stored at -30°C for 7 and 12 months were approximately twentyfold higher than for sarcoplasmic proteins. This may reflect a more intensive protein oxidation in

myofibrillar proteins compared to sarcoplasmic proteins. This is in agreement with the results of Tokur and Polat, who reported higher content of protein carbonyls in myofibrillar proteins compared to sarcoplasmic proteins in thin-lipped mullet (*Liza Ramada*) during refrigerated storage at 4℃ for 10 days [213]. Yang et al. found higher carbonyl level in myofibrillar proteins compared to sarcoplasmic proteins in minced Porcine samples stored at 2±1℃ up to 10 days under high oxygen atmosphere packaging [214]. In contrast, Rowe and coworkers reported approximately twofold higher carbonyl content in sarcoplasmic proteins compared to myofibrillar proteins of beef stored for 14 days at 4℃ [13].

Since the composition and properties of sarcoplasmic and myofibrillar proteins are different, one could therefore expect a difference in their susceptibility to protein oxidation. Diversity in amino acid composition of myofibrillar and sarcoplasmic proteins could contribute to the differences in carbonyl content. In fact, all amino acids are not equally susceptible to protein oxidation. Lysine, threonine, arginine and proline are the amino acids which are generally more susceptible to protein carbonylation [215]. This could be because of higher content of amino acid residues with metal binding sites in myofibrillar proteins which are sensitive to metal catalyzed- oxidation by a site specific mechanism.

Myofibrillar proteins are more susceptible to degradation during chilled and frozen storage of fish compared to sarcoplasmic proteins [216] [217] [213] [218]. Denaturation of protein involves disruption of secondary and tertiary structures of protein. Denatured unfolded proteins might be more accessible for ROS attacks.

Protein solubility results in section 3.3 shows lower solubility in myofibrillar proteins compared to sarcoplasmic proteins. This could indicate higher protein denaturation in myofibrillar proteins compared to sarcoplasmic proteins.

Since few studies have been performed on protein oxidation in myofibrillar and sarcoplasmic protein simultaneously in muscle tissues especially in fish, more studies on myofibrillar and sarcoplasmic protein oxidation is advised.

The higher content of carbonyls in the sarcoplasmic and myofibrillar protein in black muscle compared to white muscle could be due to the higher amount of heme proteins, fat and transition metal like iron and copper in dark muscle. Undeland et al. reported eightfold higher content of iron in dark muscle of herring *(Clupea harengus)* compared to white muscle [219]. Hypervalent myoglobin species such as perferrylmyoglobin and ferrylmyoglobin are formed in muscle tissue after death in the presence of H_2O_2 and have been found to be a good initiator of protein oxidation [29]. Studies indicated that dark muscles which are rich in

oxidative fibers are more susceptible to oxidative reactions than white muscles which are rich in glycolytic fibers [220] [221].
The higher sarcoplasmic and myofibrillar protein carbonyl content of mixed muscle compared to white muscle could be due to the presence of skin in the mixed muscle. Skin is rich in transition metals. A 32 times higher content of organic iron in the skin compared to the light muscle in Atlantic mackerel *(Scomber scombrus)* was reported by Ke and Ackman [222]. Undeland and coworkers found almost 2 times higher content of non-hem iron and copper in skin compared to the light muscle in herring (*Clupea harengus*) [219]. Transition metals could promote protein carbonylation through a metal catalyzed oxidation system.
The relatively stable sarcoplasmic and myofibrillar proteins carbonyl content during chilled storage of samples indicates that carbonyl protein oxidation was not developed during storage of mackerel at 4°C. This is in agreement with the results of Tokur and Polat [213] who found that carbonyl contents of proteins did not change during refrigerated storage of Gray Mullet fish fillets at 4°C for 10 days.
Slightly lower sarcoplasmic and myofibrillar proteins carbonyl contents in samples stored at -30° C for 1 month compared to the samples stored at 4°C for 9 days could be due to slowing down the physical and biochemical reactions during storage at low temperatures. Most chemical and physical reactions are slowed, as the temperature decrease [223].

Slightly lower myofibrillar carbonyl content of all muscle types in frozen samples stored for 12 months compared to frozen samples stored for 7 months could probably be due to interaction between protein carbonyls and other cellular constituents such as lipids, carbohydrates and nucleophilic compounds. Feeney and coworkers demonstrated that carbonyls may form Schiff bases with amino group of other proteins leading to reduction in carbonyl concentration [114].

Significantly higher myofibrillar and sarcoplasmic protein carbonyl content in samples stored for 7 and 12 months at -30°C compared to the samples stored at the same temperature for 1 month and chilled samples could be explained by the extensive cell disruption during long frozen storage times. Cell disruption leads to release of pro-oxidants such as H_2O_2, iron, and myoglobin from the cells. Free or hem-bound iron serves as some of the main pro-oxidants in muscle foods. As discussed in chapter 1, in a metal catalyzed oxidation system, transition metals such as Fe^{3+} could produce hydroxyl radicals through Fenton reaction pathway. The hydroxyl radical forms protein carbonyls by oxidizing susceptible amino acid side chains at the metal binding sites or cleavage the protein backbones [224]. Furthermore, exposure of

proteins to highly concentrated pro-oxidant solutes in unfrozen phase, creates a highly oxidative environment during frozen storage [225].

These results are in contrast with the results of Baron and colleagues who found that the carbonyl content of protein homogenate in rainbow trout (*Oncorhynchus mykiss*) was approximately stable during frozen storage at -30℃ up to 13 months [226].

Generally, the carbonyl content of salt soluble (myofibrillar) proteins measured by ELISA in the present work is relatively higher compared to the results reported by other researchers. This could be due to the considerable differences in carbonyl contents in muscle tissue depending on the muscle type, the level of oxidation, the pathway of oxidation and the methods used for detection of protein carbonyl groups[138].

Mackerel is a fatty fish species. In general, fish contains higher level of unsaturated and polyunsaturated fatty acids (PUFA) compared to land animals. Several studies have demonstrated that there is a correlation between lipid oxidation and protein oxidation [176] [226] [23] [227]. Relationship between lipid and protein oxidation will be discussed in section 3.5.

Most studies investigated carbonylation in sarcoplasmic or total homogenate proteins. Moreover, in most studies the conventional spectrophotometric method is used to measure the carbonyl contents in muscle foods. Hence, an accurate comparison of the present results with results obtained in other works is difficult. A few studies have investigated the protein carbonyls in myofibrillar proteins during storage or in model oxidation systems. Reported values for myofibrillar protein carbonyls vary from 1.48 to 27 nmol carbonyl/mg protein [18] [11] [228]. The highest value was reported by Martinaud and colleagues [11] who reported approximately 27 nmol carbonyl/mg protein in myofibrillar proteins of beef in a model oxidation system with ferrous iron and H_2O_2, and the lowest value was reported by Huang et al. [228] who reported 1.48 nmol carbonyl/mg protein in pork dumpling filler myofibrillar protein on day one of frozen storage at -7°C, and almost 5 nmol carbonyl/mg myofibrillar protein on day 180.

It should be emphasized that these studies employed the conventional DNPH spectrophotometric method to measure carbonyl contents. Compared to the conventional method, the ELISA method used in the present work is a more accurate and sensitive assay for measuring protein carbonyls. The higher sensitivity and accuracy of the ELISA method

compared to the conventional method could be due to minimal interferences of free DNPH and other non-protein constituents with the assay [229] . As an excess level of DNPH is used during labeling of the carbonyl adducts in the conventional method, further extensive washing of pellet results in protein losses and underestimation of protein carbonyls. Lyras et al. [230] found variability in the reported level of human brain tissue protein carbonyls measured by the different protocols of conventional carbonyl assay in various studies. Alamdari and coworkers demonstrated that TCA precipitation which used in conventional method, causes 20% lower carbonyl values compared to the analyzing the samples directly and without precipitation with TCA in ELISA method. This could be due to the modification of the adsorption characteristic of the protein by TCA. They found that precipitation with TCA also leads to 10-20 % loss of proteins [144]. Mohanty et al. reported significantly higher levels of carbonyls (~8 fold) measured by Cell Biolabs OxiSelect protein carbonyl ELISA kit (USA) compared to the level detected by the conventional spectrophotometric method in the same human plasma samples [231]. The kit used in their study is the same as the kit used in the present work.

Yan and Forster demonstrated that the ELISA results are sometimes not correlated with the results from conventional method [232]. However, as the conventional method was not working out in our case, we are not able to compare ELISA results with the conventional one in the same samples.

As discussed in chapter 1, while ELISA is a highly sensitive and accurate method, it has the disadvantages of bonding DNA and small molecules to the ELISA wells in addition to the desired proteins [233]. To remove DNA, streptomycin sulfate was added to the protein extracts but no precipitate was observed in the extract. This showed that the extract contains no or very little nucleic acids.

Jongberg et al. reported an unsuccessful attempt to develop an ELISA method for determination of carbonyl content in meat. The aim of their study was to modify the ELISA methods developed by Alamdari et al. They reported that unspecific binding of DNPH to the wells in the ELISA method developed by Alamdari and coworkers, results in an artificially high absorbance in reduced BSA which contains no carbonyl groups and blank samples. To modify the ELISA method, the reduced concentration of DNPH was used and unspecific binding sites were blocked using phosphate buffered saline containing Tween 20 before and after derivatization with DNPH [234]. They demonstrated that the modified ELISA did not

work for meat proteins as the myofibrillar proteins are highly insoluble in water and needs salts to get them in solution (unpublished results). Salts may interfere with the ELISA methods and give some artifact results.

However, in our case, the kit producer claimed that the salts used to extract myofibrillar proteins does not interfere with the carbonyl results. They also claimed that the DNPH concentration used to derivatize the carbonyl groups are low enough and the blocking step is efficient enough to prevent interference of remaining DNPH with the ELISA assay.

In conclusion, further investigation on employing the ELISA carbonyl method in muscle tissues would be of interest.

Table 3-2 Carbonyl content (µmol/gr wet weight) of sarcoplasmic and myofibrillar proteinof black, white and mixed mackerel muscle stored for 4 and 9 days at 4°C (chilled) and 1, 7 and 12 month at -30°C (Frozen).

Muscle type	Storage time				
	Chilled 4 days	Chilled 9days	Frozen 1month	Frozen 7 months	Frozen 12 months
Black(sarcoplasmic protein)	0.07±0.01	0.07±0.01	0.07±0.01	0.08±0.01	0.08±0.01
Black(Myofibrillar protein)	0.34±0.05	0.39±0.06	0.33±0.01	1.12±0.03	0.79±0.06
White(sarcoplasmic protein)	0.04±0.01	0.05±0.01	0.04±0.01	0.05±0.01	0.06±0.01
White(Myofibrillar protein)	0.23±0.04	0.25±0.04	0.23±0.04	0.88±0.09	0.70±0.05
Mixed(sarcoplasmic protein)	0.06±0.01	0.06±0.01	0.06±0.02	0.05±0.01	0.06±0.01
Mixed(Myofibrillar protein)	0.26±0.04	0.35±0.01	0.27±0.01	0.86±0.07	0.73±0.09

Table 3.2 shows concentration of sarcoplasmic and myofibrillar protein carbonyl in µmol carbonyl per gram wet weight of mackerel.

Myofibrillar and sarcoplasmic proteins carbonyl content in wet weight of mackerel was relatively stable during chilled and 1 month frozen storage. The same trend was observed for carbonyl content given in nmol per milligram protein in extract.

Myofibrillar and sarcoplasmic proteins carbonyl content in wet weight of mackerel was significantly higher in frozen samples stored for 7 and 12 months compared to frozen samples stored for 1 month and chilled samples. The trend was the same as for nanomol carbonyl content in milligram protein in extract. As could be seen from protein solubility results in

section 3.3 protein solubility of sarcoplasmic and myofibrillar proteins significantly decreased after 7 and 12 months of frozen storage. The observed decrease in protein solubility could also be due to the formation of carbonyl products leading to the formation of cross-linkages between polypeptides and proteins. Cross linked proteins and polypeptides are responsible for the formation of protein aggregates which is associated with reduction in protein solubility.

3.4.2 Content of total thiol groups

In addition to content of carbonyl groups, the content of total thiol groups was used to determine protein oxidation. The analysis was performed in triplicate and results are presented in Figure 3.7 and 3.8 for sarcoplasmic (water soluble) and myofibrillar (salt soluble) proteins, respectively. The results are expressed as nanomole thiol per milligram protein. Content of thiol groups were also calculated in µmol of thiol per gram wet weight of mackerel and the results are presented in table 3.3 for sarcoplasmic and myofibrillar proteins.

Sarcoplasmic (water soluble) proteins

Figure 3.7 shows the thiol content of sarcoplasmic (water soluble) proteins.

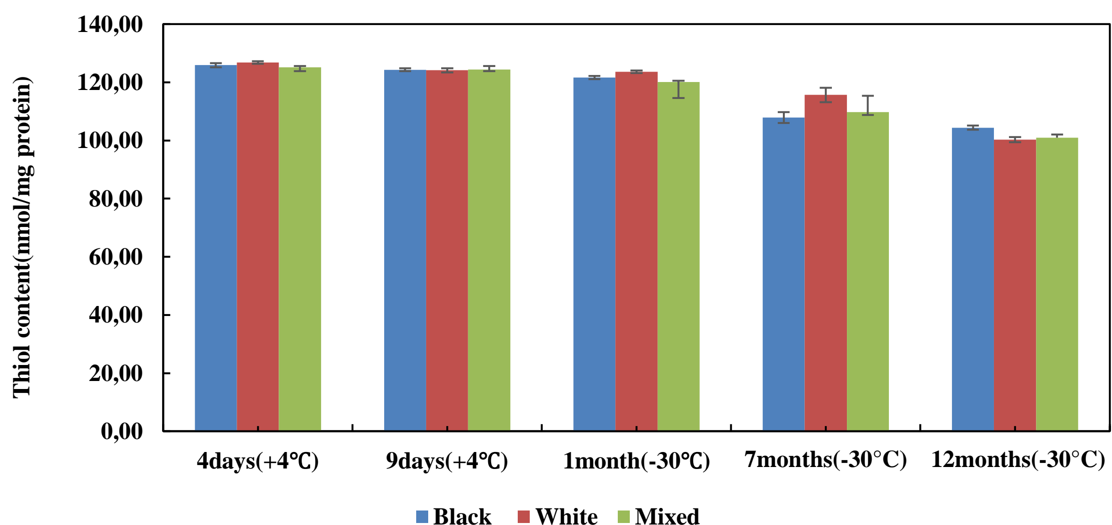


Figure 3-7 Sarcoplasmic protein thiol content (nmol thiol/mg protein)of black, white and mixed mackerel muscle stored for 4 and 9 days at 4°C and 1, 7 and 12 months at -30°C. Error bars indicate standard deviation(n=3).

No significant difference was observed in sarcoplasmic protein thiol content between black, white and mixed muscle in chilled samples. Thiol content of sarcoplasmic white muscle was significantly higher than black and mixed muscle in frozen samples stored for 1 and 7 months ($p < 0.05$). Thiol content of sarcoplasmic black muscle was significantly higher than white and mixed muscle in frozen samples stored for 12 months ($p < 0.05$).

Sarcoplasmic protein thiol content of all muscle types was significantly lower in frozen samples compared to chilled samples except for black and white muscle in 1 month frozen sample which was approximately the same as for chilled muscle. Sarcoplasmic thiol content of all muscle types decreased significantly during frozen storage ($p < 0.05$).

Myofibrillar (Salt soluble) proteins

Figure 3.8 shows the total thiol content of myofibrillar (salt soluble) proteins.

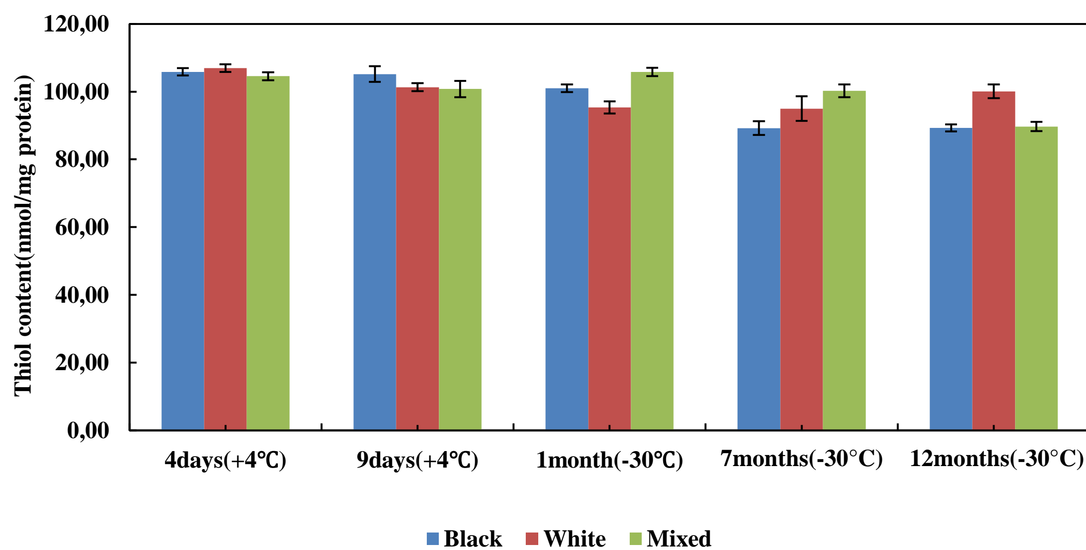


Figure 3-8 Myofibrillar protein thiol content(nmol thiol/mg protein) of black, white and mixed mackerel muscle stored for 4 and 9 days at 4°C and 1, 7 and 12 months at -30°C. Error bars indicate standard deviation(n=3).

No significant difference was observed in myofibrillar protein thiol content between black, white and mixed muscle in chilled samples. Mixed muscle had a significantly higher myofibrillar protein thiol content compared to white and black muscle in frozen samples stored for 1 and 7 months ($p < 0.05$). White muscle had a significantly higher myofibrillar protein thiol content compared to black and mixed in samples stored for 12 months at -30° C ($p < 0.05$).

Myofibrillar protein thiol content of white and mixed muscle decreased significantly during chilled storage of samples ($p < 0.05$).

All muscle types had significantly lower myofibrillar protein thiol content in frozen samples compared to chilled samples with the exception of mixed muscle in 1 month frozen sample which had higher thiol content compared to the same muscle in chilled samples.

Thiol content of black and mixed muscles decreased significantly during frozen storage ($p < 0.05$).

As can be seen from Figure 3.7 and 3.8, myofibrillar proteins had significantly lower thiol content compared to sarcoplasmic proteins. This could implicate that protein oxidation occur faster and more intensively in myofibrillar protein compared to sarcoplasmic protein or that less thiol groups is found in the myofibrillar fraction compared to the sarcoplasmic fraction. Differences in the structure of myofibrillar and sarcoplasmic proteins could be another reason. Similar results have been reported by Grossi et al. who found lower thiol content in myofibrillar compared to sarcoplasmic protein during high pressure treatment of pork [235].

Significantly lower myofibrillar protein thiol content of white and mixed muscle stored for 9 days at 4ºC compared to the same sample types stored for 4 days at the same temperature could indicate that there was thiol oxidation in myofibrillar proteins during chilled storage of samples. The maximum loss of thiol group during chilled storage was 2.1% and 5.3% for white muscle in sarcoplasmic and myofibrillar protein, respectively. Similar results have been reported by Eymard et al. who found a decrease in thiol content of protein homogenate (myofibrillar plus sarcoplasmic proteins) in mackerel mince after 1 day storage at 5 ºC and Nieto et al. who reported a decrease in thiol content of protein homogenate of pork patties during 9 days storage at 4ºC under aerobic conditions [236] [237].

Reduction in thiol groups during storage could be attributed to the susceptibility of myofibrillar proteins to oxidation after death due to depletion of endogenous antioxidants and

production of ROS, non-oxygen free radicals and non-radical species such as H_2O_2 and ROOH [115]. Cysteine residues are highly susceptible to oxidation by H_2O_2 [238]. Oxidation of cysteine sulfhydryl groups will result in the formation of disulfide linkages and cysteine sulfonic acid [9]. Also, a net accumulation of metmyoglobin [MbFe(III)] after death have been reported leading to oxidation of myosin thiols [17] [239]. Martinaud et al. reported 30% and 37% decrease in thiol content of myofibrillar protein in *Longissimus lumborum* and *Diaphragma pedialis* bovine muscles after one hour incubation with Met Mb/H_2O_2 in a model system [240]. Besides, cysteine could undergo oxidative modification in the presence of lipid oxidation products such as lipid hydroperoxides. The oxidation process is much faster in the presence of transition metals such as Fe^{3+} and Cu^{2+} [241]. The relationship between lipid and protein oxidation is discussed in section 3.5.

The significantly lower thiol content of sarcoplasmic and myofibrillar proteins in some of the frozen samples compared to chilled samples could be due to ice crystal formation and growth, leading to mechanical damage to cells that would, in turn, facilitate the exposure of pro-oxidants with proteins. Furthermore, protein denaturation during frozen storage would results in higher exposure of thiol groups towards pro-oxidants [242]. Increasing the concentration of solutes including pro-oxidants around protein molecules in the unfrozen phase could be another reason.

Losses of thiol group generally increased with duration of frozen storage especially in sarcoplasmic proteins. Similar results have been reported by Soyer and others [176] who found increased loss of total thiol group of chicken leg and breast meat with increasing duration of frozen storage at -7°C,-12°Cand -18°C and Benjakul et al. [243] who found a continued decrease in total thiol group in lizard fish, croaker, threadfin bream and bigeye snapper during frozen storage for 6 months at -18°C.

Table 3-3 Total thiol content (µmol/gr wet weight) in sarcoplasmic and myofibrillar protein of black, white and mixed mackerel muscle stored for 4 and 9 days at 4°C (chilled) and 1, 7 and 12 months at -30°C (Frozen).

Muscle type	Storage time				
	Chilled 4 days	Chilled 9days	Frozen 1month	Frozen 7 months	Frozen 12 months
Black(sarcoplasmic protein)	4.59±0.03	4.38±0.02	4.40±0.02	3.01±0.05	2.53±0.02
Black(Myofibrillar protein)	1.69±0.02	1.56±0.03	1.57±0.02	1.50±0.03	1.13±0.01
White(sarcoplasmic protein)	4.80±0.02	4.48±0.03	4.54±0.02	3.74±0.08	2.97±0.03
White(Myofibrillar protein)	1.61±0.02	1.44±0.02	1.39±0.03	1.47±0.06	1.27±0.03
Mixed(sarcoplasmic protein)	4.62±0.02	4.43±0.04	4.25±0.02	2.92±0.15	2.66±0.03
Mixed(Myofibrillar protein)	1.52±0.02	1.47±0.04	1.46±0.02	1.34±0.03	1.05±0.02

Table 3.3 shows concentration of sarcoplasmic and myofibrillar protein thiols in µmol thiol per gram wet weight of mackerel. White muscle had a significantly higher sarcoplasmic protein thiols in gram wet weight of mackerel compared to black and mixed muscle at all storage times. Black muscle had a significantly higher myofibrillar protein thiols in gram wet weight of mackerel compared to white and mixed muscle at all storage times except for samples stored for 12 months at -30̊ C.

Frozen samples had significantly lower sarcoplasmic and myofibrillar protein thiol content compared to 4 days chilled samples. This was also the case for nmol/mg protein thiol in extract with the exception of 1 month frozen samples which was approximately the same as for chilled samples. At the same time sarcoplasmic protein solubility decreased significantly after 7 months of frozen storage. Myofibrillar protein solubility of all muscle types also decreased after 12 months of frozen storage. The decreased solubility could be due to the formation of disulfide bridges resulting from oxidation of thiol groups.

Sarcoplasmic protein thiol content in gram wet weight of mackerel significantly decreased during frozen storage, the same as for nmol thiol content per mg protein in extract.

Myofibrillar protein thiols per gram wet weight of mackerel decreased significantly during frozen storage except for white muscle of 7 months frozen sample which had higher thiol content compared to the white muscle of 1 month frozen samples. As can be seen from Figure 3.8 the trend was the same for thiol content in nmol/mg protein in extract except for

white muscle which had an increase in myofibrillar thiol content during frozen storage. Myofibrillar proteins had significantly lower thiol content per gram wet weight of mackerel compared to sarcoplasmic proteins. The same trend was observed for sarcoplasmic and myofibrillar protein thiol content in nmol/mg protein in extract (see Figure 3.7 and 3.8). In addition to the factors contributing to these results discussed above, higher protein solubility of sarcoplasmic protein compared to myofibrillar protein per gram wet weight fish (see section 3.3) could also be a reason for obtained results.

3.5 Relationship between lipid and protein oxidation

In the storage trial on mackerel both lipid and protein oxidation was measured on the same samples. In this chapter, the results from the lipid and protein oxidation will be compared and discussed. Lipid oxidation was investigated by measuring primary (conjugated dienes) and secondary (thiobarbituric acid reactive substances) lipid oxidation products during storage of mackerel in a master project done in cooperation with SINTEF by Iversen [244].

Lipid oxidation may impact protein functionality because of its effects on protein structure [245]. Primary and secondary lipid oxidation products may react with proteins and peptides, resulting in the loss of solubility and reduced water holding capacity. The decreased protein solubility of myofibrillar proteins (see section 3.3) and decreased water holding capacity (see section 3.2) of mackerel samples during frozen storage could also be due to the interaction between proteins and amino acids with lipid oxidation products, resulting in cross-linking and aggregation of proteins.

The primary lipid oxidation products reached a peak on the 9th day of chilled storage, but it was relatively stable during frozen storage of mackerel. Relatively stable conjugated dienes values during frozen storage could indicate that the rate of formation of primary lipid oxidation products is equal to the rate of degradation. Primary lipid oxidation products could contribute to the formation of secondary lipid oxidation products [246] or/and protein radicals [185]. Protein thiol content results in section 3.4.2 of the present study showed a gradual decrease in thiol content of myofibrillar proteins during frozen storage of mackerel. The factors contributing to these results have been discussed in that section. In addition to the factors discussed in section 3.4.2, the decrease found in thiol content of proteins during frozen storage could also be the result of the reaction between primary lipid oxidation

products and sulfhydryl containing proteins like cysteine. Primary lipid oxidation products (lipid peroxides) could abstract hydrogen from sulfhydryl groups in proteins, resulting in the oxidation of protein thiols [247] [248].

Carbonyl content of proteins especially myofibrillar proteins also increased during frozen storage of mackerel (see section 3.4.1). Peroxyl radicals formed during the initial phase of the propagation step of lipid peroxidation could attack side chain or backbone of proteins, resulting in structural modification of side chains and protein carbonylation [249] .

TBARS values were relatively stable during chilled and 1 month frozen storage, after which they decreased dramatically after 7 and 12 months frozen storage of mackerel samples. Interestingly, at the same time, the carbonyl content of myofibrillar proteins increased sharply (see Figure 3.6). The fact that the reduction in TBARS values occurred at the same time as the increase in carbonyl content in myofibrillar proteins may show that TBARS contributed to the formation of protein oxidation products. Proteins containing reactive carbonyl groups can be generated by Michael-addition reactions of histidine, lysine or cysteine residues with α, β aldehydes formed during the peroxidation of poly-unsaturated fatty acids [250] [251] [252] [123]. α, β aldehydes are among several homologous families of aldehydes which is formed on decomposition of lipid peroxides during autoxidation of polyunsaturated lipids [253]. Secondary lipid oxidation products such as malondialdehyde could also react with sulfhydryl group of cysteine, resulting in the formation of disulfide bonds and loss of sulfhydryl groups [130]. The decreased thiol content during frozen storage of mackerel in sarcoplasmic and myofibrillar proteins (see section 3.4.2) could confirm this hypothesis.

3.6 Suggestions for future work

During the work on this master thesis, some difficulties observed in measuring protein carbonyls using the conventional DNPH-based method. So, using alternative methods for measuring protein carbonyls in fatty fish is advised.

The total carbonyl group measurement methods such as ELISA, which is used in this thesis, are simple and useful methods to measure total carbonyl derivatives formed by various unspecific pathway, but they just give the quantities of total carbonyl which could be present in muscle resulting from different pathways. Therefore, using more specific methods for the determination of particular carbonyl derivatives is advised in future studies in order to

understand the chemical nature and oxidation pathway. An alternative could be the use of α-aminoadipic and γ-glutamic semialdehydes (AAS and GGS, respectively) as oxidation markers of specific amino acids. They have been detected in food proteins and highlighted as oxidation markers in food system. They are not affected by the composition or structure of the food and are a very specific measurement that provides precise information about the fate of certain amino acids during processing and storage of muscle food products.

4 Conclusions

The main aim of this work has been to study the oxidation of sarcoplasmic and myofibrillar proteins of white, black and whole mackerel muscle during chilled (+4°C) and frozen storage (-30°C). The samples were either chilled stored for 4 and 9 days or frozen stored for 1, 7 and 12 months. The contents of protein carbonyl and total thiol were used to measure the extent of protein oxidation during fish storage. Protein carbonyl was measured by the use of a conventional DNPH-based and an ELISA method. The second aim was to evaluate the effects of protein oxidation on water holding capacity and protein solubility. The lipid oxidation during chilled and frozen storage of the same samples as used in the present work, have been evaluated in a master project and the results was used to consider the relationship between lipid and protein oxidation.

The conventional DNPH-based method was found not to be a useful method for measuring the contents of protein carbonyls in fatty fish like mackerel due to the incomplete resolubilization of the protein pellet in guanidine hydrochloride.

Carbonyl contents of sarcoplasmic and myofibrillar proteins of all muscle types were relatively stable during chilled and 1 month frozen storage of samples. This could implicate that protein carbonylation was not developed in samples during refrigerated storage. However, the total thiol group generally decreased in myofibrillar proteins during chilled and 1 month frozen storage, which could be an implication of protein thiol group oxidation in those samples. WHC was relatively stable during chilled and 1 month frozen storage. Sarcoplasmic protein solubility was also stable during this period of storage while the myofibrillar protein solubility generally decreased.

Frozen storage for 7 and 12 months significantly influenced the oxidative stability of sarcoplasmic and myofibrillar proteins of all mackerel muscle types. Sarcoplasmic and myofibrillar protein carbonyl contents increased significantly during frozen storage at -30°C for 7 and 12 months compared to the chilled and 1month frozen storage. At the same time, the total thiol group content decreased significantly. These results show that duration of frozen storage has a strong impact on protein oxidation in mackerel.

Increased protein carbonyl contents and decreased thiol group contents in 7 and 12 months frozen samples occurred concomitantly with a loss of WHC and protein solubility. It could be concluded that protein oxidation had a significant effects on the loss of WHC and protein solubility after 7 months frozen storage of mackerel.

Myofibrillar proteins had significantly higher carbonyl content, lower total thiol group content and lower protein solubility compared to the sarcoplasmic proteins in both chilled and frozen stored samples. This may implicate the higher stability of sarcoplasmic proteins with regard to protein oxidation and freeze denaturation compared to myofibrillar proteins.

While the black and mixed muscle had a significantly higher sarcoplasmic and myofibrillar carbonyl contents compared to the white muscle, no clear relationship between muscle types and loss of thiol groups, WHC and protein solubility was observed.

The results from the lipid oxidation study on the same mackerel samples show decreased level of TBARs after 7 months frozen storage. The increased carbonyl contents and decreased thiol group contents at the same time may indicate a correlation between protein and lipid oxidation progress in mackerel.

Appendix

Raw data and calculations used to obtain the results showed in this thesis can be found as several Microsoft excel worksheets on the attached CD. The files are named after their content and divided by type of experiment.

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