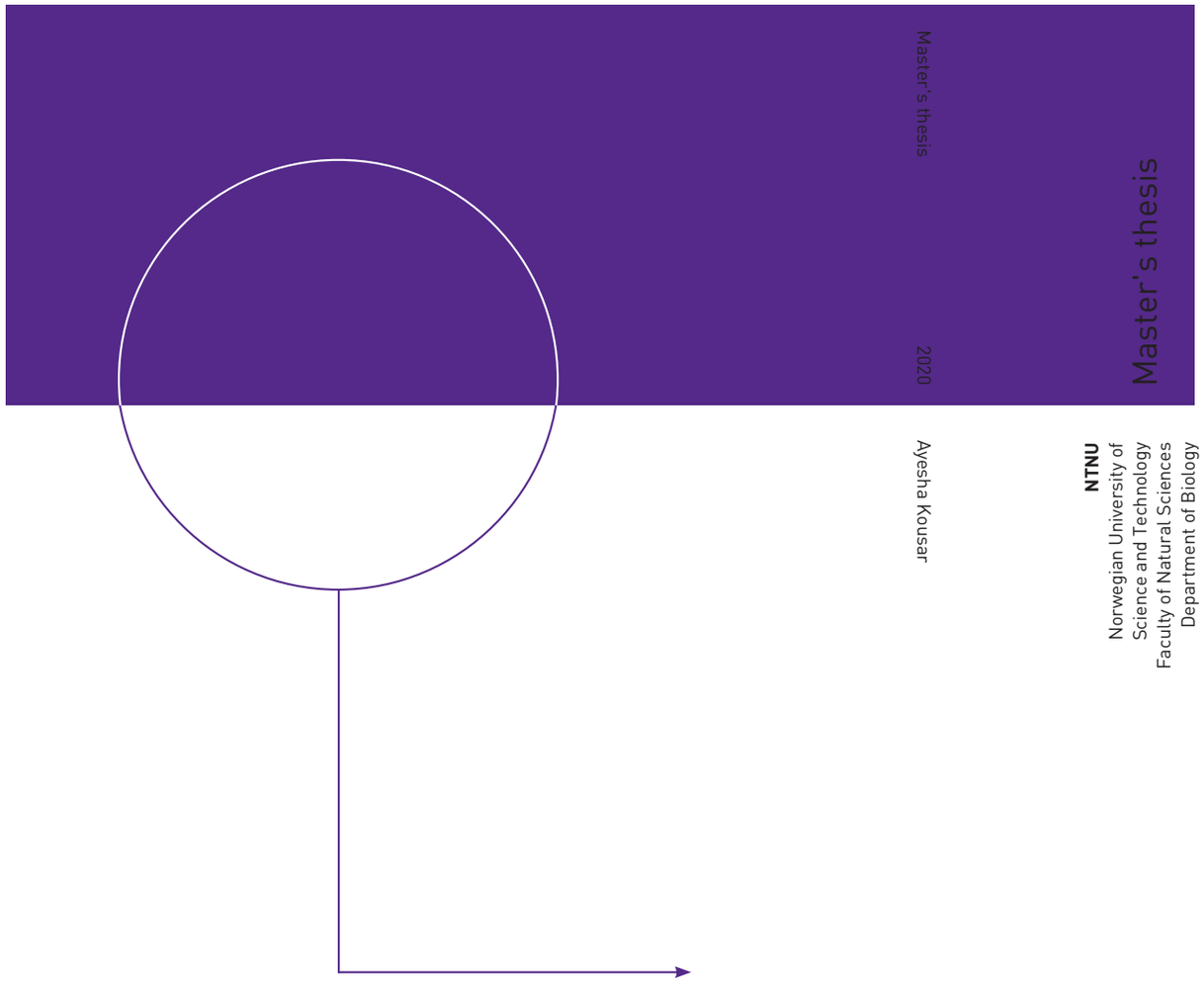

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Quality Characteristics of Sous-vide Cooked Atlantic Herring - Effect of Natural Plant Antioxidants and Frozen Storage

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Molecular Cell Biology
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Abstract

Atlantic herring has a high nutritional value and is a good source of lipids with a high content of PUFAs (polyunsaturated fatty acids) in addition to protein, minerals (calcium, phosphorus, magnesium) and vitamins (A, D). Herring has a high content of EPA (eicosapentaenoic acid) DHA (docosahexaenoic acid) as well as some monounsaturated fatty acids. Numerous health benefits are related to the high lipid content and nutritional profile of herring.

Partially cooked ready to eat, finished products of fish are preferred more over fresh fish due to their palatability and convenience. Unfortunately, auto-oxidation is a huge problem in finished and frozen fish products particularly in fatty fish such as herring. Lipid oxidation causes organoleptic changes and results in loss of nutritional quality of fish products. Therefore, it is of utmost importance to explore ways to increase stability during prolonged storage of the finished products.

The main aim of this study was to evaluate the effects of different natural antioxidants (oregano, nettle, dill, olive leaf extract) on preserving the quality of sous vide cooked herring fillets during frozen storage (at -20°C). Peroxide value (PV), conjugated dienes (CDs), conjugated tetraenes (CTe) and TBARS were performed to determine the status of primary and secondary oxidation in frozen herring samples. The reduced peroxide content, TBARS values and CDs compared to the Control indicated the effect of the antioxidants used, particularly nettle and oregano that more efficiently reduced the values.

The same fillets were further frozen stored for 5 months and analyzed for the sarcoplasmic protein solubility and thiol content to study the effects on solubility and oxidation of proteins. The increased solubility and decreased thiol content indicated that structural changes in proteins had taken place during the frozen storage.

Additionally the two antioxidant extracts (oregano, dill) were investigated for the antioxidant potential using ABTS, DPPH, Folin Ciocalteau assays. Oregano was found to be a more potent antioxidant than dill and its PG equivalence was considerably higher than dill in all three assays. Oregano and nettle can be effective in increasing the oxidative stability in partial cooked ready to eat fish products.

Preface

This study is done in fulfillment of the masters degree requirement in 2 years international master program in Biology at the Norwegian University of Science and Technology, NTNU. The research was performed at the department of Biotechnology & Food Sciences at NTNU. The research work was performed during September-1st 2019 to 15 August-2020 and counts for 60 credit points.

Also, I'd like to thank number of people for their continuous support who made this experience of work and life fascinating. I am highly obliged to my supervisor Professor Turid Rustad (Department of Biotechnology & Food Science, NTNU, Trondheim) for the knowledge, continuous guidance, support, encouragement and availability which made it possible for me to complete my work within due time. I am cordially thankful to her for the precious time she gave, for discussions, for all the corrections and comments, for her cooperation and understanding and especially her encouraging attitude which made me keep working in all circumstances.

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Ayesha Kousar
Trondheim, August 15, 2020

List of Acronyms

ABTS	2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CDs	Conjugated dienes
CTe	Conjugated tetraenes
DHA	Docosahexaenoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5, dithiobis (2 nitrobenzoate)
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
FCA	Folin Ciocalteu Assay
FCR	Folin Ciocalteu reagant
FRS	Free radical scavengers
GSE	Grape seed extract
GTE	Green tea extract
HAT	Hydrogen atom transfer
KI	potassium iodide
MDA	Malondialdehyde
OE	Oregano extract
OLE	Olive leaf extract
PG	Propyl gallate
PUFA	Polyunsaturated fatty acids
PV	Peroxide Value
ROS	reactive oxygen species
SET	Single electron transfer
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid

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Chapter 1

Introduction

1.1 Background

Fisheries and aquaculture have a great influence on the economy of many countries and besides providing essential food nutrients, fisheries and aquaculture provide employments to millions of people living in coastal regions. According to FAO Report 2018 [1], in the year of 2016, approximately 59.6 million people worldwide were employed in the primary capture sector. Fish and its products are prepared and exported on an international level. About 60 million tons of fish and its products were traded in the year 2016. In the year 2017, the export was increased by 7% and reached a peak value of USD 152 Billion. China is at the top in world fish trade, producing and exporting seafood at the highest rate annually. Other main producers are Norway, Vietnam and Thailand [1]. Norway generates a large portion of its export revenue from fisheries and seafood products. It became the worlds second biggest exporter of fish products in 2017 and produced 3.5 million tons of fish which are approximately half of the total generated by EU, which was 6.7 million tons. In the same year Norway generated the worlds' highest revenue of 94.5 billion NOK which is 3% increase in value as compared to 2016. Europe and Asia are the big markets for Norwegian fish and finished products [2]. EU is a major seafood market and the fifth big exporter of fish, it generated 3% of global fish, both from fisheries (80%) and aquaculture (20%), in year 2017. Its annual apparent consumption in the same year, was 12.45 million tonnes which made about 24 kg per capita. The species most commonly consumed were tuna, cod, herring, shrimps, mussel and Alaska pollock. Herring (100% wild) was the seventh most consumed specie (1,23 kg per capita of the total 24,33 kg per capita apparent consumption of the fisheries and aquaculture products) in the year 2016. In the year 2016, herring was recorded as one of the main commercial species, 258.544 tonnes (live equivalent weight) herring was imported which was 72% more than, that was imported in 2015. This import of herring significantly contributed to the decline in self sufficiency of the EU in fisheries and aquaculture products [3].

Besides direct human consumption, seafood sources are prepared for the in-

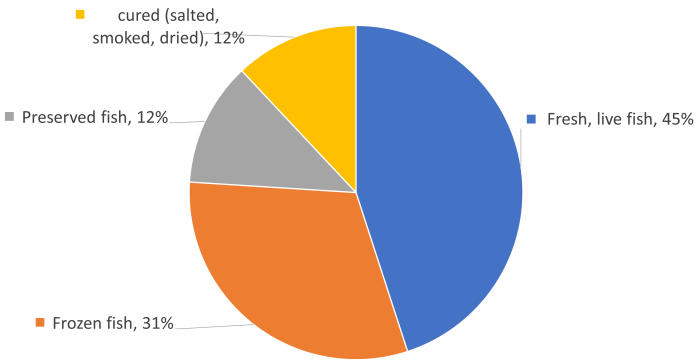

Figure 1.1: Global fish utilization in year 2016 [1].

direct consumption as well. Total fish production in 2016 was 171 million tonnes, of which 151 million tons was utilized for direct human use and 20 million tonnes is processed to be utilized for non-food purposes. By-products and remaining parts of fish are treated as rest raw materials and are processed to make supplementary products such as fish silage, fish meal and fish oil, thus minimizing seafood loss [1].

Fish is preferably consumed in fresh form, however, a big percentage is also preserved by frozen storage for commercial usage. A fresh fish is preferred over chilled and frozen fish in Asia and Africa, while preserved or frozen fish is consumed in Europe and US. People in developing countries have higher liking for the fresh fish while consumers in developed countries utilize frozen, preserved or cured fish as shown in Figure. 1.1.

With increasing awareness about the nutritional importance of fish components, people throughout the world are preferring to add fish and related products to their diet. Additionally, advanced processing and improved refrigeration technologies have facilitated transportation and distribution of fish and variety of its products in the past few decades. In developed countries, fish is mostly sold in frozen, processed or preserved form, the proportion of frozen fish for direct human consumption increased from 27% in 1960s to 58% in 2016. The trend of using frozen fish products is also changing in the developing countries, it was 3% in 1980s and increased to 26% in 2016.

1.1.1 Health benefits and Nutritional significance of Marine Fish

Over the last few decades, there has been a conscious global rise in the consumption of seafood sources. A large number of fish species have been studied and their potential health benefits have been widely explored. Various studies on the com-

positional significance of marine fish species have affirmed the idea that it is much more than a food source and contain essential omega 3 fatty acids, fat soluble vitamins (vitamins D and E) and digestible peptides [4–8]. Intake of about 150g of fish can fulfill 50-60% of our daily protein requirement [1]. Fish proteins are easily digestible, have good and well-balanced amino acid composition. It contains the essential amino acids such as lysine, leucine as well as some non-essentials as glutamic acid and taurine in considerable amount [9]. In addition to being a good source of amino acids, fish derived protein hydrolysates have also been shown to give biologically active peptides. Bioactive peptides have 3-20 amino acids and bioactivity depends on the composition and sequence of amino acids. Bioactive peptides obtained from fish are involved in metabolic functioning like reducing hypertension [10], improving blood glucose metabolism and altering intestinal microbiota, prevent the obesity linked insulin resistance [11].

The marine fish are enriched with the long chains of polyunsaturated fatty acids (PUFAs). The unsaturated fatty acids contain one or more double bond. The double bonds facilitates the removal of H+ atom, thus making these unsaturated and even more vulnerable to rancidity. The PUFAs have preliminary role in strengthening immune system and the intake is prerequisite to prevent body from certain serious disorders [8, 12]. The PUFA content of marine fish is usually higher than in freshwater fish [13]. This is because of the food they consume. The fish feed on zooplanktons which usually eat autotrophic green sea algae or phytoplankton. Phytoplankton have short chains fatty acids which are elongated inside body of fish, to form (20-22 C) long chain PUFAs [14, 15].In pelagic fish such as herring the lipids are mainly stored in muscles, viscera, under skin and around the liver of pelagic fish [16].

The two essential unsaturated fatty acids, omega 6 and omega 3 are found in fatty fish. Both are essentially required in diet because these cannot be synthesized by animal body. Both of these are involved in the formation of eicosanoids but different types with different effects on inflammation and blood pressure. For example, the linoleic acid is a shortest chain omega-6 fatty acid and it is a precursor of arachidonic acid. The arachidonic acid is an integral part of the cell membranes. It is important for the biosynthesis of eicosanoids such as leukotrienes, prostaglandins and thromboxanes. The eicosanoids are involved in cell to cell signaling and in the promotion of inflammatory immune response [17–19]. Alpha linolenic acid is omega-3 fatty acid from which other omega-3 fatty acids like eicosapentaenoic acid (EPA/C20-5) and docosahexaenoic acid (DHA/C22-6) are derived. The importance of omega 3 fatty acids for cardiovascular diseases is well established [20]. EPA and DHA bind to cell membranes, regulate inflammatory responses and ensure healthy fetal development, and prevent heart diseases. DHA is mainly found in nerve cells and in retina so, it is important for eyesight and brain functioning [12]. Seafood and fatty fish are rich in DHA, mammalian blubber and animals brain, have it in high amount. Feeding mothers from coastal

areas have DHA in higher percentage in their milk. Its deficiency in infants or during pregnancy causes vision and brain problems, 15-22mg/ week in last 3 months of pregnancy [21].

1.1.2 Fish as a raw material

Fish is highly perishable and fragile, a large portion (35%) of the total global catch is usually lost during different stages from harvest to consumption, of which 9-15% is lost at the sea [22]. Fish is easily degraded by the metabolic activity of microorganisms. Microbial spoilage depends upon the storage conditions and the type of the metabolites produced leading to to the organoleptic changes in the fish. Lipid oxidation is a serious problem for storage products in seafood industry, as it causes deterioration and reduced acceptability of the products Pelagic fatty fish have higher concentration of unsaturated fatty acids compared to lean fish, so they are more exposed to lipid oxidation [13]. Lipid oxidation results in loss of the sensory quality, flavor, color, taste and smell of fish making it unacceptable as food and causes the huge economic loss for the industry. Hence, it is very important that these products should not get oxidized or degraded during storage [1]. Researchers have been working on the development of advanced preservation techniques that will best preserve the nutrients and nutritional quality. Various studies have shown that the use of antioxidants, chilling, MAP (Modified Atmosphere Packaging), vacuum packaging and use of some preservatives are beneficial for controlling autooxidation in fish [23, 24]. Sous-vide cooking is important when it comes to making convenient food products-ready to eat/ ready to heat. It enhances stability and increases shelf life of the products thereby causing minimal changes [23]. Sous-vide cooking is discussed further in section. 1.6.

1.1.3 Fatty Fish Muscle

General Composition

Composition of fish muscle is influenced by its age, size, sexual maturity, feed, seasonal changes and its origin [25]. Fish muscle has highly digestible amino acids because it has less connective tissue. Fresh lean meat in general has 70% water, 18-23% proteins, vitamins (A, D) and ashes (minerals; selenium, phosphorus, magnesium, calcium)are 1-1.2% [26]. Water in fish is usually 70-84%. The lipids and water together make 80% of fish but the ratio between lipids and water may differ depending upon fat content of fish for example the fatty fish usually has more lipids and less water while lean fish has more water and less lipids. Water in fish is bound to the proteins but if fish is stored for longer periods, bonding between water and amino acids is weakened and water along with the dissolved salts is released as drip [27]. Lean fish such as cod has protein as the main energy reserve, ranging from 17-20% [28]. All fish have less than 100mg/100g cholesterol and sodium levels, but in some shellfish and processed products sodium levels may be increased. Other minerals are present in small or moderate amounts

in fish and seafood [29]. Fish muscle is divided into dark muscle and white muscle. Dark muscle has high lipid content, glycogen, vitamin B, nucleic acids myoglobin, mitochondria and oxidative enzyme systems [30, 31]. White muscle has low fat content and has glycolytic enzymes. Fatty fish (herring, mackerel) have more dark muscle as compared to lazy bottom dwellers (cod fish).

Fish Lipids

On the basis of fat content, fish can be divided into lean fish (low fat content below 2 g/ 100g), low fat fish (2-5g), medium fatty fish (5-10g) and higher fatty fish (over 10g). Lean fish such as cod, tuna, pollock and shrimps are lighter in color as compared to fatty fish. Higher fatty fish like salmon, herring and mackerel have 15% fats [29] however, some variation occurs within the individuals of same specie due to different diet, fishing ground and season of capture [32]. In fatty fish fat deposits are found under the skin, in viscera and in or around the muscle tissues while in lean fish, fats are deposited in liver [21]. Lipids in viscera may vary depending upon the feeding state and the last food eaten. Lipids in lean fish are mostly phospholipids (65%), and neutral lipids (35%) whereas the fatty fish have triacylglycerols as main lipid [33, 34]. Studies have shown that water content of fish is inversely related to the lipid content. For instance, fatty fish has less water and more fat while lean fish has more water and less lipids [25, 26].

Fish Muscle proteins

Muscle Proteins can be divided int to three groups. Myofibrillar proteins, sarcoplasmic proteins and connective tissue proteins. Myofibrillar proteins comprise 65-70% of total muscle proteins, are salt soluble, unstable and are easily denatured during frozen storage. Connective tissue proteins are called insoluble proteins comprising 3% of total proteins in meat. Sarcoplasmic proteins are called water soluble proteins because these are easily dissolved in water. These make around 20% of total muscle protein. Water soluble protein mostly include enzymes and also the chromoproteins like myoglobin. The concentration and state of myoglobin effects the color of meat. Usually actively swimming fish species such as sardines and mackerel possess a high content of myoglobin as compared to stationary fish. The sarcoplasmic proteins are stable and frozen storage have little effects on the properties of these proteins. Pelagic fish has comparatively higher content of water-soluble proteins than demersal fish [35].

1.1.4 Aims and Objectives Of the Study

The main aim was to study the effects of natural antioxidants obtained from Mediterranean plants on lipid and protein oxidation of sous-vide cooked Atlantic herring during frozen storage. The fish was sous vide cooked, treated with antioxidants and frozen stored at -20°C for about two months. Total lipid content was determined, effect of sous vide and antioxidants was evaluated by analyzing primary

and secondary oxidation products. The antioxidant potential of the two antioxidants, dill and oregano extracts was determined. Protein solubility was measured. To study the effect of frozen storage on proteins from antioxidant treated, sous-vide cooked herring, protein solubility and quantification of thiols was performed. The main objectives of our research work were:

1. Determination of total fat content
2. Determination of primary and secondary lipid oxidation products to study the oxidation status of sous-vide cooked herring
3. Comparing the effectivity of natural antioxidants including oregano and dill extract using standard assays., DPPH, FCA and ABTS
4. Determination of changes in protein solubility and protein oxidation of sous-vide treated frozen herring fillets

1.2 Atlantic Herring

Figure 1.2: Clupea harengus [36]

1.2.1 Habitat, distribution and life cycle

The biological name for Atlantic herring is *Clupea harengus*, it belongs to the family *Clupeidae*. Atlantic herring eat small fish, it also feeds on the tiny aquatic animals, called zooplanktons which include some crustacean species like krill and copepods. Herring itself is eaten by whales, cod and seals, so because of its availability and abundance this make it an influential organism in the food web. Herring exhibits a wide geographic distribution and can migrate to distant areas, so it is found in pelagic waters as well as in the coastal areas. It lives in depths of 2-400 meters, under the sea surface. Sometimes, these migrations are attributed to their sensitivity to sunlight and temperature changes, but they also migrate in search of food. It is caught in large numbers because they form bigger schools so thousands can be captured at a time [37].

Herring species are inhabiting both sides of North Atlantic Ocean. It is found in waters of north-east Atlantic expanding from Norwegian water to the Bay of Biscay in south and in north-west Atlantic, it can be located from Greenland to South Carolina in USA [3]. They are the most abundantly found fish species as they reproduce at a fast rate and produce eggs in a large number. Reproduction is external and first female lays eggs in ocean and then these are covered by milt

by the male partner. Thousands of eggs are laid and are deposited in the bottom of ocean in such a way that they form few centimeters thick layer on the seabed. Usually it spawns in summer, eggs hatch out in 7 to 10 days. Larvae then grow into juveniles. These juveniles then group together to form schools and swim farther towards coastal zones. Unfortunately, most of these juveniles are eaten up by the other marine fishes and sea birds, those which escape, survive and grow to become adult. Generally, a 4-year herring, is considered as an adult and is able to reproduce [37].

1.2.2 Significance and Consumption

The fish has a long body with silver shiny belly and the upper part is of deep blue or dark green colour. The head region is black colored, and mouth has weak tooth. The skin is covered with loosely attached scales. Its average length is 30 cm and can reach up to the maximum length of 39 cm. Generally, its average weight is around 0.68 kg and has an age limit of 15-18 years at maximum [38].

Atlantic herring is called silver of the sea because of its colour and also it is an important fish which drives the economy of many coastal countries. Atlantic herring constitutes 2% of the total global catch [1]. It is the seventh most common utilized specie. According to Norwegian fisheries report total catch (herring and other pelagic fatty fish) in the year 2019 was 130,2284 tonnes, out of which Norwegian spring spawning herring made 430,506 tonnes and Atlantic herring was 130793 tonnes. Value of total catch was 21166 million NOK, of which pelagic fish was 6712 million NOK and Atlantic herring gave 690 million NOK in the year 2019. The main exporters of herring in Europe are Denmark and Netherlands while main consumers in E.U are Germany, Netherlands and Sweden [3]. People like to eat herring due to its taste and high nutritional values. The importance of marine lipids for improving human health status has become more evident due to more work on nutritional status of pelagic fishes. Likewise, herring is enriched with essential fatty acids, such as omega-3 PUFAs in particular EPA (20:5n-3) and DHA (22:6n-3) [8].

1.2.3 Potential Health benefits

Nutritional health benefits have been widely studied [39, 40]. It has minerals as calcium, phosphorus and magnesium. It has low level of saturated fats therefore it can reduces the chances of heart diseases. Fatty fish such as herring and mackerel are a rich source of EPA and DHA so increasing its intake in diet can provide with the related health benefits for example EPA and DHA can help relaxing the joints to cure rheumatoid arthritis and can also reduce the autistic spectrum disorder and can reduces the risks of heart linked diseases [41]. Vitamin A and D, and omega 3 are vital for vision and health, effective against depression, dementia, ulcerative colitis and has healthier effect on lungs [39]. n-3 PUFAs not only have effects on the immunosuppressive and anti-inflammatory responses but also have the influence on body glucose and lipid metabolism [40].

Table 1.1: Nutritional values of Raw Atlantic herring/100g

Nutritional value	Raw
Kcal	158
KJ	661
Water (g)	72
Protein (g)	17.96
Carbohydrates (g)	0.00
Lipids (g)	9.04
Minerals (mg)	Moderate
Vitamin B12 (ţg)	13.67
Vitamin D (IU)	167
Sodium (mg)	90

Herring has soft tissues and fatty muscles. Its lipids are highly vulnerable to oxidation therefore careful handling and chilling procedures are needed to be followed for safe storage and transportation. It is sold in raw form, but a bigger quantity is also processed to make finished products, so it is smoked, canned, salted, pickled and marinated. Therefore, it is of utmost importance to protect it from rancidity and oxidation. Fat and protein content of the herring also varies with maturity and water content. Most of the herring is composed of fatty muscles and adult fish has more than 20% lipid content while small fish at spawning stage has 1% fat [38]. But there are seasonal variations in lipid content in herring for instance in summer the lipid content is maximum whereas in winters the lipid content is reduced to its lowest. It was found that herring lipids were around 14% in January to March and were 23% in June to August [42].

1.3 Lipid oxidation

Fish is regarded as healthy food because of the high nutritional value of its components, digestibility and flavor, availability and abundance. Autoxidation ruins the acceptability and nutritional values of fish lipids thus giving huge losses to the food industry [43]. Marine fish have a high content of PUFAs, but they also contains metal ions and heme pigments which make it more vulnerable to lipid oxidation [42]. Additionally, endogenous enzymes in the tissue such as lipooxygenases and myeloperoxidases speed up oxidation by inserting O_2 into fatty acids chains [44]. Lipid oxidation rate increases with increase in degree of unsaturation [45, 46]. Rancidity leads to deterioration of the product quality by changing its taste, colour, smell and texture [43]. It produces compounds which are carcinogenic and can speed up aging process [47]. In fatty fish such as herring, dark muscle is more susceptible to spoilage by lipid oxidation as compared to light muscle. So, nutrients and shelf life can be sustained by removing the dark muscle. Moreover Dang et al. [48] proposed that light muscle should be preferably used for making

finished products and dark muscle can be used as rest raw material.

There are some external factors that are affecting oxidation in fish lipids like mishandling on shore, storage time, high temperatures during transport and storage, processing and packaging [8]. However, the fatty acid composition, number and position of double bonds determining the degree of unsaturation intrinsically contribute to the process [49]. If the fish is stressed during capture this can lead to earlier onset of oxidation in the unprocessed fish and also even when fish is still breathing. But during the processing of its fillets as it becomes more exposed to the atmospheric oxygen and other prooxidants, the susceptibility to oxidize and rate of oxidation is also doubled [50, 51].

1.3.1 Mechanism of oxidation

Lipid oxidation can be divided in to three main steps. Initiation, propagation and termination. Initiation begins when PUFA enriched products are exposed to the molecular oxygen in the presence of catalysts (1.1). Light, heat, photosensitizers or metal ions act as catalysts and promote the reaction [45, 46]. As soon as these fats come into contact with oxygen, in the presence of a catalyst, a hydrogen atom from the double bonds is removed producing an alkyl free radical $R^{\bullet}$ (1.1).

$$RH \longrightarrow R^{\bullet} + H^{\bullet} \tag{1.1}$$

An alkyl free radical ($R^{\bullet}$) is unstable due to the presence of an unpaired electron [45]. So, it rapidly reacts with the triplet oxygen ($3O_2$) to form a peroxy free radical ($R00^{\bullet}$), (1.2). This further extracts a hydrogen from an unsaturated fatty acid to form a hydroperoxide and another new $R^{\bullet}$ radical (1.3).

$$\mathrm{R}^{\bullet} + \mathrm{O}_2 \longrightarrow \mathrm{ROO}^{\bullet} \tag{1.2}$$

$$\mathrm{ROO}^{\bullet} + \mathrm{RH} \longrightarrow \mathrm{ROOH} + \mathrm{R}^{\bullet} \tag{1.3}$$

These peroxides are the primary oxidation products [45] and the production of a new $R^{\bullet}$ reinitiates the process and, in this way, lipid oxidation propagates and continues until no free radicals are left behind or the process is stopped by the formation of a nonradical product (1.4, 1.5, 1.6) [46].

$$R^{\bullet} + R^{\bullet} \longrightarrow RR \tag{1.4}$$

$$R^{\bullet} + R00^{\bullet} \longrightarrow ROOR \tag{1.5}$$

$$R00^{\bullet} + R00^{\bullet} \longrightarrow ROOR + O_2 \tag{1.6}$$

1.3.2 Analytical measurement methods for lipid oxidation

Different methods can be used depending upon which end product we want to analyze because lipid oxidation produces a number of end products at different

stages of oxidation. Most commonly aldehydes and peroxides are selected as indicators of lipid oxidation in food [52]. Commonly used methods for the detection of primary and secondary oxidation products are PV, conjugated dienes and tetraenes and TBARS. Some other methods have been developed which give interesting results for determination of oxidation products. These include Ferrous oxidation method, Liquid and gas Chromatography, Fluorescence spectroscopy, Infrared spectroscopy, Raman spectroscopy, Nuclear magnetic resonance and Electron paramagnetic resonance. The ferrous oxidation method can be used to determine peroxide content, in the presence of thiocyanate or xylenol orange which gives absorption at 500-560nm [53]. Chromatographic methods are specific and accurately determine the lipid hydroperoxides but the methodology is tedious and data processing is complicated [54]. Gas chromatography coupled with mass spectrometry are used to quantify and identify the volatile oxidation compounds [52]. Fluorescence spectroscopy and infra red spectroscopy are helpful in detecting lipid oxidation [55]. Infrared spectroscopy has been used for the detection of peroxides, unsaturation and MDA (malondialdehyde) in lipids [52]. Zhang et, al. [56] developed a method for detecting MDA-TBA in lipids using Raman spectroscopy. Raman spectroscopy can analyze peroxides and unsaturation. Nuclear magnetic resonance has been utilized to determine the oxidation status of lipids in food systems. NMR can detect peroxides, aldehydes and dienes, it is specific, sensitive but expensive technique and involves a complicated data interpretation system. [52].

Different compounds can be the markers of lipid oxidation but the most commonly detected are hydroperoxides and aldehydes. Each one can indicate the oxidation state of lipids so combining different compounds for the analysis of the oxidation status can be beneficial [52]. Of the most commonly used methods for the detection of primary and secondary oxidation products, only those which are followed in this study will be discussed here.

Peroxide value:

There are different methods to determine peroxide value but the iodometric titration of sodium thiosulphate is used in this study [57]. The reaction of iodide ion with peroxides in the product gives iodine which is titrated with sodium thiosulphate. The amount of titrated iodine gives the estimate of peroxides present in lipids. The peroxide value (PV) is concentration of active peroxides present in 1kilogram lipids, that can oxidize KI [58]. Potassium iodide solution is used as an indicator. The yellow color of the iodide solution is decolorized as it is exposed to peroxides in the presence of sodium thiosulphate. As peroxides are highly unstable and they are further oxidized or degraded reacting to form secondary oxidation products. [52]. The limit of PV for the rancidity of fish lipids is less than or equal to 10 meO_2/kg lipids [59]. Details of the procedure are discussed in material and methods chapter 2.

Conjugated dienes/ trienes:

The unsaturated fats are oxidized to form hydroperoxides, the double bonds in the molecules are rearranged to form conjugated double bonds resulting in the formation of conjugated dienes and trienes. These conjugated dienes and trienes are stable and give UV absorbance at 233 nm and 315 nm which can be detected using a spectrophotometer [60]. This method is simple and rapid, however over or under estimation can occur because of interference. Still this method has been used in many studies [52]. More details are discussed in materials and methods (chapter. 2, section. 2.8.3).

Primary oxidation product can produce secondary oxidation products under favourable conditions. A number of compounds are produced and most commonly aldehydes are used as markers of secondary oxidation. Different methods have been designed to determine the concentration of secondary oxidation products. Only the method used in this study is discussed here.

Thiobarbituric acid reactive substances (TBARS)

The TBA assay is used for determination of Malondialdehyde (MDA) which is main marker of secondary oxidaton. The monoenolic form of MDA reacts with methylene groups of TBA at acidic pH and high temperature resulting in the fromation of compound which gives absorption at 532 nm 535 nm. There are various modifications in this method, including centrifugation, homogenization, reaction with TCA and heating at 90°C. TBA can react with aldehydes and other compounds causing overestimation. This method has some limitations but it is still considered for determining the extent of lipid oxidation. It is suggested that TBARS should be used along with other assays. A detailed methodology is given in material and methods (chapter. 2, section. 2.8.4).

1.4 Protein Oxidation

Fish is highly perishable food because of its high water content, endogenous enzymes, prooxidants, polyunsaturated fatty acids as well as high content of easily degradable proteins. The oxidation of proteins can be initiated either by direct exposure to reactive oxygen species or indirectly by secondary by-products of oxidative stress [61]. It can also be induced either through free radicals like hydroxyl ($OH^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$) or superoxide (O_2-) or non-radical species like transition metals, hydrogen peroxide and singlet oxygen [62]. The haem proteins, transition metals (Fe, Cu), lipoxygenases, reducing agents and peroxidases present in fish muscle act as catalysts and can induce protein oxidation. Free radicals induce oxidation in peptide backbone and amino acid side chains leading to the formation of cross linkages and carbonyl groups [63]). Cleavage of peptide backbone and binding with nonprotein carbonyl components (malondialdeyhde) from lipid oxidation can form carbonyl residues. Protein carbonylation can also

be induced by heme proteins (hemoglobin and myoglobin), when free iron is released from these proteins, Fe+ reacts with H_2O_2 to form hydroxyl radical. This hydroxyl radical($OH^{\bullet}$) initiates protein oxidation [64]. Studies have shown that endogenous enzymes can also contribute to oxidation and deterioration of fish proteins during storage ([65–68]. Protein oxidation can also be caused by reactions with intermediates in lipid oxidation. Research suggests that there exists a correlation between lipid and protein oxidation and the products of lipid oxidation promote the oxidation of amino acids in fish, more noticeably during frozen storage [69]. Some external factors like temperature, pH and water activity may also stimulate the process of protein oxidation [70]. Protein oxidation results in loss of protein solubility, decreased water holding capacity, amino acid modifications like formation of protein carbonyls, crosslinkages like disulphides, protein aggregation and changes in texture [62].

Mechanism

Protein oxidation proceeds in same way as lipid oxidation through a free radical chain reaction. When a reactive oxygen specie removes a hydrogen atom, a protein carbon centered radical ($P^{\bullet}$) is formed. On exposure to oxygen, it is converted into peroxyl radical ($POO^{\bullet}$), which further forms alkyl peroxide (POOH) upon abduction of another hydrogen. Afterwards alkoxyl (PO) radical is formed which is converted to its hydroxyl derivative (POH). The products formed as are result of oxidation are dependent on the amino acids involved. Usually microfibrillar proteins are most vulnerable to oxidation. When amino acids like cysteine, methionine is involved then sulphur derivatives are detected as reduction in SH groups or thiols and when arginin, lysine are involved there is a formation of carbonyl groups [62].

1.4.1 Detection

Carbonyls and thiol detection are carried out to analyze oxidative stability of proteins [71]. About 70% of the carbonyls formed in animal protein are due to alpha-aminoadipic (AAS) and alpha glutamic acid (GGS) semialdehydes [72]. Thiol groups of amino acid cysteine are easily oxidized in the presence of hydrogen peroxide [62]. Thiol oxidization produces a number of products via a series of complex reactions. For example, sulphenic acid (RSOH), sulphinic acid (RSOOH) and disulphide (RSSR) cross links. The quantification of thiol groups is an indicator of protein oxidation because the total thiol content does not increase or decrease after death and remains the same as before. Thiol content is determined on the basis of 5,5'- Dithiobis-(2-nitrobenzoic acid) (DTNB or Ellmans reagent [62]. About 0-40% decrease in total thiol content, in washed products of different fish species was reported [62]. A 50% decrease in thiol content of horse mackerel following storage for about 96 hours at 5°C was also observed [53]. The details of protein solubility and loss of thiol groups is further discussed in section 3.5 and 3.6.

1.5 Antioxidants

The antioxidants can be used to prevent or slow down the process of lipid oxidation. The efficacy and potential of antioxidants have been explored through various studies and a number of compounds have been found effective in inhibiting and slowing down the process of oxidation [23, 24, 73]. Antioxidants are the compounds that have the ability to retard the process of oxidation in lipids including omega 3 PUFAs, DHA and EPA and can protect the product from getting deteriorated [74]. High temperature, oxygen and light can induce lipid oxidation in products so PUFA containing products should be stored in dark and cool place in order to avoid photooxidation. Antioxidants may be indigenous to the food products or can be applied externally. Antioxidants have several functions; antioxidants can act as free radical scavengers (FRS) or some act as metal chelators. These are classified as primary and secondary antioxidants depending upon their mode of action [23]. Primary can directly inhibit oxidation while secondary antioxidants can react indirectly like they can react with prooxidants [75].

1.5.1 Primary antioxidants

Primary antioxidants act as free radical scavengers, as these can scavenge the alkoxy $RO^{\bullet}$ and peroxy $ROO^{\bullet}$ free radicals and donate hydrogen to these radicals making them stable, unreactive radicals, which are no longer able to carry out the process of oxidation. Each FRS can inactivate two free radicals, first when it donates hydrogen to the peroxyl radical and second when it reacts with the lipid radical to form a nonradical product during termination. Phenols are the best FRS and donate the hydrogen of its hydroxyl group. Phenolics can act as primary antioxidants either by donating hydrogen (HAT) or by single electron transfer (SET) [75]. In food products the efficiency of phenolic FRS is affected by polarity, volatility and pH of the product. Tocopherols are natural phenolic FRS isomers. Carotenoids and alpha tocopherol can quench singlet oxygen [74, 76].

1.5.2 Secondary antioxidants

Secondary antioxidants control the catalysts that help in accelerating the process of oxidation. These prooxidants or catalysts are metal ions, lipoxygenases and singlet oxygen. Usual metal chelators are citric acid and EDTA that bind to the metal ions, making them unavailable to initiate the process of oxidation or to decompose the hydroperoxides. Metal chelators can behave as prooxidants as well as antioxidants depending upon their concentration [76]. Synthetic compounds BHT, BHA and PG are some efficient antioxidants for food systems[24]. When Sodium erythrobate and polyphosphate were applied on frozen fillets of Atlantic mackerel, the stability of the products was increased for 15 months [77]. Although synthetic antioxidants are effective, due to different side effects, these are not favored by consumers. So natural antioxidants are being explored so that the finished fish products could be safely preserved and consumed [78]. Decker

[76] claimed that carotenoids especially beta carotene acts as FRS in the presence of singlet oxygen [76]. Application of the plant based, green tea extract (GTE) and grape seed extract (GSE) was found effective against lipid and protein oxidation in frozen mackerel [73]. Similarly rosemary extract, nettle, oregano, dill and olive leaf extract have been used in various experiments and their effects are studied [79].

1.5.3 Plant based natural antioxidants

Plants contain the phenolic compounds in their leaves or stems or even in the fruits. These antioxidants are of natural origin and can be extracted and used in the food systems to avoid or minimize the negative effects that are produced by the use of synthetic antioxidants (Figure 1.3).

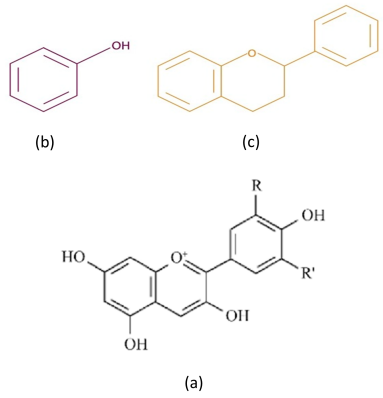

Figure 1.3: (a) Structure of Anthocyanidins (b) Phenol (c) Flavanoid [80]

DILL

Anethum graveolens commonly known as dill, is a green colored aromatic herb, used as a spice in food and has a great medicinal value. It is cultivated in Mediterranean countries and southeast Europe, southwest Asia and US. Its leaves contain essential minerals such as P, Ca K. Traditionally dried leaves of dill are also used for treating various stomach related problems like intestinal spasms and flatulence [81]. Dill possesses free radical scavenging ability and can chelate with metals, thereby reducing lipid oxidation [82]. It is an is effective diuretic, can be used as potential food supplement [81]. Its leaves, flowers, fruit and seeds show strong antioxidant and antibacterial properties. However, a study showed that its flowers have stronger anti-oxidant potential as compared to its seeds and leaves. It was found that the flowers had more phenols, flavonoids and proanthocyanidin

content (Figure 1.3) which was considered to be related to the antioxidant property [83]. Dill extract in 20% concentration was found most effective for frozen mackerel fillets during storage [24, 78]. Antioxidant potential of the dill depends upon the technique used for its extraction, for instance the dill extract obtained in aqueous solution by Soxhlet extraction method showed maximum activity as compared to reflux and ultrasonic extraction [24]. The phenols and flavonoids in the methanol extract are mainly responsible for the antioxidant activity of dill [84].

OREGANO

Oreganum vulgare. L belongs to the *Labiatae family*. It is a natural herb and it is used as a common spice in Mediterranean food. Dried leaves of oregano are used for flavoring pizza, sausages etc [85]. Oregano oil has phenols which are responsible for its antibiotic properties [86]. Oregano oil has 78-82% carvacrol and thymol, which are mainly responsible for its antioxidant potential (Figure 1.4) [85, 87]). Essential oils and extracts from oregano, have shown the radical scavenging properties higher than BHT. Higher content of flavonoids, turpenoids and phenolics are found in essential oils [88]. The phenolic compounds donate electrons to free radicals and make them nonreactive and stable. The antioxidative potential of oregano is proportional to the amount and concentration of phenolics present [88]. Its biochemical properties may vary depending upon vegetative state, season of harvest and cultivation technique [89]. A number of studies have been conducted to explore the antioxidant potential of oregano extracts in food products. Oregano in low concentrations has shown antimicrobial effect in meat products [86, 90]. Because of its antioxidant potential, it enhanced stability of products like mayonnaise [88], minced beef during refrigeration [87] and stored meat products [91]. Oregano can retard autooxidation in lipid containing food ([85, 92]. When OE was added to soybean oil, lipid oxidation was slowed down. It was recommended as an effective alternative to synthetic antioxidants [89].

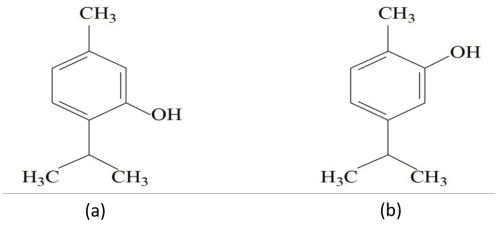

Figure 1.4: Structure of (a) Carvacrol (b) Thymol [93]

NETTLE

It is a Mediterranean herb (*Urtica. Dioica. L*) and belongs to the family Urticacae. It is found in north America, northern Europe and Asia [94]. Nettle has antibac-

terial, antiviral properties, its bright green leaves are rich in essential amino acids, polyphenols, ascorbic acid and minerals. Nettle has ursolic acid in its roots, as a main component and quercetin is accumulated in its leaves (Figure 1.5). Ursolic acid has anti-elastase activity and quercetin is an antioxidant in nature. Both these compounds enable its extract to be used in anti-aging cosmetic products [95]. Because of its biochemical properties, it has long been used as a traditional medicine for treating urinary tract problems, eczema and anemia [96]. Nettle extract can be used as an antioxidant and as preservative in meat products. Nettle extract has the potential to inhibit the formation of free radicals [95, 97]. Water extract of nettle are more potent antioxidants then quercetin, BHA and alpha-tocopherols [98]. Application of herbs like nettle and dandelion in processed seafood can prolong the quality and shelf life of the products [99]. Hydroalcoholic extracts of nettle are proposed as an effective natural preservative for kilka fish products [100]. Besides 0.4% nettle extract reduced lipid oxidation in chilled stored trout fillets [101]. Nettle water extract reduced TBARS and increased color stability in cooked sausages during storage. Several other studies also suggested that it could be used as an antioxidant to retard lipid oxidation including; meat products [94], kavurma (cooked meat product)[79], aquacultured seabass [99] and chocolates [96].

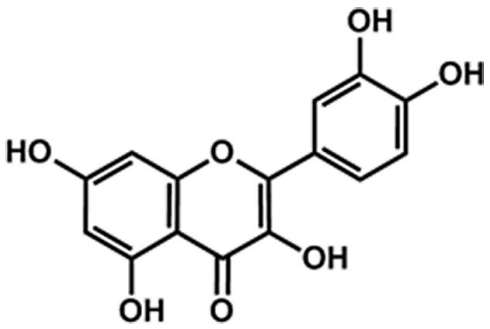

Figure 1.5: Structure of Quercetin [102]

OLIVE LEAF EXTRACT

Olea europaea. L, commonly known as olives belongs to the *Oleaceae family* [103]. It is the most studied plant due to the high medicinal values of its leaves, fruit and oil. Olives are grown in the Mediterranean region and in Arabia, Asia and India [103, 104]. Olives are also effective in treating obesity [104].

OLE has a deep brown color and a bitter taste [105]. The nutritional content of the extract obtained from olive leaves (OLE) may vary depending upon the different processing techniques used in drying and extraction. Olive leaf extract has high concentration of phenols and shows strong antimicrobial activity. It can be used as an antioxidant in the food industry and it can increase shelf life of food products without producing any sensory effects [106]. A study was conducted on addition of OLE to HMB (Halal minced beef) and it was concluded that the

OLE was highly effective in reducing oxidation and retarding microbial activity in packed minced meat [107].

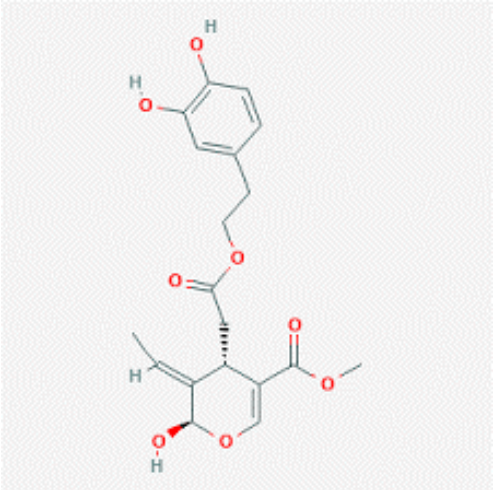

Figure 1.6: Structure of Oleuropein [108]

A number of beneficial health effects of OLE have been studied. Olive leaf extract (OLE) has anti-inflammatory, antimicrobial, antiallergic hypoglycemic and hypercholesterolemic properties [103]. All these properties are attributed to the polyphenolic compounds. Most abundant phenolic compound found in OLE is Oleuropein (Figure 1.6). Olive oil has 0.005-0.12% whereas olive leaves have 1-14% oleuropein [104]. Olives have higher concentration of oleuropein, other polyphenols such as hydroxytyrosol, verbascoside are present in lower amounts. It was concluded that the total phenol content and antioxidant activity are actually inter-related [109]. OLE shows antimicrobial activity against a number of bacteria. Its antibacterial potential against food spoilage bacteria have been reported [106]. The use of OLE in food as a preservative for long term storage of products, has been recommended in various studies [103, 106, 110].

1.5.4 Antioxidant activity analysis

The antioxidant capacity can be determined by SET(single electron transfer) assays or HAT (Hydrogen atom transfer) assays. SET assays are based on the principle of transfer of single electron from antioxidants to the oxidant present in the system and reducing it. These assays similar to the redox reactions. The most common assays include DPPH, ABT and FCA.

DPPH Assay

It is the most commonly used method which is based on the ability of antioxidant to reduce DPPH. The DPPH radical either undergoes hydrogen atom transfer or the single electron transfer in the presence of an antioxidant [75]. $DPPH^{\bullet}$ is

the 2,2 diphenyl 1 picryl hydrazyl radical and it is a stable organic nitrogen radical (Figure 1.7). It has a dark purple colour and gives the highest absorption at 515 nm. Its radical loses colour when it reacts with antioxidant. This loss in colour is measured using spectrophotometer [111]. The reaction mechanism in both cases, HAT and SET can be seen as:

Hydrogen atom transfer:

$$\mathrm{DPPH}^{\bullet}(Violet\ colour\ at\ 515nm) + \mathrm{ArOH} \longrightarrow \mathrm{DPPH\,(H)(colorless)} + \mathrm{ArO}^{\bullet} \tag{1.7}$$

Singlet electron transfer:

$$\mathrm{DPPH}^{\bullet}(violet\ colour\ at\ 515nm) + \mathrm{ArOH} \longrightarrow \mathrm{DPPH-(colorless)} + \mathrm{ArO}^{\pm} \tag{1.8}$$

The loss of colour is measured in DPPH assay. The percentage of DPPH remaining after loss is related to the antioxidant concentration. The antioxidant concentration that decreases the initial concentration of DPPH radical by 50% is termed as EC50. This assay has some challenges also, like interference by some other compounds that have overlapping spectrum at 515 nm. DPPH radical is not as reactive as the peroxy radicals so the highly active antioxidants may not react or react very slowly in this assay [111].

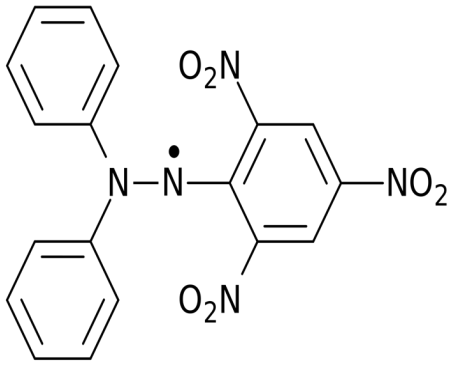

Figure 1.7: Structure of 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$)

ABTS Assay

This assay is based on the radical scavenging ability of antioxidants. The electron transfer is facilitated by acidic pH. This gives maximum absorption spectra at wavelength 415 nm and 734 nm. The reaction between the antioxidant and the radical (Figure 1.8) is monitored by a spectrophotometer. This gives speedy reaction with antioxidants with in 30 minutes. It is effective on both acidic and

basic pH. It can be used to determine antioxidant capacity of body fluids due to its high solubility [111]. The compounds which have a redox potential lower than the redox potential of ABTS (0.68V) can also react with ABTS radical.

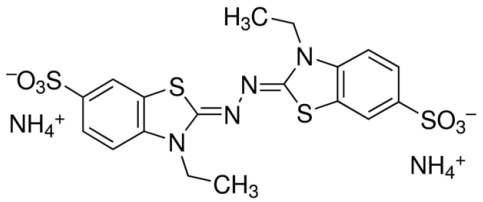

Figure 1.8: Structure of 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) [112]

Folin Ciocalteau Assay

This assay is based on the redox reaction between the folin reagent and the phenolics of antioxidants. It is a sensitive and precise method, but it has been modified a number of times to make it a standard method. This assay is used to determine the total content of phenolics in naturally occurring compounds. The FCR is reduced by the phenolic anions at pH 10 and the yellow colored FCR, loses its color and turns blue. This method has some drawbacks associated with it like a number of compounds can cause interference with FCR. The reagent is soluble in water so it cannot be used for lipid soluble antioxidants. The structure of FCR can be seen in Figure 1.9. Gallic acid is used as a reference in FC assay [111].

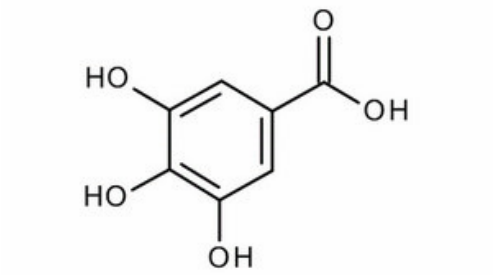

Figure 1.9: Structure of Folin Ciocalteau Reagent [113]

1.6 Sous-vide cooking

For the last several decades, sous-vide has been extensively studied and emphasized as a food preservation technique. Conventional cooking methods usually involves high temperature heating, which promote oxidation and affect the quality of a product. Such high temperature cooking ruptures the membranes, degrade

heme proteins and release free iron that stimulates oxidation of lipids [114]. In contrast to traditional cooking, in sous vide, raw materials or the products are first vacuum packed and then heated slowly at a controlled temperature (below 100°C) for a longer time [115]. The quality of the finished products is enhanced due to low level of oxygen inside vacuum packs. There is decreased flavor loss and texture is also retained. The product remains juicy and tender. Cropotova et al. (2019) [116] suggested the most effective optimal temperature for sous-vide in regards to oxidative stability of Atlantic mackerel (Scomber scombrus) is 70°C.

There are a number of benefits of vacuum packaging and controlled temperature. Vacuum packaging ensures even and efficient heat transfer to the product. The moisture content is retained and there is no loss of volatile flavoring compounds. Additionally, the product is protected from the invasion of aerobic microorganisms, so the chances of microbial spoilage are reduced. Subsequently the quality of the product is stabilized, and nutritional status is maintained. Controlled heating in sous vide makes it possible to obtain reproducible product and the precise control of temperature helps keeping a sharp check on all fast and slow changes taking place in the product [115]. The compounds like vitamins, nutrients and essential amino acids are not denatured or lost during sous-vide or at least destroyed to a small extent [23].

Vacuum sealing should not be so forceful that it damages the texture of the food, but it should be done in a way that it can save the cook-chilled product from aerobic contamination during long term storage. Myhrvold et al.(2011) [117] recommended a pressure of 30-50 mbar/0.4-0.7 psi is suitable for vacuum sealing. Baldwin [115] proposed the water displacement method as an option for vacuum sealing and the use of a proper zip lock bag for keeping the product in it. The sous vide cooked products which need prolonged chill storage, should be chilled immediately at 0-4°C, until further storage to ensure complete pasteurization [23, 115]. Pelagic fish is highly susceptible to spoilage during storage and treating it with a proper cooking technology can increase the shelf life and quality [23]. Cooking temperature and time both effect lipid oxidation in perishable fatty fish. Sous vide becomes less significant if the products are chilled stored for too long so it was recommended that applying antioxidants prior to sous-vide would enhance shelf life of mackerel fillets [23].

1.7 Chilled/ frozen storage

1.7.1 Chilling

Large amounts of ice are used for chilled storage of seafood for transporting large amounts over long distances, it increases the net weight and the transportation costs as well [118]. Chilled storage temperature for food products is +8°C to -1°C [119]. Different methods include use of refrigerated sea water which is cooled below 0°C [120], use of ice slurry and cold air storage in cold rooms at temperatures 0-4°C used for chilling fish [121]. During chilled storage the main problem

in fish is, quality deterioration by lipid oxidation, microbial spoilage and activity of endogenous enzymes. The endogenous enzymes present in meat or fish product start proteolysis and make it vulnerable to lipid oxidation [118]. Cropotova and coworkers (2019) [23] studied the effects of chilled storage on the mackerel fillets and confirmed that the prolonged chilled storage negatively affected the physiochemical properties of the fish. The duration of chilled storage contributes mainly to the carbonylation and protein losses [23, 122]. The protein oxidation and proteolysis were found to increase, and meat turned pale. It was further suggested that the use of antioxidants during chilled storage could be beneficial to increase the shelf life [23, 116].

1.7.2 Freezing

It has been a common practice to freeze the meat and related products for a long time. Fish proteins are highly digestible, and its lipids are also susceptible to oxidation, so preserving fish for long becomes quite challenging. Freezing has been found as an effective preservation technique for fish and can slow down the physical and chemical processes that can cause deterioration [123]. However, some physical and chemical reactions are still going on during frozen storage that can cause changes in texture and physiochemical properties of products. Freezing can convert water content of fish to ice crystals [48]. This can change the pH [124, 125] and decrease water holding capacity [126] and protein solubility [127]. Temperature stability is very important for long term storage of a frozen product, fluctuation in temperature can speed up the rate of deterioration [48].

Degree of sensitivity of fish products to lipid deterioration depends upon various factors, fish species, water in muscle, temperature of frozen storage and duration of fluctuation [48, 123]. A temperature range -35°C to -24°C is considered ideal for preserving pelagic fatty fish like herring and mackerel [43]. A larger fluctuation in temperature can increase unfrozen water in muscles, deform ice crystals and can cause recrystallization. These changes in frozen product begin proteolysis and lipid oxidation and as a result the quality of product is spoiled [48]. Dang et al. (2017) [48] concluded that storage conditions are very important for long term storage, especially the temperature fluctuations should be avoided to keep the quality and stability of fish. Fish products can be safely stored for longer time at freezing temperature -18° C and -40°C. But the problem is that it requires more energy and the sensory quality might get affected [23]. In another study Cropotova and coworkers investigated the effects of frozen storage duration on Atlantic mackerel and reported that as protein oxidation increased with time. However frozen storage combined with sous vide, was found effective for stability [122, 128].

Chapter 2

Materials and Methods

2.1 Overview

Atlantic Herring fillets were treated with four different natural antioxidants (Oregano, Nettle, Dill, Olive leaf extract), samples were vacuum packed and sous vide cooking of fillets at 70°C was carried out. The samples were initially stored at -40°C and further at -20°C during analysis in order to determine the oxidative stability of lipids and proteins during storage.

The primary and secondary oxidation products were analyzed. Additionally, the antioxidant activity of extracts of Dill and Oregano, was analyzed through the three standard assays ABTS, DPPH and FCA. The content of water soluble protein was determined, and quantification of thiols was done to determine protein oxidation.

2.2 Experimental Setup

The Figure 2.1 shows the flowchart of experimental setup.

2.3 Chemicals

All chemicals used were of analytical grade.

- Antioxidants solutions 1% (four antioxidants; oregano, nettle, dill, olive leaf extract)
- Acetic acid, CH_3COOH
- Chloroform, $CHCl_3$
- Methanol, CH_3OH
- Sodium thiosulphate, $Na_2S_2O_3$ (0.01 for 1 g oil or more and 0.001 mol/L for 0.8 g oil and less)
- Potassium iodide, KI (0.046 mM)
- Thio barbituric acid, TBA

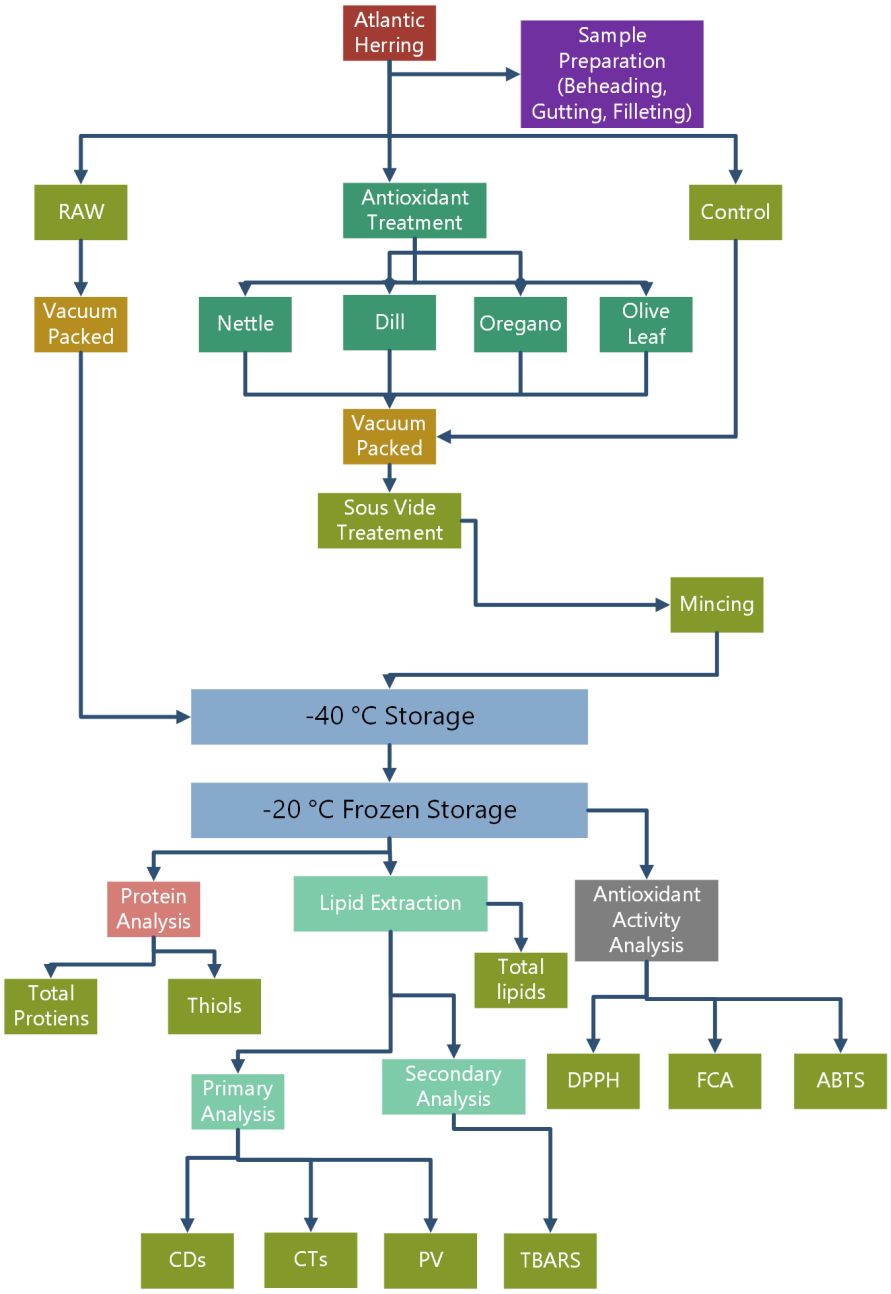

Figure 2.1: Flowchart of experimental setup

- Butylated hydroxy toluene, BHT (3% in alcohol)
- Tri chloracetic acid, TCA (0.28 M)
- 1,1,3,3- Tetraethoxypropane, TEP (0.01 mM)
- 2% Diluted antioxidant extracts (methanol based)
- Folin Ciocalteu phenol reagent, FCR (2M)
- Sodium carbonate Na_2CO_3 (20%)
- Propyl gallate (10 mM)
- Methanol
- Ethanol (96%)
- DPPH
- ABTS (7 mM)
- $K_2S_2O_8$ (140 mM)
- Sodium hydrophosphate, Na_2HPO_4
- Sodium di hydrogen phosphate, $NaH_2PO_4.H_2O$
- Urea
- Dithiobis2 nitrobenzoic acid DTNB
- EDTA

2.4 Equipment

- Ultra Turrax
- SI Analytics titrator mode TL 6000/7000 (France), consisting of, Platinum electrode Pt 62/61 with cable L1A and Basic device (titrator unit) 10 mL exchange unit WA 10, with fitted in titrant bottle
- Magnetic stirrer TM235
- Centrifuge (Universal 16A-Hetlich Zentrifugen)
- Centrifuge (Heraeus Multifuge X1R).
- Erlenmeyer flasks with stoppers (100 250 mL)
- Webomatic Advanced vacuum packaging systems machine
- Genesys 10S UV-VIS spectrophotometer (Thermo Scientific, USA)
- MS2 Minishaker IKA (Vortex)
- PH Meter Framo M21/1

2.5 Preparation of Antioxidant Extract

The antioxidants used for the study were obtained from the University of Zagreb, Department of Food Engineering, Croatia in February 2019. The dried leaves (3g) of the antioxidant plants were added to a mixture of water-ethanol (ratio 1:1, 40 mL) to prepare the solvent extract. The antioxidant extracts were obtained by high voltage electrical discharge (100 Hz) using Argon (20 kV) for 10 minutes in a microwave Milestone oven at 110°C with 60% stirring power (SR- 12 rotor).

2.6 Herring Fillets Preparation

The fresh Atlantic Herring (*Clupea harengus*) was obtained from Ravnkloa and Meny (Trondheim, Norway) and it was stored in cold room at 4±1°C for 24-48 hours. The next day the fishes were washed, beheaded, gutted and filleted, the fillets were blotted dry on paper (Figure 2.2) and kept on ice. The fillets were then divided into six groups. Each group was treated with one antioxidant extract, such as oregano extract, nettle extract, dill extract and olive leaf extract. For the treatment of the samples we sprayed antioxidant extract on the surface of each fillet (3 sprays for each fillet). Of the remaining two groups, one was taken as Control (without antioxidants) and other was kept Raw (without antioxidants and without sous vide).

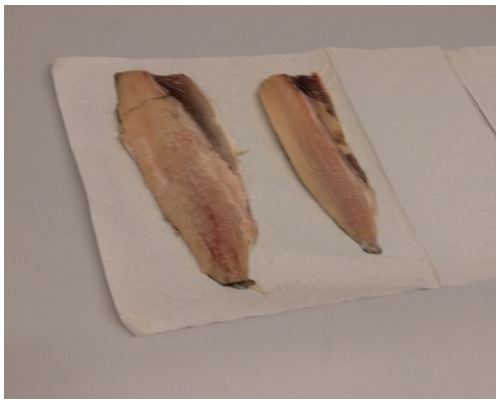

Figure 2.2: Prepared fillets arranged in a group and dried on paper to remove excess water

2.7 Sous Vide Cooking

All antioxidant treated fillets and control samples were cooked in water bath set at 70 °C for 10-minutes(Figure 2.3). They were taken out of water bath and were chilled immediately on ice in the cold room and further stored these at 4±1 °C for 1-2 days with changing of ice twice a day.

2.8 Initial Analysis

Initial analysis on the day 1, 4, 6, 8, 12, 14, 18 and 20 from the freshly sous-vide treated fish was performed during cold storage by a fellow student, Dat Trong Vu [129]. After analysis the minced samples were frozen at -40°C for about one month. After one month the samples were taken out and analysis was carried out for determination and analysis of oxidation stability of stored samples. During the analysis all the samples were kept at -20°C for about two months. Lipids from the

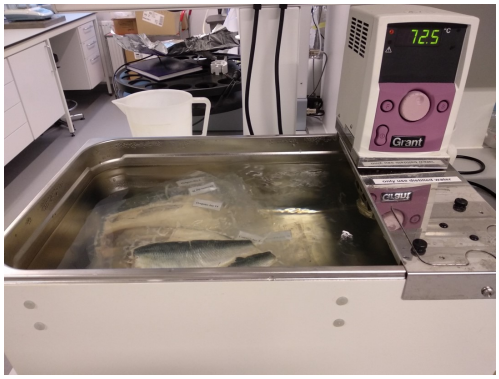

Figure 2.3: Sous-vide cooking of all vacuum-packed herring samples

minced frozen samples were extracted on the day 27, 29, 32, 34, 36, 41, 45 and 48 of sous vide cooking for further studies.

2.8.1 Lipid extraction

The Bligh and Dyer [130] method was used to extract lipids. The extraction was performed in two parallels for each sample. Lipids in chloroform phase were extracted.

Approximately 10 g of minced fish was taken in each centrifugation bottle which was kept on ice during the procedure. 10 ml of water, 40 ml of methanol and 20 ml of chloroform were added to sample and it was homogenized for 2 minutes using an Ultra-Turrax (9000 rpm). Further, another 20 ml of chloroform was added followed by subsequent homogenization for 30 seconds. Finally, 20 ml of distilled water was added and homogenized again for 30 seconds. The weight of centrifuge bottles was adjusted with distilled water before centrifugation at 9000 g for 10 minutes. The lipids in chloroform phase were collected in small chloroform resistant tubes, flushed with nitrogen to avoid contamination and stored at -20°C for further analysis.

Empty kimax tubes were weighed and 2 ml chloroform extract from each chloroform resistant tube was taken in each of the tube. These tubes were then placed in heating block in the evaporation unit (60°C) under a stream of nitrogen for about 30-45 minutes. When chloroform from the sample in tube was completely evaporated then the samples were allowed to cool down at room temperature by incubating overnight in a desiccator. The cooled tubes with the chloroform extracts were weighed again to obtain the total lipid content in sample. The total lipid content was determined using the formula:

$$Total\ lipids\ \% = \frac{lipid\ in\ tube \times volume\ chloroform\ in\ total\ \times\ 100}{Amount\ of\ sample\ (g) \times\ chloroform\ evaporated(ml)} \tag{2.1}$$

2.8.2 PV Analysis

Peroxide Value was measured by iodometric titration method described in AOCS official methods (2003) , Cd 8b-90 [131]. It gives a procedure to determine the peroxides in fats and oils. It is used to determine the extent to which oil is oxidized. It gives the primary oxidation products in unsaturated fats and oils. All values were measured in meqO_2/kg lipids (milliequivalents of peroxide per kg). The end point of titration was measured potentiometrically by an automated titrator (SI Analytics Titroline 7000) connected with a platinum electrode (Figure 2.4). The analysis was performed in two parallels.

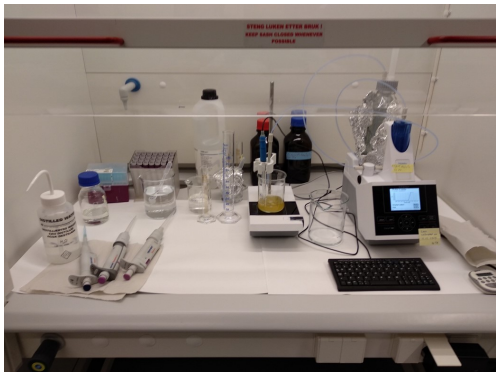

Figure 2.4: Titration equipment set up with PV analysis of one sample in progress

Titration equipment:
- Analytical balance with ±0.0001 g sensitivity
- SI Analytics titrator mode Titroline 7000 with four basic units connected together for functioning.

(a) Titration unit
(b) Magnetic stirrer
(c) Exchange unit with a dark glass bottle for titrant
(d) Platinum electrode

Preparation of solutions

Sodium thiosulphate solution was prepared to be used as titration agent, in two concentrations, 0.01 mol/L for extracts containing more than 0.8 g oil and 0.001 mol/L for those estimated to have less than 0.8 g oil sample. Glacial Acetic acid (CH_3COOH) was used as a solvent, saturated potassium iodide solution (KI) was used as a titration indicator, KI (1 g) dissolved in distilled water (1.3 g) to prepare KI. Solvent mixture was prepared by acetic acid (600 ml) and chloroform (400 ml).

Procedure

Titrating solution ($Na_2S_2O_3$) was added to the bottle in the exchange unit. After rinsing the titrator, PV of blank was performed by chloroform (12 ml) and glacial acetic acid(18 ml) taken in a beaker, then KI (0.5 mL) solution was added to it. The stirrer was kept in the solution and placed on the magnetic stirrer for 1 minute. Further distilled water (30 ml) was added to it and the probe was immersed into the solution while placing the titration tip beside it. For performing the iodometric titration of sample 12 ml chloroform is substituted with 12 mL oil sample (lipids extracted in chloroform phase). The result of PV is calculated as average ± standard deviation in milliequivalents O_2 per kg lipid sample.

Equation used for PV

$$PV(m_{eq}O_2/kg) = \frac{(V-B) \times T \times M \times F}{W \times F2} \tag{2.2}$$

Where,
V=volume of the titrant consumed during titration of sample, mL
B=volume of the titrant consumed during titrating blank, mL
T=Titre concentration, (0.001M or 0.01M)
M=Molarity, 1000
F1=factor 1(1.0000)
F2=factor 2(1.0000)
W=oil sample weight, g.

2.8.3 Conjugated Dienes, (CDs) / Conjugated Tetraenes, (CTs)

The chloroform phase which was initially obtained from [130] was used as sample for detecting conjugated dienes and tetraenes. Sample (1 mL) was transferred to cuvettes and UV absorption is measured at 233 nm for CDs and at 315 nm for measuring CTs using GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific, USA) for detecting the conjugated structures in sample according to AOCS standard method (1998). Chloroform was used as blank. The analysis was performed with two replicates. The average with standard deviation was calculated in terms of mL CD/CTe mL per gram of lipid. The CDs, CTs were calculated as:

$$CD/CTe(mL/g) = \frac{Average\ absorbance \times volume\ of\ sample(mL)}{weight\ (g)\ of\ oil\ content\ in\ sample} \tag{2.3}$$

2.8.4 TBARS

Thiobarbituric acid reactive substances were analyzed using the procedure as described by Ke and Woyewoda (1979) [132]. The analysis was performed in two parallels. All the solutions needed for the TBARS analysis were prepared, TBA

stock solution, Sodium sulphite solution (Na_2SO_4). TCA solution, TEP stock solution. Finally, tertiary butyl alcohol (TBA) work solution was prepared using 180 ml TBA stock solution, 120 mL chloroform, 15 mL sodium sulphite solution and 9.45 mL BHT (3% BHT in ethanol).

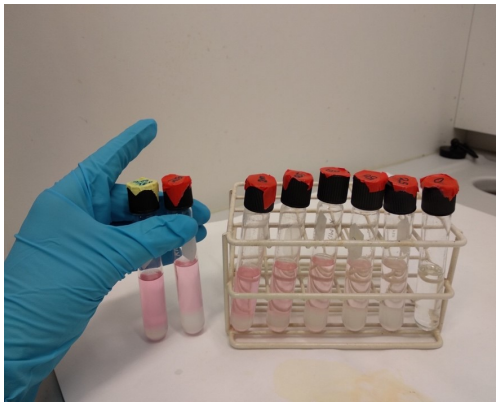

Figure 2.5: TBARS

First the standard curve was obtained using TEP solution in concentrations: 0, 25μl, 50μl, 100μl, 150μl, 200μl, 250μl (0.1 mM) (Figure 2.5). The standard curve was repeated each time when a new solution was made. TBARS analysis was performed in duplicate. The chloroform resistant tubes containing the lipids in the chloroform phase were taken and placed on ice during the procedure. Kimax tubes were labelled for each of sample then 200μl sample was pipetted into kimax tubes and 5ml TBA work solution was added and vortexed for 15 seconds (using MS2 Minishaker VWR). kimax tubes were heated for 45 minutes in a boiling water bath. Tubes were then cooled for about 10 minutes, further 2.5 ml TCA solution was added into the tubes then each tube was inverted once to ensure uniform mixing. Finally, the tubes were centrifuged at 900 g for 10 minutes (Heraeus Multifuge X1R). After centrifugation the lower phase containing the lipids was taken into cuvette and absorbance was measured at 538 nm using the UV Visible spectrophotometer Genesys 10S UV-VIS spectrophotometer (Thermo Scientific, USA). Distilled water was used as a blank. TBARS were calculated as average ± standard deviation μMol TBARS per gram lipid. The following equation was used:

$$\mu Mol\ TBARS/g\ lipid = \frac{Absorbance\ of\ sample - intercept\ of\ standard\ curve}{Slope\ of\ the\ curve \times\ total\ lipid\ content\ in\ sample \times\ 1000} \quad (2.4)$$

Total lipid content is in μL/ g lipid, 1000 is the conversion factor used to obtain μmoles/ g.

2.9 Analysis of Antioxidant Activity

Antioxidant potential of the two antioxidants dill and oregano extracts (the olive leaf extract and nettle were not available so were excluded) was analyzed using three standard assay, DPPH, ABTS and FCA.

2.9.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

DPPH free radical method is used to evaluate antioxidant activity by spectrophotometry. This free radical is stable at room temperature but is reduced in the presence of antioxidant producing a colorless solution. When it reacts with an antioxidant which donates a hydrogen atom it gets reduced which results in change in colour of the solution from violet to light blue and this change in colour can be seen at an absorbance of 517 nm [133, 134].

80% methanol, 96% ethanol, 2% diluted antioxidant extracts (Dill, Oregano) 0.15 mM DPPH in 96% ethanol,10 mM propyl gallate in 80% methanol, stock solution used for analysis. Firstly, all antioxidant extracts were diluted with methanol (2%). The solutions were covered with aluminium foil and stored at 4°C in cold room. 0.53 g Propyl Gallate was dissolved in 250 mL of 80% methanol. The solution was stirred until completely dissolved. DPPH solution was prepared (5.92 mg DPPH in 100 mL of 96% ethanol). The solution was kept in the dark or wrapped in Aluminium foil and stored in a cold room (4°C) on a magnetic stirrer overnight. For the standard curve, propyl gallate was serially diluted with 80% methanol, in concentrations of 10 μM, 15 μM, 20 μM, 25 μM and 30 μM using 10 mL volumetric flasks. 1.5 mL from each standard solution was taken in separate glass tubes and then 1.5 mL DPPH was added to each tube. The tubes were vortexed for a few seconds and incubated for 30 minutes in dark at room temperature. The UV absorbance was read at 517 nm. Antioxidant extract ana-

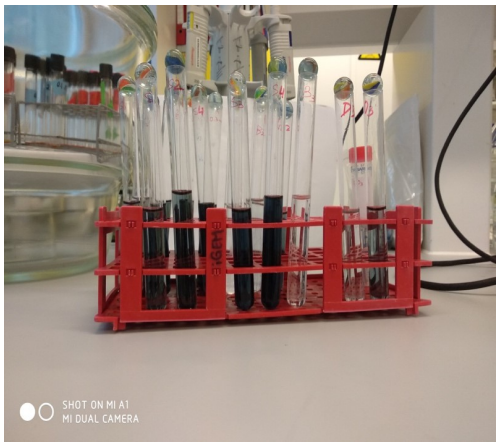

Figure 2.6: Antioxidant activity analysis using DPPH assay

lysis was performed in three parallels (Figure 2.6). For this DPPH (1.5 mL) was added to a tube containing 1.5 mL of antioxidant. The tubes were vortexed to mix the solutions. 80% methanol was used to prepare blank. These were incubated in dark for 30 minutes and afterwards the absorbance at 517 nm was measured using GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific, USA). 96% ethanol was used as a reference in spectrophotometric analysis. The blank is used

to calculate IC_{50} using the following equation:

$$IC_{50} = \left(1 - \frac{A}{A}\frac{sample}{blank}\right) \tag{2.5}$$

Where A sample is the absorbance of sample and A blank is absorbance of the blank. Average values of PG equivalence were calculated using the formula

$$PG\ equivalence = \frac{A-b}{a} \tag{2.6}$$

Here, A is the average absorbance, b is the intercept of the curve and a is the value of the slope.

2.9.2 Folin Ciocalteu Assay

The FCA was performed using the standard procedure described by Singleton et al. (1999) [135] with some modifications from [133]. Folin-ciocalteu phenol reagent (FCR), 20% Na_2CO_3 (20 g in 100 mL), propyl gallate (10 mL) in 80% methanol (stock solution) were used for the assay. Propyl gallate solution was prepared by dissolving propyl gallate (0.53 g) in 250 mL of 80% methanol in a volumetric flask and the solution was stored in dark at 4°C±1. Serial dilutions with different concentrations 0.5 mM, 1 mM, 1.5 mM, 2 mM and 2.5 mM of propyl gallate, were prepared in small volumetric flasks to obtain the standard curve using eppendorf tubes (1.5-2 mL).

Distilled water (5 mL), FCR (0.5 mL) and 0.5 mL from each of the extracts was added to the tubes. Extract was replaced by respective standard solutions (0.5 ml) and then by blank (80% methanol) in the other tubes, keeping the rest composition the same as that for the extracts. Each tube was vortexed and exactly after 3 minutes, 20% Na_2CO_3 (1 ml) was added to it. Mixed and made a final volume up to 10 mL by adding 3ml water. Tubes were vortexed again and incubated for one hour at room temperature. Finally, the absorbance was measured at 725 nm using GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific, USA) and keeping water as a blank. The assay was repeated thrice with each compound and the average slope value ± standard deviation was measured. The equation used for calculating average PG equivalence was

$$PG\ equivalence = \frac{A-b}{a} \tag{2.7}$$

Where A is the average absorbance, b is the intercept of the curve and a is the slope.

2.9.3 ABTS Assay

ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] assay was carried out as explained by Nenadis et al. (2004, 2007) [133, 134] with small modifications, methanol was used in place of ethanol and the sample 200 ul was used

for the analysis. Antioxidants can react with this radical decreasing the absorbance which can be detected. This radical is chemically stable and can give strong absorption in a range of 600 nm to 700 nm. It is soluble in both organic solvents and water so can be used for analyzing the antioxidant activity in both lipids and water-soluble samples. The ABTS radical can be generated by reaction with potassium per sulphate and it is chromophore blue/ green that gives absorption at 645, 734 and 815 nm. When antioxidant is added to it initiates an electron transfer which can be detected by decolorization when it is reduced by an antioxidant. Depending on the time and concentration of antioxidants different degree of decolorization is observed [133, 134]. 25 ml of 7 mM ABTS (0.36 g in 100 ml), 440 μl 140 mM $K_2S_2O_8$ (0.378 g in 10 ml), 10 mM propyl gallate in 80% methanol (stock solution), Diluted extracts (oregano and dill) were used.

The ABTS and $K_2S_2O_8$ solutions were mixed together and allowed to react in the dark at room temperature for 24 hrs. The next day this reaction mixture was diluted up to 1:90 using 80% methanol to obtain the absorbance of 0.75 ±0.05 at 734 nm. Distilled water was used as a reference. Propyl gallate solution was prepared by dissolving propyl gallate (0.53g) in 80% methanol (250 mL) and stored in dark at 4°C±1. A series of dilutions were made from PG stock solution and methanol with five concentrations 10 μM, 20 μM, 30 μM, 40 μM and 50 μM, in 10 ml volumetric bottles. ABTS (2 ml) was mixed with 200μl extract sample, then it was vortexed and incubated for 6 minutes at room temperature. Same procedure was repeated for all standards as well as for blank by replacing extracts with standard solutions or blank (80% methanol) in the mixture. The absorbance of all compounds was measured at 734 nm using GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific, USA). The deionized water was taken as a reference. The whole procedure was repeated three times for every compound. Average values of PG equivalence were calculated by the formula

$$PG\ equivalence = \frac{A - b}{a} \tag{2.8}$$

Here, A is the average absorbance, b is the intercept of the curve and a is the value of the slope.

2.10 Protein Solubility Analysis

The total water-soluble protein content was determined after extraction with phosphate buffer using the method given by Anderson and Ravesi (1968) [136] and Licciardello et al, (1982)[137] . The procedure involved three steps; in first step all the required solutions were prepared. The minced samples after the lipid oxidation analysis had been frozen stored for 5 months at -80°C. Then the samples were taken out and made ready. Finally, the water-soluble proteins were extracted.

2.10.1 Preparation of solutions

In the first step all the solutions were prepared.

(i) Buffer A was prepared using 0.1M sodium phosphate buffer [3.1g Na_2HPO_4.H2O, 10.0g Na_2PO_4 in 800 mL distilled water] with pH adjusted to 7.4. The EDTA III (1mM) was added to it and was shaken to dissolve.
(ii) Urea solution was made by taking urea solution (8M) in buffer A and it was then heat mixed using a magnetic stirrer. The pH was adjusted to 7.42.
(iii) 0.1% DTNB solution was prepared and its pH was adjusted to 7.45. This solution was added to small aliquots and stored at -20°C.

2.10.2 Sample preparation

In the second step samples were taken out a day before from -80°C and these were kept at -20°C and were used later for protein extraction.

Fish samples were weighed (4.0 g) out in centrifugation bottles. Samples were homogenized for a few seconds using Ultra-Turrax (9000 rpm) after addition of 40 ml distilled water in each sample. Then all the samples were centrifuged for 15 minutes at 5000 g. The supernatant containing the protein extract was decanted in volumetric flasks using glass wool. Finally, deionized water was used to bring total volume to 50 ml. This was the water-soluble fraction. The protein content was determined using the Bio Rad assay [138]. Dye reagent was prepared by adding 1 part to 4 parts of distilled deionized water (DDI). It was then filtered using Whatman # 1 filter. 5 dilutions of standard protein (IgG) were made. Each standard and sample was pipetted into a test tube. Three replicates of each were made. Diluted dye reagent (5 ml) was added to each tube and vortexed. The tubes were incubated for 5 minutes and the absorbance was measured at 595 nm.

2.11 Analysis of Protein Oxidation - thiols

Urea used as a denaturant in the procedure while Ellmans reagent, 5,5, dithiobis (2 nitrobenzoate) DTNB is used which readily gives a thiol disulphide interchange reaction in the presence of a free thiol [139, 140]. 100 μl sample was taken in eppendorf tubes (in 3 parallels). Water was used as blank. 800 μl urea and 100 μl DTNB was added to all tubes including blank. Vortexed and incubated at room temperature for 30 minutes. All tubes were centrifuged for 3 minutes at 12000 g at room temperature. Finally, the absorbance was read at 412 nm with the blank as reference. The dianion TNB shows absorption at 412 nm.

Chapter 3

Results

This chapter includes all the results of sous vide cooking, primary and secondary lipid oxidation, antioxidant activity analysis and protein analysis obtained through Total lipid content, PV, conjugated dienes, tetraenes, TBARS, total protein content, thiol analysis and then the antioxidant assays including DPPH, ABTS and FCA. The four antioxidants are used oregano (O), nettle (N), dill (D), olive leaf (OL) and C is the control (without any antioxidant).

According to the initial findings from day 1 till day 20 by Vu [129], the ash content of raw sample was 0.97% and ash content of treated samples was about 1.6%. There was a little variation in color development through the initial days of storage from the fresh red color of raw sample to the lighter pale yellowish color in the antioxidant treated sous-vide cooked herring samples [129]. The same samples were subjected to frozen storage for the investigations of stability of antioxidant treated samples.
All the results obtained in this study are shown by graphs and the raw data can be found in the Appendix.

3.1 Total Lipid Content

Total variation of lipid content (%) of herring was between 21 and 35% during the storage period of 22 days (Figure 3.1). The initial lipid content of sample Raw was 24 ± 1,00 % (Figure 3.1). Results showed that there was no significant variation (>0.05) found in the lipid content of all the samples oregano, nettle, dill, olive leaf except Control from day 46. On the 27th day of storage all the samples had lipid content below 25% but along the axis it continues to show little variations, followed by a sharp increase on day 29 and then a little decrease in values on the next two days and then all antioxidant treated samples excluding oregano, exhibited similar values. Oregano and nettle treated fillets showed high value of 30 % towards the end of storage period. Overall all the values seem to lie less or more in the same range around 25-30% throughout the period.

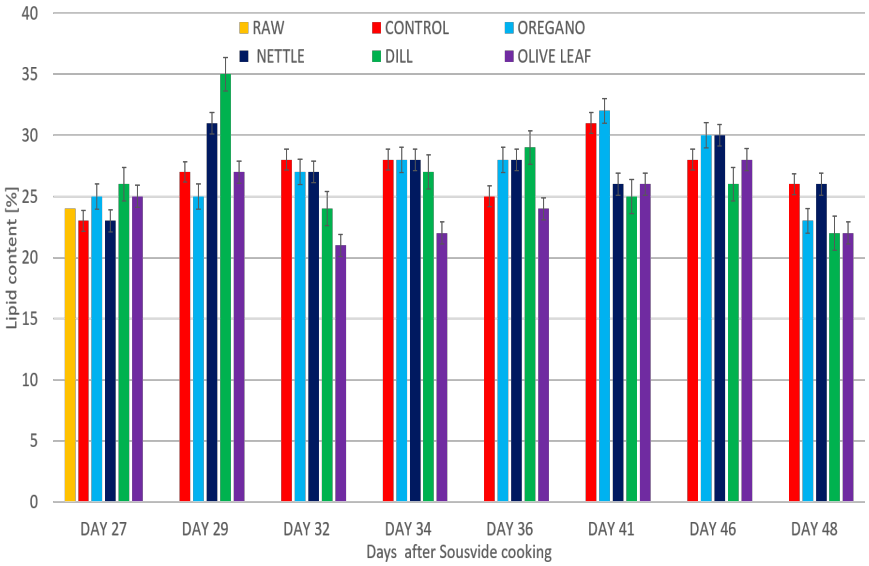

Figure 3.1: The total lipid content [%] of herring samples as function of storage days after sous-vide cooking. Raw represent raw herring samples (orange). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). O (sky blue), N (deep blue), D (green) and OL (purple) are herring fillets with addition of one of the antioxidants, either oregano extract (O), nettle extract (N), dill extract (D) or olive leaf extract (OL), and subjected to sous-vide cooking. The x-axis shows the number of days after sous-vide cooking (day 27 to day 48, the sampling days with intervals). The y-axis shows lipid content in % of wet weight of herring fillets. The values are given as mean value with error bars as standard deviation.

3.2 Primary Oxidation Products

Determination of peroxides formed during storage, by PV analysis, along with the quantification of total conjugated dienes and tetraenes to get an estimate of the primary lipid oxidation in herring samples. PV determines the peroxides in early stages of the lipid oxidation whereas the conjugated systems involves quick estimation of dienes and tetraenes through UV absorbance. [26, 49]. The results of PV, CDs, CTs are shown here.

3.2.1 Peroxide Value, PV

The peroxide value is calculated following Iodometric titrations in terms of meqO_2/kg lipid. The PV values in this study was in the range of 0.07 to 77.7 meqO_2/kg lipid of herring fillets (Figure 3.2). PV of sample Raw was 21.9± 19 meqO_2/kg lipid (Figure 3.2). (Raw analysis was not repeated due to the limited sample amount). All the antioxidants showed some variability (>0.05) in peroxide content. The Control showed an ascending trend throughout, except day 36, where it showed

a sudden fall to a value of 9.6 and afterwards it rose again till it reached the maximum value (77.7) of peroxides by the end of storage period. Besides, the sample treated with oregano showed a prominent variation in values throughout the storage period. None of the changes in PV value during the storage period were significant (>0.05) except Nettle day (42).

The Control showed higher PV-values as compared to all other antioxidants throughout the period except day 27 and 36. In contrast Oregano showed an opposite trend to the Control sample, it continued to descend till day 32 (7.6) started to rise till it reached a peak value (54.9) on day 36 and started to decrease again till it gave value (11.8) on day 48, lower than all other antioxidants as well as Control.

Figure 3.2: The peroxide value [meqO_2/kg lipid] of sous-vide cooked herring samples following the storage of 27-48 days. Raw represent untreated herring samples. Control are sous-vide cooked herring fillets without pre-treatment with antioxidants. O= Oregano, N= Nettle, D= Dill and OL=Olive Leaf are antioxidants treated herring. The x-axis shows the number of days after sous- vide cooking (day 27 to day 48, the sampling days). The y-axis shows the peroxide value in milliequivalents of peroxide oxygen per kg lipid [meqO_2/kg lipid]. The values are given as mean value with error bars indicating standard deviation, n = 2.

3.2.2 Conjugated dienes (CDs)

The CDs values ranged from 6.6 to 24.06 mL/g (Figure 3.3). The initial CD values for raw herring was 15.4± 0.42 ml/g. Olive leaf sample from the day 27 was not enough, so it could not be analyzed for CDs and CTs value, so its value is missing in Figures 3.3 and 3.4. The sample Control gave highest values 24.06 mL/g on

the day 27 and then it showed a decreasing trend.

No significant (>0.05) changes were found among CDs in the samples O, N, D, OL and C except O and OL (day 34), O (day 36), C and O(day 41), D and OL (day 45), N and OL (day 48). All samples seem to follow the same trend throughout the storage period. For example, oregano showed a value 18.7 ml/g and then it remained around 13 throughout the period. Similarly nettle values started from 14.7 ml/g on day 27 and it was 15.5 ml/g on day 48 in the end. The sample dill gave 14. ml/g on day 27 and was 18.5 ml/g on day 48. The sample OL gave little high values in mid of period but then it followed same pattern towards the end of period and reached the same point as nettle to a value of 15.4 ml/g.

In comparison to Control values all samples except oregano showed similar effect on lipid stability, olive leaf extract showed a different pattern as it can be seen in Figure 3.3. In the beginning of the period all other samples had values lower than control then all values were above control. Oregano had similar values in the start but showed lowest values as compared to all other antioxidants in the rest of the period.

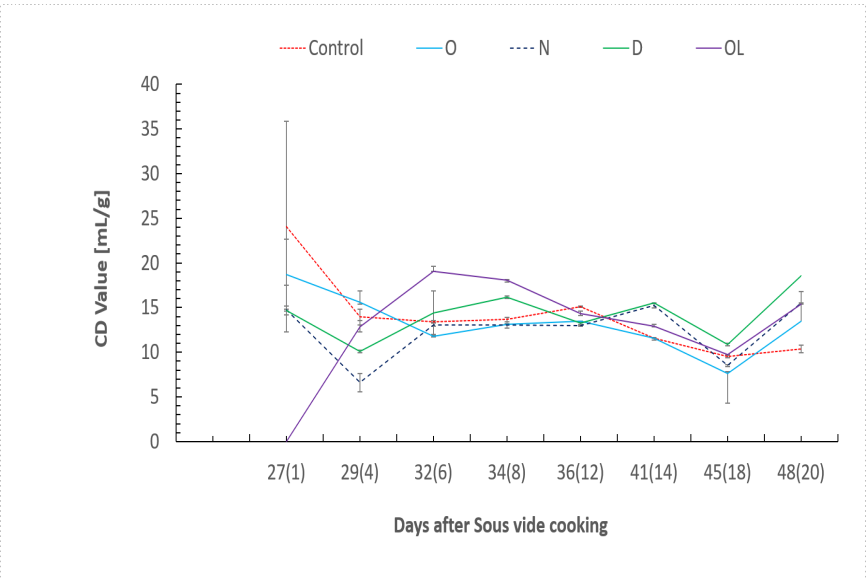

Figure 3.3: The conjugated dienes value [mL/g] of all four antioxidant treated herring samples as function of storage days after sous-vide cooking. Control are herring fillets subjected to sous-vide cooking without prior treatment with antioxidants (red). Oregano (sky blue), Nettle (deep blue), Dill (green) and Olive Leaf (purple) are antioxidant treated fillets which are then sous-vide cooked. The x-axis represents the number of days after sous- vide cooking (day 27 to day 48). The y-axis shows the values of conjugated dienes in milliliter per gram lipid [mL/g]. The values are given as average ± standard deviation, n = 2.

3.2.3 Conjugated tetraenes (CTe)

The CTe values varied from 1.29 to 25.48 mL/g (Figure 3.4) and the raw sample initial values were 14.5 ±0.175 ml/g. The Control values were almost stable from 23.6 in start of storage till the end to 25.48 mL/g. All the samples treated with antioxidants showed lower CTe values throughout storage period as compared to the Control. All the antioxidant treated samples showed insignificant (< 0.05) variations in CTe values except N (day 27),O (day 34), N (36), C,D and OL (day 41), N (day45) and N (day48) through out the storage period.

Oregano gave 18.7 ml/g on the day 27 then its value decreased to the lowest value of 5.5 ml/g on the day 32 and then with some fluctuations it reached the same point as dill (around 18 ml/g). As compared to all other antioxidants, oregano showed a sharp pattern with highest and lowest values as compared to all other antioxidants throughout the period.

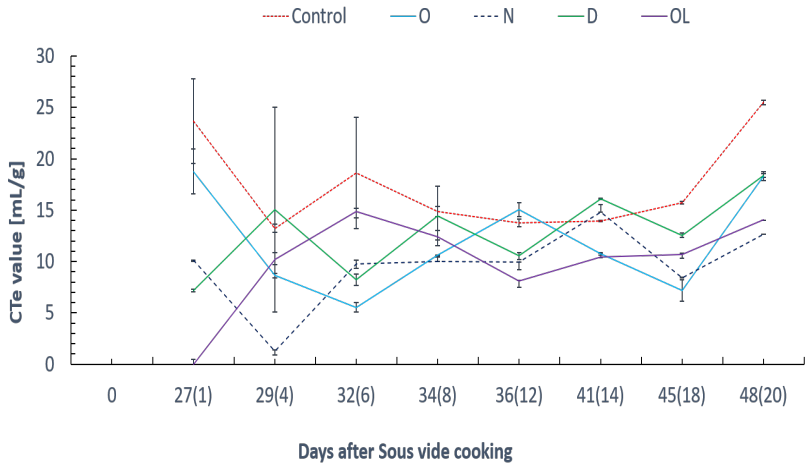

Figure 3.4: The conjugated tetraenes calculated after of storage or sous-vide cooked herring. X-axis represents the days after sous-vide and antioxidant treatment, y-axis represents the values of CTe expressed in mL/g. Control (sous-vide cooked), O= Oregano, N= Nettle, D=Dill, OL=olive leaf treated, and sous-vide cooked herring. All values are expressed in average ± SD

3.3 Secondary Oxidation Products

For determination of secondary oxidation products samples were analyzed for thiobarbituric acid reactive substances which mainly indicate the total aldehydes produced during secondary lipid oxidation. TBARS in chloroform phase were determined.

3.3.1 TBARS

TBARS values are given as μMol TBARS/g lipid in herring samples. The values of Raw initial was 69.55 ±13.4 μMol TBARS/g lipid (0.07 mmol/g) as shown in Figure 3.5. The trend of TBARS values of all samples and the control were similar till the middle of the storage period but later on it showed an increase in all, samples and the control. Control values remained higher than all other antioxidants throughout the storage period. Initially from day 27 to day 34, C gave lower values, all lying below 400 μmol TBARS/g lipids, it was then followed by a sudden increase in values to 869.8 and ending in 1377.4 towards the end of storage period. None of the TBARS values obtained were significant (>0.05).

The sample oregano followed the same trend as control till the middle of period then it showed a sharp peak on day 36 with a value of 841.14 μmol TBARS/g lipids, higher than control and then giving the lowest value (49.37) of all on day 46. There was no significant difference in the values except that olive leaf gave the lowest values till day 48, on the day 48 OL gave a higher value above 800 μmol TBARS/g lipids.

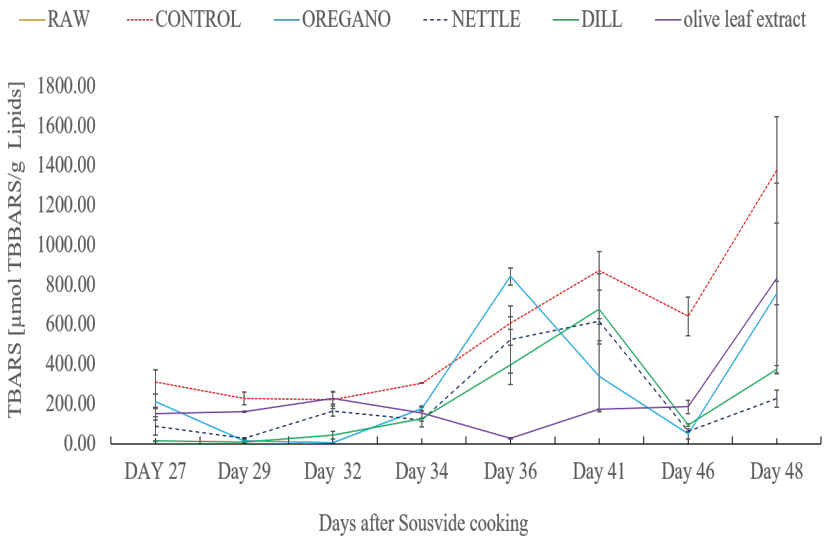

Figure 3.5: TBARS values of sous vide cooked herring (μ Mol TBARS/g lipid) subjected to storage from day 27 to 48. Raw represent raw herring samples (orange). Control are sous vide cooked herring fillets without prior treatment with antioxidants (red). O (blue), N (deep blue), D (green) and OL (purple) are herring treated with antioxidants. The x-axis shows the number of days after sous-vide cooking (day 27 to day 48, the sampling days). The y-axis indicates the value of TBARS in μmoles per gram lipid [μ Mol TBARS/g lipid]. The values are given as average value ± standard deviation (n = 2).

Table 3.1: IC_{50} and PG equivalence value of Dill and Oregano by DPPH assay

Antioxidant	IC_{50}	PG equivalence value
Dill	0.37	9.56
Oregano	0.88	26.02

Table 3.2: PG equivalence value of Dill and Oregano obtained by ABTS ASSAY

Antioxidant	PG equivalence value
Dill	36.97
Oregano	383

3.4 Antioxidant Activity Analysis

Antioxidant activity is analyzed by the different assays, DPPH, ABTS and FCA. The two antioxidant extracts were used, dill extract (2%) and oregano extract (2%). At first the standard curve was calibrated using a spectrophotometer and then the PG equivalence was calculated.

3.4.1 DPPH Assay

The radical scavenging activity of the antioxidants was determined through DPPH assay Standard curve with varying concentrations of PG was obtained (see Appendix E, Figure E.1) and then IC_{50} and PG equivalence was calculated using dill and oregano extracts as shown in Table 3.1.

3.4.2 ABTS Assay

The standard curve in the ABTS assay was obtained (see Appendix E, Figure E.2). The standard linear curve was obtained by plotting absorbance values obtained against standard concentrations of propyl gallate. The PG concentrations 0 - 50μM showed the corresponding absorbances ranging from 0.707 to 0.29. The two antioxidants were analyzed for their PG equivalence and values were found as shown in Table 3.2.

3.4.3 Folin-Ciocalteu Assay

The FCA was performed to determine the total phenolic content of the antioxidant extracts. The standard curve was obtained using absorbance obtained for different concentrations of propyl gallate solutions in a spectrophotometer (can be seen in Appendix E, Figure E.3). The PG equivalence values of both the extracts were calculated as shown in Table 3.3.

In all three assays the PG equivalence of the oregano extract was considerably higher than the dill extract. The PG equivalence of oregano in DPPH was 26.016, in ABTS it was 383 and in FCA it was 0.577 while dill PG equivalence values were

Table 3.3: PG equivalence value obtained using FCA

Antioxidant	PG equivalence value
Dill	0.034
Oregano	0.577

9.5, 36.93, 0.034 respectively. The sample (antioxidant in this case) concentration which is required to convert half of the DPPH radicals to stable molecules is IC_{50}. So IC_{50} of dill and oregano extract obtained through DPPH assay 0.36 and 0.87 respectively.

3.5 Protein Solubility

In the present study the content of water soluble proteins was analyzed in the frozen herring samples.

3.5.1 Water Soluble Proteins Content

The three samples from the day 27, 36 and 48 which showed distinct results in lipid oxidation analysis were selected, so that the these could be investigated further for protein solubility and oxidation. Two extracts were made from each sample and the content of sarcoplasmic proteins was determined by extracting the water soluble proteins in 0.05 phosphate buffer and determining the protein content in the extract. All the analysis was done in three replicates and the results for protein solubility/sarcoplasmic (water soluble) proteins content were expressed in % weight in the mean value ± standard deviation.

The results showed that the Raw herring had the highest content of water soluble proteins (1.8%) compared to other frozen stored samples. The values of the other samples ranged from 0.2% to 0.7% on storage days 27, day 36 and day 48.

The water-soluble protein content gradually increased in the Control (without antioxidants) with storage time, as it can be seen from the Figure 3.6, it was 0.2 % on the day 27, rose a little to 0.3%, on the day 36 and was doubled to 0.7% on the day 48. A little variation was observed among the rest of the samples which were all treated with the respective antioxidants, such as nettle extract, dill extract, oregano extract and olive leaf extract. There was a minor decrease of 0.02% in nettle extract on day 36 while oregano, olive leaf and dill increased with storage time. All the values didnot vary much on the day 48 , that is between 0.6-0.7% so, no noticeable variation could be found among the different antioxidant samples except the thing that these were all much lower compared to raw sample. All the values were significant except C, O, OL (day 27) C, D (day 36) and D(day 48) (see Appendix F).

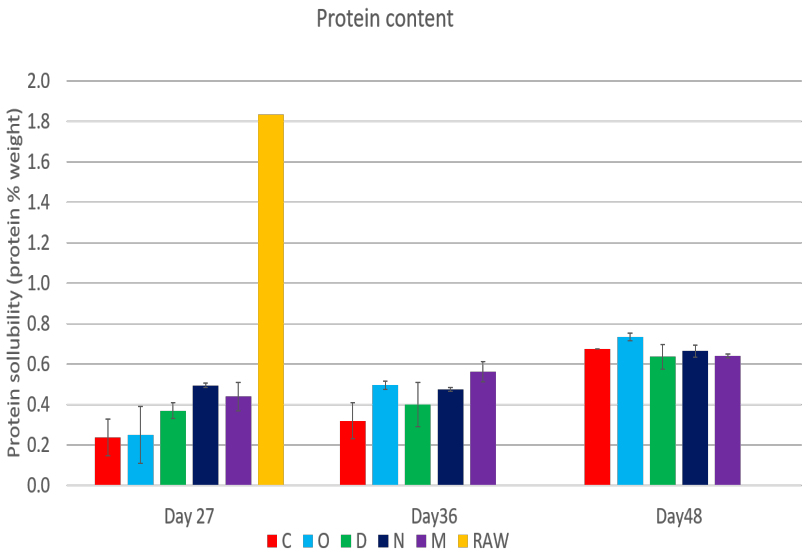

Figure 3.6: Water soluble proteins in % of wet weight of herring in samples Raw initial, control C (without antioxidants), herring treated with O (oregano extract), N (nettle extract), D (dill extract), OL (olive leaf extract) from samples collected on days 27, 36 and 48 of sous vide cooking. Error bars indicate SD (n=2).

3.6 Protein Oxidation

The protein oxidation can be quantified by the carbonyls or disulphide groups. In the present study, the thiols were determined.

3.6.1 Quantification of Thiols

Analysis of the thiol content of water-soluble proteins was performed to determine the protein oxidation in herring samples. Thiol content is an indicator of protein oxidation because the amino acids containing the sulphur (cysteine and methionine) are the ones that are most vulnerable to the protein oxidation [141]. Protein oxidation causes the formation of cross linkages which are characterized by disulphide bond formation and loss of thiol groups [69]. The loss of thiol groups is used as a marker to determine the extent of protein oxidation [62]. A decrease in thiol content indicate the increased oxidation in the samples. The analysis was performed in parallels with three replicates and the results for thiol content are shown in Figure 3.7 (The dill values from day 36 are missing because the amount of sample was not enough). All the values obtained were significant (<0.05) except Dill (day 27). The results are expressed in μmole per milligram (μmol/mg) of proteins.

The value of Raw initial was 0.079 μmol/mg and the remaining samples ranged from 0 to 0.508 μmol/mg (Figure 3.7). Dill from the day 36 was -0.022 which is not correct. The results show that the Control and dill values were max-

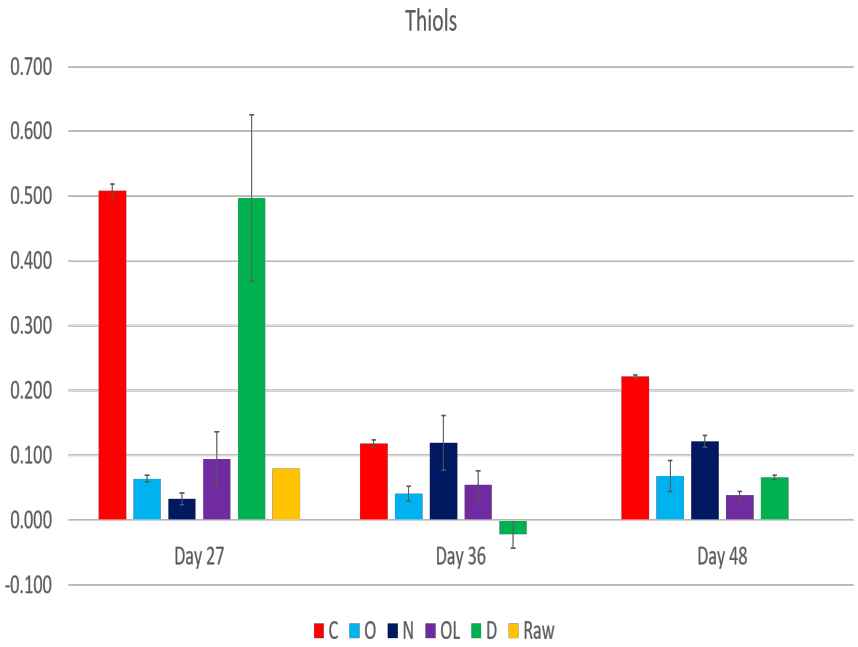

Figure 3.7: Thiol content of water- soluble proteins of herring frozen stored for 2 months at -20°C expressed in μmol thiol/mg

imum on the day 27 (0.508 & 0.497 μmol/mg respectively). The values of Control were decreased to about less than half of the day 27 values (0.222 μmol/mg), on the day 48. The nettle followed the increasing pattern while olive leaf extract showed the decreasing trend. The oregano showed the decrease in thiols but the decrease was more pronounced on day 36. There was a large difference in dill values from day 27 and day 48 (0.497 to 0.066 μmol/mg).

Chapter 4

Discussion

The lipid content in herring fillets in this study upon frozen storage for 1 month was found to be 21-35% (as shown in Figure 3.1) and most of the values were lying in the range of 25-30%. These results are in accordance with previous studies. Romotowska et al. (2016) [43] stated that lipid content in herring and other pelagic fish varies with season. Lipid content in Atlantic mackerel varies from 10-15% in June and 25-30% in September [43]. The average lipid content of Norwegian spring spawning herring caught in June, July was reported by Slotte in 1996 was 26 to 30% which are exactly similar to the results obtained in this study. Total lipid content in mackerel was 20.5 ± 2.7g/100g following the frozen storage at -25°C [77] which is also in accordance with the study. There was no significant effect on lipid content of frozen minced herring fillets which is in agreement with [142]. However, the small variations observed during the storage period might be attributed to the differences in size of the fillets [143] or individual variations.

Peroxide value, Conjugated dienes, trienes and TBARS were measured to determine the oxidation stability of the herring during whole period of frozen storage. In this study the aim was to find out which changes take place during the frozen storage of sous-vide cooked Atlantic herring. In the Primary oxidation step production of hydroperoxides takesplace which are determined by PV value. This is a secondary stage analysis on the sous vide cooked herring fillets after a frozen storage for 1-2 months at -20°C. The peak PV obtained in initial analysis done by Vu [129] was 36 meqO_2/kg lipid (during first month of frozen storage at -20°C) while the peak values obtained in this study (after 2-3 months of frozen storage at -20°C) are 77.7 meqO_2/kg lipid. The results obtained in this study showed a large ($>$0.05) increase in the degree of oxidation compared to the results obtained by Vu, (2020) [129] from the initial stage analysis of the same sous-vide treated herring fillets analysed fresh. The increased oxidation can be because this study was conducted on minced samples which were frozen and remained stored at -20°C further during the whole analysis procedure. So, the cell membranes were ruptured during mincing and during storage the pro-oxidants might have leaked out leading to the increased lipid oxidation. Herring muscle is rich in heme proteins,

so mincing might have denatured heme proteins and released free iron. Both the free iron and the heme group promote lipid oxidation [142]. Similar results with an increase in lipid peroxide values in herring and mackerel during frozen storage were reported by Cropotova and Rustad (2020) [144].

The results obtained in this study showed that the PV of all the samples treated with the antioxidants was lower than control (Figure 3.2) indicating that antioxidants used in this study exerted their effect in slowing down the process of primary oxidation in herring lipids. All the samples showed one common trend that they reach a peak and then enter a decline phase before starting a new ascend (Figure 3.2). A similar trend was seen in the findings of [145–147]. Initial rise in PV values may refer to the ongoing primary oxidation which is occurring at different rates in different samples. Then the peroxides number is reduced causing the fall in PV because there is an onset of secondary oxidation and the peroxides from primary oxidation are decomposed to form the secondary oxidation products [146].

The samples which have the PV value under 10 meqO_2/kg oil were considered acceptable [148]. On the day 27 only dill showed a value (3.89) lower than 5 meqO_2/kg lipid but almost all other samples showed values higher than this, so the samples which had higher peroxide content above 10 meqO_2/kg oil were considered rancid. Nettle from day 41 also showed a value 0.07 after reaching to a peak value of 35.34 meqO_2/kg lipid. Nettle showed a gradual consistent effect on lipid peroxides. The antioxidant activity of Nettle is due to the content of quercetin present in it. Quercetin is an antioxidant but also acts as a pro-oxidant [95, 149]. In the beginning dill was much effective and kept the peroxide content to a low level but a little instability was also seen with dill (Figure 3.2). The water and alcohol extracts of dill have high antioxidant activity against linoleic acid oxidation [150]. The antioxidant activity of dill is due to the presence of flavonol, flavonoids and phenols. The PV of the Raw initial was lower than others, this was in accordance with the findings of Cropotova [23]. It was reported that the higher values of sous vide cooked samples were due to the effect of heating and longtime cooking which increased the rate of lipid oxidation.

The CDs are also markers of primary lipid oxidation, these are produced during the formation of peroxides. The methylene interrupted double bonds are converted to conjugated double bonds which can absorb UV in 233 nm and 315 nm. Absorbance is directly related to the concentration of CDs. The CDs and CTe values indicated that there was no significant difference in the values of raw and the treated samples. The control CD values (on the day 27) and CTe values, in all days, were comparatively higher, nettle showed slightly low values of both CDs and CTe compared to other antioxidants (except day 41 samples) but overall, all the values were in the same range (Figure 3.3). These results are similar to earlier findings. Standal et al., (2018) [142] reported similar results indicating that there

were no significant changes found in the conjugated systems during the chilled and frozen storage of mackerel fillets.

TBARS are the markers for secondary oxidation products in lipid oxidation. All the samples showed values insignificantly higher(> 0.05) than the acceptable limit (2 μmol TBARS/g lipids) given by de Oliveira et al(2016) [151], so all treated and untreated samples (raw) following a frozen storage (-20°C) of two months were considered rancid. The increase in time and temperature of sous-vide cooking can increase the TBARS content [23]. The TBARS values increased with storage time, this is in accordance with the results obtained in previous studies. Similar results with TBARS were found in the dark muscle of pelagic fish after storage [152]. As far as the effect of antioxidants is concerned, it can be seen in the Figure 3.5 that the TBARS values increased with time but values in the samples treated with antioxidants were lower as compared to Control (without antioxidants). Similar results were observed by [78] while studying the antioxidant effects of dill extract obtained by microwave oven boiling (MOB). Dill extract increased the stability of refrigerated mackerel fillets and the treated fillets showed reduced values compared to the control. It was further suggested that antioxidant activity of dill was due to the presence of phenolic compounds [78]. The nettle extract gave reduced levels of TBARS till day 34 then it started to increase. Ahmadi et al. (2014) [100] found that nettle delayed lipid oxidation in first 8 days and then the TBARS gradually increased in kilka fish samples during superchilled storage (at -2°C) of 28 days so, the trend was exactly in accordance with the TBARS results obtained with nettle in this study. Latoch and Stasiak (2015) [94] observed that after a cold storage of 14 days, the TBARS in nettle treated meat sausages, was reduced. The olive leaf extracts showed the reduction in TBARS for a relatively longer period of time (till the day 46 of frozen storage), thus proving its high efficiency in retarding lipid oxidation. This was in consistence with the results obtained by Lins et al. (2018) [103]. The antioxidant activity of olive leaf extracts is related to the high phenolic content as investigated by Nicoli et al., (2019) [109] evaluation, in 15 leaf extracts of Italian olive cultivars. Djenane et al [107] observed the similar results with 5% OLE in halal minced beef during refrigerated storage. Vogel et al., (2015) [104] suggested that the phenolic oleuropein in particular have a strong antioxidant activity and these show radical scavenging capacity superior to the vitamin C and E.

The stable low values of the TBARS in initial days of the storage period (from day 27 to day 34) can be explained as, the rate of decomposition of the peroxides is equal to the rate of formation of secondary oxidation products. Increase in values after the middle of the period, can be attributed to the accelerated secondary oxidation accompanied by production of increased number of secondary oxidation products. Besides the accelerated oxidation might be due to the activity of prooxidants that are exposed in minced meat [153]. The peak value recorded by Vu in initial stage analysis was 13.15 μMol/g lipids [129]. All the samples in this

study showed high values of TBARS compared to TBARS values obtained in initial stage analysis before frozen storage. The initial value of sample Raw, obtained initially was 20 μ Mol/g lipids and in this study (after 2 months frozen storage) Raw value was increased to 69 μMol/g lipids. These higher TBARS could be attributed to the prolonged storage of the frozen samples. Moreover, the rate of lipid oxidation increases with the storage duration, which was seen as increased PV, CDs and TBARS during chilled storage of Atlantic mackerel [23].

The fillets treated with antioxidants including Oregano showed lower TBARS values as compared to Control in the first half of frozen storage period (Figure 3.5). This is in agreement with findings of [90] where similar results were observed with oregano application on frozen storage of black wilde beest muscle. Similar observations were made in earlier studies [91, 154] Oregano showed a higher potential among all the other antioxidants. Antioxidant activity of oregano is due to the content of thymol and carvacrol which scavenge free radicals and convert these into stable products. Moreover, long time heating did not have any noticeable effect on the phenolic content of oregano [92]. This may be the reason that it exhibited more potent results as compared to all other antioxidants during the period of frozen storage. Furthermore, the instabilities found during the second half of the period might be due to the pro-oxidants like iron and heme proteins which can speed up the reaction by reacting with free radicals.

Antioxidant assays DPPH, ABTS and FCA were performed to assess the radical scavenging activity of two antioxidant extracts dill and oregano. Numerous studies have been conducted involving these assays for the evaluation of antioxidant activity of the synthetic antioxidants as well as natural extracts [24, 78, 81, 82]. When the DPPH radical is reduced by the antioxidant, a loss of colour in the absorbance spectrum occurs. This loss of colour is detected by electron spin resonance (ESR) or by measure of absorbance at 515 nm [111, 155]. The linear curve using absorbance values and antioxidant concentration is made. The slope of the linear curve gives the reducing power of the antioxidant. The results obtained are expressed in terms of gallate equivalence. Gallic acid is used as a reference because of its high phenol content and because its antioxidant activity is well documented [111, 135]. The antioxidant activity of plant based natural phenolics, (such as dill and oregano) is mainly attributed to the content of phenols. The Folin Ciocalteau method has been used to determine total phenol content in the natural products. The gallic acid is recommended as standard to obtain reliable results in FC assay [135]. Some studies have been carried out utilizing the ABTS assays to evaluate other natural herbs for their antioxidant potential [83, 96]. The ability of phenolic compounds to scavenge ABTS radical is affected by the position and number of hydroxyl groups [156].

The results obtained with the oregano extract showed a higher antioxidant activity in terms of PG equivalence as compared to dill extract and IC_50 in all

the three assays (Table 3.2, Table 3.1, Table 3.3). The results obtained in this study are in accordance with the previous studies [87–89, 91, 157]. When 3000 mg/kg oregano was added to the soybean oil, it slowed down the process of oxidation and showed maximum antioxidant activity [89]. In another study meat was treated with 1% oregano extract, it increased the shelf life by 3 days following the aerobic storage [90]. The oregano extract was found to contain the highest phenols in it. The strong antioxidant activity of oregano extract might be attributed mainly to thymol and carvacrol which are the major constituents of oregano oil [154]. Antioxidant potential varies with the different concentrations of thymol and carvacrol. Different factors like climate, soil composition, harvest season and method of cultivation causes variability in chemical composition and biological properties of the natural antioxidants [158, 159]. The phenolic content of the extract varies depending upon the solvent used for its extraction for instance polar solvents will facilitate the extraction of phenols from the raw source [160]. Antioxidant activity of the dill extract have been investigated in numerous studies but in this study the dill could not gave much effective results in all the assays, a possible explanation could be the concentration of active compounds that might got effected during the extraction, processing or during the assays [161]. Another possibility could be that the selected concentration of dill extract might not be suitable enough to reflect its maximum activity in the assays. Kannaiyan et el., [78] reported that dill extract showed the highest radical scavenging activity in DPPH assay when its concentration was increased from 10% to 20%. So, 20% concentration of dill extract with a higher phenolic content, was considered ideal for the dip treatment of fish fillets. When antioxidant activities of Dill leaf extract in water, ethanol and acetone was studied in different assays it was found that water extract showed best antioxidant activity [81].

Besides there are some documented limitations in these assays for instance the 6 minutes incubation might be a hindrance in correctly evaluating the activity of those compounds which react comparatively slow to reach an end point of a reaction [111]. So ABTS assay cannot accurately perform the quantitative estimation but be used to rank different compounds according to their activities [111]. The results in the present study suggested that oregano can be regarded as 10 times more potent than dill with respect to the PG equivalence obtained through ABTS assay (Table 3.2).

The changes in protein solubility is an indicator of protein denaturation [162]. The protein solubility is commonly used to determine the quality of myofibrillar proteins during frozen storage [127]. Fish proteins are denatured during frozen storage [31]. This results in loss of functionality and hence the quality of muscle foods is affected as well [163]. Normally the three-dimensional structure of proteins is maintained by the hydrogen bonding. During frozen storage as water is removed because of crystallization, the hydrogen bonding is broken and the hydrophobic regions are exposed on the amino acid surfaces, so the nonpolar

amino acids interact and aggregate together. This protein aggregation results in decreased protein solubility [164]. Besides hydrophobic interactions and hydrogen bonding, the disulphide bond formation also contributes to the protein aggregation [127]. Moreover, protein oxidation in meat also causes changes in conformation, solubility and hydrophobicity [165], resulting in decreased digestibility and nutritional value of meat proteins [166].

Protein solubility is important for functional properties of fish like gelation, emulsification and foaming [167]. Freezing denatures the proteins [168] decreasing the solubility. The results obtained in the present study showed that almost all the cooked samples treated with antioxidants gave the significant lower solubility values(<0.05) compared to Raw, indicating that frozen storage caused protein denaturation. Gokoglu et al. (2018) [169] reported that the significant differences in protein solubility were found in the squid, mussels before and after, a month frozen storage at -18°C. The protein solubility is usually decreased with duration of frozen storage, longer the storage lower is the protein solubility but in the present study the protein solubility is lower on the day 27, only a little rise was seen by the end of the period (Figure 3.6). The small variations in values can be due to the lower sarcoplasmic protein content in fillets as explained by Tejada [170]. The small changes can be associated with the antioxidants which exhibited a their effect on the proteins and protected them from denaturation during freezing or it might be due to the reason that water soluble proteins are not much affected by the frozen storage as compared to the microfibrillar proteins [171].

The results are supported by Farouk et al., (2004) [172] who observed an increase in the solubility of sarcoplasmic proteins in beef after freezing. The storage temperature and time have a great impact on the protein denaturation, higher effects were reported at a temperature of -10°C to -20°C than at -30°C [173]. The results can be compared to the observations made by Cropotova et al., (2019) [23] where a decrease in sarcoplasmic protein solubility was reported in Atlantic mackerel following a frozen storage of 12 months.

Protein oxidation is initiated by the metal ions [174], lipid oxidation products [69, 152] or directly by ROS or reactive nitrogen species [62]. When proteins are exposed to radicals in the presence of oxygen, protein oxidation is initiated causing changes in the protein chemistry, structure and functionality. The functional groups in the backbone and side chain are more prone to ROS [69]. The main consequences of protein oxidation are peptide bond cleavage, amino acid side chain modification and formation of cross linkages. The cross linkages lead to the formation of disulphide bonds [69]. The results obtained in this study showed that the values thiols in Control were high on day 27 but the values decreased during storage (Figure 3.7). Similarly, dill extract showed a large reduction on the day 48. It can be said that the thiol values of all the samples were significantly reduced (<0.05) along the duration of frozen storage with small variations in values indic-

ating that all the samples were highly oxidized except dill and control samples on day 27. The decrease in -SH content could be linked to the formation of disulphide bonds due to oxidation of SH groups. The results were in accordance with the previous findings in Pacific whiting, where thiol content was increased on initial days then it continued to decrease for rest of the period during frozen storage [175, 176] . Eymard et al., (2009) [53] found the similar results with washed minced of horse mackerel after cold storage. It was proposed that the protein oxidation is sensitive to the conformation of proteins for instance the randomly coiled proteins are more susceptible to the oxidation as compared to globular protein structure. According to Eymard et al., (2009) [53] the oxidation is affected by the nature of protein, accessibility, conformation and exposure to prooxidants. The ice crystals formed during frozen storage release prooxidants in the sample. The protein denaturation also promotes the loss of thiol group [174]. In addition, the frozen lipid samples were also highly oxidized so the secondary oxidation products like aldehydes might be involved in initiating or promoting the protein oxidation and loss of thiol groups as explained by Hematyar et al., (2019) [69].

Moreover, comparing the results for protein solubility and thiols, it can be seen that there is a significant (<0.05) reduction in solubility of the samples and at the same time there is an increased protein oxidation. This tendency is in agreement with the earlier findings which proposed that protein hydrophobicity and solubility are directly dependent on protein oxidation [62], when side chains are oxidized the carbonyl groups are formed. Carbonylation causes loss of amino groups so the charges on the proteins are changed. The oppositely charged amino acids attract each other, thus the proteins aggregate and the solubility decreases [177]. So, the higher is the protein oxidation, lower is the protein solubility.

Chapter 5

Conclusion

This study showed different effects of plant-based antioxidants on lipid and protein stability during frozen storage of sousvide cooked Atlantic herring at -20°C. The preservative effects of oregano, nettle, dill and olive leaf extract were studied. Among all antioxidants, nettle and oregano extracts were found to be more effective than dill and olive leaf extract. The antioxidants did not effectively reduce the values during the period to an acceptable limit and all the samples were considered rancid. The changes produced in herring lipids were determined by the primary and secondary analysis and, in proteins by analyzing protein solubility and thiol content. Peroxide value (PV), conjugated dienes (CDs), conjugated tetraenes (CTe) and TBARS were performed to determine the status of primary and secondary oxidation in frozen herring samples. The lipid content ranged between 21-35% and no significant difference in the lipid content of herring samples was found during storage.

The PV values in all the frozen samples were measured using iodometric titration method. PV values were in the range of 0.07 to 77.7 meqO_2/Kg lipid. The reduced peroxide content in samples treated with Nettle,oregano and dill extracts indicated their effects as antioxidants.

TBARS in all the antioxidants treated samples were initially in a similar range and all the values showed an increase towards the end of the period. The TBARS ranged from 46.4 to 1377 μmol TBARS/g lipids. No significant differences were found ($>$0.05). All the antioxidants Nettle, oregano, olive leaf and dill showed values lower than control indicating effect as antioxidants. Olive leaf showed a prolonged effect and oregano showed lowest values in the first half of the period.

The CDs values ranged from 6.6 to 24.06 mL/g. No significant changes were found. Nettle and oregano gave the low values in CDs and Cte. The CTe values varied from 1.29-25.5 ml/g.

The results of primary and secondary oxidation indicate a large increase in the degree of oxidation during frozen storage compared to the initial stage analysis (performed on the fresh samples). Additionally the antioxidant activity analysis was performed to analyze the activity of the antioxidant extracts. In all three antioxidant assays oregano was proved more potent antioxidant than dill and its PG

equivalence was considerably higher than dill. In DPPH, ABTS and FCA, Oregano gave 26.02, 383 and 0.58 values respectively whereas the values of dill was much lower (9.56, 36.9 and 0.034) in all three assays.

To determine the effects on protein solubility and oxidation, the fillets were analyzed after frozen storage for 5 months and the sarcoplasmic protein solubility and thiol content were analyzed in sous-vide cooked treated herring. The content of water soluble proteins in all the samples were significantly lower compared to raw samples and were in a range of 0.2-0.7%. Protein oxidation was quantified by analysis thiols. Thiol values ranged from 0 to 0.5 μmol/mg. Nettle showed an increase in thiol content during frozen storage.

Among all the antioxidants nettle and oregano were found more effective but even that effect was not so pronounced because the samples showed the values of PV and TBARS that was above the acceptable limits for lipid oxidation. The samples were in the secondary oxidation stage as indicated by the results of Lipid oxidation analysis. Proteins solubility was reduced and thiol content indicated that protein oxidation had also taken place. Oregano and nettle can be effective in increasing the oxidative stability in partial cooked ready to eat fish products and further investigations are needed to explore the significance.

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Appendix A

Total Lipid Count

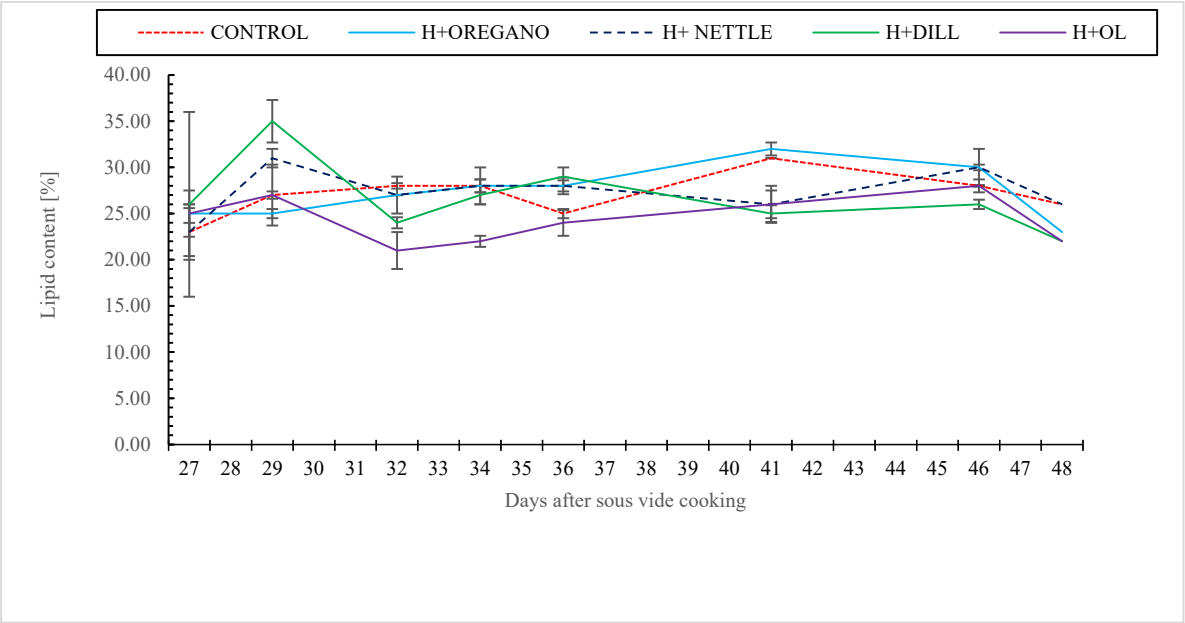

Figure A.1: The total lipid content [%] of herring samples as function of storage days after sous-vide cooking. Control are sous-vide cooked herring without any antioxidants (red). Oregano (sky blue), Nettle (deep blue), Dill (green) and Olive Leaf (purple) are antioxidant treated sous vide cooked herring fillets. The x-axis shows the number of days after sous-vide cooking (day 27 to day 48, the sampling days at intervals). The y-axis shows the percentage of lipid content of mass of herring fillets [%]. The values are given as mean value with error bars representing standard deviation.

Data collected for the Total Lipid content of sous vide cooked frozen stored herring on day 27, 29, 32, 34, 36, 41, 46 and 48 after sous vide cooking is shown in the table A & B. The results are collected by using the equation (1). Each sample had two parallels.

Table A.1: Data for the determination of total lipid content (%)

Samples	26-Sep-19	Tube + lid weight (g	Tube + Chloroform extract sample wt (g	Chloroform extract sample wt (g)	Tube + lipid sample wt (g) after evaporation	mg/g extract	% Total lipids	Wt of lipid sample (g) / mL chloroform
Raw (1)	Day:27th	10.7185	13.3136	2.5951	10.8367	45.55	23.64	0.0591
Raw (2)		10.6622	13.5487	2.8865	10.7878	43.51	25.12	0.0628
C-1 (1)		10.3575	13.0229	2.6654	10.4649	40.29	21.48	0.0537
C-1 (2)		10.5665	13.4479	2.8814	10.6890	42.51	24.50	0.0613
O-1 (1)		11.3948	14.1375	2.7427	11.5087	41.53	22.78	0.0569
O-1-(2)		11.5396	14.4592	2.9196	11.6725	45.52	26.58	0.0664
N-1 (1)		10.4483	13.0464	2.5981	10.5631	44.19	22.96	0.0574
N-1 (2)		10.7499	13.7062	2.9563	10.8672	39.68	23.46	0.0587
D-1 (1)		10.5523	13.1024	2.5501	10.6795	49.88	25.44	0.0636
D-1 (2)		11.3288	14.2254	2.8966	11.4578	44.53	25.80	0.0645
M-1 (1)		10.6408	13.2365	2.5957	10.7660	48.23	25.04	0.0626
M-1 (2)		10.7550	13.6519	2.8969	10.8814	43.63	25.28	0.0632
C-4 (1)	27-Sep-19	10.7586	13.4223	2.6637	10.8790	45.20	24.08	0.0602
C-4 (2)	Day:29th	10.6257	13.5531	2.9274	10.7757	51.24	30.00	0.0750
O-4 (1)		10.5977	13.4991	2.9014	10.7179	41.43	24.04	0.0601
O-4-(2)		11.2601	14.2650	3.0049	11.3908	43.50	26.14	0.0654
N-4 (1)		10.5480	13.2171	2.6691	10.7177	63.58	33.94	0.0849
N-4(2)		10.3852	13.8669	3.4800	10.5283	41.12	28.62	0.0716
D-4 (1)		10.6351	13.1243	2.4892	10.7578	49.29	24.54	0.0614
D-4 (2)		10.6659	13.2350	2.5691	10.8920	88.01	45.22	0.1130
M-4 (1)		11.0708	13.7606	2.6800	11.1955	46.53	24.94	0.0623
M-4 (2)		9.9479	12.8043	2.8556	10.0973	52.32	29.88	0.0747
C-6 (1)	30-Sep.19	10.7468	13.3872	2.6404	10.8711	47.08	24.86	0.0621
C-6 (2)	Day: 32	10.5723	13.3957	2.8234	10.7293	55.61	31.40	0.0785
O-6 (1)		10.6614	13.3364	2.6750	10.7975	50.88	27.22	0.0680
O-6 (2)		13.2977	16.2124	2.9147	13.4292	45.12	26.30	0.0657
N-6 (1)		10.5315	13.2292	2.6977	10.6712	51.78	27.94	0.0699
N-6 (2)		10.5581	13.4919	2.9338	10.6858	43.53	25.54	0.0639
D-6 (1)		10.7512	13.4543	2.7031	10.8605	40.44	21.86	0.0546
D-6 (2)		10.5715	13.3444	2.7729	10.7036	47.64	26.42	0.0660
M-6 (1)		10.1161	12.7112	2.5951	10.2195	39.84	20.68	0.0517
M-6 (2)		10.3368	13.1185	2.7817	10.4447	38.79	21.58	0.0539
C-8 (1)	2-Oct-19	10.2529	13.0176	2.7647	10.3921	50.35	27.84	0.0696
C-8 (2)	Day:34	10.5598	13.5549	2.9950	10.7017	47.38	28.38	0.0710
O-8 (1)		10.6647	13.2890	2.6200	10.7927	48.85	25.60	0.0640
O-8 (2)		10.5587	13.3675	2.8088	10.7099	53.83	30.24	0.0756
N-8 (1)		10.6584	13.4493	2.7909	10.8000	50.74	28.32	0.0708
N-8 (2)		10.6685	13.4831	2.8150	10.8108	50.55	28.46	0.0712
D-8 (1)		10.5651	13.3820	2.8200	10.6954	46.21	26.06	0.0652
D-8 (2)		10.6224	13.5982	2.9760	10.7585	45.73	27.22	0.0680
M-8 (1)		10.6335	13.3297	2.6960	10.7312	36.24	19.54	0.0488
M-8 (2)		12.5293	15.3352	2.8000	12.6466	41.89	23.46	0.0587
C-12 (1)	Day:36	9.9281	12.6961	2.7680	10.0516	44.62	24.70	0.0618
C-12 (2)	4-Oct-19	13.1607	16.1263	2.9600	13.2917	44.26	26.20	0.0655
O-12 (1)		10.6646	13.3204	2.6558	10.7936	48.57	25.80	0.0645
O-12 (2)		10.6203	13.3881	2.7678	10.7695	53.91	29.84	0.0746
N-12 (1)		11.3944	14.0027	2.6083	11.5372	54.75	28.56	0.0714
N-12 (2)		11.6464	14.4627	2.8100	11.7823	48.36	27.18	0.0679
D-12 (1)		11.3595	14.0356	2.6761	11.4977	51.64	27.64	0.0691
D-12 (2)		10.2859	13.1824	2.8965	10.4341	51.17	29.64	0.0741
M-12 (1)		11.0434	13.6967	2.6500	11.1615	44.57	23.62	0.0591
M-12 (2)		10.2288	13.0967	2.8679	10.3532	43.38	24.88	0.0622
C-14 (1)	Day:41	9.8617	12.6187	2.7570	10.0147	55.50	30.60	0.0765
C-14 (2)	9-Oct-19	11.2217	13.8450	2.6233	11.3799	60.31	31.64	0.0791
O-14 (1)		10.5231	13.2216	2.6985	10.6860	60.37	32.58	0.0815
O-14 (2)		10.6741	13.6836	3.0095	10.8327	52.70	31.72	0.0793
N-14 (1)		11.6332	14.2496	2.6164	11.7580	47.70	24.96	0.0624
N-14 (2)		10.5171	13.4815	2.9644	10.6512	45.24	26.82	0.0671
D-14 (1)		10.6932	13.2970	2.6038	10.8148	46.70	24.32	0.0608
D-14 (2)		10.4124	13.2675	2.8551	10.5440	46.09	26.32	0.0658
M-14 (1)		10.9964	13.6281	2.6317	11.1217	47.61	25.06	0.0627
M-14 (2)		10.5447	13.4940	2.9493	10.6839	47.20	27.84	0.0696

C-18 (1)	day:46	10.6309	13.2663	2.6354	10.7716	53.39	28.14	0.0703
C-18 (2)		10.5671	13.3742	2.8071	10.7078	50.12	28.14	0.0704
O-18 (1)		10.6307	13.3553	2.7246	10.7779	54.03	29.44	0.0736
O-18 (2)		11.5124	14.4344	2.9220	11.6672	52.98	30.96	0.0774
N-18 (1)		10.5491	13.2101	2.6610	10.6874	51.97	27.66	0.0692
N-18 (2)		10.6262	13.5110	2.8848	10.7875	55.91	32.26	0.0806
D-18 (1)		10.0612	12.7667	2.7055	10.1855	45.94	24.86	0.0621
D-18 (2)		10.5377	13.4182	2.8805	10.6707	46.17	26.60	0.0665
M-18 (1)		10.7312	13.2238	2.4926	10.8657	53.96	26.90	0.0673
M-18 (2)		10.5446	13.4592	2.9146	10.6940	51.26	29.88	0.0747
C-20 (1)	day: 48	10.6347	13.4425	2.8078	10.7625	45.52	25.56	0.0639
C-20 (2)		10.4282	13.4323	3.0041	10.5629	44.84	26.94	0.0674
O-20 (1)		11.2669	13.9627	2.6958	11.3852	43.88	23.66	0.0591
O-20 (2)		10.3499	13.3080	2.9581	10.4653	39.01	23.08	0.0577
N-20 (1)		11.1752	13.7767	2.6015	11.2947	45.94	23.90	0.0598
N-20 (2)		10.6013	13.5183	2.9170	10.7407	47.79	27.88	0.0697
D-20 (1)		10.3135	13.0474	2.7339	10.4194	38.74	21.18	0.0530
D-20 (2)		10.9097	13.8895	2.9798	11.0210	37.35	22.26	0.0557
M-20 (1)		10.6232	13.5917	2.9685	10.7342	37.39	22.20	0.0555
M-20 (2)		10.4393	13.3443	2.9050	10.5518	38.73	22.50	0.0563
repeated								
D1		10.4463	13.2440	2.7977	10.5524	37.92	21.22	0.0530
D2		10.5578	13.4037	2.8459	10.6835	44.17	25.14	0.0629

Table A.2: The average of two parallels are calculated and then total lipid content is determined in percentage with the standard deviation values of each as shown in the table B. The samples after sous-vide cooking period are labelled such as samples from day 27 as 1, day 29th as day 4, day 32 as 6, day 34 as 8, day 36 as12, day 41 as14, day 46 as 18, day 48 as 20.

Sample	Average of sample %	Average weight of lipid sample (g) / mL	SD of Total lipid content
Raw	24.38	0.06	0.74
C-1	22.99	0.06	1.51
O-1	24.68	0.06	1.90
N-1	23.21	0.06	0.25
D-1	25.62	0.06	0.18
M-1	25.16	0.06	0.12
C-4	27.04	0.07	2.96
O-4	25.09	0.06	1.05
N-4	31.28	0.08	2.66
D-4	34.88	0.09	10.34
M-4	27.41	0.07	2.47
C-6	28.13	0.07	3.27
O-6	26.76	0.07	0.46
N-6	26.74	0.07	1.20
D-6	24.14	0.06	2.28
M-6	21.13	0.05	0.45
C-8	28.11	0.07	0.27
O-8	27.92	0.07	2.32
N-8	28.39	0.07	0.07
D-8	26.64	0.07	0.58
M-8	21.50	0.05	1.96
C-12	25.45	0.06	0.75
O-12	27.82	0.07	2.02
N-12	27.87	0.07	0.69
D-12	28.64	0.07	1.00
M-12	24.25	0.06	0.63
C-14	31.12	0.08	0.52
O-14	32.15	0.08	0.61
N-14	25.89	0.06	0.93
D-14	25.32	0.06	1.00
M-14	26.45	0.07	1.39
C-18	28.14	0.07	0.00
O-18	30.20	0.08	0.76
N-18	29.96	0.07	2.30
D-18	25.73	0.06	0.87
M-18	28.39	0.07	1.49
C-20	26.25	0.07	0.69
O-20	23.37	0.06	0.29
N-20	25.89	0.06	1.99
D-20	21.72	0.05	0.54
M-20	22.35	0.06	0.15

Appendix B

Peroxide Value

The raw data obtained for determination of peroxide value of sous vide cooked Atlantic herring after frozen storage on the day 27, 29, 32, 34, 36, 41, 46 and 48 is shown in the Table B.1. The results were calculated according to the equation (2). The values are expressed in meqO_2/kg lipids. The PV values along with the standard deviation are calculated.

Table B.1:

DAY 27(1)	Vol1 tirant(ml)	Titre Conc (M)	Blank	B(ml)	V-B	g/ml lipids	oil sample wt(g)	PV1=(V-B)*C*1000\Sample wt	Average PV	STD DEV
Raw 1	25.581	0.001	0.013	25.568	0.06	0.72	35.51	21.90	19.26	
Raw 2	5.974	0.001	0.013	5.961	0.06	0.72	8.28			
C1	8.695	0.001	0.013	8.682	0.06	0.72	12.06	11.09	1.37	
C2	7.299	0.001	0.013	7.286	0.06	0.72	10.12			
O1	28.789	0.001	0.013	28.776	0.06	0.72	39.97	35.05	6.95	
O2	21.71	0.001	0.013	21.697	0.06	0.72	30.13			
N1	18.621	0.001	0.013	18.608	0.06	0.72	25.84	22.32	4.99	
N2	13.553	0.001	0.025	13.528	0.06	0.72	18.79			
D1	2.804	0.001	0.035	2.769	0.06	0.72	3.85	3.89	0.06	
D2	2.862	0.001	0.035	2.827	0.06	0.72	3.93			
M1	20.625	0.001	0.025	20.6	0.06	0.72	28.61	24.93	5.20	
M2	15.326	0.001	0.025	15.301	0.06	0.72	21.25			
DAY 29(4)										
C1	3.1	0.01	0.026	3.074	0.07	0.84	36.60	40.54	5.58	
C2	3.763	0.01	0.026	3.737	0.07	0.84	44.49			
O1	14.485	0.001	0.015	14.47	0.06	0.72	20.10	15.27	6.82	
O2	7.563	0.001	0.04	7.523	0.06	0.72	10.45			
N1	2.165	0.01	0.015	2.15	0.08	0.96	22.40	24.49	2.97	
N2	2.579	0.01	0.026	2.553	0.08	0.96	26.59			
D1	1.429	0.01	0.026	1.403	0.09	1.08	12.99	11.40	2.25	
D2	1.085	0.01	0.026	1.059	0.09	1.08	9.81			
M1	2.534	0.01	0.026	2.508	0.07	0.84	29.86	30.20	0.48	
M2	2.591	0.01	0.026	2.565	0.07	0.84	30.54			
DAY 32(6)										
C1	3.997	0.01	0.026	3.971	0.07	0.84	47.27	40.48	9.60	
C2	2.856	0.01	0.026	2.83	0.07	0.84	33.69			
O1	0.633	0.01	0.026	0.607	0.07	0.84	7.23	7.61	0.54	
O2	0.697	0.01	0.026	0.671	0.07	0.84	7.99			
N1	2.665	0.01	0.026	2.639	0.07	0.84	31.42	28.77	3.75	
N2	2.22	0.01	0.026	2.194	0.07	0.84	26.12			
D1	5.868	0.001	0.035	5.833	0.06	0.72	8.10	6.17	2.74	
D2	3.083	0.001	0.035	3.048	0.06	0.72	4.23			
M1	7.191	0.001	0.035	7.156	0.05	0.6	11.93	11.63	0.41	
M2	6.839	0.001	0.035	6.804	0.05	0.6	11.34			
DAY 34(8)										
C1	3.911	0.01	0.026	3.885	0.07	0.84	46.25	49.08	4.00	
C2	4.386	0.01	0.026	4.36	0.07	0.84	51.90			
O1	4.217	0.01	0.026	4.191	0.07	0.84	49.89	42.64	10.25	
O2	2.999	0.01	0.026	2.973	0.07	0.84	35.39			
N1	2.599	0.01	0.026	2.573	0.07	0.84	30.63	31.46	1.18	
N2	2.739	0.01	0.026	2.713	0.07	0.84	32.30			
D1	3.173	0.01	0.026	3.147	0.07	0.84	37.46	34.73	3.87	
D2	2.713	0.01	0.026	2.687	0.07	0.84	31.99			
M1	5.383	0.001	0.035	5.348	0.05	0.6	8.91	8.05	1.22	
M2	4.346	0.001	0.035	4.311	0.05	0.6	7.19			
DAY 36(12)										
C1	7.833	0.001	0.018	7.815	0.06	0.72	10.85	9.58	1.81	
C2	5.994	0.001	0.018	5.976	0.06	0.72	8.30			
O1	4.968	0.01	0.015	4.953	0.07	0.84	58.96	54.95	5.68	
O2	4.297	0.01	0.019	4.278	0.07	0.84	50.93			
N1	3.07	0.01	0.019	3.051	0.07	0.84	36.32	35.34	1.39	
N2	2.901	0.01	0.015	2.886	0.07	0.84	34.36			
D1	2.513	0.01	0.015	2.498	0.07	0.84	29.74	28.32	2.00	
D2	2.279	0.01	0.019	2.26	0.07	0.84	26.90			
M1	4.363	0.001	0.018	4.345	0.06	0.72	6.03	6.81	1.09	
M2	5.476	0.001	0.018	5.458	0.06	0.72	7.58			
DAY 41(14)										
C1	4.936	0.01	0.015	4.921	0.08	0.96	51.26	50.87	0.55	
C2	4.865	0.01	0.019	4.846	0.08	0.96	50.48			
O1	3.362	0.01	0.019	3.343	0.08	0.96	34.82	33.65	1.66	
O2	3.137	0.01	0.019	3.118	0.08	0.96	32.48			
N1	0.077	0.001	0.018	0.059	0.06	0.72	0.08	0.07	0.01	
N2	0.064	0.001	0.018	0.046	0.06	0.72	0.06			
D1	9.147	0.001	0.018	9.129	0.06	0.72	12.68	15.03	3.33	
D2	12.536	0.001	0.015	12.521	0.06	0.72	17.39			
M1	3.17	0.01	0.019	3.151	0.07	0.84	37.51	37.02	0.69	
M2	3.088	0.01	0.019	3.069	0.07	0.84	36.54			
DAY 46(18)										
C1	4.936	0.01	0.015	4.921	0.07	0.84	58.58	58.92	0.48	
C2	4.997	0.01	0.019	4.978	0.07	0.84	59.26			
O1	1.73	0.01	0.019	1.711	0.08	0.96	17.82	29.14	16.01	
O2	3.903	0.01	0.019	3.884	0.08	0.96	40.46			
N1	1.989	0.01	0.019	1.97	0.07	0.84	23.45	25.47	2.85	
N2	2.328	0.01	0.019	2.309	0.07	0.84	27.49			
D1	5.922	0.001	0.018	5.904	0.06	0.72	8.20	8.30	0.14	
D2	6.067	0.001	0.018	6.049	0.06	0.72	8.40			
M1	3.134	0.01	0.019	3.115	0.07	0.84	37.08	38.48	1.98	
M2	3.369	0.01	0.019	3.35	0.07	0.84	39.88			
DAY 48(20)										
C1	7.165	0.01	0.019	7.146	0.07	0.84	85.07	77.71	10.41	
C2	5.928	0.01	0.019	5.909	0.07	0.84	70.35			
O1	10.805	0.001	0.018	10.787	0.06	0.72	14.98	11.87	4.40	
O2	6.322	0.001	0.018	6.304	0.06	0.72	8.76			
N1	6.958	0.001	0.018	6.94	0.06	0.72	9.64	18.72	12.85	
N2	20.038	0.001	0.015	20.023	0.06	0.72	27.81			
D1	21.294	0.001	0.026	21.268	0.05	0.6	35.45	32.21	4.58	
D2	20.885	0.001	0.026	20.859	0.06	0.72	28.97			
M1	29.886	0.001	0.018	29.868	0.06	0.72	41.48	40.37	1.57	
M2	28.288	0.001	0.018	28.27	0.06	0.72	39.26			
RAW day 27	not enough to repeat									

Appendix C

Conjugated Dienes & Tetraenes

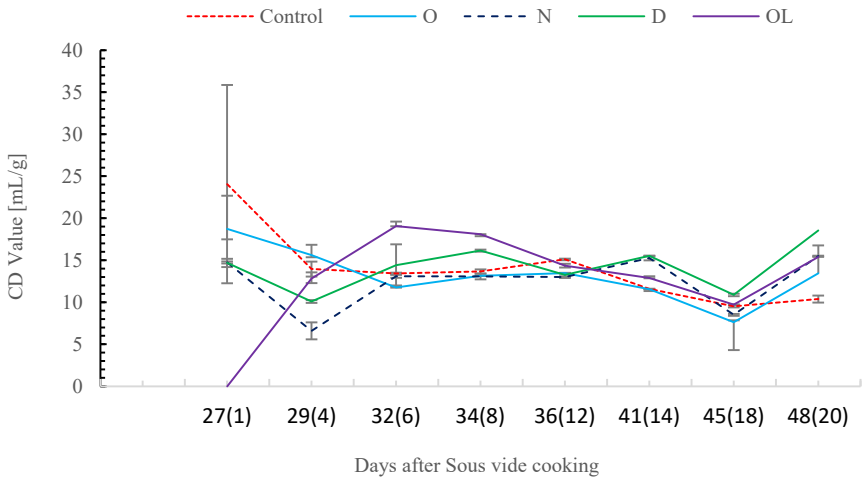

Figure C.1: The conjugated dienes value [mL/g] of all four antioxidant treated herring samples as function of storage days after sous-vide cooking. Control are herring fillets subjected to sous-vide cooking without prior treatment with antioxidants (red). Oregano (sky blue), Nettle (deep blue), Dill (green) and Olive Leaf (purple) are antioxidant treated fillets which are then sous-vide cooked. The x-axis represents the number of days after sous- vide cooking (day 27 to day 48). The y-axis shows the values of conjugated dienes in milliliter per gram lipid [mL/g]. The values are given as average± standard deviation, N = 2.

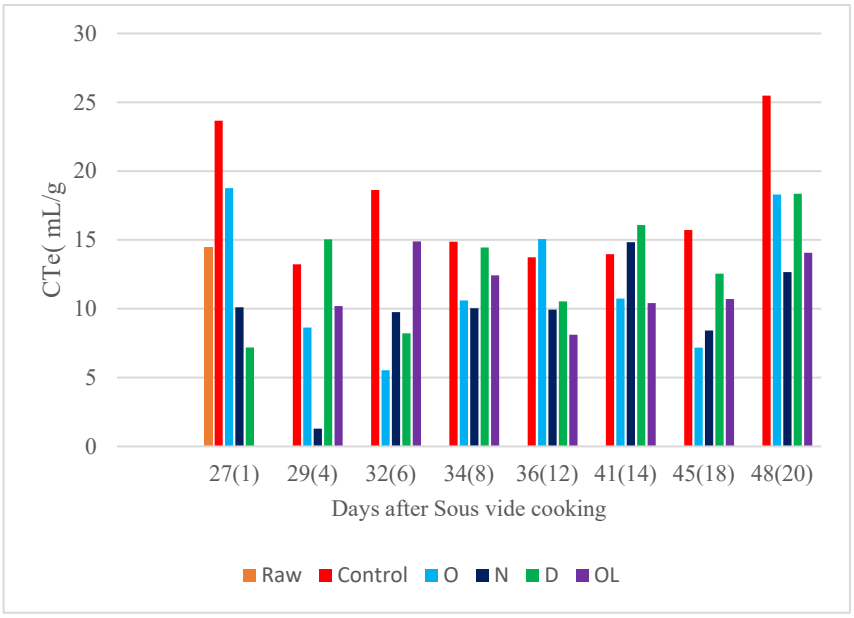

Figure C.2: The conjugated tetraenes values calculated as a function of storage days. X-axis respresents the days after sous-vide and antioxidant treatment, y-axis represents the values of CTe expressed in [mL/g]. Raw (untreated), control (sous-vide cooked), O= Oregano, N= Nettle, D=Dill, OL=olive leaf treated herring. All values are expressed in average.

Table C.1: The raw data obtained for the calculation of CDs is shown. The results were expressed in {mL/g} and calculated according to equation (3). The absorbance was noted at 233nm.

DAY27 (1)	DAY 27 (Dat's day1)	Wt of lipid sample in 1ml chloroform	Absorbance 1	Absorbance 2	Absorbance 1 corrected with Blank	Absorbance 2 corrected with Blank	CD primary oxidation products sample 1	CD primary oxidation products sample 2	Average	SD
	Raw	0.06	0.943	0.907	0.941	0.905	15.683	15.083	15.383	0.424
	CONTROL (1)	0.06	0.945	1.946	0.943	1.944	15.717	32.400	24.058	11.797
	OREGANO-(10	0.06	1.293	0.958	1.291	0.956	21.517	15.933	18.725	3.948
	NETTLE .(1)	0.06	0.891	0.881	0.889	0.879	14.817	14.650	14.733	0.118
	DILL.(1)	0.06	0.916	0.850	0.914	0.848	15.233	14.133	14.683	0.778
	OLIVE LEAF .(1)	0.06	SAMPLE NOT ENOUGH							
DAY 29 (4)	CONTROL.(4)	0.07	1.023	0.935	1.021	0.933	14.586	13.329	13.957	0.889
	Oregano (4)	0.06	0.887	0.991	0.885	0.989	14.750	16.483	15.617	1.226
	NETTLE (4)	0.08	0.473	0.587	0.471	0.585	5.888	7.313	6.600	1.008
	DILL (4)	0.09	0.879	0.942	0.877	0.940	9.744	10.444	10.094	0.495
	OLIVE LEAF (4)	0.07	0.936	0.862	0.934	0.860	13.343	12.286	12.814	0.748
DAY 32 (6)	CONTROL (6)	0.07	0.946	0.936	0.944	0.934	13.486	13.343	13.414	0.101
	OREGANO (6)	0.07	0.812	0.837	0.81	0.835	11.571	11.929	11.750	0.253
	NETTLE (6)	0.07	0.927	0.909	0.925	0.907	13.214	12.957	13.086	0.182
	DILL (6)	0.06	0.873	0.859	0.871	0.857	14.517	14.283	14.400	0.165
	OLIVE LEAF (6)	0.05	0.974	0.936	0.972	0.934	19.440	18.680	19.060	0.537
DAY34 (8)	CONTROL (8)	0.07	0.971	0.947	0.969	0.945	13.843	13.500	13.671	0.242
	OREGANO (8)	0.07	0.922	0.923	0.92	0.921	13.143	13.157	13.150	0.010
	NETTLE (8)	0.07	0.933	0.900	0.931	0.898	13.300	12.829	13.064	0.333
	DILL (8)	0.07	1.256	1.009	1.254	1.007	17.914	14.386	16.150	2.495
	OLIVE LEAF (8)	0.05	0.906	0.907	0.904	0.905	18.080	18.100	18.090	0.014
DAY36(12)	CONTROL(12)	0.06	0.904	0.912	0.902	0.910	15.033	15.167	15.100	0.094
	OREGANO(12)	0.07	0.945	0.943	0.943	0.941	13.471	13.443	13.457	0.020
	NETTLE(12)	0.07	0.917	0.907	0.915	0.905	13.071	12.929	13.000	0.101
	DILL(12)	0.07	0.936	0.924	0.934	0.922	13.343	13.171	13.257	0.121
	OLIVE LEAF (12)	0.06	0.873	0.853	0.871	0.851	14.517	14.183	14.350	0.236
DAY 41(14)	CONTROL (14)	0.08	0.927	0.930	0.925	0.928	11.563	11.600	11.581	0.027
	OREGANO(14)	0.08	0.928	0.924	0.926	0.922	11.575	11.525	11.550	0.035
	NETTLE(14)	0.06	0.929	0.906	0.927	0.904	15.450	15.067	15.258	0.271
	DILL(14)	0.06	0.943	0.922	0.941	0.920	15.683	15.333	15.508	0.247
	OLIVE LEAF (14)	0.07	0.893	0.914	0.891	0.912	12.729	13.029	12.879	0.212
DAY 45(18)	CONTROL (18)	0.07	0.675	0.662	0.673	0.660	9.614	9.429	9.521	0.131
	OREGANO(18)	0.08	0.6	0.625	0.598	0.623	7.475	7.788	7.631	0.221
	NETTLE(18)	0.07	0.592	0.603	0.59	0.601	8.429	8.586	8.507	0.111
	DILL(18)	0.06	0.653	0.656	0.651	0.654	10.850	10.900	10.875	0.035
	OLIVE LEAF (18)	0.07	0.684	0.681	0.682	0.679	9.743	9.700	9.721	0.030
DAY 48(20)	CONTROL (20)	0.07	0.708	0.749	0.706	0.747	10.086	10.671	10.379	0.414
	OREGANO(20)	0.06	0.668	0.950	0.666	0.948	11.100	15.800	13.450	3.323
	NETTLE(20)	0.06	0.93	0.934	0.928	0.932	15.467	15.533	15.500	0.047
	DILL(20)	0.05	0.934	0.924	0.932	0.922	18.640	18.440	18.540	0.141
	OLIVE LEAF (20)	0.06	0.924	0.926	0.922	0.924	15.367	15.400	15.383	0.024

Table C.2: Data used for calculation of conjugated tetraenes in the herring samples.

Day 27(1)	DAY 27 (Dat's day1)		Absorbance 1	Absorbance 2	Absorbance 1 corrected with blank	Absorbance 2 corrected with blank	CTs primary Oxidation products sample 1	CTs primary products sample 2	Average	SD
	Raw	0.06	0.858	0.879	0.858	0.879	14.3	14.65	14.475	0.175
	CONTROL (1)	0.06	1.174	1.665	1.174	1.665	19.567	27.75	23.658	4.092
	OREGANO-(10	0.06	1.256	0.995	1.256	0.995	20.933	16.583	18.758	2.175
	NETTLE .(1)	0.06	0.605	0.608	0.605	0.608	10.083	10.133	10.108	0.025
	DILL.(1)	0.06	0.439	0.423	0.439	0.423	7.317	7.05	7.183	0.133
	OLIVE LEAF .(1)	0.06	SAMPLE FINISHED							
day 29(4)	CONTROL.(4)	0.07	0.953	0.899	0.953	0.899	13.614	12.843	13.229	0.386
	Oregano (4)	0.06	0.531	0.505	0.531	0.505	8.85	8.417	8.633	0.217
	NETTLE (4)	0.08	0.11	0.096	0.11	0.096	1.375	1.200	1.288	0.088
	DILL (4)	0.09	0.457	2.250	0.457	2.250	5.078	25.000	15.039	9.961
	OLIVE LEAF (4)	0.07	0.749	0.679	0.749	0.679	10.7	9.700	10.200	0.500
day 32(6)	CONTROL (6)	0.07	0.924	1.683	0.924	1.683	13.2	24.043	18.621	5.421
	OREGANO (6)	0.07	0.356	0.419	0.356	0.419	5.086	5.986	5.536	0.450
	NETTLE (6)	0.07	0.708	0.657	0.708	0.657	10.114	9.386	9.750	0.364
	DILL (6)	0.06	0.525	0.461	0.525	0.461	8.75	7.683	8.217	0.533
	OLIVE LEAF (6)	0.05	0.7778	0.711	0.7778	0.711	15.556	14.22	14.888	0.668
day 34(8)	CONTROL (8)	0.07	1.074	1.007	1.074	1.007	15.343	14.386	14.864	0.479
	OREGANO (8)	0.07	0.744	0.741	0.744	0.741	10.629	10.586	10.607	0.021
	NETTLE (8)	0.07	0.731	0.674	0.731	0.674	10.443	9.629	10.036	0.407
	DILL (8)	0.07	1.214	0.809	1.214	0.809	17.343	11.557	14.450	2.893
	OLIVE LEAF (8)	0.05	0.637	0.606	0.637	0.606	12.74	12.12	12.430	0.310
day 36(12)	CONTROL(12)	0.06	0.803	0.846	0.803	0.846	13.383	14.1	13.742	0.358
	OREGANO(12)	0.07	1.1	1.008	1.1	1.008	15.714	14.4	15.057	0.657
	NETTLE(12)	0.07	0.697	0.694	0.697	0.694	9.957	9.914	9.936	0.021
	DILL(12)	0.07	0.762	0.713	0.762	0.713	10.886	10.186	10.536	0.350
	OLIVE LEAF (12)	0.06	0.524	0.450	0.524	0.450	8.733	7.5	8.117	0.617
day 41(14)	CONTROL (14)	0.08	1.121	1.113	1.121	1.113	14.013	13.909	13.961	0.052
	OREGANO(14)	0.08	0.867	0.852	0.867	0.852	10.838	10.65	10.744	0.094
	NETTLE(14)	0.06	0.936	0.850	0.936	0.850	15.6	14.167	14.883	0.717
	DILL(14)	0.06	0.968	0.962	0.968	0.962	16.133	16.033	16.083	0.050
	OLIVE LEAF (14)	0.07	0.725	0.732	0.725	0.732	10.357	10.457	10.407	0.050
day 45(18)	CONTROL (18)	0.07	1.093	1.108	1.093	1.108	15.614	15.829	15.721	0.107
	OREGANO(18)	0.08	0.49	0.657	0.49	0.657	6.125	8.2125	7.169	1.044
	NETTLE(18)	0.07	0.586	0.593	0.586	0.593	8.371	8.471	8.421	0.050
	DILL(18)	0.06	0.765	0.741	0.765	0.741	12.75	12.35	12.550	0.200
	OLIVE LEAF (18)	0.07	0.777	0.722	0.777	0.722	11.1	10.314	10.707	0.393
day48(20)	CONTROL (20)	0.07	1.768	1.799	1.768	1.799	25.257	25.7	25.479	0.221
	OREGANO(20)	0.06	1.124	1.072	1.124	1.072	18.733	17.867	18.300	0.433
	NETTLE(20)	0.06	0.76	0.759	0.76	0.759	12.667	12.65	12.658	0.008
	DILL(20)	0.05	0.908	0.927	0.908	0.927	18.16	18.54	18.350	0.190
	OLIVE LEAF (20)	0.06	0.839	0.849	0.839	0.849	13.983	14.15	14.067	0.083

Appendix D

TBARS

The raw data for the determination of TBARS is shown in table and the standard curves calibrated are also shown in the tables D.1, D.2 and D.3. The TBARS were calculated according to equation (4) and the results are expresses in µMol TBARS/g lipid.

Table D.1: data from the standard curve. Each time a fresh solution was used a new standard curve was obtained. The values obtained from the curve are and the data used are shown in the tables. The A1 is the average absorbance obtained at 538 nm and the A2 is the absorbance with background correction. The curve was calibrated using A2 values

TEP conc(ug/mL)	Avg Absorbance A1	Absorbance A2
200	0.265	0.264
150	0.22	0.219
100	0.139	0.138
50	0.071	0.07
25	0.035	0.034
0	0.001	0

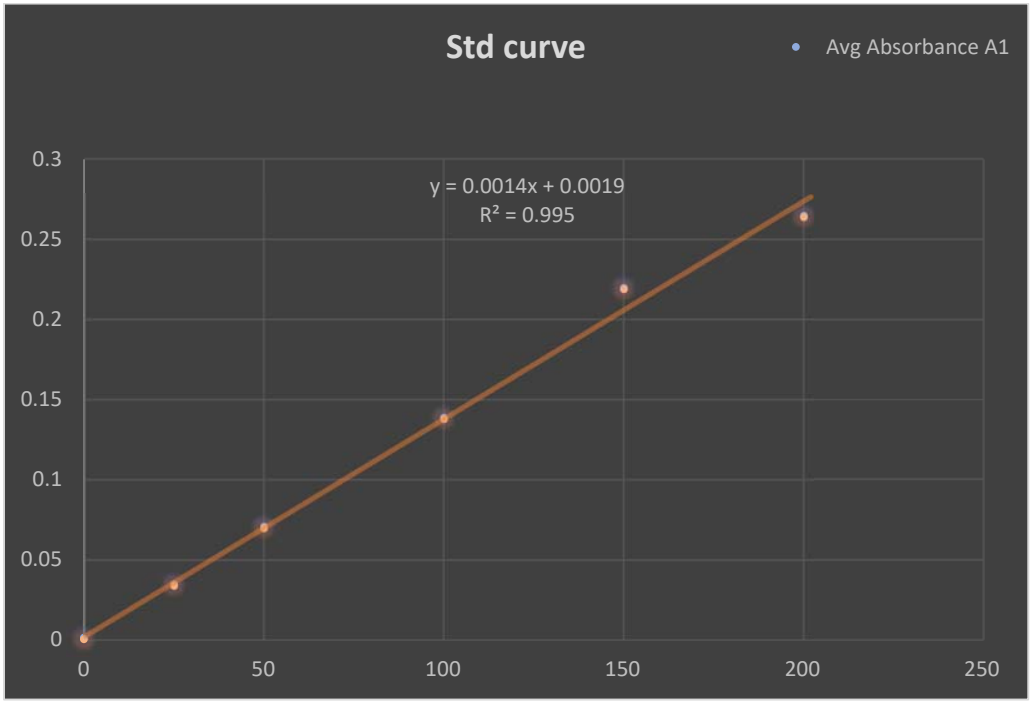

Figure D.1: Standard curve obtained for TBARS, intercept= 0.0019 and slope value=0.0014.

Table D.2: The Standard curve obtained with freshly prepared TEP concentrations

TEP Conc.(uL)	AVG. absorbance
0	0
25	0.047
50	0.079
100	0.16
150	0.232
200	0.301
250	0.293
300	0.413

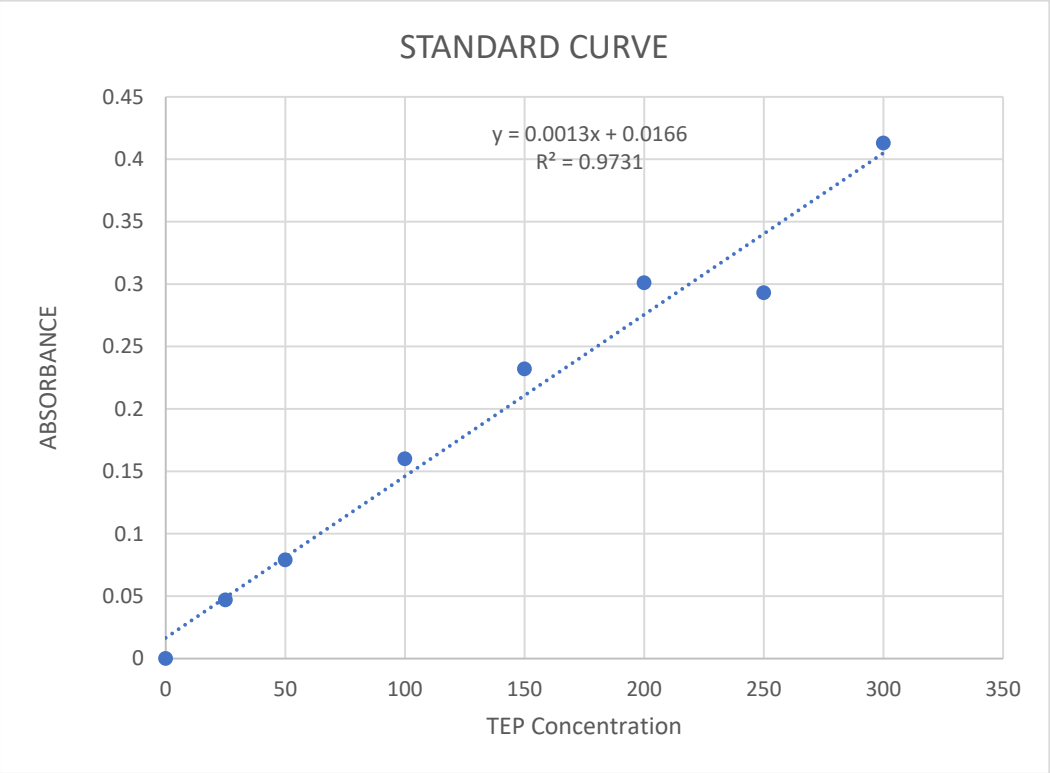

Figure D.2: Standard curve obtained for TBARS, intercept= 0.0019 and slope value=0.0014.

Table D.3: The data used for calibrating the standard curve.

TEP Conc.(uL)	AVG. absorbance
0	0.000A
25	0.038
50	0.079
100	0.152
150	0.223
200	0.276
250	0.309
300	0.427

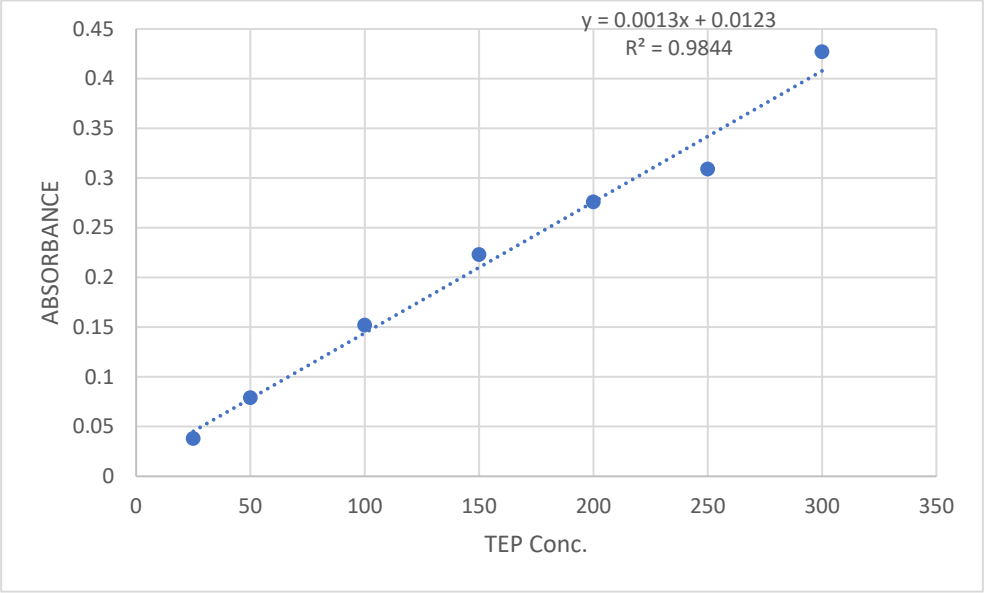

Figure D.3: The standard curve obtained indicating the values of Intercept = 0.0123 and slope = 0.0013.

TBAR Tables

Table D.4: Raw data used for the determination of TBARS.

DAY 27			Absorbance of Chloroform phase samples		
dilutions	samples	g lipid /mL chloroform	Absorbance 1	Absorbance 2	Total lipid content in sample / ML
100ul	RAW	0.06	0.254	0.334	0.006
50ul	CONTROL	0.06	0.284	0.372	0.003
50ul	OREGANO	0.06	0.198	0.254	0.003
100ul	NETTLE	0.06	0.241	0.256	0.006
200ul	DILL	0.06	0.259	0.243	0.012
50ul	olive leaf extract	0.06	0.185	0.137	0.003
DAY 29					
50uL	CONTROL	0.07	0.254	0.309	0.0035
100UL	OREGANO	0.06	0.071	0.063	0.006
100UL	NETTLE	0.08	0.17	0.146	0.008
200ul	DILL	0.09	0.165	0.291	0.018
50uL	olive leaf extract	0.07	0.197	0.203	0.0035
DAY 32					
50ul	CONTROL	0.07	0.234	0.309	0.0035
200ul	OREGANO	0.07	0.123	0.11	0.014
50ul	NETTLE	0.07	0.181	0.224	0.0035
100ul	DILL	0.06	0.24	0.131	0.006
50ul	olive leaf extract	0.05	0.183	0.221	0.0025
DAY 34					
50ul	CONTROL	0.07	0.365	0.363	0.0035
50ul	OREGANO	0.07	0.208	0.229	0.0035
50ul	NETTLE	0.07	0.161	0.147	0.0035
50ul	DILL	0.07	0.192	0.125	0.0035
50ul	olive leaf extract	0.05	0.159	0.125	0.0025

DAY 36					
25ul	CONTROL	0.06	0.155	0.166	0.0015
25ul	OREGANO	0.07	0.243	0.26	0.00175
25ul	NETTLE	0.07	0.127	0.195	0.00175
25ul	DILL	0.07	0.145	0.105	0.00175
100ul	olive leaf extract	0.06	0.245	0.198	0.012
DAY 41					
25ul	CONTROL	0.08	0.273	0.317	0.002
25ul	OREGANO	0.08	0.082	0.164	0.002
25ul	NETTLE	0.06	0.165	0.16	0.0015
25ul	DILL	0.06	0.147	0.208	0.0015
50ul	olive leaf extract	0.07	0.21	0.209	0.0035
DAY 46					
25ul	CONTROL	0.07	0.214	0.175	0.00175
100ul	OREGANO	0.08	0.178	0.36	0.008
100ul	NETTLE	0.07	0.297	0.28	0.007
100ul	DILL	0.06	0.398	0.365	0.006
50ul	olive leaf extract	0.07	0.196	0.251	0.0035
DAY 48					
25ul	CONTROL	0.07	0.35	0.458	0.00175
25ul	OREGANO	0.06	0.187	0.207	0.0015
50ul	NETTLE	0.06	0.204	0.262	0.003
50ul	DILL	0.05	0.303	0.328	0.0025
25ul	olive leaf extract	0.06	0.133	0.298	0.0015

Table D.5:

DAY 27						
samples	µmol TBARS/ g lipid (1)	µmol TBARS/ g lipid (2)	Average µmol TBARS / g lipid	SD	Intercapt of standard curve	Slope of standard curve
RAW	60.02	79.07	69.55	13.47	0.0019	0.0014
CONTROL	268.67	352.48	310.57	59.26		
OREGANO	186.76	240.10	213.43	37.71		
NETTLE	56.93	121	88.96	45.31		
DILL	15.30	14.35	14.83	0.67		
olive leaf extract	174.38	128.67	151.52	32.32		
DAY 29						
CONTROL	205.80	250.69	228.24	31.75		
OREGANO	16.45	14.55	15.50	1.35		
NETTLE	30.02	25.73	27.88	3.03		
DILL	6.47	11.47	8.97	3.54		
olive leaf extract	159.27	164.16	161.71	3.46		
DAY 32						
CONTROL	189.47	250.69	220.08	43.29		
OREGANO	6.18	5.52	5.85	0.47		
NETTLE	146.20	181.31	163.76	24.82		
DILL	56.69	30.74	43.71	18.35		
olive leaf extract	206.97	250.4	228.69	30.71		
DAY 34					0.0166	0.0013
CONTROL	306.29	304.53	305.41	1.24		
OREGANO	168.26	186.73	177.49	13.05		
NETTLE	126.95	114.64	120.79	8.70		
DILL	154.20	95.30	124.75	41.65		
olive leaf extract	175.26	133.42	154.34	29.59		

DAY 36						
CONTROL	585.44	630.56	608.00	31.91	0.0123	0.0013
OREGANO	811.25	871.03	841.14	42.27		
NETTLE	403.34	642.46	522.90	169.08		
DILL	466.64	325.98	396.31	99.46		
olive leaf extract	29.83	23.81	26.82	4.26		
DAY 41						
CONTROL	802.15	937.54	869.85	95.73		
OREGANO	214.46	466.77	340.62	178.41		
NETTLE	626.46	605.95	616.21	14.50		
DILL	552.62	802.87	677.74	176.96		
olive leaf extract	173.80	172.92	173.36	0.62		
DAY 46						
CONTROL	709.27	572.13	640.70	96.97		
OREGANO	31.87	66.87	49.37	24.75		
NETTLE	62.57	58.84	60.70	2.64		
DILL	98.90	90.44	94.67	5.98		
olive leaf extract	161.49	209.85	185.67	34.19		
DAY 48						
CONTROL	1187.52	1567.30	1377.41	268.55		
OREGANO	716.72	798.77	757.74	58.02		
NETTLE	196.62	256.10	226.36	42.06		
DILL	357.78	388.55	373.17	21.76		
olive leaf extract	495.18	1172.10	833.64	478.66		

Appendix E

Antioxidant Activity Assays

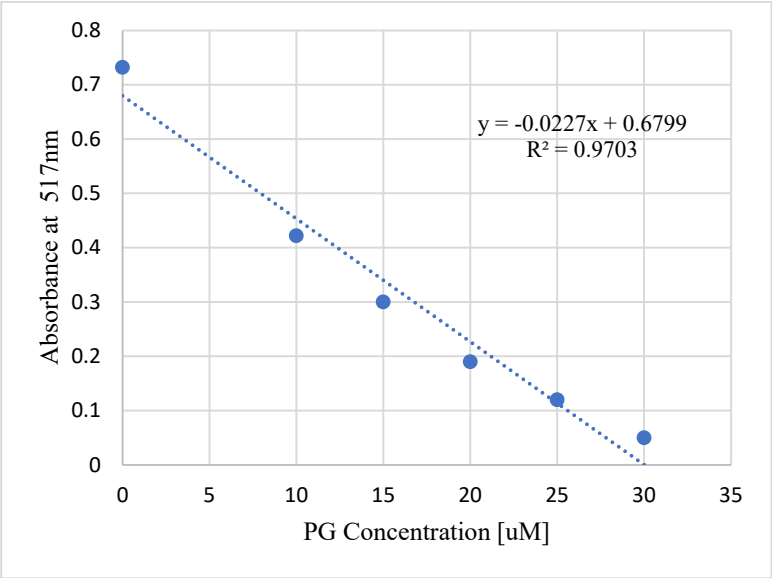

Figure E.1: Standard curve obtained at 517 nm indicating absorbance values by standards PG concentrations in DPPH Assay. Blank was methanol 80%. Intercept and slope values are shown by the equation obtained from the curve (a=slope= -0.0227, b=intercept= 0.6799). R2 value (0.9703) indicates a good correlation

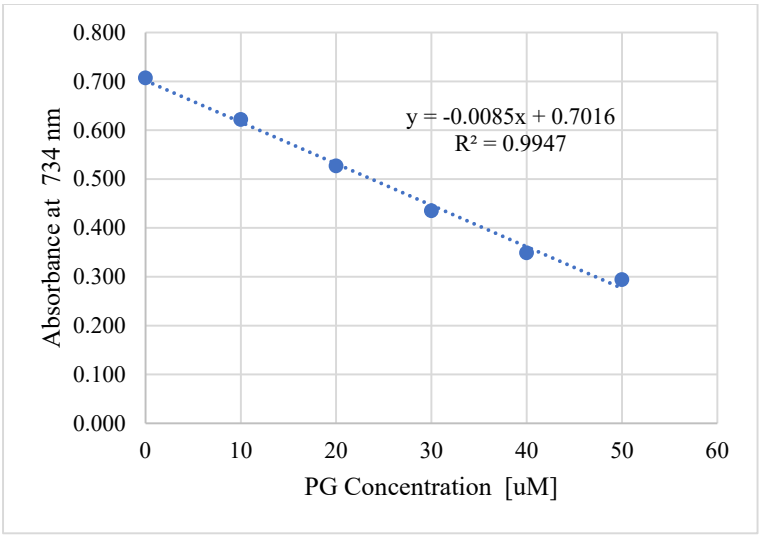

Figure E.2: Standard curve obtained at 734 nm, indicating absorbance values against different concentrations of PG obtained by ABTS Assay. Methanol 80% was used as blank. Equation gives the values of intercept and slope (a=-0.0085, b= 0.7016). R2 value (0.9947) indicates a good correlation.

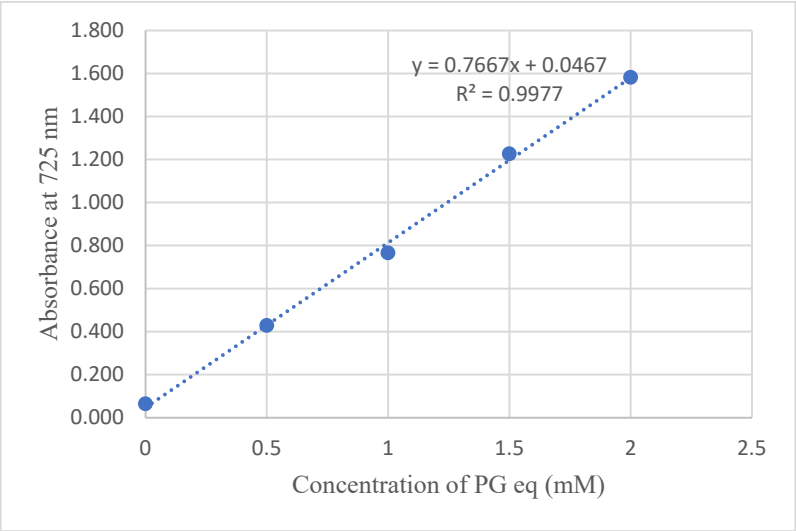

Figure E.3: Illustrates the absorbance values given by the different concentrations of propyl gallate in FC Assay. Methanol 80% was used as a blank. Equation gives the values of intercept and slope (a= 0.7667 , b= 0.0467).R2 value (0.9977) indicates a good correlation

Table E.1: DPPH Assay

Conc.PG	Absorbance
0	0.732
10	0.422
15	0.3
20	0.19
25	0.12
30	0.05

Table E.2a: The raw data used for the ABTS Assay

		Abs.1	Abs.2	Abs.3	Avg.Abs.(A)
BLANK	0	0.707	0.709	0.704	0.707
Standards	10	0.635	0.619	0.613	0.622
	20	0.534	0.526	0.521	0.527
	30	0.449	0.429	0.427	0.435
	40	0.369	0.344	0.334	0.349
	50	0.311	0.291	0.279	0.294

Table E.2b: The raw data used for the ABTS Assay

STDS	Abs
0	0.707
10	0.622
20	0.527
30	0.435
40	0.349
50	0.294

Table E.3: The raw data for the FCA

	mM	Abs.1	Abs.2	Abs.3	Avg. Abs
BLANK	0	0.064	0.001	0.288	0.118
STANDARDS	0.5	0.413	0.451	0.422	0.429
	1	0.786	0.847	0.665	0.766
	1.5	1.391	1.343	0.945	1.226
	2	1.838	1.778	1.13	1.582

Appendix F

Protein Content

The raw data for watersoluble protein content is shown in the tables and the standard curve obtained between Absorbance at 595nm and concentration of IgG is shown in the figure.

The standard curve was straight linear indicating the linear correlation between the varying concentrations of bio rad dye and the absorbance.

Table F.1: The raw data obtained for the calibration of standard curve using the Bio rad assay.

std curve protein content	(mg/mL)			
	Absorbance	A2	A3	Avg. A
0.2	0.636	0.651	0.655	0.647
0.4	0.845	0.8	0.81	0.818
0.6	1.003	0.928	0.956	0.962
0.8	1.148	1.097	1.085	1.110
1	1.296	1.249	1.244	1.263

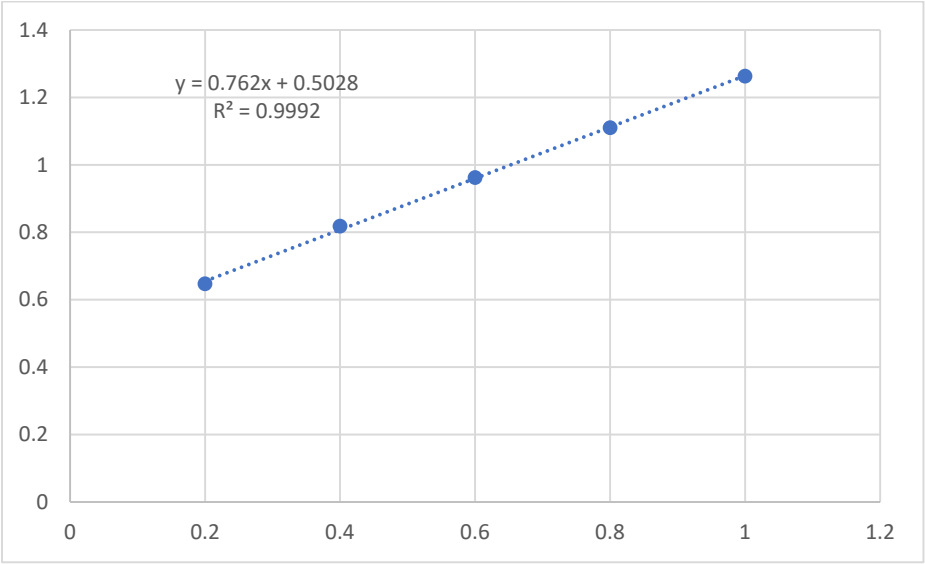

Figure: F.1: The standard curve obtained for the determination of protein content in the herring samples.

samples	A1	A2	A3	Avg. A	SD	A1	A2	A3	avgA
Raw (diluted 1:5)	1.048	1.088	1.049	1.062		0.715	0.768	0.717	0.733
C1	0.594	0.618	0.609	0.607	0.06	0.120	0.151	0.139	0.137
C2	0.704	0.688	0.673	0.688		0.264	0.243	0.223	0.243
O1	0.717	0.718	0.714	0.716	0.09	0.281	0.282	0.277	0.280
O2	0.604	0.593	0.581	0.593		0.133	0.118	0.103	0.118
D1	0.718	0.715	0.704	0.712	0.02	0.282	0.278	0.264	0.275
D2	0.761	0.78	0.693	0.745		0.339	0.364	0.250	0.317
N1	0.833	0.777	0.815	0.808	0.00	0.433	0.360	0.410	0.401
N2	0.843	0.782	0.779	0.801		0.446	0.366	0.362	0.392
M1	0.833	0.785	0.784	0.801	0.04	0.433	0.370	0.369	0.391
M2	0.742	0.741	0.739	0.741		0.314	0.313	0.310	0.312
DAY 36 (12)									
C1	0.662	0.666	0.652	0.660	0.05	0.209	0.214	0.196	0.206
C2	0.74	0.736	0.731	0.736		0.311	0.306	0.299	0.306
O1	0.802	0.786	0.804	0.797	0.01	0.393	0.372	0.395	0.387
O2	0.81	0.807	0.821	0.813		0.403	0.399	0.418	0.407
D1	0.745	0.843	0.79	0.793	0.07	0.318	0.446	0.377	0.380
D2	0.695	0.709	0.698	0.701		0.252	0.271	0.256	0.260
N1	0.805	0.827	0.75	0.794	0.00	0.397	0.425	0.324	0.382
N2	0.822	0.848	0.698	0.789		0.419	0.453	0.256	0.376
M1	0.815	0.826	0.829	0.823	0.03	0.410	0.424	0.428	0.421
M2	0.903	0.857	0.84	0.867		0.525	0.465	0.443	0.478
DAY 48(20)									
C1	0.856	0.925	0.963	0.915	0.00	0.464	0.554	0.604	0.541
C2	0.898	0.914	0.928	0.913		0.519	0.540	0.558	0.539
O1	0.93	0.975	0.908	0.938	0.02	0.561	0.620	0.532	0.571
O2	0.972	0.979	0.939	0.963		0.616	0.625	0.572	0.604
D1	0.905	0.934	0.907	0.915	0.04	0.528	0.566	0.530	0.541
D2	0.884	0.858	0.854	0.865		0.500	0.466	0.461	0.476
N1	0.894	0.896	0.891	0.894	0.02	0.513	0.516	0.509	0.513
N2	0.901	0.923	0.94	0.921		0.523	0.551	0.574	0.549
M1	0.895	0.892	0.881	0.889	0.01	0.515	0.511	0.496	0.507
M2	0.921	0.876	0.897	0.898		0.549	0.490	0.517	0.519

Table F.2: raw data for the calculation of protein content from the day 27, day 36 and day 48 samples of sous vide cooked frozen herring samples. The raw sample from the day 27 was diluted with a dilution factor 5 so the protein content in the undiluted sample was calculated. The percentage protein was calculated using the equation. The average of two parallels was calculated and then the standard deviation was measured. Then the % protein content was used in mg/ml.

samples	mg/ml undil	% protein	AVG %	std.dev	mg/ml
Raw (dilutec	3.667	1.834	1.834	0.00	18.34
C1	0.137	0.171	0.238	0.09	2.38
C2	0.243	0.304			
O1	0.280	0.350	0.249	0.14	2.49
O2	0.118	0.147			
D1	0.275	0.344	0.370	0.04	3.70
D2	0.317	0.397			
N1	0.401	0.501	0.495	0.01	4.95
N2	0.392	0.490			
M1	0.391	0.489	0.439	0.07	4.39
M2	0.312	0.390			
DAY 36 (12)					
C1	0.206	0.258	0.320	0.09	3.20
C2	0.306	0.382			
O1	0.387	0.483	0.496	0.02	4.90
O2	0.407	0.508			
D1	0.380	0.476	0.400	0.11	4.00
D2	0.260	0.325			
N1	0.382	0.478	0.474	0.01	4.74
N2	0.376	0.470			
M1	0.421	0.526	0.561	0.05	5.61
M2	0.478	0.597			
DAY 48(20)					
C1	0.541	0.676	0.675	0.00	6.75
C2	0.539	0.673			
O1	0.571	0.713	0.734	0.02	7.34
O2	0.604	0.755			
D1	0.541	0.677	0.636	0.06	6.36
D2	0.476	0.595			
N1	0.513	0.641	0.664	0.03	6.64
N2	0.549	0.687			
M1	0.507	0.634	0.641	0.01	6.41
M2	0.519	0.648			

Appendix G

Thiols Content

Table G.1: The raw data for the estimation of Thiols in the sousvide cooked herring from the day 27, day 36 and day 48.

samples	A1	A2	A3	SAMPLES	A1	A2	A3	V2(ml)	V1	C1	C2	C3	Avg C	S.DEV of 3 absorbances
C1 dAY 27	0.105	0.106	0.092	C1 dAY 27	0.105	0.106	0.092	1	0.1	7.35E-05	7.42E-05	6.44E-05	7.07E-05	5.47E-06
C2	0.198	0.102	0.221	C2	0.198	0.102	0.221	1	0.1	1.39E-04	7.14E-05	1.55E-04	1.22E-04	4.42E-05
O1	0.028	0.025	0.02	O1	0.028	0.025	0.02	1	0.1	1.96E-05	1.75E-05	1.40E-05	1.70E-05	2.83E-06
O2	0.012	0.012	0.01	O2	0.012	0.012	0.01	1	0.1	8.40E-06	8.40E-06	7.00E-06	7.93E-06	8.08E-07
N1	0.028	0.029	0.01	N1	0.028	0.029	0.01	1	0.1	1.96E-05	2.03E-05	7.00E-06	1.56E-05	7.48E-06
N2	0.013	0.015	0.017	N2	0.013	0.015	0.017	1	0.1	9.10E-06	1.05E-05	1.19E-05	1.05E-05	1.40E-06
M1	0.036	0.036	0.036	M1	0.036	0.036	0.036	1	0.1	2.52E-05	2.52E-05	2.52E-05	2.52E-05	0.00E+00
M2	0.017	0.081	0.067	M2	0.017	0.081	0.067	1	0.1	1.19E-05	5.67E-05	4.69E-05	3.85E-05	2.35E-05
R1	0.427	0.538	0.28	R1	0.427	0.538	0.28	1	0.1	2.99E-04	3.76E-04	1.96E-04	2.90E-04	9.06E-05
D1	0.093	0.097	0.503	D1	0.093	0.097	0.503	1	0.1	6.51E-05	6.79E-05	3.52E-04	1.62E-04	1.65E-04
D2	0.201	0.218	0.134	D2	0.201	0.218	0.134	1	0.1	1.41E-04	1.53E-04	9.38E-05	1.29E-04	3.11E-05
C1 DAY 36	0.072	0.077	0.085	C1 DAY 36	0.030	0.035	0.043	1	0.1	2.10E-05	2.45E-05	3.01E-05	2.52E-05	4.59E-06
C2	0.069	0.134	0.073	C2	0.027	0.092	0.031	1	0.1	1.89E-05	6.44E-05	2.17E-05	3.50E-05	2.55E-05
O1	0.072	0.067	0.067	O1	0.030	0.025	0.025	1	0.1	2.10E-05	1.75E-05	1.75E-05	1.87E-05	2.02E-06
O2	0.057	0.065	0.062	O2	0.015	0.023	0.020	1	0.1	1.05E-05	1.61E-05	1.40E-05	1.35E-05	2.83E-06
N1	0.144	0.114	0.112	N1	0.102	0.072	0.070	1	0.1	7.14E-05	5.04E-05	4.90E-05	5.69E-05	1.25E-05
N2	0.085	0.09	0.095	N2	0.043	0.048	0.053	1	0.1	3.01E-05	3.36E-05	3.71E-05	3.36E-05	3.50E-06
M1	0.067	0.082	0.103	M1	0.025	0.040	0.061	1	0.1	1.75E-05	2.80E-05	4.27E-05	2.94E-05	1.27E-05
M2	0.068	0.067	0.069	M2	0.026	0.025	0.027	1	0.1	1.82E-05	1.75E-05	1.89E-05	1.82E-05	7.00E-07
D1	0.036	0.035	0.043	D1	-0.006	-0.007	0.001	1	0.1	-4.20E-06	-4.90E-06	7.00E-07	-2.80E-06	3.05E-06
D2	0.028	0.028	0.029	D2	-0.014	-0.014	-0.013	1	0.1	-9.80E-06	-9.80E-06	-9.10E-06	-9.56E-06	4.04E-07
blank	0.042			blank				1	0.1					
C1 DAY 48	0.225	0.189	0.251	C1 DAY 48	0.176	0.140	0.202	1	0.1	0.00012316	9.79706E-05	0.00014	0.0001208	2.18E-05
C2	0.2	0.24	0.217	C2	0.151	0.191	0.168	1	0.1	0.00010567	0.00013366	0.00012	0.0001190	1.40E-05
O1	0.111	0.114	0.13	O1	0.062	0.065	0.081	1	0.1	4.3387E-05	4.54864E-05	0.00006	0.0000485	7.15E-06
O2	0.086	0.096	0.096	O2	0.037	0.047	0.047	1	0.1	2.58922E-05	3.28901E-05	0.00003	0.0000306	4.04E-06
N1	0.125	0.144	0.131	N1	0.076	0.095	0.082	1	0.1	5.3184E-05	6.64801E-05	0.00006	0.0000590	6.80E-06
N2	0.171	0.166	0.112	N2	0.122	0.117	0.063	1	0.1	8.53744E-05	8.18754E-05	0.00004	0.0000704	2.29E-05
D1	0.102	0.089	0.114	D1	0.053	0.040	0.065	1	0.1	3.70889E-05	2.79916E-05	0.00005	0.0000369	8.75E-06
D2	0.112	0.037	0.129	D2	0.063	-0.012	0.080	1	0.1	4.40868E-05	-8.3975E-06	0.00006	0.0000306	3.43E-05
M1	0.081	0.077	0.082	M1	0.032	0.028	0.033	1	0.1	2.23933E-05	1.95941E-05	0.00002	0.0000217	1.85E-06
M2	0.079	0.07	0.074	M2	0.030	0.021	0.025	1	0.1	2.09937E-05	1.46956E-05	0.00002	0.0000177	3.16E-06

Table G.2: Using values of water-soluble proteins to calculate thiols content in all the samples from day 27, 36 and 48. The thiols values are calculated in micromole/mg and the same values are plotted in the graphs with the standard deviation of the mean values.

SAMPLES	Protein content (mg/ml)	mmol/mg	micromol/mg	SDEV micromol/mg	Average micromol/mg of two parallels
C1 dAY 27	0.137	0.000516	0.5159	0.011	0.508
C2	0.243	0.000500	0.5001		
O1	0.280	0.000061	0.0608	0.005	0.064
O2	0.118	0.000067	0.0672		
N1	0.401	0.000039	0.0390	0.009	0.033
N2	0.392	0.000027	0.0268		
M1	0.391	0.000064	0.0644	0.042	0.094
M2	0.312	0.000123	0.1234		
R1	3.667	0.000079	0.0792		
D1	0.275	0.000588	0.5878	0.128	0.497
D2	0.317	0.000407	0.4069		
C1 DAY 36	0.206	0.000122	0.1223	0.006	0.118
C2	0.306	0.000114	0.1143		
O1	0.387	0.000048	0.0482	0.011	0.041
O2	0.407	0.000033	0.0332		
N1	0.382	0.000149	0.149	0.042	0.119
N2	0.376	0.000089	0.089		
M1	0.421	0.000070	0.070	0.022	0.054
M2	0.478	0.000038	0.038		
D1	0.380	-0.000007	-0.007	0.021	-0.022
D2	0.260	-0.000037	-0.037		
blank			0.000		
C1 DAY 48	0.541	0.000223	0.223	0.002	0.222
C2	0.539	0.000221	0.221		
O1	0.571	0.000085	0.085	0.024	0.068
O2	0.604	0.000051	0.051		
N1	0.513	0.000115	0.115	0.009	0.122
N2	0.549	0.000128	0.128		
D1	0.541	0.000068	0.07	0.003	0.066
D2	0.476	0.000064	0.06		
M1	0.507	0.000043	0.04	0.006	0.038
M2	0.519	0.000034	0.03		