Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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Sous-vide cooking of Atlantic herring with addition of natural antioxidants extracted from Mediterranean herbs

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Janna Cropotova and Turid Rustad
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Preface

This Master's thesis is the result of my final semester of my Master's degree in Chemical Engineering and Biotechnology. The thesis is written at the Department of Biotechnology and Food Science at the Norwegian University of Science and Technology in the time period 29th of August 2019 to 23rd of January 2020.

I would like to thank my supervisors, laboratory technicians and fellow students for all their help, guidance and support during this project work. This work would not have been completed without them.

Firstly, I would like to thank my supervisors, Professor Turid Rustad (Department of Biotechnology & Food Science, NTNU, Trondheim) and Associate Professor Janna Cropotova (Department of Biological Science Ålesund, NTNU). Together we defined an aim and objectives for the master thesis. Both are great supervisors, who ensures progression and are very engaging. Thank you all for your encouragement, guidance, and support of this work both academically and morally during this time period.

Secondly, I would like to thank the laboratory technician Siri Stavrum (Department of Biotechnology & Food Science, NTNU, Trondheim). She provided me with all the chemicals and equipment I needed for my experiments. Thank you for all the help in the laboratory and your guidance.

Thirdly, I would like to thank Trond Viggo Pettersen (Department of Biotechnology & Food Science, NTNU, Trondheim) for his guidance and for his permission to use the vacuum-packing machine at Kalvskinnet campus.

I would like to thank all my friends and all people from the Food Chemistry group for a good social working place both in the laboratory and in the office during the semester. I also want to thank Ayesha Kousar for helping me with the preparation of the Atlantic herring samples during filleting, vacuum packing and sous-vide cooking.

Finally, I want to thank my parents for their support, care and love.

Dat Trong Vu
Trondheim, January 23rd, 2020

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Abstract

Oregano extract showed its efficacity to retard lipid oxidation in fish sample, while olive leaf extract, nettle extract and dill extract did not show any effect. Oregano extract was the only extract showing its antioxidant potential to decrease lipid oxidation in fish samples during cold storage.

The peroxide value varied between 3,56 and 36,95 $m_{eq}O_2$/kg lipid during the storage period. The PV-value limit of acceptability was 5 $m_{eq}O_2$/kg lipid. Some herring samples were below the PV-value limit of acceptability and are regarded as not rancid, while other samples were over the limit and are regarded as rancid. The content of conjugated dienes and conjugated tetraenes varied between 7,68 and 14,53 mL/g and 1,40 and 5,83 mL/g, respectively.

The thiobarbituric acid reactive substances value varied between 1,47 and 13,15 µMol TBARS/g lipid. All herring samples surpasses 2 µMol TBARS/g lipid TBARS-limit of consumption, except of herring sample with addition of nettle extract from day 18 which was below the TBARS-limit of consumption. The Schiff base values of herring samples displayed a variation ranging from 0,31 to 0,88 mL/kg lipid during the storage period.

The water content varied between 56,24 and 63,79%, and the ash content varied between 0,57 and 1,62% of wet weight herring samples. The cook loss varied between 4,25 and 12,24% and the lipid content varied between 17,93 and 29,91% of herring samples. No correlations were found between water content and cook loss. The total variation of L*-value was between 63 and 75 CIE units, the a*-value between -2,07 and 2,54 CIE units, and the b*-value between 14,67 and 18,66 CIE units. No correlations were found between L*-value and cook loss of the different sample groups. No correlations were found between a*-value and peroxide value, conjugated dienes, conjugated tetraenes and Schiff bases. No correlations were found between b*-value and conjugated dienes, conjugated tetraenes, TBARS and Schiff bases. Only the statistical analysis of peroxide value and b*-value for herring sample with addition of olive leaf extract showed a significant ($p < 0,05$) correlation (R = 0,70).

Further investigations are needed to study the influence of the concentration effect of the antioxidant extracts on lipid oxidation stability in fish products. Overall, the herring samples are considered to be in the propagation step. After 20-days of cold storage, all herring samples are regarded as rancid.

Sammendrag

Oregano-ekstrakt var den eneste antioksidant ekstraktet som viste potensiale til å forsinke lipidoksidasjonen i fisk under lagring. Oregano-ekstrakt hadde effektivitet til å forsinke lipidoksidasjon i fisk, mens olivenblad-ekstrakt, brennesle-ekstrakt og dill-ekstrakt ikke viste noen effekt på lipidoksidasjon.

Peroksidverdier varierte mellom 3,56 – 36,95 $m_{eq}O_2$/kg lipid under lagringsperioden. Noen sildeprøver lå under peroksidverdi grensen for akseptabilitet 5 $m_{eq}O_2$/kg lipid og regnes som ikke harske, mens andre prøver som lå over grensen regnes som harske. Innholdet av konjugerte diener og konjugerte tetraener varierte henholdsvis mellom 7,68 – 14,53 mL/g, og mellom 1,40 – 5,83 mL/g.

Tiobarbitursyre-reaktive-stoffer (TBARS) varierte mellom 1,47 – 13,15 µMol TBARS/g lipid. Alle prøver av sild hadde TBARS-verdier høyere enn TBARS-inntaksgrensen på 2 µMol TBARS/g lipid, utenom sildeprøver med tilsatt brennesle-ekstrakt fra dag 18 som var den eneste prøven som lå under TBARS-inntaksgrensen. Skift base verdier måler mengden av protein-lipid komplekser dannet mellom sekundære lipid oksidasjonsprodukter og proteiner i sildeprøver under lagring. Skift base verdien varierte mellom 0,31 – 0,88 mL/kg lipid under lagringstiden.

Vanninnholdet varierte mellom 56,24 – 63,79% og askeinnholdet varierte mellom 0,57 – 1,62% av våtvekten i sild. Koketapet varierte mellom 4,25 – 12,24% og fettinnholdet varierte mellom 17,93 – 29,91% i sild. Ingen korrelasjoner ble funnet mellom vanninnhold og koketap. Total variasjonen av L*-verdier var mellom 63 – 75 CIE enheter, a*-verdier mellom -2,07 – 2,54 CIE enheter og b*-verdier mellom 14,67 – 18,66 CIE enheter. Ingen korrelasjoner ble funnet mellom L*-verdi og koketap. Ingen korrelasjoner ble funnet mellom a*-verdi og peroksidverdi, konjugerte diener, konjugerte tetraener og Skift baser. Ingen korrelasjoner ble funnet mellom b*-verdi og konjugerte diener, konjugerte tetraener, TBARS og Skift baser. Den eneste signifikante korrelasjonen mellom peroksidverdier og b*verdier ble funnet for sildeprøver med tilsatt olivenblad-ekstrakt som viste en signifikant ($p < 0,05$) korrelasjon ($R = 0,70$).

Videre studier behøves for å studere innflytelsen av økt konsentrasjon av antioksidant ekstrakter på lipidoksidasjon stabilitet i fiskeprodukter. Alle sildeprøver er vurdert til å være i propageringsfasen. Etter 20-dagers lagring, er alle sildeprøver vurdert som harske.

Table of content

1. Introduction

1.1 Background

Pelagic fish per definition means fish which can be found in the middle depths or near the ocean surfaces. These areas are referred to as pelagic zones and are the largest habitats on earth, encompass a wide variety of different pelagic fish species.

Pelagic fish species have high nutritional value due to their high content of polyunsaturated fatty acids (PUFAs), selenium, vitamin D and proteins. These nutrients have positive benefits on the human health and may reduce the risk of cardiovascular diseases. Atlantic herring is a pelagic fish caught along the Norwegian coast, and is also found on both sides of the North Atlantic Ocean. Atlantic herring can contribute to a healthy diet. However, the nutrients in Atlantic herring, especially marine lipids and proteins are susceptible to oxidation during handling, transportation and processing. This leads to loss of fish quality and results in formation of undesirable off-flavours, odour and taste with negative impact on consumer acceptance. Optimisation of processing along the value chain is important to obtain high quality products and prolong the shelf-life.

The dietary patterns and the lifestyle of people are changing. Previously, due to a time-stressed lifestyle, the consumers had a high intake of fast and processed food. However, today the consumers have a more negative perception of industrial food and prefer to consume less processed foods. Therefore, there is an increasing consumer demand for healthy and convenient fish products which at the same time are more fresh-like (less processed) but have a long shelf-life and are safe. Today people eat mackerel and herring either frozen, canned, marinated or fresh. To increase the consumption and receive the healthy benefits of pelagic fish, sous-vide cooking treatment might be one way to make new consumer-friendly ready-to-eat products by retaining high quality, improved shelf-life of fish products and make them more convenient for the consumer.

The use of antioxidants is a strategy to extend the shelf-life of fatty fish species. The food industry uses synthetic antioxidants like BHT and BHA which have good antioxidant properties. However, consumers are becoming more aware of the safety and health in their diets. Due to suspicion of potential effects of synthetic antioxidants, the consumers want the use of natural antioxidants.

This master thesis is a continuation of the specialization project, "Processing to retain quality and stability of healthy nutrients in model herring products" (Vu, 2018). In the project work, herring fillets with and without addition of Fortium TR25 antioxidant (based on ascorbyl palmitate, rosemary extract and tocopherol) were subjected to sous-vide cooking and stored for 13 days in a cold room at +4°C. Colour, lipid content, primary lipid oxidation products (peroxide value (PV-value), conjugated dienes and conjugated tetraenes (CD and CTe)) and secondary lipid oxidation products (thiobarbituric acid reactive substances (TBARS)) were measured and the extent of lipid oxidation was determined as a function of storage time.

The aim for this continuation of the work is to study the influence of different natural antioxidants obtained from Mediterranean plants on lipid oxidation stability of sous-vide treated Atlantic herring during chilled storage. During the cold storage, water and ash content, cook loss, lipid content, primary lipid oxidation products (peroxide value (PV-value), conjugated dienes and tetraenes (CD and CTe)), secondary lipid oxidation products (thiobarbituric acid reactive substances (TBARS) and Schiff bases (SBs)), and colour were measured. Herring fillets without addition of antioxidants and herring fillets with addition of antioxidants were compared in terms of quality parameters during 20 days of storage in the cold room at +4°C. The extent of lipid oxidation was determined as a function of storage time.

The introduction in this master thesis is built on and is an expansion of the introduction presented in the specialization project performed in Autumn 2018.

1.2 Atlantic herring

The Atlantic herring (*Clupea harengus*) is a pelagic fish living in the North-East Atlantic (Stenevik, 2019). The herring has a streamlined, silver-coloured body (Figure 1). The Norwegian herring spawns in February to March in a region in Norway called Møre. The herring can also spawn along the coast of Vesterålen and Nordland (Stenevik, 2019). The Norwegian spring-spawning herring have a maximum lifespan of 25 years and may reach a maximum size of 40 cm in length and weight 0,5 kg (Havforskningsinstituttet, 2019).

Figure 1: The Atlantic herring (Clupea harengus) consist of important components such as proteins, omega-3, vitamin D, selenium and fatty acids. Adapted from SFN (Seafood from Norway) (2019).

The Atlantic herring is a fatty fish and is a good source of polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA, C22:6n-3), eicosapentaenoic acid (EPA, C20:5n-3) and the monosaturated fatty acid, cetoleic acid (C22:1n-11) (Jensen et al., 2007; Stankova et al., 2018). According to Delgado-Lista et al. (2012), DHA and EPA have beneficial effects on human health with regard to cardiovascular and inflammatory diseases. However, a strong limiting factor during storage and processing of herring is the oxidation of lipids which causes discolouration, loss of nutritional value, formation of off-flavour and have the potential to generate toxic compounds which may have negative effects on human health (Erickson, 1997; Lauritzsen et al., 1999; Vu, 2018). Lipid oxidation during storage and processing will be further described in section *1.4.3 Factors influencing lipid oxidation during storage and processing*.

1.3 Chemical composition of fish muscle

The chemical composition of herring varies both with maturity and season (FAO, 2001). When herring approaches spawning, the lipid is being used to build up gonads and the lipid content is low. After spawning, the lipid content starts to increase (Røjbek et al., 2013). The lipid content of herring varies over a wide range. In a batch of herring caught at the same time, the lipid content may vary from 1 – 20% (Anthony et al., 2000), while Aro et al. (2005) gives a variation

range of 12 – 17%. Over 50% of the lipid consists of monounsaturated fatty acids and over 12% of the lipid is made up of EPA and DHA (Aro et al., 2005).

In herring muscle, the amount of fat and proteins together makes up around 80% of the wet weight. This means that as the fat content increases, the water content in herring decreases (FAO, 2001). The results of water, protein and fat content from the study of Bisenius et al. (2020) are consistent with the range reported in FAO (2001). The composition of whole herring and in herring flesh is given in Table 1. Table 2 shows the composition of untreated herring caught from the North and Baltic sea. For more details, the reader is referred to the paper of Bisenius et al. (2020).

Table 1: The main components in whole Atlantic herring and in herring flesh. The amount of each component is given in percentage of mass (FAO, 2001).

Components	Whole herring [%]	Herring flesh [%]
Water	60 – 80	57 – 79
Fat	0,4 – 22	0,8 – 24,9
Protein	16 – 19	14 – 17

Table 2: The main components of untreated herring (n = 48). B09 and B11 refers to the Baltic sea area where the fish was caught. Adapted from Bisenius et al. (2020).

Components	North Sea [%]		Baltic Sea [%]	
	May n = 12	November n = 12	December n = 12 B09	December n = 12 B11
Water	67,3	70,9	77	70,9
Fat	13,0	9,6	3,5	10
Protein	17,3	18,3	18,3	18,4

Herring muscle also contains some carbohydrates, fat soluble vitamins (A, D and E), water soluble B vitamins (riboflavin, folate, niacin, pyridoxine, cobalamin, pantothenic acid and thiamine) and minerals (iodine, selenium, chromium, zinc and iron) (Havforskningsinstituttet, 2019; Vu, 2018).

Pelagic fish like mackerel and herring have a high proportion of dark muscle which contains non-enzymatic (e.g. heme proteins like myoglobin and haemoglobin) and enzymatic (lipoxygenases) prooxidants, low-molecular-weight (LMW) metals and microsomal enzymes (Undeland et al., 1998; Abdollahi and Undeland, 2019; Hematyar et al., 2019). The dark muscle is therefore more susceptible towards lipid oxidation and results in a faster degradation compared to the light muscle (Dang et al., 2017).

1.4 Handling and transportation

1.4.1 Handling at sea

Herring needs careful handling and rapid cooling after catch because it is a highly perishable fish (Nielsen and Hyldig, 2004; Vu, 2018). The same fishing method is applied for both herring and mackerel (McDonald, 2001). The mackerel quality starts to deteriorate already in the fishing net during capture and continues throughout the handling and storage process (Digre et al., 2006; Digre and Jes, 2005; Sone et al., 2019). Fishing gear, hauling time, storage temperature, pumping and weather conditions are the factors which may influence the mackerel and herring quality onboard fishing boats. According to Pedersen (1989), the fish should be chilled as fast as possible to achieve long shelf-life. Delayed cooling at different temperature levels right after catch affects the shelf-life of the product (Figure 2).
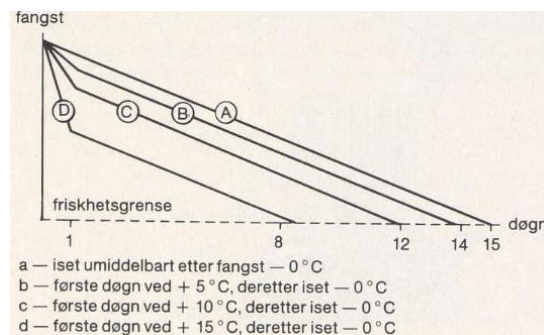
Figure 2: Shelf-life of fish as a function of storage temperature right after capture. (a) Fresh fish stored at 0°C right after capture have a shelf-life of 15 days. (d) Fresh fish stored at 15°C the first 24 hours after capture then stored at 0°C have a shelf-life of 8 days. Adapted from Pedersen (1989).

Efficient chilling methods are used to prevent spoilage of fresh herring after capture (Huss, 1995). The traditional methods used to chill the fish have been with ice, but this could damage the fish. The fish is stowed with layers of ice between, below and above the fish. If the catching is performed in larger amounts, the herring may sometimes be stowed in bulk in the storage

room without and with ice. The fish will not be iced adequately and some fish may be damaged in deep bulk stowage (Stroud, 2001).

A more efficient chilling method is to store fish in tanks onboard the fishing vessels filled with refrigerated sea water (RSW) (Thorarinsdottir et al., 2008). However, according to Madmud et al. (2018) the RSW-system have some drawbacks. Loss of proteins, excessive uptake of salt, problems with anaerobic bacteria, uptake of water by low fat species and the chance of spreading spoilage microbes in the circulating water are potential problems. According to Graham et al. (1992), RSW also has many advantages. The RSW-system has: 1) quicker handling of fish in large quantities with less labour involvement and little delay, 2) greater speed of cooling, 3) low holding temperature, 4) reduced pressure on the fish and 5) improved handling and reduced quality loss of fish products. RSW has been shown to be more efficient in preserving fish than the use of ice (Rustad, 2019).

1.4.2 Handling after capture and freezing storage

Whole ungutted herring that is properly stored at -30°C within a few hours after capture may retain high quality up till 7 months (Dang et al., 2017; Vu, 2018). The fat content of the herring determines how long after capture it could be stored at -30°C before lipid oxidation becomes prominent. A herring with high fat content should be frozen no later than 18 hours after catch, and the herring should be properly chilled between catching and freezing (Rahaman, 2014). According to World Food Logistics Organization (WFLO, 2008), fatty fish stored at -18°C have a shelf-life of 6 months without commercially significant loss in quality. By storing a fatty fish at
-30°C, its shelf-life may be between 6 – 18 months (WFLO, 2008). Aubourg et al. (2002) found that storing horse mackerel on ice for 3 – 5 days before freezing at -20°C have reduced the shelf-life by 4 months compared to fish frozen at -20°C, 0 – 1 days after slaughter which had a shelf-life up to 7 months. In the study of Tenyang et al. (2019), red carp (*Cyprinus carpio*) immediately stored at -18°C had a shelf-life of 3 months. However, the shelf-life varies from species to species, fat and water content due to seasonal variations and greatly depends on how the product has been packaged (Johnston, 1994; Huss, 1995).

1.4.3 Factors influencing lipid oxidation during storage and processing

There are many factors that influence lipid oxidation during storage and processing of fish. These includes sexual maturity, size, fishing ground, storage time before freezing and fish species (Makris et al., 2019; Pedersen, 1989; Undeland et al., 1998; Vu, 2018). The proportion of dark muscle, the amount of unsaturated fat, the amount of heme proteins and the activity of enzymes are factors that are directly linked to the fish itself (Undeland et al., 1998; Hematyar et al., 2019), while exposure to light, oxygen, transition metals and temperature fluctuations are processing factors (Singh et al., 2018; Mozuraityte, 2007; Dang et al., 2017).

In the study of Hematyar et al. (2019), haemoglobin and myoglobin are prooxidants which may be released during the post-slaughter process. Haem iron (ferrous (2+)) is converted to ferric (3+) which may increase the lipid oxidation progress in fish fillets and other muscle foods (Kanner, 1994). Furthermore, the protein oxidation of myoglobin, from ferrous oxymyoglobin (Fe^{2+}) oxidized to ferric metmyoglobin (Fe^{3+}) leads to a discolouration reaction in fish fillets (Papuc et al., 2017). Moreover, secondary lipid oxidation products (e.g. aldehydes) may alter the myoglobin structure via covalent bonds and change the fillets colour during storage (Lynch and Faustman, 2000).

Richards & Hultin (2003) found that hemolysate from pelagic fish species like mackerel and herring was a more powerful prooxidant than cod samples containing trout blood. Fatty fish fillets (e.g. herring) containing blood residue after filleting may catalyse lipid oxidation during cold storage (Hematyar et al., 2019). Furthermore, some enzymes like myeloperoxidase and lipoxygenases are capable of catalysing lipid oxidation. Myeloperoxidase initiates lipid oxidation in presence of hydrogen peroxide and halides. Lipoxygenases exist in the fish gills and skin, and catalyse the incorporation oxygen into an unsaturated fatty acid and generate hydroperoxide (ROOH) (Hematyar et al., 2019).

There are several processing factors that may influence lipid oxidation (Vu, 2018; Singh et al., 2018; Taylor, 1987; Undeland et al., 1998; Mozuraityte, 2007; Cropotova et al., 2019a). The rate of lipid deterioration depends on the exposure of light and oxygen on the fish muscle (Singh et al., 2018). Oxygen reacts with the unsaturated fatty acid chain, producing radicals that further reacts with other unsaturated fatty acid chain. The lipid oxidation mechanism is further described in section *1.6 Lipid oxidation*. Transition metals may enter the fish by the water used in processing equipment and via salt and spices (Taylor, 1987). Transition metals such as copper, iron and cobalt may enhance and initiate lipid oxidation by catalysing cleavage of hydroperoxides. These metals are facilitating the transfers of electrons which increases the rate

of free radical formation (Mozuraityte, 2007). Transition metals will be further described in section *1.6.2 Propagation*. Temperature fluctuations may increase lipid oxidation and enzymatic activity in the fish muscle. Olafsdottir et al. (2006) found that in the storage of superchilled cod (Gadus morhua), the temperature fluctuations during processing of cod samples affects the shelf-life. The processed cod samples stored at 0,5°C had shorter shelf-life compared to cod samples stored at -1,5°C. In the study of Mu et al. (2017) on chilled saithe (*Pollachius virens*) fillets, it was confirmed that the higher temperature levels during processing before cold storage affects the shelf-life of saithe fillets. The first group of saithe fillets were stored on slurry ice after filleting before being packed in polystyrene (EPS) boxes with zero holding time at 16°C, and stored in the cold room at 2°C. The second and the third group of saithe fillets had a holding time of 1 and 2 hours at 16°C before packing and cold storage at 2°C. The first group of saithe fillets had a shelf-life of 14 days compared to the second and the third group which had a reduction in shelf-life of 2 days and 4 days, respectively.

1.5 Sous-vide cooking

The French term "sous-vide" means "under vacuum" cooking. The cooking is defined as thermal treatment of raw materials under controlled temperature and time inside a heat-stable vacuumized pouch. This method uses low heating temperature (below 100°C), have longer cooking times compared to traditional cooking, with a rapid cooling to 0 – 3°C and is stored in the cold room (Schellekens, 1996; Vaudagna et al., 2002; Vu, 2018).

The sous-vide process has been actively studied by food scientists since the 1990s (Mossel and Struijk, 1991; Ohlsson, 1994; Schellekens, 1996; Cropotova et al., 2019a; Cropotova et al., 2018; Cropotova et al., 2019b; Cropotova et al., 2019c; Aftret, 2018; Vu, 2018; Baldwin, 2012; del Pulgar et al., 2012), and there has been increasing interest to use sous-vide cooking to produce consumer friendly products with an extended shelf-life. However, the success of sous-vide food relies on high hygienic standard and also time-temperature regimes (Smith and Sewell, 2006; Baldwin, 2012).

Sous-vide cooking differs from traditional cooking in two fundamental ways (Schellekens, 1996; Baldwin, 2012). The raw materials are sealed in heat-stable vacuumed pouches and cooked under precise temperature control. The vacuum pouch has many benefits. It can increase the food's shelf-life by eliminating contamination and recontamination during storage, allows the heat to be transferred efficiently from steam or water to the food, prevents loss of moisture

and evaporation of flavour volatiles, protects the food and makes a consumer friendly product which have a reduced rate of lipid oxidation, and ensures high quality of the fish product due to reduced oxygen concentration inside the vacuum pouch (Church and Parsons, 2000; Baldwin, 2012; Oz and Seyyar, 2016; Díaz et al., 2009).

Raw materials which has gone through a sous-vide cooking process can have a shelf-life between one to four weeks depending on the composition of the product and type of food (Rosnes et al., 2011; Aftret, 2018; Vu, 2018; Cropotova et al., 2019b; Cropotova et al., 2019c; Cropotova et al., 2018). However, there is still oxygen in the fish muscle after vacuum packing that may affect the oxidation and aerobic microbial growth (Mbarki et al., 2009).

1.6 Lipid oxidation

Lipid oxidation is a complex process and is usually divided into initiation, propagation and termination steps (Figure 3) (also presented in Vu (2018)). In the initiation step, a labile hydrogen atom is abstracted from a site on the fatty acyl chain (RH) and produces a free lipid radical (R*). In the propagation step, the free lipid radical (R*) reacts with oxygen and produces a peroxyl radical (ROO*). The peroxyl radical (ROO*) may react further and subtract a hydrogen atom from another fatty acid chain (RH), producing another lipid hydroperoxides (ROOH) and another free lipid radical (R*). In the last step, the termination step, the radicals will react with each other to form non-radical products (Pearson et al., 1977; Mozuraityte, 2007).

Initiation:	$RH \rightarrow R* + H*$
Propagation:	$R* + O_2 \rightarrow ROO*$
	$ROO* + RH \rightarrow ROOH + R*$
Termination:	$ROO* + ROO* \rightarrow ROOR + O_2$
	$R* + R* \rightarrow R_2$
	$R* + ROO* \rightarrow ROOR$
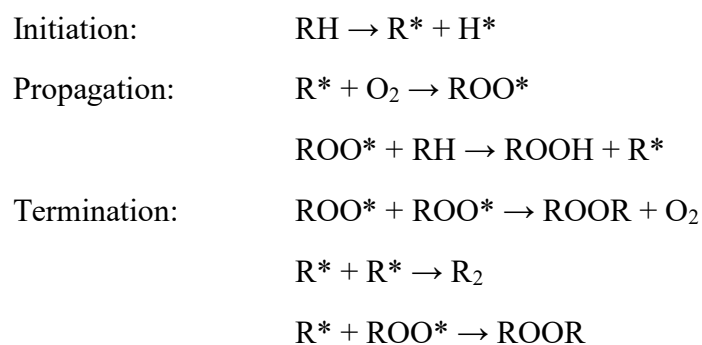
Figure 3: The general autooxidation of unsaturated fatty acids by a radical mechanism in an initiation, propagation and termination steps. Adapted from Paquette et al. (1985).

1.6.1 Initiation

During the initiation step, formation of highly unstable free radicals (R*) takes place. Atmospheric oxygen or triplet oxygen ($^3O_2$) have electrons spinning in the same direction while electrons in the double bond (C=C) of PUFAs spin in the opposite direction (Figure 4). The spin barrier between triplet oxygen and the fatty acid chain may be readily overcome in the presence of initiators (transition metals or reactive oxygen species) (Mozuraityte, 2007). This will overcome the dissociation energy of the allylic bond and cause abstraction of hydrogen from the fatty acid chain (RH) to form an alkyl radical (R*) (Figure 5) (Mozuraityte, 2007). The more unsaturated fatty acid, the higher formation of fatty acid radicals (Damodaran and Parkin, 2017). Once a lipid radical is formed, the chain reaction or the propagation begins (Mozuraityte, 2007).
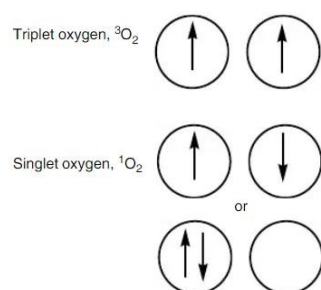
Figure 4: The triplet oxygen ($^3O_2$) is biradical due to two electrons in the antibonding 2p orbital which have the same (antiparallel or parallel) spin direction. Singlet oxygen ($^1O_2$) have electrons with opposite spin in the antibonding 2p orbital (Damodaran and Parkin, 2017). Adapted from Parkin (2008).
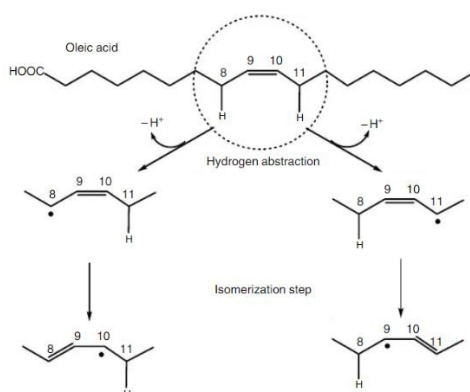
Figure 5: Oleic acid reacting in the initiation step to give cis or trans configuration of alkyl radical. Adapted from Damodaran & Parkin (2017).

1.6.2 Propagation

In the propagation step, the produced alkyl radical (R*) may react with a diradical triplet oxygen to form a peroxyl radical (ROO*) (Mozuraityte, 2007). The reaction between oxygen (O_2) and alkyl radical (R*) has a very low activation energy (Min, 1998). The reaction rate is also very fast (Ivanov, 1985). The high energy of peroxyl radicals (ROO*) allows them to abstract hydrogen from another fatty acid molecule (RH). This results in a formation of fatty acid hydroperoxide (ROOH) and the occurrence of a new alkyl radical (R*) on the fatty acid molecule (Damodaran and Parkin, 2017). Therefore, a single initiation event may result in conversion of hundreds of fatty acid side chains into lipid hydroperoxides, accompanied by oxygen consumption (Gutteridge and Halliwell, 1990). Since the carbon-hydrogen covalent bond of unsaturated fatty acids is weak, they are susceptible to attack from peroxyl radicals (Figure 6) (Mozuraityte, 2007). Therefore, PUFAs such as DHA and EPA in herring are so susceptible to lipid oxidation (Undeland et al., 1998).

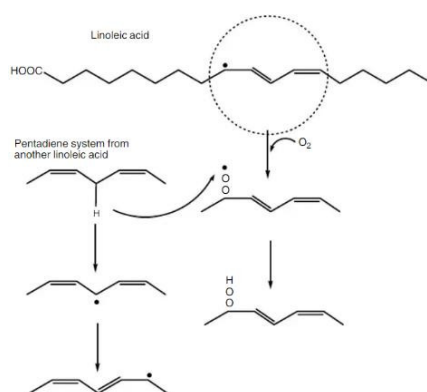*Figure 6: Linoleic acid reacting with oxygen in the propagation step to form peroxyl radicals. Adapted from Parkin (2008).*

Hydroperoxides may further be degraded (due to heat, UV-light or transition metals) and produce secondary lipid oxidation products (Frankel, 1984). These secondary oxidation products (e.g. aldehydes, alcohol and ketones) give a rancid smell (Figure 7) (Mozuraityte, 2007).
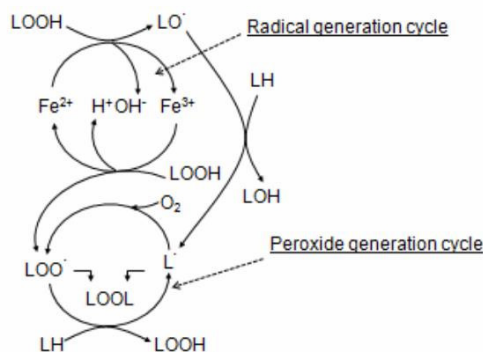
Figure 7: Radical generation cycle further degrades primary lipid oxidation compounds to form secondary lipid oxidation products (top cycle). Peroxide generation cycle produces primary lipid oxidation products from PUFAs and oxygen due to initiators (transition metals, bottom cycle). Adapted from Mozuraityte (2007).

As mentioned in section *1.4.3 Factors influencing lipid oxidation during storage and processing*, transition metals like iron and copper may enhance lipid oxidation. Two reactions may occur as seen in equation a and b, and Figure 7. In the first reaction (equation a), a transition metal (in this example iron(II)) is reacting with a hydroperoxide compound (ROOH). Iron(II) is oxidized to iron(III) creating an alkoxide radical compound (RO*) and a hydroxide (OH$^-$). In the second reaction (equation b), the oxidized iron(III) can then react with another hydroperoxide compound, be reduced to iron(II) which creates another radical (RO$_2$*) and a proton (H$^+$) (Mozuraityte, 2007). The relative effectiveness of transition metals to degrade hydroperoxides, when added to fish homogenate is: $Fe^{2+} > Cu^{2+} > Fe^{3+}$ (from high effectiveness to low effectiveness) (Mizushima et al., 1977).

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO* + OH^- \qquad (a)$$

$$Fe^{3+} + ROOH \rightarrow Fe^{2+} + RO_2* + H^+ \qquad (b)$$
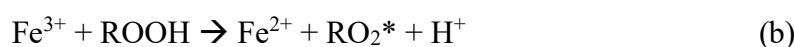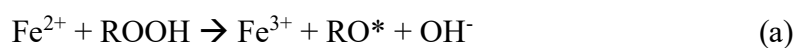
1.6.3 Termination

In the termination step, peroxyl radicals (ROO*, R*) will react with each other forming bridged dimer compounds such as fatty acid dimer (R-R) and peroxide bridge dimer (ROOR or ROOR + O$_2$) due to high concentration of formed radical (ROO*, R*) (Figure 3) (Gill and Tuteja, 2010). Thus, under atmospheric conditions, the termination reaction will occur between two peroxyl radicals and the sequence of the autooxidation reaction will continue (Damodaran and Parkin, 2017; Colakoglu, 2007; Mozuraityte, 2007). In the next section *1.7 Antioxidant*, antioxidants which have the ability to reduce the rate of lipid oxidation formation are described.

1.7 Antioxidants

To overcome the challenges with lipid oxidation and the problems with quality loss, antioxidants can be used to retard oxidation of lipids in fish products (Choe and Min, 2006; Aftret, 2018). The desirable properties of an antioxidant is to have no objectionable colour, odour or flavour, be nontoxic, effective in low concentration in a wide of variety of fats, be allowed to be used in food products, process versatility for use in a variety of food applications, be heat stable and have a high "carry-through-ability" (able to withstand frying and baking), be economical (easy to make and available in quantities), and fat and water-soluble (Shahidi, 2015). Synthetic antioxidants are chemically synthesized compounds obtained under laboratory conditions (Figure 8), while natural antioxidants occurs in the nature (Figure 9) (Atta et al., 2017). Due to the inherent instability of natural antioxidants, synthetic antioxidants like BHT and BHA are being used to stabilize fats and oils (Simec and Porter, 1980). Some studies have shown that BHT and BHA may have adverse health effects when used at concentration above the safety limit (Pereira et al., 2015). Despite that BHA and BHT are "generally accepted as safe", some chronic toxicity studies have implicated BHT as a potential tumour promoter when fed at high levels (Kaczmarski et al., 1999; Witschi, 1981). In addition, BHA and BHT may cause mutation and damage in DNA, and trigger the onset of neoplasia (Pereira et al., 2015). Furthermore, blood cholesterol level may increase and the risk of destruction of important compounds such as vitamin D may occur (Aun et al., 2011). Therefore, there have been attempts to remove these antioxidants and replace them with natural antioxidants (Benson et al., 1979). Desirable properties of natural antioxidants are to increase the shelf-life of food by reducing the oxidation rate of pigments, proteins and lipids, while preserving attributes such as aroma, taste, texture, colour, and without negative impacts on the human health (Ordóñez and García de Fernando, 2005; Fennema et al., 2010; Pizzino et al., 2017).
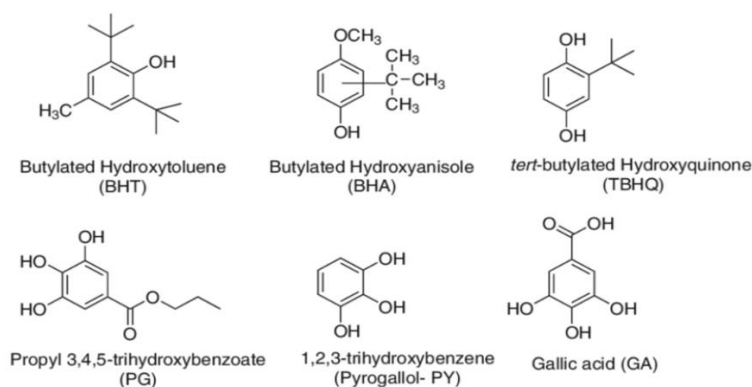

Figure 8: Some synthetic antioxidants obtained under laboratory conditions in the food industry e.g. from vitamin E (Vitalend, 2019). Adapted from Martins et al. (2018) and Bhalerao (2016).
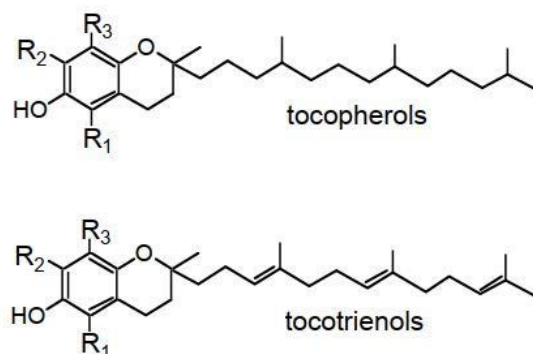
Figure 9: Natural oxidants tocopherols and tocotrienols. Adapted from Bartosińska et al. (2016).

There are several mechanisms by which an antioxidant acts to reduce oxidation rate in foods. These include free radical scavenging, metal chelating, singlet oxygen quenching, photosensitizer inactivation and inactivating lipoxygenase (Choe and Min, 2009). The most important antioxidant mechanisms in food are free radical scavenging and metal chelating (Shahidi, 2015). A combination of two or more antioxidant mechanisms could give a synergetic effect. Synergism can be reached in a system of antioxidants with several different antioxidant mechanisms (Choe and Min, 2009).

Free radical scavenger antioxidants (chain breaking antioxidants) e.g. tocopherol can transfer its own hydrogen to a free radical. The chain breaking antioxidants repair the oxidizing radical directly (Hatwalne, 2012). In the first reaction, a radical (RO_2*) is reacting with an unsaturated fatty acid chain (RH) producing a hydroperoxide (ROOH) and a new radical (R*) that may further react with another unsaturated fatty acid chain to produce a new radical (Figure 10). In the second step, with an antioxidant (AH) added to the system, the antioxidant can transfer its hydrogen by sacrificing itself to a radical (RO_2*), creating a hydroperoxide (ROOH), but stops a new radical from being formed (Figure 10). The antioxidant which gave away its own hydrogen is now a resonance stabilised radical (A*)**,** a non-propagating compound (Choe and Min, 2009).

$$RO_2* + RH \rightarrow ROOH + R* \qquad\qquad k_3$$

$$RO_2* + AH \rightarrow ROOH + A* \qquad\qquad k_7$$

Figure 10: An example of a chain breaking antioxidant (free radical scavenger). An antioxidant (AH) in step k_7 reacting with a radical (RO_2*) and stopping the autoxidation. $k_7 >> k_3$ leads to successful interference on the autoxidation. A is a non-propagating species (stable product). Adapted from Jain & Sharma (2011).*

Metals chelators decrease oxidation by preventing metal redox recycling, providing steric hindrance between food components and metals by forming insoluble metal complexes (Graf and Eaton, 1990). Citric acid and EDTA are the most common metal chelators used in food (Choe and Min, 2009). EDTA is a synthetic chelating agent which can bind transition metals (e.g. Cu^{2+}, Fe^{2+}) on its negatively charge site to retard lipid oxidation in food (McClements and Decker, 2000). However, there are some antioxidants like propyl gallate which act as an antioxidant in the absence of iron and as a prooxidant in the presence of iron. Propyl gallate binds iron(II) and in the presence of oxygen, iron(II) rapidly oxidizes to iron (III) making the iron(III)-propyl gallate complex acting as a prooxidant (Perron et al., 2010).

Essential oils (EO) are complex mixtures with low molecular weight, obtained from plant substances by solvent extraction, water distillation or steam distillation (Gomes et al., 2016). The terpenoids, phenolic and flavonoids components of EO have significant antioxidant effects (Raut and Karuppayil, 2014). Essential oils of olive leaf, oregano, nettle and dill will be further described in the next sections.

1.7.1 Olive leaf

Olea europaea L. tree, known as olive, is one of the most important components in the Mediterranean diet due to its nutritional properties, cultural influences and organoleptic characteristics (Polari et al., 2018; García-Vico et al., 2017; Fernández et al., 2018). Olive leaf extract (OLE) has many benefits for the human body, through protective effects against cardiovascular and neurogenerative diseases and protect the body from oxidative stress (Visioli, 2012; Olmo-García et al., 2017; García-Rodríguez et al., 2015; Cicerale et al., 2010; Hayes et al., 2011).

OLE is a bitter tasting, dark brown liquid and is derived from the leaves of the olive tree (Figure 11) (Şahin et al., 2017; Difonzo et al., 2017). The term "olive leaves" refers to a mixture of branches from both the harvesting and cleaning of olives, and the pruning of olive trees and leaves (Molina-Alcaide and Yáñez-Ruiz, 2008).

The health benefits of OLE are partly attributed to phenolic compounds (Rafiee et al., 2018; Quintero-Flórez et al., 2018). OLE contains different phenolic groups. This includes secoiridoid derivatives (oleuropein (aldehydic form of elenolic acid linked to hydroxytyrosol, the corresponding derivatives linked to tyrosol and dialdehydic)), phenolic alcohols (tyrosol and hydroxytyrosol), lignans (1-acetoxypinoresinol and pinoresinol), flavonoids (luteolin and

apigenin) and phenolic acids (vanillic and p-coumaric acids) (Vogel et al., 2015; Japón-Luján et al., 2006; Mateos et al., 2001; Ivanov et al., 2018; Djenane et al., 2019).

As mentioned, olive oil has a high content of polyphenols, but it has also a high content of polyunsaturated fatty acids (PUFAs). A combination of high amounts of polyphenols and unsaturated fatty acids leads to an enormous antioxidative capacity (Žanetić and Gugić, 2006; Krzeminski et al., 2003; Ferrara et al., 2000). Due to antioxidant and antimicrobial activity of OLE, it is being used as food additive (Lee and Lee, 2010; De Leonardis et al., 2018).

Figure 11: An example of an olive branch, a part of the olive tree. Adapted from Shay and Company Inc. (2019).

Olive leaf extract showed good antioxidant capacity in different fish species. OLE was found to delay lipid oxidation in fresh carp fish (*Caprinus caprio*) during cold storage (Khidhir, 2015). The antioxidant capacity was measured by determining the thiobarbituric acid reactive substances (TBARS) and free fatty acids (FFA). The fish sample treated with OLE had a TBARS-value of 2,275 mg MDA/kg lipid which was below the 8 mg MDA/kg lipid limit of acceptability (Shormüller, 1968). While, the control sample had a TBARS-value of 5,050 mg MDA/kg lipid. The batch of fish sample was stored for 7 days in a cold room at 4°C. OLE also showed good antioxidant activity with its phenolic compounds in marinated anchovies (Testa et al., 2019). The result of TBARS-value was 5,68 mg MDA/kg lipid which was below the 8 mg MDA/kg lipid limit of acceptability (Shormüller, 1968). The samples where kept in a plastic container with marinated solution and with addition of OLE, and stored for 22 days at a temperature below 5°C. These two studies show that OLE may be used to extend shelf-life of different fish products.

1.7.2 Oregano

Oregano (*Origanim vulgare L.*) is a perennial herb, containing a mixture of natural antioxidants and is one of the most favourite herb plants for production of natural antioxidant extracts (Figure 12) (Yan et al., 2016). The herb is traditionally used for flavouring meats, salads, sausages, pizza, soups, sauces and stews (Yan et al., 2016). The antioxidant effect of oregano has attracted the interest of food producers since the herb has been used for many years in traditional food without concern over safety (Camire and Dougherty, 1998). Oregano extract is a natural antioxidant which can replace synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Camire and Dougherty, 1998).

Oregano has also drawn attention of consumers due to the antifungal, antioxidative and antimicrobial effect (Bozin et al., 2006; Chun et al., 2005; Bakkali et al., 2008). The essential oil of oregano (OEO) has traditionally been used in folk medicine for indigestion, rheumatoid arthritis and respiratory disorders (Ninfali et al., 2005; Henning et al., 2011; Ivanova et al., 2005).

The main components in OEO is thymol and carvacrol which have antioxidant and antibacterial properties, followed by sabinere hydrate, terpinen-4-ol, p-cymene, linalool and γ-terpinene (Azizi et al., 2009a; Azizi et al., 2009b; Skoula and Haboerne, 2002; d'Antuono et al., 2000; Can Baser, 2008; Huang et al., 2018). The important antioxidant components in oregano are flavonoids and phenolic acids such as apigenin, hispidulin, rosmarinic acid and caffeic acid (Zheng and Wang, 2001). Based on quantity, rosmarinic acid is the dominant antioxidant among phenolic acids in oregano herb (Pizzale et al., 2002; Ranucci et al., 2015).

Figure 12: An example of an oregano plant. Adapted from Alibaba (2019).

In both meat and fish products, OEO has shown good antioxidant activities. In frozen stored chicken meat patties, OEO was found to be effectively delaying lipid oxidation (Al-Hijazeen, 2018). The antioxidant activity of OEO was due to the high content of thymol and carvacrol. At the end of the storage days (day 7), samples treated with OEO had lower TBARS-value than

samples treated with ascorbic acid and BHA, indicating that OEO could be added to reduce lipid oxidation rate in meat during storage. In cold storage of vacuumed packed tuna, the oregano extract showed good antioxidant properties (Lahreche et al., 2019). A combination of vacuum packing with addition of oregano extract to tuna fillets, showed lower TBARS-value (mg MDA/kg lipid) compared to control samples of tuna without addition of oregano extract during 18 days of storage at 3°C. Thus, oregano extract may be used by the meat processing industry and by the seafood industry to extend shelf-life of meat and seafood products (Lahreche et al., 2019; Al-Hijazeen, 2018).

1.7.3 Nettle

Nettle (*Urtica dioica L.*) is a widespread wild plant throughout the world (Latoch and Stasiak, 2017; Bourgeois et al., 2016). The word "nettle" is derived from the Anglo-Saxon word "noedl" meaning "needle". It's Latin name "*Urtica*" means "burn" which refers to the stinging effects of the tiny hairs on the leaves and stems. The plant is dark green, bright and serrated leaves in addition to stem which are covered with erect glandular hairs containing histamine and formic acid (Figure 13). Those two components give unpleasant effects. Nettle has been used to cure a wide range of diseases such as eczema, rheumatisms and arthritis (Upton, 2013).

The leaves of nettle have important antimicrobial and antioxidant effect in foods (Aksu, 2003; Ahmadi et al., 2014; Külcü et al., 2019; Jan and Singh, 2017). Components which have high antioxidant activity are polyphenols. The antioxidant activity of nettle is attributed mainly to the presence of rutin, quercetin and ascorbic acid; thus water extract of nettle (WEN) can be used to extend shelf-life of food (Jan and Singh, 2017). However, according to Pinelli et al. (2008), the antioxidant activity of nettle leaves are mainly attributed to phenolic compounds such as isorhamnetin 3-O-rutinoside, kaempferol 3-O-rutinoside, quercetin 3-O-glucoside, rutin, 2-O-caffeoymalic acid, chlorogenic acid and caffeic acid derivative.

Figure 13: An example of a nettle plant. Adapted from HerbaZest (2019).

Nettle extract has shown good antioxidant activity in different fish species. In cold storage of rainbow trout, samples treated with nettle showed good antioxidant activity compared to samples of rainbow trout treated with propyl gallate (Arashisar et al., 2008). Rainbow trout fillets with addition of nettle had lower TBARS-value compared to fillets with addition of propyl gallate during cold storage at 4°C. In superchilled minced meat of common kilka, samples treated with water extract of nettle (WAN) showed good antioxidant capacity compared to samples treated with hydroalcoholic extract of nettle (HAEN) and control sample during cold storage at -2°C for 28 days (Ahmadi et al., 2014). However, no significant differences between WAN and HAEN in terms of TBARS-values were found. The study of Ahmadi et al. (2014) concludes that WAN may be used as an effective preservative for storage of kilka fish products. However, both studies mention that more research on different fish, especially fatty fish is needed before a conclusion can be made due to indices such as peroxides, conjugated dienes and fluorescent compounds during frozen storage (Arashisar et al., 2008; Ahmadi et al., 2014).

1.7.4 Dill

Dill (*Anethum Graveolens L.*) is an important aromatic herb and is native to Mediterranean countries and south-eastern Europe (Figure 14) (Selen Isbilir and Sagiroglu, 2011). It is used in flavouring food such as sauces, seafoods, pickles, salads and soups (Słupski et al., 2005; Shyu et al., 2009). In traditional medicine, dill is used to solve gastrointestinal problems such as intestinal spasms and flatulence and to relieve fatigue in sleep disorders (Hosseinzadeh et al., 2002; Koppula and Choi, 2011). Dill essential oil (DEO) is extracted from both seeds and leaves.

The leaves of dill contain minerals (magnesium, potassium and phosphorus) and the major component of essential oil of dill are carvone, and in food industry the essential oil is used to give flavour and aroma (Jirovetz et al., 2003; Słupski et al., 2005). Besides carvone, α-terpinene, α-pinene, α-phellandrene, p-cymene, dihydro carvone, 1,8-cineole, limonene and dill apiole are present in dill seeds essential oil (Pino et al., 1995; Leung and Foster, 2003). These bioactive compounds are responsible for pharmacological effects of dill such as anti-bacterial and antioxidant activity (Stavri and Gibbons, 2005; Stanojevic et al., 2016).

Figure 14: An example of a dill plant. Adapted from Click&Grow (2019).

In both fish and meat products, dill extract has shown good antioxidant properties. In cold storage of mackerel fillets, samples treated with dill extract obtained by microwave oven boiling (MOB) displayed good antioxidant effect (Kannaiyan et al., 2015). The mackerel samples were placed in polythene bags and stored in a cold room at 4°C for 12 days. The TBARS-value of mackerel fillets with addition of dill extract were below 5 mg MDA/kg lipid, the TBARS-limit of acceptability (Shormüller, 1968). In cold storage of beef, plantago major seed mucilage (PMSM) containing DEO enhanced the shelf-life of beef (Behbahani et al., 2017). The beef was stored at 4°C for 18 days. The TBARS-value of PMSM with 1,5% coating of dill extract was 0,31 mg MDA/kg lipid and the peroxide value was 4,7 m_{eq} peroxide/kg beef after 18 days of storage. However, the low TBARS and peroxide value may be due to both PMSM and DEO containing phenolic compounds where both contributed to antioxidant activity. These two studies results showed that dill essential oil could be used to extend shelf-life of fish and meat products.

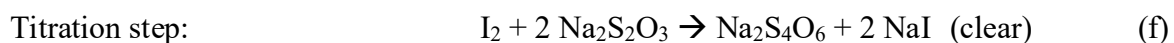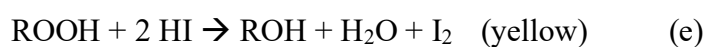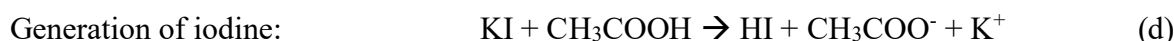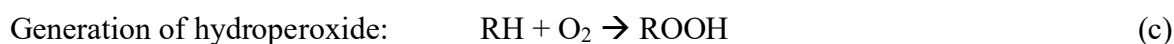## 1.8 Analytical methods for determination of lipid oxidation products

The measurement of lipid oxidation can be difficult because of formation of many different oxidation products. Since many oxidation products are formed, it is necessary to measure both primary and secondary lipid oxidation products.

Peroxide value (PV) analysis measures the amount of hydroperoxides (primary lipid oxidation products) in samples (Schaich, 2016). Conjugated dienes (CD) and conjugated tetraenes (CTe) measures the amount of primary lipid oxidation products by UV-absorption (Undeland et al., 1998). Thiobarbituric acid reactive substances (TBARS) value measures secondary lipid oxidation products, the amount of aldehydes (Frankel, 1998). Schiff bases (SBs) analysis is used to measure secondary lipid oxidation products, the cross-linking between protein and secondary lipid oxidation products (Børresen, 2008; Pokorny et al., 1974). Colour measurement is used to monitor changes of colour in fish samples during storage (CIE, 2001). Each method will be described more in the next section.

1.8.1 Determination of primary and secondary lipid oxidation products

Primary lipid oxidation products may be determined by measuring PV. There are many methods to measure PV. However, titration of sodium thiosulfate against sample containing potassium iodine is commonly used (Toledo, 2019).

A saturated potassium iodine solution is used as indicator. The generation of hydroperoxides can be seen in equation c. Equation d shows the dissociation of potassium iodine in acid. Hydrogen iodine (HI) reduces the lipid hydroperoxides (ROOH) to hydroperoxyl derivatives (ROH), giving a yellow colour to the solution (equation e). The amount of iodine consumed is equal to the amount of hydroperoxides present in the sample. In the titration step the solution goes from a yellow to a clear solution when sodium thiosulphate reacts with dissolved iodine (equation f). (Schaich, 2016).

Generation of hydroperoxide: $\quad RH + O_2 \rightarrow ROOH$ $\qquad\qquad\qquad\qquad$ (c)

Generation of iodine: $\quad KI + CH_3COOH \rightarrow HI + CH_3COO^- + K^+$ $\qquad$ (d)

$\qquad\qquad\qquad\qquad ROOH + 2\,HI \rightarrow ROH + H_2O + I_2$ (yellow) $\qquad$ (e)

Titration step: $\quad I_2 + 2\,Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2\,NaI$ (clear) $\qquad$ (f)

An increasing PV is an indicator of a beginning rancidity in fats and oils (Schaich, 2016). According to CODEX STAN 329 (2017), the PV-limit for fish lipid is ≤ 5 $m_{eq}O_2$/kg lipid. Lipids over 10 $m_{eq}O_2$/kg lipid are considered rancid (Schaich, 2016). Low PV may indicate low oxidation or high oxidation levels (Figure 16).

Other methods are the measurement of conjugated dienes (CD) and conjugated tetraenes (CTe) by UV-absorption. Non-conjugated double bonds that are presented in natural unsaturated lipids are converted to conjugated double bonds (primary lipid oxidation products) after the formation of peroxides. CD and CTe absorb UV-light at 233 nm and 315 nm respectively and this can be measured using a spectrophotometer (Jackson, 2016; Undeland et al., 1998).

Thiobarbituric acid reactive substances (TBARS) value is a measure of secondary lipid oxidation products. TBARS measures the amount of malondialdehyde (MDA) or to be more specific, measures the amount of aldehydes present in the sample. The aldehydes are generated from unstable lipid hydroperoxides (Corongiu and Banni, 1994; Frankel, 1998; Janero, 1990). Malondialdehyde (MDA) reacts with thiobarbituric acid and forms a pink Schiff base complex which can be measured in the wavelength region between 532 – 535 nm (Figure 15) (Antolovich et al., 2002).

TBA   Malondialdehyde
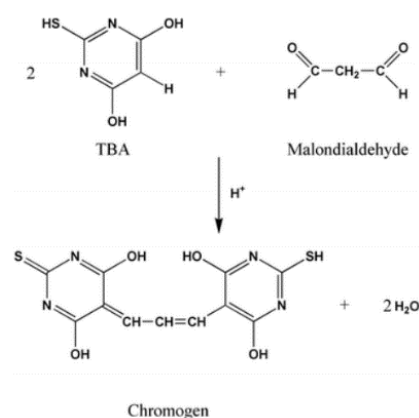
H^+

Chromogen

Figure 15: Malondialdehyde (MDA) reacts with thiobarbituric acid (TBA) which forms chromogen. Chromogen is a pink Schiff base complex and have an absorption between 532 – 535 nm (Antolovich et al., 2002). Adapted from Antolovich et al. (2002).

The TBARS analysis may lead to formation of other complexes such as acetyl aldehydes, alkenes and alkanes. Acetyl aldehydes reacts with TBA under heat and an orange complex is formed which can be measured at 496 nm. When TBA reacts with alkanes, a yellow complex is formed which can be measured between 450 – 455 nm. To prevent those side reactions, it is more suitable to perform the analysis with lipid extracts (Schaich, 2016). In this analysis, the chloroform phase is measured at 538 nm and the results may be presented either in mg MDA/kg lipid or in µMol TBARS/g lipid (Romotowska et al., 2016; Ke and Woyewoda, 1979).

The TBARS concentration in chilled and frozen fish or fish stored on ice is usually between 5 – 8 mg MDA/kg lipid. TBARS values over 8 mg MDA/kg lipid are regarded as the limit of acceptability for most species (Shormüller, 1968; Secci and Parisi, 2016). According to a more strict TBARS concentration of Ke et al. (1984), TBARS-value for fish with 0,58 mg MDA/kg lipid was not rancid, fish between 0,58 – 1,51 mg MDA/kg lipid was slightly rancid and fish with over 1,51 mg MDA/kg lipid was rancid. The TBARS-value limit for consumption is between 1 – 2 µMol TBARS/g lipid according to de Oliveira et al. (2016). In this report, the results of TBARS-value will be compared to the TBARS-value variation range of de Oliveira et al. (2016).

Schiff base (SB) measurement is used for determination of secondary lipid oxidation products. Schiff base is a tertiary product which is formed from some secondary lipid oxidation products reacting further with free amino groups of proteins. These products may polymerize into brownish-yellow pigments (Pokorny et al., 1974; Børresen, 2008). Proteins may also interact with lipid radicals or oxygen, forming a protein-lipid or protein-cross-linking (S-S bridges) complexes. Both formations may reduce the solubility of proteins and water holding capacity (WHC) (Liu and Wang, 2005; Børresen, 2008).

Colour measurement is used to monitor changes of colour in fish samples during storage. It is associated with quality changes in the fish, i.e. lipid and protein oxidation in the muscle of herring (CIE, 2001). If the colour of the herring flesh is yellow it may give an indication that lipid oxidation has taken place (Hamre et al., 2003).

As mentioned in the specialization project (Vu, 2018), Figure 16 shows an example of concentration of polyunsaturated fatty acids (PUFAs), concentration of primary and secondary lipid oxidation products as a function of time. As seen in the figure, as the amount of PUFA's decreases in the initiation step, primary and secondary lipid oxidation products are formed.

At the beginning of the initiation step, the formation of primary oxidation products increases and is higher than the degradation rate of primary oxidation products. During the propagation step, the formation of secondary oxidation products increases. The formation rate of primary oxidation products reaches a maximum before it starts to decrease. At the termination step, the formation of secondary oxidation products reaches a maximum before it evens out. The formation rate of primary oxidation products is slower than the degradation rate of primary oxidation products (Prime, 2018).
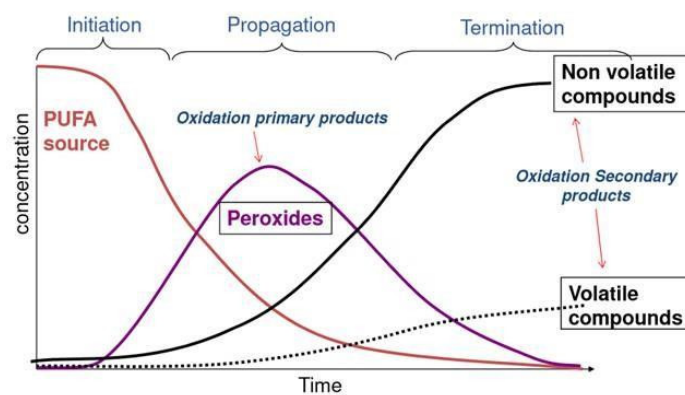
Figure 16: The graph shows the concentration of polyunsaturated fatty acids (PUFAs), the concentration of primary and secondary lipid oxidation products as function of time during initiation, propagation and termination step. Adapted from Prime (2018).

1.9 Aim

The aim is to study the influence of different natural antioxidants obtained from Mediterranean plants on lipid oxidation stability of sous-vide treated Atlantic herring during chilled storage.

1.10 Objectives

- To apply different methods to determine primary and secondary lipid oxidation products and study lipid oxidation stability of sous-vide cooked herring fillets during chilled storage.
- To compare lipid oxidation stability of the fish samples prepared with and without addition of natural antioxidants during chilled storage.
- To identify the effect of lipid oxidation on changes in colour of the fish tissue.

1.10 Flowsheet of experimental set up

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                          ┌─────────────────────┐
                          │  Fillets of herring │
                          └─────────────────────┘
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| Raw (Fillets without antioxidant or sous-vide cooking | Control (Fillets without antioxidant) | Herring + Oregano extract (fillets with antioxidant) | Herring + Nettle extract (fillets with antioxidant) | Herring + Dill extract (fillets with antioxidant) | Herring + Olive leaf extract (fillets with antioxidant) |

Vacuum sealing (Raw branch)

Vacuum sealing (Control/extracts branch)

→ **Sous-vide cooking (10 minutes, 70°C)**

→ **Stored at 4°C on ice (Change ice twice every day)**

→ **Cook loss and colour measurements**

→ **Fillets cut with a knife and minced with a mixer**

→ **Lipid extraction** → **Determination of lipid content in sample**

Remaining minced fish meat of herring stored at -20°C for other analysis

→ **Water and ash content measurement**

Chloroform extract from the samples added to separated tubes and stored at -40°C for later determination of primary and secondary lipid oxidation products

- **Peroxide value measurement (Primary lipid oxidation products)**
- **CD and CTe measurement (Primary lipid oxidation products)**
- **Schiff bases measurement (Secondary lipid oxidation products)**
- **TBARS-value measurement (Secondary lipid oxidation products)**

2. Materials and methods

This section describes how the herring fillets were prepared, what equipment was used and what chemicals were used to perform the analysis of herring samples.

2.1 Equipment

- Spray bottles (100 mL)
- Konica Minolta CR-400 colorimeter
- Benchtop mixer (Bosch)
- Ultra Turrax
- SI Analytics titrator mode TL 6000/7000 (France), consisting of:
  - Platinum electrode Pt 62/61 with cable L 1 A.
  - Basic device (titrator unit)
  - 10 mL exchange unit WA 10, with born glass bottle for titrant
  - Magnetic stirrer TM235
- Erlenmeyer flasks with stoppers (100 – 250 mL)
- Webomatic Advanced vacuum packaging systems machine
- GENESYS 12S UV-VIS spectrophotometer (Thermo Scientific, USA)
- MS2 Minishaker IKA (Vortex)
- Universal 16A-Hetlich Zentrifugen (Centrifuged)
- Luminescence spectrometer (LS 50B Perkin-Elmer, Waltham, MA, USA)
- Porcelain crucibles
- Drying oven
- Muffle furnace

2.2 Chemicals

All chemicals, unless otherwise stated, were of analytical grade.

- Antioxidant solutions (oregano, nettle, dill and olive leaf essential oil extracts, 1% antioxidant in distilled water, 100 mL)
- Sodium thiosulphate, $Na_2S_2O_3$ (0,001 M for less than 0,8 g oil and 0,01 M for more than 0,8 g oil)
- Acetic acid, CH_3COOH
- Chloroform, $CHCl_3$
- Methanol, CH_3OH
- Nitrogen, N_2-gass
- Potassium iodine, KI (0,046 mM)
- Tert-butyl alcohol (TBA)
- Butylated hydroxytoluene (3% BHT in ethanol)
- Sodium sulphite, Na_2SO_3 (0,3 M)
- Trichloroacetic acid (TCA, 0,28 M)
- 1,1,3,3 – Tetraethoxypropane (TEP, 0,01 mM)

2.3 Preparation of antioxidant extracts

The antioxidant extracts were prepared at the University of Zagreb, Department of Food Engineering, Croatia in February 2019. Dried leaves of the antioxidant samples (3 g) were prepared in a water-ethanol (ratio 1:1, 40 mL) mixture which was used as solvent. 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).

2.4 Preparation of herring fillets, vacuum packing and sous-vide cooking

Atlantic herring (*Clupea harengus*) was bought fresh from Ravnkloa and Meny (Trondheim, Norway), and stored on ice in a cold room (4 ± 1°C) for 1 – 2 days before the experiment. On Thursday the 29.08.2019, the fishes were cut open, gutted, filleted, washed, blotted dry with paper and placed on ice.

The fillets were divided into six groups: 1) Raw (N = 2), 2) Control (N = 12), 3) Herring with addition of oregano extract (N = 16), 4) Herring with addition of nettle extract (N = 16), 5) Herring with addition of dill extract (N = 16) and 6) Herring with addition of olive leaf extract (N = 16). Two fillets were placed into each of the VAKPAK-TM type vacuum bags from Lietpak (Lietpak, Čekoniškių settlement, Vilnius district, Lithuania LT-14207) with the following parameters: thickness 80 – 130 ± 5% μm, heat sealing temperature 140 – 170°C, gas permeability (O_2) 27 – 52 cc/m^2/24 h by 23°C/75% RH, and vapour permeability 1,4 – 2,3 cc/m^2.24 h. Four vacuum bags contained only one fillet due to a limited amount of herring samples. Four antioxidant solutions were prepared in a spray flask (1% antioxidant extracts in distilled water, 100 mL). The antioxidants were sprayed 4 – 5 times onto the surface of the fish fillets. The fillets were placed into the vacuum bags and transported on ice to Kalvskinnet for vacuum packing. The samples were vacuum-packed by using a Webomatic advanced vacuum packaging systems machine with vacuum extent of 99,5% (remaining air in the bag 0,5%). The vacuum-packed herring samples were transported back to Gløshaugen. All herring samples except for sample Raw were subjected to sous-vide cooking in a water bath at 70 ± 2°C. The samples were placed in the water bath and held there for 10 minutes. The cooked fish samples were rapidly chilled on ice and further stored in the cold room at 4 ± 1°C for 1, 4, 6, 8, 12, 14, 18 and 20 days. The ice was changed twice a day during the storage period (20 days). At each sampling day, one vacuum bag from each group were selected and analysed.

2.5 Analytical methods

2.5.1 Cook loss

The cook loss was determined as described by Cropotova et al. (2019c). The sous-vide cooked herring fillets were first weighed in the vacuum bags. Then the fish samples were removed from the vacuum bags. Both the fillets and the vacuum bags were blotted dry with tissue paper. The vacuum bag and the fish samples were weighed again separately. The cook loss was calculated as a percentage of initial fish weight (equation 1).

$$Cook\ loss\ \% = \frac{(BFBO-EB)-(BFAD-EB)}{(BFBO-EB)} * 100\% \qquad (1)$$

BFBO – Weight of bag + fish before opening
BFAD – Weight of bag + fish after drying
EB – Weight of empty bag

2.5.2 Colour measurement

After determination of cook loss, the colour of herring samples were determined as described by Cropotova et al. (2019c). The sealed vacuumed pouches containing fillets were cut open, the fillets were blotted dry with paper and the colour was measured at three different places of each fillet using a Konica Minolta CR-400 colorimeter (Figure 17) and the average was calculated. Before starting the analysis, the instrument was calibrated by using a standard white plate.

The data were recorded in colour coordinates of L*, a* and b*. The tristimulus measure mode relates to the human eye response colour (Hamre et al., 2003). Variable L* represents the lightness (L* > 0 for white and L*< 0 for black), variable a* represents the red to green region (a* > 0 for red and a* < 0 for green) and variable b* represents the yellow to blue region (b* > 0 for yellow and b* < 0 for blue) according to the Commission Internationale de l'Eclairage Lab scale (2001).

After the colour measurement, the fillets were cut with a knife, minced using a benchtop mixer (Bosch), transferred into a zip lock bag and stored at -20°C for further analyses.
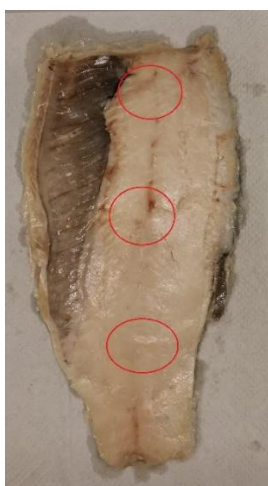
Figure 17: An illustration of how the colour of a herring fillets was measured at three different places.

2.5.3 Lipid content

Lipid content of herring samples was determined by the Bligh & Dyer (1959) method. Chloroform – methanol was used as an extraction medium. The analysis was performed in duplicate.

Minced herring samples were weighed (10 g) in centrifuge tubes. The centrifuge tubes were kept on ice during the whole procedure. Then, distilled water (10 mL), chloroform (20 mL) and cold methanol (40 mL) were added to each tube. The mixture was homogenized using Ultra Turrax (2 minutes, 9000 rpm). Additional amount of chloroform (20 mL) and distilled water (20 mL) was added separately, and the mixture was homogenized again using Ultra Turrax (30 seconds) after each addition. The tubes were centrifuged for 10 minutes at 11 800 g. Chloroform phase (2 mL, the bottom phase) was transferred into a pre-weighed Kimax glass tube (10 mL). The Kimax glass tube was weighed again containing chloroform phase before it was placed in an evaporation unit and heated (60°C) with N_2-stream for 1 hour. After the evaporation, the tubes were corked, cooled down to room temperature and weighed again. The remaining chloroform phase in the centrifuge tubes was transferred to a plastic tube resistant to chloroform, streamed with N_2-gas and stored at -40°C for further analysis of primary and secondary lipid oxidation products. The results are expressed as total lipid (average ± standard deviation) in percentage of wet weight fish (equation 2) (Bligh and Dyer, 1959).

$$Total\ lipid\ \% = \frac{((Tube + lipid\ sample\ after\ evaporation) - (Tube\ weight\ (g))}{Fish\ weight\ (g)} * 20 * 100\% \quad (2)$$

2.5.4 Peroxide value

The procedure for peroxide value (PV) measurement was performed in duplicate by using the iodometric titration method with SI Analytics titrator. The analysis was performed as described in the AOCS official methods (2003), Cd 8b-90.

The previously prepared chloroform extracts (12 mL, 0,480 – 1,080 g lipid per sample) were used to determine the peroxide value.

For the blank titration, a solvent mixture containing acetic acid and chloroform (18 mL acetic acid, 12 mL chloroform) was mixed with potassium iodide solution (0,5 mL, 0,046 mM) in an Erlenmeyer volumetric flask, stirred (60 seconds), filled with distilled water (30 mL) and auto titrated with $Na_2S_2O_3$ (0,001 M) with an electrode and a titration tip placed into the solution. For the sample titration, the procedure was followed as the blank titration where chloroform was replaced by chloroform extract samples. For those samples that had a higher lipid content than 0,800 g, a new blank was titrated with $Na_2S_2O_3$ (0,01 M) before the sample titration. The results are expressed as average ± standard deviation in milliequivalents of peroxide oxygen per kg lipid ($m_{eq}O_2$/kg lipid, equation 3).

$$PV \left(\frac{m_{eq}O_2}{kg\ lipid} \right) = \frac{(V-B)\ x\ T\ x\ M\ x\ F_1}{w\ x\ F_2}$$

(3)

V – Volume of titrant consumed for iodometric titration of sample (mL)
B – Volume of titrant consumed for iodometric titration of blank (mL)
T – Titre (0,001 M) (mol/L)
M – Molarity (1000)
F_1, F_2 = 1
W – Weight of oil sample (g)

2.5.5 Conjugated dienes and conjugated tetraenes

Spectrophotometric determination of conjugated dienes (CD) and conjugated tetraenes (CTe) was performed in duplicate by measuring UV absorption of the chloroform phase (1 mL, obtained from the Bligh & Dyer (1959) method). The samples were read at 233 nm and 315 nm respectively, by using a GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific, USA) according to AOCS standard method 2.501 (1998). Chloroform was used as blank. The results are expressed as average ± standard deviation in mL CD/CTe per gram lipid (mL/g) (equation 4).

$$CD/CTe\left(\frac{mL}{g}\right) = \frac{Absorbance * volume\ of\ Chloroform\ sample\ (mL)}{Weight\ of\ lipid\ (g)\ in\ Chloroform\ extract} \qquad (4)$$

2.5.6 Determination of TBARS

The determination of thiobarbituric acid reactive substances (TBARS) was performed in duplicate as described by Ke & Woyewoda (1979). The TBA stock solution was made by dissolving tert-butyl alcohol (1,44 g) in a volumetric flask (250 mL) with distilled water (50 mL) and diluted with glacial acetic acid to the calibration ring mark.

The tert-butyl alcohol (TBA) working solution was made by mixing TBA stock solution (180 mL), chloroform (120 mL), sodium sulphite (15 mL, 0,3 M) and butylated hydroxytoluene (9,45 mL, 3% BHT in ethanol).

Chloroform extract (200 µL) and TBA working solution (5 mL) were added to Kimax tubes (15 mL) and vortexed for 15 seconds. The Kimax tubes were incubated in a boiling water bath (45 minutes) and cooled down in cold water. TCA-solution (2,5 mL, 0,28 M) was added to each tube, inverted and centrifuged 5 – 10 minutes at 900 g. The samples were read at 538 nm by using a GENESYS 10S UV-VIS spectrophotometer (Thermo Scientific, USA).

TEP was used to generate the standard curve. Following TEP volumes were used: 0, 25, 50, 100, 150 and 200 µL (0,1mM). The generation of the standard curve was performed as the sample analysis where the chloroform extracts were replaced by TEP solution. The results are expressed as average ± standard deviation in micromol TBARS per g lipid (µMol TBARS/g lipid, equation 5)

$$\frac{\mu Mol\ TBARS}{g\ lipid} = \frac{(A_s - I)}{(S * L * 1000)} \tag{5}$$

A_s – Absorbance of sample
I – Intercept of standard curve
S – The slope of standard curve
L – Weight lipid sample used (g)

2.5.7 Schiff bases

Schiff bases (SBs) was determined according to the method describe by Cropotova et al. (2019c) with some modifications as follows: a chloroform lipid extract obtained by the Bligh & Dyer (1959) method (section *2.5.3 Lipid content*) was used to measure Schiff bases instead of chloroform-methanol extract (Cropotova et al., 2019c). The chloroform extract (3 mL) was measured spectroflourimetrically using a luminescence spectrometer (LS 50B Perkin-Elmer, Waltham, Ma, USA) at 360 nm excitation and 430 nm emission wavelength. The measurement was performed in duplicate, and the results are expressed as average ± standard deviation in millilitre Schiff bases per kilogram lipid (mL/kg lipid) (equation 6).

$$SBs\frac{mL}{kg\ lipid} = \frac{(Fluorescence * Volume\ of\ chloroform\ extract\ (mL))}{Weight\ of\ lipid\ (g)\ in\ chloroform\ extract * 1000} \qquad (6)$$

2.5.8 Water content and ash content

The water content was determined as described in the official method (AOAC, 2005) and were run in duplicate. 2 g samples were transferred to a pre-weighed porcelain crucible and dried at 105°C for 24 h to a constant weight. The sample was weighed again after drying and were further ashed at 550°C overnight in a muffle furnace. The sample was weighed again to determine the ash content. The results are expressed as average ± standard deviation of water content and ash content in percentage of wet weight herring (equation 7 and 8).

$$\% \ Water\ content = \frac{(WWS-WSAD)}{WWS} * 100\% \qquad (7)$$

$$\% \ Ash\ content = \frac{(WSAD-WSAB)}{WWS} * 100\% \qquad (8)$$

WWS – Wet weight of sample
WSAD – Weight of sample after drying
WSAB – Weight of sample after burning

2.5.9 Statistical analysis

The statistical analysis was performed in Microsoft Excel 2013. The results are presented as average $\pm$ standard deviation. Significance was analysed by using two-sided student t-test with a significance level of 5% ($p < 0,05$). Linear correlation coefficient was determined by trying to find correlations between the results.

3. Results and discussion

This section displays results of determination of water and ash content, cook loss, lipid content, peroxide value (PV-value), thiobarbituric acid reactive substances value (TBARS-value), conjugated dienes and tetraenes (CD and CTe), Schiff bases (SBs) and colour development of herring samples. HO, HN, HD and HOL refers to herring samples (H) subjected to one of the treatments with antioxidant extracts, either oregano extract (O), nettle extract (N), dill extract (D) or olive leaf extract (OL). The raw data and calculations of the analysis are attached in a compressed zipped-folder, see *Appendix A – Raw data and calculation of analysis*.

3.1 Water content

The total variation range for water content was from 56,24 to 63,79% of wet weight herring (Figure 18). The initial water content of sample Raw was $62,62 \pm 0,03\%$. The results agree with FAO (2001) which give a water content in the range from 57 to 79% in herring flesh. However, sample Control from day 8 (Figure 18) had a lower water content compared to the water content range suggested by FAO (2001).

No significant changes were found in the water content of samples Control, HO, HN, HD and HOL on the last day of storage compared to the first day of storage. In addition, no significant variations were found in water content between the samples throughout the 20-days of cold storage. Control had the highest variation of water content throughout the cold storage. Samples treated with antioxidant solutions, except sample HD, had higher water content compared to Control on the first day of storage. This could be because the antioxidant solutions were sprayed on the surface of herring fillets, resulting in additional water adsorbed on the fish surface. On day 12 of storage, samples treated with antioxidant solutions had lower water content than Control. By the end of the storage, samples treated with antioxidant solutions had higher water content than Control.

The variation in water content could be due to the sexual maturity and the age of herring. However, the herring samples were all caught at the same time. Aftret (2018) found that the water content varies with the amount of fat in the Atlantic mackerel samples. The same result was also observed from the study of Laub-Ekgreen (2018) on Atlantic herring.

Water content is a good indicator of the relative content of energy, lipid and protein in fish species (Olagunju et al., 2012). However, high water content could lead to increase in susceptibility to microbial spoilage and consequently decrease in quality of the fish (Oluwaniyi and Dosumu, 2009).
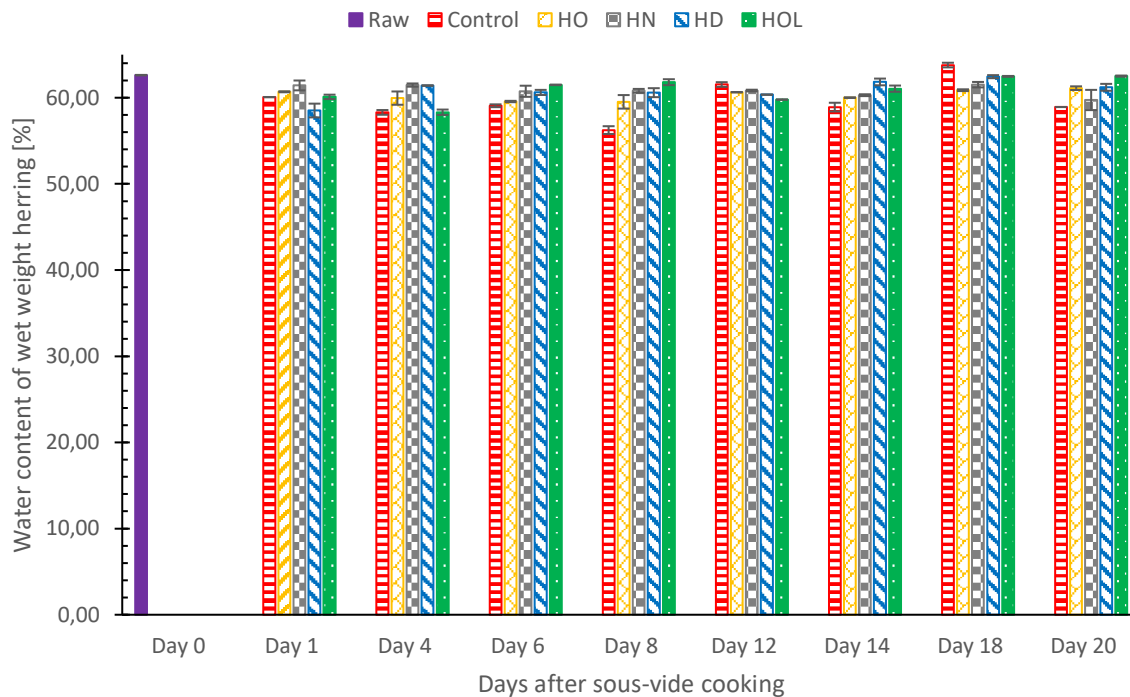

Figure 18: Water content [%] of wet weight herring samples as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days). The y-axis shows the amount of water content in wet weight herring samples in percentage [%]. The values are given as mean value with error bars as standard deviation, N = 2.

3.2 Ash content

The total variation range of ash was from 0,57 to 1,62% of wet weight herring (Figure 19). The initial ash content of sample Raw was $0,97 \pm 0,00\%$ (Figure 19). No significant changes were found in ash content of samples Control, HO, HN, HD and HOL on the last day of storage compared to the first day of storage (Figure 19). In addition, no significant variations were found between the samples throughout the 20-days of cold storage. In the study of Cropotova et al. (2019a), no significant changes were found in ash mass fraction of different mackerel samples, which is in agreement with the result in this study. Aftret (2018) suggested that the variation of ash content between Atlantic mackerel samples could be due to variations in bone content. This may also explain the variations of bone content in this study.

On the first day of storage, the ash content of samples treated with antioxidant solutions (HO, HN, HD and HOL) were lower compared to Control. On day 14 of storage, the ash content of samples treated with antioxidant solutions were higher compared to Control. By the end of storage, HD and HN had higher ash content compared to Control, while HO and HOL had lower ash content. Sample HD from day 8 had the lowest ash content and could be an outlier compared to other fish samples. This could be due to a limited amount of fish sample available for determination of ash content.

The ash content is a measure of the mineral content in food (Olagunju et al., 2012). The ash content between 1,17 – 1,79% in marine fish species is a source of minerals such as iron, magnesium, zinc, potassium and calcium (Olagunju et al., 2012).
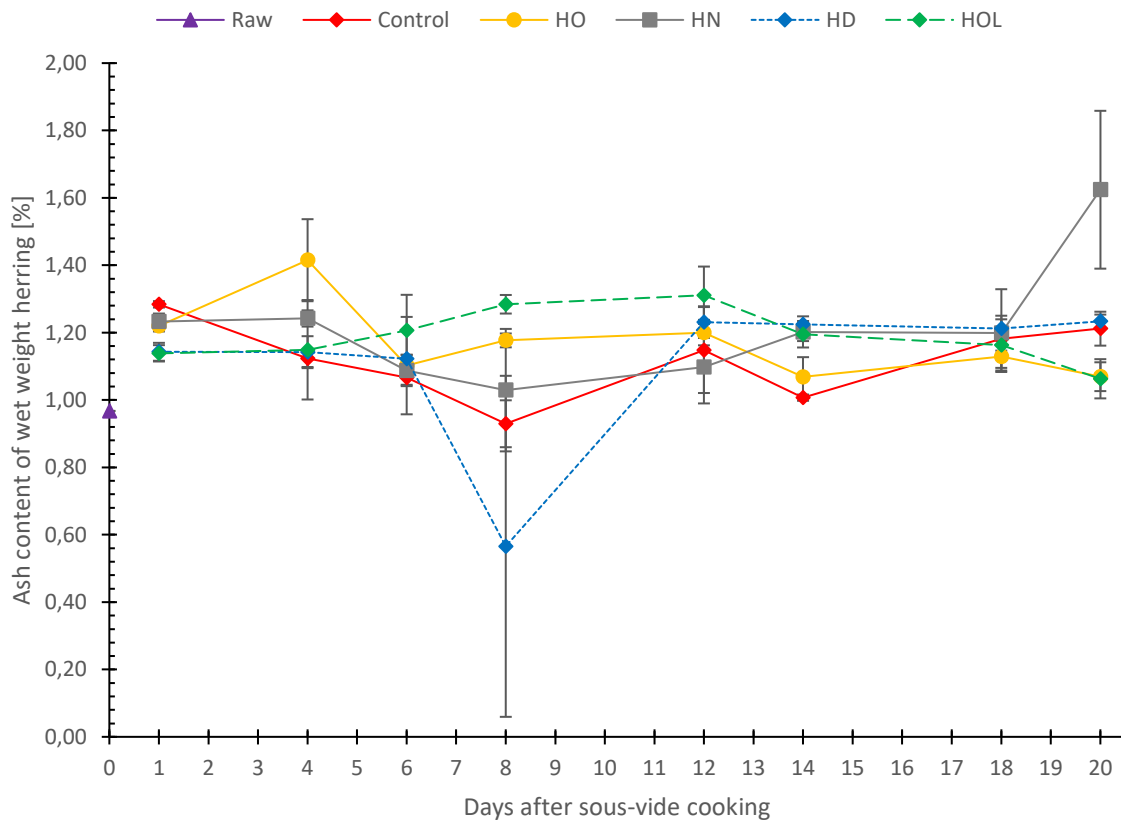

Figure 19: Ash content [%] of wet weight herring samples as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days). The y-axis shows the amount of ash content in wet weight herring samples in percentage [%]. The values are given as mean value with error bars as standard deviation, N = 2.

3.3 Cook loss

The total variation range of cook loss (%) was from 4,25 to 12,24% of herring mass (Figure 20). The initial drip loss of sample Raw was 4,71%. Sample Raw was not subjected to sous-vide cooking. The term "drip loss" will be used for sample Raw and the term "cook loss" will be used for samples subjected to sous-vide cooking.

No significant changes were found on cook loss of samples Control, HO, HN, HD and HOL on the last day of storage compared to the first day of storage. In addition, no significant variations were found in cook loss between the samples throughout the 20-days of cold storage. Sample Raw was not subjected to heat treatment, which is the main reason why the drip loss was one of the lowest compared to samples subjected to sous-vide cooking. Another explanation of why some samples of HO (e.g. day 14), HN (e.g. day 12) and HOL (e.g. day 18) had lower cook losses compared to Raw, could be due to different sizes of herring fillets after filleting (uneven thickness and length) (Ofstad et al., 1996). No correlations were found between water content and cook loss.

On the first day of storage, all samples treated with antioxidant solutions had lower cook losses compared to Control. On day 6, samples treated with antioxidant solutions had higher cook losses compared to Control. By the end of storage, HO, HN and HD had higher cook losses compared to Control, while HOL had lower cook losses.

The cook loss is mainly affected by water holding capacity (WHC) which influences the loss of liquid from muscle during storage and heat treatment (Cropotova et al., 2018). Hermansson and Lucisano (1982) found that the amount of water released from the fish tissue was influenced by WHC and by the magnitude of micro structural changes. The tendency for reduction of cook loss in sous-vide treated fish samples during chilled storage may be due to the reabsorption of liquid released during cooking where the myofibrillar proteins unfold and water distributes between the extra- and intracellular space (Ofstad et al., 1993). The muscle structure is modified after sous-vide treatment which affects the WHC and water leaks out from the fish muscle (Baldwin, 2012; Aftret, 2018). This could be another explanation of the variations of cook losses in this study.
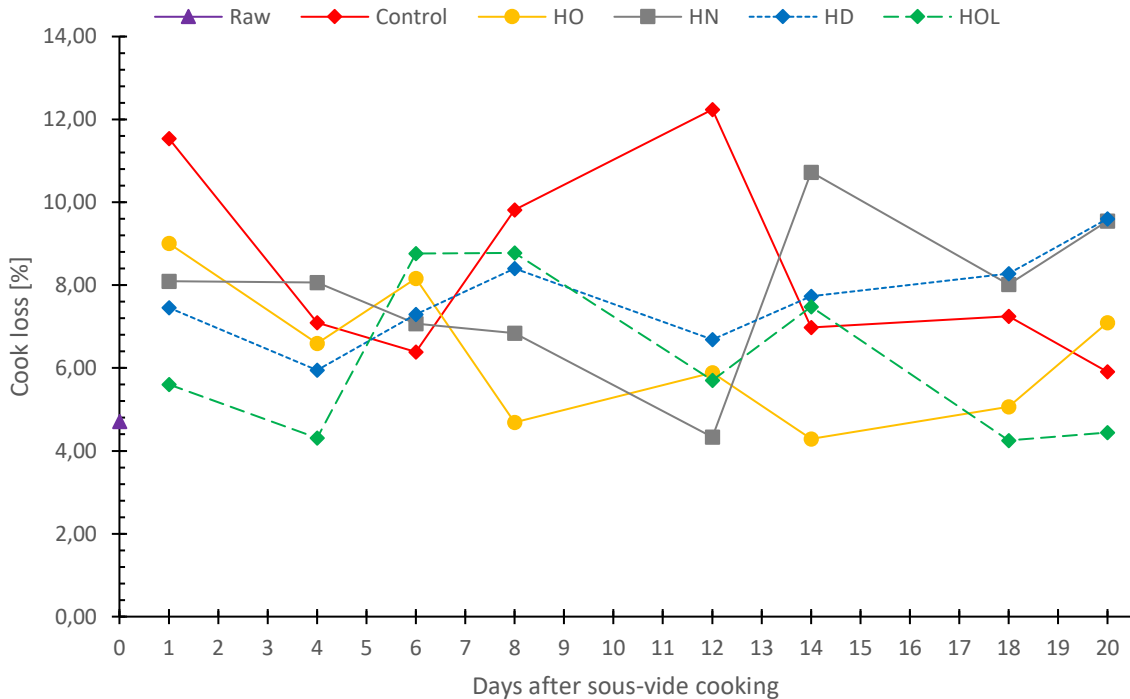
Figure 20: The cook loss [%] of herring samples as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days). The y-axis shows the percentage of cook loss of mass of herring fillets [%], N = 1.

The herring samples did not show a stable reduction of cook loss throughout the storage period, but a fluctuating trend. These results do not agree with the study of Cropotova et al. (2019a) or Cropotova et al. (2019c) on Atlantic mackerel. According to those two studies, the cook loss should have been reduced during cold storage of the sous-vide cooked samples. Even though herring is a pelagic fish it clearly behaves differently from mackerel with regard to cook loss. This may explain the deviation of the results in this study compared to the studies of Cropotova et al. (2019a) and Cropotova et al. (2019c).

The heat treatment causes denaturation of myofibrillar proteins and the connective tissue that holds water. The proteins may coagulate on the surface of the fish (Aftret, 2018; Skipnes et al., 2011). During the storage period, gelation and precipitation of some proteins were observed on the surface of the herring samples. This may be connective tissue and myofibrillar proteins that were denatured during heat treatment. The same result was also observed in the study of Aftret (2018) on Atlantic mackerel fillets.

3.4 Lipid content

The total variation of lipid content (%) was between 17,93 and 29,91% of herring mass (Figure 21). The initial lipid content of sample Raw was 18,70 ± 1,00% (Figure 21). No significant changes were found on lipid content of samples Control, HO, HN, HD and HOL on the last day of storage compared to the first day of storage. In addition, no significant variations were found on lipid content between the samples throughout the 20-days of cold storage.

On the first day of storage, sample HO, HN and HD had higher lipid content compared to Control, while HOL had lower lipid content. On day 8 of storage and by the end of storage, all samples treated with antioxidant solutions had lower lipid content compared to Control.

Herring is a fatty pelagic fish in which lipid content varies with season. It is usually at late-summer and autumn where the lipid content of herring is at its maximum (Bigelow and Schroeder, 1953). According to the study of Slotte (1996) on Norwegian spring spawning herring, the average fat content of whole herring was between 26 – 30%, recorded in June and July in 1994. This is in agreement with the results in this study.
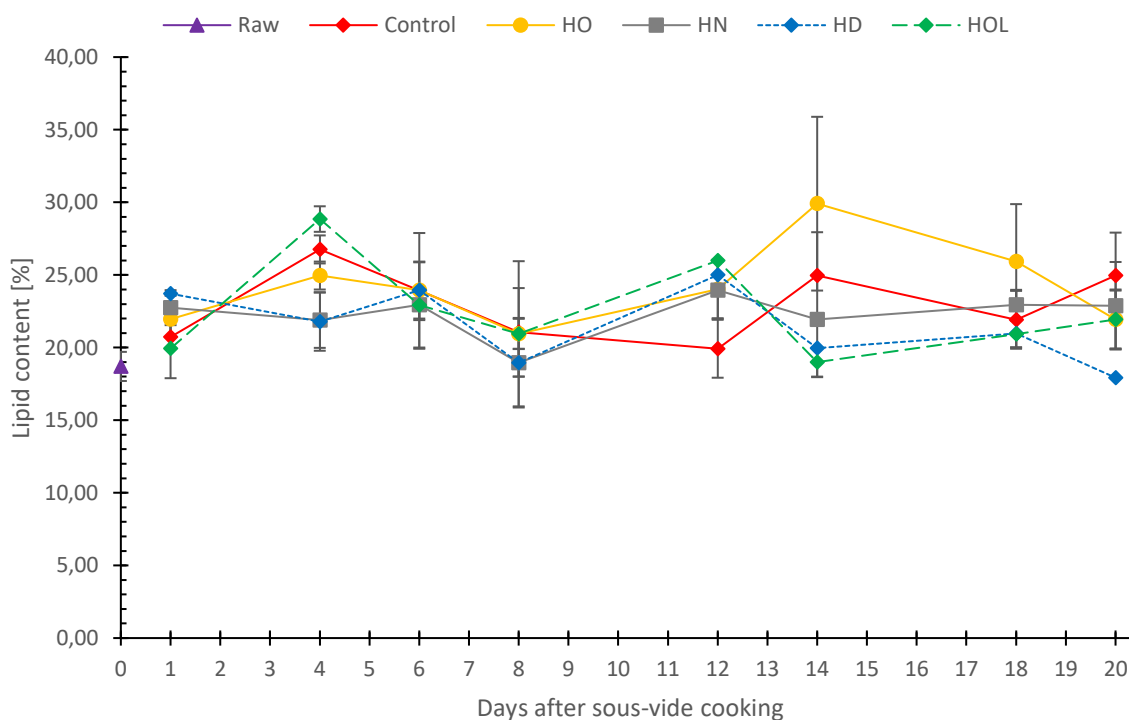
Figure 21: The total lipid content [%] of herring samples as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days). The y-axis shows the percentage of lipid content of mass of herring fillets [%]. The values are given as mean value with error bars as standard deviation, N = 2.

In a study on Atlantic mackerel, Aubourg et al. (2005) found that the lipid content was stable during cold (4°C) and frozen storage. However, the lipid content of herring samples in this study showed some variation during the storage period. A possible explanation could be a large variation of lipid content among herring samples within one batch due to different sizes of fish fillets (Anthony et al., 2000). Other possibilities could be due to leakage of the fat along with the cook loss after the sous-vide cooking. The oil may have leaked out from the fillets into the vacuum pouches, which could be one of the reasons for the decreased lipid content.

3.5 Primary lipid oxidation products

Lipid peroxides and conjugated systems (dienes and tetraenes) were measured to get a full overview of the formation of primary lipid oxidation products during cold storage of sous-vide cooked herring samples. Lipid peroxides are unstable and their determination will not give the full overview of the formation of primary oxidation products (Brash et al., 1988). In this section, the results from peroxide value (PV-value), conjugated dienes (CD) and conjugated tetraenes (CTe) as primary lipid oxidation products will be discussed.

3.5.1 Peroxide value

In this study, the PV-value was measured by iodometric titration and expressed in $m_{eq}O_2$/kg lipid. The total variation in PV-value was from 3,56 to 36,95 $m_{eq}O_2$/kg lipid (Figure 22) of herring samples. The initial PV-value of sample Raw was $3,59 \pm 0,68$ $m_{eq}O_2$/kg lipid (Figure 22). No significant changes in PV-value were found between samples Control, HO, HN, HD and HOL on the last day of storage compared to the first day of storage, except sample Control from day 1. This sample had a significantly ($p < 0,05$) higher PV-value compared to other samples from the same day. This phenomenon probably occurred after the repeated extraction of lipids from the fish samples (due to limited amount of sample available for the analysis) stored at -40°C. No significant variations were found between the samples throughout the 20-days of cold storage. No effect was found on lipid oxidation stability between samples HN, HD, HOL and Control. HO was the only sample group which showed a potential on lipid oxidation stability of herring sample compared to Control during storage. The antioxidant activity of these extracts will be discussed more in detail in section *3.8 Effects of antioxidant extracts on lipid oxidation stability*.

When PUFAs are exposed to prooxidants, the lipid oxidizes to hydroperoxides, primary oxidation products that may be determined by measuring PV-value (Schaich, 2016). On the first day of storage, samples treated with antioxidant solutions had a lower PV-value compared to Control. On day 6, samples treated with antioxidant solutions had higher PV-value than Control. By the end of storage, samples treated with antioxidant solutions had lower PV-value compared to Control.
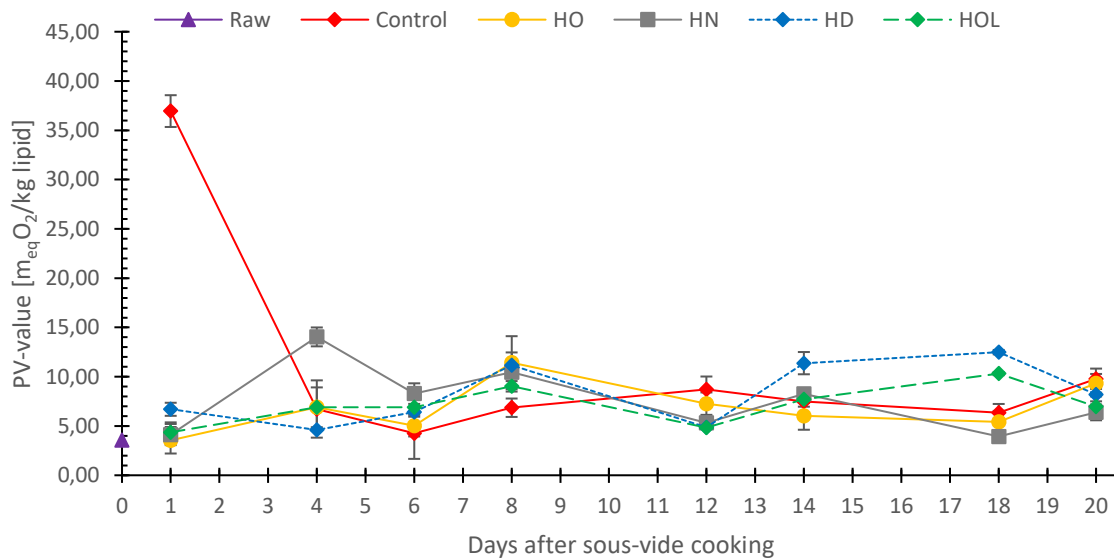

Figure 22: The peroxide value [$m_{eq}O_2$/kg lipid] of herring samples as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days). The y-axis shows the peroxide value in milliequivalents of peroxide oxygen per kg lipid of herring fillets [$m_{eq}O_2$/kg lipid]. The values are given as mean value with error bars as standard deviation, N = 2.

Some fish samples including Control (e.g. day 6), HO (e.g. day 1), HN (e.g. day 18), HD (e.g. day 4) and HOL (e.g. day 1) had PV-values lower than 5 $m_{eq}O_2$/kg lipid (Figure 22). Those samples were below the PV-value limit of acceptability and not considered rancid (CODEX-STAN-329, 2017). Other samples such as Control (e.g. day 1), HO (e.g. day 8), HN (e.g. day 4), HD (e.g. day 8) and HOL (e.g. day 18) had PV-values above 10 $m_{eq}O_2$/kg lipid (Figure 22) which was above the PV-value limit of acceptability (CODEX-STAN-329, 2017). Those samples were considered rancid. The rest of the samples from Control (e.g. day 12), HO (e.g. day 4), HN (e.g. day 14), HD (e.g. day 6) and HOL (e.g. day 4) had PV-values between 5 – 10 $m_{eq}O_2$/kg lipid, which was also above the limit of acceptability for PV-value. Those samples are also regarded as rancid (CODEX-STAN-329, 2017).

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3.5.2 Conjugated dienes and conjugated tetraenes

The content of CD varied between 7,68 and 14,53 mL/g (Figure 23), and the content of CTe varied between 1,40 and 5,83 mL/g (Figure 24). The initial CD- and CTe-value of sample Raw was $13,04 \pm 0,31$ mL/g and $4,33 \pm 0,68$ mL/g (Figure 23), respectively. The CD- and CTe-value of sample Control did not have a stable increase during cold storage, but a fluctuating trend. Samples HO, HN, HD and HOL had the same trend.

No significant changes in CD- and CTe-value were found between samples Control, HO, HN, HD and HOL on the last day of storage compared to the first day of storage. In addition, no significant variations were found on CD- and CTe-value between the samples throughout the 20-days of cold storage. No effect was found on lipid oxidation stability between samples HN, HD, HOL and Control. HO was the only sample group which showed a potential on lipid oxidation stability of herring sample compared to Control during storage. The antioxidant activity of these extracts will be discussed more in section *3.8 Effects of antioxidant extracts on lipid oxidation stability*.

On the first day of storage, sample HO and HOL had higher CD-values compared to Control, while samples HN and HD had lower CD-values. On day 12 of storage, Control had higher CD-value compared to samples treated with antioxidant solutions. At the end of storage, all samples treated with antioxidant solutions had higher CD-values compared to Control.

Figure 23: The conjugated dienes value [mL/g] of herring samples as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days). The y-axis shows the content of conjugated dienes in millilitre per gram lipid [mL/g]. The values are given as mean value with error bars as standard deviation, N = 2.

However Cropotova et al. (2019b) found that when adding antioxidant to Atlantic mackerel, the CD- and CTe-values increased throughout the cold storage. This is not what one would expect, showing that the antioxidants did not have a large effect. The Atlantic mackerel samples from day 1 in that study had fourteen times higher CD-value and about ninety times higher CTe-value compared to sample HOL from day 1 in this study (Figure 23 and Figure 24). In addition, the raw sample from that study had about four times higher CD-value and about seventy times higher CTe-value compared to the initial herring sample Raw from day 0 (Figure 23 and Figure 24) in this study. The CD- and CTe-values of all herring samples in this study were lower compared to the study of Cropotova et al. (2019b) on Atlantic mackerel.

The initial high value of lipid oxidation products generated in some mackerel samples could be due to the freshness of the fish. In addition, due to the transportation and the storage time before the sous-vide cooking experiment of mackerel fillet (Cropotova et al., 2019b). The same situation may have occurred in Atlantic herring samples explaining the high initial value of CD- and CTe-value from sample Raw (Figure 23) compared to other antioxidant treated samples.
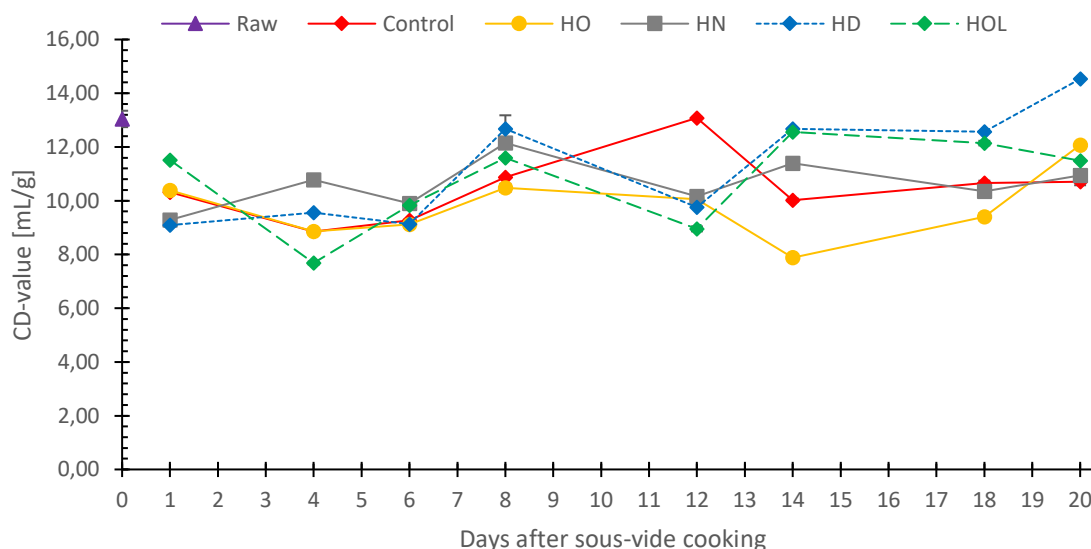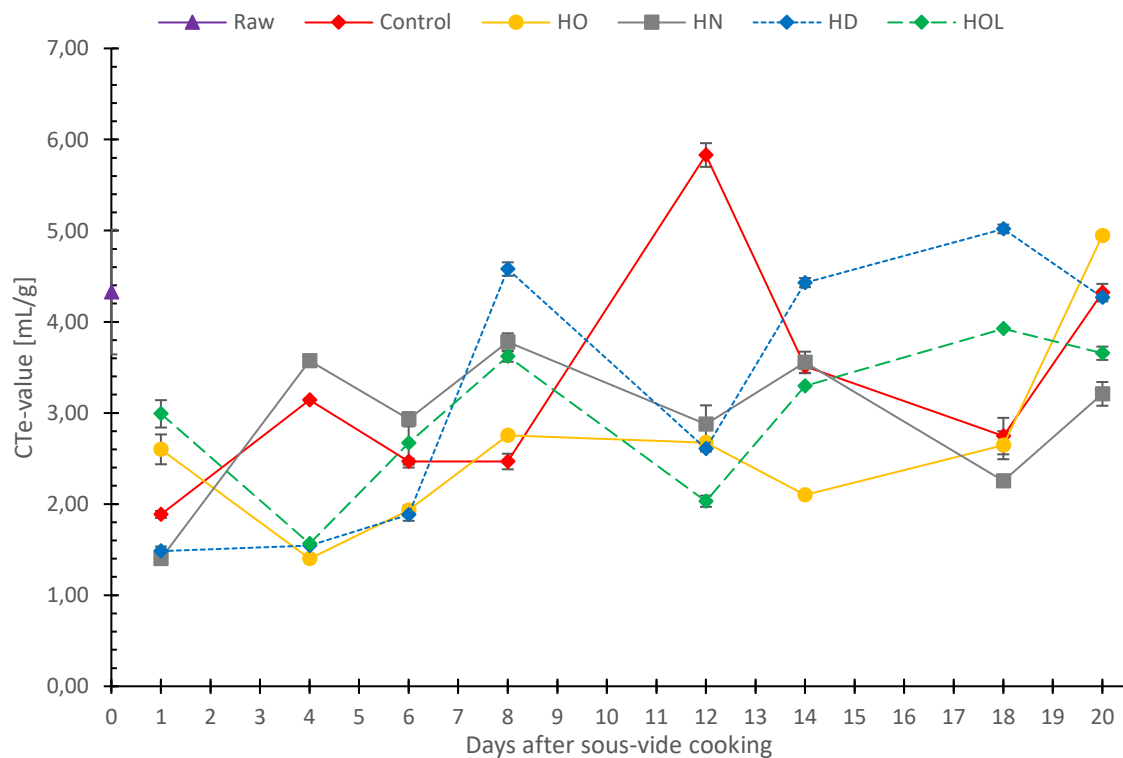

Figure 24: The conjugated tetraenes value [mL/g] of herring samples as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days). The y-axis shows the content of conjugated tetraenes in millilitre per gram lipid [mL/g]. The values are given as mean value with error bars as standard deviation, N = 2.

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Sample HO and HOL had higher CTe-values compared to Control on the first day of storage, while sample HD and HN had lower CTe-values. On day 8 of storage, all samples treated with antioxidant solutions had higher CTe-values than Control. On day 12 of storage, all samples treated with antioxidant solutions had lower CTe-values compared to Control. In addition, sample Control from day 12 had the highest CTe-value compared to other samples. By the end of storage, HO was the only sample which had a higher CTe-value compared to Control, while sample HD, HD and HOL had lower CTe-value.

The conjugated dienes (CD) and conjugated tetraenes (CTe) are formed as a result of lipid oxidation during cold storage. The formation of CD and CTe may be due to elevated temperature where lipids undergo thermo-oxidation, isomerization, cyclization, hydrolysis and polymerization (Lalas, 2009). During oxidation of for example Atlantic herring, due to isomerization, lipids containing polyenes or methylene-interrupted dienes get a shift in the position of the double bond forming CD or other conjugated systems such as CTe (Lalas, 2009).

3.6 Secondary lipid oxidation products
3.6.1 TBARS

Thiobarbituric acid reactive substances (TBARS) measures the amount of secondary lipid oxidation products (Frankel, 1998). In this study, TBARS-value was measured spectrophotometrically and expressed as µMol TBARS/g lipid. The total variation of TBARS-value was between 1,47 and 13,15 µMol TBARS/g lipid of herring samples (Figure 25). The initial TBARS-value of sample Raw was $20,73 \pm 2,55$ µMol TBARS/g lipid (Figure 25). No significant changes were found in TBARS-value between samples Control, HO, HN, HD and HOL on the last day of storage compared to the first day of storage. In addition, no significant variations were found in TBARS-values between the samples and Control throughout the 20-days of cold storage. No effect was found on lipid oxidation stability between samples HN, HD, HOL and Control. HO was the only sample group which showed a potential on lipid oxidation stability of herring sample compared to Control during storage. The antioxidant activity of these extracts will be discussed more in section *3.8 Effects of antioxidant extracts on lipid oxidation stability*.

All samples from Control, HO, HN, HD and HOL, except sample HN from day 18, had TBARS-values higher than 2 µMol TBARS/g lipid (Figure 25). Sample HN from day 18 was the only sample which had a TBARS-values below 2 µMol TBARS/g lipid. The TBARS-value

limit for consumption was claimed to be 2 µMol TBARS/g lipid in the study of de Oliveira et al. (2016). All samples except sample HN from day 18 may be considered not consumable due to high TBARS-value.

On the first day of storage, sample HO and HOL had higher TBARS-values compared to Control, while sample HD and HN had lower TBARS-values. Sample HN from day 4 had the highest TBARS-value compared to other samples. On day 8 of storage, samples treated with antioxidant solutions had higher TBARS-values compared to Control. On day 12 and at the end of storage, samples treated with antioxidant solutions had lower TBARS-value than Control.
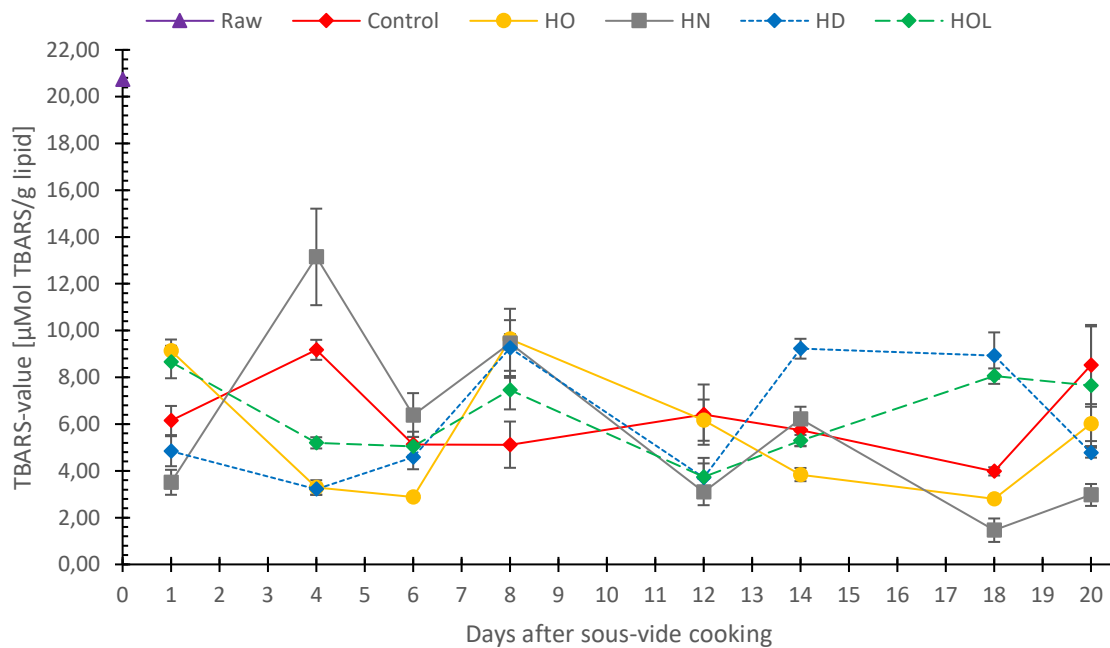

Figure 25: TBARS measurement of herring samples [µMol TBARS/g lipid] as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days). The y-axis shows the amount of micromol TBARS per gram lipid [µMol TBARS/g lipid]. The values are given as mean value with error bars as standard deviation, N = 2.

As mentioned in section *3.5.2 Conjugated dienes and conjugated tetraenes*, the study of Cropotova et al. (2019b) claimed that the initial high value of lipid oxidation products generated in some mackerel samples may be due to the freshness of the fish. In addition, due to the transportation and the storage time before the sous-vide cooking experiment of mackerel fillet. The same event may have occurred in Atlantic herring samples which may explain the high initial value of TBARS of sample Raw.

3.6.2 Schiff bases

The total variation of Schiff bases value (SBs-value) of herring samples was between 0,31 and 0,88 mL/kg lipid (Figure 26). The initial SBs-value of sample Raw was $0,58 \pm 0,06$ mL/kg lipid (Figure 26). All sample groups had a significant ($p < 0,05$) increase in SBs-value on the last day of storage compared to the first day of storage. The increase of SBs-value of sous-vide cooked herring samples could be associated with the denaturation of myofibrillar proteins which lead to impaired functionality (Cropotova et al., 2019c). No significant variations were found in SBs-values between samples treated with antioxidant solutions and Control throughout the 20-days of cold storage. No effect was found on lipid oxidation stability between samples HN, HD, HOL and Control. HO was the only sample group which showed a potential on lipid oxidation stability of herring sample compared to Control during storage. The antioxidant activity of these extracts will be discussed more in section *3.8 Effects of antioxidant extracts on lipid oxidation stability.*
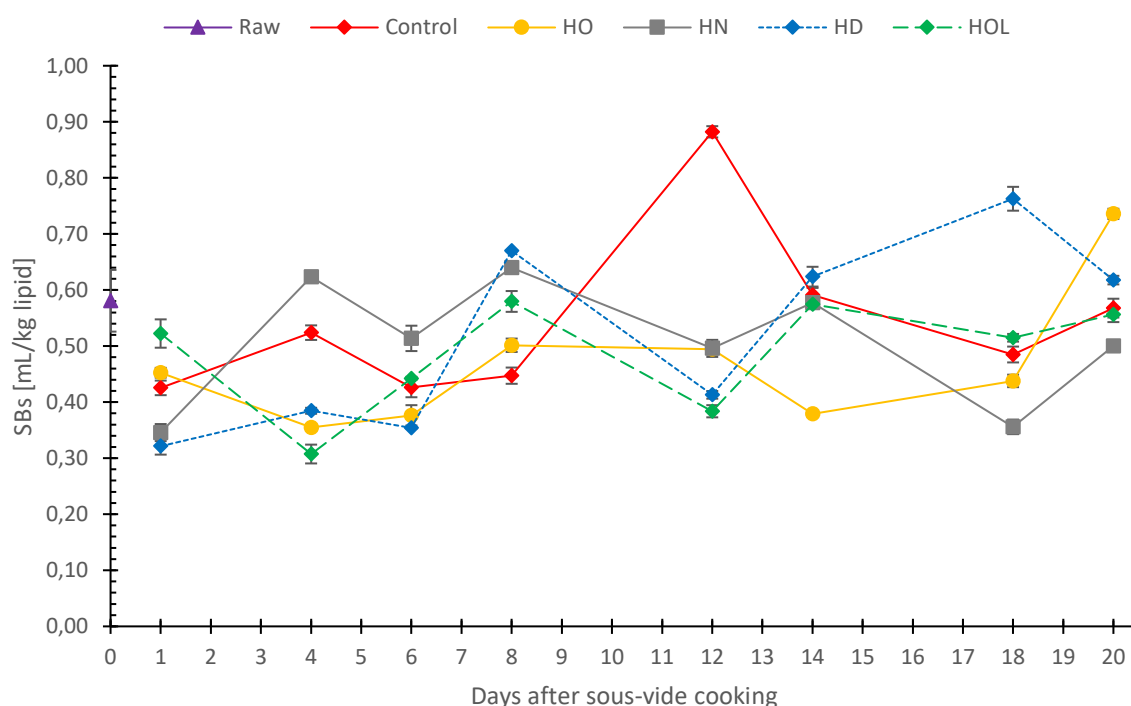
Figure 26: Schiff bases measurement [mL/kg lipid] of herring samples as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days). The y-axis shows the amount of Schiff bases per kg lipid [mL/kg lipid]. The values are given as mean value with error bars as standard deviation, N = 2.

On the first day of storage, sample HO and HOL had higher SBs-values compared to Control, while sample HN and HD had lower SBs-values. On storage day 8, all samples treated with antioxidant solutions had higher SBs-values compared to Control. On day 12 of storage, samples treated with antioxidant solutions had lower SBs-value than Control. In addition, sample Control from day 12 had the highest SBs-value compared to other antioxidant treated samples. At the end of storage, sample HO and HD had higher SBs-values compared to Control, while HOL and HN had lower SBs-values.

The formation of Schiff bases (SBs) involve progressive cross-linking, leading to polymerization during storage, proteins denaturation leads to impaired functionality, including the loss of water holding capacity (WHC) (Estévez, 2011). The formation of SBs is an indicator of lipid-protein oxidation (for example in Atlantic herring in this study during cold storage) resulting from a cross-linking reaction between alkaline amino acids of proteins and aldehyde moiety from protein carbonyls or an aldehyde formed during secondary lipid oxidation reactions (Estévez, 2011).

3.7 Colour development

The colour development of L*-value (lightness, Figure 27), a*-value (redness/greenness, Figure 28) and b*-value (yellowness, Figure 29) of herring samples had some fluctuations during cold storage. The total variation range in L*-values of all sous-vide treated herring fillets were between 63 and 75 CIE units. No significant changes in L*-value were found between samples Control, HO and HD on the last day of storage compared to the first day of storage. In addition, most of the herring samples had L*-values over 70 CIE units. Some samples during cold storage had L*-value less than 70 CIE units. A significant (p < 0,05) difference in L*-value was found between samples HN and HOL throughout the 20-days of cold storage. No correlations were found between L*-value and cook loss of the different sample groups.

The a*-value of herring samples varied along the storage period. The variation of a*-value for some sous-vide cooked herring samples were between 0,02 and 2,54 CIE units (Figure 28) which corresponds to the red colour area. However, most of the sous-vide cooked samples had a variation in a*-value between -0,08 and -2,07 CIE units which corresponds to the green colour area. The same result was observed in the specialization project (Vu, 2018). Moreover, significant (p < 0,05) differences in a*-value between samples HN and HOL, and between samples HD and HOL were observed throughout the 20-days of cold storage. No significant

changes in a*-value were found between the samples on the last day of storage compared to the first day of storage. No correlations were found between a*-value and PV-, CD-, CTe- and SBs-value.

During the cold storage, the total variation of b*-values of sous-vide cooked herring samples were between 14,67 and 18,66 CIE units (Figure 29). All herring fillets subjected to sous-vide cooking, with and without addition of antioxidant had higher b*-value compared to sample Raw from day 0 (Figure 29). Moreover, significant ($p < 0,05$) differences in b*-value between samples Control and HN, HO and HN, HO and HD, and HN and HOL were observed throughout the 20-days of cold storage. All sample groups had a significant ($p < 0,05$) increase in b*-value on the last day of storage compared to the first day of storage. Lipid oxidation may have contributed to the significant ($p < 0,05$) increase in b*-value at the end of storage. However, no correlations were found between b*-value and CD-, CTe-, TBARS- and SBs-value. Only the statistical analysis of PV-value and b*-value for sample HOL showed a significant ($p < 0,05$) correlation ($R = 0,70$). This was probably because primary lipid oxidation products further generated secondary lipid oxidation products that contributed to an increase in yellowness of the fish muscle. It could also be due to contribution of antioxidant solution to the yellowness of the fish flesh.

In this study, the colour parameters of raw herring were L* = 50,86 ± 3,11 CIE units, a* = 4,02 ± 2,33 CIE units and b*= 8,05 ± 1,37 CIE units (Figure 27, Figure 28 and Figure 29). This means the raw fish had lower lightness, were a little yellow and red in colour compared to other heat-treated samples. In addition, it was revealed that L*-value (lightness), a*-value (redness) and b*-value (yellowness) were not affected by the addition of antioxidants in form of solutions made of 1% antioxidant extract in distilled water due to low concentration.
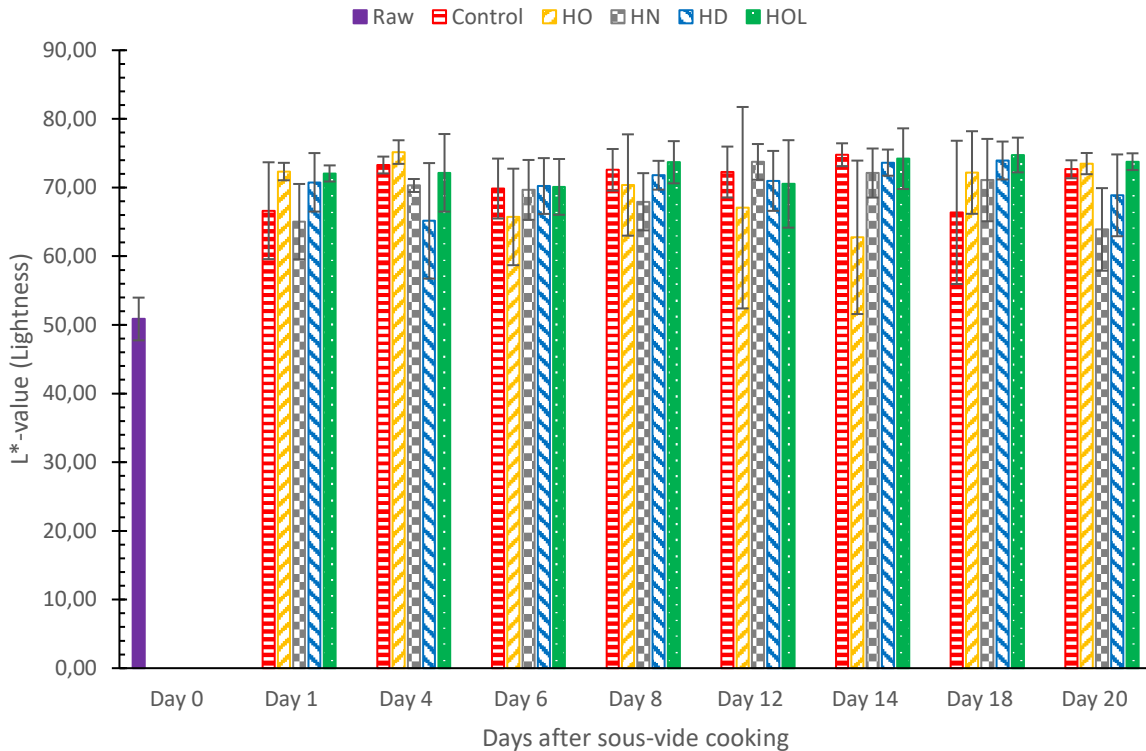
Figure 27: Colour parameters of herring samples where L* > 0 is whiteness of herring as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days). The y-axis shows the lightness of herring fillets. The values are given as mean value with error bars as standard deviation, N = 2.

On the first day of storage, samples HO, HD and HOL had higher L*-values compared to sample Control, while sample HN had lower L*-value. On day 14 of storage, samples treated with antioxidant solutions had lower L*-values compared to Control. At the end of storage, samples HO and HOL had higher L*-values compared to Control, while samples HN and HD had lower L*-value. The L*-values for all cooked samples were significantly ($p < 0,05$) higher compared to the lightness of Raw sample due to the cooking process. The same result was observed in the specialization project (Vu, 2018). The changes may be due to structural changes in the fish muscle tissue, where the cooking changes the light scattering of the muscle, leading to changes in lightness of the muscle (Chan et al., 1992). This could also be explained by the heat denaturation of myoglobin, changing the fish flesh colour from pale-red to white-yellowish as reflected by an increase in lightness accompanied by a simultaneous decrease in redness (Haard, 1992; Vu, 2018).

During storage it was observed that the blood was oxidized to a very dark colour which may explain the variations of L*-values between the samples. This phenomenon could be explained by metmyoglobin formation. Metmyoglobin formation is caused by the autooxidation of myoglobin leading to the darkening of fish flesh during frozen and iced storage. Aldehydic lipid oxidation products such as hexenal and hexanal may potently induce the formation of metmyoglobin lowering whiteness of fish muscle (Chaijan et al., 2005).
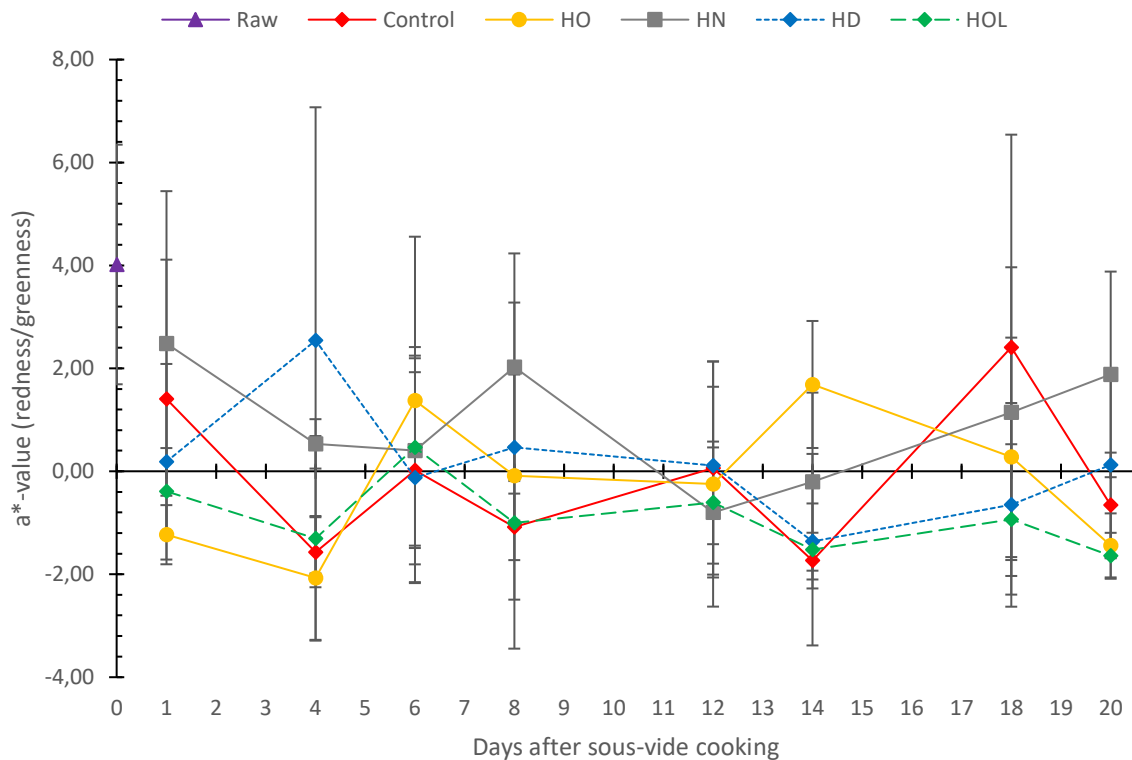
Figure 28: Colour parameters of herring samples: redness (a* > 0) and greenness (a* < 0) as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days) and the y-axis shows the redness (positive a*-value) or greenness (negative a*-value) of herring fillets. The values are given as mean value with error bars as standard deviation, N = 2.

The decrease in a*-value may be due to degradation of haemoglobin, the denaturation of heme-ring after cooking leading to decreased redness of the fish muscle compared to other samples (Akoh, 2017; Frankel, 2005). Moreover, the variation in redness may also be due to the oxidation of myoglobin, therefore the colour of the fish fillets (mainly redness) was changing during storage (Haard, 1992; Vu, 2018). This may explain why sample Raw from day 0 had the highest a*-value compared to the heat-treated samples.

The a*-value is an indicator of redness. The pigment myoglobin is predominant in pelagic fish and gives red colour to the fish muscle (Checmarev et al., 2017). In addition, a green pigment could be produced during heat processing of dark fish muscle when myoglobin reacts with other muscle components including trimethylamine oxide (TMAO) and cysteine. After heating, a single type of green pigment is formed resulting in discoloration of cooked fish flesh. The green pigmentation of fish flesh could be formed during the reaction of myoglobin with hydrogen peroxide, a by-product of lipid and myoglobin oxidations (Naughton et al., 1958; Chaijan et al., 2005). This phenomenon may explain why most of the samples had a*-values below 0, in the green colour area. In the study of Sánchez-Escalante et al. (2003), myoglobin and oxymyoglobin oxidized to brown metmyoglobin which is associated with reduction of reddish colour. This may describe why some herring sample had low a*-values.
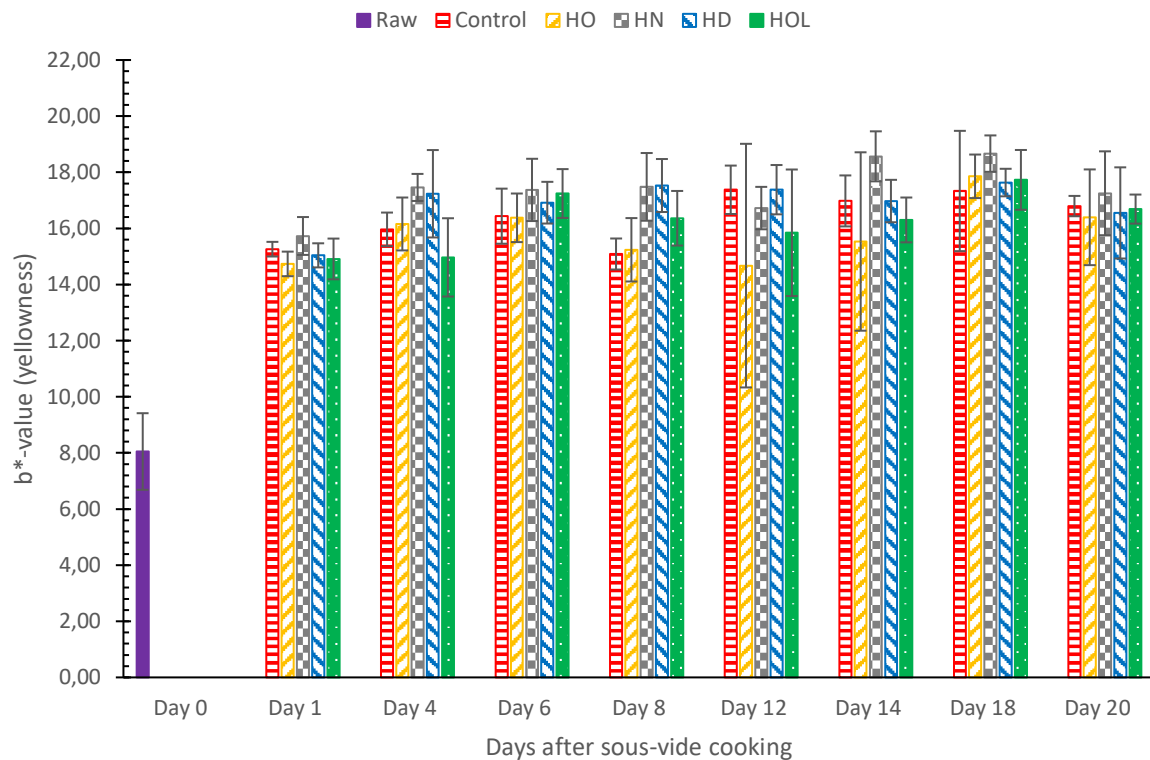
Figure 29: Colour parameters of herring samples: yellowness (b-value) as function of storage days after sous-vide cooking. Raw represent raw herring samples (purple). Control are herring fillets subjected to sous-vide cooking without pre-treatment with antioxidants (red). HO (orange), HN (grey), HD (blue) and HOL (green) are herring fillets (H) 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 0 to day 20, the sampling days) and the y-axis shows the yellowness of herring fillets. The values are given as mean value with error bars as standard deviation, N = 2.*

The yellowness of fish muscle is usually caused by lipid oxidation and the formation of secondary lipid oxidation products, but could also be due to the formation of protein carbonyls (Aftret, 2018). The b*-value (yellowness) may give an indication of lipid oxidation occurrence in fish, since the oxidation products often result in formation of yellow pigments in the fish muscle (Hamre et al., 2003; Aftret, 2018). An increase in b*-value during the storage process may indicate lipid oxidation (Aftret, 2018).

Colour is among the main quality parameters affecting the perception of food by consumers. Colour change in fish may indicate loss of quality. Heat treatments of fish meat would lead to colour change (Beltran et al., 2004; Zamora and Hidalgo, 2005). A change in L*-value (lightness), a*-value (redness) and b*-value (yellowness) may give an indication that food has undergone rancidity and may have lost quality during cold storage (Aftret, 2018).

3.8 Effects of antioxidant extracts on lipid oxidation stability

Oregano extract showed the best potential to prolong lipid oxidative stability as measured by PV-, CD-, CTe-, TBARS- and SBs-value. Overall, in this study the fish samples treated with oregano extract showed the highest lipid oxidation stability during cold storage of 20-days. Olive leaf extract, nettle extract and dill extract did not show good lipid oxidation stability during storage of herring samples. Oregano extract was the only extract showing its antioxidant potential to retard lipid oxidation in fish samples during cold storage. In the PV-value measurement, some samples had PV-values below 5 $m_{eq}O_2$/kg lipid. Those samples are considered not rancid, while other samples had PV-values between 5 and 10 or over 10 $m_{eq}O_2$/kg lipid. Those samples are considered rancid according to the requirements established by CODEX STAN 329 (2017). In the TBARS-value measurement, the herring samples had a TBARS-value which were higher than 2 µMol/g lipid, except sample HN from day 18. Those samples are not ideal for consumption (de Oliveira et al., 2016). Overall, the lipid oxidation of all samples subjected to sous-vide cooking and with addition of one of the antioxidant solutions were considered to be in the propagation step after the 20-days of cold storage.

Further investigation is needed to see if oregano extract, nettle extract, dill extract or olive leaf extract could be used to increase the shelf-life of Atlantic herring when added in higher amounts. In this study, 1% antioxidant extract in distilled water was used. Thus, one suggestion could be to investigate the concentration effect of the antioxidant extracts to see if this could work at a higher concentration.

The antioxidant activity of oregano extract is due to the presence of phenolic compounds in the extract (Gomes et al., 2016). The phenolic compounds act as antioxidants by interrupting the chain reaction of oxidation or of chelating metals by donating a hydrogen atom which acts as a receiver of free radicals (Choe and Min, 2009). Carvacrol and thymol (Figure 30) are the main compounds which have antioxidant properties in oregano extract (Shange et al., 2019). Carvacrol and thymol react with hydroxyl radicals and lipid and converts them to stable products, non-propagating species (Ariza-Nieto et al., 2011).
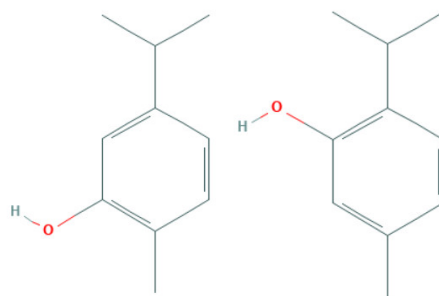
Figure 30: The chemical structure of carvacrol (left) and Thymol (right) in oregano extract. Adapted from PubChem (2019c; 2019j).

The dill extract did not show potential of increasing lipid oxidative stability of sous-vide cooked herring samples during cold storage according to results of PV-, CD-, CTe-, TBARS- and SBs-value. Thus, in the present study, dill extract cannot be considered to be used as an antioxidant in sous-vide cooked herring. Stanojevic et al. (2016) found that the antioxidant activity of dill extract may be due to the presence of non-volatile phenol compounds. In addition, there may be some small amounts of compounds with different polarities which are contributing to the antioxidant activity. Oshaghi et al. (2016) found that dill extract showed metal chelating capacity, ferrous chelating activities, oxygen – scavenging ability and reducing power. Furthermore, the radical scavenger ability of dill extracts are due to the synergistic effect between limonene and carvone with minor components such as linalool, trans- and cis-carveol and dihydrocarvone (Figure 31) (Stanojevic et al., 2016). However, polyphenols in dill may act as a prooxidant in the presence transition metals (e.g. Fe and Cu) and oxygen molecules (Figure 32) (Eghbaliferiz and Iranshahi, 2016). This may explain why samples containing dill extract had higher PV-, CD-, CTe-, TBARS-, and SBs-value compared to other samples containing oregano extract.
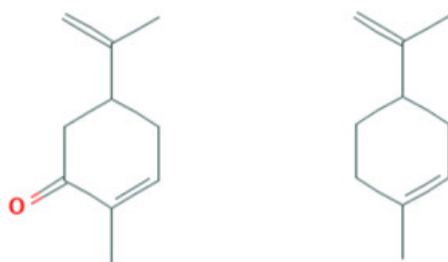
Figure 31: Chemical compounds in dill extract. Carvone (left) and limonene (right). Adapted from PubChem (2019d; 2019h).
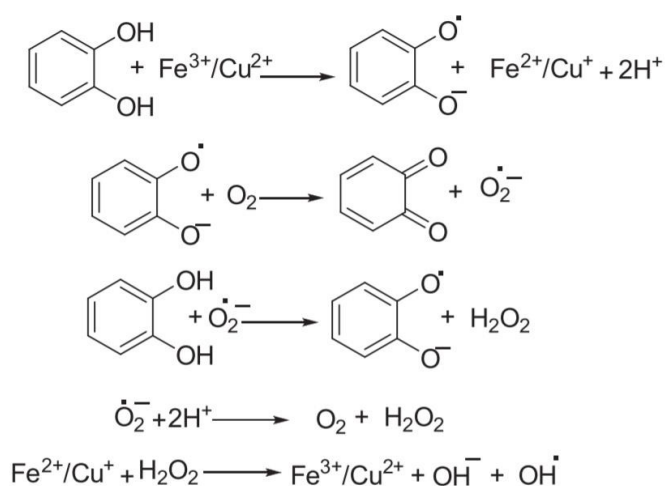
Figure 32: The prooxidant activity mechanisms of phenolics in the presence of iron(III) (Fe^{3+}), copper(II) (Cu^{2+}) and oxygen (O_2). Adapted from Eghbaliferiz and Iranshahi (2016).

Olive leaf extract did not show a potential to increase lipid oxidative stability of sous-vide cooked herring samples during cold storage according to results of PV-, CD-, CTe-, TBARS- and SBs-value. Thus, in the present study, olive leaf extract cannot be considered to be used as an antioxidant in sous-vide cooked herring during 20-days of cold storage. The antioxidant activity of OLE is due to the high amount of polyphenols (Ferrara et al., 2000; Krzeminski et al., 2003; Žanetić and Gugić, 2006). In addition, according to the study of Rafiee (2018) on the effect of olive leaf extract on sunflower oil, the antioxidant activity of OLE is not only from phenolic compounds, but also to other compounds such as vitamins, carotenoids and volatile oils. Hayes et al. (2011) found that OLE consist of oleuropein, hydroxytyrosol, verbascoside, apigenin-7-O-glucoside, luteolin-7-O-glucoside, tyrosol, and lutein (Figure 34). Furthermore, oleuropein and hydroxytyrosol are the main components possessing antioxidant activity (Djenane et al., 2019). The study of Altemimi (2017) showed that the free radical scavenging activity increased with an increase in amount of phenolic compounds.

The antioxidant activity of lutein could be due to its unique structure (Hayes et al., 2011). Lutein has conjugated double bonds which provides free radical quenching activity (Figure 34). In addition, lutein has two phenolic hydroxyl groups on both ends of its chemical structure and is thus a strong antioxidant compared to other carotenoids (Miki, 1991). Furthermore, carotenoids are excellent antioxidants due to their ability of trapping peroxyl radical and quench singlet oxygen (Figure 33) (Burton and Ingold, 1984). Moreover, the antioxidant activity of lutein depends on its chemical structure and the concentration of oxygen (Krinsky, 1993). According to the study of Ivanov et al. (2018), the antioxidant activity may be from the oleuropein which is the most abundant phenolic compound in OLE. Oleuropein has the ability to chelate metals ions (e.g. Cu^{2+} and Fe^{2+}) which catalyse free radical generation reaction (Suchal et al., 2016; Omar, 2010).

$$CAR + ROO^{\cdot} \rightarrow CAR^{+\cdot} + ROO^{-}$$
$$CAR + ROO^{-} \rightarrow CAR^{\cdot} + ROOH$$
$$CAR + ROO^{\cdot} \rightarrow ROOCAR^{\cdot}$$
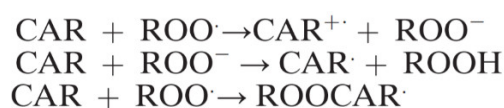
Figure 33: The carotenoids (CAR) scavenger radical mechanism. Adapted from Eghbaliferiz and Iranshahi (2016)

The antioxidant activity of OLE is a combination of oleuropein with other active polyphenol groups which has a synergism behaviour (Benavente-Garcıa et al., 2000). In addition, the polyphenols may act as a prooxidant in the presence of Fe or Cu (Figure 32) (Eghbaliferiz and Iranshahi, 2016). Due to this complex system of polyphenols, some polyphenol compounds may have acted as a prooxidant, which could be the reason why some samples with addition OLE had higher PV-, CD-, CTe, TBARS- and SBs-value compared to other samples containing oregano extract.
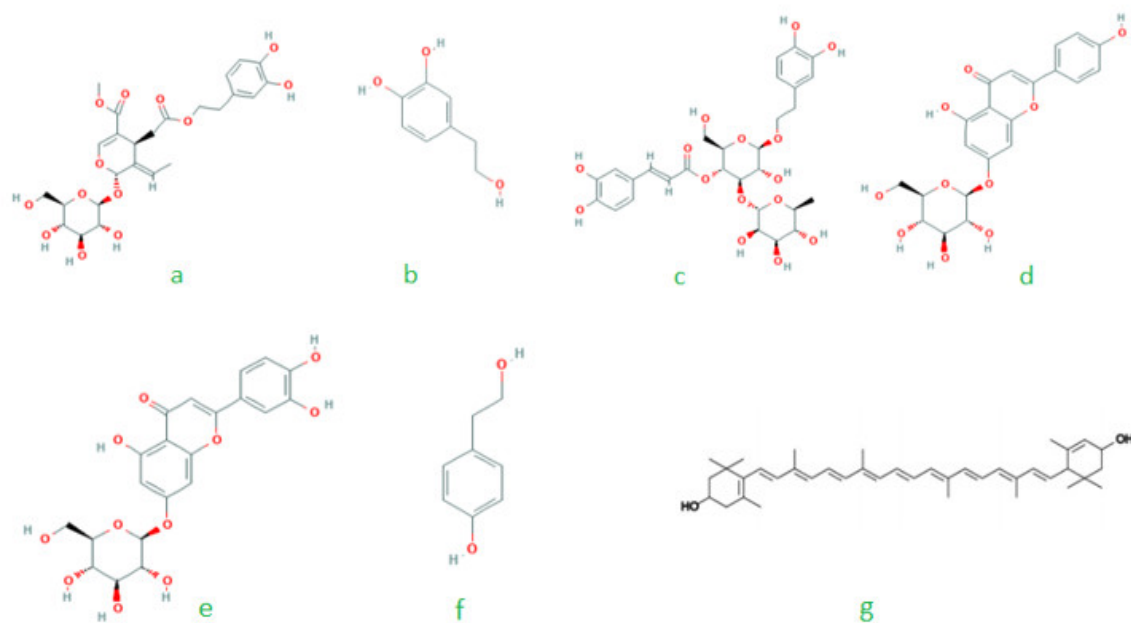
Figure 34: Chemical compounds in OLE. a) Oleuropein, b) hydroxytyrosol, c) verbascoside, d) apigenin-7-O-glucoside, e) luteolin-7-O-glucoside, f) tyrosol and g) lutein. Adapted from PubChem (2019i; 2019g; 2019b; 2019e; 2019f; 2019a) and Hayes et al. (2011).

Nettle extract did not show potential to increase lipid oxidative stability of sous-vide cooked herring samples during cold storage according to results of PV-, CD-, CTe-, TBARS- and SBs-value. Thus, in the present study, nettle extract cannot be considered to be used as an antioxidant in sous-vide cooked herring during cold storage of 20 days. The antioxidant activity of nettle extract is mainly due to rutin, querceting and ursolic acid (Figure 35) (Bourgeois et al., 2016). The same result has also been confirmed by the study of Jan and Singh (2017). However, quercetin may act as a prooxidant in certain circumstances (Choi et al., 2003; Fiorucci et al., 2007). Schmalhausen et al. (2007) found that the prooxidant activity of quercetin is connected to the presence of metals. The results of Schmalhausen et al. (2007) showed that the presence of iron(II) (Fe^{2+}) significantly increases the prooxidant activity of quercetin due to reaction between hydrogen peroxide (H_2O_2) and iron(II) (Fe^{2+}) yielding a highly reactive hydroxyl radical. In the work of Cao et al. (1997), quercetin was oxidising beta-phycoerythrin in the presence of copper (II) (Cu^{2+}). In addition, the presence of other phenolic compounds may be important in nettle extracts due to the stabilization of the antioxidant action of quercetin. This agrees with the study of Orčić et al. (2014) and Otles et al. (2012) that the presence of other potential antioxidant compounds such as lignans (secoisolariciresinol) or flavonoids (kaempferol derivatives) is stabilizing the antioxidant action of quercetin. Nettle extract may have acted as a prooxidant in this study (Figure 32), which may explain why herring samples

subjected to nettle extract had higher PV-, CD-, CTe, TBARS- and SBs-value compared to other samples containing oregano extract.
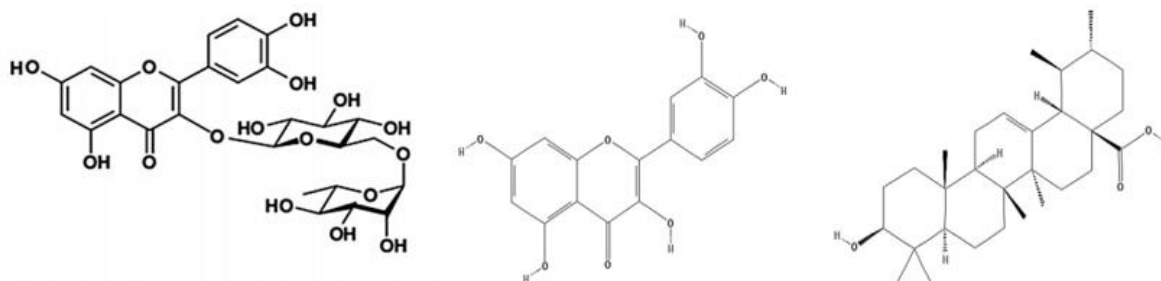
Figure 35: Antioxidant compounds in nettle extract. Rutin (left), quercetin (middle) and ursolic acid (right). Adapted from Jan and Singh (2017) and Bourgeois et al. (2016).

According to the obtained results, it was revealed that oregano extract has a potential to act effectively in fish products to retard lipid oxidation. The results have shown that both primary and secondary lipid oxidation products decreased or insignificantly varied in herring samples treated with oregano antioxidant extracts compared to sample Control. The rest of the antioxidant extracts did not show the same efficiency to retard lipid oxidation in herring samples, probably because they lost their antioxidant activity during storage and started to act as prooxidants (Ingold et al., 1993).

Conclusion

Oregano extract showed its efficacity to retard lipid oxidation in fish sample, while olive leaf extract, nettle extract and dill extract did not show any effect. Oregano extract was the only extract showing its antioxidant potential to decrease lipid oxidation in fish samples during cold storage.

The peroxide value varied between 3,56 and 36,95 $m_{eq}O_2$/kg lipid during the storage period. The PV-value limit of acceptability was 5 $m_{eq}O_2$/kg lipid. Some herring samples were below the PV-value limit of acceptability and are regarded as not rancid, while other samples were over the limit and are regarded as rancid. The content of conjugated dienes and conjugated tetraenes varied between 7,68 and 14,53 mL/g and 1,40 and 5,83 mL/g, respectively.

The thiobarbituric acid reactive substances value varied between 1,47 and 13,15 µMol TBARS/g lipid. All herring samples surpasses 2 µMol TBARS/g lipid TBARS-limit of consumption, except of herring sample with addition of nettle extract from day 18 which was below the TBARS-limit of consumption. The Schiff base values of herring samples displayed a variation ranging from 0,31 to 0,88 mL/kg lipid during the storage period.

The water content varied between 56,24 and 63,79%, and the ash content varied between 0,57 and 1,62% of wet weight herring samples. The cook loss varied between 4,25 and 12,24% and the lipid content varied between 17,93 and 29,91% of herring samples. No correlations were found between water content and cook loss. The total variation of L*-value was between 63 and 75 CIE units, the a*-value between -2,07 and 2,54 CIE units, and the b*-value between 14,67 and 18,66 CIE units. No correlations were found between L*-value and cook loss of the different sample groups. No correlations were found between a*-value and peroxide value, conjugated dienes, conjugated tetraenes and Schiff bases. No correlations were found between b*-value and conjugated dienes, conjugated tetraenes, TBARS and Schiff bases. Only the statistical analysis of peroxide value and b*-value for herring sample with addition of olive leaf extract showed a significant ($p < 0,05$) correlation (R = 0,70).

Further investigations are needed to study the influence of the concentration effect of the antioxidant extracts on lipid oxidation stability in fish products. Overall, the herring samples are considered to be in the propagation step. After 20-days of cold storage, all herring samples are regarded as rancid.

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Appendix A – Raw data and calculation of analysis

The raw data and calculations used to obtain the results of this work can be found in the attached compressed zipped-folder with 10-excel files. The excel-files are numbered and named after content.