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Oxidation of Fish Oil

The Influence of Aroma Compounds on
Determination of PV and AV

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Summary

The consumption of fish oil as a nutraceutical has increased extensively in the recent years, due to their high content of health beneficial omega-3 fatty acids. The omega-3 fatty acids in fish oil are highly unsaturated and therefore particularly prone to oxidation, the deterioration reaction that give rise to off-flavors associated with rancid fish oil. Many people find the flavor of fish oil rejecting, thus the industry commonly add fruit flavorings to make the fish oil more appealing.

The most commonly used methods for assessment of oxidation status in fish oil today are peroxide value (PV) method (iodometric titration method) and anisidine value (AV) method that provide determination of primary and secondary oxidation products respectively. Flavorings were recently found to interfere with these traditional methods. The aim of this thesis was to study the influence of flavorings on PV and AV methods further and to investigate if different concentrations of aldehydes in the flavorings give different effects.

The Iodometric Titration Method were found to be affected by the lemon flavorings, giving slightly elevated peroxide values. The interference is possibly caused by the presence of oxidizing compounds in the lemon aroma interfering with the redox method for measuring hydroperoxides. The anisidine value method was found to be highly affected by the aldehydes in the flavorings giving extremely elevated anisidine values. A significant correlation ($p < 0.05$) between the aldehyde content and the anisidine values was found.

Both PV and AV are widely used methods for determining lipid oxidation status in fish oil, both in industry and research. They are also used as parameters for recommendations of limits of oxidation status by expert organizations and regulatory authorities. Both methods were found to be influenced by compounds that were not part of the lipid oxidation process, and especially the AV method gave highly unreliable results in fish oils with flavoring. There is a need for development and implementation of methods that are less sensitive to aroma compounds for the assessment of oxidations status in flavored fish oils.

The more advanced and specific method, ^{1}H NMR spectroscopy made it possible to distinguish the aldehydes from the aroma and the oxidation-derived aldehydes. The aldehydes from the lemon aroma gave signals in another region than the oxidation-derived aldehydes in the ^{1}H NMR spectrum. Dynamic Headspace GC-MS was used to study the development of volatile

secondary oxidation products. An increase in the amount of (E,E)-2,4-heptadienal with storage time was found, but that there was a clear decrease in the amount of the volatiles (E)-2-pentenal, (E)-2-hexenal, (Z)-4-heptenal with storage time was unexpected.. The inconsistency of the findings of development of volatiles shows how complex lipid oxidation and volatile formation in fish oil is and more studies should be performed to study the potential of volatiles serving as parameters of oxidation status in fish oil.

In addition, the influence of some minerals and vitamins on the oxidation process were investigated. Of the minerals Potassium Iodide, Folic Acid, Zink Oxide, Magnesium Oxide and Calcium Carbonate examined using the Oxidative Stability Index method, Calcium Carbonate and Potassium Iodide were found to have small pro-oxidative effects in fish oil without antioxidant, but no effects were observed in fish oil with antioxidant.

The Vitamin K2, Ascorbic Acid, Vitamin A, Vitamin D and Vitamin E were also studied using the Oxidative Stability Index method, and no significant effects were found. A slight anti-oxidative effect was found for Vitamin A and E on the development of secondary oxidation products measured by the anisidine value method in fish oil with and without antioxidant. However, for fish oil without antioxidant, there was observed a pro-oxidative effect at day 20, the last day of measurements, but this effect was not seen in the fish oil with antioxidant. Since the fish oil on the market is always added with antioxidant, these findings are not critical.

Sammendrag

Fiskeolje som kostholdstilskudd har økt i popularitet de siste årene på grunn av det høye innholdet av sunne umettede omega-3 fettsyrer. Høyt innhold av umettede fettsyrer gjør fiskeolje spesielt utsatt for oksidasjon, reaksjonen som fører til forringelse og utvikling av uønskede smaksstoffer assosiert med oksidert fiskeolje. Som følge av at mange ikke liker smaken av fiskeolje tilsetter industrien ofte fruktsmak til fiskeoljen for å gjøre produktene mer appellerende.

De mest brukte metodene for å bestemme oksidasjonsstatus i fiskeolje er peroxide value (PV) med iodometrisk titrerings metode og anisidine value (AV) metode som bestemmer henholdsvis primære og sekundære oksidasjonsprodukter. Smakstilsetningene i fiskeolje er nylig funnet å interferere med disse tradisjonelle metodene. Målet med denne masteroppgaven var å finne ut mer om smakstilsetningenes påvirkning på PV og AV, og videre om ulik aldehydkonsentrasjon i smakstilsetningene gav ulik effekt.

Sitronaroma ble funnet å interferere med PV bestemmelse i fiskeolje, hvor sitronaroma gav litt forhøyede verdier for primære oksidasjonsprodukter. Effekten var trolig forårsaket av oksiderende stoffer i sitronaromaen som interferer med redoksmetoden for å måle hydroperoksidene. Aldehydene i aromastoffene ble funnet å interferer i høy grad med AV metoden og det gav utslag i ekstremt forhøyede anisidine verdier. Signifikant korrelasjon ($p < 0.05$) ble funnet mellom mengden aldehyder i aromaene og anisidine-verdiene. Både AV og PV er vanlige metoder for å bestemme oksidasjonsstatus i fiskeolje, både i industrien og i forskning. I tillegg er anbefalinger om grenser for oksidasjonsstatus i stor grad basert på disse parameterne. Begge metodene har vist seg å bli påvirket av forbindelser som ikke har noe med lipidoksidasjon å gjøre, og spesielt AV-metoden gir svært upålitelige resultater i smakstilsatt fiskeolje. Metoder som er mindre sensitive for smaksstoffer må utvikles og implementeres for å kunne vurdere oksidasjonsstatus i smakstilsatt fiskeolje.

Den mere avanserte og spesifikke metoden ^{1}H NMR-spektroskopi gjorde det mulig å skille sitronaromaaldehydene fra oksidasjonsaldehydene. Aldehydene fra aromaen gav signaler i et annet område i NMR-spektrumet enn der aldehyder fra oksidasjonen er forventet å gi signaler. Dynamic Headspace GC-MS ble brukt til å studere utviklingen av de sekundære volatile oksidasjonsproduktene. En økning av (E,E)-2,4-heptadienal ble funnet med økt lagringstid i motsetning til (E)-2-pentenal, (E)-2-hexenal, (Z)-4-heptenal som ble funnet å minke med økt

lagringstid. De inkonsekvente resultatene viser hvor kompleks lipidoksidasjon og dannelse av volatile forbindelser er i fiskeolje. Flere studier kreves for å evaluere potensiale for å bruke sekundære volatile oksidasjonsprodukter som parametere for å bestemme oksidasjonsstatus i fiskeolje.

I tillegg ble noen mineraler og vitaminer undersøkt for potensiell påvirkning på oksidasjonsprosessen i fiskeolje. Mineralene kaliumjodid, folsyre, sinkoksid, magnesiumoksid og kalsiumkarbonat ble undersøkt ved å bruke Oxidative Stability Index metoden. Kalsiumkarbonat og kaliumjodid ble funnet å ha en prooksidative effekt i fiskeolje uten antioksidant. Vitaminene vitamin K2, vitamin C, vitamin A, vitamin D og vitamin E ble også undersøkt med OSI metoden, men ingen signifikante effekter ble funnet. Vitamin A og E gav en liten antioksidativ effekt på utviklingen av sekundære oksidasjonsprodukter målt med AV metoden i fiskeolje med og uten antioksidant. Videre ble det funnet en prooksidativ effekt for vitamin A og E i fiskeolje uten antioksidant i siste fase av lagringsforsøket, men denne effekten ble ikke observert i fiskeolje med antioksidant. Siden fiskeolje på dagens marked alltid er tilsatt antioksidant, er ikke disse funnene kritiske.

Preface

This master thesis was completed as part of the MSc Biotechnology at Norwegian University of Science and Technology, NTNU. The experiments of the thesis were mainly carried out at SINTEF Fisheries and Aquaculture but also at the Department of Biotechnology, NTNU, both in Trondheim, Norway.

The supplement company is thanked for providing the fish oil, antioxidants, vitamins and minerals used in the experiments.

I want to express my gratitude to my main supervisor Professor Turid Rustad at Department of Biotechnology, NTNU for always being available to answer my questions, for her guidance in scientific writing, for her constructive feedbacks through the whole process and proofreading in the end.

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Karoline Kaknita Eap

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Abbreviations

ALA – α-linolenic acid

AOCS – American Oil Chemists' Society

AV – Anisidine value

DHA - Decosahexaenoic acid

DPA – Docosahexaenoic acid

EDTA – Ethylenediaminetetraacetic acid

EFSA – European Food Safety Authority

EPA – Eicosapentaenoic acid

GOED – Global Organization for EPA and DHA Omega 3s

LA – Linoleic acid

n-3 LC PUFA – omega 3 long chained poly unsaturated fatty acid

n-6 LC PUFA – omega 6 long chained poly unsaturated fatty acid

NMR – Nuclear Magnetic Resonance

OSI – Oxidative Stability Index

OUR – Oxygen Uptake Rate

PV – Peroxide value

ppb – parts per billion

ppm – parts per million

TMS - Tetramethylsilane

UV – Ultraviolet

Contents

1. Introduction

The Danish researchers, Bang and Dyerberg, discovered in 1971 that ischemic heart disease is very rare in the Greenlandic Eskimos. They made further investigations showing that the Eskimos had remarkably low levels of plasma-lipids in their blood, despite their diet consisting almost entirely of meat and fish, including considerably high amounts of animal fat, compared to a healthy Danish control group (Bang et al., 1971) as shown in figure 1. These findings led to studies of the Eskimos' diet, and significant differences were found between the diet of Eskimos and Danes regarding the fatty acid distribution of the fats in their food. The fat in the Eskimos diet was largely made up of omega-3 long chained polyunsaturated fatty acids (n-3 LC PUFA) compared to the Danish diet that was in contrast very high in omega-6 fatty acids. Of the total fatty acid consumption, the n-3 LC PUFAs: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) constituted 13,1% for the Eskimos, and only 0,8% for the Danes, as a result of the Eskimos' high intake of marine food (Bang et al., 1980).

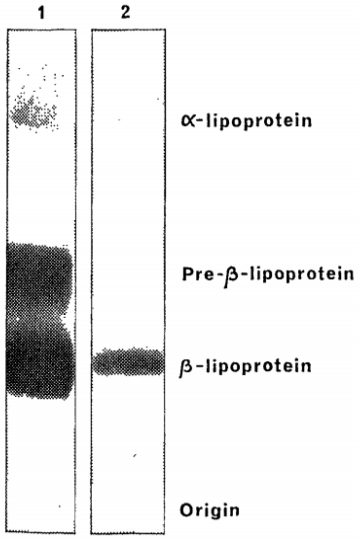

Figure 1: Big differences were observed in the typical lipoprotein electrophoresis strip from a healthy Dane (1) and a Greenland Eskimo (2) (Bang et al., 1971)

From the first publication of the n-3 LC PUFAs possible role in reducing the risk of cardiovascular disease, extensive research has been carried out and by now n-3 LC PUFA is proven to have a number of beneficial health effects. EPA and DHA are reported to have preventive effect on coronary heart disease (Wesley, 1998) (Leaf et al., 2003) (CALDER,

2004), anti-inflammatory effects (Calder, 2013), and to be important in development and maintenance of normal function of brain and retina (Swanson et al., 2012).

1.2 Fish oil as a nutraceutical

There are no human enzymes capable of introducing a double bond at n-3 and n-6 positions, thus omega-3 and omega-6 fatty acids need to be supplemented through the diet. The challenge however is that the modern western diet has evolved into containing a highly unbalanced ratio of omega-3 and omega-6 fatty acids, due to increased consumption of vegetable oil and grains (Simopoulos, 2002).

α-linolenic acid (ALA), the precursor of the n-3 LC PUFAs EPA and DHA can be found in some plants, seeds and nuts, and the human body is able to convert ALA into the longer fatty acids DHA and EPA. However, the conversion are inefficient, and only about 8% of ALA is believed to be converted to EPA (Burdge and Calder, 2005). The metabolic pathway of both n-6 PUFA and n-3 PUFA, depend on the same enzymes to elongate the fatty acids. Even though the enzymes have greater affinity for ALA than for the n-6 PUFA precursor, Linoleic acid (LA), the western diet consists of about ten times more of LA compared to ALA, thus the overall conversion is greater for n-6 PUFAs (Burdge and Calder, 2005).

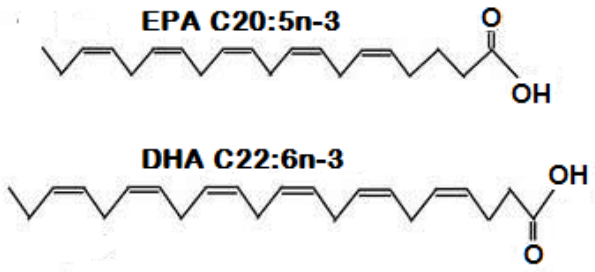

Figure 2: The n-3 LC PUFAs EPA and DHA

EFSA (2010) recommends 1-2 meals of fatty fish per week, or equal supplement of 250mg of EPA + DHA per week. The easiest way of implementing EPA and DHA into the diet is obviously the natural way, by eating fatty fish like mackerel, sardines, anchovy and herring, but many people do not have easy access to fatty fish, do not like the taste or simply think it is more convenient to take a supplement. The demand for fish oil therefore increased, and today about 1 million tons are produced annually, and of this approximately 70 000 tons are used for human consumption (Carvajal and Mozuraityte, 2015).

EFSA (2010b, 2010a, 2009) have evaluated the substantiation of health claims related to DHA and EPA and concluded that a cause and effect relationship is possible to establish between the consumption of DHA and EPA and the following health claims, when 200mg-300mg/day are consumed.

DHA/EPA contribute to:

- The maintenance of normal blood concentrations of triglycerides
- The maintenance of normal brain function and vision
- The maintenance of normal cardiac function and blood pressure

Marine oils are the predominant sources for n-3 LCPUFAs and especially DHA and EPA. The raw-material primarily used for fish oil production are the body of fatty fish, fish liver of white lean fish, and the blubber from marine mammals, like seal and whale, but also algae (Shahidi, 2007). The EPA and DHA content in various fish is shown in table 1.

Table 1: The content of EPA and DHA in various Fish (% of total fatty acids) Adapted from (Shahidi, 2007)

	EPA (%)	**DHA (%)**
Sardine	3	9-13
Pacific anchovy	18	11
Mackerel	8	8
Capelin	9	3
Herring	3-5	2-3

1.3 Oxidation

Lipid oxidation causes decomposition of the fatty acids, resulting in nutritional losses, development of undesirable flavor, odor and colour, and may give rise to compounds associated with negative biological effects. Lipid oxidation describes a series of complex chemical changes from the reaction of lipids with oxygen. Different forms of lipid oxidation are autoxidation, thermal oxidation, photo-oxidation, and enzymatic oxidation. In the case of fish oil, the autoxidation is of most concern, because most of the enzymes such as the lipoxygenases are inactivated and metal ions are removed during the refining process (Aursand et al., 2011), and the thermal oxidation and photo-oxidation is prevented by storing the oil in a cold, dark place and using dark-coloured containers.

1.3.1 Autoxidation

Autoxidation is the natural, autocatalytic chain reaction occurring between atmospheric oxygen and unsaturated fatty acids. The chain mechanism can be divided into three steps: initiation, propagation and termination (Shahidi and Wanasundara, 2002).

Initiation – The Formation of the Lipid Radical

Autoxidation requires the fatty acids to be in a radical form. Because fatty acids normally are non-radical, and the reaction with atmospheric triplet oxygen is thermodynamically unfavorable, the reaction depends on an initiator. Initiation reactions involve for example fatty acids reacting with singlet oxygen or other activated oxygen species or reacting with metals ions, to produce free radical inititators, which catalyze the reaction. The radicals formed can easily abstract a hydrogen from a fatty acid containing an allylic center, resulting in a hydrogen radical ($H^{\cdot}$) and a lipid free radical called an alkyl free radical ($R^{\cdot}$) (Frankel, 2005). The formation of the alkyl radical leads to electron delocalization and double bond rearrangement to form conjugated dienes and trienes (Shahidi and Zhong, 2010).

$$RH \xrightarrow{initiator} R^{\cdot} + H^{\cdot}$$

Propagation – The Formation of Lipid Hydroperoxides

In the following step, the alkyl free radical reacts with atmospheric oxygen to form a hydroperoxy free radical ($ROO^{\cdot}$), which may abstract a hydrogen from another unsaturated fatty acid, leading to the formation of a hydroperoxide (ROOH) and a new alkyl free radical. The new alkyl free radical may in turn react with atmospheric oxygen, and in this manner continue the chain reaction or decompose to other free radicals, which consequently results in accumulation of free radicals in the oil (Shahidi and Wanasundara, 2002) (Coultate, 2009).

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$$

$$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$$

Termination – The Formation of End Products

The chain reaction may be terminated, when the concentration of free radicals is so high that two radicals interact with each other to form a non-radical product (Coultate, 2009).

$$R^{\cdot} + R^{\cdot} \rightarrow RR$$

$$R^{\cdot} + ROO^{\cdot} \rightarrow ROOR$$

$$ROO^{\cdot} + ROO^{\cdot} \rightarrow ROOR + O_2$$

1.3.2 Kinetics

The oxidation process in lipids starts out with an induction period where the oxidation products develop slowly, before the formation increases exponentially later on as illustrated in figure 3. This induction period is very important in the industry, because this is also expressed as the shelf life, the storage time before the quality is unacceptable to the consumers. Thus, the induction period is a phase of oxidation, which is particularly important to control and often measures are taken to prolong it, by reducing temperature, remove oxygen access, adding antioxidants and reducing prooxidant activity. Later on, the decomposition of hydroperoxides, into secondary oxidation products, will be higher than the hydroperoxide formation and there will be a drop in hydroperoxide concentration. Taken together this means that the primary oxidation products will dominate initially, while the secondary oxidation products eventually dominates in a later phase (Frankel, 2005).

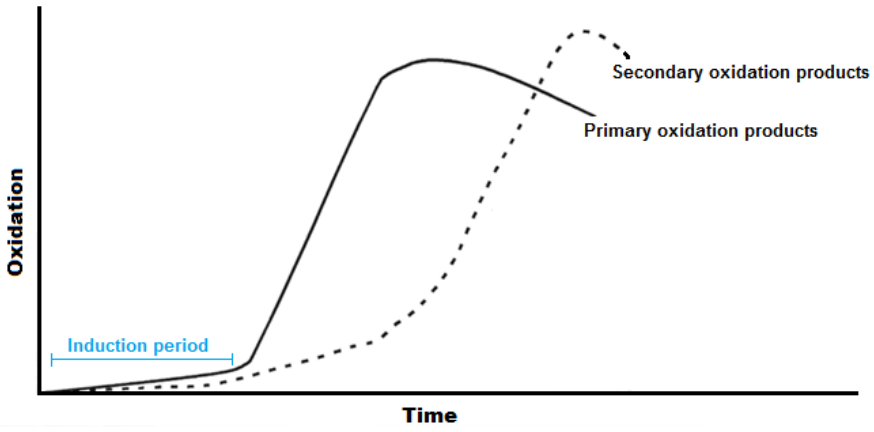

Figure 3: After an induction period where the formation of oxidation products develop slowly, the formation rate accelerates. First the primary oxidation products will dominate before secondary oxidation products dominate later on.

The oxidative stability of oils and the length of the induction period depends on environmental factors such as light, heat, types of oxygen accessible, the processing of the oil, fatty acid composition and small substances present, such as metals, free fatty acids and antioxidants (Choe and Min, 2006). Fish lipids are very rich in long-chained polyunsaturated fatty acids (30-40%), thus highly susceptible to oxidation. The first step of lipid oxidation is removal of hydrogen from the fatty acid to form a lipid radical. In n-3 LC PUFA the hydrogen is abstracted from one of the bis-allylic position, because the double bonds on each side withdraw the electrons, leaving the C-H bond in the middle weakened. One of the bis-allylic positions in DHA is shown in figure 4. For example the hydrogen at C11 in linoleic acid require 50kcal/mol to be removed, while the hydrogens at C8 and C14 require 100kcal/mol (Min and Boff, 2002). Frankel (2005) reported that the oxidizability of the fatty acid esters 18:2, 18:3, 20:4 and 22:6 was proportional to the number of bis-allylic positions present in the fatty acids. Oxidizability is a term used to express the reactivity of unsaturated lipids to undergo oxidation. As the number of conjugated double bonds increases in PUFA, the more bis-allylic positions are available for hydrogen abstraction, resulting in a huge variety of primary oxidation products that are further decomposed to an even more complex mixture of secondary oxidation products that are challenging to analyze quantitatively (Kobayashi et al., 2003).

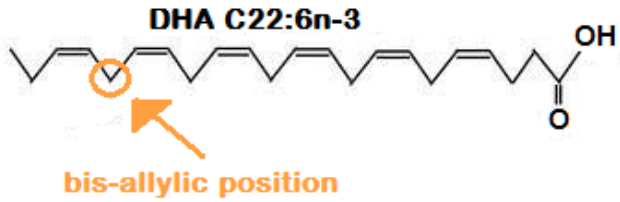

Figure 4: One of the bis-allylic positions between two conjugating double bonds in DHA.

1.4 Oxidation products

The lipid hydroperoxides are relatively stable in the absence of prooxidants, but since it is impossible to eliminate prooxidants completely the hydroperoxides are decomposed further. The most common decomposition pathway is cleavage of the oxygen-oxygen bond, which require the least activation energy. The cleavage leads to formation of the decomposition products; alcoxyl radical and hydroxyl radical. The alcoxyl radical can enter a number of different reaction pathways, but is eventually cleaved by β-scission on the carbon-carbon bond.

The decomposition products from the carboxylic end is esterified to the glycerol of triacylglycerols and therefore do not contribute to the volatile compounds. The decomposition product from the methyl end will result in formation of secondary oxidation products such as aldehydes, ketones, alcohols, furans, hydrocarbons and acids (Chaiyasit et al., 2007).

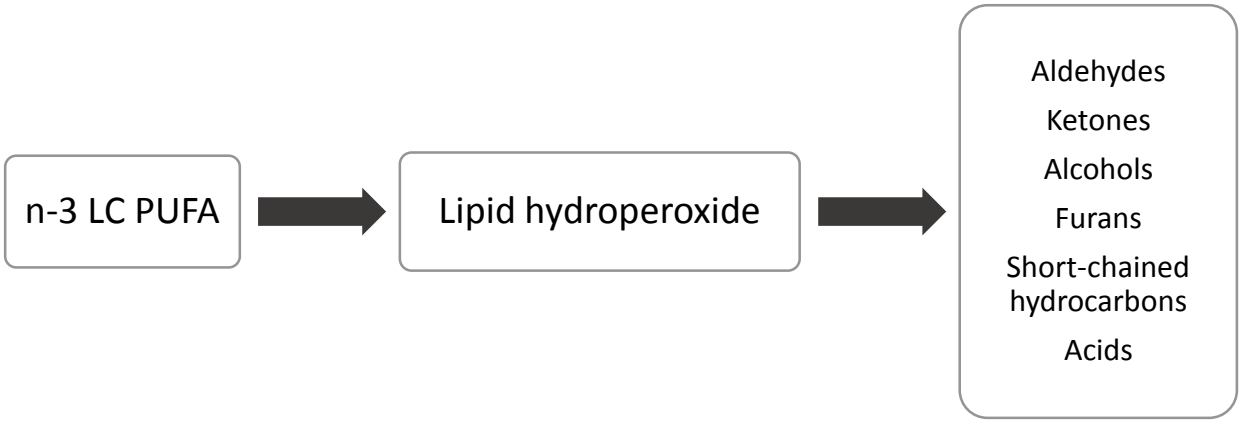

Figure 5: The oxidation products formed from the n-3 LC PUFA

The secondary oxidation products comprise the volatile compounds giving off-flavors in oxidized fish oil. The odors and flavors of oxidized fish oil have been described as rancid, painty, fishy, green and burnt (Karahadian and Lindsay, 1989). The flavor compounds have very low thresholds, perceptible at concentrations as low as parts per billion (ppb). These volatile compounds are challenging in the industry, because they are produced at very low levels of oxidation (Frankel, 2005).

1.5 Antioxidants

Addition of antioxidant is an important measure to extend the induction period and slow down the rate of the autoxidation process in fish oil, with the aim to prevent quality deterioration. Antioxidant activity involve free radical scavenging, inactivation of peroxides and other reactive oxygen species, chelation of metal ions and inhibition of pro-oxidative enzymes (Shahidi and Zhong, 2010). Antioxidants can be divided into two groups, the primary antioxidants, those which break the chain reaction of oxidation by scavenging free radicals, and the secondary antioxidants which inhibit the oxidation by suppression of oxidation initiators or by regeneration of primary antioxidants (Shahidi and Zhong, 2010).

The primary antioxidants such as phenolic compounds scavenge free radicals like alkyl radicals ($R^{\cdot}$), alkoxy radicals ($RO^{\cdot}$) and Hydroperoxi radicals ($ROO^{\cdot}$) and do so by neutralizing them through donating a hydrogen.

$$R^{\cdot} + AH \rightarrow RH + A^{\cdot}$$

$$ROO^{\cdot} + AH \rightarrow ROOH + A^{\cdot}$$

$$RO^{\cdot} + AH \rightarrow ROH + A^{\cdot}$$

The antioxidant radicals formed, undergo stabilization through delocalization of the unpaired electron around the phenol ring. The stabilized antioxidant radicals will usually not react further to generate new free radicals, and consequently break the propagation step. In addition, the antioxidant radicals can scavenge free radicals and contribute to formation of stable non-radical end-products, thereby having a role in the termination of oxidation (Shahidi and Zhong, 2010).

$$ROO^{\cdot} + A^{\cdot} \rightarrow ROOA$$

$$RO^{\cdot} + A^{\cdot} \rightarrow ROA$$

$$A^{\cdot} + A^{\cdot} \rightarrow AA$$

The secondary antioxidants act by a range of mechanisms. Some antioxidants suppress initiators such as metal ions, singlet oxygen or prooxidative enzymes, others are reducing agents and are able to reduce lipid peroxides to form stable products. Metal chelators such as citric acid, phosphoric acid and ethylenediaminetetrascetic acid (EDTA) can inhibit metal ions' well known ability to initiate oxidation, by incorporating them in a stable complex. Other secondary antioxidants can regenerate primary antioxidants from their radical forms by providing hydrogen atoms, and thereby help maintain the supply of primary antioxidants. In example ascorbic acid can regenerate tocopherols from their radical form (Shahidi and Zhong, 2010).

1.6 Determination of oxidation status in fish oil

Lipid oxidation is the main cause of quality deterioration and limited shelf life of fish oil, and may generate compounds with negative health effects, but information on the latter is scarse. Both EFSA (2010) and Norwegian Scientific Committee for Food Safety (2011) concluded in their reports that the available information on the relation between oxidized fish oil and negative health effects is insufficient to carry out a health risk assessment. The information is insufficient

both with respect to the lack of information on toxicity of individual oxidation products and at what oxidation level that will potentially give a negative effect in humans.

Regarding recommended limits on oxidation in fish oil for human consumption, EFSA (2010) concluded that it is not possible to establish a maximum tolerable level of Peroxide value and Anisidine value, based on the very few studies available on negative health effects of oxidized fish oil where PV and AV are the parameters used. However, CODEX Alimentarius Comission states in the Standard for Fish Oil that PV should not exceed 5 and AV should not exceed 20 in fish oils, fish liver oils and concentrated fish oil intended for human consumption (Codex Alimentarius Commission, 2015). And The Global Organization for EPA and DHA omega-3s (GOED) sets the limit for EPA and DHA oils to have PV ≤ 5 and for AV ≤ 20 ((GOED), 2015).

However, humans have a very low threshold for detection of these volatiles, due to the natural avoidance mechanism for oxidized fats (Frankel, 2005). The Global Organization for EPA and DHA Omega 3s (GOED) reports that surveys show that between 7% and 23% of non-users answers that fishy flavor is the reason they are not willing to try an omega-3 supplement (GOED, 2015). Hence both from a health and a economical point of view, it is of great interest to the industry to monitor the oxidation status, in order to offer high quality fish oil products, with low oxidation status to the consumer marked.

1.7 Methods for determining oxidation status

As mentioned previously the primary oxidation products and the secondary oxidation products dominate at different stages in the oxidation process, thus it is not sufficient to determine either of the oxidation products alone. To get a total picture of the oxidation status it is necessary to use a combination of methods.

One of the most commonly used methods for determining primary oxidation products involve utilizing the redox properties of hydroperoxides. One of the most used methods is the iodometric titration method for determining peroxide value, which is based on the oxidation of iodide to iodine. Another method for determining primary oxidation products is measuring conjugated dienes spectrophotometrically at 233nm. To measure the secondary oxidation products some colorimetric methods are commonly used. The anisidine value method is the most popular method, where aldehydes react with p-anisidine to generate a yellow compound measured spectrophotometrically at 350nm. An alternative approach to study lipid oxidation is to test their stability under conditions that favor oxidation, with for example increased

temperature and increased oxygen access. Oxygen absorption measurements involve methods monitoring the oxygen consumption using for example an oxygraph system that measures the oxygen concentration with time as the lipid oxidation proceeds in the sample and gives the oxygen uptake rate (OUR). Oil Stability Index is a method that measures the secondary oxidation products by detecting an increase in conductivity, caused by the formation of volatile organic acids, when effluent from oxidizing oil is passed through distilled water.

1.7.1 Peroxide Value (PV) – Iodometric Titration method

The iodometric titration method is based on redox properties of the hydroperoxides and is a method for determining the total amount of the primary oxidation products: hydroperoxides. The hydroperoxides in the oil sample react with iodide ions (I^-) in a saturated solution of potassium iodide (KI) to produce iodine (I_2), which is then titrated against a standardized sodium thiosulfate solution ($Na_2S_2O_2$) as displayed in equation 1 and 2. The end point can be determined with a starch indicator, but nowadays the end point is more commonly determined potentiometrically by measuring liberated iodine electrochemically by reduction at a platinum electrode (Frankel, 2005). The PV is given as milliequivalents of peroxides per kilogram of sample (meq/kg) (Shahidi and Zhong, 2005).

$$ROOH + 2H^+ + 2KI \rightarrow I_2 + ROH + H_2O + 2K^+ \quad (1)$$

$$I_2 + 2NaS_2O_3 \rightarrow Na_2S_2O_6 + 2NaI \quad (2)$$

The popularity of the iodometric titration method is probably due to the simplicity of the experimental procedure, but even though the method is widely used, it has some disadvantages. The method is time-consuming and labor-intensive, requires a relative high amount of sample, and produces a large amount of waste. And more importantly absorption of iodine at unsaturated positions of the fatty acids and liberation of iodine from the potassium iodide by oxygen present in the solution to be titrated, may give falsely low and falsely high PV, respectively (Shahidi and Wanasundara, 2002). Furthermore, the peroxide value is highly influenced by factors such as reagent reaction time and the stirring procedure used, and therefore the method is highly dependent on the technique of the performer (Semb, 2012,

Frankel, 2005). The sensitivity of the method is 0,5 meq/kg, but are improved by the potentiometric end point determination (Frankel, 2005).

1.7.2 Anisidine Value (AV) Method

The Anisidine Value method is a spectrophotometric method where aldehydes, primarily 2-alkenals and 2,4-alkadienals) in the oil sample, are detected. The method is based on the reaction between the aldehydic carbonyl and the amine group of p-anisidine, which produces yellow compounds that absorb at 350nm in acidic conditions (Shahidi and Zhong, 2005) as illustrated in figure 6. The anisidine value is defined as the absorbance of a solution of 1g of oil dissolved in 100mL of solvent (iso-octane) and added reagent (0,25% p-anisidine, dissolved in glacial acetic acid) (IUPAC, 1979).

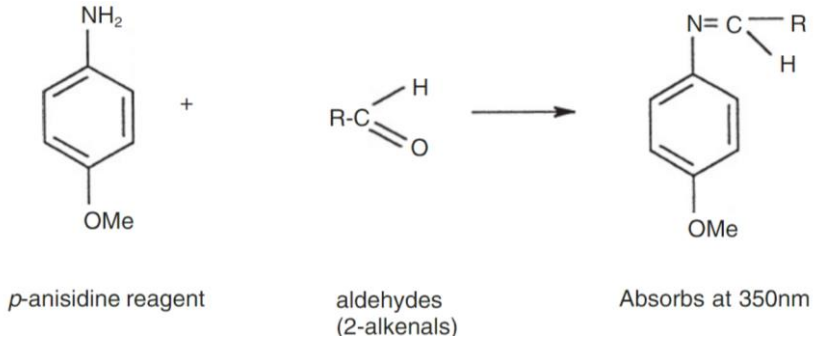

Figure 6: The aldehyde reacts with the anisidine reagent to form a yellow compound that has absorbance peak at 350nm.

The experimental procedure is simple in the AV method, but due to the toxicity of the anisidine reagent, precautionary measures needs to be taken. The colour intensity of the yellow compound produced is not only dependent on the amount of aldehydes present in the sample but also on the structure of the aldehydes. Hydrocarbon chains with a double bond conjugated with the carbonyl double bond have been found to increase the molar absorbance four to five times. As a result unsaturated aldehydes give higher AV than saturated aldehydes, and therefore the oxidation products 2-alkenals and 2,4-alkadienals largely contribute to the anisidine value given by the AV method (IUPAC, 1979). However, the anisidine value is found to correlate well with the total number of volatiles in the sample (Doleschall et al., 2002).

1.7.3 ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H NMR spectroscopy is an analytical method, which in recent years has become important in the determination of lipid oxidation products on a research basis. Nuclear magnetic resonance is a phenomenon that arises when nuclei, such as protons, are exposed to an external magnetic field and irradiated with a specific radio frequency. This only occurs to nuclei with the spin property, because the spin give the nuclei magnetic moment. ^{1}H has nuclear spin because of its unpaired proton, and is useful in NMR due to its high abundance in organic molecules, such as fatty acids (Keeler, 2011).

In the presence of a strong external magnetic field the spin states with lower energy orientation will align with the field and the ones with higher energy orientations, will oppose the field. The absorption of energy will transform the nuclei from the lower energy level spin orientation to the higher energy level spin orientation. Each energy transition give a reversal of the spin orientation and the following changes in the magnetic field depend on nuclear shielding (Carey et al., 2011). Nuclear shielding is what enables NMR spectroscopy to be utilized to identify a specific molecular structure. All atom nuclei are surrounded by electromagnetic fields from their own electrons, electrons in covalent bonds and other atoms in the molecule. All together, these small magnetic fields shield the nucleus, and its experience of the applied magnetic field is decreased compared to if the nuclei were all alone in the sample (Keeler, 2011).

The resulting NMR spectrum is recorded. Each proton with a specific nuclear shielding will give rise to peaks at different frequencies in the spectrum. The area underneath each peak is directly proportional to the number of protons with that frequency. The frequencies are directly proportional to the strength of the magnetic field, so to be able to compare different NMR spectra obtained using different magnetic field strengths, it is convenient to use the chemical shift scale. The chemical shift scale give peak positions that are field strength independent with the unit parts per million (ppm). To set a point of zero, a reference compound is used. Tetramethylsilane (TMS) is commonly used for ^{1}H NMR (Keeler, 2011).

The use of NMR Spectroscopy is a relatively new approach to study lipid oxidation. The main advantages are that the NMR spectroscopy provides specific information and can detect the primary oxidation products and the secondary oxidation products simultaneously (Shahidi and Zhong, 2005). Since the integral of the peak is proportional to the amount of protons, the concentration can be determined. Other advantages are that the methodology is rapid, non-destructive and requires minimal sample preparation and no toxic chemicals. The ^{1}H NMR spectroscopy are found to correlate well with the traditional method PV (Falch et al., 2004) but

was found to correlate even better with the TOTOX value, the total oxidation value = 2 PV + AV which includes both the primary and secondary oxidation products (Shahidi et al., 1994). The disadvantages are that the chemical shift values are highly dependent on the operating conditions. Other disadvantages are that it is expensive and requires special skills to interpret the spectra (Barriuso et al., 2013). NMR Spectroscopy is found to have a relatively high detection limit <0.01 mM (Falch et al., 2004) thus relatively high levels of lipid oxidation need to occur before any changes are observed in the NMR spectra.

Most studies published addressing lipid oxidation, uses ^{1}H NMR spectroscopy to monitor the changes in lipid oxidation during storage (Claxson et al., 1994) (Guillén and Ruiz, 2005) (Saito, 1987). Very recently, articles addressing the potential of ^{1}H NMR spectroscopy for the determination of the oxidation status in fish oil have been published. Falch et al. (2004) reported good correlations between ^{1}H NMR and the traditional methods for determining primary oxidation products: conjugated dienes analysis and PV. Skiera et al. (2012b) presented a ^{1}H NMR method for the quantification of aldehydes as an alternative to the AV method in edible oils. Giese et al. (2016) used ^{1}H NMR spectra to predict the classical parameters PV and AV.

1.7.4 Dynamic Headspace Gas Chromatography-Mass Spectroscopy (GC-MS)

Gas chromatography is another advanced method for quantification of secondary volatile oxidation products and together with mass spectrometry they can be identified. Some volatile lipid oxidation products are detectable by sensory analysis at concentrations as low as ppb range (Frankel, 2005). Gas chromatography provide the sensitivity and specificity that methods like anisidine value lack, and is a valuable tool for detecting volatiles in the early stages of lipid oxidation.

Due to the complicated oil sample material direct injection methods are generally considered to be unsuitable, thus different methods are developed to collect the volatiles prior to GC-MS analysis. The automated dynamic headspace sampling method is one of the newer methods used for collecting the volatiles. The volatiles are released from the fish oil by purging the headspace of the sample with an inert gas such as nitrogen. The volatiles are then trapped in a tube packed with an absorbent. The porous polymer Tenax is commonly used. The volatiles are desorbed and transferred directly to the GC/MS system from the Tenax tube via a small thermal desorption unit. The volatiles are separated in the GC column and analyzed by MS. The only manual step in the procedure is weighing the sample into the vial. (Lerch and Gil, 2008).

Several studies have been published using dynamic headspace GC-MS and purge-and-trap method to identify volatiles contributing to the off-flavors in oxidized fish oil. Karahadian and Lindsay (1989) found hexanal, (E,Z)-2,6-nonadienal, 2,4-decdienal, 2,4-heptadienal, (E)-2-hexenal, (Z)-4-heptenal, 1,5-octadien-3-one, (E,Z,Z)- and (E,E,Z)-2,4,7-decatrienal to be the major volatiles contributing to off-flavors in oxidized fish oil. Hsieh et al. (1989) found that (Z)-2-pentenal, (E)-2-hexenal, 2,4-heptadienal, 2,4-decadienal, and (E,Z,Z)- and (E,E,Z)-2,4,7-decatrienal in fish oil.

1.8 Aim of thesis

The oxidation parameters widely used in routine analysis to monitor oxidation status in fish oil are today based on the PV and AV method, which measure the primary and secondary oxidation products, respectively. These traditional methods hold certain limitations and is recently found to be interfered by flavorings commonly added to fish oil. The aim of the thesis was to investigate further how aroma compounds influence the traditional oxidation parameters PV and AV, and if different aldehyde content would give different effects. Additionally were more specific and advanced methods such as ^{1}H NMR and dynamic headspace GC-MS were examined to see if these methods could give more accurate information on the oxidation status in fish oil.

Today fish oil is commonly fortified with minerals and vitamins to make it a more complete dietary supplement. Additionally these additives were investigated to see if they had any impact on the oxidation process of fish oil.

2. Materials and Methods

2.1 Oils

The fish oil used in the experiments was donated from a known fish oil supplement company in Norway. The crude oil was made from sardines and anchovy originating from South America, and refined in their factory in Norway. Two oils were used, one fish oil without added antioxidant and one fish oil with added antioxidant. The antioxidants added were a mixture of natural tocopherols: D-α-tocopherols, D-β-tocopherols, D-γ-tocopherols and D-δ-tocopherols.

The fish oil was stored in dark, green colored bottles in a -20 freezer, and nitrogen flushed after every sampling to limit unintentional oxidation. The fish oil was divided into several tubes assigned the different analysis, upon arrival, to avoid freezing/thawing more than one time.

2.2 Reagents and solvents

The reagents and solvents used in the experiments listed with source in parentheses:

PV measurements by iodometric titration: acetic acid (glacial) 100%(Merck, Germany), iso-octane(Merck, Germany), potassium iodide(Merck, Germany), distilled water.

AV measurements: p-anisidine reagent(Sigma Aldrich, Germany), acetic acid (glacial) 100%(Merck, Germany), iso-octane(Merck, Germany)

Oxygen Absorption measurement by Oxygraph system: ethanol (Kemetyl, United Kingdom), Potassium chloride(Merck, Germany),

Methylation of fatty acids, sample preparation prior to determination of fatty acid composition on GC: Chloroform(Merck, Germany), Sodium hydroxide(Merck, Germany), Sodium chloride(Merck, Germany), Methanol(Sigma Aldrich, Germany), BF3 methanol(Sigma Aldrich, Germany), Hexane(Merck, Germany).

2.3 Experimental design

2.3.1 Pre experiment

To start with, a pre experiment was set up to acquire knowledge on how the oxidation process in the fish oil proceeded. The fish oil without added antioxidant was chosen, and the oil was kept in ambient environment with free access to oxygen to oxidize. Samples of the fish oil was collected every second day and AV and PV was determined.

100mL of fish oil was transferred to a 250mL beaker placed on the laboratory bench in ambient environment with access to oxygen to oxidize for a total of 12 days. Samples were collected about every other day, flushed with nitrogen gas and stored in -80 °C until analyzed. PV and AV were used to determine the oxidation status in the fish oil samples. The experimental set up is schematically illustrated in figure 7.

Figure 7: The experimental set up of the oxidation of fish oil in the pre experiment

2.3.2 Aroma experiment

To investigate how the aroma components influence determination of oxidation status by PV and AV methods, the oxidation status in fish oil with added aroma components was compared to oxidation status in fish oil without aroma component. Some samples were in addition analyzed with ^{1}H NMR spectroscopy to compare the aldehydes originating from the aroma component with the aldehydes formed in the oxidation process.

1,1g of the respective aroma component were added to 110g of fish oil and stirred with magnet at 750 rpm for 1 minute. The control oil without added aroma were treated the same way as the oil with added aroma to prevent any effects from the handling. The 110g of oil was divided into 9 tubes with approximately 10g of oil in each. The prepared samples were left on the laboratory bench to oxidize for up to 19 days. To obtain samples with different oxidation levels, tubes were collected about second other day, divided into new tubes assigned the different planned analytical analysis, to avoid freezing/thawing more than once. The samples were finally flushed

with nitrogen gas and stored at -80°C until analyzed. The experimental set up is schematically illustrated in figure 8.

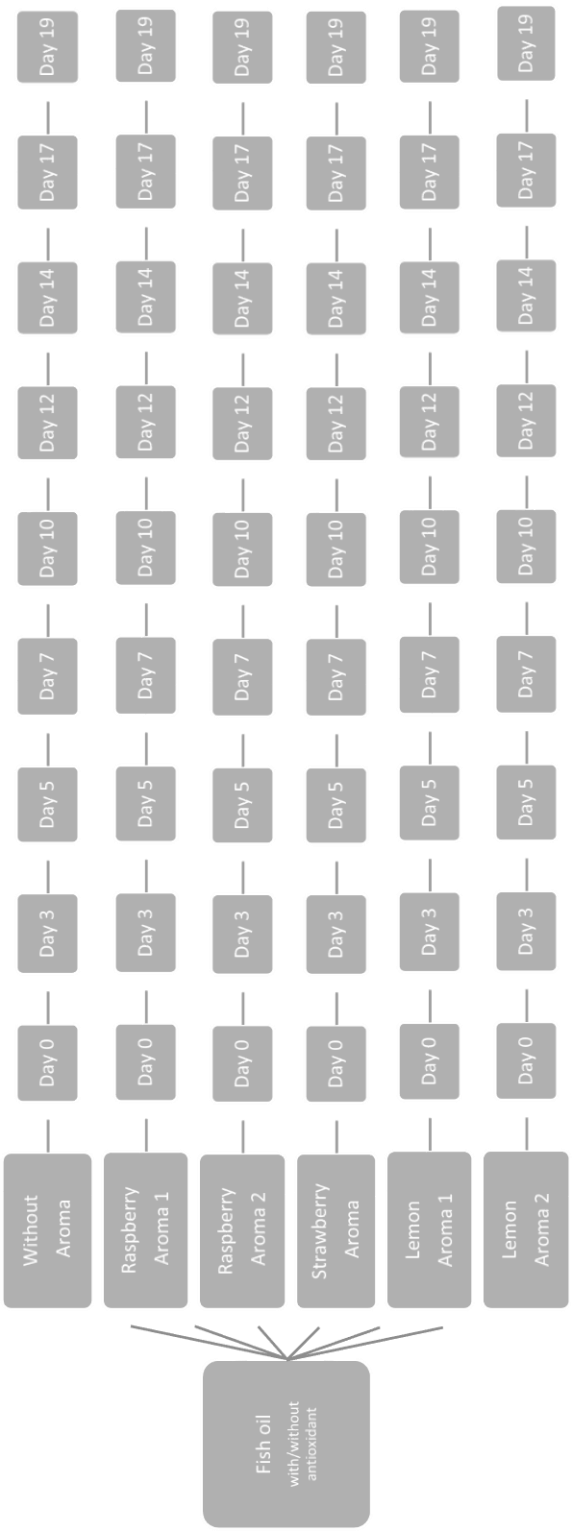

Figure 8: The experimental set up of the oxidation of fish oil in the aroma experiment

2.3.3 Vitamin experiment

To investigate what impact the addition of the vitamins A, D and E to fish oil, have on the determination of oxidation by PV and AV, fish oil with added vitamin were set on the laboratory bench to oxidize for up to 20 days. The concentrations used for the vitamins A, D and E were 250µg/5mL, 10µg/5ml, and 10mg/5mL respectively. The concentrations were chosen based on the concentrations found in the fish oil on the market. The control oil without added vitamin were treated the same way as the oil with added aroma to prevent any effects from the handling. The vitamin concentration were chosen based on the concentration of the vitamins used in the commercial fish oil on the market.

To obtain samples with different oxidation levels, tubes were collected about every fifth day, divided into new tubes assigned the different planned analytical analysis to avoid freezing/thawing more than once. The samples were finally flushed with nitrogen gas and stored at -80°C until analyzed. The experimental set up is schematically illustrated in figure 9.

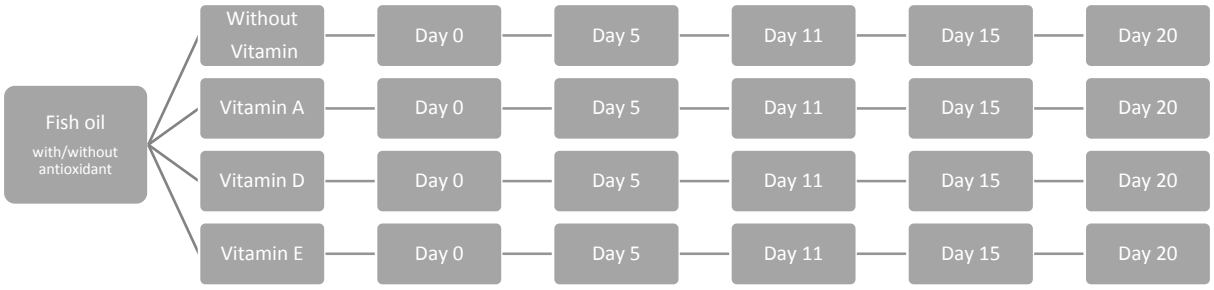

Figure 9: The experimental set up of oxidation of the fish oil in the vitamin experiment.

2.3.4 Mineral experiment

To investigate if any of the commonly added vitamin/minerals in commercial fish oil have any influence on the lipid oxidation process, the oxygen absorption of fish oil with added vitamin/mineral was measured in an oxygraph system and the oxidative stability determined using the Oxidative Stability Index (OSI) method.

2.4 Analytical methods

The PV and AV measurements, GC analysis of fatty acid composition, conjugated dienes, Oil Stability Index and Oxygen absorption measurements were carried out at SINTEF, Department of Fisheries and Aquaculture, Trondheim. Dynamic Headspace GC-MS were carried out at Department of Biotechnology, NTNU, Trondheim.

2.4.1 Fatty acid composition on GC

Sample preparation prior to the GC analysis involve methylation of the fatty acids. 10 mg of oil was transferred to a stoppered centrifuge glass tube before addition of 1 mL of 1 mg of internal standard dissolved in 1mL of chloroform. The chloroform was evaporated at 60°C with N_2 gas. Then, 1 ml of 0,5M NaOH dissolved in methanol, was added to the lipids and the mixture was heated at 100°C for 15min and cooled. Then, 2mL of BF3 methanol was added before the mixture was heated at 100°C for 5min and cooled. Lastly, 1mL of hexane was added before the mixture was heated at 100°C for 1min and cooled.

Further, 1mL of hexane and 2mL of saturated NaCl solution were added, the mixtures vortexed and centrifuged for 3min. The hexane phase was collected before a new 1mL of hexane was added, the mixture vortexed and centrifuged again. The hexane phase was collected and 2mL of hexane was added, vortexed and centrifuged and the hexane phase collected for the last time. The combined three hexane phases were analyzed on GC by Merethe Selnes, Engineer at SINTEF Fishery and Aqualculture.

The methylated fatty acids were analyzed according to Daukšas et al. (2005) with the following modifications: Agilent Technologies 7890A gas chromatograph was used with a flame ionisation detector (FID). The detector temperature was held at 250°C.

The column used was a Cp-wax 52CB, 25m x 0.25mm x 0.2µm column (part no. CP7713, Agilent Technologies) and hydrogen was used as the carrier gas at a flow rate 1.5 mL/min. At first, the oven temperature was held at 80°C and increased to 180°C at 25°C/min with a 8 min hold, secondly the temperature was increased to 205°C at 2.5°C/min with a 2 min hold, then finally the temperature was increased to 215°C at 2.5°C/min, with a 5 min hold. The fatty acids were identified by the comparison of the retention times, with those of a commercial standard and quantified by using an internal standard.

2.4.2 Peroxide Value, Iodometric titration method

The iodometric titration method for Peroxide Value (PV) measurements was performed in accordance with the AOCS Official Method Cd 8-53. The end point was determined potentiometrically by using an automatic titrator (TitraLab980) with a platinum electrode and a reference electrode.

Approximately 0.6 – 1.5 grams of oil was weighed in plastic cups(amount was adjusted depending on expected PV-value) and dissolved in 25 mL of a iso-octane/acetic acid solution(2:3). 250 µL of saturated Potassium Iodide solution was added and the mixture stirred with a magnet stirrer for 1 minute at 250rpm. Lastly, 15 mL of distilled water was added before the sample was placed in the titrator.

The peroxide value was calculated from the following formula:

$$\text{Peroxide Value(mEq peroxide } kg^{-1}oil) = \frac{C(V_t - V_b)}{m} \times 1000 \quad (3)$$

C= titrant concentration(mol/L)

V_t = titrant volume(mL)

V_b =blank volume

m = mass of oil sample(g)

1000 = unit conversion factor

The analysis was preformed in triplicate and the results reported in mmol hydroperoxide /kg oil.

2.4.3 Conjugated Dienes

The conjugated dienes analysis was performed as described in Pegg (2001), using a Spectronic Genesys 10 Bio, Thermo Scientific. 20-160mg of oil was dissolved in 10mL of isooctane and the absorbance measured at 233 nm in quarts cuvettes.

The conjugated dienes (mmol/kg) was calculated from the following formula:

$$C_{CD}\ (mol/L) = \frac{A_{233}}{\varepsilon \times l} \quad (4)$$

$$CD\ value\ (mmol/g) = \frac{C_{CD} \times 10^4}{C} \quad (5)$$

A_{233} = the absorbance of the sample at 233nm

ε = the extinction coefficient = 2.525 x 10^4 M^{-1} x cm^{-1}

l = the length of the cuvette (cm)

C = the concentration of the sample (g/L)

10^4 = factor to obtain mmol unit

2.4.4 Ansidine Value

The p-anisidine value (AV) was determined according to the AOCS Official Method Cd 18-90, using a UVmini 1240 Spectrophotometer, Shimadzu.

The 0.25% p-anisidine reagent was prepared every working day. 0.25 grams of p-anisidine was dissolved in 100 mL of 100 % acetic acid and the absorbance measured to ensure a value below Abs = 0.2.

To analyze the samples, about 0.1 grams of oil were weighed directly in test tubes and dissolved in 5 mL of iso-octane. 2.5mL of sample was transferred to a cuvette and the absorbance was measured at 350 nm against pure iso-octane as blank. Then, 0.5 mL of p-anisidine reagent was added and the cuvette shaken by hand. The cuvette was kept in the dark for 10 minutes before the second absorbance measurement was made. The measurements were performed in triplicates.

The AV was calculated using the following formula:

$$Anisidine\ Value(AV) = D \times \left(\frac{1.2 \times (A_{S2} - A_{B2}) - (A_{S1} - A_{B1})}{m}\right) \quad (6)$$

D = Volume (mL) of iso-octane used to dissolve the oil sample

A_{S1}= First spectrophotometric measurement of sample

A_{S2}= Second spectrophotometric measurement of sample

B_{S1}= First spectrophotometric measurement of blank

B_{S2}= Second spectrophotometric measurement of blank

m = mass (g) of oil sample

2.4.5 Oxygen Absorption Measurements

The analysis was conducted according to the method described in earlier studies: Mozuraityte et al. (2006a), Mozuraityte et al. (2008), Mozuraityte et al. (2006b). The oxygen uptake was measured using an oxygraph system (Hansatech Instrument Ltd.). The oxygraphic cell is made up of a reaction chamber, surrounded by a water jacket to ensure a constant temperature (40°C), equipped with magnetic stirring and closed with a plunger with a capillary opening to allow injection of solutions. The dissolved oxygen concentration was continuously measured by a polarographic oxygen electrode and plotted against time, in the Oxyg32 software, giving oxygen uptake curves with oxygen uptake rates (OUR).

The system was calibrated with oxygen saturated distilled water and with distilled water where all oxygen was removed by adding a small amount of dithionate. To prepare the samples, 100 mg of the mineral was dissolved in 10mL of ethanol. To analyze the OUR 2mL of fish oil was transferred to the reaction chamber. First, the background oxygen uptake rate was recorded for about 10min until stable. Then, 20µL of the mineral dissolved in ethanol was injected. The OUR was recorded for approximately 20min after injection to detect any effect of the mineral. Four parallels were used in the analysis.

2.4.6 Oil Stability Index

The Oil Stability Index was determined according to the AOCS Official Method Cd 12b-92, using Oxidative Stability Instrument (Omnion, ADM).

To prepare the samples, 0.01grams of the mineral/vitamin and 5grams of oil were transferred to glass tubes and placed in the instrument. Instrument parameters were 60°C and an airflow of 5.5 psi. The samples were analyzed in four parallels.

2.4.7 Dynamic Headspace Gas Chromatography – Mass Spectrometry

Agilent Technologies 7890 A gas chromatograph equipped with a 7000 Series Triple Quadrupole GC/MS system (Agilent Technologies) together with the autosampler: HT3™ Static and Dynamic Headspace System (Teledyne Tekmar). The only manual step was weighing of 0.5g of sample into 20mL vials The autosampler was controlled with the software HT3 TekLink and the instrument was controlled with the software MassHunter. The column used was Agilent J&W DB-624 UI, 30 m × 0.25 mm, 1.4µm with Helium as carrier gas at constant flow, 1.8 mL/min and the following temperature program was used: 40°C (1 min),

3°C/min to 120°C, 8°C/min to 170°C, 30°C/min to 240°C (5min). The Inlet temperature was 220°C, split 10:1. MSD: SIM The experimental conditions for dynamic headspace system were:

- Trap: Purge trap A Tekmar®, Tenax®, 24 cm (Supelco)
- Valve oven temp.: 110°C
- Transfer line temp.: 115°C
- Standby flow rate: 25mL/min
- Trap standby temp.: 30°C
- Platen/Sample temp.: 70°C
- Sample preheat time: 10 min
- Sweep flow rate, time: 50mL/min, 10 min
- Dry purge: 1 min
- Desorb preheat: 175°C
- Desorb temp., time: 180°C, 4 min
- Trap bake temp., time: 180°C, 5 min
- Trap bake flow: 200 mL/min

Identification were based on the retention time of purchased standards.

2-(E)- pentenal: t_R 11,1 min, 55,0 m/z, 83,0 m/z,

2-(E)-hexenal: t_R 16,3 min, 69,0 m/z, 83 m/z,

2,4-(E,E)-heptadienal: t_R 25,3 min, 81,0 m/z, 110,0 m/z.

4-(Z)-heptenal: t_R 18,3 min, 68,0 m/z, 94,0 m/z.

The Dynamic Headspace GC-MS analysis was preformed by Zdenka Bartosova, Researcher at Department of Biotechnology, NTNU.

2.4.8 ^{1}H NMR Spectroscopy

Approximately 100 mg of the sample was dissolved in 250 µL of a mixture of deuterated chloroform containing tetramethylsilane (TMS) as an internal standard, before transferred to 3 mm NMR-tubes. Skiera et al., 2012 described this solvent for optimal resolution of aldehyde signals. NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer (Bruker Biospin GmbH) at 25°C with cryo-probe operating at a ^{1}H frequency of 600.23 MHz.

For ^{1}H-NMR the following acquisition parameters were used: pulse program zg30, time domain 64k, spectral width 20.5 ppm, acquisition time 3.0 s, relaxation delay 2.0 s, number of scans 128, dummy scans 4. Zero filling and exponential line broadening (0.30 Hz) was applied before Fourier transformation. The chemical shift scale is referred to TMS at 0 ppm. Peak assignments were done according to literature(Skiera et al., 2012b) (Mozuraityte et al., in prep). The NMR

spectroscopy was performed by Inger Beate Standal, research scientist at SINTEF, Fishery and Aquaculture.

2.5 Equipment

The equipment used in the experiments is listed in the following table 4.

Tabel 1 The equipment used listed with name and source.

Weight	**AG204 Delta Range**	**Mettler Toledo**
Vortex mixer	MS2 Minishaker	IKA
Magnet Stirrer	Heidolph MR 3001K	Heidolph
Autotitrator	TIM 980 Titrator Manager	Radiometer Analytical
Reference electrode	REF 921	Radiometer Analytical
Detection Electrode	M2IPt	Radiometer Analytical
Oxygraph system	Oxygraph Plus System	Hansatech Instrument Ltd.
Oxidative stability instrument	Oxidative Stability Instrument	Omnion, ADM
Centrifuge	Universal 16A	Hettich
Heating unit	Reacti-Therm	Pierce
Spectrophotometer	UVmini 1240 Spectrophotometer	Shimadzu
Spectrophotometer	Spectronic Genesys 10 Bio	Thermo scientific
Cuvettes	10mm QS	Hellma GmbH & Co. KG
Cuvettes	10mm OP	Hellma GmbH & Co. KG
NMR Spectrometer	Bruker Avance 600 MHz spectrometer	Bruker Biospin GmbH
Gas chromotopraph	GC- System 7890A	Agilent Technologies

2.6 Statistics

To calculate the precision of the parallels, the standard deviation was used, calculated by the formula:

$$Standard\ deviation(s) = \sqrt{\frac{\sum_{i=1}^{n}(x - \bar{x})^2}{n - 1}}$$

$x = measurement$

$\bar{x} = mean\ value$

$n = number\ of\ measurements$

Significant differences between measurement a and measurement b were determined by using the formula:

$$t = \frac{|a - b|}{\sqrt{{s_a}^2 + {s_b}^2}}$$

$x_1 - x_2 = the\ diffrence\ between\ the\ measurements\ compared$

$\sqrt{{s_a}^2 + {s_b}^2} = the\ uncertainty\ of\ the\ standard\ deviations\ of\ the\ measurements\ compared$

The t- value was used to find the probability of measurement a being significantly different from measurement b from a table of normal error intergral (Taylor, 1997). The confidence interval was set to 95%.

Rejection of measurements were done by using Chauvenet's Criterion (Taylor, 1997).

All calculations were performed in Microsoft Excel.

3. Results and discussion

3.1 Fatty acid composition

The fatty acid composition of the fish oil was determined through gas chromatography. The fatty acid composition is important for the development of oxidation products.

Table 2: Fatty acid composition of the fish oil used in the experiments as well as EPA and DHA content. The values are percentages of the total fatty acids determined through gas chromatography. The values are means of two parallels.

Fatty acid composition		
	Content (%)	Standard Deviation
Saturated	42.38	0.02
Monosaturated	13.28	0.09
Polysaturated	35.78	0.05
Omega 3	30.54	0.03
Unknown	8.56	0.12
C20:5n3 (EPA)	0.25	0.00
C22:6n3 (DHA)	0.77	0.00

The DHA and EPA content was determined to 0.77 % and 0.25 % of the total fatty acid content, respectively as shown in table 2. The total amount of omega-3 fatty acids was found to be 30.54 % and the total amount of polyunsaturated fatty acids was found to be 35.78 % of the total fatty acid content. More details on the fatty acid composition is given in appendix A.

3.2 Pre experiment

To start with, a pre experiment was set up to acquire knowledge on how the oxidation process in the fish oil (without antioxidant) proceeded. The fish oil was kept at ambient temperature with access to oxygen in order to oxidize. To obtain samples with different oxidation status, samples of the fish oil were collected every second day. To determine the oxidation status in the samples PV and AV were used to determine the primary and secondary oxidation products, respectively.

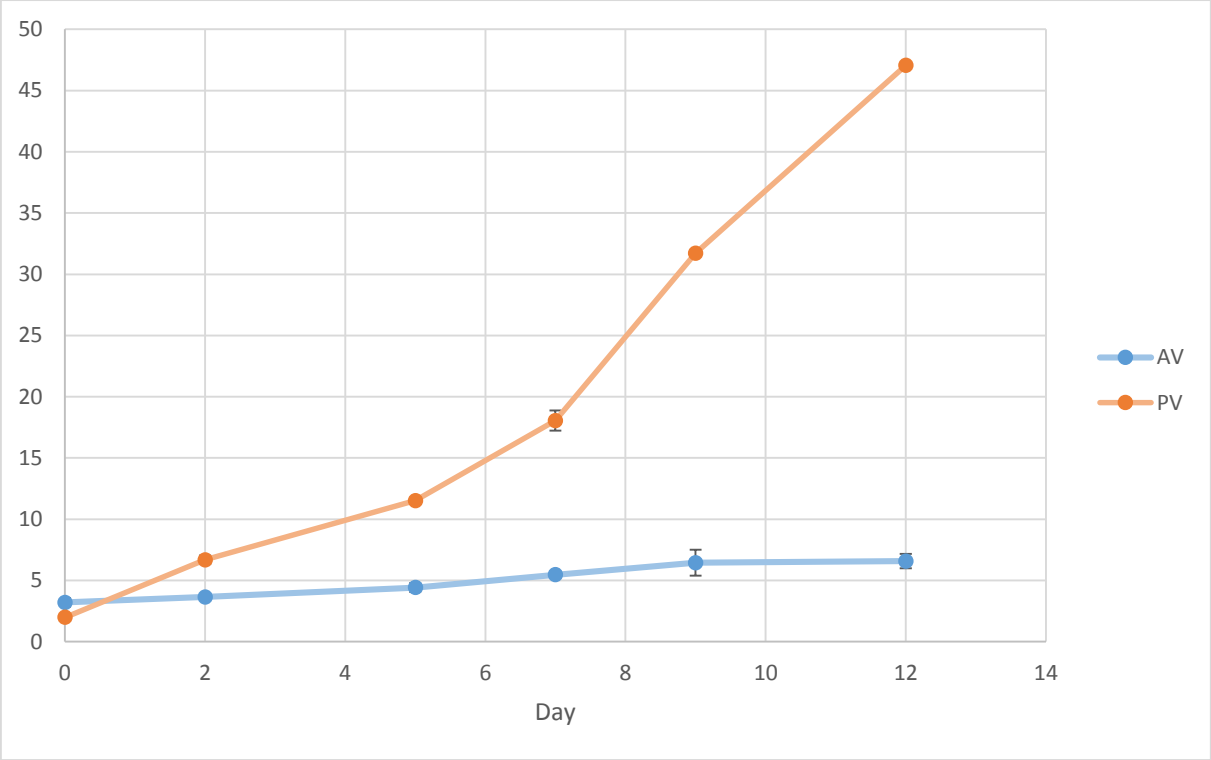

Figure 10: The development of primary and secondary oxidation products determined by peroxide value and anisidine value methods respectively in fish oil (without antioxidant). The values are means of three parallels and the error bars represent the standard deviations.

The development of primary oxidation products given by the peroxide value showed a relatively constant rate for the first days of the experiment, but after day 5 there was about a twofold increase in the rate as illustrated in figure 10. The PV and AV limits recommended by expert organizations are PV less than 5meq/kg and AV less than 20 ((GOED), 2015) (Codex Alimentarius Commission, 2015). At day 5, the PV had already exceeded the limit of 5, but the secondary oxidation products were found to develop much slower, and only increased from 3.20 to 6.58 at day 12, far below the limit of 20. The further experiments were planned based on the results in this experiment.

3.3 Aroma experiment

Today fruit flavoring is commonly added to fish oil to make the fish oil more appealing. Recently however, some compounds in flavorings have been found to interfere with the most common methods for determining oxidation status in fish oil, the PV and AV methods (Semb, 2012). Aldehydes are commonly accepted to be important constituents in aroma formulas and are also the analytes detected in the anisidine value method. To study the influence of aroma on PV and AV methods further, aromas with different concentrations of aldehydes were added to both fish oil with and without antioxidant and left at ambient temperature with access to oxygen in order oxidize. To obtain samples with different levels of oxidation, samples were collected every second day.

The five aroma components examined were: two raspberry aromas named 1 and 2 with 0.5 % and 7.6 % aldehyde content respectively, one strawberry aroma with an aldehyde content of 2 % and two lemon components named 1 and 2 with respectively 41 % and 11.19 % aldehyde content. In addition, some samples were analyzed with ^{1}H NMR spectroscopy and dynamic headspace GC-MS to see if these methods gave a more detailed picture of the oxidation status.

3.3.1 Influence of Aroma the Iodometric Titration Method

Fish oil with aroma additions were analyzed of different levels of oxidation to study the potential effect of different aromas with different aldehyde concentrations on the iodometric titration method for determining peroxide value. Five aroma components with different aldehyde concentrations were added to both fish oil without and with antioxidant and the PV was determined.

Fish oil without antioxidant

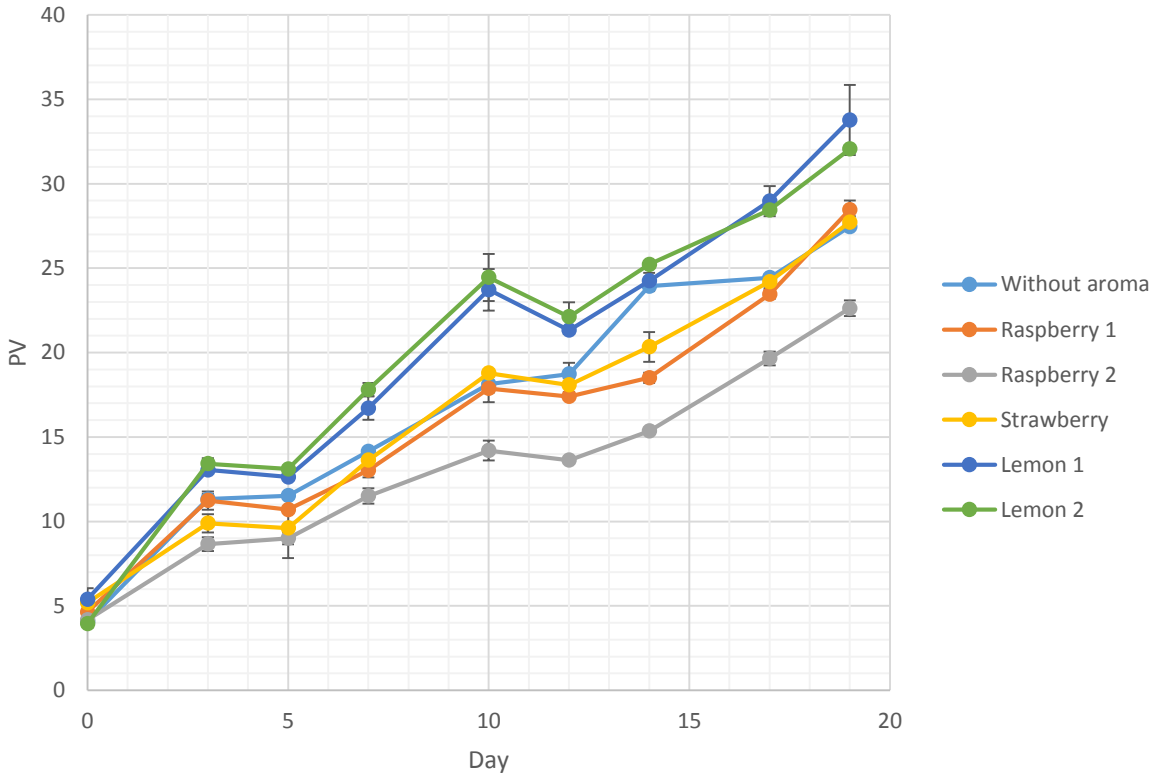

Figure 11: The effect of aroma components on PV measurments in fish oil (without antioxidant) with increasing oxidation status. The PV measurements are shown as the mean of three paralells and the error bars represent the standard deviation between the parallels.

As illustrated in figure 11, fish oil with both lemon aromas showed consistently elevated peroxide values compared to the fish oil without aroma. Fish oil containing the Lemon 1 and Lemon 2 aromas gave rather similar peroxide values, despite having different concentrations of aldehydes, 41% and 11.19% respectively. PV measurements in fish oil with the Lemon 2 aroma were significantly higher than in the fish oil without aroma for all days, while fish oil with the Lemon 1 are significantly higher for all days except day 14 and 19. In the case of fish oil with the Strawberry and the Raspberry 1 aromas, they showed peroxide values very close to the peroxide values of fish oil without aroma. For fish oil with Raspberry 1, all peroxide values were significantly different from the fish oil without aroma, except at day 3 and 10 and for fish oil with the Strawberry all days were significantly different except at day 5, 12 and 17. When it comes to the fish oil with the Raspberry 2, the peroxide values were consistently lower than the peroxide values of the fish oil without aroma, and the values are significantly different from the fish oil without aroma for all days.

The difference between the PV of fish oil without aroma and the PV of fish oil with lemon aromas and Raspberry 2 aroma are small in the beginning, but increases with time, as the oxidation status in the fish oil increases. All the fish oil aromas followed the same pattern of oxidation as the fish oil without aroma. It would have been interesting to reanalyze the fish oil without aroma at day 14, because of the observed deviation from the trend. However, this was not possible due to problems with the PV electrode. Taken together, the fish oil with Raspberry 1 and the Strawberry aroma showed peroxide values close to the fish oil without aroma. Raspberry 2 gave lowered PV levels in fish oil, while the lemon aromas gave elevated PV, when compared to fish oil without aroma, and it seem like the effect becomes more prominent with time, as the oxidation status increases.

Fish oil with antioxidant

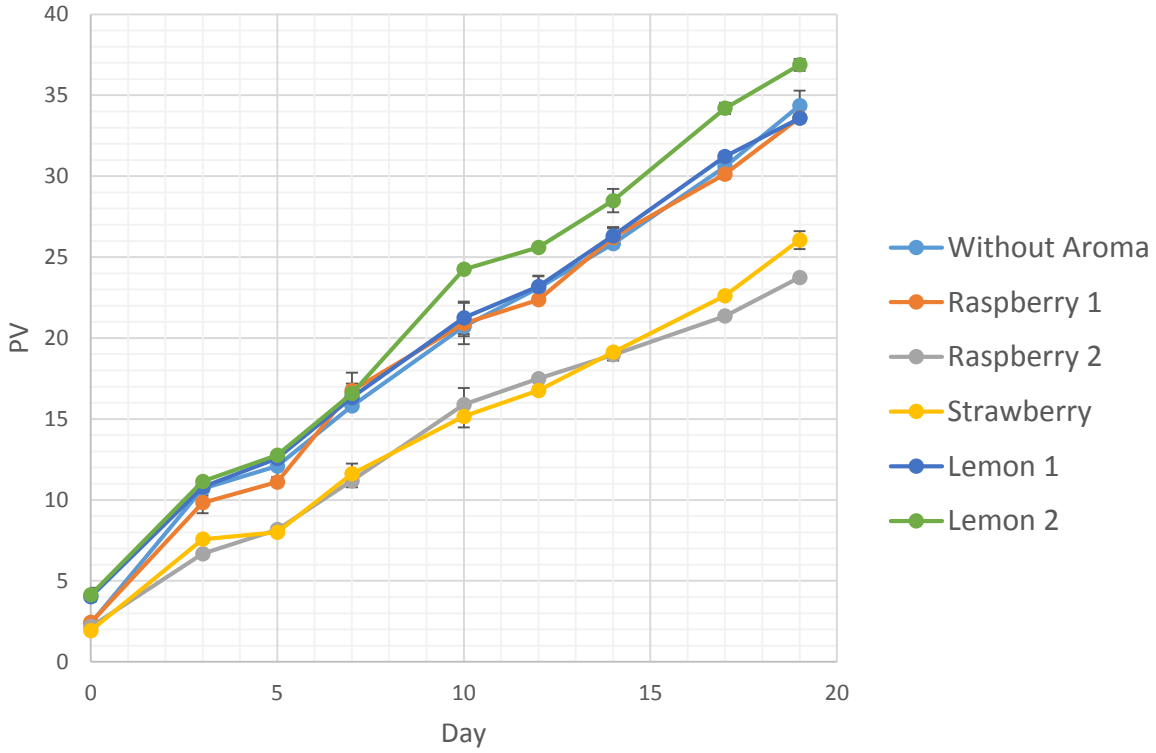

Figure 12: The effect of aroma components on the PV measurements in fish oil (with antioxidant) in samples with increasing oxidation status. The PV measurements are shown as the mean of three paralells and the error bars represent the standard deviation between the parallels.

As illustrated in figure 12, between day 0 and 7 all fish oils with Raspberry 1 and Lemon 1 and 2 showed peroxide values close to the ones of fish oil without aroma. After day 7 the PV of fish oil with Lemon 2 increases to a higher level, the same tendency as in the fish oil without

antioxidant, and for the rest of the time measured the peroxide values were elevated, just above the peroxide values of Raspberry 1, Lemon 1 and fish oil without aroma, which continues to lie closely together. The PV measurements of fish oil with Lemon 2 are significantly different from the fish oil without aroma after day 7. For fish oil with Lemon 1 and Raspberry 1 there were no significant difference. Fish oil with Strawberry and Raspberry 2 showed peroxide values consistently below the PVs of fish oil without aroma, and all peroxide values measured were significantly different from the fish oil without aroma, except for day 0. Also here, the effect seen in fish oil with Raspberry 2, Strawberry and Lemon 1 increased with time.

The peroxide values at day 0 were lower in the pre-experiment compared to the aroma-experiment. The 2 minutes magnet stirring of the oil to blend in the aroma might explain the slightly increased peroxide values in the aroma experiment at day 0. The fish oil without aroma was treated in the same way as for the fish oil with aroma. The development of primary oxidation products was faster in the pre-experiment than in the aroma experiment. One explanation can be that the oil oxidation was performed in a beaker in the pre-experiment, and not small tubes as in the aroma-experiment, and therefore had a larger surface to react with oxygen.

The reduced peroxide values in fish oil observed in samples containing berry flavoring, compared to the fish oil without aroma, can be explained by the high content of polyphenolic compounds in berry aroma. Phenolic compounds are commonly known to have good antioxidant activity. Phenolic compounds are hydrogen donors as described in Shahidi and Zhong (2010).The main polyphenolic compounds found in raspberry and strawberry extracts are anthocyanins and ellagitannins (Kähkönen et al., 2001).

The anti-oxidative effect of the berry aroma, especially Raspberry 2 is more distinct in the fish oil with antioxidant than in the fish oil without antioxidant. The difference between the PV of fish oil without aroma and the PV of Raspberry 2 in fish oil without antioxidant is 4.34, but the difference between the PV of fish oil without aroma and the PV of Raspberry 2 in fish oil with antioxidant is 10.62. One possible explanation might be that there is a synergistic effect between the tocopherols added as antioxidants and the phenolic compounds in the berry aroma. A variety of berry phenolic compound have been found to enhance the anti-oxidative effect of α-tocopherol (Marinova et al., 2008, Pedrielli and Skibsted, 2002).

The fish oils with lemon aroma gave elevated peroxide values, in compliance with the findings of Semb (2012). The high peroxide values compared to the fish oil without aroma suggest that

it is not only the hydroperoxides originating from lipid oxidation, but also other oxidizing compounds in the lemon aroma that contributes to the oxidation of iodide to iodine and that this cause erroneously high PV. A recent study by Skiera et al. (2012a) reported that the terpenes thymoquinones in black seed essential oil gave elevated PV in black seed oil, due to the commonly known redox properties of quinones. Terpenes are the major components of essential oils from citrus (Vekiari et al., 2002) and some of these may have redox properties and contribute to the oxidation of iodide in the same manner as for the thymoquinones in black seed oil. This is not possible to determine, due to the compounds of the aroma not being known.

To sum up, the lowered PV seen in Raspberry 2 in fish oil without antioxidant and Raspberry 2 and Strawberry in fish oil with antioxidant is suggested to be a real reduction of the hydroperoxides due to the anti-oxidative effect of polyphenolic compounds in berry aroma. The elevation of PV seen with the Lemon 2 in fish oil without antioxidant and with Lemon 1 and Lemon 2 in fish oil with antioxidant is suggested to be caused by interference with the iodometric titration method due to the presence of oxidizing compounds in lemon aroma, which will contribute to the oxidation of iodide to iodine.

The peroxide value is a widely used measure of lipid oxidation both in industry and for regulatory purposes. Since the PV iodometric titration method is shown to be affected by compounds were not part of the lipid oxidation process, it is necessary to investigate what compounds that might interfere and to what degree. It is suggested that the determination of primary oxidation products in flavored fish oil is done with an alternative method. Analysis of conjugated dienes is a spectrophotometric method for determination of primary oxidation products which is found to have good correlation with the peroxide value method (Marmesat et al., 2009, Wanasundara et al., 1995) and might be a useful alternative method.

3.3.2 Influence of Aroma on the Anisidine Value method

To investigate the potential effect of aroma addition to fish oil on the anisidine value method of determining secondary oxidation products, five aroma components with different aldehyde concentrations were added to both fish oil without and with antioxidant, and the anisidine values were determined.

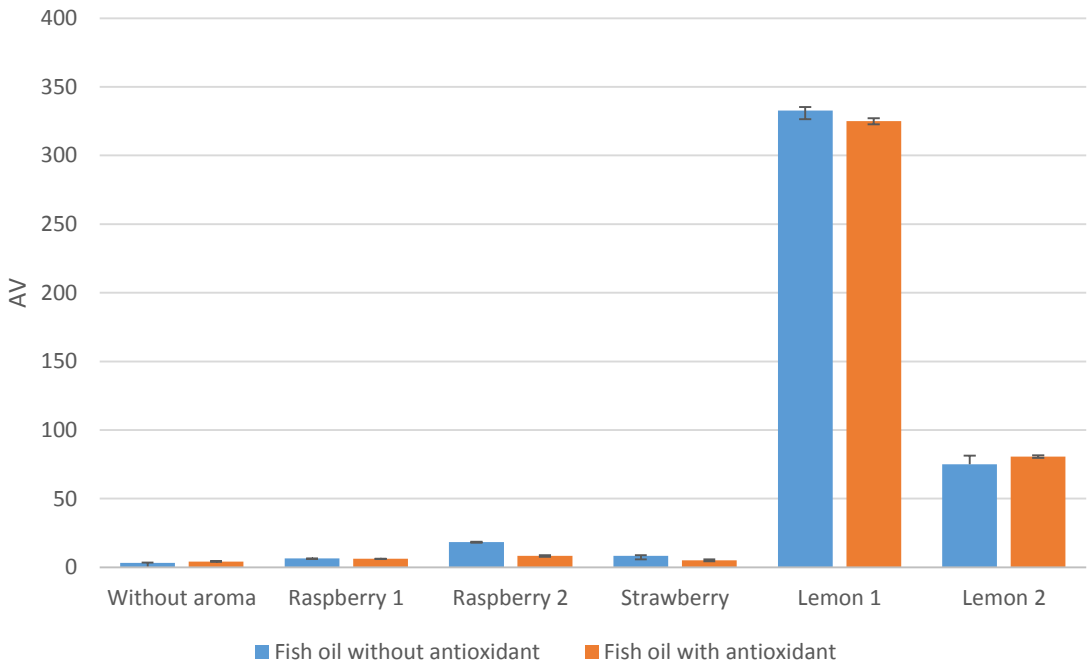

Figure 13: Anisidine value of fish oils without and with addition of the five aromas with different content of aldehydes. The values are means of three parallels.

The anisidine values of fish oil with lemon aromas 1 and 2 were clearly elevated compared to the anisidine values of fish oil without aroma as illustrated in figure 13, with Lemon 1 showing the absolute highest effect. The effect of the lemon aromas was similar in fish oil with and without antioxidant.

To show how the berry aromas influenced the AV measurements more clearly, figure 14 illustrates the aromas' influence on the AV measurements with fish oil containing the two lemon aromas left out.

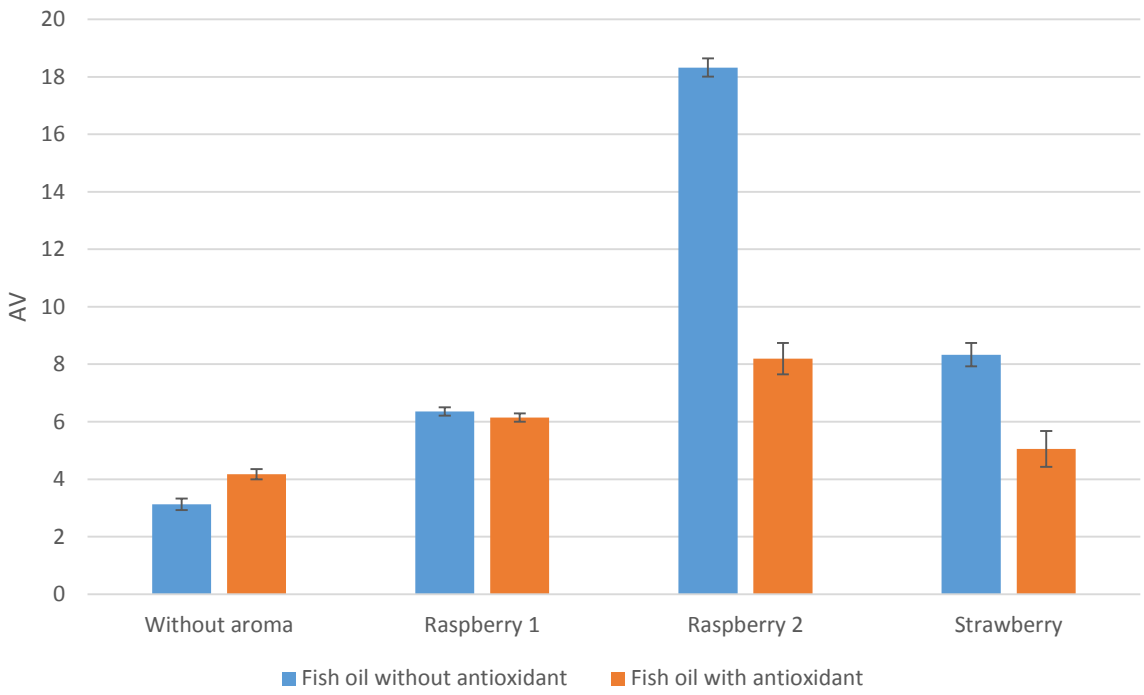

Figure 14: Anisidine value of fish oils without and with addition of the berry aromas with different content of aldehydes. The values are means of three parallels.

The measurements of fish oil with the berry aromas also showed elevated anisidine values compared to the fish oil without aroma. In fish oil with Raspberry 1, the anisidine values in fish oil with and without antioxidant were similar, in contrast to fish oil with Raspberry 2 and Strawberry where the fish oil without antioxidant showed higher levels than in fish oil with antioxidant.

The elevated AV measurements of fish oil with addition of aroma is presumably due to the p-anisidine reagent reacting with the aldehydes in the aroma and not only with aldehydes originating from lipid oxidation. When adding aroma components containing aldehydes to the fish oil, more aldehydic carbonyls will be present and available to react with the amino groups of the p-anisidine reagent. This results in more yellow compounds produced that will have absorption in the UV region at 350nm giving elevated AV measurements and a falsely high status of secondary oxidation products.

The specific compositions of the aromas used in this experiment were not known, but some information of the general compositions of aromas are available. In the case of lemon flavorings, aldehydes are the compound contributing the most to the characteristic flavor, and predominately the aldehyde citral, a mixture of the isomers neral and geranial, are the most important (Ikeda et al., 1962). Citral has a double bond conjugated with its carbonyl double bound, which is the structure largely contributing to the anisidine value (IUPAC, 1979). Other

aldehydes found present in lemon oil are heptanal, octanal, nonal, decanal and citronellal, among others (Bernhard, 1960, Ikeda et al., 1962). In raspberry aromas, ketones and esters are the most important, but some aldehydes are of relevance as well. In addition to acetaldehyde, citral is present in 13 parts per weight, reported in Fenaroli (1971). Strawberry aroma consists mainly of alcohols, ketones and organic acids, but interestingly, also here citral is present, but in lower quantity than in raspberry aroma. Fenaroli (1971) reported that citral is present in 1 part per weight in strawberry aroma. Further investigations are necessary in order to determine whether it is citral or other aldehydes that are responsible for the elevated AV measurements observed.

Correlation between AV and Aldehyde Concentration in Aroma

To have a closer look on the relationship between aldehyde content and AV measurements, correlations were calculated for both fish oil without and with antioxidant.

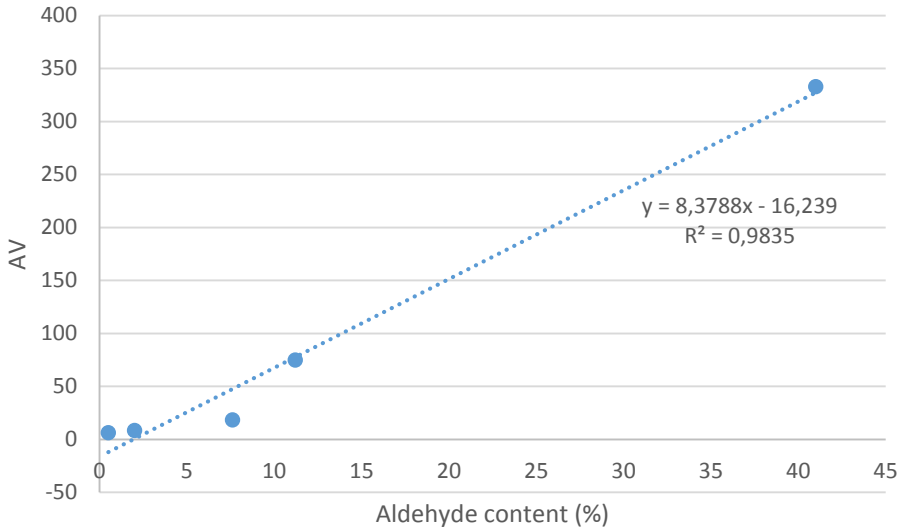

Figure 15: Relationship between AV measurements and the aldehyde content (%) of aroma components in fish oil (without antioxidant) at day 0.

Significant correlations ($p \leq 0.05$) were found between the AV measurements and the percentage aldehyde content in fish oil with the different aroma components at day 0 ($R = 0.9917$) as illustrated in figure 15 and also at day 10 ($R = 0.9878$) and at day 19 ($R = 0.9862$).

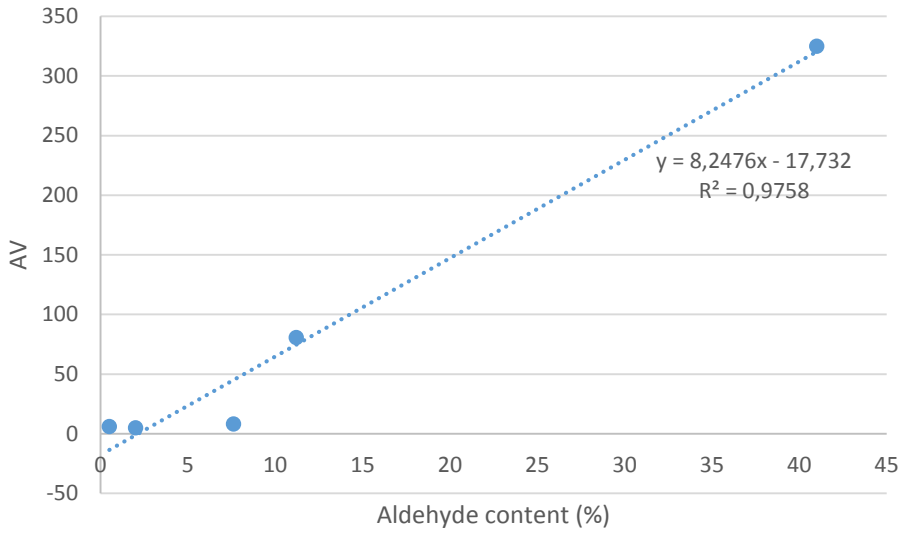

Figure 16: The relationship between AV measurements and the aldehyde content (%) of aroma compounds in fish oil (with antioxidant) at day 0.

As displayed in figure 16, significant correlations ($p \leq 0.05$) were found between the AV measurements and the percentage aldehyde content in the fish oil with additions of the different aroma components at day 0 (R = 0.9878), but also at day 10 (R = 0.9804) and at day 19 (R = 0.9936).

The AVs increase with increasing aldehyde content of the aroma component added. This is another indication of the aldehydes in the aroma interfering with the AV method, giving rise to falsely high AV measurements, and that the degree of elevation corresponds to the amount of aldehydes in the aroma added to the fish oil. The degree of interference of the aroma compound was found to be the following: Lemon 1 (41 %) > Lemon 2 (11.19 %) > Raspberry 2 (7.6 %) > Strawberry (2 %) > Raspberry 1 (0.5 %)

The influence of aroma on the development of secondary oxidation products with time

Fish oil without antioxidant

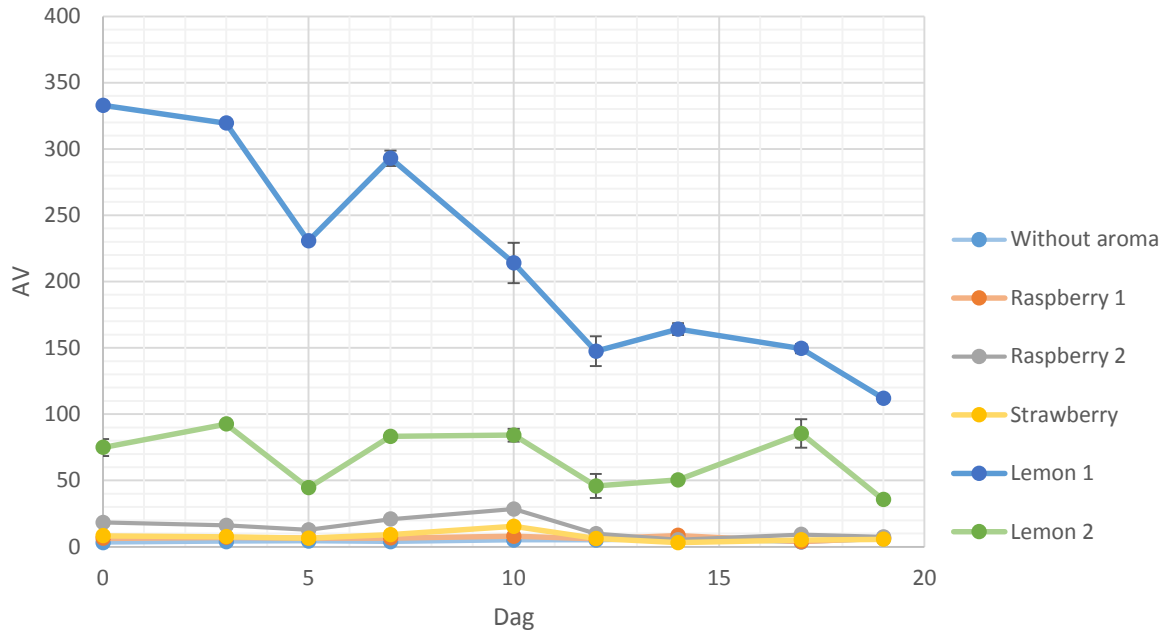

Figure 17: Effect of added aromas with different aldehyde content on the AV measured in fish oil (without antioxidant) with incresingly oxidation status. The AV measurements are shown as the mean of three parallels and the error bars represent the standard deviation.

The fish oils with the two lemon aromas showed highly elevated AV measurements for all samples measured, when compared to the fish oil without aroma as illustrated in figure 17 Lemon 1 aroma with the highest content of aldehydes (41%) gave higher AV measurements than Lemon 2 aroma containing the lower content of aldehydes (11.19%). For the two lemon aromas, the difference from the fish oil without aroma is significant for all measurements.

To show how the other aroma components influenced the AV measurements more clearly, figure 18 illustrates the aromas' influence on the AV measurements with fish oil containing the two lemon aromas left out.

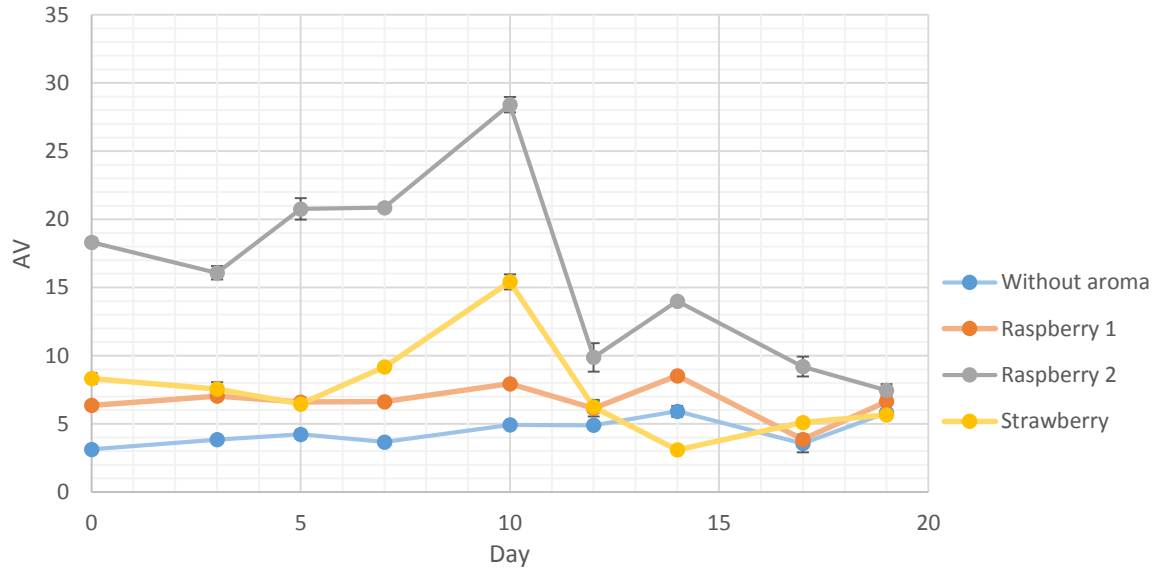

Figure 18: Effect of added aromas with different aldehyde content on the AV measured in fish oil (without antioxidant) with increasing oxidation status. The AV measurements are shown as the mean of three parallels and the error bars represent the standard deviation.

The fish oil with Raspberry 2 aroma also showed clearly elevated AVs compared to the fish oil without aroma, and the difference is significant for all measurements. The fish oil with the Raspberry 1 showed significantly higher AV for all samples measured except for the sample collected from day 17. Fish oil with Strawberry aroma had significantly higher AVs for all levels of oxidation with exception of the samples collected from day 14 and day 19, where the measurements are below the measurements of the fish oil without aroma. This could be due to poor precision of the parallels, and more parallels could have been used to be able to exclude this possibility.

The AV of the fish oils with aroma seem to decrease with time. This is especially visible in the aromas with high concentration of aldehydes, such as the lemon aromas and also the Raspberry 2 aroma. A likely explanation could be that since the aldehydes in the aromas are volatile, they will volatilize, and the resulting decrease in aldehyde concentration will consequently give a decreased AV.

Because the fish oil with Raspberry 2 aroma showed large differences in anisidine values, some of the lowest anisidine values (from day 5 and 14) were reanalyzed to verify the results.

Table 3: Reanalysis of anisidine value in fish oil added Raspberry 2 aroma samples collected from day 5 and 14. Precision is given by standard deviation.

	Day 5		**Day 14**	
	AV	SD	AV	SD
Original measurements	5.21	0.12	12.80	1.00
Reanalysis	21.95	0.17	28.72	0.58

The new anisidine values were highly deviating from the original values as displayed in table 3. The second measurements used oil from the same sample tube as the first measurements, thus the oil had already been exposed to oxygen and thawing-freezing one more time than the first measurements, but this cannot fully explain the deviating results.

Fish oil with antioxidant

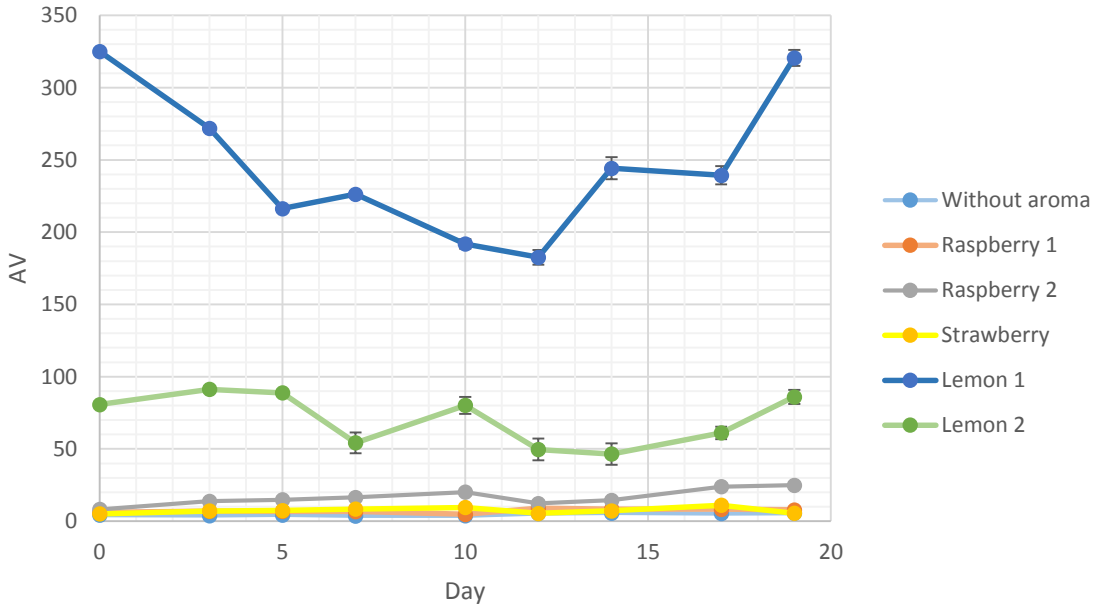

Figure 19: Effect of added aromas with different aldehyde content on the AV measured in fish oil (with antioxidant) with increasing oxidation status. The AV measurements are shown as the mean of three parallels and the error bars represent the standard deviation.

The AV measurements of fish oil with the lemon aromas are highly elevated for all levels of oxidation measured, compared to the fish oil without aroma as illustrated in figure 19. Lemon aroma 1 with the higher concentration of aldehydes (41 %) gave higher AV measurements than Lemon aroma 2 containing the lower concentration of aldehydes (11.19 %). There is a

significant difference for all measurements of the two lemon aromas compared to the fish oil aroma.

To show how the other aroma components influenced the AV measurements more clearly, figure 20 illustrates the influence on the AV measurements with the fish oil containing the lemon aromas left out.

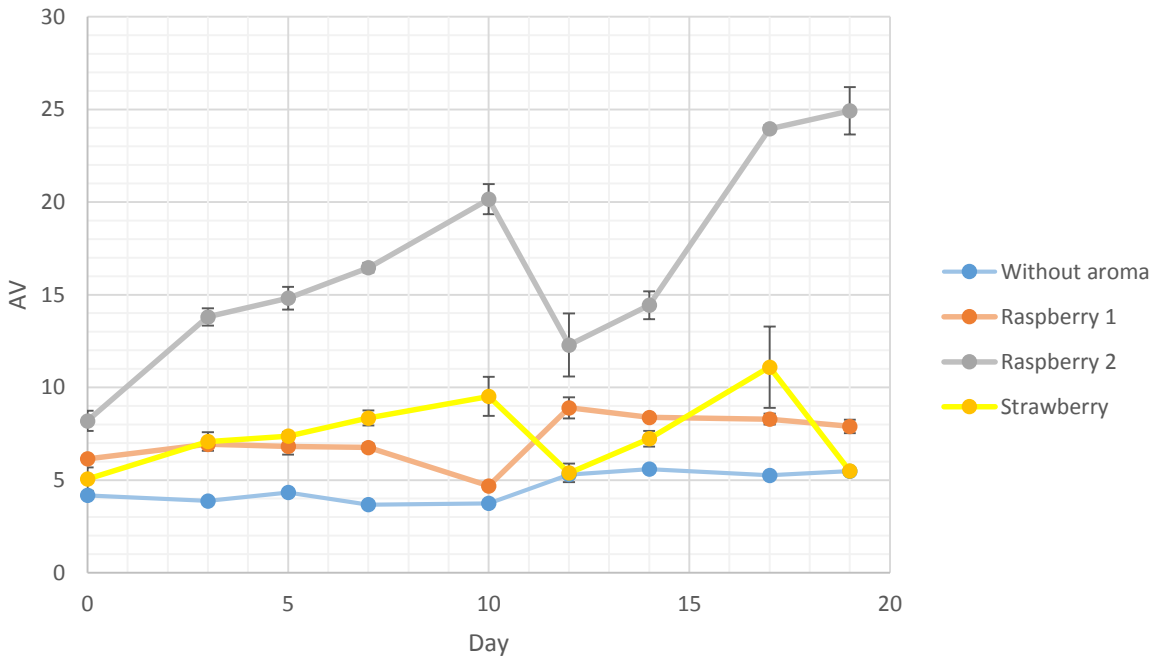

Figure 20: Effect of added aromas with different aldehyde content on the AV measured in fish oil (with antioxidant) with increasing oxidation status. The AV measurements are shown as the mean of three parallels and the error bars represent the standard deviation.

Also for fish oil with Raspberry 2 aroma the elevation of the AV measurements are clearly visible, as shown in figure 17. The AV measurements of fish oil added Raspberry 1 and 2, all showed significantly higher AV compared to the fish oil without aroma. The fish oil with the Strawberry aroma showed significantly higher AV for all oxidation levels except the samples from day 0, 12 and 19. The fish oil with Raspberry 1 and Strawberry aromas, have similar anisidine values, and also have similar content of aldehydes in the aroma.

The AVs of the fish oil with aroma decrease with time until day 12. It is especially evident in the aromas with high concentration of aldehydes, such as the lemon aromas and also the Raspberry 2 aroma. This pattern can be explained by the volatility of the aldehydes in the aroma. At first, the elevated AVs are caused by high levels of aldehydes from the aroma. Since the

aldehydes in the aroma are volatile, they will volatilize, and the concentration of aldehydes will decrease giving a decrease in AV. After day 12 the AV measurements shift and start to increase, at least for the lemon aromas and Raspberry 2. One explanation can be that compounds in the aroma interact with the antioxidants in the fish oil which leads to the formation of compounds with structures absorbing at the 350nm.

Comparison of development of secondary oxidation product in fish oil with and without antioxidant with addition of aromas with high concentration of aldehydes

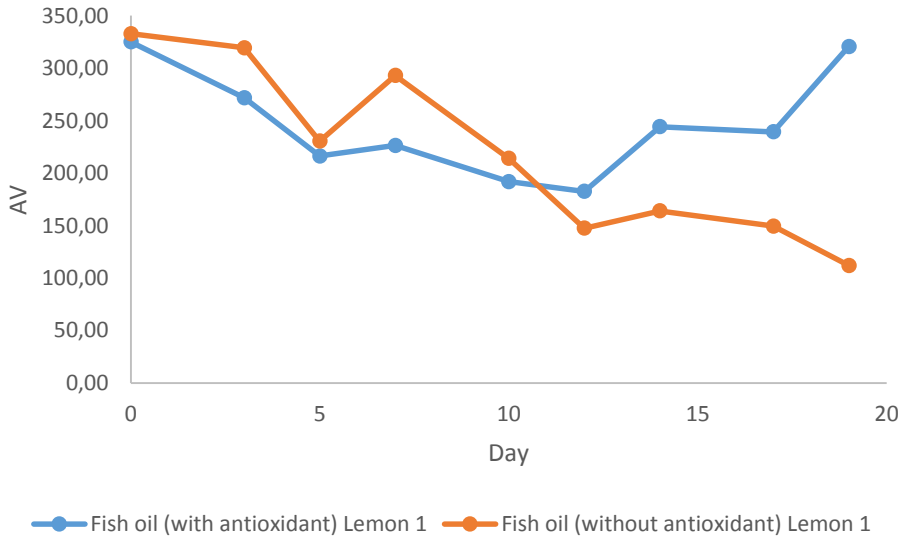

Figure 21: The development of secondary oxidation products determined by the AV method for fish oil with antioxidant and fish oil without antioxidant with addition of Lemon 1 aroma.

From day 0 to day 12, the development of secondary oxidation products measured by the AV method is rather similar it both fish oil with antioxidant and fish oil without antioxidant in fish oil with addition of Raspberry 2, Lemon 1 and Lemon 2. However, after day 12, there is a difference in the development. In fish oil without antioxidant, the AVs continued to decrease, but in fish oil with antioxidant the development shifted and started to increase. The difference increased with increasing concentration of aldehydes in the aroma, that is to say the largest difference was seen for Lemon 1 with 41% aldehyde content as illustrated in figure 21.

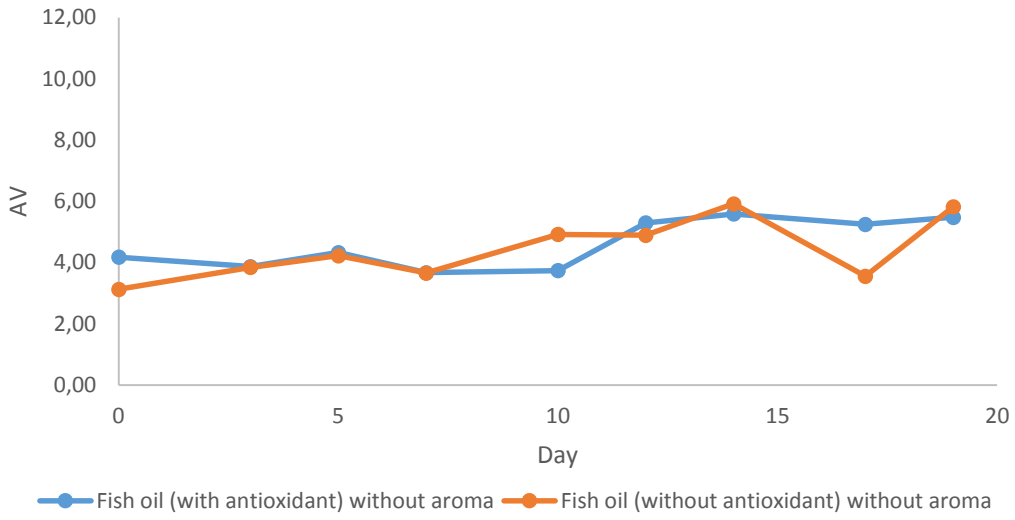

Figure 22: The development of secondary oxidation products determined by the AV method for fish oil with antioxidant and fish oil without antioxidant, without addition of aroma.

The development of the secondary oxidation products as measured by the anisidine value method was very similar in fish oil with and without antioxidant and overall there is not much development of secondary oxidation products as illustrated in figure 22. Hence, the difference seen in AV after day 12 between the effect of Lemon 1 aroma in fish oil with antioxidant and in fish oil without antioxidant is likely to be caused by something only being present in the fish oil with antioxidants - the antioxidants. The antioxidants added in fish oil with antioxidant were a mixture of the natural tocopherols: D-α-tocopherols, D-β-tocopherols, D-γ-tocopherols and D-δ-tocopherols. The increase in AV after day 12 might be due to some sort of interaction between the aldehydes from the aroma or their degradation products and the tocopherols, which leads to the formation of compounds with structures absorbing at 350nm, and consequently increase AV with time. It could also be other compounds in the aroma, but it is likely to be the aldehydes, because the effect corresponds to the aldehyde content. With Lemon 1 showing the highest effect, Lemon 2 showing the second highest effect and the Raspberry 2 showing the third highest effect. No studies addressing interactions between aldehydes and tocopherols were available.

The anisidine value, together with PV are commonly used methods for determination of oxidation status in fish oil. The AV method is popular in the industry and research as well as being the parameter used for setting limits of secondary oxidation products by regulatory authorities. The AV method is found to be highly influenced by the aldehydes in the flavoring, the influence being proportional to the content of aldehydes. Based on the results in this thesis the AV method is not a good method for measuring secondary oxidation products in flavored

fish oil. More advanced and specific methods like NMR and headspace can be useful tools in determining secondary oxidation products and overall oxidations status in flavored fish oil.

3.3.3 ^{1}H NMR Spectroscopy of Fish Oil with Lemon Aromas

The fish oil with the two lemon aromas that gave highly elevated AV measurements were analyzed with ^{1}H NMR spectroscopy to see if this method could give a more detailed picture of the oxidation status in the fish oil. The carbonyl protons of autoxidation derived aldehydes in edible oil give signals in the downfield region of the spectra, between 9.4 and 9.8 ppm (solvent CDCl3) (Guillen and Goicoechea, 2009) and peroxides at 8.5 - 8.9 ppm (solvent CDCl3) (Claxson et al., 1994).

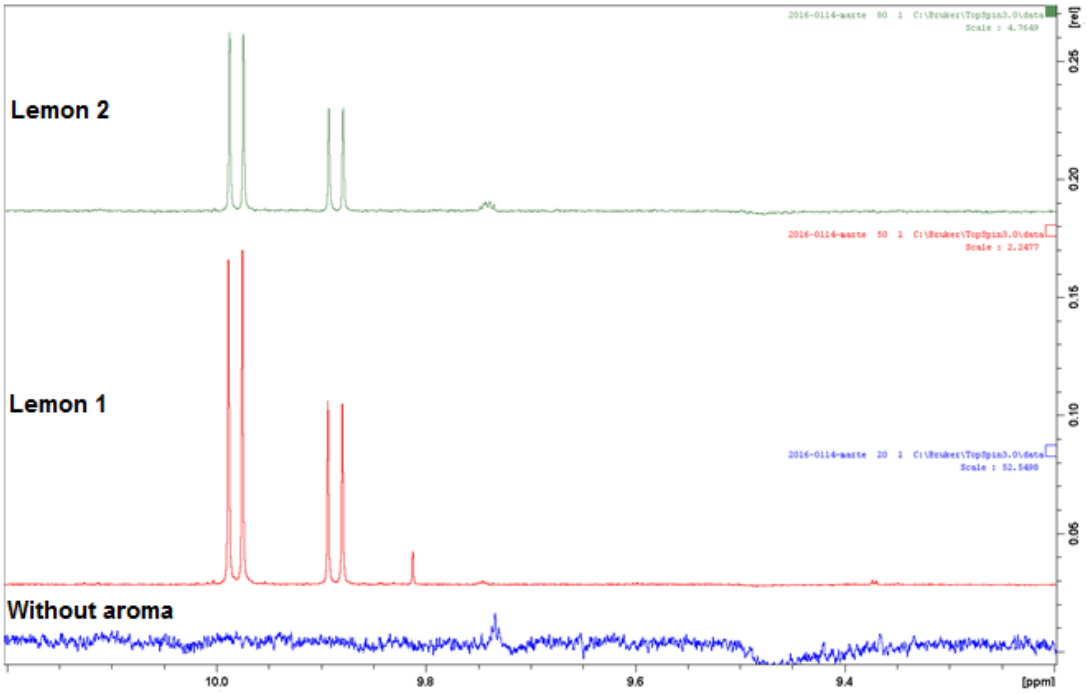

Figure 23: Chemical shifts of aldehydes in fish oil with lemon aroma. No aldehydes detected in the fish oil without aroma.

The chemical shifts of the aldehydes in the aroma components are shown in figure 23. It appears to be the same aldehydes in both lemon aromas, with Lemon 1 having one peak more than Lemon 2, the amount of aldehyde giving rise to this peak may be lower than the detection limit in Lemon 2. The difference in amount of aldehydes is clearly visible and correspond with the aldehyde content of the respective aroma, with Lemon 1 having 41% aldehydes and larger peaks than Lemon 2 with 11.19 % aldehydes.

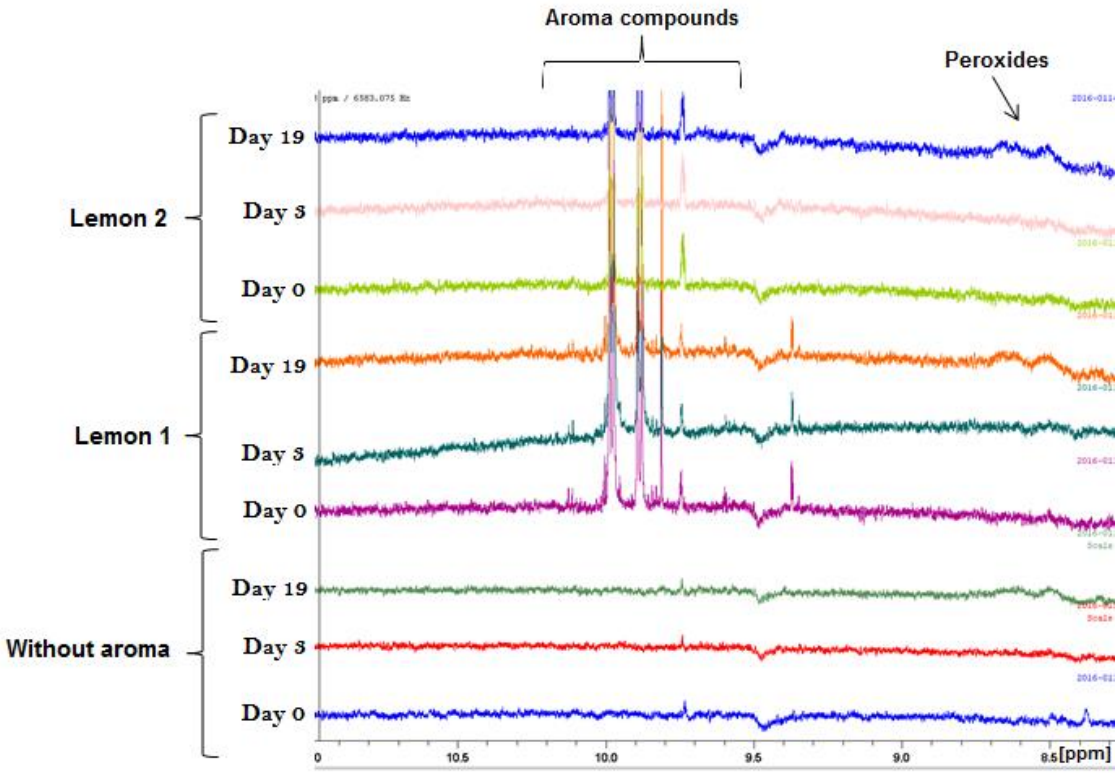

Figure 24: ^{1}H NMR spectra for fish oil without aroma compared to spectra with addition of two lemon aromas with different concentration of aldehydes.

In the fish oils with Lemon 1 and 2, there is observed a variety of clear peaks with strong signals in the region where aldehydes are expected as shown in figure 24. The aldehyde content does not seem to increase through day 3 and day 19, thus the contribution of aldehydes from the lipid oxidation seem to be less important. For the fish oil samples without aroma, no aldehydes were detected, with the number of scans used in neither of the samples from day 0, 3 and 19. As measured by the AV method, the development of secondary oxidation products in the fish oil without aroma are rather low, and the amount of aldehydes is possibly being lower than the limit of detection for the NMR analysis.

The development of peroxides is barely detected in the samples from day 19. The amount of peroxides seem to correspond well with the peroxide values for day 19 where the fish oil without aroma have lower peroxide value (27.46) than the Lemon 1 and Lemon 2 (33.77 and 32.06). However, the differences are not clearly visible in the NMR spectra. The set up in this experiment were optimized for the study of aldehydes and another solvent would have been more suitable for the studies of peroxides.

The aldehydes from the lemon aroma gave signals downfield for the region where autoxidation-derived aldehydes give signals. For example citral give signal closer to 10ppm ((Aldrich). There

might be other aldehydes in other aromas giving rise to peaks at other locations in the spectra. The specific analysis provided by NMR spectroscopy is very useful in distinguishing the aldehydes from the aroma component with the aldehydes from the lipid oxidation, when comparing fish oil with and without addition of aroma.

3.3.4 Dynamic Headspace (GC-MS) analysis of fish oil

There was no significant development of secondary oxidation products measured by the anisidine value method, thus Dynamic Headspace GC-MS was used to study the volatile secondary oxidation products: (E)-2-pentenal, (E)-2-hexenal, (Z)-4-heptenal and (E,E)-2,4-heptadienal to see if these volatiles could be used as a more sensitive measure of lipid oxidation. Samples from day 0 and day 19 were compared for fish oil without aroma and fish oil with Lemon 1 and Lemon 2 in fish oil without antioxidant.

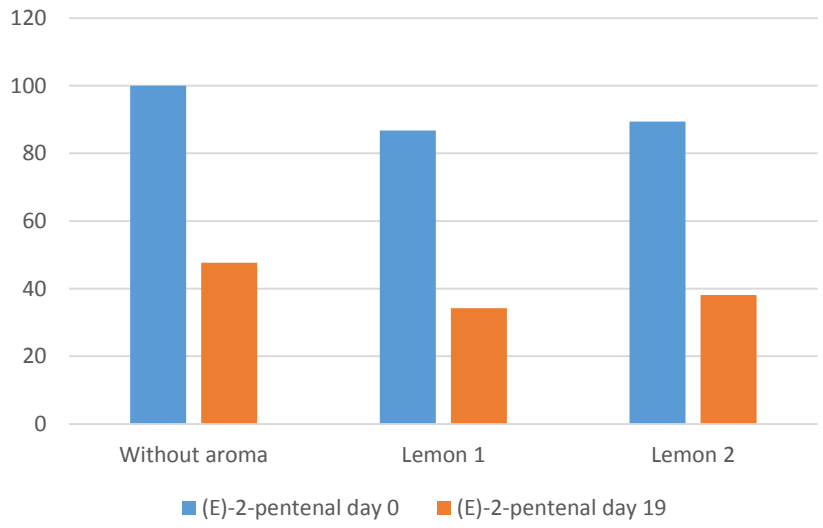

Figure 25: The relative amount (E)-2-pentenal in fish oil without aroma and fish oil with Lemon 1 and 2 aroma in samples from day 0 and day 19.

As illustrated in figure 25. the (E)-2-pentenal amount was reduced about a twofold in day 19 compared to day 0 in fish oil without aroma. In fish oil with lemon aromas, the amount of (E)-2-pentenal were reduced even more.

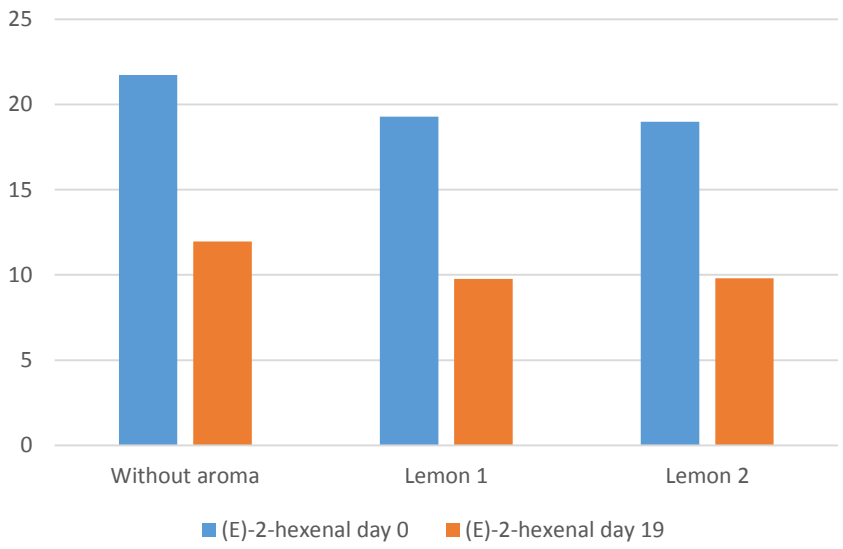

Figure 26: The relative amount (E)-2-hexenal in fish oil without aroma and fish oil with Lemon 1 and 2 aroma in samples from day 0 and day 19.

In fish oil without aroma the (E)-2-hexenal amount was reduced almost twofold in day 19 compared to day 0. Similar trends were seen in fish oil with lemon aromas as illustrated in figure 26.

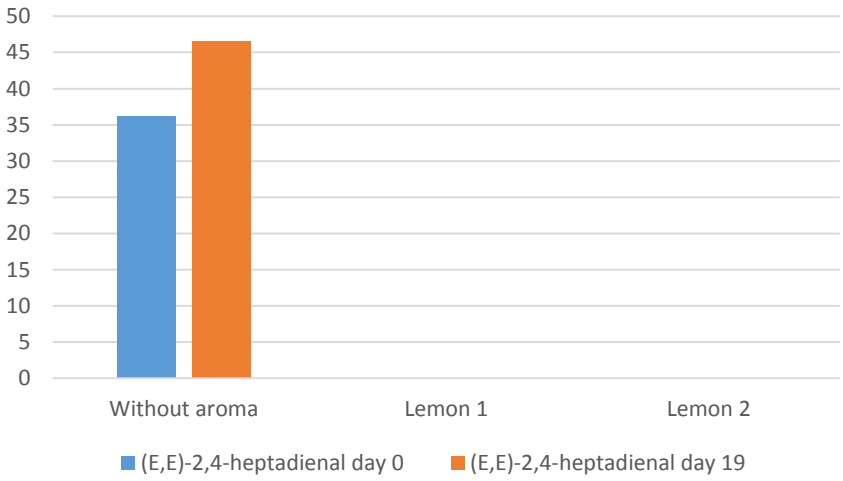

Figure 27: The relative amount (E,E)-2,4-heptadienal in fish oil without aroma and fish oil with Lemon 1 and 2 aroma in samples from day 0 and day 19.

(E,E)-2,4-heptadienal in fish oil without aroma clearly increased from day 0 to day 19. No (E,E)-2,4-heptadienal were detected in fish oil with the lemon aromas as illustrated in figure 27.

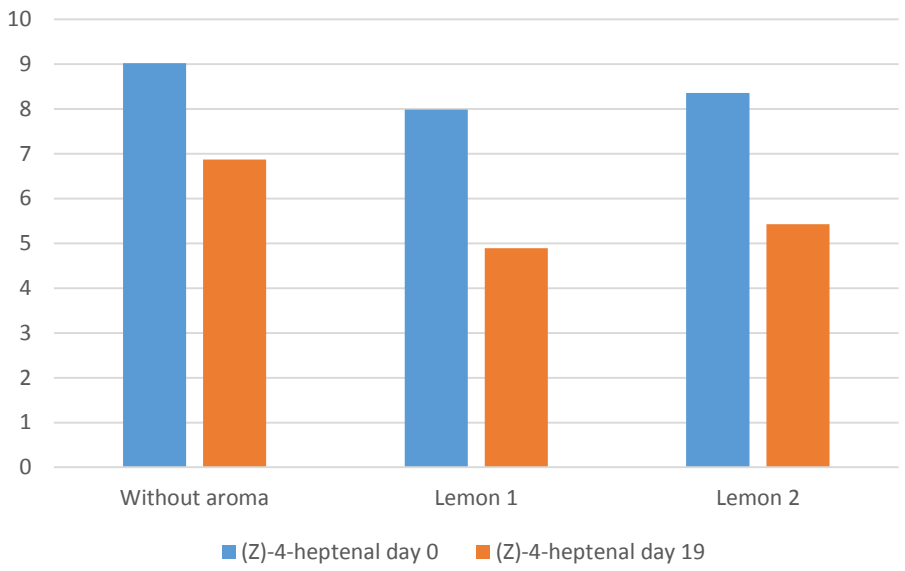

Figure 28: The relative amount (Z)-4-heptenal in fish oil without aroma and fish oil with Lemon 1 and 2 aroma in samples from day 0 and day 19.

As illustrated in figure 28 there was a decrease in the amount of (Z)-4-heptenal in fish oil without aroma from day 0 to day 19. There is a slightly larger decrease in the amount of (Z)-4-heptenal in fish oil with lemon aromas from day 0 to day 19. The retention time of heptanal was found to be very similar to the retention time of (Z)-4-heptenal, therefore the values of (Z)-4-heptenal obtained can have contribution from heptanal as well although the detector was set for detection of (Z)-4-heptenal.

That there were no increase in the amount of the volatile aldehydes (E)-2-pentenal, (E)-2-hexenal, (Z)-4-heptenal from day 0 to day 19 in the fish oil without aroma as well as in fish oil with Lemon 1 and Lemon 2 aroma was unexpected. Contrary, a clear decrease in the amount of the mentioned aldehydes were found. These findings were not expected, but they are supported by another study which reported a decrease of (E)-2-pentenal and (E,E)-2,4-heptadienal among other volatiles with storage time (Lee et al., 2003). They explain the reduction with the use of an open-air system which contributed to the removal of the oxidized volatile compounds generated in early stages in the oxidation process. They also said that the removed volatiles could not be replaced due to the decreasing content of original intact fatty acids in the oil during storage time.

The case was different for (E,E)-2,4-heptadienal where the amount increased from day 0 to day 19 in fish oil without aroma. (E,E)-2,4-heptadienal were not detected in fish oils with lemon aroma. This finding is in compliance with another study that followed the development of (E,E)-

2,4-heptadienal during storage in accelerated conditions and suggest to use this volatile as a measure of oxidation status in fish oil together with 1-penten-3-one (Aidos et al., 2002).

The inconsistency in the findings of volatiles shows how complex lipid oxidation and volatile formation in fish oil is and more studies should be preformed to investigate the potential of volatiles serving as parameters of oxidation status in fish oil.

3.4 Vitamin Experiment

The vitamins A, D and E are commonly used to fortify fish oil, and to study their potential effect on the lipid oxidation process in fish oil, primary and secondary oxidation products were determined in fish oil with the added vitamins in different levels of oxidation. To reach the different levels of oxidation, the fish oil with and without added aroma components were left in ambient environment with access to oxygen to oxidize for 20 days. Samples were collected about every fifth day. The vitamins studied were vitamin A (retinyl palmitate), vitamin D (cholecalciferol) and vitamin E (DL-α-tocopheryl acetate).

3.4.1 Influence of Vitamin A, D and E on Primary Oxidation Products

The primary oxidation products were determined in fish oil with addition of vitamin A, D and E by the conjugated dienes method. This method was chosen as a measure of primary oxidation products instead of the peroxide value method, due to problems with the PV electrode.

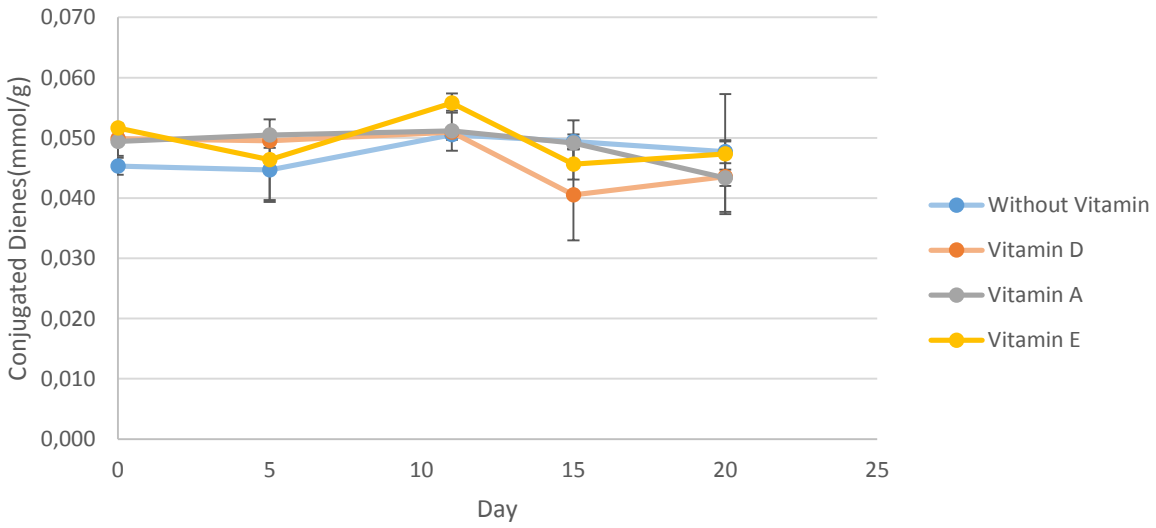

Figure 29: Conjugated dienes in different levels of oxidation in fish oil (without antioxidant) with addition of vitamin A, D and E. The values are means of three parallels and the error bars represent the standard deviation between the parallels.

The conjugated diene values in fish oils with vitamin are close to the values for fish oil without vitamin as shown in figure 29. From day 0 until day 11, the fish oils with vitamins lie just above the values for the fish oil without addition, but after day 11 and the rest of the time measured, there seem to be a decrease and they stabilize to lie just below the fish oil without vitamin. Significant differences were found for vitamin A at day 0, and for vitamin E at day 0 and 11, but overall the conjugated diene values are very similar for all samples for all days.

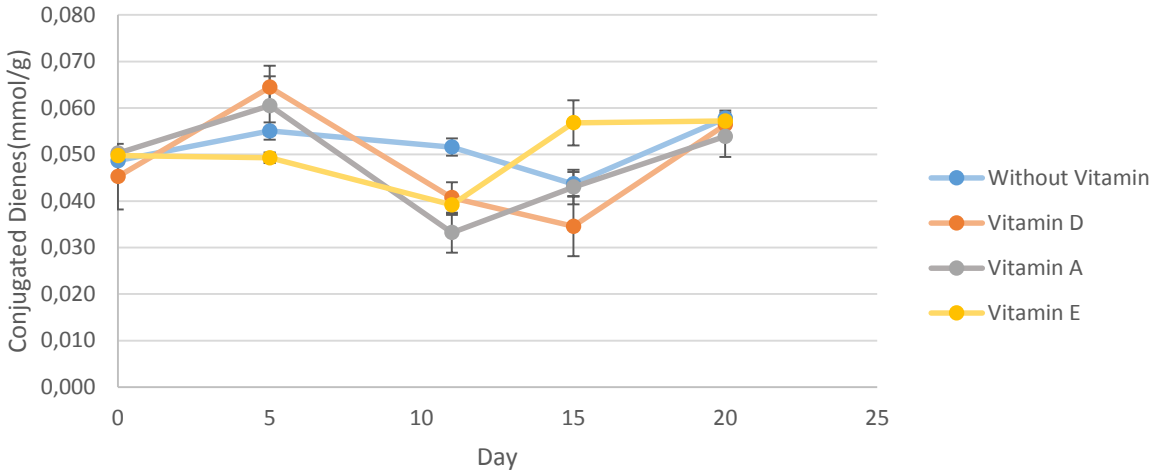

Figure 30: Conjugated dienes in different levels of oxidation in fish oil (with antioxidant) with addition of vitamin A, D and E. The values are means of three parallels and the error bars represent the standard deviation between the parallels.

For the fish oil with antioxidant, the conjugated dienes values in the fish oils with vitamin showed no clear trend as displayed in figure 30. Until day 5, there is only the fish oil with vitamin E that had lower conjugated dienes values than the fish oil without vitamin. Somewhere between day 5 and 11, the fish oils with vitamin D and A shift and are also measured to be below the fish oil without vitamin. Their values stay below for the rest of the time measured, while the conjugated dienes in fish oil with vitamin E increase after day 11 and lie above the fish oil without vitamin for the rest of the time measured. Vitamin D showed a significant difference at day 5 and 11, vitamin A was significant different at day 0 and 11 and vitamin E showed significantly different levels at day 5, 11 and 15, when compared to the fish oil without vitamin.

There was close to no development of primary oxidation products in the experiment in neither of the fish oils with or without antioxidant measured by the conjugated dienes analysis. Based on previous experiments the development was expected to be more pronounced. Usually the conjugated dienes are found to correlate well with PV (Shahidi et al., 1994), but unexpectedly this correlation is not found here when compared to the PV values of the pre-experiment and the aroma experiment. Although it is not the same samples analyzed, it is the same fish oil. This low development of primary oxidation products and the poor correlation give rise to doubt about whether the results from the conjugated dienes analysis are reliable or if something went wrong during the analysis.

3.4.2 Influence of Vitamin A, D and E on Secondary Oxidation Products

The secondary oxidation products were determined in fish oil with addition of vitamin A, D and E by the anisidine value method in different levels of oxidation, to study their influence on the oxidation process.

Fish oil without antioxidant

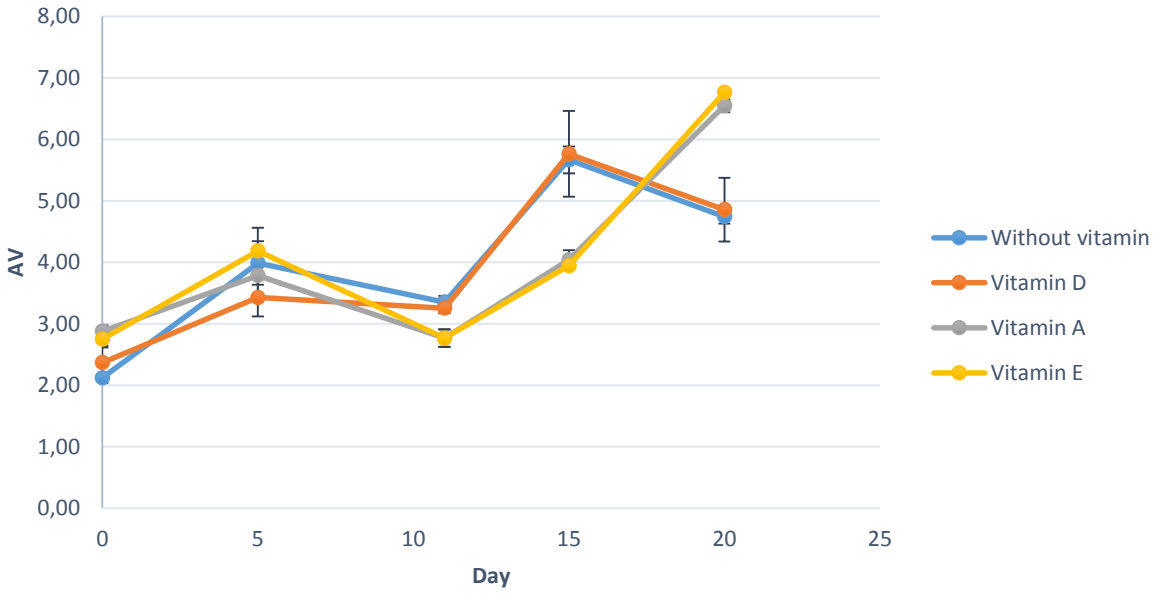

Figure 31: The effect of vitamin A, D and E on the AV measurements in fish oil (without antioxidant) in samples with increasing oxidation status. The AV measurements are shown as the mean of three parallels and the error bars represent the standard deviation.

Figure 31 illustrates the anisidine value measured in fish oils with vitamin at different levels of oxidation. The fish oils with vitamin showed anisidine values similar to the anisidine values of the fish oil without vitamins until day 11. After day 11, the fish oil with vitamin D followed the same pattern as the fish oil without vitamins, while fish oil with vitamin E and vitamin A followed their own, but similar pattern of oxidation. Anisidine values of all fish oils increased, but the measurements of fish oil with vitamin A and E lay below. Between day 15 and day 20 the anisidine values of fish oil with vitamin D and fish oil without vitamin decreases, while the fish oil with vitamin A and E increase. Fish oil with vitamin A and E showed significant differences for all days except day 5 when compared to the fish oil without vitamin.

Vitamin A and vitamin E provide a small anti-oxidative effect when added to fish oil (without antioxidant), when it comes to development of secondary oxidation products measured by the anisidine value method. This effect was observed between day 11 and 15. At day 20, the vitamin A and vitamin E seem to give a pro-oxidative effect in the fish oil.

Fish oil without antioxidant

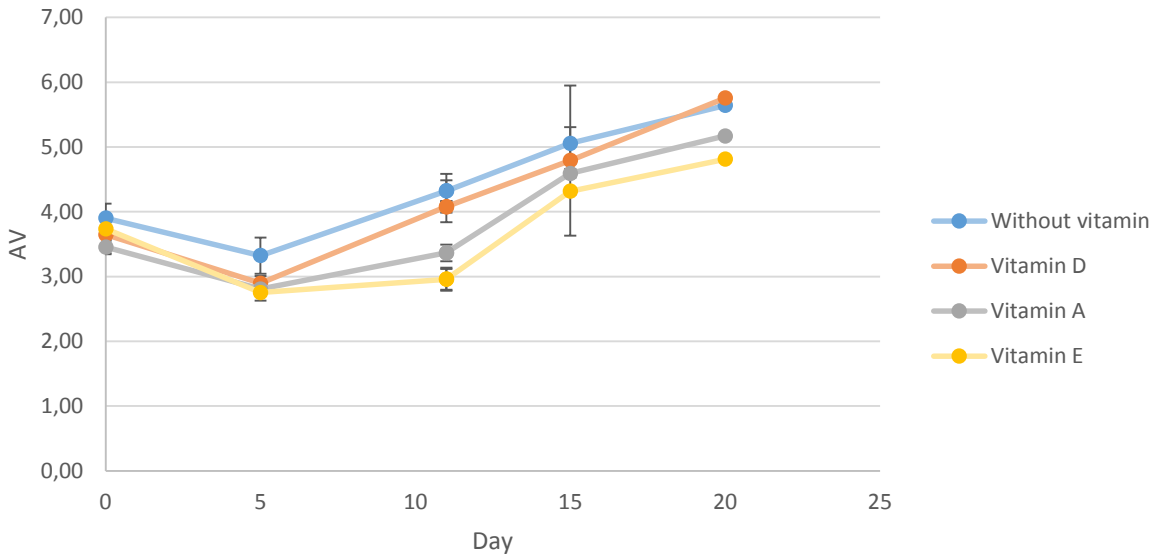

Figure 32: The effect of added vitamin A, D and E to fish oil with antioxidant on the AV method in samples with increasing oxidation status. The AV measurements are shown as the mean of three parallels and the error bars represent the standard deviation.

As displayed in figure 32, the fish oil without vitamin have slightly higher anisidine values than the fish oils with vitamins for all days, except for day 20, where the anisidine values are very alike.

The anisidine values of all fish oils with vitamins are very similar until day 5. After day 5, as observed in the fish oil with antioxidant as well, there was a splitting of the AV patterns. Fish oil with vitamin A and E showed similar behavior while vitamin D followed just below the fish oil without vitamin. Fish oil with vitamin D showed a significant difference at day 0, fish oil with vitamin A showed significant differences at 0, 11 and 15 and fish oil with vitamin E showed significant differences for all days, compared to the fish oil without vitamin. Vitamin A and vitamin E gave an slightly anti-oxidative effect when added to fish oil (with antioxidant), when it comes to development of secondary oxidation products measured by anisidine value method, with vitamin E giving the most notable effect.

3.4.3 Influence of Vitamin A, D and E on Stability of Fish Oil measured by Oil Stability Index

To investigate the stability of fish oil with vitamin addition, the Oil Stability Index analysis was used. This is an accelerated oxidation test, with elevated temperature and oxygen bubbling, which facilitate oxidation. Fish oil with the vitamins A, D and E were investigated.

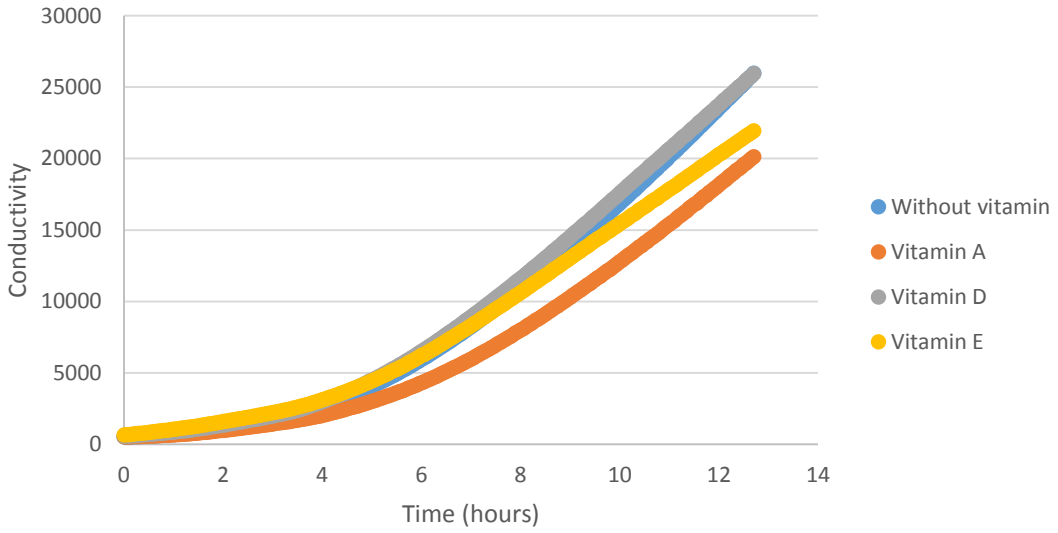

Figure 33: The increase in conductivity in fish oil without and with vitamin A, D and E. The values represent the mean of four parallels.

As displayed in figure 33, the increase in conductivity for fish oil with vitamin A seem to occur a little later than the fish oil added vitamin D and E and the fish oil without vitamin. However, there are no significant differences for either of the fish oils with added any of the vitamins compared to the fish oil without any addition. The OSI values are given in table 4.

Table 4: OSI values of vitamin A, D and E.

	OSI value	**Standard deviation**
Without Vitamin	7.01	0.16
Vitamin A	8.77	1.15
Vitamin D	7.03	0.73
Vitamin E	6.83	0.33

The OSI value is defined as the point of maximal change in conductivity caused by the increase in formation of volatile organic acids during lipid oxidation. The tendencies of vitamin A giving a small antio-xidative effect is in line with the results found using the AV method.

The vitamin E used in this experiment, the same used in the fish oil on the market is α-tocopherol which is commonly known to have good antioxidant properties (Frankel, 1996). Vitamin E can break the chain reaction of lipid oxidation by donating their phenolic hydrogens to lipid free radicals and thereby put a stop to the propagation. The antioxidant properties of vitamin E is well recognized, but the antioxidant effect of vitamin A is less investigated. A study has reported the effect of vitamin A on oxidation in bulk linoleic acid and found it to inhibit oxygen uptake (Monaghan and Schmitt, 1932). Some reviews describe the structure of vitamin A and their potential antioxidant effect (Palace et al., 1999), and others report on vitamin A's role in protecting human low density lipoproteins against copper-stimulated oxidation (Livrea et al., 1995). The antioxidant effect of vitamin A is thought to be conferred by its conjugated hydrocarbon chain that can quench singlet oxygen and stabilize peroxy radicals. The hydroperoxy radicals in lipid oxidation can be stabilized through combination with vitamin A before they have time to propagate in the oxidation process by abstracting a hydrogen from another unsaturated fatty acid and generate hydroperoxides and new alkyl free radicals (Palace et al., 1999). By this mechanism vitamin A can break the chain reaction of lipid oxidation in the propagation step

Both vitamin E and vitamin A are chain-breaking antioxidants that interfere with the propagation step of the oxidation process. This may explain the similar oxidation patterns of the fish oils added vitamin A and vitamin E in the AV determinations and partly in the OSI measurements.

At the end of the experiment, a pro-oxidative effect was seen in fish oils without antioxidant with vitamin A and vitamin E. In addition to the well known anti-oxidative activity, vitamin E have been found to have neutral and even pro-oxidative effect under certain conditions. Publications are available on the pro-oxidative effect of vitamin E in human lipoproteins (Bowry et al., 1992), but not in bulk fish oil.

3.5 Mineral Experiment

A range of vitamins and minerals are commonly added to fish oil to make a more total nutritional supplement. In this experiment, Potassium Iodide, Folic Acid, Vitamin K2, Zink Oxide, Ascorbic Acid, Magnesium Oxide and Calcium Carbonate were studied to examine their potential effect on the oxidation process in fish oil.

3.5.1 Influence of Vitamins and Minerals on Oxygen Absorption Measurements

To study the vitamins and minerals influence on the oxidation process in fish oil, oxygen absorption measurements were done in an oxygraph system and the oxygen uptake rate (OUR) was measured in fish oil (without antioxidant) upon addition of minerals and vitamins.

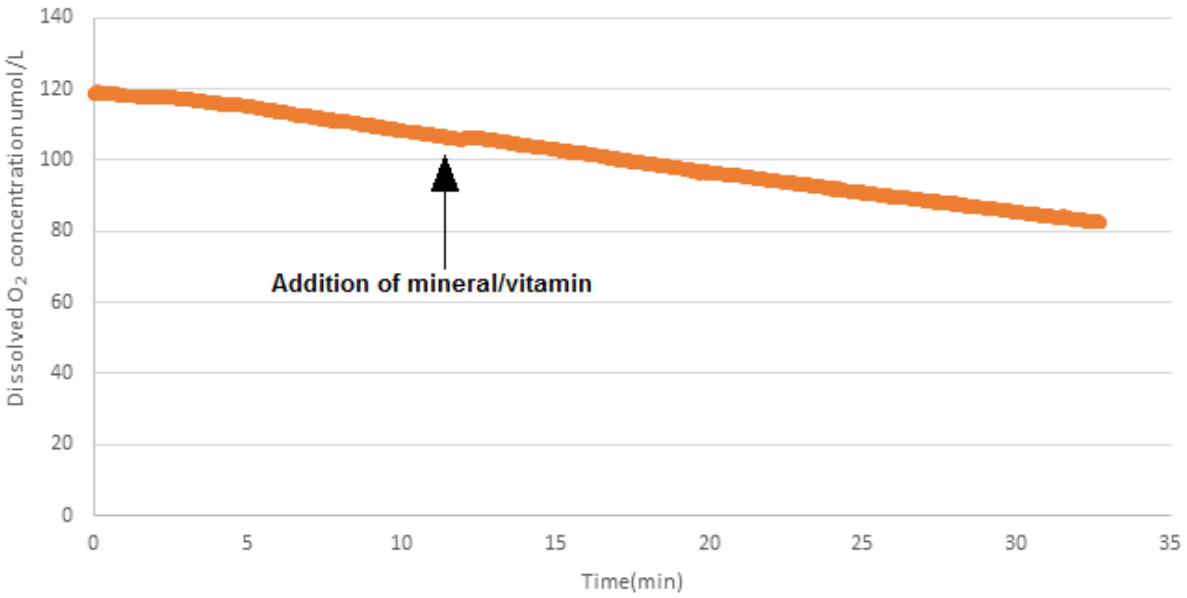

Figure 34: The oxygen uptake rate of fish oil prior to and after addition of Calcium Carbonate.

Initially, the background OUR was measured for about 10 minutes before the additive was injected, followed by about 20 minutes of OUR measurement to detect any pro-oxidative or anti-oxidative effect. As shown in figure 34 there was no observed change in OUR after the injection compared to prior to the injection. This curve is representative for all the minerals/vitamins tested. The OURs of all vitamins and minerals are displayed in table 5.

Table 5: Oxygen uptake rate measured before and after addition of the minerals to the oxygraphic cell containing fish oil.

	Background OUR (µM/min)	**OUR after addition (µM/min)**	**Difference**
Ethanol control	1.178	1.049	-0.130
Ascorbic acid	1.200	1.111	-0.089
Potassium Iodide	1.491	1.612	0.122
Folic Acid	1.348	1.222	-0.126
Vitamin K2	1.343	1.424	0.081
Zink Oxide	1.376	1.302	-0.074
Magnesium Oxide	1.075	1.079	0.004
Calcium Carbonate	1.292	1.389	0.097

The additives were dissolved in ethanol before they were injected into the fish oil, thus the fish oil with only ethanol injection served as a control to ensure that the solvent did not affect the measurements. The injection with only ethanol did not show any change in OUR, thus it was concluded that the ethanol did not interfere with the measurements and was suitable as a solvent.

A slight change in OUR was seen in fish oil with Potassium Iodide, Calcium Carbonate and possibly in Vitamin K2, but since there was doubt about whether or not the additives were entirely dissolved in the fish oil, it is not possible to draw any clear conclusions from this analysis.

3.5.2 Influence of Vitamins and Minerals on Oil Stability Index

Oil stability index was in this experiment used to study the stability of fish oils with addition of vitamins and minerals when exposed to accelerated oxidation conditions. First the impact of the additives were analyzed in fish oil without antioxidant, then the additives that showed any effect were analyzed in fish oil with antioxidant.

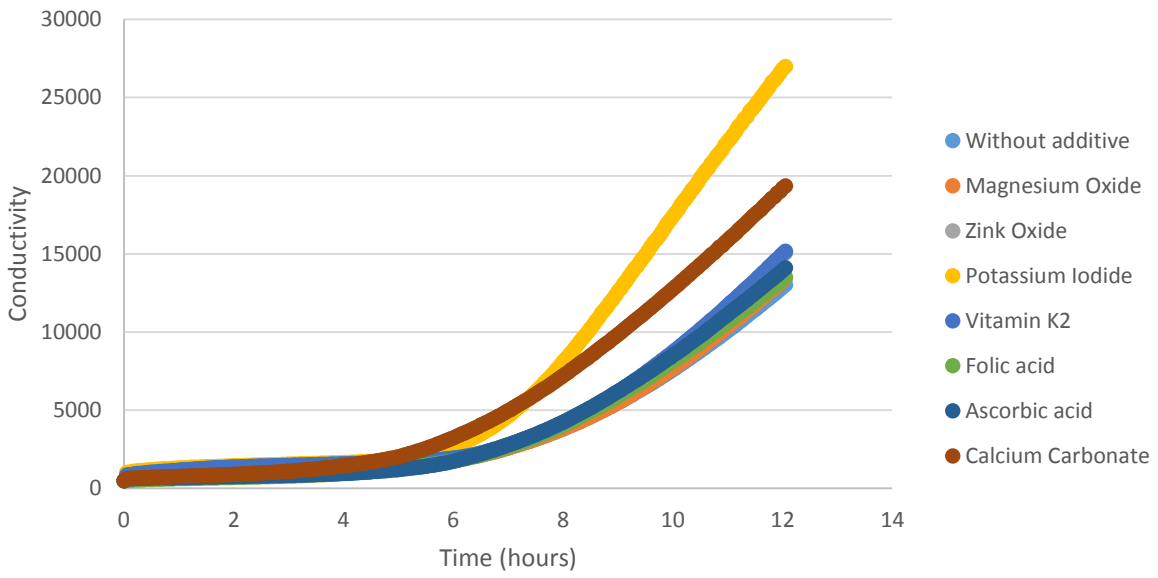

Figure 35: The increase in conductivity for fish oil added a variety of additives under accelerated oxidation conditions. The values represented is the mean of four parallels.

Of the seven additives investigated, an effect was seen in the fish oil with Potassium Iodide and Calcium Carbonate, they showed a pro-oxidative effect as shown in figure 35 in accordance with the findings from the oxygen absorption measurements.

The Potassium Iodide and the Calcium Carbonate were analyzed in fish oil with antioxidant.

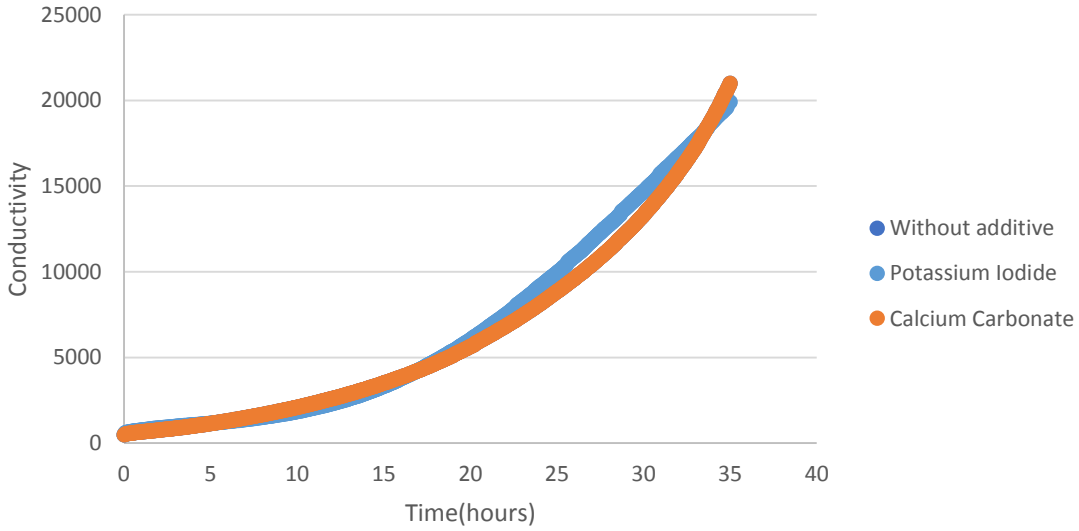

Figure 36: The increase in conductivity for fish oil (with antioxidant) added Potassium Iodide and Calcium Carbonate, under accelerated oxidation conditions. The values represented is the mean of four parallels.

There were no effects observed for addition of Potassium Iodide and Calcium Carbonate in fish oil with antioxidant as illustrated in figure 36.

The observed pro-oxidative effect of potassium iodide and calcium carbonate in fish oil without antioxidant and the absence of effect in fish oil with antioxidant might be explained by the antioxidants, the tocopherols. Tocopherols have a hydroxyl group on their ring structure that can donate a hydrogen to free radicals, thereby inactivating them resulting in inhibited development of the secondary oxidation products, the organic acids detected in the OSI.

There are no available information on calcium carbonate and potassium iodide in regard to their effect on the lipid oxidation process. The pro-oxidative effect of these two additives should be confirmed through other methods, before any conclusions can be drawn. Although these finding are interesting, they are not of high relevance to the industry because antioxidants such as tocopherols are always added to the oil available on the market today.

4. Conclusion

The main aim of this thesis was to study the influence of aldehydes in flavorings on the Av and PV method (Iodometric Titration method) in fish oil. Additionally ^{1}H NMR spectroscopy and dynamic headspace GC-MS were applied to see if these methods could give a more informative picture of the oxidation status in fish oil.

Raspberry 1, Raspberry 2 and Strawberry were found to give reduced PV values in fish oil, believed to be a real lowering of the primary oxidation products caused by an antioxidative effect of the polyphenolic compounds in the berry aroma and not interference with the method. Lemon aromas on the other hand gave elevated peroxide values, with a higher effect in fish oil without antioxidant. This effect is thought to be the result of oxidizing compounds in the lemon aroma interfering with the method, contributing to the oxidation of iodide to iodine.

The aldehydes in the aroma compounds were found to interfere highly with the AV method, giving extremely elevated anisidine values in the fish oils. A significant correlation ($p < 0.05$) between the aldehyde content and the anisidine values was found. Both AV and PV are widely used methods for assessment of oxidation status in fish oil, both in industry and research, and they are also used as parameters for recommendations of limits of oxidation status by expert organizations and regulatory authorities. Both methods were found to be influenced by compounds that were not part of the lipid oxidation process, and especially the AV method gave highly unreliable results in fish oils with flavoring. There is a need for development and implementation of methods that are less sensitive to aroma compounds for the assessment of oxidations status in flavored fish oils.

^{1}H NMR spectroscopy made it possible to distinguish the aldehydes from the aroma and the oxidation-derived aldehydes. The aldehydes from the lemon aroma gave signals in another region than the oxidation-derived aldehydes in the ^{1}H NMR spectrum. By using Dynamic Headspace GC-MS an increase in the amount of (E,E)-2,4-heptadienal with storage time was found, but that there was a clear decrease in the amount of the volatiles (E)-2-pentenal, (E)-2-hexenal, (Z)-4-heptenal with storage time was unexpected. The inconsistency of the findings of development of volatiles shows how complex lipid oxidation and volatile formation is in fish oil and that more studies are needed to evaluate the potential of volatiles serving as parameters of oxidation status in fish oil.

In addition, the impact of some minerals and vitamins on the oxidation process were investigated using the OSI method. The minerals Folic Acid, Zink Oxide, Magnesium Oxide

were found to have no effect, while Calcium Carbonate and Potassium Iodide were found to have a pro-oxidative effect in fish oil without antioxidant, but no effect in fish oil with antioxidant. Vitamin K2, C, E and A were also studied using the OSI method, and no significant effects were found. A slight anti-oxidative effect was found for Vitamin A and E on the development of secondary oxidation products measured by the anisidine value method in fish oil with and without antioxidant. However, for fish oil without antioxidant, there was observed a pro-oxidative effect at day 20, the last day of measurements, but this effect was not seen in the fish oil with antioxidant. Since the fish oil on the market is always added with antioxidant, these findings are not critical.

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Appendix

Apendix A: Fatty acid composition

Detailed fatty acid composition of the fish oil used in the experiments found using gas chromatography. The values are % of total fatty acids.

	1	2	avg	std
C14:0	3,26	3,27	3,27	0,01
C14:1	0,11	0,11	0,11	0,00
C15:0	0,24	0,24	0,24	0,00
C16:0	7,78	7,79	7,78	0,01
C16:1 n 9	3,97	3,99	3,98	0,02
C16:1 n 7	0,47	0,48	0,48	0,00
C17:0	0,56	0,56	0,56	0,00
C17:1	3,58	3,60	3,59	0,01
C18:0	7,88	7,84	7,86	0,03
C18:1n11 + n9	2,66	2,73	2,69	0,05
C18:1n7	1,13	1,14	1,13	0,00
C18:2n6	0,23	0,24	0,24	0,00
C18:3n6	0,81	0,82	0,82	0,01
C18:3n3	2,37	2,38	2,38	0,01
c18:4n3	0,34	0,34	0,34	0,00
C20:0	2,00	2,00	2,00	0,00
C20:1n11+ n9	0,50	0,51	0,50	0,00
C20:1n7	0,29	0,29	0,29	0,00
C20:2n6	0,30	0,30	0,30	0,00
c20:3n6	0,20	0,20	0,20	0,00
C20:4n6	0,02	0,01	0,02	0,00
C20:3n3	1,16	1,16	1,16	0,00
c20:4n3	25,63	25,66	25,65	0,02
C20:5n3	0,25	0,25	0,25	0,00
C22:0	1,26	1,25	1,25	0,01
c22:1n11	0,36	0,36	0,36	0,00
C22:1n9	0,14	0,15	0,15	0,00
C22:2	0,16	0,16	0,16	0,00
C22:3	0,16	0,16	0,16	0,00
C22:4	3,35	3,36	3,36	0,01
c22:5n3	0,00	0,00	0,00	0,00
C24:0	19,42	19,42	19,42	0,01
C22:6n3	0,77	0,77	0,77	0,00
C24:1n9	0,00	0,00	0,00	0,00
Saturated	42,39	42,37	42,38	0,02
Monosaturated	13,21	13,34	13,28	0,09
Polysaturated	35,74	35,81	35,78	0,05
omega 3	30,52	30,57	30,54	0,03
Unknown	8,65	8,48	8,56	0,12

Apendix B: Experimental data from the Pre-experiment

Experimental data obtained from PV and AV measurements in the pre-experiment. The values are the means of three parallels and the precision is given as standard deviations.

Day	PV (meq/kg)	STD	AV	STD
0	1.99	0.20	3.20	0.36
2	6.69	0.36	3.65	0.21
5	11.52	0.08	4.42	0.36
7	18.05	0.83	5.47	0.24
9	31.73	0.20	6.45	1.06
12	47.05	0.17	6.58	0.60

Appendix C: Experimental data from the determination of PV in the Aroma Experiment

Experimental data from the determination of peroxide value using the iodometric titration method in the aroma experiment. The values are means of three parallels and the precision is given as standard deviation.

Fish oil without antioxidant

Day	Without aroma	STD	Raspberry 1	STD	Raspberry 2	STD	Strawberry	STD	Lemon 1	STD	Lemon 2	STD
0	4.07	0.36	4.63	0.24	4.17	0.22	5.18	0.19	5.39	0.66	3.96	0.19
3	11.33	0.30	11.24	0.54	8.66	0.41	9.89	0.10	13.06	0.15	13.42	0.32
5	11.52	0.17	10.70	0.11	9.00	0.36	9.61	1.77	12.64	0.12	13.11	0.19
7	14.15	0.19	13.03	0.42	11.51	0.46	13.63	0.31	16.71	0.69	17.81	0.38
10	18.13	0.50	17.88	0.81	14.20	0.60	18.79	0.20	23.72	1.24	24.45	1.39
12	18.73	0.66	17.40	0.03	13.63	0.16	18.08	0.41	21.32	0.21	22.12	0.87
14	23.93	0.28	18.51	0.30	15.35	0.15	20.34	0.89	24.25	0.47	25.22	0.10
17	24.44	0.16	23.45	0.26	19.66	0.40	24.20	0.38	28.97	0.90	28.44	0.22
19	27.46	0.05	28.46	0.54	22.63	0.47	27.72	0.35	33.77	2.08	32.06	0.22

Fish oil with antioxidant

Day	Without aroma	STD	Raspberry 1	STD	Raspberry 2	STD	Strawberry	STD	Lemon 1	STD	Lemon 2	STD
0	2.40	0.25	2.43	0.23	2.18	0.16	1.93	0.13	4.03	0.00	4.14	0.42
3	10.68	0.41	9.83	0.65	6.68	0.12	7.58	0.12	10.80	0.25	11.14	0.14
5	12.09	0.68	11.10	0.10	8.17	0.19	8.00	0.19	12.56	0.24	12.76	0.14
7	15.80	0.12	16.75	1.12	11.18	0.40	11.61	0.64	16.33	0.68	16.57	0.62
10	20.71	0.60	20.90	1.28	15.89	1.02	15.16	0.67	21.25	1.00	24.25	0.23
12	23.10	0.76	22.37	0.17	17.49	0.16	16.76	0.22	23.19	0.63	25.60	0.20
14	25.82	0.26	26.22	0.57	18.97	0.34	19.13	0.18	26.31	0.55	28.49	0.72
17	30.59	0.16	30.13	0.29	21.36	0.19	22.61	0.18	31.22	0.27	34.19	0.35
19	34.36	0.92	33.61	0.09	23.74	0.13	26.05	0.56	33.58	0.27	36.88	0.37

Appendix D: Experimental data from the determination of AV in the Aroma Experiment

Experimental data from the determination of anisidine value in the aroma experiment. The values are means of three parallels and the precision is given as standard deviation.

Fish oil without antioxidant

Day	Without aroma	STD	Raspberry 1	STD	Raspberry 2	STD	Strawberry	STD	Lemon 1	STD	Lemon 2	STD
0	3.13	0.20	6.36	0.14	18.32	0.32	8.33	0.41	332.80	2.48	74.92	6.37
3	3.13	0.06	7.03	0.22	16.07	0.49	7.55	0.27	319.43	1.26	92.58	1.12
5	4.23	0.31	6.60	0.14	20.76	0.79	6.46	0.22	230.55	1.62	44.44	1.38
7	3.66	0.17	6.62	0.12	20.85	0.71	9.17	0.25	292.96	5.73	83.27	0.57
10	4.92	0.17	7.94	0.04	28.41	0.55	15.42	0.55	213.99	15.30	84.23	4.84
12	4.90	0.02	6.14	0.61	9.88	1.05	6.23	0.42	147.48	11.28	45.84	9.13
14	5.92	0.40	8.53	0.16	5.21	0.14	3.09	0.16	164.07	4.45	50.42	0.74
17	3.56	0.65	3.85	0.36	9.20	0.74	5.10	0.22	149.50	3.53	85.43	10.68
19	5.82	0.04	7.46	0.11	7.46	0.45	5.65	0.18	111.96	2.55	35.58	0.99

Fish oil with antioxidant

Day	Without aroma	STD	Raspberry 1	STD	Raspberry 2	STD	Strawberry	STD	Lemon 1	STD	Lemon 2	STD
0	4.18	0.18	6.14	0.15	8.20	0.55	5.05	0.63	325.02	2.19	80.68	0.94
3	3.87	0.18	6.93	0.03	13.80	0.46	7.08	0.50	271.85	1.92	91.24	0.79
5	4.33	0.40	6.82	0.44	14.82	0.61	7.37	0.23	216.31	2.13	88.76	0.26
7	3.67	0.19	6.76	0.21	16.46	0.26	8.35	0.41	226.29	0.02	54.26	7.16
10	3.74	0.09	4.69	0.06	20.15	0.81	9.51	1.06	191.85	3.35	80.13	5.94
12	5.30	0.19	8.90	0.57	12.28	1.70	5.39	0.50	182.60	5.12	49.57	7.54
14	5.59	0.26	8.38	0.11	14.43	0.34	7.23	0.42	244.18	7.61	46.47	7.42
17	5.25	0.15	8.29	0.29	23.95	2.43	11.09	2.20	239.31	6.32	61.08	4.41
19	5.48	0.50	7.89	0.37	24.92	0.52	5.49	0.52	320.58	5.55	85.98	4.84

Appendix E: Experimental data from Dynamic Headspace GC-MS

Experimental data from the Dynamic Headspace GC-MS analysis of volatiles in fish oil without antioxidant and in fish oil with Lemon 1 and Lemon 2. The values are % relative to the signal with the highest response.

	(E)-2-pentenal		**(E)-2-hexenal**		**(Z)-4-heptenal**		**(E,E)-2,4-heptadienal**	
	Day 0	Day 19	Day 0	Day 19	Day 0	Day 19	Day 0	Day 19
Without aroma	100.00	47.69	21.74	11.97	9.02	6.87	36.22	46.49
Lemon 1	86.77	34.21	19.29	9.76	7.98	4.89	-	-
Lemon 2	89.40	38.15	19.00	9.80	8.36	5.43	-	-

Appendix F: Experimental data from determining CD in the vitamin Experiment

Experimental data from the determination of conjugated dienes in the vitamin experiment. The values are means of three parallels and the precision is given as standard deviation.

Fish oil without antioxidant

Day	Without vitamin	STD	Vitamin D	STD	Vitamin A	STD	Vitamin E	STD
0	0.045	0.001	0.050	0.001	0.049	0.002	0.052	0.001
5	0.045	0.005	0.050	0.001	0.050	0.000	0.046	0.007
11	0.050	0.001	0.051	0.000	0.051	0.003	0.056	0.002
15	0.049	0.001	0.041	0.008	0.049	0.004	0.046	0.003
20	0.048	0.002	0.044	0.006	0.043	0.001	0.047	0.010

Fish oil with antioxidant

Day	Without Vitamin	STD	Vitamin D	STD	Vitamin A	STD	Vitamin E	STD
0	0.049	0.001	0.045	0.007	0.050	0.000	0.050	0.000
5	0.055	0.002	0.064	0.005	0.061	0.006	0.049	0.001
11	0.052	0.002	0.041	0.003	0.033	0.004	0.039	0.002
15	0.044	0.003	0.035	0.006	0.043	0.004	0.057	0.005
20	0.058	0.001	0.056	0.003	0.054	0.004	0.057	0.001

Appendix G: Experimental data from the determination of AV in the vitamin experiment

Experimental data from the determination of AV in the vitamin experiment. The values are means of three parallels and the precision is given as standard deviation.

Fish oil without antioxidant

Day	Without Vitamin	STD	Vitamin D	STD	Vitamin A	STD	Vitamin E	STD
0	2.12	0.08	2.37	0.25	2.88	0.09	2.75	0.11
5	3.99	0.35	3.43	0.31	3.79	0.43	4.19	0.38
11	3.35	0.10	3.25	0.08	2.77	0.14	2.77	0.14
15	5.67	0.22	5.77	0.70	4.04	0.16	3.94	0.04
20	4.74	0.11	4.86	0.52	6.55	0.10	6.77	0.06

Fish oil with antioxidant

Day	Without Vitamin	STD	Vitamin D	STD	Vitamin A	STD	Vitamin E	STD
0	3.90	0.06	3.65	0.07	3.45	0.10	2.75	0.39
5	3.32	0.28	2.89	0.11	2.81	0.04	2.75	0.13
11	4.32	0.26	4.08	0.24	3.37	0.05	2.96	0.16
15	5.06	0.16	4.79	0.09	4.59	0.13	4.59	0.18
20	5.64	0.25	5.75	1.16	5.17	0.06	4.81	0.05

Appendix H: Experimental data from the OSI analysis of fish oil with minerals

Experimental data from the OSI analysis of fish oil with different minerals. The values are means of four parallels and the precision is given as standard deviation.

In fish oil without antioxidant

Additive	OSI value	Standard deviation
Without	9.61	0.53
Magnesium Oxide	9.61	0.79
Zink Oxide	8.99	0.17
Potassium Iodide	6.95	0.40
Vitamin K2	8.89	0.37
Folic Acid	9.58	1.33
Ascorbic Acid	9.35	1.45
Calcium Carbonate	7.80	0.69

In fish oil with antioxidant

Additive	OSI value	Standard deviation
Without	29.75	0.30
Potassium Iodide	36.23	3.31
Calcium Carbonate	27.47	1.58