Antioxidants stabilizing fish oils - effect of antioxidant, storage temperature and type of fish oil

Antioksidanter for stabilisering av fiskeoljer - effekt av antioksidant, lagringstemperatur og type fiskeolje

Mari Kristine Kasbo

Norwegian University of Life Sciences
Department of Chemistry, Biotechnology and Food Science (IKBM)
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Abstract

The aim of this study was to investigate four commercially available antioxidants in two fish oils. The antioxidants were investigated to see which one of them is most efficient in preventing oxidation. Antioxidants were added in three levels to find the optimal concentration. In addition a possible synergistic effect between the antioxidants was investigated.

Two different oils with different concentration of EPA and DHA were added antioxidants. Four of the antioxidants were single antioxidants. Two mixes with two antioxidants were added to investigate possible synergistic effect. The antioxidants were added in three different concentrations. The oils were stored at two different temperatures with access to oxygen. Peroxide value (PV), anisidine value (AV) and gas chromatography-mass spectroscopy (GC-MS) were methods used to evaluate extent of oxidation. Total oxidation value (TOTOX) was calculated based on PV and AV measurements. Gas chromatography was used to identify and quantify 14 selected volatile organic compounds (VOC) used to describe extent of oxidation. In addition the fatty acid profiles of the two oils were analyzed by gas chromatography.

The storage temperature and type of oil showed to have significant effect on TOTOX. These two factors also influenced the concentration of VOC in the oil samples. The three levels of antioxidants showed no significant effect on TOTOX. Only the middle level of antioxidant was used when analyzing VOC. Ascorbyl palmitate and propyl gallate distinguished from the rest of the antioxidants. Oil with ascorbyl palmitate showed great variation and high values for PV and AV. Some of the oil samples with ascorbyl palmitate gave higher areas for specific VOC compared to reference samples without antioxidant. Ascorbyl palmitate distinguished as the least efficient antioxidant with regard to specific VOCs. Oil with propyl gallate showed less variation and lower PV and AV than the other antioxidants. Samples with propyl gallate distinguished from the other oil samples, including reference samples, with regard to VOC. Mixed tocopherol, either as single antioxidant or in mix with rosemary extract, also distinguished with regard to VOC.

The reference samples were stored under the same conditions and were not added antioxidants. These samples were not significantly different from the other samples with antioxidants added with regard to PV and AV. A possible prooxidative effect was seen for some of the antioxidants. Total area of VOC, on the other hand, revealed higher areas for most of the reference samples. Evaluation of VOC revealed antioxidative effect of all the antioxidants, in more or less degree. The efficiency of the antioxidants applied in this study depended on type of oil and storage temperature. Due to ambiguous results no specific antioxidant can be claimed the most efficient in this study.

Sammendrag (Norwegian summary)

Hensikten med studien var å vurdere fire antioksidanter tilgjengelige på markedet i to fiskeoljer. Antioksidantene ble vurdert for å se hvilken av de som var mest effektiv i å forhindre oksidasjon. Antioksidantene ble tilsatt i tre ulike nivåer for å finne optimal konsentrasjon. I tillegg ble mulig synergistisk effekt mellom antioksidanter undersøkt.

To forskjellige oljer med ulikt innhold EPA og DHA ble tilsatt antioksidanter. Fire av antioksidantene ble tilsatt som single antioksidanter. To blandinger med to antioksidanter i hver ble tilsatt for å undersøke mulig synergistisk effekt. Antioksidantene ble tilsatt i tre forskjellige nivåer. Oljene ble lagret ved to forskjellige temperaturer med tilgang til oksygen. Peroksidverdi (PV), anisidinverdi (AV) og gass kromatografi-masse spektroskopi (GC-MS) var metoder brukt for vurdering av graden av oksidasjon. Total oksidasjonsverdi (TOTOX) ble beregnet basert på PV og AV målinger. Gass kromatografi ble brukt til identifisering og kvantifisering av 14 utvalgte flyktige organiske komponenter (VOC). Disse flyktige komponentene ble brukt til å beskrive graden av oksidasjon. I tillegg ble fettsyresammensetningen for de to oljene analysert ved bruk av gass kromatografi.

Lagringstemperatur og type olje viste signifikant effekt på TOTOX. Disse to faktorene påvirket også konsentrasjonen av flyktige komponenter i oljeprøvene. De tre nivåene av antioksidanter viste ikke signifikant effekt på TOTOX. Kun middelverdiene for antioksidantene ble brukt ved vurdering av flyktige komponenter. Askorbyl palmitat og propyl gallat skilte seg fra de andre antioksidantene. Oljer med askorbyl palmitat viste stor variasjon og høye verdier for PV og AV. Noen av oljeprøvene med askorbyl palmitat ga høyere arealer for visse flyktige komponenter sammenliknet med referanseprøver uten antioksidanter. Askorbyl palmitat skilte seg ut som den minst effektive antioksidanten med hensyn på visse flyktige komponenter. Olje med propyl gallat viste mindre variasjon og lavere PV og AV enn de andre antioksidantene. Prøver med propyl gallate skilte seg fra de andre prøvene, inkludert referanseprøver, med hensyn på flyktige komponenter. Miks av tokoferoler, enten alene eller i blanding med rosmarin, skilte seg også fra de andre prøvene med hensyn på VOC.

Referanseprøvene ble lagret ved de samme betingelsene og var ikke tilsatt antioksidanter. Disse prøvene var ikke signifikant forskjellig fra de andre prøvene med antioksidanter tilsatt med hensyn på PV og AV. Antioksidantene kan ha hatt en prooksidativ effekt i fiskeoljene. Totalt areal av flyktige komponenter viste derimot høyere arealer for de fleste referanseprøvene. Vurdering av flyktige komponenter viste antioksidativ effekt for alle antioksidantene i større eller mindre grad. Effekten av antioksidantene benyttet i denne studien viste seg å avhenge av type fiskeolje og lagringstemperatur. Grunnet tvetydige resultater kan ingen antioksidant påstås å være den mest effektive i denne studien.

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Abbreviations

AOCS	American Oil Chemists' Society
AV	anisidine value
DHA	docosahexaenoic acid (C22:6 n-3)
EPA	eicosapentaenoic acid (C20:5 n-3)
FA	fatty acid
FFA	free fatty acid
FID	flame ionization detector
FRS	free radical scavenger
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GC-FID	gas chromatography-flame ionization detector
GOED	Global Organization for EPA and DHA omega-3s
meq	milli equivalent
n	omega
n-3	omega-3 fatty acids
PCA	principal component analysis
ppm	parts per million (mg/kg)
PUFA	poly unsaturated fatty acid
PV	peroxide value
TOTOX-value	total oxidation value (2PV + AV)
VOC	volatile organic compounds

1. Aim of study

This study focuses on reduction of the extent of oxidation in fish oil for use in foods and supplements. We are recommended to include higher amount of polyunsaturated fatty acids (PUFA) of marine origin in our diet to reduce health risks. However, PUFAs found in fish oil are prone to oxidation and might be decomposed to carbonyl compounds, alcohols and hydrocarbons such as aldehydes, ketones and alkyls. The volatile lipid oxidation products have great impact on taste and smell at extremely low levels (Frankel 2005). Oxidized oils are known for their unpalatable taste and smell which results in shorter shelf life of products containing marine lipids (Burkow et al. 1995). This contributes to extensive economic loss for the industry (Dubois et al. 2006).

Fortification of foods with fish oils is increasing, trying to make new, healthy and convenient functional foods (Norwegian Scientific Committee for Food Safety 2011; Olsen 2005). The development of these products has been slow, due to difficulties with oxidation (Frankel 2007). Together with the increased use of PUFAs in various food products, there is an enlarged need for improved stabilization methods (Frankel 2005).

Antioxidants are added to fish oils to maintain oxidative stability of the oil during storage. There are several antioxidants available on the market. Some of the antioxidants are naturally occurring, while others are synthetically produced. There is now reduced use of synthetic antioxidants and a drive for using only natural antioxidants. In this study three natural antioxidants and one synthetic antioxidant were investigated. These four antioxidants are much used in the industry. Antioxidants may act differently in different fish oils. Two fish oils with different content of omega-3 fatty acids were applied to investigate antioxidative effect.

The aims of this study were to:

1. Examine four antioxidants on the market for stabilizing two fish oils with different omega-3 PUFA content; mixed tocopherol, rosemary extract, ascorbyl palmitate and propyl gallate.

2. Investigate which of the above mentioned antioxidant is most efficient at what dosage.
3. Examine possible synergism when combining two antioxidants to further improve the effect in fish oils.

2. Introduction

2.1 Fish oils and fatty acid nomenclature

Lipids can be divided into different lipid classes; among them triglycerides and phospholipids. Triglycerides are storage lipids, while phospholipids are structural lipids in foods and cell membrane (Frankel 2005). Refined and deodorized fish oil[1] consists mainly of triglycerides. Phospholipids are minor components in crude oil and are removed by a degumming process during refining of the crude oil (Allen 1995; Frankel 2005). Triglycerides consist of a glycerol molecule with three fatty acids attached. The three hydroxyl groups in the glycerol molecule are esterified with one fatty acid each to make up a triglyceride (Christie 2011b). The triglycerides generally contain a mixture of different fatty acids on the glycerol backbone. There are eight fatty acids usually dominating in fish oil from fatty fish (Kulås et al. 2003). Typical content of fatty acids in fish oil is C14:0, C16:0, C16:1, C18:1, C20:1, C22:1, C20:5 and C22:6 (Allen 1995).

Fatty acids are named with trivial names. Systematic names are reflecting their structure. In addition the fatty acids may be named with abbreviations with numbers. International Union of Pure and Applied Chemistry (IUPAC) have developed a nomenclature giving basis for a structured way of naming chemical compounds. The fatty acid (5Z, 8Z, 11Z, 14Z, 17Z)-eicosa-5, 8, 11, 14, 17-pentaenoic acid has the abbreviation eicosapentaenoic acid, or short EPA, trivial name timnodonic acid and also C20:5 n-3 is used.

Unsaturated fatty acids consist of varying numbers of double bonds. The double bonds are found at specific positions along the carbon chain. Unsaturated fatty acids are named after the length of the carbon chain, the number and position of double bonds, or with the position of the double bond closest to the methyl end of the carbon chain. Omega-3 fatty acids are a group of fatty acids with their last double bond positioned on the third carbon atom from the methyl end. The omega-3 fatty acid EPA can also be written as C20:5 n-3. C20 means 20 carbon atoms in the fatty acid chain and 5 is the number of double bonds. n-3 means that the last double bond is located on the third carbon atom from the methyl end,

[1] The term fish oil covers oils from both whole fish and liver oil from fish.

also called the omega end. In the same way docosahexaenoic acid (DHA) is also known as C22:6 n-3 from the same basis.

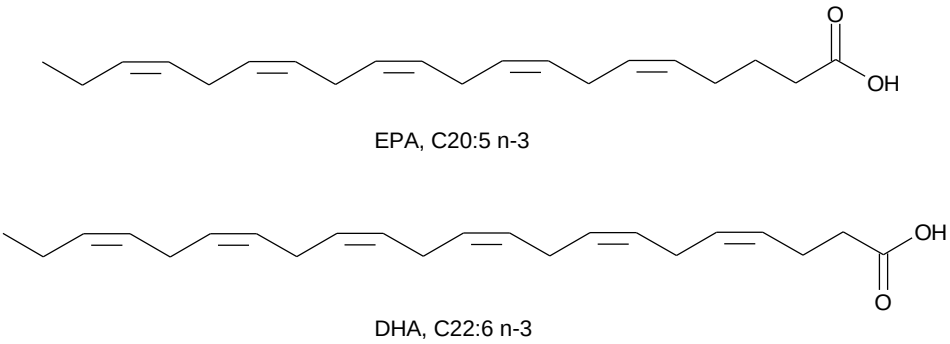

Figure 1: Molecular structure of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Kulås et al. 2003)

2.2 Omega-3 fatty acids and health

Omega-3 fatty acids are normally found in marine sources, such as fish oil. Polyunsaturated fatty acids (PUFAs) found in vegetable oils are primarily omega-6 fatty acids. The structural difference between omega-3 and omega-6 fatty acids has great impact physiologically speaking (Rice 1995). EPA and DHA are both omega-3 fatty acids and are used to characterize fish oils. These fatty acids are well documented with regard to positive impact on health (Eritsland 2000; Hu et al. 2002; Lopez-Huertas 2010; Rice 1995). The content of EPA and DHA in fish oils is dependent on the type of fish, the fish diet, seawater temperatures and geographic location of the catch (Allen 1995). The carbon chains of fatty acids in fish oils vary greatly. Fish oils often have higher degree of unsaturation than oils from plants and other animals (Allen 1995).

Dyerberg and Bang published their work with Greenland Eskimos in the mid-1970s. This study highlighted the positive effect of including omega-3 fatty acids from marine sources in the diet (Dyerberg et al. 1975). Marine oils can be obtained from the body of fatty fish, the liver of lean fish and blubber layer of marine mammals such as whale and seal. This constituted an important part of the Greenland Eskimo's diet. Their diet showed to have an impact on morbidity caused by coronary atherosclerosis. The rate of incidences of coronary atherosclerosis was much higher in Denmark where a typical Western diet was consumed. This was applicable for both Danish and Greenland Eskimos living in Denmark (Dyerberg et al. 1975). Fish used for obtaining fish oils for commercial production today is herring, cod,

salmon, sardine, anchovy, menhaden, horse mackerel, eel and capelin (Allen 1995; Kulås et al. 2003). Fish accumulate omega-3 fatty acids from microalgae or smaller prey fish that have accumulated the fatty acids from microalgae. Fatty predatory fish is not recommended for consumption due to possible accumulation of toxic substances over time.

PUFAs have been recognized for their nutritional health benefits, especially cardiovascular benefits (The World Health Organisation 2003). Linoleic acid (LA; 18:2 n-6) and α-linolenic acid (ALA; 18:3 n-3) are long chain fatty acids considered as essential fatty acids. Essential fatty acids have important biological functions and have to be included in the diet. LA and ALA are precursors for other fatty acids such as arachidonic acid (ARA; 20:4 n-6), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3). Both omega-3 and omega-6 fatty acids are essential to health (Simopoulos 1999). In theory our bodies can produce adequate amounts of ARA, EPA and DHA from the shorter fatty acids like LA and ALA. EPA is thought to be biologically more active than ALA and the conversion is favorable. Due to our modern diet this conversion is limited (Hamilton & Rice 1995; Simopoulos 1999). After the agricultural revolution 10 000 years ago there has been a shift from equal amounts of omega-6 and omega-3 fatty acids to 10-20 : 1 ratio in the diet. High amounts of LA limits the conversion of ALA to EPA because of competition among the enzymes involved in the elongation and desaturation of LA and ALA. The optimal ratio of LA to ALA 4 : 1 has been shown to be crucial for normal metabolism (Simopoulos 1999).

There has been conducted several studies documenting the health effect of EPA and DHA when included in the diet (Norwegian Scientific Committee for Food Safety 2011; Simopoulos 1999). Long chain omega-3 fatty acids may prevent or ameliorate the following diseases: Coronary heart disease and stroke, essential fatty acid defiency in infancy, autoimmune disorders, Crohn disease, breast cancer, colon cancer, prostate cancers, mild hypertension and rheumatoid arthritis (Connor 2000; Hu et al. 2002; Simopoulos 1999). Fish oils are rich in these essential fatty acids and it is recommended to include fish oil in the diet.

There is increasing interest for use of functional foods and functional ingredients[2]. These foods and ingredients are believed to have a positive impact on one or more health indicators (Thomas & Earl 1994). Essential nutrients are often used and vegetable and marine polyunsaturated oils have been used for a larger extent the last decades. Polyunsaturated oils are used both as supplement and as ingredient in different foods. Nutritionists are advising us to replace vegetable oils with marine oils to decrease the ratio omega-6/omega-3. The benefits from a low ratio omega-6/omega-3 diet were also shown in Dyerberg and Bang's study of the Greenland Eskimos earlier mentioned. The Eskimos had a much higher proportion of EPA (C20:5 n-3) and a markedly lower concentration of linoleic acid (C18:2 n-6) in their diet than the references living in Denmark (Dyerberg et al. 1975). The promotion of omega-3 fatty acids is especially due to the essential long chain omega-3 fatty acids, DHA and EPA. Marine oils including fish oils, krill oils, cod liver oils and seal oils are the product group which constitutes the largest sales volume of food supplements (Norwegian Scientific Committee for Food Safety 2011). These marine oils are documented as good sources of long-chain omega-3 fatty acids.

Oxidation of these highly unsaturated fatty acids causes oxidative deterioration and reduces food quality. Studies have shown that there may be consequences occurring also in our biological systems when consuming oxidized oil. Nutritional problems may be caused by lipid oxidation and oxidative stress resulting from reactive oxygen species. Oxidation of low-density lipoproteins (LDL) has been recognized as promoter for coronary heart diseases (CHD) (Frankel & Meyer 2000; Halver 1980; Holvoet 2004; Mertens & Holvoet 2001; Visioli & Galli 1994). A report published in June 2011 by the Norwegian Scientific Committee for Food Safety assessed the negative and positive health effects from intake of oxidized PUFAs. This assessment was requested by Norwegian Food Safety Authority (Norwegian Scientific Committee for Food Safety 2011). The report indicated that positive health effects from consuming fish oils exceed the possible negative health effects from oxidized oils. We still need to prevent oxidation of PUFAs to minimize the possible health risks. More research is needed to fully understand the effects of consuming oxidized PUFAs.

[2] The Institute of Medicine's Food and Nutrition Board has defined functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Thomas & Earl 1994).

2.3 Lipid oxidation

Oxidation of lipids produces undesirable flavors in foods. It may also reduce the nutritional quality and safety of the oils. Lipid oxidation is a complex topic and much research is still being conducted (Tall & Harris 1995). There have been identified three types of oxidation; autoxidation, photooxidation and enzymatic oxidation. Autoxidation occurs in presence of oxygen, resulting in an autocatalytic chain reaction which proceeds through free radical intermediates (Frankel 2005). The term autoxidation is used to describe the self-perpetuating generation of free radicals (Fennema 2008). Photooxidation and enzymatic oxidation is driven by exposure to light and enzymes respectively (Frankel 2005). In this study there will be focus on autoxidation reactions where the oils have access to oxygen during storage.

Much research has been conducted to understand the processes of lipid oxidation, the effects of decomposition products and the action of antioxidants. Unfortunately the complex mechanisms of oxidation are still not clear. The basis of oxidation reactions is the molecular species known as free radicals (Fennema 2008). Free radicals are atoms or molecules with an unpaired electron. Free radicals vary greatly in their energy. Hydroxyl radical (•OH) has high energy level and is able to oxidize nearly any molecule. Molecules such as antioxidants can also form free radicals, but with low energy. Radicals with low energy are less susceptible to attack molecules such as unsaturated fatty acids (Fennema 2008). This favors the use of antioxidants in lipid systems.

The pathway of an oxidation reaction generally includes three steps: Initiation, propagation and termination. LH is an unsaturated lipid in the following reactions.

2.3.1 Initiation

The initiation process describes the abstraction of a hydrogen atom from a fatty acid with one or more double bonds. This forms a fatty acid radical (alkyl radical; L•). The alkyl radical is stabilized by delocalization over the double bond resulting in double bond shifting. Oxidation of PUFAs results in formation of conjugated double bonds. Due to the shift in location there can be either *cis* or *trans* configuration of the double bonds. *Trans*

configuration is predominating due to greater stability than *cis* configuration (Fennema 2008).

α-Bonds which bind carbon atoms together are stronger than the π-bond which binds carbon atoms together in presence of a double bond. π-electrons are less firmly held and they are therefore more prone to reaction (Frankel 2005). In an aliphatic chain the bond dissociation energy for a carbon-hydrogen covalent bond is 98 kcal/mol. With a carbon atom next to an electron-rich double bond, the carbon-hydrogen covalent bond has dissociation energy of 89 kcal/mol. When there are more double bonds the dissociation energy is reduced even further. The carbon-hydrogen covalent bond is weakened by double bonds, which in turn makes hydrogen abstraction easier and oxidation rate faster (Fennema 2008). Oxidizability of fatty acids can be related to how easily the allylic hydrogens are abstracted. The oxidizability of PUFAs increases approximately two fold for each doubly allylic position available. Thus the relative oxidation rates are 1, 2, 3, 4 and 5 for 18:2, 18:3, 20:4, 20:5 and 22:6 respectively (Frankel 2007). Fatty acids with high degree of unsaturation are more subjected to oxidize due to more weakly bound carbon atoms (Frankel 2005; Tall & Harris 1995).

The initiation process is not fully understood yet. Initiators are believed to be light, oxygen, heat, peroxides or hydroperoxides and transition metals such as copper and iron (metal catalysts). Fatty acids can decompose to lipid radicals (alkyl) and a hydrogen atom:

$$LH \rightarrow L\bullet + \bullet H \quad (1)$$

(Frankel 2007; Smith 1995)

2.3.2 Propagation

The activation energy of the propagation reactions are estimated to zero. This witnesses an autocatalytic reaction with fast reaction time (Frankel 2005). The first step of propagation involves oxygen addition to the alkyl radical formed under the initiation step (Fennema 2008). The autoxidation reactions are propagated when free radicals abstract hydrogen atoms from methylene groups and yields new alkyl radicals. The odd electron in the free radical intermediate weakens the surrounding bonds, which facilitates the further reaction (Frankel 2005). Alkyls reacting with molecular oxygen result in peroxyl radicals (LOO•):

$L\bullet + O_2 \leftrightarrow LOO\bullet$ (2)

(Frankel 2005; Smith 1995)

Peroxyl radicals have high energy which allows further promotion of hydrogen abstraction from other molecules. Carbon-hydrogen covalent bonds in PUFAs are weak and an easy target for radicals to attack (Fennema 2008). Peroxyl radicals selectively abstract the most weakly bound hydrogen atom from the fatty acid (Frankel 2007). When peroxyl radicals are reacting further with a fatty acid abstracting the hydrogen, primary hydroperoxides and a new alkyl radical are formed:

$LOO\bullet + LH \rightarrow LOOH + L\bullet$ (3)

(Frankel 2005; Smith 1995)

The new alkyl radical can react further with oxygen (2) and so the reaction process continues (Frankel 2007).

Hydroperoxides can be further reduced to an alkoxyl radical (LO•) and hydroxyl radical (•OH) by scission of the hydroperoxide:

$LOOH \rightarrow LO\bullet + \bullet OH$ (4)

(Frankel 2007)

Once hydroperoxides are decomposed into alkoxyl radicals, numerous further reaction pathways are possible (Fennema 2008). The alkoxyl radical (LO•) has higher energy than either the alkyl (L•) or peroxyl (LOO•) radicals. The alkoxyl radical has enough energy to abstract an electron from the adjacent covalent bonds, which causes cleavage of the aliphatic chain of fatty acid. This reaction is known as the β-scission reaction. It results in aldehydes and a radical on the aliphatic chain (e.g. alkyl radical) (Fennema 2008; Frankel 2005). Oxidation products can be further decomposed if they have intact double bonds. The new alkyl radical (L•) can further react with hydrogen radical (H•) to form a hydrocarbon (LH), oxygen to form a peroxide (LOO•) or hydroxyl radical (•OH) to form an alcohol (LOH) (Fennema 2008). These reactions give rise for a wide variety of lipid oxidation products which contribute to the rancidity of fish oils. The oxidation products from these reactions depend on the fatty acid that is decomposed and the location of the hydroperoxide on the

fatty acid. The molecules are of low molecular weight and responsible for rancid flavors. Oxidation products from long chain omega-3 fatty acids give rise to volatiles described as "fishy" flavors (Fennema 2008).

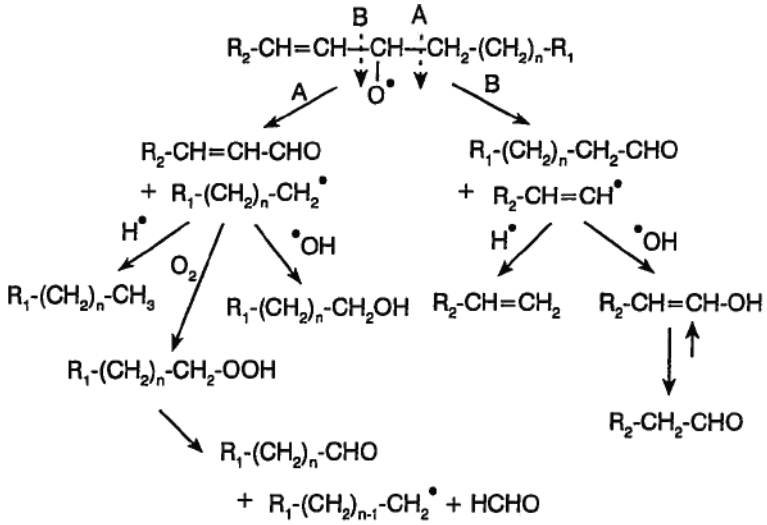

Figure 2: Decomposition of hydroperoxides by homolytic β-scission (Frankel 2005).

Figure 2 illustrates the many possible reaction pathways from hydroperoxides. Decomposition of hydroperoxides yields a number of low-molecular weight volatile compounds. The volatiles formed are determined by fatty acid, temperature, presence of antioxidants etc.

2.3.3 Termination

When radicals accumulate to a sufficient level, they will eventually interact with each other. Two free radicals can react with each other to a non-radical product. This terminates two chains and forms stable molecular products. In presence of oxygen, peroxyl radical (LOO•) will be the predominant free radical. In that case oxygen is added onto alkyl radicals (2) (Fennema 2008). In absence of antioxidants under atmospheric conditions, the peroxyl radicals self-react by the following reaction:

$$2\ LOO\bullet \rightarrow [LOOOOL] \rightarrow >C=O + O_2 + >CH\text{-}OH \quad (5)$$

(Frankel 2005)

Other molecules such as antioxidants are able to form relatively stable radicals which end the propagation. The remaining antioxidant radical will have lower dissociation energy and will not react with double bonds on PUFAs that easily.

$$LOO\bullet + AH \rightarrow LOOH + A\bullet \quad (6)$$

(Frankel 2007)

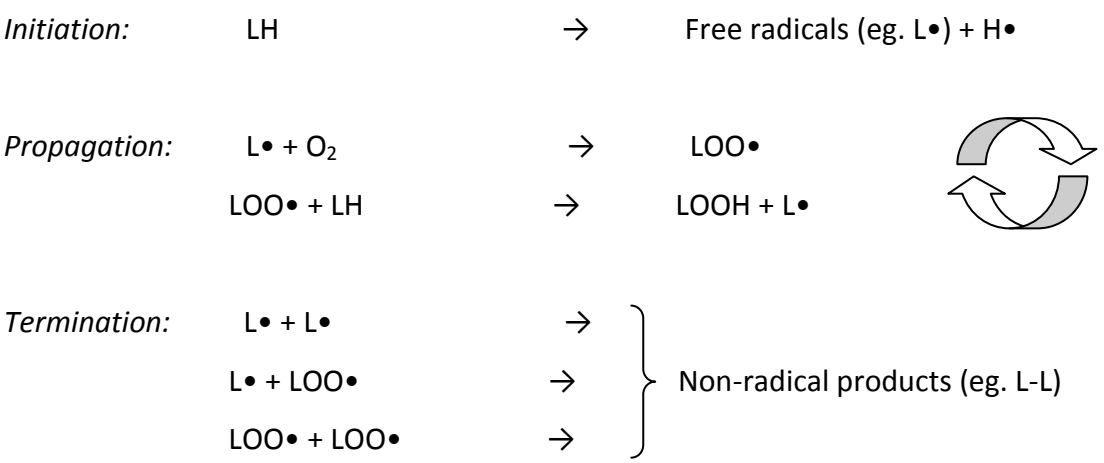

Figure 3: Sum up of three stages of lipid autoxidation. Autoxidation includes initiation, propagation and termination reactions (Frankel 2005; Shahidi & Zhong).

2.3.4 Oxidation products

Hydroperoxides are the primary oxidation product and are free for odor and flavor. Primary oxidation products can be further decomposed to secondary oxidation products. These products can be low molecular weight, volatile compounds or high molecular weight, non-volatile compounds. Non-volatile secondary products can further undergo decomposition to volatile products. The volatiles have direct impact on odor and flavor even in low doses. These volatiles are seen as the main source of rancidity in oxidized food lipids. The oxidation products formed depends on the structure of the original hydroperoxide. The secondary oxidation products can further undergo complex decomposition. Increased number of double bonds creates more sites available for free radical attack. This results in more complex mixes of hydroperoxides.

There is a wide array of possible pathways from different fatty acids and hydroperoxides. Oxidation products can also interact with other components in food systems which will indirectly affect the food quality (Frankel 2005). This makes it even more difficult to understand the complex oxidation processes and to predict the development of rancidity in food (Olsen 2005). When the triglycerides are broken down the parent triglyceride will be left partly decomposed; this is called a core aldehyde. The impact of these compounds on odor, flavor and further decomposition of these compounds is still to be explored (Frankel 2005).

Storage under different temperatures will have an impact on which volatiles are produced and the rate of decomposition. Elevated temperatures and excessive oxidation may give rise to different reaction pathways. In an ideal accelerated system, the oxidation reactions should be the same, but the rate of oxidation should be faster (Olsen 2005). The rate of oxidation can be seen as exponentially related to the temperature of storage (Frankel 2005). In reality there is also a considerably risk for changing the reaction pathway and therefore also the oxidation products (Olsen 2005). Different hydroperoxides are the basis for different volatiles, and hydroperoxides are decomposed to different extent at different temperatures. This may lead to non-consistent results when comparing results from oils stored at different temperatures. Antioxidants have different temperature stability. One antioxidant may be efficient at 20 °C and not at 40 °C, while another is most efficient in the opposite conditions (Frankel 2005). When analyzing oils to evaluate oxidation reactions, the oils should be stored under different conditions to get a more complete picture.

2.4 Prevention of oxidation

Autoxidation of lipids is initiated by abstraction of hydrogen from an unsaturated fatty acid, resulting in a free radical. Addition of oxygen to the alkyl radical forms a peroxyl radical. The following abstraction of hydrogen from another fatty acid forms a lipid hydroperoxide. These reactions do not result in a net increase of free radicals. If the autoxidation reactions were the only one occurring, the formation of lipid oxidation products would increase linearly from the start. When fatty acids are oxidized in foods there is often a lag phase followed by

an exponential increase in oxidation rate. This illustrates that there are other reactions occurring under lipid oxidation (Fennema 2008).

As long as the oxidation remains in the lag phase, the rancidity of the food is not detected. Once the exponential phase is reached, the reactions of oxidation advance and oxidation products are formed and detected. By considering all intrinsic and extrinsic factors that can influence oxidation and act as promoters, the lag phase can be extended. The longer the lag phase of oxidation, the longer the lipid containing food maintains good quality (Fennema 2008). This is important for the producers, distributers and also the consumers of the food products.

There are a number of factors regarding both handling and the environment that affects the oil quality (Vinter 2007). From the moment the fish is harvested precautions need to be made to ensure quality of the fish oil. Fish oils consist of more than just the fatty acids and the oil is also subjected to external factors that may induce oxidation. Internal and external factors include exposure to light and heat, access to oxygen, the physical state of the lipids (liquid vs. solid), pH (availability of free trace metals, surface activity), the fatty acid composition and pro- and antioxidants. Prooxidants are compounds that can either cause or accelerate lipid oxidation by interacting directly with the fatty acids or promoting formation of free radicals (Fennema 2008).

With regard to autoxidation which is induced by access to oxygen the majority of oxygen should be removed from the system. It is difficult to keep the oil completely free from oxygen. Replacing oxygen with inert gas (e.g. nitrogen) or using vacuum packaging is possible (Fennema 2008; Frankel 2005). Unfortunately this is not applicable for all food products. A large surface area between oil and air is crucial for the rate of oxidation. More fatty acid molecules are exposed to air and prooxidants when the surface area is large compared to a smaller surface (Fennema 2008; Frankel 2005).

Higher temperatures generally increases the rate of oxidation (Fennema 2008; Frankel 2005). In addition new reaction pathways are enabled (Olsen 2005). Elevated temperatures can degrade and volatilize the antioxidants present (Fennema 2008). Water activity in food

systems should be lowered to reduce oxidation rate. This reduces the mobility of reactants such as trace metals and oxygen. In bulk oils water should be avoided completely. In some foods water acts as a protective water salvation layer surrounding lipid hydroperoxides (Fennema 2008). Trace metals like iron and copper can act as prooxidants by catalyzing decomposition of hydroperoxides and promote formation of free radicals. These metals act highly accelerating on the oxidation rate and work as catalysators promoting oxidation. The mixture of trace metals and hydroperoxides are the most important initiator of oxidation caused by free radicals and rancidity of oils (Frankel 2005). It is difficult to remove all traces of metals, but their presence should be limited.

Refining of crude oil is crucial for the final oil quality. The main steps during refining include degumming, neutralization, bleaching and deodorization (Fennema 2008). Degumming removes phospholipids which would cause formation of water-in-oil emulsions. Phospholipids are not desirable in the final product. The fatty acids associated with health benefits are present as triglycerides. Neutralization removes free fatty acids which are known as promoters for oxidation of fatty acids. The bleaching step also removes free fatty acids, together with residues of phospholipids. The crude oil contains undesirable flavor compounds which are removed by deodorization. The flavor compounds are molecules such as aldehydes and ketones (Fennema 2008). These compounds are identified as reasons for the characteristic taste and smell of oxidized fish oils.

There are several methods that can be used to control and prevent rapid oxidation of oils with the above mentioned factors in mind. The most common methods are use of metal inactivators (chelators[3]), minimizing exposure to air, heat and light, minimizing the loss of naturally occurring antioxidants and adding additional antioxidants. Antioxidants are added to refined fish oils to prevent the oil from oxidizing. The oil has to be of high initial quality with regards to oxidative deterioration. Once the autoxidation process has started the process cannot be reversed by adding antioxidants (Sherwin 2007). The oil should be freshly produced and subjected to a gentle refining process. Deodorization removes secondary

[3] Chelators are able to bind to metal ions resulting in inactivation of the metal ion which could act as a prooxidant.

oxidation products. Oils already oxidized will still rapidly develop rancid taste and smell. Analyses done straight after refining may indicate good quality of the oil. Short time after analyses will reveal much higher values for oxidation. This will be noticed by consumers when applied in foods and omega-3 supplements (Kulås et al. 2003).

2.5 Antioxidants

Antioxidants are able to prevent or decrease the rate of oxidation in fish oils. The antioxidants consumed may also increase the stability of LDL in the human body and reduce risk of coronary heart disease (Frankel & Meyer 2000). Further studies will probably identify the outcome of consuming antioxidants. Antioxidants may act different in our body than in the food itself. Until now most of the studies have been conducted *in vitro* and not *in vivo*. *In vivo* studies are important to observe and investigate the actual effect of consuming antioxidants.

Antioxidants minimize oxidation of fatty acids by delaying the initiation step, slowing down the rate of propagation or terminating the chain reaction (Frankel 2005). Antioxidants are able to prolong the lag phase of autoxidation. There is no particular definition of antioxidants due to the variety of possible reaction mechanisms involved. The antioxidant naturally present in fish oil is generally α-tocopherol (Ackman & Cormier 1967). The refining and deodorization process of fish oil reduces the content of α-tocopherols. Additional antioxidants must be added to oils intended for human consumption. Addition of antioxidants to the refined oil is a crucial step for preventing the oil from oxidizing (Fennema 2008). This is an efficient and relatively simple way to prevent oxidation of PUFAs. There has been conducted several studies looking into antioxidants and their ability to prevent oxidation. The refined oil must be of good quality; otherwise the quality is already impaired before adding antioxidants. Autoxidation can be inhibited and postponed, but not reversed.

Antioxidants increase the overall activation energy which results in a lower rate of oxidation. There will be required more energy for lipid oxidation in presence of antioxidants (20-25 kcal/mole), than in their absence (18 kcal/mole). The effect of antioxidants is influenced by the surrounding conditions which is important to recognize. The effectiveness of

antioxidants increases when the temperature decreases. At higher temperatures the effectiveness may vanish and the antioxidants may start acting as prooxidants. Fish oils should be tested at temperatures at 40 °C or less (Frankel 2005). The effects of antioxidants are also dependent on the concentration. Too high concentration of antioxidants may change the action of antioxidants to work as prooxidants.

Antioxidants can impact the oxidation process on all three stages of autoxidation with different mechanisms. Antioxidants can inhibit formation of free radicals by chelating metals, scavenge free radicals, or inhibit formation of or quench singlet oxygen[4]. A chain-breaking antioxidant can react with peroxyl radicals (LOO•) and terminate the chain reaction. Any compound with a reduction potential lower than the reduction potential of the free radical is able to donate hydrogen to the free radical. This requires the reaction to be feasible. Free radical scavengers (FRS) react faster with free radicals than with unsaturated fatty acids. The transfer of the hydrogen from a FRS to the free radical is more kinetically favorable and thus a more rapid reaction. The efficiency of a FRS is dependent on the ability to donate hydrogen to a free radical (Fennema 2008).

An effective FRS forms radicals (FRS•) with low energy due to resonance delocalization. Phenolic compounds are effective in scavenging free radicals due to their ring structure. The hydrogen from the hydroxyl group is donated to a free radical. The remaining phenoxy radical[5] can delocalize the extra electron throughout the phenolic ring structure. In this way the antioxidant radical do not have any sites available for oxygen attack and is relatively stable (Fennema 2008).

[4] Singlet oxygen ($^1O_2$) is very reactive and is able to react directly with the unsaturated fatty acid forming a fatty acid peroxide (LH + $^1O_2$ → LOOH). Singlet oxygen can be transformed back to normal oxygen ($^3O_2$) by antioxidants (quenching) (Olsen 2007).

[5] A phenoxy radical is a phenol with an unpaired electron on the oxygen atom (Fennema 2008)

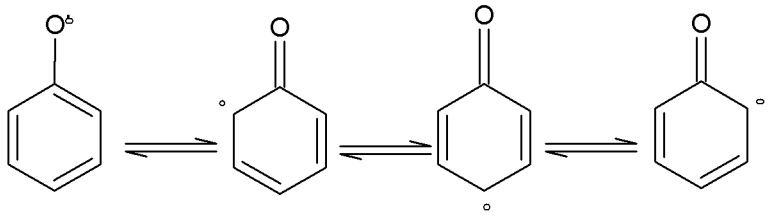

Figure 4: Phenoxy radicals possess antioxidative properties. A hydrogen atom is donated to the free radical. Delocalization of the extra electron throughout the ring structure makes the phenoxy radical relatively stable (ACD/ChemSketch).

FRS• may also be involved in termination of autoxidation reacting with other FRS• or lipid radicals. The antioxidant radical can either react with another peroxyl radical and form a stable peroxide (LOOA) or react with another antioxidant radical to form a dimer (A-A) (Frankel 2005). Free radical scavengers (FRS) or chain breaking antioxidants (A) are able to interact with peroxyl radicals (LOO•) and alkoxyl radicals (LO•) by the following reactions:

LOO• or LO• + FRS → LOOH or LOH + FRS• (7)

LOO• or LO• + FRS• → LOO-FRS or LO-FRS (8)

(Fennema 2008; Frankel & Meyer 2000)

For each FRS at least two free radicals can be inactivated. The first radical being inactivated is peroxyl or alkoxyl radicals (7). The second radical is inactivated when FRS• is involved in a termination reaction (8) (Fennema 2008).

Antioxidants should not generate new lipid radicals. Some antioxidants may nevertheless act as prooxidants by reinitiating the chain by producing lipid radicals:

A• + LH → AH + L• (9)

(Frankel 2007)

There are found antioxidants naturally occurring in nature. Fat-soluble antioxidants are found in fats and oils and water-soluble antioxidants are found in fruits, berries, herbs etc. Unfortunately there are few naturally occurring antioxidants which are allowed by the authorities (Kristinova et al. 2009). Rosemary extract and tocopherols are natural antioxidants commercially available (Frankel 2005). Synthetic antioxidants have been used due to limited access and fluctuation in supply of natural antioxidants. Synthetic antioxidants are often more efficient in lower doses, thus more cost efficient than the natural

alternatives. Propyl gallate (PG), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butyl-hydroquinone (TBHQ) and ethylenediaminetetraacetic acid (EDTA) are synthetic antioxidants commercially available (Frankel 2005). Ito et al. published a study in 1982 investigating potential side effects of some synthetic antioxidants (Ito et al. 1982). The use of synthetic antioxidants has been questioned since (Kristinova et al. 2009). Their use is declining due to possible health issues and the trend of using all-natural ingredients in foods. There are regulations for use of antioxidants as additive in foods. Synthetic antioxidants usually have more restrictions than natural alternatives (Lovdata 2011).

The decision of choosing the right antioxidant should be based on several factors. Antioxidants may act different in different matrixes. Complex foods may contain constituents that affect the action of antioxidants. The physical nature of the antioxidant may affect the effectiveness in a food system. Lipophilic antioxidants have shown to be less effective in bulk oils than hydrophilic antioxidants (Fennema 2008; Frankel 2005). This is known as the polar paradox[6] (Frankel 2005). The effect of an antioxidant in one matrix may differ from another matrix. One should always test the antioxidants in the actual food product under relevant conditions to evaluate the antioxidant ability. The antioxidants must be safe for consumption and the dose should not exceed the limitations of concentration. It is favorable if the antioxidant does not affect flavor, color or odor of the final product. They should be robust and withstand the external and internal changes that may occur during processing and storage (pH, oxygen, temperature, other constituents, water activity etc.). From a practical point of view the antioxidants should be easy to handle and the cost should be acceptable (Fennema 2008; Frankel 2005)

2.5.1 Rosemary extract

Rosemary extract is a natural plant phenolic. It is the most commercially important natural phenolic used in foods to inhibit lipid oxidation by FRS (Fennema 2008). Rosemary extract can be used in a wide variety of foods, including bulk oils and lipid emulsions due to amphiphilic[7] structure. The major FRSs in rosemary extract are carnosic acid, carnosol and

[6] Polar paradox is based on the observation that non-polar antioxidants are more effective on polar lipids in emulsion. Polar antioxidants have shown to be more effective on non-polar lipids (Frankel 2005).

[7] Amphiphilic molecules possess both lipophilic and hydrophilic properties.

rosmarinic acid, but the extract also contains several other compounds. The active ingredients in rosemary extract has the structural requirements of FRS including phenolic ring structure (Fennema 2008; Frankel 2007). The phenolic ring structure of the active ingredients allows resonance delocalization of radical which gives the FRS radical low energy and higher stability (Fennema 2008).

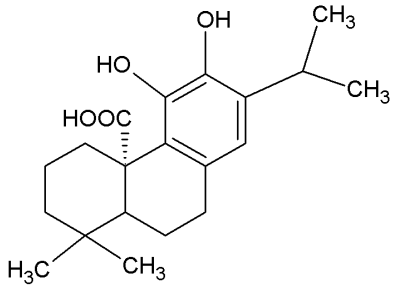

Figure 5: Molecular structure of carnosic acid. Carnosic acid is one of the active molecules in rosemary extract with regard to antioxidative effect (ACD/ChemSketch)

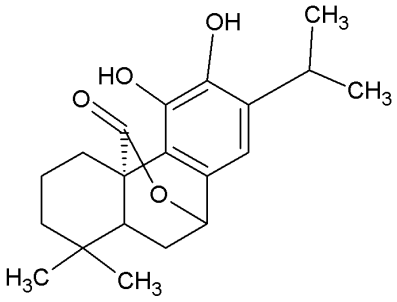

Figure 6: Molecular structure of carnosol. Carnosol is one of the active molecules in rosemary extract with regard to antioxidative effect (ACD/ChemSketch)

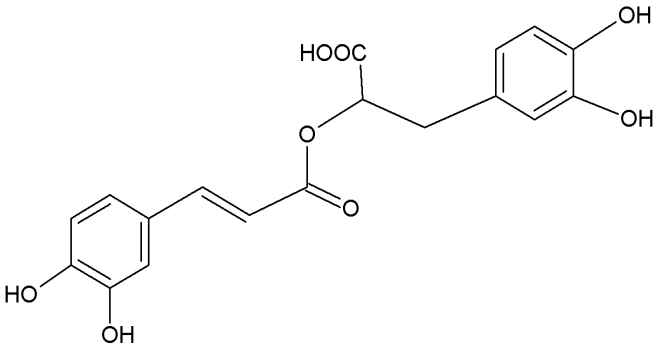

Figure 7: Molecular structure of rosmarinic acid. Rosmarinic acid is one of the active molecules in rosemary extract with regard to antioxidative effect (ACD/ChemSketch)

The antioxidant activity is influenced by degree of hydroxylation, solubility, polarity, reducing potential, stability of the phenolic to processing conditions and stability of the phenolic radical (Fennema 2008). The highly polar rosemary compounds showed in a study to be more active in bulk oil than in oil-in-water emulsion systems (Frankel et al. 1996). In the same study pH was shown to influence the antioxidant performance of carnosol and carnosic acid in oil-water emulsions. There is still investigation being conducted to understand these mechanisms and to find the most efficient lipid system for this antioxidant (Frankel et al. 1996).

The taste and smell of rosemary extract may influence the flavor of the final product. Thus the use of rosemary extract is dependent on the end product. The extraction of rosemary extract and the phenolic levels in the plants may vary due to plant variety, harvesting, storage, growth conditions, plant maturity (Fennema 2008; Howard et al. 2002). The flavor impact and the variation in phenolic level may influence the usage of rosemary extract as an antioxidant in fish oils.

2.5.2 Mixed tocopherols

Tocopherols are the most important natural antioxidants found in foods based on vegetable oil (Frankel 1996). Tocopherols are also known as vitamin E which has biological importance. Their function as antioxidant *in vitro* is well documented and the use in non-biological systems such as food is extensive (Christie 2011a). Tocopherols are lipophilic and most effective as an antioxidant in lipid emulsions. This observation is known as the "polar paradox" described by Edwin N. Frankel (Frankel 2005). This is due to the physical location in an oil-in-water systems (Fennema 2008). Tocopherol as a lipophilic antioxidant will be oriented in the oil-water interface in oil-in-water emulsions protecting the fatty acids (Frankel 1996).

Tocopherols constitute of several related benzopyranols which occur in plants and vegetable oils. The molecules consist of a 20 carbon phytyl tail including the pyranol ring. Tocopherols occur as four different homologs; α-, β-, γ- and δ-tocopherols. Fish oils and animal fat contain mainly α-tocopherols (Christie 2011a; Fennema 2008). Soy is often used as source for mixed tocopherols, but sunflower and olive oils are also rich in α-tocopherols (Christie

2011a). The four homologs of tocopherols constitute of varieties in methylation on the chromanol ring. α-Tocopherol has three methyl groups positioned at carbon 5, 7 and 8 in the chromanol ring. β-Tocopherol is dimethylated at positions 5 and 8, γ-tocopherol is dimethylated at positions 7 and 8, and δ-tocopherols are monomethylated at position 8. The number of methyl groups in the chromanol ring affects the polarity and surface activity, which may impact the antioxidant activity (Fennema 2008).

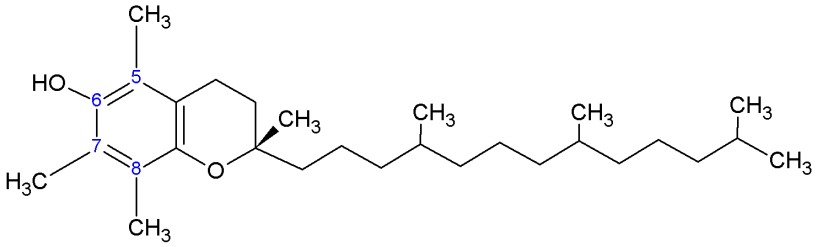

Figure 8: Molecular structure of α-tocopherol. Three methyl groups are positioned at carbon 5, 7 and 8 in the chromanol ring (ACD/ChemSketch).

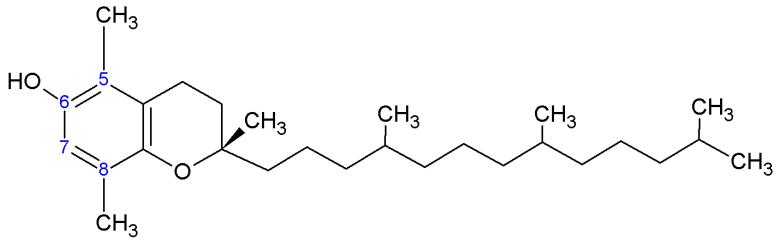

Figure 9: Molecular structure of β-tocopherol. Two methyl groups are positioned at carbon 5 and 8 in the chromanol ring (ACD/ChemSketch).

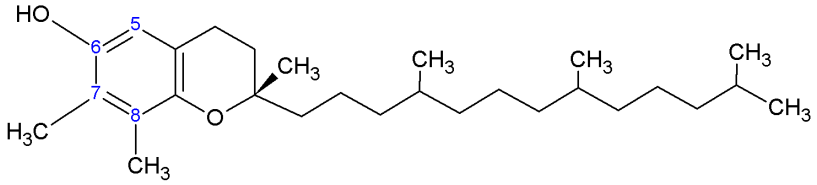

Figure 10: Molecular structure of γ-tocopherol. Two methyl groups are positioned at carbon 7 and 8 in the chromanol ring (ACD/ChemSketch).

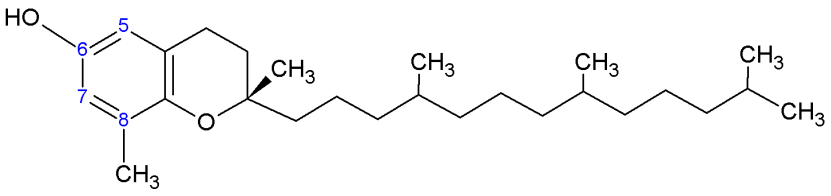

Figure 11: Molecular structure of δ-tocopherol. One methyl group is positioned at carbon 8 in the chromanol ring (ACD/ChemSketch).

α-Tocopherol is highly reactive toward singlet oxygen ($^1O_2$) and forms stable reaction products (Frankel 1996; Frankel 2005). Tocopherols are also able to inactivate metal initiators and interfere with lipid autoxidation. When interfering with autoxidation chain propagation or the decomposition processes are disturbed. They are acting as FRS donating a hydrogen atom from the hydroxyl group on the chromanol ring (Frankel 1996; Frankel 2005). It is suggested by Kulås et al. that the hydrogen-donating capacity of tocopherol will influence the further decomposition of primary hydroperoxides and already formed unsaturated aldehydes (Kulås et al. 2003). Studies have shown that α-tocopherol in model systems scavenge peroxyl radicals faster than peroxyl radicals can react with a new lipid (Christie 2011a). Tocopherols reacting with lipid peroxyl radicals form lipid hydroperoxides and several resonance structures of tocopheroxyl radicals. The unpaired electron is delocalized in the ring structure which in turn makes the tocopheroxyl radical relatively stable (Christie 2011a). The tocopheroxyl radicals can react further with other lipid radicals terminating lipid oxidation reaction (Fennema 2008). α-Tocopherol at high concentration inhibits decomposition of hydroperoxides, but also promotes formation of hydroperoxides. Hydroperoxides are further decomposed to aldehydes which are major contributors to rancidity of foods containing PUFAs (Frankel 1996).

Tocopherols can act both as antioxidant and prooxidant depending on the concentration and test system. A high concentration of tocopherols may change the action from antioxidant to prooxidant (Frankel 2005; Hamilton et al. 1998). A study conducted by Kulås et al. showed that a relatively high level of α-tocopherol (1000 ppm) in fish oil induced rapid increase in formation of hydroperoxides (Kulås et al. 2002). γ-Tocopherols may be present in higher concentration in oils than α-tocopherols without acting as a prooxidant.

α-Tocopherol is generally the antioxidant naturally present in fish oil (Ackman & Cormier 1967). The α-tocopherol level in crude fish oil varies according to the type of fish and the season for harvesting. The range is normally 25 – 60 ppm, but oil of good quality prepared from fresh fish may contain up to 350 ppm (Allen 1995).

Mixed tocopherols are often a mix of the four homologs; α-, β-, γ- and δ-tocopherols. Studies are indicating that mixed tocopherols have higher antioxidant activity than α-tocopherols alone (Liu et al. 2002). γ- and δ-tocopherols have relatively more antioxidant effect than α- and β-tocopherols (Frankel 2005). Analysis of antioxidative effect however is influenced by the test system, the methods used for analysis and the stage of oxidation when tested (Frankel 2005). The distribution of the four homologs reflects the distribution in the source. A blend of tocopherols may from this reason constitute of varying amounts of the four homologs. The distribution of the homologs may influence the action of oxidation and also distribution of the volatile secondary oxidation products formed (Kulås et al. 2002; Kulås et al. 2003).

2.5.3 Ascorbyl palmitate

Ascorbyl palmitate is an ester formed from ascorbic acid and palmitic acid. It is also known as a fat-soluble form of vitamin C. Ascorbyl palmitate can be used as an antioxidant, working as metal chelator inactivating metals such as iron and cupper in lipid systems. Metals are known as great contributors to initiation of lipid autoxidation. Ascorbyl palmitate is also believed to work as an oxygen scavenger[8] in the same way as ascorbic acid. This means that the antioxidant is able to remove oxygen in head space and in solution (Cort 1974). The mechanism of oxygen scavenging is not yet understood. Ascorbyl palmitate may also reduce hydroperoxides to stable alcohols (Frankel 2005).

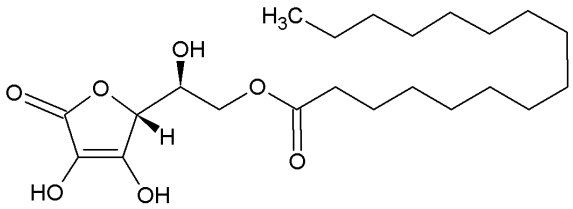

Figure 12: Molecular structure of ascorbyl palmitate (ACD/ChemSketch)

[8] Oxygen scavenging implies ascorbate releasing two electrons to oxygen resulting in formation of water molecule: Ascorbate + ½ O_2 → dehydroascorbate + H_2O

Ascorbyl palmitate is more effective as antioxidant in lipid emulsion systems (oil-in-water), while ascorbic acid is more effective in bulk oils and (Fennema 2008; Frankel 1996). This observation is known as the “polar paradox”. The difference in effectiveness in bulk oils and oil-in-water emulsions are due to their physical location in the two systems (Fennema 2008). Ascorbyl palmitate is a lipophilic antioxidant and is therefore oriented in the oil-water interface in oil-in-water emulsions. Ascorbic acid is hydrophilic and is protective in air-oil interface (Frankel 1996). Ascorbyl palmitate has greater solubility in oil than ascorbic acid. Ascorbyl palmitate still needs to be heated to over 100 °C to dissolve properly in oil. This may cause an extra step in the production process.

Ascorbyl palmitate is physiologically accepted even though it is not found in nature (Cort 1974). Ascorbyl palmitate is hydrolyzed to ascorbic acid and palmitic acid in the gastrointestinal tract, thus there are no restrictions on the level of usage in food (Fennema 2008). Ascorbyl palmitate may be used in higher concentrations than legal limits of other frequently used antioxidants. It has not been extensively applied due to lower prices and easier access of other antioxidants, such as BHT. Ascorbyl palmitate has shown better results for preventing oxidation at lower concentration than BHT (Cort 1974). Studies have shown ambiguous effects of ascorbyl palmitate as an antioxidant.

Both ascorbyl palmitate and ascorbic acid have shown to be able to regenerate tocopherols (Frankel 2005). By combining ascorbyl palmitate or ascorbic acid with tocopherols the antioxidative effect may be prolonged. This synergistic effect will be discussed in *‘2.5.6 Synergism between antioxidants’*.

2.5.4 Propyl gallate

Propyl gallate is a synthetic antioxidant commercially prepared by esterification of gallic acid with propyl alcohol. This is followed by distillation for removing the excess alcohol (Shahidi & Naczk 1995). Propyl gallate is a phenolic antioxidant. It consists of a ring structure with three OH-groups and one $COOC_3H_7$-group in the para-position.

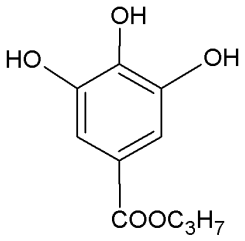

Figure 13: Molecular structure of propyl gallate. The ring structure allows resonance delocalization (ACD/ChemSketch).

The phenol ring facilitates the mechanism of free radical scavenging. A hydrogen atom is donated to a free radical. The ring structure allows resonance delocalization which makes the antioxidant radical relatively stable. Propyl gallate acts as antioxidant also by chelating metals. Metals acting as prooxidants are inhibited by propyl gallate. Synergism is obtained with butylated hydroxyanisole (BHA) and butylated hyroxytoluene (BHT) which are well known synthetic antioxidants (Shahidi & Naczk 1995). Propyl gallate is efficient at low doses and has a relatively low cost in use. Gallates have lower volatility and thus less phenolic odor than other phenols. This may be an advantage when considering sensory attributes of the final product.

Propyl gallate and other synthetic antioxidants such as BHA and BHT have been widely used in foods containing oil. These antioxidants have shown good effect on prevention of oxidation (Kristinova et al. 2009). After a study published by Ito et al. in 1982 the side effects of these antioxidants have been questioned (Ito et al. 1982; Kaitaranta 2007). Propyl gallate is still regarded as safe for human consumption by the Norwegian Food Safety Authorities. It is listed as a permitted additive for food in Norway (E310) and can be used in a range of food products (Lovdata 2011).The use of propyl gallate to stabilize foods is regulated by the FDA (UnitedStatesEnvironmentalProtectionAgency 2005).

2.5.5 Comparison of antioxidants

Antioxidants have different sources, structures and mechanisms. To choose the right antioxidant advantages and disadvantages of the antioxidants need to be considered. Table 1 shows a sum up of some of the properties of the four antioxidants described. In addition to the criteria in the table, price, availability, handling and storage requirements need to be

considered. Consumers are aware of the trend of using all-natural ingredients for food. The consumers' requirements need to be considered in a competitive market.

Table 1: Sum up of properties for the four antioxidants applied in this study.

	Rosemary extract	**Mixed tocopherol**	**Ascorbyl palmitate**	**Propyl gallate**
Natural/synthetic	Natural	Natural	Synthetic, but no restrictions	Synthetic
Mechanism	FRS	FRS, singlet oxygen quencher, metal chelator	Metal chelator, oxygen scavenger (?)	FRS, metal chelator
Structure	Amphiphilic	Lipophilic	Lipophilic	Amphiphilic
System	Bulk	Lipid emulsion	Lipid emulsion	Bulk
Solubility	Fat soluble	Fat soluble	Poorly soluble, but fat soluble when heated	Fat soluble
Advantage	Synergism with tocopherol (two different amphiphilic properties gives better effect)	Withstands high temperatures	Oxygen scavenger? Synergism with tocopherol	Very effective in low doses
Disadvantage	Impact on taste/smell. Chlorophyll present + UV-light may result in photosyntesis	Prooxidative effect when over dosed	Poorly soluble due to polar end	Restricted use

2.5.6 Synergism between antioxidants

Antioxidants can be divided into groups indicating their way of acting: Chelating antioxidants, oxygen scavengers and radical quenchers. By combining more than one antioxidant the total effect may be greater than the effect when used separately. Synergistic effect between antioxidants is shown as a greater effect of the antioxidant combination than the sum of the two individual antioxidants (Fennema 2008). Two antioxidants may act in different ways and therefore be more efficient when added together. Presence of several antioxidants will enhance the oxidative stability of the product due to their interactions (Fennema 2008). Synergistic effect can be calculated by the

following formula by the basis of induction periods (IP)[9] observed for the different antioxidant additions:

$$\% \text{ synergism} = \frac{(\text{IP AOX1 + AOX2 combined}) - (\text{IP AOX1 alone + IP AOX2 alone})}{(\text{IP AOX1 alone + IP AOX2 alone})}$$

Often the effect is shown to be equal or less than the sum of the individual antioxidants. Utilizing the synergistic effect may be an effective way of preventing lipid oxidation, but one should be careful claiming this effect (Fennema 2008).

Studies have shown that some antioxidants are able to regenerate each other (Frankel 1996; Mukai et al. 1991; Olsen et al. 2005). Regeneration of α-tocopherol is seen in biological systems where vitamin C and A extends the biological potency of tocopherol (Christie 2011a). In food systems with presence of more than one FRS, one of them might react faster with the lipid radical. This is due to different bond dissociation energies or location closer to the site where the radicals are formed. In systems where one FRS reacts faster than the other, the first FRS may be regenerated by the second FRS. The free radical from the second FRS will be transferred to the first FRS (Fennema 2008). In this way the prevention of lipid oxidation will be more efficient and also prolonged.

This regeneration mechanism is seen with α-tocopherol and ascorbic acid which illustrates synergistic effect in edible oils (Fennema 2008; Frankel 1996). Ascorbic acid and α-tocopherol are able to interfere with the chain propagation or the decomposition process. In this way the lipid autoxidation is interrupted (Frankel 2005). In the system α-tocopherol is the first FRS to react with the lipid radical due to the presence in the lipid phase (Fennema 2008). Ascorbic acid may then regenerate and recycle the tocopheroxyl radical back to α-tocopherol (Frankel 2005). α-Tocopherol can thus be held in an active state and keep scavenging free lipid radicals for a prolonged time (Fennema 2008).

[9] The induction period is the time for the oxidation being inhibited by an antioxidant (Frankel 2005).

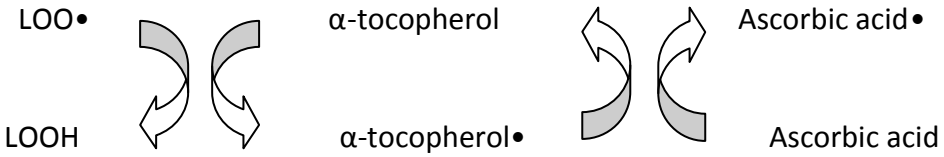

Figure 14: Ascorbic acid is able to regenerate α-tocopherol. These reactions enables synergism between the two antioxidants (Frankel 2005).

Lecithin is functioning as an emulsifier binding ascorbic acid and tocopherol when added to the mix (Frankel & Meyer 2000). This addition further improves the antioxidative effect. Blend of ascorbyl palmitate, tocopherol and lecithin is being investigated showing promising results (Kulås et al. 2003). The lipophilic ascorbyl palmitate has also shown to increase the effect of tocopherol in oils. Ascorbyl palmitate is capable of incapacitate oxygen radicals, and it can regenerate tocopherol in the same way as ascorbic acid. Ascorbyl palmitate mixed with propyl gallate and thiodipropionic acid have also shown good results in preventing oxidation (Cort 1974). Tocopherols mixed with rosemary extracts have shown to act synergistically in bulk oil. This is probably due to their mutual reinforcing activity (Frankel 2005).

Metal chelating agents and free radical scavengers together may be more efficient than adding them separately. The metal chelating agent is able to deactivate and decrease the amount of trace metals that can catalyze formation of free radicals. Oxidation catalyzed by metals will be inhibited, which in turn slows down inactivation of FRS. If the trace metals working as catalysts are deactivated, the activity of FRS might increase and last longer (Fennema 2008). The most commonly used metal chelators are citric acid and phosphoric acid. They are often used in food together with a chain breaking antioxidant such as tocopherol (Frankel 2005). Phosphatides, phosphoric acid and purified lecithin are also effective metal chelators. These compounds need to be added in low concentrations to prevent undesirable colors or off-flavors (Frankel 2005).

For natural alternatives an often used combination of antioxidants is tocopherol and ascorbic acid. These two antioxidants will react with the alkyl chain at different positions which may amplify the antioxidative effect. For a blend of natural and synthetic combination of antioxidants, tocopherol and propyl gallate is used. Synthetic antioxidants can often be

used in lower doses than natural alternatives and still be effective (Helm New York 2011). This is often an important factor for food producers when choosing antioxidants.

Antioxidants should be combined in the right relation to achieve an optimal synergistic effect. The antioxidants used in a mix should have different reaction mechanisms and/or physical properties (Fennema 2008). It is desirable to optimize the blend of antioxidants and find the optimal relation between these antioxidants. One should consider factors such as physical state of the food/matrix, type of oxidation catalysts and other factors that may influence the antioxidants itself (e.g. temperature, pH and other antioxidants or constituents in the food) (Fennema 2008).

2.6 Methods for analyzing oxidation products

Several different lipid oxidation pathways and products can result from one fatty acid. Primary oxidation products may still contain double bonds and can be further decomposed to secondary oxidation products. Food lipids contain various fatty acids and can be exposed to different prooxidants and conditions. The variety and complexity of lipid oxidation products makes evaluation and analysis of lipid oxidation demanding (Fennema 2008). One should consider the food system and other external factors such as pH and temperature when evaluating oxidation. These factors may influence the mechanism of an antioxidant and also the final results.

There are several different methods available for measuring oxidation level in oils. Even if there are a great number of methods, there are no approved, standardized methods. The significance and relevance of evaluation of antioxidants highly depend on test methods (Frankel & Meyer 2000). Burkow et al. showed that ranking of antioxidants in cod liver oil strongly depended on the method used for evaluation. Their studies showed that an increased temperature during storage made the applied method less useful. The conditions relevant for normal storage is important when evaluating antioxidative effect (Burkow et al. 1995). Inconsistent results for evaluation of antioxidants have been obtained due to varying methods and conditions used for testing the effect. Antioxidants are often multifunctional

and their actions are complex. Ambiguous interpretations of their effects should be avoided by using measurements for different types of oxidation products (Frankel & Meyer 2000).

The most common methods for analyzing oils for lipid oxidation are peroxide value (PV) and anisidine value (AV) (EFSA & BIOHAZ 2010). These two methods measure high molecular weight compounds in oxidized oil. There are some limitations with these methods. The data from the analyses will vary according to which method is used and how and where the procedure is performed (Frankel & Meyer 2000). To evaluate oxidation of fish oils best possible, several methods should be combined to illustrate the complete picture. Both primary and secondary oxidation products should be assessed due to the development of the oxidation (see figure 15).

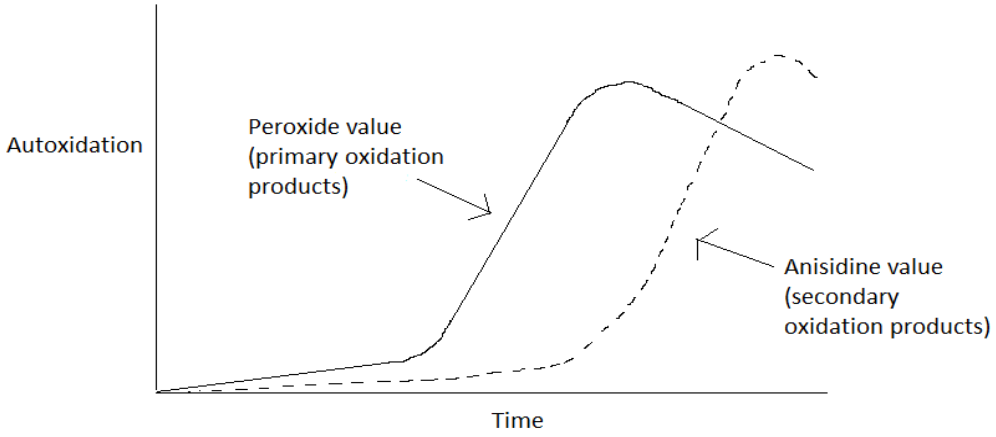

Figure 15: Development of hydroperoxides and aldehydes during autoxidation of unsaturated fatty acids. These oxidation products can be measured by peroxide value (PV) and anisidine value (AV) respectively (Frankel 2005). Time for development depends on several factors during processing and storage. The first phase is known as lag phase which lasts until exponential phase begins. Oxidation products are formed rapidly in the exponential phase. Autoxidation occur with access to oxygen.

The methods PV and AV are both described in European Pharmacopeia (EuropeanPharmacopeia 2005). Together with gas chromatography-mass spectrometry (GC-MS) these methods work as a basic platform. The methods used to evaluate lipid oxidation are chosen based on what information one would like to achieve and the specific needs of

the study (Olsen 2005). As mentioned there are a number of different methods for analyzing oxidized oils and foods. Only a few of the methods will be described here.

2.6.1 Measuring primary lipid oxidation products

Primary lipid oxidation products are compounds formed during the initiation and propagation steps of autoxidation. The concentration of primary oxidation products will be highest early in the oxidation progress as seen in figure 15. The rate of formation of primary oxidation products will eventually decrease and they may decompose to secondary oxidation products (Fennema 2008). Peroxides are primary oxidation products and their formation is the net result of the production rate and decomposition rate (Frankel 2005). The rate of decomposing primary oxidation products is high when the oil is exposed to high temperatures or high amounts of reactive transition metals. This is a disadvantage when measuring primary oxidation products for evaluation of oxidation. Primary oxidation products are non-volatile compounds and do not give rise to off flavours. This method may give inconsistent results for oxidation status (Fennema 2008). Peroxide value (PV) is one of the methods often used to measure primary oxidation products. This method is based on the reduction of hydroperoxides by iodide ions and measures mainly hydroperoxides (Frankel 2005).

2.6.2 Measuring secondary lipid oxidation products

Secondary oxidation products are formed due to further decomposition of primary oxidation products. Secondary oxidation products constitute of both volatile and non-volatile compounds. The volatile compounds are known to give rise to the characteristic off-flavours associated with oxidation of lipids. The secondary oxidation products are formed from different primary oxidation products and from different reaction pathways and conditions. EPA and DHA are found in high concentrations in fish oils. They have four and five double bonds respectively. When hydrogen is abstracted a 4- and 5-pentadienyl radical can be formed at each double bond. Oxygen may attach on either side of these pentadienyl radicals. A mixture of eight positional hydroperoxide isomers of EPA and ten positional hydroperoxide isomers for DHA can be formed. Each hydroperoxides may have either *cis, trans* or *trans, trans* configuration. A complex system of 16 and 20 monohydroperoxide isomers may be formed from autoxidation of EPA and DHA, respectively (Kulås et al. 2003).

Numerous amounts of oxidation products may be formed which makes it challenging to evaluate the complete distribution of oxidation products in fish oils.

Methods for analyzing secondary oxidation products often focus on a specific compound or class of compounds. Food matrixes are even more difficult to analyze for secondary oxidation products due to interaction with other constituents such as proteins. When measuring secondary oxidation products, the compounds responsible for the off-flavours are measured. This will correlate more with sensory analysis[10] compared to measurement of non-volatile primary oxidation products (Fennema 2008). Anisidine value (AV) is an often used method for measuring secondary oxidation products. AV is often attributed to 2-alkenals present, but no direct evidence is published (Frankel 2005). The aldehydes in the oil react with p-anisidine and a spectrophotometer is used to detect the colour intensity (A.O.C.S. 1990b). This intensity is affected by both the amount of aldehyde components and their structure.

When the consumer perception is important for a food product, sensory analysis provides important information. Secondary oxidation products give rise to unfavourable odor and flavor which is easily detected by human perception. Sensory evaluation will provide important information about the sensory characteristics of oil. This can be used to evaluate the quality of food by several parameters. Sensory analysis can help identify the consumer's threshold for compounds associated with rancid odor and taste. This is done by combining assessment results from a trained sensory panel and consumer tests (Lawless & Heymann 1999). Sensory analysis demands a trained sensory panel and is not suitable for rapid, extensive analysis. If cost and time is not an issue, sensory analysis will give valuable information about lipid oxidation in that particular product.

Volatile oxidation products only represent a small portion of all the oxidation products. Still volatile oxidation products are the greatest contributors to rancid flavor (Pokorny et al. 2001). Presence of some volatile secondary oxidation products has shown to correspond to "fishy" flavor easily detected by consumers in rancid cod liver oil (Kulås et al. 2003). There

[10] Sensory evaluation comprises a set of techniques for accurate measurement of human responses to foods and minimizes the potentially biasing effects of brand identity and other information influences on consumer perception (Lawless & Heymann 1999).

has been identified over 150 volatile compounds using headspace gas chromatography, but only 20 of them correlated with sensory panel response. The following compounds give rise to flavors associated with “fishy” flavor in cod liver oil:

- Cis-heptanal
- Trans, cis, cis-decatrienal
- Trans, trans, cis-decatrienal
- Trans-2-hexanal
- Trans, cis-decadienal
- Trans, cis-nondienal
- Cis-3-hexen-1-ol

(Frankel 2005)

Gas chromatography (GC) is a method that can be used for measuring volatile secondary oxidation products. This method is a good alternative when details concerning the oxidation products are of interest. The method has strong correlation with sensory analysis and may replace sensory assessment. GC contributes with valuable information by identifying and quantifying volatiles. Low levels of oxidation in oils and food lipids can be detected by use of this method (Frankel 2005). Identification and quantification of oxidation products are important to understand the reaction pathways in oxidation reactions.

Chromatography is methods based on separation of compounds between mobile and stationary phase. The components in the lipid are separated in a volatile equilibrium state between a mobile gas phase (carrier gas) and a stationary non-volatile liquid phase. It is distributed on a non-reactive base in a chromatographic system (Christie 1989). The different components in a sample moves with different velocity through the column, which facilitates separation of components. Varying velocity is due to the variation in affinity to the stationary phase. This affinity is affected by chain length, degree of unsaturation and varying polarity. Different components in the sample will diffuse into the stationary phase in different degree. The velocity through the column is determined by the distribution of components in the mobile and stationary phase at equilibrium. The stationary phase is not able to move and the velocity of the compounds is therefore determined by the mobile

phase. If the mobile fraction is large, the compound will move fast through the column and contrary if the stationary fraction is large. The compounds will move along the column with varying velocities, which causes dispersion of the compounds. This is the fundament for chromatographic separation of compounds (Christie 1989).

GC provides a lot of detailed information, but requires much chemical and analytical knowledge. Expensive instruments are required to do the analysis. Much time and effort is necessary to analyze the large amount of information resulting from this method (Fennema 2008). These factors restrict the extent of use of this method.

There have been used a number of methods for measuring oxidation and antioxidant activity. This makes it challenging to compare results between studies. Different methods and varying conditions lead to inconsistent results. There is need for well-designed studies *in vitro* and reliable biomarkers for demonstrating the effects *in vivo*. This will allow results from different studies to be compared and may give more consistent and reliable results.

2.7 Quality criteria

There are no set quality criteria for oxidation level of PUFAs in foods. There are great differences between fish oils due to fatty acid composition, seasonal variations etc., which makes it demanding to set quality standards (NUTRAingredients-USA.com 2011). However there are two voluntary guidelines recommended to follow for refined oil. Global Organisation for EPA and DHA Omega-3s (GOED)[11] maintains and updates the GOED Voluntary Monograph quality standards for products containing EPA and DHA. GOED spun off from the Omega-3 Working Group at the Council for Responsible Nutrition (CRN) which developed this voluntary monograph. GOED has together with their members set quality standards for omega-3 products which are the strictest quality and purity standards today. Maximum values for PV and AV are 5 meq/kg; AOCS Official Method Cd 8-53 and 20; AOCS Official Method Cd 18-90 respectively. Total oxidation value (TOTOX)[12] has a maximum

[11] GOED is an international association of producers, retailers, distributers, promoters and other contributors to products with EPA and DHA (GlobalOrganisationForEPAandDHAomega-3s 2010).

[12] TOTOX is total oxidation level based on PV and AV measurements (AV + 2PV) (GlobalOrganisationForEPAandDHAomega-3s 2006)

accepted value of 26 as a result of calculation 2PV + AV (GlobalOrganisationForEPAandDHAomega-3s 2006). The TOTOX maximum value indicates that AV and PV should not both be of maximum value. All oils containing EPA and DHA in triglycerides or ethyl esters from fish, plant or microbial sources are covered in GOED's monograph, except from cod liver oil (GlobalOrganisationForEPAandDHAomega-3s 2006).

The European Pharmacopoeia has a monograph specific to cod liver oil (EuropeanPharmacopeia 2003; GlobalOrganisationForEPAandDHAomega-3s 2006). The European Pharmacopoeia has published official standards to provide a legal and scientific basis for quality control. These include both qualitative and quantitative measurements for quality control of medicines, raw material used for production of medicines and on intermediates of synthesis. European Pharmacopoeia has set quality criteria for cod liver oil in Supplement 4.8. This includes criteria for AV and PV, but also composition of fatty acids in cod liver oil (EuropeanDirectorateforthe QualityofMedicines&HealthCare 2011)

3. Materials and methods

3.1 Oils, antioxidants and storage conditions

The oils were provided by Maritex AS, Norway (cod liver oil; CLO) and GC Rieber Oils AS, Norway (concentrated triglycerides; TG). CLO had minimum 22 % total omega-3 fatty acids. TG had minimum 65 % total omega-3 fatty acids. The oils were sampled from commercially processes after refining. No antioxidants were added prior to sample preparation at Nofima AS.

Fenchem Biotek Ltd., China supplied the following commercially available antioxidants:

- EsseRose 5 % CA oil
- Mixed Tocopherols 70 %
- Ascorbyl Palmitate
- Propyl Gallate

The rosemary extract consisted of 5 % carnosic acid which is one of the active ingredients in rosemary extract (Frankel et al. 1996; Frankel 2007). The mixed tocopherol had a concentration of tocopherols of 70 %. The propyl gallate and ascorbyl palmitate were both powders.

Four antioxidants were added to two different fish oils; cod liver oil (CLO) and concentrated triglycerides (TG). In addition two mixes of antioxidants were added to the oils to investigate a possible synergistic effect between the antioxidants. The antioxidants were added in three different levels in both oils (range 50 - 1200 ppm). The levels of antioxidants were based on earlier studies (Cort 1974; Frankel et al. 1996; Hamilton et al. 1998; Jacobsen et al. 1998; Kaitaranta 2007; Kulås et al. 2002; Olsen et al. 2005; Sherwin 2007). The antioxidants are listed in table 2 showing abbreviations used further in this study.

Table 2: Six antioxidants (both singles and mixes) were added to CLO and TG and stored at 40 °C and 20 °C.

	Abbreviations	Cod liver oil (CLO)	Concentrated triglycerides (TG)
Ascorbyl palmitate	AP	40 °C and 20 °C	40 °C and 20 °C
Mixed tocopherol	toco.	40 °C and 20 °C	40 °C and 20 °C
Propyl gallate	PG	40 °C and 20 °C	40 °C and 20 °C
Rosemary extract	rose.	40 °C and 20 °C	40 °C and 20 °C
Propyl gallate/ascorbyl palmitate	PG/AP	40 °C and 20 °C	40 °C and 20 °C
Mixed tocopherol/rosemary extract	toco./rose.	40 °C and 20 °C	40 °C and 20 °C

After preparing the oils by adding antioxidants, the bottles were flushed with nitrogen gas. The nitrogen gas displaced the oxygen from the bottles, which created a protecting atmosphere in the headspace of the bottles. When all the oils were distributed in the respective bottles, the lids were loosened to allow oxygen to access the headspace in the bottles stored at 20 °C. The headspace volume in the bottles was about 15.07 cm^3 and the surface area 12.56 cm^2. The samples stored at – 40 °C was placed in a dark room with temperature control and limited access to oxygen until exposure to accelerated conditions. The oil samples were stored at two different temperatures; 20 °C for 17 weeks and 40 °C for 14 days both with access to oxygen. By exposing the samples to different temperatures the effect of the antioxidants stored under different storage conditions could be compared. Samples of cod liver oil and concentrated triglycerides without added antioxidants were stored under the same conditions as the samples added antioxidants. These samples were used as references and enabled evaluation of the effect of antioxidants in the fish oils.

Chemicals and instruments used for conducting the analyses are listed in Appendix 1.

3.2 Samples analyzed

76 oil samples were measured by use of peroxide value (PV) and anisidine value (AV). Due to time constraints, only the samples at middle level of antioxidants were analyzed for VOC by using headspace GC-MS. This included 28 oil samples. Level of antioxidant showed not to have significant effect on TOTOX ($P > 0.05$) and the middle level of antioxidant was assumed to be fairly representative. Two parallels for each sample were analyzed by PV, AV and GC-MS. Fatty acids of selected samples from both cod liver oil (CLO) and concentrated

triglycerides (TG) were analyzed as FAME with GC-FID (n = 14). The selection of samples represented both storage temperatures, both oils, all three levels and four antioxidants together with reference samples.

3.3 Oxidation analysis

After 17 weeks the oil samples stored at 20 °C was analyzed. In this paper two of the most commonly used methods, PV and AV, were used. The methods PV and AV are used as quality specifications in the European Pharmacopoeia 5.0. To be able to compare the results with other studies, these two well know methods are applied in this study. GC is a descriptive method and yield more detailed information about the oxidation process and products. The bottles were flushed with nitrogen and stored at 0 °C with the lid tightly on between the analyses. Parallel measurements were conducted for all the samples. The mean of the parallels were used for comparison of antioxidative effects.

3.3.1 Peroxide value (PV)

The level of primary oxidation products, hydroperoxides, can be measured using peroxide value (PV) as a method. This is the oldest and most commonly used method for evaluating oxidative status in oils (Frankel 2005). GOED has defined a limit of PV 5 for accepted quality of oil, while European Pharmacopoeia has set the limit to PV 10 (EuropeanPharmacopeia 2003; GlobalOrganisationForEPAandDHAomega-3s 2006).

When analysing fats and oils PV is often measured using an iodometric method. This method is based on the reduction of hydroperoxides by iodide ions (I^-). There will be released iodide equivalent to the concentration of peroxides in the oil. The amount of iodide which is released can be measured by titration to a standard solution of sodium thiosulfate, by using a starch solution as indicator (Frankel 2005). Iodide binds to starch and gives a dark blue colour. When all the iodide has been consumed by sodium thiosulfate, the blue colour disappears and the end point of the titration is reached.

Fatty acid peroxides is oxidized to fatty acid alcohols with iodide. Iodide is reduced to a molecular iodide, I_2:

$LOOH + 2KI + H_2O \rightarrow LOH + I_2 + 2KOH$

The formed iodide (I_2) is then titrated with a standard sodium thiosulfate solution to determine how much of the oxidation reagent (I^-) is consumed:

$I_2 + 2Na_2S_2O_3 \rightarrow 2I^- + Na_2S_4O_6 + 2Na^+$

(Olsen 2007)

Measuring of peroxide value using titration

Five grams of the oil was weighed into a 250 ml Erlenmeyer bulb. 30 ml of acetic acid (*Merck*) and chloroform (*Merck*) solution (3:2) was added to the bulb. The bulb was placed on a magnet mixer to mix the content. Adding 0.500 ml saturated potassium iodide (*Merck*) resulted in a colour change due to oxidation by hydroperoxides. After exactly one minute of stirring, 30 ml of distilled water was added to end the reaction. Sodium thiosulfate (*Merck*) with concentration of 0.01 N was added by titration to the solution until a light yellow colour was observed. 0.500 ml starch indicator (*Merck*) was added which resulted in a colour change to dark blue. The solution was titrated further with sodium thiosulfate until the blue colour disappeared and the solution had a clear appearance (A.O.C.S. 1990b).

PV was calculated using the following formula:

$$PV = \frac{(S - B) \times N \times 1000}{m}$$

S is ml sodium thiosulphate titrated in the sample, B is the ml sodium thiosulphate titrated in a blank sample, N is the concentration of the sodium thiosulphate solution and m is the weight of the sample. PV is expressed as milli equivalent iodide per kg of oil (meq/kg).

With differences of more than 10 % between the parallels, the samples were analyzed a second time.

The method was conducted according to AOCS Official Method Cd 8-53.

3.3.2 Anisidine value (AV)

Measure of anisidine value (AV) is a common method for determining the level of secondary oxidation products in pure fats and oils. The method is an unspecific estimate of the amount of secondary oxidation products, or non-volatile carbonyl compounds (Frankel 2005). These compounds mainly include aldehydes that are decomposed from hydroperoxides. The two aldehydes 2-alkenals and 2.4-dienals are believed to have the major impact on AV (A.O.C.S. 1990a). Later studies have shown that the method might not be that specific regarding which secondary oxidation products are measured (Frankel, 2005; Olsen, 2005). It has not been scientifically proven which compounds the AV-method measures (Frankel 2005). Fish oils containing long-chain omega-3 PUFAs are particularly susceptible for oxidation during refining of the oil. These oils often have high initial AV (Frankel 2005). AV can be used as a rough predictor of future storage stability of freshly processed oil (Frankel 2005). GOED has defined a limit of AV 20 for accepted quality of oil, while European Pharmacopeia has set the limit to AV 30 (EuropeanPharmacopeia 2003; GlobalOrganisationForEPAandDHAomega-3s 2006).

A sample of oil is dissolved in isooctane before p-anisidine in an acetic acid solution is added. The aldehydes in the oil react with p-anisidine in glacial acetic acid. After 10 minutes the absorbance of the reaction products can be measured at 350 nm in a spectrophotometer (A.O.C.S. 1990a). The colour intensity is affected by both the amount of aldehyde components and their structure. P-anisidine measures both saturated and unsaturated aldehydes (Dubois et al. 2006).

Purification of p-anisidine

P-anisdine tends to darken during storage because of oxidation. The crystals should be stored in a dark bottle not exposed to strong light at 0 – 4 °C. The crystals should be used before any colour change occurs. The reagent was already discoloured and the p-anisidine needed to be reduced and decolourized.

4.0 g of p-anisidine (*Merck*) was dissolved in 100 ml of distilled water at 75 °C. 0.2 g of sodium sulphite (*Merck*) and 2.0 g of active carbon (*Merck*) was added to the solution and

stirred for five minutes. The solution was filtered through two filter papers (150 mm) and then cooled at 0 °C for three hours. During the cool storage crystals were formed. The crystallized p-anisidine was filtered off and washed with a small amount of water at 0 °C. The crystals were dried in a vacuum desiccator over night with no exposure to light. The dry material was then dissolved in acetic acid (*Merck*) in the relation 0.25 g p-anisidine per 100 ml glacial acetic acid.

P-anisidine was purified according to AOCS Official Method Cd 18-90 (A.O.C.S. 1990a).

Measuring of anisidine value by spectrophotometer

One gram of the oil sample was weighed into 25 ml volumetric flask. Isooctane (*Merck*) was added up to 25 ml and the content was mixed. The absorbance of the solution was registered in 2.5 ml solvent resistant cuvettes at 350 nm wavelength in a spectrophotometer in 1 cm cells. A reference cell with isooctane (solvent) was used as a blank.

5 ml of the fat solution was transferred to a test tube with a pipette. 1 ml p-anisidine dissolved in acetic acid (*Merck*) was added to the test tube and the content was mixed by shaking. In presence of acetic acid p-anisidine reacts with aldehyde components which can be observed as a yellow colour. After 10 minutes the absorbance was registered. A reference cell with isooctane (*Merck*) with added p-anisidine was used as a blank.

AV was calculated using the following formula:

$$AV = \frac{25 \times (1.2As - Ab)}{m}$$

Ab is the absorbance for sample dissolved in isooctane, As is the absorbance for fat solution (sample and isooctane) after reaction with p-anisidine, and m is the mass of oil in 25 ml isooctane. The factor 1.2 is due to the solution containing the oil together with p-anisidine is diluted as a result of the anisidine reagent, while the solution with oil in isooctane, Ab, is not diluted (A.O.C.S. 1990a). AV is defined as 100 times the absorbance in a solution where one gram of oil is dissolved in a 100 ml solution of solvent and p-anisidine, registered at a wavelength of 350 nm in a 1 cm cell (A.O.C.S. 1990a). The AV has no term.

With differences of more than 10 % between the parallels, the samples were analyzed a second time.

The method was conducted according to A.O.C.S. Official Method Cd 18-90.

3.3.3 Total oxidation value (TOTOX)

PV and AV will change over time as hydroperoxides are produced and decomposed. Total oxidation value (TOTOX) gives a more complete picture of the oxidative status of an oil. This value combines the history of the oil (AV) with the present status (PV). TOTOX considers both primary and secondary oxidation products and is calculated based on PV and AV. One PV-equivalent is believed to give rise to two AV-equivalents.

TOTOX is calculated by the following formula: 2PV + AV

The GOED monograph states TOTOX-value of 26 as the limit for oxidative status. (GlobalOrganisationForEPAandDHAomega-3s 2006).

3.3.4 Gas chromatography (GC)

The basis for gas chromatography (GC) is described in "2*.6.2 Measuring secondary lipid oxidation products"*. GC results in qualitative and quantitative information about compounds in the oil. GC combines a separation and identification technique. Compounds in the sample material are collected and further separated. Different detectors result in different chromatograms with different information. In this study both mass spectroscopy (MS) and a flame ionization detector (FID) were applied. Figure 16 illustrates a schematic presentation of GC.

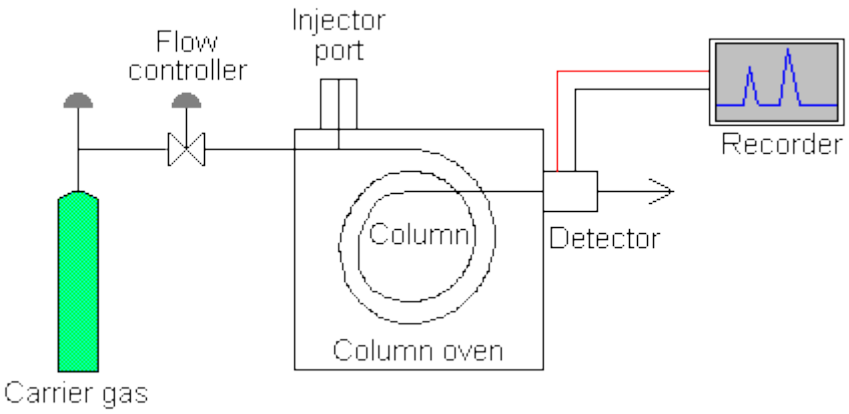

Figure 16: Schematic presentation of gas chromatography (GC) (SheffieldHallamUniversity)

The sample solution is vaporized when it is injected to the gas chromatograph. The mobile phase (carrier gas) carries the vapor onto the column where it is divided into a stationary phase and mobile phase. The carrier gas is inert and is not reacting with the sample. Commonly used gases for this purpose is nitrogen, helium and hydrogen. The mobile phase carries the sample compounds through the column. The solvent in which the sample is dissolved is the first one out of the column due to little interaction with the stationary phase. The different compounds react differently with the stationary phase. Some compounds are retained in the column for longer time than others. In this way the different components are separated through the column. At the end of the column a detector produces an electrical response when an organic substance passes through. When only the carrier gas passes by a straight line will appear on the chromatogram. A chromatogram is based on the records of the detector response and the retention time.

Many types of detectors may be applied when performing GC-analysis, resulting in different types of information. A mass spectrometry (MS) detector is often used when the compounds in the sample are not known. This detector is often applied when performing a headspace GC analysis investigating volatile organic compounds. GC-MS will provide information about the fingerprint (mass spectra) for each volatile in addition to retention time in the column. The mass spectra can be compared to spectra in databases and/or spectra of pure standard compounds and identified (Kulås et al. 2002). It also provides quantitative information about the same compounds (Watson 1997). Retention time is compared to retention time of known compounds to identify the compound in question (Christie 1989). With regard to

headspace GC-MS the sample amount, nitrogen flow, GC-column, absorber and temperature and time program are selected based on the type of compounds of interest.

GC can also be used to identify and quantify fatty acids in oils. GC allows the fatty acids to be studied in detail and facilitates comparison of different oils based on fatty acid content. When investigating the fatty acid composition the most common detector is flame ionization detector (FID). GC-FID provides information about retention time of compounds, which can be compared to retention time of known compounds and identified (Christie 1989). Temperature and time program in GC is selected based on type of compounds of interest.

Identifying volatile secondary oxidation products using headspace GC-MS

The volatile secondary oxidation products were investigated using dynamic headspace GC-MS. 2 grams of oil was weighed into Erlenmeyer bottles before they were placed in water bath at 70 °C and purged for 15 minutes with 100 ml/min Nitrogen. The volatiles were trapped on an adsorber (*Tenax GR*) which had been conditioned at high temperature before use. The volatiles were desorbed at 280 °C for five minutes in *Markes Ultra Thermal Desorber* and transferred to an *Agilent HP 6890 A* with an *Agilent 5973 Mass Selective Detector (EI, 70 eV)*. The volatiles were separated on a *DB-WAXetr column (30 m, 0.25 mm i.d., 0.5 µm film)*. The temperature program started with 30 °C for 10 minutes. The temperature then increased 1 °C per minute until the temperature reached 40 °C, 3 °C per minute to 70 °C, and 6.5 °C per minute to 230 °C. The temperature was held at 230 °C for five minutes. The peaks were integrated and the compounds were tentatively identified with *Agilent MSD ChemStation* and *NIST 20d. Des. 2 2005 Mass Spectral Library*. The system performance was checked with blanks before and after analysis (Olsen et al. 2005), (Kulås et al. 2002). The samples used for this method of analysis were parallels at middle level of antioxidant from oil samples stored at both temperatures.

Identifying fatty acids using GC-FID

To perform a gas chromatographic analysis of fatty acid profile, the fatty acids in the oil needs to be derivatized. This increases the volatility and enables separation of compounds. The derivates needs to be non-polar with low molecular weight when analyzed. The fatty acids are usually esterified with methanol to form fatty acid methyl esters (FAME). FAME are

non-reactive derivates of the fatty acids, which are more volatile (Christie 1989). Fatty acids are derivatized with the following procedure:

50 µl of oil was dissolved in 1 ml benzene (*Merck*). 3 ml of metanolic HCl (*Merck*) was added as a methylation reagent. 200 µl of 2.2-dimethoxypropan (*Sigma-Aldrich*) were added to the solution to shift the equilibrium towards complete. The samples were mixed and held on waterbath at 80 °C for 20 minutes for complete derivatization. After cooling 1 ml isooctane *(Merck)* and 1 ml sodium chloride (*Merck)* were added to the solution. This enhanced the phase separation. The samples were centrifuged for 5 minutes at about 2500 rpm at room temperature. The organic phase was transferred to a new tube and 1 ml 2 % sodium bicarbonate (*Merck*) was added to the solution to neutralize excess HCl. Centrifugation induced a new phase separation and the organic phase was transferred to GC-vials. Water free sodium sulfate (*Merck*) was added to the vials to attract potential residues of water. The GC-vials were properly sealed. This method is a standard method at *Nofima AS* to derivatize fatty acids.

The prepared samples were injected split 50 : 1 at 240 °C with *Agilent HP 6890 Injector* on an *Agilent HP 6890 A*. Helium was used as carrier gas. The samples were separated on *SGE BPX-70 capillary column (60 m, 0.25 mm i.d, 0.25 µm film)*. The temperature program started at 70 °C for one minute. The temperature increased with 30 °C for each minute until 170 °C was reached, then 1.5 °C per minute until 200 °C, then 3 °C per minute until 220 °C was reached. The temperature was held at 220 °C for five minutes. The fatty acids were detected by a flame ionization detector (FID) (*Agilent G1530A)*. Chromatographic peaks were integrated by *Agilent GC ChemStation* software. The peaks were identified by comparing them with retention time of known fatty acids from the extern standard solution *68D* (*Nu-Check Prep Inc*). Blank samples (isooctane) and standard solutions were run to calibrate and control the system. The results were used for comparing the fatty acid composition of the two oils used in this study.

3.4 Statistic analysis

3.4.1 ANOVA

ANOVA was used for statistic analyses based on results from PV, AV and TOTOX. The results from this study is presented by descriptive analysis. General linear model, main effects plot and interactions plot were used to investigate the results. The results are presented with median, minimum and maximum values to illustrate dispersion. Oil, temperature, antioxidant and level of antioxidant were the four factors first observed. Level of antioxidant showed not to have significant effect ($P > 0.05$) on TOTOX-value. Due to this finding the three levels of inclusion were merged when further discussing the results. A three-factor model was used investigate main effects and interactions between oil, temperature and antioxidant. There were not enough observations to use a fourth order interaction. A third order interaction was used. Tukey Method was used for comparing the six antioxidants. This allowed looking into grouping information of the antioxidants. Statistic significance is defined as $P \leq 0.05$. The analyses of PV, AV and TOTOX were conducted by use of *Minitab version 16*.

3.4.2 Principal component analysis

For the volatiles obtained from GC-MS analyses *The Unscrambler version 9.8* (*Camo Software*) was used for processing the results. This allowed us to investigate the variations in areas of the volatiles examined. GC-MS creates complex data sets and principal component analysis (PCA) was used to evaluate variations in the data sets (Kulås et al. 2003). Multivariate analysis was used to find trends and relationships between the variables (multiple responses). Principal component analysis (PCA) is a descriptive method which yields information about the correlations in the data set. “Score plots” revealed variation between the samples in each plot. “Loading plots” illustrated which VOC were the major contributors to variation in the score plots. This enabled the major contributors to rancidity to be identified. The volatiles were evaluated according to area of each volatile and the relative relation of volatiles between the samples. Area of VOC in a chromatogram represents the concentration of secondary oxidation products in an oil sample. In plots based on areas of volatile organic compounds (VOC), compounds with high areas dominated the sample variation in the plot. By using standardized areas (1/sd), also possible systematic variance contained in the low area VOCs was revealed. Thereby VOCs with low areas could

also be identified as contributors to rancidity. 14 volatile organic compounds (VOC) were chosen to be investigated in detail by PCA. Specific VOCs can be used as markers for concentration of secondary oxidation products.

4. Results

4.1 Sample selection

PV and AV results from ten out of 38 CLO samples stored at 20 °C showed abnormally high levels of oxidation (see figure 17). The TOTOX-values based on PV and AV showed values ranging from 43.1 to 90.6. It was only one of the two parallels for the oil samples indicating such high levels of oxidation.

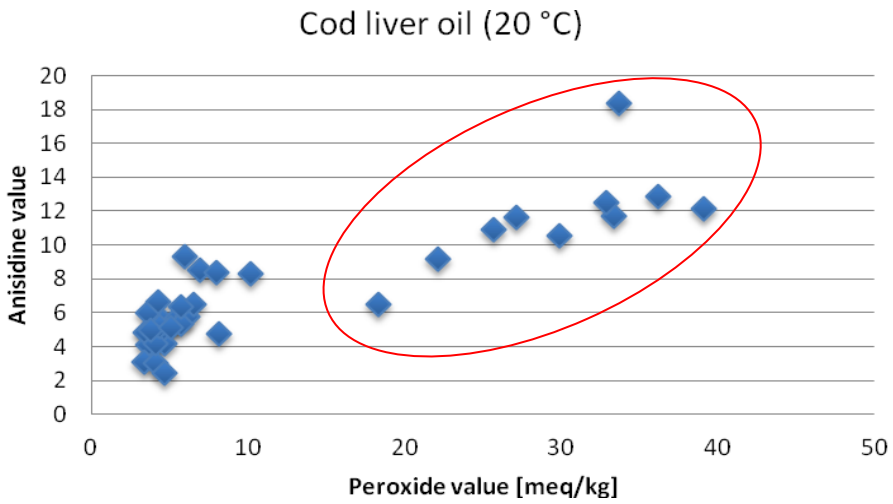

Figure 17: Scatter plot showing cod liver oil (CLO) samples stored at 20 °C. Ten out of 38 parallels measured showed abnormally high levels for both peroxide value (PV) and anisidine value (AV). These ten samples were excluded when further discussing the results (n = 38 including two parallels).

The samples with abnormally high PV and AV were analyzed further to identify possible sources for deviation. The type of oils was confirmed by looking at fatty acid (FA) composition with GC-FID. Thin layer chromatography revealed no free fatty acids (FFA). FFA could have been a sign of hydrolysis due to presence of water. Further GC-MS was applied to identify volatile compounds present in the samples. The results from GC-MS indicated two different reaction pathways in the ten samples showing high PV and AV. This might have been due to different antioxidants present. The abnormally high levels of oxidation could not be responded to after this trouble shooting. The unexpected results did not repeat for the CLO samples stored at 40 °C. Only the parallel indicating “normal” oxidation level was used for further discussion of the results. The remaining parallels were counted twice to be able

to calculate mean values for all the samples (n = 76). Possible sources for deviation are listed in Appendix 2.

Eleven other samples had parallels showing very divergent results for both PV and AV. Due to this observation these samples were analyzed a second time. The very divergent results were consistent for the second analysis, with difference up to 40 % between parallels. It was decided to take this into consideration when further discussing the results. The reason for the divergent parallels is not known. To be able to get a more correct picture of the antioxidative effect, the outliers were excluded in addition to the ten samples mentioned above. Nine CLO parallels and two TG parallels were excluded. Only the parallel indicating "normal" oxidation level was used for further discussion of the results. The remaining parallels were counted twice to be able to calculate mean values for all the samples. 131 parallels remained for further evaluation. The mean values for the parallels were used when evaluating and presenting the results. This gave 76 mean values. Incorrect decisions may have led to false interpretation.

4.2 Peroxide value, anisidine value and total oxidation value

A sum up of PV, AV and TOTOX for each antioxidant in different oils and storage temperatures are presented in Appendix 3; table A2.

ANOVA was used to examine results based on PV, AV and TOTOX. Main effects and potential interactions were investigated. A four factor model was applied with the following factors: Oil, storage temperature, antioxidant and level of antioxidant. Tukey Method with grouping was used to compare the six antioxidants. Tukey method could reveal possible significant differences between the antioxidants. The results are illustrated in figures below (figure 18-21).

Comparison to quality criteria

Only the oil stored at 20 °C can be compared to quality criteria in the monographs described. Storage temperature of 40 °C with access to oxygen was used as accelerated storage condition and will not be representative for normal storage. Storage temperature of 20 °C is

more relevant to normal storage conditions. From the oils stored at 20 °C for 17 weeks with, 29 out of 38 samples were within the limits from GOED (PV 5, AV 20). All the TG samples stored at 20 °C were within the limits from GOED. The limits from both GOED and European Pharmacopoeia are shown as lines in figure 18 and 20.

Effect of level of antioxidant

The six antioxidants were added to the fish oils in three different levels. The three levels indicated low, middle and high level of antioxidants. ANOVA four-factor model (antioxidant x oil x temperature x level of antioxidant) was used to evaluate the effect of level of antioxidant. The different levels showed no significant effect on oxidation based on TOTOX-values ($P > 0.05$). Level of antioxidant as a factor in ANOVA analysis was excluded in further statistic analyses; the three levels were merged.

Effect of oil and storage temperature

Both CLO and TG were used for evaluation of the six antioxidants. They were stored under two different conditions; 40 °C and 20 °C with access to oxygen.

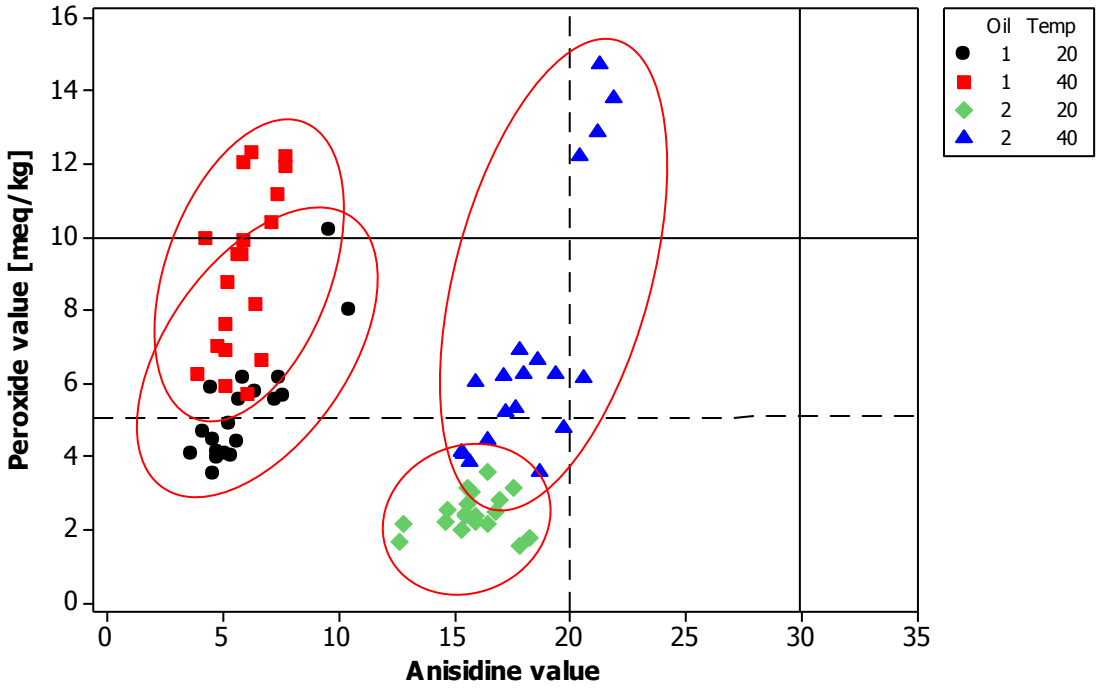

Figure 18: Scatter plot shows peroxide values (PV) and anisidine values (AV) for oil samples stored at 40 °C and 20 °C. Six antioxidants at all three levels are included together with reference samples.

Four groupings appear in the plot due to two different fish oils and two different storage temperatures. Oil 1 is cod liver oil (CLO). Oil 2 is concentrated triglycerides (TG). The limits given in European Pharmacopoeia is indicated by continuous lines in the plot (PV 10 and AV 30). The limits given by GOED's monograph is indicated by dotted lines in the plot (PV 5 and AV 20) (n = 76).

Four groupings can be seen in figure 18. This is due to different oils and storage temperatures. The CLO samples have higher PV than TG at both storage temperatures. There are only a few exceptions where samples of TG have higher PV than CLO. These samples were stored at 40 °C. TG had higher AV than CLO at both storage temperatures. Storage temperature of 40 °C gave basis for higher PV values for both CLO and TG. AV was not affected markedly by the different temperatures. TG showed some higher AV at 40 °C than at 20 °C. The effect of oil type and temperature is also illustrated in 'interactions plot' based on TOTOX in figure 19.

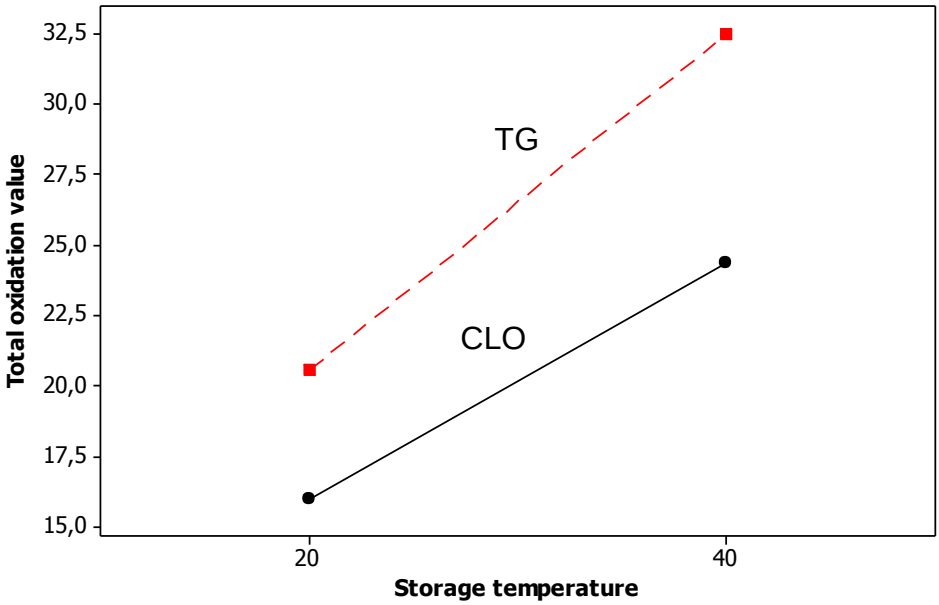

Figure 19: Interactions plot for all oil samples based on total oxidation values (TOTOX). TOTOX-values are calculated based on peroxide value (PV) and anisidine value (AV). One PV-equivalent is believed to give rise to two AV-equivalents (TOTOX = 2PV + AV). Oil 1 is cod liver oil (CLO). Oil 2 is concentrated triglycerides (TG). The two storage temperatures are 20 °C and 40 °C. Two parallels for each sample are included (n = 76).

The interaction plot in figure 19 illustrates the effect of type of oil and temperature on TOTOX. CLO samples had lower mean TOTOX values than TG samples at both temperatures.

The mean TOTOX value of CLO samples at 20 °C was 16.0. The mean TOTOX value of TG samples at the same storage temperature was 20.6. CLO samples at 40 °C gave a mean TOTOX value of 24.4. The mean TOTOX value of TG samples at the same storage temperature was 32.5. Storage temperature of 20 °C resulted in lower mean TOTOX values than temperatures of 40 °C for both oils.

Analysis of variance performed with ANOVA three factor model (antioxidant x oil x temperature) confirmed that both oil and temperature had significant effect on TOTOX-value ($P < 0.05$).

Effect of antioxidant

Six different antioxidants were investigated. Four of the antioxidants were added as single antioxidants. Two mixes consisted of two antioxidants. The effect of antioxidant on PV and AV is illustrated in figure 20.

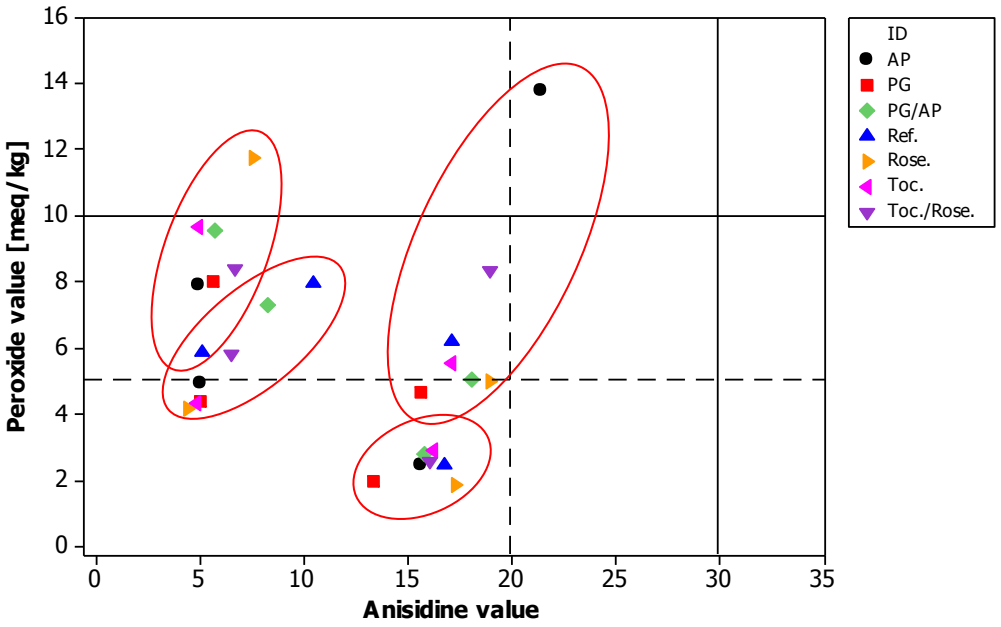

Figure 20: Scatter plot of six different antioxidants together with reference samples. The plot is based on peroxide value (PV) and ansidine value (AV) for each oil sample. The antioxidants are ascorbyl palmitate (AP), mixed tocopherol (toco.), propyl gallate (PG), rosemary extract (rose.), propyl gallate/ascorbyl palmitate (PG/AP), mixed tocopherol/rosemary extract (toco./rose.). The values for each antioxidant are a mean of results from all three levels of antioxidants. The limits given in

European Pharmacopoeia is indicated by continuous lines in the plot (PV 10, AV 30). The limits given by GOED's monograph is indicated by dotted lines in the plot (PV 5, AV 20) (n = 28).

Figure 20 shows no clear differences in effect of the six antioxidants. Oils with propyl gallate however seemed to have relatively low PV values compared to the other antioxidants in the four different groupings. The AV values for oils with propyl gallate were the lowest measured for the TG samples. Propyl gallate samples had relatively low AV also in CLO samples. The four groupings are also illustrated in figure 18. TG sampel with ascorbyl palmitate stored at 40 °C had the highest measured PV and AV of all the samples. This sample had PV higher than limits from both GOED and European Pharmacopoeia, PV 5 and 10 respectively. The AV for this sample was higher than GOED's limits for but not European Pharmacopoeia, AV 20 and 30 respectively. For CLO stored at 40 °C AP had the lowest measured PV and AV values together with the reference values.

The findings for propyl gallate and ascorbyl palmitate in figure 20 were confirmed when processing the results by ANOVA. Grouping information from Tukey Method revealed that these two antioxidants distinguished the most from the other antioxidanats (95.0 % confidence[13]). Type of antioxidant showed significant effect on TOTOX-value ($P < 0.05$) in the three factor model of variance of analysis (antioxidant x oil x temperature).

Sum up of peroxide value, anisidine value and total oxidation value

Sum up of PV, AV and TOTOX values for oils with the six antioxidants are presented as an overview in figure 21.

[13] For each pair wise comparison between antioxidant blends, the following hypotheses are tested: H0: There is no difference between AOX 1 and AOX 2, and H1: There is difference. Tukey Method takes into consideration that there are several hypotheses tested simultaneously. 95 % confidence means that there is 95 % probability that there is an actual difference between the antioxidants. The Tukey Method secures that probability of doing a type 1 error (rejecting H0 when it is actually true) is 0.05 for all the tests. This is a joint confidence level for all the comparisons. For the individual comparisons the probability might be even higher.

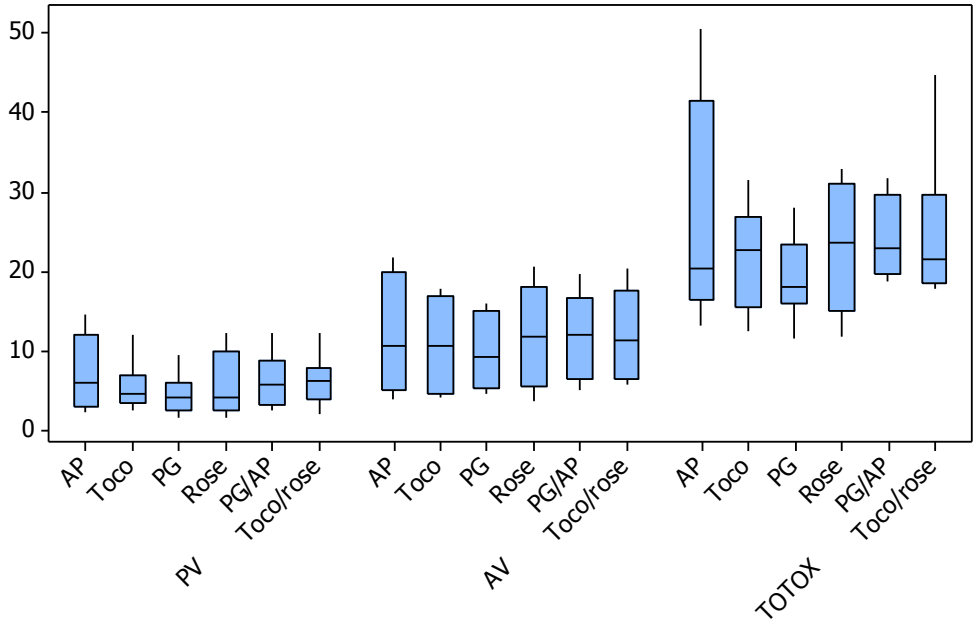

Figure 21: Box plot illustrating variation in measurements of oxidation for all oil samples. The plot presents results for peroxide values (PV), anisidine values (AV) and total oxidation values (TOTOX) for oils with six different antioxidants. TOTOX-values are calculated based on peroxide value (PV) and anisidine value (AV). One PV-equivalent is believed to give rise to two AV-equivalents (TOTOX = 2PV + AV). The antioxidants presented are ascorbyl palmitate (AP), mixed tocopherol (toco.), propyl gallate (PG), rosemary extract (rose.), propyl gallate/ascorbyl palmitate (PG/AP), mixed tocopherol/ rosemary extract (toco./rose.). The values for each antioxidant are a mean of results from all three levels. The values represent both storage temperatures and both types of oil. Median, 25- and 75-persentils are given as middle, lower and upper lines in the boxes. The lowest and highest values are given by the lines under and above the boxes (whiskers). PV, AV and TOTOX have different terming, but are presented in the same box plot as an overview. The median, minimum and maximum values are listed in Appendix 4; table A3 (n = 28).

Ascorbyl palmitate distinguished from the other antioxidants with the highest variation in measurements of oxidation. Some of the samples with ascorbyl palmitate were about twice the recommended maximum level of TOTOX based on GOED's recommandations (TOTOX ≤ 26). Rosemary extract, mixed tocopherol, propyl gallate/ascorbyl palmitate and mixed tocopherol/rosemary extract also gave rise to much variation in PV, AV and TOTOX values. Propyl gallate seems to result in the lowest measurements of both PV, AV and TOTOX. Propyl gallate also had less variation in measurements of PV, AV and TOTOX. The box plot includes

both oils and storage temperature and variation within each antioxidant was expected. The reference samples were are not included in this box plot due to imbalance in data set. As of figure 20 the reference samples did not distinguish from samples with added antioxidants.

4.3 Gas chromatography for identifying volatile organic compounds

Different antioxidants in oils give raise to different development of secondary oxidation products. These oxidation products were illustrated by volatile organic compounds (VOC). Gas chromatography – mass spectroscopy (GC-MS) was used to identify VOCs in oil samples. 14 VOCs were identified as markers of formation of volatile secondary oxidation products. The middle level of each antioxidant was chosen for GC-MS analyses. The volatiles are known secondary oxidation products contributing to rancidity. The volatiles were propanal, butanal, pentanal, 1-penten-3-one, 2-butenal, hexanal, 2-hexenal, 1-penten-3-ol, 4-heptenal, 2-penten-1-ol, nonanal, 2,4-hexadienal, 2-octenal and 2,4-heptadienal. These volatiles were chosen based on earlier studies identifying volatiles responsible for off-flavors caused by lipid oxidation (Frankel 2005; Olsen et al. 2005). Area of both cis and trans isomers in the chromatogram were included.

4.3.1 Total volatile organic compounds

Total area of VOCs may provide information about the extent of oxidation for each sample. High areas of VOC in the chromatogram imply high concentration of VOC in a sample. The variation in total VOCs may illustrate possible effect of antioxidants added to the oils. The sum of total area of the 14 VOCs selected is presented in figure 22.

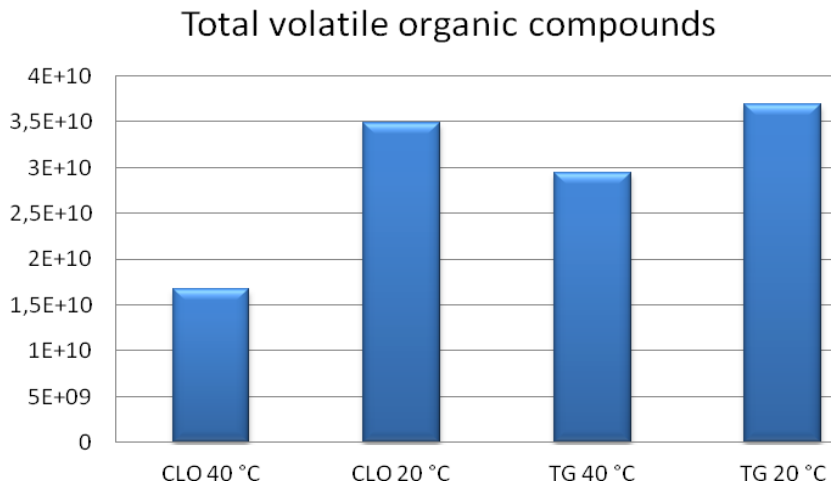

Figure 22: Sum of total volatile organic compounds (VOC) for all the samples at both 40 °C and 20 °C. Total areas of VOCs are based on the 14 selected volatiles. Both cod liver oil (CLO) and concentrated triglycerides (TG) are presented in the diagram. The four columns represent the four groupings earlier described due to type of oil and different storage temperature (see figure 18) (n = 28).

The diagram in figure 22 illustrates the effect of type of oil and storage temperature on total volatile organic compounds (VOC). The highest total VOC-areas were measured for CLO and TG at storage temperature 20 °C. Storage temperature of 40 °C resulted in lower total areas of VOCs for both CLO and TG. TG samples had higher total area of the selected VOCs than CLO at both storage temperatures.

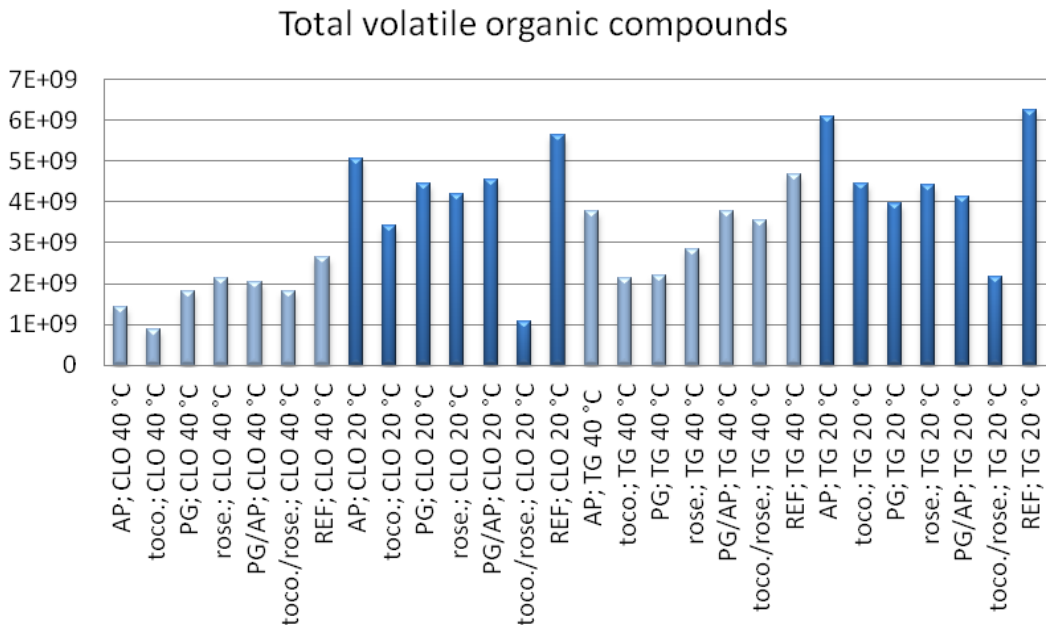

Figure 23: Total area of volatile organic compounds (VOC) in all the samples based on the 14 volatiles selected. Columns represent area of total VOCs in all the samples. The antioxidants are ascorbyl palmitate (AP), mixed tocopherol (toco.) propyl gallate (PG), rosemary extract (rose.), propyl gallate/ascorbyl palmitate (PG/AP) and mixed tocopherol/rosemary extract (toco./rose.). The reference samples are included (REF).The first seven columns from the left being CLO at storage temperature of 40 °C, the next seven columns being CLO at 20 °C, the next seven columns TG at 40 °C and the last seven columns TG at 20 °C (n = 28).

Figure 23 shows the total area of VOCs specific for each antioxidant in CLO and TG at both storage temperatures. High total area of VOCs indicated high concentration of secondary oxidation products in the sample. The reference values in each group had the highest total area of VOCs. Ascorbyl palmitate in both CLO and TG at 20 °C resulted in high areas close to the area of the references in the same two groups. CLO and TG samples with mixed tocopherol showed the lowest total area of VOCs at storage temperature of 40 °C. Mix of mixed tocopherol and rosemary extract showed lowest total area of VOCs at storage temperature of 20 °C.

4.3.2 Evaluation of volatile organic compounds by principal component analysis

14 variables from 56 fish oil samples were evaluated by principal component analysis (PCA) using *The Unscrambler.* These oil samples were all blends with middle level of antioxidant.

Variations between samples due to VOCs are presented in score and loading plots (figure 26, figure 29-33, appendix 7-10). The principal component (PC) 1 explained the majority of the variation in the data set. PC2 explained the rest of the variation after PC1 variation was removed.

Some of the volatiles dominated the loading plots when the areas of VOCs were used as basis. Dominating volatiles were 1-penten-3-ol, 1-penten-3-one and 2-hexenal. These volatiles described much of the variation between the samples. These specific volatiles had higher concentration in oxidized oils, represented by higher areas in the gas chromatograms. Compounds with the highest areas explain most of the variation in a score plot. Other VOCs may have less area in a gas chromatogram and will not be prominent in plots with areas as basis. Still these compounds may be source to differentiation between samples. VOCs with low concentrations may also be major contributors to rancidity of fish oils.

Other VOC than 1-penten-3-ol, 1-penten-3-one and 2-hexenal got more prominent when using the relation between VOC in the oil samples as basis. 2-octenal was one of the VOCs that got more prominent. 1-penten-3-ol was still a major contributor to variation. 1-penten-3-one did not contribute to variation in the same extent when the relations between VOCs were used.

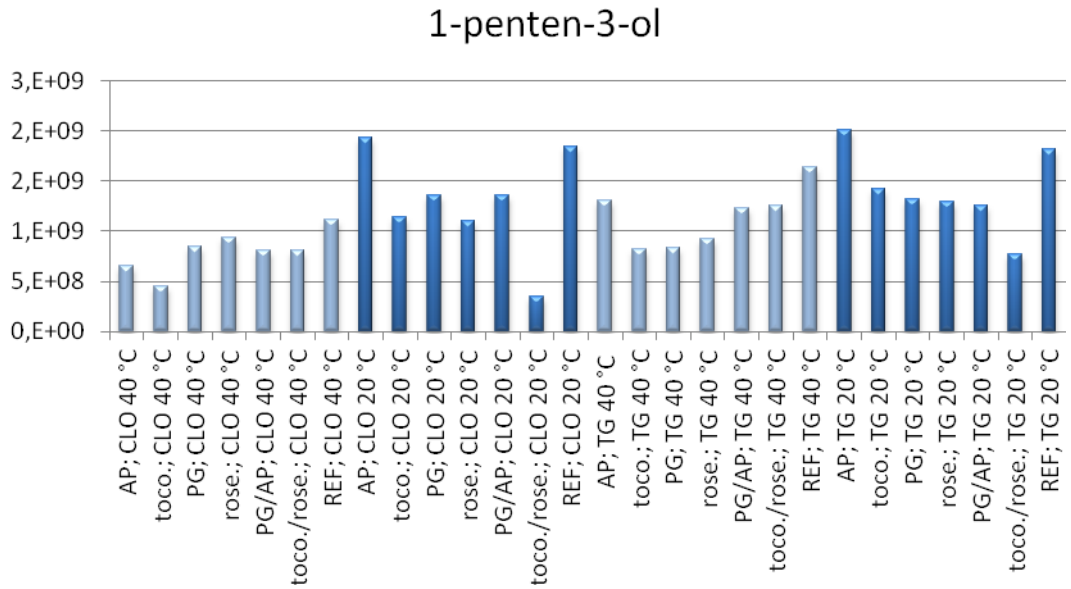

Figure 24: Columns represent area of 1-penten-3-ol for all the samples. 1-penten-3-ol distinguishes samples due to different antioxidant. The antioxidants are ascorbyl palmitate (AP), mixed tocopherol

(toco.) propyl gallate (PG), rosemary extract (rose.), propyl gallate/ascorbyl palmitate (PG/AP) and mixed tocopherol/rosemary extract (toco./rose. The reference samples are included (REF). The first seven columns from the left being CLO at storage temperature of 40 °C, the next seven columns being CLO at 20 °C, the next seven columns TG at 40 °C and the last seven columns TG at 20 °C (n = 28).

As an example of VOC as major contributor to variation between samples, areas for 1-penten-3-ol are presented in figure 24. 1-penten-3-ol was a major contributor to variation both when based on areas of VOCs and the relation between the areas for each VOC (1/sd). The diagram in figure 24 shows the variation in 1-penten-3-ol content in samples with each antioxidant. Comparing total areas of VOCs with areas of 1-penten-3-ol revealed many similarities (see figure 23 and 24). Samples with high area of 1-penten-3-ol also had high total area of VOC. The differences between total VOCs and 1-penten-3-ol are explained by the remaining 13 VOCs investigated.

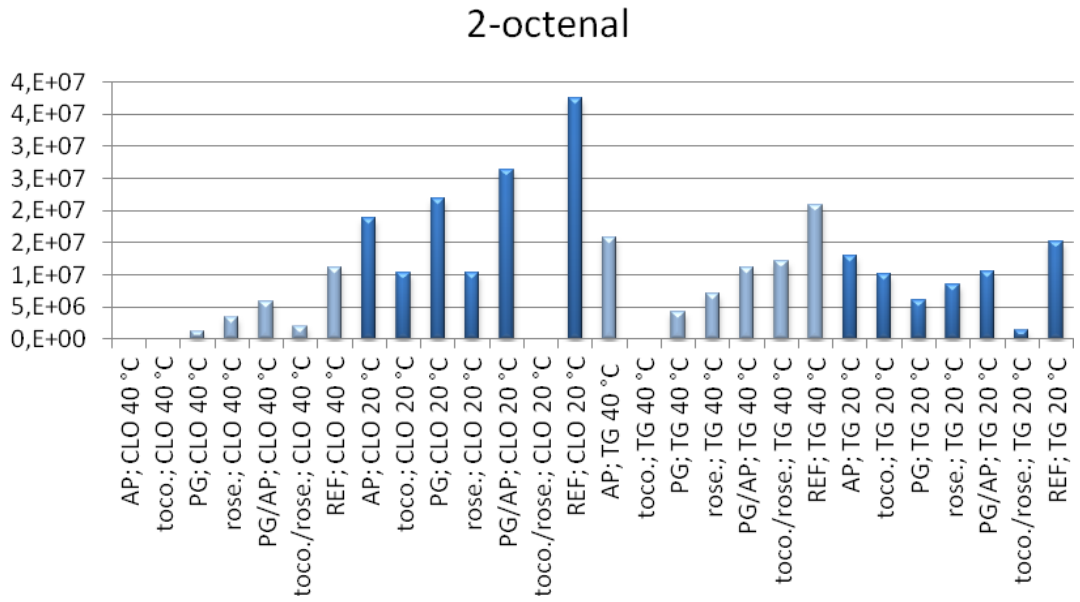

Figure 25: Columns represent area of 2-octenal based on oil samples with all six antioxidants. There is great variation in areas between the samples due to different antioxidants. The antioxidants are ascorbyl palmitate (AP), mixed tocopherol (toco.) propyl gallate (PG), rosemary extract (rose.), propyl gallate/ascorbyl palmitate (PG/AP) and mixed tocopherol/rosemary extract (toco./rose. The reference samples are included (REF). The first seven columns from the left being CLO at 40 °C, the

next seven columns being CLO at 20 °C, the next seven columns TG at 40 °C and the last seven columns TG at 20 °C (n = 28).

The variation between samples with regard to 2-octenal is presented in the diagram in figure 25. The areas for 2-octenal were in general lower than areas for 1-penten-3-ol. Still 2-octenal was a prominent contributor to variation in score plots based on relation between areas (1/sd). In samples were 1-penten-3-ol was high, area of 2-octenal was also high for most samples. Comparing areas for 2-octanal with total areas of VOCs reveals many similarities (figure 23 and 25).

Effect of storage temperature

PCA plots revealed differences between oil samples stored at different temperatures. As example grouping based on temperature was seen in the score plot in figure 26.

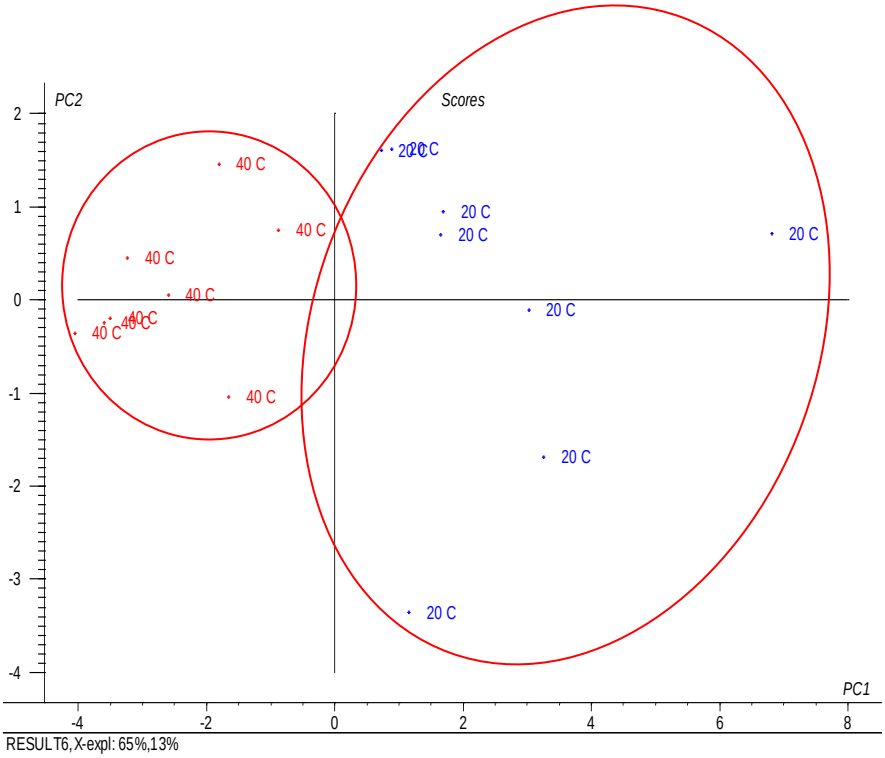

Figure 26: Score plot illustrating variation between samples of cod liver oil (CLO) stored at 40 °C and 20 °C. The antioxidants used are ascorbyl palmitate, propyl gallate and propyl gallate/ascorbyl palmitate. Reference samples are also included. In this plot 65 % of the variance is explained by PC1. 13 % of the variance was explained by PC2 (n = 16 including two parallels).

PC1 explained the major variation within the data set. The variation between samples was mainly due to the different storage temperatures. Temperature affected the concentration of volatile organic compounds (VOC) in the oil samples. Samples stored at 20 °C seemed to be more oxidized than samples stored at 40 °C. There was more variation between the samples stored at 20 °C than samples stored at 40 °C.

According to the loading plot for samples of CLO stored at 40 °C and 20 °C the major variance in figure 26 was explained by certain VOC. These were 2-hexanal, hexanal and 1-penten-3-ol and 2,4-heptadienal. See appendix 7; figure A1 for loading plot.

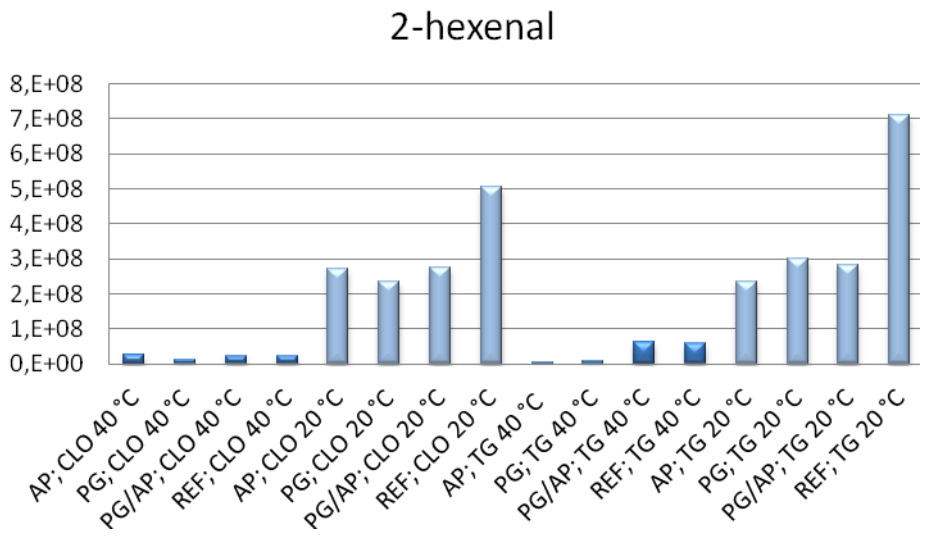

Figure 27: Columns represent areas of 2-hexenal for three of the antioxidants. The diagram illustrates the effect of storage temperature. The diagram is based on samples with ascorbyl palmitate (AP), propyl gallate (PG) and mix propyl gallate/ascorbyl palmitate (PG/AP). Both types of oils and storage temperature are presented (n = 16).

The areas of 2-hexanal in figure 27 illustrate the effect of temperature in the oil samples. For this specific VOC the areas were higher for both CLO and TG samples stored at 20 °C than 40 °C. The effect of temperature on specific VOC was also seen by evaluation of other VOCs.

Effect of type of oil

Comparing the VOCs describing most of the variation in the plots demonstrated a difference between the oils. CLO samples containing the same antioxidants as TG samples were explained by other VOCs. In CLO with tocopherol, rosemary extract and tocopherol/

rosemary extract the VOCs explaining most of the variation were 2,4-hexadienal, 1-penten-3-ol, 2-penten-1-ol, 2-octenal, 2,4-heptadienal and nonanal (appendix 10; figure A15 and A17). In TG with the same antioxidants 2-butenal, hexanal, nonanal, 2-octenal, pentanal and propanal explained most of the variation in the plots (appendix 10; figure A19 and A21). In CLO samples with ascorbyl palmitate, propyl gallate and propyl gallate/ascorbyl palmitate, the variation was mostly described by 2-butenal, 1-penten-3-ol, 2-penten-1-ol and 2,4-heptadienal (appendix 9; figure A7 and A9). Variations in TG samples with the same antioxidants were mostly described by 1-penten-3-ol, 2-octenal, propanal and hexanal (appendix 9; figure A11 and A13). Some of the dominating VOCs were seen as major contributors to variation in both mixes. A few of the major contributors were more specific to the oil type. Different oils gave rise to different VOC as major contributors to variation. These findings suggest an effect of type of oil on development of VOC.

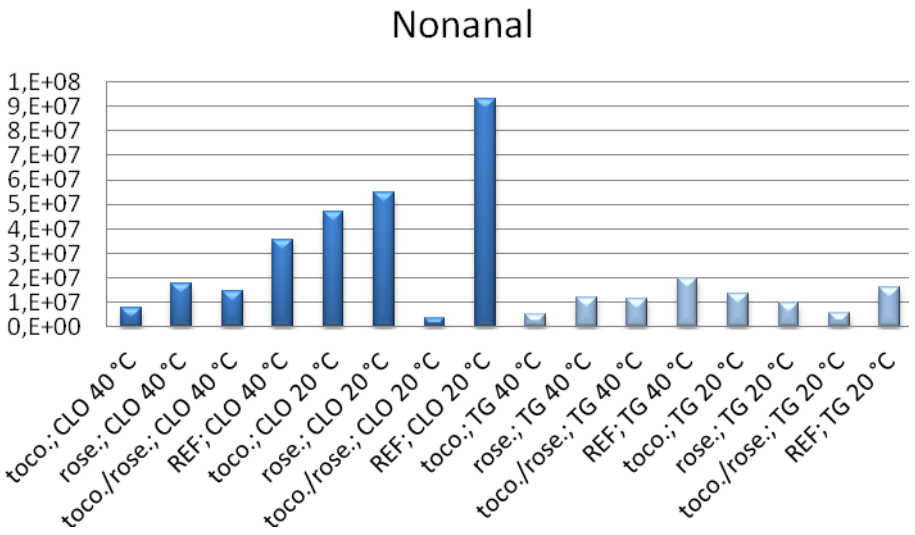

Figure 28: Columns represent areas of nonanal for three of the antioxidants. The diagram illustrates the effect of type oil. The antioxidants applied in these samples are mixed tocopherol (toco.), rosemary extract (rose.) and mixed tocopherol/rosemary extract (toco./rose.). Samples from both types of oil and storage temperature are presented. The first columns in the diagram are CLO samples, while the last samples are TG samples (n = 16).

CLO and TG have different fatty acid compositions. Effect of type of oil on concentration of VOC in oil samples is illustrated by nonanal in figure 28. The concentration of nonanal was higher in CLO samples than TG samples. For the other VOCs measured, TG samples had higher concentrations of specific VOCs than CLO. There was also observed an effect of type

of oil in oil samples with the other antioxidants added (ascorbyl palmitate, propyl gallate and ascorbyl palmitate/propyl gallate added) (see appendix 9).

Effect of antioxidants

Reference samples were often discriminated from the other samples in the score plots evaluated (see figure 29-33). The references had the highest total areas for VOC and were expected to distinguish from the other samples. Reference samples had higher areas for specific VOCs than most of the samples with added antioxidants. The score plots show how samples with different antioxidants distinguished from the reference samples. A clear difference between the reference samples and samples with antioxidants indicates antioxidative effect. The loading plots identify the major contributors to these variations. The loading plots are attached in appendix 8 (figure A2-A5).

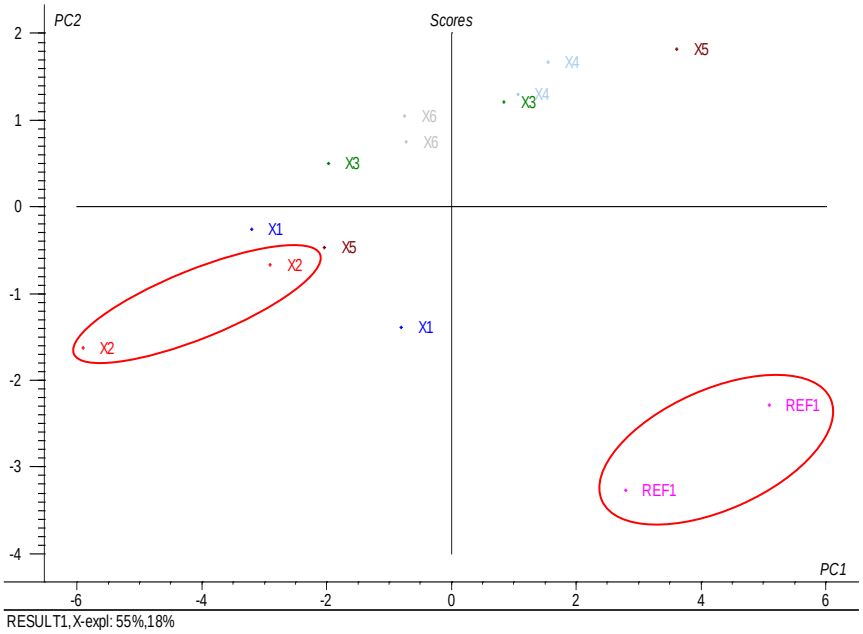

Figure 29: Score plot of cod liver oil (CLO) at 40 °C. Oil samples with six antioxidants and the reference samples are illustrated in the plot. The antioxidants are labelled X1 to X6. The antioxidants in the figures are ascorbyl palmitate (X1), mixed tocopherol (X2), propyl gallate (X3), rosemary extract (X4), propyl gallate/ascorbyl palmitate (X5) and mixed tocopherol/rosemary extract (X6). The reference samples are labelled as 'REF'. Both parallels of each sample are included (n = 14 including two parallels of each blend).

55 % of the variation in CLO stored at 40 °C is explained by PC1 and 18 % of the variation is explained by PC2 (see figure 29). The reference samples distinguished from the samples with added antioxidants. The reference values had the highest content of total VOC indicating greatest extent of oxidation of the samples analyzed. PC1 implies that mixed tocopherol (X2) was less oxidized than the rest of the samples. Due to variation from the reference samples, all the antioxidants seem to have had effect on CLO at this storage temperature. Rosemary extract (X4) and propyl gallate/ascorbyl palmitate (X5) in CLO at 40 °C have had least effect according to the score plot (figure 29).

From the loading plot for CLO samples at 40 °C the major contributors to variation can be explained. 2-penten-1-ol and 1-penten-3-ol explain most of the variation in these samples. These two VOC are oriented along PC1. Pentanal and 2,4-hexadienal are oriented along PC2 and explain some variation between the CLO samples. See appendix 8; figure A2 for loading plot.

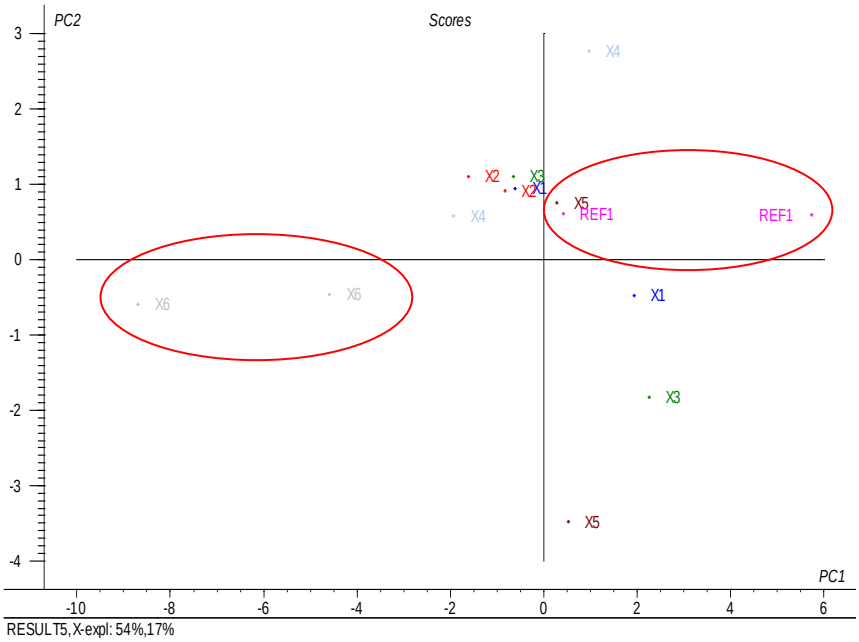

Figure 30: Score plot of samples of cod liver oil (CLO) stored at 20 °C. Oil samples with six antioxidants and the reference samples are included in the plot. The antioxidants are labelled X1 to X6. The antioxidants in the figures are ascorbyl palmitate (X1), mixed tocopherol (X2), propyl gallate (X3), rosemary extract (X4), propyl gallate/ascorbyl palmitate (X5) and mixed tocopherol/rosemary extract (X6). The reference samples are labelled as 'REF'. Both parallels of each sample are included (n = 14 including two parallels for each blend).

54 % of the variation in CLO samples stored at 20 °C is explained by PC1. 17 % of the variation is explained by PC2 (see figure 30). Oil samples with tocopherol/rosemary extract (X6) distinguished from the other samples. This antioxidant mix seemed to act antioxidative in the CLO samples at this storage temperature. Samples with ascorbyl palmitate (X1), propyl gallate (X3) and propyl gallate/ascorbyl palmitate (X5) were closest to the reference samples in the score plot. According to this plot these three antioxidants have had little effect in CLO at storage temperature of 20 °C.

Most of the variation in CLO samples stored at 20 °C is explained by PC1. According to the loading plot 2,4-heptadienal and 2-octenal explain most of the variation along PC1. 4-heptenal contributes to variation along PC2. See appendix 8; figure A3 for loading plot.

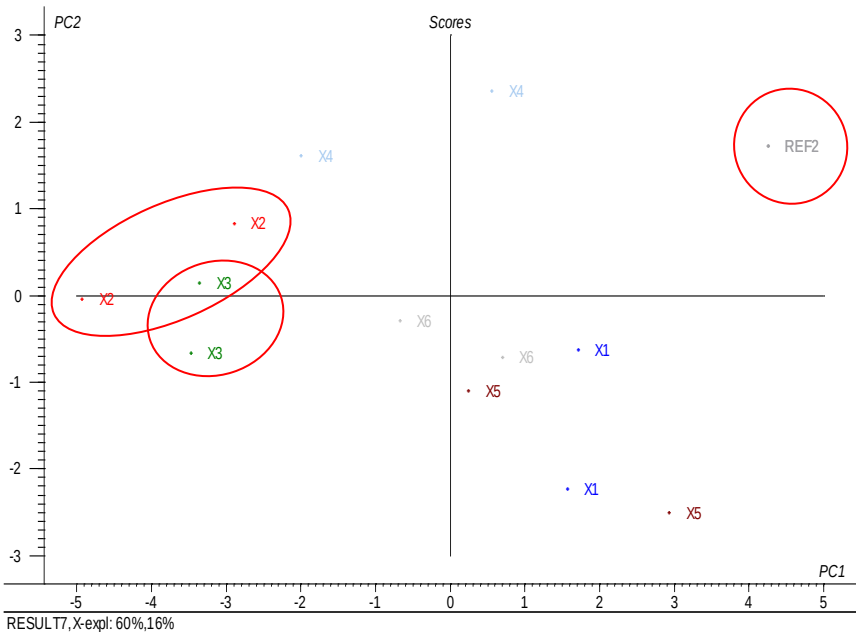

Figure 31: Score plot of samples of concentrated triglycerides (TG) stored at 40 °C. Oil samples with six antioxidants and the reference samples are included in the plot. The antioxidants are labelled X1 to X6. The antioxidants in the figures are ascorbyl palmitate (X1), mixed tocopherol (X2), propyl gallate (X3), rosemary extract (X4), propyl gallate/ascorbyl palmitate (X5) and mixed tocopherol/rosemary extract (X6). The reference samples are labelled as 'REF'. Both parallels of each sample are included (n = 14 including two parallels of each blend).

60 % of the variation in TG stored at 40 °C is explained by PC1. 16 % of the variation is explained by PC2 (see figure 31). The reference samples are believed to be most oxidized due to high concentration of VOC. Samples with mixed tocopherol (X2) and propyl gallate (X3) seemed to be less oxidized than the other samples with different antioxidants. These two antioxidants seemed to have had an effect on TG stored at 40 °C. The other antioxidants had less effect on the TG samples at this storage temperature.

The major contributors to variation in TG samples stored at 40 °C are located along PC1. According to the loading plot the main contributors were 1-penten-3-ol, 2-octenal and hexanal. The variation along PC2 was due to differences in concentrations of 4-heptenal in the samples. See appendix 8; figure A4 for loading plot.

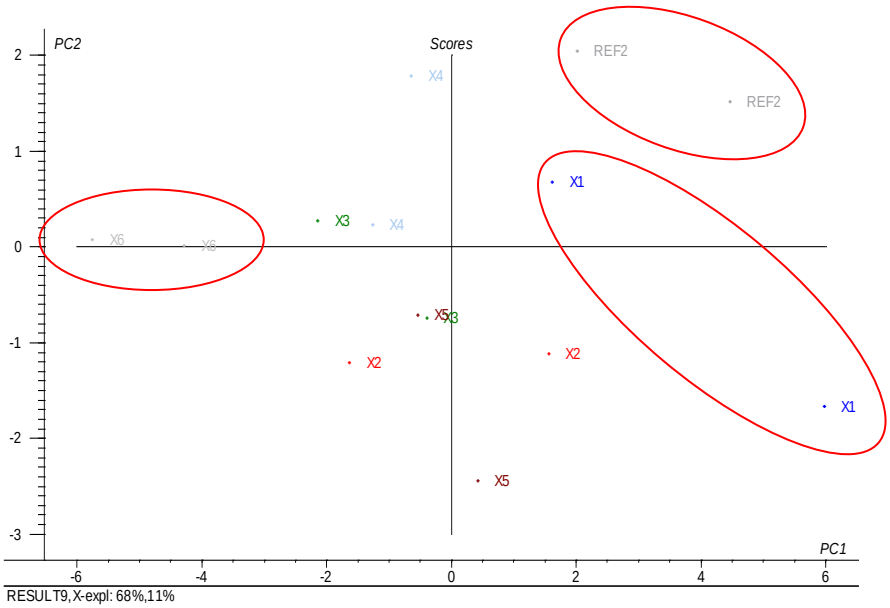

Figure 32: Score plot of samples of concentrated triglycerides (TG) stored at 20 °C. Oil samples with six antioxidants and the reference samples are included in the plot. The antioxidants are labelled X1 to X6. The antioxidants in the figures are ascorbyl palmitate (X1), mixed tocopherol (X2), propyl gallate (X3), rosemary extract (X4), propyl gallate/ascorbyl palmitate (X5) and mixed tocopherol/rosemary extract (X6). The reference samples are labelled as 'REF'. Both parallels of each sample are included in the plot (n = 14 including two parallels of each blend).

68 % of the variation in TG stored at 20 °C was explained by PC1. 11 % of variation was explained by PC2 (see figure 32). The reference samples had the highest areas of total volatile organic compounds and are believed to be most oxidized. Ascorbyl palmitate (X1)

was closest to the reference samples in the score plot. Mixed tocopherol/rosemary extract (X6) had most effect as antioxidant in TG at this temperature.

According to the loading plot there were several VOCs contributing to variation between samples in TG stored at 20 °C. 2-penten-1-ol, 1-penten-3-ol, 2-octenal and hexanal were the main contributors along PC1. 2-hexenal explained variation along PC2. See appendix 8; figure A5 for loading plot.

Effect of antioxidant mixes

Based on PCA plots a few VOC were identified as major contributors to variation between the samples. Tocopherol/rosemary extract is a mix of two antioxidants. The mix and the two individual antioxidants were compared in the same plot. This was done to reveal any effect by the mix in comparison with the individual antioxidants. Propyl gallate/ascorbyl palmitate is also a mix of two antioxidants. Propyl gallate/ascorbyl palmitate was compared with the individual antioxidants propyl gallate and ascorbyl palmitate (see figure 33 and appendix 9). Differences between the mix and the individual antioxidants could reveal a potential effect of the mix.

Ascorbyl palmitate, propyl gallate and propyl gallate/ascorbyl palmitate

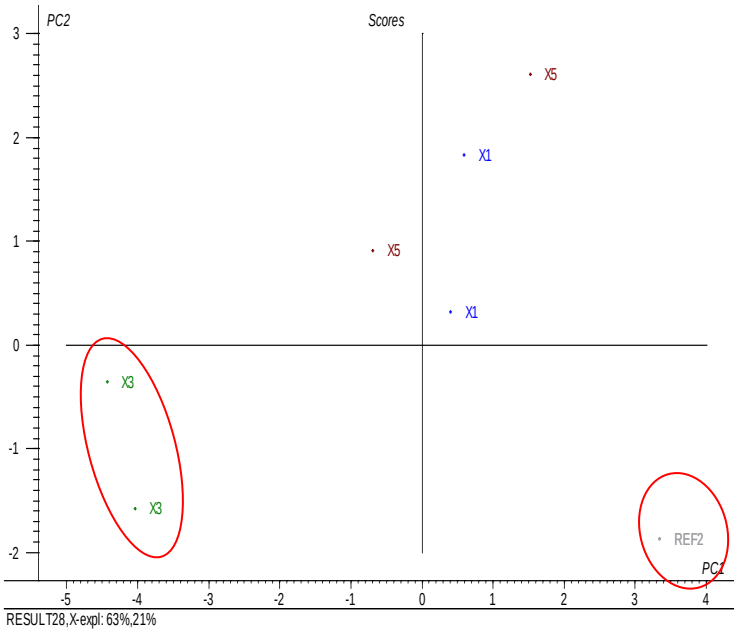

Figure 33: Score plot of samples of concentrated triglycerides (TG) stored at temperature of 40 °C. The antioxidants in the score plot are ascorbyl palmitate (X1), propyl gallate (X3) and the mix propyl

gallate/ascorbyl palmitate (X5). The reference samples are labeled 'REF' (n = 14 including two parallels of each blend).

Figure 33 shows the score plot for TG stored at 40 °C. 63 % of the variation in the plot was explained by PC1. Samples with propyl gallate were clearly different from the reference samples along PC1. Ascorbyl palmitate and propyl gallate/ascorbyl palmitate were less different from the reference samples. Ascorbyl palmitate seemed to have had little antioxidative effect on TG at 40 °C. Ascorbyl palmitate lowered the antioxidative effect of the mix propyl gallate/ascorbyl palmitate (X5).

According to the loading plot hexanal and 1-penten-3-ol explained the variation between the TG samples stored at 40 °C (see appendix 9; figure A11). Hexanal and 1-penten-3-ol also explained the variation in TG stored at 20 °C (see appendix 9; figure A13). In addition 2-penten-1-ol contributed to variation in the plot. In the score plot for TG samples at 20 °C propyl gallate differed most from the reference samples. The mix propyl gallate/ascorbyl palmitate also differed in some extent from the reference samples. Ascorbyl palmitate as single antioxidant varied less from the reference samples. This suggests that ascorbyl palmitate lowers the antioxidative effect of the mix propyl gallate/ascorbyl palmitate.

The score plots for CLO samples showed no apparent differences between the samples with added antioxidants (see appendix 9). The CLO reference samples stored at 40 °C distinguished from the samples with antioxidants (figure A6). This showed an effect of the antioxidants added in the CLO. For the CLO reference samples stored 20 °C there were no apparent difference from the samples with added antioxidants (figure A8). One of the two reference parallels distinguished from the other samples, while the other parallel did not. The VOC explaining variation between CLO samples were mainly 1-penten-3-ol according to the loading plot (see appendix 9). For CLO samples stored at 40 °C nonanal, 1-butenal, butanal and 2-hexenal also contributed to variation in the plot. For CLO samples stored at 20 °C 2,4-heptadienal and 2-penten-1-ol contributed to variation between the samples in addition to 1-penten-3-ol.

Propyl gallate seemed to be the most efficient antioxidant from the antioxidants evaluated in these plots. This was more apparent in TG samples than in CLO samples. See appendix 9 for score and loading plots.

Mixed tocopherol, rosemary extract and mixed tocopherol/rosemary extract

Mixed tocopherol, rosemary extract and the mix of mixed tocopherol/rosemary extract were evaluated in the same score and loading plots (see appendix 10). The reference samples were different from all the oil samples with added antioxidants. The antioxidants had an effect in both CLO and TG at both storage temperatures. In CLO samples stored at 40 °C mixed tocopherol differentiated from the reference samples along PC1. 57 % of the variation in the plot was explained by PC1 (figure A14). According to the loading plot of CLO samples at 40 °C, 1-penten-3-ol was the major contributor to variation between the samples (figure A15). Samples with mixed tocopherol distinguished from the reference samples also in TG samples stored at 40 °C (figure A18). PC1 explained 75 % of the variation between the samples. Rosemary extract and mixed tocopherol/rosemary extract were less different from the reference samples in TG. According to the loading plot for TG stored at 40 °C propanal, nonanal, pentanal and 2,4-heptadienal were main contributors to variation in the plot (figure A19).

For both CLO and TG samples stored at 20 °C with mixed tocopherol/rosemary extract distinguished the most from the reference samples (figure A16 and A20). According to the loading plot, the major contributors in CLO were 2-octenal and 2,4-heptadienal (figure A17). In TG the major contributors were 2-octenal, hexanal, pentanal and 2-butenal (figure A21). Rosemary extract as individual antioxidant showed antioxidative effect together with the two other antioxidants evaluated. Mixed tocopherol and mixed tocopherol/rosemary extract distinguished more from the reference samples than rosemary extract. This indicates that mixed tocopherol might be the most efficient antioxidant between the three antioxidant evaluated in these plots. See Appendix 10 for score and loading plots.

In plots with both CLO and TG samples several VOC described the variation. The main VOC describing variations between samples in both plots were hexanal, 2,4-hexadienal, 2-penten-1-ol, pentanal, 1-penten-3-ol, 2,4-heptadienal, nonanal, butanal and 2-hexenal. In addition

2-butenal was a major contributor to variation in plot with mixed tocopherol, rosemary extract and mixed tocopherol/rosemary extract. 2-octenal was major contributor to variation in plot with ascorbyl palmitate, propyl gallate and propyl gallate/ascorbyl palmitate. This indicates that different antioxidants promote formation of different secondary oxidation products. Presence of antioxidants interferes with the formation of volatiles. Formation of volatiles may be promoted or prevented according to the antioxidant present. Due to different major contributors to variation between samples there is reason to believe that two reaction pathways may have occurred in the oil samples in this study.

4.4 Gas chromatography for identifying fatty acid composition

Gas chromatography with flame ionization detector (GC-FID) revealed the fatty acid composition of CLO and TG. CLO and TG were compared on the basis of the eight fatty acids usually dominating in fish oil. Typical content of fatty acids in fatty fish is C14:0, C16:0, C16:1, C18:1, C20:1, C22:1, C20:5 and C22:6 (see page 15). TG had twice the concentration of EPA and DHA than CLO. This was expected due to concentration of these two fatty acids in TG. The other fatty acids were also present in TG, but in much lower concentrations than in CLO. The other fatty acids had a relative content five times higher in CLO than TG. The fatty acid content is shown in Appendix 5; table A4.

5. Discussion

5.1 Peroxide value, anisidine value and total oxidation value

Comparison to quality criteria

Quality criteria from the European Pharmacopoeia and the GOED monographs were used to evaluate the oxidative status of the oil samples. These are the only existing quality criteria concerning oxidation of fish oils. In this study the oil samples were exposed to oxygen as part of the experimental set up. In addition one of the batches was held at storage temperature of 40 °C. Normally oils will have limited access to oxygen to prevent oxidation. From these two batches, the one held at 20 °C was closer to conditions of normal storage. The oils stored at 40 °C may have undergone different reaction pathways in addition to faster rate of oxidation. This storage condition will not show results representative for normal conditions. Only the batch held at 20 °C was compared to the quality criteria in the monographs.

From the oils stored at 20 °C for 17 weeks with access to oxygen, ten CLO samples and all the TG samples (n = 19) were within the limits of GOED (PV 5, AV 20). The remaining nine CLO samples stored at 20 °C had PV higher than the limits of GOED. Except from samples with propyl gallate/ascorbyl palmitate at middle level, the nine remaining samples stored at 20 °C were within the limits of European Pharmacopoeia (PV 10, AV 30). Fish oils as supplement normally have shelf life of two years. These oils have limited access to oxygen which acts as major contributor to oxidation. The oil stored at 20 °C is believed to have had a faster reaction rate due to access to oxygen. The quality of the oils with regard to oxidation should be on acceptable level through the whole storage period meeting the quality criteria described. The oil samples should be analyzed several times for a longer time period to see the oxidative status at the end of shelf life.

Total oxidation value

TOTOX value is based on both PV and AV to give a better description of the oxidative status of oils high in PUFA. This represents a measure of the non-volatile carbonyls present in processed oils (PV), together with any further oxidation products which develop during storage (AV) (Frankel 2005). One PV-equivalent is believed to give rise to two AV-equivalents (TOTOX = 2PV + AV). Oil of satisfactory quality should have TOTOX value less than 26

(GlobalOrganisationForEPAandDHAomega-3s 2006). As seen in figure 15 the content of primary and secondary oxidation products develop with time. The time for analysis will be crucial if only primary or secondary oxidation products are measured. Combining PV and AV by calculation of TOTOX will provide a better picture of the oxidation status in the oils. TOTOX was used for evaluation of the antioxidants in ANOVA.

Effect of type of oil

The fatty acid compositions of CLO and TG were confirmed by gas chromatography. Oil composition is known to be an important factor in terms of oxidation. Type of oil had significant effect on TOTOX in this study. Of the eight fatty acids investigated in CLO and TG, EPA and DHA had most double bonds. Double bonds on the alkyl chain facilitate sites for radicals to attack. The relative content of EPA and DHA was higher in TG than in CLO. The EPA content in TG was about twice as high as in CLO. The DHA content in TG was about five times higher than in CLO. CLO had higher relative content of the six remaining fatty acids investigated. These fatty acids accounted for saturated and monounsaturated fatty acids.

TG had higher total degree of unsaturation than CLO, due to higher concentration of the poly unsaturated omega-3 fatty acids, EPA and DHA. TG was expected to oxidize more rapidly due to the fatty acid composition. CLO had higher PV values than TG, while TG had higher AV values than CLO (see figure 18). The peak of primary oxidation products might be reached earlier for oils with higher degree of oxidation. The primary oxidation products will eventually be decomposed to secondary oxidation products. TG had higher degree of unsaturation than CLO and may have reached the exponential phase of oxidation faster. Primary oxidation products might decompose to secondary oxidation products earlier in TG than in CLO. This will be illustrated by an earlier shift from high PV (primary oxidation products) to high AV (secondary oxidation products) for oils with high degree of unsaturation. The effect of oil composition resulted in PV and AV as expected for CLO and TG. TG is more prone to oxidation, which was shown in this study. TOTOX-values for TG samples were higher than for CLO samples at both storage temperatures confirming effect of oil composition (see figure 19).

Effect of storage temperatures

Temperature is known to influence development of oxidation considering both oxidation rate and reaction pathways. The oils stored at 40 °C showed higher levels of oxidation with regard to PV than the oils stored at 20 °C (see figure 18). AV was not affected markedly by the higher temperature, but TG showed some higher levels for AV at 40 °C compared to 20 °C. Storage temperature at 40 °C gave higher TOTOX-values than storage temperature of 20 °C in general (see figure 19). Storage temperature had significant effect on TOTOX in this study. Storage time with accelerated conditions was much shorter, but still influenced TOTOX values in this study. The results correlates with the fact that oxidation is promoted by increased temperature (Fennema 2008). Formation and decomposition of hydroperoxides are promoted by accelerated conditions. Higher temperatures may give rise to new reaction pathways and oxidation products which can be illustrated by volatile secondary oxidation products.

The effect of antioxidants may also have been reduced at the higher temperature. In general antioxidants are more effective at lower temperatures, but different antioxidants may respond to temperatures in different ways. Tocopherols are known to withstand high temperatures, but this was not seen according to PV and AV in this study (appendix 3; table A2).

Effect of level of antioxidants

Level of antioxidants applied had no significant effect on TOTOX in this study. The concentration of antioxidants is important due to possible change in effect. Both ascorbyl palmitate and tocopherols have shown to act prooxidative in fish oil at certain concentrations (Hamilton et al. 1998). Different concentrations of antioxidants, both as singles and mixes, may cause different effect in fish oil. The levels of antioxidants chosen in this study were based on levels used in studies earlier published. The concentration of antioxidants could have been source to differences in the oxidation development. Analyses during the storage period may reveal potential differences due to different concentrations.

Effect of antioxidant

Type of antioxidant had significant effect on TOTOX in this study. Two of the antioxidants were different from the other antioxidants; propyl gallate and ascorbyl palmitate. Oils with propyl gallate added as antioxidant showed the lowest values of oxidation based on TOTOX. Propyl gallate was the only antioxidant which resulted in oils with TOTOX values less than 26 for both oils under both storage conditions. Ascorbyl palmitate possessed the lowest antioxidant efficiency in this study based on TOTOX. Oils with ascorbyl palmitate showed great variation in PV, AV and TOTOX as seen in figure 21. Ascorbyl palmitate gave basis for the highest measured PV and AV of all the samples (see figure 18). However, ascorbyl palmitate also resulted in the lowest measured CLO sample at 40 °C together with the reference sample. Ascorbyl palmitate has also shown to be prooxidative in certain concentrations. The antioxidant effect of ascorbyl palmitate has shown to increase when added together with lecithin (Hamilton et al. 1998). Ambiguous results have been shown for both ascorbyl palmitate and propyl gallate in fish oil (Jacobsen et al. 1998). Data vary between studies due to differences in sampling and analysis techniques.

Two mixes of antioxidants were applied to investigate a possible synergistic effect. These two mixes were not different from the single antioxidants based on TOTOX. The total concentration of the two antioxidants may have been too high or low. The relation between antioxidants in a mix has shown to be crucial. This might be more important than the absolute level of antioxidants (Hamilton et al. 1998). It would be interesting to test other concentrations and relations between the two antioxidants in mix.

5.2 Volatile organic compounds

This study showed that concentration of VOCs in fish oils was affected by oil composition, temperature and presence of antioxidants. Both total volatile organic compounds and specific compounds are discussed.

Total volatile organic compounds

Evaluating the effect of antioxidants based on total content of certain volatile organic compounds (VOC) gives an indication of the extent of oxidation. Total areas of VOCs were

high in reference samples compared to most samples with added antioxidants. This suggests that the antioxidants had a favorable effect on extent of oxidation. There were some exceptions were the reference samples did not have the highest concentration of specific volatiles. Ascorbyl palmitate was the antioxidant responsible for most of the samples with high areas of VOCs. Areas higher than those of reference samples occurred in either TG or CLO at one of the storage temperatures. High areas were not consistent for all the samples with ascorbyl palmitate. High areas for ascorbyl palmitate regarded eight of the 14 VOC investigated; hexanal, pentanal, 4-heptanal, 2-penten-1-ol, 2,4-heptadienal, propanal, butanal and 1-penten-3-one. In addition propyl gallate/ascorbyl palmitate gave higher areas than references for butanal, pentanal, 1-penten-3-one, 2,4-hexadienal and 2,4-heptadienal. Rosemary extract, tocopherol and the mix mixed tocopherol/rosemary extract also showed a few higher areas than the references. Propyl gallate did only show higher area than references for measurements of nonanal. Nonanal had higher values for CLO than TG. For all the other 13 VOC TG had higher values than CLO. Nonanal seemed to differ from the other measured VOCs according to these observations.

Specific volatile organic compounds

By investigating specific volatile organic compounds more detailed information was obtained. Different VOCs were acting as major contributors to variation between samples. This indicated different reaction pathways due to different temperature, oil composition and antioxidants present.

Some of the VOCs with major impact on rancidity were present in low concentrations. This was illustrated by small areas in chromatograms. These compounds had little effect on a diagram showing total area of VOC (see figure 23). Compounds with small areas also contributed to variation between samples and described variation in the data set. 2-octenal in figure 25 is used as example. Standardized areas (1/sd) were used to better illustrate variation between compounds with low areas.

1-penten-3-ol was one of the major contributors to variation in the data set based on both area and standardized area (1/sd) (see figure 24 and loading plots in appendices 7-10). This VOC alone indicated that mixed tocopherol and mixed tocopherol/rosemary extract were

most efficient due to low areas in the chromatogram. Other VOCs evaluated indicated different antioxidants to be most efficient.

Effect of type of oil

Effect of type of oil was expected to have major impact on formation of specific VOCs. The oxidizability of fatty acids is highly dependent on number of doubly allylic positions available (Frankel 2007). EPA and DHA have five and six double bonds in the alkyl chain respectively and are prone to oxidation. These fatty acids were present in both CLO and TG, confirmed by GC-FID. TG had much higher content of EPA and DHA than CLO and was more prone to oxidation. CLO had higher relative values of the six other fatty acids examined, which consisted of saturated and monounsaturated fatty acids. A clear difference between TG and CLO samples with the same antioxidants were shown for most VOCs. Most volatiles were present in higher concentrations in TG than CLO which indicates greater extent of oxidation. This correlated with the literature. For nonanal as VOC, the concentration was higher in CLO than TG. Figure 28 illustrates the effect of type of oil on formation of nonanal.

Types of volatiles are related to the fatty acid composition of the oil. Fatty acids are precursors for different oxidation products. EPA and DHA were expected to be precursors for the major oxidation products in the two oils applied in this study. Different VOCs were seen as major contributors in the two oils. For both CLO and TG stored at 40 °C, 1-penten-3-ol was a major contributor. In addition 2-penten-1-ol was major contributor in CLO, and 2-octenal and hexanal major contributors in TG. Different major contributors for CLO and TG were also seen at storage temperature 20 °C. The variation between major contributors in the two oils can be explained by the different fatty acids acting as precursors for volatiles.

Effect of storage temperature

Temperature has shown to influence development of oxidation products. The rate of oxidation is increased by increased temperature. There was observed differences in areas of VOCs between samples stored at different temperatures. The oils stored at 20 °C showed higher concentration of 13 selected volatiles than the oils stored at 40 °C (see figure 27). Propanol had higher content of volatiles at 40 °C and differed from the other volatiles.

Increased temperature may also change the reaction pathways. There were identified different VOCs explaining variation in the score plots for CLO and TG at 40 °C and 20 °C (see loading plots in appendix 8; figure A2-A5). Storage temperature of 40 °C resulted in other VOCs as major contributors to variation than storage temperature of 20 °C. This illustrates a possible change in reaction pathways due to elevated temperatures.

Different antioxidants withstand temperature in different degree. Tocopherols are able to act efficiently as antioxidants also in higher temperatures. Figure 23 shows the total areas of VOCs in all oil samples at both temperatures. Mixed tocopherol has the lowest total areas of VOCs in both oils at 40 °C. This may illustrate the efficiency of tocopherols at higher temperatures.

Effect of antioxidant

Effect on volatile organic compounds

Tocopherols, propyl gallate, carnosic acid, carnosol and rosmarinic acid constitutes of a six-ring structure. They all act as chain breaking antioxidants donating a hydrogen atom to radicals. Ascorbyl palmitate has a five-ring structure and is believed to possess different reacting mechanisms than the hydrogen donors. Ascorbyl palmitate is mainly an oxygen scavenger and metal chelator. The different reaction mechanisms with radicals are believed to influence the outcome with regard to oxidation products. 2,4-heptadienal may further decompose to 1-penten-3-ol as a tertiary oxidation process. Hydrogen donors are able to protect unsaturated aldehydes from further oxidative degradation. This may lead to lower levels of 1-penten-3-ol which was shown in a study by Olsen et al. (2005). In this study oils with ascorbyl palmitate had high levels of 1-penten-3-ol close to reference samples. This was seen for TG samples at both temperatures and CLO samples stored at 20 °C also in this study (see figure 24). The other antioxidants were either in mix with a hydrogen donating antioxidant or were able to donate hydrogen itself. These antioxidants resulted in lower areas for 1-penten-3-ol possibly due to protection of unsaturated aldehydes from degradation. 1-penten-3-ol often distinguished reference samples from less oxidized samples in this study. 1-penten-3-ol was used as marker for oxidation in oil samples.

Mixed tocopherol commercially available differs in composition of the four homologs; α-, β-, γ- and δ-tocopherol. Not only will the antioxidant used affect the formation of volatiles. The composition of mixed tocopherol may also direct formation of certain volatiles (Kulås et al. 2002; Kulås et al. 2003). In a study by Kulås et al. (2003) α-tocopherol was found to promote formation of *cis, trans* isomer of 2,4-heptadienal. δ-tocopherol on the other hand promoted formation of shorter and more saturated aldehydes, such as propanal (Kulås et al. 2003). Mixed tocopherol was not seen to promote formation of the 14 specific VOCs chosen for evaluation in this study. Other commercially available mixes of tocopherol homologs may show different results than those presented in this study.

Effect on configuration of volatile organic compounds

Antioxidants may have an effect on the formation and decomposition of lipid hydroperoxides (Olsen et al. 2005). Antioxidants can be grouped according to their reacting mechanisms as mentioned previously. *cis,trans*-Hydroperoxides are able to isomerize to *trans, trans*-hydroperoxides via peroxyl radical. Tocopherols may inhibit this reaction depending on their hydrogen donating capacity and concentration (Olsen et al. 2005). The four different tocopherol homologs have different donating capacity. Composition of mixed tocopherol may influence these reactions. The areas presented for the 14 selected volatiles in this study were based on both *cis* and *trans* isomers. This limits the possibility to further investigate which isomers were present.

Effect on reaction pathways due to different mechanisms

Results from GC-MS indicated different oxidation reaction pathways due to different antioxidants present. The two mixes of antioxidants were compared with the associated two single antioxidants. Score and loading plots showed that many of the major contributors to variation were the same in both mixes (appendix 9 and 10). Plots also revealed different major contributors to variation in the different mixes (appendix 9 and 10). The variation in major contributors to variation between samples may demonstrate the effect of using different antioxidants which posess different reacting mechanisms. A fatty acid may be cleaved at different positions in the alkyl chain in presence of different antioxidants. Antioxidants may interact with the scission pathways. Thus antioxidants may be involved in creating different oxidation products. In a mix of two antioxidants two reaction mechanisms

may be involved. This might create smaller amounts of each oxidation product, but also greater variety of oxidation products.

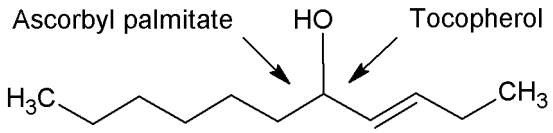

Figure 34: Cleavage of alkyl chain may occur at different positions in presence of different antioxidants. Antioxidants may promote or prevent certain reaction pathways. This gives rise to a great variety of oxidation products. Ascorbyl palmitate and tocopherol are used as example of antioxidants in this figure.

The presence of antioxidants may have an effect on formation and decomposition of hydroperoxides. This is due to different types of antioxidant activity. Certain volatiles are promoted by presence of specific antioxidants. A study by Olsen et al. (2005) investigated ascorbyl palmitate and tocopherol in CLO. They could see from the study that ascorbyl palmitate had little or no effect with regard to scavenging of peroxyl radicals in CLO. The main effect of ascorbyl palmitate in CLO seemed to be specific interactions with one or more scission pathways (Olsen et al. 2005). In the same study ascorbyl palmitate promoted formation of hexanal, 2-hexenal, propanal and 2,6-nonadienal. High areas of hexanal for samples with ascorbyl palmitate as individual antioxidant were also shown in this study (data not shown). Samples with ascorbyl palmitate had high areas of propanal in CLO and TG at 20 °C, and 2-hexenal to some extent. 2,6-nonadienal was not investigated in detail in this study.

Effect of solubility of antioxidants

Lipophilic antioxidants have shown to be more effective in lipid emulsions than in bulk oils. Hydrophilic antioxidants are more effective in bulk oils. This is known as the polar paradox described by Frankel (Frankel 1996). Rosemary extract and propyl gallate possess both hydrophilic and lipophilic properties. These amphiphilic properties make the antioxidants most efficient in bulk oils. Mixed tocopherol and ascorbyl palmitate are lipophilic and will be most active in lipid emulsion. Propyl gallate and mixed tocopherol distinguished from the other antioxidants based on score plots (appendix 9 and 10). These two antioxidants showed greatest variation from the reference samples. The antioxidants were probably the most

efficient antioxidants, together with mixed tocopherol/rosemary extract (appendix 9; figure A16 and A20). These were antioxidants of both amphiphilic and lipophilic character. The effect of solubility of antioxidants in oil was not shown in this study.

5.3 Synergistic effect

Two mixes of antioxidants were prepared and added to the oils to investigate a possible synergistic effect. The four antioxidants have been documented as effective in fish oils in earlier studies, either as single antioxidants or in mixes. Different methods and lipid systems used in studies results in ambiguous results.

Mixed tocopherol/rosemary extract and the two single antioxidants mixed tocopherol and rosemary extract were evaluated in the same PCA plots (appendix 10; figure A14-A21). Mixed tocopherol/rosemary extract was the most efficient antioxidant in TG and CLO samples stored at 20 °C. Mixed tocopherol and rosemary extract have different structural properties which are believed to give better total antioxidative effect. Mixed tocopherol as single antioxidant was the most efficient antioxidant in oils stored at 40 °C. Rosemary extract as single antioxidant showed no apparent antioxidative effect on the oil samples. Rosemary extract was more efficient when added together with mixed tocopherol. It seems from these results that the synergistic effect between mixed tocopherol and rosemary extract is temperature dependent. Storage temperature of 20 °C favored the mix of mixed tocopherol/rosemary extract. Storage temperature of 40 °C favored mixed tocopherol as single antioxidant. Tocopherols have shown to withstand higher temperatures, which might have been demonstrated in this study.

Propyl gallate/ascorbyl palmitate and the two single antioxidants propyl gallate and ascorbyl palmitate were evaluated in the same PCA plots (appendix 9; figure A6-A13). Propyl gallate had best antioxidative effect in TG samples stored at both 40 °C and 20 °C. The antioxidants in CLO samples showed no apparent variation in the score plot (figure A6 and A8). Neither ascorbyl palmitate nor propyl gallate/ascorbyl palmitate showed apparent antioxidative effect. Propyl gallate alone was more efficient than in mix with ascorbyl palmitate. Ascorbyl

palmitate reduced the antioxidative effect of the propyl gallate/ascorbyl palmitate mix. No synergistic effect was shown of propyl gallate and ascorbyl palmitate in this study.

Based on the theory about synergistic effect of antioxidants, the mixes were believed to prevent oxidation of fish oils efficiently. A possible synergistic effect was only observed for mixed tocopherol/rosemary extract at 20 °C in both CLO and TG in this study. The antioxidants may not have been mixed in the right relation. The ratio between the antioxidants have shown to be crucial for the synergistic effect (Hamilton et al. 1998).

5.4 Evaluation of methods used

Reference samples were expected to be more oxidized than samples with added antioxidants. Based on PV and AV the reference samples were not oxidized as much as some of the samples with added antioxidants. Results even showed possible prooxidative effect from adding certain antioxidants. The reference samples without added antioxidants should be significantly higher to claim a significant antioxidative effect. The antioxidants were compared to each other relatively when evaluating PV, AV and TOTOX results. The least oxidized samples based on TOTOX were also most different from the reference samples in TG stored at 40 °C with regards to VOCs (figure 31).

Gas chromatography revealed high total areas of VOC in reference samples without antioxidants. Reference samples were expected to have the highest measured areas of VOCs. This implies high concentration of volatile secondary oxidation products. The PCA plots showed that reference values were different from most of the other samples for most volatiles. The presence of VOCs may provide a valuable tool for revealing information of the course of oxidation. In addition, measuring VOCs may better illustrate the oxidation progress with regard to development of sensory attributes. When evaluating extent of oxidation and antioxidative effect in fish oils, the sensory attributes are essential. Sensory rancidity related attributes are best explained by identification and quantification of volatile compounds by GC. VOCs represent volatile secondary oxidation products which have direct impact on taste and smell of fish oils. Both methods are useful tools that might reveal different reaction pathways in presence of antioxidants.

Primary oxidation products measured by PV have no effect on taste and smell. AV measures secondary oxidation products, but not all the compounds responsible for rancid taste and smell. Compared to PV and AV, headspace gas chromatography will provide more detailed information of volatiles responsible for rancidity. The three methods used for evaluation of oxidation reveal different types of information and therefore the methods may not always correlate. In this study reference samples without antioxidants were not among the samples with the highest PV and AV. GC on the other hand showed reference samples to have the highest content of most VOCs investigated. In a study by Hamilton et al. (1998) α-tocopherol, ascorbyl palmitate and lecithin showed no significant oxidation determined by PV the first six months of storage at 20 °C with access to oxygen. Certain volatiles, however, developed within three weeks (Hamilton et al. 1998). Traditionally PV and AV are used as quality criteria of fish oils. The study by Hamilton et al. (1998) illustrated the necessity of using more than PV and AV for measuring oxidation level in fish oils. Together the three applied methods give a more complete picture of extent of oxidation and antioxidant efficiency.

5.5 Further studies

This study was conducted with a time limit which restricted the extent of the study. Additional tasks can be done in further studies;

In this study some of the samples showed great variation between parallels. Due to time limitations there were only two parallels of each sample. It would be favorable to repeat the trial with a third parallel of all the samples. A third parallel would give more confidence to the data. A third parallel could also reveal more information securing right interpretation of results. When choosing one of the parallels for further discussion, incorrect interpretations may have occurred. The reasons for the divergent results earlier described would be interesting to know. With this information this can be prevented in further studies contributing to more consistent results.

No analyses were done directly after preparation of the samples. The reference samples may have been oxidized to a certain level faster than samples with antioxidants. This was shown

in a study done by Kulås et al. (2002). Several analyses could be conducted during the storage period to investigate development of volatiles. Antioxidants may give rise to different development of secondary and tertiary oxidation products. Tocopherol and tocopherol/ascorbyl palmitate were shown to inhibit formation of 1-penten-3-ol and 2-propenal (Olsen et al. 2005). The volatiles developed rapidly in the beginning of oxidation course, followed by decreased rate of formation. The results are dependent on time for analyses especially when the analyses are only conducted once. The results are only applied to the time of measurement. Antioxidants are able to direct the oxidative processes in fish oil. In this way they modify flavor development during storage (Kulås et al. 2003). Flavor compounds contribute to sensory attributes differently. Some volatiles are easier detected by human perception. Investigating development of volatiles during storage may provide important information with relevance to sensory acceptance. Analyses could be conducted several times during the storage period, including directly after preparation. More analyses may contribute to a better picture of the complex oxidation progress.

Cis and *trans* isomers may give more detailed information of course of oxidation. With more time available isomers of the selected VOCs can be investigated. This may give further information of development of oxidation and better understanding of oxidation mechanisms. Investigation of more than the 14 selected VOCs in this study may give relevant information. Other VOCs may also contribute to variation between samples. These may reveal even more information not detected in this study.

Both ascorbyl palmitate and tocopherol have shown to be dependent on concentration in their way of acting. Hamilton et al. (1998) demonstrated that ratio, rather than the total concentration of antioxidants in mixes, was crucial for synergistic effect (Hamilton et al. 1998). Other ratios within the mixes could be applied in a further study.

A study by Mäkinen et al. (2001) assumed that the effect of ascorbyl palmitate was due to its synergistic interactions with other antioxidants (Mäkinen et al. 2001). Ascorbyl palmitate has shown to be efficient as antioxidant in mix with tocopherol and lecithin (Kulås et al. 2003). Ascorbyl palmitate did not show synergistic effect with propyl gallate in this study. A mix with tocopherol and lecithin would be interesting to investigate in detail.

It would be interesting to compare the GC-MS results with sensory analysis to see the effect of two reaction pathways. This could answer the question whether greater amounts of a few volatiles give better sensory quality, rather than smaller amounts of several volatiles. Different antioxidants have shown to result in different volatiles present in oil. The antioxidants applied may give rise to variation in sensory attributes. GC-MS results have previously been shown to correlate well with sensory analysis and is a useful tool when consumer perception is important (Olsen et al. 2005).

Normal shelf-life of omega-3 products is two years. It would be interesting to evaluate the quality at the end of shelf-life. In this study the storage period consisted of 17 weeks with access to oxygen for the oils stored at 20 °C. The oxygen is believed to promote rate of oxidation to some extent. Storing oil samples at conditions close to real conditions for two years would give better picture of the effect of antioxidants. Samples from this study are stored further for later analyses to see effect of long-term storage. Further results will be correlated with results from this study as extension of the project run by G. O. Johnsen AS.

6. Conclusions

The study conducted investigated four commercially available antioxidants. The antioxidants were added to different fish oils both as single antioxidants and in mixes. The antioxidants were evaluated by three methods much used for evaluation of fish oils. There was less extent of oxidation in cod liver oil which had less concentration of omega-3 fatty acids. This was confirmed by the fatty acid compositions of cod liver oil (CLO) and concentrated triglycerides (TG). Accelerated storage conditions promoted high peroxide values (PV) and anisidine values (AV). Total oxidation values (TOTOX) are based on the history of the oil and the present state represented by PV and AV respectively. TOTOX values for both fish oils tested were higher at accelerated storage conditions (40 °C) compared to storage at room temperature (20 °C). The level of antioxidants had no significant effect on TOTOX.

The effect of ascorbyl palmitate and propyl gallate differed from the other antioxidants with regard to TOTOX. Ascorbyl palmitate in oil samples showed great variation in TOTOX. It

resulted in the highest measured TOTOX of all the samples. However, it also resulted in the lowest measured CLO sample together with the reference sample stored at 40 °C. As a sum up, this antioxidant was probably less efficient than the other antioxidants applied. Propyl gallate was more efficient than the other antioxidants in TG at both storage temperatures. In CLO stored at 20 °C rosemary extract gave the lowest measured TOTOX. Ascorbyl palmitate resulted in the lowest measured TOTOX in CLO stored at 40 °C. No synergistic effects of the mixes were seen on PV, AV or TOTOX.

14 volatile secondary oxidation products were chosen to be investigated in detail. The samples were evaluated in PCA plots to identify the most efficient antioxidants and contributors to variation between samples. 1-penten-3-ol was prominent as major contributor to variation in the data set based on both area and standardized area (1/sd). Other VOCs were also prominent depending on antioxidant, type of oil and storage temperature. Different VOCs indicated different antioxidants to be most efficient.

Propyl gallate and mixed tocopherol as single antioxidants were more efficient than the other antioxidants with regard to the 14 selected VOCs. Mixed tocopherol/rosemary extract showed synergistic effect in both CLO and TG at storage temperature of 20 °C. Synergistic effect of propyl gallate/ascorbyl palmitate was not observed in this study. The efficiency of the antioxidants applied seemed to depend on both oil type and storage temperature. The analyses of VOCs were useful for investigating effect of antioxidants in the two fish oils. Variation in VOC reflected the complexity in lipid oxidation processes. Due to time constraints the data generated could not be investigated further with regard to lipid chemistry.

There are many aspects connected to oxidation of fish oils and one has to consider each of them when choosing an antioxidant. The effects of antioxidant, type of fish oil and storage temperature have been demonstrated in this study.

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8. Appendices

Appendix 1

Table A1: Listing of chemicals and instruments used for measuring peroxide value (PV), anisidine value (AV), volatile compounds (GC-MS) and fatty acid composition (GC-FID):

Chemicals	Producer
Acetic acid (glacial)	*Merck*
Chloroform	*Merck*
Sodium thiosulfate solution 0.1 N	*Merck*
Starch ($C_6H_{10}O_5$)	*Merck*
Potassium iodide	*Merck*
p-Anisidine	*Merck*
Sodium sulphate (water free)	*Merck*
Active carbon	*Merck*
Isooctane	*Merck*
Benzene	*Merck*
Hydrochloride	*Merck*
2.2-dimethoxy propan	*Sigma-Aldrich*
Extern standard (68D)	*Nu-Check-Prep. Inc.*
n-Hexan	*Merck*
Sodium chloride (NaCl)	*Merck*
Sodium bicarbonate ($NaHCO_3$)	*Merck*
Formic acid	*Merck*
Iodide	*Merck*

Instruments	Producer
Magnet mixer	*Heigar/Framo-Gerätetechnik M21/1*
Weight	*Bergman/PG503-S Delta Range Mettler Toledo*
Spectrophotometer	*Agilent/8453 (2002)*
Gas chromatograph (GC-MS)	*Agilent/HP 6890 A (G1530A) (2000)*
Gas chromatograph (GC-FID)	*Agilent/HP 6890 A (2000)*
Mass spectrometer	*Agilent/5973 Mass Selective Detector(EI, 70eV) (2000)*
Flame ionization detector	*Agilent/G1530A (2000)*
Injector (GC-FID)	*Agilent/HP 6890 Injector (2000)*
Desorber (GC-MS)	*Markes/Ultra Thermal Desorber (2007)*
Adsorber (GC-MS)	*Tenax GR*
Column (GC-MS)	*DB-WAXetr column (30 m, 0.25 mm i.d., 0.5 µm film)*
Column (GC-FID)	*SGE BPX-70 capillary column (60 m, 0.25 mm i.d, 0.25 µm film)*
Software (GC-FID)	*Agilent/GC ChemStation Rev B.01.01 [164]SR1*
Software (GC-MS)	*Agilent/MSD ChemStation E.02.00.493*
Mass spectral library (GC-MS)	*NIST 20d. Des. 2 2005 Mass Spectral Library*
Flowmeter	*Agilent/ADM 1000*
Waterbath	*Geseltchaft für Labortechnik (GFL) D-30938 BurgWedel 1013*
Waterbath	*Grant Instruments*
Vortex mixer	*IKA/MS2 Minishaker*
Centrifuge	*Heraeus Sepatech Minifuge RF*
Numeric calculation (GC)	*Microsoft Excel 2007*
Statistics program (GC)	*The Unscrambler 9.8 (Camo Software)*
Statistics program (PV, AV, TOTOX)	*Minitab 16*

Appendix 2

Possible sources for deviation:

Some of the parallels showed much higher PV and AV values than the other parallel from the same sample. Ten out of 38 CLO samples stored at 20 °C showed abnormally high levels of oxidation. The TOTOX-values were ranging from 43.1 to 90.6. The reason for these high measured values is not known.

- Residues of water in the sample bottles and glasses → checked; no FFA present
- Time to oxidize before antioxidants were added or the bottles were flushed with nitrogen → would not give such an effect. Same treatment of oils and bottles, but the divergence was not seen in the other batch.
- Sunflower oil used for dissolving ascorbyl palmitate. Might have been a source of already oxidized products which are able to work as prooxidants → would not give such an effect
- The temperature was above 100 °C when dissolving ascorbyl palmitate to keep it dissolved. High temperatures may promote rapid formation of oxidation products → would not give such an effect
- Difficulties with dissolving ascorbyl palmitate may have led to little dispersion of ascorbyl palmitate in the oil → one of the parallels with much divergence was a reference sample
- Not correct concentration of antioxidant mixes → these samples did not have higher values than the reference samples
- Different exposure to air when preparing the samples → would not give such an effect
- Minor refined fish oil components may include cholesterol/cholesterol esters, α-TOH, free fatty acids, mono- and diacylglycerols, phospholipids, hydrocarbons, wax esters, alcohols, as well as oxidation products not removed in the refining and deodorization processes (Kulås et al. 2003). Compounds like these may cause higher levels of oxidation → would affect both parallels, not just one of them

Appendix 3

Table A2 Sum up of peroxide value (PV), anisidine value (AV) and TOTOX-value for each oil sample, including three levels of antioxidants. Results for cod liver oil (CLO) and concentrated triglycerides (TG) at both temperatures (40 °C and 20 °C) are shown separately. The values are based on three levels of antioxidants with two parallels of each blend. The antioxidants are ascorbyl palmitate (AP), mixed tocopherol (toco.), propyl gallate (PG), rosemary extract (rose.), mixed tocopherol/rosemary extract (toco./rose.) and propyl gallate/ascorbyl palmitate (PG/AP).

			PV	AV	TOTOX
AP	**CLO**	**40 °C**	7,92	4,92	20,91
	CLO	**20 °C**	4,92	5,01	14,86
	TG	**40 °C**	13,79	21,42	48,99
	TG	**20 °C**	2,47	15,62	20,55
Toco.	**CLO**	**40 °C**	9,67	4,96	24,29
	CLO	**20 °C**	4,33	4,84	13,50
	TG	**40 °C**	5,53	17,13	28,18
	TG	**20 °C**	2,91	16,19	22,01
PG	**CLO**	**40 °C**	8,02	5,60	21,63
	CLO	**20 °C**	4,37	4,99	13,74
	TG	**40 °C**	4,66	15,61	24,94
	TG	**20 °C**	1,99	13,31	17,30
Rose.	**CLO**	**40 °C**	11,78	7,55	31,11
	CLO	**20 °C**	4,18	4,41	12,77
	TG	**40 °C**	5,02	18,94	28,99
	TG	**20 °C**	1,84	17,31	20,99
PG/AP	**CLO**	**40 °C**	9,58	5,70	24,86
	CLO	**20 °C**	7,32	8,21	22,86
	TG	**40 °C**	5,05	18,11	28,22
	TG	**20 °C**	2,79	15,78	21,37
Toco./Rose.	**CLO**	**40 °C**	8,40	6,66	23,45
	CLO	**20 °C**	5,80	6,49	18,09
	TG	**40 °C**	8,36	18,96	35,69
	TG	**20 °C**	2,56	16,02	21,14
REF	**CLO**	**40 °C**	5,90	5,08	16,89
	CLO	**20 °C**	7,98	10,45	26,41
	TG	**40 °C**	6,21	17,10	29,51
	TG	**20 °C**	2,48	16,73	21,69

Appendix 4

Table A3: Sum up of all peroxide values (PV), anisidine values (AV) and total oxidation values (TOTOX) for each antioxidant. The antioxidants are ascorbyl palmitate (AP), mixed tocopherol (toco.), propyl gallate (PG), rosemary extract (rose.), mixed tocopherol/rosemary extract (toco./rose.) and propyl gallate/ascorbyl palmitate (PG/AP). The values from the two oils, each level of inclusion and both storage temperatures are merged. This table shows the variation in PV, AV and TOTOX values. These values are the basis for the box plot in figure 20.

	PV	**AV**	**TOTOX**
AP	6.07 (2.35-14.73)	10.62 (3.87-21.83)	20.44 (13.31-50.69)
Toco.	4.55 (2.44-12.05)	10.66 (4.17-17.84)	22.86 (12.64-31.66)
PG	4.09 (1.67-9.56)	9.23 (4.55-15.91)	18.18 (11.55-28.02)
Rose.	4.25 (1.54-12.22)	11.80 (3.63-20.57)	23.76 (11.71-32.88)
PG/AP	5.89 (2.55-12.30)	12.16 (5.08-19.66)	23.06 (18.82-31.86)
Toco./Rose.	6.19 (2.00-12.23)	11.26 (5.89-20.35)	21.67 (17.84-44.81)

Appendix 5

Table A4: Fatty acid composition of a selection of samples from both cod liver oil (CLO) and concentrated triglycerides (TG). The selection represents both storage temperatures, both oils, all three levels and four antioxidants and reference samples are represented. The fatty acids studied are eight of the most common fatty acids present in fish oil. There is especially focus on the fatty acids 20:5 n-3 (EPA) and 22:6 n-3 (DHA). This is due to exposure to oxidation and association to health benefits.

		14:0	**16:0**	**16:1**	**18:1 n-9**	**18:1 n-7**	**20:1 n-9**	**22:1**	**20:5 n-3**	**22:6 n-3**
CLO	Toco.	4,9	12,4	11,4	24,0	6,7	14,7	0,8	11,8	13,4
	Toco.	4,9	12,4	11,4	24,1	6,7	14,6	0,8	11,8	13,3
	Toc/rose	4,8	12,5	11,2	24,1	6,7	14,9	0,8	11,6	13,4
	Toc/rose	4,8	12,4	11,3	24,1	6,7	14,7	0,8	11,7	13,4
	REF	4,9	12,4	11,5	24,3	6,8	14,7	0,8	11,7	13,0
	REF	4,8	12,6	11,3	24,2	6,7	15,0	0,8	11,4	13,2
	REF	4,7	12,4	10,9	24,1	6,7	15,3	0,9	11,4	13,6
TG	AP	1,0	2,0	1,5	3,9	1,2	2,4	0,9	22,4	64,7
	AP	1,0	2,0	1,5	3,9	1,2	2,4	0,9	22,3	64,7
	Rose.	1,0	2,0	1,5	3,9	1,3	2,4	0,9	22,3	64,8
	Rose.	1,0	2,0	1,5	3,9	1,2	2,4	0,9	22,3	64,9
	REF	1,0	2,0	1,5	3,9	1,3	2,4	0,9	22,2	64,7
	REF	1,0	2,0	1,5	3,8	1,2	2,3	3,2	21,8	63,2
	REF	1,0	2,0	1,5	3,8	1,3	2,3	3,2	21,8	63,2

Appendix 6

Statistical analysis of total oxidation values (TOTOX). TOTOX is based on peroxide value (PV) and anisidine value (AV) and gives a more complete picture of the status of oxidation. A general linear model with three factors was used to evaluate oxidation status (antioxidant x oil x temperature). Tukey Method with groupings was used to see if the antioxidant blends had different effect on TOTOX value.

General Linear Model: TOTOX_1 versus A; O; T

```
Factor  Type   Levels  Values
A       fixed       6  1; 2; 3; 4; 5; 6
O       fixed       2  1; 2
T       fixed       2  20; 40


Analysis of Variance for TOTOX_1, using Adjusted SS for Tests

Source  DF   Seq SS   Adj SS   Adj MS       F      P
A        5   345,77   345,77    69,15    5,60  0,000
O        1   727,43   727,43   727,43   58,89  0,000
T        1  1863,00  1863,00  1863,00  150,81  0,000
A*O      5   484,60   484,60    96,92    7,85  0,000
A*T      5   302,03   302,03    60,41    4,89  0,001
O*T      1    56,31    56,31    56,31    4,56  0,038
A*O*T    5   496,95   496,95    99,39    8,05  0,000
Error   48   592,96   592,96    12,35
Total   71  4869,05


S = 3,51472   R-Sq = 87,82%   R-Sq(adj) = 81,99%


Unusual Observations for TOTOX_1

Obs  TOTOX_1      Fit  SE Fit  Residual  St Resid
 14  30,8360  24,8643  2,0292    5,9717      2,08 R
 15  18,9340  24,8643  2,0292   -5,9303     -2,07 R
 32  29,9830  22,8550  2,0292    7,1280      2,48 R
 52  44,8140  35,6853  2,0292    9,1287      3,18 R

R denotes an observation with a large standardized residual.


Grouping Information Using Tukey Method and 95,0% Confidence

A   N  Mean  Grouping
1  12  26,3  A
6  12  24,6  A B
5  12  24,3  A B
4  12  23,5  A B C
2  12  22,0    B C
3  12  19,4      C

Means that do not share a letter are significantly different.
```

Appendix 7

Loading plot illustrating major contributors to variation which are seen in the related score plot.

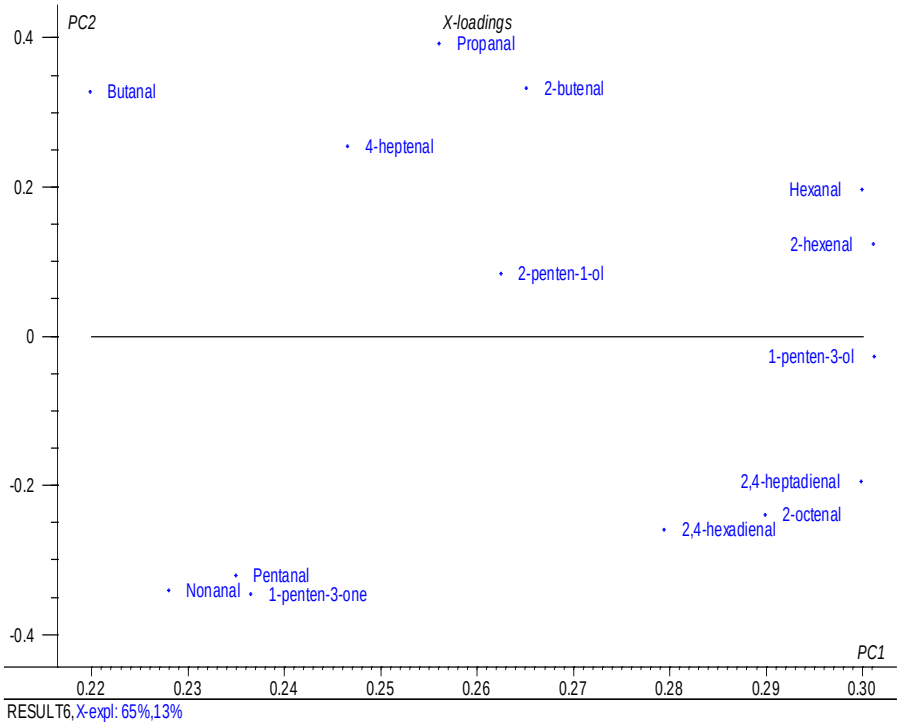

Figure A1 Loading plot illustrating variation between the samples of cod liver oil (CLO) stored at 40 °C and 20 °C. The antioxidants used are ascorbyl palmitate, propyl gallate and propyl gallate/ascorbyl palmitate. Reference samples are also included.

Appendix 8

Loading plots including all the antioxidants in both types of oil and storage temperature. The loading plots are presenting the contributors to variation between samples.

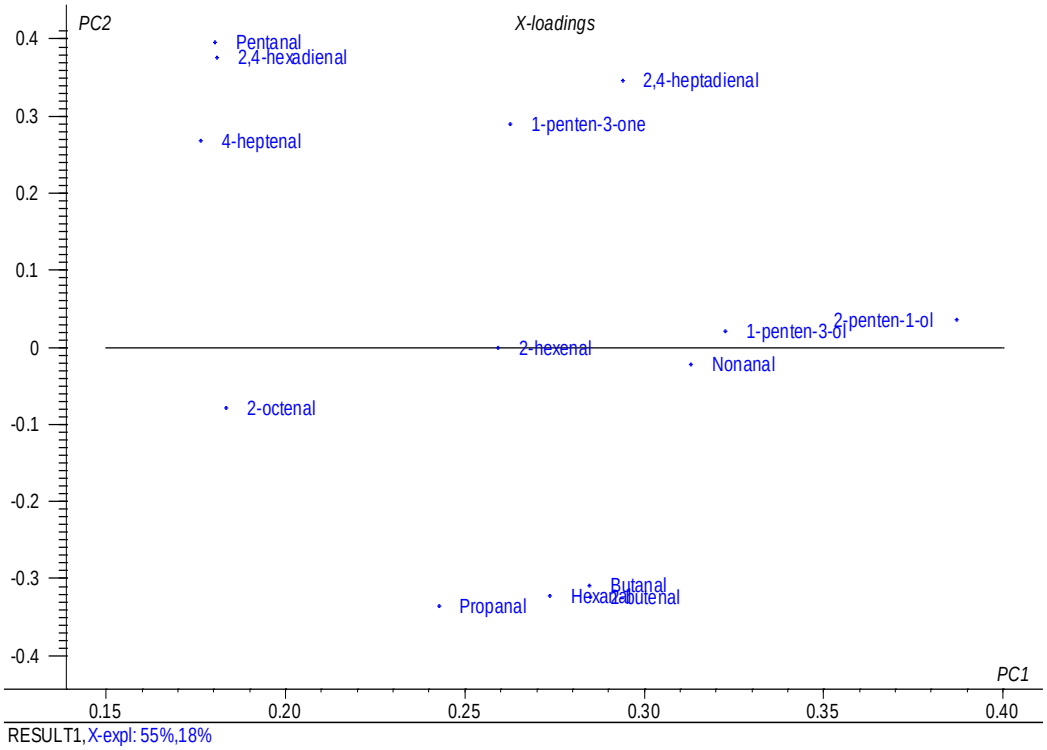

Figure A2: Loading plot for volatile organic compounds (VOC) explaining the variation in cod liver oil (CLO) samples stored at 40 °C. The plot includes all six antioxidant blends together with the reference samples.

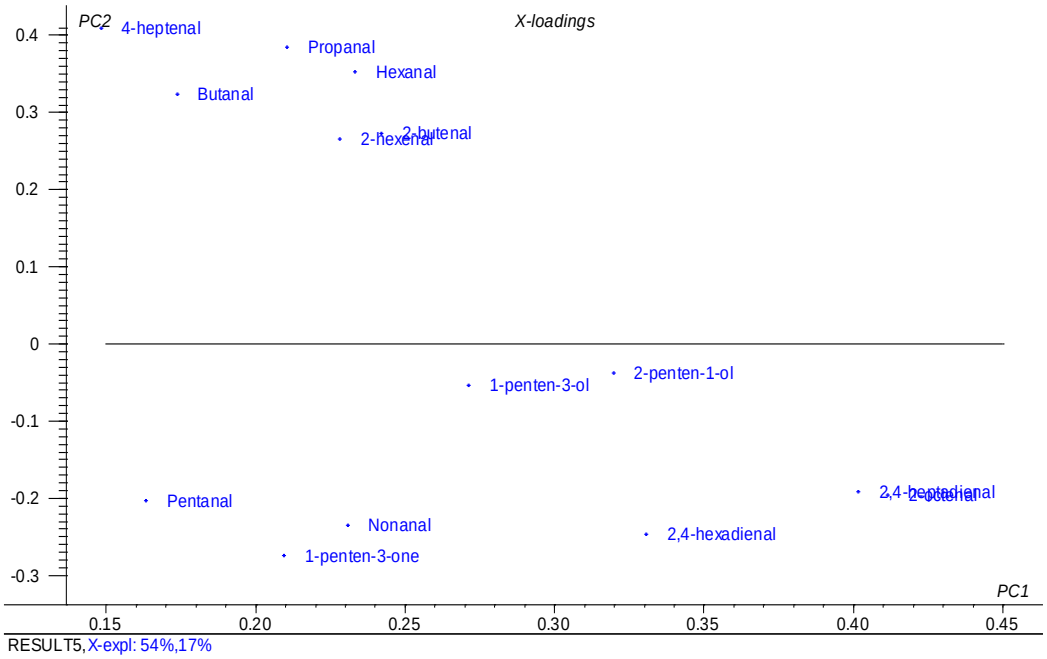

Figure A3: Loading plot for volatile organic compounds (VOC) explaining the variation in cod liver oil (CLO) stored at 20 °C. The plot includes all six antioxidant blends together with the reference samples.

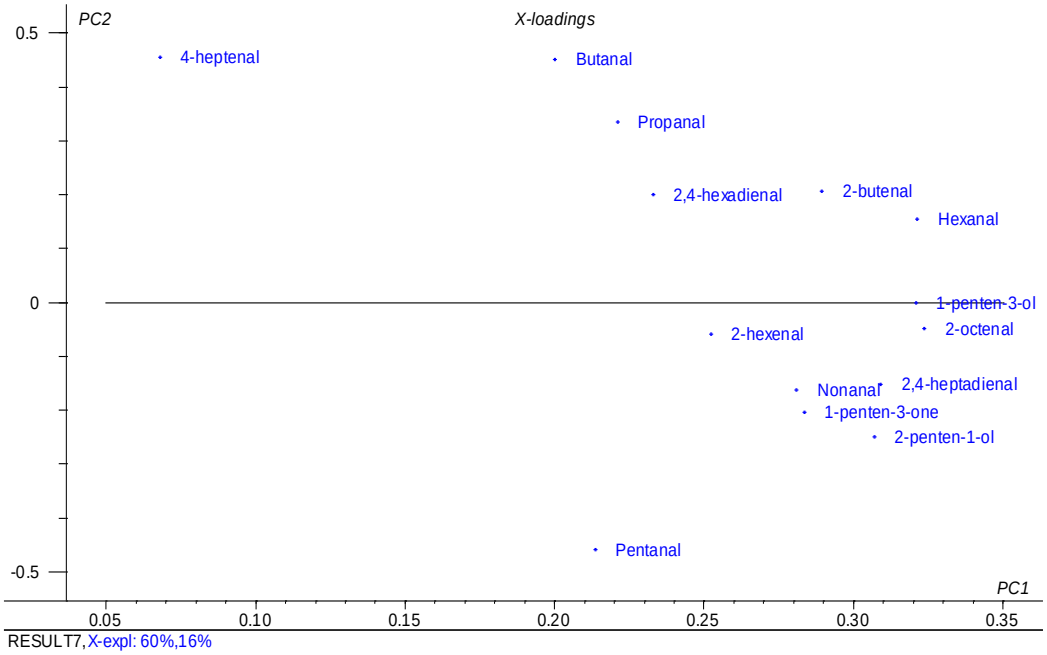

Figure A4: Loading plot for volatile organic compounds (VOC) explaining the variation in concentrated triglycerides (TG) stored at 40 °C. The plot includes all six antioxidant blends together with the reference samples.

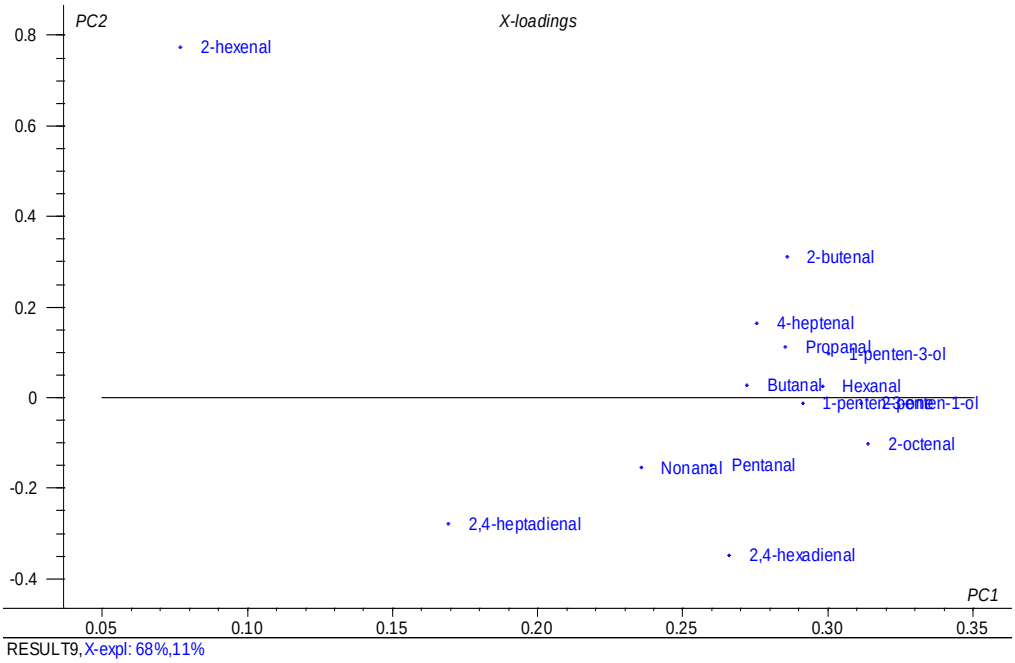

Figure A5: Loading plot for volatile organic compounds explaining the variation in concentrated triglycerides (TG) stored at 20 °C. The plot includes all six antioxidant blends together with the reference samples.

Appendix 9

PCA plots with attention to the mix propyl gallate/ascorbyl palmitate are presented in figures below. Both score plots and loading plots are presented. This illustrates the variation between the samples due to specific volatile organic compounds (VOC).

Ascorbyl palmitate (X1), propyl gallate (X3) and propyl gallate/ascorbyl palmitate (X5):

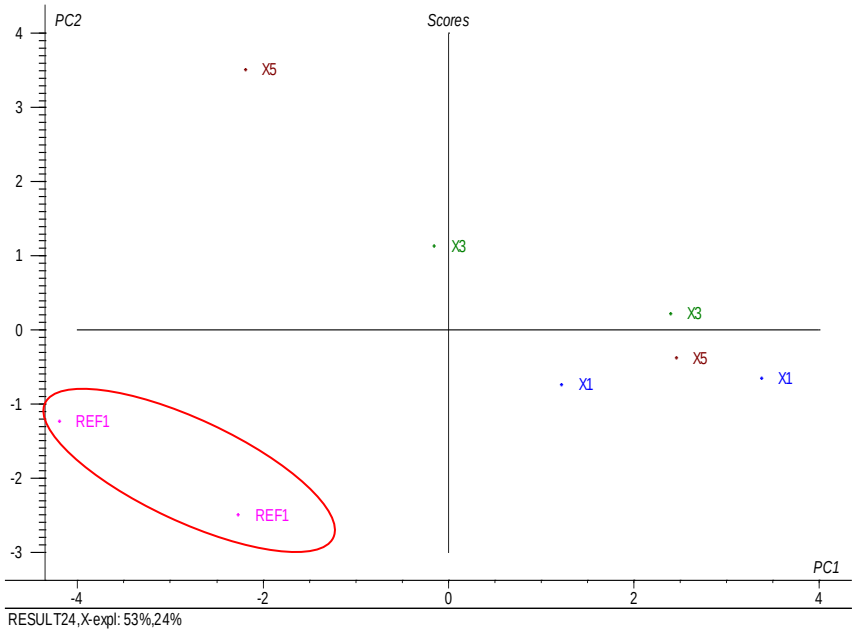

Figure A6: Score plot of cod liver oil (CLO) samples stored at 40 °C. Ascorbyl palmitate (X1), propyl gallate (X3) and propyl gallate/ascorbyl palmitate (X5) are presented in the plot together with reference samples.

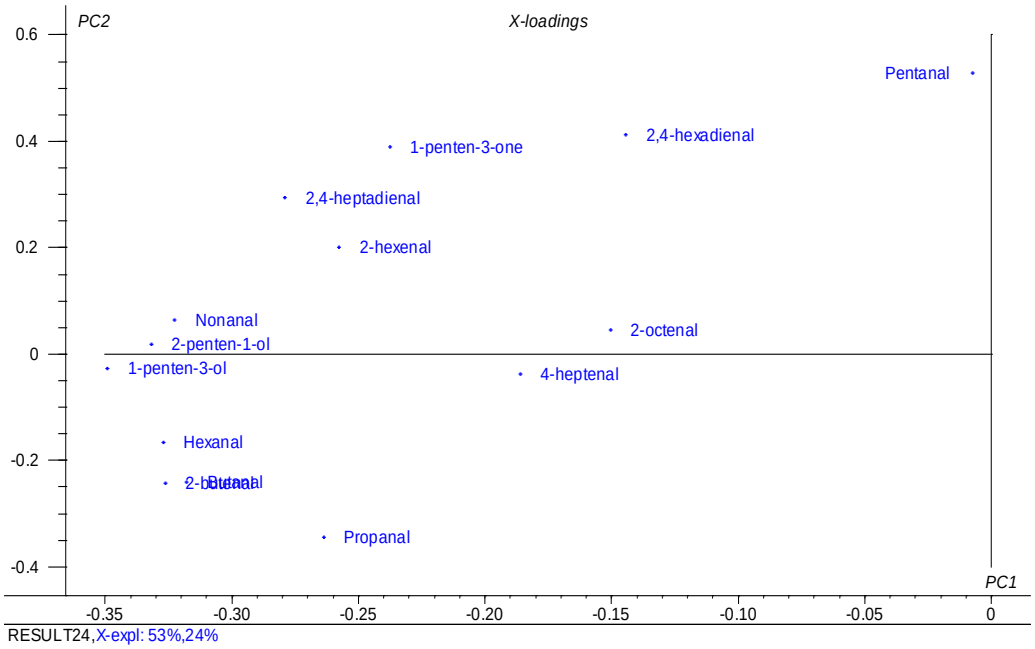

Figure A7: Loading plot of cod liver oil (CLO) samples stored at 40 °C. The plot is based on samples with ascorbyl palmitate (X1), propyl gallate (X3) and propyl gallate/ascorbyl palmitate (X5) together with reference samples. The volatile organic compounds (VOC) explain the variation in the score plot (see also figure A6).

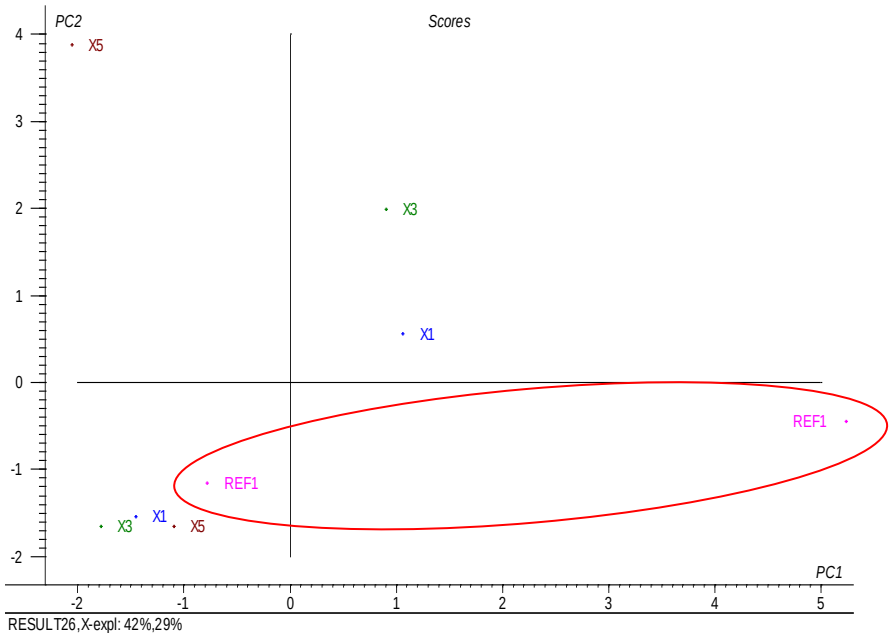

Figure A8: Score plot of cod liver oil (CLO) samples stored at 20 °C. Ascorbyl palmitate (X1), propyl gallate (X3) and propyl gallate/ascorbyl palmitate (X5) are presented in the plot together with reference samples.

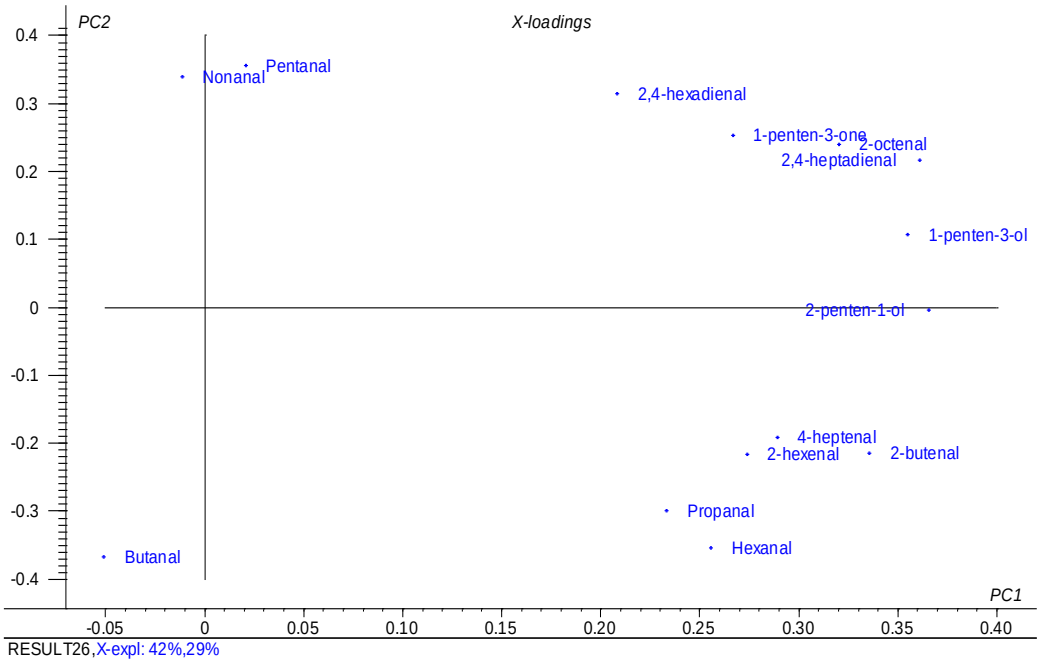

Figure A9: Loading plot of cod liver oil (CLO) samples stored at 20 °C. The plot is based on samples with ascorbyl palmitate (X1), propyl gallate (X3) and propyl gallate/ascorbyl palmitate (X5) together with reference samples. The volatile organic compounds (VOC) explain the variation in the score plot (see also figure A8).

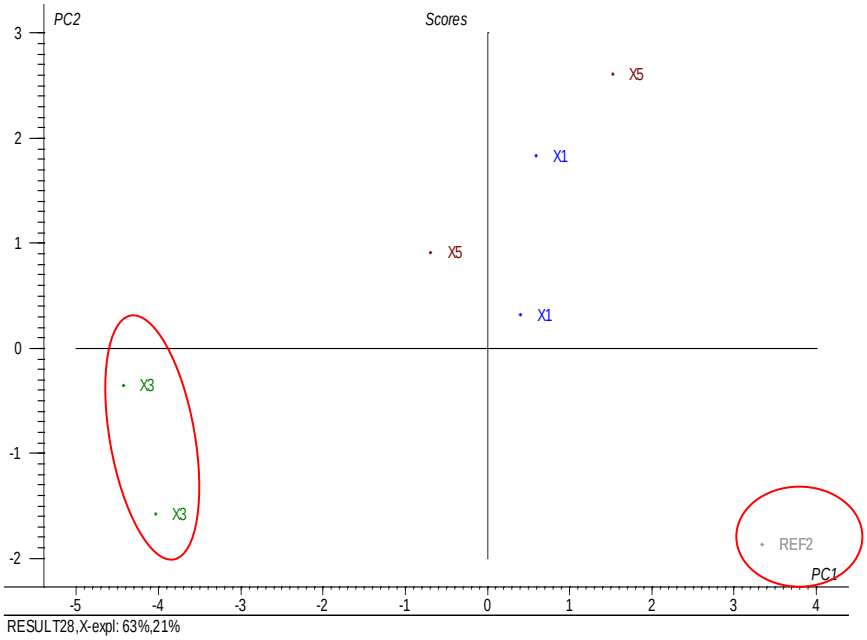

Figure A10: Score plot of concentrated triglycerides (TG) stored at 40 °C. Ascorbyl palmitate (X1), propyl gallate (X3) and propyl gallate/ascorbyl palmitate (X5) are presented in the plot together with reference samples.

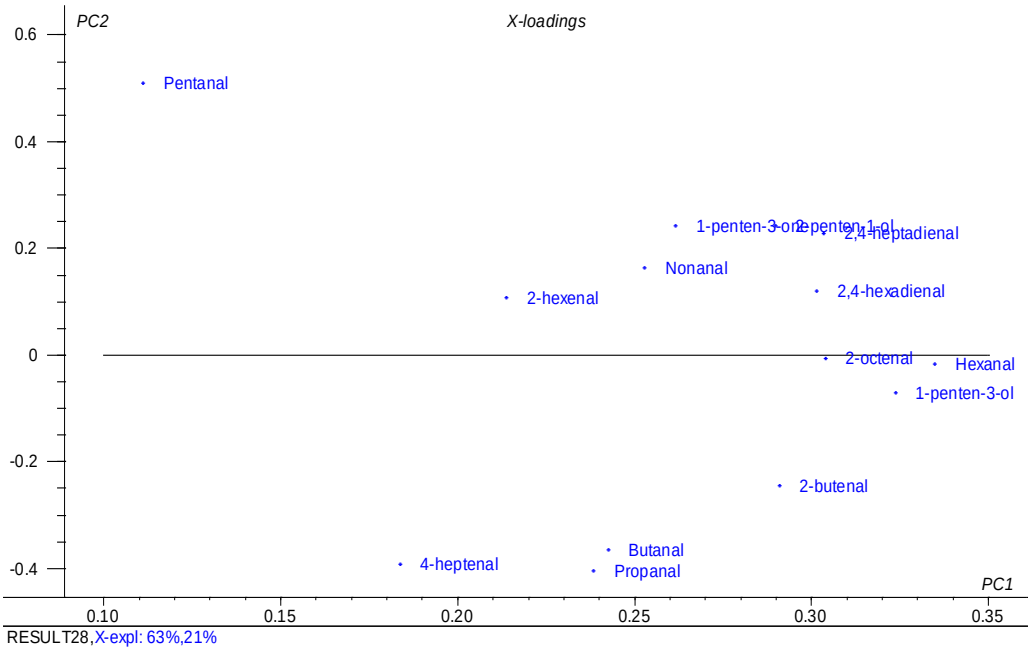

Figure A11: Loading plot of concentrated triglycerides (TG) stored at 40 °C. The plot is based on samples with ascorbyl palmitate (X1), propyl gallate (X3) and propyl gallate/ascorbyl palmitate (X5) together with reference samples. The volatile organic compounds (VOC) explain the variation in the score plot (see also figure A10).

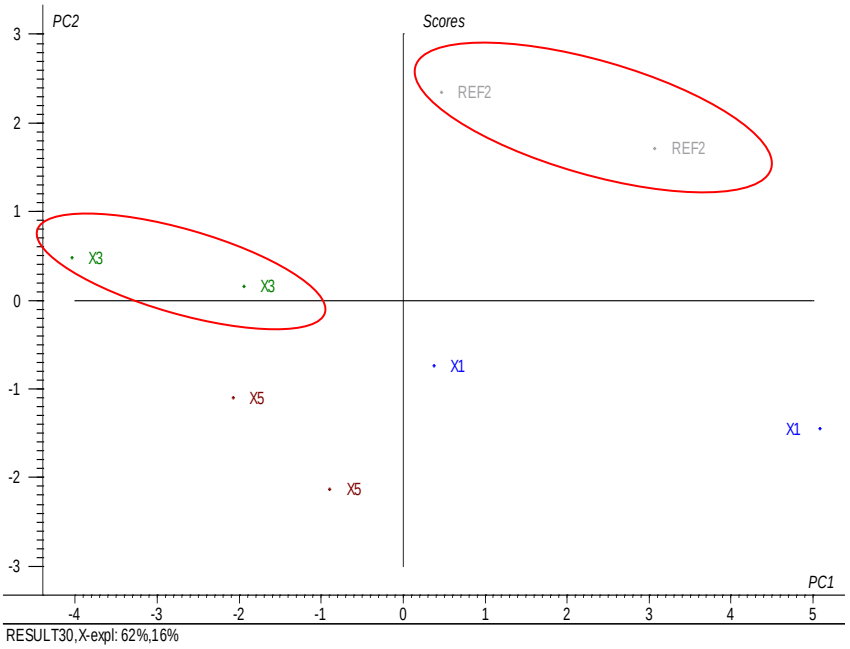

Figure A12: Score plot of concentrated triglycerides (TG) stored at 20 °C. Ascorbyl palmitate (X1), propyl gallate (X3) and propyl gallate/ascorbyl palmitate (X5) are presented in the plot together with reference samples.

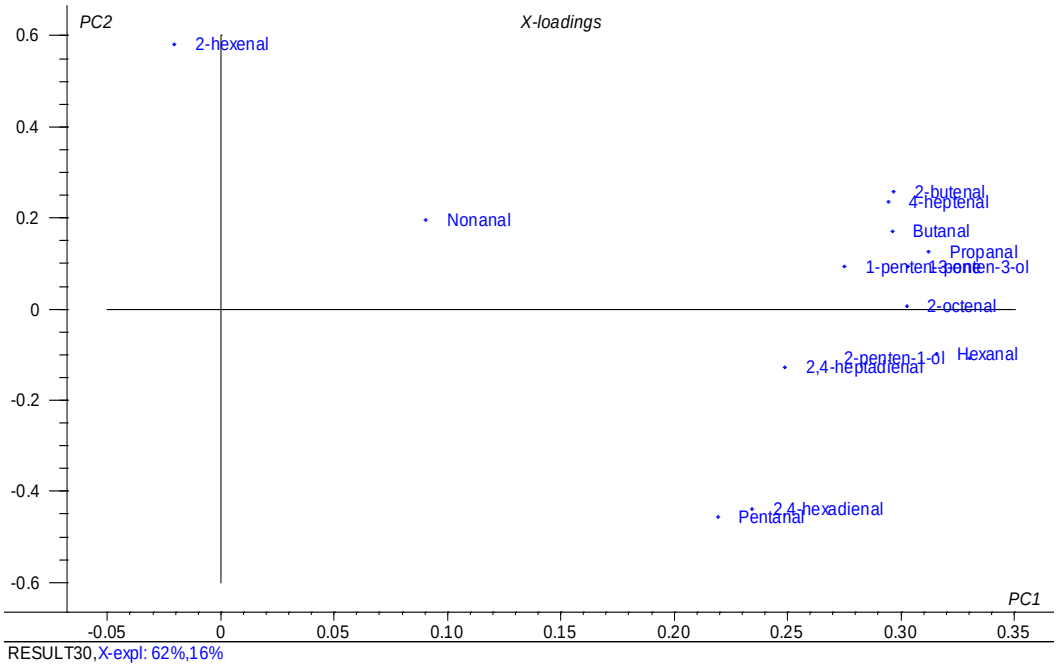

Figure A13: Loading plot of concentrated triglycerides (TG) stored at 20 °C. The plot is based on samples with ascorbyl palmitate (X1), propyl gallate (X3) and propyl gallate/ascorbyl palmitate (X5) together with reference samples. The volatile organic compounds (VOC) explain the variation in the score plot (see also figure A12).

Appendix 10

PCA plots with attention to the mix mixed tocopherol/rosemary extract are presented in figures below. Both score plots and loading plots are presented. This illustrates the variation between the samples due to specific volatile organic compounds (VOC).

Mixed tocopherol (X2), rosemary extract (X4) and mixed tocopherol/rosemary extract (X6):

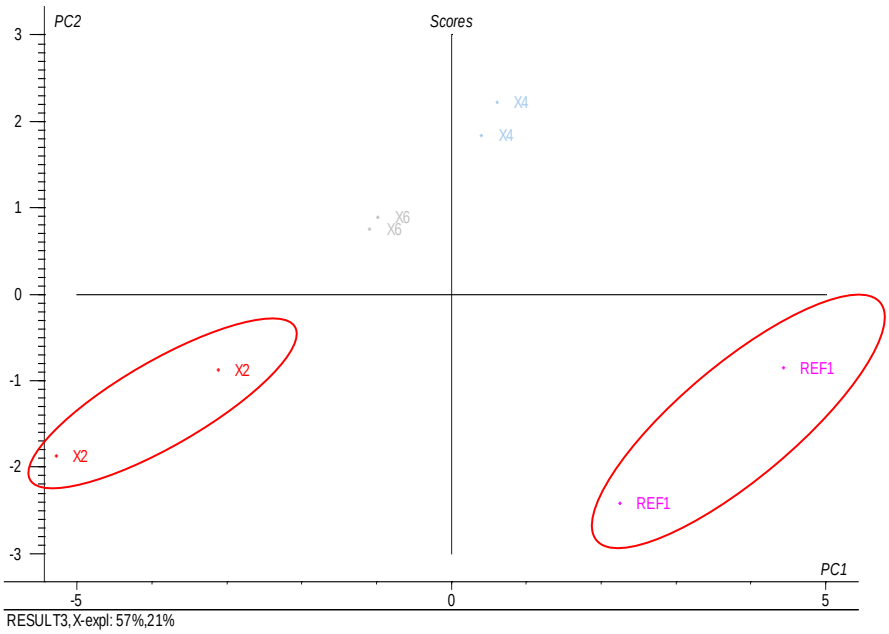

Figure A14: Score plot of cod liver oil (CLO) samples stored at 40 °C. Mixed tocopherol (X2), rosemary extract (X4) and mixed tocopherol/rosemary extract (X6) are presented in the plot together with reference samples.

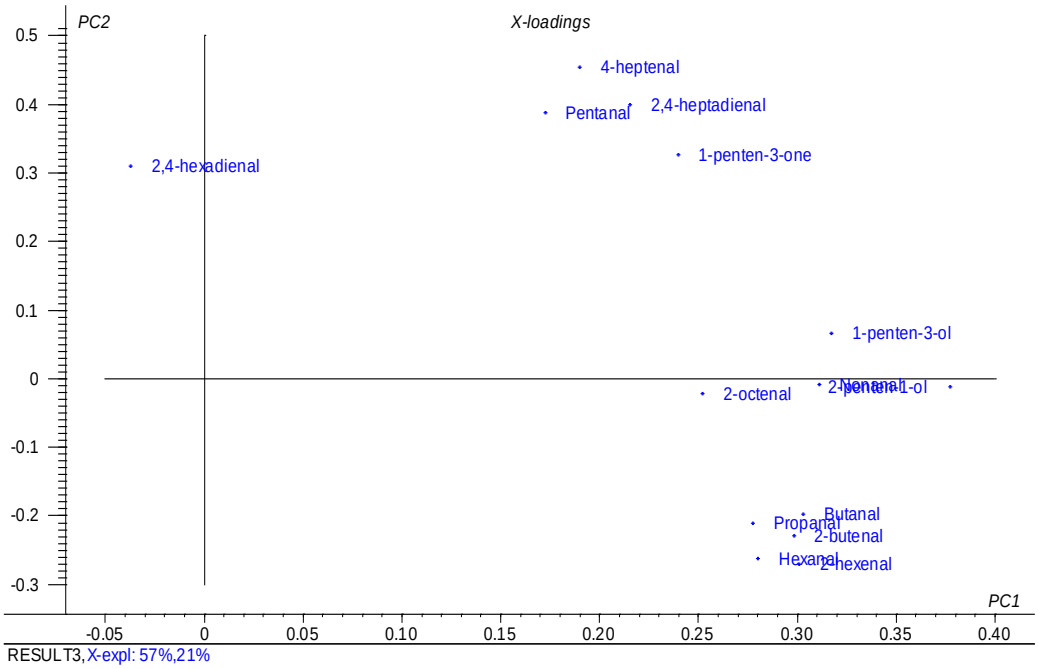

Figure A15: Loading plot of cod liver oil (CLO) samples stored at 40 °C. The plot is based on samples with mixed tocopherol (X2), rosemary extract (X4) and mixed tocopherol/rosemary extract (X6) together with reference samples. The volatile organic compounds (VOC) explain the variation in the score plot (see also figure A14).

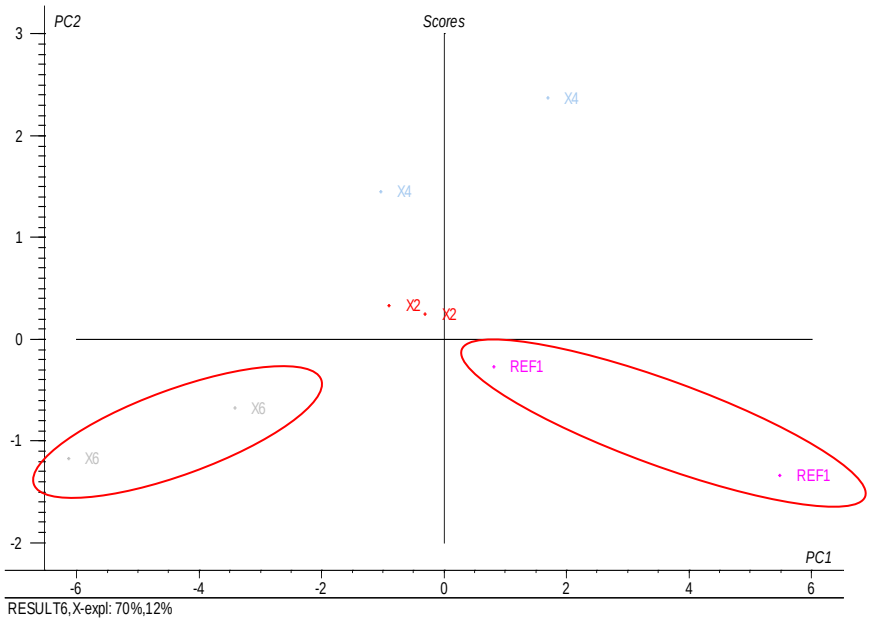

Figure A16: Score plot of cod liver oil (CLO) samples stored at 20 °C. Mixed tocopherol (X2), rosemary extract (X4) and mixed tocopherol/rosemary extract (X6) are presented in the plot together with reference samples.

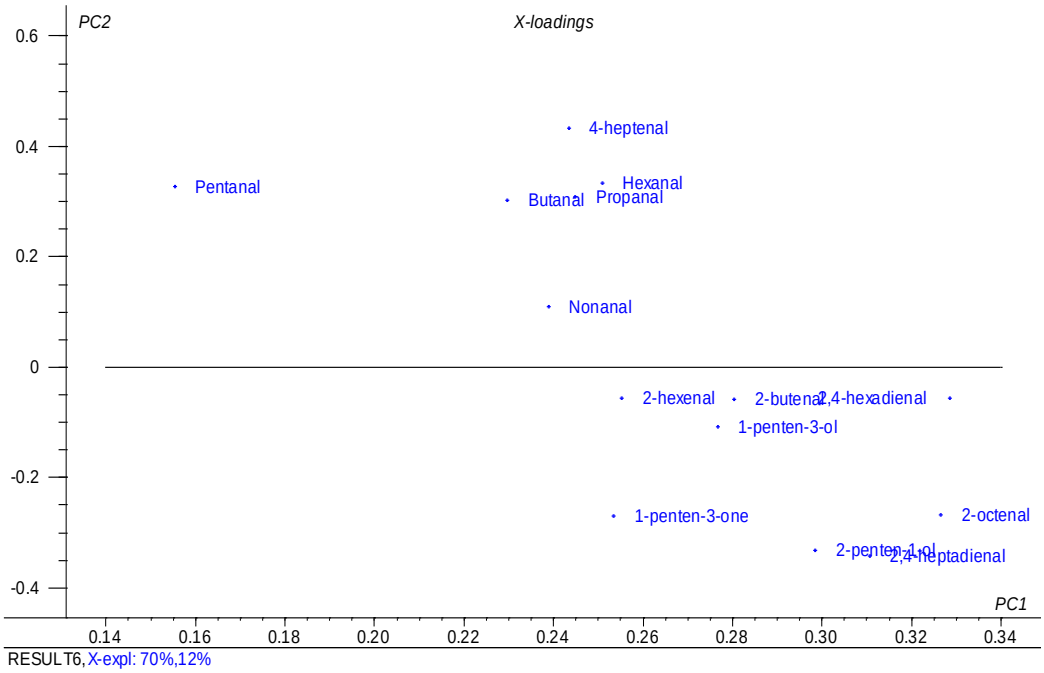

Figure A17: Loading plot of cod liver oil (CLO) samples stored at 20 °C. The plot is based on samples with mixed tocopherol (X2), rosemary extract (X4) and mixed tocopherol/rosemary extract (X6) together with reference samples. The volatile organic compounds (VOC) explain the variation in the score plot (see also figure A16).

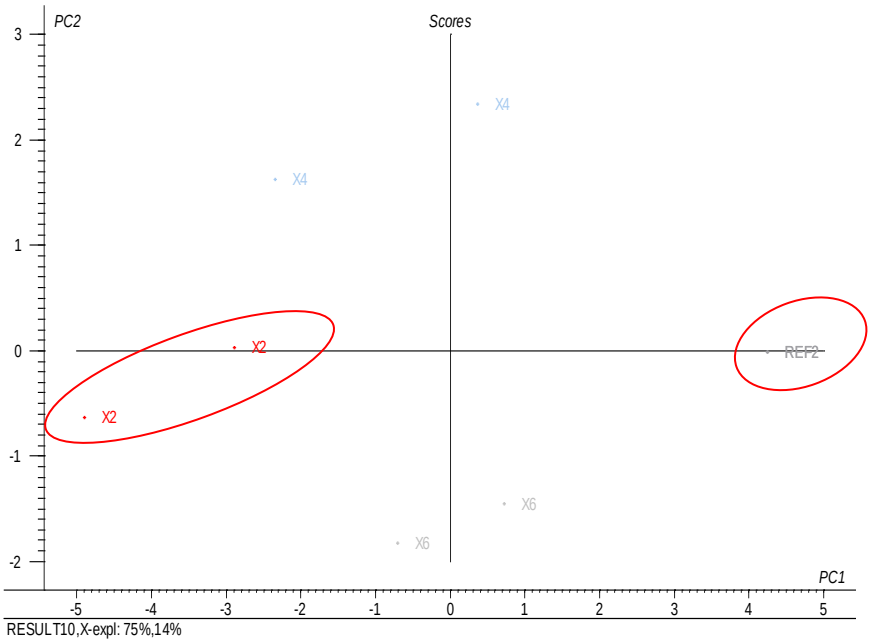

Figure A18: Score plot of concentrated triglycerides (TG) samples stored at 40 °C. Mixed tocopherol (X2), rosemary extract (X4) and mixed tocopherol/rosemary extract (X6) are presented in the plot together with reference samples.

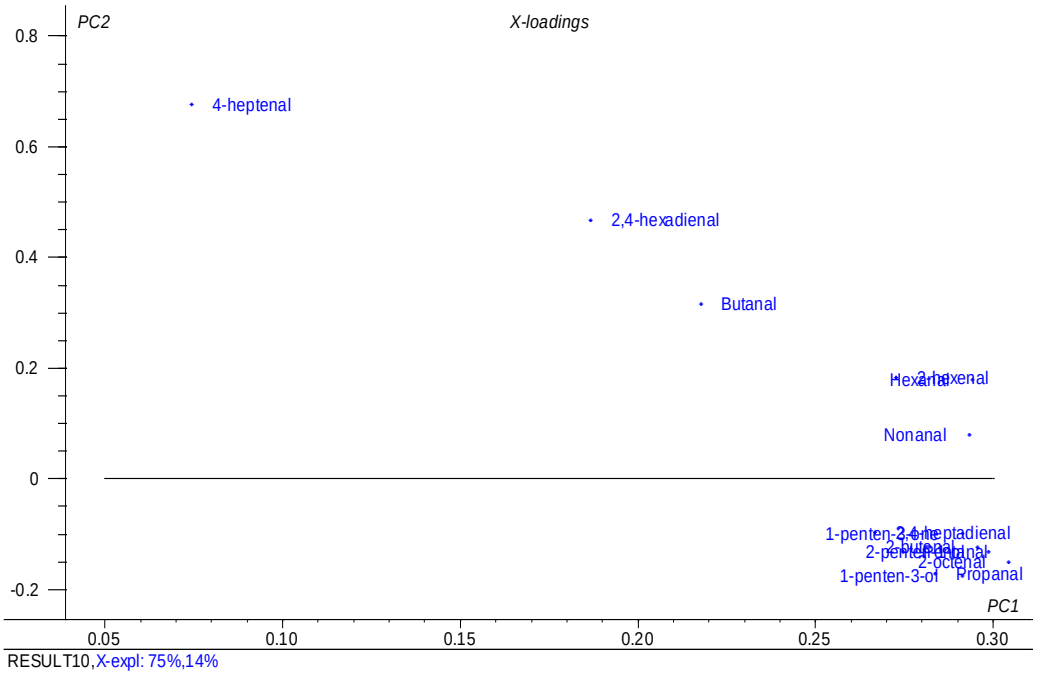

Figure A19: Loading plot of concentrated triglycerides (TG) samples stored at 40 °C. The plot is based on samples with mixed tocopherol (X2), rosemary extract (X4) and mixed tocopherol/rosemary extract (X6) together with reference samples. The volatile organic compounds (VOC) explain the variation in the score plot (see also figure A18).

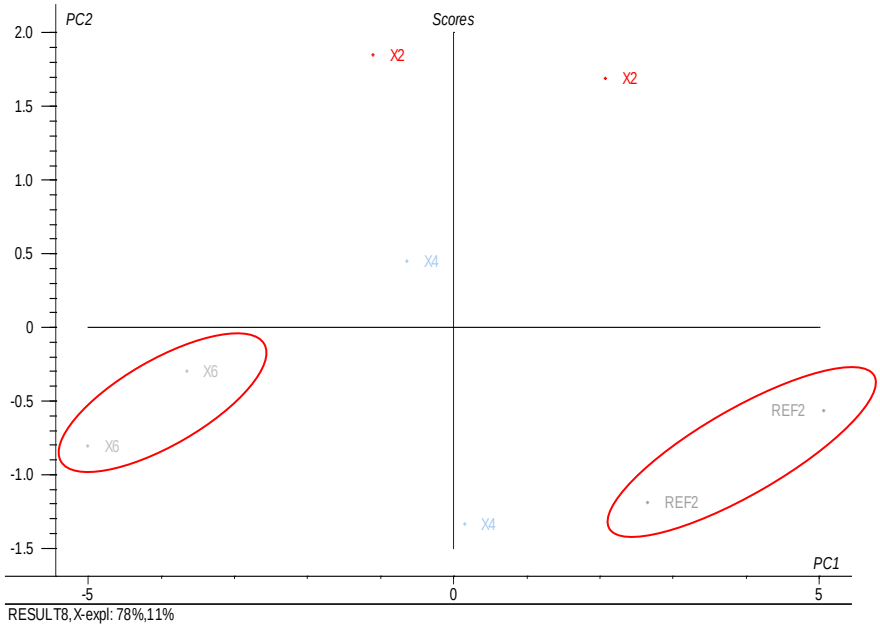

Figure A20: Score plot of concentrated triglycerides (TG) samples stored at 20 °C. Mixed tocopherol (X2), rosemary extract (X4) and mixed tocopherol/rosemary extract (X6) are presented in the plot together with reference samples.

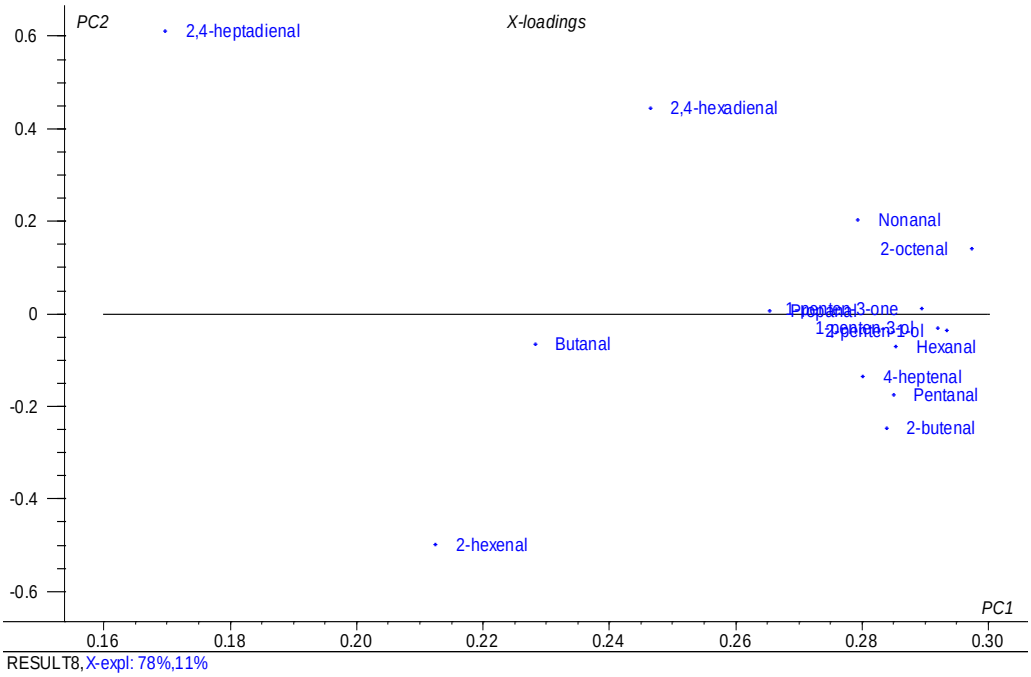

Figure A21: Loading plot of concentrated triglycerides (TG) samples stored at 20 °C. The plot is based on samples with mixed tocopherol (X2), rosemary extract (X4) and mixed tocopherol/rosemary extract (X6) together with reference samples. The volatile organic compounds (VOC) explain the variation in the score plot (see also figure A20).