Master's thesis

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Oxidation of Fish Oil and Powder

Exploring Suitable Methods for the Determination of Oxidative Changes in Fish Oil and Powder

Master's thesis in Food Science, Technology and Sustainability
Supervisor: Eva Falch
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Thea Vikingstad

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Summary

Lipid oxidation is one of the major causes of deterioration of foods, limiting the ability for enrichment of marine n-3 fatty acids in food products. The beneficial health effects of long-chain n-3 PUFA are well documented, but many people do not meet their daily need for n-3 due to the undesirable fishy flavor and smell caused by lipid oxidation. Analytical methods that can detect oxidative changes in complex food matrixes are highly needed, and the interest in rapid methods is great. However, there are extensive challenges and limitations associated with the traditional methods.

This study investigated suitable methods for the determination of oxidative changes in fish oil and powder by conducting a storage experiment for up to 4 weeks at 40 °C with oxygen access. This research contributes to filling an information gap regarding a method of measuring oxidative changes in fish powder without extracting the powder, the main goal of this study. GC-MS was found to be a suitable method for directly determining the oxidative status in fish powder without an extraction step that may aggravate the oxidative state.

The analyzes in this study found no evidence that the powder was more stable than the oil, or that the powder protected and stabilized the oil in the powder. The powder seemed initially more oxidized compared to the oil and oxidized faster than the oil. Both the powder and oil were stored with oxygen access (to capture the volatile compounds), which may have aggravated the oxidative state during storage in the freezer compared to if the samples had been flushed with nitrogen before storage. Based on the results of this study, dynamic headspace GC-MS analysis provided useful information in the research on oxidative changes in fish oil and powder. In the process, GC-MS method parameters for the analysis of volatile components in fish oil and powder were optimized.

This study also compared the traditional methods for determining PV and AV with the rapid method, CDR FoodLab, to examine how suitable the rapid method is against the traditional methods. Strong correlations were found between the methods.

Peroxide value (PV) was used as a parameter for primary oxidation products, and Anisidine value (AV), Thiobarbituric acid reactive substances (TBARS), and Gas Chromatography-Mass Spectroscopy (GC-MS) were used to analyze secondary oxidation products.

Sammendrag

Lipidoksidasjon (harskning) er en av hovedårsakene til kvalitetsforringelse i mat, noe som begrenser muligheten for anrikning av marine n-3 fettsyrer i matvarer. De gunstige helseeffektene av langkjeda n-3 flerumettede fettsyrer er godt dokumentert, men mange mennesker dekker ikke sitt daglige behov av n-3 på grunn av den uønskede fiskesmaken og lukten forårsaket av lipidoksidasjon. Analysemetoder som kan måle oksidative endringer i komplekse matmatriser er høyst nødvendig, og interessen for raske metoder er stor. Det er imidlertid omfattende utfordringer og begrensninger knyttet til de tradisjonelle metodene.

Denne studien undersøkte egnede metoder for å bestemme oksidative endringer i fiskeolje og pulver ved å utføre et lagringseksperiment i opptil 4 uker ved 40 °C med oksygentilgang. Denne forskningen bidrar til å fylle et behov for informasjon angående en metode til å måle oksidative endringer i fiskepulver uten å ekstrahere ut oljen i pulveret, hovedmålet med denne oppgaven. GC-MS ble funnet til å være en egnet metode for å bestemme den oksidative statusen i fiskepulver direkte, uten et ekstraksjonstrinn som kan forverre den oksidative tilstanden.

Analysene i denne studien fant ingen holdepunkt for at pulveret var mer stabilt enn oljen, eller at pulveret beskyttet og stabiliserte oljen i pulveret. Pulveret virket i utgangspunktet mer oksidert sammenlignet med oljen, og oksiderte raskere enn oljen. Både pulveret og oljen ble lagret med oksygentilgang (for å fange de flyktige forbindelsene), noe som kan ha forverret den oksidative tilstanden under lagring i fryser sammenlignet med om prøvene hadde blitt flushet med nitrogen før lagring. Basert på resultatene i denne studien ga dynamisk headspace GC-MS-analyser nyttig informasjon i forskningen på oksidative endringer i fiskeolje og pulver. I prosessen ble GC-MS metodeparametere for analyse av flyktige komponenter i fiskeolje og pulver optimalisert.

Denne studien sammenlignet også de tradisjonelle metodene for å bestemme PV og AV med den raske metoden, CDR FoodLab, for å undersøke hvor egnet den raske metoden er mot de tradisjonelle metodene. Det ble funnet sterke korrelasjoner mellom metodene.

Peroksidverdi (PV) ble brukt som en parameter for primære oksidasjonsprodukt, og Anisidinverdi (AV), Tiobarbitursyrereaktive stoffer (TBARS), og Gasskromatografi-Massespektroskopi (GC-MS) ble brukt til å analysere sekundære oksidasjonsprodukter.

Abbreviations

AHA – American Heart Association

ANOVA – Analysis of Variance

AOCS – American Oil Chemists' Society

AV – Anisidine value

BHT – Butylated hydroxytoluene

DHA – Docosahexaenoic acid

DPA – Docosapentaenoic acid

EE – Ethyl ester

EPA – Eicosapentaenoic acid

FFA – Free fatty acids

GC-MS – Gas Chromatography-Mass Spectroscopy

GOED – The Global Organization for EPA and DHA omega-3s

HS – Headspace

IS – Internal standard

ISO – International Organization for Standardization

MA – Malonaldehyde

meq – milliequivalent

n-3 fatty acids – Omega-3 fatty acids

nm – nanometer

ppb – parts per billion

PV – Peroxide value

SDG – Sustainable Development Goals

TBA – Thiobarbituric acid

TBARS – Thiobarbituric acid reactive substances

TCA – Trichloroacetic acid

TEP – Triethyl Phosphate

TG – Triacylglycerol

TOTOX – The total oxidation value

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1. Introduction

The ocean has great potential to provide healthy and sustainable foods to a growing population as the ocean covers 70% of the planet, but only provides 5% of our food (MarineStewardshipCouncil, 2021). The population growth and the increasing life expectancy will lead to a growing need for food. To minimize food loss, a better and more sustainable use of the earth's resources is needed, especially in the oceans.

There are several definitions of marine rest raw materials. Usually meaning viscera, heads, cut-offs, bone, skin, fish that is damaged or unsuitable for human consumption or further processing, and bycatch (Rustad, 2002). Today 35% of global catches are lost or wasted and therefore not utilized when discards (rest raw material) before landing are included (Ana Carvajal et al., 2020). In the whitefish industry, a significant part of the rest raw material goes to waste in the sea or early in the value chain (Jouvenot, 2015) and ends up as low-value products. Only 50% of the whitefish is put to profitable use, meaning that there is an immense potential for higher utilization in the whitefish sector (Ana Carvajal et al., 2020), e.g., the production of fish oils from rest raw materials.

Today, we miss out on large, valuable resources along the Norwegian coast, which we buy elsewhere. Increased utilization and value creation from the rest raw materials from whitefish can contribute to improving the circular economy, which will lead to less pressure on nature, lower greenhouse gas emissions, and less pollution. Rest raw material contains valuable nutrients such as lipids (n-3 fatty acids) and vitamins that have positive effects on human health (Rustad et al., 2011). There is great value in taking advantage of the lipids in the rest raw material, which are important macromolecules in food. Lipids affect nutritional values in foods, such as their flavor, texture, general palatability, and storage stability (Frankel, 2014; Nawar, 1996). Studying the physical and chemical challenges associated with lipids to gain more knowledge about which parameters affect lipid oxidation will allow us to make better use of lipids.

Currently, few countries meet their daily need for n-3 and do not eat the amount of fish recommended to utilize the health effects (Gunstone, 2009). The world's population should consume more n-3 fish oils, which research has shown numerous benefits to our health (Elmadfa & Kornsteiner, 2009; Forsyth et al., 2016; Hosomi et al., 2012). The most known benefit is probably their vital role in the cardiovascular health of humans, but they can also have a positive effect on immune response disorders and mental illnesses (Fang et al., 2018;

Gunstone, 2009; Rimm et al., 2018; Simopoulos, 2004). It is also known that n-3 fatty acids are beneficial for infants and pregnant women (Coletta et al., 2010). The long-chained n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are reported to help keep the cell surface soft and contribute to the normal function of the heart. Moreover, DHA will help maintain normal brain function and normal visual function. These fatty acids are mainly found in fatty fish and fish oils, and many people have a low or non-existent intake of these (Gunstone, 2009; Klinkesorn et al., 2005; Lee et al., 2006).

A better understanding of lipid oxidation, and functional foods enriched with n-3 fatty acids, could contribute to increased consumption of n-3-fish oils in the world's diet. However, fatty acids containing many double bonds are highly prone to oxidation, which leads to deterioration of the odor and flavor as well as the nutritional quality of the food (Frankel, 2014). Analytical methods that can detect lipid oxidation at an early stage are valuable in the research and product development of foods with high n-3 content because such methods can provide rapid and qualitative results to ensure the production of quality and stable foods.

1.2 Aims and Objectives

Oxidation of unsaturated lipids produces offensive odors and flavors in foods, and can in some cases also affect the color (Frankel, 2014). Considerable attention has been given to the evaluation and assessment of oxidative and flavor deterioration, but there is still a need for knowledge about the basic sources of offensive odors and flavors in oxidized fat, and the strategies that can be implemented to counteract lipid oxidation. This study includes information and carries out research on the primary and secondary oxidation products in fish oil and powder with the belief that new knowledge within these areas could lead to useful information when choosing methods of controlling lipid deterioration. Further, this can lead to higher consumer acceptance and increasing n-3 uptake in the world’s diet, better utilization of rest raw materials, and less food loss in the future. The overall objective of the project fits into the Sustainable Development Goals (SDG) 2, Zero Hunger; 12, Responsible Consumption and Production; and 14, Life below water (UnitedNations, 2022).

One objective of this work was to study and optimize the method parameters for the analysis of fish oil and powder using Gas Chromatography-Mass Spectrometry (GC-MS). This was to find a simplified and suitable method for measuring volatile compounds and oxidation products, with minimal sample preparation and solvent waste. This study also explored the possibility of

measuring the oxidation status directly in powder using GC-MS, without an extensive extraction step that can lead to an aggravated oxidation state of the product.

Other objectives of this study included studying and comparing the stability of the oil and powder by exposing them to conditions that favor oxidation. To get a complete picture of the oxidation process in the oil and powder, the peroxide value (PV) was used as a parameter for primary oxidation products, and anisidine (AV), thiobarbituric acid reactive substances (TBARS), and GC-MS were used to analyze secondary oxidation compounds. Lastly, this work studied the possibility of using a method that is less time-consuming and uses fewer solvents, by comparing traditional analysis methods with the rapid CDR FoodLab method for the determination of PV and AV.

The main goal of this study was to determine if GC-MS was a suitable method for determining the oxidative status directly in fish powder without extracting the oil from the powder. In this process, GC-MS method parameters for the analysis of volatile components in fish oil and powder were optimized.

Sub-goal 1: compare the traditional methods for determining PV and AV with the rapid method, CDR FoodLab, and examine how suitable the rapid method is against the traditional methods.

Sub-goal 2: study the oxidative stability of the fish oil and powder.

Sub-goal 3: examine if the powder is more stable than the oil, with the hypothesis of whether the powder protects the oil or not.

2. Background

This section will provide background information on lipid oxidation, formation of oxidation products, volatile compounds, and methods for determining oxidation status.

2.1 Lipids in Food

Lipids consist of a large group of compounds that are important elements in all plant and animal tissues, and together with carbohydrates and proteins, they are the primary structural components of all living cells (Nawar, 1996). Diverse compounds such as fatty acids and their derivatives, steroids, carotenoids, terpenes, and bile acids all consist of lipids (Christie, 2003). The physical properties, stability, and nutritional value of lipids are determined by the fatty acid (FA) composition. All lipids of natural origin consist of saturated FA, monounsaturated FA, and polyunsaturated fatty acid (PUFA) in various proportions (Sikorski & Kolakowska, 2010). The principal classes of lipids that are of concern in terms of food quality include free fatty acids, triacylglycerols (triglycerides) that consist of fatty acids esterified to glycerol, and phosphoglycerides consisting of fatty acids esterified to glycerol containing phosphoric acid and organic bases (Frankel, 2014).

Lipids are vital components in nearly all kinds of foods, except for most fruits, sweets, and beverages, and contribute significantly to the nutritional and sensory value of the food (Sikorski & Kolakowska, 2010). Food lipids also have important functional and structural properties. The structure and palatability of the food are highly influenced by its composition, and their association with other food components, in addition to their melting and crystallization behavior. Lipids are also a nutritional source of energy, essential fatty acids, and fat-soluble vitamins (Nawar, 1996). They contribute to odor, flavor, color, and texture in addition to a feeling of satiety and palatability (Frankel, 2014). Lipids' effect on food quality is primarily related to the contents of lipids in the food, distribution in the food matrix, their chemical composition, and reactivity of the lipids (Sikorski & Kolakowska, 2010). Properties of food constituents such as proteins, sugars, pigments, and vitamins can be negatively modified by interaction with lipid oxidation intermediate products (free radicals) and end products (reactive aldehydes) (Mozuraityte et al., 2016). Lipids' physical changes due to processing, and the interactions with other components can also affect the food quality (Sikorski & Kolakowska, 2010).

In foods, triacylglycerols (triglycerides) and phosphoglycerides (phospholipids) are the two most abundant lipid classes. Triglycerides containing three fatty acids linked to glycerol, usually referred to as fats and oils, are storage lipids of food energy (Nawar, 1996; Sikorski & Kolakowska, 2010).

2.2 Lipid Oxidation

Lipid oxidation is an irreversible phenomenon that causes major changes in food (Laguerre et al., 2020). The highly complex set of free radical reactions between fatty acids and oxygen leads to oxidative degradation of lipids, also known as rancidity (Mozuraityte et al., 2016). Further, the decomposition of fatty acids results in nutritional losses, deterioration of sensory properties, and development of undesirable odor, flavor, and color (Aursand, 2011).

When lipids react with oxygen, a series of complex chemical changes occur. A chain of reactions that firstly yields primary products, gives rise to secondary oxidation products when exposed to extended oxidation conditions. Most of these produce undesirable sensorial and biological effects (Kanner, 2007; Márquez-Ruiz et al., 2007).

Different mechanisms of lipid oxidation include autoxidation, thermal oxidation, photo-oxidation, and enzymatic oxidation. Regarding fish oil, autoxidation is of most concern, as most of the enzymes (e.g., lipoxygenases) are inactivated, and metal ions are removed during the refining process. Thermal oxidation and photo-oxidation can be prevented by storing the oil in a cold, dark place in dark-colored containers (Aursand, 2011). Due to heat treatment which inactivates lipoxygenase enzymes, enzymatic oxidation is also of minor importance. Therefore, some relevant aspects of autoxidation are described in the next chapter, while photooxidation and enzymatic oxidation are extensively described elsewhere (Frankel, 2014).

2.2.1 Autoxidation

Autoxidation is the natural, autocatalytic chain reaction and one of the most important processes by which lipids degrade. It occurs when unsaturated fatty acids are exposed to atmospheric oxygen (Shahidi & Wanasundara, 2002) and require initial activation energy for the removal of a hydrogen atom. Thus, it is enhanced by high temperatures and the presence of double bonds (Barriuso et al., 2013). During the early stages of this autocatalytic free-radical chain reaction, the positions of double bonds in unsaturated fatty acids are changed and hydroperoxides are

produced. The initial mechanism of lipid autoxidation consists of three stages: Initiation, the formation of free radicals; Propagation, free-radical chain reactions; and Termination, the formation of nonradical products (Shahidi & Wanasundara, 2002), as seen in Figure 1.

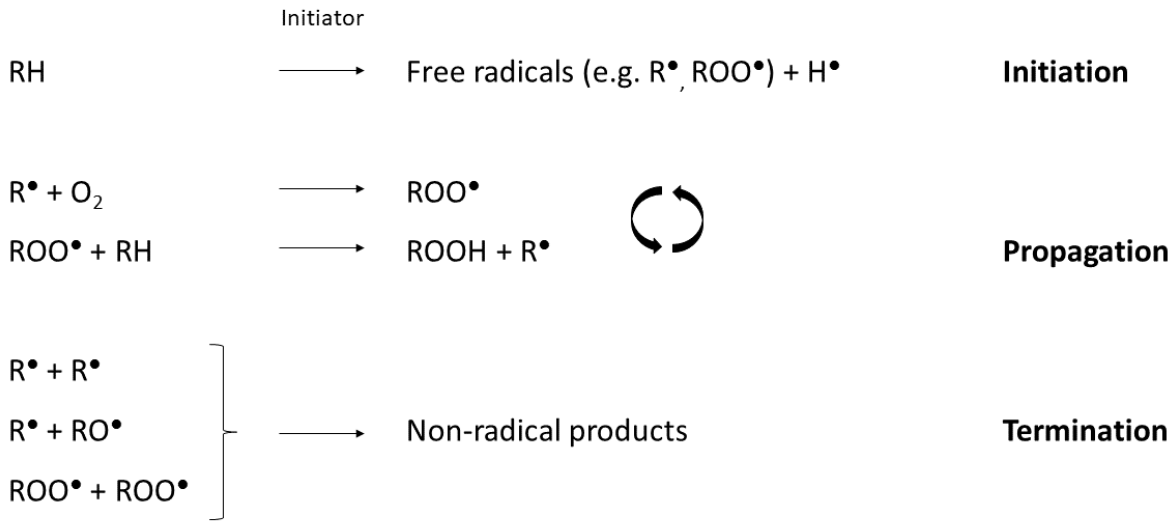

Figure 1. The different stages of autoxidation: initiation, propagation, and termination (adapted from Nawar (1996) and Frankel (2014)).

Initiation – Formation of free radicals

In the first stage, unsaturated lipid molecules lose a hydrogen atom to form lipid free radicals. To initiate the reaction, the fatty acids are required to be in a radical form. The reaction depends on initiators (e.g., heat, light/ionizing radiation, metal ions/metalloproteins) because fatty acids normally are non-radical and the reaction with atmospheric triplet oxygen is thermodynamically unfavorable (Shahidi & Zhong, 2010). The radicals formed can easily abstract hydrogen from a fatty acid containing an allylic center, resulting in a hydrogen radical (H˙) and a lipid-free radical called an alkyl free radical (R˙) (Frankel, 2012).

Propagation – Free-radical chain reactions

In the next stage, the alkyl free radical (the lipid radical) reacts with oxygen to form a hydroperoxyl free radical (ROO˙). This peroxyl radical can easily abstract hydrogen from another unsaturated fatty acid, forming a hydroperoxide (ROOH) and a new alkyl free radical (R˙). The latter may further react with oxygen and continue the chain reaction, or decompose into other free radicals, resulting in the accumulation of free radicals in the oil (Coultate, 2009;

Shahidi & Wanasundara, 2002). The reaction may continue and repeat itself several thousand times during propagation until the hydrogen source is unavailable or the chain is interrupted e.g., by antioxidants. Lipid oxidation is therefore a self-propagating and self-accelerating process (Shahidi & Zhong, 2010).

Termination – Formation of nonradical products

In the termination stage, certain molecules can end the propagation. Non-radical products are in this stage formed by free radicals reacting with each other and forming a bond in a step called annealing. Free radicals can also react with other molecules (e.g. antioxidants), forming stable radicals that do not further propagate (Nawar, 1996). The chain reaction may be terminated when the concentration of free radicals is high enough for two radicals to interact with each other to form a non-radical product (Coultate, 2009).

2.2.2 Kinetics

The start of a lipid oxidation process is referred to as the induction period where the oxidation products initially develop slowly. Later, the formation increases exponentially after some time, as illustrated in Figure 2.

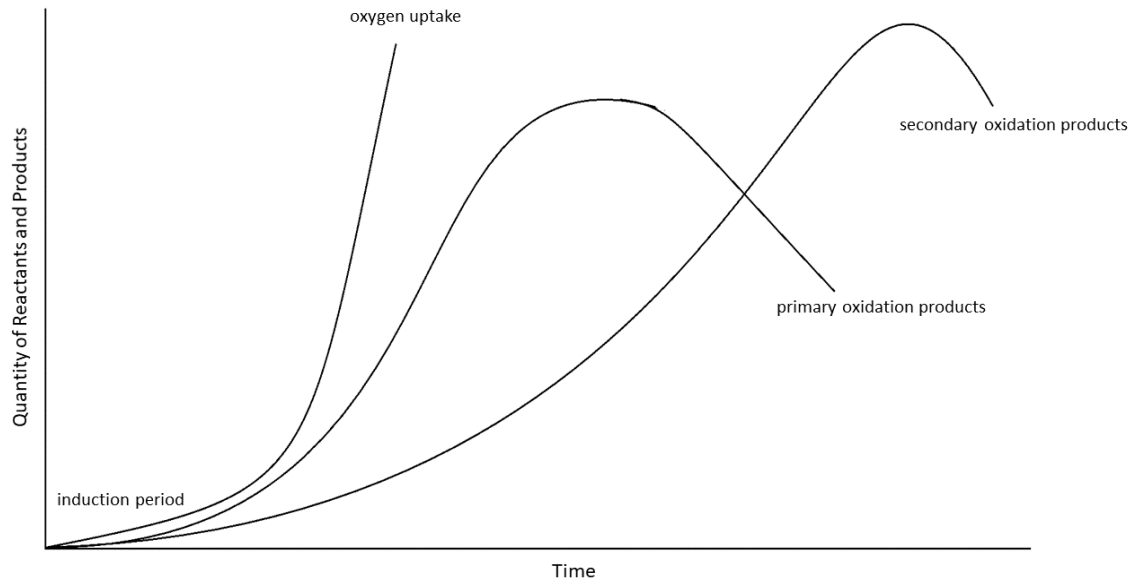

Figure 2. Illustration of changes in quantities of lipid oxidation reactants and products over time. The induction period can be seen before the formation of oxidation products accelerates. Primary oxidation products dominate in the early stages, and secondary oxidation products will dominate in a later phase (adapted from Pike and O'Keefe (2017)).

The induction period is very important in the industry, as this period is known as the shelf life and the quality of the food product before it becomes undesirable to the consumers. Thus, this phase in lipid oxidation is particularly important for controlling food quality. Measures are often taken to prolong this phase, by reducing the temperature, removing oxygen access, adding antioxidants, and reducing prooxidant activity. During the lipid oxidation process, the decomposition of hydroperoxides (primary oxidation products) into secondary oxidation products will be higher than the hydroperoxide formation, which leads to a drop in hydroperoxide concentration. The primary oxidation products will dominate initially, and the secondary oxidation products will eventually dominate in a later phase (Frankel, 2005).

2.2.3 Formation of Primary Oxidation Products

Peroxides, and especially hydroperoxides are the first compounds formed during the autoxidation process; hence, the name primary oxidation products. Primary oxidation products accumulate during the initiation and propagation of autoxidation (Barriuso et al., 2013). When lipid oxidation is extensive or carried out at elevated temperatures, hydroperoxides are believed to decompose by many routes leading to the formation of a variety of volatile and non-volatile secondary products (Paquette et al., 1985).

In oleic acid (18:1 n-9) during oxidation, hydrogen can be abstracted from the methylene groups at each side of the oleic acid, which have only one double bond. Two three-carbon radical intermediates are formed, which further give rise to four hydroperoxides after oxygen addition (Frankel, 2014; Nawar, 1996). In linoleic acid (18:2 n-6) and other polyunsaturated fatty acids, the double bonds are separated by bis-allylic methylene groups. The double bonds on each side withdraw the electrons, leaving a weakened C-H bond in the middle. The hydrogen atoms in these methylene groups are more susceptible to hydrogen abstraction than those placed on the "outside" of the double bonds. This increases the oxidizability of polyunsaturated fatty acids (Frankel, 2014), which is a term used to express the reactivity of unsaturated lipids to undergo oxidation (Kobayashi et al., 2003).

The various bis-allylic methylene groups react independently of each other in fatty acids containing more than two double bonds. Different pentadienyl radicals are then formed. The resulting mixture of hydroperoxides grows more complex with an increasing number of double bonds (the number of bis-allylic sites) in the fatty acid (Frankel, 2014).

2.2.4 Formation of Secondary Oxidation Products

Foods are affected through odor, flavor, and texture by oxidation products. Hydroperoxides are odorless and flavorless (Frankel, 2014), but usually, hydroperoxides suffer further oxidation forming hydroperoxide degradation products or secondary oxidation products. There is a wide variety of secondary oxidation products produced by oxidation, including aldehydes, ketones, epoxides, hydroxy compounds, and oligomers. Among them, both low molecular weight, volatile compounds, and relatively high-molecular-weight, non-volatile compounds can be found (Barriuso et al., 2013). Volatile secondary compounds belong to the following chemical groups: hydrocarbons, unsaturated hydrocarbons, alcohols, esters, saturated and unsaturated aldehydes, ketones, aromatic compounds, etc. (Karleskind & Wolff, 1996).

The volatile components have a direct impact on the sensory properties of the fish oil and are produced at very low levels of oxidation. The flavor compounds, having very low threshold levels, are perceptible at concentrations as low as parts per billion (ppb) (Frankel, 2014). Hexanal and 1-penten-3-one have flavor thresholds as low as 0.06 ppm and 0.003 ppm, respectively (Karleskind & Wolff, 1996). Rancid, painty, fishy, green, and burnt are some descriptions of the odors and flavors of oxidized fish oil (Karahadian & Lindsay, 1989).

There are several possible reaction pathways from primary lipid oxidation products to further reactions. Cleavage of the O-O bond require the least activation energy and are the most common decomposition pathway. Hydroperoxides start to get decomposed by scission of the hydroperoxide group, forming the decomposition products; an alkoxyl radical ($RO^{\bullet}$) and a hydroxyl radical ($^{\bullet}OH$). Homolytic β-cleavage of the C-C bonds next to the alkoxyl group can then give rise to aldehydes and alkyl- or olefinic radicals, which undergo further reactions. This results in the formation of other types of compounds, e.g., hydrocarbons and alcohols. Homolytic β-scission occurs during metal-catalyzed or thermal decomposition of hydroperoxides, but other decomposition reactions can also occur (Frankel, 2014; Nawar, 1996)

The secondary oxidation products can undergo further oxidation to a wide array of several decomposition products, which are mentioned below. The origin is often difficult to explain. Decomposition of unsaturated aldehydes can e.g., form lower aldehydes and other components (Frankel, 2014), and saturated aldehydes can easily be formed from the corresponding acids (Nawar, 1996).

When a hydroperoxide is decomposed to give a low-molecular-weight, volatile compound, the parent triglyceride is left with a shorter fatty acid than the original one. The short stump may

contain a terminal carbonyl group, and the molecule is called a core aldehyde. Core aldehyde's and other partly decomposed triglycerides' impact on odor, flavor, and further deterioration of the oil/food has been slightly explored. High molecular weight dimers or polymers linked by peroxyl- or ether-bonds can easily be formed by polyunsaturated methyl esters. Polymerization of triglycerides is mainly linked to high-temperature refining processes, frying, or storage of highly unsaturated oils under harsh conditions like elevated temperatures (> 100 °C) and/or exposure of large surfaces to light or air. (Frankel, 2014)

2.3 Volatile Compounds in Fish Oil

Even at low oxidation levels, fish oil rich in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) is known to have an unpleasant smell and taste (Hashim et al., 2021; Shibata et al., 2018). The development of fishy and metallic off-flavors in these oils is due to the extremely low stability of EPA- and DHA-OOH. Even at low ROOH levels, they are easily decomposed into volatile oxidation products. The rapid formation of volatile compounds is the most important limitation of the addition of fish oil to food products. Hence, it is highly important to have knowledge about the major volatiles formed during the early stages of fish oil oxidation and identify the volatile compounds responsible for flavor deterioration in fish oil oxidation. (Shibata et al., 2018)

Many different fatty acid hydroperoxide positional and geometric isomers are formed during the oxidation of n-3 polyunsaturated fatty acids. These give rise to a complex mixture of secondary oxidation products, many of which are low molecular weight compounds with strong olfactory attributes, which create the characteristic odor of rancid fish oil (Kulås et al., 2002). Most likely, a complex mixture of oxidation products gives rise to unpleasant flavors, particularly carbonyl compounds (Hsleh et al., 1989; Karahadian & Lindsay, 1989), which are mentioned below.

Very potent odorants which contribute to the unpleasant rancid and fishy off-flavor in bulk fish oil, fish oil-enriched mayonnaise, and fish meat are 1-penten-3-one (pungent, green odor), 4-(Z)-heptenal (fishy odor), (E,E)-2,4-heptadienal, and (E,Z)-2,6-nonadienal (cucumber odor) (Hartvigsen et al., 2000; Karahadian & Lindsay, 1989; Milo & Grosch, 1995, 1996). Among the numerous carbonyl compounds in oxidized fish lipids, Shibata et al. (2018) and Olsen et al. (2006) also identified 1-penten-3-one and 2,4-heptadienal as the flavor deterioration indicators of oxidizing fish oil, in addition to good marker compounds for early lipid oxidation in n-3

containing matrixes. 4-heptenal, 2,6-nonadienal, 2,4,7-decatrienal, 1-octen-3-one, and 1,5-octadien-3-one have also been proven to be major off-flavor contributors (Shibata et al., 2018). Several of the mentioned volatiles can be expected to be formed from EPA during autoxidation and can be seen in Figure 3.

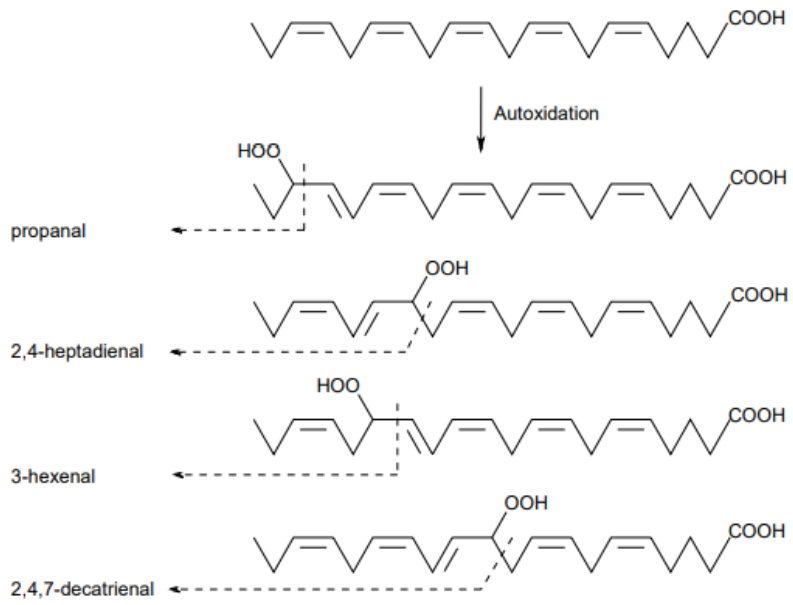

Figure 3. Autoxidation sites that are expected to be formed from EPA, are associated with major aldehydes (borrowed from Kulås et al. (2003)).

Karahadian and Lindsay (1989) stated that hexanal, 2,4-heptadienal, and 2,4-decadienal caused oxidized, rancid, and painty flavors in fish oils. Gunstone (2009) reported compounds such as 4-heptanal, and the 2,6- and 2,3-nonadienals to be considered the most significant flavor notes, although many of the volatile materials have a little sensory effect. The major volatiles found by Kulås et al. (2002) were propanal, 2-propenal, 1-penten-3-ol, and the two 2,4-heptadienal geometrical isomers. Certain volatile oxidation products that are expected to be formed from 2,4-heptadienal can be seen in Figure 4.

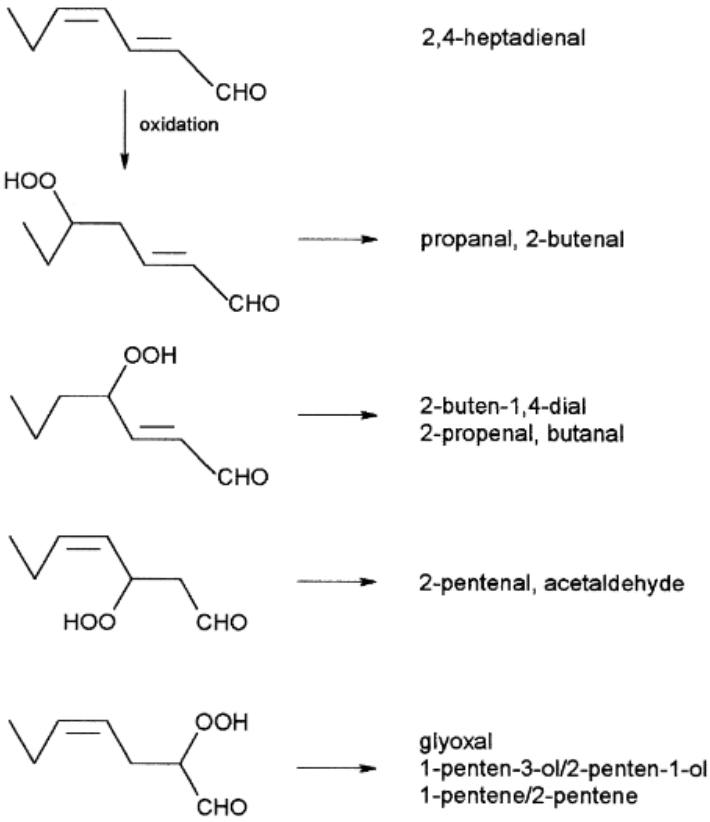

Figure 4. Volatile oxidation products expected to be formed from 2,4-heptadienal (borrowed from Kulås et al. (2003)).

2.4 Factors Influencing Lipid Oxidation in Foods

Factors influencing lipid oxidation (in foods) are many. Both intrinsic elements in the material such as the type of lipid (triacylglycerols, phospholipids, and others), the physical state of the lipid, the fatty acid composition (especially the proportion of double bonds), pH, and the presence of prooxidants and antioxidants, in addition to external factors like storage conditions, e.g., oxygen availability, temperature, water activity, and light exposure can all have an impact. The physical state of the lipid can also influence the oxidation process as liquid lipids are more mobile than solids (Mozuraityte et al., 2016; Nawar, 1996).

As mentioned in Chapter 2.2.3, the number of bis-allylic groups present in the unsaturated fatty acid highly affects the oxidizability and increases approximately two-fold for each additional bis-allylic state (Frankel, 2014). More bis-allylic positions will be available for hydrogen abstraction as the number of conjugated double bonds increases in PUFA. This results in a large selection of primary oxidation products which further decomposes to an even more complex mixture of secondary oxidation products (Kobayashi et al., 2003).

In foods, lipids may be present with other food components such as carbohydrates, vitamins, proteins, and water. In these systems, the lipids and lipid oxidation products interact not only with each other, but also with other types of molecules, and thus have a more complex deteriorative effect than the bulk lipid (Frankel, 2014; Nawar, 1996). This makes the oxidation processes and the predictability of the development of rancidity in the food exceedingly challenging to understand. Water activity and pH are other factors that can affect oxidation rates. It is suggested by several investigators that lipid oxidation in foods is lowest at water activity close to the water monolayer (between 0.2 and 0.4 for most foods), and the rate of lipid oxidation increases rapidly as the water activity is either decreased below or increased above the monolayer (Klinkesorn et al., 2005). pH correlates with the availability of free trace metals, surface activity, and ionic interactions with e.g., proteins (Nawar, 1996).

Traces of metals such as iron and copper are found in all foods and can act as effective prooxidants as they can catalyze the decomposition of hydroperoxides and promote the formation of free radicals. Antioxidants, found in nature, can either be fat-soluble and occur naturally in fats and oils, or water-soluble and common constituents of e.g., fruits, berries, and herbs. They can inhibit oxidation by delaying initiation or reducing the rate of propagation. Unfortunately, the use of synthetic antioxidants has declined due to health issues (Frankel, 2014). Therefore, antioxidants must be chosen after careful consideration (Nawar, 1996).

An external factor that is crucial for autoxidation reactions to take place is oxygen access. In foods, it is especially complicated to remove all the oxygen present, even if the food is packaged in a vacuum or modified atmosphere. The contact area between lipids and air is also an important factor as large surface-volume ratios leave relatively more fatty acid molecules easily exposed to air than if the material is more compact. The temperature has an important effect on the oxidation rates, as it generally doubles for every 10 degrees the temperature rises. Exposure to light is another very important factor, as it leads to photooxidation. It occurs when unsaturated fatty acids are exposed to light in the presence of oxygen and a photosensitizer (e.g., pigments such as riboflavin, hemeproteins, and chlorophyll present in food). (Frankel, 2014).

2.5 Health Effects of n-3 Fatty Acids

Some fatty acids required for our health and welfare cannot be synthesized by humans themselves, making n-3 polyunsaturated fatty acids essential dietary requirements that must be obtained from plant and animal sources through the diet (Shaw et al., 2007). Two families of essential fatty acids of major importance for animal and human health are linoleic acid (n-6), and those based on α-linolenic acid (n-3) (Gunstone, 2009).

n-3 fatty acids have positive health effects, as shown by many in relation to beneficial growth and development throughout the life cycle (Klinkesorn et al., 2005; Lee et al., 2006; Luten, 2009), reducing plasma cholesterol levels and serum triacylglycerol levels (especially n-3 from marine sources) (Gunstone, 2009), and playing an important role in the prevention and treatment of different diseases: coronary artery disease (arteriosclerosis), hypertension, arthritis, and immune response disorders (Klinkesorn et al., 2005).

The most significant metabolites in n-3 fatty acids are eicosapentaenoic acid (EPA, 20:5), docosapentaenoic acid (DPA, 22:5) and docosahexaenoic acid (DHA, 22:6). Direct consumption of fish lipids is more effective than the slow process of converting α-linolenic acid to EPA and DHA in vivo (Gunstone, 2009).

EPA and DHA are widely known for their beneficial health attributes, and their dietary sources are seafood as well as refined marine and algal oils (Shaw et al., 2007). These long-chain n-3 fatty acids have been reported by many as beneficial for brain and retinal development (Coletta et al., 2010; Klinkesorn et al., 2005), playing a vital role in the cardiovascular health of humans (Fang et al., 2018; Lee et al., 2006; Rimm et al., 2018), and decreasing inflammatory responses in humans (Ismail, 2005; Nestel, 2000; Simopoulos, 2002).

Even though research reveals these health benefits of consuming n-3 fatty acids, and current health advisories recommend a higher intake of unsaturated acids, the average dietary intake is especially low in the Western world. Due to humans´ natural prevention mechanism for oxidized fats, we have a very low threshold for detection of the volatiles formed during oxidation (Frankel, 2014). The Global Organization for EPA and DHA Omega-3s (GOED) refers to surveys showing that between 7-23% of non-users of n-3 supplements are not willing to try the supplement because of the fishy flavor (Ismail et al., 2016). Consequently, attempts have been made to increase the consumption of marine lipids by incorporating fish oil into different functional food products (Hartvigsen et al., 2000). Unfortunately, the addition of these

oils into foods is an incredibly challenging task due to the oil's instability to oxidative rancidity, which causes loss of food quality (Shaw et al., 2007).

EFSA Panel on Dietetic Products and Allergies (2010) and American Heart Association (AHA) recommendations is that the consumers´ beneficial effect is achieved by a daily intake of 250 mg EPA and DHA to make the health claim. This recommendation can be met by consuming fish 1 to 2 times per week (Rimm et al., 2018). The simplest way of implementing EPA and DHA into the weekly diet is by eating fatty fish like mackerel, anchovy, sardines, herring, and salmon. Nevertheless, a large part of the population does not have easy access to fatty fish, or simply does not like the taste or thinks it is more convenient to take a supplement.

The total fat intake across most of the world´s population ranges from 15-40 % energy and Gunstone (2009) recommends the following dietary intakes for healthy adults, summed up in Table 1.

Table 1. Recommendations for dietary intakes of saturated fatty acids, trans acids, and polyunsaturated fatty acids, suggested by Gunstone (2009).

Saturated fatty acids	max. 10 energy %
Trans acids	should not exceed 2 energy %
Polyunsaturated acids	
omega-6 acids	around 6 energy % (3-10 % representing a safe range)
omega-3 acids	
linolenic acid	1 energy % within a range of 0,5-2,5 %
EPA/DHA	0.5 % within a range of 0-2.0 %
Cis monounsaturated acids	
oleic acid	should represent 11-16 energy % of dietary intake, and should not exceed this upper limit

*The growing opinion that these levels might be too high has led to arguments for an intake of only 2% and a maximum of 3% (Gunstone, 2009).

The balance of dietary fat should consist of *cis* monounsaturated acids, almost entirely oleic acid. This becomes especially important when total fat intake exceeds 30 energy % (Gunstone, 2009).

2.6 Methods for Determining Oxidation Status

The basic characteristics of certain foods, such as edible oils, will be dependent upon their source. Factors degrading the quality of the oil must be ascertained before the oils are used in other foodstuffs. Consequently, knowledge of the lipid's quality before shipping the product to the market, or before use in fabricated foods is of great interest to the industry. Monitoring the oxidation status to offer high-quality fish oil products with low oxidation status to the consumer market is of both economical and health value.

Evaluating lipid oxidation status in fish oil is a challenging task due to the complex process. Proper measurement of lipid oxidation depends on the type of lipid substrate, the oxidation agents, and the environmental factors. Today, there is a wide range of developed and implemented methods for determining both primary and secondary oxidation products. As primary and secondary oxidation products dominate at different stages in the oxidation process, it is not sufficient to determine only one of the oxidation products alone. Some of the most common methods and classical procedures for measuring lipid oxidation will be briefly described in this chapter, concentrating on the methods that have been used in this study. The methods include Peroxide value (PV), Anisidine value (AV), Thiobarbituric acid reactive substances (TBARS), and Gas chromatography-mass spectrometry (GC-MS). Suitable measurements of lipid oxidation remain a challenging task but combining a variety of these methods can give a total picture of the oxidation status.

2.6.1 Peroxide Value (PV) – Iodometric Titration Method

Peroxide value (PV) is one of the classical methods for measuring oxidative status, more specifically to which extent an oil sample has undergone primary oxidation. There are several analytical methods available, but for fats and oils, a titration method is often used for the measurement of hydroperoxides (Gunstone, 2009).

The iodometric titration method is based on the oxidation of iodide to iodine. The oil sample is often dissolved in a chloroform-acetic acid solution. Hydroperoxides in the oil react with potassium iodide added to liberate iodine. The amount of iodine present is determined volumetrically by titration with a sodium thiosulphate solution with starch as the indicator. This method is detecting all substances that oxidize potassium iodide under acidic conditions. Therefore, the purity of the reagents is critical. The peroxide value (PV) of an oil or fat is

defined as the quantity of peroxide oxygen present in the sample and can be expressed in meq peroxide/kg sample (Hoover et al., 2005).

The iodometric titration method is a widely used popular method due to the simple experimental procedure. However, it has some disadvantages considering the large amounts of solvent waste produced, and the high amount of sample needed (Crowe & White, 2001). It is also a time-consuming and labor-intensive method, which may give falsely low and falsely high PV. This is due to the absorption of iodine at unsaturated positions of the fatty acids and the liberation of iodine from potassium iodide by oxygen present in the solution to be titrated, respectively (Shahidi & Wanasundara, 2002).

Gunstone (2009) reported that freshly refined oils should have a peroxide value below 1, and that fat is rancid at a peroxide value of 10. The results from different peroxide determinations vary between the different methods, and according to how the procedure is performed, the technique of the performer, and by which matrix is measured (Frankel, 2014).

It is challenging to establish a maximum tolerable level of Peroxide value and Anisidine value based on the few studies available on the toxicity of individual oxidation products and at what oxidation level that will potentially give a negative effect in humans, but The Global Organization for EPA and DHA Omega-3s (GOED) sets the maximum limit for EPA and DHA oils to have PV 5 meqO_2/kg (Ismail et al., 2016).

2.6.2 Anisidine Value (AV)

The formation of secondary oxidation products is often measured by the anisidine value (AV). This is a popular method to determine the number of aldehydes (mainly 2-alkenals and 2,4-dienals) in fats and oils. The oil sample is dissolved in isooctane before p-anisidine in an acetic acid solution is added. Aldehydes react with p-anisidine giving a yellow compound that is measured spectrophotometrically at 350 nm (Barriuso et al., 2013; Shahidi & Zhong, 2005).

The amount of aldehydes present in the sample in addition to the structure of the aldehydes affects the color intensity of the yellow compound produced. Unsaturated aldehydes give higher AV than saturated aldehydes because hydrocarbon chains with a double bond conjugated with the carbonyl double bond have been found to increase the molar absorbance four to five times. This results in the oxidation products, 2-alkenals and 2,4-alkadienals, largely contributing to the anisidine value (IUPAC, 1979). However, the anisidine value correlates well with peroxides

content, TBARS, and volatile aldehydes analysis, and is a recommended method and a good control parameter for secondary oxidation (Barriuso et al., 2013).

The anisidine value is only strictly comparable across results for a single oil because of the variations depending on the enals-compounds present. An anisidine value of 1 corresponds with around 0.1% of oxidized material (Gunstone, 2009) and is defined as 100 times the absorbance of a solution containing 1 g of fat in 100 mL of solvent (Barriuso et al., 2013). The maximum limit for AV in EPA and DHA oils set by GOED is 20 AnV (Ismail et al., 2016).

The spectroscopic method of determining the anisidine value is one of the oldest methods for evaluating secondary lipid oxidation. The methodology is considered to be very simple and rapid (Barriuso et al., 2013), but precautionary measures need to be taken due to the toxicity of the anisidine reagent (IUPAC, 1979).

2.6.3 Thiobarbituric Acid-Reactive Substances (TBARS)

Thiobarbituric acid-reactive substances, also called TBARS (or, simply TBA), is one of the oldest and most common analyzes for determining secondary oxidation products (aldehydes) (Shahidi & Wanasundara, 2002). This method has been used to measure the degree of autoxidation of oils and lipids and the rancidity of various fat-containing foods. It is used as a routine method by food technologists to determine the quality of lipids and foods (Ke & Woyewoda, 1979).

The TBARS value, the extent of lipid oxidation, can be expressed as μmoles MA/g sample (Shahidi & Wanasundara, 2002). The reaction between thiobarbituric acid (TBA) and the oxidation products of unsaturated lipids produces a red color, which is determined spectrophotometrically as the TBARS value. A compound prepared from malonaldehyde (MA), which gives a red color, has been identified as the reaction product given by oxidized lipids and TBA reagent (Ke & Woyewoda, 1979). Standard curves of malonaldehyde are used for standardization and calculations by acid hydrolysis of 1,1,3,3-tetraethoxypropane (the tetraethoxyacetal of malonaldehyde) (Frankel, 2014).

Although the method for determining the TBARS value has had several analytical improvements and modifications, Ke and Woyewoda (1979) reported that the results from marine lipids and oil samples, especially for highly oxidized samples, have had less reproducibility than desired. Some common problems associated with TBARS determinations

are the mixing of two-phase reaction systems, the formation of a yellow pigment that interferes in the reaction, and the complexity of the carbonyls from the oxidation of polyunsaturated fatty acids (Ke & Woyewoda, 1979). Also, several types of components present in food samples can react with TBA or otherwise interfere with the results. Components that can influence the results are proteins, amino acids, nucleic acids, nitrite, sugars, browning reaction products, antioxidants, and trace metals (Frankel, 2014).

2.6.4 CDR FoodLab

Kit-based colorimetric methods have recently offered several advantages for PV and AV determination, including simple and rapid detection procedures, short analysis time, the requirement of small quantities of samples, and the ability to produce small amounts of residues (Zhang et al., 2021). CDR FoodLab is an instrument for fast and accurate analyzes of foods, drinks, and oils. The instrument can perform the following analyzes: peroxide value (PV), free fatty acids (FFA), and anisidine value (AV) in addition to testing the oxidation status of fat in finished products (Labolytic, 2022).

The instrument is based on photometric technology with a thermostat and built-in printer and is delivered together with ready-made kits with reagents. This makes the instrument easy to use, even by people without laboratory experience. The analyzes require small sample volumes, and the sampler is exposed to low toxicity as the reagents are delivered in pre-filled cuvettes (Labolytic, 2022).

Considering the limitations related to the traditional methods for determining PV and AV, rapid instrumental techniques have been considered reliable alternatives. Martín et al. (2012) used CDR FoodLab to measure PV and AV in “ultra-high” n-3 concentrates (80%) from fish oils. Similarly, PV and AV were determined in high-oleic rapeseed oil by Petersen et al. (2012). Nader et al. (2021) used the same instrument for the analysis of PV, and AV in peanut kernels.

2.6.5 Headspace Gas Chromatography-Mass Spectroscopy (GC-MS)

Gas chromatography combined with mass spectrometry is an advanced method for the quantification and identification of secondary volatile oxidation products (Frankel, 2014). The separation of a mixture of compounds into individual components is called chromatography and includes three steps: injecting a sample into the GC instrument, separating the sample into

individual components, and finally detecting and identifying compounds in the sample. Mass spectrometry is required for the identification of the compounds (AgilentTechnologies, 2010).

Volatile oxidation products from seafood have been analyzed by various headspace analyzes through the years (Karahadian & Lindsay, 1989). Headspace (HS) is a term referring to the volume of vapor above the condensed phase sample (Bruno & Harries, 2019). Static headspace and solid-phase microextraction (SPME) are less sensitive headspace GC techniques than purge-and-trap techniques, that has similarities with the dynamic headspace sampling. Both static HS sampling and SPME differ from dynamic HS sampling in that an aliquot of the headspace of a closed vial is analyzed (Kolb & Ettre, 2006). The sample and its headspace in static HS sampling reach a static equilibrium before the volatile compounds are transferred to a gas chromatograph-mass spectrometer (GC-MS), while the dynamic headspace sampling represents exhaustive gas extraction. In dynamic HS, the headspace above the sample is flushed or purged with an inert gas (e.g., nitrogen) onto an adsorbent trap, releasing the volatiles from the fish oil. The volatiles from the sample are commonly trapped in a tube packed with the porous polymer, Tenax. Further, the volatiles are desorbed and transferred directly from the Tenax tube to the GC-MS system passing through a small thermal desorption unit. The volatiles are separated in the GC column and analyzed by MS after further concentration (Bardsley, 2015; Lerch & Gil, 2008).

Dynamic headspace GC-MS is a widely used, highly sensitive isolation method for identifying volatile compounds responsible for off-flavors in fish oil (Jónsdóttir et al., 2005). The volatile compounds can be identified by comparing mass spectra of the compounds to spectra in databases and/or spectra of pure standard compounds (Hübschmann, 2015).

It is possible to include an internal standard (IS) in the GC-MS analysis to achieve quantitative results. In the internal standard method, the target component concentration is calculated based on the relationship between the peak area ratio and concentration ratio of the target component and the internal standard. In a GC chromatogram, the size (peak area) of the component is proportional to the amount of the component reaching the detector. (SHIMADZU)

The selection of the internal standard can be challenging as it must fulfill several requirements. It must be separated almost completely from all components in the sample, elute close to the target component, have similar chemical properties to the target component, and be chemically stable (K. A. Kristiansen, personal communication, December 3, 2021; SHIMADZU).

3. Materials and Methods

This section will provide a short background of the chosen materials and methods in this study.

3.1 Fish Oil and Powder

The fish oil and powder used in the experiments were supplied from a fish oil refining company in Norway. The fish oil was made from fish species (mainly sardines) containing high amounts of n-3 polyunsaturated fatty acids and was up-concentrated to > 60% EPA/DHA (10:50% EPA:DHA). The fatty acid composition in the oil can be seen in Table 2. The specification is a min. 90% TG product with 3.0-4.0 mg/g of mixed tocopherols added. The oil was refined, distilled, bleached, and deodorized. The oil had a yellow color (4 on the Gardner scale), almost without odor, and had a peroxide value (PV) of 0.9 meqO_2/kg, and an anisidine value (AV) of 3.0 AnV.

Table 2. Fatty acid composition in the oil delivered from the fish oil refining company.

	Area%	mg/g as TG	mg/g as FFA
EPA (C20:5 n-3)	11	100	100
DHA (C22:6 n-3)	58	510	490
DPA (C22:5 n-3)	9	80	80
Total n-3	80	720	690

The powder was called an absorbed powder, which had a composition of 35% oil blended with a powder matrix. The absorbent in the powder consisted mainly of a starch portion with a small proportion of protein and had a large surface area. At the company's request, the further composition of the powder was confidential. The oil was evenly and well distributed on the powder by good and intensive mixing. The powder was flushed with nitrogen in the process. Both the oil and powder were stored in dark, tight bottles in a -20 °C freezer. If necessary, the bottles were flushed with N_2 after sampling to limit unintentional oxidation before further analysis.

The fish oil and powder used in the pre-experiments (Chapter 3.2.1) were made from the same raw material and the same supplier company, but the powder consisted of 50% oil, and not 35%.

3.2 Experimental Design

The experiments in this study were divided into two groups; GC-MS pre-experiments to optimize the method parameters, and the main storage experiment including oxidative analyzes of PV, AV, TBARS, and volatile compounds (GC-MS).

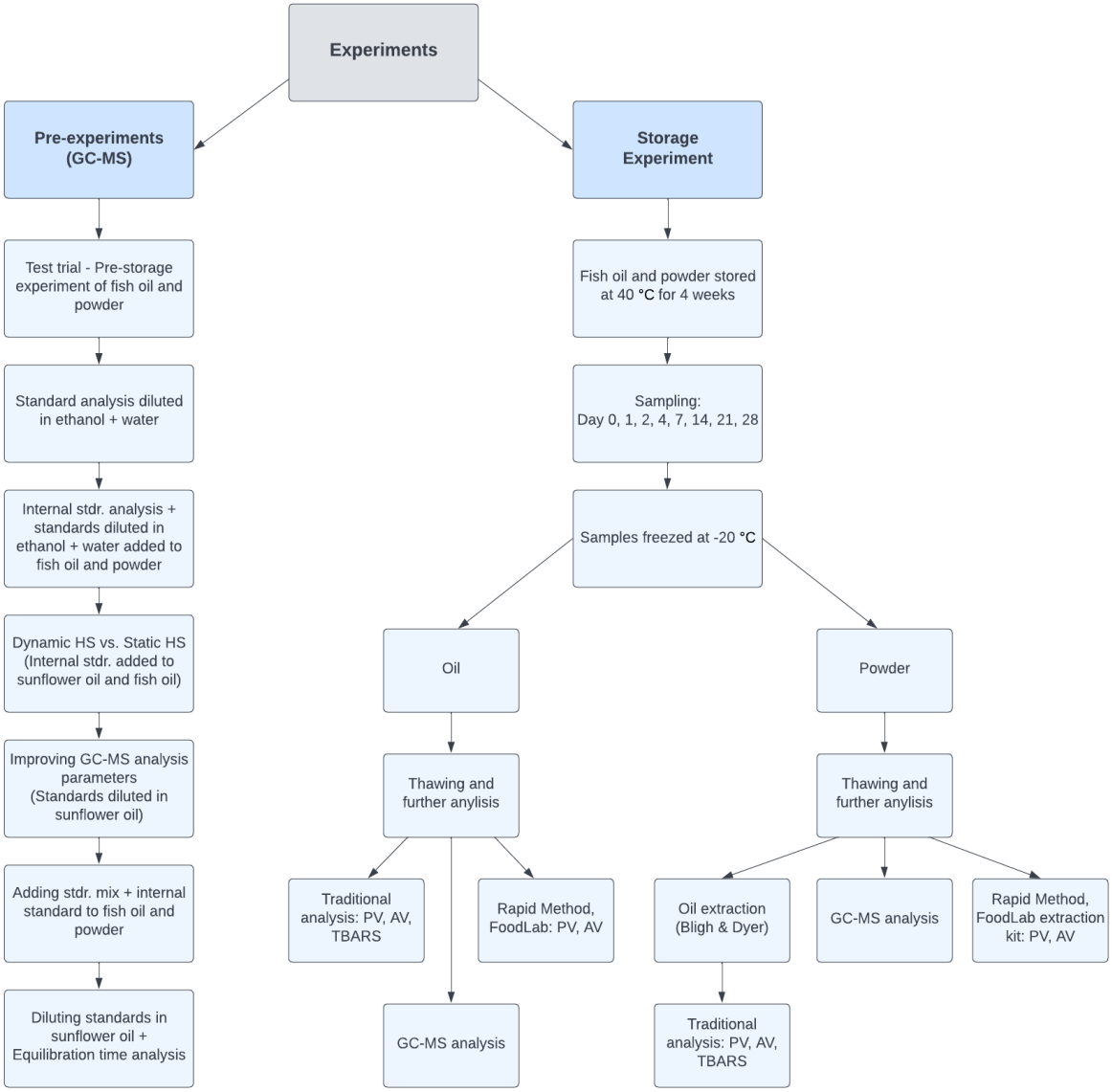

Figure 5. The experimental setup of the pre-experiments to optimize the GC-MS analysis parameters, and the storage experiment including oxidative analyzes of PV, AV, TBARS, and volatile compounds (GC-MS).

3.2.1 Pre-Experiments

The pre-experiments consisted of preparatory GC-MS analysis to adjust and optimize the method parameters for the storage experiment to be analyzed.

Pre-Storage Experiment

A pre-storage experiment of stored fish oil and powder was performed on dynamic HS GC-MS to test the analysis parameters and to study the fish oil and powder. 2 g oil and 2 g powder were weighed in 10 mL dark glass bottles (diameter 2 cm). The stored samples were left without a cap in a heating cabinet at 44.5 °C for 2 days, then placed in the fridge at 4 °C for 3 days. 40 °C was used as the storage temperature later in the main storage experiment and was chosen to accelerate lipid oxidation, in line with literature (Aidos et al., 2002; Frankel, 2014; Lee et al., 2006; Sullivan et al., 2011). To keep the formed volatiles in the headspace, it was also decided that the bottles should keep the cap on during the next storage experiment.

0.50 g of oil and 1.0 g of powder were weighed into 22 mL GC vials (diameter 2 cm). For the analyzed samples to contain the same amount of oil, half as much oil as powder was weighed since the powder contained 50% oil. Fresh and stored oil and powder were analyzed in addition to fresh and stored powder samples diluted in distilled water. These samples were analyzed with a platen temperature of 60 °C. One last sample containing fresh oil was analyzed with a platen temperature of 70 °C. One blank injection was analyzed before the sample series.

Quality Indicator Standards and Internal Standard

To be able to identify, and at best quantify oxidation products in the fish oil and powder, pre-purchased standards were analyzed: 2,3-butanedione, 1,3-hexadiene, 2-ethyl-furan, 1-penten-3-ol, 2,3-pentanedione, (E)-2-octene, (E)-2-pentenal, (E,E)-2,4-heptadienal, 2-pentene and 2-methyl-1-butene. CAS numbers and manufacturers for the standards used in the experiments can be seen in Table 3. First, experiments of all the 10 standards diluted in ethanol and water were performed.

Table 3. CAS number and manufacturer of the standards used in the experiments.

Standards	CAS nr.	Manufacturer
1-Penten-3-ol	616-25-1	Merck, Darmstadt, Germany
(E)-2-Pentenal	1576-87-0	Alfa Aesar, Massachusetts USA
2-Ethyl-furan	3208-16-0	Merck, Darmstadt, Germany
(E)-2-Octene	133389-42-9	Merck, Darmstadt, Germany
1,3-Hexadiene	592-48-3	Merck, Darmstadt, Germany
2,3-Pentanedione	600-14-6	Merck, Darmstadt, Germany
2,3-Butanedione	431-03-8	Alfa Aesar, Massachusetts USA
(E,E)-2,4-Heptadienal	4313-03-5	Merck, Darmstadt, Germany
2-Methyl-1-butene	563-46-2	Merck, Darmstadt, Germany
2-Pentene	646-04-8	Merck, Darmstadt, Germany

Sunflower oil (Eldorado) was later used as a diluent as it had a similar matrix to fish oil. Quantification requires that the analytes (standards) must be dissolved in the same matrix as the sample i.e., oil. Neutral sunflower oil with few chemical double bonds was chosen for dilution as it was expected to oxidize minimally. An equal amount (20 µM) of all standards diluted in sunflower oil were spiked to 1 mL fish oil in 22 mL GC vials. All vials that contained oil samples were vortexed before analysis.

An internal standard was also spiked to the fish oil samples to increase the possibility of obtaining quantitative results. The internal standard should be stable, representative of all analytes, and have small variations in response (nearly constant in area). 4-Methyl-2-pentanol (CAS nr. 108-11-2, Merck, Darmstadt, Germany) was chosen to be the internal standard as it is not that structurally different from oxidation products (1-penten-3-ol, (E)-2-pentenal) that were expected to be detected in the fish oil and powder. IS was diluted in sunflower oil and added to fish oil at a concentration of 20 µM. Attempts were also made to add the internal standard and standards to the powder in the same concentration (20 µM).

Comparing Dynamic HS and Static HS method

An experiment comparing Dynamic headspace and Static headspace was conducted as several of the standards were not detected or found in a reasonable concentration range (2-20 µM). Pure sunflower oil, sunflower oil spiked with internal standard (2 µM and 20 µM), fresh fish oil, and fish oil spiked with internal standard (2 µM and 20 µM) were analyzed by both methods. All samples contained 1 mL of oil. All vials that contained oil samples were vortexed before

analysis. System performance was checked with blank injections before and after the sample series.

Improved GC-MS Analysis Parameters

An experiment changing the method parameters was done to develop an efficient extraction method. The aim was to be able to detect several of the standards in a reasonable concentration range (around 2-20 µM) before analyzing the samples from the storage experiment. Jeleń et al. (2017) compared a static headspace method with a combination of a headspace method in which compounds could be extracted multiple times from a headspace vial into a Tenax trap before transferring them into a gas chromatograph. A similar GC-MS method was used in this study. Shaking intensity inside the sampler, higher transfer line temp. and desorb temp., and higher trap bake temp. and time were tested, based on Jeleń et al. (2017) findings of higher peak areas of the compounds and higher repeatability of the peak areas.

A standard mix of (E,E)-2,4-heptadienal, 1-penten-3-ol, (E)-2-pentenal, and 2-ethyl-furan with a concentration of 20 µM was added to 1 mL sunflower oil, vortexed and analyzed. The first analysis parameters and the optimized analysis parameters are shown in Table 4.

Table 4. The first analysis parameters, and the optimized analysis parameters for dynamic HS GC-MS.

	Parameters 1.	Optimized parameters
Variable	**Value**	
Valve Oven Temp.	110 °C	150 °C
Transfer Line Temp.	115 °C	160 °C
Standby Flow Rate	25 mL/min	50 mL/min
Trap Standby Temp.	30 °C	30 °C
Trap Sweep Temp.	0 °C	0 °C
Platen/Sample Temp.	60 °C	70 °C
Sample Preheat Time	10.0 min	10.0 min
Preheat Mixer	Off	On
Preheat Mixing Level	Level 0	Level 5
Preheat Mixing Time	2.0 min	2.0 min
Preheat Mixer Stabilize Time	1.0 min	1.0 min
Sweep Flow Rate	75 mL/min	50 mL/min
Sweep Flow Time	5.0 min	10.0 min
Dry Purge Time	1.0 min	1.0 min
Dry Purge Flow	50 mL/min	50 mL/min
Dry Purge Temp.	25 °C	25 °C
Desorb Preheat	175 °C	200 °C
Desorb Temp.	180 °C	220 °C
Desorb Time	3.0 min	1.0 min
Trap Bake Temp.	180 °C	230 °C
Trap Bake Time	4.0 min	10.0 min
Trap Bake Flow	200 mL/min	200 mL/min

Equilibration Time

The equilibration time was tested by adding a known concentration (20 µM) of the internal standard to five different vials containing fish oil (1 mL), analyzed at different times on the platen (70 °C): 5, 10, 15, 30 and 45 min. The samples were vortexed before analysis. This experiment aimed to find conditions that achieved equilibrium for the analytes in addition to obtaining maximum response and sensitivity. It was also important to learn whether oxidation occurred while the sample was on the platen. Therefore, this experiment sets the conditions for achieving the best possible equilibrium (response) of the analytes in addition to avoiding oxidation during the analysis (while the sample is on the platen).

3.2.2 Storage Experiment

The oil and powder were exposed to conditions that favor oxidation (high temperature and oxygen access) to study lipid oxidation and test the stability. The bottles were not flushed with nitrogen before they were placed in the heating cabinet, giving the oil and powder access to oxygen in the headspace of the bottle.

The oil and powder were thawed at room temperature with minimal light exposure. The samples were weighed immediately after thawing, and light exposure was minimized during the process. 10 g of oil and 10 g of powder were stored in dark, tight 100 mL Kimble Kimax bottles (diameter 4 cm): one bottle for GC-MS analysis and one bottle for other analyzes such as PV, AV, and TBARS. The oil samples had more headspace (5/6 parts air) in the storage bottle than the powder samples (2/6 parts air). All samples were placed tight capped in a heating cabinet at 40 °C at the same time. The exception was the fresh samples (day 0) that were placed directly in the freezer at -20 °C. Temperature loggers (EBI11-P100, Ebro) were used to monitor the temperature every 15 minutes throughout the whole period. The mean temperature was 40.26 °C ± 0.55.

The fish oil supplier company suggested to extend the experiment for up to 4 weeks. For sampling, the bottles were taken immediately from the heating cabinet to the freezer on days 1, 2, 4, 7, 14, 21, and 28 at the same time of the day. The bottles were flushed with N_2 after analysis, and the remains of the samples were put back in the freezer at -20 °C. The powder samples may have oxidized more easily in the bottles than the oil during storage in the freezer, as it can be more challenging to remove all the oxygen with N_2 in a powder with a large surface area. It was ensured that the samples were completely thawed before analysis (GC-MS, PV, AV, TBARS), which took approx. 15 minutes at room temperature.

All samples for GC-MS analysis were weighed in 22 mL vials (diameter 2 cm). Four different standard samples diluted in sunflower oil were analyzed containing the same concentration of the standard mix and IS: 10 µM, 20 µM, 50 µM, and 80 µM, respectively. The standard samples were analyzed to have the ability to calculate the concentrations of the individual volatiles.

The oil samples (1 mL) containing 20 µM IS were vortexed and analyzed, followed by the powder samples (1 g), which all were analyzed in triplicates. As 1 mL of oil corresponds to approximately 1 g of oil, it was chosen to add 1 g of powder to the vials. However, the powder contained 35% oil, and the response (area) from the powder samples had to be corrected from

35% to 100% oil for the analyzed powder samples to contain an equal amount of oil as the oil samples (formula 1). This is explained in Chapter 4.1.3.

All samples were prepared and placed in the GC-MS instrument during the analysis to minimize exposure to light and room temperature before analysis. System performance was checked with blank injections before and during the sample series.

100 % oil in the powder samples =

$$the\ response\ (area)\ of\ the\ powder\ sample\ *\ (\frac{100\%}{35\%}) \quad (1)$$

3.2.3 Lipid extraction – Bligh & Dyer method

The fish powder samples were lipid extracted before analysis of PV and AV. It was done according to the Bligh and Dyer (1959) method. This method is used especially on frozen fish, homogenizing the wet tissue with a mixture of chloroform, methanol, and water in proportions that forms a miscible system with the water in the tissue (Bligh & Dyer, 1959).

In this study, 10 g of fish powder was homogenized (POLYTRON PT 3100 D) (20 000 rpm) for two minutes with 16 mL distilled water, 40 mL methanol (CAS nr. 67-56-1, Merck, Darmstadt, Germany), and 20 mL chloroform (CAS nr. 67-66-3, Merck, Darmstadt, Germany). Then the mixture was homogenized for 40 sec after the addition of 20 mL chloroform, followed by 30 sec homogenizing after the addition of 20 mL distilled water. The samples were then centrifuged (ROTINA 420R) at 5000 rpm for 10 min. The samples were protected from oxidation by placing them on ice in a dark cabinet between homogenization and centrifugation. The dilution with chloroform and water separates the homogenate into two layers, one layer containing all the lipids (the chloroform layer), and one layer containing all the non-lipids (the methanolic layer).

The lower layer (chloroform phase) was pipetted out, and a pure lipid extract was obtained by isolating the chloroform layer. With the addition of N_2, the chloroform was evaporated off on a heating block (60 °C).

3.3 Analytical Methods

PV and TBARS measurements, FoodLab measurements (PV and AV), Bligh & Dyer lipid extraction, and GC-MS analysis were carried out at the Department of Biotechnology and Food Science, NTNU, Trondheim. PV and AV measurements were also carried out at the quality assurance laboratory at the fish oil refining company.

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

An Agilent Technologies 7890A gas chromatograph equipped with a 7000 Series Triple Quad GC-MS system (Agilent Technologies) was used. The autosampler HT3™ Static and Dynamic Headspace System (Teledyne Tekmar) was equipped with a Supelco K Trap. The only manual step was preparing samples into 22 mL vials. The autosampler was controlled with the software HT3 TekLink and the instrument was controlled with the software MassHunter Workstation Software, Qualitative Analysis (version B.05, 2011, Agilent Technologies Inc.). The volatiles were separated on an Agilent J&W DB-624 UI column, 30 m × 0.25 mm × 1.4 μm, with Helium as carrier gas at constant flow, 2.2 mL of helium/min. The following temperature program was used: initially set at 35 °C for 6 min, increased to 100 °C at a rate of 9 °C/min, followed by 13 °C/min raise to 220 °C, and a final hold at 250 °C for 4 min. The inlet temperature was 220 °C, split 10:1. The mass detector ion range was 35 to 300 m/z. The experimental conditions for the Dynamic Headspace system are shown in Table 4.

1-Penten-3-ol, (E)-2-pentenal, 2-ethyl-furan, and (E,E)-2,4-heptadienal were selected as quality indicators to monitor the lipid oxidation during storage conditions of the samples as they were expected to be found in fish oil and powder and is common oxidation products in the literature. 4-Methyl-2-pentanol was used as an internal standard.

The software Agilent MassHunter Qualitative Analysis and NIST Mass Spectral Library (version 2.0, 2011, US Secretary of Commerce/Agilent) were used for performing the identification of compounds and the Integration of peaks. Identification of the oxidation products and volatiles was achieved by comparison of retention times (t_R) and mass spectra against the sample peaks of the indicator standards, analyzed under identical conditions:

(E)-2-Pentenal: t_R 11.32 min, 55.0 m/z, 84.0 m/z,

2-Ethyl-furan: t_R 8.64 min, 81.0 m/z, 53.0 m/z,

1-Penten-3-ol: t_R 9.19 min, 57.0 m/z, 29.0 m/z,

(E,E)-2,4-Heptadienal: 81.0 m/z, 110.0 m/z,

4-Methyl-2-pentanol (internal standard): t_R 11.43 min, 45.0 m/z, 43.0 m/z.

The Dynamic Headspace GC-MS analysis was performed by Kåre Andre Kristiansen, Senior Engineer at the Department of Biotechnology and Food Science, NTNU. I attended and was present for each set of samples that were analyzed.

3.3.2 Peroxide Value (PV)

The peroxide value (PV) measurements were performed by the iodometric titration method in accordance with the AOCS Official Method Cd 8-53. The titration endpoint was determined potentiometrically by an automatic titrator (TitroLine 7000, SI Analytics) connected to a platinum electrode and a reference electrode. The titrator agent was a 0,01 M $Na_2S_2O_3$ (CAS nr. 7772-98-7, Merck, Darmstadt, Germany) solution. 0.10-1.05 grams of oil was weighed in beakers (amount of sample was adjusted depending on expected PV) and dissolved in 30 mL of a chloroform (CAS nr. 67-66-3, Merck, Darmstadt, Germany) / acetic acid (CAS nr. 64-19-7, Darmstadt, Germany) solution (2:3). Further, 0.5 mL of a potassium iodide (CAS nr. 7681-11-0, Merck, Darmstadt, Germany) solution was added to the mixture and stirred with a magnetic stirrer for 1 minute. Lastly, 30 mL of distilled water was added before the sample was placed in the titrator. The blank titration was carried out by the same procedure, without the addition of the oil sample. The analysis was performed in duplicate, and the results were reported in milliequivalent hydroperoxide per kg oil. The PV was calculated using the following formula:

$$\text{Peroxide Value (meq peroxide kg}^{-1}\text{ oil)} = \frac{C(V_t - V_b)}{m} \times 1000 \qquad (2)$$

C = titrant concentration (mol/L)

V_t = titrant volume (mL)

V_b = blank volume (mL)

m = mass of oil sample (g)

1000 = unit conversion factor

3.3.3 Anisidine Value (AV)

The anisidine value (AV) was performed according to the AOCS Official Method Cd 18-90. Instruments and chemicals are not further specified as the analysis was conducted at the quality assurance laboratory at the supplement fish oil company. The p-anisidine reagent (0.25%) was prepared fresh every day of analysis. The absorbance of 0.25 g of p-anisidine dissolved in 100 mL of 100% acetic acid was measured to ensure a value below Abs = 0.2. 0.196-0.523 g of oil (amount of sample was adjusted depending on expected AV) were weighed directly in test tubes and dissolved in 5 mL of iso-octane. After transferring 2.5 mL of sample to a cuvette, the absorbance was measured at 350 nm against pure iso-octane as a blank. 0.5 mL of p-anisidine reagent was then added and the cuvette was shaken by inversion. The second absorbance measurement was made after the cuvette had been in the dark for 10 minutes. The measurements were performed in duplicate and expressed as AnV.

The AV was calculated using the following formula:

$$\text{Anisidine Value (AV)} = D \times \frac{1.2 \times (A_{S2} - B_{S2}) - (A_{S1} - B_{S1})}{m} \quad (3)$$

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

A_{S1} = First spectrophotometric measurement of sample

A_{S2} = Second spectrophotometric measurement of sample

B_{S1} = First spectrophotometric measurement of blank

B_{S2} = Second spectrophotometric measurement of blank

m = mass of oil sample (g)

3.3.4 Thiobarbituric Acid-Reactive Substances (TBARS)

The measurements of TBARS were determined by the spectrophotometric method in accordance with Ke and Woyewoda (1979). For analysis, 10-12 mg of oil sample was weighed into a 25 mL Kimax tube with a Teflon-lined screw cap. 5 mL of TBA working solution was added and the solution was mixed on a Vortex mixer (VWR International) for 10-15 sec to dissolve the lipids in the TBA reagent. The TBA working solution was made 30 minutes before analysis and was a mixture of 180 mL 0,04 M TBA stock solution (2-thiobarbituric acid (CAS

nr. 504-17-6, Sigma-Aldrich, Missouri, USA)), 120 mL chloroform (CAS nr. 67-66-3, Merck, Darmstadt, Germany), 15 mL 0,3 M sodium sulfite (CAS nr. 7757-83-7, Merck, Darmstadt, Germany) reagent, and 9.45 mL 3% BHT (CAS nr. 128-37-0, Merck, Darmstadt, Germany) solution in ethanol (CAS nr. 64-17-5, Merck, Darmstadt, Germany).

A standard graph was prepared by adding 1, 3, 5, 10, 15, and 20 nmol/MA of the TEP (CAS nr. 122-31-6, Sigma-Aldrich, Missouri, USA) working standard solution (0.1 mM) to 5 mL of the TBA working solution. All tubes containing lipid samples, blank, and TEP working standard solution were then heated for 45 min in a 90 °C boiling water bath (VWR International). Next, the tubes were cooled in tap water, and 2.5 ml of TCA solution (0.28 M trichloroacetic acid (CAS nr. 76-03-9, Sigma-Aldrich, Missouri, USA)), was added to each tube. The tubes were mixed by inversion, and centrifuged (Multifuge X1R, Thermo Scientific) for 10 min at 2500 rpm so that the pink aqueous phase became completely separated from the chloroform layer. The absorbance of the top aqueous layer was measured at 538 nm in 10-mm cuvettes by a spectrophotometer (Ultrospec 2000, Pharmacia Biotech). For the highly oxidized lipid samples, the aqueous phase was diluted (1:100) with 55% (v/v) acetic acid (CAS nr. 64-19-7, Darmstadt, Germany) in distilled water.

The analysis was performed in triplicate and expressed as µmol MA (malonaldehyde) per gram lipid. A linear calibration curve was obtained for the range of 1.0-20.0 nmol malonaldehyde (MA), and the TBARS value was calculated from the following formula:

$$\text{TBARS (µmol MA/g lipid)} = \frac{A_S - I}{S \times L \times 1000} \quad (4)$$

A_s = Absorbance of sample

I = Intercept of the standard curve

S = Slope of the standard curve

L = Weight lipid sample (g)

3.3.5 Other equations

The total oxidation value (TOTOX) was calculated according to Shahidi and Wanasundara (2002) as:

$$\text{TOTOX} = (2 \times PV) + AV \quad (5)$$

The iodine number was calculated with the following simplified formula:

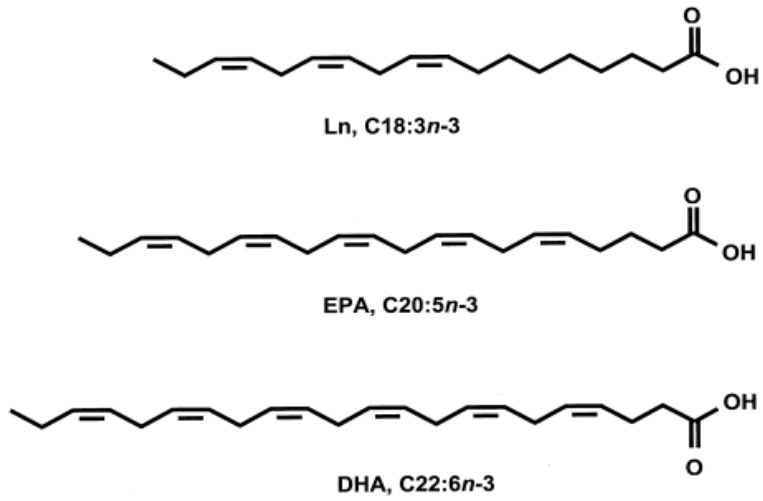

Figure 6. Chemical structures of n-3 PUFA: α-linolenic, eicosapentaenoic (EPA), and docosahexaenoic acid (DHA) (borrowed from Kamal-Eldin and Yanishlieva (2002)).

Eicosapentaenoic acid (EPA) has a molecular weight of 302.5 $gmol^{-1}$. As seen in Figure 6, 1 mol of EPA has 5 mol of double bonds. 1 mol of double bonds reacts with 1 mol of I_2 (M = 254 $gmol^{-1}$).

5 mol of double bonds react with $5 \times 254 = 1\ 270$ g I_2

1 mol (302.5 $gmol^{-1}$) EPA reacts with 1 270 g I_2

100 g EPA reacts with: $\frac{100}{302.5} \times\ 1\ 270$ (6)

3.3.6 CDR FoodLab

PV and AV were also measured using the CDR FoodLab reagent kit-based (CDR s.r.l, Firenze, Italy) spectrophotometric method. PV in oil measured in the range 0.3-25 meqO_2/kg required cuvettes with R1 reagents (*300154) and a R2 reagent (*300155). For measuring PV in highly oxidized samples out of the measuring range, a diluent (*300144) was also needed. AV in oil was measured in the range 0.5-100 AnV with cuvettes containing a R1 reagent (*300503). For powder measurements, the same reagents as for PV and AV determination were used in addition to an extraction solution (*300136). The concentration range was 0.05-25 meqO_2/kg and 0.5-100 AnV for PV and AV, respectively. Procedures used in the experiments were received from the supplier (Labolytic AS, Trondheim, Hilde Bremseth), and are referred to below. The analyzes were performed in duplicate. All (R1) reagent cuvettes were placed in incubation cells in the instrument to warm up for at least 5 minutes before analysis.

For PV measurements of the oil samples, procedures in the range 0.3-25 meqO_2/kg (CDR FoodLab, 700.02, Peroxides [0.3-25] meqO_2/Kg | PEROXIDES | OIL FATS) and 7-550 meqO_2/kg (CDR FoodLab, 750.02, Peroxides [7-550] meqO_2/Kg | PEROXIDES | OIL FATS) were used. The PV determination by the FoodLab method is based on a colorimetric reaction where peroxides (R-O-O-R) oxidize the Fe^{2+} ions. These complexed ions form a red color having an intensity that was measured at 505 nm, which is directly proportional to the concentration of peroxides in the sample (Aprea et al., 2006). The amount of oil sample added to the reagent depended on the concentration range measured. There were two photometric readings in the PV method: one after the addition of the oil sample to the R1 reagent, and one after adding the R2 reagent to the same test tube. A blank reactive was carried out the first time the test was performed. The highly oxidized samples measured in the range of 7-550 meqO_2/kg required a diluent. The oil sample was diluted with an appropriate amount of dilution solution, incubated for 2 minutes and this solution was added to the reagent cuvette and read photometrically. Further, an appropriate amount of R2 was added before the second photometric reading. The PV was expressed in meqO_2/kg.

The AV determination by the FoodLab method is based on a colorimetric reaction of aldehydes, derived from the secondary oxidation of fat matrix that reacts with the p-anisidine determining a variation in the absorbance, forming a yellow color that was measured at 336 nm (Labolytic, 2022). The AV method only required the oil sample added in the same quantity to all reagent cuvettes as this method only had a concentration range of 0.5-100 AnV (CDR FoodLab, 1350.04, p-Anisidine fish oil | p-ANISIDINE | OIL FATS). The AV was expressed in AnV.

PV and AV measurements of the powder samples required an extraction solution before analysis. Procedures for powders (CDR FoodLab, SAMPLE PREPARATION | OIL AND FAT, Fluors) were used for AV (CDR FoodLab, 1250.09, p-Anisidine flours | p-ANISIDINE | OIL FATS) and PV (CDR FoodLab, 600.07, Peroxides flours | PEROXIDES | OIL FATS).

1 g powder was weighed and added to 3 mL extraction solution before mixing it for 5 min with a vortex mixer (VWR International). The solution was centrifuged (Eppendorf Centrifuge 5804) for 10 min at 5000 rpm. The clear solution was extracted in an appropriate amount to analyze. The result of this analysis was relative to the fat content in the sample of flour, or this case powder. The percentage of fat (35%) was therefore specified in the first step of the analysis. Further, the PV and AV were measured in the same procedure as the oil samples.

3.4 Statistical Analysis

The experiments were conducted in duplicate (PV and AV) and triplicate (GC-MS and TBARS), and the mean values and standard deviations are reported using Microsoft Excel.

Statistical analysis was performed using IBM SPSS statistics software (v. 28.0, SPSS Inc., Chicago, IL). All values were assumed to be normally distributed. Differences were considered significant at $p \leq 0.05$. A t-test was performed to determine the differences between the traditional method and the rapid method for measuring PV and AV. A two-way ANOVA (Univariate Analysis of Variance) was used to establish the relationship between the different methods and the oil and powder. A comparison between the oil and powder was determined by a t-test. The TOTOX data, TBARS data, and the total area of volatile components were analyzed by one-way ANOVA and Tukey's test with a 0.05 level of significance to determine significant differences between the samples throughout the storage period. A correlation analysis between traditional methods and FoodLab was carried out for the oil. Correlation was also tested between volatile compounds and PV and AV. The Pearson correlation coefficient, *r*, was used to verify the presence of a correlation (significant at 0.01 level (2-tailed)) between the data. P-values are shown in Appendix A.

4. Results and Discussion

Lipid oxidation consists of very complex reactions with several possible interactions between influencing factors, which makes it almost impossible to fully predict what will happen in a complex food system. This chapter includes the storage experiment of the oil and powder by exposure to conditions that favor oxidation; high temperature and oxygen access. PV was used as a parameter for primary oxidation products, and AV, TBARS, and GC-MS were used to analyze secondary oxidation products. The rapid methods for the analysis of PV and AV using CDR FoodLab are also included. Finally, this chapter will address the pre-experiments that led to the optimized GC-MS method parameters.

4.1 Storage Experiment

One sub-goal of this study was to analyze the oxidative stability of the oil and powder. Thereupon, the oil and powder were stored at 40 °C with a headspace of oxygen for up to 4 weeks. The storage temperature of 40 °C was chosen as a compromise between accelerating lipid oxidation and not risking other reactions to occur in the oil and powder in line with literature (Frankel, 2014; Sullivan et al., 2011). Frankel (2014) stated that the same reactions occur at 60 °C as well as lower temperatures, hence, the oxidation state at lower temperatures can be estimated. As the stability of the oil and powder had not been tested at high temperatures over a long period of time, the experimental design had to ensure that the oil and powder oxidized as well as having the opportunity to study oxidative development. Sullivan et al. (2011) research indicated that accelerated testing of fish oil should be conducted at temperatures no higher than 40 °C as this temperature follows the same kinetics as lower temperatures. This also agrees well with the recommendation by Frankel (2014). To monitor oxidative volatile compounds, Lee et al. (2006) and Aidos et al. (2002) used accelerated temperatures of 50 °C and 80 °C, respectively.

This chapter also includes the last sub-goal, exploring whether the powder is more stable than the oil. Moreover, the storage experiment will determine whether GC-MS was a suitable method for measuring oxidative status directly in powder, without an extraction step (main goal). The results from the traditional methods and CDR FoodLab are compared as one sub-goal of this study was to examine how suitable the rapid method is against the traditional methods.

4.1.1 Volatile Oxidation Products

Fish oil and powder were analyzed with dynamic headspace GC-MS at different levels of oxidation to study and compare the oxidative changes and stability. The GC-MS analysis of volatile oxidation products detected oxidative changes as early as the first day (sub-goal 2) and was able to detect several of the targeted indicator compounds and volatile oxidation products in the fish oil and powder during the storage period.

The chromatogram peaks were essentially the same in oil and powder and had very similar retention times. This research contributes to filling an information gap regarding a method of measuring oxidative changes in fish powder without extracting the powder (main goal). The oil samples represented 1 g of 100% oil, and the powder samples represented 1 g of powder containing 35% oil. As the addition of the internal standard to the samples was only partially successful considering IS did not have the same response (area) for each parallel of samples, or for each sample if the oil changed over time, the concentration of the volatiles is expressed in peak areas only.

As illustrated in Figure 7, 9 volatile secondary oxidation products were identified in the oil, and 11 were identified in the powder, in addition to two furan compounds. The volatiles are listed in Appendix B and shown in Figure 7. Major volatiles found, which can be possible markers for oil quality, were 1-penten-3-ol, 1-penten-3-one, 2-ethyl-furan, 2-pentenal, and hexanal compounds. The volatile oxidation products found in this study were mainly in good accordance with compounds previously detected in comparable materials (Fang et al., 2018; Hsleh et al., 1989; Karahadian & Lindsay, 1989; Kulås et al., 2002; Lee et al., 2006; Olsen et al., 2006; Shibata et al., 2018). However, qualitative, and quantitative comparison of headspace data obtained with different methods and sample matrixes is complicated.

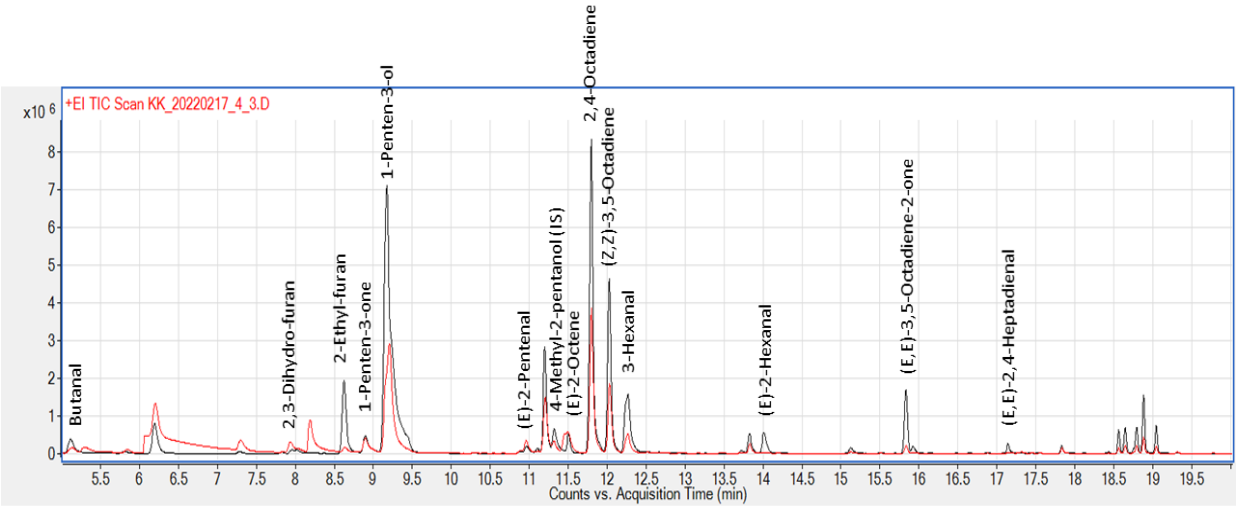

Figure 7. Identification of oxidation products and volatile compounds in the fish oil (red) and powder (black) by GC-MS analysis. The chromatogram shows samples on day 14.

Three quality indicators detected in previous trials (Figure 17) were found in the fish oil and powder. 1-Penten-3-ol was one of the most abundant volatiles from n-3 fatty acids and was a good marker compound. 2-Ethyl-furan appeared at a retention time of 8.63 min, and 1-penten-3-ol had a retention time of 9.20 min in both the oil and powder. (E)-2-Pentenal had a retention time of 10.96 min in the oil, and 10.95 min in the powder (Appendix B). Figure 8 shows an increasing trend of the area for 2-ethyl-furan, (E)-2-pentenal, and especially 1-penten-3-ol, during the storage period of 4 weeks.

The last quality indicator, (E,E)-2,4-heptadienal, wasn't detected when diluted in ethanol and water, nor sunflower oil. Proper identification of volatile oxidation products requires a GC retention time identical to that of a standard, in addition to a match with the MS specter of the standard or with that of a library spectrum. The peak at 17.83 min got a hit on (E,E)-2,4-heptadienal in the NIST Spectral Library. The mass spectra of the sample peak had strong similarities to the mass spectra of (E,E)-2,4-heptadienal. (E)-2-Octene, a targeted indicator standard detected in previous trials (Figure 17), was detected in the powder (t_R 11.50 min). Thus, identification by mass spectra was confirmed by the comparison of the retention times with standards of four compounds.

Which of the identified volatile compounds contribute to the unpleasant smell and taste of rancid fish and fishy off-flavor? Jónsdóttir et al. (2005) reported 1-penten-3-one (pungent, green odor) and (E,E)-2,4-heptadienal as very potent odorants which contribute to the unpleasant rancid and fishy off-flavor in bulk fish oil. Shibata et al. (2018) and Olsen et al. (2006) also

identified 1-penten-3-one and (E,E)-2,4-heptadienal as the flavor deterioration indicators of oxidizing fish oil, and good marker compounds for early lipid oxidation in n-3 containing matrixes. Karahadian and Lindsay (1989) stated that hexanal and (E,E)-2,4-heptadienal caused oxidized, rancid, and painty flavors in fish oils.

1-Penten-3-one, (E,E)-2,4-heptadienal, and (E)-2-hexanal appeared in relatively small concentrations in the fish oil and powder relative to other volatile compounds in the chromatogram. None of these compounds were identified by analysis of standards, but were found in the NIST Spectral Library, and were compared with their mass spectra. However, Olsen (2005) stated that the rapid formation of 1-penten-3-ol and other compounds may indicate that oxidation of less abundant n-3 fatty acids may also contribute to the deteriorated odor and flavor of the materials.

In accordance with Kulås et al. (2002) (fish oil stored at 30 °C), none of the expected 2,4,7-decatrienal isomers were detected, possibly due to rapid further oxidation to molecules of lower molecular weight.

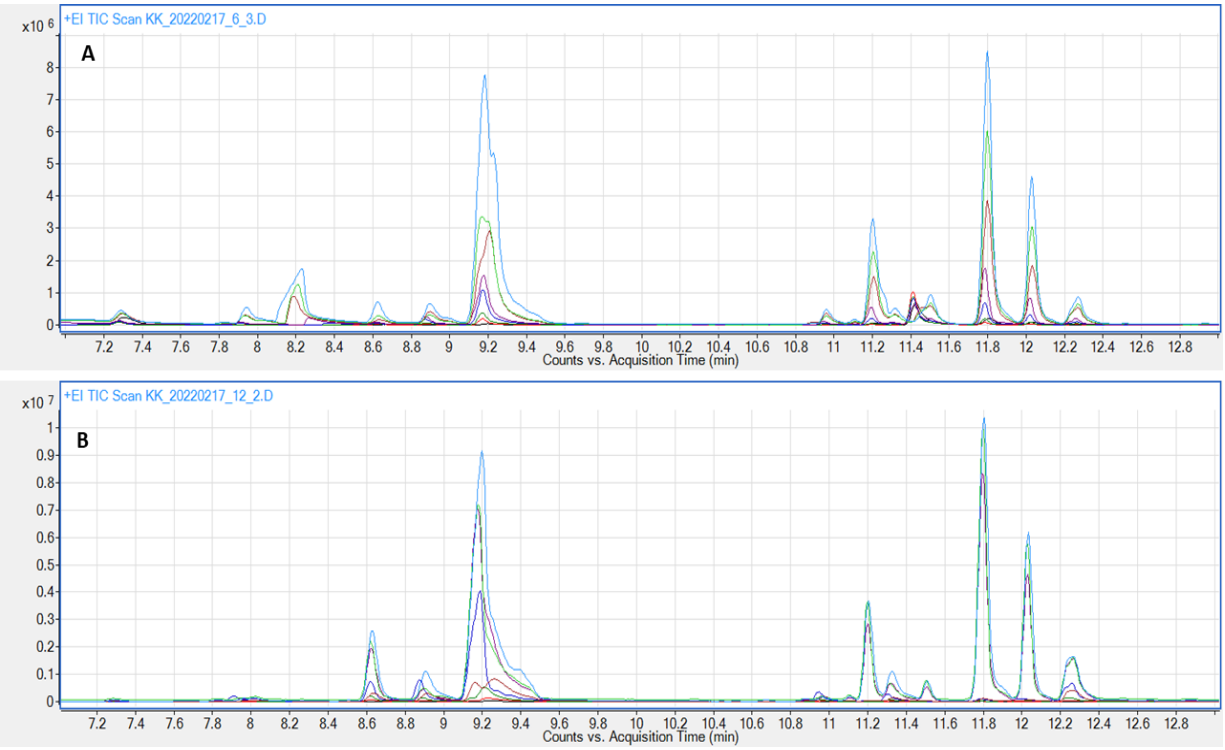

Figure 8. Formation of oxidation products and volatile compounds in the fish oil and powder during 4 weeks of storage (in the retention time range with the most activity). Sampling days 0, 1, 2, 4, 7, 14, 21, and 28, respectively. ***A*** *Chromatogram of the oil samples (100% oil).* ***B*** *Chromatogram of the powder samples (35% oil).*

Figure 8 shows that the total amount of volatiles, and their area, increased during the storage time. The oxidation products and the volatile compounds identified in the oil and powder occurred approximately at the same retention times (± 0.02). It is also illustrated in the chromatogram that there are more peaks in the oil than in the powder, but two more oxidation products were identified in the powder samples (Appendix B).

By studying the shape of the peaks, it appeared that especially 1-penten-3-ol (t_R 9.19 min) got an overload on day 14 in the oil and day 7 in the powder. In these samples, the NIST Spectral Library got a hit of pentanal (t_R 9.18 min) in the same peak as 1-penten-3-ol.

In Figure 9, the area in the powder samples is corrected from containing 35% oil to 100% oil for the analyzed powder samples and oil samples to contain the same amount of oil (formula 1 Chapter 3.2.2). This was done to create equal conditions for the oil samples and the powder samples, as it is the oil that oxidizes and forms the volatile compounds that are analyzed.

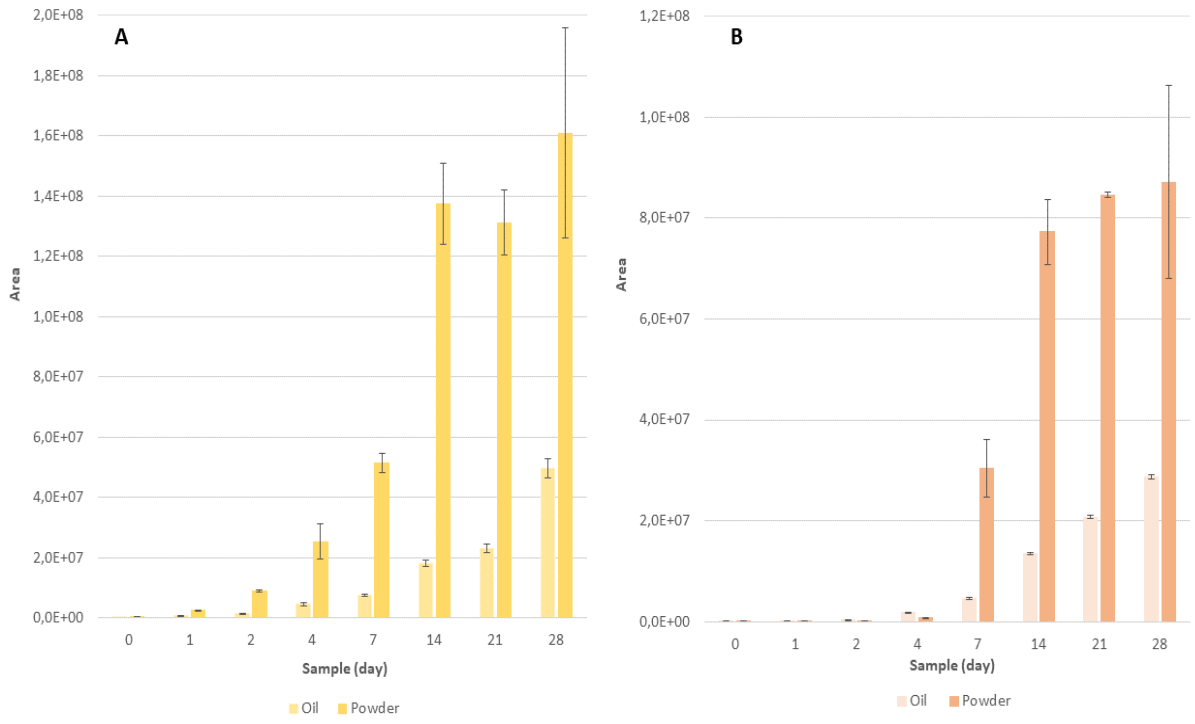

Figure 9. Comparison of two oxidation products´ areas in the fish oil and powder throughout the storage period analyzed with GC-MS. The area in the powder samples is corrected from containing 35% oil to 100% oil. Data are presented as mean values (n=3) ± standard deviations. ***A*** *The area of 1-penten-3-ol (t_R 9.2 min) in oil and powder.* ***B*** *The area of 2,4-octadiene (t_R 11.8 min) in oil and powder.*

Significant development ($P < 0.05$) of secondary oxidation products throughout the storage period in the oil and powder was found, based on the total area of 2,4-octadiene and 1-penten-3-ol. Figure 8 and Figure 9 show that oxidation products were developed at different reaction rates. Figure 9 illustrates that the development of 2,4-octadiene increased more gradually than for 1-penten-3-ol. The area of 1-penten-3-ol had a stable increasing rate in both the oil and the powder throughout the storage period. A clear peak of 1-penten-3-ol occurred in the chromatogram already on days 1 and 2 for the oil and powder, respectively. A significant increase ($P < 0.05$) can be seen on day 2 in the oil, and day 4 in the powder. The area of 2,4-octadiene in the oil had a slight increase in the first days, but a clear peak in the chromatogram can be seen on day 4, with an area significantly higher ($P < 0.05$) than the prior days. In the powder, a clear peak for 2,4-octadiene does not occur until day 7, which had a significantly higher ($P < 0.05$) area than the days before. Data can be seen in Appendix C.

Based on the area of 2,4-octadiene and 1-penten-3-ol, there were found significant differences ($P < 0.05$) between the oil and powder. The oxidation products in the powder generally gave a higher oxidation products area than in the oil through several of the GC-MS analysis in this study, despite the powder samples containing only 35% oil. Looking at the overall powder analyzes in this study it appears that the powder led to an increased degree of oxidation, which is further discussed below.

A substantial parameter regarding the differences in the area of the volatile compounds between the oil and powder was the difference in sample matrixes. The powder is less compact (bigger surface area), filling a larger amount of the GC vial, as well as giving a smaller headspace compared to the oil. The surface volume ratio is a very important parameter for how much of the analyte goes into headspace (G. Vogt, personal communication, January 14, 2022), and may be a reason why the powder samples got a higher area than the oil samples. This is in accordance with theory (Chapter 2.4) by Frankel (2014) explaining that large surface-volume ratios between lipids and air leave relatively more fatty acid molecules easily exposed to air than if the material was more compact.

There were probably large quantities of oxygen inside the powder analyzed in this study, which made it more exposed to oxidation than if it had been a microencapsulated powder where the wall material act as a physical barrier to oxidation in protected microcapsules (Jiménez-Martín et al., 2015; Jónsdóttir et al., 2005). If the oil was absorbed into the powder without oxygen and air pockets, the powder would probably be more protected against oxidation. There is generally

little dissolved oxygen in oils, which means that the oxidation mainly took place on the surface of the oil.

To study volatile components, Lee et al. (2006) accelerated the oxidation in two types of DHA-enriched fish oil, triacylglycerol (TG) and ethyl ester (EE), during storage at 80 °C with aeration. 31 volatile compounds were identified in EE, and 23 volatile compounds in TG. (E)-2-Pentenal, 1-penten-3-ol, and (E,E)-2,4-heptadienal were some of the commonly detected oxidized volatile compounds from TG and EE fish oil in Lee et al. (2006) study, which are the same compounds found in the fish oil and powder in this study. Aidos et al. (2002) measured volatile oxidation products in crude herring oil under accelerated oxidative conditions (50 °C). Their results suggested that 1-penten-3-one could be a useful volatile to follow, due to its good correlation with PV and AV. This finding gave a good indication of the hydroperoxides formed and decomposed. Together, 1-penten-3-one and (E,E)-2,4-heptadienal, were found to be good indicators of the oxidative status in fish oil.

Parallels in the powder samples varied more than parallels in the oil samples, which can be seen from the standard deviations in Figure 9. The powder sample on day 21 was re-analyzed because the GC chromatogram confirmed that there were contaminations in the bottle the first time the sample was analyzed. The sample also smelled of chemicals. Figure 9 shows that this sample was analyzed fresher, and especially the samples for days 14 and 28 have continued to oxidize when left in the autosampler for a couple of hours before being analyzed.

4.1.2 Primary and Secondary Oxidation Products

Primary and secondary oxidation products were measured in fish oil and powder stored with restricted access to oxygen at 40 °C to support the results of oxidation products and volatile compounds analyzed with GC-MS. PV and AV were determined using both traditional methods and the rapid method CDR FoodLab. TBARS was measured with a traditional method.

In the fish oil, PV was measured with the traditional iodometric titration method both at The Department of Biotechnology and Food Science, NTNU, and by the supplier fish oil company, due to complications with the method and high standard deviations when performing the method at NTNU. The traditional methods of determining PV and AV (measured by the supplier fish oil company) were compared with the rapid PV and AV measurements by the CDR FoodLab method. Peroxide values and anisidine values in fish oil and powder from the storage experiment can be seen in Figure 10.

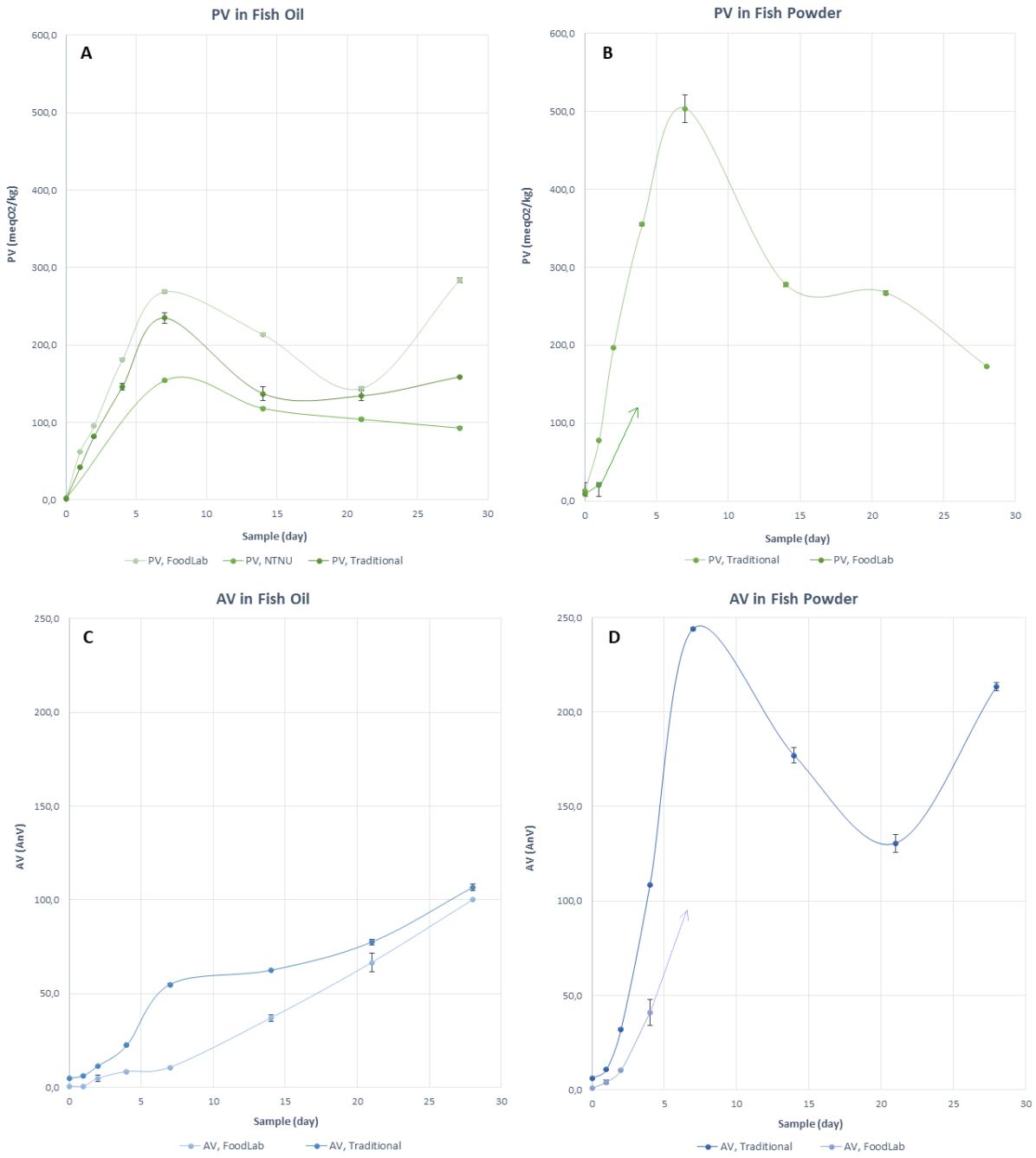

Figure 10. Formation of primary and secondary oxidation products in fish oil and powder stored at 40 °C for up to 4 weeks. Sampling days 0, 1, 2, 4, 7, 14, 21, and 28. Data are presented as mean values (n=2) ± standard deviations. ***A*** *Peroxide values in fish oil determined by traditional methods and a rapid method (FoodLab).* ***B*** *Peroxide values in oil extract from fish powder determined by a traditional method and a rapid method (days 0 and 1).* ***C*** *Anisidine values in fish oil determined by a traditional method and a rapid method.* ***D*** *Anisidine values in oil extract from fish powder determined by a traditional method and a rapid method (days 0, 1, 2, 4).*

For both PV and AV, the traditional methods and CDR FoodLab show significant differences ($P < 0.05$) throughout the storage period in the oil and powder. Appendix D shows more detailed data. Both the traditional methods and CDR FoodLab show that the oil is significantly different ($P < 0.05$) from the powder, using the TOTOX parameter. The TOTOX value was used as it includes the total amount of oxidation products (PV and AV).

The peroxide values showed immediate oxidative changes, which indicate high amounts of primary oxidation products. The high temperature during storage caused no induction period as illustrated in Figure 2 (Chapter 2.2.2). The PV in both fish oil and powder increased rapidly in the first week (sub-goal 2), reaching their highest values on day 7 (Figure 10, A, and B). PV measured in the fish oil day 7 with CDR FoodLab reached 268.9 $meqO_2/kg$, while PV with the traditional method (supplier fish oil company) was 234.8 $meqO_2/kg$. PV in the oil extract measured with the traditional method in the powder was 503.4 $meqO_2/kg$ on day 7. The TOTOX values on day 7 were significantly higher than values for the remaining days for both methods in the oil, and the traditional method in the powder. The peroxide values reached their saturation point on day 7 and shows a decreasing trend for the rest of the period. The decreasing peroxide values after day 7 may indicate that the hydroperoxides have been converted to secondary oxidation products, in line with Velasco et al. (2010) findings.

The fresh samples (day 0) for oil, FoodLab and traditional, and powder, FoodLab and traditional, had PV values of 2.1, 1.0, 8.6, and 12.6 $meqO_2/kg$, respectively. It appeared that the powder was initially more oxidized compared to the oil, which may have given the powder a different starting point for further oxidation. The powder oxidized faster than the oil. This suggested that the powder was less stable than the oil and that the powder did not protect the oil in the powder (sub-goal 3). These findings were consistent with the results of the volatile compounds by GC-MS analysis mentioned in the previous chapter. As the bottles were not flushed with nitrogen before they were placed in the heating cabinet or the freezer, the oil and powder had access to oxygen in the headspace of the bottle. The oil and powder were stored in bottles with equal surface area. However, it seemed like the powder may have oxidized more easily in the container than the oil during the storage period, as the powder had a bigger surface area, and more oxygen was therefore dissolved on the powder´s surface (Figure 15). Hence, the powder samples may have been more easily affected by the platen temp. (70 °C) and by room temperature and light exposure during sample preparation and analysis.

Furthermore, the PV in the fish oil decreased before an increasing value can be observed towards day 28 for two of the methods. It was found significant differences ($P < 0.05$) between the traditional method and the rapid method in the determination of PV.

Immediate production of hydroperoxides or a very short induction period was expected considering the high abundance of EPA and DHA in the fish oil, as the reactivity for oxidation in refined n-3 long-chained PUFA depends mainly on the level of polyunsaturated fatty acids and the degree of polyunsaturation. The unsaturation of the fish oil (>60% EPA/DHA) was calculated as the iodine number (Fang et al., 2018). For EPA ($M = 302.5\ gmol^{-1}$) and DHA ($M = 328.5\ gmol^{-1}$), the iodine number was calculated to be 420 and 464 respectively, using a simplified formula (formula 6). The greater the iodine value, the more unsaturation, meaning higher susceptibility to oxidation (Sanders, 2003). The fish oil used in this study (made from mainly sardines and up-concentrated at the fish oil factory) contained high amounts of n-3 (>70%). The producer of the oil calls it "Ultrahigh" as it has amongst the highest iodine values in the market. Sardine oils (crude oil) were reported to have an iodine value between 165-185 by Thomas (2012). Cod liver oil was reported to have an iodine value of 159 by Aure et al. (1959).

The (restricted) access to oxygen in the bottle headspace and the storage temperature at 40 °C also promoted the high production of hydroperoxides. This is in accordance with data for oxidation of fish oil stored at 40 °C in the dark (Sullivan et al., 2011). With free oxygen access, lipid oxidation continues regardless of oxygen pressure, but if oxygen availability is limited, the oxidation rate becomes oxygen-dependent (Frankel, 2014). The restricted oxygen availability (samples stored in tight containers) could be a limiting factor for the formation of oxidation products during the later stages of the storage experiment, in line with previous studies by Olsen et al. (2005). Sullivan et al. (2011) found fish oil concentrate to demonstrate first-order kinetics at temperatures ≥ 4 °C and ≤ 40 °C. At 60 °C, the oil was found to oxidize more rapidly, likely because of hydroperoxides breaking down faster than they could form.

The decomposition of hydroxyperoxides into alkoxyl radicals initiates a variety of reaction pathways and products, for both volatile and nonvolatile compounds. As this decomposition may take place at a higher rate than the formation of new ones, it can result in falsely low levels of PV, even if the oil already is in an aggravated oxidative state (Matthäus, 2010). Therefore, PV measurement should be considered in combination with measurements of secondary oxidation products.

As illustrated in Figure 10, the secondary oxidation products (AV) were found to develop much slower than the primary oxidation products (PV). This correlates with Figure 2 (Chapter 2.2.2) as the primary oxidation products will dominate initially, and the secondary oxidation products will eventually dominate in a later phase (Frankel, 2014). Generally, a progressive decrease in primary oxidation compounds (PV) is normally detected as the secondary oxidation compounds (AV) start to emerge (Martín et al., 2012).

It was found significant differences ($P < 0.05$) between the traditional method and the rapid method in the determination of AV. In the fish oil, the anisidine values measured by CDR FoodLab and the traditional method (by the supplier fish oil company) were < 0.5 and 4.5 AnV on day 0 and reached their highest value on day 28 with values of > 100 and 106.7 AnV, respectively (Figure 10, C). The AV in the oil extract from the powder reached its saturation point on day 7 (244.1 AnV) and had a decreasing trend through day 21 before the value increased on day 28. AV day 0 in the powder sample was 6.0 AnV measured with the traditional method, and 0.9 AnV measured with FoodLab (Figure 10, D).

The upper PV and AV limits recommended by GOED are PV 5 meqO_2/kg and AV 20 AnV (Ismail et al., 2016). The PV in oil (41.9 meqO_2/kg) and powder (12.6 meqO_2/kg) had already exceeded the limit of 5 meqO_2/kg after 1 day and 0 days of storage at 40 °C, respectively. The secondary oxidation products were found to develop slower, especially in the first 2 days. The AV in oil (22.3 AnV) and powder (32.1 AnV) exceeded the limit of 20 AnV on day 4 and day 2, respectively. However, PV and AV are expected to be high as 40 °C is a fairly high temperature for storing fish oil that is greatly susceptible to oxidation. As mentioned in Chapter 3.2.1, this temperature was chosen in this study to accelerate lipid oxidation.

The Arrhenius equation can be used to calculate what the PV and AV would be if the oil and powder were stored at e.g., room temperature (25 °C) on the kitchen counter, or in the fridge (4 °C) over the same period. The widely used Arrhenius equation is a model of the temperature effect on the rate of chemical reactions and biological processes in foods (Peleg et al., 2012). Peleg et al. (2012) demonstrate that The Arrhenius equation has been widely used in food research.

In this study, based on the principle that the oxidation reaction rate doubles for every 10-degree increase in temperature (Frankel, 2014), a rough estimate was made for each of the peroxide and anisidine values up to day 7. The constants in the Arrhenius equation were not included as all conditions are the same except the temperature between storage time. The estimated PV and

AV for 0, 10, 20, and 30 °C can be seen in Figure 11. This rough estimate shows that the fish oil exceeds GOED´s upper recommended PV (5 meqO_2/kg) on day 1 (10.5 meqO_2/kg) if stored at room temperature (approx. 20 °C), and the upper recommended AV (20 AnV) is not yet reached at day 7 (13.7 AnV). If stored in the fridge (approx. 0 °C), the fish oil exceeds the upper recommended PV on day 2 (5.2 meqO_2/kg), and the AV on day 7 is 3.5 AnV. Subject to the presence of oxygen in the oil during storage, this highly oxidizable oil should not be stored in the fridge for more than 1 day if its use is for human consumption, even though the AV is only 0.4 AnV.

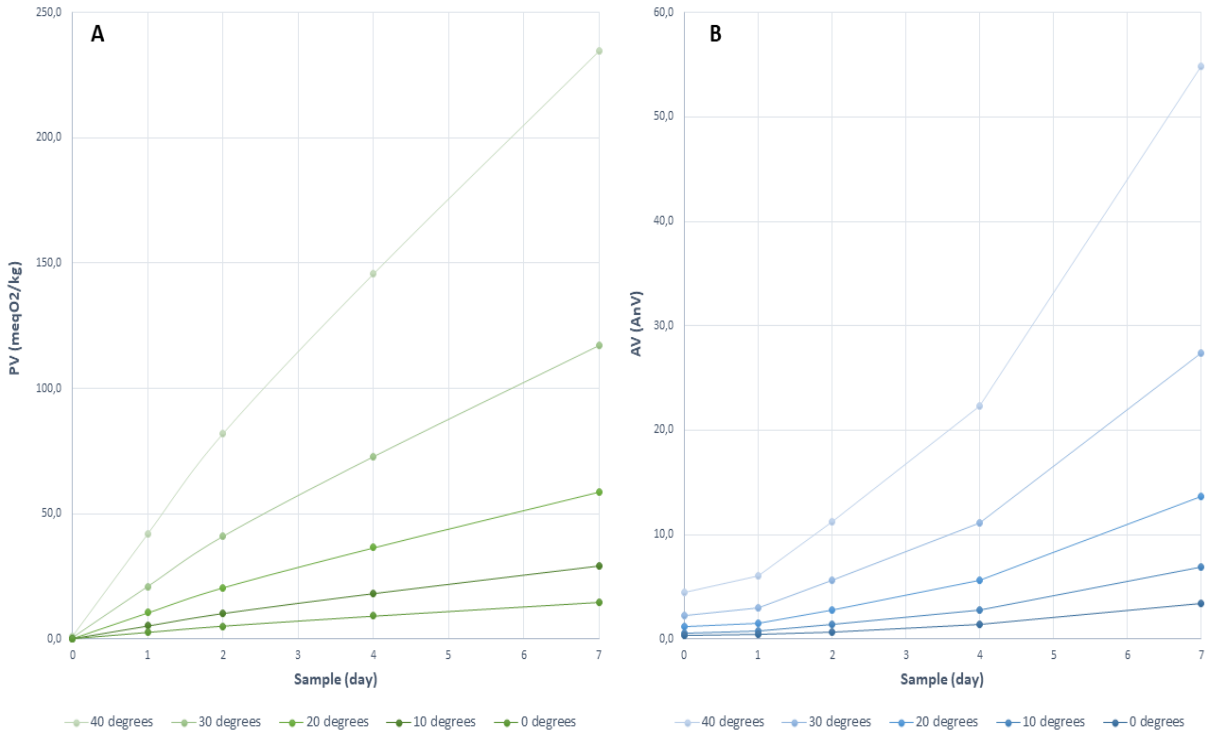

Figure 11. A rough estimate of the reaction rates for every 10-degree increase in the fish oil. ***A*** *The reaction rate of the peroxide values through the first week of storage.* ***B*** *The reaction rate of the anisidine values through the first week of storage.*

The oil from the powder samples had to be extracted before the determination of PV and AV, which made the analysis relatively time-consuming. The Bligh & Dyer extraction method was undertaken with care by placing the samples on ice in the dark between homogenizing and centrifugation. However, the formation of new hydroperoxides or decomposition of existing ones during the procedure was possible, regarding the PV. It was expected that the oil extracts from the powder samples had been subjected to oxidation through the extraction process,

especially at the step of chloroform evaporation at 60 °C even though there was a supply of N_2. The samples were left on the heating block for 7 hours for the chloroform to evaporate. Therefore, the powder samples got considerably higher PV and AV than the oil samples. It could be expected that the AV would be even higher as the aldehydes in the oil could evaporate into the water phase, which was not included further in the analysis. Only the chloroform phase is taken on to evaporation in the Bligh & Dyer method.

The extraction method with CDR FoodLab did not involve an evaporation step with a heating source. However, as it is a new method for flours, it is not fully developed. The concentration range for PV (0.05-25 meqO_2/kg) was low, and a dilute solution corresponding to the extraction solution was not developed. Therefore, most samples ended up outside the concentration range for the method. However, it can be seen in Figure 10 that PV (8.6 meqO_2/kg) and AV (40.9 AnV) measured with this method exceeds GOED´s upper limits on day 0 and 4, respectively. Furthermore, Figure 10 shows that the powder samples generally have lower values measured with CDR FoodLab than with the traditional PV and AV methods after the Bligh & Dyer extraction.

CDR FoodLab required minimal sample preparation. Sample preparation by this method with the right equipment can be approximately 1 hour with the number of samples prepared in this experiment. Also, the analysis time was very short and both PV and AV with the same number of samples could be completed in 1-2 hours (triplicates). The Bligh & Dyer method required 3-4 hours of sample preparation for the same number of samples and 6-7 hours of evaporation time of the chloroform phase. In addition, the time it took to perform the traditional methods of PV and AV must be included. It would have been of great health and economic value to the industry to have a fully developed rapid method for measuring and monitoring oxidation status in both fish oil and powder to offer products of the highest possible quality with low oxidation status to the consumer market.

The analyzes for determining PV and AV in fish oil and powder using CDR FoodLab were performed in duplicate. The standard deviations for PV and AV in the oil samples ranged from 0.14 to 4.88, with only two samples that got standard deviations over 1.70. The analyzes of the oil extract from the powder had standard deviations between 0.42-1.25. One sample got a standard deviation of 6.79. As expected, most of the samples ended up outside the concentration range for both PV (0.05-25 meqO_2/kg) and AV (0.5-100 AnV) (Appendix D). Martín et al. (2012) performed PV and AV analyzes on CDR FoodLab in duplicates. Petersen et al. (2012)

performed the analyzes in triplicate and got standard deviations between 0.1 to 1.8 for the determination of PV and AV.

The formation of secondary oxidation products measured by the Thiobarbituric acid-reactive substances (TBARS) method in the fish oil with weekly sampling can be seen in Figure 12. Especially in fish products, TBARS values can be a good indicator of rancidity (Dalsvåg et al., 2021).

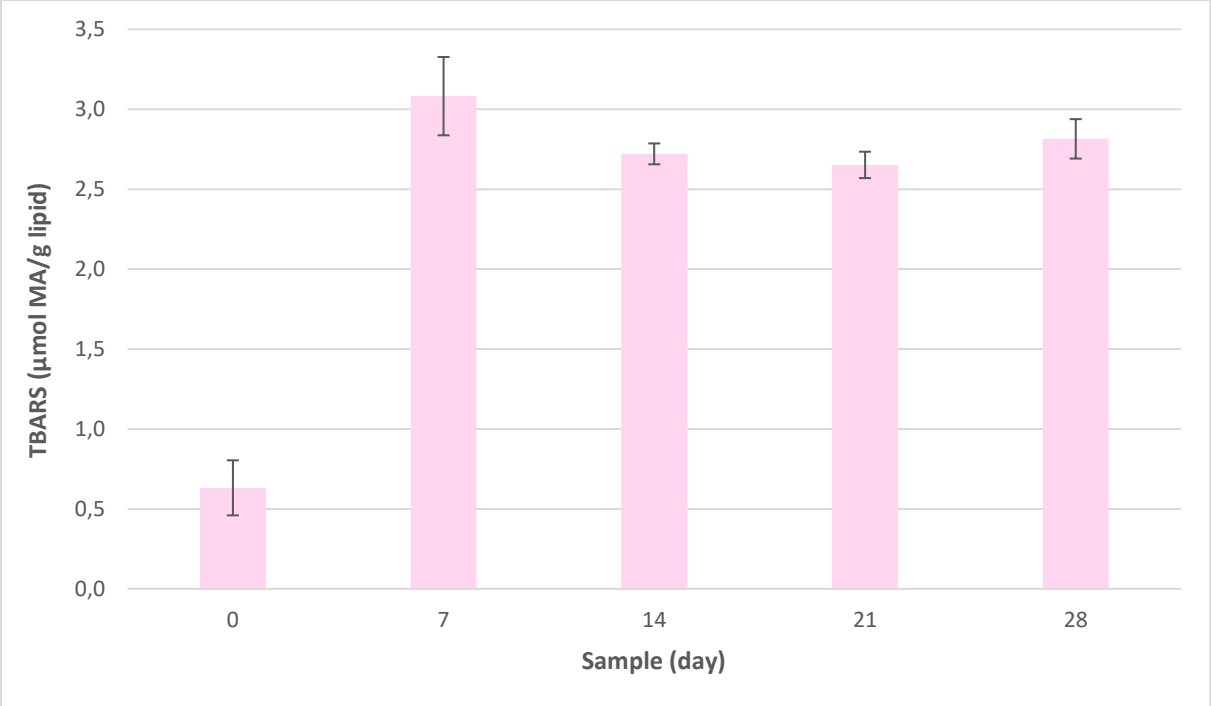

Figure 12. The extent of lipid oxidation in the oil expressed as TBARS values at weekly sampling: days 0, 7, 14, 21, and 28. Data are presented as mean values (n=3) ± standard deviations.

Significant development ($P < 0.05$) of secondary oxidation products (TBARS) in the oil was found throughout the storage period. The rapid increase in the development of oxidation products during the first week can, as for the PV and AV, also be observed for the TBARS values. The highest value (3.08 µmol MA/g lipid) can be seen on day 7, significantly higher ($P < 0.05$) than the remaining values, followed by a slightly decreasing trend. An increase in the TBARS value on day 28 shows the same trend as PV in fish oil and AV in the oil extract from powder. The TBARS value in the fresh sample (day 0) was 0.63 µmol MA/g lipid. The lipid samples got a dark orange color when it was supposed to be pink (Ke & Woyewoda, 1979). The color got light pink/orange by diluting the samples 1:100 with 55% (v/v) acetic acid in distilled water (Figure 13).

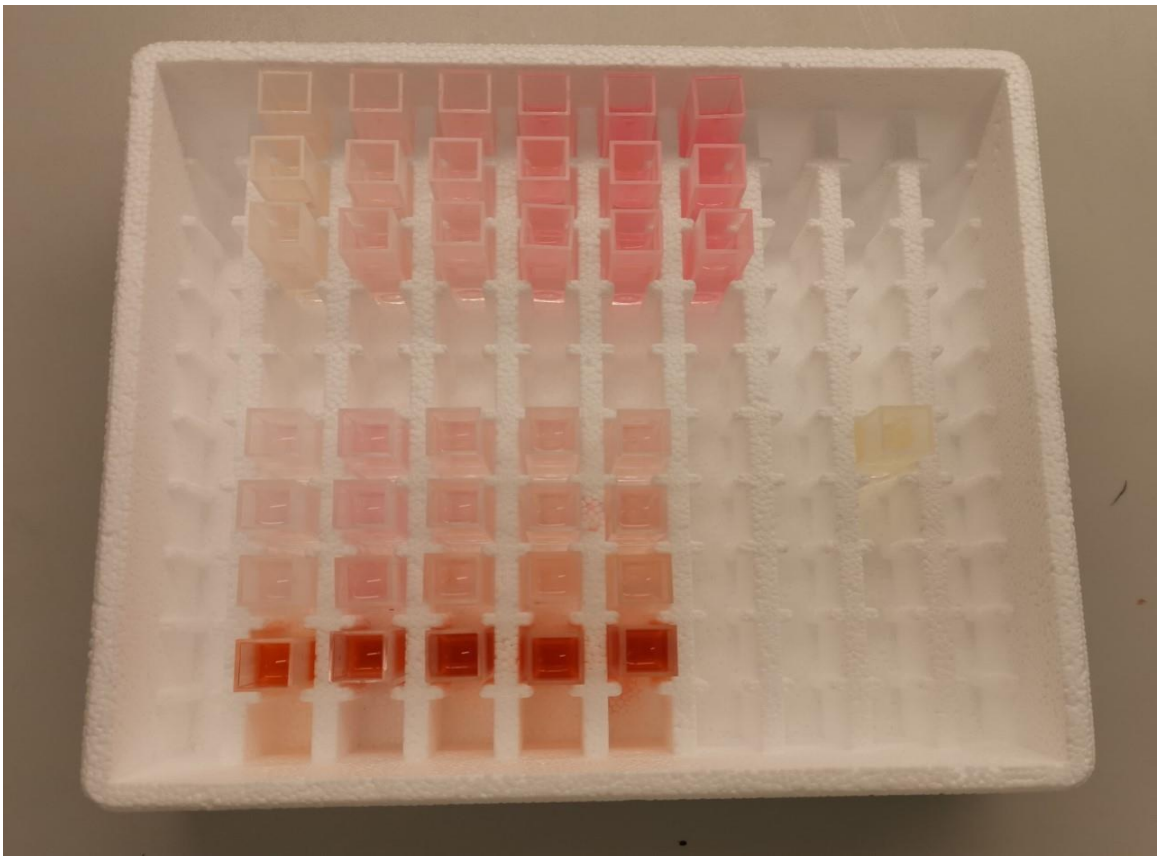

Figure 13. Cuvettes containing TBARS samples ready for spectrophotometric reading. The TEP standard graph (1, 3, 5, 10, 15, and 20 nmol/MA) can be seen in triplicates at the top. The blank sample is located to the right. The bottom row shows the oxidized oil on sampling days 0, 7, 14, 21, and 28, respectively. The top row in the same bulk shows the same samples diluted 1:100, which were analyzed in the spectrophotometer.

Kannaiyan et al. (2015) stated that several studies consider TBARS values less than 5 mg MDA/kg to be the limit of good quality, and the maximum acceptable limit for chilled fish for human consumption to be 8 mg MDA/kg. Converted to units used in this study, the limit values are 0.069 and 0.111 μmol MA/g lipid, respectively. As seen in Figure 12, all the oil samples got TBARS values above these recommended values throughout the storage period. This is not surprising since the oil was containing high amounts of n-3 polyunsaturated fatty acids (10:50% EPA:DHA), and was stored at 40 °C. As TBARS is a summarized measurement of several thiobarbituric acid-reactive oxidation products, it would be expected to detect early oxidative changes, which is in accordance with research by Olsen (2005).

Comparing results with other studies and food systems can be misleading as TBA reacts with other substances in food. TBARS values will vary among different food systems, and the fatty acid composition is found to influence the TBARS levels of foods (Irwin & Hedges, 2004). The recommended limits for TBARS values should therefore be interpreted with caution. Other

types of compounds than lipid oxidation products can influence the TBARS value, which is a well-known challenge (Frankel, 2014; Ke & Woyewoda, 1979). Interfering compounds can cause increased noise and lower sensitivity in some cases. This subject might be a bigger challenge for detection of smaller oxidative changes than in the oil used in this study.

Ke and Woyewoda (1979) reported that one common problem with highly oxidized samples from marine lipids and oil is the formation of a yellow pigment that interferes in the reaction. This yellow pigment was formed in the analysis in this study and can be seen in the bottom row in Figure 13. As aldehydes are involved in secondary oxidation reaction pathways and are hydroperoxide decomposition products, they may influence the TBARS levels (Hu & Jacobsen, 2016; Mariutti et al., 2009). Also, aldehydes from the fish oil may have evaporated into the water phase during the heating for 45 min in a 90 °C boiling water bath. The water phase was not included in the spectrophotometric reading.

4.1.3 Limitations of the Study, and Comparison of the Methods

The methods showed similar trends of oxidative changes. The rapid increase in the development of oxidation products in the first week was observed for all the traditional methods (PV, AV, TBARS, and GC-MS) in the fish oil and powder. For the fish oil, the highest values for PV and TBARS were reached on day 7, followed by a slightly decreasing trend. Later, on day 28, both the TBARS value and PV increased. The same trend can be seen in AV in the oil extract from the powder. Also, PV and AV in the oil extract from the fish powder had their highest values on day 7, followed by a decreasing trend. The area of volatile compounds detected by GC-MS also increased rapidly during the first week but continued to increase throughout the storage period.

It was found significant correlations ($P < 0.01$) between volatile compounds (1-penten-3-ol and 2,4-octadiene) and primary and secondary oxidation products (PV and AV) measured with traditional methods in oil and powder during the first week (day 0-7). The correlation analysis found evidence for good correlations between PV and AV in oil ($r = 0.955$), and PV and AV in powder ($r = 0.948$). Good correlations were also found between 1-penten-3-ol and 2,4-octadiene in oil ($r = 0.970$), and powder ($r = 0.872$).

Development of 1-penten-3-one had strong correlations with PV and AV in oil ($r = 0.984$ and $r = 0.965$, respectively), and powder ($r = 0.975$ and $r = 0.994$, respectively). Strong correlations were also found for development of 2,4-octadiene with PV and AV in oil ($r = 0.952$ and $r =$

0.998, respectively), and powder (r = 0.773 and r = 0.918, respectively). These findings are consistent with research by Aidos et al. (2002). They found that development of 1-penten-3-one correlated well with peroxide and anisidine values (r = 0.938 and r = 0.931, respectively). Correlations between the volatiles in their study were also found.

To obtain the most representative results that can best be compared between the different methods, the analyzes of PV and AV performed by the traditional methods should have been performed on the same day as the analyzes on CDR FoodLab. One sub-goal of this study was to compare the traditional methods with the rapid method, CDR FoodLab, and examine how suitable the rapid method is against the traditional methods.

To see if there was a statistically significant relationship between the methods, a correlation analysis between the traditional methods and CDR FoodLab was carried out for the oil, as the oil had a full data set on the FoodLab method. It was found significant correlations ($P < 0.01$) between PV determined by the traditional method and PV determined by the FoodLab method (r = 0.927). Significant correlations ($P < 0.01$) were also found between AV determined by the traditional method and AV determined by the FoodLab method (r = 0.932). This analysis found evidence for a significant relationship between the traditional methods and the rapid FoodLab method for determining PV and AV (sub-goal 1).

A similar conclusion was reached by Koohikamali and Alam (2019), which stated that the CDR methods' accuracy has been validated to achieve a high correlation between individual curves from the CDR method and the results from the American Oil Chemists' Society (AOCS) official methods (i.e., Ca 5a-40, Cd 8b-90 and Cd 18–90 ($R^2 > 0.99$)). Kwon et al. (2016) also found good agreement ($R^2 = 0.998$ and $p < 0.05$) between AV and PV in edible oils obtained from FoodLab and those obtained from the official methods (ISO 660 and 3960 protocols). It was also reported that this method had many advantages compared with the official methods because of its rapidness, simplicity, and sensitivity of the reagent kit based on spectrophotometry. The ISO protocols regarding the official methods have a probability of human error in manual titration and are more time-consuming (Kwon et al., 2016).

A two-way ANOVA was conducted to determine the relationship between the oil and powder and the different methods (traditional and CDR FoodLab) used for measuring PV and AV. TOTOX was used as the dependent variable, and the test was conducted for the first week (day 0-7) while the TOTOX values still had an increasing trend. The test showed no significant differences between oil and powder ($P > 0.05$) but found significant differences between the

different methods ($P < 0.05$). For the total oxidation value (TOTOX), the traditional methods had an increasing effect, while the FoodLab method had a decreasing trend. The highest TOTOX values occurred in the powder measured with traditional methods, and the lowest TOTOX values occurred in the powder measured by FoodLab. The latter is affected that several of the powder samples ended up outside the concentration range for the FoodLab method and did not have increasing TOTOX values (Appendix D). Figure 14 shows two crossing insignificant lines. Thus, both methods are functioning for determining PV and AV in the oil, but neither of the methods are preferred for analyzing the powder.

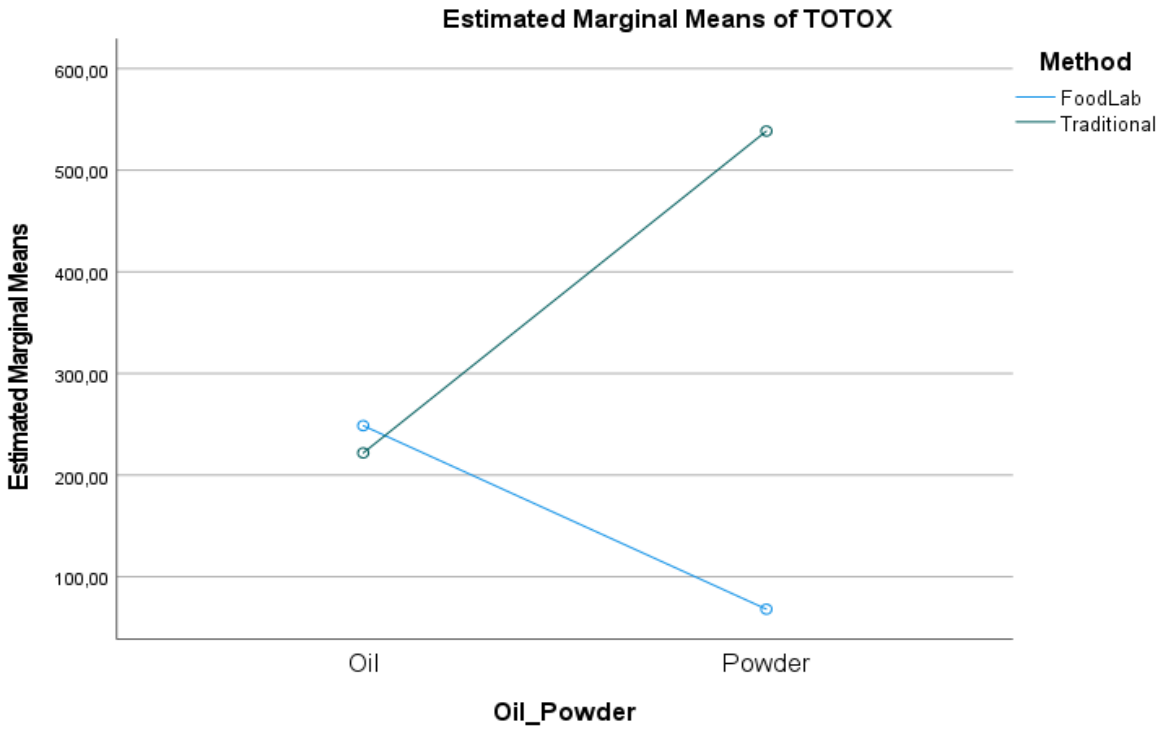

Figure 14. A two-way ANOVA determining the relationship between the oil and powder and the different methods (traditional and CDR FoodLab) used for the determination of PV and AV the first week (days 0, 1, 2, 4, and 7).

Barriuso et al. (2013) reported that the anisidine value correlates well with peroxides content, TBARS, and volatile aldehydes analysis. The traditional methods for determining PV and AV have been used for 100 years and are standardized methods. The iodometric titration method for determining PV is the only hydroperoxide assay that is chemically accurate and absolutely quantitative, and the only assay that can handle high oxidized lipids (Hu & Jacobsen, 2016). However, the methods have some disadvantages regarding time consumption, the high sample

volume needed (PV), the large amount of solvent waste produced (PV), and the toxicity of the anisidine reagent (AV). Also, the methods can give falsely low and falsely high PV and AV as mentioned in Chapters 2.6.1 and 2.6.2.

Hu and Jacobsen (2016) implied that the anisidine reaction is neither quantitative nor specific giving only relative aldehyde concentrations. This is because unsaturated aldehydes have a higher color response than saturated aldehydes. Also, reactions between hydroperoxides and anisidine are slow, making it challenging to distinguish primary oxidation products from secondary. Therefore, the method should not be used to analyze oil with PV above 5 meqO_2/kg (Hu & Jacobsen, 2016). A well-known challenge regarding these traditional methods for determining PV and AV is how to compare results from different oils.

TBA results are reported to correlate well with sensory analyzes, which support the involvement of TBA reactants in processes generating off-flavors and odors detected by consumers (Hu & Jacobsen, 2016). Ke and Woyewoda (1979) reported that the results from highly oxidized samples from marine lipids and oil have had less reproducibility than desired. Some of the common problems mentioned are the mixing of two-phase reaction systems, the complexity of the carbonyls from the oxidation of polyunsaturated fatty acids, and the formation of a yellow pigment that interferes in the reaction (Ke & Woyewoda, 1979). Hu and Jacobsen (2016) questioned the long heating step in the analysis, which can contribute to the decomposition of hydroperoxides and artifactual increases in secondary products rather than measuring compounds already present. Also, aldehydes being well-known indicators of the oxidation of fish oil may have evaporated into the water phase of the sample. This implies that the TBARS values can get falsely low.

Olsen (2005) concluded that sensory analysis yields information that is difficult to fully replace with any instrumental method. Therefore, sensory research should be included when consumer-related aspects of lipid oxidation are an issue.

A becoming mainstay in the understanding of lipid oxidation analyzes is the analysis of volatile components by Gas-Chromatography (Hu & Jacobsen, 2016). GC-MS is a flexible method that can be adapted according to needs by changing method parameters like the sample amount, sampling temperature and time, and the flow of the inert gas. Also, the GC column, temperature program, and the adsorber can be selected based on the types of volatiles to be analyzed. Olsen (2005) highlighted dynamic headspace GC-MS as a method with generally high sensitivity, excellent specificity, and that no other method can give so much information in one analysis.

However, there are some disadvantages to the method, including the great chances of large variations in the data due to small variations in the sampling procedure and the complicated and tedious data handling. Quantification of headspace data is challenging, and the results are mainly comparable within sample series of the same material analyzed with the same method. This type of method generally requires highly chemical and analytical skills of the user, and it is not a rapid method. Each sample can have an analysis time of up to 1 hour. Although the analysis time can be long, one can save a lot of time on sample preparation, compared to the traditional methods of AV and TBARS determinations.

Olsen (2005) stated some of the challenges regarding the sensible use of dynamic headspace GC-MS:

- Finding the sampling conditions that are well adapted to the matrix in question and yield a sufficient, but not an excessive amount of volatiles. Also, not cause further oxidation of the sample.
- The set-up of the chromatography system and MS adapted to the needs of such an analysis.
- The tedious and complicated data handling.

Some theoretical evaluations were made before the GC-MS analysis of the storage experiment. The powder and the oil had very different compositions, as mentioned in Chapter 4.1.1. The powder was looser than the oil in addition to having a larger surface area. The powder samples filled more of the vial than the oil samples (Figure 15) and contained most probably more oxygen within the sample. It was considered whether the GC vials should contain the same amount of headspace in the oil and powder samples for GC-MS analysis. This became a challenge as there was only 35% oil in the powder. However, as the He-gas in the vial was replaced and flushed several times, the oil and powder samples should give the same response (area) if there is an equal amount of oil in the vials.

To obtain the same amount of oil in the vials, either 1 g of powder (maximum volume of powder in the 22 mL vial) had to be weighed, and the corresponding weight of 0.35 g of oil. This is a small sample amount for the oil samples, which can lead to fewer peaks or lower responses in the chromatogram. It was also considered to weigh 1 g of oil (enough sample material), but then, there was no space for 2.85 g of powder in the vial without compressing the powder. This high sample amount would lead to the sample needle being pressed into the powder. The

maximum sample amount in the 22 mL vials was a 17 mL liquid sample for the needle to not come in contact with the sample. The solution was to use an equal amount of sample in the vials. 1 mL of oil was used as it corresponds to approx. 1 g of oil. Then, 1 g of powder was added to the vials. As the powder contained 35% oil, the area from the powder samples had to be corrected from 35% to 100% oil for the vials with powder samples to contain an equal amount of oil as the oil samples.

Figure 15. Illustration of GC-MS vials containing 1 mL of oil (left), and vials containing 1 g powder (right).

Miguel et al. (2019) used 4 g of neat fish oil for dynamic HS GC-MS sampling. The fish oil was weighed in a 100 mL purge bottle connected to the flow of N_2 in a water bath at 60 °C. This method made a higher surface volume ratio, which is a very important parameter for how much of the analyte goes into headspace (G. Vogt, personal communication, January 14, 2022; Frankel (2014)). For dynamic HS GC-MS, Olsen et al. (2006) used five-gram aliquots of the homogenized samples, which were distributed to 250 mL Erlenmeyer flasks. The samples were heated to 50 °C in a water bath and purged with N_2.

The results from the storage experiment showed that the oil and powder oxidized much faster than expected, and a lower storage temperature could probably have been used in further experiments. As mentioned in Chapter 4.1, the storage temp. of 40 °C was used to be sure to

observe oxidative changes and volatile compounds in the oil and powder. The powder was assumed to protect the oil better by reducing the oxidation process, which made it important for the experimental design to ensure sufficiently oxidation of the powder, and that it was possible to measure the volatile components with GC-MS. As described in Chapter 3.2.2, the oil and powder were stored in capped, tight bottles to keep the formed volatiles in the headspace. The bottles were not flushed with nitrogen before they were placed in the heating cabinet, or the freezer, giving the oil and powder access to oxygen. This means that the oil and powder most likely continued to oxidize during storage and further processing and analysis. If it were not primarily volatile compounds that were to be analyzed in this study, all the samples would have been flushed with nitrogen before storage. However, the aim of this study was not to measure the shelf life of the oil and powder, and the choice to have oxygen access in the samples during storage enabled the formation of volatile compounds in the oil and powder during storage, which was the goal of this study.

4.2 Pre-Experiments to Optimize the GC-MS Parameters

This chapter will include the choices that were made, and the associated results, to optimize the GC-MS method parameters for the analysis of volatile compounds in fish oil and powder. The pre-experiments led to improved analysis parameters to find out if GC-MS is a suitable method for determining the oxidative status in fish oil, and especially the powder without an extraction step, the main goal of this study.

4.2.1 Pre-Storage Experiment

A pre-storage experiment of stored fish oil and powder were performed to test the GC-MS analysis parameters and to study the oxidative stability of the fish oil and powder by investigating peak development and the formation of volatile components. For the analyzed powder samples (50% oil) and oil samples to contain the same amount of oil, 0.5 g of oil and 1 g of powder were added to the GC vials. The quality indicators used in later experiments in the study were selected based on which oxidation products were found in this experiment.

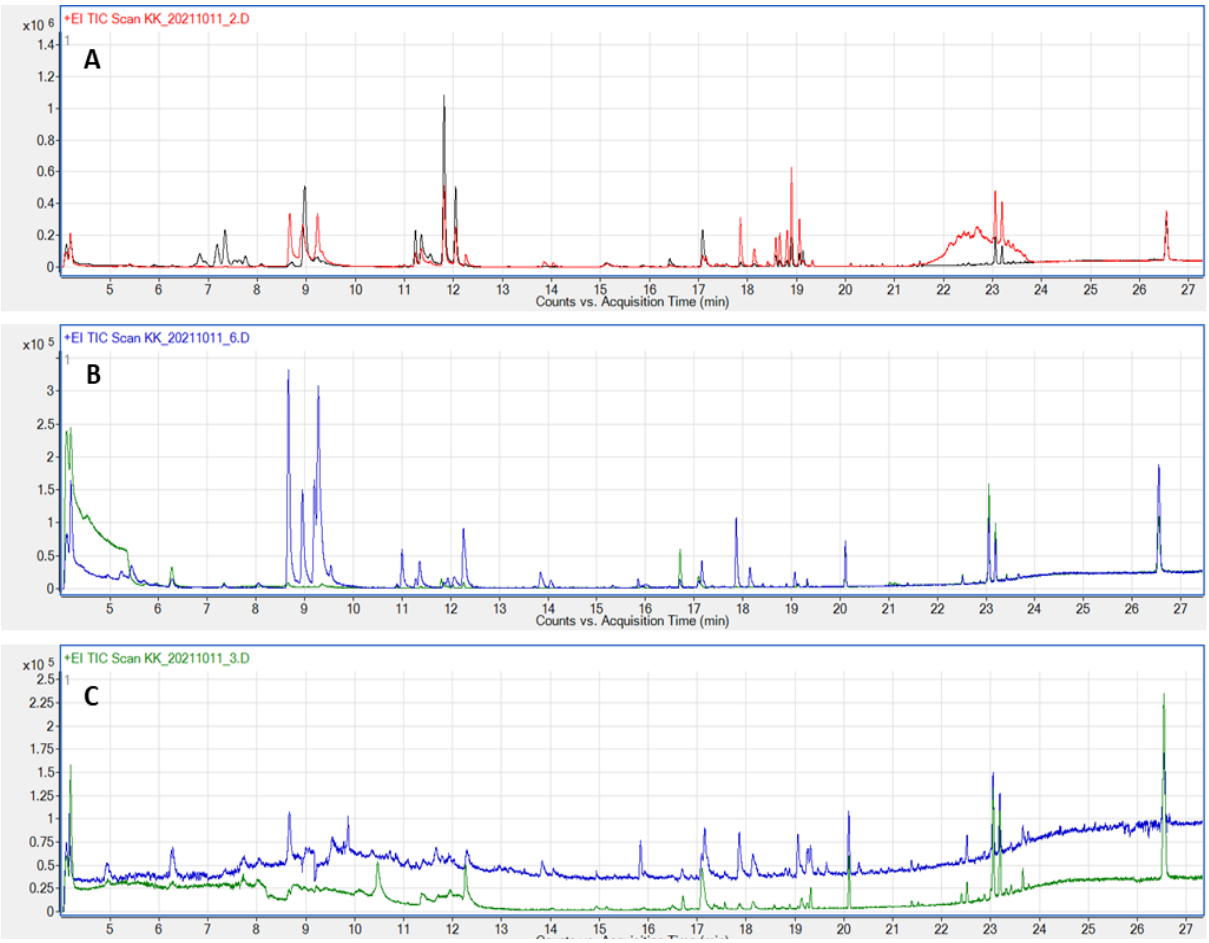

Figure 16. Comparison of GC-MS chromatograms of fresh and stored fish oil and powder from the pre-storage experiment. ***A*** *Fresh fish oil (black) and stored fish oil (red).* ***B*** *Fresh fish powder (green) and stored fish powder (blue).* ***C*** *Fish powder diluted in distilled water: fresh (green) and stored (blue).*

As seen in Figure 16, many of the same oxidation products and volatile components appeared in the fish oil and powder, at approx. equal retention times. The largest difference in area can be seen between fresh and stored fish powder (Figure 16, B).

The powder samples diluted in distilled water did not dissolve. No attempt was made to mix the powder samples diluted in distilled water since mixing with e.g., a vortex mixer could form an emulsion. New chemical bonds that are not in the powder originally could be formed, which are more difficult to break for going into the gas phase (headspace in the GC vial). The water settled on top in the vial, leading to poor chromatography and high baseline levels (Figure 16, C). There were no major differences between fresh and stored powder in these samples. As the water settled as a blocking layer on top, the volatile components from the oil did not escape into the headspace. The powder was therefore analyzed without diluent in further analyzes.

2-Ethyl-furan, 1-penten-3-ol, and (E)-2-pentenal were found in the chromatograms with retention times of 8.66 min, 9.23 min, and 11.34 min, respectively. These volatiles were chosen as quality indicators, in accordance with Miguel et al. (2019). 2,4-Heptadienal was also included as it was expected to be found in the fish oil and powder and is a common oxidation product in literature (Jónsdóttir et al., 2005; Miguel et al., 2019; Olsen et al., 2006; Shibata et al., 2018). Fang et al. (2018) reported that (E,E)-2,4-heptadienal and (E)-2-pentenal had a fishy and paint odor, respectively, and could be ascribed to the oxidation of n-3 fatty acids. 1-Penten-3-ol was one of the major alcohols detected in the fish oil, which imparted a desirable burnt odor, and 2-ethyl-furan was reported as a common oxidation product of n-3 fatty acid with a pungent rubber odor (Fang et al., 2018).

Platen temperatures of 60 and 70 °C were analyzed and compared in the pre-experiment. With a temperature below 60 °C, one can expect that the same reactions occur as at lower temperatures in the oil, in line with Frankel (2014). However, there is a risk by increasing the platen temperature as a temperature between 60 to 100 °C can lead to other reactions that give rise to cyclic compounds which cause oxidation of the samples (Frankel, 2014). An increase in the sampling temperature from 60 to 75 °C was reported to have small effects on the AnV in sand eel oil by Rørbæk and Jensen (1997), while an increase to 90 °C caused oxidation of the samples.

In this study, 70 °C gave a slightly higher response to some oxidation products and somewhat better chromatography (peaks with Gaussian peak shape, and less background noise). This temperature was selected as a compromise between the risk of inducing oxidation in the oil and collecting a suitable amount of volatiles, in accordance with literature (Jeleń et al., 2017; Kulås et al., 2002; Olsen et al., 2005), (G. Vogt, personal communication, December 6, 2022). However, the powder samples may have been more easily affected by the platen temperature than the oil samples, explained by the higher area of the powder samples compared to the oil samples, as seen in Figure 18 and Figure 19.

4.2.2 Quality Indicator Standards and Internal Standard

GC-MS analyzes were performed on standards both diluted in ethanol and water, and a neutral oil (sunflower oil). All standards were always analyzed in the same concentration. The standards 2,3-butanedione, 1,3-hexadiene, 2-ethyl-furan, 1-penten-3-ol, 2,3-pentanedione, (E)-2-octene and (E)-2-pentenal were found in the chromatogram, but (E,E)-2,4-heptadienal, (E)-

2-pentene and 2-methyl-1-butene were not detected (Figure 17). The same standards were not detected by dilution in ethanol and water. (E,E)-2,4-Heptadienal is soluble in ethanol but not in water, and 2-methyl-1-butene and 2-pentene are not soluble in water. Still, standards such as 1-penten-3-ol, (E)-2-pentenal, and 2-ethylfuran, which were detected, are slightly soluble or insoluble in water (Appendix E). However, the extent to which it is possible to find a method that "captures" all analytes is a challenging task. It is not given that all standards analyzed in this study will be detected in this particular method, using e.g., one type of column and one type of adsorber.

IS was diluted in sunflower oil and detected in fish oil with a good response at a concentration of 20 µM with a retention time of 11.43 min (Figure 18) using Dynamic HS analysis. Achieving stable results of IS diluted in both sunflower oil and fish oil that could be used for quantitative calculations was very challenging. The GC peaks (area) of the oxidation products could, at least, be assessed against IS. A challenge, however, was that IS did not have the same area for each parallel of samples, or each sample if the oil changed over time.

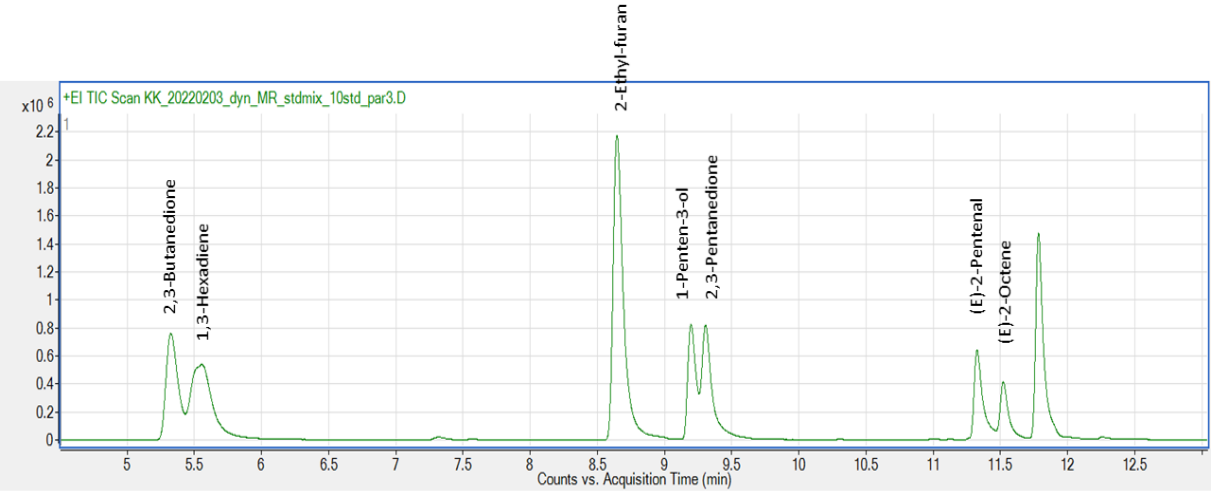

Figure 17. GC-MS chromatogram of the detected quality indicator standards diluted in sunflower oil.

The different standards were analyzed with the same concentration (20 µM), but there were large differences in area. This suggested that the analytes (standards) have different volatilities, depending on the size of the molecules. Different analytes that have different vapor pressures could explain why they were released into the headspace in different quantities. Oxidation products are also very different, leading to different responses (areas) in the chromatogram. Attempts to analyze undetected standards at higher concentrations were not performed.

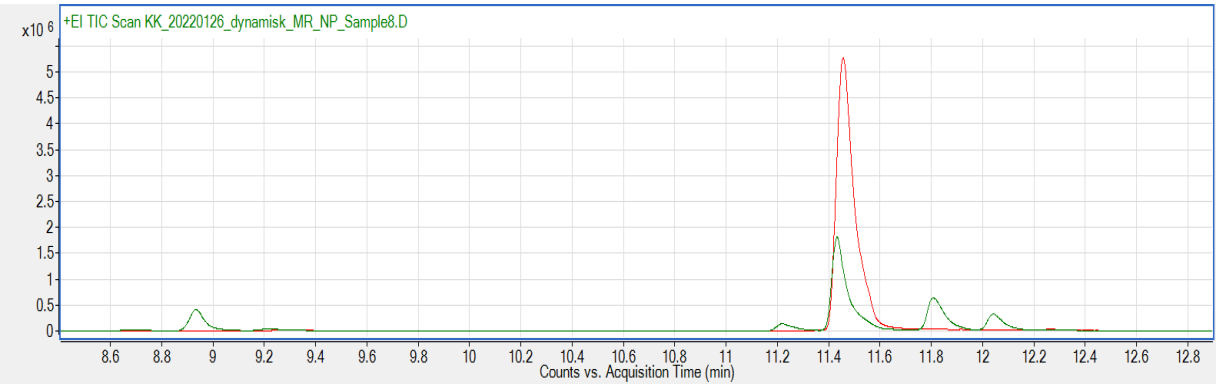

Figure 18. GC-MS chromatogram of 20 µM internal standard (t_R 11.43 min) added to fish oil (green) and powder (red).

The quality indicators and the internal standard added to the oil and powder appeared at the same place in the chromatogram with nearly the same retention times, making the fish oil and powder comparable. This was an important finding in the understanding of how to analyze volatile oxidation products in fish powder using GC-MS, without extracting the oil.

As illustrated in Figure 18 and Figure 19, the internal standard and the quality indicators were released in different quantities to the column in fish oil and powder (Appendix F). IS and the standard mix (quality indicators) got a weaker response (area) when added to the oil as the standards got blended in the oil and could also lie as drops protected by the oil if they didn´t dissolve in the oil. IS and standard mix added to the powder were more accessible on the powders´ surface area. However, this is only correct if a certain amount of headspace is taken out of the vial, but in the system used for these analyzes, the gas is replaced and flushed several times. An equal amount of IS and the quality indicators was added to the oil and powder and should therefore give the same area in the oil and powder samples.

A critical parameter is the different sample matrixes of the oil and powder samples. This is discussed in Chapter 4.1.1. The powder analyzes did not tell anything about the condition of the oil in the powder, which may be protected inside the powder, but to what extent the powder could protect the oil, and how stable the oil was in the powder.

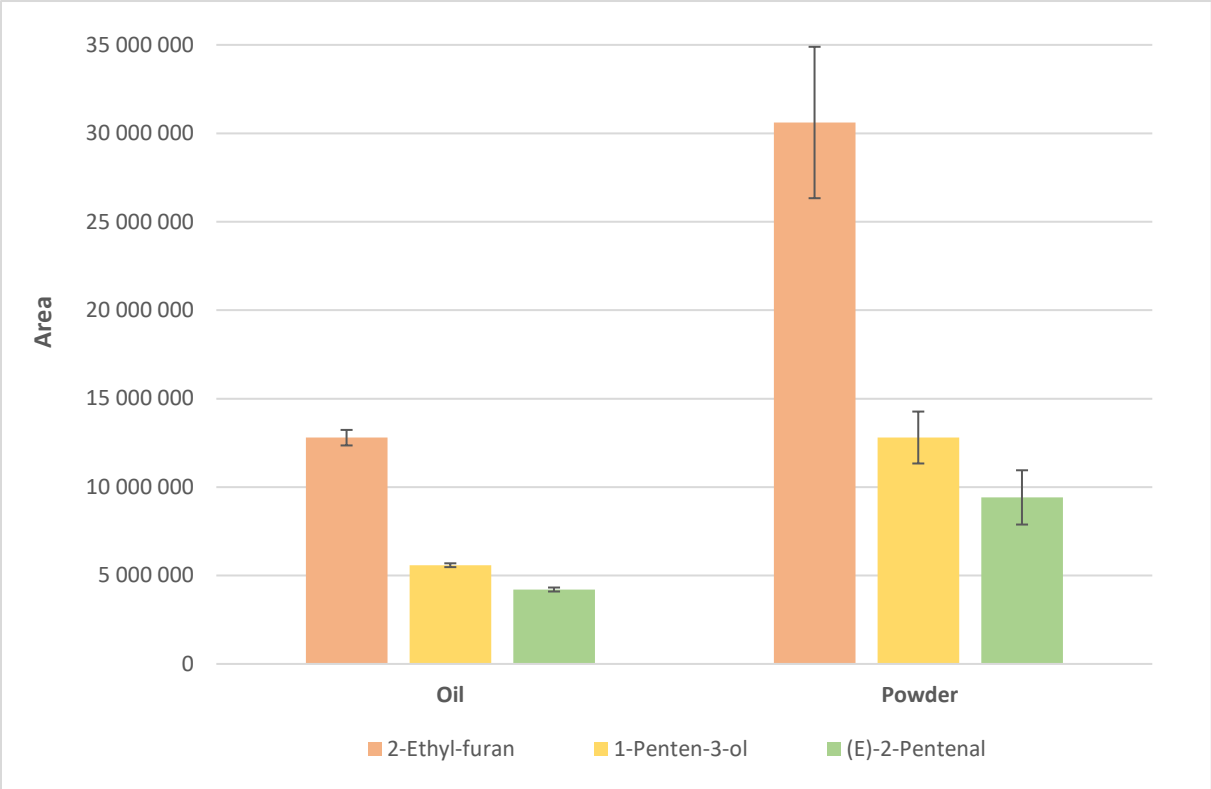

Figure 19. Comparison of the quality indicators´ area in fish oil and powder (with optimized analysis parameters with shaking). Data are presented as mean values (n=3) ± standard deviations.

It was decided that the internal standard and the quality indicator standards should not be added to the powder samples when analyzing the storage experiment. Shen et al. (2010) also concluded not to use an internal standard to quantify the volatile compounds as it was not easy to homogenously spike liquid IS solution into a powder.

The pure powder (without oil) that was mixed with oil in the powder samples was also analyzed, and the chromatogram can be seen in Figure 20. These compounds have not appeared in high concentrations or been disruptive throughout the storage experiment. As the composition of the powder was confidential at the company's request, analyzes of the pure powder were not further investigated in this study and are mentioned in Future work in Chapter 4.3. In experiments with fish powder or microcapsules, analyzes of the pure powder could be important for exploring if the powder or wall material contains volatile components that can interfere with the measurements.

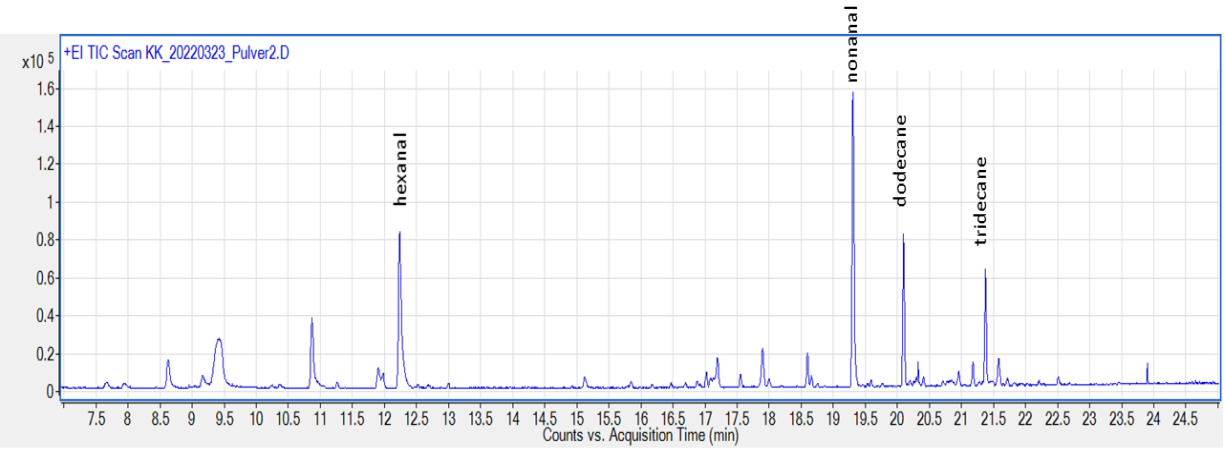

Figure 20. The chromatogram of pure powder (without oil) analyzed by GC-MS, with associated volatile components.

It was suggested that (E)-2-pentene, 1,3-hexadiene, and 2-methyl-1-butene were highly volatile as they evaporated at weighing before the amount of substance met the vial from the pipette tip. This may explain why (E)-2-pentene and 2-methyl-1-butene were not found in the chromatogram. However, 1,3-hexadiene did also evaporate by weighing but was found in the chromatogram. (E,E)-2,4-Heptadienal was not detected even though the compound did not evaporate by weighing. Injection of the pipette tip containing the standard solution into the sunflower oil could have been tried to avoid evaporation.

4.2.3 Comparing Dynamic and Static Headspace Method

Difficulties arose regarding a relatively high level of background noise by the Dynamic HS method. The blank injections performed before the analyzes contained many ghost peaks, in a relatively high response. Also, several standards were not detected, and it was assumed that many compounds were not “captured” well enough on the trap column by Dynamic HS. Static HS was therefore compared to Dynamic HS.

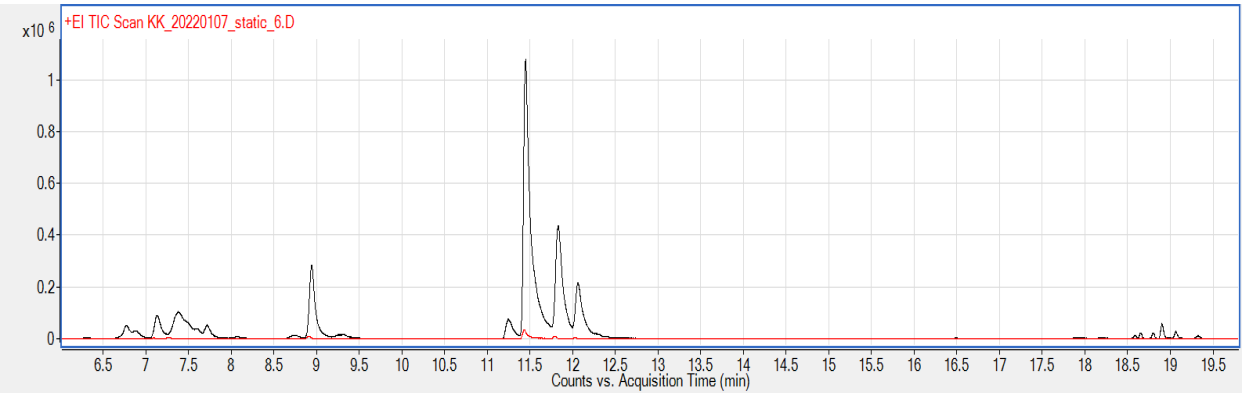

Figure 21. GC-MS chromatogram of 20 µM internal standard (t_R 11.43 min) added to fresh fish oil using Dynamic HS (black) and Static HS (red) analysis.

As seen in Figure 21, valuable information can be lost using the Static HS method. This experiment showed that the detection level is significantly lower in Dynamic HS. Jeleń et al. (2017) stated that authors have noted lower area counts for static HS compared to dynamic HS methods, even though it was said that static HS can be a method capable of determination of oxidation levels.

Ghost peaks in the blank injections may be due to all the various analyzes the column (Tenax trap) had been used for in the past. The column was, therefore "baked out". Later, when three blank injections were run before analysis, the ghost peaks were essentially gone in the last blank injection, and the analysis could continue without fear of carryover. It was decided that each future analysis should start with three blank injections before analysis of the oil and powder samples.

4.2.4 Optimized GC-MS Analysis Parameters

Challenges arose regarding the volatility of the analytes and getting the analytes into headspace from the oil and powder matrixes. The analyte might not dissolve in the oil but may remain as small, dispersed droplets protected by the oil, which could lead to reduced parts going out into the headspace. Changing the GC-MS method parameters was done to develop an efficient extraction method and be able to detect several of the standards in a reasonable concentration range (around 2-20 µM). Based on Jeleń et al. (2017) findings, shaking intensity inside the sampler, higher transfer line temp. and desorb temp., and higher trap bake temperature and time were tested.

Samples were analyzed with and without shaking inside the sampler, in addition to the first parameters and the optimized parameters (Table 4). A standard mix of (E,E)-2,4-heptadienal, 1-penten-3-ol, (E)-2-pentenal, and 2-ethyl-furan was added to sunflower oil at a concentration of 20 µM.

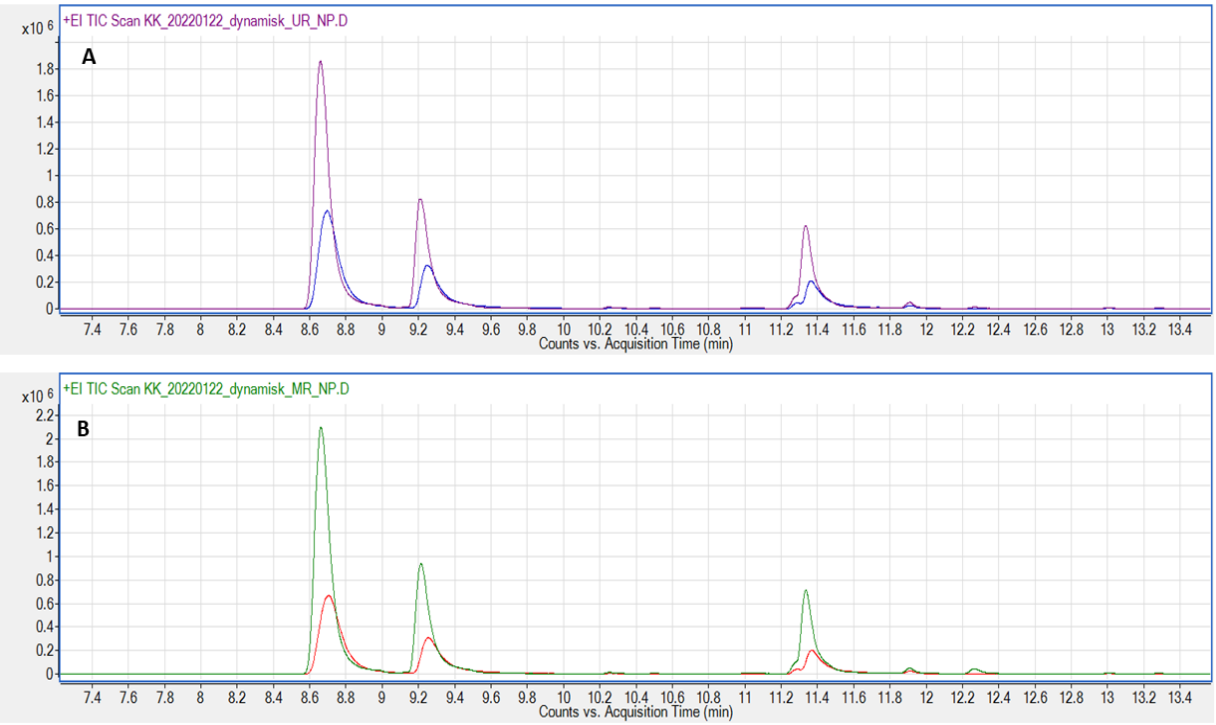

Figure 22. GC-MS chromatogram comparing the quality indicator´s signal in fish oil analyzed with the first parameters and the optimized parameters. ***A*** *Parameters 1. (blue) and optimized parameters (purple) without shaking.* ***B*** *Parameters 1. (red) and optimized parameters (green) with shaking.*

Figure 22 shows that all detected quality indicators (1-penten-3-ol, (E)-2-pentenal, and 2-ethyl-furan) achieved the highest response (area) with optimized parameters with shaking (Appendix G). A similar conclusion was reached by Rørbæk and Jensen (1997) who found the response with HS-GC to increase with increased temperature and time.

The migration of volatiles from the matrix is influenced by mixing. Jeleń et al. (2017) tested shaking levels in the autosampler to enable a faster transfer to the headspace. Their results showed a slightly decreasing trend in response (total peak area) with a higher shaking level, but the repeatability of peak areas was the highest with the highest degree of shaking.

There were smaller differences between the quality indicators areas analyzed with the first analysis parameters with and without shaking. The difference in area for 1-penten-3-ol, 2-ethyl-

furan and (E)-2-pentenal with and without shaking were 138 970, 213 000, and 96 890, respectively. The shaking parameter gave a greater response of the quality indicators with optimized parameters, compared to analysis without shaking. 1-Penten-3-ol, (E)-2-pentenal and 2-ethyl-furan got a difference in area of 753 990, 430 670, and 1 712 630, respectively (Appendix G). Therefore, shaking level 5 was used further for analysis in this study.

4.2.5 Equilibration Time.

The time the sample is heated before it is analyzed is an important factor in how much of the analyte goes into headspace (G. Vogt, personal communication, January 14, 2022). The equilibration time was tested by adding a known concentration (20 µM) of IS to fish oil. It was expected that the response should stop increasing over time. One can see from Figure 23 that equilibrium was not reached after 45 min. Jeleń et al. (2017) also experienced that the equilibration time was long (40 minutes). This could also be expected in the system used in this study, working with a viscous oil in addition to not knowing if the oxidation products are "dissolved" in the oil.

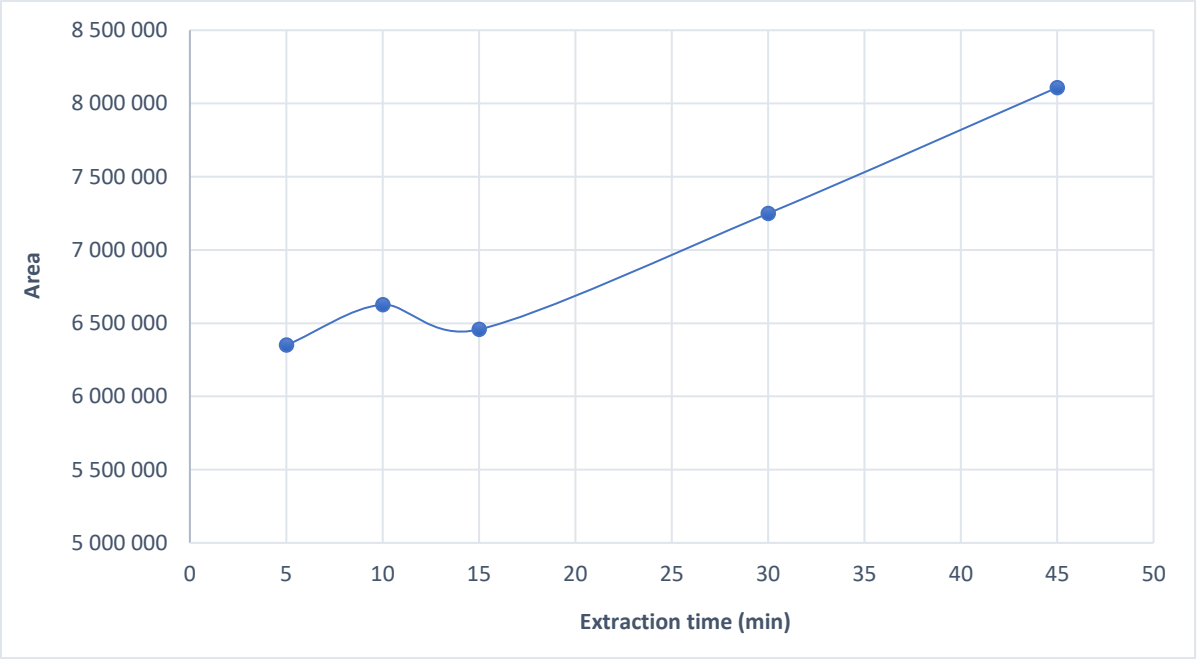

Figure 23. Influence of extraction time, finding conditions to achieve equilibrium for the analytes. The area of 20 µM internal standard added to 5 different vials containing 1 mL fish oil and analyzed with different extraction times (extraction temperature 70 °C): 5, 10, 15, 30, and 45 min.

Equilibrium was not reached, and in practice, it was not an option to leave the samples for longer than 45 minutes on the platen as there was a risk that samples in later experiments (fish oil and powder) could start oxidizing. It was found that an extraction time of 10 min was a compromise to not risk oxidation while the sample was on the platen, and to achieve the best possible response (equilibrium) of the analytes.

The internal standard was diluted in sunflower oil. If IS had been dissolved in a pure solvent, the analyte might have extracted more easily into the headspace and thus reached equilibrium earlier compared to when IS was dissolved in sunflower oil.

4.3 Summary of Optimized GC-MS Analysis Parameters

Rapid methods have recently offered several advantages for PV and AV determination, including simple and rapid detection procedures, short analysis time, the requirement of small quantities of samples, and the ability to produce small amounts of residues. As mentioned in Chapters 2.6.1, 2.6.2, and 4.1.3, oxidation measurements using traditional methods (PV and AV) can be challenging based on which raw material is analyzed and can have low reproducibility, especially in highly oxidized samples. Consequently, there is still a need for better analytical methods to measure oxidative changes.

This chapter includes a summary of the GC-MS analysis parameters that were successful with a type of oil containing high amounts of n-3 polyunsaturated fatty acids (> 60% EPA/DHA), which may be the start of a protocol for measuring volatile oxidation products in oils and powders using Dynamic headspace GC-MS. A selection of the optimized parameters for dynamic HS GC-MS is summed up in Table 5. However, qualitative, and quantitative comparison of headspace data obtained with different methods and sample matrixes can be complicated.

Based on the results and literature mentioned in this study, it appears that GC-MS has the potential to analyze oxidative changes in fish oil and powder, and volatile compounds that can influence odor and flavor properties. This method can be particularly useful in the study of oxidation in fish oils if the internal standard can successfully contribute to quantification of the volatile compounds that influence the flavor and taste of the food products. The results of this study show that GC-MS was a suitable method for directly determining the oxidative status in fish powder without extracting the oil from the powder. This is useful information as the result

shows that the powder does not need to be extracted before analysis. GC-MS analysis of fish oil and powder detected several volatile oxidation products during the storage period.

Shibata et al. (2018), Olsen et al. (2006), and Aidos et al. (2002) identified 1-penten-3-one and 2,4-heptadienal as the flavor deterioration indicators of oxidizing fish oil, and to be useful volatile indicators for the oxidative status in n-3 containing matrixes. In this study, using an oil containing high amounts of n-3 (>70%), 2-ethyl-furan, 1-penten-3-ol, (E)-2-pentenal, and (E,E)-2,4-heptadienal were used as quality indicators, in accordance with Miguel et al. (2019). Aidos et al. (2002) used the standards 1-penten-3-one, (E,E)-2,4-heptadienal, nonanal, (E,Z)-2,6-nonadienal, hexanal, and (Z)-4-heptenal for quantification of volatiles. Their results suggested that 1-penten-3-one and (E,E)-2,4-heptadienal were good indicators of the oxidative status in fish oil.

Findings in this study show that dynamic headspace GC-MS is a sensitive and specific method for identifying volatile compounds responsible for undesirable smell and flavor in fish oil, in accordance with what others have found (Aidos et al., 2002; Jónsdóttir et al., 2005; Karahadian & Lindsay, 1989; Morales et al., 1994; Olsen, 2005). Dynamic headspace methods have the great advantage of effectively concentrating the sample so that it is possible to detect compounds present at low concentrations (Morales et al., 1994).

A Tenax trap was used in this study, which is the most widely used trap sorbent in flavor analysis (Qian & Reineccius, 2003). Qian and Reineccius (2003) stated that the Tenax trap has a high affinity for a wide range of hydrophobic organic compounds. However, they found that the Tenax trap had its limitations of efficiently retaining acetaldehyde and other highly volatile compounds. The standards (E,E)-2,4-heptadienal, (E)-2-pentene and 2-methyl-1-butene were not detected in this study. A Tenax trap has also been used by others for trapping volatile compounds (Hartvigsen et al., 2000; Hsleh et al., 1989; Olsen, 2005).

The different standards and the internal standard diluted in sunflower oil were detected in fish oil with a good response at a concentration of 20 µM. Experiments in this study showed that both 0.5 and 1 g of oil were sufficient sample material to detect volatile compounds with GC-MS. 1 g of powder detected higher concentrations of volatile compounds compared to the oil. Given that the powder had a higher surface area than the oil, it may be possible to use a smaller amount of powder in GC-MS analysis.

Table 5. A selection of the optimized analysis parameters for dynamic HS GC-MS.

Variable	Value
Valve Oven Temp.	150 °C
Transfer Line Temp.	160 °C
Platen/Sample Temp.	70 °C
Preheat Mixing Level	Level 5
Sweep Flow Time	10.0 min
Desorb Preheat	200 °C
Desorb Temp.	220 °C
Trap Bake Temp.	230 °C
Trap Bake Time	10.0 min

The time the sample is heated before it is analyzed is an important factor in how much of the analyte goes into headspace (G. Vogt, personal communication, January 14, 2022). Results from the pre-experiments (Chapter 4.2) led to optimized analysis parameters, shown in Table 5. The platen temperature of 70 °C was selected as a compromise between the risk of inducing oxidation in the oil and collecting a suitable amount of volatiles, in line with what others have used (Jeleń et al., 2017; Kulås et al., 2002; Olsen et al., 2005), (G. Vogt, personal communication, December 6, 2022). Results in this study found the response of quality indicators to increase with higher temperature and extended time, similar to Rørbæk and Jensen (1997). Thus, valve oven temp. and transfer line temp. were increased, in addition to desorb temp. and trap bake temp. Sweep flow time and trap bake time were also increased to get a higher response (area) of the volatile compounds. A higher shaking level (level 5) in the autosampler was included to enable a faster transfer to headspace, in accordance with Jeleń et al. (2017).

4.4 Future Work

Analysis with GC-MS, in particular, has great potential and needs further research. This chapter will mention some methods and improvements that were not made in this study due to lack of time, but which may increase the level of knowledge about analysis of oxidation products and volatile compounds in fish oil and powder.

1. Perform GC-MS experiments with an equal amount of headspace in the vial for both oil and powder. It could be interesting to dilute the powder in a buffer solution to enable the powder to have equal headspace in the vial as the oil, in addition to making the powder´s matrix more equal to the oil matrix.
2. Make standard solutions (quality indicators) in a powder form (e.g., encapsulation) to be able to spike the fish powder with standards with a similar matrix. It could also be an idea to find a suitable buffer solution that can dissolve the powder so that it has a more similar matrix to the standards and the oil.
3. Study the powder and gain more knowledge about the condition of the oil in the powder. Is the oil protected by the powder? How well do the oxidation products diffuse out of the oil in the powder? Perform GC-MS analysis of powders using less sample volume than 1 g in the vials as this seemed to give very high signals (areas) due to the high surface area of the powder.
4. Perform GC-MS analysis using a larger surface area. GC vials used in this study were 22 mL with a diameter of 2 cm. Eurofins uses 1-5 grams of oil in 250 mL Erlenmeyer flasks, which are further transferred to the GC column. The surface volume ratio is a very important parameter for how much of the analyte goes into headspace (G. Vogt, personal communication, January 14, 2022). This method may not be relevant for highly oxidized oils, as the up-concentrated oil used in this study. However, this method may be of importance for standard analyzes (quality indicators).
5. Perform more sensitive and specific spectrometric measurement methods on selected standards (quality indicators) to detect all the standards in the chromatogram and quantify compounds in more complex solutions - MRM mode (multiple reaction monitoring).

5. Conclusion

The results of this study show that GC-MS was a suitable method for directly determining the oxidative status in fish powder without extracting the oil from the powder. GC-MS may provide a more accurate insight into the oxidation of the product compared to the traditional methods (PV and AV) as the powder did not go through an extraction step that may aggravate the oxidative state in the product.

Dynamic headspace GC-MS is a widely used, flexible, and sensitive method for identifying volatile compounds responsible for undesirable smell and flavor in fish oil. Based on the results of this study, dynamic headspace GC-MS analysis provided useful information in the research on oxidative changes in fish oil and powder. GC-MS may not replace the traditional methods for determining PV, AV, and TBARS in fish oils, but could be an addition to gain a full understanding of the oxidative changes in the product. The lipid oxidation processes are probably too complex for using just one method.

This study also compared the traditional methods for determining PV and AV with the rapid method, CDR FoodLab, to examine how suitable the rapid method is against the traditional methods. Strong correlations were found between the methods. CDR FoodLab can provide useful data of great health and economic value to the industry, but it remains to see whether this method can be relied on alone.

The analyzes in this study found no evidence that the powder was more stable than the oil, or that the powder protected and stabilized the oil in the powder. The powder seemed initially more oxidized compared to the oil and oxidized faster than the oil. Both the powder and oil were stored with oxygen access (to capture the volatile compounds), which may have aggravated the oxidative state during storage in the freezer compared to if the samples had been flushed with nitrogen before storage.

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Appendix

Appendix A: P-values

	P-value
Comparing traditional methods and FoodLab	
PV (Traditional/FoodLab)	0.004
AV (Traditional/FoodLab)	0.022
Oil/Powder	0.437
Oil/Powder*Method	0.007
Sig. differences (TOTOX) in the storage exp.	
Traditional	
Oil	<0.001
Powder	<0.001
FoodLab	
Oil	<0.001
Powder	<0.001
Comparing oil and powder (TOTOX)	
Traditional	0.006
FoodLab	<0.001
Sig. differences (GC-MS) in the storage exp.	
Traditional	
Area 2,4-octadiene	<0.001
Area 1-penten-3-ol	<0.001
FoodLab	
Area 2,4-octadiene	<0.001
Area 1-penten-3-ol	<0.001
Comparing oil and powder (GC-MS)	
Area 2,4-octadiene	0.003
Area 1-penten-3-ol	<0.001
Sig. differences (TBARS) in the storage exp.	
TBARS	<0.001
Correlation analysis	
Oil	
PV (Traditional/FoodLab)	<0.001
AV (Traditional/FoodLab)	<0.001
PV_traditional/AV_traditional	<0.001
PV_traditional/Area_1-penten-3-ol	<0.001
PV_traditional/Area_2,4-octadiene	<0.001
AV_traditional/Area_1-penten-3-ol	<0.001
AV_traditional/Area_2,4-octadiene	<0.001
Area_1-penten-3-ol/Area_2,4-octadiene	<0.001
Powder	
PV_traditional/AV_traditional	<0.001
PV_traditional/Area_1-penten-3-ol	<0.001
PV_traditional/Area_2,4-octadiene	0.009
AV_traditional/Area_1-penten-3-ol	<0.001
AV_traditional/Area_2,4-octadiene	<0.001
Area_1-penten-3-ol/Area_2,4-octadiene	0.001

Appendix B: Identification of Oxidation Products

Oxidation products identified in the oil and powder from the storage experiment. Retention times (RT) are presented in minutes.

RT (min)	Oxidation Products	
Oil Samples		
7.91	2,3-Dihydro-furan	
8.63	2-Ethyl-furan	
8.88	1-Penten-3-one	
9.20	1-Penten-3-ol	Pentanal was formed on day 14
10.96	(E)-2-Pentenal	
11.43	4-Methyl-2-pentanol	1-methoxy-octane (?) was formed on day 7.
11.80	2,4-Octadiene	
12.03	(Z,Z)-3,5-Octadiene	
12.27	3-Hexanal	
15.83	(E,E)-3,5-Octadiene-2-one	
17.83	(E,E)-2,4-Heptadienal	
Powder Samples		
5.12	Butanal	
7.91	2,3-Dihydro-furan	
8.63	2-Ethyl-furan	
8.88	1-Penten-3-one	
9.20	1-Penten-3-ol	Pentanal was formed on day 14
10.95	(E)-2-Pentenal	
11.50	(E)-2-Octene	
11.80	2,4-Octadiene	
12.03	(Z,Z)-3,5-Octadiene	
12.26	3-Hexanal	
14.02	(E)-2-Hexenal	
15.83	3,5-Octadien-2-one	
17.83	(E,E)-2,4-Heptadienal	

Appendix C: The area of 1-penten-3-ol and 2,4-octadiene

Area of 1-penten-3-ol and 2,4-octadien from the storage experiment. Data are presented as mean values (n=3) ± standard deviations. Retention times (RT) are presented in minutes.

1-Penten-3-ol

	Oil		Powder	
Sample (day)	**RT (min)**	**Area**	**RT (min)**	**Area**
0	9.19	46 942^{a} ± 4 334	9.23	385 451^{a} ± 68 871
1	9.17	704 307^{a} ± 40 763	9.22	2 508 942^{a} ± 137 117
2	9.17	1 449 837^{ab} ± 60 978	9.19	8 957 062^{a} ± 388 571
4	9.17	4 635 315^{bc} ± 453 034	9.23	25 367 192^{ab} ± 5 920 470
7	9.18	7 588 395^{c} ± 450 242	9.19	51 316 975^{b} ± 3 212 437
14	9.21	1 809 5351^{d} ± 1 170 569	9.18	137 353 988^{c} ± 13 417 866
21	9.17	23 087 930^{e} ± 1 348 732	9.18	131 089 238^{c} ± 10 767 492
28	9.18	49 680 163^{f} ± 3 081 794	9.19	160 886 240^{c} ± 34 834 874

2,4-Octadiene

	Oil		Powder	
Sample (day)	**RT (min)**	**Area**	**RT (min)**	**Area**
0	11.78	106 536^{a} ± 7 466	11.79	76 550^{a} ± 14 752
1	11.78	188 694^{a} ± 17 714	11.79	85 020^{a} ± 8 477
2	11.78	463 988^{a} ± 19 655	11.78	102 493^{a} ± 10 303
4	11.78	1 804 703^{b} ± 73 454	11.78	724 482^{a} ± 63 121
7	11.78	4 686 887^{c} ± 193 039	11.79	30 383 911^{b} ± 5 684 123
14	11.80	13 542 928^{d} ± 224 238	11.79	77 212 357^{c} ± 6 468 363
21	11.80	20 755 477^{e} ± 302 218	11.80	84 557 776^{c} ± 521 552
28	11.80	28 687 837^{f} ± 347 412	11.80	87 132 165^{c} ± 19 133 189

Appendix D: PV, AV, and TBARS in Fish Oil and Powder

PV, AV and TBARS measurements in the fish oil and powder from the storage experiment. Data are presented as mean values (n=2 and 3) ± standard deviations.

	Rapid Method; CDR FoodLab			Traditional Method; supplier fish oil company			Traditional method; NTNU	
Sample (day)	**PV (meqO2/kg)**	**AV (AnV)**	**TOTOX**	**PV (meqO2/kg)**	**AV (AnV)**	**TOTOX**	**PV (meqO2/kg)**	**TBARS (µmol MA/g lipid)**
0, oil	2.09 ± 0.15	< 0.5	4.67^{a} ± 0.30	1.00 ± 0.034	4.48 ± 0.19	6.44^{a} ± 0.14	1.84 ± 0.13	0.63^{a} ± 0.17
1, oil	62.00 ± 1.13	< 0.5	124.50^{b} ± 2.26	41.91 ± 0.66	6.00 ± 0.30	89.81^{b} ± 1.60		
2, oil	95.55 ± 1.06	4.70 ± 1.56	195.80^{c} ± 3.68	81.96 ± 0.80	11.24 ± 0.03	175.16^{c} ± 1.53		
4, oil	181.05 ± 1.91	8.30 ± 0.14	370.40^{e} ± 3.96	145.74 ± 4.04	22.30 ± 0.18	313.77^{d} ± 8.22		
7, oil	268.85 ± 1.77	10.55 ± 0.21	548.25^{g} ± 3.32	234.76 ± 6.94	54.78 ± 0.81	524.32^{f} ± 13.04	154.20 ± 1.44	3.08^{c} ± 0.24
14, oil	213.65 ± 1.63	36.90 ± 1.70	464.20^{f} ± 1.56	137.07 ± 8.61	62.26 ± 0.12	336.38^{d} ± 20.72	118.10 ± 1.55	2.72^{bc} ± 0.07
21, oil	144.00 ± 1.70	66.55 ± 4.88	354.55^{d} ± 1.48	134.14 ± 6.22	77.45 ± 1.38	345.72^{d} ± 11.09	104.08 ± 2.42	2.65^{b} ± 0.83
28, oil	283.90 ± 3.11	> 100	667.80^{h} ± 6.22	158.80 ± 0.45	106.65 ± 1.58	424.24^{e} ± 0.74	92.75 ± 1.76	2.82^{bc} ± 0.12
0, powder	8.63 ± 1.01	0.90 ± 0.56	18.15^{a} ± 2.59	12.60 ± 0.71	6.04 ± 0.19	31.24^{a} ± 1.60		
1, powder	20.71 ± 1.25	4.15 ± 1.20	45.56^{b} ± 3.71	78.20 ± 0.13	10.95 ± 0.11	167.35^{b} ± 0.37		
2, powder	> 25	10.40 ± 0.42	60.40^{b} ± 0.42	196.69 ± 1.15	32.06 ± 0.69	425.44^{c} ± 2.98		
4, powder	> 25	40.90 ± 6.79	90.90^{c} ± 6.79	354.67 ± 2.02	108.53 ± 0.14	817.86^{g} ± 3.89		
7, powder	> 25	> 100	$> 150^{d}$	503.41 ± 17.82	244.10 ± 0.80	1250.92^{h} ± 36.43		
14, powder	> 25	> 100	$> 150^{d}$	277.51 ± 2.48	176.88 ± 4.11	731.90^{f} ± 0.84		
21, powder	> 25	> 100	$> 150^{d}$	266.84 ± 2.65	130.49 ± 4.71	664.16^{e} ± 10.02		
28, powder	> 25	> 100	$> 150^{d}$	172.53 ± 1.24	213.50 ± 2.27	558.55^{d} ± 0.21		

Appendix E: Properties of the Standards

Different properties of all the standards used in the experiments.

Standards	Boiling Point (°C at 1 mm Hg)	Solubility Water	Solubility Ethanol	Solubility Others	Molecular Weight	Chemical Formula	RT (min)	Mass Spektrum (mz)
Detected								
1-Penten-3-ol	114	slightly	miscible at room temp.	miscible in ether	86,13	C5H10O	9,55	57, 29
(E)-2-Pentenal	80-81 °C/160 mmH	no	yes	PG, and most mixed oils	84,12	C5H8O	11,39	55, 84, 83, 41, 29, 39, 27
2-Ethylfuran	92-93	no	yes	oils	96,13	C6H8O	8,66	81, 53, 96
(E)-2-Octene	124				112,21	C8H16	11,51	55(+), 41(+), 70, 29, 112
1,3-Hexadiene	72-76?				82,14	C6H10	5,54	67, 82, 39, 41
2,3-Pentanedione	108-110	yes	miscible	miscible with dietylether and acetone	100,12	C5H8O2		43, 29, 57, 27
2,3-Butanedione	87-88	yes		glycerole, and soluble with alcohol, propylene glycol and most solid oils	86,09	C4H6O2	5,40	43, 86
Not detected								
(E,E)-2,4-Heptadienal	84-84,5	no	yes	mixed oils	110,16	C7H10O		81, 110, 53, 41
2-Methyl-1-butene	31,2	no		alcohol, ether, benzene	70,13	C5H10		55, 70, 42, 39, 41, 29
Propionaldehyde (Propanal)	48-49	yes	miscible	miscible with ethyl ether	58,08	C3H6O		58, 29, 28, 27, 57
Crotonaldehyde (2-Butenal)	102-104	yes	very	soluble in chloroform; very soluble in ethyleter, acetone; miscible in benzene	70,09	C4H6O		41, 39, 70, 69
2-Pentene	36,73	no			70,13	C5H10		55, 70, 42, 41, 39, 29

(National Institute of Standards and Technology, 2022; National Library of Medicine, 2022)

Appendix F: The area of the quality indicators with optimized parameters

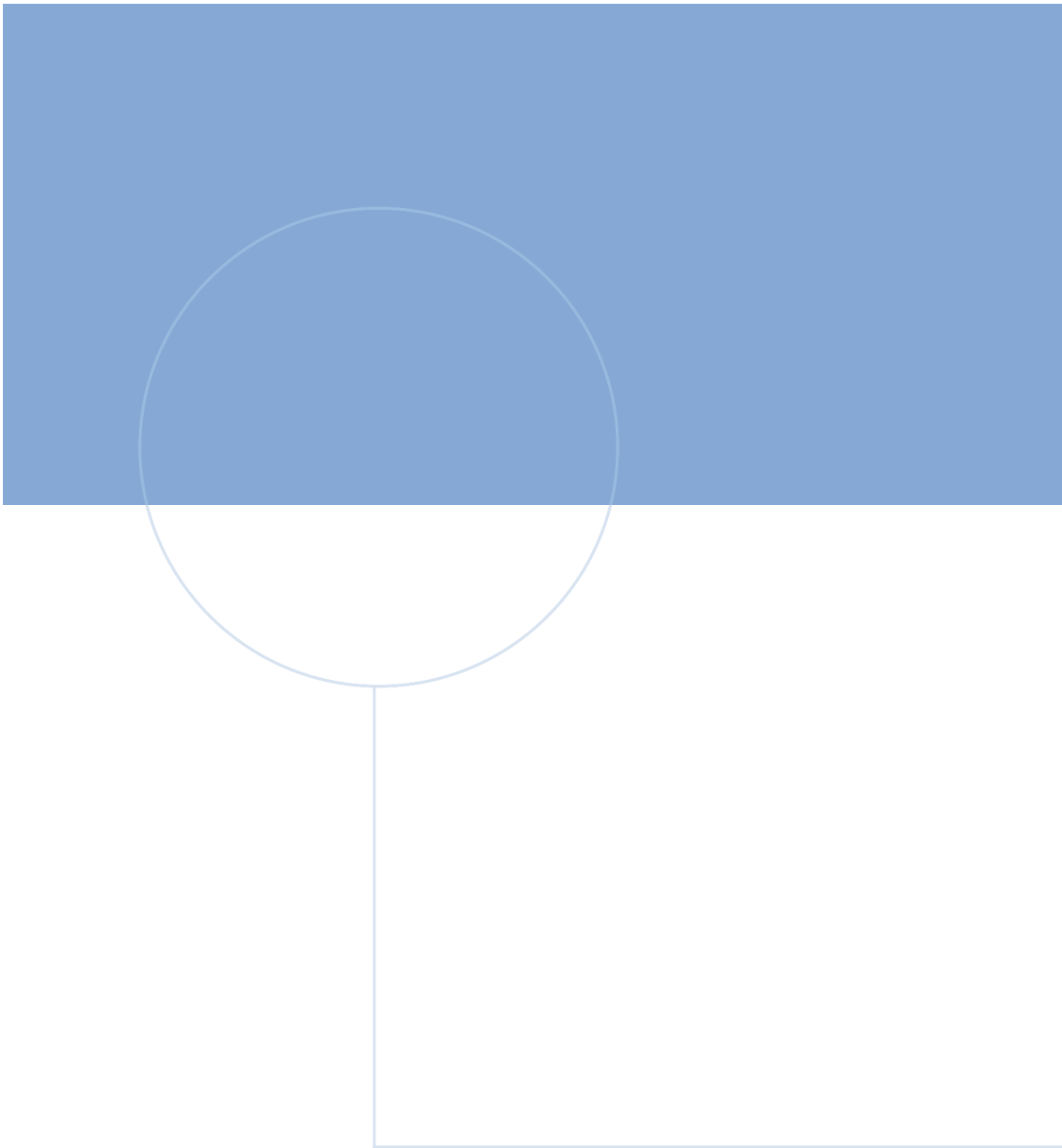
The area of the detected quality indicators (1-ethyl-furan, 1-penten-3-ol and (E)-2-pentenal) in the fish oil and powder with optimized GC-MS analysis parameters. Data are presented as mean values (n=3) ± standard deviations. Retention times (RT) are presented in minutes.

	Oil		Powder	
Quality Indicator	**RT (min)**	**Area**	**RT (min)**	**Area**
2-Ethyl-furan	8.66	12 792 402 ± 439 532	8.65	30 611 648 ± 4 279 623
1-Penten-3-ol	9.21	5 581 534 ± 107 896	9.26	12 801 297 ± 1 468 145
(E)-2-Pentenal	11.33	4 207 905 ± 112 302	11.33	9 415 361 ± 1 534 215

Appendix G: Parameters 1. vs. Optimized parameters

The first experiment optimizing the GC-MS analysis parameters. Data show the area and retention time (min) of the quality indicators with the first analysis parameters with and without shaking, and optimized analysis parameters with and without shaking.

Quality Indicator	Analysis Parameters		RT (min)	Area
2-Ethyl-furan	Parameters 1.	With shaking	8.71	6 027 691
		Without shaking	8.70	6 240 645
	Optimized parameters	With shaking	8.66	13 245 092
		Without shaking	8.66	11 532 462
1-Penten-3-ol	Parameters 1.	With shaking	9.26	2 853 483
		Without shaking	9.25	2 714 511
	Optimized parameters	With shaking	9.22	5 628 883
		Without shaking	9.21	4 874 891
(E)-2-Pentenal	Parameters 1.	With shaking	11.37	1 640 042
		Without shaking	11.36	1 736 932
	Optimized parameters	With shaking	11.33	3 806 001
		Without shaking	11.33	3 375 327



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