CHEMICAL MEASURES OF FISH OIL QUALITY: OXIDATION PRODUCTS AND SENSORY CORRELATION

by

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ABSTRACT

Although quality of commercial fish oil is of the upmost importance to both suppliers and consumers, it can be difficult to maintain due to rapid lipid oxidation attributable to the high levels of EPA and DHA. Fish oil quality can be assessed in a number of ways; this paper focuses on ethyl ester (EE) content and oxidation products. Fish oil supplements are sold as both triacylglycerols (TAG) and EE. TAG products are more resistant to oxidation, have better bioavailability and are generally considered to be of higher quality. Here, a method is described to quantify EE in fish oil using solid phase microextraction (SPME) headspace analysis and gas chromatography-mass spectrometry (GCMS). A related aspect of quality is state of oxidation and although the causes of oxidation in fish oil are well known, there is little research on the kinetics of the oxidation process. The work presented here monitors hydroperoxides to model the kinetics of oxidation in two commercially available fish oil supplements by fitting the data to the Arrhenius model. It was determined that the same mechanisms of oxidation hold at temperatures ≤ 40 °C and thus, this temperature was used in the final stages of this work where accelerated stability testing of fish oil was conducted. Currently, taste panels are the only reliable method to assess the sensory properties of fish oil, but these are costly and subjective. Described here is an alternative method using SPME-GCMS to monitor volatile oxidation products. Two different statistical methods were used to identify oxidative volatiles that correlate with sensory characteristics of fish oil. First, stepwise discriminant function analysis (DFA) was used to identify volatiles that could be used to classify oil as acceptable or unacceptable based on sensory analysis. Principal component analysis (PCA) and linear regression were then applied with greater success. Both techniques identified similar oxidative volatiles as being important to sensory properties. It is anticipated that these methods could be adopted by fish oil manufacturers as measures of quality.

LIST OF ABBREVIATIONS USED

AOCS	American Oil Chemists' Society
AV	Anisidine value
C	Concentration
CAR	Carboxen
DA	Discriminant analysis
DAG	Diacylglycerol
DFA	Discriminant function analysis
DHA	Docosahexaenoic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DVB	Divinylbenzene
E_a	Activation energy
EE	Ethyl ester
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAST	Fatty Acid Smell and Taste Index
FFAP	Free fatty acid phase
FID	Flame ionization detector
GC	Gas chromatograph
GCMS	Gas chromatography-mass spectrometry
GOED	Global Organization for EPA and DHA
GRAS	Generally regarded as safe
I.D.	Internal diamter
MAG	Monoacylglycerol
MANOVA	Multivariate analysis of variance
ME	Methyl esters
MEQ	Milliequivalents
MCR	Multivariate curve resolution
NIH	National Institute of Health
NIST	National Institute of Standards
NMR	Nuclear magnetic resonance
O/W	Oil-in-water
OSI	Oxidative Stability Index
PV	Peroxide value
PDMS	Polydimethylsiloxane
PC	Principal component
PCA	Principal component analysis
PUFA	Polyunsaturated fatty acids
RI	Retention index
SIMCA	Soft independent modeling of class analogy
SPME	Solid phase microextraction
TAG	Triacylglycerol
TBME	*tert*-butyl methyl ether
WHO	World Health Organization

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CHAPTER 1 INTRODUCTION

1.1 DISSERTATION OVERVIEW

The health benefits of long chain polyunsaturated fatty acids (PUFA) are well recognized, specifically those attributed to the omega-3 fatty acids (FA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These fatty acids are essential for development of the brain, heart and other systems (Leaf 2006). EPA and DHA are transferred from mother to fetus during pregnancy and breast feeding, and are considered essential for proper development of the retina. Deficiencies in omega-3 FA have been linked to cardiovascular problems, dermal conditions, behavior and mood disorders, diabetes, arthritis, and respiratory conditions (Lands 2005, Horrocks and Yeo 1999). The best dietary source of omega-3 PUFA is fatty fish; however, many people do not eat the recommended two servings of fatty fish per week (Health Canada 2008, World Health Organization 2008). Fish oil is commonly used as a dietary supplement by those who do not regularly consume fatty fish. Though fish oil is a very good source of EPA and DHA, the high number of double bonds present in these fatty acids makes them highly susceptible to oxidation which can produce fishy flavors and odors that make fish oil supplements unpalatable and discourage people from taking them.

Fish oil is found naturally in the form of triacylglcerols (TAG). In nature, fish oil contains only trace amounts, if any, of ethyl esters (EE) (Carlier et al.1991). However, EE supplements are readily available on the market and are often sold very cheaply. EE oxidize more rapidly than TAG, leading to faster formation of off-flavors and odors, and potentially degradation of EPA and DHA (Yoshii et al. 2002). Recent studies have shown that EE may not provide the same health benefits as TAG products because they are not

absorbed as efficiently by the human body (Dyerberg et al. 2010, Neubronner et al. 2010). For these reasons, TAG oils are generally considered to be higher quality than EE and are usually sold at a premium price. The work presented here focuses on TAG oils, as raw materials and supplements, with different levels of EPA and DHA (Table 1.1), and concentrates on issues of fish oil quality, specifically EE content and oxidation.

Table 1.1 Percentage of EPA and DHA in oils studied.

Oil	Raw Material or Dietary Supplement	Approximate EPA (%)	Approximate DHA (%)	Approximate Total EPA+DHA (%)
NutraSea (Ascenta Health)	Dietary Supplement	32	11	43
NutraSea HP (Ascenta Health)	Dietary Supplement	16	11	27
Fish Oil 1	Raw Material	18	12	30
Fish Oil 2	Raw Material	33	22	55
Fish Oil 3	Raw Material	55	10	65

To produce fish oil with higher levels of EPA and DHA, base-catalyzed transesterifcation with ethanol is used, which creates ethyl esters (EE) from fish oil. EE are then fractionated using molecular distillation or supercritical extraction to selectively reduce the levels of shorter chain fatty acids, and to control the amount of PUFA (Breivik 2007). In some cases the product is sold as an EE but an additional processing step can convert EE back into the TAG form. This concentration process can be used to create TAG oils that contain > 65% EPA and DHA, and EE containing > 95% EPA and DHA. Because the presence of EE can decrease the quality of TAG fish oil, most supplement manufacturers who purchase TAG fish oil specify an EE proportion of <3% in raw material.

A related concern regarding fish oil quality is oxidation. Oxidation reactions take place in all oils but are of higher concern in fish oils due to the high number of double bonds present in EPA and DHA. The classical description of fish oil oxidation describes the oxidation process as a free radical chain reaction consisting of initiation, propagation and termination steps. More specifically, it is initiated by the reaction of singlet oxygen with lipids (Frankel 2005, Min and Boff 2002). This generates free radicals that start chain reactions of oxidation. The primary products of oxidation are hydroperoxides, which then decompose into secondary oxidation products, mainly aldehydes, ketones and alcohols. The Voluntary Monograph on Long Chain Omega-3 EPA and DHA set out by the Global Organization for EPA and DHA (GOED, GOED 2008) is regarded as an "industry standard" and is used by many companies to assess fish oil quality and for shelf life validation. Some of the specifications set by this monograph can be seen in Table 1.2.

Table 1.2 Guidelines for fish oil quality set out by the GOED Voluntary Monograph on Long Chain Omega-3 EPA and DHA

Test	**Max**	**Method**
Peroxide Value	5 meq/kg	AOCS Official Method Cd 8-53
Anisidine Value	20	AOCS Official Method Cd 18-90
TOTOX	26	(2*Peroxide Value)+Anisidine Value

Measurement of hydroperoxides is the basis of the peroxide value (PV) test, a standard test to monitor oxidation that is commonly used as the primary indicator of oil shelf life. In oils containing large amounts of PUFA, such as fish oil, the maximum PV may occur at early stages of oxidation due to the instability and rapid decomposition of hydroperoxides into secondary oxidation products (Frankel 2005). This means that oils

can have very low PV but still be oxidized. Elevated temperatures and the presence of transition metals accelerate hydroperoxide decomposition (Choe and Minn 2006). Numerous other factors, including lipid class composition, concentrations and type of oxygen present, presence of antioxidants and light can also influence hydroperoxide formation and degradation (Choe and Minn 2006). Being primary oxidation products, hydroperoxides have very little impact on flavor of oils and are primarily measured because they are precursors to compounds that negatively affect sensory characteristics. Despite this, PV often serves as the basis to assess oxidative state because it is easy to measure and is recommended by GOED. In order to complete shelf life testing in a reasonable amount of time, elevated temperature is frequently used to accelerate oxidation with the goal being to obtain results that can then be used to predict the shelf life of fish oil products that are stored under normal conditions (Frankel 2005). For this to be possible it is essential to understand the kinetics of fish oil oxidation and its relationship with temperature so that results of accelerated shelf life studies can be reliably extrapolated to lower temperatures.

Measurement of secondary oxidation products is also used to assess oxidation. For instance, anisidine value (AV) measures aldehydes with α- and β-unsaturation; however, this is not a sensitive method and there is some uncertainty whether those specific components are linked to oil flavor. It is well documented that sensory properties of fish oil are highly affected by the presence of volatile ketones and alcohols, in addition to aldehydes, that are also breakdown products of hydroperoxides (Frankel 2005). In some cases aldehyde and ketone compounds have a taste and/or odor threshold of less than 1 ppm, well below the detection limits of AV testing (Table 1.3). The totox value of fish oil is calculated by doubling the PV and adding the AV, and attempts to predict the total

amount of oxidation products present. This number is arbitrary, as neither the primary or secondary oxidation products being measured correlate with the sensory characteristics of the oil. The poor relationship between conventional oxidation testing and sensory parameters draws attention to the need for an alternative method to monitor oil quality.

Table 1.3 Common oxidation products found in fish oils and their sensory thresholds.

Compound	Odor/Flavor	Sensory Threshold (ppm)
1-octen-3-ol[a, b]	Mushroom[a]	0.01[a]
(*E*)-2-heptenal[a, b, c]	Fatty, bitter almond[e]	0.20[a]
(*E, E*)-2,4-heptadienal[a, c, d]	Rancid, fatty[a]	0.055[a]
(*E,Z*)-2,6-nonadienal[a, c, d]	Cucumber[e]	0.022[a]
2-nonenal[a, b, e]	Tallow, cucumber[e]	0.1[a,b]

a- Frankel 1985 b- Jacobsen et al. 2000. c- Karahadian and Lindsey 1989 d-Karahadian and Lindsey 1990 e-Belitz et al. 1999

Taste panels are the most accurate method of evaluating sensory qualities of oils, as humans can detect lower levels of volatile components that traditional tests of oxidation cannot. Unfortunately, the high costs associated with establishing and maintaining a panel makes their use unattractive. In many cases it is difficult to recruit panelists as people are reluctant to participate if the product being tasted has poor sensory properties. An alternative to sensory panels are methods that monitor amounts of volatile secondary oxidation products in the headspace of samples, as these are the compounds most responsible for oil flavors, using techniques such as solid phase microextraction (SPME). While over 200 volatiles have been detected in fish oils, so far only about 20 have been shown to have a relationship with sensory characteristics (Frankel 2005). These volatiles are present at such low levels that they are impossible to detect using conventional quality testing.

The overall goal of this project is to accurately assess the quality of commercially available fish oil and develop new methods to make quality monitoring more efficient. The specific objectives of this study are as follows:

1. To develop a method to assess fish oil for adulteration with EE using SPME-GCMS.
2. To determine if fish oil oxidation follows the Arrhenius model and if the kinetic model for oxidation at elevated temperatures is the same as at room temperature, then to use this information to predict the shelf life of fish oil.
3. To identify the key oxidation products in fish oils containing different levels of EPA and DHA that are important in distinguishing between acceptable and poor quality fish oils.
4. Use this information to create a method to monitor fish oil oxidation that correlates well with sensory characteristics of oils.

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CHAPTER 2 DETERMINING ETHYL ESTERS IN FISH OIL USING SOLID PHASE MICROEXTRACTION AND GCMS

2.1 PUBLICATION INFORMATION

This chapter is a modified version of a manuscript, reworked here for formatting consistency. Originally published as:

Sullivan JC, Budge SM, St-Onge M (2009) Determining ethyl esters in fish oil using Solid Phase Microextraction and GC-MS. JAOCS 86:643-748. Suzanne Budge and Marc St-Onge contributed to the study design and manuscript preparation. Jenna Sullivan Ritter was the principal investigator and lead author.

2.2 ABSTRACT

The long chain PUFA found in fish oil, specifically EPA and DHA play an important part in human health. As a result, fish oil supplements are commonly consumed by people around the world. Supplements in the form of TAG can be sold at a premium price, compared to those in the EE forms. Producers of TAG supplements require a simple, rapid method to determine the authenticity of their raw material. Here, we describe a method to quantify EE in fish oil using solid phase microextraction headspace analysis and GCMS. Despite the variation in linear ranges of the calibration curves with volatility of the EE, 30 individual FA were quantified including common saturated FA such as palmitic and stearic acid, as well as longer chain PUFA, such as EPA and DHA. The method was then applied to three commercial fish oils in the TAG form and two of the products were found to contain EE, with one containing EE above 1.5% w/w, indicating that contamination had occurred. With growing consumer interest in fish oil products, the method proposed here will help resolve future issues of authenticity in fish oils.

2.3 INTRODUCTION

The health benefits of long chain PUFA have long been recognized, specifically the omega-3 FA, EPA and DHA. They are essential for the growth and development of the brain, heart and other systems (Leaf 2006). Adequate PUFA supplies are essential during retina development as well, and can be transferred from mother to infant during pregnancy and through breast milk. Deficiencies in omega-3 FA have been associated with cardiac problems, hypertension, dermal conditions, impairment of adult brain function, attention deficit disorder, diabetes, arthritis, asthma, inflammation and clinical depression (Land 2005, Horrocks et al. 1999). The best dietary source of PUFA is fatty fish; however many people to do not consume the recommended two servings of fatty fish per week (Health Canada 2008, World Health Organization 2008). Fish oil can be used as a dietary supplement for those who do not consume adequate amounts of fatty fish.

Fish oil is naturally found in the form of TAG (Carlier et al. 1991), and that obtained from anchovy, sardine and herring naturally contains approximately 30% total EPA and DHA. During processing of pharmaceutical grade fish oil, TAG undergo base-catalyzed transesterifcation with ethanol to create EE and a product commonly known as "EE oil". The EE can be fractionated, usually by molecular distillation to selectively reduce the levels of particular FA, such as saturates, and to control the amount of PUFA, ensuring that the oil contains the desired 30% PUFA (Breivik 2007). An additional processing step can convert EE back into the TAG form, creating a "TAG oil". Through distillation of EE and modification of PUFA content, fish oil concentrates, sometimes containing more than 65% EPA and DHA, may be created. These concentrates are often left as EE as the conversion from an EE oil to a TAG oil is quite costly and for this reason, a large number of commercially available fish oil supplements are made primarily

with EE. However, TAG oils are available, and fish oil refiners and producers generally specify an EE content of 3% w/w or less in their TAG products. Since natural fish oil has only trace amounts, if any, EE present, a measureable amount of EE indicates either accidental or deliberate contamination of the product. Refiners usually produce both products so accidental contamination is always a possibility. More worrisome is the potential for deliberate addition of EE to TAG oils to modify FA composition. For instance, fish oil manufacturers may dilute TAG oil concentrates that have high levels of PUFA with shorter chain EE in order to lower the EPA and DHA content, while others have been suspected of spiking TAG fish oil with EE-EPA and EE-DHA to increase their levels and reduce costs (Ackman and Timmins 2007). Unfortunately, EE are much less resistant to oxidation than TAG, which can lead to a lower quality product due to the production of fishy off-flavours (Yoshii et al. 2002). Interestingly, some ethnic communities have been known to test fish oil deemed to be of poor quality by placing it in a polystyrene cup. If the cup dissolves, the oils would be considered to be of inferior quality. Ackman and Timmins (2007) have shown that neither TAG nor free FA will dissolve polystyrene, but EE lead to rapid destruction.

In addition to the decrease in oxidative stability, there is evidence to suggest that supplements containing EE may not provide the same health benefits as TAG products. For instance, a number of studies have shown that TAG oils are better absorbed in the body than EE. Beckermann et al. (1990) found that consumption of TAG fish oil resulted in 50% more plasma EPA and DHA than with EE supplements, while Lawson and Hughes (1988) showed that EPA and DHA in TAG form were more efficiently absorbed by 48% and 36% respectively, when compared to EE forms. Plasma lipid concentrations of EPA and DHA were significantly higher in subjects who consumed salmon than those

who consumed supplements of EE (Visioli et al. 2003). Animal studies also suggest that the ethanol molecule from the EE can be released into the liver and pancreas when EE are digested, resulting in organ damage, particularly in those with diseases that affect the liver (Yuan et al. 2006, Werner et al. 1997, Ikeda et al. 1995). Because of the negative aspects of EE, fish oil companies often promote TAG supplements as being superior and therefore charge a premium price. Thus, it becomes important to develop rapid methods to test for the presence of EE from both an authenticity and quality perspective.

Although a literature search did not return any documented methods specifically for quantifying EE in TAG oils, there are a number of chromatographic methods that may be used to determine EE in lipid mixtures. For instance, Bernhardt et al. (1996) used solid phase extraction and HPLC to isolate and quantify EE from blood serum. In the same paper they also described a separation using thin layer chromatography (TLC) with petroleum ether and ethyl ether (75:5, v/v) as developing solvent. The major difficulty with these techniques is the separate steps of isolation and quantification of EE that may result in loss of EE and inaccurate recoveries. Furthermore, methods developed for biological samples are often designed to separate very different proportions and types of lipids than are found in fish oils and difficulties are often encountered when attempting to adapt these methods for analysis fish oil. Here we describe a new method to test commercial fish oil supplements for adulteration with EE using SPME and GCMS.

2.4 Experimental Procedures

2.4.1 Materials

Fish oil EE were obtained from Ocean Nutrition Canada Ltd. (Dartmouth, NS, Canada). Commercially available refined canola oil was purchased from a grocery store.

Spectrophotometric grade 1,2-dichlorobenzene (99%) was used as internal standard (Aldrich, Oakville, ON, Canada).

SPME fibers with divinyl benzene/ polydimethylsiloxane/Carboxen coating (50/30 μm), a SPME fiber holder for manual sampling, 22 ml glass vials, polytetrafluoroethylene/silicone rubber septa and phenolic screw caps were purchased from Supelco. A custom-made heating block designed to accommodate 22 ml glass vials was used to control temperature.

2.4.2 Fatty acid analysis

EE in the EE oil to be used as a standard material were quantitatively converted to methyl esters (ME) using the Council for Responsible Nutrition Voluntary Monograph for Omega-3 (2008) and analyzed by GC-FID. ME, rather than EE, were analyzed because accurate correction factors to account for the differential response of FA structures by FID were only available for ME. ME were separated on a DB-23 column (30 m x 0.25 mm x 0.25 μm film thickness) and helium was used as the carrier gas, at a flow rate of 1.0 ml/min. The oven temperature was initially held for 2 min at 153 °C then increased at 2.3 °C/min to 174 °C and held for 0.2 min. The temperature was then increased at a rate of 2.5 °C/min to 205 °C and held for 8.3 min. The total run time was approximately 32 min. The FID was maintained at 270 °C, and the injector (split mode 1:100, 250°C, 4 mm liner) at 250 °C.

2.4.3 Calibration Curve

Varying amounts of EE oil ranging from 0.70 ml to 1.40 ml (62.0 mg to 1250 mg) were added to a 22 ml glass vial containing a stir bar. Samples were then diluted to exactly 14 ml with canola oil and 1.0 μl dichlorobenzene was immediately added as an

internal standard. Typical odd carbon-numbered FA were not used as internal standards because these fish oils contained traces of such structures. Dichlorobenzene was chosen instead because it had been successfully employed as an internal standard to monitor other volatile components using SPME (Beltran et al. 2005). Vials were capped with phenolic screw caps containing PTFE/silicone rubber septa and samples were stirred at 80°C for exactly 15 min. During this equilibrium period, the SPME fiber was placed in the hot injector port of the GC to desorb any volatiles that may have accumulated between uses. After the 15 min equilibration time, the SPME fiber was inserted into the vial to a depth of 2.0 cm and exposed to the headspace for exactly 45 min. The equilibration and fiber exposure times were based on an optimized procedure developed to extract volatiles from fish oil. Extracted compounds were analyzed by GCMS in electron ionization mode. The fiber was then inserted in the injector port (splitless mode, 250 °C, 0.75 mm liner) to a depth of 5.0 cm, to the center of the injector, and remained there for 15 min. Volatile analytes were separated on a free FA phase (FFAP) column (30 m x 25 mm x 0.25 um film coating) and helium was again used as the carrier gas at a flow rate of 1.0 ml/min. The oven temperature was held initially at 40 °C for 5 min, then increased at a rate of 10 °C/min to 220 °C and held for 10 min (total run time of 38 min). Volatiles were identified using library matches from NIST library and standards. The ratio of the area counts of each EE to the internal standard was then calculated and standard curves of ratio vs. EE were constructed. Samples of commercially available fish oil supplements, purchased at retail outlets, were analyzed using the same method as for the standard curve, that is 14 ml of sample was added to a 22 ml glass vial along with 1.0

μl internal standard. Analysis was conducted in the same manner as for the standard curve.

2.5 Results and Discussion

FA present in the standard EE oil were typical of nutraceutical grade fish oil, containing the major constituents of EPA and DHA, as well as smaller amounts of monounsaturated, saturated and branched-chain FA. The concentrations of EE in the standard material (Table 2.1) as determined by GC FID, combined with the EE concentrations from the SPME GC-MS analysis were used to create a standard curve (mg/g of EE) for each FA EE present, in order to compensate for the varying volatilities of EE with different structures. Because of the range in molecular masses with different FA structures, not all EE present in the oils could be quantified by SPME. Some were simply not volatile enough and/or were not present at concentrations sufficient for quantification. For example, 16:3n-3 was quantified in trace amounts as ME by GC in the standard oil, but did not adsorb to the SPME fiber in large enough amounts to allow for quantification. Similarly, nervonic acid (24:1) was not volatile enough to be quantified, despite being easily detected as ME by GC. However, in many situations it is only EPA and DHA that need to be verified as TAG since they are generally considered the active ingredients in fish oil supplements and are likely to be added as EE to meet product specifications. Both of these FA were easily quantified using SPME. Alternatively, if TAG is diluted with EE in order to decrease the PUFA content, some EE likely to be used would be myristic acid EE (14:0), palmitic acid EE (16:0) and stearic acid EE (18:0). These FA were accurately quantified by SPME with high coefficients of

determination because of their relatively low molecular weights and high volatility. Lower volatility of longer chain PUFA does, however, become a problem when combined with low concentrations of those EE in the standard material, resulting in calibration curves with poorer fit. For instance, 20:1n-7 and 20:3n-6 were present in the standard at levels < 10 mg/g and all had $r^2 < 0.96$; however, levels of myristic acid (14:0) and 15:0 were similarly low in the standard (concentrations of 3.73 and 0.50 mg/g, respectively) but both r^2 values were 1.00. Longer chain PUFA also have greater detection limits due to their low volatility; for example, the lower detection limit for EPA is 5.08 mg/g, while shorter chain FA such as myristic acid can be detected at 0.04 mg/g. If one requires a lower detection limit for specific long chain FA, selection of standard material with a higher PUFA concentration will be necessary.

Table 2.1 Concentration of individual EE (mg/g ± SD) in standard EE oil used to construct the calibration curves.

Fatty Acid	**Concentration (mg EE/g EE oil)**	**Fatty Acid**	**Concentration (mg EE/g EE oil)**
14:0	3.73 ± 0.09	18:2n-4	4.42 ± 0.04
14:1n-9	0.10 ± 0.01	18:3n-6	2.08 ± 0.04
14:1n-7	0.01 ± 0.03	18:3n-4	1.68 ± 0.01
i-15:0	0.22 ± 0.03	18:3n-3	8.53 ± 0.04
ai-15:0	0.07 ± 0.01	18:3n-1	0.61 ± 0.04
15:0	0.50 ± 0.01	18:4n-3	21.7 ± 0.2
i-16:0	0.46 ± 0.01	18:4n-1	3.5 ± 0.3
16:0	29.0 ± 0.3	20:0	3.1 ± 0.1
16:1n-11	1.10 ± 0.01	20:1n-11	2.9 ± 0.7
16:1n-9	0.53 ± 0.01	20:1n-9	19 ± 1
16:1n-7	13.4 ± 0.1	20:1n-7	5.93 ± 0.06

Fatty Acid	Concentration (mg EE/g EE oil)	Fatty Acid	Concentration (mg EE/g EE oil)
16:1n-5	0.37 ± 0.01	20:2n-9	0.7 ± 0.2
i-17:0	0.76 ± 0.01	20:2n-6	4.4 ± 0.3
16:2n-6	0.26 ± 0.01	20:3n-6	2.96 ± 0.07
ai-17:0	0.30 ± 0.06	20:4n-6	16.31 ± 0.06
16:2n-4	1.84 ± 0.03	20:3n-3	2.03 ± 0.07
17:0	2.20 ± 0.03	20:4n-3	11.8 ± 0.1
16:3n-4	1.69 ± 0.01	20:5n-3	265 ± 1
17:01	0.88 ± 0.05	22:0	1.2 ± 0.2
16:3n-3	0.19 ± 0.04	22:1n-11	14.7 ± 0.1
16:4n-3	1.52 ± 0.04	22:1n-9	2.6 ± 0.1
16:4n-1	2.86 ± 0.01	22:1n-7	2.5 ± 0.1
18:0	37.2 ± 0.1	22:2n-6	0.8 ± 0.2
18:1n-13	1.04 ± 0.07	21:5n-3	8.2 ± 0.2
18:1n-11	0.8 ± 0.3	22:4n-6	1.3 ± 0.6
18:1n-9	75.2 ± 0.4	22:5n-6	4.3 ± 0.2
18:1n-7	37.1 ± 0.2	22:4n-3	1.04 ± 0.07
18:1n-5	1.63 ± 0.02	22:5n-3	26.7 ± 0.1
18:2Δ5,11	0.40 ± 0.07	22:6n-3	172.7 ± 0.7
18:2n-7	0.42 ± 0.02	24:1	6.87 ± 0.05
18:2n-6	12.9 ± 0.2		

Of the three samples tested, two were found to contain ethyl esters. Commercial Sample A was a liquid fish oil product, purportedly containing a TAG concentrate. It was found to contain 1.6% w/w (16 mg/g) EE with the primary EE detected as EPA (20:5n-3), DHA (22:6n-3) and oleic acid (18:1n-9) (Table 2.2). As EPA and DHA were the major EE present in this product, it seems likely that they were added as an inexpensive way to

increase the PUFA content. The presence of shorter chain EE in the product suggests that an EE concentrate was added to the product, as opposed to only EPA and DHA being added.

Table 2.2 Structures and concentrations of EE in Commercial Sample A. Upper and lower limits of linear range and r^2 values of the calibration curves are included.

Ethyl Ester	Amount EE (mg/g)	Lower Limit (mg/g)	Upper Limit (mg/g)	r^2
20:5n-3	6.6 ± 0.2	5.08	25.38	0.99
22:6n-3	4.8 ± 0.2	3.28	16.40	0.98
18:1n-9 and 18:1n-7	1.1 ± 0.1	0.72	7.23	0.99
18:0	0.6 ± 0.1	0.36	3.57	0.97
18:4n-3	0.52 ± 0.06	0.42	2.09	0.99
22:1n-11	0.37 ± 0.07	0.28	1.39	0.96
20:4n-6	0.35 ± 0.09	0.16	1.56	0.93
16:0	0.34 ± 0.09	0.00	2.81	0.98
16:1n-7	0.18 ± 0.05	0.00	1.30	0.99
18:3n-3	0.15 ± 0.05	0.08	0.82	0.97
20:1n-7	0.11 ± 0.05	0.06	0.57	0.95
18:2n-4	0.10 ± 0.03	0.09	0.43	0.98
18:2n-6	0.09 ± 0.03	0.09	0.43	0.99
20:3n-6	0.08 ± 0.03	0.06	0.28	0.95
20:0	0.07 ± 0.03	0.06	0.30	0.97
22:1n-9	0.06 ± 0.03	0.05	0.24	0.96
16:4n-1	0.05 ± 0.03	0.01	0.28	0.96
18:3n-6	0.05 ± 0.02	0.04	0.20	0.98
14:0	0.04 ± 0.03	0.04	0.37	1.00
18:3n-4	0.04 ± 0.02	0.03	0.16	0.98
16:2n-4	0.04 ± 0.02	0.02	0.18	0.99

Ethyl Ester	Amount EE (mg/g)	Lower Limit (mg/g)	Upper Limit (mg/g)	r^2
16:3n-4 and 17:1	0.04 ± 0.01	0.03	0.16	1.00
i-17:0	0.01 ± 0.01	0.01	0.07	1.00
22:1n-7	0.01 ± 0.02	0.01	0.24	0.99
16:2n-6	0.01 ± 0.00	0.01	0.03	1.00
15:0	0.01 ± 0.01	0.00	0.05	1.00
Totals	**15.86 ± 1.53**			

Commercial Sample B caused some difficulties during analysis. The product was an encapsulated fish oil concentrate product with an enteric coating and an alginate/glycerol based capsule that claimed to be the natural form of fish oil. Upon analysis it was clear that the sample contained EE as well as two large peaks, not typically found in fish oil (Fig.2.1a and 2.1b). A NIST library search suggested that these peaks were glycerol and caprylic acid (8:0). Free FA are commonly determined by SPME using a variety of fibers (Mondello et al. 2005, Jelén et al. 2007, Gonzlez-Crdova and Vallejo-Cordoba 2001, Tomaino et al. 2001), while glycerol determination with SPME is rarer. It has been quantified in at least one study using a similar SPME fiber coating of carboxen/polydimethylsiloxane (Biswas and Staff 2001) and Goicoechea et al (2008) identified it using the same fiber as this study. The capsule material seems the most obvious source of glycerol. Caprylic acid, on the other hand, can arise from TAG breakdown; however, it is unlikely that substantial amounts of a single, very short chain FA would arise from that process, particularly from degradation of fish oil. It seems more likely that the free FA is also associated with the capsule material or the enteric

coating. Unfortunately it was impossible to quantify the EE in this sample because of the rising baseline associated with the glycerol and caprylic acid peaks.

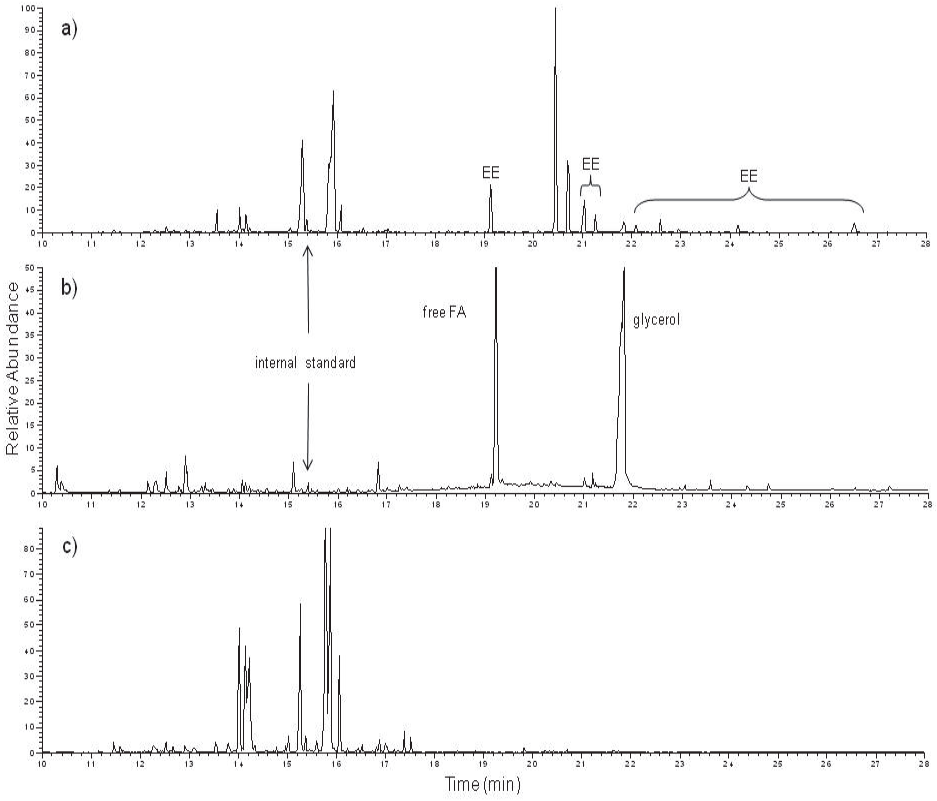

Figure 2.1 Partial chromatograms of volatile compounds in TAG oils detected by SPME. a) Commercial Sample A containing EE. The majority of compounds with large peak areas eluting before 19 min are flavours added to the product by the producer to mask fishy off-odours; b) Commercial Sample B, an encapsulated product, containing traces of EE as well as glycerol and free FA; and c) Commercial Sample C, also an encapsulated produce, without EE, glycerol or free FA. Compounds present in this sample are flavour components or products of lipid oxidation.

Commercial Sample C consisted of a capsule without enteric coating. This sample did not contain EE, glycerol or caprylic acid (Fig 2.1c). The absence of these peaks in Sample C suggests that the glycerol and caprylic acid in Sample B were likely derived from the enteric coating of the capsule, rather than the capsule itself. This also indicates that the method described here may require modification to avoid the

chromatography problems encountered with analysis of glycerol, caprylic acid and EE if it is to be applied to fish oil products in capsules that contain such coatings. We did not pursue this issue further, but it could likely be resolved by selecting a SPME fiber that retains EE but has little affinity for caprylic acid and glycerol (Harmon 2001). In addition, we envision this method as being useful for fish oil supplement producers who wish to test the integrity of the fish oil they are using in their products. Thus the testing of raw materials purchased from third parties would be carried out before encapsulation and introduction of the enteric coating, avoiding the problem of interference by capsule materials. A particular advantage of this method is that it will simultaneously extract volatile oxidation products from oils so that it is possible to test raw materials for the presence of both EE and oxidation products at the same time.

The method described here to requires very little sample preparation and is therefore, very simple to perform. Minimal sample manipulation also avoids losses of analytes that are often encountered with multi-step methods, while avoiding solvent use is particularly attractive in light of heightened awareness of solvent toxicity. Additionally this method is inexpensive and reproducible, making it ideal for testing fish oil authenticity. We found this method particularly useful in that we could monitor both EE content and volatile lipid oxidation products in a single run. We manually sampled headspace, but the method could easily be automated with the use of a SPME autosampler. In fact, the step requiring the greatest time investment is supervising the integration of peak areas and manipulating data.

2.6 Conclusions

This method offers a reliable alternative to HPLC and TLC for the detection of EE, with the important benefits inherent in all SPME techniques of avoiding the use of solvents, and being highly sensitive. We anticipate that this method will be particularly useful for fish oil supplement manufacturers as a means to test the quality of their source oil.

2.7 Acknowledgements

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CHAPTER 3 MODELING THE PRIMARY OXIDATION IN COMMERCIAL FISH OIL PREPARATIONS

3.1 PUBLICATION INFORMATION

This paper is a modified version of a manuscript, reworked here for formatting consistency. Originally published as:

Sullivan JC, Budge SM (2011) Modeling the primary oxidation in commercial fish oil preparations. Lipids 46:87-93. Suzanne Budge contributed to the study design and manuscript preparation. Jenna Sullivan Ritter was the principal investigator and lead author.

3.2 ABSTRACT

The quality of commercial fish oil products can be difficult to maintain because of the rapid lipid oxidation attributable to the high number of PUFA, specifically EPA and DHA. While it is known that oxidation in fish oil is generally the result of a direct interaction with oxygen and fatty acid radicals, there are very few studies that investigate the oxidation kinetics of fish oil supplements. This study uses hydroperoxides, a primary oxidation product, to model the oxidation kinetics of two commercially available fish oil supplements with different EPA and DHA contents. Pseudo-first order kinetics were assumed, and rate constants were determined for temperatures between 4 and 60 °C. These data were fit to the Arrhenius model, and activation energies (E_a) were determined for each sample. Both E_a agreed with values found in literature, with the lower PUFA sample having a lower E_a. The oil with a lower PUFA content fit the first-order kinetics model at temperatures ≥20 °C and ≤ 40 °C, while the higher PUFA oil demonstrated first-

order kinetics at temperatures ≥4 °C and ≤ 40 °C. When the temperature was raised to 60 °C, the model no longer applied. This indicates that accelerated testing of fish oil should be conducted at temperatures ≤ 40 °C.

3.3 Introduction

Fish oil dietary supplements have been gaining popularity in recent years due to the health benefits provided by PUFA they contain. The primary PUFA in fish oil are EPA and DHA. These FA have been shown to be important factors in cardiovascular health as well as brain and eye development in babies (Simopoulos 1991, Hu et al. 2002, Kris-Etherton et al. 2002, Lands 2005, Leaf 2006, Innis 2008) while deficiencies in PUFA have been associated with a number of negative health conditions including dermal conditions, attention deficit disorder and clinical depression (Lands 2005). Because most people do not consume the recommended 2-3 servings of fatty fish per week as recommended by the World Health Organization (2008), fish oil supplements have become a popular alternative. Unfortunately, due to their large number of double bonds, PUFA in fish oil are subject to rapid oxidation which produces fishy off-flavors and can make supplements unpalatable.

The type of oxidation most commonly seen in commercial fish oil products is a result of direct interactions between fatty acid radicals and molecular oxygen. This process is initiated by reaction of singlet oxygen with lipids (Min and Boff 2002) to generate free radicals that in turn, initiate chain reactions of oxidation. Oxidation of PUFA leads to the formation of the primary oxidation products, lipid hydroperoxides, which then break down into secondary oxidation products including aldehydes, ketones,

acids and alcohols. Hydroperoxides are stable at room temperature, but readily decompose at elevated temperatures or in the presence of transition metals (Choe and Min 2006). The rate of formation and degradation of hydroperoxides increases with increasing temperature (Shahidi and Spurvey 1996). Hydroperoxides are an important measure of oil quality as they are an indicator of the future levels of secondary oxidation products that negatively impact sensory parameters. There are a variety of other factors, including FA composition, lipid class composition, concentrations of singlet and triplet oxygen, antioxidant concentration and light exposure, that can influence oxidation, and make accurate comparisons between oxidation studies difficult (Choe and Min 2006).

Tests of oxidative stability are commonly used to evaluate the shelf life of fish oils, but in order to complete testing in a reasonable amount of time, elevated temperature is frequently used to accelerate oxidation. The goal of this accelerated testing is to obtain results that can then be used to predict the shelf life of fish oil products that are stored under normal conditions (Frankel 2005). For this to be possible, the kinetics of the oxidation reaction must be determined. Theoretically, oxidation rates can be monitored by following the degradation of specific FA. Though this has been attempted in fish oils (Bórquez et al. 1997), these oils have a complex FA profile and application of these techniques may not give an accurate representation of oxidation that is occurring in the oil as a whole. At normal oil storage temperatures, the change in FA profile happens very gradually and FA analysis may not be sensitive enough to detect the minute changes in FA concentration, making this method impractical to use for monitoring oxidation of fish oils. Rather than monitoring FA composition of fish oils, other studies use oxygen concentrations to assess oxidation (e.g. Cho et al. 1987, Yoshii et al. 2002), a technique

that may be more accurate for fish oil oxidation, though it does not directly take into account the formation of oxidation products that could impart negative flavors into the oil. No studies could be found that attempt to decipher the kinetics of fish oil oxidation using a common oxidation indicator. The present study uses peroxide values (PV) to assess oxidation because these compounds are formed directly from lipids and therefore the amount of hydroperoxides present can be directly related to the amount of oxidized lipid present at early stages of oxidation.

This study evaluates the stability of two commercially available liquid fish oil supplements for oxidative stability by monitoring hydroperoxide formation at a number of different temperatures, ranging from 4 °C to 60 °C, to determine if oxidation of fish oil follows the Arrhenius model. This information is important to fish oil manufacturers as it will enable the application of accelerated stability data to real-time conditions, thereby reducing the time required to perform stability studies from years to weeks.

3.4 Experimental Procedures

3.4.1 Materials

Two different types of commercially available liquid fish oil supplements were obtained from a retail outlet. The first was NutraSea, a typical "18:12" fish oil, containing approximately 18% EPA and 12% DHA as proportions of total FA. The second was NutraSea HP, a fish oil "concentrate" containing approximately 30% EPA and 10% DHA. Both products were produced by Ascenta Health and contained winterized fish oil (97.86%), natural flavour (2%), alpha tocopherol (0.04%) and green tea catechins (0.1%). Amber bottles (200 ml) and lids were supplied by Ascenta Health.

These products were marketed as fish oils and contained TAG as the primary lipid at 70-75%. Monoacylglycerol (MAG) and diacylglycerol (DAG) were also preset at 20-25% and < 5%, respectively.

Potassium iodide, 1% starch indicator, sodium chloride, butyl hydroxytolulene, boron trichloride-methanol, anhydrous sodium sulphate, Optima acetic acid and Optima iso-octane were obtained from Fisher Scientific (Ottawa, ON). Optima chloroform was obtained from VWR (Mississauga, ON). An Isotemp 100 Series Model 126G oven (Fisher Scientific) was used to incubate samples. Methyl tricosonate, methyl eicosapentaenoate and methyl docosahexaenoate were obtained from Nu-Chek Prep (Elysian, MN).

3.4.2 Fatty acid analysis

Both fish oils were analyzed for EPA and DHA via GC-FID. TAG were converted to methyl esters (ME) following the modified GOED Voluntary Monograph for Omega-3 (2008), using methyl tricosonate as an internal standard, as well as external standards for EPA and DHA. ME were separated using a column coated with (50% cyanopropyl)-methylpolysiloxane (30 m x 0.25 mm x 0.25 μm film thickness) and helium was used as the carrier gas at a flow rate of 1.0 ml/min. The oven temperature was initially held for 2 min at 153 °C then increased at 2.3 °C/min to 205 °C and held for 8.3 min. The total run time was approximately 32 min. The FID was maintained at 270 °C, and the injector (split mode 1:100, 4 mm liner) at 250 °C.

3.4.3 Stability Studies

Both oils were incubated in the dark at 4, 20, 40 and 60 °C. The 18:12 oil was also incubated at 30°C. Three bottles were used for each incubation temperature. Bottles

were capped but not purged with nitrogen after the initial opening. PV were measured in triplicate, with each bottle sampled at each time point, following AOCS Official Method Cd 8-53 (1997). Samples stored at 4 °C were initially tested weekly, but then were tested monthly after 3 months of testing. Samples stored at 20 °C were tested weekly, while samples stored at 30 and 40°C were tested every 3 days. Samples stored at 60 °C were analyzed daily. Different sampling periods were necessary to capture the variation in PV with changing oxidation rates. After removing an aliquot for sampling, each bottle was recapped and returned to the test temperature. Testing was stopped when an average peroxide value of 5 meq/kg was reached, as this is the maximum accepted value for fish oil as recommended by GOED (2008).

3.4.4 Determination of rate constants and shelf life prediction

Kinetic analysis of data was carried out using methods adapted from Labuza (1971), Labuza and Bergquist (1983), Spears et al (1987) and Tan et al (2001). Though kinetics analysis is often conducted by monitoring the degradation of reactants, it is well established that formation of products can also be monitored as was the case in this study (Labuza and Bergquist 1983, Labuza 1984, Macebo Campos et al 2008, Gomez-Alonso et al 2004). Pseudo-first order conditions for the formation of an undesirable quality factor, as described by Labuza (1984), were assumed with the oil substrate in excess so that:

$$-d[O_2]/dt = d[C]/dt = kC \qquad (1)$$

where C is the concentration of oxidation products, in this case represented by the PV, and k is the rate constant. Integration leads to the following relationship:

$$\ln C = \ln C_o + kt \qquad (2)$$

where C_o is the initial concentration of oxidation products (PV at initial times) and t is time in days. Plots of ln PV versus time were linear with slopes of k for most trials (Table 3.2, Appendix A). Rate constants derived from linear plots were then fit to an Arrhenius model:

$$\ln k = \ln A - E_a/RT \quad (3)$$

where A is the pre-experimental factor, R is the universal gas constant, T is the absolute temperature and E_a is the activation energy in J/mol.

Because the ultimate goal of this study was to investigate the validity of shelf life prediction by extrapolating rate constants from high temperature to low temperature studies, we therefore examined the exponential relationship between the time require to reach the upper limit of acceptability (t_{rej}) and temperature, according to Mancebo-Campos (2008):

$$t_{rej} = a\mathrm{e}^{T} \quad (4)$$

where a is a constant and t_{rej} is the time required to reach PV= 5 meq/kg, as the upper limit of acceptability for fish oil oxidation. Integration of this relationship gives

$$\ln t_{rej} = \ln a + T \quad (5)$$

suggesting that a plot of ln t_{rej} versus T should be linear.

3.5 Results and Discussion

3.5.1 Fatty Acid Analysis

The amounts of EPA and DHA present in both the 18:12 and concentrate oils (Table 3.1) differed, as expected from their label claims. The 18:12 oil had a label specification of 269 mg/g while the concentrate specified 430 mg/g. Upon testing, both

samples exceeded label claims for EPA and DHA with values of 286 mg/g and 499 mg/g respectively. We therefore considered these oils to have significantly different PUFA contents. Complying with label claims was also important to establish that these oils were typical of commercial products currently available; the purpose of this study was to monitor oxidation in commercial products with added antioxidants and flavors that contained FA at expected levels.

Table 3.1 EPA and DHA content (mean ± SD) of 18:12 oil and concentrate oil compared to the label claim.

	18:12	**Concentrate**
Omega-3 Claim (mg/g)*	269	430
Actual EPA content (mg/g)	151 ± 1	349 ± 1
Actual DHA content (mg/g)	135 ± 1	150.5 ± 0.4
Total EPA+DHA content (mg/g)	286 ± 2	499 ± 1

*Manufacturer guarantees only the total sum of EPA+DHA, not the individual fatty acid content.

3.5.2 Stability Studies

In the experimental design, pseudo-first order kinetics was assumed. Lipid oxidation is affected by the amount of oxygen dissolved in the oil, equilibrium between oxygen dissolved in the oil and that in the headspace was assumed. For the initial setup, each bottle contained approximately 180 ml of fish oil with approximately 20 ml of headspace. This corresponds to approximately 0.6 mol of FA and 2×10^{-4} mol of O_2, with FA present in excess of 3500 times the amount of O_2. This ensures that O_2 is limiting, a necessary criteria of pseudo-first order reactions. Even in the samples that had the longest testing period (22 time points, with 5 ml of oil being used at each point), this situation was maintained with 70 m of oil, and 130 ml of air. This is equivalent to 0.23 mol of FA

and 1.3 x10^{-3} mol of O_2, ensuring that FA were still present in excess of 176 times the amount of O_2. Resulting plots of PV versus time were therefore expected to increase exponentially over time (Fig 3.1a and 3.1b) with linear fits for the corresponding regressions of ln PV versus time (Appendix A, Table 3.2).

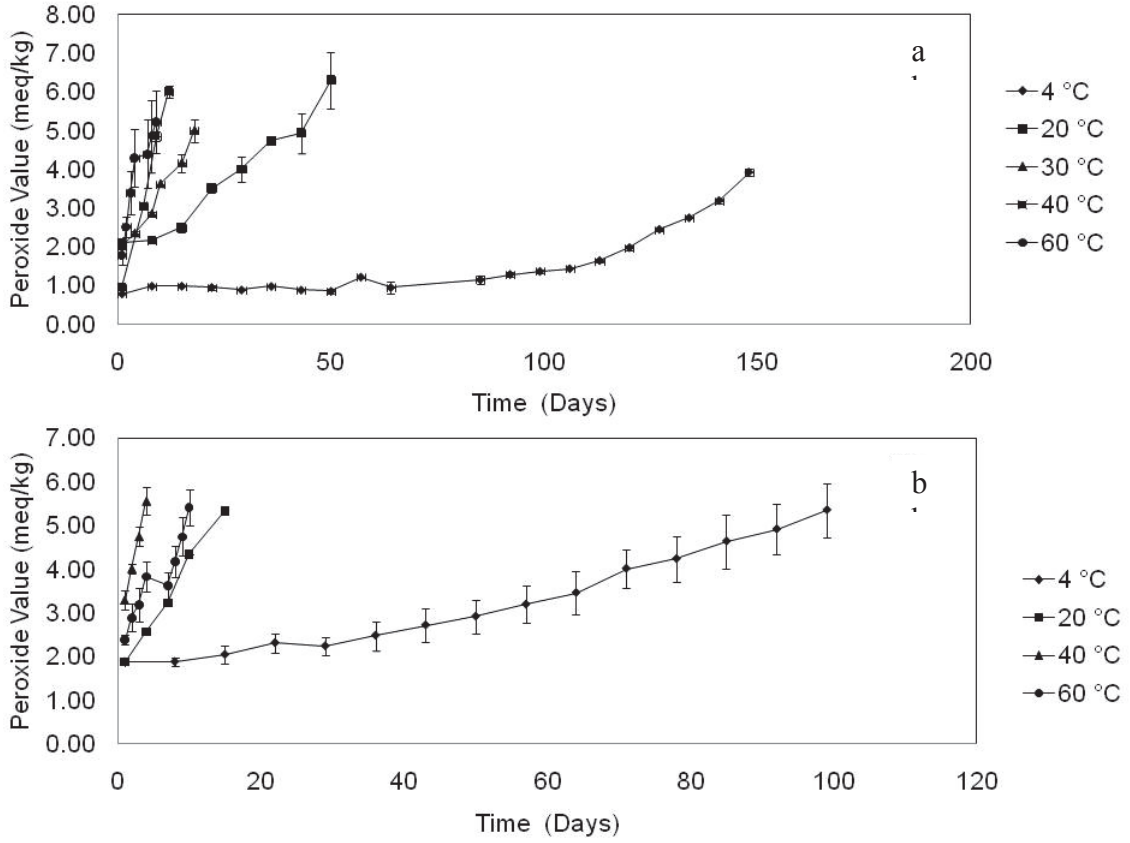

Figure 3.1 Change in hydroperoxide value (mean ± SD, n=3) at different temperatures over time. a) 18:12 oil and b) fish oil concentrate.

It should be noted that kinetics are often modeled by monitoring the consumption of reactants, while this study monitors the formation of oxidation products. Oxidation products have been used by a number of research groups including Labuza and Bergquist (1983), Mancebo Campos et al (2008), and Gomez-Alonso et al (2004) to successfully model oxidation kinetics.

Despite ensuring that the necessary conditions for pseudo-first order reactions were met, the reaction was obviously not first order for several of the sample-temperature combinations. For example, at 4 °C the 18:12 oil showed a clear lag in the onset of oxidation (Fig 2.1a) that did not fit the expected model. In fact, modeling with zero and second order kinetics did not show any improvement at that temperature. Interestingly, the concentrate oil did follow a first order model quite well at 4 °C (Fig 3.1b and Table 3.2), likely because of the absence of a prolonged lag phase.

Table 3.2 Testing temperatures, rate constants, and coefficient of determinations of Arrhenius plots for 18:12 oil and concentrate oil.

Temperature (K)	**18:12 oil**		**Concentrate oil**	
	Rate constant (k $days^{-1}$)	Std. Dev.	Rate constant (k $days^{-1}$)	Std. Dev.
277	0.009	0.000	0.011	0.001
293	0.023	0.003	0.075	0.000
303	0.053	0.003	N/A	N/A
313	0.170	0.006	0.174	0.008
333	0.111	0.008	0.073	0.007
$r^{2\,(a)}$	0.822	-	0.524	-
$r^{2\,(b)}$	0.981	-	0.938	-

(a) Includes 60 °C data. (b) Excludes 4 and 60 °C data (18:12) or 60 °C data (concentrate).

Both oils also deviated from the expected first-order model at 60 °C. This was less surprising as it is well accepted that the mechanisms for a complex chain reaction such as lipid oxidation may vary with increasing temperature (Frankel 2005). For the purpose of this study, it was assumed that peroxide derivatives of fatty acids do not degrade; however, a large variety of secondary oxidation products are formed from these compounds as oxidation progresses. It may be that the increase in temperature increased

the rate of both peroxide formation and breakdown; thus, the low rate constant derived at 60 °C for both oils suggests that peroxides are decomposing faster than they are being formed (Choe and Minn 2006, Frankel 2005). Additionally, at elevated temperatures, lipid oxidation is more dependent on the concentration of oxygen. At high temperatures, the solubility of oxygen decreases, and becomes a limiting factor in lipid oxidation reactions as oxygen is rapidly consumed (Frankel 2005).

First-order kinetics were expected because of the experimental design, but all data were also evaluated for fit to a zero- and second-order model (Table 3.3).

Table 3.3 Coefficients of determination for 18:12 and concentrate fish oils when zero, first and second-order models were considered.

18:12 Oil		**r^2**		
Temperature (°C)	**# of points**	**Zero- Order**	**First -Order**	**Second- Order**
277	20	0.6993	0.8142	0.8648
293	8	0.9596	0.9429	0.9703
303	6	0.9752	0.9801	0.9572
313	4	0.9941	0.9539	0.8546
333	7	0.8777	0.8027	0.7052
Concentrate Oil		**r^2**		
Temperature (°C)	**# of points**	**Zero- Order**	**First -Order**	**Second- Order**
277	15	0.9561	0.9876	0.9863
293	5	0.9429	0.9706	0.9105
313	4	0.9993	0.9969	0.9817
333	8	0.8812	0.8866	0.8603

For a zero-order model, regression of PV versus time is linear, while for second-order models, one expects plots of 1/PV versus time to be linear. Coefficients of determination were simply used to assess fit (Table 3.3). In most cases it is quite obvious that a first-

order model is as good as or better than other models (e.g. 18:12 oil at 20 and 30 °C). In other cases it is less obvious. For example, at both 40 and 60 °C for the 18:12 oil, a zero-order model has a slightly better fit to the data, while at 4 °C, the second order model has the best fit for the same oil. This is likely related to the high content of rapidly oxidizing PUFA that are present in fish oil. All trials were stopped when the upper level for acceptability for fish oil, PV = 5 meq/kg, so that the duration of the experiment grew shorter as temperature increased. With daily sampling, this meant that fewer data points were acquired as temperature increased, making it difficult to fully capture the change in oil quality with time. The situation reached an extreme at 40 °C with both oils only requiring 4 days to exceed the upper limit of acceptability. Had sampling continued beyond this time point, the change in slope that is expected with first-order kinetics may have been captured. As plotted here, it is likely that only a small linear portion of a larger curve is being shown. Because we were only trying to model kinetics until the quality limit was reached and first-order kinetics fit well for 20 and 30 °C, it seemed appropriate to continue to model with first-order kinetics at the other temperatures. In addition, we were very reluctant to fit the data to a zero-order model in any situation. Zero-order kinetics dictates that reaction rate is independent of substrate concentration. Though both oils contain the same amount of fatty acid structures, the concentrate sample contained more PUFA, or substrate, that could be oxidized. Rates were obviously higher in the concentrate oil (Fig 3.2, Table 3.2) so zero-order kinetics were ruled out immediately as an increase in PUFA increased the rate of peroxide formation. Finally, a first-order model was expected because others have found that oils containing antioxidants follow

such models (Labuza 1971). All this evidence pointed to the use of a first-order model when any ambiguity in model fit, as seen in Table 3.3, was encountered.

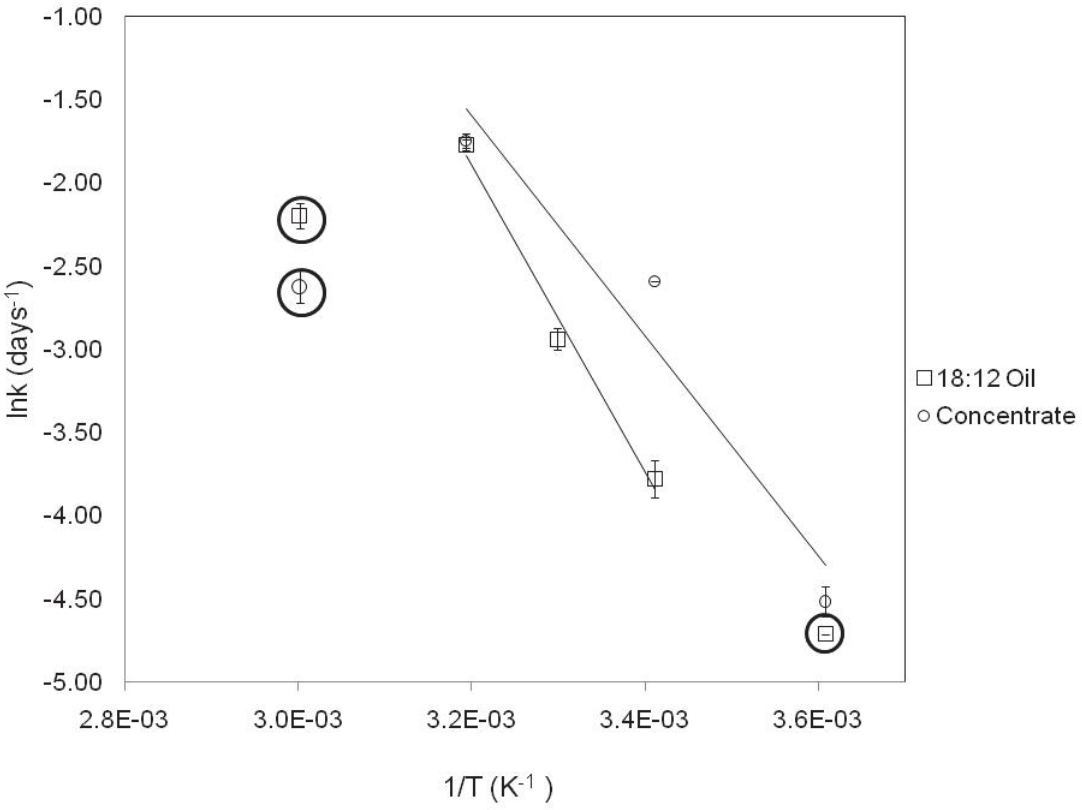

Figure 3.2 Arrhenius plots for 18:12 oil (R^2 0.9871) and concentrate oil (R^2 0.9384). Circled points are not included in regression. Data are mean ± SD.

3.5.3 Arrhenius Behavior and Shelf Life Prediction

Rate constants for both oils were lower at 60 °C than at 40 °C; this result, combined with their poor fit to the first-order model, led to their exclusion from the Arrhenius plot. Similarly, because of the obvious lag time for onset of oxidation of 18:12 oil at 4 °C, the data collected at this temperature was also omitted from the plot for 18:12 oil, leaving only 3 data points for each oil. Despite the low sample numbers, we still see a good fit for both oils, with differing slopes. From the slopes, E_a of oxidation for the

18:12 and concentrate oils were calculated as 76±9 and 55±14 kJ/mol, respectively. The lower E_a for the concentrate oil was expected because less energy should be required to initiate oxidation due to the higher PUFA content. If the 4 °C data point is included in the 18:12 analysis (data not shown), the slopes are virtually identical, giving a very similar and highly unlikely E_a. This further points to the appropriateness of omitting the 4 °C data point in the 18:12 set. The E_a determined here are similar to those reported by Labuza (1984) for lipid oxidation by free radical mechanisms (63-105 kJ\mol). With pure TAG, consisting of esterified DHA, Yoshii et al (2002) found similar E_a ranging from 77 to 97 kJ/mol, depending on the level of antioxidant added, and clearly showed that E_a increases with increased concentration of rosemary extract. With added antioxidants, both the fish oils examined here and those containing high levels of PUFA studied by Yoshii et al (2002), had E_a more similar to the stable vegetable oil from canola (Orlien et al 2006), pointing to the clear advantage of employing antioxidants to prevent oxidation.

The objective of this study was to determine the real time shelf life of these products by extrapolating from accelerated data. Data from experiments above 40 °C were therefore omitted from the shelf life plots (Fig 3.3).

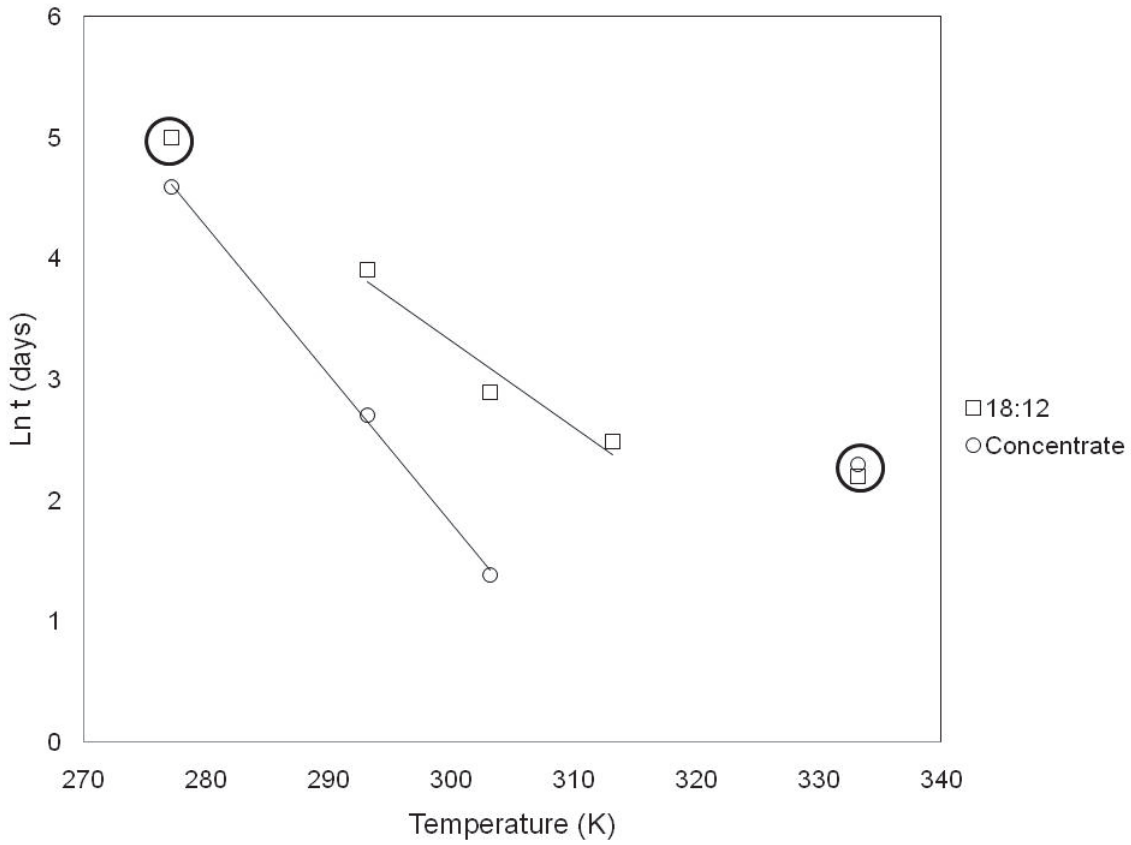

Figure 3.3 Shelf life plots for 18:12 and concentrate fish oils. Circled points are not included in regression.

This also agrees well with the recommendation by Frankel (2005) that the temperature used for accelerated fish oil stability studies should not exceed 40 °C. This has obvious implications for fish oil stability studies that involve the use of oxygen absorption methods at elevated temperatures such as such as the Rancimat and Oxidative Stability Index (OSI), which typically use temperatures > 100 °C (Méndez et al 1996, Luther et al 2007, Yu et al 2002). It is clear from the present study that PV of oils stored under Rancimat or OSI conditions will not be related to the PV of the same oils stored at temperatures less than 60 °C. Knowing that the data for 4 °C in the 18:12 oil did not follow first order kinetics also led to its omission from the shelf life plot. Without that

data, it is of course not possible to predict shelf life at temperatures < 20 °C and > 4 °C by interpolation. However, if the 4 °C data was included, the slope of the line would only change subtly so that the shelf life would be predicted at 143 days rather than the measured 148 days. This represents an error < 4% and when considering data between 4 and 20 °C, the impact would be proportionally and absolutely less. Thus, one may cautiously extrapolate beyond the linear portion of the curve for 18:12 oil, knowing that the predicted shelf life would be under-estimated by no more than 4%. It is important to note that these equations only hold true for these specific products. Oils that have different fatty acid or lipid class profiles or use different antioxidants will likely have different rates of oxidation.

This study was designed to mimic the oxidation that might take place after a consumer has purchased a bottle of fish oil and has begun consuming it. The shelf lives measured here for storage at 4 °C for both oils (148 and 99 days for 18:12 and concentrate, respectively) agrees well with typical manufacturers' recommendations of 90 days in refrigeration. An obvious contrast is with freshly bottled fish oils, purged with inert gas, usually nitrogen. Exclusion of air in such products promotes much longer-term oxidative stability and we would not expect the kinetics of oxidation to be similar to those reported here. Studies to monitor such products would be logistically difficult to organize simply because sealed containers of oil could only be sampled once; after opening, the sample would be in contact with air and, even if again purged with nitrogen, rates of oxidation would be expected to vary. Thus, a very large amount of individually bottled oil would be required.

The lack of kinetic data between 4 and 20 °C is the largest flaw in this study. Had we included at least one data point in this range, we would have been much better able to characterize both the Arrhenius behavior and shelf life prediction. This is critical because it would have set a lower limit on temperatures to which we could interpolate without introducing a known minimum error. It would also have been useful to examine other measures of oxidation. Frankel (1993) used volatile oxidation products to examine the kinetics of fish oil oxidation. Anisidine values are also recommended in the GOED Voluntary Monograph (2008) as a quality measure of fish oil. These tests could allow us to investigate the relationship between primary and secondary oxidation and could help to determine if hydroperoxides are in fact breaking down at 60 °C. A substantial increase in either anisidines or volatile oxidation products would support our hypothesis of peroxide breakdown. Additionally, monitoring secondary oxidation would allow for the creation of shelf life plots that could potentially correlate with sensory characteristics of the oils and give a better indication of how consumers will perceive the oils. However, both parameters are difficult to accurately measure in commercial oils that have added flavors. Our experience has shown that flavor compounds co-elute with oxidation products in gas chromatographic analysis of volatiles, especially when headspace analysis techniques are utilized. While selective ion monitoring could potentially be used to monitor oxidation in co-eluting peaks, these techniques are beyond the scope of this paper. Flavor compounds also interfere with the *p*-anisidine test, causing drastic over-estimation of the measure, sometimes outside the range of measurement. Thus, monitoring change in secondary kinetics with *p*-anisidine test would only be feasible in unflavored oils, which would not necessarily be relevant for shelf life studies of commercial dietary supplement.

3.6 CONCLUSIONS

Fish oil is a popular dietary supplement taken by many people for its health benefits. Because of the high PUFA content, the oil oxidizes rapidly. At temperatures ≥20 °C and ≤ 40 °C, 18:12 fish oil appears to follow first-order kinetics. Fish oil concentrate demonstrates first-order kinetics at temperatures ≥4 °C and ≤ 40 °C. At 60 °C concentrate oil oxidized more rapidly than at 40 °C, likely because of hydroperoxides breaking down faster than they could form. This confirms that accelerated stability studies using fish oil should be conducted at temperatures no higher than 40 °C. Accelerated temperature data can then be used to predict shelf-life at lower temperatures; however extrapolation of data should be done with caution as the rate of reaction may not hold true at low temperatures. This was the case for 18:12 oil as clearly demonstrated in this study.

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CHAPTER 4 FISH OIL SENSORY PROPERTIES CAN BE PREDICTED USING KEY OXIDATIVE VOLATILES

4.1 PUBLICATION INFORMATION

This paper is a modified version of a manuscript, reworked here for formatting consistency. Originally published as:

Sullivan JC, Budge SM (2012) Fish oil sensory properties can be predicted using key oxidative volatiles. Eur J Lipid Sci Techol DOI: 10.1002/ejlt.201100330. Suzanne Budge contributed to the study design and manuscript preparation. Jenna Sullivan Ritter was the principal investigator and lead author.

4.2 ABSTRACT

The high level of PUFA in fish oil, primarily EPA and DHA result in rapid oxidation of the oil. Current methods used to assess oxidation have little correlation with sensory properties of fish oils. Here we describe an alternative method using SPME combined with GCMS to monitor volatile oxidation products. Stepwise discriminant function analysis (DFA) was used to classify oils characterized as acceptable or unacceptable based on sensory analysis; a cross validated success rate of 100% was achieved with the function. The classification function was also successfully validated with tasted samples that were not used to create the method. A total of 14 variables, primarily aldehydes and ketones, were identified as significant discriminators in the classification function. This method will be useful as a quality control method for fish oil manufacturers.

4.3 INTRODUCTION

Fish oil is a rich source of the long chain omega-3 PUFA EPA and DHA. These FA have been shown to have numerous health benefits, including lowering blood pressure and reducing the risk of cardiovascular disease (e.g. Morris et al. 1993, Mozafarrian et al. 2008). This has led to a surge in the popularity of fish oil products as dietary supplements. Unfortunately, the high levels of PUFA cause fish oil to be highly prone to oxidation, which produces negative off-flavors and odors. These fishy flavors that form over time often discourage people from consuming the oils.

The most common way to assess oxidation in fish oil is through the measurement of hydroperoxides, the primary products of lipid oxidation. PV are a measure of the level of hydroperoxides and GOED sets a PV limit of 5 meq/kg for fish oils in their Voluntary Monograph for Omega-3 (2008). Hydroperoxides themselves have very little impact on oil flavor, but are precursors to the volatile secondary oxidation products that negatively impact sensory properties of fish oil. Frankel (2005) states that undesirable flavors can be detected in fish oils with PV of less than 1 meq/kg, as the unstable nature of hydroperoxides leads to rapid decomposition into secondary oxidation products. These secondary oxidation products are usually determined using AV, which reflect the content of aldehydes with α- and β-unsaturation. The AV limit set by GOED is 20. Unfortunately, this is not a sensitive method and there is some uncertainty that the specific components measured with this test are linked to oil flavor (Frankel 2005). It is well documented that both of these measures of oxidation have little relationship to the sensory properties of fish oil (Frankel 2005, Jacobsen 1999). The poor relationship

between conventional oxidation testing and sensory parameters draws attention to the need for an alternative method to monitor oil quality.

The most accurate way to evaluate sensory qualities of oils is to use a taste panel, as humans can be trained to detect low levels of volatile components that traditional tests of oxidation cannot. Unfortunately, these panels are expensive to establish and maintain which makes their use unattractive. An alternative to a sensory panel is the monitoring of the amounts of volatile oxidation products in the headspace of samples, as these are the compounds most responsible for oil flavors. SPME, coupled with GCMS, is often used to determine the levels of volatile components in fish oils (Lee et al. 2003, Guillén et al. 2009, Serfert et al. 2010). The types and number of volatiles detected depends on experimental methods and materials, including oil type, SPME fiber type, extraction temperatures and times, etc.

The aim of this study, therefore, is to compare sensory panel assessment of quality with levels of oxidation products determined using PV, AV and headspace analysis of fish oil. The goal was to identify the key oxidation products that are important in distinguishing between acceptable and poor quality oils in order to create a method to monitor fish oil oxidation that correlates well with the sensory characteristics of the oil.

4.4 Experimental Procedures

4.4.1 Materials

SPME fibers (divinylbenzene/Carboxen/polydimethylsiloxane, 50/30 um coating), a SPME fiber holder for manual sampling, 22 ml glass vials, polytetrafluroethylene/silicone rubber septa and phenolic screw caps were purchased

from Supelco (Oakville, Canada). A custom-made heating block designed to accommodate 22 ml glass vials was used to control temperature. Fish oil containing a blend of mixed natural tocopherols was obtained from Ocean Nutrition Canada Ltd. (Dartmouth, Canada). Optima chloroform was obtained from VWR (Mississauga, Canada). Methyl tricosonate, methyl eicosapentaenoate and methyl docosahexaenoate were purchased from Nu-Chek Prep (Elysian, USA). An Isotemp 100 Series Model 126G oven was used to incubate samples and was obtained from Fisher Scientific (Ottawa, Canada), along with all other chemicals and glassware.

4.4.2 Experimental Design

Amber bottles (250 ml) were filled with 200 ml of fish oil each and placed, uncapped, in an oven held at 40°C. Oils were removed from the oven at varying time intervals, ranging from 2 to 15 hours, over the course of 14 days, with a new bottle being used at each sampling point. Aliquots of 14.0 ml of each sample were placed in 22 ml glass vials and then capped with phenolic screw caps with PTFE/silicone septa and analyzed with SPME-GCMS. Approximately 15 ml of each sample was removed for PV and AV testing, and the remainder of each 200 ml sample was either stored at -80 °C under nitrogen or evaluated by a sensory panel. One sample per 24 hour time period underwent PV and AV analysis and was tasted by a sensory panel. All samples were analyzed by SPME-GCMS. The experiment was conducted in triplicate.

4.4.3 Measures of Oil Quality

The FA profile of the fish oil was analyzed using the method described by Sullivan et al (2009). The PV and AV of each sample was measured in triplicate

following AOCS Official Method Cd 8-53 (1997) and AOCS Official Method Cd-18-90 (1997), respectively.

The SPME method was adapted from Lee et al. (2003). SPME samples were placed in a heating block set to 80°C and allowed to equilibrate for exactly 15.0 min. Meanwhile, the SPME fiber was placed in the injector port of the GC at 250 °C to desorb any volatiles that had accumulated during storage or between samples. After the equilibration period, the SPME fiber was inserted into the vial at a depth of 2 cm and exposed to the sample headspace for exactly 45.0 min. Extracted volatiles were analyzed by ion trap GC-MS in electron ionization mode (200 °C). The fiber was inserted into the injector of the GC to a depth of 5 cm (splitless mode, 250°C; 1 mm liner) and left for 5 min. Volatile analytes were separated on a free fatty acid phase column (30 m x 0.25 mm x 0.25 um film thickness, Agilent, Mississauga, Canada). Helium was used as a carrier gas (1.0 ml/min). The oven temperature was initially held at 40°C for 5 min and increased at a rate of 10°C/min to 250°C and held for 5 min (total run time 29 min). Data were acquired as area counts and converted to area percent.

4.4.4 Sensory Assessment of Oil Quality

An untrained, descriptive sensory panel was used to assess samples for quality (Warner 1995). Between ten and twelve samples from each replicate were tasted by the sensory panel. Potential panelists were initially screened to eliminate those volunteers who could not distinguish fishy flavors by challenging them with a triangle test, consisting of acceptable, fresh fish oil with a low volatile content and fish oil that had been incubated at elevated temperatures to force the formation of fishy volatiles. Those who could not distinguish between the fishy sample and the fresh sample were removed

from the panel. The final panel consisted of 10 volunteers. Each panelist was asked to rank samples on a scale of 10, with 10 being "excellent" and 1 being "terrible" and to rank fishy flavors, using a fresh vegetable oil sample as a reference. These classifications were then used to group samples into acceptable and unacceptable categories. Each sample evaluated by the final panel was tasted by a minimum of three people. Design and training of the sensory panel as well as the score sheets used to evaluate the samples were adapted from guidelines provided by the American Oil Chemists Society (1995). Of the 75 samples analyzed with SPME-GCMS, 31 were evaluated for sensory characteristics, leaving approximately 59% untasted.

4.4.5 Statistical Analysis

Sensory panel results and peak area percent data from GCMS analysis of volatiles were analyzed using SPSS 11.0 (SPSS Inc. Chicago, Ill) statistical software to perform forward stepwise DFA. This created a function to classify samples as acceptable or unacceptable and also identified the volatiles most useful for oil classification based on sensory characteristics. To improve normality, data were first transformed using a geometric mean function (Aitchison 1983), following Equation 1:

$$x_n = ln \frac{a_i}{\sqrt[n]{a_1 a_2 \cdots a_n}} \qquad (1)$$

where a_i is the proportion in area percent of each peak identified in the sample. To ensure that the data set was concise, a filtering step was conducted where correlations between the three replicates were examined for each peak. Peaks that had strong negative correlations to each other were removed from the analysis, because they could not be

responsible for differences between acceptable and unacceptable samples if they were not varying in a consistent manner among replicate experiments. These peaks were attributed to noise. The reduced dataset, made up of 78 oxidative volatiles, was combined with sensory classifications to create a discriminant function (DF), using an F-value for entry into the function of 3.84 and a value for removal of 2.71, corresponding to a *p*-value of 0.05 Stepwise DFA was essential in this study as a function that uses all the 78 of oxidative volatiles present in the reduced data set for these fish oil samples would be difficult and impractical to apply. The classification method was created using the proportion of volatiles as the independent variable and sensory assessment (acceptable or unacceptable) as the grouping variables for 28 fish oil samples that underwent sensory testing. Leave-one-out cross-validation of the data was used to assess the accuracy of classification with the DF. As a further test of accuracy, the DF was applied to three tasted samples, one from each replicate, that were excluded from the data set when the DF was created. This corresponds to 10% of the tasted samples. Last, the samples that were not tasted were classified using the DF. The variables selected by DFA as being useful to discriminate between acceptable and unacceptable samples were also identified.

4.4.6 Peak Identification

The peaks recognized by DFA as being important discriminators between acceptable and unacceptable samples were identified using mass spectra library matches (National Institute for Standard Technologies), potential fragmentation patterns as predicted by HighChem Mass Frontier 4.0 (HighChem Ltd. Bratislava, Slovakia) and external standards, where possible.

4.5 Results and Discussion

4.5.1 Fatty Acid Analysis

The levels of EPA and DHA in the fish oil tested were found to be 166 ± 1 mg/g and 125.4 ± 0.5 mg/g respectively. These results are typical of commercial fish oils.

4.5.2 Hydroperoxide and Anisidine Value Testing

PV increased for the duration of the study (Fig 4.1a). The initial PV for all three experiments was quite low (average 1.0 ± 0 meq/ kg), indicating that it was initially only slightly oxidized, and well below the GOED limit of 5 meq/kg. This limit was exceeded in ≤ 48 hours for all experiments, with the highest PV of 28.1 meq/kg reached after ~312 hours. The AV for all three experiments started at an acceptable level (average 9.6 ± 0.4) and increased over time, though not as rapidly as PV (Fig 4.1b). None of the three trials exceeded the GOED limit of 20 for AV during the study. The PV and AV for replicate II increased more rapidly than replicates I and III, though the general trend of all studies was the same. Despite the more rapid increase in PV and AV for replicate II, sensory ratings agreed with the other two replicates. It is likely that slight variations in incubation and ambient temperatures, or differences in the relative humidity during incubation resulted in differences between experiments. Interestingly, the samples were not rejected by the sensory panel until the PV reached an average of 11.9 ± 3.5 meq/kg, more than double the 5 meq/kg maximum for PV set by the GOED (2008). The average AV when samples were rejected was 11.6 ± 1.4, which is lower than the GOED maximum of 20. Inconsistencies between PV and AV results and sensory scores have been found in other studies. For instance, MacFarlane et al. (2001) found that oil samples with acceptable PV and AV results were deemed to be fishy and unacceptable. Similarly, little correlation was found between PV, AV and sensory testing in fish oil-enriched mayonnaise and spreads (Jacobsen 1999), with samples with high AV often having high levels of

acceptability in sensory testing. In the present study PV and AV values at the point of sensory rejection clearly indicate that current guidelines for fish oil quality do not correlate well with sensory properties.

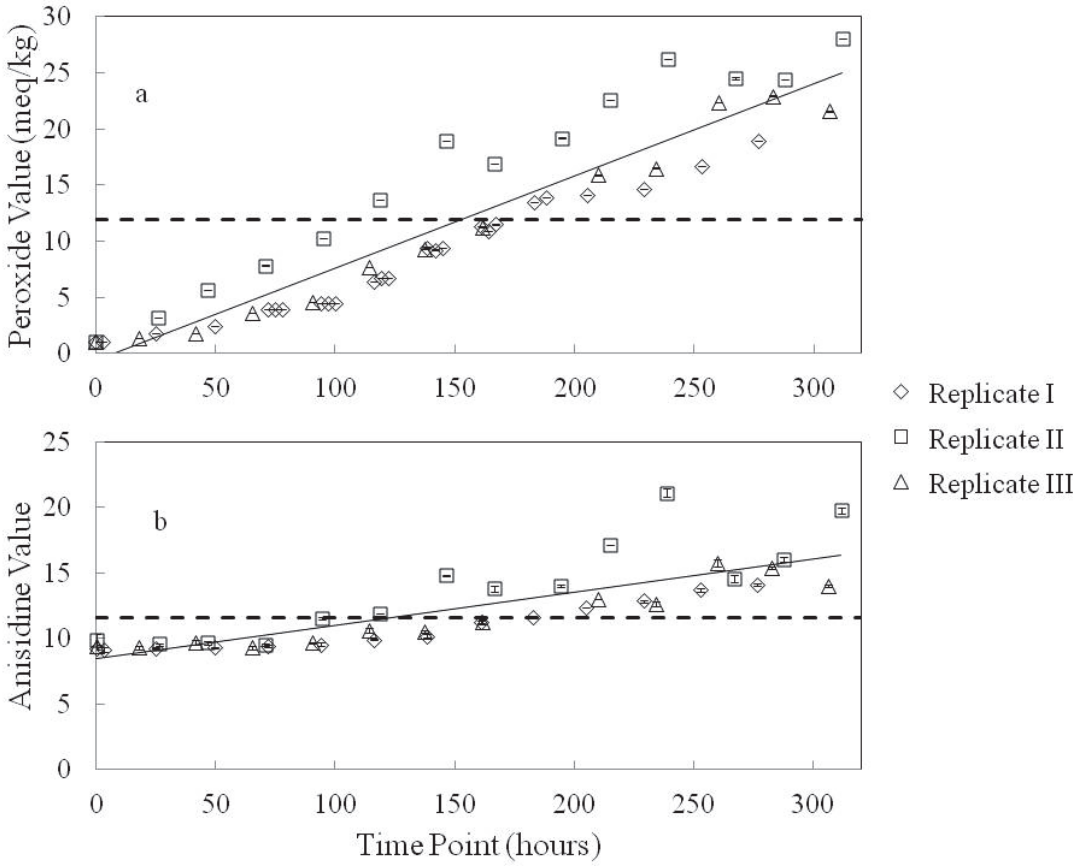

Figure 4.1 Variation in (a) peroxide and (b) anisidine (mean ± SD) values of fish oil over time. The level at which samples were rejected by the sensory panel are indicated by the dashed line.

4.5.3 Classification by DFA

DFA was chosen for statistical analysis because it classifies samples into different categories using descriptors selected by the researcher. This allowed tasted fish oil samples to be used to create a method that would identify the key differences in oxidative volatiles between acceptable and unacceptable samples. Because of the large number of volatile oxidation products, stepwise DFA was used, as this method uses only those

variables which differ significantly between categories to create the DF, rather than using all variables in the classification scheme. This was an important consideration because the goals of this study were to develop a method where levels of only a small number of oxidation products must be determined to assess oxidation, and to identify what these key volatiles are. Stepwise DFA identifies the components important to classification and creates a function that can easily be applied for classification of future samples. The DFA method yielded 100% correct classification of all samples used to build the method. Similarly, leave-one-out cross-validation resulted in perfect classification of all samples. When applied to the three tasted samples excluded from the model, all were classified correctly, giving confidence that the method will hold for other, untasted samples.

The group centroids for the DF were found to be 11.86 for acceptable samples and -11.86 for unacceptable samples, with a sample classified as unacceptable when the DF score was <0 and acceptable when >0. The variables and coefficients that make up the DF can be seen in Table 4.1.

The distribution of DF scores for all samples were compared by calculating the squared Mahalonobis distance of each score from the group centroid of acceptable or unacceptable classifications. The smaller the distance to the centroid, the more likely a sample belonged to that group. When the DF was applied to the tasted samples it was clear from the small squared Mahalonobis distances (Fig 4.2a) for most samples that the DF was likely successful at classifying samples. There were, however, some untasted samples that did not fit well with either the acceptable or unacceptable classification, evident in their great distances from the group centroid (Fig 4.2b). These samples were predominantly taken near the midpoint of the time courses, where the oils were

experiencing a transition from relatively unoxidized to more rancid conditions and therefore would not necessarily fit into the simple classification scheme of acceptable and unacceptable.

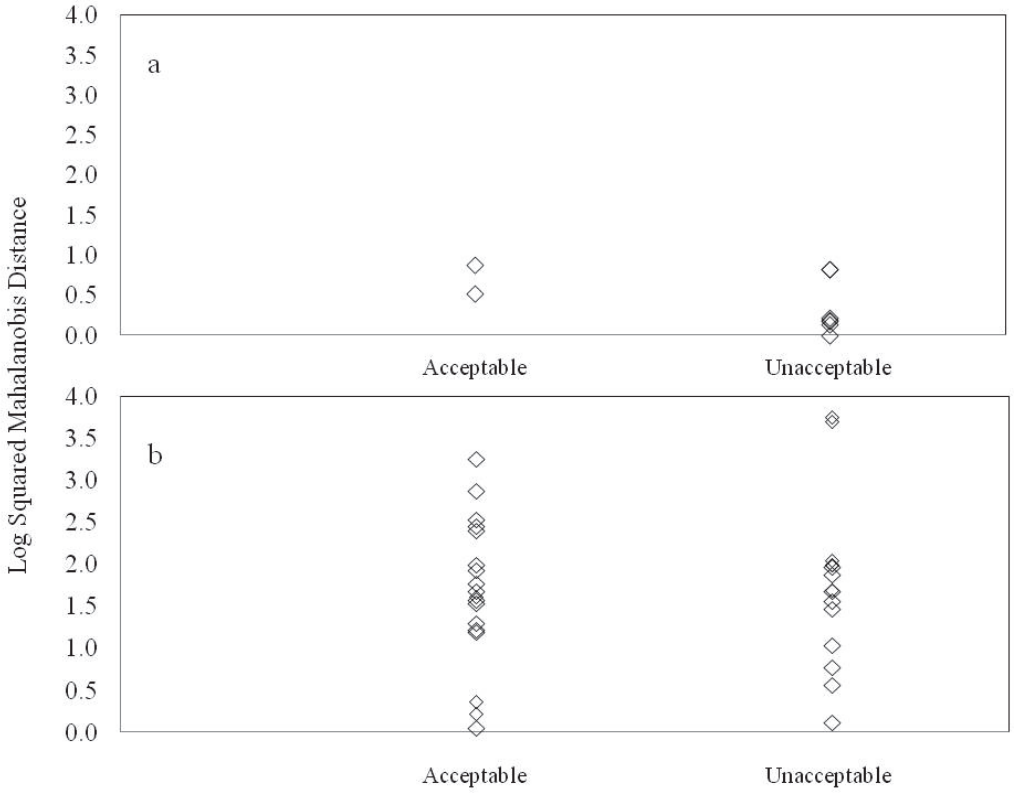

Figure 4.2 Log of the squared Mahalanobis distance from the centroid for a) tasted and b) untasted samples of fish oil.

4.5.4 Compounds Important in Classification

The function created using DFA identified the variables, in this case the volatile oxidation products, useful in differentiating between acceptable and unacceptable samples. A total of 14 oxidation products were identified as contributing to differences between acceptable and unacceptable samples (Table 4.1, Fig 4.3). The majority of the compounds increased in proportion over time, but the direction of proportion change was not necessarily indicative of the type of effect the volatile had on the sensory properties of oil. The DF coefficients better indicated those effects, for instance, if a volatile is

increasing in proportion over time, and has a coefficient >0, it drives the DF in a positive direction, closer to 11.86, and therefore results in an acceptable sensory classification. A volatile that increases in proportion but has a DF coefficient <0 would drive the DF score to more negative values, and closer to the group centroid for unacceptable classification. Table 4.1 lists the identities of these compounds, their DF coefficients and the direction of their proportion changes. The majority of the compounds identified were aldehydes, ketones and alcohols. In addition, a hydrocarbon, acid, benzene derivative and ethyl ester were also identified. Many of these structures have been previously reported in fish oil.

Table 4.1 Standardized canonical DF coefficients of oxidative volatiles used to differentiate between acceptable and unacceptable fish oils.

Volatile	**Standardized Canonical DF Coefficient**	**Direction of Proportion Change**	**Method of Identification**
2-butanone	14.47	Decrease	a, b
1,3,5-octatriene	-7.52	Increase	b, c
4,6,8-nonatrien-3-one	-10.38	Decrease	c
3-hexen-1-ol	-25.34	Decrease	a, b
3,6-nonadienal	13.74	Increase	a, c
(*E,E*)-2,4-heptadienal	-7.74	Increase	a, b
(*E,Z*) 2,4-octadienal	6.79	Increase	a, b
(*E,E)* 2,4-octadienal	12.34	Increase	a, b
Nonatrienone (undetermined isomer)	-4.14	Increase	c
2,4-heptadien-1-ol	-3.10	Increase	a, b
2-decanol	6.59	Increase	a, c
Ethyl benzaldehyde	21.92	Decrease	c
Tetradecanoic acid ethyl ester	2.26	Decrease	a

Volatile	Standardized Canonical DF Coefficient	Direction of Proportion Change	Method of Identification
Octanoic acid	-7.89	Decrease	a, b

a- Retention time and MS fragmentation pattern compared with an external standard. b- NIST library match. c- probable ion fragmentation predicted using Mass Frontier 4.0.

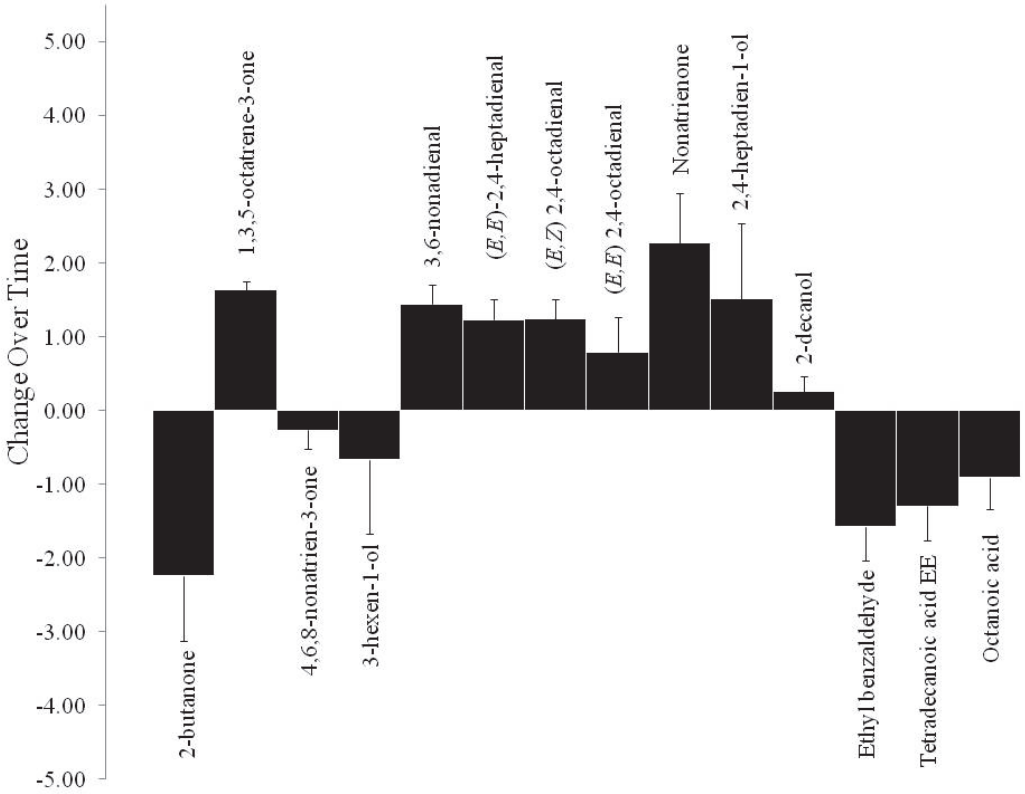

Figure 4.3 Change in levels of volatiles (mean ± SD) identified as significant in the classification function over the duration of the study. Data were transformed following $x_n = ln \frac{a_i}{\sqrt[n]{a_1 a_2 \ldots a_n}}$

Aldehydes are known to have an effect on the sensory quality of fish oil. All aliphatic aldehydes identified in this study as important indicators of sensory properties

increased over time. (*E,E*)-2,4-heptadienal was one such compound and it has previously been found in oxidized fish oil (Jacobsen 1999, Lee 2003, Venkateshwarlu et al. 2004). Its isomer, (*E,Z*)-2,4-heptadienal, has been associated with oxidized fish oil flavors (Serfert et al. 2010, Venkateshwarlu et al. 2004) and was also identified in this study. However, here it was found to have little correlation with off-flavors, despite a dramatic increase in proportion over the course of the study, demonstrating that oxidation products present in high proportions may not necessarily be most useful in predicting sensory qualities of oil. Both isomers of 2,4-heptadienal have been linked to general rancid and painty flavor in oils (Venkateshwarlu et al. 2004, Karahadian and Lindsey 1999), though Hartvigsen et al. (2000) found that (*E,Z*)-2,4-heptadienal had a distinctively fishy odor. (*E,E*) and (*E,Z*)-2,4-octadienal were also identified here as useful in classification of fish oil. 2,4-octadienal has been found in menhaden oil previously by Hseish et al. (1989), though the geometric isomer was not specified. Hartvigsen et al.(2000) identified both the (*E,E*) and (*E,Z*) isomers in fish oil-enriched mayonnaise using GC-MS combined with GC-Olfactory and found that the (*E,E*) isomer had a deep-fried odor while the (*E,Z*) isomer was associated with green odors. Interestingly, both isomers of 2,4-octadienal were found to increase over time and had a positive DF coefficient, suggesting that they had a positive impact on sensory qualities over the course of the experiment as their proportion increased. The final aliphatic aldehyde that was found to play a role in the sensory properties of fish oil, 3,6-nonadienal, is a decomposition product of the 11- and 12- hydroperoxide derivitatives of EPA and has been associated with a green flavor (Frankel 2005). Like the octadienal isomers, its level increased with oxidation and it had a positive DF coefficient, suggesting a positive influence on sensory characterization.

3,6-nonadienal is a key oxidation product used by MacFarlane et al. (2001) to predict the quality of fish oil samples, as it could be detected in oxidized oil. However, our results and the association with 3,6-nonadienal with a green flavor suggest that this compound may not be contributing to a fishy flavor.

Alcohols are formed from primary oxidation products and also influence the sensory parameters of oxidized fish oils, though their taste and odor thresholds are so high that they are often disregarded (Ho et al. 1996). 3-hexen-1-ol, identified in this study as being important in oil classification, had been identified previously by Karahadian and Lindsay (1989), who believed that this compound contributed to a green flavor in the presence of nonadienal; it had no sensory impact on its own. We also found 2,4-heptadien-1-ol useful in differentiating between acceptable and unacceptable fish oil samples. This is the first report of it in oxidized fish oil. Similarly 2-decanol has not been found in other oxidized fish oils but was useful in oil classification. Other studies may have failed to identify this alcohol because of column and temperature program differences that resulted in either co-elution of compounds, or a temperature program that was insufficient to elute this alcohol. Both 2,4-heptadien-1-ol and 2-decanol had DF coefficients <0, suggesting that they have negative impacts on sensory characteristics.

An interesting alkene, 1,3,5-octatriene, was linked to oxidation and is described as smelling like solvent or plastic by Hailler et al. (2004). Unspecified isomers of octatriene were identified by Kulås et al. (2002) in purified fish oil and Guillén (2009) in cod liver oil. 1,3,5-octatriene was also identified in fish oil-enriched mayonnaise by Hartvigsen et al. (2000) and in various fish oils by Giogios (2009). These authors, along

with Karahadian and Lindsay (1989) also identified another isomer, 1,3,6-ocatriene, in oxidized oil, though it was not detected in the present study.

Karahadian and Lindsay (1989) identified octanoic acid as a fish oil oxidation product, and it was found in this study as well. In this case, the acid decreased in proportion over time, suggesting it was present in the oil at the start of the experiment. It is also possible that octanoic acid was a decomposition product of a fatty acid or another oxidation product that was initially present in small amounts. If that was the case, then as the precursor to this acid dissipated, the levels of octanoic acid would also decrease. Octanoic acid is generally associated with a goaty, musty smell, agreeing with its negative DF coefficient, and pointing to poor sensory qualities. Other studies have found shorter chain acids, including propanoic, butanoic and hexanoic acid (Hseih et al. 1989, Kulås et al. 2002). These acids were also present in the oil used in this study but did not play a significant role in differentiating between acceptable and unacceptable samples.

Three ketones were recognized for influencing sensory quality of oils. 2-butanone, which has a buttery taste, was an early-eluting ketone that decreased over time. This compound had a DF coefficient >0, indicating it positively influences sensory characteristics, so as this compound decreased in proportion, the sensory quality of the oil also decreased. It has been identified in a number of studies as a fish oil oxidation product (Guillén et al. 2009, Venkateshwarlu et al. 2004, Kulås et al. 2002, Giogios et al. 2009). 4,6,8-nonatrien-3-one and an undetermined nonatrienone isomer were also deemed to be important in differentiating between acceptable and unacceptable oils. Neither of these ketones has been identified in fish oil studies previously but they are both probable lipid oxidation products.

Ethyl benzaldehyde was found to be useful for classification of fish oils, and decreased in proportion over time. Its DF coefficient was >0, suggesting that its positive impact on sensory characteristics decreased as oxidation progressed. It has not been previously identified as a fish oil oxidation product but other authors have found benzene derivatives, such as ethyl benzene (Guillén et al. 2009, Hartvigsen et al. 2000, Giogios 2009). The decrease in proportion of this compound with time, along with its chemical structure, suggests that it is not formed as a result of lipid oxidation but instead was present in the oil initially and degraded over the course of the experiment. Though benzene itself is unlikely to be formed during lipid oxidation, it is possible that ethyl benzaldehyde was generated through decomposition of a larger benzene derivative that was initially present in the oil. Giogios et al. (2009) speculated that benzene compounds could result from degradation of sugars or amino acids, explaining their small proportions in fish oils. Though not important to sensory parameters, xylene, another benzene derivative, was found in this study and in studies of fish oil-enriched mayonnaise (Hartvigsen et al. 2000, Jacobsen et al. 1999, Jacobsen et al. 2000). Jacobsen et al. (1999, 2000) attributed xylene formation to the presence of other ingredients, but the current study shows that benzene-containing compounds, including xylenes, exist in fish oil when only lipid ingredients are present.

The final compound of interest in distinguishing between acceptable and unacceptable oils was tetradecanoic acid EE. This compound decreased over time, likely because it is a component formed during fish oil manufacturing rather than via oxidation. Most commercially available fish oils undergo base-catalysed transesterification, creating EE. These EE are fractionated to manipulate the fatty acid profile of the fish oil, then re-

esterified into triglycerols (Breivik 2007). This final step often leaves traces of EE in the oil, and fish oil manufacturers usually specify a maximum of 3% EE in their triglycerol oils for this reason (Sullivan et al. 2009). The unstable nature of EE results in rapid oxidation and may explain the decrease in this compound over time.

The majority of compounds identified by DFA as being important in the classification scheme were present only in low proportions, and did not vary greatly in proportion over time. There were six oxidation products that decreased over time: 3-hexen-1-ol, ethyl benzaldehyde, 2-butanone, 4,6,8-nonatrien-3-one, octanoic acid and tetradecanoic acid EE. It is possible that these compounds were decomposing into other oxidation products that were then detected by sensory analysis. This is most likely with 3-hexen-1-ol and tetradecaonic acid EE as they had the largest proportions of the four volatiles and were therefore more likely to decompose into other oxidation products that might be present in sufficient proportions to influence the flavor profile of the fish oil. However, even though decomposition products of other volatiles are often present at low levels, it is the compounds present in the smallest proportions that seem to be the most important variables in sensory quality. The volatiles present in larger amounts, including in this case (*E,Z*)-2,4 heptadienal, were not found to play an important role in oil quality. It is possible that some oxidation products have little contribution to sensory properties on their own, but when combined with other volatiles, have a large impact as a result of a synergistic relationship. The potential relationship between 3-hexen-1-ol and nonadienal is an example. These types of relationships highlight the importance of monitoring as many volatiles as possible when attempting to describe off-flavors in oils.

The individual volatiles identified in fish oil and fish oil-enriched foods are consistent from study to study, though those deemed to have the greatest impact on sensory characteristics vary. MacFarlane et al. (2001) developed a method to monitor fish oil quality using dynamic headspace sampling, known as the Fatty Acid Smell and Taste (FAST) Index. This method monitors levels of 4-heptenal, 2,6-nonadienal and 3,6-nonadienal, all of which are products of EPA oxidation, and uses a simple mathematical formula generated from principal component analysis of sensory data to give oil samples a numerical rating of fishy taste. While this method can be very useful, it does not take into account the possibility that volatiles derived from fatty acids other than EPA are also likely to play a role in the formation of off-flavors in fish oil. By limiting the method to only three oxidation products, all of which come from EPA, this method may be missing other potent volatile oxidation products derived from other fatty acids that could be linked to oils with poor sensory properties. The authors also note that dynamic headspace method for the FAST Index test is time consuming and difficult to perform.

While studies relating fish oil oxidation products to sensory perceptions are rare, there are numerous studies that have attempted to associate sensory properties of fish oil-enriched food products with levels of volatile oxidation products. For instance, Jimenez-Alvarez et al. (2008) attempted to correlate the proportions of volatiles in cod liver oil-enriched milk with sensory testing through the use of a triangle test where participants were asked to smell emulsion samples, some of which were spiked with (*Z*)-2-hexenal and (*E*)-4-heptenal, the oxidation products they had identified as having the largest increases in proportion over time. Only half of the panelists could differentiate between spiked and unspiked samples, and no panelists associated (*Z*)-2-hexenal or (*E*)-4-heptenal

with lipid oxidation descriptors, highlighting the importance of screening sensory panelists for their ability to taste certain indicators. That study (Jimenez-Alvarez et al. 2008) also illustrates that it is not necessarily the oxidation products with the greatest increase in proportion over time that are important to sensory properties of oils. The sensory threshold of oxidation products varies amongst compounds (Frankel 2005), so it is also probable that some oxidation products that show large increases have very high sensory thresholds and thus cannot be tasted until levels are very high.

4.5.5 Applications

The method described here was originally designed as a tool in product development to test the efficacy of new antioxidants. Rather than using chemical tests that are not correlated with sensory properties of oil, this procedure will provide results that directly relate to the taste and odor of fish oil. Ideally, this method will substantially reduce pricey, subjective sensory panels but still provide the same type of information. In an industrial setting, this method could be used to monitor the quality of raw materials or finished products. However, this technique was developed using unflavored fish oil and has not been validated with flavored oil; our experience has shown that the compounds added to mask fishy flavors overload the SPME fiber, resulting in detection of only those compounds. This may limit its use as a method in evaluating finished products. Further, the oil used in the development and testing of this method had a specific fatty acid profile, roughly 30% EPA and DHA. Other oils such as fish oil concentrates with different fatty acid profiles, fish oil emulsions or complex fish oil products have not yet been tested. It is uncertain if the same volatiles will be linked to flavor degradation in fish oils with differing fatty acid profiles.

Currently, to use the technique developed here, data must be collected for all volatile peaks as area percent data from chromatograms. It was essential to monitor all volatiles during the construction of this method as there were uncertainties as to which volatiles might influence sensory properties of the oils. With the volatiles known to be important to classification selected, it may not be necessary to collect information on all oxidation products. Thus, this method may be easier to implement if an internal standard were used so that ratios of selected peaks relative to the internal standard could be compared, allowing only data for the significant peaks to be collected.

4.6 CONCLUSIONS

In conclusion**,** the technique described in this study identifies oxidative volatiles that can be used to distinguish between acceptable and unacceptable fish oils, as an alternative to sensory evaluation. DFA was used to classify fish oil samples and to identify volatile oxidation products that were important to classification. The DF scheme was validated on tasted samples that were not used to build the DF. The volatiles identified are primarily aldehydes and ketones, though other classes of compounds were also found to be important. The relationship between oxidation product proportion and sensory characteristics is not straightforward, and the DF coefficient of the compound must be taken into consideration when examining the relationship between a compound and sensory quality. Volatiles with DF coefficients >0 had a positive impact on sensory characteristics, while those with coefficients <0 negatively affected sensory perceptions. This method may be useful as a quality control method in industrial fish oil operations. Future research will attempt to apply this method to other fish oils and fish oil emulsions.

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CHAPTER 5 KEY LIPID OXIDATION PRODUCTS CAN BE USED TO PREDICT SENSORY QUALITY OF FISH OILS WITH DIFFERENT LEVELS OF EPA AND DHA

5.1 Publication Information

This chapter is a modified version of a manuscript submitted for publication for publication in Lipids on May 15, 2012. It has been reworked here for formatting and consistency. Suzanne Budge contributed to the study design and manuscript preparation. Jenna Sullivan Ritter was the principal investigator and lead author.

5.2 Abstract

Despite its many health benefits, many consumers avoid fish oil supplements due to fishy tastes and odors. Common chemical measures of oxidation have little correlation with sensory properties, making it difficult to determine the sensory quality of fish oil without the use of an expensive sensory panel. Here we investigate an alternative method to assess oxidation using SPME and GCMS. Fish oils containing different amounts of EPA and DHA were oxidized, and headspace volatiles were monitored over time and compared to sensory evaluations by a taste panel. Peroxide value and anisidine value were also measured. Sensory panel scores and headspace volatile data were analyzed using principal component analysis (PCA) and linear regression to identify key volatiles responsible for changes in sensory degradation of oils over time. A total of eight compounds were identified, primarily aldehydes and ketones. By monitoring these volatiles, it may be possible to create a simple method to assess oxidation in fish oils that correlates well with sensory properties of the oil without the use of a sensory panel.

5.3 INTRODUCTION

The strong link between the use of fish oil supplements and decreases in cardiovascular disease has resulted in a strong demand for fish oil supplements. However, the fishy flavor often found in fish oil can dissuade people from consuming them. The long chain PUFA found in fish oil, specifically EPA (20:5n-3) and DHA (22:6n-3), oxidize rapidly due to the large number of double bonds they contain producing negative off-flavors. It follows that oils with higher concentrations of these PUFA oxidize more rapidly than those with low concentrations.

Fish oil oxidation is commonly assessed by measuring levels of hydroperoxides, in oils. GOED specifies in their Voluntary Monograph that the PV of fish oils should be < 5 meq/kg (GOED 2008). Hydroperoxides are primary oxidation products that have little effect on the sensory properties of fish oil as they are inherently unstable and rapidly degrade into volatile secondary oxidation products that are responsible for off-flavors in fish oils. Thus, it is possible to have oils with very low PV that taste very rancid. Secondary oxidation products are primarily assessed through the use of AV for which GOED specifies a limit of 20 (GOED 2008). AV reflects the level of aldehydes with α- and β-unsaturation. There is very little evidence that AV and sensory properties are correlated; it may be that the sensitivity of this method is not high enough to detect minute changes in aldehyde concentrations that could affect sensory properties (Frankel 2005). The poor relationship between PV, AV and sensory properties highlights the need for new methods to assess oil quality that correlate well with sensory parameters.

Sensory panels are the most accurate method for assessing fish oil quality as humans are capable of tasting certain compounds at concentrations much lower than

chemical techniques are able to detect (Frankel 2005). Unfortunately, sensory panels can be cost prohibitive due to the expenses associated with their set up and maintenance. An alternative to sensory panels is the use of SPME, coupled with GCMS, which can be used to monitor the presence of volatile secondary oxidation products in the headspace of fish oil. These volatile compounds are responsible for the off flavors and odors caused by oxidation.

The aim of this study is to compare the results of sensory panel assessment of three fish oils with different concentrations of EPA and DHA with PV, AV and proportions of volatile oxidation products. The goal is to create a robust method that can be used to predict sensory quality of fish oil and to identify key oxidative volatiles associated with those sensory properties.

5.4 Materials and Methods

5.4.1 Materials

SPME fibers (divinylbenzene/Carboxen/polydimethylsiloxane, 50/30 um coating), a SPME fiber holder for manual sampling, 22 ml glass vials, polytetrafluoroethylene/silicone rubber septa and phenolic screw caps were purchased from Supelco (Oakville, ON). A custom-made heating block designed to accommodate 22 ml glass vials was used to control temperature. Optima chloroform was obtained from VWR (Mississauga, ON). An Isotemp 100 Series Model 126G oven was used to incubate samples and was obtained from Fisher Scientific (Ottawa, ON) along with all other glassware. Methyl tricosonate, methyl eicosapentaenoate, methyl docosahexaenoate and 1,2-dipalmitin were purchased from Nu-Chek Prep (Elysian, MN). 1,3-dipalmitin

was purchased from Doosan Serdary Research Laboratories (Etobicoke, ON). Tripalmitin was purchased from Sigma Aldrich (Oakville, ON), along with all other chemicals. Two fish oils containing a blend of mixed natural tocopherols were obtained from Ocean Nutrition Canada Ltd. (Dartmouth, NS) and a third oil was obtained from EPAX (Oslo, Norway). Oil 1 contained approximately 30% EPA and DHA, while Oil 2 contained roughly 50%. Oil 3 contained approximately 65% EPA and DHA. All oils were marketed as triacylglycerols and all were blends of anchovy and sardine oil sourced from Peru.

5.4.2 Experimental Design

Amber bottles (250 ml) were filled with 200 ml of fish oil each and placed, uncapped, in an oven held at 40 °C. Oils were removed from the oven at varying time intervals, ranging from 2 to 15 hours, over the course of 10-14 days, with a new bottle being used at each sampling point. From each bottle, aliquots of 14.0 ml were placed in 22 ml glass vials and then capped with phenolic screw caps with PTFE/silicone septa and analyzed with SPME-GCMS. Approximately 15 ml of each sample was used for PV and AV testing, and the remainder of each 200 ml sample was either stored at -80 °C under nitrogen or evaluated by a sensory panel. One sample per 24 hour time period underwent PV and AV analysis and was tasted by a sensory panel. All samples were analyzed by SPME-GCMS. The experiment was conducted in triplicate for each of the three oil types.

5.4.3 Measures of Oil Quality

The fatty acid profile of the fish oil was analyzed using the method described by Sullivan et al. (2009). The PV and AV of each sample was measured in triplicate following AOCS Official Method Cd 8-53 (1997) and AOCS Official Method Cd-18-90 (1997), respectively.

5.4.4 Lipid Class Analysis

Lipid classes were analyzed using HPLC. Aliquots of each oil were diluted with $CHCl_3$/MeOH (2:1 by vol) to achieve a final concentration of 0.25 mg/ml. Samples were filtered using 0.45 µm filters and transferred to autosampler vials. Analysis was done in triplicate using a Thermo Finnigan Surveyor HPLC with autosampler (Thermo Fisher Scientific, Mississauga, ON) coupled to a Sedex 80 low temperature evaporative light scattering detector (Sedere, North York, ON) set to 30 °C with a gain of 8. The injection volume for each sample was 10 µl. The mobile phase was made up of hexane and *tert*-butyl methyl ether (TBME) (Table 5.1) at a flow rate of 2 ml/min and the total run time was 10 min. Samples were first analyzed on a YMC-Pack PVA-Sil column (5 µm particles, 100 mm x 30 mm I.D.) with matching guard cartridge (2.0x20 mm I.D.) to confirm that samples did not contain any MAG. Because of inconsistent retention times when the YMC-Pack PVA-Sil column was used, a silica column (Waters Spherisorb, 5 µm particles, 250 mm x 4.6 mm) column with matching guard column was used to quantify DAG and TAG. The column was held at 20±1 °C. Standard curves were prepared using tripalmitin, 1,2-dipalmitin and 1,3-dipalmitin with concentration ranges between 0.63 and 6.25 mg/ml depending on the standard.

Table 5.1 Composition of mobile phase for gradient elution with HPLC analysis

Time (min)	Hexane (%)	TBME (%)
1	98	2
5	100	0
7	100	0
8	98	2
10	98	2

5.4.5 SPME Analysis

The SPME method is described by Sullivan and Budge (2012). SPME samples were placed in a heating block held at 80°C and allowed to equilibrate for exactly 15.0 min while the SPME fiber was placed in the injector port of the GC at 250 °C to desorb any volatiles that had accumulated during storage. The SPME fiber was then inserted into the vial at a depth of 2 cm and exposed to the sample headspace for exactly 45.0 min. Extracted volatiles were analyzed by ion trap GCMS in electron ionization mode (200 °C). The fiber was inserted into the injector of the GC to a depth of 5 cm (splitless mode, 250°C; 1 mm liner) and left for 5 min. Volatile analytes were separated on a free fatty acid phase column (Nitroterephthalic acid modified polyethylene glycol 30 m x 0.25 mm x 0.25 um film thickness, Agilent, Mississauga, Canada). Helium was used as the carrier gas (1.0 ml/min). The oven temperature was initially held at 40°C for 5 min and increased at a rate of 10°C/min to 250°C and held for 5 min (total run time 29 min). Data were acquired as area counts and converted to area percent. Retention indexes were calculated by comparing retention times of oxidative volatiles to those of the two closest eluting n-alkanes in a C8-C20 retention index (RI) standard. Volatile oxidation products

were identified by comparison to pure external standards. When standards were not available, compounds were tentatively identified by matching them with mass spectral data in the NIST/EPA/NIH Mass Spectral Library (National Institute of Standards and Technology), through matching of fragmentation patterns generated by HighChem Mass Frontier 4.0 (HighChem Ltd., Bratislava, Slovakia) and through comparison to RI and fragmentation patterns available in the literature.

5.4.6 Sensory Assessment of Oil Quality

Between ten and twelve samples from each replicate were assessed for quality using an untrained, descriptive sensory panel (Warner 1995). To assess tasting abilities of potential panelists, volunteers were screened to eliminate those who could not distinguish fishy flavors. This was accomplished by challenging them with a triangle test, consisting of acceptable, fresh fish oil with a low volatile content and fish oil that had been incubated in an open container at 40°C for 7 days to force the formation of fishy volatiles. Those who could not distinguish between the fishy sample and the fresh sample were removed from the panel. The final panel consisted of 10 volunteers. Each panelist was asked to rank samples on a scale of 1-10, with 10 being "excellent" and 1 being "terrible", using a fresh vegetable oil sample as a reference. These classifications were then used to assign a scalar value to each tasted sample. Samples with sensory panel scores ≤ 7 were considered unacceptable, based on the descriptions of the scalar values. Each sample evaluated by the final panel was tasted by a minimum of three people. The design and screening of the sensory panel as well as the score sheets used to evaluate the samples were adapted from guidelines provided by the AOCS (Warner

1995). Of the 184 samples analyzed with SPME-GCMS, 93 were evaluated for sensory characteristics, leaving approximately half untasted (Appendix C).

5.4.7 Statistical Analysis

To improve normality of SPME-GCMS data, area percent data were first transformed using a geometric mean function (Aitchison 1983), following Equation 1:

$$x_i = ln \frac{a_i}{\sqrt[n]{a_1 a_2 \cdots a_n}} \qquad (1)$$

where a_i is the amount, in area percent, of each peak quantified in the sample. This data was then combined with sensory panel results. Mahalonobis distances were calculated for all samples as described by Filzmoser and Hron (2008) and those with χ^2 statistic greater than the critical value for $p \leq 0.001$ were eliminated. Following this elimination, 10% of remaining tasted samples were removed from the data set to be used for method validation. Each replicate of each of the three oils then underwent principal component analysis (PCA) of the covariance matrices. PCA is a multivariate data analysis technique that can be used to reduce the number of variables in a dataset into a smaller number of uncorrelated variables. The new variables, called principal components (PC), are linear combinations of the original variables and maximize the variability they represent. Scores are the transformed variable values corresponding to a specific data point, while loadings are the coefficients of the principal components. In the present study, the original data set contained 106 volatile oxidation products that were highly correlated and PCA was used to reduce the number of variables into PC. After outliers were removed,

ach dataset contained between 13 and 20 samples. The PC for each data set then underwent backwards linear regression, using sensory score as the dependent variable, to create a function that could be used to predict the sensory score of fish oil samples. Backwards linear regression begins by entering all predictor variables, in this case PC, into a regression model. The weakest predictor is then removed and the regression is recalculated. If the removal of a variable significantly weakens the model to the point where it is no longer a significant model, then that variable is added back in. This is repeated until only significant variables remain in the model. In many cases, more than one significant model ($p < 0.05$) was generated. In these cases, the model that had the highest R^2 and adjusted R^2, and lowest standard error of the estimate was selected. To validate the function, a sensory score was calculated for the samples that were not used to create the regression by calculating the PCA scores for each sample that was not used in the original PCA and regression function and inputting them into the regression equation. The calculated sensory score was then compared to the actual sensory score assigned by the sensory panel. The final step in this analysis was to test the omitted samples in other trials of the same oil to check for robustness. SPSS 11.0 (SPSS Inc. 1999) statistical software was used for all statistical analysis.

5.5 RESULTS

5.5.1 Fatty Acid Analysis

All three oils met suppliers' specifications for EPA and DHA (Table 5.2) and all three have different levels of these fatty acids.

Table 5.2 EPA and DHA concentrations (n=3, mean ± SD) of fish oils

Oil	EPA (mg/g)	DHA (mg/g)	Manufacturers Specification (mg/g)*
Oil 1	166 ± 1	125.4 ± 0.5	260
Oil 2	312 ± 6	225 ± 4	500
Oil 3	561 ± 3	157.3 ± 0.5	600

*- Manufacturer only guarantees an amount for combined EPA+DHA

5.5.2 Peroxide and Anisidine Values

As expected, PV and AV increased over time for all three oils. Initial PV was low (2.0 ± 1.3 meq/kg) for all samples, indicating that samples were not oxidized at the beginning of the experiment. As the study progressed, the PV increased for all oils, far exceeding the 5 meq/kg limit specified by GOED (Fig 5.1). Initial AV were also below the GOED limit of 20, with an average starting value of 9.4 ± 1.4. These values increased over time, but only one sample exceeded this limit at the end of the experiment (Fig 5.2). Though the rate of increase of PV and AV differs from oil to oil, the sensory ratings for each oil are consistent within oils of the same type. The average PV at which samples were deemed unacceptable by a sensory panel was 14.9 ± 6.5 meq/kg which is almost 3 times the PV limit of 5 meq/kg specified by GOED. The average AV at the sensory rejection point was 14.2 ± 3.7, almost 6 units below the GOED limit.

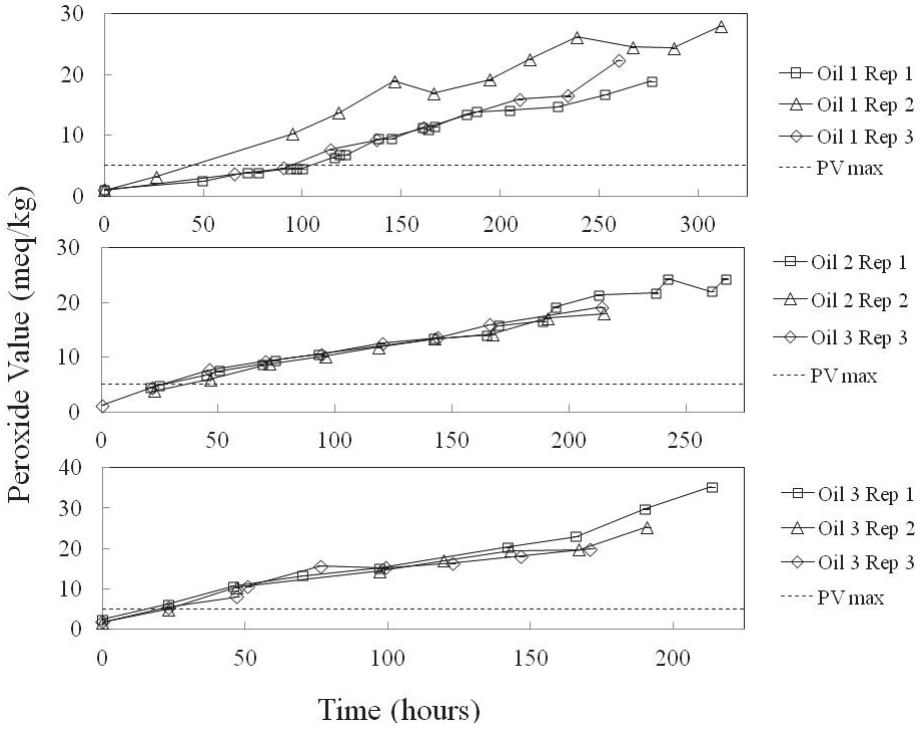

Figure 5.1 Variation in peroxide value (mean ± SD) in fish oil over time. The PV limit set by GOED is represented with a dashed line.

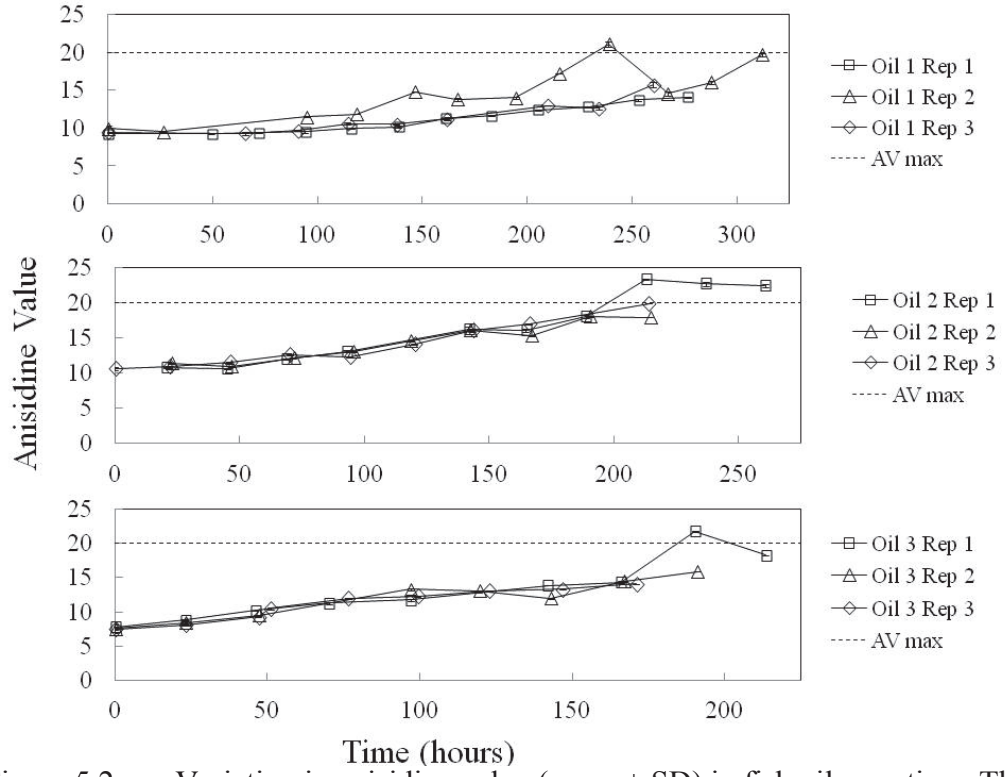

Figure 5.2 Variation in anisidine value (mean ± SD) in fish oil over time. The AV limit set by GOED is represented with a dashed line.

5.5.3 Lipid Class Analysis

TAG produced a linear standard curve while polynomial curves were the best fit for 1,2-DAG and 1,3-DAG. Oil 1 had higher TAG and lower DAG levels than specified by the manufacturer (Table 5.3), but Oil 2 and Oil 3 agreed with manufacturers' specifications. In the case of Oil 1, the TAG value was likely higher because the oil did not undergo a concentration step during processing. The FA profile of Oil 1 with EPA and DHA levels of 16.6% and 12.5%, respectively, was what one would expect to find naturally in a sardine-anchovy oil blend that has not undergone concentration during refining (Finley and Shahidi 2001).

Table 5.3 Lipid class composition for fish oil (n=3, mean ± SD)

Oil	TAG (%)	Suppliers TAG Specification	DAG (%)	Suppliers DAG Specification
Oil 1	96.7 ± 12.0	70-75	3.35 ± 2.18	20-25
Oil 2	72.3 ± 13.1	70-75	27.72 ± 4.74	20-25
Oil 3	91.5 ± 10.0	90-91	8.55 ± 2.11	8

TAG-triacylglycerol, DAG-diacylglyerol

5.5.4 Statistical Analysis

PCA of SPME-GCMS data generated from five to nine PC for each of the nine oil trials. When these PC's were used to develop multiple linear regression models, from two to five PC were needed to create a significant regression function, depending on the oil type and replicate (Table 5.4). Though the percent variance explained by the PC used in the regression models may appear low it is possible to achieve a high R^2 value (e.g. Oil 1 Trial 1). The reverse is also true (e.g. Oil 1 Trial 2). Regression of principal components gave well-fitting, significant (p <0.05) models with for all but one oil replicate for Oil 3 (Table 5.4). The failure of the Oil 3 trial is a result of contamination of volatiles from another study that were absorbed by the SPME fiber and interfered with SPME-GCMS analysis. Further statistical analyses on this trial were not carried out.

Table 5.4 Regression models for fish oil samples

Oil	# Samples in data set (outliers removed)	PC's in Regression Model	% Variance Explained by PC's	p	R^2	Adjusted R^2	Std Error of Est.
Oil 1 Trial 1	20	1, 3	56.2	0.001	1.00	1.00	0.05
Oil 1 Trial 2	18	1, 4	71.9	0.033	0.68	0.57	1.07
Oil 1 Trial 3	13	1, 2, 4	78.6	0.026	0.98	0.96	0.41
Oil 2 Trial 1	17	1, 2, 4, 5	87.7	0.037	0.88	0.77	0.76
Oil 2 Trial 2	18	1, 2, 3, 6	85.4	0.026	0.95	0.89	0.53
Oil 2 Trial 3	18	1, 3, 4	62.9	0.036	0.86	0.75	0.90

Oil	# Samples in data set (outliers removed)	PC's in Regression Model	% Variance Explained by PC's	*p*	R^2	Adjusted R^2	Std Error of Est.
Oil 3 Trial 1	18	1, 3, 4, 5, 6	78.4	0.015	0.99	0.98	0.20
Oil 3 Trial 2	16	1, 2, 3, 5	92.7	0.040	0.98	0.94	0.40

All samples that had not been used to create the regression functions were assigned sensory ratings using the appropriate regression function (Table 5.5). The calculated sensory scores for all three replicates of Oil 1 were quite close to the scores given by the sensory panel. All three samples had PV and AV levels below the average rejection values. The regression functions for two of the three replicates of Oil 2 also produced sensory scores that were very close to those given by the sensory panel. The sample with the poor result had a PV of 11.8 ± 0.0 meq/kg and an AV of 14.6 ± 0.1. The AV of the sample was very close to the average AV score at the sensory rejection point, while the PV was approaching the rejection point. The other two replicates of Oil 2 had PV and AV values well below the rejection thresholds. For Oil 3, the regression equation for the first replicate gave scores that were different from those assigned by the sensory panel. This replicate had a PV (23.0 ± 0.0 meq/kg) much higher than the average PV at the rejection point. The AV (14.4 ± 0.1) of this sample was also close to the rejection value for AV. In contrast, the regression equations for the second replicate of Oil 3 gave very accurate scores when compared to those given by a sensory panel. This samples had PV (5.0 ± 0.0 meq/kg) and AV (8.4 ± 0.1) that were well below the average rejection values. Finally, when regression equations were tested with other replicates of the same oil type the calculated sensory scores agreed with the actual scores for all but Oil 3 Replicate 2 (Table 5.5). When the sensory score for the omitted sample from Oil 3

Replicate 2 was calculated using the regression function for Oil 3 Replicate 1 a score of -1 was obtained, a value that is not valid. All other samples were within three sensory units of their taste panel score and most were within one unit.

Table 5.5 Sensory scores given by a taste panel and calculated sensory scores, PV and AV (n=3, mean ± SD) for select samples.

Oil	Sensory Panel Score	Regression Equation used to Calculate Sensory Score	Calculated Sensory Score	Avg. PV (meq/kg)	Avg. AV
Oil 1 Trial 1	6	Oil 1 Trial 1 Oil 1 Trial 2 Oil 1 Trial 3	7 4 5	2.5 ± 0.0	9.3 ± 0.0
Oil 1 Trial 2	7	Oil 1 Trial 1 Oil 1 Trial 2 Oil 1 Trial 3	7 6 7	10.3 ± 0.0	11.5 ± 0.1
Oil 1 Trial 3	8	Oil 1 Trial 1 Oil 1 Trial 2 Oil 1 Trial 3	8 5 7	4.6 ± 0.0	9.7 ± 0.0
Oil 2 Trial 1	9	Oil 2 Trial 1 Oil 2 Trial 2 Oil 2 Trial 3	9 9 9	8.66 ± 0.0	12.0 ± 0.1
Oil 2 Trial 2	9	Oil 2 Trial 1 Oil 2 Trial 2 Oil 2 Trial 3	9 8 9	11.8 ± 0.0	14.6 ± 0.1
Oil 2 Trial 3	7	Oil 2 Trial 1 Oil 2 Trial 2 Oil 2 Trial 3	7 4 5	10.5 ± 0.0	12.3 ± 0.0
Oil 3 Trial 1	8	Oil 3 Trial 1 Oil 3 Trial 2	5 8	23.0 ± 0.0	14.4 ± 0.1
Oil 3 Trial 2	8	Oil 3 Trial 1 Oil 3 Trial 2	-1 9	5.0 ± 0.0	8.44 ± 0.1

PCA and regression were used instead of other multivariate techniques because of the characteristics of the SPME-GCMS data. The high number of oxidative volatiles (>100) resulted in a situation where there were many more variables than tasted samples, which eliminated the use of many common statistical methods such as multivariate

analysis of variance (MANOVA). The levels of volatile oxidation products were highly correlated as most compounds were either increasing or decreasing over time making regression on raw data impossible. The changes in volatile levels occurred gradually so there was no clear divide between acceptable and unacceptable samples, instead there seemed to be an indistinct area where samples were neither good nor bad, making it difficult to obtain valid results using other classification techniques such as discriminant function analysis (Sullivan and Budge 2012). This was evident when scores of PC1 versus PC2 were plotted for each oil trial separately; samples showed a clear transition with time, with samples taken early in the time course in one quadrant and late samples in another quadrant (Fig 5.3). Samples in the middle of the time course grouped near the center of the plot, emphasizing the lack of a distinct separation between new and old, or acceptable and unacceptable, indicating that the transition from acceptable to unacceptable happened gradually rather than suddenly. This gradual transition made it difficult to pinpoint the exact time when fish oil quality was considered unacceptable.

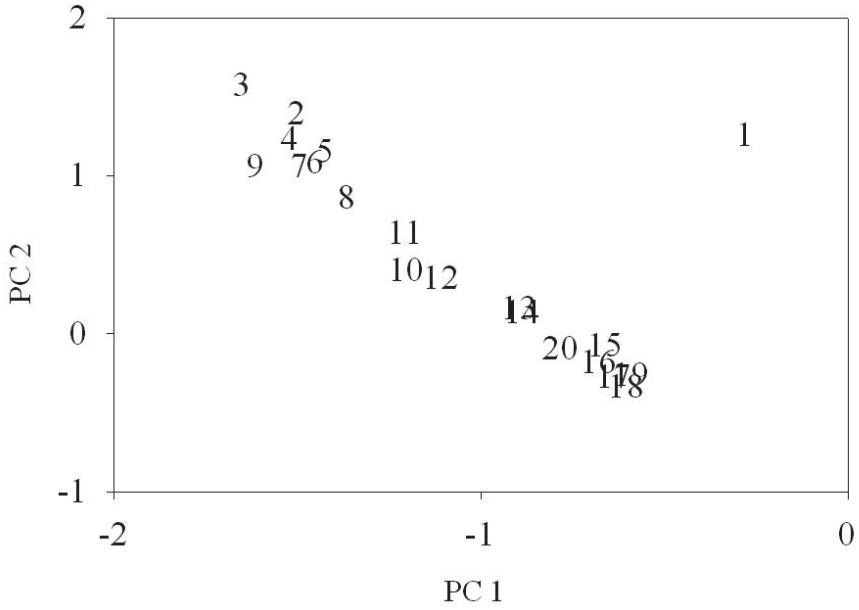

Figure 5.3 Scores for principal components for Oil 2 Rep 1. Numbers indicate sequence of samples.

As an alternative, multiple linear regression was considered. This is a useful technique for characterizing samples, but only if variables are uncorrelated. The volatile oxidation products studied here were highly correlated, so PCA was used to generate uncorrelated variables. PCA also greatly reduced the number of variables to < 10. Linear regression of these variables generated a simple equation to assign sensory scores to fish oil samples. One tasted sample, selected at random, was left out of each trial with removal occurring before PCA analysis. Removal at this early point in data analysis allowed the samples to truly serve as a validation of the regression functions. One sample per oil trial corresponded to roughly 10% of tasted samples being omitted during construction of the regression methods.

The combination of PCA and regression employed in this study allowed all data to be capitalized on as PCA transforms highly correlated variables, in this case volatile oxidation products, into uncorrelated linear combinations of the original variables, while still capturing all variability in the original data set. Initially, SPME-GCMS results of all replicates of all oils were combined and PCA was applied. When PC scores were examined, oils were clearly separated by oil type rather than state of oxidation as was expected due to differences in fatty acid and lipid class profiles between oils. It is well documented that PUFA, including EPA and DHA, oxidize more rapidly than other fatty acids (Frankel 2005, Sullivan et al. 2011) so Oil 2 and Oil 3 which contained higher levels of these fatty acids were expected to oxidize at a different rate than Oil 1, with lower PUFA content. There is some evidence that DAG and MAG oxidize more rapidly than TAG (Wang et al. 2005, Kristensen et al. 2006), so different levels of these structures in the oils may also be responsible for their clear separation based on volatile secondary oxidation products. Additionally, Oil 1 and Oil 2 were produced by the same manufacturer, while Oil 3 came from another, so slight differences in oil refining processes could affect the oxidation patterns and stability of the oils. For example, differences in the winterization process may result in oils having higher levels of shorter chain saturated fatty acids, which are more resistant to oxidation. Because PCA results were not useful when applied to all trials of all oils combined in a single group, data were separated by oil type and PCA was applied to Oil 1, 2, and 3 separately. PCA then detected the variance between oil replicates, rather than changes occurring in the same oil due to oxidation, resulting in three distinct groupings of samples according to replicate within a time course. Slight differences between replicates of the same oil were expected,

as all trials were done sequentially rather than concurrently. This was done intentionally in order to develop a more robust method but unfortunately prevented all time courses of the same oil from being analyzed as one unit. These results demonstrate the sensitivity of PCA to small changes in volatile composition among replicates of the same oil. Thus, it was necessary to treat each replicate of each oil as an individual experiment.

When regression equations were tested with samples from other replicates of the same oil type, Oils 1 and 2, along with the first replicate of Oil 3 gave results consistent with the sensory panel. The results of the second replicate of Oil 3 were poor, giving an impossible score of -1. It is unclear what caused this odd result as no inconsistencies in the data were noted. With the exception of Oil 3 Trial 2, the consistency of the calculated scores compared to the sensory panel scores suggests that this classification method is robust and will hold true for oils of the same type, despite the need to develop separate regressions for each time replicate.

5.5.5 Key Oxidative Volatiles and Sensory Characteristics

When PCs and sensory panel scores were used to build regression equations, it became clear that the first PC (PC1) had a large effect on the reliability of the equation as all equations incorporated PC1. This is logical as PC1 is the component that describes the most variability in a given data set. Because PC are linear combinations of the original variables, each PC is comprised of many oxidation products. For all trials, the compounds that had the highest correlations ($R \geq 0.80$) with each PC used in the regression equations were examined and compared. Since PC1 described such a large proportion of the change that occurred in the data set and was incorporated into each regression equation, the replicates of the same oil type had many peaks in common, that were also highly

correlated with PC1. When all replicates of all three oil types were compared there were eight peaks with high correlations with the PC used in the regression common to all trials (Table 5.6). These consisted of four aldehydes, one ketone, one benzene derivative and one hydrocarbon all of which were all previously identified in oxidized fish or fish oil. It is interesting to note that not all of these compounds increased in proportion over time; three actually decreased.

Table 5.6 Oxidative volatiles correlated with sensory properties of all three oils

Retention Index	Compound	Direction of proportion change	Method of identification
963	2-ethyl furan	Decrease	b, c
996	octene	Decrease	b, c, d
1385	3,6-nonadienal	Increase	b, c
1565	3,5-octadien-2-one	Increase	b, c
1573	benzaldehyde	Decrease	a
1608	(*E,Z*)-2,4-octadienal	Increase	a
1820	(*E,E*) 2,4-decadienal	Increase	a
1879	(*E,E,Z*) 2,4,7-decatrienal	Increase	c, d

a- Retention index and MS fragmentation pattern compared with an external standard. b- NIST library match. c- probable ion fragmentation predicted using Mass Frontier 4.0. d-comparison to retention index and spectral data from literature

It is well known that aldehydes play a role in the sensory properties of fish oil, and all four aldehydes identified here increase over time. (*E,Z*)-2,4-ocatdienal has been identified previously as a fish oil oxidation product and is associated with green odors (Sullivan and Budge 2012, Hartvigsen et al. 2000). The second aldehyde, 3,6-nonadienal, is a degradation product of EPA and is associated with a green odor (Frankel 2005). It has also been identified by MacFarlane et al. (2001) as an oxidative volatile associated with sensory properties, and is a key component in the Fatty Acid Smell and

Taste (FAST) method of predicting oil quality. The third aldehyde identified was (*E,E*)-2,4-decadienal, a compound with a deep-fried odor that is often identified in oxidized fish oil (Hartvigsen et al. 2000, Jacobsen et al. 2000, Venkateshwarlu et al. 2004). Jacobsen et al. (2000) found that an increase in this oxidation product was correlated with an increase in rancid, fishy flavors in fish oil enriched mayonnaise. The final aldehyde associated with sensory properties of fish oil, (*E,E,Z*)-2,4,7-decatrienal, has been identified in oils and emulsions containing omega-3 fatty acids and is believed to have green, plant-like odors (Karahadian and Lindsey 1989, Hartvigsen et al. 2000, Venkateshwarlu et al. 2004].

Only one ketone, 3,5-octadien-2-one, which increased over time, was shown to be strongly related to sensory parameters of all three oil types. The configuration of the double bonds could not be determined. Both the (*E,Z*) and (*E,E*) isomers of this compound have been identified in fish oil-enriched mayonnaise by Jacobsen et. al (2000) and Hartvigsen et al. (2000), who found that both isomers were strongly correlated with oxidaiton. Venkateshwarlu et al. (2004) identified this ketone in fish oil-enriched milk. The (*E,Z*) isomer has been linked to green, fruity and fatty odors as well as plastic and synthetic odors, while the (*Z,Z*) isomer is associated with fresh, green and fruity odors (Hartvigsen et al. 2000, Venkateshwarlu et al. 2004).

The ether 2-ethylfuran was also identified as being a significant indicator of sensory characteristics, and decreased as oxidation progressed. This is a common oxidation product of omega-3 fatty acids and can be formed from the 12-hydroperoxide of EPA and the 16-hydroperoxide of DHA (Medina et al. 1999). Venkateshwarlu et al. (2004) identified this compound as having a sweet odor. Since sweet odors are

commonly associated with positive sensory properties, a reduction in this compound could mean that positive sensory attributes are being eliminated as oxidation progresses, allowing negative attributes to become more noticeable.

An unknown isomer of decene had a strong relationship to sensory properties of oils and decreased over time. 1-decene can be formed during oxidation of oleic acid which is present in small amounts in fish oil (Min and Boff 2002). Chung et al. (2011) identified both 1-decene and 3-decene in mackerel, though no relationship with sensory properties was mentioned. Though hydrocarbons are generally thought to be flavorless, the importance of this oxidative volatile to predicting sensory quality suggests that it plays a role in the flavor of fish oil.

Benzaldehyde was strongly associated with sensory properties of fish oil and decreased with time. This compound has a sweet odor and has been detected in fish oil-enriched mayonnaise (Hartvigsen et al. 2000), tuna oil (Roh et al. 2006) and cod liver oil (Guillén et al. 2009). It is unlikely that benzaldehyde is a direct product of fish oil oxidation, but it could be an oxidation product of a larger benzene derivative that was initially present in the oil. Giogios et al. (2009) hypothesized that benzene compounds could be decomposition products of amino acid or sugars, explaining their low levels in fish oils.

PV and AV are convenient ways to monitor the formation of oxidation products in fish oil, but this work supports the well-known issue with these measures: limits, including those specified by GOED do not correlate with sensory properties of the oil. Hydroperoxides are primary oxidation products, which then degrade into volatile secondary oxidation products that are responsible for off-flavors and odors in oils.

Frankel (2005) has said that sensory panels can detect off-flavors in oils with PV < 1 meq/kg. This is supported by results collected by MacFarlane et al. (2001) that showed freshly refined fish oil samples with PV of < 1 meq/kg had strong fishy tastes. In some cases this might be because the hydroperoxides increased to their maximum and had now begun to degrade into secondary oxidation products. In that situation elevated AV would be expected. Conversely, the results of this study suggests that oils with high PV do not necessarily have poor sensory properties, likely because hydroperoxides have not yet degraded to sufficient extent to form detectable levels of secondary oxidation products.

A number of samples in this study had PV > 10 meq/kg and were classified as acceptable by the sensory panel and the PV at the point of rejection was 14.5 ± 6.5 meq/kg, suggesting that the commonly used limit of 5 meq/kg is not appropriate. Although there is little evidence that AV is related to sensory properties of fish oils (Frankel 2005), a value of 20 is commonly used as the rejection value when assessing fish oil quality. Because the regression model was created using data produced by monitoring proportions of volatile oxidation products produced over time, these results suggest that AV may be of more importance to sensory quality than was previously thought, with an average AV of 14.2 ± 3.7 at the sensory panel rejection point being lower than the limit of 20 specified by GOED. Interestingly, MacFarlane et al. (2001) found that fish oil samples that had poor sensory properties with PV < 1 meq/kg also had AV < 20 indicating that hydroperoxides had likely not yet reached their maximum and begun to degrade. The regression models created from SPME-GCMS data classified samples with AV > 14 as unacceptable, with predicted sensory scores ≤ 7, despite sometimes positive ratings given by the sensory panel. This also suggests that the current

limit of 20 is too high. At least one study (Thomkins and Perkins 1999) found that AV was strongly correlated with sensory evaluation and headspace volatiles in partially hydrogenated soybean oil used for frying. The results of this study suggest that PV and AV may both be accurate and reliable methods to assess sensory quality but the limits that are currently used are not ideal, with the PV limit too low and the AV limit too high.

Sensory panels have long been recognized as the best way to monitor fish oil quality; however, the expenses associated with them often prevent them from being implemented. It is costly to train and maintain panels. Often people do not want to participate in a panel that involves tasting foods with poor sensory characteristics, so compensation is essential. A proper sensory panel requires specially designed tasting booths, lighting and air flow systems. Despite the best training, sensory assessment is still highly subjective and even trained panelists can give inaccurate results on occasion. Our results offer support for this. When our regression model was used to assign a sensory score to samples, the majority of the results were within one unit of the score given by the sensory panel. There were three samples that were assigned a high score (8 or 9) by the sensory panel, but when classified using the regression model, received scores of 5 suggesting that the sample was of poor quality. On closer examination, it was found that all three were samples that were taken late in the time course, and all had AV above 14.2, the average AV at the point of sensory panel rejection. Two of the three samples also had PV > 20 meq/kg indicating that oxidation had progressed considerably. The combination of these factors suggest that the regression models may actually be more accurate than the sensory panel in predicting the quality of highly oxidized oil samples; however, for the regression model to be accurate the sensory panel must also provide

accurate initial assessments as this is the source of the data for the regression equations. Because of the cyclic relationship, it may be more logical to use PV and AV to evaluate the quality of very oxidized samples, or to present the taste panel with more samples to evaluate so that more data is available to build the regression model. This study was very small and limited samples were tasted by the sensory panel. Future research will focus on expanding the sensory panel and the number of oil samples evaluated to improve the robustness of this method.

5.6 CONCLUSIONS

The method presented here can be used by fish oil refiners and dietary supplement manufacturers to determine if fish oil has acceptable sensory parameters without the need to maintain an expensive sensory panel. Though a panel is required to develop the method, after the initial set up there should be no further need to maintain the sensory panel. Because the PV and AV limits currently used to determine oil quality seem to be of little use in predicting sensory quality, this method will provide more accurate results; however, in a commercial setting it should be possible to determine PV and AV limits that correlate with sensory parameters and then use these as an additional measure of quality. Based on results in this study, the PV and AV limits typically used by industry should be reevaluated for usefulness

In conclusion, PCA and linear regression can be used in conjunction with SPME-GCMS of oxidative volatiles to predict the sensory quality of fish oil. The method presented here suggests that the PV and AV values typically used to indicate fish oil quality have little relationship with sensory properties.

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CHAPTER 6 CONCLUSIONS

6.1 General Conclusions

Fish oil is high in the omega-3 fatty acids EPA and DHA, and is a very popular dietary supplement. Thus, it is important to be able to accurately assess the quality of fish oil as both a raw material and a finished product. The work presented here focused on two specific aspects of quality: EE and oxidation products.

Fish oil EE are considered to be of lower quality than TAG fish oil as they are prone to more rapid oxidation, resulting in formation of fishy flavors and odors. Consequently, it is important to have a reliable method to detect and quantify EE in fish oils claiming to be TAG. Current methods employ HPLC and/or TLC, and are often tedious and time consuming. A simple, reliable method was needed to detect EE in fish oil. A SPME and GCMS method was developed to quantify the level of EE present in fish oils sold as TAG products. This method used a standard curve to quantify amounts of EE, and is simple, solvent free, and highly sensitive. Because of this, it can be used as an alternative to traditional methods for detection of EE. It is anticipated that this method could be implemented as a quality control measure by fish oil supplement manufacturers to test EE content of their raw materials.

Though TAG fish oil is more stable than EE, it is still susceptible to oxidation due to the high level of PUFA it contains. Because of this, it is important to evaluate levels of various oxidation products when determining the shelf life of fish oil products. Standard methods exist to determine the extent of oxidation products in fish oil. One such technique to assess shelf life is by monitoring PV, using high temperature storage conditions to accelerate oxidation, and to then extrapolate these results to lower

temperatures. The present study modeled the kinetics of two commercially available fish oil supplements with different EPA and DHA concentrations to determine if results of accelerated stability studies could be extrapolated to oils stored at lower temperatures. Fish oils were incubated at temperatures from 4 °C to 60 °C and PV was measured over time to determine if fish oil oxidation follows the Arrhenius model. First-order kinetics were followed at temperatures ≥20 °C and ≤ 40 °C for 18:12 fish oil and at temperatures ≥4 °C and ≤ 40 °C for fish oil concentrate. Both oils oxidized more rapidly at 60 °C and did not follow first-order kinetics. Because of this, it is recommended that accelerated stability studies using fish oil should be conducted at temperatures ≤ 40 °C.

To investigate alternative measures of oxidation, accelerated stability studies were conducted on three fish oils, each with different levels of EPA and DHA in an attempt to develop a method to distinguish between acceptable and unacceptable fish oils based on their sensory characteristics. Volatile secondary oxidation products were measured using SPME-GCMS and samples were tasted by a sensory panel. PV and AV were also monitored. DFA was used to analyze data for Fish Oil 1 (18:12 fish oil) and to identify key volatile oxidation products, primarily aldehydes and ketones, that were important in classifying samples as either acceptable or unacceptable. Volatiles with DF coefficients >0 had a positive impact on sensory characteristics, while those with coefficients <0 negatively affected sensory perceptions. However, a limitation of DFA is that it classifies samples into distinct groups, and cannot be used to monitor subtle changes in quality. For instance, DFA cannot differentiate between samples that do not fit well in a defined group, and, in this case, all samples are forced into the one of the two groups, even if the fit is poor.

In situations where changes in properties are gradual, such as fish oil oxidation, PCA becomes a better alternative to DFA. Because of this, PCA and linear regression were carried out on three fish oils with different EPA and DHA levels (Fish Oils 1-3). One sample from each trial was omitted from data analysis and used to validate the regression equations. These samples were then tested in regression equations for the other trials of the same oil type. Significant regression equations were found for all but one trial of Oil 3, and this was a result of contamination from another study. No further analysis was conducted on that trial. When sensory scores were calculated for the omitted samples from Oil 1 and Oil 2, results were consistent with those given by the sensory panel. Oil 3 results were less consistent, with one equation resulting in an impossible sensory score of -1. It is unclear why this occurred as no inconsistencies in that data were found. When the regression equations were examined in more detail, eight compounds, primarily aldehydes and ketones, were found to be strongly associated with the principal components that were used in the regression to relate to the sensory characteristics of all three oils. Two of these compounds, 3,6-nonadieanal and (*E,Z*)-2,4-octadienal, are also important with the DFA classification of oils. When PV and AV results were examined, it was found that the limits commonly used in industry had no relationship with sensory characteristics. Instead, a PV limit of 15 meq/kg and an AV of 14 appeared to be appropriate for the oils used here. It is anticipated that this method could be used to generate information on fish oil quality that would correlate well with results of sensory evaluation, without the need to use a sensory panel except for method development, saving both time and money. Additionally, it is hoped that this work will

highlight the discrepancies between the GOED limits for oxidation markers and actual sensory quality.

6.2 Future Work

Future work will focus on modeling the kinetics of lipid oxidation using a wider range of temperatures and more sampling point. A major flaw in this study is that it did not include sampling at temperatures 4 °C > 20 °C, or temperatures 40 °C > 60 °C, in the future temperatures within these ranges will be included. Oils will also be sampled more frequently at higher temperatures to generate more data.

In future research it will be of benefit to repeat the above experiments using a larger sensory panel, with more tasted samples. A larger taste panel will have the capability to taste more samples, providing more data on which a method can be built. Additionally, the study can be extended over a longer time period so a more complete picture of oxidation can be captured. This will allow for a more robust method that will be accurate over a wider quality range. The use of a trained sensory panel, rather than the untrained panel used in this study, could also be beneficial. Panelists could be trained to detect specific off flavors as opposed to assessing overall quality, helping to identify the oxidative compounds that are responsible for specific off flavors.

Future work will include classifying oil samples using the eight compounds identified here as being important contributors to sensory characteristics. This could be accomplished by performing multiple linear regression with just these compounds.

It is recommended that other marine oils that are high in omega-3 also undergo similar studies. These could include fish oils with different fatty acid profiles, as well as

seal oil, krill oil, algal oil and squid oil. This would provide insight into differences in oxidation processes in oils from different sources as well as identify oxidation products that are specific to certain oils.

Lastly, the development of a similar method for fish oil emulsions would also be useful. Fish oil emulsions are gaining popularity as dietary supplements because their flavor and texture that are more appealing than traditional fish oil supplements. In addition to fish oil emulsions usually contain many other ingredients such as water, sweeteners, thickeners and flavoring agents, which are likely to also contribute to oxidation. Thus different regressions would be required even if the same oils as examined here were used.

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APPENDIX A PLOTS OF LN PV VERSUS TIME

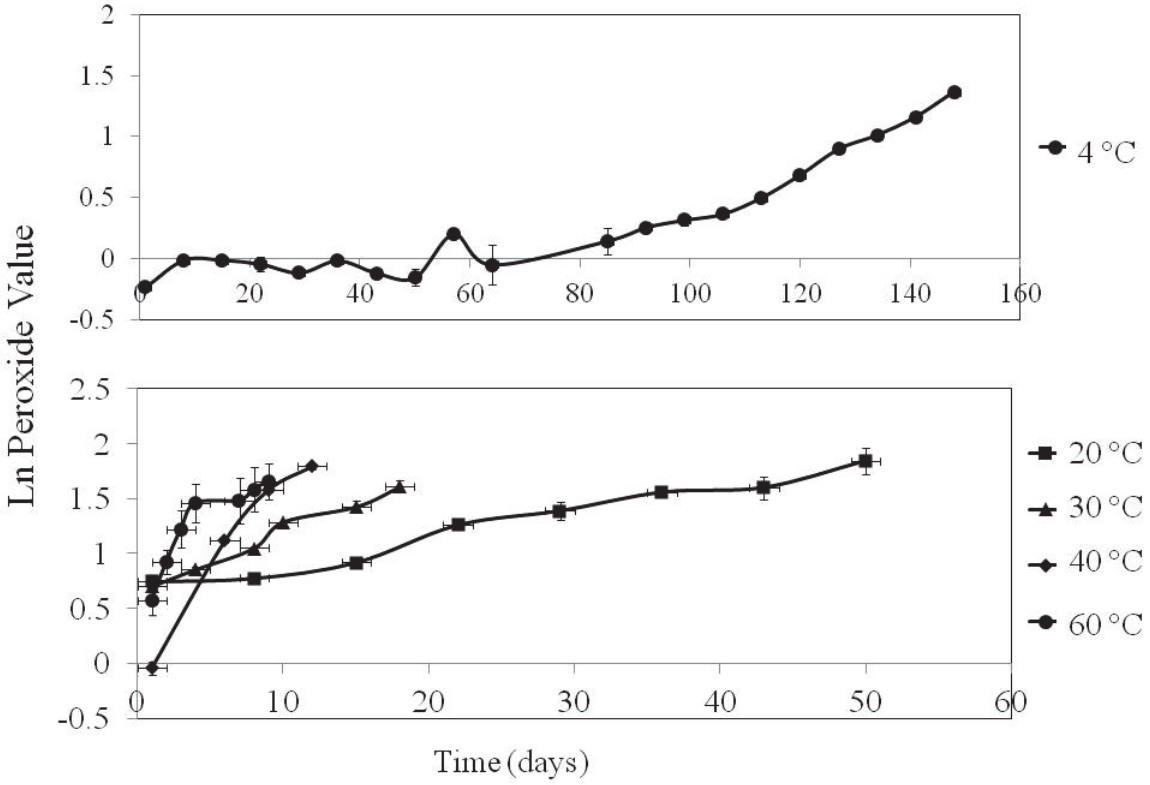

Figure A1 Ln PV versus Time for 18:12 oil at 4 °C (top plot), 20 °C, 30 °C, 40 °C and 60 °C (bottom plot).

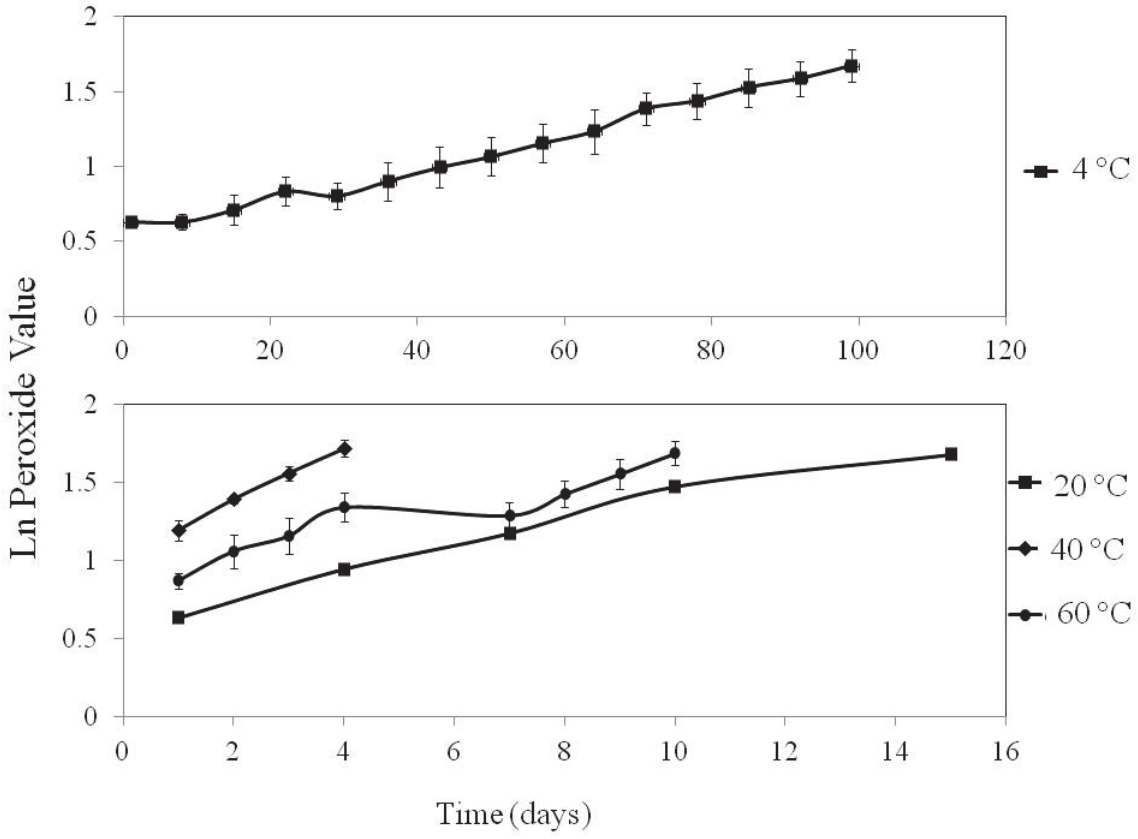

Figure A2 Ln PV vs Time for concentrate oil at 4 °C (top plot), 20 °C, 40 °C and 60 °C (bottom plot).

APPENDIX B HISTOGRAMS OF SENSORY PANEL SCORES

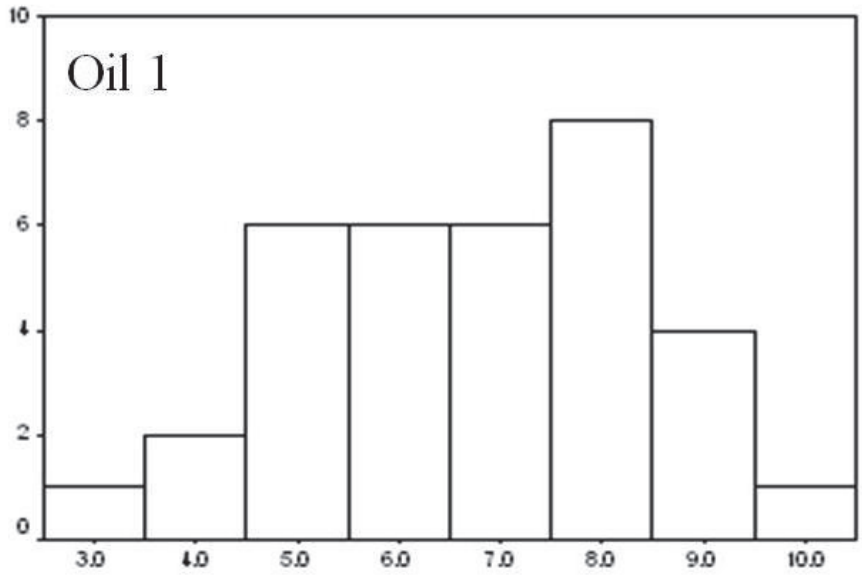

Figure B1 Distribution of sensory panel scores for Oil 1.

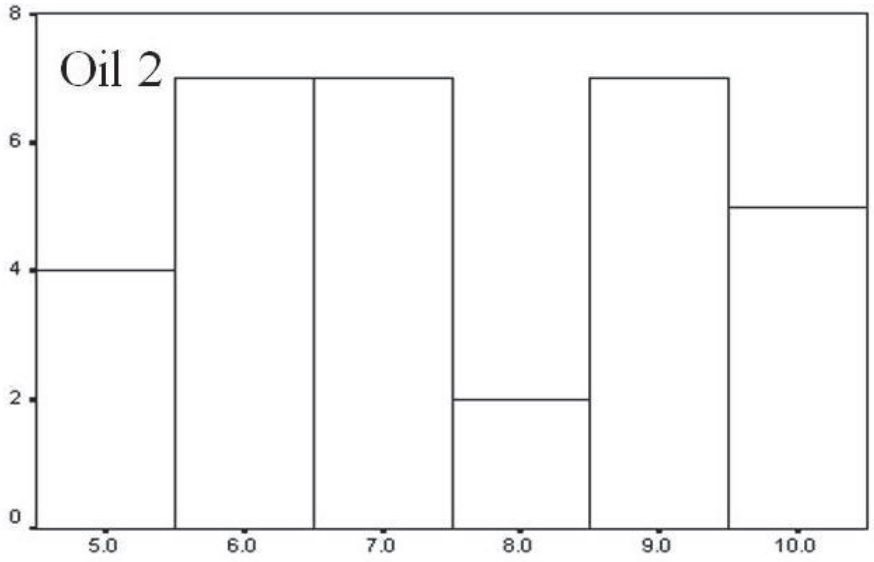

Figure B2 Distribution of sensory panel scores for Oil 2.

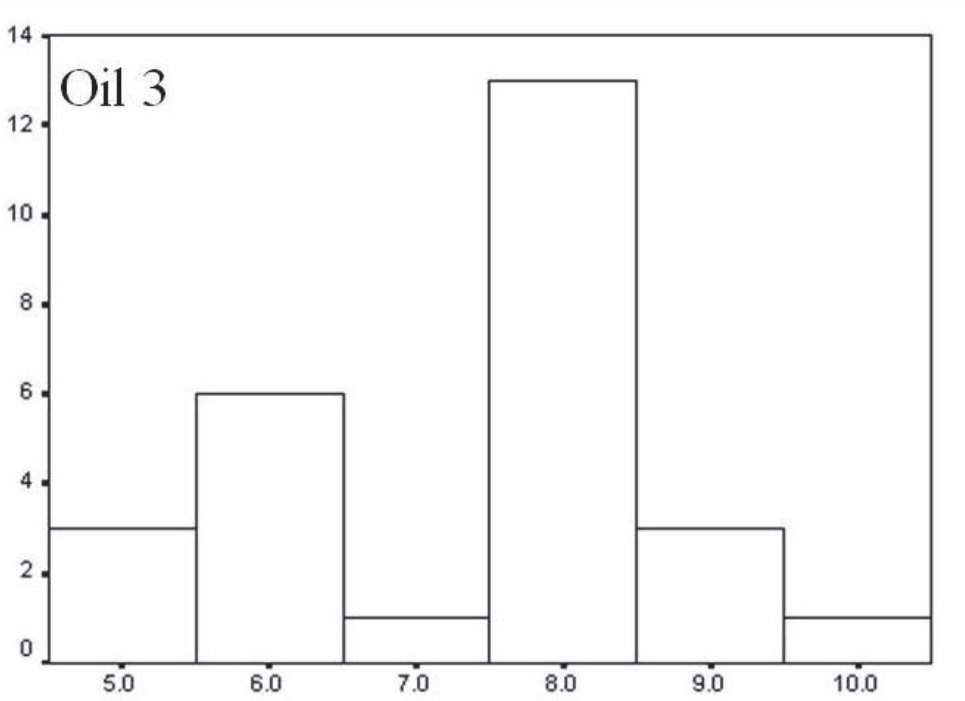

Figure B3 Distribution of sensory panel scores for Oil 3.

APPENDIX C SENSORY PANEL SCORE SHEET

Oil Sensory Panel

Instructions

Take oil into mouth; pull air through the oil and exhale through the nose. Rate samples for overall flavour quality on a 10-point scale (10 being excellent, 1 being bad). Identify fishy flavour as weak (W), moderate (M) or strong (S). If the flavour is not present, leave space blank. Finally, rate each sample as "accept" (would purchase or consume again) or "reject" (would not purchase or consume again). Any additional comments can be written on the back of the evaluation sheet.

Please do not talk or communicate with other panelists during tasting. Unsalted crackers and carbonated water have been provided to rinse mouth between samples. Spit cups have been provided if necessary.

Each panelist is required to fill out a consent form before participating. This information will remain anonymous.

Quality		Overall Quality Scores			
	SAMPLE				
10	Excellent				
9	Good				
8					
7	Fair				
6					
5	Poor				
4					
3	Very Poor				
2					
1	Bad				
Fish flavor intensity					
Accept					
Reject					

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