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Trans isomers of EPA and DHA in refining and concentration of fish oils.

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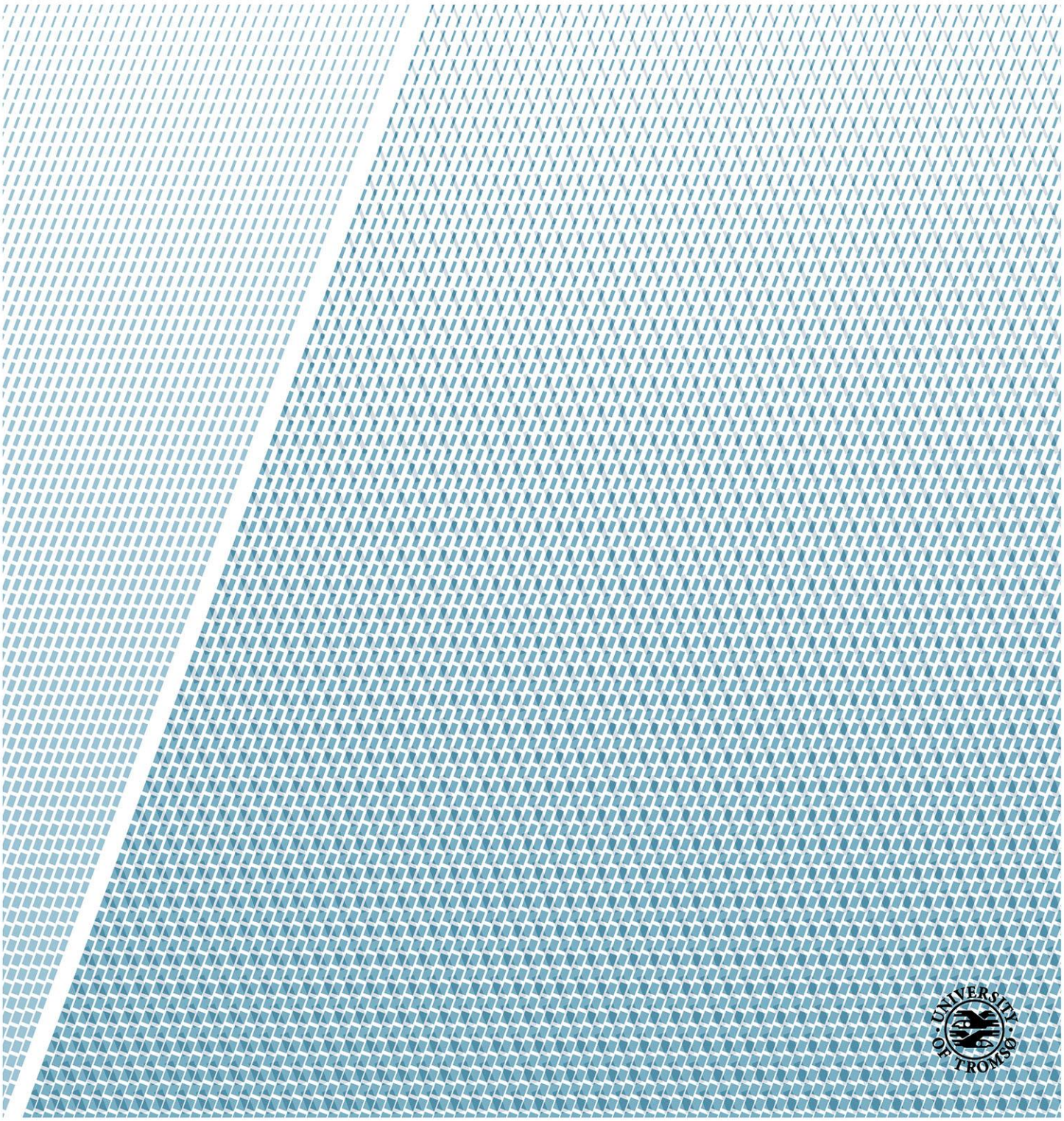

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Lars Dalheim

Abstract

The omega-3 long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are strongly recommended to be part of a normal diet due to the many positive health effects in humans. Health authorities in many countries recommend an average daily intake of 0.25 – 0.5 grams of EPA and DHA by eating fish at least twice a week or alternatively, consume fish oil supplements. The unsaturated fatty acids in native marine and vegetable lipids are all present as *cis*-isomers while *trans*-isomers are found in industrially hydrogenated oils and in minor amounts in fat from ruminants. The intake of large amounts of *trans*-fatty acids is considered to have serious negative health effects and the advices are to reduce the consumption as much as possible.

Several processing steps are necessary to produce high quality fish oil supplements, including concentrated forms of EPA and DHA. These steps include high temperature processes that might induce transformation of *cis*-double bonds to *trans*-double bonds in the unsaturated fatty acids. The objectives of this thesis was determine if the processing conditions used at Nordic Pharma Inc. resulted in the formation of *trans* fatty acids in both natural "1812" fish oils (18 % EPA, 12 % DHA) and EPA/DHA concentrates and to investigate how time and temperature used in the processing steps affected the formation of *trans* fatty acids in fish oil concentrates. Initially, a method for the analysis of *trans* fatty acids in fish oils was established.

Methylated standards of *trans* EPA and DHA were produced using *p*-toluenesulfinic acid as catalyst and separating the different isomers on high performance thin layer chromatography (HPTLC) plates impregnated with silver nitrate. A 100 meter SLB-IL111, the most polar gas chromatography (GC) column commercially available, was used to analyze the samples. The preparation and the separation of the *trans* standards on HPTLC plates was successful and gave sufficient amounts to optimize a temperature program for GC separation. The results from samples of processed fish oil showed that only minor amounts of *trans* fatty acids were formed during the processing conditions applied at Nordic Pharma Inc. The content of *trans* fatty acids was far below the amount allowed for such products. The SLB-IL111 column worked well as a tool for analysis of *trans* fatty acids in fish oil, but some further investigations are needed for this analysis system to be optimized. The temperature experiment showed that 200 °C for more than an hour was needed for the formation of larger amounts of *trans* LC-PUFA to occur.

Keywords: Fish oil, industrial processing, *trans* fatty acids, EPA, DHA, SLB-IL111, silver ion TLC

Sammendrag

De langkjedede, flerumettede omega-3-fettsyrene eikosapentaensyre (EPA) og dokosaheksaensyre (DHA) er sterkt anbefalt som en del av det normale kostholdet på grunn av de mange positive helseeffektene disse har. Helsemyndighetene i flere land anbefaler at en bør få i seg 0,25-0,5 gram EPA og DHA daglig, enten ved å spise fisk to ganger i uka eller alternativt å få i seg tilsvarende mengder fra fiskeoljetilskudd. De umettede fettsyrene som forekommer i naturlig marint og vegetabilsk fett foreligger som *cis*-isomerer, mens *trans*-isomerer finnes i industrielt herdet fett og i små mengder i fett fra drøvtyggere. Et høyt inntak av *trans*-fettsyrer er ansett å ha en negativ effekt på helsa, og inntaket anbefales å reduseres til et minimum.

For å oppnå høy kvalitet på fiskeoljetilskuddene, inkludert konsentrater av EPA og DHA, må flere raffineringstrinn gjennomføres. Flere av disse prosessene involverer høye temperaturer som potensielt kan indusere omdannelse av *cis*-dobbeltbindinger til *trans*-dobbeltbindinger. Målene med denne oppgaven var å finne ut om prosessbetingelsene som brukes hos Nordic Pharma Inc. resulterte i dannelse av *trans*-fettsyrer, i både naturlige «1812»-oljer (18% EPA og 12% DHA) og EPA/DHA-konsentrater, og å finne ut hvilken effekt tid og temperatur, i prosesseringen, har på dannelsen av *trans*-fettsyrer i fiskeoljekonsentrater. Først ble en metode for analyse av *trans*-fettsyrer etablert.

Metylerte standarder av *trans* EPA og DHA ble laget ved hjelp av *p*-toluensulfinsyre som katalysator og separert ved hjelp av tynnsjiktskromatografiplater (HPTLC) impregnert med sølvnitrat. En 100 meter lang SLB-IL111 kolonne, den mest polare kolonnen som er kommersielt tilgjengelig for gasskromatografi (GC), ble brukt for å analysere prøvene. Tillagingen og separasjon av *trans*-standardene på HPTLC-platene fungerte godt, og gav tilstrekkelige mengder til å optimalisere et temperaturprogram for separasjon på GC. Resultatene fra de prosesserte fiskeoljeprøvene viste at bare små mengder *trans*-fettsyrer ble dannet under de prosessbetingelsene som brukes ved Nordic Pharma Inc. Innholdet av *trans*-fettsyrer var langt lavere enn hva som er tillatt i slike produkter. SLB-IL111-kolonnen fungerte bra som et verktøy for analyse av *trans*-fettsyrer i fiskeolje, men det trengs videre undersøkelser for å optimalisere dette analysesystemet. Temperatureksperimentet viste at fiskeoljekonsentratet måtte varmes til 200 °C i over en time for at det skulle dannes større mengder av *trans*-fettsyrer.

Nøkkelord: fiskeolje, industriell prosessering, *trans*-fettsyrer, EPA, DHA, SLB-IL111, sølvion-kromatografi

Abbreviations

ALA – α-linolenic acid
AOCS – American Oil Chemists Society
ARA – arachidonic acid
BHT – butylated hydroxytoluene
BLF 1812 – bleached fish oil containing 18% EPA and 12% DHA
DAG – diacylglycerol
DEO 1812 – deodorized fish oil containing 18% EPA and 12% DHA
DHA – docosahexaenoic acid
DPA – docosapentaenoic acid
DTD 3020 – concentrate from SPD containing 30% EPA and 20% DHA
DTL – volatile fraction distillate from SPD
DTR – heavy fraction residue from SPD
ELOVL2 – elongation of very long chain fatty acids 2 enzyme
ELOVL5 – elongation of very long chain fatty acids 5 enzyme
EPA – eicosapentaenoic acid
ETY 2412 – fish oil in ethyl ester form containing 24% EPA and 12% DHA
FA – fatty acid
FAEE – fatty acid ethyl ester
FAME – fatty acid methyl ester
FID – flame ionization detector
GC – gas chromatography
HDL – high density lipoproteins
HEPT – height equivalent per theoretical plate
HPTLC – high performance thin layer chromatography
IL – ionic liquid
LA – linoleic acid
LC-PUFA – long chain polyunsaturated fatty acids
LDL – low density lipoproteins
MAG – monoacylglycerol
MS – mass spectrometry
PHFO – partially hydrogenated fish oil
PHVO – partially hydrogenated vegetable oil
PLC – preparative layer chromatography
POPs – persistent organic pollutants
PTSA – *p*-toluenesulfinic acid
rt – retention time
SPD – short path distillation
STF 1812 – stripped fish oil containing 18% EPA and 12% DHA
TAG – triacylglycerol
VKM – Norwegian Scientific Committee for Food Safety
VNT 1812 – winterized fish oil containing 18% EPA and 12% DHA

Contents

1 Introduction

In recent years, the importance for the general health of including sufficient long-chain omega-3 fatty acids in the diet and balancing the ratio between omega-6 and omega-3 fatty acids have received a lot of attention, both as subject for research and in the media. The term omega in relation to fatty acids refers to the carbon atom in the hydrocarbon chain furthest away from the carboxyl group. An omega-3 fatty acid has the last double bond inserted between the third and the fourth carbon atom counted from the omega end and likewise, the omega-6 fatty acid has the last double bond inserted between carbon six and seven from the omega end.

For mammals, including humans, the fatty acids (FA) linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3) are essential. Plants and phytoplankton are the only organisms with the enzymes to synthesize double bonds at the omega-3 and omega-6 site of the fatty acid, and are therefore the only ones who are able to synthesize LA and ALA. Every human need these fatty acids to function optimally. Our main sources of ALA are currently soybean oil, linseed oil and rapeseed oil (Gunstone 2012), while our main sources of LA are soybean oil, corn oil and safflower oil (Schmitz & Ecker 2008). From the omega-6 fatty acid LA, humans are able to synthesize arachidonic acid (ARA; 20:4n-6), and from the omega-3 fatty acid ALA, humans are able to synthesize eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). ARA, EPA and DHA are long chain polyunsaturated fatty acids (LC-PUFA), which are precursors of important signaling molecules (Calder 2009), and important for cell structure and function (Sidhu 2003). Both LA and ALA use the same elongation and desaturation enzymes (Schmitz & Ecker 2008), which presents a bottle neck to the production of ARA, EPA and DHA. If omega-6 fatty acids make up a larger proportion of our diet than omega-3 fatty acids then larger amounts of ARA will be produced than of EPA and DHA. In addition to the competition for the enzymes, the synthesis of ARA, EPA and DHA in mammals is slow compared to ingesting these fatty acids directly. A diet containing sufficient amounts these long chain polyunsaturated fatty acids is therefore recommended (Jain *et al.* 2015).

The physiological effects and characteristics of omega-6 and omega-3 fatty acids are quite different. The inflammatory activities of omega-6 derived signaling molecules are generally, but not always, recognized as more potent than the ones derived from omega-3 (Calder 2009), and it is therefore important with a balanced ratio between these two groups. Simopoulos (2006) claims that human beings evolved on a diet with, roughly, equal amounts of omega-6

and omega-3, but during the last century the western diet has shifted towards a ratio of at least 15 to 1. This shift towards a diet rich in omega-6 fatty acids may have a negative effect on several aspects of health, including diseases with an inflammatory component (Calder 2006). An excellent way to even out the ratio between omega-6 and omega-3 fatty acids is to consume fatty fish or take fish oil supplements. Consumption of seafood or marine oils rich in omega-3 fatty acids may also help prevent heart disease and help patients recover after such illnesses (Kris-Etherton *et al.* 2002; Salem *et al.* 2015). Kris-Etherton, Grieger and Etherton (2009) mention recommendations made by several health authorities, and most of them agree on two servings of fatty fish per week, equivalent to 250-500 mg of EPA + DHA per day, or consuming the equivalent amount of fish oil supplements.

The annual production of fish oil is relatively small compared to major vegetable oils like palm oil and soybean oil (1.1 versus 42.4, and 37.7 million metric tonnes, respectively, in 2007-2008) (Gunstone 2011). Out of this relatively modest production volume of fish oil, 75% is used for fish feed, 22% for human consumption (supplements and functional foods), and 3% for other uses (Tocher 2015). The production of fish oils can be divided into two steps; extraction of the oil from the fish or parts of it, and the refining of the fish oil. The different refining processes have different goals, like removal of undesirable taste and odor, removal of environmental pollutants, and change of fatty acid composition of the oil. Some of these processing steps, although meant to improve the quality of the fish oil, may have a detrimental side effects if used erroneously. Some of the processing steps apply a high temperature to the oil, thereby inducing the transformation of *cis*-configured double bonds into *trans*-configured double bonds.

Trans fatty acids have received a lot of attention since the 1990's because of their negative effect on health. Most of the attention has been devoted to the shorter, less unsaturated fatty acids, which have been shown to cause heart disease (Oomen *et al.* 2001), metabolic disease (Menaa *et al*. 2013) and inflammation diseases (Lopez-Garcia *et al*. 2005). A few studies (e.g Chardigny *et al.* 1995) have focused on *trans* isomers of EPA and DHA and found that they have different functions from their *cis* isomers. This is to be expected because the characteristics of *trans* and *cis* isomers are very different. It is therefore important to be able to analyze how much *trans* isomerization occurs under the processing of fish oils, and the *trans* content of the final product.

While there are official methods for quantification of *trans* isomers in milk fat and vegetable oils, there is no official method for the analysis of *trans* isomers in fish oil (Mjøs & Haugsgjerd 2011). The composition of fatty acids in fish oils is quite different from the

composition in milk fat and vegetable oils, as the former contains more of the longer and more unsaturated fatty acids than the latter two. This makes it difficult to apply exactly the same methods for analysis of the fatty acid composition. From 16 January 2014 the governmental health regulations in Norway states that no more than 2 g *trans* fatty acids per 100 g fat is allowed (Helse- og omsorgsdepartementet 2014).

The primary objective of this master thesis was to investigate whether *trans* isomers of EPA and DHA fatty acids were formed during normal processing conditions at the fish oil refining company Nordic Pharma Inc. The second objective was to establish a method for the analysis of *trans* fatty acids in fish oil. The third objective was to explore how temperature and time affected the formation of *trans* isomers of EPA and DHA.

2 Background

2.1 Fatty acids

2.1.1 The structure of fatty acids

Fatty acids are hydrocarbon chains with one methyl (-CH_3) end and one carboxyl (-COOH) end. They are often esterified to other molecules, forming for example triacylglycerols or phospholipids, depending on where they occur and what function they serve. A triacylglycerol has three fatty acids esterified to a glycerol molecule, whilst a phospholipid has two fatty acids esterified to a glycerol molecule, with a phosphate group attached to the third glycerol hydroxyl group.

The hydrocarbon chain of the fatty acids may contain no double bonds (saturated fatty acids) or they may contain one or more double bonds (unsaturated fatty acids). Fatty acids with two or more double bonds are called PUFA (polyunsaturated fatty acids). In nature the double bonds usually have a *cis* (Z) configuration, which means that the hydrogen atoms are found on the same side of the planar double bond (figure 1). The alternative are the *trans* (E) configuration, where the hydrogen atoms are found on the opposite sides of the planar double bond (figure 1). Most of the naturally occurring *trans* fatty acids are found in ruminants and are formed by bacteria in the rumen via biohydrogenation, whereas most of the *trans* fatty acids formed industrially comes from partially hydrogenated fats and oils. Carbon atoms with double bonds cannot rotate freely, so a spontaneous switch between the two isomers is not possible. However a shift will occur if enough energy is applied, for instance by heating the oil to a certain temperature. A *cis* double bond will turn into a *trans* double bond if the oil is heated to a high temperature since the *trans* double bond is energetically more favorable due to less steric strain.

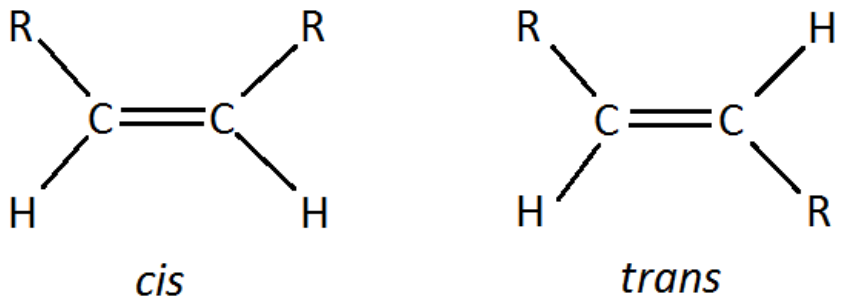

Figure 1. The difference between a *cis* and a *trans* double bond. The straight lines are the bonds, C = carbon, H = hydrogen, and R = the hydrocarbon chain.

The double bonds affect the structure of the fatty acids. A *cis* double bond will give the hydrocarbon chain a bend, while a *trans* double bond will not. This bending of the structure has implications for the melting point of the fatty acids, because the *trans* fatty acids can pack more tightly together so they crystallize more easily. Generally, the longer the carbon chain in the fatty acid, the higher the melting point, but an unsaturated fatty acid will have a lower melting point than its saturated counterpart. For fatty acids with the same chain length, *cis* unsaturated fatty acids have the lowest and saturated fatty acids the highest melting point. In between these two, but closer to the saturated fatty acid lay the *trans* unsaturated fatty acids melting points.

There are several ways to name fatty acids. Sometimes the trivial name is used, e.g. eicosapentaenoic acid (EPA) and sometimes the number of carbons and double bonds, e.g. C20:5n-3, is used. This nomenclature tells us that there are 20 carbon atoms in the chain, with five double bonds where the last double bond occurs at the omega-3 site. Another way of naming fatty acids is by the IUPAC system, (5Z, 8Z, 11Z, 14Z, 17Z)-5,8,11,14,17-eicosapentaenoic acid. The first two mentioned will be the ones used in this thesis. When referring to *trans* isomers the number of *trans* double bonds will be indicated before the trivial name, e.g. mono-*trans* EPA, which implies one *trans* double bond. The IUPAC name could have been used, e.g. (5Z, 8Z, 11Z, 14Z, 17E)-5,8,11,14,17-eicosapentaenoic acid which specifies EPA with a *trans* double bond at the omega-3 site of the hydrocarbon chain.

2.1.2 The roles of fatty acids in the body

Fats and oils are a good source of energy in the cell respiration, and have a higher energy yield per carbon atom than carbohydrates (Campbell & Farrell 2008). But energy generation is not the only function of the fatty acids; they also have important structural and physiological functions. Particularly important fatty acids are the essential linoleic acid (LA) and α-linolenic acid (ALA). Only plants and phytoplankton are able to synthesize them, because they are the only organisms with the enzymes delta-12- and delta-15-desaturase, which insert double bonds at the omega-6 and omega-3 sites in the C18 fatty acid chain (Napier & Sayanova 2005). An overview of the synthesis can be seen in the figure 2.

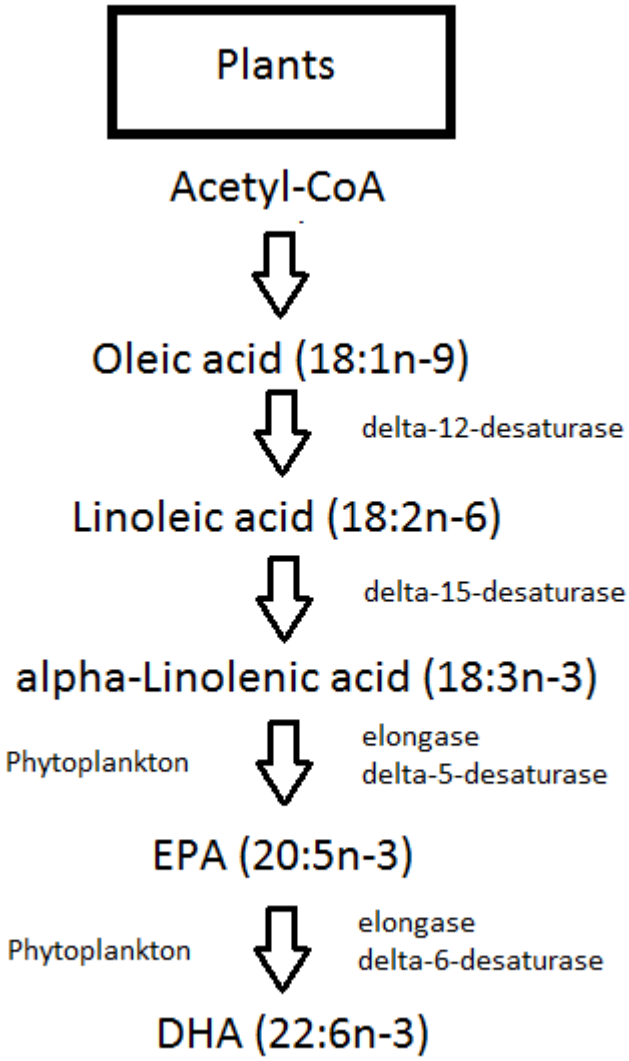

Figure 2. An overview of the enzymatic pathway for the insertion of double bonds at the omega-6 and omega-3 sites of the fatty acid hydrocarbon chain in all plants, and the ability of phytoplankton to produce the longer and more unsaturated fatty acids EPA and DHA. Delta-12- and delta-15-desaturase insert double bonds at the omega-6 and omega-3 carbons, respectively, of the 18 carbon fatty acid.

When mammals ingest LA and ALA they can synthesize longer and more unsaturated fatty acids. This is done through a series of enzymes, known as desaturases and elongases (figure 3). LA or ALA are ingested and stored before being metabolized in cells. First the delta-6-desaturase inserts an extra double bond into the fatty acids, and then the chain is elongated to a 20-carbon chain. After the elongation, a new double bond is introduced into the carbon chain via delta-5-desaturase. At this point, LA and ALA have been turned into ARA and EPA, respectively. Through further elongation, via two steps involving elongases, the carbon chain now consists of 24 carbons. At this point, delta-6-desaturase introduces yet another double bond before a β-oxidation cycle removes two carbon atoms from the chain and DPA (n-6) and DHA are produced. The process of synthesizing ARA, EPA and DHA in

mammals is slow compared to marine algae. There is a competition between omega-3 and omega-6 fatty acids for the enzymes involved (Calder 2014), and therefore it is recommended that humans get supplementary EPA and DHA from their food.

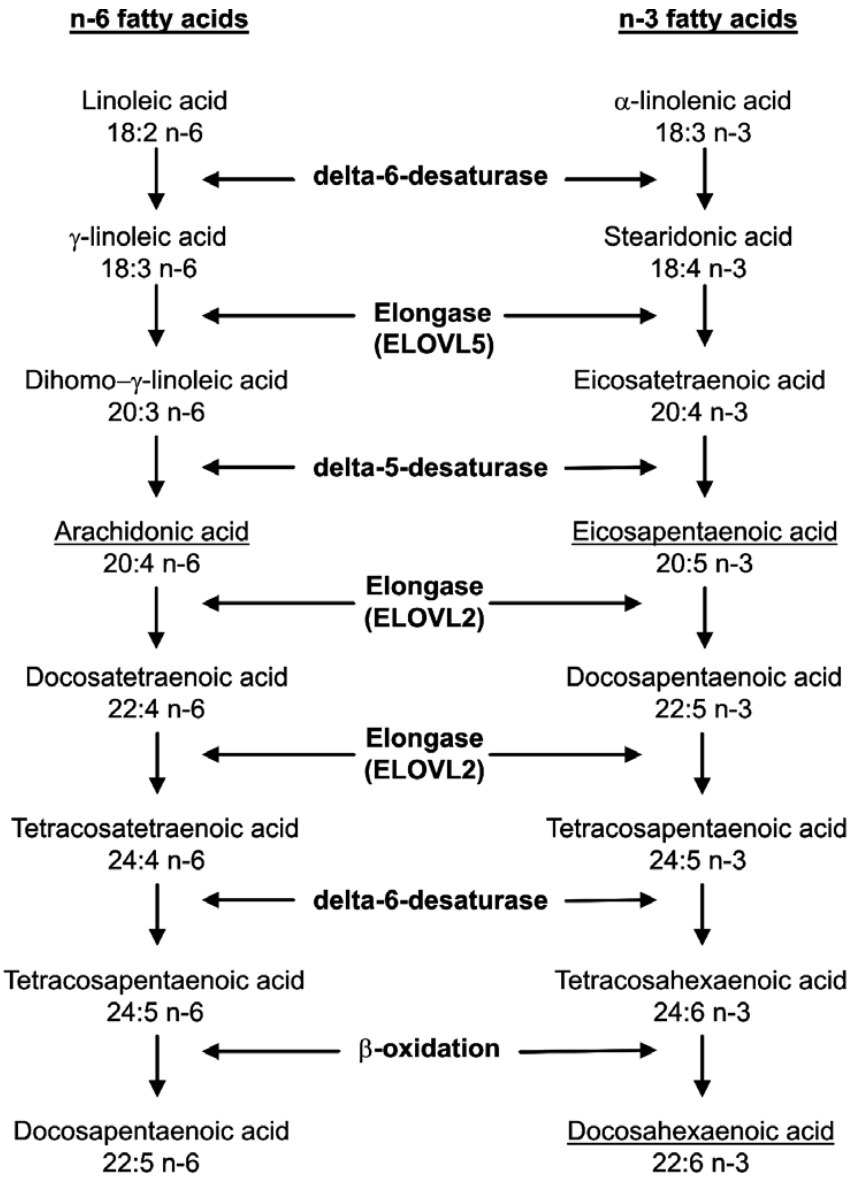

Figure 3. The enzymatic pathway of synthesizing LC-PUFA from LA and ALA. ELOVL = elongation of very long chain fatty acid enzyme (modified from Schmitz and Ecker (2008)).

2.1.2.1 Signaling molecules

Several diseases have an inflammatory component, and it is important that the body is capable of properly regulating and adjusting the inflammatory responses to maintain homeostasis (Calder 2009). LC-PUFAS are the precursors of many substances involved in inflammation processes. One of these lipid derived substances is the group of eicosanoids, consisting of prostaglandins, thromboxanes and leukotrienes (Wall *et al*. 2010). Eicosanoids are synthesized from 20-carbon PUFA, usually ARA and EPA (figure 4). The synthesis involves

cyclooxygenase and lipoxygenase enzymes. This is yet another example where n-3 and n-6 fatty acids compete for the same enzymes. Eicosanoids derived from ARA are usually more potent than the ones derived from EPA, and often the EPA-derived eicosanoids are referred to as anti-inflammatory (Calder 2012). This effect might be ascribed to the competition for enzymes. The more EPA that is present the less cyclooxygenase will be available for ARA and hence, there will be milder inflammatory reactions. Other groups of molecules involved in inflammation are resolvins, protectins (figure 4), lipoxins and maresins, which are also synthesized from LC-PUFAs. These substances have anti-inflammatory functions, as well as being tissue protective and resolution stimulating (Bannenberg & Serhan 2010).

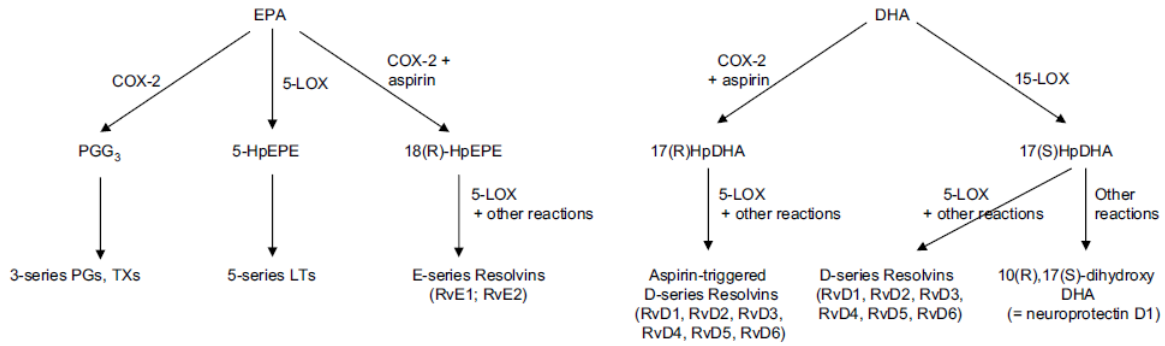

Figure 4. The synthesis of eicosanoids, resolvins and protectin via cyclooxygenase (COX) and lipoxygenase (LOX) from EPA and DHA, with and without aspirin present (Calder 2009).

Peroxisome proliferator activated receptors (PPARs) are closely connected to lipids, and play a part in regulating inflammatory responses. The PPARs can interpret fatty acid signals from dietary lipids, pathogenic lipoproteins and essential fatty acid metabolites, and respond accordingly (Varga *et al.* 2011).

2.1.2.2 Structural fatty acids

DHA is an important fatty acid in the cell structures of the brain, retina, and spermatozoa because of its physiochemical properties. For proper development of organs such as the brain and eyes, one needs adequate amounts of omega-3 fatty acids from the diet, or else DHA will be replaced by the omega-6 fatty acid docosapentaenoic acid (DPA) (Connor *et al.* 1992). Studies on the effect of omega-3 deficiency in rhesus monkeys have shown that low levels of DHA leads to reduced function of the eyes (Neuringer *et al*. 1986). The reason for this might come from DHA being more flexible than DPA, which may affect membrane proteins such as rhodopsin (Eldho *et al*. 2003). Rhodopsin is a G-protein coupled receptor (GPCR) in the retina, which is light sensitive. Feller & Gawricsh (2005) found that flexibility and adaptability in the lipid bilayer of the cell favored the activation of GPCR rhodopsin. The flexibility and adaptability is enhanced when DHA is present. It seems that omega-3

deficiency is most harmful for the development of eyes and brain for fetuses and infants, because the nervous system is good at retaining DHA (Salem *et al*. 2001).

2.1.2.3 Trans fatty acids

As mentioned, *trans* fatty acids are unsaturated fatty acids with one or more double bonds having a *trans* configuration. The *trans* configuration is energetically favored compared to *cis*, because of steric strain. The larger carbon chains come very close to each other in the *cis* configuration while in the *trans* configuration, they are further apart (McMurry 2008). In nature, the double bond of fatty acids are synthesized as *cis*. The double bond cannot freely switch between cis and trans, but if enough energy is applied (e.g. high temperature) the fatty acid can be converted to its more energetically stable *trans* isomer. The activation energy for this reaction can be reduced in the presence of a strong acid (McMurry 2008). In thermal isomerization, there is mostly a switch between *cis* and *trans* as the double bond does not change its position (Sciotto & Mjøs 2012). This means that EPA, that has five double bonds, can have 2^5 or 32 different isomers after thermal isomerization.

Most of the research on the health impacts of *trans* fatty acids has focused on the mono- and polyunsaturated isomers of 16 and 18 carbon chain fatty acids. They have been found to increase the risk of heart disease (Oomen *et al*. 2001, Laake *et al*. 2012). Intake of *trans* fat increases the plasma activity of cholesteryl transfer protein, the enzyme that transfers HDL cholesterol to LDL and VLDL cholesterol, and so, the ratio of LDL to HDL cholesterol in blood serum gets higher. This is a predictor of coronary heart disease (CHD) risk (Mozaffarian *et al*. 2006). Almendingen *et al*. (1995) compared partially hydrogenated fish oil (PHFO) to partially hydrogenated soy bean oil (PHSO) and butter and saw how these affected the serum lipoproteins and Lp[a]. This study reported that PHFO had the worst effect of all three on cholesterol levels, and the authors suggested that this effect might come from the LC-PUFA *trans* isomers, but could not conclude on this. Laake *et al* (2013) found that *trans* fatty acids from PHFO increased the risk of cancer, whereas *trans* fatty acids from partially hydrogenated vegetable oil did not. This article hypothesized that *trans* isomers of EPA and DHA were the cause of increased risk of cancer because of metabolic effects caused by their chemical structure, and that modified eicosanoids might be produced from these isomers. It has also been found that *trans* fatty acids might have a negative effect on endothelial function and inflammation. Lopez-Garcia *et al*. (2005) found higher levels of biomarkers related to inflammation and endothelial dysfunction in people with diets containing higher levels of *trans* fatty acids.

Trans isomers of fatty acids may also be recognized as other fatty acids with fewer double bonds. EPA with a *trans* double bond at the omega-3 site might for example be recognized as ARA. In an experiment focusing on the effects of mono-*trans* isomers of EPA on bovine endothelial cells, Loi *et al*. (2000) found that they were incorporated into cells and were metabolized by cyclooxygenase, like ARA and EPA, thus inhibiting the synthesis of prostacyclin. This inhibition could come from the isomers of EPA competing for the cyclooxygenase pathway and/or that incorporation of these isomers resulted in less ARA being available.

DHA is, as previously explained, important for the development and functionality of the central nervous system and the eyes. It has been found that the eyes of rats fed *trans* isomers of ALA are susceptible to *trans* isomers of DHA (Acar *et al*. 2006). The incorporation of these isomers into the eyes reduces the eyes' functionality.

2.2 Industrial Processing and Refining of Fish Oil

The fish meant for fish oil production is first caught and stored aboard fishing boats, then cooked and pressed. This results in crude fish oil which can be used for example, for fish feed. For crude fish oil to be suitable for human consumption the crude fish oil has to undergo several processing steps. Many different products of fish oil are available on the market; the natural types such as cod liver oil and Anchoveta oil, or concentrates having various compositions of EPA and DHA. Fish oils often contain numbers in their names, like "1812" or "3020", these names refer to the fraction of EPA and DHA in the fish oil. The "1812" oil contains 18% EPA and 12% DHA, likewise the "3020" contains 30% EPA and 20% DHA. The different processing steps (figure 5) are applied to both concentrates of fish oil (red arrows) and 1812 fish oils which have the fatty acid composition common to fish caught outside South America (blue arrows). The processing is done to enhance the quality and edibility of the oil. In this section, the most common processing steps will be described, with emphasis on the steps that may involve the formation of *trans* isomers of EPA and DHA.

Neutralization and washing involves heating the oil to 80-90 °C and mixing it with caustic soda, before adding hot water. This removes free fatty acids (FFA), reduces the content of phospholipids, pigments, trace metals, and water soluble components like free amino acids, oxidation products, trace metals, and soap residuals (VKM 2011).

Winterization removes components with high melting points, e.g. waxes and the stearin fraction. By reducing the temperature to 0-2 °C these high melting point components crystallize and settle, so they can be filtered from the oil (VKM 2011). Samples from the olein fraction after the winterization process are named VNT 1812 in this thesis.

The bleaching process utilizes the adsorptive properties of activated carbon, bleaching earth, and silica to remove pigments (De Greyt 2012), reduce the amount of FFA, oxidation products (García-Moreno 2013), trace metals (Bimbo 2012), and environmental pollutants (Usydus *et al*. 2009, Ortiz *et al*. 2011). Activated carbon and bleaching earth are added to the oil and the mixture is heated (VKM 2011). The negative consequences of bleaching is the removal of the antioxidants that are naturally present in the fish oil, but bleaching improves the oxidative stability of the oil, and antioxidants can be added to the oil in later processing steps, such as final refining and formulation. Samples from the bleaching process are named BLF 1812 in this thesis.

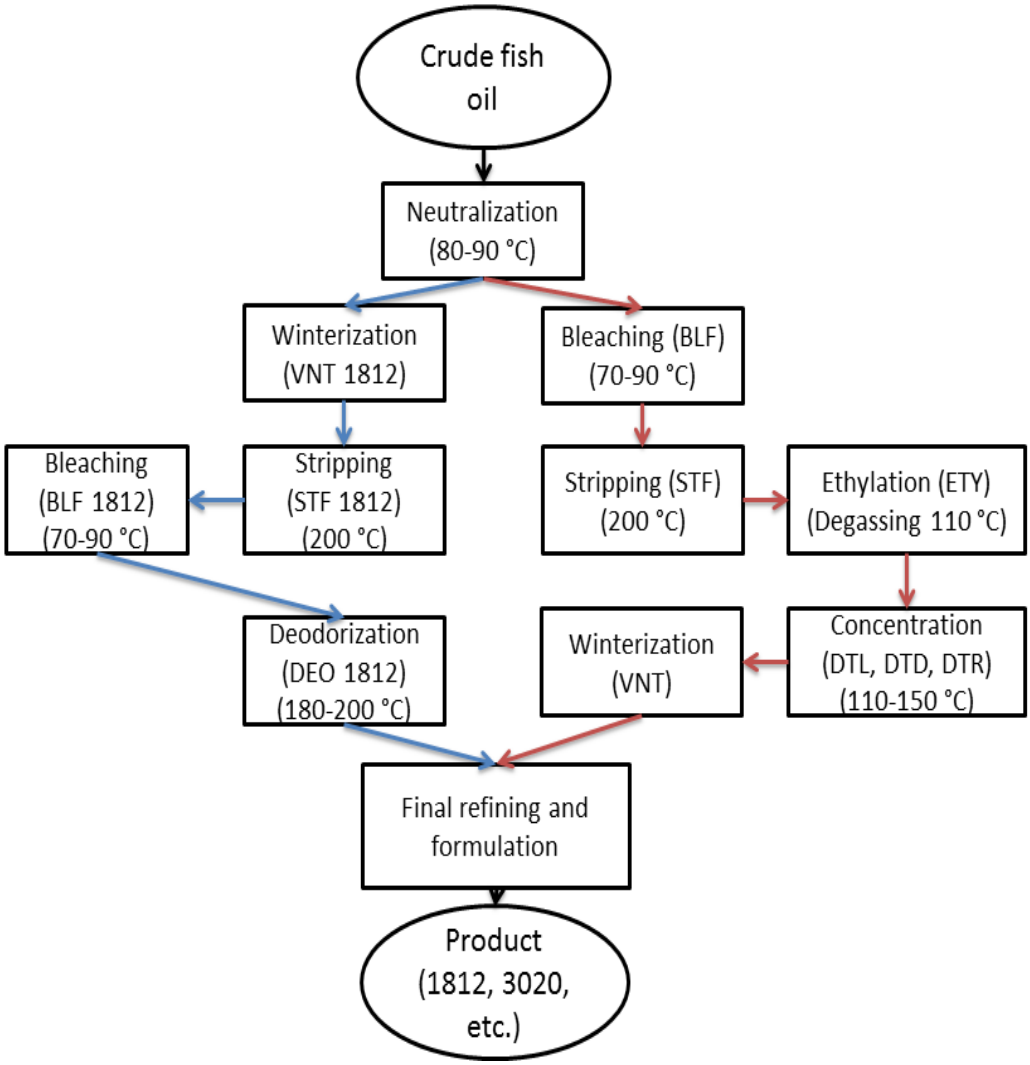

Figure 5. Flowchart showing the processing of fish oils. The black arrow shows processes that are necessary for all the different products. The red arrow shows the processing pathway for concentrates. The blue arrow shows processing pathway for "natural" oils. 1812 implies 18% EPA and 12% DHA, while 3020 implies 30% EPA and 20% DHA.

Deodorization combines high temperature, low pressure and steam to remove volatiles from the oil by continually removing the gas phase. These volatiles include FFA and oxidation products (Crexi *et al*. 2009). The temperature range is between 150-250 °C, which are quite high temperatures. Fournier *et al*. (2006 B) found that *trans* isomers of EPA and DHA were formed at temperatures above 180 °C, at first only mono-*trans*, but at higher temperatures (220, 250 °C), di- and tri-*trans* fatty acids were formed, as well (Fournier *et al*. 2006 A). Fournier *et al.* (2006 A) also found that EPA and DHA were isomerized to a much higher degree than LA and ALA when they compared their results with other studies. Mjøs & Solvang (2006) found that the fatty acids were more isomerized by increasing number of double bonds, giving DHA>EPA>ALA>LA in fractions of *trans* isomers formed. Samples from the deodorization process are named DEO 1812 in this thesis.

Short path distillation (SPD) can be used for a number of different tasks, e.g. removal of persistent organic pollutants (POPs) (Olli *et al.* 2013), removal of other volatile compounds, such as aldehydes, ketones, etc. (VKM 2011), and for concentration of EPA and DHA. In the fish oil industry the SPD unit consists of two columns, their functions will be described in the following paragraphs. Samples from the SPD stripping process are named STF 1812, and samples from the SPD concentration process are named distillate light fraction (DTL), distillate residual fraction (DTR) and distillate-distillate fraction (DTD).

For purification purposes (removal of POPs and other unwanted volatile compounds) the bleached fish oil is run through one SPD-column. In the column the fish oil is heated under vacuum (>0.001 mbar). The impurities will evaporate from the BLF and condense on the colder inner surface of the column. The high temperature applied in this process may remove desired compounds such as anti-oxidants and induce isomerization of PUFAs (Oterhals & Berntsen 2010). The two fractions, condensate and residue, are collected for further treatment/processing. The product (STF) is the residue fraction.

For concentrating purposes, the SPD unit has to be able to separate EPA and DHA from other fatty acids present in the oil, so TAGs must be esterified to fatty acid ethyl esters (FAEE). By mixing TAG with ethanol and the catalyst sodium ethylate, glycerol, FAEE, some DAG (< 6%) and MAG (< 4%) are formed (VKM 2011). Samples taken after the ethylation process are named ETY2412 (24% EPA and 12% DHA). The shorter FAEEs and the heavier DAGs and MAGs have different boiling points from EPA and DHA. Hence by adjusting temperatures, vacuum and flow in and through the two SPD columns in series the desired proportions of EPA and DHA will be obtained by removal of the volatile fraction in the first column and the heavy fraction in the second column.

After the concentration process, the FAEE might be re-esterified to TAG. This is done in part because TAG is considered more natural (VKM 2011) and because the oxidative stability of the fatty acids in TAG is better than in the form of FAEE. To re-esterify the fatty acids to TAG one mixes FAEE with glycerol in the presence of a catalyst or an enzyme. The outcome is mostly TAG, but also some DAG, MAG and FAEE.

2.3 Analysis of fatty acids

There are several ways to analyze fatty acids, depending on the purpose of the investigation. In this section two of the most common methods for analysis of fatty acid isomers will be described.

2.3.1 Chromatography

Both methods applied in this study are chromatographic methods. Chromatography is a separation method. Common to all chromatographic systems are the mobile and stationary phase. Analytes can be separated due to differences in the solubility or affinity to either the stationary or mobile phase. For example two components, X and Y, are present in a sample, X has a high and the Y has little affinity to the stationary phase, they are both soluble in the mobile phase. In this sample X will be retained more strongly than Y, and so Y will move further than X in a given time. In thin layer chromatography (TLC), this difference is referred to as the retention factor. In for example gas chromatography (GC), the difference in speed of travel for the components is referred to as retention time.

2.3.2 Silver ion chromatography

L.J. Morris (1966) published a review on the analysis of lipids using silver ion chromatography, where he discussed the history, mechanisms, and the uses. The method of separating lipids with silver ion chromatography was developed in the early 1960's, and rapidly became a popular field of research. The use of transition metals for separating lipids by number, type, and position of double bonds was an excellent tool for scientist in the field of lipids. Among the transition metals, silver has some major advantages. It is relatively inexpensive compared to e.g. gold, and compared to the other, less expensive, transition metals such as copper it is more stable (AOCS 2011).

The Dewar concept is generally accepted as the explanation of the mechanisms controlling the separation of unsaturated compounds (figure 7) (Morris 1966; Nikolova-Damyanova 2009). A carbon-carbon double bond consists of two sp^2-hybridized carbon atoms (figure 6). In the sp^2-hybridized carbon atom the 2s orbital combines with two of the three available 2p orbitals, forming three hybrid sp^2-orbitals and leaving one 2p orbital. The three sp^2 orbitals lie flat with an angle of 120 ° to each other, while the 2p orbital is situated at a 90 ° angle to the plane. In the carbon-carbon double bond there is a strong sp^2-sp^2 σ bond and a weaker 2p-2p π bond. In an unsaturated fatty acid the remaining sp^2 orbitals are bonded to either hydrogen or carbon atoms. The σ bond is centered between the nuclei of the carbons, while the π bond occupies the regions above and below the σ bond. In a single bond the

carbon atom is sp^3 hybridized. The sp^2 hybridized carbon atom attracts electrons more strongly than sp^3 hybridized carbons, which means that surrounding sp^3-hybidized carbon atoms gives a charge to the sp^2 hybridized carbon atoms.

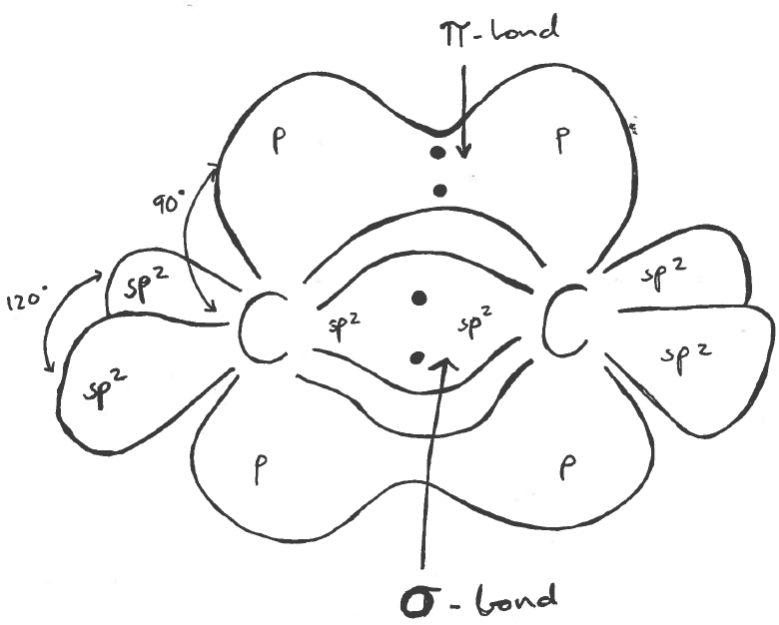

Figure 6. Two sp^2 hybridized carbon atoms bound together through a σ-bond and a π-bond. It also shows the angle between sp^2 orbitals and p orbitals.

When unsaturated compounds bind with silver ions a σ-bond is formed by overlap of the filled π orbital of the carbon-carbon double bond with the free s orbital of the silver ion, and a π bond formed by overlap of the vacant antibonding π orbital of the carbon-carbon double bond with the filled d orbitals of the silver (figure 7). The bonding is reversible, and will be affected by the availability of electrons in the filled orbitals and the ease of overlap between these orbitals. This is determined by steric factors, and therefore one can separate different compounds by number, position, and type of the double bonds (Morris 1966).

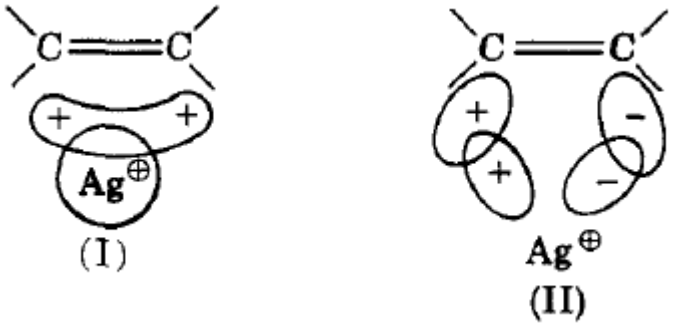

Figure 7. The Dewar concept. Figure is taken from Morris (1966).

As mentioned, the availability of electrons is important to the binding between silver ions and carbon-carbon double bonds. In *cis* double bonds, the electrons are more available than in the *trans* double bonds because of steric factors (larger carbon chains hiding the electrons in the *trans* configuration), causing *cis* isomers of one compound to be more strongly retained than its *trans* isomer (Nikolova-Damyanova 2009). The position of the double bond plays an important role as well; Gunstone *et al*. (1967) proved that fatty acids of the same length and same unsaturation but with the double bond at different positions had different retention factors. This showed that the fatty acid interacted with two different components of the stationary phase; the silver ions form a complex with the carbon-carbon double bond, while the silica particles of the stationary phase forms a complex with the oxygen in the ester bond of the fatty acid methyl ester. For both complexes to form there must be a certain distance between the double bond and the ester, i.e. if the double bond is placed too close to the ester bond the component will elute faster than if it is placed a bit further away. A double bond placed at carbon number six from the ester group is retained most strongly (Gunstone *et al*. 1967). The mobile phase should also be considered, as it will increase or decrease the retention time of the analyte in the chromatographic system according to its polarity. The most common mobile phases usually consist of a mixture between two of the following components; toluene, methanol, chloroform, diethyl ether and others. Silver ion chromatography for separation of unsaturated fatty acids should be performed at low temperatures, because the stability of the silver ion-double bond complex increases with decreasing temperature, and a temperature of about -20 ° C was found to be the best (Morris *et al.* 1967).

Chromatography with silver ions in the stationary phase can be performed using TLC or HPLC. It is not suitable for GC because of the low temperatures needed. Silver ion HPLC has a couple of drawbacks as well. Firstly the column is rapidly deteriorating and therefore the retention time of the analytes will change over time making it difficult to automate this method (Fournier *et al*. 2006 A). Secondly an HPLC system is quite expensive compared to the much cheaper TLC plates. Pre-impregnated TLC plates can be bought, but can just as easily be made in the laboratory.

2.3.3 Gas chromatography

The gas chromatography (GC) system can be used to analyze substances that are volatile or can be made volatile by heating. A schematic overview of a GC system is shown in figure 8. As with other chromatographic systems there is a mobile and a stationary phase that is used to

separate the analytes. The mobile phase in the GC is a gas, called the carrier gas. This gas must be inert. The gas is delivered from a high pressure cylinder and passes through a reduction valve that reduces the pressure before it enters the GC instrument where the flow and temperature are regulated to make sure that a constant inlet pressure is maintained. The gas goes via an injector that is either a split/splitless injector or a "cool on column" injector. Split/splitless injectors are the most common and makes it possible to decide whether one wants the whole sample to be injected onto the column or just a small fraction of it. These injectors are heated so the sample turns into gas immediately after it is injected. From the injector, the sample moves into the column, which is a long tube made of fused silica or metal, where the inside wall is lined with a stationary phase. There are lots of different stationary phases that can be used, and they can be non-volatile liquids or solids. The solid stationary phases are made from polymers and the analytes are separated by differences in adsorption to the surface of the polymer or by sieving through pores in the polymer. Non-volatile organic liquids are the most popular stationary phases; here the analytes are separated by differences in distribution between the gas phase and the stationary liquid phase. The column is placed in an oven with a fan; this provides good circulation and an even distribution of the heat. The detector, which is situated at the end of the column, often is a flame ionization detector (FID). The temperature of the detector is often a bit higher than the temperature in the oven to make sure analytes do not condense in the detector. In the FID, there is a flame tip and a constant stream of hydrogen, air and a make-up gas. When the analyte enters the FID it reacts with the flame and the hydrogen and ions are formed, the electrical tension is measured and a signal is sent to a computer.

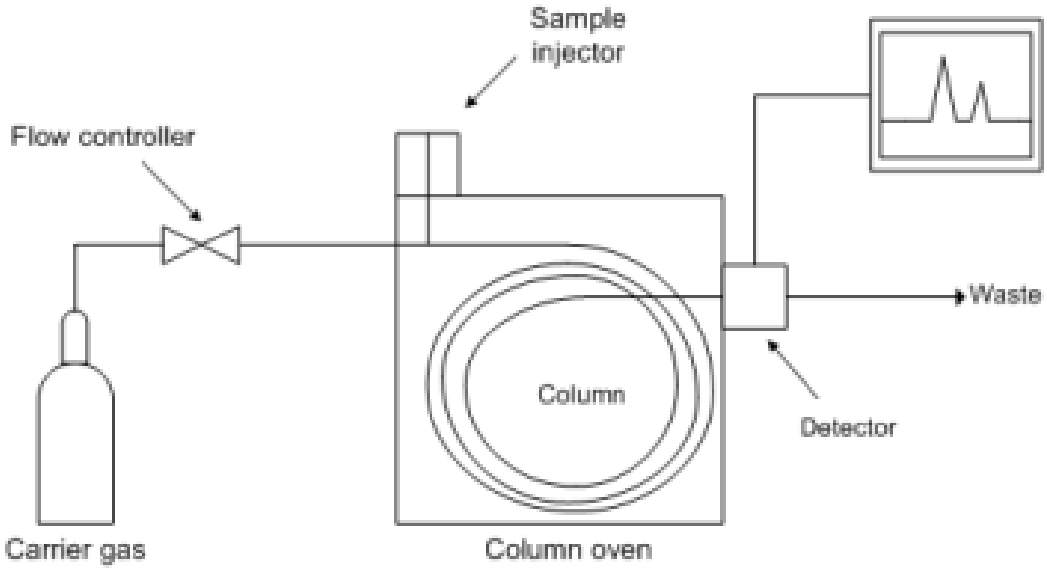

Figure 8 Schematic overview of the GC system. Picture taken from http://www.chromatographer.com/gas-chromatography/ (11/03/2015)

The main parameters deciding the retention time of the analytes are:

1. Temperature is the most important parameter. The analytes can only move forward in the column when they are in their gas phase. An increase in temperature increases the volatility of the substances. A higher temperature drives the distribution towards the gas phase instead of the stationary phase, and the retention time is reduced. The temperatures in the GC can be regulated, and one has the choice of an isothermal run or temperature programming. In an isothermal run, the temperature is kept constant from start to finish. This works very well when the analytes in the sample are similar to each other in size and volatility, but can produce broad peaks when some analytes in the sample are much less volatile than the others. A temperature program is often used, and the temperature is increased throughout the GC run, producing peaks with better shapes when there are big differences between the volatility of the substances.
2. Type and amount of stationary phase is important for the retention time of analytes, because different analytes have different solubility in different stationary phases. The stationary phase is made from temperature stable liquids with very low vapor pressure. The basis of the stationary phase is often polysiloxanes or polyethylene glycols with various functional groups attached, depending on the desired characteristics. The solubility of the analytes in the stationary phase is dependent on these characteristics. A polar substance will for example be more soluble in a polar stationary phase than in an apolar stationary phase, due to intermolecular interactions. One should not operate the GC outside the maximum or minimum temperatures of the column. The first case will lead to the destruction of the stationary phase and the latter case will lead to the stationary phase becoming more viscous and the analytes will travel too slowly through the column, leading to broad peaks. Alternative columns for the analysis of *trans* fatty acids will be discussed in detail later.
3. Column dimensions are also very important. Columns can be either capillary or packed. Nowadays, the capillary columns are most widely used. They are most often made of fused silica which is inactive and very robust. The inner diameter ranges from 20 to 500 µm. The stationary phase sits as a thin film on the inside wall of column, and the thickness of this film is 0.05-10.0 µm. Analytes are retained more strongly in thick films than in thin films. Capillary columns are very effective in separating analytes, and the longer the column the better the separation. However, a column with twice the length does not have twice as good separations. One must decide whether

increasing column length is worthwhile because the analysis time increases with increasing column length.

4. Type and speed of carrier gas affects the separation of the analytes. The carrier gas must not react with the analytes in the sample or the stationary phase. Nitrogen, helium and hydrogen can all be used, and they have different uses because of different optimum velocities. If the speed of the gas is too low or too high the peaks in the chromatogram will become broader. Nitrogen is mostly used in packed columns because the optimum velocity for this gas is very low compared to the other gases (figure 9). Both helium and hydrogen works well at high velocities, and has broad ranges of optimum velocities. Hydrogen is best at the highest speeds, but helium is used more often for safety reasons.

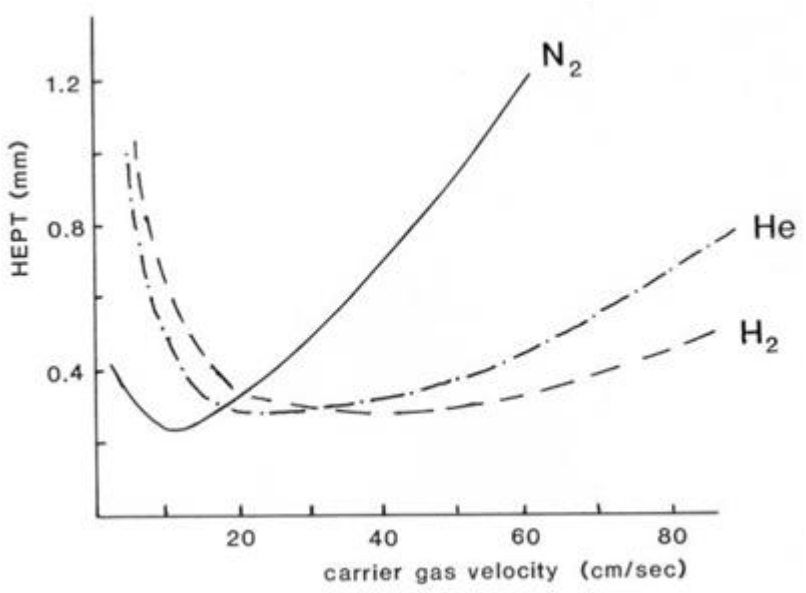

Figure 9. van Deemter plot for nitrogen, helium, and hydrogen. The Y-axis show the height equivalent to a theoretical plate (mm) and the X-axis has the average velocity of the gas (cm/sec). A lowest possible height equivalent per theoretical plate (HEPT) is desired to obtain the best separation between the analytes.

Several different columns have been used for the analysis of *trans* fatty acids in fish oil. The results have been variable considering the separations of isomers of EPA and DHA. The current method, recommended by AOCS, for determination of *trans* isomers in fats and oils from non-ruminant sources (AOCS Ce 1h-05), recommends using the highly polar BPX-70, CP-Sil88, or SP-2560 columns. Ratnayake *et al*. (2006) evaluated this method and found that, under the right chromatographic conditions, the CP-Sil88 and SP-2560 could separate the different isomers of the 18:1, 18:2, and 18:3 fatty acids. This means that this method is suitable for analysis of *trans*-isomers in vegetable oils and fats. However this evaluation did

not study the longer, more unsaturated fatty acids that are present in fats and oils of marine origin. According to Mjøs & Solvang (2006), the longer and more unsaturated fatty acids are isomerized much faster than the shorter ones, so in oils of marine origin, one would expect to find more *trans*-isomers of EPA and DHA than *trans*-isomers of LA and ALA. Therefore, the columns must also be able to separate the peaks of the different EPA and DHA isomers. By using a 60 m BPX-70 column, Sciotto & Mjøs (2012) found that the mono-*trans* isomers of EPA eluted as three peaks, where one of the peaks co-eluted with a different fatty acid, the mono-*trans* isomers of DHA eluted as five peaks with no overlap with other fatty acids. Fournier *et al*. (2006 A) used a 100 m CP-Sil88 column for determination of *trans*-fatty acids in deodorized fish oil and were able to obtain four peaks for the mono-*trans* isomers of EPA and five for DHA, but one of the *trans* EPA peaks overlapped with the all-*cis* EPA peak. The authors concluded that a better stationary phase for analyzing *trans* isomers in fish oil was needed.

The SLB-IL111 column from Supelco (Bellefonte, PA, USA) is the most polar column commercially available (Ragonese *et al*. 2012), and it is therefore of interest for the analysis of *trans* fatty acids. An ionic liquid (IL) is defined as salts that are liquids below an arbitrary temperature (Weber & Anderson 2014). One of the advantages of IL stationary phases is their low volatility and high thermal stability (Twu *et al*. 2011), and this allows for separation of compounds that require a high temperature to evaporate. ILs also have a high peak capacity so that complex samples can be analyzed with little or no overlap between peaks (Ho *et al*. 2013). The mechanisms of interactions between analyte and the stationary phase that are most important in the SLB-IL columns have been thoroughly studied, using both Rohrschneider/McReynolds constants and Abrahams solvation parameter model. There are a couple of different IL columns available, and Weber & Andersson (2014) found that a significant difference between these columns is the hydrogen-bond acidity. This study also reported that the most important factor for separations in these columns is the hydrogen-bond basicity. Another interesting thing is the ability of ILs to separate non-polar molecules, meaning that ILs can act as relatively non-polar stationary phases when the analytes are non-polar (Anderson *et al*. 2002). The structure of the stationary phase in SLB-IL111 (1,5-Di(2,3-dimethylimidazolium)pentane bis(trifluoromethylsull)imide) can be seen in figure 10. SLB-IL111 is the column utilized in the present study. The imidazolium in the stationary phase increases the interaction with polar compounds, such as π-electrons in double bonds (Zeng *et al*. 2013) which makes it possible to separate *cis* and *trans* isomers of fatty acids. The SLB-

IL111 column also show dipole-dipole and dipole induced dipole interactions, along with cavity formation and dispersion interactions (Zeng *et al*. 2013).

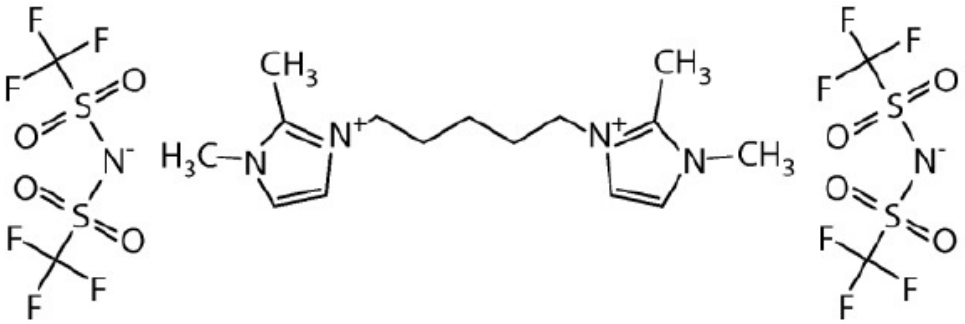

Figure 10. The structure of the stationary phase in the SLB-IL111 column (1,5-di(2,3-methyl imidazolium)pentane bis(trifluoromethylsulfonyl)imide). From: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/Posters/1/ISCC_2013-Ionic-Liquid-GC.pdf

A couple of studies on separations of *trans* fatty acids have been performed utilizing this column, with promising results. Delmonte *et al*. (2012) managed to separate the *trans* isomers usually found in milk fat using a 200 meter SLB-IL111 column, and Fardin-Kia *et al*. (2013) managed to separate 125 FAMEs from menhaden oil using the same GC conditions. By applying the same conditions yet again, Srigley & Rader (2014) were able to separate five peaks of mono-*trans* EPA and four peaks for mono-*trans* DHA, where one of the DHA-isomer peaks co-eluted with another fatty acid. The GC conditions applied in these studies were optimized for milk fat, and could perhaps be improved.

3 Materials and methods

3.1 Chemicals, standards and equipment

Table 1 list of chemicals, standards and equipment. SRM 3275, GLC 68D and supelco 37 collectively contained C14:0, C15:0, C14:1, C16:0, C17:0, C16:1n-7, C18:0, C17:1n-11, C18:1n-9t, C18:1n-9c, C18:1n-7, C18:2n-6t, C18:2n-6c, C20:0, C20:1n-11, C20:1n-9, C21:0, C18:3n-6, C18:3n-3, C22:0, C20:2n-6, C22:1n-9, C20:3n-6, C20:3n-3, C20:4n-6, C24:0, C22:2, C24:1, C20:5n-3, C22:5n-3, C22:6n-3.

Chemicals	**Quality**	**Supplier**
1,4-dioxane	99,8%	Sigma-Aldrich (St. Louis, MO, USA)
2',7'-dichlorofluorescein solution	<10% in isopropanol	Sigma-Aldrich (St. Louis, MO, USA)
Acetonitrile	≥ 99.9%	Sigma-Aldrich (St. Louis, MO, USA)
BHT		Sigma-Aldrich (St. Louis, MO, USA)
Copper(II)sulfate-pentahydrate		Merck (Darmstadt, Germany)
Diethyl ether	≥ 99.8%	Sigma-Aldrich (St. Louis, MO, USA)
Hexane	≥ 99%	Sigma-Aldrich (St. Louis, MO, USA)
Hydrochloric acid	≥ 37%	Sigma-Aldrich (St. Louis, MO, USA)
Isooctane	≥ 99%	Merck (Darmstadt, Germany)
Methanol	≥ 99.8%	Sigma-Aldrich (St. Louis, MO, USA)
Na_2SO_4		VWR Chemicals (Leuven, Belgium)
Phosphoric acid	≥ 85%	Sigma-Aldrich (St. Louis, MO, USA)
Silver nitrate		VWR Chemicals (Leuven, Belgium)
Sodium *p*-toluenesulfinate	95%	Sigma-Aldrich (St. Louis, MO, USA)
Sodium methylate	30% in methanol	Merck (Darmstadt, Germany)
Toluene (anhydrous)	99.8%	Sigma-Aldrich (St. Louis, MO, USA)
Standards	**Quantity**	**Supplier**
FAME EPA	1 g	Nu chek prep (Elysian, MN, USA)
FAME DHA	1 g	Nu chek prep (Elysian, MN, USA)
FAME C23:0	1 g	Nu chek prep (Elysian, MN, USA)
SRM 3275 (FAME standard)	7.2 ml	National institute of standards and technology (NIST)
GLC 68D (FAME standards)		Nu chek prep (Elysian, MN, USA)
Supelco 37 (FAME standards)		Sigma-Aldrich (Bellefonte, PA, USA)
Plate	**Size**	**Supplier**
HPTLC Silica gel 60	10 x 10 cm	Merck (Darmstadt, Germany)
PLC Silica gel 60 (0.5 mm thick)	20 x 20 cm	Merck (Darmstadt, Germany)

3.2 Isomerization of EPA and DHA standards

The method for isomerization of EPA and DHA was taken from Snyder and Scholfield (1982), with minor modifications. In this method, *p*-toluenesulfinic acid (PTSA) was used as a catalyst for isomerization of the fatty acids.

Firstly, the sodium *p*-toluenesulfinate had to be acidified to *p*-toluenesulfinic acid, as described by Delmonte *et al*. (2008).

3.2.1 Acidification of sodium *p*-toluenesulfinate

Deionized water (30 mL), 2 mL 37% HCl, 30 mL diethyl ether and 1 g of sodium *p*-toluenesulfinate were added to a 100 mL separatory funnel. The contents were then mixed until the sodium *p*-toluenesulfinate was fully dissolved and the organic phase appeared clear. The aqueous phase was then discarded. 30 mL of deionized water and 1 mL of 37% HCl was once again added to the organic phase and mixed, and the aqueous phase was again discarded. The organic phase was then transferred to a round bottom flask, and the contents were dried under vacuum using a rotary evaporator (IKA, Staufern im Breisgau, Germany). After the liquid had been evaporated 125 mg of the PTSA was dissolved in 25 mL of 1,4-dioxane (5 mg/ml), and stored in a dark flask (Mjøs 2005).

3.2.2 Isomerization

Approximately 5 mg of all-*cis* EPA or all-*cis* DHA FAME were dissolved in 1 ml dioxane with 5 mg of *p*-toluenesulfinic acid in a test tube. The contents were then heated for 60 minutes (Mjøs 2005) or 120 minutes at 60 °C. After the heat treatment, the reaction was stopped by adding 1 ml of 1 M NaOH. The oil was extracted with 1 ml hexane. The organic phase was transferred to a dark vial, flushed with nitrogen and stored in a freezer at -30 ° C.

3.3 Silver ion TLC

The methods applied in this section are partly taken from the AOCS web sites and partly from Fournier *et al*. (2006 A).

3.3.1 Preparation of TLC plates

Plates were impregnated with $AgNO_3$ by immersing them in an $AgNO_3$ solution (10%, w/v in acetonitrile) for 30 minutes in a glass container (Fournier *et al*. 2006 A). Afterwards the plates were dried, put in plastic bags and kept in the dark at room temperature.

3.3.2 Applying sample to TLC plate

For the 10 x 10 cm HPTLC plate used as a test to see if the isomerization was successful, a straight line was drawn about 1.5 cm above the bottom of the plate. Points where the samples were to be applied was marked and numbered, and 5 µl of the sample was applied to the plate using a micropipette.

HPTLC plates were also used for separation of the isomer standards. Here larger samples were applied. The isomerized standards were dissolved in hexane (approximately 20 mg/ml). Six spots of 10 µl were applied to the plate, 5 µl at a time.

3.3.3 Developing the plates

Freshly prepared and well-mixed toluene/methanol (85:15, v/v) was used as mobile phase (Srigley & Rader 2014, Fournier *et al*. 2006 A). The mobile phase was poured into the developing tank before the plate with the samples was inserted. The sample application line was above the level of the mobile phase. The atmosphere in the tank was not saturated with mobile phase prior to developing the plate because avoiding saturation seems to give better separations between the analytes (AOCS 2009)

The plates were developed at -25 °C. The HPTLC plates for testing the isomerization reaction was developed for 1.5 hours, while the HPTLC plates for preparing the standards were developed for 4 hours, to ensure appropriate separation between the different isomers.

3.3.4 Method testing

The 10 x 10 cm HPTLC plates were sprayed with a 10% copper sulfate in an 8% phosphoric acid solution. Afterwards they were heated to 160 °C for approximately 30 minutes or until brown spots appeared on the plate.

3.3.5 Sample recovery

The plates were sprayed with 2',7'-dichlorofluorescein in isopropanol as a ready-to-use solution. After being sprayed, the plate was viewed under UV light at 366 nm, and the boundaries of the different *cis*- and *trans*-bands were marked with a scalpel and scraped off onto filter paper, and then transferred to a test tube. In the test tube the fractions were recovered by adding 5 mL of 1% NaCl in methanol:water 90:10 (v/v) solution, mixed thoroughly, then extracted twice with 2 ml of hexane (Fournier *et al*. 2006 A). The latter step was performed to remove silver ions and 2',7'-dichlorofluorescein. After having cleaned up the samples they were transferred to dark vials and placed in the freezer for later analysis.

3.4 Column installation and conditioning

The installation of the SLB-IL111 column (100 m length, 0.25 mm inner diameter and 0.2 µm film thickness) (Supelco, Bellefonte, PA, USA) was performed according to the instruction papers delivered with the column.

1. The GC system was first turned off.
2. About 1 cm of one end of the column was cut off using a ceramic glasscutter. The nut and the ferrule were fitted onto the column and the column was cut so that it measured 3.7 cm from nut to column end.
3. The cut end was then attached to the split/splitless injector.
4. The other end of the column was cut off and the flow of the GC system was turned on. The loose end was dipped in methanol to see if a constant stream of gas bubbles came out.
5. After the flow had been checked, a nut and a ferrule was fitted onto the loose end and cut, measuring 9.5 cm from nut to column end.
6. The column was then attached to the FID. All the nuts were then checked to see if they were tightly enough fitted and a gas leak check was performed.
7. After the gas flow had been allowed to run through the column for 30 minutes the GC oven was turned on. The temperature was gradually increased up to 220 °C, and kept at that temperature for some hours before it was reduced to 170 °C. The conditioning of very polar columns such as the SLB-IL111 takes a while, so bleeding and spikes were observed after several days.

3.5 Optimization of temperature programs for analysis of *trans* fatty acids

The hexane was evaporated from the *trans* standards. Then 250 µl of isooctane with BHT (0.15 g/liter) was added before the sample was transferred to GC-vials with inserts. For each temperature program one sample of mono-*trans* EPA, di-*trans* EPA, mono-*trans* DHA, di-*trans* DHA and GLC 68D was tested.

Table 2, 3, 4 and 5 shows the different temperature programs that were analyzed. In temperature program 4 the flow was 1 ml/min, in the other three temperature programs it was 1.5 ml/min.

Table 2. Temperature program 1.

Rate (° C/min)	Temp (° C)	Time (min)	Total time (min)
Initial	110	4	4
20	150	0	6
2	170	20	36
2	185	20	63,5

Table 3. Temperature program 2.

Rate (° C/min)	Temp (° C)	Time (min)	Total time (min)
Initial	110	4	4
20	150	0	6
1	170	20	46
2	185	10	63,5

Table 4. Temperature program 3.

Rate (° C/min)	Temp (° C)	Time (min)	Total time (min)
Initial	110	4	4
10	150	0	8
2	170	20	38
2	185	15	60,5

Table 5. Temperature program 4 with carrier gas flow 1.5 ml/min.

Rate (° C/min)	Temp (° C)	Time (min)	Total time (min)
Initial	110	4	4
20	150	0	6
2	168	20	35
2	180	10	51

3.6 Fatty acid composition

The FAME standards SRM 3275, GLC 68D and Supelco 37 were used to identify the peaks of the chromatogram.

3.6.1 Methylation

The methylation process was performed according to the in-house method at Nordic Pharma Inc. (Tromsø, Norway):

1. Approximately 200 mg of oil was weighed into a test tube, and the exact weight noted.
2. 1.5 ml of toluene containing 11.24 mg of C23:0 (internal standard) was added to the test tube, using a syringe.
3. 1.5 ml sodium methylate (3% in methanol) was added to the test tube, using a pipette, and the contents were mixed thoroughly.
4. Test tubes were then placed in a water bath at 60 °C.

5. After ten minutes the test tubes were removed from the water bath and cooled down to approximately 40 °C in room temperature before 5 ml of isooctane with BHT (butylated hydroxytoluene) (0.15 g/liter) and 3 ml of distilled water was added. The contents were then shaken vigorously again.
6. After the aqueous and the organic phases had separated into two layers, 2 ml of the organic phase was transferred to a new test tube with some Na_2SO_4 at the bottom for water removal and shaken.
7. When the liquid in the new test tubes appeared clear, 200 µl were pipetted to a vial and 1 ml of isooctane with BHT was added. The mixture was properly mixed and ready for the GC.

3.6.2 GC instrumentation and programming

The GC system was a Scion 436-GC (Bruker). The column was a 100 meter Ionic Liquid Stationary phase (SLB-IL111). Injector temperature was 250 °C, and was run in split mode 1:50. The FID was kept at 270 °C. Carrier gas was hydrogen, and column flow was 1 ml/min. 1 µl of the sample was injected. Temperature program 1 was used (table 2).

3.7 Oil samples from production

For additional information about the different oil samples in table 6 and 7 see chapter 2.2.

Table 6. Samples of naural fish oil.

Sample name	Treatment/processing conditions	Number of replicates
VNT 1812	Winterized fish oil. Before stripping.	3
STF 1812	Stripped fish oil. Temperature = 199 °C	3
BLF 1812	Bleached fish oil. Before deodorization.	3
DEO 1812	Deodorized fish oil. Temperature = 190 °C	3

Table 7. Samples from the concentration process.

Sample name	Treatment/processing conditions	Number of replicates
ETY 2412 FEED	After ethylation, before degasser	3
ETY 2412 DEGASSER	Before SPD, after degasser. Temperature = 110 °C	3
DTL EE	Light fraction from SPD. Temperature = 127.7 °C	3
DTD 3020 (R1)	After the first column in SPD. Temperature = 127.7 °C	3
DTR EE	Heavy residue fraction from SPD. Temperature = 143 °C	3
DTD 3020 (R1D2)	Product after the two columns of SPD. Temperature = 143 °C	3

3.8 Temperature experiment

Samples of fish oil concentrates, approximately 5 ml each, were transferred to dark vials and wrapped in aluminum foil. Samples were inserted into the oven at 180, 190 or 200 °C. For each temperature the samples were heated for 1, 15, 30 or 60 minutes. One sample remained untreated. After heating, the samples were stored in a fridge at 4 °C, before being analyzed in the GC.

3.9 Calculations

The calculation of mg FA in the oil was performed using C23:0 as internal standard in the oil sample using equation 1.

$$\frac{mg\ IS}{area\ IS} = \frac{mg\ FA}{area\ FA} \quad (1)$$

In this equation IS = internal standard, FA = fatty acid. The amount of IS is known, the area for both IS and FA are found by integrating the peaks in the chromatogram. By multiplying both sides with area FA one achieves mg FA. Approximately 200 mg of sample is weighed in, so by multiplying by a factor of approximately 5 (depending on the exact weight of the sample) one gets mg FA/g fish oil.

4 Results

4.1 Separation of FAME isomers on HPTLC plates

HPTLC plates (10 x 10 cm) were used to check whether the reaction of all-*cis* FAME with *p*-toluenesulfinic acid (PTSA) at 60 °C had formed *trans* isomers, and to see what happened to the FAME if only PTSA or only heat was applied. Figure 11 shows different samples of EPA after being developed on the HPTLC plate and charred in the oven. The spot for EPA without any treatment (1) was similar to the spots for EPA that had only been treated with heat (2) and only PTSA (3). Sample 4, which is EPA FAME treated with PTSA for one hour at 60 °C contained all the different isomers of EPA, from all-*cis* to all-*trans*, with mono-, di- and tri-*trans* being most abundant. Sample 5, which contains EPA FAME treated with PTSA at 60 °C for two hours, also showed all the different isomers, but here there was almost no all-*cis* left and the more *trans*-isomerized molecules were the most abundant.

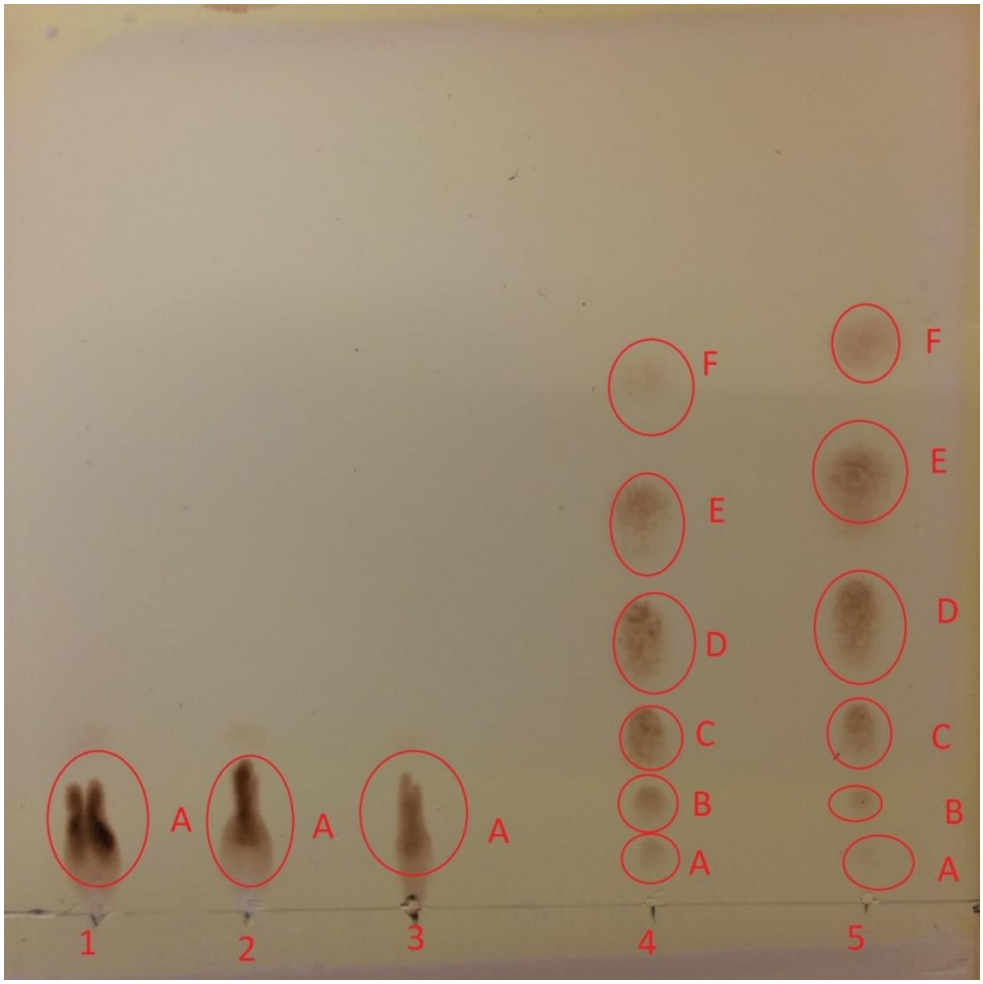

Figure 11: Shows 10 x 10 HPTLC plate impregnated with $AgNO_3$. 1 = EPA FAME no treatment, 2 = EPA FAME heated for one hour at 60 °C without PTSA, 3 = EPA FAME with PTSA for one hour without heat treatment, 4 = EPA FAME heated for one hour at 60 °C with PTSA, 5 = EPA FAME heated for two hours at 60 °C with PTSA. A = all-*cis*, B = mono-*trans*, C = di-*trans*, D = tri-*trans*, E = tetra-*trans*, and F = all-*trans*. The plate was charred in the oven to visualize the isomers.

Separation of DHA FAME isomers (not shown) gave similar results as for EPA FAME isomers. Based on visual inspection the amount of different isomers was approximately the same as for EPA. Heating for one hour at 60 °C with PTSA was judged to be the best approach for making *trans* FAME standards.

4.2 Preparation of *trans* FAME standards on PLC

To obtain larger amounts of isomers, the use of preparative layer plates (PLC; 20 x 20 cm) was attempted. Several experiments were carried out, with for example different methods for applying samples. The results were always a smeared sample where no separation had occurred (figure 12). A dark band alongside the sample spots appeared when viewed under UV-light, and the elution of the sample was bended. Also the whole plate showed a bright green glow after it had been sprayed with 2',7'-dichlorofluorescein, the same bright green glow that should be seen from the fatty acids. No bands or spots of separation were detected.

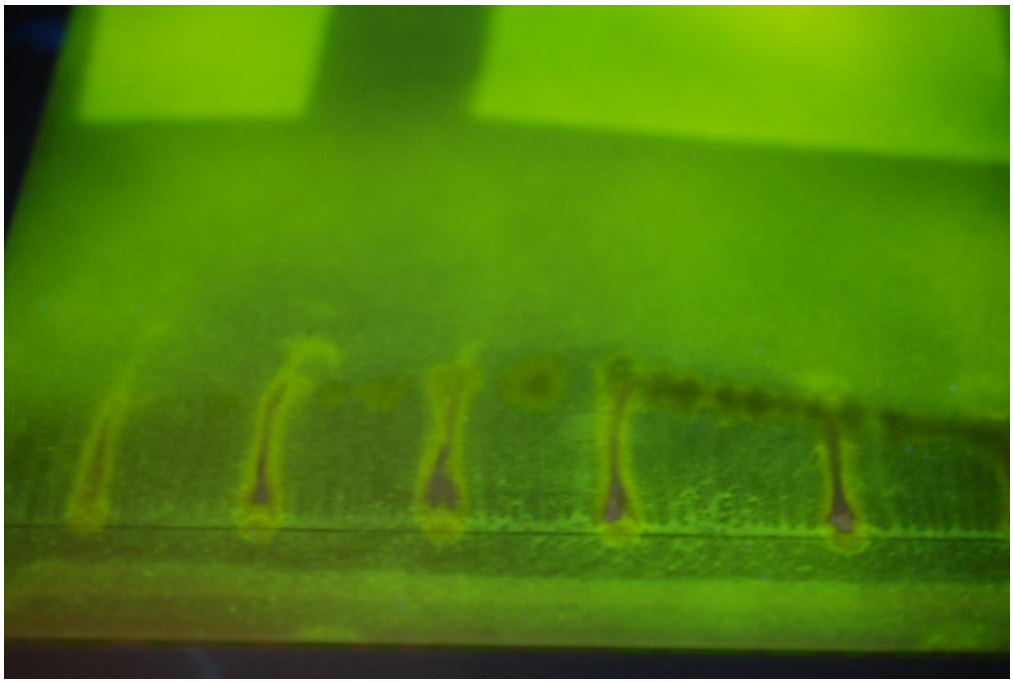

Figure 12. Part of a PLC plate after development, watched under UV light after being sprayed with 2',7'-dichlorofluorescein spray. The sample is smeared out, and no separation occurs.

Preparation of trans FAME standards was also studied using the 10 x 10 cm HPTLC plates in the large development chamber. As can be seen in figure 13 the entire plate showed a bright green glow and a darker mark some distance up the plate, just like the PLC plate. No separation between isomers could be spotted.

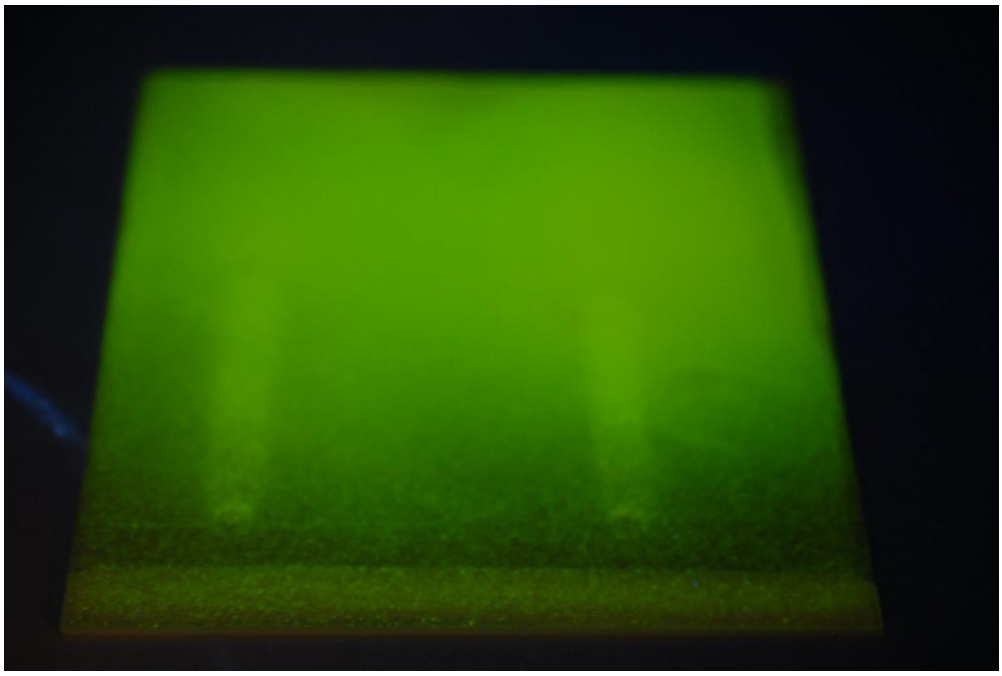

Figure 13. HPTLC plate developed in the large chamber, watched under UV-light after being sprayed with 2',7'-dichlorofluorescein. The background has the same bright green color as the fatty acids are supposed to have. A slightly brighter green stripe can be seen, but no separations between the different isomers.

Figure 14 shows the size of the plates and the development chambers. The volume of the large chamber is 4.1 liters, whereas the volume of the small chamber is 0.46 liters. The lid for the small chamber is made of metal, whereas the lid for the large chamber is a glass plate.

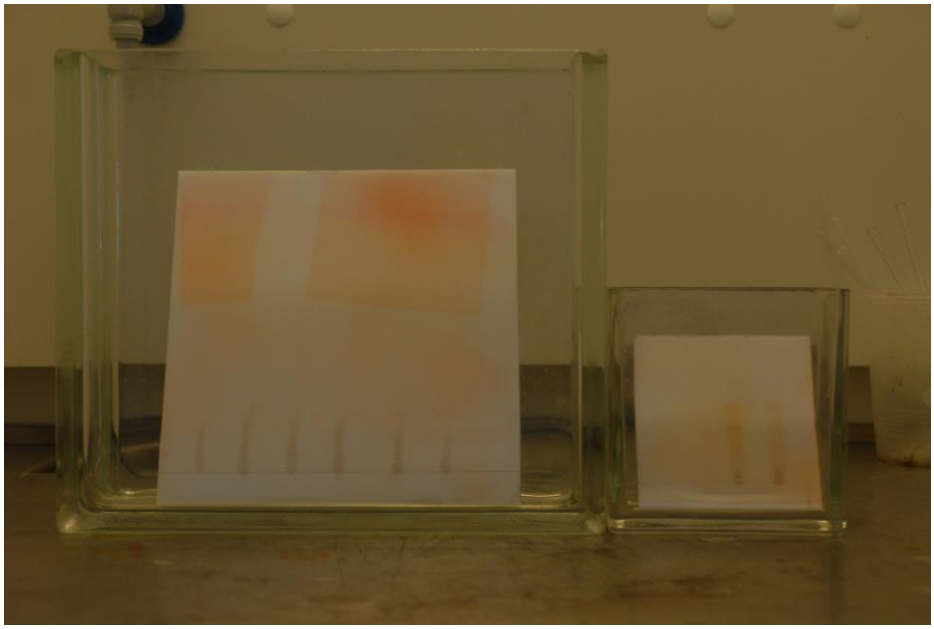

Figure 14. The large and small developing chamber with their respective plates.

Because of these problems it was decided to use the small chamber and HPTLC plates to prepare isomerized standards.

4.3 Preparation of *trans* FAME standards on HPTLC

Figure 15 shows separations of isomerized EPA FAME, as visualized by 2',7'-dichlorofluorescein under UV-light. Five spots can be seen as bright green spots on a darker background, the most highly isomerized is barely visible. There was separation between all spots, and increasing resolution with increasing number of *trans* double bonds. The separation between the first and the second spot, containing all-*cis* and mono-*trans*, was very narrow. The separations for DHA were similar, but the resolution was not as good as for the different EPA isomers.

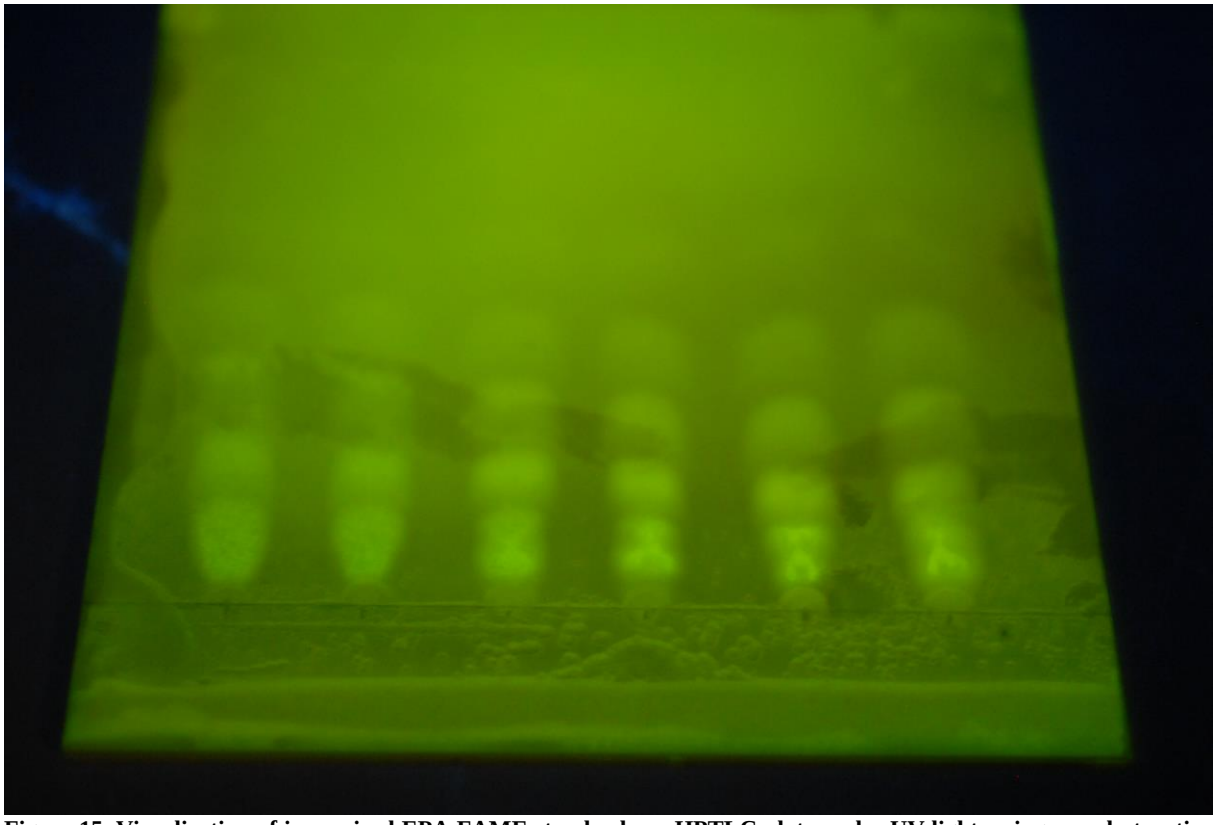

Figure 15. Visualization of isomerized EPA FAME standards on HPTLC plate under UV light, using non-destructive 2',7'-dichlorofluorescein. Six replicate samples treated with PTSA at 60 °C for one hour were applied to the plate along a line 1.5 cm above the bottom of the plate.

The samples were scraped off the plate using a scalpel (figure 16). The blade was replaced after each individual isomer had been scraped off. Isomers containing three or more *trans* double bonds were pooled. All-*cis*, mono- and di-*trans* isomers were collected individually.

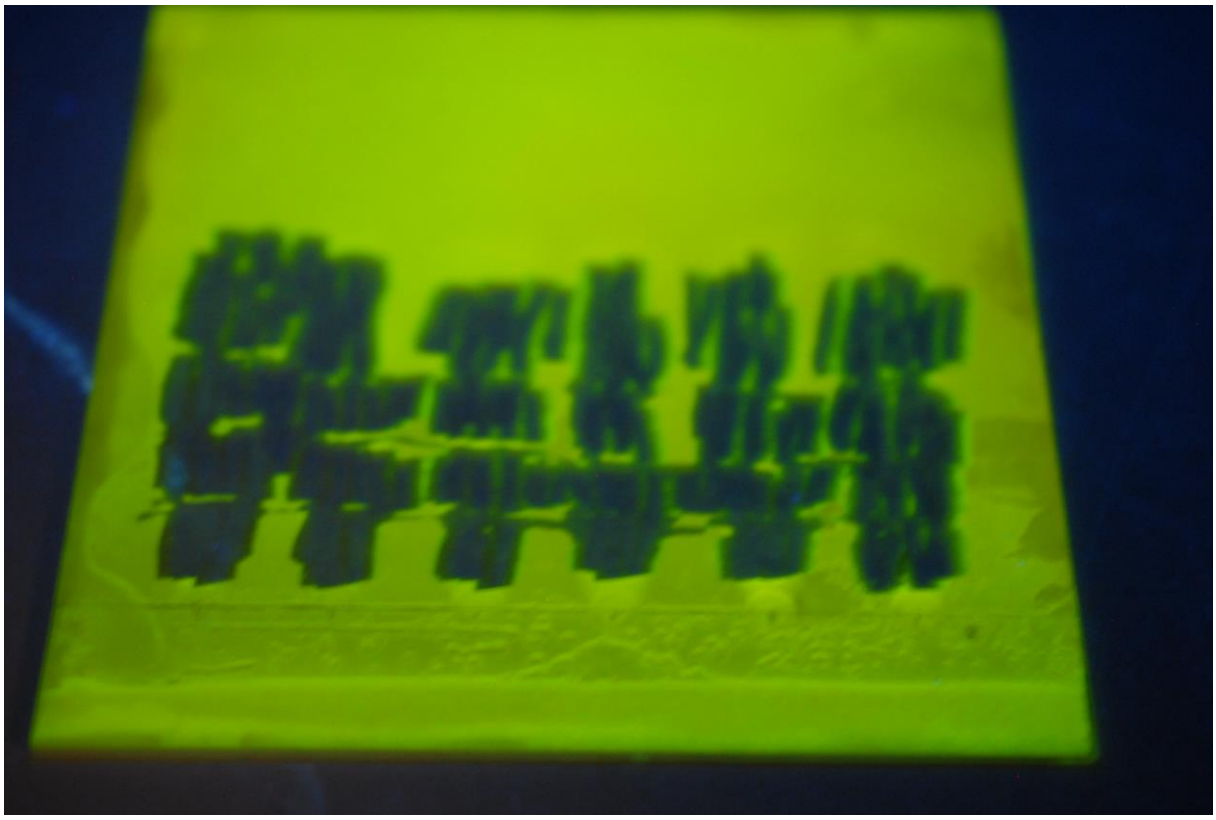

Figure 16 The plate after the isomers have been scraped off.

4.4 Separations of *trans* isomers on GC column using different temperature programs

In this section the ability of the different temperature programs to separate *trans* isomers from each other and from compounds present in the GLC 68D standard, were investigated.

Chromatograms with mono-*trans* isomers of EPA and DHA were superimposed onto the chromatogram for the standard GLC 68D (figure 17). All chromatograms were obtained using temperature program 1. As can be seen in (A) there was almost no co-elution of mono-*trans* isomers of EPA and the fatty acids in GLC 68D. However, one minor peak co-eluted with all-*cis* EPA (peaks 4). Peak 5 probably contained three isomers of mono-*trans* EPA based on calculations of area percent of the integrated peaks, but was integrated as two peaks in the computer program. The peak for C24:1 (2) eluted between the first and the second mono-*trans* EPA peak. For DHA and GLC 68D (B) there was again almost no co-elution of mono-*trans* isomers and components of GLC 68D. As in 17A a small peak co-eluted with all-*cis* DHA. Peak 9 and 10 probably contains two isomers each, based on calculations of area percent of the integrated peaks.

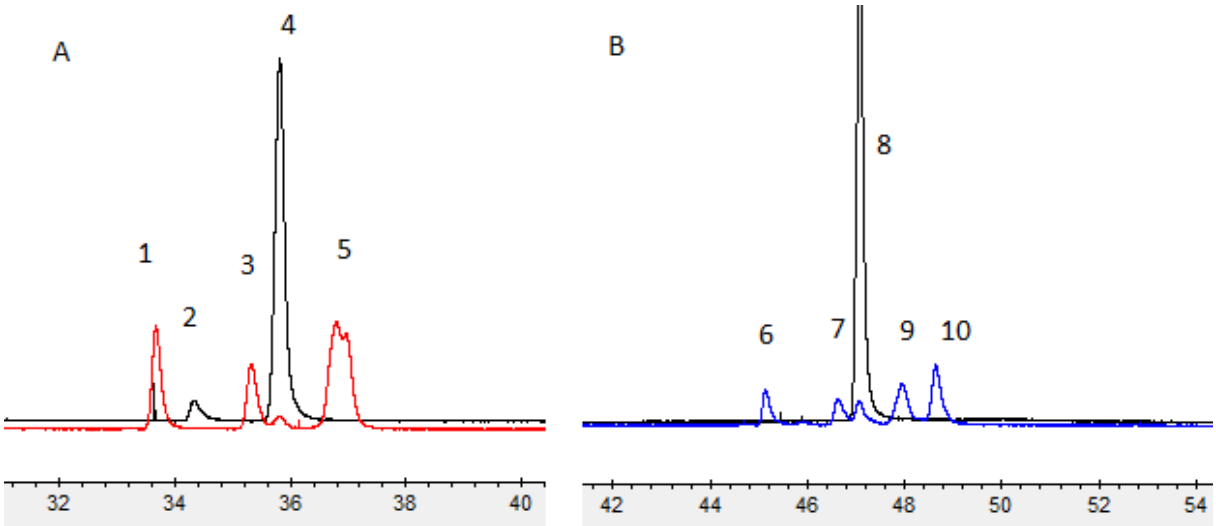

Figure 17. Chromatograms with retention time (rt) in minutes, for mono-*trans* EPA (red line) and GLC 68D (black line) (A) and mono-*trans* DHA (blue line) and GLC 68D (black line) (B) under temperature program 1 (gas flow 1ml/min). 1 = mono-*trans* EPA, 2 = C24:1, 3 = mono-*trans* EPA, 4 = all-*cis* EPA, 5 = mono-*trans* EPA, 6 = mono-*trans* DHA, 7 = mono-*trans* DHA, 8 = all-*cis* DHA, 9 = mono-*trans* DHA, and 10 = mono-*trans* DHA.

The chromatograms for di-*trans* isomers of EPA and DHA obtained using temperature program 1 can be seen in figure 18. The di-*trans* isomers of EPA (A) were not completely separated from each other, and some peaks had the same retention time (rt) as all-*cis* EPA (rt = 35.82 minutes). In addition, two of the peaks most likely contained three isomers of di-*trans* EPA each, based on calculations of the area percent of the integrated peaks. Some of the di-*trans* isomers of DHA (B) co-eluted with all-*cis* DHA at approximately 47 minutes. Two the

peaks most likely contained two isomers each, and two of the peaks contained four isomers each, based on calculations of area percent.

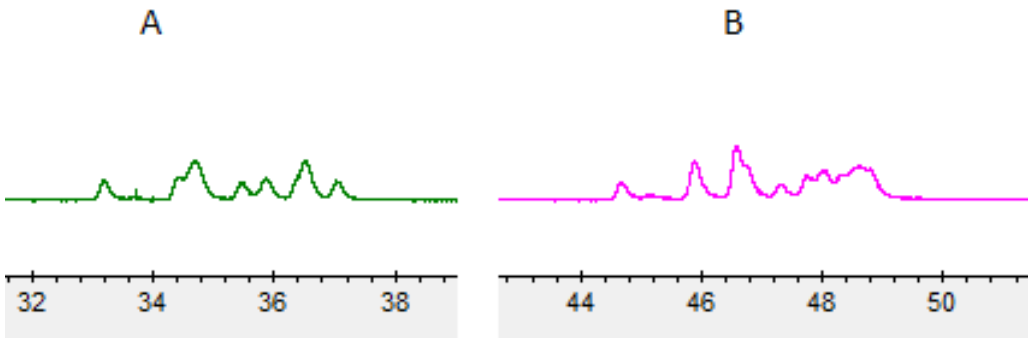

Figure 18. The chromatograms with retention time (rt) in minutes, for di-*trans* EPA (A) and di-*trans* DHA (B) obtained under temperature program 1.

Figure 19 contains the chromatograms for GLC 68D with mono-*trans* EPA (A) and mono-*trans* DHA (B) obtained using temperature program 2. The peaks showed more tailing than in the previously shown chromatogram, which made the separation between the different analytes poorer. Peak 2 (C24:1) was very broad and dragged into peak 3 (mono-*trans* EPA), and the tail of peak 3 was barely separated from peak 4 (all-*cis* EPA). Peak 5 most likely contained three mono-*trans* isomers of EPA, based on calculations of the integrated area of the peaks, but was integrated as two peaks in the computer program. In (B) peak 7 (mono-*trans* DHA) went into peak 8 (all-*cis* DHA). Peak 9 and 10 contained two mono-*trans* DHA isomers each, based on calculations of integrated peak area. As with temperature program 1, a small peak was found to co-elute with all-*cis* EPA and all-*cis* DHA.

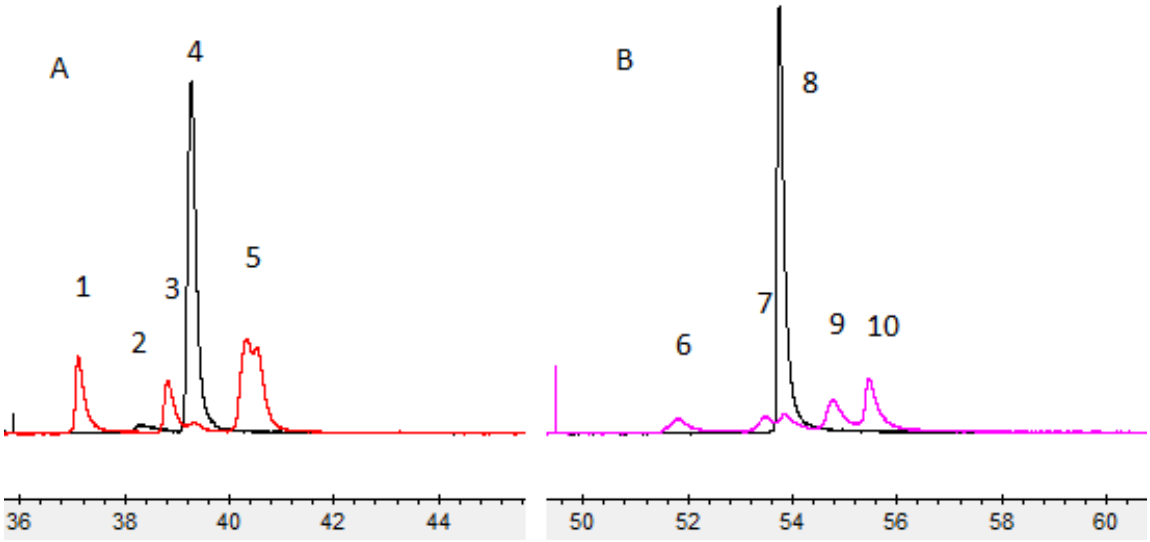

Figure 19. Chromatograms with retention time (rt) in minutes, for mono-*trans* EPA (red line) and GLC 68D (black line) (A) and mono-*trans* DHA (pink line) and GLC 68D (black line) (B) under temperature program 2 (gas flow 1ml/min). 1 = mono-*trans* EPA, 2 = C24:1, 3 = mono-*trans* EPA, 4 = all-*cis* EPA, 5 = mono-*trans* EPA, 6 = mono-*trans* DHA, 7 = mono-*trans* DHA, 8 = all-*cis* DHA, 9 = mono-*trans* DHA, and 10 = mono-*trans* DHA.

The chromatograms for di-*trans* EPA (A) and di-*trans* DHA (B) obtained using temperature program 2 are shown in figure 20. The separation was quite similar to that obtained using temperature program 1. The resolution between the peaks was not as good as in temperature program 1. All the components could not be completely separated, and some of the components would co-elute with the all-*cis* isomers.

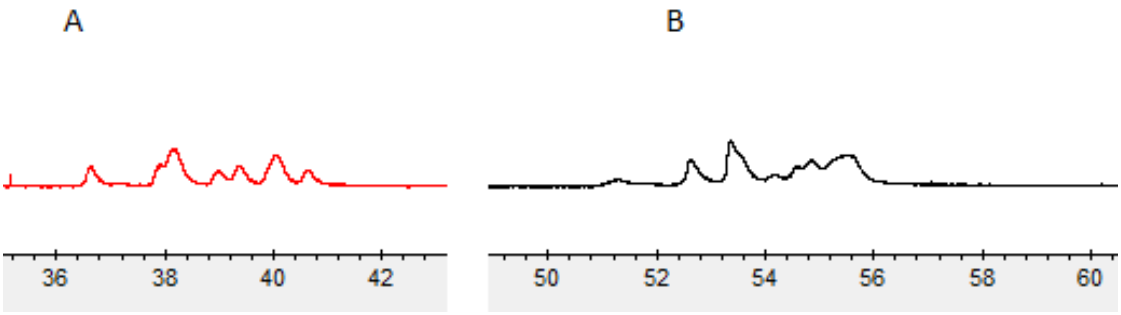

Figure 20. The chromatograms with retention time (rt) in minutes, for di-*trans* EPA (A) and di-*trans* DHA (B) obtained under temperature program 2.

The chromatograms for mono-*trans* EPA (A) and mono-*trans* DHA (B) together with GLC 68D, obtained using temperature program 3 are shown in figure 21. There was little co-elution of compounds using this temperature program. As with the previously shown chromatograms, small peaks co-eluted with the all-*cis* isomers. The peaks were quite sharp, and little to no tailing was observed. The chromatogram was similar to that obtained using temperature program 1, except the retention time was a bit longer in temperature program 3.

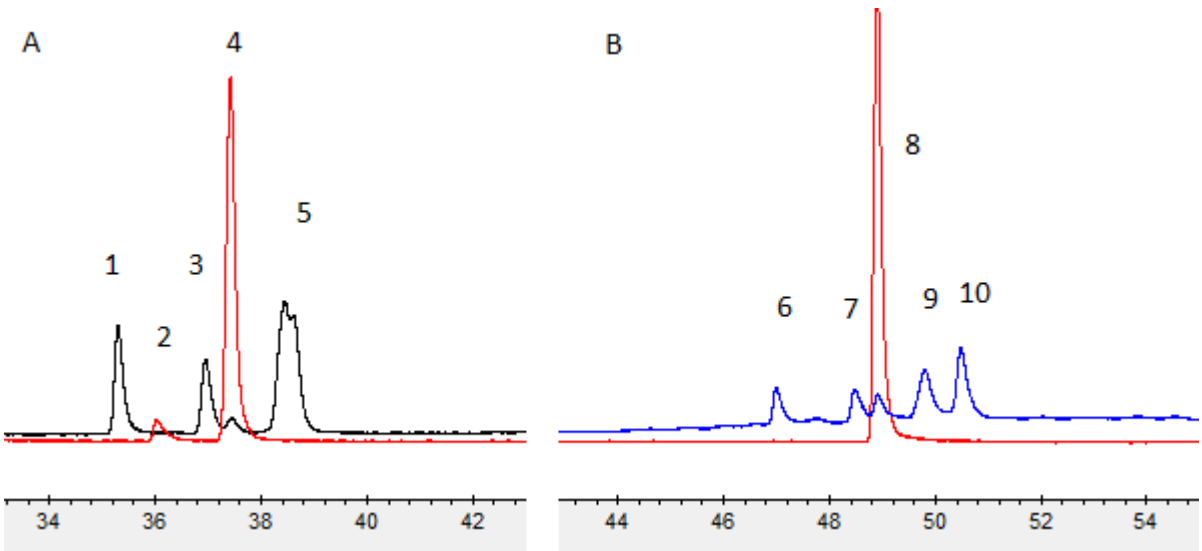

Figure 21. The chromatograms with retention time (rt) in minutes, for mono-*trans* EPA (black line) GLC 68D (red line) (A) and mono-*trans* DHA (blue line) and GLC 68D (red line) (B) under temperature program 3 (gas flow 1ml/min). 1 = mono-*trans* EPA, 2 = C24:1, 3 = mono-*trans* EPA, 4 = all-*cis* EPA, 5 = mono-*trans* EPA, 6 = mono-*trans* DHA, 7 = mono-*trans* DHA, 8 = all-*cis* DHA, 9 = mono-*trans* DHA, and 10 = mono-*trans* DHA.

The chromatograms for di-*trans* EPA (A) and di-*trans* DHA (B), obtained using temperature program 3 can be seen in figure 22. Again the chromatograms were quite similar to those obtained using temperature program 1. There was, again, not complete separation between all the components present, and co-elution between some of the di-*trans* isomers and all-*cis* isomers would occur.

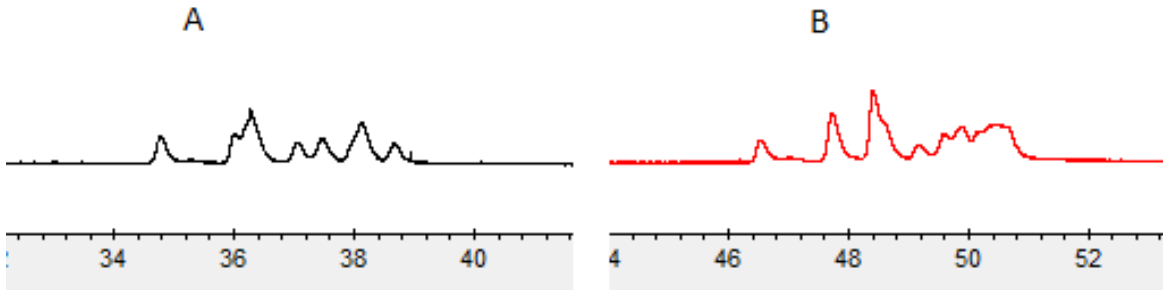

Figure 22. The chromatograms with retention time (rt) in minutes, for di-*trans* EPA (A) and di-*trans* DHA (B) obtained under temperature program 3.

Figure 23 contains the chromatograms for GLC 68D, mono-*trans* EPA (A) and mono-*trans* DHA (B) obtained using temperature program 4 were the flow had been changed to 1.5 ml/minute. The resolution between peak 2 (mono-*trans* EPA) and peak 3 (all-*cis* EPA) was not good. C24:1 was not detected using these temperature conditions. In addition, peak 4, which in the previously shown equivalent chromatograms eluted as a peak with a split in the middle, eluted more like a single peak using this temperature and flow program. The mono-*trans* isomers of DHA appeared more stretched, and one of the peaks co-eluted with all-*cis* DHA (peak 6). The di-*trans* isomers were not tested in this temperature program.

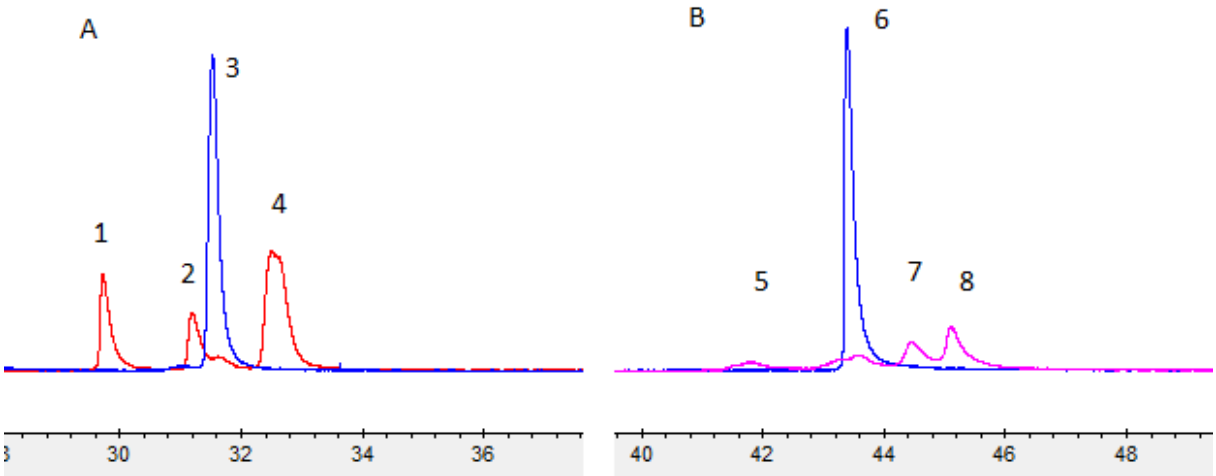

Figure 23. The chromatograms with retention time (rt) in minutes, for mono-*trans* EPA (red line) and GLC 68D (blue line) (A) and mono-*trans* DHA (pink line) and GLC 68D (blue line) (B) under temperature program 4 (gas flow 1 ml/min). 1 = mono-*trans* EPA, 2 = mono-*trans* EPA, 3 = all-*cis* EPA, 4 = mono-*trans* EPA, 5 = mono-*trans* DHA, 6 = all-*cis* DHA, 7 = mono-*trans* DHA, and 8 = mono-*trans* DHA.

4.5 Trans fatty acids formed during processing of fish oil

In this section the results from the analysis of oils from different processing steps are presented (figure 5). All the samples with "1812" in their names follow the pathway of the blue arrow in figure 5, whereas all the other samples follow the pathway of the red arrow in figure 5. The tables (6-10) only contain results of integrated peaks for which standards were available, leaving out a few peaks from the chromatograms.

4.5.1 VNT 1812 and STF 1812

VNT 1812 (table 8) had the fatty acid composition of anchoveta oil. The amounts of C14:0, C16:0, C16:1n-7, C18:1n-9c, EPA and DHA were high. The *trans* fatty acid 18:1n-9t was detected as 0.82 ± 0.71 mg/g. One isomer of mono-*trans* EPA was also detected, at 1.51 ± 0.11 mg/g, giving a total *trans* fatty acid content of 2.32 ± 0.59 mg/g. The standard deviance for most fatty acids in these samples was relatively low, except for C24:1, which was detected at 0.40 ± 0.70 mg/g.

STF 1812 is the same oil as VNT 1812, but after the SPD stripping process. The fatty acid composition of STF 1812 is shown in table 8. In STF 1812 the *trans* fatty acid 18:1n-9t was detected at 0.73±0.64 mg/g. One isomer of mono-*trans* EPA was also detected in this sample, the amount was 1.62±0.05 mg/g. Total *trans* fatty acid content was 2.35 ± 0.64 mg/g. C24:1 had a very high relative standard deviance compared to the rest of the fatty acids present in this sample.

Table 8 The fatty acid composition of VNT 1812 and STF 1812. Values are expressed as mean of three replicates ± standard deviation. "t" refers to *trans* isomers, "c" refers to *cis* isomers. ND = not detected. The results for each analyzed fatty acid are given as area percent and mg/g. Sum *trans* FA = the sum of *trans* FA for which standards were available in this study, i.e. 18:1n-9t, 18:2n-6t, mono-*trans* EPA and mono-*trans* DHA.

	VNT 1812		STF 1812	
FA	**Area percent**	**Mg/g**	**Area percent**	**Mg/g**
C14:0	8.27 ± 0.08	81.64 ± 3.52	8.38 ± 0.08	84.06 ± 2.05
C15:0	0.68 ± 0.01	6.68 ± 0.30	0.69 ± 0.00	6.87 ± 0.12
C16:0	16.58 ± 0.07	163.56 ± 5.84	16.45 ± 0.07	164.99 ± 3.10
C17:0	0.29 ± 0.00	2.86 ± 0.12	0.29 ± 0.00	2.93 ± 0.06
C16:1n-7	9.37 ± 0.05	92.45 ± 3.43	9.50 ± 0.05	95.33 ± 1.89
C18:0	3.16 ± 0.03	31.20 ± 0.79	3.14 ± 0.03	31.46 ± 0.71
C17:1n-11	0.38 ± 0.03	3.79 ± 0.45	0.38 ± 0.01	3.86 ± 0.09
C18:1n-9t	**0.08 ± 0.07**	**0.82 ± 0.71**	**0.07 ± 0.06**	**0.73 ± 0.64**
C18:1n-9c	8.12 ± 0.02	80.08 ± 2.59	8.10 ± 0.02	81.21 ± 1.13
C18:1n-7	3.21 ± 0.03	31.63 ± 1.29	3.18 ± 0.02	31.92 ± 0.68
C18:2n-6t	**ND**	**ND**	**ND**	**ND**
C20:0	0.22 ± 0.01	2.18 ± 0.08	0.21 ± 0.00	2.09 ± 0.02
C18:2n-6c	1.28 ± 0.01	12.62 ± 0.45	1.29 ± 0.01	12.95 ± 0.16
C20:1n-11	2.14 ± 0.02	21.07 ± 0.61	2.09 ± 0.05	20.92 ± 0.52
C20:1n-9	0.97 ± 0.00	9.52 ± 0.33	0.99 ± 0.06	9.88 ± 0.63
C21:0	0.27 ± 0.01	2.70 ± 0.06	0.27 ± 0.01	2.73 ± 0.03
C18:3n-6	0.26 ± 0.01	2.53 ± 0.11	0.25 ± 0.01	2.54 ± 0.05
C18:3n-3	0.65 ± 0.01	6.39 ± 0.13	0.65 ± 0.01	6.48 ± 0.10
C20:2n-6	0.13 ± 0.00	1.24 ± 0.03	0.13 ± 0.01	1.29 ± 0.05
C22:1n-11	0.40 ± 0.03	3.93 ± 0.14	0.40 ± 0.02	4.04 ± 0.28
C20:3n-6	0.28 ± 0.02	2.81 ± 0.13	0.27 ± 0.02	2.75 ± 0.27
C20:4n-6	1.00 ± 0.01	9.83 ± 0.36	1.00 ± 0.01	10.02 ± 0.24
Mono-t EPA	**0.15 ± 0.01**	**1.51 ± 0.11**	**0.16 ± 0.00**	**1.62 ± 0.05**
C24:1	0.04 ± 0.07	0.40 ± 0.70	0.11 ± 0.10	1.14 ± 0.99
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
C20:5n-3	18.82 ± 0.11	185.68 ± 5.16	18.74 ± 0.08	187.94 ± 2.38
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
C22:5n-3	1.77 ± 0.03	17.41 ± 0.43	1.80 ± 0.04	18.02 ± 0.17
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
C22:6n-3	11.75 ± 0.12	115.90 ± 2.70	11.68 ± 0.15	117.10 ± 0.69
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
Sum *trans* FA		**2.32 ± 0.59**		**2.35 ± 0.64**

4.5.2 BLF 1812 and DEO 1812

Fish oil bleaching (BLF 1812) is the processing step preceding deodorization. BLF 1812 (table 9) had the same natural fatty acid composition as the other "1812" oils. The only *trans* fatty acid detected in this sample was C18:1n-9t, which was present at 1.99±0.09 mg/g. No *trans* isomers of EPA or DHA were detected. The standard deviation was relatively low for every fatty acid except C20:3n-3.

Deodorized fish oil DEO 1812 (table 9) had, like the other "1812"-oils, a natural fish oil fatty acid composition. 1.87±0.11 mg/g of 18:1n-9t was detected, but no *trans* isomers of EPA or DHA were observed. The standard deviation for C20:3n-3 was also very high in DEO 1812.

Table 9 The fatty acid composition of BLF 1812 and DEO 1812. Values are expressed as mean of three replicates ± standard deviation. "t" refers to *trans* isomers, "c" refers to *cis* isomers. ND = not detected. The results for each analyzed fatty acid are given as area percent and mg/g. Sum *trans* FA = the sum of *trans* FA for which standards were available in this study, i.e. 18:1n-9t, 18:2n-6t, mono-*trans* EPA and mono-*trans* DHA.

	BLF 1812		**DEO 1812**	
FA	**Area percent**	**Mg/g**	**Area percent**	**Mg/g**
C14:0	6.65 ± 0.09	64.53 ± 1.18	6.70 ± 0.10	64.24 ± 3.34
C15:0	0.61 ± 0.01	5.88 ± 0.14	0.61 ± 0.01	5.88 ± 0.27
C16:0	16.29 ± 0.13	158.03 ± 1.75	16.62 ± 0.12	159.41 ± 7.02
C17:0	0.39 ± 0.05	3.81 ± 0.50	0.42 ± 0.00	3.99 ± 0.17
C16:1n-7	8.40 ± 0.04	81.47 ± 1.00	8.37 ± 0.05	80.27 ± 3.49
C18:0	3.17 ± 0.04	30.78 ± 0.48	3.23 ± 0.03	30.95 ± 1.11
C17:1n-11	0.33 ± 0.01	3.22 ± 0.12	0.32 ± 0.02	3.09 ± 0.27
C18:1n-9t	**0.21 ± 0.01**	**1.99 ± 0.09**	**0.19 ± 0.01**	**1.87 ± 0.11**
C18:1n-9c	8.97 ± 0.06	86.99 ± 0.55	8.87 ± 0.01	85.05 ± 3.16
C18:1n-7	3.03 ± 0.04	29.34 ± 0.20	2.97 ± 0.02	28.47 ± 1.17
C18:2n-6t	**ND**	**ND**	**ND**	**ND**
C20:0	0.42 ± 0.00	4.09 ± 0.04	0.40 ± 0.03	3.84 ± 0.26
C18:2n-6c	1.45 ± 0.01	14.02 ± 0.12	1.42 ± 0.01	13.64 ± 0.47
C20:1n-11	1.86 ± 0.02	18.04 ± 0.22	1.87 ± 0.06	17.91 ± 1.20
C20:1n-9	2.06 ± 0.01	20.02 ± 0.26	2.01 ± 0.08	19.22 ± 0.65
C21:0	0.22 ± 0.01	2.14 ± 0.09	0.21 ± 0.02	2.01 ± 0.18
C18:3n-6	0.26 ± 0.01	2.52 ± 0.07	0.26 ± 0.01	2.46 ± 0.05
C18:3n-3	0.86 ± 0.02	8.33 ± 0.17	0.85 ± 0.01	8.12 ± 0.32
C22:0	0.25 ± 0.01	2.44 ± 0.08	0.26 ± 0.01	2.46 ± 0.10
C20:2n-6	0.17 ± 0.02	1.63 ± 0.17	0.17 ± 0.01	1.59 ± 0.04
C22:1n-11	0.54 ± 0.02	5.23 ± 0.18	0.53 ± 0.01	5.08 ± 0.27
C20:3n-6	0.24 ± 0.03	2.28 ± 0.27	0.23 ± 0.01	2.23 ± 0.18
C20:3n-3	0.03 ± 0.06	0.32 ± 0.55	0.07 ± 0.06	0.67 ± 0.58
C20:4n-6	1.03 ± 0.02	9.96 ± 0.19	1.04 ± 0.05	9.92 ± 0.63
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
C24:1	0.17 ± 0.01	1.67 ± 0.06	0.19 ± 0.01	1.78 ± 0.07
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
C20:5n-3	18.02 ± 0.16	174.78 ± 1.17	17.77 ± 0.11	170.38 ± 5.79
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
C22:5n-3	1.55 ± 0.01	15.03 ± 0.15	1.57 ± 0.02	15.08 ± 0.37
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
C22:6n-3	11.47 ± 0.05	111.20 ± 0.68	11.32 ± 0.09	108.49 ± 3.40
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
Sum *trans* FA		**1.99 ± 0.09**		**1.87 ± 0.11**

4.5.3 ETY 2412

Oils from after the ethylation process and before concentration in SPD (ETY 2412) were also analyzed for the presence of *trans* fatty acids. Samples were taken after the ethylation before the degasser (ETY 2412 FEED) and after degassing before concentration in SPD (ETY 2412 DEGASSER). ETY 2412 FEED (table 10) contained high amounts of C14:0, C16:0, C16:1n-7, C18:1n-9c, EPA and DHA. Of *trans* fatty acids, C18:1n-9t, C18:2n-6t and one mono-*trans* EPA isomer were detected. The amounts were 0.31±0.54 mg/g, 0.68±0.60 mg/g and 1.89±0.07 mg/g, respectively. The total *trans* fatty acid content was 2.88 ± 0.82 mg/g. The standard deviations were in general relatively small for most fatty acids, except the two aforementioned 18-carbon *trans* fatty acids and C20:0.

ETY 2412 DEGASSER (table 10) was similar to ETY 2412 FEED in fatty acid composition. The same *trans* fatty acids were detected, but the means and standard deviations were a bit different for 18:1n-9t (0.65±0.56 mg/g) and 18:2n-6t (1.13±0.20 mg/g). The mono-*trans* isomer of EPA was similar in amount to ETY 2412 FEED at 1.86±0.08 mg/g. The total amount of *trans* fatty acids was 3.63 ± 0.61 mg/g. The relative standard deviations were in general a bit lower for ETY 2412 DEGASSER than for ETY 2412 FEED.

Table 10 The fatty acid composition of ETY 2412 (FEED), ETY 2412 (DEGASSER) and ETY 2412. Values are expressed as mean of three replicates ± standard deviation. "t" refers to *trans* isomers, "c" refers to *cis* isomers. ND = not detected. The results for each analyzed fatty acid are given as area percent and mg/g. Sum *trans* FA = the sum of *trans* FA for which standards were available in this study, i.e. 18:1n-9t, 18:2n-6t, mono-*trans* EPA and mono-*trans* DHA.

	ETY 2412 FEED		ETY 2412 DEGASSER	
FA	**Area percent**	**Mg/g**	**Area percent**	**Mg/g**
C14:0	6.06 ± 0.18	56.32 ± 3.47	5.89 ± 0.09	53.00 ± 1.35
C15:0	0.48 ± 0.01	4.47 ± 0.21	0.47 ± 0.01	4.22 ± 0.14
C16:0	14.95 ± 0.22	138.84 ± 6.29	14.74 ± 0.12	132.66 ± 2.46
C15:1	0.22 ± 0.01	2.07 ± 0.15	0.20 ± 0.01	1.83 ± 0.11
C17:0	0.30 ± 0.00	2.80 ± 0.10	0.34 ± 0.00	3.06 ± 0.04
C16:1n-7	7.18 ± 0.11	66.66 ± 3.08	6.71 ± 0.04	60.45 ± 1.02
C18:0	4.21 ± 0.02	39.12 ± 1.33	4.25 ± 0.03	38.28 ± 0.29
C17:1n-11	0.27 ± 0.03	2.50 ± 0.31	0.25 ± 0.01	2.24 ± 0.05
C18:1n-9t	**0.03 ± 0.06**	**0.31 ± 0.54**	**0.07 ± 0.06**	**0.65 ± 0.56**
C18:1n-9c	8.99 ± 0.06	83.42 ± 2.85	8.94 ± 0.04	80.51 ± 0.96
C18:1n-7	3.49 ± 0.05	32.40 ± 1.31	3.45 ± 0.01	31.09 ± 0.33
C18:2n-6t	**0.07 ± 0.06**	**0.68 ± 0.60**	**0.13 ± 0.02**	**1.13 ± 0.20**
C20:0	0.21 ± 0.18	1.93 ± 1.67	0.57 ± 0.02	5.12 ± 0.10
C18:2n-6c	1.30 ± 0.01	12.03 ± 0.35	1.30 ± 0.02	11.68 ± 0.16
C20:1n-11	2.88 ± 0.05	26.76 ± 0.48	2.84 ± 0.02	25.58 ± 0.40
C21:0	0.32 ± 0.02	2.95 ± 0.07	0.31 ± 0.00	2.81 ± 0.05
C18:3n-6	0.34 ± 0.01	3.14 ± 0.04	0.33 ± 0.01	2.97 ± 0.10
C18:3n-3	0.86 ± 0.07	7.94 ± 0.62	0.85 ± 0.09	7.70 ± 0.92
C22:0	0.20 ± 0.04	1.87 ± 0.33	0.24 ± 0.03	2.16 ± 0.29
C20:2n-6	0.16 ± 0.01	1.50 ± 0.05	0.19 ± 0.01	1.68 ± 0.11
C22:1n-11	0.39 ± 0.01	3.60 ± 0.05	0.40 ± 0.01	3.58 ± 0.06
C20:3n-6	0.20 ± 0.01	1.89 ± 0.07	0.19 ± 0.01	1.75 ± 0.09
C20:4n-6	1.15 ± 0.07	10.64 ± 0.49	1.20 ± 0.00	10.81 ± 0.10
Mono-t EPA	**0.20 ± 0.01**	**1.89 ± 0.07**	**0.21 ± 0.01**	**1.86 ± 0.08**
C24:1	0.29 ± 0.05	2.71 ± 0.34	0.34 ± 0.01	3.08 ± 0.07
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
C20:5n-3	21.01 ± 0.27	194.96 ± 5.07	21.05 ± 0.14	189.53 ± 1.62
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
C22:5n-3	1.94 ± 0.05	17.96 ± 0.24	1.98 ± 0.01	17.85 ± 0.14
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
C22:6n-3	10.81 ± 0.22	100.30 ± 1.55	11.00 ± 0.09	98.98 ± 0.73
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
Sum *trans* FA		**2.88 ± 0.82**		**3.63 ± 0.61**

4.5.4 DTL EE and DTR EE

DTL EE (table 11) is a volatile fraction that is removed from the fish oil concentrate during the short path distillation (SPD). High amounts of the short fatty acids, such as C14:0, C16:0 and C16:1, and low amounts of LC-PUFA were observed in the chromatograms of DTL EE. No *trans* EPA or DHA was observed in DTL EE, however, 0.66±0.58 mg/g of 18:1n-9t and 2.52±1.92 mg/g of 18:2n-6t was detected. Total *trans* fatty acid content was 3.18 ± 1.63 mg/g. For most of the fatty acids detected, the standard deviations were relatively small, except for C18:1n-9t, C18:2n-6t, C20:0 and C22:0.

DTR EE (table 11) is the heavy fraction of the oil. The sum of FAME made up only 504.6 mg/g in this sample compared to 834.3 mg/g in DTL EE (data not shown). No *trans* isomers of any fatty acid was detected in DTR EE. The standard deviations were relatively small in two-thirds of the detected fatty acids, whereas C15:0, C20:0, C21:0, C18:3n-6, C22:0, C22:1n-11 and C24:0 had large standard deviations.

Table 11. The fatty acid composition of DTL EE and DTR EE. Values are expressed as mean of three replicates ± standard deviation. "t" refers to *trans* isomers, "c" refers to *cis* isomers. ND = not detected. The results for each analyzed fatty acid are given as area percent and mg/g. Sum *trans* FA = the sum of *trans* FA for which standards were available in this study, i.e. 18:1n-9t, 18:2n-6t, mono-*trans* EPA and mono-*trans* DHA.

	DTL EE		DTR EE	
FA	**Area percent**	**Mg/g**	**Area percent**	**Mg/g**
C14:0	9.49 ± 0.31	90.83 ± 4.17	3.87 ± 0.20	21.45 ± 1.17
C15:0	0.74 ± 0.02	7.07 ± 0.26	0.12 ± 0.20	0.66 ± 1.14
C16:0	22.19 ± 0.50	212.30 ± 7.58	10.26 ± 0.38	56.85 ± 2.28
C15:1	0.33 ± 0.02	3.16 ± 0.12	ND	ND
C17:0	0.42 ± 0.05	3.97 ± 0.41	0.07 ± 0.12	0.38 ± 0.65
C16:1n-7	10.65 ± 0.18	101.86 ± 3.01	5.07 ± 0.20	28.07 ± 1.29
C18:0	4.21 ± 0.05	40.24 ± 0.81	2.88 ± 0.08	15.93 ± 0.30
C17:1n-11	0.37 ± 0.02	3.54 ± 0.29	0.23 ± 0.02	1.26 ± 0.12
C18:1n-9t	**0.07 ± 0.06**	**0.66 ± 0.58**	**ND**	**ND**
C18:1n-9c	9.70 ± 0.16	92.80 ± 2.72	6.46 ± 0.21	35.81 ± 1.33
C18:1n-7	3.69 ± 0.09	35.26 ± 1.27	2.44 ± 0.07	13.51 ± 0.57
C18:2n-6t	**0.26 ± 0.20**	**2.52 ± 1.92**	**ND**	**ND**
C20:0	0.49 ± 0.33	4.68 ± 3.13	0.09 ± 0.15	0.50 ± 0.86
C18:2n-6c	1.53 ± 0.16	14.60 ± 1.51	0.92 ± 0.02	5.10 ± 0.15
C20:1n-11	3.52 ± 0.09	33.63 ± 1.29	2.26 ± 0.05	12.55 ± 0.44
C21:0	0.18 ± 0.00	1.72 ± 0.04	0.13 ± 0.22	0.71 ± 1.23
C18:3n-6	0.35 ± 0.00	3.33 ± 0.06	0.08 ± 0.15	0.48 ± 0.83
C18:3n-3	0.95 ± 0.02	9.12 ± 0.27	0.61 ± 0.03	3.36 ± 0.23
C22:0	0.08 ± 0.07	0.76 ± 0.66	0.30 ± 0.27	1.66 ± 1.49
C22:1n-11	0.32 ± 0.01	3.04 ± 0.09	0.08 ± 0.14	0.45 ± 0.78
C20:3n-6	0.13 ± 0.01	1.21 ± 0.10	ND	ND
C20:4n-6	0.71 ± 0.01	6.80 ± 0.14	0.97 ± 0.02	5.40 ± 0.13
C24:0	ND	ND	0.35 ± 0.30	1.92 ± 1.67
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
C24:1	ND	ND	4.80 ± 0.15	26.61 ± 0.42
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
C20:5n-3	13.15 ± 0.09	125.73 ± 2.47	19.07 ± 0.48	105.66 ± 3.27
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
C22:5n-3	0.53 ± 0.02	5.08 ± 0.16	4.74 ± 0.14	26.28 ± 0.48
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
C22:6n-3	3.17 ± 0.06	30.35 ± 0.26	25.27 ± 0.49	140.02 ± 3.79
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
Sum *trans* FA		**3.18 ± 1.63**		**0**

4.5.5 DTD 3020 (R1) and DTD 3020 (R1D2)

DTD 3020 (R1) (table 12) contained the heavier fraction from the first column of the SPD. Small amounts of the short fatty acids, such as C14:0 and C16:0 were detected. EPA and DHA were the most abundant fatty acids. In DTD 3020 (R1) only one *trans* isomer of EPA was detected, and the amount, 2.24 mg/g, was quite low. Generally the standard deviance for most fatty acids was relatively small, except in the case of C20:0, C21:0 and C22:0.

DTD 3020 (R1D2) (table 12) contained the heavy fraction from the first column of the SPD, which had been distilled in the second column, thereby removing the heavier unwanted compounds, such as cholesterol, DAG and MAG. In this sample, only one isomer of mono-*trans* EPA was detected and the amount was 2.64 mg/g.

The fatty acid composition of DTD 3020 (R1) and DTD 3020 (R1D2) was approximately the same. Only small differences in the amount of short fatty acids could be observed.

Table 12 The fatty acid composition of DTD 3020 (R1) and DTD 3020 (R1D2). Values are expressed as mean of three replicates ± standard deviation. "t" refers to *trans* isomers, "c" refers to *cis* isomers. ND = not detected. The results for each analyzed fatty acid are given as area percent and mg/g. Sum *trans* FA = the sum of *trans* FA for which standards were available in this study, i.e. 18:1n-9t, 18:2n-6t, mono-*trans* EPA and mono-*trans* DHA.

	DTD 3020 (R1)		DTD 3020 (R1D2)	
FA	**Area percent**	**Mg/g**	**Area percent**	**Mg/g**
C14:0	0.38 ± 0.01	3.48 ± 0.19	0.13 ± 0.01	1.18 ± 0.09
C16:0	3.19 ± 0.08	29.11 ± 1.24	2.78 ± 0.07	25.29 ± 1.08
C17:0	ND	ND	0.17 ± 0.03	1.58 ± 0.29
C16:1n-7	1.51 ± 0.01	13.73 ± 0.30	1.08 ± 0.04	9.78 ± 0.56
C18:0	4.85 ± 0.06	44.26 ± 1.30	4.74 ± 0.14	43.06 ± 2.02
C18:1n-9t	**ND**	**ND**	**ND**	**ND**
C18:1n-9c	8.23 ± 0.09	75.08 ± 2.13	8.23 ± 0.11	74.78 ± 2.38
C18:1n-7	3.66 ± 0.19	33.42 ± 2.17	3.51 ± 0.04	31.92 ± 0.98
C18:2n-6t	**ND**	**ND**	**ND**	**ND**
C20:0	0.38 ± 0.22	3.44 ± 1.96	0.49 ± 0.01	4.46 ± 0.14
C18:2n-6c	1.11 ± 0.06	10.12 ± 0.46	1.13 ± 0.01	10.23 ± 0.33
C20:1n-11	0.38 ± 0.05	3.43 ± 0.50	0.28 ± 0.04	2.51 ± 0.29
C20:1n-9	1.25 ± 0.03	11.39 ± 0.25	1.25 ± 0.15	11.37 ± 1.25
C21:0	0.36 ± 0.31	3.26 ± 2.82	0.50 ± 0.12	4.56 ± 1.09
C18:3n-6	0.35 ± 0.05	3.20 ± 0.49	0.28 ± 0.10	2.54 ± 0.87
C18:3n-3	0.71 ± 0.03	6.50 ± 0.24	0.74 ± 0.02	6.72 ± 0.27
C22:0	0.04 ± 0.06	0.34 ± 0.59	0.12 ± 0.02	1.07 ± 0.16
C20:2n-6	0.18 ± 0.03	1.67 ± 0.25	0.22 ± 0.02	1.98 ± 0.14
C22:1n-11	0.56 ± 0.09	5.08 ± 0.84	0.47 ± 0.04	4.27 ± 0.32
C20:3n-6	0.72 ± 0.08	6.54 ± 0.82	0.51 ± 0.05	4.62 ± 0.45
C20:4n-6	1.77 ± 0.09	16.15 ± 0.64	1.92 ± 0.02	17.40 ± 0.18
Mono-t EPA	**0.25 ± 0.01**	**2.24 ± 0.09**	**0.29 ± 0.01**	**2.64 ± 0.07**
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
C20:5n-3	36.54 ± 0.60	333.26 ± 9.82	36.49 ± 0.30	331.56 ± 9.21
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
Mono-t EPA	**ND**	**ND**	**ND**	**ND**
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
C22:5n-3	3.32 ± 0.26	30.25 ± 1.99	3.69 ± 0.05	33.50 ± 0.31
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
C22:6n-3	23.61 ± 0.18	215.29 ± 3.67	23.44 ± 0.06	212.95 ± 4.48
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
Mono-t DHA	**ND**	**ND**	**ND**	**ND**
Sum *trans* FA		**2.24 ± 0.09**		**2.64 ± 0.07**

4.6 Temperature experiments

Heating a fish oil concentrate (30% EPA and 20% DHA) for 1, 15, 30 and 60 minutes at 180, 190 and 200 °C was executed to see how time and temperature affected the formation of *trans*-fatty acids in the fish oil. The results are found in table 13.

All the samples contained the same amount of C18:1n-9t, of approximately 0.06-0.07%. There was no increase in the amount of C18:1n-9t with increasing temperature or time. In addition to C18:1n-9t, all the samples contained the same peak, with a retention time corresponding to an isomer of mono-*trans* EPA. All the heated samples had a larger area percent of the mono-*trans* EPA than the control sample (no heat). However, this peak seemed to decrease in size as time of heating increased. The only sample to contain two peaks corresponding to the retention time of isomers of mono-*trans* EPA was the one that had been heated for 60 minutes at 200 °C, so there was an increase in the area percent of mono-*trans* EPA in this sample compared to the sample that had been heated for 30 minutes at 200 °C.

Table 13. The area percent (A%) of *trans* fatty acids in oil samples that had been heated for 1, 15, 30 and 60 minutes at 180, 190 and 200 °C. Two replicates were tested for each sample. Total *trans* FA = the sum of *trans* FA for which standards were available in this study, i.e. 18:1n-9t, 18:2n-6t, mono-*trans* EPA and mono-*trans* DHA.

Oil sample	18:1n-9t (A%)	Mono-*trans* EPA (A%)	Mono-*trans* DHA (A%)	Total *trans* FA (A%)
No heat (control)	0,06	0,27	0	0,33
1 min 180 °C	0,06	0,37	0	0,42
15 min 180 °C	0,06	0,35	0	0,40
30 min 180 °C	0,06	0,32	0	0,37
60 min 180 °C	0,06	0,29	0,03	0,38
1 min 190 °C	0,06	0,36	0	0,41
15 min 190 °C	0,06	0,32	0	0,37
30 min 190 °C	0,07	0,29	0	0,35
60 min 190 °C	0,06	0,29	0,13	0,48
1 min 200 °C	0,06	0,39	0	0,44
15 min 200 °C	0,06	0,31	0,05	0,42
30 min 200 °C	0,06	0,29	0,12	0,47
60 min 200 °C	0,06	0,34	0,24	0,64

The sample which had been heated for one hour at 180 °C contained 0.03% mono-*trans* DHA. The samples which had been heated for 15 and 30 minutes at 200 °C, contained 0.05 and 0.12% mono-*trans* DHA, respectively. The sample that was heated for one hour at 190 °C contained 0.13% mono-*trans* DHA, distributed over two peaks corresponding to the retention time of mono-*trans* DHA. The sample which had been heated for one hour at 200 °C also contained two peaks corresponding to the retention time of mono-*trans* DHA, the area percent of these peaks amounted to 0.24%.

The total amount of *trans* fatty acids in the control sample was 0.33%. The content of *trans* fatty acids in most of the heat-treated samples were around 0.4%. The sample that had been heated for 60 minutes at 190 °C and the sample which had been heated for 30 minutes at

200 °C contained a total of 0.48 and 0.47% *trans* fatty acids, respectively. The total amount in the sample which had been heated for 60 minutes at 200 °C was 0.64% *trans* fatty acids.

5 Discussion

The concentration of PTSA in 1,4-dioxane described by Mjøs (2005) worked well for producing *trans* isomers from all-*cis* FAME standards of EPA and DHA. The tests showed that 60 minutes at 60 °C produced a proper amount of mono-*trans* isomers, which were considered the most important ones. Applying 60 °C for 120 minutes also worked well, but produced much higher amounts of the poly-*trans* isomers. Hence 60 minutes of heat treatment was considered the best approach. The smudged appearance of all-*cis* EPA, EPA + PTSA (no heat) and EPA without PTSA for one hour at 60 °C (figure 11), could be explained by sample overload, as there was only one component in the applied samples that appeared smudged and the amount applied to the plate was as large as the samples containing multiple components (different *trans* isomers).

Several experiments were carried out using the 20x20 cm PLC plates in the large development chamber to obtain larger amounts of *trans* FAME standards. The results were always smudged samples, no separation, and a dark band following the eluting streaks. Using 10x10 cm HPTLC plates in the large chamber under the exact same conditions as in the small chamber, revealed that the development chamber, not the 20x20 cm PLC plates, was the source of error. It may be attributed to its relatively larger size in relation to the plates, and that this relatively larger volume resulted in the atmosphere of the tank not being saturated with mobile phase at the low temperature. This, however, is unlikely, since the large chamber is made for the development of 20x20 cm plates and several studies have reported using 20x20 cm PLC with success (Fournier *et al.* 2006 A; Fournier *et al.* 2006 B; Srigley & Rader 2014). Another possible explanation might be found in the material of the lid. The large chamber had a lid made of glass, whereas the small chamber had a lid made from metal. Metal and glass react differently to temperature, i.e. they have different thermal expansion; metal contracts more at low temperatures than glass does. The metal lid could have made the small chamber more gas tight at -25 °C than the glass lid made the large chamber. This was not tested, as there were no metal lids available for the large chamber and no glass lids available for the small chamber.

Using 10x10 cm HPTLC plates for preparation of *trans* isomer standards of EPA and DHA FAME worked well. It was expected that not enough standard would be produced and that several plates would be needed to obtain enough sample for GC separation. This was not the case, however, and one plate was sufficient to test the different temperature programs and to check later whether the retention time of the *trans* isomers had been altered after all the sample runs. The gap between all-*cis* and mono-*trans* isomers on the plates was narrow, and

the small apparent *trans*-isomers co-eluting with all-*cis* isomers in the optimization of GC conditions are probably all-*cis* isomer residuals from scraping off the plates, contaminating the *trans* FAME standards.

It seems that the usual approach to the analysis of *trans* fatty acids is running the GC oven isothermally. This was also tested in an early stage of this study, but it was found to give poorer separation between the low boiling point fatty acids. In addition, the antioxidant BHT was added to the solvent used for sample preparation. This led to BHT being eluted very close to C14:0. For these reasons, the temperature programs were made.

The different temperature programs were tested to achieve the best possible separation of isomers of EPA, DHA and other compounds with closely coinciding retention times. GLC 68D which contained C24:1, EPA and DHA, was compared with the previously made *trans* isomer standards of FAME EPA and DHA. Under the conditions of temperature program 1, sharp peaks with no co-elution between the compounds of GLC 68D and the mono-*trans* isomers were observed. Some co-elution was observed between the di-*trans* isomers and the all-*cis* isomer, but this may not be overcome by any temperature program. Temperature program 2 did not show as sharp peaks and good resolution as temperature program 1. In the chromatograms for temperature program 2, the peaks were more tailed and more broadened, which led to components eluting closer to each other, especially in the DHA-region, where peak 7 eluted into all-*cis* DHA. Temperature program 3 very closely resembled temperature program 1, with sharp peaks and resolution between the peaks. The main difference between temperature program 1 and 3 was the longer retention times of the components in the latter program. The di-*trans* isomers were once again not properly separated. Temperature program 4 did not give useful results as the chromatograms showed poor separations and the C24:1 peak did not show up. It had probably become too broad for detection. One of the mono-*trans* EPA peaks eluted halfway into the all-*cis* EPA peak. The peaks in the DHA region of the chromatogram showed both tailing and broad peaks, and one of the mono-*trans* DHA isomers co-eluted with all-*cis* DHA. Because the mono-*trans* isomers showed such poor results it was decided not to check the di-*trans* isomers in this temperature program. The reason that temperature program 1 and 3 gave similarly good results could probably be ascribed to the temperature increase from 150 °C to 170 °C by 2 °C/min. This seems to be important for the sharpness of the peaks. With a slower increase in temperature between 150 °C and 170 °C band-broadening became a problem. The rate of increase from 110 to 150 °C by 10 °C/min or 20 °C/min did not seem to have any large impact on the shape of the peaks. A carrier gas flow of 1 ml/min gave better results than 1.5 ml/min, as can be seen in the results of temperature

program 4. It could however, be argued that temperature program 4 should have been tested with the same flow as the other temperature programs.

Temperature program number 1 was chosen for the subsequent analyses. It showed good separations and relatively short retention times compared to temperature program 3, which also showed good separations. When standards which contained DPA (C22:5n-3) was tested, it was found that one mono-*trans* DHA isomer co-eluted with this fatty acid. A standard containing this fatty acid should have been used during the testing of temperature programs, and this presents another weakness to the study.

Compared to the BPX-70 used by Sciotto & Mjøs (2012) the SLB-IL111 gave better results for mono-*trans* EPA with four peaks, and little apparent co-elution with other compounds. For mono-*trans* DHA the results were better on the BPX-70 column with five peaks and no co-elution compared to the SLB-IL111, where four peaks were detected and one peak co-eluted with DPA. The CP-Sil88 column, which was used in Fournier *et al.* (2006 A), separated four peaks of mono-*trans* EPA and five peaks of mono-*trans* DHA, but one of the EPA peaks co-eluted with all-*cis* EPA. This meant that the 100 meter SLB-IL111 showed better results for the EPA region, but worse results for the DHA region. Mjøs (2008) investigated the use of a 50 meter polyethylene glycol (PEG) stationary phase column for determination of *trans* isomers of EPA and DHA. Four peaks were observed for mono-*trans* EPA, where one isomer co-eluted with C22:0 and one isomer co-eluted partially with C22:1n-9. Four peaks were also observed for mono-*trans* DHA, and no co-elution was observed. Srigley & Rader (2014) also used the SLB-IL111 column, but they used 200 meters instead of the 100 meters that were used in this present study. In their study they were able to separate five peaks of mono-*trans* EPA and four peaks for mono-*trans* DHA, where one of the mono-*trans* DHA peaks co-eluted with DPA. The longer column therefore gave the same results for mono-*trans* DHA, but better results for mono-*trans* EPA, at the cost of a longer analysis time.

Fardin-Kia *et al.* (2013) found 125 FAMEs in their samples, using a 200 meter SLB-IL111 column. In the present study, many peaks were also detected, but not presented here because a limited selection of standards for identifying the peaks was available at the laboratory. It would also have been useful to know which *trans* isomer eluted in the different peaks, i.e. which double bond had isomerized. Finding out which compounds were present in the samples could have been investigated with the use of mass spectrometry (MS), but such equipment was not available at the laboratory where the present study was executed.

Samples from different processing steps were analyzed for fatty acid composition, with emphasis on *trans* fatty acids. None of the samples from the processing of fish oils contained large amounts of *trans* fatty acids.

The fish oil before stripping (VNT 1812) was similar to the stripped fish oil (STF 1812). The stripping process was performed at 199 °C, which is a very high temperature, and one might expect some *trans* fatty acids to form (Oterhals & Berntsen 2010). However, the fatty acid composition of the oil before and after stripping was the same. VNT 1812 contained 0.82±0.71 mg/g of 18:1n-9t, but this *trans* fatty acid was not detected in one replicate, and if that replicate was removed the average was 1.23 mg/g. If the "outlier" was removed from STF 1812, the result for 18:1n-9t was the same as for VNT. One isomer of mono-*trans* EPA was also detected at similar levels in VNT 1812 and STF 1812. The total amount of *trans* fatty acids was far below the allowed content of 2% (Helse- og omsorgsdepartementet 2014). The reason that no increase in *trans* fatty acid content was observed, even though the fish oil had been processed at 199 °C, might be attributed to the short residence time of the oil in the SPD-column.

DEO 1812 was deodorized at 190 °C and had the same fatty acid composition as BLF 1812, which was sampled before deodorization. The only *trans* fatty acid detected was 18:1n-9t, and the concentrations were approximately the same in both samples. Deodorization has been mentioned in several studies as a potential source of *trans* fatty acid formation (e.g. Kemény *et al.* 2001, Fournier *et al.* 2007), but the deodorization temperature and time applied to this sample seemed not to be sufficient to isomerize detectable amounts of *cis* double bonds to *trans* double bonds. At Nordic Pharma Inc. they perform continuous deodorizing, as opposed to traditional batch deodorizing, thereby exposing the oil to the high processing temperature for a shorter time.

The ETY 2412 samples had similar fatty acid compositions; the degassing did not seem to alter the amount of any fatty acid to any notable extent. The temperature of the degasser was 110 °C. Three different *trans* fatty acids were detected in the ETY 2412 samples, namely 18:1n-9t, 18:2n-6t and one isomer of mono-*trans* EPA. 18:1n-9t was not detected in two of the replicates of the FEED samples, but was detected as 0.93 mg/g in one of the replicates. The same *trans* fatty acid was detected in two of the replicates of the DEGASSER samples, with an average of the two amounting to 0.97 mg/g. The fatty acid 18:2n-6t was detected in two of the replicates from the FEED sample, the average of the two replicates being 1.03 mg/g, whereas all the replicates of the DEGASSER sample contained this *trans* fatty acid, at an average of 1.13 mg/g. Both ETY samples contained approximately

1.80 mg/g of mono-*trans* EPA. There was little difference between the two samples, regarding the fatty acid composition. The true amount of *trans* fatty acids is probably close to the values presented in this paragraph, where the outliers have been removed, though one should be careful with removing potential outliers when so few replicates have been tested. The total amount of *trans* fatty acids in the ETY 2412 samples was close to 4 mg/g, which means that these samples have the highest content of *trans* fatty acids of all the different process samples in this study. However, 4 mg/g is still low compared to the allowed content in oils of 20 mg/g.

If there were any "short" *trans* fatty acids in the samples they were expected to be found in DTL EE, the distillate light fraction, which is a concentrate of the low boiling point fatty acids. The highest temperature for this process was 127.7 °C. The *trans* fatty acids present in DTL EE were 18:1n-9t and 18:2n-6t, amounting to 0.66±0.58 mg/g and 2.52±1.92 mg/g respectively. The standard deviations were relatively high, and implied some uncertainty on the real amounts. However, in one of the replicates 18:1n-9t was not detected, and the other two had peak areas just above the set integration level. The average of the two replicates in which 18:1n-9t was detected equaled 1.00 mg/g. For 18:2n-6t one of the replicates showed three times more of this fatty acid than the two other replicates, and if this high value was removed from the calculations the amount of 18:2n-6t where calculated to be 1.40 mg/g. If the extreme values for each fatty acid was removed the total *trans* fatty acid content was 2.40 mg/g, which is about a tenth of the allowed content.

The highest temperature exerted in the DTR EE process was 143 °C. No *trans* isomers of any fatty acid was detected in DTR EE. Only 50% of the weight in this sample was attributed to fatty acids, the rest probably being heavy compounds such as cholesterol, MAG, DAG and pigments. Because only half the sample was FAEE the *trans* fatty acids present in this fraction might have been below the limit of detection. The DTR fraction is not meant for human consumption directly, but some of the desired fatty acids from this sample, such as EPA and DHA, can be distilled from the heavier compounds. Such a distillate, having a higher ratio of FAEE, might have contained some *trans* fatty acids, even though they were not detected in this sample. The large standard deviations observed for some of the fatty acids in DTR EE arose from the FAME not being detected in some replicates and detected in others. The heavier compounds present in the sample may have interfered with the methylation of the FAEE, making the injected samples heterogeneous. Since it was known that DTR EE had a large fraction of heavier compounds blanks were injected between each replicate. This was done to prevent heavy compounds from interfering with the subsequent analysis.

DTD 3020 (R1) and (R1D2) was treated with maximum 127.7 °C and 143 °C, respectively. R1 was previously separated from DTL EE and contained R1D2 and DTR EE before entering the second SPD-column, where the latter two fractions were separated. R1 and R1D2 had approximately the same fatty acid composition. Only one *trans* fatty acid was detected in both samples. This was one isomer of mono-*trans* EPA. In R1, the amount of this *trans* fatty acid was 2.24 ±0.09 mg/g and in R1D2 it amounted to 2.64±0.07 mg/g. This is a slight difference, just as there is a slight difference observed for the other fatty acids in these samples. The total amount of *trans* fatty acids was low, especially compared to the large proportions of the healthy omega-3 fatty acids.

By comparing the ETY 2412 samples to DTL EE and the DTD 3020 samples one gets an indication of whether the *trans* fatty acids are concentrated along with the other fatty acids. The same amount of C18:1n-9t was detected in ETY 2412 DEGASSER and DTL EE, as they both contained approximately 1 mg/g, if disregarding the outlier as discussed previously in this chapter. C18:1n-9t was not detected in any of the DTD 3020 samples or in the DTR EE sample. In ETY DEGASSER, 1.13 mg/g of C18:2n-6t was found. The same *trans* fatty acid was not detected in the DTD 3020 samples and DTR EE. In DTL EE, 1.40 mg/g of C18:2n-6t was detected, if the outliers were removed. This indicated a small increase of this *trans* fatty acids in the volatile fraction of the distillate. The amount of mono-*trans* EPA detected in ETY 2412 DEGASSER was 1.86 mg/g, whereas it was not detected in DTL EE or DTR EE. In DTD 3020 R1, 2.24 mg/g of mono-*trans* EPA was detected and in DTD 3020 R1D2 2.64 mg/g was detected. There was a slight increase of mono *trans* EPA in the concentrate. However, the total content of identified *trans* fatty acid was reduced in all samples compared to ETY 2412 degasser. These comparisons of the product entering the concentration process and the different fractions produced indicate that there is no real danger of concentrates having very high amounts of *trans* fatty acids under the present processing conditions. The *trans* fatty acids analyzed in this thesis were divided into the two fractions, the shorter *trans* fatty acids entered the volatile fraction and the ones entered the concentrate. In total, the amount of *trans* fatty acids is reduced from ETY to fish oil concentrate.

Analyses of *trans* fatty acid content of commercially available marine oils have been performed. Sciotto & Mjøs (2012) found low amounts of *trans* fatty acids in all the oil samples of marine origin. The 1812 oils contained an average of 0.3 ± 0.2%, and fish oil concentrates contained an average of 0.7 ± 0.6% *trans* fatty acids. Srigley & Rader (2014) found an average of 0.4 ± 0.3% *trans* EPA + *trans* DHA in their samples of "natural" fish oil supplements and an average of 0.6 ± 0.4% *trans* EPA + *trans* DHA in their samples of fish oil

concentrates. Srigley & Rader (2014) did not comment the amount of other *trans* fatty acids present in their samples. In the present study, the 1812 fish oils contained an average of 0.2% *trans* fatty acids and the concentrates (3020) contained 0.2-0.3% *trans* fatty acids. The samples from the processes at Nordic Pharma Inc. were in the lower range compared to samples of commercially available fish oils.

It has become clear that values of about to 1 mg/g or below, of *trans* fatty acids, are very uncertain values because the area is sometimes detected and sometimes not. This may be solved in two possible ways: one is closer scrutiny of the chromatograms by more manual integration or setting a lower minimum area. The other solution could be to have more concentrated samples. The problem with having samples that are too concentrated is the possibility of broader peaks that may interfere with the integration of neighboring fatty acids with only a slight difference in retention times.

It has also become clear that the processing conditions applied to the fish oils at Nordic Pharma Inc. do not have a detrimental effect on the oil when it comes to the formation of *trans* fatty acids. A remaining question is where the observed *trans* fatty acids originate from, as there was no samples showing any clear increase in *trans* fatty acids during a specific processing step. The *cis* double bonds are the naturally occurring ones in fatty acids, not the *trans* double bonds. This means that at some processing point, before the ones that have been tested here isomerization must have occurred. *Trans* fatty acids might have been formed during cooking, pressing, neutralization or they might even have come from the diet of the fish. This study contained samples from all the high temperature processes at this fish oil plant and samples from the processes directly preceding the high temperature processes. However, this study did not have samples of fish oil from processes preceding the bleaching step. Other negative impacts might arise from the processing conditions applied, such as oxidation. Oxidation measurements were not part of this thesis, but internal results from Nordic Pharma Inc. show that under correct and well-controlled production processes, oxidation is and kept at a minimum and not a quality issue (personal communication Lødemel).

In the temperature experiment, high but realistic processing temperatures were tested against time to see how they affected the formation of *trans* fatty acids in a fish oil concentrate. The experiment gave clear indications that both time and temperature had an effect. All the samples that had been heated for one hour showed an increase in the amount of mono-*trans* DHA, although the increased amount in the sample that had been heated at 180 °C was very small. At 200 °C an increase in mono-*trans* DHA could be observed after only

15 minutes, indicating that 200 °C is a critical temperature for the formation of *trans* isomers from the LC-PUFAs. After one hour at 200 °C more isomers of mono-*trans* EPA also started to form. Mjøs & Solvang (2006) performed a similar temperature experiment with fish oil concentrates and temperatures ranging from 140 to 240 °C at intervals of 2 hours. Their study found an increase in the amount of mono-*trans* EPA after 4 hours at 180 °C, and after 2 hours at 200 °C. Similar results were found for mono-*trans* DHA, but these started to form after 4 hours at 160 °C and 2 hours at 180 °C. Mjøs & Solvang (2006) did not investigate how one hour of heat treatment affected the formation of *trans* EPA and DHA, but data from the present study, having oils heated for a shorter time, seemed to agree with their data on the formation of *trans* fatty acids.

My findings that mono-*trans* isomers of DHA started to increase before mono-*trans* isomers of EPA fits well with the findings in Mjøs & Solvang (2006) and Fournier *et al.* (2006 A), but leaves the question whether the mono-*trans* isomer of EPA observed in several samples from the processing of fish oil and in all the samples in the temperature experiment really is mono-*trans* EPA or some other compound having the same retention time? The fact that this peak is present in so many samples without accompanying mono-*trans* DHA, and the fact that the area of the peak seemed to decrease with increased time of heating, underlines this uncertainty. One way this could be determined is by mass spectrometry (MS), a tool that offers a way of confirming the identity of the compound that is detected. The FID detector has many benefits. It is easy to use, it gives a clear indication of the amount of each compound that is detected and it is versatile. However, one must rely on the retention time of standards to identify the compounds in the samples. Several compounds may have the same retention time on a given stationary phase with a given temperature program. By using MS and referring to a library of the mass spectra of the possible analytes, one could probably be more certain of the identity of the compounds being eluted. To verify the increase and decrease of the different fatty acids, and to be able to calculate the quantities in mg/g, an internal standard should have been added to the samples in the temperature experiment.

From the results of the temperature experiment, it seems that the conditions applied to the fish oil in the processing plant do not result in detrimental effects regarding the oils' content of *trans* fatty acids. The only process with temperatures close to 200 °C is the stripping, but the residence time of the oil in the SPD column is very short, and the time of heating clearly affects the formation of *trans* fatty acids. The fish oil has a longer residence time in the deodorizer, but evidently not long enough for the formation of *trans* fatty acids to occur. Higher temperatures than 200 °C should have been investigated in the temperature

experiment to achieve a better understanding of how temperature and time would have affected the content of *trans* fatty acids in the fish oil concentrate, and to establish a maximum operating temperature. For example one might want to apply higher temperatures in the SPD stripping for removal of cholesterol. Hence it could be interesting to see how a short time interval at a higher temperature would have affected the formation of *trans* fatty acids.

The total *trans* fatty acid that was measured and reported in this thesis was not complete, because standards were not obtained for important *trans* fatty acids such as C18:3n-3t. This may have led to an under-estimation of total amount of *trans* fatty acids. More comprehensive *trans* fatty acid standards should have been bought to give a more complete picture, and more certainty. In addition, standards containing a broader selection of fatty acids should have been tested to see if there were any rare fatty acids that could have co-eluted with the different *trans* standards in the sample. Mjøs & Haugsgjerd (2011) mention the possibility of co-elution between C16:4n-1, C16:4n-3 and C18:1n-9t on highly polar stationary phases. The reported amount of C18:1n-9t may be false, the peak may in fact contain a completely different fatty acid. Even though they did not test SLB-IL111, it is still a possibility that this could have happened in the present study since no standards containing these highly unsaturated C16 fatty acids were available.

There are several sources of error in this study that, in hindsight, could have been checked. A detection limit of the GC peaks should have been investigated using different concentrations of fatty acids with varying volatility. A response factor between the internal standard and different fatty acids at varying concentrations should also have been established, since this would have given a lot more certainty to the calculated amounts of fatty acids present in the samples. Testing the recovery of samples with known amounts of fatty acids should also have been performed, to see whether over- or under-estimation was inherent in the system. There is always some uncertainty involved in chemical analysis, but the goal should be to reduce this uncertainty as much as possible. No statistical tests were performed on the data, because the data sets were too small. If more replicates had been tested analyses of variance could have been performed to establish whether the observed differences were significant or not. With no such tests it is difficult to conclude on the results obtained, especially when the amount of *trans* fatty acids were as small as they were in basically all the samples.

6 Conclusions and further work

The main objective of this study was to investigate whether or not *trans* fatty acids were formed during the processing of fish oils at Nordic Pharma Inc. The analyses of the samples indicated that *trans* fatty acids are not formed under the current processing conditions. *Trans* fatty acids were detected, or at least components with the same retention time as the standards of certain *trans* fatty acids, but these were present in all the samples in comparable amounts and therefore one can conclude that the processing conditions did not contribute to the formation of *trans* fatty acids. The total *trans* fatty acid content of concentrates was lower than the content of the fish oil before concentration. In addition, the observed amounts of *trans* fatty acids were well below the permissible amount, set by the authorities in several countries.

The secondary objective was to establish a method for the analysis of *trans* fatty acids present in fish oils. The method for producing *trans* standards of FAME EPA and DHA, using PTSA, was successful. The use of HPTLC plates for separation and preparation of the aforementioned standards also worked out well, so there was no real need for the bigger PLC plates. The use of a 100 meter SLB-IL111 column proved as efficient as previously reported columns for separation of *trans* isomers in fish oil. Some co-elution was observed, but this has been reported for other columns as well. For the method described in this thesis to be able to report the total *trans* fatty acid content, additional standards should be tested that contain a wider spectrum of all-*cis* and *trans* fatty acids.

The temperature experiment showed that high temperatures and long residence time were necessary for *trans* fatty acids to form. After one hour at 200 °C, i.e. the harshest conditions applied, several *trans* isomers started to form. No process at Nordic Pharma Inc. applies such high temperatures for this long period of time.

There are a lot of interesting aspects that should be included in future work in this field. Firstly, a detection limit should be established by testing different concentrations of known components with different volatilities. Recovery of samples with known amounts of different fatty acids should be measured against the internal standard, both to see if the system under- or over-estimates the contents and to see if C23:0 is the best suited internal standard for this analysis. FAME standards containing a larger variety of fatty acids should be tested to see if there are more co-elutions and to be able to identify every peak in the chromatogram. It would also be very interesting to use a GC-MS to gain more certainty to which compounds were present and to determine if peaks eluting really are *trans* fatty acids. The temperature experiment should also be extended to higher temperatures than the ones tested in this study

to see how the fish oil concentrates react to different time intervals. This is interesting because there is a need to test if higher temperatures in the stripping process could remove cholesterol from the fish oil. Isomerizing for example the GLC 68D standard, which contains many fatty acids but not DPA, would be very interesting. By doing this one could investigate at what rate the different isomers of DHA form and establish a correction factor for the isomer co-eluting with DPA.

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