Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

Description of the processes in the value chain and risk assessment of decomposition substances and oxidation products in fish oils

Opinion of Steering Committee of the Norwegian Scientific Committee for Food Safety

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Assessed by

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PREFACE

The Norwegian Food Safety Authority has requested the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) to make a risk and benefit assessment of marine oils. Marine oils (fish oils, cod liver oils, krill oils and seal oils) represent the product group accounting for the largest sales volume of food supplements, and fortification of regular foods with these oils is increasing.

The task from the Norwegian Food Safety Authority is divided in three separate parts:

Part 1: Risk Assessment of decomposition substances and oxidation products in fish oils

Part 2: Evaluation of negative and positive health effects of n-3 fatty acids as constituents of food supplements and fortified foods

Part 3: Risk- and benefit assessment of marine oils.

This report answers the terms of reference in the assessment of Marine oils - Part 1:

In accordance with the terms of reference, the main focus is on production of fish oil and on fish oil used as food supplements (i.e. in bottle or encapsulated). Other marine sources for oil production such as krill and seal blubber, as well as emulsions and microencapsulation of marine n-3 fatty acids for fortification of regular food, are not evaluated in detail. Furthermore, food supplement based on oil derived from the crustaceans *Calanus finmarchicus*, which is a growing business, has not been included.

Major parts of this evaluation are descriptions of marine oil production and description of oxidation of marine oils as asked for by the Norwegian Food Safety Authority (Mattilsynet).

The term “fish oil” is used when the oil in question is derived from fish sources, while the term “marine oil” is used when the oil is derived from fish or other marine organisms, including seal and krill.

Please note that it is beyond the scope of this evaluation to address environmental contaminants in marine raw materials/oils and in final products.

SUMMARY (EXTENDED)

The Norwegian Scientific Committee for Food Safety (VKM) has been asked by the Norwegian Food Safety Authority to perform a health risk assessment on decomposition substances and oxidation products in fish oils intended for human consumption except pharmaceuticals. Seal and/or krill oil intended for human consumption might be included but in separate chapters. EU's hygiene regulations for the production of fish oils intended for human consumption were strengthened from 2008 in the EU (from March 2010 in Norway), but concerns regarding the possible presence of decomposition substances and oxidation products in the oils and their possible consequences on human health were raised.

In the request from the National Food Safety Authority, VKM was asked to include descriptions of the substances resulting from decomposition of the various raw material used for fish oil production, and the products formed during oxidation of marine oils. Additionally, a description of the substances formed and/or eliminated during the various processing steps in the production of marine oils intended for human consumption and to take raw material, processing conditions, storing and transporting conditions of raw material and fish oil into account. Depending on the detectability of the identified substances/products of concern in n-3 fatty acid food supplements, a qualitative, and if possible quantitative, health risk assessment should be performed.

The main focus is on production of fish oil and on fish oil used as food supplement (i.e. in bottle or encapsulated). Other marine sources for oil production such as krill and seal blubber, as well as emulsions and microencapsulation of marine n-3 fatty acids used for fortification of regular food, are not evaluated in detail. Food supplements based on oil derived from the crustaceans *Calanus finmarchicus*, which is a growing business in Norway, has not been included. It was beyond the scope of this evaluation to address environmental contaminants in marine raw materials/oils and in final products.

Marine oil products on the Norwegian marked

The main commercial source of long chained polyunsaturated (n-3) fatty acids (eicosapentaenoic acid; EPA, docosapentaenoic acid; DPA, and docosahexaenoic acid; DHA) is marine oils. The addition of marine n-3 fatty acids in regular food and food supplements is increasing due to the putative positive health effects. Marine oil products such as bottled cod-liver oil and capsules containing fish oil (natural or concentrated), are considered both by the national and EU Food Safety Authorities as food supplements, i.e. concentrated sources of nutritients that are intended to supplement the consumers' daily basic diet. In Norway, products of krill oil for human consumption are food supplements and not considered as novel food whereas EU defines and regulates krill oil as novel food. The most common categories of marine oil supplements on the Norwegian market are cod-liver oil; salmon oil from farmed salmon, fish oil rich in omega-3 (i.e. n-3) fatty acids, and concentrated fatty acids (60% n-3 fatty acid ethyl esters or triglycerides). The highest concentration, n-3 fatty acid ethyl esters 90%, is generally found in pharmaceuticals. There are also n-3 supplements consisting of EPA and DHA as free fatty acid available via internet, but to the best of our knowledge, these are not marketed in Norway.

In the following, the term n-3 fatty acid food supplement includes cod-liver oil, fish oil, krill oil, seal oil or concentrated n-3 fatty acids. Furthermore, the term marine oil includes oil derived from marine organisms like fish, seal, krill or other.

Legislation of quality criteria for marine oils

Currently, for fish oil only the hygienic quality is regulated by the EU's hygiene regulations. The present "*Codex standard for edible fats and oils not covered by individual standard*" is not exclusively related to marine oils and is aimed for voluntary application by commercial partners and not for application by governments, but work to address fish oils has been initiated by the Codex Alimentarius. The [1]European Pharmacopoeia (Ph. Eur.) has quality criteria for some types of marine n-3 oils and these standards are mandatory for medicinal products marketed within the signatory states. The Ph. Eur. standards are however increasingly being used as a reference point for pricing during purchase and sale of bulk marine oils (i.e. crude, refined and concentrated oils) for human consumption. The Global organization for EPA and DHA (GOED) have prepared recommendations for evaluation of oxidative status as well as values for some other quality parameters for some marine oils/concentrates, which also is for voluntary application by commercial partners.

Storage and transport of marine oils in bulk

Refined and concentrated oils ready for packaging (e.g. encapsulation) are stored in bulk using airtight and well-filled containers, protected from light, under an inert gas and transported in flexi bags or in stainless steel or mild steel construction tanks flushed with an inert gas such as nitrogen. The international code of practice for transport of marine oils recommended by Codex Alimentarius applies.

The value chain from raw material to the consumer

Norway is the largest global importer of fish oil (about 200 000 tons in 2005), followed by Chile, United Kingdom (UK) and Canada. Approximately 40% of the world marine n-3 fatty acid products for human consumption are manufactured in Norway.

The marine oil industry in Norway (mainly refiners) exploit crude fish oil mainly imported from Chile, Peru and Morocco, or crude fish/cod-liver oil produced in Norway. The latter is mainly produced from cut-offs from herring and farmed salmon, and from cod liver. Refined and concentrated marine oils in bulk are, as mentions above, usually stored and traded in airtight and well filled containers, protected from light and sealed under an inert gas. They usually have a defined fatty acid profile and known e.g. oxidation status and content of free fatty acids until the seal is broken. In Norway, the production of refined/concentrated marine oil in bulk and the encapsulation/packaging/distribution of products ready for human consumption are mostly handled by different operators. Some refiners may bring their products directly on the market, but most of the refiners sell their product either to value-added resellers (e.g. marketers, distributors, retailers) or to encapsulators. Oil of a certain composition and fatty acid profile is ordered from the buyer. The buyers might store the load, transport it abroad for encapsulation, split the load (involving seal breaking) and/or further trade it. The number of distributions steps involved before the product is available for consumer is unknown.

In connection with the bottling or encapsulation processes, vitamins, antioxidants, stabilisers, colour and flavourings (e.g. lemon, peppermint, orange) may be added to the oil and vegetable oils may be mixed in. Detailed descriptions of the processing steps during encapsulation are not available in scientific literature.

[1] The European Pharmacopoeia is a single reference work for the quality control of medicines in the signatory states of the Convention on its elaboration. Additional information on background and legal framework can be found at http://www.edqm.eu/en/Background-amp-Legal-Framework-50.html

The production line for n-3 fatty acid food supplements may consist of many production steps depending on the composition and quality of the raw material and the final concentration of n-3 fatty acids in the food supplements (e.g. natural oil as in bottles of cod liver, or capsules containing 60% n-3 fatty acid). The presence of decomposition substances, oxidation products and process-generated substances in n-3 fatty acid food supplements are dependent on the composition and treatment of the raw materials, processing parameters for extraction of crude oil, refining and concentration techniques, potential processing techniques prior to encapsulation (e.g. microencapsulation), encapsulation techniques and the storage and transport conditions throughout the whole value chain.

Processing of marine oil

The raw material for marine oil production is composed of three major fractions: solids (fat-free dry matter), oil (i.e. lipids) and water. The purpose of the first processing steps is to separate the lipid fraction from the solids, as completely as possible. The raw material is cooked and/or enzyme-treated, centrifuged for removal of solids and washed with hot water to obtain *crude fish or crude cod-liver oil*. To be suitable for human consumption, crude fish/cod-liver oil has to undergo different refining processes for decreasing or removing impurities. *Refined oil* is crude fish/cod-liver oil that usually has been neutralised, washed with hot water, cooled down (winterisation), bleached, deodorised and distilled, and thus is suitable for human consumption. During the refining process, unwanted components naturally found in the raw material and decomposition substances arising during storage of the raw materials as well as during processing and storage of the crude marine oil, are decreased or removed. The effectiveness depends on the combination of processing steps and conditions used (e.g. equipment, temperature, time, pressure). *Refined natural fish oil/cod-liver oil* contains about 10-30% EPA and DHA depending on the fish species used as raw material, and the EPA and DHA occur as triacylglycerols just as in the living fish. *Concentrated fish/cod-liver oil* contains up to 60-90% n-3 fatty acids and is obtained by chemical modification of refined/semi-refined marine oils. The naturally occurring triacylglycerols are broken down and re-build to achieve higher concentrations of the desired fatty acids EPA and DHA. In the concentrates, the EPA and DHA thus exist either as ethyl esters or as mixed acylglycerols (i.e. mono, di and triacylglycerols) dependent on the concentration technique used. Because of the complex fatty acid composition of fish/cod-liver oils, highly purified long-chained n-3 fatty acids are not easily prepared by any single fractionation technique and a combination of techniques is often used. Traditional refining steps (i.e. bleaching, winterisation and deodorisation) are commonly used as an end procedure of the up-concentration.

During the refining and concentration procedures, the oil is exposed to temperatures that may cause formation of non-volatile decomposition products such as polymers, cyclic fatty acid monomers (CFAM) and geometrical isomers (mostly mono and di-*trans*-fatty acids) of EPA and DHA, i.e. process-generated substances. However, scientific literature on the influence of processing steps and conditions on the content of decomposition substances, oxidation products and process-generated substances in the n-3 fatty acid food supplements, in addition to detailed descriptions of the whole industrial processing line for n-3 fatty acid supplements, is more or less lacking.

Production of krill and seal oil

Krill and seal blubber as raw materials for production of n-3 fatty acid food supplements are not evaluated in depth in this risk assessment. Norway imports small volumes of crude seal oil for further refining and processing and export the refined seal oil. Refining and processing crude seal oil is very similar to that of fish oils. Krill catch is semi-processed onboard and

most of the following oil processing takes place onboard or on shore in South America, but processing details for krill oil is very limited.

Oxidation products and decomposition substances in marine oils

The long chained n-3 polyunsaturated fatty acids in marine lipids are highly susceptible to oxidation, which is an on-going process creating a complex mixture of primary, secondary and tertiary products with different characteristics. Lipid oxidation starts in raw material and continues throughout the value chain of marine oil. The amount and sort of oxidation and decomposition products that may develop in natural and concentrated fish/cod-liver oils are dependent on freshness/oxidative quality of the raw material, storage temperatures and processing parameters such as time, temperature, pressure and catalyst.

The *primary oxidation products* are tasteless and odourless lipid peroxides traditionally determined as peroxide value (PV). Breakdown of the primary oxidation products form mixtures of volatile and non-volatile *secondary oxidation products*. A large variety of secondary oxidation products can be formed (ketones, alcohols, acids etc.), but the most numerous are aldehydes. The volatiles are responsible for the fishy odours and flavour of oxidised fish oil while the non-volatiles are tasteless and odourless compounds. The content of secondary oxidation products, mostly aldehydes, is expressed by the anisidine value (AV), which is given without any unit, but can give an impression of the oxidation status of oil at the time of analysis The *tertiary oxidation products* are relatively unreactive compounds including high molecular weight products such as dimeric and trimeric triacylglycerols (i.e. polymeric compounds).

Throughout the value chain of the marine n-3 fatty acid food supplement production, the main challenges are to reduce the risk of oxidation and thermal transformation of the n-3 fatty acids and subsequently to minimise the content of oxidation products and process-generated substances such as polymers, *trans*-fatty acids and cyclic fatty acid monomers (CFAM) in the n-3 fatty acid supplements. Several of the oxidation products are removed or reduced during refinement. However, oxidation products can be formed in the period between the end of the final refining/concentration process and until the n-3 fatty acid food supplement is consumed, e.g. during transport, encapsulation, packaging, at the retailer and during storage of the product at home.

Data on the influence of raw fish material freshness on the oxidative quality of extracted oil and n-3 fatty acid food supplements for human consumption is lacking. Quantitative data on concentration and occurrence as well as absence of such substances in n-3 fatty acid food supplements are very limited in open scientific literature. Data from open studies of food supplements collected from the supermarket indicate that the products contain both primary and secondary oxidation products in variable amounts. Storage conditions (e.g. time, temperatures) were unknown for all the examined products. The commonly used methods which were used for analysing these substances in n-3 fatty acid food supplements are far from optimal.

Krill and seal oil: the same decomposition substances, oxidation products and substances formed due to processing/thermal treatment of fish oil may also occur in krill and seal oil. Due to higher proportion of phospholipids and cholesterol in the raw krill material, the decomposition substances lysophospholipids and oxysterols might be present at higher concentration in krill oil than in fish oil. No occurrence data of any of the substances associated with processing of krill oil were found.

Analytical methods commonly used to evaluate marine oils

Peroxides (primary oxidations products) and aldehydes (secondary oxidation products) are commonly measured by standard methods such as peroxide value (PV) and anisidine value AV), respectively. However, these methods do not give a true quantitative measure of the oxidative status of the oil. In addition, some food additives and flavourings in n-3 fatty acid food supplements may distort the results. To provide more detailed information about lipid oxidation products in n-3 fatty acid food supplements, advanced methods such as GC-MS and LC-MS should be used, but standardisation of these methods is needed. Additionally, optimisation is needed for methods determining tertiary oxidation products in n-3 fatty acid food supplements. Methods for determination of process-generated substances (e.g. *trans*-isomers) are either at a developing stage or not used to an extent that has revealed the reliability of the method (e.g. cyclic fatty acid monomers). The lack of adequate analytical methods hampers the control of oxidation level in microencapsulated marine oils used for food fortification and in some n-3 fatty acid food supplements.

Currently there are no standard accelerated stability methods that would be enabling the determination of the shelf-life of n-3 fatty acid food supplements. Accelerated stability methods such as measurement of oxidative stability index are needed.

Exposure characterisation: Consumption of fish oil supplements and intake of oxidation products in Norway

Consumption of fish oil supplements in the Norwegian population is high compared with the rest of Europe because of recommendations from the Norwegian Directorate of Health.

The consumption varies in the population but the most recent consumption data are for pregnant women. In pregnancy cohort studies, the use of fish oil/cod-liver oil supplementation among the participants in Norway, Denmark and Iceland is reported to be 59%, 4.3% and, 23%, respectively.

A cod-liver oil consumption of 15 ml/day represents a high consumption and exceeds the daily volume expected from consumption of marine n-3 oil in capsules. Provided that the concentration of potential toxic substances is not substantially different in cod-liver oil and capsules with marine oils, a worst case scenario exposure could have been calculated. It is, however, not possible to calculate exposure to oxidation products in fish oil based on peroxide and anisidine values because these values are relative and not quantitative measure of oxidation.

Form of n-3 fatty acid supplement and sensory acceptability for the consumer

The natural avoidance reaction should to some extent prevent humans from consuming rancid marine oils as the volatile oxidation products produce bad smell and taste with very low thresholds.

Encapsulation or microencapsulation and/or addition of food additives and flavouring may camouflage the content of volatile secondary oxidation products responsible for the rancid odour and taste of the product and the consumers will experience the n-3 fatty acid food supplement as sensory acceptable. In bottled n-3 fatty acid food supplements, the consumer detects the presence of rancid odour and taste and can choose to avoid consumption. VKM notes that surpass of the natural avoidance reaction due to encapsulation or microencapsulation and/or addition of food additives and flavouring might represent a consumer risk and could therefore be of concern.

A number of other oxidation products do not have such properties. Consequently, the consumers may experience the n-3 fatty acid food supplement (bottled or encapsulated) as

sensory acceptable despite possible presence of the odourless and tasteless non-volatile primary, secondary, tertiary oxidation products and process-generated products like *trans*-fatty acids, polymers and cyclic fatty acid monomers, which all may represent a health concern.

Health risk assessment

A quantitative health risk assessment cannot be performed because of insufficient information to perform a full hazard identification and characterisation and lack of quantitative occurrence data for products consumed for an exposure characterisation. Consequently, a qualitative risk assessment has been performed where it was possible.

Hazard identification, characterisation and qualitative health risk assessment of substances of potential concern in n-3 fatty acid food supplements

The raw material used for marine oil production mainly consists of protein, lipid and water and small amount of e.g. glycogen, vitamins and minerals. The raw fish material contains many microorganisms already present in the living fish (especially from the gut), but may also be contaminated by bacteria during handling and processing.

Microorganisms present in the raw material are not likely to be carried over to oil for human consumption due to considerable heat-treatment both of the raw material and the oil during processing. Additionally, the growth requirements of the toxin producing *B. cereus* are unlikely to be fulfilled in the lipid fraction of raw materials and the lipophilic toxin cereulide has to our knowledge not been reported in refined marine oils so far.

Decomposition substances from proteins in the raw material, such as di-peptides, free amino acids, trimethyl amine and biogenic amines, as well as processing aids and substances used in preserving the raw materials such as formic acid, are water soluble and largely removed by several washing step during the refining processes and will normally not be detectable in the n-3 fatty acid food supplements.

Decompositions substances from lipids in raw material that may be present in n-3 fatty acid food supplements include free fatty acids (FFA) and mono/diacylglycerols, lysophospholipids (lysoPL) and oxidised cholesterol.

FFA and mono/diacylglycerols can be present in low concentrations in n-3 fatty acid food supplement. Different guidelines for free fatty acid concentrations have been suggested by CODEX, the Ph. Eur. and GOED. The concentration of FFA is reduced during the different processing steps and mainly during refining by the neutralisation step. However, FFA and mono/diacylglycerols may be formed in production of n-3 fatty acid concentrates in the form of ethyl esters or glycerols. At low concentrations, free fatty acids are not expected to pose any health risk because they are absorbed and esterified in the enterocyte. However, safe levels of concentrations cannot be derived based on the current knowledge. It is desirable that the concentration of free fatty acids remains as low as possible due to their potential harmful effects on the intestinal mucosa. It might be a health concern if products that rapidly release pure free fatty acids are consumed.

Lysophospholipids (lysoPL): Phospholipids represent a minor part of lipids from fish raw materials. The amounts of lysoPL possibly formed are mostly removed during oil processing. No information on content of lysoPL in n-3 fatty acids food supplements has been found, but the concentration of LysoPL in fish and cod-liver oils is most probably very low, and not of any health concern.

Oxidised cholesterol: Concentration of cholesterol in fish oil is low and the fraction of oxidised cholesterol even lower. As the intake of marine oil is low compared with fat from the

rest of the diet, the presence of low amounts of oxidised cholesterol in n-3 fatty acid food supplements is not expected to pose any additional health risk.

Oxidation products in n-3 fatty acid food supplements

Both *primary oxidation substances* such as peroxides, *secondary volatile oxidation products* (short-chained cleavage product such as aldehydes, ketones etc.) responsible for the rancid odour and unpleasant taste of the products, the odourless and tasteless *secondary non-volatile* and *tertiary oxidation products* have been detected in n-3 fatty acid food supplements. The tertiary oxidation products, which are relatively stable high molecular substances such as dimeric and trimeric triacylglycerols (i.e. polymeric compounds), may also be formed as a result of thermal treatment of oil during processing. Even though the concentrations of oxidation products are aimed to be minimised during the oil processing steps and in particular during refining, they may be formed as a result of autooxidation during transport and storage of bulk oil, during mixing and encapsulation, and during transport and storage of n-3 fatty acid food supplements before consumption. At present there is no available information in the open literature on the shelf-life duration of n-3 fatty acid food supplements and way of assessment of shelf-life duration.

It should be noted that there is very little or no information in the public domain with respect to composition of oxidation products in food supplement of marine oil origin, as well as the related toxicological effect of oxidation products in humans. However, animal studies with whole oxidised vegetable oils indicate that high doses can affect health negatively, but the data were not sufficient for risk assessment. Based on the very limited information available, VKM concludes that there is some concern related to regular consumption of oxidised marine oils.

It is desirable that the concentration of primary oxidation products (peroxides) remains as low as possible as it is not clear to what extent the conversion of hydroperoxides to hydroxyl fatty acids during absorption reduces the adverse effects of dietary oxidised lipids. Long-term exposure to dietary lipid peroxides may also have negative effects locally in the gastrointestinal tract.

Some of the secondary oxidation products are absorbed and may have negative effects on health. However, the methodological limitations in isolation and quantification of these compounds from other oxidation products, hampers a risk assessment.

Very little is known about both concentration of aldehydes/core aldehydes and other secondary oxidation products in n-3 fatty acid food supplements and their potential health effects. Therefore a risk assessment cannot be performed.

Little is known about the concentration of tertiary oxidation products such as polymers of oxidised fat in n-3 fatty acid food supplements and their potential health effects. Therefore a risk assessment cannot be performed.

Substances formed from lipids due to thermal treatment during processing that can be present in n-3 fatty acid food supplements include the odourless and tasteless *trans-fatty acids, polymers* and *cyclic fatty acid monomers (CFAM).*

Based on limited information indicating low concentrations of *trans*-fatty acids in marine oils, intake of *trans*-fatty acids from n-3 fatty acid food supplements is low compared with the amount from rest of the diet and is not expected to add significantly to the health risk.

The lack of available data on both occurrence of polymers and CFAM in n-3 fatty acid food supplements and possible health effects made it impossible to perform a risk assessment of polymers and CFAM.

Knowledge gaps: In the risk assessment of decomposition substances and oxidation products in marine oils, knowledge gaps were identified along the whole value chain for production of marine n-3 fatty acid food supplements.

NORSK SAMMENDRAG

Vitenskapskomiteen for mattrygghet (VKM) har fått i oppdrag fra Mattilsynet å utføre en helserisikovurdering av nedbrytningsstoffer og oksidasjonsprodukter i fiskeoljer, samt eventuelt krill og selolje, til humant konsum, med unntak av legemidler. Sel- og krillolje skulle omtales i egne kapitler. EUs hygieneregelverk for produksjon av fiskeoljer til humant konsum ble styrket i 2008 (fra mars 2010 i Norge), men det ble uttrykt bekymring knyttet til eventuell forekomst av nedbrytningsstoffer og oksidasjonsprodukter i oljene og hvilke eventuelle betydninger disse kan ha for folkehelsen.

I oppdraget fra Mattilsynet ble VKM bedt om å inkludere beskrivelse av nedbrytningsstoffene fra ulike råmaterialer brukt ved produksjonen av marine oljer til humant konsum og produktene som dannes ved oksidasjon. Beskrivelse av hvilke stoffer som kan dannes og som elimineres under produksjonsprosessen av marine oljer til humant konsum, skulle også inkluderes. I denne forbindelse skulle ulike forhold ved råmaterialet, produksjonsmåten, lagring og transport av råmateriale og oljen(e) tas i betraktning. Avhengig av om identifiserte bekymringsfulle stoffer foreligger i marine kosttilskudd, skulle en kvalitativt, og om mulig kvantitativt, helserisikovurdering utføres.

Hovedfokus er på produksjon av fiskeoljer og bruk av disse som kosttilskudd; enten i flytende form eller som kapsel. Andre kilder for marin oljeproduksjon, slik som krill og selspekk, og andre produkter i form av emulsjoner og mikroinnkapslet av marint fett som blir brukt til beriking av mat, er ikke omtalt detaljert. Kosttilskudd med Callanusolje, som er en økende industri i Norge, er ikke inkludert. Mattilsynets oppdrag omfatter ikke eventuelt innhold av miljøgifter i marine oljer og kosttilskudd av disse.

Marine kosttilskudd på det norske markedet

Marine oljer er hovedkilden til de kommersielt tilgjengelige langkjedede polyumettede (n-3) fettsyrene eikosapentaensyre (EPA), dokosapentaensyre (DPA) og dokosaheksaensyre (DHA). Marine n-3 fettsyrer blir i økende grad tilsatt vanlig mat og kosttilskudd på grunn av sine omtalte positive helseeffekter. Matmyndighetene definerer både tran på flaske og n-3 kapsler (naturlig eller konsentrert fiskeolje) som kosttilskudd, dvs. konsentrerte kilder av ernæringsstoffer som er tiltenkt å supplere den daglige kosten. Produkter med krillolje ansees som kosttilskudd og ikke som "ny mat" i Norge, mens i EU defineres og reguleres krilloljeprodukter som "ny mat". De vanligste kosttilskuddene med marine oljer på det norske markedet er tran, lakseolje fra oppdrettslaks, fiskeolje rike på omega-3 (dvs. n-3) fettsyrer og konsentrerte fettsyrer (60 % n-3 fettsyre etylestere eller triglyserider). Produkter med den høyeste konsentrasjonen, 90 % n-3 fettsyreetylestere, er gjerne legemidler. Det finnes også n-3 kosttilskudd med EPA og DHA som frie fettsyrer tilgjengelig via internett, men etter det vi kjenner til blir disse ikke markedsført i Norge.

I det følgende omfatter begrepet n-3 kosttilskudd både tran, fiskeolje, krillolje, selolje og konsentrerte n-3 fettsyrer. Begrepet marine oljer omfatter olje utvunnet fra marine organismer som fisk, sel, og krill eller annet.

Regulering av kvalitetskriterier for marine oljer

Per i dag er det kun den hygieniske kvaliteten av fiskeoljer til humant konsum som blir regulert av EUs hygieneregelverk. Codex Alimentarius har en standard for spislig fett og oljer som ikke er regulert annet sted ("*Codex standard for edible fats and oils not covered by individual standard"*), men denne er ikke myntet spesielt på marine oljer. Den er for frivillig bruk av kommersielle partnere, og er ikke for myndigheter. Det er nylig startet et arbeid i Codex for å definere fiskeoljer og utarbeide kvalitetskriterier. Den Europeiske farmakopen

(European Pharmacopoeia, Ph. Eur.) har utarbeidet kvalitetsparametre for noen typer marine oljer. Hvis slike oljer skal markedsføres som legemidler i de landene som er tilsluttet Ph. Eur. samarbeidet, må denne standarden følges. I forbindelse med kjøp og salg av marine oljer på bulk, både råolje, raffinert olje og oppkonsentrert oljer, blir i økende grad Ph. Eur. standarden brukt som referansepunkt for pris. Den globale organisasjonen for EPA og DHA (Global Organization for EPA and DHA; GOED) har laget anbefalinger for oksidasjonsstatus og for enkelte andre kvalitetsparametre for marine oljer. Det er frivillig for de kommersielle partnerne å følge disse anbefalingene.

Lagring og transport av marine oljer på bulk

Raffinerte og konsentrerte oljer som er klar for emballering blir lagret i bulk under en inert gass i lufttette konteinere som beskytter mot lys. Oljene transporteres i fleksibager eller i tanker av rustfritt stål under en inert gass slik som for eksempel nitrogen. Transporten skal foregå i henhold til Codex sin internasjonale anbefaling om transport av marine oljer.

Verdikjeden fra råmateriale til konsument

Norge er verdens største importør av fiskeolje (ca. 200 000 tonn i 2005). Deretter kommer Chile, Storbrittania og Canada. Omtrent 40 % av verdens produksjon av marine oljeprodukter for humant konsum foregår i Norge.

Den marine oljeindustrien i Norge (flest raffinerier) bruker mest marin råolje importert fra Chile, Peru og Marokko, men også rå fisk/fiskelever olje produsert i Norge. Norsk råstoff er avskjær fra sild og oppdrettslaks og torskelever. Raffinerte og oppkonsentrerte marine oljer på bulk blir, som nevnt over, lagret og omsatt i lufttette konteinere forseglet under en inert gass. Vanligvis har da oljene en definert fettsyreprofilen og innholdet av for eksempel frie fettsyrer og oksidasjonsstatus er kjent. Når forseglingen brytes kan oksidasjonsstatus endres. I Norge er stort sett de som produserer raffinerte/oppkonsentrerte marine oljer på bulk og de som videreforedler oljene og distribuerer produkter for humant konsum, ulike aktører. Enkelte raffinerier markedsfører sine egne produkter, men de fleste selger olje i bulk til andre aktører distributører, videreformidlere) eller til aktører som kapsler inn produktene. Kjøper bestiller da olje med en bestemt sammensetning og fettsyreprofil. Kjøper kan lagre lasten, transportere/eksportere den for innkapsling, dele den (innebærer at forseglingen brytes) og/eller videreselge den. Et ukjent antall distribusjonsledd kan være involvert før kosttilskuddet er tilgjenglig for forbruker.

Vitaminer, antioksidanter, stabilisatorer, farge og smaksstoffer (f. eks. sitron-, peppermynte-, appelsinsmak) tilsettes ofte oljene/konsentratene i forbindelse med tapping på flaske eller innkapsling, og vegetabilske oljer kan blandes inn. Detaljert beskrivelse av produksjonstrinnene ved innkapsling er ikke funnet i vitenskapelig litteratur.

Det kan ligge mange produksjonstrinn bak et n-3 kosttilskudd avhengig av sammensetningen og kvaliteten på råvaren samt den endelige konsentrasjonen av n-3 i kosttilskuddet (for eksempel naturlig olje som i tranflasken eller kapsler med 60 % n-3 fettsyrer). Hvor mye nedbrytningsstoffer, oksidasjonsprodukter og prosessgenererte stoffer det kan være i kosttilskuddene er avhengig av sammensetning og behandling av råstoffet, prosesseringsparametre ved ekstraksjon av råoljen, prosesseringsparametre under raffinering og oppkonsentrering, eventuelle prosesseringsteknikker før innkapsling (f. eks. mikroinnkapsling), innkapslingsteknikk, samt lagrings- og transportbetingelser gjennom hele denne kjeden.

Prosessering av marine oljer

Råstoffet for marine oljeproduksjon er sammensatt av tre hovedfraksjoner: fast stoff (fettfritt tørrstoff), olje (dvs. lipider) og vann. Første del av prosesseringen skal skille lipid og fast stoff så fullstendig som mulig. *Råolje* fremkommer ved at råstoffet blir kokt og/eller behandlet med enzymer, sentrifugert for å felle ut fast stoff og vasket med varmt vann. Råoljen må raffineres før den kan brukes til kosum. *Raffinert olje* er råolje som vanligvis er blitt nøytralisert, vasket med varmet vann, nedkjølt (”winterisation”), bleket (”bleached”), deodorisert og destillert. Raffineringen reduserer eller fjerner innholdet av uønskede komponenter som naturlig finnes i råstoffet; av nedbrytningsstoffer som oppstår under lagring av råstoffet og av nedbrytningsstoffer som oppstår under prosessering og lagring av råoljen. Hvor effektiv raffinering er, avhenger både av hvilke prosesseringstrinn som inkluderes og hvilke forhold som brukes (type utstyr, temperatur, tid, trykk). *Raffinert naturlig fiskeolje/tran* inneholder 10-30 % EPA og DHA avhengig av hvilken fiskeart som brukes som råstoff. I slike oljer foreligger EPA og DHA som triacylglyseroler, slik som i levende fisk. *Konsentrert fiskeolje/tran* inneholder 60-90 % n-3 fettsyrer og lages ved kjemisk endring av raffinerte/halvraffinerte marine oljer. De naturlig forekommende triacylglycerolene blir brutt ned og gjenoppbygget for å oppnå høy konsentrasjon av de foretrukne fettsyrene EPA og DHA. I konsentrater foreligger EPA og DHA som etylestere eller som en blanding av acylglyseroler (mono-, di- og triacylglyceroler). Fordi fettsyresammensetningen i fiskeolje/tran er kompleks, må vanligvis en kombinasjon av ulike kjemiske teknikker brukes for å oppkonsentrere de langkjedede fettsyrene. Tradisjonelle raffineringstrinn som bleking, nedkjøling og deodorisering, er vanlig å bruke avslutningsvis i oppkonsentreringen.

Under raffinering og oppkonsentrering blir oljene utsatt for temperaturer som kan forårsake dannelse av ikke-flyktige nedbrytningsprodukter som polymerer, sykliske fettsyremonomerer (CFAM) og geometriske isomere av EPA og DHA (mest mono og di-transfettsyrer). Dette kalles prosessgenererte forbindelser. Det er imidlertid sparsomt med vitenskapelig litteratur om hvilken innflytelse ulike prosesstrinn og prosesseringsforhold kan ha på innholdet av nedbrytningssubstanser, oksidasjonsprodukter og prosessgenererte forbindelser i n-3 kosttilskudd. Det er også mangelfull beskrivelse av hele den industrielle prosesseringslinjen bak n-3 kosttilskudd i vitenskapelig litteratur.

Produksjon av krill- og selolje

Krill og selspekk som råstoff for produksjon av n-3 kosttilskudd er ikke inngående evaluert i denne risikovurderingen. Norge importerer et mindre volum av rå selolje som raffineres og prosesseres, og så hovedsakelig eksporteres. Raffinering og prosessering av selolje er tilsvarende som for fiskeolje. Krill semiprosesseres i tilknytning til fangsten på båten. Videre prosessering av krill skjer også vanligvis på båten eller på land i Sør-Amerika. Opplysninger om prosesseringsdetaljer for krillolje er begrenset.

Oksidasjonsprodukter og nedbrytningsstoffer i marine oljer

De langkjedede n-3 polyumettede fettsyrene i marint fett oksideres lett. Oksidasjonen er en kjedereaksjon hvor det dannes en kompleks blanding av primære, sekundære og tertiære oksidasjonsprodukter med ulike egenskaper. Oksidasjon av fett starter i råstoffet og fortsetter gjennom hele verdikjeden til marine oljer. Hvilke mengder og typer av oksidasjonsprodukter som dannes i naturlig og oppkonsentrerte oljer, er avhengig av ferskhet og oksidasjonsstatus i råstoffet, lagringstemperatur og prosesseringsparametre som tid, temperatur, trykk og katalysator.

Primære oksidasjonsprodukter er smak og luktfrie lipidperoksider som tradisjonelt måles ved bruk av peroksid verdi (PV). Nedbrytning av de primære oksidasjonsproduktene resulterer i

en blanding av flyktige og ikke-flyktige *sekundære oksidasjonsprodukter*. Både ketoner, alkoholer og syrer kan dannes, men ulike typer aldehyder utgjør hovedtyngden av sekundære oksidasjonsprodukter. De flyktige produktene gir den karakteristiske lukten og smaken av oksidert (harsk) fiskefett, mens de ikke-flyktige er smak og luktfrie. Innholdet av sekundære oksidasjonsprodukter, og da mest aldehyder, uttrykkes ved anisidin verdi (AV). AV oppgis uten benevning, men kan likevel gi et inntrykk av oljens oksidasjonsstatus på analysetidspunktet. De *tertiære oksidasjonsproduktene* er relativt lite reaktive forbindelser. Eksempler er dimere og trimere triacylglyseroler med høymolekylær vekt (dvs. polymere forbindelser).

Hovedutfordringene i produksjonskjeden av n-3 kosttilskudd er å minske oksidasjon og temperaturavhengig transformasjon av fettsyrene slik at innholdet av oksidasjonsprodukter og prosessgenererte stoffer som polymere, transfettsyrer og sykliske fettsyremonomere i kosttilskuddene blir så lavt som mulig. Mange av oksidasjonsproduktene reduseres eller fjernes helt under raffinering. På den annen side kan oksidasjonsprodukter også dannes i oljene etter raffinering og frem til kosttilskuddet er konsumert; både under transport, innkapsling, emballering, i butikken og hos forbruker.

Det er mangelfulle vitenskapelige data om hvilken innflytelse ferskhetsgraden av råstoffet har på oksidasjonsstatus til den påfølgende oljen og videre på n-3 kosttilskuddet. Kvantitative data som viser tilstedeværelse og konsentrasjon av nevnte stoffer i n-3 kosttilskudd, eller som viser fravær av slike stoffer, er svært begrenset i vitenskapelig litteratur. Det er publisert noen studier som har analysert n-3 kosttilskudd som finnet i butikker, og som viser at de inneholdt variable mengder av både primære og sekundære oksidasjonsprodukter. Hvordan disse n-3 kosttilskuddene var lagret (f. eks. hvor lenge, hvilken temperatur), var ukjent. Det ble brukt tradisjonelle metodene for å analysere kosttilskuddene, men slike metoder er langt fra optimale.

Krill og selolje: De samme nedbrytningsstoffene, oksidasjonsproduktene og prosessgenererte stoffene som dannes ved fremstilling av fiskeolje, kan dannes ved fremstilling av krill og selolje. Fordi krill inneholder mer fosfolipider og kolesterol enn fisk, kan det være mer av nedbrytningsstoffene lysofosfolipid og oksisterol i krillolje enn i fiskeolje. Det er ikke funnet data på noen av stoffene som kan forekomme i forbindelse med fremstilling av krillolje.

Vanlig brukte analysemetoder for vurdering av oljekvalitet

Peroksider (primære oksidasjonsprodukter) og aldehyder (sekundære oksidasjonsprodukter) blir vanligvis målt ved standardmetoder, henholdsvis som peroksid verdi (PV) og anisidin verdi (AV). Disse metodene imidlertid gir ikke et reelt kvantitativt mål på oljens oksidasjonsstatus. Tilsetnings- og aromastoffer i n-3 kosttilskudd kan også forstyrre analysene. Mer avanserte analysemetoder, som GC-MS og LC-MS, vil fremskaffe mer detaljert informasjon om oksidasjonsprodukter i n-3 kosttilskudd og bør derfor brukes. Slike metoder må standardiseres. Det er også behov for å optimalisere metoder for å måle tertiære oksidasjonsprodukter i n-3 kosttilskudd. Metoder som måler prosessgenererte stoffer, f eks transfettsyrer, er enten under utvikling eller ikke brukt tilstrekkelig til å ha vist pålitelighet (f eks for sykliske fettsyremonomerer). Mangel på adekvate analysemetoder er til hinder for kontroll av oksidasjonsnivå i mikroinnkapslete oljer som blir brukt i beriking av mat, samt i enkelt n-3 kosttilskudd.

Per i dag er det ingen standardisert akselerert stabilitetsmetoder som kan bestemme eventuelt forutsi holdbarhet på n-3 kosttilskudd. Det er behov for akselererte stabilitetsmetoder som f. eks. måling av oksidativ stabilitetsindeks.

Eksponeringskarakterisering: Konsum av kosttilskudd med fiskeolje og inntak av oksidasjonsprodukter i Norge
Den norske befolkningen har et høyt konsum av kosttilskudd med fiskeolje sammenlignet med det øvrige Europa på grunn av slik anbefaling fra Helsedirektoratet.

Inntaket varierer i befolkningen, men de nyeste inntaksdata er for gravide. Kohortstudier med gravide viser at 50 % av de gravide i Norge tar kosttilskudd med fiskeolje eller tran, mens andelen i Danmark og Island er hhv. 4,3 % og 23 %.

Et konsum på 15 ml tran per dag representerer et høyt konsum og overstiger det forventede daglige volumet fra n-3 kosttilskudd i kapsel. Hvis man forutsetter tilnærmet lik konsentrasjonen av potensielt toksiske stoffer i tran og n-3 kapsler, kunne et verstefalls-scenario blitt beregnet. Det er imidlertid ikke mulig å beregne eksponering av oksidasjonsprodukter i fiskeolje basert på peroksid- og anisidinverdier fordi disse tallene er relative, og ikke kvantitative, mål på oksidasjon.

Type n-3 kosttilskudd og sensorisk aksept av forbruker
De flyktige oksidasjonsproduktene i oksiderte marine oljer gir dårlig lukt og smak som oppfattes selv ved svært lave konsentrasjoner. Mennesker har en instinktiv motvilje – naturlig unngåelsesreaksjon – mot å spise harske (oksiderte) marine oljer.

Innkapsling eller mikroinnkapsling og/eller tilsetnings- og aromastoffer kan kamuflere et eventuelt innhold av de flyktige sekundære oksidasjonsproduktene, som gir oljeproduktet harsk smak og lukt, og forbruker vil oppleve produktet som sensorisk akseptabelt. Når kosttilskuddet er på flaske, kan forbruker oppdage harsk lukt og smak og velge ikke å konsumere produktet. VKM merker seg at den naturlig unngåelsesreaksjon hos forbruker omgås ved innkapsling eller mikroinnkapsling av, og/eller tilsetnings- og aromastoffer i, n-3 kosttilskudd. Dette kan medføre en risiko for forbruker og kan derfor være til bekymring.

Et utall andre oksidasjonsprodukter er uten lukt eller smak. Forbruker kan oppleve n-3 kosttilskuddet (på flaske eller kapsel) som sensorisk akseptabelt til tross for innhold av lukt eller smakløse ikke-flyktige primære, sekundære, tertiære oksidasjonsprodukter, eller prosessgenererte stoffer (transfettsyrer, polymere og sykliske fettsyremonomere), som alle kan ha uheldige helsemessig effekter.

Helserisikovurdering
Det har ikke vært mulig å gjennomføre en kvantitativ helserisikovurdering, fordi informasjonsgrunnlaget for å utføre fullstendig fareidentifisering og -karakterisering har vært utilstrekkelig, og fordi det manglet kvantitative data på innhold i kosttilskudd til bruk i eksponeringskarakterisering. Det er i stedet utført en kvalitativ risikovurdering der det har vært mulig.

Fareidentifisering, farekarakterisering og kvalitativ helserisikovurdering av mulig helseskadelige stoffer i n-3 kosttilskudd
Ferskt råstoff som brukes til marin oljeproduksjon består hovedsakelig av proteiner, fett (lipid) og vann, samt små mengde av f. eks. glykogen, vitaminer og mineraler. Fiskeråstoff inneholder mikroorganismer fra fisken (særlig fra tarm), men kan også bli forurenset bakterielt under håndtering og prosessering.

Mikroorganismer i råstoffet blir sannsynligvis ikke overført i ferdig olje for humant konsum ettersom både råstoffet og oljen blir varmebehandlet. Den toksinproduserende bakterien *B. cerus* klarer antagelig ikke å vokse i råstoffets lipidfraksjon og dens lipofile toksin cereulid er etter det vi vet, ikke blitt rapportert i raffinerte marine oljer.

Nedbrytningsstoffer fra proteiner i råstoffet, slik som dipeptider, frie aminosyrer, trimetylamin og biogene aminer, i tillegg til prosesshjelpemidler og konserveringsmidler for råstoff, f. eks. maursyre, er vannløselige. Vannløslige stoffer fjernes av vasketrinnene under raffinering og vil normalt ikke være påvisbare i n-3 kosttilskudd.

Nedbrytningsstoffer fra fett i råstoffet som kan gjenfinnes i n-3 kosttilskudd er frie fettsyrer, mono- og diacylglyseroler, lysofosfolipider og oksidert kolesterol.

Frie fettsyrer (FFA), mono og diacylglyseroler kan gjenfinnes i lave konsentrasjoner i n-3 kosttilskudd. Den Europeiske farmakopen, GOED og Codex har ulike anbefalinger om konsentrasjon av frie fettsyrer. Under raffinering blir frie fettsyrer særlig fjernet under nøytraliseringstrinnet. Både fettsyrer og mono- og diacylglyseroler kan imidlertid bli dannet under oppkonsentrering av n-3 fettsyrene til etylestere eller glyserol. Frie fettsyrer blir tatt opp og esterifisert i tarmcellene og er ikke antatt å representere noen helserisiko i lave konsentrasjoner. Basert på nåværende kunnskap er det imidlertid ikke mulig å angi hvilket innhold som kan anses trygt. Det er ønskelig at konsentrasjonen av frie fettsyrer er så lav som mulig fordi de kan skade tarmslimhinnen. Konsum av produkter med frie fettsyrer som frigis raskt, kan innebære en helserisiko.

Lysofosfolipider: Kun en liten del av fiskefettet består av fosfolipider. Mengden lysofosfolipid som eventuelt dannes, blir hovedsakelig fjernet under prosessering av oljene. Det er ikke funnet noe informasjon om konsentrasjon av fosfolipider i n-3 kosttilskudd. Antagelig er konsentrasjonen av lysofosfolipid i fiskeolje og tran meget lav og utgjør ingen helserisiko.

Oksidert kolesterol: Konsentrasjonen av kolesterol i fiskeolje er lav og konsentrasjonen av oksidert kolesterol enda lavere. Inntak av marine oljer er lavt sammenlignet med fett fra øvrig kost og mengden oksidert kolesterol fra n-3 kosttilskudd er derfor ikke antatt å bidra til økt helserisiko.

Oksidasjonsprodukter i n-3 kosttilskudd

Både *primære oksidasjonsprodukter* slik som peroksider, *flyktige sekundære oksidasjonsprodukter* (kortkjedede spaltingsprodukt som aldehyder) som gir harsk lukt og smak på produktene, og smak- og luktfrie *ikke-flyktig sekundære* og *tertiære oksidasjonsprodukter,* er påvist i n-3 kosttilskudd. De tertiære oksidasjonsproduktene (dimere og trimere triacylglyceroler; polymere forbindelser), som er relativt stabile høymolekylære stoffer, kan også kan dannes på grunn av varme under prosessering av oljene.

Selv om konsentrasjonen av oksidasjonsprodukter så langt som mulig forsøkes å bli minimalisert under prosesseringen av oljene, kan de på grunn av autooksidasjon bli dannet under lagring og transport av olje på bulk, under blanding og innkapsling, og under transport og lagring av ferdig kosttilskudd før konsum. I åpen litteratur er det ingen tilgjengelig informasjon om holdbarhetstiden til n-3 kosttilskudd eller hvordan denne skal vurderes.

Det bør bemerkes at det er lite til ingen offentlig tilgjengelig informasjon om sammensetningen av oksidasjonsprodukter i kosttilskudd av marin opprinnelse, samt hvilke toksiske effekter disse oksidasjonsproduktene kan ha i mennesket. Dyrestudier utført med hele oksiderte vegetabilske oljer viser imidlertid at høye doser har uheldige helseeffekter, men dataene var ikke tilstrekkelige for en risikovurdering. Basert på den meget begrensede tilgjengelig informasjonen konkluderer VKM med at det er noe bekymring knyttet til jevnlig inntak av oksiderte marine oljer.

Det er ønskelig at konsentrasjonen av primære oksidasjonsprodukter (peroksider) forblir så lav som mulig. Det er uklart i hvilken grad omdanning av hydroperoksider til hydroksylfettsyrer under absorpsjonen kan minske uheldige effekter av oksidert fett fra

kosten. Langvarig eksponering av oksidert fett fra kosten kan også ha uheldige effekter lokalt i tarmen.

Noen av de sekundære oksidasjonsproduktene blir absorbert og kan ha uheldige helseeffekter. En risikovurdering forhindres imidlertid av de metodologiske begrensningene forbundet med isolering og kvantifisering disse forbindelsene adskildt fra andre oksidasjonsprodukter.

Veldig lite er kjent både om konsentrasjonen av aldehyder/core aldehyder og andre sekundære oksidasjonsprodukter i n-3 kosttilskudd, og hvilke eventuelle helseeffekter disse forbindelsene kan ha. En risikovurdering kan derfor ikke utføres.

Lite er kjent om konsentrasjonen av tertiære oksidasjonsprodukter, slik som polymerer av oksidert fett i n-3 kosttilskudd, og hvilke eventuelle helseeffekter disse forbindelsene kan ha. En risikovurdering kan derfor ikke utføres.

Stoffer dannet fra lipider på grunn av varmebehandling under prosessering og som kan være i n-3 kosttilskudd, omfatter smak og luktfrie *transfettsyrer, polymere* og *sykliske fettsyremonomere (CFAM).*

Basert på begrenset informasjon som indikerer lave konsentrasjoner av transfettsyrer i marine oljer, er inntak av transfettsyrer fra n-3 kosttilskudd lavt sammenlignet med mengden fra øvrig kost og antas derfor ikke å bidra vesentlig til helserisikoen.

Mangel på tilgjengelige data både med hensyn til forekomst av polymerer og CFAM i n-3 kosttilskudd og eventuelle helseeffekter har gjort det umulig å utføre risikovurdering av polymerer og CFAM.

Kunnskapsmangler: Under arbeidet med risikovurdering av nedbrytningssubstanser og oksidasjonsprodukter i marine oljer, ble det identifisert mangel på kunnskap langs hele produksjonskjeden for marine n-3 kosttilskudd.

CONTENT

ABBREVIATIONS

AA or ARA - arachidonic acid
ALA - alpha linolenic acid
ALAT - alanine amino transferase
ALE - advanced lipoxidation end products
ALP - alkaline phosphatase
AOCS - American Oil Chemists' Society
ASAT - aspartate amino transferase
AV - anisidine value *(see also "Definitions and terms")*
BHA - butyl hydroxyanisole
BHT - butyl hydroxytoluene
CCAMLR - Convention on the Conservation of Antarctic Marine Living Resources
CCFO - Codex Committee on Fats and Oils
CFAM - cyclic fatty acid monomers
CHD - coronary heart disease
CM - chylomicron
DHA - docosahexaenoic acid
DMA - dimetylamin
DPA - docosapentaenoic acid
d.w. - dry weight
EEA – European Economic Area
EPA - eicosapentaenoic acid
EQ - ethoxyquin
ES-TOF-MS - Electrospray Time-of-Flight Mass Spectroscopy
FA - fatty acid
FAO - Food and Agriculture Organization (USA)
FAME - fatty acid methyl esters
FFA - free fatty acids
Fig. - figure
GC - Gas Chromatography
GC-FID - Gas Chromatography - Flame Ionization Detector
GC-MS - Gas Chromatography – Mass Spectrometry
GSH - glutathione
GSSG - oxidised glutathione
GPx - glutathione peroxidase
GOED - Global Organization for EPA and DHA
GMO - gene modified organism
Hb - haemoglobin
HDL - high density lipoprotein
HPLC - High-performance liquid chromatography **or** High-pressure liquid chromatography
HPLC-MS see LC-MS
HPMC - hydroxypropyl methylcellulose
4-HNE - 4-hydroxynonenal
4-HHE - 4-hydroxyhexenal
13-HODE - hydroxy linoleic acid
13-HPODE - linoleic acid hydroperoxyd
IFFO - International Fish meal and Fish oil Organization
LA - linoleic acid
LC-MS - Liquid Chromatography- Mass-Spectrometry (*see also "Definitions and terms")*

LC PUFAs - long chained polyunsaturated fatty acids
LDL - low density lipoprotein
lysoPL - lysophospholipid; partly hydrolysed PL
MDA - malondialdehyde (*see also "Definitions and terms")*
MDA-TBA - reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA)
mM – millimole
N - nitrogen
NMR - Nuclear magnetic resonance
NF-κB - nuclear factor kappa B
NZW - rabbits: New Zeeland White rabbits
NPN - non protein nitrogen
PAH - polycyclic aromatic hydrocarbons
PAF - platelet activating factor
PC - phosphatidylcholine
Ph. Eur. - European Pharmacopoeia
PL - phospholipids
PLA_2 - phospholipase A_2
PPAR - peroxisome proliferator activated receptor
ppm - parts per million
PUFA - polyunsaturated fatty acid
PV - peroxide value *(see also "Definitions and terms")*
RCS - reactive carbonyl species
RSW - refrigerated sea water
SEC - size-exclusion chromatography
SFE - supercritical fluid extraction
SPD - short path distillation
SSO - specific spoilage organism
TAG - triacylglycerol
TBA - thiobarbituric acid
TBARS - thiobarbituric acid reactive substances, measure mainly of MDA
TBHQ - tert-butylhydroquinone
TMA t- rimetyl amine
TMAO - trimetyl amine oxide
TOTOX - total oxidation value of oil (*see also "Definitions and terms")*
TVB-N - total volatile basic nitrogen (*see also "Definitions and terms")*
UV - light ultraviolet light

DEFINITIONS AND TERMS

Acid value: common term used in the fishery industry to describe the amount of free fatty acid in fish oil. The acid value is typically twice as high as the percent free fatty acid in the fish oil.

Acylglycerol: an ester formed from glycerol and fatty acids (previous term glyceride). Mono, di, or triacylglycerol is formed depending on whether one, two, or three of the hydroxyl functional groups of the glycerol molecules is esterified with fatty acid.

Anisidine value (AV): is defined as 100 times the absorbance (at 350nm) of a solution resulting from the reaction of 1g of fat with 100mL of p-anisidine and acetic acid mixture. The compounds react with p-anisidine to form coloured complexes that absorbs at 350nm (spectrophotometric method). The value expresses the amount of aldehydic compounds in the oil and is given without any unit. Other oxidation compounds such as peroxides and polymeric compounds may also contribute to AV.

α-linolenic acid (ALA): Essential polyunsaturated fatty acid in the n-3 series (18:3n-3) that must be supplied through the diet. Main dietary sources are terrestrial plants, nuts and plant oils such as linseed and rapeseed oils. ALA is often referred to as plant n-3 fatty acid.

Astaxanthin is a carotenoid (pigmented molecule) naturally occurring in e.g. krill and shrimps and in wild salmon and trout because their diet is based on zooplankton and krill. Several isomers of astaxanthin exist. Commercial production of astaxanthin comes from both natural and synthetic sources. In Norway, astaxanthin is a feed additive, an ingredient in food supplements and novel foods.

By-products are according to Regulation (EC) No 1069/2009 raw animal materials characterized as unsuitable for human consumption, even when handled according to food hygienic standards.

Core aldehydes are aldehydes left on the triacylglycerol or phospholipid molecule during oxidation of marine oils and belong to the non-volatile secondary oxidation products.

Crude fish oil is fish oil processed from raw fresh fish by cooking and different processing steps to separate oil from protein and water fraction, but not suitable for human consumption.

Cut-offs is other fish parts apart from the fillet for human consumption.

Diacylglycerol is an acylglycerol (previous term diglyceride) consisting of two fatty acid chains covalently bonded to a glycerol molecule through ester linkages. See *acylglycerol*.

DHA (docosahexaenoic acid): Polyunsaturated fatty acid in the n-3 series (22:6n-3) that can be synthesized from ALA. Main sources are marine plants, fish and other seafood and food supplements.

DPA (docosapentaenoic acid): Polyunsaturated fatty acid in the n-3 series (22:5n-3) that can be synthesized from ALA. Main sources are marine plants, fish and other seafood and food supplements.

EPA (eicosapentaenoic acid): Polyunsaturated fatty acid in the n-3 series (20:5n-3) that can be synthesised from ALA. Main sources are marine plants, fish and other seafood and food supplements.

EPA, DPA and DHA are often referred to as marine n-3 fatty acids.

Fish meal and fish oil production: 1000 kg wild fat fish as raw material gives approximately 250 kg fish meal and 67 kg fish oil (Guide to good practice, Norwegian Seafood Federation)

Food supplements: Substances with nutritional and/or physiological effects that are intended to supplement the consumer's daily basic diet.

Glyceride is a pervious term, see *acylglycerol*.

Glycerol molecules: forms the backbone of triacylglycerols.

Liquid chromatography-mass spectrometry: an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry.

Malondialdehyde is a secondary oxidation product formed from the fatty acids containing three or more double bonds. It may be measured in oils as a marker for the aldehyde content by use of the Thiobarbituric Acid Reactive Substances (TBARS) test. Aldehydes react with thiobarbituric acid and give rise to coloured products (spectrophotometric method).

Marine n-3 fatty acids: EPA, DPA and DHA.

Marine oil: oil derived from marine organisms (e.g. fish, seal, krill, or other).

Moroctic acid (also called stearidonic acid): a vegetable n-3 fatty acid (C 18:4 n-3) biosynthesised from *alpha*-linolenic acid. Natural sources are e.g. the seed oils of hemp and corn gromwell and echium.

n-3 fatty acids: ALA, EPA, DPA and DHA.

Non-volatile decomposition products: polymers, cyclic fatty acid monomers (CFAM) and geometrical isomers of EPA and DHA.

Non-polar compounds: see polar compounds.

Norwegian Seafood Federation: Federation representing the majority of companies within the fisheries and aquaculture sectors in Norway.

Partial acylglycerols: esters of glycerol with fatty acids where not all the three hydroxyl groups are esterified, i.e. mono and diacylglycerols.

Pelagic fish: fish living in open sea/free water mass such as herring and mackerel.

Peroxide value (PV) (e.g. lipid hydroperoxide value): Primary oxidation products in oil are mainly hydroperoxides which react with potassium iodide during the liberation of iodine. PV is usually expressed as milliequivalents (meq) of peroxide, iodine or active oxygen per kilogram (kg) lipid, or as millimole (mmole) of peroxide, iodine or active oxygen per kg oil.

Polar/non-polar compounds: Fish oils can easily be separated into two fractions: non-polar compounds (mainly triacylglycerols; e.g. unaltered glycerol molecules) and polar compounds including polymerized triacylglycerols, oxidised triacylglycerols, diacylglycerols and free fatty acids.

Polymeric oxidation compounds (polymeric compounds, polymerized triacylglycerols): high molecular weight tertiary oxidation products such as dimeric and trimeric triacylglycerols.

Post mortem: after death.

Postprandial: After mealtime; e.g. a postprandial rise in the blood glucose level is one that occurs after eating.

Process-generated substances: such as polymers, cyclic fatty acid monomers (CFAM) and *trans*-fatty acids results from thermal exposure of oils with high amounts of EPA and DHA.

Rigor mortis: muscular stiffness after death.

Schiff's base: the condensation product of the chemical reaction of an aldehyde with a primary amine.

Semi-refined marine oil: the oil has gone through only some of the refining steps

Stearin: the tristearate ester of glycerol.

Southern Ocean: also known as the Great Southern Ocean, the Antarctic Ocean and the South Polar Ocean.

"Total oxidation of oil" (TOTOX) is the sum of primary oxidation products expressed as peroxide value, PV (see PV definition) and secondary oxidation products expressed as anisidine value, AV (no unit; see AV definition). Because one unit increase in PV has been shown to correspond to an increase of about two units of AV, the parameter TOTOX = 2PV+AV has been developed. It is traditionally used in trading of fish oil but is an empirical parameter since it corresponds to the addition of two parameters with different units.

Total volatile basic nitrogen (TVB-N concentration): Nitrogen content of volatile nitrogenous bases in fish and fishery product (expressed in mg/100g) as determined by the procedure described in Commission regulation (ec) No 2074/2005 (5.12.2005). The parameter is used as an indicator of spoilage.

Trans-esterification: The process of exchanging the organic group of an ester with the organic group of an alcohol. These reactions are often catalyzed by the addition of an acid or base catalyst. The reaction can also be accomplished by use of enzymes (biocatalysts); in particular lipases

Trans-fatty acid: an unsaturated fatty acid with at least one double bond in the *trans* configuration

Triacylglycerol is an acylglycerol (previous term triglyceride) consisting of three fatty acid chains covalently bonded to a glycerol molecule through ester linkages. See *acylglycerol*.

Volatile nitrogenous bases: see *Total volatile basic nitrogen*.

BACKGROUND

The addition of n-3 fatty acids in regular food and food supplements is increasing due to the putative positive health effects. The main commercial source of marine n-3 fatty acids (EPA, DPA and DHA) is marine oils. Marine oils include oils derived both from whole fish and cut-offs from wild and aquaculture fish, as well as oils derived from seal and krill. EUs hygiene regulations[2] for the production of fish oils intended for human consumption have been implemented in Norway. In brief, this legislation includes processing steps (i.e. from fish catch to storage of the refined oil) in the production of fish oils intended for human consumption.

Even though the hygienic requirements for the production of fish oils intended for human consumption were strengthened from 2008 in the EU (from March 2010 in Norway), there are concerns regarding the possible presence of decomposition- and oxidation products in the oils and their possible consequences on human health.

In order to obtain a scientific background on the stability of fish oil products and their safety on human health, the Mattilsynet asked the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) to conduct a risk assessment on decomposition substances and oxidation products in fish oils intended for human consumption. The risk assessment should focus on fish oils, but if considered relevant by VKM, marine oils derived from seal or krill might be included, but in separate chapters.

VKM appointed an ad hoc group consisting of VKM members and external experts to prepare a draft response to the request from the Mattilsynet.

The Scientific Steering Committee of VKM is responsible for the outcome of the work. During the drafting of the report, comments and suggestions from the Panel on Contaminants and Panel on Animal Feed have been incorporated by the ad hoc group. This risk assessment report has been evaluated and approved by the Scientific Steering Committee of VKM.

[2] EUs hygiene regulations comprehend Regulations (EC) no. 852/2004, 853/2004 and 854/2004. Regulation no. 852 concerns hygienic regulations of food items, while no. 853 gives specific demands concerning food items of animal origin. No. 854/2004 concerns control of food items and is not relevant in this connection.

TERMS OF REFERENCE

The Norwegian Food Safety Authority has asked the VKM to conduct a health risk assessment on decomposition substances and oxidation products in fish oils intended for human consumption except fish oils used as pharmaceuticals.

The risk assessment should focus on fish oils, but if considered relevant by VKM, the oils derived from seal or krill may be included, but in separate chapters.

The following topics should be addressed:

1. a. Description of substances that are produced during decomposition of the raw material for fish oil production, such as whole fish or fish cut-offs (for example muscles, liver, bone). If relevant, raw materials like krill and/or seal may be included[3].

   b. A qualitative and, if possible, quantitative health risk assessment of the decomposition substances, depending on their detectability in the end product.

2. a. Description of products that are produced during oxidation of fish oils. If oils from seal and/or krill are included, describe which products are produced during oxidation of these oils.

   b. A qualitative and, if possible, quantitative health risk assessment of the oxidation products, depending on their detectability in the end product.

3. a. A qualitative and, if possible, quantitative description of the substances that are produced and/or eliminated during the various processing steps in the production of marine oils intended for human consumption:

   i. Raw material (examples of factors that may be taken into consideration are freshness of the raw material, time, temperature and geographical location of the fish catch)

   ii. The conditions under which the oils are processed (examples of factors that may be taken into consideration are pressure, temperature, methods of chemical extraction)

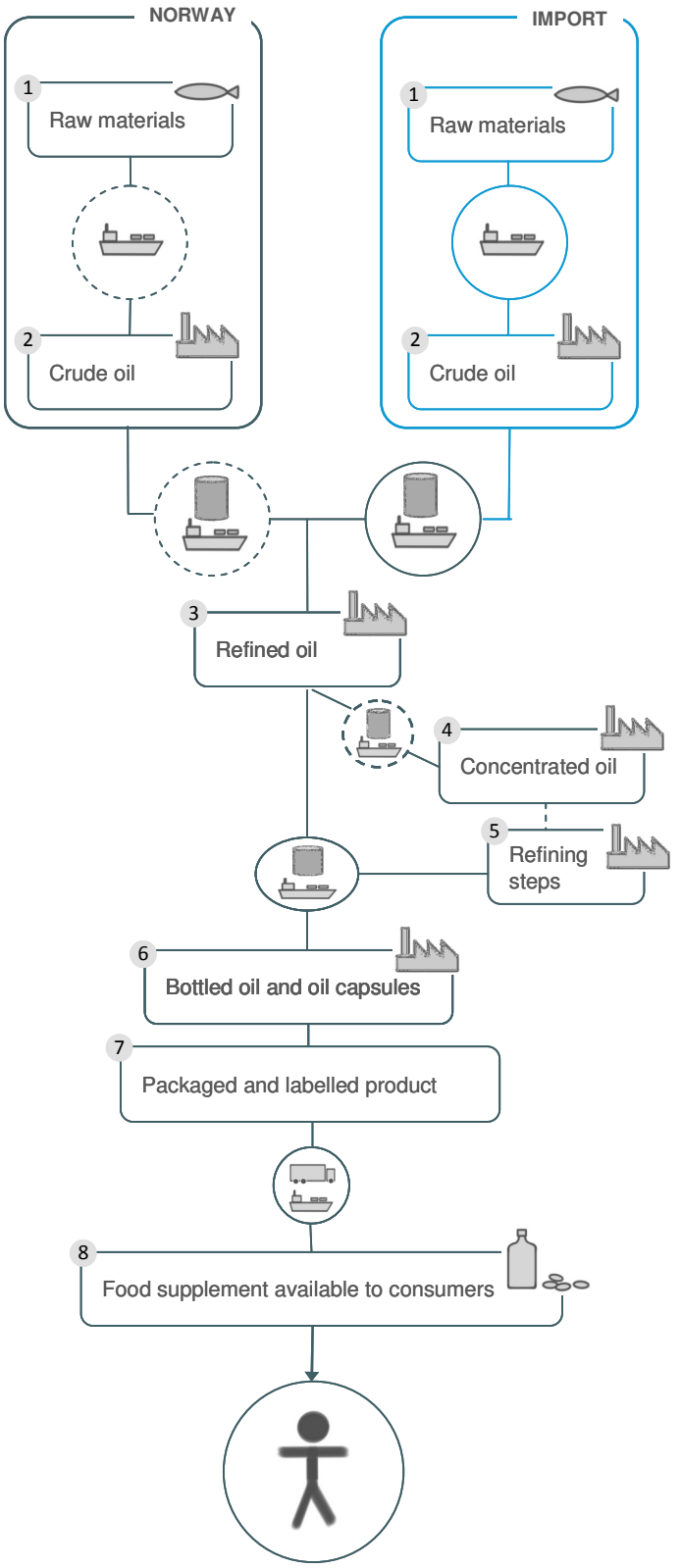
   iii. Storing (examples of factors that may be taken into consideration are time, temperature and quality of the storing containers)

   iv. Transport (examples of factors that may be taken into consideration are time, temperature and transport conditions)

   b. A qualitative and, if possible, quantitative health risk assessment of the substances which are described under question 3a, depending on their detectability in the end products.

[3] In case seal and/or krill are included, please mention them in separate chapters.

Overview of the value chain from fish to food supplement



(1) Raw materials used for fish oil production include fish, fish liver and cut-offs and trimmings from the fish processing industry. All raw materials should be produced, stored and transported according to European hygiene regulations.

(2) Raw materials are processed into crude fish oil. Cut-offs and trimmings from the fish processing industry in Norway is not stored but directly processed into crude fish oil.

(3) Crude fish oil has to be refined to be suitable for human consumption. The composition and quality of the crude oil and of the refined oil is made to order.

(4) Refined fish oil for human consumption can also undergo a concentration process.

(5) Concentrated fish oil may undergo additional refining steps.

(6) Ingredients such as vegetable oils, antioxidants, vitamins, colour, flavourings and stabilizers can be mixed into the oil before bottling or encapsulation. Capsules can be liquid filled or filled with paste. n-3 fatty acid food supplements can among others also be produced as tablets or as chewable gel.

(7) The bottled fish oil and capsules are then packaged and labelled, and finally shipped to wholesalers and/or sales points.

(8) Fish oil products are marketed as food supplements for consumers.

1. Introduction

The total catch and aquaculture production of seafood was about 145.1 million tons in 2008, of which 117.8 million tons was used directly for human consumption. In 2008, 20.8 million tons of seafood production was used for fish oil and fish meal production (FAO, 2010). The quantity used for fish oil and fish meal production has not varied much in recent years and was 21 million tons in 2005 (Tacon et al., 2006). The subsequent global productions of fish oil and fish meal are estimated to be about 1 million ton and 6-7 million tons, respectively (Breivik, 2007; Nichols, 2007; Tacon et al., 2006). The global production of fish oil has decreased during the last 20 years from about 1.6 million tons in the late eighties. Out of the estimated 1 million ton fish oil production in 2012, about 70 000 tons (7%) has been projected to be used for human consumption (Figure 1) (Tacon et al., 2006).

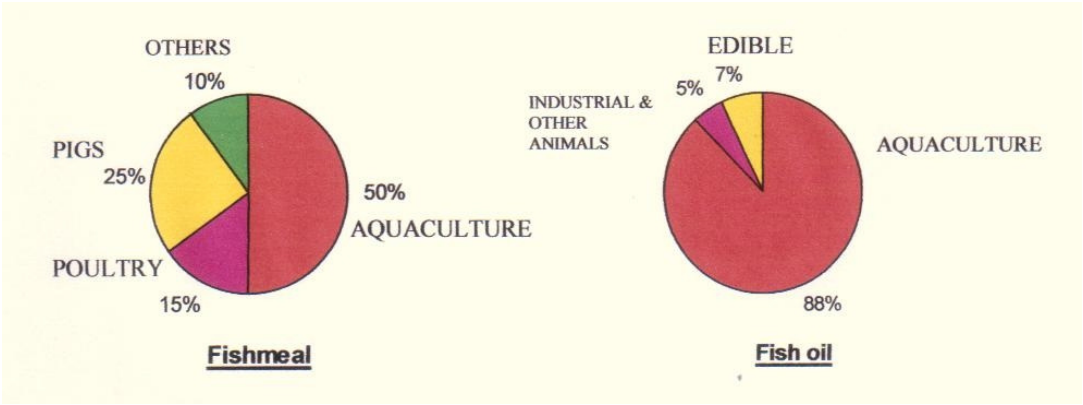


Figure 1 Estimated global use of fish meal and fish oil in 2012 (in Tacon et al., 2006; modified from Pike, 2005). The edible part of fish oil production = estimated human consumption = 7%.

Norway is the largest global importer of fish oil (approximately 200 000 tons in 2005), followed by Chile, United Kingdom (UK) and Canada (Nichols, 2007). Approximately 40% of the world n-3 related oil products for human consumption are manufactured in Norway. Between 2001 and 2007, there has been approximately 25% mean annual market growth for marine n-3 oils (RUBIN report no. 167, 2008).

Fish oil products based on natural fish oil or their derivatives are marketed internationally as food supplements or pharmaceutical products (i.e. medicines). Microencapsulated fish oil has been introduced for food fortification (e.g. bread, pizza, infant formula, baby food), as well as fish oil emulsions for diary product, fruit juice, among others (Hjaltason and Haraldsson, 2007; Kolanowski and Laufenberg, 2006; RUBIN report no. 167, 2008).

Consumption of fish oil supplements is high in the Norwegian population compared with rest of Europe because of recommendations from the Norwegian Directorate of Health (Helsedirektoratet, 2011). The consumption varies in the population but the most recent consumption data are for pregnant women. In a study based on 40,108 pregnant women participating in the Norwegian Mother and Child Cohort Study (MoBa), 58.6% reported consumption of cod-liver oil or fish oil during the first 4-5 months of pregnancy (Haugen et al., 2008). The high use of fish oil/cod-liver oil supplementation during pregnancy is unique to Norway. For comparison, use of these supplements was reported by 4.3% of the women in a Danish pregnancy cohort (Olsen et al., 2007) and by 23% of the women in a cohort in Iceland (Olafsdottir et al., 2006).

The global volume of marine oils for human consumption in recent years is given in Table 1, however due to continuing marked growth the volume today (2011) is probably higher.

Table 1 Global market volume of marine oils for human consumption

Source	Volume, ton/year	Source
Cod-liver oil	4000-6000	*RUBIN report no. 167, 2008
Refined oil, 30% n-3	30 000 – 35 000	
Refined oil for food fortification	3000-4000	
Concentrate, 50-60% n-3	6000-7000	
Concentrate, ca. 90% n-3	1500	
Tuna oil	1000	
DHA/algae oil	1100	
Seal oil	4500	**Wahren, 2004
Krill oil	<2400	***Nichols, 2007
Total	**55 000 – 60 000**	

* first half of 2008, ** estimated per 2004, *** estimated per 2007

1.1 Marine sources for production of marine oils

Marine sources used for oil production may be divided in four categories:

1. Fish caught for the sole purpose of fish meal/fish oil production (for example by Chile, Peru, Norway, Denmark, South Africa and the USA) or by-catches from other fishery.
2. Fish cut-offs and offal from the processing industry (wild and farmed fish).
3. Cod liver (from *Gadus morhua L.* or other *gadidae* species)
4. Marine resources other than fish (seal, krill etc).

Examples of fish from different stocks used for meal- and oil production are given in Table 1.1. For biochemical composition of these fish species, see Table 3.1.1-1. Carapaces and shells (crustaceans) are also used for production of meal and oil, especially small crustaceans that are unmarketable for direct human consumption.

Natural fish oil reflects the amount and composition of EPA and DHA in the raw material the oil is derived from. The total amount of n-3 fatty acids vary according to fish species and ranges from about 10 - 30% EPA/DHA.

Concentrated n-3 fatty acids contain higher proportions of polyunsaturated fatty acids than the oil used as the starting material. EPA and DHA exist as ethyl esters or as mono, di and triacylglycerols with EPA/DHA concentration up to 60-90%. Such concentrated EPA/DHA fatty acids are obtained by chemical modification of refined natural marine oils (see Chapter 2.5). The highest concentration (90%) is generally found in pharmaceutical n-3 fatty acid products.

Food supplements: Oil products such as bottled cod-liver oil and capsules containing fish oil (natural or concentrated) are considered both by the national and EU Food Safety Authorities as concentrated sources of nutritients that are intended to supplement the consumers daily basic diet. Thus, these products are defined as “food supplements”.

Table 1.1 Examples of fish from different fish stocks (FAO, 1986)

Fish species	Comments
AMMODYTIDAE Sand eels (Tobis) (North Sea)	**Sand eel (tobis in Norwegian)** are fatty fishes mostly used for industrial purposes (meal and oil production).
ELASMOBRANCHS Dogfish	**Elasmobranchs** (the sharks and the rays) are not specially caught for meal and oil production. Some species, however, provide raw material as trash fish and as cut-offs from processing.
CLUPEIDS Anchoveta Herring (spring) Herring (winter) Anchovy (South Africa) Sprat (Atlantic) Pilchard (South Africa)	**Clupeids** (the herrings) provide the largest single source of raw material for production of fish meal and oil. They may be classified as fatty although the fat content may vary from 2% to 30%, depending on species and season. The fat is not, as in lean fish, concentrated in the liver, but generally distributed throughout the body.
GADOIDS Blue whiting (North Sea) Hake (South Africa) Norway pout	**Gadoids** (the cod-like fishes) comprise a number of fish species which can be classified as lean. Characteristic of these species is that most of their fat is located in the liver. Fish meal made from these lean species of fish is called "white fish meal".
SCOMBROIDS Mackerel (spring, North Sea) Mackerel (autumn, North Sea) Horse mackerel (North Sea) Horse mackerel (South Africa)	**Scombroids** (the mackerels) are fatty fish species and the fat is generally distributed throughout the body
SALMONOIDS Capelin (Norway)	**Salmonoids** (the salmons and other closely related fish) are generally not harvested for fish meal production, but salmon cut-offs are used. However, there is one species, the capelin, which has become a considerable source for fish meal and - oil production.

The most common marine oil supplements are (for definition, see Table 1.4-1):

- Cod-liver oil
- Salmon oil, farmed
- "Fish oil, rich in omega-3 acids"
- "Omega-3-acid ethyl esters 60"
- "Omega-3-acid triglycerides" (i.e. concentrated n-3 fatty acid products)

Salmon oil produced from cut-offs from farmed fish is used both as feed ingredient (i.e. ensilaged and fresh) and for food industry purposes (i.e. fresh).

In Norway, products of krill oil for human consumption are defined as food supplement and not novel food whereas EU defines and regulates krill oil as novel food (EFSA, 2009). Norway has not yet implemented the EU Novel Food Regulation, but has a corresponding national provision.

The main focus in this evaluation is on production of fish oil used as food supplements (i.e. in bottle or encapsulated). Other marine sources for oil production such as krill and seal blubber as well as the production of emulsions and microencapsulation of marine n-3 fatty acids for fortification of regular food are not evaluated in detail. Furthermore, food supplement based

on oil derived from the crustaceans *Calanus finmarchicus*, which is a growing business, is not included.

Illustration of the value chains for production of the above mentioned n-3 fatty acid oils, is given in Figure 1.1.

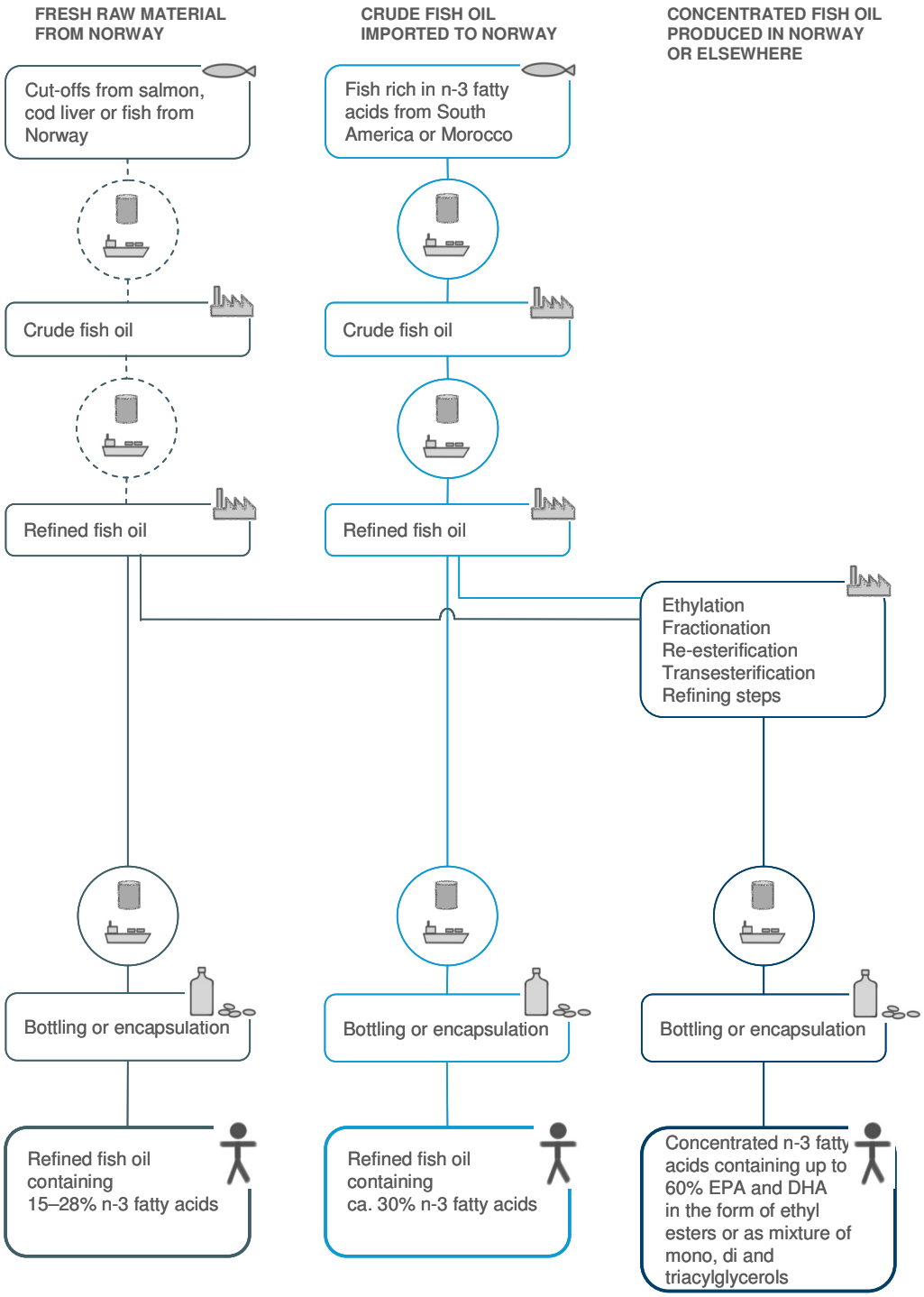


Figure 1.1 Illustration of the value chains for production of salmon/cod-liver oil, "Fish oil rich in omega-3 acids" and "Omega-3 acid ethyl esters 60" / "Omega-3-acid triglycerides" (see Table 1.4-1 for definition of the products). Refined oil produced in Norway may be based on raw material from Norway or on imported crude oil. Refined oil is suitable for human consumption and can be offered as food supplements. Alternatively, refined oil may be concentrated to achieve higher n-3 fatty acids content, see Chapter 2.5, and then sold as food supplements.

1.1.1 FISH OIL DERIVED FROM WHOLE FISH

Most of the raw materials used for the production of fish oil are pelagic fish such as anchovy, sardine, horse mackerel, and menhaden, which are usually caught off the coasts of South-America, while herring, sprat, capelin, mackerel, and sand eels (tobis) are relevant species caught in the Atlantic Ocean and used for fish oil production in Norway (see Table 1.1).

1.1.2 FISH OIL DERIVED FROM CUT-OFFS

Salmon oil can be produced from either fresh or silage (mainly as feed ingredient, see Chapter 2.1) raw material. Norway produces salmon oil from 33 000 tons of fresh cut-offs/year which is subsequently used in n-3 fatty acid supplements and functional food (RUBIN Varestrømanalyse, 2010). Due to an increase in the use of filleting and half-fabricates of both farmed salmon and pelagic fish in Norway, the possibility to produce n-3 oils from raw fresh materials is expected to increase (Aas et al., 2009). However and to our knowledge, pelagic fish cut-offs is at present not used for commercial production of fish oil in Norway.

1.1.3 FISH LIVER OIL

Cod-liver oil has been produced in Norway for more than 100 years. Cod is a lean fish where most of the body fat is stored in the liver. The liver from lean species such as tuna, hoki and shark are also sources of oil production. The production of oil from liver is estimated to be around 30 000 tons/year; which is less than 5% of the global fish oil production. Iceland, Norway, and Japan are the main suppliers of cod-liver oil (RUBIN report no. 144, 2007).

1.1.4 OILS FROM OTHER MARINE SOURCES

1.1.4.1 Oil derived from seal blubber

Seal oil is produced from blubbers harvested mostly in Canada (annual catch of 300 000 animals). Blubber is thicker and more vascularised (i.e. rich in blood vessels) than other adipose tissue. In 2004 the global harvest of seal was about 400 000 animals (Wahren, 2004). The hunted species is mainly harp seal (*Pagophilus groenlandicus*) with a mean body weight of 90 kg containing about 25% blubber. Thus, one hunted seal produces about 22kg blubber (Wahren, 2004). The Canadian hunting of around 300 000 animals could theoretically give about 6 000 tons of blubber, from which about 4500 tons of oil could be derived (100kg blubber gives approx. 75kg oil). Seal oil is used as pet food, but it is also available for human consumers as a source of n-3 fatty acid (Aagesen, 2007; Health Canada, 2009; Wahren, 2004) except in EU (see Chapter 1.3).

1.1.4.2 Oil derived from krill

Krill is a crustaceans and one of the most abundant species in the oceans, but only Antarctic krill (*Euphausia superba*) and Pacific krill (*Euphausia pacifica*) have been harvested to any significant degree for human consumption (Tou et al., 2007). The Convention on the Conservation of Antarctic Marine Living Resources (CCAMLR), manages the marine living resources in Antarctica including recommendations for krill harvesting. Commercial fishing of krill is done in the Southern Ocean and in the waters around Japan. Since 1992/93, the catches of krill have been approximately 100–130 000 ton/year. Japan, South Korea, Poland, Ukraine, Uruguay, USA, Chile and Norway (since 2006/07) participate in krill fisheries (Antarktis polaråret 2007-2008). In total, most of the krill catch is used for aquaculture and aquarium feeds, but some is used as bait in sport fishing, or in the food industry as a source for polyunsaturated n-3 fatty acid. The lipid composition and fatty acid profile of krill varies markedly with the region and season of harvest (Färber-Lorda et al., 2009; Gigliotti et al., 2011; Saether et al., 1985; Winther et al., 2010).

1.2 Lipid composition

1.2.1 CLASSIFICATION AND NOMENCLATURE OF FATTY ACIDS

Fatty acids are molecules consisting of a linear hydrocarbon chain (-CH2-) varying in length from 2-22 with an acid or carboxyl group (-COOH) at one end and a methyl group (-CH3) at the terminal end (Fig. 1.2.1-1). Fig. 1.2.1-1 illustrates how the carbon atoms in fatty acid are numbered (marked red) from the carboxyl group (COOH) and where the last carbon atom has designation n or omega (ω) (marked blue). For example, EPA, which has 20 carbon atoms and 5 double bonds, with the first one located at n-3, can also be written as 20:5 n-3. The Greek nomenclature is often used (e.g. as ω, blue in the figure below). Note that according to the former nomenclature, it is carbon atom number 2 that is α. The two designations (n-3 and ω-3 fatty acids) are the same, meaning that the first double bond counted from the n or ω–end is positioned between carbon atoms three and four. If the first double bond had been between carbon atoms 6 and 7 or 9 and 10 counted from the ω–end, it would have been an n-6 or n-9 fatty acid, respectively.

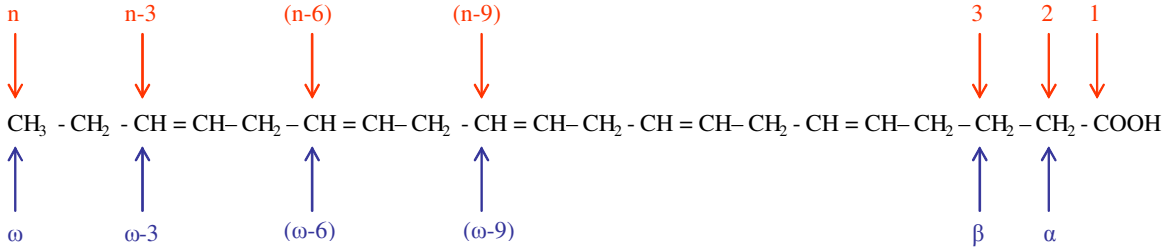


Figure 1.2.1 A schematic outline of the structure and nomenclature of the polyunsaturated n-3 fatty acid eicosapentaenoic acid with 20 carbon atoms and 5 double bonds, often abbreviated EPA (20:5 n-3). *(Adopted from VKM, 2006)*

The most common fatty acids in foods have an even number of carbon atoms ranging from 12-18. Fatty acids are mainly categorized according to chain length, number - and configuration of the double bond(s).

Fatty acids carrying the maximum number of hydrogen atoms are termed saturated. A fatty acid becomes unsaturated when a pair of hydrogen atoms is removed, thereby creating a double bond between the adjacent carbon atoms where the hydrogen atoms disappeared. In the human body, enzymes with high specificity regarding position catalyze desaturation at certain positions, and therefore the location of double bonds is tightly controlled.

Except for linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), humans and animals can synthesise most of the needed fatty acids. As a result, these two fatty acids are called essential and must be supplied through the diet.

The different types of fatty acids are present in many types of fat, but the ratios may vary considerably. It is common to divide fatty acids into saturated, monounsaturated (one double bond) and polyunsaturated (two or more double bonds). Examples are given in Table 1.2.1 and Figure 1.2.1, which illustrates the structure and nomenclature of fatty acids.

Table 1.2.1 Examples of polyunsaturated fatty acids (PUFAs) and abbreviation

Trivial name	Short name*	Abbreviation
Linoleic acid [1]	[5]18:2 n-6	LA
Arachidonic acid [2]	20:4 n-6	ARA
α-Linolenic acid [3]	18:3 n-3	ALA

Trivial name	Short name*	Abbreviation
Eicosapentaenoic acid [4]	20:5 n-3	EPA
Docosapentaenoic acid [4]	22:5 n-3	DPA
Docosahexaenoic acid [4]	22:6 n-3	DHA

*Length of the carbon chain, number and location of double bound; [1] Mainly plant oils (e.g. soybean oil, sunflower oil and corn oil); [2] Animal meat, eggs, dairy products; [3] Plant oil (e.g. linseed oil, rapeseed oil and walnut oil); [4] Fish and seafood; [5] Number of carbon atoms is 18; number of double bindings is 2, and the first double bound is located at carbon atom 6 (n-6). *(Adopted and modified from VKM, 2006)*

1.2.2 LIPID CLASSES AND FATTY ACID PROFILE

Lipids are composed of fatty acids or closely related compounds such as the corresponding alcohols or sphingosine bases. Lipids are commonly divided into two categories: neutral and polar lipid. Major neutral lipid classes are wax esters, triacylglycerols (TAG), diacylglycerol ethers, free fatty alcohols and sterols, while polar lipids are mainly phospholipids (PL) and sphingolipids (Gunstone, 1996).

Fatty and lean fishes, and seal, have their lipids mainly stored as triacylglycerols in muscle and adipose tissue, liver tissue and blubber, respectively. In fish and sea mammals, phospholipids are located in cell membranes providing structure and fluidity. The lipids in krill are deposited both as phospholipids and triacylglycerols (Phleger et al., 2002; Ju et al., 2009, see Tabel 3.1.1-2 for overview of fatty acid composition).

Triacylglycerols

The triacylglycerols consist of fatty acids esterified at three different positions on a glycerol molecule, namely sn-1, sn-2 or sn-3 (Fig. 1.2.2-1). This distribution of fatty acids in the glycerol molecule is more or less unique for different types of natural fat (Aursand et al., 1995, Standal et al., 2009). In order to minimise the risks of blends and adulteration, the European Pharmacopoeia (Ph. Eur.) has in its definition of salmon oil (farmed salmon) and cod liver oil (farmed cod) included the distribution of fatty acids in triacylglycerols (sn-2 position specificity) as determined by ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy (see Table 1.4-1).

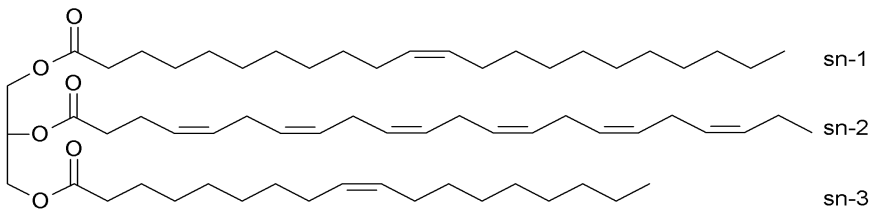


Figure 1.2.2-1 Structure of a triacylglycerol (TAG) molecule; three fatty acids esterified to the positions sn-1, sn-2 and sn-3

Phospholipids

Most phospholipids head groups belong to phosphoglycerols, with a glycerol joining the head and tail ends.

A phospholipid molecule consists of a hydrophilic polar head group and a hydrophobic tail (see Fig. 1.2.2-2). The polar head group contains one or more phosphate groups. Examples of phosphoglycerols include phosphatidylcholine, phosphatidylserine, phosphatidyl-ethanolamine, and phosphatidylinositol.

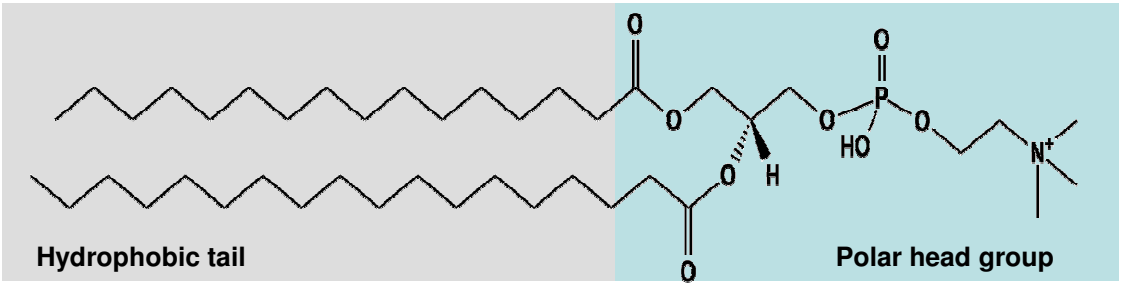


Figure 1.2.2-2 Structure of a phospholipid (PL) molecule; two fatty acids esterified to the positions sn-1 and sn-2 in a glycerol molecule and a hydrophobic head group with phosphoric acid linked.

1.3 Regulatory framework of fish oils intended for human consumption

Production, storage and transport of the oils

The legislative framework in EU and Norway for fish oils intended for human consumption is well described in the recently published EFSA report on Fish oil for human consumption (EFSA, 2010). Briefly, fish oil intended for human consumption has to come from an establishment approved by the competent authority to fulfil the Hygiene Regulations and the raw material has to be derived from fishery products that are fit for human consumption and similarly handled throughout the production chain (see Appendix A). These Regulations provide that operators of food business that produce fish oil intended for human consumption are subjected to strict rules covering production, storage and transport as far as hygienic requirements are concerned.

Beside the above mentioned Regulation (EC) 853/2004, which requires fish oils for human consumption to be transported and stored in hygienic conditions (Appendix A), [4]Codex Alimentarius Commission have worked out a "*Recommended International Code of Practice for the Storage and Transport of Edible Oils and Fats in Bulk*" (CAC/RCP, 2005) which includes a List of Acceptable Previous Cargoes. The worldwide trade of edible fats and oils in bulk implies their transport by road, railroad, inland waterways and sea.

A phytosanitary certificate should follow all produced fish oils (crude and refined). In Norway, the Food Safety Authority approves this certificate that the refined oil is pure fish oil for human consumption and that these are produced according to given legislation.

Oils derived from fish by-products

The utilisation of fish by-product for fish oil production for **pharmaceutical purposes** is possible, but the European Union (EU) Regulation governing Animal By-Products (ABPs) requires that the starting material must be derived from "Category 3 materials" i.e. materials

[4] The Codex Alimentarius Commission was created in 1963 by the Food and Agricultural Organization of the United Nations (FAO) and World health Organization (WHO) to develop food standards, guidelines and related texts such as codes of practice under the Joint FAO/WHO Food Standards Programme. The main purposes of this Programme are protecting health of the consumers and ensuring fair trade practices in the food trade, and promoting coordination of all food standards work undertaken by international governmental and non-governmental organizations.

from animals fit for human consumption following veterinary validation. The Regulation (EC) No 1774/2002 governing ABPs, which was implemented in Norway in October 2007, lays down strict animal and public health rules for the collection, transport, storage, handling, processing and use or disposal of all ABPs.

Quality criteria and compositional factors of the oils

Currently, there is no European or International standard for quality criteria or standard compositional requirements for different types of marine oils. The [5]Codex standard for edible fats and oils not covered by individual standards does not adequately cover the specific nature of marine oils (also see Chapter 1.4). Additionally, some detailed quality and composition factors in this standard are intended for voluntary application by commercial partners and not for application by governments (see Table 1.4-3). However, international work has been initiated on these aspects (see Chapter 1.5).

Processing aids

Processing aids are used during production and handling of foodstuff, and should avoid technological effect on the final food. Regulation (EC) No 1333/2008 on food additives refers to processing aids as "*Substances not consumed as food itself, but used intentionally in food processing, which only remain as residues in the final food and do not have a technological effect in the final product.* This implies that substances that have an effect on the final food are **not** regarded as processing aids, but as food additives.
The use of processing aids under conditions of good manufacturing practice should result in the removal of all or major part of their residues from the foodstuff or food ingredient. Under such conditions, the presence of residues or derivatives in the final foodstuff or food ingredient may be unintentional, but technically unavoidable. There are special legislative regulations for some processing aids such as extraction solvents Ekstraksjonsmiddelforskriften, 2010).

Food supplements

Food supplements is defined as foodstuffs which purpose is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional physiological effect. Regulation (EC) 853/2004 includes a clause stating that, pending the establishment of specific Community legislation, Member States/food business operators must ensure compliance with national rules for fish oil placed on the market for the final consumer. Member States may lay down limits for the final products, but has according to the Food Safety Authority Norway not used this opportunity so far.

Norway has adopted the notification for food supplements but has yet not implemented it. The notification means that food supplements have to be reported to the Food Safety Authority by the producer/importer prior to be placed on the market and includes name and address of the notifier, a list of all ingredients (also added vitamins and minerals), and the total content (sum of natural and added amounts) of all vitamins and minerals per recommended daily dose. The Food Safety Authority may request additional documentation beyond this notification. However, there are regulatory limits for content of environmental contaminants; for maximum and minimum content of vitamins and minerals, as well as for type and amount of added antioxidants.

[5] The Codex Alimentarius Commission: Current official standards can be found at http://www.codexalimentarius.net/web/standard_list.do

Food supplements have to be labelled according to the legislation for food supplements (EU Directive 2002/46). In this context, the product has to be labelled with the duration of its shelf-life and the Food Safety Authority may require documentation when inspecting the product.

Health claims accepted by the Food Safety Authorities to be used in the marketing of the food supplements is the same in all EU/EEA countries.

Fortified food

In food manufacturing, a wide range of nutrients and other ingredients including essential fatty acids might be used. Their addition to foods is regulated by the Regulation (EC) No 1925/2006 on the addition of vitamins and minerals and of certain other substances to foods, which is implemented in Norway.

Specific national regulation

In Norway, fish oils intended for human consumption is in addition to the EU Hygienic legislation regulated by Fiskekvalitetsforskriften (http://www.lovdata.no/for/sf/fi/xi-19960614-0667.html).

In August 2010, trade and import of seal products in the EU was banned (Regulation (EC) No 1007/2009 of the European Parliament and of the Council on trade in seal products). The regulation is not a part of the EEA-agreement and has not been implemented in Norway.

1.4 Quality criteria for marine oils and derived products for human consumption – existing recommendations

For the time being, there is no adequate international, European or national legislation on standard for origin, quality and/or composition of marine n-3 oils and their concentrates for use as food/food supplement, but work has been initiated (see Chapter 1.5). However, the [6]European Pharmacopoeia (Ph. Eur.) contains quality criteria for some types of marine n-3 oils (Table 1.4-1). These standards are mandatory for medicinal products marketed within the signatory states. The various quality criteria have been developed by a group of European scientific experts and are mainly based on analytical batch-data of the marine oil products and current processing technologies. Additionally, traditional human consumption of cod-liver oil without reported adverse effects to the medicinal agencies has been taken into account. According to the Norwegian Seafood Federation, these Ph. Eur. requirements are increasingly being used as a reference point for pricing during purchase and sale of marine oils for human consumption.

Oxidised fish oils have an unpleasant, rancid odour and taste which affects the consumption acceptability for humans, and also for animals (for details, see Chapters 4.1.3 and 5.1). Oxidised fish oils are more costly and difficult to make suitable for human consumption because they need more processing (see Fig. 2.3 for overview of refining of crude oil).

[6] The European Pharmacopoeia is a single reference work for the quality control of medicines in the signatory states of the Convention on its elaboration. Additional information on background and legal framework can be found at http://www.edqm.eu/en/Background-amp-Legal-Framework-50.html

Table 1.4-1 Definition of some marine oil products for human consumption according to the European Pharmacopoeia 7.0

Oil	Scientific name of the oil	Definition and/or composition of the oil		Content of [a] EPA and DHA	[b] Positional distribution β (2)-acyl	Other conditions
Cod-liver oil type A and B	*Iecoris aselli oleum A and B*	Purified fatty oil obtained from fresh livers of **wild** *Gadus morhua* L. and other species of *Gadidae*.	Solid substances being removed by [c] cooling and filtering. Suitable antioxidant may be added.	as triacylglycerols EPA: 7-16% DHA: 6-18% Max. content of linoleic acid (C18:2 n-6) is 3.0%.		Composition of fatty acids determined by GC is given with lower- and upper limit areas (per cent).
Cod-liver oil, farmed	*Iecoris aselli oleum domestici*	Purified fatty oil obtained from fresh livers of **farmed** *Gadus morhua L.*	As above	as triacylglycerols 10-28% Max. content of linoleic acid (C18:2 n-6) is 11%.	DHA: 71-81% EPA: 32-40% C18:4 n-3: 28-38%	The composition of the fish feed shall be in accordance with the relevant EU or other applicable regulations.
Salmon oil, farmed	*Salmonis domestici oleum*	Purified fatty oil obtained from farmed *Salmo salar*.	As above	as triacylglycerols 10-28% Max. content of linoleic acid (C18:2 n-6) is 11%.	DHA: 60-70% EPA: 25-35% C18:4 n-3: 40-55%	Fish feed as above. The oil is to be produced by mechanical extraction of fresh raw materials (whole fish or fish cut-offs) at a temperature not exceeding 100˚C, and without using solvents.
Fish oil, rich in omega-3 acids (type 1)	*Piscis oleum omega-3 acidis abundans*	Purified, winterised and deodorised fatty oil obtained from the families *Engraulidae, Carangidae, Clupeidae, Osmeridae, Scombridae (except genera Thunnus and Sarda), Ammodytidae*.	As above	as triacylglycerols: sum EPA+DHA: min 28% EPA: min 13% DHA: min 9%		
Fish oil, rich in omega-3 acids (type 2)	As the row above	Oil prepared as type 1, but obtained from the genra *Thunnus* and *Sarda*.	As above	as triacylglycerols: sum EPA+DHA: min 28% EPA: 4 -12% DHA: min 20%		
Omega-3-acid ethyl esters 60	*Omega-3 acidorum esteri ethylici 60*	Esters of n-3 fatty acids[c]; C18:3, C18:4, C20:4, C20:5, C21:5, C22:5, and C22:6 (n-3). Obtained by trans-	The physico-chemical purification processes should include [d] molecular distillation.	Total n-3 fatty acid ethyl esters min. 60%. Sum EPA- and DHA ethyl esters min. 50%, and content of DHA ethyl esters min. 40%.		

Oil	Scientific name of the oil	Definition and/or composition of the oil		Content of [a] EPA and DHA	[b] Positional distribution β (2)-acyl	Other conditions
Cont. Omega-3-acid ethyl esters 60		esterification of the body oil of fat fish species from families mentioned in the above two rows, in addition to salmonidae and animals of the class *Cephalopoda*.				
Omega-3-acid ethyl esters 90	*Omega-3 acidorum esteri ethylici 90*	Esters of the same n-3 fatty acids[c] as specified above.	As for "Omega-3-acid ethyl esters 60", except that [e] urea fractionation should be performed prior to [d] molecular distillation.	Total n-3 fatty acid ethyl esters min. 90%. Sum EPA and DHA ethyl esters min. 80%, with EPA ethyl esters min. 40% and DHA ethyl esters min. 34%.		
Omega-3-acid trigly-cerides	*Omega-3 acidorum triglycerida*	Mixture of mono, di and triesters of n-3 acids with glycerol containing mainly triesters obtained either by esterification of concentrated and purified n-3 acids with glycerol, or by transesterification of the n-3 acid ethyl ester with glycerol. From body oil of the fish species defined in the above last three rows, containing the [f] n-3 fatty acids as specified above.		Total n-3 acids, as triacylglycerols; min. 60%. Sum EPA and DHA as triacylglycerols; min. 45%.		

[a] EPA: the polyunsaturated fatty acid eicosapentaenoic acid (22:5 n-3), DHA: the polyunsaturated fatty acid docosahexaenoic acid (22:6 n-3). Vegetable oil is used in feed to farmed cod or salmon and in order to distinguish oil from farmed vs. wild cod/salmon, different limits for content of linoleic acid (C18:2 n-6) is given.

[b] Positional distribution of fatty acids is used to identify oil derived from farmed cod and salmon and specific limits are given for EPA, DHA and the vegetable oil moroctic (also named stearidonic) acid (C18:4 n-3), see Chapter 1.2.2.

[c] "Winterisation" is the process of cooling down oil for the removal of unwanted substances; see Chapter 2.3.3 and Figure 2.3.

[d] Molecular distillation = Short path distillation; see Chapter 2.3.6, and Figure 2.3.

[e] Urea fractionation is a processing method for up-concentration of oils but is mainly used for pharmaceutical production.

[f] Ethyl esters of *alpha*-linolenic acid (C18:3 n-3, ALA), moroctic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), eicosapentaenoic acid (C20:5 n-3; EPA), heneicosa-pentaenoic acid (C21:5 n-3), clupanodonic acid (C22:5 n-3) and docosahexaenoic acid (C22:6 n-3; DHA).

min. – minimum, max. – maximum

Both the Ph. Eur. and the Global organization for EPA and DHA (GOED) have prepared recommendations for evaluation of oxidative status ("rancidity") (Table 1.4-2), as well as criteria for some other quality parameters (Table 1.4-3) for some marine oils/concentrates.

Table 1.4-2 Recommended criteria for oxidative status of marine oil products for human consumption

[a] Type of marine oil	[b] Ph. Eur. 7.0 (2011) maximum values		[c] GOED monograph (2006) maximum values	
	[d] Peroxide value	[e] Anisidine value	[f] Peroxide value	[g] Anisidine value
Cod-liver oil (type A)	10	30	none	none
Cod-liver oil (type B)[h]	10	none		
Cod-liver oil, farmed	5	10		
Salmon oil, farmed	5	10		
Fish oil, rich in omega-3 acids (type 1)	10	30	5	20
Fish oil, rich in Omega- 3 acids (type 2)[i]	5	15		
Omega-3-acid triglycerides	10	30		
Omega-3-acid ethyl esters 60	10	20		
Omega-3-acid ethyl esters 90	10	20	none	none

[a] See Table 1.4-1 for description of the oils. [b] Ph. Eur. - European Pharmacopoeia [c] GOED: Global organization for EPA and DHA, [d] Peroxide value (milliequivalents of active oxygen/kg oil) determined according to Ph. Eur. Monograph 2.5.5, Method A, [e] Peroxide value (milliequivalents of active oxygen/kg oil) determined according to AOCS (American Oil Chemists' Society) Official Method Cd 8-53, [f] Anisidine value determined according to Ph. Eur. Monograph 2.5.36, [g] Anisidine value determined according to AOCS Official Method Cd 18-90, [h] Cod-liver type B is identical to type A except that anisidine value is not given. [i] Type 2 of "Fish oil, rich in omega-3 acids" is intended used as medical nutrition of infants and thus lower peroxide- and anisidine values than for type 1 is required.

The "*Codex standard for edible fats and oils not covered by individual standard*" proposes an oxidation status of maximum 10 milliequivalents (meq) of active oxygen/kg oil (Codex Alimentarius, Codex stan 19-1981, rev 2, 2009). However, this is for voluntary application by commercial partners and not for application by governments. As previously mentioned in this document (Chapter 1.3), the Codex standard is not exclusively related to marine oils but applies to "*oils and fats that have been subjected to processes of modification (such as trans-esterification or hydrogenation) or fractionation*". This standard is not directly applicable to marine oils as is reflected in the proposed maximum content of iron (see Table 1.4-3), which is not acceptable for marine oil in view of increased oxidation risk (see Chapter 4.2.4 Prooxidants).

During purchase and sale of crude fish oil, the industry uses a standard where the free fatty acid content (acid value) and unsaponifiable matter (i.e. mainly cholesterol, but also pigments, hydrocarbons or alcohols) should be below certain levels (see Table 1.4-3). Unsaponifiable matter may be used as a marker for contamination of the fish oil during transport. Free fatty acids are more readily oxidised than fatty acids in the form of acylglycerols or ethyl esters, and free fatty acids may also act as prooxidants. For marine oils/concentrates, the free fatty acid content should for example be a maximum of 2 for cod liver oil or 0.5 for fish oil rich in omega-3 acids. In the concentrated products "omega-3-acid triglycerides" and "omega-3-acid ethyl esters 60", the free fatty acid content should be a maximum of 3 and 2, respectively (Table 1.4-3). The acid value is typically twice as high as percent free fatty acid in oil.

For inclusion in the human diet, some fish/krill oils are generally recognized as safe (GRAS)[7] by the US Food and Drug Administration (FDA). The n-3 fatty acids quality specifications are mainly according to Ph. Eur. recommendations.

Table 1.4-3 Recommended maximum concentrations of quality parameters for edible oil and marine oil products

Parameter	Codex	European Pharmacopoeia	GOED
Colour	See [a)]	See [b)]	none
Matter volatile at 105°C	*0.2% m/m	none	none
Insoluble impurities	*0.05% m/m		
Soap content	*0.005% m/m		
Unsaponifiable matter [c)]	none	1.5% (cod-liver oil A/B; salmon oil, farmed; "fish oil, rich in omega-3 acids")	none
Acid value [d)]	*0.6mg KOH/g fat or oil	2% (cod-liver oil A/B; salmon oil, farmed; "omega-3-acid ethyl esters 60 and 90") 0.5% ("fish oil, rich in omega-3 acids") 3% ("omega-3-acid triglycerides")	3mg KOH/g
Oligomers [e)]	none	1.0% ("omega-3-acid ethyl esters 90") 1.5% ("fish oil, rich in omega-3 acids") 3.0% ("omega-3-acid triglycerides") [f)] 7.0% ("omega-3 acid ethyl esters 60") [g)]	none
Lead (Pb)	**0.1mg/kg	none	0.1mg/kg
Arsenic (As)	**0.1mg/kg	none	0.1mg/kg
Iron (Fe)	*2.5mg/kg	none	none
Copper (Cu)	*0.1mg/kg		
Cadmium (Cd)	none	none	0.1mg/kg
Mercury (Hg)	none	none	0.1mg/kg

[a)] List of specified colours which are permitted for the purpose of restoring natural colour lost in processing or for the purpose of standardizing colour is given in the standard, [b)] For cod-liver oils and "fish oil rich in omega-3 acids": not more intensely coloured than a specified reference solution, [c)]"Unsaponifiable matter" includes substances like cholesterol, pigments, waxes, hydrocarbons or alcohols, [d)]"Acid value" is measured as the mass of potassium hydroxide in milligrams that is required to neutralise one gram of oil and reflects the content of free fatty acids in oils. An acid value of X corresponds approximately to a per cent free fatty acid of ½ X, [e)]"Oligomers" are tertiary oxidation products such as polymers (see Chapter 5.1.3), [f)] For "omega-3 acids triglycerides" an additional limit of maximum 50% partial acylglycerols is recommended [g)] The given limit concerns the sum of partial acylglycerols and oligomers because of methodologically limitations in measuring oligomers only (see Chapter 5.1.3)

* It is stated in the Codex standard that these proposed maximum values are for voluntary application by commercial partners and not for application by governments.

** Maximum values in force (adopted in Codex).

1.5 Ongoing international framework for marine oils – quality standards

The Codex Committee on Fats and Oils (CCFO) has decided to start the process to establish an overall standard containing origin, quality and composition factors for different marine oils (e.g. fish oils) intended for human consumption (Codex Committee of fats and oils, 2011). Essential background for Codex to prioritize this work is that the marine oil supply chain is international where the major fisheries sites might be located in regions distant from refineries and producers of consumer products. Marine oils or products usually cross several national boarders before reaching the consumer. The CCFO work is expected to conclude in 2015.

[7] The definition of GRAS is available on the homepage of FDA: http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/default.htm. GRAS is a FDA designation that a chemical or substance added to food is considered safe by experts, and so is exempted from the usual Federal Food, Drug, and Cosmetic Act food additive tolerance requirements.

1.6 Previous risk assessments of fish oils for human consumption

In 2010, EFSA published the risk assessment "*Scientific opinion on fish oil for human consumption (Food hygiene, including rancidity*": EFSA, 2010). The assessment includes an evaluation of hygiene and rancidity of fish oil intended for human consumption from fish catch until the refined oil is stored as a bulk product. Due to extensive heating as well as alkali/acid treatments and removal of the water phase during processing of refined fish oil, the report concluded that the food safety risk was negligible and that the potential chemical hazard in bulk stored refined fish oil was oxidation products. However, the opinion stated that - no qualitative or quantitative risk assessment of hazards in relation to rancidity of fish oil intended for human consumption could be carried out based on currently available information.

Furthermore, the opinion recommended sensory assessment for evaluation of freshness of raw material for fish oil production for human consumption. The justification for this statement is that the freshness criterion of 60 mg total volatile basic nitrogen (TVB-N)/100 g for whole fish, laid down in the EU-legislation 2074/2005, is not based on scientific evidence.

Additionally, the opinion concluded that determination of peroxide and anisidine values are the most reliable chemical methods for rancidity measurements in bulk fish oils. However, it was also pointed out that due to lack of information on oxidation status in consumed fish oil (measured as peroxide and anisidine values) and related toxicological effects in humans as well as lack of toxicological information of individual oxidation products of fish oils in humans, setting and recommending the maximum acceptable peroxide and anisidine values based on science for the large variety of refined fish oils was not possible.

In June 2011, the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) published an "Evaluation of negative and positive health effects of n-3 fatty acids as constituents of food supplements and fortified foods" (VKM, 2011). The conclusions are based on a recent systematic literature search for mainly human studies and previous assessments from official organisations together with intake assessments of n-3 fatty acids based on nationally representative dietary surveys in Norway. The evaluation concludes, among other things, that it is possible to obtain positive health effects in the Norwegian population from intake of EPA and DHA, including from n-3 fatty acid food supplements, without any appreciable risk of negative or adverse health effects. The report addresses lipid peroxidation that may occur in the body (i.e. *in vivo*) as a result of consumption of EPA and DHA, but notes that oxidative stress biomarkers are yet not established as defined risk factors for disease. However, risks associated with consumption of rancid fish oils or oxidation products in oil that may occur during oil processing and storage of food supplements and fortified foods were not part of the mandate and therefore not addressed in the evaluation.

2. Processing of marine oils – from catch to final product

Marine oil processing from raw materials to refine oil/concentrated n-3 fatty acids in bulk involves several steps and operators. The final use of the oil generally defines the extent of processing. Oils intended for human consumption require more extensive processing and higher quality control than those intended for use in animal nutrition.

The raw material used for marine oil production is composed of three major fractions: solids (fat-free dry matter), oil and water. The purpose of the first processing steps is to separate the oil fraction from the others, as complete as possible. The oil obtained is named *crude fish/cod-liver/marine oil* or in short *crude oil*. The crude fish oil is sold as a commodity and usually priced according to the concentration of free fatty acids, moisture, presence of impurities, colour and peroxide and anisidine value (see Table 1.4-3). The quality and content of decomposition substances of crude marine oil can vary significantly based on source and storage of the raw material and production methods. The crude marine oil is usually not ready for human consumption and needs to undergo further processing to remove unwanted substances such as free fatty acids, oxidation products, colour pigments and environmental pollutants (see Fig. 2.3). The processed oil is thereafter referred to as *refined fish/cod-liver/marine oil* or in short *refined oil*.

Refined marine oil may be further processed for different applications such as concentrates for n-3 fatty acid food supplements and pharmaceutical products, or as ingredient for food fortification.

Good manufacturing practice should result in the removal of all or the major part of processing aids residues from the final n-3 fatty acid product (see Chapter 1.3).

2.1 Storage and transport of the raw material before processing

The raw material for the refining industry in Norway is mainly crude fish oil produced prior to importation into Norway, based on ungutted wild fish caught off the coast of South America or Morocco (see Fig. 1.1, p. 34, and Chapter 2.6 for details). However, cut-offs from processing of farmed salmon/cod, herring and liver from wild *Gadus morhua* (cod) and other species of *Gadidae* are raw materials for production of crude fish/cod-liver oil production locally **in** Norway.

The EU hygienic regulations include requirements for equipments onboard fishing vessels, as well as for storage and transport conditions when the catch is intended used to produce oil for human consumption (see Appendix A). Storage and transport should be under hygienic conditions e.g. using tanks of stainless or mild steel construction. Furthermore, the catch has to be chilled within 24 hours. The fishing of pelagic fish species used for meal and oil productions is mainly done by large purse seiners and the fish are stored in bulk in RSW (refrigerated sea water) tanks at minus 1.5^0C on-board. An exception from the chilling rule is whole fish being processed within 36 hours; on the condition that freshness is acceptable determined as the nitrogen content of volatile nitrogenous compounds (TVB-N concentration) (see Appendix A and Chapter 1.3 Regulatory framework).

The cut-offs may be freshly handled directly from the slaughtering line to oil production, but in the event transportation is needed, the cut-offs are chilled and transported in closed containers that are customized for this purpose. Presently, the majority of salmon cut-offs are conserved using organic acids to produce fish silages (Espe et al., 1989; Espe and Haaland, 1992; Shalaby, 1996). The low pH reduces bacterial growth, but both decomposition of

proteins and lipids will commence in the silage. The rate of decomposition increases with increasing temperatures. Oil derived from ensilaged raw material mainly goes to other industrial purposes than directly to human consumption (RUBIN report no. 177, 2009; Bekkevold and Olafsen, 2007) and the content of free fatty acids (measured as acid value) in crude fish oil from ensilaged material will be higher than in oil from fresh raw materials.

Krill is caught in the Southern Ocean and most of the oil processes take place onboard or on land in South America.

Seal blubber is frozen onboard and stored as such until destination on land for oil processing

Table 2.1 Schematic outline of storage and transport of raw material for production of marine oils for human consumption

Raw material	Storage before processing	Transport to processing plants
Whole fish	On board, on shore in tanks. Chilled in RSW[1] tanks if possible.	In tanks/containers by boat/car
Cut-offs from fresh fish	Storage in processing plants. Transport by trucks. Production directly from the slaughtering/processing line where that is possible. Chilled.	In containers by boat or car
Cut-off conserved by formic acid	On shore in tanks	In containers by car or boat
Fish liver	On board, on shore in tanks. Chilled.	In tanks by boat
Seal blubber	Frozen on board	By boat
Krill	Produced as fresh on board and stored as meal or oil in tanks	For further oil extraction/processing, transport by boat to onshore facilities.

[1]RSW - refrigerated sea water

2.2 Production of crude fish/cod-liver oil

Raw materials such as whole fish, cut-offs, fish liver etc can be processed to fish oil/liver oil in a number of ways. Common to all processing methods of practical importance is the following processing steps (Bimbo, 2007; Breivik, 2007, Rubio-Rodriguez et al., 2010):

- ***Cooking***, which coagulates the protein, ruptures the fat depots and liberates oil and physico-chemically bound water
- ***Enzyme treatment*** in a reactor where common enzymes (i.e. proteases) are added to assist the release of fish oil from the solid part in lower processing temperature (when producing hydrolysate)
- ***Pressing or centrifugation***, which removes a large fraction of the liquids from the mass
- ***Separation*** of the liquid into crude marine oil and water (stick water)

The content of decomposition substances, oxidation products and process-generated substances in crude fish/cod-liver oil is dependent on the freshness and composition of the raw material and the processing parameters such as time, pressure and temperature (Aidos et al., 2003) (see Table 6.1 for overview). Further refining is essential in production of food supplements (see Chapters 2.3 and 2.5).

2.2.1 COOKING

The purpose of the heating process is to coagulate the protein, rupture the fat depots and liberate oil and physico-chemically bound water and to condition the material for the subsequent treatment in the various processing units of the plant. "Cooking", as this operation traditionally has been called, is therefore a key process of utmost importance. The most common practice is to cook the fish in a steam cooker, through which it is conveyed continuously with a heated rotary screw conveyor. Coagulation of the fish protein is completed at about 75°C, but the most common practice is to heat to 95°-100°C for 15-20 minutes. The process must be controlled to ensure sufficient cooking, but overcooking must be avoided (Bimbo, 2007; Dijkstra and Segers, 2007). Oliveira (2010) has studied Pollock *(Heragra chalcogramma)* liver oil showing that cooking time combined with exposure to air seem to be more critical for formation of primary oxidation products (peroxides) than cooking temperature. Most of the microorganisms possibly present in the raw material (see Chapter 3.1.4) will be inactivated during this processing step (EFSA, 2010).

2.2.2 ENZYME TREATMENT IN A REACTOR

To reduce cooking temperature and to assist the release of the oil to improve marine oil quality, addition of enzymes may replace heating or lowering the processing temperature (Xu et al., 2007). The fish paste is exposed to enzymes (proteases) at 50-60°C in a reaction vessel for about 1.5 h. This can be done in a screw conveyor. During this step of protein hydrolysis, the fish material is solubilised to different fractions. Depending on enzymes, temperature ranging from 75-100°C for 5-30 min is used to terminate the enzymatic reaction (Kristinsson and Rasco, 2000), but typically temperature above 85°C for at least 10 minutes is applied (Kristinsson, 2007).

Majority of salmon oil at the food supplement market are produced using an initial hydrolysis step on fresh cut-offs from salmon processing industry.

2.2.3 PRESSING OR DECANTING/CENTRIFUGATION

The purpose of the press/centrifugation step is to squeeze out as much liquid ("press liquor") as possible from the solid phase. This step is important to improve the oil yield (Bimbo, 2007). The efficiency of separation depends upon both design and mode of operation of the centrifuge. Accordingly, good temperature control is required; the temperature should be maintained at about 95°C, but not less than 90°C.

2.2.4 SEPARATION OF PRESS LIQUOR (SOLIDS REMOVAL)

The liquor coming from the press and pre-strainer consists of water, oil and dry matters (Bimbo, 2007). The oil content is related to the proportion of lipid in the raw material (Bockisch, 1998). The separation of the three fractions of the press liquor (solids, oil and water) is based on their different specific gravities using centrifugation. An important prerequisite for efficient separation is high temperature, implying that the press liquor should be reheated to 90°-95°C before entering the centrifuge. This applies to sludge removal as well as separation of oil and water.

After the pressing step, the processing of fish meal and fish oil continues in separate lines.

2.2.5 FIRST OIL POLISHING (OIL-WATER SEPARATION)

Oil polishing (oil-water separation), which is carried out in special separators, is the final step before the crude fish oil is pumped into steel storage tanks. Polishing is facilitated using hot water that extracts impurities (e.g. prooxidants) from the oil, ensuring stability during storage.

2.2.6 CRUDE FISH/LIVER OIL OIL: COMPOSITION AND QUALITY

Crude fish/cod-liver oil may contain minor amounts of water and unwanted substances such as insoluble impurities, waxes, phospholipids, mono and diacylglycerols, free fatty acids, cholesterol, proteinaceous compounds, trace metals, pigments and/or pigment decomposition substances and oxidation products (primary and secondary). Before the crude marine oil can be used for human consumption, it should go through different refining processes to remove such impurities. Low amount of free fatty acids (i.e. low acid value) in crude fish oil is an important quality parameter influencing the yield and economy for the refining industry. The natural content of antioxidants such as carotenoids and tocopherol is however often intact and contributes to stabilising crude fish oil during storage until further refining (Jacobsen et al., 2009; Hamre, 2011; Wanasundara, 1998; also see Chapter 2.3.4).

2.2.7 STORAGE/TRANSPORT OF CRUDE FISH OIL

Crude fish oil is one of the most internationally traded and transported commodities because the main production centres (i.e. South America, Africa, Scandinavia and Asia) often are far away from the refining plants and main consumption centres. It is estimated that each ton of fish oil and fish meal travels an average of 5000km to reach the end-user. Majority of the volume are shipped by bulk vessels, although a growing share of higher-quality products are moved to the final destination in containers (Nichols, 2007).

Fish oil imported to Norway from places such as South-America or Morocco is shipped in bulk storage installations/tanks onboard either on bulk tankers/parcel tankers, coasters or container vessels.

The use of tanks of stainless steel construction or mild steel coated with epoxy resin when transporting fully refined oil for human consumption are recommended.

Crude fish oil is stored before transport in steel tanks (preferably coated to prevent corrosion of the tank by the cargo). When possible, tall narrow tanks are preferred to minimise the surface areas of the contents and to minimise contact between oils. The "*Recommended international code of practice for the storage and transport of edible fats and oils in bulk*" (CAC/RCP, 2005; see also Chapter 1.3) provides guidance for tank material as well as recommendations for storage, transport, loading and discharge temperatures and oxygen. Additionally, tank bottoms should be conical (with a sump) to facilitate draining.

2.2.8 SUMMARY OF CRUDE FISH/COD LIVER OIL

Crude fish or cod liver oil results from raw material that is cooked and/or enzyme-treated, centrifuged for removal of solids, and washed with hot water. The processing parameters as well as the composition and freshness of the raw materials influence on the crude fish/liver oils content of decomposition substances, oxidation products and process-generated substances. Crude fish/liver oil contains minor amounts of water and of unwanted substances (e.g. waxes, phospholipids, mono and diacylglycerols, free fatty acids, cholesterol, proteinaceous compounds, trace metals, pigments and/or pigment decomposition products, primary and secondary oxidation products, environmental contaminants) and needs to go through different refining processes before it is suitable for human consumption. The natural content of antioxidants like vitamin E and tocopherols is however often intact and contributes to stabilizing the crude fish oil during storage and transport until further refining. The quality of crude fish oil, in particular the content of free fatty acids, influence on the yield and the economy for the refining fish oil industry. Crude fish oil is one of the most internationally traded and transported commodities because the main production centres (i.e. South America, Africa, Scandinavia and Asia) often are far away from the refining plants.

2.3 Production of refined fish and cod liver oils

In general, the content of decomposition substances, oxidation products and process-generated substances of refined oil is dependent on refining techniques and processing conditions.

The types of refining steps that are included depend on the requirement from the purchaser. Refined or semi-refined (i.e. the oil has gone through only some of the refining steps) oil is used as raw material for production of concentrated n-3 fatty acid oils (see Chapter 2.5).

The refining process of crude fish/cod-liver oils can include the following steps: neutralisation, washing, bleaching, winterisation (cool-down), deodorisation and molecular distillation/short path distillation. Short path distillation (SPD) is commonly used in the refining process to assist in the reduction of free fatty acids, oxidation products (Oliveira, 2010) and of contaminants including environmental pollutants. A flow diagram illustrating the most important processing steps from crude fish/cod-liver oil to refined natural oil is shown below (Fig. 2.3).

During the refining process, the oil is under vacuum and/or nitrogen atmosphere to prevent oxidation.

2.3.1 NEUTRALISATION

Neutralisation is a process that removes most of the free fatty acids (i.e. lowering the oils' acid value) and reduces the content of phospholipids, pigments and trace metals in the oil (Bimbo, 2007; Cmolik and Pokorny, 2000; Dijkstra and Segers, 2007; Noriega-Rodríguez et al., 2009). Free fatty acids can cause darkening of the oil, foaming and possible smoke during heating (see Table 1.4-3 for recommended maximum content of free fatty acid).

During the neutralisation process, the oil is mixed with a caustic soda solution, resulting in the formation of soaps that can be separated. The oil is initially heated to the optimum processing temperature of 80-95°C and thereafter, caustic soda is added to neutralize the free fatty acid. Hot water is also added to the oil, intensively mixed and the soapy wash water is removed in a separator. Washing with water also leads to the removal of water-soluble components that may be present in the oil.

Oterhals and Berntssen (2010) observed that PV (peroxides) and AV (aldehydes etc.) values in crude fish (*Sprattus sprattus*) oil were reduced by the neutralisation step from respective 1.8 and 10.4 meq/kg to 1.7 and 6.0 meq/kg (see Chapter 4.1 for description and evaluation of the methods used).

The major by-product of the neutralisation step is fatty acid soaps which are diluted by water and split with acid to form free fatty acids. These free fatty acids are sold to blenders, soap makers, or feed manufactures (Bimbo, 2007).

2.3.2 WASHING

During the washing step with hot water, water-soluble components from decomposition of proteins such as di-peptides, free amino acids, trimethyl amines and histidine and decomposition products from lipids such as partly hydrolysed phospholipids (lysophospholipids, lysoPL) and/or oxidation products of lipids will be removed together with trace metals and residual soaps.

2.3.3 WINTERISATION

In the winterisation step, high melting point triacylglycerols and waxes are reduced. The winterisation process is conducted in tanks (inert conditions) and the oil is slowly chilled to 0-2°C allowing the formation of triacylglycerol crystals with higher melting points to settle (stearin fraction; stearin is the tristearate ester of glycerol) for subsequent filtration. The winterisation process prevents clouding of the oil at refrigeration temperatures (Bimbo, 2007).

2.3.4 BLEACHING

In addition to the removal of pigments, the bleaching step further reduces the amount of free fatty acids, residual trace metals and oxidation products (peroxides) left from the neutralisation step. Removal of phospholipids, soaps (if neutralised), and sulphur containing materials also occur during this process. Bleaching with activated carbon reduces residual traces of environmental pollutants such as dioxins, furans, and polycyclic aromatic hydrocarbons (PAHs). Unfortunately, activated carbon also reduces the concentration of fat soluble retinol, tocopherol, cholecalciferol and astaxanthin (Jacobsen et al, 2009; Hamre, 2011). Tocopherol acts as an antioxidant in marine oils. A study on cod-liver and seal oils indicated that the respective crude fish oils were more stabile before than after bleaching and deodorisation due to the removal of natural antioxidants such as α-tocopherol (Wanasundara, 1998). However, Bimbo (2007) concluded that bleaching is a process that improves the colour and oxidative stability of the oil. Bleaching earth and activated carbon are added to the oil through dosing unit. Bleaching is usually conducted at 130-150°C for 30-60 min under vacuum.

2.3.5 DEODORISATION

The main purpose of the deodorisation step is to remove, as much as possible, volatile compounds (i.e. volatile secondary oxidation products such as aldehydes, ketons, etc.) that are responsible for the unwanted odour and taste of the oil. In addition, some free fatty acids and pigment decomposition products will be removed. The non-volatile secondary and tertiary oxidation products that are eventually formed will still be in the oil after the deodorisation process. See Chapter 4.1.3 Autooxidation, for detailed information about oxidation products

In general, the oil is steamed under vacuum at high temperature (150-250°C) to keep the oxygen level low, minimizing oxidative damage despite the applied heat in the process. The stripping operation, also called desorption or steam distillation, is defined as purification of the liquid oil phase and this is performed by mixing stripping gas with the oil, to facilitate mass transfer of the volatile impurity to the gas phase. The gas phase is continuously removed from the processing chamber, thus preventing the volatiles from re-entering the liquid. For a deodoriser, up to 200°C is used for 2-3 hours at vacuum below 2 mbars. For thin film counter current distillation technology, the bleached oil is heated to relatively lower temperature with significantly shorter deodorisation time (Bimbo, 2007; Dijkstra and Segers, 2007).

The n-3 fatty acids are very sensitive to oxygen and temperature (Mjøs and Solvang, 2006) and the extreme processing conditions may lead to the occurrence of chemical degradation reactions that will influence on the final oil quality. Formation of non-volatile decomposition products that may take place during heat treatment in the absence of air includes **polymers, cyclic fatty acid monomers (CFAM) and trans-fatty acids** (geometrical isomers of EPA and DHA). Isomerisation of EPA and DHA may theoretically give 32 and 64 isomers, respectively. In EPA, the central double bond (Δ11), while in DHA, the two central double bonds (Δ10 and Δ13) are more prone to isomerisation (Fournier et al., 2007). At 180°C or above, there is an increased risk of isomerisation of *cis*-fatty acids to *trans*-fatty acids, and oil deodorised at 220 and 250°C contained 4.2 and 7.2% geometrical isomers, respectively.

Polymers are the major decomposition products generated at high deodorisation temperatures, with 19.5% oligomers formed in fish oil deodorised at 250°C. A significant amount of CFAM was formed at 220°C or above.

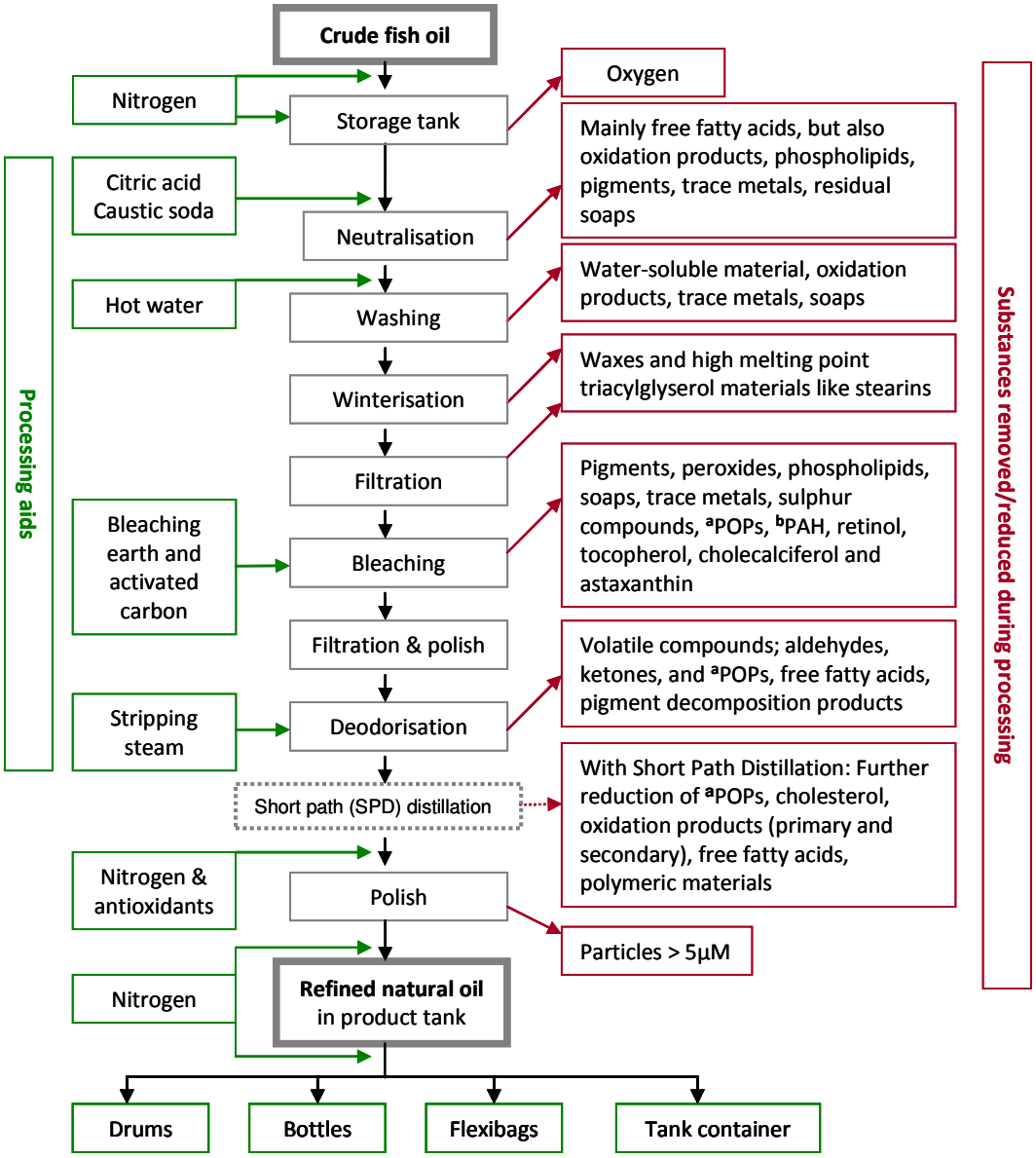


Figure 2.3 Flow diagram illustrating the general processing steps that can be employed to progress crude fish oil to refined natural fish oil. Processing aids are listed at the left in the figure, while main components that will be removed or reduced during each processing step are listed at the right in the figure. [a] POPs: persistent organic pollutants, [b] PAH; polycyclic aromatic hydrocarbons. (*Modified from Bimbo, 2007*).

The samples deodorised at 220°C consisted of 23.9mg/g C20 and C22 CFAM, while the samples deodorised at 250°C contained 66.3mg/g C20 and C22 CFAM (Fournier, et al. 2006a; Fournier et al. 2006b; Fournier et al., 2007, Berdeaux et al., 2007).

It seems that deodorisation of fish/cod-liver oil should be conducted at maximum recommended temperature of 180^0C, which is putatively lower than the activation energy required for polymerization (intra and inter) and geometrical isomerisation (see also Chapter 4.2 for factors influencing on oxidation). The deodorisation process at 250°C was found to reduce the concentration of n-3 fatty acids in fish oil with more than 60% (Fournier et al., 2006a).

2.3.6 MOLECULAR (SHORT PATH) DISTILLATION

In a crude fish oil refining process, molecular distillation or more correctly named short path distillation (SPD) is used to reduce trace amounts of persistent organic pollutants such as dioxins, PCBs and furans (Xu, 2005). In addition, it also leads to the reduction of odour compounds and free fatty acids, oxidation products and polymers formed during decomposition/oxidation of the oil. The use of SPD reduced peroxid and anisidine values in crude fish oil from respective 1.8 and 10.4 meq/kg to 0.7±0.4 and 5.5±0.2 meq/kg (an average of 11 different experiments; Oterhals and Berntssen, 2010) (see Chapters 5.1 for description and evaluation of the analytical methods used).

Separation by distillation depends on the relative volatility of the components in a mixture. The molecular size of the components strongly influences the degree of fractionation. Depending on the operation purpose, the operation conditions vary significantly. SPD is used at vacuum conditions (0.001–1 mbar), resulting in the use of low evaporator temperature and short residence time, and therefore a process that is more suitable for thermal sensitive products such as fish/liver oil. However, when removing pollutants, temperature up to 200°C is usually preferred (Bimbo, 2007; Xu, 2005)

Note that SPD also is used as a fractionation/concentration step in the production of n-3 fatty acids ethyl esters (see Chapter 2.5).

2.3.7 SUMMARY OF REFINED FISH/COD LIVER OIL

Refined oil is crude fish/cod-liver oil that usually has been neutralised, washed with hot water, cooled down, bleached, deodorised and distilled, and thus is suitable for human consumption. The refining processes decrease or remove unwanted components naturally found in the raw material, decomposition substances arising during storage of the raw materials as well as during processing and storage of the crude marine oil. The effectiveness of the refining process depends on the combination of processing steps and conditions (equipment, temperature, time, pressure etc.). During the refining process, the oil is exposed to temperatures that may cause formation of non-volatile decomposition products such as polymers, cyclic fatty acid monomers (CFAM) and geometrical isomers (mostly mono and di-*trans*-fatty acids) of EPA and DHA, i.e. process-generated substances. Optimisation of the processing parameters (e.g. temperatures, time and pressure) in combination with Short path distillation minimise unwanted process-generated substances due to the refining processing steps.

2.4 Production of krill and seal oils

2.4.1 PRODUCTION OF KRILL OIL

Oil from krill (*Euphausiacea*) contains triacylglycerols, phospholipids and astaxanthin, with phospholipids being a significant part. In krill oil intended as a direct food ingredient, the content of phospholipids was equal to or higher than 40% (GRAS Notice Inventory, 2008), whereas the content of cholesterol was 0.8-1.2 g/100 g oil and total *trans*-fat < 0.1g/100g oil.

The high content of polar lipids (i.e. phospholipids, see Chapter 3.2.1) in krill produces emulsions that impede fractionation during processing. In recent years, the krill industry has developed and optimized the processing of krill, resulting in different processing steps and end products (e.g. oil, meal, pasta, etc). Processing details are often confidential and little is published in the literature.

A general overview of processing of krill to oil is given in Fig. 2.4.1. The most common process is to pump live krill onboard the factory trawler. The catch is either deep frozen

onboard or directly produced to krill meal, resulting in dried meal with high fat content. The krill meal can then be transported to a shore side facility where solvent extraction methods can be used to extract the oil and produce a high-protein meal. The oil can further be processed to separate the phospholipids from the acylglycerol fraction. Ethanol, acetone and/or ethyl acetate can be used (repeatedly) in the extraction of phospholipids/triacylglycerols from krill (Beaudoin and Martin, 2004; Gigliotti et al., 2011). The solvent-oil solution is clarified by centrifugation and solvent is then evaporated from the final oil. The deep frozen krill is crushed and subjected to solvent extraction to obtain oil (GRAS Notice Inventory, 2008; EFSA, 2009). The lipid extract is filtrated to remove proteins and krill material, and solvent and residual water are removed by evaporation.

The exoskeleton (i.e. shell, carapace and crust of crustaceans) contains high levels of fluorine (see Chapter 3.2.2). The fluorine mainly follows the protein fraction during processing and is recovered in krill meal (Måge et al. 2010). Use of krill meal in animal feed is challenged towards the maximum level for fluorine in animal feed. Efficient removal of the exoskeleton decreases the diffusion of fluorine from the shell materials and increases the oil yield. According to a recently published patent, an alternative processing way is initial enzymatic treatment of the catch, inactivation of added enzymes by heating >90°C and mechanical separation of oil and solids (Patent cooperation treaty, 2010).

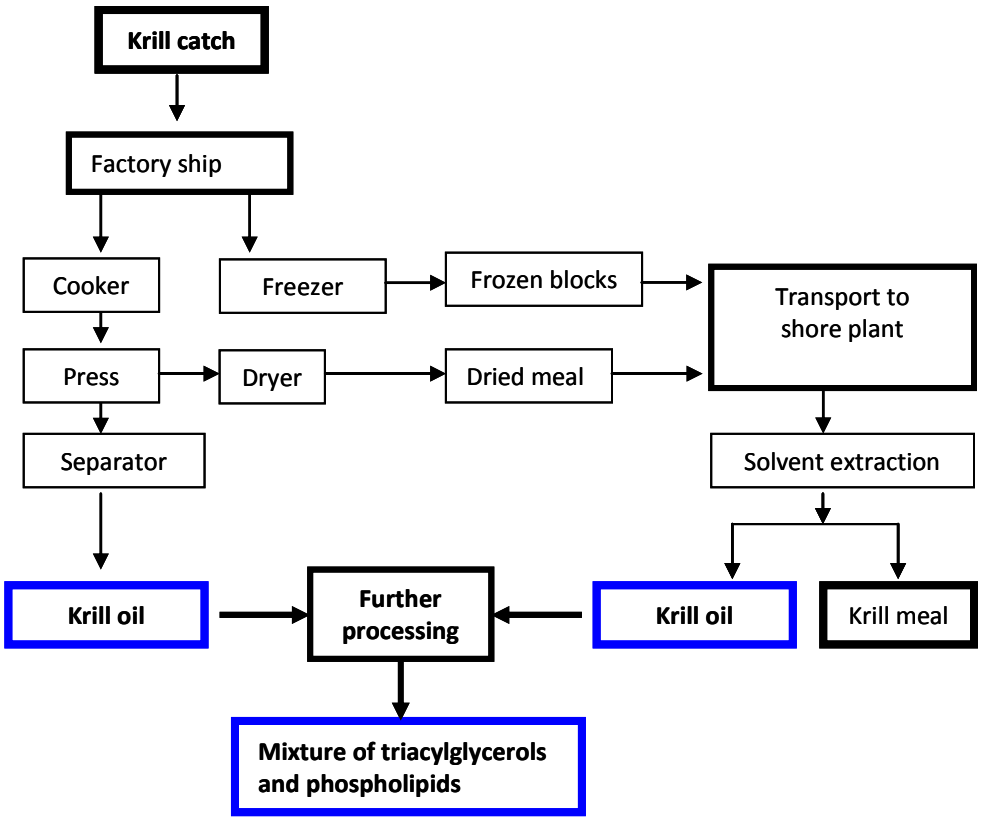


Figure 2.4.1: Overview of processing of krill to oil (*adopted from Bimbo, 2007*). Krill is pumped alive onboard the factory trawler. The catch is either deep frozen onboard or directly produced to krill meal, resulting in dried meal with high fat content and an oil fraction rich in triacylglycerols. The krill meal can then be transported to a shore side facility where solvent extraction methods can be used to extract oil rich in phospholipids and produce a high-protein meal. The deep frozen krill is crushed and subjected to solvent extraction to obtain oil. The lipid extract is filtrated to remove proteins and krill material, and solvent and residual water are removed by evaporation.

2.4.2 PRODUCTION OF SEAL OIL

Raw seal oil is produced from blubber by heating at 80-90°C followed by centrifugation. The raw seal oil is filtered to remove proteins and other tissue remnants and then stored for further processing (Wahren, 2004). There are about 5-10 processors that produce crude seal oil and to our knowledge, most of these are located in Canada. Norway imports an insignificant volume of crude seal oil for further refining, which is very similar to refining of crude fish oils (Wahren, 2004), and export the refined seal oil.

2.5 Production of n-3 fatty acid concentrates

Concentrated marine oil is obtained by modification of refined/semi-refined natural marine oils (see Chapter 2.3). Concentrated n-3 fatty acids consists of EPA and DHA either as ethyl esters or as mixed acylglycerols (mono, di and triacylglycerols) dependent on the concentration processes that have been used in their production (Kralovec et al., 2011).

The most common concentrated n-3 products on the Norwegian market are:

"Omega-3-acid ethyl esters 60": Esters of the following n-3 fatty acids; C18:3, C18:4, C20:4, C20:5, C21:5, C22:6 obtained by trans-esterification. According to the Ph. Eur. recommendations, the physico-chemical purification processes should include molecular distillation (see Table 1.4-1).

"Omega-3-acid triglycerides": Mixture of mono, di and triesters of n-3 fatty acids with glycerol containing mainly triesters, obtained according to the Ph. Eur. recommendations either by esterification of concentrated and purified n-3 fatty acids with glycerol, or by trans-esterification of the n-3 fatty acid ethyl ester with glycerol (see Table 1.4-1).

Because of the complex fatty acid composition of marine oils, highly purified long-chained n-3 fatty acids are not easily prepared by any single fractionation technique. Therefore, a combination of techniques is used, depending on the fatty acid composition of the refined or semi-refined oil and the desired concentration and purity of the n-3 fatty acids in the final concentrated product (Kralovec et al., 2011; Rubio-Rodriguez et al., 2010).

Available concentrated n-3 fatty acid production methods include adsorption chromatography, fractional- or molecular (short path) distillation, enzymatic splitting, re- or inter-esterification (chemical or enzymatic), low-temperature crystallization, supercritical fluid extraction (SFE) and urea complexation (Breivik, 2007). According to the Norwegian Seafood Federation (personal communication), urea fractioning is used in order to achieve concentrated n-3 fatty acid **above** 70% that is mainly used in the production of pharmaceutical products (i.e. 90% n-3 fatty acids). This method will therefore not be further discussed in this assessment.

Although numerous methods can be used for production of n-3 fatty acid concentrates, only few are suitable for large-scale production.

In general, all concentration procedures represent an oxidation risk (see Chapter 4.2 for factors influencing the oxidation status of marine oils). Throughout the whole processing line, the oil will be protected from oxygen by processing under vacuum and if possible under an inert gas. Decomposition of the oil that may take place will mostly occur during heat treatments of the thermo labile polyunsaturated n-3 fatty acids. However, heating of oils with high amounts of EPA and DHA may result in increased content of process-generated substances such as polymers, cyclic fatty acid monomers (CFAM) and *trans*-fatty acids (see Chapter 2.3.5).

The content of decomposition substances, oxidation products and process-generated substances in the end products for human consumption depend on the content of

decomposition/oxidation products from lipids, adequate protection against oxidation during production and storage of the crude fish oil, and during all subsequent processing/concentration steps that may be influenced by temperature and time. Rubio-Rodriguez et al. (2010) has reviewed the production process for concentrated n-3 fatty acid. A detailed description of the industrial processing steps is not available in scientific literature.

2.5.1 PRODUCTION OF ETHYL ESTERS

The first step in the production line of concentrated n-3 fatty acid is often to produce fatty acid ethyl esters by a chemical process: refined oil is mixed with ethanol and a catalyst (e.g. sodium ethylate or sodium methylate) at approximately 80°C under vacuum resulting in glycerol, some mono and diacylglycerols and ethyl esters. Glycerine is a by-product that will be separated from the ethyl ester fraction.

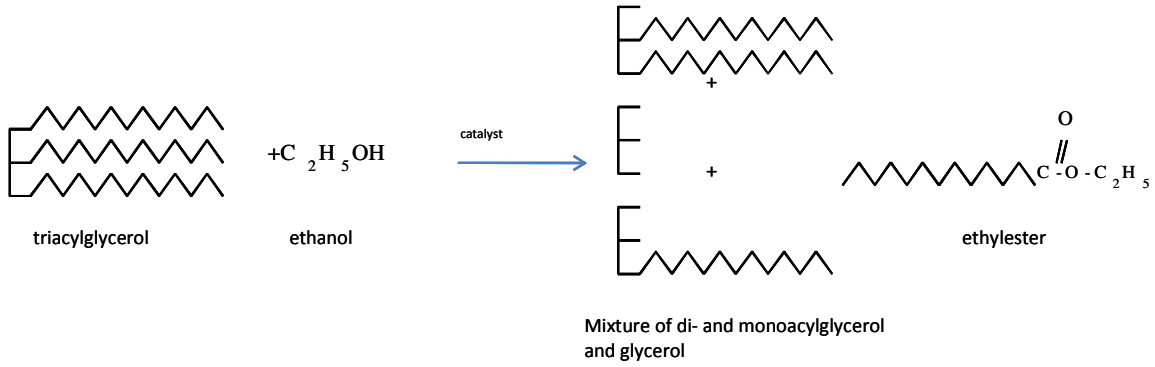


Figure 2.5.1 Esterification of refined marine oil produces n-3 fatty acid ethyl esters and a mixture of mono and diacylglycerols and glycerols. Examples of catalyst used are sodium ethylate or sodium methylate.

2.5.2 UP-CONCENTRATION OF ETHYL ESTER – SHORT PATH DISTILLATION (SPD)

Molecular distillation is often used to separate fatty acid ethyl esters where the molecular size of the components strongly influences the degree of fractionation. Presently, SPD is one of the most common fractionation techniques used for the production of concentrated n-3 fatty acids. The use of SPD in refining process is previously described in Chapter 2.3.6. However, in connection with up-concentration, distillation takes advantage of the differences in boiling point and molecular weight of fatty acids ethyl esters under reduced pressure. SPD may be performed using lower temperature and shorter heating intervals than traditional molecular distillation, and can thus be employed to concentrate polyunsaturated fatty acids without any apparent decomposition. During the production of concentrates, SPD can be used twice or more. The concentrated n-3 fatty acid may contain up to 30% EPA and 20% DHA after a second SPD treatment (Finley and Shahidi, 2001).

SPD treatment partly separates fatty acid esters from oligomeric or polymeric material that might have been generated during thermal treatment and/or autooxidation of the original oil (for detailed of autooxidation see Chapter 4.1.3) or during the preceding refining or concentration steps (Breivik, 2007).

2.5.3 PRODUCTION OF ACYLGLYCEROLS

For market purposes, triacylglycerols are viewed as being more "natural" than other fatty acid derivatives, such as ethyl esters. Re-esterification and trans-esterification are processing steps

often included in the production of concentrated n-3 fatty acid in the triacylglycerol form. The stability against oxidation seems higher when the n-3 fatty acids located in the sn-2 position of the triacylglycerol molecule (Fig. 1.2.2-1) are in the form of acylglycerols, than as ethyl esters (Boyd et al., 1992, Wijesundera et al., 2008). The production of concentrated acylglycerols with n-3 fatty acids in the sn-2 position has therefore acquired great interests.

Re-esterification: To produce concentrated n-3 triacylglycerols, the concentrated ethyl ester fraction (concentrated by using SPD or other fractionation techniques) with high amount of (EPA and DHA) will be re-esterified via the reaction of glycerol with the ethyl esters where chemical catalysts and ethanol are included. A continuous two-step supercritical fluid extraction process can be used to remove both volatile and heavy by-products (product from partial decomposition or isomerisation of the chemically unstable n-3 fatty acids) from re-esterified triacylglycerol concentrates (Breivik, 2007; Perrut, 2006).

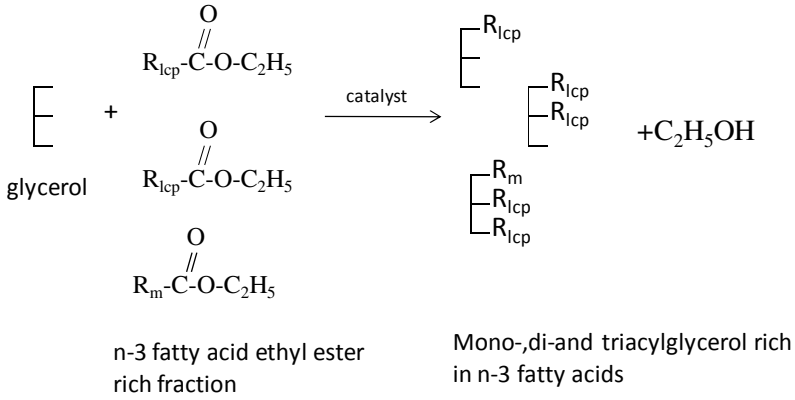


Figure 2.5.3-1 Re-esterification of fatty acid ethyl esters to produce triacylglycerols rich in EPA and DHA. R_m symbols a monounsaturated fatty acid and R_{lcp} a long chained polyunsaturated fatty acid.

Trans-esterification: In general, trans-esterification can involve three reaction types where the esterified oil reacts with fatty acids, alcohols or esters. The reaction between an ester and an acid is known as acidolysis; the reaction between an ester and an alcohol is known as alcoholysis (e.g. ethanolysis or methanolysis) and the reaction between an ester and another ester is known as inter-esterification (Gunstone, 2006). Inter-esterification is a reaction that exchanges carbonyl groups of fatty acids within and between triacylglycerol molecules. It is used to modify the structure and composition of oils to improve the physical and nutritional properties of triacylglycerol (Basheer et al., 1995). Chemical inter-esterification has been used to produce tuna oils enriched with n-3 fatty acids using sodium methoxide as catalyst (Klinkesorn et al., 2004).

Enzyme catalysed trans-esterification/re-esterification can also be used for the production of triacylglycerols that are highly enriched with n-3 fatty acids (Breivik, 2007; Haraldsson et al., 1989; Kralovec et al., 2011; Linder et al., 2002; Rubio-Rodriguez et al., 2010; Shahidi and Wanasundara, 1998; Xu et al., 1999).

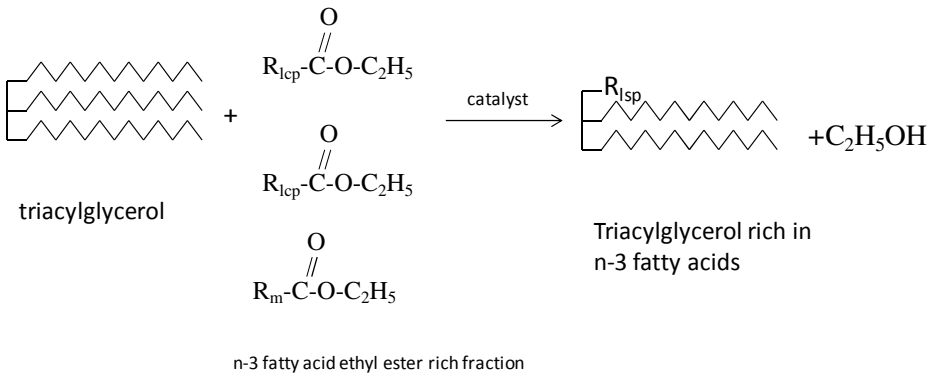


Figure 2.5.3-2 Trans-esterification of fatty acid ethyl esters to produce triacylglycerols rich in EPA and DHA. R_m symbols a monounsaturated fatty acid and R_{lcp} a long chained polyunsaturated fatty acid

The resulting products of both re- and trans esterification will contain a mixture of mono, di and triesters of n-3 fatty acids with high proportion of triesters (minimum 60% triacylglycerol, see Table 1.4-1). The amount and sort of decomposition products in the concentrated acylglycerol will depend on which processing steps and parameters (temperature, pressure and catalyst) that were used. Thermal treatment during the concentration steps can results in the same decomposition product that previously has been described under the deodorisation process (see Chapter 2.3.5).

VKM has received information from a producer about production of concentrated n-3 fatty acids from cod-liver oil in Norway. Following chemical trans-esterification, distillation (SPD) may be used for further up-concentration and additionally some of the traditional refining steps (bleaching, winterisation and deodorisation) may be included to decrease the level of oxidation products, trace metals etc.

Antioxidants (e.g. α-tocopherols) and nutrients (e.g. vitamin D) are added to the refined oil/n-3 fatty acid concentrate prior to storage and marketing.

2.5.4 SUMMARY OF PRODUCTION OF N-3 FATTY ACID CONCENTRATES

Concentrated fish/cod-liver *oil* is obtained by chemical modification of refined/semi-refined natural marine oils. Concentrates may contain up to 60-90% n-3 fatty acids. In general, concentrated n-3 fatty acids for consumption consist of EPA and DHA either as ethyl esters or as mixed acylglycerols (mono, di and triacylglycerols) dependent on the concentration processes that have been used. Because of the complex fatty acid composition of fish/cod-liver oils, highly purified long-chained n-3 fatty acids are not easily prepared by any single fractionation technique. Therefore, a combination of techniques is often used, depending on the fatty acid composition of the refined or semi-refined oil and the desired concentration and purity of the n-3 fatty acids in the final concentrated product.

Processing steps that can be included for concentration are:

- Production of ethyl ester from refined oil (basis is often fish oil from South America with approx 30% n-3 fatty acid content).
- Concentration of the fatty acid ethyl esters with focus on increasing the n-3 fatty acids content where SPD (Short path distillation) technique is one of the concentration steps of importance.

- Re-esterification or trans-esterification (chemical or enzymatic) to produce a concentrate where the fatty acids will be in mono, di and triacylglycerols form.

The amount and sort of decomposition substances, oxidation products and process-generated substances in the concentrates will depend on which process steps that is included in addition to process parameters such as time, temperature, pressure and catalyst. The traditional refining steps (bleaching, winterisation and deodorisation) are commonly included in the production line for concentrates to decrease the level of oxidation products etc. However, detailed descriptions of the whole industrial processing line and the influence of different processing steps and parameters on the product quality/content of decomposition substances, oxidation products and process-generated substances are not available in scientific literature.

2.6 Transport and storage of bulk refined oil and concentrated n-3 fatty acid

Refined and concentrated marine oils are stored in bulk using airtight and well-filled containers, protected from light, under an inert gas (see Chapter 1.4 for Ph. Eur. recommendations). Refined and concentrated marine oils ready for packaging (e.g. encapsulation) are transported in flexi bags or in stainless steel or mild steel construction tanks flushed with an inert gas such as nitrogen. The previously mentioned international code of practice for transport of marine oils recommended by Codex (CAC/RCP, 2005) is also applied (see Chapter 1.3).

2.7 Bulk refined/concentrated marine oil via packaging to food supplement

The producers of refined and concentrated oil in bulk can usually document the fatty acid profile and e.g. oxidation status, content of free fatty acids of the cargo and may issue a guarantee for the product until the seal is broken. According to the Norwegian Seafood Federation (personal communication), the production of refined/concentrated marine oil in bulk and the encapsulation/packaging/distribution of products ready for human consumption are mainly handled by different operators. The sale is performed on order from the buyer. In case of sale, the producers of refined/concentrated marine oil in bulk have no influence on the further handling, possible mixing in of other substances and packaging of the n-3 fatty acid food supplements.

According to a presentation at GOED conference in Salt Lake City 2011, the same value chain for marine food supplements is found in other parts of the world as well (Fig 2.7-1, also see Fig. 1.1, p. 34, for overview).

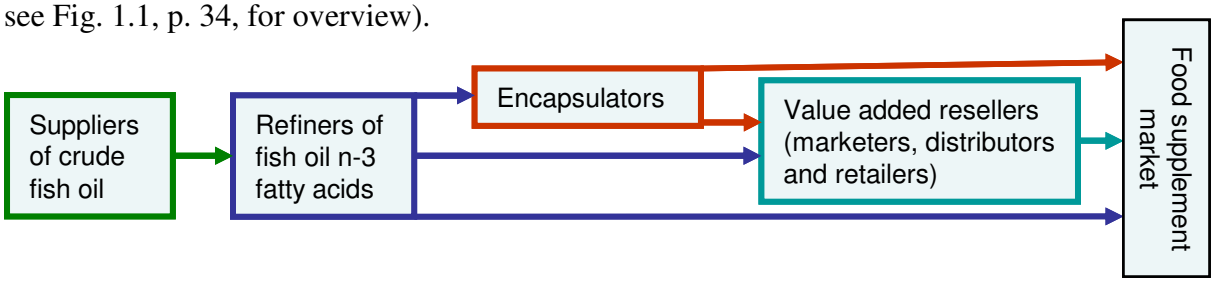


Figure 2.7-1 Illustration of the value chain for n-3 fatty acid food supplements in North America (adopted from GOED conference 2011 where source is given as Frost and Sullivan, and the base year is 2009). According to the presentation, the number of suppliers of crude fish oil was not applicable, but the number of refiners, encapsulators and value added resellers were higher than 35, 45 and 200, respectively. Suppliers of crude fish oil may sell their product to refiners; refiners may sell their product either to encapsulators, to value added resellers or bring their product directly on the market.

To the best of our knowledge there are only a few establishments for encapsulation of marine oils in Norway. Refined and concentrated oils ready for packaging may be shipped abroad for microencapsulation and/or encapsulation; mostly in soft gelatine capsules (see Chapter 4.2.3 for a more detailed description of the encapsulation material commonly used). During the encapsulation process, vitamins, food additives such as antioxidants, stabilisers and colour, as well as flavourings (e.g. lemon, peppermint, orange) may be added to the oil. Additionally, vegetable oils may be mixed in. The product should be labelled according to the given legislation (see Chapter 1.3).

In summary, refined and concentrated marine oil in bulk has defined fatty acid profile and known e.g. oxidative status, content of free fatty acids until the seal is broken. Some refiners may bring their products directly on the market, but most of the refiners sell their product either to value added resellers (e.g. marketers, distributors, retailers) or to encapsulators. The sale is performed on order from the buyer. The buyers might store the load; transport it abroad for encapsulation; divide it (seal breaking) and/or further trade it. Detailed descriptions of e.g. the processing steps during encapsulation are not available in scientific literature. The number of joint distributions involved before the product is available for consumer is unknown (see Fig. 2.7-2).

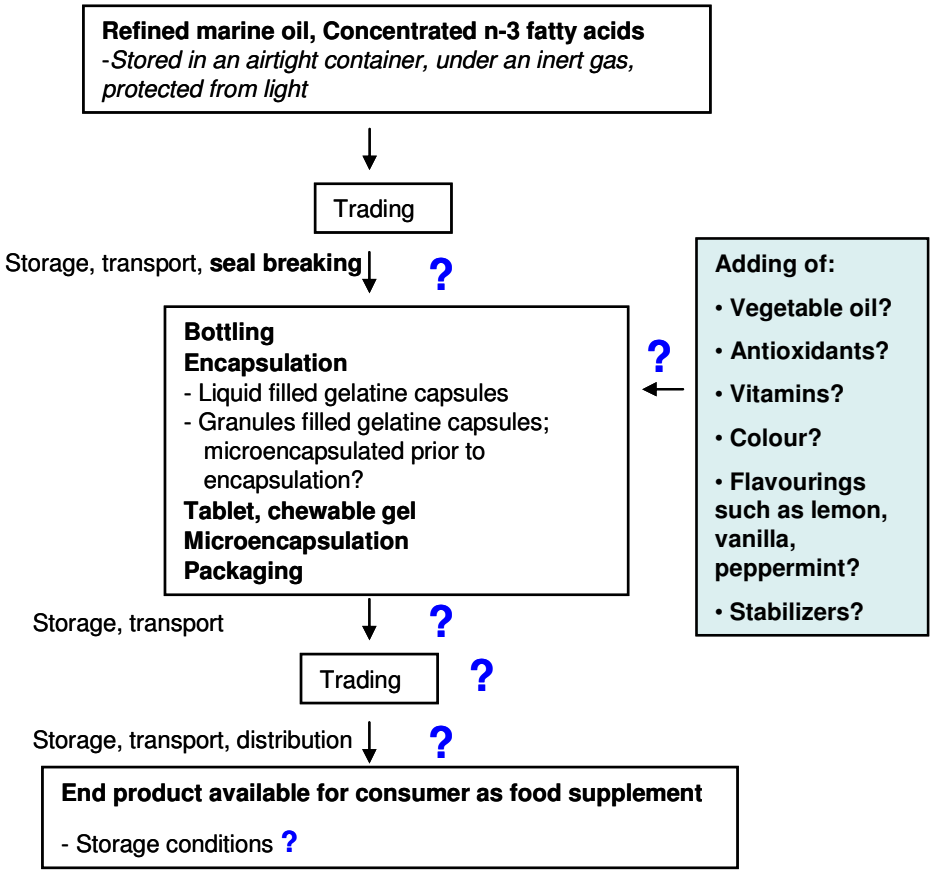


Figure 2.7-2 Illustration of the possible further destinations of traded refined marine oils and concentrated n-3 fatty acids before the products are available for consumers as food supplements.
Question marks (?) indicate parts of the distribution chain where no scientific literature has been found.

2.8 End products for consumption

The application of refined fish/marine oil and its concentrates can be divided into three main categories:

Food supplements
Refined fish oil, krill oil, seal oil and concentrated n-3 fatty acids are examples of products sold to consumers as food supplements. They may be in the form of e.g. oil in bottles (the classical cod liver oil), liquid- or micro granulated-filled capsules, "jelly" pastilles, chewable gel tablets, film-coated tablets (i.e. fish meal). Products with a combination of krill oil and n-3 fatty acid concentrates are also available. Generally, n-3 products are sold as natural triacylglycerols (max. 30% n-3), ethyl esters (60% n-3) or as mixed acylglycerols (min. 60% n-3). There are also n-3 supplements consisting of EPA and DHA as free fatty acid available via internet, but to the best of our knowledge, these are not marketed in Norway.

Enriched food
A consumer's requirement is that food products enriched with n-3 fatty acids are indistinguishable from non-fortifies food with regard to odour and taste. Refined fish oil/n-3 fatty acid concentrates in liquid form may be added directly to short shelf-life products. N-3 fatty acids may also be encapsulated as emulsions prior to food incorporation (Kolanowski and Laufenberg, 2006). Emulsifiers create smaller oil particles that are more available for absorption. However, the most commonly used way to incorporate fish oil into food is to convert the liquid form of oil to powder by using microencapsulation (Kolanowski and Laufenberg, 2006). In principle, microencapsulation creates a barrier between the fish oil and the environment in order to protect the oil against oxidation during storage or further food processing (see also Chapter 4.2.3). In addition, microencapsulation protects the fish oil from interaction with other food ingredients, and limits the odour and taste of the oil. There are many different options for coatings and these include proteins, natural and modified polysaccharides, sugars, synthetic polymers, and fats. The size of the granules is very small (from less than 1 to 250 microns). There are also several options as to how the ingredients are encapsulated, but the most popular way is by spray-drying. When using microencapsulation, the industry needs to face three challenges; (1) the amount of carrier used, (2) quality and stability of the oil, and (3) bioavailability. Extended discussion on the different coating materials that can be used is beyond the scope of this report. But, it should be noted that the use of "strong" coating materials to minimise interaction with the food matrix, decreases the bioavailability of the oil.

Pharmaceuticals
Pharmaceutical products are concentrates with a minimum of 90% total n-3 fatty acid ethyl esters and a minimum of 80% of combined EPA and DHA ethyl esters (see Table 1.4-1). In Norway (and Europe), to the best of our knowledge there is only one company, which produces n-3 fatty acid liquid-filled capsules that are approved as a pharmaceutical (i.e. Omacor). Approval by the Medicines Agency is based on documentation about clinical effect, clinical and preclinical safety and product quality.

2.8.1 STORAGE OF FOOD SUPPLEMENTS

In view of their high susceptibility to oxidation, which is an on-going process (see Chapter 4), shelf-life durability of marine n-3 fatty acid food supplements as well as storage conditions both in the store and the consumers are essential.

According to the legislation for food supplements, the product should be labelled with the duration of its shelf-life time (see Chapter 1.3). Shelf-life durability for n-3 fatty acid food

supplements is generally given as 1-2 years, but information on how the shelf-life is set by the industry has not been found (see Chapter 5.5 for Oxidative stability testing).

Information about storage condition varies depending on product. Bottles with cod-liver oil should be stored in refrigerator and kept for not more than 3 months after opening. Boxes with gelatine capsules should generally be stored at room temperature and some are labelled with explicit information about **not** to be stored in a refrigerator (i.e. softening of the gelatine capsules due to moisture permits oxygen penetration).

For factors influencing the oxidation status of marine oils, see Chapter 4.2.

2.9 Summary of processing of marine oils from catch to final product

In general, the decomposition substances, oxidation products and process-generated substances that can be detected in the marine oil end products for human consumption are dependent on the composition and the treatment of the raw materials, the processing parameters for extraction of the crude oil and the subsequent refining and concentration techniques and storage/transport conditions throughout the whole value chain.

Before the crude marine oil/cod liver oil can be used for human consumption, it needs to go through different refining processes to decrease or remove impurities such as free fatty acids, oxidised compounds, metals, colorants, and environmental contaminants. At the same time, it is important to retain the desirable nutrients like the n-3 fatty acids and natural content of antioxidants. Which kind of production steps that is included for refined marine oils for human consumption depends on the requirement from the purchaser. Refined or semi-refined oils (the oil has gone through only some of the refining steps) are raw material for production of concentrated n-3 fatty acid.

The raw material for fish oil refining/concentration in Norway is mainly crude fish oil processed before transported to Norway from ungutted wild fish caught in South America or Morocco. Cut-offs from processing of farmed salmon/cod, and herring and liver from wild *Gadus morhua* (cod) and other species of *Gadidae* are raw materials for crude fish oil production in Norway. Krill catch is semi processed on board in the Southern Ocean and most of the following oil processing takes place onboard or on shore in South America.

Crude oil results from marine raw material that is cooked and/or enzyme-treated, centrifuged for removal of solids and washed with hot water. The content of decomposition substances oxidation products and process-generated substances of the crude oil is dependent on the composition and freshness of the raw materials in combination with processing parameters used during the processing. Crude oil may contains minor amounts of water, insoluble impurities, waxes, phospholipids, mono and diacylglycerols, free fatty acids, cholesterol, proteinaceous compounds, trace metals, pigments/pigment decomposition products and oxidation products (primary and secondary) etc. Often the natural content of antioxidants is intact in crude oil resulting in relatively stable oil for storage until refining/concentration.

Refined oil is crude oil that has been neutralised, washed with hot water, cooled down, bleached, deodorised and distilled, and thus is suitable for human consumption. The content of decomposition substances, oxidation products and process-generated substances in refined oil is dependent on the content in crude oil, refining techniques and processing conditions. The refining process will remove or decrease the concentrations of unwanted components naturally found in the raw material, and decomposition products arising during storage of the raw materials and processing and storage of crude oil. The effectiveness of the refining process depends on the combination of processing steps and parameters (equipment, temperatures, pressure etc.).

During the refining process the water-soluble decomposition components will be removed by the washing step with hot water. According to the Norwegian Seafood Federation (2010), the resulting **refined oil** preferably has a water content ≤1% (0.3% dissolved in oil phase) and a content of impurities below 0.2%. Most of the free fatty acids generated during hydrolytically decomposition in the raw material will be removed during the neutralisation, deodorisation and the molecular distillation step. Trace metals contamination during extraction or storage/transport will be minimised during neutralisation, washing and the bleaching process. The concentration of both the primary and some of the secondary oxidation products will decrease throughout the whole refining process. Washing, bleaching, deodorisation and SPD (Short Path Distillation) will reduce the concentration of the oxidation products. The deodorisation step reduces the volatile secondary oxidation products (short chain cleavage product as aldehydes, ketons etc) that are responsible for the fishy flavour. The non-volatile oxidation substances will still be in the oil. SPD can, to a certain degree, separate fatty acid esters from oligomeric or polymeric material that might have been generated during autooxidation of the original oil.

The n-3 fatty acids are very sensitive to oxygen and temperature and processing conditions with thermal treatment may lead to the occurrence of chemical degradation reactions that will influence the final oil quality. Formation of non-volatile decomposition products which include polymers, cyclic fatty acid monomers (CFAM) and *trans*-fatty acids (geometrical isomers of EPA and DHA) may take place during heat treatment in the absence of air (i.e. process-generated substances). Polymers are the major decomposition products generated at high deodorisation temperatures. Non-volatile secondary oxidation products will still be in the refined oil, but have no impact on the sensory quality of the oil. Recommendations for upper limits of oxidation status and some decomposition substances in marine oils are given both by the Ph. Eur. and by GOED (see Chapter 1.4, Tables 1.4-2 and -3).

Marine oil concentrates contain higher proportions - up to 60-90% - of polyunsaturated fatty acids than the refined/semi-refined oil employed as the starting material. Thus, the concentrates are obtained by modification of refined/semi-refined marine oils and consist of EPA and DHA as ethyl esters or mixed acylglycerols (mono, di and triacylglycerols) dependent on the concentration processes that have been used in their production.

Generally, processing steps that can be included for concentration are:

- Production of ethyl ester from refined oil (basis is often fish oil from South America with approx 30% n-3 fatty acid content)
- Concentration of the fatty acid ethyl esters with focus on increasing the n-3 fatty acids content where SPD (Short path distillation) technique is one of the concentration steps of importance
- Re-esterification or trans-esterification to produce a concentrate where the fatty acids will be in mono, di and triacylglycerols form.

The amount and sort of decomposition products in the concentrates will depend on which process steps that is included in addition to process parameters such as time, temperature, pressure and catalyst. The traditional refining steps (bleaching, winterisation and deodorisation) are commonly included to decrease the content of oxidation products, trace metals, etc. (see Fig. 2.3).

However, detailed descriptions of the industrial processing steps and the influence of process parameters on the product quality during the concentration process are not available in scientific literature.

3. Composition and decomposition of raw materials for marine oil production

3.1 Whole fish as raw material

3.1.1 BIOCHEMICAL COMPOSITION OF THE FISH

Whole fish mainly consists of protein, lipid and water (Table 3.1.1-1) while the glycogen, vitamins and minerals constitute less than 4% on a wet weight basis. The protein content in whole fish is normally about 18-20% as in mammals, but lower values can be found in some species (Table 3.1.1-1). The lipid and water constitute about 80% of the fish. Fatty fishes such as mackerel and herring contain more lipid than lean species such as gadoids which contain more water (i.e. less dry matter). Some oily species, such as mackerel, show rather huge annual variation in lipid content due to seasonal variation of feed availability, spawning and migration, while the protein content is more or less constant (Huss, 1988). The protein contents (measured as nitrogen x 6.25) and total amino acid profiles in terrestrial animals and fish are rather similar (Espe, 2008). In all vertebrates, protein is mainly stored in muscle tissues, as actinomyosin genetically determined amino acid composition. In both fish and mammals, free amino acids constitute less than 1% of the total amino acids.

The total free amino acid content is slightly lower in fish than in mammalian meat, while there is relatively more of the non protein nitrogen (NPN). NPN is low molecular weight nitrogen compounds which are not part of the protein. Examples are trimethylamin oxide (TMAO), betaine, phosphocholine (lecithin), sarcosine, spermine, spermidine and taurine, all being a part of the normal metabolism in fish as in other vertebrates (Brosnan et al., 2004; Finkelstein, 1990; Huss, 1988; Mato et al., 1997; Treberg and Driedze, 2007). Free methionine and cystine may be metabolized to taurine or glutathione (Finkelstein, 1990) and influence the oxidative status in fish muscle both *in vivo* and *post mortem* (Hansen et al., 2006). Fish tissues also contain di-peptides, such as anserine (β-Ala-3-methyl-His) and carnosine (β-Ala-His), which have osmolytic functions in living tissues and both contain the amino acid, histidine. Different fish species contain different amounts of these osmolytes (van Warde, 1988). Generally, the Scombroidea fishes (e.g. mackerel and tuna), as well as sardine, pilchard, anchovies and herring have high concentrations of free histidine that is involved in histamine poisoning (reviewed by Shalaby, 1996).

The lipid content in fish fillets varies greatly from less than 1% in lean fish to more that 30% in mackerel. In whole herring, the seasonal and individual variation in lipid content is reported to be approximately 5-30% of wet weight (Slotte, 1999). The lipid is present as neutral and polar classes (see Chapter 1.2.1), where the polar phospholipids (PL) constitute biological membranes and the neutral triacylglycerol (TAG) mainly serves as an energy store. Lean fishes such as cod, store fat as TAG in the liver, while fatty fishes store TAG in the muscle. The PL level in fish tissues is coupled to the amount of biological membranes and is more or less constant, while the TAG content varies depending on the energy status of the fish. The TAG fraction is the main component in fish oil. The fatty acid composition in fish (Table 3.1.1-2) depends on the fatty acid profile in the diet. Therefore, wild-caught fish are typically rich in the long chain n-3 fatty acids of marine origin. Depending on the use of vegetable oil ingredients in the fish feeds, farmed fish may have a different fatty acid profile than their wild counterparts (Torstensen et al., 2008).

The amount of cholesterol in fish is generally less than 1% of the lipid level (Saldanha and Bragagnolo, 2007).

Glycogen constitutes less than 1% of tissue wet weight in fish, but the level of glycogen at death is important in the timing of *rigor mortis* (see Chapter 3.1.2).

Of the ***micronutrients,*** the fat-soluble vitamins (i.e. retinol, tocopherol, cholecalciferol) as well as astaxanthin are the most interesting in the context of fish oil production, since they are extracted with the oil. The levels of astaxanthin in salmon and trout are reported to be 3-11 mg/kg and 9-25 mg/kg, respectively, depending on fish size and the diet. In salmon fillet and roe, astaxanthin exist as free form but esterified form(s) is found in the skin (Christiansen, 2001). Since the vitamins are unstable and easily transformed into inactive substances, their concentrations may decrease during storage of raw fish material (Hamre, 2011).

Undesirable substances: Even though the presence of arsenic in marine raw material has been known for decades the structure of some of the lipid soluble arsenic compounds (arsenolipids) has only been elucidated recently. Lipids from capelin have been shown to contain three arsenic-containing hydrocarbons (Taleshi et al, 2008) and cod liver has been shown to contain arsenic fatty acids (Rumpler et al, 2008). Mean levels of arsenic in fish oil as high 10 mg As/kg has been reported (Måge et al., 2010) but possible biological effects are still unknown.

Raw fish materials may also contain lipid soluble undesirable substances depending on the degree of environmental contamination in the fish catch areas. The fat soluble substances will mainly follow the oil fraction whilst inorganic substances mainly follow the protein fraction. The extent to which the undesirable substances reach the final consumer product depends on the processing steps employed during manufacturing. In general, the typical fat-soluble environmental contaminants such as PCB, dioxins and chlorinated pesticides are found at higher concentrations in oils produced from the North Atlantic than South American Oceans (see Chapter 2.3 for special procedural steps which reduce oil content of environmental contaminants).

Table 3.1.1-1 Composition of whole fish (% of wet weight)

Values were averaged from published data (FAO, 1986; Norita, 2002, and RUBIN report no. 144, 2007)

[1)] Fish species	Protein	Fat	[2)] Ash	Water
AMMODYTIDAE				
[3)] Sand eels (Tobis) (North Sea)	15.4	3.7	2.6	78
ELASMOBRANCHES				
Dogfish	19	8.9	2.3	70
CLUPEIDS				
Anchoveta	18	6	2.5	78
Herring (spring)	18	8	2	72
Herring (winter)	18.2	11	2	70
Anchovy (South Africa)	17	10	3	70
Pilchard (South Africa)	18	9	3	69
Sprat (Atlantic)	16	11	2	71
GADOIDS				
Blue whiting (North Sea)	17	5	4	75
Hake (South Africa)	17	2	3	79
Norway pout	16	5.5	3	73
SCOMBROIDS				
Mackerel (spring, North Sea)	18	5.5	1.6	75
Mackerel (autumn, North Sea)	15	27	1.4	57
Horse mackerel (North Sea)	16	17	3.8	63
Horse mackerel (South Africa)	17	8	4	72
SALMONOIDS				
Capelin (Norway)	14	10	2	75

[1)] See Table 1.1 for characteristics of the different fish species. [2)] Ash is the mineral containing remnant after heating of biological material. [3)] Data from Norita, 2002, and RUBIN report no. 144, 2007.

Table 3.1.1-2 Fatty acid composition (selected fatty acids, per cent of total fatty acids) of different marine species used for oil production (adapted from Nichols, 2007).

Fatty acid	Farmed Atlantic Salmon	Tuna	Jack mackerel	Herring	Atlantic cod liver	Atlantic menhaden	South American anchovy	Capelin	Sardine	Seal	Krill
14:0 (myristic acid)	4.2	3.9	7.3	7	3.3	7.3	7.5	7	8	5.0	9.5
16:0 (palmic acid)	15.7	17.6	15.7	16	13.4	19	17.5	10	18	11.3	20.8
16:1 n-7	5.1	5.4	5.1	6	9.6	9.1	9	10	10	14.3	9.9
18:0 (stearic acid)	4.2	4.1	3.1	-	2.7	4.2	4	-	-	1.1	0.9
18:1 n-9	16.5	12.4	9.9	13	23.4	13.2	11.6	14	11	22.3	10.5
18:1 n-7	3.5	2.4	2.9	-	-	-	-	-	-	4.9	10.3
20:1 n-9	3.3	1.3	8.3	12	7.8	2	1.6	17	4	7	<1
22:1	2.5	0.5	5.8	20	5.3	0.6	1.2	14	3	2.3	<0.5
18:2 n-6 [1] (LA)	6.6	1.9	1.7	-	-	1.3	1.2	-	-	1.1	2.3
20:5 n-3 [2] (EPA)	7.1	12.4	10.9	5	11.5	11	17	8	18	6.6	18.2
22:6 n-3 [3] (DHA)	15.7	27.8	11.5	6	12.6	12.6	8.8	6	9	8.7	9.5
22:5 n-3 [4] (DPA)	3.9	1.7	2	-	1.6	1.6	1.6	-	-	4.4	-
Total n-3 [5] LC-PUFA	**26.7**	**41.9**	**24.4**	**11**	**25.7**	**22**	**27.4**	**14**	**27**	**19.7**	**27.7**

[1] LA – linoleic. [2] EPA – eicosapentaenoic. [3] DHA – docosahexaenoic acid. [4] DPA – docosapentaenoic acid. [5] LC-PUFA: long chained polyunsaturated fatty acids.
Note that seasonal variances in lipid content may occur in all species listed above.

3.1.2 DECOMPOSITION PRODUCTS FROM PROTEIN AND OTHER NITROGEN CONTAINING COMPOUNDS

Rigor mortis is characterized by muscle contraction due to depletion of energy in the muscle tissue. When the oxygen supply to the muscle stops at death glucose is metabolized to lactic acid which delays the onset of *rigor mortis*. The nutritional status of fish and the stress level (i.e. glycogen catabolisms during harvesting determine the amount of glycogen in the muscle at the time of death. The glycogen level determines the amount of lactic acid produced and thereby the *post mortem* pH. Fillet has a pH of about 7.2 in a living fish. A *post mortem* pH as low as 6.5 increases the shelf-life of fish fillet (Huss, 1988; Roth et al., 2006).

The start of deterioration in *post rigor* fish is due to autolysis caused by endogenous enzymes present in the tissues. The protein is hydrolysed and amino acids are modified, mostly by lysosomal cathepsins (Haard, 1994). Early in the storage period, enzymatic activity will contribute positively to taste by production of inosine, but if the fish is stored for longer period of time, the production of hypoxanthine and uric acid will negatively affect the quality. After harvest, trimethyl amine oxide (TMAO) present in fish muscle is converted to trimethyl amine (TMA) which contributes to the odour of spoiled fish. Elasmobranches are known to have the highest levels of TMAO, flatfishes the lowest and pelagic- and cod fish have intermediate levels (Hebard et al., 1982). In gadoid species, TMAO may be further metabolised to dimetylamin (DMA) and formaldehyde (FA) by the enzyme aldolase (Badii and Howell, 2002; Nielsen and Jørgensen, 2004). The sum of ammonia, DMA and TMA (total volatile base, TVB) are normally analysed and compared with the upper limit established for evaluating fish freshness (see Appendix A).

During fish or fish products storage, histidine may be decarboxylated to the corresponding biogenic amine, histamine. The growth rate of the fish as well as the storage temperature and duration affect the amount of NPN in the raw material. In capelin stored at 2°C the amount of

non-protein nitrogen per 100 gram total protein increased *post mortem* from below 2 gram on day 0 to 10 gram on day 14 (Espe and Haaland, 1992; Haaland et al., 1990). As a result of these factors, different nitrogen conversion factor should be used for fish and fish products (Falch et al., 2010). Several fish studies have shown lower values with a more specific conversion factor of 5.8 (nitrogen to net protein) for fish fillet (Gnaiger and Bitterlich, 1984; Sosulski and Imafidon, 1990) and a factor of 4.94 (SaloVaananen and Koivistoinen, 1996) for protein estimates for fish and fish products.

Later in the storage period, microorganisms present in fish are the major factor limiting the shelf life, in a process termed putrefaction (see Chapter 3.1.4). Free amino acids present in *post mortem* fish are decarboxylated by bacteria, giving rise to products collectively known as biogenic amines (Shalaby, 1996). Only the free amino acids can be decarboxylated since the carboxyl group otherwise is part of the peptide and not accessible by bacteria. Decarboxylase activity is found in bacteria from several different groups including members of the family Enterobacteriaceae and the genera *Clostridium* and *Lactobacillus* (see Chapter 3.1.4). Bacteria may possess a variety of amino acid decarboxylases and thus many different amines may be present in food (Lovenberg, 1973). Histidine is the precursor for the most common biogenic amine, histamine. Lysine gives cadaverine, arginine gives agmatine and putrescine, phenylalanine gives phenetylamine, and tyrosine gives tyramine.

However, degradation products from protein are mainly water-soluble and only limited amounts will be extracted in crude fish oils.

3.1.3 DECOMPOSITION PRODUCTS FROM LIPIDS

Autolysis: *Post mortem* autolysis of fish tissues includes lipolysis, such as the hydrolysis of fatty acids from TAG and PL molecules. The process liberates free fatty acids (FFA), lysophospholipid (lysoPL, partly hydrolysed PL), partly hydrolysed TAG and glycerol. The activities of lipases are highest in viscera and liver of fish (Sidell and Hazell, 2002), therefore cut-offs containing viscera produce higher amounts of FFA upon storage. The activities of lipolytic enzymes in fish and cut-offs vary with both the fishing grounds and season (Søvik, 2005). The hydrolysis of lipids is rather slow and FFA was found to be <2% of total lipid after storing Coho salmon for 14-19 days on ice (Aubourg et al, 2005). Wu and Bechtel (2008) reported that storage temperature significantly affected the FFA production in cut-offs from the filleting line of Alaskan salmon. In fresh cut-offs, FFA constituted about 1% of the total lipid content, but when stored at 15°C for 4 days, the FFA increased to 6%. On the other hand, when storage temperature was lowered to 6°C, the FFA only increased to 2-3% of the total lipid. Upon storage at high temperature, the lipolytic activity of microorganisms contributed to a higher FFA production in raw fish materials and fish oil (DeKoning, 1999).

Espe and Lied (1999) reported a slow increase in total oxidation and FFA upon storage of silage from whole herring and mackerel, but the higher the storage temperature the higher the FFA concentration. The maximum amount of FFA was however 4 mg g^{-1} total lipid in silage stored for 48 days at 50°C.

An increase in FFA of the lipid fraction will usually be accompanied with an increase in lysoPL and partly hydrolysed TAG, where FFA and lysoPL may have negative influence on human health (see Chapter 7.1). Recommend upper limits for acid content in edible oils is given by Codex, Ph. Eur. and GOED (see Chapter 1.4 and Table 1.4-3).

Oxidation of lipids: Lipid oxidation is a dynamic process where the formation and breakdown of lipid oxidation products occurs at the same time (see Chapter 4). Free fatty acids are more readily oxidised than fatty acids in the form of acylglycerols, and free fatty acids may also act

as prooxidants. It is common knowledge that the starting raw fish material is critical for producing high quality oils. There are rapid changes in fish after harvesting due to endogenously enzymatic processes that can lead to oxidation compounds formations (see Chapter 4.1.1 Enzymatic oxidation). The pH-decrease in *post mortem* fish muscle activates haemoglobin as prooxidant (Richards et al., 2002) and may lead to increased lipid oxidation (see Chapter 4.2.4). The susceptibility towards lipid oxidation in fish fillets was observed to increase in the following order: white muscle<dark muscle<skin (Undeland et al., 1998). The higher susceptibility of dark muscle to lipid oxidation is mainly due to higher content of prooxidative haemoproteins, but also higher content of phospholipids than light muscle (Undeland et al., 1998).

Moreover, the concentration of endogenous antioxidant substances in fish muscle, such as ascorbic acid, glutathione peroxidase, and tocopherol (see Chapter 4.2.5.1), decrease during storage time (Undeland and Lingnert, 1999). Therefore, lipid oxidation in fish muscle develops during storage and may vary with fish species (e.g. lipid content and composition of muscle, type and level of prooxidants) as well as the storage conditions and time. To the best of our knowledge, only few studies have examined the oxidation changes with storage of the starting raw fish material prior to production of crude fish oil. Notably, the focus in these studies is production of fish oil from cut-offs (termed "by-products" in the articles referred).

In order to meet the increasing demand for n-3 fatty acids, research efforts have targeted the valorisation of solid wastes to increase the benefits to the fish industry. Aidos (2002) studied the influence of cut-offs freshness to crude fish oil quality. Herring cut-offs stored at 2^{o}C and 15^{o}C for up to 72h, respectively, showed acceptable storage stability (PV<5.3 meq/kg fat and AV<4) (for description and definition of PV and AV, see Chapter 5.1 Methods to detect and measure oxidation). Wu and Bechtel (2009) studied the oxidative changes in Walleye Pollock cut-offs during storage at respective 6^{o}C and 15^{o}C for up to 10 and 4 days. Oxidation products measured as peroxide value and thiobarbituric acid reactive substances (TBARS) value increased initially followed by a decrease with storage time. Both Aidos (2002) and Wu and Bechtel (2009) found lower peroxide and TBARS values in crude fish oils extracted from the raw fish material which had been stored at the highest storage temperature (15^{o}C) compared to the lower storage temperatures (2^{o}C-6^{o}C). Various explanations for this finding were postulated, but none were conclusive.

According to Osada et al. (1993), oxidised cholesterol is usually not obtainable in raw fish samples (see Chapter 4.1.4 Oxidised cholesterol). More knowledge is needed to understand the influence of raw fish material freshness on the oxidative quality of extracted oil.

3.1.4 MICROBIAL OCCURRENCE AND GROWTH IN THE RAW MATERIAL

In general, raw fish material contains many microorganisms already present in the living fish (especially from the gut), but may also be contaminated by bacteria during handling and processing.

Not all bacteria growing on fish will lead to the production of objectionable characteristics. A minority of the bacteria species are often associated with majority of the spoilage, often termed the Specific spoilage organisms (SSOs) (Gram and Huss, 1996). In fish from temperate waters stored and chilled under aerobic conditions, the most active SSO are shown to be *Shewanella putrefaciens* (Gram, 1992). In many cases, *S. putrefaciens* constitutes 1 - 10% of the total bacteria flora of newly caught fish from temperate marine waters. *S. putrefaciens* is able to reduce trimethylamine oxide (TMAO) which is naturally found especially in gadoid fish species, to trimethylamine (TMA). *S. putrefaciens* is also able to produce hydrogen sulphide (H_2S) and a range of other off-odour compounds. Another

important effect of bacterial activity in raw materials is the possible production of biogenic amines. The formation of these amines is mediated by bacteria able to decarboxylate amino acids (Shalaby, 1996). Decarboxylase activity is found in several bacterial groups, including members of the family Enterobacteriaceae and the genera *Clostridium* and *Lactobacillus*. Histamine forming bacteria isolated from Scombroidea fish involved in food poisoning are *Morganella* (formerly *Proteus*) *morganii*, *Klebsiella pneumonia* and *Hafnia alvei*. Furthermore *Proteus vulgaris*, *Proteus mirabilis*, *Clostridium perfringens*, *Enterobacter aerogenes* and *Vibrio alginolyticus* have been isolated from fish and shown to decarboxylate amino acids (Shalaby, 1996). In addition to these, the following bacteria have been found to have histamine decarboxylase activity in fish: *Acinetobacter lwoffi*, *Shewanella putrefaciens*, *Aeromonas hydrophila* and *Plesiomonas shigelloides*. Of special concern is the histamine production by psychrotolerant bacteria as *Morganella psychrotolerans* and *Photobacterium phosphoreum* (Emborg and Dalgaard, 2008). For these bacteria, chilling of fish to 0-5°C alone is not sufficient to prevent formation of histamine.

As part of the oil and meal production process, the raw fish material is heat treated (see Chapter 2.2), at rather high temperature. The presence of bacteria is therefore not generally considered as a problem in n-3 oils. However, if growth of toxin-producing bacteria occur during the period from catch to processing (max 36 hours for whole fish; see Appendix A), the presence of lipophilic and heat stable toxins in oil may theoretically be possible.

3.1.4.1 Lipophilic microbial toxins

When certain bacteria, mainly Gram positives as *Bacillus* sp., *Clostridium* sp. and *Staphylococcus* sp., grow in foods, toxins may be produced as part of their metabolism. Formation of such toxins may be a potential problem when foodstuffs are stored under improper temperature or time conditions, and may lead to food borne intoxications. The growth limits for some relevant toxin producing bacteria are given in Table 3.1.4.1.

B. cereus is a facultative anaerobic, Gram positive spore forming bacterium. They are widely distributed in the environment, and have been isolated from a wide variety of foods, especially of plant origin, but also from meat, fish, and dairy products (Farrar and Reboli, 2006; Jay et al., 2005; Lunestad at al., 2011). Most *B. cereus* strains are mesophilic and are able to grow in low-acid foods at temperature down to 15^{o}C and up to 55^{o}C (optimum 30 to 40^{o}C). During the last decade, psychotrophic strains of *B. cereus* have been recognized as able to grow at temperature down to 4^{o}C. There have also been a number of reports of food borne intoxications caused by other *Bacillus* species, such as *B. subtilis, B. licheniformis*, and *B. pumilis*. *B. cereus* is known to give food-borne disease in two different ways, *i.e.* emetic and diarrhoeal type. In the emetic type of intoxication, heat stable toxins of proteinous nature are produced by the bacterium in the food. More than 10^5 *B. cereus* cells per g food product are considered to be necessary in producing sufficient amounts of toxins to give intoxications (Adams and Moss, 2008). The emetic toxin is termed cereulide, and is heat stable and thus tolerates autoclaving (90 min at 121°C), is stable in pH range of 2 -11 and is not inactivated by proteolytic enzymes. Cereulide is of hydrophobic nature and will be associated with lipid environments (Granum, 2001). However, the minimum growth requirements of *B. cereus* is considered to be pH 4.3 at 4°C and a water activity ($_{aw}$) of at least 0.95 (Martins, 1997; Adams and Moss, 2008). Such growth conditions could periodically be present in the water phase of raw material, or in the inter phase between the lipid and the water fractions, if such a biphase is present in the raw materials. The presence of cereulide in refined oils has to our knowledge not been reported so far.

Table 3.1.4.1 Relevant toxin-producing bacteria and their growth limit (Martins, 1997).

Microorganism	Minimum temp. (°C)	Minimum pH	Minimum water activity	[1)]Aerobic/ anaerobic
Bacillus cereus	4	4.3	0.95	[1)]Facultative
Clostridium botulinum ([2)]mesophilic, [3)]proteolytic)	10	4.6	0.93	Anaerobic
C. botulinum ([4)]psychrotrophic, [5)]non-proteoloytic)	3	5.0	0.97 (or ≥ 5.5% NaCl) [6)]	Anaerobic
Staphylococcus aureus	6 (10 for toxin)	4.0 (4.5 for toxin)	0.83 (0.9 for toxin)	Facultative

[1)] aerobic – requiring oxygen for growth; anaerobic – able to grow in absence of oxygen; facultative – able to grow both under aerobic- and anaerobic conditions; [2)] mesophilic – optimal growth at moderate temperatures, [3)] proteolytic – contain enzymes that degrade proteins, [4)] psychrotrophic – optimal growth in cold environments; [5)] non-proteolytic – do not contain enzymes that degrade proteins. [6)] NaCl – sodium chloride.

Clostridium botulinum is a Gram positive, anaerobic spore forming bacteria that is naturally found in soil, sediments and water including fish on a worldwide basis (Fach et al., 2002; FDA, 2009; Gram, 2001; Hielm et al., 1998; Huss, 1980). The organism and its spores occur in both cultivated and forest soils, bottom sediments of streams, lakes, and coastal waters, and in the intestinal tracts of fish and mammals, and in gills and viscera of crabs and other shellfish. Most strains of *C. botulinum* are able to produce very potent neurotoxins, and the presence of such bacteria is of great concern in the assessment of food safety. *Botulinum* toxins are thermally unstable, and will generally be inactivated at temperature >85°C for 5 minutes and the risk of finding active components in fish meal and oil after processing is therefore rather small.

The genus *Staphylococcus* comprises several species of which *S. aureus* is most often associated with food borne disease. The limits for growth and toxin production of *S. aureus* are 6°C and 10°C, respectively. *S. aureus* are rarely found on newly caught fish from temperate waters, but may be isolated from fish in warm waters. The most likely contamination route for *S. aureus* is from food production personnel and handlers with infections on the skin or in mucosal linings. The Staphylococcus enterotoxins are heat and protease stable single polypeptides of 25-28 kDa size (Jablonsky and Bohach, 2001). The toxins are however water soluble, and will normally not accumulate in the oils (Sutherland and Varnam, 2002).

3.2. Krill, seal blubber or cod liver as raw material

3.2.1 BIOCHEMICAL COMPOSITION

Krill, both Antarctic and temperate species[8], contain respective 10-50% and 5-20% of lipid (phospholipids and triacylglycerols) dry matter (d.w.), with high seasonal variation (Ju et al., 2009). The dominating lipid classes are PLs in temperate species and neutral lipid classes in the Antarctic species (Ju et al., 2009). In some species of Antarctic krill the neutral lipids contain wax esters while others mainly contain TAG. Dominating fatty acids are 16:0, 18:1 n-9, 20:5 n-3 and 22:6 n-3 (see Table 3.1.1-2). The cholesterol content is higher than in fish and

[8] The dominant temperate krill species in the Northeast Pacific are *Euphausia pacifica* and *Thysanoessa spinifera*, wereas the two major Antarctic species are *Euphausia superba* and *E. crystallorophias* (Ju et al., 2009). The arctic krill species *Meganyctiphanes norvegica* occur in the North Atlantic and the Pacific.

is reported to range from 62.1 to 71.6 mg/100 g krill tissue, but about two-thirds of sterols in shellfish are non-cholesterol sterols which may inhibit intestinal absorption of cholesterol in humans (Tou et al., 2007).

The content of astaxanthin, the principal carotenoid pigment (i.e. antioxidant) varies considerably but is generally 150-200 µg/g dry matter for *E. superba*, while *E. pacifica* may contain even more (Nicol et al., 2000). Yamaguchi et al. (1983) found that frozen Antarctic krill *Euphausia superba* contained 3-4 mg carotenoid per 100 g and astaxanthin diester, astaxanthin monoester, unbound astaxanthin and unidentified ones accounted for 40-50%, 30-40%, 15-25%, and 5-15%, respectively.

The fluorine of krill is concentrated in the exoskeleton (i.e. shell, carapace and crust), where it may reach concentrations of 350 mg/100g dry weight (Tou et al., 2007). Adelung et al. (1987) found that *E. superba* and *Meganyctiphanes norvegica* contained 2600 µg/g and 3300 µg/g dry weight fluorine in pleon cuticle, respectively. *Nyctiphanes australis* (a coastal species of krill) has been found to contain fluorine with a seasonal range between 277 and 3507 µg/g dry weight (Virtue et al., 1995).

Marine mammals: Seal is composed of approximately 44% carcass, 29% blubber, 18% viscera and 8% skin (Shahidi and Wanasundara, 1998). The lipids are mainly stored as subcutaneous fat (i.e. blubber) in the form of TAG (see Chapter 1.2.1). Neutral lipid from fish have the long chained polyunsaturated fatty acids (PUFA) mainly in the sn-2 position of the TAG, while TAG from marine mammals has the long chained polyunsaturated fatty acid in the sn-1 and sn-3 positions. Human pancreatic- and lipoprotein lipases are sn-1/sn-3 specific. Therefore the n-3 PUFA in TAG from marine mammalian oil may be more readily available for lipolysis than n-3 PUFA from fish oils (Brunborg, 2006). The fatty acid profile of seal oil can be distinguished from the other types of marine oils by the higher content of docosapentaenoic acid (DPA; 22:5 n-3) (see Table 3.1.1-2).

The liver is the main lipid storage organ in gadoid species and cod liver therefore contains very high levels of TAG (see Table 3.1.1-2). Liver from the wild caught cod has traditionally been used to prepare cod-liver oil because it provides long chained n-3 fatty acids and retinol and cholecalciferol. The composition varies greatly, but according to the Norwegian food table the cod liver contains averagely 60% lipids, 6% protein and 28% water. Moffat (2009) refers to an oil content of 50-75% in cod liver (*Gadus marhua*). In a recent study of cod from the North Sea the individual variation in lipid content was from 5% to 78% (Green et al., 2011). The fatty acid composition in the liver of cod reflects the fatty acid composition of the diet (Lie et al., 1986).

3.2.2 DECOMPOSITION OF KRILL, SEAL BLUBBER AND COD LIVER

Krill: Muscle proteins in krill have low heat stability and are extremely sensitive to enzymes, i.e. the protein easily hydrolyses after capture (Ju et al., 2009). Upon storage of krill the non protein nitrogen increases exponentially (Ju et al., 2009; Kolakowski, 1986). Both TAG and PL were also extensively hydrolysed during *post mortem* storage at 0°C, whereas wax esters were not hydrolysed (Saether et al., 1986). Free astaxanthin is unstable and easily transformed into inactive substances. According to Yamaguchi et al. (1983) all forms of astaxanthin except the free form were detected in krill meal. The content in krill meal was 15-20mg astaxanthin per 100gram and the diester form comprised 65-75%. There have been concerns about elevated levels of fluorine in krill meal, but as fluorine mainly follows the protein fraction during processing, it does not concern oil production (see Chapter 2.4.1).

Seal Blubber: Scientific publications on decomposition of seal blubber during storage were not identified.

Cod liver: Since liver has high activity of hydrolytic enzymes, it will go into autolysis quite rapidly if not stored at low temperatures. The cod-liver oil production can either be from fresh or frozen raw material. In fresh cod liver stored on ice (i.e. 0°C), the amount of free fatty acids was below 1% at storage day 4, and at storage day 10 PV was below 3meq/kg fat and the AV below 2 (RUBIN report no. 199, 2011) (see Chapter 5.1 "Methods to detect and measure oxidation" for description and definition of PV and AV). The effect of storage temperature (-18°C/-24°C), storage time (2 – 4 months) and packaging methods (vacuum packing/regular plastic bags and cardboard boxes) on quality of cod liver were evaluated by Thorarinsdottir et al. (2004). The storage time before further processing should be minimised and packaging methods limiting access of oxygen should be used (Rustad et al., 2004).

3.3 Summary of composition and decomposition of raw material

The composition of whole industrial fish used for meal and oil production varies with species and season. The protein content is normally around 20% w.w. while the lipid content varies from as little as around 2% to around 30% mainly in the form as neutral triacylglycerol (TAG). Lean fishes such as cod, store fat as TAG in the liver, while fatty fishes store TAG in the muscle. The level of cholesterol is less than 1% of the lipid level. The content of carbohydrates (i.e. glycogen) in fish is relatively small compared to protein and lipid but may influence on the *post mortem* pH and thus e.g. on the shelf-life of fish fillet.

During storage, prior to oil and meal production, there will be both enzymatic and bacterial breakdown of the raw material. The concentrations of fat-soluble vitamins A, D, E, and K may decrease during storage of raw fish material but are extracted with the oil.

The rate of decomposition (e.g. proteolysis and lipolysis) increases with increasing temperature and duration of storage. The decomposition products from protein in stored fish and fish cut-offs are water soluble free amino acids, trimethyl amine and biogenic amines, such as histamine, limited amounts will be extracted in marine oils. Breakdown products from lipids include free fatty acids (FFA), lysophospholipid (lysoPL, partly hydrolysed PL), partly hydrolysed TAG (mono and diacylglycerols) and glycerol. Decomposition of lipids, lipolysis, is usually slow in fish. The products of lipolysis are more polar than TAG, which is the main component of fish-, seal- and cod-liver oils. Lipolytic products such as LysoPL may be present in extracted marine oil (see Chapter 2.3.2). Lipid oxidation in fish muscle develops during storage but varies with fish species and storage conditions and time. More knowledge is needed to understand the influence of raw fish material freshness on the oxidative quality of extracted oil. Lipid soluble undesirable substances present in marine raw material are extracted in oils, but special procedural steps are taken to reduce oil content of environmental contaminants (see Fig. 2.3).

Microbial occurrence in raw material is not likely to be carried over to oil for human consumption due to considerable heat treatment both of the raw material and the oil during processing. Prior to heat treatment, growth of toxin-producing bacteria which produce heat-stable and lipophilic toxins could be a potential problem when the foodstuffs are stored under improper temperature or time conditions. Three types' toxin-producing bacteria are identified as possible sources, but since the toxins from two of these, namely *C. botulinum* and *S. aureus*, are water soluble, these toxins are not likely to be concentrated in oil to amounts possessing any health hazard. The toxin cereulide produced by *B. cereus* is of hydrophobic nature, and thus able to associated with lipid environments. However, the minimum growth requirements of *B. cereus* are unlikely to be fulfilled in the lipid fraction of raw materials for fish oil production intended for human consumption. Presence of cereulide in refined marine oils has to our knowledge not been reported so far.

Other raw materials such as krill and seal blubber also show variability in content of major components. Lipids in seal blubber are stored as TAG. Krill may contain up to 50% lipid dry matter (mixture of TAG and PL) and the level of cholesterol is reported to be higher than in fish. The exoskeleton of krill contains fluorine which is deliberated during oil processing but mainly follows the protein fraction. Proteolysis and lipolysis in krill is rapid after catch and the same decomposition products as described for fish apply. Due to higher content of phospholipid in krill than in fish, more lysoPL may be formed. Literature on lipolysis in blubber from marine mammals was not found.

4. Oxidation of marine oils

Lipid oxidation is a set of complex chemical reactions that degrade oils. Fatty acids in triacylglycerol, phospholipids or fatty acid ester participate in the oxidation reactions. Therefore, the oxidation processes described in this chapter are the same for triacylglycerol, phospholipid or fatty acid esters. The oxidative stability of oils depend on many factors such as fatty acid composition, amount of prooxidants and antioxidants in the oils and storage conditions such as temperature, light and oxygen availability (Chaiyasit et al., 2007, see Chapter 4.2). As the degree of unsaturation of fatty acids increases, the susceptibility to oxidation also increases (Table 4). Marine lipids contain high amounts of long chained n-3 polyunsaturated fatty acids (LC-PUFAs) and are therefore highly susceptible to oxidation (composition of lipid from different fish species are given in Table 3.1.1-2).

Table 4 Relative oxidation rates of unsaturated fatty acids subjected to photooxidation or autooxidation (Van Dyck, 2007).

Type of oxidation (oxygen)	Relative oxidation rate		
	[1] C18:1 n-9	[2] C18:2 n-6	[3] C18:3 n-3
Autooxidation (triplet)	1	27	77
Photooxidation (singlet)	$3x10^4$	$4x10^4$	$7 x10^4$

[1] Oleic acid: the carbon (C) chain is 18 C long and has one double bond (C18:1) at carbon number nine counted from the acid part of the chain (n-9). [2] Linoleic acid, two double bonds. [3] α-Linolenic acid; three double bonds.

The number of double bond in the carbon chain of the fatty acid determines the degree of unsaturation. The table above shows that an increasing degree of unsaturation causes increasing susceptibility to oxidation.

4.1 Mechanisms and oxidation products

The direct interaction between fatty acids and oxygen (oxidation substrates) cannot occur. Therefore, the reaction between fatty acids and oxygen can only occur when one of the oxidation substrates is converted into the activated form: a fatty acid radical or activated oxygen forms. According to the mechanism, the oxidation of lipids can be classified as follows:

- *Enzymatic oxidation,* which is catalysed by enzymes and is important for oxidation of marine lipids in raw marine material (e.g. harvested fish)
- *Light induced oxidation (photooxidation)*, catalysed by exposure to light in the presence of photo-sensitizers, and thereafter, activated oxygen (singlet oxygen) can directly interact with the fatty acids.
- *Autooxidation*, which is a spontaneous reaction that occurs via activation of fatty acids into fatty acid radicals, which can directly react with atmospheric oxygen.

4.1.1 ENZYMATIC OXIDATION

There are several enzymes present in the raw marine materials that may catalyse enzymatic oxidation:

- Lipases and phospholipases (widely distributed in living organisms) catalyse the splitting of fatty acids from triacylglycerol molecule leading to the formation of free fatty acids that are more prone to oxidation than the intact lipids (Refsgaard et el., 1998).
- Lipoxygenases are iron-containing enzymes present in tissues (Harris and Tall, 1989). These enzymes catalyse the insertion of O_2 to an unsaturated fatty acid (Belitz, 1987) producing lipid peroxides that are primary oxidation products.
- Myeloperoxidase, can initiate lipid oxidation in the presence of hydrogen peroxide and halides such as bromide and iodide (Kanner and Kinsella, 1983) and can be critical during processing of fish, when interaction between air (oxygen), blood and lipid (oxidation substrates) occurs.

4.1.2 LIGHT INDUCED OXIDATION (PHOTOOXIDATION)

The direct oxidation of fatty acids by atmospheric triplet oxygen ($^3O_2$) is not possible as it is a spin forbidden reaction (Choe, 2006). In the presence of photo-sensitizers (e.g. porphyrins, riboflavins) and exposure to light, the atmospheric triplet oxygen can be converted to the singlet state of oxygen ($^1O_2$). Singlet oxygen can directly bind to C-C double bond and thereby initiate *photooxidation* leading to the formation of both conjugated and non-conjugated peroxides.

One oxidizing fatty acid can give a range of different hydroperoxides through oxidation. In studies using the C_{22} docosapentaenoic acid (DPA) ethyl ester oxidation, the formation of eight isomeric hydroperoxides (7-, 10-, 11-, 13-, 14-, 16-, 17-, and 20- LOOH) was observed during autooxidation (oxidation experiment was performed in the dark). Light induced oxidation may in addition lead to the formation of 8- and 19-hydroperoxy DPA, which are characteristic hydroperoxide isomers generated by singlet oxygen-mediated oxidation (Pan et al., 2005). Hydroperoxides formed by singlet oxygen decompose through the same mechanisms as for the peroxides formed by triplet oxygen during autooxidation (see Chapter 4.1.3).

Light induced oxidation of polyunsaturated fatty acids is a **direct** addition of singlet oxygen to an unsaturated fatty acid. Singlet oxygen is formed by excitation of triplet oxygen by photo-sensitizers.

4.1.3 AUTOOXIDATION

Autooxidation is the most important mechanism in the oxidation of n-3 LCPUFAs. It occurs via the free radical chain mechanism in an autocatalytic reaction. It also involves the reaction of unsaturated fatty acids with oxygen and occurs in three phases: *initiation, propagation and termination*. At the initiation step, fatty acid radicals ($L^{\bullet}$) may be formed by thermal cleavage or due to chemical oxidizers (activated oxygen species such as $OH^{\bullet}$, $O_2^{\bullet-}$ etc), or by transition metals (Kolanowski, 2006). Fatty acid radicals ($L^{\bullet}$) can react with atmospheric triplet oxygen, generating peroxy radicals. These act as chemical oxidizers and react with a new unsaturated fatty acid, thereby propagating the chain of autooxidation (Fig. 4.1.3).

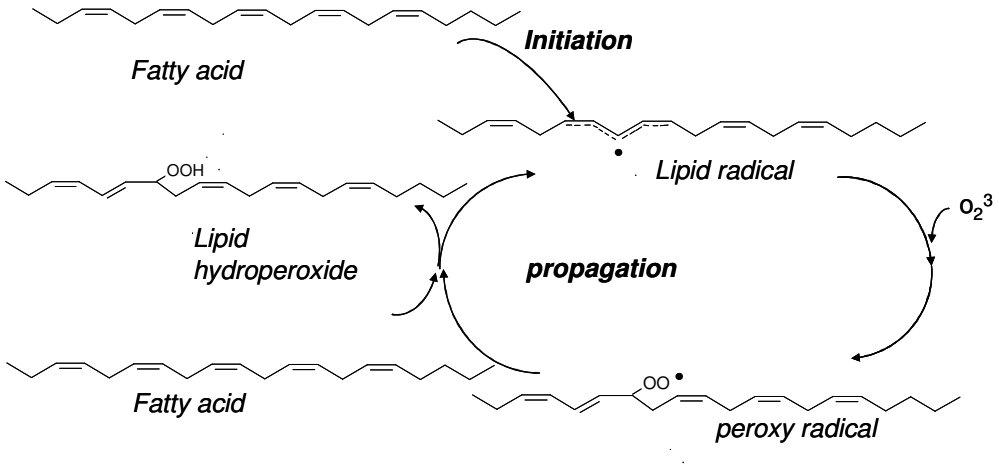


Figure 4.1.3 Simplified scheme of autooxidation of fatty acids, which is a free radical chain reaction, includes initiation, propagation, and termination steps. **Initiation**: Lipid radicals are formed due to chemical oxidizers (activated oxygen species such as OH•, O2•- etc), by thermal cleavage, by transition metals)**. Propagation**: The formed lipid radicals react with atmospheric triplet oxygen and generate peroxy radicals which act as chemical oxidizers and react with another unsaturated fatty acid, thereby propagating the chain of autooxidation. (Adopted from Frankel, 2005).

Autooxidation of polyunsaturated fatty acids is a free radical chain reaction with the following steps: initiation, propagation and termination.

4.1.3.1 Primary oxidation products

The *primary product* of the autooxidation cascade is the fatty acid peroxide (LOOH) and if a polyunsaturated fatty acid is oxidised, each peroxide group is accompanied by a conjugated diene. The chain also produces intermediate and short lived fatty acid radicals such as $L^{\bullet}$ and peroxy radical ($LOO^{\bullet}$). As the number of double bonds in PUFA increases, more complex mixtures of LOOH may be produced. For example, autooxidation of eicosapentaenoic acid (EPA) may produce eight type of **peroxides**; 5-, 8-, 9-, 11-, 12-, 14-, 15-, and 18-LOOH, while in docosahexaenoic acid (DHA), ten possible peroxides can be formed; namely 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17-, and 20- LOOH (Lyberg and Adlercrautz, 2006). These hydroperoxides have either *cis*-, *trans*- or *trans-trans* configuration, and a complex system of 16 and 20 monohydroperoxide isomers is therefore expected from the autooxidation of EPA and DHA, respectively. In studies of DHA peroxidation, it was observed that double bonds located nearest to C-4 and farthest to C-20 from the carboxyl group were the most prone to hydroperoxide formation (Lyberg and Adlercrautz, 2006). The amount of lipid hydroperoxides increases in the beginning of an oxidation cascade, but decreases later as rate of breakdown of hydroperoxides to secondary oxidation products become higher than the rate of formation (Gardner, 1983) (Fig. 4.1.3.1). The oxidation temperature may generally affect the highest amount of monohydroxyperoxides reached (before the amount of lipid peroxides starts to decrease). Lyberg and Adlercrautz (2006) observed that monohydroperoxy-DHA reached 7% and 4% mol concentration at 25°C and 40°C, respectively. In addition, it was observed that during the decrease in the total amount of DHA monohydroperoxides, the C4-monohydroperoxide decreased faster than the other isomers, indicating that it was the most sensitive to further oxidation.

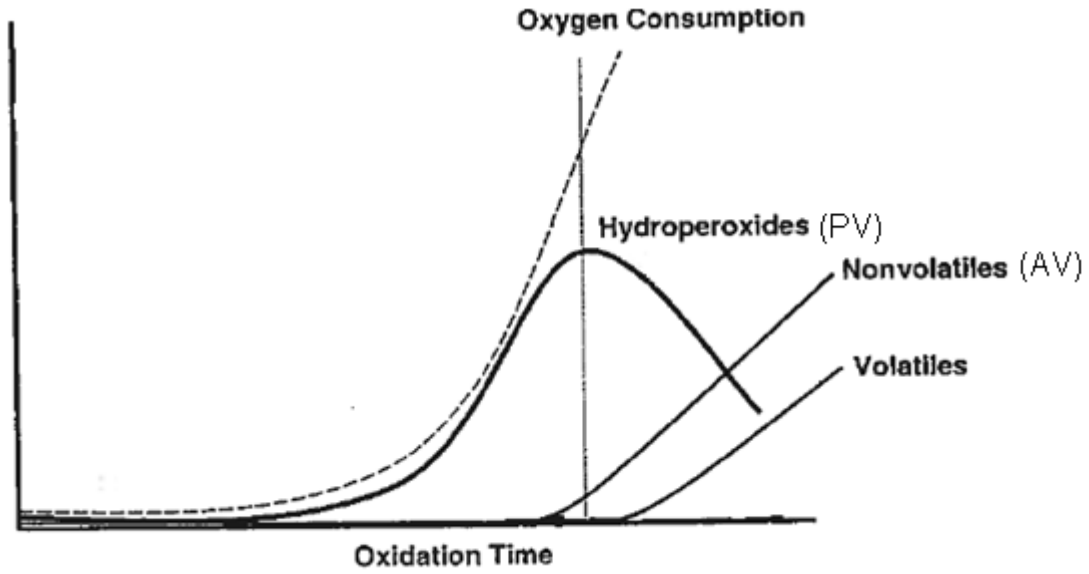


Figure 4.1.3.1 The kinetic curve of autooxidation of polyunsaturated fatty acids as modified from Kamal-Eldin et al., (2003). PV: peroxide value, AV: anisidine value. Initially in the autooxidation cascade, primary oxidation products (hydroperoxides) are generated but progressively broken down to secondary oxidation products (non-volatile- and volatile compounds). As illustrated in the figure, with increasing duration of oxidation the initially generated amount of hydroperoxides (measured as peroxide value) is decreasing while the amounts of non-volatile and volatile oxidation compounds increase as a marker for secondary oxidation products.

Primary oxidation products are taste- and odourless **lipid peroxides** (LOOH; lipid hydroperoxides). In the presence of metals such as heme and non-heme iron in fish tissue, or at high temperature, these lipid hydroperoxides breakdown to an array of non-volatile and volatile *secondary oxidation products*.

4.1.3.2 Secondary oxidation products

Peroxides are tasteless and odourless. However, in the presence of metals and at high temperature, fatty acid hydroperoxides break down to an array of non-volatile and volatile secondary oxidation products. In fish tissue, heme and non-heme iron may be present at concentrations of 6-15 µg/g and 10 µg/g, respectively (Jacobsen et al., 2008). As a result, new radicals, $^{\bullet}$OH, alkoxyl radical (LO$^{\bullet}$) and LOO$^{\bullet}$, capable of re-initiating the autooxidation chain reaction (chain branching reactions) may be formed. Alkoxyl radical (LO$^{\bullet}$) can also be cleaved in a β-scission reaction to various non-volatile and volatile secondary oxidation products.

Peroxyl radicals (LOO$^{\bullet}$) of fatty acids or esters containing three or more methylene-interrupted double bonds (such as EPA and DHA) can be oxidised by cyclization to hydroperoxy **epidioxides** and **bicycloendoperoxides**, which may be broken down to **malondialdehyde** (Fig. 4.1.3.2-1). As reported by Falch and coworkers (2004), nuclear magnetic resonance studies showed that a cyclization peak appears during the oxidation of marine oils, and that the intensity was reduced by the presence of tocopherol.

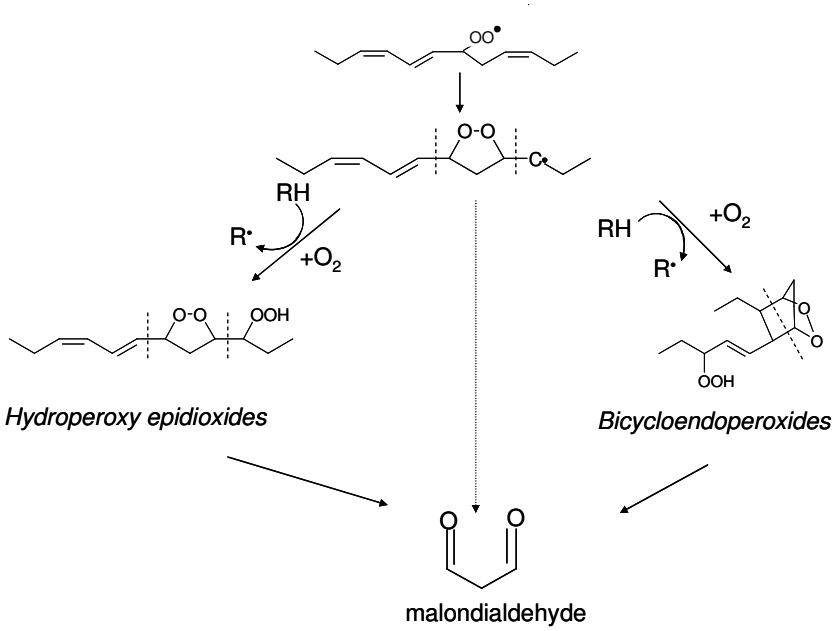


Figure 4.1.3.2-1 Formation of malondialdehyde from lipid peroxides (Frankel, 2005). Peroxyl radicals (LOO•) of eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA) can be oxidised by cyclisation to hydroperoxy epidioxides and bicycloendoperoxides, which may be broken down to malondialdehyde as illustrated in the figure.

Due to the variety of lipid hydroperoxides that can be formed during autooxidation of EPA and DHA, complex mixture of secondary oxidation products are produced after breakdown of these hydroperoxides. Analysis of fish oils by dynamic headspace gas chromatography resulted in over 150 volatile compounds (Frankel, 2005). The compounds detected were hydrocarbons, alcohols, acids, ethers, ketones, furanones, aldehydes and other compounds. **Aldehydes** are observed to be the most abundant group of volatile compounds in the headspace of fish oil (Guillen et al., 2009; Frankel, 2005).

The structure of some of the cleavage products is known (Fig. 4.1.3.2-2), but the exact mechanism for their formation is yet to be completely understood.

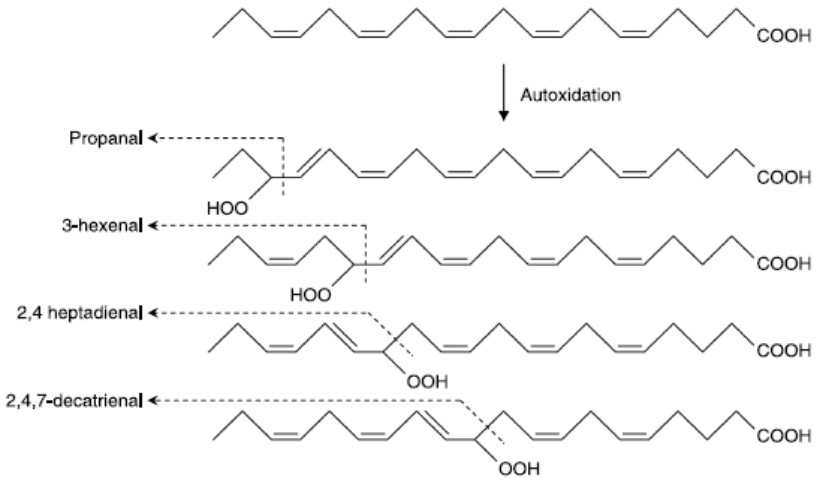


Figure 4.1.3.2-2 Autooxidation sites on eicosapentaenoic acid (EPA) associated with the formation of major secondary oxidation aldehyde products (Kulås et al., 2003). Autooxidation of fatty acids is a free radical chain reaction and includes initiation, propagation, and termination steps. The primary products of the cascade, lipid hydroperoxides (LOOH), are broken down to secondary products among which aldehydes are the most numerous group.

Volatile decomposition products from long chain n-3 polyunsaturated fatty acids in fish and fish oil have extremely low flavour threshold values at concentration of gram per kg (or parts per billion, ppb) with readily detectable odours at very low oxidation levels. 1,5-octadien-3-one is detected at concentration as low as 0.001µg/kg oil, making oxidation a large sensory problem in seafood (Jacobsen et al., 2009). Volatile compounds in fish oil have been characterized in several studies (Aidos, 2002; Jacobsen and Nielsen, 2007; Olsen et al., 2005), but the data vary between studies due to sample differences (e.g. type of added antioxidants, type of fish oil), oxidation levels and analytical methods. Important flavour compounds formed from the autooxidation of n-3 fatty acids are 1-penten– 3– one, 4-heptenal, 2,4-heptadienal 2,4,7-decatrienal and others. Formation of these compounds leads to the fishy flavour of oxidised fish oil (Aidos, 2002; Jacobsen and Nielsen, 2007; Kulås et al., 2003). Decatrienals are usually detected only in highly oxidised products.

4-hydroxy-(E)-2-hexenal (HHE) and 4-oxo-(E)-2-hexenal are *α,β*-unsaturated and oxygenated aldehydes (Fig. 4.1.3.2-3) and products of n-3 PUFAs oxidation. However, we have found only one study that analysed the presence of these compounds in fish oil. Guillen et al. (2009) observed the formation of ***α,β*-unsaturated aldehydes** (e.g. 4-oxo-(E)-2-hexenal, 4-hydroxy-(E)-2-hexenal) in cod-liver oil samples. *α,β*-unsaturated aldehydes having six and seven carbon atoms are a result of n-3 fatty acid (i.e. EPA and DHA in fish oil) oxidation, while the oxidation of vegetable oils, rich in n-6 fatty acids have predominantly *α,β*-unsaturated aldehydes with nine and ten carbon atoms.

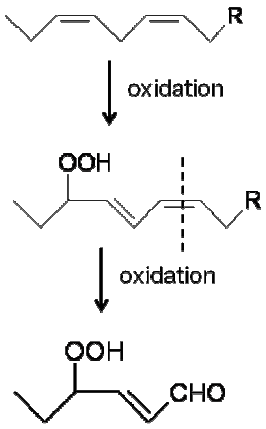


Figure 4.1.3.2-3 Formation of 4-hydroxy-(E)-2-hexenal (4-HHE) – a secondary oxidation product in the autooxidation cascade of long chained polyunsaturated fatty acids (Kasai and Kawai, 2008).

During homolytic β-cleavage of fatty acid peroxides, aldehydes are left on either triacylglycerol, phospholipid or cholesteryl ester molecules and these aldehydes are called **core aldehydes** (Fig. 4.1.3.2-4). Core aldehydes are non-volatile compounds that are included in the measured anisidin value of marine oils (see Chapter 5.1.2) and that have no impact on the sensory quality of the oils. This might be the reason why no studies investigating core aldehydes in details in fish oil have been found, but one in shark oil published by Hartvigsen et al. (2006).

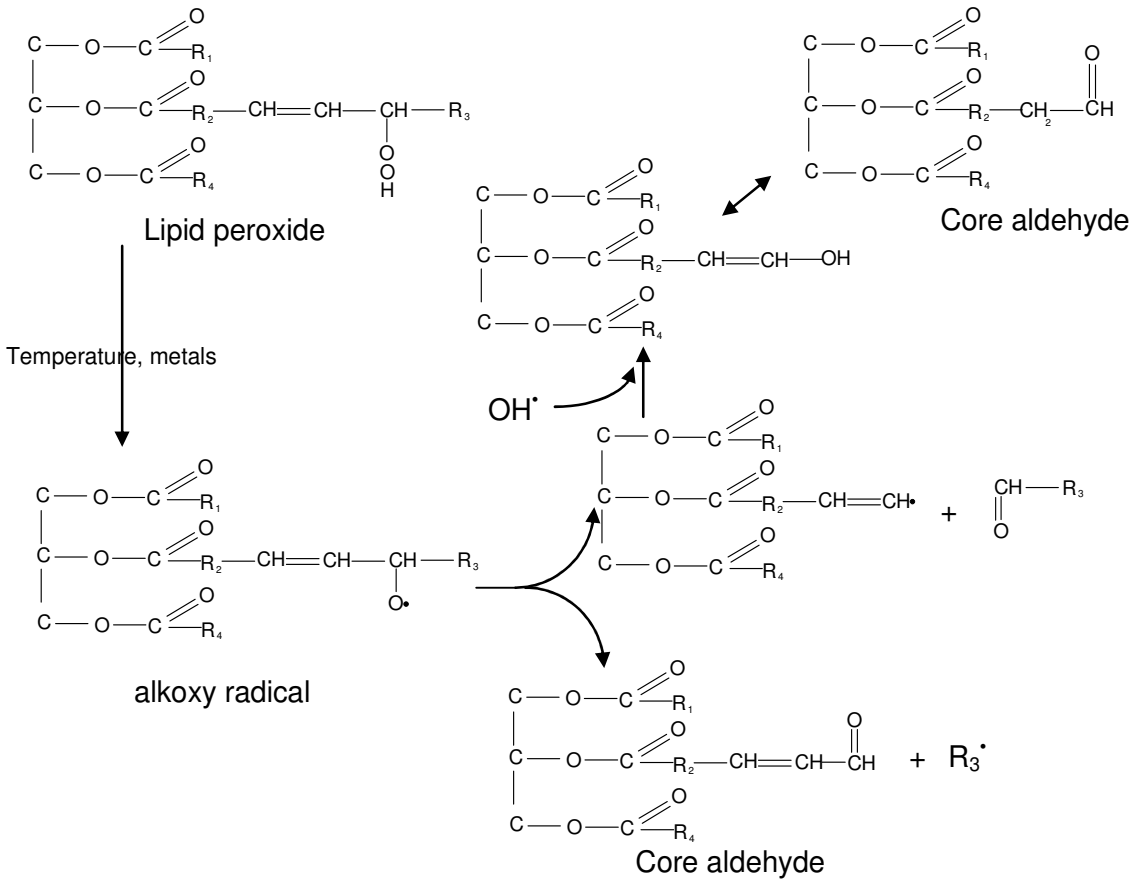


Figure 4.1.3.2-4 Formation of core aldehydes via β-cleavage of lipid peroxide – a secondary oxidation product in the autooxidation cascade of long chained polyunsaturated fatty acids (Kuksis et al., 2003). During homolytic β-cleavage of the peroxides, aldehydes are left on the triacylglycerol or phospholipid molecule and these aldehydes are called **core aldehydes** as illustrated in the figure.

Core aldehyde containing triacylglycerols have been isolated from autooxidised vegetable oils (Byrdwell and Neff, 2002; Sjövall et al., 2003) and from shark oil (Hartvigsen et al., 2006). The oxidation of 22:6 n-3 may yield a wide spectrum of short chain core aldehydes $C_{4:0}$, $C_{7:1}$, $C_{8:2}$ and $C_{10:2}$ (Hartvigsen et al., 2006).

Prostaglandin-like compounds, called isoprostanes are generated by peroxidation of arachidonic-, eicosapentaenoic- and docosahexaenoic acids (Leonardis and Macciola, 2006). Formation and effect of endogenous isoprostanes have been studied *in vivo* (see Chapter 7.2.1.6), but to the best of our knowledge, formation of isoprostanes are not studied in food items. Leonardis and Macciola (2006) and Leonardis et al. (2008) have suggested that EPA- and DHA-oxidative derivatives, "isoprostane-like" compounds, may also be formed as a result of oxidation in fish oil.

Secondary oxidation products may be volatile **or** non-volatile.

Volatile compounds (short chain cleavage products) are responsible for the fishy flavour of oxidised fish oil and aldehydes are the most abundant group.

Non-volatile compounds are taste- and odourless and therefore have no impact on the sensory quality of oxidised oils.

"Core aldehydes" (i.e. non-volatile compound) are aldehydes left on the triacylglycerol or phospholipid molecule.

4.1.3.3 Tertiary oxidation products

In the final **termination stage**, two radical compounds fuse together to form relatively stable high molecular weight compound. When ethyl esters of EPA and DHA were oxidised at 5°C in the dark, 50-70% of the consumed oxygen was used in the formation of lipid hydroperoxides, while the remaining 30-50% of consumed oxygen was used to form dimers and polymers (Cho et al., 1987). High molecular weight products may be formed as a result of thermal treatment during processing of the oil such as deodorisation (se Chapter 2.3.5), or as a result of autooxidation of oil prior to and/or after encapsulation of oil. Shukla and Perkins (1991) observed that locally purchased encapsulated fish oil samples (6 samples) contained high molecular weight material (1-10% dimeric compounds) composed of triacylglycerols bonded via peroxy bridges. Burkow and Henderson et al. (1991) detected polymeric compounds (approx. 2%) in fish oil samples, and the amount of polymeric compounds increased with increasing level of oxidation. Cho et al. (1987) observed that the major tertiary oxidation products in ethyl linolenate were hydroxyl cyclic peroxides, while those in ethyl EPA and DHA were polymers.

Tertiary oxidation products are not very reactive compounds that are formed during the final termination stage of autooxidation of polyunsaturated fatty acids. Tertiary products include high molecular weight products such as dimeric and trimeric triacylglycerols (i.e. polymeric compound).

4.1.3.4 Summary of autooxidation

Lipid oxidation includes initiation, propagation, and termination steps and is a dynamic process creating a complex mixture of primary, secondary and tertiary products with different characteristics. Since several substances are intermediate; they are formed and then broken down again during marine oil processing, and there is not always a linear increase in the oxidation-products with time of oxidation. For example, the concentrations of taste- and odourless lipid hydroperoxides (primary oxidation products) increase slowly during the initiation phase but exponentially during the propagation phase. When degradation of the lipid peroxides is more rapid than formation, the amount of these products will decline. High variety of volatile and non-volatile secondary oxidation products may be formed during the oxidation of marine oil. Volatile products are responsible for the fishy flavour while non-volatile compounds (e.g. core aldehydes) have no impact on the sensory quality of oxidised oils. Among carbonylic derivatives, aldehydes are the most predominant and studied group of oxidation products. Secondary oxidation products can be lost by reaction with other molecules and aldehydes are especially very reactive. Schematically, an increase in secondary products follows after the increase in lipid hydroperoxides (Fig. 4.1.3.1) and an increase in the concentration of tertiary products follows thereafter. However, in practice, these processes often run in parallel to each other.

Furthermore, the ultimate oxidation products formed depend on the oxidative conditions: the fatty acid composition of the oxidising oil, temperature, light, presence of metals in fish tissue (enzymes and other pro- and antioxidants), oxygen concentration, etc. Based on current knowledge, it is almost impossible to foresee which oxidation products that may be formed under the various conditions.

4.1.4 OXIDISED CHOLESTEROL (OXYSTEROL)

Cholesterol autooxidation proceeds by radical processes similar to autooxidation of polyunsaturated fatty acids. The amount of cholesterol oxidation products in marine oils has been reported to correlate with the content of polyunsaturated fatty acid in the oils (Li et al., 1996). As previously mentioned, PUFAs are very susceptible to autooxidation and the primary oxidation products formed (lipid peroxides) might also accelerate the oxidation of cholesterol.

Cholesterol oxidation is initiated when a hydrogen atom is subtracted from the molecule forming cholesterol radical, which binds oxygen during formation of monohydroperoxide such as 7α- and 7β-hydroperoxycholesterol (Fig. 4.1.4). Light induced oxidation of cholesterol by singlet oxygen leads to the formation of 3β-hydroxy-5α-cholest-6-ene 5 hydroperoxide and small amounts of 3β-hydroxy-4-ene 6 hydroperoxide, while no 7α- or 7β-hydroperoxycholesterol are formed.

Cholesterol hydroperoxides may further be decomposed into complex mixtures of hydroxy, keto, epoxyene and ketodiene secondary oxidation products.

In raw fish samples, oxidised cholesterol is usually not detected (Osada et al., 1993) (see also Chapter 3.1.1). Generally, the amount of cholesterol in fish oil is very low. To the best of our knowledge, only two studies have analysed oxidised cholesterol levels in fish oils. In herring fish oil and menhaden fish oils, respective 0.8% and 0.5% cholesterol was observed (Pickova and Dutta, 2003). The amount of cholesterol oxidation products in the two commercial herring fish oil samples and in the menhaden oil is given in Table 4.1.4.

The concentrations of oxidised cholesterol products increase during storage of fish oil. In the fish oil, the most prevalent oxides formed were shown to be 7-ketocholesterol (Li et al, 1996).

Oxidised cholesterol: The content of cholesterol in fish oils is generally low. Autooxidation of cholesterol follows the same pattern as described for autooxidation of polyunsaturated fatty acids.

Table 4.1.4 Content of cholesterol oxidation products in fish oil samples (Pickova and Dutta, 2003)

Cholesterol oxidation products, µg/g oil									
Sam-ple	**7α-OH**	**7β-OH**	**7-Keto**	**5α, 6α-epoxy**	**5β, 6β-epoxy**	**Triol**	**20-OH**	**25-OH**	**Total**
1	0.32±0.03	0.58±0.01	1.62±0.21	0.44±0.03	1.55±0.02	0.12±0.01	0.73±0.06	0.59±0.03	5.95±0.33
2	0.53±0.03	0.98±0.07	1.37±0.06	0.64±0.01	2.77±0.08	Trace	1.29±0.12	0.61±0.01	8.27±1.12
3	0.95±0.25	0.92±0.24	0.49±0.17	0.27±0.07	0.47±0.21	1.81±0.85	0.87±0.85	2.58±1.15	8.36±3.20
4	na	na	6.0	1.2	4.3	na	na	na	na

Samples 1 and 2 - Norwegian experimental herring fish oil nos 1 and 2, respectively, submitted to the author by the Norwegian Herring Oil and Meal Industry research institute, Bergen, Norway. Sample 3 – menhaden oil. Sample 4 – fish oil of edible quality from a local market (Li et al., 1996). na - not analysed

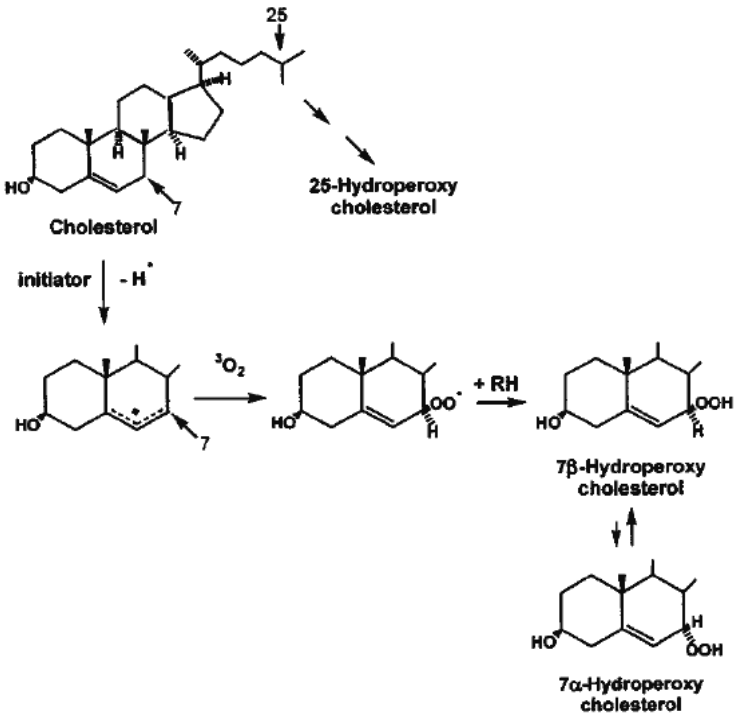


Figure 4.1.4 Products of cholesterol autooxidation (Frankel, 2005). Cholesterol oxidation is initiated by hydrogen atom rearrangement which produces a delocalized three carbon allylic radical. Both epimeric 7α- and 7β-hydroperoxycholesteron are formed following oxygen attack (at carbon no. 7). Following interconversion the more thermodynamic and stable 7β-hydroperoxide predominates.

4.1.5 OXIDATION OF PHOSPHOLIPIDS

Oxidation of phospholipids follows the same pattern as oxidation of triacylglycerols and fatty acids/esters (described above) including increase in oxidation with increasing degree of unsaturated fatty acids. The primary oxidation products are *phospholipid hydroperoxides* that may break down to a variety of secondary oxidation products.

Marine phospholipids contain a high percentage of LCPUFAs and, therefore, are highly susceptible to oxidation. In addition, phospholipids are charged molecules and may form complexes with prooxidative metals (such as iron), which in turn promote oxidation (Nwosu et al., 1997, see Chapter 4.2.4). Thus, removal of phospholipids and trace metals during processing of fish oils (see Chapter 2.2) has been suggested to improve oxidative stability of the oil (Bimbo, 2007). However, several studies obtained greater oxidative stability of polyunsaturated fatty acids in the form of phospholipids than in the form triacylglycerols (King et al., 1992; Lyberg et al., 2005; Moriya et al., 2007; Song et al., 1997).

Proposed theories explaining the higher stability of fish oils containing phospholipids include anti-oxidative synergistic effects between tocopherols and phospholipids and that the association of phospholipids in bilayers may work as isolation toward lipid radicals and limits interaction with the oil phase. Due to conflicting results about the oxidative stability of phospholipids, the oxidative behaviour of phospholipids in phospholipid-containing marine oils is complicated and not completely understood (Schaich, 2006). More research is needed to fully understand the oxidation processes of marine phospholipids.

4.2 Factors influencing the oxidation status of marine oils

Generally the susceptibility of oils to oxidative deterioration depends on a number of factors such as fatty acid composition of the marine oil, presence of impurities that in many cases may act as pro-oxidants, antioxidant protection, access to light and oxygen and processing and storage temperature. This should be considered when processing, packaging, storing and transporting the oil.

4.2.1 OIL PROCESSING

Crude fish oil is refined prior to human consumption. The refining process reduces the content of undesirable constituents and impurities such as environmental pollutants, traces of heavy metal and oxidation products such as peroxides and aldehydes. The concentration of naturally occurring antioxidants such as tocopherol in marine oils is also reduced during the refining processes (Oterhals and Berntssen, 2010; Shahidi and Kim, 2002). This may render the refined oil more susceptible to oxidation and addition of antioxidants to refined oils is required for stabilization (Shahidi and Kim, 2002). See Chapter 2.3 for additional information.

4.2.2 PHYSICAL ENVIRONMENT (OXYGEN, LIGHT, TEMPERATURE, TIME)

Oxidation of oils is a dynamic process that develops during storage time. The rate of oxidation depends on many factors such as physical environment, packaging and composition of the oils. Therefore, it is difficult to predict the oxidative stability of the oils. Oxidative stability test could be performed in order to predict the stability of the oils and the durability of the consumer product (described in Chapter 5.5).

Oxidation rate of oils and decomposition of lipid peroxides increase with increasing temperature. The oxidation rate doubles for each 10^{o}C increase (Jacobsen et al., 2009). Therefore, a 10^{o}C increase in the storage temperature of an oil product will lead to a 50% decrease in its shelf-life. The use of increased temperature (180-200^{o}C), usually under vacuum conditions, is generally necessary during processing and purification of marine oils. Even if the thermal exposure is under vacuum conditions, the susceptibility of the lipids to oxidation will increase depending on the temperature, exposure time, level of prooxidants etc.

Singlet oxygen (i.e. light induced) oxidative changes are directly related to the light-source, wavelength, intensity and exposure time. UV light was observed to be more harmful than visible light. Packaging of oil using coloured (dark) glass or plastic with UV absorbers reduces the light induced oxidation.

Oxygen is one of the lipid oxidation substrates. Therefore, the concentration of peroxides in oil is dependent on the amount of available oxygen, increasing the oxidation of oil with increased amount of dissolved oxygen. The concentration of dissolved oxygen in oil is dependent on the oxygen partial pressure in the headspace of the oil. During processing and storage, oxygen can be avoided by diminishing the empty space in pipelines, reactors and storage containers and/or flushing these spaces with an inert gas, such as nitrogen (CAC/RCP, 1987).

4.2.3 ENCAPSULATION AND PACKAGING

Encapsulation of marine oils in soft gelatine capsules or microencapsulation of oil are methods that help to control oxidative stability of the oil by protecting it against the physical environment of the oil, including oxygen, light and humidity. However, the physical properties of the encapsulation material used, as well as the operating conditions of the encapsulation process have a major influence on the oxidative stability of the oil.

Gelatine film coating acts as a barrier against oxygen and light (Bao et al., 2009). However, significant increases in peroxide values in commercially available fish oil capsules during the accelerated storage test (e.g. storage at elevated temperature) suggest that the oxidative stability of the products might be limited despite the thick gelatine capsule cover (Kolanowski, 2010). Glycerol is a central additive for the production of soft gelatine capsule. The inclusion of glycerol to hot dried film transforms gelatine from a comparatively brittle and stiff material to a highly extensible rubber. The oxygen permeability of gelatine films is shown to vary depending on the glycerol amounts added. For example, the oxygen permeability decreases by the addition of 20% glycerol but increases if more glycerol is added (Kim et al. 2007). The metal ion content of the encapsulation material is an additional factor that may influence the oxidative stability of the capsule content (Shahidi and Kim, 2002).

Microencapsulation is a process where small amounts of fluids (in this case marine oils) are packed into a matrix designed to protect the core from deterioration. The microencapsulation of active components in powders has become a rapid expanding technology. Microencapsulated fats usually consist of emulsion, stabilised by modified starch or hydrocolloids and/or proteins, which either are spray- or freeze-dried, to produce a powder. Processing conditions, as well as the choice of the emulsifiers and antioxidants influence the oxidative stability of the microencapsulated oil. The individual processing steps are shown to stress the oil, resulting in increased peroxide value and conjugated diene levels (Jacobsen and Nielsen, 2007). Similar to marine oils in soft gelatine capsules, the oxidative stability of microencapsulated oil is dependent on oxygen permeability through the protective wall matrix, and through the food matrix where microencapsulated oil is incorporated.

Packaging used for storage of marine oils is also important for oxidative stability of the oils. Packaging material should not be permeable to oxygen or UV light. In studies on oxidative stability of encapsulated fish oil, Pacheco and Regitano-D'Arce (2009) observed that the highest amount of peroxides was produced in fish oil capsules stored in polyvinylchloride (PVC-250µ) film blister. The best oxidative stability results were observed when encapsulated oil was stored in high-density polyethylene flasks with silica bags. Akhtar and coworkers (2010) showed that white, red and yellow edible colour pigments added to hydroxypropyl methylcellulose (HPMC) packaging of salmon oil gave good control of photooxidation. However, the addition of blue or green colour pigments did not provide better protection against photooxidation than transparent HPMC films.

4.2.4 PROOXIDANTS

Prooxidants are compounds that initiate, facilitate or accelerate lipid oxidation. Transition metals, such as iron and copper ions, are common catalysts of lipid oxidation. Crude fish oil usually contains metal ions (a copper concentration in µg/kg level and iron concentration in the mg/kg level) and trace levels will remain even after refining and deodorisation (Jacobsen et al., 2009). Iron and copper are naturally occurring elements (e.g. included as part of protein structure) of the raw material for marine oils, but iron can also come from production equipments.

The mechanism of iron-catalysed lipid peroxidation has been proposed to depend on the presence or absence of preformed lipid hydroperoxide (LOOH) and is divided into LOOH-independent- or LOOH-dependent mechanisms. The mechanism of LOOH–independent initiation is still not clear. Transition metals are capable of directly breaking down unsaturated lipids (LH) to alkyl radicals ($L^{\cdot}$), but this reaction occurs slowly and is therefore considered less important in promoting lipid oxidation (Reische et al., 1998). Several studies (Fukuzawa et al., 1988, Fukuzawa et al., 1993, Mozuraityte et al., 2008; Tadolini et al., 1997) have shown that when peroxides were removed from the lipids, lipid peroxidation was not induced by

iron. However, peroxide dependent oxidation reactions may become dominant even with low amounts of peroxides. As almost all lipids contain traces of peroxides, decomposition of peroxides by iron can be the most important cause of oxidation in many food items (Decker and McClement, 2001).

Iron catalyses oxidation via decomposition of lipid peroxides to free radicals by redox cycling pathways (Fig. 4.2.4). Free lipid radicals would therefore lead to formation of new peroxides in the presence of oxygen. In the absence of oxygen, metal ions may still produce the breakdown of pre-existing peroxides (reduction of PV value) and formation of secondary oxidation products (β-scission) such as aldehydes.

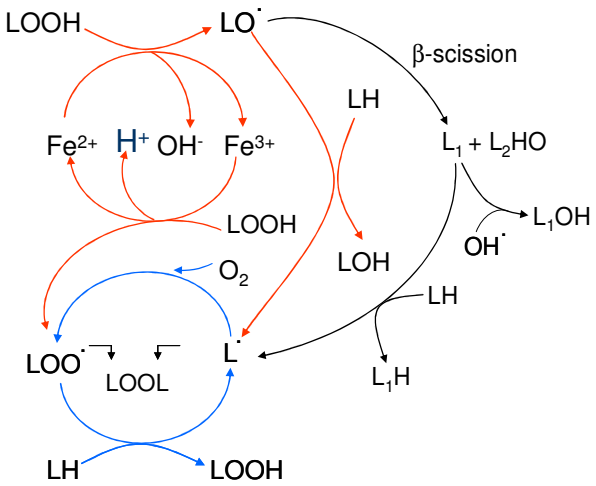


Figure 4.2.4 Iron catalysed lipid oxidation (modified from Mozuraityte et al., 2008). Iron catalyses oxidation via decomposition of lipid peroxides (LOOH) to alkoxyl radicals (LO) or peroxy radicals (LOO) through redox cycling pathways. Alkoxyl- and peroxy radicals lead to further propagated lipid peroxidation by chain branching with formation of several peroxides accompanied by oxygen consumption.

Usually trace levels of iron remain after refining and deodorisation of the oils. Since metal ions are very important for oxidation reactions, it is important to minimise the contact between these ions and the oil. This can be done by using chelating antioxidants (see chapter 2). It has been suggested that heme Fe is even a stronger prooxidant than low molecular iron (Carvajal et al., 2009). Several different mechanisms of the prooxidative activity of haemoglobin (Hb) have been proposed. However, Hb-catalyzed oxidation is more important for lipid oxidation in raw material of marine oils, than for marine oil itself.

4.2.5 ANTIOXIDANTS

4.2.5.1 Natural antioxidants

Antioxidants are compounds that prevent lipid peroxidation by preventing free radical formation. EDTA and citric acid are examples of antioxidants that form complexes with iron and copper ions and thereby prevent the formation of reactive oxygen species and metal catalyzed lipid oxidation. Carotenoids absorb energy from singlet oxygen, transferring it to triplet oxygen, without changing their chemical structure (quenching) and therefore protect oil against light induced oxidation (Frankel, 1998).

Hydrogen donors (such as vitamin E) are the most abundant group of antioxidants. Vitamin E is the most important natural antioxidant in the lipid phase, donating a hydrogen atom to the lipid peroxyl radical and preventing it from reacting with a new fatty acid, and blocking the chain reaction of lipid peroxidation. Vitamin E radical is formed as a result of this reaction

(Fig. 4.2.5.1-1). Vitamin E can be regenerated from the radical by recruiting a hydrogen atom from vitamin C or other antioxidants with similar red-ox properties as vitamin C (Fig. 4.2.5.1-2). In fish oil for human consumption, mixed tocopherols, which is a blend of vitamin E compounds (α-, β-, γ- and δ-tocopherol), are added to refined oils for protection against lipid oxidation. Vitamin C can also be added to the oil as the lipid soluble ascorbyl palmitate (Frankel, 1998).

Plant extracts are another form of natural antioxidants that contain compounds with multiple OH groups, indicating that they may act as hydrogen donors. Examples of these are rosemary extract where the active compounds are carnosol, carnosolic acid and rosmarinic acid, flavonoids found in multiple plant foods and catechins found in green tea.

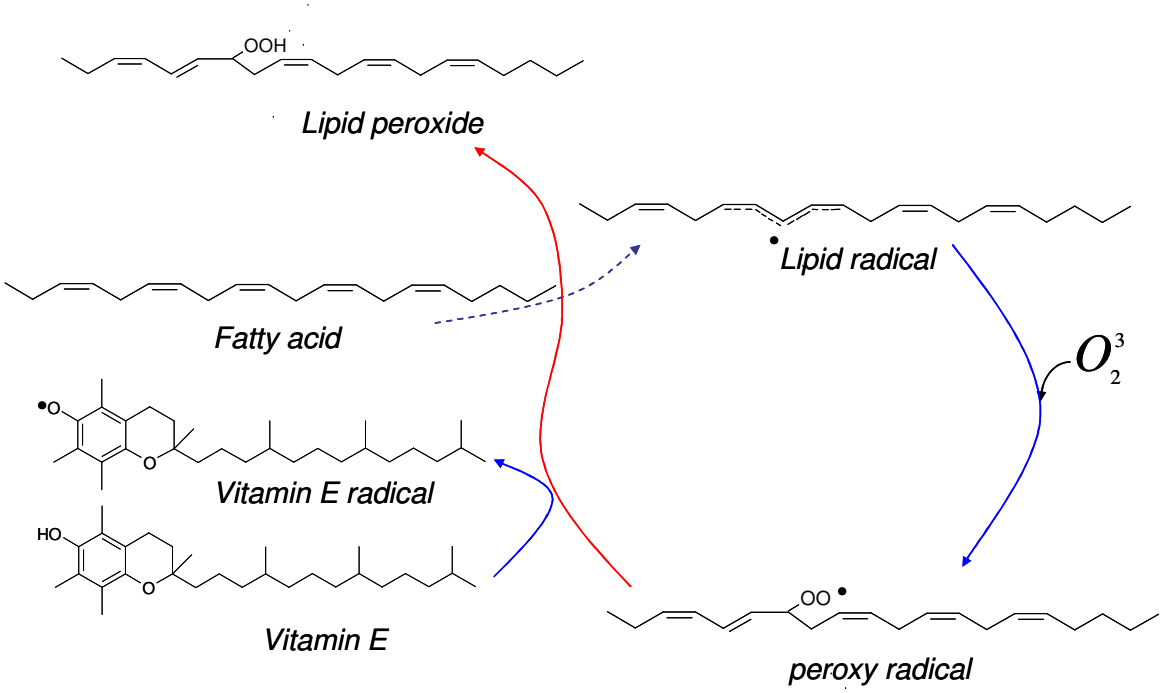


Figure 4.2.5.1-1 The mechanism of antioxidant effects of vitamin E (Hamre, 2011). The figure illustrates how vitamin E blocks the chain reaction of lipid peroxidation by donating a hydrogen atom to the lipid peroxyl radical thereby preventing it from reacting with a new fatty acid. As a result of this reaction a vitamin E-radical is formed.

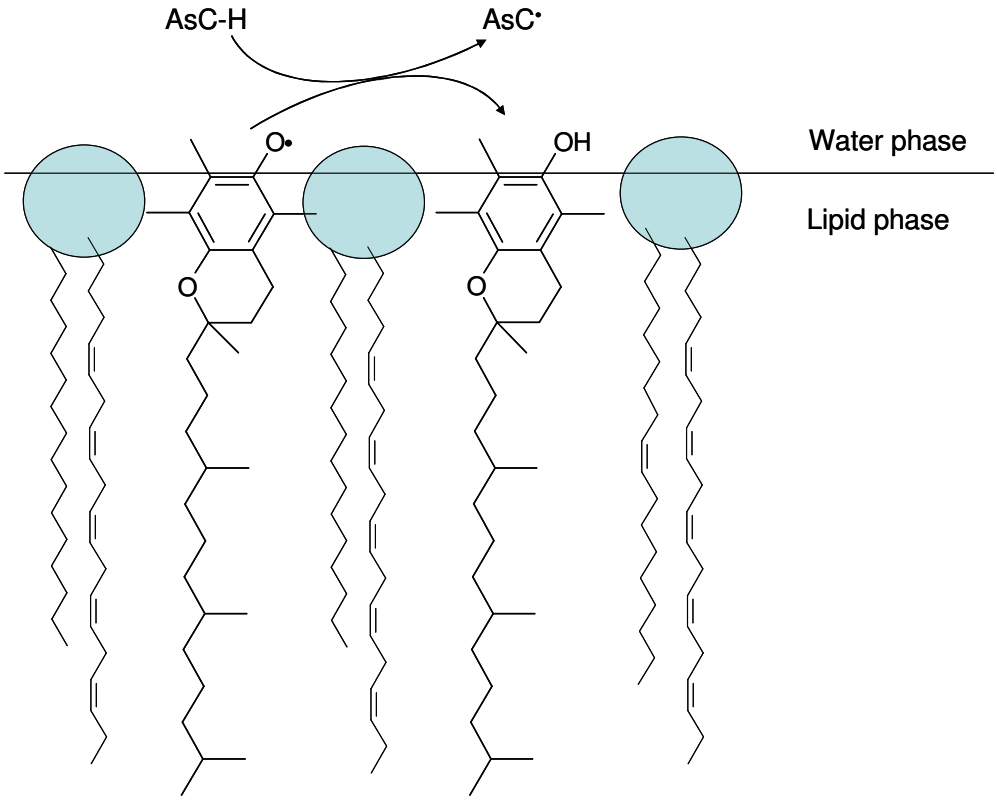


Figure 4.2.5.1-2. Vitamin E can be regenerated by vitamin C (Ascorbic acid, AsC) (Hamre, 2011). Vitamin E acts as an antioxidant by donating a hydrogen atom to the lipid peroxyl radical thereby preventing it from reacting with a new fatty acid. The figure illustrates how the resulting vitamin E-radical may be regenerated to vitamin E by a hydrogen atom donated from vitamin C.

4.2.5.2 Synthetic antioxidants

Fish oil for human consumption may also be stabilized with synthetic antioxidants that donate hydrogen atoms. The most commonly used is butyl hydroxytoluene (BHT) (Måge et al., 2008), but butyl hydroxyanisole (BHA) and the gallates (propyl-, octyl- and dodecyl-gallate) are also in use. The Norwegian Food Safety Authority approves these antioxidants as food additives with an upper addition limit in the oil. In addition, an Acceptable Daily Intake (ADI; www.efsa.europa.eu) is established. BHA-residues are rarely detected (Måge et al., 2008) and to our knowledge, gallates have not been used in fish oil. The synthetic antioxidants ethoxyquin (EQ) and tert-butylhydroquinone (TBHQ) are prohibited in food for human consumption in Europe.

4.2.5.3 Addition of antioxidants to oils – influence on the oils oxidative status

Antioxidants assist in maintaining low oxidative status of oils when added after an effective refining process, after most oxidation products and prooxidants have been removed from the oil. If the oxidation process in the refined oil has already started (i.e. oil is exposed to oxygen in presence of catalysts), addition of an antioxidant can only slow down, or even have no effect on the oxidation process.

4.3 Summary of lipid oxidation

Marine oils are highly susceptible to oxidation due to high content of polyunsaturated fatty acids. Lipid oxidation can be catalyzed by enzymes like lipoxygenases and myeloperoxidase, but this is only relevant for oxidation in the raw material. Generally, lipid oxidation starts when fatty acids interact with atmospheric oxygen. Interaction between fatty acids and oxygen occurs when one of the reaction substrates is in activated form: oxygen – singlet oxygen (light induced oxidation) or fatty acids are in radical form (autooxidation), which is the most prominent oxidative mechanism in marine oils. **Lipid peroxides** are primary oxidation products in activated oxidation. Due to high content of long chain polyunsaturated fatty acids, with several unsaturated double bonds, marine oils may contain a great variety of lipid peroxides as a result of lipid oxidation.

The different lipid peroxides decompose to a complex mixture of secondary oxidation products, where **aldehydes** are shown to be the most abundant group. During homolytic-cleavage of the peroxides, aldehydes are left on the triacylglycerol or phospholipid molecule and are called **core aldehydes**. However, few studies have determined core aldehydes in fish oil. This is probably due to the fact that they are non-volatile compounds and have no impact on the sensory quality of the oils.

Several shorter chain **volatile** compounds are formed as a result of decomposition of lipid peroxide. Volatile decomposition products from long chain n-3 polyunsaturated fatty acids have extremely low flavour threshold values and are readily detectable by sensory analysis at low oxidation levels. Important flavour compounds formed from the autooxidation of n-3 fatty acids are 1-penten–3–one, 4-heptenal, 2,4-heptadienal 2,4,7-decatrienal, among others. **Malondialdehyde** is another well-known secondary oxidation product that can be formed in fish oils. Additionally, *α,β*-**unsaturated aldehydes** such as 4-hydroxy-(E)-2-hexenal and 4-oxo-(E)-2-hexenal may be formed as a result of oxidation of long chain polyunsaturated fatty acids. However, only one study that analysed the presence of these compounds in fish oil was found.

Prostaglandin-like compounds, called isoprostanes may be generated by peroxidation of eicosapentaenoic- and docosahexaenoic acids in fish oils, but is, to the best of our knowledge, not detected or studied in marine oils.

High molecular weight polymeric oxidation products (i.e. tertiary oxidation products) are formed during oxidation of n-3 fatty acids, and have been detected in fish oil samples.

Fish oil may contain low phospholipids and cholesterol levels. Cholesterols may be oxidised to hydroperoxycholesterol, which later may decompose to complex mixtures of hydroxy-, keto-, epoxyene- and ketodiene secondary oxidation products (Table 4.1.4). To our knowledge, only two studies have analysed oxidised cholesterol levels in fish oils. Concerning phospholipids, more research is needed to fully understand the oxidation processes and its influence on fish oil oxidation.

Lipid oxidation is a dynamic process creating a complex mixture of primary, secondary and tertiary products. Since several products are intermediate; they are formed and then broken down again during marine oil processing, and there is not always a linear increase in the oxidation-products with time of oxidation. In practice, formation and breakdown of lipid peroxides, secondary and tertiary oxidation products occurs at the same time. It is impossible to give a quantitative and qualitative description of the oxidation products possibly formed because the formation depends of too many factors.

Furthermore, oxidation status of oils is influenced by processing and storage conditions. Anoxic production and storage of oils prevent oxidation. The oxidation rate of fish oil may be

reduced by low temperature. Generally, each 10°C decrease in storing temperature doubles the shelf-life of the oil. Trace amounts of prooxidants (catalysts of lipid oxidation) such as transition metals are usually present in fish oils. Therefore, in order to reduce oxidation, antioxidants that inactivate metals or lipid radicals are added to the fish oils. Encapsulation of n-3 fatty acids reduce the contact between lipids and oxygen and contribute to protect against oxidation. Packaging in material non-permeable for light and oxygen also contributes to protect against oxidation.

5. Methods commonly used to evaluate marine oil

For details about data source, see Appendix B.

5.1 Methods to detect and measure oxidation

The oxidative state of oil is defined as the sum of all oxidation products present. Since there are so many oxidation products, measuring the oxidative state of oils is quite complicated. One approach is to measure representatives of different product categories (e.g. malondialdehyde as a representative of aldehydes/secondary products). Another strategy is to take bulk measurements of groups of compounds, e.g. peroxide value (PV), which gives the sum of lipid hydroperoxide groups, or anisidine value (AV), which ideally measures the bulk of aldehydes/secondary oxidation products. Traditionally, an estimate of primary (PV) and secondary products (AV) is applied. More advanced techniques (described below) can be employed for more detailed analyses of oxidation products, but they require special equipments and experience.

5.1.1 DETERMINATION OF PRIMARY OXIDATION PRODUCTS

One of the oldest and most common methods for determination of primary oxidation products is the assessment of PV and/or conjugated dienes. PV analysis can be performed using iodometric titration methods or spectrophotometric ferro methods.

Iodometric titration method is the one referred to both by Ph. Eur. (Monograph 2.5.5 Method A) and by GOED (AOCS procedure Cd 8-53). The weaknesses of the method are that (a) iodine may react with other double bonds under certain conditions, (b) the presence of oxygen may lead to overestimation; especially at low PV, and (c) more stable peroxides may not react with iodide according to the standard procedure.

PV is an empirical measure of oxidation and is useful for samples that are oxidised to relatively low levels (Frankel, 1998). The measured PVs are relative because they differ for different methods (Nielsen et al., 2003). The peroxide value (PV) is usually expressed as milliequivalents (meq) peroxide, iodine or active oxygen per kg **lipid**, but the value is the same independent of reagent quoted. Milliequivalents may further be expressed as millimoles (mM) per kilogram **oil**, which is half the corresponding meq-value.

Primary oxidation products are traditionally determined as peroxide value (PV) expressed as milliequivalents (meq) of peroxide, iodine or active oxygen per kilogram (kg) lipid, or as millimole of peroxide, iodine or active oxygen per kg oil (mM).

5.1.2 DETERMINATION OF SECONDARY OXIDATION PRODUCTS

Anisidine value (AV) is the most commonly used method for determination of the amount of aldehydes formed as a result of breakdown of lipid peroxides in the oils. The method is based

on the colour reaction between aldehydic compound and *p*-anisidine. AV is defined as 100 times the absorbance (350nm) of a solution resulting from reaction of 1g of fat with 100mL of solvent (AOCS procedure Cd 7-58, Ph.Eur. Monograph 2.5.36). Several high molecular weight decomposition products, including dimers of triacylglycerols, aldehydoacylglycerols or core aldehydes also contribute to the measured AV. The response also depends on the level of unsaturated aldehyde.

The method has some weaknesses: (a) It is not suitable for salmon oil since the presence of astaxanthin influences the result (RUBIN report no. 173, 2009); (b) the use of AV for determination of oxidative quality of ethyl esters has also been observed to give overestimated values, and additionally, (c) food additives (e.g. colour, Q10) and/or flavourings (e.g. lemon, orange) in n-3 fatty acid supplements may influence on the measurements (RUBIN report no. 196, 2010; Thorkildsen, 2010) (see Chapter 6.2).

Holm (1972) showed that an increase by one unit of lipid hydroperoxide corresponds to an increase of about two units of AV. Therefore, the expression "total oxidation of oil", TOTOX = 2PV+AV, was suggested. However, the TOTOX value is an empirical parameter since it corresponds to the addition of two parameters with different units.

Anisidine value (AV) expresses the amount of secondary aldehydic oxidation products and give as such an impression of the historical oxidation of oil. The AV does not distinguish between low molecular (i.e. volatile) or high molecular aldehydes. Other oxidation compounds such as peroxides and polymeric compounds may also contribute to the measured AV. The AV is given without any unit.

Volatile oxidation products are low molecular weight oxidation compounds responsible for off-flavours in oils, which are usually determined by gas chromatography (GC). Due to the large number of secondary oxidation products in fish oils, a complicated spectrum is usually observed; more than 100 peaks representing different low molecular weight oxidation compounds. The results vary between studies due to sample differences (e.g. type of added antioxidants and fish oil), degree of oxidation and chromatographic techniques (Kamal-Eldin, 2002). Since GC method is widely used for qualification of marine oils in scientific lipid oxidation studies, standardisation of the method should be performed.

Malondialdehyde (MDA) is formed as a secondary volatile oxidation product from the fatty acids containing three or more double bonds, and is often measured as a marker for the aldehydes in the Thiobarbituric Acid Reactive Substances (TBARS) test. MDA reacts with two molecules of thiobarbituric acid (TBA), forming a pink coloured compound that can be measured spectrophotometrically. Several versions of the method exist. Sample distillation (MDA distils together with other volatile products), lipid extraction with organic solvents or acid extraction of MDA, followed by acid reaction with TBA can be employed for the determination of TBARS value. The TBARS test has been criticised for lack of sensitivity and specificity since a large number of secondary oxidation products and a number of other compounds may contribute to the determined value (Pokorny et al., 2003). Therefore, it is preferable to quantify the extent of lipid oxidation by a complementary analytical procedure in order to verify the results. Modifying the extraction procedure and/or subjecting the MDA-TBA adduct to HPLC has been suggested in order to increase the specificity of the method (Mendes et al., 2009).

Core aldehydes: The interest in studies of core aldehyde formation has increased in medicine and in studies of food quality (Kuksis et al., 2003). New techniques such as Liquid

Chromatography - Mass Spectroscopy (LC-MS), Electrospray Time-of-Flight Mass Spectroscopy (ES-TOF-MS) among others, have been used in studies of core aldehydes. To our knowledge, these advanced techniques are not yet employed for routine measurements of oxidation compounds in bulk oil.

High-resolution nuclear magnetic resonance (NMR) can provide information on important changes in chemical composition during oxidation. Due to low sensitivity, the method is not suitable for early stage oxidation studies when the concentration of oxidation compounds is low (Curtis, 2007). The ratio between olefinic and aliphatic proton decreases during the oxidation and was observed to correlate with peroxides and TOTOX values. As a result, NMR has been suggested as a rapid and non-destructive technique for estimating the oxidative state in edible oils during processing and storage (Shahidi and Wanasundara, 2002).

5.1.3 DETERMINATION OF TERTIARY OXIDATION PRODUCTS

Polymeric oxidation compounds can be formed as a result of the condensation of peroxyl, alkoxyl and alkyl radicals during autooxidation of marine oils (see Chapter 4.1.3.3). Moreover, polymeric compounds may also be formed when oil is exposed to high temperatures, even in the absence of oxygen (for example during deodorisation; see Chapter 2.3.5). Therefore, the content of polymeric compounds can be used as a quality parameter for marine oils.

Among various methods, size-exclusion chromatography (SEC) seems to be most promising for measuring polymeric compounds, due to its simplicity and rapidity. In SEC, compounds with the largest hydrodynamic radius elute first: oligomers elute before triacylglycerols, which in turn elute before ethyl esters and free fatty acids. Oligomers are usually present at lower abundance than the monomers. Therefore, obtaining accurate peak areas for oligomers can be difficult (Curtis, 2007). In the case of oils containing ethyl ester, separation and identification of oligomers resulting from oxidation of ethyl esters, traces of triacylglycerols and partial acylglycerols, is complicated. Therefore, the Ph. Eur. for ethyl esters 60 (European Pharmacopoeia, 2009) defined the content of "oligomer and partial glycerides" as the sum of partial acylglycerols and oligomers and the allowed limit is set to 7% (see Table 1.4-3). Burkow and Henderson (1991) observed a lack of correlation between polymer content and standard methods for oxidative rancidity assessment (i.e. PV and AV), which indicate insufficiency of standard method for quality assessment of fish oils.

Polar compounds: The oils can easily be separated into two fractions: non-polar compounds (mainly triacylglycerols) and polar compounds including polymerized triacylglycerols, oxidised triacylglycerols, diacylglycerols and free fatty acids. Quantification of polar components is a standard test used in the evaluation of **frying oils** for human consumption and adsorption chromatography is used for isolation and quantification. The content is expressed as weight percentage of the starting oil and is calculated from the weights of the starting sample and non-polar fraction as stated in the Standard Method (IUPAC, 1987). In most regulations, the maximum accepted level of polar components is set to about 25%. The method is not widely used for quality determination in marine oils. To our knowledge, only Shukla and Perkins (1991) and Fantoni et al. (1996) have determined the content of polar components in encapsulated fish oils and found that all the samples analysed contained less than 8.3%. In early stage of oxidation, the amount of polar fraction (including polymeric compounds) in oils could be relatively small. Therefore, direct analysis of polymeric compounds could be imprecise. Thus, in early oxidation studies, the separation of polar fractions for further analysis by high-performance size-exclusion chromatography could be useful because of the methodologically up-concentration effect. The combination of two

methods, namely gravimetric determination of polar compounds by silica column, plus further analysis of polar fractions by high-performance size-exclusion chromatography, has been proposed by IUPAC (Waltking et al., 1981; Wolff et al., 1991).

5.2 Methods to detect and measure *trans*-fatty acids

Oxidation and thermal isomerisation during processing steps such as deodorisation can lead to the formation of *trans*-isomers of fatty acids. There are some official methods and recommendations for analysis of *trans*-fatty acids on GC-FID, but all of these methods are intended for analysis of other type of oils than fish oils. Since the number of possible *trans*-isomers rises geometrically with the number of double bonds, it is complicated to resolve all *trans*-isomers of EPA and DHA.

GC is the only routine method that is able to quantify *trans*-fatty acids at levels below 5%. Fournier et al. (2007) showed that the limits for quantification of geometrical isomers of EPA and DHA were 0.16 and 0.56g/100g of fish oil, respectively. This GC method leads to an underestimation of about 10%, which is similar to the method's uncertainty. Mjøs and Haugsgjerd (2011) tested different chromatographic conditions for analyses of *trans*-fatty acids of marine origin and concluded that it is not possible to find a single condition that eliminates the overlap risk between *trans*-fatty acids and interferents such as 18:3 n-4 and 18:1 n-11. Such compounds will most likely contribute to incorrect assessments of *trans*-fatty acids in marine lipids. However, a Norwegian fish oil refiner advertises on their website that *trans*-fat constitutes maximum 0.9% of the total fatty acids in their products, analysed by GC-MS.

Separation of fatty acid methyl esters (FAME) fraction containing isomers of EPA and DHA has been successfully performed by argentation thin layer chromatography (Ag-TLC) (Fournier et al., 2006a, b, Wijesundera et al., 1989) and by argentation reversed-phase high performance liquid chromatography (Ag-HPLC) (Fournier et al., 2006a, b). The separated isomers were subsequently quantified by GC analyses. However, a mono-*trans* isomer of DHA formed during chemical and thermal treatment produced overlap with all-*cis*-DHA in the chromatogram resulting in lower *trans*-fatty acid content (Fournier et al., 2006b).

As described above, due to challenges with interfering oil compounds, the methods for *trans*-isomer analyses of EPA and DHA are still in developing stages.

5.3 Methods to detect and measure cyclic fatty acid monomers (cfam)

Analyses of cyclic fatty acid monomers usually involve transformation of the sample into fatty acid methyl esters (FAME) that are further hydrogenated after addition of an internal standard. Reverse phase high performance liquid chromatography is used to obtain a fraction consisting of CFAM and the internal standard. This fraction is further analysed by GC on a polar column (Berdeaux et al., 2009).

For structural identification, Berdeaux et al. (2007) isolated C20- and C22-CFAM from fish oil by saponification, esterification, urea fractionation, hydrogenation and column chromatography. The separation by reverse phase high performance liquid chromatography was performed in order to overcome overlap during structural identification by GC-EI-MS analyses.

To our knowledge, no official methods for determination of CFAM in oils exist, and only a limited number of studies have been found on the evaluation methods for CFAM determination.

5.4 Method to detect and measure free fatty acids

Free fatty acids may be formed in the raw materials due to hydrolyses of TAG and PL (see Chapter 3.1.3), and due to incomplete re-estrification during production of n-3 fatty acid concentrates in the form of ethyl esters or glycerols (see Chapter 2.5). The quantification of free fatty acids is based on a simple acid-base titration using potassium hydroxide solution (KOH). The acid value is expressed as milligram KOH required to neutralize the free fatty acids in 1 g of oil. Recommendations for upper limits of acid content in marine oils are given by the Ph. Eur. and GOED (see Chapter 1.4, Table 1.4-3).

5.5 Oxidative stability testing of marine oil

In order to predict the shelf-life of oils, testing of its oxidative stability is necessary. Because of time constraints, the most commonly used method is to test the oil under accelerated oxidative conditions ("Accelerated method") such as increased temperature, elevated oxygen pressure, and by addition of prooxidants etc. The use of such accelerated methods have been criticized because other types of oxidation reactions could be involved compared to "normal" storage conditions (Verleyen et al., 2005) and produce bias on the predictability of the oil's shelf-life.

Sullivan et al. (2011) modelled the primary oxidation of two commercial fish oil preparations with different EPA and DHA contents. The formation of fatty acid hydroperoxide followed first order kinetics for "18/12" oils at temperatures 20°C-40°C, while n-3 fatty acid concentrates demonstrated first order kinetic at temperatures 4°C-40°C. Sullivan et al. (2011) concluded that accelerated testing of fish oil should be conducted at temperatures below or equal to 40°C.

Oxidative Stability Index utilizing either Rancimat or Oxidative Stability Instrument (OSI) is based on volatile acids formation monitored by conductivity measurement. The stability index is determined as a time point when the induction period characterized by low conductivity is interrupted by a rapid increase in conductivity as a result of accelerated oxidation. Both instruments (Rancimat and OSI) are incorporated into the Official and recommended Analysis Manual of the American Oil Chemists' Society (AOCS). Fish oils showed good linear relationship between oxidation stability index and the temperature from 55°C-90°C (Mendez et al., 1997). Although the correlation between OSI induction time and sensory or chemical analysis were obtained, interpretation of accelerated data should always be performed carefully (Verleyen et al., 2005).

5.6 Summary of methods commonly used to evaluate oil

Oxidative status and oxidative stability of fish oil are very important parameters in marketing of marine oils. However, a wide variety of lipid oxidation products can be formed during the oxidation of marine lipids. Therefore, qualification and quantification of all of the oxidation products may be time- and cost intensive.

For the time being, estimates of primary and secondary oxidation products are applied to define the oxidative state of marine oils. The peroxide value (PV) expressed the content of primary oxidation products as meq of peroxide, iodine or active oxygen per kg lipid. The anisidine value (AV) is a single value for an unknown combination of saturated and unsaturated aldehydes (secondary oxidation products), with the response being dependent on the extent of the aldehyde unsaturation. The content of volatile secondary oxidation substances will not be examined by AV. Several high molecular weight decomposition products, including dimers of triacylglycerols, aldehydoacylglycerols or core aldehydes also

contribute to the measured AV. The AV is expressed as absorbance of a solution resulting from reaction between fat and a solvent, and is thus given without any unit. Recommendations for upper PV and AV limits for marine oils are given by the Ph. Eur. and by GOED (see Table 1.4-2). Determination of PV is specified in Ph. Eur. Monography 2.2.5 Method A and AOCS Official method Cd 8-53. Determination of AV is specified in Ph. Eur. Monography 2.5.36 and in AOCS Official method Cd 18-90. The peroxide- and anisidine values give a relative quantitative evaluation for groups of lipid oxidation products **at the time of analysis**. PV and AV give limited information about the oxidation rate and stability of the oil because the increases in the analytes, especially PV, are not linear and may even decrease with oxidation time. Moreover, some naturally occurring antioxidants such as astaxanthin (e.g. in salmon- and krill oil), food additives (e.g. colour) and flavourings (e.g. lemon) in n-3 fatty acid supplements have been observed to interfere with the measurements (see Chapter 6.2). Therefore, the results of PV and AV for n-3 fatty acid supplements should be assessed carefully. The same limitations as for PV and AV applies to the term TOTOX (2PV+AV), which notably is just an empirical parameter since it corresponds to the addition of two parameters with different units.

TBARS measures the amount of malondialdehyde and is another method that may be used to evaluate secondary oxidation products status in oils. However, the measured TBARS value may be affected by the presence of colour compounds (natural occurring or food additives) in the sample. Lipid hydroperoxides and other oxidation compounds can also react with TBA and therefore interfere with the results. There is no standard TBARS method for marine oils. In addition, different TBARS methods employ different analytical conditions and the results are usually incomparable.

The above mentioned PV and AV methods are simple, fast, require low-cost equipments and are commonly used for oxidative quality determination of marine oils by the industry. Advanced techniques such as GC-MS, LC-MS could provide more detailed information about lipid oxidation products in the sample. GC-MS can be employed for analysis of volatile oxidative decomposition products (low molecular weight volatiles). LC-MS can be used for analysis of non-volatile oxidation products (e.g. core aldehydes), and would enable to describe more specific secondary oxidation products then AV value. However, the analytical equipments are expensive and require highly competent professionals. Moreover, some method development and standardisation also would be needed.

The content of polymeric compounds (tertiary oxidation products) has been considered as a useful quality parameter for marine oils. Size-exclusion chromatography (SEC) elutes compounds according to molecular size (i.e. hydrodynamic radius), and is recommended by the Ph. Eur. for evaluation of oligomers' content in marine oils. But, there is methodological challenges concerning analysis of ethyl ester concentrates. This is reflected in the higher upper limits for oligomers content than for other marine n-3 fatty acid products in the recommendations given by the Ph. Eur.

Compounds that are formed as a result of lipid oxidation are usually higher polarity molecules. Quantification of polar compounds could also be a possible parameter for quality determination of marine oils. A standard IUPAC test for this purpose is available for **frying oils** for human consumption. Adsorption chromatography is used for isolation and quantification of polar compounds and the content is expressed as weight percentage of the starting oil. The method may not be useful for marine oils rich in polar phospholipids because it may lead to underestimation of oil quality. However, this method has not been routinely applied for marine oils and should therefore be validated further.

Several studies have shown that *trans*-isomer and CFAM may be formed in marine oils during processing steps that involve temperatures above 180°C. However, due to variety of possible *trans*-isomers that can be formed it is difficult to overcome overlapping of the peaks in the chromatogram, and therefore the analytical methods for such compounds are still in the developing stages.

The quantification of free fatty acids is based on a simple acid-base titration using potassium hydroxide solution (KOH). Recommendations for upper acid limits in marine oils are given by the Ph. Eur. and GOED (see Chapter 1.4, Table 1.4-3).

All the above discussed methods are used to describe the quality status of marine oil. However, lipid oxidation develops over time. Therefore, oxidative stability is the parameter that describes the shelf-life of the oil. Oxidative Stability Testing of oils is a challenging process. In order to complete it in relatively short time, accelerated oxidation conditions that may limit the applicability of the results are used. The results obtained by accelerated Oxidative Stability Testing should be interpreted with caution.

6. Hazard identification

6.1 Substances of potential concern possibly present in refined/concentrated marine oils in bulk as a result of raw material decomposition and oil processing

The decomposition substances, oxidation products and process-generated substances that may be found in crude marine oil are listed in Table 6.1. The occurrence and concentration depend on the composition of the raw materials, decomposition degree of lipids and proteins and processing parameters. The crude fish oil is usually not ready for human consumption and needs to undergo further processing including refining and deodorisation to remove the unwanted decomposition substances (see Fig. 2.3). The process parameters such as time, temperature, pressure and equipment in addition to refining/concentration steps that are included will have influence on the concentration of the decomposition substances, oxidation products and process-generated substances in the bulk end product. Table 6.1 includes substances that more or less are eliminated during the refining process and substances that can be formed during the refining and concentration processes (process-generated substances). At the time being, there is no adequate international, European or national legislation on standard for origin, quality and/or composition of marine oils and their concentrates. However, the Ph. Eur. and GOED (see Table 1.4-2 and 1.4-3) have prepared recommendations for quality criteria mainly based on technical/analytical and practical approaches.

6.1.1 SUBSTANCES IN FISH OIL AND COD-LIVER OIL

6.1.1.1 Processing aids

Cut-offs from the salmon industry can be conserved by adding organic acid (e.g. formic acid) (Chapter 2.1). Formic acid is water soluble and will mostly be removed during the oil polishing step (see Chapter 2.2.5) and the washing step (Chapter 2.3.2) in the refining process. The processing aids that may be used in refining and/or eventually in the concentration process will probably be removed during the different fractionation steps during processing, e.g. by precipitation, distillation. See Figure 2.3 for overview of refining process and Chapter 1.3 for definition and legislation for processing aids.

6.1.1.2 Decomposition substances from proteins

The protein decomposition substances resulting from autolysis or bacterial decomposition in stored fish and fish cut-offs can be di-peptides, free amino acids, trimethyl amine and biogenic amines, such as histamine (see Chapter 3.1.2). These components are water soluble. During the refining process the water soluble decomposition substances will be removed by the washing step with hot water. The resulting refined oil preferably will have a water content ≤1%.

Table 6.1 Review of potential decomposition substances, oxidation products and process-generated substances in crude fish oil, refined marine oil and concentrated n-3 fatty acids.

Substances	Crude fish oil	Refined marine oil	Concentrates
From decomposition of proteins			
Amino acids	**(X)**		
Biogenic amines (e.g. histamine)	**(X)**		
From bacterial growth			
Bacterial toxins; lipid soluble	**(X)**		
From decomposition of lipids			
Free fatty acids	**XX**	**(X)**	[a]
Lyso-phospholipids (lysoPL)	[b]		
Mono and diacylglycerols	**XX**	**(X)**	**X** [c]
Primary oxidation products			
Peroxides	**XX**	**X**	**X**
Secondary oxidation products			
Volatile substances; e.g. low molecular weight aldehydes, alkans, alkens, ketones	**XX**	**(X)** [d]	**(X)** [d]
Non-volatile substances; e.g. core aldehydes, isoprostanes, high molecular weight aldehydes, alkans, alkens, ketones	**X**	**X**	**X**
Tertiary oxidation products			
Polymeric substances	**(X)**	**X** [e]	**X** [e]
Process-generated substances			
Cyclic fatty acids (CFAM)	-	**X** [f]	**X** [f]
Trans-fatty acids	-	**X** [f]	**X** [f]
Processing aids			
Formic acid	**(X)**		

X indicates the degree of decomposition substances, oxidation products and process-generated substances. The concentration of the substances will vary dependent on the treatment of the crude oil in combination with processing parameters throughout the refining and concentration processes. XX indicates relative high concentration compared to X or (X).

[a] Assumed low concentration of free fatty acids in concentrates. [b] Scientific literature is not identified.

[c] Mono and diacylglycerols may be formed during production of n-3 fatty acid concentrates. [d] Dependent on the inclusion of deodorisation during refinement; which reduces the content of volatile oxidation products. [e] The European Pharmacopoeia for "Omega-3-acid ethyl esters 60" defines the content of "oligomers and partial glycerides" (i.e. mono and diacylglycerols) as the sum of acylglycerols and oligomers and the upper limit is set to 7% due to analytical limitations (see Chapter 5.1.3). [f] Formation of CFAM and *trans*-fatty acids is dependent on temperatures during processing.

6.1.1.3 Decomposition substances from lipids

The decomposition substances resulting from autolysis and/or bacterial decomposition of lipids in stored fish and fish cut-offs, include free fatty acids (FFA), partly hydrolysed TAG (mono and diacylglycerols), partly hydrolysed phospholipids (lysoPL), glycerol and oxidations products.

These substances will be present in the extracted crude oil, but more knowledge is needed to understand the influence of raw fish material freshness on the oxidative quality of extracted oil.

Free fatty acids (expressed as acid value): Most of the FFA generated during hydrolytically degradation in raw material and eventually during processing of crude oil, is removed during refining through the neutralisation, deodorisation and molecular distillation (SPD) steps. However, FFA may be formed due to incomplete re-estrification during production of n-3 fatty acid concentrates in the form of ethyl esters or glycerols (see Chapter 2.5). For refined oil/concentrated n-3 fatty acid, the FFA content should be in the range of 0.5 to 3mg KOH/g, dependent on the concentration of n-3 fatty acids, according to recommendations for upper limits of FFA content in marine oils given by the Ph. Eur. and by GOED (see Chapter 1.4, Table 1.4-3).

Glycerol and mono and diacylglycerols: Because high concentration of n-3 fatty acid in the form of triacylglycerols is desirable; the content of mono and diacylglycerols is aimed to be as low as possible during marine oil processing. The concentration of these substances will be decreased during refining (Chapter 2.3) via the neutralisation, washing, winterisation, filtration and the bleaching steps, but may be formed during production of n-3 fatty acid concentrates (Chapter 2.5).

LysoPL: In fish raw materials, phospholipids represent a minor part of the lipids. Data regarding occurrence of LysoPL in fish oil and possible changes in concentration during oil processing has not been found in scientific literature.

6.1.1.4 Oxidation products

Oxidations products (primary, secondary, and tertiary oxidation products) may be present in the raw fish material dependent on composition and storage condition (e.g. time, temperatures) before extraction to crude oil. During crude oil extraction, primary-, secondary- and tertiary oxidation products can be produced due to thermal treatment in combination with inflow of oxygen (air) (see Chapters 2.3, 4.2.1 and Table 6.1). Dependent on the composition of the raw material, natural antioxidants such as tocopherol may remain in the crude oil and stabilise against lipid oxidation during storage and transport of crude oil, but the natural antioxidants are removed during the refining steps.

The content of primary oxidation products, and some of the secondary oxidation products, decreases throughout the whole refining process by the washing, bleaching, deodorisation and SPD steps. The deodorisation step reduces the content of volatile secondary oxidation products (i.e. short chain cleavage product such as aldehydes, ketones) that are responsible for the fishy flavour and unpleasant odour which normally indicate the presence of these oxidation products in refined oil and/or concentrated n-3 fatty acids food supplements. Non-volatile secondary oxidation products (i.e. high molecular weight aldehydes, dimers of triacylglycerols, aldehydoacylglycerols or core aldehydes) may still be present in the n-3 fatty acid product but have no impact on taste and odour of the products. Consequently, an odour- and tasteless marine oil can still have relatively high amounts of secondary oxidations products.

Tertiary oxidation products are odour and tasteless relative stable high molecular substances like dimeric- and trimeric triacylglycerols (i.e. polymeric compounds), that will not be removed throughout the refining process and will be present in the refined oil/concentrated n-3 fatty acids in bulk (see Table 6.1).

Oxidation products can be formed in the period between the end of the refining process and until the food supplement is consumed, e.g. during storage of the bulk refined or concentrated oil, during encapsulation, packaging, at the retailer, and during storage of the product at home.

6.1.1.5 Substances formed due to thermal treatment (process-generated substances)

The n-3 fatty acids are very sensitive to oxygen particularly in combination with high temperatures. Extreme processing conditions may lead to chemical reactions and formation of non-volatile products, which include **polymers, cyclic fatty acid monomers (CFAM) and *trans*-fatty acids** (geometrical isomers of EPA and DHA). At temperature at 180°C or above there is an increased risk of isomerisation of *cis*-fatty acids to *trans*-fatty acids. Polymers are the major products generated at high deodorisation temperatures (250°C). It seems that the deodorisation of fish/marine oil should be conducted at a maximal temperature of 180^0C, a temperature that seems to be lower than the activation energy required for polymerization (intra and inter) and geometrical isomerisation (see also Chapter 4.2 for factors influencing on oxidation).

However, detailed descriptions of the industrial processing steps and their influence on the occurrences of decomposition substances, oxidation products and process-generated substances in marine n-3 oils for human consumption are not available in scientific literature.

6.1.2 DECOMPOSITION SUBSTANCES IN KRILL AND SEAL OIL

Krill and seal as raw materials for production of n-3 fatty acid for human consumption are only to a limited extent covered in this risk assessment. Seal oil is derived from seal blubber and follows the same processing steps as described for fish oil. Except for decomposition products from proteins, the same decomposition substances, oxidation products and substances formed due to thermal treatment of fish oil may also take place in seal oil. Information on processing details for krill oil is very limited. The same decomposition substances, oxidation products and substances formed due to thermal treatment of fish oil may also apply to krill oil. Due to higher proportions of phospholipids and cholesterol in the raw material, LysoPL and oxysterols might be present in higher concentration in krill oil than in fish oil (see chapter 3.2.1). No occurrence data were found on any of the substances in commercial krill oil.

6.2 Substances of potential concern detected in n-3 fatty acid food supplements – examples from open literature

An overview of the methods used to determine oxidation products, polymers and polar compounds in n-3 fatty acid food supplements in references referred to in Tables 6.2.1, 6.2.2 and 6.2.3 of this chapter is provided in Table 6.2.4. Most of the methods applied in the studies described below are following the official IUPAC or AOCS standards. For comments on methods commonly used to evaluate marine oils, see Chapter 5.

6.2.1 CONTENT OF PRIMARY AND SECONDARY OXIDATION PRODUCTS

Results from studies of oxidation substances in commercial products of refined or concentrated marine oils are given in Table 6.2.1.

The **peroxide level** in n-3 fatty acid supplements varies considerably between studies as well as within studies due to factors such as; 1) different degree of lipid oxidation during oil extraction and refining processes, 2) that lipid oxidation products are not removed during the refining process, 3) that further oxidation takes place during packaging and storage of the final food supplement product.

In the eight reported studies, 28 of 116 samples analysed had peroxide values higher than the upper limit 10 meq/kg fat recommended by the Ph. Eur. (see Table 1.4-2). Lipid hydroperoxides are primary oxidation products that may be decomposed by high temperatures or by prooxidants such as transition metals (see Chapter 4.2.4). Therefore, low peroxide value is not unambiguously the same as low oxidised oil.

In total, 80 samples were analysed for **secondary oxidation products** by the anisidine value (AV) method, which measure mostly aldehydes, and large variation in the results is observed. However, the mean anisidine values reported for all supplement categories were below the upper limit 30 recommended by the Ph. Eur. (see Table 1.4-2). Only one of the analysed samples had an AV above 30.

TBARS is another method that is used to examine the secondary oxidation products content in oils. No recommendations for TBARS values are given by Ph. Eur. or by GOED, and the method has its limitations (see Chapter 5.1.2). Two different methods were used to measure TBARS value in the three studies presented but the results were comparable. The TBARS values varied from 0.1 to 3.7 mMol/kg oil.

In general, it is difficult to conclude about quality of the analysed food supplements based on the available results.

All the studied food supplements were within the shelf-life limits. However, not all authors gave information about the actual shelf-life date. Therefore, it is possible that some of the samples analyzed were relatively fresh, while others were at the end of their shelf-life. PV and AV evaluate oxidative quality of oil only at the moment of analyses. Samples that exceed the recommended maximum values may not be able to maintain the acceptable oxidative status throughout the shelf-life. Shelf-life studies of n-3 fatty acid food supplements where not available in the scientific literature.

6.2.1.1 Challenges concerning analytical tools for measuring oxidation products

As described in Chapter 5.1, several analytical problems are associated with analyses of lipid oxidation products. For the commonly used PV and AV methods, additives in n-3 fatty acid supplements may influence on the measurements (RUBIN report no. 196, 2010; Thorkildsen, 2010). Thorkildsen (2010) sampled 113 different liquid n-3 food supplements (i.e. capsules) but after the screening phase only 56 samples were further analysed. The excluded samples showed very high PVs and AVs and even negative AVs and were mostly capsules containing food additives (e.g. colour, Q10) and/or flavourings which were assumed to interfere with the analyses and bias the results. The composition (i.e. food additives and flavourings) of the n-3 fatty acid food supplements analysed and presented in Table 6.2.1 is partly unknown. It is not possible to draw any conclusion on the oxidative status of the n-3 fatty acid food supplements at the market today based on this review. The data presented in Table 6.2.1 gives only an illustration of the PV and AV values in commercially available n-3 fatty acid food supplements.

Table 6.2.1 Oxidation status in marine oil food supplements
(Methods and value units are given in Table 6.2.4)

Type of marine oil	No.	[a] PV (meq/kg) mean (range)	[b] Anisidine mean (range)	[c] TBARS mean (range)	Source
18/12 – fish oils that contained approx. 18% EPA and 12% DHA	3	2.9 (2.7-3.2)	20.5 (14.3-30.2)	[d] na	Shukla and Perkins, 1991
	5	7.7 (3.4-20.3)	na	na	Fantoni et al, 1996
	2	3.1-9.6	6.7-13.6	0.1-0.2	[e] Lystad et al., 1999
	1	2	19	1.7	[e] Hammervold, 2008
	4	4.5 (2.4-9.8)	na	na	Kolanowski, 2010
	[f] 2	1.3 and 2.1	na	na	Kolanowski, 2010
	3	3.9 (3.2-4.8)	25.3 (17.1-30.2)	na	Kragballe and Shukla, 1990
	8	15.8 (7.3-39.8)	15.9 (8.7-34.0)	na	[e] Thorkildsen, 2010*
Concentrate – oils where % EPA + %DHA > 40%	2	1.6-20.8	27.6-17.9	na	Shukla and Perkins, 1991
	1	<1	na	0.5	Burkow and Henderson, 1991
	6	5.4 (2.8-8.6)	(n=4) 6.3 (1.6-16)	1.6 (0.7-2.7)	[e] Lystad et al., 1999
	8	5.8 (0.0-17.2)	16.5 (7-27)	na	Fierens and Corthout, 2007
	5	4.5 (2.5-6.7)	6.5 (4.9-8.1)	2.2 (1-3.1)	[e] Hammervold, 2008
	4	1.6 (1.2-1.8)	na	na	Kolanowski, 2010
	4	4.9 (1.3-9.1)	30.5 (13.1-60.4)	na	Kragballe and Shukla, 1990
	18	9.6 (5.3-27.9)	8.7 (6.2-19.9)	na	[e] Thorkildsen, 2010
Cod-liver oil	9	7.3 (2.1-15.5)	na	na	Fantoni et al., 1996
	1	2.6	na	0.9	Burkow and Henderson, 1991
	3	5.3 (1.9-9.1)	5.4 (3.7-8.5)	0.3 (0.1-0.5)	[e] Lystad et al., 1999
	2	1.1-3.7	8.2-9.8	1.1-1.3	[e] Hammervold, 2008
	4	2.5 (1.0-4.4)	na	na	Kolanowski, 2010
	2	6.7-9.7	7.5-6.8	na	Kragballe and Shukla, 1990
	[g] 5	7.4 (4.0-23.5)	8.0 (2.1-11.2)	na	[e] Thorkildsen, 2010
Plant oil/ fish oil	2	2.8-7.5	3.5-18.7	-	Fantoni et al., 1996
	1	2.2	na	na	Kolanowski, 2010
Seal oil	2	2.7-4.9	4.2	1.3-1.2	[e] Hammervold, 2008
	13	8.4 (0.8-16.1)	4.9 (3.1-6.7)	na	[e] Thorkildsen, 2010
Shark-liver oil	2	5.3-2.3	11.3-15.6	0.4-0.2	[e] Hammervold, 2008
	4	3.0 (1.7-5.5)	na	na	Kolanowski, 2010
	3	7.3 (5.3-7.4)	6.5 (2.9-9.6)	na	[e] Thorkildsen, 2010
Krill oil	2	7.3-6.4	20.1-21.4	2.7-3.4	[e] Hammervold, 2008
Salmon oil	1	6.5	54.3	na	Kragballe and Shukla, 1990

[a] **PV** - peroxide value (milliequivalents of iodine/kg oil; **mEq I/kg)**. [b]**Anisidine**: is defined as 100 times the absorbance (350nm) of a solution resulting from reaction of 1g of fat with 100mL of solvent and the value is given **without any unit**. [c] **TBARS** - Thiobarbituric Acid Reactive Substances (**mmole/kg lipid**). [d] na - not analysed. [e] Master theses. [f] Declared as salmon oil, but from fatty acid profile looks as 18/12 oil. [g] One of the products was interpreted to be salmon oil, but because the product had similar oxidation profile as cod-liver oil, it was included in cod-liver oil category.

6.2.2 CONTENT OF POLYMERS

Polymeric compounds can be formed as a result of the condensation of peroxyl, alkoxyl and alkyl radicals during autooxidation of marine oils (i.e. tertiary oxidation products) and/or when oil is exposed to high temperatures, even in the absence of oxygen. Results from studies that have measured the content of polymers in n-3 oils are given in Table 6.2.2. It is not possible to conclude that these results are representatives for the level of polymers in n-3 fatty acid food supplement on the market today. Kragballe and Shukla (1990) obtained 16.8% polymeric compounds in their sample although the same sample showed very low peroxid value (PV 1.6 meq/kg). Burkow and Henderson (1991) also analysed the amount of polymers in crude cod-liver oil and found it to be around 2%. The content of polymeric compounds can be used as a quality parameter for marine oils (see also Chapter 5.1.3).

Table 6.2.2 Polymers and polar compounds in n-3 fatty acid food supplements.
Mean per cent of total fat and range (Methods are given in Table 6.2.4)

Type of marine oil	No	Dimers % mean (range)	Trimers % mean (range)	Polymers % mean (range)	Polar compounds % mean (range)	Source
18/12 – fish oils (about 18% EPA and 12% DHA)	1	na	na	traces	na	Kragballe and Shukla, 1990
	3	1.4 (1-2)	-	-	-	Shukla and Perkins, 1991
	5	na	na	na	5.4 (3.1-7.2)	Fantoni et al., 1996
Concentrated oil (i.e. % (EPA + DHA) > 40%	1	na	na	16.8	na	Kragballe and Shukla, 1990
	2	(0-10)	(0-6.3)	(0-3.1)	36.3 (one sample)	Shukla and Perkins, 1991
	1	-	-	2.1	-	Burkow and Henderson, 1991
Cod-liver oil	9	na	na	na	5.4 (2.6-8.3)	Fantoni et al., 1996
	1	-	-	2.1	-	Burkow and Henderson, 1991

All the samples analysed were within the shelf-life limits, but information about the products' shelf-life duration was not avaiable. na – not analysed.

6.2.3 CONTENT OF *TRANS*-FATTY ACIDS

Trans-isomers of fatty acids can be formed as a result of high temperatures during processing of oils (see Chapters 2.3.5). However, as shown in Table 6.2.3, the amounts of *trans*-isomers of EPA and DHA in seven commercial marine oil samples were very low; less than 2% of the EPA content and less than 3% of the DHA content. For evaluation and comments concerning the method used, see Chapter 5.2. No limits for content of *trans*-fat is given by Codex, Ph. Eur. or GOED.

Table 6.2.3 Content of *trans*-isomers of EPA and DHA in commercially available marine oils. Mean value ±standard deviation (Fournier et al., 2007)

[1)] Sample	Gram (g) EPA/100g of fatty acids		Gram (g) DHA/100g of fatty acids	
	All cis	Trans	All cis	Trans
A	-	-	40.98±0.19	0.86±0.02
B	0.34±0.01	-	41.61±0.37	0.37±0.03
C	0.38±0.00	-	43.51±0.21	0.62±0.03
D	16.37±0.14	0.31±0.03	10.66±0.07	0.31±0.00
E	16.49±0.05	0.34±0.03	11.30±0.07	0.35±0.04
F	6.60±0.03	0.13±0.02	22.97±0.17	0.57±0.00
G	6.34±0.10	0.12±0.04	22.10±0.04	0.62±0.04

[1)] Sample A, B and C: oil derived from algae. Sample D, E, F, and G: semi-refined and refined fish oils from different countries.

Table 6.2.4 Overview of methods to determine oxidation products, polymers and polar compounds in n-3 food supplements in the references referred to in this chapter.

Study	*)Aim	Units	Method	Reference for method
Kragballe and Shukla, 1990	PV	meq/kg	IUPAC (iodometric titration method)	Paquot C, Hautfenne A. Standard Methods for the Analysis of Oils, Fats and Derivatives, seventh ed. Oxford, UK: Blackwell Scientific, 1987.
	AV	No unit	IUPAC	
Shukla and Perkins, 1991	PV	meq/kg	IUPAC (iodometric titration method)	
	AV	No unit	IUPAC	
	Polymers	%	HP size exclusion chromatography	No further details given
	Polar compon-ents	%	IUPAC	Paquot C, Hautfenne A. Standard Methods for the Analysis of Oils, Fats and Derivatives, seventh ed. Oxford, UK: Blackwell Scientific, 1987.
Burkow and Henderson, 1991	PV	meq/kg	AOAC Official Method (iodometric titration method)	[aa)]AOCS
	TBARS	nmol TBA/mg		[g)] Ke and Woyewoda, 1978
	Polymers	%	HP gel permeation chromatography	Burkow and Henderson, 1991
Fantoni et al., 1996	PV	meq/kg	AOAC Official Method (iodometric titration method)	[a)]AOCS
	Polar compon-ents	%	IUPAC-AOAC Method	*Official Methods of Analysis of the Association of Official Analytical Chemists,* edited by K. Helrich, 15th edn., Vol II, Association of Official Analytical Chemists, Arlington, 1990, Method 982.27
Lystad et al., 1999	PV	meq/kg	AOCS Official Method (iodometric titration method)	[a)]AOCS
	AV	No unit	AOCS Official Method	[b)]AOCS
	TBARS	nmol TBA/mg		[c)] Dulavik B., 1997
Fierens and Corthoud, 2007	PV	meq/kg	AOCS Official Method (iodometric titration method)	[a)]AOCS
	AV	No unit	AOCS Official Method	[b)]AOCS
Hammervold, 2008	PV	meq peroxide/kg	Ferrothiocyanate method	[d)] International Dairy Federation, modified by [e)] Ueda *et al.,* 1986, and [f)] Undeland *et al.,* 1998
	AV	No unit	AOCS Official Method	[b)]AOCS
	TBARS	[g)] µg TBARS/g		[g)] Ke and Woyewoda, 1978
Kolanowski, 2010	PV	meq of active oxygen per kg of oil (meq O/kg)	ISO 3960, (iodometric titration method)	International Standard Organization. *ISO 3960—Animal and vegetable fats and oils – determination of peroxide value*; ISO: Geneva, Switzerland, 2001, 7pp.
Thorkildsen 2010	PV	meq/kg	AOCS Official Method PeroxySafe STD kit	[a)] AOCS
	AV	No unit	AOCS Official Method PeroxySafeTM STD Kit	[b)] AOCS

*) PV- peroxide value (milli-equivalents of iodine or oxygen /kg oil), AV- anisidine value (no unit), TBARS - thiobarbituric acid values (mmol TBA/kg) [aa)] Official and Tentative Methods of the American Oil Chemists' Society, Cd 8-53, 3th edn., edited by D. Firestone, AOCS, Champaign, 1981. [a)] Official Methods and Recommended Practices of the American Oil Chemists' Society, Cd 8-53, 4th edn., edited by D. Firestone, AOCS, Champaign, 1989. [b)] Official Methods and Recommended Practices of the American Oil Chemists' Society, Cd 18-90, 4th edn., edited by D. Firestone, AOCS, Champaign, 1989. [c)] Dulavik B. "Seifilet – Kvalitetsendringer under fryselagring", Fiskerikandidatoppgave i næringsmiddelkjemi, 1997, Norges Fiskerihøgskole, UIT. [d)] International Dairy Federation, International IDF Standard 74A, 1991, Belgium. [e)] Ueda S, Hayashi T, Namiki M. Effect of ascorbic acid on lipid autooxidation in a model food system. Agric Biol Chem 1986; 50: 1-7. [f)] Undeland I, Stading M, Lingnert H. Influence of skinning on lipid oxidation in different horizontal layers of herring (*Clupea harengus)* during frozen storage. J Sci Food Agric 1998; 78: 441-50. [g)] Ke PJ and Woyewoda AD. Microdetermination of thiobarbituric acid values in marine lipids by direct spectrophotometric method with a monophasic reaction system. Analytica Chemica Acta 1978; 106: 279-84. **Note** that TBARS values in the referred thesis were given in µg/g, but by looking into the calculations it was obvious that the TBARS values should have been given in mmol/kg oil.

6.2.4 COMMENTS TO SUBSTANCES OF POTENTIAL CONCERN DETECTED IN N-3 FATTY ACID FOOD SUPPLEMENTS

The occurrence and concentration of decomposition substances in commercial n-3 food supplements are not very well documented in the scientific literature. The examples presented are based on bachelor/master's degree work and some studies published in the scientific literature. The n-3 fatty acids products examined are mainly collected from health food store, pharmacies or supermarkets. When possible, the results are compared to Ph. Eur. and GOED standards (Table 1.4-2 and 1.4-3) that have prepared recommendations for quality criteria mainly based on technical/analytical and practical approaches.

The oxidation products measured by the PV, AV, and TBARS methods, in addition to measurements of polymer content do not express the whole lipid oxidation picture that may occur in marine oils. The methods used to evaluate the above-mentioned oxidation product also have limitations (see Chapter 5.1). Therefore, it is difficult to draw any conclusions on the real picture of the oxidative quality of marine n-3 fatty acid food supplements on the market. Regular monitoring, standardised sample collection and more sensitive and advanced techniques could more precisely express the oxidative status of the n-3 fatty acid food supplements available on the market.

6.3 Summary of substances of potential concern possibly present in refined/concentrated marine oil as a result of decomposition and processing and detected in n-3 fatty acid food supplements

In total, the occurrence and concentration of primary, secondary and tertiary oxidation products in addition to polymeric-, cyclic- and trans-fatty acids depend on storage conditions/freshness of the raw fish/liver materials, processing parameters and which processing steps that is included, antioxidants added and storage conditions throughout the whole value chain.

***Free fatty acids (FFA*)**: The FFA concentration is minimised during refining and mainly during the neutralisation step, but may be formed due to incomplete re-estrification during production of n-3 fatty acid concentrates in the form of ethyl esters or glycerols. For refined oil/concentrated n-3 fatty acids in bulk, the FFA content should be in the range of 0.5 to 3 mg KOH/g dependent on the concentration of n-3 fatty acids according to recommendations for upper limits given by the Ph. Eur. and by GOED (see Chapter 1.4, Table 1.4-3).

Mono and diacylglycerols will be minimised during refining by the neutralisation, washing, winterisation, filtration, bleaching, and filtration/polishing steps, but may be formed during production of n-3 fatty acid concentrates (Chapter 2.5).

LysoPL: Since phospholipids represent a minor part of fish lipids only small amounts of lysoPL is expected to be formed. However, data regarding occurrence and concentration of lysoPL in fish oil has not been found.

Primary oxidation substances such as peroxides can be detected in n-3 fatty acid food supplements. The concentration will be reduced throughout the refining process. Peroxide value (PV) indicates the amount of these substances (see Table 6.2.1).

Secondary volatile oxidation products can be detected in n-3 fatty acid food supplements. The content of volatile secondary oxidation products, short chain cleavage product such as aldehydes, ketones etc. that are responsible for the fishy flavour in crude fish oil, is reduced during refining by the deodorisation- and short path distillation steps. Sensory evaluation (i.e. of taste and odour) normally indicates the content of these oxidation products in refined

oil/concentrated n-3 fatty acids in bulk, but it may be circumvented by encapsulation or microencapsulation and by the addition of food additives and flavourings. No commercial standard methods for exclusively analysing volatile secondary oxidations substances are available.

Secondary non-volatile oxidation products can be present in n-3 fatty acid food supplements. The non-volatile oxidation products such as core aldehydes, isoprostanes, high molecular weight aldehydes, alkans, alkens, and ketones, have no impact on the sensory quality of the fish oil products and are thus not detected by consumers. Use of short path distillation during refining may reduce the content of non-volatile oxidation products.

Anisidin value (AV) will indicate the amount of aldehydes (see Table 6.2.1) in marine oils. However, the method is not specific with regard to type of aldehydes and since the AV is expressed without unit, quantification is difficult.

Tertiary oxidation products are relative stable high molecular substances like dimeric and trimeric triacylglycerols (polymeric compounds) that may be detected in refined oil/concentrated n-3 fatty acids in bulk as well as in food supplement (see Table 6.2.2). No commercial standard methods for analysing tertiary oxidations substances are available.

Substances formed due to thermal treatment. The n-3 fatty acids are very sensitive to oxygen particularly at high temperatures and extreme processing conditions may lead to the occurrence of chemical degradation reactions. Formation of non-volatile degradation products includes polymers, CFAM and *trans*-fatty acids. Sensory evaluations (i.e. taste and odour) of the refined oil/concentrated n-3 fatty acids in bulk as well as of food supplement will not indicate the content of these degradation substances even if they occur in high concentrations. No commercial standard methods for analysing polymers, cyclic fatty acid monomers and *trans*-fatty acids in marine oils are available.

Seal and krill oils: The same decomposition substances, oxidation products and substances formed due to thermal treatment of fish oil may also take place in seal and krill oils. Due to higher proportions of phospholipids and cholesterol in raw krill material, lysoPL and oxysterols might be present in higher concentration in krill oil than in fish oil (see Chapter 3.2.1). No occurrence data were found on any of the substances in commercial krill oil.

7. Hazard characterisation

7.1 Decomposition substances (FFA and lysoPL)

FFA and lysoPL in the diet will in most cases be absorbed and esterified in the enterocytes to TAG or PC (Velasques et al., 1994) therefore there is limited effect of these components on inner organs. However, the intestine can be affected even at concentrations of FFA associated with normal digestion (Velasques et al., 1993a). The effects are dependent on fatty acid concentration and the carbon chain length. Concentrations of 5 mM (1,6g/l) oleic acid perfused into pig intestine caused increased membrane permeability, cell membrane disruption and cell lysis (Velasques et al., 1993a; Velasques et al., 1994). Mucosal injury severity due to FFA is dependent on age, for example are 1 day old piglets more susceptible to injury than one month old piglets (Velasques et al., 1993a; Velasques et al., 1994). In humans, 4g/day of free EPA and DHA given as slow-release soft gelatine capsules to patients with Crohn disease for one year, did not cause adverse effects, such as bleeding problems (Feagan et al., 2008), while capsules with 2 g/day free EPA and DHA caused eructation in a cross-over study with 8 female subjects (Beckermann et al., 1990).

The toxicity of FFA is most likely coupled to their detergent function, leading to increased permeability of biological membranes and to cell lysis at high concentrations. Normally FFA is kept at a very low level in animal and human tissues due to their toxic effects. Any increase in circulating FFA will be met by increased TAG production both in liver and white adipose tissue, keeping the circulating levels within narrow limits (Meshkani and Adeli, 2009).

The acid group of the fatty acid is important in the mechanism of toxicity, since oleic acid, but not ethyl oleate had a toxic effect on developing piglet intestine (Velasques et al., 1994). Toxicity of FFA has been shown in intestine (Gaginella and Phillips 1976; Kvietys et al., 1991: Velasques et al., 1993a, b; Velasques et al., 1994), erythrocytes (Raz et al., 1973), tumour cells (Zhu et al., 1989) and heart muscle cells (Wenzel and Hale, 1978). The severity of the toxic effect is concentration dependent and greater with long than with short chain FFA. The number of double bonds in the FFA does not seem to influence the toxicity (Kvietys et al., 1991; Velasques et al., 1993a).

Reports on adverse health effects of dietary lysoPL were not found, but the amphiphilic character of the molecules is likely to give them cytotoxic properties.

7.2 Oxidation products

For details about data source, see Appendix B.

7.2.1 STUDIES IN EXPERIMENTAL ANIMALS

7.2.1.1 Studies with oxidised oil as a dietary ingredient

In many studies, oils oxidised by aeration and/or heat treatment have been used and the whole range of oxidation products described in Chapter 4 may be present. In these studies, one cannot assign health effects to one or a few oxidation products. The measured oil rancidity represented by PV and TBARS cannot be predicted by the conditions used during oxidation, as indicated by the results of Eder et al., 2003 (see Table 7.2.1), and the measurements are not strictly quantitative. The quantification of polar compounds in the oil is also indicative of the oxidative status and seems to correspond better with the biological responses, such as differences in body weight gain (Eder et al., 2003, Table 7.2.1 and Chapter 5.1.3). Secondly, factors such as fatty acid composition and antioxidant levels, which are also affected by oxidation of the dietary oils, may affect animal performance. This complexity may explain why measured effects of oxidised oils often vary from one experiment to another. Table 7.2.1 sums up a number of studies where rats were fed oxidised lipid.

An important protection mechanism against oxidised fat is avoidance, which is an inherited property in all mammalian, including humans. The short chain secondary oxidation products are volatile and some of them, especially the aldehydes, have very low threshold levels with respect to odour and taste in mammals. These products are therefore the primary source of rancid odour and taste of marine oils (Frankel, 1998; Chapter 4.1.3.2).

Effect studies using oxidised oil

In vivo

Most of the effect studies with lipid oxidation products have been conducted with vegetable oils that have been heated or used for deep-frying. Vegetable oils are less susceptible to oxidation than fish oils. However, the products formed during oxidation are to some extent

Table 7.2.1 Characteristics of fresh and oxidised oils and effects on feed intake and growth in rats in different experiments

Body weight is the chosen endpoint since it has been measured in all the studies. Several other effects of oxidised oil are described in the text. The differences in measured oxidation in the study of Eder et al. (2003), illustrates how difficult it is to standardize the experimental conditions and run dose response studies in this field. The doses used in these animal studies are high compared to the exposure of humans with an intake of maximum 15 ml oil per day with a maximum PV 30 meq/kg (Chapter 8 and Table 6.2-1, respectively).

Experimental	**Diet content**	**Measured characteristics of the oils**				**Experimental animal (rat)**		**References**
oil used	of oil (g lipid /kg feed)	PV (meq/kg)	TBARS (nmol/g)	Polar comp. (%)	Vit E (mg/kg oil)	Feed intake (g/day)	Final body weight (g)	
Fish oil								
Control	50	15	0.24	na	na	na	360 [b)]	Hwang et al., 2000
Oxidised	50	328	12.3	na	na	na	290 [a)]	
Sunflower oil								
Control	150	na	na	4	478	12.2	181[a)]	Garrio-Polonio et al., 2004
Oxidised	150	na	na	28	44	11.7	144[b)]	
Plant oil blend								
Control	100	20	1.0	7	na	na	Ca. 230	Brandsch and Eder, 2004
Oxidised	100	727	37	37	na	na	Ca. 230	
Sunflower/lard								
Control	100	21	2.3	3	250	14	388 [a)]	Eder et al., 2003
50°C, 38d	100	918	22	54	250	14	364 [b)]	
105°C, 81h	100	231	2.2	53	250	14	376 [b)]	
190°C, 24h	100	39	0.2	46	250	14	362 [b)]	
Soy oil								
Control	200	2.9	na	2	na	16	411 [a)]	Chao et al., 2001
Oxidised	200	6.3	na	54	na	11.2	270 [b)]	
Soy oil								
Control	50	2.9	na	2	na	17.2	394	Chao et al., 2001
Oxidised	50	6.3	na	54	na	20.1	385	
Sunflower/palm								
Control		33	0.7	3	514	Restricted	273	Skufca et al., 2003
Oxidised		877	25.1	41	514		263	
Sunflower/lard								
Control	100	6.6	1.0	na	50	14-17	342	Ringseis et al., 2007a
Oxidised	100	230	19	na	50	14-17	331	

PV: peroxide value; TBARS: thiobarbituric acid reactive substances. Polar comp.: polar compounds. Analytical methods for these substances are described and evaluated in Chapter 5. na: Not analysed/measured. Restricted feeding: Feeding less than to satiation to control feed intake. [a) or b)] statistically significant differences

similar because they belong to the same categories, e.g. lipid hydroperoxides, aldehydes and so forth, but differ in specific compound distribution within each category.

Intake of oxidised feed, especially when combined with low levels of vitamin E, produces reduced body weight gain, increased kidney and liver weight, higher mortality and pathologies in a number of animal species (Baker and Davis 1997; Garrio-Polonio et al., 2004; Hwang et al., 2000; Ichinose et al., 2004; Tacon 1996;). In studies that have used experimentally oxidised oils (vegetable or marine), there is a production of lipid oxidation products in the oils, combined with a shift in fatty acid composition towards less polyunsaturated fatty acids and loss of antioxidants such as vitamin E (see e.g. Garrio-Polonio et al., 2004). If feed intake is not controlled, study animals tend to eat less of oxidised feed than control feed due to reduced palatability, and reduced growth is a possible consequence, partly or wholly of reduced feed intake. Therefore, in studies where effects of whole oxidised oils on animal health are investigated, the effects cannot be assigned only to the presence of lipid oxidations products, unless the confounding factors are corrected.

Of the studies presented below, those which control the confounding factors of reduced vitamin E and PUFA levels and lower feed intake, are of very high quality and measure mainly the effects of lipid oxidation products. Those studies with less control of these factors show more severe effects but may have included vitamin E deficiency and differences in feed intake in their independent variables.

When low vitamin E level, unsaturated fatty acids and feed intake were not corrected, several effects of oxidised plant oils in the rat model were observed, including reduced body weight (Table 7.2.1), increase in relative liver and kidney weight, reduced liver lipid, increased amounts of peroxisomes and activation of PPARα responsive genes in the liver, increased membrane fluidity and shift in membrane bound enzyme activities, increased levels of organ specific enzyme activities in plasma alanine transaminase, aspartate transaminase, and alkaline phosphatase (ALAT, ASAT, ALP), indicating cell damage and leakage, increased liver and serum TBARS and reduced liver glutathione (Chao et al., 2001; Garrio-Polonio et al., 2004; Hochgraf et al., 1997; Hwang et al., 2000). In a study where thermoxidised plant oil (PV 727 meq/kg) was fed to female rats during pregnancy and rearing, the body weight of the mothers was not affected, but the weight of the litters and growth of the pups was decreased by the oxidised plant oil. Furthermore, there was an increase in liver TBARS, concentrations of lipid hydroperoxides and oxidised cholesterol in both mothers and foetuses. Milk antioxidant status and hydroperoxide contents were not affected, but milk triacylglycerol was reduced. In this study, feed intake and dietary α-tocopherol concentration were not controlled or measured (Brandsch and Eder, 2004).

Ringseis et al. (2007a) fed rats with oxidised vegetable frying oils (PV 230 meq/kg) that were added vitamin E and lard up to control diet levels to compensate for the loss of polyunsaturated fatty acids and vitamin E by oxidation. In addition, restricted feeding at rations below satiation were used, so that rats fed fresh and oxidised food consumed equal amount of feed. The authors found no effects on body weight gain, but the relative liver weight was increased and the amount of liver triacylglycerol was reduced by oxidation (Ringseis et al. 2007a). Furthermore, PPARα responsive genes were activated in mothers and foetuses. These findings are in accordance with another study by Skufca and co-workers (2003) using a similar design in rats. The oxidised frying oil (PV 877 meq/kg) also modified thyroid hormone metabolism, causing an increase in plasma total and free thyroxine, without altering the plasma thyrotropin concentration. On the other hand, several characteristics of the thyroid gland were altered. There was an increase in epithelial height, decrease in follicle volume, a large decrease in the mRNA of the NaI symporter, the transporter for iodine into

the thyroid, and an increase in thyroxine peroxidase mRNA, an enzyme active in thyroxine synthesis (Scufca et al., 2003). Similarly, Eder and Stangl (2000), fed sunflower oil oxidised by heating (PV 144 meq kg^{-1}) to miniature pigs, with appropriate additions of vitamin E and lard. They found no effect on body weight but increased levels of plasma total and free thyroxin. There was also a non-significant trend of higher level of thyroid stimulating hormone (TSH). The changes in thyroid hormones correlated negatively with plasma, HDL and LDL cholesterol. Tocopherol concentrations in plasma and LDL were reduced and Cu^{++} stimulated oxidation of plasma gave higher levels of conjugated dienes in plasma from pigs fed the oxidised oil than in the controls. In another study, TBARS in intestinal epithelial cells was increased, even though the level of vitamin E was similar in pigs fed oxidised frying (PV 10) and fresh oils (Ringseis et al., 2007b). These results can largely be explained by the presence of oxidation products alone. However, since lipid oxidation gives a multitude of products (see Chapter 4) it is difficult to make predictions about the underlying mechanisms for these responses.

Sülze et al. (2004) fed rats fresh and thermally oxidised fats (mixture of sunflower oil and lard, PV 918 meq/kg) at two vitamin E levels and monitored gene expression profile in the liver using microarray technology. They found that a series of PPARα downstream target genes were up-regulated in rats fed the oxidised oils, regardless of vitamin E level. Genes involved in xenobiotic metabolism and stress responses were also up-regulated and increased peroxisome proliferation in the liver was confirmed by marker analyses. The amounts of fat and level of oxidation vary between the different studies. Furthermore, the lack of correlation for example between oxidation time and accumulation of products that can be measured (Chapter 4) makes quantification of oxidation product levels that may produce adverse health effects a very difficult task.

Vitamin E breaks the chain of lipid auto-oxidation, by reacting with the lipid peroxy radical, preventing it to react with a new polyunsaturated fatty acid (see Chapter 4.2.5). Vitamin E may be consumed in the oxidation process and oxidised oil may therefore reduce vitamin E levels in fish and rat tissues (Baker and Davis, 1997; Hung et al., 1981; Liu and Hung, 1995; 1996). Dietary oxidised lipids reduced the concentrations of plasma tocopherol and LDL tocopherol in rats (Turner et al., 2006) and the concentration of tocopherol in pig intestinal epithelial cells (Ringseis et al., 2007b). These effects may be attributed to reduced vitamin E levels in the oxidised oil. In the cases of corrected vitamin E loss during oxidation, the oxidised oil may still lead to decreased available vitamin E in the gut and therefore to secondary vitamin E deficiency. Furthermore, if oxidation products are taken up into the body, they may initiate lipid oxidation and subsequently increase *in vivo* consumption of vitamin E. Several vitamin E deficiency symptoms, such as increased tissue TBARS, auto-fluorescent material in the cells, anaemia and muscle degeneration, are presumably due to *in vivo* lipid oxidation which modifies cell membranes and protein, leading to leaky cells and sometimes to cell lyses (Hamre, 2011). Accordingly, effects of oxidised oils on different organisms can be partly reversed with increased vitamin E supplementation (Baker and Davis, 1997; Hung et al., 1981; Ringseis et al., 2007b; Skufca et al., 2003).

The one study found on effects of oxidised fish oil (Hwang et al., 2000) gave similar results as the studies on oxidised vegetable oils. Fresh (PV 15 meq/kg) and oxidised (PV 328 meq/kg) oils were given to male weanling Wistar rats with and without additional taurine supplementation. Feeding of oxidised fat led to decrease in body weight, increased liver and kidney weights relative to body weight, decreased concentration of glutathione and increased TBARS levels in the liver. The plasma concentrations of the tissue specific ALAT, ASAT and ALP also increased, indicating cell leaching. These effects were all partly restored by taurine

supplementation. In this study (Hwang et al., 2000); there was no control of food intake, fatty acid composition of the diets or vitamin E supplementation.

In summary, feeding highly oxidised oils to animals clearly has negative health effects, both on the macroscopic level, such as changes in body weight and organ indices and at the biochemical level with changes in markers of oxidative stress, organ damage and change in thyroid hormone metabolism and expression of PPARα responsive genes. However, it is difficult to say at which doses the effects appear, since the measurements of oxidation used in the studies are not quantitative. Because the whole oxidised oil contains a complex mixture of oxidation products and process generated products it is also not possible to say which products cause the adverse health effects.

7.2.1.2 Studies on primary oxidation products (hydroperoxides)

Lipid hydroperoxides are unstable compounds and decompose readily to fatty acid alkoxy radicals, which react strongly with various biomolecules, such as protein and DNA. They also evoke new chain reactions of lipid oxidation (Frankel, 1998). These compounds can therefore be characterised as potentially toxic.

Absorption of primary oxidation products

Whether lipid hydroperoxides are absorbed from the gastrointestinal tract of vertebrates is still subject to ongoing discussions. A substantial fraction of linoleic acid hydroperoxides or tri-lineoylglycerol hydroperoxides administered by an intragastric procedure to rats, decomposed to epoxy ketones and aldehydes prior to reaching the intestine (Cohn, 2002; Kanazawa and Ashida 1998a; 1998b; Mohr et al., 1999; Soumela et al., 2005). Small amounts of hydroperoxides were detected in the intestine when administered at higher doses. A possible mechanism of detoxification of lipid hydroperoxides is the direct conjugation with glutathione (GSH) in the intestinal lumen. GSH is excreted in bile to the small intestine and Samiec et al. (2000) found that in the rat small intestine, the mucus contained ample amounts of glutathione transferase, an enzyme that conjugates electrophilic compound with GSH at a high rate. This mechanism may also be active in neutralising oxidised lipids.

Vertebrates are protected from absorption of lipid hydroperoxides from the intestine to the body by glutathione peroxidase (GPx) that is present in the enterocytes. GPx reduces fatty acid hydroperoxides to hydroxy fatty acids at the expense of reduced GSH, thereby preventing the decomposition to fatty acid alkoxy radicals (Aw 1998; 2005; Esworthy et al., 1998; Miyamoto et al., 2003). This process appears to be subject to limitations by the availability of GSH, which can be supplied by intracellular synthesis through the reduction of oxidised glutathione (GSSG) by NADPH or by GSH transferred from the blood or the intestinal lumen. GSH in the lumen can originate from the bile or from the diet (Aw, 2005). Using a rat lymph fistula model, it has been shown that absorption of lipid hydroperoxides from the intestine depends on the GSH/GSSG redox status of the enterocytes; at low levels of GSH, lipid peroxides are absorbed into the lymph and retained in the intestinal lumen, while at high levels, the peroxides are drained from the lumen appearing as modified hydroxyl fatty acids in the lymph (Aw, 2005). This model would also imply that absorption of peroxides is dependent on dietary dose. It is therefore possible that at low doses and in animals with a good redox balance, fatty acid hydroperoxides are transformed to hydroxy fatty acids in the enterocyte before transport into the lymph and blood, while at the opposite conditions, the hydroperoxides will cross the intestinal border.

Effects of primary oxidation products

In accordance with the above mentioned hypothesis, there are animal studies showing that dietary peroxides may lead to increased tissues and body fluids concentrations of these

compounds in experimental animals (Brandsch and Eder, 2004). However, other studies (Kanazawa and Ashida 1998a; 1998b; Mohr et al., 1999; Soumela et al., 2005) reported no effect on tissue peroxides by oxidised oil consumption. Dietary hydroxy fatty acids situated in triacylglycerols seem to be treated similarly to native fatty acids in the human systems for digestion and absorption of fat (Wilson et al., 2002) and in mono-layers of Caco-2 cells; a cell-line expressing characteristics of mature intestinal cells (Müller et al., 2002).

At a high enough lipid peroxides dose to compromise the GSH/GSSG redox balance of the enterocytes, there may be a shift in cellular proliferation, growth arrest and apoptosis as shown in cell culture studies (Aw, 2005, and references therein). The intestinal mucosa is a labile organ where enterocytes originate from proliferation of stem cells in the crypts, differentiate as they migrate over the villi and are shed by apoptosis from the villi tips into the lumen. The epithelium turns over every 4-5 days in the rat (Aw, 2005). Rats fed lipid peroxide diets exhibited suppressed mucosal proliferative and apoptotic activity which correlated with a shift in the GSH/GSSG ratio (Aw, 2005). Low levels of vitamin E, often associated with oxidised lipid, may also compromise the redox-balance of the enterocytes. In rats fed oxidised sunflower oil, without correction for loss of vitamin E, both the activities of antioxidant enzymes and the GSH/GSSG balance were modified compared to controls (David et al., 2010). There are hypotheses that lipid oxidation products initiate inflammation and cancer in the gut (Biasi et al., 2008), but these have not been proven *in vivo*. However, the viability of colon cells in culture, both normal cells and carcinoma cells, was compromised when linoleate hydroperoxide was added to the medium, but NF-κB pro-inflammatory gene transcription was not activated by oxidised fat in primary porcine intestinal epithelial cells (Ringseis et al., 2007b). In another study, the viability of Caco-2 cells was reduced in response to the addition of oxidised fish oil, oxidised methyl linoleate, MDA or 4-hydroxylnonenal (4-HNE) to the culture medium (Algazeer et al., 2008).

Khan-Merchant et al., (2002) found that dietary supplementation of enzymatically oxidised linoleic acid, with conversion to linoleic acid hydroperoxide (13-HPODE) and further to hydroxy linoleate (13-HODE), produced an increase in fatty streak area in aorta in LDL knockout mice. There was also an increase in auto-antibodies against proteins modified by oxidative stress. The effect was not seen when the mice were fed a high fat (210 g/kg) and cholesterol (1.5 g/kg) diet, but only with medium and low fat and cholesterol (149/1 g/kg; 70/0.5 g/kg), indicating an interaction between fresh dietary fat and oxidised components after ingestion. Expression of vascular cellular adhesion molecules was increased in pig and humans in response to dietary lipid hydroperoxides but not to hydroxyl fatty acids. NADS(P)H oxidase and production of superoxide anion, which induces cytotoxicity, are also stimulated by lipid hydroperoxydes but not by hydroxy fatty acids (Turner et al., 2006). From the available literature, it is not clear to what extent the conversion of hydroperoxides to hydroxyl fatty acids during absorption reduces the adverse effects of dietary oxidised lipids on vascular health.

A review on cholesterol ester oxidation products (Leitinger, 2003) shows that fatty acid hydroperoxides bound to cholesterol, present in plasma and tissues are efficiently degraded by similar mechanisms as those shown for other lipid hydroperoxides in the intestinal mucosa. Still oxidised cholesterol esters, comprising hydroperoxides, hydroxides and core aldehydes, are abundant in atherosclerotic lesions. It is not known whether these products were absorbed from the intestine or generated *in vivo*.

Further literature focussing on effects of dietary lipid hydroperoxides after absorption from the intestine seems to be lacking, perhaps because of the labile nature of the compounds, the

difficulty of measuring them, the difficulty in isolating only these compounds to be fed to experimental animals and their biotransformation to hydroxyl fatty acids during absorption.

7.2.1.3 Effect studies of secondary oxidation products

The lipid hydroperoxides eventually decompose to shorter chain compounds such as aldehydes, ketones, alcohols, alkans and alkens (see Chapter 4.1.3). Some of these secondary oxidation products, especially the aldehydes, are toxic due to their ability to bind to the amino groups of protein, nucleic acid bases, the N bases of phospholipids and the -SH groups of sulfhydryl compounds (Draper et al., 1986; Esterbauer, 1993; Mahmoodi et al., 1995).

Absorption of secondary oxidation products

Malondialdehyde (MDA; Draper et al., 1986; Draper and Hadley 1990; Hamre et al., 2003) and *trans*-2-alkenals (Grootweld et al., 1998) seem to be readily absorbed from mammalian and fish gastrointestinal tracts. Furthermore, low molecular weight oxidation products from methyl linoleate were readily absorbed in rats (Oarada et al., 1986; Kanazawa and Ashida 1998b). The literature therefore indicates that secondary lipid oxidation products are absorbed, but we do not know if this is the case just for the selected aldehydes, or if other products than those specifically measured are absorbed.

Effects of secondary oxidation products

The term reactive carbonyl species (RCS) has been introduced to describe mainly α,β-unsaturated aldehydes (*trans*-2 alkenals), di-aldehydes and ketones, e.g. secondary oxidation products, because they have the ability to bind to different cellular constituents, such as aminophospholipids, protein and DNA, forming so-called advanced lipoxidation endproducts, ALE (Pamplona, 2008). The RCS group includes the aldehyde 4-hydroxynonenal (4HNE) formed from oxidation of n-6 fatty acids, and the aldehyde 4-hydroxyhexenal (4HHE) formed from oxidation of n-3 fatty acids, among a range of other compounds. In some cases, the carbonyl is esterified on phospholipid molecules, forming core aldehydes. Protection of cells against RCS is achieved by several repair mechanisms such as membrane remodelling, including lipid repair and replacement, and degradation of ALE-modified molecules (Pamplona, 2008). Strong electrophilic carbonyl compounds may induce rapid depletion of intracellular GSH concentration, due to the conjugation reaction with GSH. This reaction can occur spontaneously, but is also catalyzed by GSTs (Pamplona, 2008). RCS are also detoxified by oxidation with aldehyde dehydrogenases and through reduction by aldo-keto reductases, while modified proteins may be degraded by the proteasomes. Pamplona (2008) therefore proposed to view the formation and degradation of RCS and ALE as a dynamic steady state situation. Oxidative damage due to absorbed secondary oxidation products may occur if the protective mechanisms are saturated.

The aldehyde 4-hydroxynonenal (4HNE) is a widely studied molecule, resulting from oxidative stress, which also could be present in rancid food containing vegetable n-6 fatty acids, and is possibly absorbed (see Fig. 4.1.3.2-3). Conjugation of this molecule with protein has been demonstrated using immunohistochemistry towards 4HNE-protein adducts. 4HNE reacts at a high rate with cysteine and the reaction is reversible, giving 4HNE a potential role in protein signalling. It also forms conjugates with histidine and lysine (Petersen and Doorn, 2004). 4HNE, as other RCS compounds, is metabolized through oxidation, reduction and conjugation with GSH. Different aldehydes activate the detoxification pathways to different extents (Petersen and Doorn, 2004).

When aldehydes obtained after cleavage of lipid hydroperoxides, remain esterified to glycerolipids or cholesterols, the products are termed core aldehydes (see Fig. 4.1.3.2-4).

Oxidative cleavage of the arachidonic acid ester of glycerophosphocholine generated 5-oxovaleric acid ester of glycerophosphocholine, a core aldehyde that mimics platelet-activating factor (PAF) that activates endothelial cells to bind monocytes (Kuksis et al., 2003). Core aldehydes, including aldehydes esterified to cholesterol, have been identified in oxidised human LDL and aortic plaques. Some of these aldehydes induced platelet aggregation and thrombosis in a similar manner as PAF (Kamido et al., 2002; Leitinger, 2003). It is not clear in which concentrations such substances are present in oxidised oils. If ingested, they will probably enter the systemic pathways only after hydrolysis in the sn-2 position by digestive PLA_2. However, Hartvigsen et al. (2006) point out that lipid molecules with short fatty acid chains are absorbed directly from the intestine without prior digestion and that this could be the case also for short chain core aldehydes.

Studies on secondary oxidation products can be viewed as research on single compounds (mainly 4-HNE and MDA) representing a large range of diverse low molecular compounds as described in Chapter 4.1.3.2 The representatives are absorbed and seem to have potentially adverse health effects, since they bind to and modify protein and consume reduced GSH. There are no studies known to us that have performed quantitative toxicokinetics on secondary lipid oxidation products.

7.2.1.4 Effect studies of tertiary oxidation products

Literature on health effects of tertiary oxidation product, which are mainly polymers of oxidised fat or adducts of oxidised fat with protein and nucleic acids, seems to be lacking. However, these kinds of molecules seem to be digested, as shown by Henderson et al. (1993) who worked with polymers of triacylglycerols. If tertiary oxidation products contain radicals, they may cause injuries to the digestive tract. Digestion of tertiary oxidation products may give monomeric compounds with similar properties and effects as primary and secondary oxidation products, but other groups of products, such as fatty acid dimers from polymers of triacylglycerols, are also formed (Henderson et al., 1993).

7.2.1.5 Effect studies of oxidised cholesterol

Since fish oil contains cholesterol (4.7 g/kg, Torstensen, pers. com.), oxidised fish oil may contain oxidised cholesterol. When animals or humans are fed oxidised cholesterol, oxysterols appear in the plasma and are distributed in lipoproteins, indicating absorption from digestive tract (Linseisen and Wolfram, 1998; Staprans et al., 2000; 2003; Vine et al., 1998). It is important to discriminate between oxidised cholesterol and cholesterol bound to an oxidised fatty acid (Leitinger, 2003).

Focusing on cholesterol, a possible connection between oxidised dietary lipid and atherosclerosis has been investigated suggesting that intestinal absorption may cause lesions in internal organs. Plant and fish oil contain 3 and 5 g phyto- and zoo-sterols/kg, respectively, (Torstensen pers. com.), and high intake of dietary fat will be accompanied by high sterol intakes where a fraction may be oxidised. High (0.5-1% of diet) cholesterol levels that had been subjected to dietary oxidation produced increased oxidative susceptibility of plasma and lipoproteins and increased fatty streak formation in animal models of atherosclerosis, and in LDL knockout and apolipoprotein E deficient mice (Vine et al., 1998; Staprans et al., 2000). Repeated intravenous injections of NZW rabbits with oxidised cholesterol at 450 mg (similar to human plasma cholesterol level) over a 74-day period increased areas of vascular foam cell lesions, compared with animals that were injected fresh cholesterol (Rong et al., 1999). Furthermore, hamsters fed oxidised cholesterol at 0.1 and 0.05% of feed weight showed

increases in plasma cholesterol, aortic cholesterol and aortic plaque area, compared with animals fed a fresh cholesterol diet (Ng et al., 2008).

7.2.1.6 Effect studies of isoprostans

Another group of molecules resulting from lipid oxidation that have wide biological impact are the isoprostanes. These are cyclic oxidation products formed by non-enzymatic pathways, many of which are isomers to eicosanoids, and may mimic eicosanoid functions. The isoprostanes are formed in biological membranes from esterified fatty acids, and are then released by hydrolysis, probably through phospholipase A2 activity (Jahn et al., 2008; Belik et al., 2010). Research is focused on isoprostanes formed *in vivo.* Because isoprostanes have been reported to be e.g. mediators of inflammatory response and to have vasoactive properties, they may be involved in a number of physiological and/or pathological processes. Isoprostanes have also been suggested as a marker of oxidative stress in humans (Belik et al., 2010; Jahn et al., 2008; Nourooz-Zadeh et al., 1997; VKM, 2011). However, even though it has been suggested that EPA- and DHA-oxidative derivatives, "isoprostane-like" compounds also may be formed as a result of oxidation in fish oil (see Chapter 4.1.3.2), it is not clear to what extent isoprostanes are present in oxidised oils or absorbed by the intestinal tract.

7.2.2 EFFECT STUDIES IN HUMANS

Generally, in most of the human effect studies of n-3 fatty acids, the degree of fish oil oxidation and the influence of the decomposition substances on the observed effects have not been discussed. The evidence concerning possible adverse effects of fish oil lipid oxidation products in human intervention studies is therefore lacking. However, to the best of our knowledge, only a few postprandial studies have been performed by investigating the effects of dietary oxidised vegetable oils and these are summarized below. The long term effects of oxidised oils have, however, not been investigated. It is mainly one group who has worked with the issue of oxidised vegetable oils. Out of nine papers reviewed; six are from New Zealand and two from California/USA and one from Poland (Naruszewicz et al 1987; Staprans et al., 1994, 1999; Sutherland et al., 1999, 2002, 2010; Wallace et al., 2001; Williams et al., 1999, 2001).

Effects of oxidised vegetable oil

In order to study if oxidised PUFAs are associated with increased risk of CHD, randomized postprandial test meals studies were performed. In three of the nine studies, the aims were to study if oxidised PUFAs can be absorbed by measuring oxidised lipids, such as conjugated dienes and TBARS in chylomicrons (Staprans et al., 1994; 1999; Naruszewicz et al., 1987). In summary, when healthy males or diabetic subjects were fed oxidised corn or soy bean oil at high doses (1g/kg b.w.), oxidation products measured as increased TBARS and conjugated dienes, could be detected in chylomicrons. Patients with poor glycemic control appeared to accumulate more oxidation products in response to intake of oxidised oil. However, as described above, increased content of conjugated dienes in plasma does not prove that lipid peroxides were absorbed, since both aldehydes and hydroxyl fatty acids may contain these compounds (See Chapter 7.2.1.2, *Absorption of primary oxidation products*). The increase in TBARS is in line with animal studies, which show that secondary oxidation products are absorbed from the intestine (Draper et al., 1986; Draper and Hadley 1990; Hamre et al., 2003; Grootveld et al., 1998). In one of the papers, soybean oil was oxidised at 220°C for 7 hours and in total 100 g soybean oil were given to five healthy males, either as fresh oil or as oxidised oil (Naruszewicz et al., 1987). Chylomicrons (CMs) and plasma were isolated at baseline and 4 hours after intake of the test meals. An increase in plasma TBARS and in CM

Table 7.2.2 Overview of the oxidative status of oils/fats used in the references referred to in Chapter 7.2.2

Study	Type of experimental vegetable oil/fat	Oxidative status of the oil/fat			
		[1] Peroxides	[2] Carbonyls	[3] Conjugated diens	[4] TBARS
Naruszewicz et al 1987	Fresh soya bean oil Heated soya bean oil	[5] 1.6 meq/kg 4.8 meq/kg	[6] 7.8 meq/kg 35.6 meq/kg	na na	na na
Staprans et al., 1994	Corn oil (0.14mg vit E/g) Corn oil (no vit E) – low Corn oil (no vit E) – high	na na [8] 123 meq/kg	na na na	[7] 6.5-10 nmol/mg 30-50 nmol/mg oil 80-120 nmol/mg	na [4a] 0.041 nmol/ mg oil 0.103 nmol/ mg oil
Staprans et al., 1999	Corn oil (no vit E) Corn oil (no vit E) – high	na [8,9] 220-350 µmol/ mmol TG	na na	40-99 nmol/mmol TG [7,9] 100-200 nmol/mmol TG	na 4-10 µmol/ mmol TG[4a,9]
Sutherland et al. 1999	Unused cooking fat Used cooking fat (n=7)	[10] 0.5 meq/kg 2.1 (1.4-4.0) meq/kg	[11] 3.5 meq/kg 26.3 (12.9-34.5) meq/kg	na na	na na
Sutherland et al. 2002	Unheated safflower Heated safflower Unheated olive Heated olive	[12]12.8 meq/kg 52.8 meq/kg 13.6 meq/kg 24 meq/kg	[11] 6.0mmol/kg 30.0 mmol/kg 4.0 mmol/kg 29.4 mmol/kg	22.5 µmol/kg 57.9 µmol/kg 12.6 µmol/kg 24.1 µmol/kg	na na na na
Sutherland et al. 2010	Unheated sunflower oil (Vit E 1.62 mmol/kg) Heated sunflower oil (Vit E 1.33 ± 0.36 mmol/kg) Cream (i.e. saturated fat)	na na na	[13]4.3 mmol/kg 14.5 ± 1.2 mmol/kg (n=3) nd	17.7 mmol/kg[14] 33.4 ± 1.4 mmol/kg (n=3) [14a] 13.4 mmol/kg	na na na
Wallace et al., 2001	Thermally stressed safflower oil Thermally stressed olive oil	[10] 52.8 meq/kg 24.0 meq/kg	[11] 30.0 mmol/kg 29.4 mmol/kg	57.9 µmol/kg 24.1µmol/kg	na na
Williams et al., 1999	Unused cooking fat Used cooking fat	[12] 2.0 meq/kg 3.8 meq/kg	na na	na na	na na
Williams et al., 2001	Unheated safflower Heated safflower Unheated olive Heated olive	[10]12.8 meq/kg 52.8 meq/kg 13.6 meq/kg 24.0 meq/kg	[11]6.0 mmol/kg 30.0 mmol/kg 4.0 mmol/kg 29.4 mmol/kg	na na na na	na na na na

na – not analysed. nd – not detected. meq – milliequivalent peroxide [1] peroxides are primary oxidation products. In order to standardise the unit, some results is recalculated according to Frankel (2005): 2 x mmol/kg = meq/kg [2] carbonyls are examples of secondary oxidation products. [3] conjugated dienes are marker for lipid oxidation. [4] TBARS – thiobarbituric acid-reactive substances (marker for secondary lipid oxidation products). [4a] Measured by standard procedure according to Morel DW, Chisolm GM, J Lipid Res 1989; 30:1827-34. [5] Peroxide measured according to American oil Chemist Association, Official and Tentative Methods, 3rd edn., Am Oil Chem Soc, Chicago, IL, 1973. [6] Carbonyls measured according to Schwartz DP, Haller HS, Keeney M. Anal. Chem 1963;35:2191. [7] Conjugated diens measured by second-derivative UV spectroscopy in a Perkin Elmer 555 Spectrophotometer according to Corongiu FP, Banni S, Dessi MA. Free Radic Biol Med 1998;7:183-86. [8] Direct measurement of peroxides by use of a colour reaction with methylene blue derivatives was used, performed according to a method described by Staprans I, Rapp JH, Pan XM et al. J Clin Invest 1993; 92: 683-43. [9] TG – triglyceride. [10] Peroxide value measured according to American Oil Chemist Association, Official Method Cd 3a-63, 1989. [11] Carbonyl value determined by a modification of a spectrophotometric quinoidal ion method (Yukawa N, Takamura H, Matola T. J Am Oil Chem Soc 1993; 70:881-84). [12] Peroxide value measured according to American Oil Chemist Association, Official Method Cd 8-53, 1990. [13] Aldehyd content measured by the quinoidal ion method; described by Endo Y, Li CM, Targiri-Endo M et al. JAOCS 2001; 78:1021-4. [14] Conjugated dienes determined from the absorbance of hexane solution of the fats at 234nm; using molar absorption coefficient of 29500Lmol^{-1}cm^{-1}. [14a] Following extraction of cream (1g) with hexane/isopropanol were conjugated dienes measured in supernatant after dilution (1/100) with hexane, as described by Undeland I, Härröd M, Lingnert H. Food Chem 1998; 61:355-65.

were observed among all subjects after intake of oxidised oil, but not after intake of fresh oil (Naruszewicz et al., 1987).

In the other six studies (i.e. Sutherland et al., 1999; 2002; 2010, Wallace et al., 2001; Williams et al., 1999; 2001), the endpoints were serum paraoxonase activity, plasma MDA or TBARS level, serum lag time in conjugated diene formation, plasma fluorescent Schiff's bases (i.e. indicate aldehydes in plasma), susceptibility of isolated LDL to copper ion oxidation and impaired endothelial function. Randomized postprandial studies were performed with cross-over design using olive oil (rich in 18:1 n-9, oleic acid) or safflower oil (rich in 18:2 n-6; linolic acid) which was oxidised during use for deep-frying potato chips every hour in periods from 8h up to one week. The amount of oxidised fat was from 46g to 60g per test meal. The control meal contained either unused cooking fat or there was no control. The number of participants was 10-25 and plasma was taken at baseline and 4 hours (2.5h for diabetics) after intake of the meals.

Cooking oil that has been used for deep-frying influenced plasma paraoxonase activity (Sutherland et al., 1999; Wallace et al., 2001). Paraoxonase is a HDL-associated protein that has been shown to prevent LDL oxidation by decreasing the formation of lipid hydroperoxides (Fluiter et al., 1996). There are conflicting results as to whether intake of oxidised fat increase plasma TBARS, conjugated dienes and fluorescent Schiffs bases, since some studies showed no effects, while others showed an effect of control fat intake on these parameters (Sutherland et al., 1999; Wallace et al., 2001; Sutherland et al., 2002). LDL composition or lag time for copper induced oxidation did not seem to be influenced by the oxidised and used oils (Sutherland et al., 1999, 2002, 2010; Wallace et al., 2001).

Oxidised oil may also play a role in dilation of blood vessels (Williams et al., 1999, 2001).

An overview of the reported oxidation status of the experimental oils is given in Table 7.2.2.

7.3 *Trans*-fatty acids

Trans-fatty acids are not lipid oxidation products, but may be formed during thermal treatment during the production process of fish/marine oils (Chapter 2.3, and Tables 6.1 and 6.2.3). It is well documented that *trans*-fatty acids at high dietary intakes leads to e.g. increase in LDL cholesterol (>4% of dietary energy) and decrease in HDL cholesterol (>6% of dietary energy), factors that are associated with increased risk of coronary heart disease (Hunter, 2006; Helsedirektoratet, 2011). Additionally, dietary intake of *trans*-fat is associated with increased risk for diabetes type-2 (Helsedirektoratet, 2011). Therefore, several national health organisations including the Norwegian Directorate of Health have given recommendations to limit the intake of *trans*-fatty acids to less than 1% of energy intake or to keep it as low as possible. According to Helsedirektoratet (2011), there are no consistent data that indicate other adverse effects, such as increased cancer risk, than increased risk for coronary hearth disease and type 2-diabets of dietary intake of *trans*-fatty acids.

7.4 Cyclic fatty acid monomers

Cyclic fatty acid monomers (CFAM) are produced from polyunsaturated fatty acids during heating and may be formed in several of the steps during oil refining. CFAM are not easily removed from the oil and will be present in the final product. The studies that were found on CFAM had been using plant oil derived compounds; however, CFAM will also be formed in heated marine oils and with greater complexity than in vegetable oils, because of the longer and more unsaturated n-3, compared to n-6, fatty acids.

CFAM are absorbed from the intestine at a similar rate as native fatty acids when they are esterified in the sn-2 position of triacylglycerols, but at a slower rate when they are present as free fatty acids or esterified in the sn-1,3 positions (Martin et al., 1997). Two studies where rat cardiac myocytes were cultured with CFAM in the medium showed that the CFAM were incorporated in both triacylglycerol and phospholipids of the myocytes and had adverse effects on electromechanical properties and susceptibility to oxygen depletion of the myocytes (Ribot et al., 1992; Athias et al., 1992). A study with porcine endothelial cells showed similar results, with incorporation of CFAM into polar and neutral lipids and loss of monolayer integrity, lowered Ca^{2+} ATPase activity and increase in prostacyclin synthesis and release (Flickinger et al., 1997). Lowered activities of enzymes from rat liver involved in β-oxidation towards CFAM than towards native fatty acids, indicates that CFAM have a slower catabolism rate than native fatty acids, which may lead to accumulation and toxic effects of these fatty acids (Joffre et al., 2001). However, subsequent experiments indicate that the unaltered structures of CFAM are catabolised at the same rate as native fatty acids, but the ring structure is conjugated and excreted in the bile and urine (Bretillon et al., 2006; Joffre et al., 2004). This corresponds with the finding that CFAM stimulates the detoxification systems of the liver of rats, such as cytochrome P-450 and NADPH cytochrome P-450 reductase (Lamboni et al., 1998). Other enzyme activities are also altered in response to feeding rats with CFAM, such as enzymes involved in lipid synthesis and catabolism (Martin et al., 2000) and carbohydrate metabolism (Lamboni et al., 1998). Some of the effects on lipid metabolism are suggested to be mediated through a PPAR-α dependent mechanism (Bretillon et al., 2003; Martin et al., 2000).

The limited literature on CFAM therefore shows that these compounds may have profound effects on a wide variety of biological processes. However, we found no dose response studies or other studies that could give an indication of which quantities of CFAM that may have adverse effects on human health.

7.5 Summary of hazard characterisation

Decomposition substances like free fatty acid and lysophospholipids in the diet will in most cases be absorbed and esterified in the enterocytes to TAG or PC, therefore there is limited effect of these components on inner organs. Free fatty acids may in high concentrations excert toxicicity to the intestinal mucosa. Reports on adverse health effects of dietary lysoPL were not found, but the amphiphilic character of the molecules is likely to give them cytotoxic properties.

Only one animal study on health effects of oxidised fish oil was performed, whereas several such studies have been done with oxidised plant oils in both animal and humans. The majority of the studies performed using oxidised plant oils and the method of oxidation was mainly heating, to reproduce the effects of oils used in deep-frying of food. Heated vegetable oils contain the same groups of oxidation products as oxidised fish oil, e.g. primary lipid hydroperoxides, secondary short chain products such as aldehydes, and tertiary oxidation products such as polymers. The actual compounds within the different groups of oxidised fish oil and plant oil are, however, different. The mode of oxidation may also affect the distribution of compounds in the oxidised oils. The high complexity of the oxidation process, the large number of products formed and the difficulties in analysing the oxidation status of oils are possible reasons why dose response studies have not been conducted on effects of oxidised lipids on animal or human health. Rather the studies performed have been designed with two groups, one exposed and one control group, and it is therefore difficult to draw conclusions regarding what may be safe levels of oxidation products.

Oxidised oils at high doses (PV 200-900 meq/kg as compared to 3-33 meq/kg, Table 7.2.1) produce adverse health effects in animals. When oils oxidise, antioxidants and polyunsaturated fatty acids are consumed, concomitantly with production of lipid oxidation products. Experimental animals also decrease their feed intake when fed oxidised oil. In some studies, these factors are corrected for by addition of vitamin E to the oxidised oil and of saturated fatty acids to the fresh oil and by use of restricted feeding. In the studies where the confounding factors were not corrected, vitamin E deficiency lowered feed intake and differences in dietary fatty acid composition may have affected the results. In these studies, the effects of oxidised oils in rat models included reduced body weight, increased relative liver and kidney weight, reduced liver lipid, increased amounts of peroxisomes and activation of PPARα responsive genes in liver, increased membrane fluidity and shift in membrane bound enzyme activities, increased plasma levels of organ specific enzyme activities (ALAT, ASAT, ALP), indicating cell damage and leakage, increased liver and serum TBARS and reduced vitamin E and liver glutathione. When the confounding factors were corrected for, the number of responses was reduced. There were no effects on body weight gain, but change in relative liver weight, amount of triacyl glycerol in the livers, PPARα responsive gene expression and thyroid hormone metabolism were observed. Many of the pathological changes observed in studies with oxidised oil may therefore be effects of vitamin E deficiency. These pathological changes can be partly reversed when the animals are supplemented with extra vitamin E. The one study on health effects of fish oil used fish oil with PV 328 meq/kg at 3% in the diet (50 g lipid/kg, plant oil added). There was no control of the confounding factors. The results of this study are largely similar to those from studies with oxidised plant oils.

There are conflicting results regarding the absorption of lipid hydroperoxides in vertebrates, probably because of protective mechanisms that inhibit absorption and are overwhelmed at high doses of oxidised lipids. One of these mechanisms is degradation of lipid peroxides to aldehydes before they reach the intestinal tract. Another mechanism is reduction of fatty acid hydroperoxides to hydroxy fatty acids by the enterocytes. Hydroxy fatty acids still contain the conjugated diene bond. In many studies, conjugated dienes in plasma are used as indications of absorption of lipid hydroperoxides and it is important to interpret these results with caution. There seems to be an agreement that aldehydes and oxidised cholesterol are absorbed from the intestine in vertebrates. Due to the differences in absorption between the different lipid oxidation products, the intestinal tract will be exposed to a whole range of oxidation products, while other organs are protected from exposure to primary products, which probably are the most toxic. There are very few studies on possible effects of oxidised lipids on the gut, but compromised red-ox status of enterocytes after exposure to lipid hydroperoxides, indicate that gut injury may be a consequence of dietary oxidised oil.

Secondary oxidation products, especially the aldehydes, are toxic because they have the ability to bind to and modify different cellular constituents, such as aminophospholipids, protein and DNA. The group of products includes 4HNE, formed from oxidation of n-6 fatty acids and 4-hydroxyhexenal (4HHE), the corresponding aldehyde formed from n-3 fatty acids. Malondialdehyde and *trans*-2 alkenals appear to be absorbed from the gastrointestinal tract in vertebrates, and may affect inner organs as well as the gastrointestinal tract. Measurements of absorption of other secondary oxidation products have not been performed. When the aldehyde remains esterified to glycerolipids or cholesterol, the compound is called a core aldehyde. Several core aldehydes have similar effects on blood vessels as platelet activating factor, leading to fatty streak formation and coronary heart disease. Aldehydes, including core aldehydes, have also been identified as possible mediators of signal transduction. Animals have developed numerous protection mechanisms against secondary

oxidation products and for these compounds to cause adverse health effects, the exposed individual must be weak or the protection mechanisms must be overwhelmed by high concentrations.

Plant oil and fish oil contain 3-5 g/kg cholesterol that may be oxidised with the rest of the oil. Several studies with animal models of CHD, such as the New Zeeland white rabbit, show that the ingestion of oxidised cholesterol at high doses may increase the incidence of fatty streaks.

To the best of our knowledge, no studies have investigated the potential long term adverse effects of lipid oxidation products of marine origin on human or animal health. The studies that have been performed so far almost exclusively used thermoxidised vegetable oils, which contain other oxidation products than oxidised fish oil. The literature shows that lipid oxidation products are potentially toxic, that animals and humans have inherited protective mechanisms against dietary oxidation products and that these mechanisms are overwhelmed at high doses. The doses of oxidised oil shown to have negative health effects in laboratory animals are very high (5-20% of the dry diet at PVs of 200-900) compared to the exposure of humans by ingesting fish oil as food supplement (up to 15 ml cod liver, see Chapter 8.1). The doses of oxidation products used in the human studies (45-69 g oil/test meal with PVs 2-53 meq/kg) were generally lower than those used in studies with laboratory animals. The results were conflicting. Sometimes markers of lipid oxidation such as plasma TBARS and conjugated dienes were increased and sometimes not. The enzyme paraoxinase, which prevents lipid hydroperoxide formation in lipoproteins, showed increased activity after intake of oxidised fat, while the lag time of copper-induced LDL oxidation was not affected. The research on health effects of oxidation products has revealed that several biological processes are affected in animals consuming oxidised oil, and one can expect that more effects will be identified in the future. However, the knowledge is not sufficient to identify one or a few mechanisms that can be used as definitive toxicological endpoint, and what may be a safe level of oxidation products has not been identified.

High dietary intakes of *trans*-fatty acids have been documented to produce increases in LDL cholesterol and decrease in HDL cholesterol. These factors have been associated with increased risk of coronary heart disease.

There are few data on health effects of CFAM. However, the limited data shows that CFAM may produce profound effects on several biological processes. No dose response studies or other studies with CFAM concentrations that may produce adverse health effects on human have been identified.

8. Exposure characterisation

8.1 Consumption of fish oil as food supplement

Consumption of fish oil supplements varies in the population. In a study based on 40,108 pregnant women participating an the Norwegian Mother and Child Cohort Study (MoBa) 58.6% reported consumption of cod-liver oil or fish oil during the first 4-5 months of pregnancy (Haugen et al., 2008). National dietary surveys conducted among representative samples of Norwegian children and adolescents also showed that fish oil supplement use is widespread, especially in the youngest age groups. At 6 and 12 months age, 40% and 39% of the children received cod-liver oil (Øverby et al., 2008, Kristiansen et al., 2009). Among 4 year old children, 27% were cod-liver oil consumers, and approximately 15% consumed cod-liver oil 5-7 days a week (Pollestad et al., 2002). Among children in 4th grade (8-9 years old), 10% of the girls and 13% of the boys used cod-liver oil 5-7 days weekly. Among children in

8th grade (13-14 years old), 3% of the girls and 5% of the boys were frequent users (Øverby and Andersen, 2002).

Among consumers, it can be anticipated that majority of the cod-liver oil portion is approximately the amount recommended for the product. The volume most often recommended on bottles of cod-liver oil is 5 millilitres (ml). Traditionally a table-spoon of cod-liver oil has been used, and as the size of table-spoons varies, the consumed amount varies too. Furthermore, it is possible that some individuals consume more than one fish oil supplement, or more than the recommended amount. For exposure calculations, 5 ml cod-liver oil can be used as a mean portion for children older than 6 months old and adults. As a large portion in adults, 15 ml can be used. Among children 4 weeks to 6 months, 2.5 ml cod-liver oil (similar to the recommendation) can be used as a mean portion.

Different capsules contain different volumes of oil, but generally the oil intake is lower from capsules than from bottled cod-liver oil if the recommendation on the product is followed. For a commonly consumed capsule product the recommended amount (i.e. two capsules) corresponds to 1.2 g oil (1.3 ml) (VKM, 2006). Provided that the concentration of potential toxic substances is not substantially different in cod-liver oil and capsules with marine oils, a worst case scenario of daily consumption of 15 ml cod-liver oil would represent higher intake than expected from consumption of fish oil in capsules.

8.2 Intake of oxidation substances from marine oils

As peroxide and anisidine values are relative, not quantitative, measure of oxidation it is not possible to calculate exposure to oxidation products in fish oil based on such values.

There are insufficient or no available data on concentration of polymers, oxidised cholesterol, other lipid oxidation products, geometric isomers (including *trans*-fatty acids), polymers, cyclic fatty acid monomers or free fatty acids in fish oil, and exposure cannot be calculated.

Additionally, there is no information about unwanted substances such as e.g. oxidation products in marine oil used in fortified foods. For data on use of marine oil in fortified foods, see VKM (2011).

9. Risk characterisation

A quantitative health risk assessment cannot be performed because of insufficient information to perform a full hazard identification and characterisation and lack of quantitative occurrence data for products consumed for an exposure characterisation.

There is insufficient information on potential adverse effects of oxidised marine oils and substances of concern occurring in marine oils as a result of degradation and processing. Limited studies on oxidised vegetable oils and some oxidation products indicate that these compounds might be a matter of concern. There is however, insufficient information on which doses that adverse effects might occur. In addition, an exposure calculation could not be done because of lack of quantitative occurrence data on such substances in n-3 fatty acid food supplements at the time of consumption. The VKM also notes that the animal experiments with whole oxidised plant oils have been conducted with high amounts of oxidised oil and are thus not comparable with a potential maximally 15 ml daily consumption of marine oil, as anticipated for high consumers of such oils.

Consequently, a qualitative risk assessment has been performed where possible.

Oxidised marine oils

It should be noted that there is very little or no information in the public domain with respect to composition of the identified decomposition substances and oxidation products in food supplement of marine oil origin.

Based on the very limited information available VKM concludes that there is some concern related to regular consumption of oxidised marine oils. One of the most important protection mechanisms against oxidised lipids in animals and humans is the natural avoidance because of unpleasant taste and odour resulting from volatile oxidation products. The natural avoidance reaction should to some extent prevent humans from consuming rancid marine oils as the volatile oxidation products produce a bad smell and taste with very low thresholds. However, a number of other secondary and tertiary oxidation products, which might also represent a health concern, do not have such properties.

Encapsulation and microencapsulation of marine oils as well as addition of flavourings will conceal bad taste and smell of rancid products and thereby surpass the natural avoidance reaction. VKM notes that this might represent a consumer risk and could therefore be of concern.

Oxidation of marine oil can continue after the product is bottled or encapsulated. The lack of adequate analytical methods hampers the control of oxidation level in n-3 fatty acid food supplements with food additives and/or flavourings. The same applies for microencapsulated marine oils used for food fortification.

Oxidation products

Primary, secondary and tertiary oxidation products may occur in n-3 fatty acid food supplements. However, the occurrence and concentration of oxidation products in commercial n-3 products are not very well documented in available literature.

In randomised postprandial test meal studies in humans, conflicting effects on plasma lipoprotein, TBARS, conjugated dienes and fluorescent Schiff's bases have been reported between studies with oxidised vegetable oils and control oils. However, the lack of studies with marine oil and the lack of long-term studies anyway prelude the use of the identified human studies for risk assessment.

Only one study on effects in animal with oxidised marine oil, as an ingredient in feed, was identified. This study showed reduced body weight, but did not provide feed consumption data and did not control the content of vitamin E (i.e. antioxidant) in the feed. In most of the studies, the experiments were performed with oxidised vegetable oil that have been heated or used for deep-frying (see Table 7.2.1). The products formed during oxidation are to some extent similar, because they belong to the same categories, e.g. lipid hydroperoxides, aldehydes and so forth, but differ in specific compound distribution within each category. Several of the studies with oxidised vegetable oil that were of satisfactory quality indicate an increase in relative liver weight, increased amount of triacylglycerol in the livers, changes in PPARα responsive gene expression and thyroid hormone metabolism in experimental animals. However, none of these studies provided dose-response data or reported adverse effects in the animals, and were not useful in risk assessment.

In general it is desirable that the level of **primary oxidation products (peroxides)** remains as low as possible as it is not clear to what extent the conversion of hydroperoxides to hydroxyl fatty acids during absorption reduces the adverse health effects of dietary oxidised lipids.

Long term exposure to dietary lipid peroxides may also have negative effects locally in the gastrointestinal tract.

Secondary oxidation products are absorbed and may have negative effects on health. Especially the aldehydes are potentially toxic because they can bind to and modify different cellular constituents such as aminophospholipids, protein and DNA. Aldehydes, including core aldehydes, have also been identified as possible mediators of signal transduction and have effects on blood vessels and platelet activating factor, leading to fatty streak formation and coronary heart disease. However, reliable results are difficult to obtain since these compounds cannot be measured quantitatively by current method. Additionally, it is technically challenging to isolate these compounds from other oxidation products. Consequently, very little is known about concentration of aldehydes/core aldehydes and a quantitative risk assessment cannot be performed.

Even less is known about occurrence and possible health effects of other secondary oxidation products such as ketones, alcohols, alkans and alkens in fish oils, and risk assessment cannot be performed. The same is the case for **tertiary oxidation products** such as polymers of oxidised fat.

Decomposition substances from triacylglycerols/phospholipids in fish oil

Free fatty acids and lysoPL may be produced in the fish/cod liver raw material by lipolysis. Most of these compounds are removed from the oil during the refining process, but they may still be present at low concentration in refined fish oil.

FFA and lysoPL in the general diet will in most cases be absorbed and esterified in the enterocytes to TAG or PC. It is desirable that the concentration of FFA remains as low as possible due to their potential harmful effects to the intestinal mucosa. Different guidelines for free fatty acid concentrations have been suggested by CODEX, Ph. Eur. or GOED. At low levels free fatty acids is not expected to pose any health risk because they are absorbed and esterified in the enterocyte. However, safe levels cannot be derived based on current knowledge. It might be a health concern if products that rapidly release pure free fatty acids are consumed.

Although lysoPL might have cytotoxic properties, the concentration of LysoPL in fish oil is most probably very low since triacylglycerols are the major lipid source in fish. Additional intake of LysoPL from fish/cod-liver oil food supplements will thus not be of any health concern.

Concentration of cholesterol in fish oil is low and the fraction of **oxidised cholesterol** even lower. As the intake of oxidised cholesterol from fish oil is low compared with fat from the rest of the diet, the additional intake of oxidised cholesterol from fish oil food supplements is not expected to pose any additional health risk.

Substances formed due to thermal treatment of marine oils

Trans-fatty acids and cyclic fatty acid monomers are possibly formed by thermal treatment of polyunsaturated fatty acids during the refining and concentration processes. It is recommended by the Norwegian Directorate of Health to keep the intake of *trans*-fatty acids to less than 1% of the energy intake or as low as possible (Helsedirektoratet, 2011). Based on limited information indicating low concentrations of *trans*-fatty acids in fish oils, intake of *trans*-fatty acids from fish oils is low compared with the rest of the diet and is not expected to pose any additional health risk.

The lack of available data on both occurrence of cyclic fatty acid monomers in fish oil and possible health effects made it impossible to perform the risk assessment of CFAM in fish oil.

Substances of concern in krill oil

The risks associated with decomposition substances, oxidation products and substances formed due to thermal treatment of fish oil may also apply to oil derived from krill, but processing details about krill oil is limited. LysoPL and oxysterols might be present in higher concentration in krill oil than in fish oil due to higher proportions of phospholipids and cholesterol in the raw material. However, no occurrence data were found on any of the substances in krill oil.

Substances of concern in seal oil

The risks associated with decomposition substances (except from proteins), oxidation products and substances formed due to thermal treatment of fish oil may also apply to seal oil.

10. Conclusions

Production of n-3 fatty acid food supplement

The production line for n-3 fatty acid food supplements (i.e. cod-liver oil, fish oil, krill oil, seal oil and concentrated n-3 fatty acids) may consist of many production steps dependent on the composition and quality of the raw material and the final concentration of n-3 fatty acids in the food supplements.

To be suitable for human consumption, crude fish oil/cod-liver oil has to undergo different refining processes for decreasing or removing impurities. The industry producing marine oil for human consumption in Norway (primarily refiners) exploit crude fish oil imported mainly from Chile, Peru and Morocco or crude fish/cod-liver oil produced in Norway. The latter is mostly produced from cut-offs from herring and farmed salmon, and cod liver. Refined and concentrated marine oils in bulk are usually stored and traded in airtight and well filled containers, protected from light and sealed under an inert gas. Refined oil and concentrated n-3 fatty acids in bulk usually have a defined fatty acid profile and known e.g. oxidation status and content of free fatty acids until the seal is broken. The refiners may bring their product directly on the market or sell their product either to value added resellers (e.g. marketers, distributors, retailers), or to encapsulators. Oil of a certain composition and fatty acid profile is ordered from the buyers. The buyers might store the load; transport it abroad for encapsulation; split the load (involving seal breaking) and/or further trade it. The number of distribution steps involved before the product is available for consumer is unknown.

In connection with the bottling or encapsulation processes, vitamins, antioxidants, stabilisers and colour, as well as flavourings (e.g. lemon, peppermint, orange) may be added to the oil and vegetable oils may be mixed in.

At present, there is no available information in the open literature on the shelf-life duration of n-3 fatty acid food supplements and way of assessment of shelf-life duration.

Decomposition substances, oxidation products and process-generated substances

The presence of decomposition substances, oxidation products and process-generated substances in n-3 fatty acid food supplements is dependent on the composition and treatment of the raw materials, processing parameters for extraction of crude oil, refining and concentration techniques, potential processing techniques for encapsulation (e.g. microencapsulation) and the storage/transport conditions throughout the whole value chain.

Marine lipids contain long chained n-3 polyunsaturated fatty acids (LC-PUFAs) that are highly susceptible to oxidation, which is an on-going process. Throughout the value chain of the marine n-3 fatty acid food supplement production, the main challenges are to reduce the risk of oxidation and thermal transformation of the n-3 fatty acids to minimise the content of substances such as oxidation products, polymers, *trans*-fatty acids and cyclic fatty acid monomers (CFAM) in the n-3 fatty acid food supplements. Several of the oxidation products are removed or reduced during refinement, but may be reintroduced by oxidation in the period between the ends of the final refining/concentration process and until the n-3 fatty acid food supplement is consumed, e.g. during storage of the bulk refined or concentrated oil, encapsulation, packaging, at the retailer and during storage of the product at home. Quantitative data on concentration and occurrence of such substances in n-3 fatty acid food supplements are very limited in the open scientific literature. The commonly used methods for analysing these substances in n-3 fatty acid food supplements are far from optimal.

Data on the influence of raw fish material freshness on the oxidative quality of extracted oil and n-3 fatty acid food supplements for human consumption is lacking. Furthermore, scientific literature on the influence of processing steps and conditions on the content of decomposition substances, oxidation products and process-generated substances in the n-3 fatty acid food supplements, in addition to detailed descriptions of the whole industrial processing line for n-3 fatty acid food supplements, is more or less lacking.

Krill and seal oil

Krill and seal as raw materials for production of n-3 fatty acid food supplements are not evaluated in detail in this risk assessment. Norway imports small volumes of crude seal oil and export the refined seal oil. Seal oil is derived from seal blubber and refining and processing of seal oil is very similar to that of crude fish oils. The same decomposition substances, oxidation products and substances formed due to thermal treatment of fish oil may also apply to seal oil. Krill catch is semi processed onboard and further oil processes may take place onshore in South America. Information on processing details for krill oil is very limited. The same decomposition substances, oxidation products and substances formed due to thermal treatment of fish oil may also occur in krill oil. Due to higher proportions of phospholipids and cholesterol in the raw material, the decomposistion products lysophospholipids and oxysterols might be present at higher concentration in krill oil than in fish oil. No occurrence data of any of the substances associated with processing of krill oil were found.

Analytical methods to examine decomposition substances, oxidation products and process-generated substances in n-3 fatty acid food supplements:

- Peroxides and aldehydes are measured by standard methods such as peroxide value (PV) and anisidine value (AV), respectively. However, these methods do not give a true quantitative measurement of the oxidative status of the oil. Some food additives and flavourings in the n-3 fatty acid food supplements also distort the results
- To provide more detailed information about lipid oxidation products in n-3 fatty acid food supplements, advanced methods such as GC-MS and LC-MS should be used. Standardisation of these methods would also be needed
- Optimisation is needed for methods determining tertiary oxidation products in n-3 fatty acid food supplements
- Methods for determination of process-generated substances (e.g. *trans*-isomers) are either at a developing stage or not used to an extent that has revealed the reliability of the method (e.g. like determination of cyclic fatty acid monomers)

- Accelerated stability methods such as measurement of oxidative stability index are needed. Currently there are no standard accelerated stability methods that would be enabling the determination of the shelf-life of fish oil
- The lack of adequate analytical methods hampers the control of oxidation level in microencapsulated marine oils, which are used for food fortification, and which also may be used in some n-3 fatty acid food supplements

Exposure

An exposure calculation could not be done because of lack of quantitative occurrence data on the substances in question in n-3 fatty acid food supplements at the time of consumption.

Hazard identification, characterisation and qualitative health risk assessment of substances of potential concern in n-3 fatty acid food supplements:

A quantitative health risk assessment cannot be performed because of insufficient information to perform a full hazard identification and characterisation. Consequently, a qualitative risk assessment has been performed where possible.

- **Microorganisms present in the raw material** are **not likely to be carried over** to oil for human consumption due to considerable heat treatment both of the raw material and the oil during processing.
- **Decomposition substances from proteins in the raw material,** such as di-peptides, free amino acids, trimethyl amine and biogenic amines, as well as processing aids and substances used in preserving the raw materials such as formic acid, are water soluble and largely removed by several washing step during the refining processes and will normally not be detectable in the n-3 fatty acid food supplements.
- **Decompositions substances from lipids** in fish muscle, cut-offs and liver that may be present in n-3 fatty acid food supplements include free fatty acids, lysophospholipids, oxidised cholesterol and oxidation products:

  Free fatty acids (FFA) and mono/diacylglycerols can be presented in low concentrations in n-3 fatty acid food supplement. Different guidelines for free fatty acid concentrations have been suggested by CODEX, European Pharmacopeia or GOED. The concentration of these compounds is reduced during the different processing steps and mainly by neutralisation (for FFA). However, FFA and mono/di-acylglycerols may be formed in production of n-3 fatty acid concentrates in the form of ethyl esters or glycerols. At low concentrations, free fatty acids are not expected to pose any health risk because they are absorbed and esterified in the enterocyte. However, safe levels cannot be derived because of insufficient information. It is desirable that the level of free fatty acids remains as low as possible due to their potential harmful effects to the intestinal mucosa. It might be a health concern if products that rapidly release pure free fatty acids are consumed.

  Lysophospholipids (lysoPL): Phospholipids represent a minor part of lipids from fish raw materials. The amounts of lysoPL possibly formed are mostly removed during oil processing. The concentration of LysoPL in fish- and cod-liver oil is most probably very low, and not of any health concern. No information on levels of lysoPL in n-3 fatty acid food supplements has been found.

  Oxidised cholesterol: As the intake of n-3 fatty acid food supplements is low compared with fat from the rest of the diet, the presence of low amounts of oxidised cholesterol in n-

3 fatty acid food supplements does not add significantly to that from other fat sources and is therefore not expected to pose health risk.

- **Oxidation products:**

It should be noted that there is very little or no information in the public domain with respect to composition of the oxidation products in food supplement of marine oil origin, as well as the related toxicological effects of oxidation products in humans.

Experimental animal studies indicate that high doses of whole oxidised vegetable oils can affect health negatively. However, the data were not sufficient for risk assessment.

Based on the very limited information available, VKM concludes that there is some concern related to regular consumption of oxidised marine oils. The natural avoidance reaction should to some extent prevent humans from consuming rancid marine oils as the volatile oxidation products produce a bad smell and taste with very low thresholds. A number of other secondary and tertiary oxidation products, which might also represent a health concern, do not have such properties.

Encapsulation and microencapsulation of marine oils as well as addition of flavourings will conceal bad taste and smell of rancid products of marine oils and thereby surpass the natural avoidance reaction. VKM notes that this might represent a consumer risk and could therefore be of concern.

- **Primary oxidation substances** such as peroxides are odour and tasteless and can be detected in n-3 fatty acid food supplements. The concentration of primary oxidation products is reduced during the refining process, but peroxides may be formed as a result of autooxidation of the oil during transport and storage before consumption of n-3 fatty acid food supplements. The peroxide value (PV) will indicate the amount of these substances.

  It is desirable that the level of primary oxidation products (peroxides) remains as low as possible as it is not clear to what extent the conversion of hydroperoxides to hydroxyl fatty acids during absorption reduces the adverse effects of dietary oxidised lipids. Long term exposure to dietary lipid peroxides may also have negative effects locally in the gastrointestinal tract.

- **Secondary oxidation products** include both volatile and non-volatile oxidation products, which can be detected in n-3 fatty acid food supplements. The anisidin value (AV) indicates the content of both non-volatile and volatile aldehydes.

  The volatile secondary oxidation products, which are short chain cleavage product such as aldehydes and ketones, can also be formed as a result of autooxidation during transport and storage of oil and food supplements. These volatiles are responsible for the fishy odour and taste of the products. Encapsulation or microencapsulation and/or addition of food additives and flavouring may camouflage the content of these decomposition substances.

  The non-volatile secondary oxidation products are odour and tasteless and can also be formed as a result of autooxidation during transport and storage of oil and food supplements.

  Some of the secondary oxidation products are absorbed and may have negative effects on health. However, the methodologically limitations in isolation and quantification of these compounds from other oxidation products, hampers the risk assessment.

Very little is known about both concentration of aldehydes/core aldehydes and other secondary oxidation products in n-3 fatty acid food supplements and their potential health effects. Therefore a risk assessment cannot be performed.

- **Tertiary oxidation products** are relatively stable high molecular substances such as dimeric and trimeric triacylglycerols (i.e. polymeric compounds) that are odour and tasteless and can be present in n-3 fatty acid food supplements. Tertiary oxidation products may be formed as a result of autooxidation of oil prior to refining and after encapsulation, in addition to thermal treatment of oil during processing. Little is known about the concentration of tertiary oxidation products such as polymers of oxidised fat in n-3 fatty acid food supplements and their potential health effects. Therefore a risk assessment cannot be performed.

- **Substances formed due to thermal treatment during processing** are *trans*-fatty acids, polymers and cyclic fatty acid monomers (CFAM) that can be present in n-3 fatty acid food supplements and are odour and tasteless.

Based on limited information indicating low concentrations of *trans*-fatty acids in marine oils, intake of *trans*-fatty acids from n-3 fatty acid food supplements is low compared with the amount from the rest of the diet. The amount from n-3 fatty acid food supplements is not expected to add significantly to the health risk.

The lack of available data on both occurrence of polymers and CFAM in n-3 fatty acid food supplements and possible health effects made it impossible to perform a risk assessment of polymers and CFAM.

11. Knowledge gaps

Lack of knowledge was identified in the following areas:

Raw material

- influence of raw fish material freshness on the oxidative quality and stability of extracted oil and n-3 fatty acid food supplements for human consumption

Processing of natural fish/liver oil and concentrate

- the influence of industrial processing steps and concentration techniques on the occurrence of decomposition substances, oxidation products and process-generated substances in refined marine oil/concentrates
- the influence of phospholipids on the oxidative stability of n-3 fatty acid food supplements: both what effect the presence of phospholipids may have on the oxidative stability of the fish oil, as well as whether the oxidation pathways and resulting oxidation products are different for n-3 fatty acids in the form of phospholipids compared to in the form of triacylglycerol
- the presence and formation of isoprostanes in marine oils

n-3 fatty acid food supplement

- the qualitative and quantitative content of oxidation products (i.e. primary, secondary and tertiary oxidation products), lysoPL, FFA, *trans*-fatty acids, cyclic fatty acid monomers and polymers in commercial n-3 fatty acid food supplements
- shelf-life duration of n-3 fatty acid food supplements

- encapsulation and packaging material (i.e. composition and properties) and its influence on shelf-life duration of n-3 fatty acid food supplements

Toxicological studies

- effects of oxidised marine oils in experimental animals or in humans
- health effects of defined and isolated oxidation products, cyclic fatty acids monomers and polymers from marine oils (animal models and human studies), including long term effects on the digestive tract
- mechanisms of toxicity of, and protection against, the different categories of oxidation products

Methods

- the strength, weakness and limitations (e.g. effect of additives) of the commercial methods (PV and AV) commonly used for analysing of oxidation products in n-3 fatty acid food supplements
- the usefulness of state of the art methods to quantitatively examine the n-3 fatty acid food supplements for oxidation products, *trans*-fatty acids, cyclic fatty acid monomers and to include these as standardisation of more advanced techniques for quality evaluation of n-3 fatty acid food supplements

Krill oil production/products

- effect studies of different processing steps on the composition of n-3 fatty acid food supplements from krill (e.g. content of processing aids, decomposition substances from lipids, content of LysoPL and oxysterol)
- elucidation of the oxidation processes of marine phospholipids

12. Recommendations

Occurrence of substances of concern

- The presence of oxidation products (i.e. primary, secondary and tertiary oxidation products), lysoPL, FFA, *trans*-fatty acids, cyclic fatty acids monomers and polymers in n-3 fatty acid food supplements should be examined and documented
- The current practice of 1 to 2-years shelf-life for encapsulated products should be investigated with the aim to develop standard requirements of documentation, included the influence of encapsulation material

Toxicological studies on oxidised marine oils

- Possible adverse effects of oxidised marine oils in model animals, including long term effects on the digestive tract should be investigated
- More information on effects of defined and isolated oxidation products, cyclic fatty acids monomers and polymers from marine oils should be obtained
- Mechanisms of the biological responses from oxidation products and protective mechanisms should be investigated to facilitate future risk assessment

Chemical analysis

- The usefulness of applying existing standard analytical methods (AV, PV) on n-3 fatty acids food supplements containing food additives and flavourings on the market today should be evaluated

- Standardised state of the art analytical methods should be developed for the determination of oxidation products and other substances of concern in marine oils and n-3 fatty acid food supplements
- Standardised oxidative stability methods should be developed for assessment of shelf-life of n-3 fatty acid supplements

Develop standards for food supplements derived from marine oils

- International quality criteria concerning content of decomposition substances, oxidation products and process-generated substances in n-3 fatty acid food supplements should be developed.

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APPENDIX A

REGULATION (EC) No 853/2004 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 29 April 2004 laying down specific hygiene rules for food of animal origin

- ANNEX III; **Specific requirements**
- Section VIII; **Fishery Products**
- Chapter IV; **Requirements for certain processed fishery products**

B. Requirements for fish oil intended for human consumption

1. Raw materials used in the preparation of fish oil for human consumption must:

a) come from establishments, including vessels, registered or approved pursuant to Regulation (EC) No 852/2004 or in accordance with this Regulation;

b) derive from fishery products which are fit for human consumption and which comply with the provisions set out in this Section;

c) be transported and stored in hygienic conditions;

d) be chilled as soon as possible and remain at the temperatures set out in Chapter VII.

By way of derogation from point 1(d), the food business operator may refrain from chilling the fishery products when whole fishery products are used directly in the preparation of fish oil for human consumption, and the raw material **is processed within 36 hours after loading**, provided that the freshness criteria are met and the total volatile basic nitrogen (TVB-N) value of the unprocessed fishery products do not exceed the limits set out in point 1 of Chapter I of Section II of Annex II to Commission Regulation (EC) No 2074/2005 (▼).

2. The production process for fish oil must ensure that all raw material intended for the production of crude fish oil is subject to a treatment including, where relevant, heating, pressing, separation, centrifugation, processing, refining and purification steps before being placed on the market for the final consumer.

3. Provided that the raw materials and the production process comply with the requirements applying to fish oil intended for human consumption a food business operator may produce and store both fish oil for human consumption and fish oil and fish meal not intended for human consumption in the same establishment.

4. Pending the establishment of specific Community legislation food business operators must ensure compliance with national rules for fish oil being placed on the market for the final consumer.

▼ C1 2004R0853 — EN — 15.07.2010 — 008.001 — 56 (1) OJ L 338, 22.12.2005, p. 27:

25mg/100g for Sebastes spp, Helicolenus dactylopterus, Sebastichthys capensis
30mg/100g for Pleuronectidae family except Hippoglossus spp
35mg/100g for Salmo salar, Merlucciidae familiy and Gadidae family

APPENDIX B

Data sources

Chapter 5 Methods commonly used to evaluate marine oil

MEDLINE/EMBASE searches were performed in order to provide information on measurement/levels of oxidation products in marine oils. The map terms fish oil, cod liver oil, marine oil, or krill oil, were used in combination with cyclic fatty acid monomer/CFAM, 4 hydroxynonenal/4-hne/4 hydroxyhexenal/4-hhe, polymers/polymeric compound, core aldehyde, or free fatty acid/fatty acid. The map term core aldehyde was further combined with the following group of map terms: analytic sample preparation, method, analysis, detection, measurement, chemical analy*. The map term free fatty acid/fatty acid was outstanding with regard to several hits and thus additionally combined with "adverse effect" and limited to animal studies in order to provide additional data for Chapter 7.

Chapter 7 Hazard characterisation

Search for relevant articles for the toxicological effects described in Chapter 7, except 7.2.2, was performed in Web of Science with the map terms lipid hydroperoxides, malondialdehyd, aldehydes, lipid oxidation. Articles on oxidation products produced *in vivo* and studies designed to evaluate physiological effects of marine oils without any references to quality of the oil used, were excluded.

Additional relevant articles were identified by searching the MEDLINE database using the following map terms: lipid peroxides, malondialdehyde, trans fatty acids, oxysterol, cholesterol oxidation product, polymerization, core aldehyde, thiobarbituric acid reactive substances. These map terms linked with "or" were combined with the combination of fish oils or oxidized fat or oxidized diet or dietary fats unsaturated. The MEDLINE search was limited to year 2000 to the date of search and animal studies. A similar procedure was followed for search in the EMBASE database.

For Chapter 7.2.2 Effect studies in Human, the base was the review article by Turner et al., 2006 and the references therein.