

SCIENTIFIC OPINION

Scientific Opinion on Fish Oil for Human Consumption. Food Hygiene, including Rancidity¹

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ABSTRACT

In this document an evaluation of hygiene and rancidity of fish oil intended for human consumption was carried out until the point of the production chain for fish oil at which a product intended for human consumption is obtained as a bulk stored product. This does not include encapsulated or other consumer packages or the final product ready to be sold to the consumer. Within the scope of this document, only oxidation products may represent a potential chemical hazard in refined fish oil intended for human consumption whilst stored in bulk. The refined fish oil production process typically includes several steps such as repeated heating at high temperatures (at 90-95°C and even up to 180°C) as well as alkali/acid treatments and removal of the water phase, which reduce the biological food safety risk to negligible. Lipid oxidation in bulk stored fish oil can be prevented by cold storage in darkness, without exposure to oxygen and addition of antioxidants. Based on the currently available information, no qualitative or quantitative risk assessment of hazards in relation to rancidity of fish oil intended for human consumption can be carried out. The criterion of 60 mg total volatile basic nitrogen (TVB-N)/100 g for whole fish is not based on scientific evidence. Sensory assessment is recommended for the evaluation of the freshness of raw material for fish oil production for human consumption. At present, the methods to determine the peroxide and anisidine values are the most reliable chemical methods for rancidity measurements in bulk fish oils. The present knowledge does not allow setting and recommending of maximum acceptable peroxide and anisidine values for the large variety of refined fish oils.

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KEY WORDS

Fish oil, human consumption, hygiene, rancidity, parameters

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SUMMARY

Following a request from the European Commission for a scientific opinion on fish oil for human consumption, food hygiene including rancidity, an assessment with respect to hygiene and rancidity is carried out in this document. Based on existing knowledge this assessment was carried out until the point of the production chain for fish oil at which a product intended for human consumption is obtained as a bulk stored product.

The composition and properties of fish oil depend on the freshness and composition of the raw materials whereby by adjusting the production process, fish oil of desired properties can be obtained even from fish not meeting the current freshness criterion. However, data on the relationship between the freshness of the raw material and the level of lipid oxidation in the refined oil are missing in the literature. The refined fish oil production process typically includes several steps such as repeated heating at high temperatures (at 90-95°C and even to 180°C) as well as alkali/acid treatments and repeated removal of the water phase, which reduce the biological food safety risk to negligible. These current risk assessments relate to the bulk storage level of the fish oils and therefore hazards arising at later stages are not dealt with. The Terms of Reference (ToR) have been addressed in this document in such a way that an assessment with respect to hygiene and rancidity is carried out until the point of the production chain for fish oil at which a product intended for human consumption is obtained including bulk storage. This does not include encapsulated or other consumer packages or the final product ready to be sold to the consumer. The refined oil at bulk storage is a well defined product whereas consumer products (e.g. capsules) are complex and oil properties are influenced by numerous extrinsic factors (packaging material, other ingredients, flavourings, storage conditions and intended use).

The fish oil production was described covering raw materials, the generic crude fish oil process, the oil refining process, the production of omega-3 concentrates and antioxidant addition and other means of oxidation protection. Factors affecting fish oil properties and potential biological and chemical hazards related to oxidation were outlined and discussed. Reference was made to recently newly introduced products such as virgin fish oils or extra low oxidised fish oils where essentially lower temperatures appear to be used during processing and the raw materials seem to be of particular high quality. However, information is scattered and generally accepted definitions of these products seem to be lacking so far.

In the frame of the given mandate only oxidation products may represent a potential hazard in refined fish oil intended for human consumption whilst stored in bulk. Lipid oxidation in bulk stored fish oil can be prevented by cold storage in darkness, with no exposure to oxygen and addition of antioxidants. Information on the level of oxidation of fish oil (as measured by peroxide and anisidine values) and related toxicological effect in humans is lacking. Information on toxicity of individual oxidation products of fish oil in humans is also lacking. Based on the currently available information, no qualitative or quantitative risk assessment of hazards in relation to rancidity of fish oil intended for human consumption can be carried out.

Total volatile basic nitrogen (TVB-N) is a spoilage parameter, which was developed and defined for ice stored gutted fish and fish filets. It has not been investigated for the determination of the 'freshness' of whole fish as raw material intended to be used for production of fish oil for human consumption. The criterion of 60 mg total volatile basic nitrogen (TVB-N) /100 g for whole fish is not based on scientific evidence. Sensory evaluation gives the most reliable results for the assessment of the freshness of raw material for fish oil production for human consumption. Currently, the methods to determine the peroxide and the anisidine values are the most reliable chemical methods for rancidity measurements in bulk fish oils. However, gas chromatography methods that can measure the specific volatile oxidation products should be standardised and implemented. Current knowledge does not allow setting and recommending maximum acceptable values for these two indexes. The quantitative relationship between peroxide and anisidine values and the specific volatile oxidation products is lacking. In addition, there is no comprehensive information about the potential negative health effects of individual oxidation products originating from refined fish oil.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The Commission sent an addendum and further background information and updated terms of reference for a request for a scientific opinion on Fish Oil for Human Consumption. Food Hygiene including Rancidity.

The following issues shall be included in the scientific opinion:

1. Impact of the hygienic quality of the raw material used on fish oil for human consumption;
2. Rancidity in fish oil for human consumption.

The scientific opinion shall neither include issues related to PCBs, dioxins, etc. nor issues related to the nutritional benefits of omega-3 fatty acids, unless such issues should be directly related to hygiene issues included in the terms of reference.

The EC Hygiene Regulations on fish oil intended for human consumption

General requirements

The Hygiene Regulations make provisions for production of fish oil for human consumption. Such production and products must meet the relevant requirements for fishery products found in the Hygiene Regulations. In general this means that the raw materials used and the fish oil must:

Come from establishments, including vessels, registered or approved pursuant to the Hygiene Regulations.

Derive from fishery products which are fit for human consumption and are handled throughout the food chain as such. Animal by-products and fishery products not fit for human consumption cannot be used as raw material for fish oil for human consumption.

Additional specific requirements can be found in:

Section VIII of Annex III to Regulation (EC) No 853/2004 as amended by Commission Regulation (EC) No 1020/2008

Annex II to Regulation (EC) No 2074/2005 as amended by Commission Regulation (EC) No 1022/2008

Specific requirements for raw material for production of fish oil

According to the Regulation the raw material must be chilled as soon as possible after the catch. When chilling is not possible on board the vessel the raw material must undergo chilling as soon as possible after landing and be stored at a temperature approaching that of melting ice.

However, by way of derogation 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 the catch, provided that the freshness criterion laid down are met.

The freshness criterion is based on the total volatile basic nitrogen (TVB-N), which shall not exceed 60 mg of nitrogen/100 g of whole fishery products used directly for the preparation of fish oil for human consumption. However, where the raw material is still fit for human consumption the competent authority may set limits at a higher level for certain species.

Specific requirements for fish oil for human consumption

The Commission has the possibility to propose hygienic requirements for fish oil for human consumption, including rancidity requirements. Point 4 of Chapter VI.B of Section VIII of Annex III

to Regulation (EC) No 853/2004 includes a clause stating that, pending the establishment of specific Community legislation, Member States must ensure compliance with national rules for fish oil placed on the market for the final consumer.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

EFSA is asked to prepare an opinion covering the following issues:

ToR 1: Impact of hygiene of the raw material used for fish oil for human consumption

Qualitative and, if possible, quantitative risk assessments of hazards in relation to raw material used for the production of fish oil for human consumption;

ToR 2: Fish oil and rancidity

Qualitative and, if possible, quantitative risk assessments of hazards in relation to rancidity of fish oil;

ToR 3: Parameters

Recommendations on criteria (methods and thresholds), including those related to rancidity, which can be used to characterise the raw material for the production of fish oil and in fish oil for human consumption.

The scientific opinion shall neither include issues related to PCBs, including dioxins, etc. nor issues related to the nutritional benefits of omega-3 fatty acids, unless such issues should be directly related to hygiene issues included in the terms of reference.

ASSESSMENT

1. Preamble

1.1. Understanding of the mandate and scope of the opinion

The Terms of Reference (ToR) have been addressed in this document in such a way that an assessment with respect to hygiene and rancidity is carried out until the point of the production chain for fish oil at which a product intended for human consumption is obtained including bulk storage. This does not include encapsulated or other consumer packages or the final product ready to be sold to the consumer. The refined oil at bulk storage is a well defined product whereas consumer products (e.g. capsules) are complex and oil properties are influenced by numerous extrinsic factors (packaging material, other ingredients, flavourings, storage conditions and intended use). Some of these aspects are currently investigated by various Institutions for example by The Foundation for Scientific and Industrial Research (SINTEF) at the Norwegian Institute of Technology (NTH) and the National Institute of Nutrition and Seafood Research (NIFES) initiated by the Norwegian Food Safety Authority (Mattilsynet), and the Research Council of Norway (Forskningsrådet), respectively.

This document consequently deals with hygienic status of raw fish used for fish oil production and whether it is relevant for the safety of the oil intended for human consumption. Secondly, it addresses the rancidity of both raw material and fish oil. Recommendations for parameters to measure the oxidative status of the raw material and the fish oil are discussed and knowledge and research gaps are highlighted.

According to the request of the Commission as referred to in the ToR, issues related to chemical pollutants (including PCBs and dioxins) and issues related to the nutritional benefits of omega-3 fatty acids have been excluded from the opinion.

1.2. Legislative background

1.2.1. The EC Hygiene Regulations on fish oil intended for human consumption

As with any other food, fish oil for human consumption is covered by the Food Hygiene Regulations. In addition to general hygiene requirements as laid down in Regulations (EC) No 852/2004, (EC) No 854/2004 and their amendments, specific requirements for production of fish oil for human consumption can be found in:

- Chapter IV of Section VIII of Annex III to Regulation (EC) No 853/2004 as amended by Commission Regulation (EC) No 1020/2008.
- Annex II to Regulation (EC) No 2074/2005 as amended by Commission Regulation (EC) No 1022/2008.

Regulation (EC) No 852/2004 on the hygiene of foodstuffs establishes general hygiene requirements concerning food and a common framework to ensure that food is safe from farm to fork. It includes the general obligations of businesses in regard to food hygiene, the requirements for HACCP based food safety management procedures, hygiene requirements for facilities and equipment, staff training and personal hygiene, and processes. Regulation (EC) No 853/2004 on hygiene for food of animal origin lays down specific rules on the hygiene of food of animal origin for food business operators, and supplements Regulation (EC) No 852/2004 by adding specific hygiene requirements for products like meat, milk, fish and egg etc., as well as fish oil. This Regulation provides that food business operators producing fish oil intended for human consumption are subjected to strict rules covering production, storage and transport. In addition, suppliers' premises have to be audited to prove they meet these standards. Regulation (EC) No 854/2004 sets out specific requirements for organising official controls on products of animal origin intended for human consumption. Besides, there are

other regulations which concern contaminants (Regulation (EC) No 1881/2006) and pesticides (Regulation (EC) No 396/2005).

Due to the difficulties in implementing specific requirements of the Hygiene Regulations in some Member States and third countries the legislation provides the possibility for a derogation related to the requirement for immediate chilling of the fish after the catch. A food business operator may refrain from chilling the fishery products used in the preparation of fish oil for human consumption, if the raw material is processed within 36 hours after the catch and the freshness criterion is met (total volatile basic nitrogen limit of 60 mg nitrogen/100 g of whole fishery products) (Regulations (EC) No 1020/2008 and (EC) No 1022/2008).

1.2.2. Further information regarding fish oil to be imported from third countries into the EU

When importing fish oil as such (both crude oil and oil ready to be put on the market) for human consumption from a third country after 30 April 2009 the country must be listed in accordance with Article 11 of Regulation (EC) No 854/2004 for import of fishery products.

Furthermore, the third country establishment from which that product was dispatched, and in which it was obtained or prepared must appear on the lists of establishments from which import of specific products of animal origin are permitted (Regulation (EC) No 854/2004).

Pending the establishment of specific Community legislation on hygienic parameters for fish oil to be placed on the market for the final consumer food business operators must also ensure compliance with national hygiene rules where those exist, including rancidity parameters.

The third country's competent authority must ensure that the whole production chain, including fishing vessels and the raw materials comply with the EU requirements.

A consignment of fish oil destined for human consumption that is imported into the EU shall be accompanied by the health certificate for fishery products as laid down in Appendix IV to Annex VI to Regulation (EC) No 2074/2005, as amended.

1.2.3. Legislation in place outside the EU

A search in different databases such as Web of Science about information for non EU regulatory for fish oil intended for human consumption revealed that there are no regulations and legislation existing about requirements of the quality (freshness) of fish used for the production of fish meal and fish oil. Consequently also information about oxidation parameters for fish oil produced for human consumption is lacking. Neither in the framework of the FDA (USA) nor the Canadian Food Inspection Agency (Canada) nor in the legislations of the major fish oil producing countries (Norway, Chile, Peru) any indications could be found about this issue. Legislation about fish oil in general is in place but only regarding regulations which cover the safety of the final fish oil in respect of organic and/or inorganic residues (pollutants). Also in these regulations there is no differentiation or mentioning of differences between fish oil intended for use as a feed component or refined fish oil intended for pharmaceutical purposes or human consumption.

1.2.4. Guidelines

However guidelines for Good Manufacturing Practice of fish oil intended for human consumption exist for example from Industry organisations such as GOED (Global Organization for EPA and DHA omega-3) and Norwegian Seafood Federation (fhl) (GOED, 2006; fhl, 2010).

1.2.5. Standards

There are different standards for fish oil processing, each having their own acceptable levels of toxic metals and organic contaminants. Four main standards deserve to be mentioned as they to crude and refined fish oil: the United States Pharmacopeia (USP); the Council for Responsible Nutrition (CRN)

monograph; the International Fish Oil Standard (IFOS), and the European Pharmacopoeia (EP). For USP, no official standards for fish oil are yet developed, only a proposed monograph. CRN is assumed to be used as the basis for an official USP monograph. IFOS is a standard based on the CRN monograph. This monograph, in turn, is based on the EP standard. This standard is regarded as one of the best documents available. This monograph entails a guidance concerning stringent quality control of any product with potential pharmaceutical use (European Pharmacopoeia, 2001). In fact, for all primary raw materials and processing materials, the fish oil produced must establish a specification defining the critical physical, chemical and biological characteristics required. Furthermore, this also applies to equipment when there is a direct or indirect influence on the quality or safety of the product. Producers shall establish and verify appropriate measures and/or systems adopted by suppliers to ensure the safety and quality of raw materials. Material suppliers should operate to a relevant quality standard audited by a recognized independent qualified body. The conformity of each material must be documented by certificates of analysis archived as verification of compliance with the specification. Moreover, verification of source and preparation of the material is required.

IFOS is a standard based on the CRN monograph. This monograph, in turn, is based on the EP standard. This standard is regarded as one of the best documents available. This monograph entails a guidance concerning stringent quality control of any product with potential pharmaceutical use (European Pharmacopoeia, 2001; GOED, 2006). In fact, for all primary raw materials and processing materials, the fish oil produced must establish a specification defining the critical physical, chemical and biological characteristics required. Furthermore, this also applies to equipment when there is a direct or indirect influence on the quality or safety of the product. Producers shall establish and verify appropriate measures and/or systems adopted by suppliers to ensure the safety and quality of raw materials. Material suppliers should operate to a relevant quality standard audited by a recognized independent qualified body. The conformity of each material must be documented by certificates of analysis archived as verification of compliance with the specification. Moreover, verification of source and preparation of the material is required.

1.3. Information concerning some ongoing projects with relevance to this mandate

The Norwegian Food Safety Authority (Mattilsynet) has asked the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) to conduct a health risk assessment on decomposition substances and oxidation products in fish oils intended for human consumption. This assessment is currently ongoing.

In Norway a research project has started in which researchers from four institutions investigate how omega fatty acids from different products (e.g. fish, capsules) are absorbed into the body, as well as what kind of requirements should be stipulated in terms of freshness. The project tries to find out if it is healthier to eat fish rather than ingest special products to which omega-3 fatty acids have been added, or if it is best to down the spoonful of Norwegian cod liver oil as opposed to taking other kinds of omega-3 fatty acid supplements. In addition, the project will examine the potential negative health effects of rancid fish oils. According to I. Frøyland (NIFES), the co-ordinator of the project, rancid fish oil smells bad and tastes so awful that provokes consumer rejection. But if the fish oil is encapsulated, it is impossible to detect rancidity. That is why it is important to examine whether rancid fish oil is less beneficial to health, or, at worst, harmful to anyone who takes it (The Research Council of Norway, 2010).

2. Introduction

There is a wide range of species used in the production of fish oils. However, the biggest part derives mostly from the fatty fish and the liver of lean fish. The commonly used raw materials are menhaden, sardines, anchovies, herring, capelin, mackerel, salmon, tunas, cod liver, etc.. Besides from bony fish also some fish oil and fish liver oil can derive from cartilaginous fish as sharks. Shark and cod are the two main fish whose livers are extracted for oil. Whilst sharks are specifically targeted for their livers, cod livers are taken as a by-product along with the roe.

Fish oil intended for human consumption must live up to the requirements for fishery products found in the Hygiene Regulations. This implies that the raw materials and the fish oils (i) must come from establishments, including vessels, registered or approved pursuant to the Hygiene Regulations and (ii) derive from fishery products which are fit for human consumption and are handled throughout the food chain as such. Animal by-products and fishery products not fit for human consumption cannot be used as raw material for fish oil for human consumption.

The difference between fish oil and other oils is mainly the unique variety of fatty acids it contains. Thus, marine oils are excellent sources of polyunsaturated fatty acids (PUFAs), mainly long-chain omega-3 fatty acids, also sometimes referred to as n-3 fatty acids. The amount and variety of the fatty acids in fish oil varies from one fish species to another, and also with the biological stage, food availability, fishing location, ocean temperature, nutritional and spawning state, etc. The major omega-3 fatty acids present are the eicosapentaenoic acid (C20:5 omega-3, commonly called EPA) and the docosahexaenoic acid (C22:6 omega-3, commonly entitled DHA).

Due to the increasing body of evidence on the potential health beneficial roles of omega-3 fatty acids in humans, there has been an increasing interest in using fish oils for human consumption. To date, the human health benefits of fish oils have been dealt with in numerous papers, reviews and reports, but the most thoroughly documented effects are on the cardiovascular system (Wang et al., 2004; Psota et al., 2006; Iso et al., 2006; Mozaffarian and Rimm, 2006). Other papers, suggest that PUFAs can be used to treat CVD risk factors associated with obesity such as hypertriglyceridemia (Balk et al., 2006) or anti-inflammatory effects that may prove useful in autoimmune diseases like rheumatoid arthritis (Calder, 2006). An increase in the proportion of PUFA in the diet may delay the onset of some cancers (Simopoulos, 2006), such as colon (Chapkin et al., 2007), breast (Sun et al., 2005; Hilakivi-Clarke et al., 2005) and prostate (Istfan et al., 2007), through a variety of mechanisms (Stehr and Heller, 2006). Other plausibly beneficial effects are related to the improvement of foetal development (Joshi et al., 2004; Uauy and Dangour, 2006) and positive influence on psychiatric/psychological disorders such as schizophrenia and depression (Peet and Stokes, 2005).

2.1. World fish oil production

The market of fish oil for human consumption can be divided into three areas: as a pharmaceutical constituent, as a health food component and as an ingredient for the food industry. Further, fish oil can be an important constituent of animal feeds, contributing with essential fatty acids needed for normal growth, health and reproduction.

The production of fish liver oils and fish oils from the main species between 2005 and 2007 is shown in Table 1. The major fish oil producers are Peru (about 200,000 tonnes) followed by Denmark and United States (around 70,000 tonnes) and Iceland (approximately 60,000 tonnes).

Table 1: Production of fish oils and main species used (FAO, 2010).

| | Production (ton) | | |
|--|------------------|---------------|---------------|
| | 2005 | 2006 | 2007 |
| Fish liver oils, total | 22712 | 18264 | 25951 |
| Halibut liver oil | 25 | 36 | 8 |
| Cod liver oil | 3172 | 3428 | 4035 |
| Hake liver oil | | 23 | |
| Shark liver oil | | 15 | |
| Other fish liver oils | 19515 | 14762 | 21908 |
| Fish oils, other than liver oils, total | 690091 | 765956 | 839280 |
| Herring oil | 32192 | 49885 | 90292 |
| Menhaden oil | 39665 | 38188 | 45506 |
| Sardine oil | 3 | 201 | 3599 |
| Capelin oil | 29277 | 16126 | 9042 |
| Shark oil | 50 | 48 | 35 |
| Other fish oil | 588486 | 661508 | 690806 |

There was an important change in fish oil applications in recent years. Nowadays, most fish oil is used as part of feeds and in the production of nutraceuticals. The latter demands from the producers high standards in production processes and for the final product characteristics.

There are multiple types of finished fish oils being demanded and among them the refined omega-3 fish oils constitutes an important group. The production of these products has increased rapidly since 2001 and the utilisations are very broad. The world production of refined omega-3 fish oils has increased from 20,000 tonnes in 2001 to a volume of 85,000 tonnes in 2009. The majority (around 70%) of the finished omega-3 fish oils on the world market contain approximately 30 % of DHA+EPA. Concentrates with higher levels of DHA+EPA amount to 10 %. The remaining 20 % are other marine oils. Approximately 60 % of the omega-3 oils rich in DHA+EPA are used for dietary supplements, 20 % for functional foods, 7 % for animal feed, 6 % for pharmaceuticals and the remaining for infant and clinical nutrition.

3. Fish oil production process

3.1. Raw materials

The largest proportion of crude fish oil is produced as a by-product of fish meal production by the wet rendering process.

The fish meal and oil industry requires a regular supply of raw material, thus a wide variety of fish species is used. These may be divided into three categories: (i) catches for the single purpose of fishmeal/oil production, (ii) by-catches from another fishery or (iii) fish off cuts and offal from the processing industry. Gadoids, clupeids, scombroids and salmonoids are within the most used species. As it was already mentioned, an important part of fish oil is produced from fish liver, the so called fish liver oil, which is rich in vitamins A and D.

The composition and oxidative status of crude fish oil depends on the composition and the oxidative status of the raw material.

3.2. Generic crude fish oil process

In general, traditional processes to obtain fish oil involve two stages (FAO, 1986): oil extraction from raw material and refining (Fig.1). New oil extraction methods involving pH adjustment have been developed and compared with the traditional heat extraction method (Okada and Morrissey, 2007).

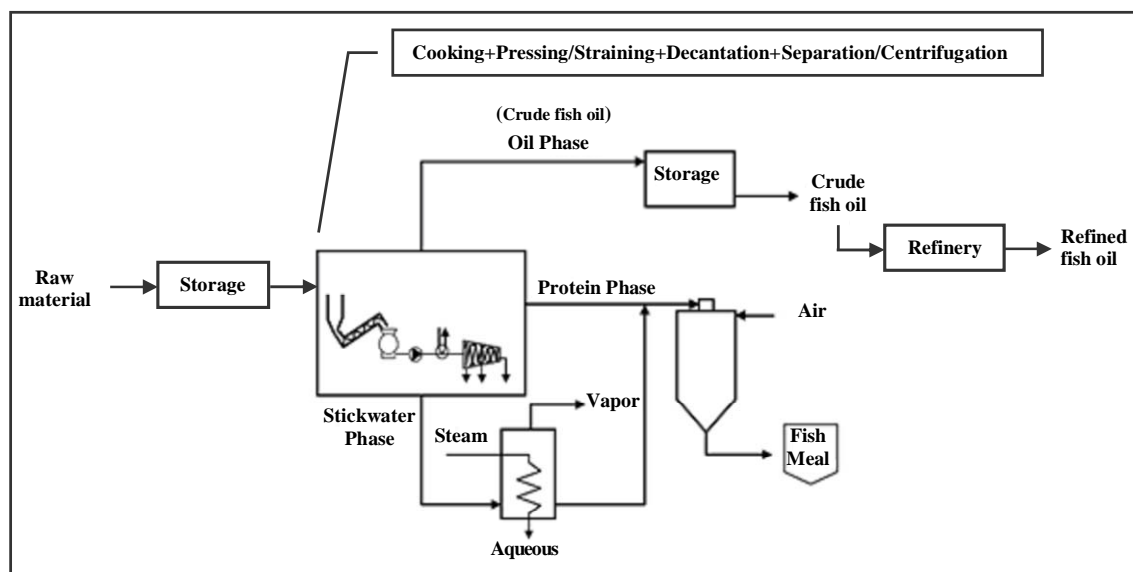


Figure 1: Flow diagram showing the typical fish meal/oil plant (adapted from: Aidos et al., 2003).

3.2.1. Oil extraction

3.2.1.1. Raw materials reception and storage before processing

The raw material is unloaded from the fishing vessel by crane, wet fish pump, pneumatic elevator or some sort of mechanical conveyor to the storage tanks or direct to lorries for transfer to the processing plants. Fish off cuts and offal from the processing industry are either stored and processed directly on the site or may be stored and transferred to fishmeal/fish oil processing facilities. Storage of some raw materials is necessary in order to allow continuous operation of the facilities. As observed in the legislation, raw materials must be chilled and kept at appropriate temperatures unless whole fishery products are used directly for preparing fish oil and the raw material is processed within 36 hours, and where the freshness criteria is met and the TVB-N values do not exceed set limits.

3.2.1.2. Cooking

The raw materials (whole fish and by-product) are conveyed to the processing plant and into a continuous cooker. The material is steam heated to 90-95 °C for approximately 10-20 minutes. This process, coagulates the proteins and disrupts the cell membranes thus allowing the leakage out of bound water and oil. This cooking stage is carried out in sealed systems.

3.2.2. Oil separation

The cooked material is then fed into a screw press where the liquid phase (comprising water, dissolved materials and oil) is squeezed from the solid phase (this press cake, which contains 60-80% of the oil-free dry matter, is further processed to fishmeal). The pressing takes place in a closed system and it takes approximately 30 minutes. The pressing step is sometimes called straining.

The liquid phase that contains water and the oil from the fish, as well as small amounts of suspended solids are then separated based on their different specific gravities. This is typically accomplished by several centrifugations. An important prerequisite for efficient centrifugation is high temperature, and the press liquid is usually reheated to 90-95 °C before entering the different centrifuges. Then the suspended solids are removed from the oil by using a decanter (a horizontal centrifuge) and transported to be mixed with the press cake, the oil/water phase is then passed forward for further separation. The separation of the water from oil takes place usually in vertical centrifuges, being the water phases discarded and the oil phase sent to storage tanks.

3.2.3. Storage of fish oil

The crude fish oil is subsequently stored in large tanks for placing on the market or export. This oil is allowed to cool to room temperature during storage. Fish oil intended for human consumption has to be stored separately from the fish oil intended for other purposes. But it may be stored in the same establishment.

3.2.4. Crude fish oil properties

The chemical composition and characteristics of crude fish oils depend on the process and the raw material. These influence the properties of oils both in regard to edible properties as well as technical applications. Thus, the condition of the fish at the time of processing affects the oil physically, chemically and nutritionally and, on the other hand, inferior quality yields malodorous oil with high contents of free fatty acids (FFA) and sulphur. As a rule, the fish oils contain variable, but small amounts of unsaponifiable components, such as hydrocarbons, fatty alcohols, waxes and ethers, which influence the properties of the oils to some extent. Whereas some of these substances, such as tocopherols, are beneficial other are objectionable because they render the oil dark-coloured, cause it to foam or smoke or are precipitated when the oil is heated in the subsequent stages.

The test methods employed by the producer/user of fish oils are often divided into two groups, the first being applied after preparation or on receipt of a consignment to check the fundamental parameters and the second examination, more detailed, as soon as possible thereafter, but in any case before the oil is refined. The purpose of this second examination is to determine refining procedures. The initial testing involves the checking of moisture, impurities, appearance (namely colour), FFA, soap and iodine value (IV). The second evaluation is usually focused on the evaluation of peroxide value (PV), anisidine value (AV), ultra violet extinction values (at 233 nm and 269 nm), trace metals (iron and copper), sulphur and phosphorus.

The properties and characteristics of crude fish oil are strongly influenced by the process and raw material. However, according to Bimbo (1998) some typical chemical and physical properties of crude fish oils can be proposed (Table 2). (See chapter 4.1.2) for information about some of the methods behind the properties mentioned in the table).

Table 2: Crude fish oil properties and physical characteristics according to Bimbo (1998).

| Quality guidelines | |
|---------------------------------|------------------------------------|
| Moisture and impurities % | Usual basis 0.5 up to 1 in maximum |
| Fatty acids, % oleic acid | Range 1-7, but usually 2-5 % |
| Peroxide value, meq/kg | 3-20 |
| Anisidine value | 4-60 |
| Totox value | 10-60 |
| Iodine value | |
| Capelin | 95-160 |
| Herring | 115-160 |
| Menhaden | 120-200 |
| Sardine | 160-200 |
| Anchovy | 180-200 |
| Jack mackerel | 160-190 |
| Sand eel | 150-190 |
| Colour, Gardner scale | Up to 14 |
| Iron, ppm | 0.5-7.0 |
| Copper, ppm | Less than 0.3 |
| Phosphorus, ppm | 5-100 |
| Physical characteristics | |
| Specific heat, cal/g | 0.50-0.55 |
| Heat of fusion, cal/g | About 54 |
| Caloric value, cal/g | About 9,500 |
| Slip melting point, °C | 10-15 |
| Flash point, °C | |
| As triglycerides | About 360 |
| As fatty acids | About 220 |
| Boiling point, °C | Greater than 250 |
| Specific gravity | |
| At 15 °C | About 0.92 |
| At 30 °C | About 0.91 |
| At 45 °C | About 0.90 |
| Viscosity, cp | |
| At 20 °C | 60-90 |
| At 50 °C | 20-30 |
| At 90 °C | About 10 |

3.3. Oil refining process

The crude fish oil is transported to the refinery for further processing. On receipt of the oil at the refinery the material is typically analysed prior to discharge into storage tanks.

The refining operation has the aim to remove undesirable components from the oil regarding human consumption and further processing. At the same time such operation has to be done in a way that fish oil components are not being damaged during the process itself, e.g. polymerisation should be avoided.

Crude oil contains varying amounts of materials that may give undesirable flavour and colour, such as small amounts of proteins, water, pigments, free fatty acids (FFA), phospholipids, and lipid oxidation products. The conventional oil refining in industry is usually made by chemical methods, which include several steps as: settling and degumming, de-acidification, bleaching, deodorization, antioxidant addition or winterisation (Fig. 2.). For this purpose hydrogenation is not included. However, traditional oil refining has been challenged by new technologies that have been shown to produce high-quality oil. Within those, supercritical fluid technology, together with membrane and enzymatic processes, has been referred. Several studies reported the application of these technologies to fish oil refining (Jakobsson et al., 1991; Catchpole et al., 2000; Kawashima et al., 2006). The latter

study proposed combining supercritical extraction with CO₂ and adsorption on activated carbon to remove PCBs and other contaminants from fish oil. Allegedly the combination of these two steps would lead to a reduction in the level of contaminants such as dioxin and PCBs of more than 95 %.

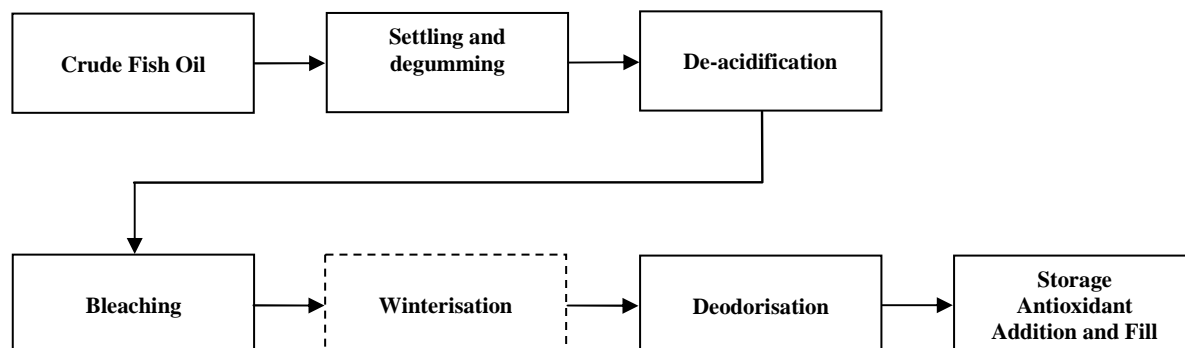


Figure 2: Flow diagram showing the conventional oil refining in the industry.

3.3.1. Settling and degumming

Settling means making the oil stand, with or without heating for the aqueous phase to separate and be withdrawn. In doing so, part of water, proteinaceous material and phospholipids are eliminated. Degumming, that can be an optional stage, consists of a treatment of oil with water or aqueous solutions of acids or salts at 30 – 100 °C to remove soluble and insoluble impurities. Typically solutions of phosphoric or citric acids are used. The oil, water and resultant soaps are then separated either by centrifugation or decantation.

3.3.2. De-acidification

De-acidification or alkali refining is performed with an aqueous alkaline solution to remove free fatty acids and reduce oil acidity.

Besides the utilization of alkali substances in the de-acidification process, which may leave residues, new de-acidification methodologies have been put forward (Rubio-Rodríguez et al., 2010). Namely, supercritical fluid technology, together with membrane and enzymatic processes, may be an alternative to classical chemical means in de-acidification (Bhosle and Subramanian, 2005). Alternatively, physical refining using thin film deodorization may be used. Thus, traditional de-acidification processes (as alkali neutralization followed by a simple aqueous/organic phase separation) are being challenged by mainly supercritical fluid and thin film deodorisation technologies.

3.3.3. Bleaching

The deacidified oil is then heated to 80-85°C and mixed with an activated bleaching clay and/or activated carbon. Soaps, trace metals, sulphurous compounds and part of the most stable pigments and pigment-breakdown products are removed. Physical adsorption on activated carbon is necessary when the removal of organic contaminants such as dioxins/furans and dioxin-like PCBs is required (Maes et al., 2005; Oterhals et al., 2007). This step also converts hydroperoxides to their respective aldehydes; ketones and other products (Hamm, 2009).

3.3.4. Deodorisation

At last, the deodorisation stage is carried out to remove specially FFA, aldehydes and ketones, which give objectionable smell and flavour characteristics (Aidos et al., 2003). Oil deodorization is

traditionally based on the application of high temperatures (Čmolík and Pokorný, 2000). In this step the volatile materials are stripped by means of a stripping gas, normally steam at high temperature (190-210 °C) and low pressure (2-5 mbar). However, the application of this method to fish oil is problematic because it has been reported that, for temperatures above 180 °C, omega-3 FA degradation occurs, involving the formation of polymers, isomers, cyclic FA monomers and other undesirable compounds (Fournier et al., 2006). Alternative methods based on vacuum steam distillation at low temperatures followed by a treatment in a silica gel column (Chang et al., 1989), adsorption with a resin (Nishimoto and Nagagawa, 1996), or treatment with diatomaceous earth (Yoshikazu et al., 1997) have been put forward for removing odours from fish oil.

Antioxidants can be added to the oil to protect it against oxidation as will be further described in 3.4.7.

3.3.5. Characteristics of refined oil

The knowledge about the characteristics of refined fish oils is very important, since it determines the uses and price. According to the quality guidelines indicated by Hamm (2009), Bimbo (1998) and the Codex Standard (1981) some characteristics are indicated in Table 3. Regarding the peroxide value, very often higher values are referred for refined oils. Bimbo (1998) used as reference 5 or 10 meq O₂/kg values indicated in Codex (not specific for fish oils – general for fats and oils) and the Global Organization for EPA and DHA omega-3 (GOED, 2006) refers to a value of 5. However, a product with a peroxide value of 5 would have some smell that makes it unsuitable for addition in food products.

Table 3: Some quality guidelines for refined fish oils (Hamm, 2009).

| Parameter | Quality guidelines |
|--------------------------|-----------------------------|
| Colour | <3.0 Red, 30 Yellow |
| Odour and taste | Bland |
| Matter volatile at 105°C | <0.2 % |
| Insoluble impurities | <0.05 % |
| Soap content | <0.005 % |
| Iron | <0.12 mg/kg |
| Free fatty acids | <0.10 % (as oleic acid) |
| Copper | <0.05 mg/kg |
| Peroxide value | <0.1 meq O ₂ /kg |
| Nickel | <0.20 mg/kg |

3.4. Production of omega-3 concentrates

The preparation of omega-3 concentrates follows a physicochemical process, comprising five steps: de-acidification (optional), transesterification, concentration, deodorisation/earth treatment, and antioxidant addition and fill off (Fig. 3). The central step of concentration is commonly done by molecular distillation. All the operations are described with greater detail in the following sections.

However, alternative methods (enzymatic or supercritical fluid technology) for various steps have been proposed. In fact, the production of omega-3 concentrates from fish oil may be achieved by a number of techniques. These concentrates may be in different forms: free FAs, methyl and ethyl esters or acylglycerols. Although concentrates can be prepared via urea complexation, products so formed are in the free acid or simple ester forms. This is a major drawback. Due to the potential benefits of having the concentrates in the acylglycerol form, re-esterification may be advisable. This problem has led to the expansion of other methods, such as enzymatic procedures (which leave the concentrated omega-3 FAs in the acylglycerol form), whose industrial production has been gaining momentum. This problem also affects other methods, namely, the supercritical fluid extraction, whose development at an industrial scale is still in its infancy (Rubio-Rodríguez et al., 2010). Therefore, there is a good number of different technologies that are being industrially applied or still under investigation.

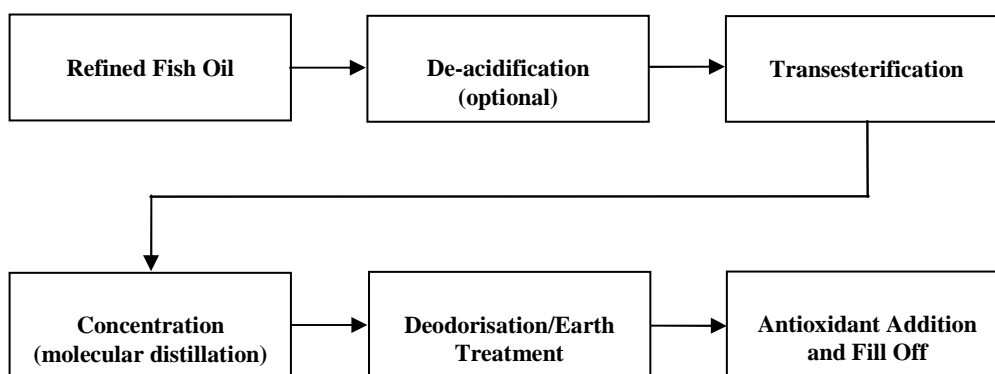


Figure 3: Flow diagram showing the common process for the preparation of omega-3 concentrates.

3.4.1. Raw material for fish oil

The raw material fish oil for the production of omega-3 FAs concentrates must have previously undergone a thorough refining process, comprising de-acidification and removal of contaminants (for instance, by physical adsorption on activated carbon). Only after such refining, it is possible to carry out successfully and with high yields the operations for achieving an omega-3 FA concentrate (Rubio-Rodríguez et al., 2010).

The raw material fish oil is transferred to the processing plant in either vessels or by road tanker depending on the location of the facility. Some processing plants purchase crude fish oil whereas others may purchase refined oil for further processing depending on their specific requirements.

3.4.2. De-acidification

If the raw material used by the facility is a crude oil then the first stage of the process is typically de-acidification in accordance with step 3.3.2.

3.4.3. Transesterification

For the most common industrial process, transesterification is a previous step to omega-3 FAs concentration itself. This is because concentration by distillation (3.4.4) requires previous conversion to methyl or ethyl esters of FAs. However, regarding transesterification, some researchers have reported that omega-3 FAs are better absorbed by human organism when they are as acylglycerides than as methyl or ethyl esters (Lawson and Hughes, 1988). Therefore, the circumvention of this step may be desirable, provided that the subsequent concentration process is able to act upon acylglycerols. This is mainly the case of the utilization of enzymatic transesterification as a concentration operation.

This transesterification may be carried out by chemical or enzymatic means (Rubio-Rodríguez et al., 2010; Shahidi and Wanasundara, 1998). The former technique uses a chemical catalyst and the latter may use non-selective lipases or not, thereby giving a contribution for the concentration process (Adachi et al., 1993; Myynes et al., 1995). The chemical method involves a sodium ethoxide-catalyzed transesterification of the oil with ethanol, Typical processing temperatures for this stage of the process would be 80-90 °C.

Lipases from microbial origin have been used for transesterification purposes (Akoh, 1993). They require the presence of water to be active, which causes hydrolysis of glycerides with resultant decreases in the transesterification yields (Hasan et al., 2006). On the other hand, enzymatic methods save energy and cause minimal thermal degradation of the very thermolabile omega-3 FAs (Hasan et al., 2006). However, the scale-up to an industrial level is still hampered by economic difficulties due

to the high costs associated to these techniques (such as the cost of the enzyme itself) (Halldorsson et al., 2003). Nevertheless, these costs can be reduced by the immobilization of the selected lipase. Thus, the simple chemical method of transesterification remains advantageous.

3.4.4. Concentration

It is important to stress that there are numerous alternative methods for the concentration of omega-3 FAs, but only a few are suitable for large-scale production, being the most widespread process the concentration of omega-3 fatty acids by molecular distillation. Other available methods less commonly used include adsorption chromatography, enzymatic splitting, low-temperature crystallization, supercritical fluid extraction, and urea complexation. Each methodology has its own advantages and drawbacks.

In fact, molecular distillation has been widely used for partial separation of mixtures of fatty acid esters. Separation of the fatty acids is done by virtue of their different boiling points. The method applies lower temperatures and shorter heating intervals than conventional distillation methods. Moreover, the use of high vacuum is very common with temperatures ranging from 100-200 °C typically being used during processing in order to minimise heat damage to the oils.

However, fractionation of marine oil esters has some problems; namely, separation of components becomes less effective with increasing molecular weight, as happens with omega-3 FAs. Moreover, concentration of omega-3 FAs in the natural triacylglycerol form (without previous transesterification) presents very important practical difficulties (Stout et al., 1990).

Depending on the physical form required by the end customer the concentrated products are sold as ethyl esters or may be converted to triglycerides or fatty acids. The conversion of the ethyl esters to triglycerides is typically done through the reaction with glycerol in the presence of a base catalyst at temperatures up to 180 °C.

An interesting technique is adsorption chromatography and is based on the potential of some appropriate adsorbents to separate fatty acids according to their carbon number or degree of unsaturation, which was already reported some decades ago (Brown and Kolb, 1955). In fact, high performance liquid chromatography (Beebe et al., 1988) and silver resin chromatography (Adlof and Emiken, 1985) have been used for the production of omega-3 FAs concentrates.

Concerning enzymatic techniques, lipases are utilized. These enzymes can catalyze esterification, hydrolysis or exchange of fatty acids in esters (Marangoni and Rousseau, 1995). Accordingly, this technology may enable the performance of the transesterification and concentration phases in a single step. The direction and efficiency of the reaction can be influenced by the choice of experimental conditions (Yadwad et al., 1991). However, enzymatic techniques present serious problems to the industry (see 3.4.3).

Low-temperature crystallization is based on the fact that solubility of fats in organic solvents decreases with increasing molecular weight and increases with higher levels of unsaturation (Chawla and deMan, 1990). Consequently, at low temperature, long chain saturated FAs which have higher melting points crystallize out and omega-3 FAs remain in the liquid fraction. The low-temperature crystallization process may be carried out in the absence of a solvent or in a selected solvent mixture (Shahidi and Wanasundara, 1998). The commonly used solvents are methanol and acetone, which pose problems in products for human consumption.

Supercritical fluid extraction (SFE) is a relatively new separation process that may circumvent some of the problems associated with the use of conventional separation techniques (Shahidi and Wanasundara, 1998). This technique is based on the ability of a number of gases to act as selective solvents, provided that they are subjected to pressure and temperature conditions that ensure their existence as supercritical fluids. For foods, CO₂ is chosen because it has mild temperature and pressure

conditions and is inert, inexpensive, non-flammable, environmentally acceptable, readily available and safe (Mishra et al., 1993). However, the industrial use of this method requires further study.

Urea complexation by itself or complexation after an enzymatic reaction offers important technical possibilities (Kojima et al., 2006; LeGoffic et al., 2000; Lin et al., 2006; Linder et al., 2002; Liu et al., 2006). This methodology is based on the crystallization of urea in hexagonal structures with channels of 8-12 Å diameter within the hexagonal crystals, provided the presence of long straight-chain molecules in the medium (Smith, 1952). The formed channels are sufficiently large to accommodate aliphatic chains. However, whereas straight-chain saturated fatty acids fit easily in the channels, the presence of double bonds in the carbon chain increases the bulk of the molecule, thereby reducing the likelihood of its complexation with urea (Schlenk and Holman, 1950).

FAs must be previously converted to free FAs or ethyl esters. These forms of the FAs are mixed with an ethanolic solution of urea with some moderate heating. Afterwards, the mixture is allowed to cool down to a particular temperature, depending on the degree of concentration desired (Shahidi and Wanasundara, 1998). The saturated, monounsaturated and, to a lesser extent, the dienoic FAs are crystallized with urea and the other FAs remain non-crystallized in the solution, being separated by filtration. Among these, a high concentration of omega-3 FAs is found.

Nevertheless, all these described techniques present important shortcomings that limit their use by the industry. The decision for one specific technology or the combination of two or more must depend on the required final characteristics of omega-3 concentrates and on the costs associated to each choice.

3.4.5. Deodorisation/earth treatment

After concentration, a thorough deodorisation and/or purification (removal of impurities) process of the oil may be carried out, especially because some of the previous steps may lead to the formation of small amounts of off-odours and leave trace amounts of some impurities.

Typically, this operation is carried out in the following way: the concentrated material is heated to 80-85 °C and mixed with an activated bleaching clay and/or activated carbon. The bleaching clay removes any oxidation products which may be present in the oil along with heavy metals. The addition of activated carbon is required when the removal of organic contaminants such as dioxins/furans and dioxin-like PCB's is necessary. Another technique uses diatomaceous earth: the FAs are brought into contact for 10 minutes or more, at less than 80 °C, with powdered or granulated diatomaceous earth (Yoshikazu et al., 1997).

3.4.6. Characteristics of omega-3 concentrates

The omega-3 concentrates must fulfil important quality requirements and, as such, their characteristics should match these requirements. The GRAS notifications concerning food-grade specifications accepted by FDA for different omega-3 concentrates offer a set of quality guidelines commonly viewed by the USA authorities as necessary for a GRAS status (Table 4).

As previously mentioned, the form of the omega-3 FAs, that is ethyl esters vs triacylglycerols, is also an important matter. Effectively, some studies have reported that omega-3 FAs are better absorbed by human organism when they are as acylglycerides than as esters (Lawson and Hughes, 1988). On the other hand, some studies have shown that their stability against oxidation is also higher when omega-3 FAs are as acylglycerides than as esters (Boyd et al., 1992). Moreover, stability is also higher when omega-3 FAs are bound to sn-2 position of the glycerol structure than to the sn-1,3 position (Wijesundera et al., 2008). Therefore, the production of acylglycerides concentrates with omega-3 in sn-2 position and the development of viable technological solutions for this purpose have been acknowledged as a wishful outcome (Rubio-Rodríguez et al., 2010). Furthermore, free FAs must be limited to a minimum for reasons identical to those presented for the esters and also because hydrolysis and acidity represent a major loss of quality for omega-3 concentrates. Another important aspect for the quality of the concentrates is their oxidative stability. All the processing steps must

reduce exposure to those factors that accelerate omega-3 FAs oxidation, such as heat, intense light or oxygen. Particularly, the peroxide (primary oxidation compounds) content must be kept low throughout all the concentration process (for this purpose, antioxidant addition may be needed, as can be seen in the next section). Finally, the levels of off-flavours and impurities in the concentrates (Rubio-Rodríguez et al., 2010) must be below human sensory perception.

Table 4: Some quality guidelines for omega-3 concentrates (FDA GRAS Notification 2002; 2006).

| Parameter | Quality guidelines |
|-------------------|--|
| Appearance | Light yellow to yellow at room temperature |
| Triglycerides | >50 % |
| Odour and taste | At worst, a slightly fishy odour and taste |
| Acid value | <1.0 % |
| Peroxide value | <2.5 meq O ₂ /kg |
| p-Anisidine value | <20 |
| Totox value | <25 |
| Density | 0.85-1.00 g/ml |
| Tocopherols | >2.0 mg/g |
| Moisture | <0.1 % |
| Unsaponifiables | <2.35 % |

3.4.7. Antioxidant addition and other means of oxidation protection

One of the major drawbacks of oil rich in omega-3 PUFA, such as fish oil, is their high susceptibility to oxidation, which involve the formation of toxic substances as peroxides or volatile compounds, held responsible for off-flavours. Therefore, storage and packaging of refined fish oils are essential to preserve omega-3 PUFA from oxidation. In addition, other strategies to improve fish oil stability and extend its shelf-life are necessary (Kamal-Eldin and Yanishlieva, 2002).

For that reason, the use of antioxidants is a common method to preserve refined fish oil, preventing its oxidation. A great number of antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA), tocopherols, ascorbic acid, ascorbyl palmitate, propyl gallate, gallic acid, lactoferrines, and others, have been tested to prevent lipid oxidation both in bulk oil (Kamal-Eldin and Yanishlieva, 2002) and in fish oil-in-water emulsions (Jacobsen et al., 2008). In the last decade, the use of natural antioxidants instead of synthetic compounds has received increasing attention and several studies regarding the efficiency of plant extracts, as oregano, parsley or rosemary, on the stabilization of bulk fish oil and fish oil-in-water emulsions have been carried out (Frankel et al., 1996; Bhale et al., 2007; Jiménez-Álvarez et al., 2008). The adequate concentration of antioxidant depends on the chosen substance, the storage conditions and specific applications for the omega-3 PUFA concentrates.

Thus, after leaving the deodoriser equipment and be cooled the fish oil or omega-3 concentrate is dosed with the suitable anti-oxidant(s) and then transferred to appropriate storage tanks. Samples are typically drawn from the oil and analysed. Preferably, storage should be in stainless steel tanks under nitrogen blanketing and at low temperature. Other storage containers than stainless steel tanks may be used as long as they are impermeable to oxygen.

Lipid oxidation of fish oils and omega-3 PUFA concentrates may also be retarded by encapsulation or microencapsulation with a coating material (Matsuno and Adachi, 1993). For this purpose, various physical and chemical processes using several wall materials have been developed to encapsulate and stabilize fish oil and concentrates (Desai and Park, 2005). Among these, spray-drying is the most common and cheaper method (Gharsallaoui et al., 2007), though other processes such as freeze drying (Heinzelmann et al., 2000), ultrasonic atomization (Klaypradit and Huang, 2008) or coacervation (Barrow et al., 2009) have also been proposed recently as alternative to reduce omega-3 PUFA oxidation, since they avoid the use of high temperatures during the drying step. Moreover, oil-in-water

emulsions are increasingly being used as delivery systems for fish oils for functional foods. If properly designed such emulsions can protect the fish oil from lipid oxidation before addition to foods (Day et al., 2007; McClements et al., 2007) and in some cases it may also improve the oxidative stability of the fish oil enriched food (Let et al., 2007).

3.5. Virgin fish oils or extra low oxidised fish oils

The production of 'extra low oxidized oils' differs from traditional production methods for fish oils and fish meals. 'Extra low oxidised oils' are produced from materials from food operations. This can for example be material after filleting of high quality (i.e. very fresh) salmon or herring. The raw material is processed very shortly after catching. The process involves heating to below 100°C, for example to a temperature around 90-95°C just for the time needed for the material to pass through an indirectly heated tubular scraped surface heat exchanger. The heated suspension is then separated in a suitable decanter in order to isolate the oil. The semisolid protein phase that is obtained from the same process can be valuable starting material, for example for production of marine protein hydrolysates. Because of the gentle processing conditions and selection of raw materials these oils are generally suitable for direct use as ingredients in food and beverages.

An example is the Norwegian virgin cod liver oil production. The preparation of this product, including winterization, distillation, blending, drumming, and bottling, is conducted in a manner that ensures the product is carefully processed to concentrate the healthy long chain omega-3 EPA and DHA fatty acids while removing any unwanted environmental chemicals and retaining the naturally occurring Vitamins A and D (www.nowfoods.com/Quality/QualityNotes/M099609.htm).

In the scientific literature references are lacking regarding the production process of virgin fish oil and it was concluded that this product category cannot be evaluated due to insufficient scientific information.

4. Factors affecting fish oil properties

Fish oil is a very delicate food commodity since it is consumed by the consumer as it is. It is not heated, otherwise prepared and the consumer has to rely on its safety. Therefore it is of utmost importance to take all possible measures during the processing of fish into fish oil to offer the consumer a safe, hygienic and high quality product.

4.1. Raw materials

The quality and freshness of the raw materials is a factor of great importance in the preparation of premium-quality fish oil and fish meal (FAO, 1986).

The by-products from the fish processing industry have especially low storage stability if not frozen and preserved (Espe and Lied, 1999). This happens because, immediately after capture, several important chemical, biochemical and microbiological changes occur in fish. Namely, after death, enzymatic activity continues in the viscera and muscle of fish. This activity results in proteolytic changes, which, in turn, lead to additional release of enzymes, not only proteases, but also lipases. The proteases catalyse the hydrolysis of the peptide bonds between the protein residues, thus freeing the amino acids, which also are prone to degradation. The latter enzymes catalyse the hydrolysis of the ester chemical bonds in lipids, such as triacylglycerides, leading to the formation of free FA, which represent a major quality problem for fish oil.

The concentration of endogenous antioxidant substances, such as ascorbic acid, glutathione peroxidase, and tocopherol, in fish muscle starts to decrease and keeps this declining trend over storage time (Undeland and Lingnert, 1999). Concomitantly, there is an increase in the levels of oxidative or catalytic substances, such as hemin, with increasing storage time (Decker and Hultin, 1990), as a result of proteolytic activity. Furthermore, there are chemical processes which lead to the oxidation of lipids (FA), which is associated with the development of rancidity.

4.1.1. Chemical status of the raw material and fish oil

4.1.1.1. Lipid oxidation in raw material (fish)

The highly unsaturated nature of the lipids present in fish makes them very susceptible to lipid oxidation. Lipid oxidation in fish muscle can be caused by non-enzymatic processes such as autooxidation and photosensitized oxidation, as well as catalysed by enzymes such as lipoxygenase. For auto- and photooxidation lipid radicals are formed in the presence of initiators such as heat, light, trace metals or heme-bound iron. The lipid radicals quickly react with oxygen, whereby peroxy radicals are formed. During the propagation phase the peroxy radicals attack intact fatty acids forming odour- and tasteless primary oxidation products, lipid hydroperoxides (LOOH). Low molecular weight (LMW) and heme-bound transition metals quickly break down LOOH to an array of new radicals (hydroxyl radicals, peroxy radicals and alkoxy radicals), which can re-initiate oxidation reactions. Alkoxy radicals can also be cleaved, whereby various secondary products like aldehydes, ketones, acids and alcohols are formed. Volatile oxidation products from omega-3 LC PUFA have extremely low odour thresholds. This makes oxidation a more severe sensory problem in seafood than in more saturated systems such as meat. Some secondary oxidation products such as aldehydes are very reactive and can for example react with free amino groups of proteins whereby tertiary products such as Schiff's bases are formed. These products can polymerize into yellow-brownish pigments (Pokorny et al., 1974).

The enzymatic lipid oxidation reaction is catalyzed by lipoxygenase enzymes. Lipoxygenase activity has been detected in various tissues of fish in blood plasma, gill, skin, fish eggs, brain, muscle, erythrocytes and platelets (Pan and Kuo, 2000). Lipoxygenases are iron containing enzymes, which are situated in the cell cytosol or microsomal fraction. The enzyme catalyses the insertion of one molecule of O₂ into an unsaturated fatty acid containing a 1,4-cis-pentadiene group (Belitz and Grosch, 1987).

In fish the LC omega-3 PUFA are found in both triacylglycerols (TAG) and phospholipids (PL). TAG are found in adipose tissue and also integrated into muscle tissue. PL are located in cell membranes giving them structure and fluidity. PL are more susceptible to lipid oxidation than TAG. This is due to the fact that PL are more unsaturated and are located closer to prooxidants in the aqueous phase. The lipid content of fish muscle affect its susceptibility to oxidation, but recent research suggests that it is not the only factor determining the lipid oxidation rate. Hence, the type and level of pro-oxidants in fish appear to be of greater importance than the lipid content (Jacobsen et al., 2008.).

In general, the susceptibility towards lipid oxidation increase in the following order; white muscle, dark muscle and skin (Undeland et al., 1998; Undeland and Lingnert, 1999). The higher susceptibility of the dark muscle towards oxidation is due to its high content of prooxidative hemoproteins like hemoglobin and its derivatives and higher concentrations of PL than the white muscle (Ingemansson et al., 1991).

The pH decreases rapidly in fish muscle post mortem and this will activate hemoglobin (Hb) as a pro-oxidant by different mechanisms. The pH decrease leads to Hb deoxygenation. Deoxy-Hb has been suggested to act as a strong pro-oxidant in itself (Richards et al., 2002a and b). Moreover, it is very willing to form the highly prooxidative met-Hb (i.e. Hb-Fe³⁺) (Livingston and Brown, 1981). At low Hb concentrations, Met-Hb can also be formed from Hb dissociation (Manning et al. 1996). Met-Hb or met-myoglobin can also react with H₂O₂ to form a hypervalent ferryl-Hb (Fe⁴⁺=O) radical, which is also capable of initiating lipid oxidation (Kanner and Harel, 1985). Apart from this, deoxy-Hb, met Hb/Mb or heme/hemin are able to break down pre-formed lipid hydroperoxides (Ryter and Tyrrell, 2000).

Low molecular weight (LMW)-Fe can be released from hemin when a certain level of lipid hydroperoxides is formed and it has previously been suggested LMW-Fe is able to act as an initiator of lipid oxidation (Puppo and Halliwell, 1988). However, several newer studies have suggested that

heme is a stronger pro-oxidant than LMW-Fe and that the LMW-Fe does not play an important role as a catalyst of oxidation in fish muscle (Chiu et al., 1996, Richards and Li, 2004).

Since oxidation is also catalyzed by heat and light, high temperatures and exposure to light will increase the oxidation rate.

4.1.1.2. Degradation of lipids in raw material by enzymes-lipolysis

Hydrolysis of lipids catalysed by lipases and phospholipases present in fish tissue is an important quality deteriorating process in fish products. The lipolysis process leads to formation of free fatty acids, diacylglycerides and monoacylglycerides, glycerol and nitrogen bases. The sensory properties are particularly affected by the level of free fatty acids. Thus, a significant correlation between off-flavours and the level of free fatty acids has been found (Refsgaard et al., 2000).

4.1.1.3. Lipid oxidation in fish oil

The lipid oxidation reactions in crude and refined fish oil are the same as those described for the raw material. However, prooxidants such as heme iron have been removed during the oil extraction and refining process, but trace amounts of low molecular weight iron and other metal ions may still be present and can catalyze oxidation. Similarly to lipid oxidation in fish, oxidation of fish oil will give rise to the formation of free radicals, lipid hydroperoxides and secondary oxidation products (aldehydes, ketones, alcohols, hydrocarbons, core aldehydes). Only the secondary oxidation products are responsible for the undesirable changes in the aroma and flavour properties of foods caused by lipid oxidation. Among these compounds the vinyl ketones and the trans, cis-alkadienals have the lowest flavour thresholds in oils (Table 5). In contrast, hydrocarbons (alkanes and alkenes) have the highest flavour thresholds (Frankel, 2005).

Table 5: Threshold values of compounds formed from oxidized oils (Frankel, 2005).

| Compounds | Thresholds (mg/kg) |
|-----------------------------|--------------------|
| Hydrocarbons | 90-2150 |
| Substituted furans | 2-27 |
| Vinyl alcohols | 0.5-3 |
| 1-alkenes | 0.02-9 |
| 2-alkenals | 0.04-2.5 |
| Alkanals | 0.04-1.0 |
| Trans,trans-2,4-alkadienals | 0.04-0.3 |
| Isolated alkadienals | 0.002-0.3 |
| Isolated cis-alkenals | 0.0003-0.1 |
| Trans,cis-alkadienals | 0.002-0.006 |
| Vinyl ketones | 0.00002-0.007 |

Lipid oxidation of oils will give rise to off-flavours ranging from nutty through green, grass cucumber to rancid and synthetic. For fish oils, the term train oil is frequently used. In cases where lipid oxidation is extremely advanced the oil may develop a painty odour. A number of different volatile oxidation compounds have been suggested to contribute to these off-flavours. Table 5 shows some of these volatiles and the odour that have been ascribed to them.

Oxidised oil will contain a mixture of different volatiles and the relationship between the concentration of these volatiles and their sensory impact is poorly understood. Only one model describing the quantitative relationship between volatiles and an important off-flavour in oxidised oils has been reported. This model describes the intensity of the fishy taste in fish oil as a function of the concentration of three volatiles, 2,6-nonadienal, 4-heptenal, and 3,6-nonadienal oil (MacFarlane et al., 2001).

Table 6: Odour threshold values of selected aldehydes produced by lipid oxidation (Belitz and Grosch, 1999)

| Aldehydes | Odor threshold in oil (ppm) | | Description |
|-------------------------|-----------------------------|------------|-----------------------|
| | Nasal | Retronasal | |
| Hexanal | 320 | 75 | Tallowy, green, leafy |
| Heptanal | 3200 | 50 | Oily, fatty |
| Octanal | 320 | 50 | Oily, fatty, soapy |
| Nonanal | 13500 | 260 | Tallowy, soapy-fruity |
| Decanal | 6700 | 850 | Orange peel-like |
| 2-t-pentenal | 2300 | 600 | Pungent, apple |
| 2-t-hexenal | 10000 | 400 | Apple |
| 3-c-hexenal | 14 | 3 | Green, leafy |
| 2-t-heptenal | 14000 | 400 | Fatty, bitter almond |
| 2-t-nonenal | 900 | 65 | Tallowy, cucumber |
| 3-c-nonenal | 250 | 35 | Cucumber |
| 2-t-decenal | 33800 | 150 | Tallowy, orange |
| 2,4-t,c-heptadienal | 4000 | 50 | Frying odour, tallowy |
| 2,4-t,t-heptadienal | 10000 | 30 | Fatty, oily |
| 2,6-t,t-nonadienal | 2500 | 460 | Fatty, oily |
| 2,6-t,c-nonadienal | 4 | 1.5 | Cucumber-like |
| 2,4-t,c-decadienal | 10 | - | Frying odour |
| 2,4-t,t-decadienal | 180 | 40 | Frying odour |
| 2,4,7-t,t,c-decatrienal | - | 24 | Cut beans |
| 4,5,2-t-epoxy-t-decenal | 1.3 | 3 | Metallic |

Due to the high number of double bonds in EPA and DHA, oxidation processes may lead to a complex mixture of hydroperoxides and a myriad of volatile, non-volatile and polymeric secondary oxidation products. The exact mechanisms for the formation of many of the observed products are not yet completely understood. Omega-3 PUFA though follows the same cleavage mechanisms as those recognized for linolenic acid. Hence, some of the oxidation products that can be expected from autoxidation of omega-3 PUFA are propanal, 2-pentenal, 3-hexenal, 4-heptenal, 2,4-heptadienal, 2,6-nonadienal, 2,4,7-decatrienal, as well as 1-penten-3-one, 1,5-octadien-3-one and 1-penten-3-ol. Fish oils not only contain omega-3 PUFA, but also omega-9 MUFA and omega-6 PUFA. Therefore, volatiles formed from these fatty acids are also found in fish oils.

Volatile compounds have different sensory threshold values. Importantly, the human sensory apparatus has a particularly low threshold for volatile off-flavours resulting from oxidation of omega-3 PUFA (Frankel 2005). Thus, 1-penten-3-one as well as (Z)-4-heptenal, (E,Z)-2,6-nonadienal and 2,4,7-decatrienals have been associated with sharp-burnt-fishy off-flavours in oxidized fish oil (Karahadian and Lindsay, 1989). However, decatrienals are usually only observed in highly oxidised products and in several studies on fish oil enriched foods this compound was not observed (Venkateshwarlu et al., 2004; Hartvigsen et al., 2000a; b).

4.1.2. Methods used to characterise raw material and fish oil

In a concerted action “Evaluation of Fish Freshness” from 1995 to 1997 methods to determine freshness of fish in research and industry were intensively studied. The results are also today the best overview about methods used for the determination of the freshness of fish (Olafsdottir, 1997a, b; Oehlenschläger, 1999). Modern methods mostly based on instrumental techniques are described in the book by Rehbein and Oehlenschläger (Rehbein and Oehlenschläger, 2009). The volatile amines (ammonia, dimethylamine, trimethylamine, total volatile basic nitrogen, TVB-N) are reviewed extensively (Howgate, 2009; Oehlenschläger, 1997a, b). TBA-value, peroxide-value and free fatty acids which are used mainly in fatty species are described (Undeland and Lingnert, 1997; Rustad, 2010). Use of biogenic amines has recently been reviewed (Mendez, 2009).

TVB-N is a spoilage indicator, low values give no indication about the freshness since this parameter does not change e.g. during iced storage for the first 10 to 12 days in ice. All literature about TVB-N is about the content in the fish muscle, which means the edible part of the fish. The method is not

validated for whole fish and there is no knowledge about the development of TVB-N in other fish parts than muscle (Howgate 2010 a; b).

4.1.2.1. Free fatty acids

As described above free fatty acids (FFA) are formed due to lipolysis. FFA content in the crude oil will depend on the fish species and season. FFA may constitute up to 12 % of total lipids but typical values are lower (Bimbo, 1998). FFA are more susceptible to lipid oxidation than Triglycerides and they will also give rise to off-flavours in the final product. FFA must therefore be removed during refining (see below). FFA are most often quantified by titration with NaOH with phenolphthalein as an indicator (AOCS, 1998). This method is valid for raw material and fish oil.

4.1.2.2. Peroxide value (PV)

Oxidation of the oil, in oily fish like herring, gives rise to rancid odours and flavours. These can limit the storage life of such species more quickly than the protein changes that govern the extractable protein value. An important stage in the oxidation is the addition of oxygen to the fatty acid molecules to form hydroperoxides; the amount of these can be used as a measure of the extent of oxidation in the early stages. The correct term lipid hydroperoxide value is frequently shortened to peroxide value.

To measure peroxide value in fish raw material the oil must first be extracted from the fish by a method that does not itself encourage further oxidation. The oil containing peroxides is treated with potassium iodide: iodine is liberated and measurement of the amount of iodine enables the peroxide value to be calculated. Other methods to determine peroxide values also exist (see below), but the method based on the reaction between peroxides and iodide is the standard method (AOCS, 1998).

Increase in the peroxide value is most useful as an index of the earlier stages of oxidation; as oxidation proceeds the peroxide value can start to fall. Therefore, a single measurement of PV can only be used as an index of current oxidation status if the peroxides formed are stable enough so that they do not decompose after formation. This is not true for most lipids. This method is valid for raw material and fish oil. The PV in crude oils will depend on the quality of fish used for oil extraction, the process of oil extraction and the storage conditions of the crude oil. As previously described elevated temperatures and high levels of Hb-Fe will catalyze oxidation. Hence, if fish have been squeezed and bruised on-board the fishing vessel and/or if the fish has been stored several days on board the vessel maybe even at a too high temperature before being processed, it may be severely oxidized when it reaches the processing plant. This will lead to crude oils with high PV, e.g. above 10 meq/kg. As mentioned above a wide range of methods have been developed for measuring PV, including both wet chemical and instrumental methods. The analytical principle of many methods is the ability of the lipid hydroperoxides to oxidize either iron or iodide ions (Nielsen et al., 2003). The main disadvantage of the AOCS standard method (AOCS, 1998) is that it requires large sample amounts. Nevertheless, this method is recommended as a standard method for PV determination in crude oils in the industry.

Most of the methods mentioned above are empirical and unspecific. Thus, other components present in the food may also react with the reagents used in the different methods. For example iodide may under certain circumstances react directly with unsaturated double bonds in the case of the titration and micro methods. Likewise, the iron salts may react with e.g. metal chelators that have been added to the food/fat to prevent lipid oxidation. Due to the lacking specificity of the various methods, different PVs may be obtained when the same samples are analyzed by different methods.

During the past decades, several new instrumental methods have been developed for measuring PV including methods such as GC-MS (Frankel et al., 1979), HPLC (Hartvigsen et al., 2000b, Müllertz et al., 1990), IR and NMR spectroscopic techniques (Khatoun and Krishina, 1998) NIR (Yildiz et al., 2001; 2002; 2003) and FTIR (Ma et al., 1997; Sedman et al., 1997) and Differential Scanning Calorimetry (Tan and Man, 2002). Some of the most promising instrumental methods for routine control analysis of peroxide values seem to be NIR, FTIR and DSC techniques. NIR spectroscopy offers several advantages over conventional methods including high speed, non-destruction of the sample, possibility of automation, on-line measurements and no reagents required/minimal sample

preparation. Yildiz et al. (2001; 2002) have developed a very simple method to measure peroxide values in soybean and corn oil by NIR spectroscopy. The only sample preparation is the transfer of a small oil sample (which has been tempered at 25 °C) to a 2 mm quartz cuvette, which subsequently is placed in the cuvette holder of the NIR instrument. The PV in a set of test samples predicted by NIR were compared to PV measured by the AOCS titration method and the correlation between the two methods was good ($r > 0.99$). The NIR method and the other spectroscopic methods can be used as long as they are calibrated against the standard method.

4.1.2.3. Thiobarbituric acid value (TBA value)

The hydroperoxides, mentioned above, can react further to give a wide range of compounds, some of which are responsible for the rancid odours and flavours in oily fish and for cold storage odours and flavours in white fish. One such compound, called malonaldehyde, and a number of related compounds can be separated from the fish either by distillation or by preparing a protein-free extract. Reaction of these compounds with 2-thiobarbituric acid gives rise to coloured products, the amount of which, the TBA value, is measured using a spectrophotometer. The increase in the TBA value is a measure of the extent of oxidative deterioration in oily fish, but, as in the case of peroxide value, the TBA value can fall again at a later stage of spoilage. This method is only used for raw material.

4.1.2.4. Anisidine value

The anisidine test is a method commonly used in the oil industry as a measure of the level of secondary oxidation products (carbonyl compounds). The principle of the anisidine test is that the before mentioned compounds react with p-anisidine to form a colored complex that absorbs at 350 nm. The anisidine value is defined as the absorbance of a solution resulting from the reaction of 1 g fat in 100 mL of isooctane solvent and 0.25 % anisidine in glacial acetic acid (AOCS, 1994). The anisidine test has previously been attributed to the reaction between p-anisidine and 2-alkenals, but other carbonyl compounds than the 2-alkenals are able to react with p-anisidine as previously mentioned. The anisidine method is therefore an unspecific method and furthermore it is not very sensitive. The anisidine test measures the carbonyl compounds that may contribute to off-flavor formation as a result of oxidation. Therefore, results obtained from the anisidine test could be expected to correlate well with sensory data, but this is not always the case, especially not in food emulsions.

Quantitative determination of anisidine value by FTIR spectroscopy also has been shown to be feasible (Sedman et al., 1997), while NIR seems to be less suitable for a spectroscopic determination of AV (Yildiz et al., 2001). The method is used for raw material and for fish oil.

4.1.2.5. Gas Chromatographic methods for determination of volatile oxidation products

In contrast to the anisidine and TBA test, gas chromatographic (GC) methods to determine secondary oxidation products offer the sensitivity and specificity that the former methods are lacking. GC methods are thus capable of determining volatile oxidation products that either contribute to or serve as markers for the off-flavor formation in oxidized lipid containing foods. Depending on the method to collect and trap the volatiles a large number of different volatiles can be determined by GC methods including aldehydes, hydrocarbons, ketones and alcohols. The most common methods to collect the volatiles are: the static headspace, the solid phase micro extraction method, and the dynamic headspace method.

The static headspace method is based on the principle that at a given temperature and in a sealed container volatiles will diffuse from the liquid/solid phase and vaporize into the headspace above the sample and after a certain length of time an equilibrium will be established. At equilibrium the concentration in the headspace will be proportional to the concentration of the volatiles in the sample. The static headspace method is suited to highly volatile compounds and for routine consecutive analyses of many samples. It is often used as a quick method to determine hexanal originating from n-6 fatty acids and propanal originating from omega-3 fatty acids. These aldehydes are often used as markers of oxidation in vegetable oils and fish oils, respectively. The static headspace method is not

sensitive towards some of the higher molecular volatile compounds such as the 2,4-decadienals and the 2,4-heptadienals, which have low flavor thresholds and which may be important to flavor deterioration.

The solid phase micro extraction (SPME) technique is very simple: The sample is weighed into a vial, which subsequently is sealed with a cap provided with a needle-pierceable septum. After equilibration at the desired temperature a SPME fiber is inserted into the vial. Subsequently, extraction of the volatiles onto the fibre is usually conducted under stirring for a period of e.g. 10-30 minutes. Then, the fibre is conditioned in the GC injector for a certain time period before analysis. This method is less sensitive than the dynamic headspace method. Calibration curves for selected compounds can be made as described for the static headspace method.

The principle of the dynamic headspace sampling method is that the volatiles are "forced" to be released from the food matrix by purging or sweeping a liquid sample with nitrogen or helium in a tube or vessel heated in e.g. a water bath. Subsequently, the volatiles are trapped into a tube containing a porous polymer (e.g. Tenax™, Chromosorb™, Carbosieve™). The volatiles are then desorbed from the absorbent tube by heating, and depending on the instrument, the volatiles may be further concentrated by trapping on a cold trap at low temperatures (e.g. minus 30 °C). Subsequently, the cold trap is heated and the volatiles transferred by a carrier gas onto the capillary inlet of the GC, where they are separated on the GC column. After sampling of the volatiles, they are analyzed by either flame ionization detection (FID) or mass spectrometry (MS). This is also the case after static headspace and SPME sampling. Different methods are available for further identification of the volatiles (spiking with authentic standards, Kovats index and mass spectra library). The advantages of the dynamic headspace method is that it is sensitive and that a more complete picture of the volatiles present in the sample is obtained compared with the static headspace and SPME methods. The disadvantage is that the sampling procedure is laborious and time consuming, but automated systems are available for the later part of the sampling procedure, i.e. the transfer of the volatiles from the absorbent tube to the GC. Another disadvantage is that a large range of volatiles usually will be detected and therefore it can be difficult to ascribe importance to any single species. However, by the use of multivariate data analytical statistics it is possible to look at many variables at the same time and to make mathematical models that describe the relation between sensory and GC data.

4.1.2.6. Total volatile bases (TVB)

Ammonia and trimethylamine are examples of bases; another base, dimethylamine (DMA), can also be formed during spoilage of fish, together with traces of others. These bases, other than ammonia, are known chemically also as amines. The combined total amount of ammonia, dimethylamine and trimethylamine is called the total volatile base content of the fish and is a commonly used estimate of spoilage. The increase in the amount of TVB parallels the increase in TMA but the analysis is easier to carry out than that for TMA.

Alternative terms used are total volatile basic nitrogen (TVB-N) and total volatile nitrogen (TVN), since the results of the analysis are always given in terms of the nitrogen content of the bases. Corresponding French and German names, are azote basique volatil total (ABVT) and flüchtiger Basenstickstoff. TVB can be measured easily and quickly using relatively simple apparatus and, for this reason, a TVB value is often used as a rejection limit in regulations and commercial specifications. This method is only used for raw material.

4.1.2.7. Trimethylamine (TMA)

Most marine fish contain a substance called trimethylamine oxide (TMAO). Certain bacteria that occur naturally on the skin and in the guts of fish and in sea water can break down TMAO to trimethylamine. The amount of TMA produced is a measure of the activity of spoilage bacteria in the flesh and so is an indicator of the degree of spoilage. TMA can be measured by a chemical method that produces a coloured solution; the amount of the coloured product is measured using a

spectrophotometer. Alternatively, TMA can be separated from similar compounds, and its amount measured, by gas chromatography (GC). This method is only used for raw material.

4.1.2.8. Ammonia

Bacteria can generate small amounts of ammonia in spoiling fish, mainly from free amino acids; the amount of ammonia can give an indication, though not a particularly accurate one, of the extent of spoilage. Much larger amounts of ammonia are produced during spoilage of the elasmobranch fish, skate and dogfish for example, because they have large amounts of urea in their flesh. Shellfish, also, may develop more ammonia than most marine fish and at an earlier stage. There are several chemical and enzymatic methods for measuring ammonia. This method is only used for raw material.

4.1.2.9. Histamine

Certain families of fish, notably the *Scombridae*, *Scomberesocidae*, *Clupeidae*, *Engraulidae* *Coryphaenidae* and *Pomatomidae* families, contain histidine, an amino acid, in larger amounts than other families. During spoilage of these fish, especially if the temperature rises to above 10°C, histidine may be converted to histamine. To measure histamine a protein-free extract is first prepared; the histamine is separated from interfering substances by extraction first into an organic solvent followed by back extraction into an aqueous solution. The histamine is treated with a substance that gives a fluorescent product and the amount of this product is measured using an instrument called a fluorimeter. Histamine can also be measured by HPLC, along with certain other amines including putrescine and cadaverine; the term "biogenic amines" is often used to describe these substances. This method is only used for raw material.

4.1.2.10. Colour in general

Colour of refined fish oil can vary, but usually it is considered that must be pale yellow. Very often the method described in European Pharmacopoeia (2005) is used.

4.1.2.11. Water content

Depending on the number of polishing steps the crude oil will contain low levels of water. The water content in crude oils is generally below 0.5 % (Bimbo, 1998). The water content of refined fish oil is below that value. Water content is usually determined by Karl Fisher titration. This method is used for fish oil. It determines the actual water content of fats and oils by titration with Fischer reagent, which reacts quantitatively with water (AOCS, 2009).

4.1.2.12. Sensory

As it was previously referred, several analytical methods are used to assess fish freshness through the quantification of spoilage in fish, mostly based on volatile bases resulting from microbial and enzyme activity. However, such evaluation has to go together with sensory assessment. For most fish species the freshness grading schemes introduced by the EU Council Regulation (EC) No 2406/96 have been widely used. However, the three levels foreseen in such schemes (E – extra, highest quality, A – good quality, B – satisfactory) do not account for biological and nutritional differences between species. Taking into consideration this drawback of the EU freshness grading scheme the QIM method was developed.

The Quality Index Method (QIM) (Bremner, 1985) which is based on objective evaluation of the key sensory attributes of each fish species using a demerit points scoring system. QIM development for seafood involves the selection of appropriate and best fitting attributes in order to observe a linear increase in the Quality Index (QI) with storage time in ice. The main objective is to obtain a linear correlation between the sensory quality of the species expressed as the sum of demerit points and the time of storage in ice, predicting the freshness of a given fishery product. These changes, evaluated by QIM panels, are individual observations of biochemical deterioration of the species during storage time. Furthermore, the sensory evaluation provided by QIM tables and the quantification of the

changes, which result from spoilage, should be complementary disciplines used to measure seafood quality. The QIM scheme is only applicable to raw materials and not to fish oils.

As it was referred previously, fish oils in different forms are very unstable. Such instability can be reflected in colour, odour and taste. The main drivers of sensory quality changes in fish oil are the oxidation and hydrolysis processes. Namely, secondary lipid oxidation products resulting from the decomposition of hydroperoxides (see above), such as aldehydes, ketones and alcohols of various chain lengths and degrees of unsaturation (Lee et al., 2003) have a strong sensory impact, imparting off-flavours to fish oil. Moreover, some of these compounds have very low odour thresholds and thus affect sensory quality at very low levels (Frankel, 2005). Therefore, the sensory evaluation of fish oils is one of the most important analyses, together with the determination of lipid oxidation products (Jacobsen, 1999). In most studies, descriptive sensory analysis is carried out and the intensity of attributes assessed by the scaling method. It is well known that a specific training of panellists can be regarded as a prerequisite; however, in most studies the training of the sensory panel was not well documented. For microencapsulated fish oil, two studies used the same descriptors commonly used for bulk fish oil, but limited to single odour attributes, such as rancid (Baik et al., 2004; Jonsdottir et al., 2005) or metallic/painty (Keogh et al., 2001). In a more recent study (Serfert et al., 2010) different sensory attributes were proposed specifically for fish oil, such as pungent, buttery, green notes and others. Another important dimension of fish oil sensory quality relates to the absence of impurities. These are commonly considered as an aggregate and termed as unsaponifiable matter (USM). This fraction of fish oil is composed primarily of sterols, glyceryl ethers, hydrocarbons and fatty alcohols, including minor quantities of pigments and various oxidation products, and, accordingly, is responsible for off-flavours and discolouration (Boran et al., 2006). However, the USM level in fish oil is not necessarily to be interpreted as a negative trait throughout all range of values, since Young (1986) reported that USM was normally less than 2 % of fish oil, only increasing up to 8 % under certain seasonal and feeding conditions. According to other authors (Bimbo, 1998), there is no standard for USM of fish oil and this parameter varies greatly depending on fish species. It has been suggested that the upper limit for USM should be 3.5 % (FAO, 1986) or 4 % (Boran et al., 2006) in order to ensure top quality. The latter authors reported that while USM of fish oil stored at 4 °C reached the acceptability threshold for human consumption in 90 days, for fish oil stored at -18 °C, such threshold was only surpassed after 150 days. Therefore, storage temperature is a fundamental factor to be taken into account in order to ensure a high quality standard, since such a high USM level is linked to the accumulation of oxidation products and to rancidity.

4.1.3. Microbiological status of raw material

Fish raw materials contain indigenous a wide variety of microorganisms, but only a selection of these contaminants is able to colonize the material and grow to high numbers (Gram and Huss, 1996). In fact, bacteria establish themselves in the outer and inner surfaces of fish (gills, skin, gastro-intestinal tract). The autolytic processes described above make catabolites available for bacterial growth (Huss, 1995).

The bacteria associated to the spoilage of aerobically stored fish material consist typically of Gram-negative psychrotrophic non-fermenting rods (Gram and Huss, 1996). Most bacteria identified as specific spoilage bacteria produce one or several volatile sulphides. In particular, these bacteria contribute to the breakdown of various biomolecules, for instance, *Shewanella putrefaciens* and some *Vibrionaceae* also produce hydrogen sulphides from cysteine (Stenström and Molin, 1990). Hence, after sufficient storage time and depending on handling, processing and storage conditions, like temperature, microbiological processes combine with the autolytic phenomena to reduce the quality of fish raw materials.

Microbiological examination has been used in fish as a measure of keeping quality and shelf life of the product (and indirectly to indicate temperature abuse and hygiene during handling and processing), and of course to detect the possible presence of pathogens. Since microbial spoilage is mainly determined by the so-called specific spoilage bacteria, their nature and number can give an indication

of spoilage process and can be used to predict the remaining shelf life. The main component of the fish microflora (present on skin, gills and gut) is indigenous to the aquatic environment, where the fish is caught; besides some microbial groups can be present as a result of faecal contamination, or introduced during post-harvest and processing (Howgate, 1998; WHO, 1999; Huss et al., 2003). Microbial contamination from human sources (in specific coastal areas or during handling) is also considered as non-indigenous. Microbial spoilage in fish develops faster as compared to meat, because of its intrinsic properties which allow more rapid bacterial proliferation – especially since the pH remains above 6.0 (Gram and Huss, 1996). A number of variables determine the initial colonization and proliferation by spoilage microorganisms including, time after fish capture, hygiene of practices during handling and processing of fish and fish storage conditions (time, temperature and atmosphere), and additional contamination from utensils, equipment, surfaces or food handlers. Despite the heterogeneity in the initial microbial population, a reduced number of bacteria dominate in the spoilage ecology of refrigerated fresh fish and include aerobic psychrotrophs belonging to *Pseudomonadaceae* and *Moraxellaceae* (*Pseudomonas* spp., *Psychrobacter* spp., *Moraxella* spp., *Acinetobacter* spp.) and facultative anaerobes such as *Enterobacteriaceae* (*Shewanella* spp.). If stored at ambient temperature, other species of *Enterobacteriaceae* and *Vibrionaceae* can also be present. Lactic acid bacteria and some *Vibrionaceae* species (*Photobacterium phosphoreum*) are dominant in modified CO₂-rich atmospheres.

Fish spoilage can be the result of bacterial metabolism, chemical decomposition (autolysis) and lipid oxidation. According to ICMSF (ICMSF, 2005), the accumulated metabolic products of bacteria are the primary causes for the organoleptic spoilage of raw finfish. Bacteria can accelerate the spoilage process by reducing TMAO to trimethylamine, and participating in other processes such as the oxidative deamination of amino acids and peptides to ammonia, release of fatty acids, and breakdown of sulphur-containing amino acids to methyl mercaptan, dimethylsulphide and hydrogen sulphide. Typically, microorganisms use low molecular compounds as primary sources of carbon and energy, with preferential utilization of carbohydrates. However, as soon as glucose and other low-molecular weight carbohydrates are depleted, there is a shift towards NPN compounds which yield off-odours (volatile sulphides and amines) and metabolites such as TMA-N and amines.

Several microbiological tests have been proposed, to evaluate the quality of fish as related to shelf-life and keeping quality. Traditional methods rely on sample culturing and colony formation and are therefore time and effort-consuming, and need expertise for execution and interpretation. This has to do with correlation of raw numbers with spoilage processes. Some examples are Total Aerobic Count and Standard Plate Count which represent the number of bacteria capable of forming visible colonies on a culture media at a given temperature. Other groups with different significance are *E. coli*/coliform bacteria and faecal streptococci which may be also employed as indicators for hygiene and appropriate handling of fish. Alternative counting methods are based on detection of cell compounds (Limulus lysate test, bioluminescence) or the microbial activity (impedance, microcalorimetry, dye reduction).

4.1.3.1. Microbiological criteria and testing

A microbiological criterion for food, according to Codex Alimentarius Commission (CAC, 1997), “defines the acceptability of a product or a food lot, based on the absence or presence, or number of microorganisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot”. In the European Union, Regulation (EC) No 2073/2005 as amended by Regulation (EC) No 1441/2007 set down the microbiological criteria for certain microbial hazards to be complied with by food business operators. Microbiological criteria and targets for food in the European legislation have been overviewed in previous BIOHAZ opinion (EFSA, 2007).

EU Food Safety Criteria defines the acceptability of food products. These criteria apply to the products placed on the market during their shelflife. If the criteria are not met the product/batch should not be placed on the market. With respect to fishery products, level of histamine is included in the food safety criteria for fish species associated with high amount of histidine (e.g. *Scombridae*, *Clupeidae*, *Engraulidae*, *Scomberesocidae*, *Coryphaenidae* and *Pomatomidae*), and unsatisfactory levels are: if

the mean (from 9 samples) histamine level exceeds 100 mg/kg, or if more than 2 samples (out of 9) contain 100-200 mg/kg, or if in any sample is found >200 mg/kg. In case of those fishery products which have undergone enzyme maturation, the histamine limits are doubled, but those products are not used for fish oil production considered in this document. The analytical reference method for histamine is HPLC (Malle et al., 1996; Duflos et al., 1999). Furthermore, the food safety criteria include limits, sampling plan and analytical method for *E. coli* in live bivalve molluscs and live echinoderms, tunicates and gastropods but, again, those products are not used for fish oil production considered in this document.

EU Process Hygiene Criteria gives guidance on, and is an indicator of, the acceptable functioning of HACCP-based manufacturing, handling and distribution processes. It sets indicative contamination values, applicable before placing the product on the market, above which corrective actions are required in order to maintain the hygiene of the process in compliance with food law. With respect to fishery products, the process hygiene criteria are stated only for shelled and shucked products of cooked crustaceans and molluscan shellfish and the limits, sampling plans, and corresponding analytical methods, relate only to *E. coli* and coagulase-positive staphylococci. However, these fishery products are not used for fish oil production considered in this document.

4.2. Other aspects after processing including storage

Prior to further processing, packaging or distribution fish oil is bulk stored in big quantities. Bulk storage can vary from short time storage to a longer period storage. There are some parameters which influence the quality of bulk stored fish oil primarily. These parameters are: time, temperature, light and oxygen. Oxygen deteriorates bulk stored fish oil by autoxidation processes which increase with increasing time and increasing temperature and are stimulated and intensified by light. Therefore care should be taken that fish oil is stored in a dark place without exposure to light or sunlight, that no oxygen is present in the oil or has access to the surface of the oil (nitrogen flushing through the oil and stored under a nitrogen atmosphere), that the oil is stored in a cool place and that the storage time is limited to a minimum.

If no oxygen and no pro-oxidative substances are present, bulk storage in complete darkness and at low temperatures is a safe way of storing fish oil. In bulk storage the relation of surface to volume is very small in contrast to storage in small units or capsules where the surface to volume ration is extremely higher and the risk of oxidation increases. Fish oil should be stored as cold as possible since lipid oxidation rates increase with temperature. As a rule of thumb lipid oxidation rates will double every time temperature is increased by 10°C.

For the bulk storage of fish oil a detailed HACCP plan has to be set up by the processors taking into account the special problems and conditions on the premises where the fish oil is processed and stored. A plan for corrective action has to be present when alterations as oxidation occur during the bulk storage. The HACCP plan shall include information about the material of the storage tanks used and about additives added to the fish oil (e.g. antioxidants).

5. Hazards

5.1. Biological hazards in raw materials

A number of biological hazards with harmful effects on human health that can be present in fish are reviewed in a recent EFSA scientific opinion entitled 'Food Safety considerations of animal welfare aspects of husbandry systems for farmed fish' (EFSA, 2008) and other publications (WHO, 1999; Huss 2002; Huss et al., 2003) which the reader can consult for further information and literature sources. Therefore, biological hazards will not be considered here in detail, but only in brief remarks. Saltwater fish-borne biological hazards include pathogenic organisms and biological toxins that can cause human disease either via fish consumption or via other routes. Parasites that can be transmitted from fish to humans include nematodes, cestodes and trematodes. *Anisakis* spp., *Diphylobotrium latum* and *Chlonorchis* spp. are some representative examples. In addition, fish can contain certain protozoan

parasites. Some pathogenic bacteria can be found in fish such as *Clostridium botulinum*, *Vibrio cholerae*, pathogenic vibrios (eg. *Vibrio vulnificus*, *V. parahaemolyticus*), *Aeromonas hydrophila* and *Listeria monocytogenes*. Particularly relevant are pathogenic *Vibrio* spp. including *Vibrio parahaemolyticus* which are usually associated with bays and coastal waters, particularly in warm waters and during summer season. Their counts are affected also by depth of water and the tidal range. Non-indigenous bacteria include members of *Enterobacteriaceae* such as *Salmonella* spp., *Shigella* spp., and *Escherichia coli*. Fish in general, and particularly shellfish, harvested from coastal waters contaminated with human or animal faecal effluents, can contain human pathogenic viruses: hepatitis A virus, calciviruses, astroviruses and Norwalk viruses. Other possible hazards found in fish are biotoxins, toxins from phytoplankton, and biogenic amines, which are generally thermostable and water soluble.

5.2. Fate of biological hazards during fish oil processing

Biological hazards present in raw material are exposed to a range of factors during fish oil production. From perspective of biological safety of the final product, the most important factors are heat treatment, alkalization-acidification and water removal.

Several heating steps are involved in the fish oil production process. In the crude oil phase, the raw materials (whole fish and by-product) are initially steam heated to 90-95°C for approximately 10-20 minutes, and then usually reheated to 90-95°C during the decanter step. In the oil refining phase, the oil is repeatedly heated at temperatures up to 180°C (deodorisation), to approximately 80-85°C (mixing with alkali/acidic solutions and earth/carbon filtration) and to 100-110°C (drying under vacuum). It is considered that these repeated heating regimes, including combined with exposure to strong alkali/acid, inactivate all parasites, bacteria, viruses and heat-sensitive toxins initially present in raw material.

It is considered that any heat-stable toxins (e.g. microbial including histamine) or potential allergens from parasites that can be present in raw material are water-soluble and therefore removed from oil together with water phase. Water is separated from oil and discarded during the crude oil production phase, as well as again repeatedly during the oil refining phase when the oil is treated with alkali/acid solutions and the water phase separated and discarded. Furthermore, following drying, remaining moisture in the finished product is very low (0.1-0.3%) and consequently the water activity is also low.

Therefore, it is considered that biological food safety risk in fish oil at the end of its production process at bulk storage is negligible. Nevertheless, it should be noted that potential microbial cross-contamination of oil during further packaging-storage-distribution chain of events cannot be totally excluded, but these stages are not considered in this document.

5.3. Chemical hazards arising during processing

This section shall include biological and chemical hazards including cleaning agents e.g. sanitizers, disinfectant, detergents traces mineral oil whereby no method to detect it seems to be available; vegetable oil which represents a fraud problem and chemical and physical analytical methods (such as for example colour).

5.3.1. Cleaning agents for fish meal and oil production as well as fish oil refining

Generally, fish oil producers and refineries use the same cleaning agents as other food producers, i.e. sodium hydroxide and hydrochloride acid. Sodium hydroxide is used in concentrations from 3-7 %. Hydrochloride acid is used for neutralisation afterwards. Hence, the hazards are the same as for production of any other food product. In fish meal factories mineral oils are used as lubricants. There may be a risk of contamination and this may be a potential hazard in fish oils, particularly if the mineral oil used is not food grade.

5.3.2. Chemical hazards arising from lipid oxidation

As described above lipid oxidation will lead to the formation of free radicals, lipid hydroperoxides and volatile oxidation products. The relationship between the peroxide values (PV) or anisidine values (AV) and the concentration of specific volatile oxidation products or a sum of these has not been established.

5.3.2.1. Published data

Most studies have dealt with lipid oxidation products formed in the body and fewer studies have dealt with the effect of ingested oxidation products. Moreover, most studies have used pure oxidation compounds synthesized in the lab, whereas fewer studies have been performed on mixture of compounds formed in real oils/foods. Furthermore, the studies have been performed *in vitro*, in cell models or in animal studies. No published data on oxidation products derived from fish oil seem to be available.

Only few data from human studies seem to be available. This means that our knowledge about the fate and effect of ingested oxidation products from oxidised oils after digestion in the human gastrointestinal tract in humans is limited, if not non-existing. Another problem is that only limited information is available on the concentrations of the oxidation products needed to induce a pathological/toxicological effect *in vivo* in animals and even when such information is available it is not known to which extent the data can be extrapolated to humans.

Esterbauer (1993) concluded that heavily oxidized oils given orally are not acutely toxic to humans. There is an overall lack of gross pathological effects of heavily or mildly oxidized oils, probably because di- and polymeric oxidation products are not well absorbed and peroxides are detoxified by glutathione dependent enzymes to less toxic lipid alcohols. However, low molecular aldehydes are more readily absorbed and produce pathological effects such as damage to liver, thymus and kidney, but it is unlikely that humans ingest amounts similar to those which have resulted in such effects in animal studies. He also concluded that malondialdehyde and lipid hydroperoxides have been reported to induce tumours and that heavily and mildly oxidised oils can cause arterial and cardiac damage, but no indications on the level of peroxides or aldehydes required to give such effects were indicated.

Toxicological aspects of the following groups of oxidation products have been described (Esterbauer 1993) including: 4-hydroxyalkenals, malondialdehyde, 2-alkenals, other aldehydes, and lipid hydroperoxides. The major conclusions for each group except the hydroperoxides are summarised below.

4-hydroxyalkenals

These compounds have primarily been found in oils after frying. The three most important compounds are 4-hydroxy-2-trans-hexenal (HHE), 4-hydroxy-2-trans-octenal (HOE), 4-hydroxy-2-trans-nonenal (HNE). HOE and HNE have been reported to be formed after heating of methyl linoleate or methyl linolenate to 185°C for 1 hr and the amount increased with increasing heating time. (Han and Csallny, 2009). No information was given on the PV level or AV/TBARS levels in these oils. Refining is done under vacuum. It is possible to remove them as volatiles during refining (at least to some extent). Grein et al. (1993) reported that these hydroxyaldehydes can be formed from unsaturated aldehydes by water mediated reactions. This means that aldehydes such as 2-alkenals that can be formed at much lower temperatures can be transformed into hydroxyl-alkenals. To which extent this happens in real oil products and foods is not known. Esterbauer et al. (1991) reported that HNE has been identified as a major toxic product in methyl linolenate oxidised at (only) 12 °C for 72 hr to a PV of 1000 meq/kg. It seems however unrealistic that such a PV can be obtained in such a short time under these conditions unless the oil used was already highly oxidised when the experiment was initiated. The formation of hydroxy-alkenals is more complex than formation of other aldehydes. Omega-3 PUFA can give HHE but not HNE. Omega-6 PUFA can give rise to HNE. 4-hydroxyalkenals reacts with SH groups in proteins and they can react with nearly all amino acids. 4-hydroxyalkenals have been found to be

cytotoxic, growth inhibiting, genotoxic, chemostatic and to play a potential role in atherogenesis. HNE and HOE are the most toxic compounds and HHE the least toxic. HNE can in concentrations between 1-20 μM partially inhibit DNA and protein synthesis (Han and Csallany, 2009). In concentrations $> 100 \mu\text{M}$ it has the following acute effects: It will inhibit catabolic (mitochondrial respiration) and anabolic (DNA and protein synthesis) processes and thereby lead to cell death. HNE has been found to be genotoxic in concentrations above 0.1 μM .

Malondialdehyde

This aldehyde is formed from both n-3 and n-6 PUFA. It can react with amino acids, but is less reactive than HNE. Proteins react more readily with malondialdehyde than free amino acids. Malondialdehyde can also react with several nucleosides. Detection can be performed by the TBA method (see 4.1.2.3)

2-alkenals and other aldehydes

The most investigated 2-alkenal is acrolein (2-propenal). This compound can be formed during oxidation of oils and does not require high temperatures for its formation like the 4-hydroxyalkenals do. This compound was found in half of the studies (4 out of 8) on volatile oxidation compounds identified in oxidised fish oil reported in the literature ((Rørbæk 1994; MacFarlane et al., 2001; Kulås et al 2002; Karahadian and Lindsay 1989; Lee et al. 2003; Hsieh et al., 1989; Olsen et al., 2005; Horuichi et al., 1998)). Only one of the four studies had quantified the concentration of the volatiles. In this study the concentration of acrolein was 46 ppb. Acrolein reacts 110-150 times faster with GSH and with nucleophiles than HNE. It forms Schiff bases with SH groups in proteins. It also reacts with nucleic acid bases. Acrolein is highly cytotoxic towards mammalian cells. LD50 towards these cells is 20 μM acrolein. The colony forming efficiency of mammalian cells significantly decreases at a more than 10 fold lower concentration, i.e. at approximately 1 μM . The reduction in the colony forming activity is probably due to DNA damage. In rat whole embryo cultures EC50 for malformation and embryo lethality are 2.8 μM and 8.3 μM , respectively in serum free medium. In media with serum it will be 10 x lower. This is probably due to the ability of SH groups present in serum proteins to scavenge and detoxify acrolein. Cytotoxicity increases with increasing chain length of 2-alkenals. Saturated aldehydes have lower toxicity.

The mechanisms by which 2-alkenals lead to cell death is largely unknown, but is probably the same as for HNE, namely a rapid depletion of SH groups due to the reaction between the aldehydes and SH. In bronchial epithelial cells 3-10 μM acrolein causes a marked reduction of GSH, but no effect on protein SH. A substantial loss of protein SH is observed at concentrations higher than 10 μM . Acrolein also has DNA damaging effects. Acrolein is detoxified in the liver, but it also seems to stimulate lipid peroxidation in the liver by destruction of the GSH dependent protective system. HNE has similar effects. Acrolein is toxic and severely irritating to the mucosa. Its vapour causes strong eye and nasal irritation. Inhalation will inhibit mucociliary transport and cause dysfunction, hyperplasia. Ingestion leads to acute gastrointestinal distress with pulmonary congestion and edema. Acute toxicity: LD50 (rat, oral) is 46 mg/kg, which is a much higher concentration than seen in oxidized oils. Moreover, acrolein is unlikely to cause acute intoxication in humans due to a highly irritating smell and lacrimatory effect. Acrolein is mutagenic in bacteria and induces mutations in mammalian cells. Unsaturated aldehydes like hexanal can react with proteins and form fluorophores. The α,β -unsaturated aldehydes may form adducts with DNA in vivo, but this requires rather high concentrations of the aldehyde and is therefore considered to be a rare event. However, these aldehydes probably have a number of genotoxic effects. 2-nonenal has been found to be genotoxic when tested in concentrations of 10, 1 or 0.1 μM , whereas nonenal does not have any genotoxic effects up to a concentration of 100 μM .

In a recent review Turner et al. (2006) summarized reported findings on the potential of lipid oxidation products, particularly lipid hydroperoxides to affect health outcomes of omega-3 PUFA. The review reported findings from cellular, animal and a few human trials. The overall conclusion was that oxidised lipids have numerous harmful effects on health, including the potential to increase the risk of

atherosclerosis and thrombosis. These effects have been seen with relatively low levels of oxidized products, similar to those that could be obtained from the regular consumption of fish oil capsules. While oxidation products originating specifically from fish oil had not been evaluated at the time of the review, the authors of the review suggested that such oxidation products are likely to have the same health effects as those reported in the review. They also suggested that oxidation products could account for the varying degrees of effectiveness and other inconsistencies associated with fish oil supplementation that have been reported in the literature. Hence, this review clearly supports that there is a knowledge gap with respect to the health effects of oxidation products originating from fish oils and with respect to the level of oxidation required for obtaining negative health effects.

5.4. Fate of hazards arising from lipid oxidation during fish oil processing

5.4.1. Chemical hazards arising from lipid degradation

The chemical hazards that are arising from lipid degradation will to a great extent be removed during oil refining as previously described in section 3.3. Briefly, free fatty acids are removed during the deacidification step. Lipid hydroperoxides are removed during the bleaching and deodorization steps. Volatile oxidation products are removed during the deodorization step. Polymers can be formed during the deodorisation process if the temperature is too high as previously described. The polymers are not further dealt with as they are not present in the raw material and they are not directly related to rancidity.

6. Considerations for parameters

6.1. Parameters for raw materials

The only criterion in the current Hygiene Regulation is the TVB-N value at a value of 60 mg TVB-N/100 g fresh weight for whole (round) fish as an indicator for the fish being fit for human consumption. TVB-N is a spoilage parameter and in the EU legislation TVB-N may only be used as an additional quality parameter when the sensory assessment of fish gives indications that the fish may be of a quality below EU quality grade B. The TVB-N is always measured in the edible part of the fish and the limits for fresh fish species range from 25-35 mg/100 g. Fish with TVB-N in edible part above this range is spoiled and putrid. There is no scientific study which evaluates the limites for TVB-N for whole fresh fish or fish products. Therefore the freshness criterion of 60 mg TVB-N /100 g for whole fish to be fit for human consumption is not based on scientific evidence.

For the determination of the properties of raw material as being fit for human consumption other methods are more appropriate. First to mention is sensory assessment. Here the long established and mandatory EU quality grading scheme with the 3 quality classes E, A, und B, or better the scoring schemes of the Quality Index Method (QIM) (Bremner, 1985) which have been developed in the past for many fish species can be used. Also the sensory assessment of cooked samples of the edible part by using e.g. the Torry scheme is a very reliable and well tested method for the determination of the freshness of fish (Martinsdottir et al., 2009). Sensory evaluation is still the most important method for freshness evaluation in fish. Many other measurement techniques give information on parameters related to fish freshness; however, none of these methods give a unique and unambiguous answer to whether the fish is fresh.

Other more sophisticated (instrumental) methods which are able to determine the freshness of fish are measuring the electrical properties of the fish by the FISCHTESTER VI or the Torrymeter, and the Time Domain Spectroscopy (TDR) which was developed for the freshness determination for fish during an EU funded research project (Kent et al., 2005; Kent and Oehlenschläger, 2009; Oehlenschläger, 2005). However, also these methods have restrictions: methods based on electrical properties work only on fish without a freezing history and TDR is expensive.

Ideally for the assessment of the fitness of raw material for human consumption a sensory method should be used to get reliable results.

6.2. Parameters for fish oil

The reduction in the content of free fatty acids during refining will improve the sensory properties and oxidative stability of the oil. Therefore, the percentage of free fatty acids is recommended as a parameter for the refined oil. There are, however, no scientific data available which indicates that free fatty acids poses a health risk, so it is not possible to recommend a maximum level if this should be based on risk assessment. PV and AV are recommended as chemical methods for determination of the oxidative status of crude fish oil as well as the refined fish oil, as these methods are already used by the industry. However, more sensitive methods, e.g. methods based on gas chromatography, that can measure specific volatile oxidation products should be standardized and implemented

Peroxides and volatile oxidation products are removed during the refining process and one could therefore argue that PV and AV of the crude oil have no influence on the oxidative stability of the final product. However, free lipid radicals are not removed during the refining step and although they are very unstable products that will easily be converted to peroxides it is likely that oxidized crude oils will oxidize faster after refining due to the presence of higher levels of free lipid radicals than less oxidized crude oils. Nevertheless, it is impossible to give an exact recommendation for the maximum tolerable PV and AV levels in crude oils. This is due to the fact that there are no data available in the literature on the relationship between PV and AV in crude oil and PV and AV in the final oil. Research is required to establish a clear relationship between crude oil PV and the PV in the final oil.

Moreover, there are only very few studies available on negative health effects of oxidized fish oil (or other oils) in which PV and AV of the applied oil has been measured. Hence, it is not possible to recommend maximum tolerable PV and AV, which are supported by science. It should be mentioned that the European Pharmacopeia states that in refined fish oils used for dietary supplements PV should not exceed 10 meq/kg. According to the Global Organization for EPA and DHA omega-3s (2006) PV should not exceed 5 meq/kg and for food products it should not exceed 2 meq/kg. For AV the corresponding values for fish oils for dietary supplements is 20 meq/kg. It is also important to note that fish oil with a PV of 5 or 10 meq/kg will have an oxidized aroma and flavour (i.e. rancid, train oil) and that such oils cannot be used for incorporation into food products as they will give rise to undesirable rancid, metallic and train oil off-flavours. For example Let et al. (2005) found that fish oils to be used for production of fish oil enriched milk with acceptable sensory properties and shelf life must have a PV below 0.5 meq/kg.

As described in section 5.3. the compounds which seem to pose the biggest risk in terms of negative health effects are volatile oxidation products, particularly a number of different unsaturated aldehydes. Although some of these aldehydes might be detected by the anisidine value, this method is not specific and one does not really know what one is measuring. The only method which can measure specific volatile oxidation products is the GC method as described previously. The GC can either be coupled to a mass spectrometer or to a flame ionisation detector. However, this method is not yet implemented as a routine method in the industry, although most of the bigger players do have such instruments available. Similar to PV and AV too few data are available in the literature on the level of specific volatile oxidation products that have negative health effects. Therefore, it is not possible to recommend acceptable level of specific volatile oxidation products.

CONCLUSIONS

Impact of hygiene of the raw material used for fish oil for human consumption

- In principle composition and properties of fish oil depend on the freshness and composition of the raw material. However by adjusting the production process the level of oxidation products can be minimised and fish oil of desired properties can be obtained even from fish not meeting the current freshness criterion. On the other hand, data on the relationship between the freshness of the raw material and the level of lipid oxidation in the refined oil are missing in the literature.
- The refined fish oil production process typically includes several steps such as repeated heating at high temperatures (at 90-95°C and even to 180°C) as well as alkali/acid treatments and repeated removal of the water phase, which reduce the biological food safety risk to negligible. These current risk assessments relate to the bulk storage level of the refined fish oils and therefore hazards arising at later stages are not dealt with.

Fish oil and rancidity

- In the frame of the given mandate only oxidation products can represent a potential hazard in refined fish oil intended for human consumption whilst stored in bulk.
- Information on the level of oxidation of fish oil (as measured by peroxide and anisidine values) and related toxicological effect in humans is lacking. Information on toxicity of individual oxidation products of fish oil in humans is also lacking.
- Based on the currently available information, no qualitative or quantitative risk assessment of hazards in relation to rancidity of fish oil intended for human consumption can be carried out.

Parameters

- Total volatile basic nitrogen (TVB-N) is a spoilage parameter, which was developed and defined for ice stored gutted fish and fish filets. It has not been investigated for the determination of the 'freshness' of whole fish as raw material intended to be used for production of fish oil for human consumption. The criterion of 60 mg TVB-N /100 g for whole fish is not based on scientific evidence.
- Sensory evaluation gives the most reliable results for the assessment of the freshness of raw material for fish oil production for human consumption.
- Currently, the most reliable chemical methods for rancidity measurements in bulk fish oils are the peroxide and anisidine values. The quantitative relationship between peroxide and anisidine values and the specific volatile oxidation products is lacking.

RECOMMENDATIONS

Impact of hygiene of the raw material used for fish oil for human consumption

- To maintain the freshness as long as possible, the raw material should be stored at a temperature close to 0°C, preferably in melting ice.
- Research should be encouraged to establish the relationship between freshness of raw fish and refined fish oil properties (including the level of lipid oxidation) and yield of refined fish oil should be established.

Fish oil and rancidity

- Lipid oxidation in bulk stored fish oil can be prevented by cold storage in darkness, with no exposure to oxygen and addition of antioxidants. The threshold level of oxidation of refined fish oil that may result in negative impact on health (e.g. oxidative stress) should be investigated.
- The effects of individual oxidation products originating from refined fish oil on human health should be thoroughly investigated.

Parameters

- Research should be conducted to establish whether TVB-N is a suitable parameter as a criterion for whole/ungutted fish as raw material for production of fish oil intended for human consumption and if it relates to lipid oxidation. If this is the case, scientific research should be carried out to establish the foundation of setting TVB-N limits for whole fish.
- The use of sensory methods such as Quality Index Method (QIM) (where available) should be preferably considered for determination of freshness of the raw material. QIM schemes for fish species intended as raw material for fish oil production should be developed.
- If other methods such as chemical or physical methods for the determination of the freshness of the raw material intended for the production of fish oil intended for human consumption are preferred they should relate to the lipid oxidation and lipolytic processes in the raw material.
- Methods based on gas chromatography for determination of specific oxidation products should be developed and standardised to characterise the oxidative status of crude and especially of refined fish oil.

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