Hinc patriam sustinet

Instituto Superior de Agronomia
Universidade Técnica de Lisboa

Industrial Production of Omega-3 Polyunsaturated Fatty Acids from Fish Oil and Microalgae

Ana Sofia de Figueiredo Rodrigues

Dissertação para obtenção do Grau de Mestre em

Engenharia Alimentar

Orientador: Doutora Maria Suzana Leitão Ferreira Dias Vicente

Co-Orientador: Doutor Alberto José Delgado dos Reis

Júri:

Presidente - Doutora Margarida Gomes Moldão Martins, Professora Auxiliar do Instituto Superior de Agronomia da Universidade Técnica de Lisboa.

Vogais - Doutora Maria Suzana Leitão Ferreira Dias Vicente, Professora Auxiliar do Instituto Superior de Agronomia da Universidade Técnica de Lisboa;
- Doutor Vítor Manuel Delgado Alves, Professor Auxiliar do Instituto Superior de Agronomia da Universidade Técnica de Lisboa;
- Doutora Natália Maria Ferreira Rebelo de Melo Osório, Professora Auxiliar Convidada do Instituto Superior de Agronomia da Universidade Técnica de Lisboa;
- Doutor Alberto José Delgado dos Reis, Investigador Auxiliar do Laboratório Nacional de Energia e Geologia, I.P.

Lisboa, 2012

Acknowledgements

I would like to acknowledge my supervisors Professora Doutora Suzana Ferreira Dias and Doutor Alberto José Delgado dos Reis for all the support, availability, for sharing the knowledge and especially for their human side.

I am also thankful to my family and friends for their positive energy.

Abstract

ω-3 polyunsaturated fatty acids (PUFAs) ingredients, especially eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) are known for its vital and unique role in human health and well-being by an extensive scientific research. These facts are widely spread by media.

At present, the major source of ω-3 PUFAs is fish oil from oily fish like sardine (*Sardina pilchardus)*. This work proposes the use of heterotrophic microalgae such as *Crypthecodinium cohnii* as an alternative source of interest for the commercial production of ω-3 EPA and DHA. It is also suggested the use of a common process suitable for both feedstock. EPA and/or DHA production are accomplished through oil saponification and PUFAs concentration winterization and urea concentration. PUFAs purification by chromatography is only necessary when oil is extracted from fish since fractions obtained from *C. cohnii* have high proportions in DHA and they do not require further purification steps for food applications.

The combination of traditional (seasonal) and alternative sources (year-round) using a common production process shows an economic advange with increasing earnings for market development.

Keywords: docosahexaenoic acid, eicosapentaenoic acid, ω-3, polyunsaturated fatty acids, sardine, *Crypthecodinium cohnii.*

Resumo

Os denominados ácidos gordos polinsaturados ω-3 são reconhecidos através da extensa pesquisa e pelas evidências científicas que mostram o seu papel vital e único na saúde humana e no seu bem-estar.

O ácido eicosapentaenóico (EPA, 20:5ω3) tem vários efeitos benéficos ao nivel das doenças coronárias e da redução do nível de colesterol no sangue reduzindo assim o risco de arteriosclerose, inflamação e vários cancros. O ácido docosahexaenóico (DHA, 22:6ω3) é um ácido gordo estrutural que existe na matéria cinzenta do cérebro e nos tecidos da retina sendo um componente essencial do leite materno, contribuindo deste modo para o desenvolvimento normal do cérebro e do sistema nervoso.

Tem sido sugerido que o tipo de dieta praticada nos países ocidentais é desequilibrada, proporcionando níveis elevados de ácidos gordos polinsaturados ω-6 PUFAs e baixos níveis de ácidos gordos polinsaturados ω-3. A crescente consciência deste desequilíbrio nutricional e a divulgação através dos meios de comunicação social da importância dos benefícios dos ácidos gordos polinsaturados ω-3 na saúde têm contribuído para o desenvolvimento do mercado de suplementos nutricionais e, mais recentemente, de produtos enriquecidos. Isto está a levar a um crescimento substancial do mercado de ingredientes ácidos gordos polinsaturados ω-3 devido às suas aplicações nos sectores alimentar, nutricional e farmacêutico.

Actualmente, a principal fonte de ácidos gordos polinsaturados ω-3 é o óleo de peixe obtido a partir de peixes gordos como a sardinha (*Sardina pilchardus*).

Este trabalho propõe que as microalgas como a *Crypthecodinium cohnii* poderão ser utilizadas como fonte alternativa de interesse para a produção comercial de ω-3 EPA e DHA. É ainda proposto um processo industrial comum adequado para ambas as matérias-primas (sardinha e microalgas) ricas em EPA e/ou DHA em que os concentrados seriam obtidos através da saponificação dos óleos e da concentração de ácidos gordos polinsaturados por cristalização e complexação por ureia. A purificação por cromatografia só será necessária quando o óleo for extraído a partir de peixe, uma vez que as fracções obtidas a partir de *C. cohnii* têm proporções elevadas em DHA e não requerem passos de purificação adicionais para aplicações alimentares.

A combinação de fontes tradicionais e alternativas, utilizando um processo de produção comum vai resultar em vantagem económica com ganhos crescentes relativamente ao desenvolvimento do mercado.

Palavras-chave: ácido docosahexaenóico, ácido eicosapentaenóico, ω-3, ácidos gordos polinsaturados, sardinha, *Crypthecodinium cohnii.*

Table of Contents

List of Tables

List of Figures

Abbreviations

Term	Definition
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
FFA	Free fatty acids
FAMEs	Fatty acid methyl esters
MUFA	Monounsaturated fatty acid
PUFAs	Polyunsaturated fatty acids
LCPUFAs	Long-chain ω-3 PUFAs
LA	Linoleic acid
ALA	α-linoleic acid
TAG	Triacylglycerol or triglyceride
PKS	Polyketide synthase
SCO	Single Cell Oil
Acetyl Co-A	Acetyl co-enzyme A

1. Rational of the study and objectives

The importance of Polyunsaturated Fatty Acids (PUFAs) in human nutrition and disease prevention was scientifically recognized three decades ago. Both ω-3 (omega-3) and ω -6 (omega-6) PUFAs along with their precursors α- linolenic acid (ω-3) and linoleic acid (ω-6) are involved in many important biological processes in the human body (Shahidi and Wanasundara, 1998) and must be obtained from diet. It has been suggested that the typical 'Western' - type diet is imbalanced, thus providing high levels of omega-6 PUFAs and low levels of ω-3 PUFAs. The growing public awareness of this nutritional gap and the importance of ω-3 health benefits contributed to a primarily market request for nutritional supplements and more recently to enriched products. This is leading to a substantial growth in ω-3 PUFAs ingredient market, due to its applications in the food, nutrition and pharmaceutical industries.

Linoleic acid (ω-6) is the major PUFAs in westernized diets (Basua *et al*., 2006), followed by α-linolenic acid (ω-3). The major sources of linoleic acid are vegetable oils such as corn, safflower, soybean and sunflower oil, whereas α-linolenic acid can be mostly found in linseed oil, rapeseed, walnuts and blackcurrant oil, but also in dark green leaf plants (Knocha *et al*., 2009).

In order to prevent different pathologies, mainly cardiovascular diseases, and more lately some psychiatric disorders, an appropriate dietary level of ω-3 fatty acids is required, more specifically during development and aging. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the most important to humans from the nutritional point of view (Valenzuela, 2009). EPA and DHA are synthesized mainly by both uni- and multi-cellular marine plants such as phytoplankton and algae (Shahidi and Wanasundara, 1998). They are eventually transferred through the food chain and are incorporated into lipids of aquatic species such as fish mainly oily fish.

Fish oil concentrates and microbial oils are fast growing markets drove by the increase confidence and health concerns of consumers. The segments include concentrated supplements, functional foods (bread and bakery products, eggs, meat and poultry products), beverage applications, and infant formula.

In order to increase the production of concentrates with good quality at low prices, with great interest for food supplier companies, new and optimized production processes of concentrates with high percentages of EPA and DHA are necessary.

The main goals of the present work are to present:

- The importance of ω-3 PUFAs for human health;

- An overview of the actual ω-3 ingredient market;

- Microalgae, as an alternative source for ω-3 concentrates production;

- A proposal of a versatile production process for the concentration and purification of ω-3 EPA and DHA from marine sources - fish and microalgae, with economic benefits in terms of raw materials supply;

- Fish wastes as an inexpensive source of PUFAs of interest - fish by-products; the extraction of oil from fishing discards offers a large opportunity to reduce waste and increase profit from a high valuable by-product.

2. Literature Review

2.1 Chemistry of ω-3 polyunsaturated fatty acids

PUFAs constitute a large group of unsaturated fatty acids containing long-chain carbonic molecules that include ω-3-fatty acids (or n−3 fatty acids). This family is based on the position of the first double bond from the methyl end group in the fatty acid chain. This is important because the position of the double bond from the methyl end influences the biological activity of these molecules.

The term “omega”, expressed as “ω”, is related to the carbon position further from the methyl functional group $–CH_3$, as shown in figure 1. The other end of the fatty acid is the carboxyl group –COOH end. Table 1 shows the main members of the ω-3 family.

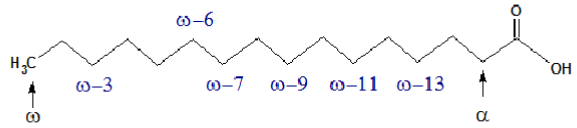


Figure1. Carbon positions in the hydrocarbonated fatty acid chain.

Table 1. Main ω-3 PUFAs.

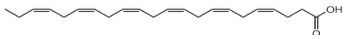
Type	Common name	Carbon number	Double bonds C-C	Formula
ω-3 PUFAs	α-Linoleic	18	3	18:3ω-3
	Stearidonic	18	4	18:4ω-3
	EPA	20	5	20:5ω-3
	DPA	22	5	22:5ω-3
	DHA	22	6	22:6ω-3

Due to their role in human nutrition and disease prevention, EPA and DHA are considered the most important PUFAs (Liu *et al*., 2005; Mendes *et al*., 2007; Reis and Silva, 2008). Their

chemical structures are shown in table 2. These two polyunsaturated fatty acids have 5 or 6 double bonds in a carbon chain of 20 or 22 carbon atoms, respectively.

Most of the naturally produced fatty acids (created or transformed in plant cells with an even number of carbon in chains) are in *cis*-configuration where the two hydrogen atoms are on the same side of the double bond. On the other hand, the trans-configuration results in much more stable chains which are difficult to break or transform, because they form longer chains that aggregate in tissues and lack the necessary hydrophilic properties. Furthermore the ω−3 have the last double bond geometrically and electrically more exposed, being more fragile than ω−6 compounds (Kapoor and Patil, 2011).

Table 2.Chemical Structure of EPA and DHA.

Chemical structure	Common name	Simplified notation	Chemical (IUPAC) name
	Eicosapentaenoic acid (EPA)	20:5 (ω-3)	*all-cis*-5,8,11,14,17-eicosapentaenoic acid
	Docosahexaenoic acid (DHA)	22:6 (ω−3)	*all-cis*-4,7,10,13,16,19-docosahexaenoic acid

2.2 Biosynthesis of ω-3 PUFAs

An understanding of the biosynthetic pathways involved in the production of these PUFAs is also highly desirable as a prerequisite to increasing their content in the oils (Ratledge, 2004) as well their productivity.

Aerobic pathway

Nearly all biological systems, including microorganisms, insects, higher plants and animals, are capable of *de novo* fatty acid synthesis from acetate to short chain fatty acids, with oleic acid (18:1ω-9) as the major product (Ratledge, 2004). The biosynthesis of PUFAs is presented in figure 2.

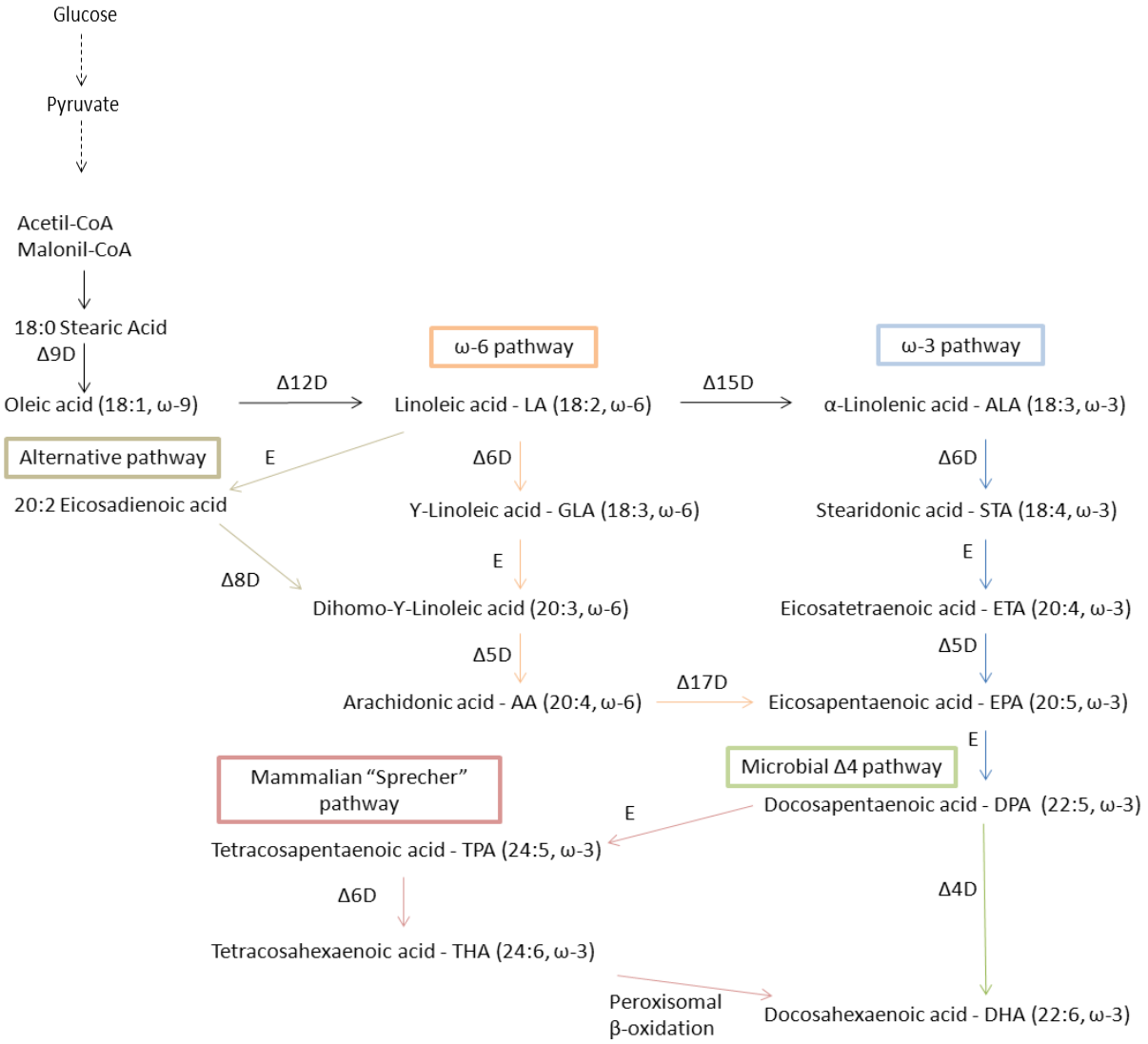


Figure 2. Biosynthesis of polyunsaturated fatty acids. Desaturases are abbreviated by D with the position of their reaction shown. E refers to elongase (Ratledge, 2004; Harwood and Gushina, 2006; Harwood and Gushina, 2009).

Both eukaryotic and prokaryotic lipid species are formed in the two pathways ω-3 and ω-6, which involved cytoplasmic and chloroplastic lipids. In the ω-6 pathway, Ƴ-linolenate is produced following desaturation of linoleic acid, which is then elongated to dihomo-Ƴ-linolenate and, subsequently, desaturated to arachidonate and further to EPA. In the ω-3 pathway, linoleate is first desaturated to a-linolenate which was then converted to 18:4 ω-3, 20:4 ω-3 and EPA (Gushina and Harwood, 2006; Knoch *et al*., 2009).

The three fatty acids - oleic acid, LA and ALA - compete with each other for the Δ6 desaturase. The affinity of the enzyme to the substrate and the amount of substrate available determine which metabolic pathway is predominant. Generally, the first Δ6 desaturation is the limiting step and ALA has the highest affinity for Δ^6 desaturase followed by LA and oleic acid (Wen and Chen, 2003).

Most algae, fungi, bacteria, mosses, insects and some invertebrates are the primary producers of these fatty acids and they use desaturase and elongase for the synthesis of various PUFAs (Wen and Chen, 2003).

The glycolytic enzymes are present in the biosynthesis of EPA which starts with the carboxylation of acetyl-CoA to form acetate or pyruvate. Then acetyl-CoA is converted into malonyl-CoA, which is used to drive a condensation reaction to extend the acyl group to stearic acid (18:0) and desaturase to oleic acid (18:1 ω-9). The next step is the conversion to linoleic acid (LA, 18:2 ω-6) by Δ12 desaturase and to α-linolenic acid (ALA, 18:3 ω-3) by a Δ15 desaturase (Wen and Chen, 2003).

Additionally, in the usual conversion of α-linolenic acid to EPA, there is an alternate pathway for the biosynthesis of arachidonic acid that elongates before a desaturation takes place (Harwood and Gushina, 2009).

By contrast, the conversion of EPA to DHA in animals involves the Sprecher pathway which produces C24:5 and C24:6 before β-oxidation shortens the acyl chain. (Harwood and Gushima, 2009).

The human body cannot synthesize ω−3 fatty acids *de novo* but it can form EPA and DHA from α-linolenic acid (Knocha *et al*., 2009). These conversions occur competitively with ω−6 compounds. These fatty acids are derived from linoleic acid. The higher ALA conversion efficiency occurs in woman. The main reason is the lower rate of utilization of dietary ALA for beta-oxidation (Kapoor and Patil, 2011).

For algae, it seems that a more direct route utilizing elongation and a Δ4 desaturation is usual (Harwood and Gushina, 2009). The conventional assumption was that C20:5ω-3 was elongated to C22:5ω-3 and then converted to C22:6ω-3 by the final Δ4 desaturation. Since Thraustochytrids accumulate large amounts of DHA and its precursor docosapentaenoic acid (DPA), they served as model organisms for studying the mechanism of DHA synthesis. The identification of a Δ4 desaturase from *Thraustochytrium* spp. finally provided unambiguous evidence for the conversion of C22:5ω-6 to DHA in this organism (Harwood and Gushima, 2006).

Anaerobic pathway

Whilst PUFAs production in most microorganisms uses a conventional fatty acid synthase (FAS) system followed by a series of desaturases and elongases, in *Schizochytrium* sp., and probably related thraustochytrid marine protists, PUFAs synthesis now appears to be via a polyketide synthase (PKS) route (Ratledge, 2004; Harwood and Gushina, 2009).

Acetyl-CoA and malonyl-CoA are the crucial base for PKS system construction (figure 3). This system does not involve *in situ* reduction of the intermediates. The synthesis of the longer chain fatty acids occurs through PKS system and involves the presence of 8 enzymes: 3-ketoacyl synthase (KS), malonyl-CoA:ACP acyltransferase (MAT), acyl carrier protein (ACP), 3-ketoacyl-ACP reductase (KR), acyltransferase (AT), chain length factor (CLF), enoyl reductase (ER) and dehydrase or isomerase (DH) (Grupta *et al*., 2012).

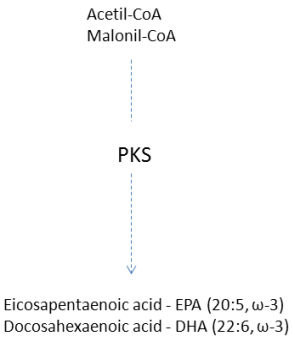


Figure 3. Polyketide synthase pathway. The dotted line indicates that this reaction has not yet been demonstrated unequivocally in algae (adapted from Harwood and Gushina, 2009).

pathway exhibited dehydration and isomerization reactions involving fatty acyl intermediates for carbon chain elongation (Grupta *et al*., 2012). Figure 4 shows the route o DHA formation by the PKS pathway in *Schizochytrium sp*.

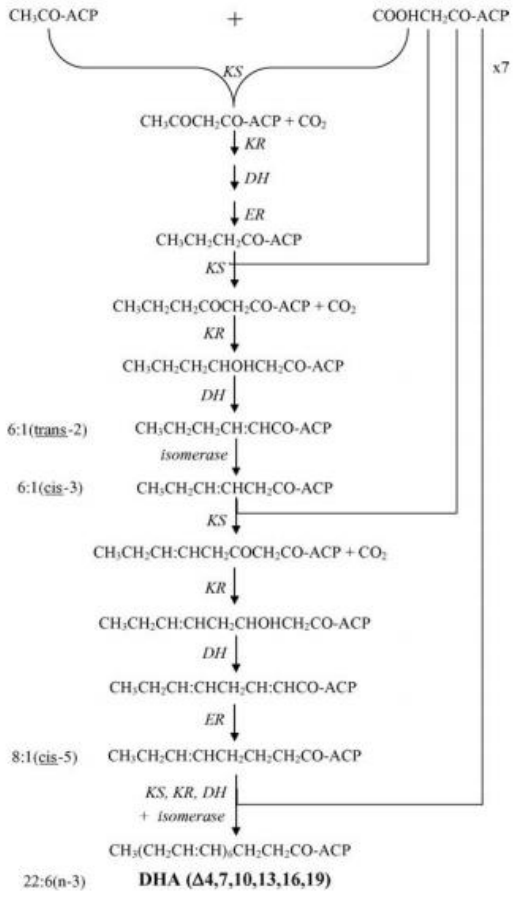


Figure 4. Formation of DHA by the PKS pathway in *Schizochytrium sp*. (Grupta *et al*., 2012).

2.3 Health benefits and nutritional recommendations

Attention to the health benefits of PUFAs first emerged when it was noted that populations deriving a substantial proportion of their food from fish had a much lower incidence of heart diseases. General recommendations for the beneficial role of EPA and DHA in human health are widely documented (Ward and Sigh, 2005).

The mechanisms by which ω-3 fatty acids reduce the risk of cardiovascular disease are well established (Ward and Sigh, 2005). EPA and DHA are important in the prevention of inflammatory diseases (Bandarra *et al*., 1997) and cardiovascular pathologies like atherosclerosis or stroke by lowering blood pressure and preventing the development of hypertension.

EPA and DHA lower the risk of cardiovascular diseases, probably by the multiple mechanisms of lowering serum triacylglycerols, improving the LDL:HDL ratio, antiarrythmic effects on heart muscle, improved plaque stability, anti-thrombotic effects and reduced endothelial activation (Wen and Chen, 2003; Mendes *et al*., 2009). EPA has several beneficial effects regarding coronary heart diseases such has hypertriglyceridemia, blood platelet aggregation and lowered blood cholesterol, thus reducing the risk of atherosclerosis, inflammation and several carcinomas (Guil-Guerrero and Belarbi, 2001; Ward and Singh, 2005) and also decreasing tumor growth and metastasis (Anderson *et al*., 2009). EPA is capable of reducing the tendency toward thrombosis by reducing the level of fibrinogen, an activation factor in the occurrence of thrombosis (Wen and Chen, 2003). EPA is also the precursor of prostaglandins, tromboxanes and leukotrienes, collectively referred to as eicosanoids, which are effective anti-aggregatory and inflammatory mediators (Liu *et al*., 2006; Anderson *et al*., 2009).

A significantly amount of references clearly establish that DHA is important for the proper neurodevelopment of the brain and visual system. Epidemiological evidence suggest that a decrease in brain DHA levels, which normally occurs during aging, and that is exacerbated by reduced dietary intake of DHA, may increase the prevalence of several neurological diseases, such as Alzheimer's disease (Valenzuela, 2009).

During the last period of gestation and during the early post natal period, neurodevelopment occurs exceptionally quickly, and significant amounts of ω-3 and ω-6 PUFAs, especially DHA, are critical to allow neurite outgrowth and the proper brain and retina development and function (Valenzuela, 2009). DHA is also an essential component of human milk, contributing

to the normal development of the brain and of the nervous system (Guil-Guerrero and Belarbi, 2001). EPA is associated with neonate growth retardation (Silva *et al*., 2006) and may contribute to thinning of artery walls in certain individuals which may cause serious bleeding problems (Ward and Singh, 2005).

Also, long-chain ω-3 PUFAs (LCPUFAs), mainly EPA and DHA, play a very important role in neuropsychiatry performance. By effectively regulating the plasma/serum cholesterol that is associated with an increased risk of depression and suicide, these LCPUFAs aid in the prevention of neuropsychiatric disorders (Mendes *et al*., 2009). Basic, clinical and epidemiologic research supports a protective effect of omega-3 LC-PUFA, particularly of DHA in mood disorders (Valenzuela, 2009). Recent reports point to possible new applications of EPA, in treatment of brain disorders including schizophrenia (Ward and Singh, 2005).

Many human studies fail to differentiate between ALA, EPA, and DHA when reporting effects of ω-3 PUFAs on cancer risk, or when a fish oil blend is used, preventing evaluation of individual effects of EPA and DHA. Despite these challenges, important mechanistic insights are continually being identified that will eventually help elucidate the individual effects of n-3 PUFAs in two of the most common forms of cancer worldwide, prostate and breast cancer. While *in vitro* and rodent studies more consistently support a potentially protective effect of EPA+DHA on prostate carcinogenesis, in breast cancer the data for EPA and DHA are equivocal (Anderson *et al*., 2009).

In addition ω-3 PUFAs are proposed to reduce the risk of insulin resistance in multiple ways, few of which seem to be differentially affected by the ω-3 fatty acids (Anderson *et al*., 2009).

The American Heart Association recommends that people without documented coronary heart disease eat a variety of fish, preferably oily fish at least twice a week. People with documented coronary heart disease are advised to consume about one gram of DHA and EPA per day, preferably from oily fish, although EPA+DHA supplements could be considered in consultation with their physicians. People who have elevated triacylglycerols may need two to four grams of EPA and DHA per day provided as capsules under a physician's care (http://www.heart.org/HEARTORG/GettingHealthy/NutritionCenter/Frequently-Asked-Questions-About-Better-Fats_UCM_305985_Article.jsp, September 2012).

3. ω-3 Ingredients Market

Food is no longer synonymous of simple nourishment. An increasingly number of consumers seeks to achieve recommended levels of nutrients, in an effort to reduce the risk or delay the probability of a number of conditions and diseases related to dietary deficits. The increasing consumer awareness has promoted important dietary changes and consequently the fast growing of functional food area- food and beverages application, infant formula, pet food and pet supplements (see table 3). This type of products is recognized to provide health benefits beyond basic nutrition through the incorporation or removal of a particular ingredient.

The ω-3 fatty acid functional products is supported by the long and safe use of fish oil supplements as medicinal products and by the extensive medical and clinical trials carried out by ingredient suppliers as well as independent organizations. The result is the consistent growing demand for commercial production of high-purity concentrates of EPA and DHA. The new challenges are novel formulation technologies required to facilitate the incorporation of DHA and EPA into numerous other delivery vehicles.

Raw materials are purchased from oil manufacturers or directly from ingredient suppliers who provide PUFAs concentrates (Figure 5).

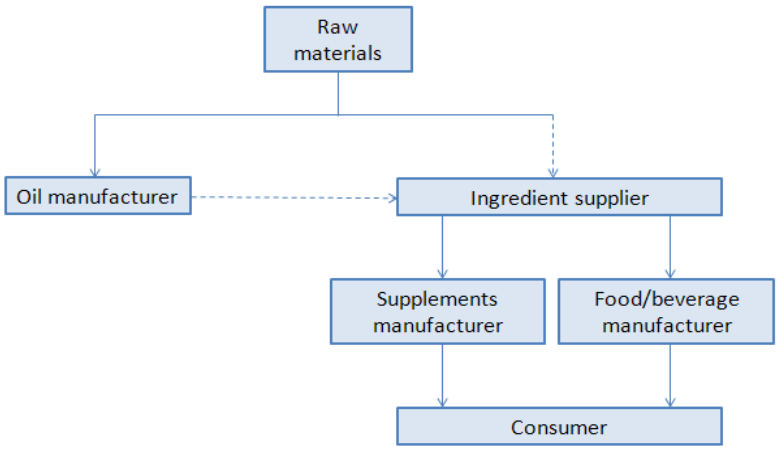


Figure 5. ω-3 PUFAs Industry.

Table 3 presents the most important world companies involved in the research, development, manufacturing or marketing of the Single Cell Oil PUFAs or PUFAs-containing products.

Table 3. Companies reported to be researching, developing, manufacturing or marketing Single Cell Oil PUFAs or PUFAs-containing products (Ward and Singh, 2005)

Aventis S.A.
BASF A.G.
Friesland Brands A.G.
Gist-brocades
Heinz-Wattie's
Hoffmann-LaRoche A.G.
Jamieson
Ordesa
Maarbarot
Martek Inc.
Mead Johnson Nutritionals
Nagase and Co.
Nestle S.A.
Novartis
Nutricia
Nutrinova Celanese A.G.
Pronova
Ross Products (Div of Abbott)
Suntory Ltd.
Walmart
Wyeth

Fish oils as raw materials are able to provide beneficial long chain PUFAS in different concentration and ratios which also leads to problems in separation of high concentration of individual fatty acids. There are also problems with undesirable taste/odors that need to be completely overcome. Despite this, fish and fish oils application as feedstock still imply economical advantage over other sources.

Despite the economic and marketing difficulties, there has been an extensive research and development of PUFAs production of microbial lipids. The key to its economical competitiveness relies on achieving the maximal PUFAs yield with the use of inexpensive substrates, the screening for more efficient strains and reduction of the processing steps

necessary for oil recovery from the cells. Microbial oils must yield to the best commercial varieties and have a broad production base to be competitive with other commodities.

The success in microbial oil production is achieved by several processes that are being used for commercial production levels. However, the supply of microbial lipids is still insufficient to meet industrial demand. Alternative strategies (for example mutation methods, molecular engineering techniques, the use of inhibitors, structuring microbial fatty acid composition by enzymatic treatment of preexisting oils) should be combined with classical fermentations (Certik and Shimizu, 1999).

At present, the high concentrations in DHA, ideal for infant formulas, are the key for microalgae market flourishing interest. Growing opportunities are mostly dependent on:

- sufficient supply of microbial lipids to meet industrial demand;
- cost of microbial oil;
- commercial production levels;
- high production yields of the top commercial microbial oils;
- further industrial process optimization related to high susceptibility to oxidation;
- solving ssafety approvals related with regulatory issues.

4. EPA and DHA commercial sources

4.1 Fish

Raw material used for the production of fish oil falls into several categories such as:

1. Fish caught specifically for fish oil - oily fish like sardine (*Sardina pilchardus);*
2. Incidental or by catch from another fishery;
3. Fish by-products from the edible fisheries such as cuttings from filleting operations, fish cannery waste, roe fishery waste and more recently surimi processing waste (http://lipidlibrary.aocs.org , July 2012).

The season, the type of fish, where the fish is harvested, and the food availability contribute to large variations in fish quality and therefore in the amount of oil and of ω-3 fatty acids in the fish oil. Moreover, marine fish stocks are subject to seasonal and climatic variations.

Health concerns about the presence of environmental manmade pollutants, such as dioxins, polychlorinated biphenyls (PCBs) and heavy metals still remain. Due to their resistance to degradation, they persist in the environmental for very long. They are fat-soluble and have a bioaccumulating effect, affecting foodchain and eventually humans. The level of pollutants is related to the geographic area and season (Certik and Shimizu, 1999; Ratledge, 2004).

Fish industry generates large quantities of the so called fish wastes - heads, viscera, skin, trimmings and fish rejects - which normally present a potential disposal problem (table 4). Fish waste management has been one of the problems having the greatest impact on the environment. It can be considered that 45% of the remaining material from the total fish capture is for non-food purposes.

Table 4. Fish species and non-fish species edible for fish oil and fishmeal production (http://lipidlibrary.aocs.org, 2012)

Species	Country
Catfish spp.	USA, Vietnam
Tuna spp.	Thailand, Japan, USA, Australia, South Korea, China, France, Ecuador, Maldives Islands and many others
Salmon, farmed	Norway, UK, Ireland, Canada, Chile, Faroe Islands, Australia
Salmon, wild	Canada, USA-Alaska, Japan, Russian Federation
Sardine	Peru, Chile, South Africa, Namibia, Japan, Spain, Mexico
White Fish spp.	UK, USA-Alaska, Canada, Chile
Dogfish	Canada, USA
Pollock	USA-Alaska, Russia
Atlantic Herring	Iceland, Norway, Denmark, UK, Faroe Islands, Sweden, Ireland, Canada
Mackerel spp.	UK, Peru, Chile, South Africa, Ireland, Norway, Denmark, Spain, Namibia, Russian Federation, China, Thailand
Horse Mackerel	Angola, Mauritania, Morocco, Namibia, South Africa, Turkey, France, Ireland, Latvia, Lithuania, Netherlands, Norway, Russian Federation, Spain, Ukraine, New Zealand
Hoki (Blue Grenadier)	Australia, New Zealand
Non-fish species	
Krill	Norway, Poland, Ukraine, Japan, South Korea
Squid	Argentina, Chile, Peru, USA, Japan, China, South Korea, Russian Federation, France, Portugal, Spain, UK, Morocco, Mexico, Hong Kong, Taiwan, Ghana, Mauritania, South Africa, Senegal, Tunisia, Falkland Islands, Indonesia, Malaysia, Philippines, Thailand, New Zealand
Single Cell Organisms	USA, Japan, Australia, Canada, USA (Hawaii), Israel, India

An important waste reduction strategy for the industry is the recovery of marketable by-products from fish wastes. Therefore, research has been carried out in order to develop methods to convert these wastes into useful products.

The three most common methods for utilization of aquatic waste (either from aquaculture or wild stock) are the manufacture of fishmeal/oil, the production of silage or the use of waste in the manufacture of organic fertilizer. The utilization of by-products is an important cleaner production opportunity for the industry, standing for environmental and public benefit besides generating additional revenue as well as reduces disposal costs for these materials. The wastes from the fishing industry could be used as a feed ingredient, as it represents a valuable source of high-quality protein and energy. Treated fish waste has other important applications such as the production of biodiesel/biogas, dietic products, natural pigments (after extraction), food-packaging applications, cosmetics (collagen), enzyme isolation, soil fertiliser and moisture maintenance in foods (hydrolysates) (Arvanitoyannis and Kassaveti, 2008).

Fish, like humans, are not capable of synthesizing EPA *de novo*. EPA in fish is derived from the microalgae consumed in oceanic environment (Wen and Chen, 2003).

Although DHA has long been known to be a major fatty acid of fish oils, it always occurs along with eicosapentaenoic acid (EPA; 20:5, n-3) which was contra-indicated to be included in infant diets as it affects the uptake of DHA which is crucial for neural development (Ratledge, 2004).

These shortcomings of fish-derived oil are diverting research towards the exploitation of other marine species for the development of a suitable alternatives.

4.2 Microalgae

Microbial oils are not produced agriculturally being intrinsically more expensive. To be perceived as economically viable, microbial oils have to be saleable at a high price in order to cover the costs of their production. PUFAs fatty acids are established as dietary important and consequently a large market is available to ensure their worldwide sales. The production of microbial oils - otherwise referred to as Single Cell Oils (SCO) - is now an economic reality (Ratledge, 2004).

Successful commercial utilization of microalgae has been established in the production of nutritional supplements, antioxidants, cosmetics, natural dyes and PUFAs. The worldwide annual production of algal biomass is estimated to be 5 million kg/year with a market value of about 269 €/kg (Hemaiswarya *et al*., 2011).

Fish obtain EPA from their diet, normally from microalgae which are the primary producers of ω-3 PUFAs. There is an increasing effort in order to develop a commercial technology to produce EPA directly from microalgae (Wen and Chen, 2003). Microalgae biomass is particularly suitable for extraction and purification of individual PUFAs due to its stable and reliable composition. PUFAs from cultured microalgae are cholesterol-free, contaminant-free (e.g. heavy metals, polychlorobiphenyls) and have a good taste. Futhermore, microalgae can be grown on low-cost nutrients (Certik and Shimizu, 1999).

The heterotrophic microalgae *C. cohnii* can accumulate lipid to over 20% of dry weight with a high content of DHA (over 30% of total fatty acid content.) Other PUFAs remain below 1% of the total fatty acid content. This characteristics make DHA separation and purification process easy and cheap, which is very attractive. For economically feasible industrial cultivations of C. cohnii, high cell densities are required so it is necessary to optimize the culture medium providing basic data for commercial production of DHA. Growth and fatty acid formation are affected by key medium components and environmental conditions (Mendes *et al*., 2009). The residual biomass that remain after fatty acid extraction from lysed cells can be used as animal feed. The whole biomass paste is suitable for use in aquaculture feed (Mendes *et al*., 2009). Moreover, fish and shrimp farming is increasing the use of DHA from microalgae rather than from fish meat and fish oil snce microalgae are a completely renewable and contaminant-free source (Mendes *et al*., 2007a).

Table 5 shows some examples algal sources for the production of EPA and DHA (Harwood and Gushina, 2009).

Table 5. Examples of microalgae as commercial sources of EPA and DHA (Harwood and Gushina, 2009).

Fatty acid	Producer	Use
EPA	*Nannochloropsis* *Nitzschia* *Phaeodactylum*	Aquaculture Nutritional supplements
DHA	*Crypthecodinium cohnii* *Schizochytrium*	Aquaculture Infant formulae Nutritional supplements

The production of DHA from *Crypthecodinium cohnii* (40-50% DHA but negligible EPA), is marketed by Martek in more than 60 countries using a number of fermenters of about 100 m^3 each (Ratledge, 2004). Smaller amounts of DHA are produced commercially from *Schizochytrium* and these are mainly used for adult dietary supplements (including cheese, yogurt, spreads, dressings, cereals) and foods for pregnant and nursing women. Both *C. cohnii* and *Schizochytrium* have potential in aquafeeds (Harwood and Gushina, 2009).

Both high biomass concentrations and high DHA productivity are difficult to reach when growing photoautotrophic cultures in photobioreactors due to problems like light limitation and oxygen accumulation (Mendes *et al*., 2009). In large-scale production of heterotrophic microalgae, such as *C. cohnii and Schizochytrium,* there is no requirement for light and algal cell density and productivity can be largely increased. This provides consistent biomass under highly controlled and monitored coditions. The result is a high quality product with no climatic or seasonal dependence (Mendes *et al*., 2007). In addition, monoseptic culture of microalgae in outdoor photobioreactors is expensive compared to heterotrophic growth of most commercial bacteria, yeast and fungi (Grima *et al*., 2003; Lee, 2004).

In heterotrophic culture, organic carbon sources such as sugars or organic acids can be used as the sole carbon and energy sources. This mode of culture eliminates the requirement for light and, therefore, offers the possibility of greatly increasing cell density and productivity in batch culture. The development of high cell density cultures for EPA production will also lead to a lower cost for EPA recovery.

Cultivation scale and volumetric productivity have been identified as major factors in determining the economic feasibility of fermentative DHA production. Determinant factors are biomass concentration, lipid fraction content of the cells, DHA content of the lipid and cultivation time (Mendes *et al*., 2009).

Although algal oils rich in DHA have been shown to be nutritionally equivalent to fish oils in several tests, they are still expensive compared to classic fish oil sources. For that reason attention is being focused on ways to decrease production costs (Harwood and Gushina, 2009).

The high production cost of microalgae remains a market constraint. Improvements in alternative diets may continue but production costs of microalgae may also decrease due to the uptake of new technology. The utilization of alternative microalgae species with improved nutritional quality or growth characteristics could improve their production rates, then producing cheaper algal biomass and therefore increasing microalgae competiveness (Hemaiswarya *et al*., 2011).

4.3 Other sources

Only a few microalgae species have demonstrated industrial production potentials. This is mainly due to a low specific growth rate and low cell density of the microalgae grown (Hemaiswarya *et al*., 2011).

Many bacterial strains, including *Shwenella* sp. and *Colwellia* sp., have been reported to produce EPA and DHA. However, bacteria are not considered viable for the production of PUFAs as they have lower lipid accumulation (2–5%). In addition, the presence and characteristics of some undesirable lipids hinders the possibility of promoting bacteria as a suitable PUFAs producer (Ratledge, 2004).

5. Preparation of EPA and DHA concentrates

.

5.1 Fish oil

5.1.1 Crude fish oil

Fish oil extraction entails the separation of fatty substances (lipids) from other constituents of the fish. There are a number of processes that can be used to extract the oil from raw fish and fish-by products.

Most industrial fish oils are obtained via a wet extraction process under inert gas or in closed containers to reduce oxidation by atmospheric oxygen. Cooking partially sterilizes the oil, denatures protein and facilitates oil release, followed by mechanical decanting and pressing, as shown in figure 6 (Ward and Singh, 2005). For improving fish oil quality, it is necessary to minimize the temperature and pressure involved.

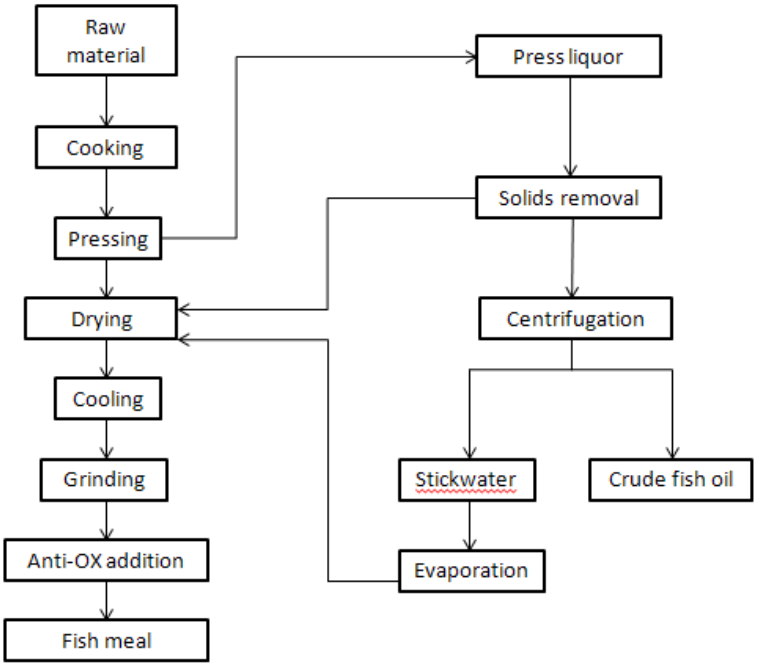


Figure 6. Wet reduction procedure for crude fish oil extraction (adapted from http://lipidlibrary.aocs.org, 2012).

The explanation and objective of the most important unit operations involved in this process are presented in table 6.

Table 6. Processing steps to obtain fish oil from fish and fish by-products (http://lipidlibrary.aocs.org, July 2012).

Unit operation	Description and objectives
Cooking	Steam cooking ruptures the fat cells, coagulates the protein and releases the oil
Pressing	Pressing mechanically expresses the free liquid from the solids producing press liquor (oil and water) and a press cake (semi-moist meat and bones).
Evaporation	After oil-water separation in a centrifuge, the stickwater (aqueous phase), containing about 8% solids, is concentrated in multiple effect evaporators to about 40-50% solids. If the factory uses steam dryers, then the waste heat from the dryer can be used to heat and evaporate the stick water.
Drying	The drying process is generally done in 2 stages. The solids obtained from the decanter separation and from the press cake are mixed and partially dried. The partially dried fishmeal is then mixed with the concentrated stick water and the drying is completed to about 10% moisture. Factories use steam and indirect hot air dryers but older factories still use the old direct fired hot air dryers.
Grinding	Reduces the particle size of the dried fishmeal.
Cooling and Stabilization	The fishmeal is cooled and antioxidant is added

There are also other methods available for fish oil production but they are not so frequent at industrial scale, such as:

- Extraction of oil with organic solvents. This process causes denaturation of proteins and loss of functional properties. In addition, the use of organic solvents is quite expensive, not totally safe for human health and not environmentally friendly (Dumay *et al*, 2006);

- 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. A large number of gases are known to possess desirable solvent properties when raised to pressure (1000 to 2000 psig) above their critical values (Shahidi and Wanasundara, 1998). The region in which a substance exists as a supercritical fluid is defined by its critical pressure (Pc) and critical temperature (Tc). Supercritical fluids have generally more gas-like transport properties, with lower viscosities and higher diffusivities, than those of typical solvents. For food commodities, CO_2 is chosen because it has moderate critical temperature and pressure (31.1°C, 1070 psig) and is inert, inexpensive, non-inflammable, environmentally acceptable, readily available and safe. Moreover, there is no solvent remaining in the product. The separation of PUFAs by SPE is dependent on the molecular size of the components involved rather than on their degree of unsaturation; therefore, a prior concentration step is needed to achieve a high concentration of PUFAs in the final product. The use of extremely high pressures and high capital costs are the major drawback of this technique (Kapoor and Patil, 2011).

- Enzymatic hydrolysis of fish-raw materials offers a rapid and reproducible method for the separation of peptide fractions, bones and oils from complex matrices (Liaset and Espe, 2008). Enzymatic oil extraction using commercial, low cost food grade proteases provides an attractive alternative as reactions can be carried out under mild conditions for short periods of time. Commercial proteases have been used to release oil from marine by-products resulting in improved yields as compared to yields obtained after heat treatment. In addition, the resulting hydrolysate provides a good source of soluble fish proteins (Mbatia, 2011).

5.1.2 Refined Fish oil

Crude fish oil obtained directly from fish meals and fish oil plants is characterized by high free fatty acid content and other impurities that limit its direct use to human and/or animal nutrition. Therefore subsequent refining steps must be carried out in order to aim the quality standards established by the food industry, namely by ω-3 ingredient suppliers.

The refining process (usually followed for fish oil) and the explanation of the most important production operations performed are present in figure 7. .

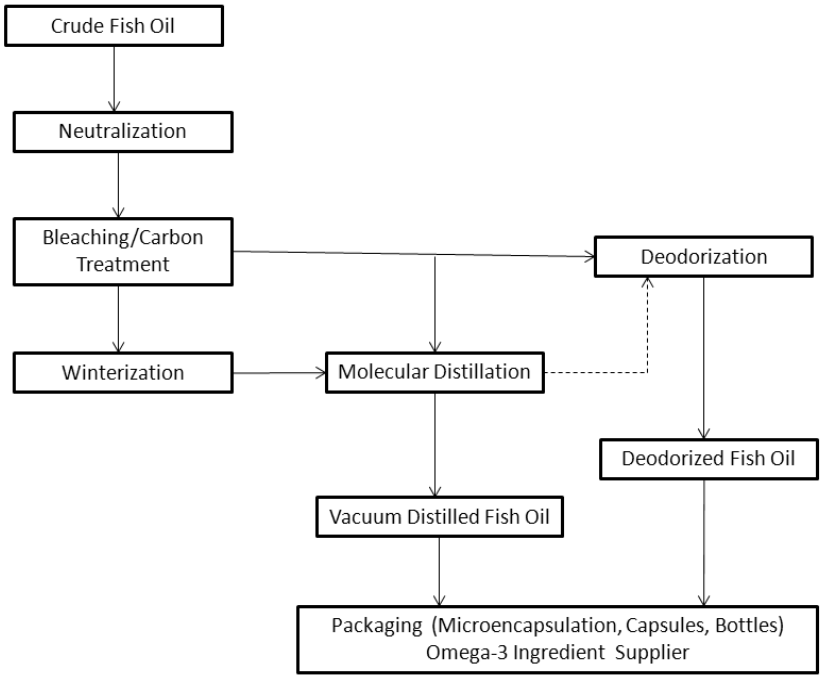


Figure 7. Production of refined fish oil (adapted from http://lipidlibrary.aocs.org, July 2012)

The most important unit operations are resumed in table 7.

Table 7. Processing steps for fish oil refining (http://lipidlibrary.aocs.org, July 2012)

Unit operation	Objectives
Oil Storage	Insoluble impurities, trace moisture and some phospholipids will precipitate out in the tanks.
Degumming	Removal of phospholipids, sugars, resins, proteinaceous compounds, traces metals and other materials.
Alkali Refining (Neutralization)	Removal of free fatty acids, pigments, phospholipids, oil insoluble material, water soluble material, trace metals
Drying	Moisture removal
Adsorptive Bleaching & Carbon Treatment	Removal of pigments, oxidation products, traces metals, sulfur compounds, dioxins and furans.
Winterization	Removal of higher melting point triacylglycerols and waxes. Used to concentrate the unsaturated triacylglycerols
Deodorization	Removal of volatiles such as short-chain free fatty acids, monoacylglycerols, aldehydes, ketones, chlorinated hydrocarbons and pigment decomposition products. This is usually the finishing step and results in bland tasting oil.
Vacuum Stripping or Thin Film, Molecular or Short Path Distillation	Removal of chlorinated hydrocarbons, fatty acids, oxidation products, PCB and free cholesterol. Sometimes this step is used to replace the deodorization step.

5.2 Microalgae oil

Industrial production of microbial oil, independently of the cell organism follows a general processing scheme separation of the biomass from the liquor, drying of the biomass, recovery of the microbial oil (figure 8). Subsequently oils can be purified by standard processes through filtration, bleaching, deodorization; polishing and antioxidants may be added to prolong shelf life (Ward and Singh, 2005).

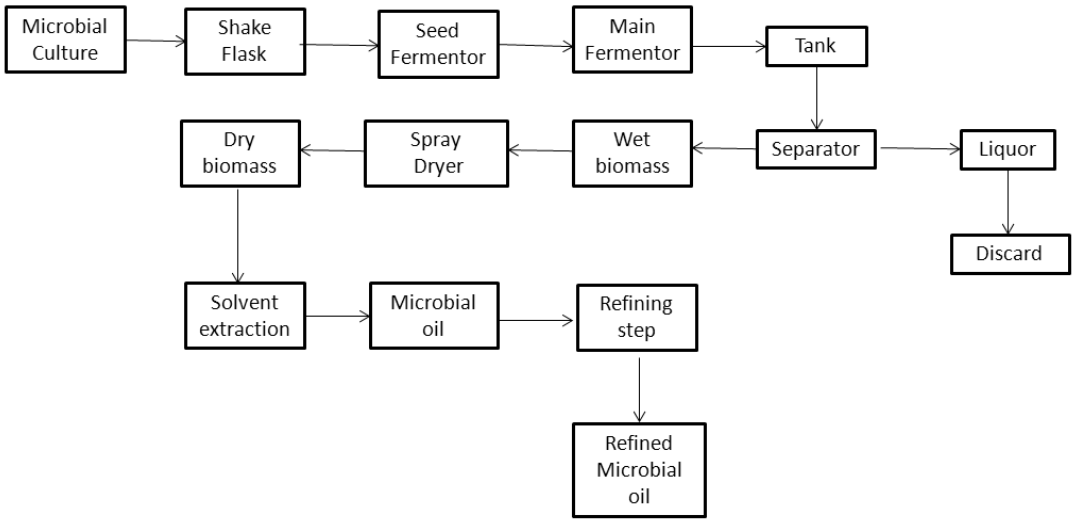


Figure 8. Production of microbial oil (adapted from http://lipidlibrary.aocs.org, July 2012).

In order to be an economic alternative method of DHA purification since the extraction step was carried out from wet biomass rather than from lyophilized cells or extracted oil, time-consuming steps that are usually used in the traditional methods of DHA extraction and purification (Mendes *et al*., 2009).

6. Proposal of a process for the production of EPA and DHA concentrates from sardine oil and *C. cohnii*

Since marine fish oil contains a large quantity of saturated and ω-6 fatty acids, the purification and concentration of PUFAs, especially EPA, is time-consuming and expensive. The undesirable taste/ odour developed by oxidation during storage are not yet solved (Gupta *et al*., 2012). Also, the growing demand for fish and fish oil is contributing to the declining in oceans of fish stocks.

All the mentioned limitations also restrain production capacity and therefore adversely affect prices. Having a wider source of raw materials, in order to build a consistent supply of feedstock, can contribute to minimize and/or overcome the production impact and prices accordingly. So, despite fish oils have a cost advantage over microalgae, these could be used to complement production.

6.1 Flow-sheet and unit operations

This section reports a proposal for the production of food grade bulk concentrates of EPA and/or DHA from (i) fish and fish by-products or *(ii) C. cohnii*.

Sardine (*Sardina pilchardus)* oil is obtained via wet extraction process.

ω-3 PUFAs, EPA and DHA concentrates from sardine oil and microalgae will be accomplished through a process where the main stages are: oil saponification, PUFAs concentration, and, when necessary, fraction separation by appropriate method. Figure 9 shows the production diagram.

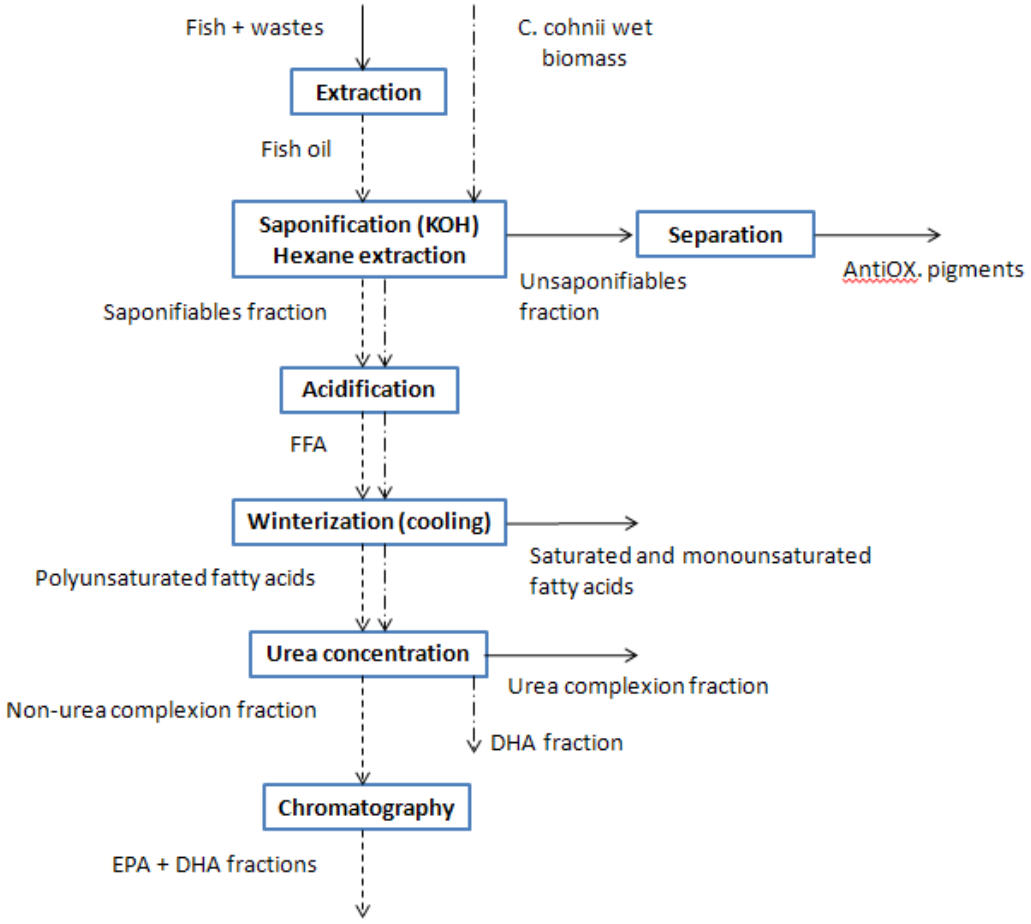


Figure 9. Scheme for the production of EPA and DHA concentrates from fish and *C. cohnii* as feedstock. Dashes corresponding to: ⇢ fish oil, ⇢ *C. cohnii*, ⟶ general process.

6.1.1 Saponification

By the action of a strong base like potassium hydroxide, yielding glycerol and the fatty acids salt (soap). The saponification is carried out in a temperature-controlled reactor with constant agitation in the presence of water, as in figure 10. The organic phase containing the unsaponifiables compounds is separated by extraction with hexane. The hydroalcoholic phase, containing the soaps, is acidified by the addition of hydrochloric acid. FFA are recovered by several extractions with hexane. The organic phase, containing the FFA is dried (Guil-Guerrero and Belarbi, 2001).

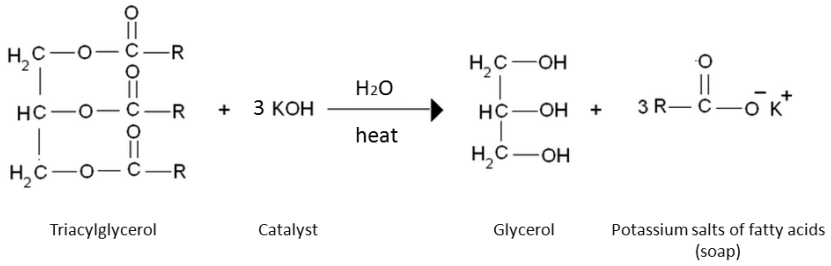


Figure 10. Saponification.

The ω-3 PUFAs incorporated in DAG or MAG are considered to be nutritionally more favorable than FFA methyl or ethyl esters of PUFAs (Shahidi and Wanasundar, 1998). This is attributed to the high susceptibility of free fatty acids to oxidation while the ethyl/methyl esters are more resistant to hydrolysis by the pancreatic lipase in the intestine then acylglycerols (Mbatia, 2011).

6.1.2 Winterization

This concentration step is essential before the urea complexation in order to concentrate the highly unsaturated fatty acids fraction, containing mainly EPA and DHA, whereby the temperature is reduced to effect precipitation of the more saturated lipids (Ward and Singh, 2005). Liquid fraction can be separated from the crystals by filtration.

The melting point of fatty acids changes considerably with the type and degree of unsaturation which allows for the separation of mixtures of saturated and unsaturated fatty acids. At low temperatures, long chain saturated fatty acids, which have higher melting points, crystallize out and PUFAs remain in the liquid form (Mendes *et al*., 2007b).

6.1.3 Urea complexation

Urea alone crystallizes in a tightly packed tetragonal structure with channels of 5.67 Å in diameter. However, in the presence of long straight-chain molecules, it crystallizes in a

hexagonal structure with channels of 8–12 Å in diameter within the hexagonal crystals. These channels are sufficiently large to accommodate aliphatic chains (Mendes *et al*., 2007b). The formation of urea inclusion compounds depends on the degree of unsaturation of the fatty acids. The tendency of fatty acids to combine with urea decreases with increasing unsaturation and decreasing chain length (figure 11).

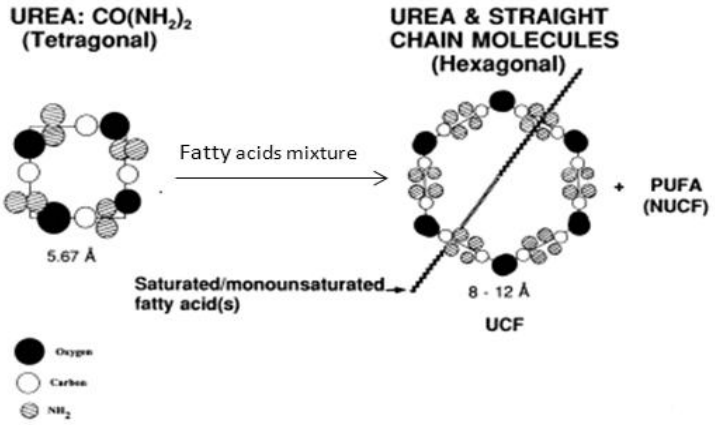


Figure 11. Formation of urea crystals in a fatty acids mixture (adapted from Shahidi and Wanasundara, 1998). UCF - Urea complexed fraction; NUCF - Non-urea complexing fraction.

The fatty acids mixture is mixed with an alcoholic solution of urea and then allowed to cool to a particular temperature depending on the degree of concentration desired. The urea complex and liquid phases can be separated by gravity filtration. The liquid fraction corresponding to the NUCF, is enriched with ω-3 PUFAs. Shorter chain saturated fatty acids may not complex with urea during the crystallization process and can be present in the non-urea complexion fraction but at very low levels (Kapoor and Patil, 2011). Nevertheless, high proportions of EPA and/or DHA are obtained in the non-urea complexion fractions. Solvent is evaporated and urea is removed in each phase by extraction with water in order to be reused in the process (Hayes *et al*., 1998).

Urea concentration is presented as the most appropriate method for ω-3 PUFAs enrichment. It permits (i) the handling of large quantities of material in simple equipment, (ii) the use of inexpensive solvents such as methanol or hexane, (iii) only mild conditions are required (e.g., room temperature), (iv) has more efficient separation when compared to other methods, (v)

is a low cost process, and (vi) a versatile since fractionation characteristics can be altered simply by changing the amounts of either solvent or urea (Guil-Guerrero and Belarbi, 2001).

6.1.4 Chromatography

This last purification step is the major concern in an industrial process since it is the most expensive. It is only necessary when sardine is used as feedstock since *C. cohnii* can accumulate relatively high amounts of lipids (up to 20% by weight) with DHA contents up to 30-50% of the total fatty acids and no other PUFAs present above 1% (Mendes *et al*., 2007).

Large-scale preparative high pressure liquid chromatography (HPLC) allows an effective and cost-efficiency separation of highly purified EPA and DHA fractions (above 95%) for the pharmaceutical and the dietary supplement industry. It is possible to separate fatty acids according to their number of carbon and their degree of unsaturation by using appropriate adsorbents. High performance liquid chromatography can be used for production of ω-3 PUFAs concentrates. Solvent choice for separation of fatty acid esters depends on the desired purity of eluted fractions and their use as well as production requirements. Ethanol and water would be the solvents of choice if the end product is to be consumed by humans (Kapoor and Patil, 2011). A preparative column is required for the production of large amounts of highly purified EPA and DHA.

Yamamura and Shimomura (1997) describe how to determine the purification conditions for industrial-scale preparative HPLC using an analytical HPLC and a small-scale preparative HPLC were used as shown in figure 11.

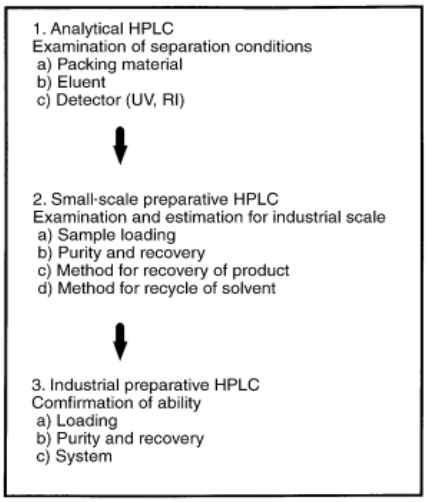


Figure 12. Determination of industrial-scale purification conditions (Yamamura and Shimomura, 1997).

First, the packing material, eluent, and detector are examined in the analytical HPLC device. The loading amounts, recovery ratio, purity, and methods of recovery of product and solvent are conducted with the small-scale preparative HPLC. Then, these conditions have to be reconfirmed with the industrial-scale preparative HPLC. The best way to achieve a scale-up purification is to use the same packing material and solvent composition. Under these conditions, the scale-up factor depends on the ratio of cross-section of the columns.

There are four major steps, namely sample dissolution, preparative HPLC, product recovery, and solvent recovery. This system includes a dissolution tank, an eluent tank, a fractionation tank, a condenser for product oil, and a distillation column for recycling the solvent. Column switching may be employed in this system.

6.1.5 Esterification of Free Fatty Acids

This step is necessary for analysis of samples. Fatty acids in fractions are analyzed by capillary gas chromatography (GC) to determine its purity (% w/w of fatty acid) and yield recovered (fatty acid in fraction/fatty acid in sample).

In the esterification reaction (figure 12), a free fatty acid reacts with a large excess of alcohol (methanol) to form an alkyl ester and water in the presence of an acidic catalyst (H_2SO_4).

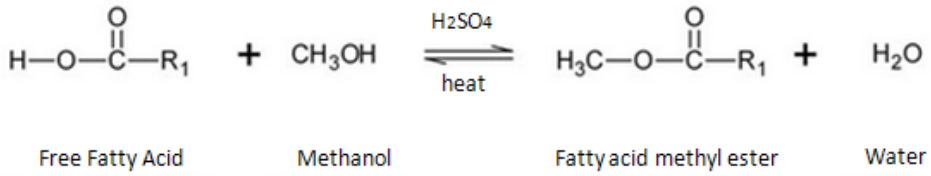


Figure 13. Acid-catalyzed esterification reaction.

It is important not to confuse the acid-catalyzed transesterification with the acid-catalyzed esterification. Transesterification refers to the reaction of an ester with an alcohol to form a different ester, while esterification refers to the reaction of an acid with an alcohol to form an ester (figure 13).

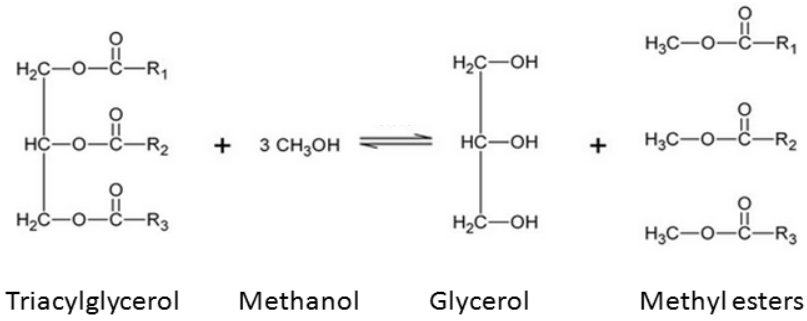


Figure 14. Transesterification reaction.

6.1.6 Other processes

There are several processes using selective and non-selective hydrolysis.

As alternative to the proposal procedure for the production of EPA and DHA concentrates, the use of lipase-catalyzed reactions might be a feasible option.

Selective lipase-catalyzed hydrolysis

Enzymatic enrichment of PUFAs may offer advantages over other methods due to regio-, stereo-, and substrate selectivity of lipases (E.C.3.1.1.3., triacylglycerol acyl-hydrolase). In addition, lipases act under mild conditions (e.g. neutral pH and low temperatures) and the process produces high quality products. The selectivity of lipases towards some fatty acids in oil is used to remove saturated and monounsaturated fatty acids from the TAG and leave PUFAs attached to the glycerol backbone (Figure 14).

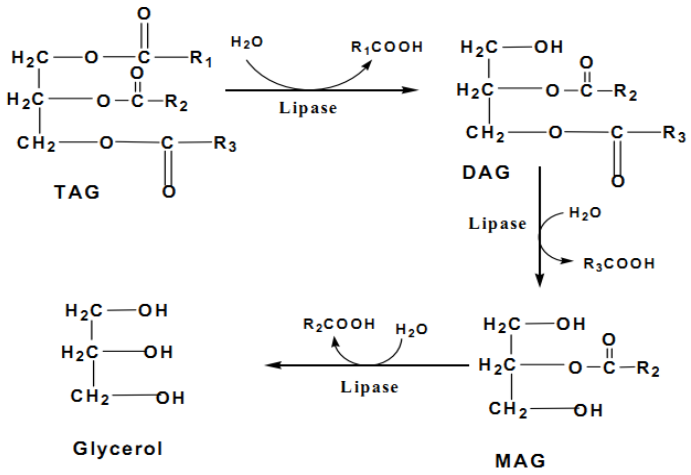


Figure 15. Hydrolysis of triacylglycerol (TAG). R_1, R_2 and R_3 are saturated, polyunsaturated (PUFAs) and monounsaturated fatty acids (Mbatia, 2011).

After separation of released free fatty acids, a concentrate of PUFAs attached to glycerol molecule in form of mono- (MAG), di- (DAG), and TAG is obtained. The percentage composition of TAG, DAG and MAG in the concentrate depends on degree of hydrolysis (Mbatia, 2011).

Non-selective esterification with alcohol

Selective esterification of FFA from fish oil or *C. cohnii* with methanol leading to the enrichment in FA-ME catalyzed by lipases is another option (Figure 15). Initially, TAG are split into their fatty acids and glycerol by alkaline hydrolysis using alcoholic KOH or NaOH.

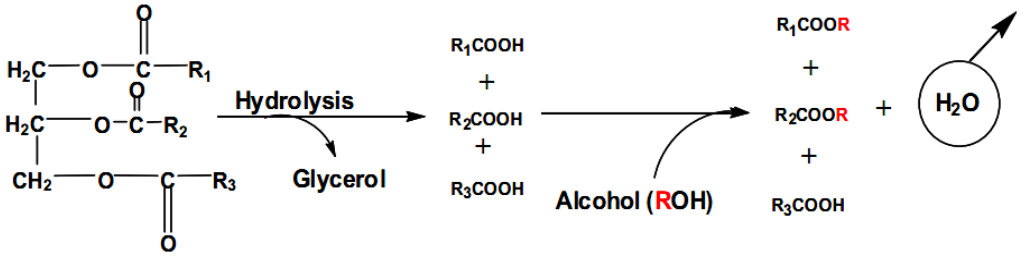


Figure 16. Lipase catalyzed esterification of fish oil FFA with alcohol (ethanol). R_1, R_2 and R_3 are saturated, monosaturated and PUFA fatty acids respectively (Mbatia, 2011).

Due to high level of unsaturation, PUFAs are in general poor substrates for the lipases while the saturated (SA) and monounsaturated fatty acids (MUFA) are preferred. Thus, most of PUFAs are left as FFA while SA and MUFAs are esterified. During esterification reaction, the water content should be kept at minimum, in order to prevent product hydrolysis, but sufficiently high in order to prevent enzyme deactivation. Thus, water released during the esterification reaction is continuously removed by use of adsorbents, vacuum evaporation or use of salt hydrates (Mbatia, 2011).

Selective lipase-catalyzed enzymatic esterification of FFA or hydrolysis of the fatty acid methyl esters (FA-ME) could be more effective than hydrolysis of fish oil TAG to concentrate PUFAs. This is because the specificity of lipases towards fatty acids bound to TAG molecules is affected by many factors such as region selectivity of the lipases toward triacylglycerols, non-homogeneous distribution of EPA and DHA into various positions of the glycerol backbone, as well as a possible triglyceride selectivity of the lipases.

On the contrary, when the fatty acid is not attached to the glycerol molecule, lipase specificity is influenced by fatty acid structure or alcohol type used during esterification reactions (Mbatia, 2011).

Nowadays, (i) the high price of commercial lipases, (ii) its low operational stability and (iii) longer reaction times, when compared to the chemically catalyzed processes, are the major constrains for the industrial implementation of enzymatic processes.

6.2 Simple mass balance and economic analysis of the process

In order to have an idea of the viability of the process and to compare the two different sources of feedstock – sardine and its wastes and *C. cohnii* a simple mass balance was carried out. These calculations are aimed at supporting a subsequent simple economic analysis.

The calculation of reagents and raw materials are based in the experimental work of Guerrero and Belarbi (2001) on the purification of EPA and DHA from cod liver oil; the proportional masses are maintained.

Table 8 shows the variable composition of EPA and DHA yields, and the body fat (%) of sardine catch in the North Atlantic as part of experimental work of Bandarra *et al.* (1997). The highest levels of total PUFAs occurred from July to December (peak season of fish catch). The EPA and DHA percentages refer to the total fatty acids composition. The average body fat (%) in these six months is 15% oil in a wet basis and 50% oil in a dry basis. All fat is considered to be 100% saponifiable oil. Moreover, the Food and Agriculture Organization of the United Nations (FAO) states that the average water content of the flesh of fatty fish is about 70% (http://www.fao.org, July 2012).

Table 8. Edible values of EPA, DHA and body fat (%) (w/w %) in sardine (*Sardina pilchardus)* (Bandarra *et al*., 1997).

	Jul	Aug	Sep	Oct	Nov	Dec	Average
EPA 20:5ω3	17.79	15.62	17.93	17.42	17.58	14.19	16.76
DHA 22:6ω3	10.82	12.38	10.49	10.87	12.08	15.3	11.99
Body fat (%)	11.1	17.6	18.4	18.2	15.8	10	15.18

For *C. cohnii,* it is assumed that 30% of the total fatty acid content is DHA and that it contains 5% of oil considering a wet basis or 20% oil in a dry basis (Mendes *et al*., 2009).

Regarding the overall process assumptions:

- In order to compare both production processes form sardine and its wastes and *C. cohnii*, the same basis of calculation was established (100kg of edible oil);
- A yield of 97% for the concentration of EPA and DHA concentrates;

- The water resulting from the extraction process is reused in the saponification, considering a loss of 5%;
- Costs regarding chromatography are not presented due to the lack of available and reliable information. Chromatography is just needed for the EPA and DHA concentrates from sardine and its waste, which would only increment the overall production costs from this raw material. Nevertheless, this assumption is taken into account in the conclusion chapter;
- The product recovery costs are identical for sardine and *C. cohnii* sources
- Prices of commodities (table 9)

Table 9. Prices of raw materials, reagents and final products.

	Reagents	€/ kg
Raw materials	**Sardine**[(1)]	1,5
	C. cohnii[(2)]	4
Reagents	**Potassium hydroxide**[(3)]	1,23
	Ethanol[(3)]	1,60
	Hexane[(3)]	0,82
	Methanol[(3)]	0,49
	Urea[(3)]	0,08
	Water[(4)]	0,001
Products	**EPA**[(2)]	570
	DHA[(2)]	570

(1) Estimated based on supermarket prices
(2) http://www.bpe.wur.nl, October 2008
(3) http://www.alibaba.com/Food-Beverage_p2, July 2012
(4) http://www.aguasgaia.eu/pt/dados.php?ref=san_tarifario, July 2012

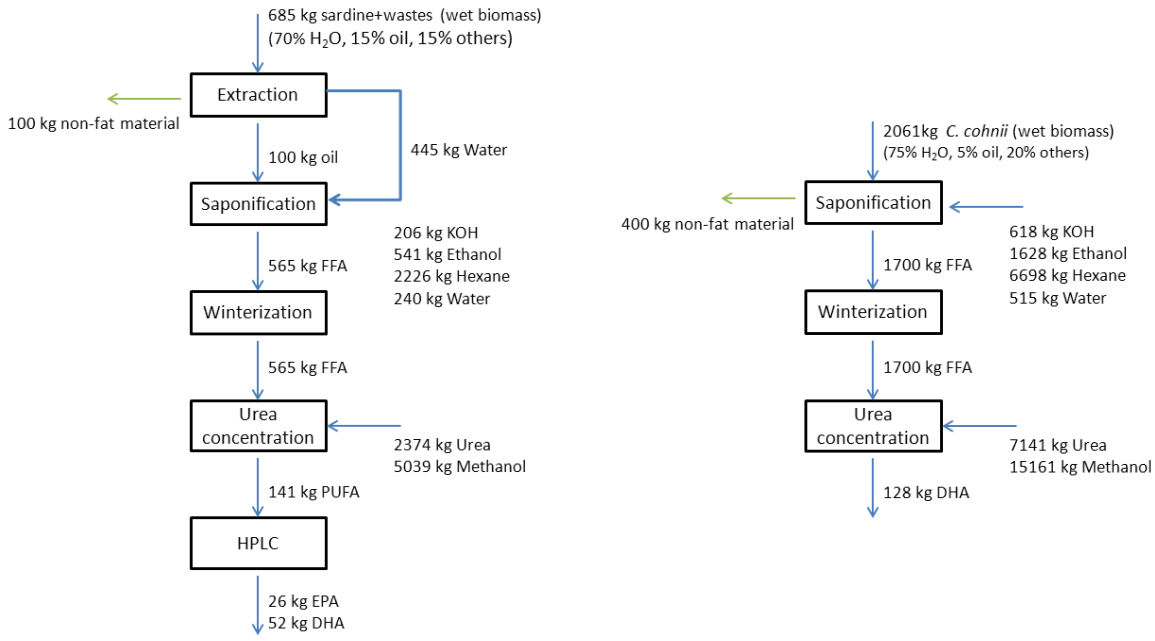
Table 10 shows the composition of water and oil in sardine and wastes and C. cohnii, used for the production of EPA and /or DHA concentrates. A simple mass balance of the process using sardine and *C. cohnii* as raw materials is demonstrated in figure 16.

Table 10. Composition of sardine and *C. cohnii* as raw materials

	Composition of Sardine + wastes[(1)]	***Composition of C. cohnii*[(2)]**
Composition raw material (wet biomass)	70% water (H_2O) 15% Oil 15% others	75% water (H_2O) 5% Oil 20% others
Raw mass for the obtention of 100 kg of edible oil	685 kg	2061 kg

[(1)]Bandarra *et al.,* 1997; http://www.fao.org/wairdocs/tan/x5916e/x5916e01.htm, 12th July 2012

[(2)]Mendes *et al.*, 2009



a) b)

Figure 17. Overall mass balance a) Sardine b) *C. cohnii*

Table 11 shows an analysis of the overall production costs and earning regarding the raw material and reagents used for the production of 100 kg of edible oil, from 685 kg of sardine and 2061 kg of *C. cohnii.*

Table 11. Simple economical balance for the use of 100kg oil from sardine or *C. cohnii* to produce EPA and/or DHA concentrates.

		Sardine (€/ 100kg oil)	*C. cohnii* (€/ 100kg oil)
Raw material		685	2061
Reagents costs	KOH	252	759
	Ethanol	866	2605
	Hexane	1823	5486
	Methanol	2474	7445
	Urea	195	586
	Water	0.2	0.5
	Total	5611	16881
Raw material + reagents total costs		6638	25125
Product revenues			
	EPA	15584	-
	DHA	30840	101764
Total revenues		39785	76640

7. Conclusions and future prospects

The beneficial role of ω-3 PUFAs, mainly EPA and DHA, in human health and nutrition has been extensively demonstrated. This recognition is leading to an increasing growth in ω-3 ingredient market and has stimulated the optimization of related industrial processes and the search for new sources.

In fact, fish and fish by-products are a readily available source of long-chain PUFAs providing significant quantities of fish oil for the production of EPA and DHA concentrates. However, there are great concerns about fish and food industries as sources of environmental contaminations. Fish waste is normally underutilized by being discarded or sold ate very low price as fertilizer or animal feed. However, waste management can be improved by converting these wastes into more useful products. High valuable compounds such as fish oils and ω-3 fatty acids, biodiesel/biogas, natural pigments, enzymes and proteins can be obtained using the appropriate techniques. Reusing process consumables also brings additional environmental and economic benefits.

Microalgae oil obtained from the heterotrophic microalgae *C. cohnii* provides a concentrated source of one PUFA - DHA avoiding time consuming and costly purification steps. However, limiting ω-3 sources may lead to a growing demand for fish oils resulting in price increasing. In addition, the use of microalgae oil can contribute to minimize production decay due to raw material fluctuations. Fermentation also offers a better control of fatty acid ratios, and contaminant levels.

Marine fish oil is actually the main source for EPA and/or DHA concentrates and therefore the cheapest one, despite its complex fatty acid composition which makes it difficult to purify. The increase fishery creates environmental imbalances. There are questions about the presence of contaminants like heavy metals, PCBs and dioxins. The microalgae *C. cohnii* is still identified as a minor source of DHA and therefore this type of concentrate is more expensive. Due to its higher DHA content it is easier to purify. It can be produced in fermenters thus the quality and quantity of the source is not dependent on environmental conditions and it is contaminant free. It is also suitable for vegetarians.

This work presents a cost-effective production method - simple, easy of scaling, and ecological friendly - that can be used to meet the growing market demand. It is showed that ω-3 concentrates - EPA and DHA can be achieved using the same production method for two different sources - fish (seasonal) and microalgae (non-seasonal). Currently the cost of

fermentation of *C. cohnii* for DHA production purposes is significantly higher than obtaining the fatty acids EPA and DHA from fish oil. However, the final production cost of high purity DHA is likely to be lower for the microalga, reflecting the cleaner fatty acid profile. Also from the economic point of view the process is feasible for both sources in an industrial scale bringing extra flexibility all year-round.

The main key for the future development of the market of high added valued products from microalgae is the reduction of the biomass production cost. This represents around 42% of the total EPA and/or DHA total production process, according to Grima et al 2003. This can be achieved, for example, by trimming culture media costs with special emphasis on the reuse of the wastewater, as well as using low cost bioreactors and taking advantage of the overproducing algal strains.

The enhancement of the use of microalgae can be achieved by trimming culture media costs with special emphasis on the reuse of the wastewater, as well as using low cost bioreactors and taking advantage of the overproducing algal strains. These are the drivers of the future trends in nutritional ω-3 PUFAs concentrates market that will contribute to lower the overall production costs.

References

Anderson B.M., Ma D.W.L. (2009) Are all n-3 polyunsaturated fatty acids created equal? Lipids in Health and Disease. *BioMed Central* 1 - 65

Arvanitoyannis I. S., Kassaveti A. (2008) Fish industry waste: treatments, environmental impacts, current and potential uses. *International Journal of Food Science and Technology* 43: 726 - 745

Bandarra N.M., Batista I., Nunes M.L., Empis J.M., Christie W.W. (1997) Seasonal Changes in Lipid Composition of Sardine (*Sardina pilchardus*). *Journal of Food Science* 62(1): 40 - 42

Basua H., Perneckya S., Senguptaa A., Liepab G. U. (1996) Coronary Heart Disease: How Do the Benefits of ω-3 Fatty Acids Compare with Those of Aspirin, Alcohol/Red Wine, and Statin Drugs? *J. Am. Oil Chem. Soc*. 83: 985 - 997

Breivik H., Haraldsson G.G., Krisfinsson B. (1997) Preparation of Highly Purified Concentrates of Eicosapentaenoic Acid and Docosahexaenoic Acid. *J. Am. Oil Chem. Soc*. 74(11): 1425 - 1429

Certik M., Shimizu S. (1999) Biosynthesis and Regulation of Microbial Polyunsaturated Fatty Acid Production. *J. Biosc. Bioeng*. 87 (1): 1 - 14

Couto R.M., Simões P.C., Reis A., Silva T.L., Martins V.H., Sanchez-Vicente Y. (2010) Supercritical fluid extraction of lipids from the heterotrophic microalga *Crypthecodinium cohnii*. *Eng. Life Sci*. 2: 158 - 164

Dumay J., Donnay-Moreno C., Barnathan G., Jaouen P., Berge J.P. (2006) Improvement of lipid and phospholipid recoveries from sardine (*Sardina pilchardus*) viscera using industrial proteases. *Process Biochem.* 41: 2327 - 2332

Guil-Guerrero J.L., Belarbi E.-H. (2001) Purification Process for Cod Liver Oil Polyunsaturated Fatty Acids *J. Am. Oil Chem. Soc*. 78 (5): 479 - 484

Grima E.M., Medina A.R., Gimenez A.G., Gonzalez M.J.I. (1996) Gram-scale purification of eicosapentaenoic acid (EPA, 20:5n-3) from wet *Phaeodactylum tricornutum* UTEX 640 biomass. *Journal of Applied Phycology* 8: 359 - 367

Gupta A, Barrow C.J., Puriet M. (2012) ω-3 biotechnology: Thraustochytrids as a novel source of ω-3 oils. Biotechnol. Advances 30: 1 - 13

Guschina I., Harwood J.L. (2006) Lipids and lipid metabolism in eukaryotic algae. *Progress in Lipid Research* 45: 160 - 186

Guschina I., Harwood J.L. (2009) The versatility of algae and their lipid metabolism.*J. Biochem.* 20: 1 - 6

Grima E.M., Belarbi E.H., Fernandez F.G.A., Medina A.R., Chisti Y. (2003) Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances* 20: 491 - 515

Hamilton R.J., Rice R.D. (1995) Proceedings of a conference organised by SCI Oils & Fats Group in Hull, UK 1 - 143

Hayes D.G., Bengtssonc Y.C., Alstineb J.M.V., Setterwall F. (1998) Urea Complexation for the Rapid, Ecologically Responsible Fractionation of Fatty Acids from Seed Oil. *J. Am. Oil Chem. Soc.* 75 (10): 1403 - 1409

Hemaiswarya S., Raja R., Kumar R.R., Ganesan V., Anbazhagan C. (2011) Microalgae: a sustainable feed source for aquaculture. *World J Microbiol Biotechnol* 27:1737 - 1746

Kapoor R., Patil U. K. (2011) MiniReview Importance and production of ω-3 fatty acids from natural sources. *International Food Research Journal* 18: 493 - 499

Knocha B., Barnett M.P.G.,Roya N.C., McNabba W.C. (2009) Study of the effects of dietary polyunsaturated fatty acids: Molecular mechanisms involved in intestinal inflammation. *Grasas Y Aceites*, 60 (1): 8 - 21

Lee Y.-K. (2004) Algal Nutrition. Heterotrophic Carbon Nutrition. *Handbook of microalgal culture: biotechnology and applied phycology* 116 - 124

Liaset B., Espe M. (2008) Nutritional composition of soluble and insoluble fractions obtained by enzymatic hydrolysis of fish-raw materials. *Process Biochem.* 43: 42 - 48

Linder M., Fanni J., Parmentier M. (2005) Proteolytic Extraction of Salmon Oil and PUFAS Concentration by Lipases. *Marine Biotechnology* 15: 70 - 76

Liu S., Zhang C., Hong P., Ji H. (2006) Concentration of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) of tuna oil by urea complexation: optimization of process parameters. *Journal of Food Engineering* 73: 203 - 209

Mbatia B.N. (2011) Valorisation of fish waste biomass through recovery of nutritional lipids and biogas - Doctoral Thesis. Lund University.

Mendes A., Reis A., Vasconcelos R., Guerra P., Silva T.L. (2009) *Crypthecodinium cohnii* with emphasis on DHA production: a review*. J. Appl. Phycol*. 21: 199 - 214.

Mendes A., Guerra P., Madeira V., Ruano F., Silva T.L.S., Reis A. (2007a) Study of docosahexaenoic acid production by the heterotrophic microalga *Crypthecodinium cohnii* CCMP 316 using carob pulp as a promising carbon source. *World J. Microbiol. Biotechnol.* 23, 1209 - 1215.

Mendes A., Silva T.L., Reis A. (2007b) DHA concentration and purification from the marine heterotrophic microalga *Crypthecodinium cohnii* CCMP 316 by winterization and urea complexation. *Food Technol. Biotechnol.* 45 (1): 38 - 44

Noriega-Rodríguez J. A., Ortega-García J., Angulo-Guerrero O., García H. S., Medina-Juárez L. A. and Gámez-Meza N. (2009) Oil production from sardine (*Sardinops sagax caerulea*) Producción de aceite a partir de sardine (*Sardinops sagax caerulea*). CyTA - *Journal of Food*, 7 (3): 173 - 179

Ratledge C. (2004) Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production*. Biochimie* 86: 807 - 815

Shahidi F., Wanasundara U. N. (1998) ω-3 fatty acid concentrates: nutritional aspects and production technologies. *Trends in Food Science & Technology* 9:230 - 240

Silva T.L., Mendes A., Mendes R.L., Calado V., Alves S.S., Vasconcelos J.M.T., Reis A. (2006) Effect of n-dodecane on *Crypthecodinium cohnii* fermentations and DHA production. *J. Ind Microbiol Biotechnol*. 33(6):408 - 16

Reis A, Silva T.L. (2008) The use of multi-parameter flow cytometry to study the impact of n-dodecane additions to marine dinoflagellate microalga *Crypthecodinium cohnii* batch fermentations and DHA production. *J. Ind. Microbiol. Biotechnol.* 35:875 - 887

Valenzuela A.B. (2009) Docosahexaenoic acid (DHA), an essential fatty acid for the proper functioning of neuronal cells: their role in mood disorders. *Grasas y aceites*, 60 (2):203 - 212

Ward O.P., Singh A. (2005) ω-3/6 fatty acids: Alternative sources of production. *Process Biochem.* 40:3627 - 3652

Wen Z.-Y., Chen F. (2003) Heterotrophic production of eicosapentaenoic acid by microalgae. *Biotechnology Advances* 21: 273 - 294

Yamamura R. and Shimomura Y. (1997) Industrial High-performance liquid chromatography purification of docosahexaenoic acid ethyl ester and docosapentaenoic acid ethyl ester from single-cell oil. *J. Am. Oil Chem. Soc.* 74: 1435 – 1440

Internet References

http://lipidlibrary.aocs.org/processing/marine/index.htm, July 2012

(http://lipidlibrary.aocs.org/processing/marine/index.htm, 2012)

http://www.fao.org/wairdocs/tan/x5916e/x5916e01.htm, July 2012