Green Technologies for Production of Oils Rich in n-3 Polyunsaturated Fatty Acids from Aquatic Sources

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ABSTRACT

Fish and algae are the major sources of n-3 polyunsaturated fatty acids (n-3 PUFAs). Globally, there is a rapid increase in demand for n-3 PUFA-rich oils. Conventional oil production processes use high temperature and chemicals, compromising the oil quality and the environment. Hence, alternative green technologies are assessed for producing oils from aquatic sources.

A critical review to identify the most promising green technologies for each of the steps in the production of oils rich in n-3 PUFAs from fish and algae species, placing special focus on research assessing green strategies in comparison with the conventional technologies was performed. The careful examination of gaps and critical challenges to be resolved by future research to facilitate green production of oils rich in n-3 PUFA indicate that most of the studies have focused on the oil extraction and enrichment of n-3 PUFAs, while less effort has been directed towards green processes for refining oils from fish and algae. Moreover, from the analytical point of view, analysis of the resulting lipid classes is of outmost importance to stablish the quality of the resulting oil. Therefore, there is still a need for improvement in some of the processing steps of n-3 PUFA-enriched.

Keywords: N-3 polyunsaturated fatty acids, Green technologies, Fish oil, Microalgae oil, n-3 PUFA-enrichment

1. Introduction

Fish oils are an important source of long-chain polyunsaturated fatty acids (PUFAs), among which eicosapentaenoic acid (EPA; 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3) are of special relevance due to their importance as structural components in synaptic membranes in the brain and the retina (Dyall & Michael-Titus, 2008) and their role in supporting the health of the heart and the cardiovascular system (Swanson, Block & Mousa, 2012). The recommended daily intake of PUFAs is continuously being revised and updated by governments and health organizations (FAO/WHO, American Dietetic Association or American Heart Association). The current recommendations for total n-3 PUFA range from 1.4 to 25 $g \cdot day^{-1}$, and for EPA + DHA from 140 to 600 $mg \cdot day^{-1}$, which means a minimum of two servings of fish per week, one being from an oily fish, such as salmon, tuna or sardine (Molendi-Coste, Legry & Leclercq, 2011). Hence, to fulfill nutritional requirements, n-3 PUFAs are of increasing demand as food ingredients and dietary supplements, as well as pharmaceutical products.

The fatty acid (FA) composition of fishes varies among the species and is affected by factors such as the environment and feed. Lower contents of lipids have been reported in fishes from tropical climate compared to fishes from the Arctic region. Marine fish species have a higher content of n-3 PUFAs due to their feed on plankton, while freshwater fish has a higher content of monounsaturated fatty acids (MUFAs) reflecting the FA composition of the vegetation and plant materials as the major feed in fresh water (Sahena et al., 2009).

According to FAO (2018), 170.9 MT of fish products were produced in 2016, of which a major part was produced in developing countries (84% of total production). Fish industry generates a high amount of by-products, of which heads, viscera, skin, and scales are the main components (Olsen, Toppe & Karunasagar, 2014). In some cases, the yield of side streams may be as high as 70% of the whole fish. Fish meal and fish oil are currently the two main products produced from the

valorization of the by-products fish processing. The production of fishmeal and fish oil have been stimulated by the significant increase in the price since the beginning of the 21st century rising from 800 to 1600 USD per ton for fishmeal and from 800 to 2400 USD per ton for fish oil by 2017 (FAO, 2018). Hence, the production of fish oil from low value fish mass and side streams not suitable for direct consumption presents a unique solution to provide valuable n-3 PUFAs for human consumption. In addition, the production of fish oil from fish side-streams fulfills the principles of circular economy stating that the wastes or by-products of one industry become the raw materials for another one (European Commission, 2015).

In addition to fish, various algae species have recently gained popularity as a source of several bioactive compounds for human consumption due to their high growth rates and high biomass production. The content of bioactive lipids in microalgae can reach up to 85% of the dry weight, being especially rich in PUFAs and they can also be grown in bioreactors under controlled conditions to maximize their performance (Gallego, Montero, Cifuentes, Ibáñez & Herrero, 2018). Moreover, research has shown potential of using microalgae (e.g. *Nannochloropsis* sp.) cultivation to recover nutrients released as wastes from industrial processing, presenting a sustainable way of producing biomass rich in EPA and DHA (Polishchuk et al., 2015). A recent study showed a lipid extraction yield of 42 wt% from *Isochrysis* biomass using pressurized liquid extraction (PLE), also known as subcritical fluid extraction, with 90% aqueous ethanol (He, Huang, Zhong, Guo & Chen, 2019). Recently, there has also been an increasing interest in macroalgae species as a source of nutrients and bioactive components for food and feed. Indeed, some species present high contents of PUFAs (Rodrigues et al., 2015); however, due to the generally low content of oil, currently oil extraction is not part of the common processing pipelines of macroalgae, which are mainly devoted to direct use as food (mostly as dried algae) as well as to production of hydrocolloids and food supplements.

Currently, several conventional techniques are applied at industrial-scale to convert aquatic sources into high-value oil. Commonly, fish oil production involves cooking at high temperature, pressing and centrifugation to separate raw oil from water and solid materials, followed by several steps of refining. Typically, refining includes degumming to eliminate the phospholipidic fraction, de-acidification by neutralization with NaOH followed by washing with water to eliminate the free fatty acids (FFAs), bleaching with an appropriate ratio of adsorbent/oil to remove the colorants and pollutants, and deodorization with steam distillation under vacuum conditions. Furthermore, to increase the content of n-3 PUFAs, fish oils are subjected to enrichment process yielding end products with the content of DHA and EPA together up to 85% of total fatty acids, either as ethyl esters (EEs) or as triacylglycerols (TAGs), the latter being established to provide a higher bioavailability for the n-3 PUFAs (Olsen et al., 2014; Neubronner et al., 2011). Moreover, acylglycerols with n-3 PUFAs bound to the *sn*-2 position have been proven to have higher oxidation stability compared to those with n-3 PUFAs bound to the *sn*-1,3 positions (Wijesundera et al., 2012).

However, the traditional oil production methods cause degradation of the labile PUFAs due to the use of harsh conditions such as high temperatures (Fournier et al., 2006). In addition, chemicals including toxic solvents are used, leaving harmful residues in the final product and causing environmental pollutions. With the aim to increase the efficiency of the whole process and to improve the quality of the final products, as well as to minimize the environmental impact (solvents and energy), it is essential to develop greener strategies for producing food and natural products (Chemat, Vian & Cravotto, 2012). The production of oil rich in n-3 PUFAs for human consumption is not an exception; there have been an increasing number of researches published on novel green technologies applied at different processing steps of fish oil production. A number of green extraction methods, including supercritical fluid extraction (SFE), PLE, enzyme-assisted processing, and fermentation, have been shown to improve the oil quality and stability by retaining

the natural antioxidants and reducing oxidation (Gallego et al., 2018; Ozogul et al., 2018; Yang, Ahotupa, Määttä & Kallio, 2011). Following the structure presented in **Scheme 1**, this review aims to summarize the current state-of-the art by looking into the technologies currently applied at the main processing steps for the extraction and refining of oils rich in n-3 PUFAs from aquatic resources including fish and microalgae. Special attention is paid to research comparing different technologies and strategies on a given processing step in terms of their impact on the composition and quality of the oil in order to guide the further development of green technologies.

2. Extraction of crude oils

The first step in oil production is the extraction of crude oil from raw materials. Currently, available techniques can be classified into “conventional” and “non-conventional” techniques. Conventional techniques refer to the ones traditionally used and globally accepted for fish oil extraction, which have been applied for many years in industrial extraction of fish oil, such as wet reduction (also known as rendering). Wet reduction starts with cooking the fish in water for a short time (*ca*. 30 min) at a temperature around 90 ºC followed by pressing and centrifuging, resulting in a 3-phase system (from the bottom to top: solid matter, water and oil), from which the top layer (oil) is separated by decantation. Although the use of water is cheap, safe, and easy to operate in industrial systems, wet reduction is not highly efficient to extract oil. Moreover, the high temperature employed in the process leads to degradation of labile n-3 PUFAs. For this reason, in recent years non-conventional green techniques have emerged. Among the green alternatives with the highest potential for industrial application and thus, investigated here in more details, are physical pretreatment with microwave (MW) or ultrasounds (US), enzymatic extraction, supercritical fluid extraction (SFE) and fermentation. In the following sections, we focus on the comparison between conventional oil extraction methods to green technologies in terms of yield and quality of the

resulting oil. Typically, the quality markers for crude oil are fatty acid composition and physicochemical parameters, including peroxide value (PV), *p*-anisidine value (AV), iodine value (IV) and acid value. These parameters are taken into account when giving an overview of each methodology as summarized in Table 1.

2.1. Microwave and ultrasound assisted extractions

Microwave assisted extraction (MAE) is based on the capacity of a system to absorb the electromagnetic radiation (requires solvents with high dipole moment) and to transform it into thermal energy, resulting in a temperature rise. Due to this temperature increase, the water in cells evaporates producing massive cell wall disruption leading to an increase in the porosity, which facilitates the mass transfer to the solvent. Ultrasound assisted extraction (UAE), on the other hand, is based on the cavitation effect of the ultrasonic waves that facilitate the extraction and mass transport by disrupting cell walls. In UAE any solvent can be used (Chemat, Zill-e-Huma & Khan 2011). Both techniques, especially the UAE, have been broadly applied for the extraction of a wide variety of compounds in food and natural products and scaling up is already ongoing (Chemat et al., 2017). Nevertheless, UAE has not been applied at industrial scale in the extraction of fish oil. Previous studies have compared the performance of MAE and UAE against extraction with Soxhlet and Bligh and Dyer methods in laboratory scale. UAE of fish oil was carried out from six fish species using ethanol as a solvent at room temperature for 90 min while MAE was applied at 600 W and 70 ºC with an extraction time of 10 min. UAE resulted in higher oil yield and higher proportions of n-3 PUFAs in the oil compared to MAE. On the other hand, when MAE was employed after optimization by central composite rotatable (CCR) experimental design, no significant differences in the yield and composition of the extract were found compared to the solvent-based Folch extraction, whereas the PV of the oil was 8-fold lower for the MAE method (Costa & Bragagnolo, 2017).

Pretreatments rupturing the cell walls of microalgae are essential for extraction of oil from microalgae. A thorough review has been previously published on the efficiency of different technologies as pretreatment for improving the extraction lipid yield from different microalgae species (Lee, Cho, Chang & Oh, 2017) While many technologies are still in the early stage of investigation, high-pressure homogenization, enzymatic treatment, ultrasonication and microwave treatment have proven to be effective techniques to break the cell walls increasing the oil extraction yield from microalgae (Lee et al., 2017; Xue et al., 2018). In the extraction of *Crypthecodinium cohnii*, a microalgae, UAE reduced the extraction time by 10-fold and increased the lipid yield by 5-fold compared to Soxhlet extraction, whereas MAE offered an increase in yield of less than 3-fold (Cravotto et al., 2008). The microalgae cells are difficult to disrupt due to the polymer network within the cell walls. Conventional extraction involves use of toxic solvents, such as chloroform, hexane and methanol, and they can be time consuming and harmful to the environment. Microwave-assisted and ultrasound-assisted extractions have shown to significantly improve the oil yield, reduce the extraction time and the environmental impact (Kapoore, Butler, Pandhal & Vaidyanathan, 2018). MAE and UAE using environment-friendly solvents represent potential green solutions for extracting n-3 rich oils from fish and algae.

A new generation of non-conventional solvents called natural deep eutectic solvents (NADES) has emerged in recent years. The most commonly studied NADES are based on choline chloride (ChCl), carboxylic acids, and other hydrogen-bond donors, e.g., urea, citric acid, succinic acid and glycerol. NADES have similar characteristics to ionic liquids but they are cheaper to produce (lower cost of raw materials), less toxic, and often biodegradable. For extracting oil from *Phaeodactylum tricornutum* (a diatom), the combination of microwave heating as a pretreatment and extraction with deep eutectic solvents resulted in total fatty acid yields and profiles (EPA, other

PUFAs, other FA and other lipids) comparable to the traditional Bligh and Dyer solvent extraction. The best results were achieved by using a NADES formed with ChCl and oxalic acid combined with MW, followed by extraction with dimethyl carbonate (DMC) as an environmentally friendly solvent. (Tommasi et al., 2017).

Based on the existing reports, UAE is a more advantageous technique than MAE, both in terms of quality of the crude oil obtained and the investment costs in instrumentation. In addition, UAE has already proven potential for scaling-up in the extraction of natural products using reactors up to 1000 L coupled with pump systems in order to fill the ultrasonic bath, to stir the mixture, and to empty the system at the end of the procedure (Chemat et al., 2017).

2.2. Enzymatic extraction

Enzyme-aided extraction of fish oil is carried out under mild temperature conditions by employing proteases together with an appropriate water/fish ratio to maximize the extraction efficiency. Senphan & Benjakul (2015) compared organic solvent extraction to wet reduction, commercial Alcalase, and powdered crude protease extract (CPE) from hepatopancrease of Pacific white shrimp yielding very similar results in terms of oil yield and FA composition. Hence, the efficiency of the enzymatic extraction was comparable to the solvent extraction resulting in an oil recovery of 95% of the total lipids. Enzyme-assisted extraction using Alcalase has also been reported to give better results for oil extraction from tuna by-products when compared to solvent and wet reduction methods by giving a crude with a higher percentage of PUFAs as well as lower degree of oxidation compared to the conventional methods of organic solvent extraction and wet reduction (de Oliveira et al., 2017). Wet reduction using low temperature (15 ºC instead of 95 ºC) did not differ from the enzymatic treatment in terms of n-3 PUFA content, PV and AV; whereas conventional wet process with high-temperature cooking resulted in higher extent of oxidation of PUFAs. Heating,

205 microwave (MW) and ultrasound (US) have also been studied as pretreatment steps before the
206 enzymatic extraction on *Labeo rohita* head, concluding that MW- and US-pretreatment improved
207 the oil yield from 55.9 to 69.8 and 68.1%, respectively (Bruno, Kudre & Bhaskar, 2019). Moreover,
208 US enhanced the content of PUFAs, whereas MW reduced the stability of the oil by significantly
209 increasing PV and AV. The same authors studied the structure of the fish mass after MW, US, and
210 heat pretreatment by scanning electron microscopy (SEM), observing that the MW- and US-
211 pretreated samples had more destroyed cells compared to the control samples. This caused an
212 increase in the porosity of the matrix, thus facilitating the hydrolysis of proteins and consequent
213 release of the intracellular lipids. Moreover, they observed that US was more efficient than MW due
214 to the thermal unfolding and further aggregation of proteins resulting from the heat released in the
215 MW-assisted procedure, which disturbs the protein hydrolysis.

216 For microalgae, a combination of different types of enzymes (cellulase, proteinase, lysozyme,
217 pectinase), acting on different components of cell walls, have shown to be most efficient pre-
218 treatments improving the extraction yield of oils (Xue et al., 2018). Ultrasound in combination with
219 enzymatic treatment has also been applied in extraction of oil from microalgae. For *Chlorella*
220 *vulgaris*, five different enzymes were investigated resulting in lipid recoveries ranging from 10%
221 using Neutrase and Protease to more than 35% using Snailase and Trypsin. The highest lipid yield
222 was achieved with a combined sonication-enzyme treatment at pH 4, which recovered 49.8% of the
223 lipids present in the microalgae. Due to the diversity of algal types, selection of the most proper
224 enzyme together with optimization of the processing conditions are of special importance in order
225 to maximize the yield with optimal quality (Liang, Zhang & Cong, 2012). In a similar work,
226 Zuorro, Miglietta, Familiari & Lavecchia (2016) optimized the lipid recovery from
227 *Nannochloropsis* resulting in oil yields around 35% using Feedlyve ALPHAGAL and Feedlyve
228 GMA, as endo-galactanase and endo-mannase, respectively.

Enzymatic treatment is a promising valorization method for a simultaneous extraction of oil, protein hydrolysate and bioactives from fish, fish by-products and algae (Araujo, Sica, Costa & Márquez, 2020). The utilization of fish discards is currently of special interest due to the circular economy approach and the Landing obligation (LO) by The European Common Fisheries Policy (CFP), which requires proper management of fish bycatch. However, the industrial processing may not be as efficient as at lab scale in the separation of the valuable oils. In their study, Vázquez et al. (2020) showed that the oil recovery in the industrial pilot plant was significantly lower than at lab scale due to the oils forming an emulsion with the hydrolysed proteins. The tricanter used instead of a centrifuge did not separate the oil phase due to a lower speed. The optimization of the process is essential to obtain the optimal yield and quality of the n-3 rich oils from specific types of raw materials, such as different fish and algae species and by products. In a study reported by Carvajal et al. (2015), an enzyme-assisted processing resulted in increased lipid oxidation compared to thermal treatment, demonstrating the importance of optimization of the process. The additions of antioxidants prior and during the processing, or running the process under anoxic conditions are important measures to decrease the level of oxidation. Despite the higher costs, enzyme treatments are increasingly used in industrial scale to separate fish oil from fish muscle. The challenge remains in the changes in structure and quality of proteins after the enzymatic treatment, which needs to be resolved by future innovations in order to obtained value added products from both the protein and oil fractions.

2.3. Supercritical and subcritical fluid extractions

In a supercritical state, with the pressure and temperature above the critical point, the solvent becomes a supercritical fluid achieving a density similar to a liquid and a viscosity similar to a gas. These properties give advantages such as better transporting properties, efficient diffusion and faster extraction. Moreover, the properties of the fluid can be modified to optimize its performance,

and the solvents used are compounds such as CO_2 generally recognized as safe (Herrero, Cifuentes & Ibañez, 2006). Supercritical fluid extraction (SFE) is used in industrial scale to produce high value berry seed oils and natural plant extracts with high contents of PUFAs and natural antioxidants (Tarvainen, Nuora, Quirin, Kallio & Yang, 2014; Yang et al., 2011). SFE using CO_2 alone or with co-solvents has been studied by several research groups to extract oil from fish products. SFE-CO_2 extraction is especially suitable for the extraction of nonpolar lipids such as TAGs, but the method is restricted to dry biomass. Thus, the raw material, such as fish, is commonly freeze-dried prior the extraction, which consumes time and energy. Pressurized liquid extraction (PLE) is a promising alternative for the SFE, and it can also be used for wet biomass, such as fish and algae. The process utilizes the subcritical state of the solvent, which remains liquid even at temperatures above the boiling point. The method can be easily modified by choosing different solvents according to their polarities; the commonly used solvents include ethanol, acetone and ethyl acetate. (Derwenskus et al., 2019).

Sahena et al. (2010) compared the performance of a Soxhlet system with various processes of SFE-CO_2, such as continuous, ethanol as co-solvent, soaking, and pressure swing techniques. The Soxhlet and SFE-CO_2 extractions resulted in a similar oil yield and composition of PUFAs with the only exception of the continuous SFE-CO_2, which resulted in the lowest values. In addition, the amount of CO_2 used for each extraction was the lowest for the pressure swing method, reducing it to half compared to the continuous system. The reason for the low consumption was long holding times, where the sample was incubated with CO_2, and thus, no CO_2 was consumed in these steps. In another study, the Soxhlet and SFE methods were applied for oil extraction from different parts of trout (head, spines and viscera). There were little variation in yield and fatty acid composition of oil between the extraction methods but a higher variability between the different parts of the fish as raw material; the oil yield (calculated as oil/fish dry weight) was around 40% for the head and spine, and 70-79% for the viscera (Fiori et al., 2012). Similarly, FA profiles found in fillets and

viscera of carp fish extracted by the SFE and Soxhlet methods were more dependent on the fish material rather than the extraction technique, reinforcing that SFE is highly efficient in extracting fish oil from fish side streams (Kuvendziev, Lisichkov, Zeković, Marinkovski & Musliu, 2018). In addition, Hao et al. (2015) compared SFE, wet reduction and enzymatic extraction of sturgeon oil in terms of oil composition and storage stability. Higher extraction yields (considering 100% for organic solvent extraction) were reported for protease and SFE: 83.6 and 97.3%, respectively, while wet reduction resulted in an extraction yield of only 52.5%. Moreover, the quality of the obtained crude oil with SFE was higher as measured by PUFA content, PV and AV.

Mendes, Reis & Palavra (2006) compared SFE-CO_2 and SFE-CO_2 using ethanol as a co-solvent (CO_2-ethanol) to Bligh and Dyer solvent extraction. The CO_2-ethanol resulted in a 40% lipid recovery of *Arthrospira maxima* biomass while CO_2 alone recovered only 32% of the lipids compared to the solvent extraction. Additionally, SFE-CO_2 with ethanol increased the recovery of γ-linolenic acid compared to pure CO_2, although both being significantly lower than achieved with the Bligh and Dyer extraction. In another study, CO_2 extraction recovered nearly 50% of the total lipids from *Crypthecodinium cohnii* biomass, of which 72% (w/w) of the total FAs were DHA (Couto et al., 2010). In contrast to other natural lipid sources, the extraction from microalgae requires higher pressures during the SFE process. The possible reason is that under low pressures, microalgae bind to the extracted lipids whereas at higher pressure the adsorption rate decreases (Sovová, Nobre & Palavra, 2016).

A recent study compared subcritical dimethyl ether extraction (SDEE) to SFE, enzymatic treatment and wet reduction in the extraction of oil from tuna liver. Dimethyl ether is a generally recognized as safe (GRAS) solvent for extraction purposes in the processing of foods. Furthermore, the low boiling point (-24.8 °C) allows it to evaporate freely from any food matrix, leaving practically no residues in the final product. The SDEE resulted in a very similar yield of PUFAs and oil to those

obtained by SFE, however, SDEE consumes less energy and time because freeze-drying of raw materials is not required, and the pressures employed are lower (Fang et al., 2019).

Pressurized liquid extraction (PLE), on the other hand, has shown over 75% w/w lipid yields from microalgae, including *Chlorella vulgaris* and *Phaeodactylum tricornutum* using a pressure of 103.4 bar and a temperature of 150°C. However, the adjustment of solvents is required for different species; medium-polar solvents such as ethyl acetate extracted lipids more efficiently from *C. vulgaris* biomass, whereas polar solvents like ethanol functioned better for *P. tricornutum.* These optimal solvents resulted in total FA yields of 85.9% for *P. tricornutum* (ethanol) and 76.5% w/w for *C. vulgaris* (ethyl acetate). *C. vulgaris* is rich in TAGs, and thus very polar or nonpolar solvents were inefficient for the extraction, the former due to poor solubility of TAGs, and the latter not being able to penetrate the water layer surrounding the cells. (Derwenskus et al., 2019). A recent study from He et al. (2019) reported that PLE with 90% aqueous ethanol resulted in a lipid extraction efficiency of 41.5 % (w/w) from *Isochrysis* sp. biomass. Furthermore, over 90% (w/w) of the extract were fatty acids, proving that PLE is an efficient method to extract lipids from *Isochrysis* biomass. In addition to fish and microalgae, PLE was also used to extract lipids from a brown macroalgae *Laminaria ochroleuca*. Among the solvents tested, ethanol:water (1:1) was the most efficient in extracting oil from *L. ochroleuca*, while ethanol, ethyl acetate and hexane were less effective. The lipid recovery was 52% using ethanol:water and extraction temperature of 160 °C, while a lower temperature of 80 °C resulted in extraction yield of 37.5% (w/w). However, both ethanol and ethyl acetate enriched more unsaturated FAs in the oil compared to the two other solvents. The ratio of n-6 to n-3 FAs was also assessed concluding that ethanol and ethyl acetate resulted in the lowest values (Otero, López-Martínez & García-Risco, 2019).

2.4.Fermentation

Fermentation occurs naturally under anaerobic conditions due to microbial activity. Fish silage technology utilizes either acid treatment (organic acid, such as formic acid) or fermentation with lactic acid bacteria to break down the fish material. Low pH produces suitable conditions for the enzymes to break down fish proteins into smaller soluble units, and the acid helps to speed up their activity while preventing bacterial spoilage (Olsen et al., 2014). Rai, Swapna, Bhaskar, Halami & Sachindra, (2010) studied the naturally present lactic acid bacteria (LAB) and added cultures (*Ent. faecium HAB01* and *Ped. acidilactici K7*) for fish ensilaging followed by Bligh and Dyer extraction method to assess the FA composition of the resulting crude oil. However, no advantages were reported over the natural fermentation in terms of oil yield and FA composition. Another study compared the natural silage process to acid silage employing formic acid (3%, v/w), and fermentation with several LAB strains *(Lb. plantarum, Pd. acidilactici, Ent. gallinarum, Lb. brevis* and *Streptococcus spp.*) supplemented with an antioxidant (BHT), a fungicide (potassium sorbate) and 15% molasses to aid in the fermentation process. The results showed lower PV and AV values in all of the added LAB fermented samples while the percentage of PUFAs did not significantly differ from the natural and acid fermentation processes (Özyurt, Özkütük, Uçar, Durmuş & Ozogul, 2019). Additionally, some LAB produce natural antioxidants which prevent the oxidation of fatty acids, and the fermentation makes the proteins more digestible than those from acid silage (Vidotti, Carneiro & Viegas, 2002). In general, fish silage requires low investment, energy and labor costs which makes it a promising technique for industrial-scale fish oil processing.

Among the extraction techniques discussed above, enzymatic extraction and especially fermentation require lower investment and energy costs making them more attractive in an industrial context. On the other hand, MAE and SFE require costly and specific instrumentation but produce high-quality oil. Thus, MAE and SFE should be regarded as technique, which needs more development in the extraction plant to make them more economically feasible. Given their proven potential to extract

high quality oil, they are probably be more suitable for the extraction of microalgae in biorefineries as they can be produced in high yield and processed *in situ*.

A number of studies have investigated various green technologies for extraction of oil from different types of fish materials and algae. Overall, the characterization of the obtained crude has largely based on the extraction yield, oxidation parameters (PV, AV) and FA composition. The lipid class composition of the resulting crudes has seldom been investigated. Distribution of lipid classes in the crude is important information guiding the optimization of oil extraction and purification processes since phospholipids have to be removed in the degumming step and FFAs in the deacidification step.

3. Technologies for refining crude oil

The crude oil obtained by any of the above detailed techniques does not yet fulfill the requirements for human consumption and further technological processing due to the presence of co-extractives, such as phospholipids (PLs), free fatty acids (FFAs), pigments. Therefore, several steps are necessary to upgrade the quality of the crude oil. These steps commonly include: 1) degumming to eliminate the phospholipids, 2) deacidification to decrease the acidity of the oil by eliminating the FFAs, 3) bleaching to remove pigments and other contaminants, and 4) deodorization to remove volatile compounds. Changes in the FA composition and physicochemical parameters through the refining process have been studied for oils extracted from different fish species including tuna and anchovies (de Oliveira, Minozzo, Licodiedoff & Waszczynskyj, 2016; Song, Dai, Shen, Peng & Zhang, 2018a), nile tilapia and hybrid sorubim (Menegazzo, Petenuci & Fonseca, 2014), sardine (Soldo et al., 2019) as well as fish by-products such as carp viscera (Crexi, Monte, Soares & Pinto, 2010). However, all the available reports rely on the conventional refining process, which includes degumming by using 1% of phosphoric acid, deacidification by neutralization with 1M NaOH followed by centrifugation, washing with hot water and drying, and bleaching with a combination

of adsorbents and deodorization by steam distillation under vacuum. In the following sections, we focus on reviewing research related to each of the above-mentioned refining steps, in which green alternatives are applied and compared with conventional methodologies.

3.1. Degumming

Degumming is the first step in the refining of the extracted crude oil. Table 2 shows the different techniques applied to remove the phospholipids (PLs) in fish oil and additional strategies that have been successfully assayed in vegetable oils. It is important to reduce the content of phospholipids in the oil because phospholipids tend to hydrolyze more easily than TAGs, generating free fatty acids and other reaction products that compromise the stability of the oil. Water degumming is the first stage of the refining process, which eliminates the hydratable fraction of PLs, i.e. phospholipids with polar moieties like hydroxyl or amino groups. In contrast, acid degumming is used for non-hydratable phospholipids, which consists primarily of phosphatidic acid having two free hydroxyl groups with high affinity for calcium and magnesium to form neutral, stable, and non-hydratable salts. Hence, the aim is to remove the phosphatidic acid yielding non-dissociated phosphatidic acid and the corresponding salts.

Although the acid degumming leads to significant loss in acylglycerols, it is still the methodology globally used and accepted due to the low cost of the chemicals used, profitable disposability of gums, and acceptable quality of oil. However, the effectiveness of the acid treatment is also dependent on the acid employed. Chakraborty & Joseph (2015) determined that the use of phosphoric acid resulted in a better quality of the oil, i.e. lower PV and AV, but lower oil yield of 86% compared to use of acetic, oxalic, or citric acids, which resulted in yield ranging from 90 to 93%. Acid and water degumming treatments have also been employed on mixed algal oil from *Chlorella* species (Paisan, Chetpattananondh & Chongkhong, 2017). The most abundant phospholipids in mixed algal oil are non-hydratable phospholipids, thus, acid degumming with

phosphoric acid resulted in a greater phospholipid reduction compared to the water degumming. The best removal up to 83% of total PLs was achieved with the following degumming conditions: 90 °C, 60 min and phosphoric acid 0.42 wt%. In contrast, the water degumming resulted in only removal of 19% of the phospholipids.

Although there are no reports comparing different degumming techniques for fish oil refining, studies comparing green alternatives have already emerged for the degumming of vegetable oils. Hence, they should be considered and assessed as possible alternatives to current conventional process applied in the fish oil degumming. Enzymatic degumming with commercial phospholipases has been examined for refining oils from corn, rapeseed and soybean. Phospholipases are a class of hydrolytic enzymes with the capacity to hydrolyze the ester bonds of phospholipids. Most commonly used phospholipases are phospholipase A_1 (PLA_1) and phospholipase A_2 (PLA_2) which catalyze the hydrolysis of fatty acids exclusively from the *sn*-1 and *sn*-2 positions, respectively. Instead, phospholipase C (PLC) is a phosphodiesterase catalyzing the cleavage of phosphatidylinositol, whereas phospholipase D (PLD) hydrolyzes the *sn*-3 phosphodiester bond of mostly phosphatidylcholine (PC) to generate a choline molecule and glycerophosphatidic acid (Richmond & Smith, 2011). Turetkan, Tasdelen-Yucedag, Ustun & Tuter, (2018) compared acid hydrolysis with citric acid to two different enzyme-based approaches, namely Enzymax process, an industrial procedure by Lurgi and Röhm GmbH, as well as a direct enzymatic process with a commercial phospholipase PLA_1 in degumming of crude corn oil. Both enzymatic methods resulted in a 10-fold enhanced performance in reducing the phosphorous content of the oil to levels of 5.7 and 6.2 ppm for the Enzymax and direct enzymatic degumming, respectively, in comparison with 54.9 ppm for the acid treatment. In another study, immobilized PLA_1 showed a reduction in the phosphorus content of crude soybean oil from 63 ppm using the non-immobilized phospholipase to 10 and 7 ppm obtained with the bio-imprinted PLA_1 (bi-PLA_1) and the immobilized bio-imprinted PLA_1 (im-bi-PLA_1), respectively (Li et al., 2016). Phospholipases have also been applied after

chemical degumming to enhance the efficiency of the process. In soybean oil, PLA_1 treatment after chemical degumming enhanced the reduction of phosphorus content from 32 to 0.7 ppm in the oil (Sampaio et al., 2015). Similarly, the use of PLC reduced the phosphorus content of corn oil to less than half compared to water degumming (Sampaio et al., 2019). Ultrasonication prior to enzymatic hydrolysis of phospholipids by PLA was found to have a positive effect on the degumming of crude soybean oil, reducing the phosphorous content by additional 4% compared to the reduction of 94% achieved by the enzymatic hydrolysis alone. Moreover, the physicochemical parameters of oil were also improved by US-treatment resulting in PV, AV, and acid value of 0.3 $mEq \cdot kg^{-1}$, 0.7 $mg \cdot g^{-1}$, and 0.7%, respectively, in comparison to 0.3 $mEq \cdot kg^{-1}$, 0.8 $mg \cdot g^{-1}$ and 1.3% in the oil obtained with the enzymatic treatment alone (More & Gogate, 2018). The optimization of the ultrasound parameters, such as temperature and power intensity together with pH and water addition for an optimal performance of the enzymes may further enhance the efficiency of the degumming process, not only in terms of the quality of the oil obtained, but also in terms of the reaction time.

Soft degumming by using a chelating agent, disodium ethylenediaminetetraacetate (EDTA) together with an emulsifying agent (sodium dodecyl sulfate, SDS) has been reported to improve the efficiency for reduction of phosphorous content of crude rapeseed oil. Optimization of the process by experimental design resulted in an additional reduction of phosphorous from 268 to 74 ppm (Szydłowska-Czerniak & Łaszewska, 2017). Moreover, the technique is cheaper than enzymatic treatment and easy to scale-up. The main drawback of this process was the high stability of the emulsion formed during the process making the separation difficult. To solve this problem Crystallization & Degumming patented a procedure without SDS, obtaining similar elimination of phospholipids as in the original method but the separation of the oil phase from water became much easier, thus reducing oil loss. Moreover, this process was scaled up to process 500 t oil/day for soybean and rapeseed oil (Deffense, 2009).

Lastly, membrane technology has also been widely investigated as a new approach to eliminate the phospholipid fraction of oils using different membranes resulting in phosphorus reduction by 85.8–92.8% in soybean oil (Subramanian et al., 1999). In combination with pretreatments of the oil with acid or alkali PL were eliminated almost completely from soya, sunflower and rapeseed crude oils (Hafidi, Pioch & Ajana, 2005). Membrane degumming is a green technology with high potential due to low consumption of energy and chemicals and low environmental impact (Chunduri, Rao, Balasubrahmanyam, & Bhowmick, 2006).

3.2.Deacidification

Deacidification removes the free fatty acids (FFAs), which are present at concentrations of 5-20% in the oils after the degumming step (Vaisali, Charanyaa, Belur & Regupathi, 2015). Table 3 shows the main alternatives for the reduction of the FFA content in fish oil. The conventional procedure for the deacidification includes the addition of NaOH to neutralize the acids, followed by precipitation of the FFAs as soap, which are then removed by centrifugation or washing. However, significant oil loss occurs during the soap formation due to alkali hydrolysis of TAGs, also known as saponification. Non-traditional neutralizing agents, such as Na_2CO_3 and $NaHCO_3$, have also been tested in vegetable oils although NaOH was proven the most efficient yielding 0.4% FFAs *versus* 0.7 and 0.8% in oils processed with Na_2CO_3 and $NaHCO_3$, respectively (De & Patel, 2010).

The main alternative to the conventional deacidification method used in fish oil processing is enzymatic esterification with ethanol or glycerol using a commercial lipase. Wang et al. (2012) reported a reduction of *p*-anisidine value and acid value from 44.4 and 10.2 to 26.4 $mg.g^{-1}$ and 0.4 mg $KOH \cdot g^{-1}$ as well as an increase in PUFA content from 32 to 82% compared to the original crude oil. Thus, in addition to removing the FFAs, enrichment of PUFAs was achieved. The same type of enzymes were applied in several studies to enrich the PUFAs in the oil at the end of the refining process by performing a selective enzymatic glycerolysis followed by molecular distillation

(Solaesa, Sanz, Falkeborg, Beltrán & Guo, 2016) or ethanolysis to achieve MAGs with a high content of PUFAs (He et al., 2017) Although in most of the commercial formulations EPA and DHA are in the form of ethyl esters, acylglycerols are the preferred forms because they confer better bioavailability and stability of EPA and DHA (Wang et al., 2012).

Charanyaa, Belur & Regupathi (2017) studied solvent extraction with methanol and membrane assisted pre-extraction combined with extraction with methanol for the deacidification of degummed fish oil. Initially, four short-chain alcohols methanol, ethanol, propanol and butanol were studied as solvents. The results showed that methanol was the most efficient by reducing the FFA content from 5.6 to 2.3%. However, the authors reported a high oil loss of 30% in the solvent extraction, whereas the MASE resulted in oil loss of 7%. In addition, the residual contents of methanol in the oil after extraction were 1% in the solvent extracted oil and 0.5% in the MASE oil. The high oil loss in the solvent extraction was probably due to the formation of stable oil-methanol micelles during the extraction. The membrane deacidification, on the other hand, occurs at 3 bars, resulting in a better separation of oil from the oil-methanol micelles. Further, the nonpolar PTEE membrane repels these micelles, leading to a reduced amount of methanol in the permeate. For green processing, the process should be optimized using ethanol to replace methanol. The use of NADES to replace traditional solvents in fish oil deacidification has not been reported yet; however, several betaine monohydrate-based NADES were studied to reduce the acidity of palm oil, obtaining a 49.4% acid reduction of palmitic acid while keeping the content of antioxidants stable (Zahrina, Nasikin, Krisanti & Mulia, 2018).

Based on the research reported on various green alternatives for conventional deacidification, esterification of the FFAs with lipases shows the highest capabilities to replace the use of alkali neutralization; however, to our best knowledge, only FA composition and oxidation status of the oils has been studied to evaluate the impact on oil quality, but no research has been carried out to

assess the oil loss. Hence, this alternative technology should be further investigated in order to fully assess its true potential.

3.3. Bleaching

Bleaching aims to remove several types of impurities, such as pigments, lipid oxidation products, and remains of phospholipids and soaps to further improve the quality and stability of the oil. Moreover, high contents of persistent organic pollutants (POPs) can be found in some fish oil products due to bioaccumulation in the fat tissue of fish in polluted marine areas and enrichment of these compounds during the oil extraction process (Rawn et al., 2009). Previously, bleaching studies (Table 4) on fish oil have pointed mainly towards two different goals. The first one aims to improve the quality of fish oil by improving its physicochemical properties such as removal of colorants and lipid oxidation products. The second is focused on the reduction of POPs such as flame retardants, dioxins and polychlorinated biphenyls (PCBs) which are highly persistent and fat-soluble environmental pollutants that bioaccumulate in the food chain. Considerable levels of POPs have been detected in some of the most important fish species in the Baltic sea such as sprat (*Sprattus sprattus*) and herring (*Clupea harengus*) (Antelo, Lopes, Franco-Uría & Alonso, 2012) and in fish oil produced from Sprat caught in the North Sea (Oterhals, Solvang, Nortvedt & Berntssen, 2007). Effective measures are necessary to remove POPs from oil in order to make these oils safe for human consumption or use as ingredient of feed.

Currently, the main methodology used for the bleaching of fish oil is the treatment with a solid adsorbent. Optimization of the process in terms of temperature, amount of adsorbent and contact time has resulted in effective reduction of oxidation products as shown in reduction of PV and AV (García-Moreno, Guadix, Gómez-Robledo, Melgosa & Guadix, 2013). Monte, Monte, Pohndorf, Crexi & Pinto (2015) carried out a similar study, where the processes using bleaching earth or activated carbon (AC) were optimized, yielding oil with a reduced content of lipid oxidation

products and an improved color. Another study compared different solid adsorbents in sardine oil with remarkable results obtained when using a combination of AC and Fuller's earth (FE). The refined oil had an enhanced PUFA content (from 25.6 to 26.6%) in addition to a reduced content of lipid oxidation products and an improved color (Chakraborty & Joseph, 2015). This combination of adsorbents was especially efficient in reducing the color-related compounds, whereas the values of oxidation parameters were not noticeably better than the ones obtained with the other adsorbents. Oterhals et al. (2007) studied the effects of alkali bleaching (AB) and the combination of alkali and active charcoal (AC) for the elimination of polychlorinated dibenzo-*p*-dioxins, dibenzofurans (PCDD/F) and PCBs from fish oil. The procedure combining AB and AC bleaching proved to be very effective in the reduction of PCDDs and PCDFs, showing a reduction rate of 99%. However, it was less effective in reducing the PCB content, probably due to the planar molecular conformation of the AC, which inevitably limits its applicability. The non-*ortho* PCB was reduced by 87% and the mono-*ortho* PCB by 21%. In addition, the authors did not observe any negative effect on the oil quality after bleaching in terms of oxidation. Similarly, Ortiz et al. (2011) studied 11 silicon- and 9 carbon-based adsorbents. The carbon-based adsorbents lead to reductions of 99, 70, and 27% of PCDDs, hexachlorobenzene (HCB), and PCBs, respectively, while treatment with the silicon-based adsorbents did not result in significant eliminations of POPs.

Ultrasound assisted bleaching (UAB) has been studied in canola oil (Icyer & Durak, 2018), but not yet in fish oil. The study compared a conventional bleaching method with a process using acid-activated bleaching earth assisted with ultrasonication. The conventional bleaching relies on mixing bleaching earth with the oil while stirring and heating under partial vacuum (70 mmHg). No remarkable differences were reported between the two methods in the final composition of the oil, except a higher reduction of yellow color in the UAB treated oil. However, the US-treatment accelerated the process resulting in 50% reduction in the contact time. Also the same bleaching

efficiency was obtained with a 25% reduction of processing temperature when compared to the non-UAB method using the same bleaching earth.

Short-path distillation (SPD) is a technique that employs short residence times and high vacuum levels (Antelo et al., 2012). The SPD technique was compared to alkali bleaching to refine sprat oil resulting in lower PV, AV, total oxidation value (TOTOX); but the reduction of the initial POP content (76%) and the loss of vitamins (20%) were similar to what observed for alkali bleaching (Oterhals & Berntssen, 2010). On the other hand, Oliveira & Miller (2014) assessed SPD in regards to the oil quality since SPD bleaching requires higher temperatures (around 200 ºC) compared to the adsorption bleaching using temperatures below 90 ºC. High temperature might lead to the degradation of the beneficial PUFAs. The authors concluded that SPD was useful in the reduction of the oxidation products and FFAs, while keeping the fatty acid profile unaltered. However, SPD involves high operating costs which have prevented the broad use of this technology by the industry to this date.

SFE with CO_2 has also been studied as an alternative bleaching technique. As CO_2 is a non-toxic and non-polar compound, it takes advantage of the rather non-polar molecular structures of many POPs to remove them efficiently from the oil. Kawashima, Watanabe, Iwakiri & Honda (2009) examined the reduction of several classes of POPs in detail, obtaining promising results especially in the reduction of PCDF and PCB contents by 84% and 93%, respectively. However, an additional step with AC adsorption was required to enhance the efficiency of the whole procedure to reduce the PCDD content by 80% compared to 35% achieved with SFE-CO_2 alone.

In conclusion, the conventional bleaching processes using solid adsorbents achieves good results and is cost-efficient for industrial applications. Additionally, the used bleaching material can be re-used as a bioorganic fertilizer (Loh et al., 2013). Hence, at this stage special attention should be

paid to the optimization of the process in terms of oil/adsorbent ratio and bleaching temperature to maximize the removal of unwanted compounds while maintaining a high content of PUFAs.

3.4. Deodorization

Deodorization is the last step in the fish oil refining which aims to remove undesirable odorous compounds. Processes studied in fish oil deodorization are shown in Table 5. Currently, steam distillation is the most commonly used process in which undesirable odorous components, mostly aldehydes and ketones (lipid oxidation products), and residual FFAs are removed (Vaisali et al., 2015). However, the use of high temperatures (180-270 ºC) can induce several chemical reactions, such as oxidation, isomerization and polymerization of lipid molecules. Additionally, high temperature and the simultaneous presence of a chloride ion source together with glycerol derivatives may favor the formation of 2-monochloropropane-1,3-diol (2-MCPD), 3-monochloropropane-1,2-diol (3-MCPD), and glycidyl esters (Zelinková, Svejkovská, Velíšek & Doležal, 2006). These compounds have been reported as possible human carcinogens (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2013), hence, deodorization should be conducted at temperatures not higher than 180 ºC (Fournier et al., 2006). Alternative deodorization strategies based on milder conditions have been a subject of research in the field of fish oil refining.

With the aim to reduce the extent of side reactions and degradation of PUFAs, Chung & Lee (2009) studied six different types of zeolites, which resulted in a reduction of 20-60% in the content of volatiles. In a recent study, Song et al. (2018b) compared the performance of conventional steam distillation with several alternative green processes, including short-path distillation (SPD), treatment with green tea polyphenol (GTP), which is a natural polymer consisting of six catechins with significant antioxidant and chelating properties (Heim, Tagliaferro & Bobilya, 2002), adsorbent-based processes such as activated clay, zeolites, and diatomite, as well as liquid-liquid

589 (L-L) extraction with alkaline ethanol. Among the methods evaluated, the most efficient reduction
590 of the volatiles was achieved with SPD, in which high vacuum was applied (around 10^{-4} mm Hg).
591 SPD reduced the content of volatile compounds in the oil to 12% of the initial level, whereas L-L
592 extraction resulted in a volatile content of 73% of the original level. The other deodorization
593 methods resulted in volatile contents of 79-98% of the level before the deodorization. Furthermore,
594 the PV and FA compositions were not largely altered by any of the aforementioned processes. The
595 PV ranged between 1.9 and 3.7 mEq $O_2 \cdot kg^{-1}$ and the percentage of PUFAs were 38% in the oils
596 after the GTP treatment and L-L extraction in comparison with 34% in the crude oil. The alkaline
597 ethanol-based L-L extraction used low temperatures, which might have reduced peroxidation and
598 degradation of the lipids, as well as the geometrical isomerization of the naturally present *cis*-
599 double bond in the lipid molecules. Other advantages of the L-L extraction were high repeatability,
600 low cost, and a simplified procedure.

601 Nanofiltration with membranes under mild operating conditions has also been studied as a low-
602 temperature alternative to steam distillation in order to minimize the thermal degradation of lipids.
603 Fang et al. (2018) used membranes with a cut-off molecular weight of 360 Da, 20 bar, and room
604 temperature in an experimental set-up to compare the performance of nanofiltration with that of
605 steam distillation in refining of tuna and squid oil. Compared to the steam distillation, a 5- to 6-fold
606 reduction in the volatile content was achieved by using nanofiltration. Additionally, the PUFA
607 content increased from 2 to 4%, improving the fatty acid profile of the oil.

608 To substitute high-temperature steam deodorization, the alternatives with a higher potential seem to
609 be techniques operating at room temperature like alkaline liquid-liquid extraction with ethanol and
610 nanofiltration, the former being less costly, while the latter giving better results in reducing the
611 volatiles but requiring high investment costs for specific installations.

4. EPA and DHA enrichment

Although the refined oil is suitable for human consumption in terms of purity, color and odor, the fish oil TAGs still contain high proportions of SFAs and MUFAs. The total content of n-3 PUFAs is typically around 20-30% of the total fatty acids in the refined oil, largely depending on the initial fish material (especially fish species), the extraction method, and the refining process applied. For this reason, enrichment of n-3 PUFAs, especially EPA and DHA, have also been an active field of research in order to obtain PUFA-concentrated products with higher added value. Most commonly used methodologies for enriching PUFAs in fish oil are urea complexation, low temperature crystallization, and enzymatic purification, although other techniques, such as liquid and supercritical fluid chromatography and supercritical fluid extraction, for concentrating n-3 PUFAs have been described as summarized in a recent review (Bonilla-Mendez & Hoyos-Concha, 2018). In addition, studies using pressurized liquids have also emerged. Urea complexation relies on the ability of SFAs and MUFAs to form complexes with urea, while PUFAs remains in the non-urea-complexed fraction, which can easily be separated by filtration. This technique has been reported to be highly efficient in the removal of SFAs, but with limited efficiency in the reduction of MUFAs (C16:1, C18:1, and C20:1) (Zheng, Dai & Shen, 2018). Urea complexation has also shown great results in enriching PUFAs of algal oil, where the concentration of the percentage of DHA was increased from 47% in the original algal oil to 97% after enrichment. Unfortunately, urea complexation does not totally fulfill the criteria of green extraction methods as urea-complexed FAs are discarded (Senanayake & Shahidi, 2000).

Molecular distillation, on the other hand, has been applied in a two-step process to increase the PUFA content of oil after the urea complexion, resulting in 2-fold increases in the contents of EPA and DHA (Magallanes, Tarditto, Grosso, Pramparo & Gayol, 2019). However, the use of urea for this purpose should be avoided due to the formation of ethyl carbamate, a human and animal

carcinogen, during the process (Canas & Yurawecz, 1999). In addition to extraction, pressurized liquids can also be used for the fractionation of different lipids, such as n-3 acylglycerols and glycolipids. In a recent study, Castejón & Señoráns (2019) extracted and enriched oil from algae species *Nannochloropsis gaditana* reaching an EPA concentration of up to 53% using PLE with hexane. In comparison, the PLE with ethanol resulted in EPA concentration of 36% of the total FAs. PLE with hexane resulted in an enriched fraction of TAGs whereas ethanol resulted in a fraction more concentrated with glycolipids and MAGs. The technique is based on different polarities of different lipid classes, which enable the fractionation of the oil into MAGs, diacylglycerols (DAGs), TAGs, FFAs and glycolipids.

SFAs can be crystallized from a solution in an organic solvent by low-temperature crystallization utilizing the high melting point of SFAs. The crystals can then be removed by filtration, and after that, the solvent is evaporated yielding FFA fractions with higher content of n-3 PUFAs (Morales-Medina, De León, Munio, Guadix & Guadix, 2016). However, crystallization requires organic solvents like hexane to solubilize the mixture, thus it should be avoided when possible. Urea complexation and low-temperature crystallization have some limitations because they are methods only efficient for FFAs or fatty acid ethyl esters (FAEEs). Therefore, additional steps are required for the transformation of lipids to FFAs or FEEs before the process as well as re-esterification or trans-esterification with glycerol afterwards to yield the n-3 PUFA enriched TAGs. It has been demonstrated by several studies that acylglycerols provide better absorption and higher bioavailability of PUFAs than ethyl esters, making acylglycerols the preferred form of PUFAs as ingredients of food and dietary supplements (Neubronner et al., 2011; Olsen et al., 2014). Hence, most of the state-of-the art research is being pointed towards the use of enzyme-catalyzed hydrolysis of TAGs to obtain PUFA-containing MAGs using refined fish oil as the substrate to reduce the number of steps of re-esterification and trans-esterification during the enrichment process.

Enzymatic production of PUFA-enriched MAGs has been carried out by three main approaches: hydrolysis, glycerolysis, and ethanolysis. Hydrolysis takes advantage of the ability of lipases to catalyze the fast removal of SFAs and MUFAs from the glycerol backbone in the presence of water. Unfortunately, this is a reversible reaction that yields low conversion rates and requires a second round of hydrolysis followed by a SPD (Kahveci & Xu, 2011). Glycerolysis consists of a lipase, a hydrophobic oil phase and a hydrophilic glycerol phase. In addition, to overcome the poor miscibility between the oil and glycerol due to the difference in polarities, tertiary alcohols are needed which are sometimes toxic and need to be removed at the end of the process, thus increasing the length and cost of the whole process. As alternatives, the use of food grade surfactants, such as lecithin, have been studied (Feltes, de Oliveira, Block & Ninow, 2013). To further increase the concentration of PUFA-enriched MAGs, molecular distillation has been applied (Solaesa et al., 2016). Although hydrolysis and glycerolysis can be utilized for the enrichment of PUFAs in fish oil, their performance is not excellent. Hence, enzyme-catalyzed ethanolysis has recently attracted the greatest research attention as it enables an irreversible reaction by using ethanol both as a reactant and solvent (He, Li, Kodali, Chen & Guo, 2016; Rodrigues et al., 2015). Moreover, ethanol is a non-toxic, cheap, and environmentally friendly solvent.

The optimization of the process by selecting the most efficient lipase has been a matter of interest, and in this regard, lipase produced by fungi such as *Candida antarctica* and *Thermomyces lanuginosus* have been widely applied and genetically engineered to increase their performance. In addition, both immobilized and liquid forms of lipases have been studied. Immobilized lipases provide higher stability and reusability, but they are more expensive. Recently, a liquid lipase Candida Antarctica Lipase (CAL-A) and a lipase NS-40116 from a genetically modified *C. antarctica* have demonstrated a superior, almost ideal, performance for the enrichment of n-3 PUFAs *via* ethanolysis using fish oil or microalgae oil as starting materials (He et al., 2016). According to the research, the most suitable lipase to obtain PUFA-concentrated glycerides *via*

686 ethanolysis should fulfill the following criteria: 1) high fatty acid selectivity to hydrolyze saturated
687 and monounsaturated FAs without releasing PUFAs from the glycerol backbone; 2) non-
688 regiospecificity in order to hydrolyze fatty acids in both *sn*-1/3 and *sn*-2 positions; 3) the ability to
689 use ethanol in excess to favor the reaction. CAL-A is such a near-ideal lipase with almost all
690 required properties. He at al. (2017) elucidated the rationale behind the highly efficient
691 concentration of n-3 PUFAs into MAGs; and they verified their conceptual hypothesis by revealing
692 the catalytic mechanism through ^{13}C-NMR analysis of starting materials and isolated products
693 (Scheme 2).

694

695 **5. Conclusions and future prospects**

696 Globally the demand for high quality oils from fish and microalgae is rapidly growing to meet the
697 recommended intake of n-3 PUFAs, which have crucial biological activities and physiological
698 effects. Application of greener techniques in production of edible oil rich in n-3 PUFAs from
699 aquatic sources is an increasingly popular field of research aiming to improve quality of the oils and
700 to reduce the impact on the environment. While most research has focused on green strategies for
701 extracting crude oil from raw materials, less effort has been directed towards the oil-refining
702 processes. Various green extraction techniques have been evaluated in comparison with the
703 conventional wet reduction and solvent extraction, among which enzymatic-aided organic solvent-
704 free extraction and ultrasound-assisted extraction are most promising ones offering higher yield and
705 improved quality of oils from fish and microalgae. Pressurized extractions using supercritical and
706 subcritical fluid of CO_2 and other solvents have proven effective for extracting lipids from
707 microalgae. Although all these processes are easy to up-scale and most of them have been applied
708 in industrial scale for extraction of oils from different sources, the processes need to be carefully
709 optimized in order to achieve the optimal performance on specific type of raw materials of fish and
710 algae. Supercritical fluid extraction requires high investment in high pressure plant, which limits the

711 industrial use. In addition, the composition of different lipid classes in raw materials and the crude
712 extract needs to be taken into consideration when selecting oil extraction and refining. In future
713 research, more attention should be placed on composition of lipid classes (phospholipids, free fatty
714 acids and acylglycerols) of oils when assessing green extraction technologies due to the importance
715 of such composition on the quality, stability, as well as nutritional and technological properties of
716 the oil.

717 Lipase-catalysed ethanolysis is a promising technology for enrichment of n-3 PUFAs from marine
718 sources producing n-3 PUFA MAGs. The method is efficient, environmentally friendly and food
719 grade; hence, active research is being conducted to improve cost-efficiency by screening for
720 effective lipases with low positional specificity (*sn*-1, 2 or 3) and high preference for non-n-3 fatty
721 acids in TAGs.

722 In contrast to oil extraction and n-3 PUFA enrichment, the green technologies for refining oils
723 extracted from fish and algae have not been investigated extensively. Traditional degumming
724 removes phospholipids from fish oil with acids, such as citric acid. However, phospholipase- and
725 membrane-assisted degumming should be tested as already investigated in vegetable oils with
726 satisfactory results. For bleaching, several combinations of solid adsorbents are commonly used to
727 obtain oils with light color and reduced content of lipid oxidation products. However, the presence
728 of POPs in the oils needs to be monitored as they are highly persistent fat-soluble compounds that
729 bioaccumulate in the food chain and concentrate during the production of fish oil. The POP content
730 is especially high in oil extracted from marine fishes, whereas the contents are lower in farmed
731 fishes (Merkle et al., 2017). In contrast to marine fishes, microalgae have great potential for
732 cultivation in bioreactors and subsequent extraction of the oil in biorefineries, obtaining oil free
733 from exogenous pollutants. Finally, at the deodorization step, alkaline ethanol liquid-liquid
734 extraction should be further investigated as a possible gentle alternative to steam extraction and

short path distillation, due to the promising results in oil quality in comparison with the steam distillation. Moreover, it is a low cost and simple procedure with high efficiency. However, nanofiltration should be the technique of choice if investment costs can be accomplished, as it provides a more efficient reduction of volatile compounds.

In addition to oil quality and environmental impact, green processes for oil production should be part of integrated processing strategies promoting sustainable utilization of fish and algae bioresources, as demonstrated in a review published on oil production from microalgae (Xue et al., 2018). Refined fish oil and algal oils and n-PUFA concentrates consist of mostly acylglycerols, whereas other lipid classes such as phospholipids, carotenoids, and sterols present in the crude extracts are removed during the oil refining processes. More effort should be directed to recovering and valorizing these fractions. Currently a dominating fraction of fish oil is refined from crude oils produced as side streams of fish meal production. There is an increasing interest in valorizing proteins of the so-called industrial fishes into high quality food products and protein concentrates due to the rapid growth of the global market for non-meat proteins. Pretreatment and oil extraction have significant impact on structure, technological properties, as well as sensory and nutritional qualities of the non-lipid fractions such as proteins of fish and algae, which should be taken into consideration to enhance circular green strategies for processing these valuable bioresources.

CRediT author statement

Alexis Marsol-Vall: Investigation, Writing – Original draft preparation, Review and editing, Visualization; **Ella Aitta:** Investigation, Writing – Original draft preparation, Review and editing; **Zheng Guo:** Visualization, Writing - Reviewing and editing; **Baoru Yang**: Conceptualization, Supervision, Writing – Reviewing and editing, Project administration, Funding acquisition.

Declarations of interest

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Tables

Table 1: A summary of different methods for oil extraction from different aquatic sources.

	Sample	Extraction method	Oil yield (%)	SFA (%)	MUFA (%)	PUFA (%)	PV (mEq $O_2 \cdot kg^{-1}$)	AV ($mg \cdot g^{-1}$)	Iodine value	Reference
physical	6 Fish species muscle	Bligh and Dyer	1.3-6.1	388-1823[1]	205 - 2237[1]	323-646[1]				(Ozogul et al., 2018)
		Soxhlet	0.2-3.9	30-1197	16 - 1305	14-381				
		MAE	1.4-2.8	542-990	325- 536	184-524				
		UAE	1.6-5.1	492-1400	295 - 2222	243-583				
	Tilapia fillets	Folch	2.3	38.2	43.9	17.9	1.8			(Costa et al., 2017)
		MAE	2.3	38.2	43.9	17.9	0.2			
	Crypthecodinium cohnii	Soxhlet	4.8	47.5	0.2	49.9				(Cravotto et al., 2008)
		UAE	25.9	47.6	0.2	49.3				
		MAE	12.5							
enzymatic	Catfish muscle	Bligh and Dyer	25.2	48.8	34.2	10.6				(Senphan et al., 2015)
		10-CPE	23.5	48.8	35.2	10.4				
		10-Alcalase	24.3	48.9	35.8	10.2				
		wet reduction	24.3	48.2	35.7	10.3				
	Yellowfish tuna by-products	Bligh and Dyer		27.4	70.8	1.8	10.8		2.3	(de Oliveira et al., 2017)
		wet reduction		44.4	24.5	31.1	10.5		4	
		Alcalase		33.3	27.6	39.1	5.1		2	
	Salmon head	high-temperature	71.1	19.7	39.9	34.5	9.2	1.3		(Głowacz-Różyńska et al., 2016)
		low-temperature	71.5	19.6	42.5	32.7	2.5	0.2		
		Alcalase	72.1	20.7	40.1	33.6	1.6	0.7		
	Labeo rohita head	control	55.9	34.4	24.9	37.6	1363	5.6<AV <8.0		(Bruno et al., 2019)
		MW[2]	60.5-69.8	34.6	24.8	37.5	1873	8		
		US[2]	58.7-68.1	31.5	26.5	39.3	1323	5.6<AV <8.0		
		heating[2]	32.0-32.3	32.9	25.6	38.3	793	5.6		
	Chlorella vulgaris	US+ 5 types of enzymes	10-35							(Liang et al., 2012)
	Nannochloropsis	6 types of enzymes	15-35							(Zuorro et al., 2016)
SFE-CO_2 & PLE	Indian mackerel skin	Soxhlet	53.6	16.7	7.7	59.6				(Sahena et al., 2010)
		SFE-CO_2 continuous	24.7	18.2	7.7	56.3				
		SFE-CO_2 with co-solvent	53.2	16.2	7.5	59.7				
		SFE-CO_2 soaking	52.8	15.9	7.5	59.7				
		SFE-CO_2 pressure swing	52.3	16.4	7.4	60.5				

	Trout by-products	Soxhlet	41-70	24.3-27.9	72.1-75.7					(Fiori et al., 2012)
		SFE-CO_2	36-79	24.7-27.4	72.6-75.3					
	Carp by-products	Soxhlet	45-50	20.8	45.3	33.4				(Kuvendziev et al., 2018)
		SFE-CO_2	28-52	19.4	46.6	34.1				
	Arthrospira maxima	Bligh and Dyer	100	41	10	48				(Mendes et al., 2006)
		SFE-CO_2	40	13	27	60				
		SFE-CO_2+ethanol	32	16	17	62				
	C. cohnii	Bligh and Dyer	19.9	41.8	8.0	50.0				(Couto et al., 2010)
		SFE-CO_2	8.6	48.5	8.4	43.1				
	Isochrysi sp,	Folch	25.4							(He et al., 2019)
		PLE with *n*-hexane	34.4							
		PLE with 90% ethanol	41.5	8.4[5]	5.4[5]	10.3[5]				
SFE-CO_2 and enzymatic	Sturgeon muscle	wet reduction	52.54	25.3	44.2	30.7	4.2	3.6		(Hao et al., 2015)
		protease	83.64	25	43.8	31.9	3.2	3.3		
		amino method	38.74	24.9	44.3	31.8	3.1	4.5		
		SFE-CO_2	97.34	20.3	45.6	33.7	2.5	0.8		
	Tuna livers	SDEE	98.64	42.7	24.5	32.8	1.8			(Fang et al., 2018)
		wet reduction	56.84	47.4	23.3	29.3	1.6			
		protease	85.34	47.6	23	29.4	3.1			
		SFE-CO_2	98.54	42.9	24.3	32.8	3.9			
fermentation	Carp viscera	wet reduction	20	40.5	48.9					(Rai et al., 2010)
		natural fermentation	85	39.9	49.2				113.3	
		LAB 1	84	39.6	50.1				117.8	
		LAB 2	83	39.7	50				117.8	
	Giblel carp	natural fermentation		35.3	31.4	19				(Özyurt et al., 2019)
		Acid fermentation	82.2	35.7	30.2	18.3	4.5	17.8		
		LAB strains	75.9-87.4	35.8-36.5	30.7-31.7	17.7-18.4	1.1-<4.5	5.7-10.3		
	Kluzinger‘s ponyfish	natural fermentation		23.2	37.1	15.4				
		Acid fermentation	76.8	21.9	40	14.6	3.4	15		
		LAB strains	70.1-76.8	22.2-23.0	14.6	14.5-15.5	1.8-<3.4	5.7-10.3		

MAE, microwave assisted extraction; UAE, ultrasound assisted extraction; CPE, crude protease extract; MW, microwave; US, ultrasound; SDEE, subcritical dimethyl ether extraction; LAB, lactic acid bacteria; PLE, pressurized liquid extraction.

[1] mg/100g of fish.

[2] Pretreatment followed by enzymatic extraction.

[3] PV calculated with the ferric thiocyanate method.

[4] % of extraction considering Soxhlet 100%.

[5] wt% of biomass

Table 2: Degumming processes applied for phospholipid (PL) removal.

Sample	Degumming process	Lipid yield (%)	SFA (%)	MUFA (%)	PUFA (%)	PV (mEq $O_2 \cdot kg^{-1}$)	AV (mg $\cdot g^{-1}$)	acidity (%oleic acid)	Phosphorous content (ppm)	Reference
Sardine oil	crude	8.3	41.0	29.2	26.5	11.9	16.2	4.4		(Chakraborty et al., 2015)
	phosphoric acid	86.0	40.2	29.5	26.0	7.2	13.6	7.9		
	acetic acid	93.1	40.6	29.7	26.4	8.0	14.1	7.0		
	oxalic acid	90.1	40.8	29.5	26.3	7.6	14.7	6.0		
	citric acid	92.6	40.4	28.9	26.2	8.3	15.0	5.6		
Crude corn oil	crude								495	(Turetkan et al., 2018)
	citric acid								55	
	Enzymax process								6	
	direct enzyme								6	
Soybean oil	crude							0.8	875	(Sampaio et al., 2015)
	citric acid							0.3	32	
	citric acid + enzyme							0.8	1	
Corn oil	crude							2.3	951	(Sampaio et al., 2019)
	water								67	
	CP + enzyme								27	
	CC + enzyme								26	
Soybean oil	water								128	(Li et al. 2015)
	citric acid								35	
	PLA_1								63	
	Bi-PLA_1								10	
	Im-bi-PLA_1								7	
Soybean oil	crude oil					3.1	2.3	1.7		(More et al., 2018)
	enzymatic					0.3	0.8	1.3	reduce 94.1%	
	UA + enzymatic					0.3	0.7	0.7	reduce 98.4%	
Rapeseed oil	crude								5655	(Szydłowska-Czerniak et al., 2017)
	acid								268	
	EDTA								74	
5 vegetable oils	crude								43-141	(Hafidi et al., 2005)
	H_3PO_4+NaOH+membrane								1.5	

CP, caustic pretreatment; CC, chemical conditioning; PLA_1, phospholipase A_1; Bi-PLA_1, bio-imprinted phospholipase A_1; Im-bi-PLA_1, immobilized bio-imprinted PLA_1, UA, ultrasound assisted.

Table 3: Deacidification processes for free fatty acids (FFA) removal.

Sample	Deacidification process	Oil yield (%)	SFA (%)	MUFA (%)	PUFA (%)	PV (mEq O_2 kg^{-1})	AV (mg · g^{-1})	acidity (%oleic acid)	acid value (mg KOH · g^{-1})	Iodine value	Reference
Rice bran oil	NaOH	80.2				2.0		0.4			(De et al. 2010)
	Na_2CO_3	83.5				3.0		0.7			
	$NaHCO_3$	85.8				3.5		0.8			
Tuna oil	crude pretreated AC				32.1	3.5	44.4	3.5	10.2	187	(Wang et al., 2012)
	lipase+ethanol+ SPD				82.2	7.6	26.4	7.6	0.4	350	
	lipase +glycerol+ SPD				80.1	8.5	11.3	8.5	1.1	345	
Sardine oil	crude		71.5	11.3	16.4			5.6			(Charanyaa et al., 2017)
	solvent	70	74.2	10.8	17.5			1.1			
	membrane + solvent	93	-	-	-			1.1			
Palm oil	betaine monohydrate-based DES							49.4% extraction of palmitic			(Zahrina et al., 2018)

AC, activated carbon; SPD, short-path distillation, DES; deep eutectic solvent

1127 **Table 4:** Bleaching processes of fish oil using various combinations of bleaching agents.

Sample	Bleaching process	SFA (%)	MUFA (%)	PUFA (%)	PV (mEq O_2 kg^{-1})	AV ($mg \cdot g^{-1}$)	acidity (%oleic acid)	Iodine value	TOTOX	hue angle	chroma	POP (ng/kg)		Reference
Sardine oil	neutralized				2.4	77.0	0.17		81.7	80.4	98.1			(García-Moreno et al., 2013)
	clay				0	21.8	0.18		21.8	89.2	81.8			
Carp oil	crude	27.4	41.3	26.0	4.0		7.3	115			16[1]			(Monte et al., 2015)
	BE+AC	27.3	41.3	25.9	2.5		0.9	114			11.4[1]			
Sardine oil	neutralized	40.2	29.0	25.6	6.1	10.0	3.2		22.2	79.3	44.4			(Chakraborty et al., 2015)
	AC (5%)				4.4	9.4	2.8		18.2	84.6	39.0			
	KA (5%				6.5	10.5	3.4		23.4	81.1	42.1			
	FE (5%)				5.0	9.5	3.2		19.6	84.7	40.0			
	CE (5%)				6.0	9.9	3.4		22.0	79.5	41.3			
	CH (5%)				5.7	9.9	3.4		21.4	83.0	40.9			
	AC+FE	38.2	28.5	26.6	3.7	9.4	2.9		16.7	38.1	84.6			
Canola oil	neutralized	7.2	47.9	32.5	12.6	5.5			30.6					(Icier et al., 2018)
	bleaching earths	10.5	46.5	32.7		9.9-18.6			23.6-31.1		6% reduction in yellow			
	UA-bleaching	9.8	48.6	32.4	7.0-7.9	10.6-20.0			26.5-35.5		6-34% reduction in yellow			
Sprat oil	crude				1.8	10.0			13.6			PCDD	7.83	(Oterhals et al., 2007)
												PCDF	27.4	
												PCB	18560	
	AB				1.6	6.6			9.8			PCDD	8.57	
												PCDF	30.2	
												PCB	20588	
	AB+ AC				1.4	6.5			9.3			PCDD	nd	
												PCDF	0.358	

									PCB	19168	
Salmon oil	11 silicon-based								no signifcant elimination		(Ortiz et al., 2011)
	9 carbon-based								PCDD	99%	
									HCB	70%	
									PCB	27%	
Sprat oil	crude				1.8	20.4		14.0			(Oterhals et al., 2010)
	AB				1.7	6.0		9.4			
	SPD				0.7	5.5		6.9	76% reduction		
Fish liver oils	Crude	13.9, 24.8	46.5, 44.0	15.6, 23.5	6.3, 10.3	20.1, 21.5	0.5, 13.8	30.2, 42.2			
	SPD	14.7, 24.9	51.0, 43.8	14.2, 23.7	0.1, 2.4	13.8, 4.6	0.1, 0.0	14.1, 9.4			(Oliveira et al., 2014)
Menhaden oil	SCE								PCDD	35%	(Kawashima et al., 2009)
									PCDF	84%	
									PCB	93%	
	SCE+AC								PCDD	80%	
									PCDF	no change	
									PCB	no change	

AB, alkali bleached; AC, activated carbon, KA, kaolin; FE, Fuller's earth; CE, cellulose powder; CH, chitin; SPD, short-path distillation; PCDD, polychlorinated dibenzo-*p*-dioxins; PCDF, polychlorinated dibenzofurans ; PCB, polychlorinated biphenyls; HCB, hexachlorobenzene.

[1] Values obatined from Lavibond color (30 Y, R)

1131 **Table 5:** Deodorization processes employed in fish oil refining.

Sample	Deororization process	SFA (%)	MUFA (%)	PUFA (%)	PV (mEq O_2 kg^{-1})	AV (mg · g^{-1})	acidity (%oleic acid)	Iodine value	AI (mg KOH· g^{-1})	SV (mg KOH· g^{-1})	Volatiles (%)	Reference
Fish oil	5 types of zeolites										deodorization 20-60%	(Chung et al., 2009)
Processed tuna and anchovies	crude	36.9	28.9	34.2	3.5			118.5	0.3	221.6	93.2	(Song et al., 2018b)
	SPD	32.0	31.8	36.2	3.7			132.8	0.1	222.1	11.7	
	steam distillation	32.4	31.9	36.3	3.7			130.2	0.1	223.3	94.1	
	GTP treatment	29.6	32.2	38.3	1.9			125.3	0.2	224.5	84.5	
	L-L (alkaline ethanol)	29.5	32.2	38.3	2.9			127.4	0.2	223.8	72.5	
	Activated clay	34.2	29.2	36.1	2.1			124.1	0.2	224.0	90.3	
	zeolites	34.6	29.3	35.8	2.3			126.6	0.2	223.6	97.5	
	diatomite	34.8	29.5	35.7	2.2			120.8	0.2	220.5	78.9	
Tuna oil	crude	15.8	35.3	49.0	1.7	0.3		186.0			125.2 (OAV)	(Fang et al., 2018)
	steam	18.0	34.9	47.3	1.8	0.3		182.0			75.1	
	nanofiltration (360 Da)	15.3	35.2	49.6	1.1	0.2		185.0			17.5	
Squid oil	crude	24.8	29.4	45.9	1.8	0.4		180.0			129.8	
	steam	27.7	28.7	43.4	2.0	0.4		176.0			68.1	
	nanofiltration (360 Da)	23.8	29.5	46.8	1.2	0.3		181.0			10.1	

1132 SPD, short-path distillation; GTP, green tea polyphenols; OAV, odor active value.

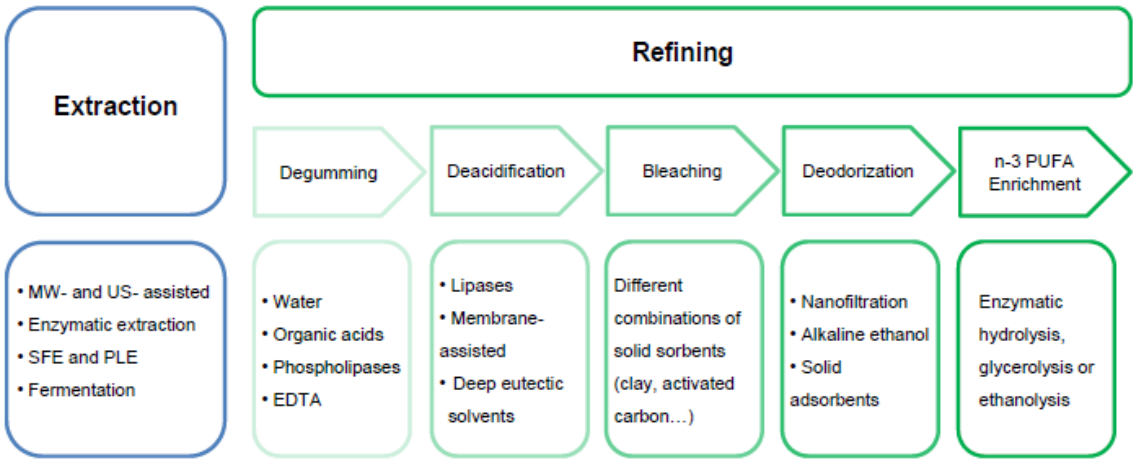
1133

FIGURE CAPTIONS

Scheme 1. Overview of the green processes with higher potential for the production of oils rich in n-3 PUFAs from aquatic sources. MV, microwave; US, ultrasound; SFE, supercritical fluid extraction; PLE, pressurized liquid extraction.

Scheme 2. (a) The rationale to design the process protocol to obtain high purity n-3 enriched monoacylglycerols from low n-3 PUFA oil by using non-regiospecific, non-n-3 PUFA preferential lipase. (b) The practical results of representative reactions as shown by spectra of ^{13}C-NMR analysis (Adopted from He et al. (2017)).

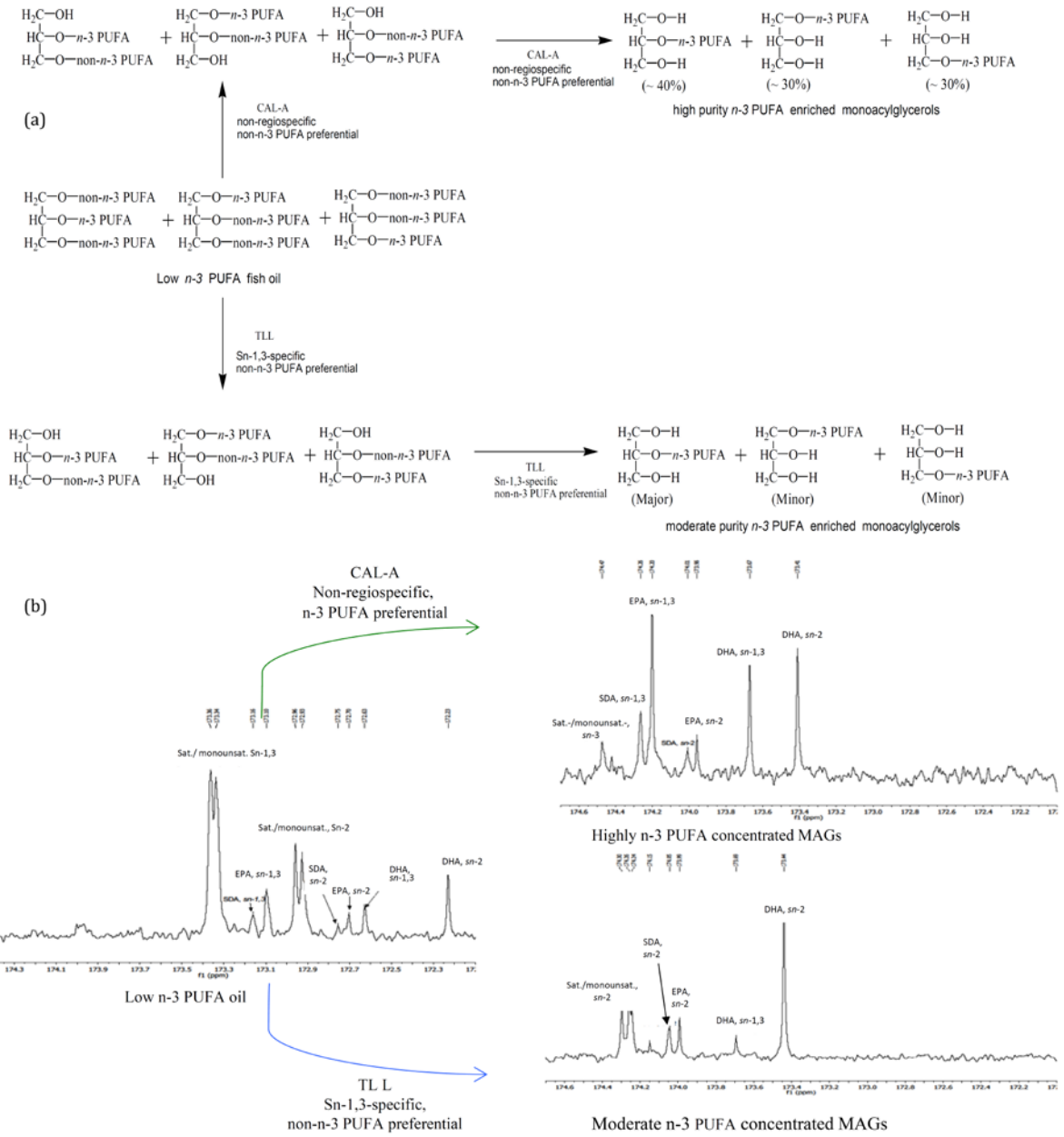
1145 **Scheme 1**



1146

1147

Scheme 2


(a)
CAL-A
non-regiospecific
non-n-3 PUFA preferential
Low n-3 PUFA fish oil
TLL
Sn-1,3-specific
non-n-3 PUFA preferential
high purity n-3 PUFA enriched monoacylglycerols
moderate purity n-3 PUFA enriched monoacylglycerols
(b)
CAL-A
Non-regiospecific,
n-3 PUFA preferential
Sat./ monounsat. Sn-1,3
Sat./monounsat., Sn-2
EPA, sn-1,3
SDA, sn-2
EPA, sn-2
DHA, sn-1,3
DHA, sn-2
Low n-3 PUFA oil
Highly n-3 PUFA concentrated MAGs
TL L
Sn-1,3-specific,
non-n-3 PUFA preferential
Moderate n-3 PUFA concentrated MAGs