Journal Pre-proof

Supercritical CO_2 processing of omega-3 polyunsaturated fatty acids – Towards a biorefinery for fish waste valorization

Rodrigo Melgosa, María Teresa Sanz, Sagrario Beltrán

PII: S0896-8446(20)30372-7

DOI: https://doi.org/10.1016/j.supflu.2020.105121

Reference: SUPFLU 105121

To appear in: *The Journal of Supercritical Fluids*

Received Date: 11 May 2020

Revised Date: 13 October 2020

Accepted Date: 11 November 2020

Please cite this article as: Melgosa R, Sanz MT, Beltrán S, Supercritical CO_2 processing of omega-3 polyunsaturated fatty acids – Towards a biorefinery for fish waste valorization, *The Journal of Supercritical Fluids* (2020), doi: https://doi.org/10.1016/j.supflu.2020.105121

Title

Supercritical CO_2 processing of omega-3 polyunsaturated fatty acids – Towards a biorefinery for fish waste valorization

Authors

Rodrigo Melgosa*, María Teresa Sanz and Sagrario Beltrán

Affiliation

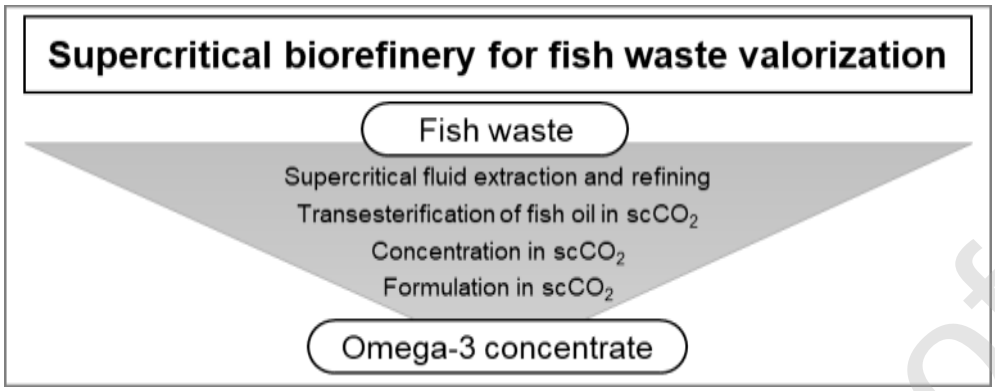
Department of Biotechnology and Food Science (Chemical Engineering Division), Faculty of Sciences, University of Burgos. Pza. Misael Bañuelos s/n, 09001 Burgos (Spain).

*Corresponding author. e-mail: rmgomez@ubu.es

Declarations of interest

None

Graphical Abstract



Highlights

- Alternative biorefinery strategy for fish waste valorization using supercritical CO_2
- Latest advances on supercritical fluid extraction of fish oil
- Production, concentration and formulation of omega-3 using supercritical CO_2

Abstract

Driven by growing prevalence of chronic diseases and consumer's health awareness, food supplements and nutraceuticals based on omega-3 polyunsaturated fatty acids (PUFAs) are extensively marketed nowadays. However, commercial omega-3 concentrates still present quality issues related to their purity, bioavailability, and easily-oxidizable nature, compelling the industry to seek for more efficient extraction, concentration and formulation methods. Technologies based on the use of supercritical CO_2 ($scCO_2$) are a promising alternative, replacing conventional organic solvents and providing milder conditions and an inert atmosphere, which reduce the risk of oxidation. The present review analyzes the latest advances on the use of $scCO_2$ for processing omega-3 PUFAs, focusing on its application in biorefinery strategies for the valorization of fish by-products. Still, further research on process fundamentals is necessary in order to increase the omega-3 yield and purity while reducing the use of energy and auxiliary materials, especially in the transesterification and concentration steps.

Keywords

fish oil; omega-3 polyunsaturated fatty acids; biorefinery; supercritical carbon dioxide; enzyme

Table of Contents

1. Introduction

Fatty acids are organic compounds, which are carboxylic acids with an aliphatic chain of variable length. Depending on the presence or absence of double covalent bonds between adjacent carbon atoms (e.g., unsaturations), fatty acids can be unsaturated or saturated, respectively. The former can be monounsaturated if they only present one double bond along its aliphatic chain, or polyunsaturated (PUFA) if they present two or more unsaturations [1]. PUFAs have important structural roles in phospholipid membranes and provide fluidity to triacylglycerol reserves. In addition, many serve as eicosanoid precursors (prostaglandins, prostacyclins, thromboxanes, and leukotrienes) with important biological functions [2].

Since vertebrates lack the Δ12 and Δ15 fatty acid desaturases responsible for converting oleic acid (C18:1 ω-9) into linoleic acid (LA, C18:2 ω-6) and α-linolenic acid (ALA, C18:3 ω-

3) [3], omega-6 and omega-3 PUFAs such as LA and ALA, respectively, are essential in the human diet [4]. Other omega-6 PUFAs as arachidonic acid (AA, C20:4 ω-6) can be synthesized by humans from LA, and omega-3 fatty acids, as eicosapentaenoic acid (EPA, C20:5 ω-3), docosapentaenoic acid (DPA, C22:5 ω-3) and docosahexaenoic acid (DHA, C22:6 ω-3), from ALA; however, the conversion of ALA into its longer-chain homologues is too low to cover the requirements of the human metabolism, especially in developmental or disease conditions; thus, EPA and DHA are considered as conditionally essential fatty acids [4].

EPA and DHA have been demonstrated to have a cardioprotective role through antiarrhythmic, blood-triglyceride-lowering, hypotensive, and antithrombotic effects [5]. Research has also shown anti-inflammatory effects including reduction of rheumatoid arthritis and Chron's disease symptoms [6]. DHA consumption is specially related to reduced risk of developing mental illnesses such as depression, Alzheimer's disease, and dementia, as well as certain forms of cancer [7–9]. Furthermore, the role of DHA in pre-natal, infant, child and adolescent development is well known [4].

In the last decades, growing prevalence of chronic diseases and increasing food and health awareness have expanded the global consumption of nutraceuticals, dietary supplements and functional foods formulated with omega-3 PUFAs. In Europe, omega-3 supplements are used by around 20 % of adult population [10], and in the USA, food products enriched with omega-3 PUFAs have been leading the functional food market in recent years [11]. However, commercial omega-3 PUFA concentrates present some inherent problems that must be addressed, such as their low oxidative stability [12] and the reduced bioavailability of the more common fatty acid ethyl ester (FAEE) form in contrast to the more natural and more easily absorbed mono- and diacylglycerides (MAGs and DAGs) and phospholipids [13]. In addition, traditional methods for production of omega-3 are also energy intensive and use organic solvents [14]. Therefore, future expansion on the omega-3 market should look for greener and

more sustainable alternatives in order to obtain highly-pure, less oxidized, and more bioavailable omega-3 concentrates.

Abundant research has been made considering alternative methods for processing omega-3 from fish oil, such as enzymatic extraction, refining and concentration; membrane methods for fish oil refining; or low-temperature crystallization and liquid chromatography for omega-3 concentration [11]. Supercritical fluid (SCF) technologies have been also proposed for extraction, refining, enzymatic transesterification in supercritical fluid media, fractionation, chromatography, and particle formulation [11], mostly using supercritical carbon dioxide ($scCO_2$) due to its unique properties as solvent. Like other SCFs, $scCO_2$ presents liquid-like density and gas-like viscosity, diffusivity, and compressibility, which provide good and tunable solvent power as well as good mass transport properties [15]. In addition, $scCO_2$ is safe (non-toxic, and non-flammable) and presents accessible critical conditions (T_C=31.1 ºC, p_C=7.38 MPa [16]). These mild critical conditions and the oxygen displacement make $scCO_2$ the solvent of choice for processing of thermolabile and easily-oxidizable compounds, such as omega-3 PUFAs, with lower risk of degradation.

In 2010, our research group [11] reviewed different alternatives for fish oil processing, focusing on the use of $scCO_2$ as an extracting solvent in the fish oil industry. More recently, Ciriminna et al. [10] made an overview of new methods for fish oil production, pointing at the development of SCF technologies as one of the milestones for omega-3 production at industrial scale. Further, Fiori et al. [17] presented a case study of a biorefinery aimed at the integral valorization of fish processing residues, obtaining omega-3 concentrates for the nutraceutical sector, fish protein destined to fish farming, and non-omega-3 FAEEs, used as biofuel.

Nowadays, biorefinery strategies are the preferred alternative for food waste and biomass valorization. Following the sustainability principles of circular economy and the zero-waste philosophy, several biorefineries have been proposed for the processing of foods and

natural products [18,19]. Based on these principles, a biorefinery for the valorization of fish by-products could follow the scheme presented in Figure 1, where $scCO_2$ processing of fish waste constitutes one of the key modules for obtaining omega-3 concentrates with high market value. Non-omega-3 lipids and glycerol can find applications in the food industry; MAG and DAG forms can be used as emulsifiers, and FAEE forms can be used as an energy source for combined heat and power plants [17]. Utilization of $scCO_2$ provides a clean process and represents an alternative to carbon capture and storage, with the possibility of internal recycling by means of decompression and recompression cycles.

Other sections of the biorefinery (not covered in this review) would deal with the fish protein and mineral fractions. Defatted fish meal rich in protein can be obtained as a solvent-free feedstock for further valorization. The protein fraction can be extracted and hydrolyzed to obtain fish protein and fish protein hydrolysates with potential bioactive applications. Conventional methods for protein extraction and hydrolysis use strong acids and alkalis, which yield low-quality products and poor control of the process [20]. Alternative and more sustainable methods for fish protein extraction such as enzymatic hydrolysis [21], ultrasound assisted extraction [22], application of pulsed electric fields [23], and subcritical water extraction and hydrolysis [24] have been extensively investigated over the last years. The remaining mineral fraction is rich in calcium phosphates such as hydroxyapatite (HAp), which can find application in the medical field thanks to its biocompatibility [25,26]. The majority of HAp is currently produced by chemical synthesis [25]. However, natural by-products and biowastes such as fish bones and scales have been investigated as alternative HAp sources [27,28], with environmental and economic advantages.

In the following sections, research dealing with fish oil extraction and omega-3 production, concentration and formulation using $scCO_2$ will be presented and discussed. The main goal of the work is to demonstrate the feasibility of biorefinery strategies for fish waste

valorization, focusing on the use of $scCO_2$ as an alternative solvent for fish oil and omega-3 processing.

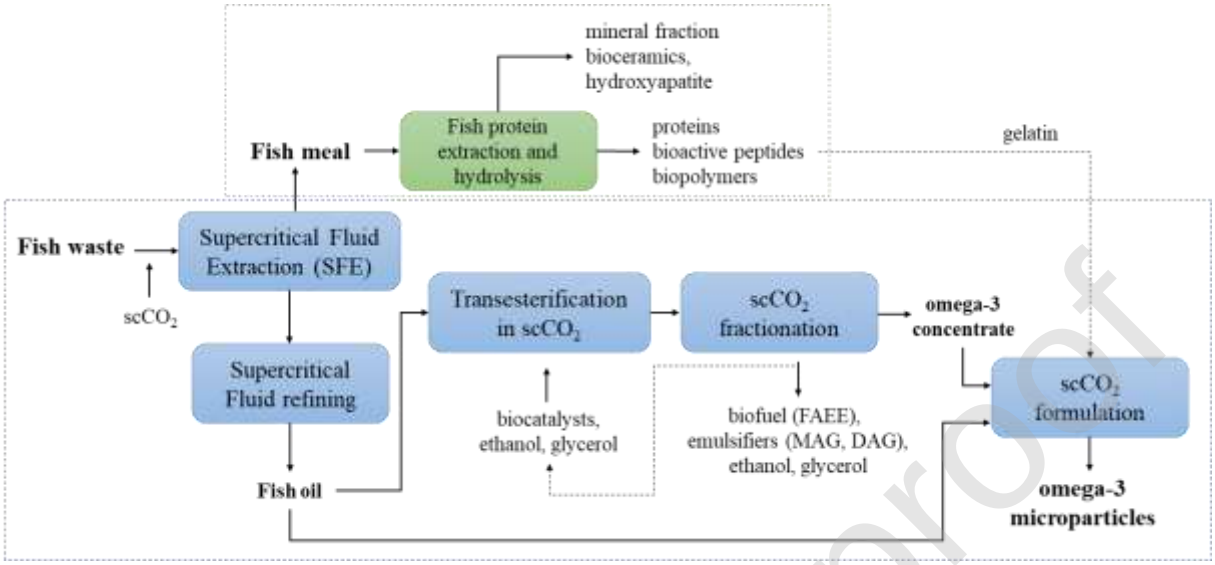


Figure 1. Proposed biorefinery strategy for fish waste valorization. Blue: Omega-3 processing using supercritical carbon dioxide ($scCO_2$) and green: processing of protein and mineral fractions, not covered in this review.

2. Sources of omega-3 polyunsaturated fatty acids: fish oil production

The most important natural sources of omega-3 PUFAs are marine organisms such as fish, seafood, and algae, that feed directly or indirectly from marine phytoplankton, which is the primary producer of omega-3 PUFAs in the trophic chain. Fish is the most common source of omega-3 PUFAs in the human diet, and blue oily fishes such as those from Scombridae, Clupeidae and Salmonidae families contain the highest percentage of omega-3 [29]. Nevertheless, it is important to point out that some fish species may contain significant levels of toxic compounds such as methylmercury, polychlorinated biphenyls (PCBs), dioxins, heavy metals and other environmental pollutants, compromising the health benefits provided by omega-3 intake if contaminated fish is consumed in a frequent basis [5]. From this point of

view, small oily fish with short life cycles are preferred as source of omega-3 PUFAs since bioaccumulation of contaminants is minimized [30]. Besides, fish allergy is increasing among the population, with prevalence rates of self-reported fish allergy ranging from 0.2 to 2.29 % in the general population, although up to 8 % can be reached among fish processing workers [31]. Microalgae, seaweed and yeast have been raised as vegan and less polluted sources of omega-3 PUFAs [32], although the lipid content, especially in seaweed, is generally lower compared to fish.

Fish oil derived from blue fish contains around 20-30 %wt. of EPA and DHA [33], and is considered the primary source of omega-3 PUFAs. Fish oil is mostly used as fish feed in aquaculture, especially for carnivorous fish from the Salmonidae family. In recent years, fish oil usage in aquaculture increased by 4-6% per year [34]. On the other hand, the use of fish oil as a dietary supplement or nutraceutical is only around 5 % of total fish oil production, although it has been increasing even more rapidly than in aquaculture, at around 15% per year [34].

Nowadays, fish oil is almost exclusively produced from small and oily species such as menhaden, sardine, sprat, herring and mackerel; and from the liver of lean fish, mainly cod [35]. These fishes or fish parts are often considered as unappetizing for human consumption and constitute a by-product in the fishery industry [34]. At industrial scale, fish oil is commonly obtained by wet pressing, an energy-intensive process that includes high-temperature boiling, centrifuging, and several drying steps [14]. Afterwards, wet-pressed fish oil is submitted to a refining process in order to remove fish oil impurities, consisting of several steps such as degumming to separate phospholipids, neutralization to eliminate FFAs, bleaching to adsorb pigments, peroxidation products and pollutants, and deodorization to remove odor compounds [11].

Alternative processes for fish oil extraction include enzymatic methods using food-grade proteases, ultrasound assisted extraction, microwave-assisted extraction, and

supercritical fluid extraction (SFE) [11]. Among them, SFE using $scCO_2$ stands out as the most promising green extraction method [36].

2.1. Supercritical fluid extraction (SFE) of fish oil

Over the last decade, extensive research has been done in the field of SFE of fish oil. Latest references deal with raw materials from different fish and seafood species, reporting variable yields depending on the nature of the raw material, the fish part, and the SFE conditions: from around 20 g oil/100 g (dry basis) in the case of hake [37,38] and tuna [39,40], up to 50 g/100 g for Indian mackerel [41–44], and 67 g oil/100 g raw material (dry basis) for African catfish [45]. Oils from sturgeon [46,47], common carp [48], striped weakfish [49], jumbo squid [38], sardine [50], orange roughy [38], yellow croaker [51], trout [52], salmon [38,53,54], sardine, and rendered fish meal [55] have been also obtained by SFE over the last decade (Table 1). Reported omega-3 PUFA contents in the extracted oil differ from 0.8 [38] up to 46 %wt [41], depending on the fatty acid profile of the original fish species and the fish part. Studies also showed that omega-3 PUFA content was not affected by the extraction method, since the fatty acid profile of $scCO_2$-extracted fish oil was very similar to that obtained by Soxhlet extraction with hexane [37]. CO_2 consumption varies from 0.03 [50] up to 2.75 kg CO_2/g oil produced [38], depending mostly on the scale of operation. Small-scale equipment requires less CO_2 to operate, although it is not useful for production purposes. In general, lower specific CO_2 consumption have been observed in SFE of fish oil, compared to other oil extraction from vegetal sources or microalgae [56], which might be due to the higher accessibility of lipids in animal cells.

Latest research on SFE of fish oil studies the influence of experimental variables affecting the extraction yield and the quality of the extract: pressure, temperature, extraction time, and solvent-to-feed ratio. Pretreatment of the raw material, which defines water content and particle size and shape, also exerts significant influence on the fish oil extraction process by SFE, as well as the use of cosolvents and mode of operation [36].

Table 1. Literature survey on SFE of fish oil over the last decade. Extractor loading and oil yield are reported in dry basis.

Raw material *Scientific name*	Extraction conditions						Oil yield (g/100 g)	omega-3 content in oil (%)	CO_2 consumption (kg CO_2/g oil)	Ref.
	p (MPa)	T (ºC)	time (h)	Extractor load (g)	CO_2 flow (kg/h)	Cosolvent				
Sturgeon muscle *Acipenser baeri*	25-35 (opt. 31.6)	n.d.	0.1-0.2 (opt. 0.18)	50	2-4 mL/min (opt. 3.5)	-	26.8			[46,47]
Common carp *Cyprinus carpio*	20-40 (opt. 40)	40-60 (opt. 60)	0.5 static + 3 dynamic	20	0.194	-	51 (viscera) 29 (fillets) 8 (caviar) (graph.)	5.12 3.48 3.45	0.06 0.10 0.36	[48]
African catfish viscera *Clarias gariepinus*	10-40 (opt. 40)	35-80 (opt. 57.5)	1-4 static (opt. 2.5)	5	0.013-0.171 (opt. 0.108)	-	67.0		0.08	[45]
Striped weakfish *Cynoscion striatus*	20-30 (opt. 25)	30-60 (opt. 45)	2.5	5.5	0.238-0.720 (opt. 0.720)	-	18.2		1.8	[49]
Jumbo squid liver *Dosidicus gigas*	25	40	3	100	13.75	-	15 (graph)	28.4	2.75	[38]
Rainbow sardine *Dussumieria acuta*	15-35 (opt. 35)	40-60 (opt. 60)	0.33 static + 0.66 dynamic	0.5	0.011-0.017	-	44.8	34.5	0.03-0.05	[50]
Orange roughy *Hoplostethus atlanticus*	25	40	3	100	13.75	-	68 (graph)	0.8	0.61	[38]
Yellow croaker muscle *Larimichthys polyactis*	15-25 (opt. 25)	35-45 (opt. 35)	1.5	30	1.670	-	21.4	12.8-21.8	0.39	[51]
Hake offcuts *Merlucius capensis* *M. paradoxus*	25	40	3	100	13.75	-	18 (graph)	13.2	2.29	[37,38]
Trout viscera, heads, and spines *Oncorhynchus* sp.	50	60	until exhaustion	80 (viscera) 35	0.6	-	79.0 (viscera) 36.0 (heads) 40.0 (spines)	6.37 EPA + 6.02 DHA 7.89 EPA +6.26 DHA 8.75 EPA +7.30 DHA		[52]
Short-bodied mackerel muscle, viscera, and skin *Rastrelliger brachysoma*	25-35 (opt. 35)	50-60 (opt. 60)	3-6 (opt. 6)	10	0.108	-	21 (muscle) 26 (viscera) 44 (skin) (graph.)	45.95	0.31 0.25 0.15	[41]

Raw material *Scientific name*	**Extraction conditions**						**Oil yield (g/100 g)**	**omega-3 content in oil (%)**	**CO_2 consumption (kg CO_2/g oil)**	**Ref.**
	p (MPa)	**T (ºC)**	**time (h)**	**Extractor load (g)**	**CO_2 flow (kg/h)**	**Cosolvent**				
Indian mackerel skin *R. kanagurta*	20-35 (opt. 35)	45-75 (opt. 75)	3	2	0.075-0.110 Continuous Cosolvent Soaking Pressure swing	 ethanol (0.019 kg/h)	 - 53.2 (cosolvent) 52.8 (soaking) 52.3 (p swing)	 29.0 (continuous) 30.7 (cosolvent) 31.0 (soaking) 31.8 (p swing)	 0.236-0.505 0.093-0.168 0.092-0.153 0.056-0.105	[42–44]
Salmon *Salmo salar*	25	40	3	100	13.75	-	45 (graph)	10	0.92	[38]
Salmon processing waste *S. salar*	15-35 (opt. 35)	40-80 (opt. 80)	2	5-8	0.18-0.48 (opt. 0.18)	-	41.0		0.11	[53]
Atlantic salmon Different parts *S. salar*	25	45	3	100	1.62	-	34.6-42.9	12.6-13.4	0.11-0.14	[54]
Longtail Tuna head *Thunnus tonggol*	20-40 (opt. 40)	45-65 (opt. 65)	2	2	0.052-0.157 (opt. 0.157)	-	35.6	36.6	0.44	[39]
Tuna (3 species) head, skin and viscera *T. tonggol, Euthynnus affinis, Auxis thazard*	40	65	2	2	0.126	ethanol (0.03 kg/h)	35.6 (*T. tonggol*) 28.4 (*E. affinis*) 29.5 (*A. thazard*)	24.1-27.9	0.35 0.44 0.43	[40]
Rendered fish meal	10-40 (opt. 30)	25-80 (opt. 40)	3	14	0.57	-	6.5 (graph.)	27.0-28.9 (incl. PUFA)	1.88	[55]

The extraction kinetics of fish oil from hake (*Merluccius capensis-M. paradoxus*) offcuts by SFE were reported in a previous work [37]. It was found that pressure positively affected extraction yield in the range 10-57.5 MPa, whereas temperature was maintained at 40 ºC in order to avoid omega-3 deterioration. Optimal extraction conditions were 25 MPa, 40 ºC and 13.75 kg CO_2/h, which yielded 10 g oil/100 g hake offcuts (dry basis) after 3 h of extraction (96.4 % of the total oil content). Only 100 g of raw material were loaded in the extractor, thus resulting in high solvent-to-feed ratios and CO_2 consumption (up to 2.3 kg CO_2/g oil). From these results, it is obvious that there is need for optimization of $scCO_2$ usage.

The water content of the raw material is also important in SFE of fish oil. Water in the sample acts as a barrier against CO_2 diffusion, reduces contact between solvent and solute and the release of lipids contained in the cells. In addition, water is partially soluble in $scCO_2$ despite its polarity (~10^{-3} molar fraction, in the usual conditions for fish oil SFE [57]), and its presence in the fluid phase can influence the solubility of oil in $scCO_2$. At industrial scale, removing water from solid raw materials is always problematic due to the associated energy costs. Since omega-3 PUFAs are highly thermosensitive, freeze-drying is the technology of choice despite its high energy and time consumption, which can be compensated by the higher extraction yield and better quality of the extract. Figure 2 shows that the extraction yield was significantly improved when the water content of the raw material was reduced from 51.5 % to 17.8 % by freeze-drying; and coextracted water significantly decreased at 8.4 % initial moisture [37].

The origin of the raw material and its particle size may also affect extraction yield due to internal mass transfer limitations and channeling phenomena. It has been reported that oil extraction was faster from hake muscle rather than from skin [37]. Regarding the extraction conditions, depressurization cycles (pressure swings) did not significantly improve the extraction yield in hake [37] and mackerel [42], probably because fish oil is not stored inside rigid structures that need to be broken in order to achieve extraction, unlike microalgae.

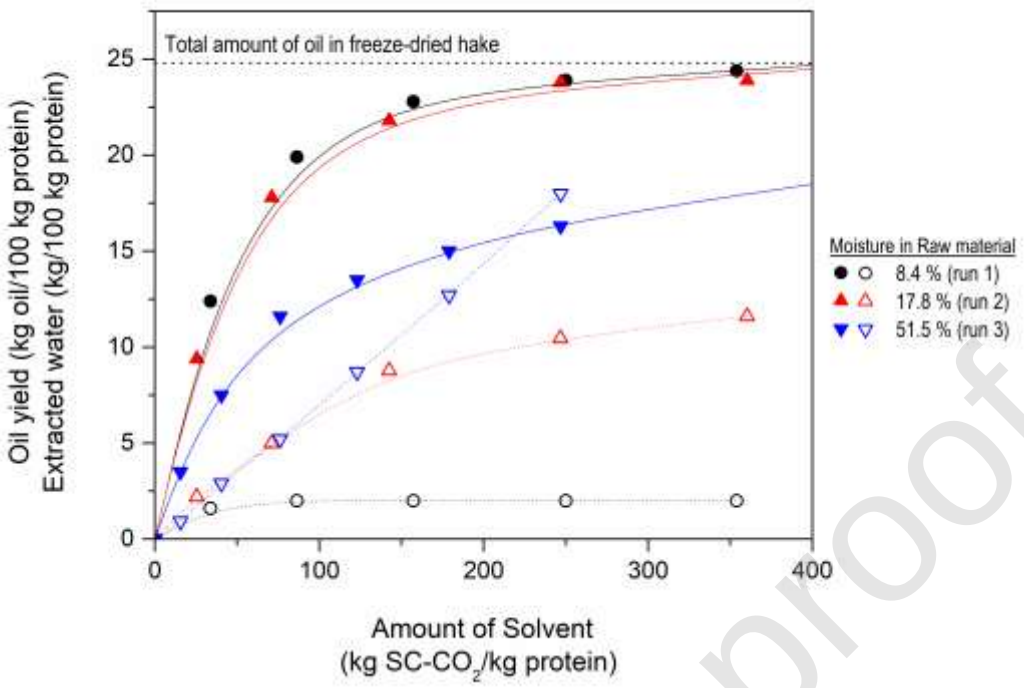


Figure 2. Effect of moisture of the raw material on the SFE extraction of hake. Solid symbols: oil yield; open symbols: amount of water extracted. Data taken from [37].

Mathematical modelling of the extraction process has been also investigated [53], finding the local adsorption equilibrium model proposed by Goto et al. [58] as the most suitable for the SFE of fish oil, since it addresses intra-particle diffusion and external mass transfer of SFE in a fixed solid bed. The presence of water in the raw material has been considered in extraction kinetic models, such as the one proposed by Dunford et al. [59], which incorporated a negative term to account for the interactions between oil and water in the supercritical phase. The Sovová's model [60] has been also applied to SFE of fish oil from fish by-products of different nature [37,49,55]. The model proposed by Kandiah and Spiro [61], which assumes that internal mass transfer resistance is significant from the beginning of the process, has led to good results for the SFE of fish oil from hake offcuts [37].

Compared to other extraction methods, similar or higher oil yields, and higher-quality extracts can be obtained by SFE. In a previous work, SFE of different fish by-products at mild conditions (25 MPa, 40 ºC) achieved similar or better yields compared to other extraction methods such as cold extraction, wet pressing, and enzymatic extraction [38]. Additionally, SFE prevented lipid oxidation, especially in fatty fish by-products such as salmon, and reduced the amount of certain pollutants [38]. The same trend was observed in the valorization of short mackerel by-products, where similar oil yield and significant reduction of pollutants (85-100% for mercury, 97-100% for cadmium, and 100 % for lead) were obtained by SFE (35 MPa, 60 ºC) compared to Soxhlet extraction with petroleum ether [41]. Inorganic or polar organometallic forms of heavy metals (e.g. arsenobetaine and most Cd and Pb forms) are not extracted by SFE due to the low polarity of $scCO_2$ [38,41]. However, non-polar organometallic forms such as arsenolipids [62] or methylmercury [63] present higher lipophilicity and may be partially coextracted with fish oil during SFE; thus, its presence in the raw material and the final products should be monitored.

Compared to ammonia extraction, wet-pressing, and enzymatic extraction, SFE achieved the highest yield (97 % of oil extracted) and lowest acidity and peroxide values (0.8 mg KOH/g and 2.52 mmol/kg, respectively) in the oil extraction from sturgeon muscle [47]. Besides, SFE-obtained oil showed better stability against oxidation during storage for 33 days at 4ºC than the other extraction methods, although some endogenous volatile compounds were co-extracted [47]. High acidity is a usual feature of crude fish oil without regard of the extraction method, and it can be linked to the co-extraction of FFA, other organic acids and water that enhances oil hydrolysis [37]. However, lower free fatty acid (FFA) content and peroxide value were found in the SFE-extracted oil from different tuna species, compared to Soxhlet extraction with hexane, and quality parameters remained acceptable even after 60 days of storage at 4 ºC [40].

Claims on the phospholipid content are one of the latest trends among omega-3 dietary supplements. Marine phospholipids present functional properties as surfactants and emulsifiers, and are considered the next-generation omega-3 supplements due to their better absorption, higher bioavailability and better stability against oxidation [64]. In the case of SFE, the low polarity of $scCO_2$ does not allow the extraction of these amphiphilic molecules, although the use of cosolvents such as ethanol can modify the solvent polarity and increase extraction yield and phospholipid content. Phospholipid-enriched fractions can be obtained by SFE following a 2-step strategy: (1) fish deoling with neat $scCO_2$, and (2) phospholipid extraction with $scCO_2$ and ethanol as an entrainer, obtaining phospholipid yields comparable to traditional methods using organic solvents [65]. The main drawback associated to the use of cosolvents is that downstream processing is required to remove the cosolvent from the extract, increasing the operational costs and the chances of omega-3 degradation, while traces might also remain in the raffinate.

Nowadays, SFE is considered a mature technology able to replace traditional extraction processes in certain applications, such as hop extraction and coffee decaffeination [15]. Results presented in this review show that SFE of fish oil has achieved important development over the last decade. However, despite the benefits regarding product quality, industrial implementation is hindered by the high initial investment. The cost of an industrial SFE plant is somewhat larger than conventional technologies, making it easier for the fish oil industry to maintain the existing solvent extraction technology with hexane or petroleum ether, which is better-known and offers similar extraction yields. Nevertheless, some studies have pointed out that SFE of essential and specialty oils requires lower processing costs because of the higher quality of the product obtained [66]. The lower complexity of the process, with no need of organic solvent evaporation and recovery units might also compensate for the higher processing costs of lyophilization,

since $scCO_2$-extracted fish oil can be perceived as a greener and more natural product, allowing for higher price tags.

2.2. Supercritical refining of fish oil

Supercritical fluid refining, together with membrane and enzymatic processes, is one of the most recently proposed alternatives to conventional oil refining using chemical products or high temperatures [11]. Several studies have taken advantage of the benefits of $scCO_2$ as a green solvent in degumming and bleaching [67], and deacidification [68,69] of different seed oils. Supercritical refining can be also applied to fish oil in order to remove coextracted impurities such as FFAs, oxidation products, and endogenous volatile compounds. Based on phase equilibria measurements that show that FFAs are more soluble in $scCO_2$ than fish oil triacylglycerides (TAGs) [70], the use of two or more separators connected in series has been proposed as a one-step strategy to refine fish oil immediately after SFE [37]. This way, a high-quality oil is obtained in the first separator, and FFA and volatile compounds precipitate in the second separator at lower pressure.

Thanks to the higher selectivity and lower intensity of the SFE process, compared to organic solvent extraction, $scCO_2$-extracted fish oil requires less downstream processing, and FFAs, peroxides and odor compounds can be removed by simple fractionation methods [37]. The major challenge, however, is still the removal of toxic contaminants such as dioxins and PCBs that might be present in the raw material [71]. Jakobsson et al. [72] studied the semi-continuous and simultaneous deacidification and removal of dioxins from cod liver oil using $scCO_2$ and ethanol as co-solvent. The different polarities of FFAs and dioxins did not allow to remove both types of compounds simultaneously, although it was possible to separate dioxins from deacidified fish oil by using pure $scCO_2$ at low pressure. In subsequent studies, the same authors implemented a countercurrent extraction column to improve dioxin removal up to 80

%, which were extracted together with 17 % of the initial oil loading [73]. A pilot countercurrent fractionation plant was also used by Catchpole et al. [74] to remove impurities such as peroxides, fatty acids and odor components from crude fish oils from different sources (orange roughy oil, deep sea shark liver oil, spiny dogfish oil and cod liver oil), by using $scCO_2$ and ethanol as co-solvent (5 %wt.). SFE of fish oil was also coupled with adsorption on activated carbon to remove PCBs, polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). The SFE process was able to effectively remove PCBs, and the adsorption method was more efficient for the removal of PCDDs and PCDFs [71,75].

Nowadays, different supercritical refining technologies are developed industrially. Some producers have opted for obtaining the fish oil by conventional methods, with no need of freeze-drying, and then refine the extract by countercurrent extraction with $scCO_2$ in order to remove FFAs, oxidation products and other coextracted impurities. This way, some of the advantages of $scCO_2$ are maintained while reducing the energy costs linked to the pretreatment of the raw material. Supercritical fluid chromatography (SFC) is also used for fish oil refining and omega-3 PUFA concentration, and will be discussed in section 3.2.2.

3. Production of omega-3 concentrates using $scCO_2$

Research on the structure of fish oil TAGs has shown that omega-3 PUFAs are mostly found in the *sn*-2 position of the glycerol backbone, where the *sn*-1,3 positions are occupied by medium-chain saturated and monounsaturated fatty acids such as palmitic acid and oleic acid, respectively [33]. In order to separate omega-3 PUFAs from the rest of the fatty acids and obtain the omega-3 PUFA concentrate, a proper strategy must be defined, which usually consists of a transesterification step followed by a suitable concentration method. Optionally, omega-3 PUFA concentrates can be further modified in order to obtain structured omega-3 lipids (Fig. 3).

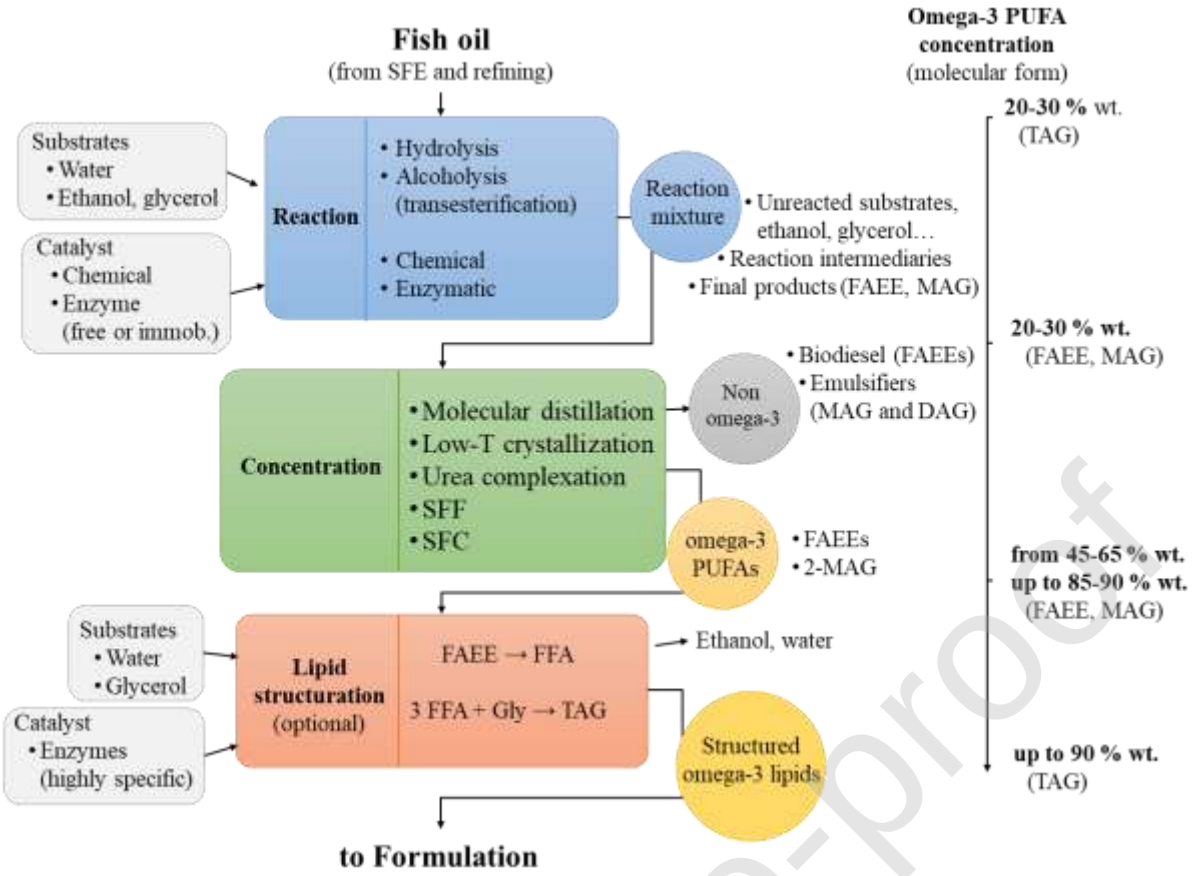


Figure 3. Schematic of the strategy for the production of omega-3 PUFA concentrates from fish oil.

3.1. Transesterification of fish oil in $scCO_2$

From an industrial point of view, transesterification (alcoholysis) with food grade alcohols such as ethanol or glycerol is the preferred pathway to separate fatty acids from the glycerol backbone in a TAG molecule. Hydrolysis reactions have been also investigated, although the produced free fatty acids may present irritant effects and low stability against oxidation [76]. In ethanolysis reactions, the final products are FAEEs and glycerol, whereas a mixture of MAGs will be obtained if glycerol is used as the acyl donor. Unreacted substrates and partial acylglycerides should be also considered in both cases.

In the food industry, chemical catalysts such as strong acids and bases, as well as sodium or sodium/potassium alkylates are often used to catalyze alcoholysis of lipids, although the low

specificity of the catalysts is not compatible with the need for structured omega-3 PUFA concentrates, and many studies have shown that chemical transesterification negatively affects oxidative stability of fats and oils [1]. Enzyme-catalyzed alcoholysis emerges as a greener and much more specific alternative to chemical catalysts for processing of omega-3 PUFA. A great number of lipases are available for the food industry to prepare structured lipids with interesting properties, such as cocoa butter and human milk fat substitutes [1]. Lipases can also operate under mild conditions, which is preferable to avoid oxidation [11,77].

Enzymatic ethanolysis of fish oil has been widely studied as a simple method to obtain FAEEs at mild conditions. Reaction products can be subsequently fractionated by different methods in order to obtain omega-3 PUFA concentrates in FAEE form, which in turn can be formulated or further reacted with glycerol and a suitable lipase to obtain omega-3 acylglycerols (see section 3.3).

Since omega-3 PUFAs are usually located in the *sn*-2 position, stereo- and regiospecificity of certain lipases can be exploited to selectively produce 2-MAGs enriched in EPA and DHA. In the case of fish oil ethanolysis by Lipozyme 435 from *Candida antarctica*, which acts as a *sn*-1,3-specific lipase at high ethanol concentrations, up to 24 % mol MAG, with 46 % being docosahexaenoyl glycerol, were obtained at 76:1 ethanol:oil (mol/mol), 30 ºC and 10 %wt. lipase [77].

Other food-grade alcohols such as glycerol can be also used as acyl donors in enzymatic alcoholysis of fish oil [78,79]. This approach is particularly advantageous because the more bioavailable and stable 2-MAGs can be obtained in a one-step reaction, although it is limited by the poor miscibility of the substrates [78]. To overcome this drawback, organic solvents such as tertiary alcohols [77–81], and/or surfactant-stabilized emulsions [82] can be used in order to achieve homogeneous reaction systems.

A greener alternative is $scCO_2$, which can replace organic solvents not only in lipid extraction, but also in enzymatic reactions. $scCO_2$ improves the miscibility of the reactants and the mass transport properties of the reaction media, while not requiring complicated operations for solvent removal and reducing production costs [83]. Lipid oxidation can be also minimized using $scCO_2$ as reaction media, as shown by the lower acidity and total oxidation (TOTOX) values of the reaction products obtained in $scCO_2$, compared to organic (*tert*-pentanol) or solvent-free reaction media [84]. An additional advantage is that $scCO_2$ physical properties can be tuned by adjusting pressure and temperature, in order to increase enantioselectivity of the lipase [85]. The use of enzymes as biocatalysts in $scCO_2$ has been widely studied in the literature, as showed by previous reviews [86–89]. In the next subsections, some basic aspects of enzymatic reactions in $scCO_2$ are presented and discussed: (1) the effects of $scCO_2$ on the enzyme activity and stability, (2) the phase behavior of the reaction system, and (3) the kinetics of the enzymatic reaction.

3.1.1. Enzyme activity and stability in $scCO_2$

Lipase behavior in $scCO_2$ has been extensively studied in recent years, finding activity changes when enzymes are exposed to $scCO_2$ at high pressures. In certain cases, $scCO_2$ exposure promotes the total deactivation of enzymes, which is the basis of HPCD (High Pressure Carbon Dioxide) technology used to preserve the organoleptic quality of fruit juice and animal foods [90–95]. In the case of enzymatic reactions in $scCO_2$, the aim is to preserve or even increase the catalytic activity of enzymes, which can be achieved by selecting proper biocatalysts and operating conditions. Our research group has demonstrated that several lipases, either in free or immobilized form, are active and stable in $scCO_2$, and retain their catalytic activity after several cycles of utilization [96].

Free lipases are known to increase its residual activity after $scCO_2$ treatment at mild conditions. Gießauf and Gamse [97] reported up to 860 % increase in catalytic activity of

porcine pancreas lipase after incubation in $scCO_2$ at 75 ºC and 15 MPa, which was likely due to extraction of impurities from the enzyme preparation, given the nature of the lipase and the reported weight loss (about 4 %wt.) after $scCO_2$ treatment. However, we have observed slight $scCO_2$-induced activity enhancements in other commercial, more purified lipase preparations, such as Lipozyme CALB L from *Candida antarctica* (112 ± 1 % residual activity after treatment at 15 MPa, 35ºC and 3 h), and Palatase 20000 L from *Rhizomucor miehei* (135 ± 2 % residual activity at 15 MPa, 50ºC and 1 h) [96]. Similar results were obtained by Liu et al. [98] for Lipozyme CALB L, with a maximum of 105 ± 4 % residual activity at 10 MPa, 40ºC and 0.5 h. In these cases, changes in the conformational structure of the enzyme due to $scCO_2$ interactions with hydrophobic enzyme residues were the most likely explanation to activity changes, as corroborated by fluorescence emission spectrophotometry [96,98]. We also found a direct relationship between residual activity and fluorescence intensity maxima of the treated enzymes [96].

Commercial immobilized lipases from *Mucor*/*Rhizomucor miehei* (Lipozyme RM IM) and lipase B from *Candida antarctica* (Lipozyme/Novozym 435) are likely the most widely studied lipases for omega-3 PUFA modification due to their commercial availability and high catalytic activity. Our research group investigated the effect of $scCO_2$ exposure at different conditions (35-70 ºC; 10-25 MPa; 1-6 h; 1-3 pressurization/depressurization cycles) on the residual activity of these lipases, finding no significant changes in most of the experiments [96]. A slight decrease in lipase activity was found at the highest temperature (70 ºC) and when increasing the number of pressurization/depressurization cycles from 1 to 3 (from 92 ± 2 % to 88 ± 3 % of the initial activity for Lipozyme RM IM and from 82 ± 2 % to 78 ± 1 % of the initial activity for Lipozyme 435) [96]. These results are similar to those reported by Oliveira et al. [99] who observed that activity of Novozym 435 decreased from 96 % to 89.5 % when submitted to 1-5 pressurization/depressurization cycles at 27.6 MPa, 75ºC and 1 h.

Different mechanisms have been proposed to explain the activity changes of immobilized enzymes after $scCO_2$ treatment. Pressure denaturation is not likely to happen at the usually mild treatment conditions [100], although the shape and surface of the support might be affected [99]. Formation of carbamates [101] and changes in enzyme structure due to $scCO_2$-enzyme interactions [98] have been also proposed. Extraction of essential water from the enzyme microenvironment, as a result of unfavorable partitioning between the support and the solvent, might also occur during depressurization [102,103]. For Lipoozyme RM IM and Lipozyme 435, this water stripping effect was assessed through the addition of water to the $scCO_2$-treated biocatalysts. Results obtained (Fig. 4) showed that Lipozyme RM IM, with a hydrophilic support, was able to recover its initial activity when low quantities of water (0.5-1 % wt. enzyme) were added [96]. This behavior was not observed on Lipozyme 435, possibly because its hydrophobic support did not allow the enzyme to recover its constitution water, nor its initial activity. Larger amounts of water lead to lower enzymatic activity due to displacement of the esterification reaction towards hydrolysis [96].

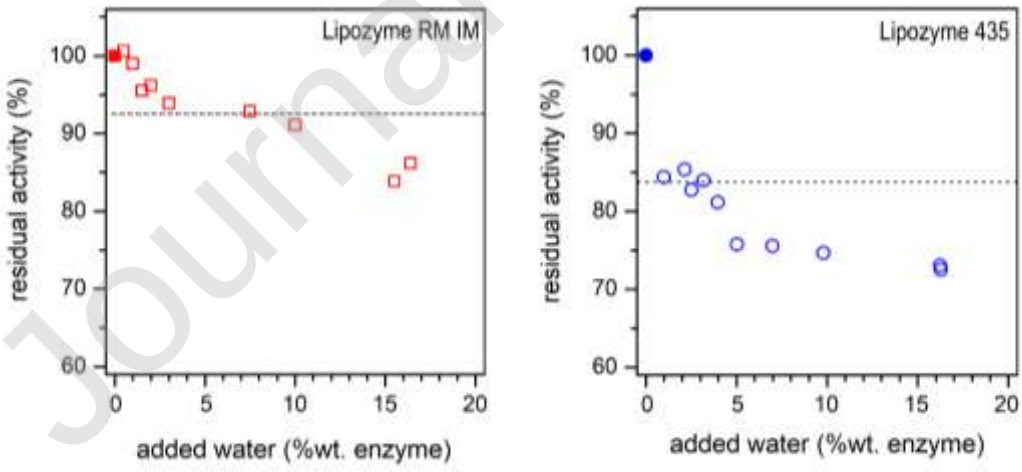


Figure 4. Residual activities of Lipozyme RM IM (red squares) and Lipozyme 435 (blue circles) after $scCO_2$ treatment (15 MPa, 50 ºC, 3 h) and different amounts of added water. Solid symbols: untreated samples; open symbols: treated samples. Dashed lines indicate the residual activity of each treated lipase when no water was added. Adapted from [96].

The results presented demonstrated that lipases are able to maintain and even increase their activity after exposure to $scCO_2$, although operating parameters must be closely observed. In practice, pressure, temperature, reaction time, stirring speed, depressurization rate, and reutilization cycles must be considered in batch reactors [102]. Additionally, the water dependence of the catalyst and the accumulation of unreacted substrates, intermediaries, reaction products, and/or impurities that may deactivate the biocatalyst or block the reactor bed should be addressed when operating continuously [104].

3.1.2. Phase behavior of the reaction media

As in any other chemical or biochemical process, phase behavior of the system is one of the basic aspects to be considered in fish oil alcoholysis in $scCO_2$. Reliable experimental phase equilibrium data and suitable equations of state would help to predict miscibility regions at different substrate ratios and operating conditions, understand the reaction kinetics of the system, prevent catalyst deactivation or optimize solvent usage.

Experimental data related with high-pressure phase equilibrium of ternary and higher systems comprising $scCO_2$ and pure TAGs, other lipid derivatives and their mixtures has been extensively investigated [105]. Phase equilibria data of pseudo-ternary mixtures of CO_2, ethanol, and edible oils is more scarcely reported and mainly focused on the oil solubility in $scCO_2$ with ethanol as cosolvent [70,106]. These data might be useful for SFE applications, but the ethanol concentrations are too low for ethanolysis reactions in $scCO_2$. Geana and Steiner [107] reported fluid phase equilibrium data for the pseudo-ternary system CO_2 + ethanol + rapeseed oil in the temperature range 40-80 ºC and at pressures from 6 MPa to 12 MPa, satisfactorily correlating the phase behavior with the Peng-Robinson equation of state (PR EoS) [108] coupled with the conventional van der Waals mixing rules with two adjustable parameters

(vdW2). Ndiaye et al [109] studied the fluid phase equilibria of binary and ternary mixtures involving CO_2, ethanol, soybean oil, castor oil, and their fatty acid ethyl esters. The pseudo-ternary system CO_2 + ethanol + castor oil was studied at fixed ethanol-to-oil ratios, temperatures ranging from 40 ºC to 70 ºC and pressures from 2.13 MPa to 27.13 MPa. Experimental data were correlated both with PR EoS vdW2 and the Statistical Associating Fluid Theory (SAFT) [110] with one binary interaction parameter. Hernández et al [111] investigated the phase behavior of the pseudo-ternary mixture CO_2 + ethanol + sunflower oil at two different conditions of temperature and pressure (40 ºC and 13 MPa; 60 ºC and 20 MPa). A group contribution equation of state (GC EoS) [112] was used to correlate the experimental data, adopting two sets of TAG-alcohol interaction parameters for the Liquid + Liquid (L1+L2) and the Vapor + Liquid (V+L2) 2-phase regions. More recently, Dalmolin et al [113] studied the phase transitions in the system CO_2 + ethanol + rapeseed oil, at temperatures in the range 40-70 ºC and pressures up to 22.53 MPa. They found a 3-phase region with a Vapor + Liquid + Liquid (V+L1+L2) phase transition that occurred at higher pressures when increasing temperature, and satisfactorily explained their experimental results with the PR EoS vdW2 model.

Our research group recently presented experimental phase equilibria data of the pseudo-ternary system fish oil + ethanol + CO_2 at high pressures and temperatures typical of enzymatic reactions (T = 50-70 ºC and p = 10-30 MPa) [114], finding homogeneous and expanded-liquid regions that may enhance reaction rates. Experimental tie-lines were obtained by means of an analytical isothermal method with recirculation of the vapor phase. Results showed two 2-phase regions (L1+L2 and V+L2) at T = 50 ºC for both pressures investigated (10 MPa and 30 MPa). An additional V+L1 and a 3-phase region (V+L1+L2) were observed at T = 70 ºC and p = 10 MPa (Fig. 5). Experimental data were correlated with the Peng-Robinson equation of state coupled with conventional two-parameter van der Waals mixing rules. The model successfully

explained the experimental results, although different sets of binary ethanol + fish oil interaction parameters were required to adequately represent the different types of phase equilibria. To give an idea of the importance of phase equilibria determination, the results obtained in this work were successfully employed to explain some particularities of the fish oil ethanolysis reaction kinetics that would allow to optimize solvent usage in a further upscaling of the process.

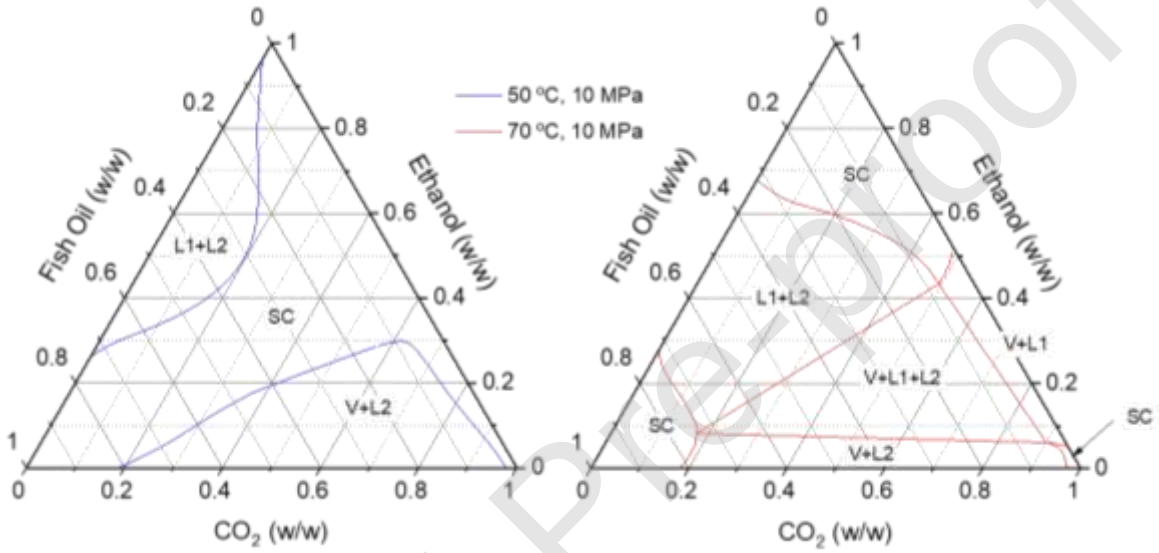


Figure 5. Effect of temperature on the phase behaviour of the pseudo-ternary system CO_2 (V) + ethanol (L1) + fish oil (L2) at 10 MPa. Data taken from [114].

3.1.3. Fish oil transesterification kinetics in scCO₂

The last basic aspect that is needed to demonstrate the industrial feasibility of enzymatic modification of fish oil in $scCO_2$ is the study of the reaction kinetics. Together with phase equilibrium of the reaction system, reaction kinetics provide tools to understand the interrelationships between operating parameters, reactant concentrations, solvent (if any) and catalyst along reaction time. Adequate kinetic models allow to predict the effect of changing

these variables in order to maximize the reaction yield or avoid catalyst inhibition, and help in the optimization and scale-up of the process. Ideally, these models should have biochemical significance, and consider the effect of operating variables (pressure, temperature, solvent, etc.), reactant intermediaries and enzyme inactivation.

In the literature, different immobilized lipases have been used to successfully conduct alcoholysis reactions in $scCO_2$, such as Lipozyme RM IM from *Rhizomucor miehei* [84,115,116], Lipozyme/Novozyme 435 from *Candida antarctica* [117–122], and Lipozyme TL-IM from *Thermomuces lanuginose* [123,124]. Marty et al. [115] compared the esterification of oleic acid and ethanol in $scCO_2$ and in hexane by an immobilized lipase from *R. miehei* (Lipozyme), finding higher initial reaction rates and lower ethanol inhibition in $scCO_2$. In subsequent works, a Ping-Pong Bi-Bi mechanism was proposed as a good correlation between experimental data and kinetic curves [116]. Oliveira and Oliveria [121,122] studied the ethanolysis of palm kernel oil in $scCO_2$ by Lipozyme IM from *R. miehei* and Novozym 435. The experiments were performed in a reactor vessel in the temperature range of 40-70°C and from 6 to 20 MPa using a water concentration of 0-10 %wt. and oil/ethanol molar ratios from 1:3 to 1:10, finding that the initial reaction rate increased with pressure and the oil/ethanol molar ratio. A simple model based on the mass balances of the initial substrates (TAG and ethanol) and final products (FAEE and glycerol) was proposed, obtaining good fitting results although reaction intermediates (DAG and MAG) and ethanol inhibition were not considered [121].

Regarding fish oil modification in $scCO_2$, the earlier publications investigated the ethanolysis of cod liver oil by Novozym 435 from *C. antarctica* in continuous and semi-continuous mode [118–120]. The reaction products, fatty acid ethyl esters (FAEEs) were continuously extracted by the flowing supercritical phase; thus, CO_2 flowrate and phase behavior of the system influenced the reaction rate and product composition [118–120]. Some years later, the transesterification of menhaden oil with excess ethanol in $scCO_2$ was

investigated [123,124], finding that reaction rates were enhanced and ethanol inhibition was reduced in $scCO_2$ when compared to organic solvent or solvent-free systems. An empirical kinetic model assuming reversible binary reactions at each transesterification step was proposed [124], obtaining good fitting results. The concentrations of all the species in the reaction system were considered in the model, except for glycerol due to the *sn*-1,3 specificity of the biocatalyst. Our research group has presented some kinetic results for the enzymatic ethanolysis of refined fish oil from tuna and sardine in $scCO_2$, using Lipozyme RM IM as catalyst [84]. Reaction kinetics were correlated to an empirical kinetic model based on the mass-balance of each compound (TAG, ethanol, DAG, MAG, FAEE and glycerol), similar to the one proposed in [124]. The effect of temperature (50-80 ºC) on the kinetic rate constants was considered by introducing an Arrhenius-type dependence in the kinetic model, whereas the volume and energy of activation of the reaction accounted for the effect of pressure (7.5-30 MPa). The highest reaction rates were found at 70 ºC and 30 MPa, since higher temperatures promoted enzyme deactivation; pressure had an overall positive effect due to the increasing solubility of CO_2 in the reaction mixture, which reduces viscosity and improves diffusion coefficients [84]. Recently, the ethanolysis of fish oil in $scCO_2$ by Lipozyme 435 has been also presented, adopting the Ping-Pong Bi-Bi model to explain the kinetic data [117]. High ethanol concentrations were chosen to enhance catalyst selectivity, based on previous studies [77]. Reaction kinetics (Fig. 6) were similar to the observed when using *tert*-pentanol and solvent-free media [77], with a rapid production of FAEEs and accumulation of reaction intermediaries in the early stages of the reaction (up to 24.1 % mol). The effect of initial phase equilibrium conditions on the reaction rate was also investigated, based on previous studies [81,114], finding much higher reaction rates when starting at homogeneous conditions, compared to expanded-liquid media [117]. Reaction kinetics also helped in the maximization of MAG

production with the lowest possible concentrations of DAG and glycerol, obtaining a more easily fractionable reaction product [117].

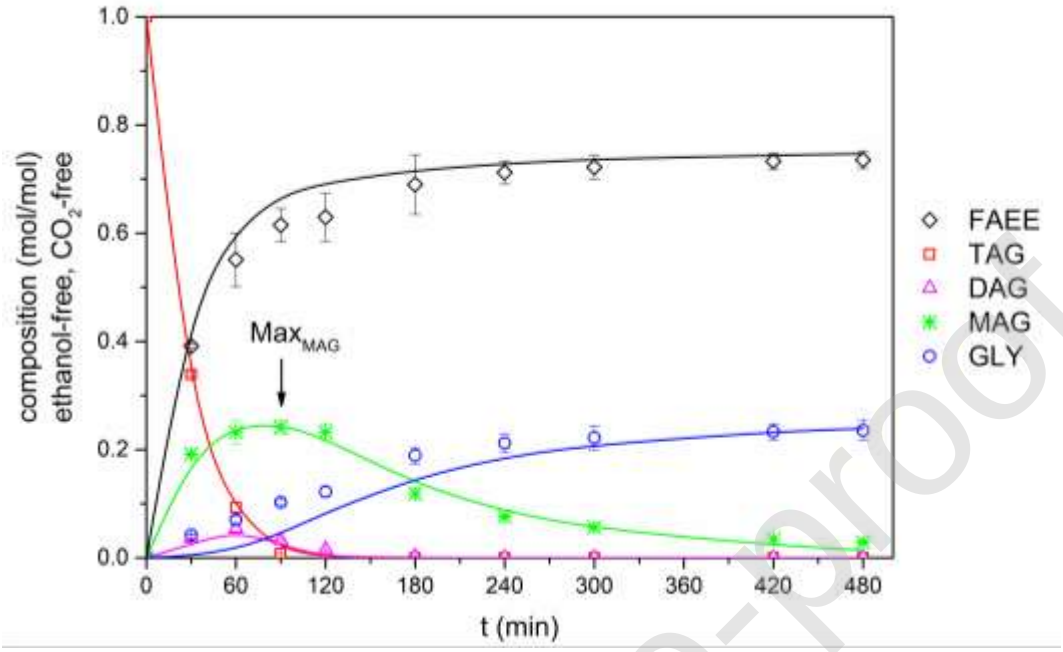


Figure 6. Time course of the ethanolysis of fish oil by Lipozyme 435 in $scCO_2$. T = 50 ºC, p = 10 MPa, 76:1 ethanol oil (mol/mol) and 10 %wt. enzyme. Lines represent the fitting of the Ping-Pong Bi-Bi kinetic model to the experimental data. Data taken from [77].

3.2. Concentration of omega-3 in $scCO_2$

The complex mixture obtained after fish oil transesterification must undergo a subsequent concentration step in order to separate the omega-3 PUFAs from the rest of the lipid fractions, ethanol and glycerol. The main properties of each fatty acid or fatty acid family, such as boiling and melting temperatures, molecular size and degree of unsaturation are considered in the separation of omega-3 PUFAs from other lipids. Industrially-developed technologies include molecular distillation, low-temperature crystallization, urea complexation, and liquid chromatography [64]. Table 2 summarizes the main features of these methods and compares

them to alternative methods using $scCO_2$, such as supercritical fluid fractionation (SFF) and supercritical fluid chromatography (SFC). Furthermore, combined fractionation techniques can be applied, depending on the desired concentration and purity in the final product [64].

Currently, molecular distillation is the most commonly applied process at industrial scale [125], although SFF and SFC offer much lower processing temperatures and higher selectivities, while maintaining high separation efficiency and very low risk of oxidation.

3.2.1. Supercritical fluid fractionation (SFF)

SFF of fish oil and omega-3 PUFA fractionation is known since the late 1970's, when Dr. Kurt Zosel, patented SFF with supercritical ethane [126]. Some years later, different authors took advantage of the interesting features of $scCO_2$ to fractionate fish oil [127–129], leading to the development of continuous counter-current SFF (CC-SFF) in multi-stage columns [130–132]. FAEE forms are preferred to feed the SFF process, since they present higher solubility in $scCO_2$ than pure TAGs, and omega-3 PUFAs can be more easily fractionated [133–135]. On the other hand, fractionation of omega-3 PUFAs in TAG form is not technically feasible, since solubility studies with Ropufa 30 oil at 28-50 ºC and 7.8 to 29.4 MPa [136] and menhaden oil at 40-70 ºC and 13.6-27.2 MPa [137] resulted in distribution coefficients for omega-3 PUFAs close to 1.

In 2010, Rubio-Rodríguez et al. [11] discussed the prospects of SFF, aiming at production at large scale and the need for phase equilibria data and models to describe the process. These models have been provided in recent years: Maschietti and Pedacchia [138] developed a predictive model for CC-SFF of fish oil FAEEs, taking into account the main operating parameters (p, T, number of theoretical stages, reflux ratio and solvent-to-feed ratio); similarly, Fiori et al. [139] developed an Aspen Plus™ model for the CC-SFF of omega-3 FAEEs from trout by-products, finding optimal operation conditions (T = 80 °C, p = 19.5 MPa, external reflux ratio = 0.92, and solvent to feed ratio = 63) and estimating operating and

investment cost as 2.30-2.50 €/kg concentrate and 2-15 €/kg concentrate, respectively; Pieck et al. [140] have recently developed a simplified equilibrium-stage model for CC-SFF of fish oil FAEEs that successfully correlates the effect of varying solvent-to-feed ratio on the yield and composition of the feed and raffinate, which in turn define the economic viability of the process [140].

Development of SFF modelling and favorable results of economic analysis should motivate the implementation of SFF plants at industrial scale. However, a recent review [141] showed that SFF is widely spread among leading research institutions, yet the actual number of industrial-scale CC-SFF applications is still low. The most likely reason is that competing processes such as molecular distillation are well known and readily available, whereas CC-SFF needs to be designed for each specific application [142], and phase equilibria and mathematical models to describe the CC-SFF process are not always available.

Table 2. Comparison of available industrial technologies for omega-3 PUFA concentration and supercritical alternatives (adapted from [64]).

	Molecular distillation	**Liquid chromatography**	**Low-temperature crystallization**	**Urea complexation**	**Supercritical fluid fractionation**	**Supercritical fluid chromatography**
Driving force	Boiling point	Chain length and degree of unsaturation	Melting point	Saturated fats	Chain length	Chain length and degree of unsaturation
Operating T (ºC)	140 to 220	20 to 50	-70 to 0	-10 to 90	35 to 50	35 to 50
Operating p (bar)	10-6	1	1	1	> 140	> 140
Use of organic solvents	No	Possible	Possible	No	No	No
Max EPA+DHA concentration (%)	65-75	> 90	> 90	45-65	75-85	> 90
Decontamination efficiency	Very high	High	Low	Low	Medium	Very high
Mode of operation	Continuous	Semi-continuous	Batch	Batch	Continuous	Semi-continuous
Risk of oxidation	Low	Very low	Possible	Possible	Very low	Very low
Flexibility to adjust EPA/DHA final concentration	Limited	Very high	Limited	Low	Limited	Very high
Capital investment	Low	High	Low	Low	High	High

3.2.2. Supercritical fluid chromatography (SFC)

Supercritical fluid chromatography (SFC) is an attractive alternative to traditional techniques such as gas chromatography (GC) or high-performance liquid chromatography (HPLC) for analytical, preparative or production purposes [143]. SCFs can be used as a mobile phase with tunable properties thanks to pressure and temperature variations, together with packed or capillary columns filled with a suitable stationary phase. Materials such as octadecyl silane, aluminum oxide, or silica gels from different nature are mainly used as the column stationary phase, whereas $scCO_2$ is the eluent solvent of choice due to its green characteristics. Co-solvents are often incorporated, most of the times with changing proportion over time [143]. The high selectivity of the mobile phase, combined with a suitable stationary phase, make SFC especially suitable for separation of omega-3 PUFAs. Since its invention [144,145], many studies have been published about SFC applied to fish oil derivatives, mainly FAEE, to obtain omega-3 PUFAs with a high purity and recovery not only in the laboratory but also at large scale [11]. From the point of view of pharmaceutical and food industries, continuous SFC with simulated moving beds (SMB) [146] constitutes a very interesting technique, able to satisfy the growing demand for ultra-pure EPA and/or DHA products. SFC processes equipped with silica xerogel columns are already installed in pharmaceutical companies such as KD-Pharma, Solutex [64] or Beps Biopharm. SFC processes can be coupled with previous SFE of fish oil in order to remove coextracted FFAs, oxidation products and odor compounds and separate the different lipid fractions and fatty acid families, allowing to obtain omega-3 PUFA concentrates up to 90-95 %wt., in FAEE or TAG form and with tailor-made EPA/DHA ratios [10].

Although less energy-intensive than other concentration techniques such as molecular distillation or SFF, industrial-scale SFC requires high initial investment and maintenance costs related to the specialized stationary phases. Since separation efficiency is inversely related to particle size of the column packing, special coating materials with 5-10 μm particle size are

recommended. However, these materials are extremely expensive at a production scale [65]. In addition, pressure drop across the column is also inversely dependent on particle size; thus, compromise solutions that result in decreased separation efficiency should be adopted.

3.3. Production of structured omega-3 lipids

The last stage in the production of omega-3 PUFA concentrates is the restructuration into the TAG form. This step can be considered as optional since omega-3 PUFA concentrates are also sold in FAEE form, although omega-3 structured lipids constitute one of the most important advances in the omega-3 industry in recent years due to the increased bioavailability and better oxidative stability of TAG forms with respect to FAEEs [10].

As in the case of fish oil transesterification, restructuration is commonly catalyzed by highly specific enzymes, such as CALB immobilized lipases. Kralovec et al. [147] described a continuous enzymatic reaction carried out in two steps: first, omega-3 FAEEs were converted to FFAs by CALB L in the presence of water; secondly, FFAs were esterified with glycerol by another lipase (CALB-FPX66). Vacuum conditions were used to remove ethanol from the system [147] and prevent omega-3 oxidation. This process could be easily implemented in $scCO_2$ media, based on the discussion in section 3.1. Moreover, omega-3 enrichment in $scCO_2$ have been already reported for menhaden oil, using Lipozyme IM-60 as the catalyst [148]. The strategy involved two consecutive reactions: (1) production of free omega-3 PUFAs from menhaden oil by urea inclusion method, and (2) transesterification reaction of menhaden oil and free omega-3 PUFAs obtained in the previous step in $scCO_2$. At optimal conditions (50 ºC, 10.3 MPa, and 1:4 substrate ratio), omega-3 PUFA content of menhaden oil increased from 20 %wt. to around 60 %wt [148], which was 40 % higher than in hexane at ambient pressure [148].

Nowadays, investigation of novel biocatalysts for the production of omega-3 structured lipids is a leading trend in the biotechnological sector. In a recent work [149], Lipozyme TL

IM has been found as the most suitable among four commercial enzymes to produce 2-monoacylglycerols from $scCO_2$-extracted oil from salmon bones. Lipase A from *C. antarctica* (CAL-A) is also very promising for the concentration of omega-3 PUFA in MAG form since it presents high activity and is able to discriminate against omega-3 PUFAs due to its non-regiospecificity and high fatty acid selectivity [150]. Ethanolysis of anchovy oil and microalgae oil (25-27 % omega-3 PUFAs) by CAL-A yielded MAGs with 90 % omega-3 PUFAs and FAEEs with application as biodiesel [150].

4. Formulation of fish oil and omega-3 concentrates in $scCO_2$

The main goal in formulation of omega-3 PUFAs is to protect them against lipid oxidation during their shelf-life. Lipid oxidation involves the formation of lipid hydroperoxides and free radicals that decompose into low-molecular-weight volatile compounds responsible for rancid odors. Oxidation products may also present potentially cytotoxic, carcinogenic and mutagenic effects [151,152]; thus, their concentration in fish oil and omega-3 supplements is strictly regulated [153]. A secondary target of formulation of fish oil and omega-3 supplements is to increase consumer's acceptance by masking the unpleasant taste and oily texture of bulk omega-3 supplements.

Preventing lipid oxidation is not an easy task due to the complex mechanisms of lipid oxidation and the multiple factors associated, such as temperature, light exposure, oxygen concentration, presence of transition metals (e.g., iron and copper), the molecular form of the lipids, and the presentation of the product [154]. Oxidation products and volatile aroma compounds can be removed in the refining and concentration steps, but the more efficient strategy is to prevent their formation. As presented in this review, $scCO_2$ technologies offer mild temperatures and inert atmosphere conditions, which are essential to avoid oxidative

degradation. The formulation step should follow the same rule, and at the same time ensure that non-oxidative conditions are maintained during shelf-life.

Addition of antioxidants is a very common practice that serves to this purpose. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and tertiary butyl hydroquinone (TBHQ) have been commonly used for the stabilization of omega-3 enriched foods [155]. Addition of ascorbic acid and its fatty acid esters (ascorbyl palmitate), in combination with metal chelators such as Ethylenediaminetetraacetic acid (EDTA) has been also reported at industrial scale [156]. However, consumer's preference for additives from natural sources compels the industry to look for novel natural antioxidants such as natural polyphenols [157], tocopherols [158,159], carotenoids such as astaxanthin [160], or plant and herb extracts [161].

The formulation step defines the final presentation of the omega-3 PUFAs. Encapsulation in gelatin soft-gel capsules is currently the most common commercial presentation of omega-3 supplements. Fish gelatin is often used as encapsulating agent, which interrelates omega-3 processing with fish protein processing in the proposed biorefinery for fish waste valorization. Other alternatives are also commercially available, such as emulsions for milk beverages and mayonnaise, gel formulations, and microencapsulation [155].

Microencapsulation has been extensively investigated for incorporation of omega-3 PUFAs in fortified foods [162]. Spray-drying is the most common method at industrial scale, although other non-thermal techniques have been proposed in recent years (Table 3), and some of them have also become commercial [163]. Among these novel alternatives, we can find microencapsulation processes based on the use of $scCO_2$, such as Supercritical Anti Solvent (SAS), Supercritical Fluid Extraction of Emulsions (SFEE), Particles from Gas-Saturated Solutions (PGSS), and PGSS-drying.

The SAS and SFEE processes are based on the quick dissolution of $scCO_2$ into an organic solution, causing the precipitation of solutes by antisolvent effect [164]. In the case of SFEE, the scale of the precipitator is reduced to an emulsion droplet, thus nanometric particles can be obtained [165]. However, considerable amounts of organic solvents and their associated drawbacks are incorporated into the process, requiring large CO_2 flows to completely remove solvent traces, and therefore high gas consumption.

Particles from gas-saturated solutions (PGSS) and PGSS-drying offer a more promising alternative for omega-3 encapsulation, since organic solvents are completely eliminated from the process. In addition, less gas is consumed since $scCO_2$ dissolves into the liquid, to give an expanded gas-saturated solution with reduced viscosity [166]. In the PGSS process (Fig. 7), the gas-saturated solution is expanded through a nozzle into a spray-tower to a lower (usually atmospheric) pressure. The sudden vaporization of the dissolved CO_2 provides a high atomization of the sprayed droplets, as well as solidification and precipitation of the particles due to the temperature decrease caused by the Joule-Thomson effect [167]. Solid particles are then separated from the gas by means of filters and/or a cyclone. Final product properties, such as particle size and morphology, can be controlled by adjusting the process parameters, such as $scCO_2$ concentration in the liquid or molten polymer, nozzle geometry, and pre- and post-expansion conditions.

Table 3. Examples of microencapsulation technologies applied to fish oil and omega-3 PUFA concentrates.

Technologies	Typical operating conditions	Typical wall materials and antioxidants	Solvent	Particle size	omega-3 loading (%wt.)	Ref.
Spray drying	T_{inlet} = 150-200 ºC; T_{outlet} = 70-100 ºC p atm; Air or N_2 atmosphere	OSA-starch, methylcellulose (MC), hydroxypropyl MC, pectin, starch, gum Arabic, lecithin, whey protein isolate, fish protein hydrolysates **Antioxidants:** tocopherols, lycopene	aqueous	50-200 μm	20-50	[168–171]
Freeze drying	T < 0ºC; vacuum conditions long processing time	Na-caseinate, carbohydrates, resistant starch	aqueous	30-100 μm	10	[172,173]
Ultrasonic atomization	RT; ultrasonic probe Ø1/32" 40 kHz, 130 W,120 V, A = 55 subsequent freeze-drying	Chitosan, maltodextrin, whey protein isolate	aqueous	0.8-15 μm	10-30	[174]
Enzymatic gelation	T = 37 ºC; 4-5 h subsequent freeze-drying	Soluble wheat protein, whey protein isolate, Na-caseinate, isolated soy protein	aqueous	30-60 μm	33	[175]
Electrospinning	RT; electric field: 20 kV	Poly(vinyl alcohol), whey protein isolate, fish protein isolate **Antioxidant:** EDTA (100 ppm)	aqueous	150-250 nm	5-10	[176]
Coacervation	T = 5-25 ºC; long processing time subsequent spray or freeze-drying	Simple: Hydroxypropyl MC Complex: Protein-polysaccharide mixtures (gelatin-gum Arabic, casein-pectin, pectin-whey protein)	aqueous	30-250 μm	20-50	[163,177]
Supercritical Antisolvent (SAS) process	T = 60 ºC; p = 15 MPa $scCO_2$ atmosphere	Hydroxypropyl methyl cellulose	organic	60 μm	20-40	[178]
Supercritical Fluid Extraction of Emulsions (SFEE)	T = 40 ºC; p = 8.0 MPa $scCO_2$ atmosphere	Polycaprolactone	organic (acetone)	<100 nm	5-15	[165]
Particles from Gas-Saturated Solutions (PGSS)	T = 40-60 ºC; p = 10-25 MPa $Ø_{nozzle}$ = 200-400 μm $scCO_2$ atmosphere	PEG 6000-8000 Solid lipid particles (SLP) **Antioxidant:** astaxanthin	aqueous/ molten polymer/ lipid	50-200 μm	15-50	[179–181]
PGSS-drying	T_{inlet} = 70-120 ºC; T_{outlet} = 30-50 ºC p = 10-15 MPa; $scCO_2$ atmosphere	Maltodextrine, OSA starch **Antioxidant:** Ascorbic acid (30 ppm)	aqueous	30-60 μm	20-50	[182,183]

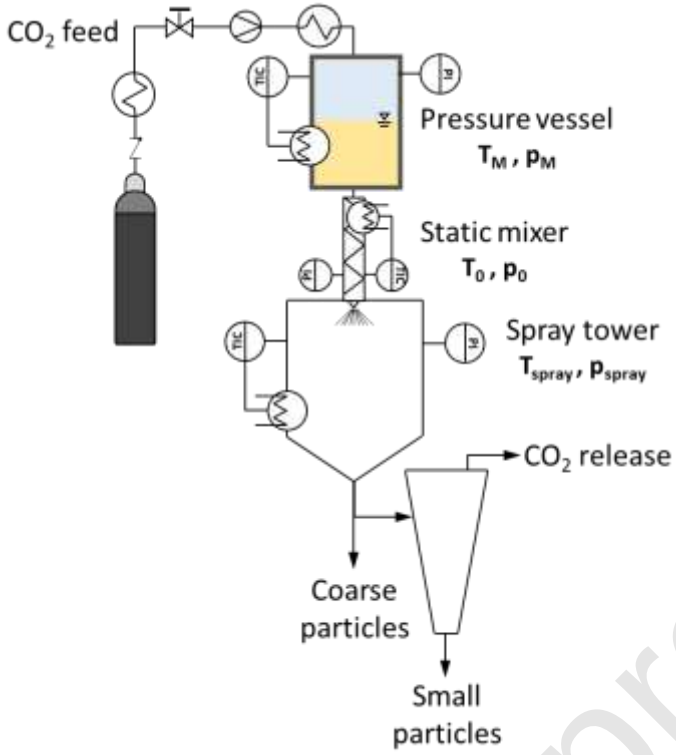


Figure 7. Schematic representation of the PGSS process. T_0, p_0: pre-expansion conditions (3-25 MPa, 40-60 ºC), T_{spray}, p_{spray}: post-expansion conditions (0.1 MPa, RT).

The PGSS process has been applied to microencapsulate FAEEs from menhaden oil into polyethylene glycol (PEG) 8000 [179], obtaining irregular particles with 16.7 % wt. FAEE and average particle size of 120 µm. Oil quality before and after PGSS process was measured through FFA determination, acid value, and peroxide value, finding no significant changes [179]. This process has been also applied to microencapsulate salmon oil with astaxanthin into PEG 6000 [180], obtaining up to 79.2 % encapsulation yield at 50 ºC, 25 MPa, 1:5 oil:polymer, and Ø400 µm nozzle. The average particle size was 166 µm and oxidative quality of the microparticles was not reported, although astaxanthin content was preserved after the PGSS process [180]. Important advances in this field can be achieved by the use of solid lipid particles (SLP) instead of PEG polymers, since hollow lipid micro- and nanoparticles with high oil loadings (up to 50 %wt.) and encapsulation efficiencies (up to 97.5 %wt.) can be obtained at 57 ºC, 20 MPa and Ø50 µm nozzle [181].

The PGSS-drying process is a modification of the PGSS process to allow processing aqueous solutions and dispersions (e.g., natural extracts, oil-in-water emulsions) [184]. PGSS-drying typically operates at pressures from 10 to 15 MPa and temperatures between 100 and 120 ºC in the static mixer, although residence time is short enough to avoid deterioration of the dissolved or dispersed bioactive compounds. Similar to classical PGSS, CO_2 is intensively mixed with the aqueous solution or dispersion in a static mixer and, subsequently, the saturated mixture is expanded through a nozzle into the spray tower, which operates at low pressure. PGSS-drying can be used as an alternative to conventional spray-drying process, especially for processing of thermolabile compounds, since the intense and deep cooling caused by the Joule-Thomson effect allows drying at lower temperatures. Nevertheless, conditions in the spray tower must be adequately controlled in order to ensure the complete drying of particles [185]. Again, phase equilibria can predict adequate drying conditions, since temperature in the spray tower and gas-to-product ratio (GPR) should be high enough to operate above the dew line of the carbon dioxide + water system [185].

Rubio-Rodríguez [182] reported the fish oil encapsulation into a maltodextrine coating using PGSS-drying, using cationic and anionic emulsifiers to obtain oil-in-water emulsions. Different operating parameters, such as emulsion formulation, nozzle geometry (wide and narrow), pre-expansion pressure (11-25.7 MPa), GPR (6-88) and expansion temperature (70-119 ºC) were studied through 21 PGSS-drying runs. Spherical microparticles with fish oil loading up to 40 mg/g and encapsulation efficiencies (EE) up to 90 % were obtained, illustrating the ability of PGSS-drying to produce fish oil microcapsules with food-grade coatings from fish oil-in-water emulsions.

In a recent work [183], a commercial omega-3 concentrate has been encapsulated into octenyl-succinic anhydride (OSA) starch and dried by PGSS-drying, obtaining microparticles with EE up to 98 % and omega-3 loading around 200 mg/g. Particle Size Distribution (PSD)

measurements and Scanning Electron Micrographs (Fig. 8) showed non-aggregated microspheres. Compared to other conventional methods such as spray-drying and freeze-drying, PGSS-drying achieved higher or similar EE and better storage stability, with lower peroxide value (PV) and TBARS (thiobarbituric acid reactive substances) content after 28 days at 4 ºC. The addition of ascorbic acid, combined with the mild processing conditions of PGSS-drying, yielded particles with a maximum PV of 2.5 meq O_2/kg oil after 28 days at 4 ºC, although higher TBARS values were found [183].

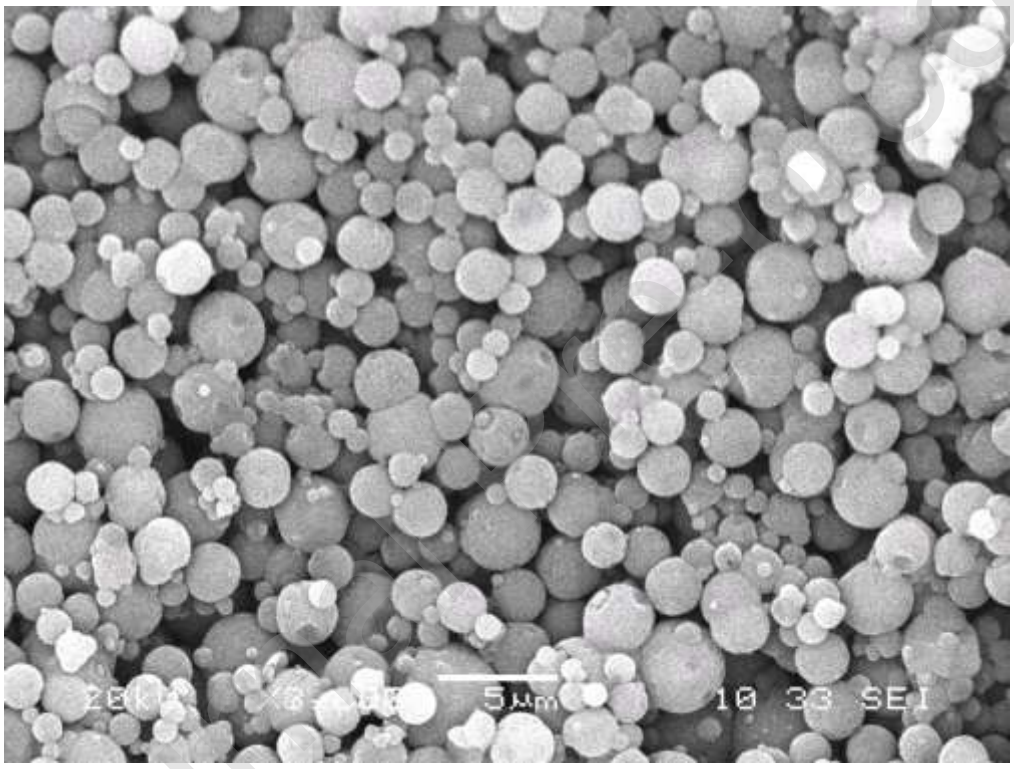


Figure 8. Scanning Electron Microscopy (SEM) magnification (3000 x) of omega-3 PUFA microparticles obtained by Particles from Gas-Saturated Solutions (PGSS) drying;
carrier: octenyl-succinic anhydride (OSA) starch; omega-3 loading: 200 mg/g.

This work demonstrated that PGSS-drying can be applied to obtain solid microparticles loaded with omega-3 PUFAs. However, the technology is still under development and further optimization is needed in order to achieve industrial-scale production. Different emulsification

methods (e.g. ultrasonic or high-pressure atomization), encapsulating materials (lecithin, caseinate, etc.), and/or addition of natural antioxidants (tocopherol, natural polyphenols, plant extracts) should be explored in order to reduce the omega-3 PUFA oxidation during emulsification and improve the stability of the PGSS-dried microparticles during their shelf life.

Outlook and Conclusions

The use of fish by-products as a source of omega-3 PUFAs has been rapidly and steadily increasing at around 15 % per year over the last decade [34]. Fish oil has become the most popular food supplement in USA and Europe, where it is used by around 20 % of adult population [10,11]. Therefore, production of human grade omega-3 concentrates constitutes a great opportunity for valorization of fish by-products, which are commonly used as low-value animal feed or disposed of as waste at high economic and environmental costs.

Development of alternative extraction, purification, concentration, and stabilization methods based on the use of green technologies and the sustainability principles, avoiding the use of organic solvents and high temperatures, is needed in order to produce non-oxidized omega-3 supplements of higher purity and efficacy. These advancements would be highly beneficial for consumers and society, since experts estimate that an adequate daily intake of omega-3 could prevent 1.5 million cardiovascular disease-attributed hospitalizations over the next five years, in Europe only [38]. Nowadays, SFE is a mature technology with several industrial applications. Among them, fish oil extraction with supercritical carbon dioxide constitutes a good alternative to conventional extraction processes in the fish oil industry such as wet-pressing or solvent extraction, since SFE has shown similar or even higher extraction yields with no solvent residues and lower amounts of impurities, especially heavy metals. Moreover, the protein fraction left after SFE can still be used as animal feed or further valorized

as a source of valuable bioactive compounds, which adheres to the zero-waste principle of the biorefinery concept.

Lipase-catalyzed transesterification of fish oil in $scCO_2$ is an attractive alternative to produce omega-3 at mild and non-oxidative conditions. Enzymatic modification of fish oil in $scCO_2$ provides enhanced reaction rates and even higher total conversion than solvent-free media, with the advantage of not using toxic organic solvents. However, kinetic models able to provide good predictions about yield and composition of the final products are needed. Enzyme stability and phase behavior of the reaction system must be considered as key parameters of the process, since they influence the reaction kinetics. Moreover, the former affects the viability of the process in continuous large-scale operation, and the latter is essential for downstream fractionation of the reaction products.

Fish oil derivatives obtained by enzymatic transesterification with ethanol could be fractionated and purified by downstream SCF technologies, such as further enzymatic concentration, supercritical fluid fractionation, and supercritical fluid chromatography. More stable and bioavailable omega-3 derivatives can be obtained with the benefits of using mild temperatures and an inert atmosphere, whereas non-omega-3 fractions may still have use as emulsifying agents in the food industry or as biofuel. Nevertheless, correct process design, considering the phase equilibria of the mixtures involved and mathematical models that describe the process, is of utmost necessity in order to replace conventional competitors such as molecular distillation.

Formulation of omega-3 into a stable form and protected from oxidation is one of the major challenges of the omega-3 industry. Encapsulation technologies using SCF such as Particles from Gas-Saturated Solutions (PGSS)-drying are capable of encapsulating high loads of omega-3 PUFAs at lower drying temperatures compared to spray drying and in an intrinsically inert atmosphere, which would avoid oxidative degradation during processing.

Furthermore, the technology is compatible with the use of natural antioxidants and plant extracts that actively protect the omega-3 PUFAs from oxidation while replacing synthetic antioxidants.

The SCF technologies presented in this review can be integrated as one of the key modules of a fish waste biorefinery, constituting a green alternative to conventional omega-3 processing. Further research should be focused on increasing omega-3 yield and purity while keeping operating costs at acceptable levels, which will make the process more economically competitive in spite of the high initial investment. Finally, the benefits of the $scCO_2$-based process should be given more prominence among the omega-3 sector and consumers, since a better-quality product obtained through a greener process constitutes a clear commercial advantage against competing technologies.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Spanish Agencia Estatal de Investigación [grant number PID2019-539 104950RB-I00 / AEI / 10.13039/501100011033] and the Junta de Castilla y León (JCyL) and the European Regional Development Fund (ERDF) [grant number BU301P18].

References

[1] C.C. Akoh, Food Lipids, Chemistry, Nutrition, and Biotechnology, 4th ed., CRC Press,

Boca Raton, 2017. doi: 10.1201/9781315151854

[2] D.E. Vance, J.E. Vance, Biochemistry of Lipids, Lipoproteins and Membranes, 5th ed., Elsevier, San Diego, 2008. doi: 10.1016/B978-044453219-0.50001-5.

[3] N. Hastings, M. Agaba, D.R. Tocher, M.J. Leaver, J.R. Dick, J.R. Sargent, A.J. Teale, A vertebrate fatty acid desaturase with Δ5 and Δ6 activities, Proc. Natl. Acad. Sci. USA 98 (2001) 14304-14309. doi: 10.1073/pnas.251516598

[4] A.P. Simopoulos, Omega-3 fatty acids in health and disease and in growth and development, Am. J. Clin. Nutr. 54 (1991) 438–463. doi: 10.1093/ajcn/54.3.438.

[5] P.M. Kris-Etherton, W.S. Harris, L.J. Appel, Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease, Circulation. 106 (2002) 2747–2757. doi: 10.1161/01.CIR.0000038493.65177.94.

[6] P.C. Calder, Mechanisms of action of (n-3) fatty acids, J. Nutr. 142 (2012) 592S-599S. doi: 10.3945/jn.111.155259.

[7] S. Kalmijn, L.J. Launer, A. Ott, J.C.M. Witteman, A. Hofman, M.M.B. Breteler, Dietary fat intake and the risk of incident dementia in the Rotterdam study, Ann. Neurol. 42 (1997) 776–782. doi: 10.1002/ana.410420514.

[8] M.C. Morris, D.A. Evans, J.L. Bienias, C.C. Tangney, D.A. Bennett, R.S. Wilson, N. Aggarwal, J. Schneider, Consumption of fish and n-3 fatty acids and risk of incident Alzheimer disease, Arch. Neurol. 60 (2003) 940–946. doi: 10.1001/archneur.60.7.940.

[9] F. Calon, G.P. Lim, F. Yang, T. Morihara, B. Teter, O. Ubeda, P. Rostaing, A. Triller, N. Salem Jr., K.H. Ashe, S.A. Frautschy, G.M. Cole, Docosahexaenoic acid protects from dendritic pathology in an Alzheimer's disease mouse model, Neuron. 43 (2004) 633–645. doi: 10.1016/j.neuron.2004.08.013.

[10] R. Ciriminna, F. Meneguzzo, R. Delisi, M. Pagliaro, Enhancing and improving the extraction of omega-3 from fish oil, Sustain. Chem. Pharm. 5 (2017) 54–59. doi: 10.1016/j.scp.2017.03.001.

[11] Rubio-Rodríguez, S. Beltrán, I. Jaime, S.M. de Diego, M.T. Sanz, J. Rovira-Carballido, Production of omega-3 polyunsaturated fatty acid concentrates: a review, Innov. Food Sci. Emerg. Technol. 11 (2010) 1–12. doi: 10.1016/j.ifset.2009.10.006.

[12] L.C. Boyd, M.F. King, B. Sheldon, A rapid method for determining the oxidation of n-3 fatty acids, J. Am. Oil Chem. Soc. 69 (1992) 325–330. doi: 10.1007/BF02636060.

[13] L.D. Lawson, B.G. Hughes, Human absorption of fish oil fatty acids as triacylglycerols, free acids, or ethyl esters, Biochem. Biophys. Res. Commun. 152 (1988) 328–335. doi: 10.1016/S0006-291X(88)80718-6.

[14] FAO, The production of fish meal and oil, FAO Fish Tech. 142 (1986).

[15] G. Brunner, Gas Extraction: An Introduction to Fundamentals of Supercritical Fluids and the Application to Separation Processes, Steinkopff-Verlag Heidelberg, Springer, Darmstadt, New York, 1994. doi: 10.1007/978-3-662-07380-3

[16] S. Angus, B. Armstrong, K.M. de Reuck, International Thermodynamic Tables of the Fluid State - 3, IUPAC Project Centre, Imperial College, Pergamon Press, London, 1976. doi: 10.1016/C2013-0-10127-4

[17] L. Fiori, M. Volpe, M. Lucian, A. Anesi, M. Manfrini, G. Guella, From fish waste to omega-3 concentrates in a biorefinery concept, Waste Biomass Valorization. 8 (2017) 2609–2620. doi: 10.1007/s12649-017-9893-1.

[18] M. Herrero, E. Ibañez, Green extraction processes, biorefineries and sustainability: Recovery of high added-value products from natural sources, J. Supercrit. Fluids. 134 (2018) 252–259. doi: 10.1016/j.supflu.2017.12.002.

[19] K. Srinivas, J.W. King, Developments in the Processing of Foods and Natural Products Using Pressurized Fluids, in: Alternatives to Conventional Food Processing, 2nd ed., The Royal Society of Chemistry, 2018: pp. 196–250. doi: 10.1039/9781782626596-00196.

[20] H.G. Kristinsson, B.A. Rasco, Fish protein hydrolysates: production, biochemical, and functional properties, Crit. Rev. Food Sci. Nutr. 40 (2000) 43–81. doi: 10.1080/10408690091189266.

[21] A. Jafarpour, R.M. Gomes, S. Gregersen, J.J. Sloth, C. Jacobsen, A.D. Moltke Sørensen, Characterization of cod (*Gadus morhua*) frame composition and its valorization by enzymatic hydrolysis, J. Food Compos. Anal. 89 (2020) 103469. doi: 10.1016/j.jfca.2020.103469.

[22] H.K. Kim, Y.H. Kim, Y.J. Kim, H.J. Park, N.H. Lee, Effects of ultrasonic treatment on collagen extraction from skins of the sea bass *Lateolabrax japonicus*, Fish. Sci. 78 (2012) 485–490. doi: 10.1007/s12562-012-0472-x.

[23] G. He, X. Yan, X. Wang, Y. Wang, Extraction and structural characterization of collagen from fishbone by high intensity pulsed electric fields, J. Food Process Eng. 42 (2019) 1–9. doi: 10.1111/jfpe.13214.

[24] R. Melgosa, E. Trigueros, M.T. Sanz, M. Cardeira, L. Rodrigues, N. Fernández, A.A. Matias, M.R. Bronze, M. Marques, A. Paiva, P. Simões, Supercritical CO_2 and subcritical water technologies for the production of bioactive extracts from sardine (*Sardina pilchardus*) waste, J. Supercrit. Fluids. 164 (2020). doi: 10.1016/j.supflu.2020.104943.

[25] S. V. Dorozhkin, Bioceramics of calcium orthophosphates, Biomaterials. 31 (2010) 1465–1485. doi: 10.1016/j.biomaterials.2009.11.050.

[26] M. Mucalo, ed., Hydroxyapatite (HAp) for Biomedical Applications, Woodhead Publishing, 2015. doi: 10.1016/C2013-0-16440-9

[27] M. Ozawa, S. Suzuki, Microstructural development of natural hydroxyapatite originated from fish-bone waste through heat treatment, J. Am. Ceram. Soc. 85 (2004) 1315–1317. doi: 10.1111/j.1151-2916.2002.tb00268.x.

[28] C. Piccirillo, M.F. Silva, R.C. Pullar, I. Braga Da Cruz, R. Jorge, M.M.E. Pintado, P.M.L. Castro, Extraction and characterisation of apatite- and tricalcium phosphate-based materials from cod fish bones, Mater. Sci. Eng. C. 33 (2013) 103–110. doi: 10.1016/j.msec.2012.08.014.

[29] J. Mataix-Verdú (ed.), Tabla de Composición de Alimentos, 5th ed., Editorial Universidad de Granada, 2009.

[30] M. Canli, G. Atli, The relationships between heavy metal (Cd, Cr, Cu, Fe, Pb, Zn) levels and the size of six Mediterranean fish species, Environ. Pollut. 121 (2003) 129–136. doi: 10.1016/S0269-7491(02)00194-X.

[31] M. Sharp, A. Lopata, Fish Allergy: In review, Clinic. Rev. Allerg. Immunol. 46 (2014) 258–271. doi: 10.1007/s12016-013-8363-1.

[32] S.E. Schober, T.H. Sinks, R.L. Jones, P.M. Bolger, M. McDowell, J. Osterloh, E.S. Garrett, R.A. Canady, C.F. Dillon, Y. Sun, C.B. Joseph, K.R. Mahaffey, Blood mercury levels in US children and women of childbearing age, 1999-2000, J. Am. Med. Assoc. 289 (2003) 1667–1674. doi: 10.1001/jama.289.13.1667.

[33] Solaesa, S.L. Bucio, M.T. Sanz, S. Beltrán, S. Rebolleda, Characterization of triacylglycerol composition of fish oils by using chromatographic techniques, J. Oleo Sci. 63 (2014) 449–460. doi: 10.5650/jos.ess13202.

[34] I.H. Pike, A. Jackson, Fish oil: Production and use now and in the future, Lipid Technol. 22 (2010) 59–61. doi: 10.1002/lite.201000003.

[35] M. Van Dijk, J. Vitek (Eds.), Fish oil: Production, Consumption and Health Benefits, Nova Science Publishing Inc., 2012.

[36] K. Ivanovs, D. Blumberga, Extraction of fish oil using green extraction methods: A short review, Energy Procedia. 128 (2017) 477–483. doi: 10.1016/j.egypro.2017.09.033.

[37] N. Rubio-Rodríguez, S.M. de Diego, S. Beltrán, I. Jaime, M.T. Sanz, J. Rovira, Supercritical fluid extraction of the omega-3 rich oil contained in hake (*Merluccius capensis–Merluccius paradoxus*) by-products: Study of the influence of process

parameters on the extraction yield and oil quality, J. Supercrit. Fluids. 47 (2008) 215–226. doi: 10.1016/j.supflu.2008.07.007.

[38] N. Rubio-Rodríguez, S.M. de Diego, S. Beltrán, I. Jaime, M.T. Sanz, J. Rovira, Supercritical fluid extraction of fish oil from fish by-products: A comparison with other extraction methods, J. Food Eng. 109 (2012) 238–248. doi: 10.1016/j.jfoodeng.2011.10.011.

[39] S. Ferdosh, I.S.M. Zaidul, N.N.A. Norulaini, S. Jinap, M.H.A. Jahurul, M.A.K. Omar, Storage stability and quality of polyunsaturated fatty acid rich oil fraction from Longtail tuna (*Thunnus tonggol*) head using supercritical extraction, CyTA - J. Food. 12 (2014) 183–188. doi: 10.1080/19476337.2013.811296.

[40] S. Ferdosh, Z.I. Sarker, N. Norulaini, A. Oliveira, K. Yunus, A.J. Chowdury, J. Akanda, M. Omar, Quality of tuna fish oils extracted from processing the by-products of three species of neritic tuna using supercritical carbon dioxide, J. Food Process. Preserv. 39 (2015) 432–441. doi: 10.1111/jfpp.12248.

[41] P. Hajeb, J. Selamat, L. Afsah-Hejri, N.A. Mahyudin, S. Shakibazadeh, M.Z.I. Sarker, Effect of supercritical fluid extraction on the reduction of toxic elements in fish oil compared with other extraction methods, J. Food Prot. 78 (2015) 172–179. doi: 10.4315/0362-028X.JFP-14-248.

[42] S. Ferdosh, I.S.M. Zaidul, S. Jinap, A.M. Yazid, A. Khatib, N.A.N. Norulaini, Fatty acid compositions of fish oil extracted from different parts of Indian mackerel (*Rastrelliger kanagurta*) using various techniques of supercritical CO_2 extraction, Food Chem. 120 (2010) 879–885. doi: 10.1016/j.foodchem.2009.10.055.

[43] S. Ferdosh, I.S.M. Zaidul, S. Jinap, M.H.A. Jahurul, A. Khatib, N.A.N. Norulaini, Extraction of fish oil from the skin of Indian mackerel using supercritical fluids, J. Food Eng. 99 (2010) 63–69. doi: 10.1016/j.jfoodeng.2010.01.038.

[44] S. Ferdosh, M.Z. Islam Sarker, N.N.N. Abd Rahman, J. Selamat, M.R. Karim, T.A. Razak, M.O. Abd Kadir, Fish oil recovery from viscera of indian mackerel (*Rastrelliger kanagurta*) by supercritical fluid: An optimization approach, J. Chinese Chem. Soc. 59 (2012) 1421–1429. doi: 10.1002/jccs.201100235.

[45] M.Z.I. Sarker, J. Selamat, A.S.M.A. Habib, S. Ferdosh, M.J.H. Akanda, J.M. Jaffri, Optimization of supercritical CO_2 extraction of fish oil from viscera of African Catfish (*Clarias gariepinus*), Int. J. Mol. Sci. 13 (2012) 11312–11322. doi: 10.3390/ijms130911312.

[46] S. Hao, H. Hui, L. Li, X. Yang, J. Cen, W. Lin, Y. Wei, Extraction of fish oil from the

muscle of sturgeon using supercritical fluids, Adv. Mater. Res. 655–657 (2013) 1975–1981. doi: 10.4028/www.scientific.net/AMR.655-657.1975.

[47] S. Hao, Y. Wei, L. Li, X. Yang, J. Cen, H. Huang, W. Lin, X. Yuan, The effects of different extraction methods on composition and storage stability of sturgeon oil, Food Chem. 173 (2015) 274–282. doi: 10.1016/j.foodchem.2014.09.154.

[48] S. Kuvendziev, K. Lisichkov, Z. Zeković, M. Marinkovski, Z.H. Musliu, Supercritical fluid extraction of fish oil from common carp (*Cyprinus carpio* L.) tissues, J. Supercrit. Fluids. 133 (2018) 528–534. doi: 10.1016/j.supflu.2017.11.027.

[49] A.C. Aguiar, J. V Visentainer, J. Martínez, Extraction from striped weakfish (*Cynoscion striatus*) wastes with pressurized CO_2: Global yield, composition, kinetics and cost estimation, J. Supercrit. Fluids. 71 (2012) 1–10. doi: 10.1016/j.supflu.2012.07.005.

[50] B. Homayooni, M.A. Sahari, M. Barzegar, Concentrations of omega-3 fatty acids from rainbow sardine fish oil by various methods, Int. Food Res. J. 21 (2014) 743–748.

[51] J.-H. Lee, A.K.M. Asaduzzaman, J.-H. Yun, J.-H. Yun, B.-S. Chun, Characterization of the yellow croaker Larimichthys polyactis muscle oil extracted with supercritical carbon dioxide and an organic solvent, Fish. Aquat. Sci. 15 (2012) 275–281. doi: 10.5657/FAS.2012.0275.

[52] L. Fiori, M. Solana, P. Tosi, M. Manfrini, C. Strim, G. Guella, Lipid profiles of oil from trout (*Oncorhynchus mykiss*) heads, spines and viscera: Trout by-products as a possible source of omega-3 lipids?, Food Chem. 134 (2012) 1088–1095. doi: 10.1016/j.foodchem.2012.03.022.

[53] I.A. Adeoti, K. Hawboldt, Experimental and mass transfer modelling of oil extraction from salmon processing waste using SC-CO_2, J. Supercrit. Fluids. 104 (2017). doi: 10.1016/j.supflu.2015.06.003.

[54] M. Haq, R. Ahmed, Y.-J. Cho, B.-S. Chun, Quality properties and bio-potentiality of edible oils from atlantic salmon by-products extracted by supercritial carbon dioxide and conventional methods, Waste Biomass Valorization. 8 (2017) 1953–1967. doi: 10.1007/s12649-016-9710-2.

[55] S.L. Bucio, M.T. Sanz, S. Beltrán, R. Melgosa, Á.G. Solaesa, M.O. Ruiz, Study of the influence of process parameters on liquid and supercritical CO_2 extraction of oil from rendered materials: Fish meal and oil characterization, J. Supercrit. Fluids. 107 (2016) 270–277. doi: 10.1016/j.supflu.2015.09.019.

[56] R.B. Gupta, J.-J. Shim, Solubility in supercritical carbon dioxide, CRC Press, Boca Raton, 2007. doi: 10.1201/9781420005998.

[57] R. Gallego, M. Bueno, M. Herrero, Sub- and supercritical fluid extraction of bioactive compounds from plants, food-by-products, seaweeds and microalgae – An update, Trends. Anal. Chem. 116 (2019) 198–213. doi: 10.1016/j.trac.2019.04.030

[58] M. Goto, M. Sato, T. Hirose, Extraction of peppermint oil by supercritical carbon dioxide, J. Chem. Eng. Japan. 26 (1993) 401–407. doi: 10.1252/jcej.26.401.

[59] N.T. Dunford, M. Goto, F. Temelli, Modeling of oil extraction with supercritical CO_2 from Atlantic mackerel (*Scomber scombrus*) at different moisture contents, J. Supercrit. Fluids. 13 (1998) 303–309. doi: 10.1016/S0896-8446(98)00064-3.

[60] H. Sovová, Mathematical model for supercritical fluid extraction of natural products and extraction curve evaluation, J. Supercrit. Fluids. 33 (2005) 35–52. doi: 10.1016/j.supflu.2004.03.005.

[61] M. Kandiah, M. Spiro, Extraction of ginger rhizome: kinetic studies with supercritical carbon dioxide, Int. J. Food Sci. Technol. 25 (1990) 328–338. doi: 10.1111/j.1365-2621.1990.tb01089.x.

[62] E. Schmeisser, W. Goessler, N. Kienzl, K.A. Francesconi, Direct measurement of lipid-soluble arsenic species in biological samples with HPLC-ICPMS, Analyst. 130 (2005) 948–955. doi: 10.1039/b502445e.

[63] H.H. Harris, I.J. Pickering, G.N. George, The chemical form of mercury in fish, Science 29 (2003) 1203. doi: 10.1126/science.1085941.

[64] F. De Meester, R.R. Watson, S. Zibadi, (eds.) Omega-6/3 fatty acids: Functions, sustainability strategies and perspectives, Humana Press, 2013. doi: 10.1007/978-1-62703-215-5.

[65] O. Catchpole, T. Moreno, F. Montañes, S. Tallon, Perspectives on processing of high value lipids using supercritical fluids, J. Supercrit. Fluids. 134 (2018) 260–268. doi: 10.1016/j.supflu.2017.12.001.

[66] C.G. Pereira, M.A.A. Meireles, Economic analysis of rosemary, fennel and anise essential oils obtained by supercritical fluid extraction, Flavour Fragr. J. 22 (2007) 407–413. doi: 10.1002/ffj.

[67] J. Čmolík, J. Pokorný, Physical refining of edible oils, Eur. J. Lipid Sci. Technol. 102 (2000) 472–486. doi: 10.1002/1438-9312(200008)102:7<472::AID-EJLT472>3.0.CO;2-Z.

[68] B.M. Bhosle, R. Subramanian, New approaches in deacidification of edible oils - A review, J. Food Eng. 69 (2005) 481–494. doi: 10.1016/j.jfoodeng.2004.09.003.

[69] L.L. Lai, K.C. Soheili, W.E. Artz, Deacidification of soybean oil using membrane

processing and subcritical carbon dioxide, J. Am. Oil Chem. Soc. 85 (2008) 189–196. doi: 10.1007/s11746-007-1182-x.

[70] O.J. Catchpole, J.B. Grey, K.A. Noermark, Solubility of fish oil components in supercritical CO_2 and CO_2 + Ethanol Mixtures, J. Chem. Eng. Data. 43 (1998) 1091–1095. doi: 10.1021/je980184l.

[71] A. Kawashima, R. Iwakiri, K. Honda, Experimental study on the removal of dioxins and coplanar polychlorinated biphenyls (PCBs) from fish oil, J. Agric. Food Chem. 54 (2006) 10294–10299. doi: 10.1021/jf061962d.

[72] M. Jakobsson, B. Sivik, P.-A. Bergqvist, B. Strandberg, M. Hjelt, C. Rappe, Extraction of dioxins from cod liver oil by supercritical carbon dioxide, J. Supercrit. Fluids. 4 (1991) 118–123. doi: 10.1016/0896-8446(91)90039-9.

[73] M. Jakobsson, B. Sivik, P.A. Bergqvist, B. Strandberg, C. Rappe, Counter-current extraction of dioxins from cod liver oil by supercritical carbon dioxide, J. Supercrit. Fluids. 7 (1994) 197–200. doi: 10.1016/0896-8446(94)90026-4.

[74] O.J. Catchpole, J.B. Grey, K.A. Noermark, Fractionation of fish oils using supercritical CO_2 and CO_2 + ethanol mixtures, J. Supercrit. Fluids. 19 (2000) 25–37. doi: 10.1016/S0896-8446(00)00075-9

[75] A. Kawashima, S. Watanabe, R. Iwakiri, K. Honda, Removal of dioxins and dioxin-like PCBs from fish oil by countercurrent supercritical CO_2 extraction and activated carbon treatment, Chemosphere. 75 (2009) 788–794. doi: 10.1016/j.chemosphere.2008.12.057.

[76] N. Ding, Y. Xue, X. Tang, Z.-M. Sun, T. Yanagita, C.-H. Xue, Y.-M. Wang, Short-term effects of different fish oil formulations on tissue absorption of docosahexaenoic acid in mice fed high- and low-fat diets, J. Oleo Sci. 62 (2013) 883–891. doi: 10.5650/jos.62.883.

[77] S.L. Bucio, Á.G. Solaesa, M.T. Sanz, R. Melgosa, S. Beltrán, H. Sovová, Kinetic study for the ethanolysis of fish oil catalyzed by Lipozyme® 435 in different reaction media, J. Oleo Sci. 441 (2015) 431–441. doi: 10.5650/jos.ess14263

[78] Á.G. Solaesa, M.T. Sanz, R. Melgosa, S.L. Bucio, S. Beltrán, Glycerolysis of sardine oil catalyzed by a water dependent lipase in different *tert*-alcohols as reaction medium, Grasas y Aceites. 66 (2015) e102. doi: 10.3989/gya.0238151.

[79] Á.G. Solaesa, M.T. Sanz, S. Beltrán, R. Melgosa, Kinetic study and kinetic parameters of lipase-catalyzed glycerolysis of sardine oil in a homogeneous medium, Chinese J. Catal. 37 (2016) 1–11. doi: 10.1016/S1872.

[80] Á.G. Solaesa, S.L. Bucio, M.T. Sanz, S. Beltrán, S. Rebolleda, Liquid-liquid equilibria

for systems glycerol+sardine oil+tert-alcohols, Fluid Phase Equilib. 356 (2013) 284–290. doi: 10.1016/j.fluid.2013.07.026.

[81] S.L. Bucio, Á.G. Solaesa, M.T. Sanz, S. Beltrán, R. Melgosa, Liquid-liquid equilibrium for ethanolysis systems of fish oil, J. Chem. Eng. Data. 58 (2013) 3118–3124. doi: 10.1021/je400573u.

[82] Á.G. Solaesa, M.T. Sanz, R. Melgosa, S. Beltrán, Substrates emulsification process to improve lipase-catalyzed sardine oil glycerolysis in different systems. Evaluation of lipid oxidation of the reaction products, Food Res. Int. 100 (2017) 572–578. doi: 10.1016/j.foodres.2017.07.048.

[83] P. Lisboa, A.R. Rodrigues, J.L. Martín, P. Simões, S. Barreiros, A. Paiva, Economic analysis of a plant for biodiesel production from waste cooking oil via enzymatic transesterification using supercritical carbon dioxide, J. Supercrit. Fluids. 85 (2014) 31–40. doi: 10.1016/j.supflu.2013.10.018.

[84] R. Melgosa, M. Teresa Sanz, Á.G. Solaesa, E. De Paz, S. Beltrán, D.L. Lamas, Supercritical carbon dioxide as solvent in the lipase-catalyzed ethanolysis of fish oil: Kinetic study, J. CO_2 Util. 17 (2017) 170–179. doi: 10.1016/j.jcou.2016.11.011.

[85] T. Matsuda, R. Kanamaru, K. Watanabe, Control on enantioselectivity with pressure for lipase-catalyzed esterification in supercritical carbon dioxide, Tetrahedron Lett. 42 (2001) 8319–8321. doi: 10.1016/S0040-4039(01)01785-3

[86] H.R. Hobbs, N.R. Thomas, Biocatalysis in supercritical fluids, in fluorous solvents, and under solvent-free conditions., Chem. Rev. 107 (2007) 2786–820. doi: 10.1021/cr0683820.

[87] Ž. Knez, Enzymatic reactions in dense gases, J. Supercrit. Fluids. 47 (2009) 357–372. doi: 10.1016/j.supflu.2008.11.012.

[88] G.R. Akien, M. Poliakoff, A critical look at reactions in class I and II gas-expanded liquids using CO_2 and other gases, Green Chem. 11 (2009) 1083. doi: 10.1039/b904097h.

[89] T. Matsuda, Recent progress in biocatalysis using supercritical carbon dioxide., J. Biosci. Bioeng. 115 (2013) 233–41. doi: 10.1016/j.jbiosc.2012.10.002.

[90] H. Briongos, A.E. Illera, M.T. Sanz, R. Melgosa, S. Beltrán, Á.G. Solaesa, Effect of high pressure carbon dioxide processing on pectin methylesterase activity and other orange juice properties, LWT - Food Sci. Technol. 74 (2016). doi: 10.1016/j.lwt.2016.07.069.

[91] Ó. Benito-román, M.T. Sanz, R. Melgosa, E. de Paz, I. Escudero, Studies of polyphenol oxidase inactivation by means of high pressure carbon dioxide (HPCD), J. Supercrit. Fluids. 147 (2019) 310–321. doi: 10.1016/j.supflu.2018.07.026.

[92] A.E. Illera, M.T. Sanz, S. Beltrán, R. Melgosa, Á.G. Solaesa, M.O. Ruiz, Evaluation of HPCD batch treatments on enzyme inactivation kinetics and selected quality characteristics of cloudy juice from Golden delicious apples, J. Food Eng. 221 (2018) 141–150. doi: 10.1016/j.jfoodeng.2017.10.017.

[93] A.E. Illera, M.T. Sanz, E. Trigueros, S. Beltrán, R. Melgosa, Effect of high pressure carbon dioxide on tomato juice : Inactivation kinetics of pectin methylesterase and polygalacturonase and determination of other quality parameters, J. Food Eng. 239 (2018) 64–71. doi: 10.1016/j.jfoodeng.2018.06.027.

[94] Benito-Román, M.T. Sanz, A.E. Illera, R. Melgosa, S. Beltrán, Polyphenol oxidase (PPO) and pectin methylesterase (PME) inactivation by high pressure carbon dioxide (HPCD) and its applicability to liquid and solid natural products, Catal. Today. 346, (2020) 112–120. doi: 10.1016/j.cattod.2018.12.051.

[95] Ó. Benito-Román, M.T. Sanz, A.E. Illera, R. Melgosa, J.M. Benito, S. Beltrán, Pectin methylesterase inactivation by High Pressure Carbon Dioxide (HPCD), J. Supercrit. Fluids. 145 (2019) 111–121. doi: 10.1016/j.supflu.2018.11.009.

[96] R. Melgosa, M.T. Sanz, Á.G. Solaesa, S.L. Bucio, S. Beltrán, Enzymatic activity and conformational and morphological studies of four commercial lipases treated with supercritical carbon dioxide, J. Supercrit. Fluids. 97 (2015) 51–62. doi: 10.1016/j.supflu.2014.11.003.

[97] A. Gießauf, T. Gamse, A simple process for increasing the specific activity of porcine pancreatic lipase by supercritical carbon dioxide treatment, J. Mol. Catal. B Enzym. 9 (2000) 57–64. doi: 10.1016/S1381-1177(99)00084-3

[98] Y. Liu, D. Chen, S. Wang, Effect of sub- and super-critical CO_2 pretreatment on conformation and catalytic properties evaluation of two commercial enzymes of CALB and Lipase PS, J. Chem. Technol. Biotechnol. 88 (2013) 1750–1756. doi: 10.1002/jctb.4037.

[99] D. Oliveira, A.C. Feihrmann, A.F. Rubira, M.H. Kunita, C. Dariva, J.V. Oliveira, Assessment of two immobilized lipases activity treated in compressed fluids, J. Supercrit. Fluids. 38 (2006) 373–382. doi: 10.1016/j.supflu.2005.12.007.

[100] K. Nakamura, Biochemical reactions in supercritical fluids, Trends Biotechnol. 8 (1990) 288–292. doi: 10.1016/0167-7799(90)90200-H.

[101] S. V Kamat, E.J. Beckman, A.J. Russell, Enzyme activity in supercritical fluids, Crit. Rev. Biotechnol. 15 (1995) 41–71. doi: 10.3109/07388559509150531.

[102] Z. Wimmer, M. Zarevúcka, A review on the effects of supercritical carbon dioxide on

enzyme activity., Int. J. Mol. Sci. 11 (2010) 233–53. doi: 10.3390/ijms11010233.

[103] K. Rezaei, E. Jenab, F. Temelli, Effects of water on enzyme performance with an emphasis on the reactions in supercritical fluids., Crit. Rev. Biotechnol. 27 (2007) 183–95. doi: 10.1080/07388550701775901.

[104] O.N. Ciftci, F. Temelli, Continuous biocatalytic conversion of the oil of corn distiller's dried grains with solubles to fatty acid methyl esters in supercritical carbon dioxide, Biomass and Bioenergy. 54 (2013) 140–146. doi: 10.1016/j.biombioe.2013.03.031.

[105] Ö. Güçlü-Üstündağ, F. Temelli, Solubility behavior of ternary systems of lipids in supercritical carbon dioxide, J. Supercrit. Fluids. 38 (2006) 275–288. doi: 10.1016/j.supflu.2005.12.009.

[106] Ö. Güçlü-Üstündağ, F. Temelli, Solubility behavior of ternary systems of lipids, cosolvents and supercritical carbon dioxide and processing aspects, J. Supercrit. Fluids. 36 (2005) 1–15. doi: 10.1016/j.supflu.2005.03.002.

[107] D. Geana, R. Steiner, Calculation of phase equilibrium in supercritical extraction of C54 triglyceride (rapeseed oil), J. Supercrit. Fluids. 8 (1995) 107–118. doi: 10.1016/0896-8446(95)90023-3.

[108] D. Peng, D.B. Robinson, A new two-constant equation of state, Ind. Eng. Chem. Fundam. 15 (1976) 59–64. doi: 10.1021/i160057a011

[109] P.M.M. Ndiaye, E. Franceschi, D. Oliveira, C. Dariva, F.W.W. Tavares, J.V. Oliveira, Phase behavior of soybean oil, castor oil and their fatty acid ethyl esters in carbon dioxide at high pressures, J. Supercrit. Fluids. 37 (2006) 29–37. doi: 10.1016/j.supflu.2005.08.002.

[110] S.H. Huang, M. Radosz, Equation of state for small, large, polydisperse, and associating molecules: Extension to fluid mixtures, Ind. Eng. Chem. Res. 30 (1991) 1994–2005. doi: 10.1021/ie00107a014.

[111] E.J. Hernández, G.D. Mabe, F.J. Señoráns, G. Reglero, T. Fornari, High-pressure phase equilibria of the pseudoternary mixture sunflower oil + ethanol + carbon dioxide, J. Chem. Eng. Data. 53 (2008) 2632–2636. doi: 10.1021/je800523j.

[112] S. Skjold-Jørgensen, Group contribution equation of state (GC-EOS): A predictive method for phase equilibrium computations over wide ranges of temperature and pressures up to 30 MPa, Ind. Eng. Chem. Res. 27 (1988) 110–118. doi: 10.1021/ie00073a021.

[113] I. Dalmolin, A.A. Rigo, M.L. Corazza, P.M. Ndiaye, M.A.A. Meireles, E.A.C. Batista, J.V. Oliveira, Phase behaviour and thermodynamic modelling for the system (grape seed

oil+carbon dioxide+ethanol) at high pressures, J. Chem. Thermodyn. 68 (2014) 71–74. doi: 10.1016/j.jct.2013.09.002.

[114] R. Melgosa, M.T. Sanz, Á.G. Solaesa, S. Beltrán, Phase behaviour of the pseudo-ternary system carbon dioxide + ethanol + fish oil at high pressures, J. Chem. Thermodyn. 115 (2017) 106–113. doi: 10.1016/j.jct.2017.07.032.

[115] R. Melgosa, M.T. Sanz, Ó. Benito-Román, A.E. Illera, S. Beltrán, Supercritical CO_2 assisted synthesis and concentration of monoacylglycerides rich in omega-3 polyunsaturated fatty acids, J. CO_2 Util. 31 (2019) 65–74. doi: 10.1016/j.jcou.2019.02.015.

[116] A. Marty, W. Chulalaksananukul, J.S. Condoret, R.M. Willemot, G. Durand, Comparison of lipase-catalysed esterification in supercritical carbon dioxide and in n-hexane, Biotechnol. Lett. 12 (1990) 11–16. doi: 10.1007/BF01028485.

[117] A. Marty, W. Chulalaksananukul, R.M. Willemot, J.S. Condoret, Kinetics of lipase-catalyzed esterification in supercritical CO_2, Biotechnol. Bioeng. 39 (1992) 273–280. doi: 10.1002/bit.260390304

[118] H. Gunnlaugsdottir, B. Sivik, Lipase-catalyzed alcoholysis of cod liver oil in supercritical carbon dioxide, J. Am. Oil Chem. Soc. 72 (1995) 399–405. doi: 10.1007/BF02636078.

[119] H. Gunnlaugsdottir, B. Sivik, Lipase-catalyzed alcoholysis with supercritical carbon dioxide extraction. 1: Influence of flow rate, J. Am. Oil Chem. Soc. 74 (1997) 1483–1490. doi: 10.1007/s11746-997-0258-y

[120] H. Gunnlaugsdottir, A.Å. Karlsson, B. Sivik, Lipase-catalyzed alcoholysis with supercritical carbon dioxide extraction. 2: Phase behavior, J. Am. Oil Chem. Soc. 74 (1997) 1491–1494. doi: 10.1007/s11746-997-0259-x

[121] J.V. Oliveira, D. Oliveira, Kinetics of the enzymatic alcoholysis of palm kernel oil in supercritical CO_2, Ind. Eng. Chem. Res. 39 (2000) 4450–4454. doi: 10.1021/ie990865p.

[122] D. Oliveira, J.V. Oliveira, Enzymatic alcoholysis of palm kernel oil in n-hexane and $SCCO_2$, J. Supercrit. Fluids. 19 (2001) 141–148. doi: 10.1016/S0896-8446(00)00068-1.

[123] S.-K. Shin, J.-E. Sim, H. Kishimura, B.-S. Chun, Characteristics of menhaden oil ethanolysis by immobilized lipase in supercritical carbon dioxide, J. Ind. Eng. Chem. 18 (2012) 546–550. doi: 10.1016/j.jiec.2011.11.065.

[124] M.-K. Roh, Y.D. Kim, J.-S. Choi, Transesterification of fish oil by lipase immobilized in supercritical carbon dioxide, Fish. Sci. 81 (2015) 1113–1125. doi: 10.1007/s12562-015-0926-z.

[125] Á.G. Solaesa, M.T. Sanz, M. Falkeborg, S. Beltrán, Z. Guo, Production and concentration of monoacylglycerols rich in omega-3 polyunsaturated fatty acids by enzymatic glycerolysis and molecular distillation, Food Chem. 190 (2016) 960–967. doi: 10.1016/j.foodchem.2015.06.061.

[126] K. Zosel, Praktische Anwendungen der Stofftrennung mit überkritischen Gasen, Angew. Chemie. 90 (1978) 748–755. doi: 10.1002/ange.19780901005.

[127] W. Eisenbach, Supercritical fluid extraction: A film demonstration (Invited Lecture), Berichte Der Bunsengesellschaft Für Phys. Chemie. 88 (1984) 882–887. doi: 10.1002/bbpc.19840880922.

[128] W.B. Nilsson, E.J. Gauglitz, J.K. Hudson, V.F. Stout, J. Spinelli, Fractionation of menhaden oil ethyl esters using supercritical fluid CO_2, J. Am. Oil Chem. Soc. 65 (1988) 109–117. doi: 10.1007/BF02542560.

[129] W.B. Nilsson, E.J. Gauglitz, J.K. Hudson, Supercritical fluid fractionation of fish oil esters using incremental pressure programming and a temperature gradient, J. Am. Oil Chem. Soc. 66 (1989) 1596–1600. doi: 10.1007/BF02636184.

[130] U. Fleck, C. Tiegs, G. Brunner, Fractionation of fatty acid ethyl esters by supercritical CO_2: High separation efficiency using an automated countercurrent column, J. Supercrit. Fluids. 14 (1998) 67–74. doi: 10.1016/S0896-8446(98)00100-4

[131] V. Riha, G. Brunner, Separation of fish oil ethyl esters with supercritical carbon dioxide, J. Supercrit. Fluids. 17 (2000) 55–64. doi: 10.1016/S0896-8446(98)00130-2.

[132] G. Brunner, Fractionation of fats with supercritical carbon dioxide, Eur. J. Lipid Sci. Technol. 102 (2000) 240–244. doi: 10.1002/(SICI)1438-9312(200003)102:3<240::AID-EJLT240>3.0.CO;2-Z

[133] W.B. Nilsson, E.J. Gauglitz, J.K. Hudson, Solubilities of methyl oleate, oleic acid, oleyl glycerols, and oleyl glycerol mixtures in supercritical carbon dioxide, J. Am. Oil Chem. Soc. 68 (1991) 87–91. doi: 10.1007/BF02662323.

[134] A. Staby, J. Mollerup, Solubility of fish oil fatty acid ethyl esters in sub- and supercritical carbon dioxide, J. Am. Oil Chem. Soc. 70 (1993) 583–588. doi: 10.1007/BF02545324.

[135] A. Staby, J. Mollerup, Separation of constituents of fish oil using supercritical fluids: a review of experimental solubility, extraction, and chromatographic data, Fluid Phase Equilib. 91 (1993) 349–386. doi: 10.1016/0378-3812(93)85109-Y.

[136] A.P.A. Corrêa, C.A. Peixoto, L.A.G. Gonçalves, F.A. Cabral, Fractionation of fish oil with supercritical carbon dioxide, J. Food Eng. 88 (2008) 381–387. doi: 10.1016/j.jfoodeng.2008.02.025.

[137] R. Davarnejad, K.M. Kassim, A. Zainal, S.A. Sata, Extraction of fish oil by fractionation through supercritical carbon dioxide, J. Chem. Eng. Data. 53 (2008) 2128–2132. doi: 10.1021/je800273c.

[138] M. Maschietti, A. Pedacchia, Supercritical carbon dioxide separation of fish oil ethyl esters by means of a continuous countercurrent process with an internal reflux, J. Supercrit. Fluids. 86 (2014) 76–84. doi: 10.1016/j.supflu.2013.12.003.

[139] L. Fiori, M. Manfrini, D. Castello, Supercritical CO_2 fractionation of omega-3 lipids from fish by-products: Plant and process design, modeling, economic feasibility, Food Bioprod. Process. 92 (2014) 120–132. doi: 10.1016/j.fbp.2014.01.001.

[140] C.A. Pieck, C. Crampon, F. Charton, E. Badens, A new model for the fractionation of fish oil FAEEs, J. Supercrit. Fluids. 120 (2017) 258–265. doi: 10.1016/j.supflu.2016.05.024.

[141] A. Bejarano, P.C. Simões, J.M. del Valle, Fractionation technologies for liquid mixtures using dense carbon dioxide, J. Supercrit. Fluids. 107 (2016) 321–348. doi: 10.1016/j.supflu.2015.09.021.

[142] G. Brunner, Applications of supercritical fluids, Annu. Rev. Chem. Biomol. Eng. 1 (2010) 321–342. doi: 10.1146/annurev-chembioeng-073009-101311.

[143] L.T. Taylor, Supercritical fluid chromatography, Anal. Chem. 82 (2010) 4925–4935. doi: 10.1021/ac101194x.

[144] M. Perrut, R.M. Nicoud, H. Breivik, Processes for chromatographic fractionation of fatty acids and their derivatives, US5719302A, 1998.

[145] G. Brunner, F. Reichmann, Process for recovering unsaturated fatty acids and derivatives thereof, US5777141A, 1998.

[146] M. Johannsen, G. Brunner, Supercritical fluid chromatographic separation on preparative scale and in continuous mode, J. Supercrit. Fluids. 134 (2018) 61–70. doi: 10.1016/j.supflu.2017.12.027.

[147] J.A. Kralovec, S. Zhang, W. Zhang, C.J. Barrow, A review of the progress in enzymatic concentration and microencapsulation of omega-3 rich oil from fish and microbial sources, Food Chem. 131 (2012) 639–644. doi: 10.1016/j.foodchem.2011.08.085.

[148] T. Lin, S. Chen, A.-C. Chang, Enrichment of n-3 PUFA contents on triglycerides of fish oil by lipase-catalyzed trans-esterification under supercritical conditions, Biochem. Eng. J. 29 (2006) 27–34. doi: 10.1016/j.bej.2005.02.035.

[149] M. Haq, P. Pendleton, B.S. Chun, Utilization of Atlantic salmon by-product oil for omega-3 fatty acids rich 2-monoacylglycerol production: Optimization of enzymatic

reaction parameters, Waste Biomass Valorization. 11 (2020) 153–163. doi: 10.1007/s12649-018-0392-9.

[150] Y. He, J. Li, S. Kodali, B. Chen, Z. Guo, The near-ideal catalytic property of *Candida antarctica* lipase A to highly concentrate n-3 polyunsaturated fatty acids in monoacylglycerols via one-step ethanolysis of triacylglycerols, Bioresour. Technol. 219 (2016) 466–478. doi: 10.1016/j.biortech.2016.08.007.

[151] S. Uluata, D.J. Mcclements, E.A. Decker, How the multiple antioxidant properties of ascorbic acid affect lipid oxidation in oil-in-water emulsions, J. Agric. Food Chem. 63 (2015) 1819–1824. doi: 10.1021/jf5053942.

[152] E. Niki, Lipid peroxidation: Physiological levels and dual biological effects, Free Radic. Biol. Med. 47 (2009) 469–484. doi: 10.1016/j.freeradbiomed.2009.05.032.

[153] Codex Alimentarius Comission, Standard for fish oils Codex Stan 329-2017, Rome, Italy, 2017.

[154] C. Wijesundera, C. Ceccato, P. Watkins, P. Fagan, B. Fraser, N. Thienthong, P. Perlmutter, Docosahexaenoic acid is more stable to oxidation when located at the *sn*-2 position of triacylglycerol compared to *sn*-1(3), J. Am. Oil Chem. Soc. 85 (2008) 543–548. doi: 10.1007/s11746-008-1224-z.

[155] C. Jacobsen, A.D.M. Sørensen, N.S. Nielsen, Stabilization of omega-3 oils and enriched foods using antioxidants, in: Food Enrichment with Omega-3 Fatty Acids, Woodhead Publishing Limited, 2013, pp. 130–149. doi: 10.1533/9780857098863.2.130.

[156] T.L. Vestland, L.B. Petersen, A.H. Myrset, J. Klaveness, Oxidative stability of omega-3 tablets, Eur. J. Lipid Sci. Technol. 119 (2017) 1–7. doi: 10.1002/ejlt.201500322.

[157] S. Maqsood, S. Benjakul, A. Abushelaibi, A. Alam, Phenolic compounds and plant phenolic extracts as natural antioxidants in prevention of lipid oxidation in seafood: A detailed review, Compr. Rev. Food Sci. Food Saf. 13 (2014) 1125–1140. doi: 10.1111/1541-4337.12106.

[158] G.M. Suárez-Jiménez, C.M. López-Saiz, H.E. Ramírez-Guerra, J.M. Ezquerra-Brauer, S. Ruiz-Cruz, W. Torres-Arreola, Role of endogenous and exogenous tocopherols in the lipid stability of marine oil systems: A review, Int. J. Mol. Sci. 17 (2016). doi: 10.3390/ijms17121968.

[159] S. Drusch, N. Groß, K. Schwarz, Efficient stabilization of bulk fish oil rich in long-chain polyunsaturated fatty acids, Eur. J. Lipid Sci. Technol. 110 (2008) 351–359. doi: 10.1002/ejlt.200700195.

[160] L. Wang, B. Yang, B. Yan, X. Yao, Supercritical fluid extraction of astaxanthin from

Haematococcus pluvialis and its antioxidant potential in sunflower oil, Innov. Food Sci. Emerg. Technol. 13 (2012) 120–127. doi: 10.1016/j.ifset.2011.09.004.

[161] S.D. Bhale, Z. Xu, W. Prinyawiwatkul, J.M. King, J.S. Godber, Oregano and rosemary extracts inhibit oxidation of long-chain n-3 fatty acids in menhaden oil, J. Food Sci. 72 (2007) C504–C508. doi: 10.1111/j.1750-3841.2007.00569.x.

[162] C. Encina, C. Vergara, B. Giménez, F. Oyarzún-Ampuero, P. Robert, Conventional spray-drying and future trends for the microencapsulation of fish oil, Trends Food Sci. Technol. 56 (2016) 46–60. doi: 10.1016/j.tifs.2016.07.014.

[163] D. Eratte, K. Dowling, C.J. Barrow, B. Adhikari, Recent advances in the microencapsulation of omega-3 oil and probiotic bacteria through complex coacervation: A review, Trends Food Sci. Technol. 71 (2018) 121–131. doi: 10.1016/j.tifs.2017.10.014.

[164] S. Yeo, E. Kiran, Formation of polymer particles with supercritical fluids: A review, J. Supercrit. Fluids. 34 (2005) 287–308. doi: 10.1016/j.supflu.2004.10.006.

[165] C. Prieto, L. Calvo, The encapsulation of low viscosity omega-3 rich fish oil in polycaprolactone by supercritical fluid extraction of emulsions, J. Supercrit. Fluids. 128 (2017) 227–234. doi: 10.1016/j.supflu.2017.06.003.

[166] M. Türk, Particle Formation with Supercritical Fluids Challenges and Limitations, Elsevier, 2014. doi: 10.1016/B978-0-444-59486-0.00009-7.

[167] E. Weidner, High pressure micronization for food applications, J. Supercrit. Fluids. 47 (2009) 556–565. doi: 10.1016/j.supflu.2008.11.009.

[168] S. Drusch, Y. Serfert, K. Schwarz, Microencapsulation of fish oil with n-octenylsuccinate-derivatised starch: Flow properties and oxidative stability, Eur. J. Lipid Sci. Technol. 108 (2006) 501–512. doi: 10.1002/ejlt.200500312.

[169] W. Kolanowski, M. Ziolkowski, J. Weißbrodt, B. Kunz, G. Laufenberg, Microencapsulation of fish oil by spray drying–impact on oxidative stability. Part 1, Eur. Food Res. Technol. 222 (2006) 336–342. doi: 10.1007/s00217-005-0111-1.

[170] S.M. Jafari, E. Assadpoor, B. Bhandari, Y. He, Nano-particle encapsulation of fish oil by spray drying, Food Res. Int. 41 (2008) 172–183. doi: 10.1016/j.foodres.2007.11.002.

[171] R. Morales-Medina, F. Tamm, A.M. Guadix, E.M. Guadix, S. Drusch, Functional and antioxidant properties of hydrolysates of sardine (*S. pilchardus*) and horse mackerel (*T. mediterraneus*) for the microencapsulation of fish oil by spray-drying, Food Chem. 194 (2016) 1208–1216. doi: 10.1016/j.foodchem.2015.08.122.

[172] K. Heinzelmann, K. Franke, B. Jensen, A.M. Haahr, Protection of fish oil from oxidation

by microencapsulation using freeze-drying, Eur. J. Lipid Sci. Technol. (2000) 114–121. doi: 10.1002/(SICI)1438-9312(200002)102:2<114::AID-EJLT114>3.0.CO;2-0.

[173] C. Chung, L. Sanguansri, M. Ann, M.A. Augustin, In vitro lipolysis of fish oil microcapsules containing protein and resistant starch, Food Chem. 124 (2011) 1480–1489. doi: 10.1016/j.foodchem.2010.07.115.

[174] W. Klaypradit, Y.-W. Huang, Fish oil encapsulation with chitosan using ultrasonic atomizer, LWT - Food Sci. Technol. 41 (2008) 1133–1139. doi: 10.1016/j.lwt.2007.06.014.

[175] Y.H. Cho, H.K. Shim, J. Park, Encapsulation of fish oil by an enzymatic gelation process using transglutaminase cross-linked proteins, J. Food Sci. 68 (2003) 2717–2723. doi: 10.1111/j.1365-2621.2003.tb05794.x.

[176] P.J. García-Moreno, K. Stephansen, J. van der Krujis, A. Guadix, E.M. Guadix, I.S. Chornakis, C. Jacobsen, Encapsulation of fish oil nanofibers by emulsion electrospinning: Physical characterization and oxidative stability, J. Food Eng. 183 (2016) 39–49. doi: 10.1016/j.jfoodeng.2016.03.015.

[177] K.G. Wu, X.H. Chai, Y. Chen, Microencapsulation of fish oil by simple coacervation of hydroxypropyl methylcellulose, Chinese J. Chem. 23 (2005) 1569–1572. doi: 10.1002/cjoc.200591569.

[178] F.T. Karim, K. Ghafoor, S. Ferdosh, F. Al-Juhaimi, E. Ali, K.B. Yunus, M.H. Hamed, A. Islam, M. Asif, I.S.M. Zaidul, Microencapsulation of fish oil using supercritical antisolvent process, J. Food Drug Anal. 25 (2017) 654–666. doi: 10.1016/j.jfda.2016.11.017.

[179] A.S.M. Tanbirul Haque, B.-S. Chun, Particle formation and characterization of mackerel reaction oil by gas saturated solution process, J. Food Sci. Technol. 53 (2016) 293–303. doi: 10.1007/s13197-015-2000-3.

[180] M. Haq, B.S. Chun, Microencapsulation of omega-3 polyunsaturated fatty acids and astaxanthin-rich salmon oil using particles from gas saturated solutions (PGSS) process, Lwt. 92 (2018) 523–530. doi: 10.1016/j.lwt.2018.03.009.

[181] J. Yang, O.N. Ciftci, Encapsulation of fish oil into hollow solid lipid micro- and nanoparticles using carbon dioxide, Food Chem. 231 (2017) 105–113. doi: 10.1016/j.foodchem.2017.03.109.

[182] N. Rubio-Rodríguez, Supercritical fluid technology for extraction, concentration and formulation of omega-3 rich oils. A novel strategy for valorization of fish by-products (PhD. Thesis), University of Burgos, 2011.

[183] R. Melgosa, Ó. Benito-Román, M.T. Sanz, E. de Paz, S. Beltrán, Omega–3 encapsulation by PGSS-drying and conventional drying methods. Particle characterization and oxidative stability, Food Chem. 270 (2019) 138–148. doi: 10.1016/j.foodchem.2018.07.082.

[184] D. Meterc, M. Petermann, E. Weidner, Drying of aqueous green tea extracts using a supercritical fluid spray process, J. Supercrit. Fluids. 45 (2008) 253–259. doi: 10.1016/j.supflu.2008.02.001.

[185] Á. Martín, E. Weidner, PGSS-drying: Mechanisms and modeling, J. Supercrit. Fluids. 55 (2010) 271–281. doi: 10.1016/j.supflu.2010.08.008.