Transformation and agro-industry

Review article

Methods of extraction, refining and concentration of fish oil as a source of omega-3 fatty acids

Métodos de extracción, refinación y concentración de aceite de pescado como fuente de ácidos grasos omega 3

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Editor temático: Miguel Ángel Rincón Cervera (Instituto de Nutrición y Tecnología de los Alimentos [INTA])

Date of receipt: 05/07/2017
Date of approval: 15/03/2018

How to cite this article: Bonilla-Méndez, J. R., & Hoyos-Concha, J. L. (2018). Methods of extraction, refining and concentration of fish oil as a source of omega-3 fatty acids. *Corpoica Ciencia y Tecnología Agropecuaria, 19*(*3*), 645-668.

DOI: https://doi.org/10.21930/rcta.vol19_num2_art:684

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Abstract

Fish oil is an industrial product of high nutritional value because of its Omega-3 polyunsaturated fatty acids content, currently valued for their beneficial effects on health. Studies and advances made since year 2000 on fish oil extraction from several fish species, its refining and polyunsaturated fatty acids concentration are reviewed in this article. Extraction techniques range from conventional technologies such as wet pressing and extraction using solvents, to more recently proposed technologies such as supercritical fluids and fish silage. Moreover, although refining is performed by traditional methods, there are new technologies with potential to be applied on fish oil. On the other hand, interest in omega-3 polyunsaturated fatty acids concentration has increased and several techniques such as winterization, enzymatic methods, supercritical fluids fractionation, complex formation with urea, fractionation by chromatographic methods and concentration by membranes have been proposed. The information collected indicates a trend to combine different conventional and emerging technologies to improve product yields and purity.

Keywords: fish oil, polyunsaturated fatty acids, extraction, lipids, purification

Resumen

El aceite de pescado es un producto industrial de alto valor nutricional, por su contenido de ácidos grasos poliinsaturados omega-3, valorados en la actualidad por sus efectos benéficos en la salud. En este artículo se revisan estudios y avances realizados desde el año 2000 en la extracción de aceite de pescado de diversas especies, su refinación y concentración de ácidos grasos. Las técnicas de extracción van desde tecnologías convencionales, como prensado húmedo y extracción por solventes, hasta otras propuestas más recientemente, como fluidos supercríticos y ensilaje de pescado. Así mismo, aunque la refinación se realiza con métodos tradicionales, existen nuevas tecnologías con potencial para aplicarse en aceite de pescado. Por otro lado, el interés en la concentración de ácidos omega-3 ha crecido y se han propuesto varias técnicas, como hibernación, métodos enzimáticos, fraccionamiento por fluidos supercríticos y por métodos cromatográficos, formación de complejos con urea y concentración por membranas. La información recopilada indica una tendencia a combinar diferentes tecnologías convencionales y emergentes, con el fin de mejorar los rendimientos y la pureza del producto obtenido.

Palabras clave: aceites de pescado, ácidos grasos poliinsaturados, extracción, lípidos, purificación

Introduction

Fish oil is an industrial product of great nutritional value due to its content of long chain omega-3 polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA), docosapentaenoic acid (DPA) and eicosapentaenoic acid (EPA), which are currently highly valued for their prophylactic and therapeutic properties in nutritional and health fields. Fish oil, which was previously a by-product of fishmeal used for animal feed, is now recognized as the primary source of these fatty acids (Valenzuela, Sanhueza, & De la Barra, 2012).

EPA and DHA content in fish oil is an important quality parameter of this product. These fatty acids are related to different neuronal functions, and their absence is associated with diverse inflammatory processes and the precarious development of neurons in human patients. Likewise, its beneficial effects in cardiovascular diseases are recognized (Coronado, Vega y León, Gutiérrez, García, & Díaz, 2006).

Fish oil can be obtained from different species depending on the production area. The raw material is comprised of three major fractions, which include solids, oil and water. The goal is to separate these components as best as possible, commonly obtaining fishmeal and fish oil (United Nations Food and Agriculture Organization [FAO], 1986). Methods to extract them include cooking, use of solvents and, recently, extraction by supercritical fluids, by enzymatic procedures and by chemical (i.e. applying acids) or biological silages (Mbatia et al., 2010; Menegazzo, Petenuci, & Fonseca, 2014).

The crude oil contains impurities, which depend on the extraction method used (Chakraborty & Joseph, 2015a), and requires a purification process to reach quality features that make it acceptable for human consumption (Crexi, Legemann-Monte, Almeida de Souza, & De Almeida-Pinto, 2010).

To achieve such characteristics, different impurities must be eliminated while maintaining the most desirable compounds such as omega-3 and other PUFAs, so the refining process is designed to achieve this, minimizing oil losses and maximizing the availability of beneficial constituents (Vaisali, Charanyaa, Belur, & Regupathi, 2015).

The interest in obtaining higher good quality PUFA concentration is evident in several investigations that aim at extracting fish oil, purifying it and increasing its PUFA content, especially EPA and DHA, using different techniques. Regarding PUFA some studies include extraction and fractionation (Rubio et al., 2010; Sahena et al., 2009), but they focus on supercritical fluid technology and only cover studies carried out until 2009.

Therefore, the objective of this study is to carry out a general review of the progress that has been made since year 2000 regarding different fish oil extraction technologies, as well as the advances in refining and fractionation, focused on the conservation and enrichment in omega-3 PUFAs.

Fish oil extraction

Several studies have been developed around the extraction and quality analysis of oil obtained from different fish species, as well as by-products of their processing, in which different techniques are used, such as supercritical fluids, wet pressing, extraction using solvents, and fish silage employing enzymes present in fish or from other sources (Adeoti & Hawboldt, 2014).

Extraction using conventional methods

The extraction of fish oil by wet pressing is the most commonly used method for production on an industrial scale, and is basically carried out in four stages: fish cooking, pressing, decantation and centrifugation (FAO, 1986).

Drastic temperature and pressure conditions used for protein coagulation and subsequent oil release may partially modify the PUFAs present, due to degradation reactions such as hydrolysis and oxidation (Linder, Fanni, & Parmentier, 2005; Mbatia et al., 2010). Table 1 shows studies that have been carried out using wet pressing in the laboratory, comparing this technique with other methods and including further test conditions.

Table 1. Fish oil extraction through wet pressing

Fish species	Temperature (°C)	Time (min)	Observations	Reference
Scomber scombrus	95-100	10-20	Extraction yield of 18.7 % Quality within standard values	Bako, Umogbai and Obetta (2014)
Cyprinus carpio	95-100	30	Quality within standard values	Crexi et al. (2010)
By-products of *Oncorhynchus gorbuscha*	95	15	Good source of DHA and EPA, even after the by-products have been stored for four days	Wu and Bechtel (2008)
Sufflamen capistratus	-	-	Yield of 40 % PUFA content of 15.74 %, i.e. lower than in other methods	Immanuel, Sathasivan, Shankar, Peter and Palavesam (2009)
By-products of *Merluccius capensis, Merluccius paradoxus, Hoplostethus atlanticus, Salmo salar, Dosidicus gigas Acipenseridae*	-	-	The method was not adequate to extract hake or squid oils, due to emulsion formation	Rubio et al. (2012)
Acipenseridae	85	60	Yield of 52.51 %, i.e. low compared to other methods Oxidative stability affected	Hao et al. (2015)
Oreochromis niloticus, Pseudoplatystoma corruscans x *P. fasciatum*	40	180	The oil obtained showed an adequate chemical quality	Menegazzo et al. (2014)
Sardinella longiceps	75	30	Yield of 8.3 % compared to the weight of the wet tissue Better quality compared to the oil extracted with solvents	Chakraborty and Joseph (2015a, 2015b)

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(Continuation of table 1)

Fish species	Temperature (°C)	Time (min)	Observations	Reference
Theragra chalcogramma	50-60	15-30	The highest yield was obtained at 60 °C during 30 min	Ribeiro, Oliveira, Bechtel and Prentice (2013)
Fish by-products	90	30	Yield of 2.13 % compared to the weight of the fish, and lower compared with solvents and microwaves	Silva-Aguiar and Soares-Goulart (2013)
Heads of *Thunnus* spp.	75-95	10-30	The highest yield was obtained at 85 °C for 30 min in non-precooked heads	Chantachum, Benjakul and Sriwirat (2000)
By-products of *Oncorhynchus mykiss*	50-90	-	Temperature had an effect on oil extracted from trout heads, bones and tails, as well as intestines	Honold, Nouard and Jacobsen (2016)
By-products of *Salmo salar*	95	30	Yield was similar to cold and enzymatic extractions, but with a higher oxidation degree	Glowacz et al. (2016)

Source: Elaborated by the authors

Another conventional procedure is the extraction using solvents, applied generally for analytical purposes but not for industrial production, because of the disadvantages of using substances with restrictions in the food industry (Rubio et al., 2010). This process is based on the solubility of lipids in organic solvents and their insolubility in water, thanks to which these, as well as its soluble components such as proteins, carbohydrates and minerals, can be separated from water.

The main limitations of this technique are that it requires a relatively dry sample that is destroyed, and it takes a long time, in addition to generating large amounts of residual solvent (Adeoti & Hawboldt, 2014; Sahena et al., 2009).

Several methods vary according to the type of solvent or treatment used on the sample. The most common are the ones of Soxhlet and Bligh-Dyer, however, others such as McGill-Moffatt and the one of Randall and Folch have also been evaluated (Fiori et al., 2012; Immanuel et al., 2009; Rincón-Cervera, Villarreal-Rubio, Valenzuela, & Valenzuela, 2017).

These methods have been applied for the extraction of fish oil as the sole technology used (Adeniyi & Bawa, 2006; Boran, Karaçam, & Boran, 2006; Kołakowska, Domiszewski, Kozłowski, & Gajowniczek, 2006; Shamsudin & Salimon, 2006). Moreover, these have also been used in comparison to other methods (Chakraborty & Joseph, 2015a; Fiori et al., 2012; Immanuel et al., 2009; Létisse, Rozières, Hiol, Sergent, & Comeau, 2006; Rincón-Cervera et al., 2017; Sahena et al., 2010; Silva-Aguiar & Soares-Goulart, 2013) (table 2).

Table 2. Conventional extraction with fish oil solvents

Method	Solvents	Material	Reference
Bligh and Dyer	Chloroform, methanol, water	Rainbow trout (*Oncorhynchus mykiss*)	Kołakowska et al. (2006)
		Alaska pollock (*Theragra chalcogramma*)	Silva-Aguiar and Soares-Goulart (2013)
		Masked Triggerfish (*Sufflamen capistratus*)	Immanuel et al. (2009)
		Sardine (*Sardinella longiceps*)	Chakraborty and Joseph (2015a); Létisse et al. (2006)
Soxhlet	Petroleum ether	Indian mackerel (*Rastrelliger kanagurta*)	Sahena et al. (2010)
	n-hexane	Horse mackerel (*Trachurus trachurus*), twait shad (*Alosa fallax*), garfish or sea needle (*Belone belone*), golden grey mullet (*Mugil auratus*)	Boran et al. (2006); Rincón-Cervera et al. (2017)
	Chloroform, methanol	*Seriola nigrofasciata*	Shamsudin and Salimon (2006)
	Petroleum ether	Atlantic mackerel (*Scomber scombrus*)	Adeniyi and Bawa (2006)
McGill and Moffatt	Acetone	Masked Triggerfish (*Sufflamen capistratus*)	Immanuel et al. (2009)
Randall	n-hexane	Rainbow trout (*Oncorhynchus mykiss*)	Fiori et al. (2012)
Folch	Chloroform, methanol	Salmon	Rincón-Cervera et al. (2017)

Source: Elaborated by the authors

Supercritical fluid extraction

Supercritical fluid extraction (SFE) is an emerging extraction technology using solvents that has gained great interest in recent years, due to advantages such as the use of a moderate temperature, an oxygen- free environment and extraction of low polarity lipids, which avoids the extraction of impurities (Rubio et al., 2012).

Supercritical fluids have a lower viscosity and a higher diffusivity than conventional solvents, improving mass transfer and, in general, reduces the time needed for extraction. Its biggest disadvantage is the high cost of applying this technology at an industrial level. The fluid that is mostly used is CO_2; it is employed as an inert solvent and is safe for oil extraction. Its main advantage is that it does not remain in the product, since at room temperature and pressure it returns to its gas state and evaporates (Rubio et al., 2010, 2012).

The parameters that are usually evaluated are pressure, temperature, CO_2 flow and time. Yet, the effect of moisture, flow direction, solvent: material ratio, and particle size have also been studied in fish

oils (Rubio et al., 2008), and the process has been modeled to find optimal conditions (Ferdosh et al., 2013; Sarker et al., 2012).

Other studies, which compare SFE in fish oils with other extraction techniques (table 3) (Ferdosh, Sarker, Norulaini, Oliveira et al., 2014; Fiori et al., 2012; Hao et al., 2015; Rubio et al., 2012; Sahena et al., 2010) show that this technology is attractive in conventional processes, to obtain oils rich in PUFA and with a reduced content of contaminants.

Table 3. Comparative studies of fish oil extraction by supercritical fluids (SFE)

Fish species	Pressure (kPa)	Temperature (°C)	Comparison with other technologies	Observations	Reference
Rastrelliger kanagurta	20,000-35,000	45-75	Comparison with Soxhlet	Best conditions: 35.000 kPa and 75 °C The yields achieved were similar compared to the ones obtained with Soxhlet (53.6 g/100 of dry sample)	Sahena et al. (2010)
Oncorhynchus mykiss	50,000	60	Comparison with Randall	Oil with high content of EPA and DHA Profile similar to that obtained with Randall	Fiori et al. (2012)
Merluccius capensis, Merluccius paradoxus, Hoplostethus atlanticus, Salmo salar	25,000	40	Comparison with cold extraction, wet reduction and enzymatic extraction	SFE allowed the reduction of contaminants such as arsenic and showed less oxidation compared to other methods	Rubio et al. (2012)
Thunnus tonggol, Euthynnus affinis, Auxis thazard	40,000	65	Comparison with Soxhlet	Performance and quality of the oil extracted with Soxhlet and supercritical CO_2 were acceptable	Ferdosh, Sarker, Norulaini, Oliveira et al. (2014)
Acipenseridae	31,600	-	Comparison with enzymatic extraction, wet reduction and addition of amino compounds	SFE showed the highest extraction yield (97.25 %), followed by enzymatic extraction Less lipid oxidation was observed	Hao et al. (2015)

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(Continuation of table 3)

Especie de pescado	Presión (kPa)	Temperatura (°C)	Otras evaluaciones	Observaciones	Referencia
-	35.000	60	Comparison with Soxhlet, wet reduction, enzymatic extraction	SFE allowed a significant reduction in mercury, cadmium and lead content, while with the other methods the oil exceeded all permitted limits	Hajeb et al. (2015)
Sardinella lemuru	20.000-40.000	40-70	Comparison with Soxhlet and solvent method chloroform: methanol	In terms of PUFA extraction efficiency and recovery, SFE and the extraction with chloroform: methanol did not show significant differences	Gedi et al. (2015)
Salmo salar	25.000	45	Comparison with hexane extraction	Using SFE premium quality oil, rich in PUFA was obtained It allowed a greater PUFA selectivity and lower oxidation, in comparison with extraction using solvents	Haq, Ahmed, Cho and Chun (2016)

Source: Elaborated by the authors

Extraction by fish silage

Fish silage is a semi-liquid product made from whole fish or parts of fish, to which acids (chemical silage), enzymes (silage or enzymatic extraction) or lactic acid bacteria (biological silage) are added, causing protein hydrolysis (Ferraz de Arruda, Borghesi, & Oetterer, 2007).

This process has been shown as a good alternative to traditional methods, since it can be simpler and cheaper in terms of investment costs and energy expenditure. In addition, this technology does not use solvents or employs high temperatures, and the physicochemical and microbiological changes caused can not only improve extraction performance, but also prevent undesirable processes, i.e. fat oxidation. Furthermore, essential fatty acids and other functional ingredients, such as protein hydrolysate and collagen, among others can be recovered (Ferraz de Arruda et al., 2007; Rai, Swapna, Bhaskar, Halami, & Sachindra, 2010; Rubio et al., 2010).

Some studies have focused on the evaluation of this procedure for the extraction of oil from whole fish or its by-products (table 4). One of the most studied techniques is enzymatic silage with different types of proteases, but biological and chemical silages are also used in various investigations for the separation of fish oil.

Table 4. Fish oil extraction by silage

Silage type	Additive	Fish species	References
Enzymatic	Alcalase®	*Sardina pilchardus*	Batista, Ramos, Mendonça and Nunes (2009)
	Protamex®	*Aphanopus carbo*	Batista, Ramos, Coutinho, Bandarra and Nunes (2010)
	ALCALASE® 2.4 L	*Salmo salar*	Gbogouri, Linder, Fanni and Parmentier (2006)
	Protamex®	*Scomber scombrus, Clupea harengus*	Laplante, Souchet and Bryl (2009)
	Alcalase® 2.4 l fg, Protamex®, Flavourzyme 500 mg	*Sardina pilchardus*	Dumay, Donnay, Barnathan, Jaouen and Bergé (2006)
	Protamex®	*Thunnus albacares*	Nguyen (2013)
	Flavourzyme	*Gadus morhua*	Slizyte, Dauksas, Falch, Storro and Rustad (2005)
	Alcalase®, Lecitase® Ultra	*Gadus morhua*	Slizyte, Rustad and Storro (2005)
	Alcalase®	*Thunnus albacares*	De Oliveira, Minozzo, Licodiedoff and Waszczynskyj (2016)
	Alcalase®	*Salmo salar*	Glowacz et al. (2016)
	Papaine	*Oncorhynchus mykiss*	Muñoz, Bucheli, Bonilla and Hoyos (2016)
	Neutral protease	*Scomberomorus commerson*	Qi-Yuan, Jun-Qing and Xiao-Ge (2016)
Biological	*Lactobacillus acidophilus*	*Oreochromis niloticus*	Llanes, Toledo, Savón and Gutiérrez (2012)
	Pediococcus acidilactici K7, *Enterococcus faecium* HAB01	*Cyprinus carpio*	Rai et al. (2010)
	Lactobacillus plantarum, Lactobacillus buchneri, Lactobacillus casei ssp. *casei, Lactococcus lactis* ssp. *lactis, Leuconostoc mesenteroides* ssp. *mesenteroides, Pediococcus acidilactici*	*Xiphias gladius, Raja clavata, Isurus oxyrinchus*	Vázquez et al. (2011)

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(Continuation of table 4)

Silage type	Additive	Fish species	References
Biológico	Yogur	*Oreochromis* spp.	Vidotti, Pacheco and Gonçalves (2011)
	Lactic acid bacteria	*Oncorhynchus mykiss*	Muñoz et al. (2016)
Químico	Acetic acid	*Cyprinus carpio*	Crexi et al. (2010)
	Formic acid	*Oncorhynchus mykiss*	Goosen, De Wet and Görgens (2014); Goosen, de Wet, Görgens, Jacobs and De Bruyn (2014)
	Sulfuric acid, formic acid	*Oreochromis niloticus*	Llanes et al. (2012)
	Formic acid, propionic acid	*Oreochromis niloticus*	Dos Santos, Da Silva, Zinani, Wander and Gomes (2015)
	Formic acid	*Maccullochella peelii*	Turchini, Gunasekera and De Silva (2003)
	Formic acid, sulfuric acid	*Oreochromis* spp.	Vidotti et al. (2011)
	Formic acid	*Oncorhynchus mykiss*	Muñoz et al. (2016)

Source: Elaborated by the authors

Fish oil refining

Once fish oils are extracted these require a purification process to achieve the quality characteristics that make it acceptable for human and animal consumption (Crexi et al., 2010), since they contain insoluble impurities, phospholipids, free fatty acids, moisture, primary oxidation products, minerals, pigments and even persistent organic pollutants (POP).

Impurities in the oil reduces its quality (Huang & Sathivel, 2010) and must be eliminated while maintaining the most desirable compounds, such as omega-3 and other PUFA, so the refining process must be designed in such a way that this goal is achieved, minimizing oil loss and maximizing the availability of beneficial constituents (Vaisali et al., 2015).

The traditional refining process includes several stages, such as degumming, neutralization, bleaching, deodorization and, in some cases, winterization, although this could be considered to a greater extent a PUFA concentration method. Each of the stages is especially important to remove the different classes of compounds (table 5) and is the most studied and industrially applied process for the refining of fish oils.

Table 5. Minor oil components, effect on the quality and refining stage for its removal

Type of component	Effect on oil quality	Refining stage for their removal
Phospholipids	Sedimentation in the product, less oxidation stability	Degumming
Free fatty acids	Acylglycerol prooxidants, lower stability to oxidation	Neutralization
Pigments	Decrease in sensory quality	Bleached
Ions and metal complexes	Harmful, prooxidants	Neutralization, bleached
Oxidation products	They cause bad taste and rancidity, as well as being harmful	Neutralization, bleaching, deodorization
Persistent organic pollutants (POP)	Toxicity	Bleached
Humidity	Prooxidant	Drying

Source: Adapted from Čmolik and Pokorný (2000) and Vaisali et al. (2015)

Several studies have been carried out to evaluate the effect of the refining process on different oil properties, through the establishment of parameters such as acidity, peroxides, thiobarbituric acid, iodine, saponification and anisidine indexes, which are some of the most important ones.

However, in all assessments, the peroxide (PI) and acidity (IA) indexes are included as essential indicators in the evaluation process (table 6). Furthermore, in most cases, these are negatively affected in the stages in which exposure to high temperatures is higher, such as neutralization, drying and deodorization, because it increases the susceptibility to oxidation and the formation of peroxides (Crexi et al., 2010).

Although the refining process has been studied for several years, many of the investigations are still directed towards oils of vegetable origin, which involves the evaluation of several stages and new techniques that have not yet been reported for fish oil, but that have the potential for its application in this material.

Some authors have carried out a review of different discovered and rediscovered technologies (as in the case of enzymatic processes) for the optimization of the refining process of edible oils (table 7) (Čmolik, & Pokorný, 2000; Kumar & Krishna, 2015; Misra, Nandi, & Nandi, 2013; Moharana, Byreddy, Puri, Barrow, & Rao, 2016; Vaisali et al., 2015).

Table 6. Evaluation parameters in fish oil refining

Oil source	Refining stages	Follow-up parameters	Reference
Oreochromis niloticus, Pseudoplatystoma corruscans + *Pseudoplatystoma fasciatum*	Degumming, neutralization, washing, drying, bleaching, filtration	IA, IP, IS, IY, IR, density, humidity	Menegazzo et al. (2014)
Cyprinus carpio	Degumming, neutralization, washing, drying, bleaching, winterization, deodorization	IA, IP, IpA, TBA, phosphorous content, color	Crexi et al. (2010)
Clupeonella delicatula	Neutralization, washing, drying, bleaching, hibernation, deodorization	IA, IP	Motalebi-Moghanjoghi, Hashemi, Mizani, Gharachorloo and Tavakoli (2015)
Sardinops sagax ssp. *caerulea*	Neutralization, bleaching, deodorization	IA, IP, IpA, IS, OSI, CA, IR, humidity, phosphorus content, metals, density, color	Noriega et al. (2009)
Sardinella longiceps	Degumming, neutralization, bleaching, deodorization	IA, IP, IpA, totox, TBA, color	Chakraborty and Joseph (2015b)
Sardinella longiceps	Degumming, neutralization, bleaching, deodorization	OSI, TBA, IP, IpA, totox	Chakraborty, Joseph and Joseph (2016)
Thunnus albacares	Degumming, neutralization, washing, drying, bleaching, deodorization	IS, IP, IA, IY, IR	De Oliveira et al. (2016)
Oncorhynchus mykiss	Degumming, neutralization, washing, drying, blanching, winterization	IA, IP, IY, color, omega-3 concentration	Díaz, Bonilla, Hoyos and Benítez (2016)

CA: conjugated acids; AI: acidity index; PV: peroxide value; IpA: anisidine index; SI: saponification index; RI: refraction index; IOI: iodine index; OSI: oxidative stability index; TBI: thiobarbituric acid index; Totox: total oxidation value.
Source: Elaborated by the authors

Table 7. Technologies with application potential in fish oil refining

Technology	Principle	Limitations
Enzymatic degumming	Modification of phospholipids with phospholipases to facilitate hydration	Possible oil instability after processing
Degumming by membranes	Retention of phospholipids through the passage of crude oil through semipermeable membranes	Process conditions must be adapted for each kind of oil
Neutralization by molecular distillation	Purification by distillation at low pressures	High implementation costs at the industrial level
Enzymatic deacidification	Esterification of fatty acids through a reaction catalyzed by lipases	Higher energy consumption compared to neutralization with alkali
Nanoneutralization	Reaction of high pressure oil in a hydrodynamic cavitation reactor (nanoreactor), where high turbulence and cutting forces are created, which mixes the caustic solution and the oil very well, and eliminates phospholipids and other impurities	Possible secondary reactions with alteration of physical and organoleptic characteristics of the oil

Source: Elaborated by the authors

The real challenge is to achieve a balance between process quality and sustainability, and savings in potential costs and oil yield, i.e. key factors for the implementation of new technologies (De Greyt, 2012).

PUFA concentration in fish oil

Several techniques have been proposed for the concentration of PUFA, and especially for omega-3, including winterization, concentration by enzymatic methods, fractionation by supercritical fluids and by chromatographic methods, formation of complexes with urea, and concentration by membranes.

Fish oil winterization

Winterization is a process that involves partial crystallization of the oil by controlled cooling, followed by filtration. Its main objective is to separate saturated fatty acids from unsaturated ones. This separation is possible due to differences in melting points of the fatty acids, which depends mainly on the chain length and the unsaturation degree.

Thus, saturated and monounsaturated fatty acids, which have a higher melting temperature, crystallize and can be separated by filtration, while PUFAs remain in liquid form in the oil (Vázquez & Akoh, 2012).

The use of organic solvents is common in the winterization process in order to increase the rate of mass transfer and fraction crystallization of saturated fatty acids, from oil dissolved in a suitable solvent (López-Martínez, Campra-Madrid, & Guil-Guerrero, 2004; Morales, Muñío, Pérez, Guadix, & Guadix, 2013). This methodology has been one of the main techniques developed for the concentration of omega-3 PUFA from triacylglycerols in natural form (Lei et al., 2016).

Hence, Cunha, Crexi and De Almeida-Pinto (2009) performed the optimization of the fish oil winterization process, evaluating the type of solvent (acetone and hexane), its proportion (40 % and 60 %), and agitation in the second cooling stage (0 and 40 rpm), and obtained a statistical model for the concentration of unsaturated and saturated fatty acids. The best conditions obtained were 40 % hexane without agitation in the second cooling stage, which increased the concentration of unsaturated fatty acids by 9.2 % and reduced the saturated fatty acids by 13.3 % compared to the bleached oil.

Likewise, Tengku-Rozaina and Birch (2013) carried out winterization of hoki or blue grenadier oil (*Macruronus novaezelandiae*) with and without hexane as solvent. They observed that the use of this solvent facilitated the separation of the oil fractions and allowed a higher concentration of omega-3 fatty acids.

Meanwhile, Homayooni, Sahari and Barzegar (2014) evaluated three different winterization temperatures (-5, 0 and 10 °C) in sardine oil (*Dussumieria acuta*) with ethanol as a solvent, and observed that at -5 °C there was a higher decrease in saturated fatty acids and an increase of the unsaturated ones. Results indicated that the concentration of omega-3 PUFA in the non-crystallized portion increased as the temperature decreased.

Nonetheless, Díaz et al. (2016) also evaluated this process in trout oil, by optimizing the percentage of acetone and the crystallization time and achieved an increase in DHA and EPA of 69 % and 51.6 %, respectively.

Fractionation by supercritical fluids (FSCF)

This technique has been proposed for the extraction of oil, as well as for the concentration of PUFA, in particular, omega-3 such as DHA and EPA, from oil extracted with conventional methods, which is also known as fractionation.

Several studies address oil fractionation in its natural state, but it seems to be ineffective, possibly due to the complex structure of fish oil, composed of a large number of triacylglycerols (Corrêa, Peixoto, Gonçalves, & Cabral, 2008; Homayooni et al., 2014).

Lopes et al. (2012) evaluated the fractionation of fish oil from fresh water with a low omega-3 content of approximately 10 %. The best fractionation was obtained in the isotherms of 33 and 40 °C at 20,000 kPa. Ferdosh, Sarker Norulaini, Akanda et al. (2014) also studied this process in tuna oil, using ethanol as a co-solvent, and found that it was highly effective in PUFA recovery.

The structural complexity of fish oil has hindered its fractionation, so the interest in the synthesis and fractionation of methyl esters and ethyl esters of triacylglycerols from fish oil has increased, being more stable compounds than fatty acids. These are obtained by hydrolysis of triacylglycerols (TAG) and alkylation of fatty acids, before the FSCF (Lopes et al., 2012; Rubio et al., 2010).

Perretti et al. (2007) studied the concentration of omega-3 from a commercial mixture of fatty acid esters obtained from fish oil, using FSCF with supercritical fluids-CO_2. They studied different pressures (10,000, 14,000, 15,000 and 30,000 kPa) and various CO_2 fluxes, maintaining the temperature of the three column sections at 40, 50 and 60 °C, respectively, to increase the concentration of DHA and reduce the EPA: DHA ratio, an important characteristic to define its functional properties.

The increase in pressure and flow rate caused an increase in DHA concentration (from 24.54 % to 49.57 %) and a desired reduction in the EPA: DHA

ratio from 1.61 to 0.65, whereas saturated and monounsaturated fatty acids decreased from an average of 3.33 % to 0.6 %.

Létisse and Comeau (2008) applied this type of fractionation in fatty acid ethyl esters of sardine oil and observed the effect of temperature when obtaining a higher concentration ratio of EPA (24.74 %) and DHA (26.02 %) at 60 °C compared to 40 °C (4.28 % and 7.53 %). Likewise, the density of CO_2 showed a higher concentration of DHA and EPA, increasing from 700 to 800 kg/m^3, reaching a composition close to 40 % and 60 % of EPA and DHA, respectively.

On the other hand, some thermodynamic models and simulations of this process have been carried out based on state equations such as the ones published by Gironi and Maschietti (2006), contribution of groups (GC-EOS) (Espinosa, Díaz, & Brignole, 2002) and the methods of McCabe-Thiele and Ponchon-Savarit (Riha & Brunner, 2000). Likewise, also relationships between top temperature, number of theoretical stages, the ratio solvent: food in countercurrent fractionation with internal reflux (Maschietti & Pedacchia, 2014), and simplified models in the equilibrium stage (Pieck, Crampon, Charton, & Badens, 2016) to design a separation process of fatty acid ethyl esters that maximizes the concentration of omega-3 fatty acids.

Fractionation by chromatography

The chromatographic methods are used for the concentration of PUFA, in particular of EPA and DHA, and include liquid chromatography (HPLC), and argentometric and supercritical fluids, to obtain products of high purity (> 95 %) (Dillon, Aponte, Tarozo, & Huang, 2013). In general, the separations are carried out through argentometric chromatography and are conducted on ethyl esters of fatty acids, in some cases combining preconcentration steps prior to the final fractionation by chromatography (table 8).

Table 8. Fractionation of fish oil by chromatographic methods (since 2000)

Chromatographic method	Source material	Product	Purity of the product	Reference
Supercritical fluid chromatography (SFC)	Fatty acid esters of tuna oil	DHA concentrate	>95 %	Alkio, González, Jäntti and Aaltonen (2000)
Argentometric chromatography	Fatty acid esters of cod liver oil	EPA concentrate	83 %	Belarbi, Molina and Chisti (2000)
Argentometric chromatography (preconcentration by saponification and inclusion with urea)	Fatty acid esters of cod liver oil	EPA concentrate	90 %-97 %	Guil-Guerrero and Belarbi (2001)

(Continue on next page)

(Continuation of table 8)

Chromatographic method	Source material	Product	Purity of the product	Reference
Argentometric chromatography (preconcentration by saponification and inclusion with urea)	Fatty acid esters of sardine oil	EPA concentrate	99,6 %	Chakraborty and Raj (2007)
Argentometric chromatography	Fatty acid esters of fish oil	DHA and EPA concentrate	EPA >95 % DHA >99 %	Dillon et al. (2013)
Combined argentometric and liquid chromatography (HPLC)	Fatty acid esters of tuna oil	DHA concentrate	90 %	Fagan and Wijesundera (2013)
Argentometric chromatography	Fatty acid esters of sardine oil	DHA and EPA concentrate	91,26 % in the seventh fraction	Chakraborty et al. (2016)

Source: Elaborated by the authors

Liquid chromatography (HPLC) is used for the separation of omega-3 fatty acids from microalgae and fish oil. However, authors have reported that it is easier to separate DHA from oil, from simple cells compared to fish oil, because the latter has a more complex composition. With this technique, the esters of fatty acids are eluted from their number of carbon equivalents. Moreover, the esters with the same number can be separated under certain optimal conditions (Fagan & Wijesundera, 2013).

Argentometric chromatography is frequently used for the concentration of fatty acids, according to the degree of unsaturation, since the ability to form complexes with silver ions increases with this grade. For this reason, highly unsaturated fatty acids such as DHA and EPA are retained more strongly than others with less unsaturation, facilitating their separation.

Different configurations have been designed in the stationary phase, in order to improve the stability of the silver ion (i) and reduce its mobility, such as the use of silver nitrate, silver covalently bound to 3-mercaptopropyl as a stationary phase, and the stationary phase thiolate-silver (Dillon et al., 2013; Fagan & Wijesundera, 2013).

On the other hand, supercritical fluids have the density and solvent capacity of certain liquids, but with lower viscosity and better diffusion, so they can be used as substance carriers such as the mobile phase in gas chromatography or dissolve them as the solvents in HPLC. This technique is known as supercritical fluid chromatography (SCFC) (Taylor, 2009).

SCFC is especially suitable to separate high purity omega-3 PUFA, since it combines the high selectivity of the supercritical fluids and the stationary phase (Rubio et al., 2010). As with other techniques, investigations are conducted mainly on ethyl esters of fatty acids.

In fish oil, Alkio et al. (2000) applied this technique for the concentration of ethyl esters of EPA and DHA, using supercritical CO_2 as a mobile phase and a stationary phase of octadecylsilane (ODS). They obtained a production rate of DHA ethyl ester with a 90 % purity of 0.85 g/(kg ODS*h) and EPA ethyl ester with 53 % purity of 0.23 g/(kg ODS*h).

Concentration using enzymatic methods

The concentration by enzymatic methods is based on the selectivity of some lipases for certain fatty acids or positions in the triacylglycerol molecules, catalyzing reactions of hydrolysis, alcoholysis or transesterification of triacylglycerols (Correa, Tejeda, Martín, García, & Noriega, 2017; Miranda, Baeza, Noriega, García, & Otero, 2013; Rubio et al., 2010).

Some studies propose the application of these processes before other stages, such as molecular distillation or filtration by membranes, to obtain concentrates of omega-3 fatty acids. Table 9 shows the materials studied and the type of enzymes used to concentrate fish oil.

Table 9. Concentration of fish oil by enzymatic methods

Material	Type of enzyme	Results	Reference
Salmon oil	*Candida rugose* lipase	EPA increased from 5.46 % to 10.00 % DHA increased 2.8 times	Kahveci and Xu (2011)
Fish oil	*Candida antarctica* lipase	Production of diacylglycerols structured with 89.37 % of omega-3 PUFA, 11.32 % of EPA, 8.34 % of DPA and 69.71 % of DHA	Miranda et al. (2013)
Sardine and tuna oil	Lipozyme TL-IM and QLG lipase	EPA increased from 19 % to 61 % DHA increased from 22 % to 69 %	Valverde et al. (2014)
Sardine oil	Lipase MTCC 2421 from *Pseudomonas fluorescens*	EPA and linoleic acid increased 1.98 times	Chakraborty and Raj (2009)
Cod, sardine, salmon and shark oil	*Cryptococcus* sp. lipase	EPA increased from 16.9 % to 30.4 % DHA increased from 6.5 % to 9.6 %	Aarthy, Saravanan, Ayyadurai, Gowthaman and Kamini (2016)
Herring oil	*Rhizopus oryzae* immobilized	Increase in selectivity with enzyme immobilization	Ashjari, Mohammadi and Badri (2015)
Sardine oil	Lipase of *Candida antarctica*	Development of a kinetic model to contribute to the design and scaling of reactors in esterification reactions	Correa et al. (2017)

Source: Elaborated by the authors

Other investigations have studied the process of alcoholysis in the presence of solvents such as supercritical CO_2, because it is green, non-toxic and can easily be removed from the reaction products, which makes it a good mean to carry out the enzymatic reactions (Lin, Chen, & Chang, 2006). Besides, it can easily be coupled with other processes such as SCFE, FSCF, SCFC and encapsulation with supercritical CO_2 (Rubio et al., 2010).

Furthermore, Lin et al. (2006) studied the concentration of omega-3 PUFA from *Sardinella aurita* oil using Lipozyme IM-60, obtaining favorable results and finding that when using supercritical CO_2 the conversion was 40 % higher than under regular environmental conditions.

However, in more recent investigations (Shin, Sim, Kishimura, & Chun, 2012; Tanbirul-Haque & Chun, 2015), no significant effect was found when using this same solvent in the alcoholysis of fish oil with Lipozyme TL-IM, Lipozyme RM-IM and Novozyme 435, although it seems to improve the thermal stability of the enzyme and reduces the oxidation of omega-3 PUFA, tuna oil (*Thunnus* sp.) and sardine (*S. pilchardus*) (Melgosa et al., 2017).

Other PUFA concentration methods

Other methods for the concentration of PUFA include molecular or short-path distillation, filtration by membranes and the formation of complexes with urea.

Molecular distillation is a technology that can be used appropriately for the separation, purification or concentration of thermolabile substances, since it operates with high vacuum pressures (lower than an absolute pressure of 1,000-500 kPa) (Cerón, Cardona, & Toro, 2012; Pramparo, Prizzon, & Martinello, 2005).

Although in some cases this technology is used as an oil purification process for the removal of organic pollutants (Olli, Breivik, & Thorstad, 2013), it has also been applied in the concentration of PUFA, particularly of EPA and DHA in free form or as ethyl esters (Oliveira & Miller, 2014; Solaesa, Sanz, Falkeborg, Beltrán, & Guo, 2016; Wang et al., 2012). Likewise, in tuna oil, Wang et al. (2012) achieved a concentration increase of the total content of EPA and DHA from 32.11 % to 82.23 %, and similarly, Solaesa et al. (2016) reached an increase in the concentration of acylglycerols of omega-3 PUFA from 63 % to 91 % in sardine oil.

Membrane filtration is characterized by the application of hydraulic pressure as a driving force for mass transfer. The nature of the membrane controls which components will permeate, and which will be retained, according to their molar mass or particle size.

This technology has been used in degumming, recovery of solvents in extraction processes, pigment removal, acidity reduction, concentration of minor components, removal of waxes and separation of emulsions (De Morais-Coutinho et al., 2009), just as in PUFA (Ghasemian, Sahari, Barzegar, & Ahmadi, 2016; Ghasemian, Sahari, Barzegar, & Gavlighi, 2015; Linder et al., 2005; Linder, Matouba, Fanni, & Parmentier, 2002).

Thus, in salmon oil, the decrease in saturated fatty acids has been observed from 27.2 % to 20.2 %, while the PUFA content increased from 41.6 % to 46.5 %, with an increase of DHA from 9.9 % to 11.6 %, and EPA from 3.6 % to 5.6 % (Linder et al., 2005).

Nevertheless, Ghasemian et al. (2015) performed an optimization study for the concentration of omega-3 PUFA by polymeric membrane in fish oil, in which they evaluated the effect of temperature, pressure and agitation speed. These authors found that the optimal conditions were 36.19 °C, 4.82 bars and 43.01 r.p.m., respectively, which resulted in a maximum omega-3 PUFA value of 35.11 %.

On the other hand, the formation of complexes with urea is the simplest and most efficient technique to obtain omega-3 PUFA concentrates as free fatty acids or ethyl esters of triacylglycerols. Saturated and monounsaturated fatty acids are separated

from polyunsaturated fatty acids starting from a saturated solution of urea, in which all the fatty acids are found.

By cooling and filtration, the compounds formed between the saturated and monounsaturated fatty acids can be removed during crystallization. PUFAs do not form inclusion complexes with urea, so they remain concentrated in the liquid fraction (Homayooni et al., 2014).

Studies on this technique mainly evaluate the relationship between urea and fatty acid, temperature and crystallization time (Gámez et al., 2003; Homayooni et al., 2014; Liu, Zhang, Hong, & Ji, 2006; Suriani, Lawalata, & Komansilan, 2014; Tengku-Rozaina & Birch, 2013).

With this method, DHA and EPA contents higher than 85.02 % in tuna oil were obtained (Liu et al., 2006), with a ratio of urea: fatty acid of 15, a temperature of -5 °C and in a time period of 20 hours. In sardine oil, the highest amounts of DHA and EPA were found at -10 °C and 1 °C, respectively, where the DHA was enriched from 17.45 % to 29.61 % and the EPA from 15.39 % to 19.76 % (Homayooni et al., 2014).

Conclusions

The omega-3 polyunsaturated fatty acids (PUFA) are highly valued at present for their beneficial effects on health. Fish oil is recognized as the primary source of these fatty acids, mainly eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids.

Some studies employ the extraction of fish oil by traditional methods, such as wet pressing and conventional solvents, but recent research is opting for the application of technologies such as supercritical fluids and fish silage. Refining methods are still based on traditional stages, although there is an important potential for the application of new technologies such as enzymatic and physical refining.

Likewise, for the concentration of PUFA different methods have been proposed, such as enzymatic and chromatographic methods, winterization, supercritical fluids, complex formation with urea and filtration by membranes. However, currently the combination of different techniques seems to offer a good alternative to increase the purity and performance of these components.

Acknowledgements

The authors wish to thank Sistema General de Regalías [General System of Royalties] for their support in the publication of this article, through the project Alternativas para el uso de subproductos derivados de la agroindustria piscícola (Altpez) [Alternatives for the use of by-products derived from the fish farming agroindustry] executed by Universidad del Cauca.

Disclaimers

The authors declare that there are no conflicts of interest.

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