Article

Sustainable synthesis of omega-3 fatty acid ethyl esters from monkfish liver oil

Johanna Aguilera-Oviedo [1,2], Edinson Yara-Varón [1,2], Mercè Torres [2,3], Ramon Canela-Garayoa [1,2,*]and Mercè Balcells [1,2]

[1] Department of Chemistry, University of Lleida, Avda. Alcalde Rovira Roure 191, 25198 Lleida, Spain; johanna.aguilera@udl.cat (J.A.-O.); edinson.yara@udl.cat (E.Y.-V.); merce.balcells@udl.cat (M.B.)
[2] Centre for Biotechnological and Agrofood Developments (Centre DBA), University of Lleida, Avda. Alcalde Rovira Roure 191, 25198 Lleida, Spain; merce.torres@udl.cat
[3] Department of Food Technology, University of Lleida, Avda. Alcalde Rovira Roure 191, 25198 Lleida, Spain.
* Correspondence: ramon.canela@udl.cat;Tel.: (+34-973702841)

Received: date; Accepted: date; Published: date

Abstract: The search for economical and sustainable sources of PUFAs within the framework of the circular economy is encouraged by their proven beneficial effects on health. The extraction of monkfish liver oil (MLO) for the synthesis of omega-3 ethyl esters was performed evaluating two blending systems and four green solvents. Moreover, the potential solubility of the MLO in green solvents was studied using the predictive simulation software COSMO-RS. The production of the ethyl esters was performed by one or two step reactions. Novozym 435, two resting cells (*Aspergillus flavus* and *Rhizopus oryzae*) obtained in our laboratory and mix of them were used as biocatalysts in a solvent-free system. The yields for Novozym 435, *R. oryzae* and *A. flavus* in the one-step esterification were 63%, 61% and 46%, respectively. The hydrolysis step in the two-step reaction led to 83%, 88% and 93% of free fatty acids (FFA) for Novozym 435, *R. oryzae* and *A. flavus* respectively. However, Novozym 435 showed the highest yield in the esterification step (85%) followed by *R. oryzae* (65%) and *A. flavus* (41%). Moreover, selectivity in front of polyunsaturated fatty acids of *R. oryzae* lipase was evidenced, since it did slightly esterified docosahexaenoic acid (DHA) in all the esterification reactions tested.

Keywords: Omega-3 ethyl esters; monkfish liver oil; COSMO-RS; fungal resting cells; selectivity

1. Introduction

Numerous scientific studies have demonstrated the health benefits of polyunsaturated fatty acids (PUFAs), in particular those known as omega-3 [1-8]. The main omega-3 fatty acids are α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The main health effects of omega-3 fatty acids can be seen in cardiovascular disease (CVD) [1,2], but beneficial effects have also been described in various diseases such as diabetes [3], immune system diseases [4] and cancer [5-6]. Likewise, positive interaction has even been found in diseases such as autism spectrum disorder (ASD) [7] and neuronal diseases such as Alzheimer [8]. Consequently, it is rather worth including omega-3 fatty acids as nutraceutic ingredients in functional food products and pharmacy [9].

The fatty acid content of marine fish, whether oily or white fish, is high in omega-3 polyunsaturated fatty acids, as these are synthesized by microalgae and they reach the fish through the food chain [10]. According to the Apromar report on aquaculture and fisheries in Spain 2019 [11], the EU consumed 13 million tons of aquatic products in 2018. Depending on the type of product the edible part, range 40 to 80%, generating by-products that can reach up to 60% of the products consumed [12]. Therefore, these by-products or residues can be used as starting materials to prepare

new commercial products within the circular economy concept [13]. Fish viscera accounts for 12 to 18% [12] of a fish and are considered ABPs (by-products of animal origin not intended for human consumption). Although monkfish liver has an important culinary value in some restaurants as a gourmet dish, it is usually considered an ABPs and is discarded with the rest of the viscera in the vast majority of cases [14,15,16]. The monkfish, a white fish, contents *c.a.* 30% oil, which fatty acid profile shows the presence of DHA, EPA, gadoleic acid, oleic acid among other characteristics of fish oils [17].

The Folch method (FM) is a very common method to determine the fat content in fatty samples [20]. This method uses a mixture of chloroform and methanol in a 2:1 ratio to extract the sample. These solvents are volatile organic compounds (VOCs) mainly sourced from non-renewable resources. They are flammable, volatile, and toxic being responsible for environmental pollution and the greenhouse effect [21]. Therefore, the search for more environmentally friendly solvent extraction processes is a priority [22,23].

2-Methyltetrahydrofuran (2-MeTHF), cyclopentyl methyl ether (CPME), dimethyl carbonate (DCM) and limonene (LMN) are considered green solvents and have been used in sustainable and environmentally friendly extraction processes [24]. Moreover, the extraction capacity of these solvents can be studied with computer tools, which allow saving time and resources in experimentation and maximize the chances of success. COSMO-RS (Conductor like Screening Model for a Realistic Solution) is a software worldwide used to predict the most suitable solvents for the extraction of natural products [23,25].

Sustainable oil extraction processes are the first step in the exploitation of fish co-products. The application and use of fish oils rich in omega-3 is quite widespread, it is usually more common to use them in the form of esters for their stability [2,10]. Free fatty acid and triacylglycerols (TAGs) are considered more susceptible to oxidation than the corresponding esters [26,27]. Ester synthesis can be performed in one-step (transesterification) or two-step (hydrolysis followed by esterification) reactions. These reactions can be catalysed by enzymes, which allow the development of efficient and fast processes. Lipases are widely used in industry because they have the capacity to catalyse different reactions. Moreover, several scientific articles have demonstrated the selectivity of some lipases for different fatty acids in both hydrolysis and esterification reactions. Moreno-Pérez et al., [28] studied the selectivity of two lipases (*Thermomyces lanuginosus* (TLL) and Lecitase Ultra, a phospholipase with lipolytic activity) immobilized in different supports (hydrophobic C18 Sepabeads and a Duolite anion exchanger) in the synthesis of ethyl esters (EE) of omega-3 fatty acids by the ethanolysis of sardine oil in solvent-free systems. They achieved an increase on the activity of TLL and lecithase. Moreover, the Sepabeads support showed high selectivity for EPA ethyl ester (EPA-EE) synthesis. Castejón et al. [29] studied the enzymatic production of enriched structured triacylglycerols of EPA and DHA (STAG) from *Camelina sativa* oil by two-stage selective hydrolysis-esterification. A, where noteworthy selectivity of the different lipases tested towards EPA-EE compared to DHA-EE was found. Zangh et al. [27] also reported selectivity between DHA and EPA in the production of DHA-rich TAGs using the commercial enzyme Novozym 435. Ranjan-Moharana et al. [30] described the use of phospholipase A1 for omega-3 enrichment in anchovy oil.

COSMO-RS software was used in this work to predict the extractive potential of monkfish liver oil (MLO) of four alternative green solvents (2-MeTHF, CPME, DCM and LMN) to replace the Folch method (a mixture of chloroform and methanol). Afterwards, the experimental extraction of MLO was carried out to develop a comprehensive, sustainable and environmentally friendly process. The recovered monkfish liver oil was used to prepare the fatty acid ethyl esters using three different lipases, a commercial enzyme (Novozym 435) and two resting cells (*R. oryzae* and *A. flavus*). In addition, we evaluated the selectivity that these biocatalysts present regarding to the different fatty acids from the MLO. In this sense, we developed a process that potentiates the use of fish co-products to synthesize products with food, cosmetic or pharmaceutical applications such as PUFAs ethyl esters.

2. Results and Discussion

2.1. COSMO-RS prediction

A COSMO-RS simulation was conducted to determine the relative solubility of the four main TAGs of monkfish liver oil in the targeted solvents. ^{1}H-NMR oil analysis allowed determining that TAGs were the main lipids present in the fish oil. ^{1}H-NMR also allowed determining the main fatty acids present in the oil. GC-FID analysis confirmed that these TAGs were mainly composed of long carbon chains such as palmitic acid (C16:0), oleic acid (C18:1n9), EPA (C20:5n3) and DHA (C22:6n3). Therefore, we decided to use the four main fatty acids in the oil to define four TAG structures: TAG-1(R1 (C16:0); R2 (C22:6n3); R3 (C16:0)); TAG-2 (R1 (C18:1n9); R2 (C20:5n3); R3 (C16:0)); TAG-3 (R1 (C18:1n9); R2 (C22:6n3); R3 (C18:1n9)); TAG-4 (R1 (C22:6n3); R2 (C22:6n3); R3 (C22:6n3)), as models for COSMO-RS analysis using (Fig. 1). These main components were modelled with ChemSketch software and used for the predictive study. COSMO-RS integrates a quantum chemistry approach that allows the calculation of several properties such as the relative solubility of a compound in several solvents. This means that analysis of the σ profile and the σ potential of the components of the mixture (TAGs and solvents) provides important information about the molecules that can be used to predict possible interactions in the fluid phase.

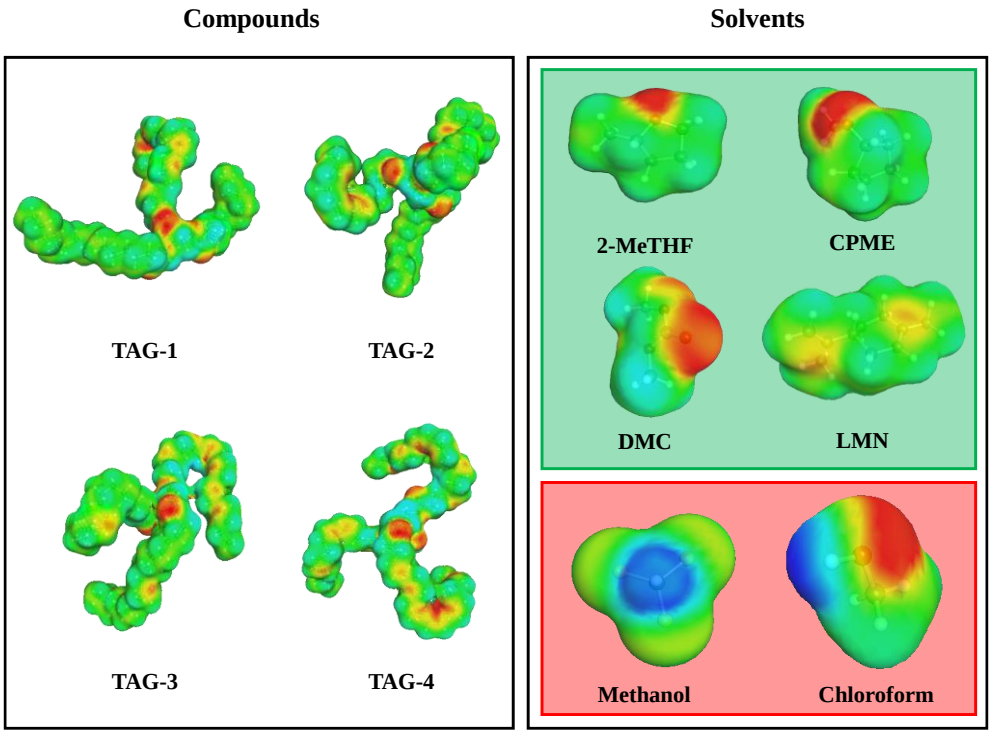

Figure 1. Modelization of α-surfaces by COSMO-RS of compounds and solvents used in the theoretical study. Compounds (TGAs): TAG-1(R1 (C16:0); R2 (C22:6n3); R3 (C16:0)); TAG-2 (R1 (C18:1n9); R2 (C20:5n3); R3 (C16:0)); TAG-3 (R1 (C18:1n9); R2 (C22:6n3); R3 (C18:1n9)); TAG-4 (R1 (C22:6n3); R2 (C22:6n3); R3 (C22:6n3)). Solvents, green solvents (green colour): (2-methyltetrahydrofuran (2-MeTHF), cyclopentyl methyl ether (CPME), dimethyl carbonate (DMC) and limonene (LMN)). Reference solvent (red colour): (Folch reagent (FR), chloroform/methanol (2:1, v/v)).

Table 1 shows the solubility of the model TAGs from monkfish liver oil in the solvents used in this study. The solubility is expressed in log10(x_solub) (best solubility is set to 0, and all solvents are given relative to the best solvent) and percentage of probability of solubility for a better understanding of the results. The solvent used as a reference was the Folch reagent (FR), a mixture of chloroform and methanol (2:1, v/v), which is considered to be the most reliable method for full recovery of total lipids [31]. Three of the green solvents tested, 2-MeTHF, CPME and LMN, showed higher probability of solubility (60-100%) than the reference solvent for the TAG-1 to TAG-3 and similar for TAG-4. Even though DMC presented low probability of solubility (0-20%) for three TAG

of the fourth model TAG, this green solvent showed better solubility percentage than the FR. Finally, taking in consideration the theoretical results obtained by the COSMO-RS computational predictive method, we decided to perform the experimental study using the four green solvents (2-MeTHF, CPME, DMC and LMN), which can have potential to replace the FR solvent in the extraction of lipids.

Table 1. COSMO-RS relative solubility (log10(x_solub)) and probability of solubility of triacylglycerides (TAGs) from monkfish liver oil using four different green solvents and FR as reference solvent.

Solvent	TAG 1		TAG 2		TAG 3		TAG 4	
	log10 (x_solub)	Probability (%)	log10 (x_solub)	Probability (%)	log10 (x_solub)	Probability (%)	log10 (x_solub)	Probability (%)
2-MeTHF	0.0000	100.00	0.0000	100.00	0.0000	100.00	0.0000	100.00
CPME	0.0000	100.00	0.0000	100.00	0.0000	100.00	0.0000	100.00
DMC	-0.9721	10.66	-0.8726	13.41	-0.6799	20.90	0.0000	100.00
LMN	0.0000	100.00	0.0000	100.00	0.0000	100.00	0.0000	100.00
FR (Reference)	-1.7547	1.76	-1.5751	2.66	-1.4406	3.63	0.0000	100.00

Green colour: high probability of solubility (60-100%). Yellow colour: medium probability of solubility (20-60%). Red colour: low probability of solubility (0-20%). Compounds (triacylglycerides): TAG-1(R1 (C16:0); R2 (C22:6n3); R3 (C16:0)); TAG-2 (R1 (C18:1n9); R2 (C20: 5n3); R3 (C16:0)); TAG-3 (R1 (C18:1n9); R2 (C22:6n3); R3 (C18:1n9)); TAG-4 (R1 (C22:6n3); R2 (C22:6n3), R3 (C22:6n3)). Solvents: green solvents (2-methyltetrahydrofuran (2-MeTHF), cyclopentyl methyl ether (CPME), dimethyl carbonate (DMC) and limonene (LMN)). Reference solvent (Folch reagent (FR), chloroform/methanol (2:1, v/v)).

2.2. Monkfish liver oil extraction

The solid-liquid extraction of oil from fresh monkfish liver using five different solvents was performed by maceration using roller mixer and ULTRA-TURRAX® systems. Folch reagent [19] was used until exhaustion (until no colour was observed in the solvent) to determine the maximum content of oil in the liver. A percentage of 39.0% w/w was the maximum content of oil in the fresh material, which contents 49.8%of moisture. This is the first determination of the oil content in monkfish liver as far as we know. This value is higher that the percentage reported for tuna liver with an oil yield of 17.5 % [32], or from salmon by-products (head, frame and viscera) with an oil content ranging 13.09 to 19.2 % [33]. Ciriminna et al. [34] reported 1.5% of oil content from anchovy heads.

2.2.1. Extraction in the two systems: Roller Mixer (RM) and ULTRA-TURRAX® (UTs)

The results presented in Table 2 showed that the extraction yields were higher when the RM was used. All green solvents showed extraction yields between 96 and 100% of the maximum oil content in monkfish liver using RM. A single extraction using FR solvent yielded 89% of oil using RM. Authors such as Fang et al. also reported the importance of this variable in their tuna oil extraction experiments [31]. The lower extraction yields obtained with some green solvents, as well as with the FR when UTs was used could be explained by the formation of emulsions due to the presence of water in the fresh monkfish liver and by the type of agitation and the high speed (4000 rpm) used in this system. 2-MeTHF and CPME extracted the highest quantity of fish oil in the two extraction systems evaluated, which coincide with the COSMO-RS prediction. In the theoretical study DCM showed the lower probability of oil solubility; however, in the experimental extraction it showed better results than expected, e.g. 99% in RM. In the case of LMN presented worst extraction yields than those predicted by COSMO-RS. Nevertheless, this experimental result it can be due to the harsh conditions used to eliminate this solvent (90ºC/ 0.3 mbar). LMN was the most difficult to

evaporate of all tested solvents. Considering the results obtained using the RM system, it can be said that the evaluated green solvents could be interesting alternatives to replace conventional solvents such as hexane, chloroform and methanol to develop more environmentally friendly extractive processes for oil samples [21,22,23].

Table 2. Monkfish liver oil extraction with four green solvents and a conventional solvent using two blending systems (mean ± standard deviations, n=2).

Solvent	Roller Mixer		ULTRA-TURRAX® system	
	Oil yield (OY) (g per 100 g FM)	OY compared to maximum oil content (%)	Oil yield (OY) (g per 100 g FM)	OY compared to maximum oil content (%)
Reference (FR)	39.0	100	39.0	100
2-MeTHF	39.0± 0.9	100± 3.0	33.9± 1.5	87.0± 2.2
CPME	39.0± 2.4	100± 0.7	39.0± 0.3	100± 2.2
DMC	38.6± 1.9	99.0± 0.4	29.3± 0.2	75.0± 5.0
LMN	37.4± 1.7	96.0± 6.7	32.0± 2.6	82.0± 4.3
FR	34.5± 1.5	89.0± 1.5	29.1± 1.5	75.0± 1.4

FM, Fresh Material; Reference (FR), maximum oil content in monkfish liver; solvents (2-methyltetrahydrofuran (2-MeTHF), cyclopentyl methyl ether (CPME), dimethyl carbonate (DMC), limonene (LMN) and Folch Reagent (FR)).

2.2.2. Analysis of the extracted oil

In the MLO ^{1}H-NMR spectra, signals corresponding to omega-3 fatty acids between 0.99 and 1.1 ppm were observed, in addition to other signals corresponding to PUFA, MUFA and saturated fatty acid (SFAs) [35,36]. Specific signal of DHA were observed at 2.4 ppm corresponding to the hydrogen bonded to both the α-carbon (=C-C-**CH$_2$**-COOR) and the allyl-carbon (=C-**CH$_2$**-C-COOR) [37]. No differences were observed between the spectra of oils obtained with different solvents or extraction methods (RM or UT).

On the other hand, the fatty acid profile of the extracted oils was determined by GC-FID (Table 3). MLO resulted to have 32.5% of SFAs, 42.1% of mono-unsaturated fatty acids (MUFAs) and 27.3% of PUFAs. According to the fatty acid profile, oleic acid is the major fatty acid with 21.1% of the total, with beneficial effects on human health as the omega-3 fatty acids [38]. Furthermore, the presence of 5.4% of vaccenic acid, an omega-7 isomer of the oleic acid, is worth to consider due to its associated with a low risk of cardiovascular disease [39,40]. Other MUFAs present are gadoleic acid (C20:1n9), which is characteristic of fish oils, and erucic acid (C22:1n9), which has also been reported that is found in fish liver [32,41]. DHA and EPA are the main PUFAs in monkfish oil with 15.2 and 4.1 % of the total, respectively. The oil content 39% w/w regarding to fresh monkfish liver and its composition confirms the potential that this by-product has as raw material to obtain products with high added value. The high content of these was expected since white fish tend to store the omegas 3 and 6 in the liver and not in the muscles as blue fish does.

Table 3. Fatty acid profile (%) of monkfish liver oil.

Common name	*Common symbol*	*FA (%w/w)*
Saturated Fatty Acid (SFAs)		
Hendecanoic	C10:0	0.3
Lauric	C12:0	0.6
Miristic	C14:0	5.8
Palmitic	C16:0	14.8
Margaric	C17:0	1.0
Estearic	C18:0	4.3

Arachidic	C20:0	1.3
Behemic	C22:0	2.9
Tetracosenoic	C24:0	1.5
	Σ SFAs	**32.5**
Mono-unsaturated Fatty *Acids (MUFAs)*		
Miristoleic	C14:1n5	0.7
cis-10-Pentadecanoic	C15:1n5	0.5
Palmitelaidic	C16:1n7t	0.4
Palmitoleic	C16:1n7c	7.1
cis-10-Heptadecenoic	C17:1n7	0.4
Elaidic	C18:1n9t	1.1
Oleic	C18:1n9c	21.1
Vacenic	C18:1n7	5.4
Gadoleic	C20:1n9	3.9
Erucic	C22:1n9	0.7
Nervonic	C24:1n9	0.8
	Σ MUFAs	**42.1**
Poly-unsaturated fatty acid (PUFAs)		
all cis-9,12-Hexadecatrienoic	C16:2n4	0.6
all cis-6,9,12-Hexadecatrienoic	C16:3n4	0.1
Linoelaidic	C18:2n6t	0.2
Linoleic	C18:2n6c	0.9
α-Linoleic	C18:3 n3	0.2
y-Linolenic	C18:3n6	0.4
Stearidonic	C18:4n3	0.5
cis-11,14-Eicosadienoic	C20:2n6	0.4
cis-11,14,17-Eicosatrienoic	C20:3n3	0.4
all cis-8,11,14-Eicosatrienoic	C20:3n6	0.2
Arachidonic	C20:4n6	1.0
Juniperonic	C20:4n3	0.5
Eicosapentanoic (EPA)	C20:5n3	4.1
cis-13,16-Docosadienoic	C22:2n6	0.7
Adrenic	C22:4n6	0.4
Clupadonic	C22:5n3	1.5
Docosahexanoic (DHA)	C22:6n3	15.2
	ΣPUFAs	**27.3**

2.3. Enzymatic preparation of fatty acid ethyl esters (FAEEs).

Looking for the best reaction conditions for the production of FAEEs using MLO and ethanol, two different procedures were performed: a) a one-step procedure (a transesterification reaction), and b) a two-step procedure through hydrolysis and esterification reactions. To catalyse these reactions a commercial enzyme (Novozym 435), two own resting cells (*R. oryzae* and *A. flavus*) and two mixtures of these resting cells (1:1 and 7:3, *R. oryzae-A. flavus*) were used in a solvent-free medium.

2.3.1. One-step synthesis: transesterification reactions.

The results of the one-step (transesterification) and two-step reactions (hydrolysis and esterification) carried out are shown in Table 4. In the transesterification and esterification reactions an increase in the yield was observed with increasing reaction time. The commercial enzyme showed the highest yield in the transesterification reaction. Yields were 44%, 61% and 63% for 24 h, 48 h and 72 h, respectively. Transesterification yield with *R. oryzae* was higher (53%) for 24 h although the yield for 72 h was slightly lower (61%). *A. flavus* showed the lowest yields (46 % for 72

h). Therefore, the resting cells from *A. flavus* cannot be considered an alternative to the commercial enzyme for this transesterification reaction. Moreover, two mixtures of the fungal resting cells (1:1 and 7:3, *R. oryzae-A. flavus*) were used for the transesterification reaction. Only the 7:3 mixture led to a 57% yield after 72 h of reaction, a lower yield than that achieved using only *R. oryzae*. Considering these low yields, the fatty acid ethyl esters profiles of these reactions were not determined.

Table 4. Yields (%) of the different reactions performed to prepare monkfish liver oil ethyl esters. (mean ± standard deviations, n = 3).

Biocatalyst	One-step			Two-step			
	Transesterification (%)			Hydrolysis (%)	Esterification (%)		
	24 h	48 h	72 h	24 h	24 h	48 h	72 h
Novozym 435	44.0± 2.8	61.0± 1.5	63.0± 0.4	83.1± 3.3	54.0± 0.5	70.0± 3.1	85.0± 1.4
R. oryzae	53.0± 4.1	54.0± 5.0	61.0± 2.3	88.1± 1.7	42.0± 2.7	55.0± 1.7	65.0± 4.0
A. flavus	32.0± 2.7	38.0± 3.4	46.0± 0.6	93.2± 0.0	37.0± 5.1	39.0± 3.1	41.0± 2.0
***R. oryzae-A. flavus* (1:1)**	34.0±4.5	38.0±3.6	45.0±1.7	87.9±5.6	32.0±3.2	37.0±4.5	37.0±4.5
***R. oryzae-A. flavus* (7:3)**	38.0±4.2	45.0±1.7	57.0±4.2	95.7±0.3	41.0±0.6	34.0±4.7	42.0±1.4

The main FAEEs obtained in the one-step reaction studied using the three biocatalysts is shown in Figure 2. The commercial enzyme and the fungal resting cells led to different polyunsaturated fatty acid ethyl esters (PUFAEEs) contents. The commercial enzyme esterified 90% of DHA and 100% of EPA regarding to the total content of the corresponding fatty acid in the MLO. This result showed that this enzyme does not discriminate between the different fatty acids to synthetize the FAEEs. Similar results have been already describer for this commercial enzyme, [28,29] which indicate that it is a suitable biocatalyst for the synthesis of these omega-3 EEs. In contrast, the resting cells showed lowest yields for the esterification of PUFAs, mainly DHA. *A. flavus* showed the highest yield of DHA-EE (38%) of the two resting cells studied. *R. oryzae* only esterified 22% of DHA present in the fish oil after 72 h reaction. No differences were observed for the esterification of EPA. Therefore, it suggests that lipases from *R. oryzae* discriminated between the different PUFAs present in the monkfish liver oil. The selectivity of enzymes has been reported by different authors who suggest that some lipases can be selective for some type of fatty acid depending on their chain length, the types of solvents used in its extraction and purification, its method of immobilization or the reaction conditions (temperature, time or substrate) [42,43,44]. The selective synthesis showed by the lipases from *R. oryzae* could facilitate the separation of the DHA from the mixture of FAEEs.

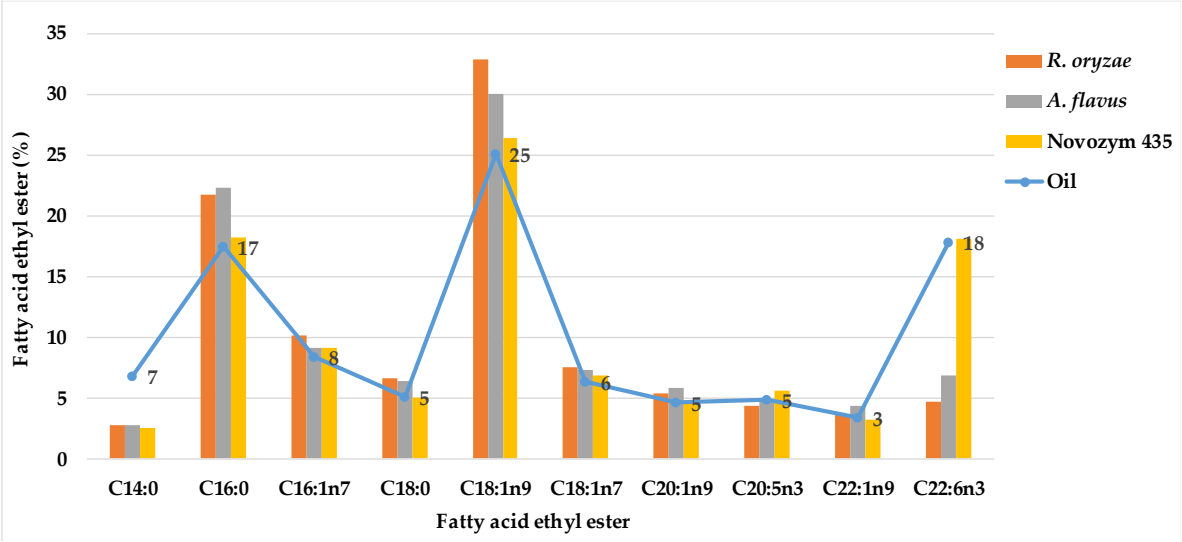

Figure 2. Effect of the biocatalyst on the profile of the main fatty acid ethyl esters obtained in the one-step reaction for 72 h. Oil: corresponds to the total content of the corresponding ethyl ester in the monkfish liver oil.

2.3.2. Two-step synthesis: hydrolysis and esterification reactions

Hydrolysis, the first step of this study, was carried out using the three previous enzymes and two mixtures (1:1 and 7:3) of the two resting cells for 24 h (Fig. 3). The resting cells of *R. oryzae* mixed with *A. flavus* in a 7:3 ratio showed the highest percentage of hydrolysis (> 95% free fatty acid (FFA)). The second biocatalyst presenting a high hydrolysis percentage was the resting cells of *A. flavus* (> 93% FFA). All resting cells studied showed a higher percentage of hydrolysis than the commercial lipase (83%), indicating that the resting cells can be a cheap alternative to immobilized commercial biocatalysts for these reactions. These hydrolysis percentages can be considered high when compared to those reported [45] for the hydrolysis of fish oil with the lipase of *Cryptococcus* sp., which yielded 25% and 66.5% of FFA for 24 h and 72 h, respectively. Furthermore, it is worth to point out that monkfish liver oil (MLO) contains more DHA than hydrolysed cod, sardine, salmon and shark liver oils (Fig. 4). A scale up of the hydrolytic process was carried out using the best reaction conditions achieved in the previous experiment. Starting from 25 g of fish oil, a mixture of *R. oryzae* and *A. flavus* (7:3) allowed the preparation of hydrolysed monkfish liver oil (HMLO) with 97.8% of FFA.

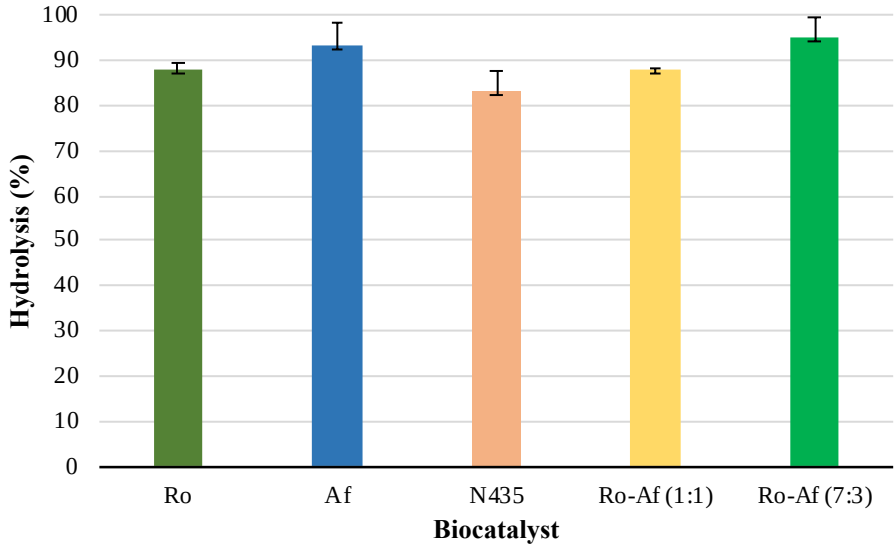

Figure 3. Hydrolysis percentage of monkfish liver oil obtained with the different enzymes (mean ± standard deviations, n=3, time reaction: 24 h). Ro, *R. oryzae*; Af, *A. flavus*; N435, Novozym 435.

The esterification reaction was performed using the hydrolysed monkfish liver oil (HMLO) obtained in the previous experiment. In these experiments, a positive correlation was found between reaction times and esterification performance as in the transesterification. The commercial enzyme showed the highest yield in the esterification reaction. Yields were 54%, 70% and 85% for 24 h, 48 h and 72 h, respectively. Esterification yield with *R. oryzae* was 65% for 72 h. *A. flavus* again showed the lowest yields (41 % for 72 h). These results confirm that *A. flavus* resting cells are not very active in the esterification of the fatty acids from monkfish oil. Regarding to the mixtures of the fungal resting cells, the yields were lower than the ones achieved with *R. oryzae* as was in the transesterification reaction (see Table 4).

In relation to the FAEEs profiles (Figure 4), these are similar to those shown in the one-step reactions. There has been an enrichment of MUFAs such as oleic acid, gadoleic acid and vacenic acid, which are beneficial to health [38]. Vacenic acid is a source of omega-7 that has been positively correlated with the presence of DHA and EPA [39,40]. The increase of palmitic acid and stearic acid observed in the assays could be due to the fatty acids present in the sunflower oil used in the production of the resting cells as lipase inductors. The microorganisms themselves can also synthesize different fatty acids, *Aspergillus* sp produces long chain fatty acids (C16:0, C16:1n7, C17:0, C18:0, C18:1n9, C18:2, C18:3 and C20:0). This fatty acid profile has been used to discriminate fungal species belonging to *Aspergillus* genus [46]. In addition, the presence of some fatty acids such as gadoleic acid (C20:1n9) may depend on the type of substrate used in the culture medium [47]. Nevertheless, the increase of these fatty acids in the final crude of the reaction cannot be higher than 4% considering the percentage of biocatalysts used (10%) and the percentage of fatty acids present in the resting cells (40%).

EPA behaved similarly to one-step reactions, in which the commercial enzyme esterified up to 100% of EPA contained in HMLO and *R. oryzae* esterified 70% and *A. flavus* esterified 60%. For DHA, Novozym 435 esterified up to 90% of the DHA contained in HMLO, while the fungal resting cells did not exceed 20%. *A. flavus* lipase esterified up to 19% of the DHA content, while *R. oryzae* lipase esterified up to 16%. These low yields seem to confirm the hypothesis of DHA selectivity shown by *R. oryzae*. Because the selectivity of *R. oryzae* lipase, DHA should be found as free fatty acid within the esterified material. The possibility of isolating these free fatty acid is also interesting as it has been shown that PUFAs ingested in the form of free fatty acids may be more bioavailable than FAEEs and therefore more assimilable in human metabolism [48].

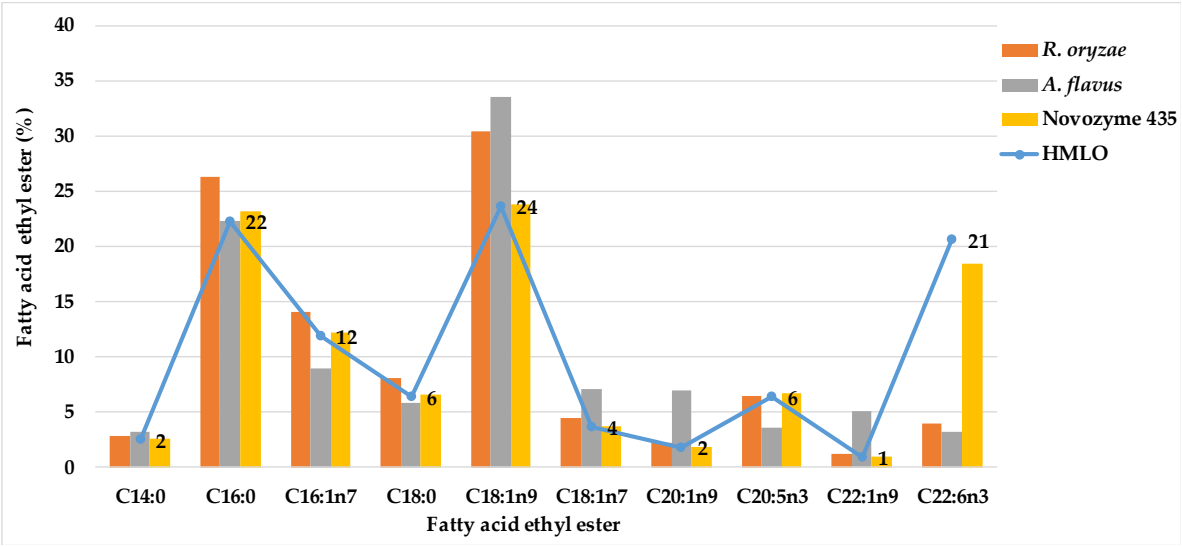

Figure 4. Effect of the biocatalyst on the profile of the main ethyl esters obtained for 72 h two-step reaction. HMLO: correspond to the total content of the corresponding ethyl ester in the hydrolysed monkfish liver oil. .

To confirm the above-mentioned hypothesis and confirm that DHA is present as an acid in the esterified fraction, these samples were subjected to total esterification through chemical catalysis (derivatisation process) using H_2SO_4. Figure 5 compares the DHA-EE obtained in the one and two-step reactions at different times (24, 48 and 72 h) using *R. oryzae* or applying chemical catalysis.

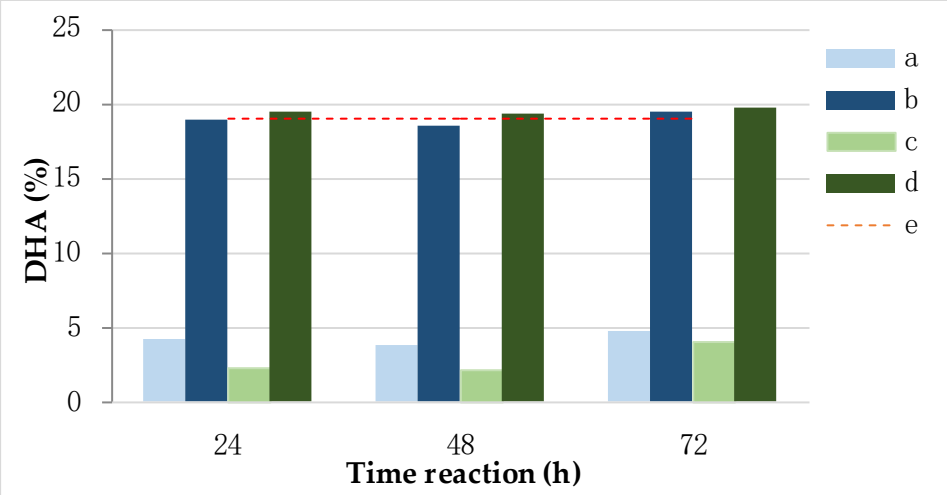

Figure 5. Percentage of DHA before and after total chemical esterification for 24, 48 and 72 h using *R. oryzae* lipase and chemical catalysis. (a) Light blue colour: ester obtained before acid catalysis, from one-step esterification with *R. oryzae*. (b) Dark blue colour: ester obtained after applying chemical catalysis to the crude resulting from one-step esterification. (c) Light green colour: ester obtained before chemical catalysis, from two-step esterification. (d). Dark green colour: ester obtained after applying chemical catalysis to the crude resulting from the two-step esterification (e) total DHA content in the MLO.

As expected, chemical catalysis achieved more than 90% esterification of the DHA contained in both monkfish liver oil and the hydrolysed monkfish liver oil. In the three reaction periods studied it was evidenced that the lipases from *R. oryzae* were able to effectively discriminate between the DHA and the rest of the fatty acids present in MLO and HMLO. In the HMLO the DHA must be present as free acid since a 97.8 of hydrolysis was achieved.

The selectivity demonstrated by the resting cells of this strain of *R. oryzae* has not previously been reported to our knowledge. However, Ashjaria et al. [49] studied the selectivity in the hydrolysis of fish oil of lipases isolated from *R. oryzae* and immobilized by different methods. All immobilized biocataysts discriminated between EPA and DHA in favour of EPA.

4. Materials and Methods

4.1. Reagents and solvents

Chloroform (purity 99%), 2-methyltetrahydrofuran, deuterated chloroform (99.9 atom % D), anhydrous sodium sulphate (Na_2SO_4), oleic acid (purity 90%), potassium hydrogen phosphate (K_2HPO_4), magnesium sulphate ($MgSO_4$) were purchased from Sigma-Aldrich (Sigma-Aldrich Química, S.A. Madrid Spain and USA). Cyclopentyl methyl ether (purity 99%) was from Zeon Corporation (Japan). Dimethyl carbonate (purity 99%), hexane (HPLC grade) and limonene (96%) were purchased from Acros Organics (USA). Ethanol absolute was purchased from Scharlau (Spain). Methanol was purchased from Fisher Scientific (Madrid, Spain). The culture media agar potato dextrosa (PDA) and yeast extracted (EY) were provided by Scharlau Microbiology (Sharlab S.L. Barcelona Spain). Lipase-B from *C. antartica* (Novozym 435) was a gift sample from Novozymes A/S (Bagsvaerd, Denmark). The *Rhizopus oryzae* (CECT20476) and *Aspergillus flavus* (CECT20475.2.1) strains are housed in the Spanish Type Culture Collection (CECT) (Burjassot, Valencia-Spain). Monkfish liver was supplied by Congelados y Especialidades Barrufet SL (Barcelona – Spain) and sunflower oil was bought at the market.

4.2. Computational method

This study has been performed by a theoretical procedure using a computational predictive method (COSMO-RS), by considering the technical properties of the solvents and via experimentation. The comparison was made considering the amount of monkfish liver oil extracted and the technical parameters of the solvents used.

4.2.1. COSMO-RS procedure

The Conductor-like Screening Model for Real Solvents (COSMO- RS) developed by Klamt and co-workers [50] is as known as a powerful method for molecular description and solvent screening based on the result of quantum chemical calculations for an understanding of the dissolving mechanism. COSMO-RS combines quantum chemical considerations (COSMO) and statistical thermodynamics (RS) to determine and predict the thermodynamic properties without experimental data. In the first step, the molecule is embedded into a virtual conductor. In such an environment the molecule induced a polarization charge density on its surface (σ-surface). Calculation was performed for each molecule of interest (Fig. 1). The second step used the statistical thermodynamic calculation. Blue is used to represent strongly positive polar regions, and red represents very negative polar surfaces. Green and yellow correspond to lower polarity. The thermodynamics of the molecular interactions that were based on the obtained σ-profile are then used to calculate the chemical potential of the surface segment (σ-potential) [21,23]. According to the composition of the monkfish liver oil determined by ^{1}H-RMN and GC-FID, we decided to use as TAG models in the COSMO-RS analysis four different models of TAG using the four main fatty acids from the oil.

In this work, the prediction of the relative solubility of the main triacylglycerides from monkfish liver oil in four green solvents 2-MeTHF, CPME, DMC and LMN and FR, that is, a mixture of chloroform and methyl alcohol (2:1, v/v) was made by implementing this COSMO-RS model in COSMOtherm software (BIOVIA COSMOthermX19; Dassault Systèmes, France). The chemical structures of the solvents and solutes were mutually transformed into their simplified molecular input line entry syntax (SMILES) notations, which were subsequently used to calculate the solubility parameters of solvents and compounds. The relative solubility is calculated from the following equation [50,51].

$$log_{10}(x_j) = log_{10}[\exp((\mu_j^{pure} - \mu_j^{Solvent} - \Delta G_{j,fusion})/RT)]$$

μ_j^{pure}: chemical potential of pure compound j (J/mol); $\mu_j^{Solvent}$: chemical potential of j at infinite dilution (J/mol); $\Delta G_{j,fusion}$: free energy of fusion of j (J/mol); x_j: solubility of j (g/g solvent); R: gas constant; T: temperature (K).

Relative solubility is always calculated in infinite dilution. The logarithm of the best solubility is set to 0 and all other solvents are given relatively to the best solvent. A solvent with a log10 (x_j) value of -1.00 yields a solubility which is decreased by a factor 10 compared to the best solvent. Also, the logarithm was transformed into the probability of solubility and was expressed in percentage.

4.3. Monkfish liver oil extraction

4.3.1. Extraction in a roller mixer

The monkfish liver oil was extracted by solid-liquid extraction with FR (chloroform-methanol 2:1 v/v) as a reference solvent and using four green solvents (2-MeTHF, DMC, CPME and LMN). An amount of 20 g of monkfish liver previously defrosted and grinded were extracted with 100 mL of each solvent at ratio 1:5 w/v. The mixture was stirred at 60 rpm for 30 min in a roller mixer. The sample was filtered through paper, and the solid residue was washed two times with fresh solvent. Solvents were joined. A solution of 1% v/v of NaCl was added to the extract obtained with FR, the mixture was shaken vigorously and left to stand for 2 h. The organic phase was recovered and dried with anhydrous sodium sulphate. It was filtered and the solid was washed with chloroform. Final

solutions were evaporated under vacuum in a rotary evaporator. Samples were dried under vacuum for 2 h and finally were stored at -20°C until analysed. All experiments were carried out in triplicates.

To perform a better comparison of the amount of monkfish liver oil obtained by every solvent, we determined the maximum content of oil in the monkfish liver by extracting with the FR one sample five times (until no colour was observed in the solvent). Solvents were joined and processed as indicated above.

4.3.2. Extraction in an ULTRA-TURRAX® system.

An amount of 2 g of ground monkfish liver, 2 g of ceramics balls and 10 ml of the solvents (green solvents or FR) were added to the ULTRA-TURRAX system. The mixture was mechanically stirred at 4000 rpm for 15 min at room temperature. The mixture was then filtered and centrifuged at 5000 rpm for 5 min to separate the supernatant. The organic phase was recovered and dried with anhydrous sodium sulphate. The mixture was filtered and then the solvent was evaporated in a rotary evaporator and the oil was dried under vacuum. The samples were stored at -20°C until analysis. All experiments were carried out in triplicate.

4.3.3. Preparation of resting cells

R. oryzae and *A. flavus* cells were grown in a synthetic liquid medium containing 2 g of asparagine, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 5 mg of thiamine hydrochloride, 1.45 mg of Fe $(NO_3)_3 \cdot 9H_2O$, 0.88 mg of $ZnSO_4 \cdot 7H_2O$, and 0.235 mg of $MnSO_4 \cdot H_2O$ per liter of distilled water. The initial pH of the medium was adjusted to pH 6.0. Next, 250-mL aliquots of the medium were sterilized at 121°C for 15 min, and 1% (v/v) of refined sunflower oil was added aseptically. The medium was inoculated with 2.5 mL of a *R. oryzae* and *A. flavus* spore suspension (1-4 × 10^6 spores/mL) and then incubated at 28°C for 5 d using an orbital shaker at 200 rpm. Mycelium was harvested from the culture medium using a Buchner funnel and washed with distilled water followed by acetone. It was then dried under vacuum for 18 h and ground to a powder. The *R. oryzae* (CECT20476) and *A. flavus* (CECT20475.2.1) strains are housed in the Spanish Type Culture Collection (CECT). The enzymatic units (U) were determined beforehand on the basis of the enzymatic esterification rate of ethyl oleate from oleic acid and ethanol. The specific activity of the resting cells of *R. oryzae* was 1.14 *U* and *A. flavus* was 0.57 *U*.

4.4. Enzymatic preparation of ethyl esters

4.4.1. One-step synthesis: transesterification reaction

A 1:3.2 mixture of monkfish liver oil (0.453 g; 0.5 mmol) and ethanol (0.0736 g; 1.6 mmol) was added to a reaction vial (5 mL) fitted with a PTFE-lined cap that contained 0.045 g of each biocatalyst (10% w/w based on the weight of monkfish liver oil), a commercial enzyme (Novozym 435) or resting cells (*R. oryzae* and *A. flavus*) or their mixtures (1:1 and 7:3, *R. oryzae-A. flavus*), respectively (Fig. 6). The mixture was stirred (220 rpm) continuously at atmospheric pressure and 28°C. Reaction progress was evaluated for 24, 48 and 74 h. Samples were collected, filtered and solvent evaporated. An aliquot of 20 mg of the crude material of the reaction was dissolved in deuterated chloroform and the resulting solution was analysed by NMR. Experiments were carried out in triplicate.

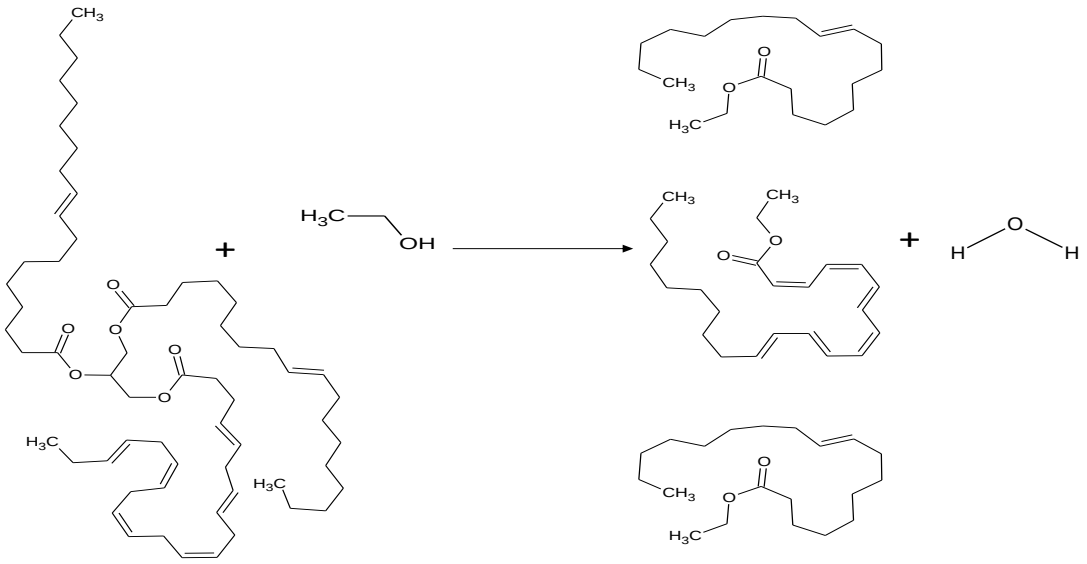

Figure 6. Synthesis performed in the one-step transesterification reaction.

4.4.2. Two-step synthesis: hydrolysis and esterification reactions

In the first step a biocatalytic hydrolysis reactions (Fig. 7) were performed using the commercial enzyme, the two resting cells and their mixtures (1:1 and 7:3, *R. oryzae* / *A. flavus*). A mixture of monkfish liver oil (0.453 g; 0.5 mmol), biocatalyst (0.045 g; 10% w/w based on the weight of monkfish liver oil) and water (0.453 g; 25 mmol) was stirred (220 rpm) at 28°C for 24 h. The sample was filtrated, centrifuged and the supernatant was collected. The resulting hydrolysate material was analysed by NMR and GC-FID, with this determining which of these tests offered the best results in terms percentage of free fatty acids. With the better biocatalyst, the hydrolysis was scaled up to obtain a minimum of 30 ml of hydrolysated, with which the esterification studies were carried out. The hydrolysate material was analysed by NMR and GC-FID to determine the hydrolysis degree and the fatty acids profile. In the second step, the hydrolysed oil was used to perform the esterification reactions using the same biocatalysts indicated above. A mixture of hydrolysed monkfish liver oil (0.300 g; 1 mmol), biocatalyst (0.03 g, 10% w/w based on the weight of hydrolysed monkfish liver oil) and ethanol (0.147 g; 3.2 mmol) were stirred (220 rpm) at 28°C. The esterification reactions were conducted for 24, 48 or 72 h. The reaction products were analysed by NMR and GC-FID. All the experiments were carried out in triplicate.

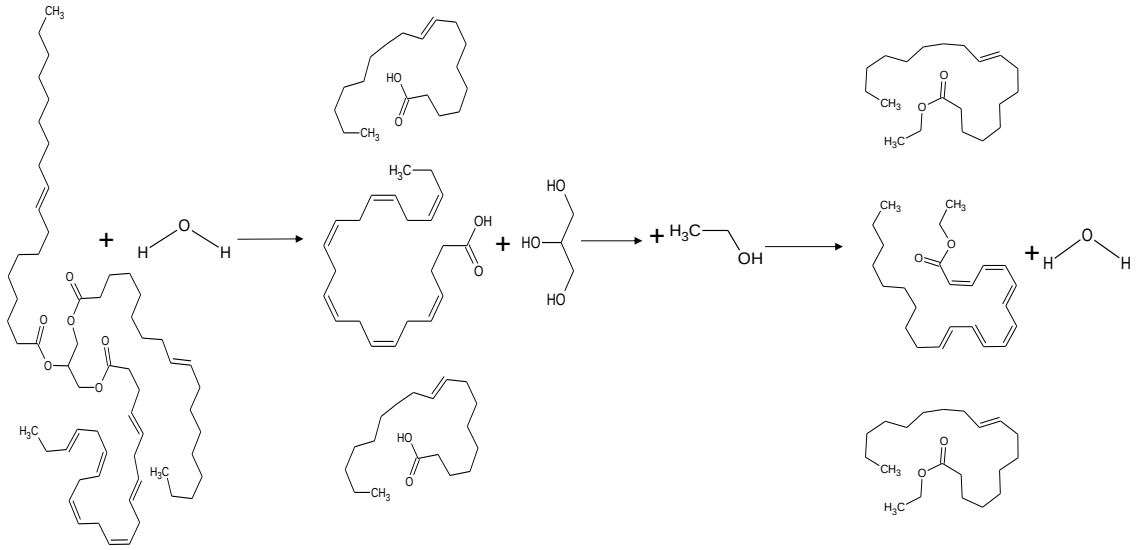

Figure 7. Synthesis performed in the two-step reaction.

4.5. Analytical Methods

Preliminary analysis of monkfish liver oil, hydrolysed oil and ethyl esters were carried out by ^{1}H NMR. Spectra were recorded with a MERCURY plus NMR Spectrometer Systems VARIAN AS 400 MHz magnet using deuterated chloroform (99.9 atom % D) as solvent. The fatty acid profiles of the initial oil and the esters after synthesis reactions were analysed using an Agilent HP6890 series gas chromatograph (Barcelona, Spain) coupled to a flame ionization detector (FID). The chromatographic column was a 30 m × 0.25 mm fused silica capillary coated with a 0.25 μm film thickness (50%-cyanopropyl)-methylpolysiloxane (DB-23; Agilent J&W, Madrid, Spain). The temperature program used was 180°C for 1 min, followed by an increase of 20°C min-1 until the final temperature of 270°C was reached, which was then maintained for 20 min. A split-less of 20 mL/ min for 0.15 min was applied. Hydrogen was used as carrier gas at a constant pressure. The injection volume was 1 μL. The injection system was maintained at 270°C and the FID at 280°C.

5. Conclusions

During this study, two extraction methods and four green solvents were studied for the extraction of MLO. The Roller Mixer was the best extraction system for all the solvents studied. All the green solvents tested (CPME, 2-MeTHF, DCM and LMN) allowed the extraction of 96 to 100% of the oil contained in this co-product. In addition, all the green solvents showed higher extraction yields than the common FR extractive method. The percentages of esters from the MLO in one-step esterification were 63%, 61% and 46% using Novozym 435, *R. oryzae* lipase and *A. flavus* lipase, respectively. In the two-step esterification reactions, the yields were 85%, 65% and only 41% using the commercial enzyme*R. oryzae A. flavus*, respectively. Consequently, the latter is definitely not a good biocatalyst for this type of reaction. Another important observation was that *R. oryzae* lipase (CECT20476), showed lowest yields for DHA-EE in both the one or two steps processed, which suggests selectivity towards this fatty acid. The resting cells of filamentous fungi used in this study unlike of commercial enzymes have not gone through any kind of extraction, purification and immobilization process. Therefore, they are biocatalysts that are considered very cheap compared to the commercial ones. Moreover, several authors report that the enzymatic activity and the selectivity of the lipases can be affected by the process of immobilization. Similarly, it is clear that the commercial lipase esterified omega-3 fatty acids optimally, with yields above 90% for DHA and 100% for EPA contained in MLO. The resting cells of *R. oryzae* and *A. flavus* tested showed good percentage of hydrolysis (88% and 93%, respectively), higher than those of the commercial enzyme (61%). These results open a way to study PUFA enrichments using these resting cells from different oil sources.

Acknowledgments: This work was supported in part by the Spanish government through the Interreg POCTEFA call (EFA253/16 BIOPLAST) and the PhD student aid programme "Programa Jade Plus" financed by Banco Santander of which Johanna Aguilera Oviedo is a beneficiary. The authors would like to thank the Vice-rector of Research of the University of Lleida for the scholarship granted to Johanna Aguilera Oviedo. The authors would also like to thank Congelados y Especialidades Barrufet SL for supplying the monkfish livers.

References

1. Puniaa, S; Sandhub, K.S.; Sirohaa, A.K.; Dhulla, S.B. Omega 3-metabolism, absorption, bioavailability and health benefits–A Review. PharmaNutrition **2019**, 10, 100162, 1-7. https://doi.org/10.1016/j.phanu.2019.100162
2. Shahidi, F.; Ambigaipalan, P.St. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. Annu Rev Food Sci Technol. **2018**, 9, 345-381. https://doi.org/10.1146/annurev-food-111317-095850
3. Golpour, P.; Nourbakhsh, M.; Mazaherioun, M.; Janani, L.; Yaghmaei, P. Improvement of NRF2 gene expression and antioxidant status in patients with type 2 diabetes mellitus after supplementation with omega-3 polyunsaturated fatty acids: A double-blind randomised placebo-controlled clinical trial. Diabetes Res. Clin. Pract. **2020**, 162, 108120, 1-9. https://doi.org/10.1016/j.diabres.2020.108120
4. Calder, P.C. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids **2007**, 77, 327-335. doi:10.1016/j.plefa.2007.10.015
5. Volpato, M.; Hull, M. A. Omega-3 polyunsaturated fatty acids as adjuvant therapy of colorectal cancer. Cancer Metastasis Rev **2018**, 37, 545–555. https://doi.org/10.1007/s10555-018-9744-y
6. Nindrea, R.D.; Aryandono, T.; Lazuardi, L.; Dwiprahasto, I. Protective Effect of Omega-3 Fatty Acids in Fish Consumption Against Breast Cancer in Asian Patients: A Meta-Analysis. Asian Pac J Cancer Prev. **2019**; 20(2), 327–332. doi:10.31557/APJCP.2019.20.2.327
7. Mazahery, H.; Conlon, C.A.; Beck, K.L.; Mugridge, O.; Kruger, M.C.; Stonehouse, W.; Camargo-Jr, C.A.; Meyer, B.J.; Tsang, B.; Von Hurst, P.R. Inflammation (IL-1β) Modifies the Effect of Vitamin D and Omega-3 Long Chain Polyunsaturated Fatty Acids on Core Symptoms of Autism Spectrum Disorder—An Exploratory Pilot Study. Nutrients **2020**, 12(3), 661, 1-17. doi:10.3390/nu12030661
8. Swanson, D.; Block, B.; Mousa, S.A. Omega-3 Fatty Acids EPA and DHA: Health Benefits Throughout Life. Adv Nutr. **2012**, 3, 1–7. doi:10.3945/an.111.000893.
9. Kwatra, B.A. Review on Potential Properties and Therapeutic Applications of DHA and EPA Ijppr.Human, **2019**;16 (4): 140-176.
10. Sahena, F.; Zaidul, I.S.M.; Jinap, S.; Saari, N.; Jahurul, H.A.; Abbas, K.A.; Norulaini, N.A. PUFAs in Fish Extraction, Fractionation, Importance in Health. Compr. Rev. Food Sci. Food Saf. **2009**, 8, 59-74.
11. Sargent, J.R.; Bell, M.V.; Bell, J.G.; Henderson, R.J.; Tocher, D.R. Origins and functions of n-3 polyunsaturated fatty acids in marine organisms. In: *Phospholipids: characterization, metabolism and novel biological applications*, Eds. G. Cevc, F. Paltauf, AOCS Press, Champaign, USA, **1995**, 248–258.
12. Informe realizado por la Asociación Empresarial de Acuicultura de España (APROMAR). España, **2019**, 1-91. Available online: http://apromar.es/sites/default/files/2019/InformeAcui/APROMAR%20Informe%20ACUICULTURA%202019%20v-1-2.pdf (accessed on 15 August 2020).
13. Iñarra, B.; Bald, C.; San Martín, D.; Orive, M.; Cebrián, M.; Zufía, J. Guía para la valorización de subproductos de la acuicultura. AZTI, Derio, España, 2018, 1-44. Available online: https://www.azti.es/wp-content/uploads/2018/12/AZTI_guia_VALACUI101218online.pdf (accessed on 15 August 2020).
14. Fisher, R.A.; DuPaul, B.A. Fisherman's Guide: Getting the Most Out of Monkfish. Marine. Resource Advisory No. 37. Virginia Institute of Marine Science, College of William and Mary. **1990**. 1-7. doi: 10.21220/m2-pjzv-6122.
15. Manipulación de la pesca del día en embarcaciones de red de enmalle. Servicio central de publicaciones Gobierno Vasco. Departamento de medio ambiente, planificación territorial y pesca. ISBN: 978-84-457-3186-433-34. Colección ITSASO 37. 2011, 33-34. Available online: http://www.euskadi.net/ejgvbiblioteka (accessed on 15 July 2020).
16. Guía de buenas prácticas de higiene para buques palanqueros y buques factoría congeladores. Organización de productores de buques Congeladores de Merlúcidos, Cefalópodos y Especies varias. OPPC-3. **2017**, 78-80. Available online:

http://www.arvi.org/publicaciones/GUIA_DE_BUENAS_PRACTICAS_DE_HIGIENE.PDF (accessed on 15 july 2020).

17. Pacetti, D.; Alberti, F.; Boselli, E.; Frega, N.G. Characterisation of furan fatty acids in Adriatic fish. Food Chem. **2010**, 122, 209–215. doi:10.1016/j.foodchem.2010.02.059
18. Asociación Española de Normalización (UNE). Available online: https://www.une.org/encuentra-tu-norma/busca-tu-norma/iso?c=006038 (accessed on 14 April 2020).
19. Luque de Castro, M.D.; Priego-Capote, F. Review Soxhlet extraction: Past and present panacea. J. Chromatogr. A, **2010**, 2383–2389. doi:10.1016/j.chroma.2009.11.027
20. Folch, A.J.; Lees, M.; Sloane- Stanlet, G.H. Simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem, **1957**, 226:497-509. Available online http://www.jbc.org/ (accessed on 16 May 2019).
21. Yara-Varón, E.; Fabiano-Tixier, A. S.; Balcells, M.; Canela- Garayoa, R.; Billy, A.; Chemat, F. Is it possible to substitute hexane with green solvents for extraction of carotenoids? A theoretical versus experimental solubility study. RSC Adv. **2016**, 6, 27750-27759. doi: 10.1039/c6ra03016e
22. Chemat, F.; Vian, M.A.; Ravi, H.K.; Khadhraoui, B.; Hilali, S.; Perino, S.; Fabiano-Tixier, A.S. Review of Alternative Solvents for Green Extraction of Food and Natural Products: Panorama, Principles, Applications and Prospects. Molecules **2019**, 24, 3007, 1-17. doi:10.3390/molecules24163007
23. Yara-Varón, E.; Selka, A.; Fabiano-Tixier, A.S.; Balcells, M.; Canela-Garayoa, R.; Bily, A.; Touaibiac, M.; Chemat, F. Solvent from forestry biomass. Pinane a stable terpene derived from pine tree byproducts to substitute n-hexane for the extraction of bioactive compounds. Green Chem. **2016**, 18, 6596–6608. doi: 10.1039/c6gc02191c
24. Chemat, F.; Vian, M.A.; Ravi, H.K.; Khadhraoui, B.; Hilali, S.; Perino, S.; Tixier, F. A-S. Review of Alternative Solvents for Green Extraction of Food and Natural Products: Panorama, Principles, Applications and Prospects. Molecules **2019**, 24(16), 3007, 1-26. doi.org/10.3390/molecules24163007
25. Yara-Varón, E.; Li, Y.; Balcells, M.; Canela-Garayoa, R.; Fabiano-Tixier, A.S.; Chemat, F. Review. Vegetable Oils as Alternative Solvents for Green Oleo-Extraction, Purification and Formulation of Food and Natural Products. Molecules **2017**, 22, 1474, 1-25. doi:10.3390/molecules22091474
26. Miyashita, K.; Uemura, M.; Hosokawa, M. Effective Prevention of Oxidative Deterioration of Fish Oil: Focus on Flavor Deterioration. Annu. Rev. Food Sci. Technol. **2018**, 9, 209–226. https://doi.org/10.1146/annurev-food-030117-012320
27. Zhang, Z.; Liu, F.; Ma, X.; Huang, H.; Wang, Y. Two-Stage Enzymatic Preparation of Eicosapentaenoic Acid (EPA) And Docosahexaenoic Acid (DHA) Enriched Fish Oil Triacylglycerols. J. Agric. Food Chem. **2018**, 66, 218−227. doi: 10.1021/acs.jafc.7b04101
28. Moreno-Perez, S.; Machado, D.F.; Pires, J.; Luna, P.; Señorans, F.J.; Guisan, J.M.; Fernandez-Lorente, G. Critical Role of Different Immobilized Biocatalysts of a Given Lipase in the Selective Ethanolysis of Sardine Oil. J. Agric. Food Chem. **2017**, 65, 117−122. doi: 10.1021/acs.jafc.6b05243
29. Castejón N.; Señoráns, F.J. Strategies for Enzymatic Synthesis of Omega-3 Structured Triacylglycerols from Camelina sativa Oil Enriched in EPA and DHA. Eur. J. Lipid Sci. Technol. **2019**, 121, 1800412, 1-9. doi: 10.1002/ejlt.201800412
30. Ranjan-Moharana, T.; Byreddy, A.R.; Puri, M.; Barrow, C., Rao, N.M. Selective Enrichment of Omega-3 Fatty Acids in Oils by Phospholipase A1. PLoS One **2016**, 11(3), 1-15. doi: 10.1371/journal.pone.0151370
31. Axelsson, M.; Gentili, F. A single-step method for rapid extraction of total lipids from green microalgae. PLoS One **2014**; 9(2), 1-14. doi: 10.1371/journal.pone.0089643
32. Fang, Y.; Gu, S.; Liu, S.; Zhang, J.; Ding, Y. and Liu, J. Extraction of oil from high-moisture tuna liver by subcritical dimethyl ether: feasibility and optimization by the response surface method. RSC Adv. **2018**, 8, 2723-2733. doi: 10.1039/c7ra12948c
33. Routray, W; Dave, D.; Ramakrishnan, V.V.; Murphy, W. Production of High Quality Fish Oil by Enzymatic Protein Hydrolysis from Cultured Atlantic Salmon By-Products: Investigation on Effect of Various Extraction Parameters Using Central Composite Rotatable Design. Waste Biomass Valor. **2018**, 9, 2003–2014. doi: 10.1007/s12649-017-9998-6
34. Ciriminna, R.; Scurria, A.; Fabiano-Tixier, A.S.; Lino, C.; Avellone, G.; Chemat, F.; Pagliaro, M. Omega-3 Extraction from Anchovy Fillet Leftovers with Limonene: Chemical, Economic, and Technical Aspects. ACS Omega. **2019**, 4, 15359-15363. doi:10.1021/acsomega.9b01168

35. Catrin, E.T.; Brecker, L.; Wagner, K.H. ^{1}H NMR spectroscopy as tool to follow changes in the fatty acids of fish oils. Eur. J. Lipid Sci. Technol. **2008**, 110, 141–148. doi:10.1002/ejlt.200700150
36. Bratu, A.; Mihalache, M.; Hanganu, A.M.; Chira, N.A.; Todaşcă, M.C.; Roşca, S. Quantitative determination of fatty acids from fish oils using GC-MS method and ^{1}H NMR spectroscopy. U.P.B. Sci. Bull. **2013**, 75, 24-31.
37. Nestor, G.; Bankefors, J.; Schlechtriem, G.; Brannas, E.; Pickova, J.; Sandstrom, C. High-Resolution ^{1}H Magic Angle Spinning NMR Spectroscopy of Intact Arctic Char (Salvelinus Alpinus) Muscle. Quantitative Analysis of n-3 Fatty Acids, EPA and DHA. J. Agric. Food Chem. **2010**, 58, 10799-10803. doi:10.1021/jf103338j
38. Gavahian, M.; Khaneghah, A.M.; Lorenzo, J.M.; Munekata, P.E.S.; Garcia-Mantrana, I.; Collado, M.C.; Meléndez-Martínez, A.J.; Barba, F.J. Health benefits of olive oil and its components: Impacts on gut microbiota antioxidant activities, and prevention of noncommunicable diseases. Trends Food Sci Technol **2019**, 88, 220–227. https://doi.org/10.1016/j.tifs.2019.03.008
39. Djoussé, L.; Matsumoto, C.; Hanson, N.Q.; Weir, N.L.; Tsai, M.Y.; Gaziano, J.M. Plasma cis-vaccenic acid and risk of heart failure with antecedent coronary heart disease in male physicians. Clin Nutr **2014**, 33, 478-482. http://dx.doi.org/10.1016/j.clnu.2013.07.001
40. Field, C.J., Blewett, H.H., Proctor, S., Vine, D.: Human health benefits of vaccenic acid. Appl. Physiol. Nutr. Metab **2009**, 34(5), 979–991. doi:10.1139/H09-079
41. Sissener, N.H.; Ørnsrud, R.; Sanden, M.; Frøyland, L.; Remø, S.; Lundebye, A.K. Erucic Acid (22:1n-9) in Fish Feed, Farmed, and Wild Fish and Seafood Products. Nutrients **2018**, 10, 1443, 1-12. doi:10.3390/nu10101443.
42. Shimada, Y.; Maruyama, K.; Sugihara, A.; Moriyama, S.; Tominaga, Y. Purification of Docosahexaenoic Acid from Tuna Oil by a Two-Step Enzymatic Method: Hydrolysis and Selective Esterification. JAOCS, **1997**, 74, 1441, 1-6.
43. Gudmundur, G.; Haraldsson, G.G.; Kristinsson, B.; Sigurdardottir, R.; Gudmundson, G.G.; Breivikb, H. The Preparation of Concentrates of Eicosapentaenoic Acid and Docosahexaenoic Acid by Lipase-Catalyzed Transesterification of Fish Oil with Ethanol. JAOCS, **1997**, 74, 1419-1424.
44. Xu, Y.; Wang, D.; Mu, X.Q.; Zhao, K.A.; Zhang, K.C. Biosynthesis of ethyl esters of short-chain fatty acids using whole-cell lipase from *Rhizopus chinesis* CCTCC M201021 in non-aqueous phase. J. Mol. Catal. B Enzym. **2002**, 18, 29-37.
45. Aarthya, M.; Saravananb, P.; Ayyaduraia, N.; Gowthamana, M.K.; Kaminia, N.R. A two-step process for production of omega 3-polyunsaturated fatty acid concentrates from sardine oil using *Cryptococcus* sp. MTCC 5455lipase. J. Mol. Catal. B Enzym. **2016**, 125, 25-33. http://dx.doi.org/10.1016/j.molcatb.2015.12.013
46. Fraga, M.E.; Santana-N., D.M.; Gatti, J.M.; Direito, G.M.; Cavaglieri, L.R.; Rosa-R., C.A. Characterization of Aspergillus species based on fatty acid profiles. Mem Inst Oswaldo Cruz, Rio de Janeiro **2008**, 103(6), 540-544.
47. Asci, F.; Aydin, B.; Akkus, G.U.; Unal, A.; Erdogmus, S.F.; Korcan, S.E.; Jahan, I. Fatty acid methyl ester analysis of Aspergillus fumigatus isolated from fruit pulps for biodiesel production using GC-MS spectrometry. Bioengineered **2020**, 11(1), 408-415. doi: 10.1080/21655979.2020.1739379
48. Schuchardt, J.P.; Neubronner, J.; Kressel, G.; Merkel, M.; Schacky, C.V.; Hahn, A. Moderate doses of EPA and DHA from re-esterified triacylglycerols but not from ethyl-esters lower fasting serum triacylglycerols in stat in-treated dyslipidemic subjects: Results from a six-month randomized controlled trial. Prostaglandins Leukot Essent Fatty Acids **2011**, 85, 381–386. doi: 10.1016/j.plefa.2011.07.006
49. Ashjaria, M.; Mohammadic, M.; Badria, R. Selective concentration of eicosapentaenoic acid and docosahexaenoic acid from fish oil with immobilized/stabilized preparations of *Rhizopus oryzae* lipase. J. Mol. Catal. B Enzym. **2015**, 122, 147-155. http://dx.doi.org/10.1016/j.molcatb.2015.08.017
50. Klamt, A. Prediction of the mutual solubilities of hydrocarbons and water with COSMO-RS. Fluid Phase Equilib. **2003**, 206, 223–235. doi:10.1016/S0378-3812(02)00322-9
51. BIOVIA COSMOtherm 2020 User guide; Dassault Systèmes, Vélizy-Villacoublay, France, 2019.