Synthesis of nutraceutics (DHA and/or EPA – nicotinol esters) by enzymatic esterification

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Abstract: Miniemulsions are promising reaction media with several advantages for lipase-catalysed esterification. In this work, lipase-assisted synthesis of nutraceutics, EPA and DHA-nicotinol esters, were carried out in miniemulsion, using fish oil supplement as substrate. Different parameters were evaluated: lipase concentration; esterification efficiency of commercial *Rhizomucor miehei*, *Candida antarctica A* recombinant in *Aspergillus oryzae*, *Candida Rugosa type VII* and Amano PS lipases; fatty acids/nicotinol molar ratio (R); EPA and DHA as pure, isolated or mixed fish oil substrates. Miniemulsions showed high potential for hydrolysis of fish oil triglycerides and subsequent esterification of EPA and DHA with nicotinol. In excess conditions of nicotinol (R=0.1), esterification yields for EPA-nicotinol and DHA-nicotinol were, 34.6% and 34.2%, respectively, for 5 mg ml^{-1} of biocatalyst. The initial reaction rate was 13.3 mol g^{-1} min^{-1}, without loss of hydrolytic activity after 72h. For R=0.2, maximum reaction rate of 21.8 mol g^{-1} min^{-1} was observed, but, at the end of reaction, Amano PS activity decreased about 57%. It was shown that high excess of nicotinol initially inhibits Amano PS activity, without compromising esterification yields of both fatty acids. The results from present work showed promising data for production of EPA and DHA-nicotinol esters in miniemulsion, needing further studies for optimization.

Keywords: Miniemulsion, EPA, DHA, Nicotinol. Esterification, Lipase, Omega-3 fatty acids.

1. Introduction

The term nutraceutic is a definition of functional food that helps in prevention and/or treatment of diseases [1].

The consumption of omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), from fish and fish oil fats is known for its healthy effects. Omega-3 fatty acids are responsible for lower the levels of plasma triglyceride, very low density lipoprotein (VLDL) -cholesterol and LDL-cholesterol [2] having a very important role for prevention and treatment of coronary artery diseases [3], in reducing inflammation and risk factors of diseases like arthritis and other inflammatory diseases [4], cancer [5], [6], hypertension [7], diabetes [8] and autoimmune disorders [9].

Nicotinol is a group-B pro-vitamin that, when in human body is rapidly metabolized to nicotinic acid. Nicotinol shows to be an efficient agent in reducing of triglycerides and LDL-cholesterol [10]. The clinical trial data support nicotinic acid as a therapeutic agent that reduces atherosclerotic coronary heart disease events and reduces atherosclerotic progression in patients that already have it or have other cardiovascular diseases [11].

The use of DHA-nicotinol in animals with heart failure showed the efficiency of this agent in the prevention of the atrial arrhythmias, proving that DHA-nicotinol is cleaved inside the body, which allows the incorporation of DHA and nicotinol in the bloodstream [12].

The intake of omega-3 fatty acids, namely EPA and DHA, is done through direct consumption of fish in daily dietary or through the supplementation of omega-3-enriched fish oils, in triglyceride form [13]. The use of enzymes, namely lipases, as biocatalysts, in the omega-3-enrichment of fish oils was already demonstrated [14]–[17].

Lipases are serine hydrolases defined as triacylglycerol acyl hydrolases (E.C. 3.1.1.3), a type of enzyme that act on carboxylic ester bonds [18]. Lipases act as biocatalyst in hydrolysis of triglycerides into free fatty acids in nature. However, in conditions of low water activity, these enzymes can catalyse synthesis reactions, such as esterification [2]. The advantage of using biocatalysis is due to high substrate-specificity of enzymes, with high enzyme activity under mild conditions [19]. The specificity of these biocatalysts, such as type-selectivity and regioselectivity make the choice of the enzyme a very important aspect when using hydrolysis/esterification reactions to selective catalysis of omega-3 triglycerides by lipases [14], [15], [20].

Reviews of enzymatic esterification catalysed by lipases, in different reaction media, have been published [21], [22]. There are advantages of using organic solvents in biocatalysis, such as, reduced water activity, which favours the synthesis reactions over hydrolysis and increased solubility of non-polar substrates [23]. However, the use of solvents in industry become inevitable to have small amounts of solvents in final product because they cannot be completely eliminated from the product by manufacturing practices. In pharmaceutical application, this needs to be minimized, since some of these solvents are toxic and reduce the therapeutical value of active substances because they accelerate decomposition of the product [24]. Solvent free systems have also been investigated as a reaction media for esterification reactions due to the absence of solvent, which facilitates downstream processing, since fewer components would be present at the end of the reaction, thus minimizing the production cost, as well as toxicity issues [21]. Reports of solvent-free synthesis of isoamyl esters of short-chain organic acids (acetic, propionic, butyric, and isobutyric) showed that mass transfer was not limiting for short-chain acid substrates. Nevertheless, for long-chain fatty acids like oleic, palmitic, and stearic acids, the problems of mass transfer may be a bottleneck during solvent-free synthesis [21]. Despite that, transesterification of omega-3 ethyl esters (EE), such as DHA-EE, showed high transesterification yields in solvent-free system [2].

Taking advantage of the enhancement of lipase activity in presence of oil-water interfaces [2], lipase catalysed esterification reactions have been carried in water-in-oil (reverse micelles) emulsion systems [25].The use of reverse micelles has proved to be advantageous, since it allows to increase the interfacial area, that is, it increases the number of substrate molecules available to react [25]. In addition, there is a higher solubilisation of both hydrophobic and hydrophilic substrates, when compared to organic solvents [25]. However, the use of reverse micelles, despite having better biocatalytic properties than organic solvents, still have organic solvents in reaction media. This aspect, considering typical pharmaceutical industries and fine chemical processes could raise safety and environmental concerns, since many of them are toxic and/or volatile [26].

Compared with organic solvents, the use of water as reaction medium would be clearly advantageous because it is an environmentally compatible solvent, it is inflammable, non-toxic, cheap and readily available. However, most organic compounds are insoluble in water, thus having inability of reactions involving organic reactants to be conducted exclusively in aqueous phase beyond to the existence of reverse esterification reactions, such as hydrolysis, in aqueous media [27]. Therefore, two-phase systems, as oil-in-water miniemulsion systems have been investigated for lipase and cutinase catalysed esterifications [26], [28]–[30]. At the same time, reports of hydrolysis of triglycerides in miniemulsion were also published [31].

Aqueous miniemulsions (oil-in-water) are two-phase systems where very small droplets (organic phase) from 30 nm to 500 nm are dispersed in a continuous phase (aqueous phase) formed using high shear forces originated by ultrasonication [30]. The miniemulsion systems are mostly composed of water (around 80 %w/w), and the oil phase is mainly made of substrates (16.5% w/w) [26]. However, to avoid the spontaneous tendency of such small emulsions to degrade and regenerate the equilibrium state, is added a surfactant (ex. Lutensol AT 50), to maintain colloidal stability of the system, by decreasing droplet collision and subsequent coalescence. Beyond coalescence, diffusional degradation of droplets does not allow maintaining osmotic stabilization of the droplets, leading to the so-called Ostwald ripening. To avoid it, is used an extremely hydrophobic agent (ex. hexadecane) [27]. After emulsification, substrates will remain inside the droplets, while surfactant will be at miniemulsion interface. Two of the key aspects of enzymatic esterification in miniemulsions are their big interfacial area, which allows efficient biocatalysed reactions at interface, and miniemulsion stability throughout the reaction, which is increased with the existence of more hydrophobic substrates. In addition, water produced during esterification reaction is expelled to the continuous aqueous phase, thus favouring product formation [30] (Figure 1).

Considering the existence of esterification and hydrolysis reactions in miniemulsion, the present work studied the possibility of having both reactions occurring sequentially in a single miniemulsion system. Initially, EPA/DHA glycerides in fish oil were hydrolysed in

free EPA/DHA, and then used as substrates in esterification with nicotinol (Figure 2).

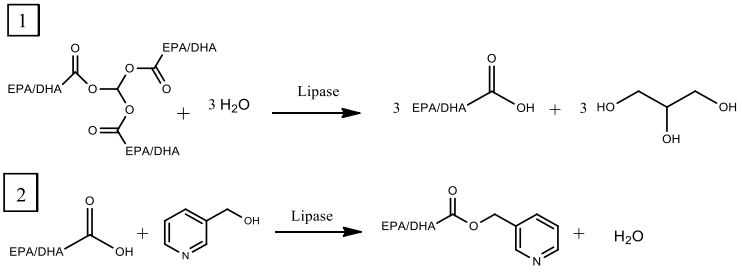


Figure 2. Reaction in miniemulsion. 1) Hydrolysis of EPA/DHA triglycerides in free fatty acids; 2) Esterification of free EPA/DHA in EPA/DHA-nicotinol ester.

2. Materials and Methods

2.1 Miniemulsion preparation

Miniemulsion (ME) systems were composed of water, Lutensol AT 50 (donation, BASF, Germany), Hexadecane (minimum 99%, Sigma-Aldrich), nicotinol (98%, Alfa Aesar) and fish oil (Omega Balance) in different relative amounts (table 1).

Table 1. Relative amounts of the compounds in ME 80% (w/v)

Reagents	% (w/w)
Water	81.2
Lutensol	1.6
Hexadecane	0.7
Omega Balance + Nicotinol	16.5

Miniemulsion system preparation started by adding the compounds shown in table 1 in their relative amounts but in two different phases, an aqueous solution with Lutensol AT 50 and an organic phase with the substrates, Omega Balance fish oil and nicotinol, and hexadecane. Lutensol was quickly solubilized in water with an agitation of 1100 rpm. After that the aqueous solution was added to organic phase, completing 10 ml of working volume, unless otherwise stated and thoroughly mixed by magnetic stirring overnight at 400 rpm. Then the two-phase systems were ultrasonicated using a probe (MS72) sonicator (SONOPLUS, BANDELIN, Germany) with ice cooling. In miniemulsion formulation different parameters were studied, particularly power, amplitude and sonication time. The best formulation was chosen to do miniemulsions for esterification reactions which was 50% amplitude, during 5 min 5 s on/10 s off.

2.1.1 Omega balance fish oil composition

Taking into consideration the potential of fish oils as nutraceutics and, at the same time, EPA/DHA commercial price of pure substrates, it was decided to use a fish oil sample that contains a mixture of these two and other omega-3 fatty acids. Omega Balance fish oil was purchased from Myprotein and it was used for reactions in miniemulsion system.

The content of fatty acids in the sample was determined by gas chromatography according to de Carvalho, 2012 [32]. This method allowed to quantify the relative composition, % (w/w), of each fatty acid in the fish oil sample. However, Omega Balance is described as having 15% (w/w) of Vitamin E, the percentage determined for each fatty acid present in the sample needed to be recalculated. At the same time, using this method it was decided to exclude every component with a %(w/w) lower than 3%. Therefore, only docosapentaenoic acid (DPA) and omega-6 arachidonic acid (AA ω-6) were quantified in addition to EPA and DHA (table 2)

Table 2. Concentration %(w/w) of fatty acids in Omega Balance.

Component	%(w/w)	M (g mol^{-1})
EPA	45.6%	302.45
DHA	30.2%	328.49
DPA	6.20%	330.56
AA ω-6	3.00%	304.47
Vitamin E	15.0%	430.71

Based on %(w/w) of each component and their molecular weight it was calculated a medium molecular weight of the sample, which is 331.36 g mol^{-1}. At the same time, Omega Balance density was determined by weighing 1 ml of fish oil, in triplicate. It was determined experimentally as 0.9530 g ml^{-1}.

2.2 Miniemulsion characterization

Miniemulsion droplets size (Z-Average) were measured by DLS under a scattering angle of 173° at a wavelength of 633 nm, at 25°C, in glass cuvettes, in a Zetasizer Nano ZS, Malvern Instruments (Malvern, UK). Particle sizes (nm) and PDI (dimensionless) are given as the average of three measurements with 12 runs each. The Z-Average diameter is the mean diameter, based on the intensity and fluctuations of the scattered light. PDI is the width of the particle size distribution, and is zero when a monodisperse particles population is measured, around 0.10-0.30 for a relatively narrow distribution and values of 0.5 and higher are obtained in case of very broad distributions.

.2.3 Characterization of lipase preparations

The hydrolytic activity of different lipases such as *Rhizomucor*. *miehei* (RM, Sigma-Aldrich), *Candida spp* recombinant, expressed in *Aspergillus oryzae* (CAL A, Sigma-Aldrich), *Candida rugosa* type VII (CR, Sigma-Aldrich) and Amano PS from *Burkholderia cepacia* (Sigma-Aldrich), was assayed using a spectrophotometric method (Hitachi U-2000 Spectrometer) based on monitoring the hydrolysis of *para*-nitrophenyl palmitate (p-NPP, Sigma-Aldrich) to *para*-nitrophenol (p-NP), a yellow compound, at 400 nm (ε = 2710 M^{-1}cm^{-1}) at 30ºC for 1 minute. For that purpose, 90 µl of a solution 7,95 mM of p-nPP in propanol (Merck) was added to a glass cuvette containing 890 µl of 50 mM Tris.HCl with pH 8, 7.11 mM Triton® X-100 (Merck) and 3.17 µM arabic gum, making a total volume of 980 µl with a final concentration of 0.730 mM p-NPP. After that, 20 µl of enzyme extract, previously diluted in Milli-Q water to work in linear variation of the concentration of p-NP, was added to the reaction mixtures being agitate by a small magnetic stirrer submitted to magnetic agitation of 700 rpm (Electronic Stirrer Model 300 Rank Brothers Ltd). The cell holder was kept at a temperature of 30ºC by circulating warm water from a thermostatic bath (Thermomix MM,B.Braun) also at 30ºC.

One unit of lipase hydrolytic activity (U) was defined as the amount of enzyme required to convert 1 µmol of p-NPP to p-NP per minute, at 30ºC.

The protein concentration was determined by the method of Pierce BCA Protein Kit (Thermofisher Scientific) with reference to Bovine Serum Albumin (BSA) as standard solution.

The specific activitiy (U mg^{-1}) of each lipase preparation was determined as the ratio between hydrolytic activity and protein concentration (*R. miehei* 25 U mg^{-1}, CAL A 193 U mg^{-1}, *C. rugosa* 30 U mg^{-1}, Amano Ps 3238 U mg^{-1}).

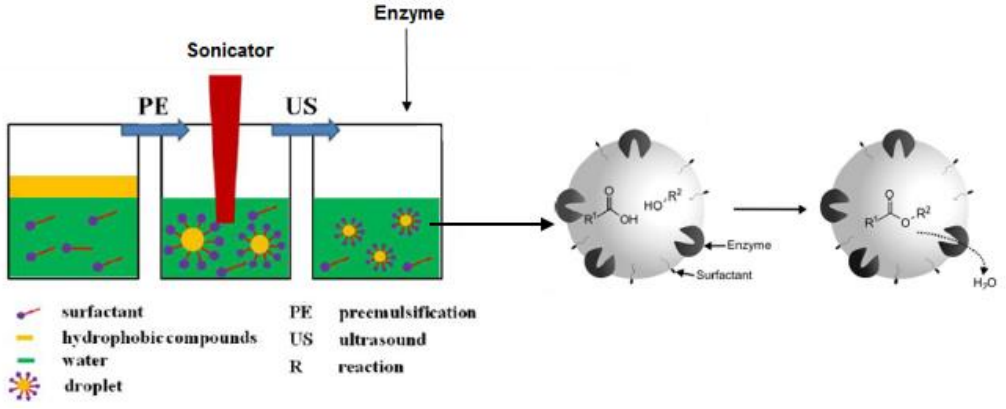


Figure 1. Miniemulsion formulation for carrying out enzymatic esterification, adapted from [28], [33]

2.4 General procedure for the chemical synthesis of esters EPA-nicotinol and DHA-nicotinol

HPLC standards of both nicotinol esters were needed and chemically synthesized according the following general procedure.

Diisopropyl carbodiimide (0.031 mL, 0.198 mmol) was added to a solution of the desired acid (0.165 mmol), Nicotinol (0.016 mL, 0.165 mmol) and dimethylaminopyridine (0.002 g, 0.0165 mmol) in dichloromethane (0.5 mL). Almost immediately a precipitate started to form. The reactions were carried out in 4 mL glass screw-cap vials under orbital stirring at room temperature for 12-16 hours. After evaporation of the solvent under reduced pressure, the crude product was purified by flash chromatography (hexane/ethyl acetate 5:1) as described in section 2.6.2.1

Ester EPA-nicotinol: Starting with EPA (0.053 mL), compound EPA-Nicotinol was obtained as a pale-yellow oil (0.054g g,83%). R_f (hexane/diethyl ether (3:1)): 0.38. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.2, 149.4, 149.3, 136.2, 132.0, 131.9, 128.9, 128.8, 128.6, 128.3, 128.2, 128.14, 128.06 127.9, 127.0, 123.5, 63.5, 33.5, 26.5, 25.63, 25.60, 25.5, 24.7, 20.6, 14.3 ppm.

2.5 Enzymatic reactions in miniemulsion systems

Enzymatic esterification reactions were carried out in flasks with 10 ml of working volume, unless otherwise stated. This volume of miniemulsion was prepared considering the relative amounts in table 1, and added to reaction vessel containing the appropriate amount of enzyme. The esterification reactions were performed in a thermostated incubator (Box cult, JP Selecta, Abrera, Barcelona, Spain), under 400 rpm magnetic stirring (Telesystem 15.20, Thermo scientific VARIOMAG®) at 40ºC or 50ºC, as specified below. This setup enabled parallel experiments, using different conditions against at least a blank without enzyme. Unless otherwise stated, all experiments were made in duplicates.

The esterification reactions were investigated in terms of different aspects, such as:

a) Enzyme Concentration: to establish the optimal amount of catalyst for carrying out the targeted esterification, different quantities of free lipase Amano PS were added to the reaction media in a linear range from 1 to 10 mg ml^{-1}. The studies were made at 50ºC, since it was described as the temperature with maximum activity for this lipase [34], with equimolar concentration of substrates Omega Balance and Nicotinol (R=1)

b) Different enzymes: to determine differences of using different lipases on esterification yield in miniemulsion, four different lipases were used: RM, CAL A, CR and Amano PS. The initial activity (191 U ml-1) in the different reactors were maintained, so it could be compared them to see if there is any difference in terms of esterification efficiency by using different biocatalysts. For that, based on specific activity, the amount of each needed enzyme was calculated and previously added to the reactors. The reactions were carried out at 40ºC since all lipases show high activity at this temperature [34]–[37].

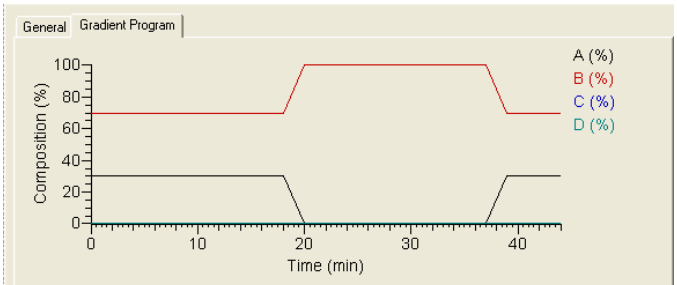
c) Fatty acids/Nicotinol molar ratio (R): effect of different fatty acid/Nicotinol molar ratios in esterification yield and reaction rate were investigated by varying R from 0.1 to 1. These studies were with Amano lipase PS concentration of 5 mg ml^{-1} at 50ºC.

d) Pure EPA/DHA vs Omega Balance fish oil: Comparative effect of using pure EPA and DHA or Omega Balance fish oil in esterification yield were investigated using R=0.1 and an Amano Lipase PS concentration of 5 mg ml^{-1} at 50ºC. Reactions were made with a working-volume of 5 ml due to the pure EPA and DHA amount constraints.

2.6 HPLC

2.6.1 Determination of the esterification yield in the miniemulsion system

In all studies, samples collected from reaction mixture were diluted in THF and analysed by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Chromolith® Performance RP-18 end capped (100mm x 4.6mm x 2µm) column. HPLC apparatus (Thermo Electron Corporation, Finnigan Surveyor) equipped with an Autosampler Plus, LC Pump Plus and a UV detector PDA Plus at 211 nm and 262 nm has been used. Samples of 20 µl were withdrawn periodically and diluted in 1480 µl of THF. After that, 10 µl of each preparation were injected in HPLC. The HPLC method used for determination of the ester yield was: flow rate of 1 ml min-1 and two mobile phases (phase A of pure water and phase B of pure acetonitrile) in different proportions over time (Figure 3).



	Time(min)	Flow(ml/min)	Water(%)	Acetonitrile(%)	Isopropanol(%)	Hexane(%)
1	0.00	1.000	30.00	70.00	0.00	0.00
2	18.00	1.000	30.00	70.00	0.00	0.00
3	20.00	1.000	0.00	100.00	0.00	0.00
4	37.00	1.000	0.00	100.00	0.00	0.00
5	39.00	1.000	30.00	70.00	0.00	0.00
6	44.00	1.000	30.00	70.00	0.00	0.00

Figure 3. HPLC method used for determination of the esterification yield.

Reaction yields of hydrolysis reactions were calculated according to the molar ratio between free EPA or DHA, determined by HPLC at each reaction sample collected, and the initial total amount (mol) of EPA or DHA used for the formation of miniemulsion reaction system. At the same time, esterification yields of EPA/DHA-nicotinol were determined as the molar ratio between EPA or DHA-nicotinol esters, determined by HPLC at each reaction sample collected, and the total amount of EPA or DHA (mol) used for the formation of miniemulsion reaction system. The initial amount of total EPA and DHA was only known through the weighted amount of fish oil and the relative amounts of each fatty acid in the fish oil (table 2). Thus, the hydrolysis yields (equation 1) with the formation of free EPA and DHA, and of esterification yields (equation 2), with the synthesis of EPA-nicotinol and DHA-nicotinol were determined in relation to the respective limiting substrate, which for this work was always EPA and DHA.

$$\eta_{hydrolysis}(\%) = \frac{mol\ free\ fatty\ acid}{mol\ total\ fatty\ acid_{initial}} \times 100 \qquad \text{Eq. 1}$$

$$\eta_{esterification}(\%) = \frac{mol\ ester}{mol\ total\ fatty\ acid_{initial}} \times 100 \qquad \text{Eq. 2}$$

The reaction rate in miniemulsion, V, was calculated as the amount of product formed per g of protein and per min (mol g^{-1} min^{-1}). For the determination of initial reaction rate, $V_{initial}$ (mol g^{-1} min^{-1}), the aliquots were collected in the first hour of the reaction, while for the determination of final reaction rate V_{final} (mol g^{-1} min^{-1}), the aliquots were collected at 72h of reaction.

2.6.2 HPLC standards of nicotinol esters

To determine concentration of both nicotinol-esters studied at a certain time of reaction it was firstly necessary synthesize them chemically as already presented. After that, the reaction products EPA-nicotinol and DHA-nicotinol were isolated to confirm their production. Therefore, the procedure steps for isolation, purification and characterization are presented below.2.6.2.1. Detection, isolation and purification of reaction productsThin layer chromatography (TLC) was applied in the analysis of reaction mixtures (see section **2.4**) and was performed in ALUGRAM® Xtra SIL G/UV254 silica gel plates (Macherey-Nagel, Germany) with detection by immersion in an ethanolic solution of phosphomolybdic acid, followed by heating.

Flash chromatography was performed on silica gel 60 (0.04 – 0.06 mm, 230 – 400 mesh ASTM) (Scharlau, Spain) under pressure and with the appropriate eluent or system of eluents (mentioned in the experimental procedures).

2.6.2.2. Characterization and analysis

The structure of all compounds synthesized (see section **2.2.4**) was confirmed by Nuclear Magnetic Resonance (NMR). NMR spectra were recorded at room temperature on a Bruker Avance II+ 300 (^1H 300 MHz, ^{13}C 75 MHz) spectrometer, using the residual solvent signal as reference, unless otherwise noted. Chemical shifts (δ) were reported in parts per million (ppm).

3. Results and discussion

3.1 Miniemulsion formulation

To prepare appropriate miniemulsion systems for enzymatic reactions, an initial study in sonication conditions was made. Thus, the influence of three different parameters in droplet size, as well as PDI, was evaluated: Power (W), Amplitude (%) and sonication time (min). For all studies, a pulse of 5s on/10 s off was maintained. All studies were done in triplicates. After that, the best formulation was used for further miniemulsion systems.

3.1.1 Power (W)

The first parameter studied was the influence of input power communicated from sonicator probe to the liquid. To do it 35 W, 45W and 55 W were tested in a sonication time of 5 min (Figure 4). Not only Z-average was analysed but also PDI and conclusions were withdrawn from there.

In figure 4 it is possible to see that for all three conditions of different input power used, Z-Average and PDI obtained were very different. In fact, the lowest Z-Average obtained was 369 ± 41 nm with a PDI of 0.32 ± 0.04, when using 35 W, as input power. After that, by increasing settled power in ultrasonication we see an increasing in droplet size, through Z-Average increase, resulting in more broad miniemulsion, in terms of droplet size, visible in the raise of PDI values.

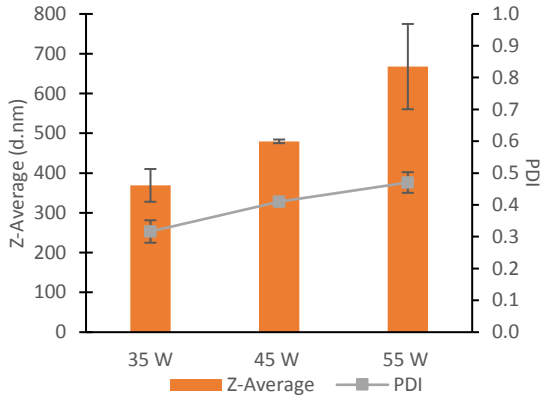


Figure 4. Z-Average (d.nm) and PDI using input powers of 35 W, 45 W and 55 W during 5 min of sonication with a 5s on/10s off pulse.

In order to see if the ultrasonication conditions could be fixed based on power input, it was repeated the analysis for the following two days, using miniemulsions with the same composition (table 1) and using the best ultrasonication conditions, such as 35 W, 5 min, 5s on/10s off pulse (Figure 5).

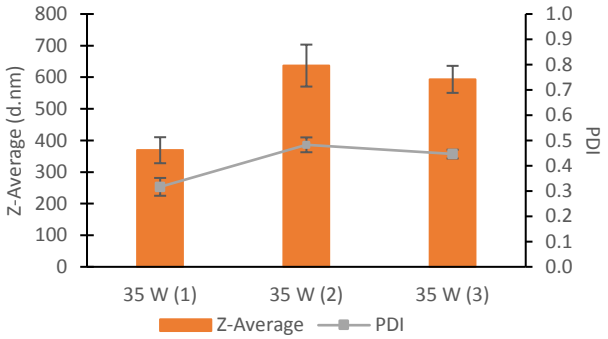


Figure 5. Z-Average (d.nm) and PDI using input power of 35 W during 5 min of sonication with a 5s on/10s off pulse in consecutive days.

It is possible to see that, despite all miniemulsions had, not only the same composition, but also the same ultrasonication conditions, it was impossible to have reproducible results. Not only droplet size was not similar, especially from the first to the remaining tests, but PDI values obtained were completely different. In fact, considering the first experiment, 35 W (1), probably could be thought that making ultrasonication based on power input would be reasonable, considering Z-Average and PDI values obtained. However, by replicating the same experiment in different days and excluding probe issues, once it was paid close attention to that, it was demonstrated that is not possible to do further experiments based on this parameter. This clearly indicates that, to create similar miniemulsion in terms of droplet size, power control in ultrasonication is not a good approach to allow having similar results in different samples, in different days.

In fact, using power input as operating mode, it was only known the energy output of the high frequency generator. However, using ultrasound what matters is the intensity, which is the energy actually applied to the sample. By maintaining ultrasound regulation based on power control, the amplitude of the tip surface from which the sound is emitted can fluctuate, thus creating fluctuations in intensity transmitted to the liquid, making difficult to have reproducible results [38].

At the same time, Z-Average obtained for sample 35 W (1) showed droplet sizes within miniemulsion definition while Z-Average of the samples 35 W (2) and 35 W (3) are clearly above miniemulsion droplet size threshold. In addition, PDI values of the last two samples are above 0.4, which indicates that the created droplets have a broad distribution, being thus very heterogenous in terms of droplet size. For that reason, it was clearly shown that for this miniemulsion system, sonication conditions cannot be chosen depending on the input power.

3.1.2 Amplitude (%)

The next parameter studied was amplitude of sonication. Because sonication probe MS72 used has a maximum amplitude of 50%, it was only studied two different values for it: 25% and 50%. To maintain the same conditions used before, sonication time was maintained at 5 min with a 5s on/10s off pulse (Figure 6).

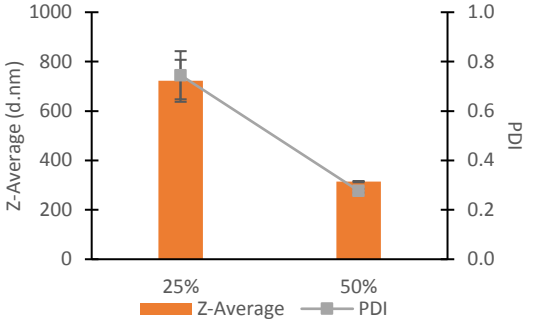


Figure 6. Z-Average (d.nm) and PDI using amplitude of 25% and 50%, during 5 min of sonication with a 5s on/10s off pulse.

In figure 6, it is seen that amplitude had a significant influence not only on droplet size, but also in PDI. To 25% amplitude case, it was obtained a Z-Average of 722 ± 86 nm and a PDI value of 0.75 ± 0.1, while for 50% case Z-Average and PDI values obtained were 314 ± 3 nm and 0.28 ± 0.0, respectively. By analysing these values, it can be seen that an amplitude condition of 25% was not enough high to create miniemulsions. In particular, it was impossible to produce homogeneous miniemulsions, since, in addition to the very high Z-Average values, PDI values were close to 1, which is indicative of a broad distribution in terms of droplet size. On the other hand, using an ultrasonication condition of 50% of amplitude, it was able to create miniemulsion, due to Z-average value obtained, with a very narrow distribution, since PDI value were below 0.3 [39].

Once again, to better see if by maintaining ultrasonication amplitude constant, reproducible results were obtained, it was done the same study previously done for power-influence study. It was repeated ultrasonication in the following two days, in different samples, but with the same composition shown in table 3 and with the same ultrasonication conditions (Figure 7). Since 50% amplitude had undoubtedly shown the better results in terms of Z-Average and PDI, it was only studied this condition.

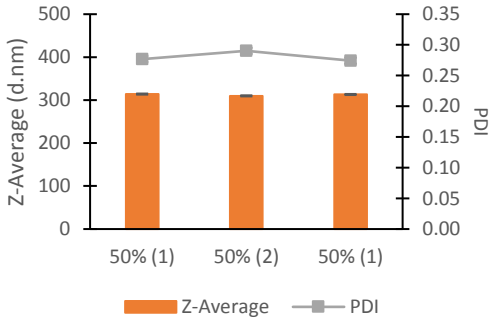


Figure 7. Z-Average (d.nm) and PDI using input amplitude 50% during 5 min of sonication with a 5s on/10s off pulse in consecutive days.

Figure 7 shows that, for all samples, Z-Average and PDI values obtained were almost the same, with similar droplet sizes and their distribution in miniemulsion system. This indicates that using these ultrasonication conditions, similar miniemulsions can be created with a very narrow distribution in terms of droplet size. Therefore, Z-Average and PDI obtained were, 312 ± 3 nm and 0.28 ± 0.01, respectively.

Using ultrasonication with amplitude directly logged in ultrasonic transducer, regardless the applied load conditions, amplitude was settled and held constant during ultrasonication. That allows the power output to fluctuate, as long as the maximum power allowed is not exceeded, and based on the physical state of the medium. Therefore, by holding constant amplitude during ultrasonication, the intensity transmitted to the fluid was also held constant. This means that it is important in the ultrasonic treatment to be able to monitor and control this amplitude, because as far as possible, the same reproducible results could be obtained [38].

Therefore, using an amplitude of sonication of 50% it can be accomplished miniemulsion standard parameters. Because of that, it turned clear that miniemulsion systems used in enzymatic reactions must be done using 50% of amplitude in sonication conditions.

3.1.3 Time (min)

Time of sonication was another parameter studied. In earlier studies, it was always used 5 min as sonication time. Although 5 min cannot be considered a high sonication time, it was evaluated the possibility of reducing this time, thus reducing overall time process but continuing to produce miniemulsion systems with a suitable droplet size and a narrow distribution in terms of droplet size, given by PDI. Thus, sonication times of 2 and 5 min were evaluated, using 50% of amplitude as sonication condition and a pulse of 5s on/10s off (Figure 8).

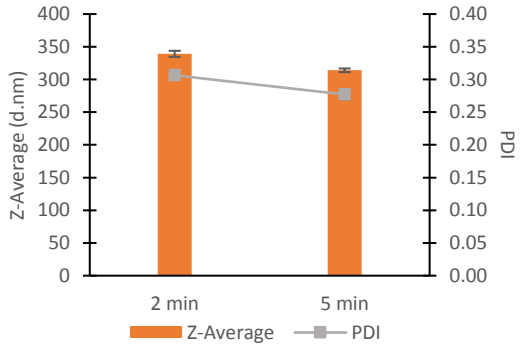


Figure 8. Z-Average (d.nm) and PDI for different sonication times, using 50% of amplitude with a pulse of 5s on/10s off.

In figure 8 it is possible to see that the Z-Average obtained in one case or the other enabled to produce miniemulsions with similar droplet size and very similar in terms of polydispersity. This is indicative of a strong non-time-dependency, since with half ultrasonication time, Z-Average and PDI values were similar. However, it is possible to see that using a sonication time of 5 min led to lower droplet sizes, but also lower PDI. In fact, using 5 min we obtained a Z-Average of 314 ± 3 nm with a PDI of 0.28 ± 0.0, while for 2 min of sonication a Z-Average and PDI values were, 339 ± 5 nm and 0.31 ± 0.0, respectively. The reason for that is probably because using 2 min as sonication time, the pulse intensity is not highly enough to sonicate the samples.

Since it was seen similar results using both conditions, it was decided to use 5 min as sonication time, due to the lower droplet size as well as the polydispersity of the samples. Probably, it could also be studied the pulse influence in miniemulsion droplet size. However, since it was achieved droplet sizes suitable for enzymatic reactions, it was chosen the following conditions for all miniemulsion systems used for enzymatic reactions in this work: Amplitude – 50%; Time – 5 min; Pulse – 5s on/10s off.

3.2 Enzymatic reactions in miniemulsion systems

As in Leticia Godoy work [2], where lipase-catalysed transesterification reactions between DHA-EE and nicotinol were done to produce DHA-nicotinol, the reactions were followed up to 72 h. Different aspects were studied, such as enzyme concentration, esterification efficiency of different lipases, different fatty acids/nicotinol molar ratio and also the influence/comparison of having esterification reactions with pure EPA/DHA instead of fish oil.

As described in **2.6.1**, the determination of hydrolysis and esterification yields were done by HPLC. With the method used, the substrates and products from resulting hydrolysis and esterification can be well identified, according specific retention times, resulting in a typical chromatogram expected to be obtained (Figure 9).

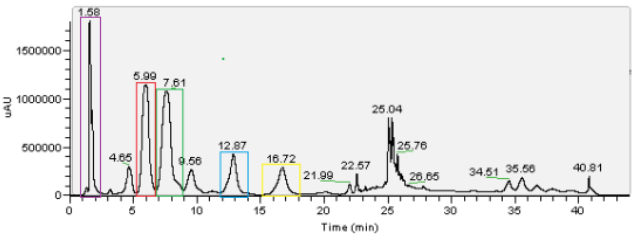


Figure 9. Resume of a typical chromatogram, with expected retention times of substrates and products resulting from hydrolysis and esterification reactions. Purple rectangle – time zone of nicotinol detection , Red rectangle – time zone of free EPA detection , Green rectangle – time zone of free DHA detection, Blue rectangle – time zone of EPA-nicotinol detection, Yellow rectangle – time zone of DHA-nicotinol detection

3.2.1 Effect of enzyme concentration in miniemulsion reactions

To establish the optimal amount of biocatalyst necessary for carrying out the targeted esterification of both EPA and DHA fatty acids, different concentrations of Amano PS lipase were used, in a linear range from 1 to 10 mg ml^{-1} and added to the reactors containing miniemulsions with substrates in an equimolar concentration of 0.369 M.

However, the initial amount of EPA and DHA in fish oil as free acids, monoglycerides, diglycerides or triglycerides were not known. That occurred because as in de Carvalho, 2012 [32], it was not possible to distinguish free fatty acids from glycerides, mono-, di- or tri-. Because of that, only the initial amount of free fatty acids and mono-/di-/triglycerides (% w/w) were known. For each fatty acid, it was observed a hydrolysis reaction, producing free EPA/DHA and an esterification reaction, producing EPA/DHA-nicotinol (Figure 2).

The objective of this study was to see if above a certain enzyme concentration, the miniemulsion interface is saturated with enzyme. It was done by varying considerably enzyme concentration and determining the initial (1h) hydrolysis and esterification rates and their equilibrium reaction yields for free EPA/DHA and EPA/DHA-nicotinol.

3.2.1.1 EPA/DHA glycerides hydrolysis into free fatty acids.

As already mentioned, two (hydrolysis and esterification) reactions occurred in miniemulsion systems. Although the objective was esterifying free EPA and DHA to their respective nicotinol esters, the hydrolysis reaction was followed by esterification. Therefore, to better understand what happens in terms of esterification, it should be quantified hydrolysis yields of free EPA/DHA formation. It was wanted to confirm that hydrolysis and esterification reactions occurred were not reverse reactions.

This experiment was started by determining the initial amounts of EPA and DHA free fatty acids, necessary to know to consider those values in hydrolysis yields calculations. After that, the hydrolysis yields were screened along 72 h of reaction, in order to determine the time of reaction in which equilibrium was reached, as well as maximum hydrolysis yields obtained for free EPA (Figure 10) and free DHA (Figure 11).

In figures 10 and 11, it was possible to see that both hydrolysis reactions occurred at least until 48h, time after reaction equilibrium seems to be established. However, in case of DHA, it clearly happened only for higher enzyme concentrations.

At the same time, in figures 10 and 11, it was possible to see that EPA and DHA triglycerides presented very different hydrolysis profile, since higher hydrolysis yields in free fatty acid form were obtained for DHA, when compared with EPA. For the same enzyme concentration, the hydrolysis yields for free DHA formation were 1.6 to 1.9 times higher, comparing with free EPA. This indicates that the hydrolysis of DHA was more complete, remaining a small percentage of mono-, di- or triglycerides. On the other hand, once the EPA hydrolysis yields were lower, a higher percentage of mono-, di- and triglycerides were remaining.

In figures 10 and 11 was also possible to see that the highest Amano PS lipase concentrations led to the highest hydrolysis yields for both acids. It was shown an increasing in the hydrolysis yield in EPA from 34.4% for an enzyme concentration of 1 mg ml^{-1}, to 48.0% for an enzyme concentration of 10 mg ml^{-1}. In case of DHA, for the same increase of enzyme concentration, hydrolysis yields increased from 59.3% to 80.2%.

The initial hydrolysis rate can also be obtained from figures 10 and 11, which was determined based in the variation of the first hour free fatty

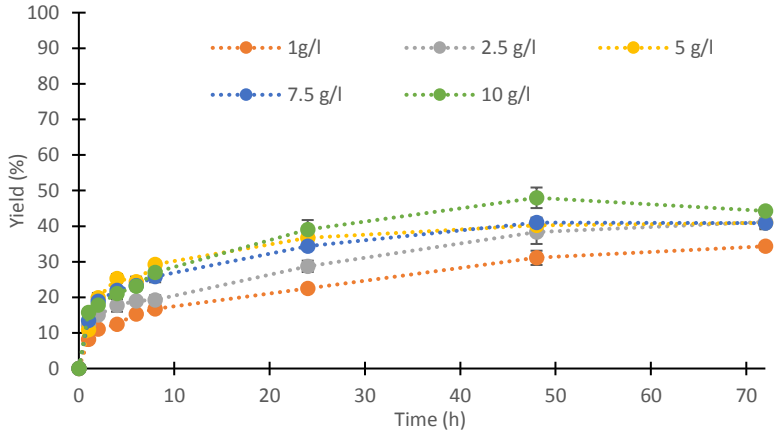


Figure 10. Effect on Amano PS lipase concentration in hydrolysis yield in free EPA at 50ºC, determined by HPLC.

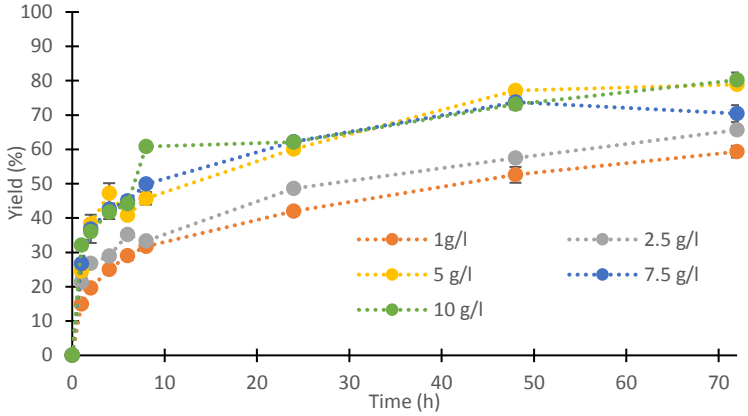


Figure 11. Effect on Amano PS lipase concentration in hydrolysis yield in free DHA at 50ºC, determined by HPLC

acid concentration. Thus, as for the hydrolysis yields, the initial free fatty acids formation rate is dependent of biocatalyst's concentration: the higher the enzyme concentration, the higher initial hydrolysis rate for both fatty acids.

The initial rate of EPA formation raised from 15.8 mM h^{-1}, for an enzyme concentration of 1 mg ml^{-1}, to 20.9 mM h^{-1} for 5 mg ml^{-1} and to 30.0 mM h^{-1} for 10 mg ml^{-1} of biocatalyst. These values corresponded to an increase around 32% and around 44%, respectively of the initial reaction rate from 1 mg ml^{-1} of biocatalyst. The hydrolysis yield of the released free EPA increased with the increase of Amano PS lipase concentration.

In case of DHA, for the same variations of enzyme's concentration, the initial esterification rate increased from 16.9 mM h^{-1} to 27.6 mM h^{-1} and 35.8 mM h^{-1}, corresponding to an increase around 63% and 30%, respectively. However, above 5 mg ml^{-1} the hydrolysis yields of the released free DHA stayed almost constant within the experimental error

Considering that Amano PS lipase is a non-regiospecific enzyme [40], it was expected that it hydrolyses indifferently each one of the *sn*-positions of the triglyceride backbone. However, the initial hydrolysis rates for free EPA and DHA formation showed the opposite. In previous work [31], it was shown that lipase Amano PS catalyses the reactions in *sn*-2 position faster and more preferred than the reactions in *sn*-1,3 positions. In fact, the results showed that by a 5-fold increasing on enzyme concentration, from 1 to 5 mg ml^{-1}, the initial rate of free DHA formation was the double of the EPA initial reaction rate. This could indicate that the initial substrate has more % of DHA in *sn*-2 position, what makes the synthesis of free DHA faster than EPA, despite the randomness of re-esterification process in fish oil supplements [41].

These evidences indicate that oil-water interface was not yet saturated with enzyme. This data is in agreement with [28], [31] where 10 mg ml^{-1} of Amano PS lipase did not saturate the reaction system.

3.2.1.2 Ester synthesis

It was monitored the evolution of the concentration of both esters over time and, through that, it was determined EPA-nicotinol (Figure 12) and DHA-nicotinol (Figure 13) esterification yields.

The results illustrated in the figures 12 and 13 show, for almost all the studied enzyme concentrations that the reaction seems to reached the equilibrium at 48h, which agrees with the previous hydrolysis studies (see section **3.2.1.1**). However, these figures showed that, after 48h of reaction, for enzyme concentrations from 5 to 10 mg ml^{-1} the esterification yields of EPA/DHA-nicotinol tended to a slight decrease, which was probably due to the beginning of the hydrolysis of EPA/DHA-nicotinol. Nevertheless, some of these variations are within the experimental error.

It was possible to see that the esterification yields were low when compared with those obtained in the hydrolysis, as shown in figures 10 and 11 (see section **3.2.1.1**).

It also showed that in a general manner, higher esterification yields for both esters were obtained when using higher enzyme concentrations. It was observed the EPA-nicotinol esterification yield increasing from 12.1%, for an enzyme concentration of 1 mg ml^{-1}, to 21.5% for an enzyme concentration of 5 mg ml^{-1}. For DHA, with the same increase of enzyme concentration, the esterification yields of DHA-Nicotinol increased from 14.5% to 20.3%.

However, for EPA-nicotinol from 5 mg ml^{-1} to 10 mg ml^{-1}, esterification yields obtained varied insignificantly, while in case of DHA-nicotinol they decreased around 4% from 5 mg ml^{-1} to 10 mg ml^{-1}.

As done previously for the hydrolysis reactions, the initial esterification rates for each ester at each used enzyme concentration were determined based in the variation of the first hour ester concentration. Since the obtained esterification yields were lower than hydrolysis yields already presented, the initial esterification rates would be, undoubtedly lower than the initial hydrolysis rates (see section **3.2.1.1.**) However, as esterification yields, the initial esterification rates showed an enzyme concentration-dependence: generally, the higher enzyme concentrations, the higher initial esterification rates obtained for both esters.

In fact, the EPA-nicotinol initial formation rate increased from 7.2 mM h^{-1}, for an enzyme concentration of 1 mg ml^{-1} to 8.2 mM h^{-1} for 5 mg ml^{-1} and to 10.2 mM h^{-1} for 10 mg ml^{-1} of Amano PS lipase. These values correspond to an increase around 14% and 24%, respectively, from 1 mg ml^{-1}.

In case of DHA-nicotinol, for the same enzyme concentrations increase, the initial ester formation raised from 6.9 mM h^{-1} to 7.3 mM h^{-1} and 9.3 mM h^{-1}, corresponding to an increase around 6% and 27%, respectively.

This shows that, despite the maximum esterification yields of both esters stayed almost constant over enzyme concentration increase, the initial esterification rates were more influenced by enzyme concentration increase, leading to higher rates in case of using higher concentrations

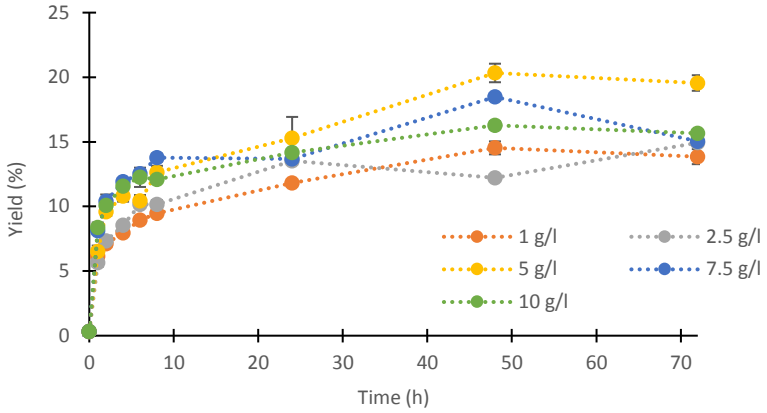


Figure 12. Effect on Amano PS lipase concentration in esterification yield of EPA-nicotinol at 50ºC, determined by HPLC

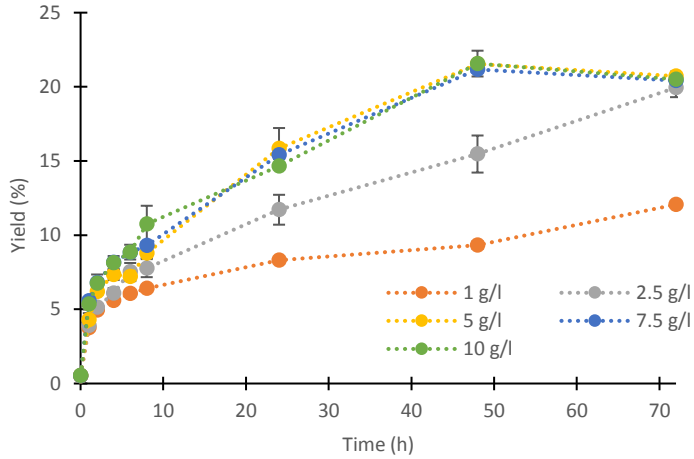


Figure 13. Effect on Amano PS lipase concentration in esterification yield of DHA-nicotinol at 50ºC, determined by HPLC

of biocatalyst. Furthermore, this effect has even higher importance in case of DHA-nicotinol

It was also noticed that there was a slightly preference of lipase PS for EPA-nicotinol synthesis rather than DHA-nicotinol. However, Haraldsson and co-workers reported preference for DHA over EPA in lipase PS-catalysed ethanolysis reaction with omega-3 substrates [17]. Nonetheless, differences in the esterification yields were almost equal within experimental error, in case of highest yield for EPA-nicotinol and DHA-nicotinol.

It was also determined the % mol of each molecular specie of EPA and DHA in miniemulsion: free fatty acid, glycerides and nicotinol-esters (data not shown). It was demonstrated that even though % mol of EPA-nicotinol and DHA-nicotinol were almost the same in miniemulsion system, there was a big difference in terms of % mol of free acid vs % mol of glycerides for each fatty acid.

In fact, there was a higher accumulation of free DHA inside the miniemulsion droplets. In theory, this accumulation should shift the equilibrium for the formation of DHA-nicotinol, but it apparently did not happen. In order to understand this evidence, enzymes with different regioselectivity, such as *sn*-1,3 regiospecific lipases, and substrate specificity should be tested to have a better comparison of this evidence between different lipase-catalysed reactions in miniemulsion. It was also presented that higher hydrolysis yields generally coincide with higher esterification yields. This finding showed that hydrolysis and esterification reactions occurring in this miniemulsion system are not reverse reactions but sequential reactions, as shown (Figure 2).

Since above 5 mg ml^{-1} there is no significant increase in esterification yields, considering that the required enzyme amount is normally a decisive aspect, if considered the overall production costs, further experiments using Amano PS lipase should be performed with 5 mg ml^{-1}.

3.2.2 Influence of different enzymes on miniemulsion reactions

In the previous study, it was seen that, despite the high hydrolysis yields obtained, the esterification failed to achieve yields higher than around 20%, when using Amano PS lipase as biocatalyst. Therefore, the results obtained for this enzyme were compared, in terms of esterification efficiency, with other enzymes, such as commercial *R. miehei*, CAL A expressed in *A. oryzae* and *C. rugosa* lipases. These lipases were chosen because some of them had already been used in the biosynthesis of alkyl esters in miniemulsion [30], others were widely used in esterification studies [42]–[44] and even in omega-3 fatty acids concentration studies with hydrolysis, esterification and transesterification reactions [15]–[17], [20], [45].

To do that, the initial activity was determined based on different Amano PS lipase concentration study, that pointed 5 mg ml^{-1} as the best enzyme concentration for carrying out esterification of EPA/DHA. Therefore, the initial activity in each reactor was approximately 191 U ml^{-1} and depending on specific activity, each enzyme amount needed was then determined. The esterification reactions were carried at 40ºC.

3.2.2.1 EPA/DHA glycerides hydrolysis into free fatty acids

Although samples were collected during reaction course, it was only represented the final hydrolysis yields for free EPA and DHA formation (Figure 14). As already seen in section **3.2.1.1** the hydrolysis reactions reached equilibrium after 48h of reaction with all lipases tested.

Is possible to say that there were obtained very different results considering the four lipases tested in the hydrolysis-biocatalysed reactions. In fact, it was seen that three in four lipases allowed to have higher hydrolysis yields for DHA than for EPA. Only *C. rugosa* had the opposite behaviour. It was also seen that, for the hydrolysis of EPA glycerides, *R. miehei* allowed to have the highest hydrolysis yield (64.8%), followed by CAL A (51.9%) and *C. rugosa* (36.7%) and Amano PS (36.5%) with almost the same yield results, within the experimental error. The same order was seen for DHA glycerides hydrolysis with *R. miehei* (75.8%), CAL A (70.9%) and Amano PS (64.0%), except for *C. rugosa*, which led to the lowest DHA hydrolysis yield (24.4%).

Considering that *C. rugosa* was the only case, in which was observed higher hydrolysis of EPA than DHA, there are reports of non-regiospecificity of this lipase [46], meaning that the hydrolysis reactions are not occurring preferentially in any of the triglyceride positions.

However, the higher hydrolysis yields obtained for EPA agrees with the statement that most lipases prefer EPA to DHA as substrate, due to the steric hindrance in lipases active site derived from DHA having one more double bound when compared with EPA [45]

The other lipases showed preferably hydrolysis of DHA rather than EPA. In case of Amano PS, the reason behind it have been already exploited in section **3.2.1.1.** The other two lipases, *R. miehei* and CAL A, have different regiospecificity: *R. miehei* preferably hydrolyses *sn*-1,3 positions [47], while CAL A has reports of *sn*-2 preference [46].

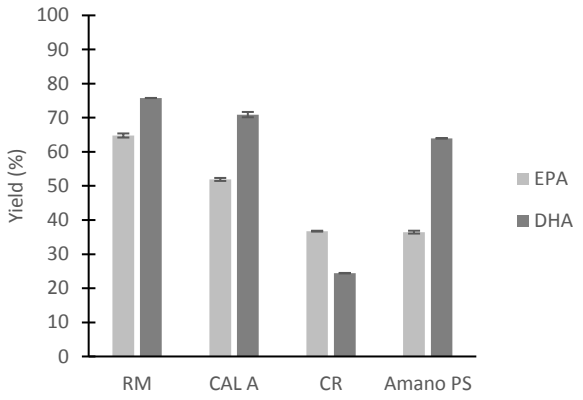


Figure 14. Hydrolysis yields, determined by HPLC, for free EPA and DHA at 48h in miniemulsion at 40ºC, using: *Rhizomucor miehei* (RM) lipase, *Candida antarctica A* (CAL A) lipase, *Candida rugosa* (CR) lipase and Amano PS lipase (Amano PS).

Despite both hydrolysed DHA over EPA, it was seen that the difference between DHA and EPA hydrolysis yields was almost double in case of CAL A (19%) when compared with *R. miehei* (11%). This clearly shows that *sn*-1,3 preference of RM lipase led to more similar hydrolysis yields. At least, the values obtained in case of using this enzyme were the most similar results obtained for hydrolysis study with different biocatalysts.

Despite all *sn*-preference considerations, it should be noticed that these miniemulsion systems are dynamic systems, where more than one reaction is occurring at the same time. This means that the hydrolysis yields obtained cannot be dissociated from further esterification of the free fatty acids produced. Therefore, it should be analysed the esterification results to have a more accurate perception of what happened in miniemulsion during reaction time course.

3.2.2.2 Ester synthesis

The same approach of section **3.2.2.1** was done for the illustration of the esterification yields. Therefore, reaction equilibrium for EPA/DHA-nicotinol synthesis was also reached at 48h (Figure 15), which had already been showed for enzyme concentration study.

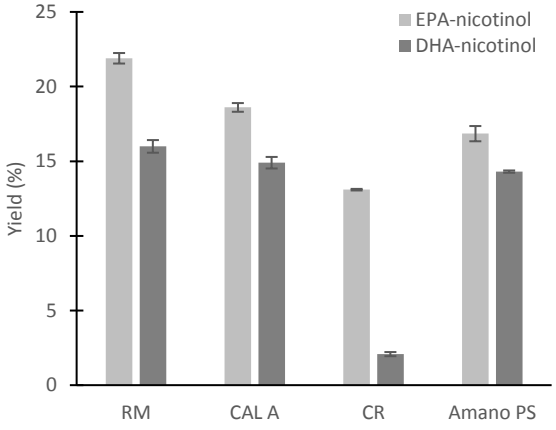


Figure 15. Esterification yields, determined by HPLC, for EPA/DHA-nicotinol esters at 48h in miniemulsion at 40ºC using: *Rhizomucor miehei* (RM) lipase, *Candida antarctica A* (CAL A) lipase, *Candida rugosa* (CR) lipase and Amano PS lipase (Amano PS).

It was possible to see in figure 41 that higher esterification yields for EPA-nicotinol instead of DHA-nicotinol were obtained for all four enzymes. At the same time, it was also possible to see that lower esterification yields were obtained in case of using *C. rugosa* lipase for both esters, when compared with other enzymes used. Even more noticing is that lipase from *C. rugosa* was the only enzyme used that showed a very low esterification yield for DHA-nicotinol.

In fact, earliers reports [15] of *C. rugosa* preference of EPA over DHA support the results obtained for esterification yields as well as hydrolysis yields (see section **3.2.2.1**) in terms of a clear preference for EPA in *C. rugosa*-catalysed reactions. At the same time, in esterification studies in miniemulsion, performed by de Barros and co-workers [30], it was demonstrated that lipase-catalysed reaction using *C. rugosa* led to slightly less esterification degree when compared with other lipases, such as Amano PS. Since it presented higher esterification selectivity for EPA compared with DHA and, considering that DHA-nicotinol ester is even more interesting as nutraceutic, as previously elaborated, it was concluded that is not advantageous using this enzyme for further esterification tests.

In figure 15, is demonstrated that for the synthesis of EPA-nicotinol, using *R. miehei* as biocatalyst allowed to have highest esterification yields (21.9%), followed by CAL A (18.6%), Amano PS (16.8%) and *C. rugosa* (13.1%). In addition, for the synthesis of DHA-nicotinol the same order was maintained, with *R. miehei* (16.0%), CAL A (14.9%), Amano PS (14.3%) and *C. rugosa* (2.1%).

As mentioned, when comparing the differences of esterification efficiency towards EPA and DHA, it was possible to see that all three enzymes allowed to catalyse more EPA-nicotinol formation than DHA-nicotinol. However, for *Rhizomucor miehei* lipase the difference between those yields was 5.9%, while for CAL A lipase and Amano Lipase PS were 3.7% and 2.5%, respectively.

Regarding *R. miehei* lipase, higher esterification degree of EPA were also expected due to the preference towards EPA over DHA in esterification studies with ethanol and fish oil free fatty acids (FFA), reported by Haraldsson and Kristinsson [48]. Furthermore, since relative higher hydrolysis yields with free EPA formation were observed (see section **3.2.2.1),** it was seen an accumulation of free EPA, pushing the esterification reaction equilibrium towards EPA-nicotinol formation. For both reasons *R. miehei* lipase demonstrated to be the biocatalyst with higher differences, in terms of esterification yield, between EPA-nicotinol and DHA-nicotinol.

CAL A and Amano PS lipases showed almost the same profile in terms of substrate preference, not only in esterification, but also in the hydrolysis studies already mentioned (see section **3.2.2.1**). In fact, to the best of our knowledge CAL A has never been reported in studies of omega-3 hydrolysis/esterification. However, since that CAL A have markedly *sn*-2 preference in the hydrolysis of triglycerides and Amano PS, although its non regiospecificity, hydrolyses *sn*-2 positions faster than *sn*-1,3 positions, this generate faster accumulation of free DHA, when compared with EPA, when using these two biocatalysts. This fact led to similar esterification yields between EPA and DHA, despite theoretical disadvantage of DHA esterification due to the steric hindrance in lipases active site derived from DHA having one more double bound [45].

With this study was possible to see that, among the four enzymes Amano PS was the one which presented less selectivity for the esterification of the fatty acids, with the difference between esterification yields to be lower using this enzyme.

In fact, if the esterification yields of EPA-Nicotinol and DHA-Nicotinol are compared using Amano PS lipase as biocatalyst at 40ºC and 50ºC, is possible to see that the difference between those yields was halved from 40ºC (2.5%) to 50ºC (1.2%). Since it was wanted, as much as possible, similar esterification yields for EPA-nicotinol and DHA-nicotinol, it has become evident that the biocatalyst that allows higher esterification yields as well as almost the same selectivity for EPA and DHA for esterification is the one that best fits our intentions. By that, it could be understood that further tests must be made using Amano PS lipase at 50ºC as biocatalyst for carrying out esterification reactions.

It was possible to demonstrate that higher yields for both reactions were correlated since cases with higher hydrolysis yields led to higher esterification yields. With that, it can be demonstrated, one more time, that both reactions were occurring sequentially, with products of hydrolysis reaction being used as reactants of esterification reactions. If it were not so, it would have seen the opposite, that is, higher esterification yields would lead to lower hydrolysis yields, for instance.

Thus, it was verified that the problem of having low esterification yields was not particularly due to the efficiency of the enzyme in the esterification reactions, because once it was obtained almost the same values in terms of hydrolysis and esterification yields. Therefore, miniemulsion systems themselves could be the reason for low esterification efficiency in all conditions tested so far.

3.2.3 Effect of fatty acids/nicotinol ratio on ester synthesis

According with what was seen until now, poor esterification yields could not be related with enzyme selection and/or concentration. In addition, it was seen that, despite the high hydrolysis yields obtained, it was not reached esterification yields higher than 22%. In theory, with the accumulation of high free EPA and DHA content in miniemulsion droplets, it should be expected that reaction equilibrium of esterification would be shifted towards esters synthesis. However, it did not happen.

One of the miniemulsion features is hydrophobicity of substrates. To have stable miniemulsions is necessary to have more hydrophobic substrates. In addition, higher hydrophobic substrates allow to have not only higher esterification yields but also faster conversions as reported by de Barros and co-workers [30] and Aschenbrenner and co-workers [28]. However, nicotinol is a very water-soluble alcohol, since it is described as fully miscible with water [49], with a maximum solubility measure of 0.1558 gm ml^{-1}.

Although none phase separation was seen until now, the fact is, being nicotinol very soluble in aqueous phase, it led to nicotinol diffusion through droplets interface, thus leaving their interior. This means that when fatty acids were available at miniemulsion interface to be esterified, there was not enough nicotinol to do that, since a part of it was in aqueous phase. Furthermore, as nicotinol was solubilized in water, when fish oil was at miniemulsion interface, glycerides were hydrolysed, in free

fatty acids, but after that they could not be esterified in EPA/DHA-nicotinol esters.

Therefore, to see if water-solubility of nicotinol could be the reason of low esterification yields, it was decided to decrease nicotinol solubility in water. The easiest way to do it is increasing alcohol's relative amount regarding to aqueous phase. Once it was desired to maintain water composition in miniemulsion, it was decided to apply excess of nicotinol regarding Omega Balance fish oil. In addition, as it is already known, to shift reaction equilibrium towards product side, one of reactants could be applied in excess. Once it was postulated that a lack of nicotinol could be the reason for low esterification yields, it was decided only to apply excess of alcohol to see if there is any improvement in the final esterification yields.

Nevertheless, increasing the amount of nicotinol above a certain concentration could result in an inhibitory effect on enzyme activity, leading to even lower reaction rates/final product yields.

Without changing the relative amounts of each miniemulsion component (table 1), fatty acids/nicotinol molar ratio (R) was decreased from 1 to 0.1 and the esterification yields, as well as reaction rates at t=0h and t=72h, were determined (Figure 16). The aim was to verify whether there would be improvement of the esterification yields and if any of those conditions would inhibit activity of lipase Amano PS.

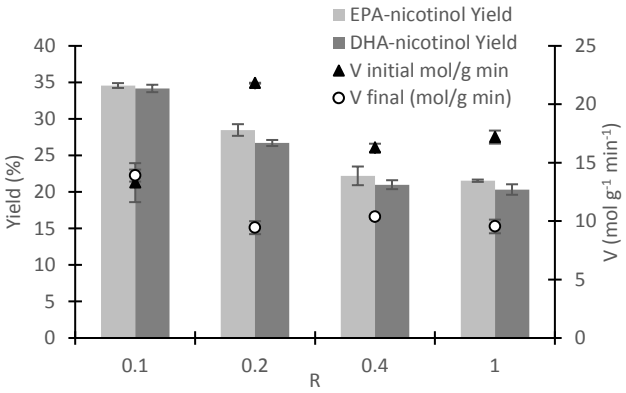


Figure 16. Effect of fatty acids/nicotinol molar ratio (R) on synthesis of EPA-nicotinol and DHA-nicotinol esters, initial and final reaction rates, at 50ºC using Amano PS lipase as biocatalyst. Yield of esterification was determined by HPLC.

The effect of nicotinol on enzyme activity was studied starting with small concentrations of EPA and DHA (R=0.1). By increasing R value from 0.1 to 0.2, the initial reaction rate increased from 13.3 mol g^{-1} min^{-1} to 21.8 mol g^{-1} min^{-1}, which was the maximum value obtained. For R=0.4, the initial rate of reaction decreased to 16.3 mol g^{-1} min^{-1} and slightly increased for 17.2 mol g^{-1} min^{-1} at equimolar concentrations (R=1) of EPA/DHA and nicotinol. On the other hand, for the final reactions rates, surprisingly, it was seen that the highest one was when R=0.1 with 13.9 mol g^{-1} min^{-1} and after that it decreased for 9.4 mol g^{-1} min^{-1}, 10.4 mol g^{-1} min^{-1} and 9.5 mol g^{-1} min^{-1} for R=0.2, R=0.4 and R=1, respectively.

Addressing final esters yield, it was clearly seen a steady decrease from R=0.1 to R=1. For R=0.1 reaction yields of 34.6% and 34.2% were obtained for EPA-nicotinol and DHA-nicotinol respectively, while for R=1 those yields became 21.5% and 20.3%.

For R=0.1, it seemed to exist an initial inhibitory effect of nicotinol, since this was the case of the lowest initial reaction rate. However, comparing the final and initial reaction rates, it was seen that they were equal within experimental error, while all other reactions rates decreased over time. For R=0.2, the reaction rate decreased 57% after 72h of reaction, while for R=0.4 and R=1 decreased 36% and 45%, respectively. This could probably mean that an excess of nicotinol in miniemulsion systems led to initial low enzyme activity, as it was shown in previous work [2].

Nevertheless, this inhibitory effect seemed to have no influence in terms of the esterification of EPA and DHA, since using miniemulsion systems with a molar ratio R=0.1 enabled to obtain the highest esterification yields seen in this study.

3.2.4 Effect of pure EPA/DHA fatty acids vs Omega Balance fish oil in ester synthesis

All enzymatic reactions made showed hydrolysis of EPA/DHA triglycerides from fish oil into free fatty acids which were then esterified in nicotinol-esters. Therefore, it was decided to examine what would be the difference in terms of the final esterification yield of starting miniemulsion reactions with only free fatty acids instead of EPA/DHA triglycerides. To do that, EPA and DHA standards were applied as substrates in different miniemulsions to have only one of these fatty acids as substrates. The idea was to see the impact of having only one of the fatty acids to be converted and the difference of having it as FFA and not as triglycerides. In this case, the reaction volumes decreased to 5 ml. Whereas miniemulsion volume has halved, it was settled to have a control exactly with the same conditions of pure EPA and DHA miniemulsions but using Omega Balance fish oil.

Hereupon, without changing the relative amounts of each miniemulsion component (table 1), mass of each component was halved, but enzyme concentration maintained. In these experiments, R=0.1 was maintained, since it was defined as the best condition to obtain higher esterification yields.

Once again, esterification yields evolution was screened over time until 72h of reaction, however, only the highest esterification yields for both acids were presented (Figure 17). It was observed that when using pure acids as substrates, the reaction equilibrium was obtained at 2h, although when using Omega Balance fish oil, the equilibrium was reached at 48h.

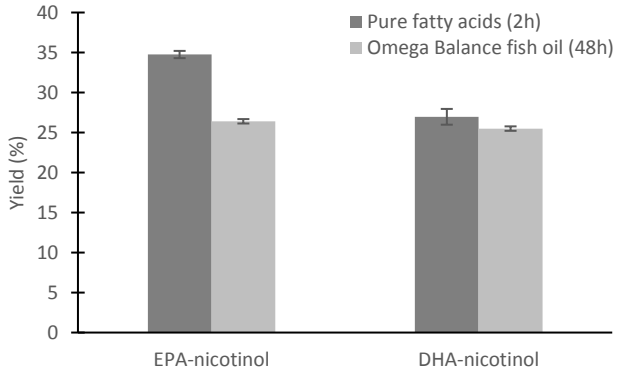


Figure 17. Effect of pure free EPA/DHA vs EPA/DHA triglycerides substrates in esterification yields,at 50ºC, determined by HPLC, using Amano PS lipase as biocatalyst.

In figure 17 is possible to see that esterification yields in case of using Omega Balance fish oil for a working-volume of 5 ml was different than those under the same conditions but with a working-volume of 10 ml with R=0.1, presented in figure 16. Since that the variation was almost equal for EPA-nicotinol and DHA-nicotinol synthesis, it can be hypothesized that it was due to scaling-down constraints, such as power/volume (W m^{-3}) in mixing [50]. By decreasing the volume of reaction to 5 ml, the power/volume value increased, once it continued to using an agitation velocity of 400 rpm. Thus, by increasing agitation power while maintaining the same enzyme concentration could lead to some loss of activity, which is correlated to the lower final ester yields obtained. However, it was not determined the reaction rate to compare with the results obtained in the same conditions using 10 ml as working volume (section **3.2.3**).

Although different esterification yields were obtained in those situations, its difference was not relevant for pure EPA and DHA vs Omega Balance fish oil comparison, because the effect in enzyme activity will be the same in both cases.

In figure 17 is seen that for EPA-nicotinol that it was possible to obtain 34.8% and 27.0% of esterification yield, when using pure EPA and Omega Balance as substrates, respectively. On the other hand, for DHA-nicotinol it was seen that the esterification yields were very similar for both cases, with 26.4% and 25.5% when using pure DHA and Omega Balance fish oil, respectively. These values were almost equal within experimental error and can be considered identical. However, the reaction equilibriums were reached at completely different times of reaction, with pure acids reactions being 24 times faster than those using Omega Balance fish oil.

In figure 17, it is seen that, in the case of EPA-nicotinol, higher esterification yields were obtained using pure acid as substrate, while this is not clear to DHA-nicotinol. A possible reason is, as it has already been discussed, that when Omega Balance fish oil was used as substrate, the hydrolysis reactions of triglycerides in fish oil to form free EPA and DHA were followed by esterification in nicotinol esters. This aspect is important because the rate of free EPA/DHA formation conditioned the formation rate of their respective esters. Therefore, the higher the rate of free acid formation, the higher were the esterification yields. Once it was seen much higher hydrolysis of DHA triglycerides comparing with EPA triglycerides, when using lipase Amano PS as biocatalyst, the free DHA formation was much faster than free EPA formation. The higher accumulation of free DHA inside miniemulsion droplets shifted reaction equilibrium towards DHA-nicotinol synthesis. Therefore, it allowed to improve esterification of free DHA, using Omega Balance fish oil, at the same level of using pure DHA, where hydrolysis reaction does not occur. In fact, by using pure acids, the reaction equilibrium was reached much faster, which is in accordance with this evidence because at the beginning of esterification reaction, EPA and DHA were already in miniemulsion as free fatty acids.

4. Conclusions and future work

In the present work was possible to conclude that lipase-catalysed esterification in this miniemulsion system should be performed with a concentration of 5 mg ml^{-1} of Amano PS lipase despite the miniemulsion interface was not saturated with enzyme.

It was also possible to conclude that except for *Candida rugosa*, all lipases tested, allowed to obtain similar esterification degree of EPA and DHA with nicotinol, demonstrating that Amano PS lipase had less selectivity between EPA and DHA.

Furthermore, it was concluded that they were some constraints due to nicotinol water-solubility. This aspect, in addition to lack of water activity control, led to medium esterification level. However, Amano PS choice as biocatalyst seemed to be successful, as it showed almost no loss of activity in catalysed reactions with molar ratios of R=0.1. At the same time, miniemulsion proved to allow sequential reactions, with high accumulation of free fatty acids and without seen enzyme's activity inhibition. This is clearly advantageous in terms of time and money spent because with only one system fish oil triglycerides can be hydrolysed in free EPA/DHA and, subsequently, esterified to nicotinol esters. This allowed to obtain same esterification levels for EPA and DHA, which would have been difficult to get, if the reactions were done with each of the fatty acids separately and, initially, as free fatty acids.

However, it was possible to conclude that making esterification of EPA/DHA in fish oil favours the synthesis of DHA-nicotinol over EPA-nicotinol, when compared with the esterification of pure EPA/DHA.

In near future, several studies should be carried out to take advantage of the beneficial aspects of using this reaction system, in order to improve esterification level of both EPA and DHA fatty acids. Concretely, the impact of diminishing water composition in miniemulsion formulation. The use of ME 50% has been proven to allow enzymatic esterification in miniemulsion. Highly hydrophobicity of fish oil, together with high solubility of nicotinol in water, make possible to believe that, by decreasing water content, the permanence of nicotinol inside miniemulsion droplets will be higher. Thus, it will increase substrate's availability to be esterified. At the same time, by doing that, water activity will be reduced, allowing thermodynamically more favourable esterification of substrates.

To the same extent, an assessment of the impact of adding nicotinol in fed-batch mode should be made. Although free fatty acids accumulation inside miniemulsion droplets is, in theory, a good approach to shift reaction towards ester synthesis, it was seen that for R=1, this was not possible to obtain. As it was discussed, it is due to lack of nicotinol inside miniemulsion droplets to react with newly formed EPA/DHA free fatty acids. Therefore, stepwise addition of nicotinol will allow instantaneous esterification of EPA and DHA, not providing diffusion of nicotinol to aqueous phase. It will improve both EPA and DHA esterification level, as it was already proven in other studies involving esterification of water-soluble alcohols with fatty acids in miniemulsion.

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