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

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Thesis to obtain the Master of Science Degree in

Biological Engineering

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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.

Resumo

Miniemulsões são meios reaccionais promissores com várias vantagens para a esterificação catalisada por lípases. Neste trabalho, a síntese de nutracêuticos, ésteres de EPA e DHA-nicotinol, foi realizada em miniemulsão e assistida por lipases, utilizando suplemento de óleo de peixe como substrato. Foram avaliados diferentes parâmetros: concentração de lipase; eficiência de esterificação de lipases comerciais de *Rhizomucor miehei*, *Candida Antarctica A* recombinante em *Aspergillus oryzae*, *Candida Rugosa* tipo VII e Amano PS; Razão molar ácidos gordos / nicotinol (R); EPA e DHA como substratos isolados puros ou misturados, em óleo de peixe. As miniemulsões mostraram alto potencial de hidrólise de triglicerídeos de óleo de peixe e posterior esterificação de EPA e DHA com nicotinol. Em condições de excesso de nicotinol (R=0.1), os rendimentos de esterificação para EPA-nicotinol e DHA-nicotinol foram 34,6% e 34,2%, respectivamente, para 5 mg ml^{-1} de biocatalisador. A velocidade inicial da reacção foi de 13,3 mol g^{-1} min^{-1}, sem perda de actividade hidrolítica após 72h. Para R = 0.2, observou-se uma velocidade inicial de reacção máxima de 21,8 mol g^{-1} $min^{-}1$, mas no final da reacção, a actividade de Amano PS diminuiu cerca de 57%. Foi demonstrado que um elevado excesso de nicotinol inibe inicialmente a actividade de Amano PS, sem comprometer os rendimentos de esterificação de ambos os ácidos gordos. Os resultados do presente trabalho mostraram dados promissores para a produção de ésteres de EPA / DHA-nicotinol em miniemulsão, necessitando de mais estudos para otimização.

Palavras chave: Miniemulsão, EPA, DHA, Nicotinol. Esterificação, Lipase, Ácidos gordos Omega-3

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ALA	α-linolenic acid	**Tris**	(hydroxymethyl)aminomethane
Ɛ	Absorption coefficient	**LA**	Linoleic Acid
AA ω6	Arachidonic Acid omega-6	**LDL**	Low Density Lipoproteins
BCA	Bicinchoninic acid	**ME**	Miniemulsion
BSA	Bovine Serum Albumin	**NAD**	Nicotinamide Adenine Dinucleotide
CAL A	*Candida Antarctica* lipase A	**NADH**	Nicotinamide Adenine Dinucleotide (reduced)
CR	*Candida rugosa*	**NADP**	Nicotinamide Adenine Dinucleotide Phosphate
CTAB	Cetyltrimethylammonium bromide	**NADPH**	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
δ	Chemical shifts	**NMR**	Nuclear magnetic resonance spectroscopy
CMC	Critical Micellar Concentration	**O/W**	Oil-in-water
d.nm	Diameter (nm)	**ω-3**	Omega-3
DHA	Docosahexaenoic acid	**p-NP**	*para*-nitrophenol
DPA	Docoapentaenoic Acid	**p-NPP**	*para*-nitrophenyl palmitate
DLS	Dynamic Light Scattering	**PDI**	Polydispersity Index
EPA	Eicosapentaenoic acid	**PCBs**	Polychlorinated Biphenyls
EE	Ethyl Ester	**PUFA**	Polyunsaturated Fatty Acids
FA	Fatty Acids	**V**	Reaction rate
R	Fatty acid/nicotinol molar ratio	**r-TG**	Re-esterified triglycerides
FFA	Free Fatty Acids	**R_f**	Retention factor
HDL	High Density Lipoproteins	**RP-HPLC**	Reverse-Phase High-Performance Liquid Chromatography
HPLC	High-Performance Liquid Chromatography	**RM**	*Rhizomucor miehei*
HLB	Hydrophilic-Lipophilic Balance	**AOT**	Sodium bis(2-ethylhexyl)sulfosuccinate

AOT	Sodium bis(2-ethylhexyl)sulfosuccinate	**UV**	Ultraviolet radiation
sn-	stereospecific numbering	**VLDL**	Very Low Density Lipoprotein
Sc-CO_2	Supercrticial carbon dioxide	**a_w**	Water activity
THF	Tetrahydrofuran	**W/O**	Water-in-oil
TLC	Thin Layer Chromatography	**w/v**	Weight/volume
TAG	Triacylglycerol	**w/w**	Weight/weight
TG	Triglyceride		

Motivation and aims of the thesis

Cardiovascular disease and other problems associated with the cardiovascular system have long been a global medical concern. However, this concern did not extend to ordinary people ever since. In fact, deaths caused by sudden or prolonged cardiovascular complications, are related with heart failure and have been a major cause of death since the beginning of the century. In fact, today, heart disease and cancer are the most common death causes in the world.

Heart complications are established to be related not only with gene predisposition but mainly with environment aspects such as, bad eating habits, lack of exercise, sedentary lifestyle, smoking and other reasons. Therefore, the source to cardiovascular diseases is very broad and intents to decrease it are intense. To do that, is very important to inform population the importance of eating healthy food, as well as practice exercise. These are key aspects regarding a healthy and prolonged life.

In Portugal, meat and fish consumption are side by side and fish consumption culture is well established along the country. Not only because Portuguese people are rooted in the sea fishing for centuries, namely through codfish fishing, but also because Portuguese people consume a lot of fish in their daily diet. However, in other points of the planet, diets tend to be less diversified, which causes nutrient decompensation, leading to the appearance of diet-related diseases, such as cardiovascular diseases. This problem is mostly seen in Western diets countries, where population tend to eat saturated fats. This type of diet increase obesity and as well health-related problems.

Hereupon, omega-3 fatty acids consumption has great importance to have healthier population. Past research showed the importance of omega-3 supplementation in healthy lifestyle. Therefore, through fish oils, the omega-3 consumption market has been growing for some years. Fish oil are more and more omega-3 concentrated to have higher potency in cardiovascular disease prevention. At the same time, omega-3 fatty acids derivatives, such as ethyl esters have been described as having similar properties in prevention of heart malfunction. However, recent studies indicated that despite such properties, the bioavailability of fish oil triglycerides is higher than ethyl esters.

In this way, there is a need to find alternatives to omega-3 fish oil source. To do that, synthesis of omega-3-nicotinol derivatives proven to be suitable to treat and prevent cardiovascular problems, namely, nicotinol esters of main omega-3 fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). To the best of our knowledge, there is only one report so far of synthesis of these nicotinol esters, with good reaction yields. It was possible due to enzyme-catalysed reactions, namely trough lipases, proven to be suitable for carrying out all type of synthesis reactions.

Therefore, the first objective of this work was to synthesized these esters through lipase-catalysed reactions in miniemulsion systems. This is an innovator approach, since it never been reported the use of this reaction media for EPA and DHA-nicotinol esters synthesis through esterification reactions.

The second objective was to optimize reaction conditions through enzyme concentration, type of enzyme and different molar ratios optimization to have high reaction productivities and ester yields.

1. Introduction

1.1 Nutraceutics

The term nutraceutic, also called nutraceutical, was coined from “nutrition” and “pharmaceutical” in 1989 by Stephen DeFelice. Per him, nutraceutical can be defined as “a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease [1]. Since this term has no regulatory definition, Ekta K. Kalra proposed a redefinition of the meaning of functional foods and nutraceutics. Thus, functional food provides the body with the required amounts of all food components needed for its healthy survival. However, when functional food helps in the prevention and/or treatment of diseases it is called nutraceutical [1].

1.2 Nutraceutics use in prevention/treatment of diseases

Nowadays, studies of the use of nutraceutics for prevent and treat diseases have emerged [2]–[9]. In most cases the disease-preventing/treating properties of these compounds are based on rather generic effects, such as antioxidant, anti-inflammatory, anti-ageing and detoxicant [5]. Because of it, some components and molecules are being studied for a nutraceutic use, normally as a dietary supplement, due to its natural properties. In these cases, the use of this components as nutraceutics is already been stablished, and the aim of research is more related to the improvement of the bioavailability of dietary supplements, namely: enhancement of biological stability, improvement of cellular transport and release of the active component inside the cells [2].

Considering oral administration as the most used way of delivering dietary supplements as well as pharmaceuticals, nutraceutics administration is also being done that way. However, alternative ways of administration are being under investigation [2].

Despite widely used, oral administration in nutraceutic intake can be considered a challenge, due to an only small proportion of molecules still available after its ingestion. This is due to an insufficient gastric residence time, low permeability and/or solubility within the gut, as well as instability under conditions encountered in food processing, such as temperature and oxygen. Instability is also experienced in the gastro-intestinal tract, limiting the activity and potential health benefits of nutraceutical molecules [4]. To overcome the disadvantages of nutraceutic administration, different delivery systems are being scrutinized, such as protein hydrogels [4], micro- and nano- particles [4], [6], [7], microemulsions [3], [7] nanoemulsions [7], liposomes and phytosomes [7].

On the other hand, although the nutraceutics source can be varied, bioactive materials from marine sources, namely from fish and shellfish source, like skin, muscle, frame, bone and internal organs are presently used to isolate several bioactive materials. At the same time fish oils from different sources are being used as dietary supplements in the liquid or capsule form, due to the presence of Omega-3 fatty acids [10].

1.3 Fatty acids

Oils and fats are derived from fatty acids (FA) and are used to store energy. Fatty acids are carboxylic acids with hydrocarbon tails from 4 to 36 carbons, with a terminal carboxyl group (-COOH). They can be saturated if they have all single bounds or unsaturated when they have from one to six

double bonds in the chain. Within unsaturated fatty acids there can be monounsaturated fatty acids or polyunsaturated. Unsaturated fatty acids naturally produced have a *cis* configuration in the double bonds. However, *trans* fatty acids are found in dairy products and meats, as well as in vegetal oils because of hydrogenation processes. High consumption of *trans* fatty acids increases the levels of low density lipoproteins (LDL) and reduces the concentration of high density lipoproteins (HDL) [11] (see section **1.3.1.1**).

Unsaturated fatty acids are found in vegetal and fish oils. Fish is rich in polyunsaturated fatty acids (PUFA), namely the omega-3 family with eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA representing the largest proportion in fish [11].

Fatty acids can form lipids that are the triacylglycerols (TAG), or triglycerides (TG). Triacylglycerols consist in three fatty acids, each one with an ester bond to a glycerol molecule. The glycerol has each one of the three hydroxyl groups esterified to the fatty acids, which can be saturated or unsaturated, being the same or different fatty acids in the three positions. These compounds are essential for the formation of more complex lipids which are stored as fats and oils [11]. Once that triacylglycerols can have 3 different fatty acids attached to glycerol backbone, is convenient to distinguish them by their structure position., according to stereospecific numbering (*sn*-). Therefore, figure 1 shows different fatty acid *sn*- position in a triacylglycerol molecule.

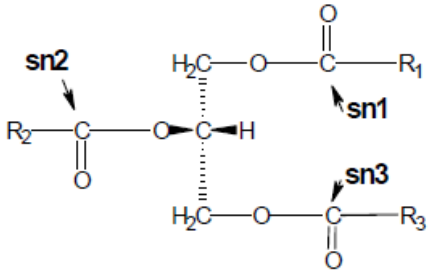


Figure 1. Identification of different ester bonds in triacylglycerol molecule

1.3.1 Omega-3 polyunsaturated fatty acids

Omega-3 is a family of polyunsaturated fatty acids (ω-3 PUFA) which belong to essential fatty acids group. This group is considered as indispensable for human health and are constituted not only of omega-3 but also omega-6 family of fatty acids. Both of these fatty acids families play an important role in brain function, as well as growth, normal development of the organism and synthesis of prostanglandins [11].

However, both omega-3 and omega-6 fatty acids are essential PUFA that cannot be made in the human body. In addition, despite omega-6 family are consumed in Western diets, namely from vegetable oils rich in Linoleic acid (LA), human body lacks the needed enzymes to convert omega-6 into omega-3 fatty acids, making the need of alternative dietary sources for omega-3 fatty acids consumption, such as fish [10].

The most important fatty acids of the Omega-3 family are the α-linolenic acid, ALA, the eicosapentaenoic acid, EPA and the docosahexaenoic acid, DHA. ALA is available from certain plants such as the seeds and oils of flax or linseed, perilla, soybean, and canola as well as walnuts. EPA and

DHA, however, are derived from marine resources, such as blue fish, salmon, tuna, halibut, herring, mackerel, anchovies and sardines [10]. Omega-3 PUFA family are presented in figure 2.

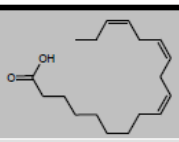
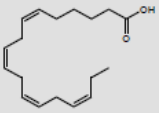
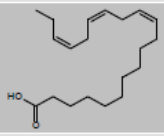
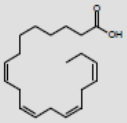
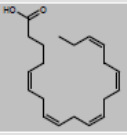
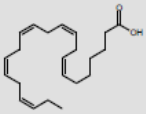
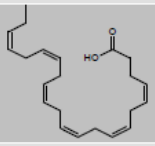
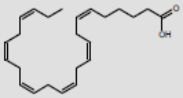
Common name	Abbreviation	Formula	Scientific name
α-linolenic acid ALA	18:3 (n−3)		*cis*-9,12,15-octadecatrienoic acid
Stearidonic acid STD	18:4 (n−3)		*cis*-6,9,12,15-octadecatetraenoic acid
Eicosatrienoic acid ETA	20:3 (n−3)		*cis*-11,14,17-eicosatrienoic acid
Eicosatetraenoic acid	20:4 (n−3)		*cis*-8,11,14,17-eicosatetraenoic acid
Eicosapentaenoic acid EPA	20:5 (n−3)		*cis*-5,8,11,14,17-eicosapentaenoic acid
Docosapentaenoic acid DPA	22:5 (n−3)		*cis*-7,10,13,16,19-docosapentaenoic acid
Docosahexaenoic acid DHA	22:6 (n−3)		*cis*-4,7,10,13,16,19-docosahexaenoic acid
Tetracosahexaenoic acid	24:6 (n−3)		*cis*-6,9,12,15,18,21-tetracosahexaenoic acid

Figure 2. Omega-3 PUFA family, from [11].

Humans can only synthesize up to approximately 5% EPA and DHA through desaturation and elongation from dietary ALA. This pathway is an important source of these long-chain ω-3 PUFA in strict vegetarians, who do not consume fish [10].

1.3.1.1 Nutraceutic applications of ω-3 PUFA

Nowadays, health benefits of ω-3 PUFA consumption, namely EPA and DHA, due to seafood's dietary are well known and had been contributing for promising developments in human nutrition and disease prevention [10]. Omega-3 fatty acids play an important role in healthy brain functions, like memory and performance, being at the same time an important structural component of the grey matter of the brain, eye retina and heart tissue [11]. At the same time, they are known to lower the

plasma triglyceride, very low density lipoprotein (VLDL) -cholesterol and LDL-cholesterol levels and to slowly raise the HDL-cholesterol [11]. By doing that, these fatty acids are very important for prevention and treatment of coronary artery diseases [12], in reducing inflammation and risk factors of diseases like arthritis and other inflammatory diseases [13], cancer [14], [15], hypertension [16], diabetes [17] and autoimmune disorders [18].

Attending to new dietary habits, omega-3 fatty acids consumption is considered as low and because of that, in countries like USA, a traditional Western diet country, guidelines recommending daily intake has been established. Therefore, in USA an intake of 0.65 g/day of DHA and EPA is recommended, which is a fourfold increase of consumption levels of today, while in Great Britain a daily intake of 0.5-1.0 g of ω-3 PUFA is recommended [10].

Despite the health benefits of consuming omega-3 fatty acids, either in marine food or as dietary supplements of purified fish oil, ω-3 PUFA have a storage problem because they are chemically unstable. In fact, fish oils are very susceptible to oxidize, forming a complex chemical soup of lipid peroxides, secondary oxidation products, thus diminishing unoxidized fatty acid concentration. EPA and DHA are highly prone to oxidize due to their large number of double bounds and their position within fatty acid chain. In each fatty acid, between two consecutive double-bonded carbon atoms, there is a carbon atom that have a low activation energy for hydrogen loss and free radical formation. Therefore, in oxidation processes of omega-3 fatty acid, peroxides are formed. As lipid peroxides, ω-3 PUFA have different shape, polarity and reactivity, making them necessarily different, in some cases ineffective, through the same cellular mechanisms when compared with fatty acid form. In agreement with this statement is the evidence that fish oil supplementation with and without the anti-oxidant vitamin E showed a higher triglycerides level decreasing in case of fish oil containing vitamin E. In addition, despite there are insufficient human studies for the consumption of oxidized marine omega-3 fatty acids, animal studies showed that oxidized lipids may cause organ damage, inflammation, carcinogenesis and advanced atherosclerosis [19].

1.3.1.1.1 ω-3 PUFA and cardiovascular disease

Cardiovascular diseases are referred to heart and circulatory system and include ischemic heart disease, non-ischemic myocardial heart disease, hypertensive heart disease and valvular heart disease [10]. Normally, heart diseases are the first cause of death in all world, particularly Western societies. In 2012, cardiovascular diseases killed 17.5 million people, meaning 3 in every 10 deaths [20]. In 2014, heart disease continued to be one of the leading death causes in the world, regardless the society, sex and race [21].

Death by cardiovascular disease has been linked to the high fat intake, particularly saturated fat, common in Western diets. The hallmark of cardiovascular disease is cardiac dysfunction, which in most cases is caused by hypertension due to the narrowing of large arteries or the total occlusion of coronary arteries (thrombus), leading to myocardial tissue necrosis. Both conditions reduce the heart's ability to pump blood and can result in either chronic or sudden heart failure [10].

On the other hand, omega-3 fatty acids induce the production of eicosanoids, compounds that act by making the blood less viscous and reducing clots formation in blood vessels. In particular, their

benefits are related to diminish blood pressure, reduction of cholesterol and triacylglycerol levels as well as prevent arrhythmia [11].

Comparative studies between different populations and their diet showed that populations with high consumption of ω-3 PUFA, normally by high consumption of fish and marine mammals, have less incidence of coronary heart disease and cardiovascular disease [22]–[25] . Despite ω-3 PUFA cardioprotective effects are not well known, it seems to be multifactorial: increasing heart rate variability (antiarrhythmic), reducing atheroma development (antiatherogenic) and decreasing platelet reactivity/aggregation (antithrombotic) [10]. Figure 3 shown omega-3 fatty acid role in cardiovascular protection.

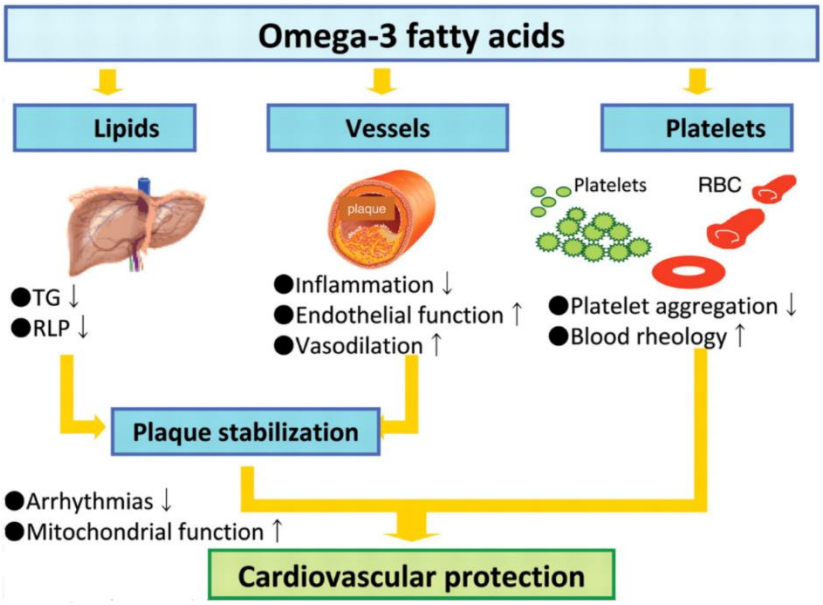


Figure 3. Beneficial effects of omega-3 fatty acids. RLP, remnant lipoproteins (LDL); RBC, red blood cells, from [26].

1.3.1.1.2 ω-3 PUFA and cancer

Cancer, as cardiovascular disease, is one of leading causes of death around the world [20]. Experimental and epidemiological studies have demonstrated that the composition of dietary fat affects the incidence and progression of some cancers. In particularly, omega-3 fatty consumption through seafood diet was classified as the reason of diminution of 70% to 80% in prostate cancer incidence due to anticarcinogenic effects [27]. In addition, other studies clearly showed that omega-3 fatty acids consumption is inversely associated to prostate cancer [28].

At the same time, effects ω-3 PUFA were also related with other types of cancer, such as colon carcinoma. Some studies strongly suggests that omega-3 fatty acids consumption have antitumor effects during early stages of this type of tumour [29], due to an inverse correlation between fish meat and fish oil consumption and colon cancer mortality [30]. In addition, some studies showed that omega-3 fatty acids reduce the proliferation of early-stage colonic cancers, allowing to reduce the progression of colorectal polyps to colorectal carcinoma and thus protect high-risk individuals from colon cancer [31].

1.3.1.1.3 ω-3 PUFA and inflammatory diseases

Omega-3 fatty acids are also known for their anti-inflammatory properties, namely for treatment of arthritis rheumatoid, psoriasis and lupus, as well as chronic inflammatory diseases of gastrointestinal tract [11].

1.3.1.1.4 ω-3 PUFA in mental health and neural functional

Omega-3 fatty acids, namely DHA have also been investigated in terms of human brain development, and in the appearance of neurological and neuropsychiatric disorders. DHA is highly concentrated in the cerebral cortex, and is, in fact, the predominant omega-3 fatty acid in brain [32].

It is well established that an appropriate DHA intake in prenatal stage helps to prevent decreased visual acuity, cerebellar dysfunction and several cognitive impairments as well as neurologic disorders [33]. That is related to an especial importance of DHA intake during pregnancy, since growing neurons need DHA as a prerequisite for formation of synapses in human brain [34]. In addition, since higher brain development starts from third month of pregnancy, a diet with low PUFA levels could result in a delaying myelination (maturation of the brainstem), contributing to sudden infant death syndrome [32]. However, DHA importance is not only in prenatal stage but also postnatally, for example in encephalization, since the greatest brain development occurs until 18 months after birth [10].

Omega-3 PUFA deficiencies are also related to attention deficit, hyperactivity disorder, or neurodevelopment disorders such as dyslexia and autism, depression, aggression and dementia. Recent epidemiological studies suggest that high fish and omega-3 fatty acid consumption prevents cognitive impairment, cognitive decline, and development of dementia or Alzheimer's disease [10] .

At the same time, for depression, psychosis and schizophrenia, studies showed that mental health is not only influenced by genetic predisposition, but also by deficit of nutritional elements, such PUFA from marine fat [32].

1.3.1.1.5 ω-3 PUFA commercialized form

As mentioned, omega-3 fatty acids consumption is healthy and because of that ω-3 PUFA have increase the interest of researchers, namely to concentrate omega-3 from marine oils. Indeed, there are several technologies that allow to increase omega-3 fatty acids content of fish oil: low-temperature crystallization, urea complexation, supercritical fluid extraction, adsorption chromatography, molecular or fractional distillation [35] or enzymatic reactions (esterification, transesterification and hydrolysis) creating conditions to have omega-3 enriched oils [35]–[40], to use as food supplement. In natural fish oils EPA and mostly DHA are present in *sn*-2 positions. The ω-3 PUFA concentration in fish oils through enzymatic hydrolysis is highly due to a higher lipase *sn*-1,3 regioselectivity, which makes the attack preferable to these triacylglycerol positions (see section **1.6.1.2.6.2**), leaving EPA/DHA in *sn*-2 position in triacylglycerol molecule, and thus increasing its content in fish oil sources [11].

Through different omega-3 concentration methodologies, there are five types of fish oil supplements used: natural TG in fish body oil or cod liver oil, ethyl esters (EE), free fatty acids (FFA) and re-esterified TG (r-TG). The r-TG are produced from initial natural TG transferred to ethyl esters and then molecularly distilled to remove the short chain and the saturated fatty acids, thus increasing

the EPA and DHA contents. After that ethyl esters are then enzymatically reconverted to glycerides [41]. Normally, re-esterified TG have triglycerides (55-60%), diglycerides (38–42%) and monoglycerides (1–3%) since European Pharmacopoeia define a ‘‘re-esterified TG’’ with a triglyceride level >50%. During production of r-TG fish oils, the omega-3 fatty acid is randomly attached to any position on the glycerol molecule (*sn*-1, 2, or 3) [41] and the statistical probability is that more omega-3 will attach at sn-1 and/or *sn*-3 than at *sn*-2 [42].

Different comparative bioavailability studies have been made with contradictory conclusions in terms of the best omega-3 supplementation form. The bioavailability measures the fraction of an administered dose of unchanged drug that reaches the systemic circulation, one of the principal pharmacokinetic properties of drugs. Some studies bring EE bioavailability to the same level FFA or TG [43]–[45], although is due to consumption of high-fat meals [41], [46]. However, other studies suggest the opposite, with EE bioavailability lower than FFA and TG [47], [48]. In, fact, other study clearly demonstrated that r-TG has superior bioavailability, whereas EE may have a lower bioavailability. FFA has medium bioavailability, which is not different from that of natural fish oil TG [41].

Fish oil supplementation is thus commercialized either as r-TG or EE, if companies do not do final step of r-TG formation because this additional process increases the cost of the final product. However, since EPA and DHA in the form of EE appear to be more prone to autoxidation, the safety of dietary supplements containing EE needs greater attention. Currently, no distinctions or labelling is required for natural triglycerides vs. re-esterified triglycerides, and companies generally refer to both forms as ‘natural’ [42].

1.4 Nicotinol

Nicotinol is an alcohol also known as nicotinyl alcohol or its IUPAC name 3-Pyridinemethanol and when in human body is rapidly metabolized to nicotinic acid [49]. Nicotinic acid is commonly found in fish, namely tuna, peanuts, mushrooms, chicken or turkey, for example. The pharmaceutical properties of nicotinic acid are extended to nicotinol. Nicotinol is a group-B pro-vitamin that shows to be an efficient agent that allows to raise HDL-cholesterol, on average by 15-30%, reducing of triglycerides by 15-30% and up to 5-10% LDL-cholesterol [50]. Its structural formula is presented in figure 4.

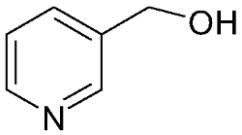


Figure 4. Structural formula of nicotinol.

Nicotinic acid mechanism is essentially related to prevention of free fatty acids release from adipose tissue depots, which is associated with cholesterol lowering levels. By preventing free fatty acids release from adipose tissue, nicotinic acid acts on lipoprotein secretion, inhibiting VLDL synthesis in the liver. Therefore, by decreasing free fatty acids availability in blood stream, we reduce the ability of liver to synthesize triglycerides for subsequent assembly into lipoproteins. Since LDL are

derived from VLDL, because of catabolic processes, decreasing VLDL levels will lead to decreasing in LDL levels as well [51], making favourable the use of nicotinic acid as an antihyperlipidemic drug. In fact, 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 [52]. Nicotinic acid role in cardiovascular prevention is shown in figure 5.

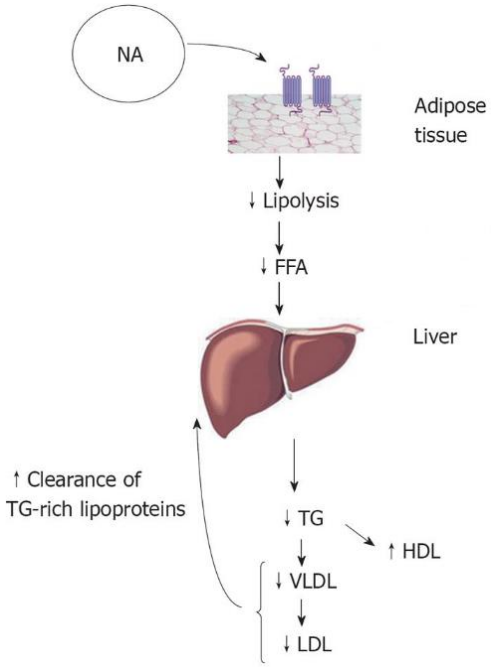


Figure 5. Effect of nicotinic acid (NA) in triglycerides and low density lipoprotein cholesterol decreasing levels, adapted from [53].

At the same time, nicotinic acid is known, in a lower scale, as a low blood pressure agent. In fact, for hypertensive subjects, after nicotinic acid intravenous infusion, they experience significant decreases in systolic as diastolic and mean arterial pressure. This blood pressure decreasing is favourable by vasodilation induced by nicotinic acid [52].

Nicotinic acid can be synthesized in human body from tryptophan and used in nicotinamide adenine dinucleotide (NAD) biosynthesis which is involved in every aspect of cellular metabolism through NAD/NADH or NADP/NADPH-linked systems. However, nutritional studies show that for 1 mg of nicotinic acid equivalent produced 60 mg of tryptophan is needed, making the quantities synthesized by the body lower than the effective therapeutic doses (several weeks with 2-6 g of daily doses) for purposes of lowering cholesterol and lipids [51].

1.5 Promising new DHA-nicotinol drug in heart failure-induced atrial fibrillation prevention

Attending in all aspects mentioned before for the advantages of using omega-3 fatty acids and nicotinol in cardiovascular diseases prevention, it was postulated that DHA-nicotinol ester would present the cumulative properties of the two reactants [11].

At the same time, among all ω-3 PUFA, DHA is suggested to be the most active compound against structural remodelling leading to the development of heart failure, and therefore in the prevention of atrial fibrillation. Atrial fibrillation consists in a quivering or irregular heartbeat (arrhythmia) that can lead to blood clots, stroke, heart failure and other heart-related complications [54].

The use of DHA-nicotinol in animals with heart failure was assessed by Le Grand and co-workers [54]. These authors showed that DHA-nicotinol could prevent the atrial arrhythmias and remodelling in animal models of heart disease. These studies provided insights into the mechanisms by which PUFA benefits may be achieved, including improvements in blood pressure and systemic vascular resistance, autonomic function, cardiac relaxation and filling, ventricular remodelling, arrhythmic risk, coagulation and thrombosis, and inflammation [54]. These results showed that nicotinyl ester of DHA is effectively cleaved and therefore, it releases DHA and nicotinol in blood stream. Thus, following a unique oral administration of DHA-nicotinol, total DHA was increased with a maximum blood concentration 6 h post-dosing whereas DHA-nicotinol was not quantifiable in blood from 0.5 h up to 24 h post-dosing. Therefore, nicotinyl ester of DHA acts as a prodrug of DHA and can be used to increase the incorporation of DHA in myocardial tissues [54].

1.6 Enzyme

Enzymes are natural proteins that can act as catalysts for specific biochemical reactions. They can increase reaction rates 10^6 to 10^{24} fold, being very effective as catalysts. They are called biocatalysts, since they are biological entities capable of catalyse conversion of substrates into products. Enzymes are substrate-specific catalysts with high activity under mild conditions, making them advantageous when compared to conventional catalysts. Most enzymes have clear temperature and pH optima, and their action can often be inhibited or enhanced by certain other compounds or co-factors. Enzymes can be separated and purified for use as processing aids, especially for natural products, or for pharmaceutical applications [55].

1.6.1 Lipases

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, catalysing hydrolysis reactions of long-chain acyl glycerides [56]. They can be distinguished from esterases (E.C. 3.1.1.1) due to interfacial activation phenomenon. The mechanism of interfacial activation is related to active site of lipase. For most known lipases, enzymes become inactive, due to the presence of a lid-like structure that does not allow the accessibility of the active site to the substrate. However, when a lipase is bound to an interface, conformational changes occur causing the lid to open, becoming the active site fully accessible. This exposes the hydrophobic side of the lid to the lipidic phase, which enhances hydrophobic interaction between the enzyme and the lipid substrate [57]. In fact, lipases act at lipid water interface, catalysing both the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids [58].

Lipases are produced by animals, plants, and microorganisms. Microbial sources of lipases are fungal, yeast or bacterial. Lipases of microbial origin represent the most widely used class of enzymes

in biotechnological applications and organic chemistry [59]. The reasons for the enormous biotechnological potential of microbial lipases include the facts that they are (1) stable in organic solvents, (2) do not require cofactors, (3) possess broad substrate specificity and (4) exhibit a high enantioselectivity. Therefore, lipases are now used in varied industrial applications, such as food technology, detergent, chemical industry and biomedical sciences [58].

1.6.1.1 Lipase-catalysed reactions

In nature, lipases catalyse hydrolysis of the ester bond of tri, di- and mono-glycerides into free fatty acids and glycerol. However, when in thermodynamic favourable conditions, such as low water activity, these enzymes also catalyse a variety of synthesis reactions of a broad range of substrates [11]. Esterification and transesterification are the two main types of reactions catalysed by lipases in these conditions. Figure 6 shows hydrolysis and esterification reactions catalysed by lipases.

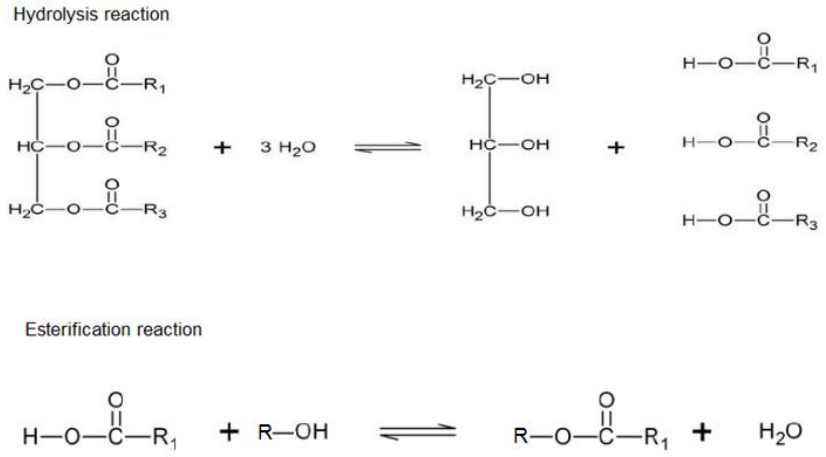


Figure 6. Hydrolysis and esterification reactions catalysed by lipases.

1.6.1.2 Lipase structure and catalytic triad

Although lipases have a very narrow origin, animal or microbial, all lipases are characterized by a common α/β hydrolase fold and a conserved catalytic triad. Most lipases also possess the consensus motif Glycine-X1-Serine-X2-Glycine. From their structures and the residues forming the oxyanion hole (amino acids of the lipase active site that stabilize the reaction intermediate) and catalytic triad, microbial lipases and esterases can be grouped in fifteen superfamilies and thirty-two homologous families [60].

1.6.1.2.1 α/β hydrolase fold

Three-dimensional structure of lipases shows a conserved α/β hydrolase fold, which is composed of a central, parallel β-sheet of eight beta-strands, with only the second strand antiparallel (β2). Strands β3 to β8 are connected by α arranged on the slides of the central β sheet. Some variations of the α / β fold were found in several lipases. The variations of the fold consist in differences in the amount of α helices, β sheets, loops length and architecture of the substrate binding

sites [61], [62]. Lipases have also present disulphide bridges which gives enzyme stability and are often important for their catalytic activity.

1.6.1.2.2 Catalytic triad

Among lipases there is a high conserved catalytic triad, which is consisted in a serine (Ser) as nucleophile, an aspartate/glutamate (Asp/Glu) as acidic residue and a histidine [63]. In the α/β hydrolase fold catalytic serine is located after the sheet β5 and before the following α-helix. The strand-nucleophile arrangement has been known for "nucleophile elbow". It positions the nucleophilic residue free of the active site surface, allowing easy access on one side by the active site histidine (His) residue and on the other by substrate. The sharp turn also optimally positions the nucleophile at the N-terminal end of the following helix, thereby helping to stabilize the tetrahedral intermediate and the ionized form of the nucleophile. The catalytic acid, aspartate or glutamate, is found after the β7 sheet and it is hydrogen-bonded to the active site histidine. The histidine is located in a loop after the β8 sheet [62] [64]. Figure 7 shows α/β hydrolase fold and catalytical triad of lipase.

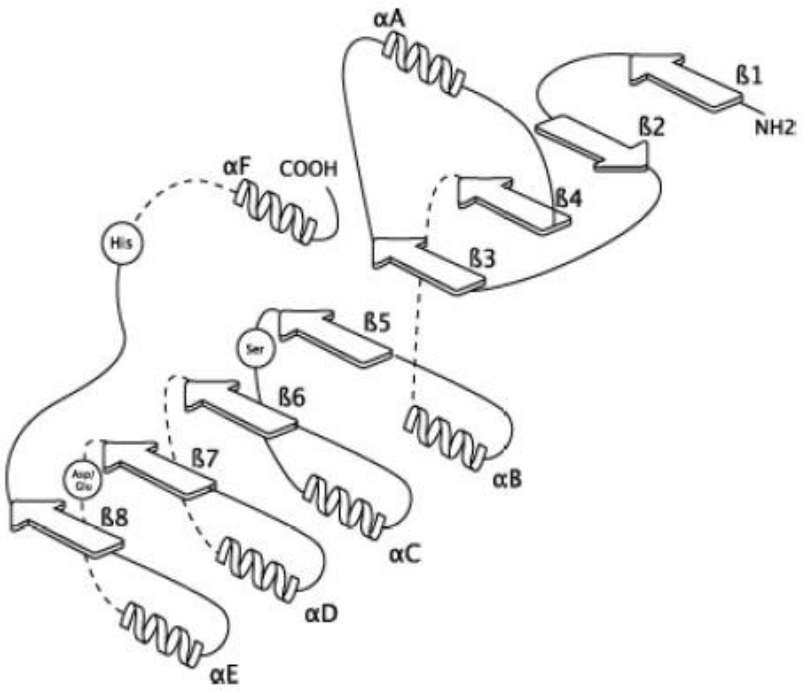


Figure 7. Lipase's α/β hydrolase fold. The spirals represent α-helices and the arrows represent β-strands. The active site residues are shown as circles, from [11].

1.6.1.2.3 The catalytic mechanism

The catalytic mechanism of lipases is formed by acylation step and a subsequent deacylation step. Figure 8 shows the catalytic mechanism of lipases where the numbers represent each of the following steps: the mechanism starts with (1) an activation of hydroxyl group of the catalytic serine by a proton transfer between the aspartate, the histidine and the serine residues of lipase. Hydroxyl residue of serine increases its nucleophilicity and, consequently, attacks the substrate's carbonyl group. (2) It is formed a transient tetrahedral intermediate with O^- in the carbonyl group. This intermediate is stabilized by hydrogen bond interactions with two peptide NH groups, the oxyanion

hole, that allows to stabilize the charge distribution and reduce state energy of the intermediate. After that, the histidine group donates a proton to the leaving alcohol component of substrate. (3) Acyl-enzyme, in which the acid component of the substrate is esterified to the enzyme's serine residue, is formed, resulting this half-mechanism in acylation step. The next stage is deacylation in which a water molecule hydrolyses the acyl enzyme. (4) The active-site histidine activates this water molecule by drawing a proton from it. The resulting hydroxyl ion, OH^-, performs a nucleophilic attack on the carbonyl group of acyl enzyme. (5) Once again, a transient negatively charged tetrahedral intermediate is formed which is stabilized by interactions with the oxyanion hole. After that, histidine residue donates a proton to the oxygen atom of the active serine, the ester bond between serine and acyl component is broken and the acyl product is released. (6) After diffusion of the acyl product, the enzyme is ready for another catalytic round. [11], [62].

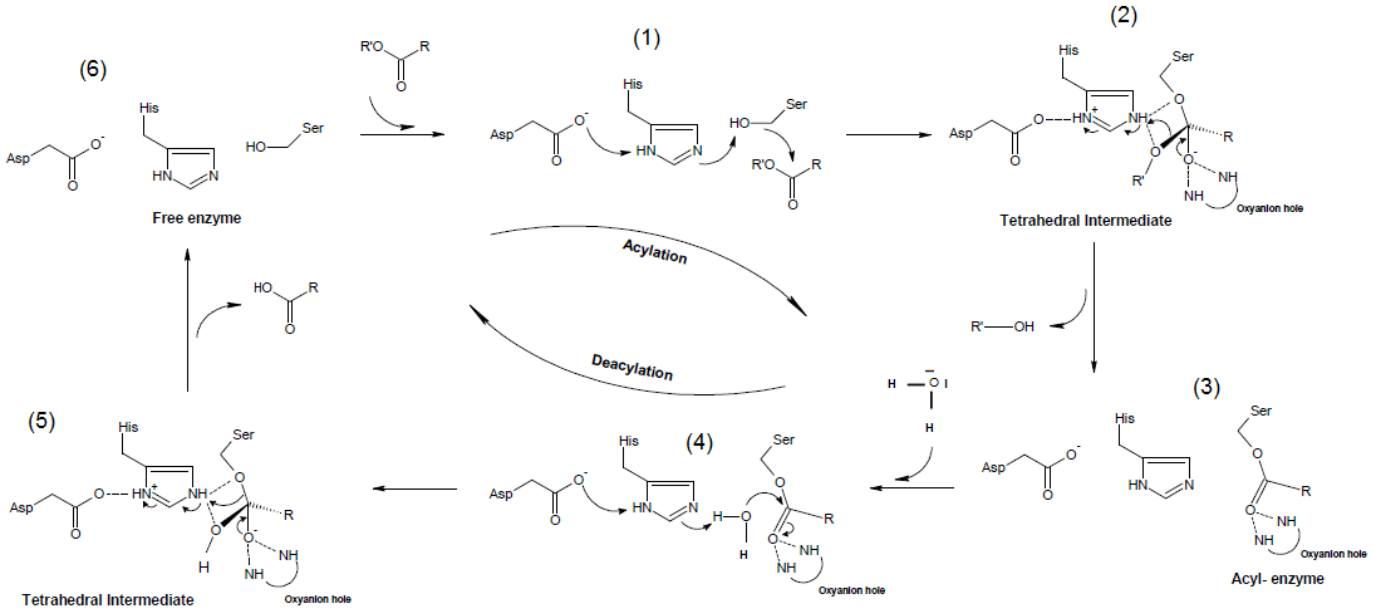


Figure 8. Catalytic mechanism of lipases, adapted from [11] .

1.6.1.2.4 Lipases α-helical loop, lid-like structure in interfacial activation

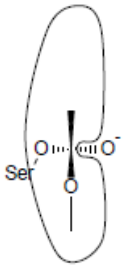
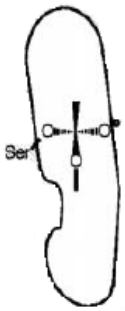
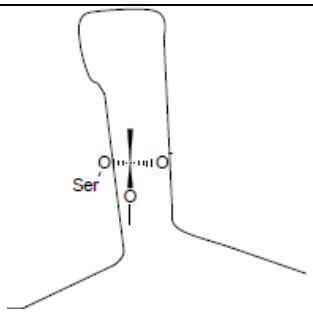
As already mentioned, lipases are characterized for an interfacial activation phenomenon related to a so-called lid over the active site, which is composed of one or two α helices or a loop region. In the absence of a lipid-water interface, the active site of lipases is covered by the lid structure and making the enzyme inactive. However, in the presence of that interface, there are structural rearrangements that allows the lid to open, making possible the access of the substrate to the active site, thus increasing lipases activity [11], [62].

Most lipases have a non Michaelis-Menten behaviour due to interfacial activation process. Indeed, lipase activity increases dramatically when the substrate concentration is high enough to form micelles and emulsions [11], [65] , giving sigmoid curves when the reaction initial rate is plotted against the substrate concentration [11].

1.6.1.2.5 Substrate binding site

The active site of lipases is in the inside of a pocket on the top of the central β sheet of the protein structure. The surface of the pocket's border is mainly consisted in hydrophobic residues to interact with the hydrophobic substrate. The active sites of lipases differ in their shape, size, deepness of the pocket and physicochemical characteristics of their amino acids [61]. Pleiss and co-workers [61] classified lipases in three groups, according to the geometry of their binding site. The first group has a hydrophobic, crevice-like binding site located near the surface of the protein. Lipases from *Rhizomucor*, for example, display such a crevice-like binding site. The second group has a funnel-like binding site. This group includes lipases from *Candida antarctica*, *Pseudomonas cepacia*, today named *Burkholderia cepacia* [58], as well as mammalian pancreas. The last group has a tunnel-like binding site and comprises lipases from *Candida rugosa* [61]. Table 1 shows the three different types of binding site of lipases, depended on its shape.

Table 1. Shape of the three different types of binding site of lipases [61].

Lipase Source	Binding site shape	Top view
Rhizomucor miehei	Crevice-like	
Pseudomonas cepacia *(Burkholderia cepacia)*	Funnel-like	
Candida rugosa	Tunnel like	

1.6.1.2.6 Selectivity

Lipase's selectivity is related to its preference to catalyse some reactions rather than others. There are three types of lipase's selectivity: type-selectivity, regioselectivity and enantioselectivity.

1.6.1.2.6.1 Type-selectivity

Type-selectivity is related to the preference for a given substrate, for example tri, di, or monoglycerides. At the same time, type-selectivity is also associated with the preference of lipases towards short, medium, or long chain fatty acids and to the degree of unsaturation and potential substitutions of the substrate. The preference of a lipase for acyl groups of different sizes is directly influenced by the shape of its binding site, and the nature of the amino acids composing this binding site [11]. In previous work it was shown that very homologous *C. rugosa* lipase isoforms differ in chain length specificity due to slight modifications of the amino acids in their tunnel-shaped binding site [66].

1.6.1.2.6.2 Regioselectivity

Regioselectivity is a type of selectivity related to the preferential attack of lipases towards a given ester bond in the glycerol backbone of triglycerides. Therefore, attending to esters-bond position, regioselectivity can be *sn*-1(3), when lipases attack preferentially these positions or *sn*-2, when is in this position. In previous work, the lipase from *Burkholderia cepacia* was crystallized with triglyceride analogues and was undoubtedly detected four binding pockets for the triglycerides [67]. The binding pockets include the oxyanion hole and three pockets that accommodate the *sn*-1, *sn*-2 and *sn*-3 fatty acid chains. The size and hydrophobicity of these different pockets will control the regioselectivity of lipases [11] .

Most lipases are *sn*-1,3 regiospecific, hydrolysing acylglycerol only in the outer (or external) positions of glycerol. The regiospecificity of lipases on *sn*-1,3 positions was explained by the binding site. Triacylglycerols are docked to the binding site in different orientations. It was shown that binding of the *sn*-1 chain to the scissile fatty acid binding site is preferred for triacylglycerols. The specificity depends much on the *sn*-2 chain. Steric conflicts between the *sn*-2 fatty acids and some amino acids were identified to mainly affect the specificity [68]. Fewer lipases show *sn*-2 regioselectivity like Staphylococcal [69]. There are some non-regiospecific (or random) lipases, which act on acylglycerol in all three positions [68].

1.6.1.2.6.3 Enantioselectivity

Lipase's enantioselectivity relates to the possibility of them to catalyse reactions with the formation of enantiomers. Enantiomers are formed in chiral molecules with an asymmetric centre. This conformation allows to obtain non-superimposable mirror images, which are called R and S enantiomers. These enantiomers share similar chemical properties, such as, melting point, solubility or reactivity. However, they normally have different biological properties. One enantiomer could present therapeutic activity, while the other might be inactive or even toxic [11]. For pharmaceutical properties, the possibility of only produce the therapeutic enantiomer is seeked.

An empirical rule, based on the relative size of the substituents at the stereocentre, was proposed for the reaction of lipases with secondary alcohols [70]. This rule was also relevant to predict

enantioselectivity of lipases for hydrolysis and transesterification reactions, the substrate being an ester or an alcohol, respectively. Unfortunately, rules predicting the enantioselectivity of lipases towards primary alcohols are far less reliable, even though natural substrates of lipases, such as esters of long chain fatty acid with glycerol, are in in this category [11].

1.6.1.2.6.4 Applications

Lipases are widely used in industry due to their stability in organic solvents, their wide variety of substrates, their selectivity and their ability to catalyse reactions without addition of expensive cofactors [11]. In fact, the commercial use of lipases is a billion-dollar business that comprises a wide variety of different applications, namely:

- **Detergents**, as additives since they are active and stable at high temperatures and alkaline pH and in presence of other detergent ingredients as surfactants. Lipases are widely used in detergent formulations to remove fat-containing stains including fried fat, salad oils, butter, fat-based sauces, soups, human sebum, and lipstick. They are also essential in the production of soap, dish washing products, dry cleaning solvents and contact lens cleaning [11], [56].
- **Food industry**, for instance in production of dairy products, namely cheese, modification of human milk fat to increase the proportion of palmitic acid at the *sn*-2 position in triglycerides for better absorption in infants, production of structured lipids, which are modified fats or oils modelled to be more nutritious and suitable for food applications, including omega-3 PUFA enriched fish oils [56].
- **Pharmaceutical industry,** as an advantageous catalyst over chemical synthesis, due to enantio- and regioselectivity, operable mild conditions which avoids isomerization, racemization, epimerization and rearrangement reactions phenomena. The ability of lipases to resolve racemic mixtures by the synthesis of a single enantiomer is currently exploited for drug production by the pharmaceutical industry. In fact, only one enantiomer of a drug is responsible for the desired therapeutic effect, and milder or fewer side effects are observed when using optically pure drug products compared with those found with the use of racemic mixtures [56].
- **Energy industry,** production of biodiesel from renewable sources by transesterification of vegetable oils [71].
- **Pulp and paper industries,** for removing the pitch from pulp produced in the paper industry. ‘Pitch’ is a term used to describe collectively the hydrophobic components of wood, namely triglycerides and waxes, which cause severe problems in pulp and paper manufacture [58].
- **Bioremediation and environmental processes**, for waste-water treatment of oil-enriched residual waters, degradation of organic debris and sewage treatment of abattoirs, food processing industries, the leather industry and the poultry waste processing. At the same time, lipases are also used for biodegradation of petroleum hydrocarbons in oil spills [72].

- **Other fine chemicals**, such as agrochemicals by enantio-pure herbicide production or cosmetics and flavour with lipase-assisted synthesis of enantiomerically pure menthol esters [71].
- **Other applications,** including production of biopolymers such as polyphenols, polysaccharides and polyesters [71], production of disposable enzyme based immunosensors or enzyme-labelled probes. Textile industry also uses lipases for enzymatic wash and jeans treatment [72].

1.7 Enzymatic esterification in different reaction media

Lipase-catalysed esterification for modification of oils and fats [37], [39], as well as ester synthesis has been documented for many years. Different reaction media has been studied for carrying out lipase-catalysed esterification reactions, to overcome yield and thermodynamic constraints. In fact, enzymatic synthesis may be carried out in several reaction media [73].

1.7.1 Aqueous reaction media

Previous work showed enzymatic ester synthesis in aqueous media [74], [75]. Different lipases were tested (*Rhizopus miehei, Rhizopus delemar,* and *C. deformans*) for esterification catalysis of free fatty acids in aqueous media. Janssen et al (1990) reported low esterification yields in enzymatic synthesis of carbohydrate esters in aqueous media. In fact, though water is essential for the preservation of catalytic activity of an enzyme, the specific activity and concordantly, reaction rates, can be reduced at high and low ends of water content. Therefore, the optimal amount of water determined by thermodynamic water activity (a_w) is often within a limited range. In dehydrative reactions such as esterification, control of water activity is essential to avoid side reactions, such as hydrolysis [76].

1.7.2 Water: water-miscible organic solvent (monophasic aqueous–organic system)

Typical systems of water-miscible organic co-solvent and water forms a monophasic solution and is mainly used for the transformation of lipophilic substrates, since its low solubility in water. Normally, addition of small amount (10%) of this water-miscible solvent has little effect on the biocatalyst activity and stability. However, in reactions where hydrophobic interactions are involved in the complex formation between enzyme and substrate, the reduction of the activity of the biocatalyst at a high concentration of water-miscible organic solvent is mainly due to changes in the affinity of the enzyme for the substrate [77]. Therefore, if the proportion of the solvent system exceeds this value, the essential bound water is stripped from the enzyme's surface leading to deactivation in most cases [78].

1.7.3 Organic solvents reaction media (monophasic system)

A typical non-aqueous reaction system is obtained when no water more than the absorptive capacity of reaction medium is added [73]. In fact, some residual water is necessary to maintain catalytic activity [79]. However, unwanted side reactions such as hydrolysis, racemization, polymerization and decomposition often occur in high water activity. Because of that, organic solvent systems with minimum water content, were studied for enzymatic synthesis [80]–[83]. There are some

potential advantages of using this reaction media, normally related to the possibility of having reduced water activity, thus thermodynamically favouring esterification reaction, over hydrolysis. In addition, by using organic solvents, the solubility of hydrophobic organic substrates is increased, improving the volumetric productivity of the reaction. At the same time, due to lower boiling points of organic solvents, recovery processes are simplified [77]. However, there are some disadvantages of using this reaction media, such as denaturation/inhibition of biocatalysts in organic solvents, due to enzyme conformational changes and deformation of active site geometry, which does not allow efficient interaction between enzymes and substrates. The partition coefficient (log P) of the organic solvents is helpful to determine the stability of enzymes in this reaction media [84]. At the same time, by using solvents in industry it 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 minimize, since some of these solvents are toxic and reduce the therapeutical value of active substances because they accelerate decomposition of the product [85]. In fact, only few solvents have low toxic potential or are classified as solvents for which no adequate toxicological data were found. In that case, FDA only approve their use with a justification by manufacturers for residual levels of these solvents in pharmaceutical products [86].

1.7.4 Anhydrous

Anhydrous systems are characterized for having a water content below 0.01%, which highly reduces water activity. Such systems have been studied using a-chymotrypsin and subtilisin, while lipase use in anhydrous media has been limited [73].

1.7.5 Supercritical fluids

Supercritical fluids are materials above their critical temperature and critical pressure having liquid-like densities and gas-like viscosity properties. Although they exhibit similar behaviour of hydrophobic solvents they have absence of toxic issues. In biocatalytic process, the main advantages of using supercritical fluids as reaction media is related to the gas-like viscosity. In fact, it enhances mass transfer rates of reactants to the active sites of enzymes that are dispersed in the supercritical fluid, which can increase bioconversion rate. At the same time, downstream processing might be simpler than in organic solvents [87].

Among the solvents that can be used in supercritical conditions to conduct biocatalytic reactions, the most studied is carbon dioxide, sc-CO_2, because of its low critical temperature. Apart from being non-toxic, readily available and cheap, many native and immobilised enzymes such as lipases, cutinases, subtilisin Carlsberg, and β-D-galactosidase have proven to be stable, active and enantioselective in sc-CO_2 [87]. Some disadvantages are the considerable initial investment for high-pressure equipment and the problem of enzyme deactivation due to the depressurization step [73].

1.7.6 Reverse Micellar systems

Reversed micelles consist of aqueous micro-droplets stabilized by surfactant (AOT or CTAB, for example) in a nonpolar organic solvent (isooctane for example), also known as water-in-oil emulsions (W/O). The enzyme is entrapped in the interior of aqueous phase of reverse micelles and degree of

interaction with the surfactant interface is variable and of significance in the case of lipase [88]. This location is assumed to protect the enzyme from the adverse effects of organic solvent also constituting an easy way to run lipase-catalysed reactions in a dominant organic solvent phase.

Reverse micelles are closed, almost spherical aggregates of surfactant molecules, the outer shell of which is formed by hydrophobic “tails” of surfactant molecules whereas the inner core is composed of polar “heads” of these molecules. Solubilized water is in the inner core of micelle and forms a microdroplet separated from the bulk organic solvent by a layer of surfactant molecule. Depending on the amount of water added, the diameter of the inner aqueous cavity of hydrated reverse micelles is of the order 10–100 Å [73]. This aspect enables to increase the interfacial area, which is of great technological interest because this results in an increase in the number of substrate molecules available to react. In addition, other biocatalytic advantages of using reverse micelles as enzymatic reaction media are: reversed micelles have relatively ordered structure, they form spontaneously reaching equilibrium in short time, solubilisation of both hydrophilic and hydrophobic substrates and products, low reaction volumes are needed, enzyme aggregation is avoided, enzyme’s stability/activity is improved, comparing with organic solvents due to water-oil interface existence, suitable for lipases. The product recovery and enzyme re-use are some disadvantages [88].

However, despite having better biocatalytic properties than organic solvents, reverse micelles 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 [89]. Nevertheless, extended work was made in enzymatic esterification reactions in reverse micelles [90], [91].

1.7.7 Solvent-free system

Several advantages are associated with the solvent-free systems. The absence of solvent 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. Solvent-free system would also permit the use of high substrate concentrations. The solvent-free system could be a reaction mixture solely composed of the substrates (alcohol and acid) in equimolar ratios or alcohol used in large excess [73].

However, problems arise when both substrates are solids and when mass transfer limitations are associated due to the high viscosity of the substrates. The 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 [73]. Despite that, transesterification of omega-3 ethyl esters, such as DHA-EE, showed high transesterification degree in solvent-free system (99% of conversion in 4 hours) [11].

1.7.8 Gas-phase

Lipase-catalysed esterification in solid-gas media has increased over the years due to a varied number of advantages, such as: very high conversion yields compatible with a high production rate for a minimal plant scale, more efficient mass transfer, reduced diffusion limitations due to low gas

viscosity and better stability of enzymes and cofactors. In addition, downstream processing is simplified through the absence of a solvent phase and the scale-up is simpler due to the use of a gaseous circulating phase [87]. Therefore, the optimisation of a solid–gas enzymatic esterification has been transferred successfully to the pre-industrial scale for fragrances and aromas production [92]. However, operation temperatures are very high (60 – 100^{o}C depending on the targeted ester) and regardless almost no loss of activity seen, this is a not omega-3-compatible process, due to temperature-susceptibility of omega-3 for peroxidation.

1.7.9 Ionic liquids

Ionic liquids are salts which have low melting points, thus being liquid over a broad range of temperature and composed only by ions. They possess essentially no vapor pressure, a high ionic conductivity, and a large electrochemical window, and are extremely good solvents for a wide range of organic, inorganic, and polymeric compounds. This solvent system combines the advantages of nonaqueous medium while being moderately polar [73].

Ionic liquids have been introduced into organic chemistry to meet the increasing demand for clean technologies in industrial processes. They are considered green solvents and by using it, organic solvents are avoided. However, despite ionic liquids use in enzymatic-catalysed reactions, even esterification reactions, the effect of ionic liquids in enzyme's activity is still unknown. Rather than overall solvent properties, such as polarity or acidity/basicity, it also depends on the contribution of individual ions, namely anions. The main advantages of using ionic liquids, in comparison with organic solvents are: higher reaction rates, improved enantioselectivity [87],and stability [93]. Although ionic liquids as enzymatic reaction media most used lipase as biocatalyst, proteases also have been studied [87].

1.7.10 Miniemulsions

Emulsion system is a typical aqueous/organic two-phase system composed by a continuous and a dispersed phase. In every emulsion, there is a continuous phase that suspends the droplets in the dispersed phase, using a surfactant. Emulsions can be water-in-oil (W/O, reverse micelles) where the continuous phase is an organic phase and aqueous phase is dispersed in it. On the other hand, in oil-in-water (O/W, normal micelles) the continuous phase is water and the dispersed phase is organic. [89].

The use of reverse micelles for lipase-catalysed esterification was already addressed, as well as its disadvantages, such as organic solvent use. At the same time, since most biological processes and enzymatic reactions take place in water, it would be clearly advantageous to use it. In addition, water use as reaction medium bring some advantages 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 inverse esterification reactions, such as hydrolysis, in aqueous media [94].

Taken that into consideration reverse micelles advantages, in terms of high interfacial area and water presence, enzymatic reactions were investigated to occur at oil-in-water interface. In that case,

the mass transfer is also limited by interface provided, namely interfacial area. Therefore, methods to increase the interfacial area and additionally provide a stable reaction environment are of high interest [94].

Normally, by stirring a two-phase system with a conventional magnetic stirring bar, an emulsion with droplet sizes of several μm is created. However, to have higher interfacial area we need to decrease droplet size, using high shear forces originated by ultrasonication. With that, miniemulsions up to 500 nm can be created [95].

At laboratory scale, ultrasonication is normally chosen to produce miniemulsions, since it is an easy and quick method, leading to low emulsification times. The mechanism of droplet breakage during emulsification using ultrasonication is attributed to intensified mechanical vibrations in the probe, which create pressure waves in the emulsion that leads to the formation of bubbles. Once the bubble reaches a critical size it will implode violently; a phenomenon commonly referred to as cavitation. At this point, a substantial amount of energy is released, consequently generating high shear rates, temperatures, pressures and shockwaves. To prevent degradation of the newly formed droplets, ultrasonication must be done using an ice-water bath [96]. Figure 9 shows a typical ultrasonicator used to making miniemulsions at laboratory scale.

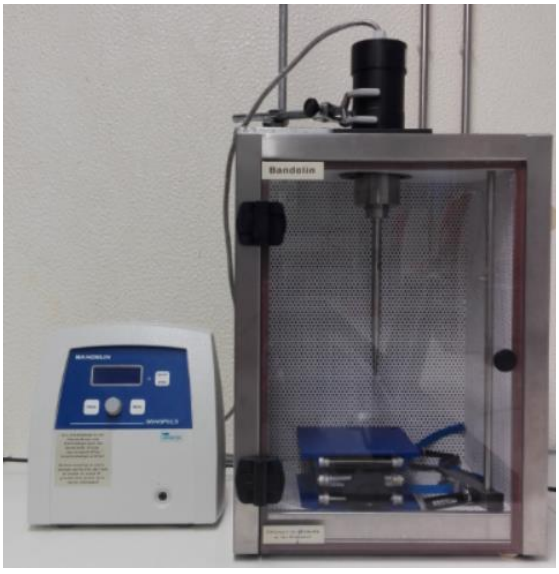


Figure 9. Ultrasonicator SONOPLUS, Bandelin, Germany used in miniemulsion formation.

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, which is essentially water and formed using ultrasonication. Nevertheless, is necessary avoid the spontaneous tendency of such small emulsions to degrade and regenerate the equilibrium state. It can be done by the addition of surfactants, Lutensol AT 50 for example, 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, for example hexadecane [89],[94],[95].

The oil phase is composed of substrates, surfactant and co-stabilizing agent [89]. During emulsification, substrates will remain inside the droplets, while surfactant will be at miniemulsion interface, since it is amphiphilic, allowing oil phase to be dispersed in aqueous phase.

After enzyme's addition, dehydrative esterification reaction starts to occur at miniemulsion interface and the water produced is expelled to the continuous aqueous phase, and thus favouring the product formation [95]. Figure 10 shows all miniemulsion formulation since its production until enzymatic esterification.

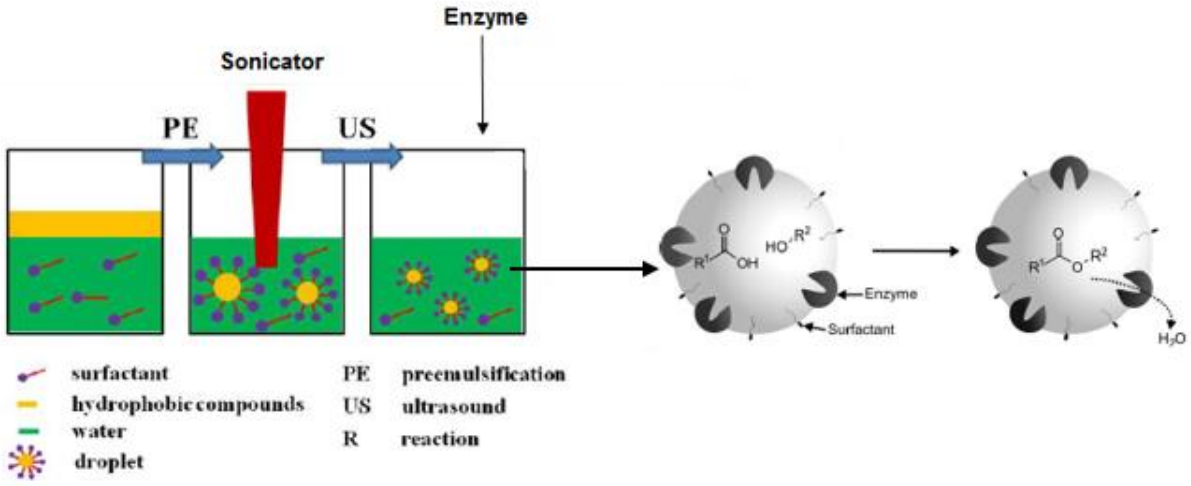


Figure 10. Miniemulsion formulation for carrying out enzymatic esterification, adapted from [97], [98]

Essentially, miniemulsion droplets can be considered as individual enclosed nanoreactors and when both degradation mechanisms are controlled droplets are stable for a long time. This provides constantly enormous interfacial area, that favour formation of esters, improving synthesis efficiency in miniemulsion [94], [95]. Miniemulsion water content %(w/v) influence water activity, however, miniemulsion with 80% w/v have been successfully used in esterification reaction conducted in miniemulsion systems [89], [95], [99].

In previous work, alkyl esters were successfully produced by lipase and cutinase catalysed reactions, using miniemulsion methodology [89], [95], [99]. By that, influence of substrate hydrophobicity in esterification yield, equilibrium reaction time and miniemulsion stability was investigated, not only in terms of fatty acid chain length, but also in terms of lower/higher alcohol hydrophobicity. The authors conclude that more hydrophobic substrates allow to have higher miniemulsion stability over time, and related to that, higher esterification yields. On the other hand, using more hydrophobic substrates enabled to reach equilibrium in less time. At the same time, substrates concentration was evaluated in miniemulsion efficiency in ester synthesis. The authors conclude that higher substrates concentration could inhibit esterification and quickly induce phase separation [95]. In more recent work, miniemulsion esterification was assessed in terms of operability conditions, such as optimal concentration of enzyme and acid/alcohol molar ratio in organic phase (R) [89].

In different work, lipase-catalysed hydrolysis of triglycerides (tricaprylin) in miniemulsion was addressed [100]. The authors concluded that hydrolysis of tricaprylin was not complete after 24h of

reaction. The reason behind that is related with dicaprylin and subsequent monocaprylin synthesis because, being these glycerides less hydrophobic than tricaprylin, it makes tricaprylin interface access restricted. After that, only dicaprylin and monocaprylin hydrolysis occurred, leading to free caprylic acid accumulation. In fact, maximum caprylic acid concentration was reached at the end of the reaction (24h) [100], suggesting that triglycerides hydrolysis in miniemulsion is a good approach for free fatty acids accumulation in reaction vessels, without enzyme deactivation or miniemulsion destabilization.

In figure 11 is shown the reaction sequence since EPA/DHA triglycerides until the formation of EPA/DHA-nicotinol esters. Thereby, in this work the reaction sequence in miniemulsion was: (1) the hydrolysis reaction of fish oil triglycerides with formation of free EPA/DHA; (2) after that, the esterification of free EPA/DHA with nicotinol in nicotinol-esters. This reaction sequence has the first reaction products being substrates of the second reaction.

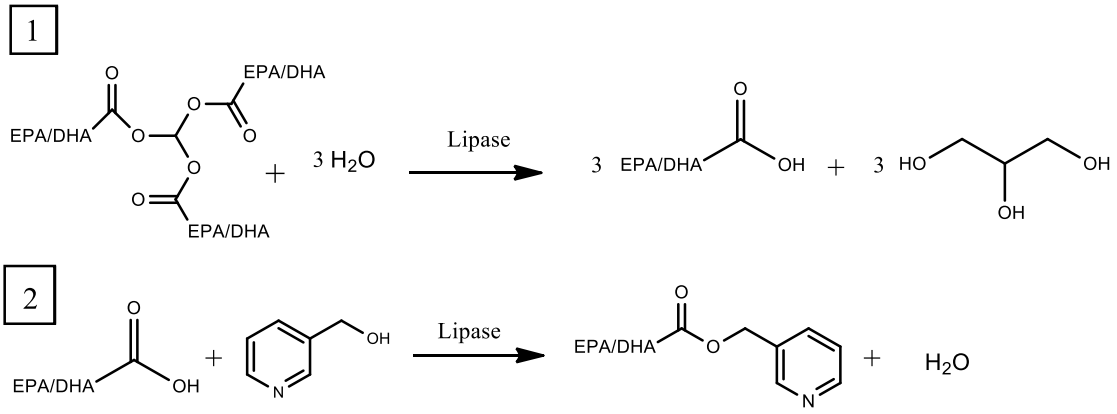


Figure 11. Reaction sequence in miniemulsion. 1) Hydrolysis of EPA/DHA triglycerides in free fatty acids; 2) Esterification of free EPA/DHA in EPA/DHA-nicotinol ester.

1.7.10.1 Influence of surfactant

Surfactants have been used as emulsifiers for miniemulsion enzymatic catalysed reactions [95], [98]–[102]. They are amphipathic molecules because they are composed of a hydrophilic head and a hydrophobic tail (lipophilic), making them soluble in both polar and non-polar solvents. Surfactants, are surface-active agents, which at low concentrations, adsorb and accumulate at emulsion interface, enabling to reduce surface or interfacial tension, by reducing surface or interfacial free energy. By reducing interfacial tension, surfactants reduce the natural tendency of coalescence of the droplets [103]. Influence of surfactants in coalescence avoidance is shown in figure 12.

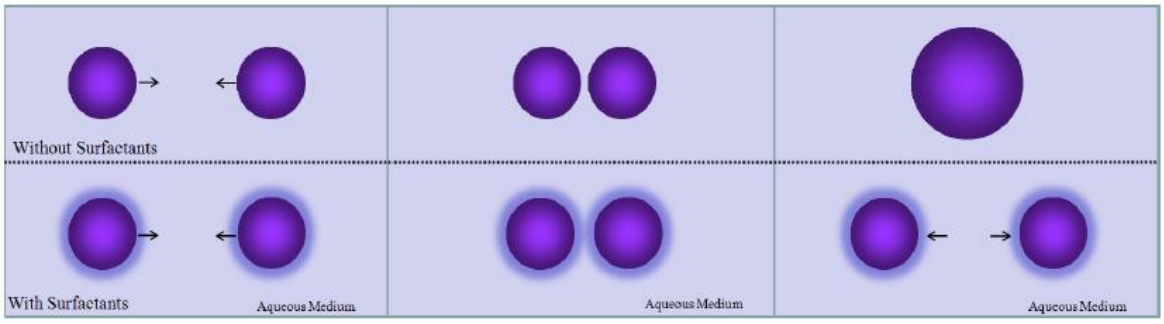


Figure 12. Effect of surfactant addition in droplets coalescence in miniemulsion, from [96].

However, in emulsification process, there is a competing process which consists in emulsifier molecules aggregation, forming micelles. The proportion of aggregated emulsifier molecules remains constant above a certain concentration, known as the critical micellar concentration (CMC). Therefore, to have better emulsification, surfactant concentrations should be higher than CMC value of each surfactant [103].

The relative and effective proportions of the surfactant two moieties are reflected in Hydrophilic-Lipophilic Balance, or HLB value, defined by William Griffin. HLB number of a surfactant is assigned based on the molecular structure of the surfactant. Hereupon, low HLB numbers indicates a more hydrophobic surfactant, pointing to a more suitable W/O emulsifier application. On the other hand, high HLB value indicates a more hydrophilic surfactant, allowing it to be solubilized in water for O/W emulsification [104].

The surfactants can be ionic, non-ionic and amphoteric surfactants. Ionic surfactants are traditionally thought to infer electrostatic stability, whilst non-ionic surfactants are traditionally thought to infer steric repulsion stability [105].

1.7.10.2 Ostwald ripening

In miniemulsion formation, although droplets of very small size are created during ultrasonication step, droplets particle size distribution is very narrow. Therefore, in addition to coalescence, diffusional degradation of oil droplets leads to Ostwald ripening phenomenon [94].

Ostwald ripening occurs because spherical droplets of radius r and surface tension γ experiences a pressure P exceeding that in the external medium according to the Laplace pressure equation (Eq.1) [106].

$$P = \frac{2\gamma}{r} \qquad \text{Eq. 1}$$

Since droplets has unstable curved surfaces, is possible to see at equation 1 that smaller (more curved) droplets will have higher pressure inside, creating a Laplace pressure gradient from droplets inside to the surrounding phase [94]. According to this, pressure gradient will be higher for smaller droplets than larger ones. The unstable surface molecules often go into solution, shrinking the smaller droplets over time and increasing the larger ones by transferring their mass into them. Thus, small particles decrease in size until they disappear and large particles grow even larger. This shrinking and growing of particles will result in a larger mean diameter of a particle size distribution [107]. Ostwald ripening is an oil-in-water miniemulsions concern because it could lead to miniemulsion instability and eventually phase separation. Ostwald ripening phenomenon is shown in figure 13.

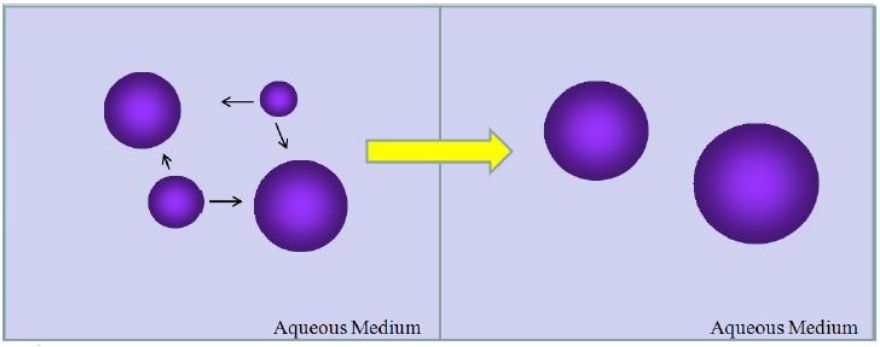


Figure 13. Ostwald ripening effect in two-phase system, from [96].

The Laplace pressure, that induce Ostwald Ripening, can be counteracted by creating an osmotic pressure inside the droplets with the addition of small amounts of a hydrophobic component, for example hexadecane. Hexadecane will act as a co-stabilizing agent since it cannot diffuse from one droplet to the other, being thus trapped inside the droplets. However, hexadecane addition does not completely block droplet growth, because of a still finite solubility, existence of droplets collisions, and surfactant-assisted transport, despite a quite slowdown in this natural process. Therefore, since the final state is obtained by osmotic pressure vs Laplace pressure balance and after miniemulsion formation, osmotic pressure is larger, miniemulsion droplet sizes tend to increase in a timescale of days to weeks. Due to this particularity, droplets growth normally have no big influence in miniemulsion synthesis [101].

1.7.10.3 Miniemulsion characterization

Dynamic Light Scattering (DLS) is a technique used to determine the mean particle size and the width of the particle size distribution, expressed as polydispersity index (PDI). The particle size is the diameter of the sphere that diffuses at the same speed as the particle being measured.

The measurement using DLS is based on the light scattering phenomena in which the statistical intensity fluctuations of the scattered light from the particles in the measuring cell are measured and after related to particle size. The intensity fluctuations are due to the random movement of the particles in the dispersion medium, called Brownian motion.

Usually a DLS device consists of a laser light which illuminates a small volume of the sample composed by a dilute suspension of particles. The light scattered from these particles is collected by a lens and its intensity is measured by a detector at a certain angle (90º or 173º). The diffusion rate of the particles depends on their size, at a known fluid viscosity and temperature. Hence, the size of these particles can be calculated from the rate of fluctuation of the scattered light intensity. When the suspended particles are small, they diffuse relatively fast, and the fluctuations in the scattered light are rapid. On the other hand, if the particles are large, they move slowly, and the fluctuations in the scattered light are slow. The detected intensity signals are used by a correlator to calculate the auto-correlation function $G(\tau)$. A correlator is designed to measure the degree of similarity between two signals, or one signal with itself at varying time intervals, being thus essentially a signal comparator. From the decay of correlation function, the diffusion coefficient (D) of the particles is obtained. Once

the diffusion coefficient is known, the hydrodynamic diameter of a spherical particle can be calculated applying the Stokes-Einstein equation (Eq.2),

$$d(H) = \frac{KT}{3\pi\eta D} \qquad \text{Eq.2}$$

where, $d(H)$ is the hydrodynamic diameter, D is translational diffusion coefficient which measures the velocity of the Brownian motion, k is the Boltzmann's constant, T is the absolute temperature and η is the viscosity of the solution.

As mentioned, small particles diffuse faster than large ones, causing a stronger fluctuation in the scattering signal and a more rapid decaying G(τ) (Figure 14). For a monodisperse particle population, G(τ) is a single exponential, but if more than one size of particles is present the function is polyexponential. Deviation from a single exponential is used to calculate the PDI, which is a measure of the width of the size distribution. The PDI value is zero when a monodisperse particles population is measured. PDI values of around 0.10-0.30 indicate a relatively narrow distribution, values of 0.5 and higher are obtained in case of very broad distributions [108].

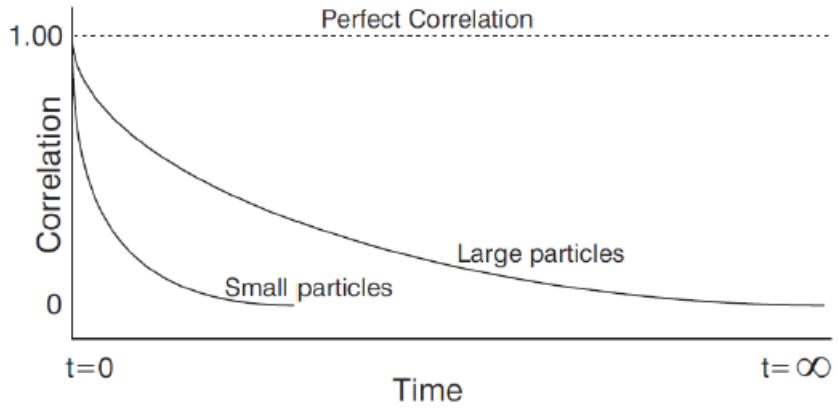


Figure 14. Correlation function for large and small particles, from [108].

2. Materials and methods

2.1 Materials

2.1.1 Miniemulsion preparation

2.1.1.1 Lutensol AT 50

The surfactant used in miniemulsions preparation was Lutensol AT 50, a donation of BASF, in form of powder. This is a non-ionic surfactant which is part of a group of surfactants denominated Lutensol AT. These surfactants are manufactured by causing the fatty alcohol to react with ethylene oxide in stoichiometric proportions. BASF ensure a high-performance product with low toxicity by using highly pure feedstocks, in combination with ethoxylation temperature kept as low as possible. In particular, Lutensol AT 50 is a poly(ethylene oxide)-hexadecyl ether with an ethylene-oxide block length of 50 units. Its chemical formula is $RO(CH_2CH_2O)_{50}H$, where R is a linear, saturated $C_{16}C_{18}$ fatty alcohol.

This surfactant, like others Lutensol AT, is colourless or slightly yellowish. It is soluble in water, ethanol or aromatic hydrocarbons. The choice of this surfactant is because it is a very effective emulsifier for hydrophobic substances, such as fatty acids, ester-type waxes, monomers for emulsion polymerization and polar solvents, because these ones require surfactants with a high HLB. Lutensol AT 50 HLB value is 18 [109], [110].

2.1.1.2 Hexadecane

Hexadecane minimum 99% was purchased from Sigma-Aldrich and it was used as a hydrophobic agent for osmotic stabilization of the miniemulsions. Hexadecane is a saturated alkane hydrocarbon, in this case, in straight chain (n-hexadecane) with molecular formula $C_{16}H_{34}$ and molecular weight 226.4 g mol^{-1}. Its melting point is 18 ^oC, while its boiling point is 287 ^oC, which means that it is liquid at room temperature (~25 °C) with a density of 0.733 g ml^{-1} [111]. Its structure is shown in figure 15.

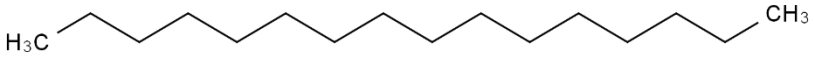


Figure 15. Structural formula of hexadecane.

2.1.1.3 Water

The water used in all experiments was purified water by reverse osmosis and was obtained from a Milli-Q Plus, Millipore system.

2.1.1.4 Nicotinol

Nicotinol, 98% was purchased from Alfa Aesar and was used in the esterification of the fatty acids in miniemulsion. Nicotinol is an alcohol analogue of nicotinic acid with molecular formula C_6H_7O and molecular weight 109.13 g mol^{-1}. It is liquid at room temperature, since its melting point is -8 to -6 ^oC, and the melting point is 154^oC. Nicotinol has a density of 1.130 g ml^{-1}, it is fully miscible in water

also have a refractive index of 1.5450 [112]. For this work, maximum solubility of nicotinol in water was determined as 0.1558 g ml^{-1}.

2.1.1.5 Omega Balance fish oil

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. It is produced by PureMax™ using a proprietary process that ensures high quality selective concentrates of EPA and/or DHA, purity and potency of fish oils that are obtained responsibly. PureMax™ brand is a technology of purification and concentration of Omega 3 developed in years of research and experience in product development and manufacturing. The PureMax™ processes ensure the best quality of fish oils by removing environmental pollutants (such as heavy metals, dioxins, pesticides and persistent polychlorinated biphenyls (PCBs), that tend to accumulate in fish up the food chain), to levels or below levels recommended by the authorities as the European Community and the World Organization for EPA and DHA [113].

The content of fatty acids in the sample was determined by gas chromatography according to de Carvalho, 2012 [114]. 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. Fish oil composition, as well as molecular weight of each pure component, is shown in 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}.

2.1.2 Lipases characterization

2.1.2.1 Commercial Lipases

Different lipases were used to catalyse enzymatic esterification of omega 3 polyunsaturated fatty acids in a miniemulsion system: Amano Lipase PS (pH=7,0, T=50ºC) from *Burkholderia cepacia*; Lipase, *from Rhizomucor miehei*; Lipase from *Candida sp* (CAL A) recombinant, expressed in *Aspergillus oryzae*; Lipase type VII from *Candida rugosa*. All lipases used were purchased from Sigma-Aldrich.

2.1.2.2 Compounds used in the lipases activity assay

In order to measure the activity of the lipases used as biocatalyst, it was used as substrate a mixture composed of *para*-nitrophenyl palmitate (p-NPP, Sigma-Aldrich], Propanol (Merck), Triton® X-100 (Merck), Arabic gum and a Tris/HCl buffer solution prepared in the lab.

2.1.3 HPLC

2.1.3.1 cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA)

Docosahexaenoic acid is an omega-3 polyunsaturated fatty acid and was purchased from Acros Organic (98%) to be used as external standard for HPLC analysis. DHA has a molecular formula $C_{22}H_{32}O_2$ with six double bonds and a molecular weight of 328.49 g mol^{-1}. It has a density of 0.95 g ml^{-1} and is a clear colourless liquid yellow oil, sensitive to light, heat and oxygen concentration, since it can peroxidise very quickly [115] [19]. DHA has a refractive index of 1.50 – 1.51 [116].

2.1.3.2 cis-5,8,11,14,17-Eicosapentaenoic acid (EPA)

Eicosapentaenoic acid is an omega-3 polyunsaturated fatty acid and was purchased from Sigma-Aldrich (purity >98.5%) to be used as external standard for HPLC analysis. EPA has a molecular formula $C_{20}H_{30}O_2$ with five double bonds and a molecular weight 302.45 g mol^{-1}. It has a density of 0.943 g m^{-1} at 25ºC. As well as DHA, EPA is a liquid oil with light, heat and oxygen concentration sensitivity. EPA has a refractive index of 1,4977 [19], [117] .

2.1.3.3 Tetrahydrofuran (THF)

It was decided to use inhibitor-free THF that was purchased from Sigma-Aldrich, 99%. Tetrahydrofuran is an organic compound and it was used to dissolve reaction samples analysed by HPLC. It has a molecular formula C_4H_8O and a molecular weight 72.11 g mol^{-1}. Since it has a melting point of -108ºC and a boiling point of 65-67ºC, it is liquid at room temperature. It is soluble in almost all organic solvents and in water, as well. THF has an UV cut-off wavelength of 220 nm [118], [119] and its structure is shown in figure 16.

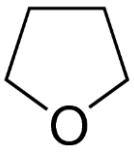


Figure 16. Structural formula of THF.

2.2 Methods

2.2.1 Miniemulsion preparation

Miniemulsion (ME) systems were studied with 80% (w/v) amount of water and prepared with a relative amount of the compounds shown in table 3.

Table 3. 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 3 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. It was studied the influence of adding the hexadecane in aqueous phase vs organic phase but no relevant differences were observed. Lutensol was quickly solubilized in water with a magnetic 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.

2.2.2 Miniemulsion characterization

In this work, a Zetasizer Nano ZS, Malvern Instruments (Malvern, UK) was used to measure the miniemulsion droplets size produced. Particle size measurements were made in glass cells at 25°C, by DLS under a scattering angle of 173° at a wavelength of 633 nm. The effective hydrodynamic diameter (Z-average) was calculated from the diffusion coefficient by the Stokes-Einstein equation (Eq. 2). It was obtained the PDI of each sample that was analysed. All measurements were performed in triplicates with 12 runs by measurement.

2.2.3 Lipases characterization

2.2.3.1 Lipases activity assay

The hydrolytic activity of different lipases was assayed using a spectrophotometric method (Hitachi U-2000 Spectrometer) based on monitoring the hydrolysis of *para*-nitrophenyl palmitate (p-NPP) to *para*-nitrophenol (p-NP), a yellow compound, at 400 nm (ε = 2710 $M^{-1}cm^{-1}$) at 30^{o}C for 1 minute. For that purpose, 90 µl of a solution 7.95 mM of p-nPP in propanol was added to a glass

cuvette containing 890 µl of 50 mM Tris.HCl with pH 8, 7.11 mM Triton® X-100 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 [120].

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.

2.2.3.2 Protein concentration assay

Even though all enzymes used in esterification studies were commercial preparations, it was necessary to determine their protein concentration.

The protein concentration was determined using the Pierce BCA Protein Kit (Thermofisher Scientific) with reference to Bovine Serum Albumin (BSA) as standard solution for the calibration curve. Thus, the calibration curve was obtained using the standards dissolved in Milli-Q water, obtaining standards with different concentrations of BSA.

The absorbance was measured in a 96-well-microplate (Greiner 96 U-Bottom) and analysed in a microplate reader (Molecular Devices, Spectra MAX 340$_{PC}$) at 562 nm. Each well in microplate consisted of 25 µl of sample or standards and 200 µl BCA-Reagent that were incubated for 30 minutes at 37ºC. Figure 17 shows the calibration curve obtained.

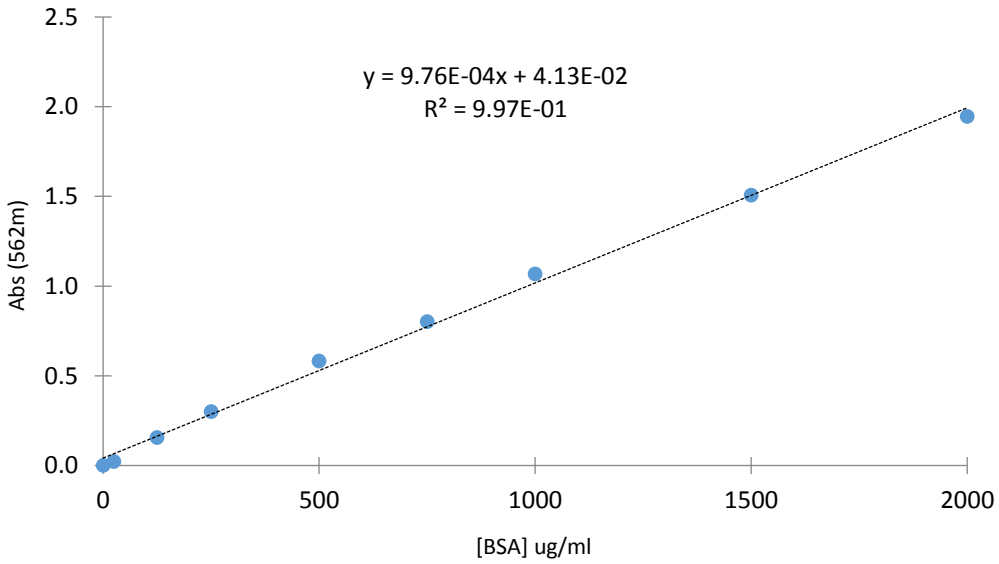


Figure 17. Calibration curve for BSA standards in Milli-Q water.

2.2.3.3 Lipases specific activity determination

The specific activity (U mg^{-1}) of each lipase preparation was determined as the ratio between hydrolytic activity and protein concentration, previously determined and is shown in table 4.

Table 4. Specific activity (U mg^{-1}) of the different lipases used

Lipase	Specific Activity (U mg^{-1})
Amano PS	3238
Rhizomucor miehei	25
CAL A	193
Candida rugosa	30

2.2.4. 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 **2.2.6.2.1**

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 (see **Appendix A**).

Ester DHA-nicotinol: Starting with DHA (0.057 mL), compound DHA-nicotinol was obtained as a pale-yellow oil (0.061 g, 88%). R_f (hexane/diethyl ether (3:1)): 0.31. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 172.7, 149.4, 149.3, 136.2, 132.0, 131.8, 129.5, 128.6, 128.31, 128.27, 128.2, 128.1, 128.1, 128.0, 127.9, 127.7, 127.0, 123.4, 63.6, 34.1, 25.63, 25.58, 25.5, 22.7, 20.5, 14.2 ppm (see **Appendix A**)

2.2.5 Enzymatic reactions in miniemulsion systems

Enzymatic 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 3, and added to reaction vessel containing the appropriate amount of enzyme. The 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 [121], 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: Amano lipase PS from *Burkholderia cepacia* (Amano PS)**; l**ipase from *Rhizomucor miehei* (RM); *Candida antarctica* A lipase recombinant from *Aspergillus oryzae* (CAL A); lipase type VII from *Candida rugosa* (CR). 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 (U mg^{-1}) presented in table 4, 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 [121]–[124].

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.

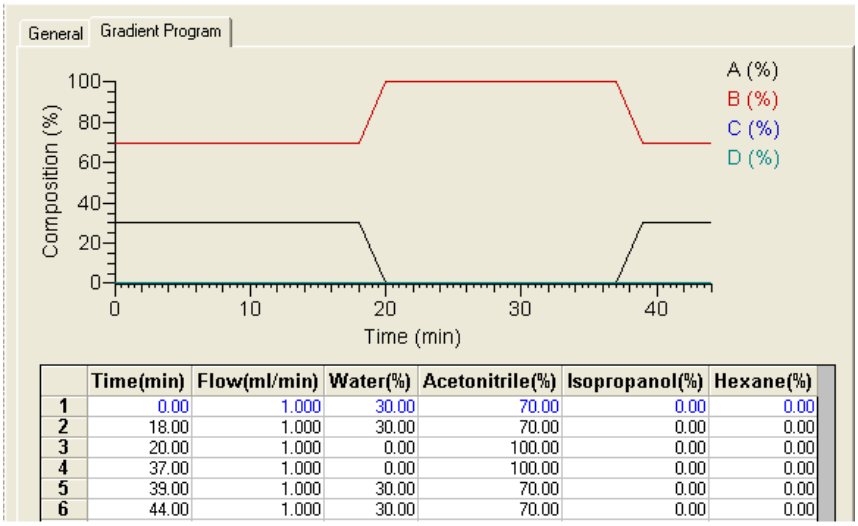
d) Pure EPA/DHA vs Omega Balance fish oil:

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.

2.2.6 HPLC

2.2.6.1 Determination of the reaction yields 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). The proportion of these two mobile phases varied with time according figure 18.



	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 18. 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 3) with the formation of free EPA and DHA, and of esterification yields (equation 4), 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.

$$\eta_{hydrolysis}(\%) = \frac{mol\ free\ fatty\ acid}{mol\ total\ fatty\ acid_{initial}} \times 100 \qquad \text{Eq. 3}$$

$$\eta_{esterification}(\%) = \frac{mol\ ester}{mol\ total\ fatty\ acid_{initial}} \times 100 \qquad \text{Eq. 4}$$

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.

At the same time, the % mol of EPA and DHA species determined in section **3.2.1.3** was determined by equation 5.

$$\%\ mol_{fatty\ acid\ specie} = \frac{mol\ specie}{mol\ total\ acid} \times 100 \qquad \text{Eq. 5}$$

2.2.6.2 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.

2.2.6.2.1. Detection, isolation and purification of reaction products

Thin layer chromatography (TLC) was applied in the analysis of reaction mixtures (see section **2.2.4**) 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).

2.2.6.2.2. 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 (^{1}H 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) (see section **2.2.4)**.

2.2.6.3 Calibration curves

HPLC standards of EPA, DHA, EPA-nicotinol and DHA-nicotinol were made to obtain calibration curves allowing to measure their concentration at a certain moment of the reaction. Thus, figures 19-28 shows calibration curves used in this work.

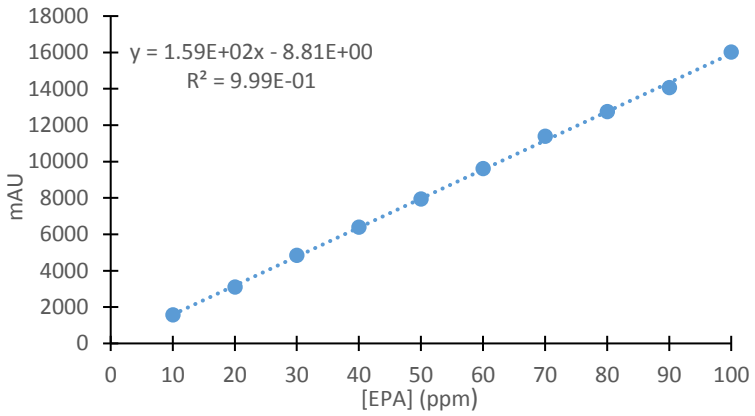


Figure 19. Calibration curve of EPA 10-100 ppm.

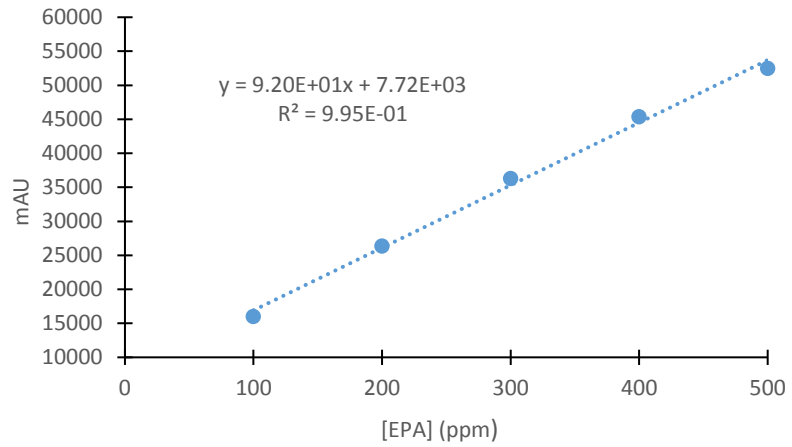


Figure 20. Calibration curve of EPA 100-500 ppm.

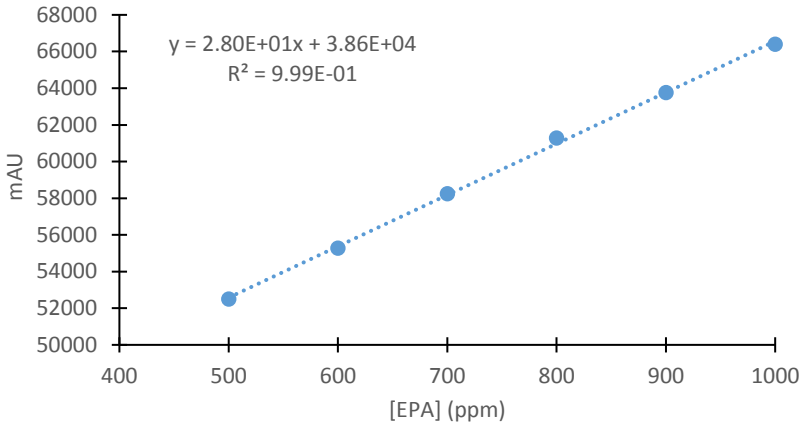


Figure 21. Calibration Curve of EPA 500-1000 ppm.

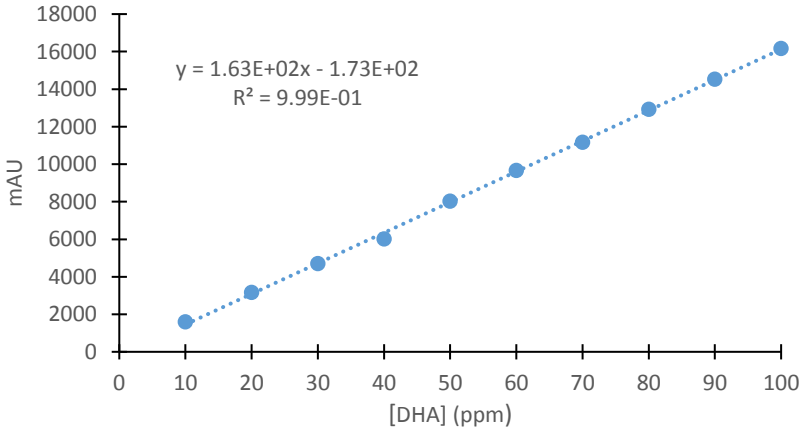


Figure 22. Calibration Curve of DHA 10-100 ppm.

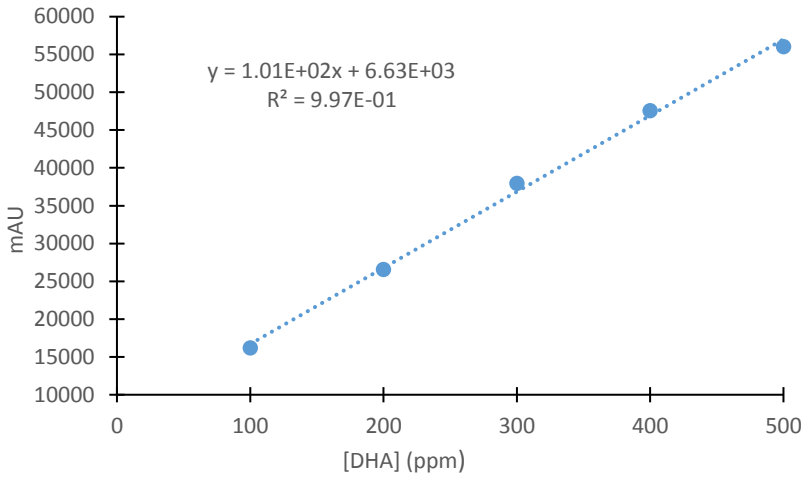


Figure 23. Calibration curve of DHA 100-500 ppm.

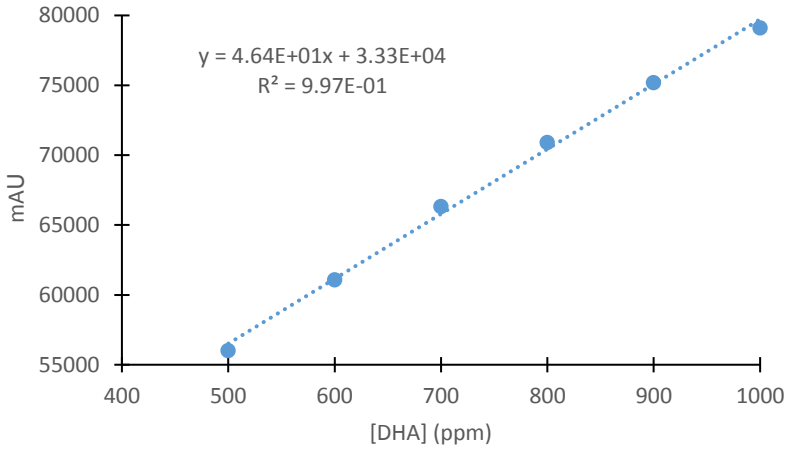


Figure 24. Calibration curve of DHA 500-1000 ppm.

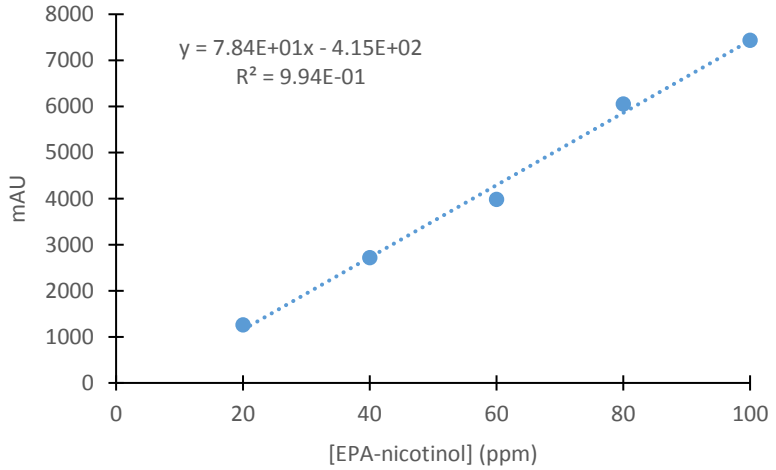


Figure 25. Calibration curve of EPA-nicotinol 20-100 ppm

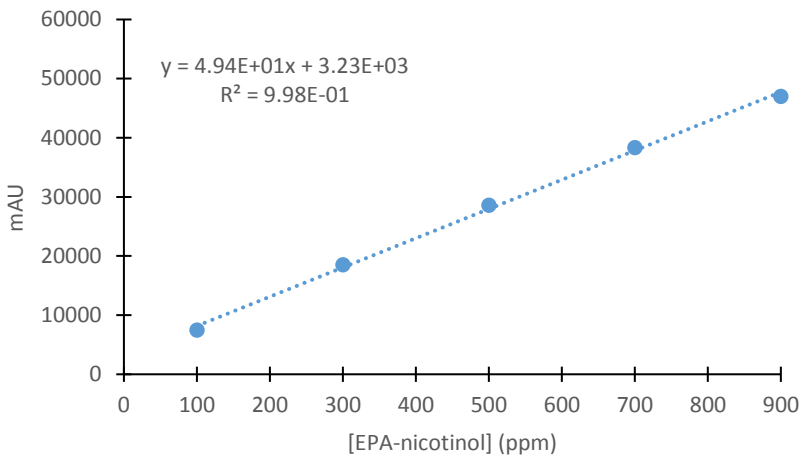


Figure 26. Calibration curve of EPA-nicotinol 100-900 ppm.

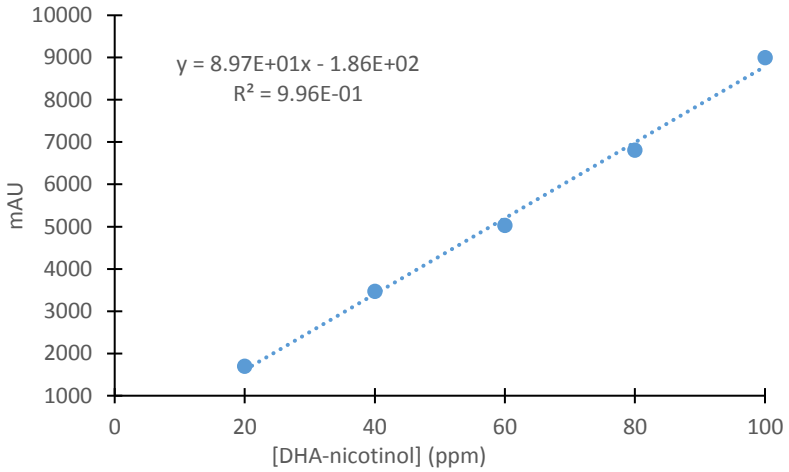


Figure 27. Calibration curve of DHA-nicotinol 20-100 ppm.

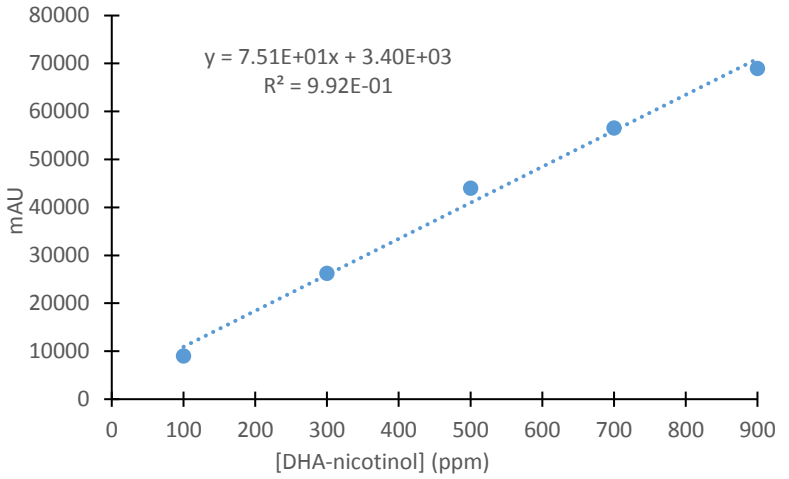


Figure 28. Calibration curve of DHA-nicotinol 100-900 ppm.

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, 45 W and 55 W were tested in a sonication time of 5 min. Not only Z-average was analysed but also PDI as presented in figure 29.

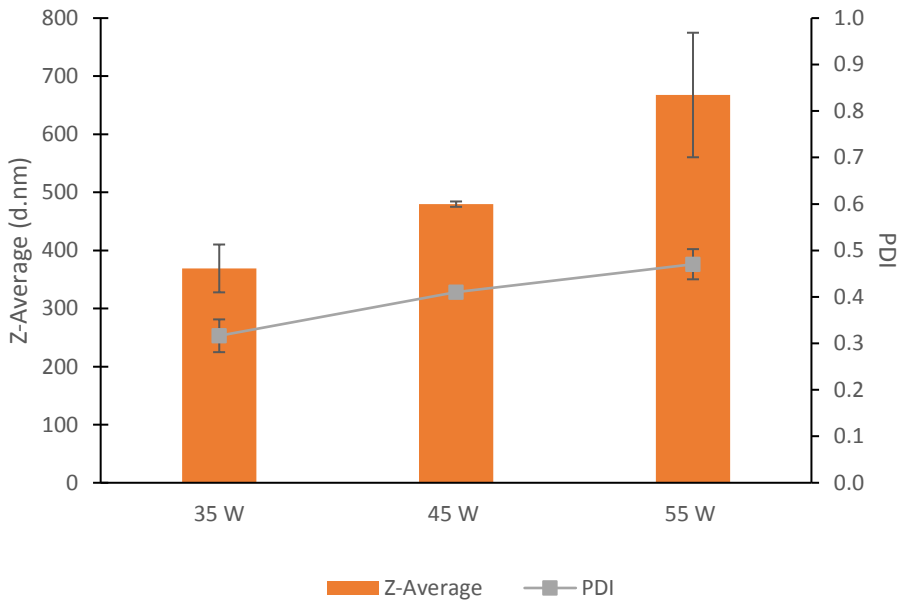


Figure 29. Z-Average (d.nm) and PDI using input powers of 35 W, 45 W and 55 W during 5 min of sonication with a 5s on/10s off pulse.

In figure 29 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.

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 3) and using the best ultrasonication conditions, such as 35 W, 5 min, with a 5s on/10s off pulse, as shown in figure 30.

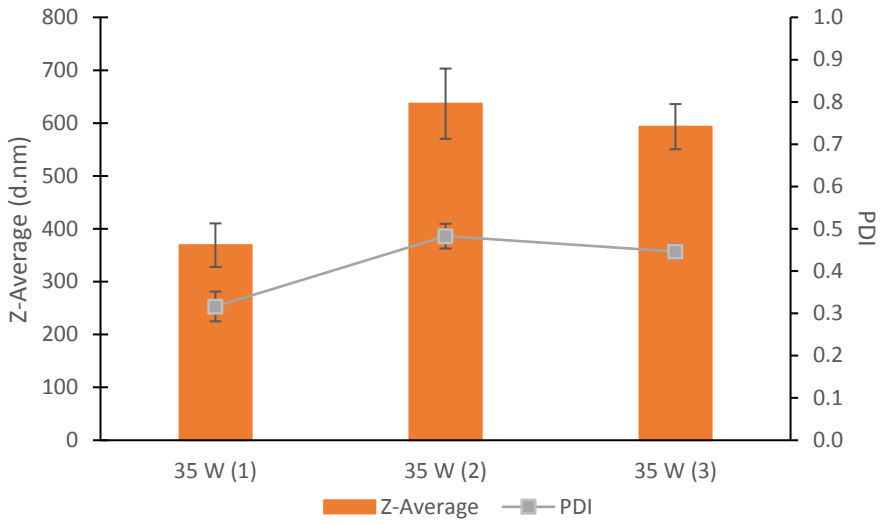


Figure 30. Z-Average (d.nm) and PDI using input power of 35 W during 5 min of sonication with a 5s on/10s off pulse in consecutive days .

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 [125].

At the same time, Z-Average obtained for sample 35 W (1) presented 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 demonstrated 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 fixed at 5 min with a 5s on/10s off pulse. Results are presented in figure 31.

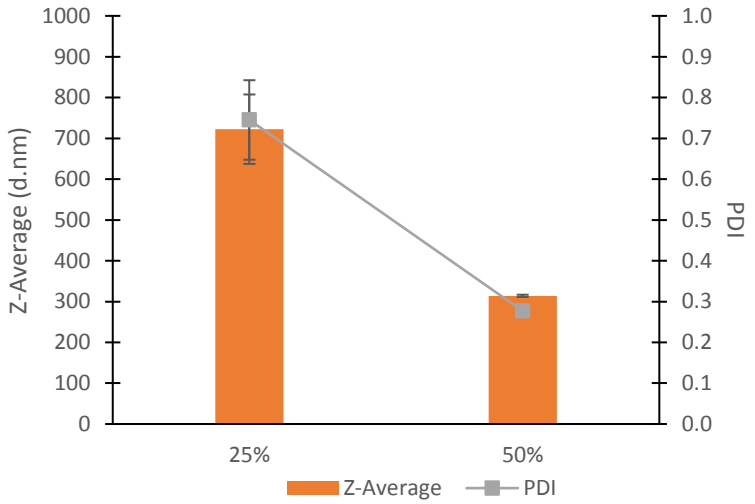


Figure 31. Z-Average (d.nm) and PDI using amplitude of 25% and 50%, during 5 min of sonication with a 5s on/10s off pulse.

In figure 31, 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 [108].

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. Since 50% amplitude had undoubtedly shown the better results in terms of Z-Average and PDI, it was only studied this condition. Results are shown in figure 32.

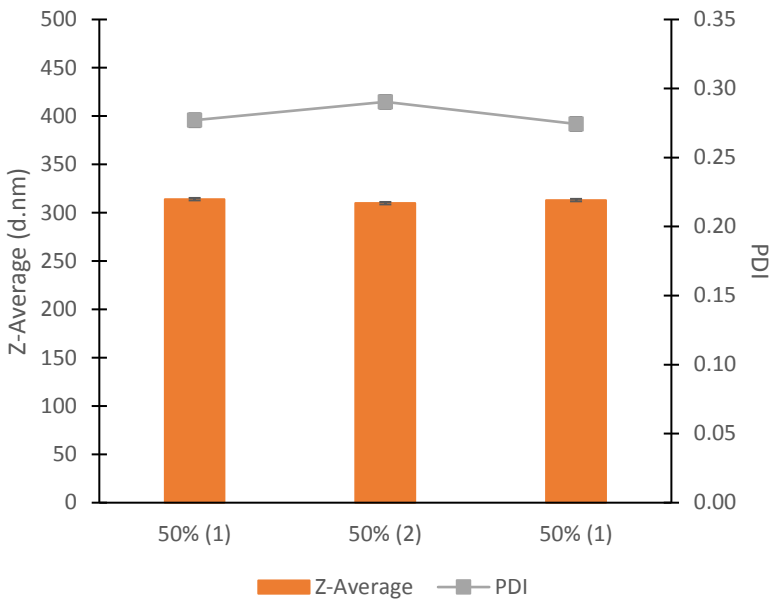


Figure 32. Z-Average (d.nm) and PDI using input amplitude 50% during 5 min of sonication with a 5s on/10s off pulse in consecutive days.

Figure 32 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 [125].

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. Results are presented in figure 33.

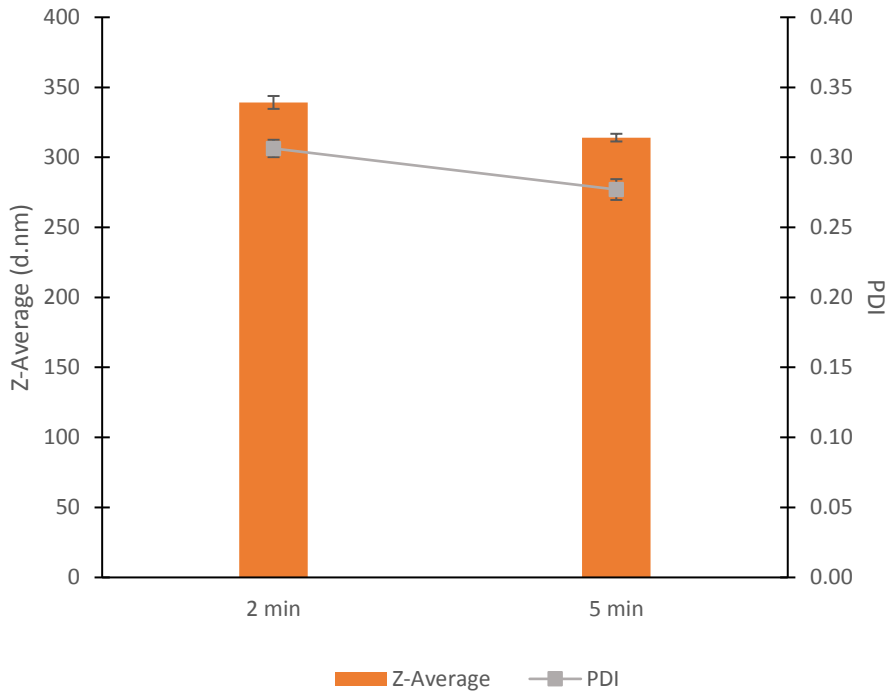


Figure 33. Z-Average (d.nm) and PDI for different sonication times, using 50% of amplitude with a pulse of 5s on/10s off.

In figure 33 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 [11], 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.

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.

The determination of composition of Omega Balance (table 2), did not allow to know the % w/w concentration of each form of EPA and DHA in fish oil: free acids, monoglycerides, diglycerides or triglycerides. That occurred because as in de Carvalho, 2012 [114], 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 and DHA and an esterification reaction, producing EPA/DHA-nicotinol, according to figure 11 (section **1.7.10**). Therefore, to understand if the hydrolysis and esterification reactions observed were reversible, hydrolysis and esterification yields were determined by HPLC (see **Appendix B**).

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. By that, it was intended to determine the optimal amount of biocatalyst, to establish further experimental procedures.

Reaction samples were taken after 1h, 2h, 4h, 6h, 8h, 24h, 48h and 72h.

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. Figures 34 and 35 shows free EPA and DHA, respectively, evolution over reaction time.

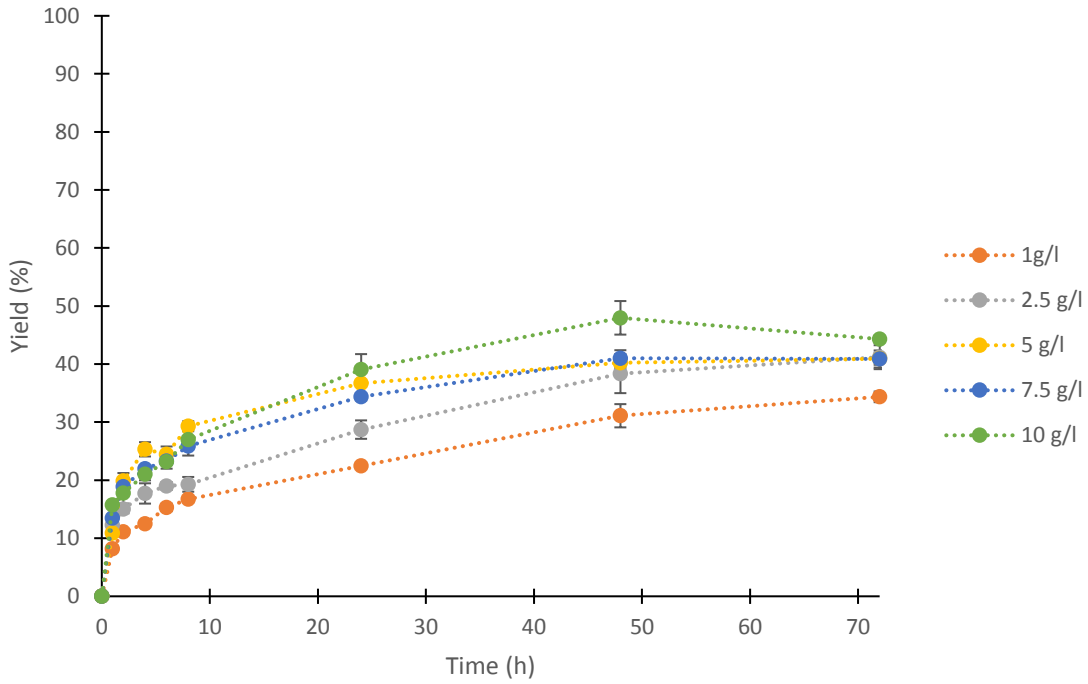


Figure 34. Effect on Amano PS lipase concentration in hydrolysis yield in free EPA at 50ºC, determined by HPLC.

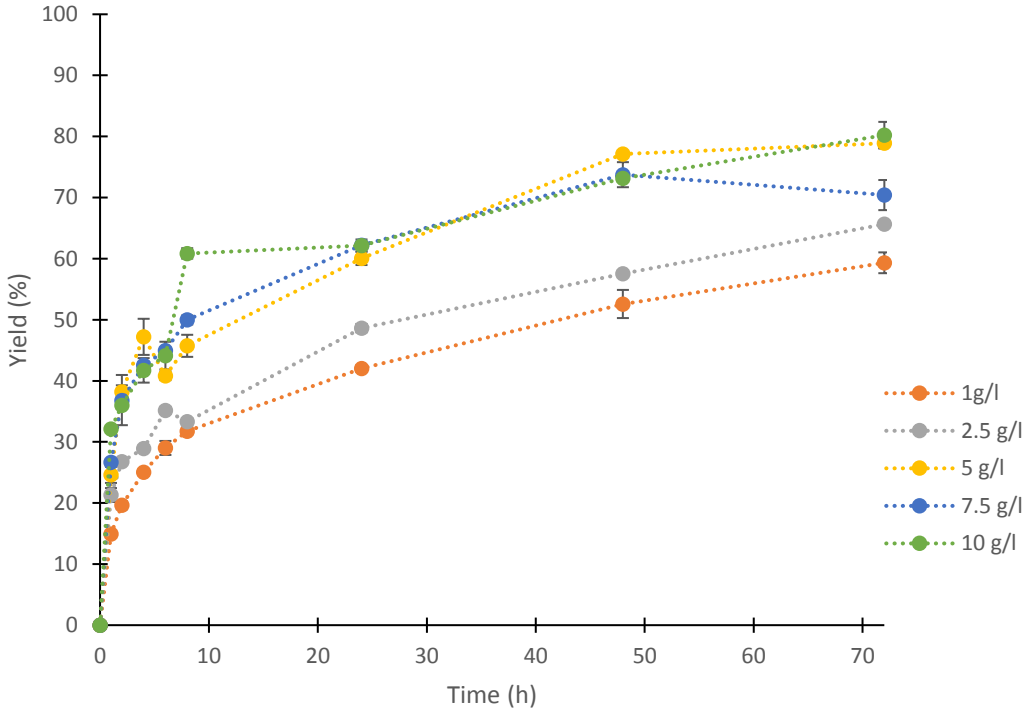


Figure 35. Effect on Amano PS lipase concentration in hydrolysis yield in free DHA at 50ºC, determined by HPLC.

In figures 34 and 35, 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 34 and 35, 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 34 and 35 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 34 and 35, which was determined based in the variation of the first hour free fatty 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 30.0 mM h^{-1} for 10 mg ml^{-1} of biocatalyst. In case of DHA, for the same variation of enzyme's concentration, the initial esterification rate increased from 16.9 mM h^{-1} to 35.8 mM h^{-1}.

Table 5 shows these results for better comparison.

Table 5. Maximum hydrolysis yields, determined by HPLC, in free EPA and free DHA for different Amano Lipase PS concentrations and initial hydrolysis rates.

	EPA		DHA	
[Amano lipase PS] (mg ml^{-1})	Yield (%)	Initial Hydrolysis rate (mM h^{-1})	Yield (%)	Initial Hydrolysis rate (mM h^{-1})
1.0	34.4 ± 0.9	15.8	59.3 ± 1.7	16.9
2.5	41.2 ± 2.1	23.5	65.6 ± 0.2	23.8
5.0	40.9 ± 1.0	20.9	78.9 ± 0.8	27.6
7.5	41.0 ± 1.4	25.9	73.7 ± 2.1	30.0
10	48.0 ± 2.9	30.0	80.2 ± 2.2	35.8

By analysing table 5 it was seen that by increasing enzyme concentration it was not obtained the same outcomes in terms of reaction yield and reaction rate. In fact, between 1 and 5 mg ml^{-1}, both parameters are influenced by the increase of the enzyme concentration. Not only reaction yield

increased for both acids, but also the initial reaction rate increased for EPA (32%) and DHA (63%) from 1 to 5 mg ml^{-1}.

Above 5 mg ml^{-1}, the hydrolysis of EPA triglycerides increased, however, that did not happen for triglyceride form of DHA. Indeed, for an Amano lipase PS concentration of 10 mg ml^{-1}, hydrolysis yield obtained was equal to that obtained for 5 mg ml^{-1}, within the experimental error. On the other hand, by increasing enzyme concentration from 5 to 10 mg ml^{-1}, the initial hydrolysis rates increased 44% and 30% for EPA and DHA, respectively.

Considering that Amano PS lipase is a non-regiospecific enzyme [126], 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 [100], 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 (table 5) that by a 5-fold increasing on enzyme concentration, from 1 to 5 mg ml^{-1}, the yield of free DHA formation was the double of the EPA initial reaction rate. At the same time, for each enzyme concentration used, the initial hydrolysis rate was always higher for the formation of free DHA.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 [98], [100] where 10 mg ml^{-1} of Amano PS lipase did not saturate the reaction system.

3.2.1.2 Ester synthesis

Given that, by NMR analysis, EPA/DHA-nicotinol esters have their structure confirmed and equal to what was expected, it was possible to quantify the esterification yields of both esters by HPLC.

It was monitored the evolution of the concentration of both esters over time and, through that, it was determined their esterification yields. The first study over time allowed to withdraw some conclusions regarding reactions system complexity, such as relative preponderance of enzyme concentration in faster/lower esters formation, reaction time with the relative highest formation of both esters in miniemulsion through esterification rates determination and confirmation of sub sequential reactions as shown in figure 11 (section **1.7.10**).

Therefore, it is possible to see in figures 36 and 37 influence of enzyme concentration in esterification yield of both esters.

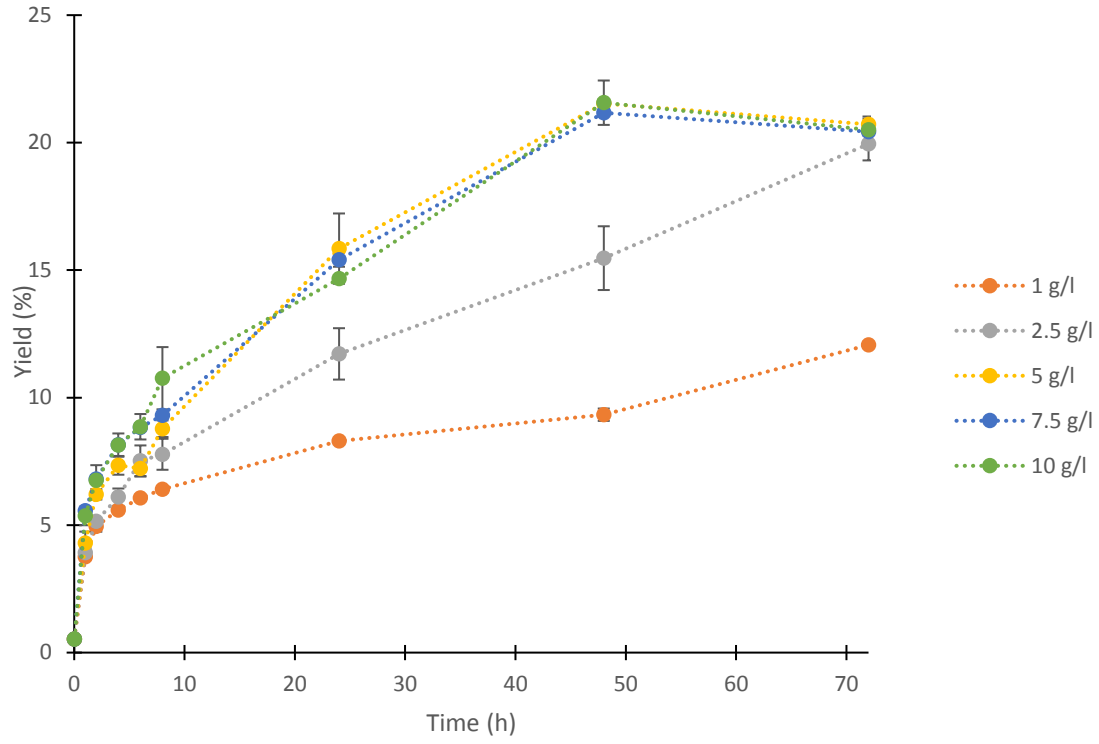


Figure 36. Effect on Amano PS lipase concentration in esterification yield of EPA-nicotinol at 50ºC, determined by HPLC.

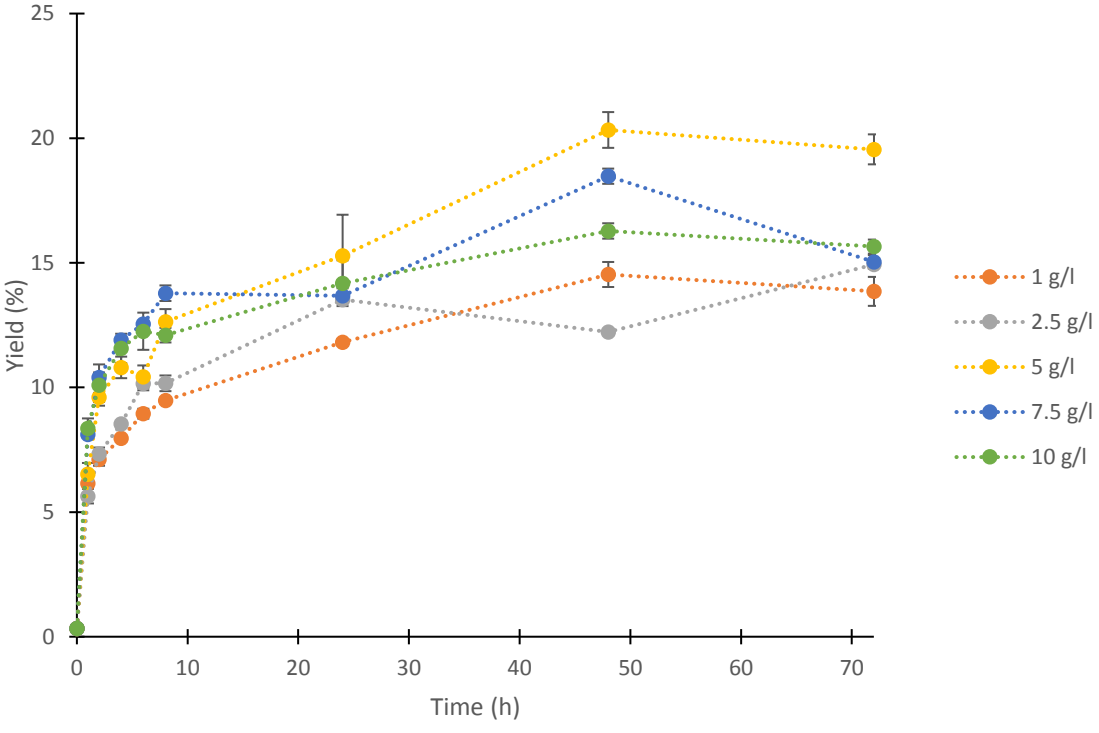


Figure 37. Effect on Amano PS lipase concentration in esterification yield of DHA-nicotinol at 50ºC, determined by HPLC.

The results illustrated in the figures 36 and 37 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 34 and 35 (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 10.2 mM h^{-1} for 10 mg ml^{-1} of Amano PS lipase. In case of DHA-nicotinol, for the same enzyme concentrations increase, the initial ester formation raised from 6.9 mM h^{-1} to 9.3 mM h^{-1}. These results are presented in table 6 for better comparison.

Table 6. Esterification yields, determined by HPLC, in EPA-nicotinol and DHA-nicotinol for different Amano PS lipase concentrations and initial esterification rates.

[Amano PS lipase] (mg ml^{-1})	EPA-nicotinol		DHA-nicotinol	
	Yield (%)	Initial esterification rate (mM h^{-1})	Yield (%)	Initial esterification rate (mM h^{-1})
1.0	12.1% ± 0.1	7.2	14.5% ± 0.5	6.9
2.5	19.9% ± 0.6	7.5	14.9% ± 0.0	6.3
5.0	21.5% ± 0.2	8.2	20.3% ± 0.7	7.3
7.5	21.2% ± 0.1	10.7	18.5% ± 0.3	9.1
10	21.6% ± 0.9	10.2	16.3% ± 0.3	9.3

Therefore, in the table 6 it can be analysed EPA/DHA-nicotinol synthesis through different approaches: maximum yield and initial esterification rate.

If, on the one hand, increasing the concentration of enzyme above 5 mg ml^{-1} does not seem to have significant influence on the final yield, on the other hand, this was not seen for the initial esterification rate, especially when the enzyme concentration increased from 5 mg ml^{-1} to 7.5 mg ml^{-1}.

On the other hand, the initial esterification rate increased 14% and 6% for EPA-nicotinol and DHA-nicotinol, respectively, when the enzyme concentration was shifted from 1 to 5 mg ml^{-1}. However, when shifted from 5 to 10 mg ml^{-1}, the initial esterification rate increased 24% and 27% for EPA-nicotinol and DHA-nicotinol, 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 of biocatalyst. Furthermore, this effect has even higher importance in case of DHA-nicotinol.

In the table 6 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 [38]. Nonetheless, differences in the esterification yields were almost equal within experimental error, in case of highest yield for EPA-nicotinol and DHA-nicotinol.

These studies about enzyme concentration in both reactions involving EPA and DHA forms show one clear certainty: above 5 mg ml^{-1} there is no significant increase in esterification yields for both cases. 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}.

3.2.1.3 Free EPA/DHA, EPA/DHA Glycerides, EPA/DHA-nicotinol evolution over time.

Since a certain number of different EPA/DHA molecular derivatives are involved in these miniemulsion systems, it is important to understand their evolution in reaction time course. Given that, there were three different species for EPA and DHA: free fatty acid, glycerides and nicotinol-esters, the concentrations of the first one and the last one were determined directly by HPLC in different times of reaction. However, the glycerides concentration was determined in an indirect way. Considering the initial Omega Balance fish oil weighted for making miniemulsion systems and the initial free EPA and DHA were determined by taking an aliquot of miniemulsion system before enzyme addition, EPA and DHA glycerides content for both cases were determined by the difference with the initial total amount of EPA and DHA.

Given that, for each enzyme concentration, the content of these three-different omega-3 types of molecules is only function of reaction rates of both hydrolysis and esterification reactions. Thus, the higher reaction rates, the faster will be the conversion of EPA and DHA glycerides in free EPA and DHA, and then in their nicotinol-esters. Because of that, it was only plotted % mol (Eq. 5) during the reaction time of the different EPA/DHA species in case of using 5 mg ml^{-1} of Amano PS lipase. In figures 38 and 39 is shown each fatty acid specie evolution over time.

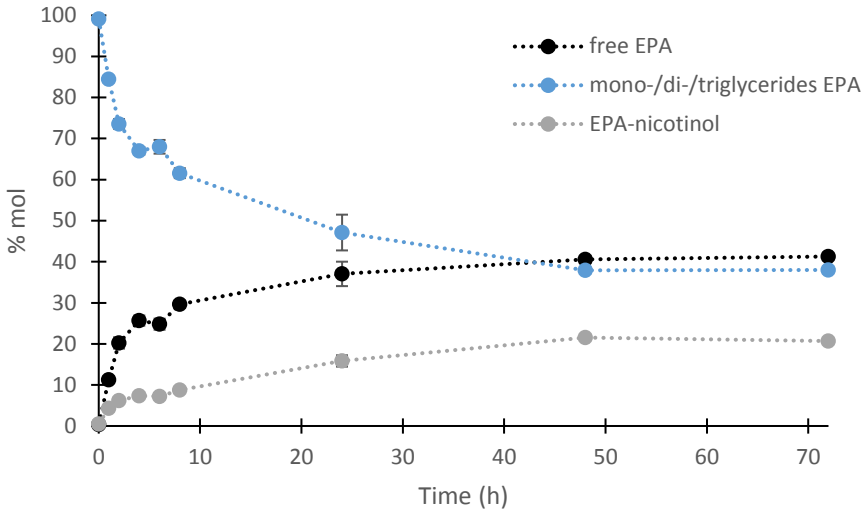


Figure 38. Evolution of % mol of EPA species over time in miniemulsion systems.

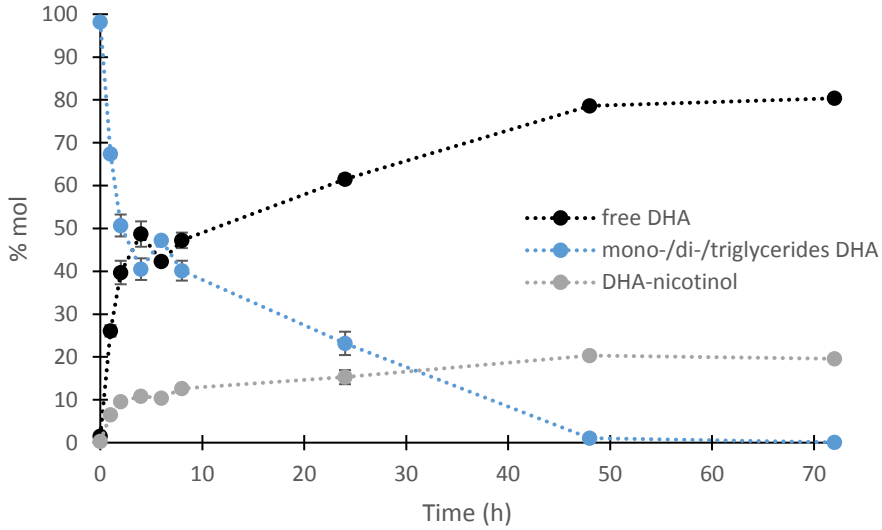


Figure 39. Evolution of % mol of DHA species over time in miniemulsion systems.

In both figures, it was possible to see that even though % mol of EPA-nicotinol and DHA-nicotinol were almost the same (20-22%) 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 (80.4%) than free EPA (41.3%) 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. Once figures 38 and 39 represents similar evolution of % mol species of EPA and DHA to the other concentrations of enzyme tested, it was noticed that the % mol of free fatty acids and their respective ester tended to increase with time. Since the % mol of both species increased with time, this finding showed that hydrolysis and esterification reactions occurring in this miniemulsion system are not reverse reactions but sequential

reactions. That is, the free EPA and DHA, reaction products formed during the hydrolysis of Omega Balance fish oil, was substrates of the esterification reaction, being esterified in nicotinol esters.

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 [95], others were widely used in esterification studies [75], [127], [128] and even in omega-3 fatty acids concentration studies with hydrolysis, esterification and transesterification reactions [37]–[40], [129] .

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 and DHA. Therefore, the initial activity in each reactor was approximately 191 U ml^{-1} and depending on specific activity (table 4), each enzyme amount needed was then determined. The esterification reactions were carried at 40^{o}C since all enzymes showed high activity at this temperature [121]–[124].

The objective of this study was to see if there is an enzyme that catalyses more efficiently the esterification of free fatty acids produced during the hydrolysis reaction, to have higher esterification yields. By the end of this experiment, it was wanted to know which is the appropriate biocatalyst for carrying out esterification reactions.

Once again, reaction samples were taken after 1h, 2h, 4h, 6h, 8h, 24h, 48h, and 72h.

3.2.2.1 EPA/DHA glycerides hydrolysis into free fatty acids

Since it was already established that hydrolysis and esterification reactions were not reverse reactions, it was desired to know the effect of having different regioselective lipases catalysing hydrolysis reactions in miniemulsion systems. By doing that, it was aimed to relate the effect of having different regiospecific lipases in the hydrolysis of EPA and DHA.

Although samples were collected during reaction course, it was only represented the final hydrolysis yields for free EPA and DHA formation. Therefore, results only showed the maximum yields obtained. As already seen in section **3.2.1.1**, when using Amano PS as biocatalyst, the hydrolysis reactions also reached equilibrium after 48h of reaction with all lipases tested. For that reason, figure 40 shows the hydrolysis yields at 48h for all enzymes used, in terms of free EPA and DHA formed.

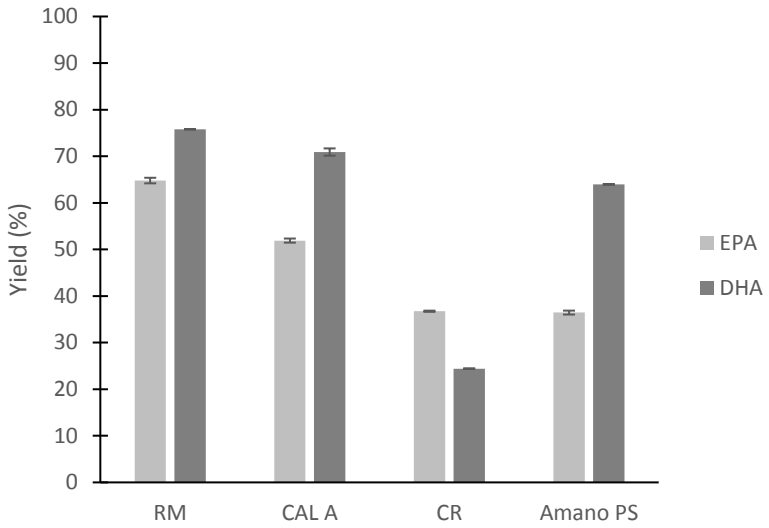


Figure 40. Hydrolysis yields, determined by HPLC, for free EPA and DHA at 48h in miniemulsion at 40^{O}C using: *Rhizomucor miehei* (RM) lipase, *Candida antarctica A* (CAL A) lipase, *Candida rugosa* (CR) lipase and Amano PS lipase (Amano PS).

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, followed by CAL A and *C. rugosa* and Amano PS with almost the same yield results. The same order was seen for DHA glycerides hydrolysis, except for *C. rugosa* which led to the lowest DHA hydrolysis yield.

To have a better overview of the hydrolysis yields for all lipases tested and for both free fatty acids formed, these results are shown in table 7 with their respective standard deviation

Table 7. Hydrolysis yields, determined by HPLC, in free EPA and DHA using four different lipases as biocatalyst.

Lipase	EPA	DHA
Rhizomucor miehei	64.8% ± 0.6	75.8% ± 0.0
Candida antarctica A	51.9% ± 0.4	70.9% ± 0.8
Candida rugosa	36.7% ± 0.1	24.4% ± 0.1
Amano PS	36.5% ± 0.4	64.0% ± 0.1

In table 7 it is seen that in case of using *R. miehei* as biocatalyst, the hydrolysis yields for free EPA were 12.9%, 28.1% and 28.3% higher when compared with CAL A, *C. rugosa* and Amano PS lipases, respectively. In terms of hydrolysis yields of free DHA, these values decreased for 4.9% and 11.8%, in case of CAL A and Amano PS lipases, respectively. Comparing with *C. rugosa*, the use of *R. miehei* as biocatalyst allowed to have 51.4% more hydrolysis of DHA.

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 [130], 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 [129].

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 [68], while CAL A has reports of *sn*-2 preference [130]. Despite both hydrolysed DHA over EPA, in table 7 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, which had already been showed for enzyme concentration study. The esterification yields for EPA/DHA-nicotinol esters at 48h are shown in figure 41.

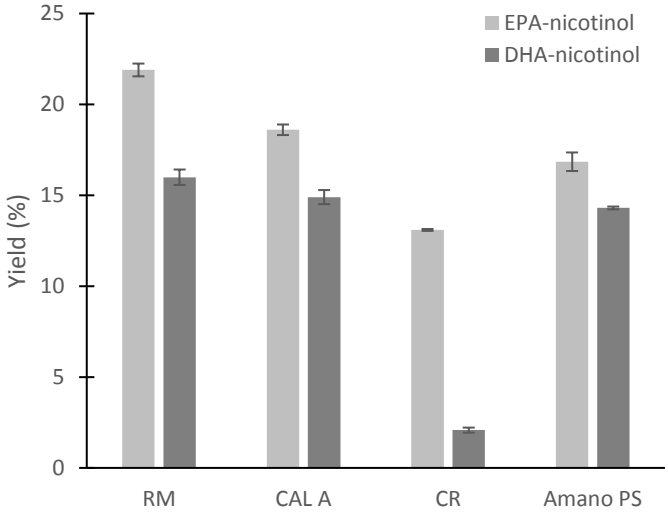


Figure 41. Esterification yields, determined by HPLC, for EPA/DHA-nicotinol esters at 48h in miniemulsion at 40ºC using: *Rhizomucor miehei* (RM) lipase, *Candida antarctica A* (CAL A) lipase, *Candida rugosa* (CR) lipase and Amano PS lipase (Amano PS).

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 [37] 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 [95], 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.

To better compare the remaining enzymes in terms of esterification efficiency, is shown in table 8 the esterification yields obtained in presence of all four lipases used.

Table 8. Esterification yields, determined by HPLC, in EPA-nicotinol and DHA-nicotinol using four different lipases as biocatalyst.

Lipase	EPA-nicotinol	DHA-nicotinol
Rhizomucor miehei	21.9% ± 0.4	16.0% ± 0.4
CAL A	18.6% ± 0.3	14.9% ± 0.4
Candida rugosa	13.1% ± 0.1	2.1% ± 0.1
Amano PS	16.8% ± 0.5	14.3% ± 0.1

Table 8, allowed to compare the esterification efficiencies of three remaining enzymes. It can be easily seen that in case of using *Rhizomucor miehei* lipase, the esterification yields for EPA-nicotinol were higher 3.3% and 5.1% when compared with CAL A lipase and Amano PS lipase, respectively. These values decreased for 1.1% and 1.7% if the same enzymes were compared in terms of DHA-nicotinol esterification yields.

At the same time, comparison of differences of esterification efficiency towards EPA and DHA are possible to be done. In fact, 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 FFA, reported by Haraldsson and Kristinsson [131]. 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 [129].

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 5 mg ml^{-1} of Amano PS lipase as biocatalyst at 40ºC (seen in table 8) and 50ºC (seen in table 6), 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.

In tables 7 and 8 was shown that higher yields for both reactions were correlated since cases with higher hydrolysis yields, 64.8% and 75.8% for free EPA and DHA formation, respectively, when using *R. miehei* as biocatalyst, led to higher esterification yields, 21.9% for EPA-nicotinol and 16.0% for DHA-nicotinol. 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 [95] and Aschenbrenner and co-workers [98] . However, nicotinol is a very water-soluble alcohol, since it is described as fully miscible with water [112].

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 huge 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 3), 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 as it is shown in figure 42. 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.

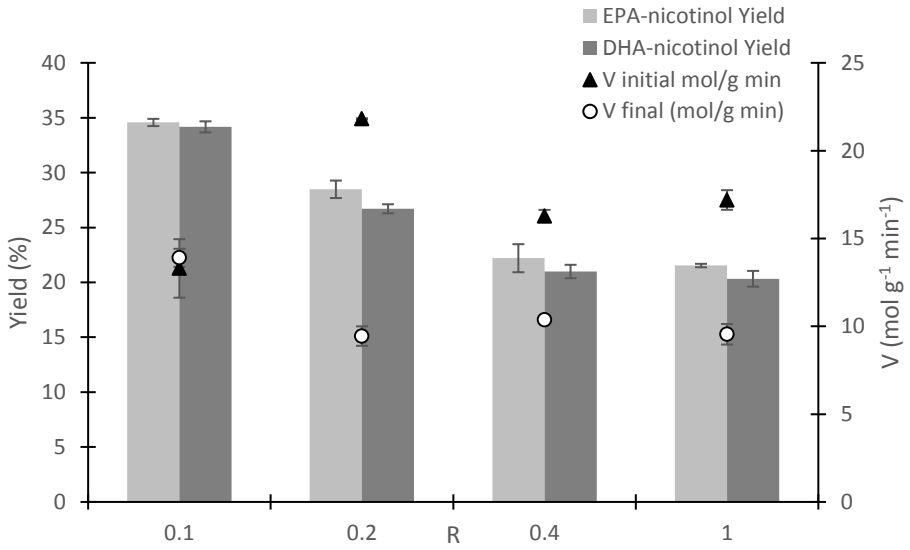


Figure 42. Effect of fatty acids/nicotinol molar ratio (R) on synthesis of EPA-nicotinol and DHA-nicotinol esters, initial and final reaction rates, at 50ºC, using Amano PS lipase as biocatalyst. Yield of esterification was determined by HPLC.

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 reaction 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 [11]. 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 3), 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. The highest esterification yields for both acids are presented in figure 43. 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.

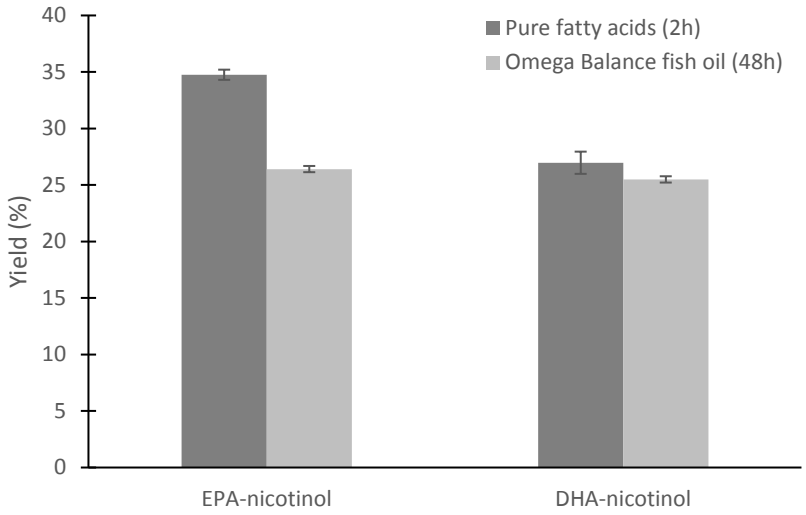


Figure 43. Effect of pure free EPA/DHA vs EPA/DHA triglycerides substrates in esterification yields at 50ºC, determined by HPLC, using Amano PS lipase as biocatalyst.

In figure 43 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 42. 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 [132]. 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 43 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 equilibrium 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 43, 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 and 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 of fish oil supplements in miniemulsion 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. In fact, maximum esterification yields obtained were 34.6% and 34.2%, for EPA-nicotinol and DHA-nicotinol, respectively. 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 and 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 and DHA in fish oil favours the synthesis of DHA-nicotinol over EPA-nicotinol, when compared with the esterification of pure EPA and 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 could 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 and 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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Appendix A

13C NMR analyses of EPA/DHA nicotinol esters standards, chemically produced

In order to confirm the formation of EPA/DHA-nicotinol by chemical synthesis (see section **2.2.4.**),13C NMR analyses were done and results are shown in figures 44 and 45.

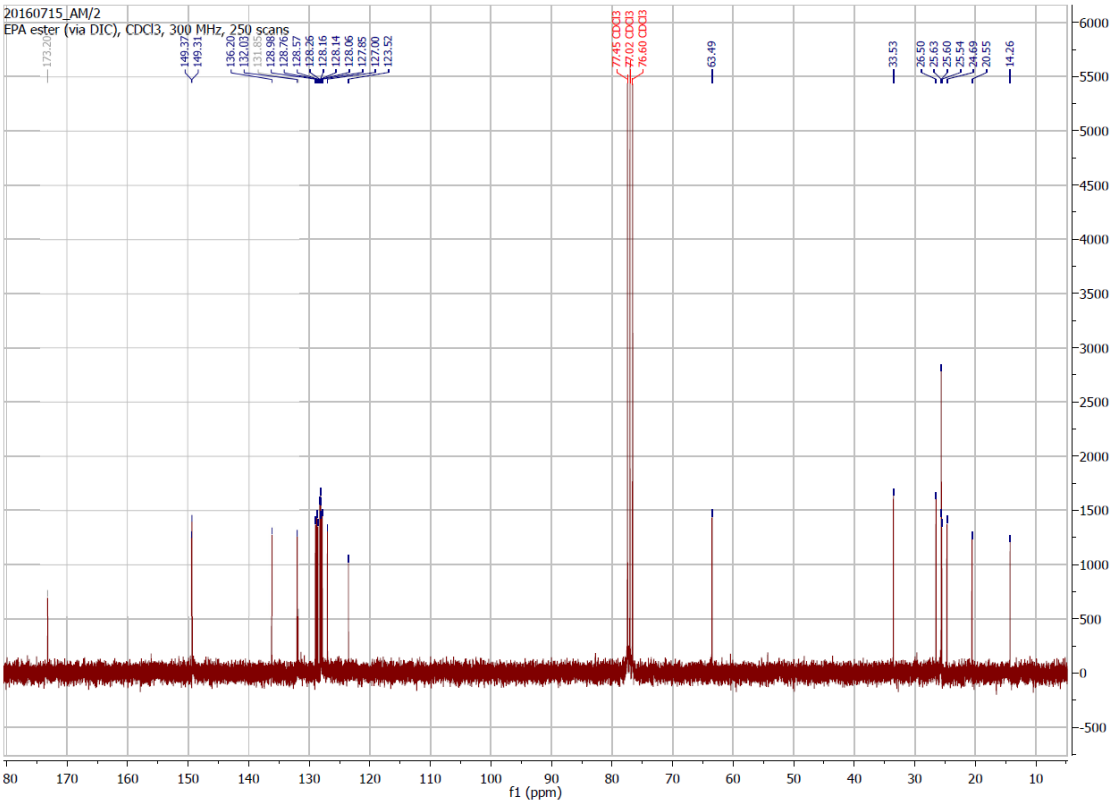

Figure 44. 13C NMR spectrum of EPA-nicotinol produced by chemical synthesis.

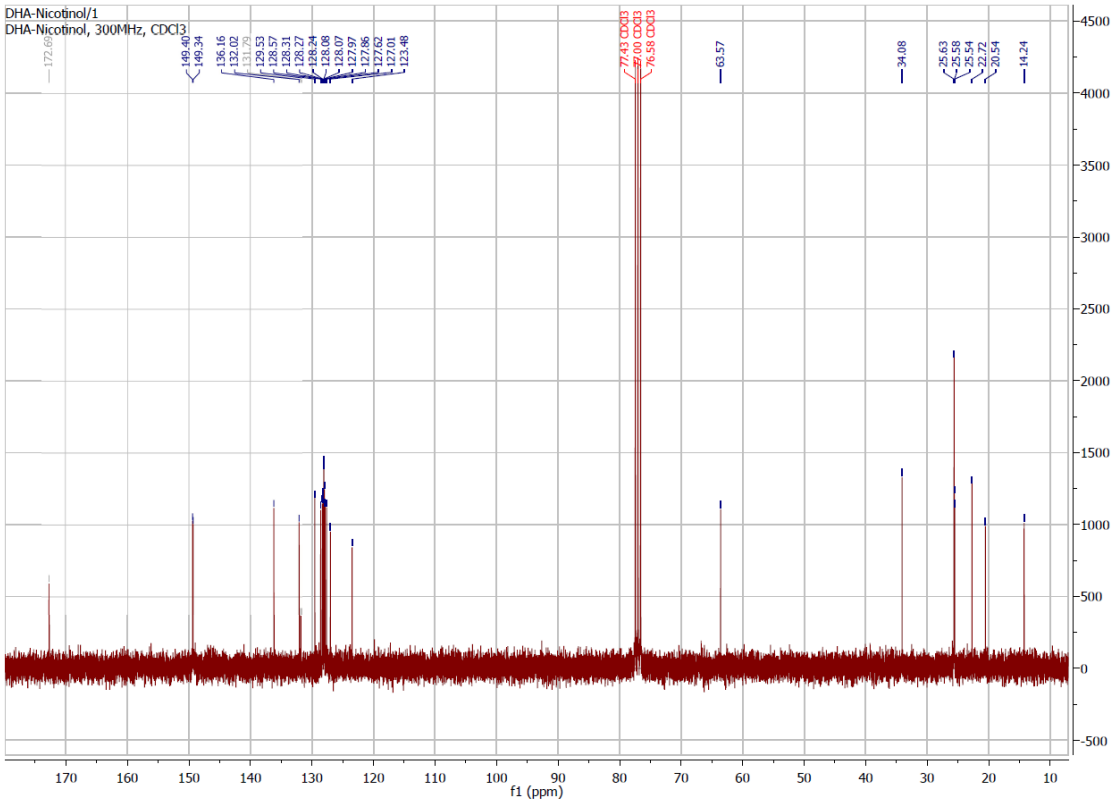

Figure 45. 13C NMR spectrum of DHA-nicotinol produced by chemical synthesis.

Appendix B

Retention times of miniemulsion components analysed by HPLC

The figure 46 shows a typical chromatogram expected to be obtained. The substrates and products from resulting hydrolysis and esterification can be well identified, according specific retention times. The figure 46 presents a typical chromatogram for the injection of samples according to section **2.2.6.1**.

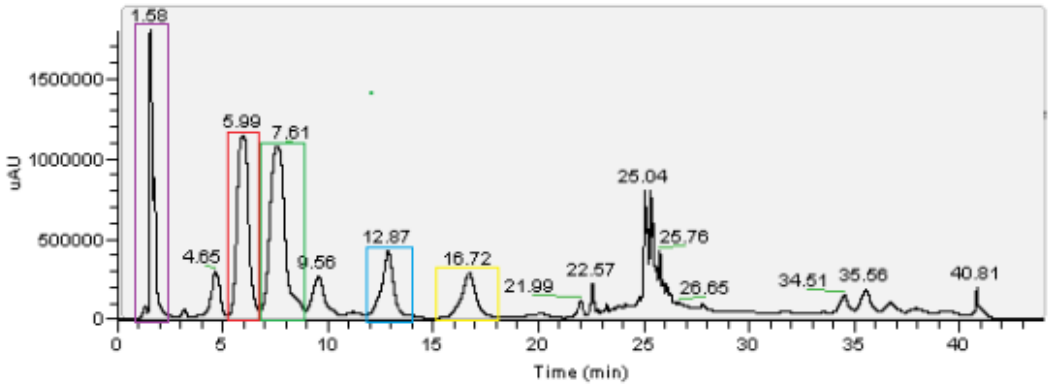

Figure 46. Resume of a typical chromatogram, with expected retention times of substrates and products resulting from hydrolysis and esterification reactions. Purple rectangle – time zone of nicotinol detection , Red rectangle – time zone of free EPA detection , Green rectangle – time zone of free DHA detection, Blue rectangle – time zone of EPA-nicotinol detection, Yellow rectangle – time zone of DHA-nicotinol detection.