The potential use of immobilized lipases in the synthesis of omega-3 monoacylglycerols enriched in stearidonic acid

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Abstract

This work presents an original approach to develop an integrated process to improve the nutritional characteristics of natural oils, starting with the extraction from the raw material by environmentally friendly methods and following with the production of novel acylglycerols using immobilized lipases. Specifically, 2-monoacylglycerols (2-MAGs) enriched in the omega-3 stearidonic acid (SDA) were synthesized by selective ethanolysis of extracted Echium plantagineum oil using the lipase from Thermomyces lanuginosus (TLL). Different reaction conditions were investigated to minimize the undesirable acyl migration and to ensure the purity of final products. The biocatalyst produced in our laboratory by the immobilization of TLL on a hydrophobic support reached the maximum theoretical amount of 2-MAG in only 2 h at mild reaction conditions, achieving a product enriched in omega 3 SDA (up to 25%). Moreover, the produced biocatalyst exhibited higher stability than commercial lipases. The average activity after 5 cycles was 71%, allowing several reutilization cycles and developing a feasible enzymatic process. Finally, 2-MAGs was used as starting material to synthesize structured triacyclglycerols (STAGs) in solvent-free systems. The use of molecular sieves in combination with the immobilized lipase from Rhizomucor miehei (RML) showed to be an extraordinarily fast strategy to produce pure STAGs (100% in 1h), 4 times higher than the activity showed by the commercial derivative. Thus, the enzymatic processes developed in this study open a range of possibilities to synthesize omega-3 acylglycerols with improved characteristics for essential biological functions and nutritional advantages, proving the usefulness of immobilized lipases to produce novel functional lipid.

Abstract:

This work presents an original approach to develop an integrated process to improve the nutritional characteristics of natural oils, starting with the extraction from the raw material by environmentally friendly methods and following with the production of novel acylglycerols using immobilized lipases. Specifically, 2-monoacylglycerols (2-MAGs) enriched in the omega-3 stearidonic acid (SDA) were synthesized by selective ethanolysis of extracted *Echium plantagineum* oil using the lipase from *Thermomyces lanuginosus* (TLL). Different reaction conditions were investigated to minimize the undesirable acyl migration and to ensure the purity of final products. The biocatalyst produced in our laboratory by the immobilization of TLL on a hydrophobic support reached the maximum theoretical amount of 2-MAG in only 2 h at mild reaction conditions, achieving a product enriched in omega-3 SDA (up to 25%). Moreover, the produced biocatalyst exhibited higher stability than commercial lipases. The average activity after 5 cycles was 71%, allowing several reutilization cycles and developing a feasible enzymatic process. Finally, 2-MAGs was used as starting material to synthesize structured triacyclglycerols (STAGs) in solvent-free systems. The use of molecular sieves in combination with the immobilized lipase from *Rhizomucor miehei*(RML) showed to be an extraordinarily fast strategy to produce pure STAGs (100% in 1h), 4 times higher than the activity showed by the commercial derivative. Thus, the enzymatic processes developed in this study open a range of possibilities to synthesize omega-3 acylglycerols with improved characteristics for essential biological functions and

nutritional advantages, proving the usefulness of immobilized lipases to produce novel functional lipids.

Keywords: omega-3 PUFAs, enzymatic modification, lipase immobilization, green process, bioactive lipids, *Echium plantagineum* L.

Introduction

In recent years, interest in omega-3 polyunsaturated fatty acids (omega-3 PUFAs) has increased because of their numerous functions in promoting human health. Omega-3 PUFAs have been associated with a lower risk of cardiovascular diseases, inflammation, cancer and neurological disorders (Bowen et al., 2016; Shahidi and Ambigaipalan, 2018). The omega-3 α-linolenic acid (ALA, 18:3 all cis-9,12,15) is considered an essential fatty acid from which all other omega-3 PUFAs are metabolically derived. ALA can be converted into stearidonic acid (SDA, 18:4 all cis-6,9,12,15), eicosapentaenoic acid (EPA, 20:5 all cis-5,8,11,14,17) and docosahexaenoic acid (DHA, 22:6 all cis-4,7,10,13,16,19) in the human body by elongation and desaturation pathways, but their conversion is often poor (less than 8%). This poor conversion can be explained by the initial Δ6 desaturase enzyme being rate limiting in humans. However, SDA is more efficiently converted to EPA than ALA because does not require the first of the rate-limiting step (Walker et al., 2013). SDA has been found to be further metabolized*in vivo* and lead to increase the conversion into EPA with approximately 20–26% efficiency (Brenna et al., 2009). Hence, SDA provide a superior strategy for the biosynthesis of omega-3 very long-chain polyunsaturated fatty acids (omega-3 VLC-PUFAs) in the human body.

On the other hand, the form of administration of omega-3 PUFAs is a crucial factor, with significant differences between different lipid classes in bioavailability, absorption, metabolism and bioactivity in the human body (Castejón and Señoráns, 2020). In natural sources, omega-3 PUFAs are primarily present as triacylglycerols (TAGs) and, to a lesser extent, as free fatty acids (FFAs), phospholipids (PLs) or other acylglycerols. It should be highlighted that the position at which the omega-3 PUFAs are attached to TAGs plays an important role in their absorption. Omega-3 PUFAs attached to the *sn* -2 position are preferentially absorbed as monoacylglycerols (MAGs) after the cleavage of fatty acids from *sn* -1 and *sn* -3 positions by pancreatic lipases, whereas the chain length and number of double bonds of resulting FFAs will influence its absorption. Unsaturated fatty acids and short- and medium-chain fatty acids (SCFAs and MCFAs) are more efficiently absorbed than VLC-PUFAs. Moreover, the presence of calcium and other ions can reduce the bioavailability of FFA by forming insoluble complex. Based on these findings, trends in food science and biotechnology have focused on the synthesis of novel functional lipids by modifying the composition and/or distribution of fatty acids in the glycerol backbone, aiming at desirable nutritional properties and improving their bioavailability.

Lipases are a versatile class of enzymes that catalyze a broad variety of reactions, being the most stablished biocatalyst used in lipid modification (Bornscheuer, 2018). Enzymes offer a range of advantages over chemical methods, such as high chemo-, regio-, and stereoselectivity, mild reaction conditions and low temperatures, minimal undesirable by-products, and less omega-3 PUFAs oxidation (Castejon et al., 2019). Moreover, the use of enzymes is considered an environmentally friendly process because enzymes are biodegradable, produce minimal waste and allow to use water or other green solvents as reaction medium (Jegannathan and Nielsen, 2013). Nevertheless, lipases, as most enzymes, need to be immobilized for their applications as biocatalysts to improve their stability and facilitate their separation from the reaction medium as well as enzyme reuse (Mateo et al., 2007). The development of stable and active biocatalyst with the possibility of using it for several cycles could significantly reduce process costs. In this sense, the immobilization of lipases via interfacial activation on hydrophobic supports has been reported to be an efficient method to immobilize lipases. This method fixes the open form of lipases via interactions between the hydrophobic surroundings of their active centre and the hydrophobic surface of the support (Fernandez-Lorente et al., 2008). Biocatalysts prepared by following this immobilization mechanism have shown enhanced activity against hydrophobic substrates and improved stability under different experimental conditions (Fernandez-Lorente et al., 2011).

In this work, enzymatic production of 2-MAGs enriched in omega-3 SDA was studied by ethanolysis using the lipase from *Thermomyces lanuginosus* (TLL) immobilized on a hydrophobic support in our laboratory. To develop an integrated process, in a first step, omega-3 oil was extracted from the highest vegetable

source of SDA, *Echium plantagineum* L., by an eco-friendly method using pressurized liquids and ethyl acetate as solvent. In a second step, to minimize the undesirable acyl migration and to ensure the purity of final products, different reaction conditions were investigated: time, temperature and type of biocatalyst. Reaction products were analyzed by HPLC-ELSD and GC-MS to evaluate the fatty acid composition of synthesized 2-MAGs. Furthermore, reutilization of produced biocatalyst was studied to assess the effect of immobilization in the stability of lipase derivatives. Finally, to show the potential application of synthesized products, 2-MAGs was used as starting material for the synthesis of structured triacyclglycerols (STAGs). Thus, the main objective of the present work was to develop an efficient and integrated process for the synthesis of omega-3 acylglycerols with high added-value using immobilized lipases for potential applications in food and nutraceutical industries.

1. **Materials and methods**
2. Materials.

Echium seeds (*Echium plantagineum* L.) were provided by Technology Crops International (Essex, United Kingdom). Seeds were ground with a particle size less than 500 μm using a grinder (Moulinex-A320R1 700 W) and stored at 4 °C until the oil extraction process.

Soluble lipases from *Thermomyces lanuginosus* and *Rhizomucor miehei* and commercial derivative Lipozyme TL IM and Novozym 40086 were kindly donated for Novozymes (Bagsvaerd, Denmark). Sepabeads-C18 was kindly donated by Resindion S.R.L. (Rome, Italy). P-nitrophenyl butyrate (pNPB) were provided by Sigma Chemical Co. (St. Louis, USA). Absolute ethanol (PRS grade), sodium hydrogen carbonate and potassium hydroxide were purchased from Panreac Quimica S.A (Barcelona, Spain). Caprylic acid ethyl esters were purchased from Sigma-Aldrich Co. LLC (Darmstad, Germany). Solid phase extraction cartridges (Bond Elut NH2) were from Agilent (Palo Alto, CA, USA). Molecular sieves pore size 4 A (pearl-shaped 2–3 mm) and n-hexane was purchased from Scharlau (Barcelona, Spain). The solvents (2,2,4-trimethyl pentane, methyl tert-butyl ether and 2-propanol) used for high-performance liquid chromatography (HPLC) analyses were HPLC-grade and purchased from LABSCAN (Dublin, Ireland). Fatty acid methyl esters standard (Supelco 37 FAME Mix) was from Supelco (Bellefonte, PA, USA). Glyceryl trilinoleate, dioleoylglycerol (mixture of 1,3- and 1,2-isomers), 1-oleoyl-rac-glycerol, oleic acid and ethyl linoleate used as HPLC standards was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents used were of analytical or HPLC grade.

Pressurized liquid extraction of *Echium plantagineum* seed oil

Pressurized liquid extraction (PLE) was carried out with an ASE 350 DIONEX (Sunnyvale, California) extractor. Oil extraction was performed using 3 g of ground echium seeds. Stainless steel extraction cells were used with a capacity of 10 mL. Extracts were collected under a nitrogen stream in different vials of 50 mL. Extraction conditions used were performed according to previous studies using ethyl acetate as solvent at 150 degC and 10 min of static time (Castejon et al., 2018).

The samples were evaporated in a rotary evaporator (Heidolph Hei-Vap Value HB/G3, Germany) under reduced pressure at 40 degC and dried under a nitrogen stream until constant weight. The oil content was determined gravimetrically and expressed as dry weight percentage. Oil obtained was stored in dark vessels with nitrogen atmosphere at 4 degC until their use.

Immobilization of lipases on Sepabeads-C18 resins

Lipase from *Thermomyces lanuginosus* and lipase from*Rhizomucor miehei* were immobilized on Sepabeads-C18 resins by hydrophobic adsorption at low ionic strength (5 mM) in sodium phosphate buffer at 25 degC and pH 7. To follow the immobilization process, the activity of the blank and the supernatant were analyzed spectrophotometrically at different times by measuring the absorbance at 348 nm ([?] = 5.150 M^{-1} cm^{-1}) produced by the release of p-nitrophenol (pNP) by hydrolysis of 0.4 mM p-nitrophenyl butyrate (pNPB) in 25 mM sodium phosphate buffer at pH 7 and 25 degC. After 24 hours, enzyme loading of lipase derivatives was 33.0 mg of TLL per gram of support and 29.6 mg of RML per gram of support.

Lipase derivatives were washed and dried, before their use in enzymatic reactions, with increasing volumes of water, water: acetone and acetone using a sintered glass funnel until derivatives were completely dried.

Enzymatic synthesis of 2-MAGs from echium oil

Enzymatic synthesis of 2-MAGs was performed by ethanolysis reaction. In a typical experiment, echium oil and ethanol (ratio 1:4 (w/w)) and 15% (w/w) of the biocatalyst Lipozyme TL IM were place in glass vials. The reaction was carried at 25 degC and 35 degC with constant stirring in an orbital shaker at 200 rpm (Unimax 1010, Heidolph, Germany) under dark conditions. A negative control without biocatalyst was carried out at the same conditions previously described. The reaction mixture was filtered to remove the biocatalyst and the excess of ethanol was removed under vacuum. The synthesis of 2-MAGs was followed by HPLC-ELSD. Reaction kinetics were done at least in duplicate. 2-MAGs obtained were stored in dark vessels with nitrogen atmosphere at 4 degC until their use.

Transesterification between 2-MAGs and caprylic acid ethyl esters

Transesterification between the synthesized 2-MAGs and caprylic acid ethyl esters was performed to produce STAGs as previously described (Castejon and Senorans, 2019). In a typical experiment, ethanolysis reaction product (2-MAGs) and caprylic acid ethyl esters ratio 1:20 (w/w), 10% (w/w) of biocatalyst and molecular sieves were place in glass vials. The reaction was carried out at 35 degC with constant stirring in an orbital shaker at 200 rpm (Unimax 1010, Heidolph, Germany) under dark conditions. A negative control without biocatalyst was carried out at the same conditions previously described. The synthesis of STAGs was followed by HPLC ELSD. Reaction kinetics were done at least in duplicate.

Reutilization cycles of lipase derivatives

For recycling studies, the enzymatic reaction was carried out in PP-Reactors, 10ml, with PTFE frit (Multi-syntech GmbH, Witten, Germany). After the optimal reaction time, reaction volume was carefully filtrated, lipase derivatives were washed with 1 mL of hexane and separated from the reaction medium. A new batch with the reutilized lipase was carried out at the same conditions previously described until lipases totally lost their activity. The 2-MAG yield of the first reaction was set as 100% and the yield in the subsequent reactions was calculated accordingly.

Lipid fractionation by Solid Phase Extraction

Reaction products were fractionated using solid phase extraction (SPE) with ISOLUTE(r) NH2 columns (aminopropyl bonded sorbent) as previously described (Castejon and Senorans, 2019). SPE columns were preconditioned with 4 mL of hexane. The sample (50 mg reaction product/500 mL hexane) was allowed to adsorb to the matrix by percolation through the cartridge by gravity. SPE column was eluted with 10 mL of solvent A (hexane), 4 mL of solvent B (hexane: isopropanol (10:1)) and 4 mL of solvent C (hexane: isopropanol (3:1)). The fractions eluted from the SPE column were dried down under nitrogen and re-dissolved to analysis by HPLC-ELSD. Fatty acid ethyl esters (FAEEs) were eluted in fraction 1, TAGs and diacylglycerols (DAGs) in fraction 2, and 2-MAGs in fraction 3.

HPLC-ELSD analysis

The lipid composition as the enzymatic reaction time proceeded was kinetically controlled and analyzed by HPLC-ELSD. Time reaction samples were taken after 0, 0.3, 0.7, 1, 2, 3, 4, 6 and 8 h. Samples were stored at -20 degC until their analysis.

HPLC-ELSD analyses were performed using an Agilent 1260 Infinity HPLC equipped with an Agilent 385 (Palo Alto, CA, USA) ELSD instrument. The chromatographic separation of FAEE, TAGs, DAGs and MAGs was performed with a silica normal-phase ACE (250 mm x 4.6 mm i.d., 5 μm) column maintained at 30 °C using a ternary gradient as follows: 0–2 min, 99.5% A and 0.5% B; at t = 6.5 min, 70% A and 30% B; at t = 11 min, 63% A, 27% B and 10% C; at t = 18 min, 99.5% A and 0.5% B; and at t = 20 min, 99.5% A and 0.5% B. Eluent A consisted of 2,2,4 trimetilpentane, eluent B consisted of methyl tert-butyl ether, and eluent C consisted of 2-propanol. The flow rate was 2.0 mL/min except for minutes 13 to 16 which was

1.0 mL/min. Optimal signal and resolution were achieved with the following ELSD conditions: evaporator temperature = 30 °C; nebulizer temperature = 30 °C; and evaporator gas N2 = 1.6 SLM.

Fatty acids composition by GC-MS

Fatty acids composition of echium oil and reaction products were analyzed on an Agilent GC MS series 5975 MSD (Palo Alto, Cal., USA) using a HP 88 capillary column (100 m x 0.25 mm, i.d. 0.2 μm) (Agilent, CA, USA). Previous to analysis, fatty acid methyl esters (FAMEs) were prepared by base-catalyzed methanolysis of the echium glycerides (KOH in methanol). 1 μL sample was injected using a split ratio of 1:100. The column was held at 175 °C for 10 min after injection, the temperature programmed at 3 °C/min to 220 °C and held for 20 minutes more. Helium was used as gas carrier, at a constant column flow rate of 1.5 ml/min. The injector temperature was 250 °C and the detector temperature was 230 °C. The mass spectrometer was operated at 70 eV with a mass range from 30 to 400 amu. Fatty acids were identified comparing their retention times and the mass spectra (NIST MassSpectral Library Version 2.0) with those obtained from the standards.

1. **Results and discussion**
2. Pressurized liquid extraction of *Echium plantagineum* seed oil

Echium oil was extracted from *Echium plantagineum* seeds by a green method using pressurized liquids. Given the fact that the development of faster, less solvent consuming, full automation and more environmentally friendly extraction methods has grown in importance during the last decade, the use of PLE has become a consolidated extraction technique. Additionally, to follow the principles of green chemistry and to avoid hazardous and toxic solvents, ethyl acetate was selected to carry out the extraction process. The maximum oil yield was achieved at the temperature of 150 °C (31.0% ± 0.7). Results were compared with an oil extraction reference solvent, hexane, achieving similar oil yield (31.1% ± 0.3) (no significant differences at 1% level). Therefore, echium oil to produce 2-MAGs was freshly extracted using PLE with ethyl acetate, providing an overall extraction process based on eco-friendly approaches.

Enzymatic synthesis of 2-MAGs from echium oil: process optimization

Enzymatic ethanolysis of TAGs is an effective method to produce 2-MAGs from natural sources. Even though, a key factor implied on the success of this reaction is the selection of the type of lipase to be used. In this study, the lipase from *Thermomyces lanuginosus* was selected due to the high regioselectivity towards the positions *sn* -1 and*sn* -3 of the glycerol backbone (Fernandez-Lafuente, 2010), producing 2-MAGs from echium oil by selective hydrolysis.

In a first stage, reaction conditions were optimized to produce the maximum efficiency yield in the shorter reaction time. Specifically, a comparison between commercial TLL from Novozyme (Lipozyme TL IM) and a biocatalyst produced in our laboratory (soluble TLL immobilized by hydrophobic adsorption on Sepabeads C-18) was carried out. To minimize the undesirable acyl migration, which can take place during hydrolysis of TAGs, different reaction conditions were investigated. Acyl migration in the glycerol backbone often leads to the increase of by-products that could significantly affect the purity of final products, like 1-MAGs that could easily hydrolyze due to the specificity of lipase used. The effect of reaction conditions on acyl migration has been elucidated and temperature is one of the most important factors that have a deep influence on the migration rate, since acyl migration is a thermodynamic process (Yang et al., 2005). Thus, in this work, ethanolysis reaction was carried out at mild reaction conditions studying two temperatures, 25 degC and 35 degC. Another critical factor in acyl migration rate is the polarity of the solvent used as reaction medium. Several authors described the synthesis of 2-MAGs by ethanolysis using acetone (Munio et al., 2009; Pfeffer et al., 2007) or hexane (Wang et al., 2014) as reaction medium. However, Li et al. concluded that solvent polarity is a crucial factor for acyl migration: decreasing solvent polarity would increase acyl migration rate (Li et al., 2010). Thus, polar solvents are highly recommended for this reaction. In the present work, ethanolysis was carried out in pure ethanol without the use of an additional solvent as reaction medium. The advantage of using ethanol, besides the minimization of acyl migration, lies in the fact that ethanol is considered a green solvent allowed in the food industry.

Figure 1 shows 2-MAGs percentage based on maximum theoretical yield of total hydrolysis products (maximum theoretical yield = 33% of initial oil TAGs) identified by HPLC-ELSD at the investigated temperatures during reaction progress. After 20 min, the commercial biocatalyst reached the maximum theoretical yield at 25 degC (100%), while the same reaction at 35 degC showed a lower production yield (74.2%). After 1 h, commercial lipase did not show differences between tested temperatures. However, a different behavior was found for TLL adsorbed on Sepabeads C-18, where the initial hydrolysis rate of the immobilized biocatalyst was lower. For instance, at the temperature of 25degC, ethanolysis reaction gave a yield of 20% after 20 min, but after 1 h, 2-MAGs yield was 89.9%. According to these results, TLL immobilized on Sepabeads C-18 needs more time to reach the maximum theoretical yield (see further discussion). Furthermore, the effect of temperature on the synthesis of 2-MAGs for TLL adsorbed on Sepabeads C-18 was more outstanding in comparison with the commercial derivative. After 1 h, 2-MAGs yield was 27.5% at 35 degC, around 3 times less than the yield reached at 25 degC. Therefore, the optimum temperature to produce 2-MAGs from echium oil was established at 25 deg C.

To fully evaluate and understand the synthesis of 2-MAGs, it is also necessary to analyze the lipid composition of the reaction mixture as the reaction time proceed, including TAGs, DAGs and 2-MAGs. Moreover, to select the optimum reaction time for the synthesis of 2-MAGs, in this work, different requirements were stablished: TAGs need to be completely hydrolyzed and DAGs need to be less than 5% of the total reaction products. For this purpose, Figure 2 shows the lipid composition analyzed by HPLC-ELSD as the reaction time proceed catalyzed by tested biocatalysts at 25 degC. As can be seen in Figure 2(a), TAGs were almost completely hydrolyzed by TLL commercial after 20 min, and the reaction mixture was composed by 50% FAEEs (data not show in Figure 2), 1% TAGs, 16% DAGs and 33% of 2-MAGs. After 2 h, commercial lipase hydrolyzed DAGs below 5%.

On the other hand, TLL adsorbed on Sepabeads C-18 (Figure 2(b)) exhibited lower initial activity in comparison with the commercial biocatalyst. This fact could be due to the different enzyme loading in terms of amount of lipase per reaction volume. Since the enzymatic load is not specified in the commercial biocatalyst and in order to minimize this effect, the amount of TLL adsorbed on Sepabeads C-18 required to achieve the same TAGs hydrolytic rate produce by the commercial biocatalyst after 20 min was calculated. As a result, the reaction kinetic of TLL adsorbed on Sepabeads C-18 using equivalent enzyme loading is shown in Figure 2(c). As expected, the use of a higher enzyme loading implied a high initial velocity for the ethanolysis reaction. After 40 min, the theoretical percentage of 2-MAGs was reached and after 2 h, DAGs were less than 5%. Thus, the optimal time for production of 2-MAGs from echium oil was established at 2 h.

Other authors reported the synthesis of 2-MAGs from echium oil using pancreatic lipase, achieving a 2-MAGs yield of 79.1% (percentage expressed as maximum theoretical yield, 33% of total reaction products) (Rincon Cervera et al., 2013). However, the production of 2-MAGs using pancreatic lipase is not recommended if the reaction products are later to be used in the synthesis of structured lipids, since water residues could favor the hydrolytic activity of lipases, carrying out the opposite reaction.

Fractionation and characterization of synthesized 2-MAGs by GC-MS

One of the objectives of the present study was to evaluate if the synthesized 2-MAGs were enriched in the omega-3 SDA. Consequently, once reaction conditions and optimal reaction time were established to synthesize 2-MAGs, reaction products from echium oil were analyzed by GC-MS to evaluate the fatty acid composition. First, the isolation of 2-MAGs from the reaction mixture was done by SPE using a polar stationary phase. Three fractionation steps were necessary to remove the excess of FAEE and to achieve a pure fraction of 2-MAGs. SPE fractions were analyzed by HPLC-ELSD, an example is shown in chromatogram in Figure 3. As can be seen, the fractionation process was successfully done. Figure 3(b) shows the reaction mixture before fractionation process (original reaction products), which were composed by 2-MAGs and FAEEs. Finally, after the fractionation process by SPE, a pure fraction of 2-MAGs was achieved (Figure 3(d)).

The purified 2-MAGs were analyzed by GC-MS to determine the fatty acids content and compare it with the

original oil. Moreover, the effect of biocatalyst type on the fatty acid composition was also studied. Table 1 shows the results of the GC-MS analysis. Fatty acid composition (% of total fatty acids) of original echium oil in growing order of abundance was stearic acid (18:0), palmitic acid (16:0), γ-linolenic acid (18:3 all-cis-6,9,12), linoleic acid (18:2 cis-cis-9,12), stearidonic acid (18:4 all cis-6,9,12,15), oleic acid (18:1 cis-9) and α-linolenic acid (18:3 all-cis-9,12,15). Echium oil was characterized by a low percentage of saturated fatty acids (SFA) (10.2%) and monounsaturated fatty acids (MUFA) (16.8%), while the percentage of polyunsaturated fatty acids (PUFA) (73.0%) was the highest of total fatty acids. Regarding to the omega-3 content, ALA and SDA were identified with a percentage of 35.0% and 15.5%, respectively. Results were in accordance with other authors (Surette, 2013) and previous studies of the research group (Castejón et al., 2018).

On the other hand, synthesized 2-MAG was composed by oleic acid, linoleic acid, γ-linolenic acid, ALA and SDA, being the omega-3 ALA and SDA the major fatty acids. Specifically, the omega-3 SDA was identified in a percentage of 25%, implying than produced 2-MAGs was enriched in the omega-3 SDA 1.6 times compared with the original oil. In addition, the effect of the type of lipase derivative used in the omega-3 composition of 2-MAGs was studied. 2-MAGs synthesized by TLL absorbed on Sepabeads C-18 was characterized by a SDA percentage of 25.5% ± 0.1, similar percentage were identified in 2-MAGs produced by commercial biocatalyst (25.0% +- 0.3) (no significant differences at 1% level). These results demonstrated that the fatty acid composition was not modified regarding the type of lipase used.

Therefore, the enzymatic production of 2-MAGs under mild reaction conditions and low temperature (25 degC) managed to enrich the omega-3 SDA, obtaining an attractive starting material to synthesize structured lipids with potential interest as functional ingredients. Other authors have investigated the enrichment of stearidonic acid from echium oil, however, following a complex process via two-step lipase-catalyzed esterification (Baik et al., 2014; Baik et al., 2015). These results show the ability of enzymatic modification in the enrichment of specific fatty acids such as stearidonic acid.

Stability of lipase derivatives in the synthesis of 2-MAGs from echium oil

Enzyme stability is a crucial factor to determine whether the application of biocatalysts will be commercially successful. Reutilization of biocatalyst produced in our laboratory and commercial derivative in the synthesis of 2-MAGs from echium oil was studied to evaluate the effect of immobilization in the stability of lipase derivatives.

2-MAGs percentage synthesized at 2 h of each lipase derivative was taken as reference value (100%), subsequent reaction yields were calculated accordingly. As can be seen in Figure 4, commercial derivatives had serious limitations for reuse in this reaction. TLL commercial lost 25% of its activity in the second cycle and 70% in the fourth cycle. However, the reuse of TLL adsorbed on Sepabeads C-18 was possible. In this case, after the second cycle the percentage of reuse was 92%, and after five reaction cycles, the average activity of the immobilized lipase was 71%. These results suggest that the immobilization process by hydrophobic interactions carried out for the lipase of *Thermomyces lanuginosus* improves the stability of the produced biocatalyst in comparison with commercial derivatives. Thus, biocatalysts developed in our laboratory seem to have reasonable industrial possibilities, since the lipases can be reused for several cycles in ethanolysis reaction with a little loss of activity.

Enzymatic transesterification of 2-MAG with caprylic acid ethyl esters

In a first approach to synthesize structured triglycerides and to show the potential application of synthesized products, transesterification between the produced 2-MAGs from echium oil with caprylic acid ethyl esters was carried out. The incorporation of short- and/or medium-chain fatty acids into TAGs is the most representative and well-known example of structured lipid. The advantage of using short- and medium-chain fatty acids relies on the fact that these fatty acids are rapidly absorbed into the bloodstream by the intestinal capillaries and efficiently converted into energy, unlike long chain fatty acids, which require bile salts for digestion. Moreover, short- and medium-chain fatty acids are more accessible to lipases due to their small size. This strategy has been used for the synthesis of STAGs with different nutritional purposes (Korma et al., 2018; Utama et al., 2019). For instance, these STAGs included essential long-chain fatty

acids located at *sn* -2 and short- and medium-chain fatty acids located at *sn* -1,3. Specifically, the STAG synthesized in this study was composed by the omega-3 stearidonic acid located at *sn* -2 and caprylic acid located at *sn* -1,3.

As it is known, under favorable conditions, lipases can catalyze esterification reactions, but it is important to control the reaction medium, since these reactions are usually reversible. In the transesterification reaction proposed in the present study, the ethanol produced during esterification must be removed from the medium since it can act as a catalyst for the hydrolysis reaction. For that reason, it is necessary to shift the equilibrium to favor the formation of desired products. The most effective strategies for this purpose are: the application of high temperatures or vacuum, induce the change of state of the product to be eliminated, the use of membrane technology (pervaporation) or the use of molecular sieves as adsorbing agents.

In this study, the use of molecular sieves in transesterification reaction was evaluated by comparing the lipase from *Rhizomucor miehei* immobilized on Sepabeads C-18 in our laboratory and the commercial derivative Novozym 40086. The selection of RML was based on lipase specificity and previous unpublished results from the research group. Moreover, to continue the efforts to promote the development of eco-friendly alternatives, the reaction was carried out in a solvent-free system, acting ethyl esters themselves as reaction medium. Figure 5 shows the reaction kinetic of transesterification between synthesized 2-MAGs and caprylic acid ethyl esters. Furthermore, Figure 6 shows an example of chromatograms corresponding to the HPLC-ELSD analysis at initial time, after 20 min and after 1 h of reaction.

It is noteworthy that the synthesis rate was extraordinarily fast for this reaction, STAGs could be observed after only 20 min (Figure 6.b) and, after 1 h, the reaction was completed, identifying only an excess of FAEEs and the targeted reaction product (Figure 6.c). In contrast with previous results reached in the production of 2-MAGs, lipase adsorbed on Sepabeads C-18 exhibited higher initial activity than the commercial derivative. After 20 min, RML adsorbed achieved a percentage yield of 64% in comparison with 2.9% reached by the commercial biocatalyst. Moreover, the reaction was complete in 1 h by RML adsorbed on Sepabeads C-18, while the commercial lipase did not reach the 100% of STAGs until 4 h. Therefore, the synthesis activity exhibited by RML adsorbed on Sepabeads C-18 was 4 times higher than the activity showed by the commercial derivative. This result suggest that the type of reaction also influences on the efficiency of each lipase derivative on the same support.

Other authors have been described the synthesis of structured lipids from echium oil containing short- and/or medium-chain fatty acids but following other strategies. For example, using free fatty acids from echium oil and tricaprylin by enzymatic acidolysis reactions, but this strategy achieved lower production yields, and also, reaction conditions were more aggressive: 6 hours and 60 degC (Yuksel and Sahin-Yesilcubuk, 2018). Similarly, the incorporation of lauric acid into echium TAGs was studied to synthesize a low-calorie structured lipid (Gokce et al., 2013). In any case, none of these studies involved the previous production of the oil to be used in the further enzymatic reactions. This is a clear advantage for developing integrated processes to improve the nutritional value of natural oils.

Conclusions

In conclusion, the present work is an original approach to develop an integrated process to improve the nutritional characteristics of natural oils, starting with the extraction of the raw material by environmentally friendly methods and following with the production of novel acylglycerols using immobilized lipases. Specifically, this work provides relevant results for the enzymatic production of 2-MAGs under mild reaction conditions and low temperature (25 degC) from echium seed oil, avoiding the undesirable acyl migration. The lipase from *Thermomyces lanuginosus* immobilized on a hydrophobic support reached the maximum theoretical percentage of 2-MAGs in only 2 h. Moreover, immobilized lipase exhibited higher stability than commercial biocatalyst in the synthesis of 2-MAGs. The average activity after 5 cycles was 71%, allowing several reutilization cycles and developing a feasible enzymatic process. Regarding to the fatty acid composition, echium oil has demonstrated to be an excellent source to enzymatically produce 2-MAGs enriched in omega-3 SDA (up to 25%). Additionally, GC-MS analysis demonstrated that the fatty acid composition

was not modified regarding the lipase used. Finally, the use of molecular sieves in combination with the lipase from *Rhizomucor miehei* immobilized on Sepabeads C-18 has shown to be an extraordinarily fast strategy to produce pure STAGs in a solvent-free system (100% yield in 1h), 4 times higher than the activity showed by commercial derivative. Hence, the enzymatic processes developed in this study open up a range of possibilities to synthesize acylglycerols high in stearidonic acid with improved nutritional properties from valuable raw materials, proving the usefulness of immobilized lipases to produce novel functional lipids. Furthermore, the improvement of biocatalyst stability and activity by lipase immobilization represents an important advance for its applications at industrial levels.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1. Fatty acid composition determined by GC-MS of original echium oil extracted with PLE using ethyl acetate and of purified 2-MAGs synthesized by TLL commercial and TLL absorbed on Sepabeads C-18 at the optimal reaction time.

		% Fatty acids[a]	**% Fatty acids[a]**	**% Fatty acids[a]**
Fatty acid	**RT (min)**	**Original oil[b]**	**2-MAGs synthesized by TLL commercial[c]**	**2-MAGs synthe**
16:0	12.8	6.5 ± 0.6	-	-
18:0	16.4	3.6 ± 0.1	-	-
18:1	17.5	16.8 ± 0.1	16.7 ± 0.2	16.0 ± 0.1
18:2	19.3	12.5 ± 0.4	16.9 ± 0.1	16.6 ± 0.2
18:3 n-6	20.5	10.0 ± 0.2	17.9 ± 0.3	18.0 ± 0.2
18:3 n-3	21.3	35.0 ± 1.1	23.5 ± 0.1	24.0 ± 0.0
18:4 n-3	22.6	15.5 ± 0.1	25.0 ± 0.3	25.5 ± 0.1
SFA	**SFA**	10.2	-	-
MUFA	**MUFA**	16.8	16.7	16.0
PUFA	**PUFA**	73.0	83.3	84.0
n-6	**n-6**	22.5	34.8	34.6
n-3	**n-3**	50.5	48.5	49.4
n-6/n-3 ratio	**n-6/n-3 ratio**	0.4	0.7	0.7

RT. retention time; SFA. saturated fatty acids; MUFA. monounsaturated fatty acids; PUFA. polyunsaturated fatty acids.

[a] Results expressed as percent over the total content (relative content). Values are the mean ± SD of two determinations.

[b] Original oil extracted using PLE with ethyl acetate as solvent at 150 °C.

[c] Pure fraction of 2-MAGs synthesized by commercial biocatalyst at optimal time (2 h).

[d] Pure fraction of 2-MAGs synthesized by TLL immobilized on Sepabeads C-18 at optimal time (2 h).

Figure legends

Figure 1. 2-MAGs percentage based on maximum theoretical yield at different reaction times using TLL adsorbed on Sepabeads C-18 and TLL commercial at studied reaction times and temperatures.

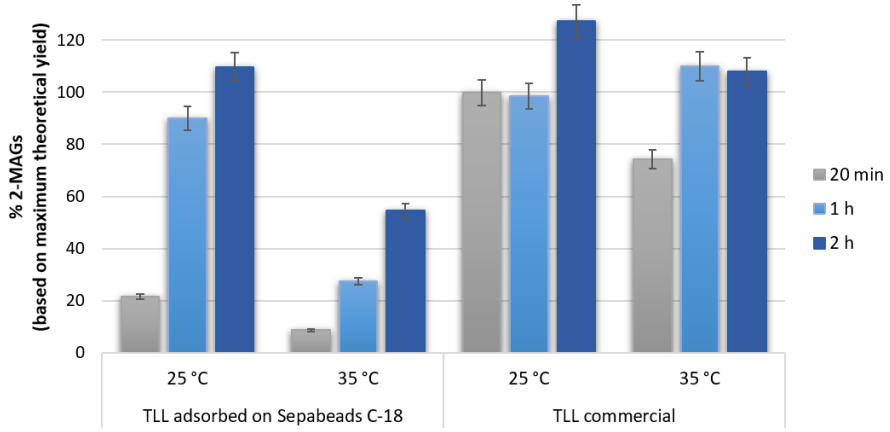
Figure 2. Reaction kinetics of 2-MAGs production from echium oil at optimum temperature (25 °C) catalyzed by TLL commercial (a), TLL adsorbed on Sepabeads C-18 (b) and TLL adsorbed on Sepabeads C-18 using equivalent enzyme loading to commercial biocatalyst (c). (*) TAGs, ($^{)}$ $DAGs$, () $2-MAGs$.

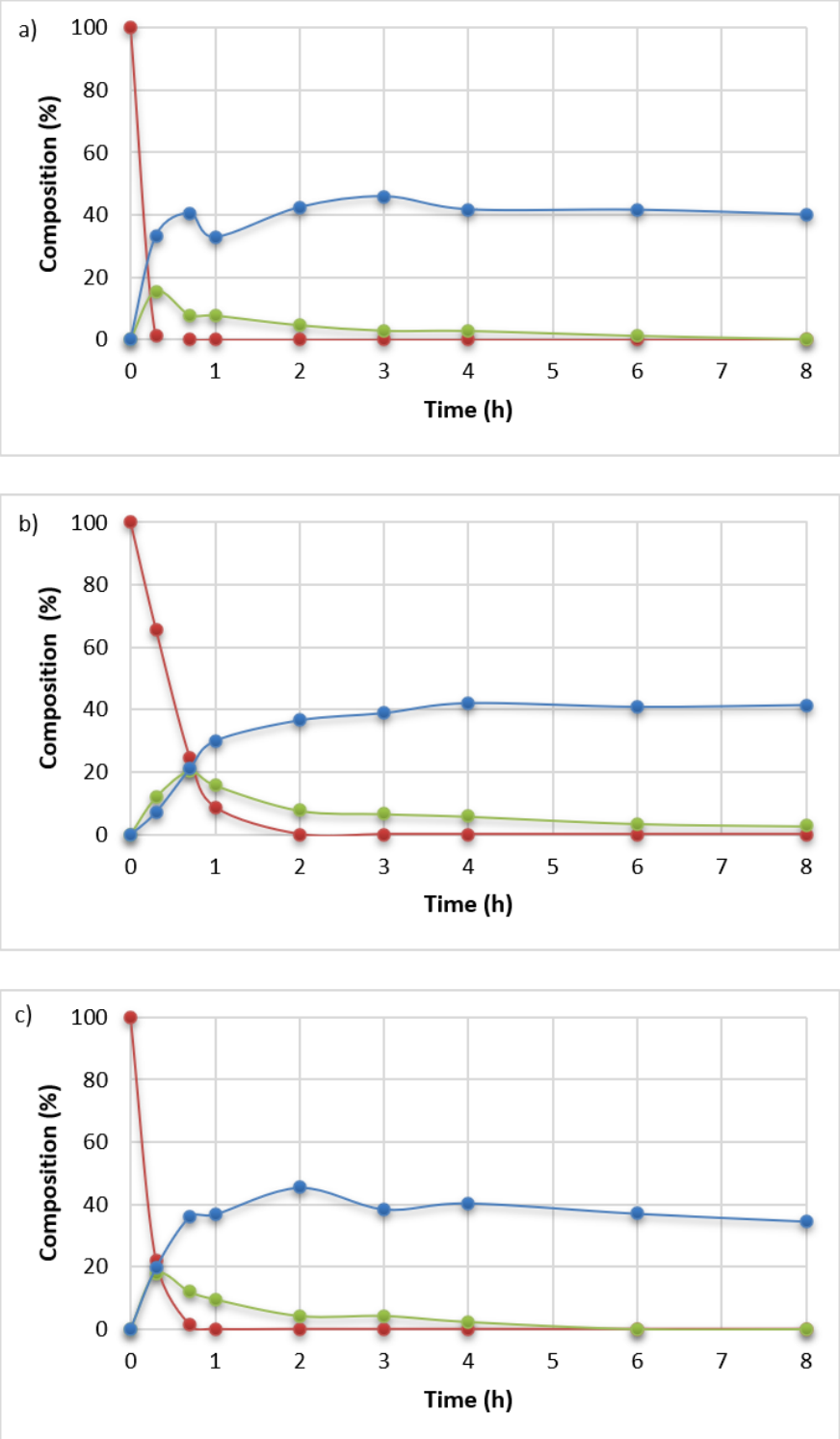
Figure 3. Fractionation of synthesized 2-MAG by SPE at optimal reaction time analyzed by HPLC-ELSD: standard mixture of different species of lipids (a), original reaction (b), SPE fraction composed by FAEEs (c) and SPE fraction composed by pure 2-MAGs (d).

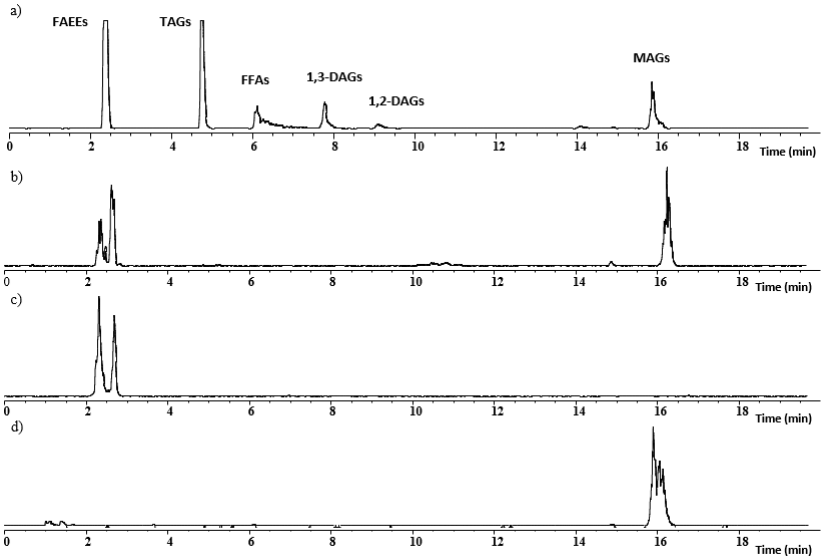
Figure 4. Recovered activity (%) of TLL adsorbed on Sepabeads C-18 and TLL commercial for 2-MAGs production after five reaction cycles. Reutilization was evaluated in reaction cycles of 2 h (optimal reaction time).

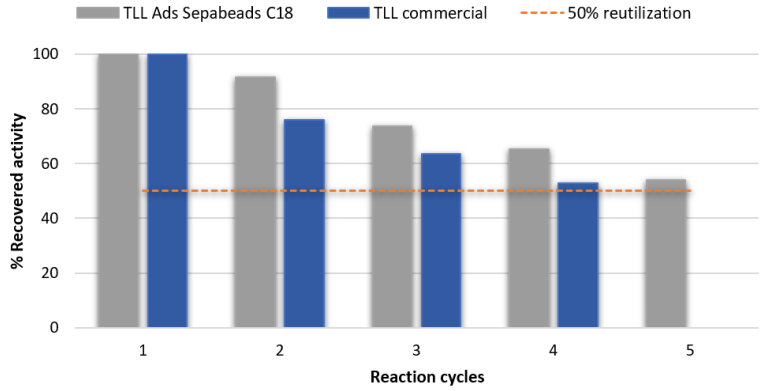
Figure 5. Reaction kinetic of transesterification reaction between synthesized 2-MAGs and caprylic acid ethyl esters catalyzed by RML commercial (a) and RML adsorbed on Sepabeads C-18 (b). (*) STAGs, ( ) $DAGs$, ( ) $2-MAGs$.

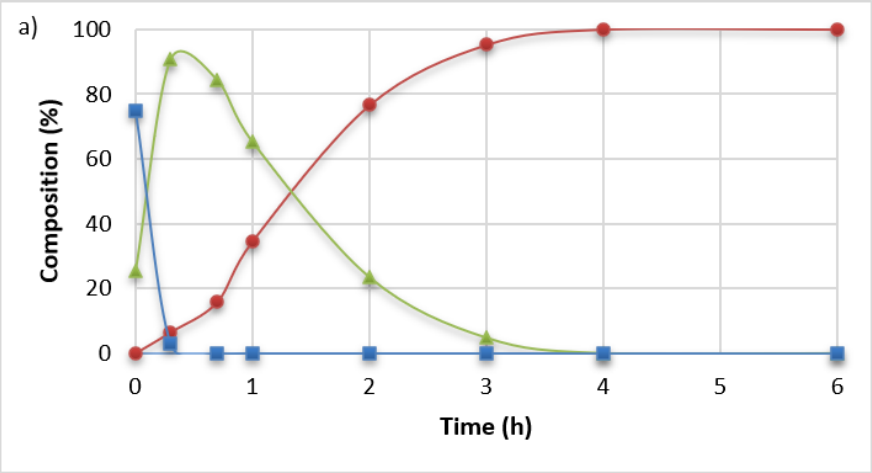
Figure 6. Chromatograms obtained by HPLC-ELSD of transesterification reaction between synthesized 2-MAGs and caprylic acid ethyl esters: reaction products at initial reaction time (a), reaction products after 20 min (b) and reaction products after 1 h (c).

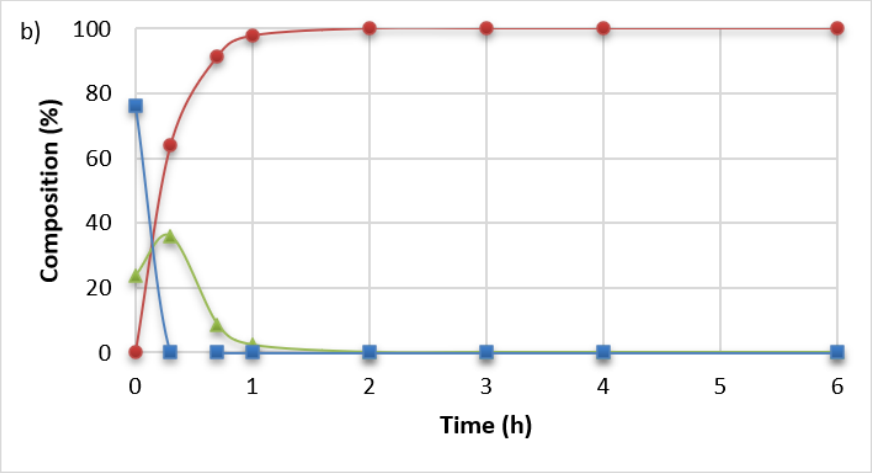












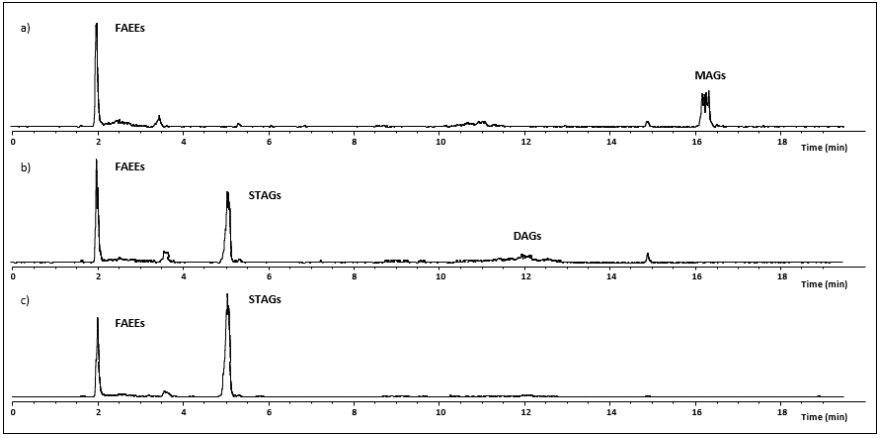


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Table 1.rtf available at https://authorea.com/users/350789/articles/475524-the-potential-use-of-immobilized-lipases-in-the-synthesis-of-omega-3-monoacylglycerols-enriched-in-stearidonic-acid