Production of structured triacylglycerols by acidolysis catalyzed by lipases immobilized in a packed bed reactor

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Abstract

The aim of this work was to produce structured triacylglycerols (STAGs), with caprylic acid located at positions 1 and 3 of the glycerol backbone and docosohexaenoic acid (DHA) at position 2, by acidolysis of tuna oil and caprylic acid (CA) catalyzed by lipases Rd, from *Rhizopus delemar*, and Palatase 20000L from *Mucor miehei* immobilized on Accurel MP1000 in a packed bed reactor (PBR), working in continuous and recirculation modes. First, different lipase/support ratios were tested for the immobilization of lipases and the best results were obtained with ratios of 0.67 (w/w) for lipase Rd and 6.67 (w/w) for Palatase. Both lipases were stable for at least 4 days in the operational conditions. In the storage conditions (5 ◦C) lipases Rd and Palatase maintained constant activity for 5 months and 1 month, respectively.

These catalysts have been used to obtain STAGs by acidolysis of tuna oil and CA in a PBR operating with recirculation of the reaction mixture through the lipase bed. Thus, STAGs with 52–53% CA and 14–15%DHA were obtained. These results were the basis for establishing the operational conditions to obtain STAGs operating in continuous mode. These new conditions were established maintaining constant intensity of treatment (IOT, lipase amount×reaction time/oil amount). In this way STAGs with 44–50% CA and 17–24% DHA were obtained operating in continuous mode. Although the compositions of STAGs obtained with both lipases were similar, Palatase required an IOT about four times higher than lipase Rd.

To separate the acidolysis products (free fatty acids, FFAs, and STAGs) an extraction method of FFAs by water–ethanol solutions was tested. The following variables were optimized: water/ethanol ratio (the best results were attained with a water/ethanol ratio of 30:70, w/w), the solvent/FFA–STAG mixture ratio (3:1, w/w) and the number of extraction steps (3–5). In these conditions highly pure STAGs (93–96%) were obtained with a yield of 85%. The residual FFAs can be eliminated by neutralization with a hydroethanolic KOH solution to obtain pure STAGs. The positional analysis of these STAGs, carried out by alcoholysis catalyzed by lipase Novozym 435, has shown that CA represents 55% of fatty acids located at positions 1 and 3 and DHA represents 42% of fatty acids at position 2.

1. Introduction

The production of structured triacylglycerols (STAGs) for clinical [1,2] and nutritional purposes [3] is a subject of interest. Most attention has focused on triacylglycerols (TAGs) with medium-chain fatty acids (M) located in positions 1 and 3 of the glycerol backbone and a functional long-chain polyunsaturated fatty acid (PUFA) in position 2 (MLM structure). This composition and structure is interesting because absorption of the fatty acids depends on their type and on their position in the TAG molecule. Thus, pancreatic lipase, which is 1,3-specific, hydrolyzes the TAG of the diet giving free fatty acids and 2-monoacylglycerol (2-MAG) [4]; in addition this lipase shows higher activity toward medium-chain fatty acids than toward long-chain ones, especially PUFAs [5,6]. The liberated medium-chain free fatty acids are directly absorbed into the portal vein and used to provide energy [7,8], while the 2-MAG (with the essential long-chain fatty acid) are well absorbed via the lymphatic route [4] and are often used in biosynthesis [7,9]. In this way STAGs may provide a balanced nutrition.

The aim of this work is to obtain STAGs rich in caprylic acid (CA), located in positions 1 and 3 of the glycerol backbone, and docosohexaenoic acid (DHA) in position 2. This fatty acid is being used as an additive to the milk formula for premature children, due to its importance in the development of the central nervous system [1,10].

The simplest and most direct route for the synthesis of STAGs of MLM type is the acidolysis between long-chain TAGs (with a high content of a functional fatty acid in position 2, L), and a medium- chain free fatty acid (M), catalyzed with a 1,3-specific lipase [11–18]. Lipases offer high catalytic efficiency, specificity and selectivity by incorporation of the required acyl group into a specific position of native TAGs. In addition, the process takes place in very mild conditions that maintain the structure of the functional fatty acid unaltered.

The production of STAGs by acidolysis has been studied in our laboratory [17–19]. The first catalyst used [17,18] was Lipozyme IM (Novo Nordisk A/S, Denmark), which is a 1,3-specific lipase, immobilized on an ion exchange resin. However, this resin may catalyze the acyl-migration of the long-chain acyl groups in theDAGs (which are intermediates in the acidolysis reaction) from position 2 to position 1 or 3 [20,21]. This undesired reaction eliminates the long-chain acyl group from position 2, decreasing the yield in STAGs of MLM type. In a posterior work [19] lipases Rd, from *Rhizopus delemar*, D, from*Rhizopus oryzae*, and AK, from *Pseudomonas fluorescens* were used immobilized on Accurel MP1000; this support is microporous polypropylene, which lacks superficial charges and as a result no acyl-migration was observed. With these catalysts STAGs were obtained with over 50% CA content at positions 1 and 3 and over 13% DHA content at position 2. These results were obtained immobilizing the lipase in a packed bed reactor (PBR) through which the reaction mixture was recirculated (operation in discontinuous mode).

The separation of the reaction products is an important aspect, which is often afforded less importance. The major products of an acidolysis reaction are FFAs and TAGs. The separation of these products is usually carried out by neutralization of FFAs with a hydroalcoholic solution of KOH or NaOH (forming soaps soluble in the hydroalcoholic phase) and extraction of TAGs by hexane [19]. However, this method can give low TAG recovery yields high amounts of FFAs lead to the formation of many soaps, in which a considerable proportion of TAGs remains occluded. In this work the separation of FFAs and TAGs has been tested by extraction of the former with water–ethanol mixtures. In this

method the most important variable to optimize is the water/ethanol ratio, which depends on the type of FFAs and TAGs [22,23].

This work, therefore, has four main objectives: (i) to immobilize the lipases Rd, from *R. delemar*, and Palatase 20000L, from *Mucor miehei*, on Accurel MP1000, (ii) to determine the activity and stability of these immobilized lipases, (iii) to obtain STAGs of MLM type by acidolysis of tuna oil (highly rich in DHA) and CA, catalyzed by these lipases immobilized in a PBR operating in discontinuous and continuous modes, and (iv) to test the purification of STAGs by extraction of FFAs by water–ethanol mixtures.

2. Materials and methods

2.1. Lipases and chemicals

The lipases used were: Palatase 20000L from *M. miehei* (kindly donated by Novozymes A/S, Bagsvaerd, Denmark) and lipase Rd from *R. delemar* (Europa Bioproducts Ltd, Cambridge, UK). These lipases are 1,3-specific. The support used to immobilize these lipases was Accurel MP1000 (Membrana GmbH, Obernburg, Germany), which is a microporous support of polypropylene with a particle size ranging between 0.4 and 1mm.

For the acidolysis reactions the following chemicals were used: caprylic acid (CA, 8:0, over 98% pure and a molecular weight of 144.2 g/mol, Sigma–Aldrich, St. Louis, MO, USA), cod liver oil (Acofarma, Terrassa, Barcelona, Spain), tuna oil (with a DHA (22:6n3) content of 20%, kindly donated by Brudy Technology S.L., Barcelona, Spain) and analytical grade hexane (95% purity, Panreac S.A., Barcelona, Spain). Table 1 shows the fatty acid composition of the cod liver and tuna oils, neither of which contained partial acylglycerols, as verified by thin layer chromatography.

Table 1. Fatty acid composition of cod liver and tuna oils (mol %).

Fatty acid	Cod liver oil (CLO)	Tuna oil
14:0	4.7	4.8
16:0	13.1	20.8
16:1n7	9.4	7.0
16:2n4	1.5	1.3
16:4n1	-	-
18:0	2.8	5.7
18:1n9	20.8	15.9
18:1n7	6.2	3.0
18:2n6	1.3	1.9
18:3n3	0.2	0.7
18:4n3	1.5	1.1
20:1n9	11.4	3.0
20:2n6	-	0.4
20:3n6	-	0.3
20:4n6	-	2.1
20:4n3	-	0.6
20:5n3 (EPA)	8.6	7.3
22:1n9	8.2	2.0
21:5n3	-	0.4
22:5n3	1.2	1.7
22:6n3 (DHA)	9.2	20.1
Average molecular weight (Da)	910.0	994.4

2.2. Immobilization of lipases

The lipases were immobilized on Accurel MP1000 at different lipase/support ratios, following the procedure described by Soumanou et al. [24]: different amounts of lipase (between 1 and 10 g, Table 2) were dissolved in 25ml phosphate buffer (20mM, pH 6). The solution was added to 1.5 g Accurel MP1000 previously mixed with 5ml ethanol. This mixture was stirred at 150rpm at room temperature for 8 h, after which 5 ml chilled acetone (−15 ºC) was added. The immobilized lipase was collected by filtration, washed three times with phosphate buffer (20mM, pH 6.0), dried at room temperature under vacuum for 48 h, and stored at 5 ºC until use. The lipase activity wasmeasured by acidolysis reactions in the batch reactor as described below.

2.3. Acidolysis in the batch reactor

The reaction mixture consisted of cod liver oil, 150mg; CA, 150mg; hexane, 5ml and immobilized lipase, 125 mg. These amounts determined a CA/cod liver oil molar ratio of m_0 = 6. This reaction mixture was placed in 50-ml Erlenmeyer flasks with silicone-capped stoppers. The mixture was incubated at 40 ºC and agitated in an orbital shaking air-bath at 400rpm for 24 h (Inkubator 1000, Unimax 1010 Heidolph, Klein, Germany). The reaction was stopped by separation of lipase by filtration and the reaction product was stored at −24 ºC until analysis. All reactions and corresponding analyses were carried out in triplicate. Standard deviations were always lower than 8%. In this way lipase activity was determined in order to choose the best lipase/support ratio and to evaluate the lipase stability. This activity was determined as the incorporation rate of CA to TAGs of cod liver oil.

Table 2. Influence of the lipase/support ratio on the molar fraction of CA incorporated to the TAG of the cod liver oil (F_M).

Lipase	Ratio lipase/support (w/w)	F_M (% mol)
Rd from *Rhizopus delemar*	0.67	47.9
	0.25	6.3
PALATASE from *Mucor miehei*	[c]0.67	14.0
	[c]1.33	34.1
	[c]2.67	37.4
	[c]4.00	42.2
	[c]5.33	45.7
	[c]6.67	48.6

[a] Weight of the dissolved protein in the buffer solution supplied by the manufacturer.

2.4. Acidolysis in the packed bed reactor (PBR)

Fig. 1 shows a scheme of the reaction system. The immobilized lipase was packed into a glass column (1.5 i.d. and 2.5 i.d. × 25cm length) covered with aluminum foil to prevent photo-induced oxidation. The enzyme bed was held between two mobile perforated disks, which permit the bed volume to be adjusted to the volume of the packed lipase. The substrate mixture and the column were jacketed to maintain the reaction temperature at 30 ºC. The content of this reservoir bottle was continuously agitated at 130rpm by a magnetic stirrer. A three-way valve was placed at the exit of the lipase bed, permitting two modes of operation (Fig. 1): (1) by recirculation of the mixture coming out of the bed to the substrate reservoir (discontinuous mode), or (2) by directing the reaction mixture leaving the bed to an additional reservoir (continuous mode). Samples were stored at −24 ºC until analysis. All analyses were carried out in triplicate. The standard deviation was always below 8%.

When the system was operated with recirculation, the reaction was monitored by sampling at different times (between1and169 h) in the substrate reservoir (Fig. 1). In a typical experiment the reaction mixture contained 20 g tuna oil, 17 g CA, 200ml *n*-hexane and 5.5 g lipase Rd or Palatase immobilized on Accurel MP1000. The reaction mixture (tuna oil, caprylic acid and hexane) was pumped up through the column (15mm×250mm) by a peristaltic pump at a flow rate of 200ml/h.

When the packed bed reactorwas operated in continuous mode samples were taken at the exit of the bed (Fig. 1). These experiments were carried out maintaining a constant CA/oil tuna molar ratio (m_0 = 6), temperature (30 ˚C) and the lipase amount immobilized in the bed (14.1 g immobilized in the column of 25mm×250 mm).

When lipase Rd was used the reaction mixture contained 200 g tuna oil, 170 g CA and 500ml hexane ($[TG]_0$ = 222.3 mol/m3); with lipase Palatase the reaction mixture contained: 20 g tuna oil, 17 g CA and 200ml hexane ($[TG]_0$ = 83.6 mol/m^3).

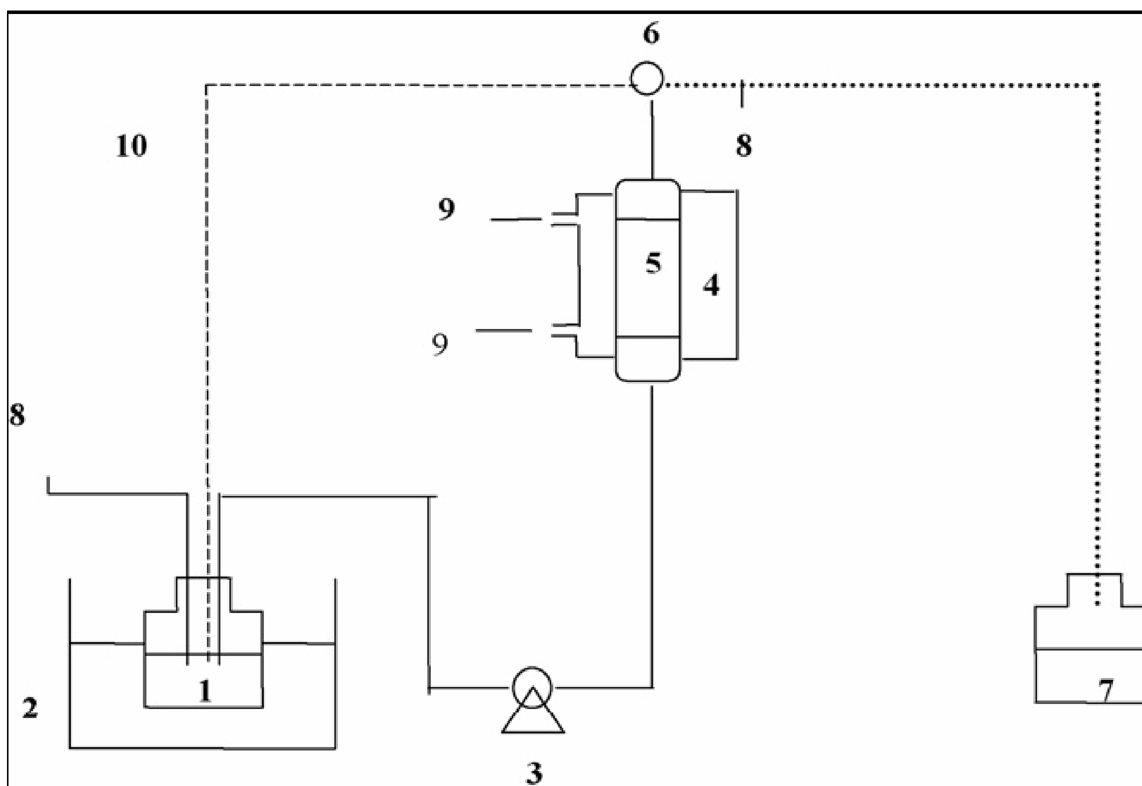

Fig. 1. Immobilized lipase packed bed reactor (PBR). (1) Substrate reservoir, (2) reactor temperature control, (3) peristaltic pump, (4)water jacket, (5) bed of immobilized enzyme, (6) three-way valve, (7) product reservoir, (8) sampling, (9) cooling/heating water, and (10) recirculation. The dashed line describes the flow of materials for recirculating operation; the dotted line describes the flow for the continuous mode.

2.5. Purification of structured triacylglycerols (STAGs)

STAGs were purified following the procedure described by Gonçalves et al. [23] and Rodrigues et al. [24]: 100 g of the final reaction mixture (STAGs and free fatty acids, FFAs) were mixed with water–ethanol solution. This mixture was shaken for 15min and then centrifuged at 3000rpm for another 15 min. Next, this mixture was decanted for at least 12 h and the two phases formed (hydroalcoholic and oily phases) were separated for posterior analysis. The variables studied in this work were: the water/ethanol ratio (from 30/70 to 45/55 (w/w)), the number of extractions (up to 5) and the reaction mixture (STAG, FFA)/hydroalcoholic solution ratio (1/1 and 1/3 (w/w)).When several extractions were carried out the oily phase was again mixed with a new water–ethanol solution (maintaining the oily/hydroalcoholic solution ratio constant) and the whole process was repeated. The STAGs purified by this method still contained a small amount of FFAs, which were eliminated by dissolving this mixture in hexane (50 ml hexane/g STAG–FFA mixture) and neutralizing the FFAs with a hydroethanolic solution (20% ethanol) of KOH 0.5N (11ml hydroethanolic solution/g STAG–FFA mixture). This mixture was agitated, the two phases decanted, the hexane from the STAG solution evaporated and the extracted STAGs

weighed. The chemicals used in this extraction were of PRS quality (Panreac S.A., Barcelona, Spain).

2.6. Determination of the positional distribution of fatty acids in STAG by alcoholysis catalyzed by Novozym 435

In order to determine the percentage of each fatty acid in position 2 of the STAG the following procedure was employed: 0.815ml of STAGs, 375mg lipase Novozym 435 and 3.8 ml of absolute ethanol were agitated at 300rpm in a batch reactor (Section 2.3 of this work) for 2 h at 35 ◦C; then this reaction was then stopped by separating the enzyme of the reaction mixture by filtration, washing the filter with *n*-hexane and fitting the final volume of the product reaction-hexane solution to 25 ml [25]. This solution was stored at −24 ◦C until analysis by TLC and GC. 2-MAGs from alcoholysis were separated by TLC and analyzed by GC. This fatty acid composition indicated the fatty acid composition at position 2 of STAGs. The fatty acid composition at positions 1 and 3 was determined from this analysis and from the composition of STAGs.

2.7. Identification of the reaction products and determination of molar fractions of fatty acids in these reaction products

The acidolysis reaction leads to a mixture of triacylglycerols (TAGs) and free fatty acids (FFAs), while the alcoholysis reaction leads to a mixture of monoacylglycerols, diacylglycerols, free fatty acids, TAGs and ethyl esters. These acylglycerols and FFAs were identified by preparative thin layer chromatography (TLC) and the fatty acid composition of each acylglycerol type was determined by gas chromatography (GC). The identification of acylglycerols by TLC was carried out on plates of silica-gel (Precoated TLC plates, SIL G-25; Macherey-Nagel, Sigma–Aldrich) activated by heating at 105 ◦C for 30 min. The samples were spotted directly on the plate by adding 0.2 ml of reaction product mixture. To identify the acidolysis products the plate was developed in chloroform/acetone/methanol (95/4.5/0.5, v/v/v) and in hexane/di-isopropil ether/glaciar acetic acid (75/25/2, v/v/v) to identify the alcoholysis products. Spots of each lipid were visualized by spraying the plate with iodine vapour in a nitrogen stream [26]. The position of the spot corresponding to each lipidic species is characterized by the response factor (RF), which is the ratio between the distances travelled by the solute and by the mobile phase. This RF is characteristic of each solute for a given chromatographic system and depends primarily on the mobile phase used.

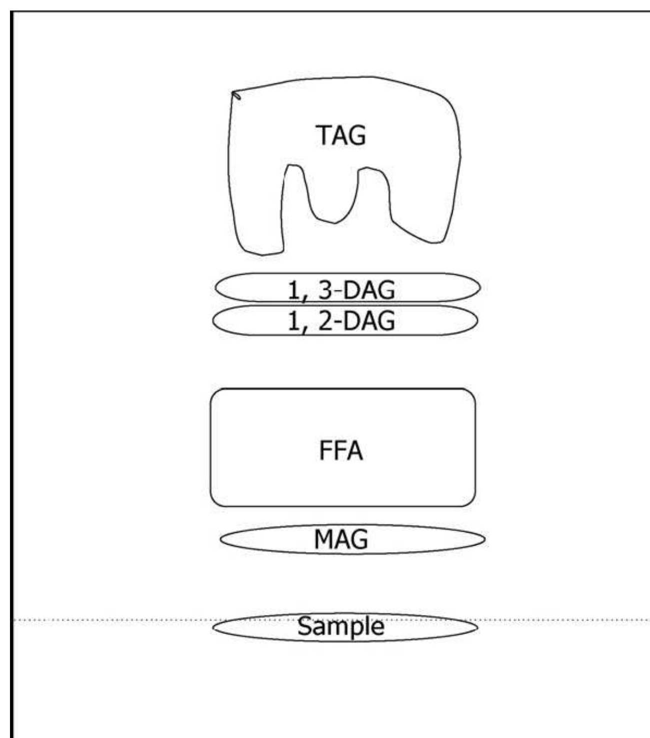

Fig. 2. Separation of lipidic species by thin layer chromatography using chloroform/ acetone/methanol (95/4.5/0.5, v/v/v) as mobile phase.

As example, Fig. 2 shows the lipid separation achieved by chloroform/acetone/methanol (95/4.5/0.5, v/v/v) in a sample with all the lipidic species (this mobile phase does not separate TAG and ethyl esters). Fractions corresponding to each lipid typewere scraped fromthe plates and methylated according to the method of Lepage and Roy [27].Methylation and methyl ester analysis by GC have been described elsewhere [17]. Nonadecanoic acid (19:0) (Sigma–Aldrich) was used as an internal standard for quantitative determination of fatty acids. The amounts of fatty acids were calculated by the equation:

$$Fatty\,acid\,(mg) = \left[19:0(mg)\right]\frac{f_X\ area_X}{area_{19:0}} \qquad (1)$$

where f_X is the fatty acid response factor of a fatty acid X. These response factors were close to 1 for all the fatty acids of molecular weight close to the internal standard, and therefore $f_X = 1$was taken for all the fatty acids, except for CA and DHA, for which f_X = 1.52 and 1.24, respectively. These factors were calculated from Eq. (1), by comparing the chromatographic areas of known amounts of 19:0, CA and DHA. Fig. 3 shows a typical chromatogram. All the chemicals used in these analytical methods were of analytical quality and acquired from Sigma–Aldrich (St. Louis, MO).

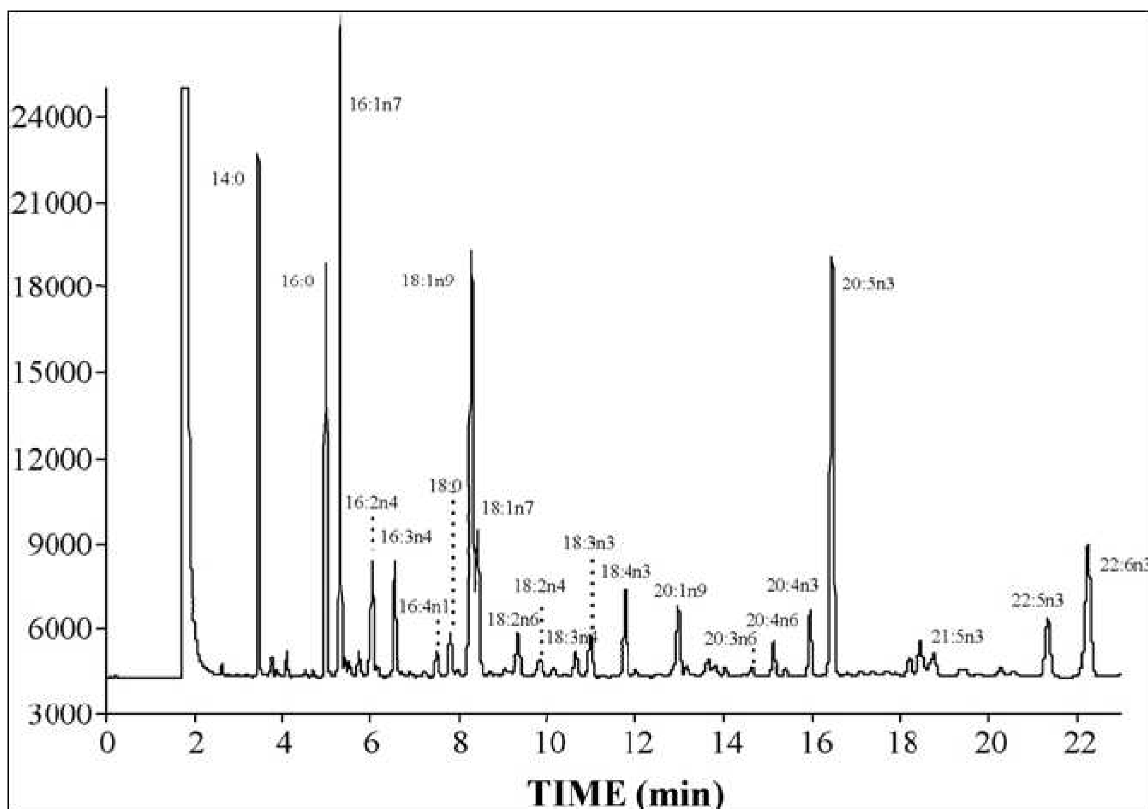

Fig. 3. Separation of methyl esters by gas chromatography.

3. Results and discussion

3.1. Immobilization of lipases: determination of the best lipase/support ratio and stability of lipases

In previous works high yields of structured triacylglycerols (STAGs) with the structure CA-PUFA-CA (MLM) were obtained by acidolysis of fish oils (cod liver and tuna oils and a commercial fish oil highly rich in eicosapentaenoic acid (EPA)) with caprylic acid (CA), catalyzed by Lipozyme IM and several lipases immobilized on Accurel MP1000 [17–19]. This latter is a microporous support of polypropylene which lacks superficial charges and does not catalyze acyl-migration, as occurs with Lipozyme IM [20,21]. Acyl-migration eliminates the long chain acyl group frompositon 2 in the acylglycerols, diminishing the yield of STAGs with long-chain fatty acids in position 2 (MLM structure).

In this work Accurel MP1000 has been used to immobilize the lipases Rd, from *R. delemar*, and Palatase 20000L, from *M. miehei*. Lipozyme IM also proceeds from *M. miehei*, but it is immobilized on an ionic exchange resin that catalyzes acyl-migration. Palatase 20000L is provided without immobilizing, which allows it to be immobilized on other supports with no superficial charges, such as Accurel MP1000. Table 2 shows the lipase/support ratios tested and the molar fractions of CA incorporated to TAGs of cod liver oil. This table shows that with lipase Rd the best result was obtained with a lipase/support

ratio of 0.67 (w/w) and that to attain similar activity with Palatase the required ratio was higher than 4. It should be noted that lipase Rdwas supplied by Europa Bioproducts Ltd in solid form with an activity ≥500,000 U/mg, while Palatase was supplied by Novozymes in liquid form with an activity of 20,000 U/g.

The stability of both lipases immobilized on Accurel MP1000 in the operational conditionswas determined by carrying out acidolysis experiments for more than 4 days. Fig. 4 shows that no decrease in activity was observed during that time. The stability of these immobilized lipases was also determined in the storage conditions (5 ºC); at different storage times lipase activity was determined by discontinuous acidolysis experiments.

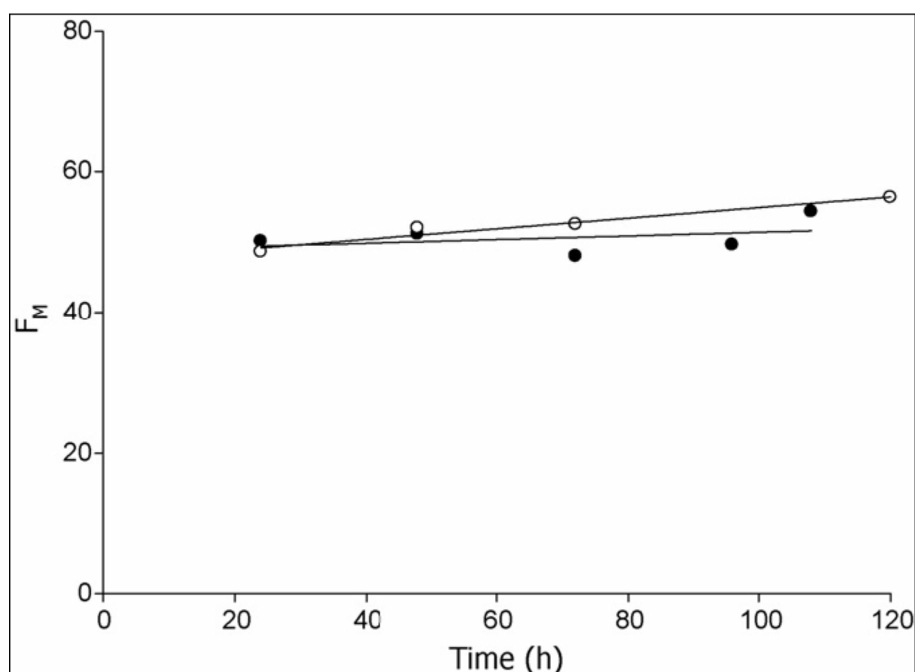

Fig. 4. Stability of lipases Rd (●) and Palatase 20000L (○) immobilized on Accurel MP1000 in lipase/support ratios 0.67 (w/w) and 6.67 (w/w), respectively, under operational conditions: influence of the reaction time on the molar fraction of CA incorporated to TAG, *F*M. *Operational conditions*: acidolysis of cod liver oil and CA in batch reactor for Palatase (125mg Palatase, 24 h, 150mg cod liver oil and 150mg CA in a bath reactor at 40 ◦C); acidolysis in the PBR, in continuous mode, for lipase Rd (60 g CLO, 60 g CA and 2.5 g lipase at 30 ºC).

Fig. 5 shows that Palatase maintained constant activity for around 30 days, but after approximately two months storage a decrease in activity was observed; however, lipase Rd maintained the activity constant over 5 months of storage at 5 ºC.

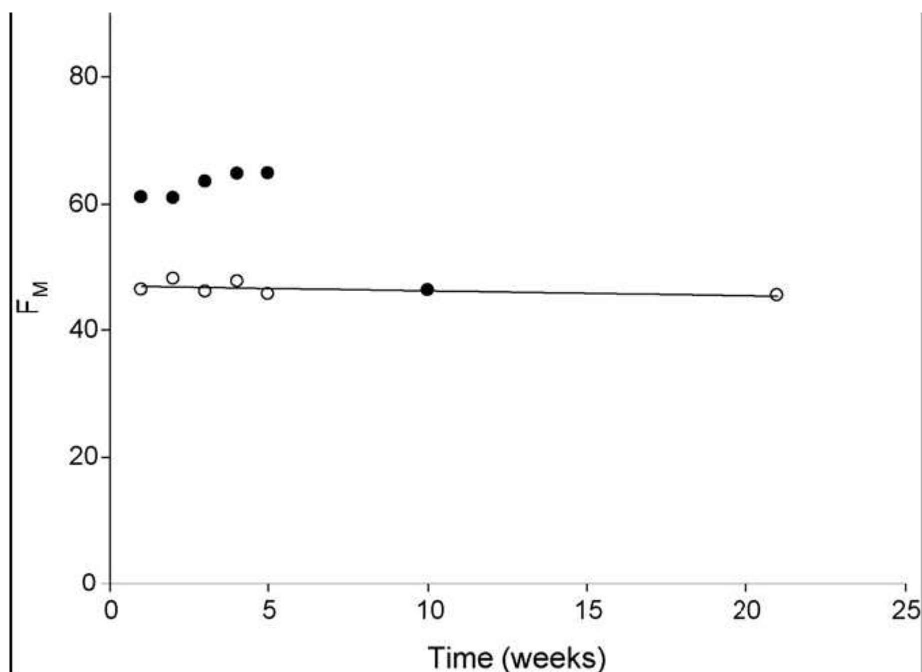

Fig. 5. Stability of lipases Rd (○) and Palatase 20000L (●) immobilized on Accurel MP1000 in lipase/support ratios 0.67 (w/w) and 6.67 (w/w), respectively, under storage conditions (5 ◦C): influence of storage time on the molar fraction of CA incorporated to TAG, *F*M. *Operational conditions*: 125mg Palatase, 48 h, 150mg cod liver oil and 150mg CA in a bath reactor; 2,5 g lipase Rd, 1 h, 3 g cod liver oil and 3 g CA in a PBR.

3.2. Production of STAGs by acidolysis of tuna oil and CA catalyzed by lipases Rd and Palatase immobilized on Accurel MP1000 in a packed bed reactor (PBR)

3.2.1. Operation in discontinuous mode

In the experiments carried out in batch reactors, the immobilized lipase particles remain suspended in the reaction mixture due to the agitation. However, one of the most common methods used for the fluid–solid contact in heterogeneous catalysis is to place the catalyst in a PBR through which the reaction mixture is pumped; this system facilitates the operation in continuous mode, the contact and separation of reaction mixture and lipase and, therefore, the reutilization of the catalyst.

First, the acidolysis process was carried out operating in discontinuous mode, i.e. recirculating the reaction mixture through the PBR and the substrate reservoir until attaining the equilibrium or a given conversion. In these conditions the operation is equivalent to the one carried out in a stirred tank reactor (STR) if the flow rate through the PBR is high and therefore the influence of the external mass transfer on the reaction rate is negligible [17,18]. To attain these conditions the pressure drop of the reaction mixture in the bed must be low and, therefore, the PBR must have a high porosity and the reaction

mixture a low viscosity. In this respect, a previous study [19] observed that the reaction rate was lower at high substrate concentrations and the lowest incorporation of CA to TAGs was obtained when no solvent was used.

Figs. 6 and 7 show the variation of the content in CA and in the major native fatty acids of tuna oil (DHA, EPA, palmitic and oleic acids) with the intensity of treatment (IOT) in the acidolysis of tuna oil and CA, catalyzed by lipases Rd and Palatase, respectively, both immobilized on Accurel MP1000, in a PBR operating with recirculation. IOT is the lipase amount×reaction time/(initial oil concentration×reaction mixture volume) ($m_L t/[TG]_0 V$) and the reaction rate is proportional to this variable if no enzyme deactivation occurs [17,28]. Fig. 6 shows that with lipase Rd a maximal equilibrium incorporation of CA to TAG of about 52% was attained at a minimal IOT of around 650 g lipase h/mol oil (5.5 g lipase×24 h/200 g oil); DHA content decreased from 24% to14% in the final STAG (144 h or IOT of 3938 g lipase h/mol TAG), although, for example, STAGs with 20% DHA and 45% CA can be obtained at a reaction time of 10 h (or IOT = 270 g lipase h/mol TAG).

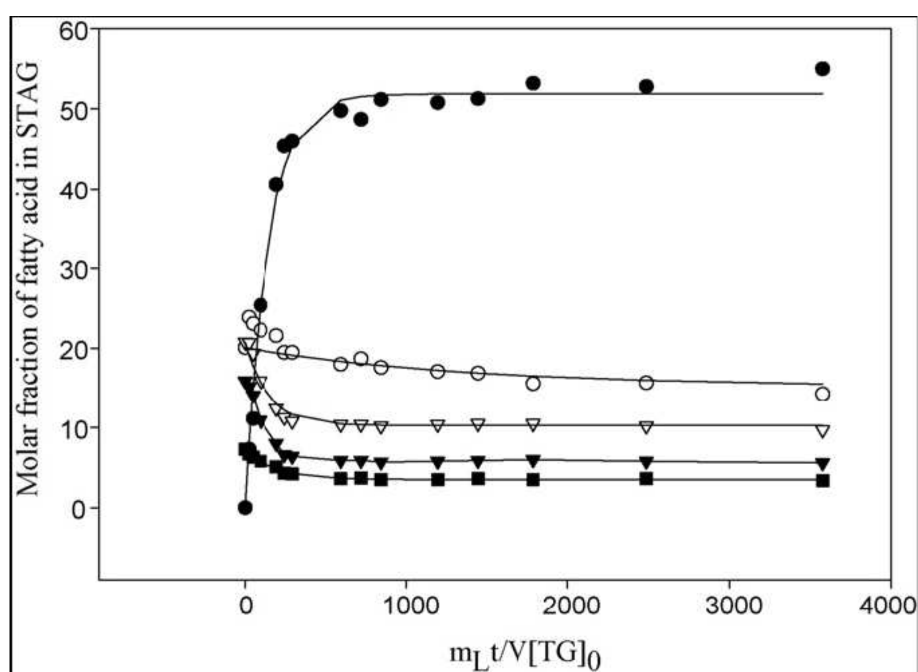

Fig. 6. Acidolysis of tuna oil and CA catalyzed by lipase from *Rhizopus delemar* immobilized on Accurel MP1000: influence of the treatment intensity on the content of CA (●), DHA (○), palmitic acid (▿), oleic acid (▼) and EPA (■) in the STAG produced. Experiments carried out with 5.5 g lipase, 200 g tuna oil, 170 g CA and 500ml hexane ($[TG]_0$ = 221.8 mol/m^3).

Fig. 7 shows that with Palatase an equilibrium maximal incorporation of 53% CA was attained from 10 h (IOT of around 2730 g lipase h/mol TAG) and a DHA content of approximately 15.5% in the final STAG.

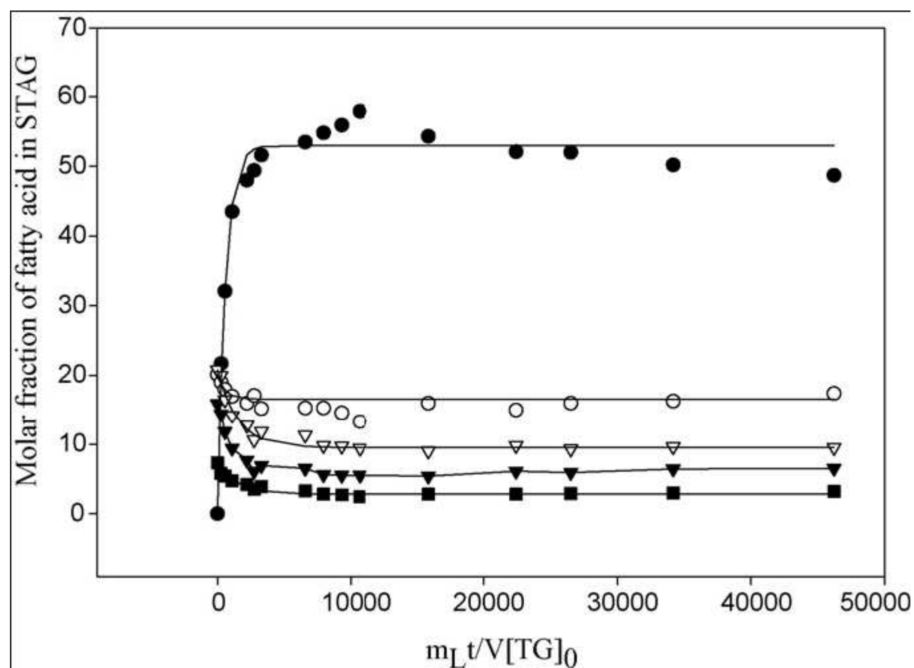

Fig. 7. Acidolysis of tuna oil and CA catalyzed by lipase Palatase immobilized on Accurel MP1000 in a packed bed reactor: influence of the treatment intensity on the content of CA (●), DHA (○), palmitic acid (▿), oleic acid (▼) and EPA (■) in the structured TAG. Experiment carried out with 5.5 g lipase, 20 g tuna oil, 17 g CA and 200ml *n*-hexane.

Table 3 shows that similar STAG compositions were obtained with both lipases, although with lipase Rd these STAGs were obtained at lower IOTs.

Table 3. Fatty acid composition (mol %) of STAGs obtained by acidolysis of tuna oil and CA catalyzed by lipases Rd from *R. delemar* and Palatase, both immobilized on Accurel MP1000 in a PBR.

Fatty acid	Lipase Rd[a]	Palatase[b]
8:0	55.0	53.5
16:0	9.7	11.3
18:1n9	5.6	6.5
20:5n3	3.4	3.3
22:6n3	14.3	15.3

[a] Experimental conditions for acidolysis: 5.5 g lipase Rd, 200 g tuna oil, 170 g CA, 500 ml *n*-hexane ($[TG]_0$ = 221.8 mol/m3), 24 h, IOT = 656 g lipase×h/mol TAG.
[b] 5.5 g Palatase, 20 g tuna oil, 17 g CA, 200ml *n*-hexane ($[TG]_0$ = 83.6 mol/m3), 10 h, IOT = 2735 g lipase×h/mol TAG.

These results are better than the one obtained in the acidolysis of an EPA enriched oil (EPAX4510TG, with 40% EPA) catalyzed by Lipozyme RMIM immobilized in a PBR, operating in discontinuous mode; in these experiment the EPA content of the final STAG was only 9.6%. This great EPA loss was explained by admitting the existence of some acyl migration of EPA from position 2 to positions 1 and 3 in the STAGs, catalyzed by the anionic exchange resin support of lipase Lipozyme RM IM [18].

The curves that fit the experimental results in Figs. 6 and 7 correspond to the equation [17,18]:

$$F_X = F_{Xe} + \left(F_{X0} - F_{Xe}\right)\exp\left(-\frac{k_M m_L t}{3[TG]_0 V}\right) \qquad (2)$$

which represents the variation in fatty acid composition of the STAGs with time in a perfectly mixed discontinuous reactor. F_X, F_{X0} and F_{Xe} are the molar fractions of any fatty acid X in the TAG at any reaction time, t, in the original TAG (tuna oil) and in the STAG at equilibrium, respectively; kX is the apparent kinetic constant for the incorporation of a fatty acid into TAG (mol/g lipase h), mL the lipase amount, $[TG]_0$ the initial TAG concentration and V the reaction mixture volume. This equation is based on the hypothesis that the system formed by the substrate reservoir and the PBR with the immobilized lipase behaves as a differential reactor when it operates with recirculation. It was obtained making mass balances to the lipase bed and to the reaction mixture reservoir and considering that the reaction rate is given by equation:

$$r_X = k_X(F_{Xe} - F_X) \qquad (3)$$

where r_X is the rate of incorporation of a fatty acid X into TAG by a unit amount of enzyme mol/h g lipase); this model considers that this reaction rate is proportional to the separation from the equilibrium for each fatty acid ($F_{Xe} - F_X$) [18]. Table 4 shows the values of the apparent kinetic constants, k_X, that fit the experimental results (Figs. 6 and 7); this table shows that the apparent kinetic constants for lipase Rd are higher than the ones for Palatase (except for DHA). These values are also similar to the ones obtained in our laboratory for other oils and lipases, such as cod liver oil and Lipozyme IM [17] or EPAX4510 and also Lipozyme IM [18].

Table 4. Apparent kinetic constants, k_X, for the exchange of CA and the tuna oil native fatty acids (palmitic acid, oleic acid, EPA and DHA).

Fatty acid	k_X (mol/g lipase h)	
	Lipase Rd from *R. delemar*	Palatase from *M. miehei*
8:0	0.0210	0.00510
16:0	0.0207	0.00198
18:1n9	0.0216	0.00276
20:5n3	0.0162	0.00207
22:6n3	0.00219	0.00525

3.2.2. Operation in continuous mode

When the system operates in continuous mode, the reaction mixture that comes out from the PBR is directed to a product reservoir (Fig. 1). In this case the relationship between the molar fraction of any fatty acid (native or CA) in the STAGs at the exit of the reactor, F_X, and the IOT ($m_L/([TG]_0 q)$) is given by the equation:

$$F_X = F_{Xe} + \left(F_{X0} - F_{Xe}\right)\exp\left(-\frac{k_M m_L}{3[TG]_0 q}\right) \quad (4)$$

where q is the reaction mixture flow rate through the PBR [18]. This equation was obtained applying the plug flow hypothesis within the bed and using the kinetic equation (3) to relate the reaction rate with the incorporation of fatty acids to TAGs. The comparison of Eqs. (2) and (4) shows that continuous and discontinuous reactor should give similar results whenever the IOT in both processes remains constant, i.e.:

$$\left(\frac{m_L t}{V[TG]_0}\right)_{discontinuous(\mathrm{Recirculation})} = \left(\frac{m_L}{q[TG]_0}\right)_{Continuous} \quad (5)$$

This equation also implies that the reaction rate is the same in both reactors and the external mass transfer resistance is not significant in the PBR.

Figs. 6 and 7 show, respectively, that the equilibrium in the incorporation of CA was attained at IOT of around 650 for lipase Rd and 2700 g lipase h/mol TAG for Palatase. Operating in continuous mode 14.1 g of both lipases were immobilized and reaction mixtures were used contained tuna oil concentrations of 222.3 and 83.6 mol/m^3 for lipases Rd and Palatase, respectively. In these conditions we can use Eq. (5) to estimate the flow rate, q, necessary to apply similar IOT to that in discontinuous mode. These flow rates are 98 ml/h for lipase Rd and 63 ml/h for Palatase. Table 5 shows the percentages of CA incorporated to STAGs at flow rates of 100 and 60 ml/h for lipases Rd and Palatase, respectively. It can be observed that the incorporation of CA at these flow rates was about 47% for lipase Rd and 44% for Palatase, less than the incorporation of CA (52–53%) attained in discontinuous mode. These differences between the incorporation of CA attained in discontinuous and continuous modes at similar IOTs can be justified taking into account the lower flow rates (and lower reaction mixture speed through the PBR) in continuous mode (200ml/h in discontinuous and 60–100 ml/h in continuous mode). These lower speeds cause less turbulence in the PBR and therefore a lower external mass transfer rate when operating in continuous mode. This lower external mass transfer rate could limit the overall reaction rate of the acidolysis process. Previous works observed that external mass transfer may not be important at high flow rates, but it is important when the flow rate through the lipase bed is low [29]. Experiments at lower flow rates were carried out to increase the percentage of CA incorporated to STAGs. Table 4 shows that with lipase Rd flow rates of approximately 40ml/h (IOT of around 1600 g lipase h/mol oil) are necessary to attain similar yields to those obtained in discontinuous mode. With Palatase flow rates lower than 25 ml/h (IOT > 6750 g lipase h/mol oil) are necessary. In any case Eq. (5) is

useful as a criterion for changing the operation mode of the reactor and for scaling up. Indeed, when it was applied STAGs with very similar fatty acid composition were obtained.

Regarding the other fatty acids, Table 5 shows that the DHA content reaches values only slightly below 20%, which is the DHA content in the original tuna oil. These contents are similar to those obtained in experiments in discontinuous mode. The contents of EPA, palmitic and oleic acids decreased to around 50% of their contents in the original tuna oil. These results could indicate that DHA preferably occupies position 2, on which 1,3-specific lipases do not act, or that DHA is more resistant to be hydrolyzed by lipases from positions 1 and 3 than the other fatty acids.

Thus, STAGs of MLM type with around 50% CA and 16–20% DHA were obtained by acidolysis of tuna oil and CA in a PBR operating in continuous mode, catalyzed by lipases Rd and Palatase 20000L.

Table 5. Acidolysis of tuna oil and CA catalyzed by lipases Rd from *R. delemar* and Palatase immobilized on Accurel MP1000 in the PBR operating in continuous mode: influence of the reaction mixture flow rate on the fatty acid composition of STAG. Experimental conditions for lipase Rd: 200 g tuna oil, 170 g CA, 500 ml hexane, ($[TG]_0$ = 222.3 mol/m3), 14.1 g lipase, 30 ºC; for Palatase: 20 g tuna oil, 17 g CA, 200ml hexane ($[TG]_0$ = 83.6 mol/m3), 14.1 g lipase, 30 ºC.

Fatty acid	Palatase 200000L				Lipase Rd from *R. delemar*			
	25 ml/h	40 ml/h	50 ml/h	60 ml/h	25 ml/h	40 ml/h	50 ml/h	60 ml/h
8:0	47.9	48.1	43.4	43.7	52.6	51.8	51.5	49.0
16:0	11.6	11.6	11.7	11.7	10.5	11.8	13.0	12.0
18:1n9	7.2	7.3	7.5	7.6	7.3	7.6	8.3	7.9
20:5n3	3.3	3.4	4.1	4.5	4.3	4.0	4.5	4.4
22:6n3	18.9	16.3	20.4	24.0	16.2	13.2	14.6	15.2

3.3. Purification of STAG

The separation of the acidolysis products (STAGs and free fatty acids, FFAs) to obtain pure TAGs with high yield is not an easy task due to the high percentage of FFAs. In a previous work [19] a good STAG extraction yield (80%) was obtained with a purity of over 99% using hexane and a KOH hydroalcoholic solution.

In this work we study the deacidification of the STAGs by using aqueous solutions of ethanol [22,23]. The procedure consists in the preferential extraction of FFAs with water–ethanol solutions due to that the FFAs are more soluble in these mixtures than the STAGs. This procedure has the advantage of not producing solid soaps in which the STAGs are occluded. In this work three variables were optimized, the water/ethanol ratio, the number of extraction steps and the hydroethanolic solution/FFA–TAG mixture ratio, the first one being the most important. The objective was therefore to extract selectively the FFAs, leaving the TAGs in the oily phase with maximal purity (minimal content of FFAs in this phase) and yield (minimal content of TAGs in the hydroalcoholic phase).

Table 6 shows the influence of the three indicated variables on purity and STAG yields. It can be observed that as the extraction number increases, so does the purity of TAGs and the yield of each extraction step; this may be because the fewer fatty acids pass to the hydroalcoholic phase, the fewer TAGs can be dragged toward that phase. Comparing the two extractions carried out with a hydroethanolic solution/FFA–TAG mixture ratio 1/1 (Table 6), it can be seen that as the water content of the hydroalcoholic phase decreases (45–30%) the TAG content (purity) in the oily phase increases, since more FFAs are extracted; also the TAG yield obtained by gravimetry increases from 71% to 81%. This separation method consumes small amounts of solvent compared with other methods (such as that used in [19]). Therefore, to increase the final purity of TAGs the hydroethanolic solution/FFA–TAG mixture ratio was increased from1/1 to 3/1. Table 6 shows the expected result of increases in both yield and purity. In these conditions TAGs of 96% purity were obtained after five extraction steps. In this case the number of extractions can be reduced because the small increase in purity obtained in the last extraction steps does not justify making these additional extractions. After this optimization, therefore, good STAG yield (84.8%) and purity (93.5%) were attained using a water/ethanol ratio 30:70 (w/w), three extraction steps and a hydroethanolic solution/FFA–TAG mixture ratio 3/1 (w/w) in each step. Extraction of 6.5% FFAs was achieved dissolving the mixture in hexane and neutralizing the FFAs with a hydroalcoholic solution of KOH (see Section 2). In this case few soaps are formed, so the amounts of STAGs occluded are also low. With this operation pure STAGs were obtained with a global yield of over 85%.

Table 6. Extraction of the FFAs contained in the TAG–FFA mixtures from acidolysis by hydroalcoholic solutions: influence of the hydroalcoholic solution/FFA–TAG mixture ratios (1/1 and 3/1, w/w), water/ethanol ratio (40 and 30%, w/w, water) and extraction number on the purity and yields of TAG.

Hydroethanolic solution/FFA–TAG mixture ratio	Water/ethanol ratio (w/w) in the hydroalcoholic solutions	Extraction number	Percentage of TAG in the oily phase (purity)[a]	Percentage of recovery TAG in the oily phase (yield)[b]	Total TAG recovery yield (% weight)
		45.7[c]			
		1	46.9	98.6	
		2	58.9	93.5	
1/1	45-55	3	62.9	98.6	70.6
		4	63.7	99.5	
		5	**64.5[d]**	99.8	
		1	45.1	83.7	
		2	62.3	94.0	
1/1	30-70	3	73.5	99.0	80.9
		4	74.9	99.7	
		5	**80.3[d]**	99.5	
		1	78.9	97.9	
		2	87.3	99.7	
3/1	30-70	3	93.5	99.6	84.8
		4	95.4	99. 8	
		5	**96.0[d]**	99.7	

[a] % weight of the fatty acids in the TAG over total fatty acids (free and in the TAG) in the oily phase.
[b] % weight of TAG in the oily phase respect the total TAG in both phases. STAG recovery yield in the oily phase respect on initial TAGs.
[c] Initial TAG content in the TAG-FFA mixture from acidolysis.
[d] Final percentage of TAG (final purity).

3.4. Positional analysis of fatty acids (acyl groups) in STAGs

Once pure STAGs are obtained, it should be checked that they have the desired structure CA-long chain fatty acid-CA (or MLM) determining the fatty acid composition of position 2 and positions 1 and 3. Due to the 1,3-positional specificity of lipases Rd and Palatase, it seems logical to expect that CA will occupy fundamentally positions 1 and 3, whereas DHA and other native fatty acids will preferably occupy position 2. To test these

hypotheses the positional distribution of fatty acids in STAGs was determined by ethanolysis with Novozym 435 following the method proposed by Shimada et al. [25] and Table 7 reflects the results. This analysis has been applied to STAGs obtained by acidolysis of tuna oil and CA, catalyzed by Palatase; these STAGs contained 39.3% CA and 17.5% DHA. Table 7 shows that CA represents 55.5% of the total fatty acids in positions 1 and 3, which means that 94% of the CA incorporated was in positions 1 and 3. Position 2 was occupied by 42.1% DHA and 6.7% EPA. Therefore this positional analysis shows that the predominant type of STAG obtained has the structure MLM, with CA as the medium chain fatty acid, M, and DHA as the long-chain fatty acid, L.

Table 10. Positional analysis of the STAG: composition (% mol) of the STAG, 2-MAG obtained by alcoholysis catalyzed by the lipase Novozym 435 and composition of positions 1 and 3 obtained from the previous one.

Fatty acid	F_M (% mol)		
	STAG	2-MAG	Fatty acids at positions 1 and 3
8:0	39.3	7.1	55.5
14:0	3.6	6.5	2.1
16:0	12.3	19.3	8.7
16:1n7	4.1	6.8	2.8
16:2n4	0.2	-	0.3
18:0	2.1	-	3.1
18:1n9	7.4	-	6.3
18:1n7	1.4	-	2.1
18:2n6	1.0	0.9	1.1
18:4n3	0.9	-	1.4
20:1n9	1.2	-	1.8
20:4n6	1.1	1.0	1.2
20:5n3	4.9	6.7	4.0
22:1n9	0.9	-	1.3
21:5n3	1.0	-	1.5
22:5n3	1.0	-	1.6
22:6n3	17.5	42.1	5.2

4. Conclusions

In this work two enzymatic catalysts have been tested which allows high CA incorporations to tuna oil at acceptable rates. These catalysts were lipases Rd and Palatase immobilized on Accurel MP1000, and they have proved to be stable under the operational conditions (over 4 days) and storage conditions (over 20 weeks lipase Rd and 5 weeks Palatase). Accurel MP1000 is an immobilization support of polypropylene which does not catalyze acyl-migration, and therefore all the PUFAs or fatty acids which occupy position 2 in the original oil continue occupying the same position in the final STAGs.

Good STAG yields were obtained by acidolysis of tuna oil (rich in DHA) and CA with these catalysts immobilized in a PBR operating in discontinuous mode (up to 50% of CA incorporated to TAG) and continuous mode (up to 47% of CA incorporated to TAG). The empirical kinetic model represented by Eq. (3) allows us to obtain a good approximation (Eq. (5)) to the change from the discontinuous to continuous operational mode of the PBR.

Pure STAGs have been obtained from the acidolysis reaction mixture, with a total yield of 85%, by the separation of FFAs in two steps: (i) extraction of FFAs with water–ethanol mixtures 30:70 (w/w) and (ii) elimination of residual FFAs by their neutralization with hydroalcoholic KOH solutions and extraction of STAGs with hexane. The structural analysis of these STAGs shows that 94% of the CA incorporated was in positions 1 and 3 and DHA represents 42% of the fatty acids present in position 2.

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