

**Production of triacylglycerols rich in palmitic acid at *sn*-2 position by
lipase-catalyzed acidolysis**

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Abstract

This paper studies the synthesis of triacylglycerols (TAGs) rich in palmitic acid (PA) at *sn*-2 position from palm stearin (PS), a vegetable oil highly rich in PA (60%, but only 12.8% of this is located at *sn*-2 position). These PA rich TAGs were obtained by lipase-catalyzed acidolysis of this oil with free fatty acids (FFAs) highly rich in PA, such as commercial PA (98% PA) and a FFA extract obtained by saponification of PS (60% PA). PA has a melting point of 63 °C and during the acidolysis reaction the substrates, highly rich in this acid, must remain liquid; therefore high temperatures or solvents must be used. An important objective of this work was to operate without solvent and at the lowest possible temperature. In this acidolysis reaction four factors were firstly studied: type of lipase, temperature, solvent amount and the intensity of treatment ($IOT = \text{lipase amount} \times \text{reaction time} / \text{PS amount}$). The influence of these variables was studied in a stirred tank reactor (STR). The lipases tested were Novozym 435 from *Candida antarctica* (immobilized on a macroporous acrylic resin), and lipases QLC (immobilized on diatomaceous earth), and QLM (non immobilized), both from *Alcaligenes* sp., and the one selected was lipase QLC. According to the manufacturer the optimum temperature for this lipase is 65-70 °C, which allows it to operate without solvent. The best results with lipase QLC (TAGs with 80% PA, both total and at *sn*-2 position) were obtained with commercial PA, at 65 °C, a 3:1 FFA/PS molar ratio (1:1 w/w), without solvent and an $IOT = 7 \text{ g lipase} \times \text{h/g PS}$ (for example 2.5 g PS, 2.5 g commercial PA, 0.75 g lipase and 24 h). These results were the basis for establishing the operational conditions to obtain PA rich TAGs with the lipase immobilized in a packed bed reactor (PBR), operating by recirculation of the reaction mixture through the lipase bed. In this system TAGs with 75% PA were obtained at an $IOT = 8 \text{ g lipase} \times \text{h/g PS}$. This result and the apparent kinetic constants obtained in both reactors show that the reaction rate is lower in the PBR than in the STR. Subsequently, PA enriched TAGs were separated from FFAs by two procedures: the first one at room temperature and in presence of hexane and the second one at 65 °C and without hexane. Using the first procedure, 95% of TAGs in the acidolysis reaction mixture were recovered with a purity of 99%. Using the second one, 98% pure TAGs were obtained with a recovery yield of 80%. Therefore, these highly rich PA TAGs can be obtained by acidolysis of PS and PA rich FFAs in solvent-free media, and then these TAGs also can be purified to 98% in absence of hexane, using only a hydroethanolic KOH solution.

Key words: palm stearin (PS), palmitic acid, triacylglycerols, acidolysis, lipase QLC, purification of triacylglycerols.

1. Introduction

The fatty acid composition of triacylglycerols (TAGs) in human diet, and especially their distribution in the TAG molecule, play an important role in the absorption of fatty acids and other nutrients [1–3]. The absorption of palmitic acid (PA) has been widely studied, since this fatty acid is important in infant nutrition [4–7]. The PA content of human milk is 20-25% of total fatty acids; 65-70% of PA at the central position of the TAG molecule [8,9]. Studies comparing PA absorption from human milk and from infant formulas with the absorption from formulas with PA mostly at the extreme positions, conclude that it is considerably higher in infants fed with human milk or infant formulas with PA at *sn*-2 position; this higher absorption also implies a decrease in the loss of calcium via faeces [10–12]. It is therefore important to synthesize TAGs with a composition and distribution of fatty acids similar to those of human milk (HMFS); these TAGs are being developed from vegetable oils, lard or tripalmitin [13–16]. In particular there is considerable interest in the synthesis of 1,3-diolein-2-palmitin (OPO), which is the most abundant TAG in human milk.

TAGs with a specific fatty acid composition can be obtained by chemical or enzymatic catalysis. Lipase-catalyzed processes have attracted attention because of the mild reaction conditions of temperature, pressure and pH under which enzyme operate, which made that they generally require less energy and are conducted in equipment of lower capital cost than many other chemical processes; also, under these milder conditions, the products are purer and less degraded than through alternative high-temperature reactions, so they are more easily purified and waste disposal is less of a problem [17]. Moreover, the regio-, acyl- and stereospecificity of enzymes result in products with better defined and more predictable chemical composition and structure [18].

Several types of enzymatic reactions appear in the literature to synthesize structured TAGs rich in PA at *sn*-2 position and in other fatty acids at *sn*-1, 3 positions (oleic acid, caprylic acid, etc.). The most direct procedure is the acidolysis of an oil rich in PA at *sn*-

2 position and oleic or caprylic acid, catalyzed by a 1,3 specific lipase. Nielsen *et al.* [15] produced HMFS by acidolysis of lard and soybean oil fatty acids. Lard oil contains 29.5% PA (74% at *sn*-2 position, similar to human milk). In this acidolysis reaction PA contents were maintained, and the contents of linoleic and linolenic acids increased from 9.2% and 0.8% to 23.8% and 2.3%, respectively; at *sn*-2 position only the linoleic acid content increased from 3.2 to 4.8%. This reaction was catalyzed by Lipozyme RM IM immobilized in a packed bed reactor. Balcao and Malcata [19] also use the acidolysis reaction to increase the level of unsaturated fatty acid of a butterfat by acidolysis of this one with oleic acid; so decreases the level of saturated fatty acids, such as myristic and palmitic acids. Schmid *et al.* [16] synthesized the structured TAG oleic-palmitic-oleic (OPO) by a two-step enzymatic process: alcoholysis of tripalmitin with ethanol to produce 2-monopalmitin and esterification of the latter with oleic acid, catalyzing both reactions with 1,3 specific lipases. These authors obtained OPO with 96% PA at *sn*-2 position and 90% oleic acid at *sn*-1,3 positions. Lee *et al.* [20] obtained the structured TAG OPO by interesterification of tripalmitin and ethyl oleate, catalyzed by lipase Lipozyme TL IM from *Thermomyces lanuginosus*. OPO-rich human milk fat substitute was synthesized with 80.6% PA at *sn*-2 position and 64.9% of oleic acid at *sn*-1,3 positions.

Most of these methods are based on TAGs rich in PA at *sn*-2 position, such as tripalmitin or lard. However in vegetable oils (such as palm oil), the main constituents of infant formulas, PA is located predominantly at the extreme positions [21]. Methods to produce TAGs rich in PA at *sn*-2 position are therefore of great potential industrial interest, since these TAGs are not produced on an industrial scale. Chen *et al.* [22] synthesized tripalmitin from glycerol and ethyl palmitate catalyzed by Novozym 435 under vacuum; about 88% conversion with 91% molar of tripalmitin was attained after 36 h of reaction. The ethyl palmitate used was previously obtained by a three-step process: (i) saponification of palm oil, (ii) low temperature fractionation of palm oil fatty acids and (iii) transformation of palmitic acid into ethyl palmitate.

Moreover, it is desirable that these products could be obtained in solvent-free media, as they are intended for infant feeding. The products used (tripalmitin, palmitic acid, palm oil, etc.) have high melting points and the PA rich TAGs present a low solubility in hexane at near room temperature. For these reasons high temperatures must be used for obtaining PA rich STAGs both in presence or absence of solvents, although higher temperatures must be used in solvent-free media; in addition, the stability of the lipases

is lower at the high temperatures needed to keep the reaction mixtures homogeneous and fluid. Some authors have already begun the search for suitable conditions to obtain these products in total absence of solvent. Thus, for example, Yang *et al.* [23] obtained TAGs with 71% PA at *sn*-2 position and 44% oleic acid at *sn*-1,3 positions, by acidolysis of lard and free fatty acids from soybean oil; this reaction was carried out at 61 °C and using Lipozyme RM IM as 1,3 specific lipase. Sorensen *et al.* [24] also produced TAGs with a molecular structure and fatty acid composition very similar to that of human milk fat, by acidolysis of butterfat and a mixture of rapeseed and soybean oil fatty acids, catalyzed by Lipozyme RM IM, at 65°C; these TAGs contained 46-56% PA at *sn*-2 position and 31-35% oleic acid of total fatty acids. After the acidolysis reaction these TAGs were purified by short path distillation and, therefore, also in absence of solvent.

The acidolysis or interesterification reactions are being carried out in several reactor types, although the most commonly used are the batch stirred tank reactor (STR) and the packed bed reactor (PBR). The former is easy to operate but the volumetric throughput is relatively low and for most large-scale catalytic reactions have traditionally been used the second one [25], because it facilitates the contact and subsequent separation, allows reuse of the enzyme without prior separation and an easy use for a continuous operation mode [26, 27].

The aim of this work was to produce TAGs rich in PA at *sn*-2 position by acidolysis of palm stearin (60% PA) and two PA enriched FFAs (commercial palmitic acid, with 98% PA, and a FFA extract obtained by saponification of palm stearin, 60% PA) in two batch reactors: STR and PBR. Three non regioselective lipases were tested to catalyze this reaction: Novozym 435, from *Candida antarctica*, and lipases QLM and QLC, both from *Alcaligenes* sp. An important objective of this work was also to produce these PA rich TAGs in absence of solvent, both in the enzymatic reaction and in the subsequent TAG purification.

2. Materials and methods

2.1. Lipases and chemicals

The chemicals used were palm stearin (PS, kindly donated by Brudy Technology S.L., Barcelona, Spain), whose fatty acid composition is shown in Table 1, commercial

palmitic acid (98% purity, Panreac S.A., Barcelona, Spain), hexane (95%) and other reagents of analytical grade (Panreac S.A., Barcelona, Spain). The lipases tested to catalyze the acidolysis reaction were: Novozym 435 from *Candida antarctica* (kindly donated by Novozymes A/S, Bagsvaerd, Denmark) and lipases QLM and QLC from *Alcaligenes* sp. (kindly donated by Meito Sangyo Co. Ltd., Japan). Novozym 435 is supplied immobilized on a macroporous acrylic resin. Usually this lipase does not show positional specificity and its recommended operating temperature range is 40-60°C. Lipase QLM was supplied as powder, it is non-specific in transesterification reactions and its optimum temperature is 65-70°C. Lipase QLC is produced by immobilizing lipase QLM on diatomaceous earth. Pancreatic lipase (type II from pig pancreas, Sigma, Spain) was used in order to determine the percentage of fatty acid at *sn*-2 position of the TAGs [28].

2.2. Preparation of the free fatty acid fraction highly rich in palmitic acid from palm stearin.

The PS saponification with a hydroalcoholic solution of NaOH was carried out following the method described in Robles *et al.* [29], obtaining FFAs extract with the same composition as the initial PS (60% PA). Fractions rich in PA and in oleic acid were obtained from these FFA extracts by crystallization in acetone following the method of Chen *et al.* [22] with some modifications, as described in Jiménez *et al.* [30]. Table 1 shows the fatty acid profile of the PA concentrate obtained by this procedure.

Table 1

Fatty acid composition (mol %) of palm stearin (PS) and a PA concentrate obtained by crystallization [26].

Fatty acid	Palm stearin (PS)		PA concentrate ^b	
	TAGs		DAGs ^a	Total ^c
	Total ^c	Position 2 ^d	Total ^c	
14:0	1.4	n.d.	n.d	1.6
16:0	60.0	23.0	58.0	75.1
18:0	5.1	0.7	n.d	6.7
18:1n9	29.0	65.2	39.5	14.5
18:2n6	4.5	11.1	2.5	2.1
Percentage of lipidic species (mol%)	95.5		4.5	

^a Diacylglycerols

^b Obtained by saponification of PS and crystallization of FFAs.

^c Fatty acid content of total fatty acids.

^d Fatty acid content of total fatty acids at sn-2 position of TAGs (composition of 2-MAGs applying the pancreatic lipase method).

2.3. Acidolysis catalyzed by lipase dispersed in a batch stirred tank reactor

A typical reaction mixture consisted of 2.5 g of PS, 2.5 g of FFAs (FFA extract of PS, PA concentrate obtained by crystallization or commercial PA), hexane (0, 2 or 4 mL) and lipase (0.25, 0.50 or 0.75 g of lipase QLM, Novozym 435 or QLC, respectively). The lipase/reaction mixture ratios for lipases QLC and QLM used in these experiments were calculated taking into account the activities given by the manufacturer (20,000 U/g for lipase QLC and 60,000 U/g for lipase QLM). The amount of Novozym 435 used was established taking into account previous studies with this lipase [30]. This reaction mixture was placed in Erlenmeyer flasks of 50 and 100 mL, in an inert atmosphere, with silicone-capped stoppers. The mixture was incubated at 50 or 65 °C and agitated in an orbital shaking air-bath at 200 rpm (Inkubator 1000, Unimax 1010 Heidolph, Klein, Germany). The reactions were stopped by separation of lipase by filtration (glass plate of porosity 4). The lipase was then washed with an acetone/ethanol mixture (1:1 v/v) and dried under vacuum. Experiments have been performed in this reactor to determine the stability of lipase QLC in the conditions of operation and storage. These experiments were carried out at the typical conditions outlined above, at

65°C and in solvent-free media. To determine the stability of lipase in the operation conditions, the same batch of lipase was used to catalyze up to 11 acidolysis reactions. All reactions and their corresponding analyses were carried out in duplicate, therefore each result is the arithmetic mean of four experimental data; standard deviations were always below 8%.

2.4. Acidolysis catalyzed by lipase immobilized in a packed bed reactor

The immobilized lipase was packed into a glass column (6.6 i.d. × 23.5 cm length). 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 reaction mixture was recirculated by a peristaltic pump (Watson-Marlow 505S, Bredel Pumps, Falmouth, U.K.) through the lipase bed at flow rates of between 85 and 200 mL/h and the reaction was monitored by sampling at different times in the substrate reservoir. The reaction mixture contained in a substrate reservoir was continuously agitated by a magnetic stirrer. This substrate mixture and the lipase column were jacketed to maintain the reaction temperature (50 or 65 °C). In the experiments carried out without solvent all the tubing, reservoir and column were located inside an incubator (Inkubator 1000 Heidolph) which allows the control of temperature. A typical reaction mixture consisted of 56 g of PS (30 g in the experiments without solvent), 56 g of commercial PA (98% PA) (30 g without solvent), hexane (0, 560 mL) and 2.5 g of lipase QLC. In this system the reactions and their corresponding analyses were also carried out in duplicate, therefore each result is the arithmetic mean of four experimental data; standard deviations were also always below 8%.

2.5. Purification of PA enriched TAGs

The acidolysis reaction products (mainly TAGs and FFAs) were separated by neutralization of FFAs with KOH 0.5 N hydroethanolic solution (30% ethanol), and subsequent extraction of TAGs. This TAG purification was carried out by two procedures.

In the first one hexane was added to the acidolysis reaction products to a concentration of 10 mg/mL (100 mL hexane/g reaction mixture), followed by 1.5 times the number of equivalents of KOH required to neutralize the FFAs. The mixture was

then shaken and left to decant for about 5 min, after which it clearly showed two phases. The upper hexanic phase was withdrawn. Then hexane was again added to the hydroalcoholic phase (50 mL hexane/g reaction mixture). This was agitated and left to stand for another 5 min to complete removal of the hexanic phase. In this process TAGs were removed in the hexanic phases, while the potassium salts of FFAs remained in the hydroalcoholic phase. This procedure was carried out at room temperature [30].

The second procedure was carried out at 65 °C and no solvent was added either before the neutralization of FFAs or to extract TAGs. In this second procedure TAGs were separated by decantation of the oily phase formed after adding the hydroalcoholic solution of KOH (also 1.5 times the number of equivalents required).

2.6. Analysis of the reaction products

The acidolysis reactions and purification processes give FFA and acylglycerol (mono-, di- and triacylglycerols) mixtures. Each of these lipidic species was identified and quantified by thin layer chromatography (TLC) with a FID detector in an Iatroscan MK-6s (New Technology System Europe, Rome, Italy) or by preparative thin layer chromatography (TLC) followed by gas chromatography (GC). In the Iatroscan equipment 1 µL of sample dissolved in hexane (at a concentration of 10 mg/mL) was deposited on the Chromarods-SIII and separation was carried out with the mobile phase benzene:chloroform:acetic acid 50:20:0.7 (v/v/v).

The identification of lipidic species by preparative TLC and their quantification by GC was carried out following the methods already described in previous articles [31,32].

2.7. Determination of the fatty acid composition at sn-2 position of TAGs

This determination was made on samples of TAGs after removing FFAs. This method is based on enzymatic hydrolysis carried out following the method previously described [33,34], which is based on the official method [28]. In this case the method was applied to 0.5 mL of hexanic solution with a TAG concentration of 0.2 g/mL and 35 mg of pancreatic lipase. The hydrolysis products were analyzed by TLC using the mobile phase chloroform/acetone/methanol 95:4.5:0.5 (v/v/v). The analysis of 2-

monoacylglycerols by GC gave the fatty acid profile at *sn*-2 position of the original TAGs.

3. Results and discussion

The aim of this work was to obtain triacylglycerols (TAGs) enriched in palmitic acid (PA) at *sn*-2 position; these TAGs will be intermediate compounds for the synthesis of structured TAGs with the structure oleic-palmitic-oleic (OPO, 1,3-diolein-2-monopalmitin), which is the major TAG in human milk, and caprylic-palmitic-caprylic (CPC), a structured triacylglycerol of easy absorption. The lipidic fraction of human milk contains about 22% PA of total fatty acids, and 72% of total PA is located at *sn*-2 position of the TAG backbone [35].

To obtain TAGs enriched in PA at *sn*-2 position, the oil selected was palm stearin (PS), which is a palm oil fraction rich in PA (Table 1, 60% PA and 23% PA at *sn*-2 position). This oil contains 95.5% of TAGs and only 4.5% of DAGs (Table 1). This oil was enriched in PA by acidolysis with several FFA fractions also rich in PA, catalyzed by positionally non-specific lipases to enrich the TAGs in PA, including those at *sn*-2 position. These acidolysis reactions were carried out in stirred tank (STR) and packed bed reactors (PBR).

3.1. Selection of lipase

The lipases Novozym 435, from *Candida antarctica*, QLM and QLC, both from *Alcaligenes* sp., were tested to catalyze the acidolysis reaction between PS and two PA enriched FFA fractions: FFA extract from PS (obtained by saponification of PS, 60% PA) and commercial PA (98% PA). To choose the operational conditions it must taken into account that PA has a melting point of 63 °C and the substrates used, highly rich in this acid, must remain liquid. However, the operating temperature can be lower if a solvent is used. In this case, low solvent amounts can be used due to the optimum temperatures recommended for the lipases, which are relatively high (see section 2.1). Thus, the first experiments (Table 2) were carried out at 50 °C and with 0.8 mL hexane/g reaction mixture.

Table 2

Palmitic acid (PA) content (mol %) of TAGs produced by acidolysis of PS and PA enriched FFAs (FFA extract from PS and commercial PA), catalyzed with several lipases, in a STR.

Lipase	FFA extract from PS (60% PA)		Commercial PA (98% PA)	
	Total ^a	Position 2 ^b	Total ^a	Position 2 ^b
N435	63.1	63.9	79.8	77.2
QLM	62.7	63.1	83.4	81.2
QLC	61.6	62.6	76.0	76.1

^a Fatty acid content of total fatty acids.

^b Fatty acid content of total fatty acids at position 2 of TAGs

Operational conditions: PS/FFA extract ratio 1:3 mol/mol, 50 °C, 0.8 mL hexane/g reaction mixture, 24 h and 200 r.p.m. Lipase amount (weight % of reaction mixture): 5% for QLM, 10% for Novozym 435 and 15% for QLC.

Table 2 shows the PA contents attained using the three lipases and the two PA rich FFA fractions. This table shows a high incorporation of PA to PS TAGs, especially at *sn*-2 position, since the original TAGs contain 23% PA at this position. When the FFA extract from PS was used, PA enrichment was limited by the PA content of this FFA extract (60%). This result shows that using only a raw material, PS, TAG with up to 63% PA at *sn*-2 position may be obtained; this TAG will be a good raw material to produce the STAG of structure oleic-palmitic-oleic by acidolysis of this TAG with oleic acid catalyzed by a 1,3 specific lipase. The incorporation of PA using commercial PA (98% PA) was higher, as expected (Table 2).

The greatest incorporations were attained with lipase QLM, although the differences between the three were small. For this reason, bearing in mind that Novozym 435 had already been tested in a previous work [30] and that lipase QLC is supplied immobilized by the manufacturer, this lipase was selected to optimize the acidolysis reaction; in addition, according to the manufacturer, lipase QLC catalyzes interesterification well up to 70 °C, which allows operation without solvent.

3.2. Influence of temperature and solvent amount

Lipase QLC was tested at temperatures between 50 and 65 °C, trying to reduce the solvent amount used at each temperature, until even eliminate it. Table 3 shows that the PA contents attained were similar at all temperatures and solvent amounts used. It was not possible to use solvent amounts lower than 0.8 mL/g at 50 °C due to the high melting point of PA. Due to these slight differences between the PA contents, 65 °C was the temperature chosen to work in absence of solvent. These results are similar to those obtained in a previous work [30] with Novozym 435 (10% w/w on reaction mixture), at 37 °C, 48 h and with 10 mL hexane/g reaction mixture (79.2% PA, 74.5% PA at *sn*-2 position). The utilization of lipase QLC at 65 °C and without solvent is, therefore, an alternative to the use of Novozym 435 at lower temperature (37 °C) and using hexane [30].

Table 3

Palmitic acid (PA) content (mol%) of TAGs produced by acidolysis of PS and commercial PA, catalyzed by lipase QLC, at several temperatures and solvent/reaction mixture ratios in a STR.

Temperature, °C	Solvent/reaction mixture ratio, mL/g	Total ^a	Position 2 ^b
50	0.8	76.0	76.1
65	0.8	76.4	77.4
65	0.4	75.3	76.8
65	0	75.7	75.1

^a and ^b as in Table 2

Operational conditions: PS/FFA extract ratio 1:3 mol/mol, 15% QLC lipase (weight % of reaction mixture), 24 h and 200 rpm.

3.3. Influence of the intensity of treatment ($IOT = \text{lipase amount} \times \text{reaction time} / \text{PS amount}$)

Figure 1 shows the influence of IOT on the PA content of TAGs. In previous works the results of acidolysis reactions were correlated to an empirical kinetic model [36, 37] and to a kinetic model based on the reaction mechanism [38]; in those works was

observed that the reaction rate is proportional to the IOT if no enzyme deactivation occurs. The lipase concentration (m_L/V) and reaction time (t) are equivalent variables and their product can be used as the intensive variable that quantifies the treatment intensity. The results shown in Fig. 1 were obtained using several lipase amounts (between 0.5 and 1.50 g) and different reaction times (between 2 and 24 h) and this figure shows that all the experimental data fitted the same line, which indicates that the $(m_L/V)t$ is actually the significant variable defining the intensity of the enzymatic reaction. $[TG]_0$ is included in the IOT because in those previous works also was demonstrated that IOT ($m_L t / (V[TG]_0)$) is an useful parameter for the scaling up of the enzymatic reactions and for the change of reactor type (SRT or PBR) or operation mode (discontinuous or continuous modes). Furthermore, it allows to compare rates of reactions carried out with different $[TG]_0$, as further shown in Fig. 2.

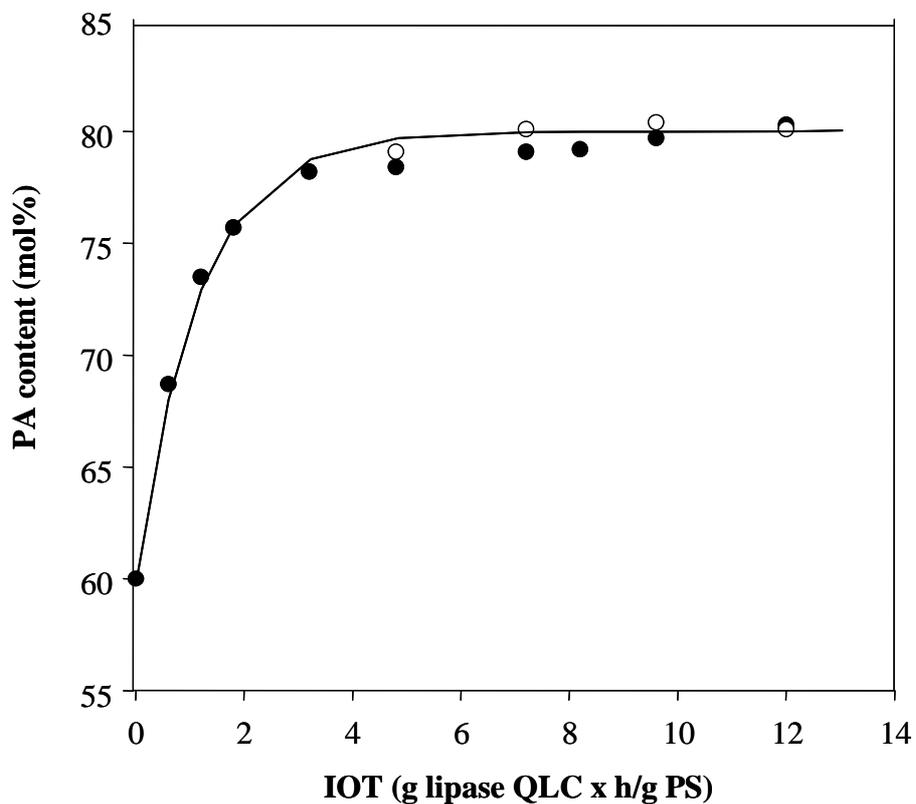


Fig. 1. Influence of the intensity of treatment (IOT) on the PA content of TAGs obtained by acidolysis of PS with commercial PA, catalyzed by lipase QLC, in the STR.
 ● Total PA content, ○ PA content at *sn*-2 position. *Operational conditions:* 2.5 g PS, 2.5 g commercial PA (1:3 PS/FFA molar ratio), 65 °C, without solvent, 200 rpm.

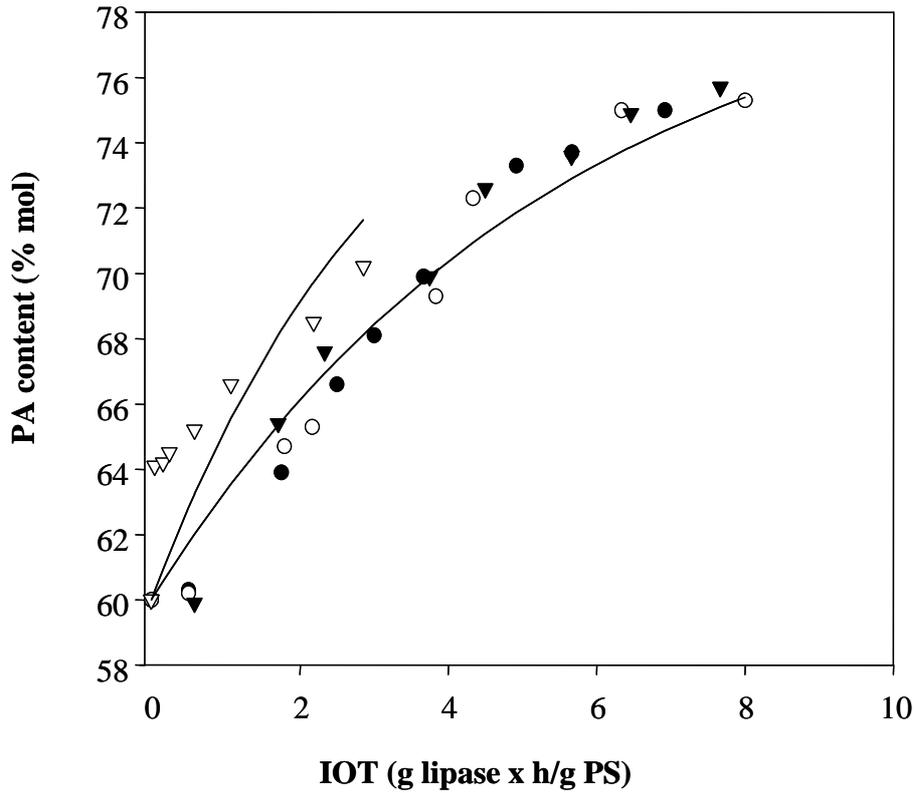


Fig. 2. Influence of the IOT and reaction mixture flow rate on the total PA content of TAGs obtained by acidolysis of PS with commercial PA, catalyzed by lipase QLC immobilized in a PBR. *Operational conditions:* ▽ Experiments with solvent: 56 g PS, 56 g commercial PA (1:3 PS/FFA molar ratio), 2.5 g lipase, 50 °C, 5 mL hexane/g reaction mixture, reaction mixture flow rate through the PBR 200 mL/h. Experiments without solvent at flow rates of 180 ●, 120 ○ and 85 mL/h ▼; 30 g PS, 30 g commercial PA (1:3 PS/FFA molar ratio), 2.5 g lipase, 65 °C.

Figure 1 shows that the PA content increases to an approximate IOT value of 7 g lipase × h/g PS (for example 0.75 g lipase, 24 h, 2.5 g PS and 2.5 g commercial PA), which can be considered the optimal IOT. At higher IOTs the PA content remains constant at around 80% (total content and at *sn*-2 position).

The curve that fits the experimental results in Fig. 1 corresponds to the equation [31,36,39]:

$$F_x = F_{xe} + (F_{x0} - F_{xe}) \exp\left(-\frac{k_x m_L t}{3[TG]_0 V}\right) \quad (1)$$

which represents the variation in fatty acid composition of TAGs with time by acidolysis in a perfectly mixed discontinuous reactor. F_{X0} is the molar fraction of any fatty acid X in the original TAG (PS), F_{Xe} is this molar fraction in the synthesized TAG at equilibrium and F_X is the molar fraction of any fatty acid X in the synthesized TAG at any reaction time, t; k_X is the apparent kinetic constant for the incorporation of PA into TAG (mol/(g lipase \times h)), m_L the lipase amount, $[TG]_0$ the initial TAG concentration and V the reaction mixture volume. Equation (1) was obtained making mass balances to the dispersion reactor and considering that the reaction rate is given by equation:

$$r_X = k_X (F_{Xe} - F_X) \quad (2)$$

where r_X is the rate of incorporation of a fatty acid X into TAG by a unit amount of enzyme (mol/(h \times g lipase)); this model considers that this reaction rate is proportional to the separation from the equilibrium for each fatty acid ($F_{Xe} - F_X$). In this case the value of k_X for the incorporation of PA to TAGs that fits the experimental results is 0.00315 mol/(h \times g lipase) for PA (Table 4), which is similar to those obtained in our laboratory for other oils, fatty acids and lipases [31,36,37].

Table 4

Apparent kinetic constants (k_X) that fit the experimental results in equation (1) ($F_{X0} = 0.60$ and $F_{Xe} = 0.801$), for the exchange of palmitic acid in the stirred tank (STR) and packed bed reactors (PBR).

Experimental conditions	k_X (mol \times g ⁻¹ lipase \times h ⁻¹)	Temperature (°C)
STR, without solvent (Fig. 2)	0.00315 \pm 0.00018	65
PBR, without solvent (Fig. 3)	0.00065 \pm 0.00005	65
PBR, with solvent (Fig. 3)	0.00109 \pm 0.00023	50

These previous experiments established the optimal conditions to obtain TAGs highly rich in PA. These conditions are: PS/commercial PA (98% PA) 1:3 molar ratio (1:1 w/w), 65 °C, 15% weight QLC lipase of reaction mixture, 200 rpm and a minimal IOT of 7.0 g lipase \times h/g PS. The acidolysis reaction in these conditions was scaled up

(by a factor of 10) and once again TAGs with around 80% PA (total and at *sn*-2 position) were obtained.

These optimized conditions were also used for the acidolysis of PS with a PA concentrate (75.1%, Table 1) obtained by saponification of PS and crystallization in acetone of FFAs (see section 2.2). With this PA concentrate TAGs with 71.4% PA (73.0% at *sn*-2 position) were obtained. These PA contents are lower than the ones obtained with commercial PA due to the lower PA content of this PA concentrate (75.1% as opposed to 98% of commercial PA). In any case this result shows that PA enriched TAGs can be obtained starting only from PS, as TAG and as source of PA enriched FFAs.

The results obtained with commercial PA are similar to the one obtained by Chen et al. [22] by alcoholysis of ethyl palmitate (98% pure) and glycerol, catalyzed with lipases Novozym 435 and Lipozyme IM 60. With Novozym these researchers attained about 88% conversion in this reaction with 91% mol of tripalmitin.

3.4. Acidolysis of PS with lipase immobilized in a packed bed reactor

The system formed by a substrate reservoir and a packed bed reactor (PBR) with the immobilized lipase behaves as a differential reactor when it operates with recirculation, like the dispersion reactor. Fig. 2 shows the variation of PA content with IOT in experiments carried out with solvent at 50 °C and without solvent at 65 °C. Also the other experimental conditions used were the optimal ones obtained from the experiments carried out in the STR; IOT was increased to a value around the optimal one (7.0 g lipase \times h/g PS). In solvent-free media the experiments were carried out keeping constant the substrate amounts and at several flow rates of the reaction mixture through the lipase bed. Fig. 2 shows that this variable did not influence the PA contents of synthesized TAGs in the range tested. Without solvent the maximum PA content attained was around 75%, at an IOT of around 8 g lipase \times h/g PS. This PA incorporation is lower than that attained in the STR (80%), which was also obtained at a lower IOT (7 g lipase \times h/g PS).

The curves that fit the experimental results in Fig. 2 also correspond to equation (1). The kinetic model represented by equation (2) fits the experimental results obtained in the STR acceptably well (Fig. 1), but does not fit the results for the PBR so well (Fig. 2); equation (1) is based on the considering that the system formed by the substrate

reservoir and the bed of immobilized enzyme, operating in discontinuous mode, behaves as a perfectly mixed reactor, which can be true at low substrate concentrations, high reaction mixture flow rate through the PBR and short length of lipase bed; moreover, the model represented by equation (1) is too simple to take into account the possible influence of the mass transfer on the kinetic process, which may be important in the PBR, as has been commented previously [39].

The apparent kinetic constant when no solvent was used (Table 3) is lower than the one obtained in the dispersion reactor, which indicates that the reaction rate is lower in the PBR than in the stirred tank reactor. This result was also obtained in previous works for this and others reaction types. For example, the maximal alcoholysis reaction rate and 2-MAG yield were attained in a STR operating in discontinuous mode, when this reactor was compared with a PBR operating in discontinuous and continuous mode [40].

Fig. 2 and Table 4 also show that in the PBR the reaction rate is lower in absence of solvent, which again indicates that when no solvent is used the reaction rate could be highly influenced by the mass transfer effects. As in previous works [38, 39], this results may be due to the influence of the diffusion within the pores of the immobilized lipase particles, which can occur when the viscosity of the reaction mixture increases appreciably. Indeed, at 65°C (the operational temperature when no solvent was used) the viscosity of the reaction mixture is about 6 mPa s or higher, whereas, at 50°C, the viscosity of the mixture hexane-PS-PA was about 0.6 mPa s. Similarly, a great decrease of the reaction rate was observed in the production of structured TAGs by acidolysis of EPAX4510 (a fish oil highly rich in EPA) and caprylic acid catalyzed by Lipozyme RM IM immobilized in a PBR, when the substrate concentration was increased and finally no solvent was used. In these cases it was concluded that in the PBR and at high substrate concentration the kinetic of the reaction can be controlled by internal mass transfer resistance [39]. Also Camacho *et al.* [38] demonstrated that in absence of solvent the sharp increase in viscosity causes a decrease in the effective diffusivity of the species during the reaction, which implies that the diffusion of substrates into the catalyst pores determines the reaction rate.

3.5. Operational and storage lipase stability

Figure 3 shows that lipase QLC remains stable for at least 11 uses in the operational conditions used in the acidolysis of PS with commercial PA, since both the total PA content and that at *sn*-2 position remained approximately constant over the 11 acidolysis reactions catalyzed by the same lipase.

The stability of lipase in the storage conditions was also tested. Acidolysis reactions were catalyzed by a lipase which was stored for 18 months at 5 °C. The PA content of TAGs obtained in three acidolysis reactions of PS with commercial PA (under the same conditions as those shown in Fig. 3) carried out at 1, 14 and 18 months of storage was the same (about 80% total and at *sn*-2 position). These experiments show the great stability of lipase QLC.

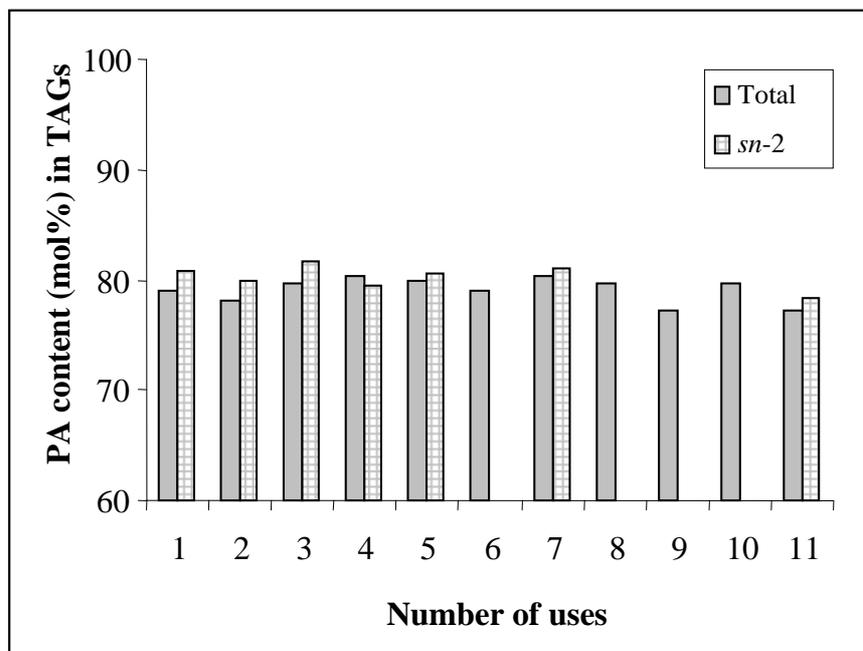


Fig. 3. Influence of the number of uses of lipase QLC on its activity, determined as the PA content in the enriched TAGs obtained by acidolysis of PS with commercial PA. *Operational conditions:* Experiments performed in the STR, PS/commercial PA ratio 1:3 mol/mol, 65 °C, no solvent, 24 h, lipase QLC/PS ratio 0.3 w/w (IOT = 7.2 g lipase × h/g PS), and 200 rpm.

3.6. Purification of PA enriched TAGs

The purification of PA enriched TAGs obtained from the acidolysis reaction, by removal of free fatty acids (FFAs), was carried out by two procedures: with and without solvent (see section 2.5).

Table 5 shows that using the first procedure almost pure TAGs can be obtained (99% purity). The TAG yield can be increased from 80 to 95% carrying out a second extraction of TAGs from the hydroalcoholic phase from the neutralization and first extraction stage. However, using this procedure high amounts of hexane are consumed. The second procedure was carried out in total absence of solvent but at 65 °C to prevent the solidification of highly PA rich TAGs. In these conditions almost pure TAGs were obtained (98% purity), but the TAG yield was lower than that attained by two extractions with hexane (procedure 1). The second procedure has the advantage of obtaining these highly rich PA TAGs, with a high purity, but without using hexane, either in the reaction medium or in the purification step. This allows great solvent savings, simplifies the production process and avoids the contact of products with this low toxic solvent, although in this case higher energy consumption is necessary, since the reaction and purification steps must be carried out at a higher temperature.

Table 5

Purities and yields obtained in the purification of PA enriched TAGs (previously obtained by acidolysis of PS and commercial PA) by two operational methods (see section 2.5): (1) neutralization of FFAs and extraction of TAGs with hexane and (2) neutralization and extraction of TAGs in absence of hexane.

Procedure	Neutralization of FFAs + Extraction of TAGs with solvent (stage I)		Extraction of TAGs with solvent (stage II)			
	Conditions	TAG ^a purity (%)	TAG ^a yield (%)	Conditions	TAG ^a purity (%)	TAG ^a yield (%)
1	Room temperature, 100 mL hexane/g reaction mixture ⁽¹⁾	99.0	80.0	Room temperature, 50 mL hexane/g reaction mixture ⁽²⁾	99.0	95
2	65 °C, without solvent ⁽³⁾	98.4	80.0			

These extractions were carried out from acidolysis reaction mixtures with FFAs/acylglycerols (TAGs and DAGs) ratios of about 1:1 w/w.

⁽¹⁾ Neutralization of FFAs with KOH 0.5 N hydroethanolic solution (30% ethanol) at room temperature and extraction of TAGs in the hexanic phase (procedure 1)

⁽²⁾ Second extraction of TAGs contained in the hydroalcoholic phase with hexane (procedure 1).

⁽³⁾ Direct extraction of TAG from the oily phase with hexane (procedure 2).

^a These TAG fractions contain less than 5% DAG (see Table 6)

Table 6 shows the fatty acid composition of the acylglycerols obtained after the acidolysis and purification steps. This product is 96% TAGs, which contains around 79% PA of total fatty acids and of fatty acids at *sn*-2 position. They also contain around 4% DAGs (similar to the original PS) and these DAGs also have been enriched in PA and have been depleted in oleic and linoleic acids.

Table 6

Fatty acid composition (mol%) of the acylglycerols mixture (TAGs and DAGs) obtained after the acidolysis and purification steps.

Fatty acid	TAG, total ^a	TAG, sn-2 position ^b	DAG
14:0	0.6	0.5	n.d.
16:0	79.1	78.5	80.5
18:0	2.7	2.9	0.1
18:1n9	15.3	15.3	19.2
18:2n6	2.3	2.8	0.2
Lipidic specie (%)		95.7	4.3

^a and ^b as in Table 2

Operational conditions for acidolysis: PS/commercial PA ratio 1:3 mol/mol, 65 °C, no solvent, 24 h, lipase QLC/PS ratio 0.3 w/w (IOT = 7.2 g lipase × h/g PS) and 200 rpm.

Purification carried out by procedure 2 (without solvent).

4. Conclusions

This work determined the optimal operational conditions for obtaining PA enriched TAGs at *sn*-2 position by acidolysis of PS (60% PA and 23% PA at *sn*-2 position) and FFAs highly rich in PA (from 60 to 98% PA), catalyzed by lipase QLC, from *Alcaligenes* sp.. This reaction was optimized in absence of solvent and operating in a STR and in a PBR. These PA enriched TAGs will be used as intermediates for the synthesis of structured TAGs. The highest PA content was attained operating in the STR at IOT of around 7 g lipase × h/g PS. In these conditions the PA content at *sn*-2 position of TAGs attained 80%. In the PBR the reaction rate was lower and TAGs with 75% PA were obtained. This result could be due to the fact that, at high substrate concentrations (in this case no solvent was used), the kinetic of reaction is controlled by the mass transfer resistance and this resistance is lower in the STR because in this system a more intense agitation is possible.

It was also proved that lipase QLC can be reused at least 10 times in the operational conditions optimized in this work (a total time of 240 h) without observing an appreciable activity loss. Furthermore, lipase QLC was shown to be stable for at least 18 months stored at 5 °C.

PA enriched TAGs were finally separated from the acidolysis reaction products by two procedures, both based on the neutralization of FFAs with a KOH hydroalcoholic solution. In one of these procedures TAGs were separated from the FFA hydroalcoholic solution without using any solvent, and therefore PA enriched TAGs were obtained without using solvents either in the acidolysis reaction or in its purification.

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