

Production of triacylglycerols rich in palmitic acid at position 2 as intermediates for the synthesis of human milk fat substitutes by enzymatic acidolysis

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Abstract

This paper studies the synthesis of triacylglycerols (TAGs) rich in palmitic acid (PA) at position 2, from palm oil stearin (POS), a vegetable oil highly rich in this acid (60%). The developed process consists of two steps: (1) obtaining PA enriched free fatty acids (FFAs), and (2) enrichment of POS in PA by acidolysis of this oil with PA enriched FFAs, catalyzed by lipase Novozym 435. In step (1) two PA enriched FFA mixtures were obtained: one by saponification of POS, and a PA concentrate (75.1% PA) obtained by crystallization at low temperature in solvents. The latter was obtained carrying out two crystallizations in acetone at -24 and -20 °C, from which PA was recovered in the solid phases with a total yield of 84%. These PA enriched FFA mixtures were used in step (2) of acidolysis of POS, along with commercial PA (98% PA). In this acidolysis step four factors were studied: temperature, hexane/reaction mixture ratio, FFA/POS molar ratio and the intensity of treatment (IOT = lipase amount \times reaction time/POS amount). The best results (TAGs with 79% PA and 75% PA at position 2) were obtained with commercial PA, at 37 °C, 10 mL hexane/g reaction mixture, a FFA/POS molar ratio 3:1 (1:1 w/w) and an IOT = 9.6 g lipase \times h/g POS (for example 48 h, 10 g lipase and 50 g POS). PA enriched TAGs were purified neutralizing the FFAs by KOH hydroethanolic solutions and extracting the TAGs with hexane. In this way 99% pure acylglycerols (TAG+DAG) were obtained; the recovery yield of this purification step was 95%. The experiments carried out with POS demonstrated that it is possible to use only this oil (60% PA, 23% PA at position 2) as a source of PA to obtain a TAG with 70.7% PA and 70.5% PA at position 2. This process consists of four steps: (1) saponification of POS, (2) crystallization of FFAs to obtain PA enriched FFAs (75.1% PA), (3) acidolysis of POS with these FFAs, catalyzed with Novozym 435, to produce PA enriched TAGs at position 2 (70.5% PA) and (4) purification of TAGs to obtain approximately 95% purity and yield. These PA enriched TAGs could be used to obtain structured TAGs rich in PA at position 2 and in oleic acid at positions 1 and 3 (OPO), which is the principal TAG of human milk fat.

Key words: palm oil stearin (POS), palmitic acid, triacylglycerols, acidolysis, lipase, Novozym 435.

1. Introduction

The fatty acid composition of triacylglycerols (TAGs) of the 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, with 65-70% of total 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 position 2; this higher absorption also involves a decrease in the loss of calcium via fecal [10-12]. It is therefore important to synthesize TAGs with a composition and distribution of fatty acids similar to those of human milk. 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.

Several types of enzymatic reactions appear in the literature to synthesize structured TAGs rich in PA at position 2 and in other fatty acids at positions 1 and 3 (oleic acid, caprylic acid, etc.). The most direct procedure is the acidolysis of an oil rich in PA at position 2 and oleic or caprylic acid, catalyzed by a 1,3 specific lipase. Sahin *et al.* [13] carried out the enzymatic acidolysis of tripalmitin and free fatty acids from hazelnut oil and stearic acid to synthesize fat substitute of human milk. Lai *et al.* [14] synthesized structured lipids by acidolysis of palm olein and caprylic acid, catalyzed by Lipozyme IM 60; they obtained a mixture of TAGs where the main one was that with the caprylic-oleic-caprylic structure; caprylic acid incorporation attained 30.5% and position 2 contained 21.3% PA and 60.7% oleic acid.

Schmid *et al.* [15] and Pfeffer *et al.* [16] synthesized the structured TAG oleic-palmitic-oleic 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. Schmid *et al.* [15] obtained the structured TAG OPO with 96% PA at position 2 and 90% oleic acid at positions 1 and 3. Pfeffer *et al.* [16] produced 2-monopalmitin with 77% purity and 73% yield in the alcoholysis reaction and, finally, OPO with 95% purity and 90% yield in the esterification reaction.

Structured TAGs can also be obtained by enzymatic interesterification between oils; this reaction causes an exchange of fatty acids and a random distribution at the

three positions, which modifies the physical properties and the posterior absorption of fatty acids [17]. Maduko and Park [18] modified the structure of TAGs in vegetable oil blends by interesterification reactions between these blends and tripalmitin, catalyzed by Lipozyme RM IM. These authors investigated the incorporation of palmitic, oleic and linoleic acids at position 2 of TAGs and obtained fatty acid profiles quite similar to those of human milk, with a high percentage of PA at position 2 (around 64-66%).

On the other hand, other authors have used animal fats as feedstock to produce human milk fat substitute (HMFS) [19-21]. Nielsen *et al.* [21] produced HMFS by acidolysis of lard and soybean oil fatty acids. Lard oil contains 29.5% PA (74% at position 2, similar to human milk). In this acidolysis reaction PA contents were maintained, but the contents of linoleic and linolenic acids increased from 9.2% and 0.8% to 23.8% and 2.3%, respectively; at position 2 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. However, consumers may be reluctant to use animal fats for infant feeding.

Most of these methods are based on TAGs rich in PA at position 2, such as tripalmitin. However in vegetable oils (such as palm oil), the main constituents of infant formulas, PA is located predominantly at the extreme positions [22]. Methods to produce TAGs rich in PA at position 2 are therefore of great potential industrial interest, since these TAGs are not produced at industrial levels. Chen *et al.* [23] 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; ethyl palmitate 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.

The aim of this work was to produce TAGs rich in PA at position 2 from a vegetable oil (palm oil stearin) by a two-step process: 1) obtaining PA enriched free fatty acids (FFAs) by low temperature fractionation and 2) enrichment of the palm oil stearin in PA by acidolysis of this oil and several PA enriched FFAs, including those obtained in step 1). In future works these TAGs will be used to synthesize TAGs with the structure oleic-palmitic-oleic (OPO) or caprylic-palmitic-caprylic (CPC).

2. Materials and Methods

2.1. Lipases and chemicals

The chemicals used were palm oil stearin (POS, Brudy Technology S.L., Barcelona, Spain), whose fatty acid composition is shown in Table 1, commercial palmitic acid (98% purity), hexane (95%) and other reagents of analytical grade (Panreac, S.A., Barcelona, Spain). The lipase used to catalyze the acidolysis reaction was Novozym 435 (kindly donated by Novozymes A/S, Bagsvaerd, Denmark); this lipase comes from *Candida antarctica* and is supplied immobilized on a macroporous acrylic resin. Usually this lipase does not show positional specificity when the substrate is a triacylglycerol (TAGs), although under certain conditions it can show 1,3 positional specificity [24]. Pancreatic lipase (type II from pig pancreas, Sigma, Spain) was used in order to determine the percentage of fatty acid at position 2 of the TAGs.

Table 1

Fatty acid composition (mol %) of palm oil stearin (POS).

Fatty acid	TAGs		DAGs
	Total ^a	Position 2 ^b	Total ^a
14:0	1.4	n.d.	n.d
16:0	60.0	23.0	58.0
18:0	5.1	0.7	n.d
18:1n9	29.0	65.2	39.5
18:2n6	4.5	11.1	2.5
Percentage of lipidic species (mol %)		95.5	4.5

^a Fatty acid content of total fatty acids.

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

2.2. Obtaining free fatty acids (FFAs) highly rich in palmitic and oleic acids

The POS saponification with a hydroalcoholic solution of NaOH was carried out following the method described in [25], obtaining FFAs extract with the same

composition as the initial POS. Fractions rich in PA and in oleic acid (OA) were obtained from these FFA extracts by crystallization in solvent following the work of Chen *et al.* [23] with some modifications (Figure 1). Figure 1 indicates the crystallization procedure; by this method PA enriched solid fractions were obtained, whereas the liquid fractions were rich in OA.

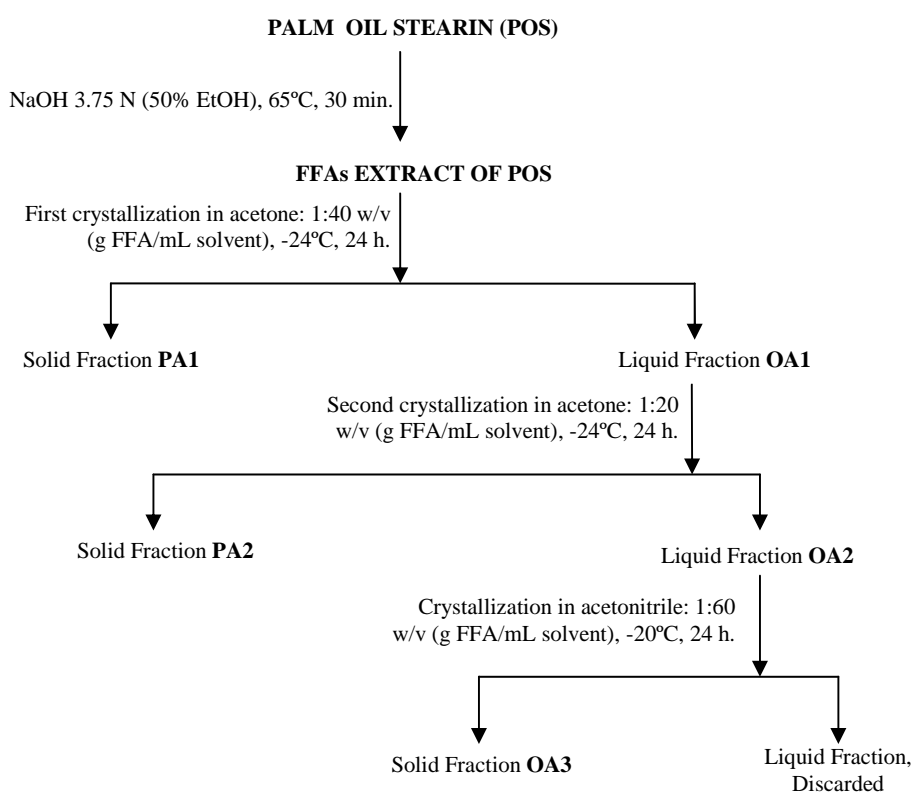
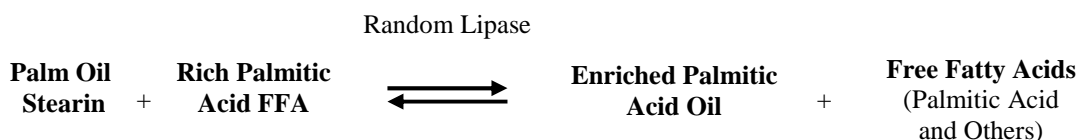


Fig. 1. Experimental procedure for obtaining palmitic acid (PA) and oleic acid (OA) enriched FFAs.

2.3. Acidolysis catalyzed by lipase Novozym 435 in a stirred tank reactor

The acidolysis reactions were as follows:



A typical reaction mixture consisted of POS, 2.5 g, FFAs (FFA extract of POS, PA concentrate obtained by crystallization or commercial PA), 2.5 g, hexane, 50 mL,

and lipase Novozym 435, 0.5 g. These amounts determined a FFA/POS molar ratio of 3:1 and a hexane/reaction mixture ratio of 10 mL/g. This reaction mixture was placed in Erlenmeyer flasks with silicone-capped stoppers. The mixture was incubated at 37 °C or 50 °C and agitated in an orbital shaking air-bath at 200 rpm for 24 h (Inkubator 1000, Unimax 1010 Heidolph, Klein, Germany). The reactions were stopped by separation of lipase by filtration. The lipase was then washed with an acetone/ethanol mixture (1:1, v/v) and dried under vacuum.

This reaction was also carried out in jacketed stirred tank reactors of 1.5 L. In this case, the reaction mixture consisted of POS, 50 g; commercial PA, 50 g; lipase Novozym 435, 10 g, and hexane, 1 L. The reaction mixture was continuously agitated to prevent deposition of the lipase. The reaction temperature was 37 °C and maximum reaction time was 96 h. All reactions and their corresponding analyses were carried out in duplicate, therefore each data is the arithmetic mean of four experimental data; standard deviations were always below 8%.

2.4. Identification of the reaction products and determination of molar fractions of fatty acids in triacylglycerols

The acidolysis reactions, crystallizations and purification processes give acylglycerols (mono-, di- and triacylglycerols) and FFAs 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). 1 μ L of sample (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 fatty acid composition of each of the lipidic species was determined by preparative TLC followed by gas chromatography (GC), following the methods already described in various articles [26-29].

2.5. Purification of PA enriched TAGs

The acidolysis reaction products (TAGs and FFAs) were separated by neutralization of FFAs with KOH 0.5 N hydroethanolic solution (30% ethanol) at room

temperature, and extraction of TAGs by hexane [26]. Figure 2 a and b show the purification process.

In the first procedure (Figure 2a) hexane was added to the acidolysis reaction products to a concentration of 10 mg/mL (volume V of hexanic solution). Then, 1.5 times the number of equivalents of KOH required to neutralize the FFAs were added (KOH 0.5 N hydroethanolic solution volume (30% ethanol) = $30 \times V \times \text{FFA percentage} / 256$, considering PA as representative of all the FFAs, molecular weight = 256). The mixture was then shaken and left to decant for about 5 min, clearly showing two phases. The upper hexanic phase was withdrawn (hexanic phase I). Hexane was added to the hydroalcoholic phase I (hexane volume $V/2$). This was agitated and left to stand for about 5 min to complete removal of the hexane phase II. In this process TAGs were removed in the hexanic phases, while the potassium salts of FFAs remained in the hydroalcoholic phase.

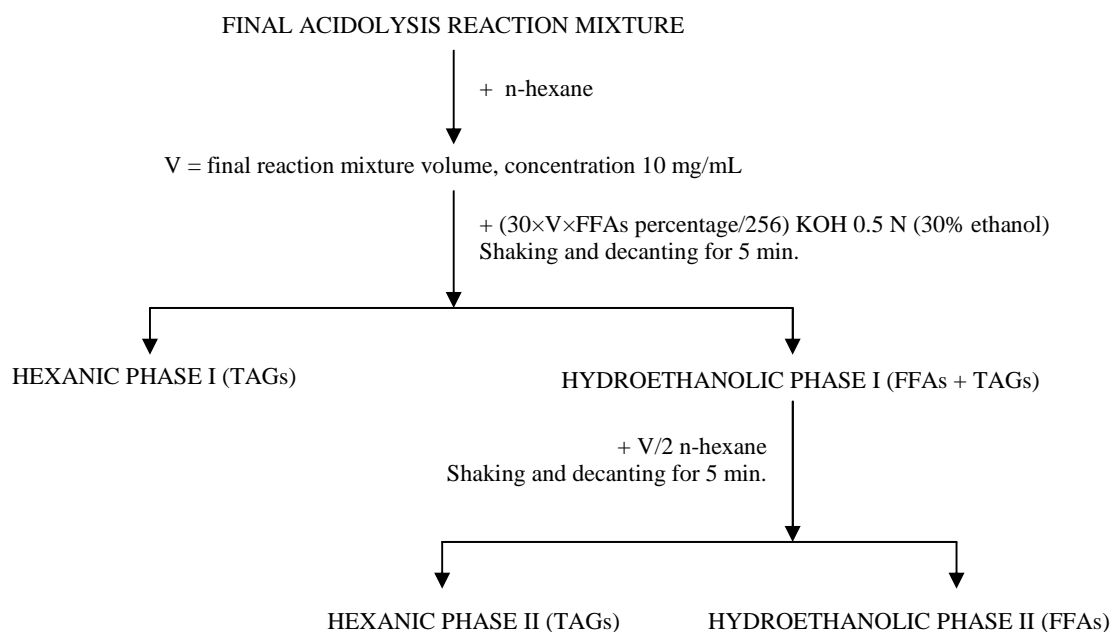


Fig. 2a. Experimental procedure I for purification of TAGs from the final acidolysis mixture.

In the second procedure (Figure 2b) the acidolysis reaction products were diluted to a concentration of 20 mg/mL by the addition of hexane (volume V' of hexanic solution). Then, 1.5 times the number of equivalents of KOH required to neutralize the FFAs were added (KOH 0.5 N hydroethanolic solution volume (30% ethanol) =

$60 \times V' \times \text{FFA percentage} / 256$). The mixture was agitated and hexane ($V'/2$ volume) and water ($V'/10$) were added; the mixture was again agitated and centrifuged at 3500 rpm for 4 min. As a result, three phases were observed, an upper hexanic phase I, an intermediate viscous phase and a lower hydroalcoholic phase. The hexanic phase was withdrawn, and $V'/10$ volumes of hexane and water were added to the other phases; after shaking and allowing it to stand, three phases were again observed. The lower hydroethanolic phase II was discarded and the others were washed 5 times with water ($V'/10$), to obtain a final hexanic phase II separated from an aqueous phase which was discarded.

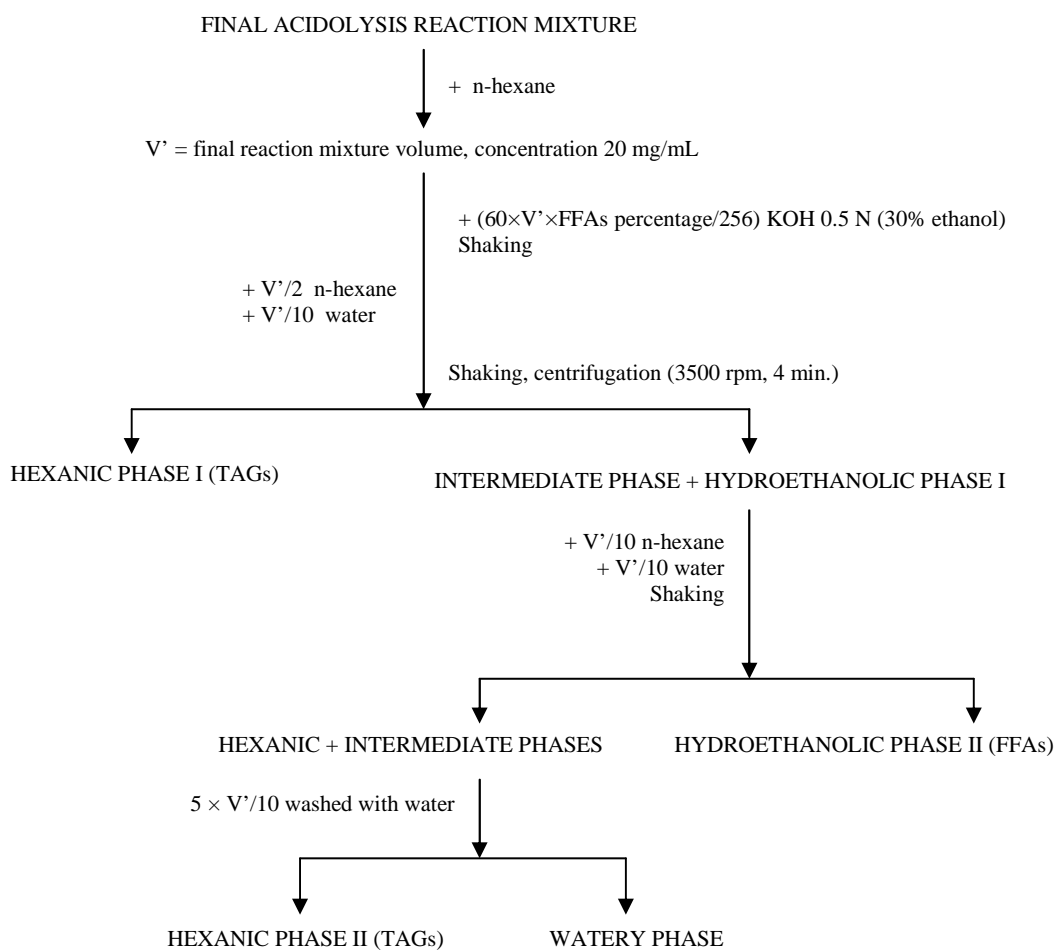


Fig. 2b. Experimental procedure II for purification of TAGs from the final acidolysis mixture.

2.6. Determination of the positional distribution of fatty acids in TAGs by pancreatic lipase method

This determination was made on samples of TAGs after removing FFAs. This method is based on enzymatic hydrolysis carried out following the method described in [28]. The method was applied to 0.5 mL of a TAG solution in hexane at a concentration of 0.2 g/mL. The extract obtained after hydrolysis was analyzed by TLC using the mobile phase chloroform/acetone/methanol 95:4.5:0.5 (v/v/v). Analysis of the 2-MAG spot by GC gave the fatty acid profile of position 2 of TAGs.

3. Results and discussion

The aim of this work was to obtain triacylglycerols (TAGs) enriched in palmitic acid (PA) at position 2; these TAGs are 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. As Table 2 shows, although the PA content is not high (21.8%), this acid account for 44.8% of total fatty acids at position 2, i. e. 68% of total PA in human milk is at position 2 of TAGs. However, although the oleic acid (OA) content is higher (33.9%), the content of this acid at position 2 is only 9.2% [30].

Table 2

Typical composition and distribution (mol %) of the most representative fatty acids in human milk triacylglycerols [30].

Fatty acid	Total ^a	Position 2 ^b	% Position 2 ^c
12:0	4.9	5.3	36.0
14:0	6.6	11.2	57.0
16:0	21.8	44.8	68.0
18:0	8.0	1.2	5.0
18:1n9	33.9	9.2	9.0
18:2n6	13.2	7.1	18.0
18:3n3	1.2	n.d.	n.d.

^{a, b} as in Table 1.

^c Fatty acid percentage at position 2 with respect to the total content of that fatty acid in the TAGs.

Natural vegetable oils contain much less PA at position 2. Although it is currently being investigated, to our knowledge there are as yet no commercially available methods for the enrichment of position 2 in specific fatty acids. For this reason, the usual methodology has consisted of obtaining TAGs rich in PA at all the three positions and then replacing positions 1 and 3 of TAGs with other fatty acids using 1,3 specific lipases. For the first step some researchers have opted for the synthesis of tripalmitin by enzymatic esterification of glycerol with PA [23]. In this work we have opted to modify a natural oil rich in PA by enzymatic acidolysis of this oil with PA concentrates. The oil chosen is palm oil stearin (POS), which contains 60% PA, of which 23% is at position 2 (Table 1).

The palmitic acid (PA) used in the acidolysis reaction was commercial PA (section 2.1) and also various PA concentrates obtained from POS by: (i) saponification of oil (the fatty acid extract obtained in this saponification has also been used to enrich the oil in PA) and (ii) crystallization at low temperature with solvents to obtain a new FFA mixture with a higher concentration of PA. In this crystallization we have also obtained a fraction rich in OA, which can be used in a second acidolysis reaction to obtain structured TAGs with the structure OPO.

The lipase chosen to obtain PA enriched TAGs was Novozym 435, which is not a 1,3 specific lipase, and so it also acts on position 2.

3.1. Obtaining FFA fractions rich in PA from palm oil stearin (POS)

The saponification of POS produces a FFA extract with 60% PA and 29% OA (Table 3), which are similar percentages to those in the original oil. The low temperature fractionation of a FFA mixture must produce a solid fraction richer in saturated fatty acids, such as PA and stearic acid, and a liquid fraction richer in unsaturated fatty acids, such as OA and linoleic acid (Table 3). The crystallization procedure shown in Figure 1 (section 2.2) was carried out to obtain FFA mixtures richer in PA and OA. Table 3 shows that the first crystallization in acetone (40 mL/g of FFA extract) gave a crystalline fraction (PA1) with 75.4% PA, recovering in this fraction 79.8% of initial PA. The second crystallization of the liquid fraction from the first crystallization (OA1, with 12.5% PA) (with 20 mL acetone/g of concentrate OA1), provides a fraction with 69.1% PA and in which 4.2% of initial PA is recovered. Thus, combining the solid fractions PA1 and PA2 a total PA recovery of about 84% was

achieved, with a purity of 75.1%. Chen *et al.* [23] enriched palm oil FFAs from 40% PA to 87.8% by a similar procedure. This greater concentration may be because palm oil contains 55% unsaturated fatty acids (45% saturated), while POS contains 33.5% unsaturated ones (Table 1). Therefore, larger amounts of unsaturated fatty acids can be removed in the liquid fractions from palm oil than from POS.

In these crystallizations the liquid fractions OA1 and OA2 and also the final solid fraction OA3 are rich in OA (Figure 1). Table 3 shows that the first crystallization in acetone produced a liquid fraction with 72.2% OA and a recovery of over 85% (fraction OA1). A second crystallization of this liquid phase gave rise to a liquid fraction with 80.3% OA and a recovery yield of 77.5% (of OA in the initial POS). This second crystallization is necessary to increase the OA concentration in the liquid phase from 72.2 to 80.3%, although the PA recovered is low (4.2%). The third crystallization was carried out in acetonitrile (60 mL/g of concentrate OA2), obtaining a crystalline phase OA3 with 87.9% OA, although the total recovery yield of OA decreased to 55.6%. Therefore this procedure increased the OA concentration threefold. Although this third crystallization achieved a higher purity of OA, the yield decreased from 77.5% to 55.6%, so it might not be justified. Chen *et al.* [23] obtained a FFA fraction with 96% OA (78% total recovery yield) starting from palm oil FFAs (42.5% OA) also using three crystallization steps; thus the OA concentration was multiplied by 2.2, although a higher OA recovery yield was attained.

Table 3 also shows that FFAs are not pure because saponification of POS was not quantitative. However, the non-saponified acylglycerols tend to remain in the liquid phases, and so they are mostly in the final liquid phase from the third crystallization, which was discarded.

Table 3

Fatty acid composition (mol %) of FFAs enriched in PA and OA obtained by saponification of POS and crystallization of FFAs mixture (Fig. 1).

Fatty acid	Initial FFAs mixture	First crystallization in acetone		Second crystallization in acetone		Third crystallization in acetonitrile
		Solid phase PA1	Liquid phase OA1	Solid phase PA2	Liquid phase OA2	Solid phase OA3
14:0	1.4	1.5	2.8	3.9	2.8	2.0
16:0	60.0	75.4	12.5	69.1	4.4	4.8
18:0	5.1	6.7	1.3	7.0	0.5	0.5
18:1n9	29.0	14.3	72.2	17.5	80.3	87.9
18:2n6	4.5	2.1	11.2	2.5	12.0	4.8
PA yield (%) ^a		79.8		4.2		
OA yield (%) ^a			85.4		77.5	55.6
FFA (mol %) ^b	95.9	98.0	91.6	93.8	91.7	93.5

^a Percentage of PA and OA recovered with respect to the PA and OA, respectively, contained in FFAs mixture obtained by saponification of POS.

^b Percentage of FFAs in each concentrated fraction.

3.2. Enzymatic acidolysis of palm oil stearin (POS) with palmitic acid (PA) catalyzed by *Novozym 435*

3.2.1. Influence of temperature, solvent amount and FFA/POS molar ratio

Usually the temperature of an enzymatic reaction is chosen bearing in mind the activity and stability of the enzyme, but in this reaction we must also take into account the fact that PA has a melting point of 63 °C and the temperature chosen must guarantee that the substrates rich in this acid remain liquid. However, this temperature can be lower if a solvent is used. Acidolysis experiments have therefore been carried out with several PA concentrates at both 37 °C and 10 mL hexane/g reaction mixture and at 50 °C and only 0.8 mL hexane/g reaction mixture. As Table 4 shows, the greater the PA contents of the FFAs the greater the enrichment in PA, both at all three positions and at position 2. At 37 °C, with the FFA extract from the saponification of POS, are obtained TAGs with 61.4% PA, which is approximately the same PA content as in FFAs and

POS (both have 60% PA). However the PA content at position 2 increases from 23% (Table 1) to 48.4% (Table 4). When FFAs enriched in PA by crystallization were used the PA content at position 2 increased to 60.3% and to 68.2% using commercial PA.

The high melting point of POS and PA concentrates makes it difficult to carry out the acidolysis reaction without solvent, since it would require a minimum temperature of about 65 °C; this temperature is too high because lipase deactivation may occur. For this reason, the reaction was made at 50 °C, since at this temperature Novozym 435 was stable [31]. A minimal amount of solvent was used to obtain a fluid mixture, which was achieved with a hexane/reaction mixture ratio of 0.8 mL/g. Table 4 shows that with the FFA extract from POS TAGs with the same total PA content (60-62.7%) were obtained, but with a redistribution of PA in the TAG molecule that increased the PA content at position 2 from 23% to 60.5%. Using commercial PA, both total PA content and that at position 2 increased, to 74.6% and 68.5%, respectively. Therefore, by acidolysis catalyzed with Novozym 435, it is also possible to modify the PA distribution in TAGs of POS.

Table 4

Acidolysis of palm oil stearin (POS, Table 1) with several PA concentrates (FFAs) to produce PA enriched TAGs: influence of temperature, hexane/reaction mixture ratio and PA concentration of FFAs on the PA composition of TAGs obtained (mol %).

T (°C)	Hexane (mL/g mixture)	Source of FFAs (PA concentration, mol %)	PA composition of TAGs obtained (mol %)	
			Total ^a	Position 2 ^b
37	10	FFAs extract of POS (60% PA) ^c	61.4	48.4
		PA concentrate (PA1 + PA2, 75% PA) ^d	64.6	60.3
		Commercial PA (98% PA)	72.5	68.2
50	0.8	FFAs extract of POS (60% PA) ^c	62.7	60.5
		Commercial PA (98% PA)	74.6	68.5

^{a, b} as in Table 1.

^c FFAs extract from the saponification of POS.

^d PA concentrate obtained from the first and second crystallizations.

Operational conditions: 2.5 g POS, 2.5 g FFAs (FFAs/POS ratio 3:1 mol/mol), 0.5 g Novozym 435 (10% w/w with respect to the reaction mixture), 24 h, 200 rpm.

The following experiments were carried out with commercial PA (98% PA) in order to attain maximum PA content at position 2. However, the results shown in Table

4 indicate that this procedure also achieved high PA enrichment at position 2 with the FFA mixture from the saponification of POS and with the PA rich FFA obtained by crystallization. With commercial PA the results at 37 °C and 10 mL/g were similar to that obtained at 50 °C and 0.8 mL/g (Table 4); for this reason it was decided to use the lower temperature (37 °C and 10 mL/g) to operate in conditions of higher lipase stability and to avoid using volatile solvents at high temperature.

With regard to the influence of FFA/POS molar ratio, in previous experiments the acidolysis reactions were carried out at the stoichiometric ratio (FFA/TAG molar ratio 3:1). In this work experiments at FFA/POS molar ratios of 1, 2, 3, 4 and 6 were carried out. Both the total content and the PA content at position 2 remained practically constant in these experiments. Taking into account this result the stoichiometric molar ratio was maintained for further experiments.

3.2.2. Influence of the intensity of treatment (IOT)

The IOT is the lipase amount \times reaction time/POS amount (limiting reagent) and it is a useful parameter for the scaling up of enzymatic reactions [32, 27]. The habitually used lipase amount ranges between 10% and 25% of the amount of reaction mixture. In these experiments 0.5 g POS and 0.5 g commercial PA (98% PA) were used and the lipase amount used was constant (0.1 g, i. e. 10% of reaction mixture); the IOT was modified by varying the reaction time. Table 5 shows the PA contents at the three positions and at position 2 of TAGs obtained from commercial PA and from PA concentrate obtained by crystallization. This table shows that the PA content increased with the IOT to values of about 10 g lipase h/g POS, remaining constant for higher IOT values. The PA content increases to 79.2%, which is an intermediate value between the PA contents in the POS (60%) and in the commercial PA (98%). The PA content at position 2 attained similar values, although somewhat lower than the total PA content. In this way, TAGs were obtained with 79.2% PA and 74.5% PA at position 2.

In the acidolysis of POS with the PA concentrate obtained by crystallization (75.1% PA) TAGs with PA contents of over 70% were obtained, at IOT of about 19-20 g lipase h/g POS. In these TAGs the PA is distributed homogeneously because position 2 also contains 70% PA.

Chen *et al.* [23] obtained tripalmitin by alcoholysis of glycerol with 98.3% pure ethyl palmitate, catalyzed by Novozym 435. These authors attained conversions of

about 88% with 91% mol of tripalmitin. However, the procedure used in this work seems more adequate to produce a PA enriched natural oil.

Table 5

Acidolysis of POS (Table 1) with commercial PA (98% PA) and a PA concentrate (FFAs) obtained by crystallization (75.1% PA): influence of reaction time and IOT (g lipase × reaction time/g POS) on the PA composition (mol %) of enriched TAGs.

IOT ^a (g lipase×h/g POS)	PA composition of TAGs obtained (mol %)			
	From commercial PA (98% PA)		From PA concentrate (75.1% PA)	
	Total ^b	Position 2 ^c	Total ^b	Position 2 ^c
0	60.0	23.0	60.0	23.0
0.4	58.6	-		
0.8	62.8	-		
1.2	63.4	-		
1.6	68.9	-		
4.8	72.5	68.2	64.6	60.3
9.6	79.2	74.5	68.1	63.5
14.4	76.4	-		
19.2	76.9	-	70.7	70.5

^a The intensity of treatment (IOT) was increased keeping constant the lipase and POS amounts and by increasing the reaction time.

^{b, c} as in Table 1.

Operational conditions: 0.5 g POS, 0.5 g commercial PA (98% PA) or PA concentrate (75.1%), 0.1 g Novozym 435, 10 mL hexane, 37 °C and 200 rpm.

3.3. Scaling up of the acidolysis reaction

After selecting the most adequate operational conditions for the PA enrichment of TAGs at position 2, the process was scaled up, carrying out experiments in a 1.5 L reactor, multiplying by 100 the reaction mixture and lipase amounts used in the experiments shown in Table 5. Figure 3 shows the influence of IOT on the PA content of the TAGs obtained. It is observed that while the total PA content increases from 60% to about 80%, the PA content at position 2 increases from 23% to almost the same final content (75%); these incorporations were achieved for an IOT of about 10 g lipase × h/g

POS (reaction time of 48 h). These results are identical to those obtained in the low-scale experiments in the same conditions (Table 5), which confirms the validity of the scaling-up approach (maintaining a constant IOT).

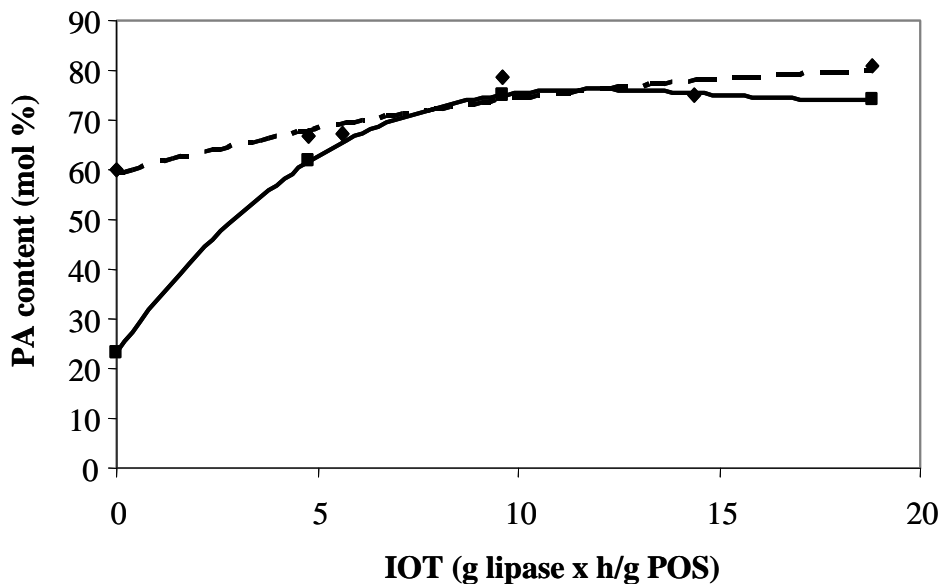


Fig. 3. Scaling up of acidolysis reaction of POS (Table 1) with commercial PA (98% PA): influence of intensity of treatment (IOT g lipase \times time/g POS) on the PA content (mol %) of enriched TAGs (◆) and on the PA content at position 2 (■). *Operational conditions:* 50 g POS, 50 g commercial PA, 10 g Novozym 435, 1 L hexane, 37 °C and 200 rpm.

3.4. Lipase stability

Figure 4 shows that lipase Novozym 435 remains stable over at least 10 uses in the operational conditions used in the acidolysis of POS with commercial PA, since both the total PA content and that at position 2 remained approximately constant over the 10 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 remained stored for 28 months at 5°C. The PA content of TAGs obtained in three acidolysis reactions carried out at 3, 16 and 28 months of storage was the same; these experiments show the great stability of Novozym 435, since after 28 months of storage its activity remained constant.

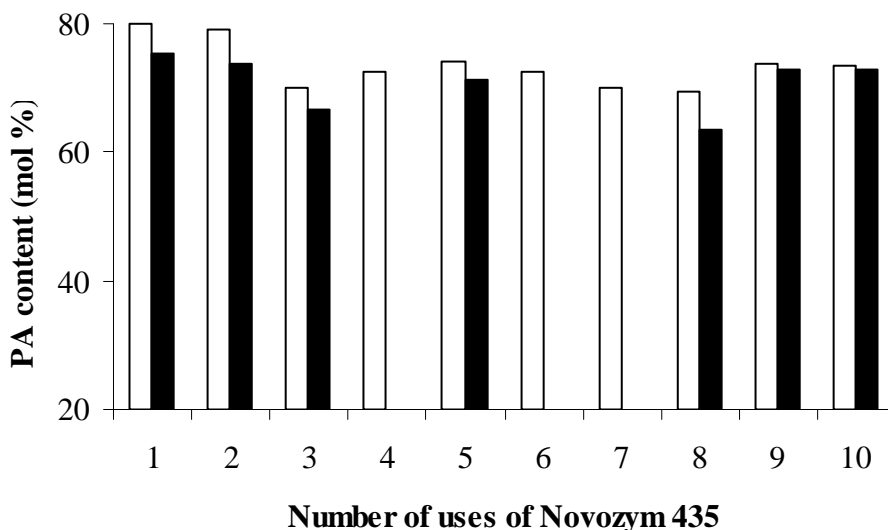


Fig. 4. Influence of the number of uses of lipase Novozym 435 on its activity, determined as the PA content in the enriched TAGs obtained by acidolysis of POS with commercial PA. *Operational conditions:* POS/commercial PA ratio 1:1 w/w, 37 °C, 10 mL hexane/g reaction mixture, IOT = 9.6 g Novozym 435 × h/g POS.

3.5. Purification of PA enriched TAGs

The acidolysis products were mixtures with approximately 50% FFAs, 2-4% diacylglycerols (DAGs) and 46-48% TAGs (weight percentages determined by TLC-FID). The purification of TAGs, by removal of FFAs, was carried out by the two operational modes described in Figures 2a and 2b. Both methods are based on saponifying FFAs with KOH hydroalcoholic solutions and extracting TAGs with hexane.

Table 6 shows that the first procedure produced 99.1% pure acylglycerols (TAGs + DAGs) with 95% yield. The second procedure also obtained 99.3% pure acylglycerols, but the recovery yield was below 82%. This second procedure consumed half of the solvents consumed in the first one, but it is more laborious, since it is necessary to carry out a phase separation by centrifugation and more hexane additions and washing with water than in procedure I. Table 7 shows the fatty acid composition of purified TAGs (which account for about 95% of final acylglycerols).

Table 6

Purification of PA enriched TAGs obtained by acidolysis of POS and commercial PA: purities and yields.

	Experimental procedure	
	I (Fig. 2a)	II (Fig. 2b)
Volume of hexane consumed per gram of mixture (FFA/acylglycerol 1:1 w/w) to separate (L)	0.15	0.08
Acylglycerol purity in the hexanic phase I (% of TAGs + DAGs on total lipids)	99.1	99.3
Recovery yield of TAGs in the hexanic phase I (%)	80.2	69.3
Acylglycerol purity in the hexanic phase II (% of TAGs + DAGs of total lipids)	99.5	99.5
Recovery yield of TAGs in hexanic phase II (%)	14.8	12.4
Recovery yield of TAGs in hexanic phases I and II (%)	95.0	81.7

Experiments carried out with 3 g TAGs, DAGs and FFAs mixture from acidolysis reaction.

Table 7

Fatty acid composition (mol %) of TAGs obtained by acidolysis of POS and PA and later purification by procedure I (Figure 2a and Table 6).

Fatty acid	TAGs obtained (mol %)			
	with commercial PA (98% PA)		with PA concentrate (75.1% PA)	
	Total ^a	Position 2 ^b	Total ^a	Position 2 ^b
14:0	0.8	n.d.	1.1	1.3
16:0	79.2	74.5	70.7	70.5
18:0	2.7	2.8	6.0	5.4
18:1n9	14.9	18.8	18.3	18.5
18:2n6	2.4	3.9	3.9	4.3

^{a, b} as in Table 1.

Operational conditions: 0.5 g POS, 0.5 g commercial PA or PA concentrate, 10 mL hexane, 37 °C and 200 rpm. IOT 9.6 g lipase x h/g POS with commercial PA and 19.2 with PA concentrate.

4. Conclusions

This work determined optimal operational conditions for obtaining PA enriched TAGs at position 2. These TAGs will be used as intermediates for the synthesis of structured TAGs. PA enriched TAGs was produced by acidolysis of POS (60% PA) and FFAs highly rich in PA, catalyzed by the lipase Novozym 435. The greater the PA content of FFAs, the greater PA content of the TAGs obtained.

Carrying out acidolysis reaction with a 3.1 PA/POS molar ratio, at 37 °C, with 10 mL hexane/g reaction mixture and an IOT of around 10 g Novozym 435×h/g POS, TAGs were obtained with about 79% PA and 75% of this fatty acid at position 2. These TAGs were separated from FFAs after the acidolysis reaction with a recovery yield of around 95%.

The acidolysis reaction was scaled up 100 times (from 0.5 g to 50 g POS), obtaining identical results. It was also proved that lipase Novozym 435 can be reused at least 10 times in the operational conditions optimized in this work without observing an appreciable activity loss. Furthermore, Novozym 435 was shown to be stable for at least 28 months stored at 5 °C.

From the experiments and results obtained in this work it can be concluded that it is possible to use only a natural vegetable oil highly rich in PA (POS, with 60% PA and 23% of this fatty acid at position 2) to obtain a TAG with 70.7% PA and 70.5% PA at position 2. This process consist of 4 steps: (1) saponification of POS, (2) crystallization of FFAs to obtain PA enriched FFAs (75.1% PA), (3) acidolysis of POS with these FFAs, catalyzed by Novozym 435, to obtain PA enriched TAGs at position 2 and (4) separation of the TAG-FFA mixtures to obtain TAGs with purity and yields of about 95%. Moreover, in steps (1) and (2) OA enriched FFAs were obtained, and these may be used later to produce structured TAGs with the structure OA – PA – OA by acidolysis of PA enriched TAGs at position 2 with OA enriched FFAs, catalyzed by a 1,3 specific lipase.

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