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Study of acyl migration during enzymatic interesterification of liquid and fully hydrogenated soybean oil

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ABSTRACT

Lipase-catalyzed interesterification has emerged as an attractive process to obtain plastic fats because of its numerous advantages compared to the chemical reaction. The use of *sn*-1,3 specific lipases adds an even more interesting feature to this process, which is related to the possibility of maintaining the *sn*-2 fatty acid composition of natural substrates unaltered. The pursuit of this characteristic in interesterified products by *sn*-1,3 specific lipases could be threatened by a chemical reaction, the acyl migration whithin mono- and diacylglycerols. The aim of this study was to evaluate the occurrence of this undesired reaction in a soybean oil:fully hydrogenated soybean oil enzymatic interesterified blend. Once acyl migration was confirmed to occur, the influence of different reaction parameters -namely enzyme type and concentration, substrate ratio, and addition of hexane and temperature effect- was studied. Lipozyme TL IM demonstrated an enhancer acyl migration effect compared to Lipozyme RM IM, effect that was hypothesized to be correlated with the immobilization support material of the former (silica gel). Acyl migration was also promoted by the increase of biocatalyst concentration in reaction media. On the contrary, the presence of hexane, together with a decrease in temperature reaction, reduced its occurrence. A temperature effect analysis performed in solvent reaction media demonstrated its promoter effect on acyl migration.

1. Introduction

In the last past years, the interesterification process has gained acceptance as a fats and oils modification technique because of its unique features. It consists in the redistribution of fatty acid moieties among and within triacylglycerols (TAG) in a mixture. Consequently, a new mixture having different chemical composition and hence enhanced physical properties is obtained. The lipase-catalyzed interesterification offers an attractive alternative to the chemical-catalyzed process [1] as it possesses several advantages: milder reaction conditions reducing product degradation, operative costs and energy consumption [2,3] and reduced use of chemical products, with a consequent increase in ambient pollution [4]. Moreover, the use of lipases in a natural environmental reaction medium diminishes the generation of certain secondary products that occur as a result of parallel degrading reactions during chemical interesterification. The consequences of these undesired reactions are multiple: reduction of the nutritional value of the product, higher tendency to oxidation due to reduction of tocopherols content, greater oil losses and increase in the number of post treatment steps for product purification [4,5].

In addition to the above-mentioned advantages, one of the most attractive features certain lipases have shown is their positional specificity. Making use of *sn*-1,3 specific lipases, only the fatty acid moieties located at the outer glycerol backbone positions are reactive. The relative distribution of the fatty acids in the TAG molecule backbone plays an important role both in the products functionality and in the way those fatty acids are metabolized when ingested. The unique distribution of the predominant cocoa butter fatty acids (stearic, palmitic, and oleic acid) is a clear example of the first implication; it defines a narrow melting point range, located just below body temperature. The way cocoa butter melts in the mouth is one of the reasons why chocolate consumption is that pleasant [6]. With respect to its nutritional implication, it is related to the pancreatic lipase action in the organism; it hydrolyzes fatty acids at *sn*-1 and *sn*-3 positions during TAG digestion [7]. The metabolic fate of fatty acids retained at *sn*-2 position is

Abbreviations: TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; SO, soybean oil; FHSO, fully hydrogenated soybean oil; FA, fatty acid; FAME, fatty acid methyl ester; S, saturated fatty acid; U, unsaturated fatty acid; GC, gas chromatography; U_2, unsaturated fatty acid at *sn*-2 position; S_2, saturated fatty acid at *sn*-2 position.

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different to that of the liberated ones. For instance, saturated fatty acids in the *sn*-2 position of dietary TAG may elevate LDL concentrations more than the same fatty acid in the *sn*-1 or *sn*-3 positions [8]. Natural vegetable oils are rich in unsaturated fatty acids (mainly essential ones) in the central position of the glycerol backbone, while the saturated fatty acids principally take the outer positions [8,9]. In the case of lipase-catalyzed interesterification oriented to obtaining products with enhanced functional properties using *sn*-1,3 specific lipases, the election of this type of enzymes is based on the intention of maintaining the *sn*-2 fatty acid profile unaltered [10].

This objective, as well as the reaction yield, could be threatened by a non-enzymatic mechanism known as acyl migration. During the interesterification process, this spontaneous phenomenon occurs due to the appearance of partial glycerides, mainly diacylglycerols (DAG) [11]. The acyl migration of this type of compounds consists of the isomerization of 1(3), 2-DAG, generated by *sn*-1,3 specific lipases, to the more thermodynamically stable form of 1,3-DAG. The main driving force for this reaction resides on a steric effect. The ester group at *sn*-2 position in 1(3), 2-DAG presents larger interactions with the adjacent ester group at *sn*-1(3) position and with the hydroxyl at *sn*-3(1) position than with the corresponding ones of 1,3-DAG. On the other hand, this effect is partially compensated by the attraction of non polar fatty acid chains due to the van der Waals forces [12]. In this manner, the reduction on the reaction yield as a consequence of acyl migration of DAG responds to the incapacity of the *sn*-1,3 specific lipase to reesterify an acyl group at *sn*-2 position.

There are different factors which can affect acyl migration. There are contradictory studies with respect to the water effect over this phenomenon. Oda et al. [13] found that the water content increased the acyl migration rate in the reaction of methanolysis of vegetable oils. However, Xu et al. [11] suggested that, in solvent free media, water does not induce this type of migration, arguing that the effects reported by other authors probably accounts for different DAG levels obtained at different media water contents. There is also controversy regarding the solvent role in acyl migration. Xu et al. [11] indicated that non-polar solvents accelerated the reaction, referencing a publication of Goh et al. [14] in which the effect of the addition of two solvents, hexane and diethyl ether, over the cocoa butter transesterification reaction was studied. The latter authors concluded that the presence of hexane in the reaction medium conducted to a significant increase in the occurrence of acyl migration, compared to diethyl ether systems. No solvent free media were evaluated in this work. Furthermore, in a subsequent work of Xu [15] it is concluded that hexane could inhibit acyl migration in acidolysis reactions of tripalmitin with conjugated linoleic acid (CLA) and capric acid.

To the authors knowledge, no study concerning acyl migration during enzymatic interesterification of refined and fully hydrogenated soybean oil, which is a commonly substrates blend, has been performed. Therefore, the objective of this work was to evaluate the occurrence of the acyl migration phenomenon during the lipase-catalyzed interesterification of the above-mentioned substrates mixture and establish the effect of certain reaction parameters.

2. Experimental

2.1. Materials

Refined soybean oil (SO) (approximate composition of predominant FA as FAMEs: 53.0% C18:2 (cis, cis-9,12-octadecadienoic acid), 20.0% C18:1 (cis-9-octadecenoic acid), 11.0% C16:0 (hexadecanoic acid), 6.0% C18:3 (cis, cis, cis-9,12,15-octadecatrienoic acid), according to AOCS Official Method Ce 2-62 and Ce 1e-91 [16]; peroxide value (PV): 1.16 meq/kg, according to AOCS Official Method Cd 8-53 [16]; calculated iodine value (IV): 129.1, according to AOCS Official Method Cd 1c-85 [16], moisture content: 0.05% (w/w), measured with a Karl Fischer titrator Mettler DL18, according to the AOCS Official Method Ca 2e-84 [16]) was provided by Molinos Río de la Plata SA (Buenos Aires, Argentina) and fully hydrogenated soybean oil (FHSO) (approximate composition of predominant FA as fatty acid methyl esters (FAME): 83.8% C18:0 (octadecanoic acid), 12.3% C16:0 (hexadecanoic acid), 1.4% C18:1 (cis-9-octadecenoic acid); PV: 0.20 meq/kg; IV: 2.14, moisture content: 0.15% (w/w)) was kindly provided by Calsa S.A. (Buenos Aires, Argentina). Immobilized lipases from *Rhizomucor miehei* (EC number 3.1.1.3, Lipozyme RM IM, immobilized on ion-exchange resin, sn-1,3 specific, with an original water content of 5.1% (w/w), determined by the Karl Fisher Titration Method) and *Thermomyces lanuginosa* (EC number 3.1.1.3, Lypozyme TL IM, immobilized on granulated silica gel, sn-1,3 specific, with an original water content of 3.6% (w/w)) was a generous gift of Novozymes Latin America Ltd. (Brazil) and they were used as received. Lipase from hog pancreas (20.3 U/mg) and *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Fluka (Buchs, Switzerland). Standards (1,2,3-trioctadecanoyl-glycerol, 1,2,3-trihexadecenoyl-glycerol, 1,2,3-trioctadecadienoyl-glycerol, 1,2,3-trioctadecenoyl-glycerol, 1,2,3-trihexadecanoyl-glycerol, 1,2,3-tridecanoyl-glycerol, 1,2-distearoyl-3-palmitoyl-rac-glycerol, 1,3-dipalmitoyl-2-oleoylglycerol, 1,3-dioleoyl-2-palmitoyl-glycerol, 1,2-dilinoleyl-3-palmitoyl-rac-glycerol, 1,2-dioleoyl-3-stearoyl-rac-glycerol, 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol, 1,2-distearoyl-3-oleoyl-rac-glycerol, 1,3-dipalmitoyl-rac-glycerol, 1-mono palmitoyl-rac-glycerol, octadecenoic acid, tetradecane) were of purity greater than 98% and were obtained from Sigma Chemical Co. (St. Louis, USA). Fatty acid methyl esters (FAME) standards were purchased from Supelco (Bellefonte, USA). Pyridine was from J.T. Baker (Philipsburg, USA). All other reagents, gases and solvents were of analytical or chromatographic grade.

2.2. Methods

2.2.1. Reaction protocol

In Table 1 the reaction conditions for all experiments are detailed. Blends constituted by SO and FHSO (total substrate weight 1 g) were placed in a screw-capped test tube, hexane was added when corresponded (3 mL/g substrates), preheated in a water bath with temperature controller and magnetic agitation (230 rpm) and, after 5 min of homogenization, biocatalyst was added to start the reaction. It is worth mentioning that for hexane-free systems a reaction temperature equal to the melting point of the FHSO (70 °C) was established. This decision was based on a previous study in which the melting point of the FHSO enriched initial blend (50:50 SO:FHSO (% w/w)) was determined as being of 64.5 °C [17]. Moreover, the melting point of mixtures arising from the lipase-catalyzed interesterification of SO and FHSO blends sharply decreased even at 1 h of reaction time with respect to the initial blend melting point [17]. In this manner, mass transfer/diffusional limitations are minimized. Reactions were stopped removing enzymes by filtering with Whatman 1 filter paper at different time intervals between 0 and 48 h. Reaction products were immediately stored below 0 °C.

2.2.2. Analytical methods

2.2.2.1. TAG, DAG, and MAG analysis. TAG, DAG, and MAG were prepared and analyzed by GC according to Pacheco et al. [17]. This method also enables the determination of glycerol. A 4890D series gas chromatograph (Agilent, Hewlett-Packard) was used with a metallic capillary column (MXT-65TG, 30 m × 0.25 mm × 0.10 μm

Table 1
Reaction conditions for all experiments.

	Hexane-free media						Hexane media[a]	
	Lipozyme RM IM				Lipozyme TL IM		Lipozyme RM IM	
Temperature (°C)	70	70	70	70	70	70	55	60
Lipase concentration (% w/$w_{subst.}$)[b]	5–10	5	5	5–10	5–10	10	5	5
SO:FHSO (% w/w)[c]	50:50	60:40	70:30	80:20	50:50	80:20	50:50	50:50

Agitation speed: 230 rpm.
[a] Hexane added: 3 mL.
[b] Mass percentage on a substrate basis.
[c] SO: refined soybean oil, FHSO: fully hydrogenated soybean oil.

Table 2
Global and positional distribution of fatty acids of reaction mixtures (% mol/mol).

		Substrate ratio SO:FHSO (% w/w)			
		50:50	60:40	65:35	70:30
Global	%S	58.44 ± 0.01	50.53 ± 0.01	46.57 ± 0.02	42.62 ± 0.02
	%U	41.56 ± 0.01	49.47 ± 0.01	53.43 ± 0.02	57.38 ± 0.02
sn-2 position	$\%S_2$	50.30 ± 3.37	41.00 ± 1.77	36.35 ± 2.86	31.70 ± 0.98
	$\%U_2$	49.70 ± 3.06	59.00 ± 1.11	63.65 ± 1.25	68.30 ± 1.12
sn-1,3 position	$\%S_{1,3}$	62.51 ± 3.37	55.30 ± 1.77	51.69 ± 2.86	48.08 ± 0.98
	$\%U_{1,3}$	37.49 ± 3.06	44.70 ± 1.11	48.31 ± 1.25	51.92 ± 1.12

SO: refined soybean oil.
FHSO: fully hydrogenated soybean oil.
S: saturated fatty acid, U: unsaturated fatty acid.

film thickness; Restek, Bellefonte, USA). Hydrogen at 41 cm/s was used as carrier gas. Split mode at 1:60 ratio was used during injection. Oven temperature was programmed as follow: 40 °C for 4 min, then increased up to 350 °C at 25 °C/min, and held at this temperature for 15 min. Injector and FID temperatures were 360 °C and 380 °C, respectively. For quantitation purposes the internal standard method was used. For both MAG and DAG determination, glyceryl tridecanoate was the internal standard used, while for TAG tripalmitolein acted as internal standard since it was not present in any of the samples. Monopalmitin, dipalmitin, and tripalmitolein were used as standards for MAG, DAG, and TAG analyses, respectively. Several commercial TAG standards were used to construct a calibration surface in order to quantify TAG concentration as a function of peak area and retention time [17]. It is important to mention that the method cannot distinguish between TAG positional isomers, although being capable of quantifying 1,2- and 1,3-DAG separately. Data acquisition and peak integration were carried out using HP 3398A GC Chemstation Software (Hewlett-Packard, 1998).

2.2.2.2. sn-*2 fatty acid composition.* Samples were deacidified according to Carrín and Crapiste [18]. The pancreatic lipase technique according to the AOCS Official Method Ch 3-91 [16] with the following modification: as some reaction products have melting points above 40 °C (reaction temperature in the original method), the reaction was developed at 52 °C (temperature at which a 50:50 (SO:FHSO, %w/w) sample in hexane is totally dissolved). Soybean oil was used to assure similar results when this modification was introduced in the procedure.

Regarding FHSO, its *sn*-2 fatty acid composition was theoretically calculated through mass balances due to its high melting point (70 °C). Experimental information about *sn*-2 fatty acid composition of SO and 50:50 SO:FHSO (% w/w) blend were used in this calculation.

2.2.2.3. Fatty acid composition. FAME from glycerides were prepared by cold transesterification with methanolic KOH according to the AOCS Official Method Ce 2-66 [16], and were analyzed by GLC with a 4890D series gas chromatograph (Agilent, Hewlett-Packard, USA) and a fused-silica capillary column (SP-2380, 30 m × 0.25 mm × 0.2 μm film thickness; Supelco Inc., USA). The carrier gas was hydrogen with a linear velocity of 17 cm/s. The injector was used in split mode with a ratio of 1:50. The oven temperature was programmed to be at 170 °C for 15 min, further to increase to 210 °C at a rate of 4 °C/min, and held for 10 min. The injector and detector temperatures were 220 °C. FAME were identified by comparing their retention times with authentic standards.

2.2.3. Statistical analysis

Data are expressed as means ± standard deviation of two replicates for *sn*-2 fatty acid composition analyses and three replicates for the remaining methods. The statistical analysis was carried out by *t*-test at the 0.05 significance level.

3. Results and discussion

The global and positional fatty acid distributions of initial reaction mixtures are presented in Table 2. Global and *sn*-2 fatty acid compositions were determined by GC according to Sections 2.2.2.3 and 2.2.2.2, respectively; while *sn*-1,3 fatty acid distribution was theoretically calculated by difference.

3.1. Interesterification reaction

The TAG distribution and composition in reaction products was analyzed. According to their constituent FA, they were classified in four groups: SSS, SUS, UUS, and UUU, regardless of the positional FA distribution within the glycerol backbone (since the analytical method did not discriminate between positional isomers). Considering the disaturated TAG group (SUS/SSU/USS) was the one showing the greatest changes in all samples, it was chosen as an indicator of the interesterification reaction. Its kinetic behaviour is shown in Fig. 1. It could be observed that, at equal substrate ratios and enzyme concentration, higher initial reaction rates were displayed by Lipozyme TL IM than RM IM ($P < 0.05$). Regarding equilibrium values, both enzymes working at the same concentration in a mixture of equal substrate ratio, led to products with similar SUS content ($P > 0.05$).

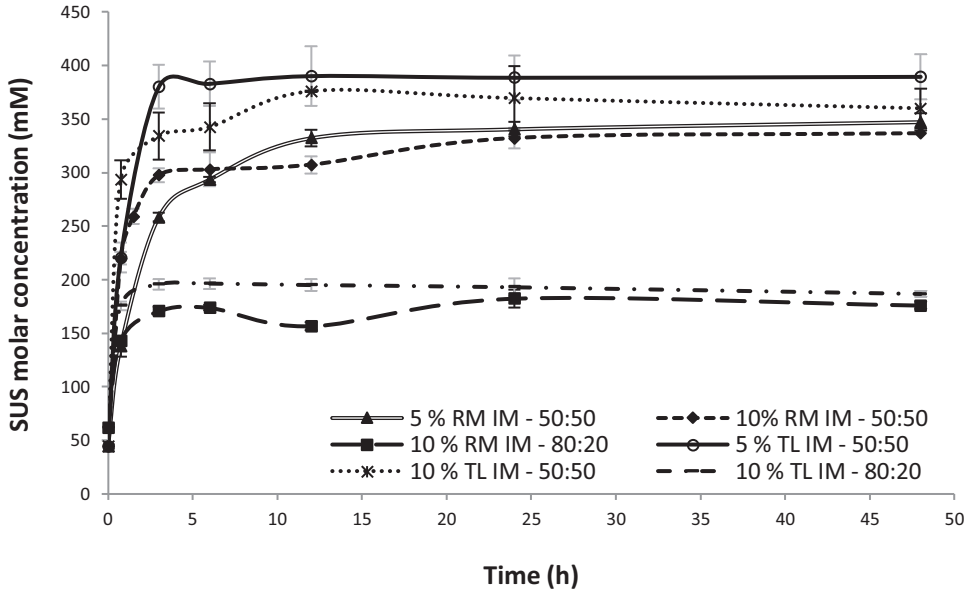

Fig. 1. Kinetic profiles of disaturated TAG (expressed as SUS, not indicating a positional preference of fatty acids over TAG) (molar concentration, mM) for different SO:FHSO (% w/w) and lipase type and concentration. Reaction conditions: 70 °C. RM IM: Lipozyme RM IM, TL IM: Lipozyme TL IM. Mean value of three independent reactions ± standard deviation.

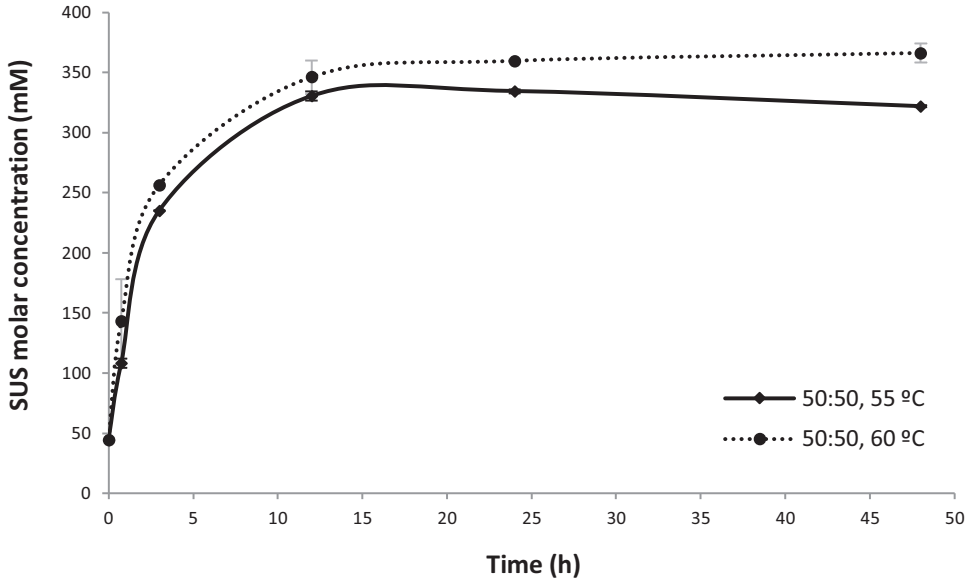

Fig. 2. Kinetic profiles of disaturated TAG (expressed as SUS, not indicating a positional preference of fatty acids over TAG) (molar concentration, mM) for different reaction temperatures. Reaction conditions: hexane media (3 mL/g substrates), 50:50 (SO:FHSO, % w/w), 5% ($w/w_{subst.}$) Lipozyme RM IM. Mean value of three independent reactions ± standard deviation.

Fig. 2 shows the kinetic behavior for disaturated TAG in hexane media at different reaction temperatures. The great effect a relatively little change in this variable (5 °C) have over the initial reaction rate and equilibrium values can be clearly observed in this figure. Moreover, in those experimental conditions, higher levels of equilibrium DAG concentration were obtained at 55 °C than at 60 °C (almost 90 mM at 60 °C against around 190 mM at 55 °C, data not shown). DAG concentration does not correlate directly with SUS concentration since there are other three groups of TAG (SSS, UUS, and UUU) which are, although individually to a lesser extent than SUS group, contributing to DAG generation.

3.2. Fatty acid profile at sn-2 position

As mentioned above, the rearrangement of FA within the glycerol backbone during interesterification reactions depends on the catalyst used. In the case of reactions mediated by chemical catalysis or non-specific lipases, the final distribution of FA is totally random. For this case, as well as for the one considering a random *sn*-1,3 acyl distribution (with unaltered *sn*-2 position), basic probability concepts can be applied in order to obtain the theoretical TAG profile at equilibrium based on the initial blend composition [19].

Table 3
Theoretical and experimental molar ratios of unsaturated (U) to saturated (S) FA at sn-2 position (%U2/%S2) corresponding to different initial substrates ratios and lipases.

SO:FHSO[a] (% w/w)	Enzyme type[b]	Theoretical distributions		Experimental values[c]
		Random acyl distribution	*sn*-1,3 acyl distribution	
50:50	RM IM	0.53	0.99	0.69 ± 0.05
	TL IM			0.58 ± 0.03
60:40	RM IM	0.66	1.44	1.14 ± 0.11
65:35	TL IM	0.73	1.75	0.96 ± 0.05
70:30	RM IM	0.81	2.15	1.13 ± 0.07

[a] SO: refined soybean oil, FHSO: fully hydrogenated soybean oil.
[b] RM IM: Lipozyme RM IM, TL IM: Lipozyme TL IM.
[c] Agitation speed: 230 rpm, 5% ($w/w_{subst.}$) lipase, 70 °C, and 48 h of reaction time.

Table 4
DAG content of both substrates and initial reaction mixtures (SO:FHSO).

	SO[a]	FHSO[b]	Blend 50:50 (% w/w)	Blend 80:20 (% w/w)
1(3), 2-DAG (mM)	0.44 ± 0.12	0.30 ± 0.03	0.37 ± 0.07	0.41 ± 0.10
1,3-DAG (mM)	0.67 ± 0.07	1.00 ± 0.09	0.83 ± 0.08	0.73 ± 0.08
% 1,3-DAG (mol/mol)	60.18 ± 3.59	76.63 ± 2.83	68.40 ± 3.21	63.45 ± 3.44

[a] SO: refined soybean oil.
[b] FHSO: fully hydrogenated soybean oil.

From these predictions, the distribution of fatty acids at *sn*-2 position and thus, the ratio of unsaturated to saturated FA in this position ($\%U_2/\%S_2$) can be estimated. This latter parameter calculated for various initial substrates ratios is listed in Table 3. It is also presented the experimental values obtained by GC at equilibrium (it was determined that at 48 h thermodynamic equilibrium is reached for all conditions). It can be clearly observed that the experimental ratios fall between both theoretical values, indicating that, even though the distribution was not totally random, FA profile at *sn*-2 position could not be maintained. Zhang et al. [20] found similar experimental values when a 75:25 (% w/w) mixture of palm stearin:coconut oil was interesterified. The corresponding ($\%U_2/\%S_2$) values for the initial reaction mixture, and both chemically and enzymatically (Lipozyme TL IM) interesterified products were: 1.02, 0.33, and 0.69, respectively.

As previously mentioned, the *sn*-2 fatty acid composition of plastic fats' glycerides possesses important nutritional implications. Therefore, it is sought to preserve it as unaltered as possible. It can be observed that different substrate ratios and immobilized lipases show rather different experimental value deviations from the theoretical one assuming maintenance of *sn*-2 position. Consequently, final products' composition will strongly depend on these two reaction conditions.

Diverse authors have indicated that both Lipozyme RM IM and TL IM can be considered as strict positional specific lipases [10,21–23]. Therefore, the modification of the FA profile at *sn*-2 position in all interesterified products was directly correlated with the acyl migration reaction occurrence.

3.3. Acyl migration study

Once the occurrence of acyl migration phenomenon was confirmed, a study of the influence of different reaction parameters was performed. The indicator used to study this undesirable side reaction was the percentage of 1,3-DAG respect to total DAG concentration in the reaction mixture. Particularly, the effect of the type and enzyme concentration, unsaturation level of FA moieties, presence of hexane in reaction medium, and temperature in the latter systems was evaluated.

DAG content of both substrates as well as the corresponding composition for the initial reaction blends which were interesterified for the analyses of the present section are presented in Table 4. Although both isomers 1,3-DAG and 1(3), 2-DAG are present in considerably low concentrations in both substrates (aprox. 1% (mol/mol)), it can be noted that the relative 1,3-DAG percentage for SO is in agreement with the equilibrium values reported for oils containing long chain fatty acids (60–70%) [11,12,24]. Regarding FHSO, its corresponding value is slightly higher than those ones. Probably the high hydrogenation temperatures have moved the equilibrium towards the formation of this isomer. Even though afterwards the product was kept at ambient temperature, the limited mobility of its molecules due to its solid character has probably maintained the equilibrium ratio unaltered.

3.3.1. Enzyme type and concentration effect

In Fig. 3 the evolution of molar percentage of 1,3-DAG with respect to total DAG during the interesterification of a 50:50 (% w/w) blend by means of both lipases is shown. Analyzing the data obtained with 5% (w/w of total substrates) enzyme concentration, it can be observed for Lipozyme TL IM that at 3 h of reaction time the percentage of 1,3-DAG is already stabilized in its final value, indicating that isomerization reaction has reached its equilibrium condition although the concentration of total DAG in the reaction medium continues changing until 12 h of reaction time (data not shown). In the case of Lipozyme RM IM at the same concentration, the stabilization of 1,3-DAG percentage is reached after 12 h of reaction time (together with the stabilization of total DAG composition (data not shown)). The fact that for Lipozyme TL IM considerably higher acyl migration rates (relative to total DAG formation rate) were obtained could be explained in terms of their corresponding immobilization supports. Lipozyme RM IM is immobilized on an ion-exchange resin, while the material support of Lipozyme TL IM is granulated silica gel. Although resins have been reported as potential acyl migration catalysts [11], there is even greater consensus about the high accelerating effect of silica gel [10,14].

Moreover, it can be observed that for 10% Lipozyme TL IM at 0.75 h of reaction time, 1,3-DAG isomers have already reached equilibrium, with respect to total DAG concentration. Therefore, the increase in the relative acyl migration rate when lipase concentration augmented 100% reaffirms the hypothesis of the importance of the silica gel catalyzing effect over the isomerization reaction under study. In the case of reactions performed with 10% of Lipozyme RM IM the isomerization equilibrium was reached between 6 and 12 h of reaction time. For this lipase, although the relative acyl migration rate increased when doubling enzyme concentration, the resulting effect was not as important as the one observed in

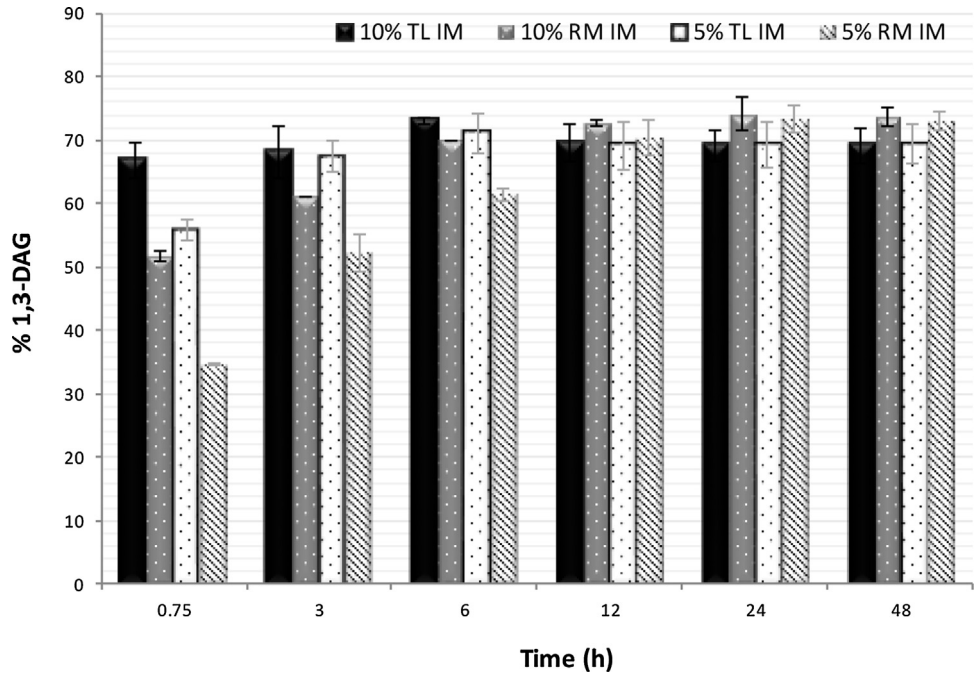

Fig. 3. Kinetic profiles of 1,3-DAG proportion (% mol/mol) of total DAG in the reaction mixture for different lipase type and concentration. Reaction conditions: 50:50 (SO:FHSO, % w/w), 70 °C. RM IM: Lipozyme RM IM, TL IM: Lipozyme TL IM. Mean value of three independent reactions ± standard deviation.

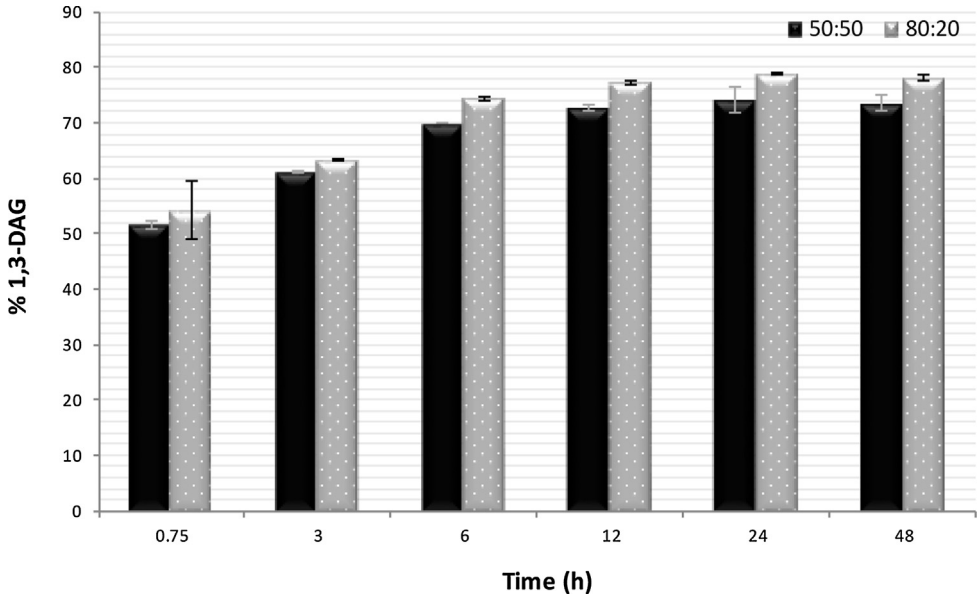

Fig. 4. Kinetic profiles of 1,3-DAG proportion (% mol/mol) of total DAG in the reaction mixture for different SO:FHSO (% w/w). Reaction conditions: 10% (w/$w_{subst.}$) Lipozyme RM IM, 70 °C. Mean value of three independent reactions ± standard deviation.

Lipozyme TL IM systems. Furthermore, Fig. 3 provides additional evidence of the predominance of chemical acyl migration over a partial *sn*-1,3 specificity of immobilized lipases. When the concentration of biocatalysts augmented, the interesterification reaction velocity increased as a consequence of the action of lipases (data not shown). If lipases were considered to be partially strict with respect to the position of the FA within the glycerol backbone, 1,2- and 1,3-DAG proportions would not change when enzyme concentration varies. However, it can be observed in Fig. 3 that 1,3-DAG percentage increased when the immobilized biocatalyst concentration was doubled. This fact further confirms the side effect of the support of the immobilized lipases over the acyl migration reaction.

3.3.2. *Substrate ratio effect*

The effect of a substrate ratio modification (related to the degree of unsaturation of their fatty acids) over the relative acyl migration rate in the interesterification reaction between SO and FHSO was evaluated. Samples with initial SO:FHSO ratios of 50:50 and 80:20 (42.1% and 65.3% (mol/mol) of unsaturated fatty acids, respectively) were chosen for the analysis. For both substrate ratios at 10% of Lipozyme RM IM total DAG concentration was similar up to 6 h of reaction time (data not shown) disregarding any enzyme substrate specificity towards saturated or unsaturated fatty acids. Isomerization equilibrium was already established at 12 h of reaction time, although for 50:50 blend its 1,3-DAG percentage stabilized in lower values ($P < 0.03$) (Fig. 4). It can be clearly noted that the 1,3-DAG

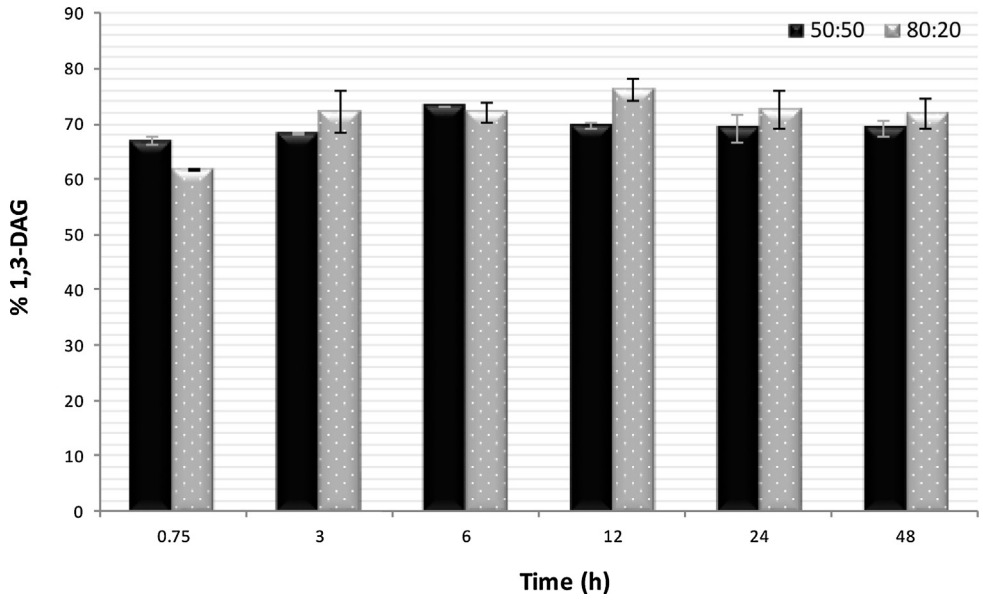

Fig. 5. Kinetic profiles of 1,3-DAG proportion (% mol/mol) of total DAG in the reaction mixture for different SO:FHSO (% w/w). Reaction conditions: 10% (w/$w_{subst.}$) Lipozyme TL IM, 70 °C. Mean value of three independent reactions ± standard deviation.

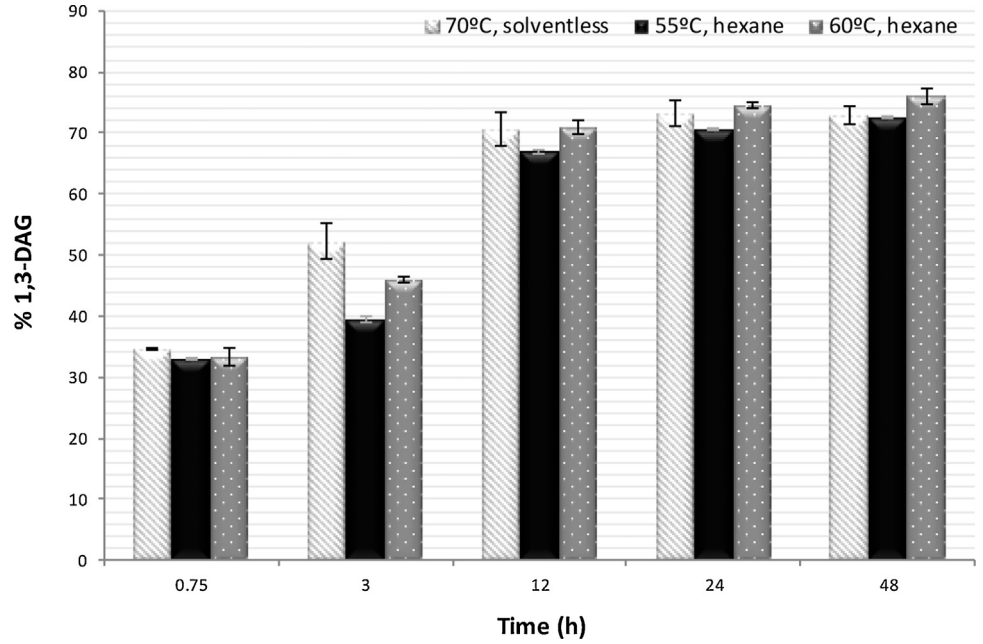

Fig. 6. Kinetic profiles of 1,3-DAG proportion (% mol/mol) of total DAG in the reaction mixture for different hexane contents (0 and 3 mL/g substrates) and temperatures. Reaction conditions: 50:50 (SO:FHSO, % w/w), 5% (w/$w_{subst.}$) Lipozyme RM IM. Mean value of three independent reactions ± standard deviation.

proportion was slightly inferior when both substrates were initially mixed in equal quantities for almost all reaction times. The higher relative acyl migration rates shown by the oil enriched initial reaction blends demonstrated that during the interesterification reaction between SO and FHSO catalyzed by Lipozyme RM IM unsaturated acyl moieties migrate faster and they also define a higher 1,3-DAG proportion at equilibrium. Similar results were obtained when 5% of Lipozyme RM IM was used (data not shown). These results are in according to previously reported ones that indicated the isomerization rate and equilibrium ratio 1(3), 2-DAG:1,3-DAG depend on the nature of the fatty acids involved in the process [12,24].

However, when both substrate ratios were tested with Lipozyme TL IM, no significant differences could be observed between 1,3-DAG percentage when equilibrium was achieved (Fig. 5) ($P > 0.05$). The same behavior was observed when 5% of enzyme was used in the reaction system (data not shown). It is to say that, in the conditions studied, fatty acid migration depends not only on the nature of these molecules but also on the biocatalyst used.

3.3.3. *Solvent and temperature effect*

Finally, the effect of the addition of hexane to the reaction medium on the acyl migration kinetics was studied. Samples obtained at the following reaction conditions were evaluated: 55 and 60 °C, SO:FHSO of 50:50 (% w/w), 5% (w/w of substrates) of Lipozyme RM IM, and 3 mL of hexane. Fig. 6 shows the corresponding results. Comparing both solvent systems at 55 and 60 °C, an

increase in the relative isomerization rate with temperature can be clearly noted ($P < 0.05$). Moreover, a decrease in the equilibrating time is observed, since at 60 °C and 24 h of reaction time both isomers concentrations are already at equilibrium, while at 55 °C it seems that even after this reaction time those compounds percentages continue changing. These results confirm the temperature dependence of the thermodynamic process in study. However, as it was mention before, the level of DAG concentration at the lower temperature was more than twice that of the highest one, indicating that the interesterification reaction was more favored than the acyl migration reaction by the increment in temperature.

If an analysis of the solvent effect over acyl migration is intended, the comparison between solvent and solventless media should be done at the same temperature. It could not be possible to find a unique temperature to perform the interesterification reaction in both systems. On one hand, due to the high melting point of FHSO, the minimum temperature at which reactions could be perform was 70 °C, the melting point of that substrate. In this manner, it is possible to assure that all species in solution will be in liquid state, avoiding thus potential diffusional limitations in the system. On the other hand, the maximum temperature for the solvent systems was chosen to be 60 °C, considering a prudential margin below hexane boiling point (69 °C). However, as one of the advantages of adding a solvent in these systems is the possibility of performing the reaction at lower temperatures, thus reducing product degradation and energy consumption, the comparison between both systems, even at different temperatures, can be profitable. In that sense, it can be observed that relative acyl migration rate in a solventless system at 70 °C is higher than that to hexane media at 60 °C, but they both reach comparable equilibrium percentages.

4. Conclusions

The analysis of the *sn*-2 fatty acid composition of enzymatically interesterified blends of SO and FHSO in different reaction conditions indicated that, in spite of using *sn*-1,3 specific lipases, initial fatty acid profiles at *sn*-2 position could not be maintained. This fact supported the idea of the occurrence of acyl migration in the studied systems.

The study was further continued analyzing the effect certain reaction parameters had over the acyl migration phenomenon, represented by the DAG distribution between 1(3), 2-DAG and 1,3-DAG isomers. In solvent-free media, the presence of Lipozyme TL IM as the biocatalyst was proven to enhance acyl migration compared to Lipozyme RM IM. This fact was hypothesized to be correlated with the support material of the former (silica gel). An increase in immobilized enzyme concentration promoted the isomerization reaction. A greater unsaturated fatty acid proportion in reactive initial blends increased the equilibrium value between DAG isomers favoring 1,3-DAG formation when Lipozyme RM IM was present in the reaction media. Conversely, the addition of hexane, which also implicated a reduction in reaction temperature, decreased the development of the undesired parallel chemical reaction. Regarding temperature, its effect was evaluated on solvent systems. The analysis permitted demonstrate the important effect this parameter has over the whole reaction system -interesterification and acyl migration kinetics- showing a positive correlation with reaction rate and improving interesterification over acyl migration yields.

Although the use of *sn*-1,3 specific lipases in oils and fats' modification processes aims at maintaining the fatty acid profile at *sn*-2 position of reacting glycerides unaltered, the results obtained herein show the impossibility of fulfilling this objective in the studied systems. Nonetheless, they also show that the use of this type of biocatalysts is still preferred compared to non specific ones -or chemical catalysts- because they maintain in a greater extent the original profile.

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