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Enzymatic synthesis of 1,3-dicaproyglycerol by esterification of glycerol with capric acid in an organic solvent system

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ABSTRACT

In this work, the esterification of glycerol with capric acid catalyzed by an immobilized form of a 1,3-positionally selective lipase (*Rhizomucor miehei*) showed to be effective for the synthesis of 1,3-dicaprin in *n*-heptane as the reaction medium.

The effects of the reaction parameters were studied using an experimental factorial design of three factors and three levels with two central points. The selected experimental variables were amount of glycerol adsorbed on silica gel (*G*), biocatalyst load (*E*) and reaction temperature (*T*), and the response variables were total conversion of capric acid, acylglycerol fractions, selectivity and yield of dicaprin, and acyl migration reaction. The range of each parameter was selected as follows: $G = 50–250$ mg, $E = 20–40$ mg and $T = 40–60\,^{\circ}C$. At optimum conditions 73% capric acid conversion was achieved, with 76% dicaprin selectivity, and selectivity to the specific 1,3-dicaprin of 70% of total products. An adequate selection of the reaction conditions is necessary not only to maximize the conversion of capric acid, but also to minimize the acyl migration reaction and the generation of undesired products. Evidence of kinetically controlled enzymatic acyl migration from sn-3/sn-1 to sn-2 is presented.

1. Introduction

In the last decades, there has been an increasing interest in the nutritional effects of food. New habits have caused the appearance of new products on the market such as those enriched with omega-3 and omega-6, foods fortified with vitamins and minerals (mainly iron, magnesium, zinc, calcium and vitamins B6 and B12), probiotic-enriched dairy products, low calorie foods and others for lowering cholesterol [1].

The increased public interest in fitness and healthy dietary habits has led to much research on the synthesis of "healthy lipids" through the modification of fats and oils. Modified lipids that have been restructured in terms of their native composition and/or distribution of fatty acids (FA) in the glycerol backbone are known as structured lipids (SL) [2]. The aim of the SL synthesis is to produce a lipid that could be used for nutritional purposes or for treating certain health problems, as well as to improve the physical and chemical properties of fats and oils.

In order to obtain lipids with specific properties, a wide range of fatty acids are incorporated in specific positions of the glycerol molecule [3–6]. They include short-chain fatty acids (SCFA or S), medium-chain fatty acids (MCFA or M), and long-chain fatty acids (LCFA or L). Structured lipids of the so-called MLM type contain medium-chain fatty acids at the sn-1 and sn-3 positions of glycerol and a long-chain fatty acid at the sn-2 position, and they are probably the structured lipids that have received the most important attention of science [5].

In this report the synthesis of 1,3-dicaproylglycerol was studied as a previous stage to the synthesis of MLM-type triglyceride. However, the synthesis of diacylglycerols (DAG) is of great importance itself. They are known to be used as additives or carriers in medicine, food and cosmetic industry, and so on [7]. The latest reports on the nutritional benefits of DAG have renewed the interest on this research topic. DAG, particularly the 1,3-isoform, has been confirmed as having certain nutritional benefits such as the ability to reduce serum triacylglycerol (TAG) concentration, body-weight and visceral fat [8–13]. There is no significant difference in energy value and absorption coefficient between DAG and TAG [14]. Numerous studies on the safety aspects of DAG on humans [14–17] and animals [18–20] demonstrated no adverse effects. With the goal of obtaining SL, the synthesis has been generally explored through acidolysis of vegetable oils or interesterification of vegetable oils. Palm oil acidolysis with capric or caprylic acid has become an option to obtain MLM SL. However, the complexity of the reaction substrates and products requires careful post-reaction

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separation steps and tedious analytical work. Undesired products are generated by acidolysis or interesterification of vegetable oils in higher relative amounts than in the case of direct esterification of glycerol with fatty acids [21].

In the present work 1,3-dicaproylglycerol was synthesized by direct esterification of glycerol with capric acid in an organic solvent using a commercial immobilized form of lipase from *Rhizomucor miehei* (Lipozyme RM IM) as catalyst. Being glycerol a low-cost by-product of the biodiesel industry, using it as a substrate is highly efficient in economic terms. Besides, as this is an esterification reaction with simple substrates such as glycerol and capric acid, the complexity of the reaction medium and analytical aspects were greatly reduced. In this paper we studied the effect of selected parameters (glycerol feed, enzyme dosage and reaction temperature) on the acid conversion, selectivity and diglyceride yield, as well as on the acyl migration process. The study was performed with a screening factorial design of three factors and three levels.

A number of studies in the literature focus on the synthesis of 1,3-DAG by esterification catalyzed by lipase, but no careful analysis of the impact of the silica (used as glycerol supplier) as fatty acid and monoglyceride adsorbent was included in those works, nor was the impact of the sampling procedure on the results considered or discussed. This impact may be huge in the case of solvent-free systems or a system with volatile substrates. No report on the subject, involving enzymes, has described the acyl migration mechanism with the level of detail presented in this manuscript. Watanabe et al. [22] studied the production of 1,3-DAG from a mixture of FA using the immobilized lipase from *R. miehei* in a solvent-free system. The kinetics of 1,3-DAG production from FA and glycerol was investigated. However, no detailed study of the acyl migration from sn-1 or sn-3 to sn-2 was included. The authors emphasized that the enzyme is regioselective for 1,3 positions, but they also reported that long reaction times, high temperatures and high concentrations of immobilized enzyme increased the acyl migration, triglyceride production and purity reduction of the 1,3-DAG. Purity was reduced from 94% to 68% when the immobilized lipase content was varied from 2.5% to 20%, but these results were not discussed. No explanation was provided, being the key question if the acyl migration responsible for the generation of triglycerides was induced by immobilized lipase support or by lipase. Other authors [23,24] studied the reaction conditions for the esterification mediated by Lipozyme RM IM. Reduced yields of the specific diglyceride were found due to acyl migration caused by the amount of biocatalyst, but no discussion of the causes of this reaction was included.

The present manuscript considers (a) the problems of the use of silica and the mistakes that may be present without careful sampling (errors as high as 20% or more in fatty acid conversion due to fatty acid adsorption on silica may be found, a phenomenon not taken into account or at least properly reported in the published literature), (b) a new explanation of the acyl migration mechanism (never presented before, to the best of our knowledge), and (c) a careful analysis of the 1,3-DAG isomeric distribution with simple gas chromatography.

2. Experimental

2.1. Materials

Lipozyme RM IM, which is a commercial form of the 1,3-specific lipase from *R. miehei* immobilized by adsorption on a macroporous anion exchange phenolic resin Duolite A-568, was kindly provided by Novo Nordisk A/S (Brazil). Glycerol, *n*-heptane, isopropyl ether and silica gel were supplied by Cicarelli Laboratorios. Capric acid, 1,2,4-butanetriol and silylation reagents were obtained from Fluka. Monocaprin, dipalmitin, trilaurin and tricaprin were supplied by Sigma–Aldrich. Absolute ethanol and ethyl ether were supplied by Dorwil, and phenolphthalein and pyridine were provided by Anedra S.A. All products were of analytical grade.

2.2. Adsorption of glycerol on silica gel

Glycerol was adsorbed as follows: 1 g of glycerol and 2 g of silica gel were mechanically mixed until total adsorption on the solid.

2.3. Lipase-catalyzed esterification

Esterification of glycerol was performed in 10 mL flasks, which were kept in a thermostatic bath with temperature control and magnetic stirring. The reaction time was 6 h. The reaction was carried out as follows: 110 mg of capric acid were dissolved in 3 mL *n*-heptane, then the amount of glycerol adsorbed onto silica fixed to each reaction under study was added. When the reactant mixture reached the selected temperature, the reaction was started by adding 50% of the total amount of enzyme to be added (time 0). The remaining 50% of the biocatalyst was added after 3 h of reaction. The values of glycerol, immobilized lipase dosage and reaction temperature were established according to the experimental design explained below. Highly hydrophilic polyols cause loss of enzymatic activity. This may be due to two factors: (1) in a hydrophobic reaction medium, polyols adhere to the support of the lipase impeding access of the acid to the active site, or (2) the hydroxyl groups of the polyol strongly interact with the active site of the enzyme.

Although silica gel behaves as a “polar substrate reservoir” and plays a protective role for the immobilized enzyme avoiding its blockage due to glycerol, the addition of the immobilized lipase in two steps minimizes the deactivation of the enzyme and maximizes the production of the desired product.

2.4. Experimental factorial design

A three-level-three-factor factorial design with two central points and a total of 10 experiments (Table 1) was applied in this study. The variables and their levels were: adsorbed glycerol on silica gel (50–250 mg), reaction temperature (40–60 °C) and immobilized lipase loading (20–40 mg). The studied responses were fatty acid conversion (mol%), enzymatic activity (μmol acid converted/mg immobilized lipase), monocaprin, dicaprin and tricaprin production (molar % of the total products), dicaprin selectivity and yield (mol%) and 1–2 dicaprin, 1–3 dicaprin and 2–3 dicaprin formation percentage (relative to total moles of produced dicaprin). All the experimental factorial design and the statistical analysis were performed using the STATGRAPHICS Centurion version XV.2 software. The factors and levels used and the obtained experimental responses are presented in Table 1. The order of the experiments was fully randomized to provide protection against the effects of lurking variables.

2.5. Statistical analysis

The complete statistical analysis was performed using the STATGRAPHICS Centurion software. The responses were adjusted by multiple regression, and the generated models were used to evaluate the effect of the selected experimental factors. The goodness of fit was assessed using the coefficient of determination (R^2). The statistically significant effect of the variables was tested using ANOVA statistical test. Non-significant coefficients were eliminated (p-value > 0.05) and the models were refined in order to consider

Table 1
Experimental factors, settings and measured response variables for the esterification of glycerol with capric acid catalyzed by Lipozyme RM IM, at 6 h of reaction.

Run number	Experimental factors			Response variables									
	G (mg)	T (°C)	E (mg)	X_{AC} (%)	MAG[a]	DAG[a]	TAG[a]	1,2-DAG[b]	1,3-DAG[b]	2,3-DAG[b]	σ_{DAG} (%)	Y_{DAG} (%)	A[c]
1	50	40	20	38	15	73	12	5	92	3	74	28	12
2	150	50	30	66	15	70	15	4	92	4	70	46	14
3	250	40	20	55	18	77	4	10	83	7	83	46	18
4	250	40	40	67	19	72	9	17	80	4	76	50	11
5	250	60	40	75	18	69	13	9	71	20	71	53	12
6	150	50	30	67	21	68	11	5	94	1	72	49	14
7	250	60	20	73	18	72	9	5	93	2	76	55	23
8	50	60	20	45	12	61	26	8	86	5	58	26	14
9	50	60	40	55	8	40	52	3	33	64	33	18	9
10	50	40	40	40	8	62	30	3	56	40	56	23	6

[a] Percentage of the total reaction product (mol%).
[b] Percentage of the total produced diglycerides (mol%).
[c] Enzyme activity evaluated as μmol converted acid/mg of immobilized lipase.

only the statistically significant effects. A multi-response analysis through desirability functions was also performed.

2.6. Analysis of samples

2.6.1. Evaluation of the capric acid adsorption on silica gel

In order to evaluate the adsorption of capric acid on silica gel, the following experiment was performed: 110 mg of capric acid, 100 mg of silica gel and 3 mL of *n*-heptane were placed in a 10 mL flask. The mixture was stirred for 3 h at 40 °C. The adsorbed FA fraction was determined by titration.

2.6.2. Capric acid desorption

Preliminary results indicated that capric acid could be adsorbed on the silica gel surface. In order to complete correctly the FA quantification, the reaction medium was sonicated for 15 min. A volume of sample (0.5 mL) was separated to analyze it by gas chromatography (GC). The remaining sample was diluted with 20 mL of 1:1 (v/v) absolute ethanol:ethyl ether. The mixture was subjected to magnetic stirring for 40 min and sonicated for 20 min. The slurry was filtrated to remove the immobilized enzyme and silica gel, and the recovered solution was ready to be titrated.

2.6.3. Capric acid determination by titration

Titration of the samples was performed with ethanolic 0.03 M potassium hydroxide to determine the amount of unreacted acid. Phenolphthalein was used as indicator. The acid conversion was obtained with an error of ±1 percentage point with the following equation

$$X_{FA} = \frac{FA_0 - FA_f}{FA_0} \quad (1)$$

2.6.4. Gas chromatography analysis

Samples were diluted with pyridine and sililated with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). The analysis of samples was performed in a PerkinElmer AutoSystem XL gas chromatograph equipped with on-column injection, a flame ionization detector (FID) and a high temperature capillary column ZB-5HT Inferno (15 m × 0.32 mm, with an ID of 0.10 μm), using H_2 as gas carrier. The detector temperature was maintained at 380 °C. The initial column temperature was held at 50 °C for 1 min, elevated to 180 °C at a rate of 15 °C/min, then the temperature was increased to 230 °C at 7 °C/min, further increased up to 370 °C at 10 °C/min, and finally held there for 5 min. This method allowed the detection of capric acid and of all the acylglycerides present in the sample in less than 25 min [25]. Results are the average of two injections with an average relative error lower than 2%. The determination of elution times of reactants and products was performed with high purity standards.

2.7. Evaluation of the role of the support in acyl migration

2.7.1. Lipase deactivation

One hundred milligrams of immobilized lipase were placed in an oven at 95 °C for 30 min to completely deactivate the RML, following previous results by our group [26]. After the lipase was deactivated, the role of the support in the reaction could be evaluated. It is worth noting that no impact of the magnetic stirring on denaturation was found in the selected conditions for the reaction here analyzed in the 200–700 rpm range (results not shown) for Lipozyme RM IM.

2.7.2. Monolein isomerization

The support-catalyzed acyl migration was studied using monoolein of chromatographic purity with 79% sn-2 monoolein and 21% sn-1 monoolein (tested chromatographically). Fifteen mg of monoolein, 2 mL *n*-heptane and 40 mg of the obtained support with the deactivated lipase were mixed, adding 20 mg initially and 20 mg after 3 h of reaction, as it was done in the esterification reaction. The reaction was carried out in 10 mL flasks with magnetic stirring at 40 °C. Total reaction time was 6 h.

2.7.3. Isomerization in the esterification reaction medium

One hundred and ten milligrams of acid, 250 mg of glycerol adsorbed on 500 mg silica gel and 3 mL *n*-heptane were mixed in 10 mL flasks at 40 °C in a thermostatic bath with magnetic stirring. The reaction was performed for 6 h, adding 20 mg Lipozyme RM IM initially, and an additional 20 mg after 3 h of reaction. The container was ultrasonicated for 15 min to desorb substrates or products, and the content was filtered to separate the silica and the immobilized lipase. The recovered volume was placed again in the bath with magnetic stirring and 20 mg of the deactivated lipase (the support of Lipozyme RM IM), and 20 mg more of the same material were added after 3 h. The initial sample for the isomerization reaction contained 16 mg capric acid, 6 mg monocaprin, 25 mg dicaprin and 8 mg tricaprin.

3. Results and discussion

3.1. Capric acid adsorption on silica gel

In preliminary studies, it was observed that capric acid is adsorbed on the surface of silica gel. If this phenomenon is not considered, an important error in the FA determination arises, with a subsequent overestimation of the FA conversion.

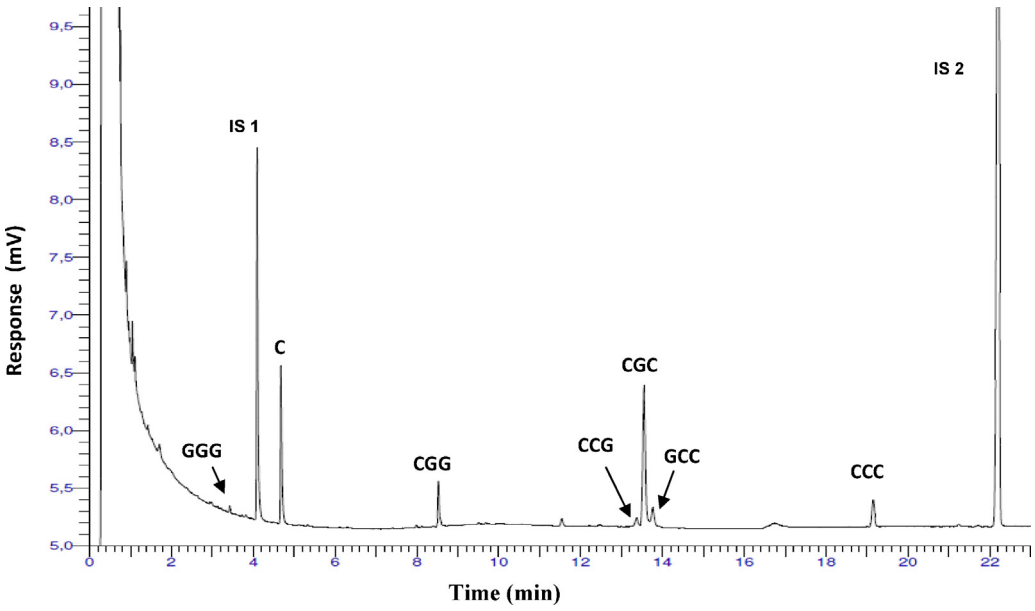

Fig. 1. Typical chromatogram of the reaction products and unconsumed reactants in the enzymatic esterification of glycerol and capric acid using Lipozyme RM IM. IS1 and IS2: Internal calibration standards. Experimental conditions: glycerol: 250 mg (adsorbed on silica gel), temperature: 60 °C, and enzyme: 20 mg, reaction time: 6 h.

The adsorption study, performed under the same experimental conditions as the reaction experiments, indicated that 12.7 mg of capric acid were adsorbed on 100 mg of silica gel. This represents an adsorption of 0.074 FA mmol per 100 mg of silica. In the literature only one article was found that reported the possibility of capric acid adsorption on silica gel [27].

3.2. Identification of products

The products of the enzymatic esterification of glycerol (GGG) with capric acid (C) are monocaprin (CGG) and 1,3-dicaprin (CGC), but also products with capric acid in the sn-2 position. Acyl migration makes it possible to obtain two dicaproyglycerol isomeric configurations: 1,2-dicaproyglycerol (CCG) and 2,3-dicaproyglycerol (GCC), which are undesirable reaction products. When those undesirable dicaproyglycerols are esterified, tricaprin (CCC) is generated as product (more information on acyl migration is presented in Section 3.3). The capillary column used differentiates positional isomers, and their identification was possible using the work by Bruschweiler and Dieffenbacher [28], who proposed a method for determining mono- and diglycerides by gas chromatography.

A typical chromatogram of the reaction products and unreacted substrates is shown in Fig. 1.

3.3. Acyl migration reaction

The positional specificity of lipases to attack the sn-1 and sn-3 positions is due to steric limitations that prevent the access of the sn-2 fatty acid to the active site of the lipase [29]. However, it can be observed in Fig. 1 that tricaprin production was not negligible. As mentioned above, the presence of CCC is explained in terms of the so-called acyl migration reaction. The acyl migration that generated tricaprin as product is the migration of fatty acids from sn-1 and sn-3 to the sn-2 position, even when thermodynamically the favored migration is from sn-2 to the sn-1/sn-3 positions [30]. In the synthesis of the MGM-type diglycerides, acyl migration involves the migration of medium-chain fatty acids from sn-1 and sn-3 to the sn-2 position and remigration from the sn-2 position to sn-1 and sn-3. Acyl migration is considered a non-enzymatic reaction [30]. There are many factors that are known to influence acyl migration, such as temperature, reaction time, acyl chain length, water activity, type of reactor, acidity of reaction medium, lipase activity, solvent polarity and type of immobilization support [30,31]. In particular, temperature has been recognized to have a profound influence on the migration rate since acyl migration is considered a thermodynamic process [30].

Fig. 2 shows the proposed mechanism for the esterification reaction between glycerol and capric acid catalyzed by immobilized lipase, based on the concepts proposed by Watanabe et al. [32]. Even using a sn1,3-specific lipase, acyl migration leads to the formation of tricaprin (CCC) and nonspecific monoglycerides and diglycerides. The grey arrow in Fig. 2 highlights the esterification route to obtain the 1,3-diglyceride.

3.4. Model fitting

The experiments were conducted under the designed conditions and responses were obtained. Factor settings and measured responses corresponding to 6 h determinations are given in Table 1.

Model results obtained for each response variable are analyzed in detail in the following subsections. Simple linear models are introduced first to identify major tendencies and correlations between factors and responses. Further analysis using quadratic models is also addressed in order to obtain better fittings of the experimental data and detect non-predictable effects.

3.4.1. Capric acid conversion (X_{AC})

After applying a linear model, the amount of glycerol was the only variable with statistically significant effects (p-values <0.05). This model could explain 86.83% of the variability of the conversion of capric acid (from the coefficient of determination R^2).

The increase in all the variables generated a higher conversion. In the selected temperature range, no lipase denaturation was observed, and no biocatalyst aggregation took place.

The highest conversion of capric acid was obtained at the maximum of the three factors (adsorbed glycerol = 250 mg, temperature = 60 °C, and immobilized lipase = 40 mg), and it did not correspond with the highest selectivity to diglyceride (as discussed in Section 3.4.2.2).

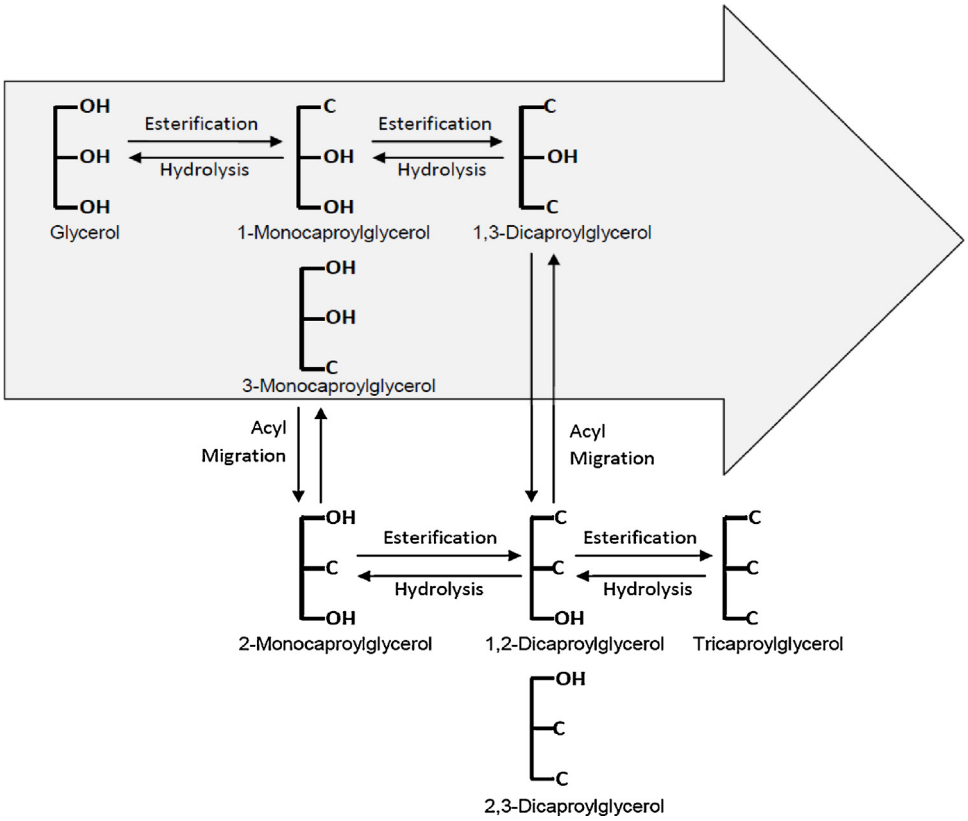

Fig. 2. Reaction scheme for the esterification of glycerol with capric acid using Lipozyme RM IM.

After major effects were identified through a simple linear model, quadratic models were also applied to the experimental data. The quadratic fit model containing all the variables and combinations thereof is represented by Equation (2)

$$X_{AC} = A_0 + A_1G + A_2T + A_3E + A_4GE + A_5GT + A_6ET + A_7G^2 + A_8E^2 + A_9T^2 \quad (2)$$

where G is glycerol, T is temperature, and E is the amount of immobilized enzyme.

This model was adjusted by stepwise selection (Fisher's test), eliminating variables in order to find the best model containing only statistically significant variables. The test begins with a model involving all the variables specified in a dialog data entry, and then it eliminates one variable at a time based on their statistical significance in the current model.

Equation (3) was obtained after proper fitting. Since the p-value for each variable was less than 0.05, there was a statistically significant relationship between the variables and the response with a confidence level greater than 95.0%. The coefficient of determination R^2 indicates that the model explains 97.33% of the variability in the conversion of capric acid. The adjusted R^2 statistic, which is more appropriate to compare models with different numbers of independent variables, was 95.20%, whereas for the linear model it was 60.49.

$$X_{AC} = -14.125 + 0.43G + 0.325E + 0.6T - 0.00105G^2 \quad (3)$$

Along with the increase in the value of R^2, the superiority of the quadratic model over the linear model was also evidenced by the analysis performed using the Fisher test.

The high F-value calculated by Fisher's test indicated a high degree of fit among the data. The F-value for the linear model was 3.30, whereas the F-value for the quadratic model was 45.65.

A response surface plot for the second-order model at 40 mg immobilized lipase is shown in Fig. 3, presenting the effects of the amount of glycerol (adsorbed on silica gel) and temperature. As for the linear model, the maximum conversion of capric acid occurred at the studied maximum value for each factor, a large region where conversions exceeded 70%.

3.4.2. Fractions of acylglycerols

3.4.2.1. Monocaprin fraction of the total product. The amount of adsorbed glycerol was the main factor positively affecting the synthesis of monocaprin. However, using a linear model, none of the factors was able to satisfy the confidence limit imposed (p-value less than 0.05), and this model could only explain 77.57% of the variability of monocaprin percentage related to the total molar amount of reaction products (from the coefficient R^2).

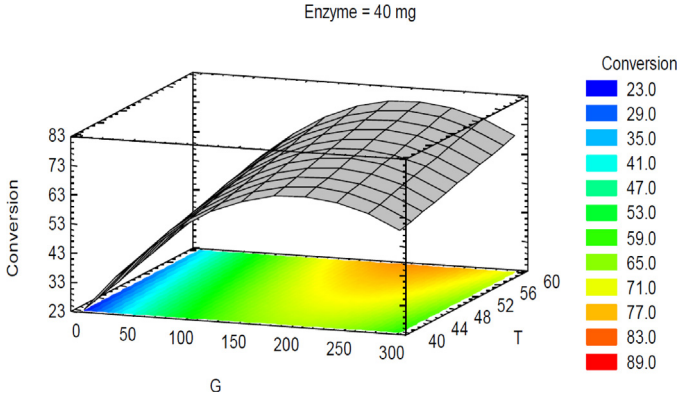

Fig. 3. Response surface for the conversion of capric acid using a quadratic model; factors glycerol and temperature E = 40 mg.

Temperature had practically no effect on the monocaprin fraction. At low concentrations of glycerol, the effect of the amount of immobilized lipase became important. Monocaprin synthesis is favored by high concentrations of glycerol, low temperatures and low doses of biocatalyst.

A second-order model was adopted as the best option. An expression equal to Equation (2) was checked by multiple regression. Refining was performed by applying Fisher's test in order to obtain an expression that contained only significant variables. Equation (4) shows the expression obtained to adjust the data of the monocaprin fraction

$$\%MAG = 15.3497 + 0.100763G - 0.352187E + 0.00142375GE - 0.000357125G^2 \quad (4)$$

The p-value for this model was less than 0.05 and there was a statistically significant relationship between the variables and the response, with a confidence level higher than 95%. The second-order model that adjusted the monocaprin fraction in the reaction product was able to explain 87.81% of the variability of this response (based on the coefficient R^2). The adjusted R^2 statistical value increased from 32.73 for the linear model to 78.05 for the second-order model.

3.4.2.2. Dicaprin fraction of the total product. The default model used by the software explained 96.18% of the variability of the results for dicaprin. The amount of glycerol, the immobilized lipase dosage and temperature were statistically significant variables. Increasing the concentration of glycerol positively influenced dicaprin synthesis, whereas the temperature increase and the amount of biocatalyst negatively affected this response.

The amount of adsorbed glycerol was the main factor affecting dicaprin synthesis. At low concentrations of adsorbed glycerol, the negative effects of increased temperature and increased amount of immobilized lipase became important. The effect of temperature and amount of immobilized enzyme were similar.

After analyzing the main factors affecting the dicaprin fraction, a second-order model to fit the data was proposed again. Equation (5) is the result of such adjustment. The coefficient of determination R^2 was 96.72, and the statistical adjusted R^2 achieved 90.16, whereas for the linear model it was 88.55. No significant improvement was achieved with the second-order model, since the linear model provided a good fit itself. It was not possible to reduce the number of variables using Fisher's test.

$$\%DAG = 132.143 - 0.096775G - 1.02756T - 0.960188E + 0.00295875GE + 0.00332625GT - 0.000302875G^2 \quad (5)$$

The largest fraction of dicaprin (for the tested conditions) obtained with the highest proportion of adsorbed glycerol, the minimum amount of immobilized lipase and the lowest temperature is presented in Fig. 4. These conditions did not match those that maximized capric acid conversion.

3.4.2.3. Tricaprin fraction of the total product. The percentage of tricaprin on the reaction product was also properly adjusted with the linear model, with a coefficient of determination R^2 of 95.04%. The main variables that increased the percentage of tricaprin were the amount of enzyme and temperature. The latter parameter did not exceed the imposed confidence limit in the linear model. On the other hand, the amount of adsorbed glycerol adversely affected the amount of produced tricaprin, and this variable was statistically significant in this model.

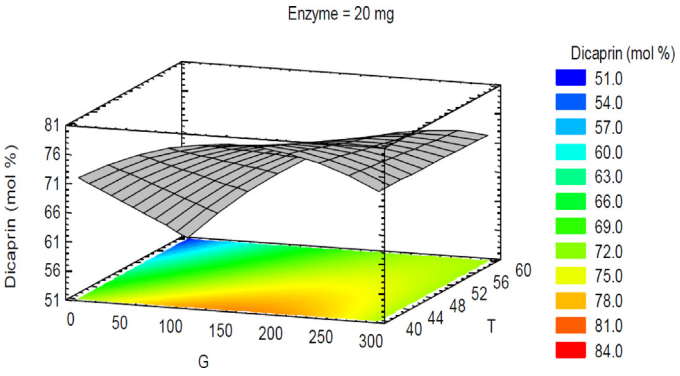

Fig. 4. Response surface for dicaprin fraction using a quadratic model: factors: glycerol (G) and temperature (T), E = 20 mg.

Glycerol had the greatest effect on the obtained molar fraction of tricaprin. The positive effects of temperature and the amount of biocatalyst were only significant when the synthesis was performed with low concentrations of adsorbed glycerol. The effects caused by temperature and the amount of immobilized lipase on the final relative amount of tricaprin were similar.

Equation (6) was obtained by fitting the data with a second-order model and refined to include only significant variables

$$\%TAG = -50.4942 + 1.09085T + 1.31034E - 0.00437391GE - 0.00345234GT + 0.000666547G^2 \quad (6)$$

The second-order model, applied to the tricaprin fraction on the reaction product, allowed a slight improvement compared to the adjustment made by the linear model. The confidence level for the relationship among variables exceeded 99.9% (p-value 0.0009). The coefficient of determination R^2 could explain 98.54% of the variability of the tricaprin fraction, and the adjusted R^2 statistic was 96.71 (it was 85.13 for the linear model).

Thus, increasing the glycerol content favored the synthesis of mono- and diacylglycerides, whereas increasing the dosage of immobilized lipase and temperature favored the acyl migration reaction and the synthesis of tricaprin.

3.4.3. Production of dicaproylglycerol

Dicaprin selectivity (σ_{DAG}) and yield (Y_{DAG}) were adjusted with a simple model that included linear variables. The role of the variables on the response can be analyzed using standardized Pareto charts (not shown).

In the case of selectivity, all the variables were statistically significant. Increased glycerol content favored selectivity to dicaprin, whereas increasing the dosage of immobilized enzyme and the increase in temperature reduced the selectivity to diglyceride.

The content of glycerol adsorbed on silica gel was the only statistically significant variable for the dicaprin yield.

The effects produced by temperature and the amount of biocatalyst on selectivity reinforces the idea of the consequences of these variables on acyl migration. These variables favored the migration of the acyl groups of the sn-1 and sn-3 positions to sn-2, allowing the generation of nonspecific diglycerides and then tricaprin, thereby reducing the selectivity towards the desired product.

Equation (7) fitted the selectivity data, and the coefficient R^2 explained 96.88% of the variability of selectivity, and the adjusted R^2 value was 92.99. Thus a good fit of the selectivity values was obtained with the simple model. In the case of the yield, the refined model only considered the initial amount of adsorbed glycerol as the independent variable (Equation (8)) and adjusted 85.07% of the

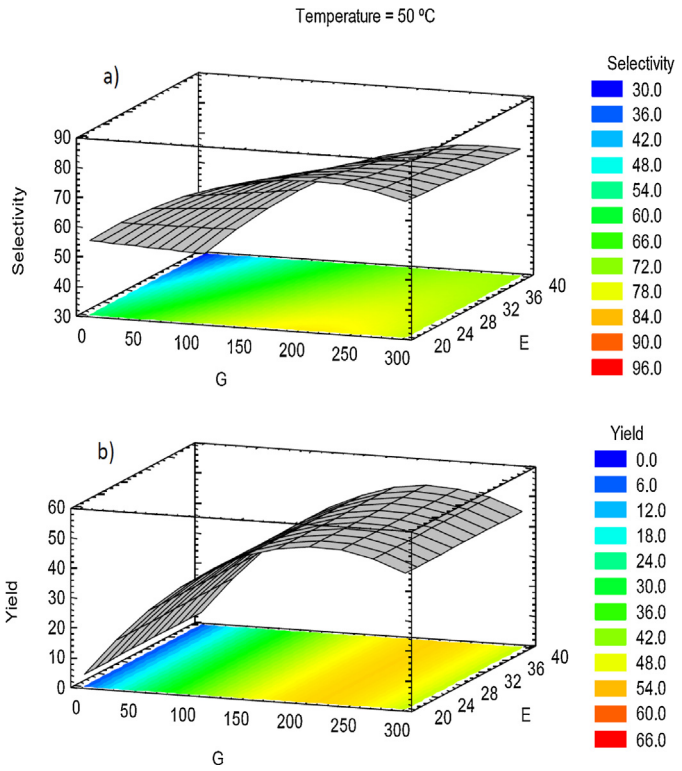

Fig. 5. Selectivity and yield to dicaprin. (a) Selectivity to dicaprin second-order model; factors: glycerol (G) and amount of enzyme (E), temperature: 50 °C, (b) dicaprin yield second-order model.

variability in yield to dicaprin (R^2), and adjusted R^2 coefficient was 83.21.

$$\sigma_{DAG} = 148.769 - 0.18635G - 1.195T - 1.26875E + 0.00354GT + 0.003815GE \quad (7)$$

$$Y_{DAG} = 18.645 + 0.1375G \quad (8)$$

A second-order model improved the adjustment made by the linear model for selectivity to dicaprin. Equation (9) was obtained after refining the multiple regression model. The coefficient of determination R^2 was 99.07, and the adjusted R^2 value achieved 97.91. Fig. 5a shows a response surface plot obtained from Equation (9), where temperature was set at 50 °C and the other factors were varied. As mentioned above, the effects caused by the amount of immobilized lipase were similar to those caused by temperature (graph not shown), and were evident at low concentrations of glycerol. The higher values of selectivity (within the range tested) were obtained by carrying out the synthesis with the highest concentration of glycerol and minimum amount of immobilized lipase and at the lower temperature values.

$$\sigma_{DAG} = 138.359 - 1.16542T - 1.251E + 0.003697GE + 0.003343GT - 0.00057516G^2 \quad (9)$$

A second-order model was proposed also for the dicaproyl glycerol yield. The adjustment provided by this model could explain 94.03% of the variation in the dicaprin yield, and the value of the adjusted R^2 coefficient was 92.32. Equation (10) was obtained as a result of this multiple regression, and Fig. 5b shows the response surface plot for this model.

$$Y_{DAG} = 4.18125 + 0.43675G - 0.0009975G^2 \quad (10)$$

The maximum yield to dicaproyl glycerol was obtained by carrying out the synthesis with the highest glycerol content studied.

3.4.4. Dicaproylglycerol isomers

Although the lipase used in this reaction is specific for the sn-1 and sn-3 positions, the reaction of acyl migration allows acyls to relocate to sn-2 position. Thus, besides the specific desired diglyceride (1,3-dicaproylglycerol), two isomeric forms of dicaprin (1,2-dicaproylglycerol and 2,3-dicaproylglycerol) were also obtained.

From the Pareto diagrams (not shown) it is evident that the isomer fractions were influenced by combinations of factors. Broadly speaking, increasing the amount of glycerol promoted the synthesis of the specific isomer, whereas increasing the biocatalyst dose and temperature favored the synthesis of the other isomers. The factors that promoted the synthesis of nonspecific isomers also favored the esterification reaction and the conversion of the isomers to tricaprin.

Fractions of each isomer are analyzed in the following sections. Temperature had no impact on the amount of 1,2-dicaprin, being the initial amount of adsorbed glycerol and immobilized lipase the factors that influenced the percentage of this isomer. On the other hand, the content of glycerol reduced the fraction of this isomer. The effects caused by the amount of biocatalyst and temperature became more important when the glycerol concentration was low. High temperatures have a negative effect on the unspecific isomer fraction. Apparently, although temperature favors the migration of acyl, it further enhances the esterification and 1,2-dicaprin is rapidly converted to tricaprin.

A simple second-order model in G adjusted 98.11% of the data obtained for the 1,2-dicaprin fraction, and the adjusted R^2 statistic was 95.76. This model is represented by Equation (11). The values of these coefficients for the linear model (default in the software) were 88.09 and 64.27 respectively.

$$\%1,2\,DAG = -4.017 + 0.375898T - 0.002273GT + 0.002138GE - 0.005318ET + 0.00025028G^2 \quad (11)$$

Fig. 6a shows the effect of glycerol and the biocatalyst dose on the 1,2-dicaprin fraction. From Fig. 6b it is evident that increasing glycerol content minimized acyl migration from sn-1 to the sn-2 position. Fig. 6c shows the results for 1,3-dicaprin. The effect of the amount of immobilized lipase can be clearly seen in combination with the impact of glycerol concentration. At low concentrations of glycerol, acyl migration became extremely important. The amount of immobilized enzyme appears as the variable that had the greatest effect on acyl migration, even more than temperature.

The response surface presented in Fig. 6b was obtained using Equation (12). This second-order model in E could explain 96.52% of the variability of the 2,3-dicaprin fraction, and the adjusted R^2 value was 92.16.

$$\%2,3\,DAG = 71.0825 + 0.20335G - 7.44106E + 0.156113E^2 - 0.0100662GE + 0.0193825TE \quad (12)$$

For the linear model, used to assess the main effects of the variables on the response under study, the R^2 was 90.03 and the adjusted R^2 was 70.10.

The synthesis of 1,3-diglyceride was favored by increasing the amount of glycerol adsorbed on silica gel as shown in Fig. 6c. The increase in temperature showed a slightly positive effect on the production of 1,3-dicaprin, whereas increasing the dose of immobilized lipase decreased the final fraction of this isomer. These aspects

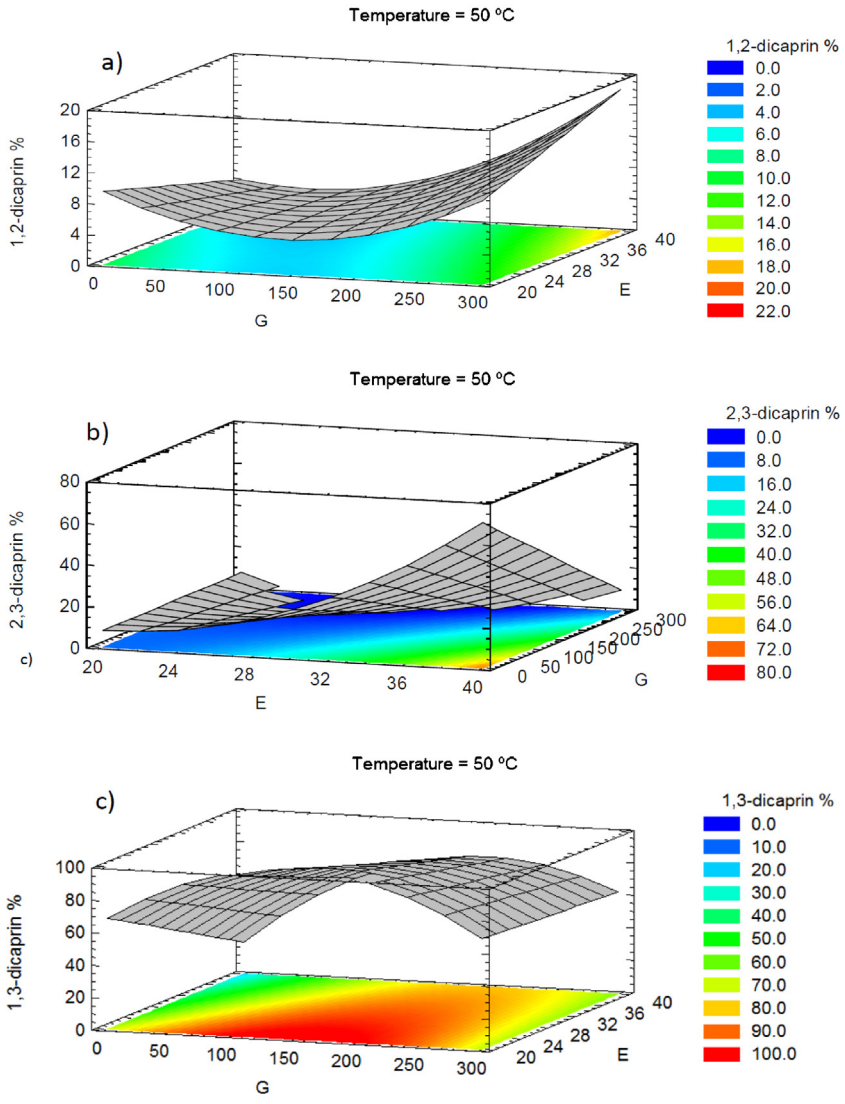

Fig. 6. Response surfaces for fractions of diacylglycerol isomers. (a) 1,2-dicaproylglycerol fraction, (b) 2,3-dicaproylglycerol fraction, and (c) 1,3-dicaproylglycerol fraction.

became even more important when carrying out the reaction with low concentrations of adsorbed glycerol.

A simple second-order model represented by Equation (13) adjusted 97.04% of the data obtained for the 1,3-dicaprin fraction, and the adjusted R^2 statistic was 94.67. The second-order model in the amount of adsorbed glycerol improved the adjustment made by the linear model (R^2 = 83.6, and adjusted R^2 = 50.79).

$$\%1,3\,DAG = 117.402 + 0.00722821GE + 0.0066921GT - 0.0489778ET - 0.00159927G^2 \quad (13)$$

3.5. Multi-response analysis using desirability functions

In the previous sections, the effects of experimental variables on different responses when performing the enzymatic esterification of glycerol with capric acid were evaluated. Regressions of varying complexity were developed for each response, and optimal conditions were obtained for the responses. In this section, responses were analyzed with the objective of obtaining a global optimum. However, when considering all the responses at the same time, it is unlikely to achieve optimality in the same place, especially when some of the responses should be maximized and others minimized.

For this analysis we used the approach based on a utility function called "desirability function". The desirability function was first introduced by Harrington [33], who suggested the calculation of desirability values associated to each outcome of an experiment.

This paper has attempted to build a model that maximizes the selected response variables (capric acid conversion, dicaprin selectivity and yield, etc.), while minimizing other variables (fraction of mono- and tricaprin, and nonspecific diglycerides).

Although the number of experiments in this paper is limited (10 experiments), there are reports in the literature on successful factorial design applications with a reduced number of experiments for analyzing multi-response with desirability functions [34].

Fig. 7a and b shows the desirability function obtained by maximization of acid conversion, selectivity and yield to dicaprin, dicaprin fraction of the reaction product and 1,3-dicaprin fraction of total isomers, and minimization of the mono- and tricaprin fraction and the percentage of nonspecific isomers of dicaprin. When carrying out the synthesis with the highest glycerol concentration studied, the desirability function had values greater than 0.63 for all the temperature range and biocatalyst doses

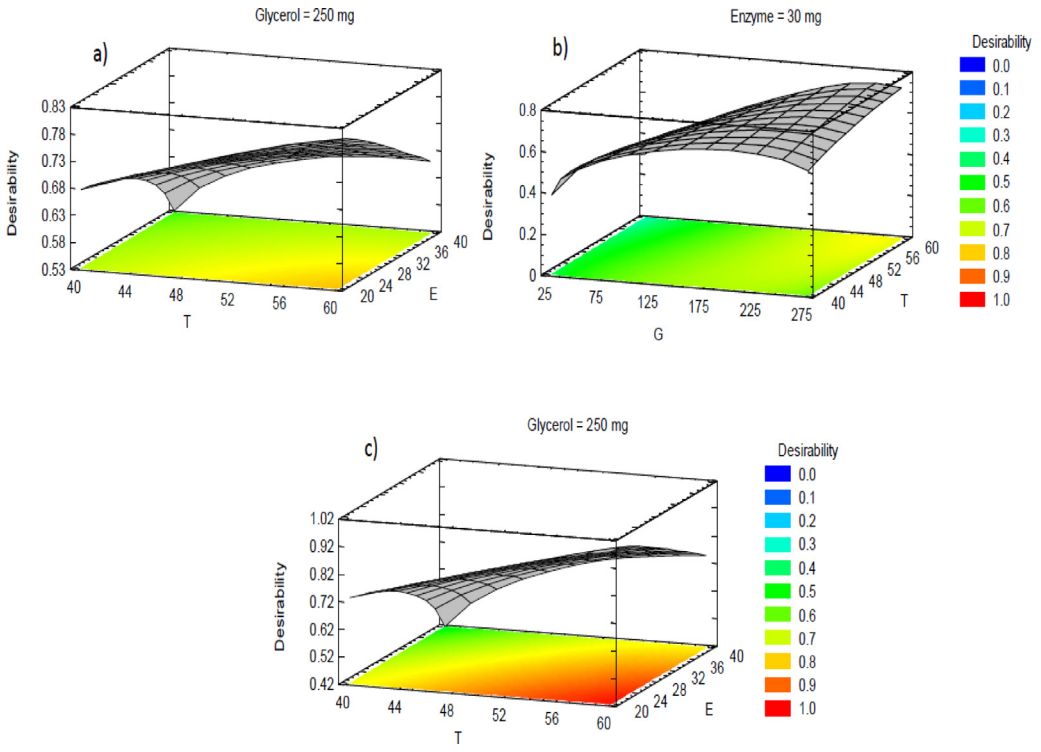

Fig. 7. Response surface for the desirability functions resulting from: (a and b) maximization of X_{AC}, Y_{DAG}, σ_{DAG}, %DAG and 1,3-DAG, and minimization of %MAG, %TAG, 1,2-DAG and 2,3-DAG, (c) maximization of X_{AC}, and 1,3-DAG and minimization of 1,2-DAG y 2,3-DAG.

(Fig. 7a), reaching a value of 0.80 for the esterification with 250 mg of glycerol adsorbed on silica gel, 20 mg of immobilized lipase and at 60 °C. Fig. 7b shows that the amount of glycerol was the main factor affecting the desirability function because it promoted the synthesis of dicaprin, but in turn minimized the acyl migration reaction and consequent production of tricaprin.

Fig. 7c shows the multivariable response resulting from maximizing conversion and the specific diglyceride fraction and minimizing the remaining isomer fractions. In this case, desirability function values greater than 0.95 were achieved when the esterification was performed with 250 mg of glycerol, 20 mg of immobilized lipase at 60 °C.

Under the above conditions, the desirability function values exceeded 0.92 for most cases. Only when the minimization of the monocaprin fraction was imposed did the desirability function decrease to 0.8. The minimization of the monocaprin fraction strongly affected the desirability function values.

However, it should be noted that all the results and the conclusions drawn from the desirability functions were absolutely conditioned by the experimental range used in this work. As desirability functions are based on models generated with the experimental data obtained within a certain range, they should be carefully analyzed before their application to real cases or extended intervals, since these models suggest that high levels of reagents and high temperatures favor the synthesis of specific diglycerides, but they do not take into account the effects of thermal inactivation, inhibition acidic, or mass transfer problems that might occur due to the high content of glycerol and silica gel.

3.6. Evaluation of the role of the support in acyl migration

3.6.1. Acyl migration in monolein

Monolein adsorbs on the support of Lipozyme RM IM mainly by hydrophobic forces. After the monolein-deactivated lipase contact, the temperature was raised to 70 °C using high magnetic stirring. After 6 h, the final composition of monolein was 71% sn-2 monoolein and 29% sn-1 monoolein (migration from the sn-2 to the sn-1 position was found). This migration was expected because the acyl migration from sn-2 to sn-1/sn-3 is thermodynamically favored. No sn1/sn-3 to sn-2 acyl migration was observed. This acyl migration was needed to explain the 1,3-dicaprin isomer and the tricaprin formation.

3.6.2. Isomerization in the esterification reaction medium

Of the total monocaprin produced, 97% corresponded to sn-1 monocaprin and only 3% to sn-2 monocaprin. For the diglyceride, 96% was 1,3-dicaprin and 4% was 1,2-dicaprin. After 6 h of reaction using the deactivated Lipozyme RM IM, temperature was raised to 70 °C at high stirring rate.

The acid concentration showed no change, confirming total lipase deactivation. The total mass of acylglycerols did not change either. Monocaprin isomers had the same initial composition, whereas in the case of dicaprin, 95% was 1,3-dicaprin and 5% was 1,2-dicaprin. The difference between initial and final composition was lower than the error detected in the experimental chromatographic measurements of the concentration (1.6%). These results showed that the support (deactivated lipase) presented no activity in acyl isomerization from sn-1/sn-3 to sn-2 position in sn-1 (sn-3) mono- or 1,3-diglycerides.

3.7. Does the R. miehei lipase catalyze the acyl migration from sn-1 (sn-3) position to sn-2 in sn-1 (sn-3) mono- and 1,3-diglycerides?

Several manuscripts reported the increase in acyl migration with temperature and lipase quantity, but no further studies have explored the topic such as it is presented in our work [22–24]. The lack of contribution of the support to acyl migration to the sn-2 position opens the possibility for lipase as an acyl migration isomerization catalyst. The reaction would be kinetically controlled

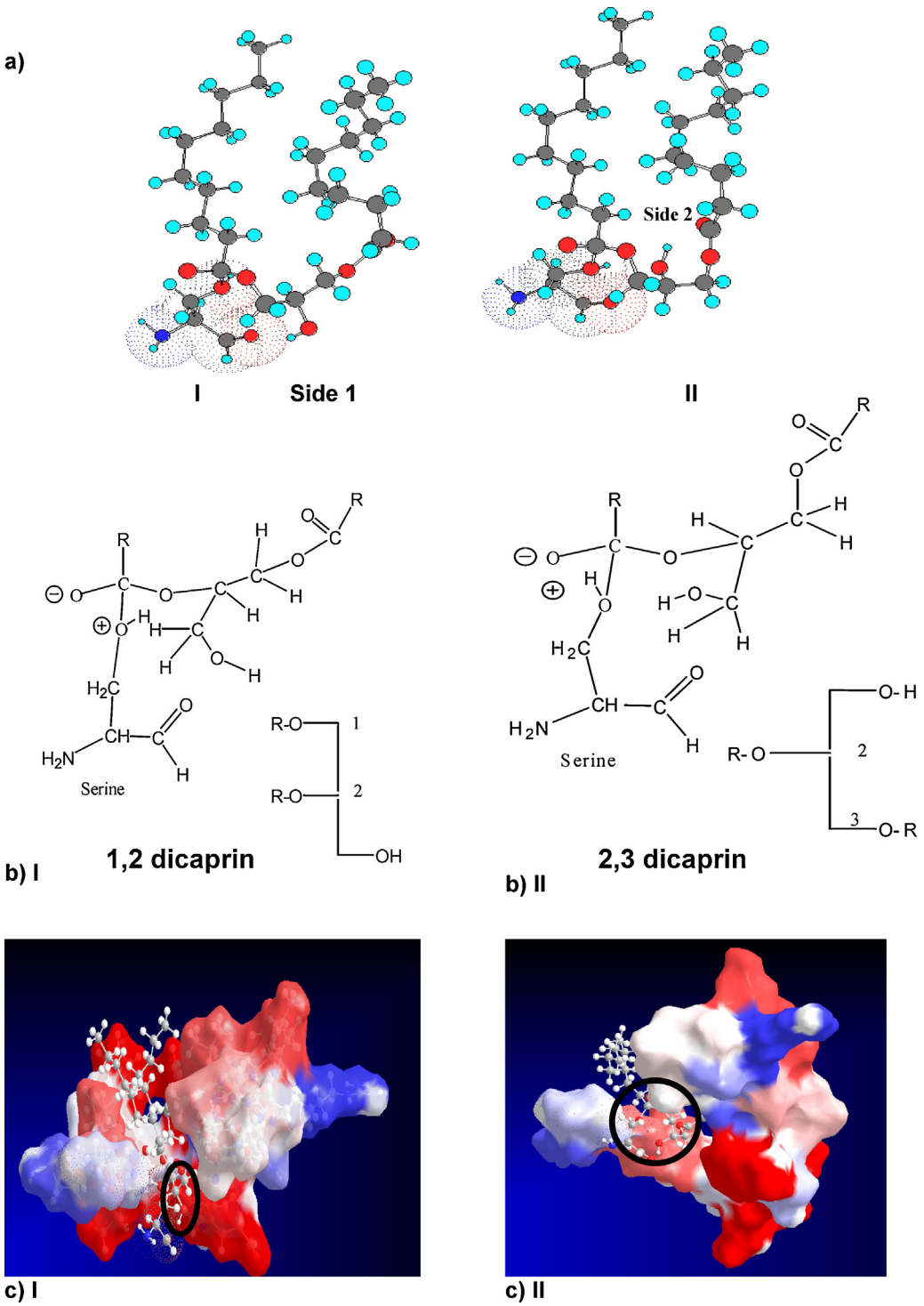

Fig. 8. Tetrahedral intermediate formed by coordination of 1,3-dicaprin or esterification of monocaprin with the acyl enzyme. (a) Structure considering the chirality of C2, with serine bonded to the intermediate, with two different orientations by side I or side II (b) after acyl migration, generation of 1,2 (I) and 2,3 (II) dicaprin, and (c) spatial distribution of the intermediate shown in (a) in the RML catalytic triad and binding cleft [36].

and it would be related to the local conditions found in the immobilized lipase. For the isomerization reaction to be operative, some conditions are important to take place, especially considering that RML is 1, 3 specific in organic solvent (as *n*-heptane):

a- the Serine with the hydroxyl group should be available to the 1,3-diglyceride and the 1(3) monoglyceride. For this reaction to be operative, the mono- or the diglyceride must be present at bonding distance to the serine group. When the acyl enzyme is present, it will probably react with glycerol or monoglyceride/diglyceride. The reaction of an acyl enzyme with sn-1 monocaprin at sn-3 generates 1,2-dicaprin, and with sn-3 monocaprin at sn-1 it generates 2,3-dicaprin by isomerization. The same transition state, as in the case of the reaction of 1,3-dicaprin, is formed (see Fig. 8, after the acyl migration).

b- a local high concentration of 1(3) monoglyceride around the catalytic triad. In the case of the monoglyceride, the preferred reaction would be the 3(1) esterification of the monoglyceride

vs. the isomerization reaction, especially at high capric acid concentration. This is the explanation of the low amount of the 2-monoglyceride found at the final esterification glycerol reaction (3% of sn-2 monocaprin).

c- a local high concentration of 1,3-diglyceride around the Serine with the hydroxyl group available instead of the acyl enzyme. This is very probable after the esterification of the sn-1 or sn-3 monoglyceride and the regeneration of the Serine group of the catalytic triad. The RML is a hydrolytic enzyme with high activity in ester hydrolysis [35]. The central carbon of 1,3-dicaprin is not chiral, therefore the hydroxyl is free in the solution, but in the lipase environment the coordination of the 1,3-dicaprin is important to understand the isomerization. With the coordination of the sn-1 or the sn-3 and the formation of an enzymatic tetrahedral intermediate, the potential of the hydroxyl shift from position sn-1 or sn-3 to sn-2 is present. When the serine attacks one of the terminal C=O groups, the C2 of the glycerol becomes chiral (see Fig. 8a).

Without the presence of the enzyme, the tetrahedral intermediate is not formed, and this is the reason for the dependence of the acyl migration from sn-1 (sn-3) to sn-2 on the amount of lipase. The formation of diglyceride isomers is a series reaction with the 1,3-dicaprin formation or by reaction of the monoglycerides with acyl enzyme. Depending on the distribution of the aminoacids from the lipase and ligands around the 1,3-dicaprin, the 2,3 or 1,2-dicaprin isomers are formed. If the hydroxyl is exchanged by the side 1, then 1,2-dicaprin is formed; if the exchange takes place from the side 2, then a 2,3-dicaprin is generated, as shown in Fig. 8a I and II. Based on the experimental results, it appears that the 2,3-dicaprin was mainly generated at high temperatures and high enzyme but low glycerol concentration, whereas the 1,2-dicaprin was generated at lower temperatures and low enzyme but high glycerol concentration (see Fig. 8b I and II, after acyl migration).

The results were mainly affected by enzyme mass and temperature, as expected if the isomerization is kinetically controlled. Acyl migration was higher when glycerol was present at low concentration. In this case, the enzyme was in the form of acyl enzyme due to capric acid at high relative concentration, and the Serine must be available for the mechanism of 1,3-dicaprin to be operative. At low glycerol concentration, the relative concentration of tricaprin was very high, and this implies that the relative concentration of 2,3-dicaprin was considerable. After the reaction time, the remaining 2,3-dicaprin was found, because part of it reacted to produce tricaprin due to the preference of the RML for the sn-1 position [37,38].

1,2-DAG is formed by reaction of the sn-3 position and migration to sn-2, whereas 2,3-DAG is formed by reaction of the sn-1 position to sn-2. The concentration of sn-1 monocaprin was higher than the concentration of sn-3 monocaprin. It is clear that the 2,3-DAG is the main precursor to TAG, and this dependence implies a preference for position 1 instead of 3. The isomerization from sn-1 to sn-2 was relatively higher than the isomerization from sn-3 to sn-2. This situation may only be operative if the lipase esterification mechanism is part of the isomerization mechanism. Therefore, the same mechanism was operative for the mono- and diglyceride synthesis and acyl isomerization through the intermediate. The spatial restriction of the enzyme is very clear in Fig. 8c I and II. The circle shows the C2 of 1,3-dicaprin and the different coordination and distributions of the hydroxyl at C2 in the tetrahedral intermediate formed by reaction with Serine of the catalytic triad of RML.

As a final comment, the acidolysis of triglycerides is by far less efficient than the glycerol esterification in terms of productivity, but there is almost no acyl migration, especially with capric acid [39]. It is evident that the presence of a hydroxyl at the sn-2 position is key to make this proposal operative.

4. Conclusions

This study showed that the commercial immobilized form of 1,3-specific lipase from *R. miehei* (Lipozyme RM IM) is a good catalyst for the synthesis of 1,3-dicaproylglycerol by the esterification of glycerol with capric acid in organic medium. The analysis of the effects of some selected reaction parameters using an experimental design showed that 1,3-dicaproilglycerol was favored by the use of high amounts of glycerol adsorbed on silica gel. The increase in temperature exhibited a slightly positive effect on the production of 1,3-dicaprin, whereas increasing the immobilized lipase dose decreased the final fraction of this isomer. The results also showed that, depending on the experimental conditions, the molar percentage of tricaprin in products varied between 52% and 4%. The maximum relative productivity of 1,3-dicaprin was 55%. Experimental data were provided to support the proposal that the tetrahedral intermediate of the enzymatic synthesis of 1,3-dicaprin may suffer acyl migration, whereas the Lipozyme RM IM support is inactive to catalyze the acyl migration, with a maximum molar percentage of dicaproylglycerol of 77% of all the products at selected conditions.

Acknowledgments

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