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Screening of Lipases with Unusual High Activity in the *sn*-2 Esterification of 1,3-Dicaprin under Mild Operating Conditions

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ABSTRACT: In this work, the synthesis of acylglycerides with high nutritional value was carried out by enzymatic esterification at *sn*-2 position of 1,3-dicaprin with palmitic acid. A comparative study of the performance of several biocatalysts according to the obtained products was carried out. The results obtained with several of the biocatalysts evaluated are very interesting, and it would be possible to use them to obtain a mixture of acylglycerides to act as a fat substitute. The final product was composed of about 90% of nutritionally attractive glycerides. These glycerides were medium-chain length triglycerides, medium−long chain triglycerides (mainly triglycerides with medium chain fatty acids at *sn*-1 and *sn*-3 positions and long chain fatty acid at *sn*-2 position), and 1,3-diglycerides. *Pseudomonas fluorescens* lipase and *Burkholderia cepacia* lipase immobilized on chitosan demonstrated unusual high activity in the *sn*-2 esterification of 1,3-dicaprin with palmitic acid at 45 °C and 12 h with 33% yield to 1,3-dicaproyl-2-palmitoyl glycerol. *Burkholderia cepacia* lipase has the advantage of being immobilized; however, BCL/chitosan has the advantages of being immobilized and therefore its easy recovery from the reaction media.

KEYWORDS: *sn-2 esterification, nutritional acylglycerides, enzymatic esterification, fat substitutes*

INTRODUCTION

Acylglycerides are esters of glycerol (GGG) and fatty acids (FA). The quantitatively most important lipid component in the human diet is the triacylglycerols (TAG), which may amount to 100 g per day or more.

The nutritional value of triglycerides and their physicochemical properties are determined not only by the fatty acid composition, but also by the positional distribution of the acyl groups bonded to the glycerol.[1]

Medium-chain triacylglycerols (MCT) are formed mainly by caprylic acid (C8) and capric acid (C10). Compared to long-chain triacylglycerols (LCT), MCTs are less likely to be deposited as body fat during metabolism.[2] MCTs are rapidly hydrolyzed to generate fatty acids and glycerol, while LCTs are converted to fatty acids and 2-monoglycerides (2-MAG). 2-MAGs are re-esterified in the small intestine.[3] Also, MCTs are metabolized as fast as glucose and have caloric densities two-times higher than protein or carbohydrate. This finding has contributed to the utilization of MCTs as rapid energy sources due to their quick absorption. It is also used as an alternative solution for consumers with digestive problems.[2] MCTs have no toxicological properties when their consumption is less than 30 g per day.[4] However, MCTs formed only by medium chain fatty acids (MCFA) are not suitable for cooking due to its low smoke point and foam generation.[5] As an alternative, long-chain fatty acids (LCFA) were incorporated into MCTs. These new glycerides were defined as medium−long chain triglycerides (MLCTs). MLCTs are similar biochemically and physiologically to MCTs, and MLCTs could be used in special cooking oils for dietary therapy.[5−8] MLCTs increase diet-induced thermogenesis, accelerate energy production, and contribute to less accumulation of body fat. The use of MLCT could prevent obesity as well as metabolic syndrome.[5−8] These triglycerides can be obtained by interesterification[9,10] or acidolysis.[11,12] A particular case of MCTs are MLM-type triglycerides, and they contain MCFAs at the *sn*-1 and *sn*-3 positions and LCFA at the *sn*-2 position. MLM-type triglycerides are an effective energy source for patients with malabsorption, for example, pancreatic insufficiency.[1] They have been synthesized using different methodologies.[13−16] However, there are few publications concerning the synthesis of structured triglycerides obtained from diglycerides (DAGs).[17−19]

DAGs are minor components of oils and fats. Normally, the level of diglycerides in edible oils is below 5%.[20,21] Several studies on the nutritional properties and effects of the consumption of DAG[22−25] have shown that diglycerides, especially 1,3-DAG, compared with triglycerides, have the ability to reduce concentrations of TAG in serum[23] and as a result decrease both body weight and visceral fat.

In this work, a screening of lipases for the synthesis of acylglycerides with high nutritional value by enzymatic esterification of *sn*-2 position of 1,3-dicaprin (CGC) with palmitic acid (P) was carried out.

MATERIALS AND METHODS

Materials. Lipozyme RM IM, which is a commercial form of the 1,3-specific lipase from *Rhizomucor miehei* immobilized by adsorption on a macroporous anion exchange phenolic resin Duolite A-568, was kindly provided by Novo Nordisk A/S (Brazil). Novozym 435, which is a commercial form of the lipase B from *Candida antarctica* immobilized on an acrylic resin, was kindly provided by Novozyme (Bagsvaerd, Denmark, Brazil branch office). Lipases from *Burkholderia cepacia*, *Pseudomonas fluorescens*, and *Candida rugosa*, commercially

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called Lipase PS "Amano", Lipase AK "Amano", and Lipase AY "Amano", respectively, were gifts from Amano Enzyme Inc. (Nagoya, Japan). Lipase from porcine pancreas was supplied by Sigma-Aldrich.

n-Heptane was supplied by Laboratorios Cicarelli. Tripalmitin and silylation reagents were obtained from Fluka. Capric acid, palmitic acid, dipalmitin, tricaprylin, tricaprin, trilaurin, trimyristin, and lipase from porcine pancreas were supplied by Sigma-Aldrich. Pyridine was purchased from Anedra S.A., and chitosan was provided by Primex S.A. (Iceland).

Immobilization of *Burkholderia cepacia* Lipase. *Burkholderia cepacia* lipase (BCL) was immobilized by physical adsorption on chitosan. For this purpose, 500 mg of commercial BCL powder was dispersed in 50 mL of twice-distilled water with pH = 6.5. The solution was stirred with a glass rod and then centrifuged to clarity. The supernatant was removed and placed in contact with 1 g of chitosan in a close beaker. Immobilization was carried out for 1 h with magnetic stirring at 400 rpm and at 35 °C. The mixture was filtered to remove the solid, which was dried for 15 h at 30 °C. This catalyst is hereafter denoted as BCL/chitosan. The degree of immobilization was determined following the methodology described by Nicolás et al.[26] This technique is based on measuring sulfur (S) in the protein solutions before and after immobilization using inductively coupled plasma-atomic emission spectrometry (ICP-AES). One molecule of BCL (34100 Da[27]) contains one unit of methionine and two units of cysteine.[28] The protein content was calculated considering that three atoms of sulfur are present per each lipase molecule.

Evaluation of Lipase Desorption in *n*-Heptane. One-hundred milligrams of BCL/chitosan and 2 mL of *n*-heptane were placed in a reaction vial. It was kept in a thermostatic bath at 45 °C for 6 h with magnetic stirring at 700 rpm. Then the biocatalyst was separated, and the content of the vial was analyzed by ICP-EAS to determine the sulfur content.

Removing Weakly Adsorbed Lipase. One-hundred milligrams of the obtained biocatalyst was placed in 5 mL of distilled water (pH 6.5) and magnetically stirred for 1 min. This washing procedure was performed twice. The biocatalyst was then dried in an oven at 30 °C for 15 h and then 24 h at 45 °C to remove the water adsorbed on the chitosan. Samples of the washing solutions were taken for sulfur analysis by ICP-AES.

Esterification Reaction. Lipases from *Candida rugosa* (CRL), *Pseudomonas fluorescens* (PFL), and *Burkholderia cepacia* (BCL) in their free form were evaluated in the esterification reaction. Lipozyme RM IM (RML) and Novozym 435 (CALB) were also tested.

The esterification of dicaprin (90% of 1,3-dicaprin and 10% of 1,2-dicaprin) was performed in 10 mL vials, which were kept in water baths with temperature control and magnetic stirring. The reaction time was 12 h, and it was carried out as follows: 45 mg of dicaprin (80:10) and 56 mg of palmitic acid were dissolved in 2 mL of *n*-heptane. The reaction temperature for all tests was set at 45 °C. When the reaction mixture reached the desired temperature, the reaction was initiated with the addition of 10 mg (50%) of the enzyme (time 0). The remaining 50% of the biocatalyst was added after 3 h of reaction. The addition of the immobilized lipase in two stages would minimize the deactivation of the enzyme and improve the yield of the desired product.[29,30]

For BCL immobilized on chitosan, 133 mg of the biocatalyst was used by carrying out the reaction under the same conditions mentioned earlier (this mass of biocatalyst provided the same protein content as that present in free BCL).

The dicaprin was obtained in optimum conditions reported in a previous work[31] and purified according to the protocol published previously.[32]

Different reaction conditions were previously evaluated. Among them, the type of acyl donor, methodologies to minimize secondary reactions, supports used to carry out the immobilization, and palmitic acid/dicaprin molar ratio were studied. All reactions were performed in triplicate, and the reported results correspond to the mean along with the standard error.

Analysis of Samples. *Gas Chromatography Analysis.* Samples were diluted with pyridine and silylated with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA). The analysis of the samples was performed in a PerkinElmer AutoSystem XL gas chromatograph equipped with on-column injection, a flame ionization detector (FID), and a high temperature ZB-5HT Inferno capillary column (15 m × 0.32 mm, with an ID of 0.10 μm) using H_2 as carrier gas. The detector temperature was maintained at 380 °C. The initial column temperature was held at 50 °C for 1 min, increased to 180 °C at a rate of 15 °C/min, then increased to 230 °C at 7 °C/min, further increased up to 370 °C at 10 °C/min, and finally maintained at 370 °C for 5 min. Reported results are the average of two injections with an average relative error lower than 2%. The determination of the elution times of the reactants and products was performed with commercial standards of high purity. Elution times for triglycerides without high purity standard were identified using another commercial standard of triglycerides with similar molecular weight (the commercial source of the standards is indicated in the Materials section). Table 1 shows the standards used and the compounds identified with them. In addition, the elution times and molecular weights are presented.

Table 1. High Purity Standards Used To Identify Compounds in the Reaction System

high purity standard	molecular weight [g/mol]	elution time [min]	compound to identify	molecular weight [g/mol]	elution time [min]
tripalmitin	807.34	27.36	tripalmitin	807.34	27.36
trimyristin	723.18	24.97	1-caproyl-2,3-palmitoyl glycerol	726.20	24.63
trilaurin	639.02	22.43	1,3-dicaproyl-2-palmitoyl glycerol 1,2.dicaproyl-3-palmitoyl glycerol	641.03	22.06
dipalmitin	568.92	21.21	dipalmitin	568.92	21.21
tricaprin	554.85	19.00	tricaprin	554.85	19.00
tricaprylin	470.69	14.84	internal standard	470.69	14.84
palmitic acid	256.43	8.55	palmitic acid	256.43	8.55
capric acid	172.27	4.69	capric acid	172.27	4.69

Identification of the Fatty Acid at sn-2 Position. Detection of the fatty acid located at the *sn*-2 position of the glycerol backbone was carried out by hydrolysis of acylglycerides using lipase from porcine pancreas (PPL) as catalyst, following the widely reported methodology[33−37] combined with gas chromatography.

Free fatty acids were previously neutralized with 2 mL of an aqueous solution of KOH 0.1 M, and the phase containing acylglycerides were recovered (procedure described in ref 32). The solvent was evaporated and 1.9 mL of tris-hydrochloric buffer (1.0 M, pH 8.0), and 0.1 mL of calcium chloride solution (220 g/L) was added to the emulsified mixture. The reaction was carried out at 30 °C with magnetic stirring at 400 rpm for 30 min and catalyzed with 20 mg of PPL. Porcine pancreas lipase is recognized as 1,3-specific. The hydrolysis reaction allows generation of 2-monoglycerides and free fatty acids. The short reaction time allows the acyl migration reaction to be avoided and correct identification of the generated monoglycerides. However, the triglyceride conversion is partial due to the short reaction time; the results are extrapolated for the total conversion. The identification of 2-MAG, free fatty acids, and diglycerides obtained by this hydrolysis reaction makes it possible to know the composition of the starting sample.

Data Analysis. A simple ANOVA procedure was performed to determine the effect of the biocatalyst on the different responses. To determine significant differences between values of the means, multiple range tests were performed. In this study, the procedure of the honestly significant difference or tukey test (Tukey HSD) was applied. This analysis is more conservative than the method of the least significant difference (LSD Fisher) since it makes it more difficult to declare significant differences between pairs of means.

The statistical analysis was performed using the STATGRAPHICS Centurion version XV.2 software.

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Table 2. Quantification of the Mass of Sulfur and Lipase in the Different Stages of the Immobilization Process

stage[a]	sulfur (mg)	lipase (mg)
onset of immobilization, end of immobilization	$0.282 \pm 3.50 \times 10^{-3}$, $0.182 \pm 5.50 \times 10^{-3}$	99.97 ± 1.24, 64.52 ± 1.95
leaching in *n*-heptane[b]	$0.00164 \pm 1.0 \times 10^{-4}$	0.58 ± 0.012
washing 1[c]	$0.00048 \pm 2.0 \times 10^{-5}$	0.170 ± 0.0071
washing 2[c]	$0.00048 \pm 3.0 \times 10^{-5}$	0170 ± 0.011
leaching in *n*-heptane[d]	0	0

[a]Measurements made in the liquid solution. [b]100 mg of unwashed biocatalyst. [c]100 mg of biocatalyst. [d]100 mg of washed biocatalyst.

Table 3. Esterification Reaction of Dicaprin with Palmitic Acid Catalyzed by Different Lipases: Initial and Final Compositions, DAG Conversion, and CPC Yield (12 h Reaction Time)

		biocatalyst/final composition (μmol)					
reagents and products	initial composition (μmol)	CRL	CALB	RML	BCL	PFL	BCL/chitosan
GGG	0.0	0.0 ± 0.0	22.3 ± 1.1	16.7 ± 1.2	11.3 ± 0.8	11.8 ± 0.9	5.2 ± 0.4
C	1.7	6.6 ± 0.6	134.3 ± 6.8	135.8 ± 5.4	109.3 ± 5.5	94.2 ± 4.7	85.5 ± 4.3
P	218.4	213.3 ± 17.1	101.8 ± 6.1	104.8 ± 5.3	119.3 ± 6.0	102.7 ± 4.1	89.1 ± 4.4
CGC[a]	112.3	91.0 ± 5.5	18.0 ± 1.5	18.6 ± 1.2	23.0 ± 1.4	27.1 ± 1.6	17.4 ± 0.9
CPG+CGP[b]	0.0	3.2 ± 0.2	24.2 ± 1.9	19.5 ± 1.2	22.7 ± 1.4	22.6 ± 1.4	17.7 ± 1.0
CCC	9.0	20.0 ± 1.4	1.0 ± 0.1	0.0 ± 0.0	6.1 ± 0.4	0.0 ± 0.0	5.3 ± 0.4
PPG+PGP	0.0	0.0 ± 0.0	12.6 ± 0.8	6.9 ± 0.4	7.8 ± 0.5	5.6 ± 0.5	7.3 ± 0.6
CPC[c]	0.0	1.9 ± 0.14	20.4 ± 1.3	30.8 ± 1.9	26.0 ± 1.6	36.3 ± 2.2	37.4 ± 2.6
CPP	0.0	0.0 ± 0.0	14.3 ± 0.9	18.6 ± 1.3	9.9 ± 0.8	14.5 ± 0.9	22.8 ± 1.6
PPP	0.0	0.0 ± 0.0	4.2 ± 0.3	3.4 ± 0.3	3.1 ± 0.3	2.1 ± 0.2	4.2 ± 0.3
X_{DAG} (%)		19 ± 1.5	84 ± 5.0	83 ± 5.0	80 ± 4.8	76 ± 4.6	85 ± 5.1
Y_{CPC} (%)		2 ± 0.2	18 ± 1.3	27 ± 1.9	23 ± 1.6	32 ± 2.2	33 ± 2.3

[a]Dicaprin mixture was composed of 90% 1,3-dicaprin and 10% 1,2-dicaprin. [b]CPG and CGP were the identified medium−long chain diglycerides. They were quantified together. [c]CPC comprises the mixture mainly of 1,3-reported dicaproyl-2-palmitoyl glycerol and minor amounts of 1,2-dicaproyl-3-palmitoyl glycerol.

RESULTS AND DISCUSSION

BCL Content Immobilized on Chitosan. The studies carried out by ICP-AES showed that 500 mg of lipase PS-amano contained 0.282 ± 0.0035 mg of sulfur, and after the immobilization procedure, 0.182 ± 0.0055 mg of sulfur was recovered. It was possible to determine that the immobilization process was initiated with 99.97 mg of lipase. Therefore, it was considered that molecular weight of BCL is 34.1 kDa and that there are three sulfur atoms per lipase molecule. After the immobilization process, 64.52 ± 1.95 mg of lipase was recovered. Thus, 35.45 ± 3.19 mg of lipase was adsorbed onto 1000 mg of chitosan (Table 2).

Leaching of Weakly Adsorbed Lipase. After 6 h of contact between the biocatalyst and the reaction solvent, the latter was recovered and analyzed by ICP-AES to determine the sulfur content. The presence of 0.00164 ± 0.0001 mg of sulfur, corresponding to 0.58 ± 0.012 mg of lipase, was determined (a loss percentage equivalent to 16.4%).

The biocatalyst was washed twice to avoid enzyme leaching in the reaction system. The results are shown in Table 2. A biocatalyst containing 3.1 wt % of lipase and that was stable in the reaction medium was obtained.

Catalytic Tests. Generally, enzyme screening is carried out by using an equal number of enzymatic activity units (U). However, in this work, the comparison of biocatalysts based on weight was not arbitrary. One of the aims of this work was the selection of a commercial biocatalyst adequate for the synthesis of high nutritional value glycerides, with good yield. From an engineering point of view, the use of mass units is more reasonable than using activity units. What is more, the biocatalysts are marketed in mass units, and in a cost estimation of the obtained product, the knowledge of the mass of the catalyst used is an essential requisite.

Moreover, units measured in a specific reaction do not provide any information on the biocatalyst activity in other reaction or even under different operation conditions.[38]

Table 3 shows the results of the catalytic test. 1,3-Dicaproyl-2-palmitoyl glycerol (CPC) and minor amounts of 1,2-dicaproyl-3-palmitoyl glycerol (CCP) were obtained by the esterification of dicaprin with palmitic acid (P). CCP is generated from the 1,2-dicaprin (CCG) present in the original reagent or from 1,2-dicaprin produced by acyl migration in 1,3-dicaprin (CGC) in the reaction medium.

The hydrolysis was an important reaction detected in this system. Free capric acid (C) and even glycerol (GGG) were obtained as products. The products of the hydrolysis reaction were re-esterified leading to different glycerides. Medium−long chain diacylglycerols (1-caproyl-2-palmitoyl glycerol (CPG) and 1-caproyl-3-palmitoyl glycerol (CGP)), long chain diacylglycerols (1,2-dipalmitin (PPG) and 1,3-dipalmitin (PGP)), medium−long chain triacylglycerols (1-caproyl-2,3-palmitoyl glycerol (CPP)), and long chain triacylglycerols (tripalmitin (PPP)) were obtained. The final product also contained tricaprin (CCC), which was present in the reagent used.

Table 3 displays that the DAG conversion was higher than 75% for the studied biocatalysts except CRL. After applying the Tukey test, no statistically significant differences were detected with a 95% confidence level in diglyceride conversion for most biocatalysts. Apparently, the CRL activity was negatively affected by the reaction medium.

CPC yields over 30% were achieved with PFL and BCL/chitosan without statistically significant differences between

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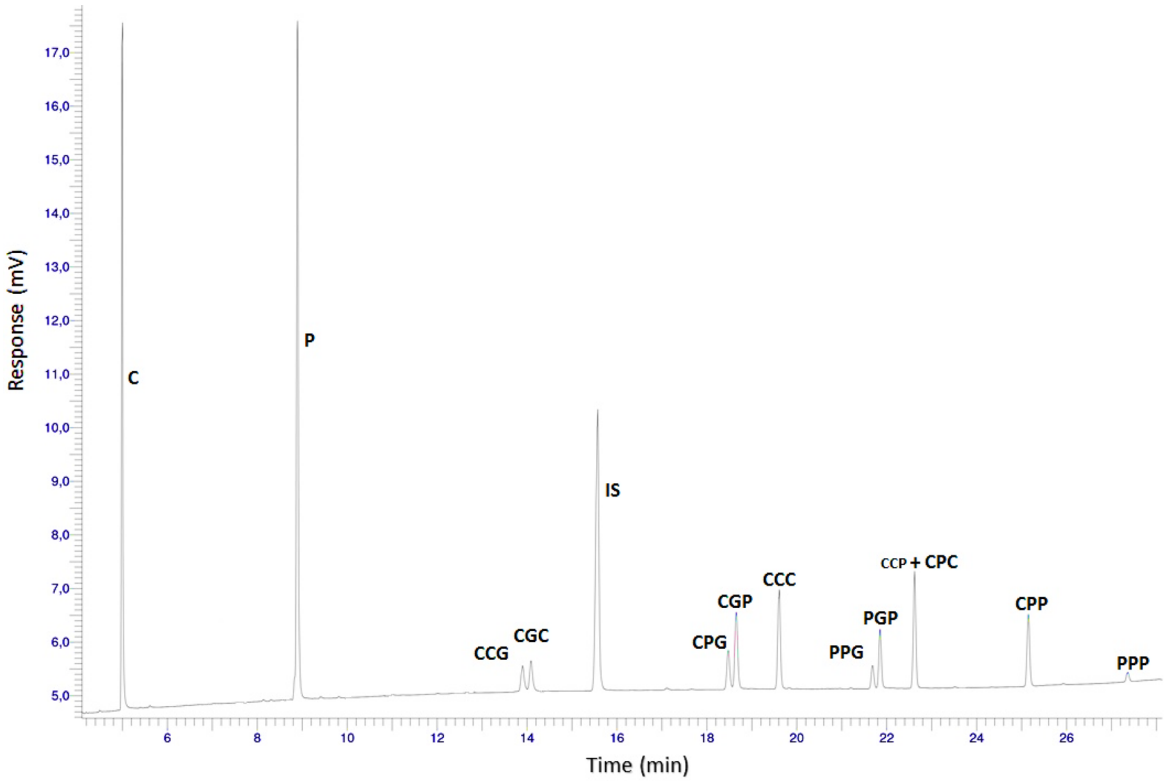

Figure 1. Typical chromatogram of the reaction products and unconsumed reactants for the enzymatic esterification of dicaprin and palmitic acid using CALB. IS: internal calibration standard. Experimental conditions: dicaprin = 45 mg, palmitic acid/dicaprina molar ratio = 2 mol/mol, temperature = 45 °C, biocatalyst load = 20 mg, and reaction time = 12 h.

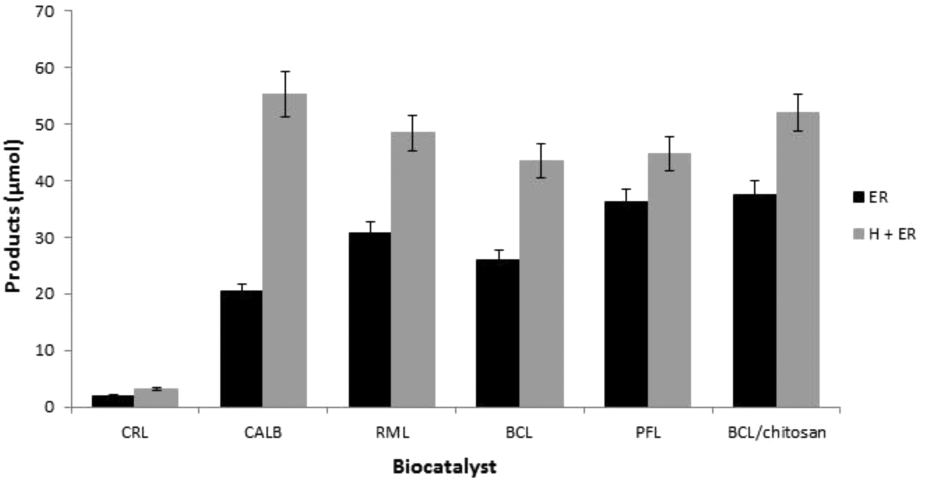

Figure 2. Products generated by esterification of dicaprin with palmitic acid. They were grouped according to the reactions that generate them. Experimental conditions: dicaprin = 45 mg, palmitic acid/dicaprina molar ratio = 2 mol/mol, temperature = 45 °C, and reaction time = 12 h.

their means. However, BCL/chitosan has the advantages of being immobilized and therefore its easy recovery from the reaction media.

The DAG conversion was obtained with the following equation:

$$X_{DAG} = \frac{DAG_0 - DAG_f}{DAG_0} \times 100 \quad (1)$$

where DAG_0 and DAG_f are the initial and final concentrations of dicaprin, respectively. The yield to CPC was defined as follows:

$$Y_{CPC} = \frac{CPC}{\text{Theoretical CPC}} \times 100 \quad (2)$$

where CPC is the actual content of the measured triglyceride, and theoretical CPC is the number of μmoles of CPC obtained if all the DAG is converted to CPC.

A typical chromatogram of the reaction products and unreacted substrates is shown in Figure 1. In this case, the esterification reaction was catalyzed by CALB.

Evaluation of Catalysts According to the Reactions Promoted by Them. As it has been mentioned above, different reactions occurred in the system under study:

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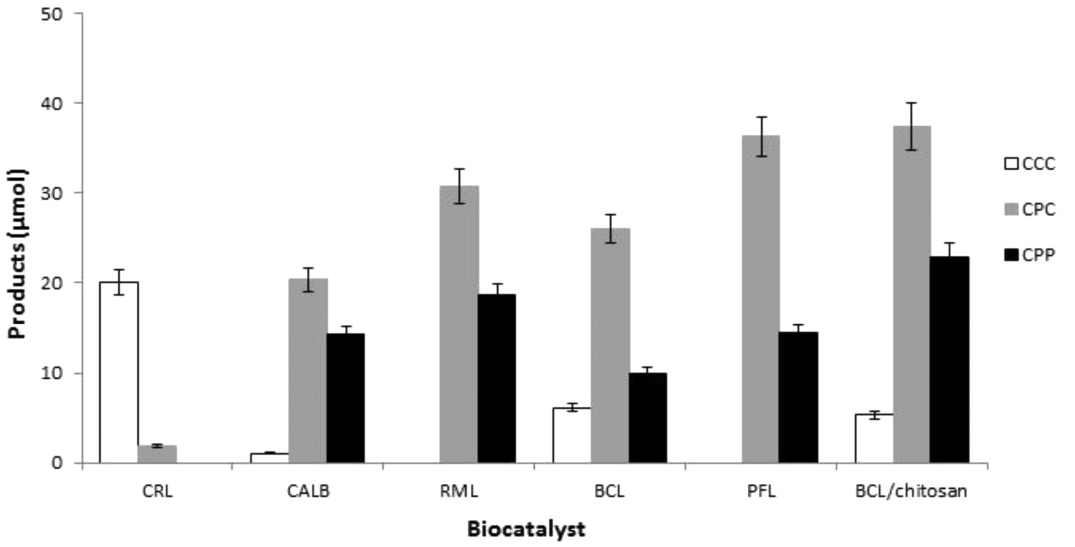

Figure 3. Triglycerides with a high nutritional value generated by esterification of dicaprin with palmitic acid. Experimental conditions: dicaprin = 45 mg, palmitic acid/dicaprina molar ratio = 2 mol/mol, temperature = 45 °C, and reaction time = 12 h.

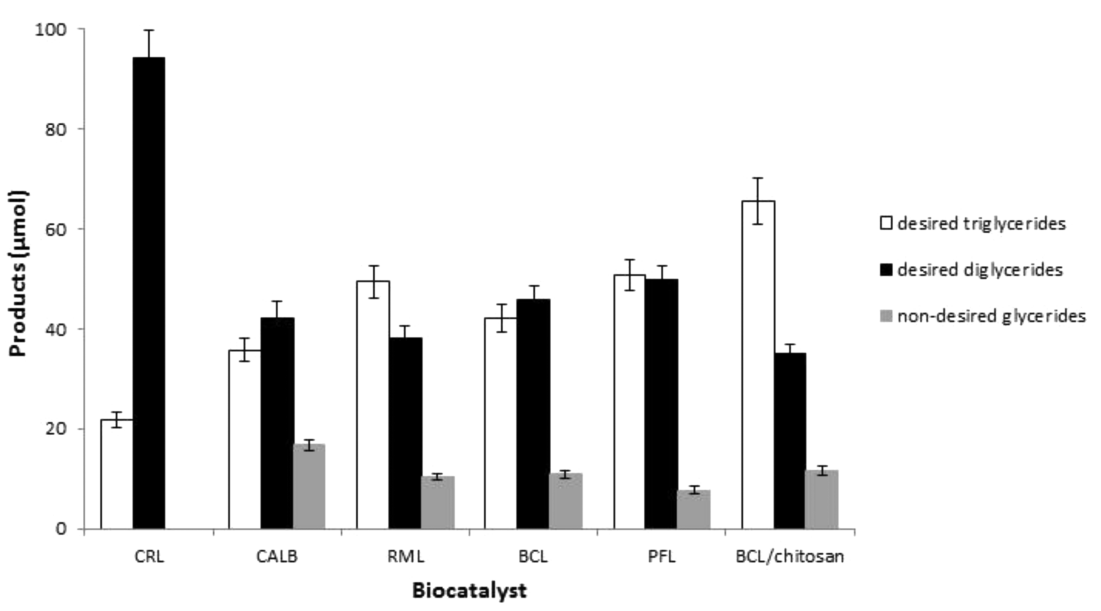

Figure 4. Glyceride composition of the reaction product by carrying out the esterification of dicaprin with palmitic acid using different biocatalysts. Experimental conditions: dicaprin = 45 mg, palmitic acid/dicaprina molar ratio = 2 mol/mol, temperature = 45 °C, and reaction time = 12 h.

esterification (ER), hydrolysis (HR), and hydrolysis followed by esterification (H + ER). The products of the HR (C and GGG) were removed using the methodology described previously.[32] Figure 2 shows the distribution of products classified according to the reaction that originated them (ER or H + ER).

BCL/chitosan and PFL were the biocatalysts that showed the highest activity in the esterification reaction without statistically significant differences between them. After CRL that showed very low activity, CALB was the biocatalyst with the lowest degree of *sn*-2 esterification, but the one that showed the highest activity for the hydrolysis reaction followed by esterification. BCL, PFL, and RML had statistically similar performances in the hydrolysis followed by esterification. The immobilization of BCL allowed an increase of diglyceride conversion, and this result was also reflected in an increase in the products generated by both reactions.

Evaluation of Biocatalysts for the Synthesis of Medium Chain Triglycerides (CCC) and Medium−Long Chain Triglycerides (CPC−CCP-CPP). In this section, the performance of the biocatalysts for the generation of triglycerides with high nutritional value is analyzed. Figure 3 shows the final concentration of CCC, CPC, and CPP (in μmoles).

CRL was the only biocatalyst that allowed the CCC generation. PFL, RML, and CALB completely hydrolyzed it. BCL and BCL/chitosan had a similar behavior, both biocatalysts partially hydrolyzed this triglyceride, obtaining little more than 5 μmoles of it at the end of the reaction.

CPC is only generated by the esterification of the *sn*-2 position of 1,3-dicaprin. As previously mentioned, PFL and

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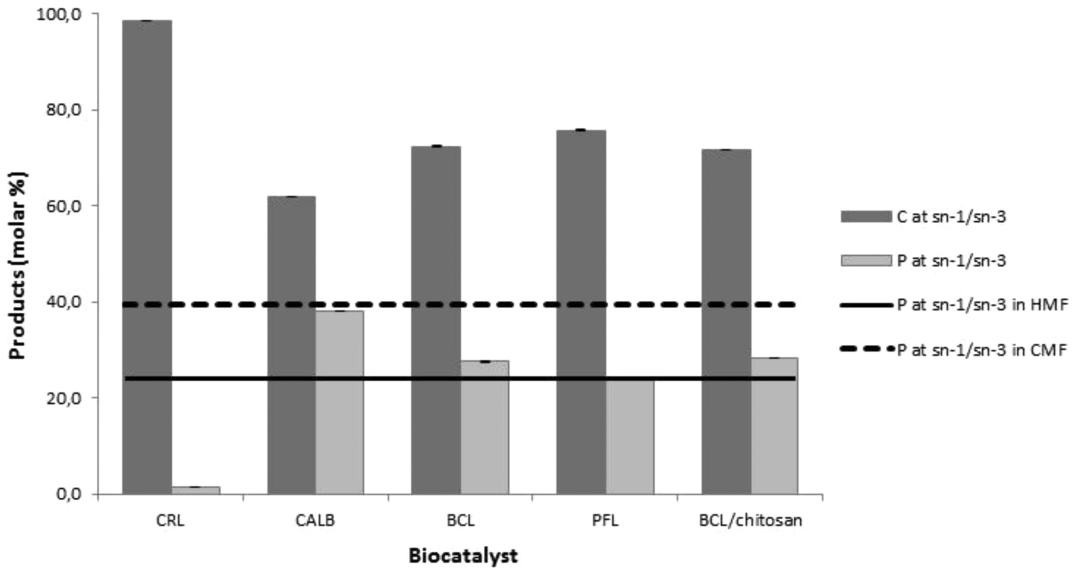

Figure 5. Distribution of fatty acids in the final reaction product by carrying out the esterification of dicaprin with palmitic acid. Experimental conditions: dicaprin = 45 mg, palmitic acid/dicaprina molar ratio = 2 mol/mol, temperature = 45 °C, and reaction time = 12 h.

BCL/chitosan were the biocatalysts with the best performance in this reaction. BCL/chitosan was also the biocatalyst that allowed the highest generation of CPP. However, the selected time could have been too high when carrying out the reaction with this biocatalyst. The increase in the molar fraction of CPP occurred at the expense of reduction of the fraction of CPC, which suggests that there was a serial reaction between CPC and CPP. Apparently, first CPC was generated, then was hydrolyzed, and the diglyceride was re-esterified with palmitic acid to give CPP. The reactions would occur as follows: CGC → CPC → CPG → CPP.

The profile of the identified diglycerides allowed support to be given to the hypothesis of these series reactions (results not shown).

Evaluation of Biocatalysts for the Synthesis of Glycerides with High Nutritional Value. The final reaction product (after neutralization of free fatty acids) was a mixture of glycerides with high nutritional value. The mixture contained medium chain diglycerides (CGC and CCG), medium−long chain diglycerides (CPG and CGP), medium-chain triglycerides (CCC), and medium−long chain triglycerides (CPC, CCP, and CPP). An important aspect of this study was to minimize the generation of dipalmitin, tripalmitin, and other glycerides with palmitic acid in the *sn*-1 or *sn*-3 position.

The composition of the final reaction mixture is presented in Figure 4. The products were classified as desired triglycerides (CCC, CPC, and CPP), desired diglycerides (CGC, CGP, and CPG), and unwanted glycerides (PPG, PGP, and PPP).

The highest proportion of triglycerides with high nutritional value was generated using BCL/chitosan. For this biocatalyst, the final concentration of desired diglycerides was one of the lowest, along with that obtained with RML. As mentioned earlier, the reaction time may have been high for this biocatalyst. Reducing the time could increase the final content of nutritionally attractive diglycerides, increase CPC concentration, and reduce unwanted glyceride content.

The highest generation of undesirable glycerides was obtained using CALB, with this biocatalyst, also the lowest proportion of high value triglycerides was obtained (after CRL, which only allowed to generate CCC).

The lowest fraction of undesired glycerides was obtained using PFL as the biocatalyst.

The metabolism of glycerides involves the attack to the *sn*-1 and *sn*-3 positions. The presence of medium chain fatty acids in such positions favors the absorption of TAG and reduces calcium losses while allowing to reduce the accumulation of fats thus contributing to weight loss. The medium chain fatty acids in *sn*-1 and *sn*-3 positions are a quick source of energy, while palmitic acid could be absorbed as *sn*-2 monopalmitin.[39]

The fatty acid distribution in *sn*-1 and *sn*-3 positions in the reaction product is presented in Figure 5. As a comparison, the fraction of palmitic acid at *sn*-1/*sn*-3 in the human milk fat (HMF) and the cow milk fat (CMF) are presented.[40]

Under the selected operating conditions, all the tested biocatalysts, with the exception of CALB, generated glyceride mixtures with proportions of palmitic acid at *sn*-1/*sn*-3 positions similar to human milk fat (Figure 5). CALB produced a glycerides mixture with a fraction of palmitic acid at *sn*-1/*sn*-3 similar to the cow milk fat.

The synthesis of glycerides with high nutritional value was carried out by esterification of 1,3-dicaprin with palmitic acid mediated by different biocatalysts in a system with an organic solvent. The diglyceride conversion was similar for all biocatalysts, except for CRL, which is an enzyme with high hydrolytic activity. The main differences were in the products generated with each biocatalyst. These results could be related to structural variations of the lipases or a different response of each lipase to the reaction medium.

Several of the catalysts tested were very interesting to synthesize a mixture of glycerides to act as a fat substitute mainly composed by MLCT, MCT, and 1,3-DAG. PFL, BCL, and RML allowed the synthesis of glycerides with interesting nutritional value. On the other hand, CRL showed low activity in this reaction system, and CALB produced the mixture with the less interesting composition. The immobilization of BCL on chitosan allowed a biocatalyst with about 3 wt % of lipase to be obtained. This simple procedure increased the lipase activity and selectivity compared with free BCL. The differences in activity between BCL and BCL/chitosan probably are due to immobilization avoiding lipase aggregation since chitosan

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showed no activity in the reaction system, and the physical adsorption does not generate conformational changes in the enzyme. BCL/chitosan is particularly interesting for the synthesis of nutritional glycerides from diglycerides, and it has not been practically studied. A single publication refers to the preparation of triglycerides by esterification of diglycerides catalyzed with BCL with considerably lower esterification levels.[18] The optimization of reaction time would be required to maximize the generation of products of interest.

BCL/chitosan demonstrated unusual high activity in *sn*-2 esterification of 1,3-dicaprin with palmitic acid at 45 °C and 12 h. Yield to CPC achieved 33%. Nonimmobilized PFL was also suitable with 32% yield but hydrolysis is higher compared with BCL/chitosan. Other manuscripts reported for a similar reaction less than 20% selectivity at 60 °C with BCL.[18]

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ABBREVIATIONS USED

BCL, *Burkholderia cepacia* lipase; C, capric acid; CALB, *Candida antarctica* lipase B; CCC, tricaprin; CCG, 1,2-dicaproyl glicerol; CCP, 1,2-dicaproyl-3-palmitoyl glicerol; CGC, 1,3-dicaproyl glicerol; CGP, 1-caproyl-3-palmitoyl glicerol; CPC, 1,3-dicaproyl-2-palmitoyl glicerol; CPG, 1-caproyl-2-palmitoyl glicerol; CPP, 1-caproyl-2,3-palmitoyl glicerol; CRL, *Candida rugosa* lipase; DAG, diacylglycerol; FA, fatty acids; GGG, glycerol; LCFA, long chain fatty acid; MCFA, medium chain fatty acid; MCT, medium chain triacylglycerol; MLCT, medium−long chain triacylglycerol; P, palmitic acid; PFL, *Pseudomonas fluorescens*; PGP, 1,3-dipalmitoyl glicerol; PPG, 1,2-dipalmitoyl glicerol; PPL, porcine pancreas lipase; PPP, tripalmitin; RML, *Rhizomucor miehei* lipase; TAG, triacylglycerol

REFERENCES

(1) Schmid, U.; Bornscheuer, U. T.; Soumanou, M. M.; McNeill, G. P.; Schmid, R. D. Optimization of the reaction conditions in the lipase-catalyzed synthesis of structured triglycerides. *J. Am. Oil Chem. Soc.* **1998**, *75* (11), 1527−1531.

(2) Lai, O. M.; Low, C. T.; Akoh, C. C. Lipase-catalyzed acidolysis of palm olein and caprylic acid in a continuous bench-scale packed bed bioreactor. *Food Chem.* **2005**, *92* (3), 527−533.

(3) Mumme, K.; Stonehouse, W. Effects of medium-chain triglycerides on weight loss and body composition: a meta-analysis of randomized controlled trials. *J. Acad. Nutr. Diet.* **2015**, *115* (2), 249−263.

(4) Marten, B.; Pfeuffer, M.; Schrezenmeir, J. Medium-chain triglycerides. *Int. Dairy J.* **2006**, *16* (11), 1374−1382.

(5) Matsuo, T.; Matsuo, M.; Taguchi, N.; Takeuchi, H. The thermic effect is greater for structured medium-and long-chain triacylglycerols versus long-chain triacylglycerols in healthy young women. *Metab., Clin. Exp.* **2001**, *50* (1), 125−130.

(6) Kasai, M.; Nosaka, N.; Maki, H.; Negishi, S.; Aoyama, T.; Nakamura, M.; Kondo, K. Effect of dietary medium-and long-chain triacylglycerols (MLCT) on accumulation of body fat in healthy humans. *Asia Pac. J. Clin. Nutr.* **2003**, *12* (2), 151−160.

(7) Shinohara, H.; Ogawa, A.; Kasai, M.; Aoyama, T. Effect of randomly interesterified triacylglycerols containing medium-and long-chain fatty acids on energy expenditure and hepatic fatty acid metabolism in rats. *Biosci., Biotechnol., Biochem.* **2005**, *69* (10), 1811−1818.

(8) Ogawa, A.; Nosaka, N.; Kasai, M.; Aoyama, T.; Okazaki, M.; Igarashi, O.; Kondo, K. Dietary medium-and long-chain triacylglycerols accelerate diet-induced thermogenesis in humans. *J. Oleo Sci.* **2007**, *56* (6), 283−287.

(9) Rønne, T. H.; Pedersen, L. S.; Xu, X. Triglyceride selectivity of immobilized *Thermomyces lanuginosa* lipase in interesterification. *J. Am. Oil Chem. Soc.* **2005**, *82* (10), 737−743.

(10) Feltes, M. M.; de Oliveira Pitol, L.; Gomes Correia, J. F.; Grimaldi, R.; Block, J. M.; Ninow, J. L. Incorporation of medium chain fatty acids into fish oil triglycerides by chemical and enzymatic interesterification. *Grasas Aceites* **2009**, *60* (2), 168−176.

(11) Camacho Páez, B.; Robles Medina, A.; Camacho Rubio, F.; Gonzalez Moreno, P.; Molina Grima, E. Production of structured triglycerides rich in n-3 polyunsaturated fatty acids by the acidolysis of cod liver oil and caprylic acid in a packed-bed reactor: equilibrium and kinetics. *Chem. Eng. Sci.* **2002**, *57* (8), 1237−1249.

(12) Foresti, M. L.; Ferreira, M. L. Lipase-catalyzed acidolysis of tripalmitin with capric acid in organic solvent medium: Analysis of the effect of experimental conditions through factorial design and analysis of multiple responses. *Enzyme Microb. Technol.* **2010**, *46* (6), 419−429.

(13) Nunes, P. A.; Pires-Cabral, P.; Guillén, M.; Valero, F.; Luna, D.; Ferreira-Dias, S. Production of MLM-type structured lipids catalyzed by immobilized heterologous *Rhizopus oryzae* lipase. *J. Am. Oil Chem. Soc.* **2011**, *88* (4), 473−480.

(14) Kawashima, A.; Shimada, Y.; Yamamoto, M.; Sugihara, A.; Nagao, T.; Komemushi, S.; Tominaga, Y. Enzymatic synthesis of high-purity structured lipids with caprylic acid at 1, 3-positions and polyunsaturated fatty acid at 2-position. *J. Am. Oil Chem. Soc.* **2001**, *78* (6), 611−616.

(15) del Mar Muñío, M.; Robles, A.; Esteban, L.; González, P. A.; Molina, E. Synthesis of structured lipids by two enzymatic steps: ethanolysis of fish oils and esterification of 2-monoacylglycerols. *Process Biochem.* **2009**, *44* (7), 723−730.

(16) Lee, K. T.; Foglia, T. A.; Oh, M. J. Medium−long−medium and medium−long−long chain acyl glycerols from beef tallow and caprylic acid. *J. Food Sci.* **2002**, *67* (3), 1016−1020.

(17) Zhang, H.; Önal, G.; Wijesundera, C.; Xu, X. Practical synthesis of 1,3-oleoyl 2-docosahexaenoylglycerol by lipase-catalyzed reactions: An evaluation of different reaction routes. *Process Biochem.* **2009**, *44*, 534−539.

(18) Wongsakul, S.; H-Kittikun, A.; Bornscheuer, U. T. Lipase-catalyzed synthesis of structured triacylglycerides from 1, 3-diacylglycerides. *J. Am. Oil Chem. Soc.* **2004**, *81*, 151−155.

(19) Blasi, F.; Maurelli, S.; Cossignani, L.; D'Arco, G.; Simonetti, M. S.; Damiani, P. Study of some experimental parameters in the synthesis of triacylglycerols with CLA isomers and structural analysis. *J. Am. Oil Chem. Soc.* **2009**, *86*, 531−537.

(20) D'Alonzo, R. P.; Kozarek, W. J.; Wade, R. L. Glyceride Composition of Processed Fats and Oils As Determined by Glass Capillary Gas Chromatography. *J. Am. Oil Chem. Soc.* **1982**, *59*, 292−295.

(21) Abdel-Nabey, A. A.; Shehata, A. A. Y.; Ragab, M. H.; Rossell, J. B. Glycerides of cottonseed oils from Egyptian and other varieties. *Riv. Ital. Sostanze Grasse.* **1992**, *69*, 443−447.

DOI: 10.1021/acs.jafc.7b01327
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

(22) Nagao, T.; Watanabe, H.; Goto, N.; Onizawa, K.; Taguchi, H.; Matsuo, N. Dietary Diacylglycerol Suppresses Accumulation of Body Fat Compared to Triacylglycerol in Men in a Double-Blind Controlled Trial. *J. Nutr.* **2000**, *130*, 792−797.
(23) Hara, K.; Onizawa, K.; Honda, H.; Ide, T.; Otsuji, K.; Murata, M. Dietary Diacylglycerol-Dependent Reduction in Serum Triacylglycerol Concentration in Rats. *Ann. Nutr. Metab.* **1993**, *37*, 185−191.
(24) Watanabe, H.; Onizawa, K.; Taguchi, H.; Kobori, M.; Chiba, H.; Naito, S.; Matsuo, N.; Yasukawa, T.; Hattori, M.; Shimasaki, H. Nutritional Characterization of Diacylglycerols in Rats. *Nihon Yukagakkaishi* **1997**, *46*, 301−307.
(25) Murata, M.; Hara, K.; Ide, T. Alteration by Diacylglycerols of the Transport and Fatty Acid Composition of Lymph Chylomicrons in Rats. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 1416−1419.
(26) Nicolás, P.; Lassalle, V. L.; Ferreira, M. L. Quantification of immobilized Candida antarctica lipase B (CALB) using ICP-AES combined with Bradford method. *Enzyme Microb. Technol.* **2017**, *97*, 97−103.
(27) Bornscheuer, U.; Reif, O. W.; Lausch, R.; Freitag, R.; Scheper, T.; Kolisis, F. N.; Menge, U. Lipase of Pseudomonas cepacia for biotechnological purposes: purification, crystallization and characterization. *Biochim. Biophys. Acta, Gen. Subj.* **1994**, *1201*, 55−60.
(28) Schrag, J. D.; Li, Y.; Cygler, M.; Lang, D.; Burgdorf, T.; Hecht, H. J.; Schmid, R.; Schomburg, D.; Rydel, T.; Oliver, J. D.; et al. The open conformation of a Pseudomonas lipase. *Structure* **1997**, *5*, 187−202.
(29) Castillo, E.; Dossat, V.; Marty, A.; Condoret, J. S.; Combes, D. The role of silica gel in lipase-catalyzed esterification reactions of high-polar substrates. *J. Am. Oil Chem. Soc.* **1997**, *74* (2), 77−85.
(30) Stevenson, D. E.; Stanley, R. A.; Furneaux, R. H. Near-quantitative production of fatty acid alkyl esters by lipase-catalyzed alcoholysis of fats and oils with adsorption of glycerol by silica gel. *Enzyme Microb. Technol.* **1994**, *16* (6), 478−484.
(31) Sánchez, D. A.; Tonetto, G. M.; Ferreira, M. L. Enzymatic synthesis of 1, 3-dicaproyglycerol by esterification of glycerol with capric acid in an organic solvent system. *J. Mol. Catal. B: Enzym.* **2014**, *100*, 7−18.
(32) Sánchez, D. A.; Tonetto, G. M.; Ferreira, M. L. Separation of acylglycerides obtained by enzymatic esterification using solvent extraction. *J. Am. Oil Chem. Soc.* **2014**, *91*, 261−270.
(33) Schmid, U.; Bornscheuer, U. T.; Soumanou, M. M.; McNeill, G. P.; Schmid, R. D. Highly selective synthesis of 1,3-oleoyl-2-palmitoylglycerol by lipase catalysis. *Biotechnol. Bioeng.* **1999**, *64*, 678−684.
(34) Miura, S.; Ogawa, A.; Konishi, H. A rapid method for enzymatic synthesis and purification of the structured triacylglycerol, 1,3-dilauroyl-2-oleoyl-glycerol. *J. Am. Oil Chem. Soc.* **1999**, *76*, 927−931.
(35) Christie, W. W. Structural analysis of lipids by means of enzymatic hydrolysis. *Lipid Anal.* **1982**, 155−166.
(36) Namal Senanayake, S.; Shahidi, F. Enzyme-catalyzed synthesis of structured lipids via acidolysis of seal (*Phoca groenlandica*) blubber oil with capric acid. *Food Res. Int.* **2002**, *35*, 745−752.
(37) Gunstone, F. D.; Harwoodand, J. L.; Padley, F. B. *The Lipid Handbook*; Chapman & Hall: London, 1994; p 231.
(38) Guauque Torres, M. D. P.; Foresti, M. L.; Ferreira, M. L. Cross-linked enzyme aggregates (CLEAs) of selected lipases: a procedure for the proper calculation of their recovered activity. *AMB Express* **2013**, *3* (1), 25.
(39) Innis, S. M.; Dyer, R.; Nelson, C. M. Evidence that palmitic acid is absorbed as *sn*-2 monoacylglycerol from human milk by breast-fed infants. *Lipids* **1994**, *29*, 541−545.
(40) Kallio, H.; Rua, P. Distribution of the major fatty acids of human milk between *sn*-2 and *sn*-1, 3 positions of triacylglycerols. *J. Am. Oil Chem. Soc.* **1994**, *71*, 985−992.

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX