SYNTHESIS OF ACYLGLYCERIDES WITH HIGH NUTRITIONAL VALUE BY AN ENZYMATIC TWO-STEP PROCESS

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***Abstract*— Acylglycerides are esters formed from glycerol and fatty acids. The structure of these glycerides affects their digestion and absorption. In this work, the synthesis of acylglycerides with high nutritional value by an enzymatic process in two stages is presented. In the first stage, the esterification of glycerol and capric acid catalyzed by Lipozyme RM IM was carried out. In this reaction, 73% conversion of capric acid and 71% selectivity to 1,3-dicaprin was achieved. The diglyceride was separated and purified efficiently by a simple liquid-liquid extraction procedure. The dicaprin was esterified with palmitic acid using *Burkholderia cepacia* lipase immobilized on chitosan as the catalyst. In this second stage conversions of 1,3-dicaprin with values between 48 and 87% and with high selectivity towards the esterification of the sn-2 position were achieved. The reaction product had between 76 and 90% of acylglycerides with high nutritional value, depending on the reaction conditions.**

***Keywords*— nutritional acylglycerides, enzymatic synthesis, lipases.**

ABBREVIATIONS

BCL = *Burkholderia cepacia* lipase
C = Capric acid
CCC = Tricaprin
CCG = 1,2-dicaprin
CCP = 1,2-dicaproyl-3-palmitoyl glycerol
CGC = 1,3-dicaprin
CGP = 1-caproyl-3-palmitoyl glycerol
CPC = 1,3-dicaproyl-2-palmitoyl glycerol
CPG = 1-caproyl-2-palmitoyl glycerol
DAG = Diacylglycerol or Diglyceride
GC = Gas chromatography
GCC = 2,3-dicaprin
GGG = Glycerol
LCFA = Long-chain fatty acid
MAG = Monoacylglyceride or Monoglyceride
MCFA = Medium-chain fatty acid
MCT = Medium-chain triacylglycerol
MLCD = Medium- and long-chain diacylglycerol
MLCT = Medium- and long-chain triacylglycerol
MLM = Triacylglycerol with medium-chain fatty acids at the sn-1 and sn-3 positions and long-chain fatty acid at the sn-2 position
PGG = 1-monopalmitin
PGP = 1,3-dipalmitin
PPG = 1,2-dipalmitin
PPL = Porcine pancreas lipase
TAG = Triacylglycerol or Triglyceride

I. INTRODUCTION

Triacylglycerols (TAG) are the main source of energy within food lipids. Medium-chain TAGs (MCT or MMM, where M is a fatty acid with a chain length of C6 to C12) have been employed as a rapid energy source due to their quick absorption, especially in infants with malabsorption and in child care (Mascioli *et al.*, 1988). It is also used as an alternative solution for consumers with digestive problems (Lai *et al.*, 2005). MCTs have no toxicological properties when their consumption is less than 30g per day (Marten *et al.*, 2006). However, MCT are not suitable for cooking due to its low smoke point and foam generation (Matsuo *et al.*, 2001). To solve this problem, long-chain fatty acids (LCFA) were incorporated into MCT. The triglycerides containing in the same molecule medium chain fatty acids and long chain fatty acids have been defined as medium- and long-chain triglycerides (MLCT). These triglycerides are similar biochemically and physiologically to MCT (Kasai *et al.*, 2003), with the advantage that they can be used as cooking oils for therapeutic purposes (Ogawa *et al.*, 2007; Shinohara *et al.*, 2005).

A particular case of MLCT are MLM-type triglycerides. They contain MCFA at the sn-1 and sn-3 positions and LCFA at the sn-2 position. These triglycerides showed metabolic benefits compared to natural triglycerides or those produced with random structure (Christensen *et al.*, 1995a, b). The MLM-type triglycerides have been synthesized using different methods (Bornscheuer *et al.*, 2002; Xu, 2000, Akoh *et al.*, 1995; Fomuso and Akoh, 1998; Shimada *et al.*, 1996; Soumanou *et al.*, 1997). However, the yields achieved were low and a large number of unwanted products were obtained with these synthesis methodologies. Obtaining MLM-type triglycerides from the esterification of the sn-2 position of 1,3-diglycerides (1,3-DAG) is an alternative but poorly studied pathway (Blasi *et al.*, 2009; Wongsakul *et al.*, 2004; Zhang *et al.*, 2009).

On the other hand, diglycerides (DAG) are found in low proportions in oils and fats (levels below 5%) (D'Alonzo *et al.*, 1982). Several studies have reported nutritional benefits associated with the consumption of DAG, particularly 1,3-DAG. The incorporation in the diet of these diglycerides have allowed to reduce the concentration of triglycerides in serum and thereby re-

duce visceral fat and body weight (Nagao *et al.*, 2000; Watanabe *et al.*, 1997; Murata *et al.*, 1994).

In this work, the enzymatic synthesis of acylglycerides with high nutritional value was carried out through an enzymatic process in two stages.

II. METHODS

A. 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). *Burkholderia cepacia* lipase was a generous donation from Amano Enzyme Inc. (Nagoya, Japan). Porcine pancreas lipase was provided by Sigma-Aldrich.

Glycerol, n-heptane and silica gel were supplied by Cicarelli Laboratorios. Capric acid, 1,2,4-butanetriol, tripalmitin and silylation reagents were obtained from Fluka. Monocaprin, dipalmitin, tricaprylin, tricaprin, trilaurin and trimyristin were supplied by Sigma-Aldrich. Absolute ethanol was supplied by Dorwil. Phenolphthalein, potassium hydroxide and pyridine were purchased from Anedra S.A., and chitosan was provided by Primex S.A. (Iceland). All the products were of analytical grade.

B. Synthesis of 1,3-Dicaprin

1,3-dicaprin (CGC) was obtained by esterification of glycerol (GGG) with capric acid (C) catalyzed by Lipozyme RM IM under previously optimized conditions (Sánchez *et al.*, 2014a). The reaction was carried out in 10 mL vials kept in a bath with temperature control and magnetic stirring. For the reaction, 110 mg of capric acid were dissolved in 3 mL of n-heptane, then 250 mg of glycerol adsorbed on 500 mg of silica gel was added. The temperature was 60 °C and the reaction started with the addition of 10 mg of Lipozyme RM IM. After 3 hours of reaction, another 10 mg of the biocatalyst was added. The total reaction time was 6 hours. A 50 µL sample was taken for analysis by gas chromatography (GC) and the remaining content was used to evaluate the acid conversion by acid-base titration with an ethanolic solution following a previously published methodology (Sánchez *et al.*, 2014a).

C. Separation and Purification of 1,3-Dicaprin

After the esterification reaction was complete, the solids were separated by filtration. Monocaprin, dicaprin, tricaprin and unreacted capric acid were dissolved in n-heptane. The capric acid not consumed in the reaction was neutralized and removed with 3 mL of an aqueous 0.1M KOH solution. The mixture was stirred vigorously and then centrifuged at 8000 rpm for 15 min. The heptane phase was recovered and the procedure repeated. The final composition of the non-polar phase was determined by GC.

An equal volume of an ethanol/water mixture (90/10 v/v) was added to the n-heptane solution containing the acylglycerides. The sample was subjected to intense agitation and then centrifuged for 20 min at 8000 rpm. The phases were separated and the heptane phase was analyzed by GC. The extraction procedure was repeated three times (Fig. 2), finally the hydroethanolic solutions were combined and the solvent evaporated to recover the diglyceride.

The selection of solvents for liquid-liquid extraction was carried out using software for the molecular design of solvents. The results of the evaluation of solvents were previously published (Sanchez *et al.*, 2014b) and the separation methodology could be used to separate other di- and triglycerides different from dicaprin and tricaprin.

D. 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 were 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 and then 24 h at 45 °C to remove the water adsorbed on the chitosan.

The obtained biocatalyst was placed in a beaker with 50 mL of distilled water and magnetically stirred at 400 rpm for 1 min. This washing was carried out twice to remove the weakly adsorbed lipase. Subsequently the solid was dried as indicated above. The characterization of the biocatalyst obtained has been previously published (Sánchez *et al.*, 2017).

E. Esterification of Dicaprin with Palmitic Acid

Dicaprin synthesized and purified by the procedures described was composed of 80% 1,3-dicaprin and 20% 1,2-dicaprin (80:20) (Sánchez *et al.*, 2014a, b). The esterification reaction was performed in 10 mL vials, which were kept in a thermostatic bath with temperature control and magnetic stirring. The reaction time was 6 h, and it was carried out as follows: 32 mg of dicaprin (80:20) were dissolved in 2 mL of n-heptane, then the given amount of palmitic acid for each reaction under study was added. When the reactant mixture reached the selected temperature, the reaction was started by adding the biocatalyst. The choice of solvent was performed according to Bi *et al.* (2015). Solvents with a log P value higher than 4.0 increased the degree of esterification at the sn-2 position. The values of temperature (T), palmitic acid/dicaprin molar ratio (R_M), and biocatalyst loading (B) were established according to a 2^3 factorial design with two central points and a total of 10 experiments.

F. Analysis of Samples by Gas Chromatography

Samples were diluted with pyridine and siliylated with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA). The analysis of the samples was performed in a Perkin Elmer 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 high purity standards. 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).

G. 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 (Miura *et al*., 1999; Schmid *et al*., 1999; Senanayake *et al.*, 2002) combined with gas chromatography.

Free fatty acids were previously neutralized with 2 mL of an aqueous solution of KOH 0.1M, and the phase containing acylglycerides was recovered (procedure described in Sánchez et al., 2014b. 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) were 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 generates 2-monoglycerides (2-MAG) and free fatty acids. The short reaction time allows avoiding the acyl migration reaction and correctly identifying 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 possible to know the composition of the starting sample.

III. RESULTS AND DISCUSSION

A. Synthesis of 1,3-Dicaprin

Under the evaluated reaction conditions, the conversion of capric acid was 73%. The final product was composed of 18% monocaprin, 72% dicaprin and 9% tricaprin (in molar percentage). Of the total dicaprin generated, 93% was 1,3 dicaprin (CGC), 5% was 1,2-dicaprin (CCG) and 2% was 2,3-dicaprin (GCC). The selectivity to 1,3-dicaprin was 71%. A typical chromatogram of the reaction products and unreacted substrates is shown in Fig. 1.

B. Separation and Purification of Dicaprin

The product of the esterification reaction was composed of monocaprin, dicaprin, tricaprin and the unconsumed fatty acid. All the compounds were dissolved in the reaction solvent (n-heptane)**.**

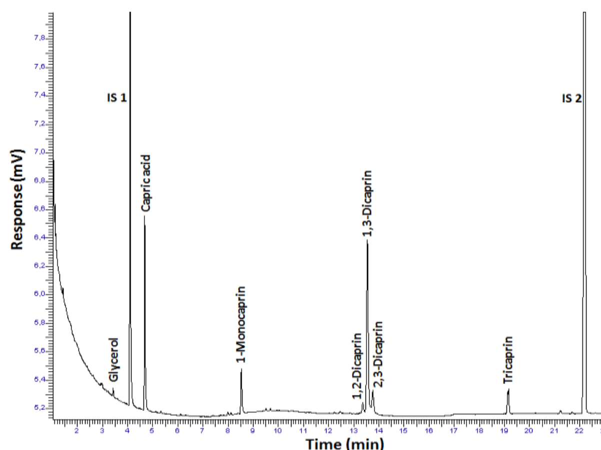

Fig. 1. Typical chromatogram of the reaction products and unconsumed reactants in the enzymatic esterification of glycerol and capric acid using Lipozyme RM IM. IS1 and IS2: Internal calibration standards.

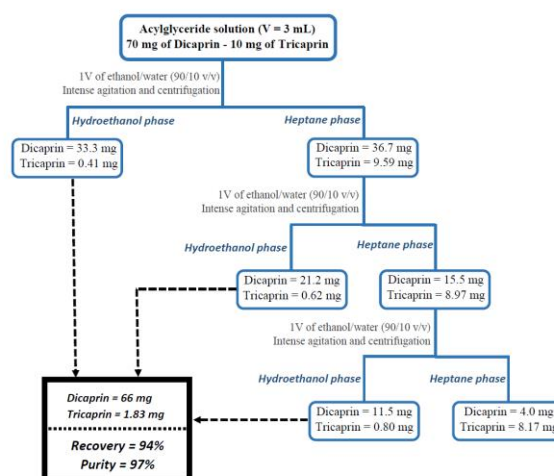

Fig. 2. Results of the liquid-liquid extraction process.

The unreacted fatty acid was neutralized with an aqueous 0.1M KOH solution. The neutralization and removal of the acid were performed by two washes with the aqueous solution. With this simple procedure, it was possible to remove 99.5% of the unreacted fatty acid. In addition, the aqueous solution was able to extract 95% of the monocaprin generated in the reaction. During the neutralization process, losses of dicaprin and tricaprin of 4.5 and 4% respectively occurred.

The liquid-liquid extraction process in three stages, shown in Fig. 2, allowed recovering 94% of the initial dicaprin with a purity of 97%.

C. Synthesis of Acylglycerides with High Nutritional Value

Using a factorial design, the reaction conditions for the esterification of dicaprin (80:20) with palmitic acid were evaluated. The reaction was catalyzed by *Burkholderia cepacia* lipase immobilized by physical adsorption on chitosan.

Esterification of 1,3-dicaprin generated 1,3-dicaproyl-2-palmitoyl glycerol (CPC), a MLM-type triglyceride with high nutritional value. Furthermore, the

esterification of 1,2-dicaprin allowed the generation of 1,2-dicaproyl-3-palmitoyl glycerol (CCP), a TCML that is also valuable from a nutritional point of view.

In addition to the esterification reaction, the hydrolysis reaction was also important in the system under study. This secondary reaction generated free capric acid and even glycerol. In addition, the products of the hydrolysis were re-esterified generating different acylglycerides, such as: 1-monopalmitin (PGG), 1,2-dipalmitin (PPG), 1,3-dipalmitin (PGP), 1-caproyl-2-palmitoyl glycerol (CPG), 1-caproyl-3-palmitoyl glycerol (CGP), tricaprin (CCC) and 1-caproyl-2,3-palmitoyl glycerol (CPP). Several of them are nutritionally valuable: CCC is a rapidly metabolizable TCM, CGP is a diglyceride of type 1,3-DAG, CPG is a diglyceride with metabolic characteristics similar to a MLM-type triglyceride but with reduced calories.

A typical chromatogram of the products of the esterification reaction and unreacted substrates is shown in Fig. 3.

Free fatty acids and glycerol were removed by performing two washes with a 0.1M aqueous solution of KOH.

Table 1 shows the factors and levels evaluated in the factorial design. The conversion of CGC and the mole fraction of valuable acylglycerides in the reaction product are presented as the response variables. CPC, CCP, CCC, CPG, and CGP were considered as the valuable acylglycerides. In addition, unreacted 1,3-dicaprin is also interesting from a nutritional point of view.

Second order models were used to adjust the data. These models were refined by applying the F-value test (F-test), also called the Fisher-Snedecor test (Besset, 2001). Equation 1 represents the quadratic model with all its variables and combinations of them.

$$X_{Ac} = A_0 + A_1B + A_2T + A_3R_M + A_4BT + A_5BR_M + A_6TR_M + A_7B^2 + A_8T^2 + A_9{R_M}^2 \quad (1)$$

where R_M is the palmitic acid/dicaprin molar ratio, T is the reaction temperature, B is the biocatalyst mass, and A_i are the regression coefficients of the model.

In Table 1a, only the reaction products were considered. The final mixture was composed of between 48 and 68% of nutritionally interesting acylglycerides. The generation of nutritionally valuable acylglycerides was favored by the increase of the biocatalyst mass and the temperature. The concentration of palmitic acid did not have a statistically significant effect on this response. The relationship between the molar fraction of these acylglycerides (excluding 1,3-dicaprin) and the studied factors was appropriately adjusted by Eq. 2 with R^2 = 97.4%.

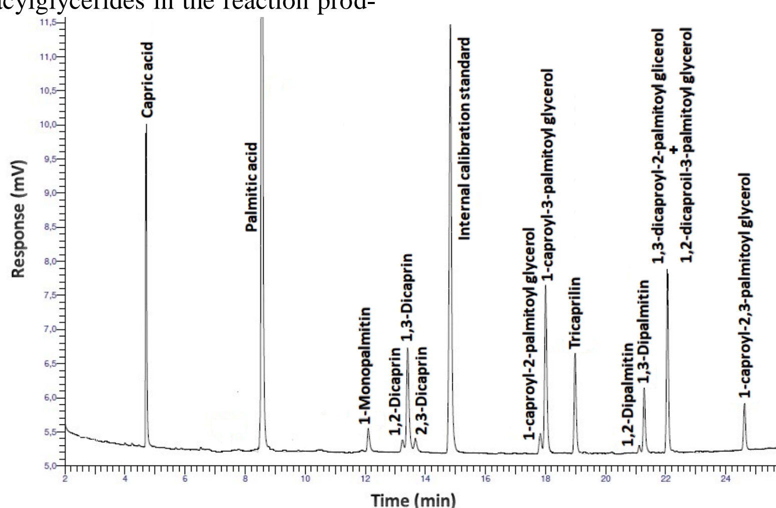

Fig. 3. Typical chromatogram of the reaction products and unconsumed reactants for the enzymatic esterification of dicaprin and palmitic acid. Experimental conditions: dicaprin = 32 mg, R_M = 3 mol/mol, temperature = 40 °C, and biocatalyst load = 150 mg. Reaction time: 6 h.

Table 1. Experimental factors and parameters evaluated during the enzymatic esterification of dicaprin with palmitic acid catalyzed by *Burkholderia cepacia* lipase immobilized on chitosan at 6 h of reaction.

Experimental factors			1,3-dicaprin conversión (%)	Valuable acylglycerides (molar %)[a]	Valuable acylglycerides (molar %)[b]
B (mg)	T (°C)	R_M (mol/mol)			
150	60	1	90	68.1	80.9
50	60	3	68	54.6	88.4
50	60	1	60	52.4	89.2
150	40	1	81	62.1	81.6
50	40	3	50	47.8	90.0
150	60	3	89	65.9	76.2
100	50	2	81	60.5	81.0
150	40	3	84	64.5	79.5
100	50	2	83	62.4	81.2
50	40	1	54	49.7	90.3

[a] Acylglycerides with high nutritional value without considering the presence of 1,3-dicaprin in the final product.

[b] Acylglycerides with high nutritional value taking into account unreacted 1,3-dicaprin in the final product.

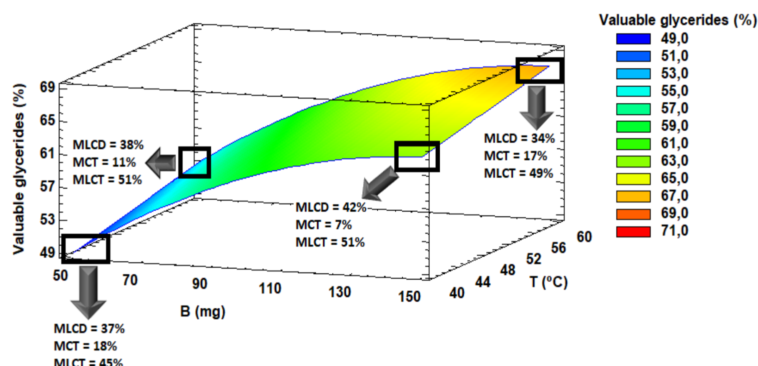

Fig. 4. Molar fraction of nutritionally valuable acylglycerides obtained from the esterification of dicaprin with palmitic acid catalyzed by the *Burkholderia cepacia* lipase immobilized on chitosan. Variables: mass of biocatalyst and reaction temperature. MLCD = medium- and long-chain diacylglycerides, MCT = medium-chain triacylglycerides, and MLCT = medium- and long-chain triacylglycerides.

$$\text{Valuable glycerides} = 23.61 + 0.405B + 0.211T - 0.00133B^2 \quad (2)$$

A surface plot for this response is presented in Fig. 4, where the variation in the composition of the glycerides with nutritional value is shown as a function of the variation in the reaction conditions. The molar percentage of medium- and long-chain diglycerides (MLCD), MCT and MLCT are shown on the vertices of the graph. It is possible to observe the change in the composition of the final product as a function of the reaction conditions. In addition, it is evident that the formation of MLCD and MLCT was favored when the reaction was carried out with the highest biocatalyst load and the lowest reaction temperature (within the range of study).

1,3-Dicaprin is also a valuable compound from a nutritional point of view. The total conversion of this diglyceride was between 50 and 90%. In this way, the presence of unreacted 1,3-dicaprin enriched the final product. Table 1b shows the percentage of acylglycerides in the final product considering unreacted 1,3-dicaprin as a nutritionally valuable acylglyceride. In this case, the final blend consisted of 76-90% acylglycerides with high nutritional value.

Equation 3 relates the fraction of nutritionally valuable glycerides to the studied factors. This equation was obtained from multiple linear regression, and only the statistically significant effects were considered. The coefficient of determination (R^2) for this model was 99.7%.

$$\text{Valuable glycerides} = 107 - 0.372B + 0.00137B^2 - 0.0233TR_M \quad (3)$$

The synthesis of acylglycerol mixtures with different compositions (of high nutritional value) could be performed according to the requirements or a desired composition by modifying the reaction conditions.

III. CONCLUSIONS

The synthesis of acylglycerides with high nutritional value was efficiently carried out by an enzymatic process in two stages, including synthesis reactions and purification processes.

The final product was a mixture of acylglycerides formed mainly by glycerol esterified with capric acid at the sn-1 and sn-3 positions and palmitic acid at the sn-2 position.

The variation in reaction conditions in the second stage allowed obtaining products with different acylglyceride composition. This process could be implemented for the production of nutritional glycerides with composition as required.

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