CHEMICAL ENGINEERING TRANSACTIONS
VOL. 64, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Antonio Marzocchella, Tajalli Keshavarz

ISBN 978-88-95608- 56-3; **ISSN** 2283-9216

Production Of Dietetic Triacylglycerols From Olive Oil Catalyzed By Immobilized Heterologous Rhizopus Oryzae Lipase

Acenini L. Balieiro[a], Natália M. Osório[b], Álvaro S. Lima[a], Cleide M. F. Soares[a], Francisco Valero[c], Suzana Ferreira-Dias[d*]

[a]Universidade Tiradentes, ITP, Av. Murilo Dantas, 300, Prédio do ITP, Farolândia, Aracaju-SE, Brazil;
[b]Instituto Politécnico de Setúbal, Escola Superior de Tecnologia do Barreiro, 2839-001 Lavradio, Portugal;
[c]Departament d'Enginyeria Quimica (EE), Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain;
[d]Universidade de Lisboa, Instituto Superior de Agronomia, LEAF, Tapada da Ajuda, 1349-017 Lisbon, Portugal.
*suzanafdias@mail.telepac.pt

In this study, low calorie triacylglycerols (TAG) of MLM type (containing a medium-chain fatty acid, M, at positions *sn*-1,3 and a long-chain fatty acid at position *sn*-2) were produced by acidolysis of virgin olive oil with caprylic (C8:0) or capric (C10:0) acids, in solvent-free media. The heterologous *sn*-1,3 regioselective *Rhizopus oryzae* lipase produced by the methylotrophic yeast *Pichia pastoris* (r-ROL) was immobilized in Amberlite IRA 96® (rROL-IRA) and used as biocatalyst. Acidolysis was optimized as a function of temperature and molar ratio (C:8/TAG or C10:0/TAG), by response surface methodology. The production of new TAG, the consumption of C8:0 or C10:0, and triolein were evaluated. From the response-surfaces fitted to the experimental data points, no optimal points were observed inside the experimental region. The highest consumption of TAGs, free fatty acids and triolein (the major TAG) were achieved at 29 °C and molar ratio (MR) of 2:1 at 24 h reaction time. For the system with virgin olive oil and caprylic acid (C8:0), the highest consumption of TAG was 76.9% while for the system with virgin olive oil and C10:0, 85.6 % of TAG consumption was observed. Also for the system with virgin olive oil and capric acid, 70.7 and 68.8% consumption of initial TAGs and triolein, respectively, were observed for MR of 1.6:1 and at 40 °C, corresponding to a star point of the experimental design. The production of structured TAG from olive oil and medium chain fatty acids (caprylic and capric acids) catalyzed by the heterologous *Rhizopus oryzae* lipase immobilized in Amberlite IRA 96 is a promising alternative to the high-cost immobilized commercial lipases for MLM production.

1. Introduction

The structured lipids (SL) of MLM-type are defined as triacylglycerols (TAG) containing medium chain (6–12 carbon atoms) and saturated fatty acids (M) in the *sn*-1 and *sn*-3 positions and with long-chain (14–24 carbon atoms) saturated or unsaturated fatty acids (L) in the *sn*-2 position. The interest on these SL has sharply increased due to their unique nutritional properties (Arifin *et al*., 2010). For clinical nutritional purposes, MLM structured lipids are of interest since they are hypocaloric (5-7 kcal/g). Furthermore, mono or polyunsaturated long-chain fatty acids are more efficiently absorbed when located at the *sn*-2 bond (Morales-Medina *et al*., 2017). Triacylglycerols of MLM type can be produced by lipase-catalyzed acidolysis between TAGs (oils or fats) and free fatty acids (FFA), either in solvent or in solvent-free media (Esteban *et al*., 2011, Nunes *et al*., 2011a, Nunes *et al*., 2011b, Nunes *et al*., 2012, Terada *et al*., 2015, Sanchez *et al*., 2017). The main problem of this method is the price of commercial enzymes. However, the use of immobilized and lower-cost non-commercial lipases has made this method potentially viable (Palla et al., 2012, Caballero et al., 2014, Kim and Akoh, 2015). The immobilization process of the enzyme may increase its operational stability and enables enzyme reutilization in repeated batches and the implementation of continuous processes, improving the cost efficiency. In most of the studies on oils and fats modification, commercial immobilized lipase preparations were used. Lipase-catalyzed acidolysis between vegetable or fish oils (used as the source of glycerol backbone and long chain fatty acids) and medium chain fatty acids (caprylic, C8:0, or capric acids, C10:0) as acyl donors, is one of the most commonly used methods to produced MLM. The *sn*-1,3 regioselective lipases, mostly from microbial sources, have been used as catalysts for the synthesis of these structured lipids. Therefore, the search for non-commercial lipases capable to catalyze reactions to produce SL with specific functional properties, greatly increased during the last decades (Nunes *et al*., 2011b, Nunes *et al*., 2012, Faustino *et al.* 2016, Costa *et al*.,

2017). The search for novel biocatalysts cheaper than those commercially available has been a challenge for the production of structured lipids. Thus, the heterologous *Rhizopus oryzae* lipase (rROL) has been produced by our group by over-expression of the corresponding gene in a mutant strain of *Pichia pastoris* (Guillén *et al*., 2011). rROL immobilized in Eupergit® C was tested as catalyst for the production of MLM by acidolysis of virgin olive oil with caprylic (C8:0) or capric (C10:0) acids, in solvent-free medium (Nunes *et al*. 2011) and the reaction optimized by response surface methodology (Nunes et al., 2012). At 40 ºC, 21.6 mol% of C8:0 and 34.8 mol% of C10:0 incorporation in TAG of virgin olive oil were attained, after 24 h reaction time. This enzyme was also immobilized in Amberlite® IRA96 resin and used as catalyst for the acidolysis of grapeseed oil with caprylic or capric acids in solvent-free media (Costa *et al*., 2017). After 24 h acidolysis, 68.5% and 52.4 % yield of new TAGs containing C8:0 or C10:0 were obtained. These results obtained with rROL are rather promising since they are similar to those attained with high-cost commercial immobilized lipase preparations (Nunes *et al*., 2011a).

The aim of the present study is to optimize the acidolysis of virgin olive oil with C8:0 or C10:0, in solvent-free media, as a function of temperature and molar ratio caprylic acid/olive oil or capric acid/olive oil, by response surface methodology (RSM), using rROL immobilized in Amberlite IRA96 as catalyst. The choice of a solvent-free system will maximize volume productivity, simplify the downstream processing and is a clean and environmentally friendly process, adequate for the food industry.

2. Materials

Extra virgin olive oil (acidity of 0.7% expressed as free oleic acid) was purchased from a Portuguese local supermarket. Caprylic acid (C8:0, octanoic acid) and capric acid (C10:0, n-decanoic acid) were purchased from TCI Europe N.V., Belgium, Amberlit IRA96® resin was obtained from Rohm and Haas, Lenntech, Philadelphia, U.S.A. The standard of monononadecanoin (minimum 99% pure) was obtained from Larodan Fine Chemicals AB, Sweden; N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (minimum 90% pure) was purchased from TCI Europe N.V., Belgium; glutaraldehyde (25 % aqueous solution) was purchased from Merck, Germany. All solvents and reagents for analyses were chromatographic or analytical grade and obtained from different sources. *Rhizopus oryzae (rROL) was produced by the Bioprocess Engineering and Applied Biocatalysis group of the Universitat Autònoma de Barcelona (UAB). The rROL was obtained by a fed-batch cultivation of a recombinant Pichia pastoris strain using methanol as inductor (Barrigón et al., 2013).*

The biomass was separated from the culture broth by centrifugation and microfiltration. The supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system equipped with an Omega membrane of 10 kDa cut-off, and subsequently dialyzed against 10mM Tris–HCl buffer pH 7.5 and finally lyophilized (Palla et al., 2012).

2.1. Methods

2.1.1. Preparation of immobilized rROL

The immobilization procedure folowed for rROL in Amberlit IRA96 was adapted from the methodology described by Wang et al., (2010), according to Costa et al. (2017). Anion resins were treated with 50 ºC deionized water for 30 min. Then, the resins were recovered by vacuum filtration and washed with 1mol/L NaOH and HCl alternately for three times and equilibrated with sodium phosphate buffer (0.2 mol/L, pH 7.5, 2 x 50 mL) in the end. The dry anionic resins (0.5 g) and lipase (5 mL) were mixed together at 28 ºC for 4 h. After the preliminar adsorption, the particles (0.5 g) above were mixed with fresh lipase (5 mL) in a solution of glutaraldehyde (0.5% v/v, 25 ml solution of glutaraldehyde/g resin), at 28 ºC and shaken at 20 min. The resin were, once again, recovered by vacuum filtration and washed twice with 50 mL of phosphate buffer solution (0.2 mol/L, pH 7.5) in order to remove the free enzyme. The immobilized lipase was dried under vacuum for approximately 24 h and stored at 4 ºC until use. After that, the beads were recovered by vacuum filtration and incubated with 25 mL of phosphate buffer solution (0.1 M, pH 7.0) containing 2.5% (v/v) glutaraldehyde aqueous solution for 2 h, under slow mixing. The beads were, once again, recovered by vacuum filtration and washed twice with 50 mL of phosphate buffer solution (0.1 M, pH 7.0) in order to remove the free enzyme. The immobilized lipase was dried under vacuum for approximately 24 min and stored at 4 ºC until use.

2.1.2. Acidolysis Reaction

The acidolysis reactions were carried out in solvent-free media, in thermostated-capped cylindrical glass vessels under magnetic stirring at 400 rpm. Reaction media consisted of 3.0 g of extra virgin olive oil and caprylic (C8:0) or capric acid (C10:0) in amounts dictated by the experimental design corresponding to different molar ratios (MR) of free fatty acid to olive oil. The temperature (T) varied according to the experimental design. In each experiment, 0.15 g of immobilized enzyme, corresponding to 5 % (w/w) of the TAGs (olive oil), were added to the reaction medium, after complete melting. After 48 h of reaction (the time needed to attain equilibrium), 0.5 mL of the reaction medium was withdrawn and the reaction medium was stored at -20 ºC until analysis. The acidolysis reaction was monitored by the consumption of TAG (consumed TAG/initial TAGs, w/w- %) from olive oil (Faustino *et al.* 2016, Costa *et al*., 2017).

2.1.3. Experimental Design and Statistical Analysis

The best reaction conditions for the acidolysis reaction were established via RSM. The statistical optimization experiments were carried out according to a Central Composite Rotatable Design (CCRD) with 11 experiments (3 center points, 4 factorial points and 4 star points), as a function of molar ratio (MR FA/olive oil;1.6:1-4.4:1) and reaction temperature (T; 29.4-55 ºC) (Table 1). For every experiment of the CCRD, the percentage (w/w) of consumed C8:0 or C10:0, TAG and triolein of olive oil were analyzed using the software ‘‘Statistica’’, version 6, from Statsoft, Tulsa, USA.

2.1.4. Quantification and analysis of reaction products

In this work, the production of dietary triglycerides of MLM type was evaluated through the consumption of medium-chain fatty acids (C8:0 or C10:0), of initial TAG and triolein.

The quantification of substrates and products was achieved by high temperature gas chromatography after derivatization of the samples with pyridine and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), according to the European Standard (EN 14105:2011), after modification (Faustino *et al.* 2016, Costa *et al*., 2017). A gas chromatograph (Agilent Tecnologies 7820A) equipped with a flame ionization detector (FID) and capillary column DB5-HT (15 m x 0.32 mm ID x 0.10 µm film), an auto sampler Agilent Tecnologies 7820 and an on-column mode injector. Injector and detector temperatures were set at 83 °C and 380 °C, respectively. Helium was used as carrier gas at a flow rate of 2.2 mL/min. Air and hydrogen were supplied to the detector at flow rates of 300 mL/min and 30 mL/min, respectively. The oven temperature program was as follows: 50 ºC for 1 min, a temperature increase to 180 ºC at 15 °C/min, followed by temperature increase at a rate of 7 °C /min to 230 °C, a final ramp at a rate of 10 °C /min to 370 °C and a final plateau at 370 °C for 12 min (total running time of 43 min). Calibration curves for C8:0 and C10:0 were used to quantify the mass percentage of consumed medium-chain fatty acids (% Consumed C8:0 (or C10:0)/Initial C8:0 (or C10:0)) and the calibration curve for triolein was used to quantify the consumed TAG (% Consumed TAG/Initial TAG).

3. Results and Discussion

3.1. Modeling MLM Production

The production of dietetic triacylglycerols of MLM type was evaluated through the consumption of capric or caprylic acid, consumption of initial TAG and triolein, after 24-h acidolysis in solvent-free media, catalyzed by rROL immobilized on Amberlite IRA96®, under the conditions dictated by the experimental design. The obtained results are presented in Table 1.

Table 1: *Experimental design matrix used (CCRD) as a function of molar ratio of medium-chain fatty acid/TAG (MR) and temperature (T) and the experimental results: percentage of consumed TAG, C8:0 (or C10:0) and triolein in the acidolysis of olive oil with C8:0 or C10:0 after 24 h reaction catalyzed by rROL immobilized in Amberlite IRA96®.*

Experiment	*Coded Matrix*		*Experimental Design* Decoded Matrix		*Experiments results*	
	(X_1)	(X_2)	Temperature (X_1, ºC)	Molar Ratio (X_2, MR)	*Consumed TAG/Initial TAG (%)*	
					C8:0	C10:0
1	-1	-1	29.4	2:1	76.9	85.6
2	-1	+1	29.4	4:1	8.6	11.5
3	+1	-1	50.6	2:1	21.4	29.4
4	+1	+1	50.6	4:1	25.8	5.2
5	$-\sqrt{2}$	0	25	3:1	52.7	48.8
6	$+\sqrt{2}$	0	55	3:1	27.9	33.9
7	0	$-\sqrt{2}$	40	1.6:1	6.9	70.7
8	0	$+\sqrt{2}$	40	4.4:1	29.8	4.1
9	0	0	40	3:1	46.2	34.1
10	0	0	40	3:1	54.9	39.0

For both systems, the highest percentages of consumption of TAG were achieved in experiment 1 at 29.4 °C and molar ratio of 2:1 after 24 h reaction. Under these conditions, 76.9 and 85.6 % of the initial TAG were consumed in the system with C8: 0 and in the system with C10:0, respectively. Also for the system with capric acid, a high consumption of initial TAG (70.7%) was observed in the experiment 7 (MR=1.6:1; T=40 °C; star point). However, under the same reaction conditions, much lower consumption values for TAG (6.9 %), was observed when caprylic acid was used. The preference towards capric acid was also observed with rROL

immobilized on Eupergit C or on modified sepiolite in the acidolysis of olive oil, in solvent-free system (Nunes *et al*., 2011). On the contrary, when rROL immobilized in Amberlite IRA96 was used as catalyst for the acidolysis of grapeseed oil in solvent-free medium, the highest yield of new TAG (68.5 %) was observed with caprylic acid (Costa *et al*., 2017). In fact, oleic acid is the major fatty acid of olive oil while linoleic acid is the major fatty acid in grapeseed oil. The oil composition and the immobilization carrier seem to affect rROL lipase selectivity.
The main effects of T and MR and of the interaction between MR and T on the acidolysis reaction were calculated (Table 2). A positive or a negative linear effect of a particular factor (MR or temperature), on the response means that an increase in the value of that factor results in an increase or reduction in the response, respectively. A negative (or positive) quadratic effect indicates that the response is described by a convex (or concave) response surface.

Table 2: *Linear and quadratic effects of factors and of interaction, and respective p-levels (values between brackets), of MR medium chain fatty acids/triacylglycerols and temperature (T) on the TAG consumption in the acidolysis of olive with with C8:0 or C10:0, catalyzed by rROL immobilized in Amberlite IRA96®.*

Factor	*TAG consumption*	
	System with C8:0	**System with C8:0**
MR (linear term)	-7.8 (0.520)	-18.6 (0.0066)
MR (quadratic term)	-27.0 (0.101)	21.5 (0.0075)
T (linear term)	-18.3 (0.167)	-19.0 (0.006063)
T (quadratic term)	-5.1 (0.718)	6.1 (0.269)
MR x T (interaction)	36.3 (0.0731)	28.5 (0.0047)

For the system with caprylic acid (C8:0), the interaction MR x T has a positive effect on the percentage of consumed TAG indicating that a simultaneous increase in these factors promotes TAG consumption. However, an increase in temperature alone promotes a decrease in TAG consumption, i.e. in acidolysis reaction. This can be ascribed to a thermal deactivation of rROL. The negative quadratic effect of MR indicates a convex response surface as a function of MR. No significant effects (p>> 0.05) of MR (linear term) and T (quadratic term) were observed.
With respect to the system with capric acid, an increase in either MR or temperature promotes a decrease in TAG consumption, while a positive effect of the interaction is observed. Again, high amounts of capric acid or higher temperature values may cause rROL deactivation. It would be expected that an increase in the molar ratio and therefore in available medium-chain fatty acids for the reaction would lead to an increased consumption of TAG. However, the experimentally observed trend was precisely the inverse. Thus, increasing the MR resulted in a decrease in consumption of TAG which may be related to an enzyme inhibition by the free fatty acids or a loss of lipase activity. High levels of free fatty acids produce high levels of carboxylic acids, which may acidify the aqueous phase in the microenvironment of the lipase, or cause water adsorption in the interphase where the lipase operates, limiting its activity.
Positive quadratic effects of both factors on acidolysis of olive oil with capric acid indicate that a convex surface as a function of MR or T is fitted to the experimental data-points.
These response surfaces, described by second order polynomial equations as a function of T and MR (Table 3) are shown in Figure 1.

Table 3: *Model equations for the response surfaces fitted to the consumption of TAG during the acidolysis of olive oil with caprylic (C8:0) or capric acid (C10:0) catalyzed by rROL immobilized in Amberlite IRA96 and respective R^2 and R^2_{adj}.*

System	**Model equations**	**R^2**	**R^2_{adj}**
Olive oil + C8:0	TAG consumed/TAG initial = -18.33 T - 5.16 T^2 - 7.85 MR - 27.0 MR^2 + 36,32 (MR x T)	0.71	0.42
Olive oil + C10:0	TAG consumed/TAG initial = -18.6 MR + 21.5 MR^2 – 19.0 T - + 6.1 T^2 28.5 (MR x T)	0.94	0.88

The coefficients of determination (R^2) and the adjusted coefficients of determination (R^2_{adj}) of these polynomials fitted to the experimental points for each system are also shown in Table 3. High values of both symbols R^2 and R^2_{adj} these models show a good fit for the consumption of TAG in the system with caprylic acid (R^2 = 0.71), as well as an excellent fit for consumption TAG (R^2 = 0.94) in the system with capric acid.

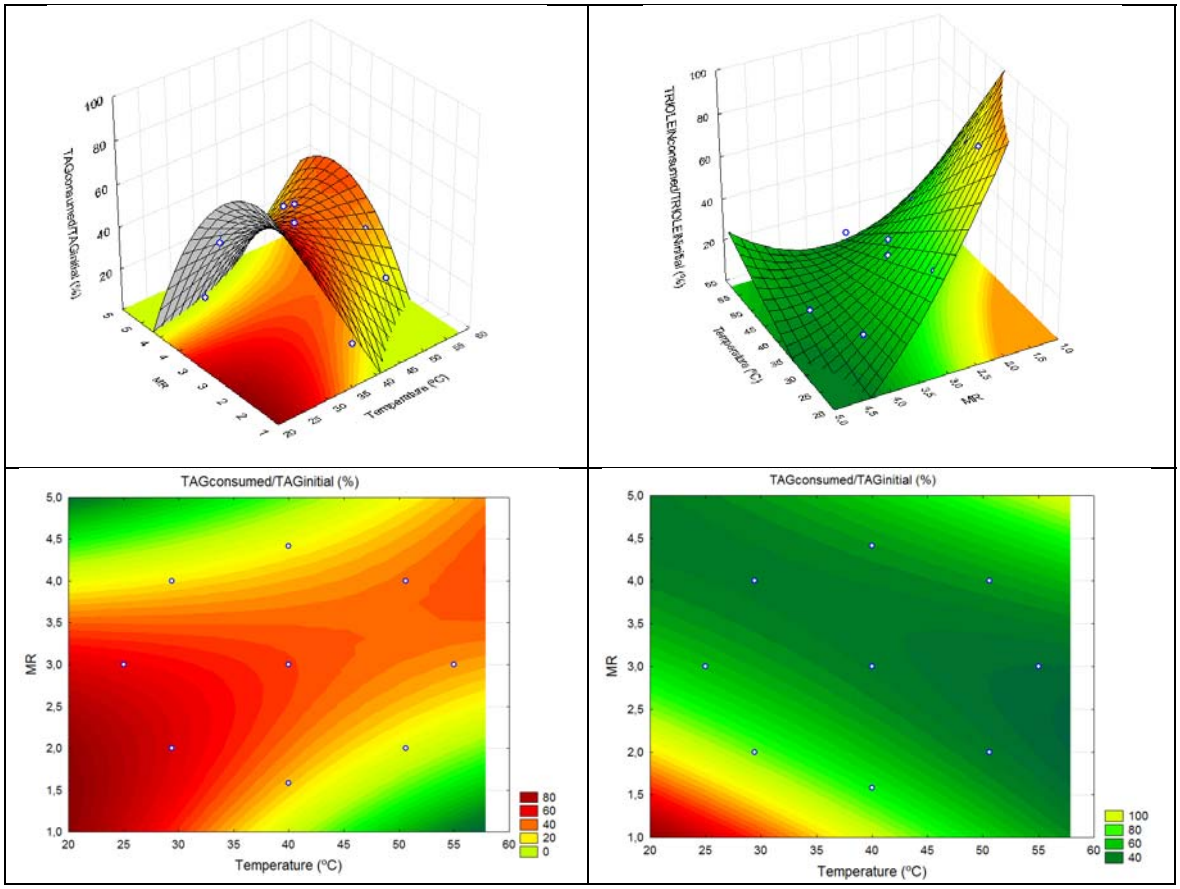

Figure 1: *Response surfaces fitted to the experimental data, and respective contour plots: a) Consumed TAG/Initial TAG (system with C8:0);b) Consumed TAG/Initial TAG (system with C10:0), as a function of the molar ratio and temperature.*

From these response-surfaces fitted to the experimental data points, no optimal points (maximum TAG consumption) were observed inside the considered experimental region. Thus, only the identification of the regions corresponding to higher TAG consumption could be achieved. For both systems, the best results are expected at low molar ratios (≤ 3 for caprylic acid; ≤ 2 for capric acid) and temperatures (lower than 30ºC- 35 ºC).

These values were similar to the optima values predicted by RSM for the production of MLM by acidolysis of virgin olive oil with caprylic or capric acids using the lipase Lip2 from *Yarrowia lipolytica*, immobilized in Accurel MP 1000 (Godoy *et al.*, 2013). Under optimized conditions (48 h reaction at 40 ˚C, with a molar ratio of 2:1 M/TAG) the highest yield was reached for C8:0 (25.51 mol %) and C10:0 (17.9 mol %).

In the production of human milk fat substitutes, rROL was (i) immobilized in Accurel® MP 1000 and used in acidolysis of lard with omega-3 polyunsaturated fatty acids (Simões *et al*., 2014) or (ii) immobilized in Lewatit VP OC 1600 and used in the acidolysis of tripalmitin with polyunsaturated fatty acids from camelina oil (Faustino *et al*., 2016). Again, the best results were obtained for lower values of temperature and molar ratios FFA:TAG .

4. Conclusions

The production of low-calorie structured triacylglycerols from olive oil and medium chain fatty acids (caprylic and capric acids) in a batch reactor was modelled by RSM. The non-commercial *sn*-1,3 regioselective rROL imobilized in Amberlite IRA96 was able to catalyze the incorporation of caprylic acid and capric into olive oil, in solvent-free media This biocatalyst showed to be a feasible option to the high-cost immobilized lipases used for SL production.

References

Arifin, N., Soo-Peng, K., Long, K., Chin-Ping, T., Yusoff, M.SA., 2010. Modelling and optimization of Lipozyme RM IM-catalyzed esterification of medium- and long-chain triacyglycerols (MLCT) using response surface methodology. Food Bioprocess Technology, 5, 216-225.

Barrigón J.M., Montesinos J.L., Valero F. , Searching the best operational strategies for Rhizopus oryzae lipase production in Pichia pastorisMut+ phenotype: Methanol limited or methanol non-limited fed-batch cultures? Biochemical Engineering Journal 75: 47-54 (2013).

Caballero, E., Soto, C., Olivares, A., Altamirano, C., 2014, Potential Use of Avocado Oil on Structured Lipids MLM-Type Production Catalysed by Commercial Immobilised Lipases. PLoS ONE 9, 1-7.

Costa, C.M., Canet, A., Rivera, I., Osório, N.M., Sandoval, G., Valero, F., Ferreira-Dias, S., 2017, Production of MLM type Structured Lipids from Grapeseed Oil Catalyzed by Non-Commercial Lipases, European Journal of Lipid Science and Technology (DOI: 10.1002/ejlt.201700320).

EN 14105:2011 (E), Fat and oil derivates – Fatty Acid Methyl Esters (FAME) – Determination of free and total glycerol and mono-, di-, triglyceride contents, European Committee for Standardization, Brussels, 21.

Esteban, L., Jiménez, M.J., Hita, E., González, P.A., Martín, L., Robles, A., 2011, Production of structured triacylglycerols rich in palmitic acid at sn-2 position and oleic acid at sn-1,3 positions as human milk fat substitutes by enzymatic acidolysis. Biochemical Engineering Journal, 54, 62-69.

Faustino, A.R., Osório, N.M., Tecelão, C., Canet, A., Valero, F., Ferreira-Dias, S., 2016, Camelina oil as a source of polyunsaturated fatty acids for the production of human milk fat substitutes catalyzed by a heterologous *Rhizopus oryzae* lipase. European Journal of Lipid Science and Technology, 118, 532-544.

Casas-Godoy, L., Marty, A., Sandoval, G., Ferreira-Dias, S., 2013, Optimization of medium chain length fatty acid incorporation into olive oil catalyzed by immobilized Lip2 from *Yarrowia lipolytica*. Biochemical Engineering Journal, 77, 20- 27.

Guillén, M., Benaiges, M.D., Valero, F., 2011, Comparison of the biochemical properties of a recombinant lipase extract from *Rhizopus oryzae* expressed in *Pichia pastoris* with a nativem extract. Biochemical Engineering Journal, 54, 117-123.

Kim, B.H., Akoh, C.C., 2015, Recent research trends on the enzymatic synthesis of structured lipids. Journal of Food Science, 80, 1713-1724.

Morales-Medina, R., Munio, M., Guadix, A., Guadix, E.M., 2017, Development of an up-grading process to produce MLM structured lipids from sardine discards. Food Chemistry, 228, 634-642.

Nunes, P.A., Pires-Cabral, P., Ferreira-Dias, S., 2011a, Production of olive oil enriched with medium chain fatty acids catalysed by commercial immobilised lipases, *Food Chemistry*, 127: 993–998.

Nunes, P.A., Pires-Cabral, P., Guillén, M., Valero, F., Luna, D., Ferreira-Dias, S., 2011b, Production of MLM-type structured lipids catalyzed by immobilized heterologous *Rhizopus oryzae* lipase. Journal of the American Oil Chemists' Society, 88, 473-480.

Nunes, P.A., Pires-Cabral, P., Guillén, M., Valero, F., Ferreira-Dias, S., 2012, Optimized production of MLM triacylglycerols catalyzed by immobilized heterologous *Rhizopus oryzae* lipase. Journal of the American Oil Chemists' Society, 89, 1287-1295.

Palla, C.A., Pacheco, C., Carrin, M.E., 2012, Production of structured lipids by acidolysis with immobilized Rhizomucor miehei lipases: selection of suitable reaction conditions. Journal of Molecular Catalysis B: Enzymatic, 76, 106-115.

Sanchez, D.A., Tonetto, G.M., Ferreira, M.L., 2017, Screening of lipases with unusual high activity in the sn-2 esterification of 1,3-dicaprin under mild operating conditions. Journal of Agricultural and Food Chemistry, 65, 5010-5017.

Simões, T., Valero, F., Tecelão, C., Ferreira-Dias, S., 2014, Production of human milk fat substitutes catalyzed by a heterologous *Rhizopus oryzae* lipase and commercial lipases, Journal of the American Oil Chemists' Society, 91: 411-419

Terada, S., Sekine, S., Aoyama, T., 2015, Dietary intake of medium- and long-chain triacylglycerols prevents the progression of hyperglycemia in diabetic ob/ob mice. Journal of Oleo Science, 64, 683-688.

Wang, Y.H., Qin, X.L., Zhu, Q.S., Zhou, R., Yang, B., Li, L., 2010, Lipase-catalyzed acidolysis of lard for the production of human milk fat substitute. European Food Research and Technology, 230, 769-777.