# Enzymatic preparation of structured triacylglycerides containing γ-linolenic acid[☆]

J.E. López-Martínez [a], Rubi Romero [a], Reyna Natividad [a], Carlos F. Torres [b], Hugo S. García [c], R. Baeza-Jiménez [a,*,1]

[a] *Centro Conjunto de Investigación en Química Sustentable UAEM-UNAM, Carr. Toluca-Atlacomulco Km 14.5, Unidad San Cayetano, 50200, Toluca, Estado de México, Mexico*
[b] *Instituto de Investigación en Ciencias de La Alimentación (CIAL, CSIC-UAM), C/Nicolás Cabrera 9, 28049, Madrid, Spain*
[c] *UNIDA, Tecnológico Nacional de México/Instituto Tecnológico de Veracruz, M.A, de Quevedo 2779, Col. Formando Hogar, 91897, Veracruz, Veracruz, Mexico*

## ARTICLE INFO

## ABSTRACT

Structured triacylglycerides (STAG) rich in γ-linolenic acid (GLA) were prepared using a three-step process. First, with the addition of molecular sieves (MS) at different addition times (3, 9, 12 and 15 h) and loads (2.5, 5, 10 and 15%, based on the total weight of substrates), the enrichment of GLA from evening primrose oil (EPO) and 1-butanol (BtOH) was improved via *Candida rugosa* lipase-catalysed esterification reactions. Secondly, the GLA-enriched fraction was separated by thin-layer chromatography (TLC) to be further set to react during the third step in the presence of glycerol and *Candida antarctica* fraction B (CALB), under different enzyme loadings (5, 10, 15 and 20%, based on the total weight of substrates), temperatures (30, 40, 50 and 60 °C) and substrates molar ratios (1:1, 2:1, 3:1 and 4:1, GLA:glycerol). 60% of STAG containing 49 wt% of GLA were produced by using 15% of CALB at 60 °C and a 3:1 molar ratio.

## 1. Introduction

The need for new and novel functional foods is in increased demand by consumers. Those products known as functional or nutraceutical foods, include oils and fats. In this regard, there are concerns related to their beneficial status against cardiovascular diseases, even though the biochemistry of lipids suggests that they could play important roles in human health (Camino-Feltes et al., 2010). In technical literature there are several studies referring the production of structured tri-acylglycerides (STAG) containing polyunsaturated fatty acids (PUFA), namely omega-3 (n-3) and omega-6 (n-6) families. Most of such studies describe the incorporation of n-3 residues to produce structured lipids, and thus we pursued the preparation of STAG containing γ-linolenic acid (GLA), an n-6 fatty acid.

GLA is formed from linoleic acid via the Δ-6 desaturase. GLA occurs naturally in oil seeds of some plant families, such as *Boraginaceae*, *Onagraceae* and *Saxifragaceae*, at low contents (no larger than 25%).

The enzymatic and chemical enrichments of GLA have been conducted by several means. López-Martínez et al. (2004) reported the enrichment of GLA from *Borago officinalis* and *Echium fastuosum* seed oils and fatty acids by low temperature crystallisation in the presence of organic solvents, reaching a maximal GLA concentration of 58.8%. Sajilata et al. (2008) reported the purification of GLA from *Spirulina platensis*, where GLA methyl esters with over 96% purity and a recovery of 66% was obtained using argentated silica gel chromatography. GLA was found to be concentrated mainly in the glycolipid fraction. Effective separation of the lipid fractions was possible using silica gel column chromatography followed by preparative thin-layer chromatography (TLC). Fregolente et al. (2009) studied the screening of microbial strains with suitable lipase activity for enrichment of GLA by selective hydro-lysis of borage oil (21.6% of GLA/total fatty acids). Only one of the lipases tested during that study showed selectivity, discriminating GLA during the hydrolysis reaction. By using the enzymatic extract from *Geotrichum candidum* as a biocatalyst, it was possible to obtain 41.7% of GLA in acylglycerols when borage oil was treated in a fixed-bed reactor for 24 h at 30 °C. Rincón-Cervera et al. (2009) carried out the

purification of GLA-triacylglycerides from evening primrose oil (EPO) by gravimetric column chromatography. Gradient elution with increasing polarity enabled separation of valuable triacylglycerides (TAG) species containing GLA. Enzymatic hydrolysis revealed the distribution of fatty acids in the isolated TAG species, with GLA located in the *sn*-2 position in different proportions.

In summary and broadly speaking, the strategies to attain enrichment of GLA are mainly based on either changing the catalyst, the purification method and/or the oil source. In this sense, it is not of the authors' knowledge that low-GLA content oil sources had been successfully assessed in the production of STAG containing GLA. Therefore, from our previous experience (Baeza-Jiménez et al., 2014), the aim of the present work was to prepare STAG with an elevated concentration of GLA using a three-step protocol, having EPO as oil source in the presence of butanol (BtOH) and *Candida rugosa* lipase during the first step, followed by the TLC step and finally, glycerolysis reactions catalysed by fraction B of *Candida antarctica* (CALB).

## 2. Materials and methods

### 2.1. Materials

EPO, BtOH (purity >99.9%), glycerol (>99.9%), molecular sieves (MS), 0.5 M sodium methoxide in methanol, as well as other reactants employed were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standards used were trilinolein (≥99%), 1,2 dipalmitin (≥99%), 1,3 diolein (≥99%), 1-monoolein (≥99%), from Sigma-Aldrich (St. Louis, MO, USA). Supelco 37 FAME Mix GC standard was purchased from Supelco (Bellefonte, PA). *C. rugosa* lipase (Lipase OF as trade name) was obtained from Meito Sangyo Co. LTD (Nagoya, Japan) while *Candida antarctica* fraction B (CALB), was purchased in a free form from Novozymes A/S (Bagsvaerd, Denmark), and immobilized in our laboratory on Duolite A568 (a gift from Rohm and Hass, Barcelona, Spain), using a previously reported protocol (Baeza-Jiménez et al., 2012).

### 2.2. 1st step – improved esterification reactions

Free fatty acids (FFA) were prepared from EPO according to our previous report (Baeza-Jiménez et al., 2014). The fatty acid composition of EPO is listed in Table 1. The trials were performed in a 25 mL glass vessel equipped with a water jacket for temperature control. The total weight of the substrate's mixture was 4 g for all reactions tested, under these operational conditions: substrate molar ratio of 1:10 (EPO-FA and BtOH), 30 °C and 10% of *C. rugosa*, stirred with a magnetic agitator at 400 rpm. For the improvement, different MS addition times (3, 9, 12 and 15 h) and MS loads (2.5, 5, 10 and 15%, based on the total weight of substrates) were explored. Individual samples were withdrawn at selected times. All trials were conducted in duplicate. Samples were further analysed by TLC and gas chromatography (GC).

**Table 1**
Fatty acid composition of the fatty acid residues (wt. %) of the starting material (EPO) and the GLA-enriched concentrates.

| Fatty acids | EPO | Concentrate 1 | Concentrate 2 |
|---|---|---|---|
| 16:0 | 6.24 | 3.66 | |
| 18:0 | 1.91 | 2.52 | |
| 18:1 (n-9) | 6.06 | – | – |
| 18:1 (n-9) | 0.63 | – | – |
| 18:2 (n-6) | 76.3 | 23.79 | 10 |
| GLA | 8.87 | 69.92 | 90 |

Concentrate 1 was prepared with a 1:10 ratio (EPO-FA:BtOH), 30 °C, 10% of the enzyme and 400 rpm, after 24 h. Concentrate 2 was prepared under identical conditions but MS were added (5 wt%, based on the total weight of substrates at 9 h of reaction), after 12 h.

### 2.3. 2nd step – separation of the esterification products

Samples (30 μL) were dissolved in chloroform (120 μL) and loaded on TLC plates from Merck (Silica gel 60 $F_{254}$) to be further developed using a mixture of petroleum ether, diethyl ether and acetic acid (100:20:1 v/v/v). The plates were subsequently subjected to ultraviolet light at 254 nm to allow the identification of both FFA and fatty acid butyl ester fractions.

For the trials related to glycerides formation, the bands corresponding to monoacylglycerides (MAG), diacylglycerides (DAG) and triacylglycerides (TAG) were scraped off and dissolved in $CHCl_3/CH_3OH$ (2:1, v/v). After that, the resultant solution was filtered and then subjected to selective derivatization for GC analysis, according to Miranda et al. (2013).

### 2.4. 3rd step – glycerolysis reaction

After recovering the GLA-rich fatty acid fraction by TLC, it was allowed to react with glycerol to prepare STAG. CALB-catalysed glycerolysis of GLA and glycerol were performed in a 25 mL glass vessel equipped with a water jacket for temperature control. The reaction was initiated by adding the enzyme while stirring with a magnetic agitator at 400 rpm. The total weight of the substrates (GLA and glycerol) mixture employed was 4 g for all reactions tested. Different enzyme loads (5, 10, 15 and 20%, based on the total weight of substrates), temperatures (30, 40, 50 and 60 °C) and substrates molar ratios (1:1, 2:1, 3:1 and 4:1, GLA: glycerol) were evaluated. Individual samples were withdrawn at selected times. All trials were conducted in duplicate. Samples were further analysed by GC.

### 2.5. Analysis of the products

To monitor the extent of both esterification and glycerolysis reactions, GC analysis was performed. For esterification reactions, one μL of the extract was injected into a Varian 3800 chromatograph (Varian Inc., Walnut Creek, CA) fitted with a HP-INNOWAX silica capillary column (30 m × 0.32 mm i.d.; Agilent Technologies). The injector and FID temperatures were set at 240 and 250 °C, respectively. The oven temperature was held at 160 °C for 10 min and then heated to 200 °C at a rate of 30 °C/min. The fatty acid methyl esters were identified by comparing their retention times with those of the true standard. Hexadecane (0.2 mg) was used as an internal reference standard.

In the case of glycerolysis reactions the same GC set up was used. However, the measurement method was according to Miranda et al. (2013). The fatty acid methyl esters were identified by comparing their retention times with Supelco® 37 FAME Mix standard.

## 3. Results and discussion

The present study deals with the preparation of STAG containing GLA. First, the enrichment of the starting material (EPO) was carried out by *C. rugosa*-catalysed esterification reactions, where from a previous study (Baeza-Jiménez et al., 2014), GLA content was increased from 8.87 to ca. 70 wt% (Concentrate 1 in Table 1). In the present work, the addition of MS allowed an improved enrichment which turned out in a higher GLA content of 90 wt% (Concentrate 2 in Table 1). After that, STAG with an elevated content of GLA were obtained.

### 3.1. Effect of molecular sieves on esterification reactions

Water is involved in all the interactions that allow an enzyme to perform its catalytical activity. Particularly, lipases require a certain amount of surface water to maintain their structure and activity (Rosu et al., 1998; Xu, 2003). However, at a certain concentration, water produced by ester synthesis decreases both reaction rate and yield. For esterification, as larger amounts of water are produced, lower

equilibrium conversions occur.

To overcome such problems, the first set of experiments consisted of improved esterification reactions by means of different MS addition times and loads, in order to minimise hydrolysis. Fig. 1 shows the effect of different addition times explored (3, 9, 12 and 15 h), using a 10% loading of MS (based on the total weight of substrates) for a 1:10 (EPO-FA:BtOH) ratio, 400 rpm, 30 °C and 10% loading of *C. rugosa* lipase (according to our previous findings). Control of the esterification reaction was conducted with no MS addition.

During the first 10 h of reaction, esterification was the controlling reaction step for all the assays, but after that time, the production of water and the effect of adding MS were evident. When MS were added at 3 h, GLA concentration reached a maximum of 67 wt% after 12.5 h. For an addition time of 9 h, the highest amount of GLA (72 wt%) was obtained after 12.5 h. When MS were added at 12 and 15 h, the maximum concentration of GLA (70 and 66 wt%, respectively) was reached after 24 h. As it can be seen, addition time of MS did not allow a higher increase in GLA content since enrichments reached are similar. However, the advantage is a shorter reaction time to achieve the highest amount of GLA. When MS was not added to the reaction system (control in Fig. 1), 24h later, 70% GLA content was achieved while with the addition of MS at 9 h; a similar GLA content (72 wt%) is obtained after 12.5 h. A reduction on reaction time is very important from an operational point of view because implies lower productions costs, energy requirements, shorter exposure time for the enzyme in a reaction system which can allow enzyme reuse.

From the above observations, it is remarkable that removing part of the formed water during esterification, allows the reaction to proceed at a faster rate because the excess of water can hydrate the enzyme while reducing its activity. Thus, the selected addition time for MS was 9 h.

After that, the effect of different MS loads was tested on GLA content. As it can be noted in Fig. 2, for 2.5 and 5% loads, a higher amount of MS increased the GLA concentration to 80 and 90 wt%, respectively. However, when 10 and 15% loadings were assayed, the GLA concentration was smaller and the contents attained were similar to control. Also, the required reaction time was 12 h to reach a maximum when 2.5,

5 and 10% loads were used. For a 15% loading the highest content was obtained after 24 h.

The results obtained during this first step are explained by these facts: (1) for larger amounts of MS mass transfer limitations can occur, affecting the interaction between the enzyme and substrates and, (2) larger amounts of MS reduce water content beyond what is necessary for enzyme performance. Therefore, water should be maintained at a concentration to permit reaction to proceed to product formation but at the same time the catalytic activity of the enzyme should not to be compromised.

The improved operating conditions for the enrichment of GLA are: 1:10 (EPO-FA:BtOH) ratio, 400 rpm, 30 °C, 10% of *C. rugosa*, based on the total weight of substrates, with the addition of 5% MS, also based on the total weight of substrates, after 9 h of reaction, reaching a 90 wt% GLA content (Concentrate 2 in Table 1).

### 3.2. Separation of the esterification products

Once the starting material (EPO) was enriched in GLA, the separation of butyl esters and FFA fractions proceeded. The latter can be removed by column chromatography, solvent extraction and short path distillation; however, TLC was selected for being a fast and simple separation method, as well as it requires low volume of solvents. The mobile phase consisted of petroleum ether, diethyl ether and acetic acid (100:20:1 v/v/v). When the GLA-enriched fraction was recovered, it was allowed to react during the third step in the presence of glycerol and CALB.

### 3.3. Preparation of STAG

As it has already been reported, production of glycerides is a complex reaction scheme where many variables affect products formation and overall reaction yield. The first variable tested was CALB loads (5, 10, 15 and 20%, based on the total weight of substrates). The amount of the enzyme present in the reaction mixture is essential to conduct the glycerolysis reaction. With sufficient amount of enzyme, the reaction proceeds to the formation of TAG, but an excess of enzyme can result in
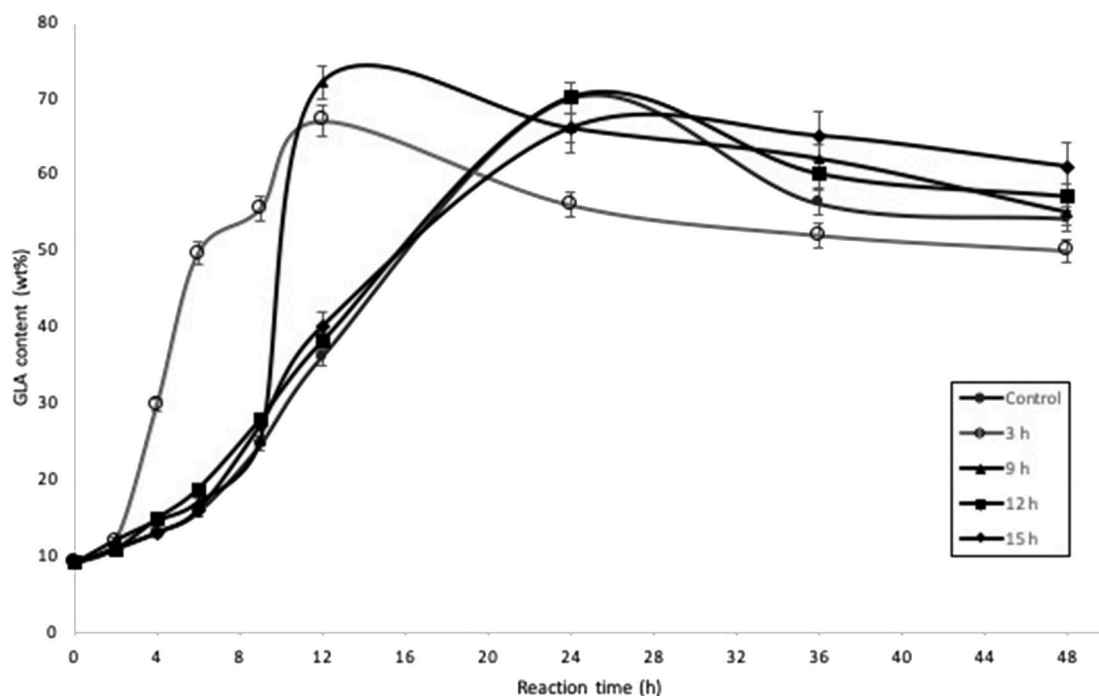


**Fig. 1.** Effect of different MS addition time on GLA content of non-esterified fraction at 30 °C, 400 rpm and 10% enzyme loading (based on the total weight of substrates). The amount of MS was 10% (based on the total weight of substrates) in all trials. The values shown in the plot are the mean of duplicate determinations from different experimental trials.
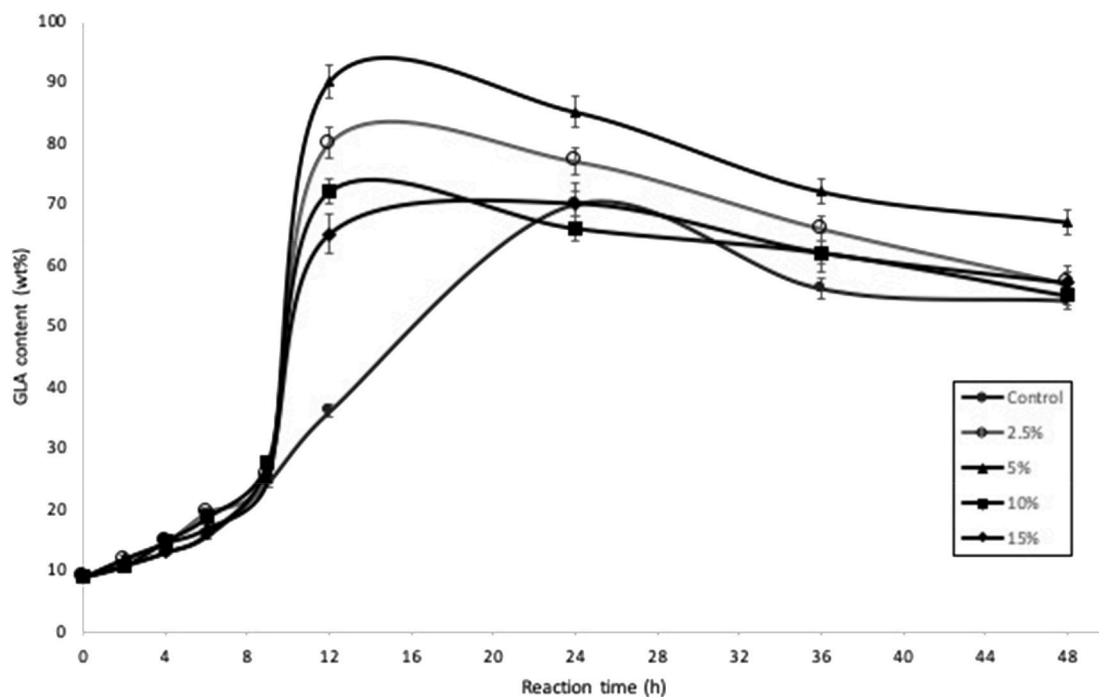
**Fig. 2.** Effect of different MS loading on GLA content of non-esterified fraction at 30 °C, 400 rpm and 10% enzyme loading (based on the total weight of substrates). The MS addition time was 9 h in all trials. The values shown in the plot are the mean of duplicate determinations from different experimental trials.

mass transfer limitations and reduce reaction yield. From Fig. 3, it can be observed that the amount of TAG formed increased as a function of loading. However, when 20% loading was tested, a lower TAG content was reached than the one yielded at a 15% load, indicating that part of the enzyme is held in the walls of the vessel, leading to a lower amount of available biocatalyst to interact with the substrates. Also, the use of high enzyme loadings produced problems with stirring and decreased mass transfer.

The percentage of TAG formation exhibited by the 15% loading was the highest content achieved: 13.9 wt%, and then, we selected such loading to evaluate the effect of temperature. In a similar report by Miranda et al. (2013), a 15% loading was selected to prepare DAG containing n-3, and is consistent with the study conducted by Camino-Feltes et al. (2010). For the production of STAG containing palmitic and docosahexaenoic acids, Liu et al. (2015), found 10% of the enzyme as the best loading for the incorporation. These authors also indicate
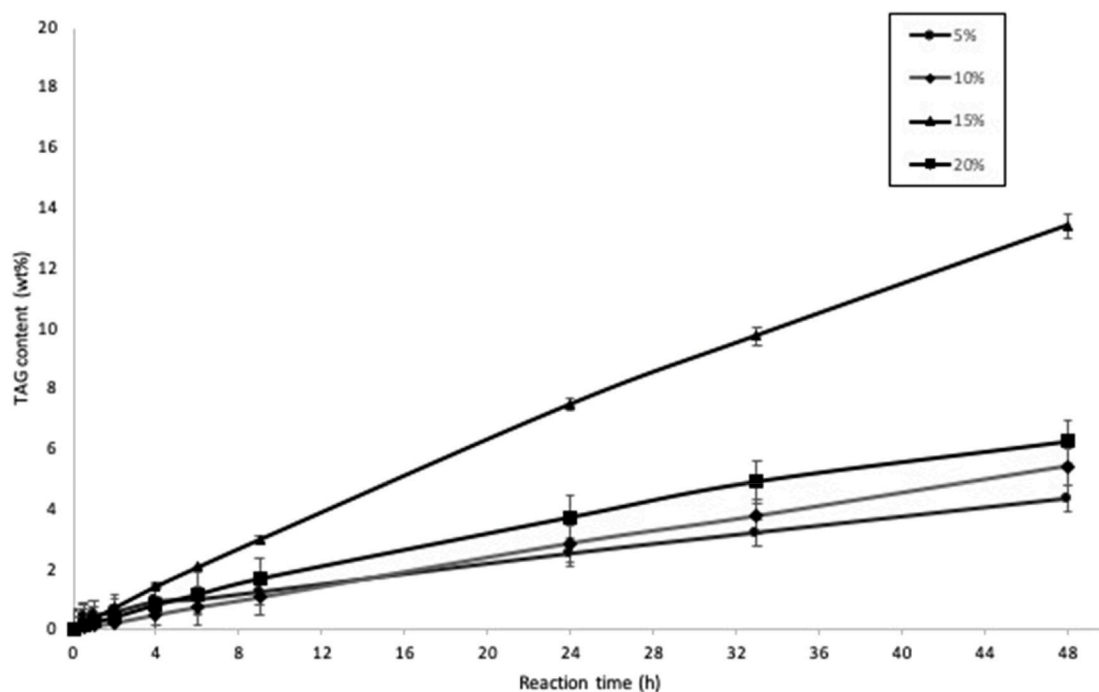


**Fig. 3.** Effect of the enzyme loading (based on the total weight of substrates) on the production of STAG at 40 °C, 1:1 GLA:glycerol, and 400 rpm. The values shown in the plot are the mean of duplicate determinations from different experimental trials.

that the enzyme dosage is restricted based on economic considerations and mass transfer limitations of the reaction system. Regarding the enzyme, the source and the supporting material for its immobilisation are remarkable parameters. Dutra Madalozzo et al. (2016), used a metagenomic lipase (LipC12) immobilized onto Immobead 150. These authors described the synthesis of flavour esters as well as STAG containing 23% of caprylic acid using that new lipase.

The second variable explored was temperature (30, 40, 50 and 60 °C). Glycerol exhibits low miscibility with oils and fats, and then, high temperatures could reduce viscosity and enhance substrates diffusion, leading up to higher amounts of TAG.

From the results obtained (see Fig. 4), as temperature increased, greater TAG contents were obtained, reaching a maximum of 43.5 wt% at 60 °C. Thus, 15% loading and 60 °C were selected to explore the effect of substrates molar ratio.

Kahveci et al. (2009), indicated that TAG conversion and the yield of total TAG increased with temperature. In a similar study, Camino-Feltes et al. (2010), attained 43% DAG at 70 °C, 15% of N435 loading for a 1:1 ratio (fish oil/glycerol). It is worth mentioning that these authors employed in one case an organic solvent (*t*-butanol) and in the other, a surfactant (Tween 65), thus their reaction proceeded in a shorter time. On the other hand, Krüger et al. (2010), used olive oil with 10% N435 loading, 70 °C and *t*-butanol, and referred that both MAG and DAG are suitable for production. For Liu et al. (2015), 60 °C was also the best temperature. These authors point out that reaction temperature should be selected with consideration of the thermostability of the enzyme because too higher temperature can inactivate the enzyme.

In general, in all our data we observed a positive effect of temperature on TAG contents. When temperature increases the viscosity of reaction mixture decreases and the solubility of substrates is higher, thus avoiding steric hindrance. Our reaction system was solvent and surfactant-free, compared to other systems reported in the technical literature.

Finally, the effect of the substrate molar ratio was evaluated (1:1, 2:1, 3:1 and 4:1, GLA:glycerol). Given the viscosity and the immiscibility of the reactants, and the increments in glycerol concentration, it was important to ensure that the agitation provided proper homogeneity of the mixture. Then, all the trials were carried out at 400 rpm. As the initial glycerol concentration increased, higher amounts of TAG were obtained. However, greater glycerol contents in the mixture turned out in mass transfer limitations and reduced the enzyme–substrate interactions.

Fig. 5 shows the effect of substrates molar ratio. By increasing the amount of GLA (1:1, 2:1 and 3:1 ratios), better acyl exchanges occurred. However, when the 4:1 ratio was employed, the smallest amounts of TAG were formed during the first 9 h, and after that time TAG concentrations were higher for 1:1 and 2:1 ratios but the 4:1 ratio did not exceed the concentration reached for a 3:1 ratio, 61 wt% of TAG.

When all these new selected conditions were assayed together, the glycerides profile shown in Fig. 6, suggest that the TAG formation was favoured. The DAG content reached a maximum of 25 wt% after 4 h. When the reaction was allowed to proceed for longer times, its yield gradually decreased to 15 wt%. On the other hand, MAG content gradually increased (ca. 15 wt%). TAG production also described this increasing trend during the whole experimental run. As it is depicted in Fig. 6, all those conditions allowed preparing 60 wt% of TAG. The GLA content for these glycerides was 49 wt%.

Reaction conditions affect the lipid profiles obtained. Also, the biocatalysts employed (in terms of activity, selectivity and mild conditions) play a crucial role. From the results obtained, GLA was found to increase to ca. 90% (wt.%). In the case of the glycerolysis reaction, the low miscibility of glycerol clearly affected the interaction of the enzyme and thus, incorporation of GLA into glycerides was low. For DAG, the amount of GLA incorporated was 17.9 wt% and for MAG was only 4.9 wt %; however, this constitutes an important effort because most of the STAG reports are related to n-3 PUFA.

## 4. Conclusions

Because *C. rugosa* exhibited a low preference for GLA, this residue was successfully concentrated during the first step, allowing the preparation of STAG. Additionally, reaction conditions affected the operational stability of the biocatalysts employed, thereby conducting to the different profiles described. The present work was carried out in solvent-
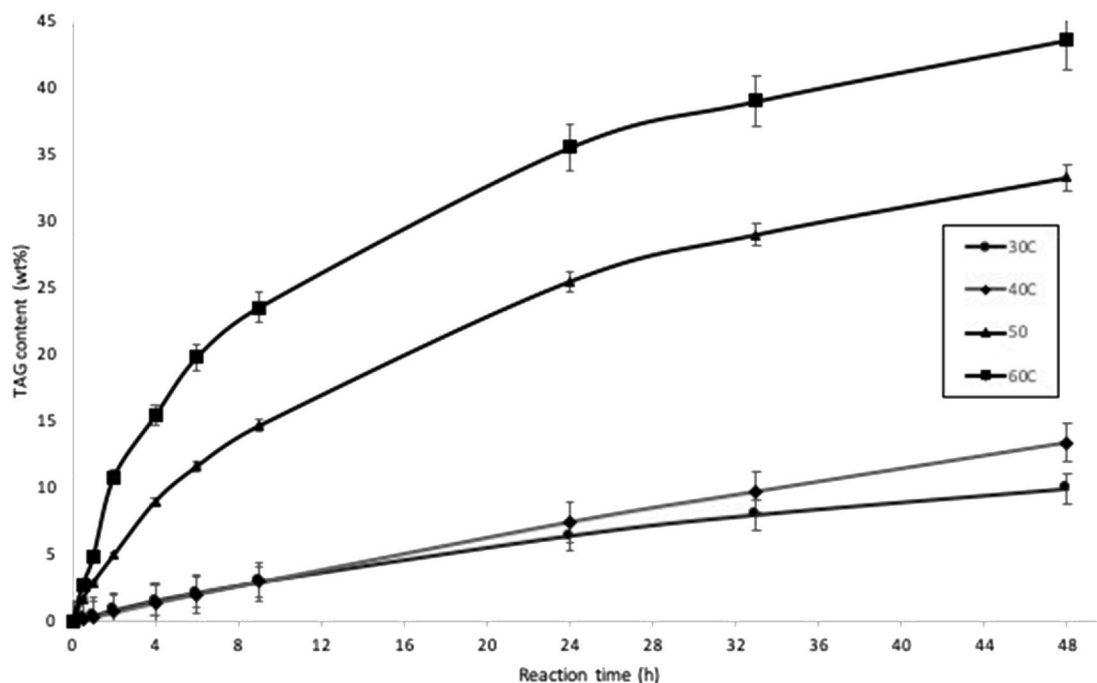


**Fig. 4.** Effect of the temperature on the production of STAG at, 1:1 GLA:glycerol, 15% of enzyme loading (based on the total weight of substrates) and 400 rpm. The values shown in the plot are the mean of duplicate determinations from different experimental trials.
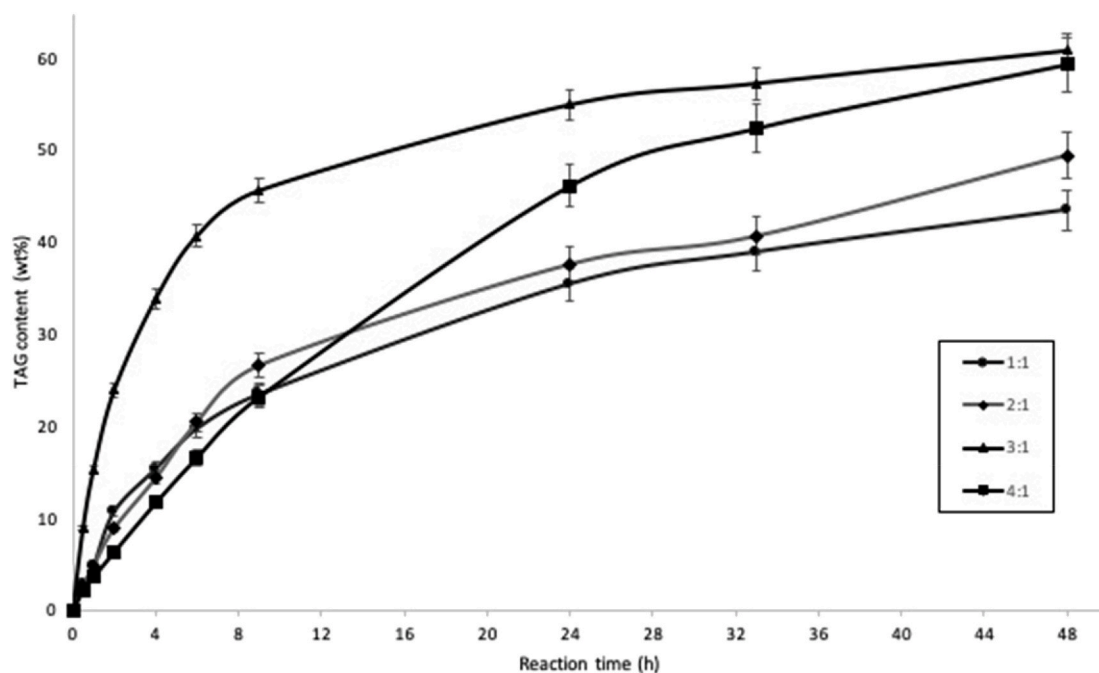
**Fig. 5.** Effect of the substrate molar ratio GLA:glycerol on the production of STAG at 60 °C, 15% of enzyme loading (based on the total weight of substrates) and 400 rpm. The values shown in the plot are the mean of duplicate determinations from different experimental trials.
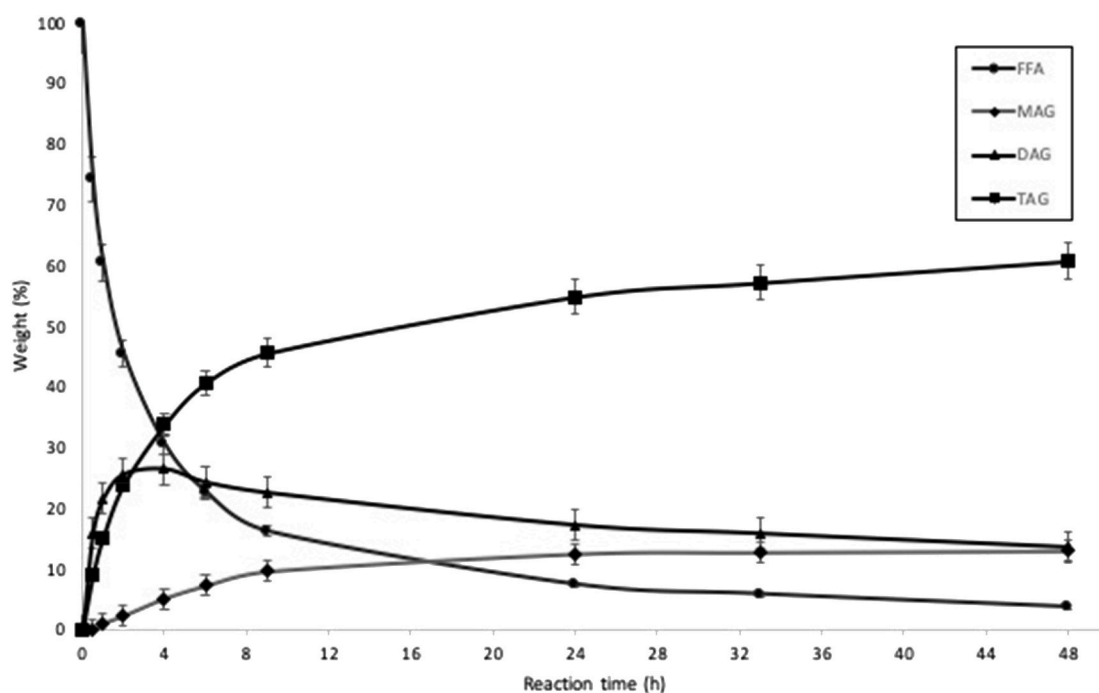


**Fig. 6.** Glycerides profile at 3:1 GLA:glycerol, 60 °C, 15% of enzyme loading (based on the total weight of substrates) and 400 rpm. The values shown in the plot are the mean of duplicate determinations from different experimental trials.

and surfactant-free system, and allowed the preparation of STAG (60%) containing 49 wt% of GLA.

Given the health benefits of MAG and DAG, we will pursue to develop further reaction schemes to reach higher amounts of those glycerides according to the effect of the corresponding parameters, in order to make them available for pharmaceutical and food applications.

## Acknowledgments

# References

Baeza-Jiménez, R., González-Rodríguez, J., Kim, I.H., García, H.S., Otero, C., 2012. Use of immobilized phospholipase A$_1$-catalyzed acidolysis for the production of structured phosphatidylcholine with an elevated conjugated linoleic acid content. Grasas Aceites 63, 44–52. https://doi.org/10.3989/gya.045211.

Baeza-Jiménez, R., No, D.S., Otero, C., García, H.S., Lee, J.S., Kim, I.H., 2014. Lipase-catalysed enrichment of γ-linolenic acid from evening primrose oil in a solvent-free system. J. Am. Oil Chem. Soc. 91, 1147–1153. https://doi.org/10.1007/s11746-014-2463-9.

Camino-Feltes, M.M., Oliveira, J.V., Treichel, H., Block, J.M., DeOliveira, D., Ninow, J.L., 2010. Assessment of process parameters on the production of diglycerides in omega-3 fatty acids through the enzymatic glycerolysis of fish oil. Eur. Food Res. Technol. 231, 701–710. https://doi.org/10.1007/s00217-010-1325-4.

Dutra Madalozzo, A., Martini, V.P., Kuniyoshi, K.K., Maltempi de Souza, E., de Olivera Pedrosa, F., Zanin, G.M., Mitchell, D.A., Krieger, N., 2016. Synthesis of flavor esters and structured lipids by a new immobilized lipase, LipC12, obtained from metagenomics. ISBAB 8, 294–300. https://doi.org/10.1016/j.bcab.2016.10.006.

Fregolente, P., Fregolente, L., Maciel, M., Carvalho, P., 2009. Screening of microbial lipases and evaluation of their potential to produce glycerides with high gamma linolenic acid concentration. Braz. J. Microbiol. 40, 747–756. https://doi.org/10.1590/S1517-83822009000400004.

Kahveci, D., Guo, Z., Özçelik, B., Xu, X., 2009. Lipase-catalyzed glycerolysis in ionic liquids directed towards diglyceride synthesis. Process Biochem. 44, 1358–1365. https://doi.org/10.1016/j.procbio.2009.07.009.

Krüger, R.L., Valério, A., Balen, M., Ninow, J.L., Oliveira, J.V., Oliveira, D., Corazza, M.L., 2010. Kinetics of lipase-catalyzed glycerolysis of olive oil in organic solvent system. Eur. J. Lipid Sci. Technol. 112, 921–927. https://doi.org/10.1021/jf901771m.

Liu, Y., Guo, Y., Sun, Z., Jie, X., Li, Z., Wang, J., Wang, Y., Xue, C., 2015. Production of structured triacylglycerols containing palmitic acids and docosahexaenoic acids at sn-1,3 positions. J. Oleo Sci. 64, 1227–1234. https://doi.org/10.5650/jos.ess15172.

López-Martínez, J.C., Campra-Madrid, P., Guil-Guerrero, J.L., 2004. γ-Linolenic acid enrichment from Borago officinalis and Echium fastuosum seed oils and fatty acids by low temperature crystallization. J. Biosci. Bioeng. 97, 294–298. https://doi.org/10.1016/S1389-1723(04)70208-X.

Miranda, K., Baeza-Jiménez, R., Noriega-Rodríguez, J.A., Otero, C., García, H.S., 2013. Optimization of structured diacylglycerols production containing n-3 via enzyme-catalysed glycerolysis of fish oil. Eur. Food Res. Technol. 236, 435–440. https://doi.org/10.1007/s00217-012-1889-2.

Rincón-Cervera, M.A., Rodríguez-García, I., Guil-Guerrero, J.L., 2009. Purification of GLA-triglycerides from evening primrose oil by gravimetric column chromatography. J. Am. Oil Chem. Soc. 86, 605–609. https://doi.org/10.1007/s11746-009-1378-3.

Rosu, R., Iwasaki, Y., Shimidzu, N., Doisaki, N., Yamane, T., 1998. Enzymatic synthesis of glycerides from DHA-enriched PUFA ethyl ester by glycerolysis under vacuum. J. Mol. Catal. B Enzym. 4, 191–198. https://doi.org/10.1016/S1381-1177(97)00035-0.

Sajilata, M.G., Singhal, R.S., Kamat, M.Y., 2008. Fractionation of lipids and purification of γ-linolenic acid (GLA) from Spirulina platensis. Food Chem. 109, 580–586. https://doi.org/10.1016/j.foodchem.2008.01.005.

Xu, X., 2003. Engineering of enzymatic reactions and reactors for lipid modification and synthesis. Eur. J. Lipid Sci. Technol. 105, 289–304. https://doi.org/10.1002/ejlt.200390059.