

# Kinetic Study of Liquid Lipase-catalyzed Glycerolysis From Olive Oil Using Lipozyme TL 100L

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## Research Article

**Keywords:** Monoacylglycerol, diacylglycerol Lipase, Surfactant, Statistical Design, Kinetic Modeling

**Posted Date:** March 10th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-275118/v1>

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22 **Abstract**

23 Monoacylglycerol (MAG) and diacylglycerol (DAG) are two natural components found in most  
24 edible oils and fats. Conventional synthesis of MAG and DAG is usually conducted by glycerolysis  
25 of triacylglycerol (TAG) at high temperatures (above 200 °C) in the presence of an alkaline  
26 catalyst. In this work, the synthesis of MAG and DAG using enzymatic glycerolysis of olive oil  
27 was investigated using Tween 80 as surfactant, *n*-butanol as co-surfactant and the novel lipase in  
28 liquid formulation Lipozyme TL 100L as catalyst. Experimental design was used to evaluate the  
29 effect of enzyme load and reaction temperature on the feedstock conversion. Enzyme load and  
30 system temperature were significant variables in the statistical design and the best condition was  
31 found at 35 °C, 7.5 vol% of Lipozyme TL 100 and 2:1 molar ratio (glycerolysis:oil) with  
32 conversion of TAG at approximately 98 % after 2 h of process. A mathematical model based on  
33 the Ping-Pong Bi-Bi mechanism was used to describe the reaction kinetics. The model adequately  
34 described the behavior of the system and can be a useful tool for the design of reactors in larger  
35 scales.

36

37 **Keywords**

38 Monoacylglycerol; diacylglycerol Lipase; Surfactant; Statistical Design; Kinetic Modeling.

39

## 40 **Introduction**

41 Monoacylglycerols (MAGs) are esters formed by fatty acids/glycerol largely utilized on  
42 industrial demands due its stabilizing, emollient and binding properties. This compound was  
43 approved by the Food and Drug Administration (FDA, United States) for use as emulsifiers in food  
44 and pharmaceuticals applications for not presenting collateral effects when ingested [1]. Within  
45 this same reasoning, diacylglycerols (DAGs) act in the treatment of cholesterol, prevention of  
46 obesity and reduction of body fat accumulation, bringing beneficial for reducing diseases related  
47 to such health problems.

48 The synthesis of MAG and DAG is performed in distinct ways. The conventional technique  
49 applied industrially occurs by triacylglycerols (TAGs) glycerolysis, usually at high temperatures  
50 (above 200 °C) in the presence of chemical (alkaline) catalyst. Under these conditions, a mixture  
51 composed around of 50 wt% of MAG, 40 wt% of DAG and 10 wt% of TAG is obtained [2, 3]. On  
52 the other hand, although it is still a scarcely explored topic, researches published recently highlight  
53 the potential of the use of lipases as biocatalysts on glycerolysis due to the reaction works at mild  
54 reaction conditions, implying a low energy demand of the process and by high selectivity of the  
55 enzymes, allowing a higher amount of product synthesis [4, 5].

56 In the enzymatic glycerolysis, an important point that should be mentioned is the presence  
57 of glycerol in the system. Frequently, the glycerol can bound with the enzyme and forming an  
58 inactive complex with the glycerol reducing the reaction efficiency once the glycerol is a  
59 competitive inhibitor [6]. Thus, ensure the system homogeneity is a crucial factor during the lipase-  
60 catalyzed glycerolysis. To aid this point, the use of a surfactant may promote higher homogeneity  
61 for the system oil-glycerol-enzyme, increasing the interfacial area, improving the immiscibility  
62 between the substrates, providing higher reaction rates and enhancing the biocatalyst efficiency,

63 mainly for lipases which are characterized as acting at the interface [4, 5, 7, 8]. There are two  
64 requirements for enzyme glycerolysis to occur: firstly, the oil-glycerol interface is formed; and then  
65 the enzyme is adsorbed in that system. Thus, the higher the interface, the higher the amount of  
66 enzyme adsorbed, which implies in lower activation energies and, consequently, higher reaction  
67 rates [9]. For this, the area of contact between enzymes and substrate is increased with the use of  
68 micelles. The micelles provide higher glycerol miscibility on apolar solvents, through the  
69 physicochemical characteristics of the micellar system and the interaction with the surface area  
70 where TAG break down and form MAG and DAG [10].

71         The majority of studies that address enzymatic glycerolysis to obtain MAG and DAG use  
72 immobilized lipases as reaction catalyst [11, 12]. The use of lipases in liquid formulations, as the  
73 novel Lipozyme TL 100L recently launched by Novozymes A/S, can be observed in scarce  
74 researches [13–15]. These papers evaluated only factors of process such as molar ratio of the  
75 system, the source and type of the enzyme activation. Furthermore, kinetic parameters estimation  
76 for this process are rare in the literature. However, mathematical modeling and knowledge of  
77 reaction kinetics are fundamental in reactors design. After all, modeling is used for the processes  
78 analysis to define strategies of operation, optimization and system control. Therefore, it is  
79 necessary to use representative, fast computing models with the good mapping of the variables and  
80 cause-effect in the processes [16]. Consequently, it is required to know the reaction kinetics to be  
81 able to reconcile the complexity of the chosen model and to better represent the reactor with the  
82 variables available and proposed [17, 18].

83         This work presents experimental results of the kinetics of enzymatic glycerolysis of olive  
84 oil in a batch reactor, using Tween 80 as surfactant, *n*-butanol as co-surfactant and the liquid lipase  
85 Lipozyme TL 100L, to evaluate the effect of temperature and enzyme concentration in the  
86 feedstock conversion. Moreover, a complete mathematical model considering the glycerolysis and

87 hydrolysis of the raw material to describe the process is also presented.

88

## 89 **Materials and Methods**

### 90 **Materials**

91 Refined olive oil (Oliva de Los Andes Premium) with 0.2 wt% of acidity was purchased  
92 from a local market and used as feedstock. Free liquid lipase from genetically modified  
93 *Thermomyces lanuginosus* microorganism (commercially named as Lipozyme TL 100L) with a  
94 nominal enzymatic activity of 100 LCLU·g<sup>-1</sup> and kindly provided by Novozymes Latin America  
95 LTDA (Araucária, Brazil) was used as biocatalyst. Glycerol and *n*-butanol (99.5 % purity,  
96 Alphatec, São Paulo, Brazil), Tween 80 PS surfactant (analytical grade, Dynamica, Indaiatuba,  
97 Brazil) were the chemicals used in the assays. Standards used in the HPLC analysis (Sigma-  
98 Aldrich, São Paulo, Brazil): linoleic acid (97 % purity), oleic acid (> 99 % purity), monopalmitic  
99 acid (> 99 % purity), dilinoleic acid (98 % purity), glycerol trioleic (> 99 % purity) and trilinoleic  
100 glycerol (98 % purity). Acetonitrile (99.9 % purity, Merck, São Paulo, Brazil) and acetone (99.8  
101 % Merck, São Paulo, Brazil) were used in the chromatographic analysis.

102

### 103 **Formation of Microemulsions**

104 Preliminary tests were performed to determine the volumetric ratios of feedstock, water and  
105 surfactant, where the follows glycerolysis to oil molar ratio was considered: 1:1; 2:1; 3:1; 1:2 and  
106 1:3, respecting a phase equilibrium diagram. After mixing the components of the microemulsion,  
107 it was subjected to constant stirring for 1.5 minutes in a Vortex homogenizer, and then stored at  
108 room temperature for 48 hours, according to the procedure described in Pescara [19].

109 **Enzymatic Glycerolysis and Factorial Design**

110 A Central Composite Rotatable Design (CCRD) plot with triplicate at the central point was  
111 used to determine the optimum conditions of MAG and DAG synthesis. Data analysis was  
112 performed using the software StatSoft Statistica, version 11.0. The effects of temperature and  
113 volumetric load of lipase on MAG and DAG yield were evaluated. The reactions were performed  
114 in a jacketed glass reactor, under constant stirring by mechanical stirrer (600 rpm) and temperature  
115 control. With the reaction parameters set, the enzyme was added and the enzymatic glycerolysis  
116 started for a period of 2 hours in pH 7.0. The values of the real and coded variables used in the  
117 statistical design are presented in Table 1. After the reaction, the enzyme separation was done by a  
118 system composed by a syringe filter, which was transferred to amber flasks brought into a  
119 Styrofoam container and then stored into the refrigerator. The filtration process was carried out to  
120 purify the sample. After separation, TAG, DAG, and MAG contents were determined by high-  
121 performance liquid chromatography.

122

123 **Table 1.** CCRD matrix, with real and coded values used in the reaction of glycerolysis catalyzed  
124 by Lipozyme TL 100L and conversions obtained to the TAG.

125

Asssay	Temperature (°C)	Enzyme Load (vol%)	Conversion (%)
1	30 (-1)	5.0 (-1)	66.61
2	40 (+1)	5.0 (-1)	70.24
3	30 (-1)	10 (+1)	82.76
4	40 (+1)	10 (+1)	86.72
5	28 (-1.41)	7.5 (0)	72.79
6	42 (+1.41)	7.5 (0)	58.28
7	35 (0)	3.97 (-1.41)	67.73
8	35 (0)	11.03 (+1.41)	97.11
9	35 (0)	7.5 (0)	98.12

10	35 (0)	7.5 (0)	98.82
11	35 (0)	7.5 (0)	98.41

126

## 127 **Sample Analysis**

128 High-performance liquid chromatography (HPLC, Thermo Scientific Ultimate 3000) was  
 129 used in the quantification of TAG, DAG MAG and FFA according procedure described by Lima  
 130 et al. [20]. Phenomenex C18 column of 5  $\mu\text{m}$  and 100  $\text{\AA}$  (250 x 4.6 mm, model 00G-4252-E0),  
 131 flow rate of 1.0  $\text{mL}\cdot\text{min}^{-1}$ , column temperature of 35  $^{\circ}\text{C}$  and detector temperature of 40  $^{\circ}\text{C}$  were  
 132 used in the analysis. The mobile phase was acetonitrile:acetone (1:1, v·v<sup>-1</sup>) and the injection volume  
 133 was 20  $\mu\text{L}$ . The content of reaction products was expressed in terms of the whole amount of MAG,  
 134 DAG, TAG and FFA as weight percent of the total sample in surfactant-free basis.

135

## 136 **Kinetic Modeling**

137 The kinetics were determined in three different conditions, according preliminary tests  
 138 performed in terms of TAG conversion and presented on Table 2, where the effect of the water  
 139 content in the reaction was evaluated and the formation of MAG, DAG and FFA was monitored.  
 140 The system temperature, concentration of olive oil and lipase load was fixed for all the experiments  
 141 at 35  $^{\circ}\text{C}$ , 10 vol% and 7.5 vol% respectively. These amounts utilized were determined through tests  
 142 of formation and stabilization of the microemulsions. The amount of enzyme and temperature were  
 143 used based on the results obtained via statistical design.

144

145

146

147 **Table 2.** Reaction conditions applied in the kinetics analysis of the system.

148

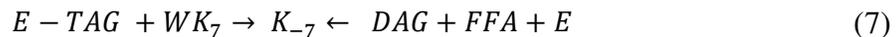
Assay	Glycerol (vol%)	Water (vol%)
1	7.40	5.0
2	7.69	10.0
3	8.00	15.0

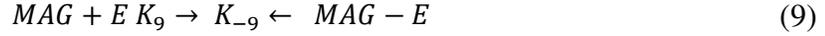
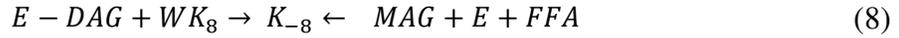
149

150 The mathematical approach to perform the modeling presented in this research considered  
 151 some factors suggested by Choong et al. [21]. These authors tested three mathematical models:  
 152 ternary model, Ping-Pong Bi-Bi and Ping-Pong-Bi complex for the glycerolysis of palm oil using  
 153 the enzyme Lipozyme TL IM in a solvent-free system. However, for all models, the hydrolysis  
 154 reactions were not considered, suggesting that the reactions were conducted under “micro-  
 155 aqueous” conditions. In this sense, this work presented the Ping-Pong Bi-Bi mathematical model  
 156 described by the set of equations – Eq. (1) to Eq (6), and also the hydrolysis reactions, characterized  
 157 by equations Eq (7) to Eq (10).

158 In the kinetic mathematical modeling of the glycerolysis reaction of olive oil, the following  
 159 elemental reactions were considered:

160





161

162 From the elementary equations, the following kinetic equations can be written:

163

$$r_1 = -K_1 C_{TAG}(t) C_E(t) + K_{-1} C_{TAG-E}(t) \quad (11)$$

$$r_2 = -K_2 C_{TAG-E}(t) \quad (12)$$

$$r_3 = -K_3 C_{E-FFA}(t) C_G(t) + K_{-3} C_{E-FFA-G}(t) \quad (13)$$

$$r_4 = -K_4 C_{E-FFA-G}(t) \quad (14)$$

$$r_5 = -K_5 C_{DAG}(t) C_E(t) + K_{-5} C_{E-DAG}(t) \quad (15)$$

$$r_6 = -K_6 C_{E-DAG}(t) \quad (16)$$

$$r_7 = -K_7 C_{E-TAG} C_w(t) + K_{-7} C_{DAG}(t) C_{FFA}(t) C_E(t) \quad (17)$$

$$r_8 = -K_8 C_{E-DAG} C_w(t) + K_{-7} C_{MAG}(t) C_{FFA}(t) C_E(t) \quad (18)$$

$$r_9 = -K_9 C_{MAG}(t) C_E(t) + K_{-9} C_{E-MAG}(t) \quad (19)$$

$$r_{10} = -K_{10} C_{E-MAG}(t) C_E(t) + K_{-10} C_{FFA}(t) C_E(t) C_G(t) \quad (20)$$

164

165 Applying the molar balance for each species and considering the productivity based on

166 stoichiometry, was obtained the enzymatic kinetics model for the several species involved in the

167 mechanism of the glycerolysis reaction, described by the Equations (21) to (31):

168

$$\frac{dC_{TAG}}{dt} = r_1 \quad (21)$$

$$\frac{dC_{DAG}}{dt} = -r_2 + r_5 - r_7 \quad (22)$$

$$\frac{dC_{MAG}}{dt} = -r_4 - r_6 - r_8 + r_9 \quad (23)$$

$$\frac{dC_G}{dt} = -r_3 + r_{10} \quad (24)$$

$$\frac{dC_E}{dt} = r_1 - r_4 + r_5 - r_7 - r_8 + r_9 - r_{10} \quad (25)$$

$$\frac{dC_{E-TAG}}{dt} = -r_1 + r_2 + r_7 \quad (26)$$

$$\frac{dC_{E-FFA}}{dt} = -r_2 + r_3 - r_6 \quad (27)$$

$$\frac{dC_{E-FFA-G}}{dt} = -r_3 + r_4 \quad (28)$$

$$\frac{dC_W}{dt} = r_7 + r_8 \quad (29)$$

$$\frac{dC_{FFA}}{dt} = -r_7 - r_8 - r_{10} \quad (30)$$

$$\frac{dC_{MG-E}}{dt} = -r_9 + r_{10} \quad (31)$$

169

170 The balance for free enzyme concentration was based on enzyme-substrate complex, where  
171 the total enzyme concentration remains constant, represented by the Equation (32):

172

$$C_E(t = 0) = C_E + C_{E-TAG} + C_{E-DAG} - C_{E-MAG} + C_{E-FFA} + C_{E-FFA-G} \quad (32)$$

173

174 The initial conditions are given by Equations (33) to (44). All concentration values are  
175 expressed in mol·kg<sup>-1</sup> and were calculated for each component based on the average molecular  
176 mass of olive oil, the mass of each element and total mass of oil and water. The enzyme  
177 concentration are expressed in g.L<sup>-1</sup>.

178

$$C_{TAG}(0) = C_{TAG,0}^{exp} \quad (33)$$

$$C_{DAG}(0) = 0 \quad (34)$$

$$C_{MAG}(0) = 0 \quad (35)$$

$$C_G(0) = 0 \quad (36)$$

$$C_E(0) = C_{E,0}^{exp} \quad (37)$$

$$C_{E-TAG}(0) = 0 \quad (38)$$

$$C_{E-FFA}(0) = 0 \quad (39)$$

$$C_{E-FFA-G}(0) = 0 \quad (40)$$

$$C_W(0) = C_{W,0}^{exp} \quad (41)$$

$$C_{FFA}(0) = 0 \quad (42)$$

$$C_{E-MAG}(0) = 0 \quad (43)$$

$$C_{E-DAG}(0) = 0 \quad (44)$$

179

## 180 **Parameter Estimation**

181           The mathematical model parameters ( $K_1, K_{-1}, K_2, K_3, K_{-3}, K_4, K_5, K_{-5}, K_6, K_7, K_{-7}, K_8, K_{-8},$   
 182  $K_9, K_{-9}, K_{10}$  e  $K_{-10}$ ) were estimated from the kinetics of the enzymatic hydrolysis of olive oil (TAG,  
 183 DAG, MAG and FFA) and minimization of the objective function represented by Equation (32).  
 184 The simplex Dowhill optimization method developed by Nelder and Mead [22] was used in the  
 185 pursuit of the objective function minimum.

186

$$ob = \sum_{j=1}^{nc} \sum_{i=1}^{nd} \left( C_{ji}^{exp} - C_{ji}^{mod} \right)^2 \quad (45)$$

187

188 where  $nc$  is the number of components (TAG, DAG, MAG and FFA);  $nd$  is the number of  
 189 experimental data;  $C_{ji}^{exp}$  is the experimental concentration of component  $j$  and;  $C_{ji}^{mod}$  is the

190 component concentration  $j$  calculated by the model;

191 Thus, from the proposed model that consists of the system of Equations (21) to (32) and the  
192 particular initial conditions, Equations from (33) to (44) were used to model the behavior of the  
193 reaction system and to determine the kinetic parameters of the hydrolysis reactions of olive oil. For  
194 the solution of the mathematical model, the numerical method of Rosenbrock [23] was used, using  
195 the software Maple<sup>®</sup>, version 13 (Waterloo Maple Inc., Canada).

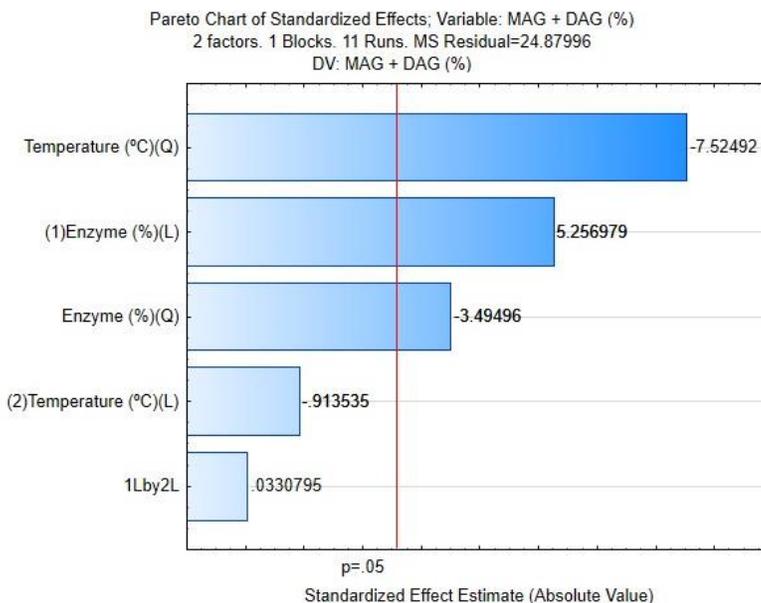
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## 197 Results and Discussion

### 198 Central Composite Rotatable Design (CCRD)

199 The results for feedstock conversion of the experimental design are presented in Table 1.  
200 The conversion of TAG was in the range of 58 % to 98 %. Table 3 presents the results of ANOVA  
201 and Figure 1 the Pareto diagram.

202



203

204 **Figure 1.** Pareto diagram obtained to the experiments performed.

205 **Table 3.** Analysis of Variance (ANOVA) to the CCRD design.

206

<b>Factor</b>	<b>Sum of Squares</b>	<b>Degree of Freedom</b>	<b>Mean Square</b>	<b>F<sub>Calculated</sub></b>	<b>p-value</b>
(1) Enzyme Load (vol%) (L)	687.58	1	687.58	27.63	0.0033 <sup>a</sup>
Enzyme Load (vol%) (Q)	303.90	1	303.90	12.21	0.0174 <sup>a</sup>
(2) Temperature (°C) (L)	20.76	1	20.76	0.83	0.4029 <sup>b</sup>
Temperature (°C) (Q)	1,408.81	1	1,408.81	56.62	0.0007 <sup>a</sup>
1L by 2L	0.03	1	0.03	< 0.01	0.9749 <sup>b</sup>
Error	124.40	5	24.88		
Total SS	2,287.70	10			

207

<sup>a</sup> Significant at “Prob > F” less than 0.05;

208

<sup>b</sup> Insignificant at “Prob > F” more than 0.05.

209

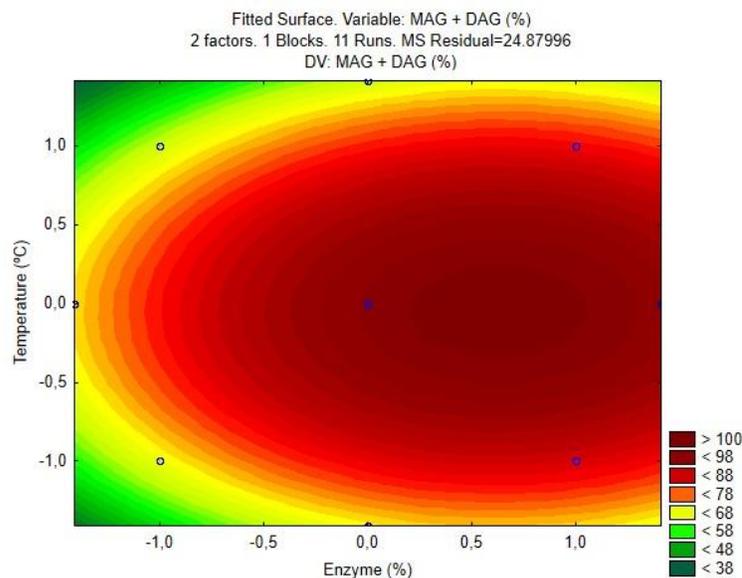
210 Significant variables presented  $p < 0.05$ . Coefficients with a positive sign indicate a  
211 synergic effect whereas negative coefficients indicate an antagonistic effect in the formation of  
212 MAG and DAG [24]. Moreover, the results presented show that any variation in the system  
213 significantly changes the conversion, statistically stating. This can be explained because an  
214 emulsion composes the system, and this emulsion needs balance and stability, which can be  
215 affected depending on the compound that is added to the medium. In turn, when using a lipase to  
216 promote the catalysis, a special attention must be given to parameter “reaction temperature”, since  
217 the enzymes are a temperature sensitive protein. This implies that any value below its optimum  
218 temperature, the enzymatic activity could be considerably reduced and, mainly, above the optimal  
219 temperature, can occurs the protein denaturation and activation loss, which is irreversible,  
220 drastically affecting the process yield. Therefore, the knowledge of the “ideal” process conditions  
221 is fundamental. Since the process variables are kept in this “ideal” range so that the synthesis of  
222 MAG and DAG is maximum, increasing the reaction efficiency [25].

223 The results presented in the Table 1 and 3, and the Pareto diagram (Figure 1), shows an  
224 interactive effect between the variables “enzyme load” and “system temperature” and an  
225 independent analysis of the parameters would not be appropriate. Therefore, a contour surface  
226 between the parameters considered is necessary to find the region with the best response and thus  
227 determine the optimum conditions of the system, where the formation of MAG and DAG is  
228 maximum. These results can be observed in the Figure 2 in terms of volumetric concentration of  
229 enzyme. The data presented shows that the region of most significant conversion of the  
230 triglycerides that composed the feedstock used is close to the central point of the statistical design.  
231 In other words, the temperature is around 35 °C and the enzyme load between 7.5 to 9 vol%.  
232 According previously discussed, temperatures above 40 °C hampered the raw material conversion  
233 since the catalytic capacity of the lipase was negatively affected due these high temperatures. On

234 the other hand, temperatures below 30 °C were not sufficient to activate the catalyst, also resulting  
235 in low yields. Lower values of temperatures tend to cause a decrease of enzymatic activity, whereas  
236 larger values can cause potential protein denaturation and in both, reduction of the activity occurs  
237 [26, 27]. Regarding the biocatalyst load, as expected, an increase in the lipase concentration used  
238 in the medium returned an increase in the observed conversions. However, it is worth noting that  
239 there was little difference in the TAG conversions when the lipase load increased from 7.5 vol%  
240 to 10 vol%, making it more economically viable to adopt the value of 7.5 vol% of Lipozyme TL  
241 100 as ideal. It is not difficult to observe in situations when an excess of enzyme is used a formation of  
242 agglomerates that result in a mass transfer resistance and consequently decreasing the  
243 conversion. Thus, the best conditions obtained from the results presented is 35 °C, 7.5 vol% of  
244 Lipozyme TL 100, a molar ratio of 2:1 (glycerolysis:oil) at 600 rpm rate and 2 h of reaction,  
245 resulting in an average conversion of 98.5 %.

246         The results found for the best conditions are in agreement with the study published by  
247 Raizer [28]. This study synthesized DAG by enzymatic glycerolysis from sunflower oil by  
248 ultrasound assistance and used a CCRD with temperature and amount of enzyme as independent  
249 variables. Through ANOVA and Pareto Diagram, the author concluded that the most significant  
250 variable was the enzyme load.

251



252

253 **Figure 2.** Contour surface for TAG conversion varying temperature and biocatalyst load.

254

## 255 **Mathematical Modeling**

256 The kinetic constants were initially estimated using 7.5 vol% of the Lipozyme TL 100  
 257 enzyme and assuming the hypothesis of having a reaction coefficient as an independent factor in  
 258 the medium. Such parameter is important since the use of excess enzyme may result in enzyme  
 259 aggregation, which ends up exposing the active site of the enzyme to substrate molecules and thus,  
 260 the model would not be efficient and would have to reformulate the conditions of analysis. The  
 261 values of the estimated parameters obtained to the model are presented in Table 4. The results of  
 262 the simulations are presented in Figures 3 to 5. It is possible to observe that the proposed model  
 263 appropriately represented the kinetics of glycerolysis of olive oil.

264

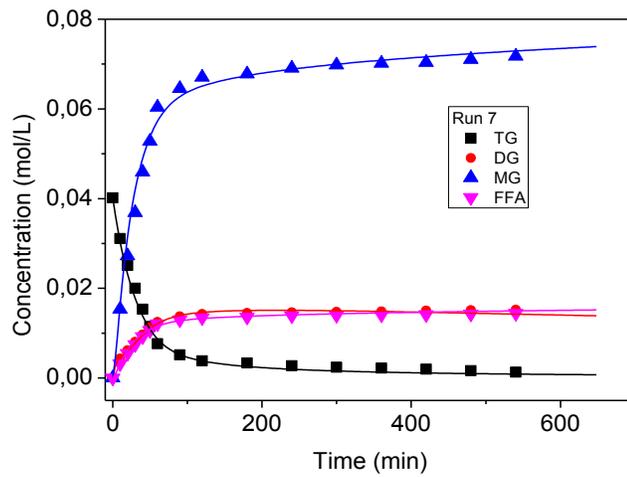
265 **Table 4.** Estimated kinetic reaction constants.

266

<b>Parameter</b>	$K_1$ (L·mol <sup>-1</sup> ·min <sup>-1</sup> )	$K_{-1}$ (min <sup>-1</sup> )	$K_2$ (min <sup>-1</sup> )	$K_3$ (L·mol <sup>-1</sup> ·min <sup>-1</sup> )	$K_{-3}$ (min <sup>-1</sup> )	$K_4$ (min <sup>-1</sup> )
<b>Estimated Value</b>	13.76	181.99	53.27	$1.108 \cdot 10^4$	$8.179 \cdot 10^3$	$2.725 \cdot 10^3$
<b>Parameter</b>	$K_5$ (L·mol <sup>-1</sup> ·min <sup>-1</sup> )	$K_{-5}$ (min <sup>-1</sup> )	$K_6$ (min <sup>-1</sup> )	$K_7$ (L·mol <sup>-1</sup> ·min <sup>-1</sup> )	$K_{-7}$ (L <sup>2</sup> ·g <sup>-1</sup> ·mol <sup>-1</sup> ·min <sup>-1</sup> )	$K_8$ (L·mol <sup>-1</sup> ·min <sup>-1</sup> )
<b>Estimated Value</b>	3.47	90.21	7.33	37.44	1.83	1.90
<b>Parameter</b>	$K_8$ (L <sup>2</sup> ·g <sup>-1</sup> ·mol <sup>-1</sup> ·min <sup>-1</sup> )	$K_9$ (L·mol <sup>-1</sup> ·min <sup>-1</sup> )	$K_9$ (L <sup>2</sup> ·g <sup>-1</sup> ·mol <sup>-1</sup> ·min <sup>-1</sup> )	$K_{10}$ (L·mol <sup>-1</sup> ·min <sup>-1</sup> )	$K_{10}$ (L <sup>2</sup> ·g <sup>-1</sup> ·mol <sup>-1</sup> ·min <sup>-1</sup> )	
<b>Estimated Value</b>	0.50	23.49	145.92	65.26	7.04	

267

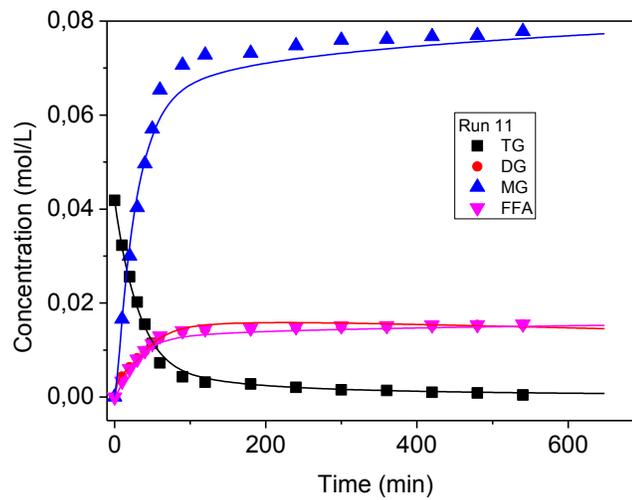
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269

270 **Figure 3.** Kinetics of glycerolysis of olive oil. Reaction conditions: 35 °C, 7.4 % of glycerol, 10  
 271 vol% of olive oil, 5 vol% of water and 7.5vol % enzyme.

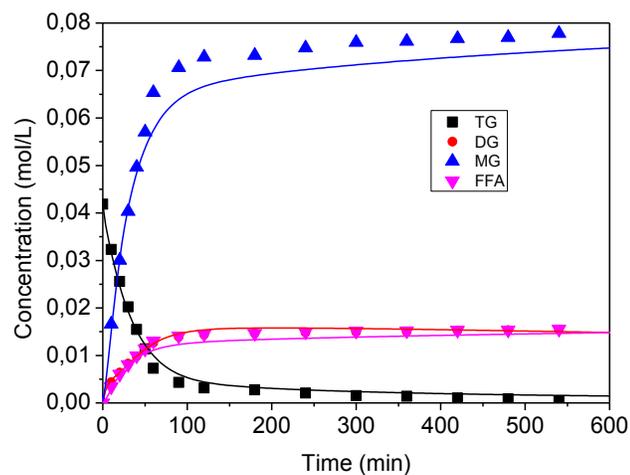
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273

274 **Figure 4.** Kinetics of glycerolysis of olive oil. Reaction conditions: 35 °C, 7.69 % of glycerol, 10  
 275 vol% of olive oil, 10 vol% of water and 7.5 vol% of enzyme.

276



277

278 **Figure 5.** Kinetics of glycerolysis of olive oil. Reaction conditions: 35 °C, 8.0 % of glycerol, 10  
 279 vol% of olive oil, 15 of vol% of water and 7.5 vol % of enzyme.

280

281 Experimental kinetics data show that the concentration of TAG had a significant reduction  
 282 between 0 and 120 minutes, indicating that the amount of enzyme used was adequate to promote  
 283 the reaction. In the same way, it is verified that the higher production of MAG and DAG was in  
 284 this period of time since such products are formed from the decomposition of the TAG. This was  
 285 also observed by Corzo-Martínez et al. [29] and Fiametti [30] that the higher formation of MAG  
 286 and DAG occurred within the first 120 minutes of reaction. It was also noted that the formation of  
 287 MAG was much higher than that of DAG, but both stabilized at similar times, which indicates  
 288 another sign that the amount of enzyme was sufficient in the process.

289 This model of curves is characteristic in TAG hydrolysis reactions, even with different  
 290 matrices. In the work of Solaesa et al. [25], the authors performed enzymatic glycerolysis tests on  
 291 several systems with sardine oil as a source of TAG. As such, they analyzed levels of MAG and  
 292 DAG in micellar systems and catalyzed by the enzyme Lipozyme 435. Comparing all the kinetic  
 293 curves of the TAG, MAG, DAG and FFA compounds, it is seen that there is the same pattern of

294 formation, with more considerable amounts of MAG formation, followed by DAG and, finally,  
295 FFA.

296         When analyzing the water fraction in the reaction medium, it did not present significant  
297 differences, since the limiting factors for the process were the amount of enzyme and temperature  
298 of the system. According studies conducted by Irimescu et. al [31] and Yang et. al [32]  
299 microemulsions with several enzymes were tested, among them lipases, immobilized or not, and  
300 achieved significant TAG conversion rates, and the amount of water was also crucial for the  
301 stabilization of the system micellar. On the other hand, in the work of Moya-Ramírez et al. [33],  
302 the authors obtained MAG and DAG from used frying oil and the reaction of glycerolysis with the  
303 use of several enzymes was strongly dependent on the amount of water inside the micellar system  
304 and the conditions with higher enzyme concentrations, better results were obtained. However, the  
305 conversion range of triglycerides was in the range of 30 % inferior, relatively low when compared  
306 to the present study. This may have occurred due to the raw material used by the authors (used  
307 frying oil) contains several impurities and contaminants due to the breakage and oxidation that the  
308 oil had undergone.

309         Another critical parameter in the glycerolysis reaction is the formation of a stable micellar  
310 system. The high conversions of consumption of triglycerides show that this happened in the cases  
311 studied. All the results obtained can be tested, for example, in the automotive lubricant industries,  
312 as an alternative emulsifier between base oil and performance additives.

313

314

315

## 316 **Conclusions**

317 In this work, the enzymatic glycerolysis reaction of olive oil was investigated using  
318 glycerolysis catalyzed by the commercial liquid enzyme Lipozyme TL 100L. The results of the  
319 experimental design showed that the amount of enzyme used and the temperature of the system  
320 were significant variables in the conversion of triglyceride. The experimental condition of 35 °C,  
321 7.5 vol% of Lipozyme TL 100 enzyme, 2:1 molar ratio (glycerolysis:oil) and reaction time of 2  
322 hours was the highest conversion of triglycerides (98.45 %). The mathematical model that  
323 considers the reaction mechanism of Ping Pong Bi Bi type and hydrolysis reactions proved to be  
324 efficient to describe reaction kinetics and can be an essential tool for reactor projects that operate  
325 at larger scales.

326

## 327 **Declarations**

328 - Ethical Approval: This article does not contain any studies with human participants or animals  
329 performed by any of the authors.

330 - Consent to Participate: All authors have consented to participate in the paper.

331 - Consent to Publish: All authors have consented to publish the paper.

332 - Authors Contributions:

333 George F. Finco: experimental data collection

334 Karina G. Fiametti: laboratory assays execution

335 Edson A. da Silva: experimental design and modeling

336 Fernando Palú: experimental data execution

337 João H. C. Wancura: manuscript writing and discussion

338 Alexsandra Valério: research planning

339 J. Vladimir Oliveira: overall research planning, writing the manuscript

340 - Funding: No funding, not applicable.

341 - Competing Interests: There is no conflict or competing interest.

342 - Availability of data and materials: Not applicable.

343

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463

## List of Figure Captions

464

465

466 **Figure 1.** Pareto diagram obtained to the experiments performed.

467

468 **Figure 2.** Contour surface for TAG conversion varying temperature and biocatalyst load.

469

470 **Figure 3.** Kinetics of glycerolysis of olive oil. Reaction conditions: 35 °C, 7.4 vol% of glycerol,  
471 10 vol% of olive oil, 5 vol% of water and 7.5vol % enzyme.

472

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477 10 vol% of olive oil, 15 of vol% of water and 7.5 vol % of enzyme.

478

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480

481 **Table 1.** CCRD matrix, with real and coded values used in the reaction of glycerolysis catalyzed  
482 by Lipozyme TL 100L and conversions obtained to the TAG.

483

484 **Table 2.** Reaction conditions applied in the kinetics analysis of the system.

485

486 **Table 3.** Analysis of Variance (ANOVA) to the CCRD design.

487

488 **Table 4.** Estimated kinetic reaction constants.

# Figures

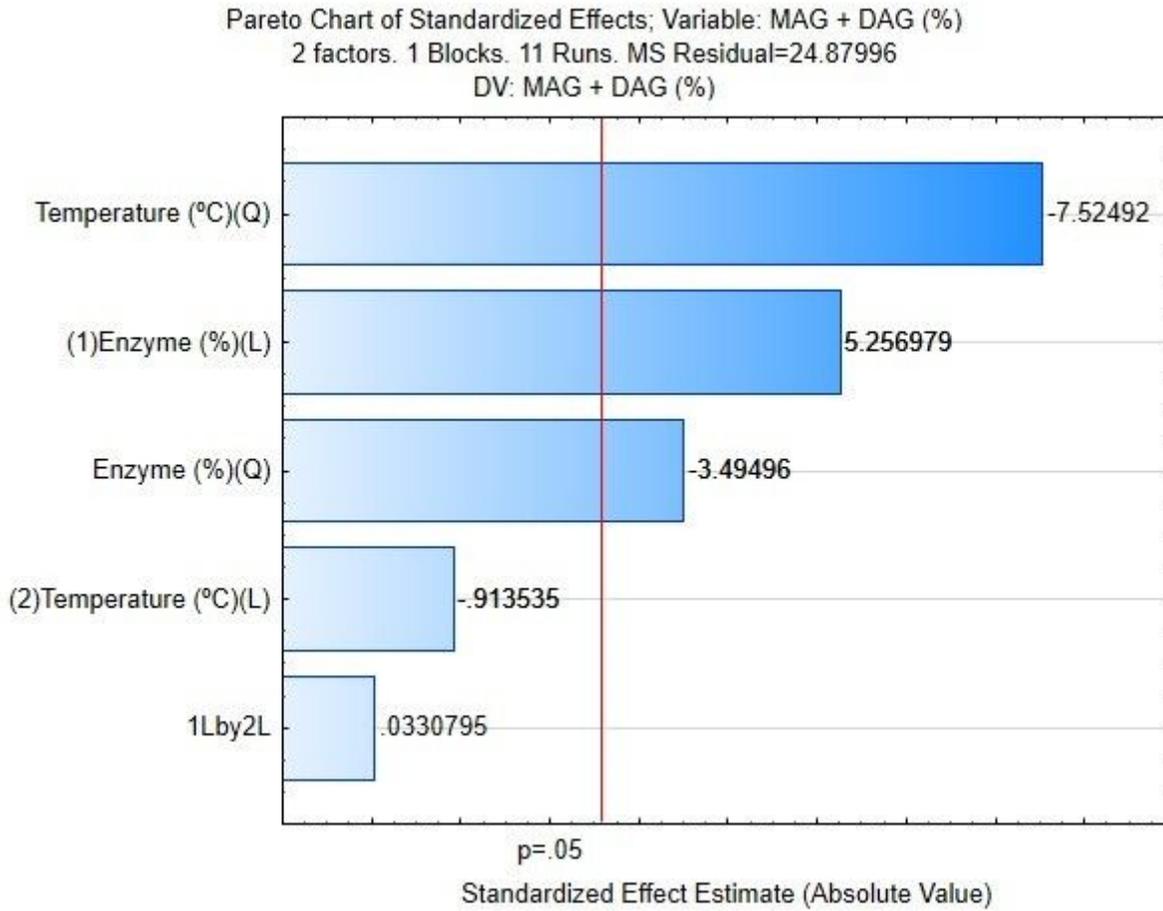


Figure 1

Pareto diagram obtained to the experiments performed.

Fitted Surface. Variable: MAG + DAG (%)  
2 factors. 1 Blocks. 11 Runs. MS Residual=24.87996  
DV: MAG + DAG (%)

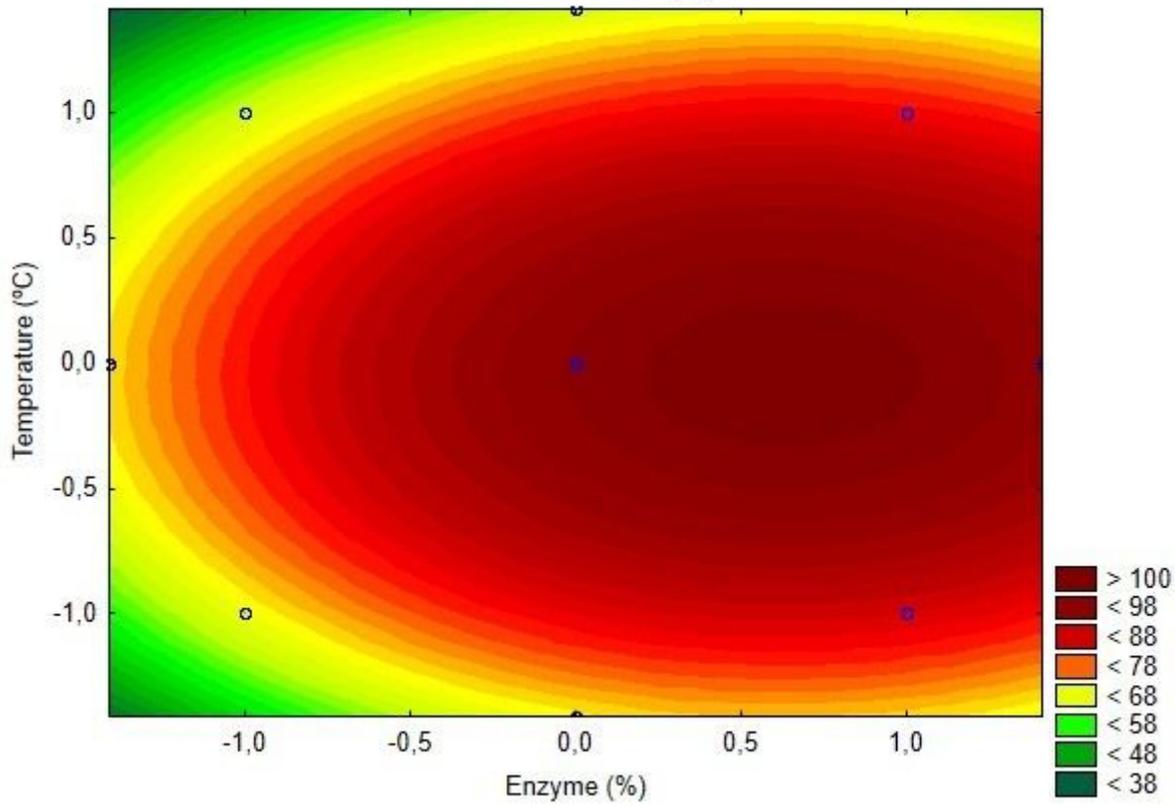


Figure 2

Contour surface for TAG conversion varying temperature and biocatalyst load.

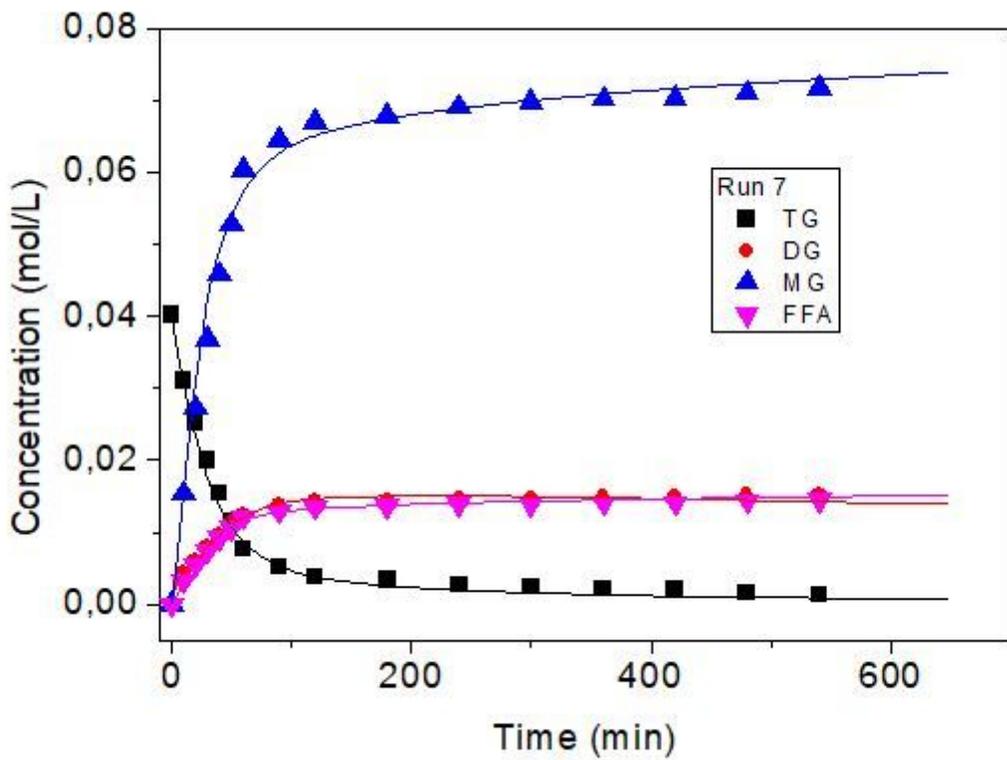


Figure 3

Kinetics of glycerolysis of olive oil. Reaction conditions: 35 °C, 7.4 vol% of glycerol, 10 vol% of olive oil, 5 vol% of water and 7.5vol % enzyme.

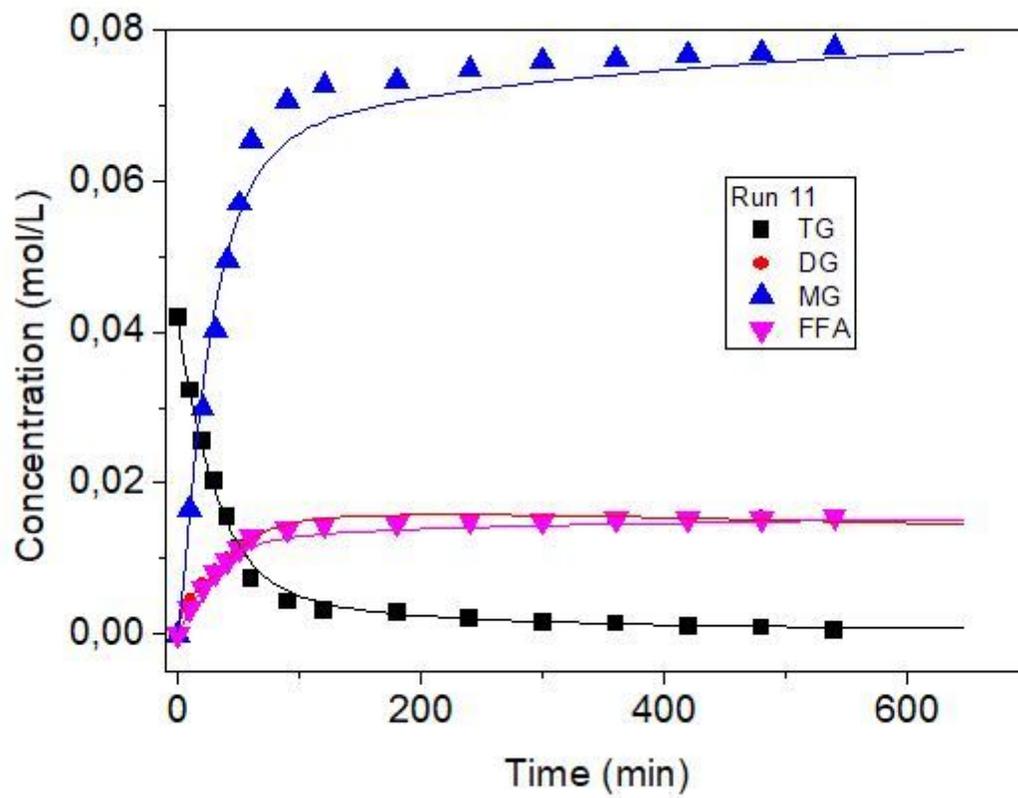
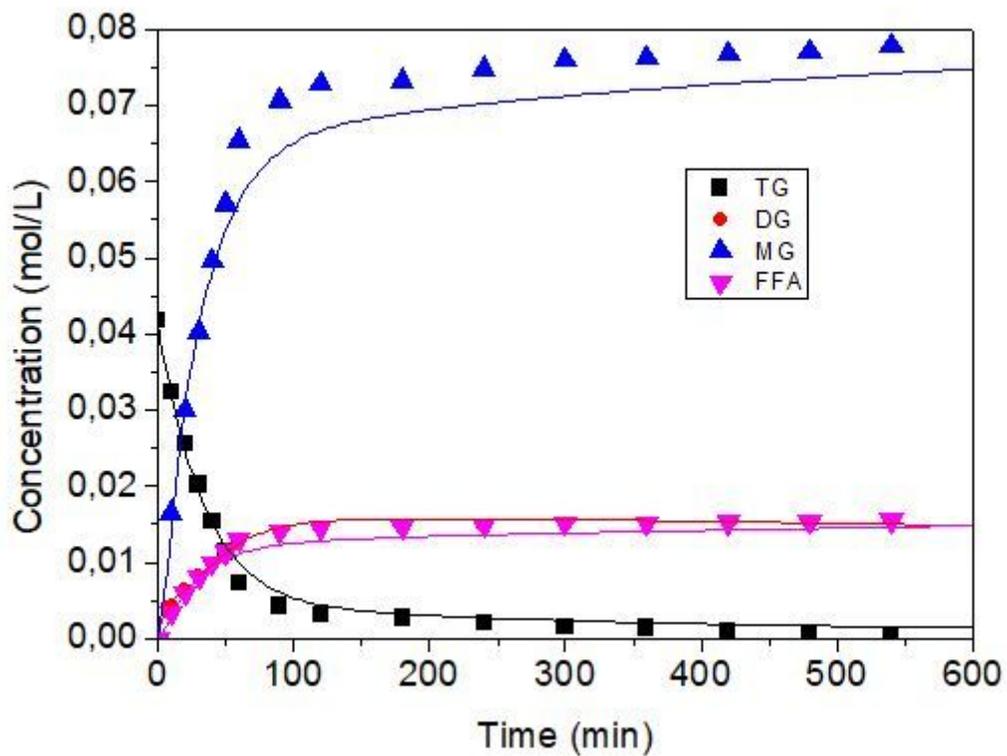


Figure 4

Kinetics of glycerolysis of olive oil. Reaction conditions: 35 °C, 7.69 vol% of glycerol, 10 vol% of olive oil, 10 vol% of water and 7.5 vol% of enzyme.



**Figure 5**

Kinetics of glycerolysis of olive oil. Reaction conditions: 35 °C, 8.0 vol% of glycerol, 10 vol% of olive oil, 15 of vol% of water and 7.5 vol % of enzyme.