Lipase-catalyzed glycerolysis of anchovy oil in a solvent-free system: Simultaneous optimization of monoacylglycerol synthesis and end-product oxidative stability

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ABSTRACT

The production of mono- and diacylglycerols rich in polyunsaturated fatty acids is achieved in this study, by solvent-free glycerolysis of anchovy oil with lipase PS-DI from *Burkholderia cepacia*. Attention is focused on the oxidative stability of the reaction products, determined in terms of induction time (I_t). The effects of glycerol/triacylglycerol molar ratio, enzyme concentration, and reaction temperature on mono- and diacylglycerol production and I_t are all assessed. The operating conditions that optimized monoacylglycerol yields and oxidative stability were a glycerol/triacylglycerol ratio of 3/1, 9.0% (w/w) Lipase PS-DI, a stirring rate of 200 rpm, and a reaction time of 4 h, at 45.8 ºC, producing a content of 24.8% and 51.9% of mono- and diacylglycerols, respectively, over an I_t of 1.41 h. The glycerolysis conditions determined by simultaneous optimization strategy increased the oxidative stability of the glycerolysis products by 68%, which rose from 0.84 h (individual optimization) to 1.41 h.

Keywords: Lipase PS-DI; Monoacylglycerols (MAG); oxidative stability; polyunsaturated fatty acids (PUFA); solvent-free glycerolysis

1. Introduction

Mono- (MAG) and diacylglycerols (DAG) are non-ionic surfactants widely used as emulsifiers in many food products (e.g. margarines, sauces, dairy and confectionery products) (Kristensen, Xu

& Mu, 2005; Panwongrat, Xu & H-Kittikun, 2007). Additionally, they are also used in cosmetic, pharmaceutical, and textile products, due to their plasticizing and texturizing properties (Chang & Bodmeier, 1998; Valério, Rovani, Treichel, de Oliveira & Oliveira, 2010). The production of MAG on an industrial scale is currently performed by chemical glycerolysis of fats and oils using alkaline catalysts under a nitrogen gas atmosphere at very high temperatures. However, rather low yields are obtained with this procedure and the aggressive reaction conditions lead to severe fatty acid oxidation. The products tend to show a dark color and unwanted byproducts are generated that require additional purification (Feltes et al., 2012; Ghamgui, Miled, Rebaï, Karrachaâbouni & Gargouri, 2006).

Enzymatic catalysis has great potential as an alternative to chemical processes, due to properties such as substrate specificity, mild operational conditions, easy control, and the reusability of immobilized biocatalysts (Weber & Mukherjee, 2004). In practice, lipase-catalyzed glycerolysis is a complex process with several equilibriums from which it is difficult to obtain only one reaction product (Cheirsilp, Kaewthong & H-Kittikun, 2007; Tan & Yin, 2005). Moreover, the high difference in polarity between glycerol (Gly) and tryacylglycerol (TAG) assists the formation of a heterogeneous reaction system with a limited mass transfer. Several alternatives, such as the addition of food additives as surfactants (Valério et al., 2009), the use of organic solvents (Cai, Gao, Liu, Zhong & Liu, 2016; Yang, Rebsdorf, Engelrud, & Xu, 2005), ionic liquids (Kahveci, Guo, Özçelik & Xu, 2010), and supercritical fluids (Moquin, Temelli, Sovová & Saldaña, 2006), have been followed to improve the miscibility of the glycerolysis reaction, thereby improving its yield. Despite the advantages of enzymatic catalysis, these technologies involve additional separation steps and recovery systems with high costs that currently hinder any commercial-scale application of this technique (Feltes et al., 2012; Fiametti, Rovani, de Oliveira, Corazza, Treichel & Oliveira, 2009). Glycerolysis in a solvent-free system represents a promising option, in view of the increasing interest in developing simple and efficient separation processes. Solvent-free systems have many

53 advantages such as reduced pollution, low costs, and simplicity in both processing and handling
54 (Tanaka & Toda, 2000).

55 Several oils and fats have been used as starting materials for MAG production. However, over
56 the last decade, fish oils have received a lot of attention because of their content in n-3
57 polyunsaturated fatty acids. PUFA are health-beneficial fatty acids, because of their role in the
58 regulation of inflammation, cholesterol metabolism, and brain functions (Nicholson, Khademi &
59 Moghadasian, 2013). Nevertheless, even frozen PUFA easily reacts with oxygen and forms
60 hydroperoxide and free radicals (Miyashita, 2014), due to the high number of its double bonds.
61 Moreover, the volatile compounds during oxidation lead to flavor deterioration reducing their
62 acceptance among consumers. More than an organoleptic problem, lipid oxidation also has
63 nutritional and health implications. In fact, the increased use of n-3 PUFA in the food industry has
64 resulted in new challenges concerning the avoidance or at least the minimization of lipid oxidation
65 (Jacobsen, 2015). Nevertheless, the measurement of lipid oxidation is a difficult task, due to its
66 complex oxidation process that depends on many factors. Numerous analytical methods for
67 determining lipid oxidation in foods have been developed, based on the measurement of oxygen
68 adsorption, the peroxide value, the formation of free radicals and primary and secondary oxidation
69 products, as well as the evaluation of the oil stability index (Shahidi, Wang & Wanasundara, 2017).
70 Rancimat is a method that companies have frequently used to determine the oil stability index. It
71 determines the conductivity of volatile oxidation products dissolved in water. The time that elapses
72 until the point when volatile acids are detected in the measuring vessel is referred to as the
73 induction time (I_t) or the oil stability index (Dabrowski, Konopka, Czaplicki & Tariska, 2017). I_t
74 characterizes the oxidation stability of oils and fats: the lengthier the I_t induction time, the more
75 stable the sample.

76 Several studies have dealt with the lipase-catalyzed synthesis of MAG and DAG enriched in
77 PUFA in non–conventional media (Fiametti et al., 2009; He, Li, Kodali, Balle, Chen & Guo, 2017;

78 Pawongrat et al., 2007). However, none of these studies considered the oxidative stability of the
79 reaction products during the design of the process.

80 Based on these considerations, the aim of this work will be to synthesize MAG and DAG
81 enriched in PUFA, in a solvent-free glycerolysis system using the immobilized Lipase PS-DI,
82 thereby maintaining the oxidative stability of the reaction end-products at a maximum. To that end,
83 the effects of reaction temperature, the Gly/TAG substrate molar ratio, and, enzyme concentrations
84 in MAG and DAG production will all be studied using response surface methodology (RSM). The
85 induction time (I_t), will also be determined to obtain the reaction conditions that minimize end-
86 product oxidation levels. Likewise, the desirability function approach of Derringer will be applied
87 for simultaneous optimization of MAG yields and oxidative stability.

88 **2. Materials and methods**

89 ***2.1. Materials***

90 Anchovy oil used in glycerolysis reactions, with a PUFA content of 34.6% (7.6%
91 eicosapentaenoic acid, EPA, and 12.7% docosahexaenoic acid, DHA), was supplied by Denomega
92 Nutritional Oils (Leknes, Norway). Lipase from *Burkholderia cepacia*, Lipase PS-DI, commercially
93 immobilized on diatomite, was provided by Sigma-Aldrich Co (St. Louis, MO, USA). The MAG,
94 DAG, and TAG standards for HPLC and Glycerol (99%) were obtained from Sigma-Aldrich Co
95 (St. Louis, MO, USA). Isooctane, methyl tert-butyl ether and acetic acid of HPLC grade were
96 purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Silica gel 60 extra pure was supplied by
97 Merck-Millipore (Madrid, Spain).

98 ***2.2. Enzymatic glycerolysis***

99 Glycerolysis was done in a screw-capped crystal flask by mixing different amounts of Gly with 6
100 g of anchovy oil to prepare molar ratios of 1/1, 2/1, and 3/1, according to the experimental design.
101 Previously, the adsorption of Gly onto silica gel had been performed, as described Berger, Laumen
102 & Schneider (1992), to overcome the solubility problems of glycerol in organic phase and to

prevent its aggregation on immobilized enzymes (Castillo, Dossat, Marty, Condoret & Combes, 1997). The enzymatic reaction was initiated by adding the Lipase PS-DI. The water content of the immobilized enzyme had previously been adjusted over saturated LiCl solution (Pawongrat, Xu & H-Kittikun, 2008) in a desiccator at 25 ºC for 24 h (a_w = 0.113). The reaction mixture was incubated, under dark conditions, at different temperatures (40, 50, and 60 ºC) with shaking at 200 rpm, over 4 h, based on previous studies performed in our laboratory. At the end of the reaction period, the immobilized lipase was removed by centrifugation (5,000 rpm for 5 min) and the products were recovered for further analysis.

2.3. Quantitative analysis of reaction products

Reaction products were quantified by using a HPLC-ELSD system from Agilent 1200 Series, with a Lichrospher 100 Diol column (4x250 mm, 5µm) at 35 ºC and 0.35 MPa. The reaction mixture was diluted in isooctane and separated using an elution gradient. The mobile phase was a mixture of isooctane and methyl tert-butyl ether/acetic acid (99.9/0.1, v/v) with a flow rate of 1 mL/min (Solaesa, Sanz, Falkeborg, Beltrán & Guo, 2016).

The reaction products are presented as mass fraction (%, w/w, based on the total oil) of MAG, DAG, TAG, and FFA. All data were the mean of two experiments with a deviation within 2.7%.

2.4. Oxidative stability

Oxidative stability in terms of induction time (I_t) was determined with a Rancimat apparatus 743 (Metrohm, Herisau, Switzerland). Samples of 2.0 g were oxidized at 110 ºC with a constant air flow of 9 L/h. Determination of I_t was based on the conductimetric detection of volatile oxidation products. The time that elapsed until these oxidation products appeared was the I_t (Dabrowski et al., 2017). All data were the mean of two experiments with a deviation within 2.9%.

2.5. Experimental design, statistical analysis, and multiple response optimization

Response surface methodology (RSM) was used for modelling the enzymatic glycerolysis and to optimize the reaction conditions that maximize MAG and DAG yields. RSM is a powerful tool that

permits a simultaneous study of several factors in a reduced number of experiments taking account of interactions between factors. A three-factor factorial design with two face-centered cube central levels was applied. The factors chosen were reaction temperature, substrate molar ratio (Gly/TAG), and enzyme concentration. Seventeen runs consisting of 8 factorial points, 6 star points, and 3 center points were performed as per the experimental design (Table 1). The response variables were MAG, DAG, and TAG contents, and induction time (I_t). The optimization of the conditions and the response surfaces were calculated using Statgraphics Centurion XVI (version 16.2.04). This software package was also used to fit the second-order model to the independent variables, by using the following equation:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i}^{i<j} \sum_{j} \beta_{ij} X_i X_j + e \quad (1)$$

Where y is the dependent variable (response) to be modelled, X_i and X_j are the independent variables (factors), β_o, β_i, β_{ii} y β_{ij} are regression coefficients, and e is the error term. The model was simplified by means of analysis of variance (ANOVA) in which statistically significant ($p>0.05$) terms were discarded.

Derringer's desirability function (Candioti, de Zan, Cámara, & Goicoechea, 2014) was used to establish the experimental conditions (factor levels) and, simultaneously, the optimal value for MAG production and oxidative stability (I_t). Individual responses are transformed into desirability functions with this method and then combined into a single one called global desirability (D) using the following equation:

$$D = \left(d_1^{r1}\, d_2^{r2}\, \ldots d_k^{rk}\right)^{1/\Sigma rk} \quad (2)$$

where, d_k is an individual desirability function of each k response and rk is the relative importance over the global desirability. The relative importance of the individual response was established by assigning different weights (very high, high, medium...) with the Statgraphics Centurion software.

3. Results and discussion

3.1. Factors affecting the MAG and DAG production

The glycerolysis yield and the relative content of the different components of the end-product are strongly dependent on several parameters such as reaction temperature, enzyme load, water activity, reaction time, and substrate molar ratio. The optimization of these parameters is an essential task to convert the glycerolysis in a suitable process for MAG and DAG production. Table 1 presents the experimental design matrix with the reaction conditions (reaction temperature, enzyme load, and Gly/TAG molar ratio) and the corresponding response variables of the 17 experiments performed in a randomized order.

Free fatty acids (FFA) were only detected in 7 out of 17 experiences, with a content of less than 4.5% (Table 1). A result that indicated that water activity limited the hydrolysis reactions and promoted the esterification of FFA produced during the glycerolysis. In this regard, von der Haar, Stäbler, Wichmann & Schweiggert-Weisz (2015) described Gly and MAG as acceptable acyl group acceptors in lipase catalyzed reactions, making the re-esterification possible of an important fraction of the FFA produced by hydrolysis.

The data in Table 1 were analyzed and fitted to second-order polynomial models. Regression coefficients (β) and significance (*P)* values of the model generated for MAG, DAG, and TAG production are given in Table 2. Analysis of variance (ANOVA) showed that the predicted models satisfactorily explained the relationship between responses and factors with a non-significant lack of fit. The coefficients of determination, R^2, for MAG, DAG, and TAG production (0.92, 0.81, 0.84, respectively) showed a good correlation between the observed and the predicted values.

The statistical analysis of the variance for MAG production showed that only four terms were significant in the model: the linear terms of the substrate molar ratio, Gly/TAG, the enzyme concentration, and the interaction of these two factors with the reaction temperature. All variables under study had a significant effect on DAG production, except for the interaction between the substrate molar ratio Gly/TAG and the reaction temperature.

177 A three-dimensional response surface plot (Fig. 1) was generated utilizing the second-order
178 polynomial functions (Table 2). An increase of enzyme load, in the area under examination, led to a
179 lineal rise of MAG content and the quadratic behavior of DAG production (Figs. 1a and b,
180 respectively). Figs. 1b and 1c show that increasing enzyme concentrations from 1% to 5%
181 improved the DAG yield and reduced the TAG content. Further increments in enzyme
182 concentration did not follow this trend, probably due to Lipase PS-DI that began to use mainly
183 DAG and Gly as substrates instead of TAG. As other authors have mentioned, initially, the DAG
184 content is higher than the MAG content in the reaction, but as it evolves, DAG as an intermediate
185 reaction product is progressively converted into MAG (Cheirsilp et al., 2007; Valério, Rovani,
186 Treichel, de Oliveira & Oliveira, 2009). Higher enzyme concentrations therefore lead to a more
187 rapid development of the reaction that yields a higher content of MAG than lower enzyme
188 concentrations can produce (Fig. 1a). This result is in agreement with previous reports showing that
189 an increase in lipase loading increased the MAG production (Watanabe et al., 2003; Yang et al.,
190 2005). Nevertheless, Valério et al. (2010) described enzyme loadings higher than 10.5 wt% with no
191 increased yields.
192 According to the stoichiometric ratio of the reaction, equilibriums can be enhanced toward MAG
193 or DAG production by controlling the substrate balance Gly/TAG that is applied. Fig. 1b shows that
194 DAG production improved when the substrate molar ratio was increased at lower enzyme
195 concentrations, while no significant differences were observed in assays with higher dosages of
196 enzymes. Otherwise, the use of a Gly/TAG molar ratio higher than 2 is a common strategy to
197 enhance the synthesis of MAG (Zhong, Li, Xu, Cheong, Zhao & Li, 2010). Thus, when the
198 Gly/TAG molar ratio was increased from 1 to 3, with 9% Lipase PS-DI at 60 ºC, the MAG content
199 improved from 14.03 to 30.50% (Table 1). Moreover, the use of glycerol absorbed on silica gel
200 facilitated the conversion of TAG (Yeoh, Choong, Abdullah, Yunus & Siew, 2009). Similar trends
201 have also been observed in recent studies, showing that the use of a relatively high content of Gly

202 rather than TAG favored TAG conversion in the glycerolysis reaction (Cai et al., 2016). On the
203 other hand, when the same reaction conditions were used, little or no difference in DAG production
204 was observed (from 46.23 to 47.46 %) (Table 1).

205 Temperature is another very important and complex factor that will, in different ways, affect
206 both the reaction yield and the relative distribution of glycerolysis products. The analysis of the
207 variance indicated that DAG production and TAG conversion were negatively affected by the
208 reaction temperature, while the same factor was not significant in the model for MAG production.
209 Fig. 2a shows no change in MAG production at increased temperatures, following the application of
210 a lipase concentration of 1% and a substrate molar ratio of 3/1. In contrast, the MAG yield was
211 raised from 21.08% to 29.67% (predicted values), with the highest enzyme content (9%) and at
212 increased temperatures (40-60 ºC). This development can be caused by a fast reaction in the
213 presence of a high enzyme concentration, while longer reaction times at lower enzyme
214 concentrations are necessary to achieve similar progress.

215 In contrast, an increase in the reaction temperature led to a slight decrease in the production of
216 DAG, regardless of the enzyme concentration (Fig. 2b). This decrease was greater for glycerolysis
217 at high enzyme concentrations.

218 Regarding substrate ratios, no apparent changes in MAG production were observed at 40 ºC,
219 with 5% immobilized enzyme Lipase PS-DI, although there was an increase of Gly/TAG (data not
220 shown). In contrast, MAG yields rose from 19.49% to 25.17% (predicted results) when the
221 temperature increased from 40 to 60ºC. According to these results, the reaction of glycerolysis was
222 facilitated at higher temperatures, mainly due to an appreciable reduction of the viscosity of the
223 reaction medium and to improvements in substrate diffusion and its solubility (Feltes, Oliveira,
224 Treichel, Block, de Oliveira & Ninow, 2010).

225 The immobilization support of the enzyme also plays an essential role in the glycerolysis
226 reaction and is strongly related to the molar substrate ratio. Hydrophilic carriers lead to higher

227 glycerol concentrations in the microenvironment of the enzyme; the formation of a glycerol layer
228 around the lipase particles reduced the area of contact between the enzyme and the hydrophobic
229 substrates (TAG and DAG) (Ferreira-Dias, Correia, Baptista & da Fonseca, 2001). The Lipase PS-
230 DI support presented low hydrophilicity, which provided good lipase-oil interaction (Fregolente,
231 Fregolente, Pinto, Batistella, Wolf-Maciel & Filho, 2008). Hence, this commercially immobilized
232 lipase dispersed easily in the reaction mixture with a relatively high glycerol content (Kristensen,
233 Xu & Mu, 2005).

234 Based on the results that have been presented, the enzymatic glycerolysis of anchovy oil in a
235 solvent-free system produced relatively high MAG and DAG contents of 30.50 and 46.23%,
236 respectively. The experimental conditions that led to these results were 60 ºC, at a stirring rate of
237 200 rpm, and with 9.0% lipase PS-DI, using a glycerol to TAG molar ratio of 3:1, over a reaction
238 time of 4 h. On the other hand, the maximum DAG content (54.8%) was predicted at 40 ºC, with
239 5.6% lipase and a substrate molar ratio of 2.6. Nevertheless, the content of MAG was reduced to
240 19.6% under the latter conditions.

241 The content of MAG and DAG obtained in the present work is higher or similar to those reported
242 in the literature for different solvent-free systems. For example, Fregolente et al. (2008), in a batch
243 system using free and immobilized lipases, obtained both MAG and DAG production in the range
244 of 28-30% and 45-48% (w/w, based on the total oil), respectively, over 24 h, at 40-70 ºC, with an
245 enzyme content (w/w) of 10%. Feltes et al. (2012) produced 25% MAG and 41% DAG over 24 h,
246 at 70 ºC, at a glycerol-to-oil molar ratio of 1:1, with 5% (w/w) Novozyme 435 in a solvent-free
247 system via glycerolysis of menhanden oil.

248 Valério et al. (2010) used lipase Novozyme 435 to catalyze the glycerolysis of olive oil in a
249 solvent-free system adding 16% of the Tween 65 surfactant. These authors obtained a content of 26
250 and 17 wt% of MAG and DAG, over 2 h, at 70 ºC, using an enzyme concentration of 9%, and a
251 glycerol/olive oil molar ratio of 6:1 under stirring (600 rpm).

252 **3.2.** ***Factors affecting the oxidative stability of the glycerolysis products***

253 In this study, the other criterion for optimization of lipase-catalyzed glycerolysis was the
254 oxidative stability of the end-product determined in terms of induction time (I_t). The results
255 obtained are shown in Table 1. The oxidative stability of anchovy oil used as starting oil was 2.07 h,
256 while the experimental design results indicated an I_t range of between 0.62 and 1.57 h. These results
257 implied a reduction in the oxidative stability of glycerolysis products. The reaction conditions,
258 temperature and agitation, and changes in the composition and structure of the oil during the
259 glycerolysis process, could explain the lower oxidative stability of the end-products. Kahveci et al.
260 (2013) described changes in the position of fatty acid in the lipid structure that affected stability
261 when the exposure time of unsaturated fatty acids to oxygen was either decreased or increased.

262 The response and the variables under study were correlated by a second-order polynomial model
263 (Table 2). The statistical analysis indicated that the quadratic model had no lack of fit and predicted
264 a significant portion of the variance ($R^2 = 0.85$).

265 According to the ANOVA results, the quadratic terms of enzyme concentration and the
266 Gly/TAG substrate molar ratio had no significant impact on the model (Table 2). The variables with
267 the most important effect on induction time (I_t) were substrate molar ratio and the interaction
268 between Gly/TAG and reaction temperature.

269 A dimensional plot obtained for the I_t as a function of Gly/TAG at different reaction
270 temperatures for a lipase concentration of 5% is shown in Fig 3a. Increased Gly/TAG had a positive
271 effect on the oxidative stability of the reaction product, with a rise in the experimental I_t from 0.62
272 to 1.57 h when the temperature was set at 40 ºC (Table 1). The surface showed a decrease in the
273 oxidative stability (i.e. a reduction of I_t) of the glycerolysis products when the reaction temperature
274 increased. Nevertheless, the negative effect of temperature on oxidative stability was partially
275 diminished by using high amounts of Gly (Fig.3a). In addition to the reaction conditions, the
276 different oxidative stabilities of reactants can modify the I_t of the end-product. Thus, the increment

277 in the proportion of Gly, a molecule of higher oxidation stability than the fish oil rich in PUFA,
278 could have provided a higher stability to the reaction mixture. In contrast, Fig. 3b shows the effect
279 of reaction temperature and enzyme concentration on the oxidative stability of the glycerolysis
280 products. The highest stability was achieved at low temperatures (± 40 ºC) and at a high enzyme
281 concentration (9%).

282 Pearson's correlation was analyzed in the experiments, to determine the impact of the different
283 components (FFA, MAG, DAG and TAG) of the glycerolysis end-product on their oxidative
284 stability. The correlation coefficients calculated using Statgraphics Centurion XVI (data not shown)
285 indicated a positive relationship between DAG content with the variable response induction time.
286 The amount of FFA detected in the samples was too low to establish any correlation with oxidative
287 stability.

288 Several studies have determined the impact of partial glycerols and FFA on the oxidative
289 stability of edible oils. Colakoglu (2007), Miyashita & Takagi (1986), and Paradiso, Caponio,
290 Bruno, Pasqualone, Summo & Gomes (2014) indicated that the presence of FFA has a negative
291 impact on oxidative stability and, although MAG and DAG are generally recognized as pro-
292 oxidants, some aspects of their behavior on oxidative process remain unclear. According to
293 Caponio, Paradiso, Bruno, Summo, Pasqualone & Gomes (2011), MAG and DAG can act as either
294 pro-oxidants or antioxidants depending on their fatty acid composition and concentration.

295 On the basis of the above-mentioned results, the optimal experimental conditions for oxidative
296 stability (I_t of 1.57 h) were determined as 9% of immobilized lipase, a Gly/TAG molar ratio of 3,
297 and a temperature of 40 ºC. Under these glycerolysis conditions, the experimental yields of MAG
298 (20.30%) and DAG (52.99%) were very close to the predicted values, respectively estimated at
299 21.10% and at 52.40%.

300 ***3.3. Simultaneous optimization of MAG synthesis and oxidative stability***

301 According to previous results (Section 3.1 and 3.2), the reaction conditions that optimized each
302 individual response were high levels of enzyme concentration (9%) and a high Gly/TAG molar ratio
303 (3/1). However, while MAG synthesis was favored by temperatures of 60 ºC, the maintenance of
304 high oxidative stability required lower temperatures (40 ºC). Therefore, the reaction conditions to
305 optimize MAG production and oxidative stability are in conflict, due to the contrary effects of
306 reaction temperature on each individual response. Thus, a low oxidative stability was achieved (I_t of
307 0.79 h) with the best reaction conditions for MAG production. In contrast, the reduction of the
308 reaction temperature led to an increase in oxidative stability (I_t of 1.57 h), but MAG production fell
309 significantly (Table 1).
310 In this context, the optimal conditions should be selected in terms of the overall performance of
311 the reaction, avoiding those combinations of factors that can lead to important characteristics of the
312 final product that are outside the desired range. Thus, Derringer's desirability function allows us to
313 calculate the operating conditions that simultaneously optimize more than one variable response by
314 converting these multiple responses into a single one. Furthermore, factor levels may also be
315 included in this optimization procedure, in order to prioritize the use of certain suitable conditions
316 within the experimental region (Candioti et al., 2014).
317 The measured values of MAG content and I_t, and temperature as adjustable reaction parameters
318 were therefore transformed into partial desirability functions (Myers, Montgomery & Anderson-
319 Cook, 2016), using the Statgraphics Centurion (version XVI) software package, in order to calculate
320 the reaction conditions that cause optimal MAG synthesis and that simultaneously increase the
321 oxidative stability of the glycerolysis end-product. Subsequently, the global desirability function
322 (D) was determined, by considering different weights of the dependent variables, MAG and I_t
323 (Table 3). The most favored solutions corresponded with medium-medium and high-medium
324 weights of MAG-I_t variables, and global desirability functions of 0.77 and 0.62, respectively. An
325 optimized MAG and DAG content of 24.58% and 51.87%, respectively, and an oxidative stability

326 of 1.41 h, were achieved taking the medium-medium weighting. An increase in MAG content
327 (28.34%) was predicted with a D of 0.62, but with a decrease in oxidative stability, at an I_t of 1.10
328 h. Oxidative stability under both optimized conditions was about 68% and 31%, respectively, higher
329 than that obtained (0.84 h) by applying the glycerolysis conditions that maximized the individual
330 MAG yield (Table 3).

331 **4. Conclusions**

332 Glycerolysis of anchovy oil in a solvent free system has been investigated to produce MAG and
333 DAG rich in PUFA using immobilized lipase PS-DI. The present study has taken into account, for
334 the first time, the oxidative stability of the glycerolysis products during the design and the
335 optimization of the process.

336 The reaction conditions that provided the maximum MAG production and that simultaneously
337 maximized the oxidative stability were a Gly/TAG molar ratio of 3/1, with 9% (w/w) Lipase PS-DI,
338 stirred at 200 rpm, over 4 h, at either 45.8 or 54.7ºC, respectively yielding MAG amounts of
339 24.58% and 28.34%.

340 **References**

341 Berger, M., Laumen, K., & Schneider, M. P. (1992). Enzymatic esterification of glycerol I. Lipase-
342 catalyzed synthesis of regioisomerically pure 1,3-sn-diacylglycerols. *Journal of the American*
343 *Oil Chemists' Society*, *69*, 955–960.

344 Cai, C., Gao, Y., Liu, Y., Zhong, N., & Liu, N. (2016). Immobilization of *Candida antarctica* lipase
345 B onto SBA-15 and their application in glycerolysis for diacylglycerols synthesis. *Food*
346 *Chemistry*, *212*, 205–212.

347 Candioti, L. V., de Zan, M. M., Cámara, M. S., & Goicoechea, H. C. (2014). Experimental design
348 and multiple response optimization. Using the desirability function in analytical methods
349 development. *Talanta*, *124*, 123-138.

350 Caponio, F., Paradiso, V. M., Bruno, G., Summo, C., Pasqualone, A., & Gomes, T. (2011). Do

monoacylglycerols act as pro-oxidants in purified soybean oil? Evidence of a dose-dependent effect. *Italian Journal of Food Science*, *23*, 239-244.

Castillo, E. Dossat, V., Marty, A., Condoret, J. S., & Combes, D. (1997).The role of silica gel in lipase-catalyze esterification reactions of high-polar subastrates. *Journal of the American Oil Chemists' Society*, *74*, 77-85.

Colakoglu, A. S. (2007). Oxidation kinetics of soybean oil in the presence of monoolein, stearic acid and iron. *Food Chemistry*, *101*, 724-728.

Chang, C., & Bodmeier, R. (1998). Low viscosity monoglycerid-based drug delivery systems transforming into a highly viscous cubic phase. *International Journal of Pharmacology*, *173*, 51-60.

Cheirsilp, B., Kaewthong, W., & H-Kittikun, A. (2007). Kinetic study of glycerolysis of palm olein for monoacylglycerol production by immobilized lipase. *Biochemistry Engineering Journal*, *35*, 71-80.

Dabrowski, G., Konopka, I., Czaplicki, S., & Tariska M. (2017). Composition and oxidative stability of oil from *Salvia hispanica* L. seeds in relation to extraction method. *European Journal of Lipid Science and Technology*, *119*, 1600209.

Feltes, M. M. C., Oliveira, J. V., Treichel, H., Block J. M., de Oliveira, D., & Ninow, J. L. (2010). Assesment of process parameters on the production of diglycerides rich in omega-3 fatty acids through the enzymatic glycerolysis of fish oil. *European Food Research and Technology*, *1*, 701-710.

Feltes, M. M. C., Villeneuve, P., Baréa, B., Barouh, N., Oliveira, J. V., de Oliveira, D., & Ninow, J. L. (2012). Enzymatic production of monoacylglycerols (MAG) and diacylglycerols (DAG) from fish oil in a solvent-free system. *Journal of the American Oil Chemists' Society*, *89*, 1057-1065.

375 Ferreira-Dias, S., Correia, A. C., Baptista, F. O., & da Fonseca, M. M. R. (2001). Contribution of
376 Response Surface design to the development of glycerolysis systems catalyzed by commercial
377 immobilized lipases. *Journal of Molecular Catalysis B-Enzymatic*, *11*, 699-711.
378 Fiametti, K. G., Rovani, S., de Oliveira, D., Corazza, M. L., Treichel, H., & Oliveira, J. V. (2009).
379 Kinetics of solvent-free lipase-catalyzed production of monoacylglycerols from olive oil in
380 aerosol-OT surfactant. *Industrial & Engineering Chemistry Research*, *48*, 708-712.
381 Fregolente, P. B. L., Fregolente, L. V., Pinto, G. M. F., Batistella, B. C., Wolf-Maciel, M. R., &
382 Filho, R. M. (2008). Monoglycerides and diglycerides synthesis in a solvent-free system by
383 lipase-catalyzed glycerolysis. *Applied Biochemistry and Biotechnology*, *146*, 165-172.
384 Ghamgui, H., Miled, N., Rebaï, A., Karra-chaâbouni, M., & Gargouri, Y. (2006). Production of
385 mono-olein by immobilized *Staphylococcus simulans* lipase in a solvent-free system:
386 optimization by response surface methodology. *Enzyme and Microbial Technology*, *39*, 717-
387 723.
388 He, Y., Li, J., Kodali, S., Balle, T., Chen, B., & Guo, Z. (2017). Liquid lipases for enzymatic
389 concentration of n-3 polyunsaturated fatty acids in monoacylglycerols via ethanolysis: catalytic
390 specifity and parameterization. *Bioresource Technology 224*, 445-456.
391 Jacobsen, Ch. (2015). Some strategies for the stabilization of long-chain n-3 PUFA-enriched foods:
392 a review. *European Journal of Lipid Science and Technology*, *117*, 1853-1866.
393 Kahveci, D., Guo, Z., Cheong, L. –Z., Falkeborg, M., Panpipat, W., & Xu, X. (2013). Oxidative
394 stability of enzymatically processed oils and fats. In A. S. Logan, U. Nienaber & X. Pan (Eds.),
395 *Lipid Oxidation. Challenges in Food Systems* (pp. 211-242). London: Elvevier Inc.
396 Kahveci, D., Guo, Z., Özçelik, B., & Xu, X. (2010). Optimization of enzymatic synthesis of
397 diacylglycerols in binary medium systems containing ionic liquids. *Food Chemistry*, *119*, 880-
398 885.

399 Kristensen, J. B., Xu, X., & Mu, H. (2005). Diacylglycerol synthesis by enzymatic glycerolysis:
400 screening of commercially available lipases. *Journal of the American Oil Chemists' Society*,
401 *82*, 329–334.
402 Miyashita, K. (2014). Paradox of omega-3 PUFA oxidation. *European Journal of Lipid Science and*
403 *Technology*, *116*, 1268-1279.
404 Miyashita, K., & Takagi, T. (1986). Study on the oxidative rate and prooxidant activity of free fatty
405 acids. *Journal of the American Oil Chemists' Society, 63*, 1380-1384.
406 Moquin, P. H. L., Temelli, F., Sovová, H., & Saldaña, M. D. A. (2006). Kinetic modelling of
407 glycerolysis-hydrolysis of canola oil in supercritical carbon dioxide media using equilibrium
408 data. *Journal of Supercritical Fluids*, *37*, 417-424.
409 Myers, R. H., Montgomery, D. C., & Anderson-Cook, C. M. (2016). *Response surface*
410 *methodology. Process and product optimization using designed experiments.* (4th ed.). New
411 Jersey: Wiley.
412 Nicholson, T., Khademi, H., & Moghadasian, M. H. (2013). The role of marine n-3 fatty acids in
413 improving cardiovascular health: A review. *Food & Function*, *4*, 357-365.
414 Paradiso, V. M., Caponio, F., Bruno, G., Pasqualone, A., Summo, C., & Gomes, T. (2014).
415 Complex role of monoacylglycerols in the oxidation of vegetable oils: different behaviors of
416 soybean monoacylglycerols in different oils. *Journal of Agricultural and Food Chemistry*, *62*,
417 10776-10782.
418 Pawongrat, R., Xu, X., & H-Kittikun, A. (2008). Physico-enzymatic production of
419 monoacylglycerols enriched with very-long-chain polyunsaturated fatty acids. *Journal of the*
420 *Science of Food and Agriculture*, *88*, 256-262.
421 Pawongrat, R., Xu, X., & H-Kittikun, A. (2007). Synthesis of monoacylglycerol rich in
422 polyunsaturated fatty acids from tuna oil with immobilized lipase AK. *Food Chemistry*, *104*,
423 251-258.

Shahidi, F., Wang, J., & Wanasundara, N. (2017). Methods for measuring oxidative rancidity in fats and oils. In C. C. Akoh (Ed.), *Food Lipids. Chemistry, Nutrition, and Biotechnology* (pp. 519-542. Boca Raton: CRC Press.

Solaesa, A. G., Sanz, M. T., Falkeborg, M., Beltrán, S., & Guo, Zh. (2016). Production and concentration of monoacylglycerols rich in omega-3 polyunsaturated fatty acids by glycerolysis and molecular distillation. *Food Chemistry*, *190*, 960-967.

Tan, T., & Yin, C. (2005). The mechanism and kinetic model for glycerolysis by 1,3 position specific lipase from *Rhizopus arrhizus*. *Biochemical Engineering Journal*, *25*, 39-45.

Tanaka, K., & Toda, F. (2000). Solvent-free organic synthesis. *Chemical Review*, *100*, 1025-1074.

Valério, A., Krüger, R. L., Ninow, J., Corazza, F. C., de Oliveira, D., Oliveira, J. V., & Corazza, M. L. (2009). Kinetics of solvent-free lipase-catalyzed glycerolysis of olive oil in surfactant system. *Journal of Agricultural and Food Chemistry*, *57*, 8350-8356.

Valério, A., Rovani, S., Treichel, H., de Oliveira, D., & Oliveira, J. V. (2010). Optimization of mono and diacylglycerols production from enzymatic glycerolysis in solvent-free systems. *Bioprocess and Biosystems Engineering*, *33*, 805-812.

von der Haar, D., Stäbler, A., Wichmann, R., & Schweiggert-Weisz, U. (2015). Enzyme-assisted process for DAG synthesis in edible oils. *Food Chemistry*, *176*, 263–270.

Watanabe, T., Shimuzu, M., Sugiura, M., Sato, M., Kohori, J., Yamada. N., & Nakanishi, K. (2003). Optimization of reaction conditions for the production of DAG using immobilized 1,3-regiospecific lipase lipozyme RM IM. *Journal of the American Oil Chemists' Society*, *80*, 1201-1207.

Weber, N., & Mukherjee, K. D. (2004). Solvent-free lipase-catalyzed preparation of diacylglycerols. *Journal of Agricultural and Food Chemistry*, *52*, 5347-5353.

Yang, T., Rebsdorf, M., Engelrud, U., & Xu, X. (2005). Enzymatic production of monoacylglycerols containing polyunsaturated fatty acids through an efficient glycerolysis

449 system. *Journal of Agricultural and Food Chemistry*, *53*, 1475-1481.
450 Yeoh, C. M., Choong, T. S. Y., Abdullah, L. C., Yunus, R., & Siew, W. L. (2009). Influence of
451 silica gel in production of diacylglycerol via enzymatic glycerolysis of palm olein. *European*
452 *Journal of Lipid Science and Technology*, *111*, 599-606.
453 Zhong, N., Li, L., Xu, X., Cheong, L. Z., Zhao, X., & Li, B. (2010). Production of diacylglycerols
454 through low-temperature chemical glycerolysis. *Food Chemistry*, *122*, 228-232.
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457 **Figure Captions**

458 **Figure 1.** Response surface plots showing the effects of substrate molar ratio and enzyme
459 concentration on (a) MAG production, (b) DAG production, and (c) TAG consumption, during
460 solvent-free glycerolysis with Lipase PS-DI. The reaction took place at 50 ºC for 4 h at 200 rpm.

461

462 **Figure 2.** Response surface plots showing the effect of reaction temperature and enzyme
463 concentration on (a) MAG and (b) DAG content obtained in the solvent-free glycerolysis with
464 Lipase PS-DI. The reaction was carried out using Gly/TAG molar ratio of 3/1 for 4 h at 200 rpm.

465

466 **Figure 3**. Response surface plot of the oxidative stability of the solvent-free glycerolysis products.
467 (a) Effect of reaction temperature and substrate molar ratio (Gly/TAG) on induction time, with 5%
468 Lipase PS-DI; (b) Effect of reaction temperature and enzyme concentration on induction time, with
469 a Gly/TAG molar ratio of 2/1.

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