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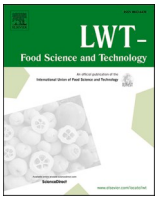

Synthesis and oxidative stability of monoacylglycerols containing polyunsaturated fatty acids by enzymatic glycerolysis in a solvent-free system

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ABSTRACT

Monoacylglycerols (MAG) containing polyunsaturated fatty acids (PUFA) have been synthesized by glycerolysis of anchovy oil using Lipozyme RM-IM in a solvent-free system. The experimental conditions, reaction temperature, substrate molar ratio (glycerol/triacylglycerol, Gly/TAG) and enzyme concentration were studied. A response surface methodology was employed to analyze the effect of the individual variables on MAG production and oxidative stability with respect to induction time. The operating conditions that simultaneously optimized MAG yields and oxidative stability were a reaction temperature of 40 °C, a Gly/TAG molar ratio of 2:1 and 6% enzyme load. The yield obtained was 20.34% of MAG (with 12.12% of 2-MAG) and an induction time of 1.88 h.

1. Introduction

Monoacylglycerols (MAG) are widely used in the food industry (e.g. margarine and ice cream production, bakery products, etc.) (Sawa, Inoue, Lysenko, Edwards, & Preston, 2009; Anese et al., 2016), as well as in many pharmaceutical and personal care products, due to their emulsifying, plasticizing and texturizing properties (Pawongrat, Xu, & H-Kittikun, 2007). In addition, MAG have been considered as functional food components due to their nutritional properties (Feltes, de Oliveira, Block, & Ninow, 2013).

Over the last few years, the positive effects of polyunsaturated fatty acids (PUFA) on human health has been recognized given their role in the regulation of inflammation, cholesterol metabolism and brain function. Recent studies have suggested beneficial effects of MAG-containing omega-3 PUFA (ω-3 PUFA) on several inflammatory diseases, as well as anti-proliferative effects in colorectal carcinoma cells (Morin, Cantin, Vézina, & Fortin, 2018; Morin, Rousseau, & Fortin, 2013). As such, the production of MAG containing these fatty acids has attracted increasing interest (Khaddaj-Mallat, Morin, & Rousseau, 2016; Mu & Porsgaard, 2005).

MAG are normally prepared on an industrial scale by chemical glycerolysis of hydrogenated fats and oils at high temperatures (220–250 °C) in the presence of an inorganic alkaline catalyst and unsaturated fatty acids (Bornscheuer, 1995). However, high temperatures are unsuitable for heat-sensitive fatty acids (unsaturated fatty acids) as they promote the *trans* isomerization of double bonds and their oxidation, thus leading to a reduction of the organoleptic, nutritional and biological properties of the starting oil. Furthermore, under these reaction conditions, PUFA are easily transformed into toxic by-products of lipid oxidation, some of which have been linked to cancer (Cao et al., 2014; Vieira, Zhang, & Decker, 2017).

A suitable approach for the preparation of oils rich in heat-sensitive PUFA is the use of lipases (EC 3.1.1.3), under mild reaction conditions, which results in high substrate specificity. The enzymatic production of MAG can be carried out by hydrolysis of TAG, esterification of glycerol (Gly) and free fatty acids, alcoholysis or glycerolysis (Phuah et al., 2015). The glycerolysis reaction is an efficient alternative due to use of the three acyl groups from TAG, although the poor miscibility between the hydrophilic Gly and the highly hydrophobic TAG limits mass transfer, thus leading to a low conversion rate for the reactants. To overcome this, food surfactants, organic solvents, ionic liquids or supercritical fluids have been used to improve the homogeneity of the reaction system. However, some of these strategies require expensive technologies or the use of toxic solvents that are unsuitable for food applications and incompatible with the principles of green chemistry (Moquin, Temelli, Sovová, & Saldaña; Cai, Gao, Liu, Zhong, & Liu, 2016; Kahveci, Guo, Özçelik, & Xu, 2010; Valério et al., 2009). In this sense, solvent-free systems are a promising alternative due to their simplicity

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Table 1
A three factors and two levels face-centered central composite design arrangement and response values (MAG and It) for glycerolysis reaction with Lipozyme RM-IM.

	Factors[a]			MAG (%)		I_t (h)	
Run	A	B	C	Experimental	Predicted	Experimental	Predicted
1	40(-1)	1:1(-1)	1(-1)	8.72	8.53	0.80	0.83
2	60(+1)	1:1(-1)	1(-1)	10.07	10.03	0.69	0.69
3	40(-1)	3:1(+1)	1(-1)	2.63	3.07	0.93	0.93
4	60(+1)	3:1(+1)	1(-1)	9.16	8.77	0.58	0.64
5	40(-1)	1:1(-1)	9(+1)	18.33	18.66	1.33	1.24
6	60(+1)	1:1(-1)	9(+1)	14.92	14.43	1.55	1.52
7	40(-1)	3:1(+1)	9(+1)	19.74	19.72	1.03	0.99
8	60(+1)	3:1(+1)	9(+1)	19.55	19.68	1.18	1.12
9	40(-1)	2:1(0)	5(0)	20.17	19.60	1.86	1.95
10	60(+1)	2:1(0)	5(0)	19.55	20.34	1.90	1.95
11	50(0)	1:1(-1)	5(0)	18.62	19.00	1.28	1.37
12	50(0)	3:1(+1)	5(0)	19.06	18.90	1.18	1.22
13	50(0)	2:1(0)	1(-1)	10.66	10.83	0.56	0.46
14	50(0)	2:1(0)	9(+1)	21.31	21.36	0.69	0.91
15	50(0)	2:1(0)	5(0)	21.77	21.07	1.56	1.47
16	50(0)	2:1(0)	5(0)	21.20	21.07	1.59	1.47
17	50(0)	2:1(0)	5(0)	20.69	21.07	1.50	1.47

[a] A: reaction temperature (°C); B: substrate molar ratio (Gly/TAG); C: enzyme concentration (%); coded variable levels: low (−1), medium (0), high (+1).

and reduced toxicity.

Despite these advantages, the enzymatic glycerolysis between TAG and Gly follows a complex ping-pong, bi-bi model comprising several equilibria, thus making it difficult to obtain a single reaction product. Additionally, the time to reach equilibrium in solvent-free systems is far too long for large-scale production processes (Cheirsilp, Kaewthong, & H-Kittikun, 2007; Choong et al., 2018). However, these equilibria can be shifted toward MAG production by controlling the Gly/TAG substrate ratio, and the reaction yield can be improved by increasing the reaction temperature or enzyme concentration (Palacios, Ortega, Rubio-Rodríguez, & Busto, 2019). To that end, response surface methodology is a powerful tool that allows the simultaneous study of several factors, while also taking into account the interactions between them, and with a limited number of experiments (Yolmeh & Jafari, 2017).

The viability of the production process for PUFA-rich MAG also depends on other factors, including the organoleptic quality of the product or economic aspects. Lipid oxidation is one of the most important reactions responsible for altering properties such as flavor or color, thereby causing nutritional losses and reducing global acceptance by consumers. Oil rancidity is a very complex process influenced by many factors in which several reactions and compounds are involved, therefore there is no single methodology for its determination (Tan, CheMan, Selamat, & Yusoff, 2002). In order to study the oxidative degree of a food matrix, methods based on the measurement of volatile compounds released by the accelerated oxidative process are one of the most popular, due to their good correlation with product quality (Matthäus, 1996; Aparicio, Roda, Albi, & Gutiérrez, 1999).

The objective of this study focuses on the production of PUFA-containing MAG with an adequate oxidative quality of the final product in a solvent-free glycerolysis system using Lipozyme RM-IM lipase as biocatalyst. To that end, the effects of reaction temperature, Gly/TAG substrate molar ratio, and enzyme concentration were screened using response surface methodology in order to determine the experimental conditions that maximized MAG production. At the same time, the induction time was evaluated as second response variable due to the importance of rancidity on final product quality. Finally, after screening the effects of the parameters studied, the desirability function was applied for simultaneous optimization of MAG yields and oxidative stability.

2. Material and methods

2.1. Materials

Anchovy oil with high PUFA content was provided by Denomega Nutritional Oils (Leknes, Norway). The fatty acids composition (wt %) was as follows: C14 (myristic acid), C16 (palmitic acid), C16:1 (palmitoleic acid), C18 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C18:3 (α-linoleic acid), C20:5 (eicosapentaenoic acid), C22:1 (erucic acid) and C22:6 (docosahexaenoic acid) (6.5, 19.4, 9.0, 4.2, 22.0, 2.8, 1.8, 7.6, 2.1 and 12.7, respectively). The starting oil used in the glycerolysis reactions had a negligible partial acylglycerol and free fatty acids content. Reference standards for MAG, diacylglycerol (DAG) and TAG were obtained from Sigma-Aldrich Corporation (St. Louis, Mo, USA). Lipase from *Rhizomucor miehei* commercially immobilized on an ion-exchange resin, Lipozyme RM-IM (Novozymes, Bagsvaerd, Denmark), was purchased from Sigma-Aldrich Corporation. Isooctane, methyl *tert*-butyl ether and acetic acid were HPLC grade and purchased from Sigma-Aldrich Corporation. Glycerol and silica were supplied by Merck-Millipore (Madrid, Spain).

2.2. Experimental design and optimization

A three-factor and two-level face-centered central composite design was used to evaluate the effect of reaction temperature (factor A: in °C), Gly/TAG substrate molar ratio (factor B: in mol:mol) and enzyme concentration (factor C: in % in solution), with MAG content (MAG: % of MAG) and induction time (in h) as response variables. This design was selected because a rotatable central composite design was not appropriate due to the experimental conditions required for the extended axial points: Lipozyme RM IM showed great tendency to form aggregates in a solvent-free system at enzyme concentrations higher than 10% and highly viscous solutions were obtained at Gly/TAG molar ratios above 3.

The experimental conditions are shown in Table 1. Seventeen experiments consisting of eight factorial points, six star points and three center points were performed according to the experimental design, and fitted to a second-order polynomial model in order to generate response surfaces and to determine the optimal conditions for each response variable.

In order to determine the reaction conditions that simultaneously optimize the production of MAG and the oxidative stability of the reaction product, both individual responses were combined into a single

one using Derringer's desirability function, which was then optimized. The global desirability values ranged from 0 (completely unacceptable) to 1 (ideal response).

An analysis of variance (ANOVA) for each regression model, and optimization of the reaction conditions using a global desirability function, was carried out with Statgraphics Centurion XVII software (Statpoint Technologies, Inc, USA).

2.3. Enzymatic glycerolysis of anchovy oil for monoacylglycerol production

The glycerolysis reaction was performed in screw-capped glass tubes by mixing 6 g of anchovy oil with different amounts of glycerol according to the experimental design to give Gly/TAG molar ratios of 1:1, 2:1, and 3:1. Glycerol was first adsorbed onto silica gel using equal amounts, as reported previously (Berger, Laumen, & Schneider, 1992). The reaction was initiated by adding varying amounts of the immobilized lipase Lipozyme RM-IM, with previously fixed water activity at 0.158, and carried out at different temperatures (40, 50 and 60 °C) in a magnetic shaker at 200 rpm, as required by the experimental design. After 4 h, the reaction was stopped by centrifugation (5000 rpm for 5 min) in order to remove the immobilized lipase, silica and glycerol from the oil.

2.4. HPLC analysis

Lipid composition (TAG, DAG, MAG, 2-MAG and free fatty acids) was quantified by normal-phase HPLC according to the method described by Solaesa, Sanz, Falkeborg, Beltrán, and Guo (2016). The analysis were carried out using an Agilent 1200 Series Model (Agilent Technologies, Folsom, CA, USA) equipped with a Lichrospher 100 Diol column (5 μm particle size and dimensions of 4 × 250 mm) and an evaporated light scattering detector, operating at 35 °C and a nitrogen pressure of 0.35 MPa. The reaction mixture was diluted in isooctane and separated using an elution gradient of isooctane (solvent A) and methyl *tert*-butyl ether/acetic acid (solvent B) (99.9:0.1, by vol) as mobile phase at a constant flow rate of 1 mL/min. Initially, solvent A was used for 10 min, then solvent B was gradually increased up to 100% for 30 min and maintained 5 min more before equilibrating again with 100% of solvent A in the mobile phase. Injection volumes of 10 μL were used in all experiments.

Individual compounds were identified and quantified using a calibration curve ($R^2 > 0.97$) of the corresponding standard compound of TAG (tripalmitin), DAG (mixture of 1,3-dipalmitin and 1,2-dipalmitin), MAG (mixture of 1-monopalmitin and 2-monopalmitin) and free fatty acids (palmitic acid) in *tert*-pentanol (Table S1). The results are given as the weight percentage of total lipid. All data were the mean of two experiments with a deviation within 2.7%.

2.5. Oxidative stability

The oxidative stability was determined as induction time index using a Rancimat 743 (Metrohm AG, Switzerland). The accelerated oxidation of 2 g of sample was carried out at 80 °C with a constant air flow of 9 L/h. The volatile compounds released during the oxidation process were absorbed in water, increasing the electrical conductivity.

3. Results and discussion

3.1. Selection of the reaction time

A preliminary study was conducted to select an appropriate reaction time for MAG production from anchovy oil rich in PUFA (12.7% docosahexaenoic acid and 7.6% eicosapentaenoic acid) with Lipozyme RM-IM in a solvent-free medium. Fig. 1 shows that the TAG content decreased (about 50%) during the first steps of the glycerolysis reaction concomitant with an increase in DAG and MAG. In fact, DAG production increased to a maximum of 39.7% after reaction for 9 h, while the MAG content remained constant (approximately 20%) after 3 h (Fig. 1). Similar results were found in other solvent-free systems, with the authors of these studies indicating that immobilized lipase Novozym® 435 reached equilibrium after 10 h at 55 °C (Feltes et al., 2012).

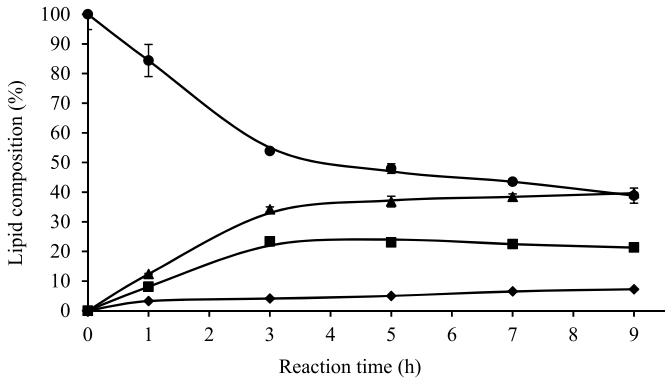

Fig. 1. Time course of Lipozyme RM-IM during solvent-free glycerolysis. TAG (●), free fatty acids (◆), DAG (▲) and MAG (■). Reaction conditions: Gly/TAG molar ratio, 1.5:1; reaction temperature, 50 °C; enzyme load, 5% and shaking at 200 rpm.

In the reaction conditions assayed (a_w, 0.158), a low amount of free fatty acids was released in the final reaction products (about 7% after reaction for 9 h; Fig. 1). This result suggests a reduced hydrolytic activity and/or rapid esterification of the free fatty acids once released. As such, the low water activity used is suitable for achieving good glycerolysis while limiting the hydrolysis side-reaction. Moreover, glycerol is a suitable acyl group acceptor in lipase-catalyzed reactions and an important fraction of the free fatty acids could be esterified, thereby increasing the MAG and DAG levels (Voll et al., 2011; Von DerHaar, Stäbler, Wichmann, & Schweiggert-Weisz, 2015). On the other hand, a decrease in free fatty acids content is very important because they are generally recognized as pro-oxidant compounds that play an important role in final oil quality (Miyashita & Takagi, 1986).

Based on these results, a short reaction time (4 h) was selected to establish the conditions for enzymatic glycerolysis of anchovy oil that optimize the production of PUFA-rich MAG rich in with maximum oxidative stability for the reaction end-products.

3.2. MAG production by enzymatic glycerolysis of anchovy oil

Glycerolysis is a complex process in which the acylglycerol profile of the final reaction product can be drastically altered by different reaction conditions. As such, a good understanding of the process is essential for the optimization of MAG synthesis. Preliminary experiments were therefore carried out to screen the parameters that influence MAG synthesis by enzymatic glycerolysis in a solvent-free system and to determine the experimental domain levels. During these experiments, three factors were investigated: reaction temperature (A), Gly/TAG substrate molar ratio (B) and enzyme concentration (C). The range and values of the variables studied are summarized in Table 1. The effect of the variables on MAG production and the optimal reaction conditions were studied by response surface methodology in Sections 3.2.1 and 3.2.2, respectively.

Table 2
Analysis of variance of the regression model for glycerolysis reaction with Lipozyme RM-IM with monoacylglycerols production as response variable.

Source	Df[a]	Sum of Squares	Mean Square	F-Ratio	*P*-Value[b]
A: Reaction temperature (°C)	1	1.33956	1.33956	4.59	0.0963
B: Substrate molar ratio (Gly/TAG)	1	0.02704	0.02704	0.09	0.7928
C: Enzyme concentration (%)	1	276.781	276.781	948.21	0.0000
AA	1	3.27518	3.27518	11.22	0.0199
AB	1	8.82	8.82	30.22	0.0017
AC	1	16.4738	16.4738	56.44	0.0003
BB	1	12.1057	12.1057	41.47	0.0007
BC	1	21.2552	21.2552	72.82	0.0001
CC	1	66.4633	66.4633	227.69	0.0000
Total error	7	2.54227	0.363181		
Total (corr.)	16	531.138			

[a] DF: degree of freedom.
[b] $P < 0.05$ indicates statistical significance at the 95.0% confidence level.

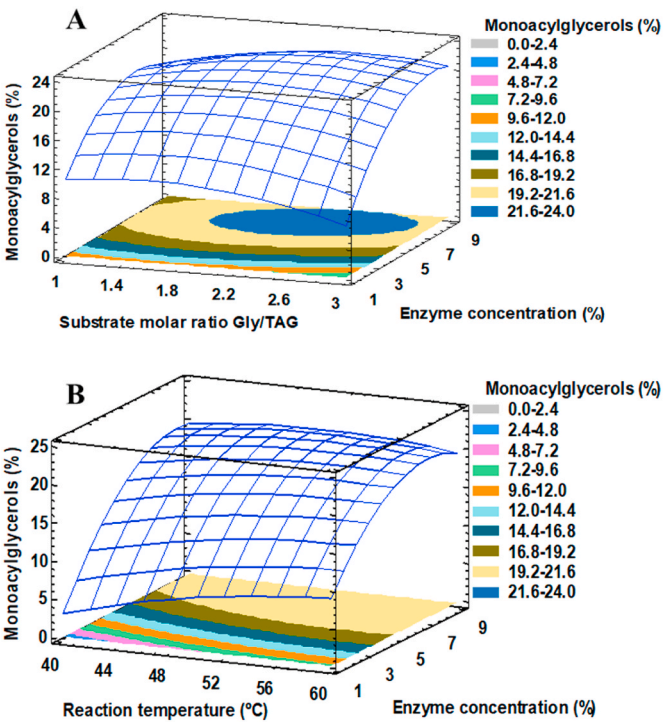

Fig. 2. Response surface plots showing the effect of (A) substrate molar ratio and enzyme concentration at 50 °C and (B) reaction temperature and enzyme concentration using Gly/TAG molar ratio of 2:1, on MAG production with Lipozyme RM-IM during solvent-free glycerolysis.

3.2.1. *Factors affecting MAG yield*

The results obtained after running the 17 experiments in order to carry out the statistical study are shown in Table 1. The analysis of variance (ANOVA) (Table 2) indicated that the model constructed was significant and did not show lack of fit ($p = 0.4791$). The coefficient of determination (R^2) was 0.9952, thus indicating an excellent correlation between experimental results and predicted values. The final response function that predicts the yield of MAG is as follows:

$$Y = -25.547 + 1.112A + 1.163B + 5.407C - 0.011A^2 + 0.105AB - 0.036AC - 2.125B^2 + 0.408BC - 0.311C^2 \quad (1)$$

where Y is % MAG, A is reaction temperature, B is substrate molar ratio and C is enzyme concentration.

Statistical analysis of the three factors explored showed that enzyme concentration was the most significant factor ($p = 0.000$) in the glycerolysis process, with a positive effect on the global reaction outcome, although its quadratic form exerted a negative effect (Eq. (1)). Also, a negative effect was observed for the quadratic term of the temperature reaction and the substrate molar ratio. Moreover, the paired interactions between independent factors also had a significant effect on MAG production (Table 2).

Fig. 2A and B shows that an increment of enzyme load led to an improvement in the yield of the glycerolysis reaction. Thus, it can be seen that, at low enzyme concentrations, the MAG yield was higher at high temperatures, although the temperature did not affect MAG production for enzyme concentrations above 6% (Fig. 2B). Moreover, at these enzyme concentrations, there was a positive interaction between the enzyme load and substrate molar ratio on MAG yield. At a constant enzyme concentration of 6%, the MAG content increased to a maximum at increasing values of substrate molar ratio, while this effect was not observed at 4% lipase, where MAG production remained more even (Fig. 2A).

However, the highest percentage of enzyme used did not increase the MAG content (Fig. 2). An excess of biocatalyst could promote aggregation of the immobilized enzyme particles, thus limiting the number of active sites available and reducing the glycerolysis activity (Choong et al., 2018). This is enhanced by the high viscosity of the solvent-free media. Similar results were found in the literature reviewed, with lower reaction yields being caused by the limiting mass transfer and blocking of the active sites, which reduced the amount of enzyme available for converting substrates into products (Naik, Naik, & Mohanty, 2014).

With regard to the substrate molar ratio (Gly/TAG), a significant negative effect of the quadratic form was observed on the MGA yield (Table 2, Eq. (1)). Theoretically, glycerolysis reaction equilibria can be displaced toward MAG or DAG production by controlling the Gly/TAG substrate ratio used. According to the stoichiometry of the reaction, the use of a high concentration of glycerol to oil will lead to an increase in MAG production. The unexpected behavior observed in our study may be due to the hydrophilic nature of the enzyme immobilization support. Lipozyme RM-IM is a *R. miehei* lipase immobilized on Duolite, a hydrophilic support that may promote the tendency of glycerol to stick to the enzyme surface. This microenvironment surrounding the enzyme restricts the entry of hydrophobic TAG to the lipase active site, thereby reducing the glycerolysis activity. As such, the nature of the immobilization support could play an important role in the glycerolysis activity of enzymes. In this regard, Zhong, Chen, Liu, and Chen (2019) observed that organic modification of the carrier SBA-15 with hydrophobic groups led to an improvement in glycerolysis activity. Conversely, hydrophilic carriers showed reduced activity due to the formation of a glycerol layer on the immobilized lipase that limits mass transfer (Kristensen, Xu, & Mu, 2005; Cai et al., 2016).

3.2.2. *Optimal conditions for MAG production*

The optimal conditions established by the model for MAG production

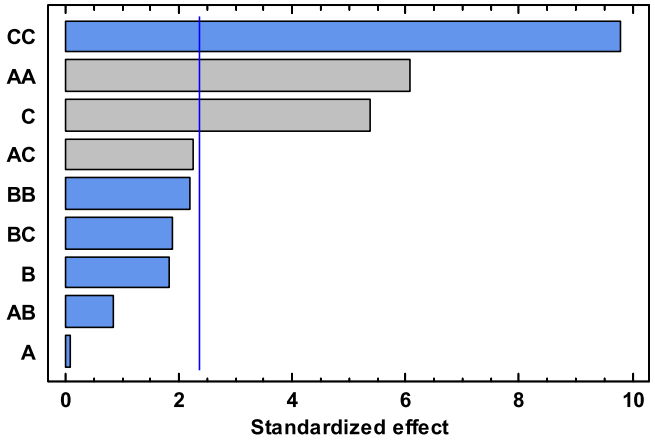

Fig. 3. Pareto chart showing the standardized effect for the variables studied on the oxidative stability (induction time). A: reaction temperature (°C), B: substrate molar ratio (Gly/TAG) and C: enzyme concentration (%). Positive effect: grey columns; negative effect: blue columns. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

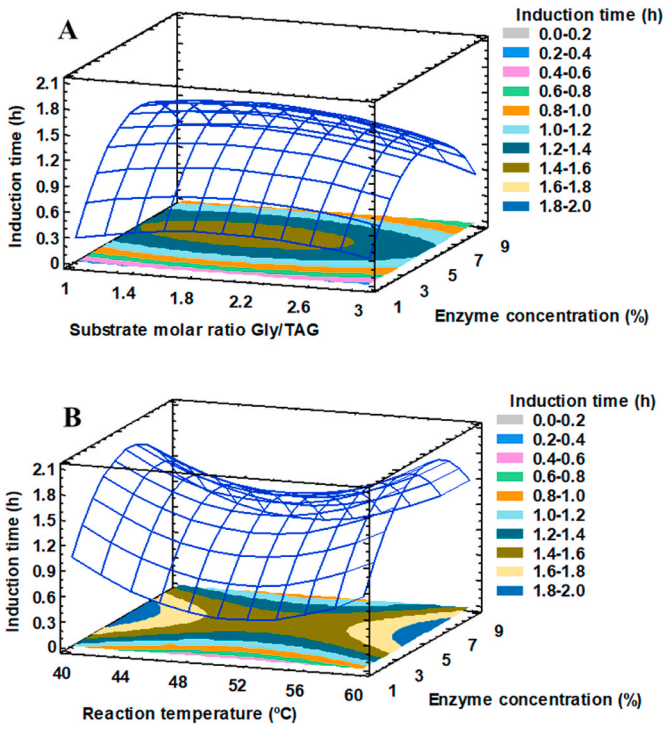

Fig. 4. Response surface plots showing the effect of (A) substrate molar ratio and enzyme concentration at 50 °C and (B) reaction temperature and enzyme concentration using Gly/TAG molar ratio of 3:1, on the oxidative stability (induction time) of the reaction products.

(Eq. (1)) were: a reaction temperature of 48.75 °C, a substrate molar ratio of 2.18:1 and an enzyme concentration of 7.31%. Under these conditions, the model predicted a MAG yield of 22.56%. Similar total MAG productivities have been achieved in solvent-free systems, using tuna oils as starting material, upon increasing the reaction time to 24 h (Pawongrat et al., 2007). Similarly, Feltes et al. (2012) reported that, at a low molar ratio (1:1) and enzyme concentrations of 5%, the commercial immobilized lipase Novozym 435 can produce a 25.93% yield of MAG when the reaction temperature is increased to 70 °C (Feltes et al., 2012). Fiametti et al. (2012) achieved a 40% MAG yield after 6 h of reaction at 70 °C, using an enzyme concentration of 7.5%, in an enzymatic ultrasound-assisted reaction. In other studies performed in different mixtures of organic solvents, the MAG yield was considerably higher, probably due to an improvement in the homogeneity of the reaction system (Pawongrat, Xu, & H-Kittikun, 2008). Despite achieving higher yields, the use of organic solvents for glycerolysis implies higher costs in the process, working under less sustainable conditions or far from green chemistry practices and, on many occasions, an increase in the amount of free fatty acids produced.

3.3. Oxidative stability of the glycerolysis products

The reaction conditions for the production of MAG can increase lipid oxidation, thus leading to the formation of dark-colored and burnt-tasting compounds that drastically affect the quality of the final product and, therefore, consumer acceptance. Indeed, if the oxidative stability is outside of an established range, the overall process will be unacceptable. In light of this, the effects of the reaction conditions (reaction temperature, Gly/TAG substrate molar ratio and enzyme concentration) on the oxidative stability (induction time) of the glycerolysis products were determined (Section 3.3.1), along with the optimal conditions to get a stable product (Section 3.3.2).

3.3.1. Factors affecting induction time of the glycerolysis products

As can be seen in Table 1, the induction times of all experiments performed ranged between 0.56 and 1.9 h, and in all cases were lower than the value of 2.25 h obtained for the starting oil. The statistical analysis indicated that the quadratic model had no lack of fit ($p = 0.0859$). Analysis of variance showed a high coefficient of determination ($R^2 = 0.9610$), thus indicating that 96% of the variability in the response can be explained by the model (Eq. (2), where Y, A, B and C are the induction time, the reaction temperature, the substrate molar ratio and the enzyme concentration, respectively).

$$Y = -11.589 - 0.490A + 0.929B + 0.457C + 0.005A^2 - 0.004AB + 0.003AC - 0.176B^2 - 0.022BC - 0,049C^2 \quad (2)$$

From analysis of the Pareto chart, it can be concluded that, in this model, the linear and quadratic terms of enzyme concentration and the quadratic term of reaction were significant (Fig. 3). The significant factors that exerted a positive effect, indicating synergistic effects, on the oxidative stability were the quadratic term of the reaction temperature (Fig. 3). All other significant factors had a negative effect (Fig. 3), thus implying an antagonist effect on the induction time.

Fig. 4A shows the interaction between substrate molar ratio (Gly/TAG) and enzyme concentration, maintaining the temperature at 50 °C. This surface response plot shows that an increase in enzyme load up to 5.08% enhanced the oxidative stability of the reaction products from 0.48 to 1.38 h, whereas higher enzyme concentrations led to a decrease in induction time. A higher enzyme concentration led to a decrease in the induction time due to a negative interaction between the molar ratio of the substrate and the enzyme concentration. This means that, when

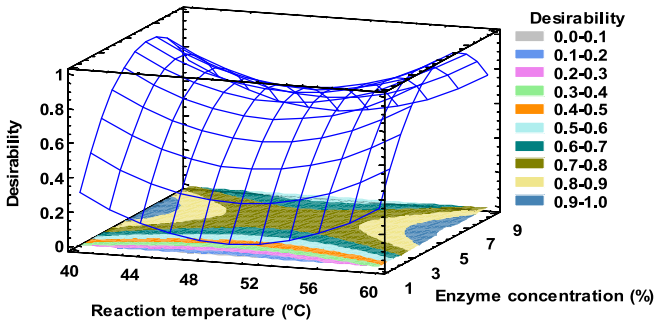

Fig. 5. Response surface plot for the overall desirability for MAG and induction time of reaction temperature and enzyme concentration keeping the Gly/TAG molar ratio constant at 2:1.

increasing the Gly/TAG ratio from 2.2 to 3.0, the positive effect of the enzyme load on oxidative stability (induction time) is reduced.

The effect of the interaction between enzyme concentration and reaction temperature on oxidative stability can be seen in Fig. 4B. Thus, the induction time increases with increasing enzyme concentration, subsequently decreasing as the lipase content increases from 5% to 9%. Indeed, this response surface has a horse saddle form, showing high values for the induction time (1.74 and 1.66 h) at both low and high temperatures (40 and 60 °C, respectively) irrespective of enzyme concentration, whereas the maximum oxidative stability is achieved with the central value of the enzyme concentration (5%). These unusual behaviors may be related to the presence of partial acylglycerols, which have a complex role in the oxidation of oils, acting as pro-oxidants or antioxidants depending on several factors, such as their fatty acids composition or concentration (Paradiso et al., 2014).

3.3.2. Optimal conditions for oxidative stability

The optimal reaction conditions established by the model described in Eq. (2) were: a reaction temperature of 60 °C, a Gly/TAG molar ratio of 1.6:1 and an enzyme concentration of 5.91%. Under these conditions, the predicted induction time for the glycerolysis product was 2.00 h. As such the model suggested that, despite the high percentage of PUFA in the starting oil, an adequate selection of reaction conditions could minimize lipid oxidation, thus resulting in a product with an oxidative stability very close to that of anchovy oil (2.25 h). This behavior can be explained by the mild reaction conditions, which lead to fewer side reactions, such as lipid oxidation, and the low amount of free fatty acids, which are generally regarded as pro-oxidants, released (Frega, Mozzon, & Lercker, 1999).

3.4. Simultaneous optimization of MAG synthesis and oxidative stability

According to previous results (Sections 3.2.2 and 3.3.2) different reaction conditions affect each of the individual responses. Thus, whereas MAG synthesis is favored by temperatures of 48.8 °C, a Gly/TAG molar ratio of 2.18:1 and an enzyme concentration of 7.3%, the maintenance of high oxidative stability requires a temperature of 60 °C, a Gly/TAG ratio of 1.6:1 and a Lipozyme RM-IM concentration of 5.9%.

When optimizing two separate incompatible responses, it is necessary to arrive at a set of compromise reaction conditions that simultaneously satisfy each individual criterion (VeraCandioti, DeZan, Cámara, & Goicoechea, 2014). The desirability function was used to maximize both responses (Khoobbakht, Kheiralipour, Yan, Seifi, & Karimi, 2020; Palacios et al., 2019), namely the MAG yield and oxidative stability (induction time), while keeping in mind that it is important to minimize both the enzyme concentration and reaction temperature as ideal conditions in order to reduce the final costs in industrial settings.

The estimated desirability function showed two high desirability regions (ranging from 0.9 to 1), at low and high temperatures (Fig. 5). In both regions, the models predicted slight differences in MAG production and oxidative stability from the most favored solution in the enzyme concentration range from 4.75% to 7.5%.

The suitability of the model for simultaneously optimizing MAG production and oxidative stability of the glycerolysis products was evaluated in the two regions predicted by the global desirability model by comparing the experimental response variables with the predicted ones (Table 3). The experimental and predicted values were found to be similar for the two conditions assayed, and the results were within the predicted interval for the model at a confidence level of 95%, with deviations in the range of 1.1–4.6% for MAG production and 1.4–4.3% for induction time. As such, the regression model for both responses was verified.

Given the similar results obtained under both optimal conditions, and in light of the desirability function and the confirmative studies of model adequacy, economic criteria were applied to select a low temperature for MAG production given the subsequent reduction in process costs. Under the optimum reaction conditions selected (40 °C, substrate molar ratio of 2:1 and 6% enzyme load), the glycerolysis product obtained from anchovy oil (100% TAG) had an induction time of 1.88 h and a composition of 20.34% MAG (with 12.12% 2-MAG), 40.15% DAG and 38.53% TAG (Fig. S1). Free fatty acids were not detected in the final product.

Studies performed previously in our laboratory with the lipase from *Burkholderia cepacia* immobilized on diatomite (Lipase PS-DI) showed that, for the best reaction conditions, MAG production yield was higher (29.67%) but the oxidative stability was lower (0.84 h) than those obtained in this study with Lipozyme RM-IM (20.34% MAG and 1.88 h induction time). Nevertheless, these results were achieved at a lower enzyme concentration and lower molar Gly/TAG ratio.

Table 3
Predicted and experimental values for MAG production and induction time (It) at different glycerolysis conditions performed for model validation.

Global desirability	Reaction temperature (°C)	Substrate molar ratio (Gly/TAG)	Enzyme concentration (%)	MAG yield (%)		It (h)	
				Predicted	Experimental	Predicted	Experimental
1.00	60	2:1	6	20.98	20.12	1.98	1.92
0.98	40	2:1	6	20.96	20.34	1.93	1.88
0.75	50	2:1	6	22.08	21.06	1.47	1.41
0.67	50	1:1	6	19.60	19.82	1.40	1.38
0.50	50	2:1	3	17.20	16.87	1.17	1.12

4. Conclusions

MAG has been successfully produced by enzymatic glycerolysis with Lipozyme RM-IM in a solvent-free system. Analysis of the results showed that enzyme concentration had a positive effect on MAG production and that the oxidative stability was strongly influenced by both enzyme load and reaction temperature. Simultaneous optimization of MAG production and oxidative stability using the desirability function gave two experimental regions, differing in terms of reaction temperature, which provided the best results for both responses. Based on these results, and given the need to establish the most profitable process, reaction conditions of 40 °C, a substrate molar ratio of 2:1 and an enzymatic load of 6%, which gave a 20.34% yield of MAG and an induction time of 1.88 h, were considered to be the most suitable for the production of PUFA-rich MAG by enzymatic glycerolysis.

Author statement

David Palacios: Methodology, Investigation, Writing-Original Draft. Maria D. Busto: Conceptualization, Supervision. Silvia M. Albillos: Visualization, Formal Analysis. Natividad Ortega: Conceptualization, Supervision, Writing-Original Draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112600.

References

Anese, M., Valoppi, F., Calligaris, S., Lagazio, C., Suman, M., Manzocco, L., et al. (2016). Omega-3 enriched biscuits with low levels of heat-induced toxicants: Effect of formulation and baking conditions. *Food and Bioprocess Technology, 9*(2), 232–242. https://doi:10.1007/s11947-015-1613-x.

Aparicio, R., Roda, L., Albi, M. A., & Gutiérrez, F. (1999). Effect of various compounds on virgin olive oil stability measured by Rancimat. *Journal of Agricultural and Food Chemistry, 47*(10), 4150–4155. https://doi.org/10.1021/jf9812230.

Berger, M., Laumen, K., & Schneider, M. (1992). Enzymatic esterification of glycerol II. Lipase-catalyzed synthesis of regioisomerically pure 1(3)-rac-monoacylglycerols. *Journal of the American Oil Chemists' Society, 69*(10), 961–965. https://doi:10.1007/BF02541058.

Bornscheuer, U. T. (1995). Lipase-catalyzed syntheses of monoacylglycerols. *Enzyme and Microbial Technology, 17*(7), 578–586. https://doi.org/10.1016/0141-0229(94)00096-A

Cai, C., Gao, Y., Liu, Y., Zhong, N., & Liu, N. (2016). Immobilization of *Candida antarctica* lipase B onto SBA-15 and their application in glycerolysis for diacylglycerols synthesis. *Food Chemistry, 212*, 205–212. https://doi.org/10.1016/j.foodchem.2016.05.167

Cao, W., Ramakrishnan, R., Tuyrin, V. A., Veglia, F., Condamine, T., Amoscato, A., et al. (2014). Oxidized lipids block antigen cross-presentation by dendritic cells in cancer. *The Journal of Immunology, 192*(6), 2920–2931. https://doi:10.4049/jimmunol.1302801.

Cheirsilp, B., Kaewthong, W., & H-Kittikun, A. (2007). Kinetic study of glycerolysis of palm olein for monoacylglycerol production by immobilized lipase. *Biochemical Engineering Journal, 35*(1), 71–80. https://doi.org/10.1016/j.bej.2006.12.024

Choong, T. S. Y., Yeoh, C. M., Phuah, E. T., Siew, W. L., Lee, Y. Y., Tang, T. K., et al. (2018). Kinetic study of lipase-catalyzed glycerolysis of palm olein using Lipozyme TLIM in solvent-free system. *PLoS One, 13*(2), Article e0192375. https://doi:10.1371/journal.pone.0192375.

Feltes, M. M. C., de Oliveira, D., Block, J. M., & Ninow, J. L. (2013). The production, benefits, and applications of monoacylglycerols and diacylglycerols of nutritional interest. *Food and Bioprocess Technology, 6*(1), 17–35. https://doi:10.1007/s11947-012-0836-3.

Feltes, M. M. C., Villeneuve, P., Baréa, B., Barouh, N., De Oliveira, J. V., De Oliveira, D., et al. (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*(6), 1057–1065. https://doi:10.1007/s11746-011-1998-2.

Fiametti, K. G., Ustra, M. K., De Oliveira, D., Corazza, M. L., Furigo, A., & Vladimir Oliveira, J. (2012). Kinetics of ultrasound-assisted lipase-catalyzed glycerolysis of olive oil in solvent-free system. *Ultrasonics Sonochemistry, 19*(3), 440–451. https://doi:10.1016/j.ultsonch.2011.09.005.

Frega, N., Mozzon, M., & Lercker, G. (1999). Effects of free fatty acids on oxidative stability of vegetable oil. *Journal of the American Oil Chemists' Society, 76*(3), 325–329. https://doi:10.1007/s11746-999-0239-4.

Kahveci, D., Guo, Z., Özçelik, B., & Xu, X. (2010). Optimisation of enzymatic synthesis of diacylglycerols in binary medium systems containing ionic liquids. *Food Chemistry, 119*(3), 880–885. https://doi:10.1016/j.foodchem.2009.07.040.

Khaddaj-Mallat, R., Morin, C., & Rousseau, É. (2016). Novel n-3 PUFA monoacylglycerides of pharmacological and medicinal interest: Anti-inflammatory and anti-proliferative effects. *European Journal of Pharmacology, 792*, 70–77. https://doi:10.1016/j.ejphar.2016.10.038.

Khoobbakht, G., Kheiralipour, K., Yan, W., Seifi, M. R., & Karimi, M. (2020). Desirability function approach for optimization of enzymatic transesterification catalyzed by lipase immobilized on mesoporous magnetic nanoparticles. *Renewable Energy, 158*, 233–262. https://doi.org/10.1016/j.renene.2020.05.087

Kristensen, J. B., Xu, X., & Mu, H. (2005). Diacylglycerol synthesis by enzymatic glycerolysis: Screening of commercially available lipases. *Journal of the American Oil Chemists' Society, 82*(5), 329–334. https://doi:10.1007/s11746-005-1074-5.

Matthäus, B. W. (1996). Determination of the oxidative stability of vegetable oils by rancimat and conductivity and chemiluminescence measurements. *Journal of the American Oil Chemists' Society, 73*(8), 1039–1043. https://doi.org/10.1007/BF02523413

Miyashita, K., & Takagi, T. (1986). Study on the oxidative rate and prooxidant activity of free fatty acids. *Journal of the American Oil Chemists' Society, 63*(10), 1380–1384. https://doi:10.1007/BF02679607.

Moquin, P. H. L., Temelli, F., Sovová, H., & Saldaña, M. D. A. (2006). Kinetic modeling of glycerolysis–hydrolysis of canola oil in supercritical carbon dioxide media using equilibrium data. *The Journal of Supercritical Fluids, 37*(3), 417–424. https://doi:10.1016/j.supflu.2006.01.009.

Morin, C., Cantin, A. M., Vézina, F. A., & Fortin, S. (2018). The efficacy of MAG-DHA for correcting AA/DHA imbalance of cystic fibrosis patients. *Marine Drugs, 16*(6), 184. https://doi:10.3390/md16060184.

Morin, C., Rousseau, É., & Fortin, S. (2013). Anti-proliferative effects of a new docosapentaenoic acid monoacylglyceride in colorectal carcinoma cells. *Prostaglandins Leukotrienes and Essential Fatty Acids, 89*(4), 203–213. https://doi:10.1016/j.plefa.2013.07.004.

Mu, H., & Porsgaard, T. (2005). The metabolism of structured triacylglycerols. *Progress in Lipid Research, 44*(6), 430–448. https://doi:10.1016/j.plipres.2005.09.002.

Naik, M. K., Naik, S. N., & Mohanty, S. (2014). Enzymatic glycerolysis for conversion of sunflower oil to food based emulsifiers. *Catalysis Today, 237*, 145–149. https://doi:10.1016/j.cattod.2013.11.005.

Palacios, D., Ortega, N., Rubio-Rodríguez, N., & Busto, M. D. (2019). Lipase-catalyzed glycerolysis of anchovy oil in a solvent-free system: Simultaneous optimization of monoacylglycerol synthesis and end-product oxidative stability. *Food Chemistry, 271*, 372–379. https://doi:10.1016/j.foodchem.2018.07.184.

Paradiso, V. M., Caponio, F., Bruno, G., Pasqualone, A., Summo, C., & Gomes, T. (2014). Complex role of monoacylglycerols in the oxidation of vegetable oils: Different behaviors of soybean monoacylglycerols in different oils. *Journal of Agricultural and Food Chemistry, 62*(44), 10776–10782. https://doi:10.1021/jf5025888.

Pawongrat, R., Xu, X., & H-Kittikun, A. (2007). Synthesis of monoacylglycerol rich in polyunsaturated fatty acids from tuna oil with immobilized lipase AK. *Food Chemistry, 104*(1), 251–258. https://doi:10.1016/j.foodchem.2006.11.036.

Pawongrat, R., Xu, X., & H-Kittikun, A. (2008). Physico-enzymatic production of monoacylglycerols enriched with very-long-chain polyunsaturated fatty acids. *Journal of the Science of Food and Agriculture, 88*(2), 256–262. https://doi:10.1002/jsfa.3081.

Phuah, E. T., Tang, T. K., Lee, Y. Y., Choong, T. S. Y., Tan, C. P., & Lai, O. M. (2015). Review on the current state of diacylglycerol production using enzymatic approach. *Food and Bioprocess Technology, 8*(6), 1169–1186. https://doi:10.1007/s11947-015-1505-0.

Sawa, K., Inoue, S., Lysenko, E., Edwards, N. M., & Preston, K. R. (2009). Effects of purified monoglycerides on Canadian short process and sponge and dough mixing properties, bread quality and crumb firmness during storage. *Food Chemistry, 115*(3), 884–890. https://doi:10.1016/j.foodchem.2009.01.010.

Solaesa, A. G., Sanz, M. T., Falkeborg, M., Beltrán, S., & Guo, Z. (2016). Production and concentration of monoacylglycerols rich in omega-3 polyunsaturated fatty acids by glycerolysis and molecular distillation. *Food Chemistry, 190*, 960–967. https://doi.org/10.1016/j.foodchem.2015.06.061

Tan, C. P., Che Man, Y. B., Selamat, J., & Yusoff, M. S. A. (2002). Comparative studies of oxidative stability of edible oils by differential scanning calorimetry and oxidative stability index methods. *Food Chemistry, 76*(3), 385–389. https://doi:10.1016/S0308-8146(01)00272-2.

Valério, A., Krüger, R. L., Ninow, J., Corazza, F. C., De Oliveira, D., Oliveira, J. V., et al. (2009). Kinetics of solvent-free lipase-catalyzed glycerolysis of olive oil in surfactant system. *Journal of Agricultural and Food Chemistry, 57*(18), 8350–8356. https://doi:10.1021/jf901771m.

Vera Candioti, L., De Zan, M. M., Cámara, M. S., & Goicoechea, H. C. (2014). *Experimental design and multiple response optimization. Using the desirability function in analytical methods development*. Talanta https://doi:10.1016/j.talanta.2014.01.034.

Vieira, S. A., Zhang, G., & Decker, E. A. (2017). Biological implications of lipid oxidation products. *Journal of the American Oil Chemists' Society, 64*, 339–351. https://doi.org/10.1007/s11746-017-2958-2

Voll, F., Krüger, R. L., de Castilhos, F., Filho, L. C., Cabral, V., Ninow, J., et al. (2011). Kinetic modeling of lipase-catalyzed glycerolysis of olive oil. *Biochemical Engineering Journal, 56*(3), 107–115. https://doi:10.1016/j.bej.2010.11.005.

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. https://doi:10.1016/j.foodchem.2014.12.072.

Yolmeh, M., & Jafari, S. M. (2017). Applications of response surface methodology in the food industry processes. *Food and Bioprocess Technology, 10*, 413–433. https://doi:10.1007/s11947-016-1855-2.

Zhong, N., Chen, W., Liu, L., & Chen, H. (2019). Immobilization of *Rhizomucor miehei* lipase onto the organic functionalized SBA-15: Their enzymatic properties and glycerolysis efficiencies for diacylglycerols production. *Food Chemistry, 271*, 739–746. https://doi:10.1016/j.foodchem.2018.07.185.