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Optimization of the Synthesis of Structured Phosphatidylcholine with Medium Chain Fatty Acid

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Abstract: Structured phosphatidylcholine was successfully produced by acidolysis between phosphatidylcholine and free medium chain fatty acid, using phospholipase A_1 immobilized on Duolite A568. Response surface methodology was applied to optimize the reaction system using three process parameters: molar ratio of substrates (phosphatidylcholine to free medium chain fatty acid), enzyme loading, and reaction temperature. All parameters evaluated showed linear and quadratic significant effects on the production of modified phosphatidylcholine; molar ratio of substrates contributed positively, but temperature influenced negatively. Increased enzyme loading also led to increased production of modified phosphatidylcholine but only during the first 9 hours of the acidolysis reaction. Optimal conditions obtained from the model were a ratio of phosphatidylcholine to free medium chain fatty acid of 1:15, an enzyme loading of 12%, and a temperature of 45℃. Under these conditions a production of modified phosphatidylcholine of 52.98 % were obtained after 24 h of reaction. The prediction was confirmed from the verification experiments; the production of modified phosphatidylcholine was 53.02%, the total yield of phosphatidylcholine 64.28% and the molar incorporation of medium chain fatty acid was 42.31%. The acidolysis reaction was scaled-up in a batch reactor with a similar production of modified phosphatidylcholine, total yield of phosphatidylcholine and molar incorporation of medium chain fatty acid. Purification by column chromatography of the structured phosphatidylcholine yielded 62.53% of phosphatidylcholine enriched with 42.52% of medium chain fatty acid.

Key words: structured phosphatidylcholine, acidolysis, medium chain fatty acid, phospholipase A_1, response surface methodology

1 Introduction

There is a constant attention focused on developing functional foods and nutraceuticals[1, 2], which are those that by the presence of physiologically active components, provide health benefits beyond basic nutrition[3]. Carotenoids, flavonoids, tannins, tocotrienols, phytosterols, polyphenols, omega-3 fatty acids, and medium chain triglycerides, are some functional components from plants or animals[4].

Researchers strive to develop more effective delivery systems for these functional compounds[5, 6]. Phospholipids (PLs), providing their physiological roles, are versatile ingredients for the formulation of systems to deliver functional compounds[7, 8], PLs possess a very low toxicity and can be used through several routes of administration[9]. PLs have been widely used for their surfactant properties in the formulation of emulsions for foods, pharmaceuticals, and cosmetics[10–12].

Structured phospholipids (SPLs), with a specific fatty acid composition, can be prepared to attain new physical properties, achieve special functionalities, and increase their ability to deliver functional compounds[13, 14]. SPLs containing medium chain fatty acid are more water soluble than natural phospholipids, have better heat stability and very long shelf life[15]. Moreover, Davidsen *et al.*[16] reported that drug release proceeds very fast when PLs containing medium chain fatty acid are incorporated into delivery systems like liposomes.

SPLs can be prepared by various synthesis routes[17, 18]. Lipases[19] and phospholipases[20] provide powerful tools for their synthesis by acidolysis. This interesterification reaction is a two-step reaction involving hydrolysis and further esterification. However, the formation of by-products, due to parallel hydrolysis reactions and acyl migration, is a serious problem in the reaction system and as consequence the yield of the acidolysis reaction is decreased[21].

In order to reduce the formation of by-products, and increase the production of SPLs, the reaction conditions should be selected with care during the reaction[22]. Several studies have been published concerning the relevant parameters for the reaction: type and amount of enzyme, reaction time, type and amount of solvents on the system, the presence of water, type and ratio of substrates, and the reaction temperature[21, 23−31].

The objective of this study was to optimize the production of modified phosphatidylcholine via the acydolisis reaction between phosphatidylcholine and free medium chain fatty acid, in order to obtain the highest yield of structured phosphatidylcholine with medium chain fatty acid. The effects and relationships among three factors, where the reaction temperature, enzyme load, and molar substrate ratio, were modeled using response surface methodology, to obtain the optimal reaction conditions.

2 Materials and Methods

2.1 Materials

Phospholipase A_1 (PLA$_1$) Lecitase® Ultra, a kind donation by NOVO (Salem, VA), was immobilized on Duolite A568, a gift from Rohm & Haas (Philadelphia, PA), according to the method described previously[26]. Soybean phosphatidylcholine (PC, 95%), was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), and HPLC standards of dioctanoyl-L-α-phosphatidylcholine (99%) and L-α-Lysophosphatidylcholine (LPC, 98%), were purchased from SIGMA-ALDRICH® (México City). A mixture of free medium chain fatty acid (MCFA), composed of caproic-C6:0 (1.65 mol%), caprylic-C8:0 (67.14 mol%), capric-C10:0 (30.57 mol%), and lauric-C12:0 (0.64 mol%) acids, was obtained by saponifying medium chain oil (Original Thin Oil®) purchased from Sound Nutrition (Dover, ID), according to the method described previously[32]. HPLC grade solvents were purchased from Tecsiquim (Mexico City) and all other reagents used were of analytical grade from SIGMA-ALDRICH® (Mexico City).

2.2 PLA$_1$ - catalyzed acidolysis reactions

Acidolysis reactions were conducted in solvent-free systems. For all trials, different enzyme loads (in%, with respect to the total weight of substrates), temperatures (℃), and molar ratios of substrates (PC/MCFA) were employed. Three g of substrates were placed in 25-mL Erlenmeyer flasks and mixed with the corresponding amount of immobilized enzyme. The reaction was carried out in a Thermo Scientific MaxQ™ 4450 orbital shaker (Thermo Fisher Scientific Inc., Waltham, MA) operating at 300 rpm and the desired temperature for a period of 72 h. Samples (50 μL) were withdrawn from the reaction mixture at appropriate time intervals. All samples were stored at −20℃ prior to HPLC analysis. The experiments were performed in duplicate.

2.3 Analysis of the PLs species by HPLC

Samples withdrawn from the reaction mixture were dissolved in methanol and diluted to a concentration of 10 mg/mL based on initial concentration of PC; 10 μL were injected into a Waters HPLC System fitted with a Partisil silica column (5 μm, 4.6×250 mm). The mobile phase consisted of acetonitrile/methanol/phosphoric acid (130:5:1.5 v/v/v) run isocratically at a flow rate of 1.5 mL/min. PLs species: unreacted phosphatidylcholine (PC), modified phosphatidylcholine (PC mod), and lysophosphatidylcholine (LPC), were detected at 205 nm with a UV–visible detector (Waters model 2487). Peaks were identified using external standards.

2.4 Experimental design and statistical analysis

Experiments were conduced using a central composite design to investigate the linear, quadratic, and cross-product effects of three factors, each varied at five levels, and also included the central point that was replicated six times. The three factors were the molar ratio of substrates (S, PC/MCFA), enzyme loading (E, % with respect to the total weight of substrates), and reaction temperature (T, ℃). The percent of modified phosphatidylcholine (PC mod) of the total PC (unreacted plus modified) was used as the response. The design of the experiments employed is depicted in Table 1. Data from the experiments were analyzed by response surface methodology (RSM), to fit a second-order model to the independent variables, using the statistical software package STATISTICA V. 6.0 from StatSoft, Inc. (Tulsa, OK). Responses were fitted to the factors by multiple regression; and model fitting were evaluated by the coefficient of determination (R^2) and analysis of variance (ANOVA). The significance of the results was established at $p \leq 0.05$.

2.5 Scale-up of the acidolysis reaction

Acidolysis was scaled-up at the optimal conditions of enzyme load, temperature, and molar ratio of substrates. 300 grams of substrates were placed in a glass reactor and mixed with the immobilized enzyme. The reaction was carried out in an orbital shaker operating at 300 rpm at the optimal temperature for 24 h. Samples were withdrawn at 1, 3, 6, 9, 12, and 24 h, to determine the percent of modi-

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Table 1 Experimental setup for the central composite design of three factors, at five levels, for the PLA$_1$-catalyzed acidolysis.

Experiment	Factors		
	S	E	T
1	1:8	16	50
2	1:16	16	50
3	1:8	32	50
4	1:16	32	50
5	1:8	16	60
6	1:16	16	60
7	1:8	32	60
8	1:16	32	60
9	1:4	24	55
10	1:20	24	55
11	1:12	8	55
12	1:12	40	55
13	1:12	24	45
14	1:12	24	65
15	1:12	24	55

Abbreviations: S, molar ratio of substrates (PC/MCFAs); E, enzyme loading (wt% based on total amount of substrates); T, reaction temperature (℃).

fied phosphatidylcholine (PC mod), the extent of incorporation of MCFA into PC, and total yield of PC (unreacted plus modified).

2.6 Recovery and analysis of structured phosphatidylcholine (SPC)

SPC, enriched with MCFA, was recovered from the acidolysis reaction mixture by column chromatography according to the method reported by Vikbjerg *et al.*[33], with slight modifications. A column (460×57 mm) was packed with 600 g of reagent grade silica (60-200 mesh, 150 Å); free fatty acids were eluted with chloroform, while chloroform/methanol (65:35 v/v) was used to elute the SPC, and pure methanol to elute LPC. SPC recovered was identified by HPLC, and its fatty acid composition was determined by GC, according to the method described previously[34].

3 Results and Discussion

3.1 PLA$_1$ - catalyzed acidolysis reaction

The use of PLA$_1$ immobilized on Duolite A568 (46 mg protein/g support, achieved from a suspension initially containing 75 mg protein/g support) for synthesis of SPC by acidolysis reaction between PC and free MCFA was the first step in the present work. This result is consistent with that reported by Garcia *et al.*[28] In order to obtain the highest production of modified PC, a central composite
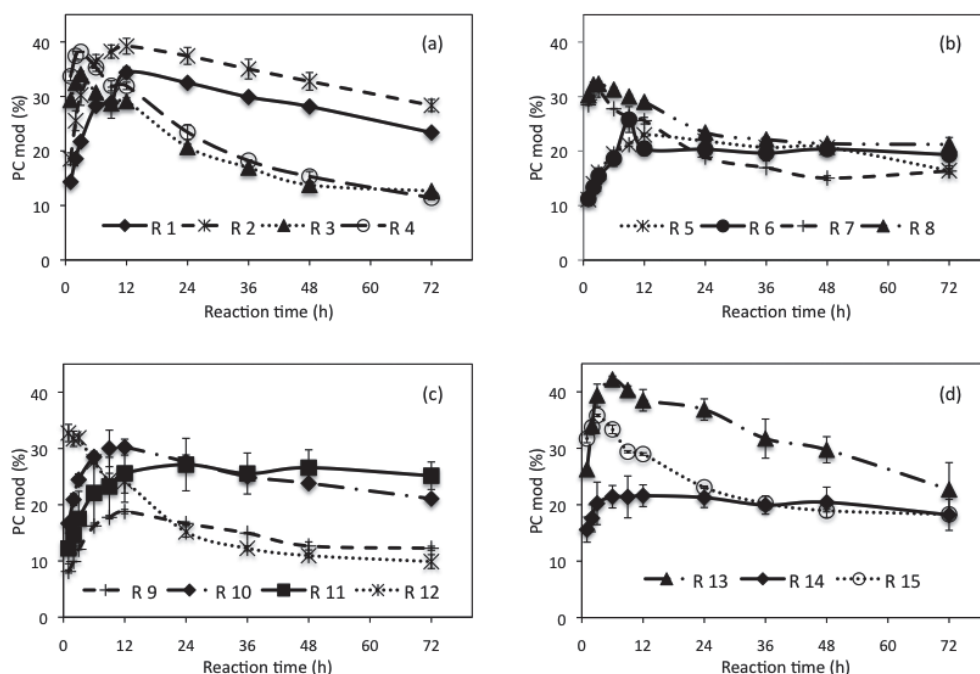
Fig. 1 Kinetics of modified PC production by acidolysis reaction between phosphatidylcholine and free medium chain fatty acid, using PLA$_1$ immobilized on Duolite, for each of the treatments (R1 – R15), according to the experimental design. Reaction conditions: S, mole ratio of substrates (PC/MCFA); E, enzyme loading (wt% based on the amount of substrates); and T, reaction temperature (℃), are shown in Table 1.

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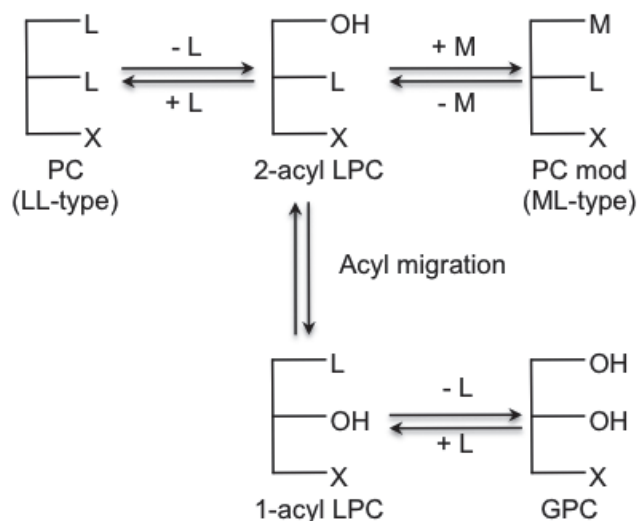
Fig. 2 Scheme of the reaction of the PLA1-catalyzed acidolysis for the production of modified phosphatidylcholine(PC mod), and side reactions (L, long chain fatty acids; M, MCFA; X, Polar head).

design was selected. The factors evaluated for the design were substrates molar ratio, enzyme load and reaction temperature. The time course of the modified PC production by PLA$_1$ Lecitase® Ultra for each of the treatments, according to the experimental design(see **Table 1**), is shown in **Fig. 1**. At first, and as expected for all the treatments evaluated, the content of modified PC increased with reaction time, whereas that of unreacted PC decreased(data no shown).

Acidolysis is a two-step reaction comprising hydrolysis of native PC to form LPC, and further reesterification of LPC formed in the first step of the reaction to obtain a modified PC with a new fatty acid[21]. HPLC analysis of the

reaction products shows how the PC composition changed in the samples taken at different reaction times. One to three peaks were observed for samples taken during acidolysis. These peaks represent, in corcondance with **Fig. 2**, to the unreacted PC(LL-type PC), modified PC(ML-Type PC)and LPC(2-acyl LPC); the retention times for each of the phospholipid species were 16.06, 18.59, and 29.48 minutes, respectively, and depends on the fatty acid composition.

Additionally, acyl migration in these types of reactions lead to the formation of byproducts, 1-acyl-LPC and, by its subsequent enzymatic hydrolysis, glycerophosphatidylcholine(GPC), as it is illustrated in **Fig. 2**. In consequence, the production of modified PC and also, the total yield of PC in the acidolysis reaction decrease. **Figure 1** shows the presence of maximal peaks of production of modified PC achieved at different reaction times(24, 12, 6, 3, and 1 h), for each of the enzyme concentrations tested(8, 16, 24, 32, and 40%, respectively)according to the experimental design.

The highest production of modified PC(42.20%)was reached after 6 h of reaction at 24% of enzyme loading, and a mole ratio of 1:12 PC/MCFA, for treatment 13(R 13), and the second-best result was obtained with treatment 2 (R 2), that produced 39.21% of modified PC after 12 h of reaction at 16% of enzyme loading, and a mole ratio of 1:16 PC/MCFA. These results are similar to those obtained by Niezgoda et al.[35], who used the term "effective incorporation" to indicate that native acyl residues in the sn-1 position have been exchanged for new fatty acid residues in the PC to obtain modified PC.

3.2 Parameters affecting the production of modified PC

The effects of molar ratio of substrates(S), enzyme loading(E), and reaction temperature(T)were evaluated on the acidolysis reaction between PC and free MCFA,

Table 2 Regression coefficients describing the influence of different variables on modified PC for each of the sampling times.

Time (h)	C	S	E	T	SS	EE	TT	SE	ST	ET	R^2
1	31.81*	3.45*	13.45*	− 4.53*	− 19.28*	− 9.20*	− 10.76*	0.88	− 3.37	3.09	94.8
2	31.72*	4.77*	11.20*	− 6.75*	− 16.12*	− 9.50*	− 7.07*	1.20	− 5.23	4.34	93.1
3	35.91*	4.75*	10.13*	− 8.44*	− 17.52*	− 11.08*	− 5.97*	− 1.26	− 6.02	5.81	92.6
6	33.47*	5.09*	4.17*	− 9.39*	− 10.76*	− 8.44*	− 1.48	0.42	− 5.06	9.79*	90.9
9	30.06*	5.70*	0.63	− 7.79*	− 5.19*	− 5.29*	1.78	− 3.50	− 2.14	7.86*	81.7
12	29.45*	3.94*	− 0.53	− 10.05*	− 4.22*	− 3.88*	3.82*	1.98	− 3.35	11.86*	88.2
24	23.42*	4.08*	− 6.20*	− 7.60*	− 0.78	− 1.77	6.14*	1.91	− 2.33	12.95*	91.2
36	20.66*	3.84*	− 7.19*	− 5.53*	− 0.04	− 1.14	5.79*	1.19	− 1.13	14.25*	90.0
48	19.18*	4.31*	− 8.46*	− 3.90*	− 0.59	− 0.07	6.25*	1.78	− 0.09	13.60*	91.6
72	18.44*	3.64*	− 7.05*	− 1.43	− 1.56	− 0.70	2.23	− 2.14	2.04	14.73*	88.1

Abbreviations: C, constant; S, molar ratio of substrates; E, enzyme loading; T, reaction temperature; R^2, coefficient of determination of the models.
* The factor is significant at the ≤ 0.05 level.

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(A)

(B)

(C)
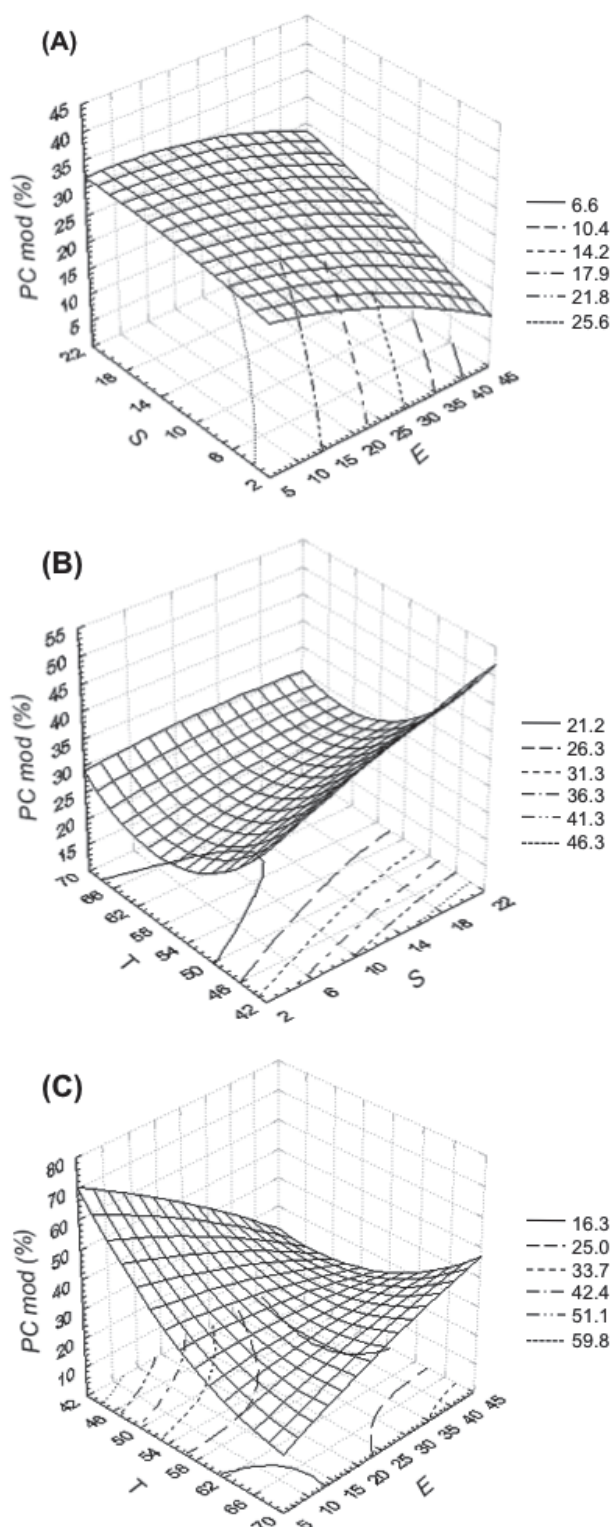
Fig. 3   Response surfaces and contour plots for modified phosphatidylcholine (PC mod) in percent (%) for acidolysis, after 24 h of reaction catalyzed by PLA$_1$ Lecitase® Ultra at: Reaction temperature (T) of 55℃, (A); enzyme loading (E) of 24%, (B); and molar ratio of substrates (S) PC/MCFA of 1:12, (C).

using immobilized PLA$_1$, in a central composite design, to obtain the highest production of modified PC. Regression coefficients of the model, their statistical significance for the response variable, and the percent of modified PC, are listed in Table 2. All the parameters evaluated (S, E, and T) showed linear and quadratic significant effects on the production of modified PC. SE and ST did not show cross-significant effects, but ET did show such effects. A significant positive effect was exhibited by S; although E, ET, and TT also had positive effects, from 1 to 6 hours of reaction, after 6 hours of reaction, and after 9 hours of reaction, respectively. T, SS and EE were the parameters that had significant negative effects. The response surfaces and contour plots associated with the model parameters and interactions of Table 2 are displayed in Fig. 3.

According to the regression coefficients of the model, enzyme loading had the most significant effect on the production of modified PC. Enzyme loading has a significant effect on the acidolysis reaction not only because it influences reaction yield but it can also cause problems with agitation, especially when a solvent-free system is used in phospholipid modification[21, 36]. Analysis of Fig. 3(A), of the response surfaces obtained after 24 h of acidolysis, reveals that enzyme loading had a negative and significant effect on the production of modified PC (PC mod). Although enzyme loading also showed positive effects on the production of modified PC during the first 9 h of the acidolysis reaction (see the regression coefficients of the model in Table 2).

These results are consistent with those reported by Niezgoda *et al.*[35] who found that althougt the increase of enzyme load had a positive effect on the incorporation of fatty acids into PC, it also resulted in higher degrees of hydrolysis of PC, and increased content of LPC and GPC in the reaction mixture. They achieved the highest effective incorporation of fatty acids to PC (32%) after 12 h of the reaction with 24% of enzyme loading, and did not increase when 30% of enzyme was applied. Chojnacka *et al.*[37], Zhao *et al.*[38], and Kim *et al.*[25], also reported that greater enzyme loadings had a negative effect on the yield of PC; they obtained an optimal enzyme loading for production of structured PC of 20, 20, and 15%, respectively.

The results of Fig. 3(B), indicate that increasing the reaction temperature decreased the production of modified PC (PC mod), but Fig. 3(C), reveals the cross significant positive effect of ET; this figure shows that both a lower reaction temperature and a lower enzyme loading, or higher reaction temperature and a higher enzyme loading, increased the production of modified PC. This parameter (T), according to the regression coefficients of the model, showed the second most significant effect on the production of modified PC.

Previous studies have also reported that elevated temperatures resulted in decreased PC concentration by a

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concomitant increase in LPC content also by acyl migration and by-product formation[21, 39, 40]. Meanwhile, Zhao *et al.*[38] observed a slightly higher PC yield at lower temperatures, although they did not observe significant differences in the tested temperature range. On the other hand, an increase in the reaction temperature of enzyme-catalyzed reactions resulted in increased reaction rates, according to Arrhenius's law, but also enzyme stability is influenced by temperature; higher temperatures reduce enzyme stability and its half-life through denaturation.

As shown in Fig. 3(A-B), the substrates molar ratio had a positive and significant effect on the production of modified PC(PC mod). No cross significant effect of SE(Fig. 3A)and ST(Fig. 3B)could bee observed. The substrates molar ratio was the parameter that showed the least significant effect on the production of modified PC. These results are consistent with those reported by Adlercreutz *et al.*[41] who noted that according to the law of mass action, which defines a fundamental equilibrium constant, K_0, the yield in the transesterication reaction is expected to increase with increasing fatty acid and/or 2-acyl LPC concentration.

Nevertheless, the increase in fatty acid concentration had two effects: it increased the yield and decreased the equilibrium constant, K_0. So that the net effect is that the yield increased with increasing fatty acid concentration, but not as much as indicated by the law of mass action. The decrease in the equilibrium constant, K_0, may have been caused by an increase in the polarity of the reaction medium upon addition of increasing amounts of fatty acids. In fact, Egger *et al.*[42] found a reaction rate reduction with increasing concentration of free fatty acids, and they suggest that this as a consequence of either an increased viscosity, a decreased solubility, or to the changes in the polarity of the reaction medium.

Niezgoda *et al.*[35] indicated that, according to the law of mass action, a high fatty acid concentration increase the yield of esterification of 2-acyl LPC, but also inhibits the hydrolysis rate of PC and formation of 2-acyl LPC, which is an intermediate in the acidolysis process to produce modified PC. They obtained the highest incorporation of conjugated linoleic acid(CLA)into PC during lipase-catalyzed acidolysis when the CLA/PC molar ratio was increased to 4:1. However, at higher CLA/PC molar ratios, there was a reduction in the extent of incorporation of CLA into PC.

3.3 Optimization of the acidolysis reaction

Optimization of PLA$_1$-catalyzed acidolysis for the production of modified PC, by incorporation of AGCM to PC, was also performed by Response Surface Methodology (RMS). RSM is a convenient method for testing multiple-process variables because fewer experimental trials are needed as compared with the study of one variable at a time. RSM answers the question of how to select the levels for the applied variables to obtain the desirable, smallest or

largest, value of the response function using a reduced number of experiments[43].

This methodology was applied to optimize the reaction system using three process parameters: molar ratio of substrates(PC to free MCFA), enzyme loading, and reaction temperature. Optimal conditions obtained from the model were a molar ratio o substrates of 1:15, an enzyme loading of 12%, and 45℃. Repeated reactions for producing SPC by acidolysis between PC and free MCFA were carried out in 25-mL Erlenmeyer flasks under the optimized conditions for verification of the model. Under these conditions, a production of modified phosphatidylcholine of 52.98% could be obtained after 24 h of reaction. The prediction was confirmed by the experiments.

The time course of the modified PC production by PLA$_1$ for the optimal conditions of reaction(R OPT)is depicted in Fig. 4. This Figure shows that the maximum production of modified PC under optimal conditions, achieved after 24 h of reaction, was 53.02%, which is greater than those levels reached under the best experimental conditions tested, 42.20% for R 13 and 39.21% for R 2. These results suggest that RSM is a reliable tool for the optimization of the production of modified PC by acydolisis between PC and free MCFA. Other related works have reported the advantages of using the RSM to determine the effects of several variables and optimize the enzyme-catalyzed synthesis of SPLs[24, 27, 30, 36, 44].

Acidolysis was scaled-up using the optimal conditions of molar ratio of substrates, enzyme loading, and reaction temperature. The mixture of substrates and immobilized enzyme were placed in a glass reactor and, the reaction was carried out by 24 h in order to obtain the highest modi-
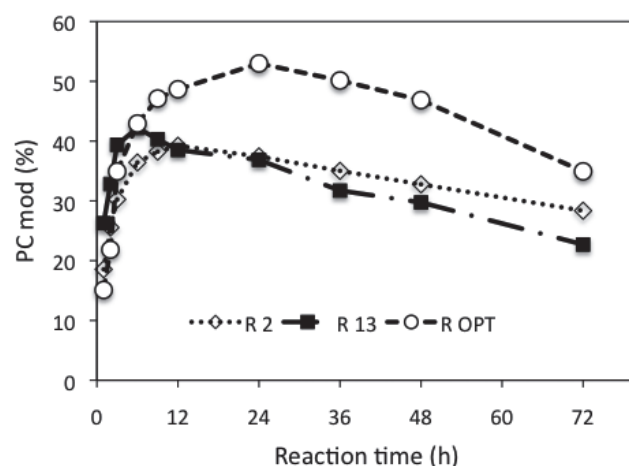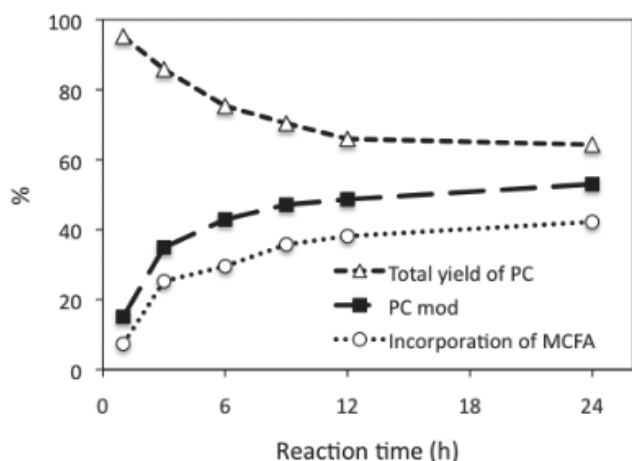

Fig. 4 Synthesis of modified PC(PC mod)by PLA$_1$ for treatments 2(R 2), 13(R 13), according to the experimental design, and optimal conditions(R OPT). Optimal conditions: mole ratio of substrates (PC/MCFA)1:15, enzyme loading 12%, and reaction temperature 45℃.

Fig. 5 Modified PC (PC mod), Incorporation of MCFA into PC, and Total yield of PC during the acidolysis reaction by PLA$_1$ for optimal conditions of reaction: mole ratio of substrates (PC/MCFA) 1:15, enzyme loading 12%, and reaction temperature 45℃.

fied PC production. **Figure 5** depicts the kinetics of production of modified PC (PC mod), incorporation of MCFA into PC, and the total yield of PC (unreacted plus modified) for the scaling-up of the reaction. Under optimal conditions for acidolysis, scaling enabled us to obtain a production of modified PC of 53.02%, a molar incorporation of MCFA to PC of 42.31%, and a total yield of PC of 64.28%, after 24 h of reaction. **Table 3** shows the yield of PC obtained in this study and in others that have been previously reported. These results reveal that we achieved a greater yield of PC at shorter reaction times; and according to Niezgoda *et al.*[36] we also attained greater production of modified PC.

SPC was separated from the reaction mixture, from free fatty acids and lysophosphatidylcholine (LPC), by column chromatography. For the separation of free fatty acids 31 volumes (125 mL each) of chloroform were required; for the separation of SPC 19 volumes of chloroform/methanol (65:35 v/v) were required; and to separate LPC 15 volumes of methanol were used. The solid phase separation yielded 62.53% of SPC that was further identified by HPLC. The fatty acid composition of the SPC fraction obtained after 24 h of acidolysis was determined by GC. The SPC was composed by 42.52 mol% of MCFA, 31.39 ± 0.81 mol% of caprylic acid, and 11.13 ± 0.43 mol% of capric acid; while the mole percentages of palmitic, oleic, and linoleic acid residues were substantially reduced. **Table 4** shows the composition (mol%) of the fatty acid residues in PC and SPC.

4 Conclusions

SPC containing high levels of MCFA was successfully prepared through acidolysis of PC and free MCFA catalyzed by immobilized phospholipase A$_1$ in a solvent-free system. RSM proved to be effective for reaction optimization. The optimum conditions were: PC to free MCFA molar ratio 1:15, enzyme loading 12% (based on the mass of substrates), and reaction temperature of 45℃. Under these conditions, a production of 53.02% of modified PC was attained after 24 h. The yield of SPC and its molar incorporation of MCFA were 62.53% and 42.31%, respectively. All parameters evaluated showed significant effects on the production of modified PC; the order of parameters affecting were enzyme loading, reaction temperature, and molar ratio of substrates.

Table 3 Enzyme-catalyzed interesterification reactions with different fatty acids for the production of structured phosphatidylcholine.

Enzyme type and Reaction time	Enzyme load, Temperature, and Substrates molar ratio	Yield of PC	Reference
Lipozyme RM-IM 70 h	40%, 55℃, 1:6 (PC/caprylic acid)	53%	Vikbjerg *et al.* (2005a)
Lipozyme TL-IM 50 h	29%, 54℃, 1:15 (PC/caprylic acid)	60%	Vikbjerg *et al.* (2005b)
Immobilized PLA$_2$ 48 h	30%, 45℃, 1:9 (PC/caprylic acid)	29%	Vikbjerg *et al.* (2007)
PLA$_1$ 6 h	10%, 55℃, 1:10 (PC/n-3 PUFA)	31.4%	Kim *et al.* (2007)
Immobilized PLA$_1$ 24 h	15%, 55℃, 1:4 (PC/CLA)	43.8%	Baeza-Jiménez *et al.* (2012)
Immobilized PLA$_1$ 24 h	20%, 55℃, 1:8	16.7%	Zhao *et al.* (2014)
Novozyme 435 72 h	20%, 50℃, 1:3 (PC/punicic acid)	36%	Chojnacka *et al.* (2016)
Lipozyme RM IM 36 h	24%, 45℃, 1:8 (PC/CLA)	39.5% Modified PC 39%	Niezgoda *et al.* (2016)
Immobilized PLA$_1$ 24 h	12%, 45℃, 1:15 (PC/MCFA)	64% Modified PC 53%	This study

Table 4 Fatty acid composition（mol％）in phosphatidylcholine（PC）and structured phosphatidylcholine with medium chain fatty acid（SPC）.

Fatty acid	PC	SPC*
C8:0		31.39 ± 0.81
C10:0		11.13 ± 0.43
C16:0	14.18 ± 0.45	2.46 ± 0.37
C18:0	3.51 ± 0.21	0.52 ± 0.08
C18:1（9）	10.65 ± 0.59	6.97 ± 0.21
C18:1（7）	1.58 ± 0.12	0.40 ± 0.05
C18:2（6）	64.03 ± 0.37	43.24 ± 0.88
C18:3（3）	6.05 ± 0.43	3.89 ± 0.08
Total MCFA		42.52 ± 0.73

*SPC obtained to the optimal reaction conditions: substrates mixture consisting of PC and free medium chain fatty acid（MCFA）in molar ratio of 1:15; a loading of 12% of phospholipase A_1 immobilized on Duolite A568; incubated for 24 h in an enclosed reactor, maintained at 45℃ and stirred at 300 rpm. Values reported are mean of duplicate determinations from different experimental trials.

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REFERENCES

1) Gul, K.; Singh, A.K.; Jabeen, R. Nutraceuticals and functional foods: The foods for the future world. *Crit. Rev. Food Sci. Nutr.* **56**, 2617-2627（2016）.

2) El Sohaimy, S.A. Functional Foods and Nutraceuticals-Modern Approach to Food Science. *World Appl. Sci. J.* **20**, 691-708（2012）.

3) Hailu, G.; Boecker, A.; Henson, S.; Cranfield, J. Consumer valuation of functional foods and nutraceuticals in Canada. A conjoint study using probiotics. *Appetite* **52**, 257-265（2009）.

4) Hasler, C.M.; Brown, A.C.; American Dietetic Association. Position of the American Dietetic Association: Functional Foods. *J. Am. Diet. Assoc.* **109**, 735-746 （2009）.

5) Momin, J.K.; Jayakumar, C.; Prajapati J.B. Potential of nanotechnology in functional foods. *Emir. J. Food Agric.* **25**, 10-19（2013）.

6) Chen, H.; Weiss, J.; Shahidi, F. Nanotechnology in nutraceuticals and functional foods. *Food Technol.* **60**, 30-36（2006）.

7) Li, J.; Wang, X.; Zhang, T.; Wang, C.; Huang, Z.; Luo, X.;

8) Deng, Y. A review on phospholipids and their main applications in drug delivery systems. *Asian J. Pharm. Sci.* **10**, 81-98（2015）.

9) Kidd, P.M. Phospholipids: versatile nutraceutical ingredients for functional foods. *Funct. Foods Nutraceut.* **4**, 30-38（2002）.

10) Fiume, Z. Final report on the safety assessment of lecithin and hydrogenated lecithin. *Int. J. Toxicol.* **20**, 21-45（2001）.

11) Klang, V.; Valenta, C. Lecithin-based nanoemulsions. *J. Drug Deliv. Sci. Tech.* **21**, 55-76（2011）.

12) Hoeller, S.; Sperger, A.; Valenta, C. Lecithin based nanoemulsions: A comparative study of the influence of non-ionic surfactants and the cationic phytosphingosine on physicochemical behaviour and skin permeation. *Int. J. Pharmaceut.* **370**, 181-186（2009）.

13) Wu, Y.; Wang, T. Soybean lecithin fractionation and functionality. *J. Am. Oil Chem. Soc.* **80**, 319-326 （2003）.

14) Guo, Z.; Vikbjerg, A.F.; Xu, X. Enzymatic modification of phospholipids for functional applications and human nutrition. *Biotechnol. Adv.* **23**, 203-259（2005）.

15) Osborn, H.T.; Akoh, C.C. Structured lipids novel fats with medical, nutraceutical, and food applications. *Compr. Rev. Food Sci. F.* **3**, 94-103（2002）.

16) Vikbjerg, A.F.; Rusig, J.Y.; Jonsson, G.; Mu, H.; Xu, X. Comparative evaluation of the emulsifying properties of phosphatidylcholine after enzymatic acyl modification. *J. Agr. Food Chem.* **54**, 3310-3316（2006）.

17) Davidsen, J.; Jørgensen, K.; Andresen, L.; Mouritsen, O.G. Secreted phospholipase A_2 as a new enzymatic trigger mechanism for localised liposomal drug release and adsorption in diseased tissue. *Biochim. Biophys. Acta* **1609**, 95-101（2003）.

18) Baeza-Jiménez, R.; López-Matínez, L.X.; García, H.S. Biocatalytic Modification of Food Lipids: Reactions and Applications. *Rev. Mex. Ing. Quím.* **13**, 29-47 （2014）.

19) Joshi, A.; Paratkar, S.G.; Thorat, B.N. Modification of lecithin by physical, chemical and enzymatic methods. *Eur. J. Lipid Sci. Tech.* **108**, 363-373（2006）.

20) Sharma, R.; Chisti, Y.; Banerjee, U.C. Production, purification, characterization, and applications of lipases. *Biotechnol. Adv.* **19**, 627-662（2001）.

21) Servi, S. Phospholipases as synthetic catalysts. *Top. Curr. Chem.* **200**, 128-158（1999）.

22) Vikbjerg, A.F.; Mu, H.; Xu, X. Parameters affecting incorporation and by-product formation during the production of structured phospholipids by lipase-catalyzed acidolysis in solvent-free system. *J. Mol. Catal. B: Enzym.* **36**, 14-21（2005）.

23) Vikbjerg, A.F.; Mu, H.; Xuebing, X. Elucidation of acyl migration during lipase-catalyzed production of structured phospholipids. *J. Am. Oil Chem. Soc.* **83**, 609-

614(2006).

23) Li, X.; Chen, J-F.; Yang, B.; Li, D-M.; Wang, Y-H.; Wang, W-F. Production of structured phosphatidylcholine with high content of DHA/EPA by immobilized phospholipase A₁-catalyzed transesterification. *Int. J. Mol. Sci.* **15**, 15244-15258(2015).

24) Baeza-Jiménez, R.; Noriega-Rodríguez, J.A.; García, H.S.; Otero, C. Structured phosphatidylcholine with elevated content of conjugated linoleic acid: Optimization by response surface methodology. *Eur. J. Lipid Sci. Tech.* **114**, 1261-1267(2012).

25) Kim, I.H.; García, H.S.; Hill, C.G. Jr. Synthesis of structured phosphatidylcholine containing n-3 PUFA residues via acidolysis mediated by immobilized phospholipase A₁. *J. Am. Oil Chem. Soc.* **87**, 1293-1299(2010).

26) García, H.S.; Kim, I.H.; López-Hernández, A.; Hill, C.G. Jr. Enrichment of lecithin with *n*-3 fatty acids by acidolysis using immobilized phospholipase A₁. *Grasas Aceites* **59**, 368-374(2008).

27) Vikbjerg, A.F.; Mu, H.; Xu, X. Synthesis of structured phospholipids by immobilized phospholipase A₂ catalyzed acidolysis. *J. Biotechnol.* **128**, 545-554(2007).

28) Awano, S.; Miyamoto, K.; Hosokawa, M.; Mankura, M.; Takahashi, K. Production of docosahexaenoic acid bounded phospholipids via phospholipase A₂ mediated bioconversion. *Fisheries Sci.* **72**, 909-911(2006).

29) Reddy, J.R.C.; Vijeeta, T.; Karuna, M.S.L.; Rao, B.V.S.K.; Prasad, R.B.N. Lipase-catalyzed preparation of palmitic and stearic acid rich phosphatidylcholine. *J. Am. Oil Chem. Soc.* **82**, 727-730(2005).

30) Vikbjerg, A.F.; Mu, H.; Xu, X. Lipase-catalyzed acyl exchange of soybean phosphatidylcholine in n-hexane: A critical evaluation of both acyl incorporation and product recovery. *Biotechnol. Progr.* **21**, 397-404(2005).

31) Vijeeta, T.; Reddy, J.R.C.; Rao, B.; Karuna, M.; Prasad, R. Phospholipase mediated preparation of 1-ricinoleoyl-2-acyl-sn-glycero-3-phosphocholine from soya and egg phosphatidylcholine. *Biotechnol. Lett.* **26**, 1077-1080(2004).

32) Kim, I.H.; Hill, C.G. Jr. Lipase-catalyzed acidolysis of menhaden oil with pinolenic acid. *J. Am. Oil Chem. Soc.* **83**, 109-115(2006).

33) Vikbjerg, A.F.; Rusig, J.Y.; Jonsson, G.; Mu, H.; Xu, X. Strategies for lipase-catalyzed production and the purification of structured phospholipids. *Eur. J. Lipid Sci. Tech.* **108**, 802-811(2006).

34) Ochoa, A.A.; Hernández-Becerra, J.A.; Cavazos-Garduño, A.; García, H.S.; Vernon-Carter, E. Phosphatidylcholine enrichment with medium chain fatty acids by immobilized phospholipase A₁-catalyzed acidolysis. *Biotechnol. Progr.* **29**, 230-236(2013).

35) Niezgoda, N.; Gliszczyńska, A.; Gladkowski, W.; Chojnacka, A.; Kielbowicz, G.; Wawrzeńczyk, C. Lipase-catalyzed enrichment of egg yolk phosphatidylcholine with conjugated linoleic acid. *J. Mol. Catal. B: Enzym.* **123**, 14-22(2016).

36) Peng, L.; Xu, X.; Mu, H.; Hoy, C.E.; Adler-Nissen, J. Production of structured phospholipids by lipase-catalyzed acidolysis: optimization using response surface methodology. *Enzyme Microb. Tech.* **31**, 523-532(2002).

37) Chojnacka, A.; Gladkowski, W.; Gliszczyńska, A.; Niezgoda, N.; Kielbowicz, G.; Wawrzeńczyk, C. Synthesis of structured phosphatidylcholine containing punicic acid by the lipase-catalyzed transesterification with pomegranate seed oil. *Catal. Commun.* **75**, 60-64(2016).

38) Zhao, T.; No, D.S.; Kim, B.H.; Garcia, H.S.; Kim, Y.; Kim, I.H. Immobilized phospholipase A₁-catalyzed modification of phosphatidylcholine with *n*-3 polyunsaturated fatty acid. *Food Chem.* **157**, 132-140(2014).

39) Kim, I.H.; Garcia, H.S.; Hill, C.G. Jr. Phospholipase A₁-catalyzed synthesis of phospholipids enriched in *n*-3 polyunsaturated fatty acid residues. *Enzyme Microb. Tech.* **40**, 1130-1135(2007).

40) Doig, S.D.; Diks, R.M.M. Toolbox for exchanging constituent fatty acids in lecithins. *Eur. J. Lipid Sci. Tech.* **105**, 359-367(2003).

41) Adlercreutz, D.; Budde, H.; Wehtje, E. Synthesis of phosphatidylcholine with defined fatty acid in the *sn*-1 position by lipase-catalyzed esterification and transesterification reaction. *Biotechnol. Bioeng.* **78**, 403-411(2002).

42) Eggert, T.; Wehtje, E.; Adlercreutz, P. Characterization and optimization of phospholipase A₂ catalyzed synthesis of phosphatidylcholine. *Biochim. Biophys. Acta* **1343**, 76-84(1997).

43) Hanrahan, G.; Lu, K. Application of factorial designs and response surface methodology in modern experimental design and optimization. *Rev. Anal. Chem.* **36**, 141-151(2006).

44) Yang, G.; Yang, L. Increase of oleic acid content in phosphatidylcholine through lipase-catalyzed interesterification: Optimization by response surface methodology. *J. Oleo Sci.* **64**, 673-682(2015).