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Enrichment of Amaranth Oil with Ethyl Palmitate at the *sn*-2 Position by Chemical and Enzymatic Synthesis

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Amaranth oil is rich in linoleic, oleic, and palmitic acids. Structured lipids (SLs) with specific functional and nutritional characteristics can be prepared through chemical or enzymatic interesterification. The aim of this study was to increase the palmitic acid content at the *sn*-2 position in amaranth oil triacylglycerols (TAG) for possible use in infant formula. Chemical and enzymatic interesterification techniques were assessed before selecting the latter for further optimization modeling. Enzymatic interesterification of ethyl palmitate and amaranth oil significantly increased the total content of palmitic acid, reduced linoleic acid content, and increased the amount of palmitic acid at the *sn*-2 position of the SL product. Even though amaranth oil content of palmitic acid (18.3%) was originally similar to that in breast milk (18.3−25.9%), the structural changes induced through enzymatic modification resulted in a SL closely resembling breast milk fat and hence its possible application as a fat substitute for infant nutrition. A second-order polynomial model was developed to predict the amount of total palmitic acid incorporated when reaction time and substrate level were manipulated, and to optimize the combination of parameters to achieve specific palmitic acid contents in amaranth oil. The resulting model is useful to develop an SL from amaranth oil enriched with palmitic acid specifically at the *sn*-2 position for possible application in infant formulas.

KEYWORDS: Amaranth oil; enzymatic interesterification; structured lipid

INTRODUCTION

Amaranth is an ancient crop originally from Meso-America where its importance was considered similar to that of corn and wheat before the colonization period. It is classified as a pseudocereal, and it is currently cultivated in warm climates with at least 18 °C soil temperature such as in Mexico, Central and South America, Africa, India, China, and the United States. The lipid content of amaranth grain ranges from 6 to 9% (*1*). Amaranth oil is a yellow liquid at room temperature, and it has a melting point of −27 °C (*2*).

Nowadays, amaranth is used in several bakery products including breads, cookies, pasta, and marzipan (*3*). Also, it has been proposed as an alternative to increase protein quality in tortillas (*4*, *5*). Other studied applications include milk-like beverages and infant formulas (*6*). Even though amaranth oil has been extensively characterized before, applications still remain underexplored. Amaranth oil contains about 18.6−23.4% palmitic, 22.7−31.5% oleic, 39.4−49.8% linoleic, and 0.5−1.4% linolenic acids (*1*, *2*, *7*−*10*). Palmitic acid, a major fatty acid (FA) in amaranth oil, also constitutes the second major FA in breast milk (∼18.3−25.9% palmitic acid) (*11*−*14*). This FA resemblance suggests that amaranth oil can be used as a raw oil to enhance palmitic acid content for infant formula applications. However, there are important compositional differences between amaranth oil and breast milk fat. Although amaranth oil contains FA levels similar to those from breast milk, in amaranth oil the main FA esterified at the *sn*-2 position is linoleic acid, followed by oleic, and palmitic acids (*10*) compared to human breast milk, which contains a relatively large amount of palmitic acid esterified at the *sn*-2 position (>60%) of the triacylglycerols (TAG) (*15*−*18*). It has been reported that about 81% of total palmitic acid is esterified at the *sn*-2 position of human milk fat (*19*). However, for adults large amounts of palmitic acid in the diet could represent higher risk for coronary heart disease (CHD) because of its atherogenic properties (*20*), but for infants, it represents two important benefits for proper nutrition. In breast milk fat, the preferential presence of palmitic acid at the *sn*-2 position improves fat and calcium absorption in infants while reducing the production and disposal of calcium soaps (*19*, *21*). Amaranth lipid profile variations greatly depend on the cultivar, extraction method, and refining process. It also contains significant amounts of squalene (∼4.2%) (*22*), sterols (∼2.5%) (*10*), and tocopherols and tocotrienols (∼0.1%) (*23*).

We do not have knowledge of any other study attempting to modify amaranth oil for infant formula applications. However, several studies had been published on the development of human milk fat analogues, from randomized oil mixtures to achieve balance in FAs, to the interesterification of substrates such as tripalmitin, hazelnut oil FA, *n*-3 polyunsaturated FAs concentrates, rapeseed oil FA, soybean FA, lard, coconut oil, safflower oil, and butter oil to resemble breast milk fat composition (*24*−*30*). Betapol (Loders Croklaan, Glen Ellyn, IL, USA) is an example of commercial breast milk fat substitutes produced

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by lipase interesterification of tripalmitin and unsaturated FAs (*12*).

Amaranth oil represents an alternative raw material that can be used to design structured lipids as milk fat substitutes for possible applications in infant nutrition. Therefore, the aim of this study was to restructure amaranth oil's TAGs by increasing its palmitic acid content at the *sn*-2 position. Chemical and enzymatic interesterification techniques were evaluated. Finally, an optimization model was developed to predict the incorporation of palmitic acid into amaranth oil.

MATERIALS AND METHODS

Materials. Amaranth oil was purchased from Nu World Amaranth Inc. (Naperville, IL). Ethyl palmitate (Kosher) and sodium methoxide (food grade) were purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized lipase, Novozym 435, was generously donated by Novozymes North America Inc. (Franklinton, NC). Supelco 37 Component FAME mix, C17:0-heptadecanoic acid (>98% purity), triolein, and 2-oleoylglycerol were used as standards and were purchased from Sigma Chemical Co. (St. Louis, MO). Other solvents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), J. T. Baker Chemical Co. (Phillipsburg, NJ), or Fisher Scientific (Norcross, GA).

Oil Mixture Preparation. For chemical and enzymatic method comparison, two mixtures (10 g) of amaranth oil (MW = 922.4 g/mol) and ethyl palmitate (MW = 284.5 g/mol) were prepared, on the basis of their molecular weight, in a 1:4 and 1:6 mol/mol ratio, respectively. Amaranth oil is a liquid at room temperature, but ethyl palmitate was melted to liquid at 38 °C before blending to ensure the uniformity of the mixtures.

Chemical Interesterification. A modified version of the method described by Lumor et al. (*31*) was used for chemical interesterification. One gram of each mixture (described above) was weighed in a labeled screw-cap test tube, flushed with nitrogen, and dried for 15 min at 110 °C. Then, 0.3% (w/w) sodium methoxide was added as a chemical catalyst. The reaction was held for 60 min at 100 °C with constant stirring at 200 rpm. The reaction product was cooled to 60−70 °C, and the catalyst was removed using hexane and filtering through an anhydrous sodium sulfate column three times.

Enzymatic Interesterification. One gram of each mixture (described above) was weighed in labeled screw-cap test tubes, and 10% (w/w) of Novozym 435 (from *C. antarctica*) was added as enzymatic catalyst. The reaction was carried out in a water bath at 60 °C for 6 h with constant stirring. The resulting product was filtrated three times through an anhydrous sodium sulfate column to remove the catalyst.

Recovery of Triacylglycerols (TAGs). After chemical or enzymatic interesterification, the resulting product was spotted onto silica gel G TLC plates, and a mixture of petroleum ether, diethyl ether, and acetic acid (90:10:0.5, v/v/v) was used to separate the TAGs. Lipid bands were visualized after spraying plates with 0.2% 2,7-dichlorofluorescein in methanol and under UV light. Ethyl palmitate and TAG separated bands were identified using ethyl palmitate and triolein as standards. The TAG band was scraped off and recovered into test tubes for fatty acid methyl ester (FAME) and positional analyses as purified representations of the structured lipid (SL) obtained after the enzymatic reactions.

Experimental Design by Response Surface Methodology (RSM). A RSM mathematical model was developed to predict the incorporation of palmitate in amaranth oil by enzymatic interesterification. Amaranth oil and ethyl palmitate mixtures were prepared on the basis of their average molecular weight. The suggested combinations resulting from the experimental design are shown in **Table 1**. The experimental design took into consideration the effect of ethyl palmitate to amaranth oil ratio (low level = 1:4; high level = 1:6, respectively) and the time of reaction (low level = 6 h; high level = 18 h) under isothermal conditions (60 °C) using 10% (w/w) Novozym 435 as catalyst. Therefore, the central composite face design included eight possible combinations and three center points. Treatments were performed in duplicate resulting in 22 experiments. The reactions took place in a water bath with constant stirring. After reaction completion, TAGs from the resulting products were recovered as described above, and analyzed for FA profile and positional analysis.

Table 1. Experimental Design of Factors and Responses for Modeling the Enzymatic Reaction by RSM

expt[a]	amaranth oil (mol)	ethyl palmitate (mol)	temp (°C)	rx time (h)	total PA[b] (mol %)	PA at *sn*-2 (mol %)
N1	1	4	60	6	39.2 ± 0.6	28.7 ± 0.7
N2	1	4	60	18	62.0 ± 0.3	57.4 ± 1.1
N3	1	6	60	6	39.7 ± 0.1	32.5 ± 0.8
N4	1	6	60	18	61.0 ± 1.7	56.0 ± 5.2
N5	1	5	60	6	41.5 ± 1.2	55.7 ± 2.9
N6	1	5	60	18	56.1 ± 6.5	46.2 ± 4.5
N7	1	4	60	12	57.4 ± 2.3	41.5 ± 5.2
N8	1	6	60	12	52.0 ± 1.5	49.5 ± 4.9
N9	1	5	60	12	55.1 ± 4.6	54.0 ± 5.7
N10	1	5	60	12	57.7 ± 0.2	46.3 ± 3.9
N11	1	5	60	12	63.5 ± 0.1	60.6 ± 0.3

[a] Mean ± SD, $n = 2$. [b] Abbreviations: expt, experiment; temp, temperature; rx time, reaction time; PA, palmitic acid.

Total incorporation of palmitate in amaranth oil TAGs and at the *sn*-2 position of the glycerol backbone were recorded as variable responses in **Table 1**, as well as the experimental conditions for each run.

Reaction Procedures for RSM. Reactions were similar to the procedure described above for enzymatic interesterification. The suggested oil blends (**Table 1**) were weighed in screw-cap test tubes, and 10% (w/w) Novozym 435 was added as enzymatic catalyst. The reaction took place in an orbital shaking water bath at constant temperature (60 °C) for 6, 12, and 18 h according to the conditions in **Table 1**. After reaction completion, TAGs were recovered according to the procedure previously described.

Mathematical Model. The response surfaces for the relationship between factors and variables from the above design were fitted to a second-order polynomial equation of the form:

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{1} \sum_{j=i+1}^{2} \beta_{ij} X_i X_j \qquad (1)$$

where Y is the dependent variable (palmitic acid incorporation); β_0 is a constant; β_i is the linear term coefficient; β_{ii} is the quadratic term coefficient; β_{ij} is the interaction term coefficient; and X_i and X_j are the independent variables. The analysis of variance (ANOVA), regression analysis, and response surfaces were obtained using MODDE 5.0 (Umetrics, Umeå, Sweden). An optimization model for palmitic acid incorporation into amaranth oil by enzymatic interesterification was determined using RSM.

Determination of Fatty Acid Profiles. Amaranth oil and SL samples were converted to FAME following the AOAC Official Method 996.01, Section E (*32*) with minor modifications. For amaranth oil sample preparation, 100 mg of oil was weighed into a Teflon-lined test tube, and 1 mL C17:0 in hexane (20 mg/mL) was added as internal standard and dried with nitrogen to remove solvent. For SL analysis, 100 μL of internal standard was added to the recovered TAG band from a previous separation step. Then, 2 mL 0.5 N NaOH in methanol was added followed by incubation for 5 min at 100 °C to saponify the lipid. After incubation, 2 mL of 14% boron trifluoride (BF_3) in methanol was added. The sample was vortexed for 1 min and incubated again for 5 min at 100 °C to allow methylation. To stop the reaction and extract the FAMEs, 2 mL of hexane and 2 mL of NaCl saturated solution were added to the sample, vortexed for exactly 2 min at room temperature, and centrifuged for 5 min at 1,000 rpm to separate the organic and aqueous phases. The upper organic layer was filtered twice through an anhydrous sodium sulfate column, recovered into a GC vial, and analyzed. The Supelco 37 component FAME mix was used as FAME external standard and run in parallel with the samples.

Positional Analysis. A modified version of the reported method (*33*) was used to perform the pancreatic lipase-catalyzed *sn*-2 positional analysis. Amaranth oil (100 mg) and SL (TAG recovered band) were collected in Teflon-lined test tubes. Two milliliter of Tris-HCl buffer (1.0 M), 0.5 mL of sodium cholate solution (0.05%), and 0.2 mL of calcium chloride solution (2.2%) were added to the samples and vortexed for 2 min.

After emulsification, 40 mg of pancreatic lipase was added, mixed, and incubated at 40 °C for 3 min. The tubes were vortexed for 2 min, and 1 mL of HCl (6 N) and 4 mL of diethyl ether were added to stop the reaction and extract the hydrolyzed TAGs. The upper layer containing the lipid components was separated, filtered twice through an anhydrous sodium sulfate column, and flushed with nitrogen to evaporate solvent until one-third of the volume was left. The dried product was spotted on silica gel G TLC plates and developed with hexane, diethyl ether, and formic acid (60:40:1.6, v/v/v). 2-Oleylglycerol was spotted in parallel as an identification standard for 2-monoacylglycerol (2-MAG). Plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and exposed to UV light to identify the different bands. The band corresponding to 2-MAG was scrapped off and converted to FAME as previously described. Three hundred microliters of C17:0 in hexane (20 mg/mL) was used as internal standard for the amaranth oil and 50 μL of internal standard for the SL. FAs esterified at the *sn*-2 position were quantified by GC, and the amounts at *sn*-1,3 were calculated.

GC Analysis. FAMEs (from amaranth oil, SL, and corresponding positional analyses) were analyzed using an Agilent Technology 6890N gas chromatograph equipped with a flame ionization detector. Separation was achieved with an SP-2560 column, 100 m × 0.25 mm i.d., and 0.20 μm film. Injection (1 μL) was performed at a split ratio of 5:1. Helium was the carrier gas, at constant pressure, and the flow rate was 1.1 mL/min. The injector temperature was 250 °C, and the FID set point was 260 °C. In the oven, the sample was held at 150 °C for 3 min, then increased up to 215 °C ramping at 10 °C/min, and held isothermally for 40 min. FAME relative content was calculated by integration using an online computer. Average of triplicate analyses were reported.

Statistical Analysis. All samples, reactions, and analyses were performed in triplicate for amaranth oil and SLs using both interesterification approaches. Average and standard deviation were calculated and reported. The analysis of variance (ANOVA) and the mathematical model for optimization by enzymatic interesterification were obtained using MODDE 5.0 (Umetrics, Umeå, Sweden).

RESULTS AND DISCUSSION.

Commercial amaranth oil FA profile was determined and shown in **Table 2**. From the FA profile obtained, our results for palmitic, oleic, and linoleic acids were in agreement and within the range established from previous studies (*1*, *2*, *8*−*10*). Our results were used to estimate the molecular weight of commercial amaranth oil (922.4 g/mol); this value was later used to determine the corresponding portion of amaranth oil in each oil blend. Linoleic acid (47.8%) was the major FA in amaranth oil, followed by oleic (28.9%) and palmitic (18.3%) acids (**Table 2**). Linoleic acid is also the major available FA at the *sn*-2 position in amaranth oil (72.2%). Conversely in breast milk, linoleic acid content is about 15.6% (*13*), while palmitic acid accounts for the majority of the saturated FA portion of human milk with over 60% by weight of its total content at the *sn*-2 position. This particular arrangement serves several nutritional purposes. For instance, the formation of calcium soaps is low, and hence, it represents a more readily absorbable source of energy for infant development and also contributes to a better absorption of calcium (*15*−*18*). From the positional analysis of amaranth oil, we determined that there was only about 2.1% palmitic acid esterified at the *sn*-2 position of the glycerol backbone. Previous studies have shown that saturated FAs tend to be exclusively located at the external positions in vegetable oil TAGs, in contrast to animal fats in which these positions are usually occupied by unsaturated FAs (*34*). Esterification techniques can be used to produce SL with improved functionality due to the incorporation of new FA into oil or fat, or the rearrangement of the existing FAs (*35*, *36*). Chemical interesterification is commonly preferred for industrial purposes because of its comparable yields and cheaper cost (*34*). However, enzymatic interesterification is more spatially selective, yielding more specific TAGs (*37*). Our aim was to modify the original amaranth oil's TAG structure to increase the palmitic acid esterified at the *sn*-2 position in order to match the recommended FAs' requirements (*38*) for breast milk fat substitutes. For that purpose, we assessed the overall performance of both chemical and enzymatic interesterification methods based on the total palmitic acid content and incorporation at the *sn*-2 position. Both interesterification reactions, carried at constant experimental conditions and using two substrate levels, increased the palmitic acid content at the *sn*-2 position at the expense of linoleic acid (**Table 3**). Even though, both techniques yielded higher total palmitic acid and higher palmitic acid at the *sn*-2 position, the increment was more significant using enzymatic interesterification. The SL products had lower total content of linoleic acid, and a lower amount of this FA esterified at the *sn*-2 position than amaranth oil. Stearic acid content was lower for enzymatically produced SLs, and linolenic acid was not detected in the lipase-catalyzed SLs in contrast to the products from chemical interesterification. As mentioned before, one of the nutritional advantages of the large amount of palmitic acid esterified at the *sn*-2 position is to prevent the formation of

Table 2. Fatty Acid Profile of Commercial Amaranth Oil

		positional distribution	
fatty acid[a]	total (mol %)	*sn*-2 (mol %)	*sn*-1,3 (mol %)[b]
16:0	18.3 ± 0.1	2.1 ± 0.0	27.1 ± 0.7
18:0	3.8 ± 0.0	ND[c]	5.7 ± 0.0
18:1	28.9 ± 0.0	26.9 ± 0.6	29.9 ± 0.3
18:2	47.8 ± 0.1	72.2 ± 2.1	35.7 ± 1.2
18:3	1.2 ± 0.0	0.7 ± 0.0	1.7 ± 0.2

[a] Mean ± SD, $n = 3$. [b] *sn*-1,3 (mol %) = [3 × total (mol %) − *sn*-2 (mol %)]/2. [c] Abbreviation: ND, not detected.

Table 3. Fatty Acid Profile of Structured Lipid Produced by Chemical and Enzymatic Interesterifications

		chemical[c]			enzymatic[e]		
substrate level[a]	fatty acid[b]	total (mol %)	*sn*-2 (mol %)	*sn*-1,3[d] (mol %)	total (mol %)	*sn*-2 (mol %)	*sn*-1,3 (mol %)
low (1:4)	16:0	20.4 ± 0.7	22.4 ± 4.2	19.4 ± 1.4	39.2 ± 0.4	28.9 ± 0.6	44.4 ± 0.4
	18:0	3.9 ± 0.2	8.4 ± 2.0	3.1 ± 2.7	2.5 ± 0.0	7.9 ± 2.5	0.6 ± 0.2
	18:1	31.5 ± 1.2	42.6 ± 2.1	26.0 ± 1.7	22.7 ± 0.1	37.3 ± 4.9	15.4 ± 2.5
	18:2	44.0 ± 1.7	29.4 ± 1.2	51.3 ± 2.0	35.5 ± 0.5	25.9 ± 6.8	40.3 ± 4.1
	18:3	0.5 ± 0.0	ND[f]	0.7 ± 0.0	ND	ND	ND
high (1:6)	16:0	19.3 ± 0.3	23.4 ± 4.7	17.3 ± 2.5	39.7 ± 0.1	33.2 ± 1.3	42.9 ± 0.6
	18:0	3.2 ± 0.1	8.9 ± 1.7	0.4 ± 0.8	2.4 ± 0.0	7.8 ± 0.6	0.0 ± 0.3
	18:1	26.9 ± 0.4	33.9 ± 3.0	23.4 ± 1.0	22.6 ± 0.1	41.6 ± 4.5	13.1 ± 2.2
	18:2	49.7 ± 0.6	33.9 ± 4.1	57.6 ± 1.5	35.4 ± 0.1	17.3 ± 5.8	44.4 ± 2.9
	18:3	0.8 ± 0.1	ND	1.2 ± 0.1	ND	ND	ND

[a] Substrate level refers to the mol ratio of amaranth oil to ethyl palmitate. [b] Mean ± SD, $n = 3$. [c] Conditions for chemical interesterification reaction: 0.3% catalyst, 100 °C for 1 h. [d] *sn*-1,3 (mol %) = [3 × total (mol %) − *sn*-2 (mol %)]/2. [e] Conditions for the enzymatic interesterification reaction: 10% catalyst, 60 °C for 6 h. [f] Abbreviation: ND, not detected.

Table 4. Analysis of Variance (ANOVA) for Total Palmitic Acid Content

total PA[a]	DF	SS	MS (variance)	*F*-value	*P*-value	SD
total	22	63931.600	2905.980			
constant	1	62235.500	62235.500			
total corrected	21	1696.120	80.768			8.987
regression	3	1443.740	481.245	34.322	0.000	21.937
residual	18	252.385	14.021			3.745
lack of fit (model error)	5	101.928	20.386	1.761	0.190	4.515
pure error (replicate error)	13	150.458	11.574			3.402
$N = 22$	$Q^2 = 0.793$		$R^2_{adj} = 0.826$			
DF = 18	$R^2 = 0.851$		RSD = 3.744			

[a] Abbreviations: PA, palmitic acid; DF, degree of freedom; SS, sum of squares; MS, mean square; RSD, relative standard deviation; N, number of experiments; SD, standard deviation; R^2_{adj}, R^2 adjusted for the number of independent factors in the model; R^2 and Q^2 are explained in the text.

calcium soaps. For the enzyme catalyzed reaction using high mol ratio (1:6, amaranth oil to ethyl palmitate), there was no major difference in overall FA composition compared to the lower substrate level, but we observed a noticeable increase in the amount of palmitic acid at the *sn*-2 position and linoleic acid at the *sn*-1,3 position. At this point, we concluded that for enzymatic reactions, no matter the higher availability of substrates, the reaction might require longer times to overcome the same level of hydrolysis and esterification reached in the low substrate level reaction, and therefore, the amount of palmitic acid esterified at any of the positions would be affected by the reaction kinetics. On the basis of our preliminary experiments (**Table 3**), we selected the enzymatic interesterification method to develop an optimization model for palmitic acid incorporation in amaranth oil when substrate availability and reaction times change. On this issue, the resulting SL from the recommended optimization reaction would be used as a fat substitute, possibly in combination with other fat sources, for infant formulas.

The experimental design included differences in substrate level and reaction time, and this was used to develop a model for future prediction of palmitic acid content. The resulting amounts of total palmitic acid and palmitic acid at the *sn*-2 position are shown in **Table 1**. For total palmitic acid response, multiple linear regressions and the backward selection method were used to fit the results to a second-order polynomial model, from which the squared term *EtP***EtP* and the interaction term *Rxt***EtP* were deleted because they were not significant at 0.05 probability level. *EtP* refers to the ethyl palmitate mole ratio, and *Rxt* refers to the reaction time.

The multiple correlation coefficient (R^2) was 0.85, corresponding to the fraction of the variation of the response explained by the model. Q^2 corresponding to the fraction of the variation of the response that can be predicted by the model was 0.79. Although R^2 is a very popular statistical value to assess the variance explanation, in planned experimentation it is more significant to support conclusions based on analysis of variance (ANOVA) statistics (*39*). The acceptable R^2 was probably adversely affected by the proximity in the range of the dependent variables (reaction time and mol ratio), therefore resulting in a smaller response difference of palmitic acid incorporation relative to the variance and hence possibly overlapping projections. However, on the basis of the acceptable value of R^2 in combination with the satisfactory results obtained in the ANOVA (**Table 4**), the RSM quadratic equation is appropriate for the modeling and optimization of palmitic acid incorporation into amaranth oil. The model showed no lack of fit ($P > 0.05$), and the *P*-value was < 0.001. The RSM quadratic equation can be expressed as follows: total $PA = 57.14 + 9.77Rxt - 0.98EtP - 7.24Rxt*Rxt$. Where total

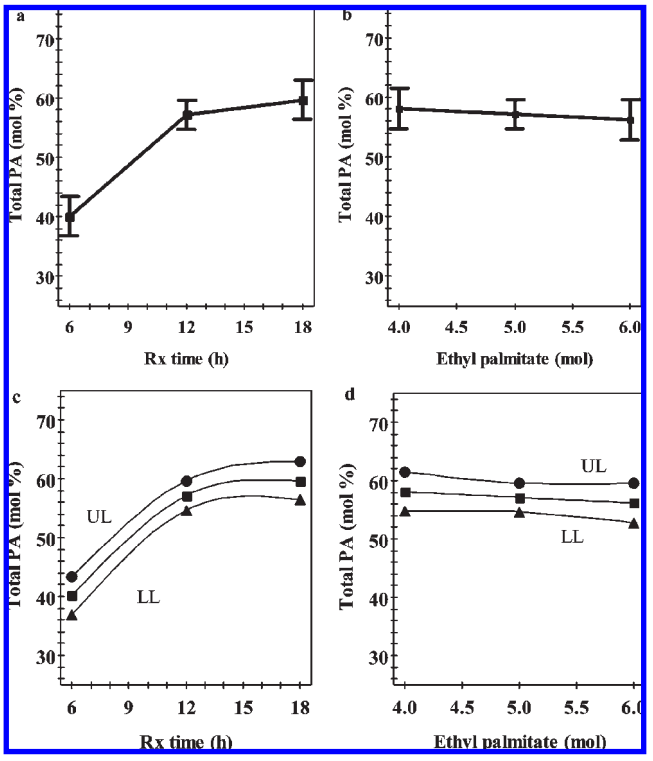

Figure 1. (**a**) Effect of reaction time on total palmitic acid content. (**b**) Effect of ethyl palmitate on total palmitic acid content. For **a** and **b**, the values plotted are the means ± SD, $n = 6$ for low and high levels, and $n = 10$ for center point. (**c**) Projected responses of total palmitic acid when reaction time is varying. (**d**) Projected responses of total palmitic acid when ethyl palmitate is varying. UL and LL refer to upper and lower confidence levels, respectively.

PA is the total content of palmitic acid in SL; *Rxt* is the reaction time at which corresponding palmitic acid incorporation is achieved; *EtP* is the ethyl palmitate mol ratio used in reaction; and *Rxt***Rxt* is the squared term of reaction time.

Reaction time had the largest effect on the amount of total palmitic acid as shown in **Figure 1a**, while ethyl palmitate availability had less effect at low substrate levels and a negative effect at high substrate levels (**Figure 1b**).

The projected responses (total palmitic acid) for variations in reaction time and ethyl palmitate availability when all but the parameter of interest remain constant are shown in **Figure 1c** and **b**. Total palmitic acid content is projected to increase when the reaction is performed for longer time periods (**Figure 1c**). However, the higher availability of ethyl palmitate showed a slightly adverse effect in total palmitic acid (**Figure 1d**). **Figure 2** shows the contour plot for the optimal combination of parameters to obtain a desired content of total palmitic acid. According to our results, palmitic acid incorporation was mostly affected by long reaction times but slightly affected by molar ratio (**Figure 2**). The highest incorporation of palmitic acid (~61.1%) can be obtained in 15−17 h of reaction using low substrate levels between 4.0 and 4.3 mol ethyl palmitate to 1.0 mol amaranth oil.

The model developed for total palmitic acid incorporation can explain a relatively high fraction of the response variations. However, the results for palmitic acid at *sn*-2 position were not efficiently fitted in a second-order polynomial equation. Only the term *Rxt*, corresponding to reaction time, was significant at $\alpha_{0.05}$. R^2 was only 0.32, and Q^2 was −0.01. The responses obtained did not show normal distribution, which means the esterification of palmitate into amaranth oil was random and nonpreferential. We believe the esterification of palmitic acid at

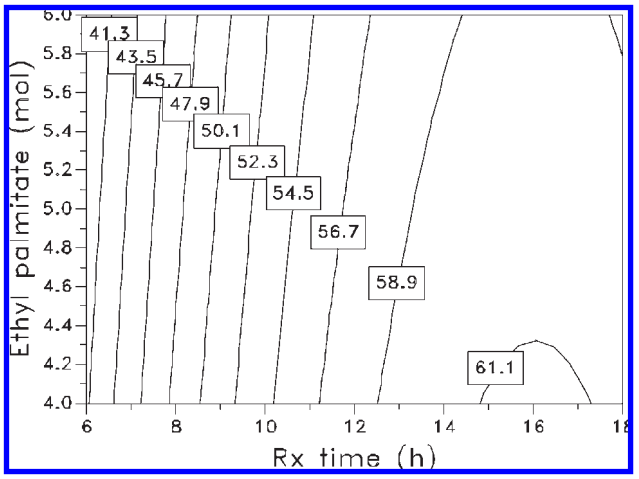

Figure 2. Contour plot showing effect of ethyl palmitate mole ratio used for the incorporation of palmitate with Novozym 435 as catalyst at 60 °C and different reaction times. The labels inside the plot indicate the total palmitic acid content (mol %).

the *sn*-2 position was not normal distribution because of the nonspecific properties of Novozym 435 used as the reaction catalyst. However, other studies have shown preference of Novozym 435 for the *sn*-1,3 position in ethanolysis reactions at a high excess of ethanol substrate (~1:20, tridocosahexanoylglycerol to ethylcaprilate) (*40*). For the interesterification reaction of ethyl palmitate and amaranth oil TAGs, we observed random interesterification when using low substrate ratios (1:4 to 1:6, amaranth oil to ethyl palmitate). The random responses obtained and the nonnormal projected responses cannot be fitted into a linear mathematical equation. Therefore, further modeling and optimization was not performed for this response.

The developed RSM model can predict the optimal parameter combination to achieve specific palmitic acid incorporation. On the basis of test tube size confirmation experiments (results not shown), we are able to design the synthesis of SL from amaranth oil that resembles breast milk fat in palmitic acid composition and with comparable or improved regiospecificity of palmitic acid at the *sn*-2 position in comparison to amaranth oil and other reported breast milk fat analogues (*24*−*30*). Our optimization model suggested that a reaction of amaranth oil and ethyl palmitate in a mole ratio of 1:4, catalyzed with 10% (by substrates weight) Novozym 435 for 3 h at 60 °C with constant stirring, will yield a SL to match the nutritional recommendations established for fat substitution in infant formula (*38*). However, further research is required to incorporate *n*-3 polyunsaturated FAs into this amaranth oil SL in order to completely resemble breast milk fat for infant formula applications. Previous studies had been successful in developing breast milk fat substitutes through enzymatic modification of vegetable oils. Sahin et al. (*25*) achieved a SL from hazelnut oil, tripalmitin, and *n*-3 polyunsaturated FAs with 76.6% palmitic acid esterified at the *sn*-2 position; however, the amount of total palmitic acid is almost double (45.5%) the normal range in breast milk (18.3−25.9%). However, linoleic acid content remained low (4.4%), but the incorporation of EPA and DHA (6.2%) might exceed the recommended contents for infant formulas (<0.25% EPA and <0.5% DHA) (*38*). In a different study, Maduko et al. (*26*) developed a SL to use with caprine milk for infant formula containing 24.6% palmitic, 29.6% oleic, and 3.4% linoleic acids similar to breast milk composition; however, the resulting SL also contained 23.6% caprylic acid. Palmitic acid is the most extensively studied saturated FA for infant formula; other saturated FAs could lead to hypercholesterolemic effects; therefore, care must be taken when increasing saturated fat level at the *sn*-2 position (*41*). Amaranth oil's SL for infant formula application is intended to achieve a balance between the palmitic acid total content and palmitic acid esterified at the *sn*-2 position, without overlooking other important FAs that contribute to the particular breast milk composition.

In conclusion, both interesterification techniques yielded high total content of palmitic acid at the *sn*-2 position, but the lipase-catalyzed reaction resulted in a higher content of palmitic acid and a better overall FA profile for the particular purpose of this research. The increment in palmitic acid was, in part, at the expense of the total content of linoleic acid, and most of the linoleic acids at the *sn*-2 position were rather substituted for palmitic acid. For the proposed application, it is important to consider that the SL produced would partially substitute for other fat sources, animal or vegetable, for commercial use. For such purposes, it is imperative to obtain the correct level of palmitic acid in SL that will enhance the desired nutritional goal while remaining technologically and economically feasible. The model obtained using enzymatic interesterification for total palmitic acid can effectively explain the responses obtained and can be used to predict the total content of palmitic acid in relation to substrate availability and reaction time. The model will be helpful in further research on the application of amaranth oil SL as a fat analogue for infant food formulations.

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