Enzymatic Acidolysis in Hexane to Produce n-3 or n-6 FA-Enriched Structured Lipids from Coconut Oil: Optimization of Reactions by Response Surface Methodology

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ABSTRACT: Response surface methodology is a statistical design that helps one to determine optimal conditions for an enzyme-catalyzed reaction by performing a minimal number of experiments. This methodology was adapted for modifying coconut oil TAG by using lipase-catalyzed acidolysis in hexane to incorporate n-3 or n-6 PUFA. FFA obtained after hydrolysis of cod liver oil and safflower oil were used as acyl donors. Immobilized lipase, Lipozyme IM60, from *Rhizomucor miehei* was used for catalyzing the reaction. The reaction conditions—substrate molar ratio, incubation time, and temperature—were optimized. The experimental data were fitted to a response function based on the central composite rotatable design. The optimal conditions generated from models indicated that maximal incorporation of n-3 PUFA occurred at a 1:4 molar ratio of TAG/FFA when incubation was carried out for 34 h at 54ºC. Similarly, maximal incorporation of n-6 FA was predicted at a 1:3 molar ratio of TAG/FFA when incubated for 48.5 h at 39ºC. Experiments conducted at optimized conditions predicted by the equation obtained from response surface methodology yielded structured lipids with 13.65 and 45.5% of n-3 and n-6 FA, respectively. These values agreed well with that predicted by the model. The reactions were also scaled up to 100 g levels in batch reactors with the incorporation level of n-3 and n-6 fatty acids agreeing closely with that observed when the reactions were carried out at lab scale (100 mg). These studies indicated that response surface methodology is a useful tool in predicting the conditions for incorporating desired levels of specific FA during the synthesis of structured lipids.

Paper no. J10198 in *JAOCS 79,* 885–890 (September 2002).

KEY WORDS: Coconut oil, cod liver oil, enzymatic acidolysis, Lipozyme IM 60, n-3, n-6 polyunsaturated fatty acid, response surface methodology, safflower oil, structured lipids.

Structured lipids with specific combinations of FA have received greater attention in recent years (1,2). Enzymatic acidolysis reactions have been employed by many investigators for the modification of the FA composition of vegetable oils to synthesize structured lipids (1–4). A number of studies have focused on obtaining TAG having a combination of medium-chain FA and PUFA (3–5). n-3 PUFA like eicosapentaenoic acid and docosahexaenoic acid have beneficial effects in controlling cardiovascular diseases, immune disorders, inflammation, renal disorders, allergies, diabetes, and cancer. These FA also are essential for the development of the brain and retina in humans (6). Similarly, linoleic acid (n-6) is an EFA. It is utilized for the synthesis of complex lipids that provide the permeability barrier to the epidermis (7). PUFA also help to maintain optimal levels of unsaturation in tissue lipids (8). Medium-chain TAG (MCT) offer numerous health benefits and have been studied extensively for medical, nutritional, and food applications. They are easily absorbed, are rapidly metabolized to yield quick energy, and are not deposited in the body as fat (9). Although a source of MCT, coconut oil cannot function as an ideal fat source for humans as it does not provide EFA, which are essential for body functions. The nutritional value of coconut oil can be improved by incorporating EFA using lipase-catalyzed interesterification reactions (10).

Lipases are enzymes that normally catalyze the hydrolysis of TAG to form FFA, DAG, MAG, and glycerol. However, when reaction conditions are modified, lipases also catalyze the reverse reaction: catalysis of the formation of acylglycerols from FFA and glycerol esters (11). Enzymatic interesterification with substrate-specific lipases provides an efficient method to improve the nutritional and physical properties of lipids. Lipases require mild reaction conditions and hence are preferred in the incorporation of the highly labile PUFA into TAG (12). Immobilized lipases have greater stability and can be reused for many cycles without losing efficiency. This is very useful when structured lipids have to be prepared in large scale (13).

Coconut oil has been incriminated as an atherogenic lipid, owing to the presence of 92% of saturated FA in the lipid. The lauric, myristic, and palmitic acids, which form around 81% of its total FA, have hypercholesterolemic effects (14,15) that are a risk factor for cardiovascular disease. We earlier reported that the saturated FA in coconut oil can be partially replaced with a long-chain saturated FA such as stearic acid (2), which has a neutral effect on serum cholesterol level. However, when similar experimental conditions were adapted for the incorporation of n-3 or n-6 PUFA into coconut oil lipids, the extent of incorporation differed considerably as compared to that of stearic acid. This underscores the need for optimizing reaction conditions for each FA to obtain desired level of incorporation into structured lipids. This could necessitate the conduct of a large number of experiments with each FA. However, these can be circumvented by using response surface methodology (RSM) to optimize reaction conditions with a minimum number of

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experiments to obtain statistically acceptable results. RSM enables evaluation of the effects of multiple parameters, alone or in combination, on response variables and also predicts their behavior under a given set of conditions (16).

In the present study, RSM was employed to optimize the conditions for the incorporation of n-3 or n-6 PUFA into coconut oil TAG by considering different variables involved in the lipase-catalyzed reaction. The study aimed at examining the influence of three variables, incubation time, temperature, and the substrate molar ratio, on the incorporation of n-3 or n-6 FA into coconut oil TAG. Based on these results, large-scale synthesis of structured lipids was undertaken.

EXPERIMENTAL PROCEDURES

Materials. Coconut oil, safflower oil (SFO), and cod liver oil (FO) were obtained from the local market. Immobilized 1,3-specific lipase, Lipozyme IM 60 from *Rhizomucor miehei,* was a gift from Novo Nordisk Bioindustrial Inc. (Danbury, CT). Silica gel G and alumina for chromatography were procured from Sisco Research Laboratories (Mumbai, India). All organic solvents were of analytical grade and distilled before use.

Enzymatic acidolysis. Enzymatic acidolysis in hexane was initially carried out at laboratory scale (2). Reaction conditions such as incubation time, temperature, and substrate ratio were optimized for the synthesis of structured lipids from coconut oil TAG and FA obtained by hydrolysis of FO and SFO (rich in n-3 and n-6 FA, respectively) as acyl donors. The reaction mixture consisted of 2 mL hexane, 100 mg of coconut oil, and different amounts of FFA from either FO or SFO, as indicated, in 25-mL stoppered conical flasks. Immobilized lipase IM 60 (5 wt% of reactants) was added. Incubation was carried out at different temperatures and for different time periods, as indicated, in an orbital-shaking incubator at a speed of 160 rpm.

Analysis of products. At the end of the incubation period, the enzyme was removed and the reaction mixture was decanted and passed over anhydrous sodium sulfate. The solvent was evaporated under a stream of nitrogen. After dissolving the sample in 0.5 mL hexane, it was applied on preparative TLC plates coated with silica gel G. The plates were developed using petroleum ether/ethyl ether/acetic acid (90:10:1, by vol) as mobile phase. Bands corresponding to different lipid fractions were visualized under UV light after spraying with 0.2% 2,7-dichlorofluorescein in methanol. The bands corresponding to TAG were eluted using chloroform/methanol (2:1, vol/vol). The TAG were saponified using methanolic KOH, and the FFA were methylated with boron trifluoride in methanol. The FAME were extracted with hexane, dried over anhydrous sodium sulfate, and analyzed by GC with FID detection. A fused-silica capillary column, 25 m × 0.25 mm (Parmabond FAP-DF-0.25; Machery-Nagel GmBH Co., Düren, Germany) was used. The injector and detector temperatures were kept at 210 and 250ºC, respectively. The initial temperature of the column was 160ºC, and it was programmed to increase at the rate of 6ºC/min to 250ºC. The individual FA were identified by comparison with the retention times of standards obtained from Nu-Chek-Prep (Elysian, MN) (2).

Experimental design for RSM studies. A five-level, three-variable central composite rotatable design (CCRD) was adopted (17). Variables considered important for the reaction were incubation time, temperature, and substrate molar ratio. The design consisted of 8 factorial points, 6 axial points (2 axial points on the axis of each design variable at a distance of 1.68 from the design center), and 5 center points. For creating response surfaces, the experimental data obtained based on the above design were fitted to a second-order polynomial equation of the form,

$$Y = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} b_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij} x_i x_j \qquad [1]$$

where Y = % incorporation of FFA; b_0 = constant, b_i = linear term coefficients, b_{ii} = quadratic term coefficients, and b_{ij} = cross-term coefficients. The regression analyses, statistical significance, and response surfaces were done using Microsoft Excel software (version 5.0). Optimization of the reaction parameters for maximal incorporation of FA was obtained through a software package "Microsoft Excel—Solver program" (version 5.0), which used Newton's search method. Optimization of reaction conditions in terms of FFA to coconut oil molar ratio, incubation temperature, and time were calculated using the predictive equation from RSM.

Large-scale preparation of structured lipids. (i) Production of FFA. FO and SFO were hydrolyzed to produce FFA rich in n-3 and n-6 PUFA, respectively (18). Briefly, 1 kg of oil was refluxed with 2 L of 0.5 N methanolic NaOH solution with EDTA for 1 h at 60ºC under a nitrogen atmosphere. The mixture was cooled to room temperature and transferred to a separatory funnel. Concentrated HCl was added till the pH of the solution reached 3. After 6 h the lower phase was discarded and the methanolic phase with the FFA was washed and then dried over anhydrous sodium sulfate. Next, methanol was evaporated in a vacuum rotary evaporator. Analysis of the resulting FFA preparations by TLC indicated an absence of TAG, DAG, and MAG. The FA compositions of the preparations, determined by methylation and GC analysis, matched those expected for FO and SFO. The FFA were stored under nitrogen at −10ºC till used for the reaction. In total, 5 kg of cod liver oil and 3 kg of SFO were hydrolyzed in batches of 1 kg each for the production of FFA.

(ii) Scale-up. Based on the optimal conditions determined with RSM, large-scale production of structured lipids was carried out. The TAG level was increased from 100 mg, used in the lab scale, to 100 g with the corresponding increase in the amount of hexane and FFA. Reactions were carried out at optimized conditions as predicted by RSM. Two types of structured lipids were synthesized from coconut oil TAG by incorporating either n-3 or n-6 FA. In the former case, coconut oil and the FFA from FO were taken in 1:4 molar ratio. Each batch consisted of 100 g of coconut oil, 240 g of FFA from FO, and 17 g of immobilized lipase taken in a 1-L stop-

pered conical flask. Incubation was carried out at 54ºC for 34 h with constant agitation. In the case of structured lipids with n-6 FA, coconut oil and FFA from SFO were taken in a 1:3 molar ratio. In each batch, 100 g of coconut oil, 135 g of safflower oil FFA, and 12 g of immobilized lipase were taken. Incubation was carried out for 48.5 h at 39ºC.

Purification of reaction products. TAG were separated from the reaction mixture by column chromatography. A mixture of 20 g each of alumina and silica gel (100–200 mesh size) was activated at 200ºC for 2 h and cooled in a desiccator. A slurry of this was made in hexane and packed in 35 × 4 cm glass columns. A 30 g sample from the reaction mixture was loaded on the column and eluted with 350 mL of hexane/diethyl ether (95:5 vol/vol). The fractions containing TAG were pooled, and solvent was removed in a vacuum rotary evaporator. The purity of TAG was checked by TLC. Petroleum ether/diethyl ether/acetic acid (80:20:1, by vol) was used as the developing solvent. The structured lipids were stored in stoppered flasks and kept in a cold room after flushing with nitrogen.

RESULTS AND DISCUSSION

Enzymatic acidolysis in hexane was employed to modify the FA composition of coconut oil. The FFA obtained from SFO contained 68% 18:2n-6 (PUFA). The FFA obtained from FO contained 13% 20:5n-3 and 8% 22:6n-3 PUFA. The TAG of unmodified coconut oil showed 12:0 as the predominant FA (48%), followed by 14:0 (24%) and 16:0 (9%). When coconut oil TAG were subjected to acidolysis with FA from FO or SFO in the presence of lipase, the resulting structured lipids were enriched in n-3 or n-6 FA, respectively. The actual values of the variables employed for the RSM experiments are given in Table 1.

Incorporation of n-3 FA. Incubation temperature, time, and substrate concentration were selected as the factors affecting the reaction. All three parameters were found to influence the incorporation of n-3 PUFA into TAG. Based on experimental results (Table 1), the regression coefficients of the response surface model as given by Equation 1 were evaluated. The responses and variable settings from Table 1 were fitted to each other with multiple regressions. A good fit was obtained, and no outliers were observed. Student's *t*-test indicated that all the linear coefficients, and all quadratic terms except temperature were highly significant ($P < 0.05$). However, none of the interaction terms was found to be significant. The regression statistics and the ANOVA are presented in Table 2. The ANOVA indicates that the model is highly appropriate for the prediction as the F_{model} value (37.0) is very high compared to the $F_{5,13}$ value (4.86). The coefficient of determination (R^2) of the model was 0.93, which indicates that the model adequately represented the relationship among the selected reaction parameters. The average absolute relative deviation of the predicted response from the experimental response is 7.8%. The normal percentage probability plot (Fig. 1) of the residuals showed a general linearity with the errors normally distributed ($R^2 = 0.96$) and independent of each other. The error variances are homogeneous, which also indicates that the model adequately represents the relationships among the selected reaction variables. The insignificant terms in the response model were eliminated after examining the coefficients and the model was finally refined. The best-fitting quadratic model by multiple regression and backward elimination was determined to be

$$Y = 13.52 + 2.08X_1 + 2.07X_2 + 1.07X_3 - 1.95X_1X_1 - 1.01X_3X_3 \quad [2]$$

TABLE 1
Actual Experimental Settings of the Factors and the Responses Therefrom for the Optimization of the Reaction by Response Surface Methodology

Substrate molar ratio (TAG/FFA)	Temp. (°C)	Time (h)	% Incorporation into coconut oil of	
			n-3 PUFA	n-6 PUFA
1.5	30	15	3.98	14.37
4.85	30	15	11.12	23.46
1.5	48	15	9.88	32.32
4.85	48	15	14.95	48.67
1.5	30	40	7.79	30.32
4.85	30	40	11.27	33.54
1.5	48	40	11.35	32.68
4.85	48	40	15.16	42.14
0.36	39	27.5	5.20	28.73
5.99	39	27.5	10.50	38.68
3.175	23.9	27.5	10.19	24.64
3.175	54.1	27.5	16.78	30.99
3.175	39	6.5	7.84	33.06
3.175	39	48.5	13.13	46.15
3.175	39	27.5	12.85	40.87
3.175	39	27.5	14.09	40.63
3.175	39	27.5	12.98	44.07
3.175	39	27.5	12.64	42.50
3.175	39	27.5	14.83	43.18

TABLE 2
Statistical Analysis of the Response Function for Incorporation of n-3 and n-6 FA in Coconut Oil

Regression statistics:	n-3 PUFA	n-6 PUFA
Multiple *R*	0.967	0.938
Standard error	1.025	3.754

ANOVA analyses for n-3 PUFA

	Degree of freedom	Sum of squares	Mean sum squares	*F* ratio	Significance *F*
Regression	5	194.05	38.81	36.96	0.000
Residual	13	13.65	1.05		
Total	18	207.70			

ANOVA analysis for n-6 PUFA

	Degree of freedom	Sum of squares	Mean sum squares	*F* ratio	Significance *F*
Regression	6	1238.18	206.36	14.65	0.000
Residual	12	169.07	14.09		
Total	18	1407.25			

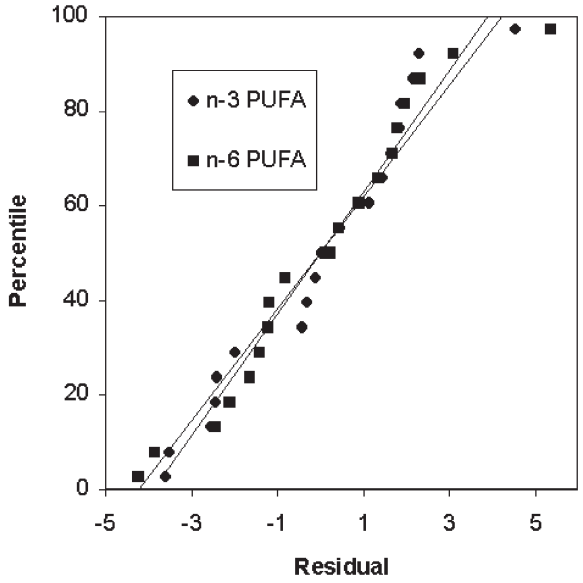

FIG. 1. Normal probability plot of residuals of the response function for the incorporation of n-3 PUFA and n-6 PUFA.

where X_1= substrate (molar ratio), X_2 = temperature (ºC), and X_3 = incubation period (h). All the linear coefficients were positive, whereas quadratic coefficients of substrate molar ratio and time were negative and there was no quadratic effect of temperature.

Figure 2A shows the effect of substrate amount and time on the incorporation of n-3 FA. At 39ºC, incorporation increased with time and substrate molar ratio. At any value of substrate molar ratio, incorporation increased with time up to 43.25 h. Similarly, at any given time, incorporation increased with substrate molar ratio till it reached 3.88 and then decreased. Incorporation was low at low values of substrate ratio and incubation time but comparatively higher at high substrate ratio and low incubation time. Incorporation was low at low substrate ratio and longer time, and it was high at high values of substrate and time. This shows that substrate level plays a more important role on incorporation than time. However, when the substrate ratio was held constant at 3.17, incorporation increased with time up to 38 h and then leveled off (Fig. 2B). At any incubation time, incorporation increased steadily with temperature. Thus, if the mixture was incubated at higher temperatures for up to 38 h, maximal incorporation was predicted at a 3.17 molar ratio of FFA to TAG. Incorporation increased linearly with temperature up to at least 54ºC although this increase was not highly significant. Figure 2C shows the effect of temperature and substrate at constant incubation time (27.5 h). At 27.5 h of incubation, temperature gave a slight linear increase in product but with an increasing in the substrate ratio, the extent of incorporation increased, reaching equilibrium at a molar ratio of 4.5. A substrate ratio of 3.17 (FFA/TAG) and high temperature of up to at least 54ºC gave high levels of incorporation of n-3 PUFA.

Maximal incorporation of 17.8% n-3 FA into coconut oil was predicted by RSM when a substrate molar ratio of 1:4.1

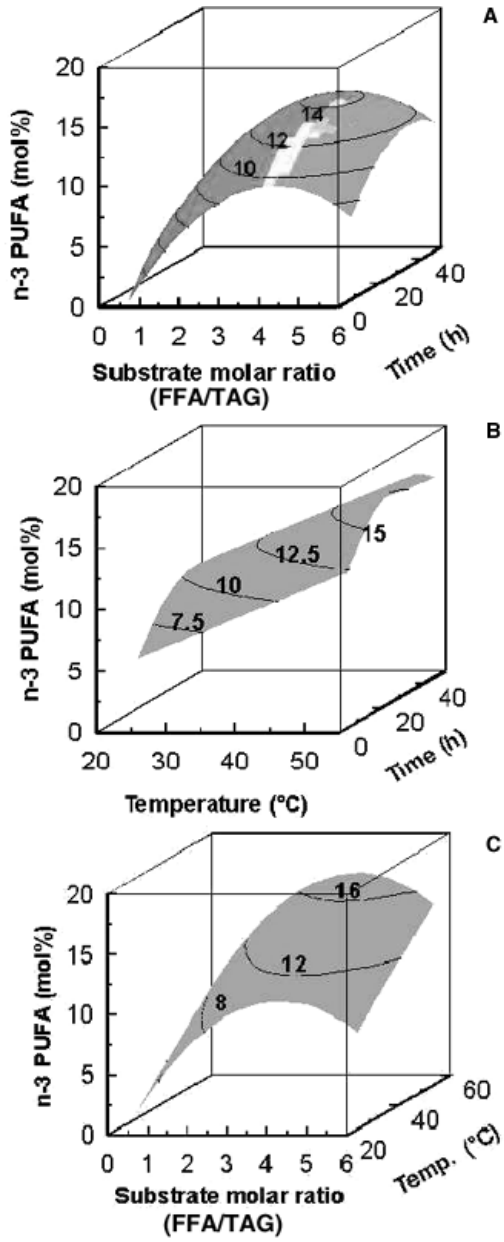

FIG. 2. Response surfaces showing incorporation of n-3 FA into coconut oil (A) at temperature = 39ºC, (B) at substrate molar ratio = 3.175, and (C) at time = 27.5 h.

of TAG/FFA, temperature of 54.1ºC, and incubation time of 34 h were used.

Incorporation of n-6 FA. The coefficients of the response surface model as given in Equation 1 were evaluated for incorporation of n-6 PUFA into coconut oil TAG. Student's *t*-test indicated that all the linear coefficients and quadratic terms were highly significant ($P < 0.05$) except time and temperature-time interaction terms. The regression statistics and the ANOVA are presented in Table 2. The ANOVA indicates that the model has high levels of significance, as the F_{model} value (14.7) is very high compared to tabular $F_{6,12}$ value (4.82). The coefficient of determination (R^2) of the model for incorporation of n-6 FA was 0.88. The average absolute rela-

tive deviation of the predicted response from the experimental response was 7.6%. The normal percentage probability plot (Fig. 1) of the residuals indicates that the errors are normally distributed (R^2 = 0.96) and are independent of each other and that the error variances are homogeneous. Neglecting the insignificant terms, the final predictive equation obtained is given as

$$Y = 41.225 + 4.019X_1 + 4.748X_2 + 3.067X_3 - 3.071X_1X_1 - 5.158X_2X_2 - 4.025X_2X_3 \quad [3]$$

All the linear coefficients are positive, and quadratic coefficients of the substrate molar ratio and temperature variables are negative. Incorporation of n-6 FA into coconut oil was affected by all three reaction variables studied. Figure 3A shows that, at a constant temperature of 39ºC, the incorporation increased with time and substrate molar ratio. But at any given time, incorporation increased with an increase in substrate only up to a ratio of 4.58, beyond which it decreased. The levels of incorporation of n-6 PUFA did not decrease even at 48 h. At a constant substrate ratio of 3.17, at any given time, incorporation of n-6 PUFA increased up to a certain temperature after which it decreased (Fig. 3B). For instance, at 3.17 molar ratio of FFA over TAG, when incubation was carried out for 27.5 h at 24, 39, and 54ºC, incorporation of n-6 PUFA was 25, 44, and 31%, respectively. Incorporation of n-6 PUFA was greater when reactions were carried out at lower temperatures for longer periods of time and for shorter time at higher temperatures. The incorporation of n-6 PUFA peaked at around 40ºC and then decreased, as shown in Figure 3B. This could be attributed to the hydrolysis of TAG at higher temperatures (2). When compared to n-3 PUFA (Fig. 2), the influence of temperature and time on incorporation of n-6 PUFA was different. As PUFA are more prone to lipid peroxidation especially at higher temperatures, the lipid peroxidation product might have reduced lipase activity, as has been observed by earlier workers (19). However, such an effect was not observed in the case of incorporation of n-3 PUFA. The FFA from SFO contained 68% PUFA while in FO it was present to an extent of 35%. Figure 3C shows the effect of temperature and substrate molar ratio on incorporation of n-6 PUFA when incubation was carried out for 27.5 h. An increase in substrate molar ratio increased the incorporation of n-6 PUFA till a temperature of 43ºC was reached beyond which it decreased. Similarly, incorporation of n-6 FA increased with an increase in substrate ratio till it reached a level of 4.6 and then decreased. Time does not seem to play a crucial role in these reactions as has been observed earlier by Huang and Akoh (16), who noticed slower incorporation of capric acid into TAG at longer incubation times. At lower temperatures, incorporation increased with time but beyond 40ºC, an increase in incubation time decreased the incorporation. The interaction of substrate molar ratio and temperature on incorporation is similar for n-6 and n-3 PUFA. It has been generally observed that the higher the incubation time, temperature and substrate, the higher the incorporation obtained. This agrees with the reports of Xu *et al.* (1), where similar observations were made for the incorporation of capric acid into menhaden oil. In the present study, values obtained from RSM predicted the synthesis of structured lipids with a maximum of 46.38% of n-6 PUFA at a substrate molar ratio of 1:3.17 of TAG/FFA, incubation temperature of 39ºC, and incubation time of 48.5 h.

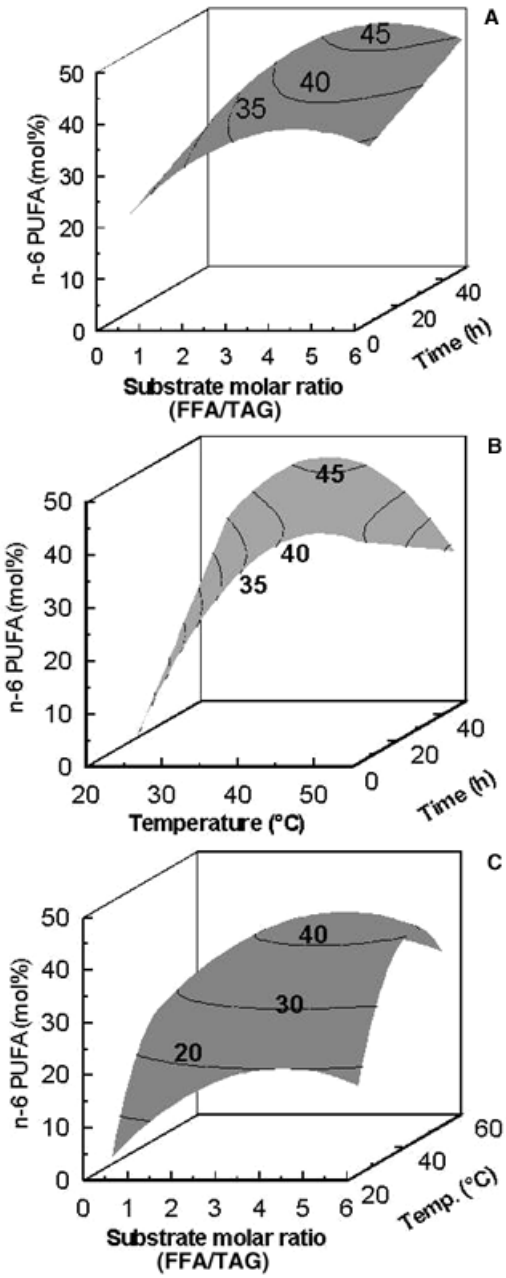

FIG. 3. Response surfaces showing incorporation of n-6 FA into coconut oil (A) at temperature = 39ºC, (B) at substrate molar ratio = 3.175, and (C) at time = 27.5 h.

Model validation. Based on the RSM studies, actual experiments were carried out at optimized conditions predicted by the model. It was observed that, at optimal conditions, the structured lipids contained 13.65% n-3 PUFA as against the predicted maximum of 17.8%. Similarly, when experiments were conducted at optimal conditions, structured lipids

TABLE 3
FA Composition (mol%) of Unmodified Coconut Oil and Structured Lipids Synthesized at Optimal Conditions

		Structured lipids synthesized from coconut oil enriched with	
FA	Coconut oil	n-3 PUFA	n-6 PUFA
8:0	2	—	—
10:0	3	—	—
12:0	48	16	17
14:0	24	11	12
16:0	9	15	10
16:1	—	3	—
18:0	3	4	2
18:1	9	25	15
18:2n-6	2	5	46
20:1 & 22:1	—	6	—
20:4	—	3	—
20:5n-3	—	10	—
22:6n-3	—	4	—

containing 45.5% n-6 PUFA were obtained as against a predicted maximum of 46% from the model.

Large-scale synthesis of structured lipids. Enzymatic acidolysis reactions were scaled up to the 100 g level under optimized conditions predicted by the RSM models. The FA composition of the resulting structured lipids is given in Table 3. The structured lipids from coconut oil contained either 14% n-3 FA when FO FA were used or 46% n-6 FA when SFO FA were used. Recovery of the purified TAG obtained was 87% for the structured lipids with n-3 FA and 90% for the ones with n-6 FA.

In the present study, linoleic acid was increased from 2% in the unmodified to 46% in the modified coconut oil lipids. Similarly, when n-3 FA from cod liver oil were used for modification, the concentration of n-3 PUFA increased from 0 to 14% in the coconut oil lipids. The structured lipids developed in the present study still retained medium-chain fatty acids and at the same time contained beneficial n-3 or n-6 FA. Thus the RSM model helped in optimizing the conditions for incorporating n-3 or n-6 PUFA into coconut oil lipids with a minimum number of experiments.

ACKNOWLEDGMENTS

The authors acknowledge the encouragement of the Director, CFTRI, and the Head of the Department of Biochemistry and Nutrition, CFTRI, for their keen interest during the course of this investigation. Reena Rao acknowledges the Senior Research Fellowship from Council of Scientific and Industrial Research, New Delhi. We also thank Rashesh Doshi, and AK. Kutty, Arun & Co, Mumbai, India, for Lipozyme IM60 from Novo Nordisk.

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[Received January 2, 2002; accepted May 30, 2002]