Enzymatic Incorporation of Stearic Acid into a Blend of Palm Olein and Palm Kernel Oil: Optimization by Response Surface Methodology

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ABSTRACT: Response surface methodology was used to model the incorporation of stearic acid into a blend of palm olein and palm kernel oil in hexane using the *sn*-1,3-regiospecific lipase Lipozyme RM IM. The factors investigated were incubation time, temperature, and substrate molar ratio. A second-order model with interaction was used to fit the experimental data. The coefficients of determination, R^2 and Q^2, were 0.96 and 0.90, respectively. The adjusted R^2 was 0.95. The regression probability was less than 0.001, and the model showed no lack of fit. Also, a linear relationship was observed between the predicted and observed values. All parameters studied had positive effects on incorporation of stearic acid, with substrate molar ratio having the greatest effect. The interaction terms of substrate molar ratio with temperature and time also had positive effects on incorporation, whereas the effect of the squared term of substrate molar ratio was negative. The quadratic terms of temperature and time, as well as their interaction term, had no significant effect on incorporation at $\alpha_{0.05}$. Model verification was done by performing a chi-square test, which showed that there was no significant difference between predicted values and a new set of observed responses.

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KEY WORDS: Acidolysis, Lipozyme RM IM, palm kernel oil, palm olein, response surface methodology, stearic acid.

The use of palm oil, palm kernel oil (PKO), and their fractions for the formulation of margarine has been on the increase because the traditional method (partial hydrogenation) of increasing solid fat content of vegetable oils leads to the production of *trans*-FA and unnatural *cis* isomers. *Trans*-FA have been suspected of having adverse effects on plasma lipoprotein levels (1), whereas unnatural *cis* isomers have an inhibitory effect on a desaturase enzyme required for prostaglandin synthesis (2). There is therefore a need to find ways of producing *trans*- and unnatural *cis*-FA-free margarine. Palm oil and its fractions have been found suitable for making margarine because they have the necessary solid fat content without hydrogenation and crystallize in the β′ form (3)—a crystal form that is associated with the organoleptic properties of margarine (4).

Physical and transesterified blends of palm oil and/or a combination of its fractions such as palm olein (PO) and stearin have been used in the formulation of zero-*trans* margarine (5–7). Other formulations contain partially hydrogenated palm oil. Transesterification results in TAG diversity, which promotes rapid crystallization and also mitigates the β′ to β transition of TAG crystals during margarine storage (8), which is often the case when physical blends of palm oil and/or its fractions are used to formulate margarine. TAG diversity is mainly achieved when the FA vary in chain length.

Preliminary study in our laboratory has shown that various combinations of PKO and PO have the appropriate crystal form necessary for margarine formulation. These combinations have also been reported to result in improved mouthfeel properties of margarine (3). PKO is rich in lauric acid and contains moderate amounts of other medium-chain FA (MCFA) such as caprylic, caproic, and myristic acids. MCFA are beneficial because they are not readily re-esterified into TAG, have higher plasma clearance, are readily metabolized, and are therefore less likely to be deposited as body fat during metabolism (9). Palm oil has about equal amounts of saturated (mainly palmitic) and unsaturated (mainly oleic) FA. Its high natural antioxidant (β-carotene, tocopherols, and tocotrienols) and moderate PUFA contents give it a good oxidative stability at high temperatures (10). These antioxidants also have been proven to fight tumor cells (11–13) and to inhibit cholesterol biosynthesis (14).

A structured lipid (SL) produced by incorporating stearic acid into a blend of PKO and PO will therefore have a diverse content of TAG as a result of the wide range of FA present in these oils and should be suitable for margarine formulation. It is expected that the diverse TAG content of the SL will reverse the β′ to β transition of TAG crystals when margarines are stored, and the health benefits associated with palm oil are not expected to be lost. Stearic acid is usually the choice of scientists for increasing the solid fat content of oils because it has been proven to play no role in increasing plasma cholesterol levels (15). It is saturated and therefore has a high m.p. The incorporation of stearic acid is expected to reduce the contents of lauric, myristic, and palmitic acids, which can increase plasma cholesterol levels, since these FA are mainly located at the *sn*-1 and -3 positions of the TAG.

The objective of this study was to model the incorporation of stearic acid into a blend of PKO and PO (50:50, vol/vol), using response surface methodology (RSM). RSM consists of a set of mathematical and statistical methods developed for modeling

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phenomena and finding combinations of a number of experimental factors that will lead to optimal response. The model developed here is expected to be used in further studies for large-scale synthesis of SL for physical characterization. SL with desirable physical properties will then be used for margarine formulation. The factors studied were incubation temperature, reaction time, and substrate molar ratio (stearic acid/oil blend).

MATERIALS AND METHODS

Materials. PO and PKO were supplied by the Malaysian Palm Oil Promotion Council (Selangor, Malaysia). Stearic acid was bought from Sigma Chemical Co. (St. Louis, MO). Immobilized 1,3-specific lipase, Lipozyme RM IM, was purchased from Novo Nordisk A/S (Bagsværd, Denmark). Organic solvents and chemicals were purchased from J.T.Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA). All other chemicals used were analytical or HPLC grade.

Experimental design for RSM. A five-level, three-variable central composite design was used (16). Variables used in this study were incubation time (t, in hours), temperature (Te, in degrees Celsius), and substrate molar ratio (Sr). 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 9 center points. The worksheet is shown in Table 1. For creating response surfaces, the data obtained based on the above design were fitted to a second-order polynomial equation of the form:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad [1]$$

where Y = % incorporation of stearic acid; β_0 = constant; β_i = linear term coefficients; β_{ii} = quadratic term coefficients; β_{ij} = interaction term coefficients, and X_i and X_j are the independent variables. Regression analyses, statistical significance, and response surfaces were done using MODDE 7.0 software (Umetrics, Umeå, Sweden). The optimal conditions for stearic acid incorporation with respect to substrate molar ratio, incubation temperature, and reaction time were determined using RSM.

Acidolysis. SL synthesis was performed in test tubes in an orbital shaking water bath at 200 rpm at the specified temperature, substrate molar ratio, and time (Table 1). One hundred milligrams of the oil blend plus different amounts of stearic acid corresponding to the various mole ratios were used for SL synthesis. The amount of enzyme used was 10% of the total weight of the substrates. The reactants were dissolved in 1.5 mL hexane. After the reaction had been stopped, 2 mL hexane was added to the reaction product, and the enzymes were filtered off by passage through a column of anhydrous sodium sulfate. All experiments were performed in duplicate and the average values reported.

FA profile of product. A general procedure similar to that described by Jennings and Akoh (17) was used for TLC analysis. The mobile phase, petroleum ether/diethyl ether/acetic acid (80:20:0.5, by vol), was used to develop the lipid bands. The bands were visualized under UV light after spraying with 0.2%

TABLE 1
Experimental Settings of the Factors and the Responses Used for the Optimization of the Reaction by Response Surface Methodology[a]

ExpNo	Incl/Excl	Te	Sr	t	Inc
1	Incl	45	2	12	44.05
2	Incl	75	2	12	33.44
3	Incl	45	5	12	30.22
4	Incl	75	5	12	44.50
5	Incl	45	2	24	31.02
6	Excl	75	2	24	
7	Incl	45	5	24	44.01
8	Excl	75	5	24	
9	Incl	34.8	3.5	18	38.97
10	Incl	75.6	3.5	18	42.96
11	Incl	60	0.977	18	22.02
12	Incl	60	6.023	18	47.12
13	Incl	60	3.5	7.908	40.08
14	Incl	60	3.5	28.092	39.65
15	Incl	60	3.5	18	41.66
16	Incl	60	3.5	18	41.26
17	Incl	60	3.5	18	39.85
18	Incl	60	3.5	18	37.67
19	Incl	60	3.5	18	40.01
20	Incl	60	3.5	18	42.39
21	Incl	60	3.5	18	40.28
22	Incl	60	3.5	18	42.21
23	Incl	60	3.5	18	41.12

[a]Abbreviations: ExpNo, experiment numbers; Incl/Excl, experiments included or excluded in model fitting; Te, temperature (°C); Sr, substrate molar ratio (= mol stearic acid to mol PO:PKO, where PO = palm olein and PKO = palm kernel oil); t, time (h); Inc, incorporation of stearic acid (mol%).

2,7-dichlorofluorescein in methanol. Bands corresponding to TAG were scraped off and the FA methylated in 3 mL methanol (containing 6% HCl) at 75°C for 2 h. The FAME were extracted twice with 2 mL hexane and dried by passing through a column of anhydrous sodium sulfate. FAME were analyzed using an Agilent Technologies 6890N gas chromatograph equipped with an FID. Helium was the carrier gas, and the total gas flow rate was 1.7 mL/min. The oven temperature was initially held at 80°C for 3 min and then programmed to 215°C at 10°C/min and held isothermally for 20 min. The different amounts of FAME (mol%) were analyzed and integrated by an on-line computer with C17:0 as internal standard.

RESULTS AND DISCUSSION

Model fitting. Incorporation of stearic acid was successful and ranged from as low as 22.02 to as high as 47.12 mol% (Table 1). Incorporation of stearic acid (Inc) was mainly at the expense of lauric, myristic, and palmitic acids, whereas the contents of oleic and linoleic acids did not change much (data not shown). The goodness of fit of the model was determined by multiple linear regression and backward elimination. The squared terms, Te*Te and t*t, and the interaction term Te*t were deleted because they were not significant at $\alpha_{0.05}$. Two outliers (Exps. no. 6 and 8) were also deleted. R^2, the fraction of the variation of the response explained by the model, was 0.96 and Q^2, the fraction of the variation of the response that can be predicted by the model, was 0.90. R^2 Adj was 0.95. The model had strong validity (0.91) and reproducibility (0.94). The normal probability plot showed a linear distribution with two close outliers—Experiments no. 12 and 18 (Fig. 1A).

The observed vs. predicted plot (Fig. 1B) also showed a linear distribution. To show that there was no significant difference between the observed and predicted values, a chi-square test was performed. The chi-squared value was 0.64, which was much smaller than the cutoff point (31.17) at $\alpha_{0.05}$ and DF_{20}. This shows that there was no significant difference between the predicted and the observed values—a further indication of a strong model. The model also showed no lack of fit ($P > 0.05$), and the P-value for the multiple regression was <0.001 (Table 2). The model equation can therefore be written as

$$\text{Inc} = 40.6289 + 1.1021\text{Te} + 6.8610\text{Sr} + 0.0995\text{t} - 2.2011\text{Sr}^2 + 6.6474\text{Te*Sr} + 7.1286\ \text{Sr*t} \quad [2]$$

Except for time (Table 3), all coefficients were highly significant

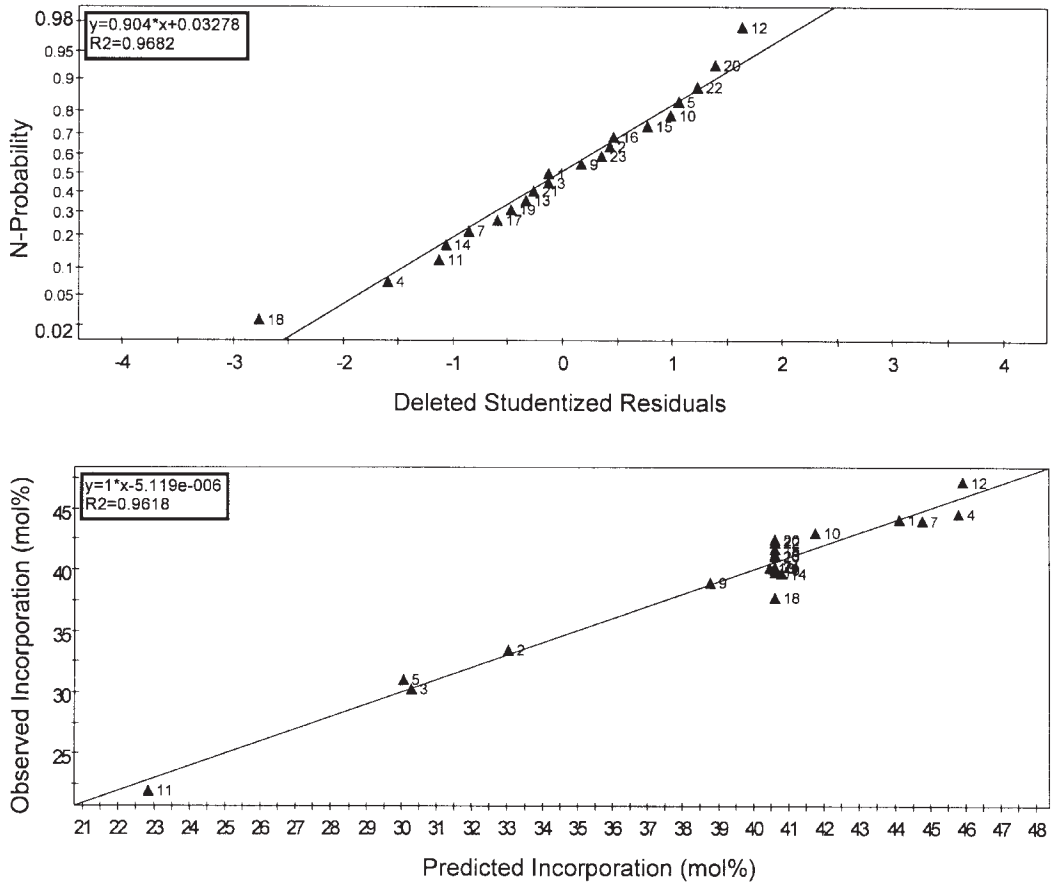

FIG. 1. (A) Normal probability plot of residuals for incorporation of stearic acid; (B) plot showing relationships between observed values and values predicted by the model. Numbers inside both graphs represent experimental numbers. The almost linear distribution of the experimental numbers is indicative of a good model.

TABLE 2
ANOVA Table for Incorporation (Inc.) of Stearic Acid

Inc. of stearic acid	DF[a]	SS	MS (variance)	*F*	*P*-value	SD
Total	21	33032.5	1572.98			
Constant	1	32366.3	32366.3			
Total corrected	20	666.178	33.3089			5.77139
Regression	6	640.707	106.785	58.6951	0.000	10.3337
Residual	14	25.4703	1.81931			1.34882
Lack of fit (model error)	6	8.38354	1.39726	0.654192	0.689	1.18206
Pure error (replicate error)	8	17.0868	2.13585			1.46145
	n = 21 DF = 14	Q^2 = 0.902	R^2 = 0.962	R^2 Adj. =	0.945	

[a]Abbreviations: DF, degrees of freedom; SS, sum of squares; MS, mean square; Q^2, R^2, and R^2 Adj, explained in text.

($P < 0.05$). Even though the coefficient of time was not significant, its interaction term with substrate molar ratio was highly significant ($P < 0.001$).

Effect of parameters. Inc was affected by all three parameters. All linear parameters had a positive influence on Inc, but Sr was most significant (Fig. 2). Temperature and time had little effect on Inc. The second-order parameter Sr*Sr had a negative influence on Inc, whereas the interaction terms of substrate molar ratio with temperature and time affected Inc positively, with Sr*t having the greatest effect.

Optimization of the reaction. The model equation shows that incorporation of stearic acid was affected not only by the first-order variables but also by the squared term of Sr and its two interaction terms (Table 3). Incorporation will therefore have a complex relationship with the parameters that encompass both the first- and second-order polynomials and may have more than one solution (18). The best way to evaluate the relationship between responses and parameters and interactions that existed therein is to analyze the contour plot for incorporation. When constructing contour plots, the variable with the greatest effect on the response is placed on the *y*-axis, the second is placed on the *x*-axis, and the one with the least effect on the response is held constant.

Figure 3 shows contour plots at three different levels of time—12, 18, and 24 h. In general, incorporation increased as the three parameters increased. However, a complex pattern of incorporation is observed when one or two of the parameters decreased or increased while the other parameters varied in the opposite direction or remained constant. At a constant time, incorporation increased or decreased with increasing temperature depending on the level of substrate molar ratio. Increasing temperature will increase the reaction rate according to the Arrhenius equation:

$$k = Ae^{-E_A/RT} \quad [3]$$

where A is a constant measured in the same units as k, E_A is the activation energy measured in kJ·mol^{-1}, R is the gas constant measured in J·mol^{-1}·K^{-1}, and T is temperature measured in K. A higher temperature is known to increase the rate of productive collisions between reactants and the enzyme. This may account for the increasing incorporation as temperature increased at substrate molar ratios above 3.25.

However, higher temperatures also accelerate the rate of enzyme inactivation (19). This rate of denaturation eventually surpasses the increasing reaction rate that is due to the increasing number of productive collisions between the enzyme and reactants (20). This therefore leads to a net reduction in the overall reaction rate. Furthermore, higher temperatures have also been reported to shift reaction equilibrium toward hydrolysis (21,22).

TABLE 3
Coefficient List for Incorporation (Inc.) of Stearic Acid

Inc. of stearic acid	Coef[a]	Std err	*P*-value[b]	Conf int (±)
Constant	40.6289	0.355271	3.35051E-022	0.761986
Te	1.10209	0.447034	0.027227701	0.958802
Sr	6.86095	0.434014	2.53696E-010	0.930876
t	0.09949	0.409070	0.811367001	0.877376
Sr*Sr	–2.20113	0.343578	1.63674E-005	0.736908
Te*Sr	6.64735	0.623067	4.16565E-008	1.33636
Sr*t	7.12860	0.623067	1.72088E-008	1.33636

[a]Abbreviations: Coef, multiple regression coefficients; Std err, standard error; Conf int, confidence interval; Te, temperature (°C); Sr, substrate molar ratio; t, time (h); Sr*Sr, quadratic term of Sr; Te*Sr, interaction term of Sr and Te; Sr*t, interaction term of Sr and t.
[b]Coefficients with *P*-value less than 0.05 are significant.

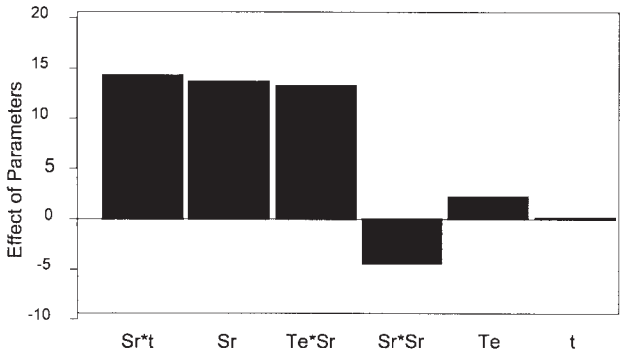

FIG. 2. Effect of parameters and their significance on incorporation of stearic acid. Sr, substrate molar ratio; Te, temperature (°C); t, time (h); Sr*Sr, quadratic term of Sr; Te*Sr, interaction term of Te and Sr; Sr*t, interaction term of Sr and t.

These explain the reduction in incorporation as temperature increased for reactions with a substrate molar ratio below 3.25. A similar observation was made by Camacho Paez *et al.* (23). That study reported a slight decrease in caprylic acid incorporation into cod liver oil as the temperature increased beyond 30°C. This shift in equilibrium toward hydrolysis as the reaction progressed also could be caused by the accumulation of water, 1 mol of which is produced for every mole of ester synthesized (24), probably the result of the reaction between stearic acid and free hydroxyl groups on DAG and MAG that were present in the original oil blend. There is, however, no net accumulation of water during ester interchange (20).

This observed disparity in the pattern of incorporation at low and high substrate molar ratios as temperature increased was probably due to the protective effect of higher substrate concentrations against thermal denaturation of the enzyme (25).

In this study, substrate molar ratio and its interactions with temperature and time were the main driving factors (Table 3, Fig. 2). The decreasing incorporation observed at lower substrate concentrations as time and temperature increased indicates that these reactions reached equilibrium faster, but this shifted toward hydrolysis as time and temperature increased. On the other hand, equilibrium was delayed for reactions with higher substrate concentrations. The increasing levels of temperature and time studied in this work favored acidolysis at high substrate concentrations.

This model presents a good picture of how the three factors studied affected stearic acid incorporation into a physical blend of PO and PKO. In choosing a set of variables responsible for a desired incorporation, it is important to factor in economic considerations. For example, an incorporation of 42% can be obtained by the following set of factors: 2.32 Sr, 50.4°C, 12 h (not shown on plot); 4.34 Sr, 67.3°C, 12 h; 3.81 Sr, 60.5°C, 18 h (not shown on plot); and 3.66 Sr, 57.8°C, 24 h. From the above four, it would be economical to choose the first set because substrate and energy requirements are significantly lower than for the other sets.

Verification of model. To verify the model, we chose five regions from the contour plot and performed experiments using the conditions specified for these regions. A chi-squared test showed that there was no significant difference between the observed and predicted values (Table 4) since the chi-squared value (1.802) was smaller than the cutoff point (9.488) at $\alpha_{0.05}$ and DF_4.

The second-order polynomial model developed in this study satisfactorily expressed the relationship between the factors studied and the incorporation of stearic acid into the oil blend. The R^2 (0.96), R^2 Adj (0.95), and regression P-value <0.001 indicate that the model well represented the relationship between the reaction parameters and the response. The model has

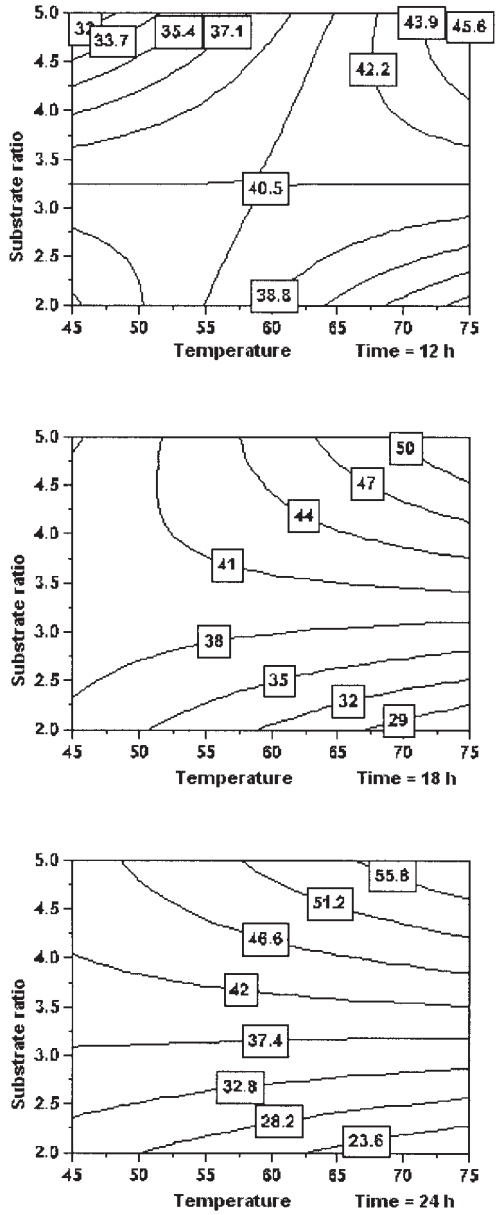

FIG. 3. Contour plots showing the effect of temperature (°C), substrate ratio (molar), and time (h) on incorporation of stearic acid. The numbers inside the contour plots indicate the level of stearic acid incorporation (mol%).

TABLE 4
Model Verification Using the χ^2 (chi-squared) Test[a]

Region[b]	Temperature (°C)	Substrate molar ratio	Time (h)	E	O	$(O - E)^2/E$
R1	70.0	2.20	18	29.94	35.54	1.045558617
R2	60.5	2.51	18	35.02	36.37	0.05165691
R3	56.1	3.41	18	40.00	42.04	0.10404
R4	62.8	4.45	18	45.07	45.80	0.01182383
R5	70.0	4.92	18	50.06	44.63	0.588991211
					χ^2 =	1.802070568

[a]$\chi^2 = \Sigma(O - E)^2/E$: E, expected incorporation (mol%); O, observed incorporation (mol%).
[b]Response surfaces/regions (R1–R5) with corresponding temperature, substrate molar ratio, and time.

predictive power too. Its Q^2 (0.90) and high reproducibility (0.94) as well as the two chi-squared tests indicate that this model will adequately describe any future incorporation of stearic acid into the oil blend and may fit a large-scale synthesis of this SL for margarine formulation as well.

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