Enzymatic Production of Infant Milk Fat Analogs Containing Palmitic Acid: Optimization of Reactions by Response Surface Methodology

C. O. Maduko,* C. C. Akoh,*¹ and Y. W. Park*†

*Department of Food Science and Technology, The University of Georgia, Athens 30602

†Agricultural Research Station, Fort Valley State University, Fort Valley, Georgia 31030

ABSTRACT

Infant milk fat analogs resembling human milk fat were synthesized by an enzymatic interesterification between tripalmitin, coconut oil, safflower oil, and soybean oil in hexane. A commercially immobilized 1,3-specific lipase, Lipozyme RM IM, obtained from *Rhizomucor miehei* was used as a biocatalyst. The effects of substrate molar ratio, reaction time, and incubation temperature on the incorporation of palmitic acid at the sn-2 position of the triacylglycerols were investigated. A central composite design with 5 levels and 3 factors consisting of substrate ratio, reaction temperature, and incubation time was used to model and optimize the reaction conditions using response surface methodology. A quadratic model using multiple regressions was then obtained for the incorporation of palmitic acid at the sn-2 positions of glycerols as the response. The coefficient of determination (R^2) value for the model was 0.845. The incorporation of palmitic acid appeared to increase with the decrease in substrate molar ratio and increase in reaction temperature, and optimum incubation time occurred at 18 h. The optimal conditions generated from the model for the targeted 40% palmitic acid incorporation at the sn-2 position were 3 mol/mol, 14.4 h, and 55°C; and 2.8 mol/mol, 19.6 h, and 55°C for substrate ratio (moles of total fatty acid/moles of tripalmitin), time, and temperature, respectively. Infant milk fat containing fatty acid composition and sn-2 fatty acid profile similar to human milk fat was successfully produced. The fat analogs produced under optimal conditions had total and sn-2 positional palmitic acid levels comparable to that of human milk fat.

Key words: enzymatic interesterification, infant milk fat, response surface methodology, sn-1,3-specific lipase

INTRODUCTION

Human milk is the best source of nutrients for infants (Megraud et al., 1990) and contains 4 to 5% fat, which

supplies the highest fraction of the infants' required dietary energy (Yang et al., 2003). The triacylglycerols (TAG) in human milk fat (HMF) constitute about 98% of total fat and are dominated by palmitic acid (23%), which also dominates the fatty acids at the sn-2 position (40 to 58%) of the glycerol backbone (USDA, 1976; Jensen, 1989; Xu, 2000).

In HMF (unlike vegetable oil, ruminant milk, and infant formula), the sn-1 and sn-3 positions are occupied by unsaturated fatty acids (Innis et al., 1995; Xu, 2000), where the location of palmitic acid at the sn-2 position of TAG in HMF increases the absorption of fatty acids in the lumen of infants and decreases the loss of calcium in feces (Quinlan et al., 1995; Kennedy et al., 1999). This is due to the preservation of the sn-2 positional fatty acid during digestion, absorption, and biosynthesis of TAG in the intestinal wall.

Milk can be modified for infant feeding by redesigning its physical, chemical, and nutritional properties. Modified lipids resembling the TAG of HMF can be produced by interesterification reactions using sn-1,3-specific lipase as the biocatalyst (Sahin et al., 2005b). Use of this lipase gives high selectivity and mimics the natural pathways of metabolic processes (Akoh et al., 2002).

Preliminary studies in our laboratory have revealed that a combination of coconut, safflower, and soybean oils in a 2.5:1.1:0.8 ratio, has a fatty acid profile similar to that of HMF (Maduko et al., 2005, 2006a,b). Coconut oil is a good source of medium- and long-chain saturated fatty acids, whereas safflower and soybean oils are 2 sources of polyunsaturated fatty acids that are suitable for infant milk formulation (Packard, 1982). However, the positions of fatty acids in TAG of vegetable oils are different from those of HMF. It is beneficial that the TAG of infant formulas produced with vegetable oils be modified to simulate that of HMF for better absorption. Previous studies by Maduko et al. (2006a) have revealed that enzymatic interesterification of tripalmitin with a vegetable oil blend containing coconut, safflower and soybean oils with Lipozyme RM IM can be successful in the simulation of infant milk fat to HMF. Lipozyme RM IM is used in interesterification reactions because of its sn-1,3-specificity, which results in the incorpora-

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¹Corresponding author: cakoh@uga.edu

tion of fatty acids at the sn-1,3 positions of the TAG backbone.

The objective of this study therefore is to model and optimize the incorporation of palmitic acid into a blend of coconut, safflower, and soybean oils (2.5:1.1:0.8 vol/vol) using response surface methodology (**RSM**) to form an infant milk fat analog. Response surface methodology consists of a set of mathematical and statistical methods developed for modeling phenomena and finding combinations of a set of a number of experimental factors that will lead to an optimal response (Lumora and Akoh, 2005). The model developed can then be used in further studies for upscaling and physical characterization of the infant formula fat. Parameters studied were substrate molar ratio (moles of total fatty acid/moles of tripalmitin), temperature, and time of reaction. The reactions were optimized with respect to palmitic acid incorporation at the sn-2 position of the glycerols.

MATERIALS AND METHODS

Materials and Experimental Procedures

Chemicals. Tripalmitin (glycerol tripalmitate, minimum purity of 85%) and porcine pancreatic lipase (type II, crude) were purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized 1,3-specific lipase, Lipozyme RM IM, was purchased from Novo Nordisk A/S, Bagsvaerd, Denmark). Organic solvents and thin-layer chromatography (**TLC**) plates were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) and Fisher Scientific (Fair Lawn, NJ), respectively. All solvents and reagents used for sample analysis were of chromatographic or analytical grade.

Preparation of Vegetable Oil Blend. Vegetable oil blend was prepared by mixing coconut oil, safflower oil, and soybean oil at a ratio of 2.5:1.1:0.8 to achieve a fatty acid profile comparable to HMF as described by Maduko et al. (2005, 2006b). Coconut oil was melted to a liquid form at 40°C before mixing. The mixture was stirred vigorously to ensure uniform distribution, and then stored at -85°C until needed for further analysis.

Experimental Design for RSM Study

The optimal conditions for palmitic acid incorporation at the sn-2 position with respect to substrate molar ratio, incubation temperature and reaction time were determined using RSM as described by Lumora and Akoh (2005), Sahin et al. (2005a, 2006). A 3-variable 5-level central composite design was used to generate factor combinations (Montgomery, 1997). The variables chosen were substrate molar ratio (**MR**; moles of total fatty acid/moles of tripalmitin), incubation time (**t**; in h) and reaction temperature (**T**; in °C), and the response

Table 1. Experimental settings of the factors and the responses used for the optimization of the reaction by central composite design experiments

Experiment no.	Experimental settings ¹			Molar percentage of C16:0 at sn-2
	Temperature (°C)	Time (h)	MR (mol/mol)	
1	55	12	4	26.265
2	65	12	4	16.925
3	55	24	4	27.95
4	65	24	4	13.35
5	55	12	10	13.32
6	65	12	10	9.365
7	55	24	10	13.22
8	65	24	10	7.945
9	51.59	18	7	13.72
10	68.41	18	7	10.085
11	60	7.908	7	16.735
12	60	28.092	7	16.94
13	60	18	1.954	55.26
14	60	18	12.046	18.465
15	60	18	7	19.63
16	60	18	7	23.295
17	60	18	7	20.855
18	55	12	4	26.755
19	65	12	4	19.22
20	55	24	4	29.44
21	65	24	4	11.78
22	55	12	10	11.8
23	65	12	10	9.775
24	55	24	10	13.55
25	65	24	10	7.935
26	51.59	18	7	25.95
27	68.41	18	7	11.095
28	60	7.908	7	15.91
29	60	28.092	7	17.065
30	60	18	1.954	51.25
31	60	18	12.046	19.68
32	60	18	7	24.615
33	60	18	7	21.48
34	60	18	7	23.08

¹Temperature = reaction temperature; Time = incubation time; MR = substrate molar ratio.

sought was molar percentage incorporation of palmitic acid at the sn-2 position of TAG. The experimental design containing the independent variables is presented in Table 1. Thirty-four runs were generated and experiments at each design point were carried out in duplicate. The design consisted of 6 factorial points, 12 axial points (2 axial points on the axis of each design variable), and 16 center points. The data developed from the design in Table 1 were used to fit a second-order polynomial function as follows:

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 \beta_{ij} X_i X_j + \varepsilon_{ij}$$

where Y = the response (percentage incorporation of palmitic acid at the sn-2 position of the TAG); β_0 = constant; β_i = linear (first-order model); β_{ii} = quadratic (second-order model); β_{ij} = interaction term coefficients;

X_i and X_j = independent variables (enzyme; vegetable oil blend); and ε_{ij} = error term.

Interesterification Reaction

The interesterification mixture, containing 3 mL of hexane, and a mixture of tripalmitin and vegetable oils at different substrate MR ranging from 1 to 12 determined by the RSM design (Table 1) were placed in screw-capped test tubes containing immobilized lipase, Lipozyme RM IM (10 wt% of total reactants). The mixtures were incubated in an orbital shaking water bath at 200 rpm. All reactions were performed and analyzed in duplicate. The enzyme and products were passed through a sodium sulfate column to stop the reaction and remove the enzyme from the reaction products, as described by Sahin et al. (2005b).

Chemical Analysis

Analysis of Products. The reaction products were applied to TLC plates (20 × 20 cm) coated with silica gel G, and were developed in a TLC tank using petroleum ether:ethyl ether:acetic acid (80:20:0.5 vol/vol/vol) as the development solvent as described by Sahin et al. (2005b). The separated bands were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The TAG band containing the new TAG product and unreacted TAG, was scraped into a screw-capped test tube, and methylated with 3 mL of 6% HCl in methanol at 75°C for 2 h. The fatty acid methyl esters (FAME) produced were extracted twice with 2 mL of hexane and dried over an anhydrous sodium sulfate column according to the method described by Jennings and Akoh (1999).

Fatty Acid Compositional Analysis. Fatty acid composition of the scraped TAG bands was quantified by gas chromatography (model 6890N GC, Agilent, Palo Alto, CA) equipped with a flame-ionization detector. Helium was the carrier gas and the 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 for 10 min at a rate of 10°C/min, and held isothermally for 20 min as described by Sahin et al. (2005a). The column used was a fused silica Heliflex capillary column (Alltech-AT-225 30 mm × 0.25 mm × 0.25 μm film thickness; Deerfield, IL). The different amounts of FAME (mol%) were analyzed and integrated by an integrator (model G2070AA, Agilent) with reference to C17:0 as an internal standard.

Sn-2 Fatty Acid Position Analysis of TAG by Pancreatic Lipase. Fatty acids at the sn-2 position were analyzed according to the method described by Sahin et al. (2005a). Twenty milligrams of pancreatic lipase,

1 mL of Tris buffer (pH 8.0), 0.25 mL of bile salts (0.05%), and 0.1 mL of calcium chloride (2.2%) were added to a test tube (25 × 200 mm) containing 0.1 g of fat sample extracted from each of the infant formula analogs as described above. The sample reaction mixture was incubated at 40°C in a water bath for 3 min. Then, 1 mL of 6 M HCl and 1 mL of diethyl ether were added, and the tube was centrifuged (800 × g). Diethyl ether layer was evaporated under a nitrogen stream (N-EVAP Organomation model No. 111) to a final volume of about 200 μL.

A 200-μL aliquot was spotted onto a silica gel G TLC plate and developed in a TLC tank by using hexane:diethyl ether:acetic acid (50:50:1 vol/vol/vol) as the developing solvent. The plate was sprayed with 0.2% 2,7-dichlorofluorescein in methanol, and the bands were visualized under UV light. The 2-monoolein standard (Sigma) was used to confirm the TLC separation of 2-monoacylglycerol (2-MAG) in the reaction products. The 2-MAG band was then scraped into a screw-capped test tube and extracted twice with 1 mL of hexane; FAME were prepared as mentioned earlier, and then analyzed by GC to evaluate the enzymatic incorporation of fatty acids at the sn-2 position of the TAG.

Statistical Analysis. Regression analysis, response surfaces, and statistical significance were performed using MODDE 5.0 Software (Umetrics, Umea, Sweden).

RESULTS AND DISCUSSION

Model Fitting

Modified lipids enriched with palmitic acid were produced by enzymatic interesterification of tripalmitin with vegetable oil blend. The incorporation level of palmitic acid at the sn-2 position of the TAG in the infant formula fat analogs ranged from 7.9 to 55.3%, and the targeted incorporation level was 40 to 55%, which is the range found in HMF (Jensen, 1989).

A 3-factor, 5-level central composite design was used for the reactions; the design points and responses are given in Table 1. Quadratic models were obtained for the incorporation of palmitic acid at the sn-2 position (response) by multiple linear regression. The regression coefficients (β) and significance (P -) values were calculated based on the results in Table 1. Time of reaction appeared to have a negative impact on palmitic acid incorporation, whereas substrate molar ratio appeared to have the most significant effect on palmitic acid incorporation followed by the temperature of reaction.

The second-order parameters of incubation time ($t \times t$), substrate molar ratio ($MR \times MR$), and reaction temperature ($T \times T$) all had significant positive effects, whereas the interaction terms of reaction temperature

Figure 1. Normal probability (N-Probability) plot of residuals for incorporation of palmitic acid. Numbers inside the graph represent experimental values. The near-linear distribution of the experimental values indicates a good model.

and incubation time ($T \times t$), reaction temperature and substrate molar ratio ($T \times MR$), and incubation time and substrate molar ratio ($t \times MR$) had no significant effects on the response and were therefore excluded from the model. The R^2 value, the fraction of the variation of the response explained by the model, was 0.845 and Q^2 , the fraction of the variation of the response that can be predicted by the model, was 0.673, whereas the value of R^2 adjacent was 0.787.

The normal prediction plot showed a linear distribution (Figure 1) and the observed vs. prediction plot (Figure 2) appeared to have a linear distribution as well.

The model had a strong reproducibility (0.948) and the P -value of the multiple regression was less than 0.001. The model equation can therefore be written as:

$$\text{Incorporation} = 22.5796 - 3.58481 T - 7.3129 MR - 3.90844T^2 - 3.39591 t^2 + 3.49712 MR^2.$$

All coefficients were highly significant ($P < 0.05$) except for the first-order parameter time, and the interac-

tion terms, which appeared to be significant only at P -value = 0.5 or higher. However, the second-order parameter time was highly significant ($P < 0.005$) and was therefore included in the model.

Optimization of the Reaction

From the model equation, it appears that the incorporation of palmitic acid at the sn-2 position was affected to a large extent by the first- and second-order variables. Palmitic acid incorporation would therefore be related to the parameters that include the first- and second-order polynomials, which may lead to more than one solution (Xu et al., 2000; Lumor and Akoh, 2005). A good way to analyze the relationships between the responses, parameters, and any interactions that may exist within is to analyze the contour plots (Lumor and Akoh, 2005) for palmitic acid incorporation. For the contour plots construction, the variable with the greatest effect on the response was placed on the y-axis, the second on the x-axis, whereas the variable with the

Figure 2. Plot of observed vs. predicted values of the generated model. Numbers inside the graph represent experimental values. The near-linear distribution of the experimental values indicates a good model.

least effect was held constant as described by Lumor and Akoh (2005). The response plots obtained by the interaction of the 3 parameters on the incorporation of palmitic acid at the sn-2 position of the TAG are given in Figure 3. The third variable (MR; levels 1 to 3) was used as intermediate level for drawing the contour plots. There appeared to be a general increase in incorporation level as the 3 variables were increased. Depending on the level of substrate MR, incorporation level increased or decreased with increasing temperature at constant time. These results are similar to those reported by Lumor and Akoh (2005) and Sahin et al. (2005a), in which higher reaction temperatures increased the rate of productive collisions between reactants and enzyme, thereby giving rise to increased reaction rate (Paivi et al., 2000). On the other hand, higher temperatures also accelerate the rate of enzyme inactivation, and thereby lead to a decrease in incorporation levels (Dordick, 1989). This is attributable to the increased rate of enzyme denaturation surpassing the rate of productive collisions between enzyme and reactants, therefore leading to an overall decrease in reaction rate (Paivi et al., 2000; Lumor and Akoh, 2005).

The optimum incubation time remained constant (18 h) with an increase in MR, whereas palmitic acid incorporation increased with decreases in MR and T. The targeted 40 to 55% incorporation of palmitic acid was observed at reaction temperatures of 55 to 61°C (Figure 3A), 55 to 61°C (Figure 3B), and 55 to 58°C (Figure 3C), thus varying with the substrate MR. The targeted range for palmitic acid incorporation was obtained at all incubation time levels in Figures 3A and 3B, whereas this target range was observed between 16.5 and 21.5 h in Figure 3C. Optimal palmitic acid incorporation (54.3%) occurred at MR 1, 16 to 21 h, and 55 to 57°C (Figure 3A).

The conditions for the targeted palmitic acid incorporation (40 to 55%) at the sn-2 position of TAG were generated by the optimizer function of the MODDE 5.0 software. The optimal conditions were calculated using the generated model equation and the targeted palmitic acid incorporation (40 to 55%) at the sn-2 position as the criteria, and are given in Table 2. The targeted range of palmitic acid incorporation can be obtained with an MR of 1 to 3, with maximum incorporation obtained at MR 1 (Table 2). The lower range for the target palmitic acid incorporation (40%) can be obtained

Figure 3. Contour plots showing effect of reaction temperature (°C), substrate molar ratio, and incubation time (h) on palmitic acid incorporation at the sn-2 position. The numbers inside the contour plots indicate the level of palmitic acid incorporation (%) at substrate molar ratios of 1 (A), 2 (B), and 3 (C).

with MR 3 at 55°C and 14.4 h, which happens to be the optimal incorporation conditions at this substrate MR. These conditions were chosen as optimum because they

meet the target level of 40 to 55% incorporation and also appear to be feasible for large-scale production of the infant formula fat analog, due to lower amounts of

Table 2. Optimal conditions (for targeted molar percentage of palmitic acid for 40%) generated by Modde 5.0 (Umetrics, Umea, Sweden) software¹

Experiment no.	Conditions			Molar percentage of C16:0 at sn-2
	Temperature (°C)	Time (h)	Molar ratio	
1	55	24	1.4	48.9
2	55	14.4	3	39.3
3	65	12	1	38.4
4	58.4	19.6	2.8	40.9
5	60	18	2	44.5
6	55	12	2	43.6
7	55	24	2	44.6
8	55	12	2	43.6

¹Parameter ranges: substrate molar ratio (1 to 3 mol/mol), reaction temperature (55 to 65°C), reaction time (12 to 24 h).

tripalmitin required to achieve up to 40% incorporation. These conditions would therefore minimize the cost of reaction materials and economize energy requirements during production, unlike that for MR 1 and 2.

Verification of the Models

To verify the model, experiments were performed using the conditions specified for 5 chosen regions from the contour plot as described by Lumor and Akoh (2005). A χ^2 test (Table 3) indicated no significant difference between the observed and predicted values. The χ^2 value (2.6237) was smaller than the cutoff point (9.488) at $\alpha_{0.05}$ and 4 df.

Enzymatic interesterification reactions were performed in test tubes using the chosen optimal conditions obtained with RSM to further verify the model as described by Sahin et al. (2005b). Table 4 shows the results of the determination of total fatty acid composition and of the fatty acids at the sn-2 position of the resulting infant formula fat analogs. The fatty acid profile of the infant formula fat analog appeared to have a resemblance to the saturated fatty acids of HMF, which contains 23% palmitic, 2% capric, 8% lauric, and 7% stearic acids. The experimental values for palmitic

Table 4. Fatty acid composition (mol %) and sn-2 fatty acid profile (mol %) of human milk and the infant formula fat analog produced under optimal conditions¹

Fatty acid	Fatty acid composition (mol %)		Fatty acid at sn-2 (mol %)	
	Formula fat analog	Human milk ²	Formula fat analog	Human milk
C8:0	1.5	—	4.1	—
C10:0	2.9	2	3.6	0.2
C12:0	23.6	7	16.3	2.1
C14:0	8.2	8	9.5	7.3
C16:0	24.6	23	40.8	40–60
C18:0	6.2	7	2.6	3.3
C18:1	29.6	38	14.3	12.7
C18:2	3.4	9	8.6	7.3
C18:3	1.1	1	0.3	0.6

¹Substrate ratio (3 mol/mol), reaction temperature (55°C), reaction time (24 0.7h).

²From Jensen (1989).

acid incorporation at the sn-2 position appeared similar to the values predicted from the model. These values resemble the sn-2 fatty acid profile of HMF, in which most of the saturated fatty acids, especially palmitic acid (40 to 60%) are at the sn-2 position and unsaturated fatty acids are mainly at the sn-1 and sn-3 positions (Innis et al., 1995; Xu, 2000).

CONCLUSIONS

The position of palmitic acid in the glycerol backbone of HMF affects fatty acid absorption, calcium absorption, and use of dietary energy (Sahin et al., 2005b). In the production of infant formula fat analog, it is therefore necessary to simulate the fatty acid structure of HMF.

The determined model successfully optimized the enzymatic reaction conditions and predicted the incorporation level of palmitic acid in the infant milk fat analog. The optimum conditions generated for the target 40 to 55% palmitic acid incorporation were at a molar ratio of 3, reaction temperature of 55°C, and incubation time

Table 3. Model verification using the χ^2 test¹

Region ²	Temperature (°C)	Substrate molar ratio	Time (h)	E	O	(O – E) ² /E
R1	65	4	24	18.71	12.46	2.09
R2	60	1.1	18	50.49	52.97	0.12
R3	55	4	12	30.92	28.08	0.26
R4	52	7	18	18.26	18.52	0.0037
R5	60	7	18	22.58	20.71	0.15
$\chi^2 =$						2.6237

¹ $\chi^2 = \sum [(O - E)^2/E]$, where E = expected incorporation (mol %), and O = observed incorporation (mol %).

²Response surface regions (R1 to R5) with corresponding temperature, substrate ratio, and time.

of 14.4 h. These conditions appear to be both feasible and economical for large-scale production. The fatty acid profile of the infant milk fat analog produced using these optimum conditions showed a palmitic acid content of 24.6%, which is comparable to that of HMF (23%). Furthermore, the sn-2 fatty acid profile of the infant formula fat analog consists of 40.8% palmitic acid, which is within the targeted range of 40 to 55% palmitic acid incorporation found in HMF.

The second-order polynomial model satisfactorily expressed the relationship between temperature of reaction, time of incubation, substrate molar ratio, and incorporation of palmitic acid at the sn-2 position of the vegetable oil blend used in this study. This model has high reproducibility and can be used to design large-scale incorporation of palmitic acid into the vegetable oil blend for infant milk fat analog production, whereby manufacturers can determine the level of incorporation desired, by increasing or decreasing the molar ratio of the substrates, while maintaining the reaction temperature and incubation time at the optimum levels.

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