Enzymatic Production of Human Milk Fat Substitutes Containing γ-Linolenic Acid: Optimization of Reactions by Response Surface Methodology

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ABSTRACT: Structured lipids resembling human milk fat and containing GLA were synthesized by an enzymatic interesterification between tripalmitin, hazelnut oil FA, and GLA in *n*-hexane. Commercially immobilized 1,3-specific lipases, Lipozyme® RM IM and Lipozyme® TL IM, were used as the biocatalysts. The effect of these enzymes on the incorporation levels was investigated. A central composite design with five levels and three factors—substrate ratio, reaction temperature, and time—were used to model and optimize the reaction conditions *via* response surface methodology. Good quadratic models were obtained for the incorporation of GLA (response 1) and oleic acid (response 2) by multiple regression and backward elimination. The determination coefficient (R^2) values for the models were found to be 0.92 and 0.94 for the reactions catalyzed by Lipozyme RM IM, and 0.92 and 0.88 for the reactions catalyzed by Lipozyme TL IM, respectively. The optimal conditions generated from the models for the targeted GLA (10%) and oleic acid (45%) incorporation were 14.8 mol/mol, 55°C, and 24 h; 14 mol/mol, 55°C, and 24 h for substrate ratio (moles total FA/mol tripalmitin), temperature and time for the reactions catalyzed by Lipozyme RM IM and Lipozyme TL IM, respectively. Human milk fat substitutes containing GLA that can be included in infant formulas were successfully produced using both Lipozyme RM IM and Lipozyme TL IM enzymes. The effect of the two enzymes on the incorporation of GLA and oleic acid were found to be similar.

Paper no. J11063 in *JAOCS 82*, 549–557 (August 2005).

KEY WORDS: Enzymatic interesterification, GLA, hazelnut oil, human milk fat substitutes, response surface methodology, *sn*-1,3-specific lipase.

Breast milk is the main and most preferred source of nutrients for infants, and human milk fat (HMF) is the component that supplies the highest fraction of the infant's required dietary energy. Human milks are characterized by the dominance of TAG (98% of HMF) in which the saturated 16-carbon palmitic acid (16:0) (20–30%) is in the *sn*-2 position (70%) of the glycerol backbone, the *sn*-1 and *sn*-3 positions being taken by unsaturated FA (1,2).

Structured lipids (SL) resembling TAG of HMF can be produced by interesterification, using *sn*-1,3-specific lipases as biocatalysts, and such TAG can be used in infant food formulations (3,4). Numerous studies have been conducted on the production of HMF substitutes (5–9). There has also been a great interest in the supplementation of infant formulas with FA such as GLA, arachidonic acid (AA), EPA, and DHA.

In infant formulas, GLA-containing oil, especially borage oil, is used for its health benefits and for its antagonist action on AA metabolism (10,11). Since GLA is rapidly elongated to di-homo-γ-linolenic acid and subsequently Δ5-desaturated to AA and since GLA is also cheaper and easier to produce than AA, an alternative and suggested way of supplementing infant formulas with AA is to use GLA instead of highly active AA (12–14). The amount of GLA in infant formulas prepared by physically adding GLA varies between 0.2 and 0.9% of the total FA (14).

In a study in which the efficacy and safety of GLA as a precursor of AA and of DHA were investigated with borage and fish oils in preterm infants, GLA used in the infant formulas as a source of AA was found to be efficacious for growth and for neurodevelopment in boys; there were no adverse effects (13). Through lipase-catalyzed reactions, HMF substitutes (HMFS) containing GLA can be produced for inclusion in infant formulas.

The aim of the present work was to use response surface methodology (RSM) to model and optimize the reaction conditions needed to synthesize SL, enriched with GLA by enzymatic interesterification using two commercial lipases, that may be used as HMFS. Models were set up by RSM based on three parameters: substrate molar ratio (moles total FA/mol tripalmitin), temperature, and time. The reactions were optimized with respect to oleic acid and GLA incorporation. Furthermore, Lipozyme® RM IM and Lipozyme® TL IM were compared in the interesterification for the synthesis of HMFS enriched with GLA.

EXPERIMENTAL PROCEDURES

Materials. Tripalmitin (glycerol tripalmitate, minimum purity 85%), borage oil, and porcine pancreatic lipase (Type II, crude) were purchased from Sigma Chemical Co. (St. Louis, MO). Refined hazelnut oil, obtained from the fruit of hazelnut trees (*Corylus avellana* L. and *C. maxima* Mill), was purchased from a grocery store in Turkey. Immobilized 1,3-specific lipases, Lipozyme RM IM and Lipozyme TL IM, were purchased from

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Novo Nordisk A/S (Bagsvaerd, Denmark). Organic solvents and TLC plates were purchased from J.T.Baker Chemical Co. (Phillipsburg, NJ) and Fisher Scientific (Fairlawn, NJ), respectively. All solvents and reagents used in analyses were of chromatographic or analytical grade.

Preparation of FFA from hazelnut oil and borage oil. Twenty-five grams of hazelnut and borage oil were saponified separately using a mixture of KOH (5.75 g), water (11 mL), and 95% (vol ethanol/total vol) aqueous ethanol (66 mL) by refluxing for 1 h at 60°C. To the saponified mixture, distilled water (50 mL) was added and the unsaponifiable matter was extracted into hexane (2 × 100 mL) to be discarded. The aqueous layer containing the saponifiable matter was acidified (pH 1.0) with 3 N HCl. The liberated FFA were then extracted with 50 mL of hexane (15). The hexane layer was dried over anhydrous sodium sulfate, and the solvent was removed with a rotary evaporator at 40°C. FA were stored in the freezer at −85°C (15).

Concentration of GLA from borage oil. Concentration of GLA from borage oil by urea complexation was carried out by mixing 10 g FFA with urea (20%, wt/vol) in 95% ethanol using a urea to FA ratio (w/w) of 3.7. The solution was heated to 60°C until clear. First, the urea–FA adduct was allowed to crystallize at room temperature, followed by crystallization for 16 h at 0–4°C. The crystals formed were separated from the liquid by filtration through a Büchner funnel. An equal volume of water was added to the filtrate and acidified to pH 4–5 with 6 N HCl. After the addition of an equal volume of hexane, the mixture was stirred for 1 h. Later the mixture was transferred to a separatory funnel. The hexane layer, containing the liberated FA, was separated from the aqueous layer containing urea. The hexane layer was washed with water to remove any remaining urea and filtered through an anhydrous sodium sulfate column to remove water (16). The solvent was removed with a rotary evaporator at 40°C. The GLA concentrate was stored at −85°C (16).

Interesterification. The interesterification mixtures, consisting of 3 mL *n*-hexane and a mixture of tripalmitin, hazelnut oil FA, and GLA at different molar substrate ratios (moles total FA/mol tripalmitin) that were determined by RSM design using Modde 5.0 (Umetrics, Umeå, Sweden) software, were placed in screw-capped test tubes. Immobilized lipases Lipozyme RM IM (10% wt of total reactants) and TL IM (6% wt of total reactants) were added. The tubes were incubated in an orbital shaking water bath at 200 rpm. All reactions were performed in duplicate and average results are reported.

Experimental design for RSM study. A three-factor, rotatable five-level central composite design (CCD) was used to generate factor combinations by using Modde 5.0 software (17,18). The three factors chosen were substrate molar ratio (S_r, total FA/tripalmitin), (12–16 mol/mol); temperature (T_e, °C), (55–65°C); and reaction time (T_i, hours), (12–24 h). The independent variables and experimental design are presented in Table 1. Experiments were run randomly, and duplicate reactions were carried out at all design points. A total of 17 runs with three center points (0,0,0) were generated.

Analysis of product. The enzyme and moisture were removed from the reaction mixture by passing the reaction product through anhydrous sodium sulfate packed in a Pasteur pipette column. A 50-μL aliquot of the reaction product was applied to TLC plates (20 × 20 cm) coated with silica gel G. The developing solvent used was petroleum ether/ethyl ether/acetic acid (80:20:0.5, by vol). The bands were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized

TABLE 1
Observed Responses in Central Composite Design (CCD) Experiments[a]

	Independent variables			Responses (incorporation, mol %)			
				With Lipozyme® RM IM[b]		With Lipozyme® TL IM[b]	
No.	S_r	T_e	T_i	GLA	Oleic acid	GLA	Oleic acid
1	12	55	12	6.2	35.5	4.9	35.8
2	16	55	12	6.9	38	6.7	40.6
3	12	65	12	7.1	37.1	5.9	36.9
4	16	65	12	7.2	40	7.1	38.6
5	12	55	24	11	39.9	9	41.3
6	16	55	24	10.4	44.3	11.4	43.5
7	12	65	24	11.3	41.1	7.4	41.6
8	16	65	24	11	46.3	10.1	44
9	10.64	60	18	9.9	39.6	7	39.3
10	17.36	60	18	9.9	44.4	8.1	42.5
11	14	51.59	18	7.9	38.8	9.4	41.3
12	14	68.41	18	10.4	43.7	6.8	41.4
13	14	60	7.91	5.4	33.6	3.9	30.9
14	14	60	28.09	9.8	45.9	10.3	46
15	14	60	18	11.3	41.3	8.1	41
16	14	60	18	10.4	40.3	7.3	41.5
17	14	60	18	11.4	40.3	7.3	43.3

[a]Abbreviations: S_r, substrate molar ratio (mol/mol); T_e, reaction temperature; T_i, reaction time; GLA, γ-linoleic acid.
[b]Enzymes were supplied by Novo Nordisk A/S (Bagsvaerd, Denmark).

under UV light. The TAG band was scraped off into a screw-capped test tube, and TAG bands were methylated with 3 mL of 6% HCl in methanol at 75–80°C for 2 h. FAME were extracted twice with 2 mL of hexane and dried over an anhydrous sodium sulfate column according to the method described earlier by Jennings and Akoh (19).

FA composition analysis. The FA composition of hazelnut oil FA and reaction products was analyzed by GLC. The gas chromatograph was an Agilent Technologies Model 6890N, equipped with a capillary column (30 m × 0.25 mm i.d., DB-225; J&W Scientific, Folsom, CA) and an FID, and operated in splitless mode. The injector and detector temperatures were maintained at 250 and 260°C, respectively. The column temperature was held at 150°C for 3 min and then programmed to 215°C for 10 min at the rate of 10°C/min. The carrier gas was helium, and total gas flow rate was 64 mL/min. A 1-mL sample was injected into the system, and the relative contents of FAME expressed as mol% were calculated by computer, using heptadecanoic acid as the internal standard.

Pancreatic lipase-catalyzed sn-2 positional analysis. Pancreatic lipase (20 mg), 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 the TAG band that was scraped from the TLC plate as described before. The mixture was incubated at 40°C in a water bath for 3 min; 1 mL of 6 M HCl and 1 mL of diethyl ether were added and centrifuged. Diethyl ether was evaporated under nitrogen gas to 200 μL volume. A 200-μL aliquot was spotted onto a silica gel G TLC plate and developed by hexane/diethyl ether/acetic acid (50:50:1, by vol) (19). The 2-MAG band was identified under UV light after being sprayed with 0.2% 2,7-dichlorofluorescein in methanol. The 2-monoolein standard (Sigma) was used to confirm the TLC separation of 2-MAG in the reaction products. The 2-MAG band was scraped off into a screw-capped test tube, extracted twice with 1 mL of hexane, then methylated and analyzed by GLC (19).

Statistical analysis. The regression analyses, statistical significance, and response surface applications were carried out using Modde 5.0 software and STATISTICA 6.0 (StatSoft Inc., Tulsa, OK) software. Second-order coefficients were obtained by regression analysis with backward elimination. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and ANOVA. A second-order polynomial model was employed to fit the data for the response by the generated model:

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

where Y is the response (response 1, incorporation of GLA; response 2, incorporation of oleic acid), β_0 is the intercept, β_i linear (first-order model), β_i quadratic, β_{ij} interaction regression coefficients, and X_i and X_j the independent variables.

RESULTS AND DISCUSSION

FA compositions of hazelnut oil FA and GLA concentrate. The FFA obtained from hazelnut oil contained 81.5% oleic, 9.6% linoleic, and 8.9% palmitic acid. GLA concentrate, obtained from borage oil by urea complexation, contained 99.4% GLA and 0.6% gondoic acid (C20:1n-9). GLA content was enriched from 21.7 to 99.4% by urea complexation of borage oil, a technique that is frequently used for obtaining GLA in the free acid and concentrated form (16).

Model fitting. Enzymatic interesterifications with tripalmitin, hazelnut oil FA, and GLA in hexane were used to produce SL resembling HMFS but enriched with GLA, the targeted incorporation of GLA and oleic acid into tripalmitin being 10 and 45%, respectively. A three-factor, five-level CCD was used for the reactions catalyzed by Lipozyme RM IM and Lipozyme TL IM, and the respective design points are given in Table 1. The responses (GLA incorporation, mol%, and oleic acid incorporation, mol%) observed for the reactions catalyzed by two enzymes are given in Table 1.

Good quadratic models were obtained for the incorporation of GLA (response 1) and oleic acid (response 2) by multiple regression and backward elimination. Based on the experimental results (Table 1), the regression coefficients (β) and significance (P) values were calculated and are given in Table 2.

It can be seen from Table 2 that, among first-order parameters, the substrate molar ratio had a negative effect on GLA incorporation by Lipozyme RM IM. Time was the most significant first-order parameter, followed by temperature and substrate molar ratio for GLA incorporation with Lipozyme RM IM. For both responses, second-order parameters, temperature × temperature and time × time, had significantly negative effects.

As can be seen from Table 2, among first-order parameters temperature had the only negative effect for oleic acid incorporation with Lipozyme TL IM. Time was the most significant first-order parameter followed by substrate molar ratio and temperature. The second-order parameter, time × time, and the interaction term, temperature × time, were significant for both responses.

ANOVA, presented in Table 3, indicates that the models are highly appropriate for the prediction since the F_{model} (24.96, 36.02, 25.20, 16.53) values are very high compared with the $F_{5,11}$ value (3.20) ($\alpha = 0.05$). As can be seen from the ANOVA table, there was no lack of fit in the models. The goodness of fit of the models was evaluated by the coefficient of determination (R^2) and the adjusted coefficient (R^2_{adj}) values of the models together with the graphic plot of predicted values by the models vs. observed experimental values. High values suggest a good fit of the models to the experimental data points. The predicted values obtained from the models had a linear relationship with the observed values. As can be seen from Figure 1, a general linearity ($R_1^2 = 0.92$, $R_2^2 = 0.94$, $R_3^2 = 0.92$, $R_4^2 = 0.88$) was obtained for the responses, which indicates that the generated models adequately represent the relationship between the response and reaction parameters.

TABLE 2
Regression Coefficients (β) and Significance (*P*-value) of the Second-Order Polynomials After Backward Elimination for Reactions Catalyzed by Lipozyme RM IM and Lipozyme TL IM

	Response 1 (GLA inc., %)		Response 2 (oleic acid inc., %)	
Variables	Coefficient (β)	*P*-value[a]	Coefficient (β)	*P*-value[a]
With Lipozyme RM IM				
Intercept	10.55	<0.0001	41.09	<0.0001
S_r	−0.001	0.969	1.69	<0.0001
T_e	0.46	0.031	1.10	0.002
T_i	1.74	<0.0001	3.05	<0.0001
$T_e \times T_e$	−0.53	0.021	−0.04	0.889
$T_i \times T_i$	−1.07	0.0002	−0.57	0.074
With Lipozyme TL IM				
Intercept	7.83	<0.0001	41.43	<0.0001
S_r	0.73	0.002	1.21	0.011
T_e	−0.43	0.035	0.01	0.990
T_i	1.76	<0.0001	3.21	<0.0001
$T_i \times T_i$	−0.18	0.348	−1.08	0.022
$T_e \times T_i$	−0.54	0.042	0.21	0.688

[a]*P*-value, level of significance. See Table 1 for abbreviations and enzyme supplier.

The model equation for the responses (% GLA incorporated and % oleic acid incorporated, respectively) catalyzed by Lipozyme RM IM can therefore be written as:

$$Y_1 = 10.55 - 0.01S_r + 0.46T_e + 1.74T_i - 0.53T_e^2 - 1.07T_i^2 \quad [2]$$

$$Y_2 = 41.09 + 1.69S_r + 1.10T_e + 3.05T_i - 0.04T_e^2 - 0.57T_i^2 \quad [3]$$

The model equation for the responses (% GLA incorporated and % oleic acid incorporated, respectively) catalyzed by Lipozyme TL IM can be written as:

$$Y_3 = 7.83 + 0.73S_r - 0.43T_e + 1.76T_i - 0.18T_i^2 - 0.54T_eT_i \quad [4]$$

$$Y_4 = 41.43 + 1.21S_r + 0.01T_e + 3.21T_i - 1.08T_i^2 + 0.21T_eT_i \quad [5]$$

Optimization of the reaction. The relationship between the responses and the parameters was examined by using contour plots. The contour plots obtained by the interaction of three parameters on incorporation of GLA and oleic acid into tripalmitin by Lipozyme RM IM and Lipozyme TL IM are given in Figures 2 and 3, respectively. Intermediate levels were used for the third variable while drawing the contour plots.

In Figure 2A–C, contour plots drawn for the interactions of reaction time with reaction temperature, of substrate molar ratio with reaction temperature, and of substrate molar ratio with reaction time on GLA incorporation by Lipozyme RM IM enzyme are shown. The reaction time and reaction temperature increased, and GLA incorporation also increased within the observed experimental ranges (Fig. 2A). The incorporation of

TABLE 3
ANOVA Analyses for the Responses for Reactions Catalyzed by Lipozyme RM and Lipozyme TL IM

	DF	Sum of squares	Mean squares	*F* ratio	R^2; R^2_{adj}[a]
GLA inc. with Lipozyme RM IM					
Total corrected	16	64.56	4.04		0.92; 0.88
Regression	5	59.33	11.87	24.96	
Residual	11	5.23	0.48		
Oleic acid inc. with Lipozyme RM IM					
Total corrected	16	198.35	12.40		0.94; 0.92
Regression	5	186.93	37.39	36.02	
Residual	11	11.42	1.04		
GLA inc. with Lipozyme TL IM					
Total corrected	16	59.70	3.73		0.92; 0.88
Regression	5	54.90	10.98	25.20	
Residual	11	4.79	0.44		
Oleic acid inc. with Lipozyme TL IM					
Total corrected	16	200.11	12.51		0.88; 0.82
Regression	5	176.61	35.32	16.53	
Residual	11	23.50	2.14		

[a]R^2, determination coefficient; R^2_{adj}, adjusted determination coefficient. For abbreviation and enzyme supplier see Table 1.

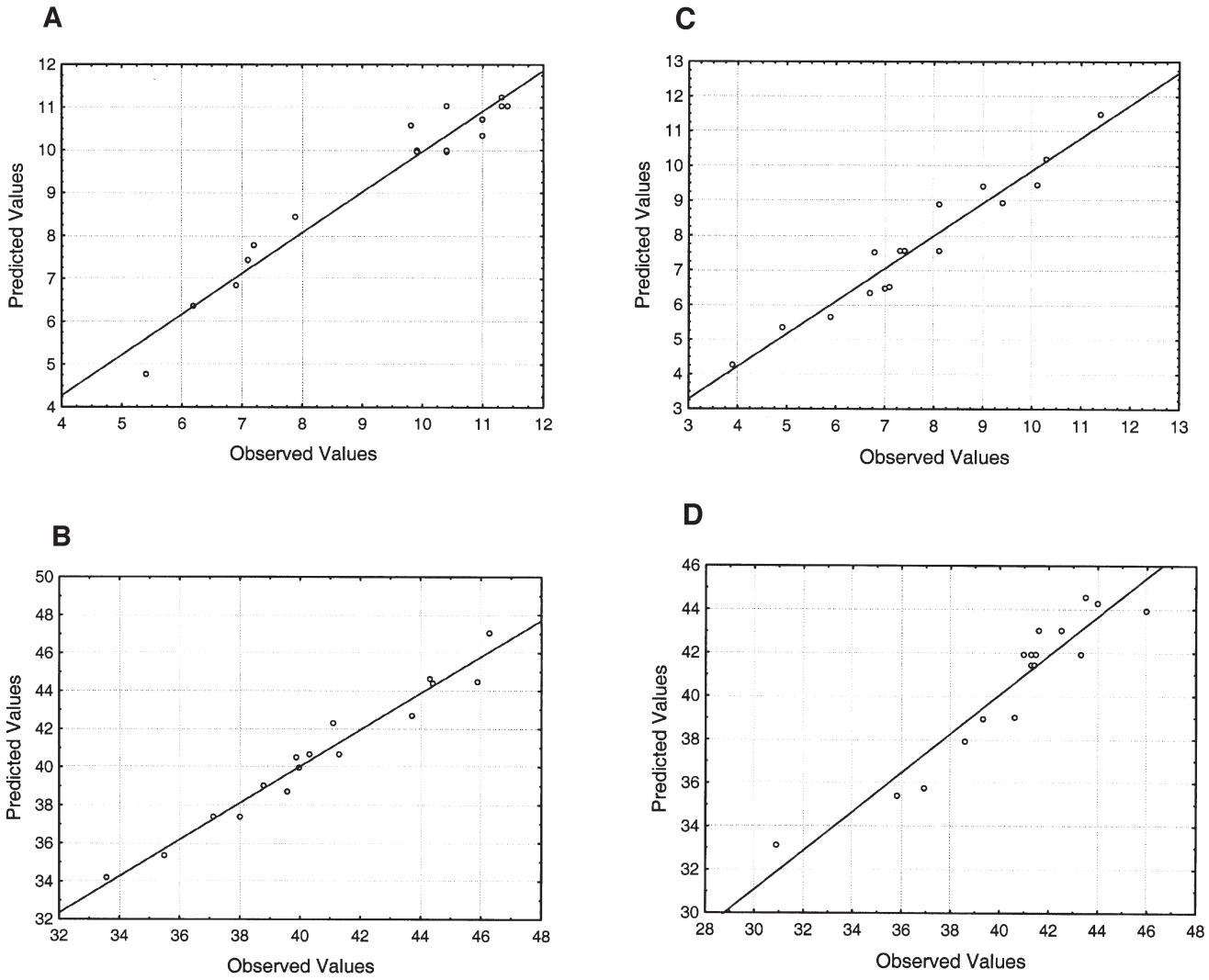

FIG. 1. The relationships between the observed results and data predicted by the models. (A) Incorporation of γ-linoleic acid (GLA) with Lipozyme® RM IM, (B) incorporation of oleic acid with Lipozyme RM IM, (C) incorporation of GLA with Lipozyme TL IM, and (D) incorporation of oleic acid with Lipozyme TL IM. Enzymes were supplied by Novo Nordisk (Bagsvaerd, Denmark).

GLA decreased from around the midpoint of the variables tested (Fig. 2B). The targeted incorporation (10%) of GLA was obtained at around 56°C. In Figure 2C, GLA incorporation decreased below and above 22 h, and substrate molar ratio had no significant effect. The targeted GLA incorporation (10%) was achieved at about 16 h. In Figure 2D–F, the interactions of reaction time with substrate molar ratio, of reaction temperature with substrate molar ratio, and of reaction temperature with reaction time on oleic acid incorporation with Lipozyme RM IM enzyme are shown. Oleic acid incorporation increased as the reaction time and substrate molar ratio increased, as the reaction temperature and substrate molar ratio increased, and as the reaction temperature and reaction time increased within the observed ranges.

In Figure 3A, the contour plot drawn for the interaction of reaction time with substrate molar ratio on incorporation of GLA with Lipozyme TL IM enzyme showed that, as the reaction time and substrate molar ratio increased, the incorporation of GLA increased.

The contour plot of the interaction of reaction temperature with substrate molar ratio (Fig. 3B) indicated the incorporation of GLA was higher at lower temperatures and higher substrate molar ratios within the observed ranges. In Figure 3C, when the reaction time was 22–24 h and the reaction temperature was kept constant at around 55–57°C, the targeted GLA incorporation (10%) was achieved. Also, GLA incorporation increased at low temperatures with the increase in reaction time.

In Figure 3D–F, contour plots for the interaction of reaction time with substrate molar ratio, of reaction temperature with

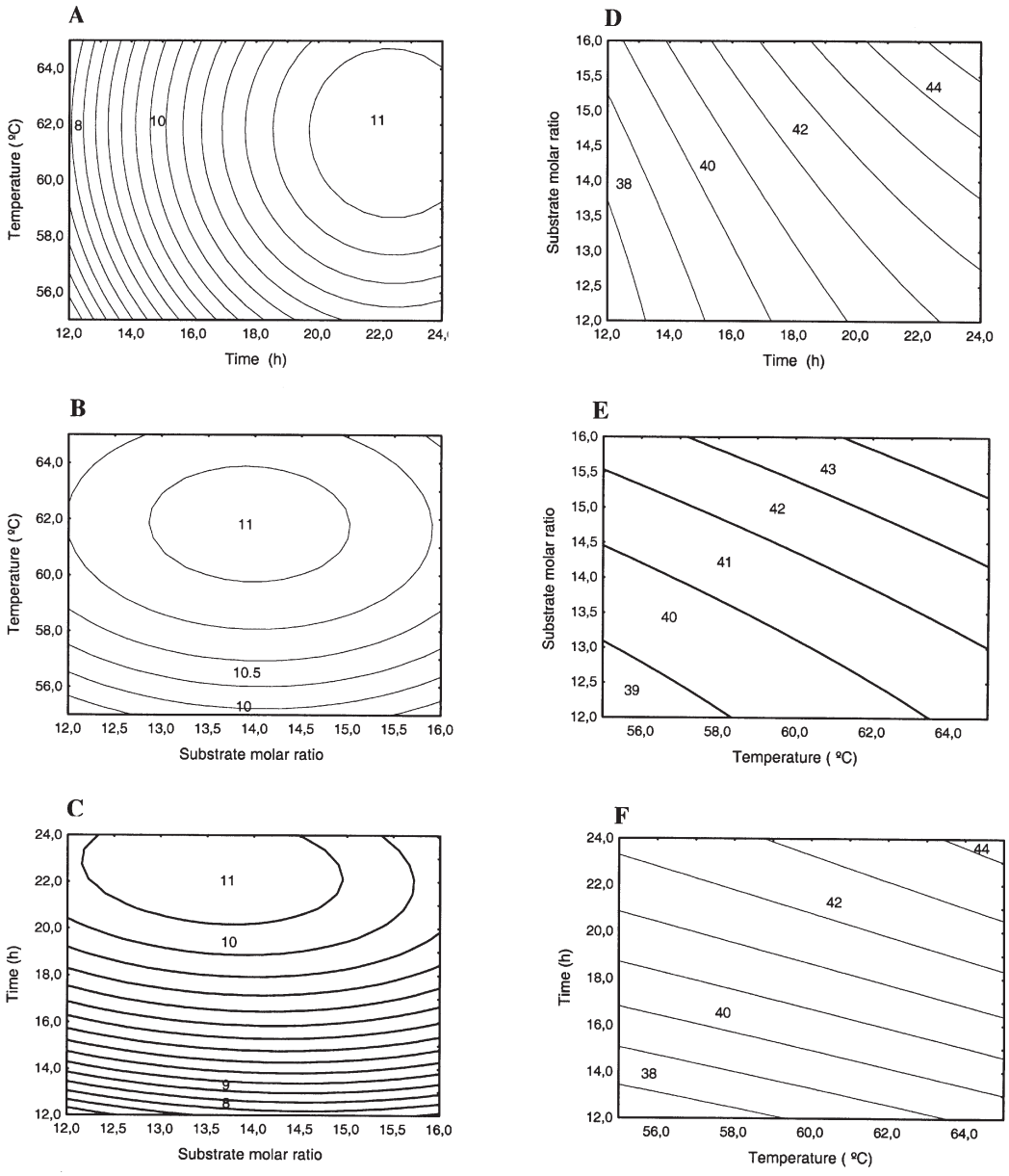

FIG. 2. Contour plots between two parameters for GLA incorporation and oleic acid incorporation with the catalysis of Lipozyme RM IM, respectively. (A) GLA inc., reaction time vs. reaction temperature, (B) GLA inc., substrate molar ratio (moles total FA/mol tripalmitin) vs. reaction temperature, (C) GLA inc., substrate molar ratio vs. reaction time, (D) oleic acid inc., reaction time vs. substrate molar ratio, (E) oleic acid inc., reaction temperature vs. substrate molar ratio, and (F) oleic acid inc., reaction temperature vs. reaction time. For abbreviation and enzyme supplier, see Figure 1.

substrate molar ratio, and of reaction temperature with reaction time on the incorporation of oleic acid with Lipozyme TL IM enzyme are shown. As the reaction time and substrate molar ratio increased, oleic acid incorporation increased within the observed ranges (Fig. 3D). The targeted oleic acid incorporation was achieved at higher substrate molar ratios independent of temperature (Fig. 3E), and targeted oleic acid incorporation was achieved at higher reaction times independent of temperature (Fig. 3F). Oleic acid incorporation decreased with the decrease in reaction time.

The contour plots for the responses shown in Figures 2 and 3 also show that substrate molar ratio, temperature, and time had positive effects on oleic acid incorporation with the two enzymes, but for GLA incorporation with Lipozyme RM IM, substrate molar ratio, and with Lipozyme TL IM, temperature, had negative effects, as discussed above.

The optimal conditions (Tables 4 and 5) for the targeted GLA and oleic acid incorporation with Lipozyme RM IM and Lipozyme TL IM enzymes were generated by the optimizer function of the Modde 5.0 software.

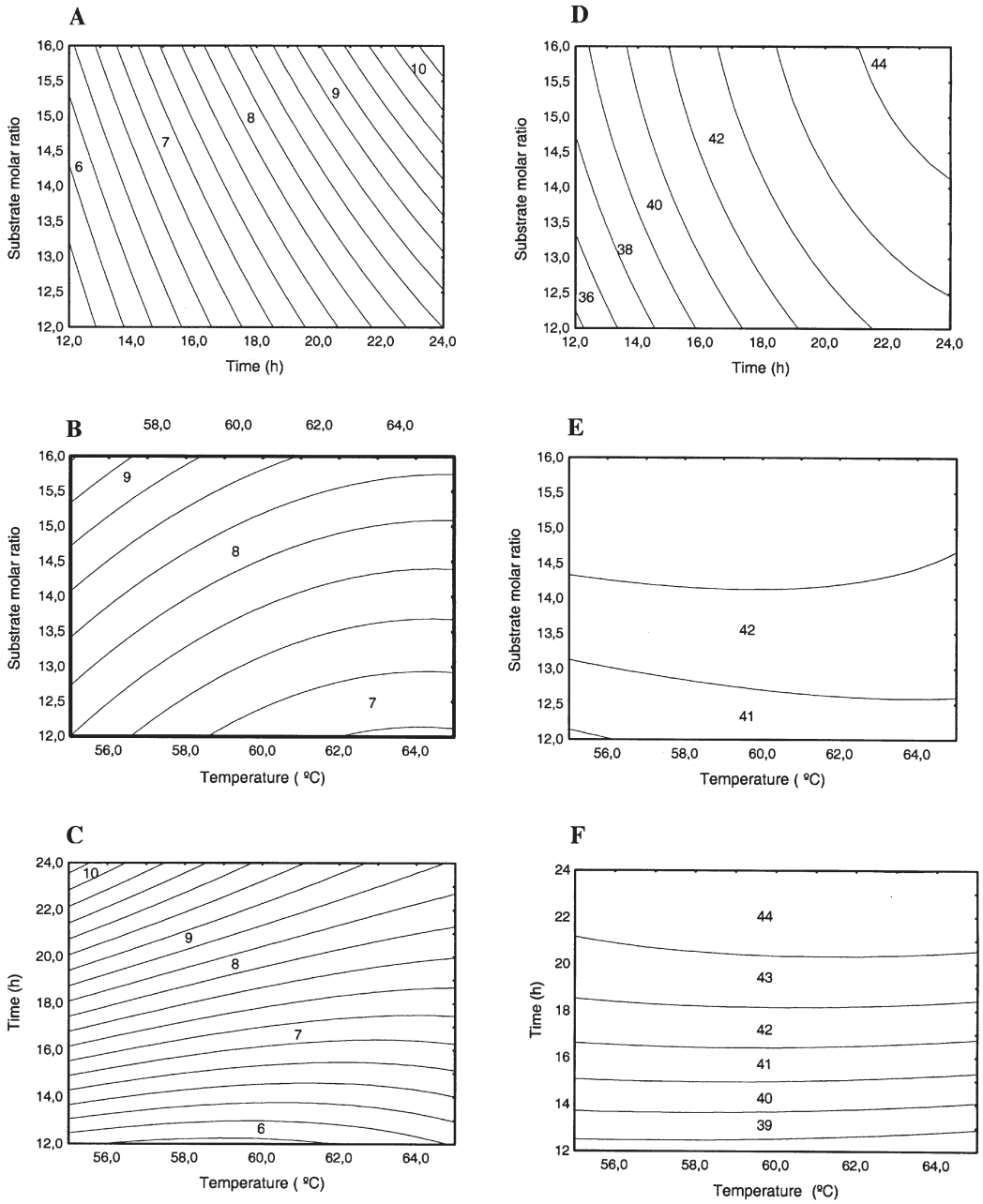

FIG. 3. Contour plots between two parameters for GLA incorporation and oleic acid incorporation with the catalysis of Lipozyme TL IM, respectively. (A) GLA inc., reaction time vs. substrate molar ratio, (B) GLA inc., reaction temperature vs. substrate molar ratio, (C) GLA inc., reaction temperature vs. reaction time, (D) oleic acid inc., reaction time vs. substrate molar ratio, (E) oleic acid inc., reaction temperature vs. substrate molar ratio, and (F) oleic acid inc., reaction temperature vs. reaction time.

The optimal conditions generated from the models for the targeted GLA (10%) and oleic acid (45%) incorporation were 14.8 mol/mol, 55°C, and 24 h and 14 mol/mol, 55°C, and 24 h for substrate ratio, temperature, and time for the reactions catalyzed by Lipozyme RM IM and Lipozyme TL IM, respectively. The lowest substrate molar ratio was chosen among the optimal conditions to decrease the cost for the substrate and the purification process (7).

Verification of the models. Enzymatic interesterifications were done in test tubes with the optimal conditions obtained with RSM to verify the model. To compare the effects of two enzymes on the incorporation of GLA and oleic acid, further interesterifications were performed using the same conditions, such as 16 mol/mol, 55°C, and 21.6 h for substrate ratio, temperature, and time, respectively.

The results of the determination of total FA composition and

TABLE 4
Optimal Conditions for Lipozyme RM IM Generated by Modde 5.0 (Umetrics, Umeå, Sweden) Software[a]

Number	S_r (mol/mol)	T_e (°C)	T_i (h)	Predicted GLA inc. (mol%)	Predicted oleic acid inc. (mol%)
1	14.8	55	24	10.2	43.1
2	15.6	56	21.3	10.5	43
3	15.6	55.1	22.8	10.3	43.4
4	16	65	24	11.1	46.3
5	15.4	55.5	22.3	10.4	43.1
6	16	55	21.6	10.2	43.3

[a]Set parameter ranges: substrate molar ratio (12–16 mol/mol), reaction temperature (55–65°C), reaction time (12–24 h). Set target: 10% for GLA, 45% for oleic acid. For abbreviations and enzyme supplier see Table 1.

TABLE 5
Optimal Conditions for Lipozyme TL IM Generated by Modde 5.0 (Umetrics, Umeå, Sweden) Software[a]

Number	S_r (mol/mol)	T_e (°C)	T_i (h)	Predicted GLA inc. (mol%)	Predicted oleic acid inc. (mol%)
1	14	55	24	10.4	43.3
2	15.2	58	23.7	10.1	44.1
3	16	59.5	24	10.2	44.7
4	16	63	24	9.6	44.9
5	15.8	58.8	23.5	10.1	44.5
6	16	55	21.6	10.3	44

[a]Set parameter ranges: substrate molar ratio (12–16 mol/mol), reaction temperature (55–65°C), reaction time (12–24 h). Set target: 10% for GLA, 45% for oleic acid. For abbreviations and enzyme supplier see Table 1.

TABLE 6
FA Composition (mol%) and FA at *sn*-2 Position (mol%) of the Structured lipids (SL) Produced Under Optimal Conditions[a]

	SL produced with Lipozyme RM IM		SL produced with Lipozyme TL IM	
FA	FA (mol%)	FA at *sn*-2 (mol%)	FA (mol%)	FA at *sn*-2 (mol%)
16:0	41.6	74.9	40.9	73.9
18:1	43.3	18.1	43.8	19.7
18:2	5.4	2.1	5.5	2.2
18:3n-6	9.7	2.0	9.8	1.4

[a]Substrate ratio (14.8 mol/mol), reaction temperature (55°C), reaction time (24 h) for SL produced with Lipozyme RM IM; substrate ratio (14 mol/mol), reaction temperature (55°C), reaction time (24 h) for SL produced with Lipozyme TL IM. For enzyme supplier see Table 1.

of the FA at the *sn*-2 position of the resulting SL are given in Table 6. The experimental values were satisfactorily close to the values predicted from the model. The FA distribution at the *sn*-2 position of SL produced with Lipozyme RM IM was similar to that of SL produced with Lipozyme TL IM (Table 6). SL resembling HMFS, produced with Lipozyme RM IM and Lipozyme TL IM contain palmitic acid at 74.9 and 73.9%, respectively. The *sn*-2 position of the SL was predominantly occupied by palmitic acid, followed by oleic acid, GLA, and linoleic acid.

These results are very close to the FA distribution of HMF, where palmitic acid is in the *sn*-2 position (70%) and unsaturated FA are in the *sn*-1 and *sn*-3 positions (1,2). Since the position of palmitic acid in the glycerol backbone of HMF affects FA absorption, calcium absorption, and use of dietary energy, it is important to mimic this unique FA structure of HMF in the production of HMFS (1,2,20).

The results from the two enzymes on the incorporation of GLA and oleic acid were very close to each other and to the predicted values for the conditions of substrate ratio of 16 mol/mol, temperature of 55°C, and reaction time of 21.6 h. The SL produced with Lipozyme RM IM contained 43.6% oleic acid and 10.1% GLA, whereas the SL produced with Lipozyme TL IM contained 44.3% oleic acid and 10.0% GLA. Both enzymes had similar effects on the production of HMFS enriched with GLA under the described test conditions.

ACKNOWLEDGMENTS

Nese Sahin was supported by a scholarship grant under the scope of the NATO Science Fellowships Programme by the Scientific and Technical Research Council of Turkey (TUBITAK) and by the University of Georgia Food Science Research fund.

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[Received February 21, 2005; accepted June 7, 2005]