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Human Milk Fat Substitutes Containing Omega-3 Fatty Acids

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Structured lipids resembling human milk fat (HMF) enriched with omega-3 fatty acids were synthesized by enzymatic acidolysis reactions between tripalmitin, hazelnut oil fatty acids (FA), and omega-3 FA concentrate. Response surface methodology was used to model and optimize the incorporation of omega-3 FA and oleic acid into tripalmitin, in hexane, using immobilized sn-1,3-specific lipase, Lipozyme RM IM. The three factors chosen were substrate molar ratio, reaction temperature, and reaction time. Good quadratic models were obtained for the incorporation of eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) (response 1) and oleic acid (response 2) by multiple regression and backward elimination. The determination coefficient (R^2) value for the models was 0.95. The adjusted R^2 values were 0.91 and 0.92 for responses 1 and 2, respectively. The optimal conditions generated from the models for the targeted total EPA and DHA (5%) and oleic acid (40%) incorporation were 12.4 mol/mol, 55 °C, and 24 h for substrate ratio, temperature, and time, respectively. The model was verified, which led to the production of a HMF ingredient with 76.6% palmitic acid at the sn-2 position.

KEYWORDS: Hazelnut oil; human milk fat substitutes; lipase-catalyzed acidolysis; Lipozyme RM IM; omega-3 fatty acids; response surface methodology

INTRODUCTION

Human milk fat (HMF) is one of the major components of breast milk for newborn, term, and preterm infants and thus supplies the highest fraction of the infant's required dietary energy as well as the required nutrients and some other vital components (*1*, *2*). Even though some small variations in breast milk fatty acid (FA) composition occur due to such factors as genetics, mother's diet, season, lactation stage, physiology, and even psychology, all human milks are characterized by the dominance of triacylglycerols (TAGs, >98% of HMF), palmitic acid (C16:0, 20−30% of total FA) being in the sn-2 position (70% of all palmitic acid) of the glycerol backbone and the sn-1 and sn-3 positions are occupied by unsaturated fatty acids. Human milk provides small quantities of eicosapentaenoic acid (EPA) (0.07−0.18%) and docosahexaenoic acid (DHA) (0.26−0.41%), usually <1% of total fatty acids (*3*, *4*).

Oleic acid is predominantly present (44%) at the sn-1 position of human milk TAG, followed by palmitic (18.7%) and stearic (14.2%) acids, whereas the sn-2 position is occupied mostly by palmitic (57.1%), myristic (15.4%), and oleic (8.1%) acids. At the sn-3 position, oleic acid (50.5%) is the predominant fatty acid followed by linoleic (12.7%) and lauric (10.4%) acids (*5*).

The position of palmitic acid at the glycerol backbone has a role in the digestion and intestinal absorption of fats in the infants. The vegetable oils that are commonly used in commercial infant formulas contain palmitic acid (>80%) esterified to the sn-1 and sn-3 positions, but they form insoluble calcium soaps (calcium palmitate) during the digestion process (*1*, *2*, *6*). Therefore, an infant formula with palmitic acid at the sn-2 position of the TAG would provide more energy to the infants and would reduce the loss of dietary fat and calcium (*2*).

EPA (20:5n−3) and DHA (22:6n−3) are long-chain (LC) omega-3 PUFAs, which are two primary components of some fish oils (*3*). Menhaden is one of the fatty fish species in which TAGs present in the fatty flesh of the fish consists of EPA (11−14%) and DHA (8−9%) (*7*, *8*).

Epidemiological studies have shown that DHA and EPA have several health benefits related to cardiovascular disease, immune and renal disorders, inflammation, allergies, diabetes, hypertension, rheumatoid arthritis, depression, and cancer (*9*−*11*). DHA is the predominant structural FA in the gray matter of the brain and retinal tissues and plays a complex role in the development and function of the nervous system (brain), photoreception (vision) of infants, especially premature ones, and inflammatory diseases (*10*, *11*). Furthermore, DHA has been reported to prevent and treat senile dementia (*9*). Studies also showed that preterm infants fed human milk during infancy exhibited a 5−12 point higher intelligience quotient (IQ) in their later lives than babies fed with conventional formulas. Moreover, the difference was also 2−5 points in term infants, and the measured IQ was positively correlated with the duration of breast-feeding. They

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showed that DHA is the substance in breast milk responsible for the difference in IQ among the children (*9*).

LC-PUFA supplemented infant formulas have gained great interest because of the fact that adequate supply of LC-PUFA is important for infants during the first year after birth and that the number of breast-feeding women is decreasing (*7*, *10*). Furthermore, term infants have a limited desaturating capacity to synthesize LC-PUFA such as arachidonic acid (AA), DHA, and EPA; they rely on the dietary supply of these fatty acids during the first months of their life (*12*, *13*). In Europe, Asia, Canada, and the United States some commercially available infant formulas for term and preterm infants contain LC-PUFA such as DHA and AA. The levels of these two fatty acids in formulas produced by different manufacturers range between 8−19 and 22−34 mg/100 calories, for DHA and AA, respectively (*14*−*17*).

When the infants were fed with formulas supplemented with LC-PUFA, it was observed that their blood patterns were similar to those of breast-fed infants (*13*). It was also reported that term and preterm infants fed with formulas lacking LC-PUFA have lower red cell and lower DHA levels in the phospholipids of the cerebral cortex than infants fed human milk (*18*). Oleic acid, the predominant monounsaturated FA in HMF, is an important source of energy for the infants. Besides this, oleic acid is a component of tissues and membranes, particularly myelin, which is mainly formed during the two years after birth (*13*).

Structured lipids (SL) that contain palmitic acid predominantly (nearly 70%) at the sn-2 position and which are also enriched with DHA and EPA can be used in infant formulas to mimic the physical and chemical structure of human milk fat, as well as to provide the health benefits associated with the omega-3 PUFAs.

Response surface methodology (RSM) is a combination of mathematical and statistical methods used to determine the effects of different variables and to model and optimize the processes in which a response is affected by several variables, alone or in combination (*19*). RSM has been widely used in the optimization of reactions for the production of SLs by several researchers (*20*−*26*). Central composite design (CCD) is one of the most widely used RSM methods (*27*). By employing RSM in the experimental design, statistically acceptable results can be obtained by reducing the number of experiments, thus providing time and cost savings (*19*, *27*).

The purpose of the present work was to produce SL resembling HMF and enriched with omega-3 fatty acids by enzymatic acidolysis reactions using Lipozyme RM IM lipase, as well as to employ RSM to optimize the reaction conditions. Models were set up by RSM based on three parameters, including substrate molar ratio, reaction temperature, and reaction time. The products were also characterized for their sn-2 positional composition.

MATERIALS AND METHODS

Materials. Tripalmitin (glycerol tripalmitate, minimum purity 85%), menhaden 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 a hazelnut tree (*Corylus avellana* L. and *Corylus maksima* Mill), was purchased from a grocery store in Turkey. Immobilized 1,3-specific lipase, Lipozyme RM IM, was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). Organic solvents and TLC plates were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) and Fisher Scientific (Fair Lawn, NJ), respectively. All solvents and reagents used in analyses were of chromatographic or analytical grade.

Preparation of Free Fatty Acids from Hazelnut Oil and Menhaden Oil. The previously described method was used for the preparation of free fatty acids from hazelnut oil and menhaden oil (*28*, *29*). Fatty acids were stored in the freezer at −85 °C.

Concentration of Omega-3 Fatty Acids from Menhaden oil by Urea Complexation. Urea complexation is a frequently used technique for the concentration of omega-3 FA from a variety of marine oils (mackerel, menhaden, Atlantic redfish, Atlantic herring, Pacific dogfish, Pacific salmon, Atlantic harp seal, seal blubber, fur seal) (*30*−*34*). Concentration of omega-3 FA from menhaden oil by urea complexation was carried out by mixing 10 g of free fatty acids with urea (20%, w/v) in 95% aqueous ethanol using a urea to fatty acid ratio (w/w) of 5 for complex formation. The solution was heated to 60 °C until it became clear. First, the urea−fatty acid adduct was allowed to crystallize at room temperature, followed by crystallization for 24 h at 0 °C. The crystals formed (the urea−fatty acid adduct), also referred to as the urea complexing fraction (UCF), were separated from the liquid [non-urea complexing fraction (NUCF)] by filtration using a Büchner funnel. An equal volume of water was added to the NUCF, which was enriched with omega-3 FA 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 fatty acids was separated from the aqueous layer containing urea. The hexane layer was washed with water to remove any remaining urea and filtered through anhydrous sodium sulfate column to remove residual water. The hexane layer was dried over anhydrous sodium sulfate, and the solvent was removed with a rotary evaporator at 40 °C. Omega-3 FA concentrate was stored at −85 °C (*30*).

Acidolysis. The acidolysis reaction mixtures consisting of 3 mL of *n*-hexane, a mixture of tripalmitin, hazelnut oil FA, and omega-3 FA at different substrate molar ratios determined by RSM design using Modde 5.0 (Umetrics, Umea, Sweden) software, were placed in screw-capped test tubes. Immobilized lipase, Lipozyme RM IM (10 wt % of total reactants), was added. The tubes were incubated in an orbital shaking water bath at 200 rev/min. All reactions were performed in duplicate, and average results were reported.

Experimental Design for RSM Study. A three-factor, rotatable five-level CCD was employed to generate factor combinations by using Modde 5.0 (Umetrics) software (*19*). The three factors chosen were substrate molar ratio (S_r, total FA/tripalmitin, 12−16 mol/mol), temperature (T, °C, 55−65 °C), and reaction time (t, hours, 12−24 h). A total number of 17 runs with 8 factorial points, 6 axial points, and 3 center points were generated by using Modde 5.0 (Umetrics) software. 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.

Analysis of Product. Fifty microliters of the reaction product, after passing through an anhydrous sodium sulfate column to remove enzyme and moisture, was applied to thin-layer chromatography (TLC) plates (20 × 20 cm) coated with silica gel G to identify and remove TAG bands for methylation (*28*, *29*).

Fatty Acid Composition Analysis. The fatty acid composition of hazelnut oil fatty acids, omega-3 fatty acids concentrate, and reaction products was analyzed by gas−liquid chromatography (GLC). The gas chromatograph was an Agilent Technologies 6890N equipped with a fused silica capillary column (DB-225, 30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA) and a flame ionization detector (FID) and operated on 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 at 10 °C/min and held isothermally for 20 min. A 1 μL sample was injected into the GLC. Relative contents of FAME as mole percent were calculated by computer, using 17:0 as the internal standard (*28*, *29*).

Pancreatic Lipase-Catalyzed sn-2 Positional Analysis. A procedure similar to that described earlier (*28*, *29*) was used for sn-2 positional analysis. The 2-MAG band identified from the TLC plate was scraped off into a screw-capped test tube, extracted twice with 1 mL of hexane, and then methylated and analyzed by GLC.

Statistical Analysis. The regression analyses, statistical significance, and response surface applications were done using Modde 5.0 (Umet-

Table 1. Observed Responses in Central Composite Design Experiments[a]

	independent variables			responses (incorporation, mol %)	
expt	S_r	T	t	EPA plus DHA	oleic acid
1	12	55	12	3.3	31.1
2	16	55	12	4.2	33.1
3	12	65	12	4.7	34.6
4	16	65	12	4.7	36.8
5	12	55	24	5.3	36.2
6	16	55	24	7.4	41.5
7	12	65	24	6.1	38.2
8	16	65	24	6.6	41.0
9	10.64	60	18	5.8	34.8
10	17.36	60	18	6.3	38.3
11	14	51.59	18	4.9	35.9
12	14	68.41	18	5.1	39.0
13	14	60	7.91	3.7	31.3
14	14	60	28.09	6.9	40.0
15	14	60	18	5.9	38.6
16	14	60	18	5.6	37.4
17	14	60	18	5.9	37.7

[a] Abbreviations: S_r, substrate molar ratio (mol/mol); T, reaction temperature (°C); t, reaction time (h).

rics) 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 the analysis of variance (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 omega-3 PUFA; response 2, incorporation of oleic acid), β_0 is the intercept, β_i linear (first-order model), β_{ii} quadratic, β_{ij} interaction regression coefficients, and X_i and X_j are the independent variables.

RESULTS AND DISCUSSION

The free FA obtained from hazelnut oil contained 81.5% oleic acid, 9.6% linoleic acid, and 8.9% palmitic acid. Omega-3 fatty acid concentrate, obtained from menhaden oil by urea complexation, contained 24.4% EPA and 30.1% DHA.

Model Fitting. The targeted TAG product was aimed to contain omega-3 FA (EPA plus DHA) and oleic acid at 5 and 40%, respectively, with the sn-2 position of TAG being occupied by palmitic acid at ~70%—similar to what is found in human milk fat. A three-factor, five-level CCD was employed for the reactions catalyzed by Lipozyme RM IM, and the respective design points together with the observed responses [response 1, incorporation (mole percent) of EPA plus DHA (omega-3 PUFA); response 2, incorporation (mole percent) of oleic acid] are given in **Table 1**.

After multiple regression and backward elimination had been applied to the design points, good quadratic models were obtained for the incorporation of omega-3 PUFA and oleic acid. The regression coefficients (β) and significance (P) values were calculated using experimental results (**Table 2**).

It can be seen from **Table 2** that among first-order parameters, time was the most significant parameter followed by substrate molar ratio and temperature for the incorporation of omega-3 PUFAs and oleic acid. For both responses, first-order parameters had positive effects, whereas second-order parameters had negative effects. The second-order parameters temperature × temperature and time × time and the interaction term substrate molar ratio × temperature were found to be significant for omega-3 PUFA incorporation. For oleic acid incorporation, the second-order parameter time × time and the interaction term temperature × time were found to be significant.

Table 2. Regression Coefficients (β) and Significance Values (P Values) of the Second-Order Polynomials after Backward Elimination

	response 1 (EPA plus DHA incorporation, mol %)		response 2 (oleic acid incorporation, mol %)	
variable	coefficient (β)	P value[a]	coefficient (β)	P value[a]
intercept	5.88	<0.0001	37.31	<0.0001
S_r	0.32	0.007	1.33	0.0003
T	0.16	0.103	1.02	0.001
t	1.02	<0.0001	2.63	<0.0001
$T \times T$	–0.33	0.007	–0.003	0.99
$t \times t$	–0.23	0.042	–0.64	0.03
$S_r \times T$	–0.31	0.027	–0.29	0.37
$T \times t$	–0.24	0.075	–0.71	0.047

[a] P value, level of significance. Ssee **Table 1** for other abbreviations.

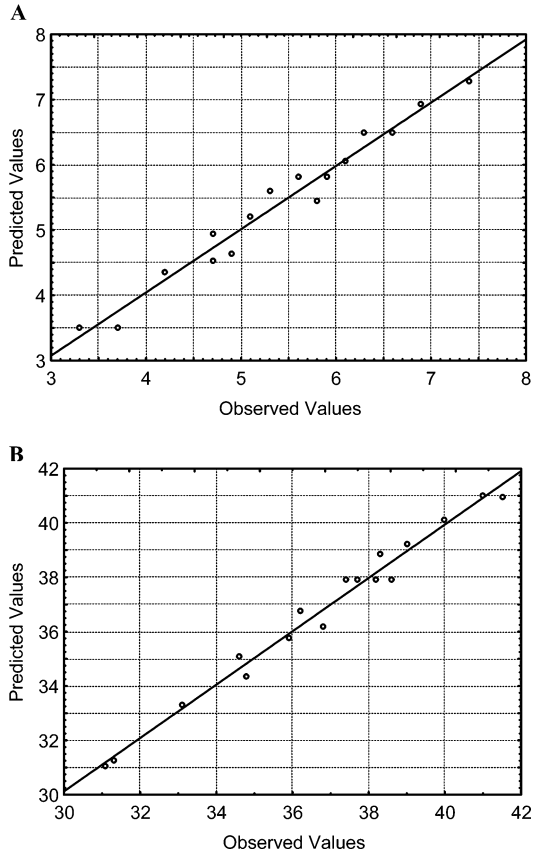

Figure 1. Relationship between the observed results and data predicted by the models: (**A**) incorporation of EPA plus DHA; (**B**) incorporation of oleic acid.

The graphic plot of predicted values by the models versus observed experimental values is given in **Figure 1**. The plot showed a linear distribution ($R_1^2 = 0.95$, $R_2^2 = 0.95$) for the responses, indicating that the predicted values obtained from the models had a linear relationship with the observed values.

The ANOVA analysis presented in **Table 3** indicates that the models were highly appropriate for the prediction because the F_{model} (23.95, 26.65) values were very high compared to the $F_{7,9}$ value (3.29) ($\alpha = 0.05$) (*24*). As seen from **Table 3**, the models showed no lack of fit because P values (0.194 and

Table 3. ANOVA Analyses for the Responses[a]

	DF	SS	MS	*F* ratio	*P* value	R^2; R^2_{adj}
EPA plus DHA Incorporation						
total	17	521.96	30.70			
constant	1	502.22	502.22			
total corrected	16	19.74	1.23			
regression	7	18.73	2.68	23.95	0.000	0.95, 0.91
residual	9	1.01	0.11			
lack of fit (model error)	7	0.95	0.14	4.50	0.194	
pure error (replicate error)	2	0.06	0.03			
Oleic Acid Incorporation						
total	17	23164.60	1362.62			
constant	1	23014.70	23014.70			
total corrected	16	149.87	9.37			
regression	7	142.97	20.42	26.65	0.000	0.95, 0.92
residual	9	6.90	0.77			
lack of fit (model error)	7	6.12	0.87	2.24	0.343	
pure error (replicate error)	2	0.78	0.39			

[a] Abbreviations: DF, degree of freedom; SS, sum of squares; MS, mean squares; R^2, determination coefficient; R^2_{adj}, adjusted determination coefficient.

0.343) were higher than $P > 0.05$, and the regression probability was <0.001.

R^2, the fraction of the variation of the response by the model ($R_1^2 = 0.95$, $R_2^2 = 0.95$), adjusted R^2 (R_{adj}^2) ($R_{1,adj}^2 = 0.91$, $R_{2,adj}^2 = 0.92$), and regression P values <0.001 and lack of fit values ($P > 0.05$) all indicate that models well fitted the experimental data points (*35*).

The model equation for the responses (EPA plus DHA incorporation percent and oleic acid incorporation percent, respectively) can therefore be written as

$$\text{EPA plus DHA incorporation} = 5.88 + 0.32S_r + 0.16T + 1.02t - 0.33T^2 - 0.23t^2 - 0.31S_r \times T - 0.24T \times t \quad (2)$$

$$\text{oleic acid incorporation} = 37.31 + 1.33S_r + 1.02T + 2.63t - 0.003T^2 - 0.64t^2 - 0.29S_r \times T - 0.71T \times t \quad (3)$$

Even though the first-order coefficient for T was not significant, it was included in eq 2 because its interaction with S_r and the second-order coefficient was significant. The $T \times t$ term was also included because it was a significant term for response 2. In the eq 3, $T \times T$ was included as it was significant for response 1.

Optimization of the Reaction. The relationship between the responses and the parameters was examined by using contour plots. The contour plots obtained by interaction of three parameters on incorporation of omega-3 PUFAs and oleic acid to tripalmitin catalyzed by Lipozyme RM IM are given in **Figures 2 and 3**, respectively. When these contour plots were drawn, the third variable was kept at its medium level.

Contour plots drawn for the interaction of reaction temperature with substrate molar ratio, the interaction of reaction time with substrate molar ratio, and the interaction of reaction time with reaction temperature on the omega-3 PUFAs incorporation are shown in panels **A**, **B**, and **C**, respectively, of **Figure 2**. As can be seen from **Figure 2A**, as the substrate molar ratio increased, the omega-3 PUFA incorporation also increased. At temperatures below 55 °C and a substrate molar ratio below 15 mol/mol, the incorporation of DHA plus EPA was smaller than

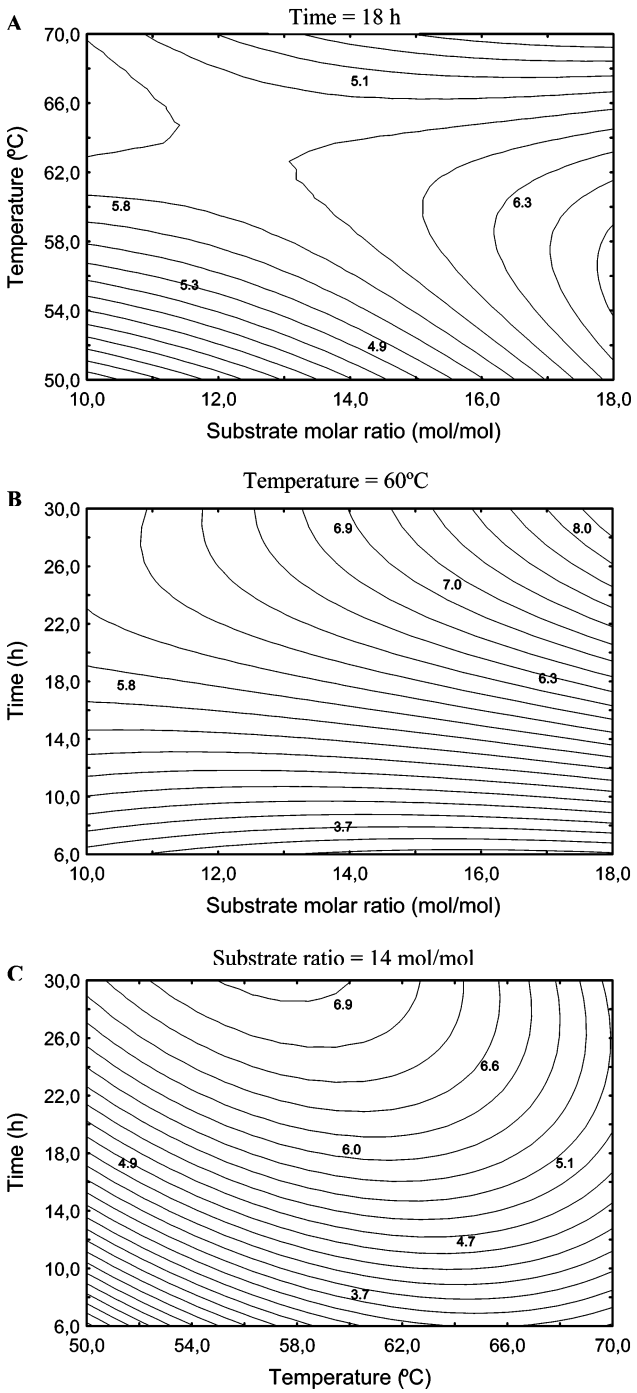

Figure 2. Contour plots between two parameters for EPA plus DHA incorporation: (**A**) reaction temperature versus substrate molar ratio; (**B**) reaction time versus substrate molar ratio; (**C**) reaction time versus reaction temperature.

the targeted incorporation level (5%) within the observed experimental ranges.

As can be seen from **Figure 2B**, substrate molar ratio did not have much of an effect at shorter reaction times. The incorporation of omega-3 PUFA increased as the substrate molar ratio increased with increasing reaction time at 18−30 h, whereas in **Figure 2C**, incorporation of omega-3 PUFA decreased from around the midpoint.

Interaction of reaction temperature with substrate molar ratio, interaction of reaction time with substrate molar ratio, and interaction of reaction time with reaction temperature on oleic acid incorporation are shown in panels **A**, **B**, and **C**, respectively,

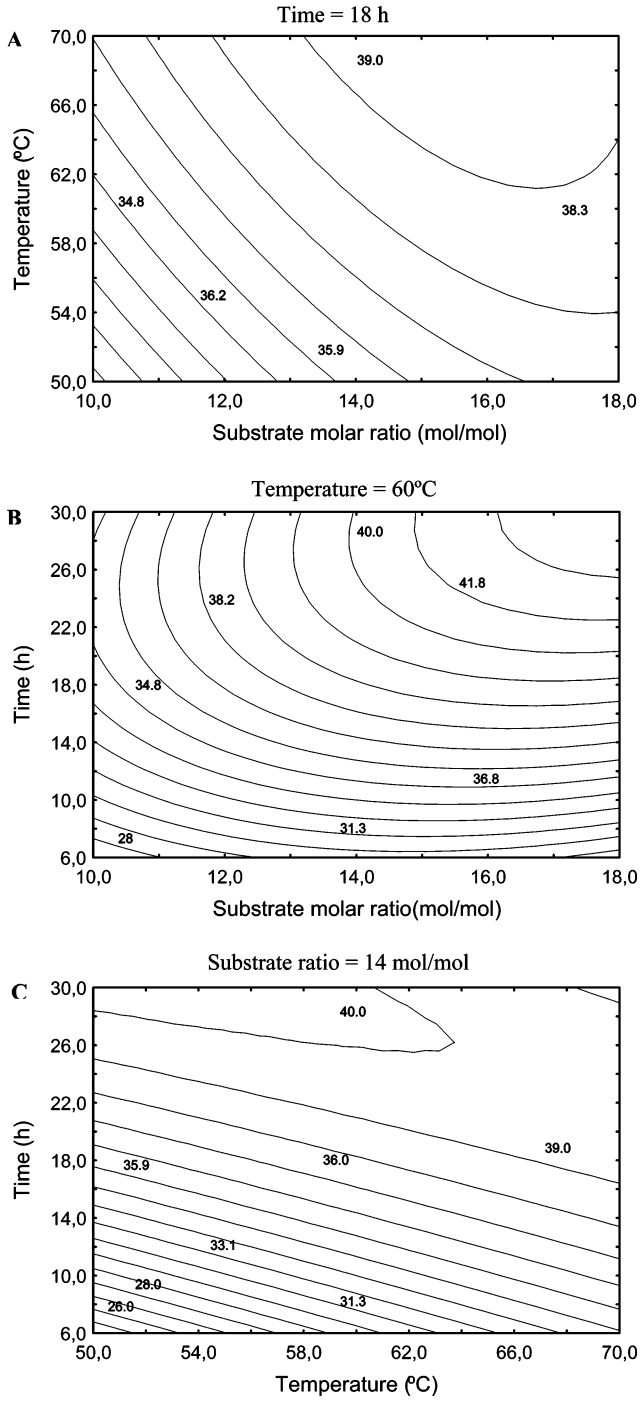

Figure 3. Contour plots between two parameters for oleic acid incorporation: (**A**) reaction temperature versus substrate molar ratio; (**B**) reaction time versus substrate molar ratio; (**C**) reaction time versus reaction temperature.

of **Figure 3**. Oleic acid incorporation increased as the reaction temperature and substrate molar ratio increased; the reaction time and substrate molar ratio as well as the reaction temperature and reaction time increased within the observed ranges.

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 omega-3 fatty acids and oleic acid incorporation.

The optimal conditions for the targeted omega-3 fatty acids (5%) and oleic acid (40%) incorporation were generated by the optimizer function of the Modde 5.0 (Umetrics) software. These were 12.4 mol/mol, 55 °C, and 24 h for substrate ratio, temperature, and time, respectively.

Table 4. FA and FA at sn-2 Position of the SL Produced under Optimal Conditions[a]

FA	FA (mol %)	FA at sn-2 (mol %)
palmitic acid (C16:0)	45.5	76.6
oleic acid (C18:1)	37.5	14.9
linoleic acid (C18:2)	4.4	2.0
EPA (C20:5n–3) plus DHA (C22:6n–3)	6.2	<1.0

[a] Substrate molar ratio, 12.4 mol/mol; temperature, 55°C; time, 24 h.

Verification of the Models. Lipase-catalyzed acidolysis reactions were then carried out in test tubes at optimal conditions obtained with RSM to verify the models.

The results of total fatty acid composition and the fatty acids at the sn-2 position of SL are given in **Table 4**. The experimental values for PUFA (6.2%) and oleic acid (37.5%) incorporation were satisfactorily close to the values predicted (5.9 and 37.7%, respectively) from the models.

The model was verified and led to our production of a HMF potential substitute with 76.6% palmitic acid at the sn-2 position, which was very close to the fatty acid distribution in HMF with 70% palmitic acid at the sn-2 position and unsaturated FAs at the sn-1,3 positions (*1*, *2*, *6*).

Thus, a human milk fat substitute SL containing EPA and DHA was successfully produced with the potential to deliver both the health benefits associated with omega-3 FAs and the absorption characteristics/fatty acid composition similar to those of human milk fat. This SL may be an important ingredient for infant nutrition and development.

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