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Stearidonic Acid Soybean Oil Enriched with Palmitic Acid at the *sn*-2 Position by Enzymatic Interesterification for Use as Human Milk Fat Analogues

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ABSTRACT: Stearidonic acid (SDA, C18:4n-3) enriched soybean oil may be added to the diet to increase intake of omega-3 fatty acids (FAs). Human milk fat has ≥60% of palmitic acid (PA), by weight, esterified at the *sn*-2 position to improve absorption of fat and calcium in infants. Enzymatic interesterification of SDA soybean oil and tripalmitin produced structured lipids (SLs) enriched with PA at the *sn*-2 position of the triacylglycerol. Reactions were catalyzed by Novozym 435 or Lipozyme TL IM under various conditions of time, temperature, and substrate mole ratio. Response surface methodology was used to design the experiments. Model optimization conditions were predicted to be 1:2 substrate mole ratio at 50 °C for 18 h with 10% (by weight) Lipozyme TL IM resulting in 6.82 ± 1.87% total SDA and 67.19 ± 9.59% PA at *sn*-2; 1:2 substrate mole ratio at 50 °C for 15.6 h resulting in 8.01 ± 2.41% total SDA and 64.43 ± 13.69% PA at *sn*-2 with 10% (by weight) Novozym 435 as the biocatalyst. The SLs may be useful as human milk fat analogues for infant formula formulation with health benefits of the omega-3 FAs.

KEYWORDS: human milk fat analogue, stearidonic acid, structured lipid, tripalmitin

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) with 18 carbons or more occur in plants and animals including fish, microalgae and fungi. However, the seeds of higher plants contain the richest source of PUFAs. The essential fatty acids (FAs), linoleic acid (LA) and α-linolenic acid (ALA), along with stearidonic acid (SDA) accumulate in plant tissues.[1] ALA can be converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by desaturases and elongases, but their conversion is often poor.[2] This poor conversion can possibly be explained by the initial Δ6 desaturase enzyme being rate limiting in humans.[3] However, the use of SDA oils could skip this rate limiting step allowing for better conversion to EPA and DHA. Miles et al.[4] observed that neither SDA nor 20:4n-3 appeared in the peripheral blood mononuclear cell when dietary SDA was ingested at a level of 1.0 g/day, indicating that SDA is readily metabolized to EPA in the body. Therefore, SDA from plant oil may efficiently increase the EPA status of immune cells, and SDA enriched soybean oil could be added to the diet to increase intake of omega-3 FAs. SDA, from borage and echium oils, has been consumed as a component of dietary supplements.[5] SDA has been noted as a possible potent inhibitor of cancer growth, inhibitor of platelet aggregation, and anti-inflammatory pharmaceutical, and it provides cardiovascular benefits.[1] The richest sources of SDA include algae and seed plants. Animal sources, such as fish oils, are mainly sources of EPA and DHA but only contain small amounts of SDA.[1] James et al.[6] suggested that the daily ingestion of fish or fish oil to obtain the health benefits of omega-3 FAs is not a sustainable long-term approach. However, increasing ingestion of land-based sources of omega-3 FAs is required to increase the tissue concentration of EPA and DHA. James et al.[6] conducted a double-blind, parallel group study to examine the effect of dietary SDA on increasing tissue concentrations of EPA in humans and compared SDA's ability with that of ALA and EPA. They concluded that SDA vegetable oils were more effective in increasing EPA tissue concentrations than the currently used ALA vegetable oils. Consuming SDA has been shown to lead to the enrichment of tissues with EPA in humans.[5,6] EPA is a long chain omega-3 polyunsaturated FA. Very long chain fatty acids are essential for the growth and development of infants. EPA has been linked to reductions in inflammation[7] and neurological disorders.[8] There are many oils on the market that contain ALA, but few that contain SDA. Dietary SDA was found to increase EPA by 3- to 4-fold more effectively than similar levels of ALA whereas SDA was approximately one-third as effective as dietary EPA.[9] Therefore, the relative effectiveness of these FAs in increasing the EPA concentrations in tissues is EPA > SDA > ALA.[6] SDA enriched soybean oil could be used in many different food products, such as infant formula, to increase the omega-3 FA intake in infants to help with their growth, development, and intestinal absorption of fat-soluble vitamins.[10] Lipids found in breast milk provide essential FAs that are required as structural cell components of membrane tissues, and they provide an essential source of energy that is approximately 50% of the total energy used by infants.[10]

SDA soybean oil contains approximately 20% stearidonic, 24% linoleic, and 12% palmitic acids.[11] Palmitic acid (PA) is the second major FA found in breast milk at approximately 18.3−25.9%.[12−14] SDA soybean oil contains lower amounts of PA than breast milk. Human breast milk contains a large portion of PA esterified at the *sn*-2 position of the triacylglycerols (TAGs). Over 60% (by weight) of PA is esterified at the *sn*-2 position of human milk

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fat, and mainly unsaturated FAs are found at *sn*-1,3 positions.[15−17] As TAGs are metabolized, the FAs esterified at the *sn*-1, 3 positions are released by pancreatic lipase during the digestion of fat molecules. The FAs esterified at the *sn*-2 position remain unhydrolyzed and are absorbed by the intestinal mucosa as *sn*-2 monoacylglycerols.[18] The large amount of PA esterified at the *sn*-2 position improves absorption of fat and calcium in an infant.[16,17,19,20] The PA in breast milk also reduces the formation and disposal of "calcium soaps" generated by the long chain saturated FA interaction with calcium.[15,20] In most infant formulas, the fat is of vegetable origin and contains mostly unsaturated fatty acids at the *sn*-2 position.[21]

Structured lipids (SLs) are TAGs that have been modified to change the FA composition and/or their position in the glycerol backbone by chemically and/or enzymatically catalyzed reactions. Currently, there have been no other studies on modifying SDA soybean oil for potential application in infant formulas. Previous studies have been published on the development of human milk fat analogues. These studies have used interesterification of randomized oil mixtures containing amaranth oil and ethyl palmitate,[22] hazelnut oil fatty acids and tripalmitin,[23] lard,[24] and butter oil[25] to produce SLs that resemble human milk fat. Betapol (Loders Croklaan, Glen Ellyn, IL, USA) was the first to commercially produce a human milk fat analogue by using a 1, 3-specific lipase to synthesize SLs by reacting tripalmitin with unsaturated FAs.[21]

SDA soybean oil may be used as an alternative substrate to produce SLs for use as human milk fat analogues in infant formulas. The objective of this study was to increase the PA content at the *sn*-2 position of SDA soybean oil to over 60% (using tripalmitin as the substrate) while obtaining a fair amount of total SDA by enzymatic interesterification. Two types of enzymes were evaluated: Lipozyme TL IM (*Thermomyces lanuginosus* immobilized on silica gel, *sn*-1,3 specific lipase, specific activity 250 IUN/g) and Novozym 435 (*Candida antarctica* immobilized on macroporous acrylic resin beads, nonspecific lipase, specific activity 10,000 PLU/g). IUN is the interesterification units novo, and PLU is the propyl laurate units. It is critical to find the optimization conditions to produce a SL from SDA soybean oil enriched with PA at the *sn*-2 position for use as a human milk fat analogue in infant formula because no such work has been done so far. The health benefits to the infant from having PA at the *sn*-2 position while containing SDA omega-3 FA are substantial. Response surface methodology (RSM) enables the evaluation of the effects that multiple parameters have on response variables[26] and limits the number of reactions to be performed. RSM was applied to determine the optimization conditions for the production of SLs with PA at the *sn*-2 position and also containing SDA by varying substrate mole ratios, time and temperature.

■ MATERIALS AND METHODS

Materials. SDA soybean oil was kindly provided by Monsanto Company (St. Louis, MO). Tripalmitin was purchased from TCI America (Portland, OR). Immobilized lipases, Novozym 435 (nonspecific lipase) and Lipozyme TL IM (*sn*-1,3 specific lipase), were obtained from Novozymes North America Inc. (Franklinton, NC). The lipid standards Supelco 37 Component FAME mix, triolein and 2-oleoylglycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), and C19:0-nonadecanoic acid was purchased from TCI America (Portland, OR). Other solvents and chemicals were purchased from Fisher Scientific (Norcross, GA) and Sigma-Aldrich Chemical Co. (St. Louis, MO).

Experimental Design by Response Surface Methodology (RSM). A RSM mathematical model (Modde 5.0, Umetrics, Umeå, Sweden) was used to predict the incorporation of PA into SDA soybean oil by enzymatic interesterification with Novozym 435 and Lipozyme TL IM lipases. The substrate mixtures of SDA soybean oil and tripalmitin were determined on the basis of their average molecular weights. Table 1 shows the resulting suggested combinations from the experimental design performed with both Novozym 435 and Lipozyme TL IM. The experimental design considered three factors: the time of the reaction (low level, 6 h; high level, 18 h), temperature of the reaction (low level, 50 °C; high level, 65 °C), and the substrate mole ratio of SDA soybean oil to tripalmitin (low level, 1:2; high level, 1:4) using Novozym 435 and Lipozyme TL IM at 10% by weight of the substrates. The central composite face design consisted of sixteen different combinations resulting in a total of eighteen experiments. Experiments were performed in triplicate resulting in a total of fifty-four total reactions. All reactions were performed in a water bath at the correct temperature with constant shaking at 200 rpm. Once the reaction was complete, the enzyme was removed by passing the SL products through an anhydrous sodium sulfate column. The resulting TAGs were recovered after TLC separation and analyzed for their fatty acid profile and positional analysis. The total amount of SDA in the SLs and the incorporation of PA at the *sn*-2 position of the glycerol backbone were recorded in Table 1 as variable responses.

RSM Mathematical Model. The 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) from Modde 5.0 (Umetrics, Umeå, Sweden). The relationships between the factors and variables from the above design were fitted into a second-order polynomial equation:

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

where Y is the dependent variable (response 1, total amount of SDA; response 2, PA incorporation at *sn*-2 position), β_0 is the intercept, β_i linear, β_{ii} quadratic, β_{ij} interaction term coefficients, and X_i and X_j are independent variables.

Enzymatic Interesterification. One hundred milligrams of each mixture (in ratios of 1:2, 1:3, and 1:4 mol SDA/mol tripalmitin) was weighed into labeled Teflon-lined test tubes, and 3 mL of *n*-hexane was added. Ten percent by weight of the enzyme (based on total substrate weight), either Novozym 435 or Lipozyme TL IM, was added to the test tube as the biocatalyst. The reaction was carried out at the temperatures indicated from the RSM conditions (50, 55, 60, or 65 °C) for the times indicated (6, 12, or 18 h) in a water bath while shaking at 200 rpm. After the reaction, the products were filtered two times through a sodium sulfate column to remove water and the biocatalyst.

Recovery of Triacylglycerols (TAGs). Silica gel G TLC plates were activated in an oven at 100 °C for 1 h to remove excess water. A mixture of petroleum ether, diethyl ether, and acetic acid (90:10:0.5, v/v/v) was equilibrated for approximately 30 min and used as the mobile phase to separate the TAGs. Following enzymatic interesterification, the products were spotted on the dried and cooled TLC plates, and placed into the TLC tanks. The lipid bands were visualized under UV light after spraying with 0.2% 2,7-dicholorofluorescein in methanol. The TAG bands were identified using triolein as the standard. The TAG bands were scraped off into test tubes for fatty acid methyl ester (FAME) analysis and positional analysis. The recovery of TAGs was performed to isolate the TAG molecules and remove free FAs from the product mixture.

Table 1. Total Incorporation of SDA (C18:4n-3) and Palmitic Acid (C16:0) at the *sn*-2 Position of Structured Lipids Produced by Enzymatic Interesterification Using RSM Conditions[a]

			Novozym 435		Lipozyme TL IM	
time (h)	temp (°C)	mole ratio[b]	total[c] (mol % SDA)	*sn*-2[d] (mol % PA)	total[c] (mol % SDA)	*sn*-2[d] (mol % PA)
6	50	1:2	4.15 ± 1.98	64.55 ± 13.73	5.37 ± 3.42	64.84 ± 3.66
18	50	1:2	9.05 ± 4.61	65.51 ± 9.61	6.78 ± 4.39	65.38 ± 9.05
12	60	1:2	4.23 ± 3.01	25.19 ± 7.26	3.55 ± 0.61	55.61 ± 1.96
6	65	1:2	1.94 ± 0.77	63.33 ± 15.10	1.90 ± 0.92	62.48 ± 6.61
18	65	1:2	2.17 ± 0.54	71.32 ± 4.03	2.82 ± 0.81	70.42 ± 1.82
12	50	1:3	7.32 ± 2.53	65.13 ± 4.03	3.32 ± 3.08	79.92 ± 11.60
18	55	1:3	2.10 ± 1.66	39.78 ± 7.01	4.02 ± 2.71	62.87 ± 13.08
6	60	1:3	2.11 ± 0.93	41.31 ± 0.58	1.24 ± 0.69	54.10 ± 6.33
18	65	1:3	1.53 ± 1.46	62.56 ± 13.21	2.82 ± 0.07	75.50 ± 2.58
6	50	1:4	4.75 ± 3.62	74.01 ± 15.42	3.05 ± 0.74	91.82 ± 2.53
18	50	1:4	7.27 ± 2.47	86.34 ± 2.47	6.14 ± 1.73	82.66 ± 8.19
6	55	1:4	3.28 ± 1.84	42.61 ± 8.12	3.29 ± 1.78	55.96 ± 13.68
18	60	1:4	3.48 ± 2.65	42.34 ± 23.31	2.55 ± 1.78	56.04 ± 7.41
6	65	1:4	0.86 ± 0.28	81.76 ± 3.38	1.51 ± 0.41	78.35 ± 3.05
12	65	1:4	1.13 ± 0.23	72.74 ± 13.21	1.70 ± 0.70	78.34 ± 4.43
18	65	1:4	2.41 ± 2.47	72.99 ± 8.34	2.63 ± 1.25	78.56 ± 3.79
18	65	1:4	3.33 ± 1.32	77.04 ± 10.56	4.84 ± 0.09	74.57 ± 16.21
18	65	1:4	2.96 ± 0.49	56.08 ± 28.50	3.72 ± 0.17	79.63 ± 0.72

[a] All experiments were performed in triplicate, and average values ± SD were reported. [b] Substrate mole ratio of SDA soybean oil (MW = 902.18 g/mol) to tripalmitin (MW = 807.32 g/mol). [c] Total content of SDA in the structured lipid. [d] Incorporated palmitic acid at the *sn*-2 position of the structured lipid.

Determination of Fatty Acid Profiles. SDA soybean oil and SL samples were converted to FAME following the AOAC Official Method 996.01, Section E,[27] with minor modifications.[22] For analysis of the SDA soybean oil, 150 mg of the oil was weighed into a Teflon-lined test tube, 100 μL of the internal standard, C19:0 in hexane (20 mg/mL), was added, and the mixture was dried under nitrogen to remove solvent. For analysis of the SL, 50 μL of the internal standard was added to the recovered TAG band (as described above). Two milliliters of 0.5 N NaOH in methanol was added, and the mixture was incubated at 100 °C for 5 min for saponification. The samples were cooled under tap water, and 2 mL of 14% BF_3 in methanol was added followed by vortexing for 1 min. Again, the sample was incubated at 100 °C for 5 min for methylation and then cooled under tap water. To stop the reaction and extract the FAMEs, 2 mL of hexane and 2 mL of saturated NaCl solution were added. The sample was vortexed for 2 min and then centrifuged at 1000 rpm for 5 min to separate the organic layer from the aqueous layer. The upper organic layer was removed and recovered in a GC vial for analysis. The FAME external standard used was the Supelco 37 component FAME mix and was run parallel with the samples.

Positional Analysis. The recovered TAGs were extracted from the silica gel with 2 mL of diethyl ether, vortexing, centrifuging at 1000 rpm for 3 min, and filtration through an anhydrous sodium sulfate column. This extraction step was repeated. The SL was completely dried under nitrogen gas. A modified version of the Luddy et al.[28] method was used to perform the pancreatic lipase-catalyzed *sn*-2 positional analysis. One hundred milligrams of SDA soybean oil and the SLs (extracted and dried TAG) were placed into Teflon-lined test tubes. Two milliliters of 1.0 M Tris-HCl buffer (pH = 8), 0.5 mL of 0.05% sodium cholate solution, and 2.2% calcium chloride solution were added, and the mixture was vortexed for 2 min to emulsify. Then 40 mg of pancreatic lipase was added, and the mixture was vortexed for 1 min and incubated in a water bath at 40 °C for 3 min while shaking at 200 rpm. The samples were vortexed again for 2 min. To stop the reaction and extract the hydrolyzed TAGs, 1 mL of 6 N HCl and 4 mL of diethyl ether were added. The samples were vortexed for 2 min and centrifuged at 1000 rpm for 3 min. The upper layer, containing the lipid components, was filtered twice through an anhydrous sodium sulfate column. The samples were concentrated under nitrogen until approximately one-third of the original volume was left. A mixture of hexane, diethyl ether, and formic acid (60:40:1.6, v/v/v) was used as the mobile phase after equilibration in the TLC tanks for approximately 30 min. The concentrated sample (~50 μL) was spotted onto the activated silica gel G dried TLC plates and placed into the tank. 2-Oleylglycerol was spotted as the standard and run parallel with the samples for identification of the 2-monoacylglycerol (2-MAG) band. The plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The 2-MAG band was scraped off and converted to FAME (as described above). One hundred microliters of the internal standard (20 mg/mL C19:0 in hexane) was used for the SDA soybean oil and 50 μL of the internal standard for the SL. The fatty acid content at the *sn*-2 position was quantified by GC, and the fatty acid content at the *sn*-1,3 positions was calculated.

GC Analysis. The FAMEs (from the SDA soybean oil, SL, and corresponding positional analyses) were analyzed using an Agilent Technology 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) with a flame ionization detector. A Supelco SP-2560 column, 100 m × 250 μm, 0.20 μm film was used to attain separation. Injection of 1 μL of sample was made at a split ratio of 20:1. Helium was the carrier gas at a flow rate of 1.1 mL/min and at a constant pressure. The injector temperature and the FID set point were 300 °C. The oven was held at 140 °C for 5 min, then increased up to 240 at 4 °C/min and held at 240 °C for 15 min. The relative FAME content was calculated using the online computer. The average and standard deviation of triplicate analyses were reported.

Statistical Analysis. All samples, reactions, and analyses were done in triplicate for SDA soybean oil and SLs. The average and standard

deviations were calculated and reported for all analyses. The analysis of variance (ANOVA) and the mathematical model for optimization were attained using MODDE 5.0 (Umetrics, Umeå, Sweden).

Verification of Model. Enzymatic interesterification reactions were carried out in test tubes at random conditions obtained with RSM to verify the model. The experimental values were then compared to the predicted values for the model, as shown in Table 3.

RESULTS AND DISCUSSION

The SDA soybean oil FA profile is shown in Table 2. The results from the FA profile were used to estimate the molecular weight of SDA soybean oil (MW = 902.18 ± 0.16 g/mol). The major FA in the SDA soybean oil was linoleic (25.78 ± 0.07%), followed by octadecatetraenoic or SDA (22.16 ± 0.23%), oleic (14.31 ± 0.17%), and PA (11.54 ± 0.04%). Linoleic acid was the major FA at the *sn*-2 position, whereas stearidonic acid was the major FA at the *sn*-1,3 positions. Linoleic acid content in breast milk is approximately 15.6%[13] which is considerably lower than the linoleic acid content in the SDA soybean oil. PA constitutes the majority of saturated FAs in human breast milk at over 60% by weight of its total content at the *sn*-2 position. This large amount of PA allows for reduced formation of calcium soaps and providing readily absorbed energy for the development and growth of the infant. It also improves fat and calcium absorption.[15−17] However, SDA soybean oil contains only a small amount of PA at the *sn*-2 position, only 4.77 ± 4.39%. SDA soybean oil itself would not be adequate for use in infant formula as a human milk fat analogue. Esterification techniques, chemical and enzymatic, are often used to produce SLs with improved functionality by incorporating new FAs into the oil or by rearranging the FAs already in the oil.[29] Enzymatic interesterification is more spatially selective producing more specific TAGs.[30] The aim of this research was to modify the SDA soybean oil's TAG to increase the PA esterified at the *sn*-2 position to meet the recommended FA requirements for human milk fat analogues.[31] Two different enzymes, Novozym 435 (nonspecific) and Lipozyme TL IM (*sn*-1,3 specific), were used for the interesterification to see if there were any differences in the incorporations of PA and SDA in the SL molecules. The same experimental conditions and substrate levels were employed for both enzymes. An increased PA content at the *sn*-2 position resulted in a lower total SDA content of the SLs, especially when the substrate mole ratio was increased (Table 1). Both enzymes provided an increase in PA content at the *sn*-2 position and a decrease in the total SDA content. The *sn*-1,3 specific lipase (Lipozyme TL IM) gave slightly higher incorporation of palmitic acid at the *sn*-2 position of SLs because SDA soybean oil contained more SDA (25.26 ± 1.40%) at the *sn*-1,3 position which were readily cleaved by Lipozyme TL IM. As previously mentioned, higher PA content at the *sn*-2 position results in the reduced formation of calcium soaps and better absorption of fats and calcium by the infant. Therefore, either of the lipases can be used to produce suitable SLs as human milk fat analogues.

The experimental design was developed using response surface methodology (RSM) to produce a model to predict PA content at the *sn*-2 position and total amount of SDA. The factors in the experimental design included time, temperature, and substrate mole ratio. The responses or resulting amounts of total SDA content and PA content at the *sn*-2 position are shown in Table 1. For incorporation of PA at the *sn*-2 position, multiple linear regression and backward selection method were used to fit the results into a second-order polynomial model, where the only significant terms were *mol* and *temp**temp* for both enzymes. The predicted values by the model versus observed experimental values are given in Figure 1 for both enzymes. The multiple correlation coefficient or R^2 was 0.906 and 0.918 for Novozym 435 and Lipozyme TL IM, respectively. This indicates that there is a linear relationship between the predicted values and the observed values at a confidence level of 95%. The Q^2 value was 0.346 and 0.525 for Novozym 435 and Lipozyme TL IM, respectively. The R^2 value is often used to assess the variance explanation. However, in planned experimentation, it is more significant to support conclusions based on analysis of variance (ANOVA) statistics.[26] The most significant first-order parameter was substrate mole ratio for PA incorporation at the *sn*-2 position. The most significant second-order parameter was temperature × temperature. Both the first-order and second-order parameters had positive effects. However, total content of SDA was less significant with R^2 values of 0.841 and 0.840 and Q^2 values of −0.392 and 0.357 for Novozym 435 and Lipozyme TL IM, respectively. Total SDA content only showed significance in a linear distribution resulting in no significant second-order parameter. The significant first-order parameters were time having a positive effect and temperature having a negative effect. However, the models for total SDA or *sn*-2 PA incorporation showed no significance for lack of fit. Statistically, the models showed no lack of fit ($P > 0.05$).

Table 2. Composition of Stearidonic Acid (SDA) Soybean Oil[a]

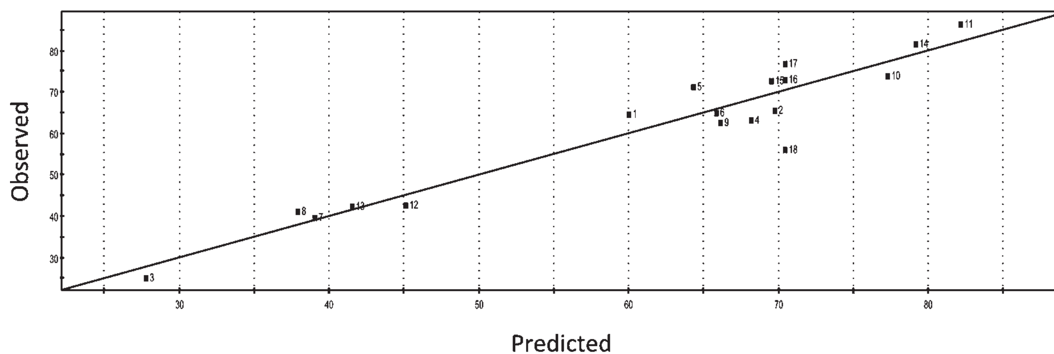
fatty acid	total (mol %)[b]	positional distribution: *sn*-2 (mol %)	positional distribution: *sn*-1,3[c] (mol %)
C16:0	11.54 ± 0.04	4.77 ± 4.39	14.92 ± 2.20
C18:0	3.80 ± 0.06	5.04 ± 4.30	4.05 ± 0.60
C18:1n-9c	14.31 ± 0.17	20.06 ± 1.19	11.43 ± 0.59
C18:2n-6t	1.44 ± 0.01	nd[d]	2.16 ± 0.00
C18:2n-6c	25.78 ± 0.07	34.11 ± 4.56	21.61 ± 2.28
C20:0	0.33 ± 0.01	nd	0.50 ± 0.00
C18:3n-6	7.65 ± 0.04	6.57 ± 3.29	8.18 ± 1.64
C18:3n-3	12.30 ± 0.06	5.74 ± 2.95	15.58 ± 1.47
C18:4n-3	22.16 ± 0.23	15.97 ± 2.80	25.26 ± 1.40
C20:1	0.39 ± 0.01	nd	0.59 ± 0.00
C22:0	0.30 ± 0.01	nd	0.46 ± 0.00

[a] Trace amounts of C15:0, C16:1, C17:0, and C24:0 were found in *sn*-2 analysis but were too small to be detected in total FA analysis. [b] Mean ± SD, $n = 3$. [c] *sn*-1,3 (mol %) = [3 × total (mol %) − *sn*-2 (mol %)]/2. [d] Not detected.

The model equation for the response of PA incorporation at the *sn*-2 position can therefore be written as follows: PA at *sn*-2 = $27.99 + 5.85S_r + 36.91T^2$ for Novozym 435, and PA at *sn*-2 = $58.21 + 6.71S_r + 22.72T^2$ for Lipozyme TL IM. For total SDA content, the model equations are as follows: total SDA = $3.25 + 0.909t - 2.19T$ Novozym 435 and total SDA = $2.02 + 0.84t - 1.35T$ for Lipozyme TL IM. T indicates the reaction temperature, S_r indicates the substrate mole ratio, and t indicates the reaction time. Total SDA is the total amount of SDA in the SL, and PA at *sn*-2 is the content of PA incorporated at the *sn*-2 position of the SLs.

The relationship between responses and parameters was examined using contour plots for *sn*-2 PA incorporation and SDA

A. Novozym 435



B. Lipozyme TL IM

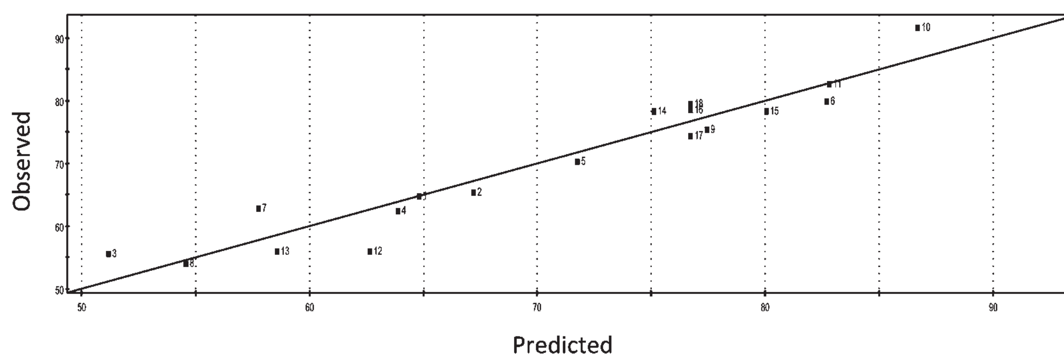


Figure 1. Relationship between observed and predicted data by the models: Incorporation of palmitic acid at the *sn*-2 position with (A) Novozym 435 and (B) Lipozyme TL IM as biocatalysts.

incorporation. The contour plots obtained by the interaction of the three parameters on the *sn*-2 PA incorporation catalyzed by either Novozym 435 or Lipozyme TL IM are shown in Figure 2. The third variable was kept at a constant when these contour plots were drawn. The contour plots drawn were for the interaction of reaction time (h) with reaction temperature (°C) when the substrate mole ratio was 1:2 (mol of SDA soybean oil/mol of tripalmitin), reaction time with substrate mole ratio at a reaction temperature of 65 °C, and reaction temperature with substrate mole ratio at a reaction time of 18 h as shown in panels A, B, and C, respectively. As shown in Figure 2A, the highest and the lowest temperatures (65 and 50 °C) gave the highest incorporation (over 60%) of PA at the *sn*-2 position for both enzymes. Temperatures below 64 °C and above 50.5 °C gave a PA content of less than 60%. Lipozyme TL IM at a 1:2 substrate mole ratio gave an overall higher incorporation of PA at the *sn*-2 position. An explanation for the Lipozyme TL IM giving a higher PA incorporation at *sn*-2 may in part be due to their regiospecificity. In Figure 2B, it can be seen that as the substrate mole ratio increases, the PA content at the *sn*-2 increases. This may be due to the substrate preference for PA by the enzymes and/or acyl migration from the 1- and 3-positions to the 2-position during the interesterification reaction. The value of lipases is related to their FA selectivity or their ability to discriminate between particular FAs or acyl moieties.[32] Different studies have shown different incorporation rates of individual FAs depending on the enzyme used and the conditions of the experiments. Different lipases show preferences for different FAs.[33] Peng et al.[34] studied the incorporations of EPA/DHA, conjugated linoleic acid (CLA), and caprylic acid. CLA and caprylic acid were found to have similar incorporation rates with the EPA/DHA mixtures having low incorporation. Lipozyme TL IM showed a slight discrimination for very long chain PUFAs in their study.[34] Another study showed that a mycelium-bound lipase demonstrated a high preference toward short chain triacylglycerols after a 20 h reaction. Also, the lipase hydrolyzed coconut oil faster than palm olein followed by rapeseed, soybean, and cottonseed oils, suggesting that the lipase has a preference for oils with saturated FAs rather than unsaturated FAs.[35] However, even at a substrate mole ratio of 1:2, the *sn*-2 PA content was still above 60%, making it possible for their use in infant formula. The Figure 2B contour plots also show a difference in the two enzymes. Novozym 435 appears to be slightly better at incorporating PA at the *sn*-2 position around 6 h, whereas Lipozyme TL IM appears to be better at 10−16 h. Again in Figure 2C, the higher *sn*-2 PA contents were at 50 and 65 °C with the higher content being achieved at a substrate mole ratio of 1:4. The higher the concentration of PA (or tripalmitin), the more likely it will re-esterify to the glycerol backbone when compared to the lower concentrations of other FAs. From the contour plots shown in Figure 2, the trend seems to be that the higher the substrate mole ratio (1:4), the more the PA will be present at the *sn*-2 position and 50 and 65 °C gave the highest incorporations. For total SDA incorporation at 65 °C, the SDA content increased with increasing time for Lipozyme TL IM in Figure 3A with a higher SDA content at 18 h. For Novozym 435, the SDA content was highest between 10.5 and 16.5 h (Figure 3A). It may be that Novozym 435 has better preference for SDA than Lipozyme TL IM since Lipozyme TL IM has been shown in previous studies to prefer shorter chain FAs over longer chain FAs.[33] Because it is a nonspecific lipase, all three positions are cleaved and re-esterified at a faster rate than the sequentially acting *sn*-1,3 Lipozyme TL IM lipase. Figure 3B demonstrates that, at a

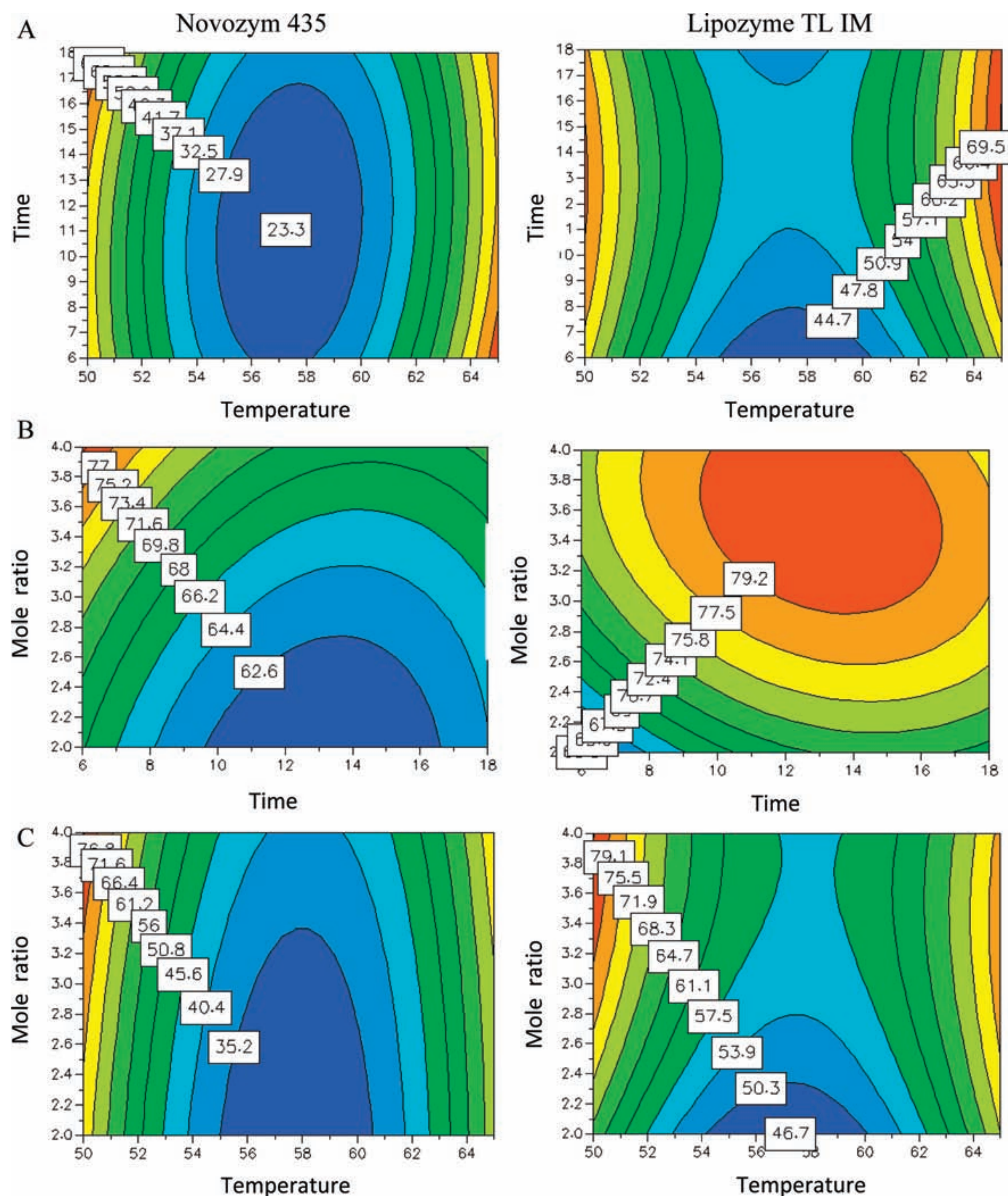


Figure 2. Contour plots between two parameters for *sn*-2 palmitic acid incorporation for Novozym 435 and Lipozyme TL IM lipases: (A) reaction time (h) versus reaction temperature (°C) when the substrate mole ratio was 1:2 (mol of SDA soybean oil/mol of tripalmitin), (B) reaction time versus substrate mole ratio at a reaction temperature of 65 °C, and (C) reaction temperature versus substrate mole ratio at a reaction time of 18 h.

substrate mole ratio of 1:2, the longer the reaction time, the better the incorporation of total SDA in the SL molecule, and this occurred at a lower temperature. Figure 3C also demonstrates that a lower temperature resulted in more incorporation of SDA, and this occurred at a lower substrate mole ratio (1:2). At a substrate mole ratio of 1:4, more PA was incorporated at *sn*-2 and less total SDA was obtained. With a higher amount of tripalmitin (PA), there was substrate competition between PA and SDA for the active site of the lipases at higher substrate mole ratios.

Verifications of the models were performed by performing enzymatic interesterification reactions at various conditions with RSM. Table 3 shows the predicted values and conditions used for the verifications. Verifications for the Novozym 435 model fell between the upper and lower limits of the predicted values of total SDA and PA at *sn*-2 position with conditions of 1:3 substrate mole ratio at 56 °C for 13 h, 1:4 substrate mole ratio at 63 °C for 18 h, 1:2.5 substrate mole ratio at 59 °C for 9 h. However, the Lipozyme TL IM model verifications did not always fall within the upper and lower limits. The values of total SDA and PA at *sn*-2 position with conditions of 1:3.5 substrate mole ratio at 59 °C for 10 h did fall within the upper and lower limits. Conditions of 1:2 substrate mole ratio at 51 °C for 8 h fell within the limits for total SDA but not for PA

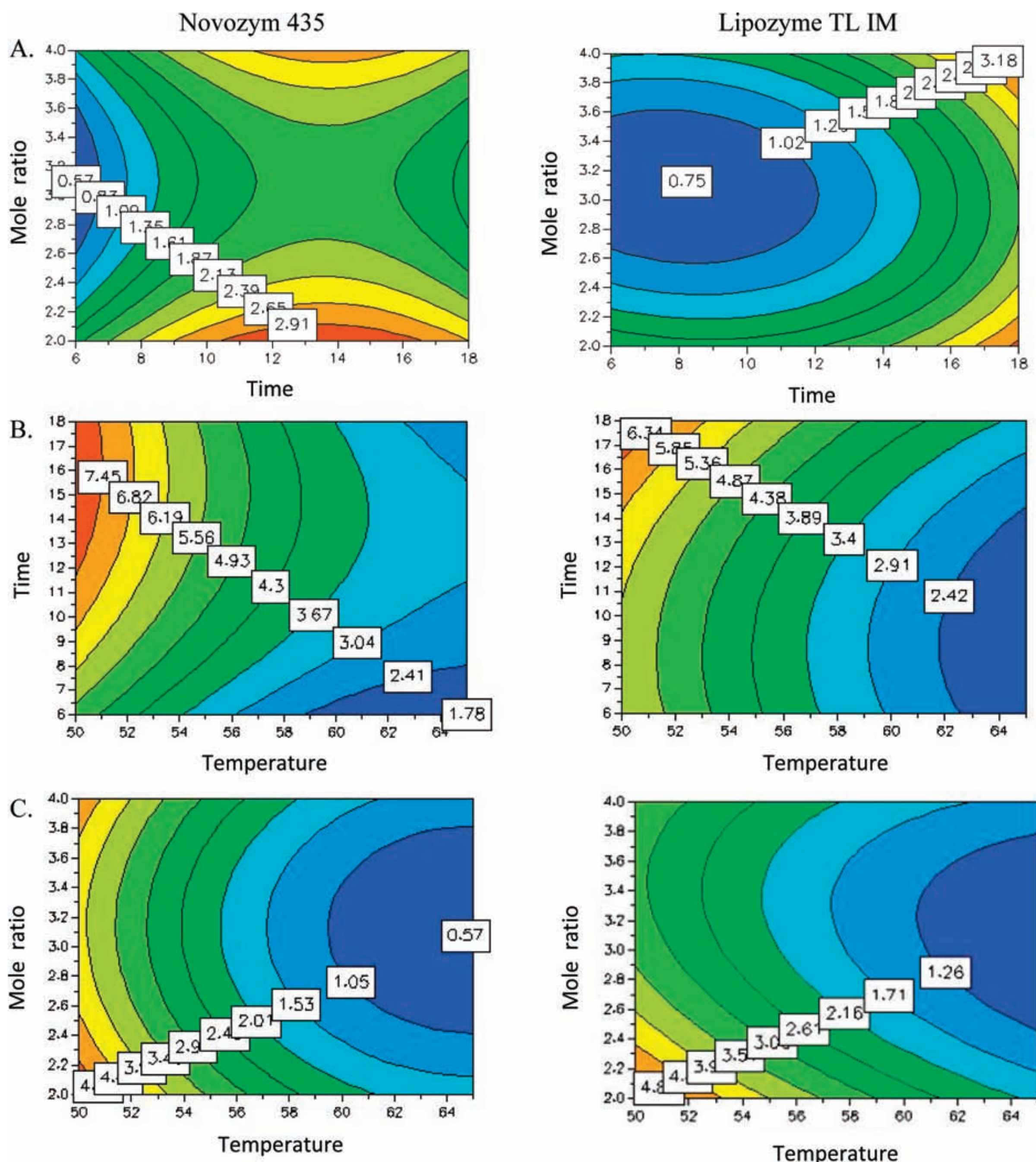


Figure 3. Contour plots of total SDA content for (A) substrate mole ratio versus reaction time at a reaction temperature of 65 °C for Novozym 435 and Lipozyme TL IM lipases, (B) reaction time versus reaction temperature at 1:2 substrate mole ratio, and (C) substrate mole ratio versus reaction temperature at a reaction time of 6 h.

at the *sn*-2 position (slightly above the upper limit), and conditions of 1:3 substrate mole ratio at 64 °C for 17 h fell within the limits for PA at the *sn*-2 position but not for total SDA incorporation (slightly above the upper limit). When taking into account the standard deviation for the 1:2 substrate mole ratio at 51 °C for 8 h for Lipozyme TL IM, the PA at *sn*-2 will fall between the upper and lower limits. The models may be used as a preliminary experimental tool to estimate the approximate optimal conditions and values of total SDA and PA at the *sn*-2 position. However, the models may result in slight error, as seen in the verification of the models, but this should not negate the usefulness of RSM predictions.

The optimal conditions for the targeted total SDA (>5%) and *sn*-2 PA incorporation (>60%) were generated by the optimizer function of the Modde 5.0 (Umetrics, Umeå, Sweden) software. These conditions were determined to be a substrate mole ratio of 1:2 at 50 °C for 18 h with 10% (by weight) Lipozyme TL IM resulting in 6.82 ± 1.87% total SDA and 67.19 ± 9.59% PA at *sn*-2, and a 1:2 substrate mole ratio at 50 °C for 15.6 h resulting in 8.01 ± 2.41% total SDA and 64.43 ± 13.69% PA at *sn*-2 with 10% (by weight) Novozym 435. These optimization conditions are used as a tool to give an estimate of the incorporation that may be obtained in SL products after interesterification. As seen in

Table 3. Predicted and Observed (mol %) from RSM Model Verification

	conditions			predicted				predicted			
enzyme	time (h)	temp (°C)	mole ratio[a]	SDA[b]	LL[c]	UL[d]	obsd SDA[b]	PA[e]	LL[c]	UL[d]	obsd PA[e]
Novozym 435	18	63	1:4	2.64	1.12	4.16	3.33 ± 1.32	54.90	46.27	63.53	56.08 ± 28.50
Novozym 435	13	56	1:3	3.86	1.57	6.15	2.19 ± 1.67	29.94	16.95	42.94	27.32 ± 11.92
Novozym 435	9	59	1:2.5	2.57	0.55	4.59	4.01 ± 0.53	28.05	16.58	39.52	38.87 ± 11.44
Lipozyme TL IM	8	51	1:2	4.96	3.38	6.55	4.30 ± 3.25	61.82	53.70	69.95	70.02 ± 5.65
Lipozyme TL IM	17	64	1:3	2.16	0.90	3.42	3.72 ± 0.17	72.72	66.28	79.17	74.57 ± 16.21
Lipozyme TL IM	10	59	1:3.5	1.60	0.15	3.04	2.79 ± 0.45	60.44	53.04	67.84	60.80 ± 10.76

[a] Substrate mole ratio of SDA soybean oil to tripalmitin. [b] Total SDA content in TAG (mol %). [c] Lower limit (mol %). [d] Upper limit (mol %). [e] Palmitic acid at *sn*-2 position (mol %).

Table 4. Fatty Acid Profiles (mol %) of a Physical Mixture and the Two SLs at Optimal Conditions[a]

	physical blend[b] (mol %)			Novozym 435 SL (mol %)			Lipozyme TL IM SL (mol %)		
FA	total	*sn*-2	*sn*-1,3[c]	total	*sn*-2	*sn*-1,3[c]	total	*sn*-2	*sn*-1,3[c]
C14:0	1.26 ± 0.02	1.95 ± 0.04	0.91 ± 0.04	0.85 ± 0.74	1.67 ± 0.37	0.72 ± 0.66	1.14 ± 0.01	1.60 ± 0.63	0.92 ± 0.31
C16:0	64.63 ± 1.86	47.05 ± 4.25	73.42 ± 0.70	71.89 ± 1.18	59.25 ± 4.14	78.21 ± 2.64	63.00 ± 1.28	62.09 ± 1.81	63.45 ± 2.44
C18:0	3.14 ± 0.07	9.59 ± 1.10	0.00 ± 0.59	3.60 ± 0.72	8.83 ± 1.03	0.98 ± 1.48	3.04 ± 0.14	6.78 ± 0.61	1.18 ± 0.12
C18:1n-9	6.90 ± 0.20	10.23 ± 1.36	5.23 ± 0.63	6.62 ± 0.65	5.78 ± 1.05	7.04 ± 1.06	6.14 ± 0.58	5.73 ± 1.36	6.34 ± 0.47
C18:2n-6	14.91 ± 0.77	20.56 ± 1.22	12.08 ± 0.96	11.92 ± 1.09	16.66 ± 1.18	9.55 ± 1.77	15.28 ± 0.50	15.05 ± 2.12	15.40 ± 0.31
C18:3n-6	2.13 ± 0.19	3.16 ± 0.24	1.62 ± 0.21	0.91 ± 0.79	3.83 ± 1.09	0.26 ± 0.41	2.54 ± 0.17	4.06 ± 1.92	1.78 ± 1.08
C20:1	3.06 ± 0.26	1.17 ± 0.15	4.01 ± 0.33	1.87 ± 0.22	0.37 ± 0.65	2.63 ± 0.58	3.44 ± 0.29	1.31 ± 0.25	4.51 ± 0.56
C18:4n-3	3.83 ± 0.43	2.56 ± 0.42	4.47 ± 0.46	2.32 ± 0.58	3.59 ± 1.65	1.69 ± 0.16	5.41 ± 0.98	3.39 ± 0.78	6.42 ± 1.86
C18:3n-3	0.14 ± 0.02	2.07 ± 1.47	0.00 ± 0.75	nd[d]	nd	nd	nd	nd	nd

[a] Trace amounts of C14:1 were also found in the *sn*-2 analysis of the physical blend but were too small to be detected in the total FA analysis. Optimal conditions for Novozym 435 SL were 1:2 substrate mole ratio, 50 °C for 15.6 h and, for Lipozyme TL IM, 1:2 substrate mole ratio, 50 °C for 18 h. [b] Physical blend of 1 mol of SDA soybean oil with 2 mol of tripalmitin with no enzyme reacted at 50 °C for 18 h. [c] *sn*-1,3 (mol %) = [3 × total (mol %) − *sn*-2 (mol %)]/2. [d] Not detected.

Table 1, higher amounts of PA at the *sn*-2 are possible, but this increase could result in a lower amount of SDA incorporation. The SL products of the two lipases at optimal conditions were produced along with a physical blend, containing no enzyme as a biocatalyst, with a substrate mole ratio of 1:2 at 50 °C for 18 h. The FA profiles of these three products are shown in Table 4. The differences in FA composition can be seen in Table 1 and Table 4. The high standard deviation in some of the experimental runs is not unusual in biocatalysis. It may be due to the enzyme specificity, substrate mole ratio, reaction temperature, and reaction time. A slight error in the observed value in Table 4 can be seen for total SDA incorporation for the Novozym 435 SL. As stated earlier, Novozym 435 (*Candida antarctica* immobilized on macroporous acrylic resin beads) is a nonspecific lipase with a specific activity of 250 IUN/g and optimum reaction temperatures of 40−60 °C. Lipozyme TL IM (*Thermomyces lanuginosus* immobilized on silica gel) is an *sn*-1,3 specific lipase with a specific activity of 10,000 PLU/g. However, Lipozyme TL IM may also be nonspecific depending on the substrates. These enzymes can be used to improve the nutritional properties of lipids. A specific lipase allows for the incorporation of an acyl group into a specific position on the TAG molecule. An *sn*-1,3 specific lipase (Lipozyme TL IM) gives specificity and selectively during esterification at the *sn*-1 and *sn*-3 positions. As seen in Table 4, the physical blend of SDA soybean oil with tripalmitin gave a lower PA content at the *sn*-2 position of only 47.05 ± 4.24%. This percentage of PA is below the over 60% of PA found at the *sn*-2 position in breast milk and would not be adequate for use as a human milk fat analogue. Metabolically, a physical mixture is not equivalent to a SL. However, using a biocatalyst can increase the amount of PA at the *sn*-2 position and hence their subsequent absorption as 2-MAG. For Lipozyme TL IM, it can increase the total SDA content and the SDA content found at the *sn*-1,3 positions due to its regiospecificity. A possible explanation for the Lipozyme TL IM SL having a higher PA incorporation at *sn*-2 and higher total SDA incorporation may be due to acyl migration and possibly the longer reaction time when compared to the Novozym 435 SL. The addition of a biocatalyst and the incorporation of PA decreased the linoleic acid content (Novozym 435, 11.92 ± 1.09%; Lipozyme TL IM, 15.28 ± 0.50%) when compared to the original SDA soybean oil (25.78 ± 0.07%), in Table 2, making it similar to breast milk, which contains approximately 15.6% linoleic acid.[13] This can be explained by PA replacing linoleic acid at the *sn*-2 position, since linoleic acid was the major FA found at the *sn*-2 position of the SDA soybean oil. Linoleic acid is one of the essential FAs, along with ALA, that the human body cannot synthesize. Both SLs contain undetectable amounts of ALA. However, SDA is a sustainable plant source of omega-3 FA that readily converts to EPA and DHA better than ALA.[4] EPA is a long chain omega-3 polyunsaturated FA that is essential for the growth and development of infants and has been linked to reductions in inflammation[7] and neurological disorders.[8] Having enough

SDA present in the SL may provide health benefits for the infant that normally would be provided from ALA.

Thus, human milk fat analogues (as SLs) containing SDA which is enriched with palmitic acid at the *sn*-2 position were successfully produced with the potential to deliver absorption characteristics and FA content similar to human milk fat with health benefits associated with omega-3 fatty acids. Using interesterification with either Novozym 435 or Lipozyme TL IM can produce a SL consisting of over 60% PA at the *sn*-2 position and containing over 6% of total SDA. Both enzymes may be used to produce a SL suitable for a human milk fat analogue. These SLs could be used as ingredients for infant formulas to help with nutrition, growth, and development.

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