JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Synthesis and Characterization of Canola Oil−Stearic Acid-Based Trans-Free Structured Lipids for Possible Margarine Application

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Incorporation of stearic acid into canola oil to produce trans-free structured lipid (SL) as a healthy alternative to partially hydrogenated fats for margarine formulation was investigated. Response surface methodology was used to study the effects of lipozyme RM IM from *Rhizomucor miehei* and *Candida rugosa* lipase isoform 1 (LIP1) and two acyl donors, stearic acid and ethyl stearate, on the incorporation. Lipozyme RM IM and ethyl stearate gave the best result. Gram quantities of SLs were synthesized using lipozyme RM IM, and the products were compared to SL made by chemical catalysis and fat from commercial margarines. After short-path distillation, the products were characterized by GC and RPHPLC-MS to obtain fatty acid and triacylglycerol profiles, ^{13}C NMR spectrometry for regiospecific analysis, X-ray diffraction for crystal forms, and DSC for melting profile. Stearic acid was incorporated into canola oil, mainly at the *sn*-1,3 positions, for the lipase reaction, and no new trans fatty acids formed. Most SL products did not have adequate solid fat content or β' crystal forms for tub margarine, although these may be suitable for light margarine formulation.

KEYWORDS: *Candida rugosa* lipase isoform 1; canola oil; interesterification; lipozyme RM IM; response surface methodology; sodium methoxide; stearic acid

INTRODUCTION

Concerns over significant consumption of trans fatty acids (TFA) has been a major health and policy issue since the past decade. Trans fatty acids occur naturally in small amounts in dairy products as a result of biohydrogenation of cis-unsaturated fatty acids by rumen microorganisms. The majority of TFA consumed, however, comes from products formulated with partially hydrogenated fats such as frying oils, margarines, spreads, shortenings, bakery products, and fast foods. The amount of TFA present in these foods is estimated to be between 0% and 35% of total fatty acids (*1*), and the mean daily intake of TFA per person in the United States population is 2.6% energy or 5.3 g (*2*).

Intake of high amounts of TFA has been correlated with increased risk of cardiovascular diseases, primarily due to their adverse effects on plasma lipid profile (*3*, *4*). These and other studies have heightened health concerns among consumers and regulatory agencies in Europe and the United States. As a result, the U.S. Food and Drug Administration (FDA) issued a final ruling requiring foods containing TFA to be labeled accordingly, effective from January 2006 (*5*). The food industry is responding to these concerns by developing processes that will produce foods with zero or reduced trans fat contents. These alternatives, among others, include the use of transesterification to make structured lipids (SL). Structured lipids are synthesized by incorporating high-melting fatty acids into oils or by blending high-melting fractions of natural oils or fully hydrogenated fats with liquid oils. This process (transesterification) therefore increases the solid fat content and oxidative stability of the

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10.1021/jf0710175 CCC: $37.00
Published on Web 12/05/2007

Table 1. Experimental Settings of Factors and Responses Used for Optimization of the Reaction by RSM

expt	enzyme	acyl donor	temp (°C)	substrate ratio	time (h)	Inc[a] (mol %)
N1	LIP1	stearic acid	35	1	14	0.01 ± 0.00
N2	LIP1	stearic acid	35	5	14	0.52 ± 0.91
N3	LIP1	stearic acid	35	3.7	6	0.58 ± 1.01
N4	LIP1	stearic acid	35	2.3	18	0.00 ± 0.01
N5	LIP1	stearic acid	65	1	10	0.01 ± 0.01
N6	LIP1	stearic acid	65	5	10	0.42 ± 0.73
N7	LIP1	stearic acid	65	2.3	6	0.00 ± 0.00
N8	LIP1	stearic acid	65	3.7	18	0.00 ± 0.00
N9	LIP1	stearic acid	45	1	6	0.00 ± 0.00
N10	LIP1	stearic acid	55	1	18	0.00 ± 0.00
N11	LIP1	stearic acid	45	5	6	0.00 ± 0.00
N12	LIP1	stearic acid	55	5	18	0.00 ± 0.00
N13	RM IM	stearic acid	35	1	6	22.68 ± 8.72
N14	RM IM	stearic acid	65	5	6	49.93 ± 3.70
N15	RM IM	stearic acid	65	1	18	23.45 ± 2.89
N16	RM IM	stearic acid	35	5	18	44.70 ± 1.16
N17	RM IM	stearic acid	50	3	12	41.37 ± 4.77
N18	LIP1	ethyl stearate	35	1	6	0.00 ± 0.00
N19	LIP1	ethyl stearate	65	5	6	23.39 ± 0.68
N20	LIP1	ethyl stearate	65	1	18	0.00 ± 0.00
N21	LIP1	ethyl stearate	35	5	18	7.92 ± 13.72
N22	RM IM	ethyl stearate	65	1	6	32.54 ± 0.92
N23	RM IM	ethyl stearate	35	5	6	48.80 ± 4.23
N24	RM IM	ethyl stearate	35	1	18	23.21 ± 1.17
N25	RM IM	ethyl stearate	65	5	18	59.10 ± 1.28
N26	RM IM	ethyl stearate	50	3	12	48.47 ± 2.44
N27	RM IM	ethyl stearate	50	3	12	52.80 ± 2.14
N28	RM IM	ethyl stearate	50	3	12	49.33 ± 2.62
N29	RM IM	ethyl stearate	50	3	12	52.29 ± 3.74

[a] Abbreviation: Inc, incorporation of stearic acid into canola oil.

product and prevents posthardening effects of margarine when stored (*6*). Moreover, this process does not result in the production of trans fatty acids, as is the case with partial hydrogenation (*1, 4*), and therefore has no adverse effects on plasma lipoprotein profile.

The suitability of fats for margarine formulation depends on their physical, crystal, and melting properties, which in turn is dependent on the amount and type of triacylglycerols (TAGs) and, to a lesser extent, the amount of diacylglycerols (DAGs) present. The aim of this study was to incorporate stearic acid into canola oil by the process of transesterification. The effects of lipase (lipozyme RM IM and *Candida rugosa* lipase isoform 1) and acyl donor (stearic acid and ethyl stearate) types, as well as temperature, substrate ratio, and time on the process, were investigated using response surface methodology (RSM). SLs produced by lipase- and sodium methoxide-catalyzed reactions were characterized for possible margarine formulation. Stearic acid was our choice of fatty acid because of its high melting point. Besides, it has no known adverse effects on plasma cholesterol levels (*7–10*). Canola oil contains α- and γ-linolenic acids which are important in reducing coronary heart disease (CHD) risk factors (*11, 12*). Our SL products were therefore expected to have the following positive health indicators: (1) low ratios of ω-6:ω-3 fatty acids, (2) reduced contents of atherogenic fatty acids, and (3) zero trans fatty acid contents.

MATERIALS AND METHODS

Materials. Stearic acid, sodium methoxide, and citric acid were purchased from Sigma Chemical Co. (St. Louis, MO). Canola oil (peroxide value 0.0, acid value 0.28) was bought from a local grocery store. Immobilized lipozyme RM IM was purchased from Novo Nordisk A/S (Bagsværd, Denmark), and unimmobilized LIP1 (genetically engineered and expressed in *Pichia pastoris*) was donated by Dr. Jei-Fu Shaw of the Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei, Taiwan. Organic solvents and chemicals were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA). All other chemicals used were of analytical or HPLC grade.

Experimental Design for RSM. The design consisted of five variables. These were enzyme (Enz), acyl donor (Acyl), temperature (Te), substrate molar ratio (Sr), and time (*t*). Enzyme and acyl donor had two factor levels while the others had five. The worksheet is shown in **Table 1**. For creating response surfaces, the data obtained based on the above design were fitted to a second-order polynomial equation of the form:

$$y = \beta_o + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1}^{5} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{ij} X_i X_j + \epsilon_{ij}$$

where y = percent incorporation of stearic acid, β_o = constant, β_i = linear term coefficients, β_{ii} = quadratic term coefficients, β_{ij} = interaction term coefficients, X_i and X_j = independent variables, and ϵ_{ij} = error term. Regression analyses, statistical significance, and response surfaces were done using MODDE 7.0 software (Umetrics, Umeå, Sweden) to obtain the relationship between the response and the independent variables.

RSM Study of Structured Lipid Synthesis. SL synthesis was performed in screw-cap test tubes incubated in an orbital shaking water bath at 200 rpm using the conditions specified in **Table 1**. The reaction mixture typically contained 100 mg of canola oil and milligrams of stearic acid or ethyl stearate corresponding to the mole ratios. The reactants were dissolved in 1.5 mL of hexane. The amount of enzyme used was 10% of the total weight of the substrates. After the reaction was stopped, 2 mL of hexane was added to the reaction products, and the enzymes were filtered off by passage through a column of anhydrous sodium sulfate. Fatty acid profiles of the products were determined as described below. All reactions were performed in triplicate and average values reported.

Determination of Stearic Acid Incorporation. About 50 μL of the reaction product was spotted onto silica gel G TLC plates. A mixture of petroleum ether, ethyl ether, and acetic acid was used to develop the plates [80:20:0.5 (v/v/v), combination for SL made with stearic acid, and 90:10:1 (v/v/v) for SL made with ethyl stearate]. Bands were visualized under UV light after spraying with 0.2% 2,7-dichlorofluo-

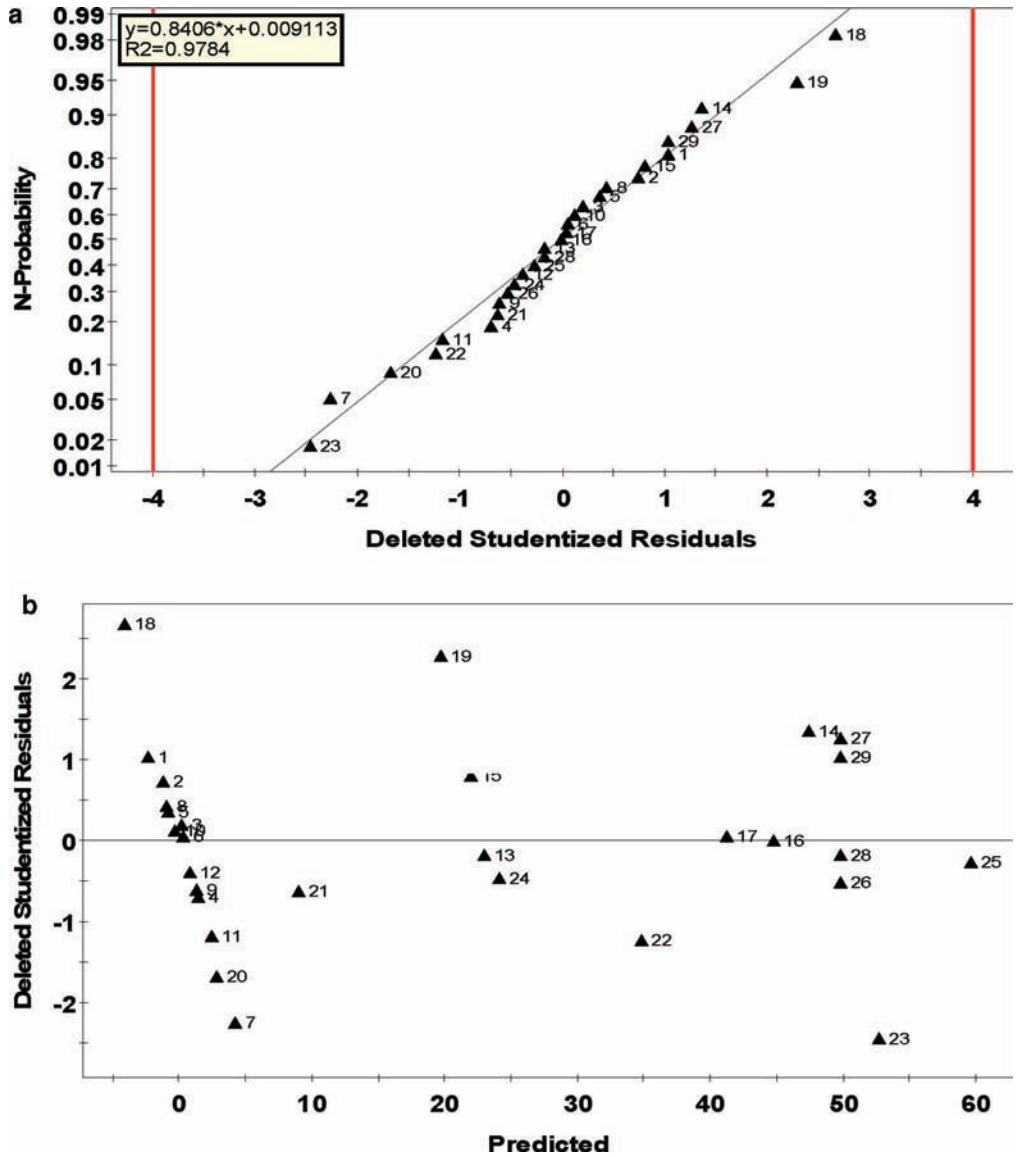

Figure 1. (**a**) Normal probability plot of residuals for incorporation of stearic acid. (**b**) Residual plot. Numbers inside both graphs represent experimental numbers. The linear (**a**) and random (**b**) distribution of the experimental numbers are indicative of a good model.

Table 2. ANOVA Table for Incorporation of Stearic Acid

Inc of stearic acid	DF[a]	SS	MS (variance)	*F*-value	*P*-value	SD
total	29	25654.9	884.653			
constant	1	11662.1	11662.1			
total corrected	28	13992.8	499.743			22.3549
regression	11	13867.5	1260.68	170.986	0.000	35.506
residual	17	125.341	7.37301			2.71533
lack of fit (model error)	14	111.57	7.9693	1.7361	0.360	2.823
pure error (replicate error)	3	13.771	4.59034			2.14251

$N = 29$; $Q^2 = 0.965$; $R^2_{adj} = 0.985$

DF = 17; $R^2 = 0.991$; RSD = 2.7153

[a] Abbreviations: DF, degree of freedom; SS, sum of squares; MS, mean square; RSD, relative standard deviation; SD, standard deviation; Q^2, R^2, and R^2_{adj}, explained in text.

rescein in methanol, and those corresponding to TAGs were scraped off and methylated in 3 mL of methanol (containing 6% HCl) at 75 °C for 2 h to form fatty acid methyl esters (FAME). The methyl esters were extracted twice with 2 mL of hexane and dried by passing through a column of anhydrous sodium sulfate. The methyl esters were separated on an AT-225, 30 m × 0.25 mm, 0.25 μm film column using an Agilent 6890N gas chromatograph equipped with a flame ionization detector (FID). Injection (1 μL) was performed in the split mode at a split ratio of 50:1. Helium was the carrier gas, the linear velocity was 18 cm/s, and the flow rate was 1.7 mL/min. The oven temperature was initially held at 80 °C for 3 min, then programmed to 215 °C at 10 °C/min, and held isothermally for 20 min. The injection port temperature was 250 °C while that of the detector was 260 °C. The different amounts of FAME were analyzed and integrated by an online computer with C17:0 as internal standard. The analyses of FAME were performed in triplicate for each SL and average values for stearic acid reported (Table 1).

Table 3. Coefficient List for Incorporation of Stearic Acid

Inc of stearic acid	coeff[a]	std error	*P*-value[b]	conf int (±)
constant	27.5609	1.09177	6.46136e-015	2.30346
Enz	DF = 1			
Enz(LIP1)	−17.9538	0.556608	1.08675e-016	1.17435
Enz(RM IM)	17.9538	0.556608	1.08675e-016	1.17435
Acyl	DF = 1			
Acyl(Stearic acid)	−4.25799	0.550759	5.79499e-007	1.16201
Acyl(Ethyl stearate)	4.25799	0.550759	5.79499e-007	1.16201
Te	2.40803	0.615244	0.0011174	1.29806
Sr	7.86007	0.628361	5.317e-010	1.32574
t	−0.954043	0.60053	0.130559	1.26702
Te*Te	−3.74231	1.29444	0.0101529	2.73105
Sr*Sr	−3.23097	1.28407	0.0221988	2.70917
Enz*Sr	DF = 1			
Enz(LIP1)*Sr	−4.71995	0.628361	8.51351e-007	1.32574
Enz(RM IM)*Sr	4.71995	0.628361	8.51351e-007	1.32574
Acyl*Te	DF = 1			
Acyl(Stearic acid)*Te	−1.98042	0.615244	0.00503818	1.29806
Acyl(Ethyl stearate)*Te	1.98042	0.615244	0.00503818	1.29806
Acyl*Sr	DF = 1			
Acyl(Stearic acid)*Sr	−2.57206	0.628361	0.000757643	1.32574
Acyl(Ethyl stearate)*Sr	2.57206	0.628361	0.000757643	1.32574
Te*t	−1.79607	0.682067	0.0174358	1.43905

$N = 29$; $Q^2 = 0.965$; $R^2_{adj} = 0.985$

DF = 17; $R^2 = 0.991$; RSD = 2.7153; conf lev = 0.95

[a] Abbreviations: coeff, multiple regression coefficients; std error, standard error; conf int, confidence interval; Te, temperature (°C); Sr, substrate molar ratio; *t*, time (h); Sr*Sr, quadratic term of Sr; Te*Sr, interaction term of Sr and Te; Sr*t, interaction term of Sr and *t*; RSD, relative standard deviation; SD, standard deviation; Q^2, R^2, and R^2_{adj}, explained in text. [b] Coefficients with *P*-value less than 0.05 are significant.

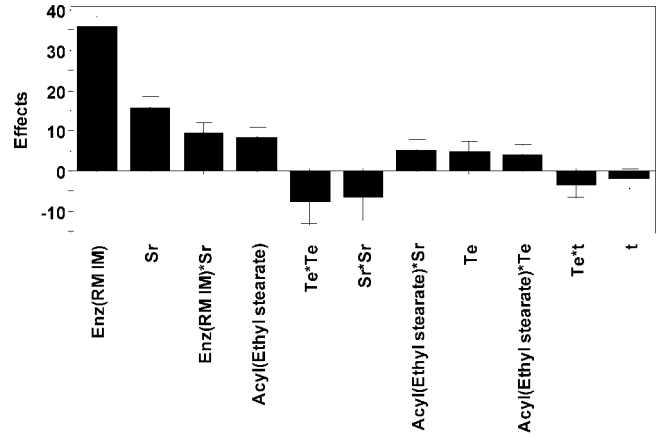

Figure 2. Effect of parameters and their significance on incorporation of stearic acid. Key: Sr, substrate molar ratio; Te, temperature (°C); *t*, time (h); Sr*Sr, quadratic term of Sr; Te*t, interaction term of Te and *t*; Enz(RM IM), lipozyme RM IM; Acyl (Ethyl stearate)*Sr, interaction term of ethyl stearate; Acyl (Ethyl stearate)*Te, interaction term of ethyl stearate and Te.

Gram Scale SL Synthesis. Structured lipid synthesis was performed in a stir-batch reactor at 50 °C for 12 h. The reaction mixture typically contained 300 g of canola oil, 10−40% stearic acid (by weight of canola oil), and 10% lipozyme RM IM (by total weight of reactants). For the anhydrous sodium methoxide-catalyzed reaction, reactants (70 g of canola oil and 7 g of ethyl stearate) were dried under nitrogen gas at 110 °C for 15 min. After the temperature was lowered to 80 °C, 0.3% sodium methoxide powder was added, and the mixture was heated to 100 °C with vigorous stirring under nitrogen gas for 1 h. The temperature was lowered to 70 °C, and 20 mL of 20% (w/v) citric acid was added to stop the reaction. Excess catalyst and citric acid were removed by warm water washes. The SL was separated and dried using anhydrous sodium sulfate, and free fatty acids were removed by short-path distillation.

Short-Path Distillation. Short-path distillation was carried out with a KDL-4 (UIC Inc., Joliet, IL) unit under the following conditions: heating oil temperature, 185 °C; cooling water temperature, 15 °C; pump vacuum, <1 mmHg; feed rate, maintained at 100 mL/h. The reaction product was passed through the system twice to reduce the free fatty acid percentage to an acceptable level. Free fatty acid content (0.13−1.08% oleic acid) was determined according to the AOCS Official Method, Ca 5a−40 (*13*).

Determination of Fatty Acid Profiles. Between 0.1 and 0.2 g of each lipid sample (in duplicate) was converted to fatty acid methyl esters (FAME) using the AOAC Official Method 996.01, Section E (*14*). Briefly, 1 mL of 20 mg/mL C13:0 (internal standard) dissolved in chloroform was added to each sample in flat-bottom flasks. Methanolic NaOH (10 mL) was added, and the mixture was refluxed for 10 min, after which time 10 mL of BF_3 reagent was added. Reflux continued for an additional 5 min. *n*-Heptane (10 mL) was then added, followed by an additional minute of reflux, after which time the reaction mixture was allowed to cool and then transferred to a measuring cylinder/centrifuge tube. The flat-bottom flask was rinsed with 10 mL of saturated NaCl solution, and the wash was transferred to the centrifuge tube. The contents of the centrifuge tube was mixed thoroughly and kept for 10 min to allow for phase separation. The organic phase containing the FAME was transferred to a GC vial and used for analysis. The FAME were analyzed in parallel with a FAME standard (Supelco 37 component FAME mix; Supelco, Bellafonte, PA) using an Agilent Technology 6890N gas chromatograph equipped with a flame ionization detector (FID). An SP-2560, 100 m × 0.25 mm i.d., 0.20 μm film column was used for separation. One injection (1 μL) per sample duplicate was performed in the split mode at a split ratio of 50:1. Helium was the carrier gas, the linear velocity was 18 cm/s, and the flow rate was 1 mL/min. The oven temperature was initially held at 120 °C for 5 min, then programmed to 240 °C at 3 °C/min, and held isothermally for 20 min. The injection port temperature was 200 °C while that of the detector was 250 °C. The different amounts of FAME were analyzed and integrated by an online computer, and values for duplicate samples were averaged to give the fatty acid profile of each sample (Table 4).

HPLC Analyses. SL products, canola oil, and margarine fats (TAG species) were analyzed by reverse-phase HPLC (RPHPLC) using an HP1050 (Agilent Technologies, Little Falls, DE) equipped with a quaternary pump, autosampler, and a Varex MKIII (Burtonville, MD) evaporative light scattering detector (ELSD). Separation was performed on a Waters Symmetry C18 (150 × 2.1 mm i.d.) column attached to a guard column (50 × 2.1 mm i.d.). Aliquots (20 μL) of each sample were dissolved in 300 μL of methylene chloride (CH_2Cl_2) and 700 μL of acetonitrile (ACN). Ten microliters of the final solution was injected into the HPLC. A binary gradient system of ACN and CH_2Cl_2 was used at 0.2 mL/min. Separation was performed using a solvent gradient profile starting with ACN:CH_2Cl_2 [65:35 (v/v)] for 10 min, followed by a linear increase of the CH_2Cl_2 concentration to 50:50 (v/v) over a period of 40 min, an isocratic period of 5 min, a linear decrease to initial conditions over a 5 min period, and a final isocratic period of 5 min.

HPLC-MS Analyses. HPLC-MS was performed on Waters 2690 separations module (Waters Corp., Milford, MA) coupled to a Micromass ZMD MS (Micromass, Manchester, U.K.) with an atmospheric pressure chemical ionization (APCI) probe. Column and separation conditions were the same as HPLC-ELSD. Acylglycerol species were identified using a database (www.byrdwell.com/Tryacylglycerols) for protonated TAGs and DAG-like fragment ions formed from TAGs in APCI-MS. Chromatograms from the HPLC-MS (APCI) and HPLC-ELSD analyses were compared, and peaks with similar retention times were analyzed to obtain qualitative and quantitative information.

^{13}C NMR Analyses. A proton-decoupled ^{13}C NMR was used to analyze the positional distribution of fatty acids on the TAG backbone. Lipid samples (50 μL) were dissolved in $CDCl_3$ (500 μL) in 5 mm NMR tubes, and NMR spectra were recorded on a 9.2 Tesla Varian INOVA spectrometer operating at 100 MHz. The ^{13}C spectra of both the lipid samples and the standard samples were acquired with a spectral

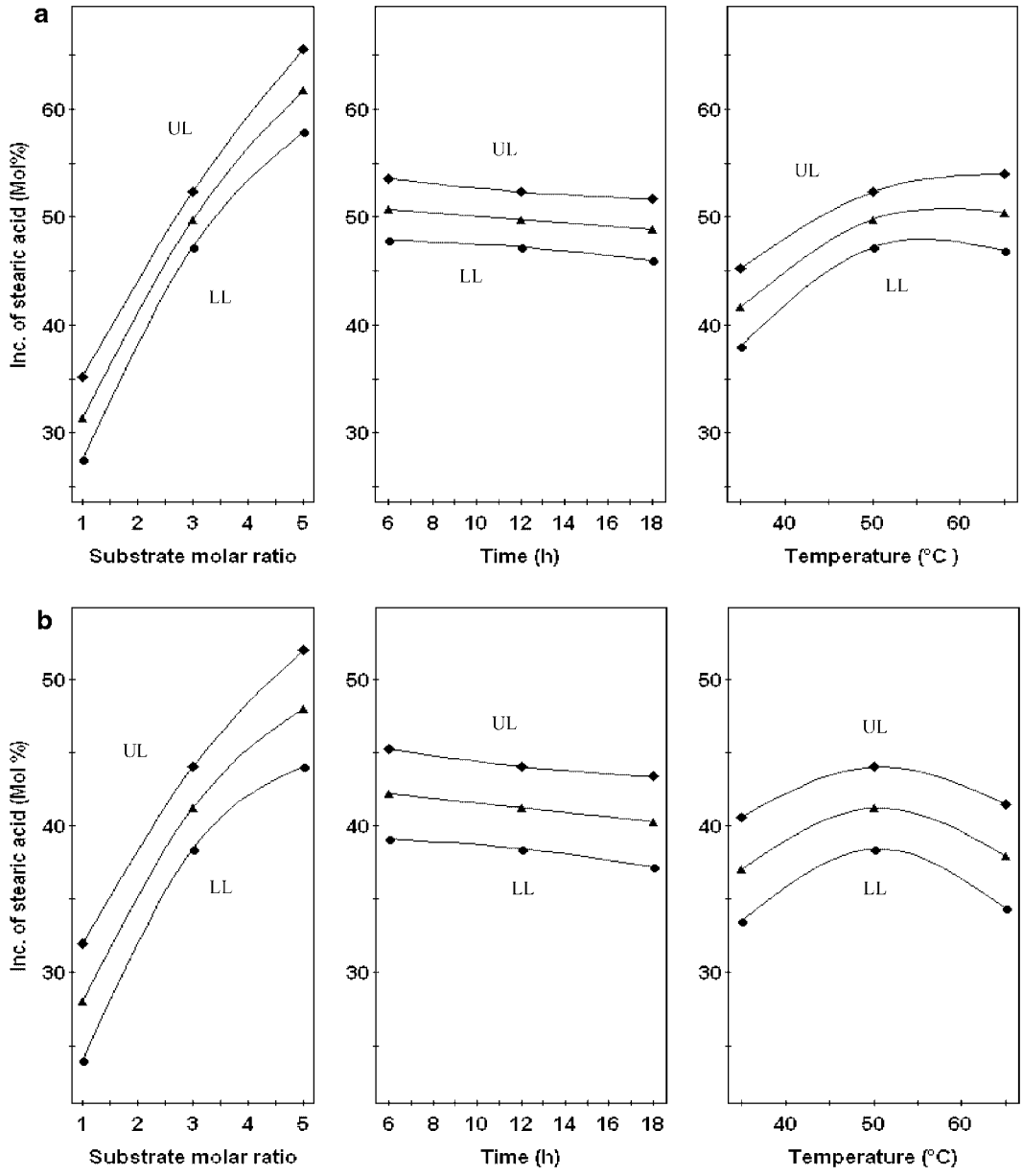

Figure 3. Projected response for substrate molar ratio, time, and temperature when all but the parameter of interest were held constant at 50 °C, 12 h, and/or substrate molar ratio 3 in both lipozyme RM IM-catalyzed interesterification (**a**) and acidolysis (**b**) reactions. UL and LL refer to upper and lower confidence limits.

width of 25000 Hz, pulse angle of 45°, a relaxation delay of 3 s, 60K data points, and 512 transients. In addition, the spectra of the lipid samples were re-acquired with a 30 s relaxation delay, 128K or 256K data points, and 1800–2400 transients. Since these ^{13}C spectra used a relaxation time that was at least 5 times the relaxation time of the carbonyls, it was possible to perform a quantitative integration of the carbonyl region to determine the relative concentrations of the components. The spectra acquired at shorter relaxation times were found to give similar relative concentrations, and hence the data were used for analyses. Prior to Fourier transformation, a TRAF apodization function with a line-broadening factor of 0.5 Hz was applied. Chemical shifts were referenced to tetramethylsilane (TMS) at 0 ppm. Tripalmitin, tristearin, triolein, trilinolien, and trilinolenin were used as standard TAGs.

Differential Scanning Calorimetry (DSC). Melting profiles of lipid samples were determined by DSC on a Perkin-Elmer model DSC1 (Norwalk, CT). The melting profiles of purified products were compared to those of fat extracted from two commercial margarine samples. Analysis was performed using a modification of the AOCS recommended procedure Cj 1–94 (*15*). Briefly, samples were held for 2 min at 25 °C, followed by rapid heating to 80 °C at 10 °C /min, and held for 10 min. The samples were then cooled to −80 °C at 5 °C/min and held for another 10 min. In the final step, samples were heated to 80 °C at 5 °C/min. Normal standardization was performed with cyclohexane (two thermal transitions; one at −87.06 °C and one at 6.54 °C) and indium (thermal transition at 156.6 °C). Liquid nitrogen (−196 °C) was used as the coolant.

Solid Fat Content Analyses. Solid fat content (SFC) was determined according to the AOCS Official Method Cd 16–81 (*16*) on a MARAN-20 pulsed NMR spectrometer (Resonance Instruments Ltd., Oxon, U.K.). Samples were tempered at 100 °C for 15 min and then kept at 60 °C for 10 min, followed by 0 °C for 60 min and finally at 30 min at each temperature of measurement. SFC was measured at intervals of 5 °C from 5 to 45 °C.

RESULTS AND DISCUSSION

Model Fitting. The amount (mole percent) of stearic acid incorporated into canola oil at different experimental conditions is shown in **Table 1**. Generally, reactions catalyzed by LIP1 produced little or no stearic incorporation, while those catalyzed

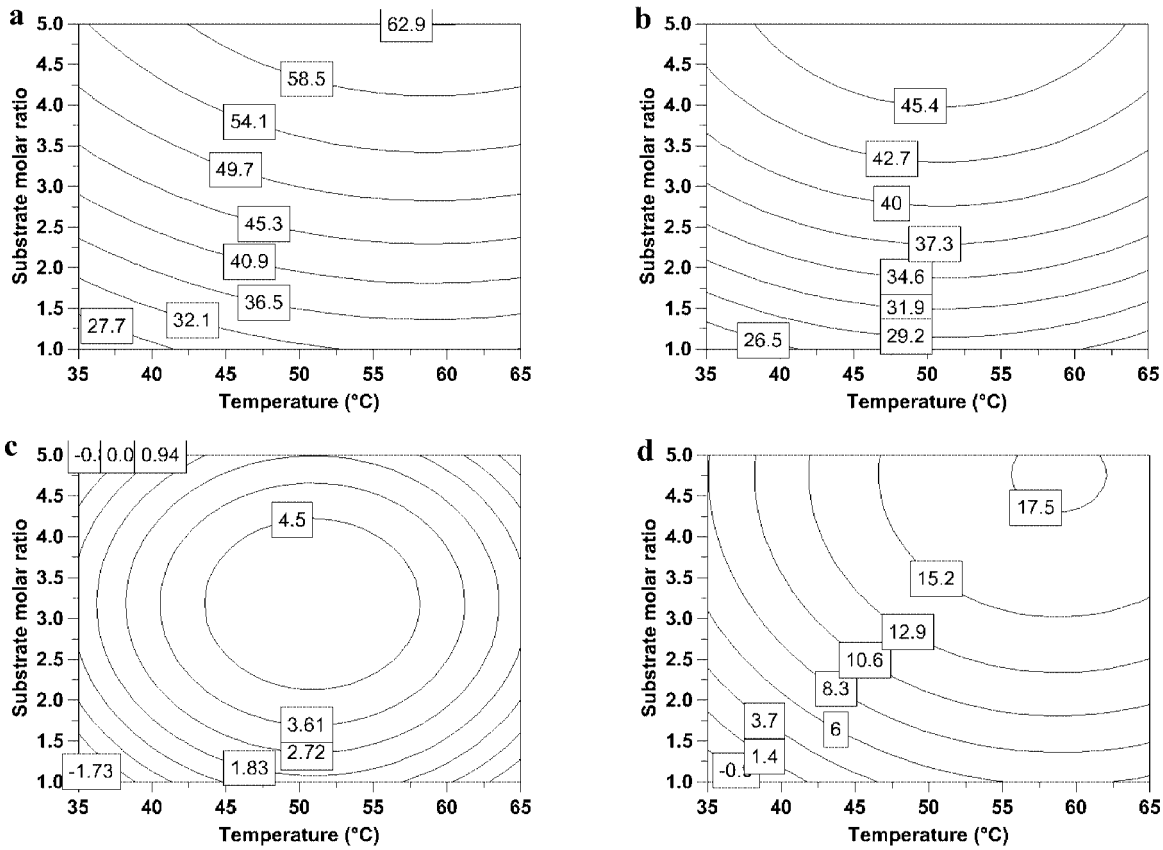

Figure 4. Contour plots showing (**a**) effects of lipozyme RM IM and ethyl stearate, (**b**) lipozyme RM IM and stearic acid, (**c**) LIP1 and ethyl stearate, and (**d**) LIP1 and stearic acid on incorporation of stearic acid at 12 h. The numbers inside the contour plots indicate the level of stearic acid incorporation (mol %).

Table 4. Fatty Acid Profile of Samples

fatty acid	canola oil	EZ-A2[a]	EZ-B2	EZ-C2	EZ-D2	CI-A	MG-B	MG-E
16:0	4.8 ± 0.00	4.17 ± 0.00	3.88 ± 0.01	3.64 ± 0.01	3.44 ± 0.00	4.55 ± 0.14	10.44 ± 0.17	26.01 ± 0.2
18:0	2.06 ± 0.00	10.71 ± 0.00	17.44 ± 0.06	22.80 ± 0.06	26.86 ± 0.00	6.27 ± 0.20	12.53 ± 0.16	4.31 ± 0.03
18:1 trans	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.01	0.07 ± 0.00	3.2 ± 0.06	0.28 ± 0.00
18:1 cis	59.72 ± 0.01	52.19 ± 0.00	47.78 ± 0.03	44.29 ± 0.03	41.65 ± 0.00	54.58 ± 1.44	24.48 ± 1.12	37.17 ± 0.13
18:2 trans							0.06 ± 0.00	0.01 ± 0.01
18:2 cis	21.29 ± 0.01	19.39 ± 0.70	18.68 ± 0.02	17.72 ± 0.02	17.04 ± 0.01	20.77 ± 0.66	40.89 ± 0.65	25.96 ± 0.2
20:0	0.67 ± 0.03	0.62 ± 0.00	0.60 ± 0.01	0.59 ± 0.00	0.58 ± 0.00	0.66 ± 0.02	0.42 ± 0.00	0.37 ± 0.00
18:3 n6	1.33 ± 0.00	1.25 ± 0.00	1.19 ± 0.00	1.12 ± 0.00	1.08 ± 0.01	1.31 ± 0.04	0.13 ± 0.00	0.43 ± 0.00
20:1	2.8 ± 0.00	2.45 ± 0.00	2.25 ± 0.00	2.10 ± 0.00	1.97 ± 0.00	2.67 ± 0.08	0.36 ± 0.01	0.35 ± 0.00
18:3 n3	7.72 ± 0.01	7.34 ± 0.01	6.92 ± 0.01	6.57 ± 0.01	6.29 ± 0.00	7.69 ± 0.24	6.07 ± 0.09	3.2 ± 0.01
other[b]	2.05 ± 0.00	1.89 ± 0.00	1.76 ± 0.00	1.64 ± 0.00	1.56 ± 0.00	2.04 ± 0.01	1.89 ± 0.00	2.25 ± 0.00
ω-6:ω-3 ratio	2.76	2.64	2.70	2.70	2.71	2.70	6.74	8.11
satd fat	8.21 ± 0.02	16.28 ± 0.11	22.57 ± 0.04	27.62 ± 0.08	31.45 ± 0.00	12.24 ± 0.39	24.02 ± 0.16	32.35 ± 0.36
unsatd fat	91.79 ± 0.02	83.72 ± 0.11	77.43 ± 0.04	72.38 ± 0.08	68.55 ± 0.00	87.76 ± 0.39	75.98 ± 0.16	67.65 ± 0.36
monounsatd fat	63.16 ± 0.02	55.17 ± 0.01	50.52 ± 0.02	46.85 ± 0.03	44.04 ± 0.01	57.82 ± 1.33	28.17 ± 1.08	37.97 ± 0.14
polyunsatd fat	28.73 ± 0.00	28.55 ± 0.12	26.91 ± 0.02	25.53 ± 0.05	24.51 ± 0.02	29.93 ± 0.94	47.8 ± 0.93	29.68 ± 0.22
% trans fat	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.00	0.08 ± 0.01	0.07 ± 0.00	3.72 ± 0.06	0.29 ± 0.00

[a] EZ-A2, EZ-B2, EZ-C2, and EZ-D2 are SL samples made from lipozyme RM IM-catalyzed reaction setups containing 10%, 20%, 30%, and 40% (by weight of canola oil) stearic acid, respectively; CI-A is SL made from the sodium methoxide-catalyzed reaction setup containing 10% stearic acid (by weight of canola oil). MG-B is a soft margarine made with a blend of soybean oil, hydrogenated soybean oil, partially hydrogenated soybean oil, and canola oil; MG-E is a soft margarine made from a blend of palm, soybean, canola, and olive oils. [b] Other refers to the sum of 12:0, 14:0, 16:1, 20:0, 21:0, 20:2, 22:0, 20:3 n6, and 22:1 n9.

by lipozyme RM IM produced appreciable levels of stearic acid incorporation: as low as 22.3 mol % and as high as 59.10 mol %. The results obtained (**Table 1**) were fitted to a second-order polynomial model by multiple linear regression and backward elimination using MODDE 7.0 software (Umetrics, Umeå, Sweden). The squared term $t*t$ and the interaction terms Enz*Acyl, Enz*Te, Enz*t, Acyl*t, Te*Sr, and Sr*t were deleted from the model because they were not significant at $\alpha_{0.05}$. R^2, the fraction of the variation of the response explained by the model, was 0.99, and Q^2, the fraction of the variation of the response that can be predicted by the model, was 0.97. R^2_{adj} was 0.99. The reproducibility and validity of the model were 0.99 and 0.74, respectively. The normal probability plot (**Figure 1a**) showed a linear distribution, indicating that our assumption of normality of model errors was not violated. Likewise, the residual plot (**Figure 1b**) showed no patterns, indicating that our model assumption of constant error variance was not violated either. Furthermore, the model showed no lack of fit ($P > 0.05$), and the multiple regression P-value was <0.001 (**Table 2**). The model equation can therefore be written as

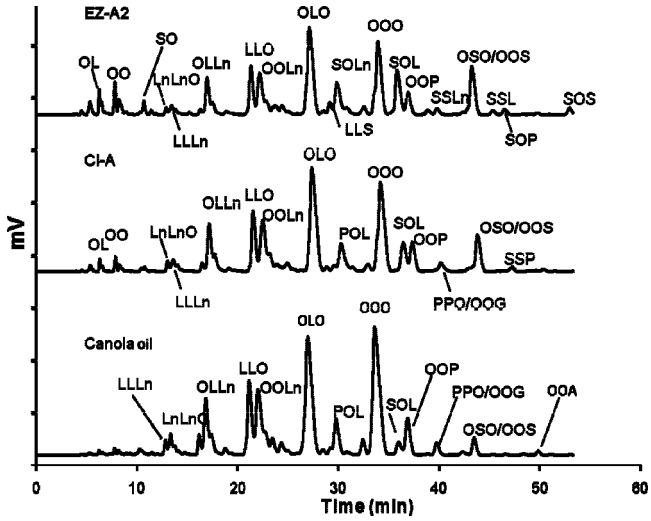

Figure 5. HPLC-ELSD chromatograms of canola oil and structured lipids produced by lipozyme RM IM-catalyzed (EZ-A2) and sodium methoxide-catalyzed (CI-A) transesterification.

$$\text{Inc} = 27.56 \pm 17.95\text{Enz} \pm 4.26\text{Acyl} + 2.41\text{Te} + 7.86\text{Sr} - 0.95t - 3.74\text{Te*Te} - 3.23\text{Sr*Sr} \pm 4.72\text{Enz*Sr} \pm 1.98\text{Acyl*Te} \pm 2.57\text{Acyl*Sr} - \text{Te*}t$$

Except for time (**Table 3**), all coefficients were highly significant ($P < 0.05$). Time (t) was kept in the model because of its significant ($P < 0.05$) interaction term with temperature (Te*t).

Effect of Parameters. Figure 2 shows the effect of reaction parameters on stearic acid incorporation. All linear parameters, except time, had a positive effect on incorporation. The squared terms of temperature and substrate ratio (Te*Te and Sr*Sr) had negative effects while the interaction terms, Enz(RM IM)*Sr, Acyl(Ethyl stearate)*Sr, and Acyl(Ethyl stearate)*Te, had positive effects. Only the interaction term of Te*t had a negative effect on incorporation. The projected response for substrate molar ratio, time, and temperature when all but the parameter of interest were held constant is shown in **Figure 3**. These were for lipozyme RM IM-catalyzed reactions. Stearic acid incorporation was higher (**Figure 3a**) when ethyl stearate was used as acyl donor than when stearic acid was used (**Figure 3b**). The substrate ratio produced the highest change in incorporation per change in factor level in both cases. The effect of time on incorporation implies that prolonging the reaction would result in a steady but nevertheless insignificant decline in the level of stearic acid incorporation ($P > 0.05$, **Table 3**). The effect of temperature on incorporation (**Figure 3**) did not follow the same pattern as did the effects of substrate ratio and time. The highest point of stearic acid incorporation was at 60 °C (**Figure 3a**) and at 50 °C (**Figure 3b**). This temperature-dependent relationship has been previously discussed by Lumor and Akoh (*17*).

Increasing temperature has two effects on reaction rate: First, it increases the rate of productive collisions between reactants and the enzyme, resulting in increased acyl incorporation. Second, it can also accelerate the rate of enzyme inactivation (*18*), thereby producing less incorporation (*19*, *20*). However, whether incorporation will increase or decrease with increasing temperature will depend on a number of factors, such as concentration and type of substrate used, and other reaction conditions, such as the presence of water. Although most lipase-catalyzed reactions require water content of less than 1% (w/v) (*21*) for effective transesterification, accumulation of water during the course of reaction, as is the case for acidolysis reactions involving oils containing partial acylglycerols (*17*), causes the equilibrium to shift in favor of hydrolysis. This phenomenon, coupled with the denaturing effect of increasing temperatures on the lipase, is mostly responsible for the disparity in the levels of stearic acid incorporated between the interesterification (**Figure 3a**) and the acidolysis (**Figure 3b**) reactions. Ester-interchange or interesterification reactions on the other hand do not involve accumulation of water.

Optimization of Reaction. As is the case for quadratic models, the response is affected not only by the first-order variables but also by second-order and interaction terms and may have more than one solution (*22*). Evaluation of the relationship between the response and parameters is best done by means of contour plots. This is done by placing the reaction parameter with the greatest effect on the y-axis, the second is placed on the x-axis, and the one with the least effect is held constant. The contour plots allow the researcher to identify parameter combinations that will produce a desired response, which, in this case, is the level of stearic acid incorporation that will produce SLs with similar melting and crystal properties as commercial margarine samples.

The contour plots are shown in **Figure 4**. Time, being the least significant variable (**Table 3**), was held constant at 12 h, while the other parameters were varied. In general, incorporation increased with increasing temperature and substrate ratio. It can be seen that both enzymes favored stearic acid incorporation more when ethyl stearate, rather than stearic acid, was used as acyl donor. This observation is consistent with other studies (*23–26*) which showed that (1) incorporation was more favored with the ethyl ester form and (2) lipozyme RM IM proved to be a better biocatalyst for the process than LIP1. The ineffectiveness of LIP1 for incorporation of certain long-chain fatty acids into TAGs has previously been reported (*27*). In a nutshell, the contour plots show that a combination of ethyl stearate and lipozyme RM IM, at any experimental conditions, will produce the highest incorporation. This is followed by the pairs, RM IM:stearic acid, LIP1:ethyl stearate, and LIP1:stearic acid, in that order.

Fatty Acid Profile of Samples. The fatty acid profiles of canola oil, SL products, and margarine samples were determined (**Table 4**). There was significant incorporation of stearic acid in both the lipase- (EZ-A2 to EZ-D2) and sodium methoxide- (CI-A) catalyzed reactions. These values ranged from 10.71% to 26.86% (in samples EZ-A2 to EZ-D2) as the substrate mole ratio increased from 10% to 40% stearic acid by weight of canola oil. Stearic acid content of CI-A was 6.27%. For the margarine samples, stearic acid content ranged from 4.31% (MG-E) to 12.53% (MG-B). The increased stearic acid content of the SL products is not expected to pose any health risks since the neutrality of stearic acid with regard to plasma cholesterol levels has been established (*7–10*). The amounts of atherogenic fatty acids such as palmitic and myristic were less than 5% in all SL samples but ranged from 10.53% to 26.6% in the margarine samples. Dietary intake of saturated fatty acids below 10% energy is recommended in order not to significantly alter plasma cholesterol levels (*28*). Oleic acid was the main fatty acid in canola oil and the SL products. Its content decreased from 59.72% in canola oil to between 41.65% and 54.58% in the SL products as stearic acid incorporation increased. Oleic acid, the main fatty acid in Mediterranean diets, has been correlated with low incidence of coronary heart disease (*29*) and is more stable to oxidation compared to polyunsaturated fatty acids.

Table 4 also gives the trans fatty acid contents of canola oil, SL products, and commercial margarine samples. Elaidic acid (18:1 trans) was detected in all samples while 18:2 trans was detected only in margarine samples (MG-B and MG-E). The

Table 5. Acylglycerol Species of Canola Oil and SLs Identified by HPLC-MS

				area (%)					
peak	acylglycerol species	diacylglycerol ions $[M - RCO]^+$	molecular ions $[M + H]^+$	canola oil	CI-A	EZ-A2	EZ-B2	EZ-C2	EZ-D2
1	OL[a]	601.52			1.0	1.4	1.1	1.3	1.1
2	OO	603.52		0.3	0.9	2.1	1.8	1.6	1.0
3	PO, OO	577, 603.52				1.7	1.8	1.4	1.3
4	SO	605.55				1.3	1.6	1.7	1.8
5	LnLnO	599.50, 595.47	878.34	0.9	0.7	1.4			
6	LLL	599.50	880.30	1.8	0.7	0.4			
7	OLLn	601.52, 597.49, 599.5	880.30	5.9	5.9	4.8	4.7	3.8	3.6
8	PLLn	575.5, 597.5, 573.5	854.28	0.8	2.0				
9	LLO	599.5, 601.52	882.26	8.6	7.8	6.6	5.1	3.8	3.0
10	OOLn	603.54, 599.54	883.26	11.9	8.7	9.0	6.9	5.2	4.1
11	LLP	575.5, 599.5	856	1.9	1.7				
12	POLn	577.52, 599.5, 573.49	856.24, 882.02	1.9		1.5	1.4	1.3	1.3
13	OLO	601.52	884.23	21.0	20.5	17.6	14.3	10.8	8.6
14	LLS	599.50, 603.54	884.23			1.3	2.1	2.4	2.6
15	SOLn, POL	601.52, 599.50, 605.55, 577.52, 575.52	884.23, 858.21	5.4	6.5	6.4	8.3	8.6	8.7
16	OLG, OLnA	599.50, 601.52, 631.57, 629.55	912.46	1.5	1.1	1.1		0.9	0.9
17	OOO	603.54	886.19	24.0	18.5	14.9	11.8	9.1	7.3
18	SOL	601.54, 603.54, 605.55	886.19	1.7	4.9	8.1	11.8	13.1	13.5
19	OOP	603.54, 577.52	860	4.7	4.5	3.5	2.9	2.4	2.1
20	SSLn, PSL	601.52, 607.57, 575.50, 579.54, 603. 54	860.17, 886.19			0.7	1.4	2.2	2.8
21	PPO, OOG	551.5, 577.52, 603.54	885.95	1.4	1.5				
22	SOL, OOG	601.52, 603.54, 605.55	886.44, 912.18			0.9	0.7	1.0	1.1
23	OSO/OOS	605.55, 603.54	888.40	1.9	6.3	8.5	12.5	13.8	14.3
24	SSP	579.54, 605.55	862.38		<1.5				
25	SSL	603.54, 607.57	888.40			0.6	1.8	3.3	4.7
26	PSO	577.52, 579.54, 605.55	862.38			0.8	1.3	1.8	2.1
27	OOA	603.54, 633.58	915.90	0.5	<1.0				
28	PoSA	577.52, 607.57, 633.58	890.12		1.0				
29	SOS	605.54	890.37			1.0	3.1	5.5	7.5
30	AAO	663.63, 633.58	945.89	<0.5	<1.0				
% TAGs containing at least one saturated fatty acid						36.4	50.9	59.1	64.6
% TAGs containing at least two saturated fatty acids						2.9	7.6	13.4	17.7

[a] Abbreviations: La, lauric; M, myristic; P, palmitic; Po, palmitoleic; S, stearic; O, oleic; L, linoleic; Ln, linolenic; A, arachidic; G, godoleic.

Table 6. Acylglycerol Species of MG-B and MG-E Identified by HPLC-MS

				area (%)	
peak	acylglycerol species	diacylglycerol ions $[M - RCO]^+$	molecular ions $[M + H]^+$	MG-B	MG-E
1	OO, PO[a]	603.54, 577.52			1.0
2	PP	551.50			0.4
3	LnLnL	595.47, 597.49	876.13	0.7	0.2
4	LLLn	599.50, 597.49	878.09	5.4	3.1
5	LLL	599.50	880.30	13.4	5.8
6	OLLn	601.52, 597.49, 599.50	880.06	3.7	2.9
7	PLLn	575.50, 579.49, 573.49	854.03	2.3	1.1
8	LLO	599.50, 601.52	882.26	13.7	7.0
9	OOLn	603.54, 599.50	882.26		1.9
10	LLP	599.50, 575.50	856.24	10.1	5.4
11	POLn	577.52, 599.5, 573.49	856.24		1.1
12	OLO	601.52	884.26	7.3	7.1
13	LLS	599.50, 603.54	883.74	3.8	1.6
14	POL	577.53, 601.52, 575.50	858.21	7.9	8.4
15	PPL	551.50, 575.50	831.7	1.8	5.1
16	OOO	603.54	886.19	2.6	7.3
17	SOL	605.55, 601.52, 603.54	886.19	6.2	2.2
18	OOP	603.54, 577.52	860.17	3.5	13.2
19	PSL	579.54, 603.54, 575.50	859.68	2.5	1.2
20	PPO	551.50, 577.52	833.90		15.0
21	OOS	603.54, 605.55	888.40	3.0	3.6
22	PSO	579.54, 605.55, 575.50	862.14	1.8	2.9
23	OOA	603.54, 633.58	916.14		0.5
24	SSO	607.57, 605.55	890.37	4.2	<0.5
% TAGs containing at least one saturated fatty acid				47	62.7
% TAGs containing at least two saturated fatty acids				10.3	25.1

[a] Abbreviations: La, lauric; M, myristic; P, palmitic; Po, palmitoleic; S, stearic; O, oleic; L, linoleic; Ln, linolenic; A, arachidic; G, godoleic.

trans fat content of the SL products was considerably lower than that in the margarine samples. The source of 18:1 trans in the SL products was most definitely canola oil since there was no significant difference between the amounts present in the SLs (0.07–0.09%) and canola oil (0.09%). However, the level of trans fatty acids in these samples may not be enough to pose

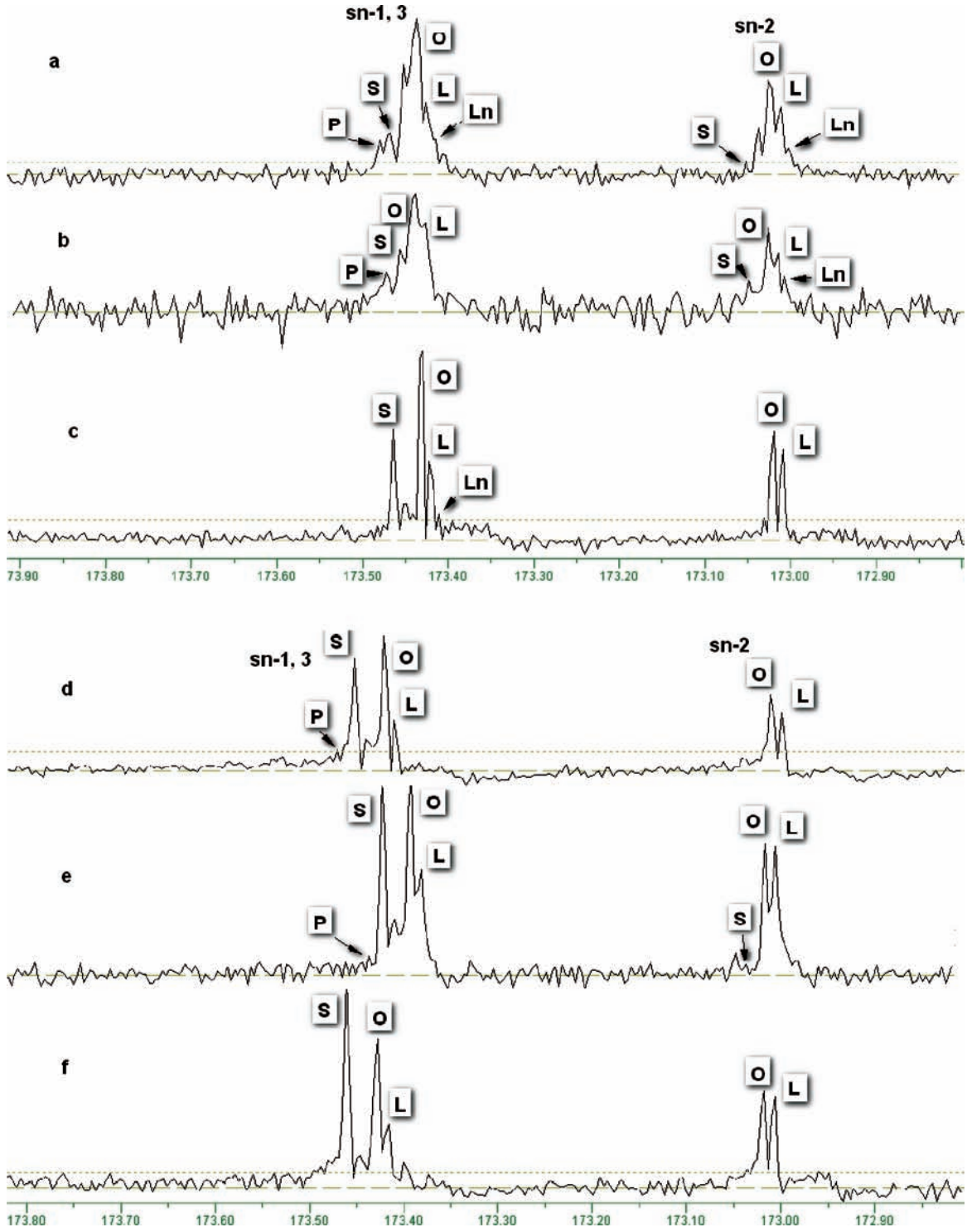

Figure 6. 100 MHz ^{13}C NMR carbonyl spectra of canola oil (**a**) and structured lipid products CI-A (**b**), EZ-A2 (**c**), EZ-B2 (**d**), EZ-C2 (**e**), and EZ-D2 (**f**). The acyl groups are palmitic (P), stearic (S), oleic (O), linoleic (L), and linolenic (Ln).

any health risks. The dietary levels of trans fatty acids required to increase LDL cholesterol is said to be approximately 4.3% energy or higher and approximately 5–6% energy or higher to decrease HDL cholesterol (*1*).

Triacylglycerol Profile. Significant amounts of stearic acid-containing TAGs were found in the SL samples (**Figure 5**). These were OSO/OOS, SSL, SOL, LLS, SOLn, SSLn, PSO, and SOS for the lipase-catalyzed reactions. Stearic acid-containing acylglycerol species increased in amount (approximately from 30.4% to 57.7%) as the amount of stearic acid used in the reactions increased from 10% to 40% (**Table 5**). For the sodium methoxide-catalyzed reaction, OSO/OOS, SOL, PoSA, and SSP were the only stearic acid-containing TAGs found. They constituted approximately 13% of the total TAGs in the product. Small amounts (3–10%) of DAGs were also found in all SL products. These were smaller (<1%) in canola oil. Major canola oil TAGs that decreased significantly with stearic acid incorporation were OOO, OLO, OOLn, LLO, and OOP. OOO and OLO decreased between 40% and 70% and between 18% and 60% of their initial amounts in lipase-catalyzed reaction products, respectively. In the sodium methoxide reaction, OOO decreased by 22% and OOLn by 27% of

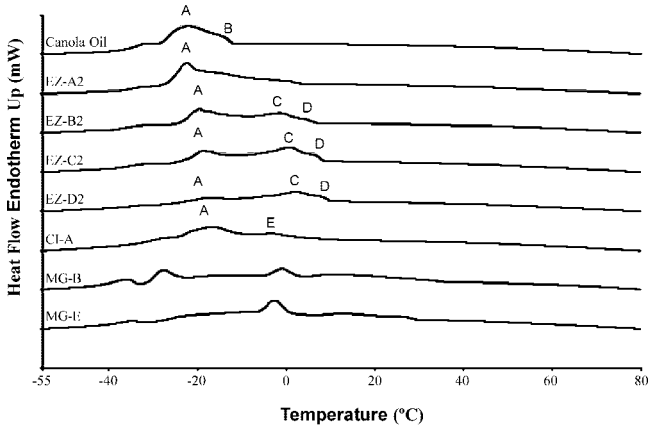

Figure 7. DSC thermograms of canola oil, structured lipid products, and margarine samples.

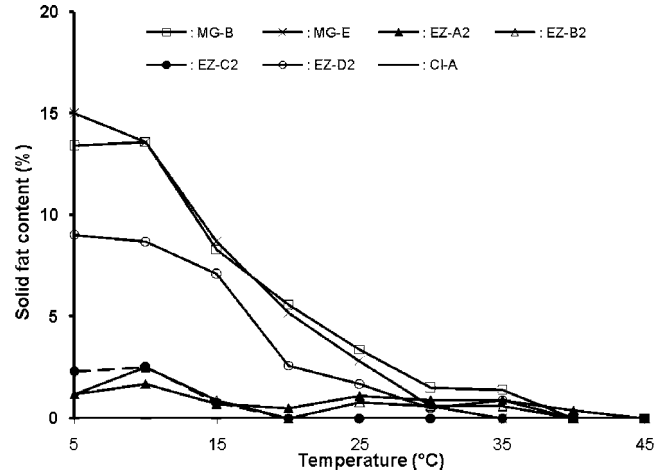

Figure 8. Curves showing solid fat content (SFC) of canola oil, structured lipid products (CI-A, EZ-A2, EZ-B2, EZ-C2, and EZ-D2), and commercial margarine samples (MG-B and MG-E).

their original amounts. TAGs containing high-melting fatty acids found in the margarine samples were PPL, POL, PPO, OOP, OOS, PSO, and POL (**Table 6**).

Positional Distribution of Fatty Acids. High-resolution proton-decoupled ^{13}C nuclear magnetic resonance spectroscopy is a rapid and nondestructive method used to determine fatty acid composition and positional distribution on the glycerol backbone (*30*–*34*). This is because the acyl carbonyl, C-2, and olefinic carbons on the triacylglycerol backbone show slightly different ^{13}C chemical shifts depending on their positional distribution (*sn*-1,3 or *sn*-2) and degree of unsaturation (*33*). Since the relaxation delay for these experiments was used consistently for all samples, and since all of the carbonyls are in similar chemical environments, the integrated intensities of these peaks could be used semiquantitatively to determine the relative concentrations of the components. The carbonyl ^{13}C chemical shifts occur usually between 172 and 174 ppm. **Figure 6** shows the carbonyl regions of canola oil and the SL products. Using the published resonance assignments, our ^{13}C NMR data showed that stearic acid was mainly incorporated at the *sn*-1,3 positions (173.30–173.55 ppm) in the lipase-catalyzed reaction products (**Figure 6c**−**f**). No significant stearic acid peaks were observed at the *sn*-2 position (172.95–173.10 ppm). On the other hand, stearic acid peaks were observed at the *sn*-1,3 and *sn*-2 positions of the sodium methoxide-catalyzed reaction product (**Figure 6b**). This observation confirms the *sn*-1,3 specificity of lipozyme RM IM and the nonspecificity of chemical interesterification. It is also significant because saturated fatty acids are better hydrolyzed and absorbed when present at the *sn*-1,3 positions while unsaturated fatty acids are better absorbed at the *sn*-2 position (*11*).

Physical Properties. The melting profiles of our products were compared to those of canola oil and commercial margarine fats. DSC thermograms (**Figure 7**) showed the emergence of high-melting endotherms (C and D) in the lipase-catalyzed structured lipid products as a result of increased stearic acid incorporation into acylglycerols, while the native peaks of canola oil (A and B) decreased. For CI-A, a broadening of peaks A and B and a small peak (E) were observed, signifying the formation of triacylglycerol species with close melting points. The melting ranges of the SLs were narrower and within those of the commercial margarine samples, indicating their suitability for formulating softer margarines. SFC data (**Figure 8**) showed that only one sample, i.e., the one containing the highest amounts of stearic acid (EZ-D2), had more than the minimum solid fat content (7.6% at 0 °C) needed to maintain a good crystal structure desirable for soft margarine formulation (*35*). The others would be suitable for liquid or light margarine formulation. The X-ray crystallography data (not shown) indicated that the crystal structures of the SLs were predominantly β. Ongoing studies in our laboratory include increasing solid fat content and regulating the crystal-forming habits of the SLs by blending with hydrogenated palm oil midfraction and/or by using emulsifiers.

We also observed that even though EZ-D2 and MG-E had comparable saturated fatty acid contents (**Table 4**), there was a noticeable difference in their SFC curves (**Figure 8**). This was most probably due to the fact that MG-E contained more TAGs (**Table 6**) with at least two saturated fatty acids than was found in EZ-D2 (**Table 5**). This indicates that the saturated fatty acid content of a fat is not enough to predict its melting behavior, but much depends on the saturated fatty acid content of the TAG species.

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Received for review April 6, 2007. Revised manuscript received September 12, 2007. Accepted November 2, 2007. This project was supported by National Research Initiative Grant 2005-35503-16186 from the USDA Cooperative State Research, Education, and Extension Service under Improving Food Quality and Value (71.1) Program.

JF0710175