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Lipase-Catalyzed Acidolysis of Tripalmitin with Hazelnut Oil Fatty Acids and Stearic Acid To Produce Human Milk Fat Substitutes

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Structured lipids (SLs) containing palmitic, oleic, stearic, and linoleic acids, resembling human milk fat (HMF), were synthesized by enzymatic acidolysis reactions between tripalmitin, hazelnut oil fatty acids, and stearic acid. Commercially immobilized sn-1,3-specific lipase, Lipozyme RM IM, obtained from *Rhizomucor miehei* was used as the biocatalyst for the enzymatic acidolysis reactions. The effects of substrate molar ratio, reaction temperature, and reaction time on the incorporation of stearic and oleic acids were investigated. The acidolysis reactions were performed by incubating 1:1.5:0.5, 1:3:0.75, 1:6:1, 1:9:1.25, and 1:12:1.5 substrate molar ratios of tripalmitin/hazelnut oil fatty acids/stearic acid in 3 mL of *n*-hexane at 55, 60, and 65 °C using 10% (total weight of substrates) of Lipozyme RM IM for 3, 6, 12, and 24 h. The fatty acid composition of reaction products was analyzed by gas−liquid chromatography (GLC). The fatty acids at the sn-2 position were identified after pancreatic lipase hydrolysis and GLC analysis. The results showed that the highest C18:1 incorporation (47.1%) and highest C18:1/C16:0 ratio were obtained at 65 °C and 24 h of incubation with the highest substrate molar ratio of 1:12:1.5. The highest incorporation of stearic acid was achieved at a 1:3:0.75 substrate molar ratio at 60 °C and 24 h. For both oleic and stearic acids, the incorporation level increased with reaction time. The SLs produced in this study have potential use in infant formulas.

KEYWORDS: Hazelnut oil; human milk fat substitutes; lipase-catalyzed acidolysis; Lipozyme RM IM; stearic acid; tripalmitin

INTRODUCTION

Structured lipids (SLs) have been defined as triacylglycerols (TAGs) that have been modified by the incorporation of new fatty acids, restructured to change the positions of fatty acids, or the fatty acid profile, from the natural state, or synthesized to yield novel TAG (*1*). SLs can be produced by interesterification reactions, either chemically or enzymatically. Typical applications of these lipase-catalyzed interesterification reactions include the production of cocoa butter substitutes, human milk fat substitutes, partial acylglycerols, modified fish oil products, margarines, structured lipids, and several lipid products.

Human milk contains nearly 3−5% total lipid. More than 98% of milk fat is TAGs, in which 90% is composed of fatty acids (FA). FA represent 88% of the total lipids (*2*). Even though some small variations in breast milk fatty acid composition occur due to such factors as genetics, mother's diet, season, lactation stage, physiology, and even psychology, the structure is very unique. Human milk fat (HMF) contains mostly long-chain fatty acids such as oleic acid (∼30−35%) followed by palmitic acid (∼20−30%), linoleic acid (∼7−14%), and stearic acid (∼5.7−8%). Palmitic acid constitutes the highest proportion of saturated fatty acids with 70% at the sn-2 position of the glycerol backbone, and the sn-1 and sn-3 positions are occupied by unsaturated fatty acids unlike in vegetable oils, cow's milk, and infant formulas (*3*, *4*). Lard is the only fat that has a structure similar to that of HMF (*2*).

Free long-chain saturated fatty acids such as palmitic acid, released from sn-1,3 positions in vegetable oils, cow's milk, and infant formulas by pancreatic lipase, can form insoluble calcium−FA complexes also referred to as "calcium soaps" in the lumen. This causes infants to have low fatty acid and calcium absorption and loss of dietary energy. Due to the formation of these calcium soaps, stool hardness, constipation, and, in some cases, bowel obstructions may occur. The location of 16:0 at the sn-2 position of human milk fat increases the absorption of 16:0 and 18:0 in the infant and decreases the loss of calcium in the feces. The sn-2 16:0 is preserved during digestion, absorption, and biosynthesis of TAGs in the intestinal wall (*3*−*7*). Oleic acid in the HMF is also an important source of energy for infants. Stearic acid exerts effects on the plasma lipoprotein profile similar to those exerted by oleic acid (*8*).

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Because the unique FA structure in HMF plays a specific and valuable function in baby's growth, SLs resembling HMF are being produced by enzymatic interesterification or acidolysis reactions, for inclusion in infant formulas (*9*). Numerous studies have been conducted on the production of a human milk fat substitute (HMFS) (*10*−*14*). In these studies, substrates such as rapeseed oil FA, soybean FA, lard, tripalmitin, *n*−3 polyunsaturated FA, and butter oil were used, and factors such as substrate molar ratio, enzyme type and amount, temperature, time, and water content that govern the reactions were investigated (*10*−*14*).

The purpose of the present work was to produce SLs resembling HMF by enzymatic acidolysis reactions of long-chain FAs obtained from hazelnut oil, stearic acid, and tripalmitin, catalyzed by Lipozyme RM IM, and to investigate the effects of different substrate molar ratios, reaction temperatures, and reaction times on the incorporation of FAs of interest. The products were also characterized for their sn-2 positional composition.

MATERIALS AND METHODS

Materials. Tripalmitin (glycerol tripalmitate, minimum purity of 85%), stearic acid (∼99%), 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 thin-layer chromatography (TLC) plates were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) and Fisher Scientific (Fair Lawn, NJ), respectively. All solvents and reagents used in analyses were of chromatographic or analytical grade.

Preparation of Free Fatty Acids from Hazelnut Oil. Preparation of free fatty acids from hazelnut oil was carried out according to the method of Senenayeke and Shahidi (*15*). Twenty-five grams of hazelnut oil was saponified using a mixture of KOH (5.75 g), water (11 mL), and 95% (v/v) aqueous ethanol (66 mL) by refluxing for 1 h at 60 °C. To the saponified mixture was added distilled water (50 mL), and the unsaponifiable matter was extracted into hexane (2 × 100 mL) and discarded. The aqueous layer containing the saponifiable matter was acidified (pH 1.0) with 3 N HCl. The liberated free fatty acids were then extracted with 50 mL of hexane. The hexane layer was dried over anhydrous sodium sulfate, and the solvent was removed with a rotary evaporator at 40 °C. FAs were stored in the freezer at −85 °C.

Acidolysis Reactions. The acidolysis reaction mixtures consisting of 3 mL of *n*-hexane and a mixture of tripalmitin, hazelnut oil FA, and stearic acid at different substrate molar ratios (1:1.5:0.5, 1:3:0.75, 1:6:1, 1:9:1.25, and 1:12:1.5), respectively, were placed in screw-capped test tubes. Lipozyme RM IM enzyme (10 wt % of total reactants) was added. The tubes were incubated at 55, 60, and 65 °C for 3, 6, 12, and 24 h in an orbital shaking water bath at 200 rev/min. All reactions were performed in duplicate, and average results are reported.

Analysis of Product. The enzyme and moisture were removed from the reaction mixture by passing the reaction product through an anhydrous sodium sulfate packed in a Pasteur pipet column. Fifty microliters of the reaction product was applied to TLC plates (20 × 20 cm) coated with silica gel G. The developing solvent used was petroleum ether/ethyl ether/acetic acid (80:20:0.5, v/v/v). The bands were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The TAG band was scraped off into a screw-capped test tube and methylated with 3 mL of 6% HCl in methanol at 70−80 °C for 2 h. The fatty acid methyl esters (FAME) were extracted twice with 2 mL of hexane and dried over an anhydrous sodium sulfate column (*16*).

Fatty Acid Composition Analysis. The FA composition of hazelnut oil fatty acids 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 in splitless mode. The injector and detector temperatures were maintained at 250 and 260 °C, respectively. The column temperature was held at 150 °C for 3 min and programmed to 215 °C for 10 min at the rate of 10 °C/min. The carrier gas was helium, and the total gas flow rate was 64 mL/min. A 1 mL sample was injected into the GLC. Relative contents of FAME as mole percent were calculated by computer, using 17:0 as the internal standard (*16*).

Pancreatic Lipase-Catalyzed sn-2 Positional Analysis. Twenty milligrams of purified pancreatic lipase (porcine pancreatic lipase, crude type II), 1 mL of Tris buffer (pH 8.0), 0.25 mL of bile salts (0.05%), and 0.1 mL of calcium chloride (2.2%) were added to the TAG band that was scraped from the TLC plate as described before. The mixture was incubated at 40 °C in a water bath for 3 min; 1 mL of 6 mol/L HCl and 1 mL of diethyl ether were added and centrifuged. Diethyl ether was evaporated under nitrogen gas to a 200 μL volume. A 200 μL aliquot was spotted onto a silica gel G TLC plate and developed with hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The 2-monoacylglycerol (2-MAG) band was visualized under UV light after being sprayed with 0.2% 2,7-dichlorofluorescein in methanol. The 2-monoolein standard (Sigma) was used for TLC confirmation of 2-MAG of reaction products. The 2-MAG band was scraped off into a screw-capped test tube, extracted twice with 1 mL of hexane, and then methylated and analyzed by GLC (*16*).

Statistical Analysis. The effect of different temperatures and substrate molar ratios studied in this work and their interaction were analyzed using ANOVA (two-way) statistical analysis.

RESULTS AND DISCUSSION

The free fatty acids obtained from hazelnut oil contained predominantly oleic acid, which accounted for 81.5% of the total fatty acids, followed by linoleic acid (9.6%) and palmitic acid (8.9%). Lipozyme RM IM enzyme was used in acidolysis reactions because of its sn-1,3 specificity that would result in incorporation of oleic, stearic, and linoleic acids at these specific positions of the glycerol moiety. It is believed that this type of structured lipid will provide higher FA and calcium absorption and therefore efficient use of dietary energy (*3*, *4*). As the reaction time increased, the incorporation of stearic acid increased as reported previously (*17*). The effect of substrate molar ratio (1:1.5:0.5, 1:3:0.75, 1:6:1, 1:9:1.25, and 1:12:1.5), reaction temperature (55, 60, and 65 °C), and reaction time (3, 6, 12, and 24 h) on the incorporation of oleic acid and stearic acid into tripalmitin was determined. As the reaction time and substrate molar ratio increased, the incorporation of oleic acid and thus the ratio of oleic acid to palmitic acid increased (**Figures 1** and **3**). The highest incorporation of stearic acid was observed at the substrate molar ratio of 1:3:0.75. As the reaction time increased, stearic acid incorporation also increased for all substrate ratios examined in this study.

Oleic Acid Incorporation. Oleic acid incorporation at different substrate molar ratios and different reaction times is given in **Figure 1**. The highest value for C18:1 incorporation (47.1%) was achieved at 65 °C and 24 h with the highest substrate molar ratio of 1:12:1.5 examined in this study (**Figure 1C**). The lowest incorporation (10%) was achieved at 65 °C with the lowest substrate molar ratio of 1:1.5:0.5 after 3 h of reaction time. It was observed that as the substrate molar ratio and reaction time increased, oleic acid incorporation increased at all three temperatures. The effects of substrate molar ratio and reaction time on the incorporation of fatty acids were also reported by several researchers (*18*, *19*).

The incorporation of oleic acid into tripalmitin increased rapidly up to a molar ratio of 1:3:0.75; the rate of incorporation of oleic acid from a molar ratio of 1:3:0.75 to 1:6:1 was slower,

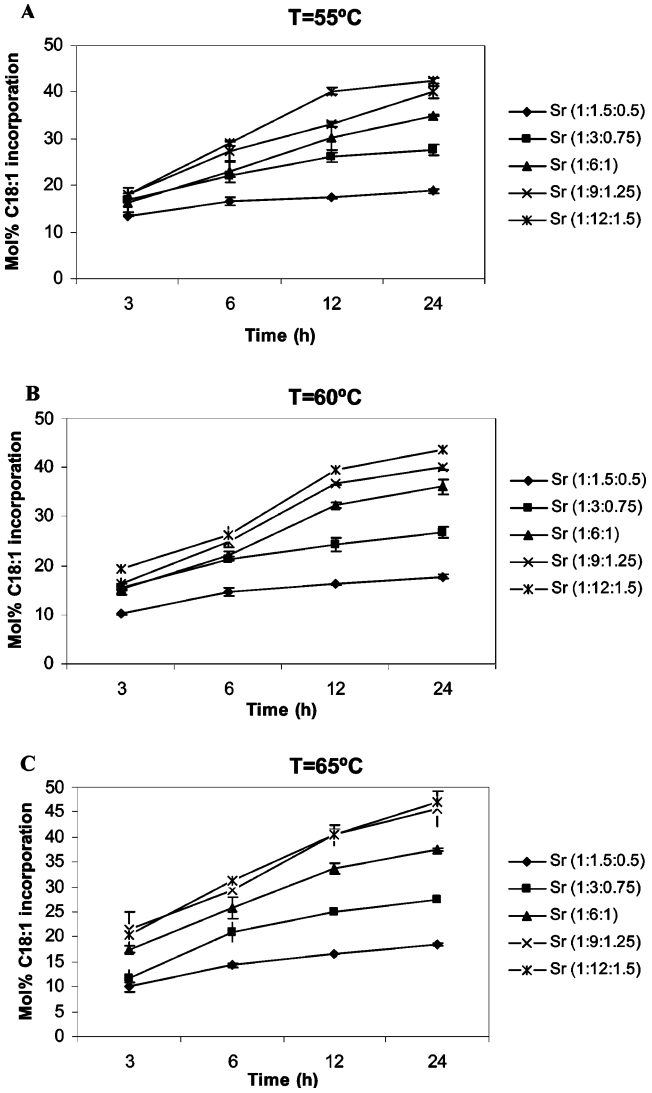

Figure 1. Oleic acid incorporation at substrate molar ratios of 1:1.5:0.5, 1:3:0.75, 1:6:1, 1:9:1.25, and 1:12:1.5 (tripalmitin/hazelnut oil fatty acids/stearic acid). Samples were analyzed at 3, 6, 12, and 24 h (*x*-axis). The *y*-axis represents the mole percent of C18:1 incorporation. Enzyme amount was 10 wt % of total substrates. Incubation was at 200 rpm in *n*-hexane. Incubations were carried out at (**A**) 55 °C, (**B**) 60 °C, and (**C**) 65 °C.

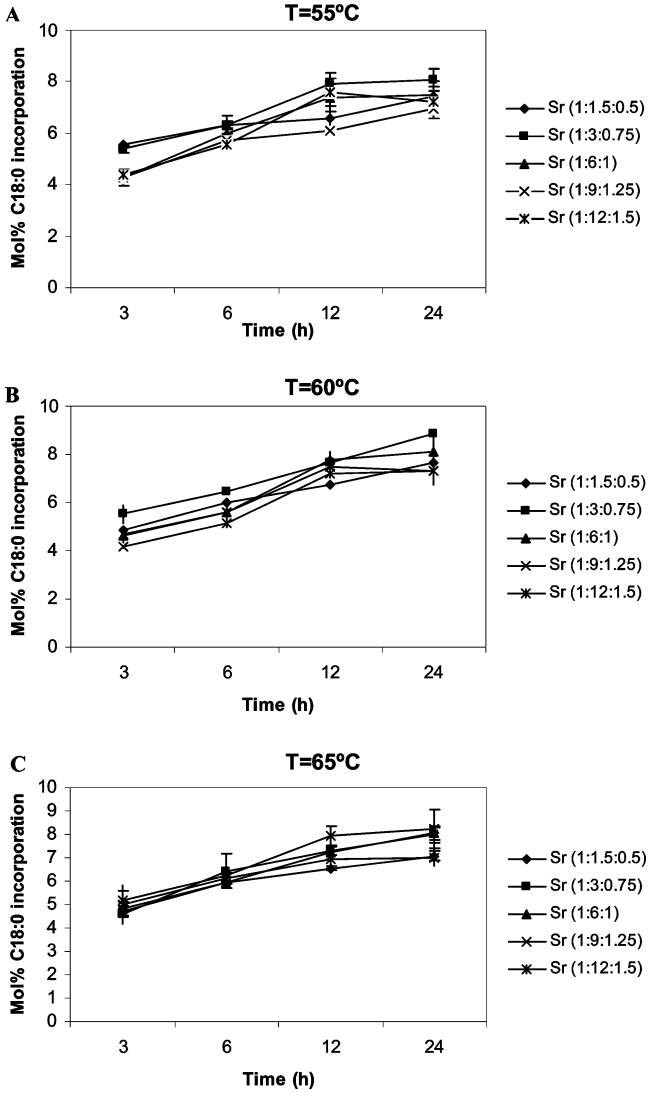

Figure 2. Stearic acid incorporation at substrate molar ratios of 1:1.5:0.5, 1:3:0.75, 1:6:1, 1:9:1.25, and 1:12:1.5 (tripalmitin/hazelnut oil fatty acids/stearic acid). Samples were analyzed at 3, 6, 12, and 24 h (*x*-axis). The *y*-axis represents the mole percent of C18:0 incorporation. Enzyme amount was 10 wt % of total substrates. Incubation was at 200 rpm in *n*-hexane. Incubations were carried out at (**A**) 55 °C, (**B**) 60 °C, and (**C**) 65 °C.

but from a molar ratio of 1:6:1 to 1:9:1.25 the rate of incorporation of oleic acid was higher. Beyond this ratio, the rate of incorporation decreased until 1:12:1.5. A further increase in substrate molar ratio would probably decrease the rate of oleic acid incorporation due to possible inhibition of lipase activity caused by higher substrate concentration (*17*).

Stearic Acid Incorporation. Stearic acid incorporation at different substrate ratios and different reaction times is given in **Figure 2**. The highest incorporation (8.9%) for C18:0 occurred at 60 °C and 24 h of incubation with a substrate molar ratio of 1:3:0.75. The lowest C18:0 incorporation (4.1%) occurred at 60 °C and 3 h of reaction time with the substrate molar ratio of 1:9:1.25 (**Figure 2B**). We observed that, as the reaction time increased, the incorporation level of stearic acid generally increased for all substrate ratios examined. In general, the highest incorporation levels were observed at the substrate molar ratio of 1:3:0.75 (**Figure 2**). The high incorporation level of stearic acid at this substrate molar ratio may be related to the concentration of stearic acid in the reaction mixture. We found that stearic acid incorporation was optimal at the substrate molar ratio of 1:3:0.75 but decreased at a 1:9:1.25 substrate molar ratio and remained constant at a molar ratio of 1:12:1.5. This situtation can be explained by the increase in oleic acid incorporation from hazelnut oil FAs as the substrate molar ratio increased. The enzyme preference for oleic acid at higher substrate molar ratios may explain, in part, the low stearic acid incorporation at these substrate molar ratios.

Oleic Acid/Palmitic Acid Ratio. The ratio of oleic acid to palmitic acid increased with increasing substrate molar ratio and reaction time as observed in oleic acid incorporation. The oleic acid/palmitic acid ratio at different substrate molar ratios and different reaction times is given in **Figure 3**. The highest ratio (1.2) was observed at a 1:12:1.5 substrate molar ratio, 65 °C, and 24 h of reaction time. The lowest ratio (0.1) was obtained with a 1:1.5:0.5 substrate molar ratio at 60 and 65 °C after 3 h of reaction. The low ratio of oleic acid to palmitic acid in the reaction products was due to a low level of oleic acid incorporation into tripalmitin.

ANOVA (two-way) was performed for stearic acid incorporation, oleic acid incorporation, and oleic/palmitic acid ratio

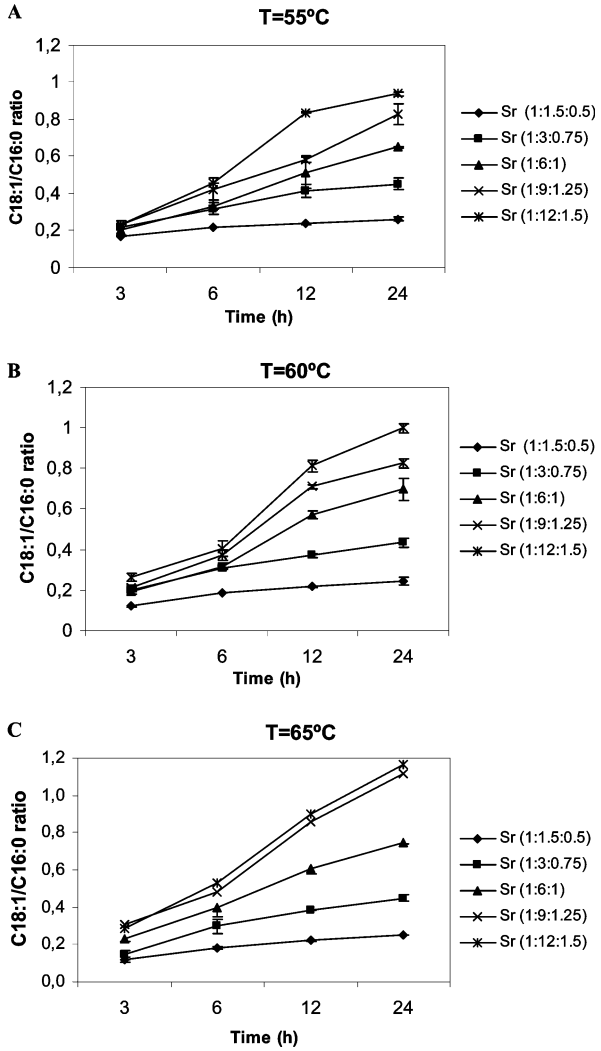

Figure 3. Oleic acid/palmitic acid ratio at substrate molar ratios of 1:1.5:0.5, 1:3:0.75, 1:6:1, 1:9:1.25, and 1:12:1.5 (tripalmitin/hazelnut oil fatty acids/stearic acid). Samples were analyzed at 3, 6, 12, and 24 h (*x*-axis). The *y*-axis represents the C18:1/C16:0 ratio. Enzyme amount was 10 wt % of total substrates. Incubation was at 200 rpm in *n*-hexane. Incubations were carried out at (**A**) 55 °C, (**B**) 60 °C, and (**C**) 65 °C.

results at 24 h of reaction time. The ANOVA indicates that the incorporation of oleic acid and the oleic/palmitic acid ratio at different substrate molar ratios (1:1.5:0.5, 1:3:0.75, 1:6:1, 1:9:1.25, and 1:12:1.5) and at different reaction temperatures (55, 60, and 65 °C) and their interaction seem to be statistically significant because F values for substrate molar ratio, temperature, and their interaction are high compared to the F table values ($F_{4,15}$, $F_{2,15}$, and $F_{8,15}$ values are 3.06, 3.68, and 2.64, respectively, at $\alpha = 0.05$).

The ANOVA for stearic acid incorporation indicates that only the substrate molar ratio is statistically significant ($\alpha = 0.05$) because the F value (7.0) is high compared to the F table value (3.06), whereas temperature and interaction of substrate molar ratio and temperaure are statistically insignificant ($\alpha = 0.05$) because F values (2.6 and 2.0, respectively) are low compared to the F table values (3.68 and 2.64, respectively).

Reaction products having an oleic acid/palmitic acid ratio of 1.2, similar to that of HMF, were considered to meet the "targeted SLs" structure (*3*).

Pancreatic Lipase sn-2 Positional Analysis. The FAs at sn-2 positions of SLs were determined for the reaction products having an oleic to palmitic acid ratio equal to or above a 0.9 limit, which were obtained after 24 h of reaction. The FA composition and the FAs at the sn-2 position of SLs are given in **Table 1**. As can be seen from **Table 1**, SLs contained more than 42.5% oleic, 7% stearic acid, and 5% linoleic acid. Palmitic acid that was found at the sn-2 position of SLs was observed to be between 69.2 and 76%. As the incorporation level of oleic acid and other FAs increased, the content of palmitic acid in the SLs decreased as a result of the action of the sn-1,3-specific lipase, Lipozyme RM IM, which incorporated FAs into sn-1 and sn-3 positions of tripalmitin. The sn-2 positions of the SLs were predominantly occupied by palmitic acid followed by oleic, stearic, and linoleic acids. Despite the specificity of the sn-1,3-specific lipase, incorporation of FAs into acyglycerols at the sn-2 position occurred due to acyl migration during interesterification (*20*).

Table 1. Fatty Acid (FA) Composition (Mole Percent) and FA at sn-2 Position (Mole Percent) of the SLs[a]

	(1)		(2)		(3)		(4)	
FA	FA	FA at sn-2	FA	FA at sn-2	FA	FA at sn-2	FA	FA at sn-2
16:0	45.3	76	43.8	70.9	41.2	69.2	40.5	70.9
18:0	7.2	5.1	7.3	6.4	8.3	5.8	7.0	6.7
18:1	42.5	17.2	43.8	20.7	45.6	22.7	47.1	20.4
18:2	5.0	1.8	5.1	2.0	5.0	2.4	5.4	2.1
C18:1/C16:0	0.9		1.0		1.1		1.2	

[a] Abbreviations: (1), SL produced under the conditions 1/12/1.5 S_r, at 55 °C for 24 h; (2) SL produced under the conditions 1/12/1.5 S_r, at 60 °C for 24 h; (3) SL produced under the conditions 1/9/1.25 S_r, at 65 °C for 24 h; (4) SL produced under the conditions 1/12/1.5 S_r, at 65 °C for 24 h.

Previous papers (*4*, *5*) have provided convincing information about higher fatty acid and calcium absorption and efficient use of dietary energy due to the specific position of palmitic acid at the sn-2 position (70%) and unsaturated FA at the sn-1,3 positions of the TAG moiety. The FA composition and structure of HMF in terms of long-chain FA were successfully reproduced in the HMFS obtained in this study using tripalmitin, hazelnut oil fatty acids, and stearic acid with Lipozyme RM IM as the biocatalyst.

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