Synthesis of Structured Lipid Enriched with Omega Fatty Acids and sn-2 Palmitic Acid by Enzymatic Esterification and Its Incorporation in Powdered Infant Formula

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ABSTRACT: Structured lipid (SL) enriched with arachidonic (ARA) and docosahexaenoic (DHA) acids was produced from tripalmitin using Lipozyme TL IM. The effects of acyl donors, that is, free fatty acids vs fatty acid ethyl esters, on the reactions were compared. The highest total incorporation of ARA and DHA was obtained when the reaction continued for 24 h, at a substrate mole ratio of 9, using free fatty acids as acyl donors (acidolysis). The SL prepared by a large-scale acidolysis reaction contained $17.69 \pm 0.09\%$ total ARA, $10.75 \pm 0.15\%$ total DHA, and $48.53 \pm 1.40\%$ sn-2 palmitic acid. SL thermograms exhibited multiple peaks indicating complexity of the triacylglycerol (TAG) distribution. RP-HPLC analysis of SL revealed nine of 26 TAG molecular species that were similar to those of human milk fat. Powdered infant formulas containing the SL were prepared by wet-mixing/spray-drying and dry-blending methods. Formula prepared with microencapsulated SL and the dry-blending method had better oxidative stability and color quality.

KEYWORDS: infant formula, structured lipid, lipase, tripalmitin, ARA, DHA, microencapsulation

■ INTRODUCTION

The type and content of lipid in infant formula are important, as they provide energy and essential fatty acids for the structure and function of the infant's cell membranes. Traditional infant formulas are formulated with vegetable oils as lipid ingredients to provide a fatty acid composition similar to human milk fat (HMF). Although the similarity could be achieved with a vegetable oil blend, the fat absorption in infants fed with vegetable-oil-based formula is still lower.[1] This lower absorption is due to the differences in the regiospecific structure of the triacylglycerols (TAG) in vegetable oils relative to those in HMF. Greater than 60% of the palmitic acid in human milk is located at the sn-2 position of TAG, whereas in vegetable oils, it is predominantly located at the outer positions (sn-1, 3).[2] Pancreatic lipase specifically hydrolyzes the sn-1, 3 positions of TAG, producing free fatty acids from these outer positions, leaving the attached sn-2 fatty acid on the glycerol backbone (2-monoacylglycerol, 2-MAG). The 2-MAG is a well-absorbed form of most fatty acids since it is conserved and readily forms mixed micelles with bile acids and cannot form insoluble soaps with divalent cations.[1] Structured lipid (SL) designed with palmitic acid esterified at the sn-2 position could therefore provide an alternative source of fat with better nutritional value for infant formula use.

Long-chain polyunsaturated fatty acids (LCPUFAs) including docosahexaenoic acid (DHA, omega-3) and arachidonic acid (ARA, omega-6) are important in neonatal development of the brain and retina. DHA and ARA constitute the majority of the fatty acids in the brain, aiding in the development and protection of neurological functions.[3] Preformed LCPUFAs are found in HMF, but traditionally are not found in infant formulas. Most traditional infant formulas rely on the bioconversion of linoleic acid and alpha-linolenic acid to LCPUFAs; however, the rates for these conversions are very limited.[4] Therefore, an alternative approach would be to use these beneficial LCPUFAs directly in infant formula. Enzymatic modification methods including acidolysis and interesterification have been employed in the production of SLs with LCPUFAs for possible use in infant formula.[5,6]

The addition of LCPUFAs oils into foods is a challenge, as their susceptibility to oxidation and development of off-flavor volatiles affect sensory properties. For microencapsulation of LCPUFA oils used in infant formula, food-grade ingredients suitable for infants and natural ingredients are desired. Milk constituents are widely used encapsulants, as they possess the ability to emulsify, build viscosity, and form gels. Recently, there has been interest in the development of protein–polysaccharide conjugates, made by the Maillard reaction, for applications in food, medicines, and cosmetics.[7] Augustin et al.[8] demonstrated the suitability of heated protein–polysaccharide mixtures for encapsulation of fish oil. In vivo and in vitro studies showed that this microencapsulation method protected fish oil from acidic conditions in the stomach and delivered and released the oil in the small intestine. More importantly, it did not compromise the bioavailability of the fish oil.[9−11]

In this study, SL enriched with sn-2 palmitate, ARA, and DHA was produced and used as lipid ingredient in infant formula. Effects of substrate mole ratio, incubation time, and type of acyl donors, i.e., free fatty acids, FFAs (acidolysis reaction), or free fatty acid ethyl esters, FAEEs (interesterification), on the total incorporation of ARA and DHA by sn-1, 3 specific immobilized Lipozyme TL IM were studied. Infant formulas were prepared using two manufacturing methods for the production of powdered formula (a wet-mixing/spray-drying process vs a dry-blending process). Prior to the dry-

blending process, SL was microencapsulated using heated protein−polysaccharide conjugates as encapsulants. Oxidative stability and color quality of the products were evaluated.

■ MATERIALS AND METHODS

Materials. DHA-rich single-cell oil (DHASCO, 40% DHA) from the algae *Crypthecodinium cohnii* and ARA-rich single-cell oil (ARASCO, 40% ARA) from the fungus *Mortierella alpina* were generously provided by DSM Nutritional Products-Martek (Columbia, MD). Immobilized lipase, Lipozyme TL IM (*sn*-1, 3 specific from *Thermomyces lanuginosus*), was obtained from Novozymes North America Inc. (Franklinton, NC). The specific activity of Lipozyme TL IM was 250 IUN/g (IUN is interesterification units novo). Tripalmitin and internal standard C15:0 pentadecanoic acid (>98% purity) were purchased from Tokyo Chemical Industry America (Montgomeryville, PA). Lipid standards, Supelco 37 component FAME mix, triolein, 2-oleoylglycerol, and ethyl oleate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). TAG standard mix (GLC reference standard) was purchased from Nu-check Prep, Inc. (Elysian, MN). Whey protein isolate (Grander Ultra WPI), maltodextrin (StarDri 100), and corn syrup solid, CSS (StarDri 42 R), were generously donated by Grande Custom Ingredients Group (Lomira, WI) and Tate & Lyle (Decatur, IL). Other ingredients including nonfat dry milk, lactose, and infant formula vitamin and mineral premix were generously donated by O-AT-KA Milk Products Cooperative, Inc. (Batavia, NY), Hilmar Ingredients (Hilmar, CA), and Fortitech, Inc. (Schenectady, NY), respectively.

Preparation of FFAs and FAEEs. DHASCO and ARASCO were mixed at a mole ratio of 1:1 prior to the preparations of FFAs and FAEEs. The mixture contained 26.01 ± 0.35% DHA, 22.56 ± 0.56% ARA, 18.65 ± 0.32% oleic acid, 8.90 ± 0.02% palmitic acid, 5.62 ± 0.02% myristic acid, 4.47 ± 0.03% stearic acid, and 2.56 ± 0.19% lauric acid. Hydrolysis and ethanolysis of the oil mixture were performed according to the methods described by Vázquez and Akoh[12] with some modifications. For hydrolysis, 150 g of oil was saponified using a mixture of KOH (34.5 g), distilled water (66 mL), 96% ethanol (396 mL), and butylated hydroxytoluene (0.03 g). All reagents were placed in a stirred batch bioreactor at 60 °C for 1 h. The reaction was stopped by adding 120 mL of distilled water and acidified to pH 2 to release FFAs. The FFAs were washed, filtered through a sodium sulfate column, and stored in an amber Nalgene bottle under nitrogen at −20 °C until use. For ethanolysis, the reaction was performed by mixing oil with sodium ethoxide (2.625%, v/v) in absolute ethanol at a ratio of 4:2 (v/v) (2.25-fold molar excess of ethanol). The mixture was heated at 60 °C with mechanical shaking for 40 min, under a nitrogen atmosphere. The product was first washed with 100 mL of a saturated NaCl solution and then washed with 100 mL of distilled water. After separation, FAEEs were dried over sodium sulfate, vacuum filtered, and stored similarly to FFAs. FFAs and FAEEs were confirmed by thin-layer chromatography (TLC) analysis using oleic acid and ethyl oleate, respectively, as standards.

Small-Scale Synthesis and Analysis of SL Products. SLs were produced using two types of reactions, acidolysis (with FFAs as substrate) and interesterification (with FAEEs as substrate). The reaction mixtures consisting of hexane (3 mL) and a mixture of FFAs or FAEEs and tripalmitin at different substrate mole ratios (FFAs or FAEEs to tripalmitin at 3, 6, and 9 mol/mol) were placed in screw-capped test tubes. Lipozyme TL IM (10% of total weight of the substrates) was added. The tubes were incubated at 60 °C for 12, 18, and 24 h in an orbital shaking water bath at 200 rpm. The products were collected and passed through a sodium sulfate column to remove moisture and enzyme. All reactions were performed in triplicate. Averages and standard deviations are reported. TLC analysis of product was carried out according to the method described by Lumor and Akoh[13] with modification. Fifty microliters of the reaction product was spotted on a silica gal G TLC plate. Petroleum ether/ethyl ether/acetic acid (80:20:0.5, v/v/v) was used to develop the plates for SL made with FFAs, and a 90:10:0.5 (v/v/v) combination was used for SL made with FAEEs. 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 for fatty acid composition analysis. The TAG sample was converted to fatty acid methyl esters (FAMEs) following AOAC official method 996.01[14] with modification.[5]

Large-Scale Synthesis and Purification of SL. The conditions giving highest incorporation of ARA and DHA were selected for 1 L-scale production of SL. The solvent-free acidolysis reaction was performed in a 1 L stirred batch reactor at 60 °C for 24 h with a substrate mole ratio of 9 (a mixture of FFAs to tripalmitin), 10% (w/w) of Lipozyme TL IM, and constant stirring at 200 rpm. The reactor was wrapped with foil to reduce exposure to light. At the end of the reaction, the resulting SL was vacuum filtered through a Whatman no. 1 containing sodium sulfate and then through a 0.45 μm membrane filter to dry and separate the SL from the enzyme. SL was stored in an airtight amber container under nitrogen at 4 °C. Purification of SL product was performed using short-path distillation followed by alkaline deacidification. Distillation was performed under the following conditions: 60 °C holding temperature; approximately 100 mL/h feeding rate; 170 °C heating oil temperature; 20 °C coolant temperature; and vacuum of <13.33 Pa. Deacidification by alkaline extraction was performed according to the method described by Lee and Akoh[15] with minor modification. Purified SL (10 g) from short-path distillation was mixed with hexane (150 mL), phenolphthalein solution, and 80 mL of 0.5 N KOH in 20% ethanol. The separation was obtained in a separatory funnel, and the upper phase was collected. The upper phase was extracted with another 30 mL of 0.5 N KOH in 20% ethanol and 60 mL of saturated NaCl solution. The hexane phase containing SL was passed through a sodium sulfate column. Hexane was evaporated to obtain the deacidified SL. The deacidification step was completed to obtain sufficient purified SL for further studies (FFAs < 0.1%). The FFA content was determined according to AOCS Official Method Ac 5-41.[16]

Positional Analysis. The pancreatic lipase hydrolysis procedure followed was as described by Pina-Rodriguez and Akoh.[17] Hydrolysis product was extracted with 2 mL of diethyl ether and concentrated with nitrogen. The concentrated extract was spotted on silica gel G TLC plates and developed with a mixture of hexane/diethyl ether/formic acid (60:40:1.6, v/v/v). 2-Oleoylglycerol was spotted in parallel as identification standard for 2-MAG. The bands corresponding to 2-MAG were collected and converted to FAMEs for fatty acid composition analysis as described above.

¹³C NMR Analysis. In addition to pancreatic lipase analysis, the regioisomeric distribution of ARA and DHA was determined by proton-decoupled ¹³C nuclear magnetic resonance (NMR) analysis. The spectrum was collected for a 200 mg sample dissolved in 0.8 mL of 99.8% CDCl$_3$ using continuous ¹H decoupling at 25 °C with a Varian DD 600 MHz spectrometer, equipped with a 3 mm triple resonance cold probe. The data were acquired at a ¹³C frequency of 150.82 MHz using the following acquisition parameters: 56 818 complex data points, spectral width of 37 879 Hz (251 ppm), pulse width 30°, acquisition time 1.5 s, relaxation delay 1 s, and collection of 20 000 scans. Exponential line broadening (1 Hz) was applied before Fourier transforming the data. ¹³C chemical shifts were expressed in parts per million (ppm) relative to CDCl$_3$ at 77.16 ppm.

Melting and Crystallization Profile. Melting and crystallization profiles were determined for tripalmitin, SL, and fat extracted from a commercial infant formula (CIFL) using a differential scanning calorimeter (DSC1 STAR^e System, Mettler-Toledo), cooled with a Haake immersion cooler (Haake EK90/MT, Thermo Scientific). Lipid extraction from infant formula was performed according to Teichart and Akoh.[6] The analysis was performed according to AOCS Official Method Cj 1-94[16] with minor modification using indium as a standard. Sample (8−12 mg) was hermatically sealed in an aluminum pan. The sample was heated from 25 to 80 °C at 50 °C/min, held for 10 min (to destroy any previous crystalline structure), cooled from 80 °C to −55 °C at 10 °C/min (for crystallization profiles), held for 30 min, and then heated from −55 to 80 °C at 5 °C/min (for melting profiles). Melting and crystallization profiles were performed in duplicate.

TAG Molecular Species. A reversed-phase HPLC (Agilent Technologies 1100) with an evaporative light scanning detector (ELSD), Sedex 55, was used to analyze the TAG molecular species of SL and CIFL on an ultrasphere C18, 250 mm, 4.6 mm, 5 μm particle size column. The column temperature was kept at 30 °C. The ELSD conditions were 70 °C, 3.0 bar, and gain of 7.[18] Sample concentration was 5 mg/mL in chloroform. The sample injection volume was 20 μL. The eluent was a gradient of acetonitrile (A) and acetone (B) at a solvent flow rate of 1 mL/min with a gradient of 0 min, 65% B; 55 min, 95% B; and 65 min, 65% B and post-run of 10 min. The equivalent carbon number (ECN) method was used to predict the elution order of TAG. Standard TAG mixtures containing trilinolenin (ECN = 36), trilinolein (42), triolein (48), tripalmitin (48), tristearin (54), and triarachidin (60), as well as palm olein, were chromatographed to help determine the TAG species.

Infant Formula Preparation. SL-containing infant formulas were prepared using two general manufacturing methods: (1) a wet-mixing/spray-drying process and (2) a dry-blending process.[19,20] For the wet-mixing/spray-drying process, nonfat dry milk (20 g), whey protein isolate (10 g), lactose (31 g), maltodextrin (30 g), and water (800 mL) were mixed at 50−60 °C. To the mixture were added SL (30 g) and vitamin/mineral premix (3.9 g), and it was homogenized using a high-speed benchtop homogenizer (Brinkmann Kinematica Polytron, Switzerland). The sample was passed through a high-pressure homogenizer (Avestin Emulsiflex-C5, Canada) in two steps at 35 MPa and subsequently at 10 MPa, pasteurized at 65 °C for 30 min, then spray-dried using a Buchi-290 mini spray dryer (Switzerland). Two different combinations of spray-drying inlet−outlet temperature (120−70 °C vs 180−80 °C) were used. The effects of these drying temperatures on product qualities were compared.

For the dry-blending process, prior to the blending step, SL was encapsulated, following the method described by Augustin et al.[8] with minor modifications. Briefly, whey protein isolate (21 g) was reconstituted in 350 mL of water at 60 °C. Corn syrup solids (42 g) was added, and the pH of the protein−polysaccharide mixture was adjusted to 7.5 using a 1 M NaOH solution. The mixture was heated in a water bath at 90 °C for 30 min and cooled to 60 °C before the addition of SL (21 g). SL from 4 °C storage was heated to 60 °C. The oil was dispersed into the aqueous protein−polysaccharide encapsulant mixture using a benchtop homogenizer. The pre-emulsion was passed through a high-pressure homogenizer (Avestin Emulsiflex-C5, Canada) in two steps at 35 MPa and subsequently at 10 MPa. The homogenized emulsion was spray-dried at an inlet temperature of 180 °C and outlet temperature of 80 °C. The microencapsulated SL (120 g) was then dry-blended with the ingredients listed above except for water.

Lipid Oxidation and Color Measurement of Infant Formulas. Lipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS) were measured according to the method of Klinkesorn et al.[21] with minor modification. Formula powder (100 mg) was reconstituted in 0.3 mL of distilled water, instead of acetate buffer, and 1.5 mL of isooctane/2-propanal (3:1 v/v) was added. The sample was vortexed for 10 s three times followed by centrifugation at 3400g for 2 min. The organic phase (0.2 mL) was added to 2.8 mL of methanol/butanol (2:1 v/v), followed by 15 μL of thiocyanate solution (3.94 M) and 15 μL of ferrous iron solution (prepared by mixing 0.132 M BaCl$_2$ and 0.144 M FeSO$_4$ in acidic solution). The solution was vortexed, and the absorbance at 510 nm was measured after 20 min. Lipid hydroperoxide concentrations were determined from a cumene hydroperoxide standard curve.

For TBARS, the formula sample (5 mg) was reconstituted with 1 mL of distilled water followed by an addition of 2 mL of thiobarbituric (TBA) solution. TBA solution was prepared by mixing 15 g of trichoroacetic acid, 0.375 g of TBA, 1.76 mL of 12 N HCl, and 82.9 mL of distilled water. One hundred milliliters of TBA solution was mixed with 3 mL of 2% butylated hydroxytoluene in ethanol prior to use. The sample mixture was vortexed and heated in a boiling water bath for 15 min, cooled to room temperature, and centrifuged at 3400g for 25 min. Absorbance of supernatant was measured at 532 nm. The concentration of TBARS was determined from a standard curve

prepared with 1,1,3,3-tetraethoxypropane (malonaldehyde diethyl acetal).

For color measurement, the L^*, a^*, b^* values were measured using a Minolta color analyzer. Chroma C^* and hue angle h^* were calculated from a^* and b^* values. The mathematic C^* and h^* are defined as $C^* = [a^{*2} + b^{*2}]^{1/2}$ and $h^* = \arctan[b^*/a^*]$.[22] All data represent an average of six measurements of two different trials, and results are reported as average and standard deviation of these measurements.

Statistical Analysis. The statistical significance of differences between samples was calculated using analysis of variance (ANOVA) and posthoc Tukey's test at a significance level of $p < 0.05$ using IBM SPSS Statistics 19.

■ RESULTS AND DISCUSSION

The effects of substrate mole ratio of acyl donors (FFA or FAEE mixture), tripalmitin, reaction time, and type of acyl donors on the incorporation of ARA and DHA were determined. In Figure 1, it can be seen that as the reaction
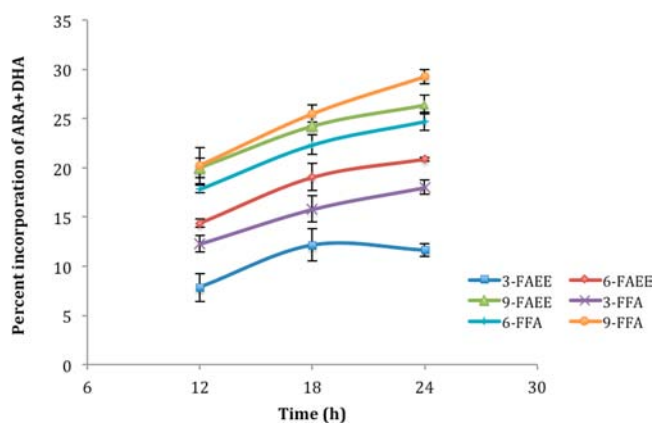

Figure 1. Percent total incorporation of ARA and DHA via acidolysis (FFAs as substrate) and interesterification (FAEEs as substrate) using different substrate mole ratios (3−9 mol acyl donor:1 mol tripalmitin) and different incubation times (12−24 h) at 60 °C.

time and substrate mole ratio increase, the total incorporation of ARA and DHA also increases. Increasing reaction times led to increased incorporation of LCPUFAs, as longer residence times allowed for prolonged contact between the enzyme and the substrates.[23] Sahin et al.[24] observed an increase in omega-3 PUFA (DHA and EPA) incorporation into tripalmitin as the reaction time increased from 18 to 30 h with increasing substrate mole ratio. In our previous study, the total incorporation of ARA and DHA also increased as reaction time (12−24 h) and substrate mole ratio (6−18 mol/mol) increased.[5] The total incorporation of ARA and DHA was significantly higher when the substrate mole ratio was 9 for both interesterification and acidolysis reactions ($p < 0.05$). The highest total incorporation for interesterification (26.38 ± 0.97%) and for acidolysis (29.27 ± 0.74%) was obtained when the reaction continued for 24 h at 60 °C at a substrate mole ratio of 9. At these conditions, the incorporations of ARA and DHA were significantly higher when FFAs (acidolysis) were used as substrate compared to FAEEs (interesterification) ($p < 0.05$). Similarly, Lumor and Akoh[13] reported a higher incorporation of LCPUFAs (GLA, an omega-6 LCPUFA) when FFAs were used as acyl donors compared to FAEEs in reactions catalyzed by sn-1, 3 specific lipase Lipozyme RM IM (donor organism: *Rhizomucor miehei*) at 45, 55, and 65 °C. At a molecular level, the interesterification process involves

Table 1. Fatty Acid Composition (%) of Structured Lipid (SL) Produced via Acidolysis of Tripalmitin and a Mixture of FFAs from DHA and ARA-Rich Single-Cell Oils, Compared to Fat Extracted from a Commercial Infant Formula (CIFL)

fatty acid	SL[a]			CIFL[c]		
	total	sn-2	sn-1, 3	total	sn-2	sn-1, 3
C12:0	1.94 ± 0.01	3.00 ± 0.13	1.12 ± 0.10	9.53 ± 0.04	13.94 ± 0.23	7.32 ± 0.06
C14:0	5.09 ± 0.02	4.84 ± 0.14	5.23 ± 0.12	4.42 ± 0.02	3.24 ± 0.08	5.01 ± 0.06
C16:0	36.70 ± 0.11	48.53 ± 1.40	30.91 ± 0.83	23.80 ± 0.04	6.02 ± 0.45	32.62 ± 0.29
C18:0	4.29 ± 0.02	4.03 ± 0.03	4.43 ± 0.02	4.00 ± 0.03	5.65 ± 0.27	3.17 ± 0.45
C18:1 ω-9	15.28 ± 0.03	9.82 ± 0.12	18.06 ± 0.04	32.55 ± 0.15	42.40 ± 0.27	27.62 ± 0.09
C18:2 ω-6	2.89 ± 0.02	1.83 ± 0.01	3.43 ± 0.03	19.21 ± 0.02	26.18 ± 0.38	15.72 ± 0.16
C18:3 ω-3	nd[b]	nd	nd	1.65 ± 0.01	1.29 ± 0.01	1.83 ± 0.01
C18:3 ω-6	0.83 ± 0.01	0.19 ± 0.00	1.16 ± 0.01	nd	nd	nd
C20:4 ω-6	17.69 ± 0.09	9.73 ± 0.13	21.73 ± 0.04	0.80 ± 0.00	0.80 ± 0.12	0.80 ± 0.06
C22:6 ω-3	10.75 ± 0.15	4.80 ± 0.03	13.76 ± 0.20	0.39 ± 0.00	0.49 ± 0.01	0.34 ± 0.00

[a]Fatty acids found in trace amounts were C8:0, C10:0, C17:0, C20:0, C20:1 n-9, C22:0, C20:3 n-6, C22:5 n-3, and C24:0. [b]nd = not detected. [c]CIFL= fat extracted from a commercially available infant formula enriched with ARA and DHA by physical blending. Other fatty acids found in trace amounts were C8:0, C10:0, C20:0, C20:1, C22:0, and C24:0.
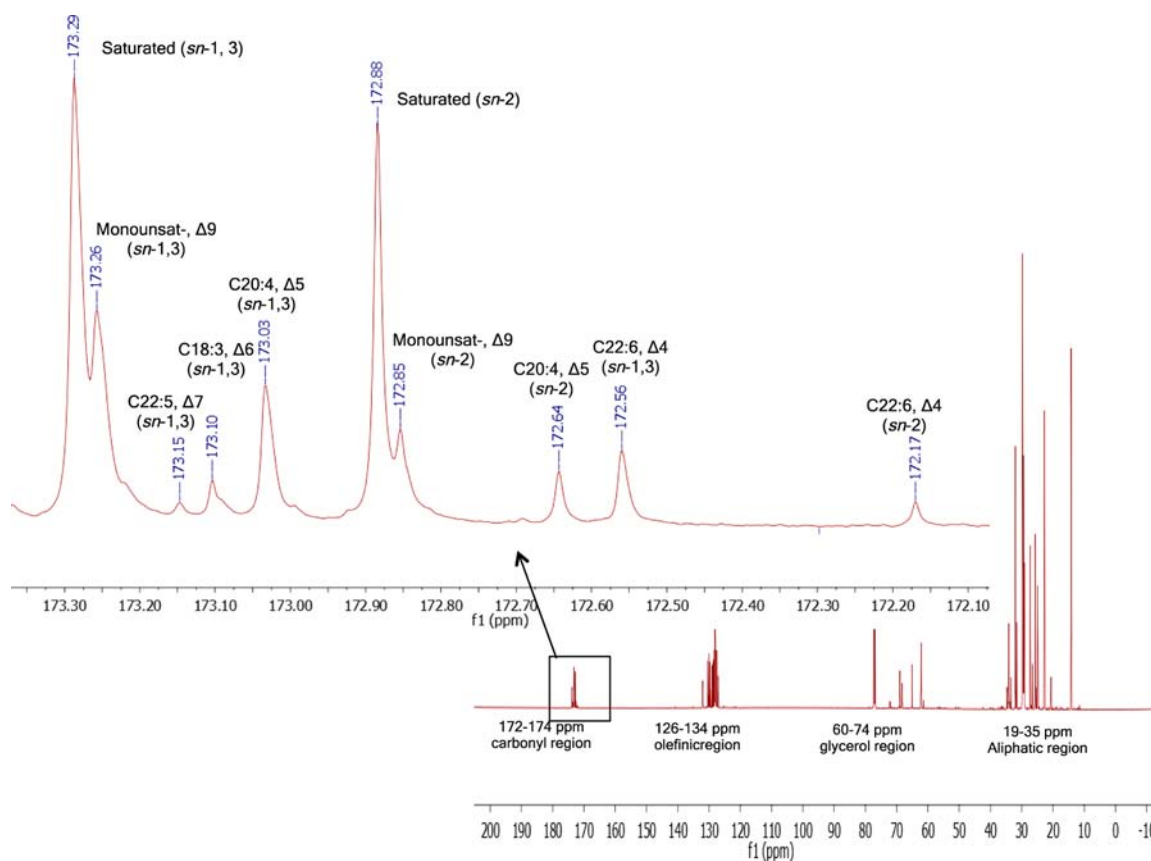
Figure 2. Carbonyl region of the broad band decoupled ^{13}C NMR spectrum of SL. The assignment of sn-1, 3 and sn-2 regioisomeric peaks to individual fatty acids is annotated.

hydrolysis of the ester molecule followed by an esterification reaction. Hydrolysis of fatty acid ethyl esters produces ethanol in the reaction, which induces a loss in enzyme activity.[25] This possibly complicated the process and led to a lower incorporation of ARA and DHA in the interesterification batch compared to the acidolysis batch.

The conditions that gave the highest ARA and DHA incorporations were used to scale-up acidolysis reaction in a 1 L stirred batch reactor. Purified SL product was obtained through short-path distillation followed by alkaline deacidification. The FFA content of purified SL was 0.01 ± 0.02%. The fatty acid

composition and positional distribution of the SL and CIFL are shown in Table 1. The major fatty acids found in SL were palmitic (36.77 ± 0.11%), ARA (17.69 ± 0.09%), oleic (15.28 ± 0.03%), DHA (10.75 ± 0.15%), and myristic (5.0.9 ± 0.02%) acids. Positional analysis showed that the sn-2 position of SL contained 48.53 ± 1.40% palmitic, 9.82 ± 0.12% oleic, 9.73 ± 0.13% ARA, and 4.80 ± 0.03% DHA. The presence of ARA and DHA at the sn-2 position was possibly due to acyl migration from sn-1, 3 to the sn-2 position during the reaction. Better absorption of palmitic acid was shown with infant formulas rich in palmitic acid esterified at the sn-2 position compared to
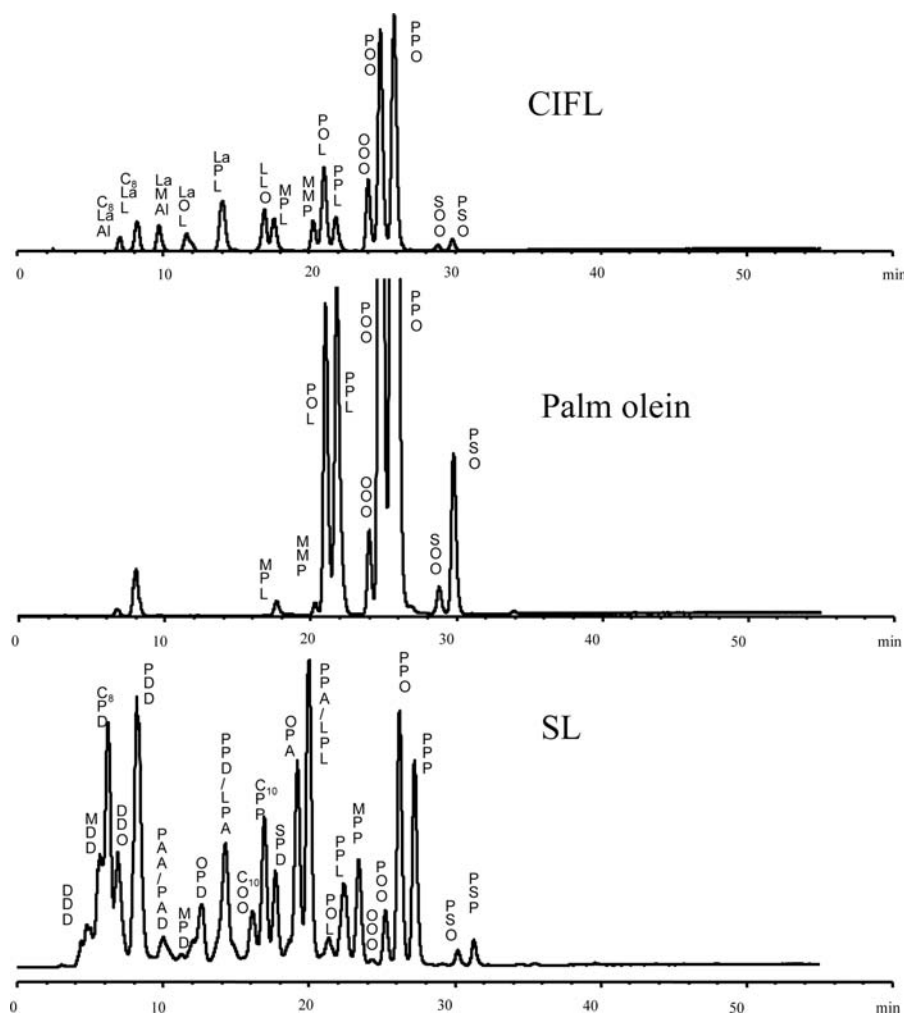
Figure 3. TAG molecular species of palm olein, CIFL, and SL determined by reversed-phase HPLC. Annotated TAG species do not reflect stereochemical configuration. C_8, caprylic acid; C_{10}, capric acid; La, lauric acid; M, myristic acid; P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Al, alpha-linolenic acid; A, arachidonic acid (ARA); D, docosahexaenoic acid (DHA).

formulas containing palmitic acid largely esterified to the sn-1, 3 positions.[26] SL appears to provide a similar level of sn-2 palmitic acid (48.53%) to that of human milk fat (51.17–52.23%).[27] The vegetable oil blend used in the commercial infant formula, as listed on the label, contained palm olein, soy, coconut, and high-oleic safflower or high oleic sunflower oils. Position analysis revealed that the content of sn-2 palmitic acid from CIFL (6.02%) was much lower than from SL. It is known that palmitic acids in vegetable oils are predominantly located at the sn-1, 3 positions, which lead to a lower fat and calcium absorption in infants fed with vegetable oil-based formulas.[2] The level of ARA in CIFL was 0.08 ± 0.00% and DHA was 0.39 ± 0.01%. CIFL must contain ARA and DHA as a physical blend. The SL produced in the current research could be used in an oil blend to increase the sn-2 palmitic acid, ARA, and DHA contents.

Positional distribution of fatty acids in SL was also determined by ^{13}C NMR spectroscopy. The chemical shift of the carbonyl carbon of fatty acids in TAGs depends on the regiospecific position (sn-1, 3 or sn-2), and for the carbonyl carbon of an unsaturated fatty acid, the chemical shift also depends on the position and number of double bonds in the chain.[26] Different carbon atoms give signals in different regions of the ^{13}C NMR spectrum. The spectrum of SL is shown in

Figure 2. The region where carbonyl carbons (C1 atoms) give signals is between 172 and 174 ppm. Assignments of resonances were made according to previous studies[29,30] on fish lipids and the fact that the distance between the sn-1, 3 and sn-2 chains is approximately 0.4 ppm.[31] The spectrum showed that saturated fatty acids, monounsaturated ω-9 fatty acids, DHA, and ARA were esterified at the sn-2 position.

TAG molecular species were determined using reversed-phase HPLC. Peak identifications were made according to published works involving palm oil and palm olein,[32,33] elution time of TAG standards, and the fact that TAG species are eluted in order of equivalent carbon number (ECN) = TC − 2 × DB. TC is the total carbon number of the acyl group, and DB is the total number of double bonds in TAG.[34] Figure 3 shows RP-HPLC chromatograms of palm olein, CIFL, and SL. Table 2 shows a comparison between TAG species and their relative percentages in the three lipid samples. PPO (61.01, 26.09%) and POO (34.23, 23.70%) constituted the majority of TAGs in both palm olein and CIFL, respectively. These TAGs were also found in the SL; however their abundances were much lower (PPO = 9.97%, POO = 1.80%). Recently, the TAG species composition of colostrum fat and transitional and mature milk fat was determined by RP-HPLC.[35] Twenty-two different TAG species were found in these milk samples, and the majority

Table 2. TAG Molecular Species of SL, CIFL, and Palm Olein Determined by RP-HPLC According to Their ECN^a

SL			CIFL			palm olein		
TAG species^b	ECN (DB)	% area	TAG species	ECN (DB)	% area	TAG species	ECN (DB)	% area
DDD	30 (18)	1.52	C$_8$LaAl	32 (3)	1.38	MPL	44 (2)	0.06
MDD	34 (12)	1.36	C$_8$LaL	34 (2)	3.46	MMP	44 (0)	0.04
C$_8$PD	34 (6)	7.40	LaMAl	38 (3)	2.87	POL	46 (3)	1.50
DDO	36 (13)	2.91	LaOL	42 (3)	2.67	PPL	46 (2)	1.76
PDD	36 (12)	15.36	LaPL	42 (2)	6.72	OOO	48 (3)	0.35
PAA/PAD	40 (8)/38 (10)	1.12	LLO	44 (5)	4.49	POO	48 (2)	34.23
MPD	40 (6)	0.18	MPL	44 (2)	3.74	PPO	48 (1)	61.01
OPD	42 (7)	3.22	MMP	44 (0)	3.02	SOO	50 (2)	0.13
PPD/LPA	42 (6)	7.38	POL	46 (3)	9.42	PSO	50 (1)	0.91
C$_{10}$OO	42 (2)	1.96	PPL	46 (2)	4.00			
C$_{10}$PP	42 (0)	5.39	OOO	48 (3)	6.78			
SPD	44 (6)	3.07	POO	48 (2)	23.70			
OPA	44 (5)	7.35	PPO	48 (1)	26.09			
PPA/LPL	44 (4)	12.33	SOO	50 (2)	0.56			
POL	46 (3)	0.65	PSO	50 (1)	1.12			
PPL	46 (2)	3.54						
MPP	46 (0)	3.82						
OOO	48 (3)	0.17						
POO	48 (2)	1.80						
PPO	48 (1)	9.97						
PPP	48 (0)	7.66						
PSO	50 (1)	0.55						
SPP	50 (0)	1.02						

^{a}Equivalent carbon number (ECN) = TC − 2 × DB; TC is total carbon number of acyl group and DB is total number of double bonds in TAG. ^{b}C$_8$, caprylic acid; C$_{10}$, capric acid; La, lauric acid; M, myristic acid; P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Al, alpha-linolenic acid; A, arachidonic acid (ARA); D, docosahexaenoic acid (DHA). TAG species do not reflect stereochemical configuration.
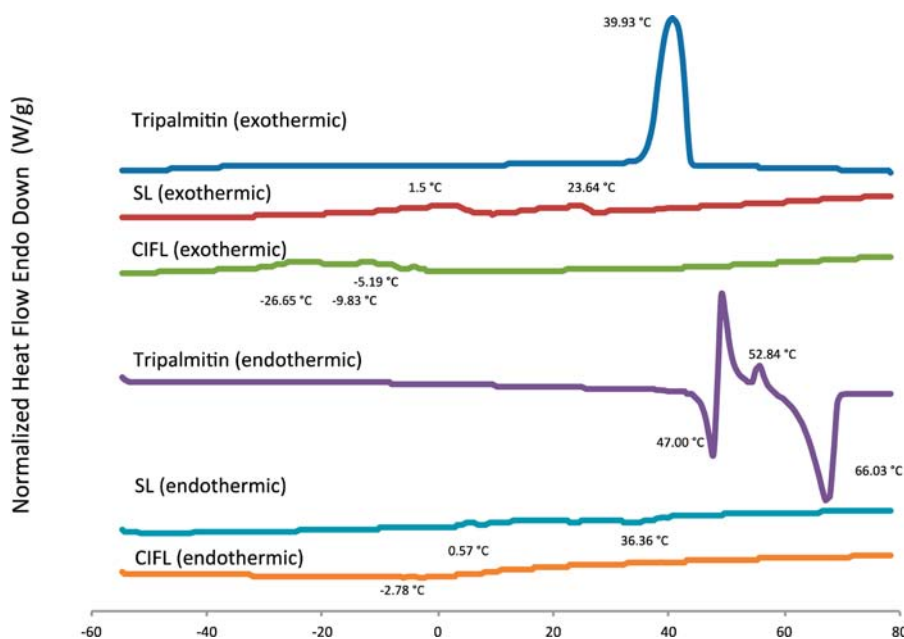

Figure 4. Crystallization (exothermic) and melting (endothermic) profile of tripalmitin, SL, and CIFL.

included POO (21.51 ± 5.39%), POL (16.93 ± 3.27%), and POLa (10.39 ± 3.02%). SL made in this study contained 26 different TAG species. Major TAG species in SL include PDD (15.36%), PPA or LPL (12.33%), PPO (9.97%), PPP (7.66%), C$_8$PD (7.40%), PPD or LPA (7.38%), and OPA (7.35%). Nine TAG species identified in the SL, including POO, OOO, POL, PPL, PPP, PPO, PSO, MPP, and SPP, were reported as HMF TAGs in the study by Zou et al.;[35] however, the amounts were considerably different. Most of the SL TAGs contained more than 3 DBs in their structures, and four contained no DB (PPP = 7.66%, C$_{10}$PP = 5.39%, SPP = 1.02%, and MPP = 3.82%). The variety of TAG species, fatty acid chain length, and degree of saturation were shown to affect the melting and crystallization profile of fat and oil.[36,37]

The melting and crystallization behaviors of SL were compared with its substrate, tripalmitin, and fat extracted

Table 3. Characterization of Powdered Infant Formulas[a]

characteristics of powdered infant formulas	wet-mixing/spray-drying at 120 °C	wet-mixing/spray-drying at 180 °C	dry-blending	commercial infant formula
peroxide (μg/mg sample)	0.18 ± 0.02 b	0.37 ± 0.02 a	0.07 ± 0.02 c	0.06 ± 0.03 c
TBARS (μg/mg sample)	0.06 ± 0.01 b	0.11 ± 0.01 a	0.04 ± 0.01 c	0.05 ± 0.01 c
color				
L^*	94.59 ± 2.46 b	95.75 ± 0.53 b	98.83 ± 0.49 a	96.35 ± 2.34 a,b
a^*	−2.28 ± 0.08 a	−3.12 ± 0.05 b	−3.80 ± 0.08 c	−5.21 ± 0.0.05 d
b^*	16.37 ± 0.34 b,c	15.30 ± 0.79 c	17.71 ± 0.55 b	19.29 ± 0.65 a
C^*	17.64 ± 0.36 b,c	16.74 ± 1.14 c	18.83 ± 0.33 b	21.25 ± 1.89 a
h^*	97.24 ± 0.32 c	100.30 ± 0.76 b	101.28 ± 0.31 b	103.89 ± 1.04 a

[a]Mean ± SD, $n = 6$; means with the same letter in the same row and category are not significantly different ($p > 0.05$).

from CIFL (Figure 4). Tripalmitin is a highly saturated TAG and melts at 66 °C. SL has lower melting points and a broader melting range, around 37 to −25 °C. The melting point of human milk fat is below 38 °C.[38] Both SL and CIFL thermograms exhibited multiple peaks, indicating the complexity of the TAG distribution. This was also shown as multiple peaks in the chromatogram from the analysis of TAG molecular species. The presence of palmitic acid in the TAGs of SL and highly saturated TAG species (PPP, C_{10}PP, SPP, and MPP) contributed to the higher temperature melting peak at 36.36 °C. Highly saturated TAGs including SPP, MPP, PPP, and SMM were also found in human milk fat samples (colostrum, transitional, and mature milk fat).[35] However, the amounts of these TAGs were rather low (with a content of <1% or in the range 1−5%). This suggested the use of this SL as a complementary fat in infant formulas with a blend containing unsaturated oils rather than a substitute for a vegetable oil blend. Crystallization thermogram showed an onset of crystallization at −6 °C, ending at 26 °C for SL. CIFL had a lower melting range of −30 to −3 °C.

Powdered infant formula is manufactured using two general types of processes: a dry-blending and a wet-mixing/spray-drying process.[19] Some manufacturers also use a combination of these processes to spray-dry the base powder (protein and fat component) then dry-blend with carbohydrate, vitamin, and mineral ingredients. To determine which process is suitable for SL application in powdered infant formula, infant formulas were prepared with SLas, the fat source using these two general processes, and the products were evaluated for oxidative stability and color scores (L^*, a^*, b^*, C^*, and h^*). PV measures the ability of lipid hydroperoxides (primary oxidation products) to oxidize ferrous ions to ferric ions, which form a red-violet complex with thiocyanate. The TBARS test measures secondary oxidation products, which form a pink color when reacted with thiobarbituric reagent. The results of these analyses of infant formulas are shown in Table 3. The dry-blending process yielded products with significantly lower PV and TBARS values compared to the wet-mixing/spray-drying process. The PV and TBARS values of dry-blended infant formulas and of the commercial infant formula were not significantly different. The higher temperature (180 °C) used in the wet-mixing/spray-drying resulted in significantly higher PV and TBARS values compared to the lower temperature of 120 °C. The color score, L^* for lightness and C^* for chroma or saturation, showed a negative correlation with PV and TBARS values (Table 3). The color of products with higher PV and TBARS values (wet-mixing/spray-drying products) was less saturated (lower C^*), meaning that the color looked dull and grayish. These products

were also darker, with lower L^* values. The hue color values of all infant formulas fall between yellow and green.

According to the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), infant formulas should provide 60−70 kcal/100 mL.[39] The preparation of infant formula in this study was aimed at a formulation that contributes 60−70 kcal/100 mL resulting from 3.3 to 6.0% fat, 1.2−3.0% protein, and 5.4−8.1% carbohydrates. Microencapsulated SL contained 25% fat (SL), 25% protein (WPI), and 50% carbohydrate (CSS). Microencapsulation of SL increased the stability of the final product; however, the energy contributed from carbohydrate and protein used as encapsulant increased the product energy contribution by 35 kcal/100 mL (Table 4). The new formulation needs adjustment in the

Table 4. Energy Contribution (in 100 mL of resuspended formula)

ingredients	composition (g)	energy contribution (kcal)
nonfat milk (fat 0%, protein 34.8%, carbohydrate 52.2%)	2	6.98
WPI (fat 0.6%, protein 92%, carbohydrate 0.5%)	1	3.93
lactose	3.1	12.4
maltodextrin	3	12
SL	3	27
total energy contribution (wet-mixing/spray-drying method)		*62.31*
microencapsulated SL (fat 25%, protein 25%, carbohydrate 50%)	12	
total energy contribution (microencapsulated SL, dry-blending)		*97.35*

protein and carbohydrate ingredients. At this stage of the study, microencapsulation with more than 25% oil is being studied to improve the use of microencapsulated SL on energy contribution in the formulation.

SL was prepared from tripalmitin and FFAs derived from DHASCO and ARASCO, in an acidolysis reaction using Lipozyme TL IM as biocatalyst. Physical and chemical analyses suggested its application in an oil blend for use in infant formula. This SL could provide a fat source with physiologically important fatty acids and serve as a good source of sn-2 palmitic acid, which can improve fat and calcium absorption. Powdered infant formulas containing SL were prepared by a wet-mixing/spray-drying and dry-blending process. SL was microencapsulated using heated protein−polysaccharide conjugates prior to dry-blending to obtain SL in dried powder form. Infant formula

prepared by the dry-blending process with microencapsulated SL had a better oxidative stability and color quality.

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Notes

The authors declare no competing financial interest.

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