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Enzymatic Synthesis of Extra Virgin Olive Oil Based Infant Formula Fat Analogues Containing ARA and DHA: One-Stage and Two-Stage Syntheses

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ABSTRACT: Structured lipids (SLs) with high palmitic acid content at the *sn*-2 position enriched with arachidonic acid (ARA) and docosahexaenoic acid (DHA) were produced using extra virgin olive oil, tripalmitin, ARA and DHA single cell oil free fatty acids. Four types of SLs were synthesized using immobilized lipases, Novozym 435 and Lipozyme TL IM, based on one-stage (one-pot) and two-stage (sequential) syntheses. The SLs were characterized for fatty acid profile, triacylglycerol (TAG) molecular species, melting and crystallization profiles, tocopherols, and phenolic compounds. All the SLs had >50 mol % palmitic acid at the *sn*-2 position. The predominant TAGs in all SLs were PPO and OPO. The total tocopherol content of SL1-1, SL1-2, SL2-1, and SL2-2 were 70.46, 68.79, 79.64, and 79.31 μg/g, respectively. SL1-2 had the highest melting completion (42.0 °C) and crystallization onset (27.6 °C) temperatures. All the SLs produced in this study may be suitable as infant formula fat analogues.

KEYWORDS: *ARA, DHA, extra virgin olive oil, infant formula, structured lipid, tripalmitin*

INTRODUCTION

Maternal breast milk is universally considered a gold standard of nutrition for infants.[1] Human milk is a complex mix of nutrients and bioactive compounds that provides balanced nutrition and also helps in building immunity. Although human breast milk is the preferred choice of nutrition for the infants, in certain cases when the mother cannot or chooses not to, or if the milk production is not sufficient, infant formulas are the ideal nutritional alternative to human breast milk. Lipids are an important constituent of human milk providing not only ∼50% energy but also essential fatty acids (EFAs) and fat-soluble vitamins. The total lipid content of human breast milk varies (3−5%), of which 98% are triacylglycerols (TAGs).[2] Human milk is a source of the EFAs linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) as well as their long-chained derivatives arachidonic (ARA, 20:4n-6) and docosahexaenoic acids (DHA, 22:6n-3). In infants, the conversion of LA and ALA to ARA and DHA, respectively, is not efficient enough to meet the requirements; therefore, most of the infant formulas are supplemented with preformed ARA and DHA. Although long chain polyunsaturated fatty acids (LC-PUFAs) account for a very small proportion of human milk fat (<1%, individually), they play an important role in proper development of the infant, especially DHA (0.32 ± 0.22%) and ARA (0.47 ± 0.13%).[3] Bioavailability of EFAs and LC-PUFAs are critical during infancy for proper brain growth and functioning, cognitive skills, motor skills, sensory functions, and neurological reflexes.[4]

In human milk, palmitic acid (16:0) is predominantly esterified at the *sn*-2 position (>50%); whereas vegetable oils or cows' milk fat contain most of their palmitic acid in the outer positions of the TAG molecules.[5] This unique fatty acid distribution of human milk TAGs greatly affects their digestion, absorption, and metabolism. All fatty acids in the *sn*-1,3 positions of TAGs are hydrolyzed during digestion, in contrast to only 22% of fatty acids in the *sn*-2 position.[6] As a result of pancreatic lipase, fatty acids at *sn*-1,3 positions are released as FFAs. If palmitic acid is predominantly esterified at *sn*-1,3 positions, it is released as free palmitic acid. At the alkaline pH of the intestine, free palmitic acid readily forms insoluble soaps with divalent cations such as calcium and magnesium, and they are excreted as hard stool. This results in unavailability of both palmitic acid and minerals to the infants.[7] Higher palmitic acid absorption has been observed in human milk compared to infant formulas[5] and also in formulas rich in *sn*-2 palmitic acid content than in formulas in which palmitic acid was mainly esterified at *sn*-1,3 positions. This has been observed in both term[8] and preterm infants.[9] Also, *sn*-2 palmitic acid rich infant formulas may improve calcium absorption.[10]

Lipids (usually TAGs) that have been structurally modified from their natural form by changing the fatty acids and/or their position, or synthesized to yield novel TAGs with desired functional and nutritional properties, are called structured lipids (SLs).[11] Positional specific TAGs suitable as infant formula fats analogues can be synthesized using lipases which are regio-and stereospecific. SLs containing palmitic acid at the *sn*-2 position are an excellent substrate for infant formula. Betapol (Loders Croklaan, Chanhannon, IL) was the first commercially available enzymatically synthesized SL for use in infant formulas.

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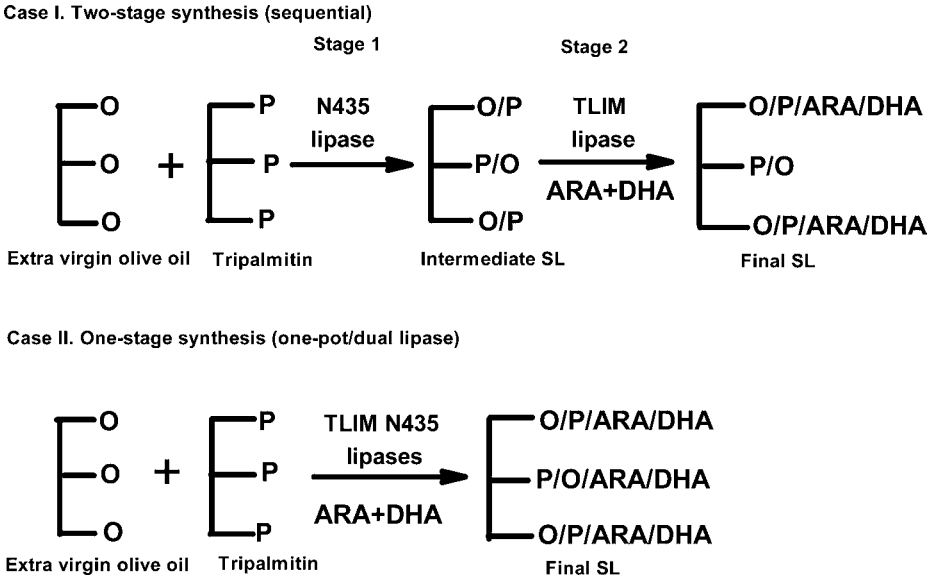

Figure 1. Reaction schemes: two-stage and one-stage syntheses. Two-stage synthesis consists of 2 stages. Stage 1 (interesterification): TP:EVOO were reacted using nonspecific lipase Novozym 435. Stage 2 (acidolysis): Stage 1 product was filtered and further reacted with AD using *sn*-1,3 specific lipase Lipozyme TL IM . In one-stage synthesis all the substrates and both lipases were added together. TP, tripalmitin. EVOO, extra virgin olive oil. AD, mix of ARASCO-FFA and DHASCO-FFA (2:1).

Although Betapol has high *sn*-2 palmitic acid, it lacks LC-PUFAs. Therefore, novel SLs with palmitic acid at the *sn*-2 position and also enriched with LC-PUFAs are required for optimal growth and development of the infant. Most symmetrical SLs are produced using a two-stage process with sequential addition of nonspecific and/or *sn*-1,3 specific lipases.[12−17] First, the acyl moiety at the *sn*-2 position is modified followed by *sn*-1,3 regioselective acylations. Few studies have been done on simultaneous use of multiple lipases for SL synthesis. Ibrahim et al. used a dual lipase system for interesterification of palm stearin and coconut oil.[18] They concluded that certain lipases when mixed in specific ratios have a synergistic effect and dual lipase system may be used for increased degree of interesterification. Türkan and Kalay also suggested the use of dual lipase reaction system instead of a single enzymatic system in biodiesel production.[19]

The objective of this study was to produce extra virgin olive oil (EVOO) based SLs containing high *sn*-2 palmitic acid and enriched with DHA and ARA as possible human milk fat analogues for infant formula application. For this we employed both a one-stage (one-pot/dual lipase) synthesis and a two-stage (sequential) synthesis. To the best of our knowledge, this is the first study on infant formula fat analogues based on EVOO and containing ARA and DHA using both syntheses. This may help to expand the application of EVOO in infant nutrition.

MATERIALS AND METHODS

Materials. Extra virgin olive oil (EVOO) was provided by Al Jouf Agricultural Development Corporation (Al-Jouf Skaka, Saudi Arabia) while ARASCO and DHASCO were kindly provided by DSM Nutritional Products (Columbia, MD). The immobilized enzymes, Lipozyme TL IM (*Thermomyces lanuginosus* lipase, *sn*-1,3 specific, specific activity 250 IUN/g: IUN = interesterification unit) and Novozym 435 (*Candida antarctica* lipase, nonspecific, specific activity 10,000 PLU/g: PLU = propyl laurate unit) were purchased from Novozymes North America Inc. (Franklinton, NC). Supelco 37 Component FAME mix, tocopherol standards, 2-oleoylglycerol, pinoresinol, gallic, ferulic, *p*-coumaric, and caffeic acids were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Hydroxytyrosol was purchased from Cayman Chemical Company (Ann Arbor, MI). Vanillic acid and tyrosol were obtained from Oakwood Products Inc. (West Columbia, SC). Luteolin and oleuropein were purchased from Indofine Chemical Company (Hillsborough, NJ). Tripalmitin and pentadecanoic acid was procured from TCI America (Portland, OR). The TAG standard mix (GLC-437) was purchased from Nu-chek Prep, Inc. (Elysian, MN). Other solvents and chemicals were from Fisher Scientific (Norcross, GA) and Sigma-Aldrich Chemical Co.

Preparation of Free Fatty Acids (FFAs) from ARASCO and DHASCO by Saponification. Saponification value (SV) was calculated based on AOCS Official Method Cd 3a-94.[20] Fatty acid profile was used to calculate the molecular weight (MW) of the substrates. The MW (g/mol) of ARASCO and DHASCO was 912.69 and 881.30, respectively. The SV (mg of KOH/g) of ARASCO and DHASCO was 183.67 and 186.18, respectively. FFAs were prepared according to a previously described method.[21] The FFAs (ARASCO-FFA and DHASCO-FFA) were mixed in the ratio of 2:1 w/w and flushed with nitrogen. This FFA mixture was termed AD and stored at −80 °C in an amber Nalgene bottle until use.

SL Synthesis. SLs were synthesized in Erlenmeyer flasks in a solvent free environment. Two types of reaction schemes were used (Figure 1). Two-stage synthesis (case I) consisted of a sequential two-stage SL synthesis. In the first stage tripalmitin and EVOO were reacted in the presence of Novozym 435. Novozym 435, which is mostly considered as a nonspecific lipase, was used in the first stage with the aim of increasing palmitic acid at the *sn*-2 position of EVOO TAGs. The product of the first stage was then filtered (to remove enzyme), and AD (ARASCO-FFA and DHASCO-FFA, 2:1) was added. The acidolysis reaction was then catalyzed by Lipozyme TL IM, an *sn*-1,3 specific lipase, to incorporate ARA and DHA into the TAG structure while conserving the palmitic acid at the *sn*-2 position. In one-stage synthesis (case II), tripalmitin, EVOO, and AD were reacted together in the presence of Lipozyme TL IM and Novozym 435 lipases. The aim was to achieve similar product as in case I and to determine if the dual enzyme system had any synergistic effect. Carrying out multiple reactions (interesterification and acidolysis) simultaneously in the presence of dual biocatalysts may help reduce the reaction time, eliminate intermediate purification steps, and result in an improved SL synthesis process. The substrate molar ratios (tripalmitin:EVOO:AD) used were 0.5:1:0.5 and 1:1:0.5. The reaction temperature was fixed at 60 °C. Preliminary small-scale reactions were

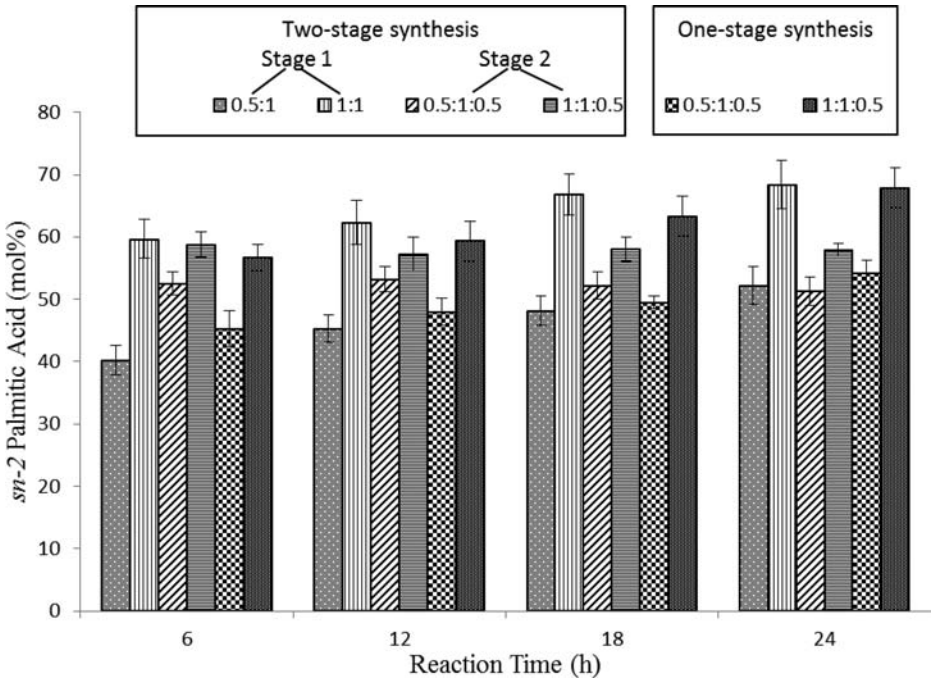

Figure 2. Small-scale study for run time optimization. Two-stage synthesis consists of 2 stages. Stage 1: TP:EVOO (0.5:1, 1:1) using Novozym 435 (10%). Stage 2: TP:EVOO(stage 1 product):AD (0.5:1:0.5, 1:1:0.5) using Lipozyme TL IM (10%). In one-stage synthesis all the substrates and enzymes were added together. TP:EVOO:AD (0.5:1:0.5, 1:1:0.5) using Novozym 435 (10%) and Lipozyme TL IM (10%) lipase. TP, tripalmitin. EVOO, extra virgin olive oil. AD, mix of ARASCO-FFA and DHASCO-FFA (2:1).

performed at 6, 12, 18, and 24 h for reaction time selection. Figure 2 shows the *sn*-2 palmitic acid content of the small-scale reaction products. Based on the small-scale results, the following conditions were selected for scale-up:

Case I. Two-Stage (Sequential) Synthesis.

SL1-1: The substrate molar ratio was 0.5:1 (tripalmitin:EVOO) and the incubation was 24 h using Novozym 435 as biocatalyst. The reaction product was filtered to remove the lipase. No further purification was done prior to the addition of the second lipase. The product was then reacted with AD for 6 h in the presence of Lipozyme TL IM lipase. The final ratio was 0.5:1:0.5 (tripalmitin:EVOO:AD).

SL1-2: Similar to SL1-1 except a substrate molar ratio of 1:1:0.5 (tripalmitin:EVOO:AD) was used and the run time for both first stage and second stage was 6 h each.

Case II. One-Stage (One-Pot) Synthesis.

SL2-1: The substrate molar ratio was 0.5:1:0.5 (tripalmitin:EVOO:AD) and the reaction time was 24 h using Novozym 435 and Lipozyme TL IM lipases as biocatalysts.

SL2-2: The substrate molar ratio used was 1:1:0.5 (tripalmitin:EVOO:AD) for 6 h using Novozym 435 and Lipozyme TL IM lipases as biocatalysts.

Each enzyme was added at 10% of the total weight of substrates. The Erlenmeyer flasks were kept in a water bath shaker at 200 rpm for the specified time and temperature. After the reaction, the extra FFAs were removed through deacidification by alkaline extraction method[22] and the purified SLs were stored at −20 °C until analysis.

Total and Positional Fatty Acids. Lipid samples were converted to fatty acid methyl esters following the AOAC Official Method 996.01[23] and analyzed with a Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) using a Supelco SP-2560, 100 m × 25 mm × 0.2 μm column. *sn*-2 positional fatty acid composition was determined following the AOCS Official Method Ch 3-91.[24] Fatty acid composition at the *sn*-1,3 position can be calculated using the following equation:

$$sn\text{-}1,3\ (\%) = [3 \times \text{total}\ (\%) - sn\text{-}2\ (\%)]/2$$

All experiments were conducted in triplicate, and average values reported.

Triacylglycerol (TAG) Molecular Species. The TAG composition was determined with a high-performance liquid chromatograph (HPLC) (Agilent Technologies 1260 Infinity, Santa Clara, CA) equipped with a Sedex 85 evaporative light scattering detector (ELSD) (Richard Scientific, Novato, CA). A Beckman Ultrasphere C18 column, 5 μm, 4.6 × 250 mm, was used with temperature set at 30 °C. The injection volume was 20 μL. The mobile phase at a flow rate of 1 mL/min consisted of solvent A, acetonitrile, and solvent B, acetone:methyl *tert*-butyl ether (90:10, v/v). A gradient elution was used starting with 35% solvent A to 5% solvent A at 42 min and then returning to the original composition in 3 min. Drift tube temperature was set at 50 °C, pressure at 4.0 bar and gain at 8. The samples were dissolved in chloroform with final concentration of 5 mg/mL. The TAG peaks were identified by comparison of retention times with those of the standards and also by equivalent carbon number (ECN). ECN is defined as CN − 2*n*, where CN is the number of carbons in the TAG (excluding the three in the glycerol backbone) and *n* is the number of double bonds. Triplicate determinations were made and averaged.

Tocopherols. HPLC (Shimadzu LC-6A pump equipped with an RF-10AXL fluorescence detector with excitation set at 290 nm and emission at 330 nm (Shimadzu Corp., Columbia, MD)) was used for tocopherol analysis. An isocratic mobile phase of 0.85% (v/v) isopropanol in hexane was used at a flow rate of 1.0 mL/min. The normal phase column was a LiChrosorb Si 60 column (4 mm, 250 mm, 5 μm particle size, Hiber Fertigsaeule RT, Merck, Darmstadt, Germany). The sample concentration was 20 mg/mL in HPLC-grade hexane. Injection volume was 20 μL. The tocopherols were identified by comparing their retention times with those of authentic standards (1.25−20 μg/mL in hexane containing 0.01% butylated hydroxytoluene). Tocopherols were quantified based on the standard calibration curves and reported as μg/g from the average of triplicate determinations.

Major Phenolic Compounds. Phenolics were extracted with methanol, water, and acetonitrile using solid phase extraction.[25] Major phenolic compounds were determined following the method described

Table 1. Total and Positional Fatty Acid Composition (mol %) of Substrates[a]

	EVOO[b]				
fatty acid	total	*sn*-2	tripalmitin	ARASCO-FFA[c]	DHASCO-FFA[d]
C8:0	nd[e]	nd	nd	nd	0.30 ± 0.00
C10:0	nd	nd	nd	nd	1.15 ± 0.02
C12:0	nd	nd	0.09 ± 0.00	nd	4.46 ± 0.07
C14:0	nd	nd	0.47 ± 0.03	0.45 ± 0.00	10.30 ± 1.02
C16:0	16.02 ± 1.21	2.31 ± 0.66	98.90 ± 1.02	8.39 ± 0.77	9.91 ± 0.79
C18:0	2.50 ± 0.62	0.10 ± 0.00	1.02 ± 0.00	8.12 ± 1.05	0.83 ± 0.04
C18:1n-9	67.81 ± 2.85	82.20 ± 2.95	nd	20.52 ± 1.86	22.17 ± 1.05
C18:2n-6*t*	nd	nd	nd	0.20 ± 0.00	nd
C18:2n-6*c*	9.56 ± 0.89	14.67 ± 1.73	nd	6.67 ± 0.59	1.04 ± 0.00
C18:3n-6	nd	nd	nd	0.82 ± 0.00	nd
C18:3n-3	0.75 ± 0.00	0.22 ± 0.00	nd	0.37 ± 0.00	nd
C20:3n-6	nd	nd	nd	1.89 ± 0.02	nd
C20:3n-3	0.89 ± 0.01	nd	nd	3.80 ± 0.01	nd
C20:4n-6	nd	nd	nd	43.22 ± 1.28	nd
C22:6n-3	nd	nd	nd	nd	44.13 ± 1.06
minor[f]	2.47	0.00	0.11	5.56	3.98

[a]Each value is the mean of triplicates ± standard deviation. [b]EVOO, extra virgin olive oil. [c]ARASCO-FFA, arachidonic acid single cell oil free fatty acids. [d]DHASCO-FFA, docosahexaenoic acid single cell oil free fatty acids. [e]nd, not detected. [f]Minor is the sum C14:1, C16:1, C17:0, C20:0, C20:1, C20:2, C22:0, C22:2, C24:0, and C24:1.

Table 2. Fatty Acid Composition (mol %) of the Structured Lipids[a]

	total				*sn*-2			
fatty acid	SL1-1[b]	SL1-2[c]	SL2-1[d]	SL2-2[e]	SL1-1	SL1-2	SL2-1	SL2-2
C8:0	nd[f]	nd	0.50 ± 0.00 a	0.60 ± 0.00 a	nd	nd	nd	nd
C10:0	1.04 ± 0.00 a	1.02 ± 0.00 a	1.11 ± 0.07 b	0.70 ± 0.02 c	nd	nd	nd	nd
C12:0	0.21 ± 0.00 a	0.18 ± 0.00 a	0.44 ± 0.00 b	0.42 ± 0.00 b	nd	nd	nd	nd
C14:0	1.97 ± 0.03 a	2.11 ± 0.21 a	2.47 ± 0.55 b	2.54 ± 0.22 b	nd	1.12 ± 0.00 a	1.44 ± 0.00 b	1.24 ± 0.01 c
C16:0	36.69 ± 2.12 a	44.23 ± 2.87 b	35.23 ± 1.78 c	40.07 ± 1.93 d	52.67 ± 3.07 a	56.25 ± 2.65 b	50.33 ± 2.84 c	55.34 ± 2.22 d
C16:1n-7	1.03 ± 0.07 a	0.87 ± 0.01 b	0.85 ± 0.00 b	0.84 ± 0.00 b	nd	nd	0.87 ± 0.00 a	0.73 ± 0.00 b
C18:0	2.82 ± 0.03 a	2.58 ± 0.05 a	3.19 ± 0.48 b	3.02 ± 0.45 b	nd	nd	2.34 ± 0.01 a	1.97 ± 0.00 b
C18:1n-9	43.22 ± 2.11 a	38.64 ± 2.06 b	38.08 ± 2.23 b	37.10 ± 1.99 c	39.64 ± 1.64 a	34.85 ± 1.98 b	34.48 ± 1.82 bc	33.50 ± 1.83 c
C18:2n-6	6.34 ± 0.04 a	5.29 ± 0.98 b	5.79 ± 0.45 b	4.09 ± 0.56 c	6.06 ± 0.78 a	6.34 ± 0.82 a	4.38 ± 0.79 b	3.91 ± 0.57 c
C20:0	0.29 ± 0.00 a	0.23 ± 0.00 a	0.33 ± 0.00 b	0.28 ± 0.00 a	nd	nd	nd	nd
C18:3n-6	0.08 ± 0.00 a	0.06 ± 0.00 a	0.46 ± 0.01 b	0.45 ± 0.01 b	nd	nd	nd	nd
C18:3n-3	0.47 ± 0.00 a	0.39 ± 0.00 b	0.33 ± 0.01 c	0.30 ± 0.00 c	nd	nd	nd	nd
C22:0	0.16 ± 0.00 a	0.12 ± 0.00 b	0.28 ± 0.00 c	0.27 ± 0.02 c	nd	nd	nd	nd
C20:3n-3	0.16 ± 0.00 a	0.11 ± 0.00 b	0.55 ± 0.00 c	0.54 ± 0.01 c	nd	nd	nd	nd
C20:4n-6	3.67 ± 0.21 a	2.97 ± 0.11 b	6.23 ± 0.96 c	5.95 ± 0.33 d	2.25 ± 0.02 a	1.09 ± 0.03 b	4.93 ± 0.22 c	4.13 ± 0.21 c
C22:6n-3	1.53 ± 0.05 a	1.39 ± 0.72 b	3.71 ± 1.01 c	2.60 ± 0.29 d	0.78 ± 0.00 a	0.83 ± 0.00 a	2.41 ± 0.01 b	2.05 ± 0.03 b
minor[g]	0.33 ± 0.00 a	0.30 ± 0.03 a	0.52 ± 0.02 b	0.53 ± 0.00 b				
n-6/n-3	4.72	4.45	2.78	3.14	10.65	8.96	3.86	3.92

[a]Each value is the mean of triplicates ± standard deviation. Values with different letter in each row within total and *sn*-2 columns separately are significantly different at $P \leq 0.05$. [b]SL1-1, structured lipid synthesized using two-stage synthesis with substrate molar ratio 0.5:1:0.5 (TP:EVOO:AD). [c]SL1-2, structured lipid synthesized using two-stage synthesis with substrate molar ratio 1:1:0.5 (TP:EVOO:AD). [d]SL2-1, structured lipid synthesized using one-stage synthesis with substrate molar ratio 0.5:1:0.5 (TP:EVOO:AD). [e]SL2-2, structured lipid synthesized using one-stage synthesis with substrate molar ratio 1:1:0.5 (TP:EVOO:AD). AD, ARASCO-FFA and DHASCO-FFA (2:1). [f]nd, not detected. [g]Minor is the sum of C17:0, C20:1, C20:2, and C22:2.

by Owen et al.[26] using a Hewlett-Packard (Avondale, PA) HP 1100 HPLC system with diode array detector. The column was Beckman Ultrasphere C18, 5 μm, 4.6 × 250 mm, with temperature set at 40 °C. The injection volume was 20 μL. The mobile phase consisted of solvent A, 2% acetic acid in water, and solvent B, methanol, at a flow rate of 1 mL/min. Gradient elution was as follows: at 2 min 5% solvent B, 10 min 25% B, 20 min 40% B, 30 min 50% B, and 100% B at 45 min. Detection was done at 260, 280, 320, and 360 nm. Identification was based on the retention times and characteristic UV spectra, and quantification was done using the external standard curves. All analysis was performed in triplicate and average reported.

Melting and Crystallization Profiles. The melting and crystallization profiles were determined using a differential scanning calorimeter DSC 204 F1 Phoenix (NETZSCH Instruments North America, Burlington, MA) following AOCS Official Method Cj 1-94.[27] 10−12 mg samples were weighed into aluminum pans and hermetically sealed. Samples were rapidly heated to 80 °C at 20 °C/min, and held for 15 min to destroy any previous crystalline structure. The samples were then cooled to −75 °C at 5 °C/min (exotherms), held for 30 min, and finally heated to 80 °C at 5 °C/min (endotherms). Nitrogen was used as the protective and purge gas. All samples were analyzed in triplicate and average values reported.

Table 3. Relative Percent (%) of Triacylglycerol (TAG) Molecular Species of Extra Virgin Olive Oil and Structured Lipids[a]

TAG	EVOO[b]	SL1-1[c]	SL1-2[d]	SL2-1[e]	SL2-2[f]
OAO	nd[g]	0.98 ± 0.02 a	0.75 ± 0.01 b	1.21 ± 0.03 c	0.74 ± 0.00 b
APA	nd	2.56 ± 0.69 a	1.78 ± 0.08 b	6.11 ± 1.04 c	5.24 ± 0.28 d
OPD	nd	1.40 ± 0.11 a	1.59 ± 0.04 b	2.36 ± 0.83 c	2.14 ± 0.19 c
ODO	nd	0.33 ± 0.00 a	0.36 ± 0.01 a	1.20 ± 0.04 b	0.98 ± 0.00 c
LOL	6.59 ± 1.21	nd	nd	2.07 ± 0.21 a	1.44 ± 0.21 b
LPL	1.97 ± 0.67	1.46 ± 0.01 a	1.14 ± 0.01 b	0.66 ± 0.00 c	0.87 ± 0.00 d
MPL	nd	0.84 ± 0.00 a	0.72 ± 0.00 b	2.35 ± 0.11 c	0.88 ± 0.01 a
POLn	nd	2.13 ± 0.18 a	1.50 ± 0.00 b	6.03 ± 0.28 c	4.56 ± 0.38 d
SMM	nd	nd	nd	1.44 ± 0.01 a	2.59 ± 0.19 b
OOL	10.20 ± 1.55	3.22 ± 0.21 a	1.68 ± 0.01 b	2.11 ± 0.19 c	2.02 ± 0.02 c
POL	nd	6.28 ± 1.01 a	4.68 ± 0.33 b	6.08 ± 2.03 a	4.34 ± 0.27 b
PLP	0.74 ± 0.01	2.53 ± 0.46 a	3.42 ± 0.19 b	2.32 ± 0.79 a	2.37 ± 0.68 a
PPM	nd	1.12 ± 0.06 a	1.11 ± 0.07 a	0.88 ± 0.04 ab	0.67 ± 0.00 b
OOO	47.19 ± 3.08	8.32 ± 0.78 a	6.12 ± 1.06 b	7.64 ± 1.44 c	6.83 ± 1.79 b
OPO	25.37 ± 2.18	25.17 ± 2.51 a	28.84 ± 2.11 b	23.00 ± 2.18 c	25.96 ± 2.79 a
PPO	2.81 ± 0.22	31.35 ± 2.49 a	33.95 ± 2.98 b	24.82 ± 1.59 c	28.64 ± 2.91 d
PPP	nd	4.50 ± 1.62 a	10.32 ± 1.70 b	4.02 ± 0.58 a	6.23 ± 1.04 c
OOS	4.47 ± 0.67	1.83 ± 0.29 a	0.86 ± 0.28 b	1.38 ± 0.00 ac	1.15 ± 0.00 c
POS	0.66 ± 0.01	4.31 ± 0.01 a	2.05 ± 0.29 b	3.80 ± 0.02 c	3.65 ± 0.00 c
PPS	nd	0.82 ± 0.00 a	0.68 ± 0.00 b	0.78 ± 0.00 a	0.82 ± 0.00 a

[a]The fatty acids are not in regiospecific order. A, arachidonic acid. D, docosahexaenoic acid. L, linoleic acid. Ln, linolenic acid. M, myristic acid. O, oleic acid. P, palmitic acid. S, stearic acid. Each value is the mean of triplicates ± standard deviation. Values with different letter in each row for SLs are significantly different at $P \leq 0.05$. [b]EVOO, extra virgin olive oil. [c]SL1-1, structured lipid synthesized using two-stage synthesis with substrate molar ratio 0.5:1:0.5 (TP:EVOO:AD). [d]SL1-2, structured lipid synthesized using two-stage synthesis with substrate molar ratio 1:1:0.5 (TP:EVOO:AD). [e]SL2-1, structured lipid synthesized using one-stage synthesis with substrate molar ratio 0.5:1:0.5 (TP:EVOO:AD). [f]SL2-2, structured lipid synthesized using one-stage synthesis with substrate molar ratio 1:1:0.5 (TP:EVOO:AD). AD, ARASCO-FFA and DHASCO-FFA (2:1). [g]nd, not detected.

Statistical Analysis. All analyses were performed in triplicate. Statistical analysis was performed with the SAS software package (SAS Institute, Cary, NC). Duncan's multiple-range test was performed to determine the significant difference ($P \leq 0.05$) between SLs.

RESULTS AND DISCUSSION

Total and Positional Fatty Acid Profiles. Table 1 shows the total and positional fatty acids of the substrates. The major fatty acids in EVOO were oleic (67.81 mol %) and palmitic acids (16.02 mol %). Tripalmitin contained 98.90 mol % palmitic acid. The major fatty acids in DHASCO-FFA were DHA (44.13 mol %), oleic (22.17 mol %), and myristic (10.30 mol %) acids, and in ARASCO-FFA, ARA (43.22 mol %) and oleic acid (20.52 mol %) were the main fatty acids. In SL1-1, oleic (43.22 mol %) and palmitic (36.69 mol %) acids were the major fatty acids (Table 2). The main fatty acids in human milk are oleic (28.30−43.83%), palmitic (15.43−24.46%), and linoleic (10.61−25.30%) acids.[28] SL1-1 and SL1-2 had 3.67 and 2.97 mol % ARA, respectively, and 1.53 and 1.39 mol % DHA, respectively. On the other hand, SL2-1 had 6.23 mol % ARA and 3.71 mol % DHA. 5.95 mol % ARA and 2.60 mol % DHA were incorporated in SL2-2. ARA and DHA are important fatty acids in infancy as they support brain development and improve visual acuity. A lower n-6/n-3 ratio is desirable for reducing the risk of several chronic diseases. SL1-1, SL1-2, SL2-1, and SL2-2 n-6/n-3 ratios were 4.72, 4.45, 2.78, and 3.14, respectively.

TAGs containing high *sn*-2 palmitic acid are preferred in human milk fat analogues as it helps in fat digestion and absorption. All the SLs had >50% palmitic acid at the *sn*-2 position. *sn*-2 palmitic acid increased from 2.31 mol % in EVOO (Table 1) to 52.67, 56.25, 50.33, and 55.34 mol % in SL1-1, SL1-2, SL2-1, and SL2-2, respectively (Table 2). The SLs were also enriched with DHA and ARA at the *sn*-2 position, where they can be better metabolized. Higher levels of DHA were found in the brain of newborn rats fed with oils containing DHA at the *sn*-2 position than those fed with oils containing randomly distributed DHA.[29] Although Lipozyme TL IM is an *sn*-1,3 specific enzyme, some ARA and DHA were also esterified to the second position of the TAGs in the two-stage synthesis (SL1-1 and SL1-2) where both enzymes were added separately and sequentially. This may be attributed to acyl migration. Acyl migration is an undesirable side reaction involving migration of acyl groups from *sn*-1,3 to *sn*-2 positions and vice versa,[30] but in this case it was desirable since fatty acids at *sn*-2 positions are better absorbed. Acyl migration mainly occurs due to the presence of partial acylglycerols, specifically diacylglycerols, which are the intermediates in enzymatic interesterification reactions.[31] Acyl migration can be affected by a number of factors. Acyl migration increases with increase in reaction temperature, run time, water content, and water activity.[31] The type of enzyme and its carrier also have an effect on acyl migration.[31] It has also been observed that the tendency to migrate increases with increasing unsaturation in fatty acids.[32] In one-stage synthesis (SL2-1 and SL2-2), since both enzymes were added at the same time, the presence of ARA and DHA at the *sn*-2 position can be attributed to the action of either enzyme.

The target (>50% palmitic acid at *sn*-2 position) was achieved at a lesser run time in one-stage synthesis than in two-stage synthesis. This may be beneficial to the industry in terms of cost. The total reaction times in SL1-1 and SL2-1 were 30 and 24 h, respectively. In the case of SL1-2 and SL2-2, the reaction run times were 12 and 6 h, respectively. Compared to SL1-1 and SL2-1, higher total palmitic acid was found when using higher substrate molar ratio of 1:1:0.5 in both two-stage

Table 4. Tocopherol Content (μg/g) of Extra Virgin Olive Oil and Structured Lipids[a]

	α-tocopherol	α-tocotrienol	β-tocopherol	γ-tocopherol	δ-tocopherol
EVOO[b]	212.34 ± 4.78	16.38 ± 2.11	4.02 ± 1.02	17.79 ± 1.68	3.28 ± 1.02
SL1-1[c]	51.83 ± 2.99 a	7.62 ± 1.09 a	2.67 ± 0.68 a	6.36 ± 1.03 a	1.98 ± 0.79 a
SL1-2[d]	50.90 ± 2.16 a	7.23 ± 1.21 b	2.37 ± 0.79 a	7.11 ± 1.01 b	1.18 ± 0.49 b
SL2-1[e]	58.84 ± 1.97 b	8.10 ± 1.77 c	2.03 ± 0.88 b	8.02 ± 0.93 c	2.61 ± 0.68 c
SL2-2[f]	56.96 ± 2.41 c	8.74 ± 1.69 c	2.11 ± 0.82 b	8.97 ± 1.11 d	2.53 ± 0.39 c

[a]Each value is the mean of triplicates ± standard deviation. Values with different letter in each column are significantly different at $P \leq 0.05$. [b]EVOO, extra virgin olive oil. [c]SL1-1, structured lipid synthesized using two-stage synthesis with substrate molar ratio 0.5:1:0.5 (TP:EVOO:AD). [d]SL1-2, structured lipid synthesized using two-stage synthesis with substrate molar ratio 1:1:0.5 (TP:EVOO:AD). [e]SL2-1, structured lipid synthesized using one-stage synthesis with substrate molar ratio 0.5:1:0.5 (TP:EVOO:AD). [f]SL2-2, structured lipid synthesized using one-stage synthesis with substrate molar ratio 1:1:0.5 (TP:EVOO:AD). AD, ARASCO-FFA and DHASCO-FFA (2:1).

(44.23 mol % in SL1-2) and one-stage syntheses (40.07 mol % in SL2-2). In one-stage synthesis, lower saturated fats and higher ARA and DHA were found compared to the two-stage synthesis. Under the reaction parameters used in this study, there seems to be a synergistic effect when using the two enzymes simultaneously. Similarly, a synergistic effect on enzymatic interesterification has been observed previously when using Lipozyme TL IM and Novozym 435 together in equal ratios.[18]

TAG Molecular Species. The TAG molecular species are shown in Table 3. The fatty acids in the TAG molecular species analyzed are not in a regiospecific order. The main TAG of EVOO, triolein (OOO), decreased from 47.19% to 8.32, 6.12, 7.64, and 6.83% in SL1-1, SL1-2, SL2-1, and SL2-2, respectively. PPO and OPO (a combination of *sn*-OPO and *sn*-POO) were the predominant TAGs in the SLs. SL1-1 had 31.35% PPO and 25.17% OPO. In SL1-2, PPO (33.95%) was followed by OPO (28.84%). SL2-1 and SL2-2 had 23.00 and 25.96% OPO, respectively. Compared to OOP, OPO is better metabolized and absorbed in infants.[33] The major TAG molecular species found in human milk are OPO (17.56−42.44%), POL (9.24−38.15%), OOO (1.61−11.96%), and LOO (1.64−10.18%).[34] All the SLs had OPO, OOO, and LOO within this range, but POL was lower than that found in human milk fat. The stereospecificity and chain lengths of fatty acids at the *sn*-1, *sn*-2, and *sn*-3 positions in TAG species determine the metabolic fate of dietary fat during digestion and absorption. Tripalmitin (PPP), which is one of the starting substrates, was also found in the SLs. SL1-1, SL1-2, SL2-1, and SL2-2 had 4.50, 10.32, 4.02, and 6.23% PPP, respectively. TAG profile greatly influences the physical properties of the SL. The SLs were composed of all four types of TAGs, namely, SSS (trisaturated), SUS (disaturated-monounsaturated), SUU (monosaturated-diunsaturated), and UUU (triunsaturated). UUU TAGs decreased from 12.85 to 8.91% in two-stage synthesis and from 14.23 to 12.01% in one-stage synthesis when substrate molar ratio increased from 0.5:1:0.5 to 1:1:0.5. SUU type TAGs were the predominant TAGs present in the SLs. SL1-1, SL1-2, SL2-1, and SL2-2 had 40.83, 40.39, 45.62, and 44.26% SUU TAGs, respectively. Compared to two-stage synthesis, one-stage synthesis resulted in higher UUU and SUU type TAGs and lower SUS and SSS type TAGs. EVOO contained 63.98% UUU, 31.81% SUU, and 4.21% SUS type TAGs. SLs also had newly formed TAGs comprising ARA and DHA such as OAO, APA, OPD, and ODO. Their relative percent was higher in one-stage synthesized SLs than in two-stage synthesized SLs.

Tocopherols. Tocopherols and tocotrienols, commonly grouped as vitamin E, are the major lipid-soluble, membrane-localized antioxidants in humans. LC-PUFAs are very susceptible to oxidation and therefore need antioxidants to protect their efficacy. Human milk contains 0.45−0.8 mg of vitamin E/100 kcal.[35] Oxidative susceptibility increases with increasing unsaturated fatty acids. The SLs were enriched with LC-PUFAs and may be prone to oxidation. Indigenous antioxidants such as tocopherols contribute to protection against oxidative deterioration. The major vitamin E isomers in EVOO were 212.34 μg/g α-tocopherol, 17.79 μg/g γ-tocopherol, and 16.38 μg/g α-tocotrienol (Table 4). The total vitamin E content of SL1-1, SL1-2, SL2-1, and SL2-2 was 70.46, 68.79, 79.64, and 79.31 μg/g, respectively of which α-tocopherol accounted for approximately 73%. Among tocotrienols only α-tocotrienol was found in the SLs. Compared to EVOO, >70% decrease was observed for α-tocopherol in the SLs. Similarly, β-tocopherol decreased 33.58% in SL1-1 and >40% in SL1-2, SL2-1, and SL2-2. The % decrease in γ-tocopherol was 64.25, 60.03, 54.92, and 49.58% in SL1-1, SL1-2, SL2-1, SL2-2, respectively. δ-Tocopherol decreased 39.63, 64.02, 20.43, and 22.87% in SL1-1, SL1-2, SL2-1, and SL2-2, respectively. Previous studies have shown that tocopherols and tocotrienols were lost mainly as tocopheryl and tocotrienyl esters during interesterification and acidolysis reactions.[36,37] Higher loss of tocotrienols and tocopherols, except β-tocopherol, was observed in the two-stage synthesis than in the one-stage synthesis.

Phenolic Compounds. The phenolics were analyzed using solid phase extraction followed by HPLC-DAD. The major phenolics in EVOO were tyrosol (18.38 μg/g), hydroxytyrosol (9.42 μg/g), pinoresinol (3.52 μg/g), and oleuropein (1.86 μg/g). The other phenolic compounds identified were luteolin, vanillic, gallic, ferulic, *p*-coumaric, and caffeic acids. Olive oil phenolics are potent antioxidants as they inhibit lipid peroxidation. This may reduce oxidative stress and related diseases such as cancer and cardiovascular diseases.[38] No peak was observed in the case of SLs, implying that the SLs lacked the indigenous phenolic compounds found in olive oil. Phenolic compounds may be lost either as esters or in free form during the interesterification and/or acidolysis reactions. The formation of phenolic acid esters changes their polarity and solubility, making it difficult to separate phenolics using solid phase extraction. It is also suggested that phenolic compounds may be lost during the alkaline deacidification process of removing free fatty acids from SLs. Further research is needed to determine where and in what form the phenolic compounds are lost.

Melting and Crystallization Profiles. The melting properties of a fat or oil can be influenced by the fatty acid chain length (increase in chain length corresponds to an increase in melting point), degree of unsaturation (increase in

unsaturation results in a decrease in melting point), and polymorphism (α, lowest melting point; β', intermediate melting point; and β, highest melting point).[39] Melting and crystallization profiles of the substrates and products are shown in Figure 3a and Figure 3b, respectively. The melting completion temperature (T_{mc}) depends on the type of fatty acids and TAGs present. Tripalmitin, consisting of SSS type TAGs, had the highest T_{mc} (72.2 °C). EVOO has mainly oleic acid and OOO as the major TAG, and it was completely melted at 12.7 °C. The T_{mc} of SL1-1, SL1-2, SL2-1, and SL2-2 were 37.1, 42.0, 35.2, and 36.1 °C, respectively. Human milk fat is completely melted at normal body temperature (about 37 °C). All the SLs except SL1-2 synthesized in this study have their T_{mc} near 37 °C, which may help in infant formula formulation to obtain proper consistency and texture. The relatively higher T_{mc} of SL1-2 may be due to high saturated fatty acids (50.60 mol %) and high concentrations of saturated TAGs (SSS 12.11%; SUS 40.14%). The complexity and wide range of TAGs in SLs resulted in gradual melting range rather than a sharp melting as in tripalmitin, which is a simple homogeneous TAG. Similarly, the crystallization onset temperature (T_{co}) of SL1-1, SL1-2, SL2-1, and SL2-2 was 23.7, 27.6, 19.8, and 22.3 °C (Figure 3b). The T_{co} of the SLs was between those of tripalmitin (42.1 °C) and EVOO (−10.2 °C) and consisted of multiple peaks due to the complexity in their fatty acid and TAG molecular species.

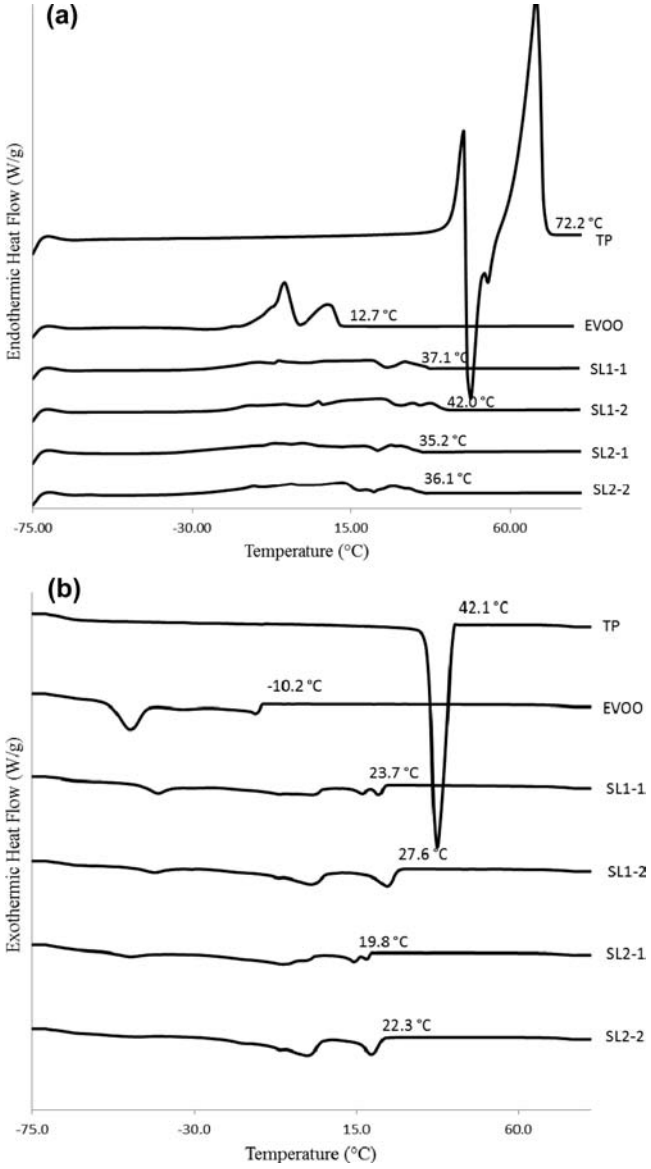

Figure 3. (a) Melting thermograms of substrates and structured lipids. The temperatures shown are melting completion temperatures. TP, tripalmitin. EVOO, extra virgin olive oil. SL1-1, structured lipid synthesized using two-stage synthesis with substrate molar ratio 0.5:1:0.5 (TP:EVOO:AD). SL1-2, structured lipid synthesized using two-stage synthesis with substrate molar ratio 1:1:0.5 (TP:EVOO:AD). SL2-1, structured lipid synthesized using one-stage synthesis with substrate molar ratio 0.5:1:0.5 (TP:EVOO:AD). SL2-2, structured lipid synthesized using one-stage synthesis with substrate molar ratio 1:1:0.5 (TP:EVOO:AD). AD, mix of ARASCO-FFA and DHASCO-FFA (2:1). (b) Crystallization thermograms of substrates and structured lipids. The temperatures shown are crystallization onset temperatures. See Figure 2a for explanation of abbreviations.

Enzyme Reusability. The enzymes' reusability was tested by performing the 1:1:0.5 reactions ten times in both two-stage and one-stage syntheses. After each run, the enzymes were washed 4−5 times with hexane and dried in a desiccator. They were stored at 4 °C until reuse. Total ARA and DHA and *sn*-2 palmitic acid (mol %) were determined as the main responses (Figure 4). For two-stage synthesis, *sn*-2 palmitic acid (about 57.0 mol %) remained fairly constant till the eighth run, after which it decreased. However, the total ARA and DHA content (about 4.4 mol %) started to decrease after the sixth run. In the one-stage synthesis, after the fifth run both palmitic acid at the *sn*-2 position (about 55.3 mol %) and the total ARA and DHA content (about 9.5 mol %) decreased and continued to decrease until the last run. The enzymes performed better in two-stage synthesis in terms of *sn*-2 palmitic acid. This may be because in the two-stage synthesis the two enzymes, Novozym 435 and Lipozyme TL IM, were separately washed, dried, and reused. On the other hand, in one-stage synthesis both enzymes were washed and reused together, which may affect their activity. An early decrease in the total ARA and DHA content was observed for both two-stage and one-stage syntheses. This

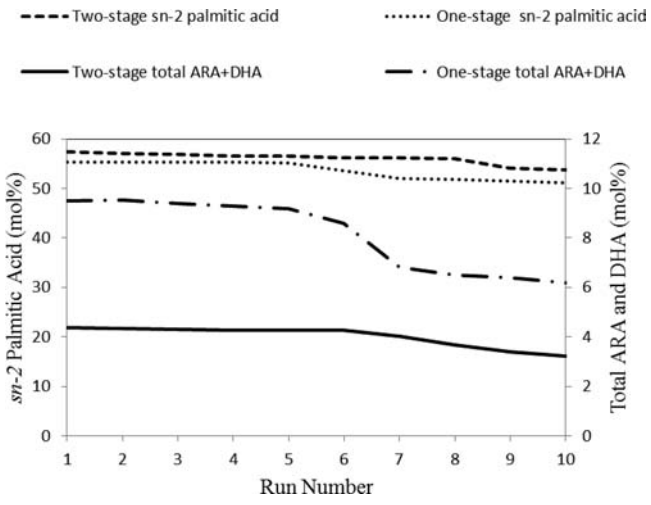

Figure 4. Mol% of *sn*-2 palmitic acid (primary *y*-axis) and mol % of total ARA + DHA (secondary *y*-axis) of structured lipids (1:1:0.5, TP:EVOO:AD) as determining factor of Novozym 435 and Lipozyme TL IM lipase reusability in two-stage and one-stage syntheses. TP, tripalmitin. EVOO, extra virgin olive oil. AD, mix of ARASCO-FFA and DHASCO-FFA (2:1).

may be due to the effect of heat on the activity and specificity of the enzyme. As the number of runs increased, the enzyme was exposed to more heat and solvent (hexane during cleaning). Moreover, the enzyme immobilization carrier properties may also have an effect on enzyme reusability. Previous studies have shown that Lipozyme TL IM absorbs less oil and was easier to clean than Novozym 435.[40,41] This difference in the absorption capacity of the enzymes may be due to the different immobilization support system of the two enzymes (granulated silica for Lipozyme TL IM and macroporous acrylic resin for Novozym 435). In one-stage synthesis, when both enzymes were used together, the difference in immobilization carrier properties may have a negative effect on their activity explaining decreased response after the fifth run. Furthermore, the immobilization carrier property may also affect the interaction and activity of two lipases when used together.[42] The enzyme activity, stability, efficiency, and selectively can also be improved through different immobilization protocols and carriers.[43,44] Although enzymes had better reusability in two-stage synthesis, one-stage synthesis was a faster reaction. One-stage synthesis also resulted in higher ARA and DHA content than two-stage synthesis.

Infant formulas based on human milk composition are the best substitutes for infant nutrition when breastfeeding may not be possible. SLs with high palmitic acid at the *sn*-2 position and also enriched with ARA and DHA can be used in infant formulas to mimic the physical, chemical, and nutritional properties of human milk fat. The SLs produced in this study may be suitable for use in infant formula as human milk fat analogues. They had the desired levels of palmitic acid at the *sn*-2 position and also contained ARA and DHA for proper growth and development of the infants.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ARA, arachidonic acid; ARASCO, arachidonic acid single cell oil; DHA, docosahexaenoic acid; DHASCO, docosahexaenoic acid single cell oil; AD, mix of ARASCO-FFA and DHASCO-FFA, 2:1 by weight; EVOO, extra virgin olive oil; FFA, free fatty acids; LC-PUFA, long chain polyunsaturated fatty acids; SL, structured lipid; TAG, triacylglycerol; SSS, trisaturated TAG; SUS, disaturated-monounsaturated TAG; SUU, monosaturated-diunsaturated TAG; UUU, triunsaturated TAG

REFERENCES

(1) Gibson, R. A.; Makrides, M. The role of long chain polyunsaturated fatty acids (LCPUFA) in neonatal nutrition. *Acta Paediatr.* **1998**, *87*, 1017−1022.

(2) Kim, J.; Friel, J. Lipids and human milk. *Lipid Technol.* **2012**, *24*, 103−105.

(3) Brenna, J. T.; Varamini, B.; Jensen, R. G.; Diersen-Schade, D. A.; Boettcher, J. A.; Arterburn, L. M. Docosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. *Am. J. Clin. Nutr.* **2007**, *85*, 1457−1464.

(4) Yehuda, S.; Rabinovitz, S.; Mostofsky, D. I. Essential fatty acids and the brain: from infancy to aging. *Neurobiol. Aging* **2005**, *26*, 98−102.

(5) Tomarelli, R. M.; Meyer, B. J.; Weaber, J. R.; Bernhart, F. W. Effect of positional distribution on the absorption of the fatty acids of human milk and infant formulas. *J. Nutr.* **1968**, *95*, 583−590.

(6) Karupaiah, T.; Sundram, K. Effects of stereospecific positioning of fatty acids in triacylglycerol structures in native and randomized fats: a review of their nutritional implications. *Nutr. Metabol.* **2007**, *4*, 1−17.

(7) Kennedy, K.; Fewtrell, M. S.; Morely, R.; Abbott, R.; Quinlan, P. T.; Wells, J. C.; Bindels, J. G.; Lucas, A. Double-blind, randomized trial of a synthetic triacylglycerol in formula-fed term infants: effects on stool biochemistry, stool characteristics, and bone mineralization. *Am. J. Clin. Nutr.* **1999**, *70*, 920−927.

(8) Carnielli, V.; Luijendijk, I.; van Goudoever, J.; Sulkers, E.; Boerlage, A.; Degenhart, H.; Sauer, P. Feeding premature newborn infants palmitic acid in amounts and stereoisomeric position similar to that of human milk: Effects on fat and mineral balance. *Am. J. Clin. Nutr.* **1995**, *61*, 1037−1042.

(9) Carnielli, V.; Luijendijk, I.; van Goudoever, J.; Sulkers, E.; Boerlage, A.; Degenhart, H.; Sauer, P. Structural position and amount of palmitic acid in formulas: Effects on fat, fatty acids and mineral balance. *J. Pediatr. Gastroenterol. Nutr.* **1996**, *23*, 554−560.

(10) EFSA Panel on Dietetic Products, Nutrition and Allergies.. Scientific opinion on the substantiation of a health claim related to beta-palmitate and increased calcium absorption pursuant to Article 14 of Regulation (EC) No 1924/2006. *Eur. Food Safety Authority J.* **2011**, *9*, 2289−2305.

(11) Akoh, C. C.; Kim, B. H. Structured lipids. In *Food lipids: chemistry, nutrition, and biotechnology*, 3rd ed.; Akoh, C. C., Min, D. B., Eds.; CRC Press: Boca Raton, FL, 2008; pp 841−865.

(12) Schmid, U.; Bornscheuer, U. T.; Soumanou, M. M.; McNeill, G. P.; Schmid, R. D. Highly selective synthesis of 1,3-oleoyl-2-palmitoylglycerol by lipase catalysis. *Biotechnol. Bioeng.* **1999**, *64*, 678−684.

(13) Soumanou, M. M.; Bornscheuer, U. T.; Schmid, R. D. Two-step enzymatic reaction for the synthesis of pure structured triacylglycerides. *J. Am. Oil Chem. Soc.* **1998**, *75*, 703−710.

(14) Negishi, S.; Arai, Y.; Arimoto, S.; Tsuchiya, K.; Takahashi, I. Synthesis of 1, 3-dicapryloyl-2-docosahexaenoylglycerol by a combination of nonselective and sn-1, 3-selective lipase reactions. *J. Am. Oil Chem. Soc.* **2003**, *80*, 971−974.

(15) Turan, D.; Şahin Yeşilçubuk, N.; Akoh, C. C. Production of human milk fat analogue containing docosahexaenoic and arachidonic acids. *J. Agric. Food Chem.* **2012**, *60*, 4402−4407.

(16) Pina-Rodriguez, A. M.; Akoh, C. C. Synthesis and characterization of a structured lipid from amaranth oil as a partial fat substitute in milk-based infant formula. *J. Agric. Food Chem.* **2009**, *57*, 6748−6756.

(17) Teichert, S. A.; Akoh, C. C. Modifications of stearidonic acid soybean oil by enzymatic acidolysis for the production of human milk fat analogues. *J. Agric. Food Chem.* **2011**, *59*, 13300−13310.

(18) Ibrahim, N. A.; Guo, Z.; Xu, X. Enzymatic interesterification of palm stearin and coconut oil by a dual lipase system. *J. Am. Oil Chem. Soc.* **2008**, *85*, 37−45.

(19) Türkan, A.; Kalay, Ş. Monitoring lipase-catalyzed methanolysis of sunflower oil by reversed-phase high-performance liquid chromatography: elucidation of the mechanisms of lipases. *J. Chromatogr., A* **2006**, *1127*, 34−44.

(20) *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 6th ed.; AOCS: Champaign, IL, 2011; Official Method Cd 3a-94.

(21) Wanasundara, U. N.; Shahidi, F. Concentration of omega3-polyunsaturated fatty acids of seal blubber oil by urea complexation: optimization of reaction conditions. *Food Chem.* **1999**, *65*, 41−49.

(22) Lee, K. T.; Akoh, C. C. Characterization of enzymatically synthesized structured lipids containing eicosapentaenoic, docosahexaenoic, and caprylic acids. *J. Am. Oil Chem. Soc.* **1998**, *75*, 495−499.
(23) *Official Methods of Analysis of AOAC International*, 19th ed.; AOAC International: Gaithersburg, MD, 2012; Official Method 996.01.
(24) *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 6th ed.; AOCS: Champaign, IL, 2011; Official Method Ch 3-91.
(25) de la Torre-Carbot, K.; Jauregui, O.; Gimeno, E.; Castellote, A. I.; Lamuela-Raventós, R. M.; Lopez-Sabater, M. C. Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS. *J. Agric. Food Chem.* **2005**, *53*, 4331−4340.
(26) Owen, R. W.; Mier, W.; Giacosa, A.; Hull, W. E.; Spiegelhalder, B.; Bartsch, H. Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignansand squalene. *Food Chem. Toxicol.* **2000**, *38*, 647−659.
(27) *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 6th ed.; AOCS: Champaign, IL, 2011; Official Method Cj 1-94.
(28) López-López, A.; López-Sabater, M. C.; Campoy-Folgoso, C.; Rivero-Urgell, M.; Castellote-Bargalló, A. I. Fatty acid and sn-2 fatty acid composition in human milk from Granada (Spain) and in infant formulas. *Eur. J. Clin. Nutr.* **2002**, *56*, 1242−1254.
(29) Christensen, M. M.; Høy, C. E. Early dietary intervention with structured triacylglycerols containing docosahexaenoic acid. Effect on brain, liver, and adipose tissue lipids. *Lipids* **1997**, *32*, 185−191.
(30) Yang, T.; Fruekilde, M. B.; Xu, X. Suppression of acyl migration in enzymatic production of structured lipids through temperature programming. *Food Chem.* **2005**, *92*, 101−107.
(31) Xu, X. Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review. *Eur. J. Lipid Sci. Technol.* **2000**, *102*, 287−303.
(32) Xu, X.; Skands, A. R. H.; Høy, C. E.; Mu, H.; Balchen, S.; Adler-Nissen, J. Production of specific-structured lipids by enzymatic interesterification; elucidation of acyl migration by response surface design. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1179−1186.
(33) Mu, H.; Høy, C. E. The digestion of dietary triacyglycerols: Review. *Prog. Lipid Res.* **2004**, *43*, 105−133.
(34) Morera, S.; Castellote, A. I.; Jauregui, O.; Casals, I.; López-Sabater, M. C. Triacylglycerol markers of mature human milk. *Eur. J. Clin. Nutr.* **2003**, *57*, 1621−1626.
(35) Barbas, C.; Herrera, E. Lipid composition and vitamin E content in human colostrum and mature milk. *J. Physiol. Biochem.* **1998**, *54*, 167−173.
(36) Zou, L.; Akoh, C. C. Identification of tocopherols, tocotrienols, and their fatty acid esters in residues and distillates of structured lipids purified by short-path distillation. *J. Agric. Food Chem.* **2012**, *61*, 238−246.
(37) Hamam, F.; Shahidi, F. Acidolysis reactions lead to esterification of endogenous tocopherols and compromised oxidative stability of modified oils. *J. Agric. Food Chem.* **2006**, *54*, 7319−7323.
(38) Owen, R. W.; Giacosa, A.; Hull, W. E.; Haubner, R.; Würtele, G.; Spiegelhalder, B.; Bartsch, H. Olive-oil consumption and health: the possible role of antioxidants. *Lancet Oncol.* **2000**, *1*, 107−112.
(39) Strayer, D.; Belcher, M.; Dawson, T.; Delaney, B.; Fine, J.; Flickinger, B.; Friedman, P.; Heckel, C.; Hughes, J.; Kincs, F.; Linsen, L.; McBrayer, T.; McCaskill, D.; McNeill, G.; Nugent, M.; Paladini, E.; Rosegrant, P.; Tiffany, T.; Wainwright, B.; Wilken, J. *Food fats and oils*; 9th ed.; Institute of Shortening and Edible Oils: Washington, DC, 2006. http://www.iseo.org/httpdocs/Publications/FoodFatsOils2006.pdf (Accessed: 5th August 2013).
(40) Teichert, S. A.; Akoh, C. C. Characterization of stearidonic acid soybean oil enriched with palmitic acid produced by solvent-free enzymatic interesterification. *J. Agric. Food Chem.* **2011**, *59*, 9588−9595.
(41) Pande, G.; Akoh, C. C.; Shewfelt, R. L. Production of *trans*-free margarine with stearidonic acid soybean and high-stearate soybean oils-based structured lipid. *J. Food Sci.* **2012**, *77*, C1203−C1210.
(42) Fernandez-Lafuente, R. Lipase from *Thermomyces lanuginosus*: uses and prospects as an industrial biocatalyst. *J. Mol. Catal. B: Enzym.* **2010**, *62*, 197−212.
(43) Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R. Improvemnet of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme Microb. Technol.* **2007**, *40*, 1451−1463.
(44) Rodrigues, R. C.; Ortiz, C.; Berenguer-Murcia, A.; Torres, R.; Fernandez-Lafuente, R. Modifying enzyme activity and selectivity by immobilization. *Chem. Soc. Rev.* **2013**, *42*, 6290−6307.