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Production of Human Milk Fat Analogue Containing Docosahexaenoic and Arachidonic Acids

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ABSTRACT: Human milk fat (HMF) analogue containing docosahexaenoic acid (DHA) and arachidonic acid (ARA) at *sn*-1,3 positions and palmitic acid (PA) at *sn*-2 position was produced. Novozym 435 lipase was used to produce palmitic acid-enriched hazelnut oil (EHO). EHO was then used to produce the final structured lipid (SL) through interesterification reactions using Lipozyme RM IM. Reaction variables for 3 h reactions were temperature, substrate mole ratio, and ARASCO/DHASCO (A:D) ratio. After statistical analysis of DHA, ARA, total PA, and PA content at *sn*-2 position, a large-scale production was performed at 60 °C, 3:2 A:D ratio, and 1:0.1 substrate mole ratio. For the SL, those results were determined as 57.3 ± 0.4%, 2.7 ± 0.0%, 2.4 ± 0.1%, and 66.1 ± 2.2%, respectively. Tocopherol contents were 84, 19, 85, and 23 μg/g oil for α-, β-, γ-, and δ-tocopherol. Melting range of SL was narrower than that of EHO. Oxidative stability index (OSI) value of SL (0.80 h) was similar to that of EHO (0.88 h). This SL can be used in infant formulas to provide the benefits of ARA and DHA.

KEYWORDS: *human milk fat analogue, ARA, DHA, hazelnut oil, interesterification, Lipozyme RM IM*

INTRODUCTION

Structured lipids (SLs) are defined as the chemically or enzymatically modified form of naturally occurring lipids. Products having different health benefits or nutritional value can be obtained through SLs.[1]

The $n-6$ and $n-3$ long-chain polyunsaturated fatty acids (LC-PUFAs) are important fatty acids for development as they play fundamental roles in the body.[2] Docosahexaenoic acid (DHA), which is an $n-3$ PUFA, is present in tissues of the central nervous system but is rarely found in other tissues.[3] It is a structural component of brain and retina and is important in terms of biological functions.[1,4,5] Arachidonic acid (ARA), an $n-6$ PUFA, is the main precursor of eicosanoids and is found in many tissues in high amounts.[3,6] DHASCO and ARASCO are single-cell oils (SCO) produced from *Crypthecodinium cohnii* and *Mortierella alpina*, respectively. For infant formulas, the addition of physical mixtures of these two products is possible, and they can be mixed with other fatty acids.[6] Since 2001, the U.S. Food and Drug Administration (FDA) accepted DHASCO and ARASCO as "generally recognized as safe" (GRAS), which means that the product is safe under the conditions of its intended use.[7]

Accretion of LC-PUFAs in tissues of infants begins with the last trimester of pregnancy, and preterm infants may therefore suffer from LC-PUFA deficiency.[4] Premature infants have higher requirement for LC-PUFAs compared to term infants. Because fast-growing organs such as the brain need LC-PUFAs, premature infants require supplemented formulas to obtain adequate amounts of these essential fatty acids.[5] According to a clinical study, infants who were fed with LC-PUFA supplemented formulas had similar LC-PUFA plasma level concentrations as compared to breastfed infants.[8] Consequently, addition of ARA and DHA to infant formulas was shown to be beneficial for visual acuity, normal growth, and development.[9]

Human milk is the most valuable source for infant nutrition as it includes all of the necessary nutrients, but for some reason it is not always possible to feed the infants with human milk.[2] The fatty acid profile of human milk is generally accepted as the "gold standard" when designing the composition of infant formulas.[4] Human milk is unique in nature because of its triacylglycerol (TAG) structure.[10] It contains 25% palmitic acid (PA), of which nearly 70% is located at the *sn*-2 position of the TAG, while the *sn*-1 and *sn*-3 positions are occupied by LC-PUFAs. This unique structure increases digestion and absorption of the fatty acids and improves calcium absorption.[4,11] DHA content varies between 0.1% and 1.0%, whereas ARA content varies between 0.5% and 1.0% in human milk. Genzel-Boroviczény et al.[12] indicated that after preterm delivery, human milk does not include enough amount of PUFAs to meet the requirement of PUFAs during the first weeks after birth when compared to human milk after term delivery.

Hazelnut is a tree nut obtained from *Corylus avellana* L. Turkey is the major producer in the world.[13] Oleic acid is the predominant fatty acid of hazelnut oil (73.6−82.6%) followed

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by linoleic (9.8−16.6%), palmitic (4.1−6.8%), and stearic (1.6−3.7%) acids.[14−21]

The lipase from *Rhizomucor miehei*, which was first described in 1973, is an enzyme previously used in lipid modifications. Lipozyme RM IM is a commercially available version of this enzyme in immobilized form.[22] Interesterification reactions are composed of, first, hydrolysis and, second, formation of a new ester bond between the exposed hydroxyl group and the new fatty acid that will be incorporated.[23]

Hazelnut oil fatty acids have been used before to modify tripalmitin to produce human milk fat substitutes (HMFS).[19−21,24] The aim of this study was to produce human milk fat analogues (HMFA) containing PA at the *sn*-2 position and DHA and ARA at the *sn*-1,3 positions by using hazelnut oil as a substrate, which is an important commercial product of Turkey. In the first step, hazelnut oil was enriched with PA at the *sn*-2 position using immobilized Novozym 435 as the enzymatic catalyst and ethyl palmitate as acyl donor. Next, DHA and ARA were incorporated into enriched hazelnut oil (EHO) using immobilized Lipozyme RM IM as the enzymatic catalyst. Furthermore, the impact of reaction variables such as temperature, substrate mole ratio, and ARA/DHA ratio on the final yield was investigated.

MATERIALS AND METHODS

Materials. Refined hazelnut oil used in this project was purchased from a grocery store in Istanbul, Turkey. DHASCO and ARASCO, which are SCOs rich in DHA and ARA, respectively, were generously donated by Martek Biosciences Co. (Columbia, MD). External (Supelco 37 Component FAME Mix) and internal (C15:0-pentadecanoic acid and C17:0-heptadecanoic acid) standards for fatty acid compositional analysis and tocopherols (α-, β-, γ-, and δ-) standards were purchased from Sigma-Aldrich Corp. (St. Louis, MO). To identify products, trilinolein and 2-oleoylglycerol standards (TAG and 2-monoacylglycerol (2-MAG)) purchased from Sigma-Aldrich Corp. (St. Louis, MO) and Avanti Polar Lipids, Inc. (Alabaster, AL), respectively, were used. Immobilized lipases, Lipozyme RM IM (1,3 specific lipase), and Novozym 435 (mostly a nonspecific lipase) was purchased from Novozymes North America Inc. (Franklinton, NC). All other chemicals and solvents (analytical and/or chromatographic grade) were purchased from Sigma-Aldrich Corp. (St. Louis, MO), Avantor Performance Materials (Phillipsburg, NJ), or Fisher Scientific (Pittsburgh, PA).

Large-Scale Production of Ethyl Palmitate. Ethyl palmitate was produced in large scale according to the method of Vázquez and Akoh.[25] PA was converted to its ethyl ester form using a round-bottom flask. 60 g of PA was mixed with sodium ethoxide (2.625%, w/v) in absolute ethanol at a ratio of 4:2 (v/v). Next, the reaction mixture was heated to 65 °C in an orbital shaking water bath at 9.4 g for 40 min. When the reaction was complete, the mixture was washed with saturated NaCl solution and then with distilled water to remove the remaining ethanol, glycerin, or any other polar compounds. Phase separation was performed using a separatory funnel. The volume of saturated NaCl solution and the distilled water used in washing step was nearly one-half of the PA. The final product was dried over anhydrous sodium sulfate and then vacuum filtered. The fatty acid ethyl ester (ethyl palmitate) content of the final product was over 95% as analyzed by gas−liquid chromatography (GLC).

Interesterification Reactions. Hazelnut oil, which was further enriched with PA at the *sn*-2 position using ethyl palmitate as acyl donor, was one of the substrates in this study. EHO was produced using stirred-batch bioreactor operated at 65 °C for 17 h. Novozym 435, mostly a nonspecific lipase, was added at 10% of total reactants as the enzymatic catalyst. The reaction product was vacuum filtered, and the excess free fatty acids were removed using short-path distillation (UIC Inc., Joliet, IL) to a level <1%. Feed vessel temperature of the unit was kept at 55 °C. The temperature of the wiper blade vessel where the oil was turning was 155 °C, and the condensation temperature was 20 °C. Feeding rate of the sample was 100 mL/h. The vacuum was kept at <135 mTorr. AOCS Official Method Ac 5-41 was used to determine free fatty acid content of the reaction product.[26]

DHA and ARA were incorporated into hazelnut oil using DHASCO and ARASCO, respectively. Interesterification reactions took place in screw-cap test tubes in an orbital shaking water bath at 9.4 g. Lipozyme RM IM was used as the biocatalyst at 10% of the total reactants. ARASCO and DHASCO were physically blended at different ratios such as 3:2 and 2:1. ARASCO:DHASCO blended ratio is designated as A:D ratio for the rest of this study. Reaction time was 3 h, and reaction temperatures for interesterification reactions were determined set at 50 and 60 °C. Substrate mole ratio was chosen as 0.1 and 0.05 (mole of A:D blend/mol of EHO). Experiments were run in triplicates, and average results were reported. Reaction products were passed through the sodium sulfate column into Teflon taped vials to remove moisture and were kept at −18 °C for further analysis.

Product Recovery by Thin-Layer Chromatography (TLC). Product recovery by TLC was performed according to the method described by Jennings and Akoh.[27] The products of interesterification reactions were spotted on TLC plates coated with silica gel G. 100 μL of product and trilinolein in hexane as a TAG standard were spotted on TLC plates and placed in TLC tank. Developing solvent was petroleum ether:diethyl ether:acetic acid (80:20:0.5, v/v/v). The plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. Corresponding TAG bands, which were identified with the standard, were scraped off into screw-capped test tubes for fatty acid profile of TAG and *sn*-2 positional analysis.[27]

Fatty Acid Methyl Ester (FAME) Preparation. FAME preparation of EHO and SL products was performed according to AOAC Official Method 996.01, Section E.[28] 100 mg of EHO was weighed into screw-capped test tubes, and 1 mL of C17:0 internal standard (20 mg/mL) was added. For the methylation of reaction products, 50 μL of C15:0 internal standard (20 mg/mL) was added to the bands scraped from the TLC plates into test tubes. Two milliliters of 0.5 N NaOH was added to each tube, vortexed, and incubated in the oven at 100 °C for 5 min to saponify the lipid. Next, 2 mL of 14% BF_3 in methanol was added, vortexed for 1 min, followed by incubation at 100 °C for 5 min again. Tubes were taken off from the oven and cooled under cold water. Later, 2 mL of hexane and 2 mL of saturated NaCl solutions were added to the sample, vortexed exactly for 2 min, and centrifuged at 188*g* for 3 min. After centrifugation, the upper hexane layer was passed through the anhydrous sodium sulfate column.

Pancreatic Lipase-Catalyzed *sn*-2 Positional Analysis. After TAG bands were transferred from TLC plates into screw-capped test tubes, they were extracted with diethyl ether two times and evaporated under nitrogen gas to obtain purified TAG. Next, 2 mL of Tris-HCl buffer (1 M, pH 8.0), 0.5 mL of 0.05% sodium cholate solution, and 0.2 mL of 2.2% calcium chloride solution were added. To emulsify the sample, tubes were vortexed for 2 min. Next, 40 mg of pancreatic lipase was added and vortexed for an additional minute. Samples were incubated in a water bath at 40 °C for 3 min. To stop the reaction, 1 mL of 6 N HCl and 4 mL of diethyl ether were added, vortexed for 2 min, and centrifuged at 188*g* for 3 min. Diethyl ether layer was passed through the sodium sulfate column, and the volume was reduced to one-third under nitrogen gas. Samples were spotted on TLC plates, which were coated with silica gel G, and placed in the tank and developed with a solvent system of hexane:diethyl ether:formic acid (60:40:1.6, v/v/v) mixture. Plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol, visualized under UV light, and 2-MAG bands were identified with 2-oleoylglycerol standard. Corresponding 2-MAG bands were scraped off into screw-capped test tubes and analyzed by GLC after methylation.[27]

Fatty Acid Composition Analysis with GLC. FAMEs of the products were analyzed using capillary GLC, Agilent Technologies 6890N (Santa Clara, CA), to determine the fatty acid profiles. Methylated samples were run with internal standard C15:0 (20 mg/mL), and Supelco FAME37 mix was used as an external standard. The instrument was equipped with Supelco SP-2560 column (100 m ×

0.25 mm i.d., 0.20 μm film thickness) and a flame ionization detector (FID). The injector and detector temperatures were 250 and 260 °C, respectively. Oven temperature was held at 150 °C for 3 min followed by an increase to 215 °C with a ramping at 10 °C/min, and temperature was held isothermally for 40 min. Injection volume was 1 μL, and the carrier gas was helium at 1.1 mL/min. Average results were calculated as mol %.

Gram-Scale Production of the SL. Gram-scale production was carried out in the water bath using round-bottom flask. After the reaction, the product was passed through sodium sulfate column and vacuum filtered. The reaction conditions were 60 °C reaction temperature, 3:2 A:D ratio, and 0.1 substrate mole ratio, because total PA, ARA, and DHA incorporations were higher at these conditions in milligram-scale reactions. Because the targeted application of the produced SL was in infant formulas, reactions were performed in a solvent-free medium. Free fatty acids from the reaction mixture were removed following the procedure of Lee et al.[29] The product was passed through the anhydrous sodium sulfate column, and the hexane layer was removed using rotary evaporator. Next, 60 mL of 0.5 N KOH (20% ethanol) solution and 120 mL of hexane were added in a separatory funnel. The upper hexane layer was collected, and 3−4 drops of phenolphthalein solution were added. The mixture was titrated with 0.5 N KOH solution (20% ethanol). Thirty milliliters of saturated NaCl solution was added and mixed. The upper hexane layer was passed through the anhydrous sodium sulfate column, and the solvent was evaporated using a rotary evaporator. Fatty acid profile and fatty acids at the *sn*-2 position were determined as described before.

Tocopherol Content Analysis. Standard preparation and tocopherol content determination of samples were performed according to the method of Shin et al.[30] Stock solutions of the standards were prepared with different concentrations and analyzed with a liquid chromatograph (Shimadzu LC-6A pump equipped with an RF-10AXL fluorescence detector (Shimadzu Corp., Columbia, MD), a SpectraSERIES AS 100 autosampler (Thermo Separation Products, Inc., San Jose, CA)). A normal-phase LiChrosorb Si 60 column (4 mm × 250 mm, 5 μm particle size; Hibar Fertigsäule RT, Merck, Darmstadt, Germany) and an isocratic mobile phase comprised of 0.85% (v/v) isopropanol in hexane at a flow rate of 1.0 mL/min were used. The excitation and emission wavelengths for the fluorescent determination of tocopherol isomers were 290 and 330 nm, respectively. Ten microliters of sample extracts or tocopherol standard solutions was injected per run. All analyses were done in triplicate, and average results were reported.

Melting Profile Analysis. AOCS Official Method Cj 1-94 was used to determine the melting and crystallization profiles of the products using a differential scanning calorimeter (DSC) instrument (Perkin-Elmer Co., Norwalk, CT).[31] As a coolant, dry ice in acetone was used. Nitrogen was the purge gas at a pressure of 10 psi. Indium was used for the calibration of the instrument. Approximately 8−12 mg of product was weighed into aluminum pans and sealed hermetically. Samples were cooled from 80 to −55 °C at a rate of 10 °C/min and heated to 80 °C at a rate of 5 °C/min. Thermograms generated by the instrument were analyzed with Syris (Perkin-Elmer, Shelton, CT) software. The samples were analyzed in duplicates, and results were obtained for melting and crystallization profiles according to DSC thermograms.

Oxidative Stability Analysis. AOCS Cd 12b-92 reference method was used for oxidative stability experiments using OSI instrument (Omnion, Rockland, MA).[32] Calibration of OSI instrument was performed with deionized water. Analysis temperature was kept at 110 °C. The oxidative stabilities of the EHO and the SL were measured with OSI instrument in duplicates, and average results of OSI values were reported.

Statistical Analysis. Interesterification reactions were done in triplicate, and average results were reported. To investigate the effects of temperature, substrate mole ratio, and A:D ratio and their interactions on total PA, ARA, and DHA incorporations, statistical analysis was performed using ANOVA statistical analysis. The significance of difference at *p*-value of 0.05 was determined by Tukey Simultaneous Tests. All data were analyzed by MINITAB 16 (PA) statistical software.

RESULTS AND DISCUSSION

Fatty acid composition, *sn*-1,3, and *sn*-2 positional analysis of commercial hazelnut oil and EHO used in this study are given in Tables 1 and 2, respectively. Hazelnut oil contained 82.3% oleic, 8.5% linoleic, and 5.7% PA. It also contained a small amount of stearic (2.9%) and palmitoleic acids (0.2%). The *sn*-2 position of hazelnut oil was generally occupied by oleic acid (86%). PA content at the *sn*-2 position of hazelnut oil was quite low (0.8%). The other fatty acids at the *sn*-2 position of hazelnut oil were linoleic and stearic acids.

Table 1. Fatty Acid Composition and *sn*-1,3 and *sn*-2 Positional Analysis of Commercial Hazelnut Oil (mol %)[a]

fatty acid	total	*sn*-2 position	*sn*-1,3 positions[b]
palmitic acid (16:0)	5.7 ± 0.0	0.8 ± 0.4	8.2 ± 0.2
palmitoleic acid (16:1*n* − 7)	0.2 ± 0.0	ND[c]	0.3 ± 0.0
stearic acid (18:0)	2.9 ± 0.0	0.6 ± 0.7	4.0 ± 0.3
oleic acid (18:1*n* − 9)	82.3 ± 0.0	86.0 ± 0.2	79.8 ± 0.1
linoleic acid (18:2*n* − 6)	8.5 ± 0.0	11.7 ± 0.3	7.0 ± 0.1
linolenic acid (18:3*n* − 6)	0.1 ± 0.0	ND	0.2 ± 0.0
arachidic acid (20:0)	0.1 ± 0.0	ND	0.2 ± 0.0
eicosenoic acid (20:1*n* − 9)	0.1 ± 0.0	ND	0.2 ± 0.0

[a]Mean ± SD, $n = 3$. [b]*sn*-1,3 (mol %) = [3 × total (mol %) − *sn*-2 (mol %)]/2. [c]Not detected.

Table 2. Fatty Acid Composition and *sn*-1,3 and *sn*-2 Positional Analysis[a] of EHO[b] (mol %)

fatty acid	total	*sn*-2 position	*sn*-1,3 positions[c]
palmitic acid (16:0)	63.5 ± 0.0	71.1 ± 6.3	59.7 ± 3.2
palmitoleic acid (16:1*n* − 7)	0.1 ± 0.0	ND[d]	0.2 ± 0.0
stearic acid (18:0)	1.3 ± 0.0	3.6 ± 0.7	0.2 ± 0.4
oleic acid (18:1*n* − 9)	31.6 ± 0.0	23.0 ± 5.8	35.9 ± 2.9
linoleic acid (18:2*n* − 6)	3.3 ± 0.0	2.4 ± 0.4	3.8 ± 0.2
linolenic acid (18:3*n* − 6)	0.1 ± 0.0	ND	0.2 ± 0.0
arachidic acid (20:0)	0.1 ± 0.0	ND	0.1 ± 0.0

[a]Mean ± SD, $n = 3$. [b]Enriched hazelnut oil. [c]*sn*-1,3 (mol %) = [3 × total (mol %) − *sn*-2 (mol %)]/2. [d]Not detected.

EHO contained primarily palmitic (63.5%) followed by oleic acids (31.6%). It also contained linoleic (3.3%), stearic (1.3%), palmitoleic (0.1%), linolenic (0.1%), and arachidic acids (0.1%) in small amounts. The *sn*-2 position of EHO was mostly occupied by PA (71.1%), oleic acid (23.0%), stearic acid (3.6%), and linoleic acid (2.4%). The EHO content of total PA was higher as compared to that of human milk fat (HMF), but their PA amount at the *sn*-2 position was very close.[4,11]

Hazelnut oil, which was enriched with PA at the *sn*-2 position, was further enriched with ARA and DHA at the *sn*-1,3 positions. The purified EHO obtained from large scale production was used as the substrate for further enzymatic interesterification reaction to incorporate ARA and DHA. Enzymatic interesterification reactions were run for 3 h in triplicates, and average results with standard deviations were calculated. Total PA at the *sn*-2 position and PA, ARA, and DHA compositions of the SL-TAGs obtained from milligram scale production are given in Tables 3 and 4, respectively. According to the tables, an increase in temperature at A:D ratio

Table 3. Total PA, ARA, and DHA Contents of Milligram-Scale Production of SLs (mol %)[a]

temperature (°C)	A:D ratio[b]	Sr[c]	PA content (mol %)	ARA content (mol %)	DHA content (mol %)
50	2:1	1:0.1	59.1 ± 0.0	3.0 ± 0.2	1.0 ± 0.2
		1:0.05	61.2 ± 0.4	1.6 ± 0.2	0.5 ± 0.2
50	3:2	1:0.1	60.2 ± 0.7	2.3 ± 0.1	0.9 ± 0.1
		1:0.05	61.2 ± 0.4	1.4 ± 0.1	0.6 ± 0.1
60	2:1	1:0.1	58.2 ± 0.4	3.4 ± 0.3	1.5 ± 0.2
		1:0.05	61.4 ± 0.4	1.6 ± 0.2	0.5 ± 0.1
60	3:2	1:0.1	58.9 ± 0.3	2.7 ± 0.1	1.5 ± 0.1
		1:0.05	61.6 ± 0.4	1.2 ± 0.1	0.5 ± 0.1

[a]Mean ± SD, $n = 3$. [b]ARASCO/DHASCO ratio. [c]Substrate mole ratio of DHASCO−ARASCO blend to EHO.

Table 4. Total PA Content and *sn*-1,3 and *sn*-2 Positional Analysis of SLs (mol %)[a]

temperature (°C)	A:D ratio[b]	Sr[c]	total PA content (mol %)	*sn*-2 position	*sn*-1,3 positions[d]
50	2:1	1:0.1	59.1 ± 0.0	60.6 ± 4.0	58.2 ± 2.6
		1:0.05	61.2 ± 0.4	58.7 ± 1.1	62.5 ± 0.0
50	3:2	1:0.1	60.2 ± 0.7	56.1 ± 1.0	62.3 ± 1.5
		1:0.05	61.2 ± 0.4	58.7 ± 0.3	62.3 ± 0.6
60	2:1	1:0.1	58.2 ± 0.4	65.1 ± 5.5	54.7 ± 2.1
		1:0.05	61.4 ± 0.4	62.5 ± 1.0	60.8 ± 1.1
60	3:2	1:0.1	58.9 ± 0.3	62.5 ± 0.5	57.1 ± 0.6
		1:0.05	61.6 ± 0.4	65.0 ± 0.9	59.9 ± 0.5

[a]Mean ± SD, $n = 3$. [b]ARASCO/DHASCO ratio. [c]Substrate mole ratio of DHASCO−ARASCO blend to EHO. [d]*sn*-1,3 (mol %) = [3 × total (mol %) − *sn*-2 (mol %)]/2.

of 2:1 and the substrate mole ratio of 0.1 affected total PA content negatively, whereas ARA and DHA incorporations and PA amount at the *sn*-2 position were affected positively (from 60.6% to 65.1%). For A:D ratio of 2:1 and the substrate mole ratio of 0.05, an increase in temperature increased total PA content in a small amount, while ARA and DHA contents remained unchanged. At the same conditions, PA difference at the *sn*-2 position was approximately 4% (increased from 58.7% to 62.5%). When A:D ratio was 3:2 and substrate mole ratio was 1:0.1, the increase in temperature resulted in lower total PA content, but higher ARA and DHA contents. Furthermore, PA amount at the *sn*-2 position increased from 56.1% to 62.5% at those conditions. If substrate mole ratio was decreased to 1:0.05, then the temperature increase affected total PA content positively but ARA and DHA incorporations negatively for A:D ratio of 3:2. PA content at the *sn*-2 position was increased from 58.7% to 65.0% at those conditions. The effects of different temperature, substrate mole ratio, and A:D ratio were analyzed using ANOVA and Tukey simultaneous tests. The results of ANOVA analysis are given in Table 5 for total PA, ARA, and DHA incorporations. In addition, ANOVA table for PA incorporation at the *sn*-2 position is given in Table 6. Table 5 shows that for total PA incorporation, temperature, substrate mole ratio, A:D ratio, "temperature*substrate mole ratio", and "substrate mole ratio*A:D ratio" were statistically significant. For ARA incorporation, except for the interactions of "temperature*A:D ratio" and "temperature*substrate mole ratio*A:D ratio", all of the factors investigated were statistically significant. For DHA incorporation, the temperature, substrate

Table 5. *P*-Values of Linear and Interaction Terms with ANOVA for Total PA, ARA, and DHA Incorporations into SLs

source	for PA incorp	for ARA incorp	for DHA incorp
T	0.024	0.021	0.000
Sr	0.000	0.000	0.000
A:D	0.009	0.000	0.765
*T**Sr	0.001	0.004	0.000
*T**A:D	0.850	0.536	0.920
Sr*A:D	0.037	0.011	0.175
*T**Sr*A:D	0.365	0.626	0.241

Table 6. *P*-Values of Linear and Interaction Terms with ANOVA for PA Incorporation at the *sn*-2 Position of SLs

source	*P*-value
T	0.000
Sr	0.881
A:D	0.296
*T**Sr	0.851
*T**A:D	0.327
Sr*A:D	0.039
*T**Sr*A:D	0.908

mole ratio, and the interaction term "temperature*substrate mole ratio" were statistically significant. Tukey Simultaneous Tests showed that PA incorporation decreased with an increase in temperature, substrate mole ratio, and A:D ratio. DHA incorporation was positively affected by increase in temperature and substrate mole ratio, whereas A:D ratio did not have a significant effect on DHA incorporation. ARA incorporation was positively affected by an increase in temperature, substrate mole ratio, and A:D ratio. *P*-Values for PA incorporation at the *sn*-2 position given in Table 6 show that temperature and "substrate mole ratio*A:D ratio" were statistically significant terms. According to Tukey Simultaneous Tests, PA incorporation at the *sn*-2 position decreased with an increase in substrate mole ratio, while incorporation increased with an increase in temperature and A:D ratio. Studies on the production of HMFA are available elsewhere.[19−21,24,33−42] Among these studies, very few of them reported DHA-enriched SLs, but none of them was enriched with both ARA and DHA that are found in similar amounts with HMF.[21,38,42,43] A commercial product of HMFS, Betapol, is available. It has the predominant glyceride moiety of 1,3-dioleoyl 2-palmitoyl TAG, but it does not contain any LC-PUFA.[44] For gram-scale production of the SL in this study, 60 °C reaction temperature, 3:2 A:D ratio, and 0.1 substrate mole ratio were chosen, because total PA, ARA, and DHA incorporations were higher at these conditions. The long-term goal of the current study was to use this SL for possible inclusion in infant formula, and so the reaction was performed in a solvent-free medium. At the end of the reaction, fatty acid profile and fatty acids at the *sn*-2 position were determined. PA, ARA, and DHA contents of the SL product were 57.3 ± 0.4%, 2.7 ± 0.0%, and 2.4 ± 0.1%, respectively. PA content of the SL at the *sn*-2 position was 66.1 ± 2.2%. At gram-scale production, DHA incorporation was higher when compared to milligram-scale production. This may be due to the reaction condition in which no solvent was used. DHA content was still lower than the level of ARA content, as recommended for infant formulas.

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Characterization of the Products. EHO and the SL were characterized for tocopherol content, melting profile, and oxidative stability. Tocopherol contents of DHASCO, ARASCO, EHO, and the SL are given in Table 7. The SL had higher tocopherol content than did the EHO. Even though tocopherols were mostly lost during enrichment reactions of commercial hazelnut oil, SL had higher tocopherol content probably due to the tocopherols present in DHASCO and ARASCO substrate oils, which are shown in Table 7. Sørensen et al.[36] added DHASCO and ARASCO to a SL obtained through enzymatic reactions from butterfat and measured the tocopherol contents. It was found that the tocopherol contents did not change after physical addition of DHASCO and ARASCO possibly due to the small addition levels (1% each).

Table 7. Characterization of the EHO and the SL[a]

	EHO	SL	DHASCO	ARASCO
tocopherol contents (μg/g oil)				
α-tocopherol	46 ± 10	84 ± 12	270	243
β-tocopherol	19 ± 3	19 ± 3	27	27
γ-tocopherol	61 ± 10	85 ± 9	345	429
δ-tocopherol	12 ± 2	23 ± 2	169	212
OSI value (h)	0.88	0.80		
melting range (°C)				
onset point	5.7	−31.9		
end point	43.0	42.1		
crystallization range (°C)				
onset point	34.4	28.5		
end point	9.3	−10.0		

[a]Tocopherol contents (μg/g oil), OSI value (h), melting range (°C), and crystallization range (°C).

Melting profiles of EHO and the SL were determined using DSC. As it can be seen from Table 7, the melting range of the SL was wider (between −31.9 and 42.1 °C) than the EHO (between 5.7 and 43.0 °C). The melting end points of EHO and the SL were close to each other, but the onset point of the SL was lower than the EHO probably due to LC-PUFA content of the SL. For crystallization, similar behavior was observed. The end point of the crystallization range was lower for the SL when compared to the EHO, possibly due to LC-PUFA content. Again, the crystallization range was wider for the SL (between 28.5 and −10.0 °C) than the EHO (between 34.4 and 9.3 °C).

OSI values indicating the oxidative stabilities of the EHO and the SL are reported in Table 7. The OSI value at 110 °C of the EHO was 0.88 h, while it was 0.80 h for the SL. A slight decrease in oxidative stability after the enzymatic reactions may be linked to the reaction conditions such as reaction temperature, reaction time, and also neutralization conditions applied for the removal of free fatty acids.

The decrease in oxidative stability after the enzymatic reactions and purification may be linked to the loss of tocopherols and phospholipids during the removal of free fatty acids from the reaction mixture and also the reaction conditions such as temperature and reaction time. The decrease in induction time of a SL produced by Teichert and Akoh[45] was predicted to be linked to the tocopherol loss during removal of free fatty acids by short-path distillation unit. As a result, the oxidative stabilities of the products were found to be very low. Tocopherol addition is suggested as a way to increase the stability of these products for infant formula formulations. Maduko et al.[34] found that adding tocopherols as antioxidants decreased products' susceptibility to oxidation. Consequently, tocopherol content present in SCOs or tocopherols/antioxidants added during production may increase the stability of the products.

HMFA containing a high amount of PA at the *sn*-2 position and ARA and DHA at the *sn*-1,3 positions was obtained as a result of this study. It is believed that this SL can be used in infant formulas to provide advantages similar to those of HMF. These SLs could also deliver the health benefits associated with ARA and DHA. Resulting SL may be used as an ingredient in infant formula applications.

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Notes

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