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ARTICLE

Synthesis and Characterization of a Structured Lipid from Amaranth Oil as a Partial Fat Substitute in Milk-Based Infant Formula

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The aim of this study was to use enzymatic interesterification techniques to modify underutilized amaranth oil as a structured lipid (SL) by increasing its palmitic acid content at the *sn*-2 position and incorporating docosahexaenoic acid (DHA). This SL can be partially or complementarily used in milk-based infant formulas to deliver a lipid component similar to that in breast milk. Amaranth oil was modified by enzymatic interesterification in two stages. First, the palmitic acid content was increased specifically at the *sn*-2 position to resemble breast milk triacylglycerols (TAGs) using Novozym 435 lipase. Then DHA was incorporated, mainly at the *sn*-1,3 positions using Lipozyme RM IM, a *sn*-1,3 specific lipase. An optimization model was developed to determine the exact parameter combinations to incorporate a specific amount of DHA (1.0−2.5%). The model suggestions were used for a gram-scale interesterification to yield the expected product. The final SL composition was as follows: palmitic acid, 33.9%; stearic acid, 2.8%; oleic acid, 23.3%; linoleic acid, 37.3%; linolenic acid, 0.7%; and docosahexaenoic acid, 1.9%. The original amaranth oil and the final SL were characterized by determining the fatty acid composition, melting profile, chemical characteristics, oxidative stability (peroxide, *p*-anisidine, and total oxidation values), and phytosterol, tocopherol, and squalene contents. The physical and chemical characteristics determined in this study support the potential application of DHA-containing customized amaranth oil (DCAO) as a partial fat substitute or complement for milk-based infant formula. Research on the application and stability of this SL used in an infant formula is being conducted.

KEYWORDS: Amaranth oil; DHASCO; enzymatic interesterification; fat substitute; infant formula

INTRODUCTION

Amaranth, an ancient Meso-American crop, is considered to be a pseudocereal and currently cultivated in warm regions such as Mexico, Central and South America, Africa, India, China, and the southern border of the United States (*1*). Amaranth grain lipid content ranges from 6 to 9% (*2*), yielding a yellow liquid oil at room temperature with a reported melting point of −27 °C. Amaranth grain has been extensively studied for its healthy nutritional properties and even as an alternative to increase the protein quality of products such as tortillas (*3*, *4*) and milk-like beverages and infant formulas (*5*). Amaranth has also been studied for its hypocholesterolemic and antioxidant properties (*6*−*8*). However, successful applications of amaranth oil are limited, and the crop remains greatly underexplored. Amaranth oil contains about 18.6−23.4% palmitic (C16:0), 22.7−31.5% oleic (C18:1n-9), and 39.4−49.8% linoleic (C18:2n-6) acids (*1*, *2*, *9*−*12*). Several studies have reported significant amounts of important unsaponifiable components in amaranth oil (*12*) such as tocopherols, phytosterols, and squalene. Tocopherols are well-known for their cardiovascular benefits and antioxidant capacity (*6*), whereas phytosterols and squalene have the ability to decrease serum total cholesterol (*13*−*15*). Squalene has also been studied for its anticarcinogenic (*16*) and antioxidant activities (*17*). Amaranth oil contains approximately 4.2% squalene (*18*), 44 mg of total tocopherols per 100 g of oil (*6*), and 834 mg of total sterols in 100 g of oil (*19*) from the *Amaranthus* variety most commonly cultivated in the United States.

The content of palmitic acid and other major fatty acids (FAs) suggests that amaranth oil might be suitable for infant formula and other infant food applications. There are few important structural differences between an oil of vegetable source such as amaranth and an animal fat such as breast milk. There are essential chemical characteristics of breast milk fat that make it highly nutritious for infants. Lipids in breast milk represent an important source of energy (approximately 50% of total energy) and essential fatty acids required as important structural cell components of membrane tissues (*20*, *21*). Lipids are also necessary for physiological functions such as fat-soluble vitamin absorption and hormone and eicosanoid syntheses. Breast milk mainly contains ∼26.7−35.5% oleic, ∼18.3−25.9% palmitic, and ∼10.2−16.49% linoleic acids (*20*, *22*−*24*). Unlike vegetable oils, the *sn*-1,3 positions are mainly occupied by unsaturated FA, whereas saturated FA are at the *sn*-2 position of TAG. Palmitic acid accounts for the majority of the saturated fat portion in breast milk with > 60% by weight esterified at the *sn*-2 position.

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Table 1. Fatty Acid Profile of Amaranth Oil, CAO[a], and DCAO[b]

	fatty acid[c] (mol %)						
	16:0	18:0	18:1n-9	18:2n-6	18:3n-3	22:6n-3	others[d]
amaranth oil							
total	18.3 ± 0.1	3.8 ± 0.0	28.9 ± 0.0	47.8 ± 0.1	1.2 ± 0.0	nd[e]	nd
sn-2	2.1 ± 0.0	nd	26.9 ± 0.6	72.2 ± 2.1	0.7 ± 0.0	nd	nd
sn-1,3[f]	27.1 ± 0.7	5.7 ± 0.0	29.9 ± 0.3	35.7 ± 1.2	1.7 ± 0.2	nd	nd
CAO							
total	34.2 ± 0.4	2.8 ± 0.0	23.3 ± 0.2	38.1 ± 0.2	0.7 ± 0.0	nd	1.0 ± 0.0
sn-2	20.8 ± 1.1	nd	23.3 ± 0.4	55.9 ± 0.7	nd	nd	nd
sn-1,3	40.9 ± 1.1	4.1 ± 0.1	23.3 ± 0.5	29.1 ± 0.7	1.0 ± 0.0	nd	1.4 ± 0.0
DCAO							
total	33.5 ± 0.0	2.8 ± 0.0	23.0 ± 0.0	36.9 ± 0.0	0.7 ± 0.0	1.9 ± 0.0	1.3 ± 0.1
sn-2	20.2 ± 0.0	0.8 ± 0.0	24.1 ± 0.0	53.3 ± 0.1	0.7 ± 0.0	0.9 ± 0.1	nd
sn-1,3	40.8 ± 0.0	3.8 ± 0.0	22.5 ± 0.0	28.7 ± 0.0	0.6 ± 0.0	2.4 ± 0.0	1.9 ± 0.1

[a] CAO, customized amaranth oil. [b] DCAO, DHA-containing customized amaranth oil. [c] Mean ± SD, $n = 3$. [d] Others include C14:0, C20:1n-9, C20:2n-6, and C20:5n-3; others for DCAO also include C12:0. [e] nd, not detected. [f] *sn*-1,3 (mol %) = [3 × total (mol %) − *sn*-2 (mol %)]/2.

The regiospecificity of palmitic acid in breast milk reduces the formation of insoluble complexes between unesterified long-chain saturated FA and calcium, also known as "calcium soaps", allowing correct energy and calcium absorption (*25*−*28*). Breast milk may also naturally contain docosahexaenoic acid (DHA) (∼0.06−0.4%), but the variations in content depend on the mother's diet and metabolism (*20*, *22*, *23*, *29*).

Infant formulas have been developed to complement and/or substitute optimum nutrition from breast milk. Infant formulas intended for healthy term infants should mimic the composition of breast milk from healthy mothers (*30*). The fat portion in commercial infant formulas is usually achieved with vegetable oils such as coconut, soybean, sunflower, and corn oils (*30*). Blends of these oils are readily used to balance the FA content of the final product. Hence, the composition of the formula depends on the type and portion of oils used. However, FA arrangement in the glycerol backbone remains similar to that of the original vegetable oils, with a low palmitic acid content at the *sn*-2 position. Other techniques involving trans- and interesterification reactions have been used to modify oils from the structural point of view. By definition, structured lipids (SLs) are novel triacylglycerols (TAG) that have been modified from natural oils and fats by incorporating a new FA or restructured to change the positions of its original FAs (*31*, *32*). The resulting SL possesses the biological, physical, and chemical characteristics of its new FA components, and so it can be manipulated to change its melting behavior, digestion, absorption, and nutrition value (*32*). The ideal SL for infant formula must contain palmitic acid mostly esterified at the *sn*-2 position and unsaturated FA at the *sn*-1,3 positions to resemble breast milk fat. No particular regulations have been reported for DHA content in the United States, but recommendations have been issued to mimic breast milk composition. The European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommends levels of 0−0.5% DHA in infant formula (*33*).

Amaranth oil represents an interesting raw material for unexplored applications, such as an SL designed to complement infant nutrition. Therefore, the purpose of this study is to use enzymatic interesterification techniques to modify underutilized amaranth oil into a SL, by increasing its palmitic acid content at the *sn*-2 position and incorporating DHA, so it can be partially or complementarily used in milk-based infant formulas to deliver a lipid component more similar to that in breast milk.

MATERIALS AND METHODS

Materials. Amaranth oil was purchased from Nu World Amaranth Inc. (Naperville, IL). DHA-containing single cell oil (DHASCO) was generously donated by Martek Bioscience Corp. (Columbia, MD). Immobilized Lipozyme RM IM, a *sn*-1,3-specific lipase from *R. miehei*, and Novozym 435 (a nonspecific lipase) were purchased from Novo Nordisk A/S (Bagsværd, Denmark). Supelco 37 Component FAME mix, C17:0-heptadecanoic acid (>98% purity), triolein, 2-oleoylglycerol, squalene (>99% purity), and tocopherols (α-, γ-, and δ-) were used as standards and were purchased from Sigma Chemical Co. (St. Louis, MO). Plant sterol mixture and 5β-cholestane were acquired from Matreya Inc. (Pleasant Gap, PA). Other solvents and chemicals were purchased from Sigma Chemical Co., J. T. Baker Chemical Co. (Phillipsburg, NJ), or Fisher Scientific (Norcross, GA).

Customized Amaranth Oil (CAO) Structured Lipid. The CAO was designed to contain increased levels of palmitic acid at the *sn*-2 position compared to the original amaranth oil. The CAO was obtained using a stir-batch reactor for a large-scale interesterification reaction of amaranth oil and ethyl palmitate (1:4 mol ratio, respectively) using 10% Novozym 435 (by total weight of reactants) as enzymatic catalyst at 60 °C for 3 h. Then, the level of free fatty acids (FFA) was reduced to an acceptable level (<1%) by purifying the CAO using a KDL-4 short-path distillation system (UIC Inc., Joliet, IL) under the following conditions: holding temperature, 50 °C; feeding rate, 150 mL/h; heating oil temperature, 191 °C; cooling water temperature, 18 °C; and pump vacuum, <100 mTorr. CAO was developed as a substrate for further interesterification with DHASCO. The FA compositions of the initial amaranth oil and CAO are shown in **Table 1**.

Experimental Design. Response surface methodology (RSM) was used to predict the optimal conditions for the interesterification of CAO and DHASCO. Two mole ratio levels (1:0.1 and 1:0.2, CAO and DHASCO, respectively) and two levels of reaction time (1 and 2 h) were studied for the reaction at 60 °C, using 10% Lipozyme RM IM as catalyst. The central composite design resulted in 20 experiments, including eight possible combinations of both independent factors, and two center points. **Table 2** shows the conditions tested and the recorded responses. The responses were fitted to a second-order polynomial equation to obtain the relationship between factors and variables and, further, to predict optimal conditions for specific incorporation objectives.

Reaction Procedure. Interesterification reactions were carried out in screw-cap test tubes using 10% (w/w) of the corresponding catalyst and incubated in an orbital shaking water bath at 200 rpm according to the conditions shown in **Table 2**. The resulting product was filtered through anhydrous sodium sulfate column three times to separate from immobilized enzyme and properly stored in Teflon-lined test tubes at −80 °C for future fatty acid methyl ester (FAME) and positional analyses.

Gram-Scale Synthesis. The prediction model was used to determine the optimal conditions for DHA incorporation into CAO. The gram-scale interesterification reaction took place in a stir-batch reactor at 60 °C for 1.6 h with constant stirring at 200 rpm. CAO and DHASCO were blended at 1:0.04 mol ratio, and 10% (w/w) of Lipozyme RM IM was used as enzymatic catalyst. By the end of the reaction, the resulting DHA-containing CAO (DCAO) SL was vacuum filtered to separate from

Table 2. Experimental Design of Factors and Responses for Modeling Enzymatic Reaction by RSM[a]

expt[b]	CAO (mol)	DHASCO (mol)	temp (°C)	reaction time (h)	total DHA (mol %)	total PA (mol %)	PA at *sn*-2 (mol %)
N1	1	0.1	60	1	7.1 ± 0.2	30.6 ± 0.0	17.7 ± 2.3
N2	1	0.1	60	2	6.8 ± 0.1	30.7 ± 0.0	20.1 ± 0.0
N3	1	0.2	60	1	12.6 ± 0.1	28.1 ± 0.0	18.1 ± 0.0
N4	1	0.2	60	2	12.6 ± 0.2	28.1 ± 0.1	18.3 ± 0.3
N5	1	0.15	60	1	10.5 ± 0.7	29.0 ± 0.3	18.4 ± 0.1
N6	1	0.15	60	2	10.1 ± 0.2	29.2 ± 0.1	18.7 ± 1.0
N7	1	0.1	60	1.5	7.0 ± 0.1	30.7 ± 0.0	20.2 ± 0.1
N8	1	0.2	60	1.5	12.5 ± 0.1	28.1 ± 0.0	18.3 ± 0.5
N9	1	0.15	60	1.5	9.8 ± 0.0	29.4 ± 0.1	19.0 ± 0.0
N10	1	0.15	60	1.5	10.0 ± 0.1	29.3 ± 0.0	18.3 ± 0.2

[a] Mean ± SD, $n = 2$. [b] Abbreviations: CAO, customized amaranth oil; DHASCO, DHA-containing single-cell oil; DHA, docosahexaenoic acid; PA, palmitic acid.

enzyme. The amount of FFAs was reduced through short-path distillation using a KDL-4 (UIC Inc.) unit under the following conditions: holding temperature, 50 °C; feeding rate, 100 mL/h; heating oil temperature, 185 °C; coolant temperature, 15 °C; and vacuum pump, < 100 mTorr. Free fatty acid content was determined according to AOCS Official Method Ca 5a-40 (*34*) as percent oleic acid.

Determination of Fatty Acid Profiles. Amaranth oil, CAO, DHASCO, and DCAO samples were converted to FAME following AOAC Official Method 996.01, Section E (*35*), with minor modifications. Briefly, 100 mg of oil was weighed into a Teflon-lined test tube, and 1 mL of C17:0 in hexane (20 mg/mL) was added as internal standard and dried with nitrogen to remove solvent. Then, 2 mL of 0.5 N NaOH in methanol was added followed by incubation for 5 min at 100 °C to saponify the lipid. After incubation, 2 mL of 14% boron trifluoride (BF_3) in methanol was added. The sample was vortexed for 1 min and incubated again for 5 min at 100 °C to allow methylation. To stop the reaction and extract the FAMEs, 2 mL of hexane and 2 mL of NaCl-saturated solution were added to the sample, vortexed for exactly 2 min at room temperature, and centrifuged for 5 min at 1000 rpm to separate the organic and aqueous phases. The upper organic layer was filtered twice through an anhydrous sodium sulfate column and recovered into a GC vial and analyzed. Supelco 37 component FAME mix was used as FAME external standard and run in parallel with the samples.

Positional Analysis. The Luddy et al. (*36*) method for pancreatic lipase hydrolysis was used to analyze the *sn*-2 position. Samples (100 mg) were collected in Teflon-lined test tubes, and 2 mL of Tris-HCl buffer (1.0 M), 0.5 mL of sodium cholate solution (0.05%), and 0.2 mL of calcium chloride solution (2.2%) were added and vortexed for 2 min to emulsify. The sample was hydrolyzed by adding 40 mg of pancreatic lipase and incubating at 40 °C for 3 min. The tubes were vortexed for 2 min, then 1 mL of HCl (6 N) was added to stop the reaction, and 4 mL of diethyl ether was added to extract the hydrolyzed product. The upper layer containing the lipid components was separated and filtered twice through an anhydrous sodium sulfate column. The extracted solution was flushed with nitrogen to evaporate solvent until one-third of the volume was left. The concentrated extract was spotted on silica gel G TLC plates and developed with hexane, diethyl ether, and formic acid (60:40:1.6, v/v/v). 2-Oleylglycerol was spotted in parallel as identification standard for 2-monoacylglycerol (2-MAG). Plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The band corresponding to 2-MAG was scrapped off and converted to FAME as previously described. Fifty microliters of C17:0 in hexane (20 mg/mL) was used as internal standard. FAs esterified at the sn-2 position were quantified by GC, and the amounts at *sn*-1,3 were calculated (*37*).

GC Analysis. FAMEs were analyzed using an Agilent Technology (Santa Clara, CA) 6890N gas chromatograph (GC) equipped with a flame ionization detector (*24*). Separation was achieved with an SP-2560 column, 100 m × 0.25 mm i.d., 0.20 μm film. Injection (1 μL) was performed at a split ratio of 5:1. The carrier gas was helium at constant pressure mode and 1.1 mL/min flow rate. The injection and detection temperatures were 250 and 260 °C, respectively. The sample was held at 150 °C for 3 min, then ramped to 215 at 10 °C/min, and held isothermally for 40 min. FAME relative content was calculated by integration using an online computer. The average of duplicate analyses was reported.

Melting Profile Analysis. The melting profiles of amaranth oil, CAO, and DCAO were determined with a differential scanning calorimeter (DSC) (model DSC7, Perkin-Elmer Co., Norwalk, CT) according to AOCS Recommended Practice Cj 1-94 (*38*). Indium (mp 156.60 °C, ΔH = 28.45 J/g) was used for instrument standardization, and dry ice was used as coolant. A sample (5−8 mg) was weighed and hermetically sealed in a 30 μL capacity aluminum pan (Perkin-Elmer), using an empty sealed pan as a reference. The thermograms were analyzed using the software provided with the DSC (Pyris software, Perkin-Elmer, Shelton, CT).

Chemical Properties Analyses. Free fatty acid, saponification, and iodine values were determined according to AOCS Official Method Ca 5a-40 (*34*) and AOCS Recommended Practices Cd 3a-94 (*39*) and Cd 1c-85 (*40*), respectively.

Tocopherol Analysis. Tocopherols were identified and quantified in amaranth oil and DCAO using a normal phase high-performance liquid chromatography (HPLC) system (Hewlett-Packard 3392A, Avondale, PA) according to the method of Ye et al. (*41*).

Squalene Determination. Amaranth oil and DCAO samples (1.0 g) were saponified according to the method described by Jekel et al. (*42*) using 0.5 mL of saturated KOH in water solution, adding 8 mL of 3% pyrogallol in ethanol to prevent oxidation and incubating in a water bath at 80 °C for 30 min. After cooling at room temperature, the unsaponifiable fraction was extracted using 20 mL of hexane and 10 mL of water. After separation, an aliquot (10 mL) was transferred to another test tube, completely dried under nitrogen, and then recovered in 1 mL of hexane. Recovered unsaponifiables were sealed in GC vials. Samples were analyzed using an Agilent Technology 6890N GC equipped with a FID and an SP-2560 column (100 m × 0.25 mm i.d., 0.20 μm film). The analysis conditions remained the same as previously described for GC analysis of FAMEs. Squalene was identified and quantified by comparing the retention response to a calibration curve of pure squalene (> 99%) (10−100 ppm).

Phytosterol Analysis. The unsaponifiable fraction was separated as previously described for sterols with some modifications (*42*). Briefly, 250 mL of sample (amaranth oil and DCAO) was weighed into a screw-cap test tube and added to 250 mL of internal standard solution (2 mg/mL of 5β-cholestane in hexane). After drying with nitrogen, the sample was saponified with 250 μL of a saturated KOH in water solution in a water bath at 80 °C for 30 min in the presence of 2 mL of 3% pyrogallol in ethanol solution (antioxidant). After cooling, 20 mL of hexane, 10 mL of water, and 2 mL of saturated NaCl in water solution were added to the sample to extract the unsaponifiables. The upper layer (about 20 mL) was transferred to a screw-cap test tube that was previously rinsed with SylonCT/toluene/methanol (glass deactivation). The extracted unsaponifiables were completely dried under nitrogen and derivatized with 150 μL of TMS/pyridine (1:1, v/v) for 1 h in a water bath at 70 °C with constant stirring. The solution was dried under nitrogen and then recovered in 1 mL of hexane into a GC vial. The analysis was performed using an Agilent Technology 6890N GC equipped with a FID and an Agilent 19091J-413 HP-5 5% phenyl methyl siloxane column (30 m × 0.32 mm i.d., 0.25 μm film, 325 °C maximum). Injection (1 μL) was at 300 °C at a split ratio of 5:1, using helium as carrier gas at 1.5 mL/min flow rate in a constant-pressure mode. The sample was heated from 260 to 300 at 3 °C/min and then held isothermally until a total time of 20 min. Detection temperature was 320 °C. Four of the most common phytosterols (brassicasterol, campesterol, stigmasterol, and β-sitosterol) found in vegetable oils, and also previously reported for amaranth oil, were identified by comparison with the GC chromatogram of a plant sterol mixture. The resulting peaks of the identified phytosterols were calculated by integration using an online computer. All TMS derivatives were prepared in duplicate, and the total amount was calculated as milligrams of sterol per 100 g of oil sample (*43*).

Oxidative Stability Experiment. Amaranth oil and DCAO (4.0 g) were weighed in screw-cap tubes and oxidized for 72 h at 50 °C in the dark using a shaking water bath (New Brunswick Scientific Co., Edison, NJ). The samples were removed from the water bath and analyzed at 0, 24, 48, and 72 h for peroxide value (PV) and *p*-anisidine value (*p*AV). PV and *p*AV were determined using AOCS Official Methods Cd 8b-90 (*44*) and Cd 18-90 (*45*) correspondingly. Total oxidation (TOTOX) value was calculated as 2 × (PV) + (*p*AV) (*46*).

Statistical Analysis. All reactions and analyses were performed in duplicate for amaranth oil, CAO, and DCAO. Average and standard deviation were calculated and reported. The analysis of variance (ANOVA) and the mathematical model for optimization by enzymatic interesterification were obtained using MODDE 5.0 (Umetrics, Umeå, Sweden).

RESULTS AND DISCUSSION

Amaranth oil was characterized and used as the initial and major substrate for this study. The FA profile for amaranth oil (**Table 1**) is in agreement and within the range established from previous studies (*1*, *9*−*12*). The major FAs in amaranth oil are linoleic (47.8%), oleic (28.9%), and palmitic (18.3%) acids. The calculated TAG average molecular mass was 922.4 g/mol. Even though oleic and palmitic acid contents resemble that in breast milk, amaranth oil significantly contains a larger amount of linoleic acid, generally esterified at the *sn*-2 position (72.2%) compared to the *sn*-2 position of breast milk fat that is mostly occupied by palmitic acid (>60%) (*26*). As mentioned before, this particular arrangement reduces the formation of calcium soaps, increases the calcium absorption, and improves the availability and absorption of long-chain FA (LCFA) for proper structural development of the newborn (*25*, *28*). Esterification techniques can also be used to produce SL with improved or customized functionality. It can also be used to modify a natural oil (amaranth oil) to meet specific characteristics as total or partial replacement of another oil or fat (breast milk fat). We developed a customized amaranth oil (CAO) with increased total palmitic acid and at the *sn*-2 position (**Table 1**). The larger incorporation of palmitic acid was mainly at the expense of the total content of linoleic acid. An optimization model (results not shown) was developed to determine the conditions for a gram-scale interesterification of amaranth oil and ethyl palmitate. Palmitic acid incorporation was significantly affected by long reaction time and slightly influenced by substrate mole ratio. After synthesis and purification through short-path distillation, the FFAs were reduced to 0.2% as oleic acid. The FA composition of CAO is shown in **Table 1**, and the calculated molecular mass was 910.2 g/mol.

Furthermore, an experiment was designed to study the effect of substrate ratio and reaction time on the incorporation of DHA into CAO. We selected DHASCO (52.6% DHA) as our raw material for enzymatic interesterification. Two levels of each factor were analyzed in a central composite design using MODDE 5.0 software (Umetrics). The reaction conditions for each experiment and the corresponding responses are shown in **Table 2**. Backward selection and multiple regressions were used to fit the results to a second-order polynomial model. The linear Rxt, squared Rxt*Rxt, and interaction Rxt*DHASCO terms were omitted because they were not significant at $\alpha_{0.05}$. The normal probability plot (not shown) showed linear distribution, and the residual plot (not shown) was equally distributed along the zero line, confirming that the assumptions made of model error and constant error variance were not violated. The resulting ANOVA is shown in **Table 3**. The multiple correlation coefficient (R^2) was 0.99 corresponding to the fraction of the variation of the response explained by the model, whereas Q^2 (0.98) corresponds to the fraction of the response that can be predicted. The model equation can therefore be expressed as follows: total DHA = 10.10 + 2.80DHASCO − 0.34DHASCO*DHASCO, where total DHA is the total content of DHA incorporated in CAO, DHASCO stands for the mole ratio used for the reaction, and DHASCO*DHASCO is the squared term of mole ratio. Substrate availability had the largest positive effect on the total DHA incorporation as shown in **Figure 1a**, whereas reaction time was slightly adverse for this response (**Figure 1b**) within the tested range. Panels **c** and **d** of **Figure 1** show the effect of each factor on the prediction of DHA incorporation. The minimum increase of the substrate ratio suggests higher DHA incorporation. The lower effect of reaction time suggests that longer times would result in steady DHA incorporation levels with nonsignificant ($P > 0.05$) variations. **Figure 2** shows the contour plot for total DHA affected by substrate ratio and reaction time. The contour plot has become a very useful tool for research to graphically identify the combined effect of the parameters tested. We observed a steady increase in total DHA as a result of changes in the DHASCO availability. On the other hand, from **Figure 2** we can also note the minimum effect reaction time seems to have on the objective response (total DHA).

Table 3. ANOVA Table for DHA Incorporation

total DHA	DF[a]	SS	MS (variance)	*F* value	*P* value	SD
total	20	2054.290	102.714			
constant	1	1958.620	1958.620			
total corrected	19	95.670	5.035			2.244
regression	5	94.684	18.937	268.925	0.000	4.352
residual	14	0.986	0.070			0.265
lack of fit (model error)	3	0.307	0.102	1.661	0.232	0.320
pure error (replicate error)	11	0.678	0.062			0.248
$N = 20$	$Q^2 = 0.980$		$R^2_{adj} = 0.986$			
DF = 14	$R^2 = 0.990$		RSD = 0.265			

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

The resulting model can be used for either prediction of responses according to specific parameter combinations or optimization of reactions to achieve desired objectives. Even though DHA incorporation was the main response studied in the experiment, the effects on the final palmitic acid content and the palmitic acid at the *sn*-2 position were also closely considered for the DCAO design.

Considering the possible application of DCAO as a partial fat replacement for milk-based infant formula, we determined that DCAO should contain from 1.0 to 2.5% DHA, and no more than 32% palmitic acid, from which at least 20% should be esterified at the *sn*-2 position. Having this objective in consideration, DCAO was developed using the optimization model previously described for DHA incorporation into CAO. The model suggested an interesterification reaction at 60 °C for 1.6 h, using a 1:0.04 mol ratio (CAO/DHASCO) and 10% (w/w) Lipozyme RM IM. The final FA composition of DCAO at gram scale is presented in **Table 1**. The final results were in agreement with those planed and set in the optimization model, with a slightly higher amount of total palmitic acid (33.9%). Palmitic acid at the *sn*-2 position accounted for 20.2%. Several studies have been conducted to support the importance of the positional distribution of palmitic acid in the dietary TAG structure (*28*, *47*, *48*). Vegetable oil mixtures commonly used for infant formula contain only about 5.0% palmitic acid at the *sn*-2 position (*47*). Formulas prepared with palmitic acid at the *sn*-2 position-enriched TAGs were better absorbed than those with significantly lower content, even when the enrichment at the *sn*-2 position was about half of the amount reported for breast milk (>60%). It is worth mentioning that it has been suggested that about 50% of the palmitic acid content at the *sn*-2 position is absorbed and conserved through TAG reassembly (*47*).

The melting behavior of amaranth oil, CAO, and DCAO was evaluated by DSC thermal profiling. **Figure 3** shows the DSC

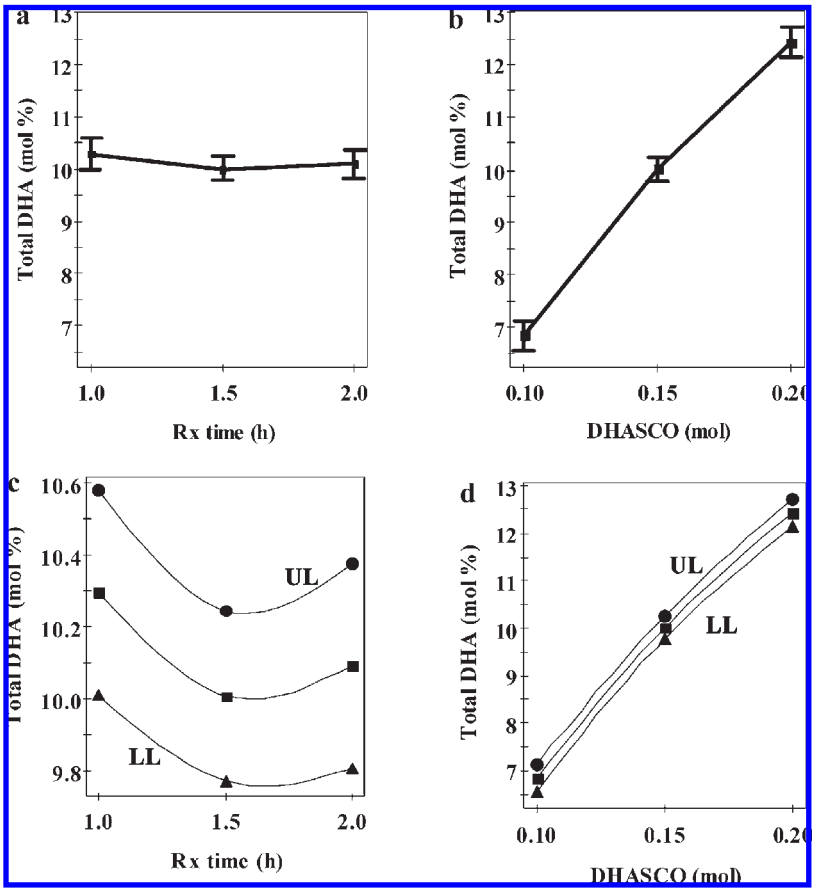

Figure 1. Effect of (**a**)reaction time and (**b**) DHASCO availability on total DHA content; values plotted are means ± SD, $n = 6$ for low and high levels, $n = 8$ for center point.) Prediction plot of total DHA content when (**c**) reaction time or (**d**) DHASCO availability varies. UL and LL refer to upper and lower confidence levels, respectively.

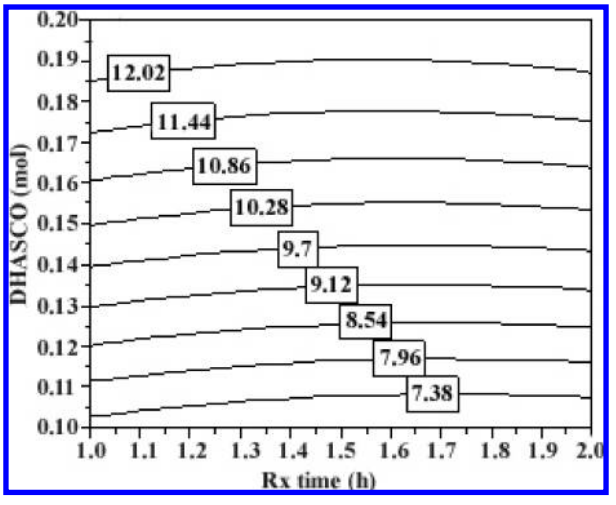

Figure 2. Contour plot showing effect of DHASCO availability (mol) and reaction time on the incorporation of DHA using Lipozyme RM IM as catalyst at 60 °C. The labels inside the plot indicate the total DHA content (mol %).

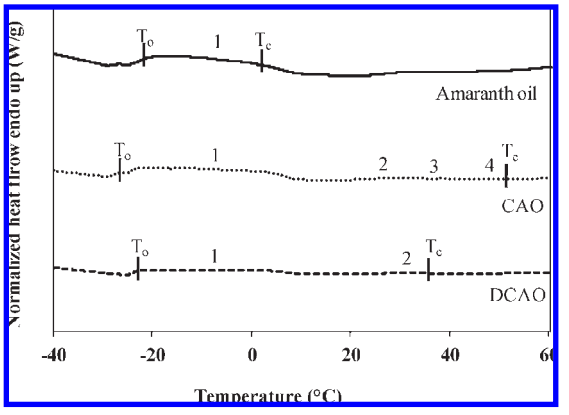

Figure 3. DSC melting thermograms of amaranth oil, CAO intermediate SL (palmitic acid enriched), and DCAO final SL (palmitic acid and DHA enriched). For each sample, T_o and T_c indicate melting onset and completion temperature points, respectively. Main endothermic peaks are consecutively numbered in each thermogram. Refer to **Table 1** for SL abbreviations.

thermograms obtained for all of the samples. Amaranth oil had only one endothermic peak resulting in a narrow melting range (**Table 4**). The themograms corresponding to CAO and DCAO showed a wider melting range due to the presence of small portions of higher melting TAG species (endothermic peaks consecutively numbered for each thermogram in **Figure 3**). CAO contains more palmitic and less linoleic acid than amaranth oil (**Table 1**); the larger saturated fat proportion is responsible for the secondary high-melting peaks 2, 3, and 4. DCAO contents of palmitic and linoleic acid remain similar to those from CAO (**Table 1**); however, the inclusion of DHA, a highly unsaturated FA, explains the lower presence of high-melting peaks (**Figure 3**) compared to CAO and hence the narrower melting range (**Table 4**). For amaranth oil, melting range was estimated from peak 1. For the melting range of CAO and DCAO, the onset

Table 4. DSC Melting Behavior of Amaranth Oil and SLs[a]

sample	onset point[b] (°C)	end point[c] (°C)
amaranth oil	−22.8 ± 0.1	2.3 ± 0.1
CAO[d]	−28.5 ± 0.7	52.0 ± 0.0
DCAO	−23.9 ± 1.3	35.3 ± 0.3

[a] Mean ± SD, n = 2. [b] Onset temperature (T_o) from the first melting peak. [c] Completion temperature (T_c) of the last melting peak. [d] Refer to **Table 1** for abbreviations.

Table 5. Chemical Properties of Amaranth Oil and SLs[a]

property	amaranth oil	CAO	DCAO
AV[b]	9.9 ± 0.2	0.4 ± 0.2	1.5 ± 0.2
FFA[b]	5.0 ± 0.1	0.2 ± 0.1	0.8 ± 0.0
IV	128.2 (93.3−100.4)[c]	120.5	118.7
SV	193.8 (217)[d]	196.4	195.8

[a] Abbreviations: CAO, customized amaranth oil; DCAO, DHA-containing customized amaranth oil; AV, acid value; FFA, free fatty acids (%, oleic acid); IV, iodine value; SV, saponification value. [b] Mean ± SD, n = 2. [c] According to manufacturer's product information. [d] From ref *59*.

temperature (T_o) was estimated from the melting start temperature of peak 1, and the completion temperature (T_c) was estimated from the melting end temperature of the last peak (peak 4 for CAO and peak 2 for DCAO).

Table 5 shows several important characteristics of amaranth oil, CAO, and DCAO. The structural and compositional changes of amaranth oil through its enzymatic modification to CAO and DCAO are also reflected in the physicochemical properties of the new oil. The interesterification reactions produced a large amount of FFAs during each SL synthesis. The amount of FFA adversely affects the oxidative stability and quality of the oil, hence the vital importance of the short-path distillation as a physical refining process for CAO and DCAO to remove the FFAs produced after interesterification. For instance, the final SL, DCAO, contained 4.2% FFA (as oleic acid) right after the end of the reaction and was reduced to 0.8% after short-path distillation. The iodine value (IV) measures the degree of unsaturation of a lipid by quantifying the grams of iodine absorbed by 100 g of lipid (*49*). The IV of amaranth oil was 128.2 (**Table 5**), which is outside the range provided by the manufacturer (Nu World Amaranth Inc.). However, other authors (*2*) reported a similar IV of 130 for amaranth oil used in feeding studies. CAO resulted in a lower IV (120.5) than its precursor amaranth oil, probably due to the larger amount of palmitic acid incorporated in the former. Even though DCAO's content of palmitic acid remained similar to that from its precursor CAO (**Table 1**), the IV of DCAO decreased to 118.7, most probably because of DHASCO's contribution of medium-chain saturated FAs (C12:0 and C14:0) to the final FA composition of DCAO (**Table 1**). On the other hand, the saponification value (SV) provides an indication of the change in molecular mass as a result of the enzymatic modifications from amaranth oil to DCAO. The wider variety of FA species in CAO and DCAO contributed to the increased SV compared to that of amaranth oil. Both SLs showed higher SV (**Table 5**), meaning that the molecular masses of both CAO and DCAO are lower than that of the original amaranth oil.

Enzymatic interesterification and purification of oils might affect the composition of the unsaponifiable fraction. The contents of tocopherols, phytosterols, and squalene in precursor amaranth oil and DCAO final product are shown in **Table 6**. The total tocopherol content of the amaranth oil used for this study was 65.3 mg/100 g of oil. The manufacturer specification for total tocopherols ranges from 50 to 120 mg/100 g of amaranth oil.

Table 6. Unsaponifiable Fraction Composition (Milligrams per 100 g) of Amaranth Oil and DCAO[a]

component	amaranth oil	DCAO[b]
tocopherol		
α	13.3 ± 0.1	0.9 ± 0.1
β	26.8 ± 0.3	1.6 ± 0.2
γ	5.7 ± 0.1	0.4 ± 0.1
δ	19.5 ± 0.2	0.4 ± 0.1
α-tocotrienol	nd	0.4 ± 0.1
total	65.3 ± 0.7	3.6 ± 0.5
phytosterols		
brassicasterol	21.0 ± 0.2	28.6 ± 0.9
campesterol	20.7 ± 0.4	36.3 ± 0.7
stigmasterol	306.6 ± 4.9	417.0 ± 2.9
β-sitosterol	497.7 ± 9.7	525.0 ± 5.6
total[c]	846 ± 15.2	1006.8 ± 4.3
squalene	4020	10

[a] Mean ± SD, n = 2. [b] Abbreviations: DCAO, DHA-containing customized Amaranth oil; nd, not detected. [c] Total refers to the sum of the four main phytosterols quantified and not to the total amount of sterols in amaranth oil.

Our result is also similar to that reported by Qureshi et al. (*6*) of 44 mg of total tocopherols per 100 g of refined and degummed amaranth oil. However, we observed a different distribution among the homologues; in this study, β-tocopherol was the major homologue identified in amaranth oil, and no tocotrienol was detected. As shown in **Table 6**, there were significant differences for all of the homologues between the amaranth oil and DCAO and an approximately 94.5% loss in total tocopherol. We believe the significant loss of tocopherols was induced by esterification of tocopherols and exposure of the samples to light during the interesterification and/or short-path distillation. Some studies reported an adverse effect of interesterification on tocopherol content (*50*,*51*). Other researchers suggested that the use of short-path distillation for purification and deodorizing purposes not only affects the FFA content but also contributes to the loss of non-TAG components such as tocopherols (*52*,*53*). In addition, it has been suggested that SLs purified by short-path distillation require supplementation with appropriate antioxidants before storage and food applications (*52*).

Several studies have reported that β-sitosterol, campesterol, and stigmasterol are the most common phytosterols found in higher plants (*54*, *55*). In this study, we identified and quantified the amount of four of the most common species of phytosterols (**Table 6**). Contrary to the effect on tocopherol content, the interesterification and purification of amaranth oil produced increased contents of all four phytosterols. The predominant sterol in both samples was β-sitosterol (497.7 mg/100 g of amaranth oil and 525 mg/100 g of DCAO), followed by stigmasterol and small amounts of brassicasterol and campesterol. The total amount of sterols increased by 19% in DCAO and contained a compositional distribution similar to that of amaranth oil, with the only difference being brassicasterol, the least available. Azadmard-Damirchi and Dutta (*50*) also reported increments in phytosterols due to interesterification. However, it is also possible that in our case the significant decrease in tocopherol content had an effect on the overall distribution of the remaining components in the unsaponifiable fraction and therefore was responsible for the apparent increase in phytosterols.

Amaranth has constantly been reported as a significant source of squalene, a biosynthetic precursor of all steroids present in food. Recently, the interest in squalene determination in food products resulted in several studies that confirmed squalene's health benefits as a chemopreventive agent (*16*) and an antioxidant that quenches singlet oxygen (*17*) and in reducing serum

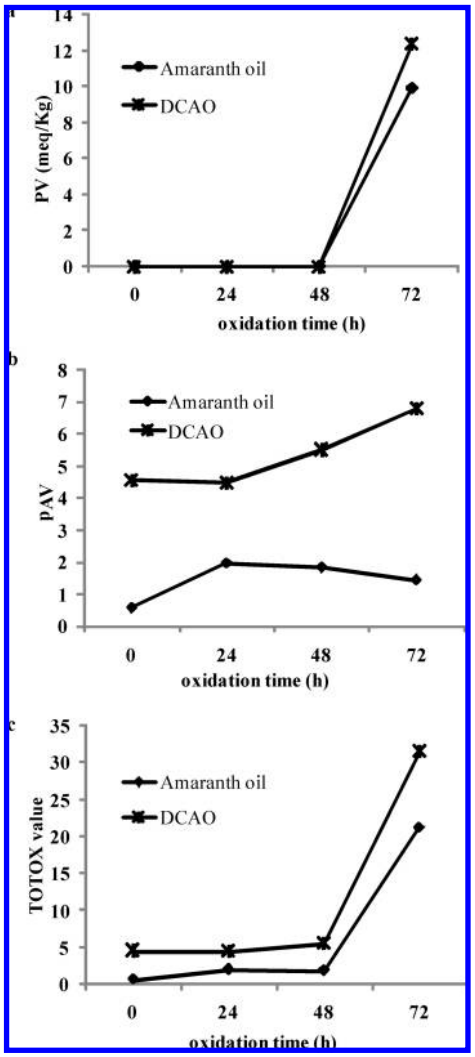

Figure 4. Changes in (**a**) peroxide value (PV), (**b**) *p*-anisidine value (*p*AV), and (**c**) total oxidation (TOTOX) values of amaranth oil and DCAO during induced oxidation at 50 °C. Refer to **Table 1** for abbreviations.

cholesterol levels (*13*). Commercially important oils such as olive, rice bran, corn, peanut, rapeseed, sunflower, and cottonseed oils contain low levels of squalene, ranging from 0.01 to 0.4% (*56*). He and Corke (*18*) reported an average 4.2% of squalene from the oil of 104 samples of 30 species of *Amaranthus* grain, in accordance with several other studies that had reported similar results (*10*, *11*). From our results shown in **Table 6**, amaranth oil contains 4.0% squalene and is similar to those previously mentioned. However, we observed that the enzymatic modification and purification through short-path distillation had a highly adverse effect on the squalene content of DCAO. We believe that the high temperatures (193 and 185 °C) used in the short-path distillation unit removed the squalene from DCAO, resulting in a squalene-rich residual distillate and a squalene-lean purified oil. Sun et al. (*57*) observed that short-path distillation at 180 °C at 3 mTorr was responsible for removing FFA and 90.5% squalene from crude amaranth oil (14.5% lost in the process). In our experiment, DCAO lost about 99.8% of the original squalene content from amaranth oil, presumably due to the two purification steps in which short-path distillationwas used (the first after CAO synthesis).

PV and *p*AV were determined to estimate the oxidation susceptibility of amaranth oil and DCAO at accelerated oxidation conditions. PV (**Figure 4a**) was used to measure the primary products of oxidation, and *p*AV (**Figure 4b**) was used to measure the production of secondary products. Oxidation was induced at 50 °C in a covered water bath for 72 h. PV and *p*AV were tested every 24 h, and results were plotted and are shown in **Figure 4**. According to our PV results, amaranth oil and DCAO were stable before 48 h. At 72 h we observed similar increases in PV for both samples (DCAO > amaranth oil). However, *p*AV through the accelerated oxidation period for DCAO was higher than the corresponding amaranth oil, which remained low and constant through the experiment. Gamel et al. (*11*) reported that crude amaranth oil was more stable to peroxide production than crude sunflower oil when incubated at 60 °C (for 30 days); because our sample had a different level of processing, we believe we registered higher PVs through a shorter period mainly because of the refining process of amaranth oil done by the manufacturer. We observed that DCAO showed good oxidative stability even though most of the natural antioxidants in the SL were removed during short-path distillation. We believe the good stability of DCAO was a result of the higher saturated FAs incorporated by interesterification and was not drastically affected by the inclusion of highly unsaturated DHA. Yankah and Akoh (*58*) reported that appreciable DHASCO oxidation occurred only after 48 h of induced oxidation at 60 °C. Obviously the stability of the raw materials (amaranth oil and DHASCO) used to synthesize DCAO played an important role in the stability of the final product, as well as the FA composition. Finally, the total oxidation value (TOTOX) was calculated from PV and *p*AV to evaluate the overall oxidative stability of amaranth oil and DCAO (**Figure 4c**). Both samples showed similar distributions. However, DCAO was in general slightly more susceptible to oxidation than amaranth oil, in part due to the loss of natural antioxidants during the purification process by short-path distillation, but mostly because of the insertion of DHA. The application of proper antioxidants will yield a more stable DCAO.

In conclusion, we developed a final SL (DCAO) from amaranth oil enriched with palmitic acid at the *sn*-2 position that in the final formulation of milk-based infant formula would yield the recommended DHA content for proper nutrition. The characteristics (physical and chemical) determined in this study support the feasibility of applying DCAO as a partial fat substitute or complement for milk-based infant formula. However, more research on the conditions for application and stability in the product should be conducted to support our result. The use of proper antioxidants would enhance the stability of DCAO and therefore its functionality in the final infant formula product.

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