STRUCTURED LIPID FROM UNDERUTILIZED AMARANTH OIL FOR POSSIBLE APPLICATION IN INFANT FORMULA

by

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(Under the Direction of Casimir C. Akoh)

ABSTRACT

The purpose of this study was to develop a structured lipid (named DCAO) from amaranth oil to resemble breast milk fat in milk-based infant formula. We increased the content of palmitic acid in amaranth oil using Novozym 435 lipase, specifically at the *sn*-2 position from 2.1 to 20.8%. Docosahexaenoic acid (DHA) was incorporated at the *sn*-1,3 positions using specific Lipozyme RM IM lipase. DCAO contained 33.0% palmitic acid and 0.9% DHA. Sterol content increased 19% compared to amaranth oil, however interesterification and purification procedures reduced the content of tocopherols (94.5%) and squalene (99.8%). Antioxidants are recommended even though we observed good oxidative stability. DCAO showed higher melting temperatures, iodine and saponification values than amaranth oil. The infant formula containing DCAO closely resembled breast milk fat composition, meeting the recommended DHA (0.2%) content, and supplying more palmitic acid at the *sn*-2 position (33.0%), for proper development and better fat absorption, respectively.

INDEX WORDS: Amaranth oil, breast milk fat substitute, infant formula, palmitic acid.

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To my supportive parents and my amazing brother

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CHAPTER 1

INTRODUCTION

Interest in lipids with specific functionality has led to the study of underutilized crops for commercial food applications. Amaranth grain has been extensively studied for its nutritional properties and as an alternative to increase the protein quality of milk-like products and infant formulas. Amaranth has also been studied for its hypocholesterolemic and antioxidant properties. However, amaranth oil applications remain unexplored. Amaranth oil is rich in linoleic (39.4-49.8%), oleic (22.7-31.4%), and palmitic acids (18.6-23.4%) (*1-6*), which are also the major fatty acids available in breast milk fat. The high content of palmitic acid suggests that amaranth oil might be suitable for infant formula and other infant food applications. There are important chemical characteristics of breast milk fat that makes it highly nutritious for infants (*7*). The positional location of the majority of palmitic acid is related to better absorption (*8-10*). Therefore, the content of this fatty acid (FA) is directly related to the high caloric energy contribution of breast milk for infants' development. Commercially available infant formula does not resemble breast milk fat. Fat analogs used for infant formula are usually mixtures of vegetable oils that do not contribute the right amount of biologically available palmitic acid. Structured lipids (SL) with desirable properties can be prepared through chemical or enzymatic interesterification, by incorporating new FAs in natural oils and fats, or rearranging the original ones (*11*). The aim of this study was to effectively modify amaranth oil via lipase catalysis for use as an alternative fat source for infant formula and other infant products.

Our research consists of a two-step modification of amaranth oil. First, a second-order polynomial model was developed using MODDE 5.0 (Umetrics, Umeå, Sweden) to predict the amount of total palmitic acid incorporated when reaction time and substrate level were manipulated in the Novozym® 435 lipase-catalyzed interesterification of amaranth oil and ethyl palmitate at 60°C; and to optimize the combination of parameters to achieve specific palmitic acid contents in modified amaranth oil. Further in the second stage of our research, the SL was interesterified with docosahexaenoic acid (DHA)-containing single cell oil (DHASCO) using Lipozyme RM IM lipase for specific incorporation of DHA at the *sn*-1,3 positions. The resulting milk fat analog was characterized and evaluated for chemical and physical properties.

Our research focused on developing a suitable SL that in combination with milk would yield a balanced FA profile that closely resembles breast milk. The most challenging aspect of our research was to design the experiment based on the final properties needed for the infant formula. An accurate balance was to be maintained between the lipid profile of the SL product and the fatty acids recommended levels for infant formula. Therefore, this research covered five specific objectives in three main phases. The first phase is the characterization of commercial amaranth oil supplied by NuWorld Amaranth (Naperville, IL, USA). The second phase is the development of a SL from amaranth oil using esterification techniques. The third phase is to confirm the application feasibility for complete or partial fat substitution in infant formulas. The specific objectives are:

1. Determine the FA profile of commercial amaranth oil and determine the content and type of FA esterified at *sn*-2 and *sn*-1,3 positions.

2. Increase content of palmitic acid esterified at *sn*-2 position by randomization of amaranth oil triacylglycerols (TAGs) with ethyl palmitate. Chemical and enzymatic methods were evaluated.
3. Develop a SL from randomized amaranth oil with DHA at *sn*-1,3 positions by specific enzymatic interesterification and using DHASCO as substrate. Lipozyme RM IM was used as catalyst for the interesterification reaction between previously modified amaranth oil TAGs and DHASCO.
4. Characterization of SL synthesized to assess *in vitro* its chemical and physical properties.
5. Formulate a balanced infant formula prototype incorporating the SL synthesized. Evaluate product suitability.

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CHAPTER 2

LITERATURE REVIEW

AMARANTH OIL

Amaranth is an ancient crop originally from Meso-America. It is classified as a pseudo-cereal and is currently cultivated in Mexico, Central and South America, Africa, India, China and the United States (*1*). It had an important economic, social and ceremonial value in the Aztec culture similar to corn. *Amaranthus* genuses differ in their chemical composition, physical characteristics and production yields. The most common species cultivated are *A. cruentus, A. hybridus, A. hypocondriacus* and *A. caudatus.* The most common species cultivated in ancient Mexico was *A. hypocondriacus,* which is still widely used by the confectionary industry to produce the popular so-called "alegria" (*2*). In the USA, amaranth is mainly cultivated in the southwest region along the Mexican border, where the most common species grown are *A. cruentus and A. hypocondriacus* (*3*).

Climatic differences result in variations in seed yield and protein content. *Amaranthus'* most common species grow better in hot-temperate and subtropical climates, *A. cruentus* yields a larger seed production in both climates, and hence a higher yield when processed (*4*). Many of these species have multiple applications as vegetables, grains and for oil production. The vegetable-amaranth crop is grown in the higher-rainfall, low elevation tropics like Africa, Indonesia, New Guinea, China and portions of South America (Peru, Argentina and Bolivia). Amaranth species are extremely adaptable in alternating wet-dry climates. As a grain, it is widely

cultivated in Western South America, Central America, Northern India, Western Nepal, Southwest US and Mexico (*5*). Amaranth grains contain significant amounts of protein, essential amino acids and lipids. Protein accounts for about 12 – 18.5% and fat content between 4 – 8.50% (*6-10*). The lipid is mainly contained in the coat-embryo fraction of the seed, which comprises about 25% of the total weight (*8*).

Amaranth oil is yellow, liquid at room temperature and has a melting point of -27° C. In a study of the optimum conditions for chemical extraction and refining of amaranth seed oil (*11*), the authors suggested the use of a pre-treatment to obtain higher yield during extraction. Abrasive milling would remove the coat-embryo fraction and further separation by screening could be used for a more efficient extraction process. Hexane extraction and distillation yield turbid oil with a dark color and a strong odor. Therefore, further processing is required to remove impurities. Degumming is done by stirring the oil with water, and centrifugation allows the separation of gums from the oil phase. The resulting oil is dewaxed by cooling and centrifuging. Alkali refining by the American Oil Chemists' Society (AOCS) method is suitable for amaranth oil. Lyon and Becker also investigated the effect of excess NaOH on the refining process. According to their results, any excess from 0.10 to 0.60% NaOH will reduce free fatty acid (FFA) content, as oleic acid, from 2.37 to about 0.30%. The oil recovery would also be reduced but the remaining oil would have a medium yellow color. The most efficient treatment, in terms of color, would be using a concentration of 16° Be' NaOH with a 0.40% excess. Bleaching is required to improve the oil color. The temperature and concentration of clay might play an important role for amaranth oil recovery and color intensity. High temperatures will reduce peroxide value but will also affect color intensity. High concentrations of clay will significantly reduce peroxide value and improve color, but it may also have an important economic impact for

processors since oil recovery is reduced and operation cost is increased due to the higher clay concentration. Lyon and Becker suggested that 110°C is the optimal temperature required to reduce peroxide value to an acceptable level; and no more than 2% of clay should be used for a significant improvement in color without major economic effect.

Amaranth oil is a good source of palmitic (16:0), oleic (18:1) and linoleic (18:2) acids. It also contains significant amounts of squalene, sterols, tocopherols and tocotrienols. The variations in the fatty acid (FA) profile of amaranth oil depend on the amaranth grain species and the processing technique used for extraction and refining. FA content variations among the main amaranth species cultivated are not significant; palmitic acid content ranges from 18.6-23.4%, oleic acid from 22.7-31.5%, linoleic acid content is about 39.4-49.8% and linolenic acid content is about 0.5-1.36% (*1, 8, 10-13*). The following tables (**Table 2.2** and **Table 2.2**) present a compilation of data from various studies on the differences in FA profile of amaranth oil from different species, using different extraction techniques and with different levels of processing.

Table 2.1. *Amanthus cruentus* Fatty Acid Profile from Raw Oil and Comparison to Processed Oil

FA (wt %)	raw oil			processed oil[c] (wt %)
	range (wt %)[a]	average (wt %)	*sn* -2 position (% of total FA)[b]	
12:0	-	0.70	-	-
14:0	0.2 - 0.27	0.24	0.1	-
15:0	-	-	-	-
16:0	13.4 - 22.2	19.80	1.1	13.9
16:1	0.11 - 0.4	0.26	0.2	-
17:0	0.95 - 1.7	0.95	-	-
17:1	-	0.70	0.1	-
18:0	2.74 - 4.5	3.57	0.3	2.61
18:1	20.4 - 32.9	30.10	35.9	19.8
18:2	38.2 - 62.1	42.20	61.3	62
18:3	0.5 - 1.12	0.70	0.9	1.09
20:0	0.66 - 1.25	0.80	-	0.61
20:1	-	0.30	-	-
22:0	0.24 - 0.3	0.27	-	-
24:0	-	-	-	-
22:1	-	-	-	-
24:1	-	-	-	-
squalene	4.6 - 9.16	5.93		8.01

For all references, oil was obtained with chemical extraction from raw *Amaranthus cruentus* seeds. [a]From references (*1, 8, 10, 11, 13*). [b]From reference (*13*). [c]Bleached and decolorized oil; From reference (*11*).

Table 2.2. Fatty Acid Profile of Other *Amaranthus Species* and Comparison to Oil Extracted by Pressing

FA (wt %)	chemical extraction			pressing extraction
	A. caudatus [a]	*A. hypocondriacus* [b]	*A. hybridus* [c]	*A. hybridus* [c]
12:0	-	-	-	-
14:0	-	0.26	0.15	0.14
15:0	-	-	0.08	0.15
16:0	20.5	23.4	18.59	19.19
16:1	-	0.15	0.08	0.13
17:0	0.9	-	1.37	1.05
17:1	-	-	-	-
18:0	2.2	3.68	4.45	3.38
18:1	25.5	31.5	22.69	22.64
18:2	49.8	39.4	48	49.89
18:3	0.6	0.65	1.27[d]	1.36[d]
20:0	0.5	0.6	0.27	0.16
20:1	-	0.24	1.49	1.04
22:0	-	0.19	0.24	0.32
24:0	-	-	0.08	0.05
22:1	-	-	0.7	0.07
24:1	-	-	0.54	0.43
squalene	4.8	9.96		5.9

[a]From reference (*10*). [b]From reference (*1*). [c]From reference (*12*). [d]Reported as 18:3n3 and 18:3n6, with an approximate ratio of 3:1.

Processing of raw oil by bleaching and decolorization substantially decrease palmitic acid and oleic acid content, while linoleic acid remains about the same. However, it has been reported that a significant increase in squalene concentration is attained after the bleaching and decolorization processes (*11*). The functional properties of amaranth oil are expected to be similar to those of buckwheat and cottonseed oil based on the tri-, di- and monounsaturation in triacylglycerols (TAGs) profile, in which the last two account for the majority (*1*). The major FAs esterified at the *sn*-2 position in TAGs are linoleic, oleic and palmitic acids, respectively (**Table 2.1**).

Chemical extraction results in a higher yield of oil production than hard pressing. Residual oil is less than 1% in meal from chemically extracted oil. In practical terms, residual oil in cake is often about 6-10% from extraction in hard pressing plants (*14*). Squalene, palmitic acid and linoleic acid are more concentrated in oil extracted from hard pressing compared with the chemical extraction profile; while oleic acid concentration remains almost unchanged (**Table 2.2**). Linolenic acid (18:3) concentration is also increased with pressing extraction, and the ratio between 18:3n-6 and 18:3n-3 remains approximately the same about 1:3 (*12*). In another study on oil yield and squalene extraction from amaranth (*15*), the authors reported an average of 4.2% of squalene content in oil chemically extracted from 30 amaranth species. Even though squalene extraction would be more effective via oil pressing extraction, further isolation and purification techniques like fractionation, removal of the saponificables fraction after saponification of amaranth oil, could increase squalene content up to an average of 94% (*16*).

Amaranth oil is believed to have cardiovascular benefits because of its vitamin E (tocopherols and tocotrienols) and phytosterol contents. Tocopherols and tocotrienols comprise a group of vitamin E homologs recognized as natural antioxidants. From the eight naturally occurring homologs, γ and δ exert the largest antioxidant activity (*17*) and are also more potent cholesterol inhibitors than α and β (*18*). It has been reported that inhibition of HMG-CoA reductase activity (cholesterol biosynthesis regulator) is directly affected by α-tocotrienols content (*19*). Amaranth's refined degummed oil tocols fraction contains 43% tocopherols (mostly δ and γ), 47% β-tocotrienol, and 8% other tocotrienols. Amaranth oil has been reported to significantly inhibit cholesterol biosynthesis in animal models (*20*). The hypothesis for cholesterol reduction is based on the balance between tocols and squalene compounds in amaranth oil (*19*). It has been reported that the hypocholesterolemic activity of amaranth oil is

also due to its phytosterol content. Phytosterols do not reduce HMG-CoA reductase activity but block cholesterol absorption by directly competing against cholesterol. Sterols are considered an important minor component in unsaponifiable fractions of vegetable oils. Amaranth oil contains about 24.6×10^3 ppm of total sterols, from which only about 20% are free compounds and about 50% of total sterols are compounds with a Δ^7-structure (*13*). β-Sitosterol is the major phytosterol present in four amaranth species (including *A. cruentus* and *A. hypocondriacus*), and it is more concentrated than in other studied plants (*21*). In a randomized placebo-controlled clinical trial in which patients with CVD (cardiovascular disease) received diets that included different amounts of amaranth oil, the authors evaluated the nutrient quality of the different diets in terms of FA content, macro and micro nutrients, and energy value. Blood pressure and cholesterol levels from patients were registered. They concluded that amaranth oil in the diet had a beneficial action by decreasing LDL (low density lipoprotein) and VLDL (very low density lipoprotein) cholesterol levels; its antioxidant activity helped in preventing oxidative damage; and its inclusion in the diet increased the concentration of polyunsaturated fatty acids (PUFAs) in the diet (*12*).

Amaranth oil is an interesting and underexplored new source of oil. Its FA composition and healthy properties make it suitable for food applications. However, little has been done to incorporate amaranth oil in successful applications. Enzymatic techniques can be used to modify amaranth oil's palmitic acid content along the glycerol backbone. That way, amaranth oil could potentially be modified to meet specific food applications. We believe amaranth oil's FA composition resembles, in part, breast milk fat composition. However, there are significant compositional differences that make breast milk fat so unique.

HUMAN BREAST MILK

Human milk composition varies according to the lactation stage. The milk produced during the first 3-4 days after birth is called *colostrum;* it is rich in protein, minerals and vitamins A, D and B_{12}, but low in fat. Most of the protein in *colostrum* is present as immunoglobulin, which would help to develop the immunity system of the newborn. After a *transitional* period which last up to day 10, the subsequent milk produced is called *mature milk*, which may vary in composition between individuals. However, fat content is generally higher and hence more energy dense (*22*). Human milk, also called breast milk, is the principal source of nutrients and immunological benefits for infants and toddlers. Breast milk composition may vary according to race, genetics, season, birth term, physiology, lactation stage, etc. However, breast milk from a healthy mother generally contains all the major and minor essential nutrients in the perfect balance for appropriate growth and development. Breast milk has been universally considered as the optimum nutrition source for full term infants up to 6 months, providing all the nutrients except for the requirements of vitamin K, vitamin D, and iron (*23, 24*). Hence the design and development of feeding substitutes for infants is based on the average composition of mature breast milk from healthy mothers. Lipids in human milk represent an important source of energy (approximately 50%) and essential fatty acids required as important structural cell components of membrane tissues (*24, 25*). Lipids are also necessary for physiological functions like fat-soluble vitamins absorption, hormone synthesis and eicosanoids synthesis.

Human milk contains approximately 3-5% total lipids (*26*), from which triglycerides (TAG) comprise about 98% (*27, 28*), phospholipids and glycolipids account for 0.5-1%, and cholesterol for approximately 0.5% (*27*). Human milk TAG includes a wide variety of long chain and medium chain fatty acids, and PUFAs (*29*) (**Table 2.3**). Fatty acids from 10:0 to 20:4

accounted for over 98% (*29, 30*). Medium chain fatty acids (MCFA) are mainly cleaved from TAG by preduodenal lipases and are quickly absorbable without requiring the formation of micelles. Long chain fatty acids (LCFA) are hydrolyzed for absorption by pancreatic lipase, producing FFA and 2-monoglycerides (2-MAG). Human milk mainly contains ~26.7–35.5% oleic acid, ~18.3–25.9% palmitic acid, and ~10.2−16.49% linoleic acid (*25, 27, 30, 31*). Unlike vegetable oils, *sn*-1 and *sn*-3 positions are mainly occupied by unsaturated FA while saturated FA are at the *sn*-2 position of TAG. Palmitic acid accounts for the majority of the saturated FA portion in human milk with over 60% by weight of its total content at *sn*-2 position. Hence the formation of an insoluble complex between unesterified long chain saturated FA and calcium also known as "calcium soaps" is significantly low, allowing correct energy and calcium absorption (*29, 32-34*).

Human milk is also the most important source of n-3 and n-6 PUFA precursors and essential FA (EFA) (*27*). Similar to human adults, infants can transform linoleic acid (LA) from n-6 family into arachidonic acid (ARA); and linolenic acid (ALA) from n-3 family into docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (*35*). ARA and EPA are precursors for the formation of eicosanoids which regulate important physiological functions. ARA is also a major structural constituent of the central nervous system as well as DHA, which is also significantly high in the retina cortex. However, this *de novo* synthesis is a limited process in which both families compete for the same enzymes. Breast milk is naturally secreted with DHA and EPA; but the variations in content depend on the mother's diet and metabolism (*27, 36*). For term infants, the long chain-PUFAs content in breast milk from well nourished mother's ranges between ~0.1−0.7% ARA, ~0.05−0.2% EPA, and ~0.06−0.4% DHA (*25, 27, 30*).

Even when breast milk from a healthy mother represents optimal nutrition for newborns, there are several factors that may lead to the necessity to complement or totally substitute human milk with infant formulas specially designed to meet the infant needs. The mother's diet affects the nutrients content in secreted milk. The infant's energy requirements change with age; in general, newborns require approximately 100kcal/kg, while adults require <41kcal/kg (*27*). During infancy and childhood, energy is highly required for structural growth and development of body functions. After the first six months of the infant's life, human milk alone cannot supply the required protein and is not longer enough to maintain infant's growth. Hence solid foods and milk formula are used to complement nutrition (*23*). There are also other cases in which milk formula are used in earlier stages as the main food supply due to maternal milk unavailability and low quality production. Sociological and psychological factors might also affect the decision of nourishing an infant. According to the United Kingdom's Office of Population Censuses and Surveys in 1990, some of the most important factors affecting the decision of breastfeeding were the mother's social class, mother's age, and education level of the mother (*22*). As a result, 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 mother's (*37*). The fat portion of infant formulas currently in the market is usually achieved with vegetable oils such as coconut, soybean, sunflower and corn oil (*37*). Blends of these oils are readily used to balance the FA content of the final product; hence composition of the formula is dependent on the type and portions of oils used. However, FA arrangement in the glycerol backbone remains similar to the original vegetable oils; it means that oil blends are a mix of the corresponding portion of oil's original TAGs. The TAG composition remains distant from what optimum human milk analogs should offer. As mentioned before,

vegetable oil differs from breast milk fat especially in the saturated fat content and position in the TAG.

Palmitic acid is the main source of energy for infants since it is primarily at the *sn*-2 position where absorption is highly achieved. In contrast with the currently used blends in which the nature of vegetable oil prevails and the *sn*-2 position is mainly occupied by unsaturated FA. Although lard is comparable to human milk fat in content and position of palmitic acid (*26*), the use of pure animal fats may not be the best way to go when developing a new infant formula. The physical characteristics of animal fats make it harder to incorporate and process it without significant changes. However, some manufacturers are using oleo oil, a structured lipid commonly derived from beef fat, to achieve saturated and monounsaturated FA contents. The use of oleo oil is an exception for the use of vegetable oils in the development of infant formulas. Even if oleo oil is able to meet the saturated FA levels to emulate breast milk, LA and EFA contents are significantly lower (*37*).

Table 2.3. Summary of Fatty Acid Profile of Human Breast Milk and Bovine Milk

FA (wt %)	total triacylglycerols			*sn* -2 position	
	human milk[a]		bovine milk[b]	human milk[c]	bovine milk[d]
	pre term (31-36 weeks)	full term (37-40 weeks)			
4:0	-	-	4.5	-	2.9
6:0	-	-	2.3	-	4.8
8:0	-	-	1.3	-	2.3
10:0	1.3±0.2	1.0± 0.3	2.7	-	6.1
12:0	6.6±0.8	4.5±1.2	3.0	-	6.0
14:0	7.6±0.9	5.7±1.4	10.6	7.5±0.9	20.4
15:0	0.3±0.1	0.3±0.1	1.0	-	-
16:0	23.2±1.5	22.2±2.3	28.2	56±1.1	32.8
16:1	2.3±0.3	3.4±0.4	1.8	-	-
17:0	0.6±0.2	0.5±0.4	0.6	-	-
18:0	7.3±1.2	7.7±1.9	12.6	1.9±0.1	6.4
18:1	33.8±1.8	35.5±2.7	21.4	15.3±0.8	13.7
18:2	13.8±1.3	15.6±2.0	2.9	9.4±0.9	2.5
18:3	0.8±0.1	1.0±0.2	0.3	1.3±0.2	-
20:0	0.1±0.1	0.3±0.1	-	-	-
20:2	0.3±0.1	0.2±0.2	-	0.2±0.0	-
20:3	0.4±0.1	0.5±0.2	-	0.2±0.0	-
20:4n-6	0.6± 0.2	0.6±0.3	0.2	0.8±0.1	-
21:0	0.1± 0.1	0.2±0.1	-	-	-
22:4	0.2±0.1	0.1±0.2	-	0.3±0.0	-
22:5n-6	0.0±0.1	0.0±0.1	-	0.1±0.0	-
22:5n-3	0.1±0.1	0.1±0.2	-	0.3±0.0	-
22:6n-3	0.2±0.1	0.2±0.1	-	0.6±0.1	-

[a]From reference (*38*). [b]From reference (*39*). [c]From reference (*40*). [d]From reference (*41*).

BREAST MILK SUBSTITUTES AND INFANT FORMULAS

Bovine Milk

Milk is ideally intended for the nutrition of young mammals, and as a consequence of animal domestication, milk from several species has been typically used for human consumption including infants feeding. In most of the world, cattle accounts for the largest source of milk for

commercial purposes, and ultimately human consumption. In fact, the Food and Drug Administration (FDA) of the United States recognizes the term “milk” as that original from healthy cows (*42*), and that is the same way we refer to cow’s milk in this study. The large availability and complex composition of cow’s milk has lead to a wide spam of modifications and food applications. Milk covers the requirements for early nutrition and development of young cattle. It is composed of a balanced mixture of lipids (3.5−4.1%), proteins (3.3−3.9%), carbohydrates from which lactose represents the largest (4.95.0%), and vitamins and minerals (0.68−0.74%) dissolved in about 85.4−87.7% water (*41, 43*). Protein and lipids are also responsible for the characteristic flavor and texture of milk. The lipid fraction is mainly composed of approximately 95.50% TAGs, 2.30% diacylglycerols, 0.08% monoacylglycerols, 0.28% FFAs, 1.11% phospholipids, 0.46% cholesterol and 0.02% cholesterol esters (*44*). The characteristic flavor of milk is related to its butyric acid content (4.5%); however, the major fatty acids contained in milk are palmitic acid (28.2%), followed by oleic acid (21.4%), stearic acid (12.6%) and myristic acid (10.6%) (**Table 2.3**). Even though milk is very similar in composition to human milk, there are significant differences especially in their fatty acid profile. Human milk contains more palmitic acid esterified at the second position and important LCFAs; while milk contains significant amounts of short chain fatty acids (SCFAs) and larger content of palmitic acid at the first and third positions of the glycerol backbone. Therefore, it is not considered as the optimum food for infant nutrition.

Recommendations for Infant Formula

The development of infant formulas and complementary infant foods has lead to the creation of nutrient specifications for the commercial manufacturing of these products that closely meet the nutritional requirements for the intended population. As in adulthood, infant

nutritional requirements vary among populations; however, in the particular case of infant formulas, the variations are minor and regulated by health and food institutions in each country (**Table 2.4**). According to FDA, infant formulas should provide at least 3.3 g of total fat for each 100kcal consumed and at least 1.8 g of total protein. Other organizations, such as the Life Science Research Office (LSRO) and the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) have also reported recommendations for energy and carbohydrates content.

Table 2.4. Recommendations for Nutritional Composition of Infant Formulas[a]

recommendation (g/100 kcal, % kcal)		FDA (1985)	ESPGHAN (1991)	expert panel LSRO
energy (kcal/100 mL)	minimum	-	64	63
	maximum	-	72	71
total fat	minimum	3.3, 30%	4.4, 40%	4.4
	maximum	6.0, 54%	6.0, 54%	6.4
total protein	minimum	1.8	1.8	1.7
	maximum	4.5	2.8	3.4
total carbohydrates	minimum	-	8	9
	maximum	-	12	13
lactose	minimum	-	-	-
	maximum	-	-	-

[a]Summarized from reference (*45*).

Commercial Breast Milk Substitutes (Benchmarking of common products)

In the United States, commercial infant formulas meet the requirements specified by FDA and CFR (Code of Federal Regulations). The same institutions and other expert associations have published recommendations for the fatty acids composition of breast milk substitutes in order to reach more resemblance of human milk composition (**Table 2.5**).

Table 2.5. Recommendations for Fatty Acid Composition of Infant Formulas[a]

FA recommendation (mg/100kcal, % kcal)		linoleic acid	α−linolenic acid	DHA	ARA	EPA
FDA (1985)	minimum	300, 2.7%	-	-	-	-
	maximum	-	-	-	-	-
CFR (1994)	minimum	300, 2.7%	-	-	-	-
	maximum	-	-	-	-	-
ESPGHAN (1991)	minimum	500, 4.5%	-	-	-	-
	maximum	1200, 10.8%	-	-	-	-
European communities	minimum	300, 2.7%	50	-	-	-
	maximum	1200	-	2	1	1
expert panel LSRO (1998)	minimum	350 (8% of FAs)	77 (1.75% of FAs) LA:ALA< 16:1	-	-	-
	maximum	2240 (35% of FAs)	256 (4% of Fas) LA:ALA> 6:1	-	-	-

[a]Summarized from reference (*45*).

DHA complemented infant formulas are available in the market place claiming to be “an ideal formula choice” in the case of Nestlé products, and the “closest formula to breast milk” in the case of Enfamil products, both brands claim to contain “DHA and ARA nutrients found in breast milk.” According to the labels on these products, both formulas contain a mixture of vegetable oils from palm olein, soy, coconut and high-oleic sunflower. In both cases, *Mortierella alpine* oil is the source of ARA, and *Crypthecodinium cohnii* oil is the source of DHA. There are slight differences in the liquid phase used, and therefore in the emulsifying and stabilizing systems. However, the nutritional information presented in both products meets the recommendations mentioned above (**Table 2.6**).

Table 2.6. Nutrient Facts of Two Widely Available Commercial Infant Formulas

nutrients		per 100 Cal (5 fl oz)	
		Nestlé	Enfamil
protein	g	2.2	2.1
fat	g	5.1	5.3
carbohydrate	g	11.2	10.9
water	g	134	134
linoleic acid	mg	900	860
vitamins			
A	IU	300	300
D	IU	60	60
E	IU	2	2
K	mcg	8	8
thiamin (B_1)	mcg	100	80
riboflavin (B_2)	mcg	140	140
B_6	mcg	75	60
B_{12}	mcg	0.33	0.3
niacin	mcg	1050	1000
folic acid (Folacin)	mcg	15	16
pantothenic acid	mcg	450	500
biotin	mcg	4.4	3
C (ascorbic acid)	mg	9	12
choline	mg	24	24
inositol	mg	6	6
minerals			
calcium	mg	67	78
phosphorus	mg	38	43
magnesium	mg	7	8
iron	mg	1.5	1.8
zinc	mg	0.8	1
manganese	mcg	15	15
copper	mcg	80	75
iodine	mcg	12	10
selenium	mcg	3	2.8
sodium	mg	27	27
potassium	mg	108	108
chloride	mg	65	63

STRUCTURED LIPIDS

By definition, structured lipids (SL) are novel TAG that have been modified from natural oils and fats by incorporating a new FA or restructured to change the positions of its original FA (*46*, *47*). 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 (*47*). SL can be produced by chemical or enzymatic interesterification for special functionality or nutritional use. Randomized SL can be produced by both methods. However, chemical randomization is commonly preferred for industrial purposes because it can be cheaper (*46*). A chemical catalyst can change the acyl chains organization into a random pattern, changing the TAG but not the FA composition. Enzymatic catalysts are more selective and yield more specific TAG. Many studies have been successfully performed with lipases from *Mucor miehei* and *Candida antarctica* to incorporate long chain EPA and DHA into vegetable oils (*48*).

From a nutritional point of view, SL development serves two main purposes: to supply energy and to provide PUFAs. Several FA can be used depending on the benefits pursued. It has been recommended to use between 2-5% of n-3, 3-4% of n-6 and 30-65% of short chain and medium chain FA when developing SL for clinical nutrition. Oleic acid (18:1), a monounsaturated fatty acid, can be incorporated in SL synthesis to promote the balance of LCFA in synthesized TAG (*47*).

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 human milk fat. SL for infant formula are typically prepared by transesterification of tripalmitin with oleic acid, PUFA or

MCFA with *sn*-1,3 specific enzymatic catalyst such as Lipozyme RM IM® from *Rhizomucor miehei* and Lipozyme TL IM® from *Thermomyces lanuginosus.*

With the intention of developing an infant formula that in nature and nutrition is closer to human breast milk, food scientists have focused their efforts in developing new products in novel presentations to achieve optimum nutrition without sacrificing sensory characteristics and product stability. New technologies such as the ones previously discussed and new sources of essential nutrients are being studied, as well as their application in common food products. Underutilized commodities might represent an unexplored source of these nutrients. So far, some human milk fats substitutes have been developed but very few have been commercialized. Most of them are vegetable oil blends which in nature do not mimic breast milk structure and hence do not meet the nutritional requirements for term infants. Betapol™, a commercially available breast milk fat substitute for infant formulas developed by Loders Croklaan and Lipid Nutrition was prepared from enzymatic transesterification of tripalmitin, from palm oil, and oleic acid or PUFA, from vegetable oil. The transesterification reaction took place using Lipozyme RM, a *sn*-1,3 specific lipase from *R. miehei*, to achieve a TAG structure similar to **Figure 2.1**.

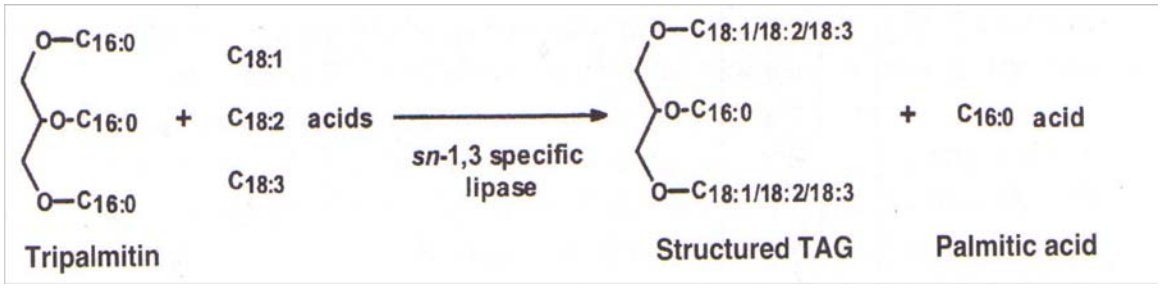

Figure 2.1. Preparation of structured TAG for use as human milk fat replacers (Betapol) (*27*)

Betapol contains up to 60% palmitic acid at the *sn*-2 position, while *sn*-1,3 positions are esterified with unsaturated FA such as oleic acid, ARA, DHA and EPA (*27*, *49*, *50*). The nutrition benefits of Betapol's application in infant formula were researched, and the authors (*40*) concluded that about 50% of the palmitic acid at the *sn*-2 position of TAGs, of breast milk and formula made with Betapol, is transferred to the chylomicron corresponding position. They also suggested that the regiospecificity of palmitic acid may have an effect on plasma lipoprotein lipid, apolipoprotein concentrations, and metabolism.

Our laboratory has previously researched on possible new fat alternatives for infant formulas, including vegetable oil blends containing palmitic acid (*51*, *52*) and structured lipids as human milk fat substitutes with palmitic acid (*53*), γ-linolenic acid (*54*) and omega-3 fatty acids (*55*). The characteristics and fatty acid profile of amaranth oil are similar to those from human milk fat. Amaranth is an underutilized crop grown in temperate climates; its oil is naturally rich in palmitic acid. Many studies have been published about the nutritional properties of amaranth, from its application as protein rich flour to its use in feeding mixes for hens to enhance n-3 FA content in eggs (*56*, *57*). Restructuring amaranth oil to match infant nutrition needs, as an alternative to vegetable oil blends with corn oil and sunflower oil, might yield a suitable SL for application in infant formulas and other infant foods.

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CHAPTER 3

ENRICHMENT OF AMARANTH OIL WITH ETHYL PALMITATE AT *SN*-2 POSITION BY CHEMICAL AND ENZYMATIC SYNTHESIS[1]

[1]Ashanty M. Pina-Rodriguez and Casimir C. Akoh. Accepted by the Journal of Agricultural and Food Chemistry, April 21, 2009.

ABSTRACT

Amaranth oil is rich in linoleic, oleic, and palmitic acids. Structured lipids (SL) with specific functional and nutritional characteristics can be prepared through chemical or enzymatic interesterification. The aim of this study was to increase the palmitic acid content at the *sn*-2 position in amaranth oil triacylglycerols (TAG) for possible use in infant formula. Chemical and enzymatic interesterification techniques were assessed before selecting the latter for further optimization modeling. Enzymatic interesterification of ethyl palmitate and amaranth oil significantly increased the total content of palmitic acid, reduced linoleic acid content, and increased the amount of palmitic acid at the *sn*-2 position of the SL product. Even though amaranth oil content of palmitic acid (18.3%) was originally similar to that in breast milk (18.3-25.9%), the structural changes induced through enzymatic modification resulted in a SL closely resembling breast milk fat and hence its possible application as a fat substitute for infant nutrition. A second-order polynomial model was developed to predict the amount of total palmitic acid incorporated when reaction time and substrate level were manipulated, and to optimize the combination of parameters to achieve specific palmitic acid contents in amaranth oil. The resulting model is useful to develop an SL from amaranth oil enriched with palmitic acid specifically at the *sn*-2 position for possible application in infant formulae.

KEYWORDS: Amaranth oil, enzymatic interesterification, structured lipid.

INTRODUCTION

Amaranth is an ancient crop originally from Meso-America where its importance was considered similar to corn and wheat before colonization period. It is classified as a pseudo-cereal and it is currently cultivated in warm climates with at least 18°C soil temperature such as in Mexico, Central and South America, Africa, India, China, and the United States. The lipid content of amaranth grain ranges from 6-9% (1). Amaranth oil is yellow, liquid at room temperature and it has a melting point of −27°C (2).

Nowadays, amaranth has been used in several bakery products including breads, cookies, pasta and marzipan (3). Also, it has been proposed as an alternative to increase protein quality in tortilla (4, 5). Other studied applications include milk-like beverages and infant formula (6). Even though amaranth oil has been extensively characterized before, applications still remain underexplored. Amaranth oil contains about 18.6-23.4% palmitic, 22.7-31.5% oleic, 39.4-49.8% linoleic, and 0.5-1.4% linolenic acids (1, 2, 7-10). Palmitic acid, a major fatty acid (FA) in amaranth oil, also constitutes the second major FA in breast milk (~18.3-25.9% palmitic acid) (11-14). This FA resemblance suggests amaranth oil can be used as raw oil to enhance palmitic acid content for infant formula application. However, there are important compositional differences between amaranth oil and breast milk fat. Although amaranth oil contains similar FA levels as those from breast milk, in amaranth oil the main FA esterified at *sn*-2 position is linoleic acid, followed by oleic and palmitic acids (10) compared to human breast milk which contains a relatively large amount of palmitic acid esterified at the *sn*-2 position (>60%) of the triacylglycerols (TAG) (15-18). It has been reported that about 81% of total palmitic acid is esterified at the *sn*-2 position of human milk fat (19). However, for adults large amounts of palmitic acid in the diet could represent higher risk for coronary heart disease (CHD) due to its

atherogenic properties (20), but for infants it represents two important benefits for proper nutrition. In breast milk fat, the preferential presence of palmitic acid at the *sn*-2 position improves infant's fat and calcium absorption while reducing the production and disposal of calcium soaps (19, 21). Amaranth lipid profile variations greatly depend on the cultivar, extraction method and refining process. It also contains significant amounts of squalene (~4.2%) (22), sterols (~2.5%) (10), and tocopherols and tocotrienols (~0.1%) (23).

We do not have knowledge of any other study attempting to modify amaranth oil for infant formula application. However several studies had been published on the development of human milk fat analogs, from randomized oil mixtures to achieve balance in FAs, to the interesterification of substrates such as tripalmitin, hazelnut oil FA, n-3 polyunsaturated FAs concentrates, rapeseed oil FA, soybean FA, lard, coconut oil, safflower oil, and butter oil to resemble breast milk fat composition (24-30). Betapol® (Loders Croklaan, Glen Ellyn, IL, USA) is an example of commercial breast milk fat substitutes produced by lipase interesterification of tripalmitin and unsaturated FAs (12).

Amaranth oil represents an alternative raw material that can be used to design structured lipids as milk fat substitutes for possible applications in infant nutrition. Therefore, the aim of this study was to restructure amaranth oil's TAGs by increasing its palmitic acid content at the *sn*-2 position. Chemical and enzymatic interesterification techniques were evaluated. Finally, an optimization model was developed to predict the incorporation of palmitic acid into amaranth oil.

MATERIALS AND METHODS

Materials. Amaranth oil was purchased from Nu World Amaranth Inc. (Naperville, IL). Ethyl palmitate (Kosher) and sodium methoxide (food grade) were purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized lipase, Novozym 435, was generously donated by Novozymes

North America Inc. (Franklinton, NC). Supelco 37 Component FAME mix, C17:0-heptadecanoic acid (>98% purity), triolein and 2-oleoylglycerol were used as standards and were purchased from Sigma Chemical Co. (St. Louis, MO). Other solvents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), J. T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA).

Oil mixtures preparation. For chemical and enzymatic methods comparison, two mixtures (10 g) of amaranth oil (M.W.=922.4 g/mol) and ethyl palmitate (M.W.=284.5 g/mol) were prepared, based on their molecular weight, in 1:4 and 1:6 mol/mol ratio, respectively. Amaranth oil is liquid at room temperature, but ethyl palmitate was melted to liquid at 38°C before blending to ensure mixtures' uniformity.

Chemical interesterification. A modified version of the method described by Lumor et al. (*31*) was used for chemical interesterification. 1 g of each mixture (described above) was weighed in labeled screw-cap test tube, flushed with nitrogen and dried for 15 min at 110°C. Then, 0.3% (w/w) sodium methoxide was added as chemical catalyst. The reaction was held for 60 min at 100°C with constant stirring at 200 rpm. The reaction product was cooled to 60-70°C and catalyst was removed using hexane and filtering through anhydrous sodium sulfate column three times.

Enzymatic interesterification. 1 g of each mixture (described above) was weighed in labeled screw-cap test tube, and 10% (w/w) of Novozym 435 (from *C. antarctica*) was added as enzymatic catalyst. Reaction was carried in a water bath at 60°C for 6 h with constant stirring. The resulting product was filtrated three times through anhydrous sodium sulfate column to remove the catalyst.

Triacylglycerols (TAGs) recovery. After chemical or enzymatic interesterification, the resulting product was spotted onto silica gel G TLC plates and a mixture of petroleum ether, diethyl ether, and acetic acid (90:10:0.5, v/v/v) was used to separate the TAGs. Lipid bands were visualized after spraying plates with 0.2% 2,7-dichlorofluorescein in methanol and under UV light. Ethyl palmitate and TAG separated bands were identified using ethyl palmitate and triolein as standards. The TAG band was scrapped off and recovered into test tubes for fatty acid methyl ester (FAME) and positional analyses as purified representations of the structured lipid (SL) obtained after the enzymatic reactions.

Experimental design by response surface methodology (RSM). A RSM mathematical model was developed to predict the incorporation of palmitate in amaranth oil by enzymatic interesterification. Amaranth oil and ethyl palmitate mixtures were prepared based on their average molecular weight. The suggested combinations resulting from the experimental design are shown in **Table 3.1**. The experimental design took into consideration the effect of ethyl palmitate to amaranth oil ratio (low level=1:4; high level=1:6, respectively) and the time of reaction (low level=6 h; high level=18 h) under isothermal condition (60°C) using 10% (w/w) of Novozym 435 as catalyst. Therefore, the central composite face design included eight possible combinations, and three center points. Treatments were performed in duplicate resulting in 22 experiments. The reactions took place in a water bath with constant stirring. After reaction completion, TAGs from the resulting products were recovered as described above, and analyzed for FA profile and positional analysis. Total incorporation of palmitate in amaranth oil TAGs and at the *sn*-2 position of the glycerol backbone were recorded as variable responses in **Table 3.1**, as well as the experimental conditions for each run.

Reaction procedures for RSM. Reactions were similar to the procedure described above for enzymatic interesterification. The suggested oil blends (**Table 3.1**) were weighed in screw-cap test tubes, and 10% (w/w) Novozym 435 was added as enzymatic catalyst. Reaction took place in an orbital shaking water bath at constant temperature (60°C) for 6, 12 and 18 h according to the conditions in **Table 3.1**. After reaction completion, TAGs were recovered according to the procedure previously described.

Mathematical model. The response surfaces for the relationship between factors and variables from the above design were fitted to a second-order polynomial equation of the form:

$$Y = \beta_o + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{1} \sum_{j=i+1}^{2} \beta_{ij} X_i X_j \quad \text{..... [1]}$$

Where Y is the dependent variable (palmitic acid incorporation); β_o is a constant; β_i is the linear term coefficient; β_{ii} is the quadratic term coefficient; β_{ij} is the interaction term coefficient; and X_i and X_j are the independent variables. The analysis of variance (ANOVA), regression analysis, and response surfaces were obtained using MODDE 5.0 (Umetrics, Umeå, Sweden). An optimization model for palmitic acid incorporation into amaranth oil by enzymatic interesterification was determined using RSM.

Determination of fatty acid profiles. Amaranth oil and SL samples were converted to FAME following the AOAC Official Method 996.01, Section E (*32*) with minor modifications. For amaranth oil sample preparation, 100 mg of oil was weighed into a Teflon-lined test tube, 1 mL C17:0 in hexane (20 mg/mL) was added as internal standard and dried with nitrogen to remove solvent. For SL analysis, 100 µL of internal standard was added to the recovered TAG band from previous separation step. Then, 2 mL 0.5N 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 hexane and 2 mL NaCl saturated solution were added to the sample, vortexed for exactly 2 min at room temperature and centrifuged for 5 min at 1,000 rpm to separate the organic and aqueous phases. The upper organic layer was filtered twice through anhydrous sodium sulfate column and recovered into a GC vial and analyzed. Supelco 37 component FAME mix was used as FAME external standard and ran in parallel with the samples.

Positional analysis. A modified version of the reported method (*33*) was used to perform the pancreatic lipase-catalyzed *sn*-2 positional analysis. Amaranth oil (100 mg) and SL (TAG recovered band) were collected in Teflon-lined test tubes. 2 mL Tris-HCl buffer (1.0 M), 0.5 mL sodium cholate solution (0.05%) and 0.2 mL calcium chloride solution (2.2%) were added to the samples and vortexed for 2 min. After emulsification, 40 mg of pancreatic lipase was added, mixed and incubated at 40°C for 3 min. The tubes were vortexed for two minutes and 1 mL HCl (6 N) and 4 mL of diethyl ether were added to stop the reaction and extract the hydrolyzed TAGs. The upper layer containing the lipid components was separated, filtered twice through anhydrous sodium sulfate column and flushed with nitrogen to evaporate solvent until one third of volume was left. The dried product 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 exposed to UV light to identify the different bands. The band corresponding to 2-MAG was scrapped off and converted to FAME as previously described. 300 μL C17:0 in hexane (20 mg/mL) was used as internal standard for the amaranth oil, and 50 μL of internal standard for the SL. FAs esterified at *sn*-2 position were quantified by GC and the amounts at *sn*-1,3 were calculated .

GC analysis. FAMEs (from amaranth oil, SL, and corresponding positional analyses) were analyzed using an Agilent Technology 6890N gas chromatograph equipped with a flame ionization detector. Separation was achieved with a SP-2560 column, 100m × 0.25mm i.d., 0.20 μm film. Injection (1 μL) was performed at a split ratio of 5:1. Helium was the carrier gas, at constant pressure and the flow rate was 1.1 mL/min. The injector temperature was 250°C and the FID set point was 260°C. In the oven, the sample was held at 150°C for 3 min, then increased up to 215°C ramping at 10°C/min, and held isothermally for 40 min. FAME relative content was calculated by integration using an online computer. Average of triplicate analyses were reported.

Statistical analysis. All samples, reactions, and analyses were performed in triplicate for amaranth oil and SLs using both interesterification approaches. 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

Commercial amaranth oil FA profile was determined and shown in **Table 3.2**. From the FA profile obtained, our results for palmitic, oleic, and linoleic acids were in agreement and within the range established from previous studies (*1, 2, 8-10*). Our results were used to estimate the molecular weight of commercial amaranth oil (922.4 g/mol); this value was later used to determine the corresponding portion of amaranth oil in each oil blend. Linoleic acid (47.8%) was the major FA in amaranth oil, followed by oleic (28.9%) and palmitic (18.3%) acids (**Table 3.2**). Linoleic acid is also the major available FA at the *sn*-2 position in amaranth oil (72.2%). Conversely in breast milk, linoleic acid content is about 15.6% (*13*), while palmitic acid accounts for the majority of the saturated FA portion of human milk with over 60% by weight of its total

content at *sn*-2 position. This particular arrangement serves several nutritional purposes. For instance, the formation of calcium soaps is low and hence it represents a more readily absorbable source of energy for infant development and also contributes to a better absorption of calcium (*15-18*). From the positional analysis of amaranth oil, we determined that there was only about 2.1% palmitic acid esterified at the *sn*-2 position of the glycerol backbone. Previous studies have shown that saturated FAs tend to be exclusively located at the external positions in vegetable oil TAGs, in contrast to animal fats in which these positions are usually occupied by unsaturated FAs (*34*). Esterification techniques can be used to produce SL with improved functionality due to the incorporation of new FA into oil or fat, or the rearrangement of the existing FAs (*35, 36*). Chemical interesterification is commonly preferred for industrial purposes because of its comparable yields and cheaper cost (*34*). However, enzymatic interesterification is more spatially selective, yielding more specific TAGs (*37*). Our aim was to modify the original amaranth oil's TAG structure to increase the palmitic acid esterified at the *sn*-2 position in order to match the recommended FAs' requirements (*38*) for breast milk fat substitutes. For that purpose, we assessed the overall performance of both chemical and enzymatic interesterification methods based on the total palmitic acid content and incorporation at the *sn*-2 position. Both interesterification reactions, carried at constant experimental conditions and using two substrate levels, increased the palmitic acid content at the *sn*-2 position at the expense of linoleic acid (**Table 3.3**). Even though, both techniques yielded higher total palmitic acid and higher palmitic acid at the *sn*-2 position, the increment was more significant using enzymatic interesterification. The SL products had lower total content of linoleic acid, and lower amount of this FA esterified at the *sn*-2 position than amaranth oil. Stearic acid content was lower for enzymatically produced SLs, and linolenic acid was not detected in the lipase-catalyzed SLs in contrast to the products

from chemical interesterification. As mentioned before, one of the nutritional advantages of the large amount of palmitic acid esterified at the *sn*-2 position is to prevent the formation of calcium soaps. For the enzyme catalyzed reaction using high mol ratio (1:6, amaranth oil to ethyl palmitate), there was no major difference in overall FA composition compared to the lower substrate level, but we observed a noticeable increase in the amount of palmitic acid at the *sn*-2 position, and linoleic acid at the *sn*-1,3 position. At this point, we concluded that for enzymatic reactions, no matter the higher availability of substrates, the reaction might require longer times to overcome the same level of hydrolysis and esterification reached in the low substrates level reaction, and therefore the amount of palmitic acid esterified at any of the positions would be affected by the reaction kinetics. Based on our preliminary experiments (**Table 3.3**), we selected the enzymatic interesterification method to develop an optimization model for palmitic acid incorporation in amaranth oil when substrate availability and reaction times changes. On this issue, the resulting SL from the recommended optimization reaction would be used as a fat substitute, possibly in combination with other fat sources, for infant formulae.

The experimental design included differences in substrate level and reaction time and this was used to develop a model for future prediction of palmitic acid content. The resulting amounts of total palmitic acid and palmitic acid at the *sn*-2 position are shown in **Table 3.1**. For total palmitic acid response, multiple linear regressions and the backward selection method were used to fit the results to a second-order polynomial model, from which the squared term $EtP^{*}EtP$ and the interaction term $Rxt^{*}EtP$ were deleted because they were not significant at 0.05 probability level. *EtP* refers to ethyl palmitate mole ratio, and *Rxt* refers to reaction time.

The multiple correlation coefficient (R^2) was 0.85, corresponding to the fraction of the variation of the response explained by the model. Q^2 corresponding to the fraction of the

variation of the response that can be predicted by the model was 0.79. Although R^2 is a very popular statistic value to assess the variance explanation, in planned experimentation it is more significant to support conclusions based on analysis of variance (ANOVA) statistics (*39*). The acceptable R^2 was probably adversely affected by the proximity in the range of the dependent variables (reaction time and mol ratio), therefore resulting in smaller response difference of palmitic acid incorporation relative to the variance, and hence possibly overlapping the projections. However, based on the acceptable value of R^2 in combination with the satisfactory results obtained in the ANOVA (**Table 3.4**), the RSM quadratic equation is appropriate for the modeling and optimization of palmitic acid incorporation into amaranth oil. The model showed no lack of fit (P>0.05) and P-value was <0.001. The RSM quadratic equation can be expressed as follows: $total\ PA = 57.14 + 9.77Rxt - 0.98EtP - 7.24Rxt^{*}Rxt$. Where $total\ PA$ is the total content of palmitic acid in SL; Rxt is the reaction time at which corresponding palmitic acid incorporation is achieved; EtP is the ethyl palmitate mol ratio used in reaction; and $Rxt^{*}Rxt$ is the squared term of reaction time.

Reaction time had the largest effect on the amount of total palmitic acid as shown in **Figure 3.1a**, while ethyl palmitate availability had less effect at low substrate level and negative effect at high substrate level (**Figure 3.1b**).

The projected responses (total palmitic acid) for variations in reaction time and ethyl palmitate availability when all, but the parameter of interest, remain constant are shown in **Figure 3.1c** and **3.1b**. Total palmitic acid content is projected to increase when reaction is performed for longer time (**Figure 3.1c**). However higher availability of ethyl palmitate showed a slightly adverse effect in total palmitic acid (**Figure 3.1d**). **Figure 3.2** shows the contour plot for the optimal combination of parameters to obtain a desired content of total palmitic acid. According to our

results, palmitic acid incorporation was mostly affected by long reaction time but slightly affected by molar ratio (**Figure 3.2**). The highest incorporation of palmitic acid (~61.1%) can be obtained in 15-17 h of reaction using low substrates level between 4.0-4.3 mol ethyl palmitate to 1.0 mol amaranth oil.

The model developed for total palmitic acid incorporation can explain a relatively high fraction of the response variations. However, the results for palmitic acid at *sn*-2 position were not efficiently fitted in a second-order polynomial equation. Only the term Rxt, corresponding to reaction time, was significant at $\alpha_{0.05}$. R^2 was only 0.32, and Q^2 was -0.01. The responses obtained did not show normal distribution, which means the esterification of palmitate into amaranth oil was random and non preferential. We believe the esterification of palmitic acid at the *sn*-2 position was not normal distribution because of the nonspecific properties of Novozym 435 used as reaction catalyst. However, other studies have shown preference of Novozym 435 for the *sn*-1,3 position in ethanolysis reactions at a high excess of ethanol substrate (~1:20, tridocosahexanoylglycerol to ethylcaprilate) (40). For the interesterification reaction of ethyl palmitate and amaranth oil TAGs, we observed random interesterification when using low substrate ratios (1:4 to 1:6, amaranth oil to ethyl palmitate). The random responses obtained and the non normal projected responses cannot be fitted into a linear mathematical equation. Therefore, further modeling and optimization was not performed for this response.

The developed RSM model can predict the optimal parameters combination to achieve specific palmitic acid incorporation. Based on test tube size confirmation experiments (results not shown), we are able to design the synthesis of SL from amaranth oil that resemble breast milk fat in palmitic acid composition, and with comparable or improved regiospecificity of palmitic acid at the *sn*-2 position in comparison to amaranth oil and other reported breast milk fat analogs (24-

30). Our optimization model suggested a reaction of amaranth oil and ethyl palmitate in a mole ratio of 1:4, catalyzed with 10% (by substrates weight) Novozym 435 for 3 h at 60°C with constant stirring will yield an SL to match the nutritional recommendations established for fat substitution in infant formula (38). However, further research is required to incorporate n-3 polyunsaturated FAs into this amaranth oil SL in order to completely resemble breast milk fat for infant formula applications. Previous studies had been successful in developing breast milk fat substitutes through enzymatic modification of vegetable oils. Sahin et al. (25) achieved a SL from hazelnut oil, tripalmitin and n-3 polyunsaturated FAs with 76.6% palmitic acid esterified at the *sn*-2 position, however the amount of total palmitic acid is almost double (45.5%) of the normal range in breast milk (18.3-25.9%). On the other hand linoleic acid content remained low (4.4%) but the incorporation of EPA and DHA (6.2%) might exceed the recommended contents for infant formulas (<0.25% EPA and <0.5% DHA) (38). In a different study, Maduko et al. (26) developed a SL to use with caprine milk for infant formula containing 24.6% palmitic, 29.6% oleic, and 3.4% linoleic acids similar to breast milk composition, however the resulting SL also contained 23.6% caprylic acid. Palmitic acid is the most extensively studied saturated FA for infant formula; other saturated FAs could lead to hypercholesterolemic effects, therefore care must be taken when increasing saturated fat level at the *sn*-2 position (41). Amaranth oil's SL for infant formula application is intended to achieve a balance between the palmitic acid total content, as well, palmitic acid esterified at *sn*-2 position, without overlooking other important FAs that contribute to the particular breast milk composition.

In conclusion, both interesterification techniques yielded high total content of palmitic acid at the *sn*-2 position, but lipase-catalyzed reaction resulted in higher content of palmitic acid and a better overall FA profile for the particular purpose of this research. The increment in palmitic

acid was, in part, at the expense of the total content of linoleic acid, and most of the linoleic acids at the *sn*-2 position were rather substituted for palmitic acid. For the proposed application, it is important to consider that the SL produced would partially substitute for other fat sources, animal or vegetable, for commercial use. For such purpose it is imperative to obtain the correct level of palmitic acid in SL that will enhance desired nutritional goal while remaining technologically and economically feasible. The model obtained using enzymatic interesterification for total palmitic acid can effectively explain the responses obtained, and can be used to predict the total content of palmitic acid in relation to substrate availability and reaction time. The model will be helpful in further research on the application of amaranth oil SL as a fat analog for infant food formulations.

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Table 3.1. Experimental Design of Factors and Responses for Modeling Enzymatic Reaction by RSM

expt[a]	amaranth oil (mol)	ethyl palmitate (mol)	temp (°C)	rx time (h)	total PA[b] (mol %)	PA at *sn*-2 (mol %)
N1	1	4	60	6	39.2±0.6	28.7±0.7
N2	1	4	60	18	62.0±0.3	57.4±1.1
N3	1	6	60	6	39.7±0.1	32.5±0.8
N4	1	6	60	18	61.0±1.7	56.0±5.2
N5	1	5	60	6	41.5±1.2	55.7±2.9
N6	1	5	60	18	56.1±6.5	46.2±4.5
N7	1	4	60	12	57.4±2.3	41.5±5.2
N8	1	6	60	12	52.0±1.5	49.5±4.9
N9	1	5	60	12	55.1±4.6	54.0±5.7
N10	1	5	60	12	57.7±0.2	46.3±3.9
N11	1	5	60	12	63.5±0.1	60.6±0.3

[a]Mean ± SD, n = 2. [b]Abbreviation: expt, experiment; temp, temperature; rx time, reaction time; PA, palmitic acid.

Table 3.2. Fatty Acid Profile of Commercial Amaranth Oil

		positional distribution	
fatty acid[a]	total (mol %)	*sn*-2 (mol %)	*sn*-1,3 (mol %)[b]
16:0	18.3±0.1	2.1±0.0	27.1±0.7
18:0	3.8±0.0	ND[c]	5.7±0.0
18:1	28.9±0.0	26.9±0.6	29.9±0.3
18:2	47.8±0.1	72.2±2.1	35.7±1.2
18:3	1.2±0.0	0.7±0.0	1.7±0.2

[a]Mean ± SD, n=3. [b]*sn*-1,3 (mol%) = [3 × total (mol%) – *sn*-2 (mol%)]/2. [c]Abbreviation: ND, not detected.

Table 3.3. Fatty Acid Profile of Structured Lipid Produced by Chemical and Enzymatic Interesterifications

		chemical[c]			enzymatic[e]		
substrate level[a]	fatty acid[b]	total (mol %)	*sn*-2 (mol %)	*sn*-1,3[d] (mol %)	total (mol %)	*sn*-2 (mol %)	*sn*-1,3 (mol %)
low (1:4)	16:0	20.4±0.7	22.4±4.2	19.4±1.4	39.2±0.4	28.9±0.6	44.4±0.4
	18:0	3.9±0.2	8.4±2.0	3.1±2.7	2.5±0.0	7.9±2.5	0.6±0.2
	18:1	31.5±1.2	42.6±2.1	26.0±1.7	22.7±0.1	37.3±4.9	15.4±2.5
	18:2	44.0±1.7	29.4±1.2	51.3±2.0	35.5±0.5	25.9±6.8	40.3±4.1
	18:3	0.5±0.0	ND[f]	0.7±0.0	ND	ND	ND
high (1:6)	16:0	19.3±0.3	23.4±4.7	17.3±2.5	39.7±0.1	33.2±1.3	42.9±0.6
	18:0	3.2±0.1	8.9±1.7	0.4±0.8	2.4±0.0	7.8±0.6	0.0±0.3
	18:1	26.9±0.4	33.9±3.0	23.4±1.0	22.6±0.1	41.6±4.5	13.1±2.2
	18:2	49.7±0.6	33.9±4.1	57.6±1.5	35.4±0.1	17.3±5.8	44.4±2.9
	18:3	0.8±0.1	ND	1.2±0.1	ND	ND	ND

[a]Substrate level refers to mol ratio of amaranth oil to ethyl palmitate. [b]Mean ± SD, n=3. [c]Conditions for chemical interesterification reaction: 0.3% catalyst, 100°C for 1 h. [d]*sn*-1,3 (mol %) = [3 × total (mol%) – *sn*-2 (mol%)]/2. [e]Conditions for enzymatic interesterification reaction: 10% catalyst, 60°C for 6 h. [f]Abbreviation: ND, not detected.

Table 3.4. ANOVA Table for Total Palmitic Acid Content

total PA[a]	DF	SS	MS (variance)	F-value	P-value	SD
total	22	63931.600	2905.980			
constant	1	62235.500	62235.500			
total corrected	21	1696.120	80.768			8.987
regression	3	1443.740	481.245	34.322	0.000	21.937
residual	18	252.385	14.021			3.745
lack of fit (model error)	5	101.928	20.386	1.761	0.190	4.515
pure error (replicate error)	13	150.458	11.574			3.402

N = 22	Q^2 =	0.793	R^2_{adj} =	0.826
DF = 18	R^2 =	0.851	RSD =	3.744

[a]Abbreviations: PA, palmitic acid; DF, degree of freedom; SS, sum of squares; MS, mean square; RSD, relative standard deviation; N, number of experiments; 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.

Figure 3.1. (a) Effect of reaction time on total palmitic acid content. (b) Effect of ethyl palmitate on total palmitic acid content. For (a) and (b) values plotted are means ± SD, n = 6 for low and high levels, n = 10 for center point. (c) Projected responses of total palmitic acid when reaction time is varying. (d) Projected responses of total palmitic acid when ethyl palmitate is varying. UL and LL refers to upper and lower confidence levels, respectively.

Figure 3.1

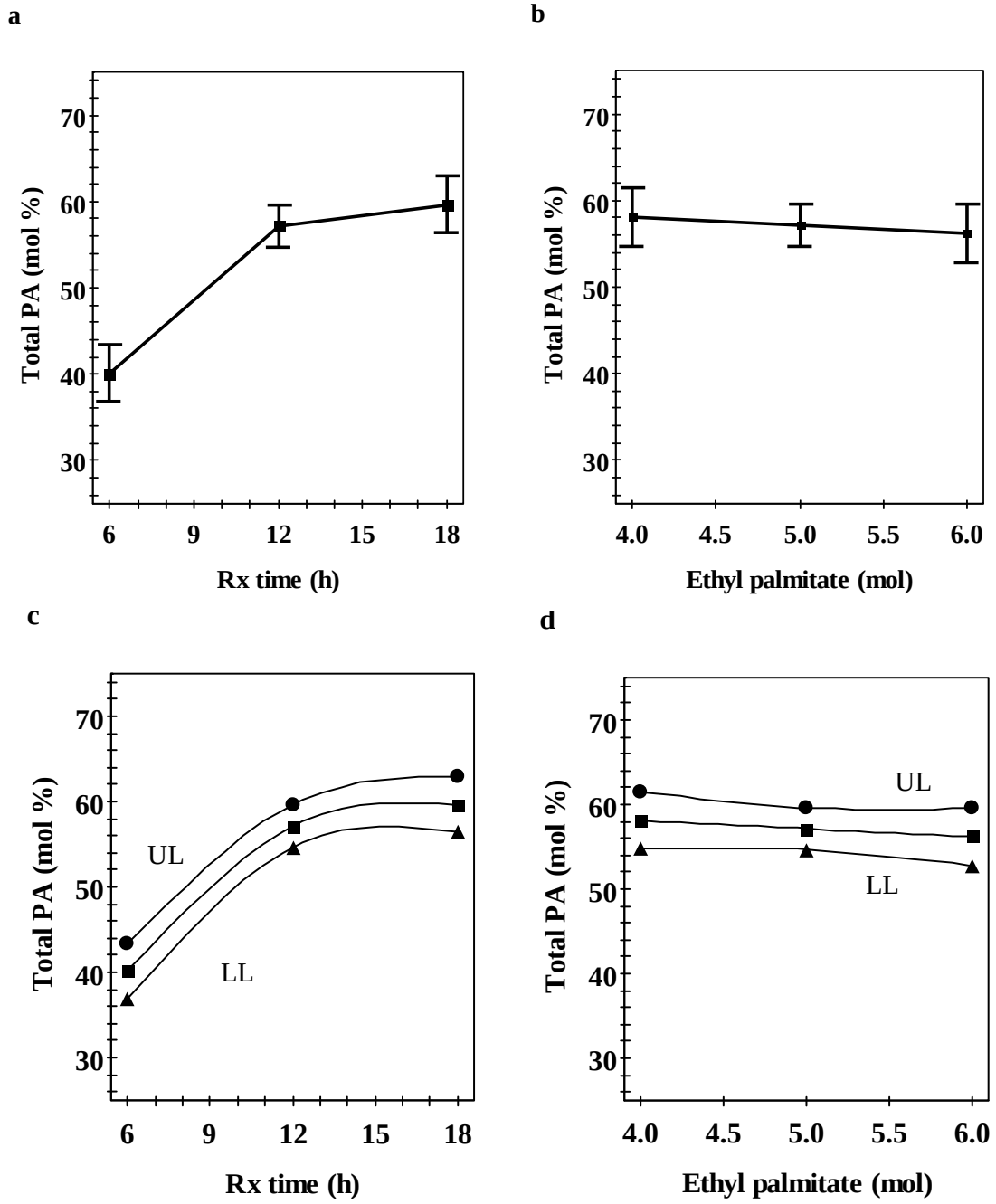
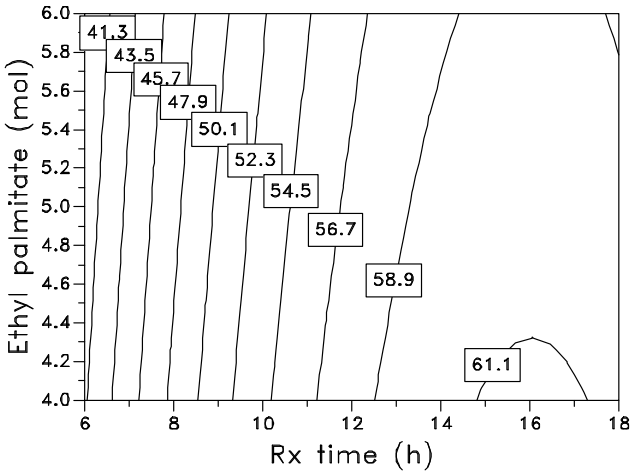

a
b
c
d
Total PA (mol %)
Rx time (h)
Ethyl palmitate (mol)
UL
LL
70
60
50
40
30
6
9
12
15
18
4.0
4.5
5.0
5.5
6.0

Figure 3.2. Contour plot showing effect of ethyl palmitate mole ratio used for the incorporation of palmitate with Novozym 435 as catalyst at 60°C and different reaction times. The labels inside the plot indicate the total palmitic acid content (mol %).

Figure 3.2


Ethyl palmitate (mol)
Rx time (h)
41.3
43.5
45.7
47.9
50.1
52.3
54.5
56.7
58.9
61.1
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
6
8
10
12
14
16
18

CHAPTER 4

SYNTHESIS AND CHARACTERIZATION OF A STRUCTURED LIPID FROM AMARANTH OIL AS PARTIAL FAT SUBSTITUTE IN MILK-BASED INFANT FORMULA[1]

[1]Ashanty M. Pina-Rodriguez and Casimir C. Akoh. Submitted to the Journal of Agricultural and Food Chemistry, March 30, 2009.

ABSTRACT

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 *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 breast milk. Amaranth oil was modified by enzymatic interesterification in two stages. First, 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: C16:0 33.9%, C18:0 2.8%, C18:1n-9 23.3%, C18:2n-6 37.3%, C18:3n-3 0.7%, and C22:6n-3 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), phytosterols, tocopherols 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. However, more research on the application and stability of the infant formula product should be conducted.

KEYWORDS: Amaranth oil, DHASCO, enzymatic interesterification, fat substitute, infant formula.

INTRODUCTION

Amaranth, an ancient Meso-American crop is considered a pseudo-cereal 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 6-9% (*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*), milk-like beverages and infant formulas (*5*). Amaranth has also been studied for its hypocholesterolemic and antioxidant properties (*6-8*). However, amaranth oil successful applications are limited, and the crop still remains greatly underexplored. Amaranth oil contains about 18.6-23.4% palmitic, 22.7-31.5% oleic, and 39.4-49.8% linoleic 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*), while phytosterols and squalene have the ability to decrease serum total cholesterol (*13-15*). Squalene has also been studied for its anticarcinogenic (*16*) and antioxidant activity (*17*). Amaranth oil contains approximately 4.2% squalene (*18*), 44 mg total tocopherols per 100 g oil (*6*), and 834 mg total sterols in 100 g oil (*19*) from the *Amaranthus* variety most commonly cultivated in USA.

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 makes 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 like fat-soluble vitamins absorption, hormone and eicosanoids 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 of breast milk fat triacylglycerols (TAGs) are mainly occupied by unsaturated FA, while saturated FAs are esterified at the *sn*-2 position. Palmitic acid accounts for the majority of the saturated fat portion in breast milk with over 60% by weight esterified at *sn*-2 position. 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 portions of oils used. However, FA arrangement in the glycerol backbone still remains similar to the original vegetable oils, with low palmitic acid content at *sn*-2 position. Other techniques involving *trans* and interesterification reactions have been used to modify oils from the structural point of view. By definition, structure lipids (SL) are novel TAG that have been modified from natural oils and fats by incorporating a new FA or restructured to change the positions of its original FA (*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 USA, 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 *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 breast milk.

MATERIALS AND METHODS

Materials. Amaranth oil was purchased from Nu World Amaranth Inc. (Naperville, IL). DHASCO (DHA-containing single cell oil) 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 (non specific 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. (St. Louis, MO), 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 mole ratio, respectively) using 10% Novozym 435 (by total weight of reactants) as enzymatic catalyst at 60°C for 3h. 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 fatty acid (FA) composition of the initial amaranth oil and CAO are shown in **Table 4.1**.

Experimental design. A RSM (response surface methodology) model was developed 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 4.2** 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 4.2**. 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 mole 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 enzyme. The amount of free fatty acids (FFAs) was reduced through short-path distillation using a KDL-4 (UIC Inc., Joliet, IL) 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 the America Oil Chemists' Society (AOCS) Official Method, Ca 5a-40 (*34*) as % oleic acid.

Determination of fatty acid profiles. Amaranth oil, CAO, DHASCO and DCAO samples were converted to FAME following the AOAC Official Method 996.01, Section E (*35*) with minor modifications. Briefly, 100 mg of oil was weighed into a Teflon-lined test tube, 1 mL C17:0 in hexane (20 mg/mL) was added as internal standard and dried with nitrogen to remove solvent. Then, 2 mL 0.5N 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 hexane and 2 mL NaCl saturated solution were added to the sample, vortexed for exactly 2 min at room temperature and centrifuged for 5 min at 1,000 rpm to separate the organic and aqueous phases. The upper organic layer was filtered twice through anhydrous sodium sulfate column and recovered into a gas chromatography (GC) vial and analyzed. Supelco 37 component FAME mix was used as external standard and ran 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 Tris-HCl buffer (1.0 M), 0.5 mL sodium cholate solution (0.05%) and 0.2 mL 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 HCl (6 N) was added to stop the reaction, and 4 mL of diethyl ether were added to extract the hydrolyzed product. The upper layer containing the lipid components was separated and filtered twice through anhydrous sodium sulfate column. The extracted solution was flushed with nitrogen to evaporate solvent until one third of 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-Oleoylglycerol 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. 50 μL C17:0 in hexane (20 mg/mL) was used as internal standard. FAs esterified at *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 GC equipped with a flame ionization detector (*24*). Separation was achieved with a SP-2560 column, 100m × 0.25mm i.d., 0.20μm 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 up to 215°C at 10°C/min, and held isothermally for 40 min. FAME relative content was calculated by integration using an online computer. Average of duplicate analyses was reported.

Melting profile analysis. The melting profile of amaranth oil, CAO and DCAO were determined with differential scanning calorimeter (DSC) (model DSC7, Perkin-Elmer Co., Norwalk, CT) according to the 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 the 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 hexane and 10 mL 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 flame ionization detector (FID) and an SP-2560 column (100m × 0.25mm i.d., 0.20μm 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 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 2mL 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 TMS:pyridine (1:1, v/v) for 1h 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 (30m × 0.32mm i.d., 0.25μm film, 325°C max).

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°C 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 the AOCS official methods Cd 8b-90 (*44*) and Cd 18-90 (*45*) correspondingly. Total oxidation (TOTOX) value was calculated as 2 × (PV) + (pAV) (*46*).

Statistical analysis. All reactions and analyses were performed in duplicate for amaranth oil, CAO and DCAO. Average and standard deviation was 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 4.1**) 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 weight was 922.4 g/mol. Even though oleic and palmitic acids content resembles that in breast milk, amaranth oil significantly contains 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 (over 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 4.1**). 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 substrates 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 4.1**, and the calculated molecular weight was 910.2 g/mol.

Further, 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, Umeå, Sweden). The reaction conditions for each experiment and the corresponding responses are shown in **Table 4.2**. Backward selection and

multiple regressions were used to fit the results to a second-order polynomial model. The interaction term *Rxt*DHASCO* was omitted because it was 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 4.3**. The multiple correlation coefficient (R^2) was 0.99 corresponding to the fraction of the variation of the response explained by the model, while Q^2 (0.98) corresponds to the fraction of the response that can be predicted. The model equation can therefore be expressed as: $total\ DHA = 10.01 - 0.10Rxt + 2.80DHASCO + 0.19Rxt * Rxt - 0.37DHASCO * DHASCO$, where total DHA is the total content of DHA incorporated in CAO, Rxt is the reaction time, DHASCO stands for the mol ratio used for the reaction, Rxt*Rxt and DHASCO*DHASCO are the squared terms of reaction time and mol ratio, respectively. Substrate availability had the largest positive effect on the total DHA incorporation as shown in **Figure 4.1a**, while reaction time was slightly adverse for this response (**Figure 4.1b**) within the tested range. **Figures 4.1c** and **4.1d** show the effect of each factor on the prediction of DHA incorporation. The minimum increase of the substrates ratio suggests higher DHA incorporation. The lower effect of reaction time suggests that longer times would result in steady DHA incorporation levels with non-significant ($P > 0.05$) variations. **Figure 4.2** 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 4.2** we can also notice the minimum effect reaction time seems to have on the objective response (total DHA).

The resulting model can be either used for prediction of responses according to specific parameter combinations, or for the optimization of reactions to achieve desired objectives. Even though DHA incorporation was the main response studied in the experiment, the effect 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 4.1**. The final results were in agreement to those planed and set in the optimization model, with slightly higher amount of total palmitic acid (33.9%). Palmitic acid at *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 only contain about 5.0% palmitic acid at *sn*-2 position (*47*). Formulas prepared with palmitic acid at *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 (over 60%). It is worth mentioning that it has been suggested that about 50% of the palmitic acid content at *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 4.3** shows the DSC thermograms obtained for all the samples. Amaranth oil had only one endothermic peak resulting in a narrow melting range (**Table 4.4**). 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 4.3**). CAO contains more palmitic and less linoleic acid than amaranth oil (**Table 4.1**); the larger saturated fat proportion is responsible for the secondary high-melting peaks 2, 3 and 4. DCAO content of palmitic and linoleic acid remain similar to those from CAO (**Table 4.1**), however the inclusion of DHA, a highly unsaturated FA, explains the lower presence of high-melting peaks (**Figure 4.3**) compared to CAO and hence the narrower melting range (**Table 4.4**). For amaranth oil, melting range was estimated from peak 1. For the melting range of CAO and DCAO, the onset temperature (T_o) were estimated from the melting start temperature of peak 1, and the completion temperatures (*58*) was estimated from the melting end temperature of the last peak (peak 4 for CAO and peak 2 for DCAO).

Table 4.5 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 on 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 amarnath oil was 128.2 (**Table 4.5**), outside of the range provided by the manufacturer (Nu World Amaranth Inc., Naperville, IL). 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 4.1**), 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 FAs composition of DCAO (**Table 4.1**). On the other hand, saponification value (SV) provides an indication of the change in molecular weight 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 4.5**), meaning that the molecular weight 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 content of tocopherols, phytosterols and squalene in precursor amaranth oil and DCAO final product are shown in **Table 4.6**. 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 50-120 mg/100 g of amaranth oil. Our result is also similar to that reported by Qureshi et al. (*6*) of 44 mg total tocopherols per 100 g of refined and degummed amaranth oil. However, we observed a different distribution among the homologs; in this study, β-tocopherol was the major homolog identified in amaranth oil, and no tocotrienol was detected. As shown in **Table 4.6**, there were significant differences for all the homologs between the amaranth oil and DCAO, and approximately 94.5% loss in total tocopherol. We believe the

significant loss of tocopherols was induced by esterification of tocopherols, exposure of the samples to light during the interesterification and/or short-path distillation. Some studies reported an adverse effect of interesterification on tocopherols content (*50, 51*). Other researchers suggested that the use of short-path distillation for purification and deodorizing purposes affect not only 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 SL purified by short-path distillation require supplementation with appropriate antioxidants before storage and food applications (*52*).

Several studies 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 4.6**). 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 conserving a similar compositional distribution as 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 squalene and tocopherols content had an effect on the overall distribution of the remaining components in the unsaponifiable fraction and therefore 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*), antioxidant by quenching singlet oxygen (*17*), and reducing serum cholesterol levels (*13*). Commercially important oils like olive, rice bran, corn, peanut, rapeseed, sunflower, and cottonseed oils contain low levels of squalene ranging from 0.01-0.4% (*56*). He and Corke (*18*) reported an average 4.2% squalene from the oil of 104 samples of 30 species of *Amaranthus* grain, in accordance to several other studies that had reported similar results (*10, 11*). From our results shown in **Table 4.6**, 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 temperature (193°C, 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 was used (the first after CAO synthesis).

Peroxide value (PV) and *p*-anisidine value (*p*AV) were determined to estimate the oxidation susceptibility of amaranth oil and DCAO at accelerated oxidation conditions. PV (**Figure 4.4a**) was used to measure the primary products of oxidation, and *p*AV (**Figure 4.4b**) 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 shown in **Figure 4.4**. According to our PV results, amaranth oil and DCAO were stable before 48 h. At 72 h we observed a similar increase 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 peroxides production than crude sunflower oil when incubated at 60°C (for 30 d); since 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 a 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 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 TOTOX was calculated from PV and *p*AV to evaluate the overall oxidative stability of amaranth oil and DCAO (**Figure 4.4c**). Both samples showed similar distributions. However, DCAO was in general more susceptible to oxidation than amaranth oil, in part, due to the addition of DHA, but mostly because of the lost of natural antioxidants during the purification process by short-path distillation. 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 to apply 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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Table 4.1. 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.

Table 4.2. Experimental Design of Factors and Responses for Modeling Enzymatic Reaction by RSM[a]

expt[b]	CAO (mol)	DHASCO (mol)	temp (°C)	rx 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: expt, experiment; CAO, customized amaranth oil; DHASCO, DHA-containing single-cell oil; temp, temperature; rx time, reaction time; DHA , docosahexaenoic acid; PA, palmitic acid.

Table 4.3. 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.

Table 4.4. 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 (*58*) of the last melting peak. [d]Refer to Table 4.1 for abbreviations.

Table 4.5. 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	120.5	118.7
	(93.3-100.4)[c]		
SV	193.8	196.4	195.8
	(217)[d]		

[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 reference (*59*).

Table 4.6. Unsaponifiable Fraction Composition (mg/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.

Figure 4.1. (a) Effect of reaction time on total DHA content. **(b)** Effect of DHASCO availability on total DHA content. For **(a)** and **(b)** values plotted are means ± SD, n = 6 for low and high levels, n = 8 for center point. **(c)** Prediction plot of total DHA content when reaction time is varying. **(d)** Prediction plot of total DHA content when DHASCO availability is varying. UL and LL refers to upper and lower confidence levels, respectively.

Figure 4.1

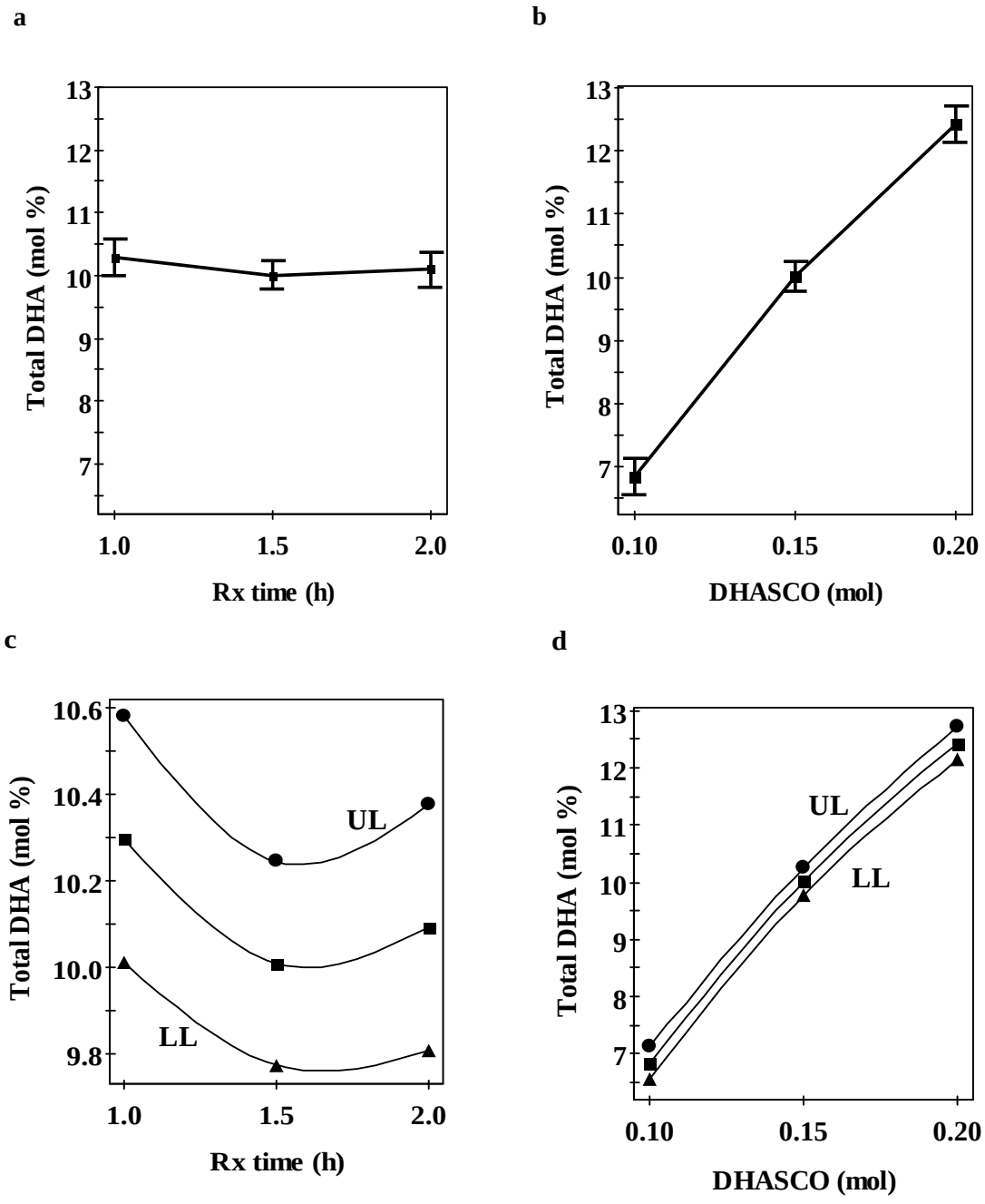

a
b
c
d
Total DHA (mol %)
Rx time (h)
DHASCO (mol)
UL
LL
13
12
11
10
9
8
7
10.6
10.4
10.2
10.0
9.8
1.0
1.5
2.0
0.10
0.15
0.20

Figure 4.2. 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 4.2

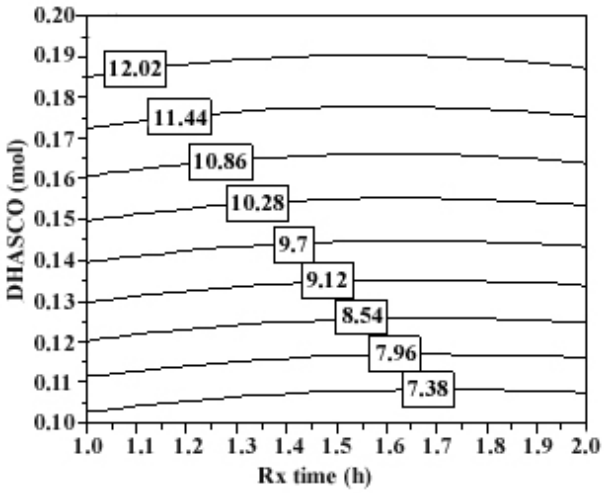


Figure 4.3. DSC melting thermograms of amaranth oil, CAO intermediate SL (palmitic acid enriched), and DCAO final SL (palmitic acid and DHA enriched). For each sample, T_o and T_c indicate melting onset and completion temperature points, respectively. Main endothermic peaks are consecutively numbered in each thermogram. Refer to Table 4.1 for SLs abbreviations.

Figure 4.3

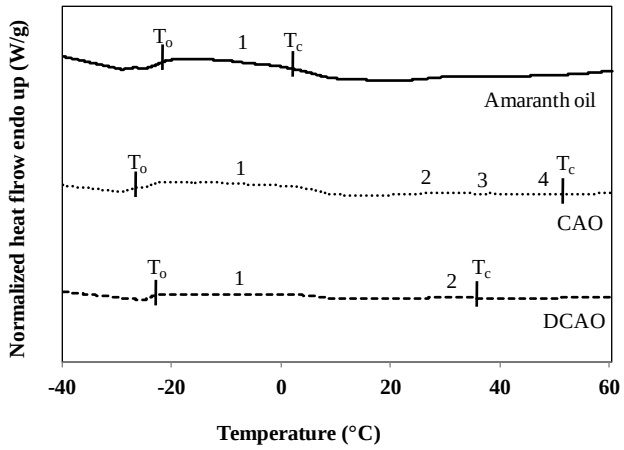
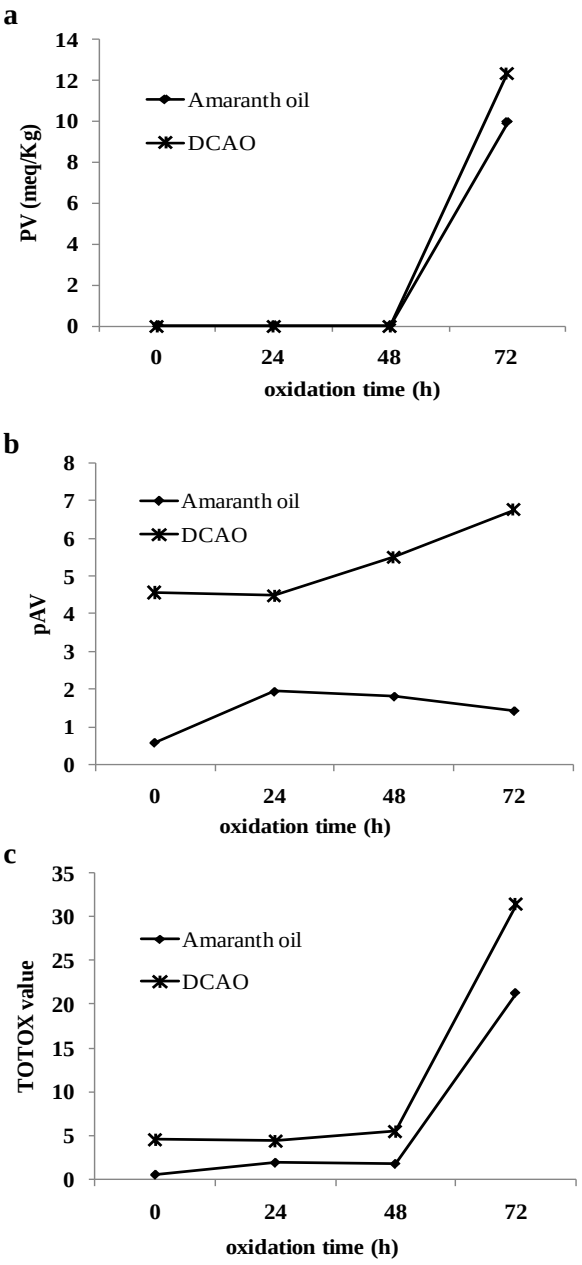

Normalized heat flrow endo up (W/g)
To
1
Tc
Amaranth oil
To
1
2
3
4
Tc
CAO
To
1
2
Tc
DCAO
-40
-20
0
20
40
60
Temperature (°C)

Figure 4.4. Changes in **(a)** peroxide value (PV), **(b)** p-anisidine value (pAV), and **(c)** total oxidation (TOTOX) values of amaranth oil and DCAO during induced oxidation at 50°C. Refer to Table 4.1 for abbreviations.

Figure 4.4


a
PV (meq/Kg)
Amaranth oil
DCAO
oxidation time (h)
b
pAV
Amaranth oil
DCAO
oxidation time (h)
c
TOTOX value
Amaranth oil
DCAO
oxidation time (h)

CHAPTER 5

FORMULATION OF MILK-BASED INFANT FORMULA USING A STRUCTURE LIPID FROM AMARANTH OIL AS PARTIAL FAT SUBSTITUTE[1]

[1]Ashanty M. Pina-Rodriguez and Casimir C. Akoh. For submission to the Journal of Agricultural and Food Chemistry.

ABSTRACT

Amaranth oil can be enzymatically modified to match breast milk fat substitute requirements. We have developed a structured lipid from amaranth oil (DCAO) that, in combination with milk fat, delivers recommended amounts of docosahexaenoic acid (DHA), and palmitic acid specifically esterified at the *sn*-2 position of the triacylglycerol (TAG) backbone. The aim of this study was to confirm the suitability of DCAO (DHA-containing customized amaranth oil) for infant formula application. DCAO was included as complementary fat in a "sample" infant formula, and prepared in parallel to a "control" infant formula under the same processing conditions, and using the same ingredients but different complementary fat source. A blend of the most commonly used vegetable oils (palm olein, soybean, coconut and high-oleic sunflower oils) for infant formula was used instead of DCAO for the "control" formula. Additionally, "sample" and "control" infant formulas were compared with a commercial product (Good Start Supreme) in terms of fatty acid (FA) composition. The fat extracted from the "sample" formula showed a superior FA composition closely resembling breast milk fat. Further research on the stability of DCAO in infant formula should be addressed in order to confirm our positive conclusion, and more completely determine its suitability in infant formula.

KEYWORDS: Amaranth oil, application suitability, infant formula, process conditions.

INTRODUCTION

Several studies have been conducted to develop a suitable breast milk fat substitute which will match the nutritional recommended requirements, as well being affordable for industrial productions. Infant formulas intended for healthy term infants should mimic the composition of breast milk from healthy mother's (*1*). The fat portion of infant formulas currently in the market is usually achieved with vegetable oils such as coconut, soybean, sunflower and corn oil (*1*). Blends of these oils are readily used to balance the fatty acid (FA) content of the final product; hence composition of the formula is dependent on the type and portions of oils used. Some of the most recently researched breast milk fat substitutes are from structured lipids (SLs) after the inter- or transesterification of substrates such as tripalmitin, hazelnut oil FA, n-3 polyunsaturated FAs concentrates, rapeseed oil FA, soybean FA, lard, coconut oil, safflower oil, and butter oil to resemble breast milk fat composition (*2-8*). Many of these products are intended to be used in combination with other vegetable oils or milk fat.

According to the US Food and Drugs Administration (FDA), infant formulas should provide at least 3.3 g of total fat for each 100 kcal consumed, and at least 1.8 g of total protein. Other organizations, such as the Life Science Research Office (LRSO) and the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) have also reported recommendations for energy and carbohydrates content (*9*).

In previous studies (*10, 11*), we developed an SL from amaranth oil, with increased content of palmitic acid at the *sn*-2 position, and with docosahexaenoic acid (DHA) esterified mainly at *sn*-1,3 positions. Our SL (called DHA-containing customized amaranth oil, DCAO) is intended to be used in combination with milk fat, in milk-based infant formulas, to meet the recommended nutritional requirements for breast milk fat substitutes. Compared to other SL developed in the

past, DCAO offers the unique promise of delivering superior balance of palmitic acid and DHA contents when solely one oil is complementing a milk-based infant formula. Therefore, no further combination with vegetable oils is required.

Previously (*11*), we described the gram scale production of DCAO, its purification process and further chemical and physical characterization. In this paper, we describe DCAO's application in milk-based infant formula, and the analysis that confirms the improved FA profile, in comparison with a control infant formula made with a blend of vegetable oils commonly used for this purpose, and a commercial product.

Infant formula is commercially available in dry-powder, concentrated-liquid, and ready-for-consumption presentations. All presentations can be achieved by a wet-mix process, in which dry ingredients are mixed in a liquid phase (water or milk), and then heat-treated (*12*). The resulting formula can be directly packaged for ready-for-consumption presentations, spray dried for powdered infant formula, or concentrated for concentrated-liquid products. The general procedure for a ready-to-feed, milk-based formula involve 4 main steps: mixing ingredients, pasteurization, homogenization, and standardization (*13*).

MATERIALS AND METHODS

Materials. DCAO was developed by two-step interesterification of amaranth oil, ethyl palmitate and DHA as described previously (*11*). High-oleic sunflower oil (Frymax Sun Supreme) and palm olein oils were generously donated by Stratas Foods (Memphis, TN) and Loders Croklaan (Glen Ellyn, IL), respectively. Evaporated whole milk (Nestlé, Glendale, CA), milk-based concentrated liquid Good Start Supreme Infant Formula (Nestlé, Glendale, CA), Coconut oil (Spectrum) and soybean oil (Publix brand) were purchased from local convenience stores in Athens, GA. Hilmar™8800 alpha-lactalbumin enriched whey protein concentrate was

donated by Hilmar Ingredients (Hilmar, CA). Supelco 37 Component FAME (FA methyl ester) mix, C17:0-heptadecanoic acid (>98% purity), triolein, and 2-oleoylglycerol were used as standards and were purchased from Sigma Chemical Co. (St. Louis, MO). Other solvents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), J. T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA).

Infant formula design and preparation. Control and sample formulas were prepared according to the design described in **Table 5.1**. Ingredients were mixed at 50-60°C using a high-speed bench top homogenizer. Standardization was calculated from ingredients composition. Samples were passed through a Gaulin homogenizer (552 bar maximum capacity) (Everett, MA) in two phases: I, 150-200 bar; II, 50 bar. Vial size samples were taken for microscopy analysis. After homogenization, formulas were collected in opaque plastic screw-cap containers (1 L), closed tightly, and quickly cooled. Control and sample formulas were stored at 4°C for 24 h to stabilize before any further analysis. The remaining containers were stored in the dark at ~~at~~ 80°C for future analysis.

Microstructural confirmation. Homogenization and sample likeness was assessed by measuring fat globule size and distribution in infant formulas (control, sample and/or commercial). Microstructural observation of fat globules was conducted using a light microscope (Leica Microsystem Inc., Allendale, NJ). Digital image was obtained using an attached Axiocam digital camera (Zess Inc., Göttingen, Germany). Stabilized samples (kept at 4°C for 24 h) were spotted on microslides, covered with coverslips, and observed under microscope using a 100x water immersion lens (Leica Microsystem Inc., Allendale, NJ).

Fat extraction. The original Bligh and Dyer method was used to extract fat from control, sample, and commercial (Good Start Supreme) formulas (*14, 15*). Briefly, 100 g of infant

formula (control, sample and/or commercial) was homogenized with 100 mL chloroform and 200 mL methanol. Then the solution was re-homogenized with 100 mL chloroform, and 100 mL weak salt solution (0.88% NaCl) was added. After homogenization, the solution was filtered through a porcelain Buchner funnel sealed with filter paper (No. 1), and under vacuum. The precipitate remaining on the filter paper was re-homogenized in 100 mL chloroform and again filtered under vacuum with the previous filtrate fraction. The final biphasic system was allowed to separate into two layers, and the lower phase (chloroform layer) collected. Excess solvent was removed using a Büchi rotovapor (Flawil, Switzerland) at 40°C. Oil was recovered in dark vials, labeled and stored at −18°C under nitrogen for future analysis.

Determination of fatty acid profiles. DCAO, oil blend, and extracted fat of infant formulas were converted to FAME following the American Oil Chemists' Society (AOAC) Official Method 996.01, Section E (*16*) with minor modifications. 100 mg of oil was weighed into a Teflon-lined test tube, 1 mL C17:0 in hexane (20 mg/mL) was added as internal standard and dried with nitrogen to remove solvent. Then, 2 mL 0.5N 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 hexane and 2 mL 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 anhydrous sodium sulfate column and recovered into a gas chromatography (GC) vial and analyzed. Supelco 37 component FAME mix was used as FAME external standard and ran in parallel with the samples.

Positional analysis. Pancreatic lipase hydrolysis was used to analyze the *sn*-2 position (*17*). Samples (100 mg extracted fat) were collected in Teflon-lined test tubes, and 2 mL Tris-HCl buffer (1.0 M), 0.5 mL sodium cholate solution (0.05%) and 0.2 mL 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 two minutes, then 1 mL HCl (6 N) was added to stop the reaction, and 4 mL of diethyl ether were added to extract the hydrolyzed product. The upper layer containing the lipid components was separated and filtered twice through anhydrous sodium sulfate column. The extracted solution was flushed with nitrogen to evaporate solvent until one third of 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-Oleoylglycerol 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. 50 μL C17:0 in hexane (20 mg/mL) was used as internal standard. FAs esterified at *sn*-2 position were quantified by GC and the amounts at *sn*-1,3 were calculated (*18*).

GC analysis. FAMEs were analyzed using an Agilent Technology (Santa Clara, CA) 6890N GC equipped with a flame ionization detector (*19*). Separation was achieved with a SP-2560 column, 100m × 0.25mm i.d., 0.20μm 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 up to 215°C at 10°C/min, and held isothermally for 40 min. FAME relative content was calculated by integration using an online computer.

Statistics. All extractions and analyses were performed in duplicate. Average and standard deviation was calculated using Excel software (Microsoft, Redmond, WA), and reported.

RESULTS AND DISCUSSION

We compared two commercial concentrated-liquid infant formulas in order to develop our own. Common ingredients of Lipil (Enfamil, Mead Johnson, Evansville, IN) and Supreme (Good Start, Nestlé, Glendale, CA) infant formulas were identified and classified according to their product functionality.

Nutrients from both commercial products were in accordance with the levels recommended by several institutions (*9*). Based on the same recommendations, and commercial products, our objective was to develop an infant formula that contributes 64-72 kcal/ 100 mL resulting from 3.3-6.0% fat, 1.2-3.0% protein, and 5.4-8.1% carbohydrates, using whole milk and whey protein concentrate (WPC) to make up for the protein difference. **Figure 5.1** shows the detailed process used for infant formulas preparation in this study. In this study, the control and DCAO-containing formula, called sample formula from now on, were prepared in parallel under the same process (**Figure 5.1**), using the same ingredients but different complementary fat source (oil blend and DCAO, respectively). We used evaporated whole milk instead of regular whole milk because it is easier to digest by infants due to the effect of processing on the protein (*20*). We also used WPC enriched with alpha-lactalbumin (Hilmar Ingredients, Hilmar, CA) specially designed for infant formula. Standardization was done by calculation of nutrients. Preparation of both formulas is presented in the **Table 5.1**.

DCAO was used as complementary fat in sample formula. In control formula, complementary fat was obtained from a blend of oils commonly used for infant formula preparation (*21*). The oil blend was made of 62% palm olein oil, 25% soybean oil, 8% coconut oil, and 5% high-oleic

sunflower oil. Coconut and palm olein oils were melted at 30°C before blending with soybean and high-oleic sunflower oils.

Homogenized samples were visualized under microscopy to assess the resemblance between control, sample and commercial infant formulas (**Figure 5.2**). The purpose of the homogenization step is to break down the fat globule into small droplets, which will make the emulsion more stable (*13*). Most of the fat globule reduction takes place in the first stage of homogenization (150-200 bar). However the smaller fat globules have a tendency to cluster. Therefore a second homogenization stage (50 bar) is recommended to separate those clusters into individual droplets (*22*). It was reported that under these homogenization conditions the average size of a milk fat globule was 0.4 μm (2 μm maximum) with a density of 12 fat globules per μm^3 (*22*).

According to our results, as shown in **Figure 5.2**, evaporated milk (**a**) fat globules vary in diameter, however the larger globule observed was about 0.01 μm diameter. The average fat globule size was similar in all three formulas. Commercial formula (**b**) showed higher density of fat globules compared to any of the infant formulas prepared for this study, because of its concentrated presentation; commercial infant formula requires the addition of water before consumption, while the formulas prepared in the lab are ready-to-use products. After analyzing these results, we concluded that the process was effective in reducing fat globule size; we also concluded that control (**c**) and sample (**d**) formulas are equivalent in processing conditions. Therefore comparison among them can be assessed exclusively in terms of their corresponding complementary fat source.

Infant formulas were analyzed 24 h after preparation. FA profile and positional analysis were determined as described above. The results are shown in **Table 5.2**. Several important

compositional differences can be observed among the samples. Both infant formulas prepared in our laboratory (control and sample) contained superior amounts of palmitic acid compared to commercial formula (Good Start Supreme). Palmitic acid regiospecificity at *sn*-2 position was more evident in sample infant formula (33.0%) than in its commercial analog (7.3%). The *sn*-2 position of commercial infant formula triacylglycerols (TAGs) was mainly occupied by oleic acid (41.6%). The higher content of palmitic acid in sample infant formula was at the expense of oleic and linoleic acids as expected from previous observations (*10*). Even though DCAO contained high level of linoleic acid (18:2n-6), when combined with milk fat it reaches a beneficial polyunsaturated FA balance (oleic>linoleic acids) similar to the control and commercial infant formulas. Although commercial infant formula is fortified with arachidonic acid (ARA) and DHA, we did not detect DHA in the fat extracted from the Good Start Supreme infant formula, but ARA and eicosapentaenoic acid (EPA) in a 2:1 ratio. Our final sample formula contained 0.2% ARA (natural from milk fat) and 0.2% DHA (incorporated by enzymatic interesterification in amaranth oil), it also contained a higher amount of palmitic acid specifically esterified at the *sn*-2 position.

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Table 5.1. Infant Formula Composition

	infant formula[a]			
		macronutrients contribution (g)		
ingredient	composition (g)	fat	protein	carbohydrates
water	550			
evaporated whole milk	450			
fat 6.6%		29.7		
protein 6.6%			29.7	
carbohydrates 10%				45.0
WPC	0.4			
fat 12%		0.0		
protein 78%			0.3	
carbohydrates 1.5%				0.0
complementary fat	6.3	6.3		
total contribution		36.0	30.0	45.0

[a]Calculations for 1,000 g control and/or sample infant formula

Table 5.2. Fatty Acid Composition of Complementary Fat Sources and Extracted Fat from Infant Formulas

fatty acid (wt %)	total (wt %)					*sn* -2 position (wt %)				
	oil blend[a]	DCAO[b]	control formula[c]	sample formula[d]	commercial formula[e]	oil blend	DCAO	control formula	sample formula	commercial formula
8:0	0.7±0.0	-	1.9±0.7	1.1±0.0	2.5±0.1	-	-	-	-	-
10:0	0.5±0.0	-	2.4±0.0	2.3±0.0	1.7±0.1	-	-	2.6±0.1	2.9±0.1	0.6±0.0
11:0	-	-	0.2±0.0	0.3±0.0	-	-	-	-	-	-
12:0	4.1±0.0	0.2±0.1	3.8±0.0	2.8±0.0	11.4±0.5	7.2±0.0	-	6.1±0.2	4.1±0.1	17.1±0.8
13:0	0.0±0.0	-	0.1±0.1	0.1±0.0	-	-	-	-	-	-
14:0	2.3±0.0	0.4±0.0	9.2±0.1	9.2±0.0	5.4±0.2	1.4±0.1	-	19.3±4.6	16.4±1.0	3.3±0.1
14:1n-5	-	-	1.4±0.0	1.5±0.0	-	-	-	-	-	-
15:0	-	-	0.0±0.0	-	0.2±0.0	-	-	-	-	-
16:0	28.5±0.0	33.5±0.0	27.0±0.2	28.0±0.0	18.5±0.2	11.1±0.0	20.2±0.0	28.7±1.8	33.0±0.4	7.3±0.4
16:1	0.1±0.0	-	1.7±0.0	1.8±0.0	0.3±0.0	-	-	1.8±0.1	2.0±0.0	-
18:0	4.5±0.0	2.8±0.0	11.7±0.1	11.9±0.0	4.4±0.1	1.2±0.0	0.8±0.0	6.9±0.1	7.4±0.0	1.1±0.0
18:1n-9	35.2±0.0	23.0±0.0	31.0±0.3	29.0±0.0	31.6±0.4	46.9±0.0	24.1±0.0	25.0±1.7	21.2±0.3	41.6±0.5
18:2n-6	20.9±0.0	36.9±0.0	7.7±0.1	10.0±0.0	20.3±0.3	30.2±0.0	53.3±0.1	9.5±0.6	13.1±0.2	27.8±0.3
18:3n-6	0.1±0.0	-	-	-	0.1±0.0	-	-	-	-	-
18:3n-3	2.2±0.0	0.7±0.0	0.5±0.0	0.6±0.0	1.8±0.1	2.1±0.0	0.7±0.0	-	-	1.5±0.0
20:0	0.4±0.0	-	0.2±0.0		0.3±0.0	-	-	-	-	-
20:1	0.3±0.0	0.3±0.0	0.9±0.0	0.6±0.0	0.2±0.0	-	-	-	-	-
20:2	-	0.3±0.0	-	-	0.0±0.0	-	-	-	-	-
21:0	-	-	-	-	0.1±0.0	-	-	-	-	-
22:0	0.2±0.0	-	-	-	0.2±0.0	-	-	-	-	-
20:3n-6	-	-	0.1±0.0	-	0.1±0.0	-	-	-	-	-
20:4n-6	-	-	0.2±0.0	0.2±0.0	0.8±0.0	-	-	-	-	-
24:0	0.1±0.0	-	-	-	-	-	-	-	-	-
20:5n-3	-	0.2±0.0	-	-	0.4±0.0	-	-	-	-	-
22:6n-3	-	1.9±0.0	-	0.2±0.0	-	-	0.9±0.1	-	-	-

[a]Oil blend composition: 62% palm oilen oil, 25% soybean oil, 8% coconut oil, and 5% high-oleic sunflower. [b]DCAO composition took from previous chapter (2). [c]Control formula made with oil blend as described before. [d]Sample formula made with DCAO as described before. [e]Commercial formula is concentrated-liquid Good Start Supreme (Nestlé).

Figure 5.1. Process diagram for infant formula preparation

Figure 5.1

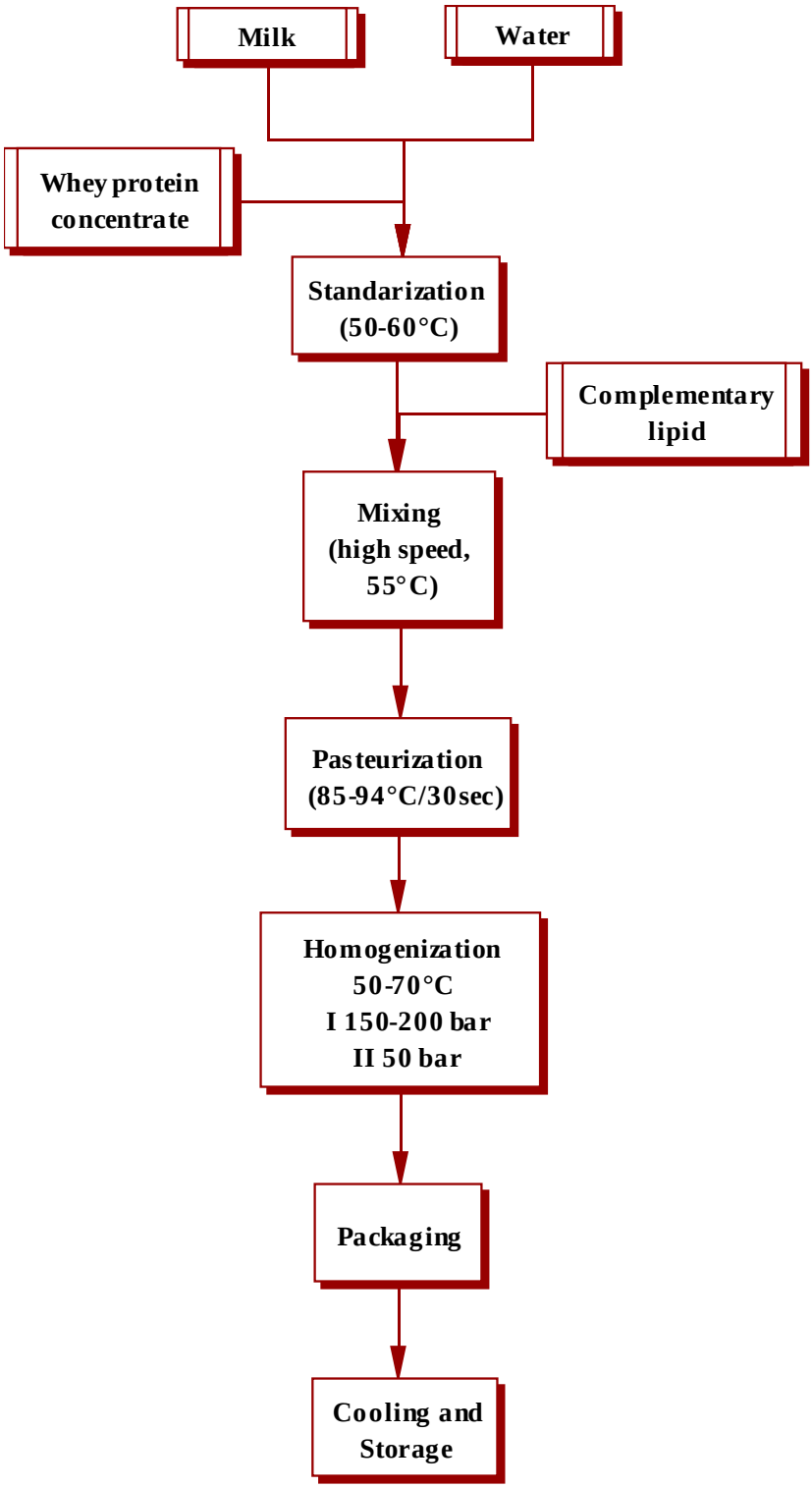
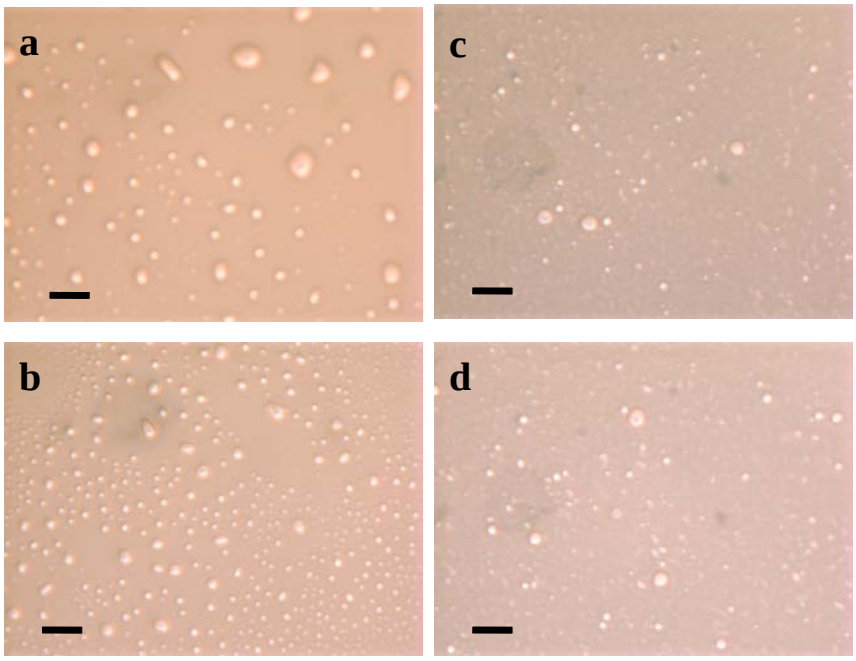

Milk
Water
Whey protein concentrate
Standarization (50-60°C)
Complementary lipid
Mixing (high speed, 55°C)
Pasteurization (85-94°C/30sec)
Homogenization 50-70°C I 150-200 bar II 50 bar
Packaging
Cooling and Storage

Figure 5.2. Phase Contrast Microscopy (PCM) images of (**a**) evaporated whole milk; (**b**) commercial infant formula (Good Start Supreme); (**c**) control formula prepared with oil blend (68% palm olein, 25% soybean, 8% coconut, and 5% high-oleic sunflower oils); (**d**) sample formula prepared using DCAO as complementary fat. The line in the bottom of each picture represents 0.01μm length.

Figure 5.2



CHAPTER 6

CONCLUSION

Amaranth oil is an underutilized crop with very limited successful food applications. Amaranth oil chemical and physical characteristics make it an interesting option to diversify our food sources. In this study, we developed a structured lipid (SL) from amaranth oil for possible application in infant formula as partial fat replacer. Infant formula is designed to emulate breast milk in order to deliver similar nutrition. Nowadays, infant formula is prepared using vegetable oil blends that do not mimic the regiospecificity composition of breast milk.

Enzymatic interesterification techniques were used to modify amaranth oil structure to match the recommended nutritional requirements for infant formula. A two-step modification was employed. First, palmitic acid was intentionally esterified at the *sn*-2 position of amaranth oil using a non-specific lipase (Novozym 435). In the second step, the customized amaranth oil enriched with palmitic acid was further interesterified with DHASCO to incorporate specific/low levels of docosahexaenoic acid (DHA). DHA levels were calculated based on nutritional recommendations and infant formula composition. Incorporation was achieved using a *sn*-1,3 specific lipase (Lipozyme RM IM) from *R. miehei*.

In conclusion, we developed a final SL (DCAO) from amaranth oil, which in the final formulation of milk-based infant formula will yield the recommended DHA content for proper infant development, and higher palmitic acid esterified at the *sn*-2 position for better fat absorption. The characteristics (physical and chemical) determined in this study support the feasibility to apply DCAO as a partial fat substitute or complement for milk-based infant

formula. Finally, DCAO was incorporated in a standardized formula, and compared to control and commercial infant formulas. Superior nutritious fatty acid (FA) balance was confirmed from the FA composition of our sample infant formula containing DCAO from amaranth oil. However, more research on the stability of DCAO in the products should be conducted to support our results. The use of proper antioxidants would enhance the stability of DCAO and therefore its functionality in the final infant formula product.

Breast milk composition is directly related to its nutritional contribution for proper infant development. Food scientists have studied possible ways to emulate such unique composition. Even though breast-feeding is the best nourishment for infants, infant formulas are close enough and considered the second best option, especially when breast-feeding is not a possibility. Research on how to improve the formulations to match infant requirements is still a popular topic among food scientist, dietitians, and medical doctors. The fat component of breast milk is of high interest because of its important energetic contribution. Nowadays, we understand that certain balance of n-3:n-6 polyunsaturated fatty acids (PUFAs) needs to be achieved, that palmitic at the *sn*-2 position is better absorbed and reduces the production of calcium soaps, and that breast milk naturally contains long chain PUFAs important for infant development and that might not be well synthesized from essential fatty acids during infancy. Therefore, some areas of opportunity observed during the development of this thesis might be of interest for future research. More clinical research needs to be done on the improved absorption of palmitic acid esterified at the *sn*-2 position. Likewise, more research is needed on the implications of DHA and ARA supplementation since information currently available is still inconclusive. On the other hand, no research has been conducted on maximum safe limits of DHA and ARA in infant formulas.