OLIVE OIL-BASED STRUCTURED LIPIDS CONTAINING PALMITIC, OMEGA-3 AND -6 FATTY ACIDS FOR POTENTIAL APPLICATION IN INFANT FORMULA

by

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(Under the Direction of Casimir C. Akoh)

ABSTRACT

In this study, refined olive oil was enriched with palmitic acid (PA) and docosahexaenoic acid (DHA) as well as gamma-linolenic acid (GLA) by lipase-catalyzed reaction using Novozym 435 or Lipozyme TL IM. The resultant structured lipid (SL) contained 47.80 mol% total palmitic aicd (PA) and 49.82 mol% at the *sn*-2 position. Total DHA and GLA were found to be at 0.73 and 5.00 mol%, respectively. In comparison, fat extract from a commercial infant formula contained 6.12 mol% PA at the *sn*-2 position and 0.26 mol% DHA. Differential scanning colorimetry showed that the SL exhibited a higher melting temperature than commercial infant formula fat and milk fat. Low pulse nuclear magnetic resonance demonstrated that the SL had a comparable solid fat content to commercial infant formula fat at temperature of 25 to 55 °C. The produced SL have the potential to be used in infant formula.

INDEX WORDS: Refined olive oil, docosahexaenoic acid, palmitic acid, structured lipids.

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DEDICATION

To mom, dad, and the living memory of grandma

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Human milk is widely regarded as the golden standard for infants' and newborns' nutrition requirement. One of the main unique characteristics of human milk is the distinctive structure of human milk fat (HMF). Studies have shown that palmitic acid is the predominant fatty acid at the *sn*-2 position of HMF triacylglycerol (TAG), while unsaturated fatty acids such as oleic acid is mainly found at the *sn*-1, 3 positions (1,2). These particular positional distributions of fatty acids have a major impact on the absorption of palmitic acid and calcium and the caloric energy contribution of human milk (3,4). However, commercial infant formulas are produced using vegetable oils where the majority of palmitic acid is located at the *sn*-1, 3 positions and oleic acid is the predominant fatty acid at the *sn*-2 position. Such oils do not provide the adequate amount of available palmitic acid for the infants.

The focus of this study was to enrich refined olive oil with palmitic acid at the *sn*-2 position and DHA and/or gamma linolenic acid (GLA) as human milk fat analogues by enzymatically catalyzed reaction for potential infant formula applications. In the first part of the study, DHA-containing single cell oil (DHASCO) was converted to DHASCO free fatty acid (DHASCO-FFA) and used as one of the substrates. Non-specific lipase Novozym 435 was used to randomly rearrange and incorporate palmitic acid and DHASCO-FFA into the glycerol backbone of refined olive oil TAGs. Response surface methodology (RSM) was employed for SL synthesis to model and optimize reaction conditions (5,6) at small scale, which were substrate molar ratio, reaction temperature, and reaction time. Three reaction responses were investigated:

total DHA incorporation, total palmitic acid incorporation, and palmitic acid content at *sn*-2 position. Milligram-scale synthesis was carried out using the conditions generated by RSM and products were analyzed for their FA composition. The results were further characterized by RSM on the predictability of the three reaction responses. In the second part of the study, tripalmitin and ethyl esters of DHASCO and GLA were used in place of palmitic acid and DHASCO-FFA. Additionally, 1, 3 specific Lipozyme TL IM was used in place of Novozym 435. Optimal reaction condition was selected for large-scale production and the product was characterized for its TAG species, oxidative stability, melting and crystallization behavior, and solid fat content. It was then compared to the total fat of commercial infant formula, milk, and physical blend of the substrates to evaluate the suitability of the product for infant formula applications.

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CHAPTER 2

LITERATURE REVIEW

OLIVE OIL

Olive oil is extracted from the fruit of the olive tree, namely *Olea europaea* L, a traditional tree that grows best between the 30° and 45° parallel (1). Accordingly, the unique warm weather and prolonged sunlight irradiation in the Mediterranean area have seen the growth of *Olea europaea* L prosper. The climate also developed the characteristic high volume of antioxidants found in olive trees (2). Consequently, the Mediterranean countries such as Spain, Italy, and Greece contribute more than 95% of the world olive oil production. With the expanding popularity of the Mediterranean diet, an increasing production of olive oil has been seen in many places in the rest of the world, such as The United States, Canada, Australia, South America, and Japan (1).

There are many varieties of olive oil available, with different chemical properties and degrees of acidity, namely virgin olive oil, refined olive oil, olive oil, crude olive-residue oil, refined olive-residue oil, and olive-residue oil (3). Virgin olive oil can be further classified into extra virgin olive oil, virgin olive oil, ordinary virgin olive oil, and lampante virgin olive oil, with extra virgin olive oil having the highest organoleptic grading and lowest free acid content and lampante virgin olive oil having the lowest organoleptic grading and highest free acid content. By this classification, extra virgin olive oil is generally recognized as the most valuable kind of olive oil as it is obtained from intact olives that are immediately processed and cold-pressed which minimizes the activation of cellular lipases and degradation of triacylglycerols (1).

On the other hand, refined olive oil is generally considered a lower value oil compared to virgin olive oil due to the loss of natural antioxidants such as α-tocopherol and phenolic derivatives, i.e., tyrosol and hydroxytyrosol during the refining process (4).

Being an important component of Mediterranean diet, consumption of olive oil have been associated with the incidence of coronary heart disease (CHD) that is considerably lower in the Mediterranean area (5). The effects of olive oil on CHD risk factors have been widely and extensively studied and are often attributed to its high levels of monounsaturated fatty acids (MUFA) such as oleic acid. In an enforcement discretion letter released on November 1st 2004, the U.S. Food and Drug Administration (FDA) states that "eating about 2 tablespoons (23 g) of olive oil daily may reduce the risk of coronary heart disease due to the monounsaturated fat in olive oil." (6). In comparison, it was revealed that the amount of olive oil consumed in Greece was 18 kg per year per capita, 13 kg per year per capita in Italy, and 11 kg per year per capita in Spain, which equals 49.3, 35.6, and 30.1 grams daily per capita in Greece, Italy, and Spain, respectively (7). However, oleic acid alone is not entirely responsible for the health benefits associated with olive oil as oleic acid is one of the primary fatty acids found in food of animal origin, such as poultry and pork, which are widely consumed in developed countries (8). This suggests that other minor components of olive oil are contributing factors for the health effects of olive oil on CHD as well. Virgin olive oil usually consists of 1-2 % of minor components, which can be classified into two groups: the unsaponifiable fraction, defined as the fraction extracted with organic solvents after saponification of the oil and the soluble fraction that includes the phenolic compounds (9). Specifically, the unsaponifiable fraction of olive oil consists of hydrocarbons (squalene), tocopherols, fatty alcohols, triterpenic alcohols, 4-methylsterols,

sterols, other terpenic compounds, and polar pigments (chlorophylls and pheophytins), with an increasing polarity (9).

The major phenolic compounds found in extra virgin olive oil are hydroxytyrosol, tyrosol, and oleuropein, which contribute to the bitter, pungent taste often associated with extra virgin olive oil (10). Numerous studies have been devoted to the structure of these phenolic compounds and their effects on human health. It has been shown that the phenolic compounds found in olive oil are strong antioxidants and radical scavengers (11). Specifically, hydroxytyrosol is a predominant antioxidant and radical scavenger while both hydroxytyrosol and oleuropein have demonstrated antimicrobial activity against some bacterial strains (11).

Oleuropein is the major phenolic compound in olive fruit, with up to 14 % in dried fruit. However, in olive oil, the main phenolic component is hydroxytyrosol (12), with a reported concentration of 1.4 – 5.6 mg/L (13), 1.63 ± 0.25 mg/kg (14), and 14.42 ± 3.01 mg/kg in extra virgin olive oil and 1.74 ± 0.84 mg/kg in refined virgin oil (15). As for tyrosol, its concentration in olive oil has been reported to be 4.69 ± 0.77 mg/kg (14) and 27.45 ± 4.05 mg/kg in extra virgin olive oil and 2.98 ± 1.33 mg/kg in refined virgin oil (15). Last but not the least, the concentration of oleuropein has been shown to be 2.3 – 9.0 mg/L (14) and 2.04 ± 0.78 mg/kg in extra virgin olive oil.

A bland-flavored or flavor-neutral, light-colored, and physically and oxidatively stable oil is desirable among consumers (16). Although virgin olive oil can be consumed in its crude form, a large proportion of olive oil nowadays is refined to render it edible (17). The end product of this refining process for virgin olive oil is often referred to as refined olive oil. The majority of phenolic compounds, as well as unsaponifiable matters such as hydrocarbons, sterols, and polar pigments are removed during the refining process, resulting in a finishing product that is odor-

less and lucid in appearance, and also worth noticing, less valuable. By colorimetrical determination, it has been shown that the total polyphenol content of olive oil is reduced to almost zero by refining (18). A common refining process can be seen in Figure 2.1.

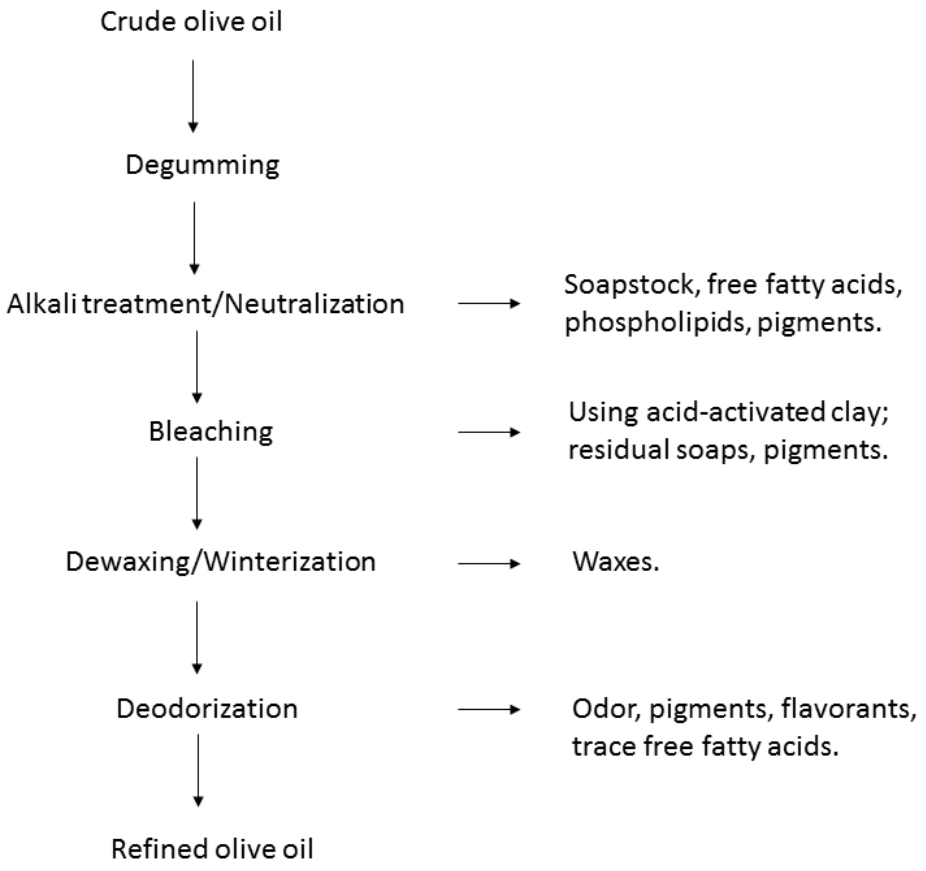

Figure 2.1. General refining process for refined olive oil production

Olive oil is a good source of oleic (18:1) acid and also contains palmitic (16:0) and linoleic (18:2) acids. At the *sn*-2 position of its triacylglyceol, oleic acid is the predominant fatty acid while very limited palmitic acid is observed. The specific total fatty acid composition and *sn*-2 fatty acid profile of the substrate refined olive oil used in this study can be seen in **Table 2.1**.

Table 2.1. Total fatty acid and *sn*-2 profile (mol%) of refined olive oil

Fatty acid	Total[a]	*sn*-2
C16:0	9.97 ± 0.08	1.49 ± 0.33
C16:1n7	1.01 ± 0.00	0.84 ± 0.01
C18:0	6.90 ± 0.15	ND
C18:1n9	73.95 ± 0.55	86.35 ± 0.32
C18:2n6	7.26 ± 0.01	10.30 ± 0.03
C18:3n3	0.51 ± 0.00	1.01 ± 0.02
C22:6n3	ND	ND

[a] Mean ± SD; ND: not detected.

Refined olive oil has a unique fatty acid profile. Its high monounsaturated fatty acid content makes it an interesting and healthy choice for food applications. However, research on exploring those potentials of refined olive oil is still limited. We believe that with enzymatic techniques, the fatty acid composition of refined olive oil can be modified to create a structured lipid (SL) that resembles HMF and consequently be used in infant formula industry.

HUMAN MILK FAT

The fat content of human milk varies between 3 to 5 % depending on the lactation stage and dietary intake of the mother (19). Milk produced during the first 5 days of postpartum is generally called colostrum milk, while transitional milk is referred to milk produced during 6 to 15 days and milk produced after 16 days of postpartum is called mature milk. Fat content of human milk increases as lactation progresses, with colostrum containing the lowest and mature milk containing the highest (20). Since fat is more energy dense (9 kcal/g) compared to carbohydrates and protein (4 kcal/g), it suggests that mature milk can provide more energy per mass than colostrum and transitional milk. Generally, as discussed above, HMF is responsible for nearly haft of energy provided for infants.

Human milk TAG consists of a wide range of medium and long chain fatty acids (21). The major fatty acids found in HMF are oleic acid, palmitic acid, and linoleic acid (22,23). Unlike vegetable oils, the *sn*-1 and *sn*-3 positions of human milk TAG are occupied by mainly unsaturated fatty acid such as oleic acid while saturated fatty acid, namely palmitic acid, is predominantly found at the *sn*-2 position. This unique structure is important as it allows adequate palmitic acid and calcium absorption (24,25). The following **Table 2.2** presents the data of the composition and distribution of some major fatty acids from a study on the lipid composition of HMF from different lactation stages (20).

Table 2.2. Total fatty acid composition and *sn*-2 profile of HMF from colostrum, transitional, and mature milk[a] (mol%)

Fatty acid	Total fatty acids[b]			*sn*-2[b]		
	colostrum	transitional	mature	colostrum	transitional	mature
C10:0	0.57 ± 0.21 a	1.56 ± 0.43 b	2.81 ± 0.93 c	0.43 ± 0.14 d	1.29 ± 0.41 e	1.55 ± 0.40 e
C12:0	3.56 ± 0.79 a	7.43 ± 1.71 b	9.84 ± 1.48 c	2.71 ± 1.07 d	7.32 ± 4.81 e	8.33 ± 2.73 e
C14:0	6.45 ± 0.57 a	9.01 ± 1.97 b	10.23 ± 1.39 b	8.44 ± 1.56 d	12.89 ± 2.43 e	14.47 ± 1.00 e
C16:0	26.59 ± 3.27 a	24.88 ± 4.49 ab	22.84 ± 2.30 b	55.31 ± 3.80 d	53.82 ± 3.10 d	52.63 ± 3.36 d
C16:1n7	1.42 ± 0.48 a	2.35 ± 0.35 b	2.11 ± 0.44 b	1.90 ± 0.30 d	1.36 ± 0.78 d	2.12 ± 1.55 d
C18:0	8.13 ± 2.17 a	6.64 ± 1.30 b	5.64 ± 0.57 b	2.47 ± 0.36 d	1.35 ± 0.37 e	1.31 ± 0.16 e
C18:1n9	36.21 ± 4.26 a	33.63 ± 3.13 ab	32.06 ± 4.55 b	13.25 ± 3.17 d	10.10 ± 2.3 e	9.25 ± 2.89 e
C18:2n6	9.67 ± 0.95 a	8.63 ± 0.46 b	8.37 ± 0.74 b	8.53 ± 2.69 d	6.74 ± 1.38 e	5.63 ± 0.94 e
C18:3n3	0.56 ± 0.09 a	0.62 ± 0.11 a	0.86 ± 0.17 b	0.70 ± 0.41 d	0.48 ± 0.24 de	0.31 ± 0.16 e
C20:4n6	0.92 ± 0.40 a	0.41 ± 0.18 b	0.36 ± 0.14 b	0.93 ± 0.33 d	0.18 ± 0.08 e	0.25 ± 0.14 e
C22:6n3	0.71 ± 0.23 a	0.54 ± 0.29 ab	0.42 ± 0.18 b	0.80 ± 0.17 de	1.20 ± 0.80 d	0.66 ± 0.38 e
SFAs	47.13 ± 6.99 a	50.17 ± 2.75 a	50.56 ± 4.34 a	70.10 ± 6.09 d	77.48 ± 3.96 e	79.38 ± 6.73 e
MUFAs	38.59 ± 6.89 a	37.01 ± 3.22 a	35.11 ± 4.96 a	16.48 ± 3.23 d	12.35 ± 1.05 e	12.28 ± 4.19 e
PUFAs	14.16 ± 0.62 a	12.22 ± 0.34 b	11.57 ± 0.50 b	13.43 ± 3.18 d	10.17 ± 2.89 e	8.31 ± 1.53 e

[a]From reference (20). [b]Mean ± SD with the same letter are not significantly different at the 0.05 probability level. SFAs: saturated fatty acids. MUFAs: monounsaturated fatty acids. PUFAs: polyunsaturated fatty acids.

It can be seen that HMF consist of nearly half saturated fatty acids and half unsaturated fatty acids. Specifically, at *sn*-2 position of human milk TAG, palmitic acid contributes approximately 50 mol% while oleic acid is found to be 9.25 mol% in mature milk and 13.25 % in colostrum. Moreover, mature milk contains 0.42 mol% DHA and 0.86 mol% linolenic acid in total, respectively. These numbers should serve as the standard when formulating human milk substitutes such as infant formulas.

Although breast milk is widely regarded as the ideal diet for newborns and infants, it alone sometimes does not provide the complete nutritional needs for infants. Six months after the infant was born, human milk alone cannot supply adequate protein content required for the infant's growth and therefore additional food and milk formula are needed to complement nutrition (26). In other cases, maternal milk can be unavailable or in low quality production due to a special condition the mother is in, thus milk formula are used as the main nutrition and

energy supply. Sociological, psychological, and cultural factors can also affect the decision of breastfeeding an infant. In 1990, the United Kingdom's Office of Population Censuses and Surveys found that the mother's social class, age, and educational background can all have an impact on the decision of breastfeeding (27). Consequently, there is a constant demand for human milk substitute that mimic the nutritional composition of human milk from healthy mothers (28).

DHA and GLA

α-Linolenic acid (ALA, C18:3n-3) is commonly considered as essential fatty acid as they cannot be synthesized in human bodies and therefore are obtained from the diet. Omega-3 (n-3) fatty acids such as DHA in the diet that are not esterified are rapidly absorbed up to over 96% and enter cells through FA transporters. Three main metabolic paths are available after n-3 fatty acids are converted to FA acyl-CoA thioesters. They can either provide quick energy supplies by the beta-oxidation pathway or become substrates for the synthesis of neutral and polar lipids or they are converted to longer-chain PUFA namely eicosapentaenoic acid (EPA, C20:5n-3) and DHA via a series of elongation and desaturation in the case of ALA (29). Delta-6-desaturase which facilitates the initial conversion of ALA to steardonic acid (SDA) is one of the critical enzymes that are involved in the conversion reactions. However, a parallel conversion of linoleic acid (LA) to gamma-Linolenic acid (GLA) where delta-6-desaturase is also involved is preferred in human bodies (30). Consequently, the production of EPA and DHA from dietary ALA is relatively suppressed and are often insufficient to meet the demands for humans. A direct dietary supplementation of EPA and DHA is therefore crucial for human health.

Lipids are the most variable constituent in human milk (31). They contain the essential polyunsaturated fatty acids linoleic acid (C18:2n-6) and ALA and their products, arachidonic

acid (ARA, C20:4n-6), EPA, and DHA, which are required in maternal diet (32). ARA, EPA, and DHA in proper balance is also required for maturation and optimal functional development of the visual, brain, and nervous system of the infants (32). Studies have shown that the amount of DHA in HMF is between 0.15-0.92 % depending on the intake of this FA by the breastfeeding mother (31). In the United States, requirements for commercial infant formulas are regulated by the Food and Drug Administration (FDA) and published in the Code of Federal Regulations (CFR). Other institutions and associations have also published recommendations for the fatty acids composition of human milk substitutes. No minimum or maximum levels for DHA in infant formula have been recommended or specified by FDA or CFR; however, the European Union have recommended a maximum level of 2 % of DHA in infant formulas (33).

Infant formulas containing DHA are available on the market in the US. The one used in this study, Gerber® Good Start® Gentle powder formula from Nestlé, claims to contain "special blend of DHA and ARA" to "support brain and eye development". According to the nutrition label of the product, the respective source of DHA and ARA are *Crypthecodinium cohnii* oil and *Mortierella alpina* oil. The specific composition of these two fatty acids in the product will be determined in this study.

Scientists have constructed several different structured lipids for infant formula use in the past, using substrates such as amaranth oil (34), hazelnut oil (35), tuna oil (36), and palm stearin (37). However, this is the first time refined olive oil was used as the main substrate while also incorporating polyunsaturated fatty acids such as DHA and GLA. Refined olive oil is a low value product compared to virgin olive oil. The addition of palmitic acid and DHA and GLA can significantly increase the value of refined olive oil and yield a suitable SL that can be used in infant formulas.

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CHAPTER 3

ENRICHMENT OF REFINED OLIVE OIL WITH PALMITIC AND DOCOSAHEXAENOIC ACIDS TO PRODUCE HUMAN MILK FAT ANALOGUE

Li, R.; Pande, G.; Sabir, J. S. M.; Baeshen, N. A.; Akoh, C. C., 2014 *J. Am. Oil Chem. Soc.* 91:1377-1385. Reprinted here with permission of JAOCS.

ABSTRACT

Refined olive oil was enriched with palmitic acid (PA) and docosahexaenoic acid (DHA) via lipase-catalyzed acidolysis reaction using Novozym 435 in hexane. The enrichment reaction was optimized by response surface methodology. Three independent variables, reaction time (12, 18, and 24 h), temperature (55, 60, and 65 °C), and substrate molar ratio (refined olive oil:DHA single cell oil free fatty acid:PA = 1:1:6, 1:1:9, and 1:1:12) and three responses, total PA and DHA incorporation, and PA content at the *sn*-2 position were investigated. Results showed that PA was incorporated into the triacylglycerols (TAGs) of refined olive oil at up to 55.79 mol% while incorporation of PA at *sn*-2 position and total DHA were found to be up to 33.63 and 3.54 mol%, respectively. Second-order models were generated for each of the three responses. A Chi-square test verified that the predicted values from the models were not significantly different from the observed ones. The prediction power of the models was further confirmed by a solvent-free scale-up reaction. The produced structured lipids have potential to be used in infant formula.

KEYWORDS

Refined olive oil; docosahexaenoic acid; palmitic acid; acidolysis; structured lipid

INTRODUCTION

Breast milk is widely considered the ideal diet source for newborns and infants and human milk fat (HMF) contributes nearly half of infant's dietary energy (1). Although the total lipid content of human milk varies between 3-5 %, it has been widely recognized that about 98 % of the lipid fraction are triacylglycerols (TAGs), which contain about 20-30 % palmitic acid (PA, C16:0), of which over 60 % is at the *sn*-2 position of the glycerol backbone and predominantly unsaturated fatty acids at the *sn*-1 and *sn*-3 positions (2). This unique structure is of great importance to infants as it helps increase the digestion and absorption of palmitic acid and calcium, which is also vital for infants' bone development (3,4). However, the majority of infant formula on the market today is produced using cow's milk and vegetable oils, where most of their palmitic acid is located at the *sn*-1, 3 positions. TAGs are hydrolyzed to FAs and 2-monoacylglycerol in the intestine by *sn*-1,3 specific pancreatic lipase. The palmitic acid located at *sn*-1,3 positions is consequently released and can further react with calcium ions to form insoluble calcium soaps in the intestine that are not digestible (5). Conversely, when palmitic acid is located at *sn*-2 position on the glycerol backbone, as found in HMF, the reaction catalyzed by pancreatic lipase leads to the production of 2-palmitoyl monoacylglycerol, and thus less amount of calcium soaps are formed compared to when consuming infant formula prepared with vegetable oils. Therefore, infant formula produced using structured lipids (SLs) that has similar TAG structure as that of HMF should be exceptionally beneficial for infants' growth and development. SLs are generally produced by chemically and/or enzymatically modifying the structure of naturally occurring lipids to alter or increase their health, functional or nutritional values (6). Previously, amaranth oil, hazelnut oil, tuna oil, and palm stearin have all been enriched to produce SLs rich in palmitic acid at the *sn*-2 position (7-10), however, the overall

amount of research devoted to the enrichment of vegetable oils or other fats with palmitic acid is very limited.

Human milk is also a major source of essential fatty acids such as linoleic acid (8-17 %) and α-linolenic acid (ALA, 0.5-1.0 %) as well as their long-chained derivatives arachidonic acid (ARA, C20:4n-6) and docosahexaenoic acid (DHA, C22:6n-3) (11). The amount of DHA in HMF varies from 0.17-0.99 % total fatty acids depending on the maternal intake of this FA (12). DHA is vital for the development of nervous system and visual ability of infants (13-15) and can be commonly found in cold water fish as well as vegetarian sources such as seaweed. It can also be synthesized from ALA in human bodies but the conversion is inefficient in infants and elderlies. Consequently, addition of preformed DHA in infant formulas has great health beneficial effects.

Another substrate used in this study, olive oil, has been a major part of the Mediterranean diet and is widely regarded for its health benefits and rich phytochemical content (16,17). Generally, olive oil is consumed in natural crude state as virgin olive oil, or in refined form as refined olive oil or as a mixture of the two (18). Crude unrefined olive oil (virgin olive oil) has a bitter taste due to its phenolic compounds (19) and therefore physical and chemical refining have been employed to reduce the acidity level and the amount of other undesirable chemical compounds such as phospholipids, lipoproteins, free fatty acids, and waxes. However, the refining processes also eliminate most of the natural antioxidants such as α-tocopherol and phenolic derivatives, i.e., tyrosol and hydroxytyrosol that were present in crude olive oil (20), rendering the refined olive oil a lower value compared to extra virgin olive oil.

The objective of this study was to enrich refined olive oil with palmitic acid at the *sn*-2 position and DHA as human milk fat analogues by acidolysis reaction for potential infant

formula applications. Non-specific lipase was used to randomly rearrange and incorporate palmitic acid and DHA into the glycerol backbone of refined olive oil TAGs to produce SL. Response surface methodology (RSM) was employed for SL synthesis to model and optimize reaction conditions (21,22), which were substrate molar ratio, reaction temperature, and reaction time. Three reaction responses were investigated: total DHA incorporation, total palmitic acid incorporation, and palmitic acid incorporation at *sn*-2 position. Milligram-scale synthesis was carried out using the conditions generated by RSM and products were analyzed for their FA composition. The results were further characterized by RSM on the predictability of the three reaction responses.

MATERIALS AND METHODS

Materials

Refined olive oil (ROO) was purchased from Columbus Vegetable Oils (Des Plaines, IL). Docosahexaenoic acid single cell oil (DHASCO) was kindly provided by DSM Nutritional Products (Columbia, MD). Palmitic acid was purchased from Alfa Aesar (Ward Hill, MA). Novozym 435 (*Candida antarctica* lipase, nonspecific, specific activity 10,000 PLU/g; PLU = Propyl Laurate Unit, immobilized on macroporous acrylic resin) was purchased from Novo Nordisk A/S (Bagsvaeard, Denmark). The lipid standards Supelco 37 component FAME mix, C15:0 pentadecanoic acid (>98 % purity), triolein, and 2-oleoyglycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Other solvents and chemicals were purchased from Fisher Scientific (Norcross, GA) and Sigma-Aldrich (St. Louis, MO). The respective fatty acid composition of ROO and DHASCO-FFA can be found in Table 3.4.

Preparation of DHASCO Free Fatty Acids

DHASCO was converted into free fatty acids (DHASCO-FFA) following the methods described by Sahin et al. (23) with minor modification. Twenty-five grams of DHASCO was treated with 5 mg butylated hydroxytoluene and then saponified using a mixture of 5.75 g of KOH, 11 mL of distilled water and 66 mL of 95 % (v/v) ethanol by refluxing for 1 h at 60 °C. 50 mL of distilled water was added to the saponified mixture and the unsaponified matter was extracted twice with hexane (100 mL) and discarded. 10 mol/L HCl was then used to acidify the aqueous layer which contains the saponifiable matter to a pH about 1.0. 50 mL hexane was used to extract the liberated free fatty acids. Subsequently, the hexane containing free fatty acids was dried over anhydrous sodium sulfate and the solvent was removed in a rotary evaporator at 60 °C. The resulting free fatty acids were flushed with nitrogen and stored in a freezer at -80 °C.

Experimental Design for RSM Study

To study the effects of experimental conditions on the incorporation of total palmitic acid, DHA, and palmitic acid at the *sn*-2 position, RSM was applied using the experimental design provided by Modde 5.0 software (Umetrics, Umea, Sweden). A mathematical model was generated by the software to predict the three reaction responses. Three factors each with three levels were taken into consideration when designing the experiments: reaction time (12, 18, and 24 h), reaction temperature (55, 60, and 65 °C), and substrate molar ratio of refined olive oil:DHASCO-FFA:palmitic acid (1:1:6, 1:1:9, and 1:1:12 mol/mol). The values of each factor were substituted by -1, 0, and 1 later in the equations by the software. Fifteen different combinations of reaction conditions were generated by the software using central composite design (CCD). Experiments were performed in triplicate resulting in forty-five total reactions.

Acidolysis Reactions

Refined olive oil (0.1 g) was mixed with DHASCO-FFA and palmitic acid at respective substrate molar ratios in screw-capped test tubes. 3 mL hexane and Novozym 435 lipase at 10 % (w/w) of the total substrate mass were also added to the reaction mix. The mixture was then incubated in a water bath shaker at its corresponding reaction temperature (55, 60, or 65 °C) with constant agitation at 200 rpm for 12, 18, or 24 h. The reactions were stopped by filtering the mixture through anhydrous sodium sulfate column and the products were analyzed as described below. All reactions were performed in triplicate and the average value and standard deviation were reported.

Pancreatic Lipase–Catalyzed *sn*-2 Positional Analysis

sn-2 Positional fatty acid composition was determined following the method described by Luddy et al. (24). All samples were analyzed in triplicate and average values were reported.

Determination of Fatty Acid Profiles

Refined olive oil, DHASCO-FFA, and the products (SLs) were converted to FA methyl esters (FAME) following AOAC Official Method 996.01 (25) with minor modifications. 0.1 g of sample (or scraped-off TLC band) was weighed into Teflon-lined test tubes and 0.25 mL internal standard (C15:0, 20 mg/mL in hexane) was added. 2 mL of 0.5 M NaOH in methanol was added and heated at 100 °C for 5 min. The samples were cooled in ice bath and 2 mL 14 % BF_3 in methanol was added and again heated at 100 °C for 5 min. The samples were cooled and finally 2 mL hexane and 2 mL saturated NaCl solution were added and the mixture was vortexed for 2 min. The upper FAME layer was collected into GC vials after centrifuging the samples at 1000 rpm for 5 min at room temperature and passed through anhydrous sodium sulfate column. The samples were analyzed with Hewlett-Packard 6890 series II gas chromatograph (Agilent

Technologies Inc., Palo Alto, CA) using Supelco SP-2560, 100 m x 25 mm x 0.2 μm column. Supelco 37 component FAME mix was used as the external standard. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. Injection volume was 1 μL and a split ratio of 5:1 was used. Detection was with flame ionization detector at 250 °C. The column was initially held at 140 °C for 5 min and then increased to 240 °C at 4 °C/min and held at 240 °C for 22 min. All samples were analyzed in triplicate and average values were reported.

Model Verification

Verification of the model was carried out by randomly selecting five regions from the contour plot and performing acidolysis reactions using the conditions corresponding to these regions. The obtained response values were compared to the predicted values from the model. A Chi-square test was done to compare the observed and predicted values.

Statistical Analysis

All the reactions were carried out in triplicates and average values were reported. Response surfaces, regression analysis, and backward elimination were performed using Modde 5.0 software (Umetrics, Umea, Sweden).

RESULTS AND DISCUSSION

Model Fitting

Table 3.1 shows the total fatty acid composition and *sn*-2 profiles of the SLs synthesized using the conditions generated by RSM. Refined olive oil was enriched with DHA and palmitic acid by acidolysis reaction. Total DHA incorporation in the SL ranged from 0-3.5 mol% while total palmitic acid incorporation ranged from 26.8-54.6 mol%. Additionally, palmitic acid at *sn*-2 position ranged from 18.0-33.6 mol%. Results were subjected to multiple linear regression and backward elimination analysis to fit into a polynomial model. The regression coefficients (β) and

significance (P) values were calculated based on the numbers in Table 3.1. The ANOVA table for the three responses can be found in Table 3.2. The R^2 value, the fraction of the variation of the response explained by the model and Q^2, the fraction of the variation of the response that can be predicted by the model were also listed for each of the three responses. The model for palmitic acid content at the *sn*-2 position is:

$$\text{PA at } sn\text{-}2 = 27.09 - 2.10\text{SR} - 1.90\text{Time} + 1.98(\text{SR} \times \text{Temp}) \quad (1)$$

where SR stands for substrate molar ratio. It can be seen that both substrate molar ratio and time had a negative impact while the interaction between substrate molar ratio and temperature had a positive impact. For total palmitic acid and DHA incorporation, the models are:

$$\text{Total PA} = 46.18 - 1.63\text{SR} + 3.29\text{Temp} + 9.08\text{Time} - 8.64(\text{Temp} \times \text{Temp}) + 4.21(\text{Time} \times \text{Time}) \quad (2)$$

$$\text{Total DHA} = 0.97 - 0.79\text{SR} + 0.44\text{Temp} + 0.65\text{Time} + 0.93(\text{SR} \times \text{SR}) - 0.85(\text{Temp} \times \text{Temp}) + 0.66(\text{Time} \times \text{Time}) + 0.40(\text{SR} \times \text{Temp}) - 0.52(\text{SR} \times \text{Time}) \quad (3)$$

Both models consisted of more significant terms than that for palmitic acid at the *sn*-2 position. Generally, substrate molar ratio consistently had a negative impact on both responses while temperature and time showed a consistent positive effect. Moreover, the effect of second-order terms of temperature and time are also consistent in both models with the former being negative and the latter being positive. In addition, the model for total DHA incorporation contained two interaction terms between substrate molar ratio and temperature and time with their effects being positive and negative, respectively.

Optimization of the Reaction

Contour plots are generated by Modde 5.0 software to display the relationships between reaction conditions and each response. The reaction factor with the greatest effect on the response was kept on the *y*-axis while the second most critical factor was placed on the *x*-axis and the factor with the least impact was held constant. The coefficients analysis (data not shown) suggested that for all three responses, reaction time was the least critical factor. As shown in Figure 3.1, time was kept constant at 18 h while substrate molar ratio and temperature were placed on *y* and *x*-axis, respectively. For PA at *sn*-2 position (Figure 3.1a), its contour plot suggests that the increase in substrate molar ratio results in a decrease in the response when time is held constant. However, as described above, the interaction between substrate molar ratio and temperature had a positive effect on the incorporation of PA at the *sn*-2 position. This positive interaction factor exhibited a much greater positive coefficient than the absolute value of the negative coefficient for the first-order term of substrate molar ratio. Consequently, the overall effect of an increasing substrate molar ratio resulted in an increase in the incorporation of PA at the *sn*-2 position. For total PA incorporation (Figure 3.1b), the effect of substrate molar ratio was more complicated, as suggested by the second-order model described above (equation 2). When time was held constant, the increase in substrate molar ratio was accompanied with a slow increase in PA incorporation while an increase in reaction temperature, until approximately 60 °C resulted in an increase in response. For total DHA incorporation (Figure 3.1c), the increase in substrate molar ratio when time was held constant resulted in a decrease in response. A probable explanation is that with an increasing amount of PA, DHASCO-FFA was competitively suppressed from reacting with refined olive oil and therefore led to a small amount of DHA esterified to the glycerol backbone. Moreover, in all three cases, the effect of temperature

displayed a similar pattern, where the response level increased as temperature became higher, and then at a certain point, as temperature continued to increase, the response level started to decrease. This phenomenon has been recorded previously where in one study an optimum 60 °C reaction temperature for Novozym 435 was reported for 1, 3-diacylglycerol preparation by esterification (26). Higher temperature is generally considered to enhance the enzyme activity and mobility of substrate molecules (27) until its maximum rate is reached. The viscosity of the mixture which promotes the contact between the substrates and enzymes may decrease and the mass transfer rate may be increased by an increasing reaction temperature. However, as temperature continued to increase, the denaturation rate of enzyme proteins increased and more enzymes became inactivated and consequently caused a decrease in response levels as seen in the contour plots. As stated by Senanayake and Shahidi (28), the temperature sensitivity of hydrogen bonds and other weak attractions hold the enzyme in its 3-D shape and contribute to the unique relationship between enzyme activity and reaction temperature. The overall effect of the temperature on responses thus depends on the nature of the reaction and the substrates used. Comparable observations between reaction temperature and response levels were also reported in another study (29).

In our study, longer reaction time was found to promote the incorporation of PA and DHA, while the level of PA at the *sn*-2 position varied depending on the substrate molar ratio and reaction temperature. This is in agreement with some previous findings (28,30). The optimal PA incorporation, DHA incorporation, and PA incorporation at the *sn*-2 position, were observed with a substrate molar ratio of 1:1:9 (ROO:DHASCO-FFA:PA), reaction temperature of 60 °C, and reaction time of 24 and the corresponding response levels were 55.79, 3.01, and 33.63 mol% for total PA incorporation, total DHA incorporation, and PA at the *sn*-2 position, respectively.

In addition, it can be seen that to achieve a certain level of response, different combinations of reaction conditions can be utilized. The complex relationship of linear and quadratic variables suggests that the cost-effectiveness of the reaction to produce the desired response values should be considered when optimizing the reaction models.

Verification of the Model

Verification of the models were performed by conducting a Chi-square test and there were no significant difference between the observed and expected values since the Chi-square value for total DHA (3.085), total PA (6.997) and PA at *sn*-2 (3.644) were all lower than the cutoff point (9.488) at $\alpha = 0.05$ and DF = 4 (Table 3.3). Interestingly, the R^2 and Q^2 values for palmitic acid content at *sn*-2 position was significantly lower than that for the other two responses, seeming to imply the prediction power of the model was low. However, the verification results showed that the model is still relatively accurate in terms of its predictability. This is likely because some second-order terms of reaction conditions were eliminated during multiple regression and backward elimination. In other words, the impact of these terms was not statistically significant. The validity of the models were further tested by conducting a scale-up reaction (total substrates, 8 g) without solvent at 60 °C with a substrate molar ratio of 1:1:6 (ROO:DHASCO-FFA:PA) for 12 h. The obtained response levels were similar to the predicted values with a total PA of 42.94 mol% (45.70 mol% predicted) of which 28.23 mol% was at the *sn*-2 position (28.20 mol% predicted) and a total DHA incorporation of 2.19 mol% (2.69 mol% predicted). These results again showed that the models possess high prediction accuracy for the three responses determined. Moreover, it is worth noting that at *sn*-2 position, more varieties of fatty acids were detected compared to that of milligram scale production, noticeably linoleic acid

(C18:2n6) and DHA (C22:6n3). This implies that these fatty acids possibly were present at milligram scale production but their signals were too weak to be detected by GC.

Fatty Acid and *sn*-2 Positional Composition of Refined Olive Oil and SL

The total and *sn*-2 positional fatty acid compositions of refined olive oil, DHASCO-FFA and SL produced at optimal reaction condition are shown in Table 3.4. It can be seen that the major fatty acids in refined olive oil were oleic (73.95 mol%), palmitic (9.97 mol%), linoleic (7.26 mol%), and stearic (6.90 mol%) acids. The dominance of oleic acid is even more striking at the *sn*-2 position with 86.35 mol% while palmitic acid was only 1.49 mol%. As discussed above, in HMF, over 60 % of palmitic acid is located at the *sn*-2 position and unsaturated fatty acids such as oleic acid are located at the *sn*-1,3 positions. Acidolysis reaction using refined olive oil, DHASCO-FFA, and palmitic acid as substrates was carried out with the aim to increase the palmitic acid content at *sn*-2 position and incorporate DHA. The resultant SL contained up to 33.63 mol% palmitic acid at *sn*-2 compared to 1.49 mol% in the refined olive oil. Acidolysis reaction normally involves free fatty acids and a triacylglycerol. With the use of Novozyme 435 which is a non-specific lipase, randomization of the free fatty acids is expected and consequently there is a 33.33 % probability that the free fatty acid can be esterified to each of the three positions on the glycerol backbone. With the optimal reaction condition reported in our study, 33.63 % of PA was found at the *sn*-2 position of the resultant SL. Additionally, DHA incorporation was 3.01 mol% under this optimal condition which further increases the nutritional value of the SL considering that DHA is normally found at 0.15-0.92 % in human milk (31).

Similar studies have been conducted to incorporate palmitic acid in vegetables oils. Hazelnut oil was previously enriched with palmitic acid or ethyl palmitate to produce HMF substitute (7). At milligram scale, reaction under optimal conditions produced SL with 48.60

mol% palmitic acid of which 35.50 % was located at the *sn*-2 position of the glycerol backbone. The findings are very comparable to what is reported here in our study. In addition, another study was carried out using a free fatty acid blend of DHA, gamma-linolenic (GLA), and palmitic acid to enrich palm olein (22). The resulting SL contained 35.11 mol% palmitic acid at the *sn*-2 position, with 3.75 mol% DHA and 5.0 mol% GLA. The palmitic acid incorporation level at *sn*-2 position and total DHA content are very similar to our results. More recently, human milk fat analogues containing DHA were synthesized from tripalmitin and extra virgin olive oil (32). Substrate ratios of 1:3:2 (tripalmitin:extra virgin olive oil FFA:DHASCO-FFA), 1:4:2, and 1:5:1 produced SLs that contained 42.23, 40.45, and 39.47 mol% total palmitic acid, of which 67.34, 63.27, and 58.78 % were found at the *sn*-2 position, respectively. DHA contents were found to be 7.54, 6.72, and 5.89 mol% for the three SLs described above. In this acidolysis reaction, tripalmitin was the starting TAG and extra virgin olive oil FFA and DHASCO-FFA were esterified to the *sn*-1,3 positions of the TAG backbone by 1,3 specific Lipozyme TL IM. Consequently, SLs with high palmitic acid content at the *sn*-2 position were produced. In another study by Pande et al. (33), ARA and DHA were incorporated into SLs rich in palmitic acid at the *sn*-2 position produced with extra virgin olive oil, tripalmitin, and ARASCO and DHASCO-FFA. Over 50 mol% palmitic acid at the *sn*-2 position was observed in all the SLs. Both interesterification and acidolysis were taking place during the reaction and tripalmitin was one of the two TAGs involved in the interesterification process which likely resulted in the high palmitic acid content at the *sn*-2 position of the produced SLs.

Although the palmitic acid incorporation at the *sn*-2 positionof the SL in our work is still lower than the level found in HMF, it is a significant 22-fold increase from what was found in refined olive oil. In order to further increase the PA content at the *sn*-2 position, a TAG that is

rich in PA at the *sn*-2 position such as tripalmitin can be added to the reaction mixture. Moreover, the oleic acid composition is now in the same range as found in HMF and the DHA content is significantly increased. It is worth noticing that the synthesized SL contained over 55 % PA content while human milk contains less than 30 %. Therefore, to make it suitable for use in infant formula, the SL can be utilized in an oil blend to produce an infant formula that contains PA content similar to that of human milk with added value of higher DHA content.

The SL produced at small-scale production contained a promising amount of DHA and PA at *sn*-2 position. The PA content at *sn*-2 position was increased compared to that of refined olive oil, and is a significant increase over standard commercial infant formula which typically contains 6.0 % of PA esterified at the *sn*-2 position (34). Moreover, the SL still has a great potential in infant formula applications and substrates such as tripalmitin that contain high amount of PA at *sn*-2 position may be added to the reaction mix to improve its composition in the final SL.

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Figure Caption

Figure 3.1 a Contour plot of the effect of substrate molar ratio and temperature on PA at sn-2 with time kept constant at 18 h. **b** Contour plot of the effect of substrate molar ratio and temperature on total PA incorporation with time kept constant at 18 h. **c** Contour plot of the effect of substrate molar ratio and temperature on total DHA incorporation with time kept constant at 18 h.

Table 3.1. Experimental settings of the factors and the responses used for optimization by RSM

	Total Fatty Acid[a] (mol%)					*sn*-2[a] (mol%)	
Reaction condition	C16:0	C18:0	C18:1n9	C18:2n6	C22:6n3	C16:0	C18:1n9
6; 55; 12[b]	28.26 ± 1.37	2.89 ± 0.53	63.26 ± 1.74	4.32 ± 2.18	1.26 ± 0.12	32.46 ± 6.36	67.54 ± 6.36
6; 55; 24	48.13 ± 2.58	ND	44.36 ± 2.60	3.97 ± 0.21	3.54 ± 0.26	19.28 ± 4.61	80.72 ± 4.61
6; 60; 18	48.32 ± 0.52	1.47 ± 0.03	43.65 ± 0.45	4.02 ± 0.04	2.54 ± 0.08	28.72 ± 3.12	71.28 ± 3.12
6; 65; 12	31.98 ± 2.13	2.53 ± 0.16	58.4 ± 1.67	5.15 ± 0.18	1.94 ± 0.20	31.57 ± 0.81	68.43 ± 0.81
6; 65; 24	54.64 ± 1.72	1.53 ± 0.34	36.95 ± 1.49	3.45 ± 0.22	3.43 ± 0.19	22.69 ± 1.37	77.31 ± 1.37
9; 55; 18	31.33 ± 0.76	2.50 ± 0.09	60.73 ± 0.79	5.43 ± 0.03	ND	25.27 ± 0.94	74.73 ± 0.94
9; 60; 12	45.52 ± 1.80	1.87 ± 0.20	48.36 ± 1.47	4.26 ± 0.14	ND	24.71 ± 1.45	75.29 ± 1.45
9; 60; 18[c]	45.13 ± 0.60	1.62 ± 0.04	47.62 ± 0.48	5.03 ± 0.65	1.46 ± 0.03	24.45 ± 3.47	75.55 ± 3.47
9; 60; 24	55.79 ± 1.22	1.29 ± 0.09	36.54 ± 1.07	3.37 ± 0.21	3.01 ± 0.13	33.63 ± 1.69	66.37 ± 1.69
9; 65; 18	44.29 ± 3.13	1.87 ± 0.23	49.42 ± 2.71	4.43 ± 0.19	ND	29.72 ± 0.67	70.28 ± 0.67
12; 55; 12	26.81 ± 0.71	2.73 ± 0.03	64.78 ± 0.66	5.68 ± 0.03	ND	27.21 ± 1.88	72.79 ± 1.88
12; 55; 24	45.70 ± 0.53	1.84 ± 0.21	48.17 ± 0.29	4.28 ± 0.29	ND	18.01 ± 0.83	81.99 ± 0.83
12; 60; 18	40.36 ± 1.85	1.93 ± 0.09	52.07 ± 1.74	4.68 ± 0.14	1.01 ± 0.12	24.88 ± 1.32	75.12 ± 1.32
12; 65; 12	31.58 ± 1.70	2.57 ± 0.08	58.64 ± 1.51	5.16 ± 0.03	2.05 ± 0.27	30.04 ± 1.26	69.96 ± 1.26
12; 65; 24	50.12 ± 1.70	1.44 ± 0.04	42.88 ± 1.32	3.90 ± 0.07	1.71 ± 0.18	23.57 ± 1.26	76.43 ± 1.26

[a] Mean ± SD, n = 3; [b]6; 55; 12: Substrate molar ratio (ROO:DHASCO-FFA:PA = 1:1:6), reaction temperature 55 °C, reaction time 12 h; 9; 60; 18[c]: center point; ND: not detected.

Table 3.2. ANOVA table for the three response variables

	DF			**SS**			**MS**			**F**			**p**			**SD**		
	PA at *sn*-2	Total PA	Total DHA	PA at *sn*-2	Total PA	Total DHA	PA at *sn*-2	Total PA	Total DHA	PA at *sn*-2	Total PA	Total DHA	PA at *sn*-2	Total PA	Total DHA	PA at *sn*-2	Total PA	Total DHA
Total	45	45	45	33781.50	82696.600	166.610	750.700	1837.700	3.7026									
Constant	1	1	1	32930.70	78734.100	96.1350	32930.7	78734.10	96.135									
Total Corrected	44	44	44	850.8010	3962.4600	70.4810	19.3364	90.0559	1.6019							4.397	9.490	1.266
Regression	9	9	9	368.5230	3638.8600	61.8260	40.9470	404.3180	6.8696	2.972	43.73	27.78	0.010	0.000	0.000	6.399	20.108	2.621
Residual	35	35	35	482.2770	323.5990	8.6554	13.7794	9.2457	0.2473							3.712	3.041	0.497
Lack of Fit (Model Error)	5	5	5	376.9630	241.4690	8.0781	75.3927	48.2937	1.6156	21.48	17.64	83.95	0.000	0.000	0.000	8.683	6.949	1.271
Pure Error (Replicate Error)	30	30	30	105.3140	82.1301	0.5773	3.5105	2.7377	0.0192							1.874	1.655	0.139

For PA at *sn*-2: N = 45, DF =35, Q^2 = 0.098, R^2 = 0.433, R^2_{adj} = 0.287; For Total PA: N = 45, DF = 35, Q^2 = 0.869, R^2 = 0.918, R^2_{adj} = 0.897; For Total DHA: N = 45, DF = 35, Q^2 = 0.808, R^2 = 0.877, R^2_{adj} = 0.846.

DF: degree of freedom; SS: sum of squares; MS: mean square; SD: standard deviation.

Table 3.3. Verification of the models using Chi-square test

Region	SR	Temp (°C)	Time (h)	O	E	O	E	O	E	$(O-E)^2/E$		
				Total DHA	Total DHA	Total PA	Total PA	PA at *sn*-2	PA at *sn*-2	Total DHA	Total PA	PA at *sn*-2
1	7	57	15	0	1.12	28.01	37.6	25.36	29.4	1.12	2.446	0.555
2	8	56	13	0	0.225	22.90	33.7	24.92	29.7	0.225	3.461	0.769
3	9	60	22	1.98	0.986	49.01	54.1	28.68	26.4	1.002	0.479	0.197
4	10	63	20	1.36	1.05	44.63	47.7	20.08	26.3	0.092	0.198	1.471
5	11	59	17	0	0.646	37.74	41.9	29.36	25.3	0.646	0.413	0.652
χ^2										3.085	6.997	3.644

SR: substrate molar ration (ROO :DHASCO-FFA : PA); O: observed response mol%; E: expected response mol%

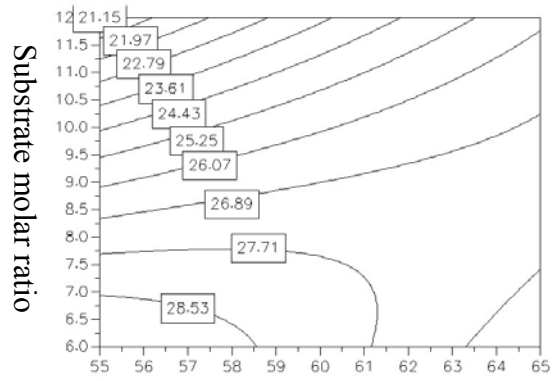
Table 3.4. Total fatty acid and *sn*-2 profile (mol%) of refined olive oil, DHASCO-FFA and SL at optimal reaction condition

Fatty Acid	Refined Olive Oil		DHASCO-FFA[a]	SL	
	Total	*sn*-2		Total	*sn*-2
C12:0	ND	ND	5.55 ± 0.37	ND	ND
C14:0	ND	ND	12.59 ± 0.13	ND	ND
C16:0	9.97 ± 0.08	1.49 ± 0.33	10.67 ± 0.12	55.79 ± 1.22	33.63 ± 1.69
C16:1n7	1.01 ± 0.00	0.84 ± 0.01	2.64 ± 0.05	ND	ND
C18:0	6.90 ± 0.15	ND	0.57 ± 0.01	1.29 ± 0.09	ND
C18:1n9	73.95 ± 0.55	86.35 ± 0.32	16.84 ± 0.41	36.54 ± 1.07	66.37 ± 1.69
C18:2n6	7.26 ± 0.01	10.30 ± 0.03	0.68 ± 0.02	3.37 ± 0.21	ND
C18:3n3	0.51 ± 0.00	1.01 ± 0.02	ND	ND	ND
C22:6n3	ND	ND	47.90 ± 1.09	3.01 ± 0.13	ND

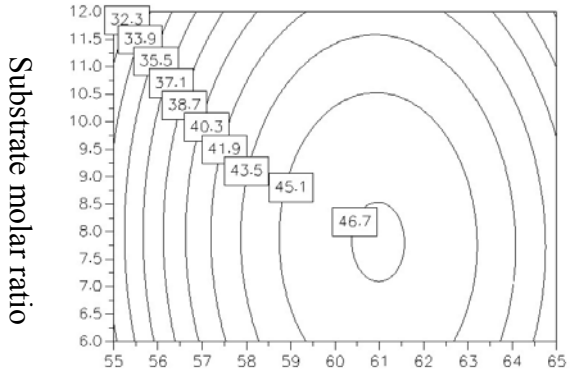
ND: not detected

[a] Others include: C8:0, C10:0, C14:1

a


Substrate molar ratio
21.15
21.97
22.79
23.61
24.43
25.25
26.07
26.89
27.71
28.53
Temperature (°C)

b


Substrate molar ratio
32.3
33.9
35.5
37.1
38.7
40.3
41.9
43.5
45.1
46.7
Temperature (°C)

c

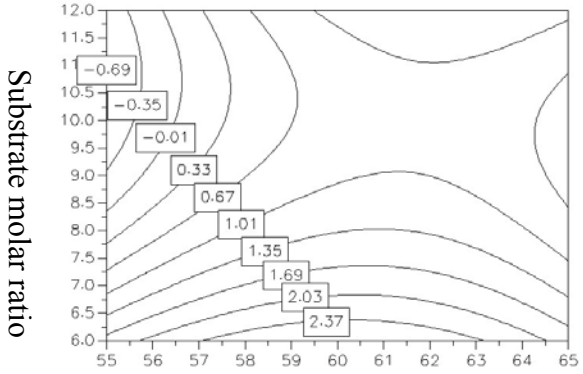

Substrate molar ratio
−0.69
−0.35
−0.01
0.33
0.67
1.01
1.35
1.69
2.03
2.37
Temperature (°C)

Fig. 3.1

CHAPTER 4

ENZYMATIC SYNTHESIS OF REFINED OLIVE OIL-BASED STRUCTURED LIPID CONTAINING OMEGA 3- AND -6 FATTY ACIDS FOR POTENTIAL APPLICATION IN INFANT FORMULA

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ABSTRACT

Structured lipid (SL) containing palmitic, docosahexaenoic (DHA), and gamma-linolenic acids (GLA) was produced using refined olive oil, tripalmitin, and ethyl esters of DHA single cell oil and GLA ethyl esters. Immobilized Lipozyme TL IM lipase was used as the biocatalyst. The SL was characterized for fatty acid profile, triacylglycerol (TAG) molecular species, solid fat content, oxidative stability index, and melting and crystallization profiles and compared to physical blend of substrates, extracted fat from commercial infant formula (IFF), and milk fat. 49.28 mol% of palmitic acid was found at the *sn*-2 position of SL TAG and total DHA and GLA composition were 0.73 and 5.00 mol%, respectively. Comparable solid fat content profiles were also found between SL and IFF. The SL produced has potential for use in infant formulas.

KEYWORDS

Docosahexaenoic acid; gamma-linolenic acid; palmitic acid; infant formula; lipase

INTRODUCTION

Maternal breast milk is widely considered as the ideal source of nutrition for infants and newborns. It contains lipids, protein, carbohydrate, minerals, and vitamins that are essential for infants' growth, development, and maturation (1,2). Although the total lipid content of human milk varies between 3-5 % (3), depending on the lactation stage of the mother, it accounts for nearly half of the energy of an infant's diet (4). These lipids consist of triacylglycerols (TAGs, 98 %), phospholipids (PL, 0.8 %), cholesterol (C, 0.5 %), and many others (3).

However, human milk is not always available or in high quality production for infants. Sociological, psychological, and cultural factors can all affect the decision of breastfeeding an infant (5). In addition, the mother could be in a special condition that renders maternal milk in low quantity or quality for infant's consumption. In such cases, alternative nutrition supply such as infant formula that resembles the nutritional composition of human milk is needed.

In human milk fat TAGs, the major fatty acids(FAs) are oleic, linoleic, stearic, myristic, and lauric acids (6). Among them, palmitic acid is the main fatty acid (FA) found at the *sn*-2 position of the glycerol backbone with over 50 mol% (7). However, in cow's milk and vegetable oils, which are fat sources used in infant formulas, palmitic acid is predominantly found at the *sn*-1, 3 positions. The FAs at the outer positions of human milk fat TAGs are hydrolyzed into free FAs by pancreatic lipases and can therefore form insoluble soaps with calcium ions in the intestines (8). This can then lead to decreased absorption of both palmitic acid and calcium by the infants. Consequently, the correct positional distribution of palmitic acid in the TAGs is critical for the infants' nutrition intake.

Long chain polyunsaturated fatty acids such as arachidonic (ARA), docosahexaenoic acid (DHA), and gamma-linolenic acids (GLA) are also present in human milk in trace amounts (9).

Concentrated DHA is found in the prefrontal cortex of brain that is associated with memory and visual development. Previous report indicated that a higher mean weight percentage of DHA and a greater proportion of DHA are found in red blood cells and brain cortex of breast-fed infants than in formula-fed infants (10).

Lipids that have been chemically and/or enzymatically modified to produce novel TAGs with desired health, functional or nutritional values are often referred to as structured lipids (SLs) (11). Betapol® was a SL that was commercially produced as human milk fat analog. It was synthesized by using a *sn*-1, 3 specific lipase, tripalmitin, and unsaturated fatty acids as substrates. Betapol® contained 53.5 % palmitic acid at the *sn*-2 position and 42.1 % total oleic acid (12). In comparison, we have synthesized SL that contained 33.6 mol% palmitic acid at the *sn*-2 position and 36.5 mol% total oleic acid using refined olive oil as the main substrate (13) and an extra virgin olive oil-based SLs that contained nearly 60 mol% palmitic acid at the *sn*-2 position and over 30 mol% total oleic acid (4). The objective of this study was to produce a refined olive oil-based SL that contains high amount of palmitic acid at the *sn*-2 position and enriched with DHA and GLA for potential infant formula application.

MATERIALS AND METHODS

Materials

Refined olive oil (ROO) was purchased from Columbus Vegetable Oils (Des Plaines, IL). Docosahexaenoic acid single cell oil (DHASCO) was kindly provided by DSM Nutritional Products (Columbia, MD) while γ-linolenic acid (GLA) in free fatty acid (FFA) form (70% GLA) was purchased from Sanmark Corp. (Greensboro, NC). Tripalmitin was purchased from Tokyo Chemical Industry America (Montgomeryville, PA). Immobilized lipase, Lipozyme TL IM® (*sn*-1, 3 specific from *Thermomyces lanuginosus*), was obtained from Novozymes North America Inc. (Franklinton, NC) with a specific activity of 250 IUN/g (IUN is the Interesterification Units Novo). Commercial infant formula, Nestle Good Start Gentle (Nestle USA, Inc., Glendale, CA), containing DHA and ARA, was purchased at a local grocery store in Athens, GA. Milk fat (MF) was purchased from Dairy Farmers of America (Winthrop, MN). The lipid standards Supelco 37 component FAME mix, C15:0 pentadecanoic acid (>98 % purity), triolein, and 2-oleoyglycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Other solvents and chemicals were purchased from Fisher Scientific (Norcross, GA) and Sigma-Aldrich (St. Louis, MO).

Preparation of Fatty Acid Ethyl Esters

Fatty acid ethyl esters (FAEEs) of DHASCO-TAG and GLA-FFA were prepared according to the methods previously described by Vázquez and Akoh (14) with minor modifications. 100 mL of DHASCO or GLA-FFA were mixed with sodium ethoxide (2.625 %, v/v) in absolute ethanol at a ratio of 4:2 (v/v). The mixture was heated at 60 °C with constant agitation at 200 rpm for 40 min under nitrogen atmosphere. The product was subsequently washed with 100 mL saturated NaCl solution, followed by a washing step with 100 mL distilled

water. After separation, the upper layer containing FAEEs was collected and passed through a sodium sulfate column under vacuum. FAEEs were then confirmed by thin-layer chromatography (TLC) using ethyl oleate as standard. DHASCO-EE and GLA-EE were finally mixed at a molar ratio of 1:2 (named DG12) and 2:3 (named DG23), respectively, and stored in amber bottles under nitrogen at -20 °C until use.

Small-scale Synthesis and Analysis of SL Products

Tripalmitin was mixed with ROO, DG12 or DG23 at different substrate molar ratios (tripalmitin to ROO to DG12 or DG23 at 1:1:1, 1:2:1, 1:3:2, 1:4:2, 1:5:2, and 1:5:1). 3 mL hexane and Lipozyme TL IM lipase at 10 % (w/w) of the total substrate mass were also added to the reaction mix. The mixture was placed in screw-capped test tubes and incubated at 65 °C for 24 h with constant agitation at 200 rpm. The products were then collected and passed through an anhydrous sodium sulfate column to remove moisture and enzyme. All reactions were performed in triplicate and the average value and standard deviation were reported. A physical blend (PB) was also prepared with a molar ratio of tripalmitin to ROO to DG23 of 1:1:1 without adding Lipozyme TL IM. The PB was subjected to the same synthesis and clean-up process as that of SLs.

Separation of SLs from FAEEs

SLs were separated from FAEEs by TLC utilizing TLC solvent systems previously described (15,16). Petroleum ether/diethyl ether/acetic acid (97.5/52.5/3, v/v/v) were firstly used to separate SLs and FAEEs from monoacylglycerols (MAGs), diacylglycerols (DAGs), and FFA. In the second TLC, petroleum ether/diethyl ether/acetic acid (75/5/1, v/v/v) were used to separate SLs from FAEEs.

Fat Extraction from Commercial Infant Formula

Fat extraction from commercial infant formula was carried out following the method previously described by Bligh and Dyer (17) with minor modifications. 100 grams of the infant formula was mixed with 100 mL of chloroform and homogenized for 30 s. 200 mL of methanol was then added to the mixture and homogenized again for 30 s. Another 100 mL of chloroform was added and the mixture was blended for 1-2 min. Finally, 100 mL of 0.88 % sodium chloride solution was added and the mixture was blended again for 1 min. A Whatman No. 1 filter paper was used to vacuum-filter the mixture through a Buchner funnel. The residue on the filter paper was transferred into a beaker and mixed with 100 mL of chloroform. The resultant mixture was vacuum-filtered again as described above and collected with the first filtrate. The entire filtrate was then transferred to a 1 L separatory funnel and allowed to separate. After clear separation was observed, the bottom chloroform layer was collected and passed through an anhydrous sodium sulfate column to remove any excess water. Chloroform was then removed using a rotovapor at 40 °C. The extracted infant formula fat (IFF) was stored in an amber bottle under nitrogen at -20 °C until use.

Determination of Fatty Acid Profiles

The substrates, namely ROO, DHASCO-EE, GLA-EE, and the products (SLs, PB, IFF, and milk fat (MF)) were converted to FA methyl esters following AOAC Official Method 996.01 (18) with minor modifications. 0.1 g of sample (or scraped-off TLC band) was weighed into Teflon-lined test tubes and 0.25 mL internal standard (C15:0, 20 mg/mL in hexane) was added. 2 mL 0.5 M NaOH in methanol was added and heated at 100 °C for 5 min. The samples were cooled in ice bath and 2 mL 14 % BF_3 in methanol was added and again heated at 100 °C for 5 min. The samples were cooled and finally 2 mL hexane and 2 mL saturated NaCl solution were

added and the mixture was vortexed for 2 min. The upper FAME layer was collected into GC vials after centrifuging the samples at 1000 rpm for 5 min at room temperature and passed through anhydrous sodium sulfate column. The samples were analyzed with Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) using Supelco SP-2560, 100 m x 25 mm x 0.2 μm column. Supelco 37 component FAME mix was used as the external standard. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. Injection volume was 1 μL and a split ratio of 5:1 was used. Detection was with flame ionization detector at 250 °C. The column was initially held at 140 °C for 5 min and then increased to 240 °C at 4 °C/min and held at 240 °C for 22 min. All samples were analyzed in triplicate and average values were reported.

Positional Analysis

sn-2 positional fatty acid composition was determined following the method previously described by Luddy et al. (19). All samples (SLs, PB, IFF, and MF) were analyzed in triplicate and average values and standard deviation were reported.

Scaled-up Production of SL

The solvent-free interesterification reaction was performed in a 1 L stirred batch reactor at 65 °C using a substrate molar ratio of 1:1:1 (tripalmitin:ROO:DG23) and Lipozyme TL IM lipase (10 % weight of total substrates) as biocatalyst. The reactor was sealed and covered with aluminum foil to minimize the impact of light and oxygen. The reaction was carried out for 24 h with constant stirring at 200 rpm. At the end of the reaction, product was vacuum-filtered through a Whatman No. 1 filter paper to separate the SLs from the enzyme. A second filtration using Whatman No. 1 filter paper and sodium sulfate was performed to remove any excess water. SLs were kept in an amber container flushed with nitrogen and stored at 4 °C until use.

Short-path Distillation

Short-path distillation was performed to remove excess FAEEs from the SLs using KDL-4 (UIC Inc., Joliet, IL, USA) system under the following conditions: holding temperature of 65 °C, feeding rate of approximately 100 mL/h, heating oil temperature of 175 °C, coolant temperature of 20-25 °C, and vacuum of <100 mTorr. SLs were passed three times and the FFA content expressed as oleic acid percentage was determined following the AOCS Official Method 5a-40 (20). The yield of the SLs after purification by short-path distillation was 89.73 % (w/w) based on the starting total substrate.

Triacylglycerol Molecular Species

The TAG composition was determined with a reverse phase HPLC (Agilent Technologies 1260 Infinity, Santa Clara CA) equipped with a Sedex 85 ELSD (Richard scientific, Novato, CA). The column was a Beckman Ultrasphere® C18, 5 μm, 4.6 x 250 mm with temperature set at 30 °C. The injection volume was 20 μL. The mobile phase at a flow rate of 1 mL/min consisted of solvent A, acetonitrile and solvent B, acetone. A gradient elution was used starting with 35% solvent A to 5% solvent A at 45 min and then returning to the original composition in 5 min. Drift tube temperature was set at 70 °C, pressure at 3.0 bar and gain at 8. The samples (SL, PB, IFF, and MF) were dissolved in chloroform at a concentration of 5 mg/mL. The TAG peaks were identified by comparison of retention times with those of the standards and also by equivalent carbon number (ECN). ECN is defined as CN – 2n, where CN is the number of carbons in the TAG (excluding the three in the glycerol backbone) and n is the number of double bonds. Triplicate determinations were carried out and averaged data reported.

Major Phenolic Compounds

Phenolic compounds were extracted from SL before and after short-path distillation, ROO, and PB using solid phase extraction (21) with methanol, water, and acetonitrile. Determination of phenolic compounds was carried out following the method described by Owen et al. (22) using a Hewlett-Packard (Avondale, PA) HP 1100 HPLC system with diode array detector (DAD). Beckman Ultrasphere C18, 5μm, 4.6 × 250 mm column was used with temperature set at 40 °C. The injection volume was 20 μL. Mobile phase consisted of solvent A, 2% (v/v) acetic acid in water, and solvent B, methanol, at a flow rate of 1 mL/min. Gradient elution was set as follows: at 2 min 5% solvent B, 10 min 25% B, 20 min 40% B, 30 min 50% B, and 100% B at 45 min. Detection was done at 260, 280, 320, and 360 nm. External standards used were hydroxytyrosol, tyrosol, oleuropein, luteolin, ferulic, cafeic, gallic, vanillic, and *p*-coumaric acids. Identification was based on the retention times and characteristic UV spectra, and quantification was done using the external standard curves. All analysis was performed in triplicate and average data reported.

Solid Fat Content

Solid fat content (SFC) was determined following the AOCS Official Method Cd 16b-93 (23) on a Benchtop NMR analyser – MQC (Oxford Instruments, Abingdon, England). Samples were tempered at 100 °C for 15 min and then kept at 60 °C for 10 min, followed by 0 °C for 60 min and finally for 30 min at each selected temperature of measurement. SFC was measured at intervals of 5 °C from 25 to 55 °C.

Oxidative Stability Index (OSI)

The OSI of the samples were determined with an Oil Stability Instrument (Omnion, Rockland, Mass., U.S.A.) at 110 °C according to the AOCS Official Method Cd 12b-92 (24).

Melting and Crystallization Profiles

The melting and crystallization profiles were determined using a differential scanning calorimeter DSC 204 F1 Phoenix (NETZSCH Instruments North America, Burlington, MA) following AOCS Official Method Cj 1-94 (25). First, 8–12 mg samples were weighed into aluminum pans and sealed. Samples were rapidly heated to 80 °C at 20 °C/min, and held for 10 min to destroy any previous crystalline structure. The samples were then cooled to -80 °C at 10 °C/min (for crystallization profiles), and held for 30 min and finally heated to 80 °C at 10 °C/min (for melting profiles). Nitrogen was used as the protective and purge gas. All samples were analyzed in triplicates and averaged values were reported.

Statistical Analysis

Statistical analyses were performed with the SAS software package (SAS Institute, Cary, NC). Duncan's multiple-range test was performed to determine the significant difference between samples.

RESULTS AND DISCUSSION

Small-scale Synthesis and Selection for Large-scale Production

Conditions for small-scale reactions were generated by randomized factorial design with three factors: substrate molar ratio (7 levels), enzymes (2 levels), and molar ratio of DHASCO-EE to GLA-EE in the ethyl ester mix (2 levels). Total palmitic acid incorporation ranged from 23.59 to 51.79 mol% in the SLs, while oleic acid incorporation ranged from 37.62 to 64.19 mol%. Total GLA and DHA contents were 1.44 to 5.29 mol% and 0 to 1.41 mol%, respectively. In general, total palmitic acid incorporation decreased with a higher substrate molar ratio. This was expected because with a higher substrate molar ratio, the molar percentage of refined olive oil in the substrates increased and since refined olive oil contained a high amount of oleic acid,

less palmitic acid would be esterified to the glycerol backbone. A similar trend was observed in the *sn*-2 positional fatty acid profiles of the SLs where decreasing palmitic acid content at the *sn*-2 position was accompanied by an increasing substrate molar ratio.

Substrate molar ratio of 1:1:1 with a 2:3 DHASCO-EE to GLA-EE molar ratio in the ethyl ester mix and Lipozyme TL IM lipase were selected for large-scale production. Under this condition, total palmitic acid was 51.79 mol% while oleic acid, GLA, and DHA were 37.62, 2.84, and 0.29 mol%, respectively. At the *sn*-2 position, 44.82 mol% of palmitic acid was found.

Total and Positional Fatty Acid Profiles

Characterization of the scaled-up SL product was carried out after short-path distillation. Three passes were required to lower the FFA value of the SL to 0.08 %. The high amount of FFA in the product was probably due to the presence of DHASCO-EE and GLA-EE, which could produce DHASCO-FFA and GLA-FFA during the hydrolysis of their respective ethyl esters. Table 4.1 shows the fatty acid composition of DHASCO-EE and GLA-EE, as well as the total and positional fatty acid composition of ROO. It can be seen that the DHASCO-EE contained 45.98 mol% of DHA while GLAEE contained 71.79 mol% of GLA. Oleic acid was the primary fatty acid found in ROO at 73.95 mol% while palmitic acid was only 9.97 mol%. At the *sn*-2 position of ROO TAG, oleic acid was 86.35 mol% while palmitic acid content was only 1.49 mol%, which is considerably lower than human milk fat which contains 50 – 60 mol% palmitic acid at the *sn*-2 position (7).

The total and positional fatty acid composition of SL, PB, IFF, and MF are shown in Table 4.2. It can be seen that at the *sn*-2 position, only 6.12 mol% of palmitic acid was found in IFF TAG while 49.28 mol% was found in the SL TAG. PB contained similar total palmitic acid content (46.60 mol%) to SL, however, at the *sn*-2 position, its 32.67 mol% was significantly

lower ($P \leq 0.05$) than that of SL. In our previous study where refined olive oil and FFAs of DHASCO and palmitic acid were used, the SL contained 33.63 mol% palmitic acid at the *sn*-2 position (13). Fatty acids located at the *sn*-1, 3 positions are released as FFA after ingestion as a result of *sn*-1, 3 specific pancreatic lipase action (4). These palmitic acid FFA can further react with calcium ions to form insoluble calcium soaps in the intestine that can lead to hard stool in infants (26). In this case, palmitic acid is not absorbed and a decrease in calcium absorption can also be expected. Consequently, TAG consisting of high palmitic acid at the *sn*-2 position is preferred as it helps increase the absorption of palmitic acid and calcium. Compared to the positional distribution of fatty acids in commercial infant formula, the SL showed a closer resemblance to the positional distribution in human milk fat.

It is also worth noticing that although the commercial infant formula claims to contain ARA and DHA, they were found to contain only 0.59 and 0.26 mol%, respectively. In comparison, the SL contained 0.73 mol% DHA, and while no ARA was found in the SL, 5.00 mol% of GLA was incorporated, and this can be converted to ARA in humans. In comparison, a palm olein-based SL previously synthesized in our lab contained 3.75 mol% DHA and 5.03 mol% GLA (27). The SL produced in the current study contained desirable palmitic acid content at the *sn*-2 position of its TAGs and were enriched with DHA and GLA. Although it has higher total palmitic acid compared to human milk fat, it can be used with other vegetable oils or SL as a blend to produce an ideal total palmitic acid content while still maintaining the *sn*-2 palmitic acid and total DHA level in the final product.

TAG Molecular Species

The TAG molecular species of SL, PB, IFF, and MF are shown in Table 4.3. The IFF and MF had much diverse TAG species than SL and PB. The predominant TAG in PB was PPP

which was expected since tripalmitin was one of the starting TAGs in the interesterification reaction. In comparison, the predominant TAGs in the SL were POP (31.91 %) and OPO (22.78 %), followed by LnDLn (10.91 %), PPP (10.18 %), LPL (10.09 %), LOO (9.83 %), and OOO (4.29 %). Besides PPP, TAGs containing palmitic acid increased from 32. 75 % in PB to 64.78 %, suggesting a potential increase in palmitic acid content at *sn*-2 position, which was in accordance with what was observed in the positional distribution of fatty acids in the SL. In contrast, the major TAG molecular species found in human milk fat are OPO (1.56-42.44 %), POL (9.24-38.15 %), OOO (1.61-11.96 %), and LOO (1.64-10.18 %) (28). The OPO, OOO, and LOO content of the SL were all within the range of that found in human milk while the OPO (3.37 %) and LOO (ND) contents of IFF were not.

Major Phenolic Compounds

Phenolic compounds in ROO, PB, and SL were analyzed using solid phase extraction followed by HPLC-DAD. The major phenolic in ROO was oleuropein (2.50 μg/g). Other phenolic compounds identified were gallic acid and luteolin. The major phenolics normally found in virgin olive oil are tyrosol, hydroxytryrosol, and oleuropein. However, phenolic compounds are removed to almost zero by the physical and chemical refining processes used to produce refined olive oil (21). In PB, oleuropein and luteolin were found in trace amounts, while in SL, no major phenolic compounds were found before and after short-path distillation. They may be lost during interesterification as esters or in free forms (29). Phenolic compounds can retard oxidation (30,31) and reduce the risk of cardiovascular disease (32). The lack of phenolic compounds in SL suggests that it is prone to oxidation and that can further affect its quality and nutritional properties. Consequently, antioxidants should be added back to the infant formula containing the SL to prolong the shelf life.

Solid Fat Content

Solid fat content (SFC) is the measure of solid/liquid ratio of a fat at various temperatures (33). It can have an impact on the physical and sensorial properties such as texture and mouthfeel of the product containing the TAG (34).

The SFC of SL, PB, IFF, and MF are shown in Figure 4.1. SL exhibited a comparable SFC profile to IFF at each temperature tested, suggesting a promising feasibility of applying the SLs in infant formula production.

Oxidative Stability Index

The OSI of the SL, PB, IFF, and MF were evaluated and results are shown in Figure 4.2. The commercial infant formula (17.08 h) and milk fat (17.50 h) showed significantly higher OSI than the SL (2.98 h) and PB (3.82h). The lower OSI observed in the SL compared to PB was probably due to the loss of natural antioxidants such as tocopherols during the interesterification process and short-path distillation (35,36). Consequently, additional antioxidants are recommended to be added to the SL to increase the oxidative stability and prolong the shelf life of the product containing the SL.

Melting and Crystallization Profiles

Melting and crystallization profiles of the SL, PB, IFF, and MF are shown in Figures 4.3 and 4.4, respectively. The melting completion temperature (T_{mc}) normally depends on the type of fatty acids present and TAG species (4). An UUU type of TAG suggests that the TAG consists of three unsaturated fatty acids while a SSS type of TAG consists of three saturated fatty acids. Since unsaturated fatty acids usually exhibit lower melting point than their saturated counterparts with the same hydrocarbon chain length, an UUU type of TAG would be expected to have a lower melting point than its SSS counterpart. In our study, SL contained 4.29 % of OOO while it

was absent in PB. In addition, SL contained significantly lower ($P < 0.05$) PPP (10.18 %) than PB (64.23 %). This could explain the lower melting completion temperature observed with SL (45.8 °C) than PB (63.1 °C). Similarly, both IFF and MF contained higher OOO (8.96 and 5.24 %, respectively) and lower PPP (ND and 8.78 %) than SL, which could result in the significantly lower ($P < 0.05$) melting completion temperatures observed in IFF (31.0 °C) and MF (34.6 °C) than SL. The SL had a broader melting curve as a result of interesterification compared to the non-interesterified PB, MF, and IFF. In addition, the SL exhibited a significantly higher crystallization onset temperature (T_{co}) (26.2 °C) than IFF (16.3 °C) and MF (17.5 °C). PB had the highest T_{co} (54.9 °C) ($P < 0.05$) compared to the SL, IFF, and MF.

Infant formulas with fat fraction that resembles human milk fat would be ideal nutrition substitute for human milk when breastfeeding is unavailable or limited. The majority of commercial infant formulas on the market are made with vegetable oils where palmitic acid is esterified to the *sn*-1, 3 positions of TAG. However, palmitic acid is predominantly found at the *sn*-2 position of human milk fat TAG. In this study, a commercial infant formula was found to contain as low as 6.12 mol% palmitic acid at the *sn*-2 position of its TAG. The SL produced in this study contained 49.28 mol% palmitic acid at the *sn*-2 position while the DHA content was also significantly higher than that found in the commercial infant formula. The SL contained an increased amount of OPO species which is desirable for better absorption of palmitic acid and calcium. In addition, the SL exhibited similar SFC to IFF. However, the absence of phenolic compounds in the SL suggests that antioxidants should be added to the infant formula to prolong its shelf life. The SL produced herein has potential to be used in infant formula applications.

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Figure captions:

Figure 4.1. Solid fat content (%) of SL, PB, IFF, and MF. SL: structured lipid; PB: physical blend; IFF: infant formula fat; MF: milk fat.

Figure 4.2. Oxidative stability index (OSI) of SL, PB, IFF, and MF. Values with the same letter are not significantly different ($P < 0.05$); SL: structured lipid; PB: physical blend; IFF: infant formula fat; MF: milk fat.

Figure 4.3. Melting thermograms of SL, PB, IFF, and MF. The temperatures shown are melting completion temperatures. SL: structured lipid; PB: physical blend; IFF: infant formula fat; MF: milk fat.

Figure 4.4. Crystallization thermograms of SL, PB, IFF, and MF. The temperatures shown are crystallization onset temperatures. SL: structured lipid; PB: physical blend; IFF: infant formula fat; MF: milk fat.

Table 4.1. Total and positional fatty acid composition (mol%) of substrates DHASCO-EE, GLA-EE, and refined olive oil

Fatty acid	DHASCO-EE*	GLA-EE*	Refined olive oil*	
			Total	*sn*-2
C12:0	6.34 ± 0.07	ND	ND	ND
C14:0	14.10 ± 0.13	ND	ND	ND
C16:0	11.97 ± 0.45	ND	9.97 ± 0.08	1.49 ± 0.33
C16:1n7	ND	ND	1.01 ± 0.00	0.84 ± 0.01
C18:0	ND	ND	6.90 ± 0.15	ND
C18:1n9	21.61 ± 0.89	1.63 ± 0.14	73.95 ± 0.55	86.35 ± 0.32
C18:2n6	ND	26.58 ± 0.41	7.26 ± 0.01	10.30 ± 0.03
C18:3n3	ND	ND	0.51 ± 0.00	1.01 ± 0.02
C18:3n6	ND	71.79 ± 0.54	ND	ND
C22:6n3	45.98 ± 1.21	ND	ND	ND

* Mean ± SD; ND: not detected.

Table 4.2. Total and *sn*-2 fatty acid composition (mol%) of the scaled-up product (SL), physical blend, commercial infant formula fat, and milk fat

	Total fatty acids*				*sn*-2*			
Fatty acids	SL	PB	IFF	MF	SL	PB	IFF	MF
C8:0	ND	ND	1.74 ± 0.04a	1.56 ± 0.03a	ND	ND	5.50 ± 1.76a	5.95 ± 0.49a
C10:0	ND	ND	1.19 ± 0.03a	3.05 ± 0.02b	ND	ND	ND	3.21 ± 0.19
C12:0	ND	0.44 ± 0.01a	9.24 ± 0.20b	3.65 ± 0.13c	ND	ND	17.56 ± 1.93d	8.02 ± 0.97e
C14:0	2.61 ± 0.93a	1.29 ± 0.03b	4.40 ± 0.06c	11.76 ± 0.45d	2.63 ± 0.54a	ND	3.18 ± 0.20d	17.54 ± 2.25e
C14:1	ND	ND	ND	0.98 ± 0.01	ND	ND	ND	1.35 ± 0.23
C16:0	47.80 ± 0.41a	46.60 ± 1.78a	21.92 ± 0.09b	31.63 ± 0.09c	49.28 ± 1.68a	32.67 ± 2.42c	6.12 ± 1.07d	33.05 ± 1.37c
C16:1	ND	0.74 ± 0.03a	ND	1.97 ± 0.04b	ND	ND	ND	ND
C17:0	ND	ND	ND	1.91 ± 0.14	ND	ND	ND	ND
C18:0	2.55 ± 0.09a	1.92 ± 0.04b	4.13 ± 0.02c	11.17 ± 0.10d	ND	ND	ND	ND
C18:1trans	ND	ND	ND	1.91 ± 0.03	ND	ND	ND	7.77 ± 0.72
C18:1cis	36.13 ± 0.37a	41.22 ± 1.41b	30.49 ± 0.20c	26.26 ± 0.12d	38.28 ± 0.84a	59.33 ± 3.62e	39.75 ± 2.47a	20.56 ± 1.15f
C18:2trans	ND	ND	ND	0.68 ± 0.01	ND	ND	ND	ND
C18:2cis	5.19 ± 0.05a	3.77 ± 0.14b	23.05 ± 0.06c	3.02 ± 0.11bd	6.29 ± 1.52e	7.99 ± 1.50f	26.22 ± 1.69c	2.55 ± 0.20d
C18:3n6	5.00 ± 0.16a	0.62 ± 0.02b	ND	ND	3.52 ± 0.37c	ND	ND	ND
C18:3n3	ND	0.51 ± 0.02a	2.99 ± 0.07b	0.46 ± 0.01a	ND	ND	1.66 ± 0.16c	ND
C20:4n6	ND	ND	0.59 ± 0.00	ND	ND	ND	ND	ND
C22:6n3	0.73 ± 0.04a	2.89 ± 0.14b	0.26 ± 0.00c	ND	ND	ND	ND	ND

* Mean ± SD; ND: not detected; SL: structured lipid; PB: physical blend; IFF: infant formula fat; MF: milk fat; Values with different letter in each row are significantly different at $P \leq 0.05$.

Table 4.3. Relative (%) of triacylglycerol (TAG) molecular species of structured lipid, physical blend, infant formula fat, and milk fat

TAG species	SL	PB	IFF	MF
LaCC	ND	ND	ND	1.25 ± 0.09
LaCLa	ND	ND	ND	3.63 ± 0.21
LnDLn	10.91 ± 0.30a	ND	2.72 ± 0.06b	ND
LaLnLa	ND	ND	ND	12.25 ± 1.35
LaLaLa	ND	ND	4.97 ± 0.18	ND
LaMLa	ND	ND	4.74 ± 0.22a	25.41 ± 0.98b
OLaM	ND	ND	ND	3.24 ± 0.67
MLaM	ND	ND	3.76 ± 0.13a	2.12 ± 0.67b
LLL	ND	ND	2.02 ± 0.14	ND
MML	ND	ND	11.93 ± 0.32a	2.26 ± 0.54b
MMM	ND	ND	ND	1.36 ± 0.35
LnLnS	ND	ND	ND	3.48 ± 1.63
LnOO	ND	ND	ND	2.62 ± 0.72
LPL	10.09 ± 0.26	ND	ND	ND
LOL	ND	ND	8.61 ± 0.43	ND
MPL	ND	ND	5.23 ± 0.47a	3.99 ± 0.63b
LOO	9.83 ± 0.96a	3.02 ± 0.41b	ND	2.25 ± 0.45c
PLP	ND	ND	5.38 ± 0.31	3.61 ± 0.48
OOO	4.29 ± 0.48a	ND	8.96 ± 0.16b	5.24 ± 0.74a
OPO	22.78 ± 0.75a	24.49 ± 1.47a	3.37 ± 0.13b	2.57 ± 0.27c
POP	31.91 ± 0.68a	8.26 ± 0.07b	ND	ND
PPP	10.18 ± 0.52a	64.23 ± 1.60b	ND	8.78 ± 0.47c
OSO	ND	ND	7.04 ± 0.63a	7.73 ± 0.76a
OSP	ND	ND	15.49 ± 0.22a	2.64 ± 0.35b
PSP	ND	ND	13.60 ± 0.02a	1.61 ± 0.23b
MSS	ND	ND	ND	3.37 ± 0.51
SOS	ND	ND	0.90 ± 0.14	ND
PSS	ND	ND	1.28 ± 0.09	ND

The fatty acids are not in regiospecific order; C: capric acid; La: lauric acid; M: myristic acid; P: palmitic acid; S: stearic acid; O: oleic acid; L: linoleic acid; Ln: linolenic acid; D: docosahexaenoic acid; Values with different letter in each row are significantly different at $P \leq 0.05$; SL: structured lipid; PB: physical blend; IFF: infant formula fat; MF: milk fat.

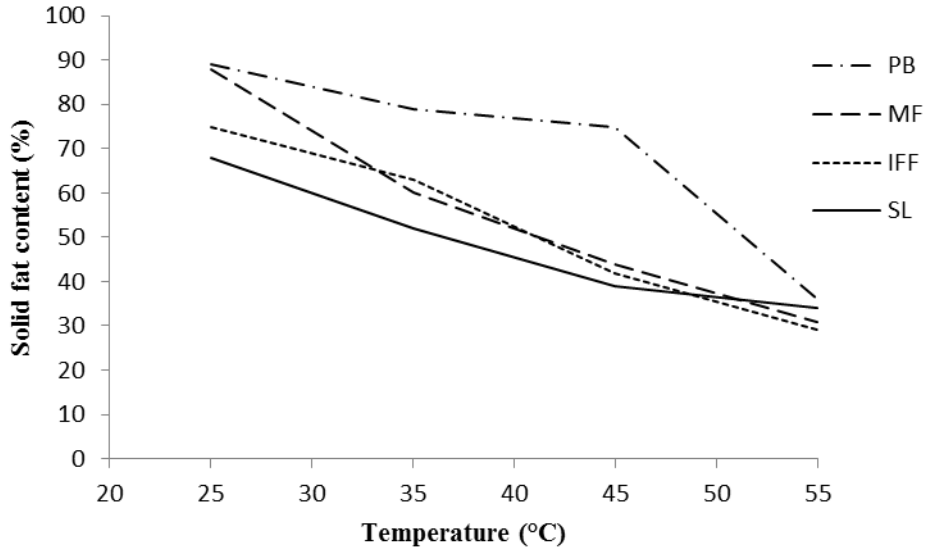
100
90
80
70
60
50
40
30
20
10
0
Solid fat content (%)
20
25
30
35
40
45
50
55
Temperature (°C)
PB
MF
IFF
SL

Figure 4.1.

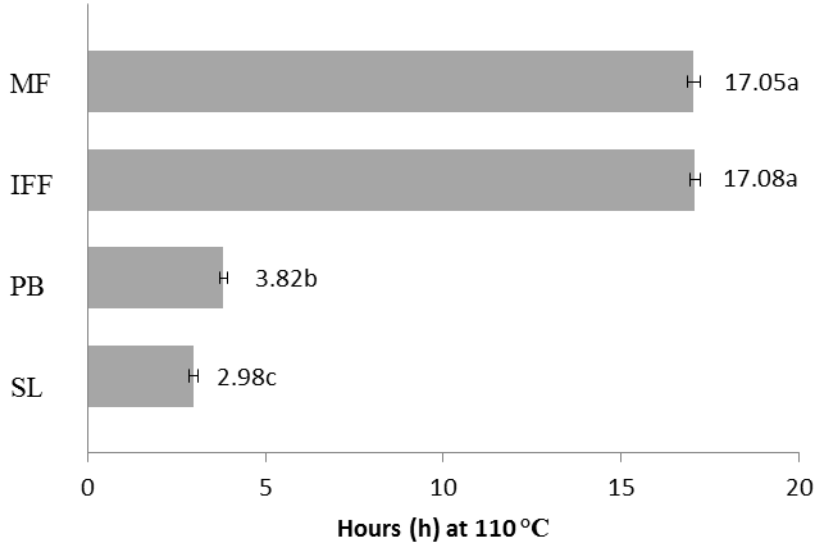

Figure 4.2.

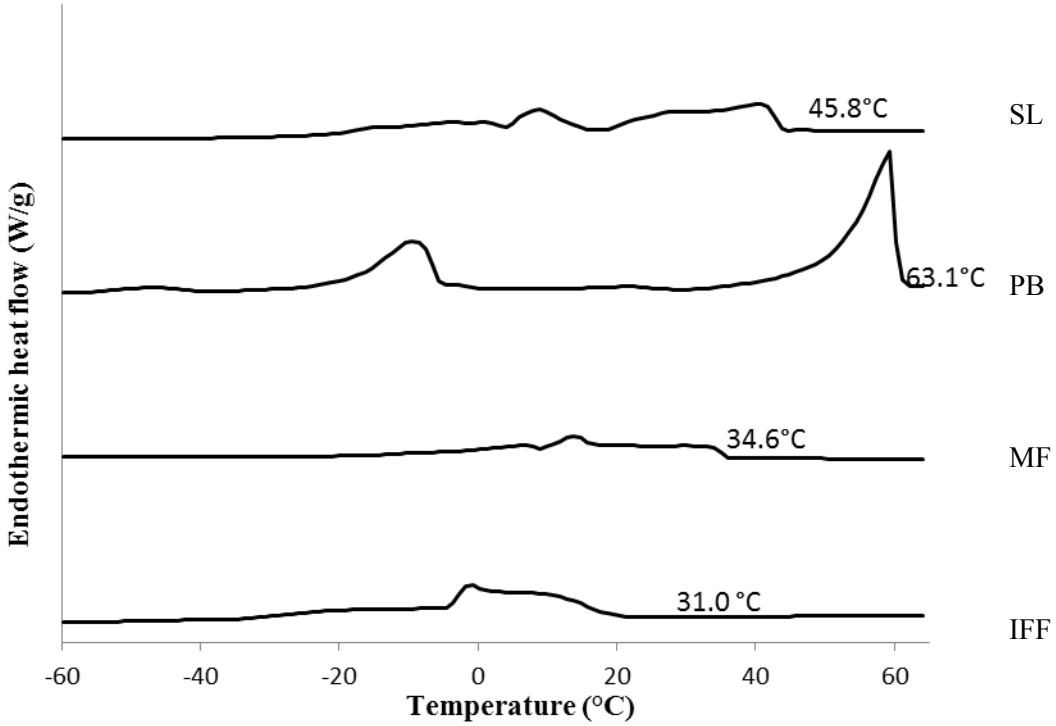
45.8°C
SL
63.1°C
PB
34.6°C
MF
31.0 °C
IFF
Endothermic heat flow (W/g)
-60
-40
-20
0
20
40
60
Temperature (°C)

Figure 4.3.

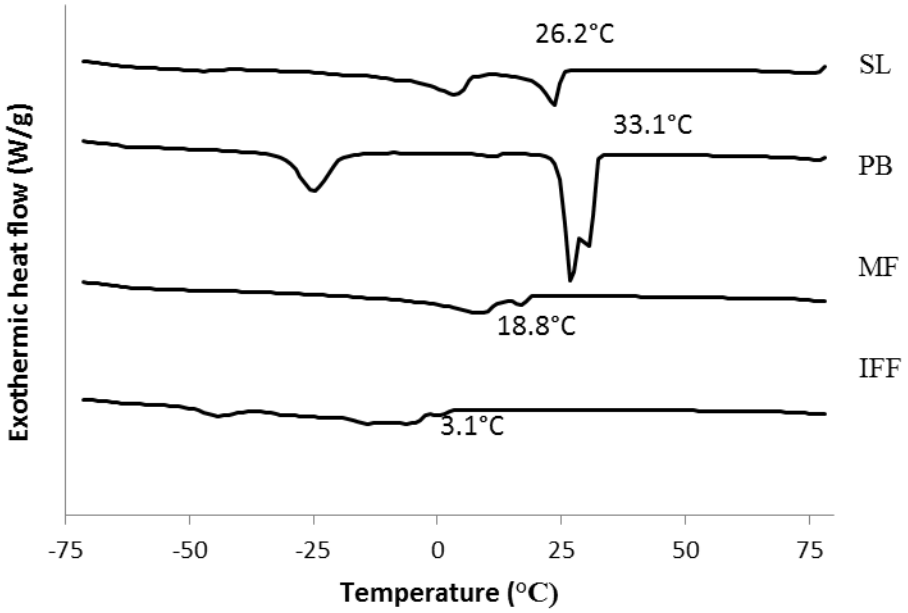
26.2°C
SL
33.1°C
PB
MF
18.8°C
IFF
3.1°C
Exothermic heat flow (W/g)
-75
-50
-25
0
25
50
75
Temperature (°C)

Figure 4.4.

CHAPTER 5

CONCLUSION

Human milk is the optimum dietary source for newborns and infants. However, due to some sociological, psychological, cultural factors and certain special conditions of the maternal mother, human milk is not always available or in high quality or quantity. Consequently, there is a constant demand for human milk substitute as the main or supplementary nutrition supply for infants. Traditional infant formulas are produced using vegetable oil blends as their fat source; however, the positional distribution of fatty acids in vegetable oils, particularly palmitic acid, is different from human milk fat. Such regiospecific composition of human milk fat is critical for the proper absorption of palmitic acid and calcium. In this study, we developed a structured lipid (SL) based on refined olive oil that has a similar positional distribution of fatty acids for possible application in infant formula.

Enzymatic acidolysis and interesterification were employed to modify the structure of refined olive oil. In the acidolysis reaction, free fatty acids of docosahexaenoic acid (DHA) single cell oil (DHASCO) and palmitic acid were used while the catalyst was a nonspecific Novozym 435 lipase. In the interesterification reaction, ethyl esters of DHASCO, gamma-linolenic acid (GLA), and tripalmitin were used but the catalyst was a *sn*-1, 3 specific Lipozyme TL IM lipase.

The SL produced contained 49.28 mol% palmitic acid at the *sn*-2 position and a total of 0.73 mol% DHA and 5.00 mol% GLA. In comparison, human milk fat usually contains 50-60 % palmitic acid at the *sn*-2 position and less than 1 % of DHA and GLA, respectively. The

commercial infant formula analyzed in this study contained 0.26 mol% of total DHA and 6.12 mol% palmitic acid at the *sn*-2 position. Our SL also showed a similar melting and crystallization profile and solid fat content as commercial infant formula fat and milk fat, suggesting the feasibility of being applied in infant formula production. Oxidative stability study did imply a lower stability for SL than commercial infant formula fat and milk fat. Therefore, the addition of appropriate antioxidants should be helpful in enhancing the stability of the SL and improving its functionality in the final infant formula product.

Scientists have come to agree that a certain balance of n-3:n-6 polyunsaturated fatty acids (PUFAs) needs to be established to achieve a proper nutrition. However, no minimum or maximum levels of PUFAs in infant formulas have been published by government regulatory agencies. There have also been inconclusive studies regarding the implications of PUFAs supplementation in infant formulas on the physiological development of infants. Future studies should be directed to these areas to improve our understanding of these issues and help create better human milk substitute for future generations.