ENZYMATIC SYNTHESIS OF STRUCTURED LIPIDS CONTAINING DHA, ARA, CAPRIC, AND PALMITIC ACIDS SUITABLE FOR USE AS FAT BLEND IN INFANT FORMULA

by

CARLOS ANDRES ALVAREZ S

(Under the Direction of Casimir C. Akoh)

ABSTRACT

Human milk fat substitutes are used in infant formulas to mimic the fatty acid composition of breast milk. The purpose of this research was first to enzymatically synthesize a high *sn*-2 palmitic acid structured lipid (SL) enriched with capric acid. Subsequently, high *sn*-2 DHA and ARA SLs were obtained by enzymatic modification of DHA and ARA single-cell oils. Finally, the SLs obtained were proportionally blended with canola (CAO) and corn (CO) oils to prepare an infant formula fat analog (IFFA) that closely matched the fatty acid profile of commercial fat blends used for infant formula. The IFFA contained 17.37 mol% total palmitic acid (nearly 35% at the *sn*-2 position). The total capric acid was 13.93 mol%. The content of DHA and ARA were 0.49 mol% (48.18% at *sn*-2) and 0.57 mol% (35.80% at *sn*-2), respectively. The IFFA obtained in this research could possibly be used for commercial production of infant formulas.

INDEX WORDS: Structured lipids, Infant formula, Capric acid, Palm stearin, Docosahexaenoic acid, Arachidonic acid, Enzymatic interesterification, Positional distribution

ENZYMATIC SYNTHESIS OF STRUCTURED LIPIDS CONTAINING DHA, ARA, CAPRIC, AND PALMITIC ACIDS SUITABLE FOR USE AS FAT BLEND IN INFANT FORMULA

by

CARLOS ANDRES ALVAREZ S

B.S., Universidad Nacional de Colombia, Colombia, 2010

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTERS OF SCIENCE

ATHENS, GEORGIA

2015

ENZYMATIC SYNTHESIS OF STRUCTURED LIPIDS CONTAINING DHA, ARA, CAPRIC, AND PALMITIC ACIDS SUITABLE FOR USE AS FAT BLEND IN INFANT FORMULA

by

CARLOS ANDRES ALVAREZ S

Major Professor:	Casimir C. Akoh
Committee:	William L. Kerr
	Fanbin Kong

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
August 2015

DEDICATION

I would like to dedicate my thesis to my guardian angel, my mother, and father for all their love and support.

ACKNOWLEDGMENTS

I would first like to thank my major professor, Dr. Casimir Akoh, for giving me advice during my entire research and graduate degree pursuit. I would also like to thank my committee members, Dr. William Kerr and Dr. Fanbin Kong, for their valuable advisement.

I would like to extend a special thank you to Jesus Antonio Jaimes and Dr. Jose Huertas from Team Foods S.A, Bogotá, Colombia for supporting this project financially and logistically.

I would especially like to acknowledge and thank my lab's research professional, Vickie Wentzel, for her constant help and support. I would also like to thank my lab co-workers Eben and Molly for their help, support, and encouragement.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Human milk is the main nutritional source for an infant. It contains lipids, carbohydrates, proteins, and micro components (such as vitamins, minerals, and hormonal factors) that nurture and help infants to build immunity against infections and other health complications (*1*). Human milk fat (HMF) counts for just 3-5% of the total composition of breast milk but contributes nearly half of the energy provided to the infants through dietary source (*2*). HMF consists mainly of triacylglycerol (TAG) molecules that are composed of a glycerol backbone containing three fatty acids (FAs) attached at three different positions (*sn*-1,2,3). Some of these FAs have a preference for a specific position on the TAG molecules. Palmitic acid is present in HMF at approximately 15.43-24.46 wt% and has a preference for the *sn*-2 position of the TAGs (57-67%) (*3*). On the other hand, unsaturated fatty acids such as oleic and linoleic are primarily found at the *sn*-1,3 positions (*4,5*). Moreover, approximately 65% of the total docosahexaenoic acid (DHA, 0.15-0.56 wt%), and 45% of the total arachidonic acid (ARA, 0.23-0.75 wt%) are present at the *sn*-2 position. FAs found in HMF provide certain health and nutritional benefits to the infants. Palmitic acid is an important source of dietary energy required for basal metabolism and body composition (*6*). DHA and ARA are critical during infancy for motor and cognitive skills development, proper brain growth, neurological reflexes, and visual acuity (*7*). Although breast milk is the preferred choice among mothers for the nutrition of their infants, breastfeeding cannot always be practiced so commercial infant formulas exist as reasonable substitutes. A similar fatty acid profile to HMF can be achieved in commercial infant formulas by combining

edible oils from vegetable and algal origins (*8*). However, the positional distribution of some of the most important FAs on the TAGs in commercial formulations differs from HMF and cannot be matched by mere blending. Palmitic acid in commercial infant formulas largely exists (about 90%) at the *sn*-1,3 positions (*9*). Furthermore, DHA and ARA in commercial formulas do not have a clear positional specificity, and their distribution in all three positions of the TAGs is almost equivalent (*10*). These differences in the placement of FAs play a crucial role in the absorption, distribution and metabolism of fat by infants (*11,12*). For palmitic acid, DHA, and ARA, a higher absorption and utilization have been observed in oils containing these FAs at the *sn*-2 position rather than randomly distributed (*13,14*).

Structured lipids (SLs) are TAGs that have been modified to change the FA composition and/or positional distribution on the glycerol backbone by chemically or enzymatically catalyzed reactions (*15*). In order to emulate both chemical composition and positional distribution of FAs in HMF, novel SLs can be synthesized to be totally or partially used in the formulation of infant formulas fat analogs (IFFAs). The ideal SL for infant formula should contain palmitic acid, DHA, and ARA mostly esterified at the *sn*-2 position and unsaturated FAs like oleic acid at the *sn*-1,3 positions to resemble HMF. It could also contain medium-chain fatty acids (MCFAs) in the outer positions of the TAGs as an excellent source of readily available energy. Numerous studies have been conducted on the production of SLs for infant formula by enzymatic esterification and/or acidolysis reactions of different substrates of vegetable origin (*16,17*). Although some of these processes successfully achieved high *sn*-2 palmitic acid levels, none of them addressed the effect of positional specificity in the absorption and utilization of DHA and ARA.

This thesis includes six chapters. The first chapter is an introduction that includes the objectives of this research. The second chapter is a literature review of related topics including HMF, infant formula, structured lipids, enzymatic interesterification, lipases, palmitic acid, capric acid, DHA and ARA, response surface methodology, and IFFAs.

The third chapter presents the synthesis of a SL containing reasonable levels of palmitic acid at the s*n*-2 position and capric acid at the s*n*-1,3 positions. The synthesis followed a two-stage enzymatic interesterification approach using a *sn*-1,3 specific immobilized lipase, as biocatalyst. In the first stage, high melting point palm stearin (HMPS) and high oleic sunflower oil (HOSO) were reacted to obtain a high *sn*-2 palmitic acid intermediate SL. In the second stage, tricaprin (TC) was interesterified with the intermediate SL to obtain the final product.

The fourth chapter presents the modification of the molecular structure of DHA and ARA-rich oils from single-cell organisms via enzymatic rearrangement of the FAs in the TAGs, so they could contain higher levels of DHA and ARA at the *sn*-2 position. The reactions were performed using a mixture of specific and non-specific immobilized lipases as biocatalyst system, and response surface methodology (RSM) was employed to model and optimize reaction conditions.

The fifth chapter presents the preparation and characterization of an IFFA containing capric and oleic acids mostly esterified at the *sn*-1,3 positions, and substantial amounts of palmitic acid, DHA, and ARA at the *sn*-2 position. The process was conducted by physically blending previously synthesized SLs from chapter three and four with canola oil (CAO) and corn oil (CO).

The sixth chapter presents the conclusions of the entire research along with possible future work.

The three objectives of this research were:

1. To synthesize a SL containing high *sn*-2 palmitic acid enriched with capric acid mostly at the *sn*-1,3 positions from HMPS, HOSO, and TC.

2. To synthesize independently two SLs containing high *sn*-2 DHA and high *sn*-2 ARA from DHA and ARA-rich single cell oils, respectively.

3. To formulate a new IFFA by physically blending SLs from objectives 1 and 2 with CAO and CO, to match as close as possible the fatty acid profile of commercial infant formulas.

REFERENCES

(1) Jensen, R.G. The lipids of human milk. CRC Press, Boca Raton, FL, **1989**.

(2) Wells, J.C.K. Nutritional considerations in infant formula design. *Semin. Neonatal.* **1996**, *1*, 19–26.

(3) López-López, A.; López-Sabater, M.C.; Campoy-Folgoso, C.; Rivero-Urgell, M.; Castellote-Bargalló, A.I. Fatty acid and *sn*-2 fatty acid composition in human milk from Granada (Spain) and in infant formulas. *Eur. J. Clin. Nutr.* **2002**, *56*,1242–1254.

(4) López-López, A.; Castellote-Bergalló, A.I.; Campoy-Folgoso, C.; Rivero-Urgel, M.; Tormo-Carnicé, R.; Infante-Pina, D.; López-Sabater, M.C. The influence of dietary palmitic acid triacylglyceride position on the fatty acid, calcium and magnesium contents of at term newborn feces. *Early Hum. Dev.* **2001**, *65*, 83-94.

(5) Martin, J-C.; Bougnoux, P.; Antoine, J-M.; Lanson, M.; Couet, C. Triacylglycerol structure of human colostrums and mature milk. *Lipids* **1993**, *28*, 637-642.

(6) De Fouw, N.J.; Kivits, G.A.A.; Quinlan, P.T.; Van Nielen, W.G.L. Absorption of isomeric, palmitic acid-containing triacylglycerols resembling human milk fat in the adult rat. *Lipids* **1994**, *29*, 765-770.

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

(8) Innis, S.M. Essential fatty acids in growth and development. *Prog. Lipid. Res.* **1991**, *30*, 39–103.

(9) Bracco, U. Effect of triglyceride structure on fat absorption. *Am. J. Clin. Nutr.* **1994**, *60*, 1002S-1009S.

(10) Myher, J.J.; Kuksis, A.; Geher, K.; Park, P.W.; Diersen-Schade, D.A. Stereospecific analysis of triacylglycerols rich in long-chain polyunsaturated fatty acids. *Lipids* **1996**, *31*, 207– 215.

(11) Lucas, A.; Quinlan, P.; Abrams, S.; Ryan, S.; Meah, S.; Lucas, P.J. Randomised controlled trial of a synthetic triglyceride milk formula for preterm infants. *Arch. Dis. Child. Fetal Neonatal* **1997**, *77*, F178-184.

(12) Ramírez, M.; Amate, L.; Gil, A. Absorption and distribution of dietary fatty acids from different sources. *Early Hum. Dev.* **2001**, *65*, S95-S101.

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

(14) Christensen, M.M.; Høy, C.E. Early dietary intervention with structured triacylglycerols containing docosahexaenoic acid. Effect on brain, liver, and adipose tissue lipids. *Lipids* **1997**, *32*, 185−191.

(15) Høy, C.E.; Xu, X. Structured Triacylglycerols. In *Structured and Modified Lipids*, Gunstone F.D., Ed.; Marcel Dekker: New York, NY, **2001**; pp. 209-239.

(16) Pande, G.; Sabir, J.S.M.; Baeshen, N.A.; Akoh, C.C. Enzymatic synthesis of extra virgin olive oil based infant formula fat analogues containing ARA and DHA: one-stage and two-stage syntheses. *J. Agric. Food Chem.* **2013**, *61*, 10590−10598.

(17) Zou, X.Q.; Huang, J.H.; Jin, Q.Z.; Liu, Y.F.; Tao, GJ.; Cheong, L.Z.; Wang, X.G. Preparation of human milk fat substitutes from palm stearin with arachidonic and docosahexaenoic acid: combination of enzymatic and physical methods. *J. Agric. Food Chem.* **2012**, *60*, 9415−9423.

CHAPTER 2

LITERATURE REVIEW

HUMAN MILK FAT

Human milk fat (HMF) is the second largest component of breast milk by concentration (3-5% in mature milk) and contributes approximately 50% of the total energy provided to the infants through dietary source (*1*). HMF contains important fatty acids (FAs) that are required as structural cell components of membrane tissues, and plays a major role in some other important physiological functions such as fat-soluble nutrient absorption and eicosanoids syntheses (*1*). HMF consists mainly of triacylglycerol molecules (TAGs) (98-99%) that are composed of a glycerol backbone containing three FAs attached at three different positions (*sn*-1,2,3) (see **Figure 2.1**). Infants absorb HMF more efficiently than milk fat from cows due to the composition and positional distribution of the FAs on the TAGs (*2*). **Table 2.1** shows the major FAs in HMF including oleic (28.30-43.83 wt%), palmitic (15.43-24.46 wt%), and stearic (4.60-8.13 wt%) acids (*3*). HMF is also a source of the essential fatty acids (EFAs) linoleic acid (10.61-25.30 wt%) and α-linolenic acid (0.41-1.68 wt%), as well as their derivatives, so called long chain polyunsaturated fatty acids (LC-PUFAs), arachidonic (ARA, 0.23-0.75 wt%) and docosahexaenoic (DHA, 0.15-0.56 wt%) acids. Some of these FAs have a preference for a specific position on the TAG molecule. Nearly 57-67% of the total palmitic acid in HMF (**Table 2.1**) is present at the *sn*-2 position of the TAGs (*3*), whereas unsaturated fatty acids such as oleic and linoleic are primarily found at the *sn*-1,3 positions (85% and 75%, respectively) (*4,5*). Furthermore, approximately 65% of total DHA and 45% of total ARA are present at the *sn*-2

position (*3*). Lipid digestion is not fully developed in the early stages of infant growth. A number of digestive enzymes are involved in the breakdown of the human milk TAGs (*6*). The positional distribution of the FAs in the TAG structure influences their breakdown and thereby the absorption of the FAs. For palmitic acid, DHA, and ARA, a higher absorption and utilization have been observed in oils containing these FAs at the *sn*-2 position rather than randomly distributed (*7,8*). HMF contains more than eighteen TAG species. The major five species are oleic-palmitic-oleic (OPO), palmitic-linoleic-oleic (PLO), oleic-oleic-oleic (OOO), oleic-linoleic-oleic (OLO), and palmitic-oleic-palmitic (POP) accounting for ~80% of the total TAGs (*9*). The diet of the mother and the stage of lactation can affect and influence the FA composition and TAGs in HMF. Different maternal intakes of FAs showed that different dietary habits greatly influenced and changed the FA composition of HMF in two groups of mothers from two different populations (*10*). Likewise, breast milk is naturally secreted with DHA and EPA but the variations in content depend on the diet followed by the mother and their metabolism (*11, 12*). According to studies for term infants, the content of LC-PUFAs in breast milk from well nourished mothers ranges between 0.1−0.7% for ARA, and 0.06−0.4% for DHA (*11, 13, 14*). HMF does not contain a significant amount of Vitamin E (less than 10 μg/g) and for that reason, is very susceptible to lipid oxidation (*15*). Additionally, the melting completion temperature of HMF is 34.7°C, which makes it completely melted at normal body temperature (about 37°C) (*7,16*).

INFANT FORMULA

Infant formulas are human milk substitutes that are intended to meet the nutritional requirements of the infants in their very first period of life (approx 0-8 months). The goal of infant formulas is to achieve as close as possible the composition present in breast milk since a

well-nourished mother's milk is considered the optimum nutrition for term infants. In the United States, commercial infant formulas meet the requirements specified by FDA and CFR (Code of Federal Regulations). Infant formulas contain a balanced formulation that include lipids, carbohydrates, proteins, vitamins, minerals, as well as nucleotides, prebiotics, carotenoids, and probiotic bacteria (*17,18*). The lipid content of infant formula is important as it provides energy and essential FAs for structure and function of cell membranes and influences physiological and metabolic processes in infants. A similar fatty acid profile to HMF is achieved in commercial infant formulas by combining edible oils from vegetable origin such as palm, rapeseed and corn with single-cell oils rich in LC-PUFAs (*18*). However, vegetable oils only contain approximately 5-15% saturated FAs at the *sn*-2 position of the TAGs, and LC-PUFAs from single-cell oils are mainly esterified at the *sn*-1,3 positions (*19,20*). **Table 2.2** shows the total and positional fatty acid composition of 11 different commercial fat blends used for infant formula (*3*). The total levels of the most relevant fatty acids present in formulas for infant nutrition are very similar to those found in HMF. Thus, commercial formulas contain predominantly oleic acid (34.34-44.69 mol%), palmitic acid (15.96-25.75 mol%), linoleic acid (8.93-17.43 mol%), and lauric acid (5.19-12.64 mol%). They also contain DHA (0.40-0.60 mol%) and ARA (0.40-0.60 mol%). Currently, preformed LC-PUFAs are added to most premium infant formulas. Nestlé and Enfamil brands claim to contain DHA and ARA nutrients found in breast milk. According to the labels on these products, both use *Crypthecodinium cohnii* and *Mortierella alpine* oils as sources of DHA and ARA, respectively. Some other formulas do not contain preformed DHA and ARA. Instead, they rely on the presence of LA and ALA for bioconversion to LC-PUFAs, such as eicosapentaenoic acid (EPA, C20:5n-3), γ-linolenic acid (GLA, C18:3n-6), DHA and ARA (*21*). In commercial infant formulas, the fraction of the total palmitic acid that is esterified at the *sn*-2

position (11.64-13.43%) is considerably lower than in HMF (57.11-67.15%). Similarly, the fraction of DHA and ARA esterified at the *sn*-2 position in commercial infant formulas (34.01-34.59% and 27.96-29.04, respectively) is lower than the corresponding FAs in HMF (56.93-64.11% and 42.22-49.75%, respectively). This difference in FAs placement on the TAGs plays a crucial role in the uptake, digestion, and metabolism of infant formulas when compared to HMF (*22*). Some formulas use modified vegetable oils in order to change their structure or the positions of the FAs in the TAG. For example, infants fed a formula with a higher *sn*-2 PA concentration (over 40%) had a higher tendency to have softer stools and less constipation problems (*23*). Constipation or hard stools are caused by calcium soaps which are generated by long chain saturated FAs (LC-SAFAs) interactions with calcium (*7, 24*). As a result of the pancreatic lipase activity, all FAs at the *sn*-1,3 positions of TAGs are hydrolyzed during digestion, in contrast to only around 20% of fatty acids at the *sn*-2 position (*25*). If LC-SAFAs are predominantly esterified at *sn*-1,3 positions, they are released as FFAs. At the alkaline pH of the intestine, saturated FFAs readily form insoluble soaps with divalent cations such as calcium that cannot be absorbed in the small intestine and are excreted as hard stools. This results in not just unavailability of both FAs and calcium for the infants but also in constipation, a consequence of the formation of soaps (*24*).

STRUCTURED LIPIDS

Structured lipids (SLs) are TAGs that have been chemically or enzymatically modified by incorporating new molecules or by rearranging the position of their original FAs. For certain applications, these novel structures can be desirable as they exhibit special biological, physical and chemical properties that affect their melting behavior, digestion, absorption, and their nutritional value (*26*). SLs can provide the desired FAs for nutrition or therapeutic purposes to

target specific functional needs, diseases, and/or metabolic conditions (*27*). SLs have a wide variety of applications in the pharmaceutical, medical and food industries. Some food applications include the production of cocoa butter substitutes, partial acylglycerols as emulsifiers, modified fish oil products, frying oils, margarines and HMF analogs (*27*). From a nutritional point of view, SLs serve two main purposes: to supply energy and to provide functional FAs such as LC-PUFAs and medium-chain fatty acids (MCFAs)(*26*). The ideal SL for infant formula should contain palmitic acid, DHA, and ARA mostly esterified at the *sn*-2 position and unsaturated FAs like oleic acid at the *sn*-1,3 positions to resemble HMF. It could also contain MCFAs in the outer positions of the TAGs as an excellent source of readily available energy. SLs can be produced by chemical or enzymatic interesterifications/acidolysis reactions. Randomized SLs can be produced by both methods. However, chemical randomization (caustic base as catalyst) is commonly preferred for industrial purposes because it is less expensive (*27*). A chemical catalyst can change the acyl chains organization into a random pattern, changing the TAGs but not the FA composition. Enzymatic catalysts are more selective and yield more specific TAGs. Many studies have been successfully performed with lipases from *Thermomyces lanuginosus*, *Mucor miehei*, and *Candida antarctica* to synthesize SLs (*28*). 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 to achieve optimum nutrition without sacrificing sensory characteristics and product stability. Betapol® (Loders Croklaan, The Netherlands) was the first SL produced by using a 1,3-specific lipase and reacting tripalmitin with oleic acid. It contained 53.5% of palmitic acid at the *sn*-2 position and 42.1 mol% of total oleic acid (*29*). The nutrition benefits of Betapol's application in infant formula were researched, and the authors (*30*) 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 positional distribution of palmitic acid may have an effect on plasma lipoprotein lipid, apolipoprotein concentrations, and metabolism (*31*).

ENZYMATIC INTERESTERIFICATION

Interesterification is a process that causes fatty acid redistribution within and among TAGs, which can lead to substantial changes in lipid functionality. Interesterification randomizes the position of the acyl chains in the glycerol backbone of TAGs changing the crystal structure, solid fat content, and melting point of fats and oils (*32*). Enzymatic interesterification (EIE) is frequently preferred over its chemical counterpart to produce high-value specialty fats. EIE occurs under milder conditions of temperature, pH, and are less likely to induce undesirable changes, especially in LC-PUFAs. It also provides a more specific positional distribution of the FAs on the TAGs when compared to chemical interesterification (*32*). EIE is accomplished using lipases, which are enzymes (biocatalysts) obtained predominantly from bacteria, yeast, and fungal sources. Lipases are differentiated from esterases in that they act only on insoluble substrates. Under appropriate experimental conditions, lipases can catalyze reversible reactions in either direction. Thus, lipases (normally associated with lipid hydrolysis) can also affect esterification, transesterification, and acidolysis. In the presence of excess water, lipases catalyze the hydrolysis of TAGs, but under water-limiting conditions, the reverse reaction (ester synthesis) can be achieved (*27*). EIE consist of three main steps: lipase activation, ester bond cleavage, and fatty acid interchange (*32*). EIE systems are composed of a continuous water-immiscible phase containing the lipid substrate and an aqueous phase containing the lipase. Lipase-catalyzed interesterifications have been extensively studied in systems using organic

solvents (mostly hexane). However, if such a process is to be used in the food industry, solvent-free systems must be developed. The type of lipase used can affect the final TAG FA composition (*33*). For example, a sn-1,3 specific lipase (i.e., Lipozyme® TL IM) acts selectively and it will cleave the FAs at the sn-1,3 positions whereas a nonspecific lipase (i.e., Novozym® 435) will cleave any of the positions resulting in a random FA distribution after re-esterification. An example of EIE reaction using a sn-1,3 specific lipase is shown in **Figure 2.2**.

LIPASES

Lipases, also known as triacylglycerol ester hydrolases (EC number 3.1.1.3), can be obtained from animal, plant, bacteria, yeast, and mold sources (*34*). Lipases are one of the most commonly used classes of enzymes in biocatalysis. Lipases catalyze the hydrolysis of TAGs to diacylglycerols (DAGs), monoacylglycerol (MAGs), FFAs, and glycerol (*35*). The reaction reverses under anhydrous conditions and the enzyme is able to synthesize new molecules by esterification, alcoholysis and transesterification (*35*). Lipases from microbial sources are more widely studied than other lipases because of their availability, easier isolation, and higher stability. They also can distinguish between enantiomers of chiral molecules (*34*). Lipases act at the oil water-interface and therefore do not strictly follow Michaelis Menton kinetics (*36*). Lipases have three-dimensional structures and their active site is buried under a short stretch of helix or hydrophobic lid. During activation at the oil-water interface, the lid opens and goes into the lipid phase and exposes the substrate to the active site (*34*). Lipases can be separated into three groups according to specificity. The first group shows no marked specificity with respect to the position of the acyl group on the glycerol molecule, or to the specific nature of the fatty acid component of the substrate. Complete breakdown of the substrate to glycerol and fatty acids occurs with nonspecific lipases. Examples of such lipases are those from *C. rugosa*, and *C. acnes*

(*34*). The second group attacks specifically at the *sn*-1,3 positions of the substrate, with mixtures of DAGs and MAGs as products. Because of the instability of intermediate 1,2-DAG, 2,3-DAG, and 2-MAG (i.e., migration of the fatty acid from the *sn*-2-position to the *sn*-1,3 positions), these lipases may catalyze the complete breakdown of the substrates. The positional specificity of the *sn*-1,3 lipases is due to steric hindrance conflict that prevents the fatty acid located at the *sn*-2-position from binding to the active sites. Most microbial lipases fall into this group. Examples include those from *A. niger*, *R. miehei*, and *T. lanuginosus* (*34*). The third group of lipases show preference for a specific fatty acid or chain length range, and are less common. The most widely studied lipase in this regard is that from *G. candidum*, which shows specificity for LC-PUFAs (*34*). Lipases are often immobilized to increase cost effectiveness and reusability (*37*). Immobilization is the conversion of enzymes from a water-soluble mobile state to a water-insoluble immobile state by the attachment of the enzyme to a solid support material (*38*). Advantages of immobilized lipases include a better performance in non-aqueous solvents compared to native enzyme formulations, an efficient recovery and separation of reaction product, reuse/recycling of enzymes, minimizing or eliminating protein contamination of products, enhanced storage and operational stability from denaturation by heat, higher catalyst productivity, and convenient and safer handling of the enzyme (*35*). Examples of hydrophobic support media used to immobilize enzymes include organic supports such as polyethylene, polypropylene, styrene, and acrylic polymers. Lipases generally retain the highest degree of activity when immobilized on hydrophobic supports (*39*). This higher activity is ascribed to increased amounts of hydrophobic substrate at the interface (*39*,*40*). Among hydrophilic supports are Duolite, Celite, silica gel, activated carbon, clay, and Sepharose (*39*). Novozym® 435 is a *Candida antarctica* lipase B (CALB) immobilized on an hydrophobic carrier (acrylic resin).

CALB is a non-specific lipase and is stable over a relatively broad pH range, especially in the alkaline pH range. The enzyme exhibits a very high degree of substrate specificity, with respect to both regio- and enantioselectivity. CALB has been used extensively in the resolution of racemic alcohols, amines, and acids, and in the preparation of optically active compounds from meso substrates. The resulting optically pure compounds are highly difficult to obtain by alternative routes and can be of great synthetic value. Likewise, CALB has been used intensively as a regio-selective catalyst in selective acylation of different carbohydrates (*35*). Lipozyme® TL IM is a *sn*-1,3 specific lipase originating from *Thermomyces lanuginosus* and immobilized on a non-compressible silica gel carrier. Lipozyme® TL IM is a highly effective catalyst for interesterification and can rearrange fatty acids preferentially, but not uniquely in the *sn*-1,3 position of TAGs. Lipozyme® TL IM exhibits a high degree of substrate selectivity allowing bulky side chains/large groups on the alcohol and acid parts of the molecules (*35*).

PALMITIC ACID

Palmitic acid is a very common saturated FA found in fats of animal and vegetable origin. Along with oleic, it is one of the largest FAs present in HMF as it counts for nearly 15-25 mol% of the total human fat composition (*3*,*13*,*14*,*41*). Palmitic acid has a preference for a specific position on the TAG molecules. Of the total palmitic acid in HMF, a very large portion (more than 60%) is esterified at the *sn*-2 position of the TAGs (*3*,*4*,*5*,*7*). When HMF is metabolized in the body, all the FAs esterified at the *sn*-1,3 positions of TAGs are hydrolyzed as a result of pancreatic lipase activity and released as FFAs. This process maintains most of the FAs at the *sn*-2 position almost intact since only 20% of them are hydrolyzed (*25*). As a consequence, the FAs at the *sn*-2 position become absorbed by the intestinal mucosa as *sn*-2 MAGs (*42*). Most FAs are better absorbed in the *sn*-2-MAG form than FFAs. This is due to the

fact that monoacylglycerols form mixed micelles with bile salts that prevent them from interacting with other molecules present in the intestine such as divalent cations, which are very reactive to generate insoluble soaps with FAs (*42*). De Fouw *et al.* (*43*) performed a study by comparing rats fed formulas with high and low *sn*-2 palmitic acid content. The study showed that the rats fed a higher *sn*-2 palmitic formula had overall higher absorption of palmitic acid than the rats fed the lower *sn*-2 palmitic formula. Another study conducted by Tomarelli *et al.* (*7*) showed that a higher palmitic acid absorption and utilization was observed in human milk rich in *sn*-2 palmitic acid when compared to formulas in which this fatty acid was mainly esterified at *sn*-1,3 positions. Another study by López-López *et al.* (*4*) was performed in 36 term infants consuming breast milk for two months (Group A, approx 66% *sn*-2 palmitic acid), commercial infant formula for two months (Group B, less than 20% *sn*-2 palmitic acid), and Group C which was fed the commercial formula from Group B for the first month and then a high *sn*-2 palmitic infant formula (approx 44% *sn*-2 palmitic acid) for the second month. After the end of the two months, they reported significantly less fecal palmitic acid in Groups A and C (which had larger intake of *sn*-2 palmitic acid for either one or two months) than Group B. The large amount of palmitic at the *sn*-2 position may improve the absorption of both fat and calcium in the infants (*5, 24, 44*). The *sn*-2 palmitic acid may also reduce the formation of calcium soaps since free palmitic acid (esterified at the outer positions of the TAGs) may be lost as calcium soaps in the feces of the infant often causing constipation problems (*7,24*). The formation of calcium soaps indicates poor palmitic acid and calcium absorption. Constipation (hard stools) is caused by calcium soaps which are generated by LC-SAFAs interactions with calcium (*7,24*). Bongers *et al.* (*23*) reported that when infants were fed a formula with a higher *sn*-2 palmitic acid concentration (over 40%), they had a tendency to have softer stools and have less constipation

than infants fed a formula with a low *sn*-2 palmitic acid concentration. Similar effects on calcium absorption with high *sn*-2 palmitic acid formulas were reported in term infants by Carnielli *et al*. (*42*). In this study, infants consuming a high *sn*-2 palmitic acid formula had significantly lower amounts of fecal calcium (43mg/kg/day) and significantly higher calcium absorption (53%) against a commercial infant formula (68mg/kg/day; 33% absorption). Calcium absorption in infants is especially important due to its critical role in bone formation and development. Constipation in infants usually occurs more in formula-fed infants than breast-fed infants. This may be due to the fact that formulas are made from vegetable oils and often contain palmitic acid at the *sn*-1,3 positions (over 85%) compared to the *sn*-2 PA in breast milk (over 60%) (*19*,*23*). Ideal infant formula fat analog would contain a large portion of palmitic acid at the *sn*-2 with unsaturated FAs such as oleic and linoleic acids occupying the *sn*-1 and *sn*-3 positions.

CAPRIC ACID

Capric acid (C10:0) is a saturated fatty acid with a chain length of 10 carbon atoms. Saturated fatty acids with 6-12 carbon atoms fall into the category of MCFAs. They are found in relatively large amounts in coconut fat, palm kernel oil, and butterfat, but also in certain SLs, parenteral nutrition formulations, and sport drinks (*45*). Capric acid has a viscosity of about 25-31 cP at 20°C and a bland odor and taste. It is also significantly stable to oxidation as a result of its saturated carbon chain (*46*). MCFAs are used to treat medical conditions such as fat absorption abnormalities in patients with cystic fibrosis (*47*). They are also considered an ideal source of energy for the growth and physiological development of newborn infants (*46*). Furthermore, it has been reported that MCFAs such as capric acid protect infants from harmful microorganisms as they exhibit antiviral and antimicrobial properties (*6*). Composition of TAGs containing MCFAs such as capric acid increases energy expenditure and decreases weight gain

in animal and human studies (*48,49*). MCFAs are metabolized through the portal system, whereas LCFAs are metabolized through the lymphatic system. As a consequence, they are more quickly absorbed by the body and not stored as fat tissue (*46*). MCFAs are more readily oxidized than LCFAs. They are not re-esterified into TAGs and they do not require chylomicron formation. Moreover, the ammonium compound carnitine is not required for transport of MCFAs into the mitochondria. Although MCFAs have more than twice the caloric density of carbohydrates and proteins, they can be absorbed and metabolized as rapidly as glucose, whereas LCFAs are metabolized more slowly. The properties that contribute to more efficient metabolism of MCFAs are lower melting point, higher solubility in water and biological fluids, and smaller molecular size when compared to LCFAs (*50*). MCFAs do not supply EFAs and can be toxic at some concentrations (*51*). Therefore, they are used in structured lipids that contain EFAs on the glycerol backbone. Capric acid is often targeted at the *sn*-1,3 positions and EFAs at the *sn*-2 position of TAGs during SL synthesis. McKenna *et al.* (*52*) reported that SLs containing MCFAs and linoleic acid bound in the TAGs are more effective for cystic fibrosis patients than safflower oil, which have about twice as much linoleic acid as most SLs. The mobility, solubility, and ease of metabolism of MCFAs were responsible for the health benefits of the SLs in these cases. McGandy and coworkers (*53*) compared the effects of MCFAs, mainly capric acid on plasma total and LDL-cholesterol levels. Eighteen physically healthy men were fed several diets, each for 4 weeks. Diets contained low-fat food items to which the experimental fats were added. It was shown that modest amounts of MCFAs in the diet have comparable effects on the plasma total and LDL-cholesterol and on TAGs concentrations. Results of two more studies (*54*,*55*), however, suggested that a mixture of MCFAs slightly increases LDL-cholesterol concentrations relative to oleic acid. No effects on HDL-cholesterol were found, whereas serum TAGs

concentrations were slightly increased. Most commercial infant formulas are enriched with lauric acid (C12:0) as a source of MCFAs in concentrations up to 13 mol% (*3*). Although lauric acid is frequently used as a MCFA for food applications, there is still disagreement within the scientific community as some researchers consider it to be at the edge of the long-chain fatty acids (LCFAs) category, and therefore its functional properties have been questioned (*56*). Instead, capric acid (C10:0) as a source of MCFAs was recommended (*49*).

DOCOSAHEXAENOIC AND ARACHIDONIC ACIDS

DHA is a LC-PUFA of the omega 3 series mainly found in fish and fish oils. ARA is a LC-PUFA of the omega 6 series mainly found in animal tissue (poultry, beef, fish). Other sources of DHA and ARA in large amounts are single-cell oils from marine algae and fungi, respectively. DSM Nutritional Products (Columbia, MD, USA) produce and commercialize high LC-PUFA oils DHASCO® and ARASCO® (see total and positional fatty acid composition in **Table 2.3**). DHASCO® is prepared by blending DHA-rich oil (50-55 mol% DHA) extracted from the unicellular algae *Crypthecodinium cohnii* with high oleic sunflower oil (HOSO). ARASCO® is prepared by blending ARA-rich oil (approx 50 mol% ARA) extracted from the unicellular fungus *Mortierella alpina* with HOSO. Alpha-linoleic acid (ALA) and eicosapentaenoic acid (EPA) are precursors of DHA in the omega-3 FA metabolic pathway. On the other hand, linoleic (LA) and gamma linoleic (GLA) acids are the precursors of ARA in the omega-6 FA metabolic pathway (*57*) (see **Figure 2.3**). Due to the high unsaturation of DHA and ARA, they are both very easily oxidized. The bioavailability of both DHA and ARA, is critical during infancy for motor and cognitive skills development, proper brain growth, sensory functions, and neurological reflexes (*17*). DHA and ARA are the predominant FAs in the structural phospholipids of the human brain and other important cell membranes and are

precursors of eicosanoids syntheses (*57*) (see **Figure 2.4**). In infants, they support nervous system development. In the case of DHA, it plays a specific function in the photoreceptor membranes of the retina, improving visual acuity (*58,59*). DHA and ARA accumulate rapidly in fetal and infant neural tissue during periods of most rapid growth and development, that is, during the last months of gestation and the first months of postnatal life (*60*). Numerous studies conducted in breastfed infants have shown that the LC-PUFAs content in HMF provides adequate DHA (0.15-0.56 wt%) and ARA (0.23-0.75 wt%) to support normal neural tissue growth (*61,62*). However, for infant formula-fed infants, reports have shown that conventional formulas, even when containing substantial amounts of ALA (0.41-1.68 wt%) and LA (10.61-25.30 wt%), which are precursors capable of endogenous synthesis of DHA and ARA, respectively, are unable to maintain postnatal LC-PUFAs levels in plasma and erythrocyte lipids compared to infants fed with human breast milk (*62,63*). Consequently, lower levels of DHA and ARA have been reported in infants fed formulas when compared to infants fed human breast milk (*62,63*). Low levels of DHA and ARA in formula-fed infants may be due to their lack of ability to desaturate and elongate ALA and LA to DHA and ARA, respectively, due to elongation and desaturation enzymes not being sufficiently active during the early stages of life to maintain tissue accumulation (*58*). Anderson *et al.* (*64*) established that preformed dietary DHA and ARA is quantitatively more effective than supplementation of ALA and LA as a source of LC-PUFAs, and therefore, adding preformed DHA and ARA to conventional infant formulas is crucial to provide levels similar to those found in HMF. Makrides *et al*. (*65*) gave one group of term infants a supplemented formula with 0.36% DHA and 0.27% GLA (precursor of ARA) and another group a formula with 1.6% ALA and neither DHA nor GLA. The group lacking DHA and GLA experienced hindered visual acuity at 4 and 6 months of age. Also, Birch

et al. (*66*) found that infants fed formula with 0.35% DHA and 0.72% ARA scored higher on the Bayley mental development index II (MDI) at 18 months than the formula fed infants with no long-chain PUFA supplementation. Jorgensen *et al.* (*60*) reported that infants fed a formula of 0.3% DHA raised DHA levels but had slightly lower levels of DHA in red blood cells than those fed breast milk containing 0.4-0.5% DHA. Koletzko *et al.* (*67*) investigated in a group of 29 premature infants if there was a correlation between birth weight and postnatal essential fatty acid status. It was found a significant positive correlation between body weight and plasma TAGs content of ARA, suggesting that during early life this FA may have a growth-promoting effect which could be related to its role as an eicosanoids precursor or to its structural function in membrane lipids. Currently, commercial infant formulas containing DHA and ARA supplementation are available in most countries and recommendations on their use have been reported by various expert bodies, including the FAO/WHO (*68*). In HMF, DHA and ARA have a preference for the *sn*-2 position of the TAG molecules (approx 60% of total DHA and 45% of total ARA) (*20*). On the other hand, the DHA and ARA from single-cell organisms used in commercial infant formulas do not display a clear positional distribution, with all the fatty acids found in all three positions almost indistinctly (*20*). DHASCO contains between 30-35% of the total DHA esterified at the *sn*-2 position, 30-40% esterified at the *sn*-3 position, and 20-30% at the *sn*-1 position. ARASCO has 25-30% of the total ARA esterified at the *sn*-2, 20-25% esterified at the *sn*-3 position, and 45-50% at the *sn*-1 position (*69*). DHASCO and ARASCO also have the unusual feature of containing significant amounts of TAGs with two or more LC-PUFAs per molecule (*20*). It has been reported that the differences in the TAG positional arrangement of DHA and ARA from dietary lipid sources such as egg yolk lipids, fish oils, and unicellular organisms, may affect their absorption, distribution and tissue uptake (*70*). Higher

levels of DHA and ARA were found in the brain of newborn rats fed with oils containing DHA and ARA at the *sn*-2 position than those fed with oils containing these but randomly distributed (*8*). Additionally, it has been reported that the presence of some LC-PUFAs, including DHA, at outer positions of the TAG molecules induce resistance to pancreatic lipase, and therefore, relatively low absorption of this FA might be expected (*71*).

RESPONSE SURFACE METHODOLOGY

Response surface methodology (RSM) is a set of mathematical and statistical procedures useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (*72*). For chemical experimentation, it enables the evaluation of the simultaneous effects that multiple parameters have on a response variable and limits the number of reactions that need to be performed (*73*). RSM can be used to suggest random combinations from the experimental design, such as reaction times, temperatures, and other factors that could affect the response. Two important models of first degree and second degree are commonly used in RSM. In general all RSM problems use either one or a combination of both of these models. In each model, the levels of each factor are independent of the levels of other factors. In order to get the most accurate model when developing the polynomial regressions, an adequate experimental design must be used to collect data. Usually it is recommended to perform screening reactions in order to have a first approximation to the reaction behavior and determine the most convenient level at which each factor is going to be tested. Once the data are collected, the Method of Least Square is used to estimate the parameters in the polynomials. The response surface analysis is performed by using the fitted surface. The response surface designs are types of designs for fitting response surface. The most common first-order designs are 2k factorial (k is the number of control variables),

Plackett–Burman, and simplex designs. The most frequently used second-order designs are the 3k factorial, central composite, and the Box–Behnken designs (*74*). The experiment is designed to estimate interaction and even quadratic effects of the variables on the response. Contour plots are the best methods to evaluate such complex relationship. Two variables are placed on the x- and y axis of the contour plot (generally the reaction parameter with the greatest effect is kept on the y-axis, the second is placed on the x-axis) and the response is presented in contour lines that separate in regions of the different levels of the response, showing "hot" and "cold" zones, according to whatever is the intent to be achieved (*73*). The contour plots help in the optimization of reactions by identifying parameter combinations that will produce a desired response. RSM designs are commonly used in industrial, biological and clinical science, social science, food science, and physical and engineering sciences for designing, formulating, developing, and optimizing new products and processes. Many studies have used RSM and contour plots to predict the optimal conditions for enzymatic synthesis of SLs. Some of these include the works of Xu *et al.* (*75*) for the production of specific structured lipids by enzymatic interesterification, Alim *et al.* (*76*) for the enzymatic *trans*esterification of fractionated rice bran oil with conjugated linoleic acid, Han *et al.* (*77*) for the synthesis of low-calorie structured lipid in a solvent-free system, Lumor *et al*. (*78*) for the enzymatic production of trans-free margarine fat, and Pina-Rodriguez *et al.* (*79*), Teichert *et al.* (*68*), Nagachinta *et al.* (*80*), Pande *et al.* (*81*), and Li *et al.* (*82*) for the enzymatic production of infant formula fat analogs.

INFANT FORMULA FAT ANALOGS

Several studies have been conducted to produce infant formula fat analogs containing high *sn*-2 palmitic acid enriched with either capric acid, DHA and/or ARA via enzymatic interesterification and acidolysis reactions. The following are the most representative: Zou *et al.*

(*83*) reported the preparation of a human milk fat substitute by blending a high *sn*-2 palmitic acid SL with sunflower, rapeseed, palm kernel, and single-cell oils. The final product contained 61.10% of total palmitic acid (23.50 mol%), 35.60% of total DHA (0.30 mol%), and 30.0% of total ARA (0.40 mol%) esterified at the *sn*-2 position. It also contained 9.60 mol% of lauric, 34.60 mol% oleic, 19.10 mol% linoleic, and 3.7 mol% of linolenic acids, mostly esterified at *sn*-1,3 positions. Pande *et al.* (*81*) reported the synthesis of infant formula fat analogs from virgin olive oil-based SLs. The final product contained 47.85% of total palmitic acid (36.69 mol%), 16.99% of total DHA (1.53 mol%), and 20.43% of total ARA (3.67 mol%) esterified at the *sn*-2 position. It also contained 43.22 mol% oleic, 6.34 mol% linoleic, and 0.47 mol% of linolenic acids, mostly esterified at *sn*-1,3 positions. Shimada *et al.* (*84*) reported the synthesis of a HMF analog by enzymatic acidolysis of tripalmitin with ARA using a *sn*-1,3 specific lipase. The product contained 28.7% of total palmitic acid (31.7 mol%) at the *sn*-2 position and 60.1% of total ARA. Sahin *et al.* (*85)* reported the production of a HMF analogs by lipase-catalyzed acidolysis of tripalmitin, stearic acid, and hazelnut oil FAs, using a *sn*-1,3 specific lipase. The HMF analogs produced contained 69.2- 76.0% palmitic acid at the *sn*-2 position. Chen *et al.* (*86*) used a three-step method to produce OPO TAGs from palm oil. The process consisted of a low temperature fractionation of palm oil FAs to separate palmitic acid, followed by ethylation and then enzymatic esterification with glycerol to produce PPP (tripalmitin). This tripalmitin was reacted with oleic acid producing OPO with 90.7% PA at the *sn*-2 position. Ilyasoglu *et al.* (*87*) produced a HMF analog by using tripalmitin, hazelnut oil FAs, and a mixture of MCFAs as substrates by lipase-catalyzed acidolysis. The total PA was 30 mol%**.** Karabulut *et al.* (*88*) obtained a HMF analog by enzymatic interesterification of a mixture including palm oil, palm kernel oil, olive oil, sunflower oil, and marine oil. The lipase used as a biocatalyst was *sn*-1,3

specific. The product contained 41.5% of total palmitic acid at the *sn*-2 position and 47.3% of total unsaturated FAs with minor amounts of DHA. Maduko *et al.* (*89*) used enzymatic interesterification with a *sn*-1,3 specific lipase to produce caprine milk infant formula analogs using tripalmitin with a vegetable oil blend. The vegetable oil blend was a mixture of coconut oil, safflower oil, and soybean oil. The resulting SLs contained *sn*-2 PA of over 60%. Zou *et al.* (*90*) reported the synthesis of a set of SLs with more than 60% of palmitic acid at the *sn*-2 position via enzymatic acidolysis of palm stearin, using a commercially available 1,3 specific lipase from *Rhizomucor miehei* (RML). Wang *et al.* (*91*), reported the production of an infant formula fat analog synthesized by lipase-catalyzed reaction of interesterified palm stearin with a blend of free fatty acids (FFAs) from rapeseed, sunflower, and palm kernel oils in a continuous packed bed reactor. The final product contained 61.6% of palmitic acid at the *sn*-2 position. Wang *et al.* (*92*) reported the synthesis of novel SLs with high content of LC-PUFAs at the *sn*-2 position by enzymatic acidolysis using a commercially available *sn*-1,3 specific lipase from *Rhizomucor miehei* (18.56% total DHA with 30.11% at *sn*-2). Iwasaki *et al.* (*93*) reported the synthesis of SLs with high *sn*-2 DHA content enriched with caprylic acid via enzymatic acidolysis of DHA single cell oil (39.01% total DHA with 36.10% at *sn*-2). Hamam and Shahidi (*94*) reported the production of high *sn*-2 DHA SLs via enzymatic acidolysis with capric acid (37.10% total DHA with 40.12% at *sn*-2). Nieto *et al.* (*95*) reported the preparation of *sn*-2 LC-PUFAs monoacylglycerols from fish oil by enzymatic hydrolysis (94.30% *sn*-2 DHA). Sahin *et al.* (*96*) used enzymatic acidolysis with tripalmitin, hazelnut oil FAs, and n-3 FA concentrate to produce a HMF analog. The SL produced contained at total PA of 45.5% with 76.6% at the *sn*-2 position and a total of EPA and DHA of 6.2% with less than 1.0% at the *sn*-2 position. Spurvey *et al.* (*97*) looked at incorporating GLA (precursor ARA) into seal blubber and menhaden oils by

enzyme-catalyzed acidolysis for use in clinical and nutritional applications. Lipase PS-30 was used as the biocatalyst. The incorporation of total GLA was 37.1% in the seal blubber SL and 39.6% GLA in the menhaden oil SL. Both SLs contained less than 10% total palmitic acid. Hamam and Shahidi (*98*) performed a study to incorporate DHA into high-laurate canola oil, Laurical 35, using AY-30 from *Candida rugosa* as a biocatalyst. The maximum total incorporation of DHA into Laurical 35 was 34.3%. The majority of DHA was esterified at the *sn*-1,3 positions of the SL. Robles *et al.* (*99*) produced a SL enriched with palmitic acid and DHA at the *sn*-2 positions and oleic acid at the *sn*-1,3 positions from tuna oil and FFAs. This process resulted in 67.0% oleic acid at the *sn*-1,3 positions, 52.1% palmitic acid at the *sn*-2 position, and 8.5% total DHA. Some other recent works include the use of either ethyl palmitate or tripalmitin with amaranth oil (*79*), stearidonic acid soybean oil (*100*), and hazelnut oil (*101*) for the production of infant formula fat analogs. Although most of these processes successfully achieved high *sn*-2 palmitic acid levels, almost none of them addressed the effect of positional distribution in the absorption and utilization of DHA and ARA in their design. Others omitted the impact of thermal behavior of the SLs on the commercial production of infant formulas and/or failed to adjust the total level of some FAs in the final composition. Furthermore, all of them lacked high reaction yields because of the nature of the substrates used (FFAs and ethyl esters), and the type of reaction conducted that promotes an excessive formation of new FFAs or their ethyl esters. The development of the present study is oriented to find reasonable solutions to these issues.

REFERENCES

(1) Wells, J.C.K. Nutritional considerations in infant formula design. *Semin. Neonatal*. **1996**, *1*, 19–26.

(2) Belavady, B. Lipid and trace element composition of human milk. *Acta. Paediatr. Scand.* **1979**, *67*, 566-571.

(3) López-López, A.; López-Sabater, M.C.; Campoy-Folgoso, C.; Rivero-Urgell, M.; Castellote-Bargalló, A.I. Fatty acid and *sn*-2 fatty acid composition in human milk from Granada (Spain) and in infant formulas. *Eur. J. Clin. Nutr.* **2002**, *56*,1242–1254.

(4) López-López, A.; Castellote-Bergalló, A.I.; Campoy-Folgoso, C.; Rivero-Urgel, M.; Tormo-Carnicé, R.; Infante-Pina, D.; López-Sabater, M.C. The influence of dietary palmitic acid triacylglyceride position on the fatty acid, calcium and magnesium contents of at term newborn feces. *Early Hum. Dev.* **2001**, *65*, 83-94.

(5) Martin, J-C.; Bougnoux, P.; Antoine, J-M.; Lanson, M.; Couet, C. Triacylglycerol structure of human colostrums and mature milk. *Lipids*. **1993**, *28*, 637-642.

(6) Francois, C.A.; Connor, S.L.; Wander, R.S.; Connor, W.E. Acute effects of dietary fatty acids on the fatty acids of human milk. *Am. J. Clin. Nutr.* **1998**, *67*, 301-308.

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

(8) Christensen, M.M.; Høy, C.E. Early dietary intervention with structured triacylglycerols containing docosahexaenoic acid. Effect on brain, liver, and adipose tissue lipids. *Lipids.* **1997**, *32*, 185−191.

(9) Morera, S.; Castellote, A.I.; Jauregui, O.; Casals, I.; López-Sabater, M. Triacylglycerol markers of mature human milk. *Eur. J. Clin. Nutr.* **2003**, *57*, 1621−1626.

(10) Peng, Y.; Zhou, T.; Wang, Q.; Liu, P.; Zhang, T.; Zetterström, R.; Strandvik, B. Fatty acid composition of diet, cord blood and breast milk in Chinese mothers with different dietary habits. *Prostaglandins Leukot. Essent. Fatty Acids.* **2009**, *81*, 325-330.

(11) Weber, N.; Mukherjee, K. D., Lipids in infant formulas and human milk fat substitutes. In *Healthful Lipids*, Akoh, C. C.; Lai, O. M., Eds. AOCS Press: Champaign, IL, **2005**; pp 607-641.

(12) Uauy, R.; Mena, P.; Rojas, C., Essential fatty acids in early life: structural and functional role. *Proc. Nutr. Soc.* **2000,** 59, 3-15.

(13) Finley, D. A.; Lönnerdal, B.; Dewey, K. G.; Grivetti, L. E., Breast milk composition: fat content and fatty acid composition in vegetarians and non-vegetarians. *Am. J. Clin. Nutr.* **1985,** 41, 787-800.

(14) Bitman, J.; Wood, D. L.; Hamosh, M.; Hamosh, P.; Mehta, N. R., Comparison of the lipid composition of breast milk from mothers of term and preterm infants. *Am. J. Clin. Nutr.* **1983,** 38, 300-312.

(15) Barbas, C.; Herrera, E. Lipid composition and vitamin E content in human colostrum and mature milk. *J. Physiol. Biochem.* **1998**, *54*, 167−173.

(16) Jensen, R.G.; Patton, S. The effect of maternal diets on the mean melting points of human milk fatty acids. *Lipids* **2000**, *35*, 1159-1161.

(17) Scott, C.E. Structured lipid: A functional and fundamental ingredient to more closely matches infant formula to breast milk. *Nutrafoods* **2010**, *9*, 7-13.

(18) Innis, S.M. Essential fatty acids in growth and development. *Prog. Lipid. Res.* **1991**, *30*, 39–103.

(19) Prosser, C.G.; Svetashev, V.I.; Vyssotski, M.V.; Lowry, D.J. Composition and distribution of fatty acids in triglycerides from goat infant formula with milk fat. *J. Dairy Sci.* **2010**, *93*, 2857-2862.

(20) Myher, J.J.; Kuksis, A.; Geher, K.; Park, P.W.; Diersen-Schade, D.A. Stereospecific analysis of triacylglycerols rich in long-chain polyunsaturated fatty acids. *Lipids* **1996**, *31*, 207– 215.

(21) Hayat, L.; Al-Sughayer, M.; Afzal, M. A comparative study of fatty acids in human breast milk and breast milk substitutes in Kuwait. *Nutr. Res.* **1999**, *19*, 827-841.

(22) Lucas, A.; Quinlan, P.; Abrams, S; Ryan, S; Meah, S; Lucas, P.J. Randomised controlled trial of a synthetic triglyceride milk formula for preterm infants. *Arch. Dis. Child. Fetal Neonatal* **1997**, *77*, F178-184.

(23) Bongers, M.E.J.; de Lorijn, F.; Reitsma, J.B.; Groeneweg, M.; Taminiau, J.A.J.M.; Benninga, M.A. The clinical effect of a new infant formula interm infants with constipation: a double-blind, randomized crossovertrial. *Nutr. J.* **2007**, *6*, 8–15.

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

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

(26) Akoh, C. C.; Kim, B. H., Structured lipids. In *Food Lipids*, 3rd ed.; Akoh, C. C.; Min, D. B., Eds. CRC Press: Boca Raton, FL, **2008**; pp 841-872.

(27) Høy, C.E.; Xu, X. Structured Triacylglycerols. In *Structured and Modified Lipids*, Gunstone F.D., Ed.; Marcel Dekker: New York, NY, **2001**; pp. 209-239.

(28) Gunstone, F. D., Why are structured lipids and new lipids sources required?. In *Structured and Modified Lipids*, Gunstone, F. D., Ed. Marcel Dekker: New York, NY, **2001**; pp 1-35.

(29) Zock, P.L.; Gerritsen, J.; Katan, M.B. Partial conservation of the *sn*-2 position of dietary triglycerides in fasting plasma lipids in humans. *Eur. J. Clin. Invest.* **1996**, *26*, 141–150.

(30) Nelson, C. M.; Innis, S. M., Plasma lipoprotein fatty acids are altered by the positional distribution of fatty acids in infant formula triacylglycerols and human milk. *Am. J. Clin. Nutr.* **1999,** 70, 62-69.

(31) O'Shea, M.; Gerritsen, J.; Mohede, I., Clinical benefits of a structured lipid (Betapol^{TM}) in infant formula. In *Handbook of Functional Lipids*, Akoh, C. C., Ed. Taylor & Francis: Boca Raton, FL, **2005**; pp. 261-278.

(32) Rousseau, D.; Marangoni, A.G. Chemical interesterification of food lipids: Theory and practice. In *Food Lipids: Chemistry, Nutrition and Biotechnology*, Akoh, C.C., Min, D.B., Eds.; Marcel Dekker, New York, **1998**, pp. 251-281.

(33) Osborn, H.T.; Akoh, C.C. Structured lipids-Novel fats with medical, nutraceutical, and food applications. *Compr. Rev. Food Sci. F.* **2002**, 3, 93-103.

(34) Weete, J.D.; Lai, O.M.; Akoh, C.C. Microbial lipases. In *Food lipids: chemistry, nutrition, and biotechnology*. 3rd ed. Akoh C.C., Min D.B. Boca Raton, FL, **2008**; pp. 767-806.

(35) Novozymes Biopharma DK A/S. Enzymes for Biocatalysis: for smarter chemical synthesis.In:*http://www.novozymes.com/en/solutions/biopharma/Brochures/Documents/20*

14-12576-01_Biocatalysis-Product-Sheet-Immobilised-Lipases-2.pdf. **2014**, accessed 20 Apr 2015.

(36) Villeneuve, P.; Foglia, T.A. Lipase specificities: potential application in bioconversions. *Inform* **1997**, *8*, 640-650.

(37) Willis, W.M.; Marangoni, A.G. Enzymatic interesterification. In Akoh CC, Min DB, editors. *Food lipids: chemistry, nutrition, and biotechnology*. 3rd ed. Boca Raton, FL, **2008**; pp. 807-840.

(38) Mustranta, A.; Forssell, P.; Poutanen, K. Applications of immobilized lipases to transesterification and esterification reactions in non-aqueous systems. *Enzyme Microb. Technol.* **1993**, *15*, 133-139.

(39) Malcata, F.X.; Reyes, H.R.; Garcia, H.S.; Hill, Jr. C.G.; Amundson, C.H. Immobilized lipase reactors for modification of fats and oils - a review. *J. Am. Oil Chem. Soc.* **1990**, *67*, 890-910.

(40) Malcata, F.X.; Reyes, H.R.; Garcia, H.S.; Hill, Jr. C.G.; Amundson, C.H. Kinetics and mechanisms catalyzed by immobilized lipases. *Enzyme Microb. Technol.* **1992**, *14*, 426-446.

(41) Fidler, N.T.; Sauerwals, A.P.; Demmelmair, H.; Koletzko, B. Docosahexaenoic acid transfer into human milk after dietary supplementation: a randomized clinical trial. *J. Lipid Res.* **2000**, *41*, 1376-1383.

(42) Carnielli, V.P.; Luijendijk, I.H.T.; Van Goudoever, J.B.; Sulkers, E.J.; Boerlage, A.A.; Degenhart, H.J.; Sauer, P.J.J. Structural position and amount of palmitic acid in infant formulas: effects on fat, fatty acid, and mineral balance. J. *Pediatr. Gastroenterol. Nutr.***1996**, *23*, 553-560.

(43) De Fouw, N.J.; Kivits, G.A.A.; Quinlan, P.T.; Van Nielen, W.G.L. Absorption of isomeric, palmitic acid-containing triacylglycerols resembling human milk fat in the adult rat. *Lipids*. **1994**, *29*, 765-770.

(44) Jandacek, R. Commercial applications of fats in foods. In *Fatty acids in foods and their health implications*, 3rd ed. Chow, C.K., Ed.; CRC: Boca Raton, FL, **2008**; pp. 473-492.

(45) Megremis, C.J. Medium-chain triglycerides: a nonconventional fat. *Food Technol.* **1991**, *45*,108-114.

(46) Babayan, V.K. Medium chain triglycerides. In *Dietary fat requirements in health and development*. Babayan V.K., Beare-Rogers, J. Eds. American Oil Chemists' Society Press, Champaign, IL, **1988**; pp. 73-86.

(47) Kennedy, J.P. Structured lipids: fats of the future. *Food Technol.* **1991**, *45*, 76-83.

(48) St-Onge, M.P.; Jones, P.J.H. Physiological effects of medium-chain triglycerides: potential agents in the prevention of obesity. *J. Nutr.* **2002**, *132*, 329-332.

(49) St-Onge, M.P.; Ross, R.; Pearsons, W.D.; Jones, P.J.H. Medium-chain triglycerides increase energy expenditure and decrease adiposity in overweight men. *Obesity Res.* **2003**, *11*, 395-402.

(50) Bach, A.C.; Babayan, V.K. Medium-chain triglycerides: an update. *Am. J. Clin. Nutr.* **1982**, *36*, 950-962.

(51) Akoh, C.C. Structured lipids-enzymatic approach. *Inform* **1995**, *6*, 1055-60.

(52) McKenna, M.C.; Hubbard, V.S.; Bieri, J.G. Linoleic acid absorption from lipid supplements in patients with cystic fribrosis with pancreatic insufficiency and in control subjects. *J. Pediatr. Gastroenterol. Nutr.* **1985**, *4*, 45-51.

(53) McGandy, R.B.; Hegsted, D.M.; Myers, M.L. Use of semisynthetic fats in determining effects of specific dietary fatty acids on serum lipids in man. *Am. J. Clin. Nutr.* **1970**, *23*, 1288-1298.

(54) Cater, N.B.; Heller, H.J.; Denke, M.A. Comparison of the effects of medium-chain fatty triacylglycerols, palm oil, and high oleic sunflower oil on plasma triacylglycerol fatty acids and lipid and lipoprotein concentration in humans. *Am. J. Clin. Nutr.* **1997**, *65*, 41-45.

(55) Temme, E.H.; Mensink, R.P.; Hornstra, G. Effects medium-chain fatty acids (MCFA), myristic acid, and oleic acid on serum lipoproteins in healthy subjects. *J. Lipid Res.* **1997**, *38*,1746-1754.

(56) St-Onge, M.P.; Mayrsohn, B.; O'Keeffe, M.; Kissileff, H.R.; Choudhury, A.R.; Laferrère, B. Impact of medium and long chain triglycerides consumption on appetite and food intake in overweight men. *Eur. J. Clin. Nutr.* **2014**, *68*, 1134-1140.

(57) Lee, S; Gura, K.M.; Kim, S.; Arsenault, D.A.; Bistrian, B.R.; Puder, M. Current clinical applications of omega-6 and omega-3 fatty acids. *Nutr. Clin. Pract.* **2006**, *21*, 323-341.

(58) Uauy, R.; Hoffman, D.R.; Mena, P.; Llanos, A.; Birch, E.E. Term infant studies of DHA and ARA supplementation on neurodevelopment: Results of randomized controlled trials. *J. Pediatr.* **2003**, *143*, S17-S25.

(59) Innis, S.M. Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. *J. Pediatr.* **2003**, *143*, 1-8.

(60) Jorgensen, M.H.; Holmer, G.; Lund, P.; Hernell, O.; Michaelson, K.F. Effect of formula supplemented with docosahexaenoic acid and (gamma) – linolenic acid on fatty acid

status and visual acuity in term infants. *J. Pediatr. Gastroenterol. Nutr.* **1998**, *26*, 412-421.

(61) Martinez, M. Tissue levels of polyunsaturated fatty acids during early human development. *J. Paediatr.* **1992**, *120*, 129–138.

(62) Carlson, S.E.; Rhodes, P.G.; Ferguson, M.G. Docosahexanoic acid status of preterm infants at birth and following feeding with human milk or formula. *Am. J. Clin. Nutr.* **1986**, *44*, 798 – 804.

(63) Pita, M.L.; DeLucchi, C.; Faus, M.J.; Gil, A. Changes in the fatty acid profiles of red blood cell membrane phospholipids in human neonates during the first month of life. *Clin. Physiol. Biochem.* **1990**, *8*, 91-100.

(64) Anderson, G.J.; Connor, W.E.; Corliss, J.D. Docosahexaenoic acid is the preferred dietary n-3 fatty acid for the development of the brain and retina. *Pediatr. Res.* **1990**, *27*, 89-97.

(65) Makrides, M.; Neumann M.A.; Gibson, R.A. Is dietary docosahexaenoic acid essential for term infants?. *Lipids* **1996**, *31*, 115-119.

(66) Birch, E.E.; Garfield, S.; Hoffman D.R.; Uauy, R.; Birch D.G. A randomized controlled trial of early dietary supply of long-chain polyunsaturated fatty acids and mental development in term infants. *Dev. Med. Child Neurol.* **2000**, *42*, 174-181.

(67) Koletzko, B.; Braun, M. Arachidonic acid and early human growth: is there a relation?, *Ann. Nutr. Metab.* **1991**, *35*, 128-131.

(68) Teichert, S.A.; Akoh, C.C. Stearidonic acid soybean oil enriched with palmitic acid at the *sn*-2 position by enzymatic interesterification for use as human milk fat analogues. *J. Agric. Food Chem.* **2011**, *59*, 5692-5701.

(69) Food Standards Australia-New Zealand. *DHASCO and ARASCO oils as sources of long-chain polyunsaturated fatty acids in infant formula*. Food Standards Australia New Zealand, Canberra, **2003**; pp**.** 3-54.

(70) Ramírez, M.; Amate, L.; Gil, A. Absorption and distribution of dietary fatty acids from different sources. *Early Hum. Dev.* **2001**, *65*, S95-S101.

(71) Bottino, N.R.; Vandenburg, G.A.; Raiser, R. Resistance of certain long-chain polyunsaturated fatty acids of marine oils to pancreatic lipase hydrolysis. *Lipids* **1967**, *2*, 489 – 493.

(72) Montgomery, D.C. Design and Analysis of Experiments: Response surface method and designs. John Wiley & Sons, Hoboken, NJ, USA, **2005**.

(73) Anderson, M.J.; Whitcomb, P.J. In *RSM Simplified: Optimizing Processes Using Response Surface Methods for Design of Experiments*, Productivity Press: New York, NY, **2005**; pp. 77-144.

(74) Khuri, A. I., Mukhopadhyay, S. Response surface methodology, *WIREs Comput Stat* **2010**, *2*, 128-149.

(75) Xu, X. Enzymatic production of structured lipids: process reactions and acyl migration. *Inform* **2000**, *11*, 1121-1131.

(76) Alim, M. A.; Lee, J. H.; Akoh, C. C.; Choi, M. S.; Jeon, M. S.; Shin, J. A.; Lee, K. T. Enzymatic *trans*esterification of fractionated rice bran oil with conjugated linoleic acid: Optimization by response surface methodology, *LWT- Food Sci. Technol.* **2008**, *41*, 764–770.

(77) Han, L.; Xu, Z.; Huang, J.; Meng, Z.; Liu, Y.; Wang, X. Enzymatically catalyzed synthesis of low-calorie structured lipid in a solvent-free system: optimization by response surface methodology. *J. Agric. Food Chem.* **2011**, *59*, 12635-12642.

(78) Lumor, S. E.; Jones, K. C.; Ashby, R.; Strahan, G. D.; Kim, B. H.; Lee, G. C.; Shaw, J. F.; Kays, S. E.; Chang, S. W.; Foglia, T. A.; Akoh, C. C. Synthesis and characterization of canola oil-stearic acid based *trans*-free structured lipids for possible margarine application. *J. Agric. Food Chem.* **2007**, *55*, 10692-10702.

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

(80) Nagachinta, S.; Akoh, C.C. Enrichment of palm olein with long chain polyunsaturated fatty acids by enzymatic acidolysis. *LWT- Food Sci. Technol.* **2012**, *46*, 29-35.

(81) Pande, G.; Sabir, J.S.M.; Baeshen, N.A.; Akoh, C.C. Enzymatic synthesis of extra virgin olive oil based infant formula fat analogues containing ARA and DHA: one-stage and two-stage syntheses. *J. Agric. Food Chem.* **2013**, *61*, 10590−10598.

(82) Li, R.; Pande, G.; Sabir, J.S.M.; Baeshen, N.A.; Akoh, C.C. Enrichment of refined olive oil with palmitic and docosahexaenoic acids to produce a human milk fat analogue. *J. Am. Oil Chem. Soc.* **2014**, *91*, 1377–1385.

(83) Zou, X.Q.; Huang, J.H.; Jin, Q.Z.; Liu, Y.F.; Tao, GJ.; Cheong, L.Z.; Wang, X.G. Preparation of human milk fat substitutes from palm stearin with arachidonic and docosahexaenoic acid: combination of enzymatic and physical methods. *J. Agric. Food Chem.* **2012**, *60*, 9415−9423.

(84) Shimada, Y.; Nagao, T.; Hamasaki, Y.; Akimoto, K.; Sugihara, A.; Fujikawa, S.;Komemushi, S.; Tominaga, Y. Enzymatic synthesis of structured lipid containing arachidonic and palmitic acids. J. *Am. Oil Chem. Soc.* **2000**, *77*, 89-93.

(85) Sahin, N.; Akoh, C.C.; Karaali, A. Lipase-catalyzed acidolysis of tripalmitin with hazelnut oil fatty acids and stearic acid to produce human milk fat substitutes. *J. Agric. Food Chem.* **2005**, *53*, 5779-5783.

(86) Chen, M-L.; Vali, S.R.; Lin, J-Y.; Ju, Y-H. Synthesis of the structured lipid 1,3-dioleoyl-2-palmitolylglycerol from palm oil. *J. Am. Oil Chem. Soc.* **2004**, *81*, 525-532.

(87) Ilyasoglu, H.; Gultekin-Ozguven, M.; Ozcelik, B. Production of human milk fat substitute with medium-chain fatty acids by lipase-catalyzed acidolysis: Optimization by response surface methodology. *LWT-Food Sci. Technol.* **2011**, *44*, 999-1004.

(88) Karabulut, I.; Turan, S.; Vural, H.; Kayahan, M. Human milk fat substitute produced by enzymatic interesterification of vegetable oil blend. *Food Technol. Biotechnol.* **2007**, *45*, 434-438.

(89) Maduko, C.O.; Akoh, C.C.; Park, Y.W. Enzymatic interesterification of tripalmitin with vegetable oil blends for formulation of caprine milk infant formula analogs. *J. Dairy Sci.* **2007**, *90*, 594-601.

(90) Zou, X.; Huang, J.; Jin, Q.; Guo, Z.; Liu, Y.; Cheong, L.; Xu, X.; Wang, X. Characterization and oxidative stability of human milk fat substitutes enzymatically produced from palm stearin. *J. Am. Oil Chem. Soc.* **2013**, *91*, 481–495.

(91) Wang, X.; Huang, J.; Jin, Q.; Song, Z.; Liu, Y.; Zou, X. Lipase-catalyzed synthesis of human milk fat substitutes from palm stearin in a continuous packed bed reactor. *J. Am. Oil Chem. Soc.* **2012**, *89*, 1463–1472.

(92) Wang, J.; Wang, X.D.; Zhao, X.Y.; Liu, X.; Dong, T.; Wua, F.A. From microalgae oil to produce novel structured triacylglycerols enriched with unsaturated fatty acids. *Bioresour. Technol.* **2015**, *184*, 405-414.

(93) Iwasaki, Y.; Han, J.J.; Narita, M.; Rosu, R.; Yamane, T. Enzymatic synthesis of structured lipids from single cell oil of high docosahexaenoic acid content. *J. Am. Oil Chem. Soc.* **1999**, *75*, 563-569.

(94) Hamam, F.; Shahidi, F. Synthesis of structured Lipids via acidolysis of docosahexaenoic acid single cell oil (DHASCO) with capric acid. *J. Agric. Food. Chem.* **2004**, *52*, 2900-2906.

(95) Nieto, S.; Gutiérrez, J.; Sanhueza, J.; Valenzuela, A. Preparation of *sn*-2 long-chain polyunsaturated monoacylglycerols from fish oil by hydrolysis with a stereo-specific lipase from *mucor miehei*. *Grasas Aceites* **1999**, *50*, 111-113.

(96) Sahin, N.; Akoh, C.C.; Karaali, A. Human milk fat substitutes containing omega-3 fatty acids. *J. Agric. Food Chem.* **2006**, *54*, 3717-3722.

(97) Spurvey, S.A.; Namal Senanayake, S.P.J.; Shahidi, F. Enzyme-assisted acidolysis of menhaden and seal blubber oils with γ-linolenic acid. *J. Am. Oil Chem. Soc.* **2001**, *78*, 1105-1112.

(98) Hamam, F.; Shahidi, F. Synthesis of structured lipids containing medium-chain and omega-3 fatty acids. *J. Agric. Food Chem.* **2006**, *54*, 4390-4396.

(99) Robles, A.; Jiménez, M.J.; Esteban, L.; González, P.A.; Martín, L.; Rodríguez, A.; Molina, E. Enzymatic production of human milk fat substitutes containing palmitic acid and docosahexaenoic acids at *sn*-2 position and oleic acid at sn-1,3 positions. *LWT-Food Sci.Technol.* **2011**, *44*, 1986-1992.

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

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

Table 2.1 Total and positional fatty acid composition (wt%) of human milk fat (HMF) (*3*)

Fatty acid	HMF (n=40)	
	Total	*sn*-2[a]
C8:0	0.11-0.36	nd[b]
C10:0	0.85-3.08	5.32-7.11
C12:0	4.05-9.35	17.18-30.65
C14:0	3.60-9.13	40.11-60.87
C16:0	15.43-24.46	57.11-67.15
C18:0	4.60-8.13	7.13-10.12
C18:1n9	28.30-43.83	8.06-15.13
C18:2n6	10.61-25.30	15.22-25.16
C18:3n3	0.41-1.68	16.18-20.65
C20:4n6	0.23-0.75	42.22-49.75
C22:6n3	0.15-0.56	56.93-64.11

[a] *sn*-2 represents the relative (%) of a particular fatty acid based on the total amount of the same fatty acid in the sample
[b] *nd*, not detected

Table 2.2 Total and positional fatty acid composition (wt%) of commercial infant formulas (*3*)

Fatty acid	Infant formulas (n=11)	
	Total	*sn*-2[a]
C8:0	0.51-1.20	1.25-3.11
C10:0	0.74-1.24	27.11-32.15
C12:0	5.19-12.64	30.01-42.56
C14:0	3.06-5.91	24.76-30.16
C16:0	17.96-25.75	11.64-13.43
C18:0	2.05-4.72	13.24-15.11
C18:1n9	34.34-44.69	27.74-39.83
C18:2n6	8.93-17.43	39.93-49.21
C18:3n3	0.67-2.83	48.14-50.21
C20:4n6	0.40-0.60	27.96-29.04
C22:6n3	0.40-0.60	34.01-34.59

[a] *sn*-2 represents the relative (%) of a particular fatty acid based on the total amount of the same fatty acid in the sample

Table 2.3 Total and positional fatty acid composition (mol%) of single-cell oils[a,b,c]

Fatty acid	DHASCO[d]		ARASCO[e]	
	Total	*sn*-2	Total	*sn*-2
C10:0	1.26 ± 0.01	nd[f]	nd	nd
C12:0	5.05 ± 0.04	17.58 ± 0.66	nd	nd
C14:0	10.83 ± 0.08	11.32 ± 0.25	0.29 ± 0.01	49.35 ± 0.83
C16:0	9.43 ± 0.06	1.19 ± 0.14	9.28 ± 0.35	3.79 ± 0.55
C16:1n7	2.73 ± 0.03	nd	nd	nd
C18:0	0.10 ± 0.02	nd	4.30 ± 0.17	3.36 ± 0.74
C18:1n9	22.38 ± 0.04	62.81 ± 0.08	22.42 ± 0.09	49.41 ± 0.71
C18:2n6	0.34 ± 0.02	43.02 ± 2.02	7.47 ± 0.52	62.49 ± 0.89
C18:3n3	nd	nd	3.46 ± 0.64	41.72 ± 0.17
C20:3n6	nd	nd	3.70 ± 0.08	25.80 ± 0.85
C20:3n3	nd	nd	1.30 ± 0.10	72.02 ± 0.35
C20:4n6	nd	nd	47.79 ± 1.40	28.50 ± 0.54
C22:6n3	47.88 ± 0.18	34.30 ± 0.29	nd	nd

[a] Each value is the mean of triplicates ± standard deviation
[b] Trace amounts of C16:1, C17:0, C17:1, C20:0 were too small to be considered
[c] *sn*-2 represents the relative percentage (%) of a particular fatty acid based on the total amount of the same fatty acid in the sample
[d] *DHASCO*, single-cell oil rich in DHA from *C. cohnii*
[e] *ARASCO*, single-cell oil rich in ARA from *M. alpina*
[f] *nd*, not detectable

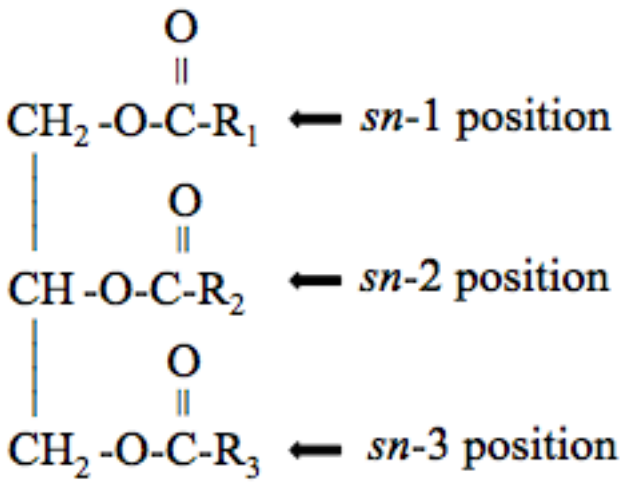


Figure 2.1 Typical triacylglycerol structure with R groups as fatty acids.

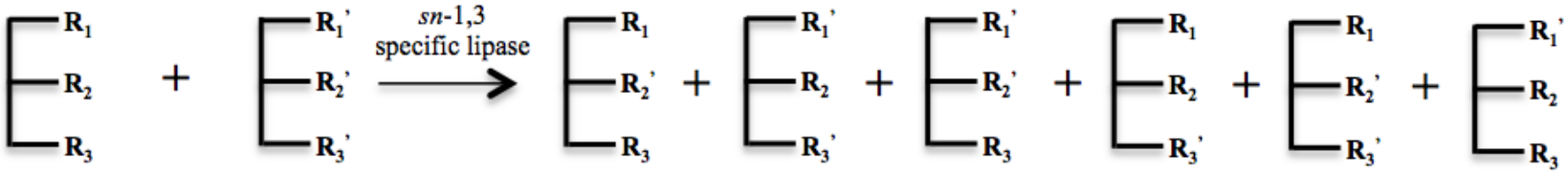


Figure 2.2 Enzymatic interesterification of triacylglycerol molecules for the production of structured lipids using *sn*-1,3 specific lipase.

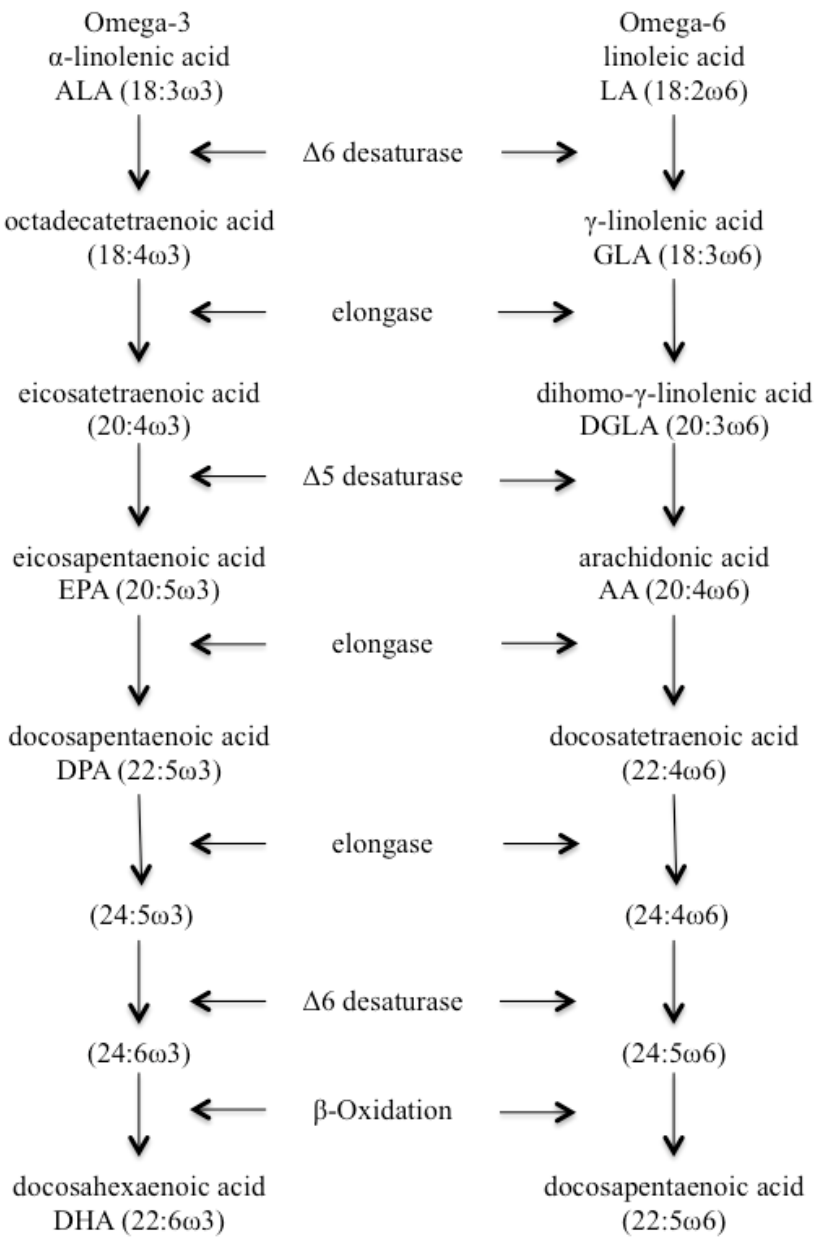


Figure 2.3 Pathway of metabolism and synthesis of omega-3 and omega-6 LC-PUFAs. Adapted and modified from Lee *et al* (*57*).

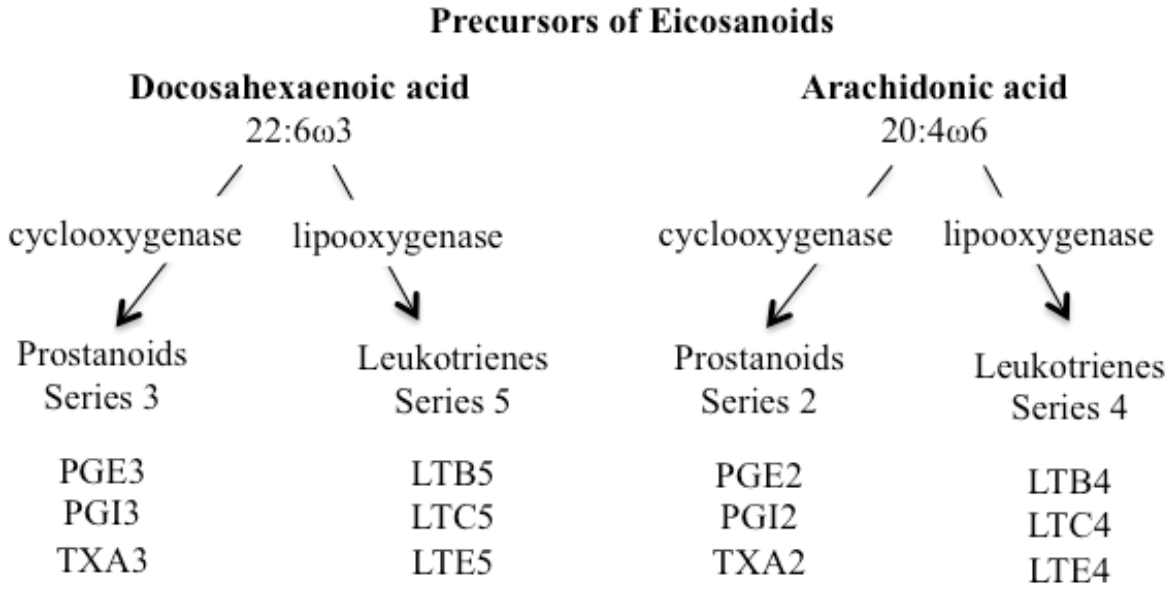


Figure 2.4 Eicosanoids synthesis from docosahexaenoic acid and arachidonic acid. Adapted from Lee *et al.* (*57*).

CHAPTER 3

ENZYMATIC SYNTHESIS OF INFANT FORMULA FAT ANALOG ENRICHED WITH CAPRIC ACID[1]

[1] Álvarez, C.A. and C.C. Akoh. 2015. *Journal of the American Oil Chemists' Society*. 92:1003-1014.

ABSTRACT

A structured lipid (SL) with substantial amount of palmitic acid at the *sn*-2 position and enriched with capric acid (C), was produced in two enzymatic interesterification stages by using immobilized lipase, Lipozyme® TL IM (Novozymes North America Inc., Franklinton, NC). The substrates for the reactions were high melting point palm stearin (HMPS), high oleic sunflower oil (HOSO) and tricaprin (TC). The SL was characterized for total and positional fatty acid (FA) profiles, triacylglycerol (TAG) molecular species, free fatty acid content, melting and crystallization profiles. The final SL contained 20.13 mol% of total palmitic acid from which nearly 40% was located at the *sn*-2 position. The total capric acid content was 21.22 mol%, mostly at the *sn*-1 and *sn*-3 positions. The predominant TAGs in the SL were OPO, POP and CLC. The melting completion and crystallization onset temperatures of the SL were 27.7°C and 6.1°C, respectively. The yield for the overall reaction was 90 wt%. This SL might be totally or partially used in commercial fat blends for infant formula.

Keywords: Structured lipids, Infant formula, Enzymatic interesterification, Capric acid, Palm stearin

INTRODUCTION

The value of the composition of maternal breast milk is widely recognized. Human milk is a complex mixture of lipids, carbohydrates, proteins, vitamins, minerals as well as growth and hormonal factors that not only nurture, but also protect infants from infections and certain chronic diseases (*1*). Lipids are the second largest component of breast milk by concentration (3-5% in mature milk). They provide essential fatty acids (EFAs) required as structural cell components of membrane tissues and count for approximately 50% of the total energy used by infants. Lipids are also necessary for physiological functions such as fat-soluble nutrient

absorption and eicosanoids syntheses (*2*). Although breast milk is the preferred choice among mothers for the nutrition of their infants, breastfeeding cannot always be practiced so commercial infant formulas exist as reasonable substitutes. Over the years, infant formulas have become more sophisticated regarding nutrient additions which naturally appear in human breast milk, including prebiotic oligosaccharides, nucleotides, carotenoids and probiotic bacteria. However, lipids as present in human breast milk have not been fully matched because of their distinctive molecular structure, which differentiates them from other common sources of lipids found in nature (*3*). A similar fatty acid profile to human milk fat (HMF) can be achieved in commercial infant formulas by combining edible oils from palm, rapeseed and corn with algal oils (*4*). However, the specific placement of palmitic acid on the TAGs in HMF differs from most commercial formulations and cannot be matched by mere blending of vegetable oils. While in HMF, palmitic acid largely exists (>60%) on the *sn*-2 position of the TAGs and the *sn*-1,3 positions are commonly occupied by oleic and linoleic acids, in most infant formulas palmitic acid exists largely (about 90%) in the *sn*-1,3 positions (*5*). This difference in palmitic acid placement on the TAGs plays a crucial role in the uptake, digestion, and metabolism of milk fat for infants (*6*). A higher palmitic acid absorption and utilization have been observed in human milk rich in *sn*-2 palmitic acid when compared to formulas in which this fatty acid was mainly esterified at *sn*-1,3 positions (*7*).

TAGs that have been chemically or enzymatically modified by incorporating new molecules or by rearranging the position of their original FAs are called structured lipids (SLs). For certain applications, these novel structures can be desirable as they exhibit special properties that affect their melting behavior, digestion, absorption, and their nutritional value. Some applications of these SLs include the production of cocoa butter substitutes, partial acylglycerols

as emulsifiers, modified fish oil products, margarines and HMF analogs (*8*). The ideal SL for infant formula must contain palmitic acid mostly esterified at the *sn*-2 position and unsaturated FAs at the *sn*-1,3 positions to resemble HMF (*9*). Betapol® (Loders Croklaan, The Netherlands) was the first HMF analog produced by using a 1,3-specific lipase and reacting tripalmitin with oleic acid. It contained 53.5% of palmitic acid at the *sn*-2 position and 42.1 mol% of total oleic acid (*10*). Following the Betapol® development, numerous studies have been conducted over the past years on the production of infant formula fat analogs by enzymatic esterification and/or acidolysis reactions. Recently, Zou *et al*. (*11*) reported the synthesis of a set of SLs with more than 60% of palmitic acid at the *sn*-2 position via enzymatic acidolysis of palm stearin, using a commercially available 1,3 specific lipase from *Rhizomucor miehei* (RML). Wang *et al.* (*12*), reported the production of an infant formula fat analog synthesized by lipase-catalyzed reaction of interesterified palm stearin with a blend of free fatty acids (FFAs) from rapeseed, sunflower, and palm kernel oils in a continuous packed bed reactor. The final product contained 61.6% of palmitic acid at the *sn*-2 position. Some other recent works include the use of either ethyl palmitate or tripalmitin with stearidonic acid soybean oil (*13*), hazelnut oil (*14*), extra virgin olive oil (*15*), and amaranth oil (*16*). Although Betapol® and the other products successfully achieved high *sn*-2 palmitic acid levels, all of them lacked high reaction yields because of the nature of the substrates used (FFAs and ethyl esters), and the type of reaction conducted that promotes an excessive formation of new FFAs or their ethyl esters. Common yields for acidolysis reactions in SL production for different applications can range from 55 to 70 wt% (*15-17*).

In the past decades, TAGs rich in medium-chain fatty acids (so called MCTs) have received considerable attention because of their distinctive metabolic pathways for digestion and

absorption when compared to long-chain triacylglycerols (LCTs). MCTs may not be assimilated via lymphatic transport like LCTs, but rather absorbed via portal transport to provide quick energy to the infants. MCTs are broken down almost immediately by enzymes in the saliva and gastric juices so pancreatic enzymes for digestion are barely required (*18*). Furthermore, it has been reported that medium-chain fatty acids (MCFAs) such as capric acid protect infants from harmful microorganisms as they exhibit antiviral and antimicrobial properties (*19*). Most commercial infant formulas are enriched with lauric acid (C12:0) as a source of MCFAs in concentrations up to 13 mol% (*20*). Although lauric acid is frequently used as a MCFA for food applications, there is still disagreement within the scientific community as some researchers consider it to be at the edge of the long-chain fatty acids (LCFAs) category, and therefore its functional properties have been questioned (*21*). Instead, capric acid (C10:0) as a source of MCFAs was recommended (*22*).

The objective of this study was to synthesize a SL at a high reaction yield that contained reasonable levels of palmitic acid at the s*n*-2 position and capric acid at the s*n*-1,3 positions. To achieve this, a two-step enzymatic interesterification approach was followed using immobilized lipase, Lipozyme® TL IM, as biocatalyst. In the first stage, high melting point palm stearin (HMPS) and high oleic sunflower oil (HOSO) were reacted to obtain a high *sn*-2 palmitic acid intermediate SL. In the second stage, tricaprin (TC) was interesterified with the intermediate SL to obtain the final product. With this SL, we propose an alternative to the fat blends currently used for the production of commercial infant formulas.

MATERIALS AND METHODS

Materials.

HMPS was provided by Team Foods S.A (Bogotá, Colombia) and HOSO was purchased under the commercial brand Frymax Sun Supreme from Stratas Foods® (Memphis, TN). The immobilized lipase, Lipozyme® TL IM (*sn*-1,3 specific *Thermomyces lanuginosus lipase*), was obtained from Novozymes North America Inc. (Franklinton, NC). The specific activity of Lipozyme® TL IM was 250 IUN/g at pH 6-8 and temperature of 50-75°C, where IUN stands for interesterification units novo, as defined by the manufacturer (*23*). The Lipid standards, Supelco 37 Component FAME mix, and 2-oleoylglycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). TC and pentadecanoic acid (C15:0) were obtained from TCI America (Portland, OR). The TAG standard mix (GLC-437) was provided by Nu-chek Prep, Inc. (Elysian, MN). Other solvents and chemicals were purchased from Fischer Scientific (Norcross, GA) and Sigma-Aldrich Chemical Co.

SL Synthesis.

The SL was synthesized in two enzymatic interesterification stages as shown in **Figure 3.1**. In the first stage, HMPS and HOSO were blended and reacted in the presence of Lipozyme® TL IM. This enzyme, which is a *sn*-1,3 specific lipase, was used with the aim of increasing the amount of palmitic acid at the *sn*-2 position, predominantly in the form of oleic-palmitic-oleic (OPO) type of TAG. For the second stage, the intermediate SL obtained in stage 1 was blended and reacted with TC in a second interesterification reaction, with the aim of incorporating capric acid into the TAG structure while conserving the palmitic acid at the *sn*-2 position. In order to establish the optimal substrate molar ratio and reaction time for both the intermediate SL in stage 1 and the final product in stage 2, a set of experiments were conducted at small scale (mg), and

samples were analyzed for total and positional fatty acid profiles. Once the reaction conditions were defined, scale-up reactions of the intermediate and final SLs were performed to determine the reproducibility of the results and to obtain enough product for further analysis of TAG molecular species, melting and crystallization profiles, free fatty acid assay, and reaction yield.

Reaction Procedure.

All the enzymatic reactions at small scale (mg) for stage 1 and 2 were performed following a modified version of the Teichert *et al.* method (*13*). Stage 1: Two hundred milligrams of blend (depending on substrate molar ratio tested) were weighed into a labeled teflon-lined test tube and 3 mL of *n*-hexane was added to reach a concentration of 0.06 g/mL. 10% by weight of the lipase, Lipozyme® TL IM, was used as the biocatalyst. The reaction was carried out at 60°C in a water bath while shaking at 200 rpm. After reaction, the product was filtered two times through a sodium sulfate column to remove both water and enzyme, and excess FFAs were removed by neutralization (as described below). The substrate molar ratios tested (HMPS:HOSO) were 2:3, 1:2, 1:3 and 1:4. The molar ratios were chosen taken into consideration that total palmitic acid in the SL should not exceed 30 mol% based on the average levels of this fatty acid in HMF (see **Table 3.1** discussion section). The resulting SLs were named SL1-1, SL1-2, SL1-3 and SL1-4, respectively. Reactions were performed independently at 4,8,12 and 24 h to determine the time at which conversion rates (in terms of *sn*-2 palmitic acid content) were the highest for each substrate molar ratio tested. Finally, SLs were compared to each other and the best product was scaled-up for further characterization and selected as the substrate for the following stage. Stage 2: The same procedure as in stage 1, but in this case the substrate molar ratios tested (SL1:TC) were 2.8:0.5, 2:0.5, 1.5:0.5 and 1.2:0.5. The molar ratios were chosen taken into consideration that total capric acid in the SL should not exceed 30 mol%. The resulting SLs were named SL2-

1, SL2-2, SL2-3, SL2-4, respectively. Reactions were performed independently at 4,8,12 and 24 h to determine the time at which conversion rates (in terms of capric acid incorporation and high *sn*-2 palmitic acid content) were the highest for each substrate molar ratio tested. SLs were compared to each other and the best product was scaled-up and selected as the final SL for further analysis and discussion.

The scale-up reaction for both stage 1 and 2 followed the Pande *et al*. method (*15*). Four hundred grams of substrate blend (at the defined substrate molar ratio) were weighed into a stirred tank reactor without solvent. 10% by weight of the lipase, Lipozyme® TL IM, was used as the biocatalyst. For this procedure the use of *n*-hexane was not recommended for safety and environmental reasons, but the tank stirrer at 200 rpm created the necessary turbulence to keep the mixture in continuous contact with the enzyme. The reaction was carried out at 60°C. After reaction, the product was filtered two times through a sodium sulfate column to remove both water and enzyme, and excess FFAs were removed by short-path distillation.

Removal of FFAs.

For small scale reactions, the FFAs were removed through deacidification by alkaline extraction method (*24*) with minor modifications. Once filtered over a sodium sulfate column, the SL (dissolved in *n*-hexane) was mixed with 3 mL of neutralized 95% ethyl alcohol and 2 ml of 1% phenolphthalein solution. Then, the sample was titrated with 0.5 N NaOH in 20% ethanol. The aqueous layer was discarded while the hexane layer was collected and once again passed through anhydrous sodium sulfate. The solvent was removed by nitrogen sparging. Finally, the SL was store at -20°C until analysis.

For the scale-up reactions, the FFAs were removed through short-path distillation (SPD) as reported previously (*15*). Once filtered over a sodium sulfate column, the SL was fed into a

KDL-4 (UIC Inc., Joliet, IL, USA) system under the following conditions: feed holding temperature of 65 °C, feeding rate of approximately 100 mL/h, evaporator heating temperature of 185 °C, condenser cooling temperature of 20−25 °C, roller speed of 200 rpm, and vacuum of <13.33 Pa. SL was passed through SPD twice to obtain FFAs of <0.1%.

Determination of Fatty Acid Profiles.

The substrates, namely HMPS, HOSO, TC, and the SLs were converted to FA methyl esters (FAME) following AOAC Official Method 996.01, Section E (*25*) with minor modifications. 0.1 g of sample was weighed into Teflon-lined test tubes and 0.20 mL internal standard (C15:0, 20 mg/mL in hexane) was added and dried under nitrogen to remove solvent. 2 mL of 0.5 N NaOH in methanol was added and heated to 100 °C for 10 min for saponification of free fatty acids. The sample was cooled under tap water and 2 mL BF_3 in methanol was added followed by vortexing for 1 min. Again, the sample was heated at 100°C for 10 min for methylation and then cooled under tap water. To stop the reaction and extract the FAMEs, 2 mL of hexane and 2 mL of saturated NaCl solution were added. The sample was vortexed for 2 min and then centrifuged at 1000 rpm for 5 min to separate the organic layer from the aqueous layer. The organic layer was passed through an anhydrous sodium sulfate column into a GC vial for analysis. The FAME external standard used was Supelco 37 component FAME mix and was run in parallel with the samples.

Positional Analysis.

The pancreatic lipase-catalyzed *sn*-2 positional analysis of substrates and products was performed following a modified version of the Luddy *et al*. method (*26*). 100 mg of sample (extracted and dried TAG) was placed into Teflon-lined test tubes. 2 mL of 1.0 M Tris-HCl buffer (pH=8), 0.5 mL of 0.05% sodium cholate solution, and 2.2% calcium chloride solution

were added, and the mixture was vortexed for 2 min to emulsify. Then, 40 mg of pancreatic lipase was added, and the mixture was vortexed for 1 min and incubated in a water bath at 40°C for 3 min while shaking at 200 rpm. The sample was vortexed again for 2 min. To stop the reaction and extract the hydrolyzed TAGs, 1 mL of 6 N HCl and 4 mL of diethyl ether were added. The sample was vortexed for 2 min and centrifuged at 1000 rpm for 3 min. The upper layer, containing the lipid components, was filtered through an anhydrous sodium sulfate column. The sample was concentrated under nitrogen until approximately one-third of the original volume was left. A mixture of hexane, diethyl ether, and formic acid (60:40:1.6, v/v/v) was used as the mobile phase after equilibration in the TLC tanks for approximately 30 min. The concentrated sample was spotted onto an activated silica gel G dried TLC plate and placed into the tank. 2-Oleyglycerol was spotted as the standard and run in parallel with the sample for identification of the 2-monoacylglycerol (2-MAG) band. The plate was sprayed with 0.2% 2,7 dichlorofluorescein in methanol and visualized under UV light. The 2-MAG band was scrapped off and converted to FAME (as described above). The determination of the fatty acid profile at the *sn*-2 position was quantified by GC analysis. The fraction of the FA that is esterified at the *sn*-2 position (out of the total content of that FA in the sample) was calculated using the following equation:

FA *sn*-2 (%)=[100*total FA at *sn*-2 (mol%)]/[3* total FA in sample (mol%)] (Eq. 1)

GC Analysis.

Fatty acid compositions of all samples were analyzed as FAME following the method of Pande *et al*. (*24*) with a Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA), and using a Supelco SP-2560, 100 m x 25 mm x 0.2 μm column. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. The injection volume was 1 μL and a split ratio

of 20:1 was used. Detection was with a flame ionization detector at 300°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 25 min. All samples were analyzed in triplicate and average values reported.

Triacylglycerol (TAG) Molecular Species.

TAG species were identified with HPLC (Agilent Technologies 1260 Infinity, Santa Clara, CA) equipped with an evaporative light scattering detector (ELSD) Sedex 85 (Richard Scientific, Novato, CA). The reverse phase column was a Beckman Ultrasphere® C18, 5 μm, 4 mm x 250 mm with the temperature set at 30°C. The sample was dissolved in chloroform with a final concentration of 5 mg/mL. 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 65 to 95% solvent B in 55 min followed by a 10 min post-run at 65% B. The drift tube temperature was set at 70°C, pressure at 3.0 bar and gain at 8. 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. Relative percent (%) of peak areas of the TAGs identified were reported as the average of three determinations.

Melting and Crystallization Profiles.

The melting and crystallization profiles were determined using a differential scanning calorimeter DSC 204 F1 Phoenix (NETZSCH Instruments North America, Burlington, MA) following AOCS Official Method Cj 1-94 (*27*). First, 8-12 mg of the sample was weighed into an aluminum pan and hermetically sealed. Sample was 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 profile), and held for 30 min and finally heated to 80°C at 10°C/min (for melting profile). Nitrogen was used as protective gas (purge). All samples were analyzed in triplicate and average values reported.

Free Fatty Acid Assay and Reaction Yield.

Free fatty acid content (FFA%) was determined following a modified version of the AOCS Official Method Ca 5a-40 (*27*) for refined and crude vegetable oils. 0.3 to 6.0 g of oil were weighed in an Erlenmeyer flask according to the range of free fatty acids expected in the sample by using the pre-established values in the method. 10 ml of neutralized 95% ethyl alcohol and 2 ml of 1% phenolphthalein solution were added, and the mixture was heated to 70°C for 3 min to keep the oil in solution. The sample was then titrated with a standardized 0.25 N NaOH solution until the appearance of the first permanent pink color indicating the end of the reaction. The volume of standardized sodium hydroxide was recorded for further calculations. All samples were analyzed in triplicate and average values were reported as percent of oleic acid.

The reaction yield of the final SL obtained in stage 2 was calculated using the following equation:

$$\text{Yield}=[1-(\text{FFA}\%_{\text{final SL}}/100)] \quad \text{(Eq. 2)}$$

Where $\text{FFA}\%_{\text{final SL}}$ corresponds to the combined FFA% of SLs in stage 1 and stage 2.

Statistical Analysis.

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

RESULTS AND DISCUSSION

Total and Positional Fatty Acid Profiles.

Table 3.1 shows the common fatty acid composition in HMF and some commercial fat blends used for infant formula as reported by López-López *et al*. (*20*). The major fatty acids in HMF are oleic (28.30-43.83 wt%), palmitic (15.43-24.46 wt%), linoleic (10.61-25.30 wt%), and stearic (4.60-8.13 wt%) acids. Commercial infant formulas achieve similar fatty acid compositions to those found in human milk by combining edible oils from vegetable origin. However, in these formulas the fraction of the total palmitic acid that is esterified at the *sn*-2 position (11.64-13.43%) is considerably lower than in HMF (57.11-67.15%). The total and positional fatty acid profiles of the substrates used for stage 1 and stage 2 are shown in **Table 3.2**. HMPS is an edible fat rich in tripalmitin (PPP>30%) obtained by the fractionation process of palm stearin, in which it is separated from a softer oleic fraction rich in POP type of TAGs (also known as PMF) (*28*). The major FA in HMPS is palmitic acid (70.98 mol%) from which 28.77% is esterified at the *sn*-2 position. Other important FAs in HMPS include oleic (17.77 mol%), and stearic (5.61 mol%) acids. HOSO contains 74.08 mol% oleic, 9.59 mol% linoleic, and 7.12 mol% palmitic acids. TC, a pure substance, contains more than 98 mol% capric acid.

Table 3.3 compares the SLs obtained in stage 1 for each substrate molar ratio tested. The SLs correspond to the reaction time at which *sn*-2 palmitic acid was the highest among independent experiments performed at 4, 8, 12 and 24 h. The major FA in all the SLs was oleic acid (59.01-71.73 mol%). This high content can be directly attributed to the high molar ratio of HOSO used as a substrate for all the SLs (>60 mol%). SL1-1, SL1-2, SL1-3, SL1-4 had 26.40, 25.14, 18.37, and 18.51 mol% total palmitic acid, respectively. Compared to the total palmitic composition of both HMF and infant formulas (**see Table 3.1**), all the SLs were reasonably

within the range. However, it was SL1-2 which showed the highest fraction of its palmitic acid esterified at the *sn*-2 position (35.21%), when compared to the values obtained in SL1-1 (31.42%), SL1-3 (32.56%), and SL1-4 (33.16%). TAGs containing *sn*-2 palmitic acid are preferred in HMF analogues as it helps in fat digestion and absorption. A number of digestive enzymes are involved in the breakdown of the human milk TAGs. As a result of the pancreatic lipase activity, all FAs in the *sn*-1,3 positions of TAGs are hydrolyzed during digestion, in contrast to only around 20% of fatty acids in the *sn*-2 position (*29*). If palmitic acid is predominantly esterified at *sn*-1,3 positions, it is released as free palmitic acid. At the alkaline pH of the intestine, free palmitic acid readily forms insoluble soaps with divalent cations such as calcium that cannot be absorbed in the small intestine and are excreted as hard stools. This results in not just unavailability of both palmitic acid and calcium for the infants but also in constipation problems (*30*). Although Lipozyme® TL IM lipase is a *sn*-1,3 specific enzyme, a considerable amount of palmitic acid was esterified at the *sn*-2 position of TAGs, which influenced the final levels found in all the SLs. This may be attributed to acyl migration. Acyl migration is considered a side reaction involving migration of acyl groups from *sn*-1,3 to *sn*-2 positions and vice versa (*31*). It mainly occurs due to the presence of diacylglycerols (DAGs) and monoacylglycerols (MAGs), which are intermediate products of enzymatic syntheses of lipids (*32*). Neither DAGs nor MAGs were analyzed in the current study. Interesterification reaction comprises two steps. First, the original TAGs are hydrolyzed into DAGs and MAGs and then fatty acid are re-esterified in other positions to form novel TAGs. Acyl migration can occur between these two steps. Furthermore, it has been found that acyl migration can be also influenced by a number of factors including temperature, degree of unsaturation of FAs, reaction time, FFA%, presence of water, and carrier of the enzyme (*33*). Acyl migration is mostly

considered an undesirable phenomenon that affects the conversion rates and the product yield in enzymatic reactions of lipids, but it this particular case it was desirable since it contributed to the *sn*-2 palmitic acid increase in the final SLs. As SL1-2 was synthesized using a molar ratio 1:2 (HMPS:HOSO), which is intermediate between the ratios used for SL1-1 and SL1-4, it is plausible that there exists a maximum value where the substrate ratio affected acyl migration the most. Because of the highest content of palmitic acid at the *sn*-2 position, and its relative lower levels of total oleic acid (66.85 mol%), SL1-2 was selected as the substrate for the stage 2 reaction.

Table 3.4 compares the SLs obtained in stage 2 for each substrate molar ratio tested. The SLs correspond to the reaction time at which capric acid incorporation was the highest (while conserving the *sn*-2 palmitic acid content achieved in stage 1) among independent experiments performed at 4, 8, 12 and 24 h. The major FA in all the SLs was oleic acid (52.98-61.85 mol%). SL2-1, SL2-2, SL2-3, and SL2-4 had 23.37, 25.12, 21.34, and 20.13 mol% total palmitic acid, respectively. The fraction of palmitic acid esterified at the *sn*-2 position in SL2-1 (38.33%), SL2-2 (38.87%), SL2-3 (38.93%), and SL2-4 (37.57%) did not show significant difference at $P \leq 0.05$. On the other hand, the amount of capric acid incorporated in SL2-1, SL2-2, SL2-3, and SL2-4 was 8.23, 11.15, 18.68, and 21.22 mol%, respectively. The levels of major FAs such as capric and oleic acid at the *sn*-2 position in all the SLs tended to remain stable during the enzymatic synthesis when compared to the values found in the physical blends of substrates before the reaction. However, in the case of palmitic acid, there was a slight increase in the fraction esterified at the *sn*-2 position, as it went from 35.21 % (level in SL1-2 from stage 1, used as substrate) to nearly 38% in the SLs of stage 2. Again, this may be attributed to acyl migration where palmitic acid displaced other fatty acids from the *sn*-2 position. TAGs rich in MCFAs

such as capric acid, have received considerable attention because of their distinctive metabolic pathways for digestion and absorption when compared to LCTs. MCTs oils are not assimilated via lymphatic transport like LCTs, but rather absorbed via portal transport to provide quick energy to the infants (*18*). Among the SLs synthesized, SL2-4 was considered the best option for a possible infant formula fat analog because of its high fraction of palmitic acid esterified at the *sn*-2 position (~38%), its relatively low content of oleic acid (52.98 mol%), and the total amount of capric acid incorporated (21.22 mol%). Whereas *sn*-2 palmitic acid level of SL2-4 appeared to be below that found in HMF (see **Table 3.1**), it still seems to be a better alternative for a human fat analog than the fats currently used in infant formulas (37.57% against 13.43%) and other edible oil-based SLs recently reported by Teichert *et al.* (*13*), Turan *et al.* (*14*), and Pina-Rodríguez *et al.* (*16*), who achieved 32.85%, 35.17%, and 29.09%, respectively (described as relative percentage of total palmitic acid). Although SL2-4 contained somewhat higher levels of total oleic acid compared to HMF (28.30-43.83 mol%), commercial infant formulas (34.34-44.69 mol%), and the SLs reported by Pande *et al.* (37.10-43.22 mol%) (*15*), Zou *et al.* (32.57-34.15 mol%) (*11*), and Wang *et al.* (34.10 mol%) (*12*), it can be physically blended with other vegetable oils in adequate proportions in order to adjust the oleic acid content, while still maintaining the desired amount of *sn*-2 palmitic acid and total capric acid in the final product. Neither HMF nor commercial formulas contain relevant amounts of capric acid as a source of MCFAs. Instead, they provided around 17 wt% of combined lauric and myristic acids, but none of them will provide the metabolic and physiologic effects of capric acid as MCFA. SL2-4 could be an alternative to provide these important fatty acids to the infants.

TAG Molecular Species.

The TAG molecular species and their relative content in substrates and SLs of stages 1 and 2 are shown in **Table 3.5**. The fatty acids in the TAG species analyzed are not in any specific order on the glycerol backbone. HMPS showed a mixture of 8 different types of TAGs. These species correspond to those concentrated originally from palm oil by fractionation process (*28*). The main TAGs present in HMPS were PPP (43.01%), POP (30.81), OPO (7.20%) and PPSt (7.28%). HOSO, which is a liquid oil, contained mostly OOO (70.73%), OPO (11.94%), and StOO (7.53%) type of TAGs. TC is a pure substance containing above 99% of tricaprin (CCC). For stage 1, PB1-2 represents the physical blend of substrates to produce SL1-2 (intermediate SL) before the enzymatic reaction occurs. This blend exhibited a more complex set of TAGs since the species found in HMPS and HOSO were combined at the established ratio. Major TAGs found in PB1-2 include OOO (48.02%), PPP (13.31%), OPO (10.67%) and POP (9.83%). As shown in SL1-2, it was found that the amount of OOO and PPP after the reaction decreased to 15.93% and 1.84%, respectively. On the other hand, the amount of OPO (a combination of *sn*-OPO and *sn*-POO) increased to 34.63% and POP increased to 22.97%. These values can be attributed to the *sn*-1,3 specific-type of interaction between OOO (from HOSO) with PPP and PPSt (from HMPS) to produce OPO and POP. In this case, OPO formation was favored over POP by using a molar ratio higher in OOO (1:2, HMPS:HOSO). Once SL1-2 was blended with TC in stage 2 (see PB2-4, **Table 3.5**), the TAG species found were CCC (20.57%), OOO (12.40%), OPO (27.75%), and POP (18.49%). After the reaction, a novel set of TAGs was synthesized as a result of the interesterification of CCC with all the pre-existing TAGs in the intermediate SL. As shown in SL2-4, the new species include CLC (11.13%), COC (7.63%), CPC (4.50%), OCO (3.46%), and OPC (2.00%), whereas CCC was almost totally depleted. The

levels of OOO (10.42%), OPO (28.06%) and POP (18.79%) remain reasonably the same before and after the enzymatic incorporation of capric acid. Other minor species present include OLO (3.58%), and PLO (2.63%). There was no evidence of unreacted PPP.

The major TAG molecular species found in HMF are OPO (17.56-42.44%), POL (9.24-38.15%), OOO (1.61-11.96) and OLO (1.64-10.18%) (*34*). SL2-4 contained all the TAGs of HMF within its levels except for POL, which fell below the range. Similar results were found by Pande *et al.* (*15*), who reported the enzymatic synthesis of extra virgin olive oil-based SLs with POL levels between 2.13 and 6.03%. This can be explained by the poor levels of linoleic acid present in both substrates used for the production of SL1-2, which predominantly contained oleic and palmitic acids. The metabolic fate of TAGs from dietary fat during digestion and absorption is determined by the position of the fatty acids on the glycerol backbone and their chain length (*15*). Compared to OOP, OPO is better metabolized and absorbed in infants (*35*). Since the fraction of total palmitic acid esterified at the *sn*-2 position in SL2-4 increased up to nearly 40%, and OOO decreased from an initial value of 48.02% in PB1-2 to 10.42%, it is plausible to assume that SL2-4 might contain higher amounts of OPO than OOP. TAG profile greatly influences the physical properties of the SLs. There are four major categories for TAG species depending on the degree of unsaturation, namely, SSS (trisaturated), UUU (triunsaturated), SUU (monosaturated-diunsaturated), and SUS (disaturated-monounsaturated). When the values of final SL2-4 are compared with PB1-2 before the reactions, it was found that SSS decreased from 15.68 to 5.33%, and UUU decreased from 54.07 to 14.00%. On the other hand SUU increased from 16.49 to 38.75%, and SUS increased from 13.76% to 41.92% as a result of the formation of the symmetric species of OPO, POP, and COC.

Melting and Crystallization Profiles.

In the crystal state, TAG molecules adopt different arrangements in relation to their neighbors to optimize intramolecular and intermolecular interactions and accomplish an efficient close-packing. These arrangements, so called polymorphs, determine the melting properties of fats and oils. There are 3 main types of polymorphs depending on their physical stability: α (unstable, lowest melting point), β' (metastable, intermediate melting point), and β (stable, highest melting point) (*36*). The occurrence of one or more polymorphs in a single fat or oil is highly influenced by the chain length and the degree of unsaturation of the FAs, which define the symmetry of the TAGs structures. In general, fats with high content of long-chain saturated fatty acids have higher melting points than those containing short and medium chains, and/or unsaturated fatty acids (*37*).

The melting profiles of substrates and SLs of stages 1 and 2 are shown in **Figure 3.2**. HMPS, which contained 50.28% of SSS type of TAGs (mostly PPP and PPSt), had a melting completion temperature (T_{mc}) of 61.6°C. On the other hand HOSO, which is liquid oil with 79.39% of UUU (mostly OOO), was completely melted at 0.6°C. Although TC contained nearly 99% of SSS (tricaprin), its T_{mc} of 41.3°C is considerably lower than that found for HMPS because of the chain length of the FAs. In both, stages 1 and 2, the melting temperature of the SLs decreased when compared to the physical blend of substrates before the reactions. For SL1-2, T_{mc} went from 54.8 to 34.2°C, and for SL2-4 went from 57.6 to 27.7°C. This behavior is highly correlated with the loss of SSS and the formation of SUS and SUU type of TAGs during the enzymatic interesterifications. Both, HMF and oil blends used for commercial formulas are completely melted at normal body temperature (about 37°C) (*7*). SL2-4 had a T_{mc} under this value, which may be helpful in the formulation of products with a proper consistency and texture

for the infants. On the contrary, HMF analogs with high T_{mc} are difficult to handle, to transport and to combine with other non-lipidic ingredients in a formulation. Such is the case of the final SLs synthesize by Pande *et al.* (*15*), which had high levels of PPP type of TAGs (up to 10.32%) and consequently, high T_{mc} values (up to 42°C). Additionally, the crystallization onset temperature (T_{co}) for HMPS, HOSO and TC were 36.3, -12.4, and 3.3°C, respectively (see **Figure 3.3**). T_{co} for SL1-2 decreased from 26.6 to 15.0°C, while SL2-4 decreased from 12.9 to 6.1°C (before and after the reactions). The complexity and wide range of TAG species in SLs resulted in multiple crystallization peaks rather than a sharp event as in TC for example, which is made of a simple homogeneous TAG.

Upscaling, Free Fatty Acid Assay, and Reaction Yield.

In order to evaluate the reaction yield of the final SL synthesized, scaled-up reactions of SL1-2 (stage 1) and SL2-4 (stage 2) were performed in a stirred tank reactor without solvent. The objective of this test was to determine the free fatty acid development in the SLs as the enzymatic reactions took place. **Figure 3.4** shows the change in FFA% (expressed as oleic acid) in SLs as a function of time. SL1-2 exhibited a high rate of FFA formation in the first 8 h of reaction as it rose from 0.16 to 5.08 wt%. After this, it continued to increase gradually until it approached the equilibrium at 24 h (6.10 wt%). The reaction time established for SL1-2 in small scale was also 24 h as the conversion of the SL at this point proved to be the highest among the range tested. In stage 2, SL2-4 also showed a high rate of FFA formation until it reached a peak at 8 h (4.00 wt%), and then it slightly dropped to find the equilibrium at 24 h (3.04 wt%). The reaction time established for SL1-2 in small scale was 8 h (higher incorporation of capric acid). In this case the SL synthesis was finished before the system approached the equilibrium (24 h).

The environment for lipase-catalyzed interesterification involves water. The water

content in a reaction system is the determining factor as to whether the reaction equilibrium will be towards hydrolysis or ester synthesis. If the water content increases in the system, the FFA content will increase (along with DAGs) and as a consequence, the yield of the new TAG products will decrease (*38,39*). Although most reactions require water content of <1% for effective interesterification, too low water activity prevents all reactions from occurring because lipases need a certain amount of water to remain hydrated, which is essential for enzymatic activity (*40,41*). Both, SL1-2 and SL2-4 produced in the stirred tank reactor were consistent with the physical and chemical properties obtained on small scale. Excess FFAs were removed from SL1-2 and SL2-4 by short-path distillation to <0.1 wt%. The combined loss (expressed as %FFAs in SL) was 10 wt% and therefore, the overall reaction yield was nearly 0.9 (90 wt%). This result is considerable higher compared to the common reaction yields observed for acidolysis reactions in SL production for different applications, such as 70 wt% (*15*), 60 wt% (*16*), and 55 wt% (*17*). A higher product yield implies less operating expenses and higher return, making the SL somewhat more economically feasible for industrial purposes.

CONCLUSION

For those health conditions where infants cannot be breastfed, commercial formulas are the most convenient alternative. However, the differences in stereospecific structure of TAGs in vegetable oils used in infant formula relative to those in HMF lead to lower energy use and less calcium absorption by formula-fed infants. SLs than contain palmitic acid predominantly at the *sn*-2 position, and which are also enriched with capric acid as a source of MCFAs can be used in the formulation of a new generation of commercial formulas, that may fit closer to the nutritional needs of the infants and enhance their physiological and metabolic functions. Therefore, SL2-4 may be suitable for use in commercial infant formulas as HMF analog as it was synthesized at a

relatively high product yield (90 wt%), and it contained substantial levels of palmitic acid at the *sn*-2 position and capric acid as a source of MCFAs for proper growth and development of the infants.

REFERENCES

(1) Jensen, R.G. The lipids of human milk. CRC Press, Boca Raton, FL, **1989**.

(2) Wells, J.C.K. Nutritional considerations in infant formula design. *Semin. Neonatal.* **1996**, *1*, 19–26.

(3) Scott, C.E. Structured lipid: A functional and fundamental ingredient to more closely matches infant formula to breast milk. *Nutrafoods* **2010**, *9*, 7-13.

(4) Innis, S.M. Essential fatty acids in growth and development. *Prog. Lipid. Res.* **1991**, *30*, 39–103.

(5) Bracco, U. Effect of triglyceride structure on fat absorption. *Am. J. Clin. Nutr.* **1994**, *60*, 1002S-1009S.

(6) Lucas, A.; Quinlan, P.; Abrams, S; Ryan, S; Meah, S; Lucas, P.J. Randomised controlled trial of a synthetic triglyceride milk formula for preterm infants. *Arch. Dis. Child. Fetal Neonatal* **1997**, *77*, F178-184.

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

(8) Høy, C.E.; Xu, X. Structured Triacylglycerols. In *Structured and Modified Lipids*, Gunstone F.D., Ed.; Marcel Dekker: New York, NY, **2001**; pp. 209-239.

(9) Akoh, C.C.; Kim, B.H. Structured Lipids. In *Food Lipids*. Akoh, C.C., Min, D.B., Eds.; CRC Press: Boca Raton, FL, **2008**; pp. 841-872.

(10) Zock, P.L.; Gerritsen, J.; Katan, M.B. Partial conservation of the *sn*-2 position of dietary triglycerides in fasting plasma lipids in humans. *Eur. J. Clin. Invest.* **1996**, *26*, 141–150.

(11) Zou, X.; Huang, J.; Jin, Q.; Guo, Z.; Liu, Y.; Cheong, L.; Xu, X.; Wang, X. Characterization and oxidative stability of human milk fat substitutes enzymatically produced from palm stearin. *J. Am. Oil Chem. Soc.* **2013**, *91*, 481–495.

(12) Wang, X.; Huang, J.; Jin, Q.; Song, Z.; Liu, Y.; Zou, X. Lipase-catalyzed synthesis of human milk fat substitutes from palm stearin in a continuous packed bed reactor. *J. Am. Oil Chem. Soc.* **2012**, *89*, 1463–1472.

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

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

(15) Pande, G.; Sabir, J.S.M.; Baeshen, N.A.; Akoh, C.C. Enzymatic synthesis of extra virgin olive oil based infant formula fat analogues containing ARA and DHA: one-stage and two-stage syntheses. *J. Agric. Food Chem.* **2013**, *61*, 10590−10598.

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

(17) Ifeduba, E.A.; Akoh, C.C. Chemoenzymatic method for producing stearidonic acid concentrates from stearidonic acid soybean oil. *J. Am. Oil Chem. Soc.* **2013**, *90*, 1011–1022.

(18) Babayan, V.K. Medium chain triglycerides. In Babayan VK, Beare-Rogers J (eds) *Dietary fat requirements in health and development*. American Oil Chemists' Society Press, Champaign, IL, **1988**; pp. 73-86.

(19) Francois, C.A.; Connor, S.L.; Wander, R.S.; Connor, W.E. Acute effects of dietary fatty acids on the fatty acids of human milk. *Am. J. Clin. Nutr.* **1998**, *67*, 301-308.

(20) López-López, A.; López-Sabater, M.C.; Campoy-Folgoso, C.; Rivero-Urgell, M. Castellote-Bargalló, A.I. Fatty acid and *sn-2* fatty acid composition in human milk from Granada (Spain) and in infant formulas. *Eur. J. Clin. Nutr.* **2002**, *56*, 1242–1254.

(21) St-Onge, M.P.; Mayrsohn, B.; O'Keeffe, M.; Kissileff, H.R.; Choudhury, A.R.; Laferrère, B. Impact of medium and long chain triglycerides consumption on appetite and food intake in overweight men. *Eur. J. Clin. Nutr.* **2014**, *68*, 1134-1140.

(22) St-Onge, M.P.; Ross, R.; Parsons, W.D.; Jones, P.J. Medium-chain triglycerides increase energy expenditure and decrease adiposity in overweight men. *Obes. Res.* **2003**, *11*, 395-402.

(23) Novozymes Biopharma DK A/S. Enzymes for Biocatalysis: for smarter chemical synthesis. In *http://www.novozymes.com/en/solutions/biopharma/Brochures/Documents/2014-12576-01_Biocatalysis-Product-Sheet-Immobilised-Lipases-2.pdf*. **2014,** accessed 20 Apr 2015.

(24) Pande, G.; Sabir, J.S.M.; Baeshen, N.A.; Akoh, C.C. Synthesis of infant formula fat analogs enriched with DHA from extra virgin olive oil and tripalmitin. *J. Am. Oil Chem. Soc.* **2013**, *90*, 1311–1318.

(25) *Official Methods of Analysis of AOAC International*, 19th ed.; AOAC International: Rockville, MD, **2012**; Official Method 996.01.

(26) Luddy, F.E.; Bardford, R.A.; Herb, S.F., Magidman, P.; Riemenschneider, R.W. Pancreatic lipase hydrolysis of triacylglycerides as a semi-micro technique. *J. Am. Oil Chem. Soc.* **1964**, *41*, 639–696.

(27) *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 6th ed.; AOCS: Champaign, IL, **2011**; Method Cj 1-94.

(28) Son, J.M.; Lee, K.T.; Akoh, C.C.; Kim, M.R.; Kim, M.J.; Lee, J.H. Optimization of tripalmitin-rich fractionation from palm stearin by response surface methodology. *J. Sci. Food Agric.* **2010**, *90*, 1520–1526.

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

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

(31) Yang, T.; Fruekilde, M.B.; Xu, X. Suppression of acyl migration in enzymatic production of structured lipids through temperature programming. *Food Chem.* **2005**, *92*, 101−107.

(32) Xu, X. Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review. *Eur. J. Lipid Sci. Technol.* **2000**, *102*, 287−303.

(33) Xu, X.; Skands, A.R.H.; Høy, C.E.; Mu, H.; Balchen, S.; Adler-Nissen, J. Production of specific-structured lipids by enzymatic interesterification: Elucidation of acyl migration by response surface design. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1179−1186.

(34) Morera, S.; Castellote, A.I.; Jauregui, O.; Casals, I.; López-Sabater, M.C. Triacylglycerol markers of mature human milk. *Eur. J. Clin. Nutr.* **2003**, *57*, 1621−1626.

(35) Mu, H.; Høy, C.E. The digestion of dietary triacyglycerols: Review. *Prog. Lipid Res.* **2004**, *43*, 105−133.

(36) Sato, K. Polymorphism in Fats and Oils In *Bailey's Industrial Oil and Fat Products*. John Wiley & Sons, Inc. Hiroshima, Japan, **2005**.

(37) Strayer, D. Food fats and oils. In *Publication of Institute of Shortening and Edible Oils*. Washington DC, United States, **2006**; pp. 1-37.

(38) Bornaz, S.; Fanni, J.; Parmentier, M. Limit of the solid fat content modification of butter. *J. Am. Oil Chem. Soc.* **1994**, *71*, 1373-1380.

(39) Malcata, F.X.; Reyes, H.R.; García, H.S.; Hill, Jr. C.G.; Amundson, C.H. Kinetics and mechanisms catalyzed by immobilized lipases. *Enzyme Microb. Technol.* **1992**, *14*, 426-446.

(40) Briand, D.; Dubreucq, E.; Galzy, P. Enzymatic fatty esters synthesis in aqueous medium with lipase from candida parapsilosis (Ashford) Langeron and Talice. *Biotechnol. Lett.* **1994**, *16*, 813-818.

(41) Svesson, I.; Wehtje, E.; Adlercreutz, P. Mattiasson, B. Effects of water activity on reaction rates and equilibrium positions in enzymatic esterifications. *Biotechnol. Bioeng.***1994**, *44*, 549-556.

Table 3.1 Fatty acid composition (wt%) of human milk fat (HMF) and commercial infant formulas (*20*)

Fatty acid	HMF (n=40)		Infant formulas (n=11)	
	Total	*sn*-2[a]	Total	*sn*-2
C8:0	0.11-0.36	nd[b]	0.51-1.20	1.25-3.11
C10:0	0.85-3.08	5.32-7.11	0.74-1.24	27.11-32.15
C12:0	4.05-9.35	17.18-30.65	5.19-12.64	30.01-42.56
C14:0	3.60-9.13	40.11-60.87	3.06-5.91	24.76-30.16
C16:0	15.43-24.46	57.11-67.15	17.96-25.75	11.64-13.43
C18:0	4.60-8.13	7.13-10.12	2.05-4.72	13.24-15.11
C18:1n9	28.30-43.83	8.06-15.13	34.34-44.69	27.74-39.83
C18:2n6	10.61-25.30	15.22-25.16	8.93-17.43	39.93-49.21
C18:3n3	0.41-1.68	16.18-20.65	0.67-2.83	48.14-50.21

[a] *sn*-2 represents the % of a particular fatty acid based on the total amount of the same fatty acid in the sample
[b] *nd*, not detected

Table 3.2 Total and positional fatty acid composition (mol%) of substrates[a,b,c]

Fatty acid	HMPS[d]		HOSO[e]		TC[f]	
	Total	*sn*-2	Total	*sn*-2	Total	*sn*-2
C8:0	nd[g]	nd	nd	nd	0.06 ± 0.07	nd
C10:0	nd	nd	nd	nd	98.95 ± 0.05	32.15 ± 0.07
C14:0	1.40 ± 0.01	46.67 ± 0.95	nd	nd	0.08 ± 0.04	nd
C16:0	70.98 ± 0.20	28.77 ± 0.00	7.12 ± 0.28	2.78 ± 0.62	nd	nd
C18:0	5.61 ± 0.05	44.38 ± 1.06	4.71 ± 0.06	13.28 ± 0.23	nd	nd
C18:1n9	17.77 ± 0.03	36.70 ± 0.61	74.08 ± 0.13	38.26 ± 0.03	nd	nd
C18:2n6	3.51 ± 0.01	3.86 ± 0.07	9.59 ± 0.03	43.84 ± 0.57	nd	nd
C18:3n3	nd	nd	1.74 ± 0.03	nd	nd	nd

[a] Each value is the mean of triplicates ± standard deviation
[b] Trace amounts of C16:1, C17:0, C17:1, C20:0 were too small to be considered
[c] *sn*-2 represent the % of a particular fatty acid based on the total amount of the same fatty acid in the sample
[d] *HMPS*, high melting point palm stearin
[e] *HOSO*, high oleic sunflower oil
[f] *TC*, tricaprin
[g] *nd*, not detected

Table 3.3 Fatty acid composition (mol%) of the structured lipids in stage 1[a,b,c]

Fatty	Total				*sn*-2			
acid	SL1-1[d]	SL1-2[e]	SL1-3[f]	SL1-4[g]	SL1-1	SL1-2	SL1-3	SL1-4
C14:0	0.40 ± 0.01a	0.48 ± 0.01b	0.34 ± 0.02c	0.35 ± 0.02c	44.04 ± 1.34a	38.41 ± 1.00b	nd[h]	nd
C16:0	26.40 ± 0.49a	25.14 ± 0.63b	18.37 ± 0.85c	18.51 ± 0.36c	31.42 ± 0.60a	35.21 ± 0.44b	32.56 ± 0.78ac	33.16 ± 0.97c
C18:0	4.73 ± 0.24a	1.77 ± 0.09b	3.74 ± 0.05c	3.78 ± 0.04c	23.80 ± 2.41a	25.00 ± 0.47ab	27.77 ± 1.93b	9.16 ± 0.28c
C18:1n9	59.01 ± 0.35a	66.85 ± 0.70b	71.73 ± 0.83c	71.62 ± 0.33c	34.28 ± 0.30a	33.07 ± 0.19b	33.84 ± 0.23a	34.88 ± 0.32c
C18:2n6	8.06 ± 0.14a	4.99 ± 0.02b	5.32 ± 0.02c	5.30 ± 0.01c	36.28 ± 0.75a	30.59 ± 0.37b	33.27 ± 0.71c	32.04 ± 1.55bc
C18:3n3	1.41 ± 0.16a	0.78 ± 0.00b	0.50 ± 0.07c	0.44 ± 0.01c	35.56 ± 2.27a	28.29 ± 0.58b	52.61 ± 1.25c	38.83 ± 1.82d

[a] Each value is the mean of triplicates ± standard deviation. Values with different letter in each row within total and *sn*-2 columns separately are significantly different at $P \leq 0.05$

[b] Trace amounts of C16:1, C17:0, C17:1, C20:0 were too small to be considered

[c] *sn*-2 represent the % of a particular fatty acid based on the total amount of the same fatty acid in the sample

[d] *SL1-1*, structured lipid synthesized using a substrate molar ratio 2:3 (HMPS:HOSO); reaction time 12 h

[e] *SL1-2*, structured lipid synthesized using a substrate molar ratio1:2 (HMPS:HOSO); reaction time 24 h

[f] *SL1-3*, structured lipid synthesized using a substrate molar ratio 1:3 (HMPS:HOSO); reaction time 24 h

[g] *SL1-4*, structured lipid synthesized using a substrate molar ratio 1:4 (HMPS:HOSO); reaction time 24 h

[h] *nd*, not detected

Table 3.4 Fatty acid composition (mol%) of the structured lipids in stage 2[a,b,c]

Fatty acid	Total				*sn*-2			
	SL2-1[d]	SL2-2[e]	SL2-3[f]	SL2-4[g]	SL2-1	SL2-2	SL2-3	SL2-4
C10:0	8.23 ± 0.51a	11.15 ± 0.50b	18.68 ± 0.39c	21.22 ± 0.74d	31.97 ± 2.59a	35.59 ± 2.11a	24.68 ± 0.52b	23.67 ± 2.13b
C14:0	0.44 ± 0.04ab	0.46 ± 0.02b	0.41 ± 0.01ac	0.38 ± 0.00c	37.87 ± 1.68a	46.62 ± 1.35b	40.23 ± 0.76a	38.62 ± 1.66a
C16:0	23.37 ± 0.64a	25.12 ± 0.12b	21.34 ± 0.75c	20.13 ± 0.26d	38.33 ± 0.21a	38.87 ± 1.15a	38.93 ± 1.61a	37.57 ± 0.80a
C18:0	1.96 ± 0.14a	2.06 ± 0.06a	1.71 ± 0.09b	1.69 ± 0.02b	32.58 ± 0.98a	36.39 ± 2.65b	22.84 ± 0.80c	28.32 ± 2.40d
C18:1n9	61.85 ± 0.39a	58.05 ± 0.87b	53.89 ± 0.68c	52.98 ± 0.67c	31.99 ± 0.54a	30.80 ± 0.87a	34.96 ± 0.60b	35.31 ± 1.19b
C18:2n6	3.88 ± 0.58a	2.21 ± 0.63b	3.31 ± 0.44ab	3.36 ± 0.53a	26.30 ± 1.73a	25.71 ± 3.86a	25.79 ± 3.29a	30.34 ± 3.93b
C18:3n3	0.27 ± 0.03a	0.38 ± 0.08b	0.27 ± 0.05a	0.24 ± 0.02a	55.51 ± 2.19a	53.64 ± 2.97a	58.47 ± 2.21a	53.36 ± 3.77a

[a] Each value is the mean of triplicates ± standard deviation. Values with different letter in each row within total and *sn*-2 columns separately are significantly different at $P \leq 0.05$

[b] Trace amounts of C16:1, C17:0, C17:1, C20:0 were too small to be considered

[c] *sn*-2 represent the % of a particular fatty acid based on the total amount of the same fatty acid in the sample

[d] *SL2-1*, structured lipid synthesized using a substrate molar ratio 2.8:0.5 (SL1-2:TC [h]); reaction time 12 h

[e] *SL2-2*, structured lipid synthesized using a substrate molar ratio 2:0.5 (SL1-2:TC); reaction time 24 h

[f] *SL2-3*, structured lipid synthesized using a substrate molar ratio 1.5:0.5 (SL1-2:TC); reaction time 12 h

[g] *SL2-4*, structured lipid synthesized using a substrate molar ratio 1.2:0.5 (SL1-2:TC); reaction time 8 h

[h] *TC*, tricaprin

Table 3.5 Relative percent (%) of peak areas of triacylglycerol (TAG) molecular species in substrates and structured lipids[a]

TAG	HMPS[b]	HOSO[c]	TC[d]	PB1-2[e]	SL1-2[f]	PB2-4[g]	SL2-4[h]
CCC	nd[i]	nd	99.00 ± 0.5	nd	nd	20.57 ± 0.79a	0.83 ± 0.02b
CLC	nd	nd	nd	nd	nd	nd	11.13 ± 0.14a
COC	nd	nd	nd	nd	nd	nd	7.63 ± 0.51a
CPC	nd	nd	nd	nd	nd	nd	4.50 ± 0.71a
LCL	nd	nd	nd	nd	nd	nd	0.84 ± 0.06a
LLLn	nd	nd	nd	nd	0.28 ± 0.03a	0.31 ± 0.02a	nd
OCL	nd	nd	nd	nd	nd	nd	2.14 ± 0.13a
LLL	nd	1.82 ± 0.06	nd	1.31 ± 0.08a	0.36 ± 0.02b	0.27 ± 0.03c	nd
OCO	nd	nd	nd	nd	nd	nd	3.46 ± 0.37a
OPC	nd	nd	nd	nd	nd	nd	2.00 ± 0.12a
OLL	nd	2.74 ± 0.20	nd	1.71 ± 0.41a	nd	nd	nd
PLL	nd	1.14 ± 0.13	nd	0.85 ± 0.13a	nd	nd	nd
MLP	nd	nd	nd	nd	0.23 ± 0.05a	0.19 ± 0.02a	nd
MOM	nd	nd	nd	nd	0.10 ± 0.02a	0.06 ± 0.01b	nd
OLO	nd	4.11 ± 0.64	nd	3.03 ± 0.09a	4.43 ± 0.46b	3.91 ± 0.19bc	3.58 ± 0.38ac
POL	nd	nd	nd	nd	6.64 ± 0.73a	5.56 ± 0.49b	2.63 ± 0.37c
PLP	3.27 ± 0.16	nd	nd	1.25 ± 0.26a	2.96 ± 0.83b	1.70 ± 0.23a	nd
MOP	3.29 ± 0.22	nd	nd	1.03 ± 0.16a	nd	nd	nd
PLSt	1.72 ± 0.05	nd	nd	0.50 ± 0.03a	nd	nd	nd
OOO	nd	70.73 ± 0.61	nd	48.02 ± 0.26a	15.93 ± 0.45b	12.40 ± 0.53c	10.42 ± 1.03d
OPO	7.20 ± 0.07	11.94 ± 0.73	nd	10.67 ± 0.49a	34.63 ± 0.58b	27.75 ± 0.52c	28.06 ± 0.15c
POP	30.81 ± 0.28	nd	nd	9.83 ± 0.31a	22.97 ± 0.19b	18.49 ± 0.55c	18.79 ± 0.18c
PPP	43.01 ± 0.12	nd	nd	13.31 ± 0.98a	1.84 ± 0.68b	1.03 ± 0.11b	nd
StOO	nd	7.53 ± 0.55	nd	4.97 ± 0.30a	3.03 ± 0.75b	2.63 ± 0.34b	1.62 ± 0.03c
POSt	3.41 ± 0.08	nd	nd	1.15 ± 0.05a	4.72 ± 0.25b	3.32 ± 1.04c	2.37 ± 0.23c
PPSt	7.28 ± 0.12	nd	nd	2.37 ± 0.16a	1.37 ± 0.33b	1.35 ± 0.58b	nd

[a] The fatty acids are not in any specific order on the glycerol backbone. *C*, capric acid. *L*, linoleic acid. *O*, oleic acid. *P*, palmitic acid. *Ln*, linolenic acid. *M*, myristic acid. *St*, stearic acid. Each value is the mean of triplicates ± standard deviation. Values with different letter in each row for SLs are significantly different at $P \leq 0.05$

[b] *HMPS*, high melting point palm stearin

[c] *HOSO*, high oleic sunflower oil

[d] *TC*, tricaprin

[e] *PB1-2*, physical blend of substrates with molar ratio 1:2 (HMPS:HOSO) before reaction

[f] *SL1-2*, structured lipid synthesized using a substrate molar ratio1:2 (HMPS:HOSO); reaction time 24 h

[g] *PB2-4*, physical blend of substrates with molar ratio 1.2:0.5 (SL1:TC) before reaction

[h] *SL2-4*, structured lipid synthesized using a substrate molar ratio 1.2:0.5 (SL1-2:TC); reaction time 8 h
[i] *nd*, not detected

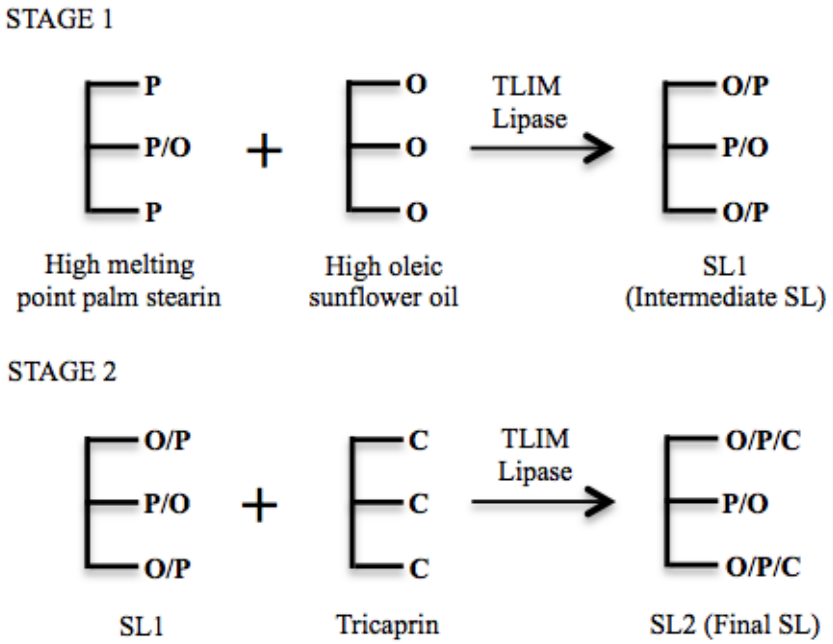


Figure 3.1 Reaction scheme for SL synthesis. Stage 1: Enzymatic interesterification of HMPS and HOSO by using *sn-1,3* specific lipase, Lipozyme® TLIM. Stage 2: Stage 1 product was filtered and excess FFAs were removed. Then, a second enzymatic interesterification with TC using Lipozyme® TLIM was conducted to obtain the final SL. P, palmitic acid. O, oleic acid. C, capric acid.

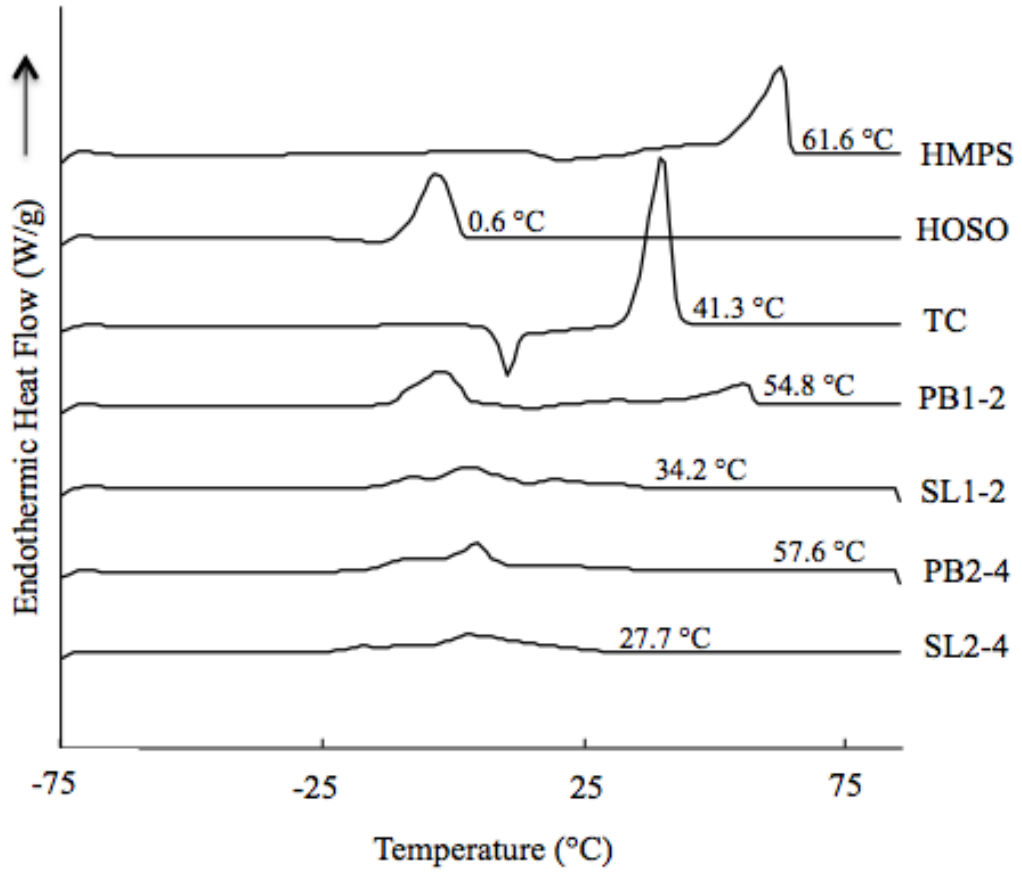


Figure 3.2 Melting thermograms of substrates and structured lipids. The values shown correspond to melting completion temperatures. *HMPS*, high melting point palm stearin. *HOSO*, high oleic sunflower oil. *TC*, tricaprin. *PB1-2*, physical blend of substrates before reaction using a molar ratio 1:2 (HMPS:HOSO). *SL1-2*, structured lipid synthesized in stage 1 (intermediate SL). *PB2-4*, physical blend of substrates before reaction using a molar ratio 1.2:0.5 (SL1-2:TC). *SL2-4*, structured lipid synthesized in stage 2 (Final SL).

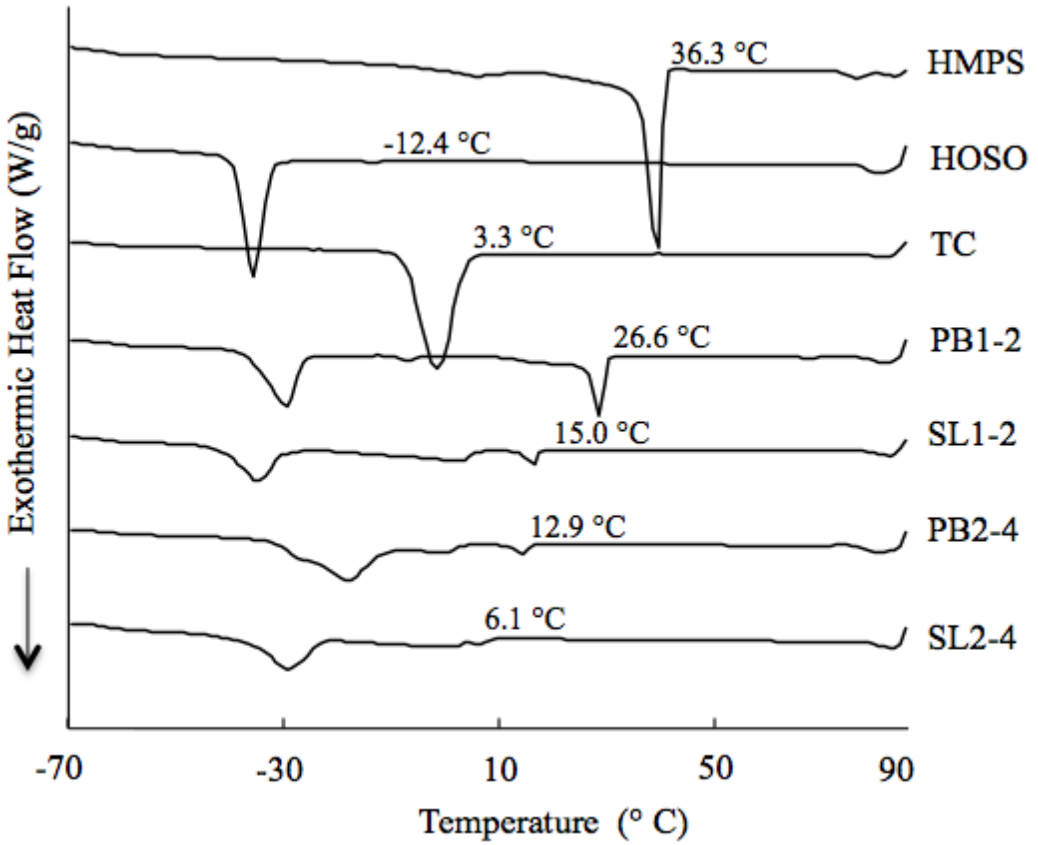


Figure 3.3 Crystallization thermograms of substrates and structured lipids. The values shown correspond to crystallization onset temperatures. *HMPS*, high melting point palm stearin. *HOSO*, high oleic sunflower oil. *TC*, tricaprin. *PB1-2*, physical blend of substrates before reaction using molar ratio 1:2 (HMPS:HOSO). *SL1-2*, structured lipid synthesized in stage 1 (intermediate SL). *PB2-4*, physical blend of substrates before reaction using molar ratio 1.2:0.5 (SL1-2:TC). *SL2-4*, structured lipid synthesized in stage 2 (Final SL).

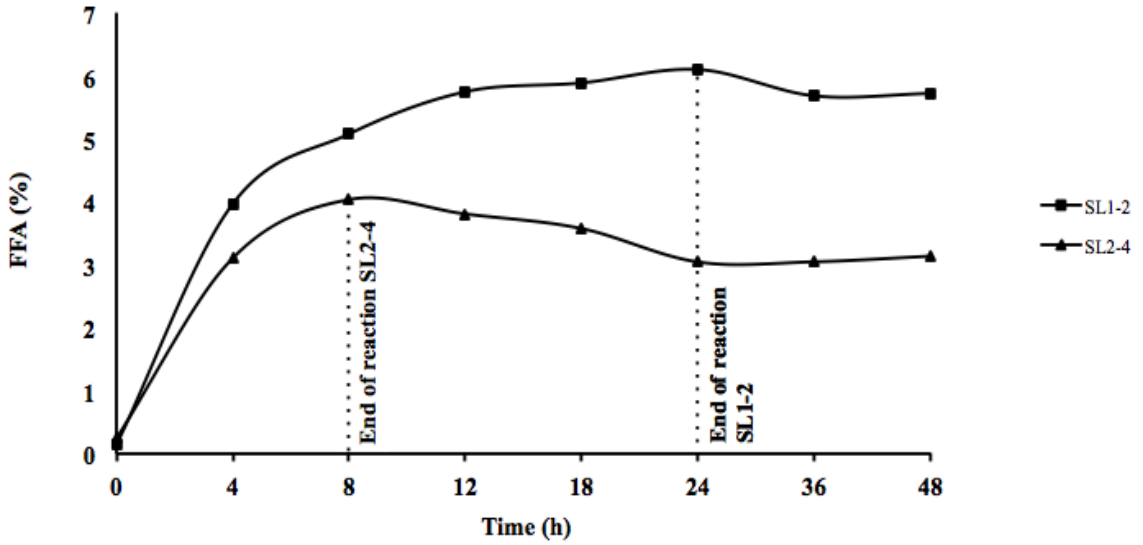


Figure 3.4 Free fatty acid development in SLs reaction. *SL1-2*, structured lipid synthesized in stage 1 using a substrate molar ratio 1:2 (HMPS:HOSO). *SL2-4*, structured lipid synthesized in stage 2 using a substrate molar ratio 1.2:0.5 (SL1-2:TC).

CHAPTER 4

ENZYMATIC PRODUCTION OF HIGH *sn*-2 DHA AND ARA MODIFIED OILS FOR THE FORMULATION OF INFANT FORMULA FAT ANALOGS[2]

[2] Álvarez, C.A. and C.C. Akoh. Submitted to *Journal of the American Oil Chemists' Society*, 06/25/15.

ABSTRACT

High *sn*-2 docosahexaenoic and arachidonic acid oils ($DHAO_m$ and $ARAO_m$, respectively), were produced independently via enzymatic interesterification of DHA-rich and ARA-rich single cell oils (DHASCO and ARASCO, respectively), using a mix of immobilized lipases, Lipozyme® TL IM and Novozym® 435 (weight ratio 1:1) as the biocatalyst system. Response surface methodology (RSM) was employed to model and optimize conditions of the reactions. Three independent variables, reaction time, reaction temperature, and enzyme load were investigated in $DHAO_m$ and $ARAO_m$ models. A Chi-square test verified that predicted responses obtained from the models and the observed ones obtained by experimentation were not significantly different at $p \leq 0.05$. The prediction power of the model was further confirmed by solvent-free scale-up reactions. Final results showed that $DHAO_m$ contained 46.53 mol% of total DHA of which 49.70% was at the *sn*-2 position, while $ARAO_m$ contained 47.25 mol% of total ARA of which 36.08% was at the *sn*-2 position. These products have potential for additions to infant formulas where DHA and ARA supplementation is required.

Keywords: Structured lipids, Docosahexaenoic acid, Arachidonic acid, Enzymatic interesterification, Positional distribution, Infant formula

INTRODUCTION

Breast milk is a complex mixture of lipids, carbohydrates, proteins, vitamins, minerals as well as growth and hormonal factors that nurture and protect infants from infections and certain chronic diseases (*1*). Human milk fat (HMF) is the second largest component of breast milk by concentration (3-5% in mature milk) and provides approximately 50% of the total energy used by infants (*2*). Some of the major fatty acids (FAs) in HMF include oleic (28.30-43.83 wt%), palmitic (15.43-24.46 wt%), and stearic (4.60-8.13 wt%) acids (*3*). HMF is also a source of the

essential fatty acids (EFAs) linoleic acid (10.61-25.30 wt%) and α-linolenic acid (0.41-1.68 wt%), as well as their derivatives, so called long chain polyunsaturated fatty acids (LC-PUFAs), arachidonic acid (ARA, 0.23-0.75 wt%) and docosahexaenoic acid (DHA, 0.15-0.56 wt%). The bioavailability of both EFAs and LC-PUFAs, is critical during infancy for motor and cognitive skills development, proper brain growth, sensory, and neurological reflexes (*4*). DHA and ARA are the predominant FAs in the structural phospholipids of the human brain and other important cell membranes. In the case of DHA, it plays a very specific function in the photoreceptor membranes of the retina, improving visual acuity (*5-7*). Studies conducted in breastfed infants have shown that HMF provides adequate DHA and ARA levels to support normal neural tissue growth (*8-10*). However, recent studies in formula-fed infants have shown that even when the conventional formulas contain substantial amounts of α-linolenic and linoleic acids (which are precursors capable of endogenous synthesis of DHA and ARA, respectively), they were unable to maintain postnatal LC-PUFAs levels in plasma and erythrocyte lipids compared to infants fed human breast milk (*10,11*). Low levels of DHA and ARA in formula-fed infants may be attributed to their lack of ability to desaturate and elongate α-linolenic and linoleic acids (to DHA and ARA, respectively), due to elongation-desaturation enzymes not being sufficiently active during the early stages of life to maintain tissue accumulation (*5*). Anderson *et al.* (*12*) established that preformed dietary DHA and ARA are quantitatively more effective than supplementation of α-linolenic and linoleic acids as a source of LC-PUFAs, and therefore, their addition to conventional infant formulas is crucial to provide levels similar to those found in HMF or better. Currently, commercial infant formulas containing DHA and ARA supplementation are available in most countries and recommendations on their use have been reported by various expert bodies, including the FAO/WHO (*13*). In commercial infant formulas,

LC-PUFAs are physically added to the fat blend by using lipids with relatively high levels of this type of FAs. While fish oil and egg yolk lipid are also used, single-cell organisms principally from algae and fungi, are the most common sources of preformed DHA and ARA for addition to the formulas. Particularly used are DHASCO containing 40-55% DHA extracted from the algae *Crypthecodinium cohnii*, and ARASCO containing 40-55% ARA extracted from the fungus *Mortierella alpina* (*14*).

In breast milk, DHA and ARA are mainly in the form of triacylglycerols (TAGs) (*15*). DHA and ARA only make up a very small proportion of the total fatty acids found in HMF and they are primarily esterified at the *sn*-2 and *sn*-3 positions (*16*). Approximately 60% of the total DHA present in HMF is esterified at the *sn*-2 position, and 30% is esterified at the *sn*-3 position. In the case of ARA, ~45% is esterified at the *sn*-2, and 40% at *sn*-3 position. On the other hand, the DHA and ARA from single-cell organisms used in commercial infant formulas do not display a clear positional distribution, with all the fatty acids found in all three positions almost indistinctly (*17*). DHASCO contains between 30-35% of the total DHA esterified at the *sn*-2 position, 30-40% esterified at the *sn*-3 position, and 20-30% at the *sn*-1 position. ARASCO has 25-30% of the total ARA esterified at the *sn*-2, 20-25% esterified at the *sn*-3 position, and 45-50% at the *sn*-1 position (*14*). DHASCO and ARASCO also have the unusual feature of containing significant amounts of TAGs with two or more LC-PUFAs per molecule (*17*). It has been reported that the differences in the TAG positional arrangement of DHA and ARA from dietary lipid sources such as egg yolk lipids, fish oils, and unicellular organisms, may affect their absorption, distribution and tissue uptake (*18*). Higher levels of DHA and ARA were found in the brain of newborn rats fed with oils containing DHA and ARA at the *sn*-2 position than those fed with oils containing these but randomly distributed (*19*). Additionally, it has been reported

that the presence of some LC-PUFAs, including DHA and ARA, at outer positions of the TAG molecules induce resistance to pancreatic lipase, and therefore, relatively low absorption of these FAs might be expected (*20*).

TAGs that have been chemically or enzymatically modified by incorporating new molecules or by rearranging the position of their original FAs are called structured lipids (SLs). For certain applications, these novel structures can be desirable as they exhibit special properties that affect their melting behavior, digestion, absorption, and their nutritional value (*21*). Numerous studies have been conducted on the production of SLs with high levels of LC-PUFAs esterified at the *sn*-2 position via enzymatic esterification (EIE) and/or acidolysis reactions. Recently, Wang *et al.* (*22*) reported the synthesis of novel SLs with high content of LC-PUFAs at the *sn*-2 position by enzymatic acidolysis. Iwasaki *et al.* (*23*) and Hamam *et al.* (*24*) reported the production of high *sn*-2 DHA SLs via enzymatic acidolysis of DHA single cell oil with caprylic and capric acids, respectively. Nieto *et al.* (*25*) reported the preparation of *sn*-2 LC-PUFAs monoacylglycerols from fish oil by hydrolysis, using a *sn*-1,3 specific lipase. Although these SLs contained higher levels of LC-PUFAs at the *sn*-2 position, all of them required blending of the single-cell oil with other substrates to achieve the reaction, changing its total fatty acid profile and losing valuable LC-PUFAs (originally esterified at the *sn*-1,3 positions) which are replaced with new FAs from the substrates.

The objective of this study was to modify the molecular structure of DHA and ARA-rich oils from single-cell organisms via enzymatic rearrangement of the FAs in the TAGs, so they can contain higher levels of DHA and ARA at the *sn*-2 position. Potential benefits include better absorption and utilization by infants when added to commercial formulas. To achieve this, independent enzymatic interesterifications of DHASCO and ARASCO were conducted using a

mixture of immobilized lipases Lipozyme® TL IM and Novozym® 435 (weight ratio 1:1). Response surface methodology (RSM) was employed to model and optimize reaction conditions.

MATERIALS AND METHODS

Materials.

Commercial DHASCO® and ARASCO® were purchased from DSM Nutritional Products (Columbia, MD, USA). The immobilized lipase, Novozym® 435 (non-specific *Candida antarctica* lipase), was obtained from Novo Nordisk A/S (Bagsvaeard, Denmark). The specific activity of this enzyme was 10000 PLU/g at pH 5-9 and temperature of 30-60°C, where PLU stands for propyl laurate unit, as defined by the manufacturer (*26*). Immobilized lipase, Lipozyme® TL IM (*sn*-1,3 specific *Thermomyces lanuginosus* lipase), was obtained from Novozymes North America Inc. (Franklinton, NC, USA). The specific activity of this enzyme was 250 IUN/g at pH 6-8 and temperature of 50-75°C, where IUN stands for interesterification unit novo, as defined by the manufacturer (*26*). The lipid standards, Supelco 37 Component FAME mix and 2-oleoylglycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Pentadecanoic acid (C15:0) was obtained from TCI America (Portland, OR, USA). Other solvents and chemicals were purchased from Fischer Scientific (Norcross, GA, USA) and Sigma-Aldrich Chemical Co.

Production of $DHAO_m$ and $ARAO_m$.

$DHAO_m$ and $ARAO_m$ were obtained from independent enzymatic interesterifications using DHASCO and ARASCO as substrates, respectively. For $DHAO_m$, a lipase mix of Lipozyme® TL IM (*sn*-1,3 specific lipase) and Novozym® 435 (non-specific lipase), was used as biocatalyst for the positional re-arrangement of the FAs attached to the TAGs in DHASCO. This process was performed with the aim of increasing the amount of DHA at the *sn*-2 position. Similarly, for

$ARAO_m$, a lipase mix of Lipozyme® TL IM and Novozym® 435 was used as biocatalyst for the re-arrangement of the FAs attached to the TAGs in ARASCO, with the aim of increasing the amount of ARA at the *sn*-2 position. In order to model and establish the optimal reaction conditions for both $DHAO_m$ and $ARAO_m$, a set of experiments were conducted at small scale (mg) using RSM, and samples were analyzed for total and positional fatty acid profiles. Once the models and reaction conditions were defined, scale-up reactions (g) for $DHAO_m$ and $ARAO_m$ production were performed to determine the reproducibility of the results.

Selection of Biocatalyst.

Preliminary small-scale enzymatic interesterifications of DHASCO were performed at 4, 8, and 12 h in order to select the lipase (or lipase mix) that will better promote the concentration of DHA at the *sn*-2 position (see **Figure 4.1**). All the reactions were conducted at 60°C using an enzyme load of 10 wt% of substrate. The lipase-systems tested were: TL (Lipozyme® TL IM alone), 435 (Novozym® 435 alone), and TL+435 (mixture of Lipozyme® TL IM and Novozym® 435, weight ratio 1:1). A synergistic effect was observed when using the two enzymes simultaneously, leading to a higher esterification of DHA at the *sn*-2 position when compared to the use of the enzymes individually (**Figure 4.1**). This can be attributed to the fact that Lipozyme® TL IM is a *sn*-1,3 specific lipase, whereas Novozym® 435 is a non-specific lipase (giving to the system a wider range of cleavage/attachment interactions, in favor of the *sn*-2 DHA content). Synergistic effects on enzymatic interesterification have been reported previously when using Lipozyme® TL IM and Novozym® 435 together in equal ratios (*27*). Since TL+435 showed higher levels of *sn*-2 DHA at the temperatures tested, this lipase system was selected as the biocatalyst for all further reactions in the study.

Reaction Screenings to Determine Temperature Ranges for RSM Studies.

In order to determine the range of reaction temperature for the RSM studies of both $DHAO_m$ and $ARAO_m$ models (Mod_{DHA} and Mod_{ARA}, respectively), small-scale screenings were conducted using a 10 wt% enzyme mix of Lipozyme® TL IM and Novozym® 435 (weight ratio 1:1) for 4 h. For Mod_{DHA}, the reaction temperatures tested were 30, 40, 50, 60, 70, and 80°C (see **Figure 4.2a**). As the highest amount of DHA at the *sn*-2 position was observed between 30 and 60°C, this range was selected for reaction temperature optimization in the RSM study for DHA. For Mod_{ARA}, the reaction temperatures tested were 20, 30, 40, 50, 60, 70, and 80°C (see **Figure 4.2b**). The highest amount of ARA at the *sn*-2 position was observed between 30 and 70°C and this range was selected for reaction temperature optimization in the RSM study.

Experimental Design for RSM Studies.

To study the effects of experimental conditions on both $DHAO_m$ and $ARAO_m$, RSM was applied using the experimental design provided by Modde 9.0 software (Umetrics, Umea, Sweden). Mathematical models were generated by the software to predict reaction responses for $DHAO_m$ and $ARAO_m$. For Mod_{DHA}: three factors, each with three levels were taken into consideration when designing the experiments: reaction time (4, 14, and 24 h), reaction temperature (30, 45, and 60 °C), and enzyme load (10, 15, and 20 wt%). The values of each factor were substituted by -1 (low), 0 (medium), and 1 (high) later in the equations by the software. Seventeen different combinations of reaction conditions were generated by the software using central composite design (CCD). Experiments were performed in triplicate resulting in fifty-one total reactions. For Mod_{ARA}: three factors, each with three levels, were taken into consideration when designing the experiments: reaction time (4, 14, and 24 h), reaction temperature (30, 50, and 70 °C), and enzyme load (10, 15, and 20 wt%). The values of each factor were substituted by -1, 0, and 1

later in the equations. Seventeen different combinations of reaction conditions were generated by the software using CDD. Experiments were performed in triplicate resulting in fifty-one total reactions.

Verification of Models.

Both Mod_{DHA} and Mod_{ARA} synthesis were verified by randomly selecting 5 points from different regions in their contour plots and performing reactions using the conditions corresponding to those points. The obtained response values were compared to the predicted values from the models. Chi-square tests were conducted to compare the observed and predicted values and determine significant differences (α=0.05).

Reaction Procedure.

All the enzymatic reactions at small scale (mg) for both Mod_{DHA} and Mod_{ARA} were performed following a modified version of the Teichert *et al.* method (*28*). For Mod_{DHA}, two hundred milligrams of DHASCO were weighed into a labeled teflon-lined test tube and 3 mL of *n*-hexane was added to reach a concentration of 0.06 g/mL. A lipase mix of Lipozyme® TL IM and Novozym® 435 (weight ratio 1:1), was used as biocatalyst (at the defined enzyme load of 10, 15, or 20 wt%). The reaction was carried out (at the defined temperature of 30, 45 or 60°C) in a water bath while shaking at 200 rpm. After reaction, the product was filtered two times through an anhydrous sodium sulfate column to remove the enzyme. Excess free fatty acids (FFAs) were removed by neutralization (as described below). Reactions were performed independently (at the defined reaction time of 4, 14 or 24 h). For Mod_{ARA}, the same procedure as for Mod_{DHA}, but in this case the reaction was carried out at 30, 50, and 70°C.

The scale-up reactions for both Mod_{DHA} and Mod_{ARA} followed the Pande *et al.* (*29*) method. One hundred grams of substrate (either DHASCO or ARASCO) were weighed into a

stirred tank reactor without solvent. For this procedure, *n*-hexane was not used for safety and environmental reasons, but the tank stirrer at 200 rpm created the necessary turbulence to keep the oil in continuous contact with the enzyme. The reaction was carried out at optimal conditions of temperature, time, and enzyme load, previously defined by RSM. After reaction, the product was filtered two times through an anhydrous sodium sulfate column to remove any trace of water and the enzyme, and excess FFAs were removed by short-path distillation (SPD).

Removal of FFAs.

For small scale reactions, the FFAs were removed through deacidification by alkaline extraction method (*30*) with minor modifications. Once filtered over a sodium sulfate column, the product (dissolved in *n*-hexane) was mixed with 3 mL of neutralized 95% ethyl alcohol and 2 ml of 1% phenolphthalein solution. Then, it was titrated with 0.5 N NaOH in 20% ethanol. The aqueous layer was discarded while the hexane layer was collected and once again passed through an anhydrous sodium sulfate column. The solvent was removed by nitrogen sparging. Finally, the oil product was store at -20°C until analysis.

For the scale-up reactions, the FFAs were removed through SPD as reported previously (*29*). After filtration over a sodium sulfate column, the reaction product was fed into a KDL-4 (UIC Inc., Joliet, IL, USA) system under the following conditions: feed holding temperature of 65 °C, feeding rate of approximately 100 mL/h, evaporator heating temperature of 185 °C, condenser cooling temperature of 20−25 °C, roller speed of 200 rpm, and vacuum of <13.33 Pa. The product was passed through SPD twice to obtain FFAs of <0.1%.

Determination of Fatty Acid Profiles.

Both, substrates and products were converted to FA methyl esters (FAMEs) following AOAC Official Method 996.01, Section E (*31*) with minor modifications. 0.1 g of sample was weighed

into Teflon-lined test tubes and 0.20 mL internal standard (C15:0, 20 mg/mL in hexane) was added and dried under nitrogen to remove solvent. 2 mL of 0.5 N NaOH in methanol was added and heated to 100 °C for 10 min for saponification of free fatty acids. The sample was cooled under tap water and 2 mL BF_3 in methanol was added followed by vortexing for 1 min. Again, the sample was heated at 100°C for 10 min for methylation and then cooled under tap water. To stop the reaction and extract the FAMEs, 2 mL of hexane and 2 mL of saturated NaCl solution were added. The sample was vortexed for 2 min and then centrifuged at 1000 rpm for 5 min to separate the organic layer from the aqueous layer. The organic layer was passed through an anhydrous sodium sulfate column into a GC vial for analysis. The FAME external standard used was Supelco 37 component FAME mix and was run in parallel with the samples.

Positional Analysis.

The pancreatic lipase-catalyzed *sn*-2 positional analysis of substrates and products was performed following a modified version of the Luddy *et al.* method (*32*). 100 mg of sample (extracted and dried TAG) was placed into Teflon-lined test tubes. 2 mL of 1.0 M Tris-HCl buffer (pH=8), 0.5 mL of 0.05% sodium cholate solution, and 2.2% calcium chloride solution were added, and the mixture was vortexed for 2 min to emulsify. Then, 40 mg of pancreatic lipase was added, and the mixture was vortexed for 1 min and incubated in a water bath at 40°C for 3 min while shaking at 200 rpm. The sample was vortexed again for 2 min. To stop the reaction and extract the hydrolyzed TAGs, 1 mL of 6 N HCl and 4 mL of diethyl ether were added. The sample was vortexed for 2 min and centrifuged at 1000 rpm for 3 min. The upper layer, containing the lipid components, was filtered through an anhydrous sodium sulfate column. The sample was concentrated under nitrogen until approximately one-third of the original volume was left. A mixture of hexane, diethyl ether, and formic acid (60:40:1.6, v/v/v)

was used as the mobile phase after equilibration in the TLC tanks for approximately 30 min. The concentrated sample was spotted onto an activated silica gel G dried TLC plate and placed into the tank. 2-Oleyglycerol was spotted as the standard and run in parallel with the sample for identification of the 2-monoacylglycerol (2-MAG) band. The plate was sprayed with 0.2% 2,7 dichlorofluorescein in methanol and visualized under UV light. The 2-MAG band was scrapped off and converted to FAME (as described above). The determination of the fatty acid profile at the *sn*-2 position was quantified after GC analysis. The fraction of the FA that is esterified at the *sn*-2 position (based on the total content of that FA in the sample) was calculated using the following equation:

FA *sn*-2 (%)=[100*total FA at *sn*-2 (mol%)]/[3* total FA in sample (mol%)] (Eq.1).

GC Analysis.

Fatty acid compositions of all samples were analyzed as FAMEs following the method of Pande *et al.* (*30*) with a Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA), using a Supelco SP-2560, 100 m x 25 mm x 0.2 μm column. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. The injection volume was 1 μL and a split ratio of 20:1 was used. Detection was with a flame ionization detector at 300°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 25 min. All samples were analyzed in triplicate and average values reported.

Statistical Analysis.

All analyses were performed in triplicate and average values were reported. Response surfaces, regression analysis, background elimination and validation of the models were performed using Modde 9.0 software (Umetrics, Umea, Sweden).

RESULTS AND DISCUSSION

Model Fitting.

Table 4.1 shows total and positional fatty acid composition of the SLs obtained via EIE of DHASCO, using the different sets of reaction conditions generated by RSM. All values for total fatty acid remained relatively stable in all the experiments, as total myristic acid ranged from 10.35 to 11.57 mol%, total oleic acid from 22.78 to 23.96 mol%, and total DHA from 46.16 to 47.44 mol%. On the other hand, values for *sn*-2 FA varied significantly as a consequence of the enzymatic rearrangement of the fatty acids in the glycerol backbone of TAGs. Thus, the amount of myristic acid, oleic acid and DHA esterified at the *sn-2* position ranged from 11.16 to 35.63%, from 17.71 to 29.20%, and from 32.91 to 49.73%, respectively. A similar behavior was observed in experiments conducted with the reaction conditions generated in EIE of ARASCO (see **Table 4.2**). The values for total fatty acid remain stable (total palmitic acid ranged from 9.16 to 10.51 mol%, total oleic acid from 22.14 to 23.17 mol%, and total ARA from 46.78 to 48.00 mol%). In contrast, the values for *sn*-2 FA varied significantly (palmitic acid ranged from 10.33 to 45.43%, oleic acid from 24.12 to 40.46%, and ARA from 30.27 to 36.08%). Each set of results were subjected to multiple linear regression and backward elimination analysis to fit into independent polynomial models. Analysis of variance (ANOVA) for the fitted models of *sn*-2 DHA (Mod_{DHA}) and *sn*-2 ARA (Mod_{ARA}) are shown in **Table 4.3**. The probability for the regression in both models was significant at 95% (*p*-value for the *F*-test statistics was lower than the significance level, $\alpha = 0.05$). The coefficient of multiple determination (R^2) that measures the proportion of the variation in the response explained by the models, was 0.896 for Mod_{DHA}, and 0.890 for Mod_{ARA}. The adjusted coefficient of multiple determination (R^2_{adj}), similar to R^2, but considers the effect of unrelated reaction factors introduced into the models, was 0.879 for

Mod_{DHA}, and 0.872 for Mod_{ARA}. Q^2, an estimate of the future prediction precision of the models was 0.866 for Mod_{DHA}, and 0.843 for Mod_{ARA}. Based on the values found for statistics F (regression), R^2, R^2_{adj}, and Q^2, it is plausible to conclude that both models are statistically good to fit the variations observed in the amount of *sn*-2 DHA and *sn*-2 ARA. However, the probability for lack of fit in both models was significant at 95% (p-value for the F-test statistics was lower than the significance level, $\alpha = 0.05$), which means that according to this test, the models have lack of fit. This could be attributed to a very high reproducibility (very low variation of replicates compared to overall variability, see low standard deviations for *sn*-2 DHA and *sn*-2 ARA in **Tables 4.1** and **4.2**, respectively). High reproducibility causes the sum of squares due to pure error to be too small, and therefore, the F-test statistics (lack of fit) to be considerably higher than 1.0 (p-value lower than $\alpha = 0.05$). Nevertheless, the software tool used for modeling (Modde 9.0, Umetrics) describes Q^2 (which was very good for the two models), as the best and most sensitive statistics for this type of regressions. The model equation that describes the final content of DHA at the *sn*-2 position was:

$$sn\text{-2 (DHA)} = 44.25 + 2.35(T) - 1.05(E_L) - 2.86(t) - 5.76(T^2) - 0.35(T \times E_L) + 0.44(T \times t) + 0.33(E_L \times t) \quad \text{(Eq. 2).}$$

The model equation that describes the final content of ARA at the *sn*-2 position was:

$$sn\text{-2 (ARA)} = 33.82 - 0.73(T) - 0.09(E_L) - 0.99(t) - 1.69(T^2) - 0.33(E_L^2) + 0.46(t^2) - 0.17(E_L \times t) \quad \text{(Eq. 3).}$$

T indicates reaction temperature (°C), E_L indicates enzyme load (wt%), and t indicates reaction time (h). For Mod_{DHA}, the most significant first-order parameter was time and the most significant second-order parameter was temperature x temperature. Both, first-order and second-order parameters had negative effects on *sn*-2 DHA content (with T representing the strongest

influence overall). Similarly, for Mod_{ARA} the most significant first-order parameter and second-order parameter were time and temperature x temperature, respectively. Both parameters had negative effects on *sn*-2 ARA content (with *T* representing the strongest influence overall).

Verification of the Model.

Verification of Mod_{DHA} and Mod_{ARA} were performed using Chi-square tests, in order to determine whether there was a significant difference between expected and observed values of the responses. To do so, five points were randomly selected from different regions of the contour plots (generated by Modde 9.0 software, see **Figure 4.3**), and small-scale reactions were performed using the conditions corresponding to those points. The obtained response values were then compared to the predicted values from the models. As shown in **Table 4.4**, there were no significant differences between the observed and expected responses in any of the models, since Chi-square values of Mod_{DHA} (1.49) and Mod_{ARA} (0.89) were lower than the critical value of the X^2 distribution (cutoff point: 9.488) at α=0.05 and *df*=4. These results confirm the relatively high accuracy of the models in terms of predictability, which was previously observed with the values for the statistics Q^2.

Optimization of the Reaction.

The relationship between responses and parameters in both models was examined using contour plots. **Figure 4.3a** shows the effect that reactions conditions have on the final content of DHA esterified at the *sn*-2 position of TAGs. In contour plot a_1, enzyme load was kept constant at 10 wt% while reaction time and reaction temperature were in y and x-axis, respectively. According to the plot, there is a range of temperature approximately between 43 and 52°C where the placement of DHA at the *sn*-2 position tends to be the highest. It also suggests that reactions conducted for periods of time longer than 6 h contain less amount of *sn*-2 DHA when compared

to those reactions that lasted shorter. Contour plot a_2 shows the interaction between enzyme load and temperature at a reaction time of 4 h. An enzyme load approximately between 10 and 13 wt% gave the highest content of DHA at the *sn*-2 position. Moreover, reaction temperatures below 43 and above 52°C, tend to be detrimental to increasing *sn*-2 DHA. According to contour plot a_3, the best set of enzyme load and reaction time to achieve high *sn*-2 DHA was the one that combined a short reaction time (4 h) with small amount of biocatalyst (10 wt%). Conversely, the least convenient was the one that combined a reaction time of 24 h with high amount of biocatalyst (20 wt%). **Figure 4.3b** shows the effect that reactions conditions have on the final content of *sn*-2 ARA. Contour plot b_1 shows the interaction between reaction time and temperature while enzyme load was kept constant at 15 wt%. Similar to the behavior observed in Mod_{DHA}, the range of temperature where the placement of ARA at the *sn*-2 position tends to be the highest was approximately between 37 and 55°C. Reactions conducted longer than 5 to 6 h contained less amount of *sn*-2 ARA when compared to shorter reactions. From plot b_2, there was no well-defined range where the amount of ARA at the *sn*-2 position can be significantly affected (increased or decreased) based on the amount of biocatalyst used. Instead, it seems that once reaction time is fixed, variable temperature governs almost entirely the variation of the response. For example, looking at the high *sn*-2 ARA region at temperatures between 37 and 55°C, there were not significant changes in the response level regardless of the use of 12, 15 or 20 wt% of biocatalyst. Although this observation suggests that the enzyme load (at the range tested in RSM, 10-20 wt%) is not as relevant as temperature or reaction time for explaining variations in the amount of *sn*-2 ARA, its significance for the model cannot be fully overlooked considering that there are three factors (first-order, second-order, and interaction term with time, see Eq.3), where enzyme load still affects the response variable. According to contour plot b_3,

the best set of enzyme load and reaction time to achieve high *sn*-2 ARA was the one that combined a short reaction (4 h) with 14 to 17 wt% biocatalyst. On the other hand, the least amount of *sn*-2 ARA was obtained with a reaction time of 24 h and high amount of biocatalyst (20 wt%).

In both Mod_{DHA} and Mod_{ARA}, the effect of temperature displayed a similar pattern. The response level first increased as temperature was increased, then reached a peak (optimal response), and finally as temperature continued to increase, it started to drop. This behavior was reported previously by Duan *et al.* (*33*) where an optimal reaction temperature of 60°C was reported for the preparation of 1,3-diacylglycerols via esterification with Novozym® 435. Reaction temperature is considered important to enhance the enzyme activity and mobility of substrate molecules (*34*). By increasing reaction temperature the viscosity of the system decreases, promoting the contact with the enzyme and therefore, increasing the mass transfer. However, as temperature continued to increase, the enzyme denaturation (inactivation) rate increases as a consequence of the thermal sensitivity of the hydrogen bonds and other weak interactions that keep the enzyme structure stable (*35*). Comparable observations between reaction temperature and response levels were also reported by Lumor *et al.* (*36*) and Li *et al.* (*37*).

The effect of time displayed a similar pattern for both Mod_{DHA} and Mod_{ARA}. The response level reached a peak at 4-5 h of reaction and then decreased gradually as time went on. In the case of Mod_{DHA}, it is worth noting that while conducting experiments at fixed conditions of temperature and enzyme load, the response level (*sn*-2 DHA) significantly decreased when the reaction went from 4 to 24 h, whereas the amount of *sn*-2 myristic acid increased during the same period of time (see **Table 4.1**). A somewhat-related behavior has been reported previously

by Long *et al.* (*38*) where a mycelium bound lipase exhibited a high preference towards short and medium triacylglycerols after 20 h of reaction. For Mod_{ARA}, the response level (*sn*-2 ARA) decreased and the amount of *sn*-2 palmitic acid increased as the reaction time went from 4 to 24 h (see **Table 4.2**). Again, Long *et al.* (*38*) reported that the mycelium bound lipase hydrolyzed oleic acid-based oils faster than palmitic acid-based oils, suggesting that even though the lipase has a preference for unsaturated rather than saturated FAs, this proportion of interactions might change with long-term reactions.

The effect of enzyme load for Mod_{DHA} displayed a pattern that suggests that higher amounts of biocatalyst lead to lower levels of response. As shown in **Table 4.1**, the response level (*sn*-2 DHA) consistently decreased when the enzyme load increased from 10 to 15, and 20 wt% (for fixed conditions of time and temperature). In the case of Mod_{ARA}, although the influence of enzyme load is not that conclusive, it still follows a pattern that suggests that an excess of biocatalyst is detrimental to the response level (*sn*-2 ARA). This behavior can be attributed to a higher rate of hydrolysis, since more enzyme is in contact with the substrate and therefore, a more random interaction with FAs from different positions can occur. This randomness allows other FAs to be placed at the *sn*-2 position of TAGs instead of those of interest.

Another possible explanation to the patterns observed for all the reaction conditions in both Mod_{DHA} and Mod_{ARA} is the occurrence of acyl migration. Acyl migration is considered an undesirable side reaction involving migration of acyl groups from *sn*-1,3 to *sn*-2 positions and vice versa (*39*). It mainly occurs due to the presence of diacylglycerols (DAGs) and monoacylglycerols (MAGs), which are intermediate products of EIE, and can be influenced by a number of factors including high temperature, degree of unsaturation of FAs, reaction time,

FFA%, amount and carrier of the enzyme, and presence of water (*40*). Thus, occurrence of acyl migration might promote other FAs different from those of interest to be placed at the *sn*-2 position of TAGs when the reaction temperature is too high, the reaction time is too long, and/or the presence of excess biocatalyst.

The optimal reaction conditions to maximize the response in both Mod_{DHA} and Mod_{ARA}, were generated by the optimizer function of Modde 9.0 software. For Mod_{DHA}, the conditions were determined to be 47.9°C (temperature), 10 wt% (enzyme load), and 4 h (reaction time), resulting in a response level of 48.72 ± 1.22% (*sn*-2 DHA). For Mod_{ARA}, the conditions were 43.7°C, 15 wt%, and 4 h, resulting in a response level of 35.33 ± 0.39% (*sn*-2 ARA). These optimization conditions are used as a tool to give an estimate of the best values that can be obtained in the final products after the enzymatic interesterifications.

Total and Positional Fatty Acid Profiles of Substrates and Final Reaction Products.

Table 4.5 shows the total and positional fatty acid composition of DHA-rich single cell oil from *C. cohnii* (DHASCO), and ARA-rich single cell oil from *M. alpina* (ARASCO) which are commonly used as sources of preformed DHA and ARA for addition to infant formulas. Results showed that DHASCO contained 47.88 mol% total DHA of which 34.30% was at the *sn*-2 position. It also contained 22.38 mol% oleic acid (62.81% at *sn*-2), and 10.83 mol% myristic acid (11.32% at *sn*-2). In the case of ARASCO, it contained 47.79 mol% ARA (28.50% at *sn*-2), 22.42 mol% oleic acid (49.41% at *sn*-2), and 9.28 mol% palmitic acid (3.79% at *sn*-2).

Scale-up reactions at optimal conditions for Mod_{DHA} and Mod_{ARA} were conducted to obtain final products with high contents of *sn*-2 DHA ($DHAO_m$) and *sn*-2 ARA ($ARAO_m$), using DHASCO and ARASCO as substrates, respectively. The results for total and positional fatty acid composition of these products are shown in **Table 4.5**. $DHAO_m$ contained 46.53 mol% total

DHA of which 49.70% was at the *sn*-2 position. This result fell within the range predicted for *sn*-2 DHA using the optimized conditions (47.50-49.95%). Other important fatty acids present in $DHAO_m$ included oleic acid (23.96 mol% of which 27.35% was at the *sn*-2), and myristic acid (10.57 mol% of which 11.16% was at the *sn*-2). In the case of $ARAO_m$, it contained 47.25 mol% total ARA of which 36.08% was at the *sn*-2 position. This result was slightly out (upper limit) of the range predicted for the optimized response of *sn*-2 ARA (34.94-35.72%), but since the model was developed with experiments conducted at small-scale (mg), some small discrepancies were expected as a consequence of changes in reactor dimensions (g scale). Consequently, this result can still be considered accurate. $ARAO_m$ also contained 22.62% total oleic acid (27.25% at *sn*-2), and 9.85 mol% palmitic acid (21.99% at *sn*-2). When comparing total and positional fatty acid profiles of the commercial single-cell oils (DHASCO and ARASCO, respectively) with the final products obtained by EIE ($DHAO_m$ and $ARAO_m$), it can be observed, in each case, that whereas total fatty acid composition was roughly the same, the specific placement of the FAs in the TAGs differed considerably. Furthermore, the amount of DHA esterified at the *sn*-2 in $DHAO_m$ surpassed the amount in DHASCO by more than 15 percentage points (a 31% increase), while the amount of ARA at the *sn*-2 in $ARAO_m$ surpassed the amount in ARASCO by almost 8 percentage points (a 21% increase). Ramirez *et al.* (*18*) reported that differences in the TAG positional arrangement of DHA and ARA oils from unicellular organisms, may affect their absorption, distribution and tissue uptake, when compared to other sources such as HMF. Christensen *et al.* (*19*) conducted a study on early dietary intervention with SLs containing LC-PUFAs and reported that higher levels of DHA and ARA were found in the brain of newborn rats fed with oils containing these FAs at the *sn*-2 position, compared to those fed with oils containing the same FAs randomly distributed. Additionally, Botino *et al.* (*20*) reported that the

presence of DHA and ARA esterified at the outer positions of the TAGs induced resistance to pancreatic lipase, and consequently poor absorption was observed. Based on this evidence, it is plausible that DHA and ARA from $DHAO_m$ and $ARAO_m$, respectively, can be absorbed more efficiently than DHA and ARA supplemented directly from single-cell oils. This could be particularly relevant for additions to infant formulas where LC-PUFAs supplementation is critical for motor and cognitive skills development, proper brain growth, sensory functions, and neurological reflexes (*4*). Some studies have been conducted over the past years on the production of modified oils with high levels of LC-PUFAs esterified at the *sn*-2 position, via enzymatic esterification and/or acidolysis reactions. For example, Wang *et al.* (*22*) reported the synthesis of novel SLs with high content of LC-PUFAs at the *sn*-2 position by enzymatic acidolysis using a commercially available *sn*-1,3 specific lipase from *Rhizomucor miehei* (18.56% total DHA with 30.11% at *sn*-2). Iwasaki *et al.* (*23*) reported the synthesis of SLs with high *sn*-2 DHA content enriched with caprylic acid via enzymatic acidolysis of DHA single cell oil (39.01% total DHA with 36.10% at *sn*-2). Hamam *et al.* (*24*) reported the production of high *sn*-2 DHA SLs via enzymatic acidolysis with capric acid (37.10% total DHA with 40.12% at *sn*-2). Finally, Nieto *et al.* (*25*) reported the preparation of *sn*-2 LC-PUFAs monoacylglycerols from fish oil by enzymatic hydrolysis (94.30% *sn*-2 DHA). Although these SLs also contained high levels of LC-PUFAs at the *sn*-2 position, none of them followed the approach described in the present study where no other substrates were used along with the single-cell oils to perform the enzymatic reactions. Our study used enzymatic interesterifications rather than acidolysis and this reduced excessive FFAs formation. Excess FFAs must be removed to increase stability of the final product (causing low reaction yields). Additionally, as $DHAO_m$ and $ARAO_m$ improved DHASCO and ARASCO (in terms of positional distribution of the FAs), while preserving the

same total fatty acid composition, they might also be used in other food applications where single-cell oils are currently used.

CONCLUSION

DHA and ARA are the predominant FAs in the structural phospholipids of important cell membranes of the body. In infants, they support proper brain growth and the development of other neurological functions. Unlike in HMF, preformed DHA and ARA from single-cell organisms used in commercial infant formulas do not display a clear positional distribution on TAG molecules, affecting their absorption, distribution, and tissue uptake. $DHAO_m$ and $ARAO_m$ containing high levels of *sn*-2 DHA and *sn*-2 ARA, respectively, can be used in the formulation of new formula fat analogs that deliver these physiologically important FAs more efficiently when provided to the infants. To the best of our knowledge, this is the first study on the EIE of single-cell oils that addressed increase in positional specificity of DHA and ARA, and proposes the potential for use of these modified oils to improve their absorption, and potentiate their metabolic benefits when added to infant formulas.

ACKNOWLEDGEMENTS

This research was supported in part by Food Science Research, University of Georgia.

REFERENCES

(1) Jensen, R.G. The lipids of human milk. CRC Press, Boca Raton, FL, **1989**.

(2) Wells, J.C.K. Nutritional considerations in infant formula design. *Semin. Neonatal.* **1996**, *1*, 19–26.

(3) López-López, A.; López-Sabater, M.C.; Campoy-Folgoso, C.; Rivero-Urgell, M.; Castellote-Bargalló, A.I. Fatty acid and *sn*-2 fatty acid composition in human milk from Granada (Spain) and in infant formulas. *Eur. J. Clin. Nutr.* **2002**, *56*,1242–1254.

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

(5) Uauy, R.; Hoffman, D.R.; Mena, P.; Llanos, A.; Birch, E.E. Term infant studies of DHA and ARA supplementation on neurodevelopment: Results of randomized controlled trials. *J. Pediatr.* **2003**, *143*, S17-S25.

(6) Innis, S.M. Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. *J. Pediatr.* **2003**, *143*,1-8.

(7) Neuringer, M; Connor, W.E.; Van Petten, C.; Barstad, L. Dietary omega-3 fatty acid deficiency and visual loss in infant Rhesus monkeys. *J. Clin. Invest.* **1984**, *73*, 272-276.

(8) Martinez, M. Tissue levels of polyunsaturated fatty acids during early human development. *J. Paediatr.* **1992**, *120*, 129–138.

(9) Makrides, M.; Neumann, M.A.; Byard, R.W.; Simmer, K.; Gibson, R.A. Fatty acid composition of brain, retina and erythrocytes in breast- and formula-fed infants. *Am. J. Clin. Nutr.* **1994**, *60*, 189–194.

(10) Carlson, S.E.; Rhodes, P.G.; Ferguson, M.G. Docosahexanoic acid status of preterm infants at birth and following feeding with human milk or formula. *Am. J. Clin. Nutr.* **1986**, *44*, 798 – 804.

(11) Pita, M.L.; DeLucchi, C.; Faus, M.J.; Gil, A. Changes in the fatty acid profiles of red blood cell membrane phospholipids in human neonates during the first month of life. *Clin. Physiol. Biochem.* **1990**, *8*, 91-100.

(12) Anderson, G.J.; Connor, W.E.; Corliss, J.D. Docosahexaenoic acid is the preferred dietary n-3 fatty acid for the development of the brain and retina. *Pediatr. Res.* **1990**, *27*, 89-97.

(13) Teichert, S.A.; Akoh, C.C. Stearidonic acid soybean oil enriched with palmitic acid at the *sn*-2 position by enzymatic interesterification for use as human milk fat analogues. *J. Agric. Food Chem.* **2011**, *59*, 5692-5701.

(14) Food Standards Australia-New Zealand. *DHASCO and ARASCO oils as sources of long-chain polyunsaturated fatty acids in infant formula*. Food Standards Australia New Zealand, Canberra, **2003**; pp**.** 3-54.

(15) Jensen, R.G. Lipids in human milk – composition and fat-soluble vitamins. In Lebenthal E (ed) *Textbook of gastroenterology and nutrition in infancy*. Raven Press, New York, **1989**; pp. 157 – 208.

(16) Martin, J.C.; Bougnoux, P.; Antoine, J.M.; Lanson, M.; Couet, C. Triacylglycerol structure of human colostrum and mature milk. *Lipids*, **1993**, *28*, 637–643.

(17) Myher, J.J.; Kuksis, A.; Geher, K.; Park, P.W.; Diersen-Schade, D.A. Stereospecific analysis of triacylglycerols rich in long-chain polyunsaturated fatty acids. *Lipids* **1996**, *31*, 207– 215.

(18) Ramírez, M.; Amate, L.; Gil, A. Absorption and distribution of dietary fatty acids from different sources. *Early Hum. Dev.* **2001**, *65*, S95-S101.

(19) Christensen, M.M.; Høy, C.E. Early dietary intervention with structured triacylglycerols containing docosahexaenoic acid. Effect on brain, liver, and adipose tissue lipids. *Lipids* **1997**, *32*, 185−191.

(20) Bottino, N.R.; Vandenburg, G.A.; Raiser, R. Resistance of certain long-chain polyunsaturated fatty acids of marine oils to pancreatic lipase hydrolysis. *Lipids* **1967**, *2*, 489 – 493.

(21) Høy, C.E.; Xu,X. Structured Triacylglycerols. In Gunstone FD (ed) *Structured and Modified Lipids*. Marcel Dekker Inc, New York, NY, **2001**; pp. 209-239.

(22) Wang, J.; Wang, X.D.; Zhao, X.Y.; Liu, X.; Dong, T.; Wua, F.A. From microalgae oil to produce novel structured triacylglycerols enriched with unsaturated fatty acids. *Bioresour. Technol.* **2015**, *184*, 405-414.

(23) Iwasaki, Y.; Han, J.J.; Narita, M.; Rosu, R.; Yamane, T. Enzymatic synthesis of structured lipids from single cell oil of high docosahexaenoic acid content. *J. Am. Oil Chem. Soc.* **1999**, *75*, 563-569.

(24) Hamam, F.; Shahidi, F. Synthesis of structured Lipids via acidolysis of docosahexaenoic acid single cell oil (DHASCO) with capric acid. *J. Agric. Food. Chem.* **2004**, *52*, 2900-2906.

(25) Nieto, S.; Gutiérrez, J.; Sanhueza, J.; Valenzuela, A. Preparation of *sn*-2 long-chain polyunsaturated monoacylglycerols from fish oil by hydrolysis with a stereo-specific lipase from *mucor miehei*. *Grasas Aceites* **1999**, *50*, 111-113.

(26) Novozymes Biopharma DK A/S. Enzymes for Biocatalysis: for smarter chemical synthesis. In *http://www.novozymes.com/en/solutions/biopharma/Brochures/Documents/2014-12576-01_Biocatalysis-Product-Sheet-Immobilised-Lipases-2.pdf*. **2014**, accessed 20 Apr 2015.

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

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

(29) Pande, G.; Sabir, J.S.M.; Baeshen, N.A.; Akoh, C.C. Enzymatic synthesis of extra virgin olive oil based infant formula fat analogues containing ARA and DHA: one-stage and two-stage syntheses. *J. Agric. Food Chem.* **2013**, *61*, 10590−10598.

(30) Pande, G; Sabir, J.S.M.; Baeshen, N.A.; Akoh, C.C. Synthesis of infant formula fat analogs enriched with DHA from extra virgin olive oil and tripalmitin. *J. Am. Oil Chem. Soc.* **2013**, *90*, 1311–1318.

(31) *Official Methods of Analysis of AOAC International*, 19th ed.; AOAC International: Rockville, MD, **2012**; Official Method 996.01.

(32) Luddy, F.E.; Bardford, R.A.; Herb, S.F.; Magidman, P.; Riemenschneider, R.W. Pancreatic lipase hydrolysis of triacylglycerides as a semi-micro technique. *J. Am. Oil Chem. Soc.* **1964**, *41*, 639–696.

(33) Duan, Z.Q.; Du, W.; Liu, D.H. Novozym 435-catalyzed 1,3-diacylglycerol preparation via esterification in t-butanol system. *Process Biochem.* **2010**, *45*,1923–1927.

(34) Can, A.; Ozcelik, B. Enrichment of hazelnut oil with long-chain n-3 PUFA by lipase-catalyzed acidolysis: optimization by response surface methodology. *J. Am. Oil Chem. Soc.* **2005**, *82*, 27–32.

(35) Senanayake, S.P.J.N.; Shahidi, F. Enzymatic incorporation of docosahexaenoic acid into borage oil. *J. Am. Oil Chem. Soc.* **1999**, *76*, 1009–1015.

(36) Lumor, S.E.; Jones, K.C.; Ashby, R.; Strahan, G.D.; Kim, B.H.; Lee, G.C.; Shaw, J.F.; Kays, S.E.; Chang, S.W.; Foglia, T.A.; Akoh, C.C. Synthesis and characterization of canola oil-stearic acid-based trans-free structured lipids for possible margarine application. *J. Agric, Food Chem.* **2007**, *55*, 10692–10702.

(37) Li, R.; Pande, G.; Sabir, J.S.M.; Baeshen, N.A.; Akoh, C.C. Enrichment of refined olive oil with palmitic and docosahexaenoic acids to produce a human milk fat analogue. *J. Am. Oil Chem. Soc.* **2014**, *91*, 1377–1385.

(38) Long, K.; Ghazali, H.M.; Ariff, A.; Che Man, Y.; Bucke, C. Substrate preference of mycelium-bound lipase from a strain of *Aspergillus flavus* link. *Biotechnol. Lett.* **1998**, *20*, 369–372.

(39) Yang, T; Fruekilde, M.B.; Xu, X. Suppression of acyl migration in enzymatic production of structured lipids through temperature programming. *Food Chem.* **2005**, *92*, 101−107.

(40) Xu, X; Skands, A.R.H.; Høy, C.E.; Mu, H.; Balchen, S.; Adler-Nissen, J. Production of specific-structured lipids by enzymatic interesterification: Elucidation of acyl migration by response surface design. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1179−1186.

Table 4.1 Experimental settings of factors and response (*sn*-2 DHA) used for optimization by RSM[a]

Reaction conditions			Total fatty acid (mol%)			*sn*-2[b] (%)		
Temp (°C)	E_L[c] (wt%)	Time (h)	C14:0	C18:1n9	C22:6n3	C14:0	C18:1n9	C22:6n3
30	10	4	11.12 ± 0.04	23.18 ± 0.03	46.31 ± 0.15	26.61 ± 2.07	27.95 ± 0.40	38.81 ± 0.21
60	10	4	10.69 ±0.26	23.60 ± 0.13	46.96 ± 0.56	19.96 ± 1.11	26.62 ± 0.07	44.28 ± 0.36
30	20	4	11.05 ± 0.02	23.43 ± 0.07	46.18 ± 0.09	25.82 ± 0.45	29.2 ± 0.39	38.20 ± 0.28
60	20	4	10.35 ± 0.07	23.54 ± 0.13	47.44 ± 0.34	29.99 ± 0.05	22.05 ± 0.33	41.11 ± 0.23
30	10	24	10.88 ± 0.02	22.78 ± 1.08	47.61 ± 0.51	35.16 ± 0.60	27.28 ± 1.68	33.32 ± 0.34
60	10	24	10.44 ± 0.01	23.48 ± 0.03	47.35 ± 0.04	31.13 ± 0.26	21.00 ± 0.34	39.43 ± 0.04
30	20	24	11.06 ± 0.09	23.13 ± 0.03	46.42 ± 0.27	31.26 ± 0.79	26.85 ± 0.64	32.91 ± 0.19
60	20	24	10.63 ± 0.24	23.38 ± 0.15	47.37 ± 0.28	33.81 ± 0.79	20.92 ± 0.59	38.73 ± 0.36
30	15	14	11.01 ± 0.01	23.19 ± 0.09	46.55 ± 0.12	27.48 ± 0.34	28.66 ± 0.65	37.44 ± 0.39
60	15	14	10.37 ± 0.06	23.53 ± 0.20	47.41 ± 0.40	29.89 ± 2.02	22.59 ± 0.97	40.68 ± 0.34
45	10	14	10.57 ± 0.09	23.96 ± 0.48	46.34 ± 0.62	16.21 ± 0.03	25.24 ± 0.47	48.32 ± 0.71
45	20	14	10.61 ± 0.08	23.64 ± 0.10	46.73 ± 0.22	27.2 ± 0.88	20.91 ± 0.57	42.71 ± 0.26
45	15	4	10.57 ± 0.05	23.96 ± 0.17	46.53 ± 0.39	11.16 ± 1.07	27.35 ± 0.44	49.73 ± 0.53
45	15	24	11.57 ± 0.78	23.24 ± 0.08	46.17 ± 0.69	35.63 ± 1.97	17.71 ± 0.12	39.07 ± 0.86
45	15	14[d]	11.05 ± 0.54	23.04 ± 0.77	46.37 ± 0.31	27.62 ± 1.39	20.53 ± 0.71	43.66 ± 0.32
45	15	14[d]	10.52 ± 0.03	23.70 ± 0.03	46.68 ± 0.02	28.84 ± 0.21	20.16 ± 0.46	43.14 ± 0.21
45	15	14[d]	10.52 ± 0.03	23.70 ± 0.03	46.68 ± 0.02	28.84 ± 0.21	20.16 ± 0.46	43.14 ± 0.21

[a] Each value is the mean of triplicates ± standard deviation

[b] *sn*-2 represent the relative percentage (%) of a particular fatty acid based on the total amount of the same fatty acid in the sample

[c] E_L, enzyme load

[d] center point

Table 4.2 Experimental settings of factors and response (*sn*-2 ARA) used for optimization by RSM[a]

Reaction conditions			Total fatty acid (mol%)			*sn*-2[b] (%)		
Temp (°C)	E_L[c] (wt%)	Time (h)	C16:0	C18:1n9	C20:4n6	C16:0	C18:1n9	C20:4n6
30	10	4	9.69 ± 0.19	22.57 ± 0.06	47.37 ± 0.56	11.73 ± 0.32	37.49 ± 0.62	33.50 ± 0.69
70	10	4	10.01 ± 0.05	23.17 ± 0.33	46.82 ± 0.31	21.64 ± 0.38	35.93 ± 0.43	32.31 ± 0.27
30	20	4	9.46 ± 0.19	22.45 ± 0.02	47.48 ± 0.30	10.33 ± 0.87	35.48 ± 1.70	34.13 ± 0.35
70	20	4	9.97 ± 0.10	22.83 ± 0.39	47.79 ± 0.27	33.56 ± 0.37	29.19 ± 0.55	32.23 ± 0.32
30	10	24	9.16 ± 0.17	22.90 ± 0.35	46.78 ± 0.41	40.44 ± 3.31	26.65 ± 0.93	32.37 ± 0.33
70	10	24	9.89 ± 0.31	22.45 ± 0.68	47.56 ± 0.46	38.45 ± 0.98	30.35 ± 1.10	30.76 ± 0.39
30	20	24	9.80 ± 0.09	22.26 ± 0.00	47.52 ± 0.08	42.55 ± 0.44	28.24 ± 0.94	32.00 ± 0.33
70	20	24	9.98 ± 0.04	22.87 ± 0.65	48.00 ± 0.19	45.43 ± 0.25	28.03 ± 0.68	30.27 ± 0.12
30	15	14	9.38 ± 0.49	22.53 ± 0.80	47.10 ± 0.43	12.28 ± 0.68	40.46 ± 1.88	32.79 ± 0.09
70	15	14	10.13 ± 0.28	22.91 ± 0.58	47.19 ± 0.58	38.63 ± 1.86	28.27 ± 0.84	31.94 ± 0.33
50	10	14	9.88 ± 0.20	22.41 ± 0.29	46.90 ± 0.22	35.42 ± 1.16	24.12 ± 0.90	34.06 ± 0.25
50	20	14	10.08 ± 0.09	22.99 ± 0.48	47.22 ± 0.38	37.72 ± 0.59	25.15 ± 0.43	33.38 ± 0.23
50	15	4	9.85 ± 0.13	22.62 ± 0.31	47.25 ± 0.26	21.99 ± 0.93	27.25 ± 0.35	36.08 ± 0.19
50	15	24	10.51 ± 0.52	22.14 ± 0.71	47.48 ± 0.52	33.01 ± 0.92	29.09 ± 0.46	32.95 ± 0.44
50	15	14[d]	10.01 ± 0.16	22.71 ± 0.13	47.32 ± 0.13	32.26 ± 1.42	28.55 ± 0.23	33.32 ± 0.15
50	15	14[d]	9.99 ± 0.09	22.76 ± 0.18	47.00 ± 0.38	31.27 ± 0.30	28.41 ± 0.30	33.67 ± 0.46
50	15	14[d]	9.99 ± 0.02	22.50 ± 0.35	47.24 ± 0.41	31.55 ± 0.30	28.86 ± 0.56	33.57 ± 0.22

[a] Each value is the mean of triplicates ± standard deviation

[b] *sn*-2 represent the relative percentage (%) of a particular fatty acid based on the total amount of the same fatty acid in the sample

[c] E_L, enzyme load

[d] center point

Table 4.3 ANOVA table for the response variables of the models[a]

	Mod_{DHA}[b]						Mod_{ARA}[c]					
	df	SS	MS	*F*	*p*	SD	*df*	SS	MS	*F*	*p*	SD
Total	51	86,134.3	1,688.91				51	55,304.4	1,084.4			
Constant	1	85,167	85,167				1	55,213.4	55,213.4			
Total Corrected	50	967.383	19.3477			4.399	50	90.9922	1.81984			1.349
Regression	7	866.75	123.821	52.9082	0.000	11.128	7	80.9538	11.5648	49.5383	0.000	3.401
Residual	43	100.633	2.34031			1.530	43	10.0384	0.233452			0.483
Lack of Fit (Model Error)	7	94.7721	13.5389	83.1593	0.000	3.680	7	6.01188	0.85884	7.679	0.000	0.927
Pure Error (Replicate Error)	36	5.86103	0.162806			0.403	36	4.02656	0.111849			0.334

[a] *df* degree of freedom, *SS* sum of squares, *MS* mean square (variance), *SD* standard deviation

[b] For Mod_{DHA}: N = 51, *df* = 43, Q^2= 0.866, R^2= 0.896, R^2_{adj}= 0.879

[c] For Mod_{ARA}: N = 51, *df* = 43, Q^2= 0.843, R^2= 0.890, R^2_{adj}= 0.872

Table 4.4 Verification of models using Chi-square test

Region	Mod_{DHA}						Mod_{ARA}					
	Temp (°C)	E_L[a] (wt%)	Time (h)	O[b] (%)	E[c] (%)	$(O-E)^2/E$	Temp (°C)	E_L (wt%)	Time (h)	O (%)	E (%)	$(O-E)^2/E$
1	48	10	5	46.63	48.42 ± 1.16	0.07	40	10	5	36.22	34.63 ± 0.41	0.07
2	40	15	7	47.11	44.94 ± 0.76	0.10	60	20	6	30.11	33.83 ± 0.39	0.41
3	55	20	14	38.22	41.98 ± 0.91	0.34	40	10	14	35.33	33.53 ± 0.38	0.10
4	45	10	22	37.74	42.74 ± 1.11	0.59	30	17	22	30.03	32.22 ± 0.37	0.15
5	32	15	23	31.22	34.96 ± 0.97	0.40	68	13	20	29.13	31.40 ± 0.32	0.16
χ^2						1.49						0.89

[a] E_L, enzyme load

[b] *O*, observed response (*sn*-2, %)

[c] *E*, expected response (*sn*-2, %)

Table 4.5 Total and positional fatty acid composition (mol%) of single-cell oils and final products[a,b,c]

Fatty acid	DHASCO[d]		ARASCO[e]		$DHAO_m$[f]		$ARAO_m$[g]	
	Total	*sn*-2	Total	*sn*-2	Total	*sn*-2	Total	*sn*-2
C10:0	1.26 ± 0.01	nd[h]	nd	nd	1.25 ± 0.01	9.31 ± 0.48	nd	nd
C12:0	5.05 ± 0.04	17.58 ± 0.66	nd	nd	5.00 ± 0.01	13.98 ± 1.09	nd	nd
C14:0	10.83 ± 0.08	11.32 ± 0.25	0.29 ± 0.01	49.35 ± 0.83	10.57 ± 0.05	11.16 ± 1.07	0.30 ± 0.02	41.70 ± 0.51
C16:0	9.43 ± 0.06	1.19 ± 0.14	9.28 ± 0.35	3.79 ± 0.55	9.22 ± 0.05	10.57 ± 0.29	9.85 ± 0.13	21.99 ± 0.93
C16:1n7	2.73 ± 0.03	nd	nd	nd	2.72 ± 0.03	21.19 ± 0.08	nd	nd
C18:0	0.10 ± 0.02	nd	4.30 ± 0.17	3.36 ± 0.74	0.26 ± 0.10	nd	4.79 ± 0.02	32.79 ± 0.67
C18:1n9	22.38 ± 0.04	62.81 ± 0.08	22.42 ± 0.09	49.41 ± 0.71	23.96 ± 0.17	27.35 ± 0.44	22.62 ± 0.31	27.25 ± 0.35
C18:2n6	0.34 ± 0.02	43.02 ± 2.02	7.47 ± 0.52	62.49 ± 0.89	0.48 ± 0.01	23.21 ± 0.27	7.16 ± 0.02	54.01 ± 1.41
C18:3n3	nd	nd	3.46 ± 0.64	41.72 ± 0.17	nd	nd	3.03 ± 0.01	42.25 ± 1.02
C20:3n6	nd	nd	3.70 ± 0.08	25.80 ± 0.85	nd	nd	3.09 ± 0.09	23.75 ± 1.16
C20:3n3	nd	nd	1.30 ± 0.10	72.02 ± 0.35	nd	nd	1.91 ± 0.18	19.91 ± 0.21
C20:4n6	nd	nd	47.79 ± 1.40	28.50 ± 0.54	nd	nd	47.25 ± 0.26	36.08 ± 0.19
C22:6n3	47.88 ± 0.18	34.30 ± 0.29	nd	nd	46.53 ± 0.39	49.70 ± 0.50	nd	nd

[a] Each value is the mean of triplicates ± standard deviation

[b] Trace amounts of C16:1, C17:0, C17:1, C20:0 were too small to be considered

[c] *sn*-2 represents the relative percentage (%) of a particular fatty acid based on the total amount of the same fatty acid in the sample

[d] *DHASCO*, single-cell oil rich in DHA from *C. cohnii*

[e] *ARASCO*, single-cell oil rich in ARA from *M. alpina*

[f] $DHAO_m$, enzymatically modified product with high content of *sn*-2 DHA using optimal reaction conditions of T (47.9°C), t (4h), and enzyme load (10 wt%)

[g] $ARAO_m$, enzymatically modified product with high content of *sn*-2 ARA using optimal reaction conditions of T (43.7°C), t (4h), and enzyme load (15 wt%)

[h] *nd*, not detectable

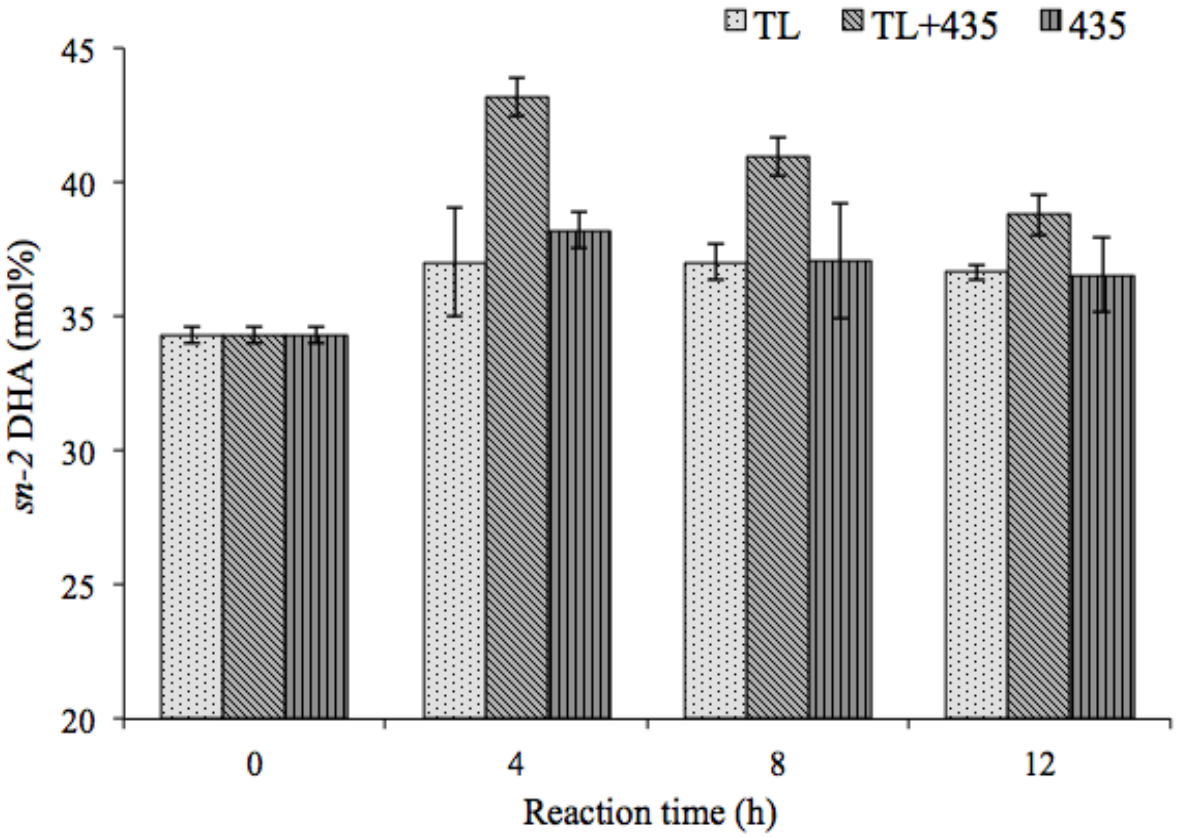
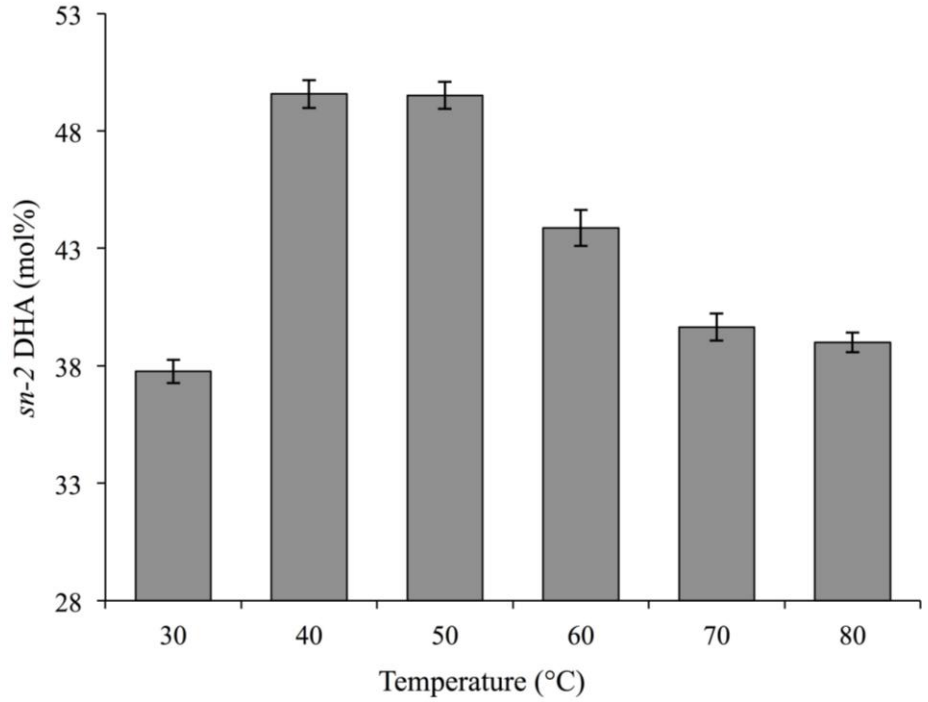


Figure 4.1 Small-scale study at different reaction times for lipase mix selection. All the reactions were conducted at 60°C, using an enzyme load of 10 wt%. *TL*, enzymatically modified DHA oil using Lipozyme® TL IM. *TL+435*, enzymatically modified DHA oil using an enzyme mix of Lipozyme® TL IM and Novozym® 435 at a weight ratio of 1:1. *435*, enzymatically modified DHA oil using Novozym® 435.

(a)



(b)

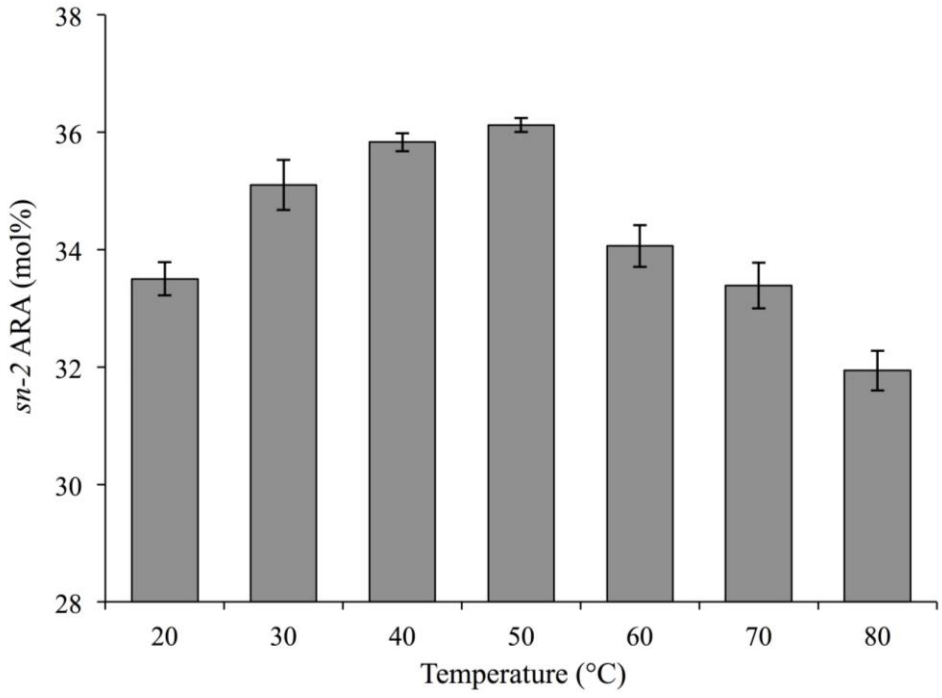


Figure 4.2 Small-scale screening for determining reaction temperature range in RSM study of (a) DHA oil and (b) ARA oil modification. The enzymatic reactions were conducted independently using a 10 wt% enzyme mix of lipozyme® TL IM and Novozym® 435 (weight ratio 1:1). Reaction time 4 h.

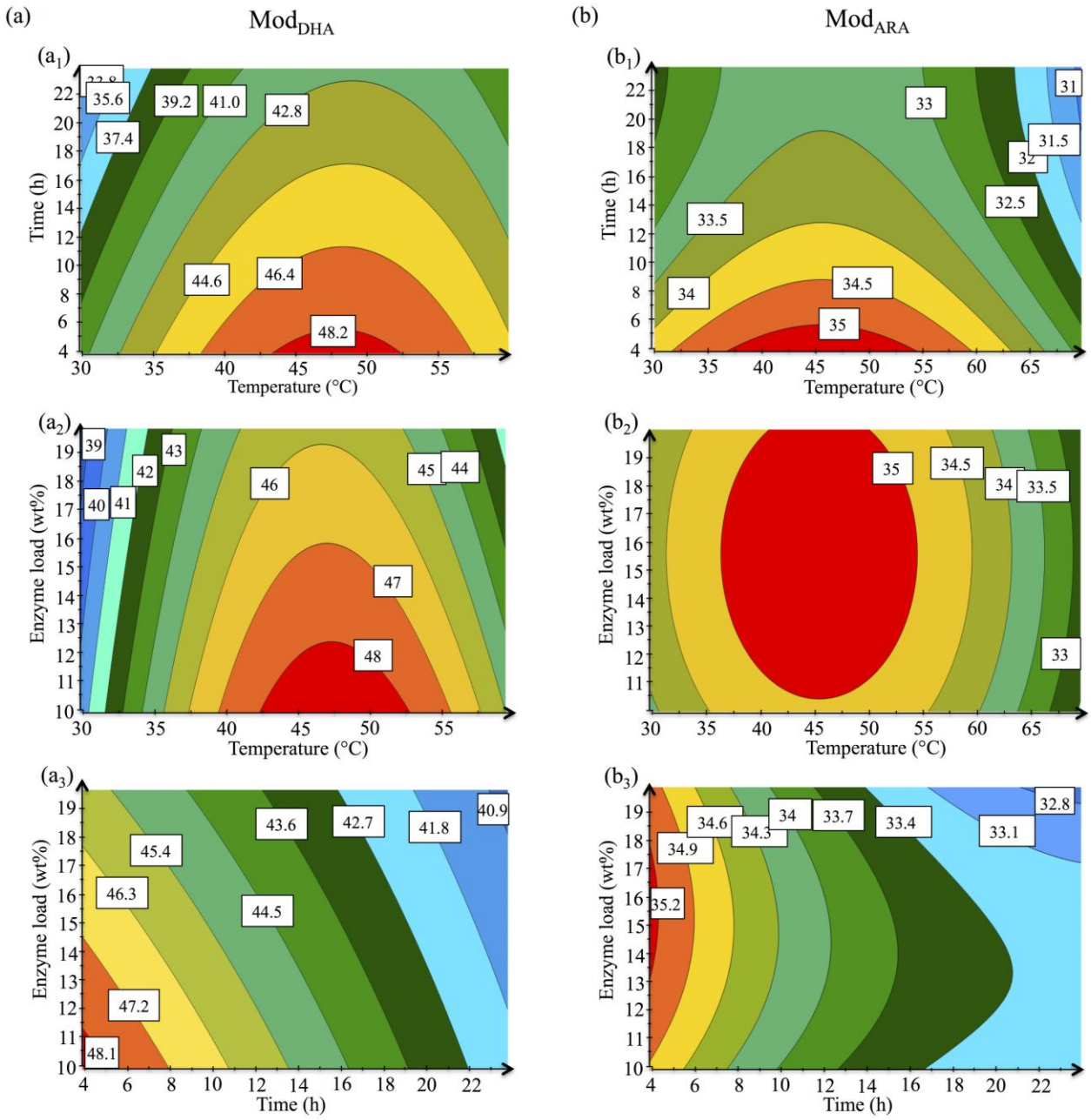


Figure 4.3 (a) Contour plots for the relationship between response (*sn*-2 DHA) and parameters (T,t, and E_L) in Mod$_{DHA}$ (b) Contour plots for the relationship between response (*sn*-2 ARA) and parameters (T,t, and E_L) in Mod$_{ARA}$.

CHAPTER 5

PREPARATION OF INFANT FORMULA FAT ANALOG CONTAINING CAPRIC ACID AND ENRICHED WITH DHA AND ARA AT THE *sn*-2 POSITION[3]

[3] Álvarez, C.A. and C.C. Akoh. Submitted to *Journal of the American Oil Chemists' Society*, 06/25/15.

ABSTRACT

An infant formula fat analog with capric acid mostly esterified at the *sn*-1,3 positions, and substantial amounts of palmitic, docosahexaenoic (DHA), and arachidonic (ARA) acids at the *sn*-2 position, was prepared by physically blending enzymatically synthesized structured lipids (SLs) with vegetable oils. The components of the blend included high *sn*-2 palmitic acid SL enriched with capric acid (SL_{CA}), canola oil (CAO), corn oil (CO), high *sn*-2 DHA ($DHAO_m$), and high *sn*-2 ARA ($ARAO_m$) modified oils. Each component was proportionally blended to match the fatty acid profile of commercial fat blends used for infant formula. The infant formula fat analog ($IFFA_1$) was characterized for total and positional fatty acids (FAs), triacylglycerol (TAG) molecular species, thermal behavior, and tocopherol content. $IFFA_1$ contained 17.37 mol% total palmitic acid of which nearly 35% was located at the *sn*-2 position. The total capric acid content was 13.93 mol%. The content of DHA and ARA were 0.49 mol% (48.18% at *sn*-2) and 0.57 mol% (35.80% at *sn*-2), respectively. The predominant TAGs were OPO (24.09%), POP (15.70%), OOO (11.53%), and CLC (7.79%). The melting completion and crystallization onset temperatures were 18.65 and -2.19°C, respectively. The total tocopherol content was 843.43 μg/g. This product might be suitable for commercial production of infant formulas.

Keywords: Structured lipids, Infant formula, Capric acid, Palm stearin, Docosahexaenoic acid, Arachidonic acid, Positional distribution

INTRODUCTION

Human breast milk is considered the ideal food for newborns. It contains macro and micro components that provide balanced nutrition and help with building immunity against infections and other chronic diseases (*1*). Human milk fat (HMF) counts for 3-5% of the total composition of breast milk and contributes nearly half of the energy provided to the infants

through dietary source (*2*). HMF is also a source of essential fatty acids (EFAs) and their derivatives, such as long-chain polyunsaturated fatty acids (LC-PUFAs), which play a major role in the development of motor and cognitive skills, neurological reflexes, visual acuity and brain growth (*3*). Although breast milk is the preferred choice of nutrients for the infants, commercial formulas have been used worldwide as the most convenient substitute, especially in situations where breastfeeding cannot be practiced. Today, infant formulas contain formulations that include probiotic bacteria, nucleotides, prebiotics, and carotenoids that closely mimic the general composition of human breast milk. However, the lipid component of HMF have not been fully matched because of their unique molecular structure that set them apart from other common sources of lipids, such as those from animal and vegetable origin (*4*). A fatty acid profile roughly identical to HMF can be achieved in commercial infant formula fats by combining edible oils from vegetable origin with single-cell oils from algae and fungi (*5*). However, the positional distribution of some of the most important fatty acids (FAs) on the triacylglycerol (TAG) molecules in HMF, differs from most commercial formulations and cannot be matched by sheer blending. While in HMF, palmitic acid is mainly located at the *sn*-2 position of TAGs (57-77%), in most infant formulas it largely exists (about 90%) at the *sn*-1,3 positions (*6*). Similarly, docosahexaenoic (DHA) and arachidonic (ARA) acids present in HMF are mostly located at the *sn*-2 position (60 and 45%, respectively), whereas in commercial formulas they do not have a clear positional distribution, and their distribution in all three positions of the TAGs is almost equivalent (*7*). These differences in the placement of FAs play a crucial role in the absorption, distribution and metabolism of fat by infants (*8,9*). In the case of palmitic acid, a higher absorption and utilization have been observed in human breast milk rich in *sn*-2 palmitic acid, when compared to formulas where it was mainly esterified at the *sn*-1,3 positions (*10*).

Likewise, higher levels of DHA and ARA were found in the brain of newborn rats fed with oils containing these FAs at the *sn*-2 position rather than randomly distributed (*11*). In addition, most of the new commercial formulas are enriched with lauric acid (C12:0) from palm kernel oil (PKO), as a source of medium chain fatty acids (MCFAs) (*12*). Because of their unusual metabolic pathway for absorption via portal transport rather than lymphatic transport, MCFAs can be assimilated faster than LCFAs, providing a more efficient and readily available source of energy to the infants (*13*). Although lauric acid is frequently used as MCFA for food applications, studies on the relationship between chain length and metabolic fate of different FAs have raised doubts about its classification, to the point that some researchers now consider it to be a LCFA rather than a MCFA (*14*). Instead, the use of capric acid (C10:0) from coconut oil and other sources, has been recommended (*15*).

In order to emulate both chemical composition and positional distribution of FAs in HMF, novel TAGs can be enzymatically synthesized by incorporating new molecules or by rearranging the position of the FAs in the original TAGs. These products, commonly known as structured lipids (SLs), can be totally or partially used in the formulation of infant formulas fat analogs. Numerous studies have been conducted over the past years on the production of SLs for infant formula by enzymatic esterification and/or acidolysis reactions of different substrates of vegetable origin (*16-20*). Although these processes successfully achieved high *sn*-2 palmitic acid levels, none of them addressed the effect of positional distribution in the absorption and utilization of DHA and ARA in their design. Others omitted the impact of thermal behavior of the SLs on the commercial production of infant formulas and/or failed to adjust the total level of some FAs in the final composition. Furthermore, all of them lacked high reaction yields because

of the nature of the substrates used (FFAs and ethyl esters), and the type of reaction conducted that promotes an excessive formation of new FFAs or their ethyl esters.

The objective of this study was to prepare an infant formula fat analog with capric acid mostly esterified at the *sn*-1,3 positions and substantial amounts of palmitic acid, DHA, and ARA at the *sn*-2 position, by physically blending previously synthesized SLs with vegetable oils. To achieve this, a proportional amount of each component was added to the blend in order to match as close as possible the fatty acid profile of commercial infant formulas. The components of the blend included an enzymatically produced high *sn*-2 palmitic acid SL enriched with capric acid (SL_{CA}), canola oil (CAO), corn oil (CO), high *sn*-2 DHA ($DHAO_m$), and high *sn*-2 ARA ($ARAO_m$) enzymatically modified oils. We propose that this product be used as an alternative to the fat blends currently used for the production of commercial formulas, because it not only contains the right balance of the important FAs but also at the desirable positions of the TAGs for proper absorption and utilization, thereby contributing to a better nutrition for the infants.

MATERIALS AND METHODS

Materials.

CAO and CO were provided by Team Foods S.A (Bogotá DC, Colombia). SL_{CA}, $DHAO_m$ and $ARAO_m$ were prepared in the laboratory following the methods and reaction conditions reported in our previous studies (*21,22*) (see detailed description below). The lipid standards, Supelco 37 Component FAME mix, α-δ tocopherol, and 2-oleoylglycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The TAG standard mix (GLC-437) was provided by Nu-chek Prep, Inc. (Elysian, MN, USA). Pentadecanoic acid (C15:0) was obtained from TCI America (Portland, OR, USA). Other solvents and chemicals were purchased from Fischer Scientific (Norcross, GA, USA) and Sigma-Aldrich Chemical Co.

Production of SL_{CA}.

A high *sn*-2 palmitic acid SL enriched with capric acid was synthesized at high reaction yield, in two enzymatic interesterification stages as previously reported by our research group (*21*). In the first stage, high melting point palm stearin (HMPS, mp=61.6°C; 70.98% total palmitic acid) and high oleic sunflower oil (HOSO), were blended and reacted in the presence of Lipozyme® TL IM (*sn*-1,3 specific *Thermomyces lanuginosus* lipase). The specific activity of this enzyme was 250 IUN/g at pH 6-8 and temperature of 50-75°C, where IUN stands for interesterification unit novo, as defined by the manufacturer (*23*). The substrate molar ratio used was 1:2 (HMPS:HOSO). The reaction temperature, reaction time, and enzyme load were 60°C, 24 h, and 10 wt% (of the substrate), respectively. For the second stage, the intermediate SL obtained in stage 1 (SL_{int}) was blended and reacted with tricaprin (TC) in a second interesterification reaction, using Lipozyme® TL IM. The substrate molar ratio used was 1.2:0.5 (SL_{int}:TC). The reaction temperature, reaction time, and enzyme load were 60°C, 8 h, and 10 wt% (of the substrate), respectively. Excess free fatty acids (FFAs) in the final product were removed through short-path distillation (SPD) as reported by Pande *et al.* (*19*). The overall reaction yield was nearly 0.9 (90 wt%).

Production of $DHAO_m$ and $ARAO_m$.

$DHAO_m$ and $ARAO_m$ were obtained in independent enzymatic interesterifications using DHA single-cell oil (DHASCO®), and ARA single cell oil (ARASCO®) as substrates, respectively. Both processes followed the conditions previously reported by our research group (*22*). For $DHAO_m$, a lipase mix of Lipozyme® TL IM and Novozym® 435 (non-specific lipase), was used as biocatalyst. The specific activity of Novozym® 435 was 10000 PLU/g at pH 5-9 and temperature of 30-60°C, where PLU stands for propyl laurate unit, as defined by the

manufacturer (*23*). The reaction was conducted for 4 h at 48°C with 10 wt% of enzyme load. For $ARAO_m$ the lipase system was the same and the reaction was conducted for 4 h at 44°C with 15% of enzyme load. Excess FFAs in the final products were removed through SPD. The reaction yields for $DHAO_m$ and $ARAO_m$ were 0.93 (93wt%), and 0.94 (94 wt%), respectively.

Preparation of Infant Formula Fat Analog by Physical Blending.

The infant formula fat analog ($IFFA_1$) was obtained by proportionally blending SL_{CA}, CAO, CO, $DHAO_m$, and $ARAO_m$, so it could closely match the fatty acid profile found in commercial fat blends used for infant formula. In order to determine the proportion at which each component was to be added, a weighted arithmetic mean approach was followed for every fatty acid, where the individual contribution of each oil to the desired fatty acid profile was counted depending on its weight in the oil blend (relative to the content of the other oils), following the equation:

$$\%FA_{Blend} = [\, FA_{SLCA} * X_{SLCA} + FA_{CAO} * X_{CAO} + FA_{CO} * X_{CO} + FA_{DHAOm} * X_{DHAOm} + FA_{ARAOm} * X_{ARAOm}] \quad \text{(Eq. 1).}$$

Where $\%FA_{Blend}$ is the amount of the corresponding FA in the blend (mol%), FA_{SLCA} is the amount of the FA in SL_{CA} (mol%), X_{SLCA} is the fraction of SL_{CA} in the blend, FA_{CAO} is the amount of the FA in CAO (mol%), X_{CAO} is the fraction of CAO in the blend, and so forth. Since X_{SLCA}, $X_{CAO,}$ $X_{CO,}$ $X_{DHAOm,}$ and X_{ARAOm} are constant in the equations generated for each fatty acid (blending proportions do not change), an iterative optimization for these values was conducted using a spreadsheet application, where fatty acids were simultaneously balanced out to fall as close as possible within the range of commercial infant formula fats. Final blending proportions were used to prepare $IFFA_1$ and compare observed and predicted fatty acid profiles. Further characterization of positional fatty acid composition, TAG molecular species, thermal behavior, and tocopherol content was also performed.

Determination of Fatty Acid Profiles.

Both blending oils and final product were converted to FA methyl esters (FAMEs) following AOAC Official Method 996.01, Section E (*24*) with minor modifications. 0.1 g of sample was weighed into Teflon-lined test tubes and 0.20 mL internal standard (C15:0, 20 mg/mL in hexane) was added and dried under nitrogen to remove solvent. 2 mL of 0.5 N NaOH in methanol was added and heated to 100 °C for 10 min for saponification of free fatty acids. The sample was cooled under tap water and 2 mL BF_3 in methanol was added followed by vortexing for 1 min. The sample was then heated at 100°C for 10 min for methylation and cooled under tap water. To stop the reaction and extract the FAMEs, 2 mL of hexane and 2 mL of saturated NaCl solution were added. The sample was vortexed for 2 min and centrifuged at 1000 rpm for 5 min to separate the organic layer from the aqueous layer. The organic layer was passed through an anhydrous sodium sulfate column into a GC vial for analysis. The FAME external standard used was Supelco 37 component FAME mix and was run in parallel with the samples.

Positional Analysis.

The pancreatic lipase-catalyzed *sn*-2 positional analysis of blending oils and final product was performed following a modified version of the Luddy *et al.* method (*25*). 100 mg of sample (extracted and dried TAG) was placed into Teflon-lined test tubes. 2 mL of 1.0 M Tris-HCl buffer (pH=8), 0.5 mL of 0.05% sodium cholate solution, and 2.2% calcium chloride solution were added, and the mixture was vortexed for 2 min to emulsify. Then, 40 mg of pancreatic lipase was added, and the mixture was vortexed for 1 min and incubated in a water bath at 40°C for 3 min while shaking at 200 rpm. The sample was vortexed again for 2 min. To stop the reaction and extract the hydrolyzed TAGs, 1 mL of 6 N HCl and 4 mL of diethyl ether were added. The sample was vortexed for 2 min and centrifuged at 1000 rpm for 3 min. The upper

layer, containing the lipid components, was filtered through an anhydrous sodium sulfate column. The sample was concentrated under nitrogen until approximately one-third of the original volume was left. A mixture of hexane, diethyl ether, and formic acid (60:40:1.6, v/v/v) was used as the mobile phase after equilibration in the TLC tanks for approximately 30 min. The concentrated sample was spotted onto an activated silica gel G dried TLC plate and placed into the tank. 2-Oleyglycerol was spotted as the standard and run in parallel with the sample for identification of the 2-monoacylglycerol (2-MAG) band. The plate was sprayed with 0.2% 2,7 dichlorofluorescein in methanol and visualized under UV light. The 2-MAG band was scrapped off and converted to FAME (as described above). The fatty acid profile at the *sn*-2 position was quantified using internal standard method after GC analysis. The fraction of the FA that is esterified at the *sn*-2 position (out of the total content of that FA in the sample) was calculated using the following equation:

FA *sn*-2 (%)=[100*total FA at *sn*-2 (mol%)]/[3* total FA in sample (mol%)] (Eq. 2)

GC Analysis.

Fatty acid composition of all samples were analyzed as FAMEs following the method of Pande *et al.* (*26*) with a Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA), and using a Supelco SP-2560, 100 m x 25 mm x 0.2 μm column. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. The injection volume was 1 μL and a split ratio of 20:1 was used. Detection was performed with a flame ionization detector at 300°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 25 min. All samples were analyzed in triplicate and average values reported.

Triacylglycerol (TAG) Molecular Species.

TAG species were identified using HPLC (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA), equipped with an evaporative light scattering detector (ELSD) Sedex 85 (Richard Scientific, Novato, CA, USA). The reverse phase column was a Beckman Ultrasphere ® C18, 5 μm, 4 mm x 250 mm with the temperature set at 30°C. The sample was dissolved in chloroform with a final concentration of 5 mg/mL. 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 65 to 95% solvent B in 55 min followed by a 10 min post-run at 65% B. The drift tube temperature was set at 70°C, pressure at 3.0 bar and gain at 8. 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. Relative percent (%) of peak areas of the TAGs identified were reported as the average of three determinations.

Melting and Crystallization Behavior.

The melting completion temperature and crystallization onset temperature of $IFFA_1$ were determined using a differential scanning calorimeter DSC 204 F1 Phoenix (NETZSCH Instruments North America, Burlington, MA) following AOCS Official Method Cj 1-94 (*27*). First, 8-12 mg of the sample was weighed into an aluminum pan and hermetically sealed. Sample was 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 profile), and held for 30 min and finally heated to 80°C at 10°C/min (for melting profile). Nitrogen was used as protective gas (purge). All samples were analyzed in triplicate and average values reported.

Tocopherol Analysis.

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

Statistical Analysis.

All analyses were performed in triplicate. Statistical analysis was performed with SAS software package (SAS Institute, Cary, NC). Duncan´s multiple-range test was performed to determine the significant difference ($P \leq 0.05$) among expected and observed fatty acid profiles of the blend.

RESULTS AND DISCUSSION

Preparation of Infant Formula Fat Analog.

$IFFA_1$ was obtained by proportionally blending SL_{CA}, CAO, CO, $DHAO_m$, and $ARAO_m$ using Eq.1. The final proportions of the components were determined to be (for 1 mol of oil blend): SL_{CA} (70%), CAO (8%), CO (20%), $DHAO_m$ (1%), and $ARAO_m$ (1%). With these proportions, the expected fatty acid profile of the oil blend generated by the model was: capric (13.87 mol%), palmitic (17.22 mol%), stearic (1.66 mol%), oleic (48.60 mol%), linoleic (15.82 mol%), linolenic (1.01 mol%), ARA (0.57 mol%), and DHA (0.47 mol%). A Duncan´s multiple-range

test was performed to determine differences between expected and observed ($IFFA_1$) fatty acid profiles (data not shown). The results showed no significant difference at $P \leq 0.05$, confirming the validity of the model used to calculate the proportions for oil blending.

Total and Positional Fatty Acid Profiles.

Table 5.1 shows the total fatty acid composition of blending oils and final product ($IFFA_1$) using the optimal proportions previously established. SL_{CA} is a high *sn*-2 palmitic acid SL enriched with capric acid (21.22 mol%), obtained by enzymatic interesterifications of HMPS, HOSO and TC. It also contained 20.13 mol% palmitic, 52.98 mol% oleic, and 3.36 mol% linoleic acids. CAO is considered a polyunsaturated oil containing 62.75 mol% oleic, 22.96 mol% linoleic, and 7.80 mol% linolenic acids. The major FA in CO was linoleic (55.28 mol %), followed by oleic (30.13 mol%), and palmitic (12.45 mol%). $DHAO_m$, which is a SL obtained by enzymatic interesterification of DHASCO® (single-cell oil from *Crypthecodinium cohnii*), contained 46.53 mol% DHA, 23.96 mol% oleic acid, and 10.57 mol% myristic acid. $ARAO_m$, obtained by enzymatic interesterification of ARASCO® (single cell oil from *Mortierella alpina*), contained 47.25 mol% of ARA, 22.62 mol% of oleic acid, and 9.85 mol% of palmitic acid. The reason for choosing these oils as components of the blend was their high content of specific fatty acids that were required to successfully match the profile of interest. Therefore, SL_{CA} mainly contributed to adjust palmitic and capric acids, CAO to adjust linolenic acid, CO to adjust linoleic acid, and $DHAO_m$ and $ARAO_m$ to adjust DHA and ARA, respectively. The total fatty acid profile of the final oil preparation ($IFFA_1$) was capric (13.93 mol%), palmitic (17.37 mol%), stearic (1.64 mol%), oleic (48.23 mol%), linoleic (15.91 mol%), linolenic (1.04 mol%), ARA (0.57 mol%), and DHA (0.49 mol%). **Table 5.1** also shows the FA content range in both commercial fat blends used for infant formula and HMF as reported by López-López *et al.* (*12*). According to

these values, all the most relevant fatty acids present in $IFFA_1$ are within ranges of commercial formulas and HMF, except for oleic, capric acids, lauric and myristic acids. In the case of oleic, it fell slightly out of the upper limit of both ranges (34.34-44.69 mol% in commercial fat blends, and 28.30-43.83 mol% in HMF). This can be attributed to the fact that the predominant component of the blend to prepare $IFFA_1$ was SL_{CA}, which contained a very high level of oleic acid (approx 53 mol%). Reducing the amount of SL_{CA} in the blend below 70% to better adjust the content of oleic acid, would have been detrimental to keeping the content of other (more important) fatty acids such as capric and palmitic acids, within the ranges of interest. The difference in content with commercial formulas and HMF was only 3.54 and 4.4 percentage points, respectively. Therefore, its impact on the whole formulation is considered minimal and may in fact be advantageous as there is no scientific evidence associated with metabolic anomalies due to consumption of oleic acid. On the contrary, it has been reported that unsaturated fatty acids are significantly better absorbed, metabolized, and utilized by infants, compared to saturated fatty acids (*6*,*28*). In the case of capric acid, neither commercial formulas nor HMF contained amounts that can be considered relevant (0.74-1.24 mol% and 0.85-3.08 mol%, respectively). $IFFA_1$ contains approx 14 mol% of capric acid as a source of MCFAs. Because of their unusual metabolic pathway for absorption via portal transport rather than lymphatic transport, MCFAs can be assimilated faster than LCFAs, providing a more efficient and readily available source of energy to the infants. Furthermore, it has been reported that MCFAs protect newborns from harmful microorganisms as they exhibit antiviral and antimicrobial properties (*29*). Unlike $IFFA_1$, HMF naturally contains lauric (4.04-9.35 mol%), and myristic (3.60-9.13 mol%) acids. Commercial infant formulas also contain lauric (5.19-12.64 mol%), and myristic (3.06-5.91 mol%) acids, both provided by adding PKO to the formulation.

Since the chain length of lauric acid (12 carbons) is considerably different from palmitic (16 carbons), and stearic, oleic, linoleic, and linoleic (18 carbons), this fatty acid is frequently used as a source of MCFAs for infant food applications. However, studies on the relationship between chain length and metabolic fate of different FAs have raised doubts about the classification of lauric acid, to the point that some researchers consider it to be a LCFA rather than a MCFA (*14*). Instead, the use of capric acid (10 carbons) has been recommended (*15*).

Table 5.2 shows the *sn*-2 positional fatty acid composition of blending oils, final product, commercial formulas, and HMF. According to the results, 37.57% of the total palmitic acid observed in SL_{CA} (20.13 mol%) was esterified at the *sn*-2 position, while nearly 76% of the total capric acid (21.22 mol%) was esterified at the *sn*-1,3 positions. In the case of CAO, around one half of the total linolenic acid (7.80 mol%) was at the *sn*-2, whereas in CO, 62% of the linoleic acid (55.28 mol%) was at the outer positions. Both $DHAO_m$ and $ARAO_m$ contained very significant amounts of *sn*-2 DHA and *sn*-2 ARA (49.70% and 36.08%, respectively). Since the process of physical blending does not involve any modifications in the structure of molecules, the placement of the fatty acids in the TAGs of blending oils remained invariable. Therefore, the positional distribution of FAs in the final product only depends on the proportions in which these oils were added. $IFFA_1$ contained 34.21% of total palmitic (17.37 mol%), 48.18% of total DHA (0.49 mol%), and 35.80% of total ARA (0.57 mol%) esterified at the *sn*-2 position. Additionally, it contained more than 80% of the total capric acid (13.93 mol%) esterified at the *sn*-1,3 positions. When comparing the amount of *sn*-2 palmitic acid in $IFFA_1$ to that found in commercial infant formulas, a very substantial difference of 20.78 percentage points (34.21% against 13.43%) can be observed. TAGs containing *sn*-2 palmitic acid are preferred in infant formula fat analogs because of their easier absorption. A number of digestive enzymes are

involved in the breakdown of the human milk TAGs. As a result of the pancreatic lipase activity, all FAs in the *sn*-1,3 positions of TAGs are hydrolyzed during digestion, in contrast to only ~20% of fatty acids in the *sn*-2 position (*30*). If palmitic acid is predominantly esterified at *sn*-1,3 positions, it is released as free palmitic acid. At the alkaline pH of the intestine, free palmitic acid readily forms insoluble soaps with divalent cations such as calcium that cannot be absorbed in the small intestine and are excreted as hard stools. This results in not just unavailability of both palmitic acid and calcium for the infants but also in constipation problems (*31*). When comparing the amounts of *sn*-2 DHA and *sn*-2 ARA in $IFFA_1$ to those found in commercial infant formulas, there are substantial differences of 13.59 (48.18% against 34.59), and 6.76 (35.80% against 29.04%) percentage points, respectively. Infant formula fat analogs containing *sn*-2 DHA and *sn*-2 ARA might be preferred against conventional formulas because of the more efficient delivery of DHA and ARA to the infants. According to Ramírez *et al.* (*9*), differences in the TAG positional arrangement of DHA and ARA from dietary lipid sources may affect their absorption, distribution and tissue uptake. This could be explained because when DHA and ARA are esterified at outer positions of the TAG molecules, they tend to induce resistance to pancreatic lipase, and therefore, their rate of absorption is reduced (*32*). Based on the results for total and positional fatty acid composition, it can be concluded that $IFFA_1$ represent a better alternative to most commercial formulas currently available in the market. It is worth noting (**Table 5.2**) that the amount of palmitic acid, DHA and ARA at the *sn*-2 position are still below those found in HMF (approximately 67% of total palmitic acid (24.46 mol%), 60% of total DHA (0.56 mol%), and 45% of total ARA (0.75 mol%) are esterified at the *sn*-2).

There are other studies on the production of infant formula fat analogs. Recently, Zou *et al.* (*20*) reported the preparation of a human milk fat substitute by blending a high *sn*-2 palmitic

acid SL with sunflower, rapeseed, palm kernel, and single-cell oils. The final product contained 61.10% of total palmitic acid (23.50 mol%), 35.60% of total DHA (0.30 mol%), and 30.0% of total ARA (0.40 mol%) esterified at the *sn*-2 position. It also contained 9.60 mol% of lauric, 34.60 mol% oleic, 19.10 mol% linoleic, and 3.7 mol% of linolenic acids, mostly esterified at *sn*-1,3 positions. Pande *et al.* (*19*) reported the synthesis of infant formula fat analogs from virgin olive oil-based SLs. The final product contained 47.85% of total palmitic acid (36.69 mol%), 16.99% of total DHA (1.53 mol%), and 20.43% of total ARA (3.67 mol%) esterified at the *sn*-2 position. It also contained 43.22 mol% oleic, 6.34 mol% linoleic, and 0.47 mol% of linolenic acids, mostly esterified at *sn*-1,3 positions. Other reports on the preparation of infant formula fat analogs include Zou *et al.* (*16*), Wang *et al.* (*17*), and Teichert *et al.* (*18*). Although most of these products achieved considerably higher *sn*-2 palmitic acid levels than $IFFA_1$, they also contained higher levels of total palmitic acid (in the case of Pande *et al.* (*19*), and Teichert *et al.* (*18*), significantly above the range of current infant formulas), which eventually would represent extra content of palmitic acid at the *sn*-1,3 positions that might not be efficiently absorbed. However, they can be used to blend with other oils prior to infant formula preparations. Additionally, none of the products addressed the effect of positional distribution in the absorption and utilization of DHA and ARA, nor did they contain relevant amounts of capric acid as a source of MCFAs. Instead, the preparation of Zou *et al.* (*20*) provided around 13 wt% of combined lauric and myristic acids from PKO, but as discussed before, they may not provide the metabolic and physiologic effects of capric acid as MCFA. Moreover, total levels of linoleic and linolenic acids reported by Zou *et al.* (*20*), lauric, linoleic, linolenic, DHA, and ARA reported by Pande *et al.* (*19*), and lauric, palmitic, linoleic, and linolenic reported by Wang *et al.* (*17*), fall outside the ranges of commercial formulas according to reference (*12*). Also, all of the products lacked high

reaction yields because of the nature of the substrates used (FFAs), and the type of reaction conducted (enzymatic acidolysis) to obtain the high *sn*-2 palmitic SLs. Acidolysis reactions promote excessive formation of new FFAs that must be removed from the SL to obtain a refined product. Lower reaction yields imply more operating expenses and lower return, making the products somewhat less economically feasible for industrial purposes.

TAG Molecular Species.

The TAG molecular species and their relative content in blending oils, final product, commercial formulas, and HMF are shown in **Table 5.3**. The fatty acids in the TAG species analyzed are not in any specific order on the glycerol backbone. SL_{CA} contained a mixture of 15 different types of TAGs as a result of the enzymatic interesterification processes that placed palmitic acid at the *sn*-2 position, and capric at the *sn*-1,3 positions. The main TAGs present in SL_{CA} were OPO (28.06%), POP (18.79%), CLC (11.13%), OOO (10.42%), and COC (7.63%). CAO, which is a liquid oil, contained mostly OOO (31.85%), OLO (21.74%), OLL (10.61%), and PLO (7.40%) type of TAGs. CO, also a liquid oil, contained mostly PLO (16.94%), SLO (14.39%), OOO (14.42%), and PLL (13.09%). $DHAO_m$, which was enzymatically structured to contain high levels of *sn*-2 DHA, showed 16 types of TAGs including PDO (21.76%), ODO (19.43%), MDO (16.16%), and DMP (7.19%). $ARAO_{m,}$ which was structured to contain higher levels of *sn*-2 ARA, contained for the most part OAL (23.09%), OAO (13.44%), PAS (12.25%), and PPA (11.30%). Since the process of physical blending does not involve any modifications in the structure of molecules, the TAGs species present in the final product depend only on the proportions in which blending oils were added. Thus, $IFFA_1$ contained 28 different types of TAGs corresponding to all the contributions made by the different oils used in the blend. The most relevant were OPO (24.09%), POP (15.70%), OOO (11.53%), CLC (7.79%), PLO (5.82%),

OLO (5.58%), and COC (5.34%). Additionally, TAGs species containing DHA and ARA included OAL (0.23%), PDO (0.22%), ODO (0.19%), and MDO (0.16%). In terms of degree of unsaturation, $IFFA_1$ contained UUU (triunsaturated, 19.03%), SSS (trisaturated, 3.15%), SUS (disaturated-monounsaturated, 41.92%), and SUU (monosaturated-diunsaturated, 43.15%).

Of all the nutrients of breast milk, HMF shows the greatest variability. The major TAG molecular species (**Table 5.3**) found in HMF are OPO (17.56-42.44%), PLO (9.24-38.15%), OOO (1.61-11.96) and OLO (1.64-10.18%) (*33*). $IFFA_1$ contained all the TAGs of HMF within levels (see **Table 5.3**) except for PLO, which fell below the range (3.42 percentage points). A similar result was found by Pande *et al.* (*19*), who reported PLO levels of 6.28% in an olive oil-based infant formula fat analog. This can be explained by the low level of PLO in SL_{CA}, which is the major component of the blend (70%). Although CO and CAO contained significant amounts of PLO (16.94% and 7.40%, respectively), their contribution to the blend was not high enough (20% and 8%, respectively), to increase PLO levels in $IFFA_1$ and fall within HMF range. In contrast, Zou *et al.* (*20*) reported a 15.40% of PLO (which fell within the range) in a HMF substitute prepared by blending a high *sn*-2 palmitic SL with vegetable oils. However, the same product contained 14.9% OPO, which fell below HMF range. Because the fatty acids are not in specific order on the glycerol backbone, different isomers exist for each TAG species, and therefore much attention should be paid to total and positional fatty acid profiles, when evaluating suitability of infant formula fat analogs. However, as discussed below, TAG profile can greatly influence the thermal properties of the oils.

Melting and Crystallization Behavior.

The melting completion and crystallization onset temperatures of blending oils, final product, and HMF are shown in **Table 5.4**. Fats with high content of long-chain saturated fatty acids have

higher completion melting temperatures (T_{mc}) than those containing short and medium chains, and/or unsaturated fatty acids (*34*). SL_{CA}, which contained 41.92% SUS, and 38.75% SUU type of TAGs, had a melting completion temperature of 27.61°C. CAO, which is liquid oil with ~70% of triunsaturated TAGs (mostly OOO and OLO), was completely melted at 3.42°C. A slightly higher melting temperature was observed for CO (7.31°C) due to its lower content of UUU (21.06%), and higher content of SUU (59.70%). $DHAO_{m,}$ which contained high levels of DHA, oleic, myristic, and palmitic acids in the form of SUU (47.08%), SUS (23.22%), and UUU (21.59%), had a T_{mc} of 4.55°C. $ARAO_{m,}$ which was rich in UUU (59.87%), and SUS (29.96%), had a melting temperature of 6.25°C. Once the oils were proportionally blended, the melting completion temperature of $IFFA_1$, which contained mainly SUS and SUU type of TAGs, was 18.65°C. Although the T_{mc} of a blend is mainly governed by the major component, some interactions between TAGs of the different blending oils can occur affecting the crystallization patterns. TAG molecules adopt different arrangements in relation to their neighbors to optimize intramolecular and intermolecular interactions and achieve efficient close-packing. The occurrence of these arrangements in a blend is highly influenced by the chain length and the degree of unsaturation of the FAs, which define the symmetry of the TAGs structures (*35*).

Both HMF and oil blends used for commercial formulas are completely melted at normal body temperature (about 37°C) (*10,36*). $IFFA_1$ had a T_{mc} significantly below this value, which may be helpful in the formulation of products with a proper consistency and texture for the infants. On the contrary, HMF analogs with high T_{mc} are difficult to handle, to transport and to combine with other non-lipidic ingredients in a formulation. This might be the case of the SLs synthesized by Pande *et al.* (*19*), and Zou *et al.* (*16*), in which high levels of PPP (10% and 5%, respectively) were found, and consequently, high T_{mc} values were expected. Additionally, the

crystallization onset temperature (T_{co}) for SL_{CA}, CAO, CO, $DHAO_m$, $ARAO_m$, and $IFFA_1$ were 6.12, -14.26, -11.0, 2.55, 3.54, and -2.19°C, respectively. As expected, SL_{CA} which contained higher levels of long-chain saturated fatty acids crystallized at higher temperature than CAO, CO, $DHAO_m$, and $ARAO_m$, which are mainly made of unsaturated molecules. The crystallization behavior of $IFFA_1$ was dependent on the proportions of the blending oils, and the interaction between the different types of TAGs present in the blend.

Tocopherol content.

Tocopherols and tocotrienols, commonly grouped as vitamin E, are the major lipid-soluble, membrane-localized antioxidants in humans. HMF does not contained a significant amount of Vitamin E (less than 10 μg/g) and for that reason, is very susceptible to lipid oxidation (*37*). **Table 5.5** shows the tocopherol content of blending oils and final product. The major vitamin E isomers in SL_{CA} were δ-tocopherol (443.68 μg/g), α-tocopherol (182.62 μg/g), and α-tocotrienol (163.24 μg/g). In the case of CAO, the major isomers were γ-tocopherol (372.36 μg/g), α-tocopherol (238.36 μg/g), and δ-tocopherol (223.26 μg/g), and for CO, the major isomers were γ-tocopherol (403.25 μg/g), α-tocopherol (22.05 μg/g), and δ-tocopherol (168.27 μg/g). The total vitamin E content of $DHAO_m$ and $ARAO_m$ were 521.02 μg/g (mostly δ-tocopherol and γ-tocopherol), and 630.0 μg/g (mostly δ-tocopherol and γ-tocopherol), respectively. The total vitamin E content of $IFFA_1$ was 843.43 μg/g (mostly δ-tocopherol and γ-tocopherol). Interestingly, SL_{CA} was the only component of the blend that showed significant amounts of α-tocotrienol. This can be attributed to the HMPS used as substrate for its production. HMPS is an edible fat obtained by the dry fractionation process of palm oil (*38*), which can contain up to 240 μg/g of tocotrienols (*39*). $DHAO_m$ and $ARAO_m$ showed high levels of total vitamin E. This also can be attributed to the substrates used for their production. As DHASCO® and ARASCO®

contain very high levels of LC-PUFA (extremely prone to lipid oxidation), they already contained indigenous tocopherols (>500 μg/g) for protection. Although previous studies have shown that tocopherols and tocotrienols were mainly lost as tocopheryl and tocotrienyl esters during interesterification and acidolysis reactions (*40,41*), SL_{CA}, $DHAO_m$, and ARAOm, still conserved a considerable amount of total vitamin E. As SL_{CA} is the major component of the blend, most of its total tocopherols and tocotrienols were transferred to $IFFA_1$. The final content of vitamin E in $IFFA_1$ prepared in this study is considered enough to protect it from oxidative deterioration. However, depending on the contents established for standardized commercial infant formulas, this level may be adjusted.

CONCLUSION

Commercial formulas are the most convenient substitutes for human milk when infants cannot be breastfed. However, differences in positional distribution of FAs in vegetable oils and HMF lead to complications related to the absorption, metabolism, and utilization of fats. Infant formula fat analogs that contain palmitic acid, DHA and ARA predominantly at the *sn*-2 position, and which are also enriched with capric acid as a source of MCFAs, can be used in the formulation of new commercial formulas that fit closer to the nutritional needs of the infants for appropriate growth and development. Since $IFFA_1$ possesses these characteristics, it may be suitable for commercial production.

ACKNOWLEDGEMENTS

We thank Team Foods S.A. for providing the canola and corn oils. This research was supported in part by Food Science Research, University of Georgia.

REFERENCES

(1) Jensen, R.G. The lipids of human milk. CRC Press, Boca Raton, FL, **1989**.

(2) Wells, J.C.K. Nutritional considerations in infant formula design. *Semin. Neonatal.* **1996**, *1*, 19–26.

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

(4) Scott, C.E. Structured lipid: A functional and fundamental ingredient to more closely matches infant formula to breast milk. *Nutrafoods* **2010**, *9*, 7-13.

(5) Innis, S.M. Essential fatty acids in growth and development. *Prog. Lipid. Res.* **1991**, *30*, 39–103.

(6) Bracco, U. Effect of triglyceride structure on fat absorption. *Am. J. Clin. Nutr.* **1994**, *60*, 1002S-1009S.

(7) Myher, J.J.; Kuksis, A.; Geher, K.; Park, P.W.; Diersen-Schade, D.A. Stereospecific analysis of triacylglycerols rich in long-chain polyunsaturated fatty acids. *Lipids* **1996**, *31*, 207– 215.

(8) Lucas, A.; Quinlan, P.; Abrams, S; Ryan, S; Meah, S; Lucas, P.J. Randomised controlled trial of a synthetic triglyceride milk formula for preterm infants. *Arch. Dis. Child. Fetal Neonatal* **1997**, *77*, F178-184.

(9) Ramírez, M.; Amate, L.; Gil, A. Absorption and distribution of dietary fatty acids from different sources. *Early Hum. Dev.* **2001**, *65*, S95-S101.

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

(11) Christensen, M.M.; Høy, C.E. Early dietary intervention with structured triacylglycerols containing docosahexaenoic acid. Effect on brain, liver, and adipose tissue lipids. *Lipids* **1997**, *32*, 185−191.

(12) López-López, A.; López-Sabater, M.C.; Campoy-Folgoso, C.; Rivero-Urgell, M. Castellote-Bargalló, A.I. Fatty acid and *sn-2* fatty acid composition in human milk from Granada (Spain) and in infant formulas. *Eur. J. Clin. Nutr.* **2002**, *56*, 1242–1254.

(13) Babayan, V.K. Medium chain triglycerides. In Babayan VK, Beare-Rogers J (eds) *Dietary fat requirements in health and development*. American Oil Chemists' Society Press, Champaign, IL, **1988**; pp. 73-86.

(14) St-Onge, M.P.; Mayrsohn, B.; O'Keeffe, M.; Kissileff, H.R.; Choudhury, A.R.; Laferrère, B. Impact of medium and long chain triglycerides consumption on appetite and food intake in overweight men. *Eur. J. Clin. Nutr.* **2014**, *68*, 1134-1140.

(15) St-Onge, M.P.; Ross, R.; Parsons, W.D.; Jones, P.J. Medium-chain triglycerides increase energy expenditure and decrease adiposity in overweight men. *Obes. Res.* **2003**, *11*, 395-402.

(16) Zou, X.; Huang, J.; Jin, Q.; Guo, Z.; Liu, Y.; Cheong, L.; Xu, X.; Wang, X. Characterization and oxidative stability of human milk fat substitutes enzymatically produced from palm stearin. *J. Am. Oil Chem. Soc.* **2013**, *91*, 481–495.

(17) Wang, X.; Huang, J.; Jin, Q.; Song, Z.; Liu, Y.; Zou, X. Lipase-catalyzed synthesis of human milk fat substitutes from palm stearin in a continuous packed bed reactor. *J. Am. Oil Chem. Soc.* **2012**, *89*, 1463–1472.

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

(19) Pande, G.; Sabir, J.S.M.; Baeshen, N.A.; Akoh, C.C. Enzymatic synthesis of extra virgin olive oil based infant formula fat analogues containing ARA and DHA: one-stage and two-stage syntheses. *J. Agric. Food Chem.* **2013**, *61*, 10590−10598.

(20) Zou, X.Q.; Huang, J.H.; Jin, Q.Z.; Liu, Y.F.; Tao, GJ.; Cheong, L.Z.; Wang, X.G. Preparation of human milk fat substitutes from palm stearin with arachidonic and docosahexaenoic acid: combination of enzymatic and physical methods. *J. Agric. Food Chem.* **2012**, *60*, 9415−9423.

(21) Álvarez, C.A.; Akoh, C.C. Enzymatic synthesis of infant formula fat analog enriched with capric acid. *J. Am. Oil Chem. Soc.* **2015**, DOI 10.1007/s11746-015-2662-z.

(22) Álvarez, C.A.; Akoh, C.C. Enzymatic production of high *sn*-2 DHA and ARA modified oils for the formulation of infant formula fat analogs. *J. Am. Oil Chem. Soc.* **2015** (submitted for review)

(23) Novozymes Biopharma DK A/S. Enzymes for Biocatalysis: for smarter chemical synthesis. In *http://www.novozymes.com/en/solutions/biopharma/Brochures/Documents/2014-12576-01_Biocatalysis-Product-Sheet-Immobilised-Lipases-2.pdf*. **2014,** accessed 20 Apr 2015.

(24) *Official Methods of Analysis of AOAC International*, 19th ed.; AOAC International: Rockville, MD, **2012**; Official Method 996.01.

(25) Luddy, F.E.; Bardford, R.A.; Herb, S.F.; Magidman, P.; Riemenschneider, R.W. Pancreatic lipase hydrolysis of triacylglycerides as a semi-micro technique. *J. Am. Oil Chem. Soc.* **1964**, *41*, 639–696.

(26) Pande, G.; Sabir, J.S.M.; Baeshen, N.A.; Akoh, C.C. Synthesis of infant formula fat analogs enriched with DHA from extra virgin olive oil and tripalmitin. *J. Am. Oil Chem. Soc.* **2013**, *90*, 1311–1318.

(27) *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 6th ed.; AOCS: Champaign, IL, **2011**; Method Cj 1-94.

(28) Mu, H.; Høy, C.E. Intestinal absorption of specific structured triacylglycerols. *J. Lipid Res.* **2001**, *42*, 792–798.

(29) Francois, C.A.; Connor, S.L.; Wander, R.S.; Connor, W.E. Acute effects of dietary fatty acids on the fatty acids of human milk. *Am. J. Clin. Nutr.* **1998**, *67*, 301-308.

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

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

(32) Bottino, N.R.; Vandenburg, G.A.; Raiser, R. Resistance of certain long-chain polyunsaturated fatty acids of marine oils to pancreatic lipase hydrolysis. *Lipids* **1967**, *2*, 489 – 493.

(33) Morera, S.; Castellote, A.I.; Jauregui, O.; Casals, I.; López-Sabater, M.Triacylglycerol markers of mature human milk. *Eur. J. Clin. Nutr.* **2003**, *57*, 1621−1626.

(34) Strayer, D. Food fats and oils. In *Publication of Institute of Shortening and Edible Oils*. Washington DC, United States, **2006**; pp. 1-37.

(35) Sato, K. Polymorphism in Fats and Oils In *Bailey's Industrial Oil and Fat Products*. John Wiley & Sons, Inc. Hiroshima, Japan, **2005**.

(36) Jensen, R.G.; Patton, S. The effect of maternal diets on the mean melting points of human milk fatty acids. *Lipids* **2000**, *35*, 1159-1161.

(37) Barbas, C.; Herrera, E. Lipid composition and vitamin E content in human colostrum and mature milk. *J. Physiol. Biochem.* **1998**, *54*, 167−173.

(38) Son, J.M.; Lee, K.T.; Akoh, C.C.; Kim, M.R.; Kim, M.J.; Lee, J.H. Optimization of tripalmitin-rich fractionation from palm stearin by response surface methodology. *J. Sci. Food Agric.* **2010**, *90*, 1520–1526.

(39) Mozzon, M.; Pacetti, D.; Giuseppe-Frega, N.; Lucci, P. Crude palm oil from interspecific hybrid *Elaeis oleifera* x *E. guineensis*: alcoholic constituents of unsaponifiable matter. *J. Am. Oil Chem. Soc.* **2015**, *92*, 717–724.

(40) Zou, L.; Akoh, C.C. Identification of tocopherols, tocotrienols, and their fatty acid esters in residues and distillates of structured lipids purified by short-path distillation. *J. Agric. Food Chem.* **2012**, *61*, 238−246.

(41) Hamam, F.; Shahidi, F. Acidolysis reactions lead to esterification of endogenous tocopherols and compromised oxidative stability of modified oils. *J. Agric. Food Chem.* **2006**,*54*,7319−7323.

Table 5.1 Total fatty acid composition (mol%) of blending oils and final product[a,b]

Fatty acid	SL_{CA}[c]	CAO[d]	CO[e]	$DHAO_m$[f]	$ARAO_m$[g]	$IFFA_1$[h]	Commercial formulas[i] (n=11)		HMF[j] (n=40)	
							Low	High	Low	High
C8:0	nd[k]	nd	nd	nd	nd	nd	0.51	1.20	0.11	0.36
C10:0	21.22 ± 0.74	nd	nd	1.25 ± 0.01	nd	13.93 ± 0.15	0.74	1.24	0.85	3.08
C12:0	nd	nd	nd	5.00 ± 0.01	nd	0.12 ± 0.00	5.19	12.64	4.05	9.35
C14:0	0.38 ± 0.00	0.07 ± 0.00	0.04 ± 0.00	10.57 ± 0.05	0.30 ± 0.02	0.47 ± 0.01	3.06	5.91	3.60	9.13
C16:0	20.13 ± 0.26	5.55 ± 0.01	12.45 ± 0.01	9.22 ± 0.05	9.85 ± 0.13	17.37 ± 0.16	15.96	25.75	15.43	24.46
C16:1n7	nd	nd	0.10 ± 0.00	2.72 ± 0.03	nd	0.16 ± 0.03	nd	nd	nd	nd
C18:0	1.69 ± 0.02	0.88 ± 0.01	0.78 ± 0.02	0.26 ± 0.10	4.79 ± 0.02	1.64 ± 0.05	1.05	4.72	4.60	8.13
C18:1n9	52.98 ± 0.67	62.75 ± 0.02	30.13 ± 0.01	23.96 ± 0.17	22.62 ± 0.31	48.23 ± 0.19	34.34	44.69	28.30	43.83
C18:2n6	3.36 ± 0.53	22.96 ± 0.01	55.28 ± 0.00	0.48 ± 0.01	7.16 ± 0.02	15.91 ± 0.08	8.93	17.43	10.61	25.30
C18:3n3	0.24 ± 0.02	7.80 ± 0.01	0.95 ± 0.03	nd	3.03 ± 0.01	1.04 ± 0.07	0.67	2.83	0.41	1.68
C20:3n6	nd	nd	nd	nd	3.09 ± 0.09	0.04 ± 0.01	nd	nd	nd	nd
C20:3n3	nd	nd	nd	nd	1.91 ± 0.18	0.04 ± 0.01	nd	nd	nd	nd
C20:4n6	nd	nd	nd	nd	47.25 ± 0.26	0.57 ± 0.13	0.40	0.60	0.23	0.75
C22:6n3	nd	nd	nd	46.53 ± 0.39	nd	0.49 ± 0.06	0.40	0.60	0.15	0.56

[a] Each value is the mean of triplicates ± standard deviation

[b] Trace amounts of C16:1, C17:0, C17:1, C20:0 were too small to be considered

[c] *SL_{CA}*, high *sn*-2 palmitic acid SL enriched with capric acid synthesized as described by reference (*21*)

[d] *CAO*, canola oil

[e] *CO*, corn oil

[f] *$DHAO_m$*, high *sn*-2 DHA oil synthesized as described by reference (*22*)

[g] *$ARAO_m$*, high *sn*-2 ARA oil synthesized as described by reference (*22*)

[h] *$IFFA_1$*, infant formula fat analog prepared by blending SL_{CA} (70%), CAO (8%), CO (20%), $DHAO_m$ (1%), and $ARAO_m$ (1%)

[i] Commercial infant formulas as reported in reference (*12*)

[j] *HMF*, human milk fat as reported in reference (*12*)

[k] *nd*, not detectable

Table 5.2 Positional fatty acid composition (*sn*-2,%) of blending oils and final product[a,b,c]

Fatty acid	SL_{CA}[d]	CAO[e]	CO[f]	$DHAO_m$[g]	$ARAO_m$[h]	$IFFA_1$[i]	Commercial formulas[j] (n=11)		HMF[k] (n=40)	
							Low	High	Low	High
C8:0	nd[l]	nd	nd	nd	nd	nd	1.25	3.11	nd	nd
C10:0	23.67 ± 2.13	nd	nd	9.31 ± 0.48	nd	14.97 ± 0.93	27.11	32.15	5.32	7.11
C12:0	nd	nd	nd	13.98 ± 1.09	nd	30.83 ± 0.39	30.01	42.56	17.18	30.65
C14:0	38.62 ± 1.66	14.85 ± 0.13	32.54 ± 1.37	11.16 ± 1.07	41.70 ± 0.51	30.25 ± 0.27	24.76	30.16	40.11	60.87
C16:0	37.57 ± 0.80	2.65 ± 0.08	4.34 ± 0.03	10.57 ± 0.29	21.99 ± 0.93	34.21 ± 0.35	11.64	13.43	57.11	67.15
C16:1n7	nd	nd	nd	21.19 ± 0.08	nd	43.94 ± 0.18	nd	nd	nd	nd
C18:0	28.32 ± 2.40	6.42 ± 0.56	7.04 ± 0.58	nd	32.79 ± 0.67	29.78 ± 0.96	13.24	15.11	7.13	10.12
C18:1n9	35.31 ± 1.19	29.14 ± 0.13	35.91 ± 0.43	27.35 ± 0.44	27.25 ± 0.35	32.02 ± 0.05	27.74	39.83	8.06	15.13
C18:2n6	30.34 ± 3.93	48.54 ± 0.24	38.75 ± 0.15	23.21 ± 0.27	54.01 ± 1.41	50.57 ± 0.33	39.93	49.21	15.22	25.16
C18:3n3	53.36 ± 3.77	46.68 ± 0.34	39.12 ± 3.43	nd	42.25 ± 1.02	58.67 ± 0.57	48.14	50.21	16.18	20.65
C20:3n6	nd	nd	nd	nd	23.75 ± 1.16	43.41 ± 1.34	nd	nd	nd	nd
C20:3n3	nd	nd	nd	nd	19.91 ± 0.21	28.19 ± 1.22	nd	nd	nd	nd
C20:4n6	nd	nd	nd	nd	36.08 ± 0.19	35.80 ± 0.76	27.96	29.04	42.22	49.75
C22:6n3	nd	nd	nd	49.70 ± 0.50	nd	48.18 ± 1.02	34.01	34.59	56.93	64.11

[a] Each value is the mean of triplicates ± standard deviation

[b] Trace amounts of C16:1, C17:0, C17:1, C20:0 were too small to be considered

[c] *sn*-2 represents % of a particular fatty acid based on the total amount of the same fatty acid in the sample

[d] *SL_{CA}*, high *sn*-2 palmitic acid SL enriched with capric acid synthesized as described by reference (*21*)

[e] *CAO*, canola oil

[f] *CO*, corn oil

[g] *$DHAO_m$*, high *sn*-2 DHA oil synthesized as described by reference (*22*)

[h] *$ARAO_m$*, high *sn*-2 ARA oil synthesized as described by reference (*22*)

[i] *$IFFA_1$*, infant formula fat analog prepared by blending SL_{CA} (70%), CAO (8%), CO (20%), $DHAO_m$ (1%), and $ARAO_m$ (1%)

[j] Commercial infant formulas as reported in reference (*12*)

[k] *HMF*, human milk fat as reported in reference (*12*)

[l] *nd*, not detectable

Table 5.3 Relative percent (%) of peak areas of triacylglycerol (TAG) molecular species in blending oils and final product[a]

TAG	SL_{CA}[b]	CAO[c]	CO[d]	$DHAO_m$[e]	$ARAO_m$[f]	$IFFA_1$[g]	HMF[h] (n=40)	
							Low	High
CCC	0.83 ± 0.02	nd[i]	nd	nd	nd	nd	nd	nd
CLC	11.13 ± 0.14	nd	nd	nd	nd	7.79 ± 0.10	nd	nd
COC	7.63 ± 0.51	nd	nd	nd	nd	5.34 ± 0.36	nd	nd
CPC	4.50 ± 0.71	nd	nd	nd	nd	3.15 ± 0.50	nd	nd
MDM	nd	nd	nd	0.80 ± 0.04	nd	nd	nd	nd
LCL	0.84 ± 0.06	nd	nd	nd	nd	0.59 ± 0.04	nd	nd
AAO	nd	nd	nd	nd	5.31 ± 0.16	nd	nd	nd
MDO	nd	nd	nd	16.16 ± 0.78	nd	0.16 ± 0.01	nd	nd
OALn	nd	nd	nd	nd	5.31 ± 0.32	nd	nd	nd
DMP	nd	nd	nd	7.19 ± 0.13	nd	nd	nd	nd
OCL	2.14 ± 0.13	nd	nd	nd	nd	1.50 ± 0.09	nd	nd
ODO	nd	nd	nd	19.43 ± 0.41	nd	0.19 ± 0.00	nd	nd
PDO	nd	nd	nd	21.76 ± 0.87	nd	0.22 ± 0.01	nd	nd
OAL	nd	nd	nd	nd	23.09 ± 0.10	0.23 ± 0.00	nd	nd
LLL	nd	5.83 ± 0.06	nd	nd	2.26 ± 0.04	0.49 ± 0.00	0.34	0.91
OCO	3.46 ± 0.37	nd	nd	nd	nd	2.42 ± 0.26	nd	nd
OPC	2.00 ± 0.12	nd	nd	nd	nd	1.23 ± 0.08	0.27	1.09
OAO	nd	nd	nd	nd	13.44 ± 0.48	0.13 ± 0.00	nd	nd
OLL	nd	10.61 ± 0.82	nd	0.28 ± 0.07	3.32 ± 0.38	0.88 ± 0.07	0.52	3.51
PPA	nd	nd	nd	nd	11.30 ± 0.93	0.11 ± 0.01	nd	nd
PLL	nd	6.11 ± 0.61	13.09 ± 0.93	nd	nd	3.11 ± 0.14	1.28	3.61
MOM	nd	nd	nd	2.32 ± 0.01	nd	nd	0.35	3.33
MMP	nd	nd	nd	5.82 ± 0.54	nd	nd	nd	nd
PPLa	nd	nd	nd	2.29 ± 0.22	nd	nd	0.4	3.9
OLO	3.58 ± 0.38	21.74 ± 0.69	6.64 ± 0.43	nd	0.33 ± 0.01	5.58 ± 0.31	1.64	10.18
PASt	nd	nd	nd	nd	12.25 ± 0.09	0.12 ± 0.00	nd	nd
PLO	2.63 ± 0.37	7.40 ± 0.17	16.94 ± 0.63	nd	nd	5.82 ± 0.21	9.24	38.15
PLP	nd	1.81 ± 0.53	3.64 ± 0.37	nd	nd	0.87 ± 0.07	1.42	5.38
MOO	nd	nd	nd	3.41 ± 1.10	nd	nd	nd	nd
MOP	nd	nd	nd	6.46 ± 0.70	nd	nd	1.12	5.14
PLSt	nd	7.40 ± 0.21	nd	nd	nd	0.59 ± 0.02	0.98	7.71
OOO	10.42 ± 1.03	31.85 ± 0.37	14.42 ± 0.53	1.89 ± 0.49	6.81 ± 0.27	11.53 ± 0.62	1.61	11.96
OPO	28.06 ± 0.15	nd	11.64 ± 0.40	4.77 ± 1.05	7.52 ± 0.63	24.09 ± 0.05	17.56	42.44
POP	18.79 ± 0.18	1.43 ± 0.11	6.69 ± 0.34	5.84 ± 0.59	3.22 ± 0.12	15.70 ± 0.19	2.15	8.49
PPP	nd	nd	1.57 ± 0.05	nd	nd	nd	nd	nd
StLO	nd	3.12 ± 0.53	15.39 ± 0.42	nd	0.16 ± 0.02	3.33 ± 0.09	1.02	3.11
StOO	1.62 ± 0.03	2.70 ± 0.20	2.65 ± 0.25	0.98 ± 0.27	2.49 ± 0.27	1.91 ± 0.08	0.91	1.95
POSt	2.37 ± 0.23	nd	1.58 ± 0.45	0.63 ± 0.04	3.19 ± 0.18	2.01 ± 0.08	2.09	7.46
StOSt	nd	nd	5.26 ± 0.44	nd	nd	1.05 ± 0.09	0.02	0.09

[a] The fatty acids are not in any specific order on the glycerol backbone. *C*, capric acid. *L*, linoleic acid. *O*, oleic acid. *P*, palmitic acid. *M*, myristic acid. *D*, docosahexaenoic acid. *A*, arachidonic acid. *Ln*, linolenic acid. *La*, lauric acid. *St*, stearic acid. Each value is the mean of triplicates ± standard deviation

[b] *SL_{CA}*, high *sn*-2 palmitic acid SL enriched with capric acid synthesized as described by reference (*21*)
[c] *CAO*, canola oil
[d] *CO*, corn oil
[e] *$DHAO_m$*, high *sn*-2 DHA oil synthesized as described by reference (*22*)
[f] *$ARAO_m$*, high *sn*-2 ARA oil synthesized as described by reference (*22*)
[g] *$IFFA_1$*, infant formula fat analog prepared by blending SL_{CA} (70%), CAO (8%), CO (20%), $DHAO_m$ (1%), and $ARAO_m$ (1%)
[h] *HMF*, human milk fat as reported in reference (*33*)
[i] *nd*, not detectable

Table 5.4 Thermal behavior of blending oils and final product[a]

	T_{mc}[b] (°C)	T_{co}[c] (°C)
SL_{CA}[d]	27.61 ± 0.22	6.12 ± 0.10
CAO[e]	3.42 ± 0.14	-14.26 ± 0.20
CO[f]	7.31 ± 0.15	-11.00 ± 0.17
$DHAO_m$[g]	4.55 ± 0.09	2.55 ± 0.15
$ARAO_m$[h]	6.25 ± 0.11	3.54 ± 0.10
$IFFA_1$[i]	18.65 ± 0.47	-2.19 ± 0.15
HMF[j]	34.7 ± 0.70	nd[k]

[a] Each value is the mean of triplicates ± standard deviation
[b] T_{mc}, melting completion temperature
[c] T_{co}, crystallization onset temperature
[d] *SL_{CA}*, high *sn*-2 palmitic acid SL enriched with capric acid synthesized as described by reference (*21*)
[e] *CAO*, canola oil
[f] *CO*, corn oil
[g] *$DHAO_m$*, high *sn*-2 DHA oil synthesized as described by reference (*22*)
[h] *$ARAO_m$*, high *sn*-2 ARA oil synthesized as described by reference (*22*)
[i] *$IFFA_1$*, infant formula fat analog prepared by blending SL_{CA} (70%), CAO (8%), CO (20%), $DHAO_m$ (1%), and $ARAO_m$ (1%)
[j] *HMF*, human milk fat as reported in reference (*36*)
[k] *nd*, not determined

Table 5.5 Tocopherol content (μg/g) of blending oils and final product[a]

	SL_{CA}[b]	CAO[c]	CO[d]	$DHAO_m$[e]	$ARAO_m$[f]	$IFFA_1$[g]
α-tocopherol	182.62 ± 0.86	238.36 ± 1.30	222.05 ± 0.09	117.00 ± 2.60	122.58 ± 1.64	179.12 ± 1.30
α-tocotrienol	163.24 ± 1.39	86.90 ± 2.86	40.66 ± 1.65	8.71 ± 0.00	7.18 ± 0.43	142.83 ± 0.78
β-tocopherol	14.32 ± 1.32	8.35 ± 0.95	9.55 ± 0.73	11.99 ± 0.07	11.99 ± 0.67	13.03 ± 0.22
γ-tocopherol	26.76 ± 2.92	372.36 ± 1.22	403.25 ± 1.29	138.66 ± 1.77	156.68 ± 0.75	199.10 ± 0.41
δ-tocopherol	443.68 ± 0.71	223.26 ± 1.16	168.27 ± 1.88	244.67 ± 2.14	331.58 ± 1.65	309.36 ± 0.31

[a] Each value is the mean of triplicates ± standard deviation

[b] *SL_{CA}*, high *sn*-2 palmitic acid SL enriched with capric acid synthesized as described by reference (*21*)

[c] *CAO*, canola oil

[d] *CO*, corn oil

[e] *$DHAO_m$*, high *sn*-2 DHA oil synthesized as described by reference (*22*)

[f] *$ARAO_m$*, high *sn*-2 ARA oil synthesized as described by reference (*22*)

[g] *$IFFA_1$*, infant formula fat analog prepared by blending SL_{CA} (70%), CAO (8%), CO (20%), $DHAO_m$ (1%), and $ARAO_m$ (1%)

CHAPTER 6

CONCLUSIONS

For those situations where infants cannot be breastfed, commercial formulas are the most convenient alternative. However, the differences in stereospecific structure of triacylglycerols (TAGs) in vegetable oils used in infant formula relative to those in human milk fat (HMF), lead to complications associated with their absorption, metabolism, and utilization. Infant formula fat analogs (IFFAs) prepared from structured lipids (SLs) that contain palmitic, docosahexaenoic (DHA), and arachidonic (ARA) acids predominantly at the *sn*-2 position, and which are also enriched with capric acid as a source of medium-chain fatty acids (MCFAs), can be used in the production of new formulas that fit closer to the nutritional needs of the infants. These IFFAs can also be produced at high reaction yields that imply less operating expenses and higher return, making the product more economically feasible for industrial purposes.

In the first part, a SL with 20.13 mol% total palmitic acid (37.57% at *sn*-2), and 21.22 mol% total capric acid (76.33% at *sn*-1,3), was successfully produced in two enzymatic interesterification (EIE) stages using an immobilized *sn*-1,3 specific lipase (10 wt% of substrate). In stage 1, high melting point palm stearin (HMPS) and high oleic sunflower oil (HOSO) were reacted using a substrate molar ratio of 1:2 (HMPS:HOSO) at 60°C for 24 h, in order to obtain a high *sn*-2 palmitic acid intermediate SL (SL_{int}). In stage 2, tricaprin (TC) was interesterified with SL_{int} using a substrate molar ratio of 1.2:0.5 (SL_{int}:TC) at 60°C for 24 h, to obtain the final product. The predominant TAGs in the SL were OPO (28.06%), POP (18.79%), and CLC (11.13%), and the yield for the overall reaction was 90 wt%. SLs with high *sn*-2 palmitic acid

content were produced in the past by enzymatic reactions of fatty acids and ethyl esters with TAGs. However, these reactions lead to a tremendous material loss (yields below 70 wt%). The present study provided a method to synthesize more efficiently these types of SLs via EIE.

In the second part, a high *sn*-2 DHA SL (46.53 mol% total DHA, 49.70% at *sn*-2) and a high *sn*-2 ARA SL (47.25 mol% total ARA, 36.08% at *sn*-2), were successfully produced via EIE of DHASCO® and ARASCO®, respectively, using a mix of *sn*-1,3 specific and non-specific lipases (weight ratio 1:1). Response surface methodology (RSM) was employed to model and optimize conditions of the reactions. For the high *sn*-2 DHA SL, the reaction was conducted for 4 h at 48°C with 10 wt% (substrate) of enzyme load. The reaction yield was 93 wt%. For the high *sn*-2 ARA SL, the reaction was conducted for 4 h at 44°C with 15% of enzyme load. The reaction yield was 94 wt%. In infants, DHA and ARA support proper brain growth and the development of other neurological functions. Unlike in HMF, preformed DHA and ARA from single-cell organisms used in commercial infant formulas do not display a clear positional distribution on TAG molecules. By modifying the molecular structure of DHASCO® and ARASCO®, a higher content of DHA and ARA at the *sn*-2 position was achieved. Potential benefits include better absorption and utilization by infants when added to commercial formulas. To the best of our knowledge, this was the first study on the EIE of single-cell oils that addressed increase in positional distribution of DHA and ARA.

Finally in the third part, an IFFA enriched with capric acid (13.93 mol% total, 85.03% at *sn*-1,3) and substantial amounts of palmitic acid (17.37 mol% total, 34.21% at *sn*-2), DHA (0.49 mol% total, 48.18% at *sn*-2), and ARA (0.57 mol% total, 35.80% at *sn*-2), was prepared by physically blending the SLs synthesized in the previous two parts of the study with canola (CAO) and corn (CO) oils. Each component was proportionally blended to match the fatty acid

profile of commercial fat blends used for infant formula. The predominant TAGs were OPO (24.09%), POP (15.70%), OOO (11.53%), and CLC (7.79%). We propose that this product be used as an alternative to the fat blends currently used for the production of commercial formulas, because it not only contains the right balance of the important fatty acids (FAs) but also at the desirable positions of the TAGs for proper absorption and utilization, thereby contributing to a better nutrition for the infants.

Future studies could evolve from this research. One possible study could involve the application of the IFFA in actual industrial production and comparing the differences with commercial fat blends currently used, in terms of oxidative stability, functionality, taste, palatability, handling, and transport. This would provide insight into a real world application and depending on the results, adjustment to formulation could be required. Another relevant study would be analyzing the metabolism, absorption, and nutritional characteristics of the IFFA on either rats or nematode models, and compare with the results using commercial fat blends and/or HMF. This would determine if the balance of FAs and our postulation regarding their positional distribution on the TAG molecules are statistically accurate. These additional studies would help to understand better the suitability of the product to be used in commercial applications and assess risks and opportunities before testing in infants.