ENHANCEMENT OF STEARIDONIC ACID SOYBEAN OIL WITH PALMITIC ACID AND

DOCOSAHEXAENOIC OR GAMMA-LINOLENIC ACIDS FOR USE AS HUMAN MILK

FAT ANALOGS IN INFANT FORMULA

by

SARAH ANN TEICHERT

(Under the Direction of Casimir C. Akoh)

ABSTRACT

Human milk fat analogs are used in infant formula to mimic the fat found in human breast milk. The purpose of this research was to produce structured lipids by enriching stearidonic acid (SDA) soybean oil with palmitic acid (PA), characterizing the resulting structured lipids (SLs), and then further enriching the SLs with GLA or DHA and characterizing the resulting SLs. Human milk fat analogs were successfully produced by enriching SDA soybean oil with over 60% PA at the *sn*-2 position of the triacylglycerol and then producing SLs with over 54% *sn*-2 PA with either over 8% GLA or 10% DHA. The addition of antioxidants improved their oxidative stability. The human milk fat analogs produced in this research could possibly be used in infant formula application.

INDEX WORDS: Docosahexaenoic acid, Gamma-linolenic acid, Human milk fat analog, Palmitic acid, Stearidonic acid soybean oil

ENHANCEMENT OF STEARIDONIC ACID SOYBEAN OIL WITH PALMITIC ACID AND

DOCOSAHEXAENOIC OR GAMMA-LINOLENIC ACIDS FOR USE AS HUMAN MILK

FAT ANALOGS IN INFANT FORMULA

by

SARAH ANN TEICHERT

B.S., The University of New Haven, 2009

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

2011

ENHANCEMENT OF STEARIDONIC ACID SOYBEAN OIL WITH PALMITIC ACID AND

DOCOSAHEXAENOIC OR GAMMA-LINOLENIC ACIDS FOR USE AS HUMAN MILK

FAT ANALOGS IN INFANT FORMULA

By

SARAH ANN TEICHERT

Major Professor: Casimir C. Akoh

Committee: Robert Shewfelt
    William Kerr

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2011

DEDICATION

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

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Human milk is the main nutritional source for an infant. However, breast feeding is not always possible due to either the health or the diet of the mother or infant. Infant formula was developed to mimic the composition of a healthy mother's breast milk. Human milk contains approximately 3.0-4.5% of fat (*1*) to provide essential fatty acids (FAs) for cell membrane structures and provides approximately 50% of the total energy used by the infant (*2*). Human milk fat (HMF) consists mainly of triacylglycerols (TAGs) that are composed of a glycerol backbone containing three FAs. In HMF, many FAs have a preference for a specific position or multiple positions on the TAG molecule. Palmitic acid (PA) is present in HMF at approximately 18.3-25.9% (*3, 4*) and has a preference for the *sn*-2 position of the TAG. PA is esterified at the *sn*-2 position in HMF at over 60% (by weight) whereas unsaturated FAs are primarily found at the *sn*-1,3 positions of the TAG (*5, 6*). In addition, docosahexaenoic acid (DHA) showed a preference for the *sn*-2 and *sn*-3 positions (*6*).

Many FAs found in HMF provide certain health and nutritional benefits to the infant. Stearidonic acid (SDA) consumption allows for better conversion to n-3 PUFAs, eicosapentaenoic acid (EPA) and DHA, when compared to α-linolenic acid (ALA). EPA is important because it is essential for growth, development, and intestinal absorption of fat-soluble vitamins in infants (*2*). DHA is another important n-3 FA for the infant. It is an essential structural component of retinal, neural and other cell membranes (*7*). DHA also supports the brain and nervous system development and improves visual acuity of infants (*7, 8*). Gamma-linolenic acid (GLA) is an n-6 FA that converts to arachidonic acid (ARA) and is needed for

brain growth and functional development of infants (*9*). GLA is thought to be safer for the infant than ARA since ARA is highly active whereas GLA is a more stable FA (*10*). PA is another important FA and its position in the TAG is vital to the nutrition of the infant. A higher content of PA at the *sn*-2 position results in higher absorption of PA in the intestinal mucosa of the infant (*11*). The PA not absorbed by the infant or PA at the *sn*-1,3 positions results in free FAs that would cause harder stools and/or constipation in the infant (*12*).

The use of SDA soybean oil in infant formula could provide better conversion to EPA than the currently used ALA vegetable oil formulas. However, SDA soybean oil is very low in *sn*-2 PA, contains no DHA, and contains approximately 7.65% GLA. Structured lipids are TAGs that have been modified to change the FA composition and/or positional distribution on the glycerol backbone by enzymatically-catalyzed reactions (*13*). Modifying SDA soybean oil by enzymatic interesterification to enrich the TAG with PA at the *sn*-2 position could produce a structured lipid that would be similar to the composition of the fat found in human breast milk. This structured lipid could provide the benefits of n-3 PUFAs and *sn*-2 PA content. Further enriching the SDA soybean oil enriched with PA at the *sn*-2 position with free FAs of either DHA or GLA by lipase-catalyzed acidolysis could produce an improved HMF analog. This structured lipid could provide the benefits of SDA, *sn*-2 PA, and GLA or DHA. There are no current studies that have modified SDA soybean oil for use as a HMF analog for possible use in infant formula. The majority of infant formulas on the market are mostly supplemented with ARA and DHA, and there are very few formulas that are supplemented with GLA.

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, interesterification and acidolysis reactions, SDA, PA, DHA, GLA, omega-6/omega-3 ratio, response surface methodology, and HMF analogs.

The third chapter presents research that used response surface methodology to generate a model to predict the incorporation of total SDA and the incorporation of PA at the *sn*-2 position into SDA soybean oil by enzymatic interesterification. The model was then optimized to produce SLs with over 60% PA at the *sn*-2 position and still retained a high amount of SDA.

The fourth chapter presents research that used the optimal conditions from response surface methodology to produce the SLs in large quantities and to analyze the physicochemical and stability characteristics of the SLs.

The fifth chapter presents research that modified the SLs containing SDA soybean oil enriched with *sn*-2 PA from chapter four by further enriching them with DHA or GLA. The resulting SLs were analyzed for their physicochemical and stability characteristics.

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

The three objectives of this research were:

1. To enrich SDA soybean oil with PA (from tripalmitin) by enzymatic interesterification with either a nonspecific or *sn*-1,3 specific lipase to produce structured lipids with a high PA content at the *sn*-2 position of the TAG using response surface methodology (RSM).

2. To produce and characterize the SDA soybean oil enriched with PA at the *sn*-2 position using optimal conditions determined from RSM from objective 1 for each lipase.

3. To enrich the structured lipids from objective 2 with free FAs of either GLA or DHA at the *sn*-1,3 positions of the TAG by enzyme-assisted acidolysis with a *sn*-1,3 specific lipase and to characterize the resulting structured lipids.

REFERENCES

(1) Hamosh, M.; Bitman, J.; Wood, L.; Hamosh P.; Mehta, N.R. Lipids in milk and the first steps in their digestion. *Pediatrics*. **1985**, *75*, 146-150.

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

(3) 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.

(4) Bitman, J.; Wood, D.L.; 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.

(5) 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.

(6) 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.

(7) 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.

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

(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) Clough, P.M. Specialty vegetable oils containing gamma-linolenic acid and stearidonic acid. In *Structured and Modified Lipids*. Gunstone, F.D., Ed.; Marcel Dekker: New York, NY, **2001**, pp. 74-117.

(11) 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.

(12) 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.

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

CHAPTER 2

LITERATURE REVIEW

HUMAN MILK FAT

Human milk fat (HMF) contains lipids that are important as sources of energy and

nutrients, in the development of the brain and retinal tissues, and in the absorption of fat-soluble

vitamins. Lipids in HMF provide approximately 50% of the total energy used by infants and the

essential fatty acids (FAs) that are required for the structural cell components of the membrane

tissues in the infant (*1*). Infants absorb fats from HMF more efficiently than the milk from a cow

due to the FA composition of the triacylglycerols (TAGs) (*2*). Mature HMF consists of 3.0-4.5%

fat and 98-99% of this fat is in the form of TAGs (*3*). **Figure 2.1** shows the basic TAG structure

with the stereospecific positions of *sn*-1, *sn*-2, and *sn*-3. The FA composition of mature HMF has

eleven major FAs with medium-chain FAs accounting for 10%, saturated FAs 42%, and

unsaturated FAs 57% of the total lipid content (*3*). HMF contains a rich source of the essential

FAs, linoleic and α-linolenic acid. Linoleic acid (LA, C18:2n-6) is found in HMF at 8-17%

whereas α-linolenic acid (ALA, C18:2n-3) is found at 0.5-1.0% (*4*). The long-chain derivatives

of these essential FAs are arachidonic acid (ARA, C20:4n-6) and docosahexaenoic acid (DHA,

C22:6n-3) and are found in HMF at 0.5-0.7% and 0.2-0.5%, respectively (*4*).

Lipid digestion is not fully developed in the early stages of infant development. Infants

are often limited in their ability to synthesize long-chain PUFAs due to their limited desaturating

capacity (*5-6*). Digestion of HMF is achieved by a number of different enzymes. The positional

distribution of the FA in the TAG structure influences the hydrolysis and absorption of the FAs.

Palmitic acid (PA, C16:0) is present in HMF at 18.3-25.9% (*7-9*) and prefers the *sn*-2 position of

the TAG at over 60% (by weight). Unsaturated FAs in HMF are primarily found at the *sn*-1,3 positions of the TAG (*10-12*). In HMF, oleic acid (C18:1n-9) showed a preference for the *sn*-1 position, LA showed a preference for the *sn*-3 position, and ARA and DHA showed a preference for the *sn*-2 and *sn*-3 positions (*12*). HMF was found to contain more than twenty-seven TAG species. The major six TAG species found were LaPP, PPO, LLP, SSLa, LaSO, and LPLa accounting for 55% of the total TAG species (La-lauric acid, P-palmitic acid, L-linoleic acid, S-stearic acid, and O-oleic acid) with PA, stearic acid (C18:0), oleic acid, and LA being the major FAs accounting for approximately 76% of the total FAs (*13*). The diet of the mother and the stage of lactation can affect and influence the FA composition and TAG species of HMF. Different maternal intakes of FAs showed that different dietary habits greatly influenced and changed the FA composition of HMF in two groups of Chinese mothers from two different provinces (*14*). The structure of TAGs is very important in HMF, and the total FA and *sn*-2 composition of HMF is shown in **Table 2.1** (*15*).

<div align="center">INFANT FORMULA</div>

Infant formula is a human milk substitute that tries to meet the nutritional requirements of the infant. The goal of infant formula is to achieve the composition present in breast milk since a well-nourished mother's milk is considered the optimum nutrition for term infants. The lipid content of infant formula is important as it provides energy and essential FAs for structure and function of cell membranes and influences the physiological and metabolic processes. Most infant formulas use vegetable oils as their fat source. However, vegetable oils only contain approximately 5 to 20% saturated FAs at the *sn*-2 position of the TAG (*16*). 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 strong tendency to have softer stools and less constipation (*17*). Usually constipation occurs more in

formula-fed infants than breast-fed since formulas usually contain PA at the sn-1,3 positions (88-94%) compared to the *sn*-2 PA in breast milk (over 60%) (*17*). Constipation or hard stools are caused by calcium soaps which are generated by long-chain saturated FA interactions with calcium (*10, 18*). Long-chain PUFAs are also important in infant formula. Preformed long-chain PUFAs are found in HMF, but traditionally are not found in infant formulas. Most traditional infant formulas rely on the presence of LA and ALA for bioconversion to long-chain PUFAs, such as eicosapentaenoic acid (EPA, C20:5n-3), DHA, γ-linolenic acid (GLA, C18:3n-6), and ARA. Infant formulas tend to resemble the FA composition of HMF, but the distribution of these FAs differs greatly. The positional specificity of FAs on the TAG has resulted in the production of structured lipids (SLs) for use as HMF analogs.

<div align="center">STRUCTURED LIPIDS</div>

SLs are TAGs modified to change the FA composition and/or positional distribution on the glycerol backbone by chemically and/or enzymatically-catalyzed reactions (*19*). SLs can provide the desired FAs for nutritive or therapeutic purposes to target specific nutritional and functional needs, specific diseases, and specific metabolic conditions. Modifying TAGs can result in the change of the chemical and physical properties such as melting point, iodine value, saponification value, crystallization profile, and oxidative stability. Many FAs are used in synthesizing SLs in order to maximize the nutritional and functional benefits. SLs have a wide variety of uses in the pharmaceutical, medical and food industries. Food uses can consist of but are not limited to margarines, modified butters, shortenings, infant formulas, and frying oils. The first commercial SL product used as a HMF analog was Betapol™ (Loders Croklaan, Glen Ellyn Ill., USA) that was produced by using a 1,3-specific lipase to synthesize SLs by reacting tripalmitin with unsaturated FAs (*20*). SLs can be produced by enzymatic interesterification and acidolysis reactions to change the total FA composition and positional FA composition.

INTERESTERIFICATION AND ACIDOLYSIS

Chemically and enzymatically-catalyzed reactions can be used to produce SLs. Enzymatic interesterification provides milder processing conditions, better FA specificity, and regiospecificity when compared to chemical interesterification (*21*). Interesterification randomizes the FA distribution in the TAG leading to the modification of the TAG composition and its physical characteristics. Enzymatic interesterification and acidolysis reactions use lipases as biocatalysts. The lipase used can affect the final TAG FA composition (*22*). For example, a *sn*-1,3 specific lipase (i.e., Lipozyme TL IM) is specific and will cleave the FAs on the *sn*-1,3 positions whereas a nonspecific lipase (Novozym 435) will cleave any of the positions resulting in a random FA distribution after reesterification. Interesterification involves exchange of acyl groups within a TAG or among TAG molecules. An example of an enzymatic interesterification reaction using a sn-1,3 specific lipase is shown in **Figure 2.2**. Acidolysis involves reacting an acid with an ester or an ester exchange. In summary, interesterification is reacting FA ethyl esters with TAGs and acidolysis is incorporating FFAs into TAGs. The basic reactions for interesterification and acidolysis are as follows:

Interesterification: $R_1\text{-CO-O}R_2 + R_3\text{-CO-O}R_4 \rightarrow R_1\text{-CO-O}R_4 + R_3\text{-CO-O}R_2$

Acidolysis: $R_1\text{-CO-O}R_2 + R_3\text{-CO-OH} \rightarrow R_1\text{-CO-OH} + R_3\text{-CO-O-}R_2$
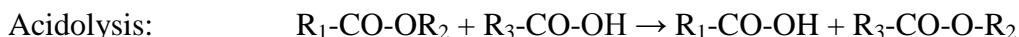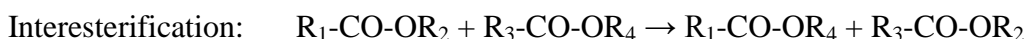
STEARIDONIC ACID

Stearidonic acid (SDA, C18:4n-3) is an n-3 PUFA with plants and algae being the richest sources. Fish is a major source of PUFAs, DHA and EPA, but only contains very small amounts of SDA (*23*). Fish oil can only contain up to 4% SDA of the total FAs (*24*). James *et al.* (*25*) suggested that a daily ingestion of fish or fish oil is not a maintainable long term approach to obtain the benefits of n-3 PUFAs. Instead, increasing the ingestion of plant-based sources of n-3 PUFAs is required to increase tissue concentrations of EPA and DHA (*25*). The metabolic

pathway (**Figure 2.3**) of n-3 PUFAs from ALA to EPA and DHA is accomplished by a series of alternating desaturation and elongation steps. However, the conversion of ALA to EPA is not very effective. The effectiveness of the conversion of ALA to EPA or SDA to EPA is dependent on the regulation of the desaturation and elongation reactions during metabolism. The reason for this ineffectiveness is due to the first desaturase step where ALA biologically converts to SDA with the Δ6 desaturase enzyme being rate limiting in humans (*26*). Therefore, the consumption of SDA instead of ALA would allow for better conversion to EPA by skipping the rate limiting step. Miles *et al.* (*27*) observed no SDA in the peripheral blood mononuclear cell with the ingestion of dietary SDA at a level of 1.0g/day. This absence of SDA indicates that SDA was readily metabolized to EPA during metabolism in the body. The dietary SDA intake increased EPA concentrations by three to fourfold more effectively than similar ALA levels, but was approximately one-third as efficient as dietary EPA (*25*). Therefore, the effectiveness of increasing EPA in the tissues is as follows: EPA>SDA>ALA. A double-blind, parallel study conducted by James *et al.* (*25*) examined the effect of dietary SDA on increasing EPA in tissues and concluded that SDA vegetable oil would be more effective at increasing EPA concentrations than the currently used ALA vegetable oils. Therefore, SDA soybean oil in infant formula may be able to enrich infants' tissue concentrations with EPA better than the currently used ALA vegetable oils in infant formulas. EPA's importance to the body is that it is essential for growth, development, and intestinal absorption of fat-soluble vitamins in infants (*1*) and is linked to reductions in inflammation (*28)* and neurological disorders (*29*). SDA could be an adequate precursor of EPA and DHA due to its position in the metabolic pathway of n-3 PUFAs and its natural occurrence in plants and algae.

Genetic modification of crops, such as oil seed crops, presents the opportunity to modify the composition of certain seed oils for their optimal FA composition, nutritional aspects, and processing characteristics. Currently, soybean oil is being genetically modified to enrich the oil with higher amounts of SDA. This synthesis of SDA in soybean oil was done by introducing genes for Δ6, Δ15 desaturases into soybeans that converted LA and ALA into SDA (*30*). SDA soybean oil's major FAs consisted of 24% LA, 20% SDA, 6% GLA, 10% ALA, and 12% PA (*30*). The differences between the soybean oil enriched with SDA and conventional soy oil are shown in **Table 2.2** (*30*). The increases of SDA, GLA, and ALA FAs in SDA soybean oil is offset by the decrease in oleic acid and LA contents when compared to regular soybean oil (*31*). SDA soybean oil has been found to significantly increase the n-3 index in clinical trials by increasing EPA concentration. However, it did not increase the DHA tissue concentrations (*32*). SDA is also more stable and has an increased shelf life in food matrices than EPA or DHA. This higher stability is due to a lower unsaturation index. The safety of the genetically modified SDA soybean oil was assessed and no treatment-related adverse effects were seen in rats at doses of 1.5, 3.0, and 4.0 g/kg body weight (*30*). This safety study performed by Hammond *et al.* (*30*) concluded that their results showed no treatment-related adverse effects for the human consumption of SDA soybean oil.

<u>PALMITIC ACID</u>

PA is a common saturated FA found in animals and plants. It is a very important FA in HMF and is the second major FA in HMF at levels between 18.3-25.9% (*7-9*). Of the total PA in HMF, a large portion (over 60%, by weight) is esterified at the *sn*-2 position of the HMF TAGs (*10-12*). When the HMF TAGs are metabolized in the body, the esterified FAs at the *sn*-1,3 positions are hydrolyzed by pancreatic lipase and are released. This leaves the *sn*-2 position unhydrolyzed and intact. Then the FAs at the *sn*-2 position become absorbed by the intestinal

mucosa as *sn*-2 monoacylglycerols (*33*). Most FAs are better absorbed in the 2-monoacylglycerol form than FFAs. This is due to the fact that monoacylglycerols form mixed micelle with bile salts that cannot form insoluble soaps with calcium or magnesium (*34*). De Fouw *et al.* (*35*) performed a study in rats by comparing rats fed a formula with high *sn*-2 PA content and rats fed formula with a low *sn*-2 PA content. The study showed that the rats fed a higher *sn*-2 PA had overall higher absorption of PA than the rats fed the lower *sn*-2 PA formula (*35*). The large amount of PA at the *sn*-2 position may improve the absorption of fat and calcium in the infants (*12, 18, 36*). The *sn*-2 PA may also reduce the formation of calcium soaps since free PA (from the *sn*-1,3 positions) may be lost as calcium soaps in the feces of the infant often causing problems (*10, 18*). The formation of calcium soaps indicates poor PA and calcium absorption. Constipation or hard stools are caused by calcium soaps which are generated by long-chain saturated FA interactions with calcium (*10, 18*). When infants were fed a formula with a higher *sn*-2 PA concentration (over 40%), they had a strong tendency to have softer stools and have less constipation than infants fed a formula with a low *sn*-2 PA concentration (*17*). 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 PA at the *sn*-1,3 positions (88-94%) compared to the *sn*-2 PA in breast milk (over 60%) (*17*). However, in the study performed by de Fouw *et al.* (*35*), they did not see a difference in calcium absorption between formulas with high and low *sn*-2 PA. Carnielli *et al.* (*34*) increased the calcium content in formula to provide more availability for calcium palmitate formation and in fecal losses for better measurement. However, even though the calcium concentration was more than double that of breast milk, the effect of fecal palmitate and *sn*-2 palmitate on calcium absorption could not be measured (*34*). Carnielli *et al.* (*34*) also found that calcium absorption was greater in the

group fed *sn*-2 PA formula and that the stools of the infants were softer in the *sn*-2 PA formula

group. Therefore, formulas with more *sn*-2 PA could result in improved calcium excretion and

absorption (34). A human milk fat analog or substitute would ideally contain a large portion of

PA at the *sn*-2 with other FAs, like PUFAs, occupying the *sn*-1 and *sn*-3 positions.

<div align="center">DOCOSAHEXAENOIC ACID</div>

DHA is an n-3 PUFA and is mainly found in fish and fish oils. However, the organoleptic

properties, such as the fishy aroma, of fish and fish oils make them very unattractive to

consumers. Other sources of DHA in large amounts are marine algae. DHASCO® is a mixture of

an oil extracted from the unicellular algae *Crypthecodinium cohnii* and high oleic sunflower oil

and is a rich source of DHA (40-45% DHA) produced by Martek (Columbia, MD). DHASCO®

is an alternate source of DHA than the more commonly used fish oils. ALA and EPA are

precursors of DHA in the n-3 FA metabolic process (**Figure 2.3**). Due to the high unsaturation of

DHA, it is very easily oxidized. In HMF, DHA is present at approximately 0.2-0.5% depending

on the diet of the mother (*4*) and has a preference for the *sn*-2 and *sn*-3 positions of the TAG

molecule (*12*). Mothers consuming either herring or menhaden oil showed increased levels of

DHA in their breast milk within 6 h and maximum levels of DHA at 24 h (*37*). DHA is an

essential structural component of retinal, neural and other cell membranes, and it supports brain

and nervous system development and improves visual acuity in infants (*38-39*). DHA is

integrated into cell membrane phospholipids during the last trimester and the first year of life of

the infant (*40*). Lower levels of DHA have been reported in infants fed formulas when compared

to infants fed human breast milk (*41-42*). Low levels of DHA in formula-fed infants may be due

to their lack of ability to desaturate and elongate ALA to DHA due to elongation-desaturation

enzymes not being sufficiently active during the early stages of life to maintain tissue

accumulation of DHA (*38*). Therefore, supplementation of preformed DHA may be critical in the

development of infant formulas. Anderson *et al.* (*43*) established that preformed dietary DHA is quantitatively more effective than supplementation of ALA as a source of DHA for brain, retina, and liver development. Makrides *et al*. (*42*) gave one group of term infants a supplemented formula with 0.36% DHA and 0.27% GLA and another group a formula with 1.6% ALA and no DHA. The group lacking DHA experienced hindered visual acuity at 4 and 6 months of age (*44*). Also, Birch *et al.* (*45*) 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. A study performed on infant rhesus monkeys deprived the infants of n-3 FAs (*46*). The infants showed no detectable DHA after twelve weeks and visual acuity was reduced by one-fourth after four weeks and by one-half after eight and twelve weeks. These results may indicate the importance of n-3 FAs as an essential nutrient and that DHA may have a specific function in the photoreceptor membranes of the retina (*46*). Jorgensen *et al.* (*40*) showed 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. Humans obtain DHA mostly from their diets since they have only a small capacity to synthesize DHA (*47*). DHA is essential since it is the predominant FA in the grey matter of the brain and can affect learning since it is involved in cell signaling (*47*). Supplementation of performed DHA is needed in order for formula-fed infants to attain DHA levels similar to infants fed breast milk. The trend is that infant formulas are now being formulated to resemble the fat content found in breast milk, and infant formulas containing DHA supplementation are now available in forty countries all over the world (*48*).

GAMMA-LINOLENIC ACID

GLA is an n-6 PUFA and is a precursor of ARA. GLA can be present in breast milk in amounts from 0.0-0.4% and usually occupies the *sn*-1 and *sn*-3 positions of the TAG molecule in HMF (*12-13*). GLA, instead of LA, bypasses the rate limiting Δ6 desaturase step in the n-6 metabolic process (**Figure 2.3**). Infants may lack the ability to desaturate and elongate LA into GLA due to impaired activity of the Δ6 desaturase enzyme (*49*). Thus, an impaired bioconversion to GLA may result in inadequate levels of GLA and ARA in the infant. As a result, GLA may be better in promoting ARA synthesis than LA. The bioconversion of GLA is accomplished by the elongation of GLA into dihomo-γ-linolenic acid (DGLA, C20:3n-6). DGLA is then desaturated by Δ5 desaturase to form ARA. DGLA is also a precursor to 15-OH-DGLA and prostaglandin (*49*). Prostaglandins are derived from n-6 PUFAs and are thought to play a role in the maturation of the immune system. It has been shown that feeding an infant GLA rather than ARA may be safer since ARA is a highly active FA (*49*). ARA is important as it is needed for brain growth and functional development of infants (*50*). GLA is anti-inflammatory and has been used in the treatment of rheumatoid arthritis, diabetic neuropathy, hypertension, asthma, atopic dermatitis, migraines, and cancer (*51-52*). Jorgensen *et al.* (*40*) showed that the addition of GLA resulted in higher ARA in red blood cells in formula containing 0.5% GLA, but it could not fully compensate for the lack of ARA in the formula. Possibly, a higher level of GLA in formula may result in sufficient levels of ARA. ARA and EPA are precursors of eicosanoids and higher levels of EPA (and other n-3 FAs) can reduce the levels of ARA in red blood cells. It is suggested that ARA levels in infants are predictors of growth (*40*). Infants fed fish oil formula with a high EPA:DHA ratio had lower scores for weight, length, and head circumference than infants fed formula without fish oil (*53*). Another study used the

supplementation of infant formula with long-chain PUFAs from tuna oil and borage oil (0.9g/100 mg fat) to study growth and mental development in infants (*54*). The conclusions of this study were that up to 9 months after term, the long-chain PUFA formula proved to be a safe strategy with benefits for growth for the entire study group and mental development in boys (*54*). Van Gool *et al.* (*55*) investigated the preventive effect of GLA on the development of atopic dermatitis in at risk infants. A reason for this investigation was due to mothers of atopic infant having lower concentrations of n-6 PUFAs in their breast milk than mothers of non-atopic infants. With a supplementation of 23.1% GLA (or 103 mg GLA per day), it was found that GLA does not prevent the expression of atopic dermatitis but does tend to alleviate the severity of atopic dermatitis later in infancy in children who are at high risk (*55*). Currently some formulas are supplemented with GLA in order to provide the benefits of ARA, but most formulas directly supplemented with preformed ARA (*49*).

<div align="center">OMEGA-6/OMEGA-3 RATIO</div>

PUFAs can be classified as n-3 or n-6 depending on the position of the first double bond in the FA. There is competition between LA (n-6) and ALA (n-3) for desaturation to ARA (n-6) and DHA (n-3), respectively (*56*). The ratio of n-6/n-3 PUFA is an important factor in the diet. However, the western diet is high in n-6 FAs often resulting in a high ratio of 15:1-16.7:1 instead of a 1:1 ratio that is the case for wild animals and presumably humans (*57*). During the late Paleolithic period, the estimated n-6/n-3 ratio of humans was 0.79 (*57*). The high ratio of n-6 FAs can result in negative effects such as increased risk of cardiovascular, inflammatory and autoimmune diseases and cancer in adults. Conversely, a lower n-6/n-3 PUFA ratio would suppress these negative effects. For example, a lower ratio of 2:1-3:1 has been shown to suppress inflammation in patients with rheumatoid arthritis, and a ratio of 5:1 was shown to have beneficial effects on patients with asthma. However, a higher ratio of 10:1 n-6/n-3 PUFA had

adverse consequences (*57*). The beneficial effects of n-3 FA intake could be achieved by

supplementation of ALA or EPA and DHA, where EPA and DHA are more potent (*57*).

Premature infants often are limited in their ability to make EPA and DHA from ALA which

often limits the supply of n-3 FAs to the infant (*41*). Thus, the direct supplementation of SDA

and DHA could increase the amount of n-3 FAs in infants. The ratio of n-6/n-3 PUFAs in

Chinese mothers' breast milk was approximately 14-16% with DHA existing in the milk fat at

approximately 0.38-0.61% and the diet of the mother greatly affected the FA content of the

breast milk (*14*). The ratio of LA/ALA in the HMF of mothers in Nordic countries was the

lowest at 7.0 followed by Canada at 8.9 and then 13-14 for the Netherlands, United Kingdom,

and the United States. The highest LA/ALA ratio was found in HMF of mothers in Spain and

France at 22 (*58*). These differences in LA/ALA ratios in mothers' breast milk may be explained

by food practices, especially the dietary intake of margarines and edible oils (*58*). Also, the ratios

of ARA/DHA differed in mothers' breast milk as 1.1-1.4 in Spain, United Kingdom,

Netherlands, and France and up to 2.0-2.6 in mothers from Canada and the United States (*58*).

Since 1995, manufacturers started limiting the amount of LA in infant formulas. The ratio of

LA/ALA before 1995 was 6.3-61.0 whereas after 1995 it was 8.5-21.7 (58). The reason for this

change was to match the ratio found in HMF. However, the amount of LA is still high and varies

greatly in most infant formulas (10-30%) resulting in a ratio much higher than the adequate

intake ratio of LA/ALA of 5:1 (*58, 59*). Ponder *et al.* (*56*) found that infants fed soy formula with

a 7:1 ratio and infants fed corn formula with a 39:1 ratio did not show a difference in ARA or

DHA levels between the two groups. Term infants received a diet of 16% LA with inclusion of

0.4-3.2% ALA lowered ARA levels and increased DHA levels giving the infant a lower body

weight (*60*). During the early stages of life, PUFAs provide insurance for normal growth, skin

physiology, and development of brain and visual functions. In Neuringer *et al.* (*46*) study on infant rhesus monkeys, a high n-6/n-3 ratio of 255:1 resulted in decreased visual acuity by one-half when compared to a formula with a ratio of 7:1. It is important to balance the n-6/n-3 ratio in infants to receive the benefits of both n-6 and n-3 FAs without the adverse effects of too much n-6 FAs. Since most mothers consume more n-6 FAs than recommended, it is likely that their breast milk may also contain more n-6 FAs than necessary. However, there is not enough clinical data to determine the minimal recommended intake of the n-6/n-3 ratio for proper nutrition of infants.

RESPONSE SURFACE METHODOLOGY

Response surface methodology (RSM) enables the evaluation of the effects that multiple parameters have on response variables (*61*) and limits the number of reactions that need to be performed. 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. The response is the outcome of the reaction that you are most interested in. In many cases, RSM can be applied to determine the optimization conditions of a response of a reaction. For example, factors could include three different temperatures, times, and substrate mole ratios with a response as the incorporation of the FA of interest into the TAG molecule. RSM can then be used to predict the optimal conditions to obtain a certain percentage of the FA of interest. The relationship between the factors and variables from the RSM design can be fit into an equation model for prediction of the responses. Contour plots can also be generated to aid in optimization. Two variables are placed on the x- and y-axis of the contour plot with contour lines from the iso-response values that are generated by computers due to their complexity (*61*).

Many studies have used RSM and contour plots to predict the optimal conditions of the incorporation of FAs into the TAG molecule. Sahin *et al*. (*62*) used RSM to predict the optimal conditions of a target of 10% GLA and 45% oleic acid incorporation between tripalmitin, hazelnut oil FAs, and GLA by enzymatic interesterification. The optimal conditions were determined for the substrate mole ratio, temperature, and reaction time producing a SL with 9.7-9.8% GLA and 43.3-43.8% oleic acid (*62*). Another study used RSM to produce a reduced calorie SL by acidolysis of tripalmitin and capric acid (*63*). The factors were substrate mole ratio (capric acid to tripalmitin), enzyme amount (weight percent of the total substrate), and reaction time with a response for maximum incorporation of capric acid into tripalmitin. The RSM optimal conditions were determined to be a 6.5 substrate mole ratio, enzyme amount of 11.6 wt%, and a reaction time of 8.9 h producing a SL containing 44.9% capric acid (*63*). Rao *et al*. (*64*) looked at the maximum incorporation of n-3 or n-6 PUFAs from FFAs obtained after hydrolysis of cod liver oil and safflower oil into coconut oil using RSM. The maximum incorporation of n-3 PUFAs occurred at a 1:4 substrate mole ratio (coconut oil TAG to FFA) at 54ºC with a reaction time of 34 h, and the maximum incorporation of n-6 FA occurred at a 1:3 substrate mole ratio (coconut oil TAG to FFA) at 39ºC with a reaction time of 48.5 h. The optimal conditions produced SLs with 13.65 and 45.5% of n-3 and n-6 FA, respectively (*64*). As shown in these studies, RSM models and contour plots can be used to predict the approximate responses of different variables.

HUMAN MILK FAT ANALOGS

Many infant formulas on the market try to mimic the composition of breast milk to provide optimal nutritional benefits to the infant. However, the fat content in infant formulas is often lacking nutritionally when compared to HMF. Many studies have been conducted to

produce HMF analogs to increase the *sn*-2 PA content, increase DHA content, and increase GLA or ARA content.

Human Milk Fat Analogs with Increased *sn*-2 PA.

With the importance of PA at the *sn*-2 position of the TAG being recognized, many studies have produced SLs with higher amounts of *sn*-2 PA than most commercial formulas contain thus producing a fat that would better resemble that of breast milk. Since HMF contains over 60% (by weight) of PA esterified at the *sn*-2 position of the total 18.3-25.9% PA, an ideal HMF analog would contain 60% or more of *sn*-2 PA (*10-12*).

A study performed by Shimada *et al.* (*65*) used acidolysis of tripalmitin with ARA using a *sn*-1,3 specific lipase to produce a HMF analog. The HMF analog contained a total of 31.7% PA and 60.1% ARA with 28.7% of PA at the *sn*-2 position. The majority of ARA occupied the *sn*-1,3 positions at 56.9% due to the *sn*-1,3 specificity of the lipase (*65*). The main TAG molecular species for this HMF analog was 1,3-arachidonoyl-2-palmitoyl-glycerol (APA). However, this SL contained a much lower amount of PA at the *sn*-2 position when compared to HMF, but did contain similar amounts of total PA. Ilyasoglu *et al.* (*66*) produced a HMF analog by using tripalmitin, hazelnut oil FAs, and a mixture of medium-chain FAs (MCFAs) as substrates by lipase-catalyzed acidolysis. At optimal reaction conditions, the total PA was 30% which is comparable to that of HMF. However, the study performed by Ilyasoglu *et al.* (*66*) did not analyze the positional composition of their HMF analog so the PA content at the *sn*-2 position is unknown.

Chen *et al.* (*67*) used a three-step method to produce 1,3-dioleoyl-2-palmitoylglycerol (OPO) from palm oil. First, low-temperature fractionation was applied to the palm oil FA to produce PA and oleic-rich fractions. The PA was transformed into ethyl palmitate and then enzymatic esterification with glycerol was used to produce tripalmitin. Tripalmitin was reacted

with oleic acid producing OPO with 90.7% PA at the *sn*-2 position (*67*). Another study

performed by Sahin *et al.* (*68*) used lipase-catalyzed acidolysis, with a *sn*-1,3 specific lipase, of

tripalmitin, stearic acid, and hazelnut oil FAs to produce HMF analogs. The HMF analogs

produced contained more than 42.5% total oleic acid, 7% total stearic acid, and between 69.2-

76.0% PA at the *sn*-2 position. As the levels of other FAs increased in the SL, the PA decreased

since the FAs were incorporated into the *sn*-1,3 positions of tripalmitin due to the specificity of

the lipase. The incorporation of other FAs into the *sn*-2 position was thought to be due to acyl

migration during the reaction (*68*). Maduko *et al.* (*69*) 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 at the ratio of 2.5:1.1:0.8. The resulting SLs contained *sn*-2 PA of over 60% with the

highest incorporation with a 1:1 ratio of tripalmitin to the vegetable oil blend and a 24 h reaction

time (*69*). An enrichment of amaranth oil with ethyl palmitate by chemical and enzymatic

synthesis was performed by Pina-Rodriguez and Akoh (*70*) to increase the PA content at the *sn*-2

position. Both reactions increased the *sn*-2 PA, but enzymatic interesterification produced a

higher significant *sn*-2 PA content. Also, a higher PA incorporation was observed at longer

reaction times (15-17 h) and there was no significant difference between the larger and smaller

substrate mole ratios (*70*). Therefore, use of a smaller mole ratio would save materials and

reduce cost while producing a similar SL. A mixture of palm oil, palm kernel oil, olive oil,

sunflower oil, and marine oil in a ratio of 4.0:3.5:1.0:1.5:0.2 was used by Karabulut *et al.* (*71*) to

produce a HMF analog by enzymatic interesterification. The enzyme used as a biocatalyst was

Lipozyme TL IM, a *sn*-1,3 specific lipase. The resulting SL had a FA profile containing 41.5%

PA at the *sn*-2 position and 47.3% of total unsaturated FAs with minor amounts of DHA (*71*).

Even though, the *sn*-2 PA is lower than that found in HMF, it is higher than what is found in most infant formulas (*71*). Jiménez *et al.* (*72*) first produced PA enriched FFAs (using two different concentrates) and then used these FFAs to enrich palm oil stearin with PA at the *sn*-2 position by acidolysis using a non-specific lipase (Novozym 435). The *sn*-2 PA increased from 23.0% in palm oil stearin to SLs with 74.5% *sn*-2 PA with a 98% commercial PA concentrate and 70.5% *sn*-2 PA with a 75.1% PA concentrate (*72*). Tecelão *et al.* (*73*) used enzymatic interesterification of tripalmitin and oleic acid to produce a HMF analog using different lipases as biocatalysts. The lipases used were Lipozyme RM IM, Lipozyme TL IM, Novozyme 435, and Lipase/acyltransferase. All lipases produced SLs containing over 60% *sn*-2 PA in a range of 61.2-87.3% (*73*). As shown in the above studies, it is possible to increase the *sn*-2 PA to over 60% of a fat source to obtain a HMF analog for possible use in infant formula.

Human Milk Fat Analogs with Increased Total DHA.

The importance of n-3 FAs in an infant's diet has become of great interest, especially with the supplementation of DHA. Many infant formulas are now being supplemented with DHA, and many studies are looking at incorporating DHA into vegetable oils to produce HMF analogs or SLs for other food uses.

One study modified trilinolein with n-3 PUFAs, EPA and DHA, by using their ethyl esters as the acyl donors by transesterification using IM60 from *Mucor miehei* and SP435 from *Candida antarctica* as biocatalysts (*74*). With the DHA ethyl ester, total DHA in the SLs were 19.0 and 26.8% while containing 81.0 and 73.2% LA with IM60 and SP435, respectively. With a combination of EPA and DHA ethyl esters and SP435 as the biocatalyst, LA was decreased to 64.5% while containing 15.3% DHA and 20.2% EPA (*74*). Even though the aim of this study was not to produce a HMF analog, it shows the possibility of incorporating DHA into TAGs in solvent-free reactions to supply essential FAs and n-3 PUFAs for use in food applications.

Hamam and Shahidi (*75*) 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% at 43.7^{o}C with a reaction time of over 44.7 h. The majority of DHA was esterified at the *sn*-1,3 positions of the SL. However, due to the higher degree of unsaturation and higher conjugated diene values, the SL was more susceptible to oxidation than the unmodified Laurical 35 (*75*). These DHA SLs contained very little total PA at 2.08% making this SL not a suitable HMF analog, but may be a used as a possible fat source in other foods to increase DHA in the diet. Robles *et al.* (*76*) produced a SL enriched with PA and DHA at the *sn*-2 positions and oleic acid at the *sn*-1,3 positions. To obtain this SL, a four step process was used. First, tuna oil and PA rich FFAs where catalyzed by Novozym 435 (nonspecific lipase) to obtain TAGs rich in PA. The resulting TAGs were purified by removing FFAs. The third step was to take the PA enriched TAGs with oleic acid FFAs with a *sn*-1,3 specific lipase as a biocatalyst and then purify the final SLs by removing any FFAs. This process resulted in 67.0% oleic acid at the *sn*-1,3 positions, 52.1% PA at the *sn*-2 position, 15.1% PA at the *sn*-1,3 positions, and 8.5% total DHA (*76*). The SL produced by Robles *et al.* (*76*) were similar to HMF's except the SL contained a higher *sn*-1,3 oleic acid and total DHA content.

A HMF analog was produced by enzymatic interesterification of amaranth oil with ethyl palmitate to increase *sn*-2 PA and interesterification of this SL with a DHA containing oil, DHASCO$^{®}$ (*77*). This HMF analog (DCAO) contained 33.5% PA, 2.8% stearic acid, 23.0% oleic acid, 36.9% linoleic acid, 0.7% linolenic acid and 1.9% DHA. Most of the DHA was found at the *sn*-1,3 positions. However, DCAO contained the majority of its PA at the *sn*-1,3 positions (40.8%) with only 20.2% PA at the *sn*-2 position (*77*). Even though there is enough total PA to resemble HMF, the positional distribution of that PA in DCAO does not match that of HMF. The

higher levels of PA at the *sn*-1,3 positions could result in harder stools in infants or more formation of calcium soaps. An infant formula was prepared with DCAO and compared to a commercial infant formula (*78*). The *sn*-2 PA in the DCAO formula (33.0%) was much higher than the *sn*-2 PA found in the commercial formula (7.3%). The total DHA found in the DCAO formula was 0.2% with the commercial formula containing no detectable DHA. The oxidative stability of the DCAO formula was significantly lower than the commercial formula possibly due to the loss of antioxidants during production of the DCAO SL (*78*). Addition of antioxidants before infant formula formulation may result in better stability and shelf life. Sahin *et al.* (*79*) 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. The optimal conditions to produce the HMF analog were a 12.4 substrate mole ratio (moles of total FAs to moles of tripalmitin) at $55^{o}C$ with a reaction time of 24 h (*79*). Sahin *et al.* (*79*) successfully produced a SL with similar *sn*-2 PA as HMF and enriched with EPA and DHA to provide n-3 FA health benefits for possible application as a HMF analog in infant formula.

Human Milk Fat Analogs with Increased Total GLA.

The importance of ARA in an infant's diet for growth has become under investigation. Instead of directly adding preformed ARA, a few studies are looking at incorporating GLA into HMF analogs in order to provide the benefits of ARA. The reasoning behind the incorporation of GLA is because feeding an infant GLA rather than ARA may be safer since ARA is highly active (*49*). There are also other studies that looked at producing SLs with GLA for other nutritional applications.

Incorporating GLA by acidolysis of borage oil and caprylic acid (C8:0) was performed to produce a SL as a possible nutritional source (*80*). The SL contained a total of 52.7% caprylic

acid mostly at the *sn*-1,3 positions, 1.6% PA and 19.6% GLA. GLA occupied mostly the *sn*-2

position (16.4%) with very little at the *sn*-1,3 positions (3.2%). The TAG molecular species of

the SL contained 1,3-capryoyl-2-γ-linolenicglycerol, 1,3-capryloyl-2-linoleoylglycerol, 1,3-

capryloyl-2-oleoylglycerol and a TAG with one molecule of caprylic acid and two molecules of

GLA (*80*). This study showed that production of a SL enriched with GLA is possible. Spurvey *et*

al. (*81*) looked at incorporating GLA 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 RSM optimal conditions were found to be a 3:1 substrate mole ratio of GLA to

seal blubber or GLA to menhaden oil at 40°C with a reaction time of 24 h. 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 PA. The SLs also contained low levels of DHA since the initial oils

were from marine animals. The seal blubber SL contained a total DHA of 4.36% whereas the

menhaden oil SL contained 6.56% (*81*). With incorporation of PA into the SL, it could produce a

FA profile that could be used in infant formula.

A different study looked at producing a SL that resembles HMF with additional GLA by

using enzymatic interesterification between tripalmitin, hazelnut oil FAs, and GLA (*62*). The

target incorporation was for the SL to contain 10% GLA and 45% oleic acid. The RSM optimal

conditions to achieve the targeted incorporation were determined to be a 14.0 substrate mole

ratio, moles of total FA to moles of tripalmitin, at 55°C with *sn*-1,3 specific Lipozyme TL IM

(*62*). The SL contained a total of 9.8% GLA and a total of 40.9% PA with 73.9% found at the *sn*-

2 position with most of the GLA occupying the *sn*-1,3 positions of the TAG (*62*). The SL was

similar in composition as HMF enriched with GLA to provide benefits of ARA upon

bioconversion during digestion in the infant. Shimada *et al.* (*65*) looked at incorporating ARA

and PA into a SL to be used as a HMF analog. The acidolysis reaction of tripalmitin with ARA successfully produced 1,3-arachidonoyl-2-palmitoylglycerol at 75.9% of the TAGs present. The ARA content of the SL was 56.9% at the *sn*-1 and *sn*-3 positions and 3.2% at the *sn*-2 position (*65*). The use of a *sn*-1,3 specific lipase was able to incorporate ARA into the *sn*-1,3 positions with only a little acyl migration into the *sn*-2 position to retain the *sn*-2 PA (*65*). The use of preformed ARA would increase levels of ARA more effectively than GLA. However, GLA is considered safer for the infant as preformed ARA is highly active (*49*). ARA has been reported to accelerate the growth of preterm infants (*40*). With similar PA levels of that found in HMF and enriched with ARA, the SL produced in this study could possibly be used as a HMF analog in infant formula.

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) Hamosh, M.; Bitman, J.; Wood, L.; Hamosh P.; Mehta, N.R. Lipids in milk and the first steps in their digestion. *Pediatrics.* **1985**, *75*, 146-150.

(4) Picciano, M.F. Nutrient composition of human milk. *Pediatr. Clin. North Am.* **2001**, *48*, 53-67.

(5) Innis, S.M. Fatty acid requirements of the newborn. *Can. J. Physiol. Pharmacol.* **1994**, *72*, 1483-1492.

(6) 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.

(7) 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.

(8) Bitman, J.; Wood, D.L.; 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.

(9) 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.

(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) 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.

(12) 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.

(13) Dotson, K.D.; Jerrell, J.P.; Picciano, M.F.; Perkins, E.G. High-performance liquid chromatography of human milk triacylglycerols and gas chromatography of component fatty acids. *Lipids.* **1992**, *27*, 933-939.

(14) 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.

(15) López-López, A.; López-Sabater, M.C.; Campoy-Folgoso, C.; Rivero-Urgel, M.; Castellote-Bergalló, 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.

(16) 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.

(17) 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.

(18) 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.

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

(20) Akoh, C. C.; Xu, X. Enzymatic production of Betapol and other specialty fats. In *Lipid Biotechnology*; Kuo, C. C., Gardner, H.W., Eds.; Dekker: New York, **2002**; pp 461-478.

(21) 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.

(22) 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.

(23) Guil-Guerrero, J.L. Stearidonic acid (18:4n-3): Metabolism, nutritional importance, medical uses and natural sources. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 1226-1236.

(24) Stoffel, W.; Ahrens, E.H., Jr. The unsaturated fatty acids in menhaden body oil: the C18, C20, and C22 series. *J. Lipid Res.* **1960**, *1*, 139-146.

(25) James, M.J.; Ursin, V.M.; Cleland, L.G. Metabolism of stearidonic acid in human subjects: comparison with the metabolism of other n-3 fatty acids. *Am. J. Clin. Nutr.* **2003**, *77*, 1140-1145.

(26) Haung, Y.S.; Smith, R.S.; Redden, P.R.; Cantrill, R.C.; Horrobin, D.F. Modification of liver fatty acid metabolism in mice by omega-3 and omega-6 Δ6-desaturase substrates and products. *Biochim. Biophys. Acta*. **1991**, 1082, 319-327.

(27) Miles, E.A.; Banerjee, T., Dooper, M.M.B.W.; M'Rabet, L.; Graus, Y.M.F.; Calder, P.C. The influence of different combinations of γ-linolenic acid, stearidonic acid and EPA on immune function in healthy young male subjects. *Br. J. Nutr.* **2004**, *91*, 893-903.

(28) Calder, P.C., *n*-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am. J. Clin. Nutr*. **2006**, *83*, 1505S-1519S.

(29) Hibbeln, J.R., Nieminen, L.R., Blasbalg, T.L., Riggs, J.A., Lands, W.E., Healthy intakes of *n*-3 and *n*-6 fatty acids: estimations considering worldwide diversity. *Am. J. Clin. Nutr*. **2006**, *83*, 1483S-1493S.

(30) Hammond, B.G.; Lemen, J.K.; Ahmed, G.; Miller, K.D.; Kirkpatrick, J.; Fleeman, T. Safety assessment of SDA soybean oil: results of a 28-day study and a 90-day/one generation reproduction feeding study in rats. *Regul. Toxicol. Pharmacol*. **2008**, *52*, 311-323.

(31) Bernal-Santos, G.; O'Donnell, A.M.; Vicini, J.L.; Hartnell, G.F.; Bauman, D.E. Enhancing omega-3 fatty acids in milk fat of dairy cows by using stearidonic acid-enriched soybean oil from genetically modified soybeans. *J. Dairy Sci.* **2010**, *93*, 32-37.

(32) Harris, W.S.; Lemke, S.L.; Hansen, S.N.; Goldstein, D.A.; DiRienzo, M.A.; Su, H.; Nemeth, M.A.; Taylor, M.L.; Ahmed, G.; George, C. Stearidonic acid-enriched soybean oil increased the omega-3 index, an emerging cardiovascular risk marker. *Lipids*. **2008**, *43*, 805-811.

(33) Iwasaki, Y.; Yamane, T. Enzymatic synthesis of structured lipids. *J. Mol. Catal. B. Enzym.* **2000**, *10*, 129-140.

(34) 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.

(35) 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.

(36) 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.

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

(38) 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.

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

(40) 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.

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

(42) 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.

(43) 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.

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

(45) 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.

(46) 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.

(47) Horrock, L.A.; Yeo, Y.K. Health benefits of docosahexaenoic acid (DHA). *Pharmacol. Res.* **1999**, *40*, 211-225.

(48) Haumann, B.R. Alternative sources for n-3 fatty acids. *Inform.* **1998**, *9*, 1108-1119.

(49) Clough, P.M. Specialty vegetable oils containing gamma-linolenic acid and stearidonic acid. In *Structured and Modified Lipids*. Gunstone, F.D., Ed.; Marcel Dekker: New York, NY, **2001**, pp. 74-117.

(50) 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.

(51) Horrobin, D.F. Manku, M.S. Clinical biochemistry of essential fatty acids, In Omega-6 Essential Fatty Acid: Pathophysiology and Roles in Clinical Medicine, edited by D.F. Horrobin, Alan Liss, New York, **1990**, 21-53.

(52) Horrobin, D.F. Clinical applications of n-6 essential fatty acids: atopic eczema and inflammation , diabetic neuropathy and retinopathy, breast pain and viral infections, In *Essential Fatty Acids and Eicosanoids*, edited by A. Sinclair and R. Gibson, American Oil Chemist's Society, Champaign, **1992**, 367-372.

(53) Clandinin, M.T.; Garg, M.L.; Parrott, A.; Van Aerde, J.; Hervada, A.; Lien, E. Addition of long-chain polyunsaturated fatty acids to formula for very low birth weight infants. *Lipids.* **1992**, *27*, 896-900.

(54) Fewtrell, M.S.; Abbott, R.A.; Kennedy, K.; Singhal, A.; Morley, R.; Caine, E.; Jamieson, C.; Cockburn, F.; Lucas, A. Randomized, double-blind trial of long-chain polyunsaturated fatty acid supplementation with fish oil and borage oil in preterm infants. *J. Pediatr.* **2004**, *144,* 471-479.

(55) Van Gool, C.J.A.W.; Thijs, C.; Henquet, C.J.M.; van Houwelingen, A.C.; Dagnelie, P.C.; Schrander, J.; Menheere, P.P.C.A.; van den Brandt, P.A. γ-Linolenic acid supplementation for prophylaxis of atopic dermatitis – a randomized controlled trial in infants at high familial risk. *Am. J. Clin. Nutr.* **2003**, *77*, 943–951.

(56) Ponder, D.L.; Innis, S.M.; Benson, J.D.; Siegman, J.S. Docosahexaenoic acid status of term infants fed breast milk or infant formula containing soy oil or corn oil. *Pediatr. Res.* **1992**, *32*, 683-688.

(57) Simopoulos, A.P. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp. Biol. Med.* **2008**, *233*, 674–688.

(58) Ailhaud, G.; Massiera, F.; Weill, P.; Legrand, P.; Alessandri, J-M.; Guesnet, P. Temporal changes in dietary fats: Role of n-6 polyunsaturated fatty acids in excessive adipose tissue development and relationship to obesity. *Prog. Lipid Res.* **2006**, *45*, 203-236.

(59) Simopoulos, A.P.; Leaf, A.; Salem Jr., N. Workshop statement on the essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. *Prostaglandins Leukot. Essent. Fatty Acids.* **2000**, *63*, 119-121.

(60) Jensen, C.L.; Prager, T.C.; Fraley, J.K.; Chen, H.; Anderson, R.E.; Heird, W.C. Effect of dietary linoleic/alpha-linolenic acid ratio on growth and visual function of term infants. *J. Pediatr.* **1997**, *131*, 200-209.

(*61*)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.

(*62*)Sahin, N.; Akoh, C.C.; Karaali, A. Enzymatic production of human milk fat substitutes containing γ-Linolenic acid: Optimization of reactions by response surface methodology. *J. Am. Oil Chem. Soc*. **2005**, *82*, 549-557.

(*63*) Bektaş, I.; Yucel, S.; Ustun, G.; Aksoy, H.A. Production of reduced calorie structured lipid by acidolysis of tripalmitin with capric acid: optimisation by response surface methodology. *J. Sci. Food Agric*. **2008**, *88*, 1927-1931.

(64) Rao, R.; Manohar, B.; Sambaiah, K.; Lokesh, B.R. Enzymatic acidolysis in hexane to produce n-3 or n-6 FA-enriched structured lipids from coconut oil: Optimization of reactions by response surface methodology. *J. Am. Oil Chem. Soc*. **2002**, *79*, 885-890.

(65) 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.

(66) 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.

(67) 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.

(68) 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.

(69) 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.

(70) Pina-Rodriguez, A.M.; Akoh, C.C. Enrichment of amaranth oil with ethyl palmitate at the sn-2 position by chemical and enzymatic synthesis. *J. Agric. Food Chem*. **2009**, *57*, 4657-4662.

(71) 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.

(72) Jiménez, M.J.; Esteban, L.; Robles, A.; Hita, E.; González, P.A.; Muñío, M.M.; Molina, E. Production of triacylglycerols rich in palmitic acid at position 2 as intermediates for the synthesis of human milk fat substitutes by enzymatic acidolysis. *Process Biochem.* **2010**, *45*, 407-414.

(73) Tecelão, C.; Silva, J.; Dubreucq, E.; Ribeiro, M.H.; Ferreira-Dias, S. Production of human milk fat substitutes enriched in omega-3 polyunsaturated fatty acids using immbolized commercial lipases and *Candida parasilosis* lipase/acyltransferase. *J. Mol. Catal. B: Enzym.* **2010**, *65*, 122-127.

(74) Akoh, C.C.; Jennings, B.H.; Lillard, D.A. Enzymatic modification of trilinolein: Incorporation of n-3 polyunsaturated fatty acids. *J. Am. Oil Chem. Soc*. **1995**, *72*, 1317-1321.

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

(76) 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.

(77) 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.

(78) Pina-Rodriguez, A.M.; Akoh, C.C. Composition and oxidative stability of a structured lipid from amaranth oil in a milk-based infant formula. *J. Food Sci*. **2010**, *75*, C140-C146.

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

(80) Shimada, Y.; Suenaga, M.; Sugihara, A.; Nakai, S.; Tominaga, Y. Continuous production of structured lipid containing γ-linolenic and caprylic acids by immobilized *Rhizopus delemar* lipase. *J. Am. Oil Chem. Soc*. **1999**, *76*, 189-193.

(81) 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.

FA	total[a]	*sn*-2[b]
C14:0 (Myristic)	3.60 – 9.13	9.66 ± 1.61
C16:0 (PA)	15.43 – 24.46	52.30 ± 4.44
C18:0 (Stearic)	4.60 – 8.13	1.71 ± 0.29
C18:1n-9 (Oleic)	28.30 – 43.83	13.97 ± 2.74
C18:2n-6 (LA)	10.61 – 25.30	10.95 ± 2.75
C18:3n-6 (GLA)	0.00 – 0.27	0.25 ± 0.09
C18:3n-3 (ALA)	0.41 – 1.68	0.59 ± 0.10
C20:4n-6 (ARA)	0.23 – 0.75	0.67 ± 0.15
C22:6n-3 (DHA)	0.15 – 0.56	0.64 ± 0.10
n-6/n-3	7.31 – 21.13	

Table 2.1 Composition of Mature Human Milk Fat (%wt/wt) of Selected FAs (*15*)

[a]Values are given as ranges. [b]Values are given as mean±SD, n=2.

Table 2.2 Fatty Acid Composition of SDA Soybean Oil and Conventional Soy Oil (*30*)		
FA (%)	SDA soybean oil (test)	conventional soy oil (control)
C16:0 (Palmitic)	12.86	10.93
C18:0 (Stearic)	4.54	4.25
C18:1n-9 (Oleic)	20.76	22.52
C18:2n-6 (Linoleic)	24.41	53.91
C18:3n-6 (γ-Linolenic)	9.86	6.01
C18:3n-3 (α-Linolenic)	6.08	ND
C18:4n-3 (Stearidonic)	19.93	ND
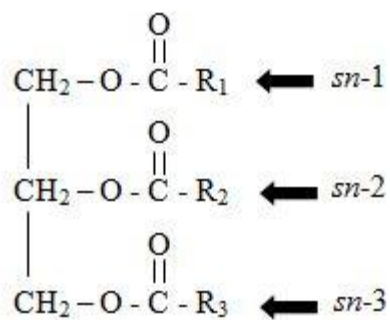
Figure 2.1 Basic triacylglycerol structure with R groups as fatty acids
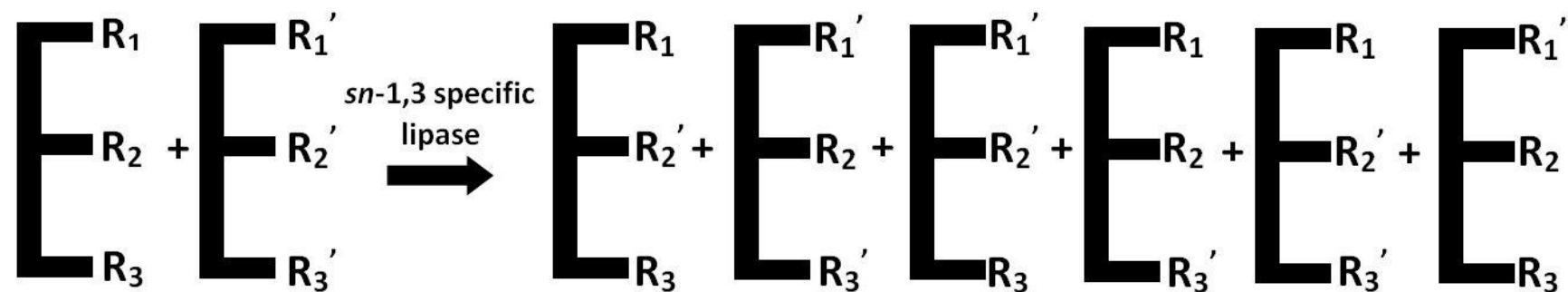
Figure 2.2 Scheme for producing structured lipids from two triacylglycerol molecules using a *sn*-1,3 specific lipase by enzymatic interesterification.
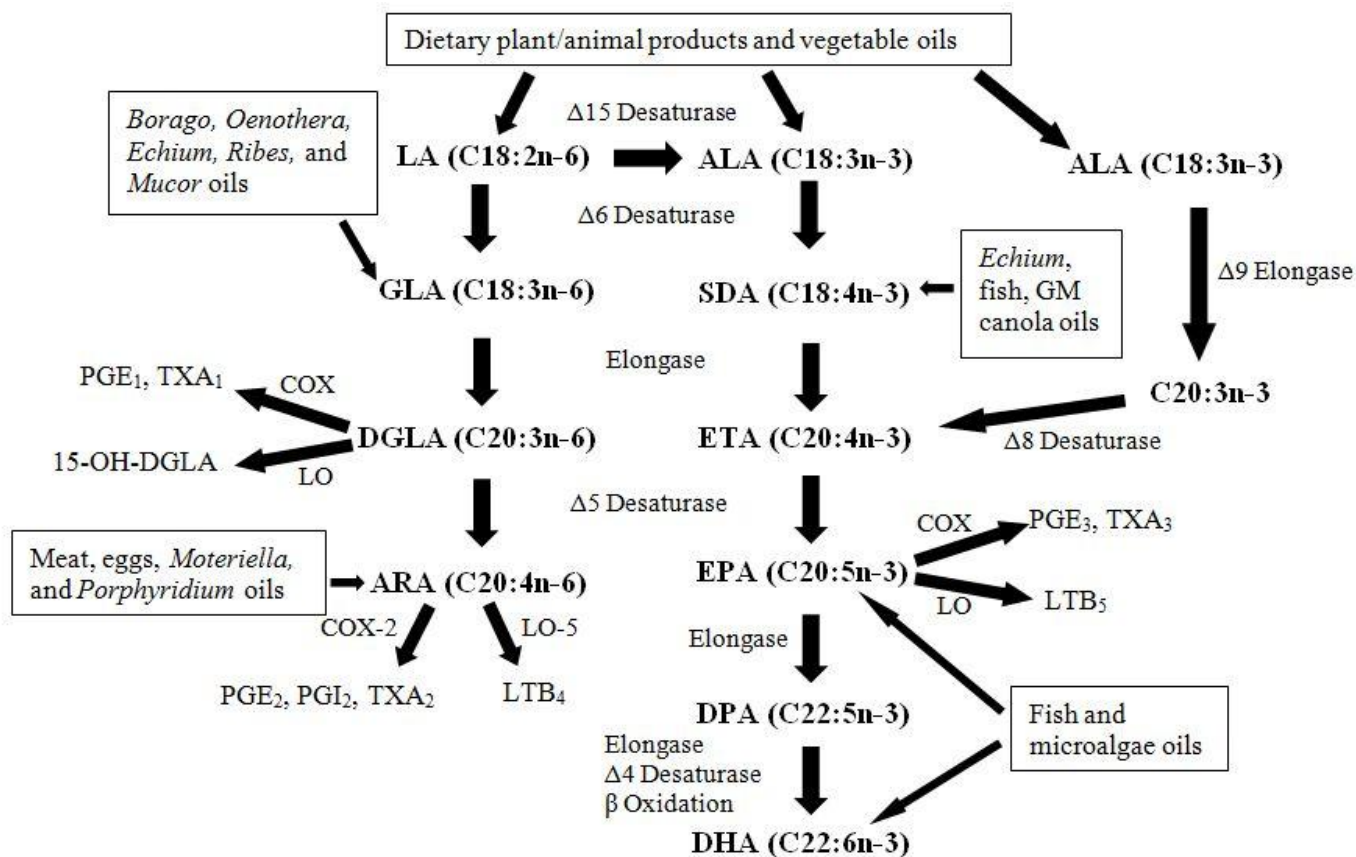
Figure 2.3 The biosynthetic pathways of omega-6 and omega-3 fatty acids. Redrawn from (*23*).

CHAPTER 3

STEARIDONIC ACID SOYBEAN OIL ENRICHED WITH PALMITIC ACID AT THE

SN-2 POSITION BY ENZYMATIC INTERESTERIFICATION FOR USE AS HUMAN MILK

FAT ANALOGUES[1]

ABSTRACT

Stearidonic acid (SDA, C18:4n-3) enriched soybean oil may be added to the diet to increase intake of omega-3 fatty acids (FAs). Human milk fat has ≥60% of palmitic acid (PA), by weight, esterified at the *sn*-2 position to improve absorption of fat and calcium in infants. Enzymatic interesterification of SDA soybean oil and tripalmitin produced structured lipids (SLs) enriched with PA at the *sn*-2 position of the triacylglycerol. Reactions were catalyzed by Novozym 435 or Lipozyme TL IM under various conditions of time, temperature, and substrate mole ratio. Response surface methodology was used to design the experiments. Model optimization conditions were predicted to be 1:2 substrate mole ratio at 50°C for 18 h with 10% (by weight) Lipozyme TL IM resulting in 6.82±1.87% total SDA and 67.19±9.59% PA at *sn*-2; 1:2 substrate mole ratio at 50°C for 15.6 h resulting in 8.01±2.41% total SDA and 64.43±13.69% PA at *sn*-2 with 10% (by weight) Novozym 435 as the biocatalyst. The SLs may be useful as human milk fat analogues for infant formula formulation with health benefits of the omega-3 FAs.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) with 18 carbons or more occur in plants and animals including fish, microalgae and fungi. However, the seeds of higher plants contain the richest source of PUFAs. The essential fatty acids (FAs), linoleic acid (LA) and α-linolenic acid (ALA), along with stearidonic acid (SDA) accumulate in plant tissues (*1*). ALA can be converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by desaturases and elongases, but their conversion is often poor (*2*). This poor conversion can possibly be explained by the initial Δ6 desaturase enzyme being rate limiting in humans (*3*). However, the use of SDA oils could skip this rate limiting step allowing for better conversion to EPA and DHA. Miles *et al*. (*4*) observed that neither SDA nor 20:4n-3 appeared in the peripheral blood mononuclear cell when

dietary SDA was ingested at a level of 1.0g/day, indicating that SDA is readily metabolized to

EPA in the body. Therefore, SDA from plant oil may efficiently increase the EPA status of

immune cells, and SDA enriched soybean oil could be added to the diet to increase intake of

omega-3 FAs. SDA, from borage and echium oils, has been consumed as a component of dietary

supplements (*5*). SDA has been noted as a possible potent inhibitor of cancer growth, inhibitor of

on platelet aggregation, as an anti-inflammatory pharmaceutical, and it provides cardiovascular

benefits (*1*). The richest sources of SDA include algae and seed plants. Animal sources, such as

fish oils, are mainly sources of EPA and DHA but only contain small amounts of SDA (*1*).

James *et al*. (*6*) suggested that the daily ingestion of fish or fish oil to obtain the health benefits

of omega-3 FAs is not a sustainable long term approach. However, increasing ingestion of land-

based sources of omega-3 FAs is required to increase the tissue concentration of EPA and DHA.

James *et al*. (*6*) conducted a double-blind, parallel group study to examine the effect of dietary

SDA on increasing tissue concentrations of EPA in humans and compared SDA's ability with

that of ALA and EPA. They concluded that SDA vegetable oils were more effective in

increasing EPA tissue concentrations than the currently used ALA vegetable oils. Consuming

SDA has been shown to lead to the enrichment of tissues with EPA in humans (*5,6*). EPA is a

long chain omega-3 polyunsaturated FA. Very long chain fatty acids are essential for the growth

and development of infants. EPA has been linked to reductions in inflammation (*7*) and

neurological disorders (*8*). There are many oils on the market that contain ALA, but few that

contain SDA. Dietary SDA was found to increase EPA by 3- to 4-fold more effectively than

similar levels of ALA whereas SDA was approximately one-third as effective as dietary EPA

(*9*). Therefore, the relative effectiveness of these FAs in increasing the EPA concentrations in

tissues is EPA>SDA>ALA (*6*). SDA enriched soybean oil could be used in many different food

products, such as infant formula, to increase the omega-3 FA intake in infants to help with their growth, development, and intestinal absorption of fat-soluble vitamins (*10*). Lipids found in breast milk provide essential FAs that are required as structural cell components of membrane tissues, and they provide an essential source of energy that is approximately 50% of the total energy used by infants (*10*).

SDA soybean oil contains approximately 20% stearidonic, 24% linoleic, and 12% palmitic acids (*11*). Palmitic acid (PA) is the second major FA found in breast milk at approximately 18.3-25.9% (*12-14*). SDA soybean oil contains lower amounts of PA than breast milk. Human breast milk contains a large portion of PA esterified at the *sn*-2 position of the triacylglycerols (TAGs). Over 60% (by weight) of PA is esterified at the *sn-2* position of human milk fat, and mainly unsaturated FAs are found at *sn*-1, 3 positions (*15-17*). As TAGs are metabolized, the FAs esterified at the *sn*-1,3 positions are released by pancreatic lipase during the digestion of fat molecules. The FAs esterified at the *sn*-2 position remain unhydrolyzed and are absorbed by the intestinal mucosa as *sn*-2 monoacylglycerols (*18*). The large amount of PA esterified at the *sn*-2 position improves absorption of fat and calcium in an infant (*16,17,19,20*). The PA in breast milk also reduces the formation and disposal of "calcium soaps" generated by the long chain saturated FA interaction with calcium (*15, 20*). In most infant formulas, the fat is of vegetable origin and contains mostly unsaturated fatty acids at the *sn*-2 position (*21*).

Structured lipids (SLs) are TAGs that have been modified to change the FA composition and/or their position in the glycerol backbone by chemically and/or enzymatically catalyzed reactions. Currently, there have been no other studies on modifying SDA soybean oil for potential application in infant formulas. Previous studies have been published on the development of human milk fat analogues. These studies have used interesterification of

randomized oil mixtures containing amaranth oil and ethyl palmitate (*22*), hazelnut oil fatty acids and tripalmitin (*23*), lard (*24*), and butter oil (*25*) to produce SLs that resemble human milk fat. Betapol (Loders Croklaan, Glen Ellyn IL, USA) was the first to commercially produce a human milk fat analog by using a 1,3-specific lipase to synthesize SLs by reacting tripalmitin with unsaturated FAs (*21*).

SDA soybean oil may be used as an alternative substrate to produce SLs for use as human milk fat analogues in infant formulas. The objective of this study was to increase the PA content at the *sn*-2 position of SDA soybean oil to over 60% (using tripalmitin as the substrate) while obtaining a fair amount of total SDA by enzymatic interesterification. Two types of enzymes were evaluated: Lipozyme TL IM (*Thermomyces lanuginosus* immobilized on silica gel, *sn*-1,3 specific lipase, specific activity 250 IUN/g) and Novozym 435 (*Candida antarctica* immobilized on macroporous acrylic resin beads, non-specific lipase, specific activity 10,000 PLU/g). IUN is the interesterification units novo and PLU is the propyl laurate units. It is critical to find the optimization conditions to produce a SL from SDA soybean oil enriched with PA at the *sn*-2 position for use as a human milk fat analogue in infant formula because no such work has been done so far. The health benefits to the infant from having PA at the *sn*-2 position while containing SDA omega-3 FA are substantial. Response surface methodology (RSM) enables the evaluation of the effects that multiple parameters have on response variables (*26*) and limits the number of reactions to be performed. RSM was applied to determine the optimization conditions for the production of SLs with PA at the *sn*-2 position and also containing SDA by varying substrate mole ratios, time and temperature.

MATERIALS AND METHODS

Materials.

SDA soybean oil was kindly provided by Monsanto Company (St. Louis, MO). Tripalmitin was

purchased from TCI America (Portland, OR). Immobilized lipases, Novozym 435 (non-specific

lipase) and Lipozyme TL IM (*sn*-1, 3 specific lipase), were obtained from Novozymes North

America Inc. (Franklinton, NC). The lipid standards Supelco 37 Component FAME mix, triolein

and 2-oleoylglycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and

C19:0-nonadecanoic acid was purchased from TCI America (Portland, OR). Other solvents and

chemicals were purchased from Fisher Scientific (Norcross, GA) and Sigma-Aldrich Chemical

Co. (St. Louis, MO).

Experimental Design by Response Surface Methodology (RSM).

A RSM mathematical model (Modde 5.0, Umetrics, Umeå, Sweden) was used to predict the

incorporation of PA into SDA soybean oil by enzymatic interesterification with Novozym 435

and Lipozyme TL IM lipases. The substrate mixtures of SDA soybean oil and tripalmitin were

determined on the basis of their average molecular weights. **Table 3.1** shows the resulting

suggested combinations from the experimental design performed with both Novozym 435 and

Lipozyme TL IM. The experimental design considered three factors: the time of the reaction

(low level, 6 h; high level, 18 h), temperature of the reaction (low level, 50°C; high level, 65°C),

and the substrate mole ratio of SDA soybean oil to tripalmitin (low level, 1:2; high level, 1:4)

using Novozym 435 and Lipozyme TL IM at 10% by weight of the substrates. The central

composite face design consisted of sixteen different combinations resulting in a total of eighteen

experiments. Experiments were performed in triplicate resulting in a total of fifty-four total

reactions. All reactions were performed in a water bath at the correct temperature with constant

shaking at 200 rpm. Once the reaction was complete, the enzyme was removed by passing the SL

products through an anhydrous sodium sulfate column. The resulting TAGs were recovered after

TLC separation and analyzed for their fatty acid profile and positional analysis. The total amount

of SDA in the SLs and the incorporation of PA at the *sn*-2 position of the glycerol backbone

were recorded in **Table 3.1** as variable responses.

RSM Mathematical Model.

The second order coefficients were obtained by regression analysis with backward elimination.

The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the

analysis of variance (ANOVA) from Modde 5.0 (Umetrics, Umeå, Sweden). The relationships

between the factors and variables from the above design were fitted into a second-order

polynomial equation:

(1) $\qquad Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_i X_i X_j$

where Y is the dependent variable (response 1, total amount of SDA; response 2, PA

incorporation at *sn*-2 position), β_0 is the intercept, β_i linear, β_{ii} quadratic, β_{ij} interaction term

coefficients, and X_i and X_j are independent variables.

Enzymatic Interesterification.

One hundred milligrams of each mixture (in ratios of 1:2, 1:3, and 1:4 mol SDA/mol tripalmitin)

was weighed into labeled Teflon-lined test tubes and 3 mL of n-hexane was added. Ten percent

by weight of the enzyme (based on total substrate weight), either Novozym 435 or Lipozyme TL

IM, was added to the test tube as the biocatalyst. The reaction was carried out at the temperatures

indicated from the RSM conditions (50, 55, 60 or 65°C) for the times indicated (6, 12, or 18 h) in

a water bath while shaking at 200 rpm. After the reaction, the products were filtered two times

through a sodium sulfate column to remove water and the biocatalyst.

Recovery of Triacylglycerols (TAGs).

Silica gel G TLC plates were activated in an oven at 100°C for 1 h to remove excess water. A mixture of petroleum ether, diethyl ether, and acetic acid (90:10:0.5, v/v/v) was equilibrated for approximately 30 min and used as the mobile phase to separate the TAGs. Following enzymatic interesterification, the products were spotted on the dried and cooled TLC plates, and placed into the TLC tanks. The lipid bands were visualized under UV light after spraying with 0.2% 2, 7-dicholorofluorescein in methanol. The TAG bands were identified using triolein as the standard. The TAG bands were scraped off into test tubes for fatty acid methyl ester (FAME) analysis and positional analysis. The recovery of TAGs was performed to isolate the TAG molecules and remove free FAs from the product mixture.

Determination of Fatty Acid Profiles.

SDA soybean oil and SL samples were converted to FAME following the AOAC Official Method 996.01, Section E, (*27*) with minor modifications (*22*). For analysis of the SDA soybean oil, 150 mg of the oil was weighed into a Teflon-lined test tube, 100 µL of the internal standard, C19:0 in hexane (20 mg/mL), was added and dried under nitrogen to remove solvent. For analysis of the SL, 50 µL of the internal standard was added to the recovered TAG band (as described above). Two milliliters of 0.5 N NaOH in methanol was added and incubated at 100°C for 5 min for saponification. The samples were cooled under tap water, and 2 mL of 14% BF_3 in methanol was added followed by vortexing for 1 min. Again, the sample was incubated at 100°C for 5 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 upper organic layer was removed and recovered in a GC vial for analysis.

The FAME external standard used was the Supelco 37 component FAME mix and was run parallel with the samples.

Positional Analysis.

The recovered TAGs were extracted from the silica gel with 2 mL of diethyl ether, vortexing, centrifuging at 1000 rpm for 3 min, and filtration through an anhydrous sodium sulfate column. This extraction step was repeated. The SL was completely dried under nitrogen gas. A modified version of the Luddy *et al*. (*28*) method was used to perform the pancreatic lipase-catalyzed *sn*-2 positional analysis. One hundred milligrams of SDA soybean oil and the SLs (extracted and dried TAG) were placed into Teflon-lined test tubes. Two milliliter 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 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 samples were vortexed again for 2 min. To stop the reaction and extract the hydrolyzed TAGs, 1 mL of 6N HCl and 4 mL of diethyl ether were added. The samples were vortexed for 2 min and centrifuged at 1000 rpm for 3 min. The upper layer, containing the lipid components, was filtered twice through an anhydrous sodium sulfate column. The samples were 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 minutes. The concentrated sample (~50 µL) was spotted onto the activated silica gel G dried TLC plates and placed into the tank. 2-Oleylglycerol was spotted as the standard and run parallel with the samples for identification of the 2-monoacylglycerol (2-MAG) band. The plates were sprayed with 0.2% 2, 7-dichlorofluorescein in methanol and visualized under UV light. The 2-MAG band was scraped

off and converted to FAME (as described above). One hundred microliters of the internal standard (20 mg/mL C19:0 in hexane) was used for the SDA soybean oil and 50 µL of the internal standard for the SL. The fatty acid content at the *sn*-2 position was quantified by GC, and the fatty acid content at the *sn*-1, 3 positions was calculated.

GC Analysis.

The FAMEs (from the SDA soybean oil, SL, and corresponding positional analyses) were analyzed using an Agilent Technology 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) with a flame ionization detector. A Supelco SP-2560 column, 100 m x 250 µm, 0.20 µm film was used to attain separation. Injection of 1 µL of sample was made at a split ratio of 20:1. Helium was the carrier gas at a flow rate of 1.1 mL/min and at a constant pressure. The injector temperature and the FID set point were 300°C. The oven was held at 140°C for 5 min, then increased up to 240°C at 4°C/min and held at 240°C for 15 min. The relative FAME content was calculated using the online computer. The average and standard deviation of triplicate analyses were reported.

Statistical Analysis.

All samples, reactions, and analyses were done in triplicate for SDA soybean oil and SLs. The average and standard deviations were calculated and reported for all analyses. The analysis of variance (ANOVA) and the mathematical model for optimization were attained using MODDE 5.0 (Umetrics, Umeå, Sweden).

Verification of Model.

Enzymatic interesterification reactions were carried out in test tubes at random conditions obtained with RSM to verify the model. The experimental values were then compared to the predicted values for the model, as shown in **Table 3.3**.

RESULTS AND DISCUSSION

The SDA soybean oil FA profile is shown in **Table 3.2**. The results from the FA profile were used to estimate the molecular weight of SDA soybean oil (MW= 902.18 ±0.16 g/mol). The major FA in the SDA soybean oil was linoleic (25.78 ± 0.07%), followed by octadecatetraenoic or SDA (22.16 ± 0.23%), oleic (14.31 ± 0.17%), and PA (11.54 ± 0.04%). Linoleic acid was the major FA at the *sn*-2 position, whereas stearidonic acid was the major FA at the *sn*-1,3 positions. Linoleic acid content in breast milk is approximately 15.6% (*13*) which is considerably lower than the linoleic acid content in the SDA soybean oil. PA constitutes the majority of saturated FAs in human breast milk at over 60% by weight of its total content at the *sn*-2 position. This large amount of PA allows for reduced formation of calcium soaps and providing readily absorbed energy for the development and growth of the infant. It also improves fat and calcium absorption (*15-17*). However, SDA soybean oil contains only a small amount of PA at the *sn*-2 position, only 4.77 ± 4.39%. SDA soybean oil itself would not be adequate enough for use in infant formula as a human milk fat analogue. Esterification techniques, chemical and enzymatic, are often used to produce SLs with improved functionality by incorporating new FAs into the oil or by rearranging the FAs already in the oil (*29*). Enzymatic interesterification is more spatially selective producing more specific TAGs (*30*). The aim of this research was to modify the SDA soybean oil's TAG to increase the PA esterified at the *sn*-2 position to meet the recommended FA requirements for human milk fat analogues (*31*). Two different enzymes, Novozym 435 (non-specific) and Lipozyme TL IM (*sn*-1,3 specific) were used for the interesterification to see if there were any differences in the incorporations of PA and SDA in the SL molecules. The same experimental conditions and substrate levels were employed for both enzymes. An increased PA content at the *sn*-2 position resulted in a lower total SDA content of the SLs,

especially when the substrate mole ratio was increased (**Table 3.1**). Both enzymes provided an increase in PA content at the *sn*-2 position and a decrease in the total SDA content. The *sn*-1,3 specific lipase (Lipozyme TL IM) gave slightly higher incorporation of palmitic acid at the *sn*-2 position of SLs because SDA soybean oil contained more SDA (25.26 ± 1.40%) at the *sn*-1,3 position which were readily cleaved by Lipozyme TL IM. As previously mentioned, higher PA content at the *sn*-2 position results in the reduced formation of calcium soaps and better absorption of fats and calcium by the infant. Therefore, either of the lipases can be used to produce suitable SLs as human milk fat analogues.

The experimental design was developed using response surface methodology (RSM) to produce a model to predict PA content at the *sn*-2 position and total amount of SDA. The factors in the experimental design included time, temperature, and substrate mole ratio. The responses or resulting amounts of total SDA content and PA content at the *sn*-2 position are shown in **Table 3.1**. For incorporation of PA at the *sn*-2 position, multiple linear regression and backward selection method were used to fit the results into a second-order polynomial model, where the only significant terms were *mol* and *temp*temp* for both enzymes. The predicted values by the model versus observed experimental values are given in **Figure 3.1** for both enzymes. The multiple correlation coefficient or R^2 was 0.906 and 0.918 for Novozym 435 and Lipozyme TL IM, respectively. This indicates that there is a linear relationship between the predicted values and the observed values at a confidence level of 95%. The Q^2 value was 0.346 and 0.525 for Novozym 435 and Lipozyme TL IM, respectively. The R^2 value is often used to assess the variance explanation. However, in planned experimentation, it is more significant to support conclusions based on analysis of variance (ANOVA) statistics (*26*). The most significant first-order parameter was substrate mole ratio for PA incorporation at the *sn*-2 position. The most

significant second-order parameter was temperature x temperature. Both the first-order and second-order parameters had positive effects. However, total content of SDA was less significant with R^2 values of 0.841 and 0.840 and Q^2 values of -0.392 and 0.357 for Novozym 435 and Lipozyme TL IM, respectively. Total SDA content only showed significance in a linear distribution resulting in no significant second-order parameter. The significant first-order parameters were time having a positive effect and temperature having a negative effect. However, the models for total SDA or *sn*-2 PA incorporation showed no significance for lack of fit. Statistically, the models showed no lack of fit (P > 0.05).

The model equation for the response of PA incorporation at the *sn*-2 position can therefore be written as: PA at *sn*-2 = 27.99 + 5.85S_r + 36.91T^2 for Novozym 435 and PA at *sn*-2 = 58.21 + 6.71S_r + 22.72T^2 for Lipozyme TL IM. For total SDA content, the model equations are: total SDA = 3.25 + 0.909t − 2.19T Novozym 435 and total SDA = 2.02 + 0.84t − 1.35T for Lipozyme TL IM. *T* indicates the reaction temperature, S_r indicates the substrate mole ratio, and *t* indicates the reaction time. Total SDA is the total amount of SDA in the SL, and PA at *sn*-2 is the content of PA incorporated at the *sn*-2 position of the SLs.

The relationship between responses and parameters was examined using contour plots for *sn*-2 PA incorporation and SDA incorporation. The contour plots obtained by the interaction of the three parameters on the *sn*-2 PA incorporation catalyzed by either Novozym 435 or Lipozyme TL IM are shown in **Figure 3.2**. The third variable was kept at a constant when these contour plots were drawn. The contour plots drawn were for the interaction of reaction time (h) with reaction temperature (^{o}C) when the substrate mole ratio was 1:2 (mol SDA soybean oil/mol tripalmitin), reaction time with substrate mole ratio at a reaction temperature of 65°C, and reaction temperature with substrate mole ratio at a reaction time of 18 h as shown in panels **A**, **B**,

and **C**, respectively. As shown in **Figure 3.2A**, the highest and the lowest temperatures (65 and 50°C) gave the highest incorporation (over 60%) of PA at the *sn*-2 position for both enzymes. Temperatures below 64°C and above 50.5^{o}C gave a PA content of less than 60%. Lipozyme TL IM at a 1:2 substrate mole ratio gave an overall higher incorporation of PA at the *sn*-2 position. An explanation for the Lipozyme TL IM giving a higher PA incorporation at *sn*-2 may in part be due to their regiospecificity. In **Figure 3.2B**, it can be seen that as the substrate mole ratio increases, the PA content at the *sn*-2 increases. This may be due to the substrate preference for PA by the enzymes and/or acyl migration from the 1- and 3-positions to the 2-position during the interesterification reaction. The value of lipases is related to their FA selectivity or their ability to discriminate between particular FAs or acyl moieties (*32*). Different studies have shown different incorporation rates of individual FAs depending on the enzyme used and the conditions of the experiments. Different lipases show preferences for different FAs (*33*). Peng *et al*. (*34*) studied the incorporations of EPA/DHA, conjugated linoleic acid (CLA), and caprylic acid. CLA and caprylic acid were found to have similar incorporation rates with the EPA/DHA mixtures having low incorporation. Lipozyme TL IM showed a slight discrimination for very long chain PUFAs in their study (*34*). Another study showed that a mycelium-bound lipase demonstrated a high preference towards short chain triacylglycerols after a 20 h reaction. Also, the lipase hydrolyzed coconut oil faster than palm olein followed by rapeseed, soybean, and cottonseed oils suggesting that the lipase has a preference for oils with saturated FAs rather than unsaturated FAs (*35*). However, even at a substrate mole ratio of 1:2, the *sn*-2 PA content was still above 60%, making it possible for their use in infant formula. The **Figure 3.2B** contour plots also show a difference in the two enzymes. Novozym 435 appears to be slightly better at incorporating PA at the *sn*-2 position around 6 h, whereas Lipozyme TL IM appears to be better at 10-16 h. Again

in **Figure 3.2C**, the higher *sn*-2 PA contents were at 50 and 65°C with the higher content being achieved at a substrate mole ratio of 1:4. The higher the concentration of PA (or tripalmitin), the more likely it will re-esterify to the glycerol backbone when compared to the lower concentrations of other FAs. From the contour plots shown in **Figure 3.2**, the trend seems to be that the higher the substrate mole ratio (1:4), the more the PA will be present at the *sn*-2 position and 50 and 65°C gave the highest incorporations. For total SDA incorporation at 65°C, the SDA content increased with increasing time for Lipozyme TL IM in **Figure 3.3A** with a higher SDA content at 18 h. For Novozym 435, the SDA content was highest between 10.5 and 16.5 h (**Figure 3.3A**). It may be that Novozym 435 has better preference for SDA than Lipozyme TL IM since Lipozyme TL IM has been shown in previous studies to prefer shorter chain FAs over longer chain FAs (*33*). Because it is a non-specific lipase, all three positions are cleaved and re-esterified at a faster rate than the sequentially acting *sn*-1,3 Lipozyme TL IM lipase. **Figure 3.3B** demonstrates that, at a substrate mole ratio of 1:2, the longer the reaction time, the better the incorporation of total SDA in the SL molecule, and this occurred at a lower temperature. **Figure 3.3C** also demonstrates that a lower temperature resulted in more incorporation of SDA, and this occurred at a lower substrate mole ratio (1:2). At a substrate mole ratio of 1:4, more PA was incorporated at *sn*-2 and less total SDA was obtained. With a higher amount of tripalmitin (PA), there was substrate competition between PA and SDA for the active site of the lipases at higher substrate mole ratios.

Verifications of the models were performed by performing enzymatic interesterification reactions at various conditions with RSM. **Table 3.3** shows the predicted values and conditions used for the verifications. Verifications for the Novozym 435 model fell between the upper and lower limits of the predicted values of total SDA and PA at *sn*-2 position with conditions of 1:3

substrate mole ratio at 56$^{\text{o}}$C for 13 h, 1:4 substrate mole ratio at 63$^{\text{o}}$C for 18 h, 1:2.5 substrate mole ratio at 59$^{\text{o}}$C for 9 h. However, the Lipozyme TL IM model verifications did not always fall within the upper and lower limits. The values of total SDA and PA at *sn*-2 position with conditions of 1:3.5 substrate mole ratio at 59$^{\text{o}}$C for 10 h did fall within the upper and lower limits. Conditions of 1:2 substrate mole ratio at 51$^{\text{o}}$C for 8 h fell within the limits for total SDA but not for PA at the *sn*-2 position (slightly above the upper limit), and conditions of 1:3 substrate mole ratio at 64$^{\text{o}}$C for 17 h fell within the limits for PA at the *sn*-2 position but not for total SDA incorporation (slightly above the upper limit). When taking into account the standard deviation for the 1:2 substrate mole ratio at 51$^{\text{o}}$C for 8 h for Lipozyme TL IM, the PA at *sn*-2 will fall between the upper and lower limits. The models may be used as a preliminary experimental tool to estimate the approximate optimal conditions and values of total SDA and PA at the *sn*-2 position. However, the models may result in slight error, as seen in the verification of the models, but this should not negate the usefulness of RSM predictions.

The optimal conditions for the targeted total SDA (>5%) and *sn*-2 PA incorporation (>60%) were generated by the optimizer function of the Modde 5.0 (Umetrics, Umeå, Sweden) software. These conditions were determined to be a substrate mole ratio of 1:2 at 50$^{\text{o}}$C for 18 h with 10% (by weight) Lipozyme TL IM resulting in 6.82 ± 1.87% total SDA and 67.19 ± 9.59% PA at *sn*-2, and a 1:2 substrate mole ratio at 50$^{\text{o}}$C for 15.6 h resulting in 8.01 ± 2.41% total SDA and 64.43 ± 13.69% PA at *sn*-2 with 10% (by weight) Novozym 435. These optimization conditions are used as a tool to give an estimate of the incorporation that may be obtained in SL products after interesterification. As seen in **Table 3.1**, higher amounts of PA at the *sn*-2 are possible but this increase could result in a lower amount of SDA incorporation. The SL products of the two lipases at optimal conditions were produced along with a physical blend, containing

no enzyme as a biocatalyst, with a substrate mole ratio of 1:2 at 50°C for 18 h. The FA profiles

of these three products are shown in **Table 3.4**. The differences in FA composition can be seen

in **Table 3.1** and **Table 3.4**. The high standard deviation in some of the experimental runs is not

unusual in biocatalysis. It may be due to the enzyme specificity, substrate mole ratio, reaction

temperature, and reaction time. A slight error in the observed value in **Table 3.4** can be seen for

total SDA incorporation for the Novozym 435 SL. As stated earlier, Novozym 435 is a (*Candida*

antarctica immobilized on macroporous acrylic resin beads*)* non-specific lipase with a specific

activity of 250 IUN/g and optimum reaction temperatures of 40-60 °C. Lipozyme TL IM

(*Thermomyces lanuginosus* immobilized on silica gel) is an *sn*-1,3 specific lipase with a specific

activity of 10,000 PLU/g. However, Lipozyme TL IM may also be non-specific depending on

the substrates. These enzymes can be used to improve the nutritional properties of lipids. A

specific lipase allows for the incorporation of an acyl group into a specific position on the TAG

molecule. An *sn*-1,3 specific lipase (Lipozyme TL IM) gives specificity and selectively during

esterification at the *sn*-1 and *sn*-3 positions. As seen in **Table 3.4**, the physical blend of SDA

soybean oil with tripalmitin gave a lower PA content at the *sn*-2 position of only 47.05 ± 4.24%.

This percentage of PA is below the over 60% of PA found at the *sn*-2 position in breast milk and

would not be adequate for use as a human milk fat analogue. Metabolically, a physical mixture is

not equivalent to a SL. However, using a biocatalyst can increase the amount of PA at the *sn*-2

position and hence their subsequent absorption as 2-MAG. For Lipozyme TL IM, it can increase

the total SDA content and the SDA content found at the *sn*-1,3 positions due to its

regiospecificity. A possible explanation for the Lipozyme TL IM SL having a higher PA

incorporation at *sn*-2 and higher total SDA incorporation may be due to acyl migration and

possibly the longer reaction time when compared to the Novozym 435 SL. The addition of a

biocatalyst and the incorporation of PA decreased the linoleic acid content (Novozym 435, 11.92 ± 1.09% and Lipozyme TL IM, 15.28 ± 0.50%) when compared to the original SDA soybean oil (25.78 ± 0.07%), in **Table 3.2**, making it similar to breast milk which contains approximately 15.6% linoleic acid (*13*). This can be explained by PA replacing linoleic acid at the *sn*-2 position, since linoleic acid was the major FA found at the *sn*-2 position of the SDA soybean oil. Linoleic acid is one of the essential FAs, along with ALA, that the human body cannot synthesize. Both SLs contain undetectable amounts of ALA. However, SDA is a sustainable plant source of omega-3 FA that readily converts to EPA and DHA better than ALA (*4*). EPA is a long chain omega-3 polyunsaturated FA that is essential for the growth and development of infants and has been linked to reductions in inflammation (*7*) and neurological disorders (*8*). Having enough SDA present in the SL may provide health benefits for the infant that normally would be provided from ALA.

Thus, human milk fat analogues (as SLs) containing SDA which is enriched with palmitic acid at the *sn*-2 position were successfully produced with the potential to deliver the absorption characteristics and FA content similar to human milk fat with health benefits associated with omega-3 fatty acids. Using interesterification with either Novozym 435 or Lipozyme TL IM, can produce a SL consisting of over 60% PA at the *sn*-2 position and containing over 6% of total SDA. Both enzymes may be used to produce a SL suitable for a human milk fat analogue. These SLs could be used as ingredients for infant formulas to help with nutrition, growth, and development.

ACKNOWLEDGEMENTS

REFERENCES

(1) Guil-Guerrero, J.L. Stearidonic acid (18:4n-3): Metabolism, nutritional importance, medical uses and natural sources. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 1226-1236.

(2) Gerster, H. Can adults adequately convert alpha-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? *Int. J. Vitam. Nutr. Res*. **1998**, *68*, 159-173.

(3) Haung, Y.S.; Smith, R.S.; Redden, P.R.; Cantrill, R.C.; Horrobin, D.F. Modification of liver fatty acid metabolism in mice by omega-3 and omega-6 Δ6-desaturase substrates and products. *Biochim. Biophys. Acta*. **1991**, 1082, 319-327.

(4) Miles, E.A.; Banerjee, T., Dooper, M.M.B.W.; M'Rabet, L.; Graus, Y.M.F.; Calder, P.C. The influence of different combinations of γ-linolenic acid, stearidonic acid and EPA on immune function in healthy young male subjects. *Br. J. Nutr*. **2004**, *91*, 893-903.

(*5*) Surette, M.E.; Edens M.; Chilton, F.H.; Tramposch, K.M. Dietary echium oil increases plasma and neutrophil long-chain (*n*-3) fatty acids and lowers serum triacylglycerols in hypertriglyceridemic humans. *J. Nutr*. **2004**, *134*, 1406-1411.

(6) James, M.J.; Ursin, V.M.; Cleland, L.G. Metabolism of stearidonic acid in human subjects: comparison with the metabolism of other n-3 fatty acids. *Am. J. Clin. Nutr.* **2003**, *77*, 1140-1145.).

 (*7*) Calder, P.C. *n*-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am. J. Clin. Nutr*. **2006**, *83*, 1505S-1519S.

(*8*) Hibbeln, J.R.; Nieminen, L.R.; Blasbalg, T.L.; Riggs, J.A.; Lands, W.E. Healthy intakes of *n*-3 and *n*-6 fatty acids: estimations considering worldwide diversity. *Am. J. Clin. Nutr*. **2006**, *83*, 1483S-1493S.

(*9*) Ursin, V.M. Modification of plant lipids for human health: development of functional land-based omega-3 fatty acids. *J. Nutr*. **2003**, *133*, 4271-4274.

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

(*11*) Hammond, B.G.; Lemen, J.K.; Ahmed, G.; Miller, K.D.; Kirkpatrick, J.; Fleeman, T. Safety assessment of SDA soybean oil: results of a 28-day study and a 90-day/one generation reproduction feeding study in rats. *Regul. Toxicol. Pharmacol*. **2008**, *52*, 311-323.

(*12*) 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.

(*13*) Bitman, J.; Wood, D.L.; 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.

(*14*) 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.

(*15*) 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.

(*16*) 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.

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

(18) Iwasaki, Y.; Yamane, T. Enzymatic synthesis of structured lipids. *J. Mol. Catal. B. Enzym.* **2000**, *10*, 129-140.

(*19*) 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.

(*20*) 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.

 (*21*) Akoh, C. C.; Xu, X. Enzymatic production of Betapol and other specialty fats. In *Lipid Biotechnology*; Kuo, C. C., Gardner, H.W., Eds.; Dekker: New York, **2002**; pp 461-478.

(*22*) Pina-Rodriguez, A.M.; Akoh, C.C. Enrichment of amaranth oil with ethyl palmitate at the sn-2 position by chemical and enzymatic synthesis. *J. Agric. Food Chem.* **2009**, *57*, 4657-4662.

(*23*) 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 substitute. *J. Agric. Food Chem.* **2005**, *53*, 5779-5783.

(*24*) Yang, T.K.; Xu, X.; He, C.; Li, L. Lipase-catalyzed modification of lard to produce human milk fat substitutes. *Food Chem.* **2003**, *80*, 473-481.

(*25*) Christensen, T.C.; Holmer, G. Lipase-catalyzed acyl-exchange reactions of butter oil. Synthesis of a human milk fat substitute for infant formulas. *Milchwissenschaft*. **1993**, *48*, 543-547.

(*26*) 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.

(*27*) *Official Methods of Analysis of AOAC International*, 17th ed.; AOAC International: Gaithersburg, MD, **1998**; Official Method 996.01.

(*28*) 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.

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

(*30*) 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.

(*31*) Koletzko, B.; Baker, S.; Cleghorn, G.; Fagundes Neto, U.; Gopalan, S.; Hernell, O.; Seng Hock, Q.; Jirapinyo, P.; Lonnerdal, B.; Pencharz, P.; Pzyrembel, H.; Ramirez-Mayans, J.; Shamir, R.; Turck, D.; Yamashiro, Y.; Zong-Yi, D. Global standard for the composition of infant formula: recommendations for an ESPGHAN coordinated international expert group. *J. Pediatr. Gastr. Nutr*. **2005**, *41*, 584-599.

(32) Borgdorf, R.; Warwel, S. Substrate selectivity of various lipases in the esterification of cis- and trans-9-octadecanoic acid. *Appl Microbial Biotechnol.* **1999**, *51*, 480-485.

(33) 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; CRC Press: New York, NY, **2008**; pp.783-786.

(34) Peng, L.; Xu, X.; Mu, H.; Høy, C-E.; Adler-Nissen, J. Production of structured phospholipids by lipase-catalyzed acidolysis: optimization using response surface methodology. *Enzyme Microb. Technol*. **2002**, *31*, 523-532.

(35) 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.

time (h)	temp (°C)	mole ratio[b]	Novozym 435		Lipozyme TL IM	
			total (mol% SDA)[c]	sn-2 (mol% PA)[d]	total (mol% SDA)[c]	sn-2 (mol% PA)[d]
6	50	1:2	4.15 ± 1.98	64.55 ± 13.73	5.37 ± 3.42	64.84 ± 3.66
18	50	1:2	9.05 ± 4.61	65.51 ± 9.61	6.78 ± 4.39	65.38 ± 9.05
12	60	1:2	4.23 ± 3.01	25.19 ± 7.26	3.55 ± 0.61	55.61 ± 1.96
6	65	1:2	1.94 ± 0.77	63.33 ± 15.10	1.90 ± 0.92	62.48 ± 6.61
18	65	1:2	2.17 ± 0.54	71.32 ± 4.03	2.82 ± 0.81	70.42 ± 1.82
12	50	1:3	7.32 ± 2.53	65.13 ± 4.03	3.32 ± 3.08	79.92 ± 11.60
18	55	1:3	2.10 ± 1.66	39.78 ± 7.01	4.02 ± 2.71	62.87 ± 13.08
6	60	1:3	2.11 ± 0.93	41.31 ± 0.58	1.24 ± 0.69	54.10 ± 6.33
18	65	1:3	1.53 ± 1.46	62.56 ± 13.21	2.82 ± 0.07	75.50 ± 2.58
6	50	1:4	4.75 ± 3.62	74.01 ± 15.42	3.05 ± 0.74	91.82 ± 2.53
18	50	1:4	7.27 ± 2.47	86.34 ± 2.47	6.14 ± 1.73	82.66 ± 8.19
6	55	1:4	3.28 ± 1.84	42.61 ± 8.12	3.29 ± 1.78	55.96 ± 13.68
18	60	1:4	3.48 ± 2.65	42.34 ± 23.31	2.55 ± 1.78	56.04 ± 7.41
6	65	1:4	0.86 ± 0.28	81.76 ± 3.38	1.51 ± 0.41	78.35 ± 3.05
12	65	1:4	1.13 ± 0.23	72.74 ± 13.21	1.70 ± 0.70	78.34 ± 4.43
18	65	1:4	2.41 ± 2.47	72.99 ± 8.34	2.63 ± 1.25	78.56 ± 3.79
18	65	1:4	3.33 ± 1.32	77.04 ± 10.56	4.84 ± 0.09	74.57 ± 16.21
18	65	1:4	2.96 ± 0.49	56.08 ± 28.50	3.72 ± 0.17	79.63 ± 0.72

Table 3.1 Total Incorporation of SDA (C18:4n-3) and Palmitic Acid (C16:0) at the *sn*-2 Position of Structured Lipids Produced by Enzymatic Interesterification Using RSM Conditions[a]

[a] All experiments were performed in triplicate, and average values ± SD were reported.

[b] Substrate mole ratio of SDA soybean oil (MW = 902.18g/mol) to tripalmitin (MW = 807.32 g/mol).

[c] Total content of SDA in the structured lipid.

[d] Incorporated palmitic acid at the *sn*-2 position of the structured lipid.

Table 3.2 Composition of Stearidonic Acid (SDA) Soybean Oil[a]		positional distribution	
fatty acid	total (mol %)[b]	*sn*-2 (mol %)	*sn*-1,3 (mol %)[c]
C16:0	11.54 ± 0.04	4.77 ± 4.39	14.92 ± 2.20
C18:0	3.80 ± 0.06	5.04 ± 4.30	4.05 ± 0.60
C18:1n-9c	14.31 ± 0.17	20.06 ± 1.19	11.43 ± 0.59
C18:2n-6t	1.44 ± 0.01	ND[d]	2.16 ± 0.00
C18:2n-6c	25.78 ± 0.07	34.11 ± 4.56	21.61 ± 2.28
C20:0	0.33 ± 0.01	ND	0.50 ± 0.00
C18:3n-6	7.65 ± 0.04	6.57 ± 3.29	8.18 ± 1.64
C18:3n-3	12.30 ± 0.06	5.74 ± 2.95	15.58 ± 1.47
C18:4n-3	22.16 ± 0.23	15.97 ± 2.80	25.26 ± 1.40
C20:1	0.39 ± 0.01	ND	0.59 ± 0.00
C22:0	0.30 ± 0.01	ND	0.46 ± 0.00

[a] Trace amounts of C15:0, C16:1, C17:0, and C24:0 were found in *sn*-2 analysis but were too small to be detected in total FA analysis.

[b] Mean ± SD, n=3.

[c] *sn*-1,3 (mol %) = [3 x total (mol%) – *sn*-2 (mol%)]/(2).

[d] Not detected.

enzyme	conditions			predicted			obsd	predicted			obsd
	time (h)	temp (°C)	mole ratio[a]	SDA[b]	LL[c]	UL[d]	SDA[b]	PA[e]	LL[c]	UL[d]	PA[e]
Novozym 435	18	63	1:4	2.64	1.12	4.16	3.33 ± 1.32	54.90	46.27	63.53	56.08 ± 28.50
Novozym 435	13	56	1:3	3.86	1.57	6.15	2.19 ± 1.67	29.94	16.95	42.94	27.32 ± 11.92
Novozym 435	9	59	1:2.5	2.57	0.55	4.59	4.01 ± 0.53	28.05	16.58	39.52	38.87 ± 11.44
Lipozyme TL IM	8	51	1:2	4.96	3.38	6.55	4.30 ± 3.25	61.82	53.70	69.95	70.02 ± 5.65
Lipozyme TL IM	17	64	1:3	2.16	0.90	3.42	3.72 ± 0.17	72.72	66.28	79.17	74.57 ± 16.21
Lipozyme TL IM	10	59	1:3.5	1.60	0.15	3.04	2.79 ± 0.45	60.44	53.04	67.84	60.80 ± 10.76

Table 3.3 Predicted and Observed (mol%) from RSM Model Verification

[a] Substrate mole ratio of SDA soybean oil to tripalmitin

[b] Total SDA content in TAG (mol%)

[c] Lower limit (mol%)

[d] Upper limit (mol%)

[e] Palmitic acid at *sn*-2 position (mol%)

Table 3.4 Fatty Acid Profiles (mol %) of a Physical Mixture and the Two SLs at Optimal Conditions[a]

FA	physical blend[b] (mol %)			Novozym 435 SL (mol %)			Lipozyme TL IM SL (mol %)		
	total	*sn*-2	*sn*-1,3[c]	total	*sn*-2	*sn*-1,3[c]	total	*sn*-2	*sn*-1,3[c]
C14:0	1.26 ± 0.02	1.95 ± 0.04	0.91 ± 0.04	0.85 ± 0.74	1.67 ± 0.37	0.72 ± 0.66	1.14 ± 0.01	1.60 ± 0.63	0.92 ± 0.31
C16:0	64.63 ± 1.86	47.05 ± 4.25	73.42 ± 0.70	71.89 ± 1.18	59.25 ± 4.14	78.21 ± 2.64	63.00 ± 1.28	62.09 ± 1.81	63.45 ± 2.44
C18:0	3.14 ± 0.07	9.59 ± 1.10	0.00 ± 0.59	3.60 ± 0.72	8.83 ± 1.03	0.98 ± 1.48	3.04 ± 0.14	6.78 ± 0.61	1.18 ± 0.12
C18:1n-9	6.90 ± 0.20	10.23 ± 1.36	5.23 ± 0.63	6.62 ± 0.65	5.78 ± 1.05	7.04 ± 1.06	6.14 ± 0.58	5.73 ± 1.36	6.34 ± 0.47
C18:2n-6	14.91 ± 0.77	20.56 ± 1.22	12.08 ± 0.96	11.92 ± 1.09	16.66 ± 1.18	9.55 ± 1.77	15.28 ± 0.50	15.05 ± 2.12	15.40 ± 0.31
C18:3n-6	2.13 ± 0.19	3.16 ± 0.24	1.62 ± 0.21	0.91 ± 0.79	3.83 ± 1.09	0.26 ± 0.41	2.54 ± 0.17	4.06 ± 1.92	1.78 ± 1.08
C20:1	3.06 ± 0.26	1.17 ± 0.15	4.01 ± 0.33	1.87 ± 0.22	0.37 ± 0.65	2.63 ± 0.58	3.44 ± 0.29	1.31 ± 0.25	4.51 ± 0.56
C18:4n-3	3.83 ± 0.43	2.56 ± 0.42	4.47 ± 0.46	2.32 ± 0.58	3.59 ± 1.65	1.69 ± 0.16	5.41 ± 0.98	3.39 ± 0.78	6.42 ± 1.86
C18:3n-3	0.14 ± 0.02	2.07 ± 1.47	0.00 ± 0.75	ND[d]	ND	ND	ND	ND	ND

[a] Trace amounts of C14:1 were also found in the *sn*-2 analysis of the physical blend but were too small to be detected in the total FA analysis. Optimal conditions for Novozym 435 SL were 1:2 substrate mole ratio, 50°C for 15.6 h and, for Lipozyme TL IM, 1:2 substrate mole ratio, 50°C for 18 h.

[b] Physical blend of 1 mole of SDA soybean oil to 2 moles of tripalmitin with no enzyme reacted at 50°C for 18 h.

[c] *sn*-1,3 (mol %) = [3 x total (mol%) – *sn*-2 (mol%)]/(2)

[d] Not detected.

A. Novozym 435
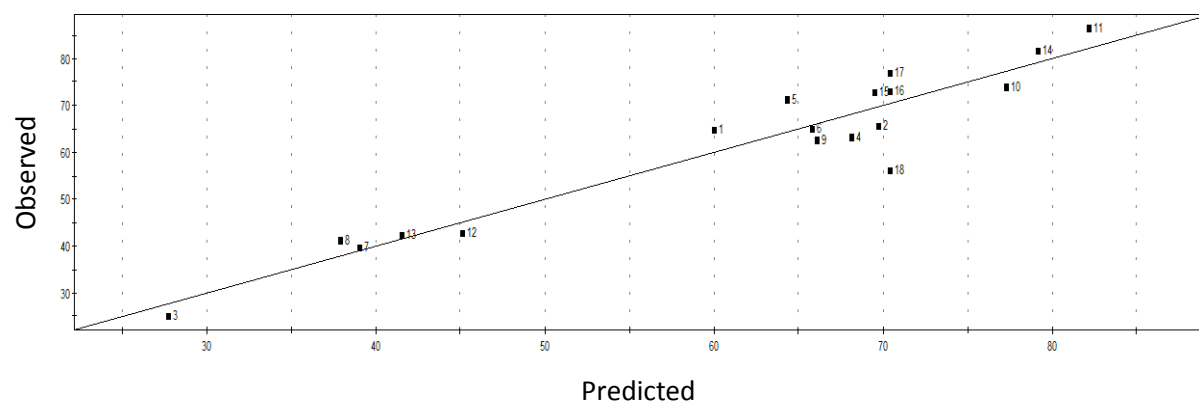
B. Lipozyme TL IM
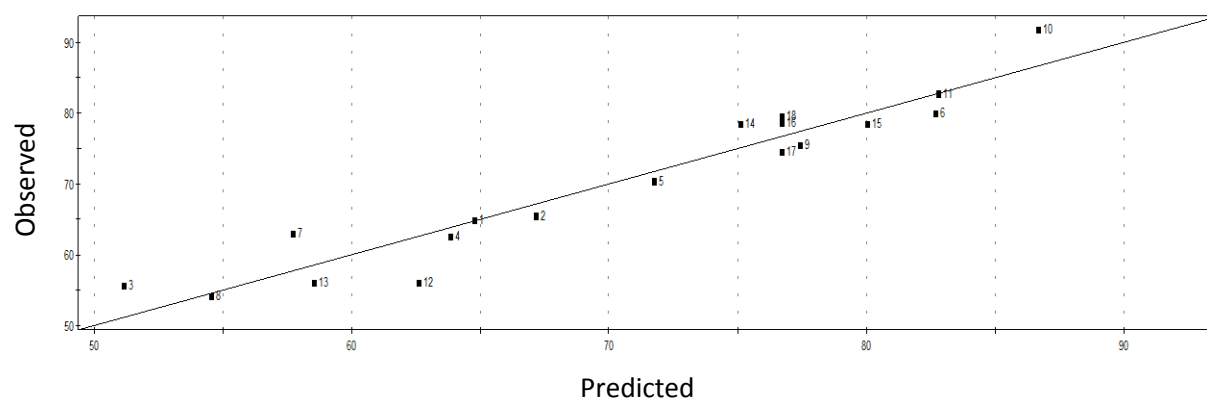
Figure 3.1 Relationship between observed and predicted data by the models: Incorporation of palmitic acid at the *sn*-2 position with (A) Novozym 435 and (B) Lipozyme TL IM as biocatalysts.

Figure 3.2 Contour plots between two parameters for *sn*-2 palmitic acid incorporation for Novozym 435 and Lipozyme TL IM lipases: (A) reaction time (h) versus reaction temperature (^{o}C) when the substrate mole ratio was 1:2 (mol SDA soybean oil/mol tripalmitin), (B) reaction time versus substrate mole ratio at a reaction temperature of 65°C, and (C) reaction temperature versus substrate mole ratio at a reaction time of 18 h.

Novozym 435   Lipozyme TL IM
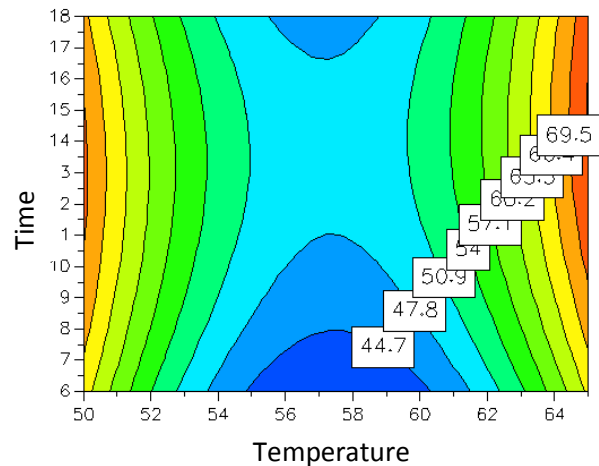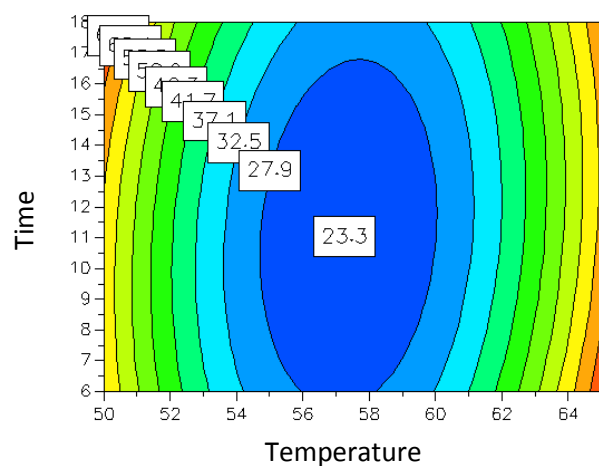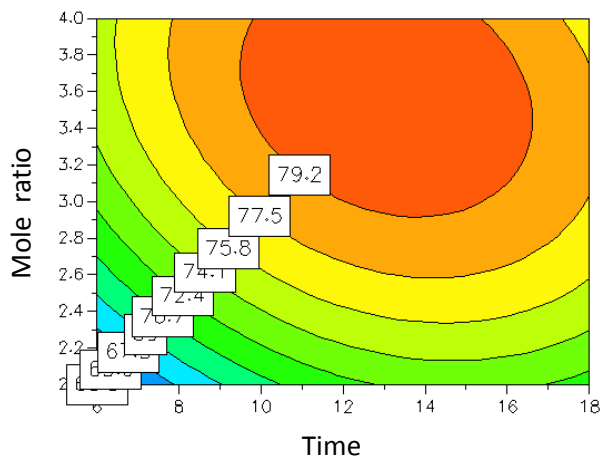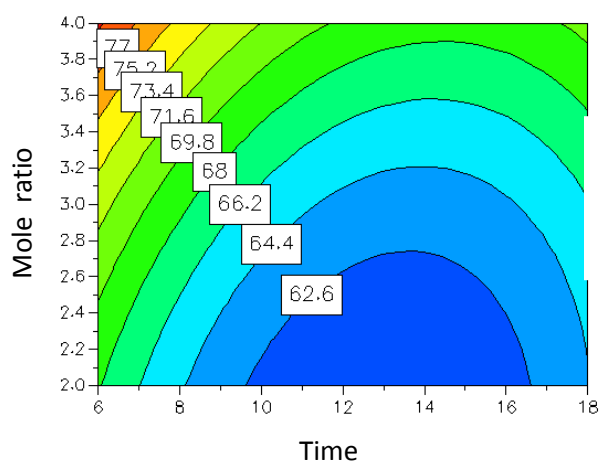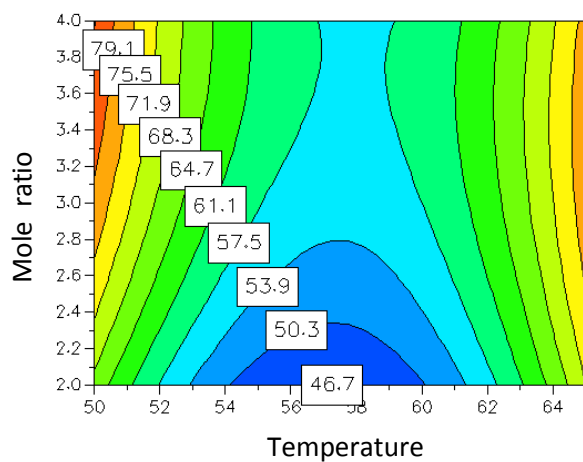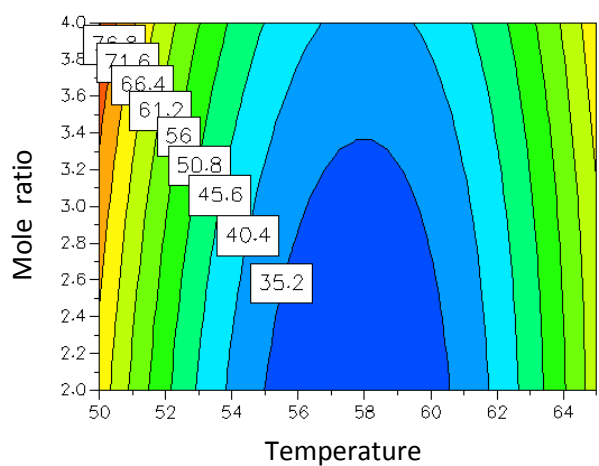
Figure 3.3 Contour plots of total SDA content for (A) substrate mole ratio versus reaction time at a reaction temperature of 65°C for Novozym 435 and Lipozyme TL IM lipases, (B) reaction time versus reaction temperature at 1:2 substrate mole ratio, and (C) substrate mole ratio versus reaction temperature at a reaction time of 6 h.
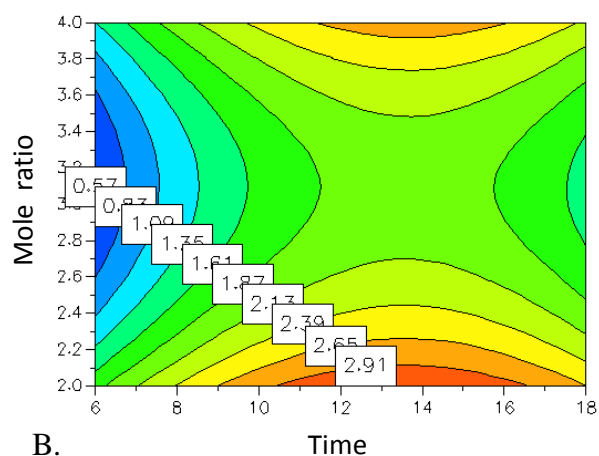
A.   Novozym 435   Lipozyme TL IM
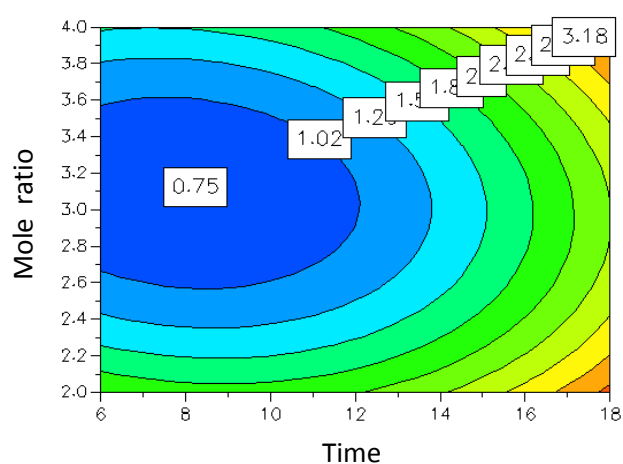
B.
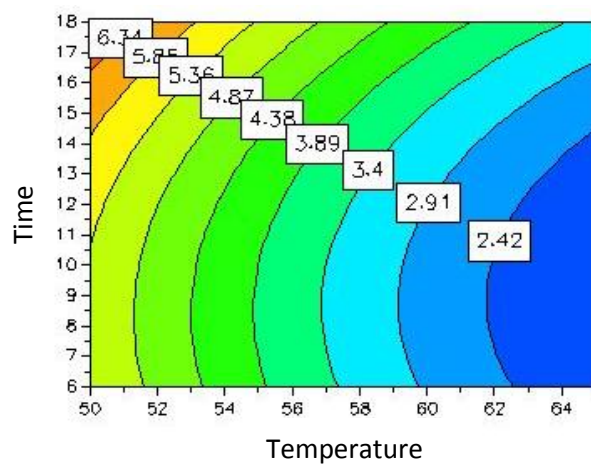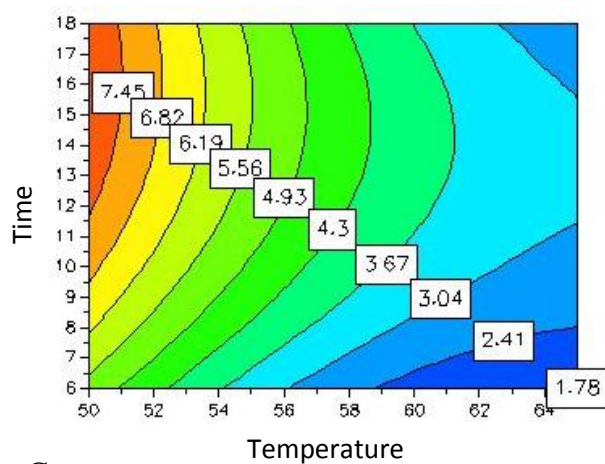
C.
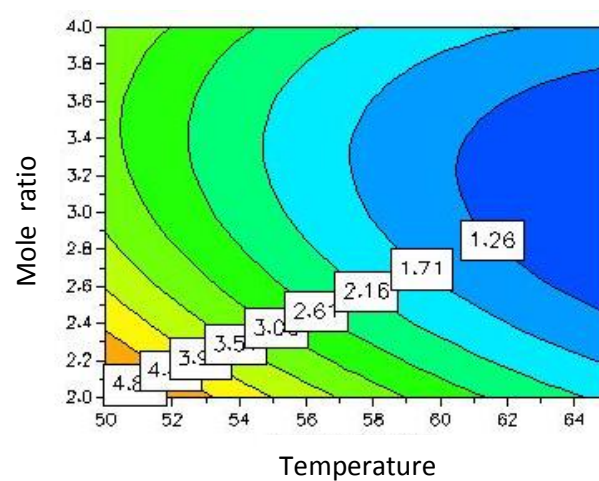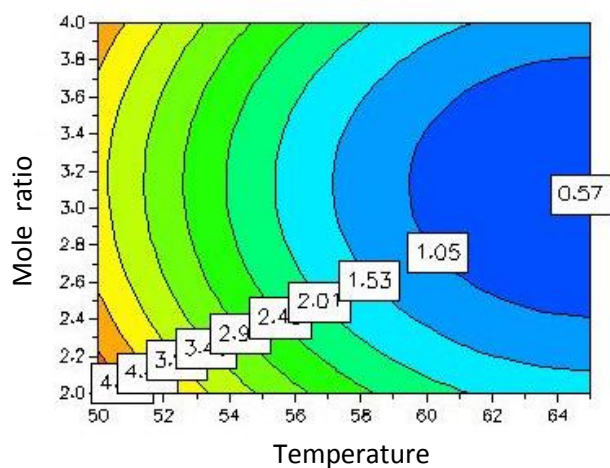
CHAPTER 4

CHARACTERIZATION OF STEARIDONIC ACID SOYBEAN OIL ENRICHED

WITH PALMITIC ACID PRODUCED BY SOLVENT-FREE ENZYMATIC

INTERESTERIFICATION [1]

ABSTRACT

Stearidonic acid soybean oil (SDASO) is a plant source of n-3 polyunsaturated fatty acids (n-3 PUFAs). Solvent-free enzymatic interesterification was used to produce structured lipids (SLs) in a 1 L stir-batch reactor with a 1:2 substrate mole ratio of SDASO to tripalmitin, at 65°C for 18 h. Two SLs were synthesized using immobilized lipases, Novozym 435 and Lipozyme TL IM. Free fatty acids (FFAs) were removed by short-path distillation. SLs were characterized by analyzing FFA and FA (total and positional) contents, iodine and saponification values, melting and crystallization profiles, tocopherols, and oxidative stability. The SLs contained 8.15 and 8.38% total stearidonic acid and 60.84 and 60.63% palmitic acid at the *sn*-2 position for Novozym 435 SL and Lipozyme TL IM SL, respectively. The SLs were less oxidatively stable than SDASO due to a decrease in tocopherol content after purification of the SLs. The saponification values of the SLs were slightly higher than that of the SDASO. The melting profiles of the SLs were similar but crystallization profiles differed. The triacylglycerol (TAG) molecular species of the SLs were similar to each other, with tripalmitin being the major TAG. SDASO's major TAG species contained stearidonic and oleic acids or stearidonic, α-linolenic and γ-linolenic acids.

INTRODUCTION

Stearidonic acid (SDA) soybean oil is a soybean oil that is enriched with SDA (C18:4n-3) consisting of approximately 20% stearidonic, 24% linoleic, and 12% palmitic acids (*1*). SDA is an n-3 polyunsaturated fatty acid (n-3 PUFA). PUFAs are often found in plants, fungi, microalgae, and fish. Fish is the main source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) but contains only small amounts of SDA (*2*). The richest sources of SDA are algae and plants. EPA is a very long chain n-3 PUFA that inhibits platelet

aggregation and lowers inflammation. James *et al.* (*3*) suggested that the daily ingestion of fish or fish oil to obtain the health benefits of n-3 PUFAs is not a sustainable long term approach. However, increasing ingestion of plant-based sources of n-3 PUFAs is required to increase the tissue concentration of EPA and DHA (*3*). SDA is the first metabolite of α-linolenic acid (ALA) in the metabolic pathway leading to EPA by desaturases and elongases (*4*). However, this conversion from ALA to EPA is often poor because the initial $\Delta 6$ desaturase enzyme is rate limiting in humans (*5*). The consumption of SDA, instead of ALA, would skip the rate-limiting step allowing for better conversion to EPA. Therefore, development of plant-based sources of n-3 PUFAs could be a solution for the supply of these fatty acids in the future. Miles *et al.* (*6*) observed that neither SDA nor 20:4n-3 appeared in the peripheral blood mononuclear cell when dietary SDA was ingested at a level of 1.0g/day, indicating that SDA was readily metabolized to EPA in the body. Dietary SDA was found to increase EPA concentrations by 3-4-fold more effectively than similar levels of ALA (*3, 7*). SDA was approximately one-third as effective as dietary EPA, (*8*) and the effectiveness of these FAs in increasing EPA concentrations in tissues is as follows: EPA>SDA>ALA (*3*). James *et al*. (*3*) conducted a double-blind, parallel group study to examine the effect of dietary SDA on increasing tissue concentrations of EPA in humans and compared SDA's ability with that of ALA and EPA. They concluded that SDA vegetable oils were more effective in increasing EPA tissue concentrations than the currently used ALA vegetable oils. SDA has been noted as a possible potent inhibitor of cancer growth, inhibitor of platelet aggregation, as an anti-inflammatory pharmaceutical, and provider of cardiovascular benefits (*2*). EPA has been linked to reductions in inflammation (*9*) and neurological disorders (*10*). A previous study reported the tocopherol content of SDA soybean as 9.6 mg/100g of α-tocopherol, 79.3 mg/100g of γ-tocopherol, and 28.8 mg/100g of δ-tocopherol (*1*). Tocopherols

are unsaponifiable materials that are well-known for their cardiovascular benefits and antioxidant capacity.

The ratio of n-6/n-3 PUFA is important in the diet. However, the Western diet is high in n-6 FAs, often resulting in a ratio of 15:1 – 16.7:1 (*11*). The high ratio of n-6 FAs can result in increased risk of cardiovascular, inflammatory and autoimmune diseases and cancer. Conversely, a lower n-6/ n-3 PUFA ratio would suppress these negative effects. For example, a lower ratio of 2:1 – 3:1 suppressed inflammation in patients with rheumatoid arthritis, and a ratio of 5:1 had a beneficial effect on patients with asthma. However, a ratio of 10:1 n-6/n-3 PUFA had adverse consequences (*11*). Premature infants often are limited in their ability to make EPA and DHA from ALA (*12*).

Structured lipids (SLs) are triacylglycerols (TAGs) that have been modified to change the FA composition and/or their position in the glycerol backbone by chemically and/or enzymatically-catalyzed reactions (*13*). SLs can be used in a wide variety of food applications such as margarines, shortenings, cookies, and salad dressings. Another possible application of SLs could be their use in infant formula as a human milk fat (HMF) analogue. Infants require proper nutrition for growth and development, and infant formulas try to mimic the fat found in breast milk. Palmitic acid (PA) is the second major FA found in breast milk at approximately 18.3 – 25.9% (*14-16*). SDA soybean oil only contains 12% PA which is lower than that of breast milk. A large portion of PA in breast milk is esterified at the *sn*-2 position of the TAGs. The amount of PA esterified at the *sn*-2 position is >60% (by weight) of HMF, whereas mainly unsaturated FAs are found at the *sn*-1,3 positions (*17-19*). As TAGs are metabolized in the body, the FAs esterified at the *sn*-1,3 positions are released by pancreatic lipase and the FAs esterified at the *sn*-2 position remain unhydrolyzed or conserved. The unhydrolyzed *sn*-2 FAs are absorbed

by the intestinal mucosa as *sn*-2 monoacylglycerols (*20*). The large amount of *sn*-2 esterified PA

helps improve the absorption of fat and calcium in infants (*18, 19, 21, 22*). Also, PA reduces the

formation and disposal of "calcium soaps" that are generated by long-chain saturated FA

interactions with calcium (*17, 22*). However, free PA may be lost as "calcium soaps" in the feces

of the infant. Most infant formulas consist of fats from vegetable oils that contain mostly

unsaturated FAs at the *sn*-2 position (*23*). Lipids are essential to the growth of an infant. In breast

milk, lipids provide essential FAs that are required as structural cell components of membrane

tissues, and they provide an essential source of energy that is approximately 50% of the total

energy used by infants (*24*). Betapol (Loders Croklaan, Glen Ellyn IL, USA) was the first to

commercially produce a HMF analogue by using a 1,3-specific lipase to synthesize SLs by

reacting tripalmitin with unsaturated FAs (*23*). We are not aware of any other studies, except our

previous research (*25*), on the enzymatic modification of SDA soybean oil to produce SLs. There

are no current studies published on the physical and chemical characterization of SDA soybean

oil SLs.

One of our objectives was to scale-up the production of two SLs at 65°C for 18 h with a

substrate mole ratio of 1:2 of SDASO to tripalmitin, catalyzed by immobilized Novozym 435

and Lipozyme TL IM lipases. These conditions were selected based on our previous research

using response surface methodology (RSM) to predict the optimal conditions (*25*). However, a

temperature above 60°C was used to conduct a solvent-free production of food grade SLs instead

of the optimal 50°C from our previous research (*25*). Our next objective was to characterize the

physical and chemical properties of Novozym 435 SL (NSL) and Lipozyme TL IM SL (LSL).

The reusability of the enzymes was also studied. The overall aim of our research was to

characterize SDASO enriched with PA (SLs) produced by solvent-free enzymatic

interesterification.

MATERIALS AND METHODS

Materials.

SDASO was kindly provided by Monsanto Company (St. Louis, MO). Tripalmitin was

purchased from TCI America (Portland, OR). Immobilized lipases, Novozym 435 (non-specific

Candida antarctica lipase) and Lipozyme TL IM (*sn*-1, 3 specific *Thermomyces lanuginosus*

lipase), were obtained from Novozymes North America Inc. (Franklinton, NC). The specific

activity of Novozym 435 was 10,000 PLU/g and Lipozyme TL IM was 250 IUN/g (PLU is the

propyl laurate units and IUN is the interesterification units Novo). The lipid standards Supelco

37 Component FAME mix, triolein, 2-oleoylglycerol, tripalmitin, tristearin, 1,2-dioleoyl-3-

palmitoyl-*rac*-glycerol, and 1-palmitoyl-2-oleoyl-3-linoleoyl were purchased from Sigma-

Aldrich Chemical Co. (St. Louis, MO) and C19:0-nonadecanoic acid was purchased from TCI

America (Portland, OR). The TAG standard mix (GLC reference standard) was purchased from

Nu-chek Prep, Inc. (Elysian, MN). Other solvents and chemicals were purchased from Fisher

Scientific (Norcross, GA) and Sigma-Aldrich Chemical Co. (St. Louis, MO).

Scaled-up Interesterification Reaction.

The solvent-free interesterification reaction occurred in a 1 L stir-batch reactor at 65°C for 18 h

with a substrate mole ratio of 1:2 (SDASO to tripalmitin) and 10% (w/w) of Lipozyme TL IM or

Novozym 435 as the biocatalyst with constant stirring at 200 rpm. The reactor was wrapped with

foil to reduce exposure to light. At the end of the reaction, the resulting SLs produced with

Lipozyme TL IM (LSL) and Novozym 435 (NSL) were vacuum filtered through a Whatman

no.1 containing sodium sulfate and then through a 0.45 μm membrane filter to dry and separate

the SL from the enzymes. If the SLs needed to be stored before short-path distillation, they were stored in an airtight amber container in the dark at 4°C after being flushed with nitrogen.

Short-Path Distillation.

Short-path distillation was used to remove liberated FFAs from the SL substrates. Short-path distillation was performed once using a KDL-4 (UIC Inc.) unit under the following conditions: holding temperature, 65°C; feeding rate, approximately 100 mL/h; heating oil temperature, 185°C; coolant temperature, 25-30°C; and vacuum, <13.33 Pa. After short-path distillation, the FFA content was then determined according to AOCS Official Method Ac 5-41 (*26*). The percent yield was calculated by using the starting weight of the substrates and the final weight of the SL after short-path distillation.

Determination of Fatty Acid Profiles.

SDASO and SL samples were converted to FAME following AOAC Official Method 996.01, Section E, (*27*) with minor modifications (*28*). For analysis of the SDASO, 150 mg of the oil was weighed into a Teflon-lined test tube and 100 µL of the internal standard, C19:0 in hexane (20 mg/mL), was added to the sample and dried under nitrogen to remove solvent. For analysis of the SL, 100 µL of the internal standard was added to the SLs after short-path distillation. Two milliliters of 0.5 N NaOH in methanol was added and incubated at 100°C for 5 min for saponification. Then the samples were cooled under tap water, and 2 mL of 14% BF$_3$ in methanol was added. The samples were vortexed for 1 min. Again, the sample was incubated at 100°C for 5 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 then centrifuged at 1000 rpm or 104.72 rad/s for 5 min to separate the organic layer from the aqueous layer. The upper organic layer was removed and recovered in an

amber GC vial for analysis. Supelco 37 component FAME mix was used as the external standard and was run parallel with the samples.

GC Analysis.

The FAMEs (from the SDASO, SLs, and corresponding positional analyses) were analyzed using an Agilent Technology 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) with a flame ionization detector. A Supelco SP-2560 column, 100 m x 250 μm, 0.20 μm film was used to attain separation. Injection of 1 μL of sample was made at a split ratio of 20:1. Helium was the carrier gas at a flow rate of 1.1 mL/min and at a constant pressure (45.0 mL/min). The injector temperature and the FID set point were $300^{o}C$. The oven was held at $140^{o}C$ for 5 min, then increased up to $240^{o}C$ at $4^{o}C$/min and held at $240^{o}C$ for 15 min. The relative FAME content was calculated using the online computer. The average and standard deviation of triplicate analyses were reported.

Positional Analysis.

A modified version of the Luddy *et al.* (*29*) method was used to perform the pancreatic lipase-catalyzed *sn*-2 positional analysis. One hundred milligrams of SDASO or the SLs were placed into Teflon-lined test tubes. Two milliliter 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 vortexed for 2 min to emulsify the oil. Then 40 mg of pancreatic lipase was added, vortexed for 1 min, and incubated in a water bath at $40^{o}C$ for 3 min while shaking at 200 rpm or 20.94 rad/s. The samples were vortexed again for 2 min. To stop the reaction and extract the hydrolyzed TAG products, 1 mL of 6N HCl and 4 mL of diethyl ether were added. The samples were vortexed for 2 min and centrifuged at 1000 rpm or 104.72 rad/s for 3 min. The upper layer, containing the lipid components, was filtered twice through an anhydrous sodium sulfate column. The samples were

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 (~50 µL) was spotted onto the activated silica gel G dried TLC plates and placed into the tank. 2-Oleylglycerol was spotted as the standard and run parallel with the samples for identification of the 2-monoacylglycerol (2-MAG) band. The plates were sprayed with 0.2% 2, 7-dichlorofluorescein in methanol and visualized under UV light. The 2-MAG band was scraped off and converted to FAME (as described above). One hundred microliters of the internal standard (20 mg/mL C19:0 in hexane) was used for the SDASO and 50 µL of the internal standard for the SL. The FA content at the *sn*-2 position were quantified by GC, and the FA content at the *sn*-1, 3 positions were calculated.

Chemical Properties.

The FFA contents, iodine values, and saponification values were determined according to AOCS Official Method Ac5-41, Cd-1c-85, and Cd 3a-94, respectively (*26*).

Tocopherol Analysis.

Tocopherols were identified and quantified in SDASO, NSL, and LSL using a normal phase high-performance liquid chromatography (HPLC) system. An isocratic mobile phase of 0.85% (v/v) isopropanol in hexane, vacuum filtered and degassed by stirring under vacuum, was used at a flow rate of 1.0 mL/min. The column was a LiChrosorb Si 60 column (4 mm, 250 mm, 5 µm particle size, Hiber Fertigsäule RT, Merck, Darmstadt, Germany) with a Shimadzu LC-6A pump equipped with a RF-10AXL fluorescence detector (Shimadzu Corp., Columbia, MD), a Spectra Series As100 autosampler (Thermo Separation Products, Inc., San Jose, CA) and Agilent Chemstation software. The samples were prepared by weighing 0.1 ± 0.001 g into a test tube and

adding 5 mL of HPLC-grade hexane. The samples were then vortexed for 30 s, capped, and centrifuged at 1000 rpm (104.72 rad/s) for 10 min at room temperature. A portion of the hexane layer was transferred into an HPLC vial for analysis. The tocopherol standards preparation was performed according to Lee *et al*. (*30*) with ~50 mg of α-, β-, and δ-tocopherols, and 100 mg of γ-tocopherol dissolved in 25 mL hexane. The standard purities were measured by pipetting 1 mL of each standard into a 25 mL volumetric flask and evaporating completely under nitrogen. The residue was diluted with 95% (v/v) ethanol and absorbance was measured with a Shimadzu model UV-1601 UV-VIS spectrophotometer and a quartz cuvette. Extinction coefficients ($E^{1\%}_{1cm}$) and maximum wavelengths (λ max) were 71.0 and 294 , respectively, for α-tocopherol, 86.4 and 297, respectively, for β-tocopherol, 92.8 and 298, respectively, for γ-tocopherol, and 91.2 and 298, respectively, for δ-tocopherol (*31*). The following equation was used to determine percent purity:

$$\% \text{ purity} = (A \times 10/C) / E^{1\%}_{1cm}$$

A is the absorbance, C is the concentration (mg/mL), and $E^{1\%}_{1cm}$ is the extinction coefficient at λ_{max} of each tocopherol. The purities of α-, β-, γ-, and δ-tocopherols were found to be 96.0, 90.0, 96.0, and 90.0%, respectively with stock concentration of 1.88, 1.80, 3.84, and 1.80 mg/mL, respectively. For the daily working standard, the stock concentrations were diluted with the mobile phase containing 0.01% butylatedhydroxytoluene (BHT). Standards and samples were injected into the HPLC at a volume of 20 μL.

Oxidative Stability Analysis.

Twelve grams of SDASO, NSL, and LSL were weighed into Teflon-lined test tubes and oxidized for 72 h at 65°C in the dark in a shaking water bath. The samples were removed from the water bath and analyzed at 0, 24, 48 and 72 h. The peroxide value (PV) and *p*-anisidine value (*p*AV)

were determined according to AOCS Official Method Cd 8b-90 and Cd18-90, respectively (*26*).

The total oxidation (TOTOX) value was calculated as $2 \times (PV) + (pAV)$ (*32*). The oxidative

stability index (OSI) was determined according to AOCS Method Cd12b-92 (*26*) using Oil

Stability Instrument (Omnion, Rockland, MA, U.S.A.) at $110^{o}C$.

Melting and Crystallization Profile.

The melting and crystallization profiles were determined for SDASO, tripalmitin, NSL, and LSL

using a differential scanning calorimeter (DSC model DSC7, Perkin-Elmer Co., Norwalk, CT).

The method was performed according to AOCS Official Method Cj 1-94 (*26*) with minor

modifications using indium and *n*-decane as standards. Sample weight ranged from 8 to 12 mg in

aluminum pans. The sample was heated from 25 to $80^{o}C$ at $50^{o}C$/min, held for 10 min (to destroy

any previous crystalline structure), cooled from 80 to $-55^{o}C$ at $10^{o}C$/min (for crystallization

profiles), held for 30 min, and then heated from -55 to $80^{o}C$ at $5^{o}C$/min (for melting profiles).

Dry ice and acetone were used as the coolant. The thermograms were analyzed with the DSC

(Pyris software, Perkin-Elmer, Shelton, CT).

TAG Molecular Species.

A reversed phase HPLC (Agilent Technologies 1260 Infinity, Santa Clara, CA) with a Sedex 85

evaporative light scanning detector (ELSD) was used to analyze the TAG molecular species of

SDASO, NSL, and LSL on a Ultrasphere C18 250 mm, 4.6 mm, 5 µm particle size column

(Beckman Coulter, Fullerton, CA). The column temperature was kept at $30^{o}C$. The ELSD

conditions were $70^{o}C$, 3.0 bar or 300,000 Pa, and gain of 8. Sample concentrations were 5

mg/mL in chloroform. The sample injection volume was 20 µL. The eluent was a gradient of

acetonitrile (A) and acetone (B) at a solvent flow rate of 1 mL/min with a gradient of: 0 min,

65% B; 45 min, 95% B; and 50 min, 65% B. The equivalent carbon number (ECN) method was

used to predict the elution order of TAG. Standards [tripalmitin, tristearin, triolein, TAG Mix, 1,2-dioleoyl-3-palmitoyl-*rac*-glycerol (OOP), and 1-palmitoyl-2-oleoyl-3-linoleoyl (POL)] were also chromatographed to help determine the TAG species.

Reusability of Enzymes.

Solvent-free interesterification was performed in a 1 L stir-batch reactor at 65°C for 18 h with a substrate mole ratio of 1:2 (SDASO/tripalmitin) with both Novozym 435 and Lipozyme TL IM for a total of five times, each while reusing the same enzymes. After each use, the enzymes were washed three to four times with hexane, dried under the hood, and placed in a desiccator. Enzymes were stored at 4°C. The FA composition and positional analyses were performed on the SLs after each use as described above.

Statistical Analysis.

All analyses, except melting and crystallization profiles, were performed in triplicate. Melting and crystallization profiles were performed in duplicate. Statistical analysis was performed with the SAS software package (SAS Institute, Cary, NC). Duncan's multiple-range test was performed to determine the significance of difference at $P \leq 0.05$.

RESULTS AND DISCUSSION

SDASO was characterized in this study since it was the starting substrate. The FA profile for SDASO was determined in our previous research (*25*) and showed that the total and positional FA composition of the oil is in agreement with previous studies (*1*). The major FAs in SDASO were linoleic acid (25.78 ± 0.07%), SDA (22.16 ± 0.23%), oleic acid (14.31 ± 0.17%), α-linolenic acid (12.30 ± 0.06), and PA (11.54 ± 0.04%). Linoleic acid was the major FA found at the *sn*-2 position (34.11 ± 4.56%), whereas SDA was the major FA found at the *sn*-1,3 positions of the TAG (25.26 ± 1.40%). SDASO contains a small amount of PA at the *sn*-2

position. Enzymatic interesterification is often used to produce SLs that have improved functionality by incorporating new FAs into the oil or by rearranging the existing FAs (*13*). The aim of this research was to produce SLs with immobilized lipases, Novozym 435 and Lipozyme TL IM, by modifying the SDASO's TAG to increase the PA esterified at the *sn*-2 position and to physically and chemically characterize the resulting SLs.

The FA profiles of the resulting SLs, NSL and LSL, are shown in **Table 4.1**. The major FA found in the SLs was PA, followed by linoleic acid, SDA, and oleic acid. Enzymatic interesterification decreased linoleic acid from $25.78 \pm 0.07\%$ in SDASO to $10.34 \pm 0.13\%$ and $10.83 \pm 0.46\%$ in the SLs, NSL and LSL, respectively. There was little to no α-linolenic acid in the SLs indicating that palmitic and other fatty acids displaced most of the C18:3n-3 during the interesterification reaction. The SDA content decreased in the SLs, from $22.16 \pm 0.23\%$ in SDASO to 8.15 ± 0.13 and 8.38 ± 0.46 in NSL and LSL, respectively. The decrease in SDA content in the SLs is a result of the incorporation of PA in the TAG. For example, the total PA content increased from $11.54 \pm 0.04\%$ to 62.55 ± 0.33 and $61.22 \pm 1.45\%$ in NSL and LSL, respectively, making PA the major FA in the SLs instead of linoleic acid which is the main FA found in SDASO. However, SDA still remained in the SLs and may provide cardiovascular benefits (*2*). The n-6/n-3 PUFA ratio for both SLs was approximately 1.6. This represents an improved ratio of n-6/n-3 PUFAs since the typical western diet has a high ratio of $15:1 - 16.7:1$ (*11*). A lower ratio can result in decreased risk of cardiovascular, inflammatory, and autoimmune diseases and cancer. Statistically, the two SLs do differ slightly. It appears that the SLs differ in the position of SDA but with a $P \leq 0.676$, they are not significantly different. The statistical differences in total FA composition were only found in C17:0 and C18:1n-9. In fact, C18:1n-9 was significantly different in the SLs at the total, *sn*-2 and *sn*-1,3 positions ($P \leq 0.05$). Another

significant difference was found for C18:3n-6 at the *sn*-2 position of the SLs. The specificity of

Lipozyme TL IM can be influenced by reaction media, substrates, and acyl migration (*33, 34*).

The PA content at the *sn*-2 position increased from approximately 4.77% in SDASO to >60% in

both NSL and LSL, resulting in SLs that were enriched with PA. The percent yields of the SLs

were 97.05% for NSL and 97.73% for LSL. This high yield is due to the substrates being both in

TAG form and not as FFAs for the interesterification reaction. An acidolysis reaction will result

in more FFA formation that must be removed by short-path distillation. Enzymatic

interesterification of two TAG substrates resulted in little unesterified FFAs, which were

removed after short-path distillation.

The physicochemical characteristics were determined for SDASO, NSL and LSL (**Table

4.2**). The iodine value (IV) was calculated, and both SLs had a lower IV than SDASO. The IV

measures the degree of unsaturation or number of carbon-carbon double bonds in relation to the

amount of fat in the oil. The higher the degree of unsaturation, the more iodine that can be

absorbed and the higher the iodine value (*35*). The SLs contained more saturated FAs and

resulted in a lower IV. The IV of SDASO, NSL, and LSL were significantly different (P ≤ 0.05).

The oxidative stability index (OSI) decreased in the SLs compared to the original, more stable

SDASO (6.13 ± 0.04 h). NSL had the lowest OSI value (1.85 ± 0.07 h), while LSL's OSI value

(3.90 ± 0.28 h) was higher than that of NSL with a significant difference at P ≤ 0.05. A lower IV

or higher amount of saturated FAs usually indicates increased oxidation stability. However, the

lower OSI of the SLs may be due to the loss of some tocopherols from the short-path distillation.

Tocopherols are antioxidants known to increase the oxidative stability of oils (*36*) and must be

added back to stabilize the oil for food use. The FFA percentage was found to be higher in the

SLs than the original SDASO. The SLs differed significantly (P ≤ 0.05) from SDASO in FFA

content because SLs were not deodorized but did not differ significantly from each other. During enzymatic interesterification, FAs are cleaved off the glycerol backbone and not all of these FAs reattach to the TAG, resulting in FFAs. Short-path distillation removed most of the FFAs, but did not lower the FFA content to the same percentage as found in the original SDASO. However, repetitive short-path distillation and/or deodorization can remove more FFAs and restore it to near fresh oil status to a FFA content of below 0.1% (*37*). Saponification value (SV) is the measure of the average molecular weight or chain length of all the fatty acids in the oil. The smaller the SV, the longer the average FA chain length is (*35*). The SVs of SDASO (59.99 mg/g), NSL (63.19 mg/g), and LSL (63.11 mg/g) were similar, with the SLs having a slightly higher SV. SDASO has a larger molecular weight (902.18 g/mol) than NSL (875.16 g/mol) and LSL (875.16 g/mol). The SV of SDASO shows that it contains a longer average FA chain length than the SLs. However, the SV of SDASO, NSL, and LSL were not significantly different (P ≤ 0.05).

Tocopherol contents were also measured and are shown in **Table 4.2**. The vitamin E family is composed of α-, β-, γ-, and δ-tocopherols that are characterized by a saturated side chain of three isoprenoid units and unsaturated tocotrienols (*35*). Tocopherols provide stability and important antioxidant properties to the oil. Compared to SDASO, α-tocopherols, γ-tocopherols, δ-tocopherols, and γ-tocotrienols decreased the most (P ≤ 0.05) after interesterification and short-path distillation. There were significant differences (P ≤ 0.05) in the total tocopherol content between SDASO (~1440.06 ppm) and the SLs, NSL (~377.84 ppm) and LSL (~390.16 ppm). The tocopherol losses were approximately 73 and 74% for LSL and NSL, respectively, when compared to SDASO. Tocopherol content was not significantly different between the SLs (P ≤ 0.05) except β-tocopherol. The difference in oxidative stability between the

SLs may be due to the loss of other antioxidants that were not tocopherols and not analyzed. This decrease may also be due to exposure to light, oxygen, and heat during interesterification and/or due to short-path distillation. However, some studies did report an increase in tocopherols after interesterification (*38, 39*). Other studies suggest that short-path distillation not only affects the FFA content but also contributes to the loss of non-TAG components such as tocopherols (*40, 41*). The loss of tocopherols by short-path distillation may be corrected by supplementation of SLs with antioxidants before food application or storage to stabilize them.

To further analyze the stability of the oils, peroxide value (PV), *p*-anisidine (*p*-AV), and total oxidation value (TOTOX) were determined and are shown in **Figure 4.1**. PV measures the amount of iodine formed by the reaction of peroxides with iodide ion and is the initial product of oxidation. PV measures hydroperoxides that increase and then decrease with time (*35*). *p*-AV measures aldehydes or the decay products of the hydroperoxides that continually increase with time. TOTOX continually increases during lipid oxidation (*35*). The TOTOX for SDASO remained constant during the 72 h oxidation period. However, the SLs experienced different levels of oxidation. NSL experienced the greatest amount of oxidation and was the least stable during this experiment. LSL oxidation values remained between the TOTOX values of SDASO and that of NSL. This may be explained in part by their tocopherol contents. SDASO had a large amount of tocopherols, followed by LSL and NSL. Tocopherols are important antioxidants that help slow or prevent lipid oxidation of oils.

The melting and crystallization behaviors for SDASO, tripalmitin, NSL, and LSL, and a physical blend (1:2 substrate mole ratio, 18 h, 65°C) without enzymatic catalysis were evaluated with DSC. The melting thermograms and crystallization thermograms are shown in **Figure 4.2** and **Figure 4.3**, respectively. SDASO is liquid at room temperature and at refrigeration

temperature (4°C). SDASO's melting thermogram showed melting from -30.36 to -24.38^{o}C and from -10.917 to -5.317^{o}C. Comparison of the SLs, NSL and LSL, to tripalmitin, reveals that the SLs' thermograms shifted to the left and were broader indicating a better plastic range (**Figure 4.2**). NSL experienced melting from 4.11 to 54.90^{o}C with melting peaks at 10.48, 43.48, and 52.22^{o}C with LSL experiencing similar melting properties. The DSC showed an onset of crystallization at -12.86^{o}C ending at -21.13^{o}C for SDASO. However, the crystallization properties of the two SLs were very different (**Figure 4.3**). NSL showed crystallization from 43.16 to 0.92^{o}C whereas LSL experienced crystallization from 29.87 to -9.407^{o}C. The difference in crystallization behaviors of LSL and NSL may be due to the molecular species of their TAGs. **Figure 4.4** shows the differences in major TAG species among the SLs. LSL has more POP (12.77%) than NSL (10.48%). The SLs will experience softening at room temperature but will not completely melt unless heated to a temperature around 54°C. According to Tan and Che Man (*42*), the presence of multiple peaks in a DSC thermogram of a vegetable oil can be attributed to the complex attributes of the TAG distribution. Saturated TAGs (like tripalmitin) tend to melt at higher temperatures than the highly unsaturated TAGs (*42*). The physical blend showed similar thermograms to tripalmitin with a shift to a slightly lower crystallization temperature (**Figure 4.3**). This similarity may be due to the fact that the physical blend contained more saturated fat (tripalmitin) than SDASO.

The TAG species were determined using reversed-phase HPLC. SDASO TAG species with relative FA percentages are shown in **Figure 4.4A (legend)**. The SLs' TAG species with relative FA percentages are shown in **Figure 4.4B (legend)** for NSL and LSL. On the basis of the relative FA percents from the chromatograms, the main TAG species found in SDASO were: StOSt/LnGSt, LnLnL, StLnLn, LnLnLn, StLnSt, OLLn, and StOO. This compares well with the

FA profile (*23*) since the major FAs were L, linoleic (26%); St, SDA (22%); O, oleic (14%); and

Ln, ALA (12%). The SLs had different major TAG species since they contained large amounts

of PA. NSL's major TAG species were: PPP, OLL, POP, PLP, and StOSt whereas LSL's major

TAG species were: PPP, POP, OLL, PLP, and LnOP. LSL did contain StOSt/LnGSt, but it was

negligible when compared to the major TAG species. This correlates with the FA profiles of the

SLs (**Table 4.1**) with mainly P, PA (>60%) occupying the TAG molecules followed by L,

linoleic (~10%); St, SDA (8%); and O, oleic (~6%). Tripalmitin or PPP, dominated both SLs at

approximately 50% of the TAG species. The second major FA in NSL, OLL, is approximately

one-fifth of PPP and the second major FA in LSL, POP, is approximately one-fourth of PPP.

This is due to the high amount of PA in the TAG molecule and the use of tripalmitin as a

substrate for interesterification.

Both Novozym 435 and Lipozyme TL IM lipases were tested for reusability. The

enzymes were reused five times under solvent-free conditions and then washed and dried after

each reuse. The *sn*-2 FA profiles remained constant over these five uses (**Figure 4.5**). The total

FA profiles also remained constant over the five reuses (not shown). However, the enzymes did

absorb some of the oil during interesterification. After the first use, Novozym 435 increased from

the initial 67.67 g to 87.65 g while Lipozyme TL IM increased from the initial 67.67 g to 83.05

g. After the fifth reuse, Novozym 435 weighed 102.64 g and Lipozyme TL IM weighed 85.62 g.

Each enzyme experienced some loss during filtration and transfer. As noted by the weight after

the first use, both enzymes did absorb oil and Novozym 435 continued to absorb more oil

through each use. This may be due to the material the lipases were immobilized onto. Novozym

435 was immobilized to macroporous acrylic resin beads whereas Lipozyme TL IM was

immobilized to silica gel. The silica gel appears to be able to release the absorbed oil better when

rinsed with hexane than the macroporous acrylic resin beads. Due to the silica gel's polarity, hexane easily extracted the oil. Macroporous resins contain a network of pathways throughout the bead, making it a sponge-like material (*43*) that appears to absorb more of the oil.

Two SLs (NSL and LSL) were developed from SDASO enriched with PA at the *sn*-2 position with physical and chemical characteristics that could be used for food application purposes, albeit, with the addition of antioxidants for stability. A possible food application may be their use as a human milk fat analogue in infant formula. The PA content at the *sn*-2 position of the SLs is >60% which improves fat and calcium absorption (*17-19*) and decreases formation of calcium soaps in infants (*17, 22*). The use of these SLs in actual food products will soon be conducted. We are also exploring the possible addition of beneficial FAs for the infant, such as DHA, into the SL. This may result in a more suitable SL for use as human milk fat analogues than our current SLs.

ACKNOWLEDGEMENTS

REFERENCES

(1) Hammond, B.G.; Lemen, J.K.; Ahmed, G.; Miller, K.D.; Kirkpatrick, J.; Fleeman, T. Safety assessment of SDA soybean oil: results of a 28-day study and a 90-day/one generation reproduction feeding study in rats. *Regul. Toxicol. Pharmacol*. **2008**, *52*, 311-323.

(2) Guil-Guerrero, J.L. Stearidonic acid (18:4n-3): Metabolism, nutritional importance, medical uses and natural sources. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 1226-1236.

(3) James, M.J.; Ursin, V.M.; Cleland, L.G. Metabolism of stearidonic acid in human subjects: comparison with the metabolism of other n-3 fatty acids. *Am. J. Clin. Nutr.* **2003**, *77*, 1140-1145.

(4) Gerster, H. Can adults adequately convert alpha-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? *Int. J. Vitam. Nutr. Res*. **1998**, *68*, 159-173.

(5) Haung, Y.S.; Smith, R.S.; Redden, P.R.; Cantrill, R.C.; Horrobin, D.F. Modification of liver fatty acid metabolism in mice by omega-3 and omega-6 Δ6-desaturase substrates and products. *Biochim. Biophys. Acta*. **1991**, 1082, 319-327.

(6) Miles, E.A.; Banerjee, T., Dooper, M.M.B.W.; M'Rabet, L.; Graus, Y.M.F.; Calder, P.C. The influence of different combinations of γ-linolenic acid, stearidonic acid and EPA on immune function in healthy young male subjects. *Br. J. Nutr*. **2004**, *91*, 893-903.

(7) Surette, M.E.; Edens M.; Chilton, F.H.; Tramposch, K.M. Dietary echium oil increases plasma and neutrophil long-chain (*n*-3) fatty acids and lowers serum triacylglycerols in hypertriglyceridemic humans. *J. Nutr*. **2004**, *134*, 1406-1411.

(8) Ursin, V.M. Modification of plant lipids for human health: development of functional land-based omega-3 fatty acids. *J. Nutr*. **2003**, *133*, 4271-4274.

(9) Calder, P.C. *n*-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am. J. Clin. Nutr*. **2006**, *83*, 1505S-1519S.

(10) Hibbeln, J.R.; Nieminen, L.R.; Blasbalg, T.L.; Riggs, J.A.; Lands, W.E. Healthy intakes of *n*-3 and *n*-6 fatty acids: estimations considering worldwide diversity. *Am. J. Clin. Nutr*. **2006**, *83*, 1483S-1493S.

(11) Simopoulos, A.P. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp. Biol. Med.* **2008**, *233*, 674–688.

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

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

(14) 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.

(15) Bitman, J.; Wood, D.L.; 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.

(16) 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.

(17) 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.

(18) 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.

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

(20) Iwasaki, Y.; Yamane, T. Enzymatic synthesis of structured lipids. *J. Mol. Catal. B. Enzym.* **2000**, *10*, 129-140.

(21) 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.

(22) 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.

(23) Akoh, C. C.; Xu, X. Enzymatic production of Betapol and other specialty fats. In *Lipid Biotechnology*; Kuo, C. C., Gardner, H.W., Eds.; Dekker: New York, **2002**; pp 461-478.

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

(25) 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.

(26) *Official Methods and Recommended Practices of the American Oil Chemists' Society,* edited by D. Firestone, Champaign, **2009**; Official Methods Ac 5-41, Cd 1c-85, Cd 3a-94, Cj 1-94, Cd 8b-90, Cd 18-90, and Cd 12b-92.

(27) *Official Methods of Analysis of AOAC International*, 17th ed.; AOAC International: Gaithersburg, MD, **1998**; Official Method 996.01.

(28) Pina-Rodriguez, A.M.; Akoh, C.C. Enrichment of amaranth oil with ethyl palmitate at the sn-2 position by chemical and enzymatic synthesis. *J. Agric. Food Chem.* **2009**, *57*, 4657-4662.

(29) 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.

(30) Lee, J.; Landen, W.O. Jr.; Phillips, R.D.; Eitenmiller, R.R. Application of direct solvent extraction to the LC quantification of vitamin E in peanuts, peanut butter, and selected nuts. *Peanut Sci.* **1998**, *25*, 123-128.

(31) Scott, M.L. Vitamin E. In *Handbook of Lipid Research*; DeLuca, H.F., Eds.; Plenum Press: New York, **1978**; Vol.2, pp. 133-210.

(32) Shahidi, F.; Wanasundra, U.N. Methods for measuring oxidative rancidity in fats and oils. In *Food Lipids*, 3rd Ed.; Akoh, C.C., Min, D.B., Eds.; CRC Press: Boca Raton, FL, **2008**; pp 387-408.

(33) Zhang, H.; Xu, X.; Nilsson, J.; Mu, H.; Adler-Nissen, J.; Høy, C-E. Production of margarine fats by enzymatic interesterification with silica-granulated *Thermomyces lanuginosus* lipase in a large-scale study. *J. Am. Oil Chem. Soc.* **2001**, 78, 57-64.

(34) Ronne, T.; Pedersen, L.; Xu, X. Triglyceride selectivity of immobilized *Thermomyces lanuginosa* lipase in interesterification. *J. Am. Oil Chem. Soc.* **2005**, *82*, 737-43.

(35) O'Keefe, S.F.; Pike, O.A. Fat characterization. In *Food Analysis*, 4th Ed.; Nielsen, S.S., Ed.; Springer: New York, NY, 2010; pp. 239-260.

(36) Turan, S.; Karabulut, I.; Vural, H. Influence of sn-1,3-lipase-catalysed interesterification on the oxidative stability of soybean oil-based structured lipids. *J. Sci. Food Agric.* **2007**, *87*, 90-97.

(37) Jennings, B.H.; Akoh, C.C. Effectiveness of natural versus synthetic antioxidants in a rice bran oil-based structured lipid. *Food Chem.* **2009**, *114*, 1456-1461.

(38) Azadmard-Dmirchi, S.; Dutta, P.C. Stability of minor lipid components with emphasis on phytosterols during chemical interesterification of a blend of refined olive oil and palm stearin. *J. Am. Oil Chem. Soc.* **2008**, *85*, 13-21.

(39) Wang, T.; Jiang, Y. Hammond, E.G. Effect of randomization on the oxidative stability of corn oil. *J. Am. Oil Chem. Soc.* **2005**, *82*, 111-117.

(40) Akoh, C.C.; Moussata, C.O. Characterization and oxidative stability of enzymatically produced fish and canola oil-based structured lipids. *J. Am. Oil Chem. Soc.* **2001**, *78*, 25-30.

(41) Akoh, C.C. Oxidative stability of fat substitutes and vegetable oils by the oxidative stability index. *J. Am. Oil Chem. Soc.* **1994**, *71*, 211-215.

(42) Tan, C. P.; Che Man, Y. B. Differential scanning calorimetric analysis of palm oil, palm oil based products and coconut oil:effects of scanning rate variation. *Food Chem.* **2002**, *76*, 89-102.

(43)Roberge, P.R. Environments. In *Handbook of Corrosion Engineering*, Roberge, P.R., Ed.; McGraw-Hill: New York, NY, **2000** pp. 85-129.

FA	total		sn-2		sn-1,3[b]	
	NSL[c]	LSL[d]	NSL	LSL	NSL	LSL
C14:0	1.21 ± 0.02A	1.18 ± 0.01A	ND[e]	ND	1.82 ± 0.03A	1.78 ± 0.01A
C15:0	0.09 ± 0.00A	0.09 ± 0.00A	ND	ND	0.14 ± 0.00A	0.14 ± 0.00A
C16:0	62.55 ± 0.33A	61.22 ± 1.45A	60.84 ± 2.26A	60.63 ± 1.54A	63.41 ± 1.44A	61.51 ± 2.19A
C17:0	0.16 ± 0.00A	0.15 ± 0.00B	ND; A	0.35 ± 0.61A	0.24 ± 0.00A	0.15 ± 0.31A
C18:0	2.93 ± 0.04A	2.88 ± 0.08A	4.34 ± 1.68A	3.10 ± 0.07A	2.22 ± 0.90A	2.78 ± 0.12A
C18:1n-9	6.11 ± 0.10B	6.53 ± 0.17A	8.32 ± 0.47A	7.07 ± 0.07B	5.00 ± 0.38B	6.25 ± 0.28A
C18:2n-6	10.34 ± 0.13A	10.83 ± 0.46A	12.39 ± 0.55A	11.49 ± 0.26A	9.31 ± 0.32A	10.50 ± 0.72A
C18:3n-6	2.89 ± 0.03A	3.01 ± 0.15A	3.28 ± 0.20B	4.03 ± 0.18A	2.69 ± 0.15A	2.50 ± 0.30A
C20:1n-9	4.73 ± 0.04A	4.90 ± 0.21A	4.24 ± 0.19A	4.50 ± 0.39A	4.97 ± 0.15A	5.10 ± 0.75A
C18:4n-3	8.15 ± 0.13A	8.38 ± 0.46A	6.59 ± 0.80A	8.82 ± 1.33A	8.93 ± 0.48A	8.16 ± 0.75A
C21:0	0.12 ± 0.01A	0.13 ± 0.01A	ND	ND	0.18 ± 0.01A	0.19 ± 0.01A
n-6/n-3	1.64 ± 0.02A	1.67 ± 0.02A				

Table 4.1 FA Composition (Mole Percent) of SLs[a]

[a] Mean $\pm$ SD, n=3. Means with the same letter in the same row and same category (i.e., sn-2 column) are not significantly different (P $\leq$ 0.05). [b] sn-1,3 (mol %) = [3 x total (mol%) – sn-2 (mol%)]/(2). [c] Novozym 435 SL. [d] Lipozyme TLIM SL. [e] Not detected.

substrate	IV	OSI (h)	FFA (%)	SV (mg/g)	tocopherols (ppm)					
					α-T	α-T3	β-T	γ-T	γ-T3	δ-T
LSL	36.80 ± 0.92A	3.90 ± 0.28B	0.34 ± 0.07A	63.11 ± 3.07A	35.73 ± 0.31B	6.93 ± 0.24A	2.65 ± 0.02A	12.55 ± 0.19B	247.19 ± 4.40B	85.11 ± 1.46B
NSL	35.17 ± 0.74B	1.85 ± 0.07C	0.35 ± 0.08A	63.19 ± 0.85A	31.62 ± 2.07B	6.26 ± 0.61A	ND; B	12.80 ± 0.48B	247.29 ± 0.07B	79.87 ± 0.06B
SDASO	111.91 ± 0.94C	6.13 ± 0.04A	0.05 ± 0.01B	59.99 ± 0.72A	107.37 ± 1.53A	6.94 ± 1.25A	2.72 ± 1.50A	28.44 ± 3.22A	945.10 ± 2.98A	349.49 ± 0.97A

Table 4.2 Physicochemical Characteristics of SDASO and SLs[a]

[a] Mean ± SD, n=3. Means with the same letter in the same column are not significantly different ($P \leq 0.05$). IV, iodine value; OSI, oxidative stability index; FFA, free fatty acid percentage; SV, saponification value; α-T, α-tocopherol; α-T, α- tocotrienol; β-T, β-tocopherol; γ-T, γ-tocopherol; γ-T, γ-tocotrienol; δ-T, δ-tocopherol; LSL, Lipozyme TL IM SL; NSL, Novozym 435 SL; [b] Not detected.

Figure 4.1 The (A) *p*-anisidine values, (B) peroxide values, and (C) total oxidation values (TOTOX) of SDA soybean oil (SDASO) (♦), Novozym 435 SL (NSL) (×), and Lipozyme TL IM SL (LSL) (▲) over a period of 72 h.
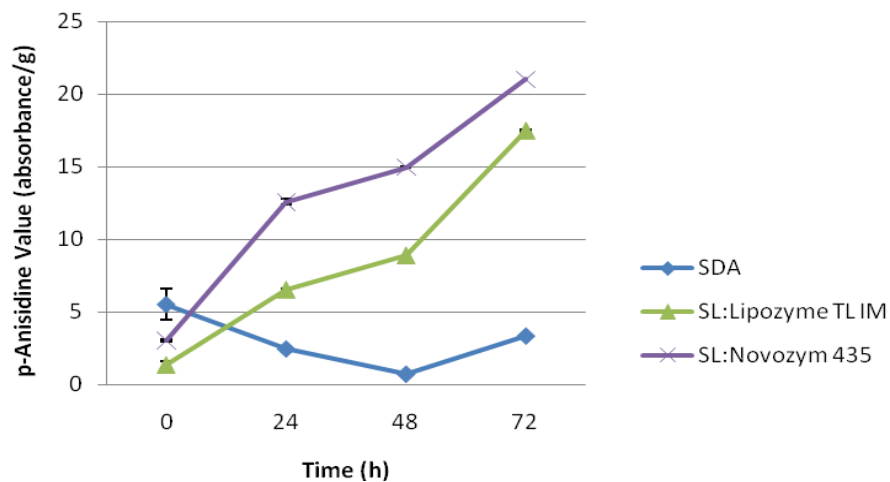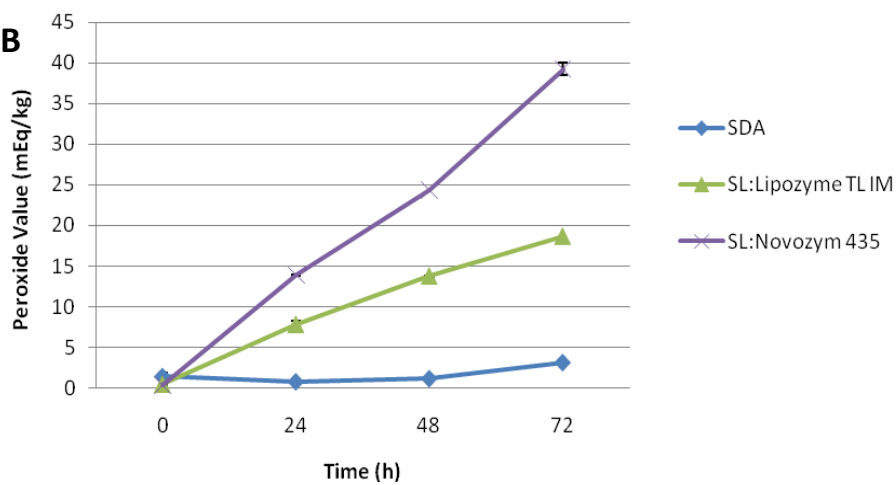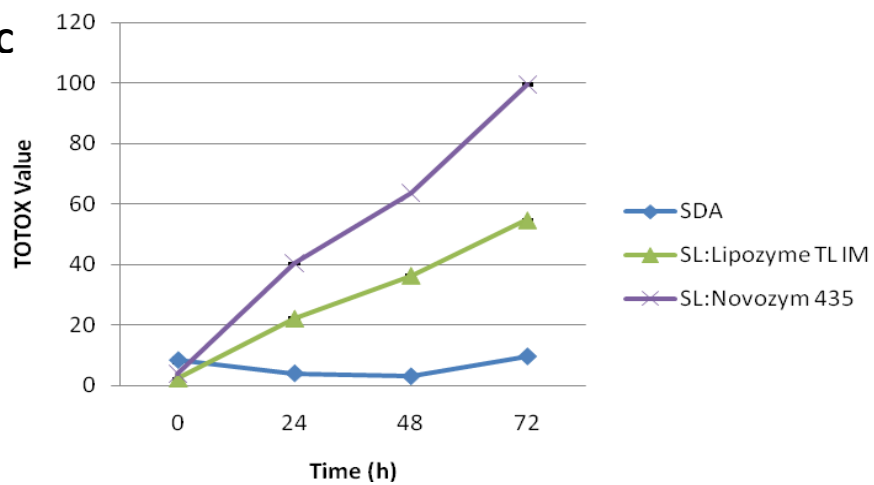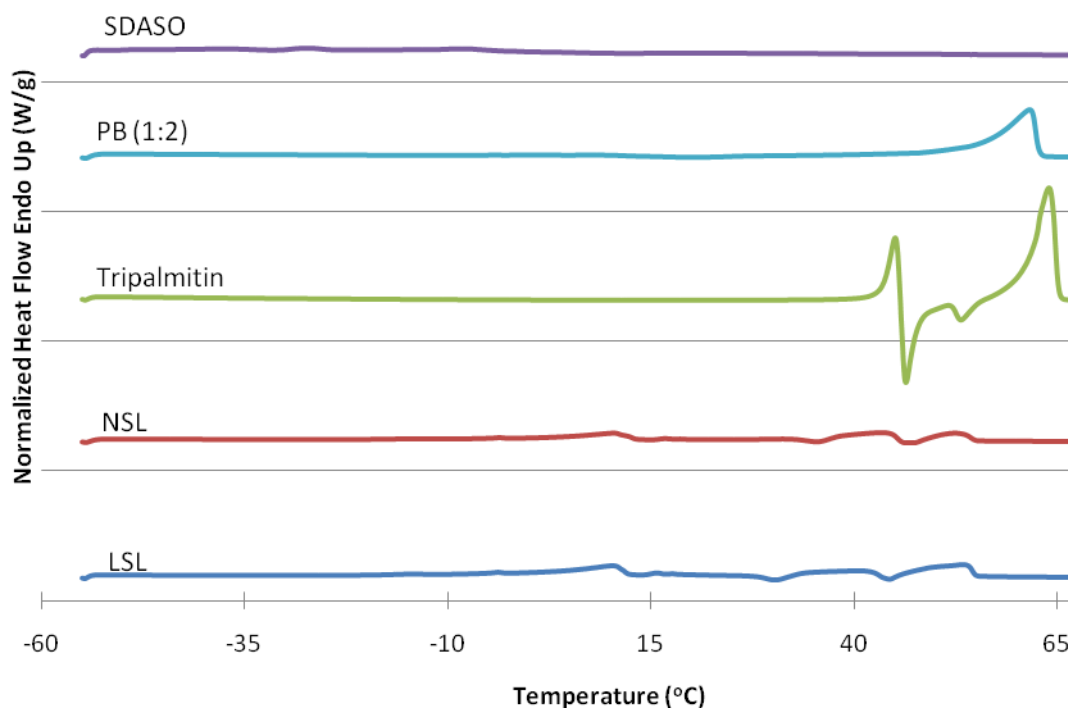
Figure 4.2 Melting curve using DSC for SDASO, tripalmitin, NSL, LSL, and a physical blend of

SDASO to tripalmitin (1:2). PB (1:2), physical blend with a 1:2 substrate mole ratio of
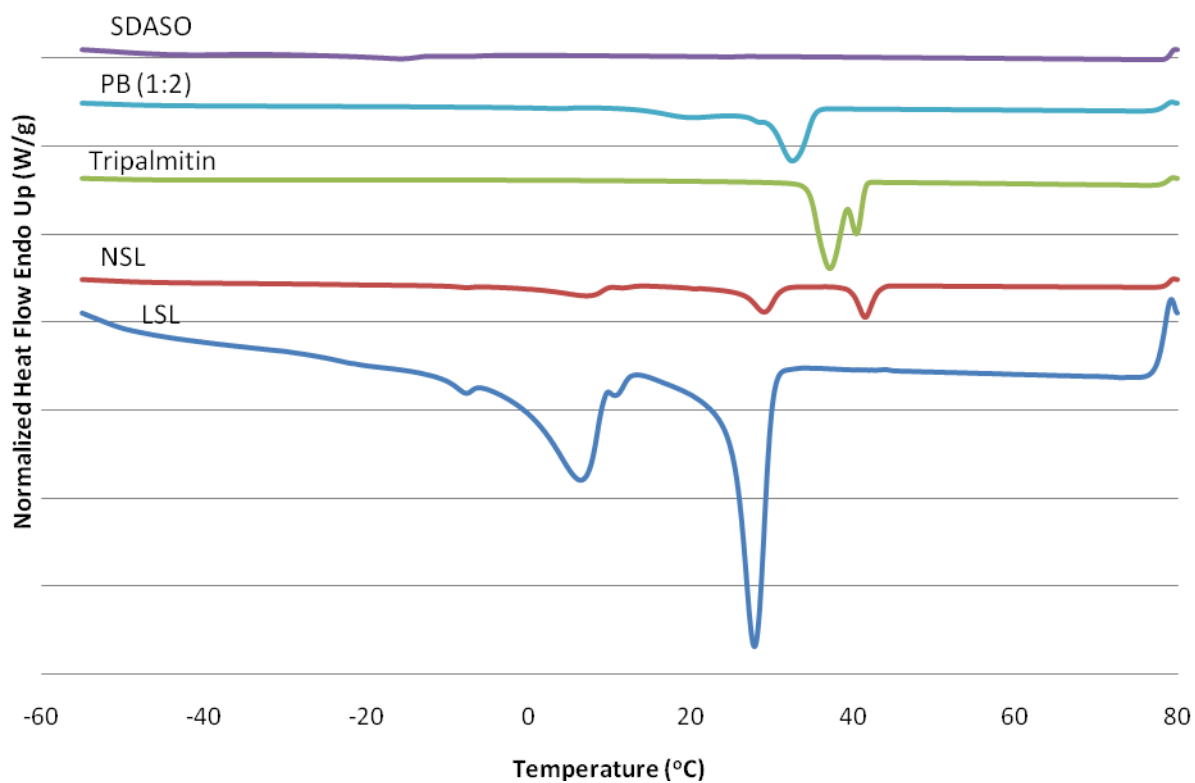
SDASO/tripalmitin.

Figure 4.3 Crystallization curve using DSC for SDASO, tripalmitin, NSL, LSL, and a physical

blend of SDASO to tripalmitin (1:2). PB (1:2), physical blend with a 1:2 substrate mole ratio of

SDASO/ tripalmitin.

Figure 4.4 (A) HPLC chromatogram of TAG molecular species of SDASO with relative FA%. Peaks: 1, StLnSt (7.21%); 2, StLnLn (12.38%); 3, StOSt/LnGSt (17.42%); 4, LnLnLn (11.37%); 5, LnGL(0.97%); 6, StLL (3.84%); 7, LnLnL (12.64%); 8, LnStO (2.10%); 9, LnStP (0.62%); 10, LLLn (1.58%); 11, LnLnO (0.53%); 12, StOO (5.38%); 13, OLLn (5.63%); 14, LLL (1.72%); 15, GLO (0.64%); 16, LnLP (2.06%); 17, LnLnS (1.42%); 18, LnGS (3.44%); 19, OLL (2.97%); 20, PLL (4.88%); 21, PLO/SLL (1.20%). (B) HPLC chromatograms of TAG and relative FA% of NSL and LSL, respectively. Peaks: 1, StLnLn (1.10/ND%); 2, StOSt/LnGSt (4.25/0.75%); 3, OLLn (1.56/1.59%); 4, LnLP (1.63/1.57%); 5, LnGS (0.70/1.43%); 6, OLL (11.04/12.53%); 7, LnOP (3.87/4.34%); 8, OOL (0.90/1.17%); 9, PLP (10.19/11.74%); 10, PPM (1.44/0.88%); 11, OOO (0.84/0.63%); 12, POO (1.78/1.72%); 13, POP (10.48/12.77%); 14, PPP (50.23/48.89%). Abbreviations: M, myristic acid; G, γ-linolenic acid; L-linoleic acid, Ln, α-linolenic acid; O, oleic acid; P, palmitic acid; S, stearic acid; St, stearidonic acid; ND, not detected.
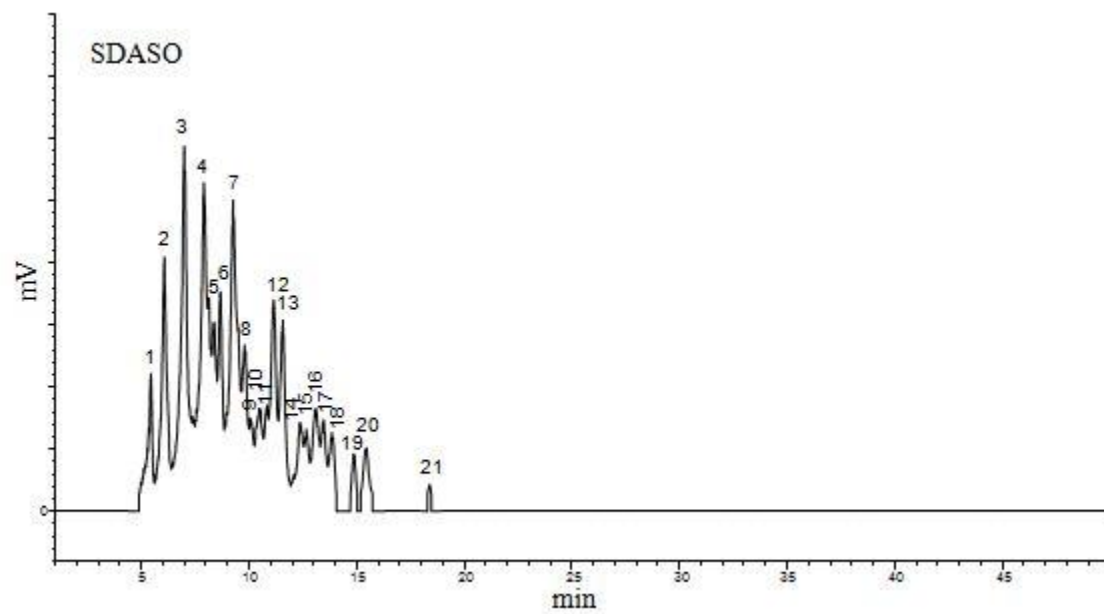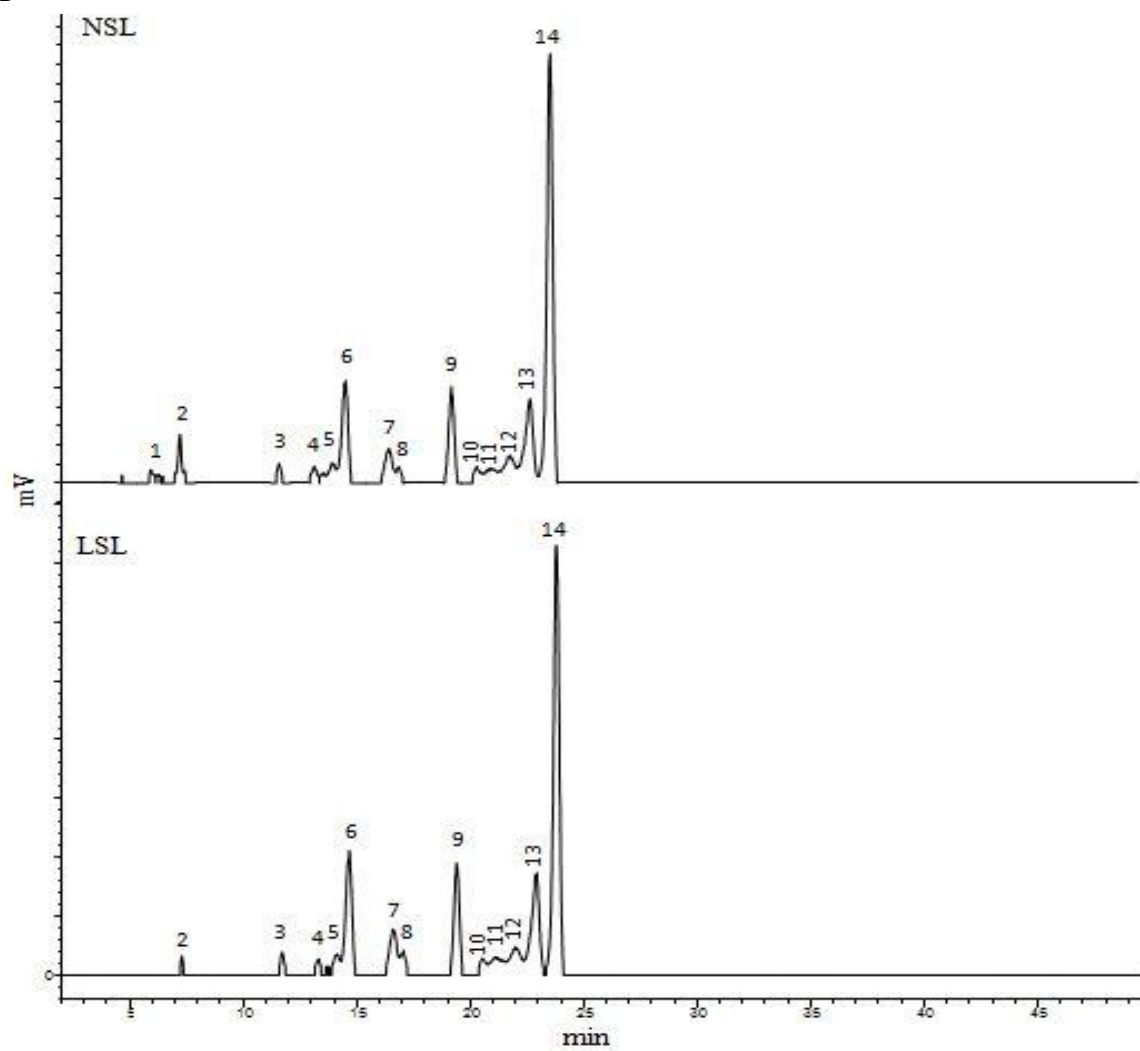
Figure 4.5 *sn*-2 positional analysis of (A) NSL and (B) LSL with C14:0 (♦), C16:0 (■), C18:0

(▲), C18:1n-9 (×), C18:2n-6 (∗), C18:3n-6 (●), C20:1 (-), and C18:4n-3 (+) after enzyme reuse.
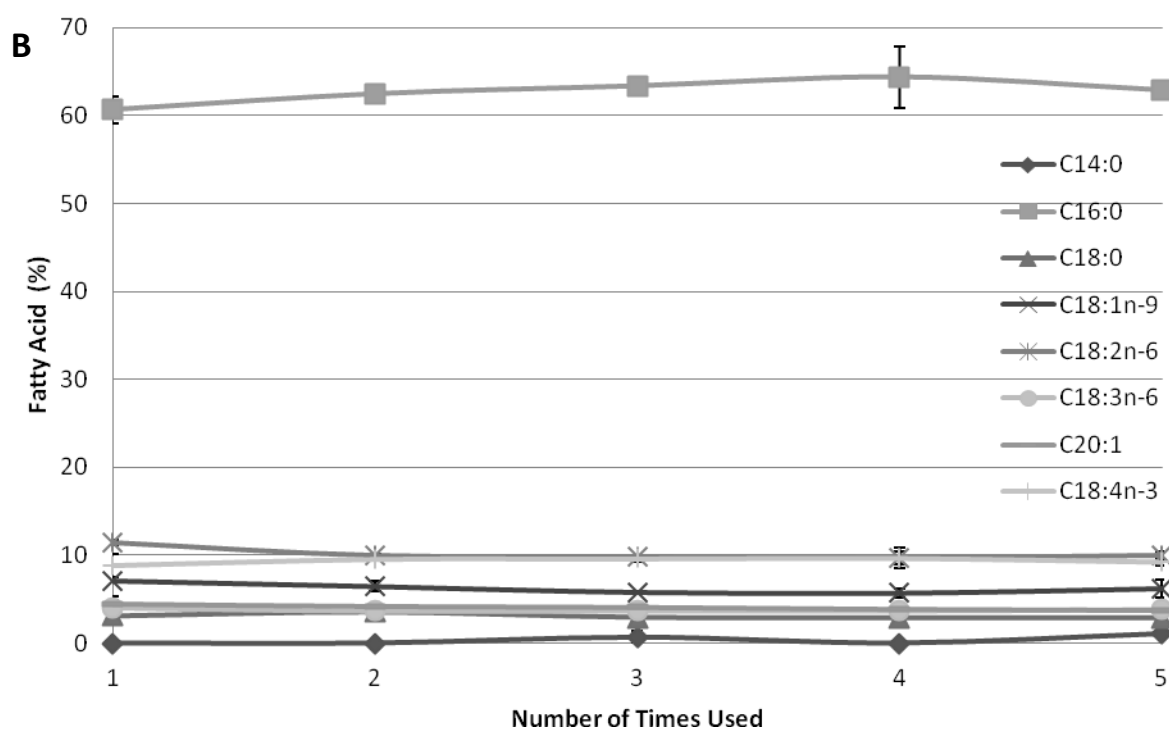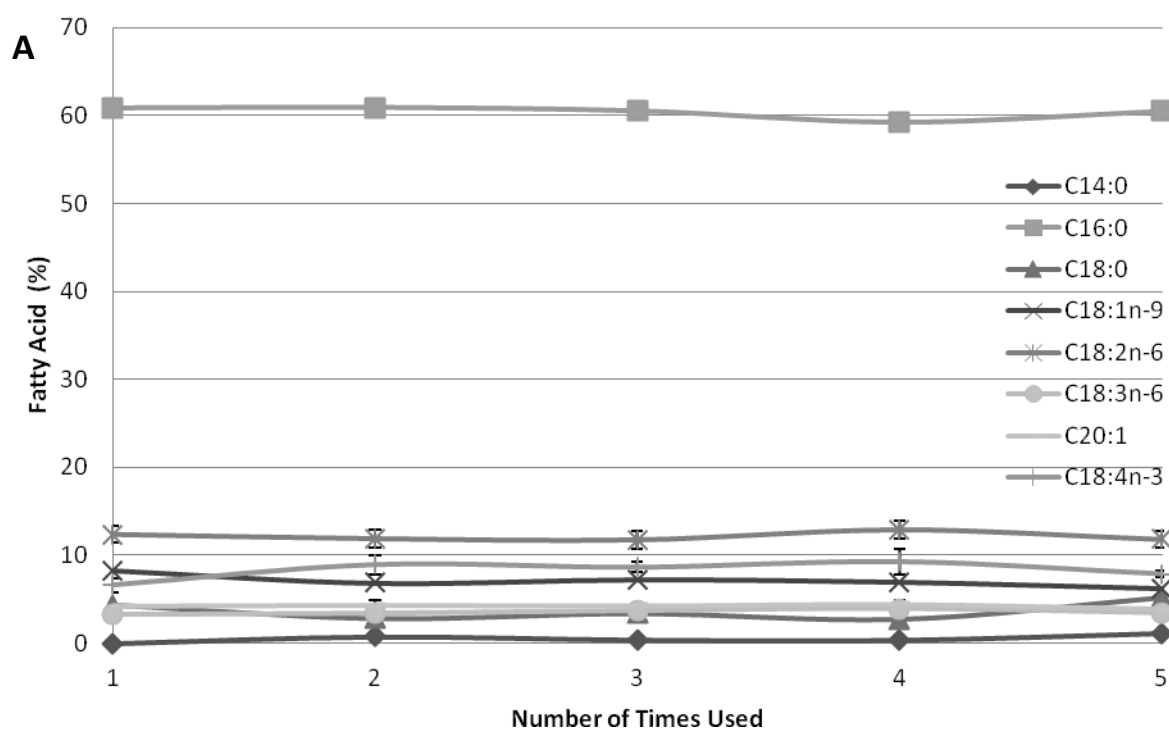
CHAPTER 5

MODIFICATIONS OF STEARIDONIC ACID SOYBEAN OIL BY ENZYMATIC

ACIDOLYSIS FOR THE PRODUCTION OF HUMAN MILK FAT ANALOGUES [1]

ABSTRACT

Structured lipids (SLs) from stearidonic acid (SDA) soybean oil pre-enriched with palmitic acid (PA) at the *sn*-2 position with Novozym 435 (NSL) or Lipozyme TL IM (LSL) from previous research were further enriched with γ-linolenic acid (GLA) or docosahexaenoic acid (DHA). Small-scale acidolysis reactions with Lipozyme TL IM were performed to determine the optimal reaction conditions as 1:1 substrate mole ratio of NSL or LSL to free DHA at 65°C for 24 h and a 1:0.5 substrate mole ratio of NSL or LSL to free GLA at 65°C for 12 h. Optimized SL products were scaled up in a 1 L stir-batch reactor, and the resulting SLs of NSL:DHA (NDHA), LSL:DHA (LDHA), NSL:GLA (NGLA) and LSL:GLA (LGLA) were chemically and physically characterized. The SLs contained >54% PA at the *sn*-2 position with GLA >8% for the GLA SLs and DHA >10% for the DHA SLs. The oxidative stabilities of the SLs were increased by the addition of 200 ppm of TBHQ with NGLA being more stable due to higher tocopherol content than the other SLs. The melting and crystallization profiles did not differ between the DHA SLs or the GLA SLs. The triacylglycerol (TAG) species were similar for the GLA SLs but differed between the DHA SLs, with tripalmitin being the major TAG species in all SLs.

INTRODUCTION

Human milk or infant formula are the only nutritional sources of energy for an infant. Human milk fat (HMF) contains 3.0-4.5% fat with 98-99% of this fat in the form of triacylglycerols (TAGs) (*1*). HMF provides essential fatty acids (FAs) that are required as structural cell components of membrane tissues and provide an essential source of energy that is approximately 50% of the total energy used by infants (*2*). Infant formulas try to mimic the composition of fat found in breast milk for proper nutrition. However, most formulas are

produced using vegetable oils that are rich in α-linolenic acid (ALA). Vegetable oils contain very few saturated FAs when compared to HMF. Biotechnology can be used to produce structured lipids (SLs) by modifying the TAG structure to change the FA composition and/or position in the glycerol backbone by chemically and/or enzymatically catalyzed reactions (*3*).

Stearidonic acid (SDA) is an n-3 polyunsaturated FA (PUFA) and is a major FA in SDA soybean oil. SDA soybean oil consists of approximately 20% stearidonic, 24% linoleic, and 12% palmitic acids (*4*). SDA is the first metabolite of ALA converted by desaturases and elongases (*5*) as shown in **Figure 5.1**. The conversion from ALA to eicosapentaenoic acid (EPA) is not effective because the initial Δ6 desaturase enzyme is rate-limiting in humans (*6*). The consumption of SDA, instead of ALA, would skip the rate limiting step for better conversion to EPA. The intake of dietary SDA was found to increase EPA concentrations by 3-4-fold more effectively than similar levels of ALA (*7*). EPA is essential for growth, development, and intestinal absorption of fat-soluble vitamins in infants (*2*). James *et al*. (*7*) conducted a study examining the effect of dietary SDA on increasing tissue concentrations of EPA and concluded that SDA vegetable oils, such as SDA soybean oil, were more effective in increasing EPA tissue concentrations than ALA-containing vegetable oils. EPA may also be converted to docosahexaenoic acid (DHA) via the n-3 biosynthetic pathway (**Figure 5.1**). However, this conversion is considered to be inefficient and most diets are now being directly supplemented with preformed DHA. DHA is an essential structural component of retinal, neural, and other cell membranes, supports brain and nervous system development, and improves visual acuity in infants (*8*). The n-6 PUFA γ-linolenic acid (GLA) is an intermediate in the bioconversion of linoleic acid (LA) to arachidonic acid (ARA). This bioconversion also includes a series of alternating desaturation and elongation steps with the initial desaturation by Δ6 desaturase being

rate limiting in humans (**Figure 5.1**). ARA is needed for brain growth and functional development of infants (*9*), and GLA is anti-inflammatory and has been used in the treatment of diabetic neuropathy, hypertension, asthma, atopic dermatitis, and cancer (*10*). The incorporation of GLA into infant formulas would bypass the rate-limiting desaturase step and increase ARA biosynthesis. Long-chain PUFA (LC-PUFA) supplementation in infant formula is important during the first year of life because infants often have a limited desaturating capacity to synthesize these LC-PUFAs (*11*). Therefore, infants need to rely on the dietary LC-PUFAs in their nutrition from either infant formula or HMF. The composition of HMF consists of approximately 0.00-0.27% GLA, 0.23-0.75% ARA, and 0.15-0.56% DHA (*12*).

Palmitic acid (PA) is important in the composition of HMF. PA is the second major FA found in HMF at approximately 18.3-25.9% (*13, 14*). However, SDA soybean oil contains only approximately 12% PA (*4*). A large portion of PA in HMF is esterified at the *sn*-2 position of the TAGs. The PA that is esterified at the *sn*-2 position is >60% (by weight), and unsaturated FAs are mainly found at the *sn*-1,3 positions of the TAGs in HMF (*15*). PA at the *sn*-2 position helps to improve the absorption of fat and calcium in infants (*16, 17*). The large amount of PA reduces the formation of calcium soaps that are generated by the long-chain saturated FA interactions with calcium (*17*).

HMF analogues can be produced by the use of enzymatically catalyzed acidolysis reactions. Acidolysis is the reaction of TAG molecules with free fatty acids (FFAs). We previously reported on the modification of SDA soybean oil to produce human milk fat analogues enriched with PA (*18, 19*). No current studies are published on the modification of SDA soybean oil enriched with PA and DHA or with PA and GLA along with characterization of their physical and chemical properties. Our first objective was to determine the optimal

conditions to incorporate either approximately 10% GLA or DHA into SLs (SDA soybean oil enriched with PA at the *sn*-2) from our previous study (*19*) by acidolysis on a small scale. Our second objective was to scale-up the production of the new SLs (containing GLA or DHA) at optimal conditions and to characterize the chemical and physical properties of the scaled-up SLs.

MATERIALS AND METHODS

Materials.

SDA soybean oil was kindly provided by Monsanto Co. (St. Louis, MO), and tripalmitin was purchased from TCI America (Portland, OR). γ-Linolenic acid (GLA) in free fatty acid form (70% GLA) was purchased from Sanmark (Greensboro, NC), and DHASCO oil (40% DHA) was purchased from Martek (Columbia, MD). Commercial milk-based infant formula, Nestle Good Start® Supreme (Nestle USA, Inc., Glendale, CA), containing DHA and ARA, was purchased from a local convenience store in Athens, GA. 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, where IUN is the abbreviation for interesterification units novo. Lipid standards, Supelco 37 Component FAME mix, triolein, 2-oleoylglycerol, tripalmitin, tristearin, 1,2-dioleoyl-3-palmitoyl-rac-glycerol, 1-palmitoyl-2-oleoyl-3-linoleoyl, and tocopherol standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), and the internal standard, C19:0-nonadecanoic acid, was purchased from TCI America. The TAG standard mix (GLC reference standard) was purchased from Nu-chek Prep, Inc. (Elysian, MN). Other solvents and chemicals were purchased from Fisher Scientific (Norcross, GA) and Sigma-Aldrich Chemical Co.

Initial Reaction Substrates.

The FA compositions of the initial substrates are shown in **Table 5.1**. NSL and LSL were two

SLs produced from our previous research (*19*) by lipase-catalyzed interesterification using SDA

soybean oil and tripalmitin as substrates. The biocatalyst for NSL was Novozym 435

(nonspecific *Candida antarctica* lipase) and Lipozyme TL IM for LSL. Both reactions were

performed at conditions of 1:2 substrate mole ratio (SDA soybean oil to tripalmitin) at 65°C for

18 h (*19*). GLA was already in FFA form when purchased, whereas free DHA was prepared from

DHASCO oil by saponification to convert it into FFA form (as described below).

Preparation of FFA from DHASCO Oil by Saponification.

One hundred and fifty grams of DHASCO oil was saponified using a mixture of KOH (34.5 g),

water (66 mL), 95% ethanol (396 mL), and butylated hydroxytoluene (0.03 g) by placing them in

a 1 L stir-batch reactor with a circulating water bath at 60°C for 1 h. After 1 h, 120 mL of

distilled water was added to the saponified mixture. The mixture was then acidified with 6 N

HCl to a pH of 1-2. A separatory funnel was used to remove the top FFA layer from the bottom

layer. The FFA layer was washed with 100 mL of distilled water and extracted. The bottom layer

was then extracted twice with 200 mL of hexane and the top hexane layer collected and pooled

together with the previously extracted FFA layer. This mixture was filtered through a sodium

sulfate column to remove any excess water. The filtrate was rotovapped with a Büchi rotovapor

(Flawil, Switzerland) at 40°C to remove hexane. The DHASCO FFA (free DHA) was stored in

an amber Nalgene bottle under nitrogen at -85°C.

Small-Scale Acidolysis Reaction.

One hundred milligrams of each mixture (in substrate mole ratios of 1:1, 1:2, and 1:3

NSL/LSL:free DHA or substrate mole ratios of 1:0.5, 1:1, and 1:1.5 NSL/LSL:free GLA) were

weighed into labeled Teflon-lined test tubes, 3 mL of *n*-hexane was added, and 10% by weight of

lipase, Lipozyme TL IM (based on total substrate weight), was added to the test tube as the

biocatalyst. The reactions were carried out at 65°C for 12 and 24 h in a water bath while shaking

at 200 rpm (20.94 rad/s). After the reaction, the products were filtered two times through a

sodium sulfate column to remove excess water and the biocatalyst.

Recovery of TAGs.

The recovery of TAGs was performed to isolate the TAG molecules and to remove FFAs from

the product mixture following our previous method using TLC (*19*). The TAG bands on the TLC

plate were scraped off into test tubes, and the recovered TAGs were extracted from the silica gel

with 2 mL of diethyl ether, vortexed, centrifuged at 1000 rpm (104.72 rad/s) for 3 min, and

filtered through an anhydrous sodium sulfate column. This extraction step was repeated for a

total of two times. The SLs were completely dried under nitrogen gas and then analyzed for fatty

acid methyl ester (FAME) analysis and positional analysis.

Determination of Fatty Acid Profiles.

The free GLA, free DHA, and SL samples were converted to FAME following AOAC Official

Method 996.01, Section E (*20*), with minor modifications (19, *21*). FAME samples were

collected in amber GC vials, and the Supelco 37 component FAME mix was used as the external

standard and was run parallel with the samples on the GC.

GC Analysis.

The FAMEs (from free GLA, free DHA, SLs, and corresponding positional analyses) were

analyzed using an Agilent Technology 6890N gas chromatograph (Agilent Technologies, Santa

Clara, CA) with a flame ionization detector (FID). A Supelco SP-2560 column, 100 m x 250 μm,

0.20 μm film, was used to attain separation. Injection of 1 μL of sample was made at a split ratio

of 5:1 with helium as the carrier gas at a flow rate of 1.1 mL/min and at a constant pressure (45.0 mL/min). The injector temperature and the FID set point were 300°C. The oven was held at 140°C for 5 min, then increased to 240°C at 4°C/min, and held at 240°C for 15 min. The relative FAME content was calculated using the online computer. The average and standard deviations of the triplicate analyses were reported.

Positional Analysis.

A modified version of the Luddy *et al.* (*22*) method was used to perform the pancreatic lipase-catalyzed *sn*-2 positional analysis as we previously described (*18*). The 2-MAG band was scraped off the TLC plates and converted to FAME (as described above). Fifty microliters of the internal standard (20 mg/mL C19:0 in hexane) was used for all samples. For better resolution, the FAME *sn*-2 samples were concentrated in the GC vials to one-third their volumes before the FA content at the *sn*-2 position were analyzed and quantified by GC.

Scaled-up Acidolysis Reaction.

The solvent-free acidolysis reaction occurred in a 1 L stir-batch reactor with a circulating water bath at 65°C for 12 h with a substrate mole ratio of 1:0.5 (NSL/LSL: free GLA) and 10% (w/w) of Lipozyme TL IM as the biocatalyst with constant stirring at 200 rpm (20.94 rad/s). The acidolysis reaction to incorporate DHA was performed for 24 h with a substrate mole ratio of 1:1 (NSL/LSL:free DHA) and 10% (w/w) of Lipozyme TL IM as the biocatalyst under the same conditions. The reactor was sealed and wrapped with foil to reduce exposure to light and oxygen. At the end of the reaction, the resulting products, two SLs containing GLA (NGLA and LGLA) and two SLs containing DHA (NDHA and LDHA), were recovered and vacuum filtered through a Whatman no.1 containing sodium sulfate and then through a 0.45 µm membrane filter to dry and separate the SLs from the biocatalyst. If the SLs needed to be stored before short-path

distillation, they were kept in an airtight amber container in the dark at 4°C after being flushed with nitrogen. Approximately 5 g of each SL sample was kept for analysis of tocopherol content before short-path distillation.

Short-Path Distillation.

Short-path distillation was used to remove liberated or excess FFAs from the SLs and substrates. Short-path distillation was performed using a KDL-4 (UIC Inc.) unit under the following conditions: holding temperature of 65°C, feeding rate of approximately 100 mL/h, heating oil temperature of 185°C, coolant temperature of 30-35°C, and vacuum of <100 mTorr or <13.33 Pa. The GLA SLs were passed through the short-path distillation once whereas the DHA SLs were passed through twice. After short-path distillation, the FFA content was determined according to AOCS Official Method Ac 5-41 (*23*). The percent yield was calculated on the basis of the starting weight of the substrates and the final weight of the SL after short-path distillation. The SLs were analyzed for FA profiles and positional analysis as stated above.

Fat Extraction from Infant Formula.

The Bligh and Dyer method (*24*) was used to extract the fat from a milk-based infant formula. One hundred grams of the infant formula was weighed into a 1 L beaker. Then 100 mL of chloroform was added and the mixture homogenized for 30 s. Two hundred milliliters of methanol was added to the mixture and homogenized again for 30 s. Another 100 mL of chloroform was added, and the mixture was blended for 1-2 min. One hundred milliliters of 0.88% sodium chloride solution was added, and the mixture was blended again for 1 min. The mixture was vacuum-filtered through a Buchner funnel containing a Whatman no.1, 9 cm, filter. The residue on the filter paper was transferred into a beaker, 100 mL of chloroform was added, and then the mixture was homogenized for 1 min. This mixture was vacuum-filtered as before

and collected with the first filtrate. The entire filtrate was transferred into a 1 L separatory funnel and allowed to separate. The bottom chloroform layer was collected and passed through a sodium sulfate column to remove any excess water. The excess solvent was removed using a Büchi rotovapor (Flawil, Switzerland) at 40°C. The extracted oil was collected in an amber bottle and stored at -20°C under nitrogen. The extracted oil was then analyzed for FA profile and positional analyses as described above. The FA profile of the commercial infant formula (IF) is shown in **Table 5.4**.

Tocopherol Analysis.

Tocopherols were identified and quantified in NDHA, LDHA, NGLA, and LGLA using a normal phase high-performance liquid chromatography (HPLC) system. The tocopherol contents in the SLs were analyzed before and after short-path distillation. An isocratic mobile phase of 0.85% (v/v) isopropanol in hexane, vacuum filtered and degassed by stirring under vacuum, was used at a flow rate of 1.0 mL/min. The column used was a LiChrosorb Si 60 column (4 mm, 250 mm, 5 µm particle size, Hiber Fertigsäule RT, Merck, Darmstadt, Germany) with a Shimadzu LC-6A pump equipped with a RF-10AXL fluorescence detector (Shimadzu Corp., Columbia, MD), a Spectra series As100 autosampler (Thermo Separation Products, Inc., San Jose, CA), and Agilent Chemstation software. The standards and samples were prepared and analyzed as we previously described (*19*).

Oxidative Stability Analysis.

Fifteen grams of NDHA, LDHA, NGLA, and LGLA was weighed into Teflon-lined test tubes and oxidized for 72 h at 65°C in the dark in a shaking water bath at 200 rpm (20.94 rad/s). The samples were removed from the water bath and analyzed at 0, 24, 48, and 72 h for both peroxide (PV) and *p*-anisidine values (*p*AV). The PV and *p*AV were determined according to AOCS

Official Method Cd 8b-90 and AOCS Official Method Cd18-90, respectively (*23*). Total

oxidation (TOTOX) value was calculated as $2 \times (PV) + (pAV)$ (*25*). The oxidative stability

index (OSI) was determined according to AOCS Method Cd12b-92 (*23*) using an Oil Stability

Instrument (Omnion, Rockland, MA) at 110^oC for each SL. *tert*-Butylhydroxyquinone (TBHQ)

was added to each of the SLs in amounts of 100 and 200 ppm (w/w) to assess the effect of

antioxidants on the OSI values of the SLs.

Melting and Crystallization Profiles.

The melting and crystallization profiles were determined for free DHA, free GLA, NDHA,

LDHA, NGLA, and LGLA using a differential scanning calorimeter (DSC 1 STAR^e System,

Mettler Toledo, Columbus, OH), and cooled with a Haake immersion cooler (Haake EK90/MT,

Thermo Scientific, Vernon Hills, IL). The analysis was performed according to AOCS Official

Method Cj 1-94 (*23*) with minor modifications using indium as a standard. Sample weight

ranged from 8 to 12 mg in 30 µL aluminum pans. The sample was heated from 25 to 80^oC at

50^oC/min, held for 10 min (to destroy any previous crystalline structure), cooled from 80 to -

55^oC at 10^oC/min (for crystallization profiles), held for 30 min, and then heated from -55 to 80^oC

at 5^oC/min (for melting profiles). The thermograms were analyzed with the DSC (DSC 1 STAR^e

System, Mettler Toledo).

TAG Molecular Species.

A reversed phase HPLC (Agilent Technologies 1260 Infinity, Santa Clara, CA) with a Sedex 85

evaporative light scanning detector (ELSD) was used to analyze the TAG molecular species of

NDHA, LDHA, NGLA, and LGLA on an Ultrasphere C18 250 mm, 4.6 mm, 5 µm particle size,

column (Beckman Coulter, Fullerton, CA) as previously described (*19*).

Statistical Analysis.

All analyses, except melting and crystallization profiles, were performed in triplicate. Melting and crystallization profiles were performed in duplicate. Statistical analysis was performed with the SAS software package (SAS Institute, Cary, NC). Duncan's multiple-range test was performed to determine the significance of difference at $P \leq 0.05$.

RESULTS AND DISCUSSION

FA Composition of Substrates.

SDA soybean oil was initially enriched with PA in our previous studies (*18, 19*) using Novozym 435 or Lipozyme TL IM as biocatalyst to produce two SLs, NSL and LSL, respectively. SDA soybean oil contained linoleic acid (25.78 ± 0.07%), SDA (22.16 ± 0.23%), oleic acid (14.31 ± 0.17%), α-linolenic acid (12.30 ± 0.06), and PA (11.54 ± 0.04%) as the major FAs in the oil (*18*). SDA soybean oil was enriched with PA because it contains less PA than HMF with very little PA at the *sn*-2 position (*18*). In our previous research (*19*), two SLs were produced from the enzymatic interesterification of SDA soybean oil with tripalmitin at a 1:2 substrate mole ratio and incubated at 65°C for 18 h. The two SLs produced were Novozym 435 SL (NSL) and Lipozyme TL IM SL (LSL). These became the initial substrates for our current research, and the NSL, LSL, free GLA, and free DHA FA profiles are shown in **Table 5.1**. The free DHA substrate contained ~44% DHA and ~28% oleic acid as its major FAs, whereas the free GLA substrate contained ~69% GLA and ~28% linoleic acid as its major FAs. The SLs from our previous work (*19*) contained no detectable DHA but contained approximately 3% GLA with n-6/n-3 ratios of approximately 1.6, with >60% of PA at the *sn*-2 position. NSL and LSL also retained SDA in their TAG composition to provide health benefits of n-3 FAs. In the present

research, acidolysis was used to incorporate the FFAs of DHA or GLA into the previously produced SLs, NSL and LSL. The resulting SLs of the acidolysis reactions are designated as follows: NSL with free DHA (NDHA), NSL with free GLA (NGLA), LSL with free DHA (LDHA), and LSL with free GLA (LGLA). The aim of the current research was to use acidolysis catalyzed by Lipozyme TL IM to enrich NSL and LSL with DHA or GLA at the *sn*-1,3 positions with approximately 10% total DHA or GLA and to retain a large amount of PA esterified at the *sn*-2 position for possible use as a HMF analogue. The second objective was to physically and chemically characterize the new SLs (NDHA, LDHA, NGLA, and LGLA). The first commercially available HMF analogue, Betapol (Loders Croklaan, Glen Ellyn, IL), was produced by using a 1,3-specific lipase to synthesize SLs by reacting tripalmitin with unsaturated FAs (*26*). Several studies have reported development of HMF analogues including modified oil mixtures containing amaranth oil and ethyl palmitate (*21*), a mixture of EPA and DHA with tripalmitin and hazelnut fatty acids (*27*), and the incorporation of tripalmitin and GLA into hazelnut oil (*28*). Currently, no studies have modified SDA soybean oil to obtain a HMF analogue enriched with *sn*-2 PA and DHA or GLA.

Small-Scale Analysis.

Small-scale acidolysis reactions were performed at 65°C for either 12 or 24 h with substrate mole ratios of 1:1, 1:2, or 1:3 for NSL/LSL:free DHA or 1:0.5, 1:1, or 1:1.5 for NSL/LSL:free GLA. Smaller ratios were used for the GLA SLs because NSL and LSL already contained small amounts of GLA (~3%). The small-scale analysis results are shown in **Table 5.2** for the DHA SLs and in **Table 5.3** for the GLA SLs. Because the aim of our research was to keep the PA at the *sn*-2 position as high as possible and also contain approximately 10% DHA or GLA, the optimal conditions were selected to be 1:1 for 24 h at 65°C for NDHA and LDHA and

were 1:0.5 for 12 h at 65°C for NGLA and LGLA. NDHA and LDHA contained >10% DHA and

the PA content at the *sn*-2 position was >55%. NGLA and LGLA contained >9.7% GLA and the

PA content at the *sn*-2 position was >51%. Even though a *sn*-1,3 specific lipase was used, the PA

content at the *sn*-2 position decreased from the 60% (*19*) observed in NSL and LSL (**Table 5.1**),

possibly due to some acyl migration during acidolysis. The position of the FA on the glycerol

backbone of the TAG is very important. Martin *et al.* (*29*) observed that there were three major

fatty acids (oleic, palmitic, and linoleic acids) which showed a specific preference in the position

of the TAG of HMF. Oleic acid showed preference for the *sn*-1 position, palmitic for the *sn*-2,

and linoleic for the *sn*-3 (*29*). Martin *et al.* (*29*) also found that ARA and DHA were located

primarily at the *sn*-2 and *sn*-3 positions.

FA Composition of Optimized SLs.

The optimal conditions were then used to scale up the acidolysis reaction in a 1 L stir-

batch reactor. The FA profiles of the scaled-up SLs are shown in **Table 5.4** along with the FA

profile of a commercial infant formula fat. The major FAs found in the GLA SLs were PA,

linoleic, GLA, SDA, and oleic acids. The conversion of GLA to ARA skips the rate-limiting

desaturase step and is important for brain growth and functional development of infants (*9*). The

GLA SLs had significant differences in their total GLA and n-6/n-3 ratios, with LGLA

containing more GLA and a higher n-6/n-3 ratio than NGLA. The major FAs found in the DHA

SLs were PA, oleic, DHA, linoleic, and SDA. The DHA SLs showed significant differences in

their DHA contents, with NDHA containing more DHA than LDHA. The DHA SLs contained

some GLA because the original SLs, NSL and LSL, contained approximately 3% total GLA. All

SLs contained SDA because both of the original SLs (NSL and LSL) had approximately 8% total

SDA. Long-chain PUFA (LC-PUFA) supplementation in infants, such as with DHA, is

important because term infants have a limited desaturating capacity to synthesize these LC-PUFAs (*11*). SDA is important because it skips the rate-limiting desaturase step, allowing for better conversion to EPA that is important to growth, development, and intestinal absorption of fat-soluble vitamins in infants (*2*). DHA is important to infants as it supports brain and nervous system development and improves visual acuity (*8*). The major FAs found in the commercial infant formula were oleic, PA, linoleic, and lauric acids. The FA profile of infant formula was significantly different from all four FA profiles of the SLs with the exception of the stearic acid content at the *sn*-2 position. The commercial infant formula also had a higher n-6/n-3 ratio than the SLs. It appears that the content of SDA helped decrease the n-6/n-3 ratio in the SLs and that the presence of DHA in the DHA SLs decreased the ratio even more significantly when compared to the GLA SLs. The n-6/n-3 PUFA ratio increased with the addition of GLA and decreased with the addition of DHA from 1.6 of the original SLs, NSL and LSL (*19*). Positional analysis is also important in HMF analogues. Lipozyme TL IM is a *sn*-1,3 specific lipase that was chosen to retain PA at the *sn*-2 position. A large portion of PA in HMF is esterified at the *sn*-2 position of the TAGs. The PA that is esterified at the *sn*-2 position is >60% (by weight), and unsaturated FAs are mainly found at the *sn*-1,3 positions of the TAGs in HMF (*15*). During metabolism, the FAs esterified at *sn*-1,3 are released by pancreatic lipase, leaving the FAs esterified at the *sn*-2 position unhydrolyzed. The unhydrolyzed *sn*-2 FAs are absorbed by the intestinal mucosa as *sn*-2 monoacylglycerols (*16*). PA at the *sn*-2 position helps to improve the absorption of fat and calcium in infants (*16, 17*). The large amount of PA reduces the formation of calcium soaps that are generated by the long-chain saturated FA interactions with calcium (*17*). The free PA, not absorbed, may be lost as calcium soaps in the infant's feces. De Fouw *et al.* (*30*) found that rats fed a formula with a high amount of PA at the *sn*-2 position had a higher

absorption of PA in the intestines when compared to formulas with low *sn*-2 PA with both groups receiving the same total PA. However, de Fouw *et al.* (*30*) did not see a difference in calcium absorption between the two formulas fed to rats. In our current study, the *sn*-2 PA content did decrease from the original SLs, NSL and LSL, possibly due to acyl migration of other FAs into the *sn*-2 position. This migration resulted in some DHA and GLA migrating into the *sn*-2 position of the TAG. A shorter reaction time could result in less migration. Also, lipases are more active in *n*-hexane and isooctane than in other solvents such as toluene and acetonitrile. The hydrophobicity of the solvent can affect the extent of acyl migration, and solvents, such as diethyl ether, can be used to reduce the possibility of acyl migration (*31*). NGLA and LGLA had similar PA contents at the *sn*-2 position. NDHA and LDHA also had similar PA at the *sn*-2 position with no significant difference. However, the PA at the *sn*-2 position was significantly different amoung all of the SLs and the commercial infant formula that was analyzed (P≤0.05). The infant formula contained only approximately 20% PA at the *sn*-2 position, whereas the SLs contained >54%. Since HMF contains >60% *sn*-2 PA, the SLs from this research appear to provide similar *sn*-2 PA content as HMF. The percent yields during the production of the SLs were also determined. NGLA and LGLA were passed through short-path distillation one time and resulted in yields of 73.62 and 68.82%, respectively. NDHA and LDHA were passed through short-path distillation twice and resulted in yields of 58.05 and 59.00%, respectively. The percent yields were lower for the DHA SLs because the reaction was between a TAG and FFA, and a higher substrate mole ratio (1:1) provided more FFA that must be removed by short-path distillation. A reaction between two TAGs would result in less FFA and a higher percent yield as in our previous research (*19*).

Physicochemical Properties.

The FFA and tocopherol contents were determined for each of the SLs and are shown in **Table 5.5**. Short-path distillation was used to remove excess and liberated FFAs in the SLs. Short-path distillation was performed once for the GLA SLs and twice for the DHA SLs because the substrate mole ratio used for the DHA SLs was higher than the ratio used for the GLA SLs (1:1 compared to 1:0.5). LGLA had the lowest FFA percentage followed by NGLA, NDHA, and then LDHA. The FFA content in LGLA was significantly lower than that found in NGLA. There was no significant difference in the FFA levels between NDHA and LDHA. FFA could be lowered even more by another pass through short-path distillation or by the use of deodorization or neutralization. Tocopherols are antioxidants and are often used to increase the oxidative stability of oils (*32*). Tocopherols are a part of the vitamin E family and are composed of α, β, γ, and δ-tocopherols that are characterized by a saturated side chain of three isoprenoid units and unsaturated tocotrienols (*33*). The tocopherol content was analyzed before and after short-path distillation. Short-path distillation involves high heat to separate the TAGs from the FFAs. The application of heat decreases the tocopherol content in the SLs (**Table 5.5**). NGLA contained a significantly higher amount of γ-tocotrienol than LGLA after short-path distillation. The higher amount of γ-tocotrienol provides NGLA with the most antioxidants of all the SLs followed by NDHA, LDHA, and then LGLA. However, there were no significant differences in tocopherol contents between the DHA SLs after short-path distillation. The loss of certain tocopherol homologues, such as β-tocopherol, may be in the FFA waste during short-path distillation. Analysis of the FFA waste could determine whether or not β-tocopherol is being destroyed by heat or just codistilled into the waste with the FFAs. Tocopherols usually decrease during the refining of vegetable oils.

The oxidative stabilities of the SLs are shown in **Table 5.6**. The oxidative stability

indices (OSI) of NDHA and LDHA were not significantly different (P≤0.05). However, the OSI

of NGLA was significantly higher than that of LGLA. This may be due to the tocopherol content

being higher in NGLA compared to the other SLs. γ-Tocotrienol may have helped stabilize

NGLA and make it more oxidative stable than LGLA with lower levels of γ-tocotrienol. The

difference in oxidatively stability may also be due to the differences in FA profiles and TAG

species. The OSI values (except in NGLA) decreased from the values of LSL and NSL due to the

additional acidolysis reaction and short-path distillation (*19*). Loss of oxidative stability can be

caused by loss of antioxidants and exposure to light, oxygen, and heat during production

(acidolysis and short-path distillation). Some studies also reported a decrease in tocopherol

content and other non-TAG components after production (*34, 35*), whereas other studies reported

an adverse effect on tocopherols (*36, 37*). The peroxide, *p*-anisidine, and TOTOX values were

also determined to analyze the oxidative stability of the SLs and are shown in **Figure 5.2**. The

peroxide value is a measurement of the initial products of oxidation (*33*). The peroxide value

measures the amount of iodine formed by the reaction of the peroxides with the iodine ion. The

p-anisidine value measures the amount of aldehydes or decay products of the hydroperoxides

that increase during oxidation (*33*). The TOTOX value takes into account both the peroxide and

p-anisidine values (*33*). Again, NGLA was the most oxidatively stable in peroxide, *p*-anisidine,

and TOTOX values followed by LGLA and NDHA. The least oxidatively stable was LDHA.

This loss of antioxidants during short-path distillation may be reversed by the addition of

antioxidants to the SLs before storage or food application for better stability. Each of the SLs

was supplemented with 100 and 200 ppm of the antioxidant THBQ. The FDA approved use of

TBHQ as a food antioxidant in 1972 and allows no more than 0.02% or 200 ppm of THBQ to be

added into the oil (*38*). The addition of TBHQ increased the OSI in each of the SLs, especially at

levels of 200 ppm (**Table 5.6**) with NGLA having the highest OSI. In a different study, a

commercialized infant formula fat was analyzed and had an OSI value of 21.7 h, and milk fat

had an OSI of 14.3 h, whereas their DHA SL contained a very low OSI value of 1.0 (*39*). The

addition of natural antioxidants lost during the SL production (tocopherols) and the addition of

200 ppm TBHQ may increase the OSI further before commercial application, as done with

vegetable oils and fish oils.

TAG Molecular Species of SLs.

The TAG molecular species were determined using reversed-phase HPLC for NGLA,

LGLA, NDHA, and LDHA. The retention times and relative FA percentages are shown in **Table

5.7**. The major TAG species for NGLA were PPP (37.75%), PPL (11.88%), LPL (8.17%), PPO

(6.89%), and StOSt/LnGSt (6.81%), and for LGLA they were PPP (34.78%), PPL (11.88%),

LPL (8.59%), PPO (7.38%), and StOSt/LnGSt (6.14%). NGLA and LGLA had the same five

major TAG species, but NGLA contained a higher percentage of PPP. This may have contributed

to its higher stability as saturated FAs are more oxidatively stable than unsaturated FAs. The

major TAG species for NDHA were PPP (23.15%), PPL (12.51%), StOSt/LnGSt (10.38%), PPO

(9.68%), and StPSt (8.39%), and for LDHA they were PPP (23.94%), StOSt/LnGSt (14.35%),

StPSt (12.22%), PPO (10.88%), and LPL (6.52%). NDHA and LDHA had the same TAG

species (except LPL/PLP) but varied in their order of abundance. All of the SLs had their major

TAG as PPP and contained other TAGs with PA in their structure because PA was the major FA

found in the SLs FA profiles. The incorporation of DHA or GLA did decrease the amount of PPP

in the original SLs (NSL and LSL) that had ~50% of their TAG species as PPP. The DHA SLs

contained DPD, OPD, and OOD, whereas the GLA SLs contained TAG species of LLG and GGO due to their higher content of GLA in their FA profile.

Melting and Crystallization Profiles.

The melting and crystallization behaviors for free DHA, free GLA, NDHA, LDHA, NGLA, and LGLA were evaluated with DSC. The melting thermograms are shown in **Figure 5.3**. The substrate, free DHA, had a melting range from -17.28 to 20.74^{o}C consisting of two sharp peaks at -6.80 and 16.38^{o}C whereas the other substrate, free GLA, had a melting range of -35.42 to -41.78^{o}C with only one peak at -36.55^{o}C. LDHA and NDHA melting thermograms both contained three peaks with similar melting ranges around 0 to 45°C. NGLA and LGLA both contained three peaks with melting ranges around 3 to 54°C indicating that the GLA SLs required a higher temperature to fully melt the SLs compared to the DHA SLs. Oils with more saturated FAs tend to melt at higher temperatures (*40*). The GLA SLs contained more total PA in their FA profile and a higher relative percentage of PPP in their TAG species than the DHA SLs. The melting profiles of the new SLs decreased slightly from the more saturated NSL and LSL from our previous research, which had an onset of 4°C and an endpoint of 55°C (*19*). The unsaturated FAs in the TAG started to melt at lower temperatures while the more saturated FAs tended to melt at higher temperatures. The modification of oils results in complex mixtures of TAGs that undergo gradual melting, whereas purer TAGs (such as PPP) undergo rapid melting. The same is true for the substrates, free GLA and free DHA. As seen in **Table 5.1**, free DHA had a more complex FA composition when compared to free GLA, which consisted of mostly GLA (~70%). The crystallization thermograms are shown in **Figure 5.4**. The substrate, free DHA, had a crystallization range of 12.06 to -28.09^{o}C with two peaks at -25.81 and 10.97^{o}C whereas the other substrate, free GLA, only had a small single peak at -37.77^{o}C. The SLs also had larger

crystallization peaks. LDHA produced 4 crystallization peaks at 5.68, 12.62, 27.0, and 30.77^{o}C in a range of -1.24 to 32.06^{o}C whereas NDHA produced 3 crystallization peaks at 6.18, 12.45, and 29.79^{o}C in a range of -0.65 to 30.87^{o}C. The extra crystallization peak may be a result of the different relative percentages of TAG species that varied between NDHA and LDHA. The presence of multiple peaks in a DSC thermogram of a vegetable oil can be explained by the complexity of the TAG distribution (*40*). NGLA and LGLA both contained three crystallization peaks at approximately 5, 8, and 29°C with approximate range of -4 to 31°C. Both of these SLs had similar relative percentages of the major TAG species with only slight differences.

Two GLA SLs (NGLA and LGLA) were produced by enriching NSL and LSL with free GLA and their physical and chemical characteristics determined for possible use as HMF analogues. The optimal conditions for the production of the GLA SLs were a 1:0.5 substrate mole ratio, 12 h of reaction time at 65°C, catalysis by Lipozyme TL IM, and producing SLs with approximately 8-9% GLA with >57% *sn*-2 PA. These GLA SLs would provide the benefits of ARA because the bioconversion of GLA to ARA skips the rate-limiting desaturase step and ARA is important for brain growth and functional development in infants (*9*). Two DHA SLs (NDHA and LDHA) were produced by enriching NSL and LSL with free DHA, and their physical and chemical characteristics were desirable as HMF analogues. The optimal conditions for production of the DHA SLs were a 1:1 substrate mole ratio, 24 h of reaction time at 65°C, catalysis by Lipozyme TL IM, yielding SLs with approximately 11% DHA with >54% *sn*-2 PA. These DHA SLs would provide benefits to infants as DHA has been found to support brain and nervous system development and improve visual acuity (*8*). The composition of HMF consists of approximately 0.00-0.27% GLA, 0.23-0.75% ARA, and 0.15-0.56% DHA with an n-6/n-3 ratio of 7.31-21.13 (*12*). Our SLs contained more GLA and DHA than are found in HMF. However,

the higher amount of GLA may increase the amount of ARA when converted metabolically in the infant without the need to directly add preformed ARA. GLA helps to regulate the body's normal inflammatory response, whereas direct addition of ARA could lead to unbalanced production of inflammatory eicosanoids. Dihomo-γ-linolenic acid (an intermediate between GLA and ARA) competes with ARA to help balance the different eicosanoids produced (*41*). Also, most Americans have a high n-6/n-3 ratio that can result in health issues, and a mother with a high n-6/n-3 ratio in her diet may produce a high n-6/n-3 ratio in her breast milk. With an increase in DHA, the ratio would decrease and may possibly eliminate the health issues associated with a higher n-6/n-3 ratio. The large amount of PA at the *sn*-2 position of all four SLs will allow for better absorption of PA (*20*) and improved absorption of fat and calcium in infants (*16, 17*). The stability of these SLs can be increased by the addition of TBHQ at 200 ppm for possible use as HMF analogues in infant formula.

ACKNOWLEDGEMENTS

ABBREVIATIONS

LSL- SDA soybean oil enriched with palmitic acid catalyzed by Lipozyme TL IM from our previous research (*19*); LDHA- LSL enriched with DHA; LGLA- LSL enriched with GLA; NSL- SDA soybean oil enriched with palmitic acid catalyzed by Novozym 435 from our previous research (*19)*; NDHA- NSL enriched with DHA; NGLA- NSL enriched with GLA

REFERENCES

(1) Hamosh, M.; Bitman, J.; Wood, L.; Hamosh P.; Mehta, N.R. Lipids in milk and the first steps in their digestion. *Pediatrics*. **1985**, *75*, 146-150.

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

(3) Akoh, C.C.; Kim, B.H. Structured lipids. In *Food Lipids: Chemistry, Nutrition, and Biotechnology*, 3rd Ed. Akoh, C.C.; Min, D.B. Ed.; CRC Press: Boca Raton, FL, **2008**; pp. 841-856.

(4) Hammond, B.G.; Lemen, J.K.; Ahmed, G.; Miller, K.D.; Kirkpatrick, J.; Fleeman, T. Safety assessment of SDA soybean oil: results of a 28-day study and a 90-day/one generation reproduction feeding study in rats. *Regul. Toxicol. Pharmacol*. **2008**, *52*, 311-323.

(5) Gerster, H. Can adults adequately convert alpha-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? *Int. J. Vitam. Nutr. Res*. **1998**, *68*, 159-173.

(6) Haung, Y.S.; Smith, R.S.; Redden, P.R.; Cantrill, R.C.; Horrobin, D.F. Modification of liver fatty acid metabolism in mice by omega-3 and omega-6 Δ6-desaturase substrates and products. *Biochim. Biophys. Acta*. **1991**, 1082, 319-327.

(7) James, M.J.; Ursin, V.M.; Cleland, L.G. Metabolism of stearidonic acid in human subjects: comparison with the metabolism of other n-3 fatty acids. *Am. J. Clin. Nutr.* **2003**, *77*, 1140-1145.

(8) 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. *The Journal of Pediatrics.* **2003**, *143*, S17-S25.

(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) Horrobin, D.F.; Manku, M.S. Clinical biochemistry of essential fatty acids, In *Omega-6 Essential Fatty Acid: Pathophysiology and Roles in Clinical Medicine*, Horrobin, D.F., Eds; Alan R. Liss: New York, **1990**, pp. 21-53.

(11) Innis, S.M. Fatty acid requirements of the newborn. *Can. J. Physiol. Pharmacol.* **1994**, *72*, 1483-1492.

(12) López-López, A.; López-Sabater, M.C.; Campoy-Folgoso, C.; Rivero-Urgel, M.; Castellote-Bergalló, 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) 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, 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) 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.

(16) Iwasaki, Y.; Yamane, T. Enzymatic synthesis of structured lipids. *J. Mol. Catal. B. Enzym.* **2000**, *10*, 129-140.

(17) 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.

(18) 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.

(19) Teichert, S.A.; Akoh, C.C. Characterization of stearidonic acid soybean oil enriched with palmitic acid produced by solvent-free enzymatic interesterification. *J. Agric. Food Chem.* **2011***, 59*, 9588–9595.

(20) *Official Methods of Analysis of AOAC International*, 17th ed.; AOAC International: Gaithersburg, MD, **1998**; Official Method 996.01.

(21) Pina-Rodriguez, A.M.; Akoh, C.C. Enrichment of amaranth oil with ethyl palmitate at the sn-2 position by chemical and enzymatic synthesis. *J. Agric. Food Chem.* **2009**, *57*, 4657-4662.

(22) 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.

(23) *Official Methods and Recommended Practices of the American Oil Chemists' Society,* edited by D. Firestone, Champaign, **2009**; Official Methods Ac 5-41, Cj 1-94, Cd 8b-90, Cd 18-90, and Cd 12b-92.

(24) Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911-917.

(25) Shahidi, F.; Wanasundra, U.N. Methods for measuring oxidative rancidity in fats and oils. In *Food Lipids*, 3rd Ed.; Akoh, C.C., Min, D.B., Eds.; CRC Press: Boca Raton, FL, **2008**; pp 387-408.

(26) Akoh, C. C.; Xu, X. Enzymatic production of Betapol and other specialty fats. In *Lipid Biotechnology*; Kuo, C. C., Gardner, H.W., Eds.; Dekker: New York, **2002**; pp 461-478.

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

(28) Sahin, N; Akoh, C.C. Karaali, A. Enzymatic production of human milk fat substitutes containing γ-linolenic acid: Optimization of reactions by response surface methodology. J. Am. Oil Chem. Soc. **2005**, *82*, 549-557.

(29) 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.

(30) 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.

(31) Marangoni, A.G. Lipases: structure, function and properties. In *Lipid Biotechnology*; Kuo, T.M.; Gardner, H.W., Ed.; Marcel Dekker: New York, NY, **2002**; pp. 357-386.

(32) Turan, S.; Karabulut, I.; Vural, H. Influence of sn-1,3-lipase-catalysed interesterification on the oxidative stability of soybean oil-based structured lipids. *J. Sci. Food Agric.* **2007**, *87*, 90-97.

 (33) O'Keefe, S.F.; Pike, O.A. Fat characterization. In *Food Analysis*, 4th Ed.; Nielsen, S.S., Ed.; Springer: New York, NY, **2010**; pp. 239-260.

 (34) Akoh, C.C.; Moussata, C.O. Characterization and oxidative stability of enzymatically produced fish and canola oil-based structured lipids. *J. Am. Oil Chem. Soc.* **2001**, *78*, 25-30.

(35) Akoh, C.C. Oxidative stability of fat substitutes and vegetable oils by the oxidative stability index. *J. Am. Oil Chem. Soc.* **1994**, *71*, 211-215.

(36) Azadmard-Dmirchi, S.; Dutta, P.C. Stability of minor lipid components with emphasis on phytosterols during chemical interesterification of a blend of refined olive oil and palm stearin. *J. Am. Oil Chem. Soc.* **2008**, *85*, 13-21.

(37) Wang, T.; Jiang, Y. Hammond, E.G. Effect of randomization on the oxidative stability of corn oil. *J. Am. Oil Chem. Soc.* **2005**, *82*, 111-117.

(38) Coppen, P.P. The use of antioxidants. In *Rancidity in Foods,* 3rd Ed.; Allen, J.C. and Hamilton, R.J. Ed.; Aspen Publishers: New York, NY, **1999**; pp.84-98.

(39) Pina-Rodriguez, A.M.; Akoh, C.C. Composition and oxidative stability of a structured lipid from amaranth oil in a milk-based infant formula. *J. Food Sci*. **2010**, *75*, C140-C146.

(40) Tan, C. P.; Che Man, Y. B. Differential scanning calorimetric analysis of palm oil, palm oil based products and coconut oil:effects of scanning rate variation. *Food Chem.* **2002**, *76*, 89-102.

(41) Russo, G.L. Dietary n-6 and n-3 polyunsaturated fatty acids: From biochemistry to clinical implications in cardiovascular prevention. *Biochem. Pharmacol.* **2009**, *77*, 937-946.

FA	NSL[a]		LSL[a]		free DHA[b]	free GLA[b]
	total	*sn*-2	total	*sn*-2		
C12:0	ND	ND	ND	ND	4.39 ± 0.10	ND
C14:0	1.21 ± 0.02	ND	1.18 ± 0.01	ND	9.39 ± 0.18	ND
C16:0	62.55 ± 0.33	60.84 ± 2.26	61.22 ± 1.45	60.63 ± 1.54	6.87 ± 0.12	0.07 ± 0.02
C18:0	2.93 ± 0.04	4.34 ± 1.68	2.88 ± 0.08	3.10 ± 0.07	0.86 ± 0.02	0.06 ±0.04
C18:1n-9	6.11 ± 0.10	8.32 ± 0.47	6.53 ± 0.17	7.07 ± 0.07	27.78 ± 0.46	1.14 ± 0.97
C18:2n-6	10.34 ± 0.13	12.39 ± 0.55	10.83 ± 0.46	11.49 ± 0.26	1.35 ± 0.04	28.15 ± 0.51
C18:3n-6	2.89 ± 0.03	3.28 ± 0.20	3.01 ± 0.15	4.03 ± 0.18	0.14 ± 0.02	69.28 ± 1.61
C20:1n-9	4.73 ± 0.04	4.24 ± 0.19	4.90 ± 0.21	4.50 ± 0.39	0.17 ± 0.04	0.39 ±0.05
C18:4n-3	8.15 ± 0.13	6.59 ± 0.80	8.38 ± 0.46	8.82 ± 1.33	ND	ND
C21:0	0.12 ± 0.01	ND	0.13 ± 0.01	ND	ND	0.59 ± 0.01
C22:6n-3	ND	ND	ND	ND	44.03 ± 1.12	ND

Table 5.1 Fatty Acid Profiles of the Substrates, NSL, LSL, free GLA, and free DHA (Mole Percent)

[a]Data from previous research (*19*). [b]Free indicates that all FAs involved are in FFA form and GLA or DHA indicates the major FA found in the substrate. [c]ND = not detected. Traces of C8:0, C10:0, C18:3n-3, and C22:0 were also detected in very small amounts.

sample	mole ratio	time (h)	total DHA (mol %)	*sn*-2 PA (mol %)
NDHA	1:1	12	6.27 ± 0.74	50.68 ± 2.86
NDHA	1:2	12	9.41 ± 0.68	42.29 ± 1.86
NDHA	1:3	12	11.48 ± 1.07	44.46 ± 1.77
NDHA	1:1	24	10.27 ± 0.24	59.75 ± 2.13
NDHA	1:2	24	15.35 ± 0.52	48.62 ± 1.08
NDHA	1:3	24	14.77 ± 0.70	41.13 ± 0.35
LDHA	1:1	12	6.20 ± 2.42	50.15 ± 2.41
LDHA	1:2	12	9.36 ± 0.85	45.48 ± 1.91
LDHA	1:3	12	10.08 ± 0.19	45.65 ± 2.60
LDHA	1:1	24	10.06 ± 0.91	55.47 ± 6.98
LDHA	1:2	24	15.79 ± 1.88	43.03 ± 1.85
LDHA	1:3	24	16.31 ± 0.68	42.03 ± 1.02

Table 5.2 Small-Scale Acidolysis Products of SLs and free DHA (~40% DHA)

Mean ± SD, n=3. NDHA = Novozym 435 SL (NSL) (*19*): free DHA; LDHA = Lipozyme TL IM SL (LSL) (*19*): free DHA.

sample	mole ratio	time (h)	total GLA (mol %)	sn-2 PA (mol %)
NGLA	1:0.5	12	9.86 ± 1.24	54.46 ± 3.97
NGLA	1:1	12	12.33 ± 0.25	52.15 ± 0.87
NGLA	1:1.5	12	15.58 ± 0.94	45.49 ± 1.64
NGLA	1:0.5	24	12.80 ± 2.29	49.13 ± 2.78
NGLA	1:1	24	15.90 ± 1.06	48.97 ± 2.04
NGLA	1:1.5	24	19.65 ± 2.04	48.94 ± 1.42
LGLA	1:0.5	12	9.78 ± 1.02	51.74 ± 3.53
LGLA	1:1	12	13.45 ± 0.97	49.41 ± 1.78
LGLA	1:1.5	12	15.22 ± 1.61	46.08 ± 1.96
LGLA	1:0.5	24	11.90 ± 1.00	49.85 ± 1.37
LGLA	1:1	24	17.58 ± 0.66	46.23 ± 0.89
LGLA	1:1.5	24	22.68 ± 1.03	44.82 ± 0.77

Table 5.3 Small-Scale Acidolysis Products of SLs and free GLA (~70% GLA)

Mean ± SD, n=3. NGLA = Novozym 435 SL (NSL) (*19*): free GLA; LGLA = Lipozyme TL IM SL (LSL) (*19*): free GLA.

Table 5.4 FA Composition (mol %) of Scaled-up SLs at Optimal Conditions

FA	total					sn-2				
	IF	NGLA	LGLA	NDHA	LDHA	IF	NGLA	LGLA	NDHA	LDHA
C8:0	1.81±0.02a	ND	ND	ND	ND	ND	ND	ND	ND	ND
C10:0	1.33±0.02c	ND	ND	0.26±0.01a	0.25±0.01a	ND	ND	ND	ND	ND
C12:0	9.16±0.09a	ND	ND	1.03±0.04b	0.98±0.02b	22.31±0.87c	ND	ND	0.89±0.15a	0.85±0.04a
C14:0	4.35±0.05d	1.16±0.03b	1.09±0.00c	3.10±0.03a	3.08±0.02 a	4.61±0.52c	1.17±0.01b	1.11±0.05b	3.12±0.27 a	3.02±0.06a
C14:1	0.03±0.00a	ND	ND	ND	ND	ND	ND	ND	ND	ND
C15:0	0.05±0.00a	ND	ND	ND	ND	ND	ND	ND	ND	ND
C16:0	21.80±0.56d	56.07±0.95a	56.13±0.86a	47.91±1.72 b	50.17±1.06c	19.97±1.30c	57.35±1.79a	58.53±1.33ab	55.01±0.79b	54.73±1.11b
C16:1n-7	0.03±0.00c	ND	ND	ND	0.71±0.02a	ND	ND	ND	ND	0.62±0.02a
C17:0	0.33±0.01d	ND	0.03±0.05;c	1.25±0.05a	0.12±0.01b	ND	ND	0.33±0.07a	0.24±0.13a	0.24±0.04a
C18:0	0.12±0.03c	2.75±0.01a	2.68±0.02a	2.28±0.33ab	1.59±0.99 b	2.85±0.38a	2.77±0.10a	2.78±0.10a	2.68±0.17a	2.76±0.12a
C18:1n-9	34.60±1.05c	6.66±0.16b	6.57±0.10b	11.53±0.60a	11.24±0.14a	30.84±1.18c	6.58±0.33b	6.16±0.18b	9.57±0.90a	9.88±0.27a
C18:2n-6	20.62±0.62c	12.39±0.30a	12.15±0.25a	8.18±0.31b	8.49±1.29b	17.91±1.19c	12.40±0.80a	11.50±0.32a	7.73±0.25b	7.42±0.18b
C20:0	0.34±0.01b	0.14±0.01a	0.14±0.01a	0.13±0.01a	0.14±0.01a	ND	0.17±0.02a	0.17±0.01a	0.15±0.01a	0.17±0.02a
C18:3n-6	0.14±0.01d	8.39±0.19a	9.12±0.26b	2.44±0.11c	2.34±0.05c	ND	6.37±0.26a	6.90±0.18b	2.72±0.03c	2.76±0.10c
C20:1n-9	0.26±0.01c	0.17±0.01a	0.17±0.00a	0.09±0.01b	0.09±0.00b	3.50±0.90d	0.13±0.01a	0.14±0.01a	0.03±0.04b	0.07±0.01c
C18:3n-3	1.93±0.05d	4.26±0.10a	4.14±0.10a	3.50±0.14b	3.33±0.06c	ND	4.29±0.27a	4.02±0.23a	3.23±0.22b	3.08±0.09b
C18:4n-3	ND c	7.89±0.20a	7.64±0.22a	6.82±0.29b	6.46±0.15 b	ND	8.44±0.55a	8.36±0.50a	7.23±0.60b	6.88±0.36b
C22:0	0.23±0.01a	0.10±0.08c	0.14±0.01bc	0.21±0.00ab	0.23±0.01a	ND	ND	ND	ND	ND
C20:3n-6	0.07±0.00a	ND	ND	ND	ND	ND	ND	ND	ND	ND
C20:4n-6	0.75±0.02a	ND	ND	ND	ND	ND	ND	ND	ND	ND
C24:0	0.15±0.10a	ND	ND	ND	ND	ND	ND	ND	ND	ND
C22:6n-3	0.52±0.01d	ND	ND	11.27±0.42a	10.71±0.35b	ND	ND	ND	6.81±0.64b	7.51±0.47b
n-6/n-3	8.81±0.08d	1.71±0.00a	1.81±0.01b	0.49±0.00c	0.53±0.05c					

Mean ± SD, n=3. Means with the same letter in the same row and category (i.e. sn-2 or total) are not significantly different (P≤0.05);

Abbreviations: IF, infant formula of Nestle Good Start® Supreme with DHA and ARA; NGLA, NSL:free GLA; LGLA, LSL:free

GLA; NDHA, NSL:free DHA; LDHA, LSL:free DHA; ND, not detected.

	NGLA		LGLA		NDHA		LDHA	
Table 5.5 Free Fatty Acid Percentage and Tocopherol Content (Before and After Short-Path Distillation) of SLs								
FFA (%)	0.11 ± 0.01b		0.01 ± 0.00c		0.15 ± 0.00a		0.14 ± 0.02a	
Toco (ppm)	before	after	before	after	before	after	before	after
α-T	38.10 ± 2.83a	27.65 ± 6.14c	35.78 ± 0.77ab	26.23 ± 5.60c	30.79 ± 5.05bc	26.65 ± 0.62c	26.29 ± 0.76c	26.75 ± 0.12c
α-T3	13.04 ± 1.52a	ND	17.42 ± 0.28b	ND	ND	ND	ND	ND
β-T	ND	ND	ND	ND	46.94 ± 13.36a	ND	47.67 ± 5.28a	ND
γ-T3	122.28 ± 12.77a	43.69 ± 2.00c	86.60 ± 7.97b	23.02 ± 1.82d	119.81 ± 7.87a	21.72 ± 0.65d	117.51 ± 10.32a	22.21 ± 2.72d
δ-T	58.30 ± 12.07a	5.23 ± 2.38ef	46.53 ± 6.55b	1.52 ± 0.56f	32.36 ± 1.00c	16.58 ± 0.81d	33.37 ± 4.54c	13.69 ± 1.52de

Mean ± SD, n=3. Means with the same letter in the same row are not significantly different (P≤0.05). Abbreviations: Toco, tocopherol content; T, tocopherol; T3, tocotrienol; ND, not detected.

sample	OSI (h)
Table 5.6 Effect of tert-Butylhydroxyquinone (TBHQ) on OSI Values (at 110°C) of SLs	
NDHA	1.10 ± 0.07f
NDHA 100 ppm	1.93 ± 0.24def
NDHA 200 ppm	2.98 ± 0.11cd
LDHA	1.18 ± 0.04f
LDHA 100 ppm	2.30 ± 0.21cdef
LDHA 200 ppm	$3.35 \pm\ 1.43$bc
NGLA	$3.22 \pm\ 0.20$bc
NGLA 100 ppm	$4.35 \pm\ 0.14$b
NGLA 200 ppm	$5.55 \pm\ 0.14$a
LGLA	$1.48 \pm\ 0.04$ef
LGLA 100 ppm	$2.18 \pm\ 0.11$cdef
LGLA 200 ppm	$2.68 \pm\ 0.25$cde

Mean $\pm$ SD, n-=3. Means with the same letter are not significantly different (P≤0.05). OSI, oxidative stability index.

TAG	relative %			
	NGLA	LGLA	NDHA	LDHA
StGSt	2.09	0.95	2.83	5.02
StLnLn/StGG	0.93	1.67	0.98	1.52
StLSt	1.73	2.32	0.59	1.28
StPSt	4.37	4.97	8.39	12.22
StOSt/LnGSt	6.81	6.14	10.38	14.35
LLG	1.19	0.81	ND	ND
DPD	ND	ND	2.68	0.66
OPD	ND	ND	1.76	0.46
OOD	ND	ND	1.74	0.52
GGO	0.98	1.18	ND	ND
OLLn	3.42	4.85	1.29	1.08
LnPL	ND	ND	1.42	1.18
LnGSt	0.53	0.69	0.78	0.65
OLL	0.39	0.58	1.37	1.74
LPL	8.17	8.59	6.55	6.52
GLS	1.10	1.03	3.23	3.53
LnPO	2.37	1.89	1.93	1.95
OOL	4.52	5.76	0.39	0.29
LPO	0.92	1.02	4.57	0.99
PPL	11.88	11.88	12.51	6.42
PPM	0.73	0.44	2.30	2.25
OPO	1.18	1.16	1.48	2.06
PPO	6.89	7.38	9.68	10.88
PPP	37.75	34.78	23.15	23.94
SPS/SPO	2.07	1.92	ND	0.48

Table 5.7 Relative Percentages of TAG Molecular Species via RP-HPLC

The TAG species do not reflect stereochemical configuration, Ln, α-linolenic acid; O, oleic acid; D, docosahexaenoic acid; P, palmitic acid; S, stearic acid; St, stearidonic acid; ND, not detected.
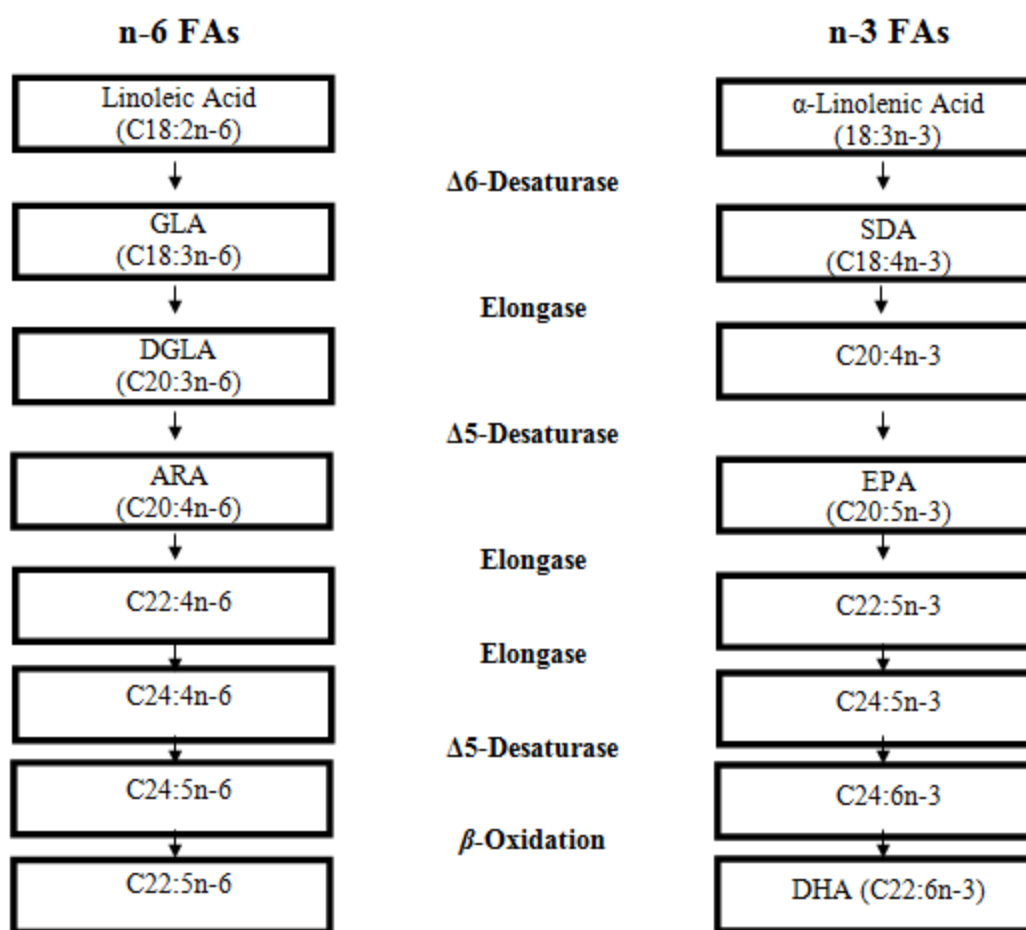
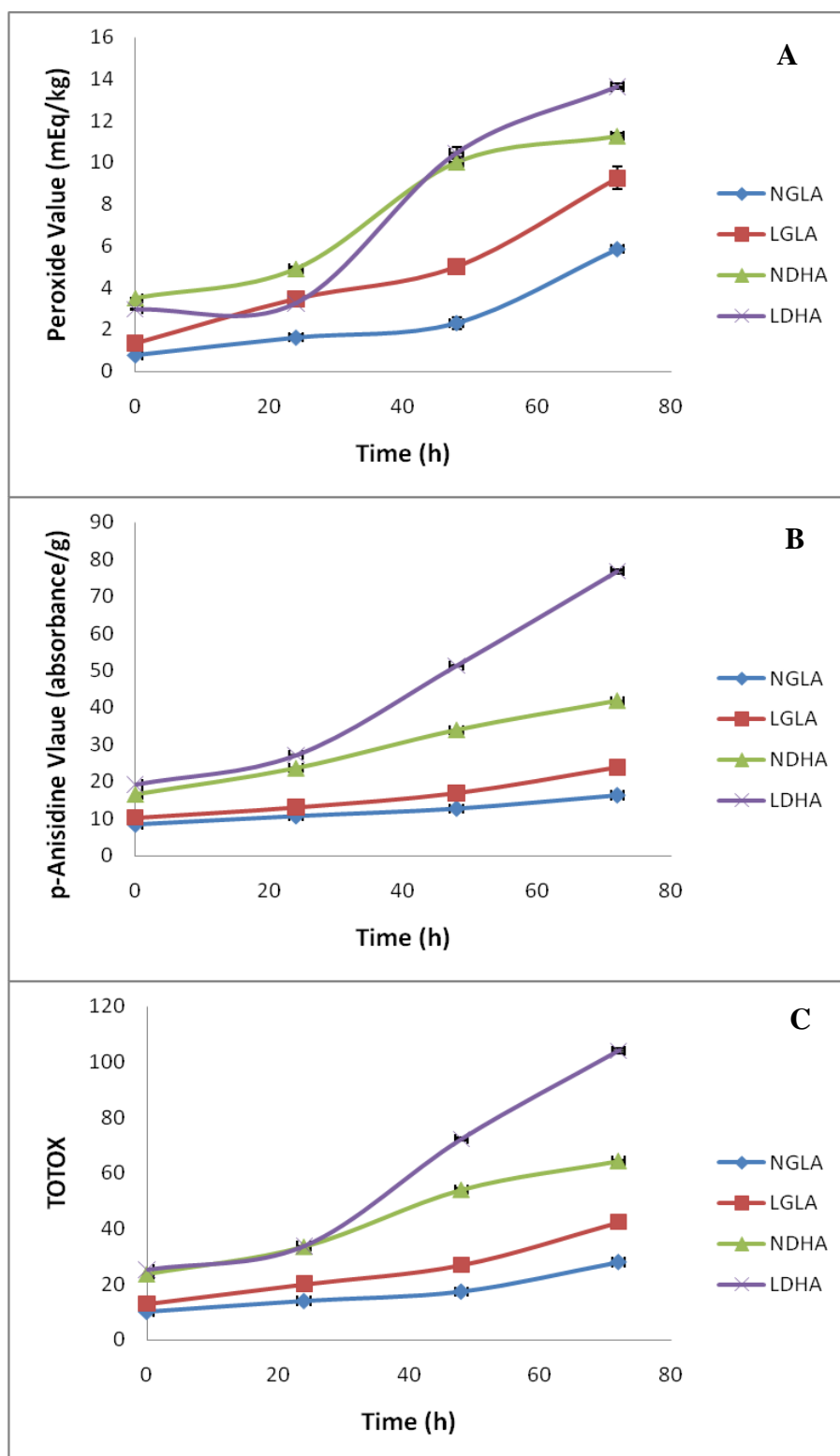**Figure 5.1** Scheme for the bioconversion of omega FAs.

Figure 5.2 The (A) peroxide values, (B) *p*-anisidine values, and (C) total oxidation values

(TOTOX) of NDHA (▲), LDHA (×), NGLA (♦), and LGLA (■) over a period of 72 h.
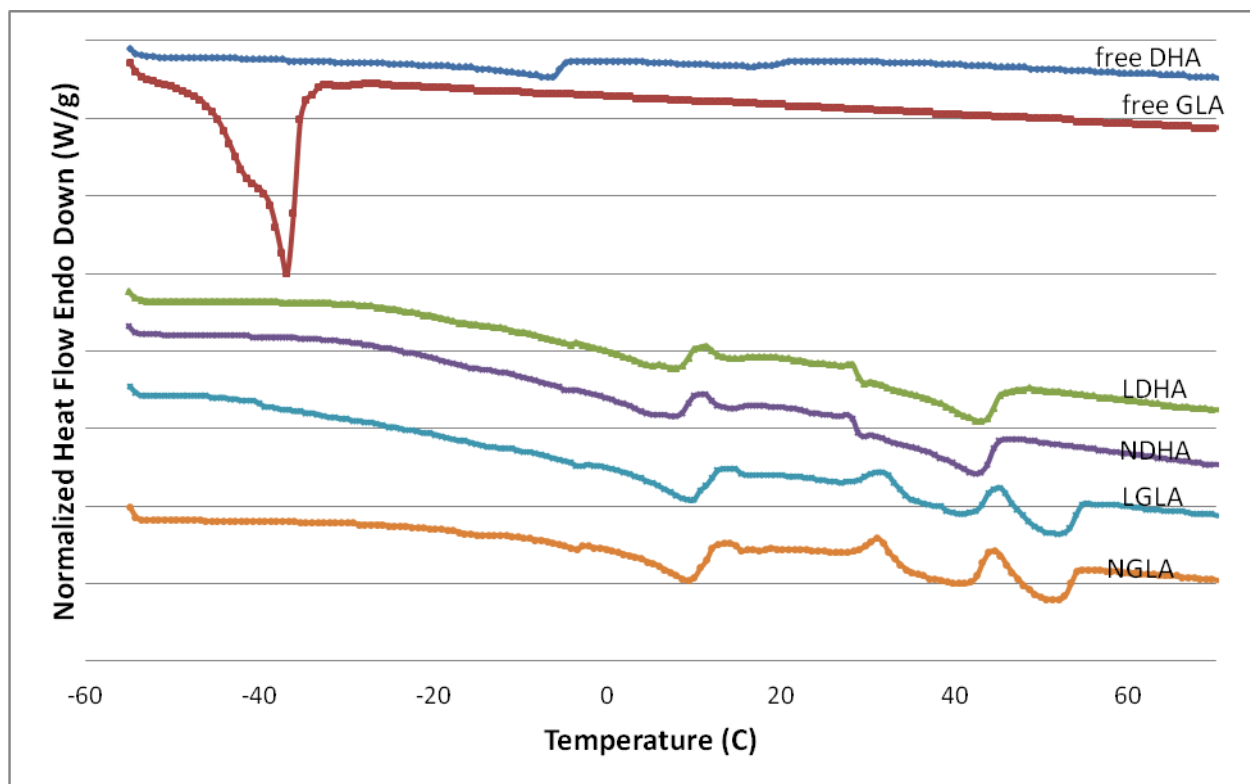
Figure 5.3 DSC melting curves of free DHA, free GLA, NDHA, LDHA, NGLA, and LGLA.
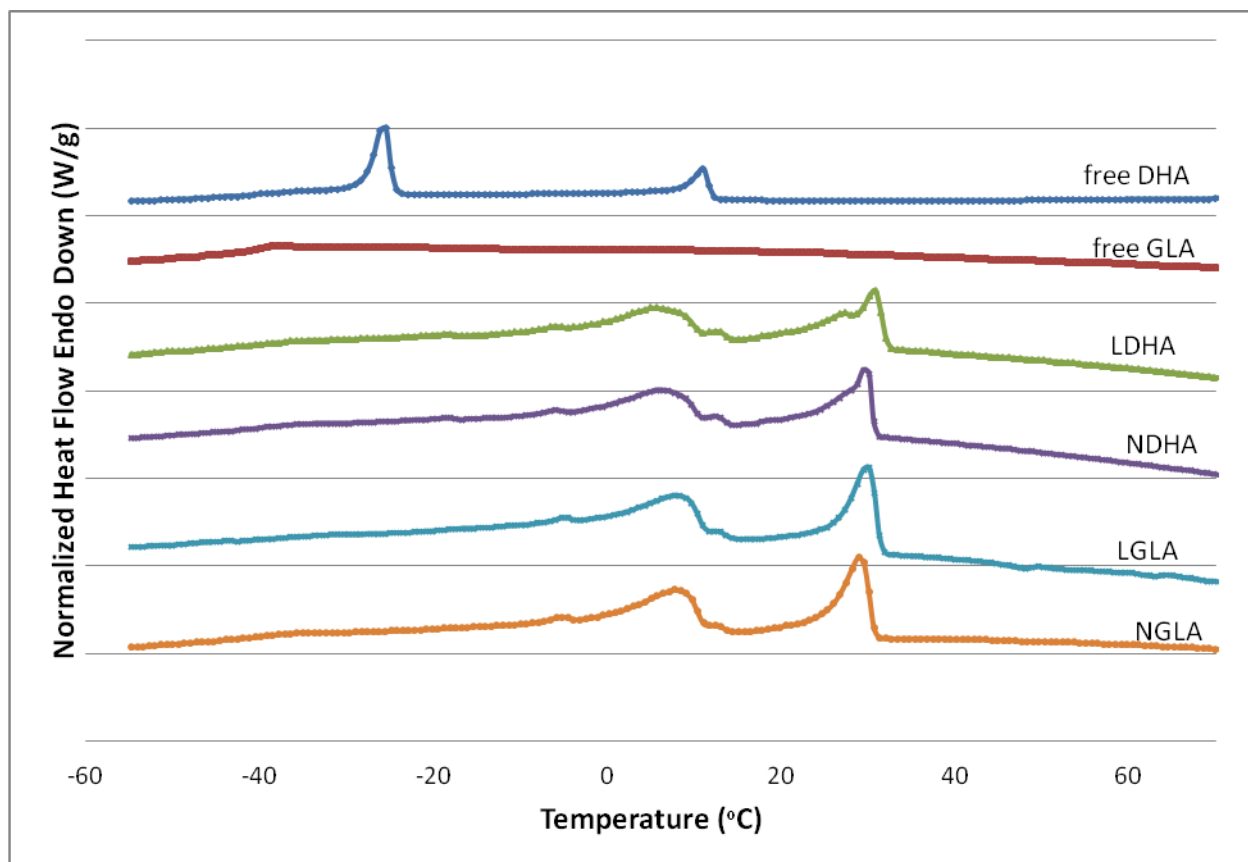
Figure 5.4 Crystallization curves using DSC for free DHA, free GLA, NDHA, LDHA, NGLA, and LGLA.

CHAPTER 6

CONCLUSIONS

Structured lipids (SLs) were successfully produced for possible use as human milk fat (HMF) analogs in infant formula. Stearidonic acid (SDA) soybean oil was enriched with palmitic acid (PA) by enzymatic interesterification in hexane. Since HMF contains over 60% of PA esterfied at the sn-2 position, the goal of this study was to produce SLs with over 60% sn-2 PA while retaining some of the SDA content in the triacylglycerol (TAG). Response surface methodology (RSM) was used to produce a model to predict the sn-2 PA and total SDA contents at various conditions. The optimal conditions were determined to be 1:2 substrate mole ratio of SDA soybean oil to tripalmitin at 50°C for 18 h with 10% (by weight) of sn-1,3 specific lipase, Lipozyme TL IM, whereas the optimal conditions for the nonspecific lipase, Novozym 435, were 1:2 substrate mole ratio at 50°C for 15.6 h. The Lipozyme TL IM SL (LSL) contained 6.82 ± 1.87% total SDA and 67.19 ± 9.59% sn-2 PA whereas the Novozyme 435 SL (NSL) contained 8.01 ± 2.41% total SDA and 64.43 ± 13.69% sn-2 PA.

In order to produce the SLs in a solvent-free environment, the optimal temperature was increased to 65°C (temperature where tripalmitin is a liquid) and the other optimal conditions were determined using the RSM model. The optimal conditions were determined to be a 1:2 substrate mole ratio at 65°C for 18 h for both lipases. LSL and NSL were scaled-up using the new optimal conditions and resulted in over 60% sn-2 PA and over 8% total SDA. The SLs were chemically and physically characterized. NSL was the least oxidatively stable followed by LSL

and then SDA soybean oil possibly due to the decrease in tocopherol contents. The melting profiles of the SLs were similar, but the crystallization profile differed possibly due to the difference in TAG species. The main TAG species in the SLs was found to be tripalmitin (PPP) at around 50%.

NSL and LSL were further enriched with either γ-linolenic acid (GLA) free fatty acid (FFA) or docosahexanoic acid (DHA) FFA by acidolysis catalyzed by a *sn*-1,3 lipase, Lipozyme TL IM. A small-scale analysis determined the optimal conditions as 1:1 substrate mole ratio of NSL/LSL to DHA FFA at 65°C for 24 h and a 1:0.5 substrate mole ratio of NSL/LSL to GLA FFA at 65°C for 12 h to retain a high amount of *sn*-2 PA and approximately 10% GLA or DHA. The SLs of NSL:DHA (NDHA), LSL:DHA (LDHA), NSL:GLA (NGLA) and LSL:GLA (LGLA) were chemically and physically characterized. All four of the SLs contained over 54% PA at the *sn*-2 position with over 8% GLA or over 10% DHA. The addition of antioxidants increased the oxidative stabilities of the SLs with NGLA being more oxidatively stable due to a higher initial tocopherol content compared to the other SLs. The iodine values were higher for the DHA SLs compared to the GLA SLs indicating a higher degree of unsaturation due to the incorporation of DHA. The melting and crystallization profiles differed between the DHA SLs and GLA SLs but did not vary between the SLs with the same substrate fatty acid (FA). The TAG species of the SLs differed significantly between the DHA SLs and differed slightly between the GLA SLs. HMF analogs were produced to retain a high PA content at the *sn*-2 position (over 54%), 6-8% SDA, and over 8% GLA or over 10% DHA for possible use in infant formula.

Many future studies could evolve from this research. One possible study could involve the application of these SLs into experimental infant formula and comparing the FA composition

and oxidative stability to the fats in commercial infant formula and human breast milk. This would provide insight into a real world application and to find out if it compares with infant formulas already on the market and more importantly to human breast milk. Another study could analyze and compare other natural antioxidants to stabilize the SLs that could also provide additional nutritional aspects. An example of a natural antioxidant with additional health benefits could be rosemary extract. Many consumers are now more concerned about what is being added into the food they purchase and many prefer finding natural ingredients on the label instead of finding chemical sounding names. Also, re-evaluating at the short-path distillation process could be very informative. The waste from short-path distillation could be analyzed to determine if the original antioxidants are removed or if they changed structurally and functionally. This analysis could determine if the antioxidants are becoming pro-oxidants and in turn are causing more harm than good. Another appropriate study would be analyzing the metabolism, absorption, and nutritional characteristics in a group of rats fed either commercial infant formula, human milk, or formula containing the SLs produced. This could determine if the percentage of GLA in the formula is adequate to supply the infant with enough ARA and if SDA is readily converted to EPA during metabolism. These additional studies could help in producing an improved HMF analog and an improved infant formula.