SYNTHESIS AND MICROENCAPSULATION OF STRUCTURED LIPIDS CONTAINING LONG-CHAIN POLYUNSATURATED FATTY ACIDS FOR POSSIBLE APPLICATION IN INFANT FORMULAS

by

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(Under the Direction of Casimir C. Akoh)

ABSTRACT

It has been well established that lower fat absorption in infants, fed with formula, is due to the difference between the regiospecificity of the palmitic acid in the vegetable oil blend versus that present in human milk fat (HMF). In HMF, most of the palmitic acids are esterified at the sn-2 position of the triacylglycerols (TAGs). However, in vegetable oil, they are predominantly present at the sn-1, 3 positions. Structured lipids (SLs), with fatty acid profiles and positional distributions similar to HMF, were developed via a single step enzymatic modification of either palm olein or tripalmitin. These SLs also were also enriched with long-chain polyunsaturated fatty acids (LCPUFAs), such as docosahexaenoic (DHA) and arachidonic (ARA), which are important for infant brain and cognitive development. SL from modified tripalmitin contained 17.69% total ARA, 10.75% total DHA, and 48.53% sn-2 palmitic acid. Response surface methodology was employed to study the effect of time, temperature, and substrate molar ratio on the incorporation of palmitic acid at the sn-2 position, and the total incorporation of LCPUFAs. Physicochemical properties of SLs were
characterized for TAGs molecular species, melting and crystallization thermograms, and fatty acid positional distribution. SLs in powdered form were obtained by microencapsulation, using Maillard reaction products (MRPs) of heated protein-polysaccharide conjugates as encapsulants, followed by spray-drying. SL-encapsulated powders had high microencapsulation efficiency and oxidative stability. Infant formula prepared with microencapsulated SL in a dry-blending method, had a better oxidative stability and color quality than the formula prepared with non-microencapsulated SL in a wet-mixing/spray-drying method.

INDEX WORDS: infant formula, structured lipid, lipase, enzymatic modification, microencapsulation, LCPUFAs
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DEDICATION

To my wonderful parents and family.
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CHAPTER 1

INTRODUCTION

Enzymatic interesterification has been applied to modify the properties of fats and oils for food and other applications. The resulting products, known as structured lipids (SLs), are altered in their fatty acid composition, and/or fatty acid positional distribution, to achieve desirable chemical and physical properties. SLs have been used in infant formulas, for example Betapol®, as a fat source that is similar to human milk fat (HMF) in palmitic acid content and positional distribution. In HMF, palmitic acid accounts for about 25% of the total fatty acids, and greater than 60% of these are attached at the sn-2 position of the triacylglycerols (TAG) (Innis and others 1995). It is well known that this unique regiospecificity is responsible for higher fat absorption in infants fed on breast milk. In contrast to HMF, infant formula fats, composed of vegetable oils, contain most of the palmitic acids at the sn-1, 3 positions (Innis and others 1995). The digestion of infant formula fats releases free palmitic acids, which tend to form insoluble complexes with calcium, lowering the fat absorption (Lien 1994). Enzymatic interesterification could improve the fat absorption by rearranging palmitic acids in vegetable oils to the preferred sn-2 position.

Long-chain polyunsaturated fatty acids (LCPUFAs) including docosahexaenoic acid (DHA C22:6 n-3), and arachidonic acid (ARA C20:4 n-6), are important in neonatal development of the brain and retina. DHA and ARA constitute the majority of the fatty acids in the brain, aiding in the development and protection of neurological functions
(Koletzko and others 2008; Ryan and others 2010). Most traditional infant formulas rely on the bioconversion of linoleic, and alpha-linolenic acids to LCPUFAs, however, the rates for these conversions are very low (Burdge and others 1998). An alternative approach would be to incorporate these beneficial LCPUFAs directly into infant formula. SLs produced for infant formula use could therefore directly incorporate the beneficial LCPUFAs, to increase their nutritional value.

Lipid oxidation is a major deterioration process, causing reduced stability, and formation of off-flavors that negatively affect shelf-life quality and consumer acceptance of the food product. LCPUFAs are susceptible to lipid oxidation, as they are highly unsaturated. Microencapsulation technology has been employed to overcome oxidative issues in food products. The technology involves packaging of a core (or mixture of active components) within a secondary material (encapsulant or coat) to form small particles that protect sensitive ingredients from oxidation, and enable delivery into foods (Augustin and Hemar 2009)

The goal of the present research was to explore the possibility of SLs application in infant formula. This approach would provide a fat source that has a similar fatty acid profile, and positional distribution, to HMF, but that delivers a higher amount of beneficial LCPUFAs. To achieve this goal, the following specific aims were developed and completed.

**Aim 1: Optimization of the reaction conditions, and blending ratios, of substrates for the synthesis of SLs.** The effects of substrate mole ratio, incubation time, temperature, and type of substrate, were determined using response surface methodology (RSM). Three SLs were developed as follows:
Design 1. SL produced from palm olein and a mixture of concentrated ARA and DHA. The SL from this design is called PDA-SL (in Chapter 3).

Design 2. SL produced from palm olein and mixture of FFAs obtained from gamma linolenic acid (GLA)-rich borage oil, and from DHASCO. The SL from this design is called PDG-SL (in Chapter 4).

Design 3. SL produced from tripalmitin and a mixture of FFAs, or a mixture of free fatty acid ethyl esters (FAEEs). Both the FFAs and FAEEs were obtained from ARASCO® and DHASCO® (ARA-rich and DHA-rich single cell oils). The SL from this design is called TDA-SL (in Chapter 5).

Aim 2: SL characterization and application in infant formula. The PDG- and TDA-SLs were characterized in terms of FA positional distribution, TAG composition profile, and thermal properties. In addition, TDA-SL (in Chapter 5) was formulated with other ingredients to produce a prototype powdered infant formula using wet-mixing/spray-drying, and dry blending processes. To determine the suitability of these methods, the resulting products were evaluated for their oxidative stability and color quality.

Aim 3: Microencapsulation of SLs using Maillard reaction products as encapsulants. For dry blending application, PDG- and TDA-SLs were encapsulated to obtain a powdered form, using heated whey protein isolate, and corn syrup solid conjugates, as encapsulants, and spray-drying method. TDA-SL powder was employed in the production of dry-blending processed infant formula (Aim 2). Both SL powders were characterized in terms of microencapsulation efficiency, chemical and physical properties, oxidative stability, and dispersibility (in Chapter 6).
REFERENCES


CHAPTER 2
LITERATURE REVIEW

Production of structured lipids (SLs) for infant formula application

Human milk fat (HMF) consists of about 20-25% palmitic acid, with greater than 60% of all palmitic acid esterified at the sn-2 position of the milk triacylglycerols (TAGs). HMFs have higher fat absorption compared to infant formula fat, despite the similarities between their fatty acid profiles (Lien 1994). Lipolysis occurs predominantly at the sn-1, 3 positions, yielding two free fatty acids (FFAs) and a 2-monoacylglycerol (2-MAG). Higher absorption of HMFs is explained by the unique TAG structure of HMF, which mainly consists of 2-monopalmitate; the well-absorbed form of palmitic acid formed after dietary fat digestion (Innis and others 1994). In contrast, vegetable oils used as infant formula fats contain mostly palmitic acids at the sn-1, 3 positions, with only 5-20% of their palmitic acid present at the sn-2 position (Mattson and Lutton 1958). Digestion of infant formula fats releases palmitic acid mainly as FFAs, which tend to bind calcium and form insoluble calcium soaps in the intestine, lowering the fat absorption and possibly causing constipation.

With the importance of palmitic acid at the sn-2 position being emphasized, different approaches have been studied to produce high sn-2 palmitic acid SLs for applications in infant formula (Chen and others 2004; Pina-Rodriquez and Akoh 2009; Shimada and others 2000). Enzymatic modification, in contrast to chemical catalysis, provides the advantages of better selectivity and milder reaction conditions (Schmid and Verger
1998). In addition, chemical catalysis requires fully-refined and anhydrous oils, and often further purification steps that lead to a higher energy cost (Holm and Cowan 2008). In the following section, enzymes, lipid substrates, synthesis procedure, and methods of evaluation of the product, are discussed.

**Lipases**

Lipases, or TAG hydrolases (EC 3.1.1.3) catalyze the hydrolysis of fats and oils to release FFAs, diacylglycerols (DAGs), monoacylglycerol (MAGs), and glycerol (Jaeger and others 1999; Wolley and Petersen 1994). These enzymes are also capable of synthesis reactions, such as esterification, acidolysis, alcoholysis, and inter-esterification (Schmid and Verger 1998). The modification of fats and oils by lipases leads to a redistribution of FAs over the three positions of the TAG backbone, as well as to a change in FA composition, ultimately altering the physical and nutritional properties of the lipid substrates. The enzymes are ubiquitous and produced by several plants, mammals, and microorganisms. The lipases from *Rhizomucor miehei, Humicola lanuginosa* (now *Thermomyces lanuginosus*), *Pseudomonas glumae, Candida Antarctica* B, and *Candida rugosa* are widely used in the food industry. The selectivity of lipases may depend on the type of substrate and the position in TAG backbone. Kleiner and others (2012) demonstrated that the substrate-selective lipase Lipomod™ 34P-L034P from *Candida cylindracea [rugosa]* has specificity for stearidonic acid (SDA, 18:4 ω-3), while Lipase AY 30 from *Candida rugosa* discriminates against it. *Sn*-1,3-regiospecific lipase preferably catalyzes reactions on the external (*sn*-1 and *sn*-3) positions of the glycerol backbone, although under certain conditions acyl migration can also occur. Examples of other regiospecific lipases include: *Rhizopus oryzae* lipase, *Rhizomucor*
Stereospecific lipases have the ability to preferentially hydrolyze either the \textit{sn}-1 or \textit{sn}-3 positions of TAGs. For example, the lipase from \textit{Carica papaya} latex has been demonstrated to have a specificity for the \textit{sn}-3 position (Villeneuve and others 1997). Parameters, such as reaction time, temperature, type of acyl donor, pH, water activity, enzyme load, and substrate ratios are important factors affecting lipase activity and the final product composition (Fickers and others 2008; Noel and Combes 2003; Xia and others 2009). Lumor and Akoh (2005) reported a higher level of gamma linolenic acid (GLA) incorporation by Lipozyme RM IM when FFAs were used as the acyl donor in the reaction (acidolysis) compared to fatty acid ethyl esters (FAEEs) (interesterification). Selection of the lipase used in the esterification process is an important step, as it can permit the manipulation of the positional distribution and incorporation of fatty acids in TAG structure.

\textbf{Lipid substrates in the production of SLs for infant formula application}

Studies have shown that FA composition and distribution are important aspects in infant formula. To mimic the FA profile and positional distribution of HMF, suitable starting materials from natural fats and oils are essential in the production of SLs. Table 2.1 shows the major TAGs present in various fats and oils studied in the production of SL for infant formula. Few natural fats and oils contain palmitic acid at the preferred \textit{sn}-2 position, for example lard and palm stearin. Production using these lipid substrates results in glycerol backbones with \textit{sn}-2 palmitic acid. However, due to some religious constraints, lard is not generally used in infant formulas (Benson and others 1992). Palm stearin is obtained after dry-fractionation of palm oil and contains 12-56\% of tripalmitin (Pantzaris 1994). Both palm stearin and tripalmitin have been successfully used in the
synthesis of HMF analogs (Teichert and Akoh 2011b; Zou and others 2012). Rapeseed, sunflower, olive, and hazelnut oils have been used in the production of HMFs as source of the major unsaturated fatty acids (oleic and linoleic acids) (Karabulut and others 2007; Turan and others 2012; Zou and others 2011).

Medium-chain FAs (MCFAs), such as capric acid, lauric acid, and myristic acid, are easily metabolized and rapidly absorbed by human body. MCFAs extracted from coconut and palm kernel oils were recently used in the synthesis of HMF analogs (Ilyasoglu and others 2011). Long-chain polyunsaturated fatty acids (LCPUFAs), such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (ARA), are important to infant brain development and are incorporated in the structure of HMF analogs (Sahin and others 2006; Zou and others 2012).

**Synthesis procedure**

Enzymatic modification of fats and oils for infant formula applications can be performed with a variety of enzymes, substrates, acyl donors, and under different conditions. The procedure can be single- or multistep. In a single-step process, acidolysis of TAG with FA, or interesterification between two TAG, or TAG with ethyl or methyl ester of FA, has been widely studied due to its simplicity. A common example of an SL, currently used in infant formulas, is Betapol® (Loders Croklaan), which consists mainly of 1, 3-dioleoyl-2-palmitoylglycerol (OPO). The production of Betapol is a single-step approach, in which a blend of palm stearin (a glycerol donor with a high level of palmitic acid at the sn-2 position), and high-oleic sunflower FAs (acyl donors) is subjected to an acidolysis reaction with an sn-1,3 specific lipase (King and Padley 1989; Quinlan and Chandler 1992). A typical two-step approach involves alcoholsysis of TAG,
using \textit{sn}-1, 3 specific lipase, to firstly obtain 2 MAG, followed in the second step by esterification of the purified MAG, with unsaturated fatty acids (Schmid and others 1999; Soumanou and others 1998). Purification is required at the completion of every enzymatic reaction, to eliminate any unreacted substrate, or by-products. By-products and unesterified FFAs are often observed in the products of acidolysis reactions. These impurities can be removed by distillation using a short-path distillation apparatus, and/or by performing an alkali deacidification step (Lee and Foglia, 2000). A three-step method has been studied to produce 1,3-dioleoyl-2-palmitoylglycerol (OPO) from palm oil (Chen and others 2004). In that study, palmitic acid was obtained from the fractionation of palm oil, transformed into ethyl palmitate, and converted into tripalmitin by enzymatic esterification with glycerol. The tripalmitin was then reacted with oleic acid from step one, producing OPO with 90.7% palmitic acid at the \textit{sn}-2 position. The requirement for a purification step at the end of each reaction makes multistep approaches less appealing for industrial application.

In many studies, experimental conditions were optimized in multiple small-scale (milligram-scale) experiments, prior to a large-scale (gram-scale) production. A mathematical response surface methodology (RSM) can be applied to predict the result of FA incorporation at different combinations of conditions. Sahin and others (2005) used RSM to predict the optimal conditions of a targeted 10% GLA and 45% oleic acid incorporation in a reaction between tripalmitin, hazelnut oil FA, and GLA. The model may result in slight errors, however, it is still useful for estimating the values of FA incorporation, and their content at the \textit{sn}-2 position (Teichert and Akoh 2011b). The optimal conditions were determined for the substrate mole ratio, temperature, and
reaction time, for producing a SL with 9.7-9.8% GLA and 43.3-43.8% oleic acid (Sahin and others 2005). Once optimal conditions are determined in the small-scale experiments, SL can then be produced in large-scale, to obtain sufficient amounts for characterization and evaluation of the SL products.

**Evaluation of SLs for infant formula application**

**Analysis of FA positional distribution**

Analysis of the positional distribution of FAs in SLs is commonly carried out by cleaving the FAs at the sn-1, 3 positions, using 1,3-regioselective porcine pancreatic lipase digestion. The resulting 2-MAGs are converted to fatty acid methyl esters (FAMEs), and analyzed by gas chromatography (GC). The analysis by pancreatic lipase is the most widely accepted method and has been demonstrated in the stereospecific analysis of SL containing LCPUFAs (Sahin and others 2006; Senanayake and Shahidi 1999, 2004). Alternatively, some authors have used different 1, 3-regioselective lipases from other sources including Rhizopus arrhizus or R. oryzae (Arcos and others 2000; Pérignon and others 2012).

Further, chemical methods can also be employed for the analysis. Several studies reported the use of Grignard reagents that react in a non-specific way on ester bonds of the TAGs (Brockerhoff 1971; Senanayake and Shahidi 2002; Straarup and others 2006). A typical Grignard reaction might involve one gram of purified SL product with 3.5 mL of 3 M methyl magnesium bromide, in 50 mL diethyl ether. After reaction completion, the ether phase is separated and dried over anhydrous sodium sulfate. The products of the Grignard reaction (MAG, DAG, and TAG) may be separated by thin layer chromatography; DAG bands may be collected and analyzed by gas chromatography.
The Grignard method requires both additional steps, and a much higher amount of the starting material (purified TAG product), compared to the pancreatic lipase method.

Both chemical and enzymatic methods are usually complicated by acyl migration, incomplete reactions, and selectivity issues (Redden and others 1996; Turon and others 2003). To avoid these problems, new analytical methods that can be performed directly on the SLs, such as high-resolution proton-decoupled $^{13}$C nuclear magnetic resonance (NMR) spectroscopy, have been successfully employed (Mannina and others 1999). This method has been applied to determine the regio-isomeric distribution of omega-3 PUFA such as EPA and DHA in the TAG of anchovy/sardine fish oil (Suárez and others 2010).

$^{13}$C-NMR methods can be rapid and non-destructive, and are applicable to the intact SL, avoiding the use of reactive chemicals or selective enzymes. The NMR chemical shift of the carbonyl carbon in fatty acids in TAGs depends on the regiospecific position ($sn$-1, 3 or $sn$-2). In addition, the chemical shift of the carbonyl carbon of the unsaturated fatty acid also depends on the position and number of double bonds in the chain (Del Coco and others 2009). The region where carbonyl carbons give signals is between 172-173.4 ppm, and the distance between the $sn$-1, 3 and $sn$-2 chain of a FA is approximately 0.4 ppm (Gunstone and Seth 1994).

**Analysis of TAG composition profile**

Recently, there has been an interest in the TAG composition profile of human milk, as a result of the imitation of HMF chemical composition. Some authors suggested that TAG composition might be the ultimate index for evaluation of SL developed as human milk fat substitute, HMFS (Zou and others 2013). Currently the separation of TAG species in fats and oils is performed by a reverse-phase high-performance liquid chromatography
(RP-HPLC) technique, often employing an evaporative light scattering detector (ELSD) (Chen and other 2007). Zou and others (2013) reported a variation in TAG composition of HMF at different lactation stages, using RP-HPLC and HPLC-atmospheric pressure chemical-ionization mass spectrometry (HPLC-APCI-MS). Chromatographic separation of TAG species is mainly performed on C18 columns, and the elution order of TAGs on such a column is in accordance with the order of their equivalent carbon numbers (ECN). 

ECN=TC-2xDB, where TC is the total carbon number of acyl group, and DA is the total number of double bonds (Ruiz-Gutierrez 1995). Combining data from HPLC, GC and mass spectrometry analysis increases the accuracy of the evaluation results when TAG isomers, or TAG species with the same ECN, coincide in the tested sample (Zou and others 2013).

**Analysis of thermal properties**

FA composition is an important basic parameter for determining the melting profiles, and crystallization characteristics of fats and oils. These thermal characteristics are usually studied in the range of -80 to 80 °C, using a differential scanning calorimeter (DSC) (Pande and others 2012, Teichert and Akoh, 2011a). The degree of saturation and unsaturation in the sample affect its thermal properties. Increases in LCPUFA, with concurrent decreases in LC-saturated FAs, results in lower melting points, and a greater proportion of low melting TAG species (Wirkowska and others 2012). Milk fat extracted from spray-dried dairy powder has a very broad melting range of between -40 to 40 °C, due to a complex mixture of TAGs composed of different FAs (Kim and others 2005).

Lipid oxidative stability is another important property of fats and oils. Oxidation reactions are exothermic, which can be measured using DSC, in an isothermal or non-
isothermal mode. Primary oxidation products, such as hydroperoxides, generated during the initial oxidation stage, react with excess of oxygen to form low molecular weight compounds, such as aldehydes and acids, which accelerate the degradation process. The oxidation onset temperature of fats extracted from powdered baby formulas were shown to depend on the ratio between unsaturated and saturated FAs (Wirkowska and others 2012). Alternatively, the oxidative stability of fats and oils can be measured using oxidative stability index (OSI) method, by monitoring the change in electrical conductivity resulting from the efflux of volatile organic acids, formed during lipid oxidation (Miura and others 2002).

**Infant formulas**

Human milk provides superior nutrition for an infant, however in situations where mothers cannot breastfeed, or choose not to, infant formulas are available and used to nourish an infant, as a sole source of nutrition. Therefore, much effort has been made to improve infant formulas so that they more closely simulate the nutrients in human milk, and it is essential therefore that infant formulas be well regulated as food products. In this section, the composition of infant formulas, recommended regulatory requirements, and manufacturing processes are discussed.

**Composition of infant formula**

The basic components in all infant formulas are proteins, fats, carbohydrates, vitamins, and minerals. Levels of these components must comply with the criteria set out in the regulations available regionally. For US manufacturers, Food and Drug Administration (FDA) requirements for nutrients in the infant formulas are listed under 21 CFR 107.100. The calories in infant formulas are provided by macronutrients (proteins, lipids, and
carbohydrates). The FDA Center for Food Safety and Applied Nutrition (CFSAN) recommends that the energy density of infant formulas fall in the range of 63 - 71 kcal/100 ml (Raiten and others 1998). Recommendations for specific compositions in infant formulas are listed in Table 2.2.

**Proteins**

Proteins provide energy and amino acids for the development and function of an infant. The essential amino acids (phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, lysine, and histidine) cannot be synthesized in human body, and must be provided from an exogenous source. In addition to the essential amino acids, cysteine, tyrosine, and arginine are considered conditionally essential. That is, they cannot be synthesized endogenously to an adequate level for some populations, and are required by infants and growing children (Imura and Okada 1998). Cow milk protein, soy protein isolates, and hydrolyzed cow milk proteins, are generally the source of protein in infant formulas. Required protein levels are based on the concentration of amino acids in mature human milk. The differences in the amino acid composition between cow and human milk are largely due to differences in their whey proteins to casein ratios, particularly in the level of α-lactalbumin, which is rich in tryptophan and cysteine contents(Hein and others 1991). Formulas with an increased proportion of the α-lactalbumin have therefore been developed.

**Fats**

The recommended dietary intake of total fat for infant populations, proposed by the Food and Agriculture Organization (FAO) is 40-60% of the total dietary energy (Table 2.3). Recommendations for omega-3 and omega-6 intakes are based on the composition of
HMF (FAO 2010). Infant formulas must provide the energy level, and fatty acid compositions, similar to those in HMF. Most conventional infant formulas are composed of fats from vegetable oils, which do not contain any ARA and DHA. These two LCPUFAs are important in neonatal development of the brain and retina, as they comprise the major fatty acids of the phospholipids in the cell membranes of these organs (Koletzko and Rodriguez-Palmero 1999; Ryan and others 2010). These fatty acids can be synthesized via desaturation and elongation of linoleic acid (LA, C18:2, n-6) and alpha-linolenic acid (ALA, C18:3, n-3) in animals, but not in plants. This is why most conventional infant formulas contain no DHA, however a very low level of ARA might be present in cow’s milk. An adequate intake (AI) of ARA and DHA for infants, has been reported to be 0.4-0.6% FA, and 0.2-0.36% FA, respectively (FAO 2010). Fortification with single cell oils that are rich in ARA and DHA, into infant formula, is becoming widely accepted.

**Carbohydrates**

Carbohydrates are an essential source of energy for the infant. Lactose is the predominant digestible carbohydrate in human milk, and is used in most infant milk. However, in the case of milk based on soy protein, glucose, maltose, or glucose polymers, such as maltodextrin, glucose syrup, sucrose, and corn syrup, are added as a source of carbohydrate.

**Vitamins and minerals**

Vitamins and minerals are essential dietary micronutrients for proper growth, maintenance, and function. The bioavailability of the added micronutrients may not be as high as that in human milk, so larger a quantity is added to the infant formula than the
amount found in human milk. Minimum and maximum levels of vitamins and minerals are specified by regulatory bodies, as some could be harmful if taken in excess.

**Infant formula manufacturing processes**

The manufacturing process of an infant formula depends on many factors, including product form (ready-to-feed or dried powder), ingredient combination, and desired type of packaging of the infant formulas. Typically, manufacturing methods involve the initial formation of a slurry containing carbohydrates, proteins, lipids, stabilizers, vitamins, and minerals. The slurry is emulsified, pasteurized, homogenized, and cooled. This could be diluted, heat-treated, standardized, packaged, and sterilized for use as a ready-to-feed or concentrated liquid, or it could be heat-treated, spray-dried, dry-mixed, and agglomerated for use as a reconstitutable powder (Barrett-Reis and others 2010; Borschel and others 2002). Supplementation of LCPUFAs in infant formulas typically involves the addition of TAG, phospholipid, fatty acid or fatty acid esters, which are all oils. The most convenient way to disperse PUFAs homogenously is by mixing them in the oil phase before homogenization. For example, formulas containing DHA and lutein may be prepared by making at least three separate slurries that are later blended together, heat-treated, standardized, packaged, and sterilized (Barrett-Reis and others 2010). The first slurry, containing carbohydrates and minerals, is prepared and held with agitation at 55-65°C. A separate protein-fat slurry, including vitamin ADEK premix, ARA oil, and DHA oil, is prepared at 55-60°C. The carbohydrate-mineral slurry is then combined with non-fat dry milk and water, and subsequently combined with the protein-oil slurry, to which lutein and beta-carotene are finally added. This mixture can be processed further into a ready-to-feed or concentrated liquid formula. For production of powdered
formulas containing DHA and lutein, a separate carbohydrate-mineral, and protein-fat, slurries are prepared in a similar manner before homogenization. The homogenized slurry passes heating and cooling processes before being pumped to the spray dryer. The product is packaged under nitrogen to maximize oxidative stability and flavor. Late addition of LCPUFAs is suggested in the preparation process, to ensure minimal exposure to conditions that induce degradation. For the production of powdered formulas, LCPUFAs can be added as a liquid to the dried material, after spray-drying, preferably while the dried material is on a fluidized bed. Alternatively, they can be added as powder in a conventional mixer, with the dried material, after microencapsulation or coating of the LCPUFAs on solid carriers (Groenendaal and van den Burg 2002).

**Microencapsulation of SLs for infant formula use**

As mentioned in the previous section, microencapsulation is one of the late-addition methods employed to deliver LCPUFAs into the infant formula mix. SLs produced for infant formula use are also designed to incorporate LCPUFAs to improve their nutritional value, in addition to an increased amount of the sn-2 palmitic acid. These LCPUFAs are susceptible to oxidation, which may lead to the development of off-flavors that would affect the sensory quality of the product. Microencapsulation involves packaging of a core (or mixture of active components) within a secondary material (encapsulant or wall) to form small particles. The purpose of microencapsulation is to protect sensitive ingredients, and facilitate their delivery into foods (Augustin and Hemar 2009).

**Core ingredients**

When oils are employed in microencapsulation, it is essential that they be high quality, with a low degree of oxidation. Refining and deodorizing processes, as well as the
addition of antioxidant mixtures to the oil, will influence the stability and the sensory properties of the microencapsulated oil products. The type of antioxidant also has an effect on stability of microencapsulated omega-3 oils; tocopherol proved to be more effective than ascorbyl palmitate for protection of microencapsulated fish oil power against oxidation (Baik and others 2004).

**Microencapsulation methods for LCPUFA oils**

Common techniques used in preparation of microcapsules include spray-drying, freeze-drying, simple and complex coacervation, and extrusion. Combinations of these techniques are used in order to obtain microcapsules with specific properties. Simple coacervation involves phase separation, by adding a competing hydrophilic substance, such as salt or alcohol. Wu and Xiao (2005) described the microencapsulation of fish oil through simple coacervation, followed by spray-drying. Their coacervation was attained by adding maltodextrin into an emulsion of fish oil and hydroxypropyl methylcellulose (HPMC). Maltodextrin being a better water-soluble substance, induced coacervation of HPMC in the emulsion. Klinkesorn and others (2005) reported a complex coacervation study using anionic lecithin and cationic chitosan in combination with a carbohydrate-coating (corn syrup solids, CSS), to create a multi-layer encapsulation for omega-3 oils. They examined both freeze-drying and spray-drying following the multi-layer encapsulation (Klinkesorn and others 2005, 2006). Gupta and others (2012) used freeze-drying to prepare microencapsulated products from an emulsion of conjugated linoleic-acid-rich pomegranate seed oil (core oil), sodium alginate (encapsulant), and calcium casein (emulsifier). Freeze-drying eliminates the use of hot air, however, the microencapsulation efficiency resulting from this method was low (Desobry and
Spray-drying is the most common method used for the production of encapsulated materials in the food industry (Reineccius 1988); although operated at a high air inlet temperature of 160 to 210°C, it is considered to be a technology with low impact on oil quality.

Encapsulating agents used in the food industry include carbohydrates, gums, lipids, and proteins (Gibbs and others 1999; Shahidi and Han 1993). Carbohydrates lack surface-active properties, and must be chemically modified, or used in conjunction with emulsifying agents, in order to encapsulate hydrophobic core materials. Sodium alginate is a water-soluble seaweed gum, and is widely used in drug delivery formations, due to its excellent biocompatibility and bioavailability (Shu and Zhu 2002). It is a plant-based GRAS ingredient, and therefore can be used in Kosher formulations. For microencapsulation of SLs to be used in infant formula, food-grade ingredients suitable for infants, and natural ingredients are desired. Milk constituents are widely used encapsulants, as they possess the abilities to emulsify, contribute to viscosity, and form gels. Previous studies demonstrated microencapsulations of oils using milk protein alone, or in combination with carbohydrates and Maillard reaction products (MRPs). MRPs are natural cross-linked products between milk proteins and reducing sugars (Kagami and others 2003, Vega and Roos 2006, Young and others 1993). Vega and Roos (2006) reported casein as a preferable encapsulant matrix, when compared to whey protein, due to its superior emulsifying properties. Whey protein isolate (WPI), combined with lactose, improved the encapsulation efficiency of WPI in the production of powders with anhydrous milk fat (Young and others 1993).
Recently, there has been interest in the development of protein-polysaccharide conjugates made by the Maillard reaction, for applications in food, medicines, and cosmetics (Kato, 2002). The Maillard reaction occurs naturally in foods, and some MRPs have specific health-promoting effects (Somoza, 2005). The MRP conjugates have superior emulsifying properties compared with protein alone (Chevalier and others 2001; Diftis and Kiosseoglou 2003; Moreno and others 2002; Shepherd and others 2000), hence they satisfy an important criterion for an encapsulant intended for microencapsulation of oils. They are stable over a greater range of pH compared with the unmodified protein (Shepherd and others 2000), making them more suitable for use in a wider range of foods. In addition, the antioxidative properties of MRPs offer protection to the unsaturated oils (McGookin and Augustin 1991). Augustin and others (2006), demonstrated the suitability of heated protein-carbohydrate mixtures for encapsulation of fish oil, and suggested that increasing the extent of the Maillard reaction confers additional stability to microencapsulated fish oil. Results from in vivo and in vitro studies showed this microencapsulation method protected fish oil from acidic conditions in the stomach, delivered and released the oil in the small intestine, and it did not compromise the bioavailability (Augustin and others 2011; Patten and others 2009, Kosaraju and others 2009).

Spray-drying

Spray-drying is commonly employed in the production of microencapsulated food ingredients. Basic microencapsulation of an oil core ingredient involves a homogenization step, in which a stable emulsion of core oil in the solution of encapsulant materials is obtained. The emulsion is then atomized into heated air to facilitate the rapid
removal of water, as the droplets are mixed with the hot air in the drying chamber. The powder particles are then separated from the drying air at the outlet at lower temperatures. During the drying step, as the core oil is dried, it is surrounded by the interfacial membrane formed in the emulsion (Augustin and Hemar 2009). It is therefore critical to obtain a stable emulsion, as the release of the core oil depends on the integrity of these interfacial membranes. Spray-drying has been applied in the production of encapsulated vitamins, minerals, flavors, PUFA oils, and probiotic microorganisms (Baranauskiene and others 2006; Fritzen-Freire and others 2012; Oneda and Ré 2003; Shu and others 2006; Tan and others 2009; Xie and others 2007). A summary of key features of the experimental conditions used in these studies is presented in Table 2.4. To obtain products with good microencapsulation efficiency, suitable encapsulant materials and optimal spray-drying conditions must be employed. Appropriate adjustment of the air inlet temperature, and flow rate, is important (Zbicinski and others 2002). Drying rate, and the final water content, are directly proportional to air inlet temperature. A low inlet temperature results in a low evaporation rate, causing the formation of microcapsules with high water content, and easiness of agglomeration. However, high inlet temperature can cause excessive evaporation, which results in cracks and premature release of core ingredients (Gharsallaoui and others 2007; Zakarian and King, 1982,)

**Selected properties of spray-dried microencapsulated powders**

The aim of the microencapsulation process is to obtain products with high encapsulation efficiency, and high oxidative stability, of the core ingredients. A high core load in the microcapsule is also desirable, however, it should not compromise the quality of the products. Moisture content and water activity (a_w) influence the rate of lipid oxidation.
The maximum moisture content of dried powder specified by the food industry is between 3-4% (Master 1991). In general, low moisture content (1-3%) and low water activity (0.1-0.25) are achieved through spray-drying, conducted at temperatures between 165-195°C (Hogan and others 2001; Klinkesorn and others 2006). The role of water in lipid oxidation depends on the structure and composition of the food. For example, lipid oxidation in milk powder, as measured by free radical concentration, or by the thiobarbituric acid-reactive substances (TBARs) value, is unaffected by water activity of between 0.11-0.22, but is stimulated by a water activity between 0.22-0.33 (Stapelfeldt and others 1997). This contradicts the generalized view that lipid oxidation in foods is at its lowest level when water activity is between 0.2 and 0.4 (close to that of monolayer water), but increases rapidly when the water activity is either decreased or increased (Hardas and others 2000; Velasco and others 2003).

During lipid oxidation, hydroperoxide primary oxidation products form continuously, and break down into a variety of non-volatile and volatile secondary products (Shahidi and Zhong 2005). The oxidative stability of microencapsulated products is characterized by the peroxide value (PV) (Bae and Lee 2008; Hardas and others 2000), the TBARS value (Klinkesorn and others 2005), and the analysis of volatile compounds using headspace gas chromatographic method (Augustin and others 2006; Rusli and others 2006; Hardas and others 2000). In addition to monitoring the oxidative products, some studies also monitored the content of specific unsaturated fatty acids. Linoleic and linolenic acid contents were monitored, along with PV, to evaluate the oxidative stability of microencapsulated milk fat (Hardas and others 2000). Headspace propanal has been used as an indicator of fish oil oxidation (Augustin and others 2006).
Microencapsulation efficiency (ME) indicates the presence of free oil on the surface of the particles, and the degree to which the wall can prevent extraction of internal oil (Hogan and others 2001). The presence of free oil in spray-dried powders adversely influences physical properties, such as flowability, bulk density and dispersibility, and enhances lipid oxidation (Granelli and others 1996; Keogh and others 2001). ME is defined as \[(\text{total oil} - \text{free oil})/\text{total oil}] \times 100.\] Previously-reported microencapsulation efficiencies, using MRPs as encapsulants, were between 80-98%, depending on the type of protein, the oil to protein ratio, and the oil load in the powder (Rusli and others 2006).

Another important property of powders is the ability to disperse in water by gentle stirring. This means that the powder should disintegrate into agglomerates, which again should disintegrate into the single primary particles. The dispersibility of the powdered emulsion can be assessed by measuring the change in mean particle diameter, and droplet concentration of the system, as a function of time (Klinkesorn and others 2006). A rapid decrease in particle size and increase in droplet concentration, indicates the majority of the powder has been dissolved quickly; hence, a homogenous suspension has been achieved.
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substitute with medium-chain fatty acids by lipase-catalyzed acidolysis:
Optimization by response surface methodology. LWT - Food Sci Technol 44:999-
1004.

148.


Table 2.1 Major TAGs in natural fats and oils used for synthesis of SLs.

<table>
<thead>
<tr>
<th>Fat or oil</th>
<th>Major TAGs</th>
<th>Major TAGs</th>
<th>Major TAGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butter fat</td>
<td>PPB</td>
<td>PPC</td>
<td>POP</td>
</tr>
<tr>
<td>Lard</td>
<td>SPO</td>
<td>OPL</td>
<td>OPO</td>
</tr>
<tr>
<td>Tallow (beef)</td>
<td>POO</td>
<td>POP</td>
<td>POS</td>
</tr>
<tr>
<td>Cocoa butter</td>
<td>POS</td>
<td>SOS</td>
<td>POP</td>
</tr>
<tr>
<td>Coconut oil</td>
<td>DDD</td>
<td>CDD</td>
<td>CDM</td>
</tr>
<tr>
<td>Palm kernel oil</td>
<td>DDD</td>
<td>MOD</td>
<td>ODO</td>
</tr>
<tr>
<td>Almond oil</td>
<td>OOO</td>
<td>OLO</td>
<td>OLL</td>
</tr>
<tr>
<td>Amaranth oil</td>
<td>PLL</td>
<td>POL</td>
<td>OLL</td>
</tr>
<tr>
<td>Corn oil</td>
<td>LLL</td>
<td>LOL</td>
<td>LLP</td>
</tr>
<tr>
<td>Cotton seed oil</td>
<td>PLL</td>
<td>POL</td>
<td>LLL</td>
</tr>
<tr>
<td>Egg TAG</td>
<td>POO</td>
<td>PLO</td>
<td>POS</td>
</tr>
<tr>
<td>Grape seed oil</td>
<td>LLL</td>
<td>OLL</td>
<td>POL</td>
</tr>
<tr>
<td>Hazelnut oil</td>
<td>OOO</td>
<td>OLO</td>
<td>POO</td>
</tr>
<tr>
<td>Olive oil</td>
<td>OOO</td>
<td>OOP</td>
<td>OLO</td>
</tr>
<tr>
<td>Palm oil</td>
<td>POP</td>
<td>POO</td>
<td>OLO</td>
</tr>
<tr>
<td>Peanut oil</td>
<td>OOL</td>
<td>POL</td>
<td>OLO</td>
</tr>
<tr>
<td>Canola oil</td>
<td>OOL</td>
<td>OOO</td>
<td>OOLn</td>
</tr>
<tr>
<td>Rice bran oil</td>
<td>PLO</td>
<td>OOL</td>
<td>POO</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>LLL</td>
<td>LLO</td>
<td>LLP</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>LLL</td>
<td>LLO</td>
<td>LLP</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>LLL</td>
<td>OLL</td>
<td>LOO</td>
</tr>
<tr>
<td>Walnut oil</td>
<td>LLL</td>
<td>OOL</td>
<td>PLL</td>
</tr>
<tr>
<td>Rapeseed oil (low Er)</td>
<td>OOO</td>
<td>LOO</td>
<td>OOLn</td>
</tr>
<tr>
<td>Rapeseed oil (high Er)</td>
<td>ErOEr</td>
<td>ErLEr</td>
<td>ErLnEr</td>
</tr>
<tr>
<td>Linseed oil</td>
<td>LnLnLn</td>
<td>LnLnL</td>
<td>LnLnO</td>
</tr>
<tr>
<td>Mustard seed oil</td>
<td>ErOEr</td>
<td>ErLEr</td>
<td>OOEr</td>
</tr>
<tr>
<td>Tea seed oil</td>
<td>OOO</td>
<td>OOP</td>
<td>OOL</td>
</tr>
</tbody>
</table>

(Small 1991; Soumanou and others 2013).

B = C4:0 Butyric, C=C10:0 Capric, D=C12:0 Dodecanoic, M=C14:0 Myristic, P=C16:0 Palmitic, S=C18:0 Stearic, O=C18:1 Oleic, L=C18:2 Linoleic, Ln C18:3 Linolenic, Er=C22:1 Erucic
Table 2.2 European union guidelines for the composition of infant milk formula.

<table>
<thead>
<tr>
<th></th>
<th>Minimum per 100 kcal</th>
<th>Maximum per 100 kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy</strong></td>
<td>60 kcal/100 ml</td>
<td>70 kcal/100 ml</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow milk protein (g)</td>
<td>1.8</td>
<td>3</td>
</tr>
<tr>
<td>Soy protein (g)</td>
<td>2.25</td>
<td>3</td>
</tr>
<tr>
<td>Protein partial hydrolysates (g)</td>
<td>2.56</td>
<td>3</td>
</tr>
<tr>
<td><strong>Fat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid (LA) (mg)</td>
<td>300</td>
<td>1200</td>
</tr>
<tr>
<td>Alpha linolenic acid (ALA) (mg)</td>
<td>50</td>
<td>6.5</td>
</tr>
<tr>
<td>Ratio LA:ALA</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>n-3 LCPUFA</td>
<td>-</td>
<td>1% of total fat content</td>
</tr>
<tr>
<td>n-6 LCPUFA</td>
<td>-</td>
<td>2% of total fat content</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>-</td>
<td>1% of total fat content</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (EPA)</td>
<td>-</td>
<td>1.5 % of total fat content</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>-</td>
<td>EPA content may not exceed DHA content</td>
</tr>
<tr>
<td><strong>Carbohydrates</strong></td>
<td>(g)</td>
<td>7</td>
</tr>
<tr>
<td>Lactose (g)</td>
<td>3.5</td>
<td>-</td>
</tr>
<tr>
<td>Saccharose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Precooked or gelatinized starch</td>
<td>-</td>
<td>20% of total carbohydrates</td>
</tr>
<tr>
<td><strong>Minerals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>60</td>
<td>145</td>
</tr>
<tr>
<td>Chloride (mg)</td>
<td>50</td>
<td>125</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Phosphorus (mg)</td>
<td>25</td>
<td>90</td>
</tr>
<tr>
<td>Magnesium (mg)</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Zinc (mg)</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Copper (mg)</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>Iodine (mg)</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Selenium (mg)</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin A (µg RE)</td>
<td>60</td>
<td>180</td>
</tr>
<tr>
<td>Vitamin D (µg)</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>Thiamin (µg)</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>Riboflavin (µg)</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>Niacin (mg NE)</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>Panthothenic acid (µg)</td>
<td>300</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin B6 (µg)</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>Biotin (µg)</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>Folic acid (µg)</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin B12 (µg)</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin C (µg)</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin K (µg)</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin E (µg α-TE)</td>
<td>0.5 mg α-TE.g LCPUFs. In no case less than 0.1 mg/100 kJ</td>
<td></td>
</tr>
<tr>
<td><strong>Nucleotides</strong></td>
<td>(mg)</td>
<td>-</td>
</tr>
<tr>
<td>Total (mg)</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Cytidine-5-monophosphate (mg)</td>
<td>-</td>
<td>2.5</td>
</tr>
<tr>
<td>Uridine-5-monophosphate (mg)</td>
<td>-</td>
<td>1.75</td>
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<tr>
<td>Adenosine-5-monophosphate (mg)</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>Guanosine-5-monophosphate (mg)</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Inosine-5-monophosphate (mg)</td>
<td>-</td>
<td>1.0</td>
</tr>
</tbody>
</table>

(Alles and others 2004; Koletzko and others 2005).
Table 2.3 Recommended dietary intakes for total fat and fatty acid intake: infant (0-24 months).

<table>
<thead>
<tr>
<th>Fat/FA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Age group (months)</th>
<th>Measure&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Numeric amount&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fat</td>
<td>0-6</td>
<td>AMDR</td>
<td>40-60%E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AI</td>
<td>Based on composition % of total fat in human milk</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>6-24</td>
<td>U-AMDR</td>
<td>&lt;15%E</td>
</tr>
<tr>
<td>LA &amp; ALA</td>
<td>0-24</td>
<td>Comment</td>
<td>Essential and indispensable</td>
</tr>
<tr>
<td>n-6 PUFA</td>
<td></td>
<td>AI</td>
<td>0.2-0.3%&lt;sup&gt;d&lt;/sup&gt; (equivalent to 0.4-0.6%FA)</td>
</tr>
<tr>
<td></td>
<td>0-6</td>
<td>U-AMDR</td>
<td>Based on human milk composition as %E of total fat</td>
</tr>
<tr>
<td></td>
<td>6-12</td>
<td>AI</td>
<td>3.0-4.5%E (6.0-9.0%FA)</td>
</tr>
<tr>
<td></td>
<td>6-12</td>
<td>U-AMDR</td>
<td>&lt;10%E (&lt;20%FA)</td>
</tr>
<tr>
<td>n-3 PUFA</td>
<td></td>
<td>AI</td>
<td>0.2-0.3% (0.4-0.6%FA)</td>
</tr>
<tr>
<td></td>
<td>0-6</td>
<td>AI</td>
<td>0.4-0.6% (0.8-1.2%FA)</td>
</tr>
<tr>
<td></td>
<td>6-24</td>
<td>U-AMDR</td>
<td>&lt;3%E (&lt;6%FA)</td>
</tr>
<tr>
<td>ALA</td>
<td>0-6</td>
<td>AI</td>
<td>0.1-0.18%&lt;sup&gt;e&lt;/sup&gt;, equivalent to 0.2-0.36%FA</td>
</tr>
<tr>
<td></td>
<td>0-6</td>
<td>U-AMDR</td>
<td>No upper value within the human milk range up to 0.75%E (1.5%FA)</td>
</tr>
<tr>
<td></td>
<td>0-6</td>
<td>Comment</td>
<td>Conditionally essential due to limited synthesis from ALA</td>
</tr>
<tr>
<td></td>
<td>6-24</td>
<td>AI</td>
<td>10-12 mg/kg</td>
</tr>
<tr>
<td></td>
<td>0-24</td>
<td>Comment</td>
<td>Critical role in retinal and brain development</td>
</tr>
</tbody>
</table>

<sup>a</sup>FA-fatty acid, PUFA-polyunsaturated fatty acid, LA-linoleic acid (C18:2, n-6), ALA-alpha linolenic acid (C18:3, n-3), AA-arachidonic acid (C20:4, n-6), DHA-docosahexaenoic acid (C22:5, n-3).<sup>b</sup>AMDRA-acceptable macronutrient distribution range, AI-adequate intake (expressed as a range).<sup>c</sup>%E-percent of energy, %FA-percent fatty acid composition.<sup>d</sup>Based on human milk composition, the amounts of AA and ALA would be expressed as 0.4-0.6%FA and for DHA as 0.2-0.36%FA. This conversion is assumes that half of the energy in human milk comes from fat. For children 6-24 months of age the estimation is based on provision of breast milk to meet half of the daily energy needs, the rest of the energy would come from non breast milk diet.

(FAO 2010).
**Table 2.4** Summary of key features of the spray-drying conditions used in the production of encapsulated vitamins, minerals, flavors, PUFA oils, and probiotic microorganisms.

<table>
<thead>
<tr>
<th>Encapsulated ingredient</th>
<th>Encapsulant/ wall material</th>
<th>Feed temperature (°C)</th>
<th>Air inlet temperature (°C)</th>
<th>Air outlet temperature (°C)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oregano, citrusellla and marjoram flavors</td>
<td>Whey proteins/milk proteins</td>
<td>NR</td>
<td>185–195</td>
<td>85–95</td>
<td>Baranauskiene and others (2006)</td>
</tr>
<tr>
<td>Lycopene</td>
<td>Gelatin/sucrose</td>
<td>55</td>
<td>190</td>
<td>52</td>
<td>Shu and others. (2006)</td>
</tr>
<tr>
<td>Vitamin A acetate</td>
<td>Starch octenylsuccinate</td>
<td>NR</td>
<td>182</td>
<td>82</td>
<td>Xie and others (2007)</td>
</tr>
<tr>
<td>Calcium</td>
<td>Cellulose and neutral polymethacrylate</td>
<td>Feeding rate 3ml/min</td>
<td>120-128</td>
<td>91-95</td>
<td>Oneda and Ré (2003)</td>
</tr>
<tr>
<td>Fish oil</td>
<td>Alginate and starch blend</td>
<td>NR</td>
<td>150</td>
<td>80</td>
<td>Tan and others (2009)</td>
</tr>
<tr>
<td><em>Bifidobacterium</em></td>
<td>Skim milk powder in combinations with inulins</td>
<td>Room temperature</td>
<td>150±2</td>
<td>55±3</td>
<td>Fritzen-Freira and others (2012)</td>
</tr>
</tbody>
</table>

NR—not reported.
CHAPTER 3

ENRICHMENT OF PALM OLEIN WITH LONG-CHAIN POLYUNSATURATED FATTY ACIDS BY ENZYMATIC ACIDOLYSIS


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ABSTRACT

Long-chain polyunsaturated fatty acids (LCPUFA) such as docosahexaenoic (DHA) and arachidonic (ARA) acids have great benefits for the development and maintenance of human brain functions. The production of structured lipid (SL) by acidolysis of palm olein with a free fatty acid mixture obtained from DHASCO® and ARASCO®, catalyzed by Novozym 435® in hexane was optimized by response surface methodology (RSM). Three independent factors chosen were substrate mole ratio of total free fatty acids to palm olein (6-18 mol/mol), reaction temperature (55-65°C), and reaction time (12-24 h). Good quadratic models were obtained for the incorporation of DHA+ARA (response 1) and their incorporation at the sn-2 position (response 2) by multiple regression and backward elimination. The models were verified by carrying out acidolysis reaction at several random combinations of the independent factors. The optimal condition generated from the models resulted in 25.25 g/100 g total incorporation of DHA+ARA and 17.20 g/100 g DHA+ARA incorporation at the sn-2 position. The fatty acid composition of SL was comparable to that of human milk fat. The SL produced in this study has potential for use in infant formulas as well as in nutraceutical applications for pregnant women.

Keywords: structured lipids, docosahexaenoic acid, arachidonic acid, palm olein, acidolysis
INTRODUCTION

Docosahexaenoic acid (DHA, C22:6 n-3) and arachidonic acid (ARA, C20:4 n-6) are long-chain polyunsaturated fatty acids (LCPUFA) derived from the elongation and desaturation of α-linolenic acid (ALA, C18:3 n-3) and γ-linoleic acid (GLA, C18:3 n-6), respectively. DHA is present at high levels in the retina, cerebral cortex and sperm, while ARA is present in all biological membrane (Neuringer, Anderson, & Connor, 1988). DHA and ARA constitute the majority of the fatty acids in the brain and their role is to aid in the development and protection of neurological functions (Bourre, 2006; Koletzko et al., 2008; Ryan et al., 2010). These fatty acids are also important in neonatal development of the brain and retina. Studies have shown that children of women receiving prenatal DHA supplements, scored higher on mental processing and visual acuity (Helland, Smith, Saarem, Saugstad, & Drevon, 2003; Williams, Birch, Emmett, Northstone, & Team, 2001). Premature infants fed with DHA and ARA supplemented infant formula, had weight gains comparable to full-term infants on breastfeeding. In contrast, premature infants fed on formula with no DHA or ARA had a lower body weight than full-term infants (Innis et al., 2002). Dietary supplementation of DHA and ARA in adults was shown to improve cognitive dysfunctions due to ageing, organic brain lesions and Alzheimer's disease (Kotani et al., 2006). Therefore, the consumption of DHA and ARA benefits people of all ages from infants, mothers of infant and aging adults.

Although conversion of ALA and GLA to LCPUFA products occurs naturally in the human body, studies demonstrated that the conversion of ALA to DHA occurs at a very limiting rate (Burdge & Calder, 2005; Gerster, 1998). Both the fetus and the newborn
depend on the maternal supply of DHA and ARA through the placenta and breastfeeding (Boehm et al., 1996; Green & Yavin, 1998). Consequently, an adequate intake of preformed LCPUFA is recommended to maintain optimal tissue function, especially for pregnant and breast-feeding women. Individuals on diets lacking meat and fish, which are primary sources of ARA and DHA, respectively, may suffer from neurological and visual disturbances. Therefore, there is an interest in creating structured lipids (SL) enriched with ARA and DHA for potential uses in the food and nutraceutical industry (Innis, 2004; Osborn & Akoh, 2002).

Palm olein, a low-melting point fraction of palm oil, has been widely used in edible-oil blends for food applications. It is liquid in warm climates and blends well with other vegetable oils. The fatty acid composition of palm olein (palmitic acid 40, oleic acid 43 and linoleic acid 11 g/100 g) makes it an excellent source of palmitic and oleic acids to be added into infant formula to mimic the fatty acid composition of human milk.

Although the fatty acid composition of vegetable oils used in infant formula are matched to human milk fat, the fat absorption in formula-fed infants is still lower (Hanna, Navarrete, & Hsu, 1970; Lien, 1994). This lower absorption is due to the differences in stereospecific structure of the triacylglycerols (TAG) of vegetable oils and human milk fat. Most of the palmitic acid in human milk (>60 g/100 g of the palmitic acid) is located at the sn-2 position, whereas in vegetable oils, it is predominantly located at the outer positions (Innis, Dyer, Quinlan, & Diersen-Schade, 1995; López-López et al., 2001; Tomarelli, Meyer, Weaber, & Bernhart, 1968). Pancreatic lipase specifically hydrolyzes the sn-1, 3 positions of TAG to produce free fatty acids from these positions and 2-monoacylglycerol. The 2-monoacylglycerol (2-MAG), is a well-absorbed form of most
fatty acids since it readily forms mixed micelles with bile acids and cannot form insoluble soaps with divalent cations (Jandacek, Whiteside, Holcombe, Volpenhein, & Taulbee, 1987; Lien, 1994). Absorption of eicosapentaenoic acid (EPA, C20:5 n-3) and DHA was higher when they were predominantly in the sn-2 position than when they were randomly distributed between the three positions (Christensen, Hoy, Becker, & Redgrave, 1995).

The levels of DHA and ARA at the sn-2 position of human milk fat are 0.4-0.7, and 0.3-0.7 g/100g of the total fatty acids in the sn-2 position, respectively. Total amount of DHA and ARA in human milk fat are 0.2-0.5, and 0.4-0.6 g/100 g of the total fatty acids, respectively. However, levels of these fatty acids vary depending on the diets of the mothers (Straaup, Lauritzen, Faerk, Hoy, & Michaelsen, 2006; Yuhas, Pramuk, & Lien, 2006).

Previously, enrichments of LCPUFA including ARA, DHA and EPA into TAG structure of borage oil, evening primrose oil and tripalmitin have been studied (Sahin, Akoh, & Karaali, 2006; Senanayake & Shahidi, 1999; Shimada et al., 2000). Stereospecific analysis of SL using pancreatic lipase is often used to provide useful information about digestion in the intestine. The aim of this study was to enrich the TAG structure of palm olein with DHA and ARA derived from single cell oil TAG (DHASCO® and ARASCO®), by an acidolysis reaction. Response surface methodology (RSM) was used to model and optimize the reaction conditions to generate high incorporation of DHA and ARA with high contents at the sn-2 position.

**MATERIALS AND METHODS**

**Materials.** Palm olein (San Trans25) was kindly donated by IOI- Loders Croklaan (Channahon, IL). DHA-containing single cell oil (DHASCO®) and ARA-containing
single cell oil (ARASCO®) from algae *Cryptocodinium cohnii* and fungus *Mortierella alpina*, respectively, were generously provided by Martek Bioscience Corp. (Columbia, MD). Novozym® 435 (a nonspecific lipase) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). The lipid standards Supelco 37 component FAME mix, C15:0 pentadecanoic acid (>98% purity), triolein, and 2-oleoylglycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Other solvents and chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), J.T. Baker Chemical Co. (Phillipsburg, NJ), or Fisher Scientific (Norcross, GA).

**Preparation of free fatty acid (FFA) from DHASCO and ARASCO.** Preparation of free fatty acids from single cell oils was carried out according to Senanayeke & Shahidi (1999). Twenty-five grams of oil (treated with 5 mg butylated hydroxytoluene) was saponified using a mixture of 5.75 g of KOH, 11 ml of distilled water and 66 ml of aqueous ethanol (95 ml/100 ml) by refluxing for 1 h at 60 °C. To the saponified mixture, 60 ml of distilled water was added, and the unsaponified matter was extracted into hexane (2x 100 ml) and discarded. The aqueous layer containing saponifiable matter was acidified to pH about 1.0 with 4 mol/l HCl. The liberated fatty acids were extracted into hexane 50 ml. The hexane containing free fatty acids was dried over anhydrous sodium sulfate, and the solvent was removed in a rotary evaporator at 40°C. Free fatty acids were flushed with nitrogen prior to storage in the freezer at -80°C.

**Preparation of DHA and ARA concentrate by urea complexation and low temperature solvent crystallization.** The separations of DHA and ARA from the hydrolyzed fatty acids mixtures of DHASCO and ARASCO, respectively were performed using urea-fatty acid complexation according to Senanayeke & Shahidi (1999)
while low temperature solvent crystallization followed a method described by Vali, Sheng & Ju (2003) with modification. Ten grams of free fatty acids was mixed with 30 g of urea in 150 ml aqueous ethanol (95 ml/100 ml) and heated at 60°C with stirring. The urea-fatty acid complex was allowed to crystallize at room temperature first and then placed in a freezer at 4°C for a period of 24 h. The crystals formed were separated from the liquid by suction filtration through Whatman® filter paper grade no. 1. The filtrate was diluted with an equal volume of distilled water and acidified to pH 4-5 with 6 mol/l HCl. An equal volume of hexane was then added and the mixture was stirred thoroughly for 1 h. Hexane layer containing liberated fatty acids was separated from the aqueous layer containing urea using a separatory funnel. The hexane layer was then washed with distilled water to remove any remaining urea and dried over anhydrous sodium sulfate. The solvent was subsequently removed at 40°C using a rotary evaporator. The method of Vali et al. (2003) for low temperature solvent crystallization was modified as given below. Four gram of free fatty acids was dissolved in 300 ml methanol. The mixture was stirred at 50°C until all dissolved. The solution was allowed to cool and stored at -80°C for 48 h. Then, the liquid and solid phases were separated immediately by vacuum filtration. Methanol was removed in a vacuum rotary evaporator at 40 °C. The concentrated fractions obtained from both methods were flushed with nitrogen and stored at -80°C. The fatty acid composition and average molecular weight of these fractions were determined using a gas chromatographic (GC) procedure as described below.

**Determination of fatty acid profiles.** Samples were converted to fatty acid methyl esters (FAME) following AOAC official method 996.01 with modification. Briefly, 100 mg of oil was weighed into a Teflon-lined test tube, and 1 ml of C15:0 in hexane (20 mg/ml)
was added as internal standard (Vali et al., 2003) and dried with nitrogen to remove solvent. Two milliliters of 0.5 mol/l NaOH in methanol was added followed by incubation for 5 min at 100°C to saponify the lipid. To the sample was added 2 ml of boron trifluoride (BF₃) in methanol (14 g/100 ml), vortexed for 1 min and incubated at the same condition to allow methylation. Two milliliters of hexane and 2 ml of NaCl-saturated solution were added and vortexed for 2 min to stop the reaction and to extract the FAME. To separate the organic and aqueous phases, the sample was centrifuged at 1,000 rpm (approximately 100x g) for 5 min. The upper layer was recovered into a GC vial and analyzed. Supelco 37 component FAME mix was used as FAME external standard and ran in parallel with the samples.

**Experiment design for RSM study.** A RSM mathematical model (Modde 5.0, Umetrics, Umeå, Sweden) was used to predict the incorporation of LCPUFA into palm olein and at the sn-2 position of the glycerol backbone for the acidolysis reaction. The experimental design considered three factors: reaction time (12-24 h), reaction temperature (55-65°C), and substrate mole ratio of total free fatty acids to palm olein (6-18 mol/mol). The central composite face design consisted of fifteen different combinations resulting in a total of seventeen different experiments (Table 3.1). Experiments were performed in triplicate resulting in fifty-one total reactions.

**Acidolysis experiments.** The concentrated DHA and ARA free fatty acids (FFA) obtained from urea complexation were mixed at a mole ratio of 2 mol/mol (DHA:ARA) to be used as total free fatty acids. The substrate mixtures of total free fatty acids and palm olein were determined on the basis of their average molecular weights. Acidolysis reactions were carried out in screw-cap test tubes using 10 mg enzyme/100 mg total
weight of reactants and incubated in an orbital shaking water bath at 250 rpm (approximately 30 x g) according to the conditions showed in Table 3.1. The resulting product was filtered through anhydrous sodium sulfate column to separate it from immobilized enzyme and properly stored in Teflon-lined test tubes at -80°C for FAME and positional analyses.

Analysis of product. After enzymatic reaction, the resulting product was spotted onto silica gel G TLC plates, and a mixture of petroleum ether:diethyl ether: acetic acid (70:30:0.5, v/v/v; 105 ml: 45 ml: 0.75 ml) was used to separate the TAG. Lipid bands were visualized after spraying plates with 2, 7-dichlorofluorescein in methanol (0.2 g/100 ml) and under UV light. TAG separated bands were identified using triolein as standard. The TAG bands were scraped off and recovered into test tubes for FAME and positional analysis.

Fatty acid composition analysis. The FA composition of single cell oils, palm olein and reaction products were analyzed by GC. The gas chromatograph was an Agilent Technologies 6890N (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector (FID). Separation was achieved with a Supelco SP-2560 column, 100 m x 250 µm, 0.20 µm film. The injector and detector temperature were maintained at 300°C. The column temperature was held at 140 °C for 5 min and increased up to 240 °C at the rate of 4 °C/min and held isothermally for 15 min. The carrier gas was helium, and the total gas flow was 1.1 ml/min at a constant pressure. A 1 µl sample was injected into the GC at a split ratio of 20. Relative contents of FAME as mole percent were calculated by computer, using C15:0 as the internal standard (Vali et al., 2003).
**Pancreatic lipase-catalyzed sn-2 positional analysis.** The recovered TAG bands were scraped from silica gel and 1.5 ml of diethyl ether was added, vortexed, centrifuged at 1,000 rpm (approximately 100x g) for 3 min and filtered through an anhydrous sodium sulfate column. The extraction step was repeated. Samples were completely dried under nitrogen gas before proceeding to the sn-2 positional analysis using a modification of Luddy, Barford, Herb, Magidman, & Riemenschneider (1964) method. Forty milligrams of purified pancreatic lipase (porcine pancreatic lipase, crude type II), 1 ml of Tris buffer (pH 8.0), 0.20 ml of sodium cholate (0.05 g/100 ml distilled water), and 0.1 ml of calcium chloride (2.2 g/100 ml distilled water) were added to the sample. The mixture was incubated at 40 °C in a water bath for 3 min; 1 ml of 6 mol/l HCl and 4 ml of diethyl ether were added and centrifuged. The upper layer containing the lipid components was separated and flushed with nitrogen to evaporate solvent until one-third of the volume was left. The concentrated extract was spotted on silica gel G TLC plates and developed with a mixture of hexane: diethyl ether: formic acid (60:40:1.6, v/v/v; 90 ml: 60 ml: 2.4 ml). 2-Oleoylglycerol was spotted in parallel as identification standard for 2-MAG. Plates were sprayed with 2,7-dichlorofluorescein in methanol (0.2 g/100 ml) and visualized under UV light. The band corresponding to 2-MAG was scrapped off and converted to FAME as previously described. Twenty-five microliters of C15:0 in hexane (20 mg/ml) was used as internal standard. Organic layer containing FAME components was concentrated with nitrogen to 200 µl before injecting an aliquot into GC.

**Statistical analysis.** All sample analysis was done in triplicate. The average and standard deviations were calculated and reported for all analyses. The analysis of variance
(ANOVA) and the mathematical model for optimization were carried out using (Modde 5.0, Umetrics, Umeå, Sweden).

**Verification of model.** Acidolysis reactions were carried out in test tubes at random conditions and the optimal condition suggested by RSM to verify the model. The experimental values were then compared to the predicted values for the model, as shown in Table 3.2.

**RESULTS AND DISCUSSION**

**Preparation of concentrated DHA and ARA free fatty acids from DHASCO and ARASCO.** Concentrated DHA and ARA substrates for acidolysis were prepared by urea complexation and low temperature solvent crystallization. By comparison, saturated and monounsaturated fatty acids were eliminated better by urea complexation method. DHA concentrate using urea complexation had almost no saturated and monounsaturated fatty acids. The major fatty acids present in the original DHASCO were DHA, oleic, palmitic, and myristic acids. DHA content was enriched from 44.89 to 98.12 g/100 g. Similar result was obtained by Senanayake & Shahidi (1999), in which the DHA content in their experiment was enriched from 47.4 to 97.1 g/100 g. The major fatty acids present in ARASCO were ARA, oleic, and stearic acids. As a result of urea complexation, saturated and monounsaturated fatty acids were eliminated while ARA was enriched from 43.27 to 78.95 g/100 g. Vali et al. (2003) reported an increase in ARA from 38 to ~65 g/100 g using similar low temperature crystallization method as in this study (methanol: free fatty acid ratio of 75 (volume/weight) at -80 °C and 48 h storage time). However, in this study ARA was increased from 43.27 to 53.46 g/100 g. The amount of DHA was only increased from 44.89 to 49.31 g/100 g using the same low temperature solvent
crystallization condition. Products from urea complexation were superior and chosen as the substrates for acidolysis as they were higher in LCPUFA concentration and contained lesser amount of saturated and monounsaturated fatty acids. These substrates were mixed at a mole ratio of 2 mol/mol (DHA: ARA) to form a total free fatty acid mixture. A higher ratio of DHA to ARA was considered in SLs design to ensure that the products will deliver the anti-inflammatory effect exhibited by consumption of DHA and a low amount of ARA (Adam et al., 2003). The total free fatty acid mixture contained DHA (70.52 ± 0.01) and ARA (23.42± 0.02 g/100 g) with an estimated molecular weight of 319.67.

Model fitting. Palm olein enriched with DHA and ARA was synthesized by acidolysis of palm olein with total FFA prepared from LCPUFA concentrated fraction of DHASCO and ARASCO. A 3-factor and 3-level central composite face design was applied for the reactions, the design points and responses are presented in Table 3.1. The higher standard deviation in some experiments is not unusual in biocatalysis. This may be due to the specificity of enzyme, substrate mole ratio, reaction time, reaction temperature, and water activity (Teichert & Akoh, 2011; Xu, 2000). The regression coefficients and p-values were calculated using experimental results. The best-fitting quadratic models were determined for responses 1, total DHA+ARA incorporation, and 2, DHA+ARA incorporation at sn-2 position. One outlier experiment (no. 15) was deleted. The model equations for the responses can be written as follows:

Total DHA+ARA incorporation (g/100g)

\[ = 16.143 + 1.778 \times Sr + 3.182 \times t - 4.980 \times T^2 + 2.840 \times t^2 + 1.218 \times Sr \times T \]  (1)
DHA+ARA incorporation at sn-2 (g/100g)

\[ = 7.858 -0.992* T + 2.011*t \]  (2)

Where Sr is substrate mole ratio, t is reaction time and T is reaction temperature.

For the total DHA+ARA incorporation, time and substrate mole ratio were the significant first-order parameters with \( p \)-value < 0.01. These parameters have a positive effect on the total DHA+ARA incorporation. The significant second-order parameters with \( p \)-value <0.05 were the second-order term of temperature \( (T^2) \), the second-order term of time \( (t^2) \), and the interaction term of substrate mole ratio and temperature \( (Sr*T) \).

DHA+ARA incorporation was negatively correlated by the second-order term of temperature, whereas the second-order term of time and the interaction term of substrate mole ratio and temperature had positive effects on the total incorporation of DHA and ARA.

DHA+ARA incorporation at the sn-2 position only showed significance in a linear distribution resulting in no significant second-order parameter. The significant first-order parameters were temperature having a negative effect and time having a positive effect.

The \( R^2 \), the fraction of the variation of the response explained by the model, were 0.96 and 0.89 for the total DHA+ARA incorporation and the DHA+ARA incorporation at the sn-2, respectively. Lack of fit values \( (p>0.05) \) indicated that both models were appropriate for the prediction.

**Response surface plots.** The relationship between responses and factors was examined using contour plots for the DHA+ARA incorporation and DHA+ARA incorporation at the sn-2 position. The contour plots describing the interaction of three factors on the incorporation of DHA+ARA and their incorporation at the sn-2 position are shown in
Figs. 3.1 and 3.2, respectively. The contour plots drawn were for the interaction of reaction time (h) with reaction temperature (°C) when the substrate mole ratio was 12 mol/mol, substrate mole ratio with reaction time when temperature was at 60°C, and substrate mole ratio with reaction temperature when reaction time was at 24 h as shown in panels A, B, and C, respectively. As shown in Figs 3.1 A and 3.1C, the incorporation of DHA+ARA increased as temperature increased until the midpoint of response surface was reached. Further increase in temperature, however decreased the incorporation of DHA+ARA (Fig 3.1C). Similar result was previously observed in the study involving the incorporation of EPA +DHA into tripalmitin using Lipozyme® RM IM. The incorporation of EPA+DHA increased as temperature increased from 55-60 °C, but decreased as the temperature increased beyond 60 °C (Sahin et al., 2006). Fig 3.1C showed an optimal condition giving the highest DHA+ARA incorporation of 23.10 g/100g (temperature 60 °C, time 24 h and substrate mole ratio of 18 mol/mol). An increase in temperature also resulted in a higher incorporation of DHA+ARA at the sn-2 position until reaching the midpoint of response surface as shown in Figs 3.2A and 3.2C. The highest DHA+ARA incorporation at the sn-2 was found between 58-60 °C at substrate mole ratio of 18 mol/mol.

As shown in Figs 3.1A and 3.1B, an increase in time after midpoint (18 h), resulted in a higher incorporation of DHA+ARA. The incorporation of DHA+ARA was also found to increase with an increase in the substrate mole ratio (Figs 3.1B and 3.1C). As can be seen in Fig. 3.1B, the substrate molar ratio did not have much effect at the shorter reaction time, but as the reaction time increased between 18-24 h, the incorporation of DHA+ARA increased. Similar characteristic of LCPUFA incorporation was previously
observed in the study by Sahin et al. (2006). Their incorporation of EPA+DHA using Lipozyme RM IM increased as the substrate mole ratio increased with increasing reaction time at 18-30 h, while at the shorter reaction time, the substrate mole ratio did not have much of an effect. The incorporation of DHA+ARA at the sn-2 position increased as time increased, however an increase in substrate mole ratio did not have much effect on the incorporation of DHA+ARA at the sn-2 position (Fig 3.2B).

Validation of model. Verification of the model was performed by carrying out acidolysis experiments at various conditions with RSM. Table 3.2 shows the predicted values and conditions used for the verifications. Verifications fell between the upper and lower limits of the predicted values of total DHA+ARA incorporation and DHA+ARA incorporation at the sn-2 position. However, only one verification value for the incorporation of DHA+ARA at the sn-2 position at 60 °C, substrate mole ratio of 18 mol/mol for 24 h, fell above the upper limit. The model may result in slight error, however, it can still be used to estimate the approximate optimal condition and values of total DHA+ARA incorporation and their content at the sn-2 position (Teichert & Akoh, 2011).

Fatty acid composition and the sn-2 positional fatty acid composition of SL product.

An optimal condition to obtain SL with the highest total DHA+ARA incorporation and the highest incorporation of DHA+ARA at the sn-2 position was generated by the optimizer function of Modde 5.0 (Umetrics, Umeå, Sweden). The condition was determined to be a substrate mole ratio of 18 mol/mol at 60 °C for 24 h with a prediction of 23.10 g/100 g total DHA+ARA incorporation, and 10.28 g/100 g incorporation of DHA+ARA at the sn-2 position. SL produced at this condition was analyzed for its fatty
acid composition and the $sn$-2 positional fatty acid composition (Table 3.3). Major fatty acids composition of the SL were oleic acid 30.33, palmitic acid 27.99, DHA 17.02, linoleic acid 9.05, and ARA 8.05 g/100 g. ARA content in the SL was about one half of the level of DHA. This was taken into consideration in the design to ensure a consumption of higher DHA and lower ARA to promote the anti-inflammatory effect of n-3 LCPUFA (Adam et al., 2003). The ratio of n-6:n-3 of this SL is 1.2. The SL contains higher amount of DHA (11.72 g/100 g) and ARA (5.47 g/100 g) at the $sn$-2 position compared to the levels of DHA (0.4-0.7 g/100 g) and ARA (0.3-0.7 g/100 g) found in the $sn$-2 of human milk fat TAG (Straarup et al., 2006).

In the study by Sahin et al. (2006), Lipozyme RM IM (an $sn$-1, 3 specific lipase) was used to incorporate EPA+DHA into tripalmitin under similar acidolysis conditions (substrate mole ratio of 12-16 mol/mol (LCPUFA free fatty acid mix: tripalmitin), temperature of 55-65 °C, time of 12-24 h, and 10 mg enzyme/100 mg total weight of reactants). A lower range (3.3-7.4 g/100 g) of total EPA+DHA incorporation was observed in their study. Higher incorporation of ARA (8.05 g/100 g)+ DHA (17.20 g/100 g) resulted in the current study and may reflect Novozym 435 as a better biocatalyst to incorporate LCPUFA, particularly the DHA. This agreed with Senanayake & Shahidi (1999) who reported that Novozym 435 gave a higher degree of DHA incorporation compared to Lipozyme RM IM. The fatty acid composition at the $sn$-2 position of SL produced in this study was considerably different from the original palm olein (Table 3.3). DHA (11.72 g/100g) +ARA (5.47 g/100 g) were incorporated in the $sn$-2 position of palm olein glycerol structure. Slightly higher palmitic acid (22.11 g/100 g), and lower oleic acid (44.24 g/100 g) were observed at the $sn$-2 position of the SL.
compared to that of palm olein. The amount of EPA and DHA incorporated into the \textit{sn}-2 of tripalmitin in the study by Sahin et al. (2006) was less than 1 g/100 g. Higher incorporation of DHA+ARA into the \textit{sn}-2 position observed in this study reflects a non-specific characteristic of Novozym 435 to attach these acyl groups to all three different positions of the glycerol backbone.

Palm olein SL enriched with DHA and ARA can be partially or complementarily used in oil blends for food and nutraceutical applications to provide LCPUFA. The content of palmitic, oleic, and linoleic acids in the SL were comparable to the values of human milk fat (23.9, 35.3, and 9.0 g/100 g, respectively) (Hanna et al., 1970). However, further study is needed to increase the content of palmitic acid at the \textit{sn}-2 position. This perhaps can be achieved by a pre-enrichment of palmitic acid at the \textit{sn}-2 position by transesterification of palm olein alone in a reaction with a nonspecific lipase. Another potential nutraceutical application of this SL will be to provide LCPUFA for pregnant women and vegans.

ACKNOWLEDGMENTS

We would like to thank Martek Bioscience Corp. (Columbia, MD) for providing single cell oils DHASCO and ARASCO and IOI Group Loders Croklaan (Channahon, IL) for providing Palm olein (San Trans25). Research was supported in part by Food Science Research, University of Georgia.
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Docosahexaenoic and arachidonic acid content of serum and red blood cell
membrane phospholipids of preterm infants fed breast milk, standard formula or
formula supplemented with n-3 and n-6 long-chain polyunsaturated fatty acids.

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Development, 45*, 581-597.

absorption and lymphatic transport of eicosapentaenoic (EPA), docosahexaenoic


Table 3.1 Central composition face design arrangement with levels of factors and the DHA+ARA incorporation (g/100 g).

<table>
<thead>
<tr>
<th>Experiment no</th>
<th>Temperature (°C)</th>
<th>Time (h)</th>
<th>Substrate mole ratio (mol/mol)(^a)</th>
<th>DHA+ARA incorporation</th>
<th>DHA+ARA at (sn)-2</th>
<th>DHA+ARA at (sn)-1 and (sn)-3(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>12</td>
<td>6</td>
<td>8.80±0.50</td>
<td>4.72±0.54</td>
<td>10.67±0.92</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>12</td>
<td>18</td>
<td>9.52±1.90</td>
<td>6.19±0.20</td>
<td>11.19±2.80</td>
</tr>
<tr>
<td>3</td>
<td>65</td>
<td>12</td>
<td>6</td>
<td>8.61±0.21</td>
<td>3.49±0.77</td>
<td>13.35±3.57</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>12</td>
<td>18</td>
<td>13.90±0.89</td>
<td>4.96±2.55</td>
<td>18.37±2.17</td>
</tr>
<tr>
<td>5</td>
<td>55</td>
<td>24</td>
<td>6</td>
<td>16.08±2.04</td>
<td>9.63±1.68</td>
<td>19.30±3.65</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>24</td>
<td>18</td>
<td>16.45±0.88</td>
<td>9.12±0.68</td>
<td>20.11±1.10</td>
</tr>
<tr>
<td>7</td>
<td>65</td>
<td>24</td>
<td>6</td>
<td>13.16±3.78</td>
<td>6.65±3.68</td>
<td>16.41±7.32</td>
</tr>
<tr>
<td>8</td>
<td>65</td>
<td>24</td>
<td>18</td>
<td>18.70±6.12</td>
<td>8.07±3.86</td>
<td>24.02±7.33</td>
</tr>
<tr>
<td>9</td>
<td>60</td>
<td>18</td>
<td>6</td>
<td>12.47±1.55</td>
<td>6.70±0.99</td>
<td>15.36±1.83</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>18</td>
<td>18</td>
<td>18.33±0.87</td>
<td>8.50±0.85</td>
<td>23.25±1.59</td>
</tr>
<tr>
<td>11</td>
<td>55</td>
<td>18</td>
<td>12</td>
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<td>7.70±1.82</td>
<td>13.39±2.45</td>
</tr>
<tr>
<td>12</td>
<td>65</td>
<td>18</td>
<td>12</td>
<td>11.01±0.75</td>
<td>4.27±0.50</td>
<td>14.38±1.37</td>
</tr>
<tr>
<td>13</td>
<td>60</td>
<td>12</td>
<td>12</td>
<td>14.94±4.04</td>
<td>4.40±0.34</td>
<td>20.21±6.23</td>
</tr>
<tr>
<td>14</td>
<td>60</td>
<td>24</td>
<td>12</td>
<td>23.20±6.71</td>
<td>10.40±1.55</td>
<td>30.05±9.32</td>
</tr>
<tr>
<td>15</td>
<td>60</td>
<td>18</td>
<td>12</td>
<td>17.15±1.51</td>
<td>10.41±0.86</td>
<td>20.52±1.92</td>
</tr>
<tr>
<td>16</td>
<td>60</td>
<td>18</td>
<td>12</td>
<td>16.88±2.40</td>
<td>8.79±0.86</td>
<td>20.93±3.56</td>
</tr>
<tr>
<td>17</td>
<td>60</td>
<td>18</td>
<td>12</td>
<td>15.06±2.79</td>
<td>8.57±0.88</td>
<td>18.31±3.75</td>
</tr>
</tbody>
</table>

\(^a\) Substrate mole ratio of DHA+ARA free fatty acids to palm olein. \(^b\) \(sn\)-1,3 (g/100g) = [3 x total (g/100g) - \(sn\)-2 (g/100g)]/2.
Table 3.2 Predicted and observed values from RSM model verification.

<table>
<thead>
<tr>
<th>Mole ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Conditions</th>
<th>Predicted</th>
<th>Observed</th>
<th>Predicted</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mole ratio&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Time &lt;sup&gt;(h)&lt;/sup&gt;</td>
<td>Temp &lt;sup&gt;(°C)&lt;/sup&gt;</td>
<td>DHA+ARA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>DHA+ARA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>17</td>
<td>65</td>
<td>11.11</td>
<td>9.05</td>
<td>13.18</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
<td>55</td>
<td>8.89</td>
<td>6.04</td>
<td>11.75</td>
</tr>
<tr>
<td>18</td>
<td>24</td>
<td>60</td>
<td>23.10</td>
<td>20.68</td>
<td>25.51</td>
</tr>
<tr>
<td>6</td>
<td>22</td>
<td>60</td>
<td>16.92</td>
<td>14.87</td>
<td>18.98</td>
</tr>
</tbody>
</table>

<sup>a</sup>Substrate mole ratio of free fatty acids to palm olein.  
<sup>b</sup>Total DHA and ARA content in TAG(g/100 g).  
<sup>c</sup>Lower limit (g/100 g).  
<sup>d</sup>Upper limit (g/100 g).  
<sup>e</sup>DHA+ARA at sn-2 position (g/100 g).
Table 3.3 Fatty acid composition of original oils, and structured lipid and their \( sn-2 \) positional fatty acid composition (g/100g).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Palm olein</th>
<th>( sn-2 ) palm olein</th>
<th>DHASCO(^a)</th>
<th>( sn-2 ) DHASCO</th>
<th>ARASCO(^b)</th>
<th>( sn-2 ) ARASCO</th>
<th>SL(^c)</th>
<th>( sn-2 ) SL(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>1.04±0.00</td>
<td>-</td>
<td>9.38±0.00</td>
<td>2.18±0.86</td>
<td>0.28±0.00</td>
<td>-</td>
<td>0.98±0.60</td>
<td>1.39±1.06</td>
</tr>
<tr>
<td>C16:0</td>
<td>43.60±0.01</td>
<td>13.79±0.18</td>
<td>7.47±0.00</td>
<td>-</td>
<td>8.19±0.00</td>
<td>-</td>
<td>27.99±5.11</td>
<td>22.11±0.78</td>
</tr>
<tr>
<td>C18:0</td>
<td>4.53±0.00</td>
<td>0.87±0.03</td>
<td>0.87±0.00</td>
<td>-</td>
<td>8.23±0.00</td>
<td>0.68±0.32</td>
<td>3.21±0.70</td>
<td>4.13±1.49</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>40.91±0.01</td>
<td>66.38±0.12</td>
<td>27.63±0.02</td>
<td>54.49±1.87</td>
<td>21.92±0.00</td>
<td>45.48±0.83</td>
<td>30.33±1.74</td>
<td>44.24±0.26</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>9.92±0.01</td>
<td>18.96±0.15</td>
<td>1.31±0.00</td>
<td>-</td>
<td>6.95±0.00</td>
<td>15.33±0.83</td>
<td>9.05±3.45</td>
<td>10.96±0.13</td>
</tr>
<tr>
<td>C18:3n-6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.73±0.00</td>
<td>4.39±0.11</td>
<td>3.17±1.15</td>
<td>-</td>
</tr>
<tr>
<td>C20:3n-6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.43±0.00</td>
<td>1.29±0.28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>43.27±0.00</td>
<td>32.83±1.72</td>
<td>8.05±0.66</td>
<td>5.47±0.29</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>-</td>
<td>-</td>
<td>44.89±0.02</td>
<td>42.64±0.89</td>
<td>-</td>
<td>-</td>
<td>17.20±2.45</td>
<td>11.72±0.21</td>
</tr>
</tbody>
</table>

\(^a\) Others for DHASCO include C8:0, C10:0, C12:0, C14:1, C17:0, C20:1, C22:0, and C20:5n-3

\(^b\) Others for ARASCO include C16:1, C17:0, C20:0, C20:1, C22:0, and C24:0

\(^c\) SL produced at 60°C, 24 h, and substrate mole ratio of 18 (mol/mol)
Figure 3.1 Contour plots of the total incorporation of DHA+ARA. (3.1A) reaction time (h) versus reaction temperature (°C) when the substrate mole ratio of free fatty acid to palm olein was 12 (mol/mol), (3.1B) substrate mole ratio versus reaction time at a reaction temperature of 60 °C, and (3.1C) substrate mole ratio versus reaction temperature at a reaction time of 24 h.
Figure 3.1

3.1A

3.1B

3.1C
Figure 3.2 Contour plots of the incorporation of DHA+ARA at the sn-2 position. (3.2A) reaction time (h) versus reaction temperature (°C) when the substrate mole ratio of free fatty acid to palm olein was 12 (mol/mol), (3.2B) substrate mole ratio versus reaction time at a reaction temperature of 60 °C, and (3.2C) substrate mole ratio versus reaction temperature at a reaction time of 24 h.
Figure 3.2

3.2A

3.2B

3.2C
CHAPTER 4

PRODUCTION AND CHARACTERIZATION OF DHA AND GLA-ENRICHED
STRUCTURED LIPID FROM PALM OLEIN FOR INFANT FORMULA USE

S. Nagachinta · C.C. Akoh. Accepted by the Journal of American Oil Chemists’ Society, 04/12/2013
ABSTRACT

Palm olein was modified via lipase-catalyzed acidolysis reaction to obtain fatty acid composition and positional distribution similar to human milk fat. In the reaction, a free fatty acid mix containing 23.23% docosahexaenoic (DHA), 31.42% gamma-linolenic (GLA), and 15.12% palmitic acid was employed. The DHA and GLA were incorporated into the structured lipid (SL) product to improve its nutritional value. Response surface methodology (RSM) was used to investigate the effects of reaction time and substrate mole ratio (palm olein to a free fatty acid mix) on the amount of palmitic acid at the sn-2 position of SL triacylglycerols (TAGs), and on the total DHA and GLA incorporation. Gram-scale production of SL was performed using the conditions predicted by RSM to maximize the content of palmitic acid at the sn-2 position. Verification of the predictions from RSM confirmed its practical utility. The resulting SL had 35.11% palmitic acid at the sn-2 position, with 3.75% DHA and 5.03% GLA. Differential scanning calorimetry and HPLC analyses of the TAGs revealed changes in their polymorphic profiles and TAG molecular species of SL compared to palm olein. The SL from this study can potentially be used in infant formula formulations.

Keywords: Acidolysis, human milk fat, palm olein, structured lipid, docosahexaenoic acid, gamma-linolenic acid
INTRODUCTION

Palm olein, a low-melting fraction of palm oil, is used in vegetable oil blends as fat source for infant formula to provide similar proportions of palmitic and oleic acids to those found in human milk fat (HMF). Despite the similarities in fatty acid profile obtained, a lower absorption of fat and calcium has been reported for infants fed with formula containing a substantial proportion of palm olein (1, 2). Lower bone mineralization, constipation, and hardening of stool were associated with the feeding of palm olein containing formula (3, 4). Contrary to HMF, which contains palmitic acid predominantly at the sn-2 position (5), palm olein has most of its palmitic acid at the sn-1,3 positions (6). Palmitic acid located at the sn-1,3 position is absorbed to a lower degree by rats (7, 8) and human infants (9, 10) than when it is predominantly present at the sn-2 position. Lower calcium absorption was presumed to be a result of insoluble calcium soaps formation (1).

Enzymatic modification of triacylglycerols (TAGs) has been used to change the fatty acid (FA) composition and/or FA position in the glycerol backbone of fats and oils. Betapol™ (IOI-Loders Croklaan, IL) is an example of a structured lipid (SL), which mimics the specific structure of HMF, and closely matches its FA composition. The predominant TAGs (1,2-dipalmitoyl 3-oleoyl and 1,3-dioleoyl 2-palmitoyl) in Betapol were obtained from acidolysis of vegetable oil and oleic acid catalyzed by an sn-1,3 specific lipase (11, 12). Previous studies on SLs production from palm olein include structural modification to produce HMF analog, incorporation of long-chain polyunsaturated fatty acids (LCPUFAs) to provide health benefits, and incorporation of medium-chain FAs to provide a rapid energy source (13-15). LCPUFAs, such as
docosahexaenoic acid (DHA) and arachidonic acid (ARA), are not traditionally present in infant formulas prepared with blends of vegetable oils. Both FAs are essential structural membrane lipids. They are present at high levels in the cerebral cortex and retina, and support the development and protection of neonatal neurological and cognitive functions (16). Studies on DHA and ARA supplementation and neurodevelopment of infants reported a significant relationship between DHA and ARA consumption and improved visual acuity, and vocabulary comprehension (17, 18). The n-6 polyunsaturated fatty acid (PUFA), gamma-linolenic acid (GLA), is an intermediate in the bioconversion of linoleic acid (LA) to ARA. Term infants fed DHA and GLA supplemented formula (DHA 0.3%, GLA 0.5 %) had a higher concentration of DHA and ARA in red blood cells than those fed a standard formula (19). The reported amounts of DHA and GLA in human milk were 0.15-0.92% and 0.06-0.13%, respectively (20). Recent studies on production of HMF analogs, include DHA and GLA-enriched HMF analog from stearidonic acid-enriched soybean oil (21), DHA and ARA-enriched HMF analog from hazelnut oil (22), and DHA-enriched HMF analog from tuna oil (23). The addition of these beneficial PUFAs to infant formula would provide infants with benefits from LCPUFAs consumption. The aim of this study was to increase the palmitic acid content at the sn-2 position of palm olein to a level close to that of HMF, and to incorporate DHA and GLA into palm olein by acidolysis reaction. The rearrangement of fatty acids in palm olein can be achieved by using a non-specific lipase, as the enzyme performs both hydrolysis and esterification. Non-specific lipase was therefore chosen to rearrange the position of palmitic acid, as well as randomly incorporate DHA and GLA into the glycerol backbone. Previously, response surface methodology (RSM) was used for SL synthesis in
other studies to model and optimize reaction conditions (15, 24). The optimal
temperature for incorporation of DHA and ARA into palm olein (60°C) was previously
reported (15). In the present study, incubation time and substrate mole ratio were
selected as the independent variables. RSM predictions were evaluated and gram-scale
production of SL was performed using the predicted optimal conditions to maximize the
palmitic acid content at the sn-2 position. The product mixture was purified by short-path
distillation. The SL product was characterized for FA composition (using gas
chromatography, GC), TAG molecular species (using high performance liquid
chromatography, HPLC), and thermal characteristics (using differential scanning
calorimetry, DSC).

**MATERIALS AND METHODS**

**Materials.** Palm olein (San Trans25) and DHA-containing single cell oil (DHASCO®,
40% DHA) from algal (*Cryptothecodinium cohnii*) source were kindly provided by IOI-
Loders Croklaan (Channahon, IL) and Martek Bioscience Corp. (Columbia, MD),
respectively. Borage oil GLA in free fatty acid form (70% GLA) was purchased from
Sanmark (Greensboro, NC). Palmitic acid (95% pure) was purchased from Alfa Aesar
(Heysham, Lancashire, UK). Novozym® 435 (a non-specific lipase) was a gift from
Novo Nordisk A/S (Bagsvaerd, Denmark). Lipid standards: Supelco 37 Component
FAME mix (≥99% pure), triolein (99% pure), 2-oleoylglycerol (95% pure), TAG
standard mix (≥99% pure) were purchased from Sigma-Aldrich Chemical Co. (St. Louis,
MO), and Nu-chek Prep, Inc. (Elysian, MN). Other solvents and chemicals were
purchased from Fisher Scientific (Norcross, GA) and Sigma-Aldrich Chemical Co.
**Preparation of FFAs from DHASCO.** DHASCO was converted to FFAs according to Vázquez and Akoh (25) with modifications. One hundred and fifty grams of oil was saponified using a mixture of KOH (34.5 g), distilled water (66 mL), 96% ethanol (396 mL), and butylated hydroxytoluene (0.03 g), by placing the reagents in a 1 L stirred batch reactor with a circulating water bath at 60 °C, for 1 h. The reaction was then stopped by adding 120 mL of distilled water. The hydroalcoholic mixture was acidified by adding 6 M HCl and adjusted to pH 2 to release the FFAs. The top layer containing the FFAs was removed and washed with 100 mL of distilled water. The bottom layer was extracted twice with 200 mL of hexane. The hexane layer was collected, pooled together with the previously extracted FFA layer, and filtered through a sodium sulfate column. Hexane was removed using a rotary evaporator at 40 °C. FFAs were stored in an amber Nalgene bottle under nitrogen at -20 °C until use.

**Miligram-Scale Acidolysis Reaction.** The acidolysis reaction mixture consisted of palm olein and FFA mix at different substrate mole ratios (Table 4.1), as previously determined by RSM, in n-hexane (3 mL). The FFA mix contained 23.23% DHA, 31.42% GLA, and 15.12% palmitic acid. The reactants were placed in screw-capped test tubes, and immobilized lipase, Novozym 435 (10% weight of total reactants) was added. The amount of lipase was selected based on our previous work (15). The specific activity of Novozym 435 was 10,000 PLU/g (PLU is propyl laurate units). The tubes were incubated in an orbital shaking water bath at 60 °C and 200 rpm (1.27 cm diameter orbit). All reactions were performed in triplicate and average results and standard deviations reported.
Experiment Design for RSM Study. RSM was applied to investigate the effects of substrate mole ratio (Sr) of palm olein to FFA mix, and reaction time (t), on the amount of palmitic acid at the $sn$-2 position of the produced SL. RSM was also employed to study the incorporation of DHA and GLA into the SL, and to predict a model for the reaction conditions. The central composite face design consisted of 11 experimental runs with 3 center points, and these were generated by using Modde 5.0 (Umetrics, Sweden) software. The levels for the two variables were: Sr (palm olein/FFA mix 0.5-2 mol/mol) and t (12-24 h). The independent variables and experimental design are shown in Table 4.1.

Analysis of Acidolysis Products. After enzymatic reaction, the resulting product was concentrated to half of its volume under nitrogen and spotted onto silica gel G TLC plates (20 x 20 cm, layer thickness 250 µm, Analtech, Inc, Newark, DE). A mixture of petroleum ether: diethyl ether: acetic acid (70:30:0.5, v/v/v) was used to separate the TAG (15) from other reaction products. The TAG band was identified using triolein as standard and visualized under UV light after spraying the plates with 0.2% 2, 7-dichlorofluorescein in methanol. The TAG band was recovered into test tube for conversion to fatty acid methyl esters (FAME) and positional analysis. TAG sample was converted to FAME following AOAC official method 996.01 (26) with modification (27). Briefly, 200 µL of C15:0 in hexane (20 mg/ml) was added as internal standard (15) to the recovered band. Sample was dried with nitrogen to remove the solvent, and 2 mL of 0.5 M NaOH in methanol was added followed by incubation for 5 min at 100°C. After the incubation, 2 mL of 14% boron trifluoride (BF$_3$) in methanol was added. The sample was vortexed for 1 min and incubated at the same condition to allow methylation. To stop
the reaction and extract the FAME, 2 mL each of hexane and saturated NaCl solution were added. The sample was vortexed for 2 min and then centrifuged for 5 min at 1,000 rpm (approximately 100x g). The upper organic layer was recovered in a GC vial for analysis. The FAME external standard, Supelco 37 component FAME mix was run parallel with the samples for FAs identification.

**Pancreatic Lipase Catalyzed sn-2 Positional Analysis.** The pancreatic lipase-catalyzed hydrolysis of TAG was as described by Pina-Rodriguez and Akoh (27). Briefly, sample was extracted twice from the recovered TAG bands on TLC using 1.5 ml of diethylether. Sample was completely dried under nitrogen. Forty milligrams of purified pancreatic lipase (porcine pancreatic lipase, crude type II), 1 ml of Tris buffer (pH 8.0), 0.20 ml of 0.05% sodium cholate, and 0.1 ml of 2.2% calcium chloride were added to the sample. The mixture was incubated at 40°C in a water bath for 3 min. Once completed, 1 ml of 6 M HCl and 4 ml of diethyl ether were added and centrifuged at 1000 rpm (approximately 100 x g). The upper layer containing lipid components was concentrated with nitrogen. The concentrated extract was spotted on silica gel G TLC plates and developed with a mixture of hexane: diethyl ether: formic acid (60:40:1.6, v/v/v). 2-Oleoylglycerol was spotted in parallel as identification standard for 2-monoacylglycerol (2-MAG). The bands corresponding to 2-MAG were collected and converted into FAME for FA composition analysis as described in the previous section.

**Fatty Acid Composition Analysis.** The fatty acid composition of single cell oils, palm olein, and acidolysis products were analyzed on a 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 for FA separation. Injection of 1
µL of sample was made at a split ratio of 20:1. Helium was the carrier gas at the 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. Relative fatty acid content was calculated on the basis of response factors and conversion factors using C15:0 as internal standard. The average and standard deviation of triplicate analyses were reported.

**Model Verification.** To verify the model, five acidolysis reactions were carried out in test tubes at random conditions, as well as at the optimal condition suggested by RSM. The experimental values were then compared to the values predicted by the model, as shown in Table 4.2.

**Gram-Scale Production of SL.** The solvent-free acidolysis reaction was performed in a 1 L stirred batch reactor at 60 °C using a substrate mole ratio of 2 (palm olein: FFA mix) and Novozym 435 (10% weight of total reactants) as biocatalyst. The reaction was incubated for 22.7 h with constant stirring, at 200 rpm. At the end of the reaction, the resulting mixture of SL and substrates was 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 enzyme. Short-path distillation (KDL-4 unit, UIC Inc.) was used to remove FFAs from the SL under the following conditions: holding temperature: 60 °C; feeding rate: ~100 mL/h; heating oil temperature: 185 °C; coolant temperature: 15-20 °C; and vacuum: <100 mTorr. After short-path distillation, the FFA content was determined according to AOCS Official Method Ac 5-41 (28). The SL obtained was stored under nitrogen at -80 °C until further use.
**TAG Molecular Species Analysis.** Reversed phase HPLC, with a Sedex 85 evaporative light-scanning detector (ELSD), was used to analyze the TAG molecular species of SL, following the method described by Pande and Akoh (29), with modifications as described here. The analysis was performed on an Ultrasphere C18, 250 mm, 4.6 mm, 5 µm particle size column. The column temperature was kept at 30 °C. The ELSD conditions were 70 °C, nitrogen pressure 3.0 bar, and gain of 7. Sample concentration was 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; 55 min, 95% B, and 65 min, 65% B, following by a delay time before next injection of 10 min. The equivalent carbon number (ECN) method was used to predict the elution order of TAG species. Standards: TAG mix containing trilinolenin (ECN=36), trilinolein (42), triolein (48), tripalmitin (48), tristearin (54), and triarachidin (60) as well as palm olein were also chromatographed to help identify the TAG molecular species.

**Melting and Crystallization Profiles.** Melting and crystallization profiles were determined for palm olein and SL using a differential scanning calorimeter (DSC 1 STAR® System, Mettler Toledo, Columbus, OH), 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 (28) with minor modifications using indium as calibration standard. The sample weight ranged from 8-12 mg and sample was sealed in aluminum pans. The sample was heated from 25 to 80 °C at 50 °C/min, held for 10 min (to destroy any previous crystalline structure), cooled from 80 to -55 °C at 10 °C/min (for crystallization profiles), held for 30 min, and then heated from -55 to 80 °C at 5 °C/min (for melting profiles).
**Statistical Analysis.** All analyses, except melting and crystallization profiles, were performed in triplicate. Melting and crystallization profiles were performed in duplicate. Average values and standard deviations were determined. The analysis of variance (ANOVA) and the mathematical model for optimization were carried out using Modde 5.0 (Umetrics, Sweden).

**RESULTS AND DISCUSSION**

**Model Fitting.** RSM experimental design was applied in this study to obtain the predictive models for palmitic acid content at the sn-2 position and the total DHA and GLA incorporation in SL. Time and substrate mole ratio were described as two independent variables. Table 4.1 shows the two responses observed in the experiments 1) palmitic acid content at the sn-2 position, and 2) the total DHA and GLA incorporation. Multiple linear regression and backward selection method were used to fit the results into a second-order polynomial model. For palmitic acid content at the sn-2 position, the first-order parameter with $p$-value $<0.01$ was time (t) and this had a positive effect. The significant second-order parameter was the second-order term of time ($t^2$), which had a negative effect. The model equation for palmitic acid content at the sn-2 position is as follows: Palmitic acid at sn-2 = $31.61 +3.85t -2.57 t^2$; where t=time. For total DHA and GLA incorporation, time and substrate mole ratio were the significant first-order parameters with $p$-value $<0.01$. Time had a positive effect on the total DHA and GLA incorporation, but substrate mole ratio had a negative effect. The significant second-order parameters were the second-order term of substrate mole ratio ($Sr^2$) and the interaction term of time and substrate mole ratio ($t* Sr$). Total DHA and GLA incorporation was negatively correlated to both of these second-order terms. The model equation for total
DHA and GLA incorporation can be written as follows: Total DHA and GLA incorporation = 11.33 +0.96t-5.90Sr+2.49Sr²-0.90t* Sr; where t=time and Sr=substrate mole ratio. The R², fraction of the variation for the response explained by the model, were 0.90 and 0.99 for palmitic acid content at the sn-2 position and total DHA and GLA incorporation, respectively. Lack of fit values (p>0.05) indicated that both models were appropriate for the prediction (30, 31).

**Optimization of the Reaction.** Contour plots describing the interaction of time and substrate mole ratio with 1) palmitic acid content at the sn-2 position, and 2) total DHA and GLA incorporation, are shown in Figures 4.1a, and b, respectively. Palmitic acid content at the sn-2 position increased as time and substrate mole ratio (palm olein: FFA mix) increased (Figure 4.1a). A higher substrate mole ratio means more palmitic acid from palm olein was present in the reaction. Higher substrate mole ratio resulted in a higher palmitic acid content at the sn-2 position of the SL. Our result is in agreement with Teichert and Akoh (24), who reported a positive correlation between the amount of palmitic acid substrate, and the level of palmitic acid incorporation at the sn-2 position. Total DHA and GLA incorporation slightly increased as time increased (Figure 4.1b). Longer residence times allow for prolonged contact between the enzyme and the substrates, leading to an increase in fatty acids incorporation (32). The total incorporation of DHA and GLA increased as the ratio of FFA mix to palm olein increased (that is, as the palm olein: FFA mix ratio decreased). Table 4.1 shows a higher incorporation of GLA compared to DHA. It is possible that the higher incorporation of GLA was due to its higher content in the FFA mix compared to DHA (23.23% DHA and 31.42 % GLA). The enzyme may have exhibited substrate preference over GLA, however due to the
difference in the amount of DHA and GLA in the reaction, we cannot conclude such activity.

The aim of this study was to increase palmitic acid content at the sn-2 position of palm olein glycerol backbone, and incorporate DHA and GLA into palm olein via acidolysis reaction. RSM predicted the highest content of palmitic acid at the sn-2 position to be 34.86%, at an incubation time of 22.7 h, and a substrate mole ratio of 2. Under these conditions, the predicted total DHA and GLA incorporation was 7.77 %. These parameters were used for model validation and gram-scale production of SL.

**Validation of Model.** Acidolysis reactions were carried out in test tubes (miligram-scale) at various conditions including the optimal conditions obtained with RSM in order to verify the model. Furthermore, the optimal conditions were used for gram-scale production of SL. The results of model verification in miligram-scale, and gram-scale production, are given in Table 4.2. Verification fell within the upper and lower limits of the predicted values of total DHA and GLA incorporation and palmitic acid content at the sn-2, indicating the usefulness of RSM prediction to estimate values of the responses (24).

**Fatty Acid and sn-2 Positional Fatty Acid Composition of Substrates and SL.** The fatty acid composition and distribution of palm olein and SL are shown in Table 4.3. Major fatty acids in palm olein were palmitic (43.60%), oleic (40.91%), and linoleic (LA) acids (9.92%). Despite being the most abundant fatty acid, palmitic acid was found at only 13.79% at the sn-2 position of palm olein glycerol backbone. The major fatty acids at the sn-2 position were the unsaturated oleic (66.38%) and linoleic acids (18.96%). HMF has most of its palmitic acid (greater than 60%) at the sn-2, whereas the unsaturated
fatty acids are located at the outer positions. Lower absorption of fat in formula-fed infants was attributed to the differences in stereospecific structure of the TAGs of vegetable oils and HMF (33). Acidolysis experiments using palm olein and FFAs mixture of DHA (23.23%), GLA (31.42%), and palmitic acid (15.12%) were performed to increase sn-2 palmitic acid content in palm olein and incorporate DHA and GLA. The resulting SL produced at the optimal conditions selected by RSM contained 35.11% palmitic acid at the sn-2 position compared to 13.79% in original palm olein. Oleic acid at the sn-2 position of palm olein decreased from 66.38 to 33.99%. The nutritional value of palm olein was improved by the addition of PUFAs including 3.75 DHA, 5.03 GLA, and 10.09% LA. DHA and GLA levels found in human milk were 0.15-0.92% and 0.06-0.13%, respectively (20). Although the content of palmitic acid at the sn-2 position of SL (35.11%) is still lower than the level of HMF (greater than 60%), it is an increase of 150% relative to the initial level in palm olein (13.79%). The three major FAs in HMF are palmitic (15.64-21.17%), oleic (28.30-38.83 %), and LA (8.80-16.59%) acids (20). These are also the major FAs in the SL (Table 4.3). The contents of oleic (36.40%) and LA (10.09%) acids in SL are in the range of those in HMF. However, palmitic acid (37.55%) is higher than the range in HMF. The possible use of this SL is therefore in an oil blend for infant formula to provide the FAs in HMF, the preferred sn-2 palmitic acid, and the beneficial DHA and GLA.

**HPLC TAG Molecular Species Identification.** TAG molecular species of palm olein and its SL product were determined using reversed-phased HPLC. Peak identifications were made according to published reports that used palm oil and palm olein (14, 34), the elution time of TAG standards, and the fact that TAG species elute in order of equivalent
carbon number (ECN)= TC-2xDB; TC is the total carbon number of acyl group and DB is the total number of double bonds in TAG) (35). Table 4.4 shows a comparison between TAG molecular species and their relative percentages in palm olein and SL. The main TAG molecular species of palm olein were PPO, POO, PPL, and POL. These TAGs were also predominant in the SL product, however their abundance changed drastically. The amount of PPO and POO were reduced from 61.01 to 28.99% for PPO and 34.24 to 24.96% for POO. PPL increased from 1.76 to 3.97%. Similarly POL increased from 1.50 to 9.58%. SL contained up to 29 different TAG molecular species. Most of these TAGs contained more than 3 DB in their structure, only two contained no DB (MMP=1.69% and PPP=5.23%). The variety of TAG species, fatty acid chain length, and degree of saturation were shown to affect melting and crystallization profile of fats and oils (14, 36-38).

SL Melting and Crystallization Profiles. Both cooling and heating thermograms of SL were broader and contained more peaks than those of palm olein. The multiple peaks observed in thermograms can be attributed to the complexity of TAGs distribution in vegetable oils (37). Palm olein exhibited one major exothermic peak (with shoulder peaks) in the crystallization profile, whereas in SL, two major peaks were observed (Figure 4.2). Palm olein major exothermic peak at 3.52 °C and its shoulder peak at -4.52 °C were close to the first major exothermic peak of SL (2.85 °C) and its shoulder peak (-5.19 °C), indicating that they both have the same types of polymorphic forms. Cooling thermogram of RBD palm olein in the study by Che Man et al (39) indicated that these low temperature peaks represented polymorphs β2' and α. The second major peak in the SL crystallization profile was new compared to palm olein and at a higher temperature
(20.29 °C), indicating a change in polymorphic profile as a result of enzymatic modification of the TAG species. TAGs species analysis by HPLC revealed a significant amount of trisaturates (PPP, 5.23% and MMP, 1.69%). These highly saturated TAGs represent this second peak at 20.29 °C. For melting profile, SL started to melt at a lower temperature (2.18 °C) compared to the onset melting temperature of palm olein (4.19 °C). This melting behavior is due to the presence of highly unsaturated (DGD, GGD) TAGs in SL. Both SL and palm olein have similar melting peaks between 4 to 12°C. However, SL had two shoulder peaks (22.97 and 39.93 °C) reflecting the presence of highly saturated TAGs.

**CONCLUSION**

In this study, palm olein was modified via lipase-catalyzed acidolysis reaction with a FFA mix of DHA, GLA, and palmitic acid. The experiment was designed to study the effect of two experimental conditions 1) substrate mole ratio and 2) reaction time, on both the content of palmitic acid at the sn-2 position and total incorporation of DHA and GLA in the obtained SL. The experimental conditions were optimized to increase the palmitic acid content at the sn-2 position using RSM. The resulting SL contained 35.11% palmitic acid at the sn-2 position with 3.75 %DHA and 5.03 %GLA.

DHA and GLA were incorporated into the TAGs of this SL to improve its nutritional value. This SL can be used in a fat blend for infant formula to provide TAGs with a higher content of palmitic acid at the sn-2 position for better fat absorption. This SL would also provide DHA and GLA to support infant development.
ACKNOWLEDGEMENTS

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REFERENCES

docosahexaenoic acids at sn-2 position and oleic acid at sn-1,3 positions. LWT - Food Sci & Technol 44:1986-1992


Table 4.1 Percent incorporation of DHA, GLA, total DHA and GLA, and \( sn-2 \) palmitic acid in SL by acidolysis using RSM conditions\(^a\)

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Time (h)</th>
<th>Mole ratio(^b)</th>
<th>DHA (%)</th>
<th>GLA (%)</th>
<th>Total DHA and GLA (%)</th>
<th>( sn-2 ) Palmitic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>0.50</td>
<td>6.55±0.51</td>
<td>10.53±0.73</td>
<td>17.08±1.22</td>
<td>23.49±1.92</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>0.50</td>
<td>9.07±0.17</td>
<td>12.29±0.75</td>
<td>21.37±0.59</td>
<td>32.00±2.38</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>2.00</td>
<td>2.84±0.12</td>
<td>4.44±0.12</td>
<td>7.27±0.25</td>
<td>25.76±1.27</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>2.00</td>
<td>3.14±0.02</td>
<td>4.82±0.19</td>
<td>7.96±0.17</td>
<td>34.88±0.41</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>1.25</td>
<td>4.22±0.18</td>
<td>6.60±0.28</td>
<td>10.82±0.46</td>
<td>27.39±0.59</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>1.25</td>
<td>4.67±0.07</td>
<td>6.92±0.10</td>
<td>11.58±0.18</td>
<td>32.89±0.63</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>0.50</td>
<td>7.89±0.19</td>
<td>12.19±0.26</td>
<td>20.08±0.43</td>
<td>32.39±0.57</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>2.00</td>
<td>3.12±0.04</td>
<td>4.78±0.10</td>
<td>7.90±0.14</td>
<td>34.12±1.07</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>1.25</td>
<td>4.37±0.09</td>
<td>6.75±0.10</td>
<td>11.13±0.19</td>
<td>30.80±0.85</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>1.25</td>
<td>4.34±0.05</td>
<td>6.74±0.03</td>
<td>11.08±0.04</td>
<td>31.62±1.11</td>
</tr>
<tr>
<td>11</td>
<td>18</td>
<td>1.25</td>
<td>4.47±0.24</td>
<td>6.95±0.40</td>
<td>11.42±0.65</td>
<td>30.20±1.27</td>
</tr>
</tbody>
</table>

\(^a\) Incubation temperature was 60 °C. \(^b\) Substrate mole ratio of palm olein to FFA mix containing 23.23% DHA, 31.42% GLA, and 15.12% palmitic acid. Reactions were carried out on the mg-scale.
Table 4.2 Predicted and observed values (%) from RSM model verification.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Predicted</th>
<th>Observed</th>
<th>Predicted</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time (h)</td>
<td>Mole ratio(^a)</td>
<td>sn-2 PA(^b)</td>
<td>LL</td>
</tr>
<tr>
<td>12</td>
<td>2.00</td>
<td>26.72</td>
<td>23.08</td>
<td>30.36</td>
</tr>
<tr>
<td>18</td>
<td>1.00</td>
<td>31.28</td>
<td>29.24</td>
<td>33.33</td>
</tr>
<tr>
<td>24</td>
<td>1.00</td>
<td>32.52</td>
<td>29.63</td>
<td>35.41</td>
</tr>
<tr>
<td>20</td>
<td>1.25</td>
<td>32.61</td>
<td>30.56</td>
<td>34.56</td>
</tr>
<tr>
<td>22.7(^f)</td>
<td>2.00</td>
<td>34.86</td>
<td>31.73</td>
<td>37.99</td>
</tr>
</tbody>
</table>

\(^a\)Substrate mole ratio of palm olein to FFA mix. \(^b\)Palmitic acid at sn-2 position (%). \(^c\)Lower limit (%). \(^d\)Upper limit (%). \(^e\)Total DHA and GLA content in SLs (%). \(^f\)Optimal conditions predicted by RSM and reaction performed in test tubes.
Table 4.3 Total and positional distribution of fatty acids (%) of substrates and produced SL.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Palm olein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>free DHA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>free GLA&lt;sup&gt;c&lt;/sup&gt;</th>
<th>free PA</th>
<th>FFA mix&lt;sup&gt;d&lt;/sup&gt;</th>
<th>SL&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>sn-2</td>
<td>Total</td>
<td>sn-2</td>
<td>Total</td>
<td>sn-2</td>
</tr>
<tr>
<td>C12:0</td>
<td>-</td>
<td>-</td>
<td>4.83±0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C14:0</td>
<td>1.04±0.00</td>
<td>-</td>
<td>10.77±0.02</td>
<td>-</td>
<td>0.64±0.00</td>
<td>5.16±0.00</td>
</tr>
<tr>
<td>C16:0</td>
<td>43.60±0.01</td>
<td>13.79±0.18</td>
<td>9.61±0.15</td>
<td>-</td>
<td>98.91±0.02</td>
<td>15.12±0.08</td>
</tr>
<tr>
<td>C18:0</td>
<td>4.53±0.00</td>
<td>0.87±0.03</td>
<td>0.92±0.10</td>
<td>-</td>
<td>0.36±0.01</td>
<td>-</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>40.91±0.01</td>
<td>66.38±0.12</td>
<td>17.80±0.12</td>
<td>1.66±0.12</td>
<td>-</td>
<td>9.14±0.03</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>9.92±0.01</td>
<td>18.96±0.15</td>
<td>1.01±0.12</td>
<td>25.45±0.07</td>
<td>-</td>
<td>11.39±0.06</td>
</tr>
<tr>
<td>C18:3n-6</td>
<td>-</td>
<td>-</td>
<td>0.17±0.01</td>
<td>71.63±0.07</td>
<td>-</td>
<td>31.42±0.09</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>-</td>
<td>47.58±0.42</td>
<td>-</td>
<td>-</td>
<td>23.23±0.19</td>
<td>3.75±0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data from previous research (15).  
<sup>b</sup> Others include: C8:0, C10:0, C16:1, C17:0, C20:1, C22:0, and C20:5n-3  
<sup>c</sup> Others include: C21:0 and C20:2.  
<sup>d</sup> FFA mix of DHA:GLA:PA, Others include: C8:0, C10:0, C12:0, C14:0, C16:1, C17:0, C21:0, and C20:1.  
<sup>e</sup> SL from gram-scale (1L) production.
Table 4.4 TAG molecular species of palm olein and SL determined by RP-HPLC according to their ECN\(^a\)

<table>
<thead>
<tr>
<th>TAG Species(^b)</th>
<th>ECN</th>
<th>DB</th>
<th>Relative %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Palm olein</td>
</tr>
<tr>
<td>DGD</td>
<td>32</td>
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\(^a\) Equivalent carbon number (ECN)= TC-2xDB; TC is the total carbon number of acyl group and DB is the total number of double bonds in TAG. \(^b\)La= C12:0, M=C14:0, P=C16:0, S=C18:0, O=C18:1 n-9, L=C18:2 n-6, G=C18:3 n-6, D=C22:6 n-3. \(^c\) Not detected.
Figure 4.1 a. Contour plots between time and substrate mole ratio for levels of palmitic acid at the sn-2 position of SL. b. Contour plots between time and substrate mole ratio for the total incorporation of DHA and GLA. Experiments were carried out using RSM conditions determined in Table 4.1 on mg-scale.
Figure 4.2 DSC melting (endothermic) and crystallization (exothermic) profiles of SL and palm olein. Endothermic peaks are shown downwards.
Figure 4.2

- SL, exothermic
- Palm olein, exothermic
- SL, endothermic
- Palm olein, endothermic

Temperature °C

Normalized heat flow (W/g)
CHAPTER 5

SYNTHESIS OF STRUCTURED LIPID ENRICHED WITH OMEGA FATTY ACIDS
AND SN-2 PALMITIC ACID BY ENZYMATIC ESTERIFICATION, AND ITS
INCORPORATION IN POWDERED INFANT FORMULA

Supakana Nagachinta and Casimir C. Akoh. Accepted by the Journal of Agricultural and
Food Chemistry, 04/17/2013.
ABSTRACT

Structured lipid (SL) enriched with arachidonic (ARA) and docosahexaenoic (DHA) acids was produced from tripalmitin using Lipzyme TLIM. The effects of acyl donor, that is, free fatty acids vs. fatty acid ethyl esters on the reactions were compared. The highest total incorporation of ARA and DHA was obtained when the reaction continued for 24 h, at a substrate mole ratio of 9, using free fatty acids as acyl donors (acidolysis). The SL prepared by a large-scale acidolysis reaction, contained 17.69±0.09% total ARA, 10.75±0.15% total DHA, and 48.53±1.40% sn-2 palmitic acid. SL thermograms exhibited multiple peaks indicating complexity of the triacylglycerol (TAG) distribution. RP-HPLC analysis revealed nine of SL twenty-six TAG molecular species that were similar to those of human milk fat. Powdered infant formulas containing the SL were prepared by wet-mixing/spray-drying and dry-blending methods. Formula prepared with microencapsulated SL and dry-blending method had better oxidative stability and color quality.

Keywords: infant formula, structured lipid, lipase, tripalmitin, ARA, DHA, microencapsulation
INTRODUCTION

The type and content of lipid in infant formula are important as they provide energy and essential fatty acids for the structure and function of infant’s cell membranes. Traditional infant formulas are formulated with vegetable oils as lipid ingredient to provide fatty acid composition similar to human milk fat (HMF). Although the similarity could be achieved with vegetable oil blend, the fat absorption in infants fed with vegetable-oil-based formula is still lower\(^1\). This lower absorption is due to the differences in the regiospecific structure of the triacylglycerols (TAG) in vegetable oils relative to those in HMF. Greater than 60% of the palmitic acid in human milk is located at the \(sn\)-2 position of TAG, whereas in vegetable oils, it is predominantly located at the outer positions (\(sn\)-1, 3)\(^2\).

Pancreatic lipase specifically hydrolyzes the \(sn\)-1, 3 positions of TAG, producing free fatty acids from these outer positions leaving the attached \(sn\)-2 fatty acid on the glycerol backbone (2-monoacylglycerol, 2-MAG). The 2-MAG is a well-absorbed form of most fatty acids since it is conserved and readily forms mixed micelles with bile acids and cannot form insoluble soaps with divalent cations\(^1\). Structured lipid (SL) designed with palmitic acid esterified at the \(sn\)-2 position could therefore provide an alternative source of fat with better nutritional value for infant formula use.

Long-chain polyunsaturated fatty acids (LCPUFAs) including docosahexaenoic acid (DHA, omega-3) and arachidonic acid (ARA, omega-6) are important in neonatal development of the brain and retina. DHA and ARA constitute the majority of the fatty acids in the brain, aiding in the development and protection of neurological functions\(^3\). Preformed LCPUFAs are found in HMF, but traditionally are not found in infant formulas. Most traditional infant formulas rely on the bioconversion of linoleic acid and
alpha-linolenic acid to LCPUFAs, however, the rates for these conversions are very limited. Therefore, an alternative approach would be to use these beneficial LCPUFAs directly in infant formula. Enzymatic modification methods including acidolysis and interesterification have been employed in the production of SLs with LCPUFAs for possible use in infant formula.

The addition of LCPUFAs oils into foods is a challenge, as their susceptibility to oxidation and development of off-flavor volatiles affect sensory properties. For microencapsulation of LCPUFA oils used in infant formula, food-grade ingredients suitable for infant and natural ingredients are desired. Milk constituents are widely used encapsulants as they possess the ability to emulsify, build viscosity, and form gel. Recently, there has been interest in the development of protein-polysaccharide conjugates, made by the Maillard reaction, for applications in food, medicines, and cosmetics. Augstin et al. demonstrated the suitability of heated protein-polysaccharide mixtures for encapsulation of fish oil. In vivo and in vitro studies showed that this microencapsulation method protected fish oil from acidic condition in the stomach, delivered, and released the oil in the small intestine. More importantly, it did not compromise the bioavailability of the fish oil.

In this study, SL enriched with sn-2 palmitate, ARA, and DHA was produced and used as lipid ingredient in infant formula. Effects of substrate mole ratio, incubation time, and type of acyl donors, i.e. free fatty acids, FFAs (acidolysis reaction) or free fatty acid ethyl esters, FAEEs (interesterification) on the total incorporation of ARA and DHA by sn-1, 3 specific immobilized Lipozyme TL IM were studied. Infant formulas were prepared using two manufacturing methods for the production of powdered formula (a wet-
mixing/spray-drying process vs. a dry-blending process). Prior to dry blending process, SL was microencapsulated using heated protein-polysaccharide conjugates as encapsulants. Oxidative stability and color quality of the products were evaluated.

**MATERIALS AND METHODS**

**Materials.** DHA-rich single cell oil (DHASCO®, 40% DHA) from algae *Crypthecodinium cohnii* and ARA-rich single cell oil (ARASCO®, 40% ARA) from the fungus, *Mortierella alpina*, were generously provided by DSM Nutritional Products-Martek (Columbia, MD). Immobilized lipase, Lipozyme TL IM® (*sn*-1, 3 specific from *Thermomyces lanuginosus*), was obtained from Novozymes North America Inc. (Franklinton, NC). The specific activity of Lipozyme TL IM was 250 IUN/g (IUN is the Interesterification Units Novo). Tripalmitin and internal standard C15:0 pentadecanoic acid (>98% purity) were purchased from Tokyo Chemical Industry America (Montgomeryville, PA). Lipid standards, Supelco 37 component FAME mix, triolein, 2-oleoylglycerol, triolein, and ethyl oleate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). TAG standard mix (GLC reference standard) was purchased from Nu-check Prep, Inc. (Elysian, MN). Whey protein isolate (Grander Ultra® WPI), maltodextrin (StarDri 100), and corn syrup solid, CSS (StarDri 42 R) were generously donated by Grande custom ingredients group (Lomira, WI) and Tate & Lyle (Decatur, IL). Other ingredients including non-fat dry milk, lactose, and infant formula vitamin and mineral premix were generously donated by O-AT-KA Milk Products Cooperative, Inc. (Batavia, NY), Hilmar Ingredients (Hilmar, CA), and Fortitech, Inc. (Schenectady, NY), respectively.
Preparation of FFAs and FAEEs. DHASCO and ARASCO were mixed at a mole ratio of 1:1 prior to the preparations of FFAs and FAEEs. The mixture contained 26.01±0.35% DHA, 22.56±0.56% ARA, 18.65±0.32% oleic acid, 8.90±0.02% palmitic acid, 5.62±0.02% myristic acid, 4.47±0.03% stearic acid, and 2.56±0.19% lauric acid. Hydrolysis and ethanolysis of the oil mixture were performed according to the methods described by Vázquez and Akoh\(^\text{12}\) with some modifications. For hydrolysis, 150 g of oil was saponified using a mixture of KOH (34.5 g), distilled water (66 mL), 96% ethanol (396 mL), and butylated hydroxytoluene (0.03 g). All reagents were placed in a stirred batch bioreactor at 60°C for 1 h. The reaction was stopped by adding 120 mL of distilled water and acidified to pH 2 to release FFAs. The FFAs were washed, filtered through a sodium sulfate column, and stored in an amber Nalgene bottle under nitrogen at -20 °C until use. For ethanolation, the reaction was performed by mixing oil with sodium ethoxide (2.625%, v/v) in absolute ethanol at a ratio of 4:2 (v/v) (2.25-fold molar excess of ethanol). The mixture was heated at 60 °C with mechanical shaking for 40 min, under nitrogen atmosphere. The product was first washed with 100 mL of a saturated NaCl solution, and then washed with 100 mL of distilled water. After separation, FAEEs were dried over sodium sulfate, vacuum filtered, and stored similarly as FFAs. FFAs and FAEEs were confirmed by thin-layer chromatography (TLC) analysis using oleic acid and ethyl oleate, respectively as standards.

Small-scale Synthesis and Analysis of SL Products. SLs were produced using two types of reactions, acidolysis (with FFAs as substrate) and interesterification (with FAEEs as substrate). The reaction mixtures consisting of hexane (3mL) and a mixture of FFAs or FAEEs and tripalmitin at different substrate mole ratios (FFAs or FAEEs to...
tripalmitin at 3, 6, and 9 mol/mol) were placed in screw-capped test tubes. Lipozyme TL IM (10% of total weight of the substrates) was added. The tubes were incubated at 60 °C for 12, 18, and 24 h in an orbital shaking water bath at 200 rpm. The products were collected and passed through a sodium sulfate column to remove moisture and enzyme. All reactions were performed in triplicate. Averages and standard deviations are reported. TLC analysis of product was carried out according to the method described by Lumor and Akoh with modification. Fifty microliters of the reaction product was spotted on silica gal G TLC plate. Petroleum ether/ethyl ether/acetic acid (80:20:0.5, v/v/v) was used to develop the plates (for SL made with FFAs), and a 90:10:0.5 (v/v/v) combination (for SL made with FAEEs). The bands were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The TAG band was scraped off into a screw-capped test tube for fatty acid composition analysis. TAG sample was converted to fatty acid methyl esters (FAMEs) following AOAC official method 996.01 with modification.

**Large-scale Synthesis and Purification of SL.** The conditions given highest incorporation of ARA and DHA were selected for 1 L-scale production of SL. The solvent-free acidolysis reaction was performed in a 1 L-stirred batch reactor at 60 °C for 24 h with a substrate mole ratio of 9 (a mixture of FFAs to tripalmitin), 10% (w/w) of Lipozyme TL IM, and a constant stirring at 200 rpm. The reactor was wrapped with foil to reduce exposure to light. At the end of the reaction, the resulting SL was 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 enzyme. SL was stored in an airtight amber container under nitrogen at 4 °C. Purification of SL product was performed using
short-path distillation and followed by alkaline deacidification. Distillation was performed under the following conditions: 60 °C holding temperature; approximately 100 mL/h feeding rate; 170 °C heating oil temperature; 20 °C coolant temperature; and vacuum <13.33 Pa. Deacidification by alkaline extraction was performed according to the method described by Lee and Akoh\textsuperscript{15} with minor modification. Purified SL (10 g) from short-path distillation was mixed with hexane (150 mL), phenolphthalein solution, and 80 mL of 0.5 N KOH in 20% ethanol. The separation was obtained in a separatory funnel, and the upper phase was collected. The upper phase was extracted with another 30 mL of 0.5 N KOH in 20% ethanol and 60 mL of saturated NaCl solution. The hexane phase containing SL was passed through a sodium sulfate column. Hexane was evaporated to obtain the deacidified SL. The deacidification step was completed to obtain sufficient purified SL for further studies (FFAs<0.1%). The FFAs content was determined according to AOCS Official Method Ac 5-41\textsuperscript{16}.

**Positional Analysis.** The pancreatic lipase hydrolysis procedure followed was as described by Pina-Rodriguez and Akoh\textsuperscript{17}. Hydrolysis product was extracted with 2 mL diethyl ether and concentrated with nitrogen. The concentrated extract was spotted on silica gel G TLC plates and developed with a mixture of hexane: diethyl ether: formic acid (60:40:1.6, v/v/v). 2-Oleoylglycerol was spotted in parallel as identification standard for 2-MAG. The bands corresponding to 2-MAG were collected and converted to FAMEs for fatty acid composition analysis as described above.

**\textsuperscript{13}C NMR Analysis.** In addition to pancreatic lipase analysis, the regio-isomeric distribution of ARA and DHA was determined by proton-decoupled \textsuperscript{13}C nuclear magnetic resonance (NMR) analysis. The spectrum was collected for 200 mg sample dissolved in
0.8 ml 99.8% CDCl₃ using continuous ¹H decoupling at 25 °C with a Varian DD 600 MHz spectrometer, equipped with a 3 mm triple resonance cold probe. The data was acquired at a ¹³C frequency of 150.82 MHz using the following acquisition parameters: 56,818 complex data points, spectral width of 37,879 Hz (251 ppm), pulse width 30 °, acquisition time 1.5 s, relaxation delay 1 s, and collection of 20,000 scans. Exponential line broadening (1 Hz) was applied before Fourier transforming the data. ¹³C chemical shifts were expressed in parts per million (ppm) relative to CDCl₃ at 77.16 ppm.

**Melting and Crystallization Profile.** Melting and crystallization profiles were determined for tripalmitin, SL, and fat extracted from a commercial infant formula (CIFL) using a differential scanning calorimeter (DSC1 STAR® System, Mettler-Toledo), cooled with a Haake immersion cooler (Haake EK90/MT, Thermo Scientific). Lipid extraction from infant formula was performed according to Teichart and Akoh. The analysis was performed according to AOCS Official Method Cj 1-94 with minor modification using indium as a standard. Sample (8-12 mg) was hermatically sealed in aluminum pan. The sample was heated from 25 °C to 80 °C at 50 °C/min, held for 10 min (to destroy any previous crystalline structure), cooled from 80 °C to -55 °C at 10 °C/min (for crystallization profiles), held for 30 min, and then heated from -55 °C to 80 °C at 5 °C/min (for melting profiles). Melting and crystallization profiles were performed in duplicate.

**TAG Molecular Species.** A reversed phase HPLC (Agilent Technologies 1100) with an evaporative light scanning detector (ELSD), Sedex 55 was used to analyze the TAG molecular species of SL and CIFL on an ultrasphere C18, 250 mm, 4.6 mm, 5 µm particle size column. The column temperature was kept at 30 °C. The ELSD conditions were 70
℃, 3.0 bar, and gain of 7\textsuperscript{18}. Sample concentration was 5 mg/mL in chloroform. The sample injection volume was 20 µL. The eluent was a gradient of acetonitrile (A), and acetone (B) at solvent flow rate of 1 mL/min with a gradient of 0 min, 65% B; 55 min, 95% B and 65 min, 65% B and post run of 10 min. The equivalent carbon number (ECN) method was used to predict the elution order of TAG. Standards TAG mix containing trilinolenin (ECN=36), trilinolein (42), triolein (48), tripalmitin (48), tristearin (54), and triarachidin (60), as well as, palm olein were chromatographed to help determine the TAG species.

**Infant Formula Preparation.** SL-containing infant formulas were prepared using two general manufacturing methods 1) a wet-mixing/spray-drying process and 2) a dry-blending process\textsuperscript{19,20}. For wet-mixing/spray-drying process, non-fat dry milk (20 g), whey protein isolate (10 g), lactose (31 g), maltodextrin (30 g), and water (800 mL) were mixed at 50°C -60°C. To the mixture was added with SL (30 g) and vitamin/mineral premix (3.9 g), and homogenized using a high-speed benchtop homogenizer (Brinkmann Kinematica polytron, Switzerland). The sample was passed through a high-pressure homogenizer (Avestin Emulsiflex-C5, Canada) in two steps at 35 MPa and subsequently at 10 MPa, pasteurized at 65 °C for 30 min, then spray-dried using a mini spray dryer Buchi-290 (Switzerland). Two different combinations of spray drying inlet-outlet temperature (120°C- 70°C vs. 180°C-80 °C) were used. The effects of these drying temperatures to product qualities were compared.

For dry blending process, prior to the blending step, SL was encapsulated, following the method described by Augustin et al.\textsuperscript{8} with minor modifications. Briefly, whey protein isolate (21 g) was reconstituted in 350 mL water at 60 °C. Corn syrup solids (42 g) was
added and the pH of the protein-polysaccharide mixture was adjusted to 7.5 using 1 M NaOH solution. The mixture was heated in a water bath at 90 °C for 30 min and cooled down to 60 °C before the addition of SL (21 g). SL from 4 °C storage was heated to 60 °C. The oil was dispersed into the aqueous protein-polysaccharide encapsulant mixture using a bench-top homogenizer. The pre-emulsion was passed through a high-pressure homogenizer (Avestin Emulsiflex-C5, Canada) in two steps at 35 MPa and subsequently at 10 MPa. The homogenized emulsion was spray-dried at inlet temperature of 180 °C and outlet temperature of 80 °C. The microencapsulated SL (120 g) was then dry-blended with the ingredients listed above except for water.

**Lipid Oxidation and Color Measurement of Infant Formulas.** Lipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS) were measured according to the method of Klinkesorn et al.\textsuperscript{21} with minor modification. Formula powder (100 mg) was reconstituted in 0.3 mL of distilled water, instead of acetate buffer, and 1.5 mL of iso-octane-2-propanol (3:1 v:v) was added. The sample was vortexed for 10 sec, 3 times and followed by centrifugation at 3400 g for 2 min. The organic phase (0.2 mL) was added to 2.8 mL of methanol-butanol (2:1 v:v), followed by 15 µL thiocyanate solution (3.94 M) and 15 µL ferrous iron solution (prepared by mixing 0.132 M BaCl\textsubscript{2} and 0.144 M FeSO\textsubscript{4} in acidic solution). The solution was vortexed and the absorbance at 510 nm was measured after 20 min. Lipid hydroperoxide concentrations were determined from a cumene hydroperoxide standard curve.

For TBARS, formula sample (5 mg) was reconstituted with 1 mL distilled water followed by an addition of 2 mL of thiobarbituric (TBA) solution. TBA solution was prepared by mixing 15 g of trichloroacetic acid, 0.375 g of TBA, 1.76 mL of 12 N HCl, and 82.9 mL
of distilled water. One hundred milliliters of TBA solution was mixed with 3 mL of 2% butylated hydroxytoluene in ethanol prior to use. Sample mixture was vortexed and heated in a boiling water bath for 15 min, cooled to room temperature, and centrifuged at 3400 g for 25 min. Absorbance of supernatant was measured at 532 nm. Concentration of TBARS was determined from standard curve prepared with 1,1,3,3-tetraethoxypropane (malonaldehyde diethyl acetal).

For color measurement, the \( L^*, a^*, b^* \) values were measured using a Minolta color analyzer. Chroma \( C^* \) and hue angle \( h^* \) were calculated from \( a^* \) and \( b^* \) values. The mathematic \( C^* \) and \( h^* \) are defined as \( C^* = [a^{*2} + b^{*2}]^{1/2} \) and \( h^* = \arctan[b^*/a^*] \). All data represent average of six measurements of two different trials, and results are reported as average and standard deviation of these measurements.

**Statistical Analysis.** The statistical significance of differences between samples was calculated using analysis of variance (ANOVA) and post-hoc Tukey’s test at a significance level of \( p<0.05 \) using IBM SPSS Statistics 19.

**RESULTS AND DISCUSSION**

The effects of substrate mole ratio of acyl donors (FFAs or FAEEs mixture), tripalmitin, reaction time, and type of acyl donors on the incorporation of ARA and DHA were determined. In Figure 5.1, it can be seen that as the reaction time and substrate mole ratio increase, the total incorporation of ARA and DHA also increases. Increasing reaction times led to increased incorporation of LCPUFAs as longer residence times allowed for prolonged contact between the enzyme and the substrates\(^{23}\). Sahin et al.\(^ {24}\) observed an increase in omega-3 PUFAs (DHA and EPA) incorporation into tripalmitin as reaction time increased from 18-30 h with increasing substrate mole ratio. In our previous study,
the total incorporation of ARA and DHA also increased as reaction time (12-24 h) and substrate mole ratio (6-18 mol/mol) increased\(^5\). The total incorporation of ARA and DHA was significantly higher when the substrate mole ratio was 9 for both interesterification and acidolysis reactions (\(p < 0.05\)). The highest total incorporation for interesterification (26.38±0.97\%) and for acidolysis (29.27±0.74\%) were obtained when the reaction continued for 24 h at 60°C at a substrate mole ratio of 9. At these conditions, the incorporation of ARA and DHA were significantly higher when FFAs (acidolysis) were used as substrate compared to FAEEs (interesterification) (\(p<0.05\)). Similarly, Lumor and Akoh\(^{13}\) reported a higher incorporation of LCPUFAs (GLA, an omega-6 LCPUFA) when FFAs were used as acyl donors compared to FAEEs in reactions catalyzed by \(sn\)-1, 3 specific lipase, Lipozyme RM IM (donor organism: \textit{Rhizomucor miehei}) at 45, 55, and 65 °C. At a molecular level, interesterification process involves hydrolysis of the ester molecule followed by an esterification reaction. Hydrolysis of fatty acid ethyl esters produces ethanol in the reaction, which induces a loss in enzyme activity\(^{25}\). This possibly complicated the process and led to a lower incorporation of ARA and DHA in the interesterification batch compared to acidolysis batch.

The conditions that gave highest ARA and DHA incorporations were used to scale-up acidolysis reaction in a 1 L-stirred batch reactor. Purified SL product was obtained through short-path distillation followed by alkaline deacidification. The FFAs content of purified SL was 0.01±0.02\%. The fatty acid composition and positional distribution of the SL and CIFL are shown in Table 5.1. The major fatty acids found in SL were palmitic (36.77±0.11\%), ARA (17.69±0.09\%), oleic (15.28±0.03\%), DHA (10.75±0.15\%) and myristic (5.0.9±0.02\%) acids. Positional analysis showed that the \(sn\)-
Positional analysis revealed the content of sn-2 palmitic acid from CIFL (6.02%) was much lower than SL. It is known that palmitic acids in vegetable oils are predominantly located at the sn-1,3 positions which led to a lower fat and calcium absorption in infants fed with vegetable oil-based formulas\(^2\). The level of ARA in CIFL was 0.08±0.00% and DHA was 0.39±0.01%. CIFL must contain ARA and DHA as a physical blend. The SL produced in the current research could be used in an oil blend to increase the sn-2 palmitic acid, ARA, and DHA contents.

Positional distribution of fatty acids in SL was also determined by \(^{13}\)C-NMR spectroscopy. The chemical shift of carbonyl carbon of fatty acids in TAGs depends on the regiospecific position (sn-1, 3 or sn-2), and for carbonyl carbon of unsaturated fatty acid, the chemical shift also depends on the position and number of double bonds in the chain\(^2\). Different carbon atoms give signals in different regions of the \(^{13}\)C-NMR spectrum. The spectrum of SL is shown in Figure 5.2. The region where carbonyl carbons (C1 atoms) give signals is between 172-174 ppm. Assignments of resonances were made
according to previous studies\textsuperscript{29, 30} on fish lipids and the fact that the distance between the 
\textit{sn}-1, 3 and \textit{sn}-2 chains is approximately 0.4 ppm\textsuperscript{31}. The spectrum showed that saturated 
fatty acids, monounsaturated n-9 fatty acids, DHA, and ARA were esterified at the \textit{sn}-2 
position.

TAG molecular species were determined using reversed-phase HPLC. Peak 
identifications were made according to published works involving palm oil and palm olein\textsuperscript{32, 33}, elution time of TAG standards, and the fact that TAG species are eluted in 
order of equivalent carbon number (ECN)= TC-2xDB. TC is total carbon number of acyl 
group and DB is total number of double bond in TAG\textsuperscript{34}. Figure 5.3 shows RP-HPLC 
chromatograms of palm olein, CIFL, and SL. Table 5.2 shows a comparison between 
TAG species and their relative percentages in the three lipid samples. PPO (61.01, 
26.09\%) and POO (34.23, 23.70\%) constituted the majority of TAGs in both palm olein 
and CIFL, respectively. These TAGs were also found in the SL, however their 
abundances were much lower (PPO=9.97\%, POO=1.80\%). Recently, the TAG species 
composition of colostrum fat, transitional and mature milk fat was determined by RP-
HPLC\textsuperscript{35}. Twenty-two different TAG species were found in these milk samples and the 
majority included POO (21.51\(\pm\)5.39\%), POL (16.93\(\pm\)3.27\%), and POLa (10.39\(\pm\)3.02\%). 
SL made in this study contained a variety of 26 different TAG species. Major TAG 
species in SL include PDD (15.36\%), PPA or LPL (12.33\%), PPO (9.97\%), PPP (7.66\%), 
C\textsubscript{8}PD (7.40\%), PPD or LPA (7.38\%), and OPA (7.35\%). Nine TAG species identified in 
the SL, including POO, OOO, POL, PPL, PPP, PPO, PSO, MPP, and SPP were reported 
as HMF TAGs in the study by Zou et al.\textsuperscript{35}, however, the amounts were considerably 
different. Most of the SL TAGs contained more than 3 DB in their structures, four
contained no DB (PPP=7.66%, C₁₀PP=5.39%, SPP=1.02%, and MPP=3.82%). The variety of TAG species, fatty acid chain length, and degree of saturation were shown to affect melting and crystallization profile of fat and oil³⁶,³⁷.

The melting and crystallization behaviors of SL were compared with its substrate, tripalmitin and fat extracted from CIFL (Figure 5.4). Tripalmitin is a highly saturated TAG and melts at 66°C. SL has lower melting points and broader melting range around 37°C to -25 °C. Melting point of human milk fat is below 38 °C³⁸. Both SL and CIFL thermograms exhibited multiple peaks indicating complexity of the TAG distribution. This was also shown as multiple peaks in the chromatogram from the analysis of TAG molecular species. The presence of palmitic acid in the TAGs of SL and highly saturated TAG species (PPP, C₁₀PP, SPP, and MPP) contributed to the higher temperature melting peak at 36.36°C. Highly saturated TAGs including SPP, MPP, PPP, SMM were also found in human milk fat samples (colostrum, transitional, and mature milk fat)³⁵. However, the amounts of these TAGs were rather low (with content of <1% or in the range of 1-5%). This suggested the use of this SL as a complimentary fat in infant formulas with a blend containing unsaturated oils rather than a substitute for a vegetable oil blend. Crystallization thermogram showed an onset of crystallization at -6 °C ending at 26 °C for SL. CIFL had a lower melting range of -30°C to -3°C.

Powdered infant formula is manufactured using two general types of processes: a dry-blending and a wet-mixing/spray-drying process¹⁹. Some manufacturers also use a combination of these processes to spray-dry the base powder (protein and fat component) then dry-blend with carbohydrate, vitamin, and mineral ingredients. To determine which process is suitable for SL application in powdered infant formula, infant formulas were
prepared with SL as the fat source using these two general processes and the products were evaluated for oxidative stability and color scores \((L^*, a^*, b^*, C^*, \text{ and } h^*)\). PV measures the ability of lipid hydroperoxides (primary oxidation products) to oxidize ferrous ions to ferric ions, which form a red-violet complex with thiocyanate. TBARs test measures secondary oxidation products which form pink color when reacted with thiobarbituric reagent. The results of these analyses of infant formulas are shown in Table 5.3. Dry-blending process yielded products with significantly lower PV and TBARs values compared to wet-mixing/spray-drying process. The PV and TBARs values of dry-blended infant formulas and of the commercial infant formula were not significantly different. Higher temperature \((180^\circ C)\) used in wet-mixing/spray-drying resulted in significantly higher PV and TBARs values compared to lower temperature of \(120^\circ C\). Color score, \(L^*\) for lightness and \(C^*\) for chroma or saturation, showed a negative correlation with PV and TBARs values (Table 3). The color of products with higher PV and TBARs values (wet-mixing/spray-drying products) was less saturated (lower \(C^*\)), meaning that the color looked dull and grayish. These products were also darker with lower \(L^*\) values. The hue color values of all infant formulas fall between yellow and green.

According to the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), infant formulas should provide 60-70 kcal/100mL\(^3\). The preparation of infant formula in this study was aimed at a formulation that contributes 60-70 kcal/100 mL resulting from 3.3-6.0% fat, 1.2-3.0% protein, and 5.4-8.1% carbohydrates. Microencapsulated SL contained 25% fat (SL), 25% protein (WPI), and 50% carbohydrate (CSS). Microencapsulation of SL increased the stability of the final
product, however, the energy contributed from carbohydrate and protein used as encapsulant increased the product energy contribution by 35 kcal/100 mL (Table 5.4). The new formulation needs adjustment in the protein and carbohydrate ingredients. At this stage of the study, microencapsulation with more than 25% oil is being studied to improve the use of microencapsulated SL on energy contribution in the formulation. SL was prepared from tripalmitin and FFAs derived from DHASCO and ARASCO, in acidolysis reaction using Lipozyme TL IM as biocatalyst. Physical and chemical analyses suggested its application in an oil blend for use in infant formula. This SL could provide a fat source with physiologically important fatty acids and serve as a good source of sn-2 palmitic acid, which can improve fat and calcium absorption. Powdered infant formulas containing SL were prepared by a wet-mixing/spray-drying and dry-blending process. SL was microencapsulated using heated protein-polysaccharide conjugates prior to dry-blending to obtain SL in dried powder form. Infant formula prepared by dry-blending process with microencapsulated SL had a better oxidative stability and color quality.

ACKNOWLEDGEMENTS

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REFERENCES


Table 5.1 Fatty acids composition (%) of structured lipid (SL) produced via acidolysis of tripalmitin and mixture of FFAs from DHA and ARA-rich single cell oils, compared to fat extracted from a commercial infant formula (CIFL).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>SL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CIFL&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>sn-2</td>
</tr>
<tr>
<td>C12:0</td>
<td>1.94±0.01</td>
<td>3.00±0.13</td>
</tr>
<tr>
<td>C14:0</td>
<td>5.09±0.02</td>
<td>4.84±0.14</td>
</tr>
<tr>
<td>C16:0</td>
<td>36.70±0.11</td>
<td>48.53±1.40</td>
</tr>
<tr>
<td>C18:0</td>
<td>4.29±0.02</td>
<td>4.03±0.03</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>15.28±0.03</td>
<td>9.82±0.12</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>2.89±0.02</td>
<td>1.83±0.01</td>
</tr>
<tr>
<td>C18:3 n-3</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td>C18:3 n-6</td>
<td>0.83±0.01</td>
<td>0.19±0.00</td>
</tr>
<tr>
<td>C20:4 n-6</td>
<td>17.69±0.09</td>
<td>9.73±0.13</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>10.75±0.15</td>
<td>4.80±0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fatty acids found in trace amounts were: C8:0, C10:0, C17:0, C20:0, C20:1 n-9, C22:0, C20:3 n-6, C22:5 n-3 and C24:0. <sup>b</sup> nd= not detected. <sup>c</sup> CIFL= Fat extracted from a commercially available infant formula enriched with ARA and DHA by physical blending. Other fatty acids found in trace amount were: C8:0, C10:0, C20:0, C20:1, C22:0, and C24:0.
Table 5.2 TAG molecular species of SL, CIFL, and palm olein determined by RP-HPLC according to their ECN

<table>
<thead>
<tr>
<th>TAG Species</th>
<th>ECN (DB)</th>
<th>%Area</th>
<th>TAG Species</th>
<th>ECN (DB)</th>
<th>%Area</th>
<th>TAG Species</th>
<th>ECN (DB)</th>
<th>%Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDD</td>
<td>30 (18)</td>
<td>1.52</td>
<td>C₈LaAl</td>
<td>32 (3)</td>
<td>1.38</td>
<td>MPL</td>
<td>44 (2)</td>
<td>0.06</td>
</tr>
<tr>
<td>MDD</td>
<td>34 (12)</td>
<td>1.36</td>
<td>C₁₀LaL</td>
<td>34 (2)</td>
<td>3.46</td>
<td>MMP</td>
<td>44 (0)</td>
<td>0.04</td>
</tr>
<tr>
<td>C₈PD</td>
<td>34 (6)</td>
<td>7.40</td>
<td>LaMAI</td>
<td>38 (3)</td>
<td>2.87</td>
<td>POL</td>
<td>46 (3)</td>
<td>1.50</td>
</tr>
<tr>
<td>DDO</td>
<td>36 (13)</td>
<td>2.91</td>
<td>LaOL</td>
<td>42 (3)</td>
<td>2.67</td>
<td>PPL</td>
<td>46 (2)</td>
<td>1.76</td>
</tr>
<tr>
<td>PDD</td>
<td>36 (12)</td>
<td>15.36</td>
<td>LaPL</td>
<td>42 (2)</td>
<td>6.72</td>
<td>OOO</td>
<td>48 (3)</td>
<td>0.35</td>
</tr>
<tr>
<td>PAA/PAD</td>
<td>40 (8)/38 (10)</td>
<td>1.12</td>
<td>LLO</td>
<td>44 (5)</td>
<td>4.49</td>
<td>POO</td>
<td>48 (2)</td>
<td>34.23</td>
</tr>
<tr>
<td>MPD</td>
<td>40 (6)</td>
<td>0.18</td>
<td>MPL</td>
<td>44 (2)</td>
<td>3.74</td>
<td>PPO</td>
<td>48 (1)</td>
<td>61.01</td>
</tr>
<tr>
<td>OPD</td>
<td>42 (7)</td>
<td>3.22</td>
<td>MMP</td>
<td>44 (0)</td>
<td>3.02</td>
<td>SOO</td>
<td>50 (2)</td>
<td>0.13</td>
</tr>
<tr>
<td>PPD/LPA</td>
<td>42 (6)</td>
<td>7.38</td>
<td>POL</td>
<td>46 (3)</td>
<td>9.42</td>
<td>PSO</td>
<td>50 (1)</td>
<td>0.91</td>
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<tr>
<td>C₁₀OO</td>
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<td>1.96</td>
<td>PPL</td>
<td>46 (2)</td>
<td>4.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₁₀PP</td>
<td>42 (0)</td>
<td>5.39</td>
<td>OOO</td>
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<td>6.78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPD</td>
<td>44 (6)</td>
<td>3.07</td>
<td>POO</td>
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<td>23.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPA</td>
<td>44 (5)</td>
<td>7.35</td>
<td>PPO</td>
<td>48 (1)</td>
<td>26.09</td>
<td></td>
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</tr>
<tr>
<td>PPA/LPL</td>
<td>44 (4)</td>
<td>12.33</td>
<td>SOO</td>
<td>50 (2)</td>
<td>0.56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POL</td>
<td>46 (3)</td>
<td>0.65</td>
<td>PSO</td>
<td>50 (1)</td>
<td>1.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPL</td>
<td>46 (2)</td>
<td>3.54</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPP</td>
<td>46 (0)</td>
<td>3.82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OOO</td>
<td>48 (3)</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPO</td>
<td>48 (1)</td>
<td>9.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPP</td>
<td>48 (0)</td>
<td>7.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSO</td>
<td>50 (1)</td>
<td>0.55</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPP</td>
<td>50 (0)</td>
<td>1.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aEquivalent carbon number (ECN)= TC-2xDB; TC is total carbon number of acyl group and DB is total number of double bond in TAG. bC₈, caprylic acid; C₁₀, capric acid; La, lauric acid; M, myristic acid; P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Al, alpha-linolenic acid; A, arachidonic acid (ARA); D, docosahexaenoic acid (DHA). TAG species do not reflect stereochemical configuration.
Table 5.3 Characterization of powdered infant formulas.

<table>
<thead>
<tr>
<th>Characteristics of powdered infant formulas</th>
<th>Wet-mixing/spray-drying at 120 °C</th>
<th>Wet-mixing/spray-drying at 180 °C</th>
<th>Dry-blending</th>
<th>Commercial infant formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxide (µg/mg sample)</td>
<td>0.18±0.02^a</td>
<td>0.37±0.02^a</td>
<td>0.07±0.02^a</td>
<td>0.06±0.03^a</td>
</tr>
<tr>
<td>TBARS (µg/mg sample)</td>
<td>0.06±0.01^b</td>
<td>0.11±0.01^a</td>
<td>0.04±0.01^c</td>
<td>0.05±0.01^c</td>
</tr>
<tr>
<td>Color</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L*</td>
<td>94.59±2.46^b</td>
<td>95.75±0.53^b</td>
<td>98.83±0.49^a</td>
<td>96.35±2.34^ab</td>
</tr>
<tr>
<td>a*</td>
<td>-2.28±0.08^a</td>
<td>-3.12±0.05^b</td>
<td>-3.80±0.08^c</td>
<td>-5.21±0.05^d</td>
</tr>
<tr>
<td>b*</td>
<td>16.37±0.34^bc</td>
<td>15.30±0.79^c</td>
<td>17.71±0.55^b</td>
<td>19.29±0.65^a</td>
</tr>
<tr>
<td>C*</td>
<td>17.64±0.36^bc</td>
<td>16.74±1.14^c</td>
<td>18.83±0.33^b</td>
<td>21.25±1.89^a</td>
</tr>
<tr>
<td>h*</td>
<td>97.24±0.32^c</td>
<td>100.30±0.76^b</td>
<td>101.28±0.31^b</td>
<td>103.89±1.04^a</td>
</tr>
</tbody>
</table>

Mean±SD, n=6. Means with the same letter in the same row and category are not significantly different (p>0.05)
Table 5.4 Energy contribution (in 100 mL of resuspended formula).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition</th>
<th>Energy contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy contribution</td>
<td>(g)</td>
<td>(kcal)</td>
</tr>
<tr>
<td>Non-fat milk (fat 0%, protein 34.8%, carbohydrate 52.2%)</td>
<td>2</td>
<td>6.98</td>
</tr>
<tr>
<td>WPI (fat 0.6%, protein 92%, carbohydrate 0.5%)</td>
<td>1</td>
<td>3.93</td>
</tr>
<tr>
<td>Lactose</td>
<td>3.1</td>
<td>12.4</td>
</tr>
<tr>
<td>Maltrodextrin</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>SL</td>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td>Total energy contribution (wet-mixing/spray drying method)</td>
<td>-</td>
<td>62.31</td>
</tr>
<tr>
<td>Microencapsulated SL (fat 25%, protein 25%, carbohydrate 50%)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Total energy contribution (microencapsulated SL, dry-blending)</td>
<td>-</td>
<td>97.35</td>
</tr>
</tbody>
</table>
Figure 5.1 Percent total incorporation of ARA and DHA via acidolysis (FFAs as substrate) and interesterification (FAEEs as substrate) using different substrate mole ratios (3-9 mol acyl donor: 1 mol tripalmitin) and different incubation time (12-24 h) at 60 °C.
Figure 5.1

Percent Incorporation of ARA+DHA

Time (h)

- 3-FAEE
- 6-FAEE
- 9-FAEE
- 3-FFA
- 6-FFA
- 9-FFA
**Figure 5.2** Carbonyl region of the broad band decoupled $^{13}$C-NMR spectrum of SL. The assignment of $sn$-1, 3 and $sn$-2 regioisomeric peaks to individual fatty acids is annotated.
Figure 5.2

- Saturated (sn-1, 3)
- Saturated (sn-2)
- Monounsat., Δ9 (sn-1, 3)
- C20:4, Δ5 (sn-1, 3)
- C22:5, Δ7 (sn-1, 3)
- C18:3, Δ6 (sn-1, 3)
- C20:4, Δ5 (sn-2)
- C22:6, Δ4 (sn-1, 3)
- C22:6, Δ4 (sn-2)

172-174 ppm carbonyl region
126-134 ppm olefinic region
60-74 ppm glycerol region
19-35 ppm Aliphatic region
Figure 5.3  TAG molecular species of palm olein, CIFL, and SL determined by reversed-HPLC. Annotated TAG species do not reflect stereochemical configuration. C₈, caprylic acid; C₁₀, capric acid; La, lauric acid; M, myristic acid; P, palmitic acid; S, stearic acid; O, oleic acid; L, linoleic acid; Al, alpha-linolenic acid; A, arachidonic acid (ARA); D, docosahexaenoic acid (DHA).
Figure 5.3

CIFL

Palm olein

SL
Figure 5.4 Crystallization (exothermic) and melting (endothermic) profile of tripalmitin, SL, and CIFL.
Figure 5.4

![Diagram showing normalized heat flow endo down (W/g) for different substances.]

- **Tripalmitin (exothermic)**
  - 23.64 °C
  - 39.93 °C

- **SL (exothermic)**
  - 0.57 °C
  - 47.00 °C

- **CIFL (exothermic)**
  - -2.78 °C
  - 36.36 °C

- **Tripalmitin (endothermic)**
  - -5.19 °C
  - 47.00 °C

- **SL (endothermic)**
  - -26.65 °C
  - 52.84 °C

- **CIFL (endothermic)**
  - -9.83 °C
  - 66.03 °C

Temperature (°C)
CHAPTER 6

SPRAY-DRIED STRUCTURED LIPID CONTAINING LONG-CHAIN
POLYUNSATURATED FATTY
ACIDS FOR USE IN INFANT FORMULAS

Supakana Nagachinta and Casimir C. Akoh. Submitted to Journal of Food Science.
04/22/13.
ABSTRACT

Human milk fat (HMF) analogs are structured lipids (SLs) modified to have palmitic acid content at the $sn$-2 position of the triacylglycerol (TAG) and fatty acid composition comparable to HMF. Some of these SLs are also designed to incorporate long chain polyunsaturated fatty acids (LCPUFAs) because of their important role in infant development. In this study, Maillard reaction products (MRPs), obtained from heated whey protein isolates and corn syrup solids (CSS) solution, were used as encapsulants for microencapsulation of two enzymatically synthesized SLs for infant formula applications. The encapsulated SL powders were obtained through spray-drying and evaluated in terms of their microencapsulation efficiency, chemical and physical properties, oxidative stability, and dispersibility. The microencapsulation efficiency of the SLs was 90%. Dispersibility test using particle size measurement demonstrated that these powders dispersed quickly into a homogenous suspension. The encapsulated SL powders had low peroxide and TBARs values. Lower oxidative stability was obtained in the powder containing SL with a higher degree of unsaturation and a lower concentration of tocopherols. The results demonstrated that the degree of fatty acid unsaturation and concentration of endogenous antioxidant in starting oils influenced the oxidative stability of the encapsulated SLs.

Keywords: Human milk fat analog, microencapsulation, spray-drying, structured lipid, oxidative stability, tocopherols
INTRODUCTION

Enzymatic interesterification has been applied to alter the properties of fats and oils for food application and other purposes. Human milk fat (HMF) analog is an example of a structured lipid (SL) resulting from such a modification that can serve as an ingredient for infant formula applications. The fat analogs resemble the fatty acid composition and triacylglycerol (TAG) structure of human milk fat. HMF analogs have been produced from various types of fat and oil substrates, including: palm stearin, soybean, and hazelnut oils (Teichert and Akoh 2011b; Turan and others 2012; Zou and others 2012). These SLs were shown to have similar fatty acid composition and levels of palmitic acid at the sn-2 position of TAG comparable to those in HMF. In addition to the regiospecificity of palmitic acid, the physiologically important long chain polyunsaturated fatty acids (LCPUFAs), such as arachidonic (ARA), docosahexaenoic (DHA), eicosapentaenoic (EPA), gamma-linolenic (GLA), and stearidonic (SDA) acids, have also been incorporated into HMF analogs to increase their nutritional value (Teichert and Akoh 2011a; Turan and others 2012; Zou and others 2012). These LCPUFAs benefit the infant’s cognitive and physical development (Horrobin 1992; Innis 2007). However, their addition into infant formulas can be a challenge, as these lipid components are susceptible to oxidation. Late addition of LCPUFAs during the production of infant food has been adopted to minimize their exposure to oxidizing conditions. In the production of powdered infant formula, LCPUFAs can be added after the spray-drying process as a microencapsulated powder using a conventional mixer (Groenendaal and van den Burg, 2002).
For microencapsulation of HMF analogs, food-grade, and preferably natural ingredients suitable for use in infant foods are desired. Milk constituents possess the ability to emulsify, contribute to viscosity, and form gels, which are suitable for microencapsulation purposes. Previous studies demonstrated microencapsulation of oils using milk protein alone (Legako and Dunford 2010), or milk protein in combination with carbohydrates (Kagami and others 2003; Young and others 1993), as well as with Maillard reaction products (MRPs) obtained from a reaction between milk protein and carbohydrate (Choi and others 2010). Augustin and others (2006) demonstrated the suitability of MRPs for encapsulation of fish oil, and suggested that increasing the extent of the Maillard reaction confers additional stability to microencapsulated fish oil. Further studies showed that encapsulation with these MRPs protected oil from stomach acidity, released the oil in the small intestine, and did not compromise the bioavailability of the fish oil (Augustin and others 2011; Kosaraju and others 2009; Patten and others 2009). Microencapsulation using MRPs, followed by spray drying was therefore selected in this study for the production of SL powders containing LCPUFAs for infant formula use. The aim of this study is to produce and characterize the properties of encapsulated SL powders containing LCPUFAs. Two SL HMF analogs were enzymatically produced in our previous studies to increase the amount of palmitic acid at the sn-2 position and containing LCPUFAs. The two SLs were microencapsulated using MRPs as encapsulants, and subjected to spray-drying. The encapsulated SL powders were evaluated in terms of their microencapsulation efficiency, chemical and physical properties, oxidative stability, and dispersibility.
MATERIALS AND METHODS

Materials. DHA-containing single cell oil (DHASCO®, 40% DHA) from algal Crypthecodinium cohnii and ARA-rich single cell oil (ARASCO®, 40% ARA) from fungal Mortierella alpina, were kindly provided by DSM Nutritional Products-Martek (Columbia, MD). GLA in free fatty acid form (70% GLA) was purchased from Sanmark Corp. (Greensboro, NC). Tripalmitin was purchased from Tokyo Chemical Industry America (Montgomeryville, PA). Palm olein (San Trans25) was generously donated by IOI-Loders Croklaan (Channahon, IL). Two structured lipids (SLs) were prepared via lipase-catalyzed acidolysis reaction in our previous studies. The fatty acid composition of these SLs are shown in Table 6.1. TDA-SL was produced from tripalmitin and a free fatty acid mix of DHASCO and ARASCO. PDG-SL was produced from palm olein and a free fatty acid mix of DHASCO and GLA. Whey protein isolate, WPI (Grander Ultra® WPI) and corn syrup solid, CSS (StarDri 42® CSS) were generously donated by Grande Custom Ingredients Group (Lomira, WI) and Tate & Lyle (Decatur, IL).

Preparation of SL powders. TDA-SL and PDG-SL were microencapsulated following the method of Augustin and others (2006) with minor modification. Whey protein isolate (21 g) was reconstituted in 350 mL water at 60 °C followed by the addition of corn syrup solid (42 g). NaOH solution (1 M) was added to the mixture to adjust the pH to 7.5. The mixture was heated in a water-bath at 90 °C for 30 min and cooled down to 60 °C before the addition of TDA-SL or PDG-SL (21 g). The oil was dispersed into the mixture using a benchtop homogenizer (Brinkmann Kinematica Polytron, Luzern, Switzerland). The pre-emulsion was passed through a high-pressure homogenizer (Avestin Emulsiflex-C5, Ontario, Canada) in two steps at 35 MPa and subsequently at 10 MPa. The homogenized
emulsion was held at 60 °C, spray-dried at inlet temperature of 180 °C and outlet temperature of 80 °C at a feeding rate of 5 mL/min.

**Microencapsulation efficiency.** Extraction of total oil was carried out according to the method of Klinkesorn and others (2006) with some modifications. Two milliliters of distilled water was added to 0.5 g powder. The mixture was vortexed for 1 min before adding 25 mL hexane/isopropanol (3:1, v/v). The tube was subsequently vortexed three times for 5 min each and centrifuged for 30 min at 3,000 g. The organic phase was collected. The aqueous phase was re-extracted twice with the same solvent mixture. After filtration through a sodium sulfate column, the solvent was evaporated at 60 °C using a rotary evaporator (Büchi Rotavapor, Flawil, Switzerland). The amount of total oil was determined gravimetrically. Free oil or hexane extractable oil was determined gravimetrically after extraction of 2.5 g powder with 15 mL of hexane. The mixture was vortexed for 3 min and centrifuged at 3,000 g for 30 min. The supernatant was filtered and the filter paper was washed twice with hexane. The filtrate was collected and hexane was evaporated at 60 °C. Microencapsulation efficiency (ME) was calculated as follows:

\[
\text{ME} = \frac{(\text{total oil} - \text{free oil})}{\text{total oil}} \times 100
\]

The units for total oil and free oil were g/g of sample.

**Water activity (a_w) and moisture content.** The water activity of SL powders were measured with Aqua Lab water activity meter (CX-2, Decagon Devices, Inc., Pullman, WA) at 25 °C. Two grams of sample was weighed into an aluminum pan and dried for 24 h at 70 °C and 29 in.Hg in vacuum oven (Fisher Scientific, Fairlawn, NJ). Moisture content was calculated from the weight difference.
**Lipid oxidation measurement.** Lipid hydroperoxide and thiobarbituric acid-reactive substances (TBARS) were measured using a modified method of (Klinkesorn and others 2005). SL powder (0.1 g) was reconstituted in 0.3 mL distilled water. The reconstituted sample was added to 1.5 mL of isooctane-2-propanal (3:1, v/v) followed by vortexing 3 times for 10 sec each and centrifuging at 3,000 g for 2 min. The organic phase (0.2 mL) was collected and added to 2.8 mL methanol-butanol (2:1, v/v), followed by 15 µL thiocyanate solution (3.94 M) and 15 µL ferrous iron solution. The solution was vortexed and the absorbance measured at 510 nm after 20 min. Ferrous iron solution was prepared by mixing 0.132 M BaCl₂ and 0.144 M FeSO₄ in acidic solution. Lipid hydroperoxide concentrations were determined using a cumene hydroperoxide standard curve.

Thiobarbituric acid (TBA) solution was prepared by mixing 15 g trichoroacetic acid, 0.375 g TBA, 1.76 mL 12 N HCl, and 82.9 mL distilled water. Three milliliters of 2% butylated hydroxytoluene (BHT) in ethanol was added to 100 mL of TBA solution and 2 mL of this solution was mixed with 1 mL of reconstituted sample (5 mg of emulsion powder in 1 mL of distilled water). The mixture was vortexed, heated in a boiling water bath for 15 min, and centrifuged at 3,000 g for 25 min. The absorbance of the supernatant was measured at 532 nm. TBARS concentrations were determined using standard curve prepared with 1,1,3,3-tetraethoxypropane.

**Accelerated oxidative tests.** The oxidative stability of SL powders was also evaluated by accelerated oxidative tests using differential scanning calorimetry (DSC). The calorimetric measurements were performed with Netzsch DSC 204 F1 Phoenix (Burlington, MA). Oxygen was used as the purge gas at a rate of 20 mL/min. The instrument was calibrated with indium using standard DSC procedure. Samples (4-5 mg)
were placed in crimped aluminum sample pans. In order to facilitate the contact of
samples to oxygen, the lid of each pan was perforated by four pinholes. Determination of
the onset oxidation temperature (OOT) was carried out in the temperature interval of 50-
300°C with a heating rate of 10°C/min. The oxidation induction time (OIT) was
determined isothermally at 200°C. All measurements were performed in triplicate and
average was reported.

**Tocopherol analysis.** HPLC (Shimadzu LC-6A pump equipped with an RF-10AXL
fluorescence detector with excitation set at 290 nm and emission at 330 nm (Shimadzu
Corp., Columbia, MD) was used for tocopherol analysis. An isocratic mobile phase of
0.85% (v/v) isopropanol in hexane was used at a flow rate of 1.0 mL/min. The normal
phase column was a LiChrosorb Si 60 column (4 mm, 250 mm, 5 µm particle size, Hiber
Fertigsaeule RT, Merck, Darmstadt, Germany). The sample concentration was 20 mg/mL
in HPLC-grade hexane. Injection volume was 20 µL. The tocopherols were identified by
comparing their retention times with those of authentic standards (0.03 to 1.25 µg/mL in
hexane containing 0.01% BHT). Tocopherols were quantified based on the standard
calibration curves and reported as parts per million (ppm) from the average of triplicate
determinations.

**Dispersibility of SL powder.** Dispersibility was determined by adding a small amount
of powder (~0.1 g) into the stirring chamber (2,000 rpm) of a laser diffraction instrument
(Malvern Laser Particle Size Analyzer, Mastersizer S, Malvern Instruments,
Southborough, MA). The measurement was performed with distilled water as dispersant.
The dispersibility was assessed by measuring the change in mean particle diameter ($d_{4,3}$)
and obscuration (the fraction of light lost from the main laser beam when the sample was introduced) as a function of time (Klinkesorn and others 2005).

**Statistical analysis.** Mean values and standard deviations of at least triplicate determinations were reported. Independent samples t-test ($\alpha = 0.05$) was performed using IBM SPSS Statistics 21 to determine a significant difference between TDA-SL and PDG-SL powders.

**RESULTS AND DISCUSSION**

**Fatty acid composition and positional distribution of TDA-SL and PDG-SL.** Tripalmitin and palm olein were modified via lipase-catalyzed acidolysis reaction with a free fatty acid mix of DHASCO and ARASCO (yielding TDA-SL), and a free fatty acid mix of DHASCO and GLA (yielding PDG-SL), respectively. The fatty acid profile of these SLs and their substrates are shown in Table 6.1. The levels of palmitic acid at the $sn$-2 position of TAGs in TDA and PDG-SLs were 48.53 and 35.11%, respectively. These levels are lower than the content of $sn$-2 palmitic acid in HMF, which are greater than 50% (Straarup and others 2006). However, these levels are higher than the levels in vegetable oils (5-20% $sn$-2 palmitic acid) (Mattson and Lutton 1958), which are commonly added as fat ingredient in infant formula mix. The levels of LCPUFAs in HMF vary and depend on the mothers’ diet. The reported value of ARA, DHA, and GLA in human milk are 0.24-1.00%, 0.06-1.40%, and 0.07-0.12%, respectively (Brenna and others 2007; Jensen 1999). TDA-SL contained 17.69% ARA and 10.75% DHA. PDA-SL contained 5.03% GLA and 3.75% DHA. These SL can be partially added in vegetable oil blend used in infant formula, to provide palmitic acid at the $sn$-2 position and the beneficial LCPUFAs.
Moisture content and water activity. Moisture content and water activity ($a_w$) affect the shelf life of food products and influence the rate of lipid oxidation. The maximum moisture content of dried powder specified by the food industry is between 3-4% (Master 1991). SL powders produced in this study have moisture contents of 1.78-1.96% and water activities of 0.15-0.16 (Table 6.2). In general, low moisture content (1-3%) and low water activity (0.10-0.25) are achieved through spray-drying conducted at a temperature between 165-195°C (Hogan and others 2001; Klinkesorn and others 2006). The role of water in lipid oxidation depends on the structure and composition of the food. For example, storage study of spray-dried tuna oil coated with a lecithin-chitosan wall, conducted at equilibrium relative humidity (RH) of 11, 33, and 52% showed a rapid oxidation at lower relative humidities (11 and 33% RH) (Klinkesorn and others 2005). This contradicts the generalized view that lipid oxidation in foods is at its lowest level when water activity is between 0.2 and 0.4 (monolayer water), but increases rapidly when the water activity is either decreased or increased (Karel and others 1967). Further evidence showed that initial powder quality of dried-whole milk powder was retained best at water activities between 0.11 and 0.23 (Stepelfeldt and others 1997).

Efficiency of microencapsulation. Microencapsulation efficiency reflects the presence of free oil on the surface of the particles, and the degree to which the wall can prevent extraction of internal oil (Hogan and others 2001). Previously reported microencapsulation efficiencies using MRPs as encapsulants were between 80-98%, depending on the type of protein, the oil to protein ratio, and the oil load in the powder (Rusli and others 2006). Microencapsulation efficiency for the SL powders was 90%,
and in the mid-range of the reported values. These lower values may be a result of the different extraction conditions used.

**Oxidative stability.** During lipid oxidation hydroperoxide primary oxidation products form continuously, and break down into a variety of non-volatile and volatile secondary products (Shahidi and Zhong 2005). The oxidative stability of dried SL powders was determined on the basis of total lipids for both lipid hydroperoxide (PV) and TBARS formation (Table 6.2). The levels of hydroperoxide and TBARs of these SL powders were comparable to fish oil powders produced in previous study (Klinkesorn and others 2005). Both TDA-SL and PDG-SL powders have low TBARS and PV values suggesting their stability to oxidative stress. MRPs possess antioxidant properties (Wijewickreme and others 1999) and may be providing protection to the unsaturated oils. Hydroperoxide concentrations in spray-dried TDA-SL powder were significantly higher than PDG-SL (p<0.05). TDA-SL powder had slightly higher TBARS concentration compared to PDG-SL powder; however, the differences were not significant (p>0.05). The oxidative stability index (OSI), determined using an oxidative stability instrument at 110 °C also indicated a higher oxidative stability for PDG-SL powder (data not shown). Both SL powders were prepared using the same microencapsulation protocol. The lower degree of oxidation for TDA-SL indicates that PDG-SL was relatively more stable to oxidizing conditions during the microencapsulation process. The amount of polyunsaturated fatty acids was greater than 30% in TDA-SL, but lower than 20% in PDG-SL (Table 6.1). Higher concentration of unsaturated fatty acids in the oil may contribute to an increase in the rate of lipid oxidation. The greater the degree of unsaturation in a fatty acid the more vulnerable it is to lipid oxidation. DHA (6 double bonds), ARA (4 double bonds), and
GLA (3 double bonds) are LCPUFAs in the SLs with high degree of unsaturation. The lower oxidative stability in TDA-SL is expected as it contained a higher amount of DHA and ARA with higher degree of unsaturation, compared to PDG-SL.

**Tocopherol analysis of oil substrates.** The oxidative stability of fats and oils depends on fatty acid composition and on the amount of antioxidant present. Antioxidant effects of tocopherols in the oils may help improve the oxidative stability of the products during microencapsulation process. Tocopherol analysis revealed that TDA-SL contained a lower amount of total tocopherols (48.19 ppm) compared to PDG-SL (147.84 ppm). The amount of each tocopherol and tocotrienol in TDA-SL and PDG-SL are shown in Figure 6.1. Tocotrienols are present at higher concentrations in PDG-SL. The substrate oil for PDG-SL is palm olein, a natural source of vitamin E. The higher oxidative stability of PDG-SL microencapsulated product is possibly due to the lower amount and lower degree of unsaturation of LCPUFAs and higher content of total tocopherols in PDG-SL compared to TDA-SL.

**Accelerated stability test.** Oxidation reactions are exothermic process, which can be measured by DSC either in an isothermal or non-isothermal mode. Oxidation onset temperature (OOT) is a relative measure of the degree of oxidative stability of the material evaluated at a given heating rate and oxidative environment. The higher the OOT value, the more stable the material (ASTM Standard E2009-2008). Similarly, oxidative induction time (OIT) is a relative measure of the degree of oxidative stability of the material evaluated at the isothermal temperature of the test (ASTM Standard E1858-2008). OOT and OIT values were determined for SL powders to obtained relative oxidative stability information. DSC measurements were conducted at a heating rate of
10°C/min for OOT and isothermally at 200°C for OIT. PDG-SL powder has a higher OOT and a longer OIT compared to TDA-SL powder (Table 6.3). Again, this is possibly due to the differences in the levels of antioxidants and unsaturated fatty acids between the two SLs powders since they were produced using the same microencapsulation and spray-drying method.

**Product dispersibility.** A small sample (~0.1 g) of the SL powder was added to a continuously stirred measurement chamber filled with distilled water. The volume-weighed average diameter \( d_{4,3} \) (the sum of the volume ratio of droplets in each size class multiplied by the mid-point diameter of the size class) is sensitive to the presence of large particles in an emulsion, and therefore sensitive to phenomena such as flocculation (Walstra 2003). The obscuration is sensitive to the total amount of material dispersed in the fluid. The change in obscuration as a function of time, and the mean particle size \( d_{4,3} \) were measured to assess the dispersibility of SL powder (Walstra 2003). Figure 6.2 showed that the droplet obscuration of both SL powders increased steeply within the first minute of agitation time, after that it reached a fairly constant value (approximately 24% for TDA-SL powder and 27% for PDG-SL powder). In addition, \( d_{4,3} \) decreased after 1 min of stirring time as shown in Figure 6.3. Both obscuration and \( d_{4,3} \) reached a relatively constant value (approximately 1.7 µm for TDA-SL powder and 2 µm for PDG-SL powder) soon after 2-3 min. Rapid decrease in particle size and increase in droplet obscuration indicated that these products dispersed quickly into homogeneous suspension (Raphael and Rohani 1996).
CONCLUSIONS

Two enzymatically synthesized SLs for infant formula use were encapsulated and spray-dried into a powder form. These SLs were encapsulated in MRPs of a heated whey protein isolates and corn syrup solid. The encapsulated SL powders resulted in 90% encapsulation efficiency, low peroxide values, and low TBARs values. These powders were rapidly dispersed in water to give a homogenous suspension. The powder containing SL with a higher degree of unsaturation and a lower concentration of tocopherols resulted in higher peroxide and TBARs values. The results suggested that the degree of unsaturation and concentration of the antioxidant present in the starting oils influence the oxidative stability of the encapsulated products.

ACKNOWLEDGEMENTS

The authors would like to thank DSM Nutritional Products-Martek (Columbia, MD), IOI-Loders Croklaan (Channahon, IL), Grande Custom Ingredients Group (Lomira, WI), and Tate & Lyle (Decatur, IL) for providing us with the materials used in this study.
REFERENCES


Table 6.1 Fatty acid composition and positional distribution (%) of enzymatically produced TDA-SL and PDG-SL

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>TDA-SL</th>
<th>PDG-SL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>sn-2</td>
</tr>
<tr>
<td>Lauric acid C12:0</td>
<td>1.94±0.01</td>
<td>3.00±0.13</td>
</tr>
<tr>
<td>Myristic acid C14:0</td>
<td>5.09±0.02</td>
<td>4.84±0.14</td>
</tr>
<tr>
<td>Palmitic acid C16:0</td>
<td>36.70±0.11</td>
<td>48.53±1.40</td>
</tr>
<tr>
<td>Stearic acid C18:0</td>
<td>4.29±0.02</td>
<td>4.03±0.03</td>
</tr>
<tr>
<td>Oleic acid C18:1 n-9</td>
<td>15.28±0.03</td>
<td>9.82±0.12</td>
</tr>
<tr>
<td>Linoleic acid C18:2 n-6</td>
<td>2.89±0.02</td>
<td>1.83±0.01</td>
</tr>
<tr>
<td>Gamma-linolenic acid C18:3 n-6</td>
<td>0.83±0.01</td>
<td>0.19±0.00</td>
</tr>
<tr>
<td>Arachidonic acid C20:4 n-6</td>
<td>17.69±0.09</td>
<td>9.73±0.13</td>
</tr>
<tr>
<td>Docosahexaenoic acid C22:6 n-3</td>
<td>10.75±0.15</td>
<td>4.80±0.03</td>
</tr>
</tbody>
</table>

*TDA-SL was produced from tripalmitin with free fatty acid mix of DHASCO and ARASCO. PDG-SL was produced from palm olein with free fatty acid mix of DHASCO and GLA. Data were obtained from previous studies.
Table 6.2 Product characteristics of microencapsulated TDA-SL and PDG-SL powder

<table>
<thead>
<tr>
<th>Product characteristics</th>
<th>TDA-SL powder</th>
<th>PDG-SL powder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total oil (g/g of sample)</td>
<td>0.2373±0.0019</td>
<td>0.2502±0.0099</td>
</tr>
<tr>
<td>Free oil (g/g of sample)</td>
<td>0.0237±0.0017</td>
<td>0.0240±0.0013</td>
</tr>
<tr>
<td>Microencapsulation efficiency (%)</td>
<td>90.00±0.73</td>
<td>90.39±0.55</td>
</tr>
<tr>
<td>Moisture content (%)</td>
<td>1.78±0.09</td>
<td>1.96±0.03</td>
</tr>
<tr>
<td>Water activity, $a_w$</td>
<td>0.15±0.02</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>Hydroperoxide value, PV (mmol/kg oil)</td>
<td>20.22±0.65*</td>
<td>4.98±0.78*</td>
</tr>
<tr>
<td>TBARS (mmol/kg oil)</td>
<td>1.00±0.14</td>
<td>0.64±0.07</td>
</tr>
<tr>
<td>Oxidative onset temperature$^b$, OOT (°C)</td>
<td>225.67±1.15*</td>
<td>239.23±0.89*</td>
</tr>
<tr>
<td>Oxidative induction time$^c$, OIT (min)</td>
<td>5.17±0.06*</td>
<td>11.60±0.00*</td>
</tr>
</tbody>
</table>

$^a$Microencapsulation was prepared using 1:1 ratio of oil to protein and 25% oil load in powder. Average values of at least triplicate measurements were reported. Asterisk indicates values with significant difference ($p < 0.05$) between the two SL microcapsules. $^b$OOT determined by DSC at a heating rate of 10°C/min. $^c$OIT determined by DSC isothermally at 220 °C.
Figure 6.1 Tocopherols concentration (ppm) in TDA-SL and PDG-SL. T, tocopherol and T3, tocotrienol.
Figure 6.1

![Bar chart showing tocopherol concentrations for TDA-SL and PDG-SL](chart.png)
Figure 6.2 Influence of stirring time on obscuration of spray-dried TDA-SL and PDG-SL powders. Obscuration was measured as a function of time after powders were added to the stirring cell of a laser diffraction instrument.
Figure 6.2

![Graph showing % Obscuration over Time (min)]

- TDA-SL powder
- PDG-SL powder
**Figure 6.3** Mean droplet diameter (µm) measured as a function of time after powders were added to the stirring cell of laser diffraction instrument.
Figure 6.3
CHAPTER 7

CONCLUSIONS

Three structured lipids (SLs), with similar fatty acid profile and positional distribution to human milk fat (HMF), were produced through a single step enzymatic modification of palm olein (PDA-SL and PDG-SL) or tripalmitin (TDA-SL). These SLs contained a relatively higher content of palmitic acid at the preferred sn-2 position compared to vegetable oils. In addition to the major fatty acids found in HMF (palmitic, oleic, and linoleic acids), these SLs also contained long-chain polyunsaturated fatty acids (LCPUFAs), which are physiologically important to infant development. To facilitate the applications of SLs into powdered infant formula, two (PDG- and TDA-SL) of the SLs were processed into dried powders using microencapsulation and spray-drying methods. PDG- and TDA-SLs were successfully microencapsulated in Maillard reaction products (MRPs) obtained from heated whey protein isolates and corn syrup solids solution with high microencapsulation efficiency. Two manufacturing methods of powdered infant formula containing SLs with LCPUFAs were investigated. TDA-SL was applied into infant formula as a fat source using wet-mixing/spray-drying (as liquid TDA-SL) and dry-blending (as TDA-SL powder) methods. Dry-blending method was a better method of production giving powdered formula with a higher oxidative stability.

In the following section, important findings and future research ideas based on the findings will be discussed according to the three specific aims of this research described in the introduction section.
**Aim 1: Optimization of the reaction conditions, and blending ratios, of substrates for the synthesis of SLs.** Selection of suitable materials from natural fats and oils is an essential step in the design of SL production. Major fatty acids (palmitic, oleic, and linoleic acids) of HMF are present in the substrates (triplalmitin, palm olein, DHASCO, ARASCO, GLA-borage oil) selected for the three SL designs, providing similarity in the FA profiles. The production of PDA-SL, from acidolysis reaction between palm olein and FFA mix of concentrated DHA and ARA, was not cost effective. This production had small yield and involved an extra concentration step of the LCPUFAs, prior to the reaction, and multiple purification steps to eliminate the non-esterified LCPUFAs, after the reaction. Response surface methodology (RSM) can be applied to predict and manipulate the incorporation of FA at different conditions. Multiple-step operation could be employed in the future study to allow additional control over types of FA incorporation in each step.

**Aim 2: SL characterization and application in infant formula.** SLs were compared to HMF and fat extracted from infant formula. Positional analysis showed a higher content of the palmitic acid at the sn-2 position of the SLs compared to vegetable oils commonly used in infant formula. Analysis of TAG molecular species revealed some similarities of TAG species present in TDA-SL, palm olein, and fat extracted from infant formula. TAG species in TDA-SL and in PDG-SL were identified in HMF, however their abundances were considerably different. Imitation of HMF chemical structure based on TAG species components is another area of research to explore. Both TDA- and PDG-SLs completely melt at temperatures close to the melting point of HMF (below 38°C). Production of powdered infant formula using SL was evaluated between wet-mixing/spray-drying
(using liquid TDA-SL) and dry-blending (using TDA-SL powder) processes. Dry-blending method involves microencapsulation of SL and milder conditions of operations, yielding products with higher oxidative stability.

**Aim 3: Microencapsulation of SLs using Maillard reaction products (MRPs) as encapsulants.** The encapsulated SL powders resulted in 90% encapsulation efficiency and high oxidative stability. MRPs have antioxidant properties and may provide SLs protection from lipid oxidation. Degree of unsaturation and concentration of endogenous antioxidant in the starting oils were shown to affect the oxidative stability of the encapsulated SL powders. Formulation of infant formula must comply with regulatory requirements for nutrients and energy contributions. Carbohydrate and protein components used as encapsulants contribute additional calorie energy in the final products. Future works are needed to increase the oil load in microencapsulated powder. This would allow minimal changes in the formulation due to the addition of encapsulated SL powders and minimize the cost of production.