ENZYMATIC PRODUCTION OF *TRANS*-FREE STRUCTURED MARGARINE FAT ANALOGS

by

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(Under the Direction of Casimir C. Akoh)

ABSTRACT

Intake of high amounts of *trans* fatty acids (TFAs) have been positively correlated with increased risk of several chronic diseases. The main aim of this research project was to produce structured lipids (SLs) suitable for formulation of *trans*-free margarines. The oils/fats used were stearidonic acid (SDA) soybean (SDASO), high stearate soybean (HSSO), cottonseed (CO), fully hydrogenated cottonseed (HCO) oils, and palm stearin (PS). The first specific aim was to optimize the reaction conditions of substrates for *trans*-free SLs production. Second specific aim was to characterize the physical and chemical properties of SLs and compare with physical blends. Third specific aim was to characterize the textural and sensory properties of margarines prepared with selected SLs and compare with commercial brands. Desirable and optimal products composition were achieved at 50 °C, 18 h, 2:1 (HSSO:SDASO) with Lipozyme TLIM, containing 15.1 mol% stearic acid and 10.5 mol% SDA. It had desirable polymorphism (β'), thermal

properties, and solid fat content (SFC, 3.2% at 10 °C and 0.1% at 33.3 °C) for formulation of soft margarine. The margarine formulated with this SL was *trans*-free, SDA-enriched with no significant difference in sensory properties. For the second combination, desirable SL containing 12.3 mol% stearic acid and no TFA was obtained at 50 °C, 20 h, 2:1 (PS:HSSO) with Novozym 435. This SL was suitable for stick/hard margarine because of its high melting completion temperature (45.4 °C). In the third combination, SL synthesized at 56 °C, 6 h, 4:1 (PS:CO), using Novozym 435, was used to formulate hard/industrial margarine with high oxidative stability and no TFA. In the fourth combination, SL synthesized at 65 °C, 16.5 h, 2:1 (PS:HCO) using Lipozyme TLIM, was selected to formulate margarine. A *trans*-free hard margarine with high melting completion temperature (50.1 °C) that may be suitable for puff pastries, cooking, or baking purposes was obtained. Different types of *trans*-free margarines were produced, the most prominent being SDA-containing soft, spreadable margarine. This research resulted in the production of *trans*-free SLs as an alternative to partially hydrogenated fat and can be used by the food industry to formulate *trans*-free foods.

INDEX WORDS: Cottonseed oil, Fully hydrogenated cottonseed oil, High stearate soybean oil, Lipases, Lipozyme TLIM, Novozym 435, Palm stearin, Stearidonic acid soybean oil, Structured lipid, *trans* Fatty acid, *trans*-Free margarines

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

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2012

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DEDICATION

To my parents

ACKNOWLEDGEMENTS

I feel a deep sense of gratitude to all the people who contributed towards the completion of this dissertation.

I want to especially thank my major professor, Dr. Casimir C. Akoh, who not only supported me in the accomplishment of this research but also encouraged and challenged me throughout my academic program towards the best realization of my goals.

I express also my deep appreciation to each professor that served on my advisory committee, Drs. Robert L. Shewfelt, Louise Wicker, William L. Kerr, and Yao-Wen Huang for their guidance. I would also like to thank USDA National Institute of Food and Agriculture for their grant to Dr. Akoh in support of this project.

I would like to thank all my lab members for their kind co-operation towards the completion of my research. A special thanks to Ms. Victoria Wentzel without whose knowledge and assistance this study would not have been successful.

I would like to thank my family and friends, especially my mother, Dr. Nutan Pande, my brother, Mr. Gaurav Pande, my husband, Mr. Rajiv Ranjan, and Eddie for their encouragement and endless love, throughout the duration of my studies.

Last but not least, thanks to the faculty and staff of the Department of Food Science & Technology, UGA, for being part of my graduate student life and for their contributions in my personal and professional development.

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CHAPTER 1

INTRODUCTION

trans Fatty acids (TFAs) are unsaturated fatty acids with double bonds in *trans* orientation. The average daily intake of TFA in North America is 3-4 g/person as estimated by food frequency questionnaire method and greater than 10 g/person by extrapolation of human milk data (Craig-Schmidt, 2006). The two main sources of TFA are ruminant animals and partial hydrogenation (Gebauer *et al*., 2007). Partial hydrogenation, a common industrial process of solidifying oils, is the main contributor of TFA (80%) in the US diet (Eckel *et al*., 2007). Products like margarines, spreads, shortenings, bakery products, and fast foods are a major source of TFA. Intake of high amounts of TFA is associated with increased risk of coronary heart disease (Hu *et al*., 1997), inflammation (Mozaffarian *et al*., 2004), and cancer (Astorg, 2005). Dietary Guidelines for Americans 2010 suggest that TFA consumption should be as low as possible. Furthermore, as per the ruling issued by United States Food and Drug Administration (FDA), all food containing $\geq$ 0.5 g TFA/serving are to be labeled accordingly (Federal Register, 2003). This has led the food industry to seek alternative processes to produce cost effective zero or reduced *trans* fat foods with acceptable functional properties. One such potential alternative is interesterification.

Interesterification is a chemically or enzymatically catalyzed ester exchange reaction between two acyl groups, thereby altering the overall chemical composition and physical properties of the interesterified fats. Although chemical interesterification

involves lower cost and is easily scalable, it lacks specificity. Lipases are preferred over chemical interesterification because they are regio-and stereo-specific and offer a better control over the final product (Marangoni & Rousseau, 1995). Structured lipids (SLs) are defined as triacylglycerols (TAGs) that have been structurally modified either by incorporation of new fatty acids (FAs) or changing the position of existing fatty acids to yield novel TAGs of desired physical, chemical, and nutritional properties (Fomuso & Akoh, 1996).

The present dissertation includes eight chapters. The first chapter is introduction which contains the overall objectives of this study. The second chapter is the literature review of topics related to interesterification, SL, TFA, substrates used, and characterization of SL. Third chapter includes enzymatic synthesis of *trans*-free structured margarine fat analogues using stearidonic acid soybean (SDASO) and high stearate soybean oils (HSSO). It discusses the optimization process at small-scale and then SL production at gram-scale and its characterization. The fourth chapter is the use of this SL in formulating a *trans*-free, n-3 FA-enriched, soft margarine.

The fifth chapter contains enzymatic synthesis of *trans*-free structured margarine fat analogues with HSSO and palm stearin (PS) and their characterization. In the sixth chapter production of *trans*-free margarine with cottonseed oil (CO) and PS by enzymatic interesterification is discussed. SLs are compared with physical blends to find a better alternative to partially hydrogenated fat. The seventh chapter includes production of hard, *trans*-free industrial margarine by enzymatic interesterification of fully hydrogenated cottonseed oil (HCO) and PS.

The last chapter covers the overall conclusions and highlights of the whole study along with some suggestions for future work.

RESEARCH QUESTION

Does enzymatic interesterification of oils result in *trans*-free SLs suitable for margarine formulation?

HYPOTHESIS

Enzymatic interesterification of oils results in *trans*-free SLs suitable for margarine formulation. The overall hypothesis was tested by lipase-catalyzed synthesis of SLs with SDASO and HSSO, HSSO and PS, CO and PS, and HCO and PS as substrates.

SPECIFIC AIMS

The overall goal of this research project is to enzymatically synthesize SL suitable for *trans*-free margarine formulation. The specific aims are:

(1) Optimization of the reaction conditions and blending ratios of substrates for *trans*-free structured margarine fat synthesis. *It is hypothesized that optimization of reaction conditions and blending ratios will result in a model that can be adapted for large-scale synthesis of trans-free SL.*

(2) Characterization of physical and chemical properties of the SLs and comparison with physical blends. *It is hypothesized that enzymatically produced SLs will have better physical and chemical properties than the physical blend and would be more suitable for margarine formulation.*

(3) Characterization of textural and sensory properties of margarines prepared with selected SL and comparison with commercial brands. *It is hypothesized that the*

margarines prepared with trans-free SL will have similar or superior textural and sensory properties when compared to commercial brands.

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CHAPTER 2

LITERATURE REVIEW

STRUCTURED LIPIDS

Lipids are hydrophobic or amphipathic small molecules that may originate entirely or partially by carbanion-based condensations of thioesters such as fatty acids (FAs) and polyketides, and/or by carbocation-based condensations of isoprene units (e.g., prenols, sterols) (Fahy *et al*., 2005). Lipids can also be defined as ‘‘a wide variety of natural products including FAs and their derivatives, steroids, terpenes, carotenoids and bile acids, which have in common a ready solubility in organic solvents such as diethyl ether, hexane, benzene, chloroform or methanol’’ (Christie, 1982). Nearly all the commercially important fats and oils of animal and plant origin consist almost exclusively of the simple lipid class triacylglycerols (TAG). TAGs are formed if all of the OH groups of a glycerol molecule are esterified by FA moieties (R1, R2, and R3) as shown in Fig. 2.1a. The essential elements of FA structure are a hydrocarbon chain and a carboxylic acid group (Fig. 2.1b). The chain length ranges from 4 to 30 carbons, 12–24 being most common. The chain is typically linear and usually contains an even number of carbons. However, in the presence of a double bond or double bonds the chain becomes kinked. Natural oils exhibit a wide range of physical properties which are influenced by the degree of unsaturation, the length of the carbon chain, the isomeric form of the FA, the molecular configurations of the TAG molecules, and the polymorphic state of the fat (Ghotra *et al*., 2002). Fat plays an important role in our daily diet:

(1) It acts as carrier for the fat-soluble nutrients such as vitamins A, D, E, and K

(2) It is the most concentrated energy source of all foods, contributing a total of 9 kcal/g compared to the 4kcal/g furnished by carbohydrates and proteins.

(3) It is the structural element of cell, subcellular components, and cell membranes

(4) It insulates the body from temperature extremes and also cushions the vital organs to prevent them from shock

(5) It is the main component of hormones and precursors for prostaglandin synthesis

(6) The essential fatty acids (EFA) are also made available to us from the fat we eat.

Besides providing specific nutritional requirements, fat plays important functional roles. It acts as an efficient heat transfer agent in frying, lubricating agent in dressings, gives a leavening effect by trapping air in baked foods, provide satiety, and contribute to the taste and aroma of food.

The majority of natural fats and oils present limited application in their unaltered form due to their particular FA and/or TAG compositions. Recent advances in oil and food chemistry make it possible to modify the structure of natural lipids for specific applications. When incorporated into food products, such custom-made lipids known as structured lipids (SLs), promote better nutrient delivery and absorption. They reduce the risk of chronic diseases and can be used for therapeutic, pediatric, supplemental, and weight management nutrition. SLs are lipids (usually TAG, but can also include monoacylglycerols (MAG), diacylglycerols (DAG), and glycerophospholipids (GPL)) that have been structurally modified by chemical or enzymatic processes (Akoh, 2008). They are restructured from their natural form by changing the positions of FAs, or the FA profile, or synthesized to yield novel TAGs (Akoh, 2008). SLs can also be defined as

TAGs containing mixtures of FAs (short chain and/or medium chain, plus long chain) esterified to the glycerol moiety, preferably in the same glycerol molecule (Akoh, 2008). Various methods like hydrogenation, fractionation, blending, interesterification, esterification, and even bioengineering have been used for modification of natural TAGs (Xu, 2000). SL can be used to produce *trans*-free margarines, cocoa butter substitutes, improve functional, physical, and nutritional properties of foods (Osborn and Akoh, 2002). SLs have been developed for nutritional and medical applications such as infant formulas, low-calorie fats, and enteral (oral tube) and parenteral (intravenous) nutrition. They are also used in food applications with specific functionalities such as plastic fats, shortenings, cocoa butter alternatives, salad oils, and coating lipids. Enzymatically produced SLs with desired nutritional quality and functionality, sometimes referred to as "nutraceutical,' has been used in various medical and food applications (Lee and Akoh, 1998). The type of FA esterified to the glycerol backbone greatly affects the properties of the final product. Therefore, SLs are designed by carefully selecting the FA to obtain application-based end product.

Short-chain FAs (SCFAs) are FAs with aliphatic tails of fewer than six carbons. These include formic, acetic, propionic, butyric, and valeric acids. SCFA-containing TAGs are neutral, chemically stable, and rapidly hydrolyzed by pancreatic and gastric lipases to glycerol and their respective FA. These are absorbed more rapidly by the stomach than other FA because of their shorter chain length, volatile nature, smaller molecular size, and higher solubility in water. SCFA have a lower heat of combustion than other FA, making them lower in calories and suitable for use in the synthesis of low-calorie SL (Devi *et al.*, 2008). Medium-chain FAs (MCFAs) are saturated FAs having 6-

12 carbon atoms. Common ones are caproic, caprylic, capric, and lauric acids. MCFA can pass directly into the portal vein and are readily oxidized in the liver to serve as a source of energy rather than being absorbed through the lymphatic system. They are consequently a source of additional and quick energy for patients recovering from surgery or illness and for athletes. MCFAs also increase metabolic rates and may serve as weight-loss ingredients in foods. They are used for enteral and parenteral nutrition and for patients suffering from fat malabsorption, maldigestion, and metabolic disorders. In infant formula, they enhance fat digestion and absorption. Long-chain FAs (LCFAs) include both saturated FA (SFA) and unsaturated FA (UFA) having more than 12 carbon atoms. Myristic (C14:0) and palmitic (C16:0) acids are considered hypercholesterolemic, whereas stearic acid (C18:0) is considered neutral. No change in blood cholesterol or TAG levels was observed when feeding cocoa butter, which had C18:0 at *sn*-1/3 positions, or an interesterified fat which had an equal distribution of C18:0 in all three positions (Grande *et al*., 1970; Hegsted *et al*., 1965). Total and LDL-cholesterol and cholesterol ester concentrations increased the most with C16:0, followed by C14:0, while remaining unchanged with C18:0 (Karupaiah and Sundram, 2007). Oleic acid (C18:1n-9) is a nonessential monounsaturated FA (MUFA) that has total and LDL-cholesterol lowering effect. Linoleic (C18:2n-6) and linolenic acids (C18:3n-3) are EFA, as they are not synthesized by mammals and must be obtained from the diet. Linolenic acid is the parent n-3 FA, which is converted to eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) by elongation and desaturation steps (**Fig. 2.2**). EPA and DHA have been associated with proper fetal development and have

cardiovascular function, weight management, and anti-inflammatory properties (Swanson *et al*., 2012).

SL containing LCFA at *sn*-2 position and SCFA/MCFA at *sn*-1,3 positions have attracted increased attention for nutritional development. SCFA are better hydrolyzed by pancreatic lipase when located at *sn*-3 position. They are a quick source of energy and have low calories (Osborn and Akoh, 2002). MCFA are used in SL with long chain polyunsaturated fatty acids (PUFA) for their mobility, solubility, and ease of metabolism. LCFA are slowly absorbed in the body and includes EFA. SL have a beneficial effect on immune system, nitrogen balance, and improved lipid clearance from the bloodstream (Xu *et al*., 1997).

METABOLISM OF SL

Fig. 2.3 shows the metabolism of long chain triacylglycerol (LCT), medium chain triacylglycerol (MCT), and SL. Lipid hydrolysis starts at the upper intestinal tract where lingual lipase hydrolyzes TAG into MAG, DAG, and free fatty acids (FFA). In the stomach, gastric lipase continues to hydrolyze SCFA and MCFA at *sn*-1,3 positions of the TAG to produce MAG, DAG, and FFA. Being more water soluble, SCFA and MCFA are transported *via* the portal system to the liver where they undergo β-oxidation to yield acetyl CoA end products. Approximately 10–30% of dietary fat is hydrolyzed in the stomach before moving to the duodenum, where the majority of TAG digestion occurs (Mu and Høy, 2004). The hydrolyzed products are then transported to the small intestine where *sn*-1,3 specific pancreatic lipase acts. It has been suggested, that pancreatic lipase shows greater affinity for ester bonds in the *sn*-1 position, compared to the *sn*-3 position (Rogalska *et al*., 1990). All FAs in the *sn*-1/3 positions of TAGs are hydrolyzed during

digestion, in contrast to only 22% of FAs in the *sn*-2 position (Karupaiah and Sundram, 2007). Mu and Hoy (2004) estimated an approximate 75% conservation of FA in the *sn*-2 position, despite acyl migration to the *sn*-1/3 positions. This is due to the regiospecificity of pancreatic lipase for FA in *sn*-1/3 positions as well as the chain length of these FA. The hydrolyzed products (FFA and MAG) are absorbed through intestinal mucosa in the form of micelles. After absorption from the intestines, the FFA and 2-MAG are re-esterified to form chylomicrons and enter the lymphatics where they reach the general circulation *via* the thoracic duct. LCT are absorbed slowly as partial acylglycerols in mixed micelles while MCT undergo near complete hydrolysis and are *trans*ported *via* the portal vein to the liver where they undergo oxidation by a carnitine-independent pathway.

PRODUCTION OF SL

Reaction schemes

SL can be produced by the following chemically or enzymatically catalyzed *trans*esterification reactions (Fomuso and Akoh, 2001):

Direct esterification: Glycerol + FA $\longrightarrow$ TAG + Water

Acidolysis: $TAG_1 + FA_1 \longrightarrow TAG_2 + FA_2$

Alcoholysis: $TAG_1 + Alcohol_1 \longrightarrow TAG_2 + Alcohol_2$

Interesterification: $TAG_1 + TAG_2 \longrightarrow TAG_3 + TAG_4$

Direct esterification

Direct esterification is the reaction between FFA and glycerol to produce TAG. An excess of water would drive the reaction towards hydrolysis and to low product yield (Iwai *et al*., 1964). Direct esterification reactions have been conducted in solvent-free conditions (Akoh, 1993; Pereira *et al*., 2004), organic media (Cloan and Akoh, 1993),

and in supercritical fluid (Marty *et al*., 1992). Torres *et al*. (2001) have used enzymatic direct esterification between glycerol and a mixture of FFA derived from saponified menhaden oil with 20% (w/w) conjugated linoleic acid (CLA), to synthesize SL rich in *n*-3 and CLA.

Acidolysis

Acidolysis is another form of *trans*esterification reaction involving the *trans*fer of acyl groups between acids and esters. This reaction has been used in the production of SL for possible use in infant formula containing more than 70% of palmitic acid esterified to the *sn*-2 position of the TAG (Sahin *et al*., 2005). Rao *et al*. (2001) produced SL with similar melting profile, spreadibility and mouthfeel as that of cocoa butter by incorporating stearic acid into TAG from coconut oil using Lipozyme IM 60. Acidolysis is one of the most commonly used methods for the incorporation of EPA and DHA into vegetable or marine oils to improve their nutritional properties. Spurvey *et al*. (2001) used *Pseudomonas* sp. and *Mucor miehei* lipases to enrich seal blubber and menhaden oils with γ-linolenic acid (GLA). Similarly, Senanayake and Shahidi (1999) modified the FA composition of borage and evening primrose oils by incorporating EPA and DHA using non-specific *Pseudomonas* lipase. Nunes *et al*. (2011) synthesized TAG containing a MCFA at positions *sn*-1,3 and a LCFA at *sn*-2 position by acidolysis of virgin olive oil with caprylic (C8:0) or capric (C10:0) acids, catalyzed by 1,3-selective *Rhizopus oryzae* heterologous lipase.

Alcoholysis

Alcoholysis is the reaction between an alcohol and an ester. It is commonly used in the production of methyl esters from the esterification reaction between TAG and methanol

(or glycerolysis, which is a type of transesterification reaction between glycerol and TAG). Enzymatic glycerolysis is one of the most preferred approaches used for the production of MAGs, which is used in the food industry as emulsifier and surface active agents. Glycerolysis have been performed in both organic solvents (Ferreira and Fonseca, 1995)) and in solvent-free conditions (Myrnes *et al*., 1995; Coteron *et al*., 1998).

Interesterification

Interesterification involves the transfer of acyl groups between two esters in the presence of a catalyst. This reaction is commonly used to alter and improve the physical properties of fat mixtures by rearranging the FA on the glycerol backbone. Chemical interesterification for synthesis of SL is usually through the hydrolysis of the acyl groups followed by reesterification after complete randomization of the acyl groups in TAG (Ferrari *et al*., 1997), catalyzed by alkali metals or alkali metal alkylates such as sodium methoxide. The effectiveness of this reaction depends on anhydrous conditions (less than 0.01% water) and elevated temperatures which can be as high as 200°C. However, in the presence of catalyst, lower reaction temperature can be used. Chemical interesterification is used to improve the textural properties of shortenings (Rodriguez *et al*., 2001) and to produce *trans*-free margarines and spreads with desired plasticity and thermal behavior (da Silva *et al*., 2010).

Although chemical interesterification involves lower cost and easy scale up process, it lacks specificity. Lipases are also used for enzymatic interesterification and are preferred over chemical interesterification because they are regio-and stereo-specific and offer a better control over the final product (Marangoni and Rousseau, 1995). Enzymatic catalyzed reactions help to save energy and minimize thermal degradation as enzymes

function efficiently under mild conditions of temperature and pH (Osborn and Akoh, 2002). Several studies have been reported utilizing enzymatic interesterification to produce desired SL. Lumor *et al*. (2007), produced SL suitable for *trans*-free margarine by enzymatic interesterification of canola oil and stearic acid using Lipozyme RM IM from *Rhizomucor miehei* and *Candida rugosa* lipases. Similarly, *trans*-free plastic shortenings have also been prepared with palm stearin and rice bran oil using enzymatic interesterification with Lipozyme RM IM as biocatalyst. This resulted in a healthier alternative to commercial shortenings with comparable SFC, melting and crystallization profiles, and polymorphism (Jennings and Akoh, 2010). Palm stearin has also been used with several other oils such as rice bran oil and coconut oil (Adhikari *et al*., 2010a), pine nut oil (Adhikari *et al*., 2010b), canola oil and palm kernel oil (Kim *et al*., 2008) using enzymatic interesterification to produce *trans*-free margarine fat analogs. Cocoa butter equivalents are also produced with soft palm mid-fraction (PMF) and stearic acid or stearic acid methyl ester by enzymatic interesterification using *sn*-1,3 specific lipases (Gibon *et al*., 2009).

Lipases

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), derived from from animal, plant, and microbial sources, the latter being the most common source, can catalyze reversal of hydrolysis in the presence or absence of organic solvents and near anhydrous conditions. Unlike esterases which act on water soluble substrates, lipases can act on the interfaces between immiscible aqueous and lipid phases (oil-water interface). Lipases can function efficiently in organic conditions as they may exhibit enhanced thermal stability, increased rigidity and modified chemo- and stereoselectivity in organic solvents (Klibanov, 1997).

Lipase from various sources display positional, FA, and stereo-specificity, and this is the main difference between enzymatic and chemical interesterification reactions. The positional specificity, i.e., specificity toward ester bonds at *sn*-1,3 positions of the TAG, of lipases is due to the steric hindrance that prevents the FA located at *sn*-2 from binding to the active site (Stadler *et al*., 1995). Some examples of 1,3-specific lipases include those from *Rhizomucor miehei, Rhizopus oryzae. R. japonicus, Thermomyces lanuginosa, Penicillium roquefortii, Aspergillus niger,* and *A. flavus* (Xu, 2000). Lipases that show selectivity towards certain FA substrates are known as FA/substrate specific lipases. The most common example would be the lipase from *Geotrichum candidum* which is known to be selective towards LCFA with a *cis*-9 double bond (Macrae, 1983). Lipases can also exhibit specificity to certain FA chain length. Certain lipases do not possess positional or FA selectivity. Such lipases are known as non-specific lipases and produces interesterified products that are similar to those obtained through chemical interesterification (Macrae, 1983). Examples of such lipases include those derived from *Corynebacterium acnes, Staphylococcus aureus* and *Candida sp. (*Macrae, 1983). Lipases that are able to differentiate between the two FA at the *sn*-1 and *sn*-3 positions are said to be stereospecific. Depending on the acyl groups, the lipases will hydrolyze the FA at different rates. *Pseudomonas* sp. and pancreatic lipase have shown stereospecificity when certain acyl groups are hydrolyzed (Uzawa *et al*., 1993).

One of the ways to reduce the cost of enzymatic interesterification in the industry is through immobilization. Immobilized enzymes are usually more thermostable than their free state. This is because immobilization restricts the degree of rotation of the enzyme and thus reduces the possibility of unfolding and denaturation that often occurs at

high temperatures (Balcão *et al*., 1996). They can be stored over a longer period of time and more importantly, they are easily recovered by simple steps such as filtration to facilitate reuse of the enzymes. Immobilized lipases offer stability and reusability to the enzymes thus lowering the cost of the reaction. Immobilization also affects the selectivity, chemical, and physical properties of the lipases (Willis and Marangoni, 2008).

Factors affecting SL production

The product yield, operational stability, equilibrium, and rate of interesterification reaction are all affected by the amount of water present in the reaction. A minimum amount of water is essential to activate the lipase but higher quantities of water favor the hydrolysis of TAG to give mixtures of FFA, MAG, and DAG that may affect the quality and yield of products (Willis and Marangoni, 2008). Higher water content in the reaction mixture also results in a higher acyl migration (Willis and Marangoni, 2008). The optimal water content for interesterification by different lipases ranges from 0.04-11% (w/v), although most reactions require water contents of less than 1% for effective interesterification (Willis and Marangoni, 2008). Temperature can have a dual effect on the rate of reaction. As temperature is increased the rate of collisions between reactants and the enzyme also accelerates, thus resulting in increased acyl exchange. Enzymes are proteins and therefore can be inactivated by high temperatures thereby producing less incorporation. When organic solvents are used the reactants and products are readily dissolved, therefore, wide temperature range can be used for catalysis. However, in solvent-free systems the temperature must be high enough to maintain the reaction mixture in the liquid state (Willis and Marangoni, 2008). The thermostability of lipases varies considerably with their origins. Animal and plant lipases usually are less

thermostable than microbial extracellular lipases. In general, the optimal temperature for most immobilized lipases falls within the range of 30–62 °C, whereas it tends to be slightly lower for free lipases (Willis and Marangoni, 2008). Lipases that can tolerate high temperatures are suitable for food industry applications where organic solvents are avoided and reaction temperatures are usually higher. Generally, microbial lipases have a broad pH activity profile and pH stability range. The pH optima for most lipases lies between 7-9, although lipases can be active over a wide range of acid and alkaline pHs, from about pH 4-10 (Willis and Marangoni, 2008). Other factors that can affect the efficiency of enzymatic transesterification are the lipase content, lipase purity and specificity, lipase loading, substrate and product composition and molar ratio, presence of surface-active agents, and reaction time.

SL synthesis and purification

SLs are synthesized in different types of reactors such as fixed bed, batch, continuous stirred tank, and membrane reactors. Stirred batch reactors are simple to operate and do not cost much. The substrates and the catalyst are fed manually in a predetermined ratio and the reaction takes place at specific temperature and time with continuous stirring. The product (SL) is removed at the end of the reaction. In continuous stirred tank reactor substrates are fed by means of a pump into the reactor containing the enzymes and the product is removed at the same rate with continuous stirring. Fixed bed reactors consist of a column packed with immobilized enzymes through which the substrates and products are pumped in and out at the same rate. They are generally used for large scale synthesis because for their efficiency and ease of operation. Membrane reactor consists of two-phase system separated by a semi-permeable membrane onto which enzymes are

immobilized. The advantage of membrane reactors is that the reaction and separation of substrate and products can be accomplished in a single unit especially in glycerolysis and acidolysis where the conversion rate is inhibited by the water formed during reaction (Willis and Marangoni, 2008). The end product obtained using any of the reaction schemes or reactors consists of a mixture of the desired SL and other by-products such as FFA, FAME, DAG or any undesired TAG that needs to be removed.

Short-path distillation is the most commonly method used for purification of the SL. The short-path distillator is a double-layered glass wall vessel which is connected to a vacuum pump, an inlet (feed vessel) and two receiving flasks – one for the purified product and the other for waste. The temperature of the distillator is controlled by circulating hot synthetic oil in between the two walls of the vessel. Fractionation is usually performed at high temperatures (185 °C) and low pressures (< 1 mm Hg). Short-path evaporators are wiped film evaporators with an internal condenser. The internal condenser drastically reduces the distance between evaporation and condensation thus enabling low operating pressures. Boiling temperature decreases with lower operating vacuum and therefore, thermolabile materials such as lipids and vitamins can be processed without product damage. To separate the FFA from the product, the incoming sample is smeared against the hot inner wall of the vessel by rotating wipers. This causes FFA and any compounds having boiling points lower than the operating temperature to vaporize. The vaporized components precipitate on a condenser (15-20 °C) and drip into the waste flask, whereas the non-volatile component (purified) remains on the inner wall of the vessel and drips into the product flask. Short-path distillation is used extensively to

remove FFA from SL products (Lumor *et al*., 2007; Kim *et al*., 2008; Pina-Rodriguez and Akoh, 2009; Xu *et al*., 2002).

trans FATTY ACIDS

trans Fatty acids (TFAs) are UFAs with carbon-carbon double bonds in *trans* orientation. This *trans* double bond configuration results in a greater bond angle than for the *cis* configuration, thus producing a more extended FA carbon chain that is more similar to that of the SFA, rather than to that of the *cis*-unsaturated double bond-containing FA (Moss, 2006; Oomen *et al*., 2001; Lee *et al*., 2007). The average daily intake of TFA in North America is 3-4 g/person as estimated by food frequency questionnaire method and greater than 10 g/person by extrapolation of human milk data (Craig-Schmidt, 2006). The two main sources of TFA are ruminant animals and partial hydrogenation (Gebauer *et al*., 2007). Partial hydrogenation, a common industrial process of solidifying oils, is the main contributor of TFA (80%) in the US diet (Eckel *et al*., 2007). Hydrogenation is a chemical process that adds hydrogen atoms to the available double bonds in the vegetable oil. As the degree of hydrogenation increases, the amount of SFA increases and MUFA and PUFA decrease. Hydrogenation is usually carried at high temperatures (100-150 °C), 2-10 atm pressure, and in the presence of a catalyst (e.g., Ni). Along with saturation of double bonds, isomerization and migration of double bonds also occur. This gives rise to TFA in partially hydrogenated fats/oils. A fully hydrogenated fat has no TFA as all FA have been converted to SFA. Factors that affect the hydrogenation process and consequently the resultant products are the temperature of the oil mixture, hydrogen gas pressure, catalyst activity and concentration, agitation of the mixture, and time or duration of the process. High temperatures, low agitation, low hydrogen gas pressure, and

high catalyst concentration favors more TFA formation (Mattill, 1964). Elaidic acid (*trans*-9 C18:1) is one of the major TFAs, although during hydrogenation, small amounts of several other TFAs are also produced, including: *trans*-9,*cis*-12 C18:2; *cis*-9,*trans*-12 C18:2; *cis*-9,*cis*-12,*trans*-15 C18:3; and *cis*-5,*cis*-8,*cis*-11, and *cis*-14,*trans*-17 C20:5 (Filip, 2010). *Trans* fats produced by partial hydrogenation are referred to as synthetic or industrial TFA and are present in margarines, snack foods, and prepared desserts. Meat and dairy products contain the naturally occurring TFA vaccenic acid and CLA. Vaccenic acid (*trans*-11 C18:1) accounts for over 60 % of the natural TFAs (Filip, 2010). The metabolic effects and health outcomes of the industrial and natural TFA is a matter of debate. Studies have indicated either no association or a potential beneficial association between ruminant TFA and coronary heart disease (CHD) risk (Ascherio *et al*., 1994). But when absolute amounts of TFA (0-2.5 g/d) were calculated and compared with these studies, no significant difference in risk of CHD for different sources of TFA was found (Weggemans *et al*., 2004).

High intake of TFA is associated with CHD (Hu *et al.,* 1997), increased LDL, decreased HDL, and increased total: HDL cholesterol (Mozaffarian and Clarke, 2009), inflammation (Mozaffarian *et al.,* 2004), cancer (Astorg, 2005), increased incidence of gallstones (Tsai *et al.,* 2005), and Alzheimer's disease in older adults (Morris *et al.,* 2003). In a meta-analysis of four large prospective cohort studies of ~140,000 participants, each 2% increase in energy intake from TFA was associated with 23% higher incidence of CHD (pooled relative risk of 1.23) (Mozaffarian *et al.,* 2006). In a nested case-control study of Nurses' Health Study, a higher multivariable relative risk of 1.33 was found for every 2% energy from TFA intake, using repeated measures (Sun *et*

al., 2007). It has been estimated that the death rates for CHD would decrease by 7% when TFA intake is reduced to 2% of calories (Ascherio *et al.,* 1999). In a randomized control trial, monkeys were fed calorie-controlled diets containing 8% of energy either from *cis* or *trans* monounsaturated fat. TFA fed monkeys had more than threefold greater weight gain than the controls with an increase in visceral fat (Kavanagh *et al.,* 2007). Rats born to TFA fed mothers showed signs of altered appetite-signaling mechanism (Albuquerque *et al.,* 2006). Similarly in humans, TFA are transported across placenta and the breast milk compositions is also affected by the dietary TFA intake, thus suggesting potential health risks in human fetuses and infants (Innis, 2006).

The *trans* fats have been shown to increase inflammatory markers, including CRP, IL-6, and TNF-α, possibly through modulation of monocyte and macrophage activity (Lopez-Garcia *et al.,* 2005). Furthermore, elevated circulating levels of soluble adhesion molecules soluble intercellular adhesion molecule-1 and soluble vascular adhesion molecule-1, as well as nitric oxide-mediated endothelial cell dysfunction, have been observed in individuals consuming large quantities of *trans* fats (Mozaffarian *et al.,* 2006). These inflammatory factors may play an important role in the development of diabetes (increases insulin sensitivity), atherosclerosis, plaque rupture, and ultimately sudden cardiac death (Mozaffarian *et al.,* 2006). Other possible adverse effects of *trans* fats include inhibition of the incorporation of other FAs into cell membranes, interference with elongation and desaturation of EFAs, increased platelet aggregation, decreased birth weight, increased body weight, decreased serum testosterone, and abnormal sperm morphology (Simopoulos, 2008).

Based on the epidemiological data of effects of TFA on human diet several expert committees have set recommended dietary TFA intake. TFA consumption should be as low as possible (Dietary Guidelines for Americans, 2010). As a result, the United States Food and Drug Administration (FDA) issued a ruling requiring all foods containing TFA to be labeled accordingly (products containing ≥0.5 g *trans* fat/serving), effective from January 2006 (Federal Register, 2003). This has led the food industry to produce cost effective zero or reduced *trans* fat foods with acceptable functional properties. In a recent Centers for Disease Control and Prevention study, it was reported that blood levels of TFA in the US white adults decreased by 58% from 2000 to 2009 (Vesper *et al.*, JAMA 2012). This reduction may be attributed to the labeling requirement which helped the consumers to make an informed decision and also increased consumer awareness. Food industry has also been responsible by providing *trans*-free and low *trans* healthy food options. There are a number of challenges faced during development of such products. The replacement ingredient must provide the functional characteristics and physical properties such as solid fat content, desired appearance, crystal polymorphism, stability, and sensory attributes while being cost effective. Some alternatives to *trans* fat are (Hunter, 2005):

(1) Use of naturally stable oils/fats, e.g., palm, cottonseed seed, palm kernel, and coconut oils.

(2) Use of trait-enhanced oils from newer oilseed varieties - use of traditional breeding techniques or biotechnological methods, e.g., high oleic sunflower and canola oils, low linolenic soybean and canola oils.

(3) Use of modified partially hydrogenated oils - modifying the hydrogenation process by altering the variables such time, temperature, and catalyst to obtain reduced *trans* fat content.

(4) Interesterification - during interesterification there is no double bond isomerization or change in unsaturation, i.e., FA remains unaltered but a redistribution of these components on the TAG molecules occurs. Thus, there is no *trans* fat formation while obtaining the desired product.

SUBSTRATES

The EFAs for humans are α-linolenic acid (ALA) (n-3) and linoleic acid (LA) (n-6). The current western n-6: n-3 ratio is 15-16.7:1 as compared to our ancestors diet with a ratio of ~1 (Simopoulos, 2008). Excessive n-6 FAs promotes the pathogenesis of many diseases such as inflammatory diseases, cancer and cardiovascular diseases (Simopoulos, 2008). Increased intake of n-3 FAs is positively associated with decreased blood pressure (Gelenjinse *et al*., 2002), reduced risk of CVD (Calder, 2004), and decreased inflammation (Calder, 2006). A lower ratio n-6/n-3 FAs is more desirable in reducing the risk of several chronic diseases. Therefore, American Heart Association (AHA) Dietary Guidelines suggest Americans consume at least two servings of fish per week and include n-3 FA, ALA, in their diets (Kris-Etherton *et al*., 2003). Soybean oil is obtained from soybean, *Glycine max*, belonging to family Fabaceae. It has been genetically modified by the Monsanto Company to contain approximately 20% stearidonic acid (SDA) resulting in a readily available and sustainable plant source of n-3 FAs (Harris *et al*., 2008). The production of SDA in soybean was achieved by introducing two desaturase genes that encode for the proteins, *Primula juliae* Δ6- desaturase and *Neurospora crassa* Δ15-

desaturase. SDA (18:4 n-3) is a Δ6-desaturase product of ALA and a metabolic intermediate between ALA and eicosapentaenoic acid (EPA) (**Fig. 2.2**). Soybeans lack Δ6-desaturase and the introduction of a gene encoding Δ6-desaturase enables the production of SDA. However, Δ6-desaturase is a rate limiting step and may also convert LA to GLA (n-6 FA). Addition of a Δ15-desaturase with similar temporal expression as that of Δ6-desaturase increases the flux of ALA to SDA (n-3 FA). The Δ15-desaturase also lowers LA levels, thus lowering the substrate pool for GLA production (http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000283.pdf).

Results of animal and human studies demonstrate that dietary SDA can increase red blood cell (RBC) concentrations of EPA 3-4 times more efficiently than ALA and approximately one-third as effective as dietary EPA than ALA (James *et al.*, 2003; Harris *et al.*, 2007). The enrichment of RBCs with EPA and DHA has been shown to reflect cardiac membrane n-3 FA content (Harris *et al.*, 2008). Expressed as a percentage of total FA, this 'Omega-3 Index' has been found to correlate with reduced risk of cardiovascular disease, particularly sudden cardiac death (Harris and Von Schacky, 2004). Soybean oil is low in SFAs and high in PUFAs and MUFAs. It is the principal source of n-3 FAs in the U.S. diet. High-stearate soybeans are designed with elevated levels of stearic acid for increased stability of foods that require solid fat functionality. High stearate soybean oil (HSSO) contains 17% stearic acid compared to 4% in regular soybean oil. HSSO may allow food companies to produce margarines and shortenings with improved texture and no *trans* fats. Soybean oil crystallizes in the β crystal form which is not the desirable crystal type in margarines. Therefore, it should be mixed with other vegetable oils that are β' forming. Cottonseed oil is obtained from *Gossypium* sp. (*G.hirsutum*) of family

Malvaceae. In the United States cotton is grown from Virginia to California, and as far north as southern Kansas. U.S. annually produces over one billion pounds of cottonseed oil. Cottonseed oil crystallizes in the β' crystal form, the desirable crystal type for margarines. The major FAs in cottonseed oil are linoleic, palmitic, and oleic acids. Palm oil is obtained from *Elaeis guineensis* belonging to the family Arecaceae. Palm stearin is the solid fraction obtained by fractionation of palm oil after crystallization at controlled temperatures containing higher proportion of SFAs and TAGs with a higher melting point of 48-50 °C. The palmitic acid content ranges from of 49-68% and oleic content from 24-34% (Sue, 2009). Palm stearin has a wide range of composition, physical properties, solid fat content, and iodine values. These variations result in different types of palm stearin which gives food manufacturers a wide choice of materials for their formulations. Palm stearin can provide the required solids in blends with unsaturated vegetable oils. It is a useful source of fully natural hard fat component for products such as shortening and pastry and bakery margarines to provide increased stability and solid fat functionality.

MARGARINE

Margarine is a water in oil (w/o) emulsion. Margarine is regulated by FDA 21CFR166.110.
(http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=166.110). Margarine (or oleomargarine) is the food in plastic form or liquid emulsion, containing not less than 80% fat. According to the FDA standard of identity margarine should be formulated with edible fats and/or oils from plant/animal/marine sources that have been affirmed as GRAS. They may contain small amounts of other lipids, such as phosphatides or unsaponifiable constituents, and FFA naturally present in the fat or oil. Emulsifiers are

multifunctional in margarine. They reduce surface tension between the aqueous and oil phases so that the emulsion forms with minimal work. Emulsifiers stabilize the finished product during storage to prevent leakage or coalescence of the aqueous phase. They also act as antispattering agents by preventing coalescence and violent eruption of steam during frying. The aqueous phase may consist of water, milk, milk products, suitable edible plant or milk based protein, or mixture of these. Vitamin A should be added in such quantity that the finished margarine contains not less than 15,000 international units (IU) per pound. The optional ingredients include Vitamin D in such quantity that the finished margarine contains not less than 1,500 IU per pound, salt (sodium chloride or potassium chloride for dietary margarine), nutritive carbohydrate sweeteners, emulsifiers, preservatives (permissible type and concentration), color additives (beta-carotene), flavoring substances, acidulants, alkalizers. Margarines were originally developed in 1869 by Hippolyte Mege Mouries, a French chemist, using tallow as an alternative to butter which was expensive and scarce (Chrysan, 2005). During the late nineteenth century, some margarine was prepared from lard or unfractionated beef fat to which liquid oils such as cottonseed or peanut were added to reduce the melting point of the blend. In the early 1900s, some 100% vegetable oil margarines were formulated with coconut and palm kernel oils (Chrysan, 2005). In the United States during the early 1950s, almost all consumer margarine was the stick variety. Since then different types of margarines and spreads (<80% fat) have been introduced. Margarines are made with vegetable oils such as soybean, corn, canola, and olive oils, and therefore have no cholesterol. Margarines also contain lower SFAs than butter. But margarines are produced by partial hydrogenation which generates TFA in the product. According to

Sundale Research (2006), the leading brands of margarines in 2005 were I can't Believe It's Not Butter (19.9%), Shedd's Country Crock (15.4%), and Land O'Lakes (7.9%). The total sale accounted for 1.2 billion USD with unit sales of 854 million pounds. Margarine is an important food and economic commodity. It is also one of the leading sources of TFA in the diet. There are three main types of margarines, table margarine which is softer and easily spreadable, a firmer industrial margarine, and puff pastry margarine with higher SFC for required plasticity (Wassell and Young, 2007). Table margarine can be further classified as tub margarine which are spreadable at refrigeration temperature and stick margarine which are spreadable at ambient temperature. Three basic steps in margarine production are: i) emulsification of the oil and aqueous phases, ii) crystallization of the fat phase, and iii) plasticization of the crystallized emulsion.

RESPONSE SURFACE METHODOLOGY

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response (Montgomery, 2005). The most extensive applications of RSM are in the particular situations where several input variables (independent variables) potentially influence some performance measure or quality characteristic of the process. Thus, performance measure or quality characteristic is called the response.

Two important models are commonly used in RSM. These are the first-degree model (d = 1),

$$Y=\beta_0+\sum_{i=1}^{k}\beta_i X_i \ +\varepsilon_i$$

and the second-degree model (d = 2)

$$Y=\beta_0+\sum_{i=1}^{k}\beta_i X_i \ +\sum_{i=1}^{k}\beta_{ii}X_i^2+\sum_{i=1}^{k}\sum_{j=i+1}^{k}\beta_{ij}X_i \ X_j \ +\varepsilon_{ij}$$

where Y = the response; β_0 = constant; β_i = linear (first-order model); β_{ii} = quadratic (second-order model); β_{ij} = interaction term coefficients; X_i and X_j = independent variables; and ε_{ij} and ε_i= error terms.

In general all RSM problems use either one or the mixture of both of these models. In each model, the levels of each factor are independent of the levels of other factors. In order to get the most efficient result in the approximation of polynomials, the proper experimental design must be used to collect data. Once the data are collected, the Method of Least Square is used to estimate the parameters in the polynomials. The response surface analysis is performed by using the fitted surface. The response surface designs are types of designs for fitting response surface. The most common first-order designs are 2k factorial (k is the number of control variables), Plackett–Burman, and simplex designs. The most frequently used second-order designs are the 3k factorial, central composite, and the Box–Behnken designs (Khuri and Mukhopadhyay, 2010). The experiment is designed to estimate interaction and even quadratic effects of the variables on the response. Contour plots are the best methods to evaluate such complex relationship. The reaction parameter with the greatest effect is kept on the y-axis, the second is placed on the x-axis, and the one with the least effect is held constant. The contour plots help in the optimization of reactions by identifying parameter combinations that will produce a desired response. RSM designs are commonly used in Industrial, Biological and Clinical Science, Social Science, Food Science, and Physical and

Engineering Sciences for designing, formulating, developing, and optimizing new products and processes. RSM has been extensively used in optimized production of SL where several variables need to be considered. Some of these are enzymatic *trans*esterification of fractionated rice bran oil with conjugated linoleic acid (Alim *et al*., 2008), production of lipase-catalyzed solid fat from mustard oil and palm stearin with linoleic acid (Alim *et al*., 2009), production of specific structured lipids by enzymatic interesterification (Xu *et al*., 1998), enzymatically catalyzed synthesis of low-calorie structured lipid in a solvent-free system (Han *et al*., 2011), enzymatic production of human milk fat analogs (Rodriguez and Akoh, 2009; Teichert and Akoh, 2011; Nagachinta and Akoh, 2012), enzymatic production of *trans*-free margarine fat (Lumor *et al*., 2007, Pande and Akoh, 2012) and enzymatic synthesis of cocoa butter analog from camel hump fat in supercritical carbon dioxide (Shekarchizadeh *et al*., 2009).

CHARACTERIZATION OF SL

Total and positional fatty acids

The composition and positional distribution of FA are important determinants of the chemical and physical properties of the SL. The type of FA and its position on the glycerol backbone greatly influences the physical, functional, and nutritional behavior of dietary fats in food products. The SFAs are better hydrolyzed and absorbed when present at *sn*-1, 3 positions while UFAs are better absorbed at *sn*-2 position (Gebauer *et al*., 2005). FA profile is determined by gas chromatography (GC) after converting the fatty acids to their methyl esters (FAME) by either acid or base catalyzed *trans*esterification. FAMEs are more volatile than FFA and therefore suitable for GC analysis. An internal standard (commonly C15:0 or C17:0) is used for quantification purpose and identification

is done by comparing retention time of individual fatty acids to those of an external standard. The internal standard chosen should behave similarly to the analytes of interest, but should not be present naturally in the sample. Positional analysis is carried out by pancreatic lipase or by Grignard degradation method. During pancreatic lipase reactions the FA at *sn*-1, 3 positions are cleaved and the *sn*-2 MAG is separated by TLC and then analyzed on GC after conversion to methyl ester. Fatty acid composition at *sn* -1,3 position are calculated using the following equation:

$$sn\text{-}1,3\ (\%) = [3 \times \text{total}\ (\%) - sn\text{-}2\ (\%)]/2$$

For Grignard reagent or Grignard degradation method, pancreatic hydrolysis is first performed. This is followed by reacting the phospholipid derivatives (phosphatidylcholine, PC) of 1,2-DAG and 2,3-DAG with phospholipase A2. As the *sn*-2 FA is already known, chemical analysis of 2,3-diacyl-PC phospholipase A2 hydrolysis product gives the FA at the *sn*-3 position. Similarly, chemical analysis of 1,2-DAG hydrolyzed product of phospholipase A2 gives the FA at sn-1 position.

Triacylglycerol (TAG) molecular species

TAG molecules making up adipose tissue of animals, largely have a SFA at the *sn*-1 position and an UFA at the *sn*-2 position whereas in most vegetables oils, either C18:1 or C18:2 are exclusively at the *sn*-2 position in TAG species. The stereospecificity and chain lengths of FAs, at the *sn*-1, *sn*-2 and *sn*-3 positions in TAG species, determine the metabolic fate of dietary fat during digestion and absorption. TAG profile of the SL greatly influences the physical properties of the SL such as solid fat content and crystal polymorphism. Fats containing highly diverse TAG profiles or greater FA chain-length diversity tend to crystallize in the β′ form, the desirable polymorph in margarines,

whereas oils with little TAG diversity crystallize in the β form (Ribeiro *et al*., 2009). In addition, some specific TAGs only crystallize in either the β or β' form. TAGs such as 1,3-dipalmitoyl-2-stearoyl glycerol (PSP) and 1,3-dipalmitoyl-2-elaidoyl glycerol (PEP), 1-palmitoyl-2,3-distearoyl glycerol (PSS) only crystallize in the β' form, whereas, 1,2,3-tripalmitoyl glycerol (PPP), and 1,2,3-tristearoyl glycerol (SSS) only crystallize in the β form. Reverse phase high performance liquid chromatography (RP-HPLC) equipped with evaporative light scattering detector (ELSD) is commonly used to determine the TAG molecular species. HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components (or analytes) are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture is resolved into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. In RP-HPLC, the stationary bed is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the analyte is, the longer it will be retained. ELSD involves nebulization of the column effluent to an aerosol, followed by solvent vaporization resulting in small solute droplets, which are then detected by light scattering detector. The TAG peaks are identified by comparison of retention times with those of the standards and also by equivalent carbon number (ECN). ECN is defined as CN – 2n, where CN is the number of carbons in the TAG (excluding the three in the glycerol backbone) and n is the number of double bonds.

Solid fat content (SFC)

SFC is a parameter that expresses the solid /liquid ratio of a fat at different temperatures (Marangoni and Rousseau, 1995) and is commonly measured by nuclear magnetic spectrometry (NMR). The basic principle of NMR is that spins of the nuclei of hydrogen atom line up in a magnetic field. These can be excited by short electromagnetic pulse and then relaxes back to their equilibrium. The relaxation rate depends on the mobility of the molecules to which the nuclear spin belongs and differs from solid to liquid phase (Ribeiro *et al*., 2009). The SFC of margarines and shortenings is responsible for their appearance, ease in filling, spreadibility, stability, and sensory properties. SFC measured at 5 °C determines the product's spreadibility at refrigeration temperature; at room temperature (25 °C) the SFC is related to the phase stability of the product; and SFC measured at 35 °C represents the texture and mouthfeel of the product at body temperature (Ribeiro *et al*., 2009). It has been reported that for a good spreadable margarine its SFC should not be greater than 32% at 10 °C (Lida and Ali, 2008). To prevent any waxy sensation in the mouth, the margarine should have SFC below 3.5% at 33.3 °C (Karabulut *et al* 2004). The temperature at which SFC is 4% is defined as the melting point of the sample (Karabulut *et al*., 2004).

Polymorphism

FA residues and their esters can occur in differentiated crystalline forms in a periodical three-dimensional pattern having the same composition but different structures. This tendency is called polymorphism. Polymorphism in fats can be studied by X-ray diffractometer (XRD). The different polymorphic states of a particular substance often demonstrate quite different physical properties (such as melting behavior and hardness),

but on melting yield identical liquids. Three specific types of crystal polymorphs exists in fats, namely: α, β', β . The α form is metastable with hexagonal chain packing. The β' form shows intermediary stability and orthorhombic perpendicular packing, while the β form shows the greatest stability and triclinic parallel packing. The melting temperature is directly proportional to their stability (Ribeiro *et al*., 2009). The basic principle of XRD consists of excitation of an anti-cathode for emission of X-rays that are diffracted at specific angles by the crystalline structure. In fats, diffractions at high angles relate to the short spacings of the sub-cells. Short spacings can be defined as the distances between the parallel acyl groups on the TAG and refer to the transversal packing of the triacylglycerol chains. The α form shows a single peak at 4.15A, β' form shows two main peaks at 3.8 and 4.2 A, and β form shows a high intensity peak at 4.6 A accompanied by other peaks of lower intensity (Ribeiro *et al*., 2009). The β' polymorph is the desired form in products such as margarines and shortenings. β' polymorph imparts smooth texture, improved spreadibility, and better mouthfeel to the product. The more diverse the structure of TAG and higher the melting point of the fat, the lower the tendency to form β polymorph (Ribeiro *et al*., 2009). The factors that influence the stability of a β' polymorph are FA chain length and diversity, TAG carbon number and diversity, TAG structure, ratio of liquid oil present in a fat system, and temperature fluctuation during processing and storage (deMan & deMan, 2001). Weiss (1983) classified a number of fats according to their crystallizing nature. Soybean, safflower, sunflower, sesame, peanut, corn, cocoa butter, palm kernel, coconut, and olive oils are oils crystallizing in β form. Cottonseed oil, palm oil, marine oils, and rapeseed oils are β' forming oils.

Thermal behavior

The melting point is usually defined as the temperature at which a material changes from the solid to the liquid state. However, fats and oils do not show a clearly defined melting point like pure substances, instead they have a melting range. They consist of complex mixtures of TAG that undergo gradual melting according to the individual melting points until they become completely liquid. During heating, fat exhibits multiple melting phases, and each recrystallization step represents the transition of a less stable polymorphic form to a more stable one. The peak transition temperature may be an indicator of the polymorphic form of a crystal, since the most stable crystalline form has a higher melting point. Differential Scanning Calorimetry (DSC) is the most common technique for studying the thermal behavior of fats and oils. DSC measures the difference in energy (heat flow) required to maintain sample and reference at the same temperature as a function of temperature or time. The thermal phenomena suffered by the sample are represented in the form of deviations of the baseline in the exo- or endothermic direction. The parameters that can be calculated from DSC thermogram are peak crystallization and melting temperatures, onset crystallization and melting temperatures, final crystallization and melting temperatures, and the peak area corresponding to the enthalpies of crystallization and melting (Ribeiro *et al.*, 2009). The melting properties of a fat or oil can be influenced by the FA chain length (increase in chain-length corresponds to an increase in melting point), degree of unsaturation (increase in unsaturation results in a decrease in melting point), presence of TFA (TFA have higher melting point than their corresponding cis form), and polymorphism (α – lowest melting point, β´ – intermediate melting point, and β – highest melting point) (Strayer *et al*., 2006).

Oxidative stability

Oxidative stability is an important characteristic of the product which determines its shelf life and flavor. Oxidative stability is measured by several methods such as peroxide value (PV), *p*-anisidne value (*p*AV), Rancimat, and oil stability index (OSI). During OSI measurement, a stream of air is passed through the oil or fat sample contained in a sealed and heated reaction vessel. Oxidation of the oil or fat molecules in the sample results in the formation of volatile oxidation products. These are transported in the stream of air to a second vessel containing distilled water whose conductivity is continuously recorded. The organic acids can be detected by the increase in conductivity. The time required for the sample to reach its induction period endpoint is termed the Oil Stability Index (OSI). The oxidative stability of fats and oils depends on the FA profile and amount of indigenous antioxidant present. SFA are less prone to oxidative deterioration compared to UFA. Tocopherols and tocotrienols are collectively known as Vitamin E. Their structure consists of a polar chromanol head and a hydrophobic prenyl tail which results in amphipathic nature of these compounds (Munne-Bosch and Alegre, 2002). The only difference between tocopherols and tocotrienols is the degree of saturation of the hydrophobic tail. Tocopherols have a 4', 8' 12- trimethyltridecylphytol chain whereas tocotrienols have double bonds at 3', 7' and 11' positions. of the chain (Munne-Bosch and Alegre, 2002). Vegetable oils, especially the seed oils, are rich sources of tocopherols. Additionally, some minor compounds such as phytosterols and phospholipids may also contribute to the oxidative stability of oils.

Microstructure

The main structural component of plastic fats is the crystalline network which can be studied by polarized light microscopy (PLM). The final texture, appearance, and functionality of the product are dependent on the crystal morphology and structure. Attributes such as spreadibility and the melting sensation depend on the mechanical strength of the crystalline network (Ribeiro *et al*., 2009). PLM can distinguish between the solid and the liquid phase based on how they refract the light. The liquid phase is isotropic in nature and due to its unique refractive index, presents the same optical properties in all directions. Thus, it appears as dark phase under PLM. On the other hand, the solid phase is anisotropic with defined molecular orientation and varying optical properties. Solid phase appears slightly brilliant under PLM. PLM also allows observing the dynamic alterations occurring during nucleation and cell growth (Ribeiro *et al*., 2009).

Texture

Texture is a sensory attribute related to consumers' preference and liking for a product. The texture of margarine depends on the fatty acid composition, crystal morphology and network, and the processing and storage conditions (Bourne, 1978). Cone penetrometer and texture analyzer are usually used for textural properties such as hardness, adhesiveness, and cohesiveness. The texture profile analysis (TPA) procedure (Bourne, 1978) is commonly used to evaluate the textural properties (hardness, adhesiveness, and cohesiveness) of margarine. A double compression test is performed using a texture analyzer. A 45º conical probe attached to a compression load cell is used to penetrate the samples at a given speed, and withdrawn at the same speed. The maximum force (N)

during the first compression is reported as hardness and the negative force area in N·s (A2) for the first compression is reported as adhesiveness. The ratio of the positive force area during the second compression (A3) to that of the first compression (A1) is indicative of cohesiveness. Hardness and spreadability are inversely related. The higher the hardness value of a margarine, the more difficult it is to spread. Cohesiveness relates to how crumbly, brittle, or crunchy a food is, while adhesiveness relates to how sticky, tacky, or gooey the food is.

Rheology

Rheology is the study of deformation and flow of matter under the influence of applied stress and strain (Borwankar, 1992). Margarine is a viscoelastic product and stress viscometry, creep, and dynamic analyses are used for evaluation of its rheological properties. In stress viscometry, stress is applied stepwise to margarine and viscosity is calculated. Samples showing high viscosities at low stresses would be more difficult to spread. In creep analysis, a constant low stress is applied to samples and deformation per unit time measured as an indicator of emulsion stability. The lower the degree of deformation, the less likely the margarine will show syneresis. In dynamic analysis, margarine samples are subjected to a sinusoidally varying stress and the strain output and phase difference between the input and output signals are measured. Storage modulus (G′) is used as a measure of solid or elastic behavior while loss modulus (G″) is used as a measure of viscous or fluid behavior. The frequency (Hz) at which G' and G" crossover is a measure of spreadibility. The higher the crossover frequency, the more spreadable the margarine is (Bohlin application note).

Sensory evaluation

Sensory evaluation is the connecting link between product attributes and consumers acceptance. The types of sensory evaluation are acceptance test, preference test, difference test, and descriptive analysis. Preference and difference tests are used to compare different products whereas descriptive analysis is used to evaluate specific attributes of a product. The three types of test used in consumer acceptance testing are the paired preference, ranking and rating tests (hedonic scale). The difference test discriminates between closely related products. The difference test could take a number of forms. In the triangle test, subjects are asked to identify the odd sample out of three, two of which are identical or duplicates. The ranking test involves simple ranking of the different products from the greatest to the least. No ties are allowed. The result is expressed as mean rankings and significant differences between the products are determined using multiple comparison tests. For descriptive analysis a trained focus group of panelists are required to evaluate specific attributes of a product (Resurreccion, 1998).

Thus, the SL produced should have the desirable FA profile, melting and crystallization temperatures, SFC, β′ polymorph, and oxidative stability to be used for formulating margarine with acceptable texture and sensory attributes.

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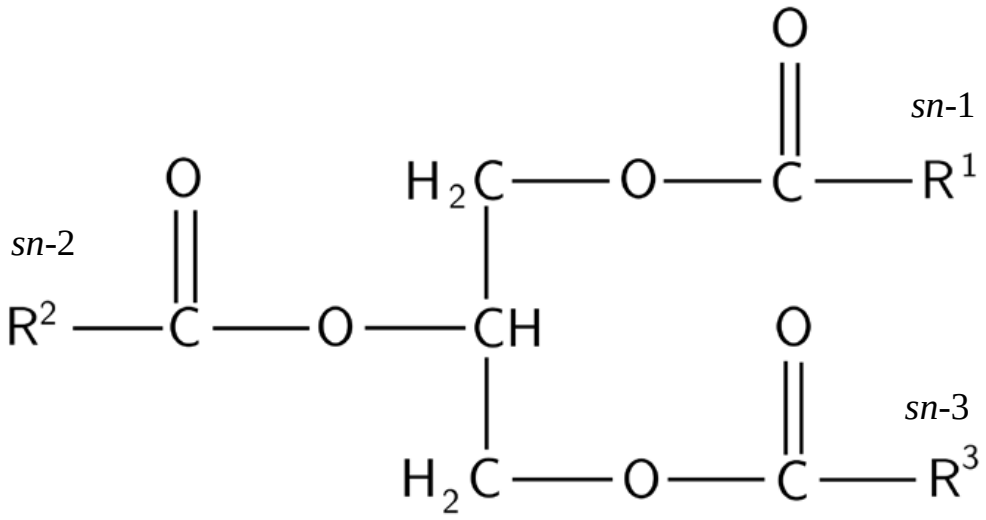

Fig. 2.1a Triacylglycerol structure
R^1, R^2, R^3, fatty acids (maybe same or different)

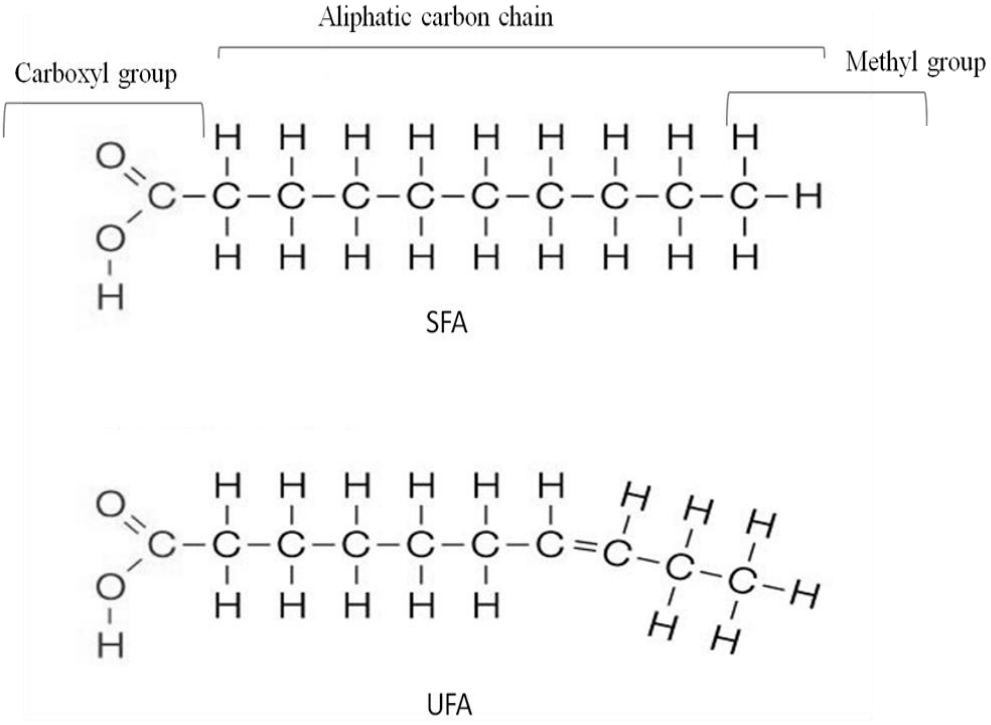

Fig. 2.1b Fatty acid structures
SFA, saturated fatty acid; UFA, unsaturated fatty acid

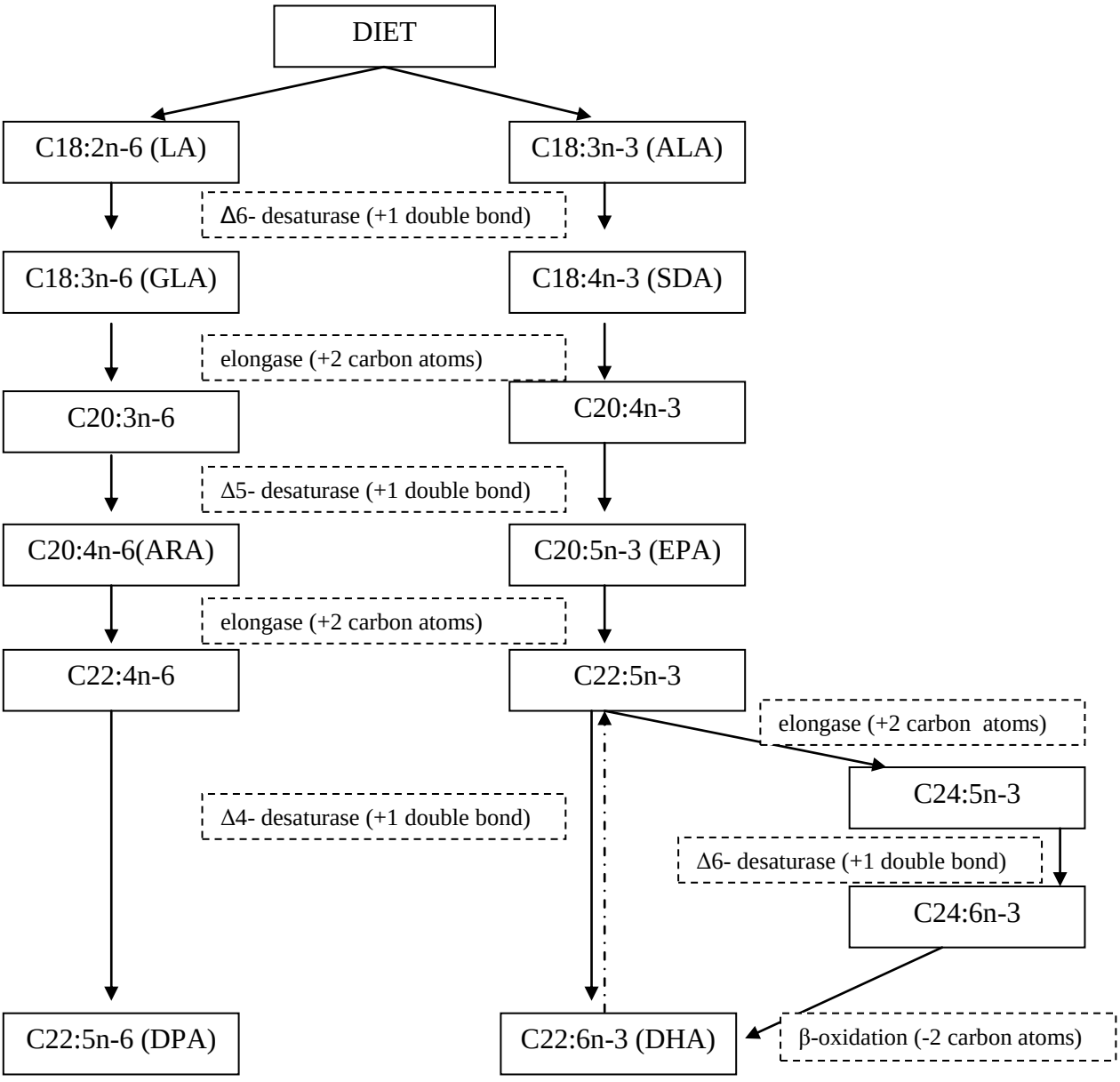

Fig. 2.2 Metabolic pathway of n-6 and n-3 fatty acids

LA, linoleic acid; ALA, α linolenic acid; GLA, γ-linolenic acid; SDA, stearidonic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid

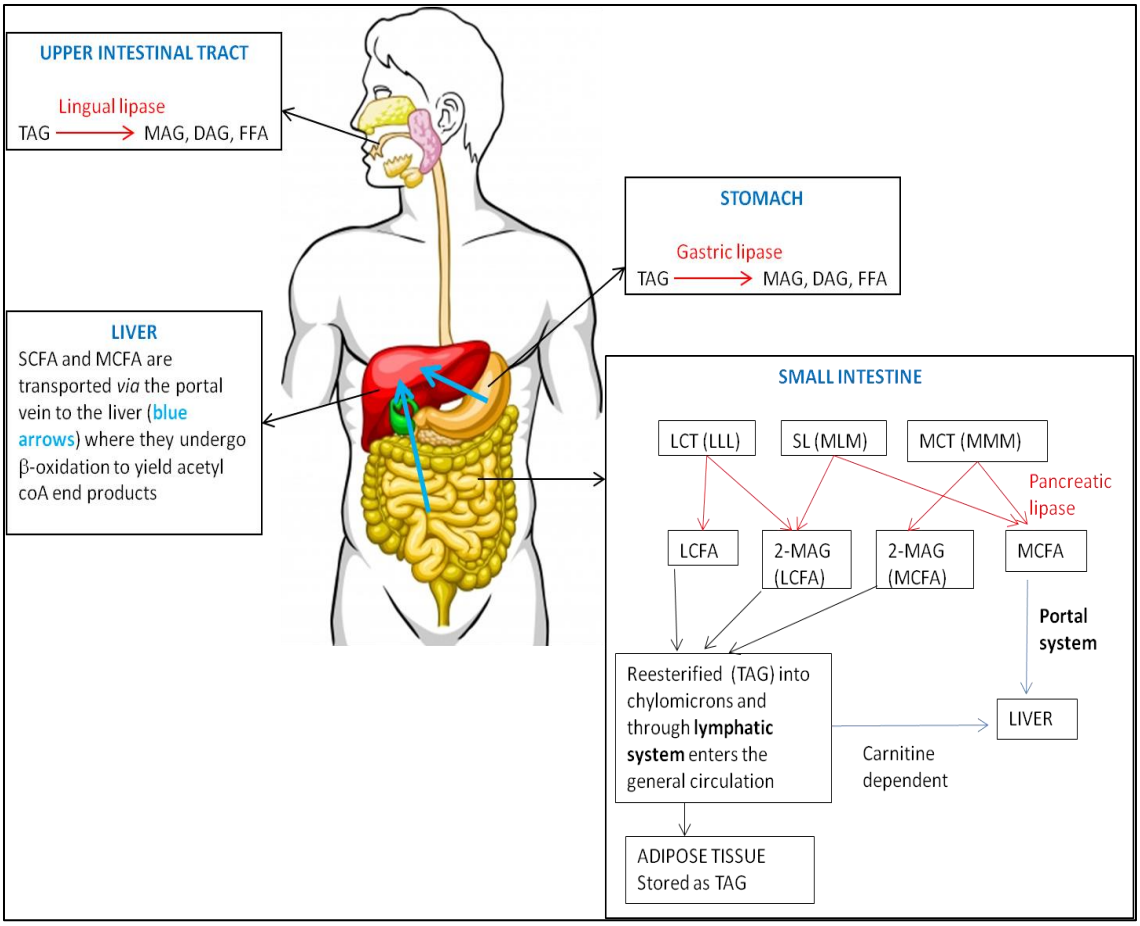

Fig. 2.3 Metabolism of SL

TAG, triacylglycerol; DAG, diacylglycerol; FFA, free fatty acid; SCFA, short-chain fatty acid; MCFA, medium-chain fatty acid; LCFA, long-chain fatty acid; 2-MAG, 2-monoacylglycerol; LCT, long-chain triacylglycerol containing LCFA at sn-1,2,3 positions or LLL; SL, structured lipid containing LCFA at the sn-2 position and MCFA at the sn-1,3 positions or MLM; MCT, medium-chain triacylglycerol containing MCFA at sn-1,2,3 positions or MMM

(Pande and Akoh, 2012)

CHAPTER 3

ENZYMATIC SYNTHESIS OF *TRANS*-FREE STRUCTURED MARGARINE FAT ANALOGUES USING STEARIDONIC ACID SOYBEAN AND HIGH STEARATE SOYBEAN OILS[1]

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ABSTRACT

Enzymatic synthesis of *trans*-free structured margarine fat analogues from stearidonic acid (SDA) soybean oil and high stearate soybean oil was optimized using response surface methodology (RSM). The independent variables considered were substrate molar ratio (HSSO:SDASO, 2-5), temperature (50-65 °C), time (6-22 h), and enzymes (Lipozyme® TLIM and Novozym® 435). The dependent variables were mol% stearic acid incorporation and mol% SDA content. A good-fit model was constructed using regression analysis with backward elimination and verified by a chi-square test. Desirable and optimal products composition were achieved at 50 °C, 18 h, 2:1, using Lipozyme TLIM, with 15.6 mol% stearic acid and 9.2 mol% SDA in the product and at 58 °C, 14 h, 2:1, using Novozym 435, with 14.8 mol% stearic acid and 6.4 mol% SDA. Using optimal conditions, structured lipids (SLs) were synthesized in a 1 L stir-batch reactor and free fatty acids removed by short-path distillation. SLs were characterized for fatty acid profile, *sn*-2 positional fatty acids, triacylglycerol profile, polymorphism, thermal behavior, and solid fat content. The SLs had desirable fatty acid profile, physical properties, and suitable β' polymorph. These SLs could be used as margarine fat analogues and an alternative to partially hydrogenated fat.

KEYWORDS

Enzymatic synthesis; high stearate soybean oil; response surface methodology; stearidonic acid soybean oil; structured lipid

INTRODUCTION

Structured lipids (SLs) are defined as triacylglycerols (TAGs) that have been structurally modified either by incorporation of new fatty acids or changing the position of existing fatty acids to yield novel TAGs of desired physical, chemical, and nutritional properties [1]. Various methods such as hydrogenation, fractionation, blending, interesterification, esterification, and even bioengineering have been used for the modification of natural TAGs. However, most SLs are produced by either chemical or enzymatic interesterification [2]. Although chemical interesterification involves lower cost and easy scale-up process, it lacks specificity. Lipases are used for enzymatic interesterification and are preferred over chemical interesterification because they are regio-and stereo-specific and offer a better control over the final product [3]. Enzyme-catalyzed reactions may help save energy and minimize thermal degradation [4]. Enzymatically produced SL with desired nutritional quality and functionality, sometimes referred to as "nutraceutical,' has been used in various medical and food applications [5].

trans Fatty acids (TFA) are unsaturated fatty acid with carbon-carbon double bonds in *trans* position. Partial hydrogenation, a common industrial process for solidifying oils, is the main contributor of TFA (80%) in the US diet [6]. Intake of high amounts of TFA has been positively correlated with increased risk of coronary heart disease [7], inflammation [8], and cancer [9]. The mean daily intake of TFA per person in the United States is 2 - 4% total food energy [10]. Dietary Guidelines for Americans 2010 recommends dietary TFA intake should be as low as possible [11]. Furthermore, the United States Food and Drug Administration (FDA) issued a ruling requiring all foods containing TFA to be labeled accordingly, effective from January 2006 [12]. This has led

the food industry to seek ways to produce cost effective zero or reduced *trans* fat foods with acceptable functional properties.

Dietary essential polyunsaturated fatty acids are fatty acids that cannot be synthesized in humans and need to be obtained from diet for normal growth and development. Omega-3 fatty acids (n-3 FAs) have been positively associated with prevention and treatment of chronic diseases like cardiovascular, inflammatory, autoimmune diseases, and cancer [13]. Therefore, American Heart Association (AHA) Dietary Guidelines suggest that Americans consume at least two servings of fish per week and include n-3 FA, α-linolenic acid (ALA) in their diets [14]. But still the American diet contains very low levels of n-3 FAs generally found in fish oil. SDA (18:4 n-3) is a metabolic intermediate between ALA and eicosapentaenoic acid (EPA) that converts more efficiently to EPA than ALA [15]. SDA soybean oil (SDASO) is a sustainable plant source of n-3 FAs. High stearate soybean oil (HSSO) was developed to contain elevated levels of stearic acid for increased stability when used in many types of foods that require solid fat functionality. This study is innovative as no previous studies have been reported on production of *trans*-free margarine fat analogues containing stearidonic acid. The first objective of this research was to optimize the reaction conditions of SDASO and HSSO for *trans*-free SLs production suitable for margarine formulation. The second objective was to scale-up the SLs synthesis and characterize them, thereby increasing the possible food applications of SDASO as a plant source of n-3 FAs.

MATERIALS AND METHODS

Materials

HSSO and SDASO were kindly provided by Monsanto Company (St. Louis, MO). The immobilized enzymes, Lipozyme® TLIM (*Thermomyces lanuginosus* lipase, *sn*-1,3 specific, specific activity 250 IUN/g: IUN=Interesterification Unit, carrier granulated silica) and Novozym® 435 (*Candida antarctica* lipase, non-specific, specific activity 10,000 PLU/g: PLU=Propyl Laurate Unit, carrier macroporous acrylic resin) were purchased from Novozymes North America Inc. (Franklinton, NC). Supelco 37 FAME mix and 14% boron trifluoride in methanol were purchased from Sigma Chemical Co. (St. Louis, MO). Nonadecanoic acid and its methyl ester were purchased from TCI America (Portland, OR). Stearidonic acid and its methyl ester were purchased from Cayman Chemical Company (Ann Arbor, MI). Organic solvents and chemicals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA).

Experimental Design for Response Surface Methodology (RSM)

RSM is a collection of statistical and mathematical techniques useful for the development, improvement and optimization of processes. RSM enables one to obtain sufficient information for statistically acceptable results using a reduced number of experiments. It is an efficient method to evaluate the effects of multiple variables and their interaction effects on the response [16]. Four independent variables were studied namely: time (6-22 h), temperature (50-65 °C), substrate molar ratio (HSSO:SDASO, 2-5), and two enzymes (Lipozyme TLIM and Novozym 435), for stearic acid incorporation and SDA content of the products as responses. RSM was done using Modde 9.0 software

(Umetrics, Umeå, Sweden) to obtain the relationship between the response and the independent variables. Twenty six runs were generated and experiments at each design point were randomly performed in triplicate. The data obtained from the design in Table 3.1 were used to fit a second-order polynomial function as follows:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i \; + \; \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i \; X_j \; + \; \varepsilon_{ij} \qquad (1)$$

where Y = the response; β_0 = constant; β_i = linear (first-order model); β_{ii} = quadratic (second-order model); β_{ij} = interaction term coefficients; X_i and X_j = independent variables; and ε_{ij} = error term.

Small-scale SL Synthesis

SL synthesis was performed in screw-cap test tubes as per the conditions generated by RSM. Briefly, 100 mg of the SDASO was weighed into the test tube and HSSO was added according to the determined respective molar ratios. 2 mL hexane was added and the substrates were thoroughly mixed while gently warming the test tubes. Specific enzymes were added at 10% total substrate weight. Test tubes were then incubated in water bath shaker at 200 rpm at time-temperature combinations generated by RSM. Reaction was stopped and filtered through anhydrous sodium sulfate column to remove the enzymes. Pure TAG bands were collected after silica gel TLC using petroleum ether:ethyl ether:acetic acid (80:20:0.5, v/v/v) as developing solvent system. TAG bands were analyzed for FA profile after conversion to methyl esters. All reactions were performed and analyzed in triplicates and average values reported.

Fatty Acid Profile

Lipid samples were converted to fatty acid methyl esters following the AOAC Official Method 996.01 [17] and analyzed with Hewlett-Packard 6890 series II gas

chromatograph (Agilent Technologies Inc., Palo Alto, CA) using Supelco SP-2560, 100 m x 25 mm x 0.2 μm column. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. Injection volume was 1 μL and a spilt ratio of 20:1 was used. Detection was with flame ionization detector at 300 ºC. The column was initially held at 140 ºC for 5 min and then increased to 240 ºC at 4 ºC/min and held at 240 ºC for 25 min. All samples were analyzed in triplicates and average values reported.

Large-scale SL Synthesis

The scaled-up reactions using the optimal conditions achieved from the small-scale reactions were performed in a stir-batch reactor. Two SL were prepared: SL1 at 50 °C, 18 h, 2:1, using Lipozyme TLIM and SL2 at 58 °C, 14 h, 2:1, using Novozym 435. Corresponding physical blends (PB1 and PB2) were also prepared at the same reaction conditions without the respective enzymes. Free fatty acids (FFAS) were removed by short-path distillation KDL-4 unit (UIC, Joliet, IL) at <0.2mmHg and flow rate 10 mL/min. FFAs were determined by AOCS Official Method Ac 5-41[18].

Fatty Acid and Positional Analysis

Fatty acids were determined as described earlier. *sn*-2 Positional fatty acid composition was determined following the AOCS Official Method Ch 3-9 [19]. Fatty acid composition at *sn* -1,3 position was calculated using the following equation

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

All samples were analyzed in triplicates and average values reported.

Triacylglycerol Molecular Species

The TAG composition was determined with a nonaqueous reverse phase HPLC (Agilent Technologies 1260 Infinity, Santa Clara CA) equipped with a Sedex 85 ELSD (Richard

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

Differential Scanning Calorimetry (DSC)

Melting and crystallization profiles of lipid samples were determined with Perkin-Elmer model DSC7 (Norwalk, CT) following AOCS Official Method Cj 1-94 [20]. 8-12 mg samples were weighed into aluminum pans and sealed. Normal standardization was performed with n-decane and indium. Samples were rapidly heated to 80˚C at 50˚C /min, and held for 10 min. The samples were then cooled to -55˚C at 10˚C/min, and held for 30 min and finally heated to 80˚C at 5˚C/min. All samples were analyzed in triplicates and average values reported.

Polymorphism

The polymorphic forms of the samples were determined with Bruker D8 Advance diffractometer (Bruker AXS, Inc., Madison, WI). The diffractometer had a 2θ configuration, a solid state detector, and a cobalt tube as the X-ray source. Corundum was

used as standard. The 2θ range was from 20 to 32°. The scan rate was 4.2°/min with a step increment of 0.01°. The samples were rotated at 15 rev/min. Samples were melted and poured into rectangular aluminum molds. They were held at room temperature for 2-3 h, at 4 °C for 4-5 h, and then stored at -20 °C overnight. The sample molds were placed over custom-designed aluminum holders containing dry ice to prevent melting of the sample during analysis. Short spacings of the major polymorphs were identified as follows: α, a single spacing at 4.15 Å; β', two strong spacings at 3.8 and 4.2 Å; and β, a very strong spacing at 4.6 and another one usually at 3.85 Å [21]. All samples were analyzed in triplicates and average values reported.

Solid Fat Content

Solid fat content (SFC) was determined according to the AOCS Official Method Cd 16-81 [22] on a BrukerPC/20 Series NMR analyzer, Minispec (Bruker Optics, Milton, On, Canada). Samples were tempered at 100˚C for 15 min and then kept at 60˚C for 10 min, followed by 0˚C for 60 min and finally for 30 min at each temperature of measurement. SFC was measured at intervals of 5˚C from 5 up to 60 ˚C.

Statistical Analysis

Regression analysis, response surfaces, and statistical significance were performed using Modde 9.0 software (Umetrics, Umea, Sweden) and backward elimination by Statistical Analysis System software (SAS 9.2, Cary, NC). SAS was also used to determine significant difference ($P \leq 0.05$.) between SLs and PBs. All samples were analyzed in triplicates and average values reported.

RESULTS AND DISCUSSION

Model Fitting

SLs enriched with stearic acid were produced by enzymatic interesterification of SDASO and HSSO in TAG forms. The incorporation level of stearic acid in the *trans*-free margarine fat analogs (SLs) ranged from 13.5-18.1% with SDA content ranging from 1.9-10.0%. Separate quadratic models were obtained for the incorporation of stearic acid and SDA content of SLs by multiple linear regression and backward elimination. The regression coefficients (β) and significance (P) values were calculated based on the results in Table 3.1. The respective ANOVA tables are shown in Tables 3.2.1 and 3.2.2. The R^2 value, the fraction of the variation of the response explained by the model, was 0.955 and 0.932 for stearic acid incorporation and SDA content, respectively. Q^2, the fraction of the variation of the response that can be predicted by the model, was 0.754 and 0.558 for stearic acid incorporation and SDA content, respectively. The observed vs. prediction plots had linear distributions for both responses (data not shown). The model for stearic acid incorporation also showed no lack of fit ($P > 0.05$), and the *P*-value for the multiple regression was <0.001 (Table 3.2.1). The model equation after backward elimination at α=0.05 can therefore be written as:

Stearic acid incorporation = 14.92 + 0.44Time + 0.51SR + 0.67(SR*SR) + 0.44(Temp*Time) + 0.43(Temp*SR) + 0.44(Time*SR) ± 0.32(SR*Enz)

The model for SDA content showed a lack of fit ($P < 0.05$) but the *P*-value for multiple regression was <0.001 (Table 3.2.2). The final model equation after backward elimination at α=0.05 can thus be written as:

SDA content = 5.90 - 1.75SR ± 1.11Enz - 1.56(Time*Time) + 0.59(Temp*Time) - 0.68(Temp*SR) ± 0.87(Temp*Enz)

where, Temp is temperature in °C, Time in hours, SR is substrate molar ratio, Enz is enzyme.

Effect of Parameters

Time and substrate molar ratio had a positive effect on the incorporation of stearic acid. Temperature had a negative effect but it was not significant. Among the linear parameters, only substrate molar ratio and enzyme were significant for SDA content. But the squared term of time (Time*Time) and the interaction term of temperature with substrate molar ratio (Temp*SR), time (Temp*Time), and enzyme (Temp*Enz) were all significant. The squared term of time (Time*Time) was not significant for stearic acid incorporation but had a negative effect on SDA content. Enzyme was not a significant factor for stearic acid incorporation but in its interaction term with substrate molar ratio, Lipozyme TLIM showed a negative effect and Novozym 435 showed a positive effect on the response. In the case of SDA content, Lipozyme TLIM had a positive effect whereas Novozym 435 had a negative effect but in its interaction term with temperature, Lipozyme TLIM showed a negative effect and Novozym 435 showed a positive effect on the response. Temperature can have a dual effect on the rate of reaction. As temperature is increased the rate of collisions between reactants and the enzyme also accelerates, thus resulting in increased acyl exchange. This may account for the increased incorporation as temperature increased at higher substrate molar ratios [23]. Enzymes are proteins and therefore can be inactivated by high temperatures thereby producing less incorporation. Thermostability of the enzyme is a crucial factor in determining its activity. The overall

effect of temperature is also affected by other factors such as the amount and type of substrate used, and presence of water in the reaction. Similar observations for the effect of temperature on stearic acid incorporation have been reported by Lumor *et al*. [24].

Optimization of the Reaction

The quadratic models obtained show that first order, second order variables, and interactions among them affect the response and may have more than one solution [25]. Contour plots are the best methods to evaluate such complex relationship. Contour plots were generated by Modde 9 software. The reaction parameter with the greatest effect was kept on the y-axis, the second was placed on the x-axis, and the one with the least effect was held constant. The contour plots help in the optimization of reactions by identifying parameter combinations that will produce a desired response. The contour plots are shown in Figure 3.1. For both stearic acid incorporation and SDA content, substrate molar ratio had the highest effect followed by temperature and time. Therefore, substrate molar ratio and temperature were varied while keeping the time constant at 18 h for Lipozyme TLIM (Figures 3.1a and 3.1b) and at 14 h for Novozym 435 (Figures 3.1c and 3.1d). As the response is a factor of a complex relationship of linear and quadratic variables, several combinations of the factors are possible to obtain desired results. Economic consideration is an important factor in optimization of the reaction parameters. In this study, higher stearic acid incorporation was obtained at the expense of SDA content. Therefore, it is important to optimize while keeping in mind both the stearic acid as well as SDA content of the products. Furthermore, enzymes are expensive and therefore should be taken into consideration. Optimal and desirable products composition were achieved at 50 °C, 18 h, 2:1, using Lipozyme TLIM, with 15.6 mol% stearic acid

and 9.2 mol% SDA and at 58 °C, 14 h, 2:1, using Novozym 435, with 14.8 mol% stearic acid and 6.4 mol% SDA. Using contour plots, these two sets of SLs were selected for scale-up process. Although fixed bed reactors are the preferred reactors, but due to the nature of the substrates (HSSO solidified easily), a stir-batch reactor was chosen for large-scale SL production.

Verification of the Model

To verify the model, five regions from the contour plot were randomly chosen to perform experiments using the conditions specified for these regions. A chi-squared test showed that there was no significant difference between the observed and predicted values (Table 3.3) since the chi-squared value for both stearic acid (0.410) and SDA (1.806) were much smaller than the cutoff point (9.488) at α=0.05 and DF 4.

Fatty Acid Profile

After purification by short-path distillation, the SLs contained 0.1% FFA which is an acceptable level to be used as ingredients for food products. Yield % was calculated as:

Yield % = (Final product weight)*100/(Total substrate weight) (3)

The yield % for SL1 and SL2 were 90.8 and 84.2%, respectively. The difference in the yield can be due to the different enzymes used. It was observed that product recovery, when using Novozym 435, was lower than Lipozyme TLIM because Novozym 435 absorbed more product than Lipozyme TLIM. This difference in the absorption capacity of the enzymes may be attributed to their carrier systems which were granulated silica for Lipozyme TLIM and macroporous acrylic resin for Novozym 435. Fatty acid compositions of SDASO, HSSO, SLs, and PBs are given in Table 3.4. The major FAs in SDASO were linoleic (24.6 mol%), stearidonic (23.5 mol%), and oleic (15.6 mol%)

acids. In case of HSSO, linoleic (50.2 mol%), oleic (16.0 mol%), and stearic (15.8 mol%) acids were the dominant FAs. Lipozyme TLIM catalyzed-SL (SL1) had 14.9 mol% stearic acid and 10.2 mol% SDA whereas the physical blend (PB1) had 14.0 mol% stearic acid and 7.1 mol% SDA. For Novozym 435, the SL (SL2) had 15.9 mol% stearic acid and 8.9 mol% SDA while the physical blend (PB2) produced under similar conditions had 15.4 mol% stearic acid and 6.8 mol% SDA. In comparison with SDASO, 275.2 and 285.0% increase in stearic acid was found in SL1 and SL2, respectively. On the other hand, SDA decreased 56.6 and 62.1% in SL1 and SL2, respectively compared to SDASO. The SLs had a wide range of FAs and no *trans* fat. The type of FA and its position on the glycerol backbone greatly influences the physical behavior of dietary fats in food products. It also determines the metabolic fate of dietary fat during digestion and absorption. Unsaturated FAs are better metabolized and utilized in our body when present at *sn*-2 position [26]. The positional distribution of FAs in the substrates and the products are shown in Table 3.5. No saturated FAs were present at *sn*-2 position of SDASO, HSSO and the PBs. However, 21.1 and 27.2% saturated fat were present at the *sn*-2 position of SL1 and SL2, respectively. 7.9 and 11.5 mol% stearic acid were found at the *sn*-2 position of SL1 and SL2, respectively. The presence of stearic acid at *sn*-2 position for *sn*-1,3 specific Lipozyme TLIM may be attributed to acyl migration. It is possible that the acyl migration occurred during interesterification reaction where specificity of Lipozyme TLIM was influenced by reaction conditions and substrates. Acyl migration, to a certain extent, can also occur during short-path distillation and/or during pancreatic lipase reaction [27]. SL1 had the highest SDA content with 9.2 mol% present at *sn*-2 position.

TAG Molecular Species

The TAG molecular species are shown in Figure 3.2. The major TAG species in SDASO were StOSt (17.8%) and StLnLn (12.6%) and in HSSO, LLL (20.3%) and SLL (16.6%). The major TAGs in SL1 were POL (14.4%), LLL (12.4%), and in PB1 were POL (19.2%), PLL (15.6%). The predominant TAG species in SL2 were LLL (15.9%) and POL (14.8%) and in PB2 were POL (23.2%) and LLL (22.6%). Only UUU (triunsaturated) (86.6%) and SUU (monosaturated-diunsaturated) (13.4%) TAGs were found in SDASO whereas in HSSO, SLs and PBs UUU, SUU, and SUS (disaturated-monounsaturated) type TAGs were present. The decrease in UUU type TAG from SDASO were 24.1, 55.5, 33.3, and 48.5% in SL1, PB1, SL2, and PB2, respectively. On the other hand, the increase in SUU type TAG from SDASO were 122.4, 276.1, 169.4, and 283.6% in SL1, PB1, SL2, and PB2, respectively. This change in TAG species affects the melting behavior of the products. The SLs had more diverse TAGs than the PBs. Fats containing highly diverse TAG profiles or greater FA chain-length diversity tend to crystallize in the β′ form, the desirable polymorph in margarines, whereas oils with little TAG diversity crystallize in the β form [21].

Thermal Behavior

Melting and crystallization profiles of the substrates and products are shown in Figure 3.3a and 3.3b, respectively. The melting completion temperature (T_{mc}) of SDASO was 1.8 °C due to its high content of UUU type TAGs. As UUU type TAGs decrease and SUU type TAGs increase in the product the T_{mc} also increase. The T_{mc} of SL1, SL2, PBs were 11.2, 12.6, 13.1 °C, respectively. Both SLs and PBs were completely melted at room temperature and therefore can be used in the production of soft spreadable

margarine. Both SLs showed broader peaks than SDASO indicating a better plastic range than SDASO which may be desirable for margarine. The crystallization onset temperature (T_{co}) for SL1, PB1, SL2, and PB2 were 32.5, 32.6, 28.6, and 26.5 °C, respectively and were completely crystallized at -10 °C , well above the freezer temperature (-20 °C). No significant differences ($P < 0.05$) in T_{mc} and T_{co} were found between SLs and PBs although significant difference ($P < 0.05$) was found between SDASO and the products.

Solid Fat Content

Solid fat content (SFC) is the measure of solid/liquid ratio of a fat at various temperatures. The SFC influences several physical characteristics of margarines. Three important SFC measurements are at refrigeration, room, and body temperatures which are related to the spreadibility, product stability and texture and mouthfeel, respectively, of margarines [21]. The SFC of SLs and PBs are shown in Figure 3.4. A desirable margarine is one that has < 32% SFC at 10˚C and <10% at room temperature [28]. Both the SLs and PBs met these requirements. In the PBs, SFC rapidly decreased between 5 and 15 °C to <1% SFC. The SFC of the SLs decreased rapidly between 5 and 10 °C and then gradually decreased to <1% SFC at 35 °C. Due to their low SFC, the SLs will be more suitable for liquid or soft margarines.

Polymorphism

Fatty acids residues and their esters can occur in differentiated crystalline forms in a periodical three -dimensional pattern having the same composition but different structure. This tendency is called polymorphism. In lipids there are three dominant crystal forms namely, α, β′, and β polymorphs [21]. Fats containing highly diverse TAG profiles or

greater FA chain-length diversity tend to crystallize in the β' form, whereas fats with little TAG diversity crystallize in the β form. Fats containing predominantly β' TAG crystals impart smooth texture or mouth-feel to margarine, whereas those with predominantly β TAG crystals impart grainy texture [21]. The polymorphic forms are shown in Table 3.6. Among all the samples, SL1 showed the most dominant β' crystal form.

CONCLUSION

High intake of TFA has been correlated with increased risk of several chronic diseases. n-3 FAs are positively associated with prevention and management of chronic diseases like cardiovascular and cancer. The second-order polynomial models developed in this study have strong predictability and reproducibility power. SDA has fewer double bonds than EPA and DHA which enhances its stability and shelf life [29]. SL1 contains 10.2% SDA so a margarine consisting of 80% SL1 will have 1.1 g SDA /serving (13 g). Relative to EPA, conversion efficiency of SDA to EPA is 17-30 % [15] therefore, 1.1 g SDA will result in 0.2-0.3 g EPA. In this study, we were able to produce SLs containing SDA and no TFA with desirable polymorphism, thermal properties, and SFC for formulation of soft margarine. The SLs from this study can be utilized for the production of *trans*-free margarine with added nutritional benefits.

ACKNOWLEDGMENT

We would like to thank Dr. Alejandro G. Marangoni for his assistance with the SFC analysis. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2009-65503-05734 from the USDA National Institute of Food and Agriculture.

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FIGURE CAPTIONS:

Figure 3.1a. Contour plots showing the effect of substrate molar ratio and temperature on incorporation of stearic acid while keeping the time constant at 18 h using Lipozyme TLIM lipase.

Figure 3.1b. Contour plots showing the effect of substrate molar ratio and temperature on SDA content while keeping the time constant at 18 h using Lipozyme TLIM lipase.

Figure 3.1c. Contour plots showing the effect of substrate molar ratio and temperature on incorporation of stearic acid while keeping the time constant at 14 h using Novozym 435 lipase.

Figure 3.1d. Contour plots showing the effect of substrate molar ratio and temperature on SDA content while keeping the time constant at 14 h using Novozym 435 lipase.

Figure 3.2a. TAG molecular species of SDASO. P is palmitic, S is stearic, O is oleic, L is linoleic, Ln is linolenic, G is γ-linolenic, St is stearidonic acid.

Figure 3.2b. TAG molecular species of HSSO

Figure 3.2c. TAG molecular species of SL1 and PB1

Figure 3.2d. TAG molecular species of SL2 and PB2

Figure 3.3a. Melting thermograms of SDASO, HSSO, SLs, and PBs. The vertical dash line represents the melting completion temperature.

Figure 3.3b. Crystallization thermograms of SDASO, HSSO, SLs, and PBs. The vertical dash line represents the crystallization onset temperature.

Figure 3.4. Solid fat content (%) of SLs and PBs

Table 3.1 Experimental settings of the factors and the responses used for optimization by response surface methodology.

Exp	Temp[1] (°C)	Time (h)	SR[2]	Enz[3]	Stearic acid inc[4] (mol%)	SDA content (mol%)
N1	55	6	2	TLIM[5]	15.4±0.0	8.5±0.8
N2	65	10	2	TLIM	14.3±0.8	7.8±1.3
N3	50	18	2	TLIM	16.0±0.0	10.0±1.0
N4	60	22	2	TLIM	15.3±1.2	7.1±0.4
N5	50	6	3	TLIM	15.3±0.0	7.1±0.0
N6	65	6	3	TLIM	13.9±2.1	4.1±0.0
N7	55	22	3	TLIM	14.8±1.1	6.5±0.5
N8	50	10	4	TLIM	14.2±1.1	6.8±0.2
N9	65	22	4	TLIM	15.9±0.9	5.3±0.0
N10	55	6	5	TLIM	15.2±0.5	4.2±0.0
N11	60	10	5	TLIM	15.5±1.8	4.1±0.1
N12	65	18	5	TLIM	16.0±0.2	3.4±0.5
N13	50	22	5	TLIM	15.9±1.6	4.3±0.0
N14	50	6	2	N435[6]	15.6±1.3	4.1±0.8
N15	65	6	2	N435	13.5±1.8	5.5±1.2
N16	50	22	2	N435	14.3±1.1	2.2±0.0
N17	65	22	2	N435	14.5±0.0	7.6±1.0
N18	55	14	3	N435	14.6±1.8	3.9±0.0
N19	60	18	4	N435	16.1±2.0	5.2±0.2
N20	50	6	5	N435	15.3±3.1	2.7±0.2
N21	65	6	5	N435	15.1±1.9	2.2±0.8
N22	50	22	5	N435	16.0±2.1	2.1±0.0
N23	65	22	5	N435	17.9±3.4	2.1±0.7
N24	65	22	5	N435	17.7±1.7	1.9±0.0
N25	65	22	5	N435	18.1±3.8	2.1±0.0
N26	65	22	5	N435	17.9±2.6	2.2±0.0

[1] Temp, temperature. [2]SR, substrate molar ratio (HSSO:SDASO). [3]Enz, enzyme. [4]Inc, incorporation. [5]TLIM, Lipozyme TLIM (*Thermomyces lanuginosus* lipase). [6]N435, Novozym 435 (*Candida antarctica* lipase). Each value is the mean of triplicates ± standard deviation.

Table 3.2.1 ANOVA table for incorporation of stearic acid

Inc[1]	DF[2]	SS[3]	MS[4] (variance)	F-value	P-value	SD[5]
Total	26	6324.97	243.268			
Constant	1	6285.93	6285.93			
Total Corrected	25	39.0352	1.56141			1.24956
Regression	13	37.2607	2.8662	19.3826	0	1.69299
Residual	12	1.7745	0.147875			0.384545
Lack of Fit (Model Error)	9	1.6684	0.185378	5.24161	0.1	0.430555
Pure Error (Replicate Error)	3	0.1061	0.035367			0.18806
N = 26		Q^2 =0.754				
DF = 12		R^2 =0.955				
		R^2Adj.=0.905				

[1] Inc, incorporation. [2] DF, degree of freedom. [3]SS, sum of squares. [4]MS, mean square. [5]SD, standard deviation.

Table 3.2.2 ANOVA table for SDA content

SDA content	DF[1]	SS[2]	MS[3] (variance)	F-value	P-value	SD[4]
Total	26	719.91	27.6888			
Constant	1	582.737	582.737			
Total Corrected	25	137.173	5.48693			2.34242
Regression	13	127.825	9.83267	12.6214	0	3.13571
Residual	12	9.34857	0.779048			0.882637
Lack of Fit (Model Error)	9	9.2941	1.03268	56.8707	0.003	1.01621
Pure Error (Replicate Error)	3	0.054475	0.018158			0.134753
N = 26		Q^2 =0.558				
DF = 12		R^2 =0.932				
		R^2Adj.=0.858				

[1] DF, degree of freedom. [2]SS, sum of squares. [3]MS, mean square. [4]SD, standard deviation.

Table 3.3 Verification of the models using chi-squared test

Region	Tem[1] (°C)	Time (h)	SR[2]	Enz[3]	E[4]		O[5]		$(O-E)^2/E$	
					Stearic acid	SDA	Stearic acid	SDA	Stearic acid	SDA
R1	52	8	2	TLIM[6]	15.9	8.8	13.9	10.2	0.257	0.234
R2	62	12	5	TLIM	15.6	3.8	14.6	4.3	0.075	0.056
R3	58	14	3.5	N435[7]	14.9	4.8	15.2	3.4	0.007	0.434
R4	63	18	4	N435	15.7	4.5	14.8	6.5	0.059	0.926
R5	56	16	2.5	N435	14.9	5.9	14.4	6.9	0.012	0.156
								χ^2 =	0.410	1.806

[1] Temp, temperature. [2] SR, substrate molar ratio (HSSO:SDASO). [3]Enz, enzyme. [4]E, expected response mol%. [5]O, observed response mol%. [6] TLIM, Lipozyme TLIM (*Thermomyces lanuginosus* lipase). [7] N435, Novozym 435 (*Candida antarctica* lipase).

Table 3.4 Total fatty acid (mol%) of the substrates, SLs, and PBs

Fatty acid	SDASO[1]	HSSO[2]	SL1[3]	PB1[4]	SL2[5]	PB2[6]
14:0	0.1±0.0a	0.1±0.0a	nd[7]	nd	nd	nd
16:0	12.2±0.1a	10.4±1.5b	15.1±2.9c	15.2±1.3c	15.3±1.3c	14.7±0.8d
16:1n-7	0.1±0.0a	0.1±0.0a	0.1±0.0a	0.1±0.0a	0.1±0.0a	0.1±0.0a
18:0	4.0±0.0a	15.8±0.9b	14.9±1.5cd	14.0±1.7c	15.9±1.2b	15.4±1.1d
18:1n-9*c*	15.6±0.1a	16.0±1.8a	25.6±3.6b	21.9±2.0c	22.6±2.0c	20.7±2.3c
18:2n-6*t*	0.2±0.0a	0.5±0.0b	nd	nd	nd	nd
18:2n-6*c*	24.6±2.3a	50.2±3.2b	19.7±2.1c	25.6±3.2ad	27.6±3.1d	33.2±3.8c
20:0	0.4±0.1a	1.1±0.0b	0.9±0.0bc	0.9±0.0bc	0.9±0.0bc	0.8±0.0c
18:3n-6	7.5±1.3a	0.4±0.0b	2.7±0.0c	2.4±0.9c	2.6±0.6c	2.4±0.0c
20:1n-9	0.5±0.0a	0.5±0.0a	0.6±0.0a	0.6±0.0a	0.5±0.0a	0.5±0.0a
18:3n-3	10.1±1.0a	4.3±1.6b	9.6±1.0c	7.3±1.0d	8.8±1.7e	7.5±0.8d
21:0	0.3±0.0a	nd	0.1±0.0b	0.1±0.0b	0.1±0.0b	nd
18:4n-3	23.5±1.9a	nd	10.2±1.1b	7.1±1.1c	8.9±0.9d	7.1±1.0c
22:0	0.3±0.0a	0.6±0.0b	0.7±0.b	0.7±0.0b	0.6±0.0b	0.6±0.0b
20:3n-6	0.1±0.0	nd	nd	nd	nd	nd
SFA[8]	17.3	28.0	31.7	30.8	32.8	31.5
UFA[9]	81.7	71.5	68.6	65.1	71.2	71.5
TFA[10]	0.2	0.5	0.0	0.0	0.0	0.0

[1] SDASO, stearidonic acid-enriched soybean oil. [2]HSSO, high stearate soybean oil. [3]SL1, Lipozyme TLIM-catalyzed structured lipid. [4]PB1, corresponding physical blend of SL1. [5]SL2, Novozym 435-catalyzed structured lipid. [6]PB2, corresponding physical blend of SL2. [7]nd, not determined. [8]SFA, saturated fatty acids. [9]UFA, unsaturated fatty acids. [10]TFA, *trans* fatty acids. Each value is the mean of triplicates ± standard deviation. a,b,c,d,e values were calculated using Duncan's multiple range test and values with the same letter in each row are not significantly different at $P \leq 0.05$.

Table 3.5 Positional fatty acid profile (mol%) of the substrates, SLs, and PBs

Fatty acid	SDASO[1]		HSSO[2]		SL1[3]		PB1[4]		SL2[5]		PB2[6]	
	sn-2	*sn*-1,3	*sn*-2	*sn*-1,3	*sn*-2	*sn*-1,3	*sn*-2	*sn*-1,3	*sn*-2	*sn*-1,3	*sn*-2	*sn*-1,3
14:0	nd[7]	0.1±0.0a	nd	0.1±0.0a	nd	nd	nd	nd	nd	nd	nd	nd
16:0	nd	18.4±1.1a	nd	15.7±1.5b	13.1±1.7a	15.0±1.7b	nd	22.7±2.7c	15.7±1.0b	15.2±1.7b	nd	22.0±2.7c
16:1n-7	nd	0.1±0.0a	nd	0.2±0.0b	nd	0.2±0.0b	nd	0.2±0.0b	nd	0.2±0.0b	nd	0.2±0.0b
18:0	nd	5.9±0.5a	nd	23.7±2.0b	7.9±1.0a	18.4±2.7c	nd	21.0±1.2d	11.5±1.8b	18.2±1.7c	nd	20.1±2.7d
18:1n-9*c*	15.5±1.3a	15.7±1.9a	12.6±1.0b	17.7±2.1b	14.8±1.0a	31.1±2.9c	19.2±2.3c	25.3±2.6d	15.1±1.7a	26.4±2.4d	18.3±2.2c	21.9±2.6e
18:2n-6*t*	nd	0.2±0.0a	nd	0.7±0.0b	nd	nd	nd	nd	nd	nd	nd	nd
18:2n-6*c*	50.3±4.9a	11.7±1.8a	84.2±5.9b	33.2±3.2b	45.3±4.9c	6.9±1.2c	68.1±5.3d	4.4±0.0d	42.5±3.7c	20.3±2.1e	68.7±5.8d	11.4±1.8a
20:0	nd	0.6±0.0a	nd	1.6±0.6b	nd	1.3±0.7c	nd	1.4±0.0c	nd	1.3±0.7c	nd	1.3±0.8c
18:3n-6	8.0±1.0a	7.2±1.7a	nd	0.7±0.0b	3.5±0.7b	2.3±0.0c	1.9±0.0c	2.7±0.0c	2.4±0.8d	2.6±0.0c	0.9±0.0e	3.1±0.3d
20:1n-9	nd	0.7±0.0a	nd	0.7±0.0a	nd	0.9±0.0b	nd	0.9±0.0b	nd	0.8±0.0b	nd	0.8±0.0b
18:3n-3	5.9±0.9a	12.1±1.0a	3.0±0.7b	5.0±1.0b	5.2±1.2a	11.8±0.7ad	4.4±0.8c	8.7±1.4c	5.2±0.6a	10.6±1.0d	4.7±0.9c	8.9±0.9c
21:0	nd	0.5±0.0a	nd	nd	nd	0.1±0.0b	nd	0.1±0.0b	nd	0.1±0.0b	nd	nd
18:4n-3	19.8±2.1a	25.3±2.8a	nd	nd	9.2±1.8b	10.8±0.8b	5.3±0.4c	8.1±0.3c	7.1±1.2d	9.8±0.9d	5.6±1.0c	7.4±1.2e
22:0	nd	0.4±0.2a	nd	0.9±0.0b	nd	1.1±0.0b	nd	1.0±0.5b	nd	0.9±0.0b	nd	0.9±0.0b
20:3n-6	nd	0.1±0.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[1] SDASO, stearidonic acid-enriched soybean oil. [2]HSSO, high stearate soybean oil. [3]SL1, Lipozyme TLIM-catalyzed structured lipid. [4]PB1, corresponding physical blend of SL1. [5]SL2, Novozym 435-catalyzed structured lipid. [6]PB2, corresponding physical blend of SL2. [7]nd, not determined. Each value is the mean of triplicates ± standard deviation. Values with the same letter in each row within *sn*-2 and *sn*-1,3 columns separately are not significantly different at $P \leq 0.05$.

Table 3.6 Polymorphic forms of the substrates, SLs, and PBs

Sample	Polymorphic form
SDASO	β′>β
HSSO	β′>β
SL1	β′>>>β
PB1	β′>β
SL2	β′>>β
PB2	β′>β

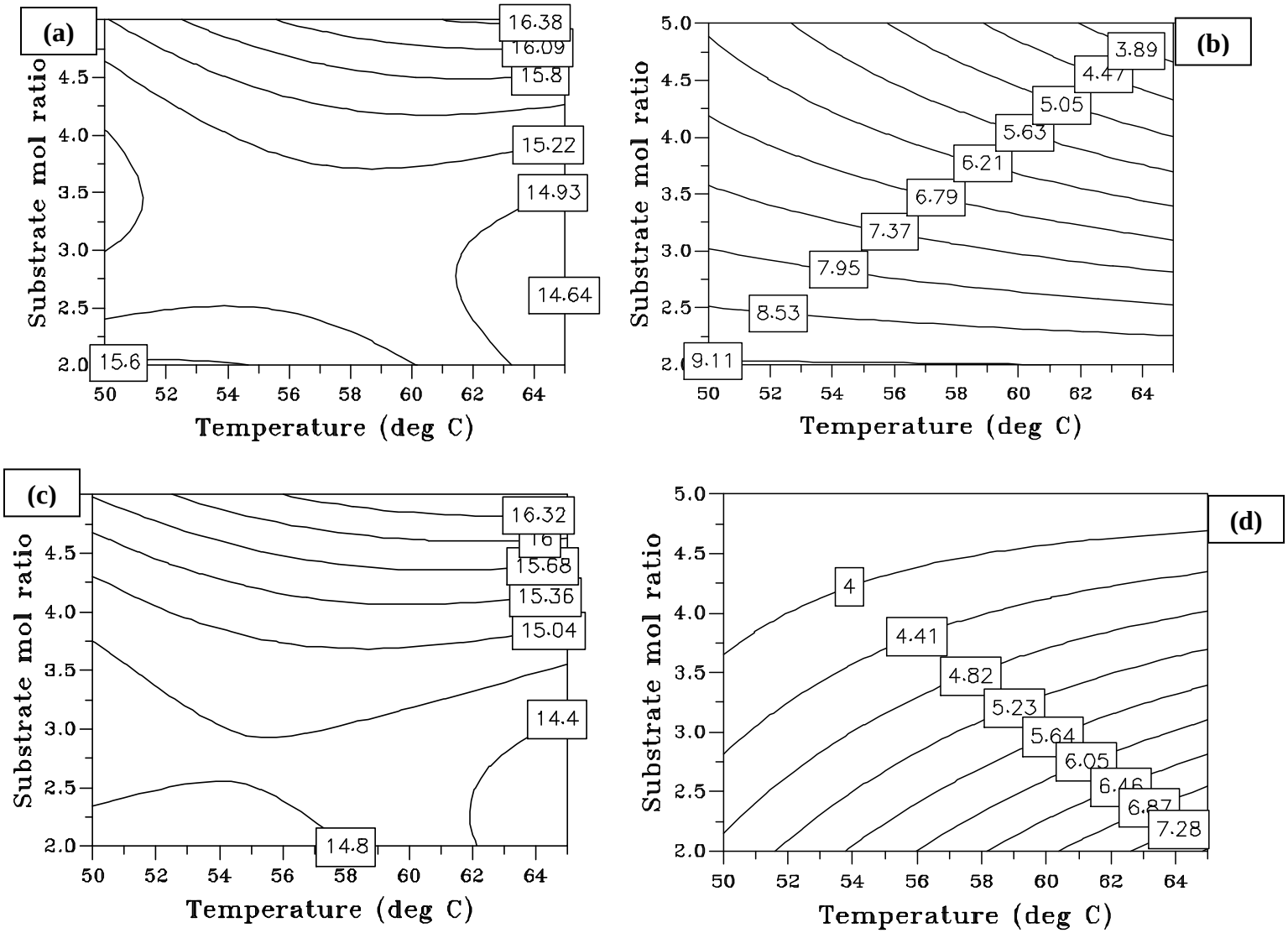
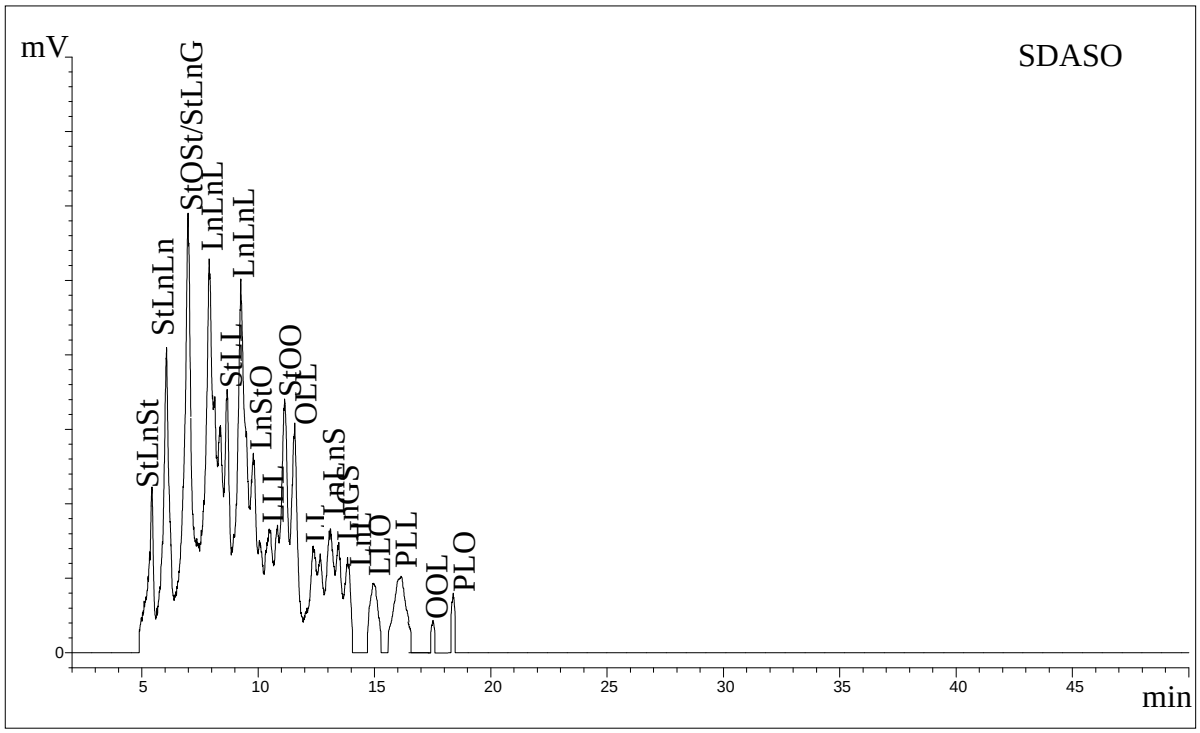

(a)
16.38
16.09
15.8
15.22
14.93
14.64
15.6
Substrate mol ratio
Temperature (deg C)
(b)
3.89
4.47
5.05
5.63
6.21
6.79
7.37
7.95
8.53
9.11
Substrate mol ratio
Temperature (deg C)
(c)
16.32
16
15.68
15.36
15.04
14.4
14.8
Substrate mol ratio
Temperature (deg C)
(d)
4
4.41
4.82
5.23
5.64
6.05
6.46
6.87
7.28
Substrate mol ratio
Temperature (deg C)

Fig. 3.1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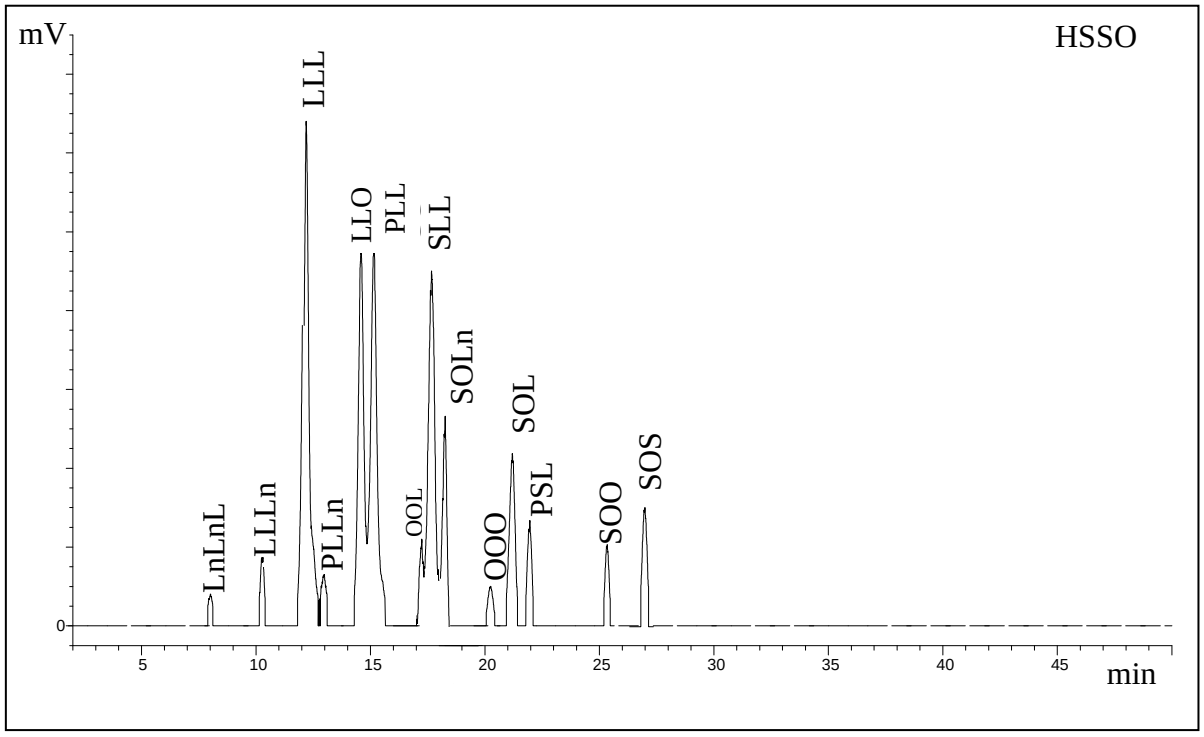
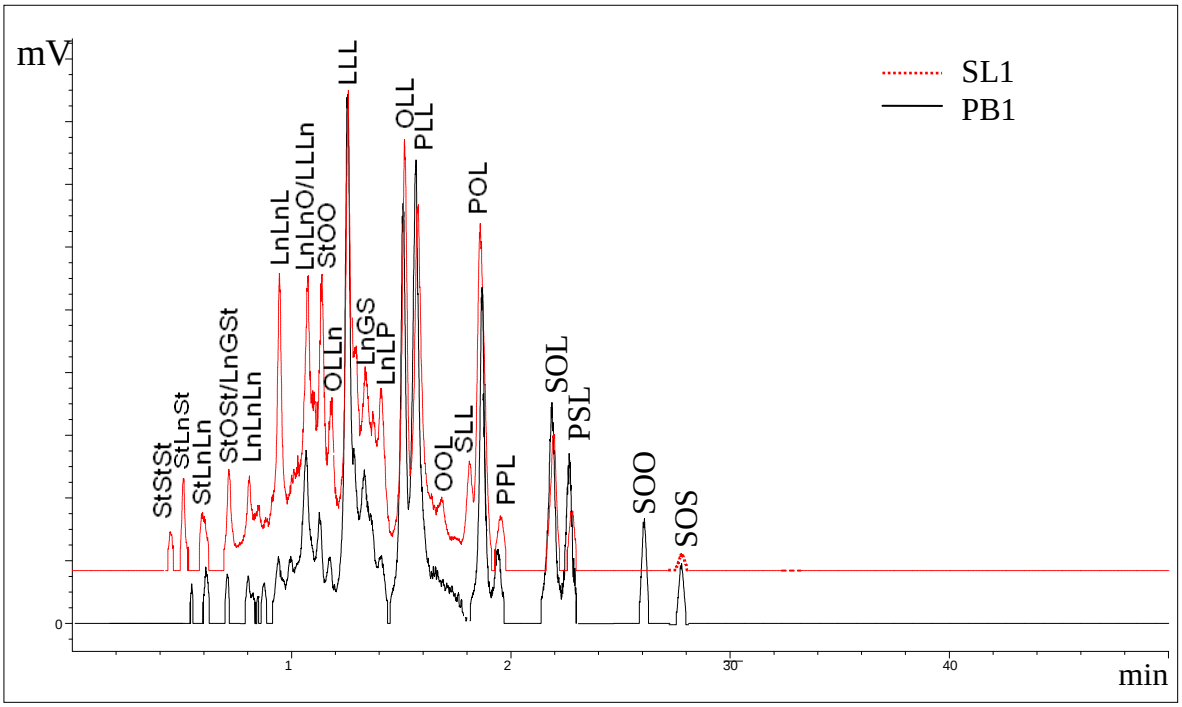
SDASO
StLnSt
StLnLn
StOSt/StLnG
LnLnL
LnLnL
StLL
LnStO
StOO
OLL
LLL
LnLnS
LLO
PLL
OOL
PLO
0
5
10
15
20
25
30
35
40
45
min

(a)

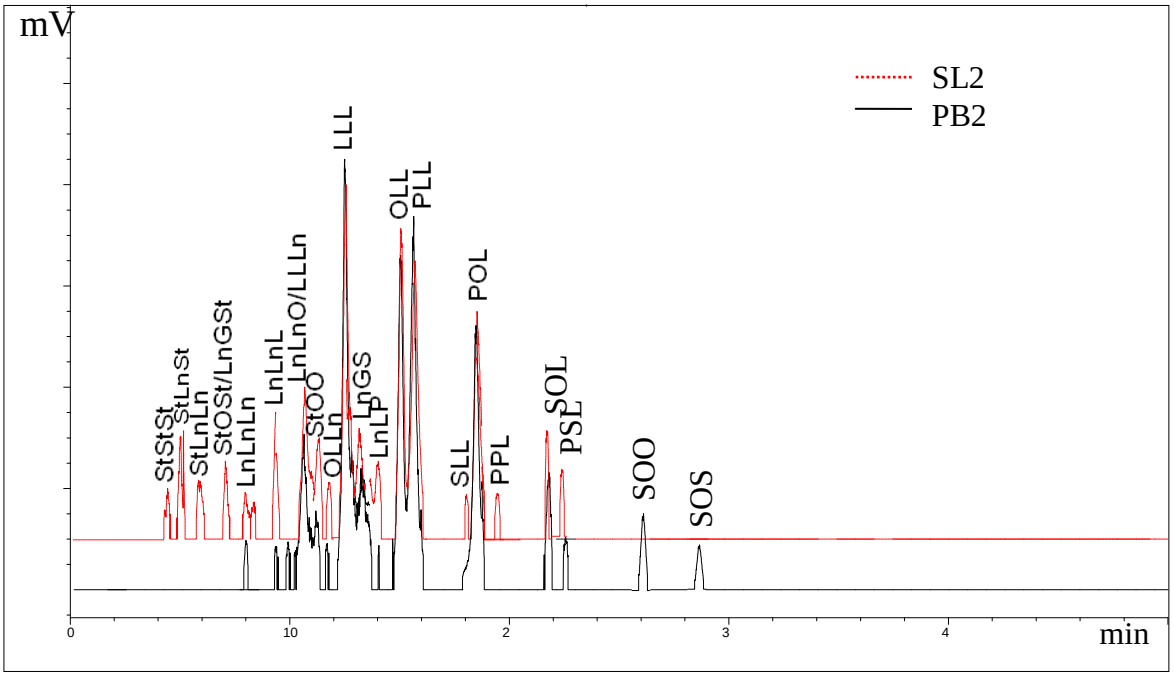

mV
HSSO
LnLnL
LLLn
LLL
PLLn
LLO
PLL
SLL
OOL
SOLn
OOO
SOL
PSL
SOO
SOS
0
5
10
15
20
25
30
35
40
45
min

(b)

Fig. 3.2

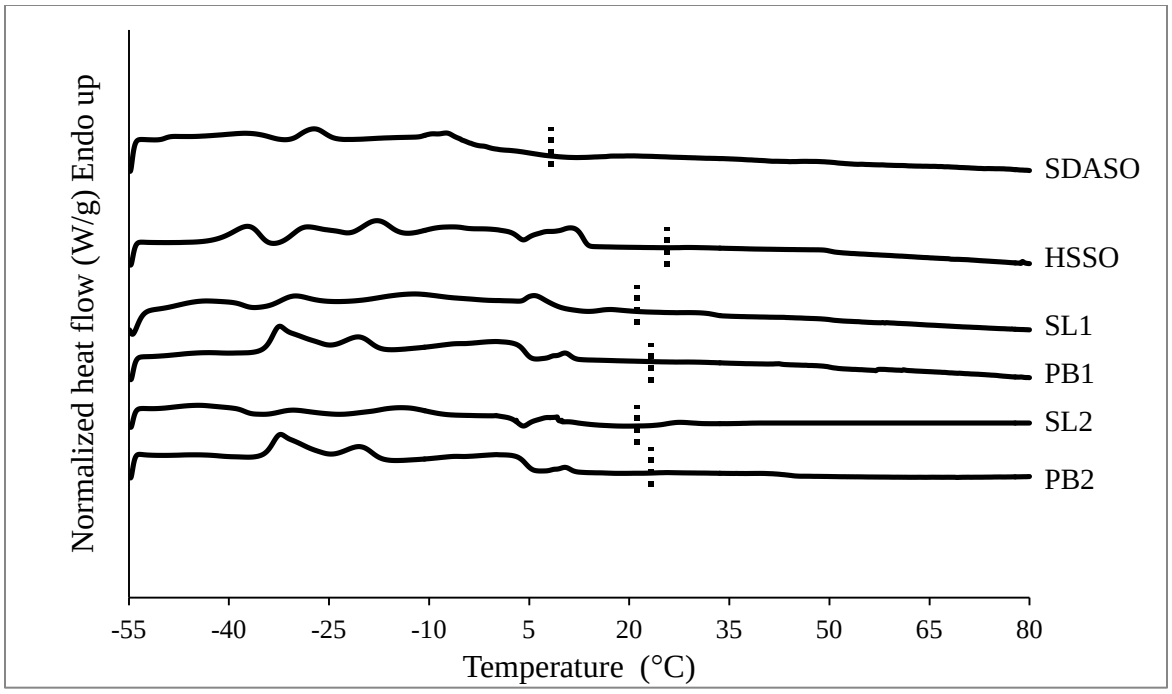

mV
SL1
PB1
StStSt
StLnSt
StLnLn
StOSt/LnGSt
LnLnLn
LnLnL
LnLnO/LLLn
StOO
OLLn
LnGS
LnLP
LLL
OLL
PLL
SLL
OOL
POL
PPL
SOL
PSL
SOO
SOS
0
1
2
30
40
min

(c)

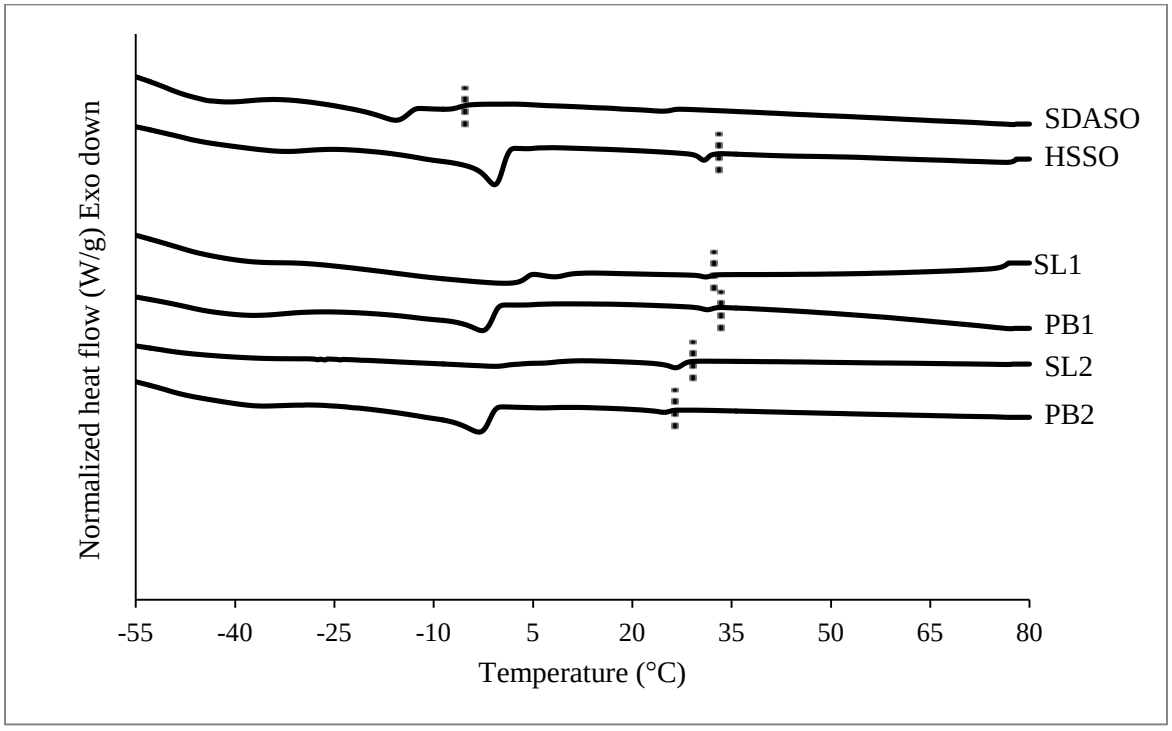

mV
SL2
PB2
StStSt
StLnSt
StLnLn
StOSt/LnGSt
LnLnLn
LnLnL
LnLnO/LLLn
StOO
OLLn
LnGS
LnLP
LLL
OLL
PLL
SLL
PPL
POL
PSL
SOL
SOO
SOS
0
10
2
3
4
min

(d)

Fig. 3.2


Normalized heat flow (W/g) Endo up
SDASO
HSSO
SL1
PB1
SL2
PB2
-55
-40
-25
-10
5
20
35
50
65
80
Temperature (°C)

(a)


Normalized heat flow (W/g) Exo down
SDASO
HSSO
SL1
PB1
SL2
PB2
-55
-40
-25
-10
5
20
35
50
65
80
Temperature (°C)

(b)

Fig. 3.3

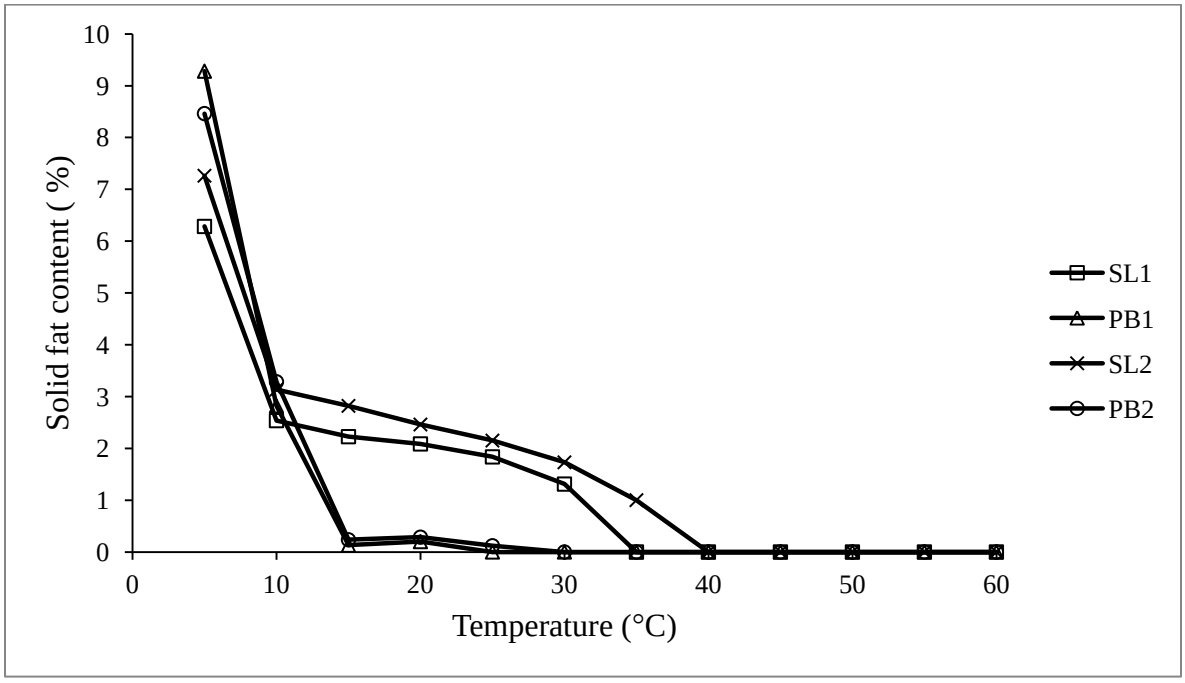

Solid fat content (%)
10
9
8
7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
Temperature (°C)
SL1
PB1
SL2
PB2

Fig. 3.4

CHAPTER 4

PRODUCTION OF *TRANS*-FREE MARGARINE WITH STEARIDONIC ACID SOYBEAN AND HIGH STEARATE SOYBEAN OILS-BASED STRUCTURED LIPID[1]

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To be submitted to *Journal of Food Science.*

ABSTRACT

Omega-3 fatty acids (n-3 FAs) have been positively associated with prevention and treatment of chronic diseases. Intake of high amounts of *trans* fatty acids (TFAs) is correlated with increased risk of coronary heart disease, inflammation, and cancer. Structured lipid (SL) was synthesized using stearidonic acid (SDA) soybean oil and high stearate soybean oil catalyzed by Lipozyme® TLIM lipase. The SL was compared to extracted fat from commercial spread (EF) for FA profile, *sn*-2 positional FAs, triacylglycerol (TAG) profile, polymorphism, thermal behavior, oxidative stability, and solid fat content (SFC). Both SL and EF had similar saturated FA (~31 mol%) and unsaturated FA (~68 mol%), but SL had much lower n-6/n-3 ratio (1.1) than EF (5.8). SL had 10.5 mol% SDA. The major TAG species in SL were POL (14.4%) and LLL (12.4%). After short-path distillation, a loss of 53.9% was observed in the total tocopherol content of SL. The tocopherols were lost as free tocopherols. SL and EF had similar melting profile, β' polymorph, and oxidative stability. Margarine was formulated using SL (SLM) and EF (RCM, reformulated commercial margarine). SLM was less hard and more spreadable than RCM. No sensory difference was observed between the two margarines. The SL synthesized in this study contained no TFA and possessed desirable polymorphism, thermal properties, and SFC for formulation of soft margarine. The margarine produced with this SL was *trans*-free, SDA-enriched with no significant sensorial difference.

KEYWORDS: high stearate soybean oil; margarine; stearidonic acid soybean oil; structured lipid; *trans*-free

PRACTICAL APPLICATION: The current research increases the food applications of stearidonic acid (SDA) soybean oil. *trans*-Free SDA containing structured lipid was synthesized with desirable polymorph, thermal properties, and SFC for formulation of soft margarine. The margarine produced with this SL had no *trans* fat and low n-6/n-3 ratio. This may help in reducing *trans* fat intake in our diet while increasing n-3 FA intake.

INTRODUCTION

The essential fatty acids (EFA) for humans are α-linolenic acid (ALA) (n-3) and linoleic acid (LA) (n-6). Increased intake of n-3 fatty acids (FA) is positively associated with decreased blood pressure (Gelenjinse and others 2002), reduced risk of CVD (Calder 2004), and decreased inflammation (Calder 2006). The American Heart Association (AHA) Dietary Guidelines suggest Americans consume at least two servings of fish per week and include n-3 fatty acid, ALA, in their diets since the current n-3 FAs intake is low (0.1–0.2 g/person/day) (Kris-Etherton and others 2002). Stearidonic acid SDA (18:4 n-3) is a Δ6-desaturase product of ALA and a metabolic intermediate between ALA and eicosapentaenoic acid (EPA). Dietary SDA increases plasma EPA 3-4 times more efficiently than ALA and approximately one-third as effective as dietary EPA (James and others 2003). SDA soybean oil (SDASO, ~23% SDA) is a readily available and sustainable plant source of n-3 FAs. Genes for Δ6- and Δ15- desaturases have been introduced into soybeans that convert LA and ALA to SDA resulting in 15-30 wt% SDA of the total fatty acids in the oil (Hammond and others 2008). High-stearate soybean oil (HSSO) contains elevated levels of stearic acid (~17%) for increased stability of foods that require solid fat functionality. High stearic acid content enables formulation of margarines and shortenings with improved texture and no *trans* fats. Furthermore, stearic acid has neutral effect on serum cholesterol levels when compared to other saturated fatty acids (SFA) (Karupaiah and Sundram, 2007).

High intake of *trans* fatty acids (TFA) is associated with increased LDL, decreased HDL, and increased total: HDL cholesterol (Mozaffarian and Clarke 2009) and cancer (Astorg 2005). The mean daily intake of TFA per person in the United States is 2-

4% of total food energy (Craig-Schmidt 2006). Partial hydrogenation is the main contributor of TFA (80%) in the US diet (Eckel and others 2007). Dietary Guidelines for Americans (2010) suggest that TFA consumption should be as low as possible. Effective from January 2006 all foods containing ≥0.5 g *trans* fat/serving must be labeled accordingly (Federal Register 2003). Interesterification is an alternative to partial hydrogenation and during interesterification reaction there is no double bond isomerization or change in unsaturation (Ribeiro and others 2009). The types of FA remain unaltered but a redistribution of these FAs on the triacylglycerol (TAG) molecules occur resulting in desired functional properties without any *trans* fat formation. Structured lipids (SLs) are lipids (usually TAG, but can also include monoacylglycerols (MAG), diacylglycerols (DAG), and glycerophospholipids (GPL)) that have been structurally modified from their natural form by changing the positions of FAs, or the FA profile, or synthesized to yield novel TAGs through chemical or enzymatic processes (Akoh, 2008).

Margarine is a water-in-oil (w/o) emulsion comprising of at least 80% lipid phase (Aini and Miskander 2007). The lipid phase consists of fat, antioxidant, and emulsifier whereas the aqueous phase contains water, preservatives and salt. There are three main types of margarines: table margarine which is softer and easily spreadable, a firmer industrial margarine, and puff pastry margarine with higher solid fat content for required plasticity (Wassell and Young 2007). Margarine is one of the major sources of TFA. Several studies have been carried out using interesterification to produce *trans*-free margarine (Adhikari and others 2010a,b; Kim and others 2008; Lumor and others 2007). The current study is innovative as no previous studies have been reported on formulation

of *trans*-free margarine containing stearidonic acid as source of n-3 FA. The objective of this study was to formulate margarine using *trans*-free SL and compare it with a commercial margarine.

MATERIALS AND METHODS

Materials

HSSO and SDASO were kindly provided by Monsanto Company (St. Louis, MO). The immobilized enzymes, Lipozyme® TLIM (*Thermomyces lanuginosus* lipase, *sn*-1,3 specific, specific activity 250 IUN/g: IUN=Interesterification Unit) was purchased from Novozymes North America Inc. (Franklinton, NC). Commercial spread and food grade soy lecithin fluid were purchased from a local grocery store. Lipid standards, Supelco 37 Component FAME mix, 14% boron trifluoride in methanol, triolein, 2-oleoylglycerol, tripalmitin, tristearin, 1,2-dioleoyl-3-palmitoyl-rac-glycerol, 1-palmitoyl-2-oleoyl-3-linoleoyl, and tocopherol standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), and the internal standard, C19:0-nonadecanoic acid and its methyl ester, were purchased from TCI America (Portland, OR). Stearidonic acid and its methyl ester were purchased from Cayman Chemical Company (Ann Arbor, MI). The TAG standard mix (GLC reference standard) was purchased from Nu-chek Prep, Inc. (Elysian, MN). Organic solvents and chemicals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA).

SL synthesis

The large-scale reaction using the conditions achieved from the optimization study (Pande and Akoh 2012) was performed in 4L stir-batch reactor at 50 °C, 18 h, 2:1 (HSSO:SDASO) using Lipozyme TLIM. The substrates were weighed and heated to 50

°C and then added to preheated bioreactor. Temperature of bioreactor was maintained by circulating water bath. Enzymes were also warmed to 50 °C before adding to the reactor to maintain the temperature. The reactants were stirred at 200 rpm. After the reaction (18 h), the product was filtered two times, both under vacuum. First through Whatman filter paper no. 1 and then through a Whatman filter paper (pore size = 0.45 μm), both sprinkled with anhydrous sodium sulfate. Free fatty acids (FFAs) were removed by short-path distillation (SPD) KDL-4 unit (UIC, Joliet, IL). The reaction products were passed through the unit under high vacuum (<0.2 mmHg absolute pressure) at the flow rate of ca. 10 ml/min. The evaporator and condenser temperatures were maintained at 185 and 25 °C, respectively. FFAs were determined by AOCS Official Method Ac 5-41 (2009). After purification by short-path distillation, the SLs contained 0.1% FFA which is an acceptable level to be used as ingredients for food products. The yield % for large-scale SL was 92.8%.

Enzyme reusability

Interesterification was performed in a 1 L stir-batch reactor at 50 °C for18 h with 2:1(HSSO: SDASO) substrate molar ratio using Lipozyme TLIM lipase for ten times. After each run, the enzyme was washed 4-5 times with hexane, dried under hood, and placed in a desiccator. The enzyme was stored at 4 °C until reuse for the next run. Since all enzyme was not fully recovered (some were lost during transferring from bioreactor and during filtering), the initial weight of the substrates were adjusted accordingly. The SL was cleaned using SPD as described above. Total and positional FA composition was analyzed using GC.

Lipid extraction

The commercial spread was melted at 80 °C. The top fat layer was decanted into a separatory funnel, washed five times with the same volume of warm water, and filtered under vacuum through a Whatman filter paper (pore size = 0.45 μm) sprinkled with anhydrous sodium sulfate. This extracted fat from commercial spread was designated EF.

Fatty acid profile

Lipid samples were converted to fatty acid methyl esters following the AOAC Official Method 996.01 (1998) and analyzed with Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) using Supelco SP-2560, 100 m x 25 mm x 0.2 μm column. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. Injection volume was 1 μL and a spilt ratio of 20:1 was used. The column was initially held at 140 ºC for 5 min, then increased to 240 ºC at 4 ºC/min, and finally held at 240 ºC for 25 min. Detection was with flame ionization detector at 300 ºC. All samples were analyzed in triplicates and average values reported. *sn*-2 Positional fatty acid composition was determined following the AOCS Official Method Ch 3-9 (2009). Fatty acid composition at *sn* -1,3 position was calculated using the following equation:

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

All samples were analyzed in triplicates and average values reported.

Triacylglycerol (TAG) molecular species

The TAG composition was determined with a nonaqueous reverse phase HPLC (Agilent Technologies 1260 Infinity, Santa Clara, CA) equipped with a Sedex 85 ELSD (Richard Scientific, Novato, CA) (Pande and Akoh 2012). Triplicate determinations were made and averaged.

Differential scanning calorimetry (DSC)

Melting and crystallization profiles of lipid samples were determined following AOCS Official Method Cj 1-94 (2009). SL and EF were analyzed on Mettler Toledo model DSC 1 STARe System (Columbus, OH). All samples were analyzed in triplicates and average values reported.

Polymorphism

The polymorphic forms of the samples were determined with Bruker D8 Advance diffractometer (Bruker AXS, Inc., Madison, WI) (Pande and Akoh 2012). Short spacings of the major polymorphs were identified as follows: α, a single spacing at 4.15 Å; β', two strong spacings at 3.8 and 4.2 Å; and β, a very strong spacing at 4.6 and another one usually at 3.85 Å (Ribeiro and others 2009). All samples were analyzed in triplicates and average values reported.

Solid fat content (SFC)

SFC was determined according to the AOCS Official Method Cd 16-93b (2009) on a BrukerPC/20 Series NMR analyzer, Minispec (Bruker Optics, Milton, On, Canada). All samples were analyzed in duplicates and average values reported.

Tocopherol analysis

Normal phase high-performance liquid chromatography (HPLC) system (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 column was a LiChrosorb Si 60 column (4 mm, 250 mm, 5 μm particle size, Hiber Fertigs€aule RT, Merck, Darmstadt, Germany). The sample

concentration was 20 mg/mL in HPLC-grade hexane. The samples were vortexed for 1 min and then centrifuged at 1000 rpm (104.72 rad/s) for 5 min at room temperature (23 °C). The top hexane layer was transferred into HPLC vials for analysis. Injection volume was 20 μL. The tocopherols were identified by comparing their retention times with those of authentic standards (α-, β-, δ-, and γ-tocopherol, 1.25-20 μg/mL in hexane containing 0.01% butylated hydroxytoluene). Tocopherols were quantified based on the standard calibration curves and reported as parts per million (ppm) from the average of triplicate determinations.

Oxidative stability index (OSI)

The OSI was determined according to AOCS Method Cd 12b-92 (2009) using an Oil Stability Instrument (Omnion, Rockland, MA) at 110 °C. All samples were analyzed in triplicates and average values reported.

Margarine formulation

The margarines were formulated as described by Kim and others (2008). Since a *trans*-free n-3 FA-containing commercial margarine could not be obtained, a commercial spread was used. After extracting the fat from the commercial spread, margarine was formulated using 80% EF. The ingredients (w/w %) were lipid phase, 80.5% (EF or SL, 80%; soy lecithin fluid, 0.5%; and TBHQ, 0.01%) and aqueous phase, 19.5% (distilled water, 18%; and table salt, 1.5%). The lipids and water were heated to 60 °C. Both phases were poured into a tabletop blender and vigorously mixed for 5 min to emulsify them. Artificial butter flavor was added to mask any difference in the aroma of the samples. The resulting liquid emulsion was then crystallized for 25 min using an ice cream maker (Krups North America, Peoria, IL). The resulting crystallized emulsion was refrigerated

overnight and then tempered at room temperature for 4 h. The margarines were vigorously mixed with a hand mixer to obtain a smooth product. The margarine sample were placed into plastic tubs and stored at 4 °C. The margarines formulated with SL and EF were designated SLM (structured lipid containing margarine) and RCM (reformulated commercial margarine), respectively.

Texture profile analysis (TPA)

Margarine samples were taken out from refrigerator and measured at room temperature (23 °C). A double compression test was performed using a TA-X2 texture analyzer (Stable Micro Systems, London, United Kingdom) (Kim and others 2008). A 45º conical probe attached to a 25 kg compression load cell was used to penetrate the samples at 1.0 mm/s to a depth of 10 mm from the sample surface, and withdrawn at the same speed. Textural properties (hardness, adhesiveness, and cohesiveness) of margarines were evaluated at 23 °C. All samples were analyzed in triplicates and average values reported.

Rheological properties

A series of tests were performed on a dynamic stress-controlled rheometer SR5000 (Rheometric Scientific, Piscataway, NJ) at 23 °C to determine the rheological properties of margarine samples. A 25 mm parallel plate (0.6 mm gap) was used for analysis. In dynamic analysis, margarine samples were subjected to a sinusoidally varying stress (0.4 kPa) and the strain output and phase difference between the input and output signals were measured. Storage/elastic (G') and loss/viscous (G'') moduli were calculated from this information. In creep analysis, a constant low stress (0.4 kPa) was applied to samples and deformation per unit time measured as an indicator of emulsion stability. In stress

analysis, viscosity was measured across different stress (0.1-2.5 kPa). All samples were analyzed in triplicates and average values reported.

Microstructural morphology

Microstructure of margarines was observed with a polarized light microscope (Leica Microsystem Inc., Allendale, NJ) attached to an SPOT Idea™ digital camera (SPOT™ Imaging Solutions, Sterling Heights, MI). Margarine samples were melted at 80 °C and using a preheated capillary a small drop was placed on a preheated microslide. The sample was carefully covered with a preheated coverslip to obtain a uniform film. The glass slides were kept in the refrigerator overnight and were measured at room temperature (23 °C). Microstructure was observed using 50x lens. All samples were observed in triplicates.

Sensory evaluation

All methods and procedures for sensory studies were approved by the University of Georgia (UGA) Institutional Review Boards on Human Subjects. Thirty five UGA staff and students above 18 yrs of age participated in the sensory evaluation. A triangle test was conducted in individual booths under fluorescent light to study the difference between experimental and commercial margarines. Each participant was provided with 3 coded margarine samples, two of which were the same and one different. Subjects were told to spread the margarine samples on the three toasts provided. They were to chew the samples but not required to swallow them. They had to identify the odd sample and explain the difference observed. They were provided with an evaluation sheet with all instructions, water, non-salted crackers (to cleanse the mouth during the test), and empty cups for expectoration.

Statistical analysis

Duncan's multiple-range test was performed using Statistical Analysis System software (SAS 9.2, Cary, NC) to determine significant difference ($P \leq 0.05$) between SL and EF, and between SLM and RCM. All samples, except SFC, were analyzed in triplicates and average values reported.

RESULTS AND DISCUSSION

Fatty acid composition

The total and positional FA compositions of SL and EF are given in Table 4.1. The type and positional distribution of FA on the glycerol backbone greatly influences the physical behavior and metabolism of dietary fats. Unsaturated FAs (UFA) are better metabolized and utilized in our body when present at *sn*-2 position (Karupaiah and Sundram 2007). The major FAs in SL were oleic (25.8 mol%) and stearic (15.0 mol%) acids whereas oleic (42.1 mol%) and palmitic (24.8 mol%) acids were the major FA in EF. SL had 10.2 mol% SDA and 9.8 mol% of it was present at *sn*-2 position. Both SL and EF had similar saturated FA (SFA) (~31 mol%) and UFA (~68 mol%) but SL had much lower n-6/n-3 ratio (1.1) than EF (5.8). The current western n-6:n-3 ratio is 15-16.7:1 as compared to our ancestors diet with a ratio of ~1 (Simopoulos 2008). A lower ratio n-6/n-3 FA is desirable for reducing the risk of several chronic diseases. n-3 FA have been positively associated with prevention and treatment of chronic diseases. On the other hand, excessive n-6 FA promote the pathogenesis of many diseases such as inflammatory diseases, cancer, and cardiovascular diseases (Simopoulos 2008). No *trans* fat was found in either samples. Oleic acid was the predominant FA at *sn*-2 postion of SL (45.8 mol%)

and EF (41.3 mol%). Lower SFA (21.0 mol%) were found at SL's *sn*-2 position than EF (27.7 mol%).

TAG molecular species

TAG profile influences the crystallization and texture of the final product. Fats containing highly diverse TAG profiles are likely to crystallize in the β′ form, the desirable polymorph in margarines, whereas those with little TAG diversity crystallize in the β form (Ribeiro and others 2009). The relative concentration (%) of TAG molecular species is given in Table 4.2. The major TAG species in SL were POL (14.4%) and LLL (12.4%). The major TAG species in EF were POO (14.4%) and POP (12.5%). SL was composed of only UUU (triunsaturated) (53.7 %), SUU (monosaturated-diunsaturated) (42.2 %), and SUS (disaturated-monounsaturated) (6.4 %) type TAGs whereas UUU (41.2%), SUU (41.1%), SUS (17.3%), and SSS (trisaturated) (1.8%) type TAGs were present in EF. This difference in TAG species influenced the thermal behavior and the physical properties of product.

Thermal behavior

Melting and crystallization profiles of SL and EF are shown in Figs. 4.1a and 4.1b, respectively. The melting completion temperature (T_{mc}) depends on the amount and type of the TAG molecular species. T_{mc} of SL and EF were 10.7 and 11.3 °C, respectively. The T_{mc} of the SL was slightly lower than EF because it contains more UUU and SUU, fewer SUS, and no SSS type TAGs compared to EF. Both lipid samples were completely melted at room temperature. Therefore, this SL can be used in the production of soft spreadable margarine. The crystallization onset temperature (T_{co}) of SL and EF were 9.1 and 10.9 °C, respectively. T_{co} depends on the TAG molecular species, if more than 30%

POP is present, products are more likely to have post hardening defect due to slow crystallization (deMan and others 1989). None of the lipid samples had more than 30% POP and therefore will maintain their texture. SL, similar to EF, had small difference between its T_{mc} and T_{co} which shortens its crystallizing time and may eventually help in margarine production.

Solid fat content

One of the important parameters of margarine is solid fat content (SFC) which is a measure of solid/liquid ratio of a fat at various temperatures. Three useful measurement temperatures are refrigeration, room, and body temperatures which are related to the spreadibility, product stability, and texture and mouthfeel, respectively, of margarines (Ribeiro and others 2009). The SFC of SL and EF are shown in Fig 4.2. A desirable margarine is one that has less than 32% SFC at 10 ˚C (Lida and Ali 1998) less than 3.5% SFC at 33.3 °C (Karabulut and others 2004). At 10 ˚C, SL had 3.2% SFC and 0.1% SFC at 33.3 °C. This will help maintain its spreadibility at refrigeration temperature and provide no waxy feel in the mouth. EF also met these criteria. Therefore, the current SL is suitable for production of soft tub margarine spreadable at refrigeration temperature.

Polymorphism

The tendency of FAs residues to occur in differentiated crystalline forms in a periodical three-dimensional pattern having the same composition but different structures is called polymorphism. In lipids there are three dominant crystal forms namely: α, β′, and β polymorphs which greatly influence the physical properties and processing of the final product (Ribeiro and others 2009). The type of crystal formed depends on the acyl groups of TAGs. Fats containing highly diverse TAG profiles or greater FA chain-length

diversity tend to crystallize in the β′ form, whereas fats with little TAG diversity crystallize in the β form. Interesterification alters the TAG composition, leading to modifications in the crystalline morphology of the fats. Fats containing predominantly β′ TAG crystals impart smooth texture and mouthfeel to margarine since they are softer and allow for good aeration and creaming properties, whereas those with predominantly β TAG crystals impart grainy texture (Ribeiro and others 2009). Both SL and EF had β′ crystal as the dominant polymorph (data not shown).

Oxidative stability and tocopherols

The tocopherols content and oxidative stability of the samples are given in Table 4.3. Oxidative stability is an important characteristic of the product which determines its shelf life and flavor. The oxidative stability of fats and oils depends on the FA profile and amount of indigenous antioxidant present. SFAs are less prone to oxidative deterioration compared to UFAs. Tocopherols (T) and tocotrienols (T3) are collectively known as vitamin E. γ-T was the major vitamin E in SDASO (867.4 ppm) and HSSO (755.8 ppm). γ-T was also the predominant vitamin E homologue present in SL (1126.3 ppm) and EF (931.6 ppm). No T3 was detected in SDASO, HSSO, and SL whereas 26.4 ppm δ-T3 was found in EF. EF had slightly higher OSI value (15.4) than SL (13.8 h). This may be due higher SFA content in EF and higher tocopherols content (1632.2 ppm) than SL (1511.4 ppm). After SPD, a loss of 53.9% was observed in the total tocopherol content of SL. α-, β-, γ-, and δ-T decreased by 57.4, 63.6, 52.6, and 57.5%, respectively. The SPD waste was also analyzed for tocopherols. It contained 126.5, 0.7, 1220.7, and 375.9 ppm α-, β-, γ-, and δ-T, respectively. As per mass balance, the percent of α-, β-, γ-, and δ-T in the waste was 95.7, 100, 97.7, and 97.1%, respectively. The total percent of tocopherols

in the waste was 97.4%. The remaining 2.6% could be tocopheryl esters as tocopherols can be lost during SL production (Hamam and Shahidi 2006). For confirmation, TLC was done to separate the tocopheryl esters and further analyze them. Silica gel plates were used after drying at 80 °C for 1 h. The developing solvent was hexane/diethyl ether/formic acid (90:10;1, v/v/v). A mix of tocopheryl linoleate and tocopheryl oleate (Tri-K Industries, Inc., Northvale, NJ) was used as standard. No tocopheryl ester band was observed in the waste sample. This indicates that during interesterification reaction of SDASO and HSSO, the tocopherols were lost mainly as free tocopherols and not as esters as in the case of acidolysis (Hamam and Shahidi 2006). This may be because the FFA content of the product before interesterification and after SPD were 0.2 and 0.01%, respectively, which is very low. Therefore, the remaining 2.6% could be the tocopherols that remained on the short-path distillator and were not fully recovered in the waste. Also, some tocopherols may be lost due to the heat or oxidized during short-path distillation.

Textural, rheological, and sensory analyses

Texture is a sensory attribute related to consumers' preference and liking for a product. The texture of margarine depends on the FA composition, crystal morphology and network, and the processing and storage conditions (Bourne 1978). Hardness was measured as the maximum force during the first compression. The higher the hardness value of a margarine, the more difficult it is to spread. Significant difference ($P < 0.05$) was found between SLM and RCM in terms of hardness (Fig. 4.3). SLM was less hard than RCM implying that it will be easy to spread. Although SL and EF have similar SFA (~31 mol%), this difference in their hardness may be due their different FAs (SL had 10.5 mol% SDA while EF had higher oleic acid 42.1 mol%), TAG species (SL had more UUU

and SUU type TAGs than EF) and/or due to their different SFC. SL also had lower SFA (21.0 mol%) at *sn*-2 position compared to EF (27.7 mol%) which may also affect the overall texture of the product. The negative force area for the first compression was reported as adhesiveness. Cohesiveness is the ratio of the positive force area during the second compression to that of the first compression. Cohesiveness relates to how crumbly or brittle a food product is, while adhesiveness relates to the stickiness of food samples. The spreadibility of margarine is affected to different extents by adhesiveness and cohesiveness. No significant difference ($P > 0.05$) was found between SLM and RCM for adhesiveness and cohesiveness suggesting that these two margarines will have similar mouthfeel (Fig. 4.3).

Rheology is the study of deformation and flow of matter under the influence of applied stress and strain (Borwankar 1992). The rheological properties of SLM and RCM are shown in Fig. 4.4. In stress viscometry, stress is applied stepwise to margarine and viscosity is calculated. Samples showing high viscosities at low stresses would be more difficult to spread. SLM had lower viscosity at low stress than RCM, meaning that it will be much easier to spread than RCM (Fig. 4.4a). In creep analysis, a constant low stress is applied to samples and deformation per unit time measured as an indicator of emulsion stability. The lower the degree of deformation, the less likely the margarine will show syneresis. RCM showed lower deformation with time meaning that it is less likely to show syneresis. SLM due to its soft texture and low SFC had more deformation (Fig. 4.4b). In dynamic analysis, margarine samples are subjected to a sinusoidally varying stress and storage modulus (G′) and loss modulus (G′′) are measured. The frequency (Hz) at which G’ and G” cross over is a measure of spreadibility. Spreadibility of margarine is

directly proportional to the crossover frequency (Bohlin application note). In Fig. 4.4c, SLM has slightly higher cross over frequency (6.8 Hz) than RCM (5.0 Hz) meaning that SLM will be easier to spread. This is in accord with the textural analysis (Fig. 4.3).

Sensory evaluation is the connecting link between product attributes and consumer acceptance. A triangle test was used to discriminate between SLM and RCM. No significant difference ($P > 0.05$) was achieved between SLM and RCM (data not shown).

Microstructural morphology

Fig. 4.5 shows the microstructural morphology of SLM and RCM. The main structural component of plastic fats is the crystalline network which can be studied by PLM. Attributes such as spreadibility and the melting sensation depend on the mechanical strength of the crystalline network. The aqueous phase is isotropic in nature and appears as dark phase under PLM. On the other hand, the lipid phase is anisotropic with defined molecular orientation and varying optical properties and appears slightly brilliant under PLM (Ribeiro and others 2009). Both individual crystals and crystal aggregates were observed in SLM. SLM had similar uniformly dispersed, sharp, and small crystals as that of RCM, implying that it will have a smooth and easily spreadable texture.

Enzyme reusability

The enzyme, Lipozyme TLIM lipase, was reused ten times and total and *sn*-2 stearic acid (mol%) and SDA (mol%) were analyzed as the main response (Fig. 4.6). The yield% after each SPD was also calculated. The total incorporation of stearic acid (~15.0 mol%) and total SDA content (~10.2 mol%) of SL did not change up to the tenth run. However,

a different trend was observed for *sn*-2 position. After the seventh run SDA content at *sn*-2 position decreased and continued to decrease till the last run whereas the stearic acid at *sn*-2 position increased after the seventh run. This may be due to the effect of heat on the activity and specificity of the enzyme. As the number of runs increased, the enzyme was exposed to more heat (during reaction) and solvent (hexane during cleaning). This might have affected the *sn*-1,3 specific nature of the enzyme leading to cleavage of *sn*-2 ester bond, acyl migration, and thereby decreasing the amount of SDA esterified to it. The yield % after the first run was 90.6% which remained unchanged till the ninth run. After the tenth run the yield % decreased slightly to 88.8%. This may be due to the enzyme immobilization carrier properties. Lipozyme TLIM was immobilized on silica gel which is highly polar and does not absorb much oil. Furthermore, washing with hexane (non-polar) removed any residual oil on the immobilized enzymes, thereby maintaining its efficiency.

CONCLUSIONS

In this study, we were able to formulate a *trans*-free margarine containing n-3 FA using enzymatically synthesized SL. The margarine had low n-6/n-3 ratio and was easily spreadable. This margarine was comparable to commercial margarine in terms of melting temperature, SFA, UFA, and sensory properties. It also had lower SFC and hardness than the reformulated commercial margarine. As high intake of TFA is correlated with increased risk of several chronic diseases and n-3 FAs are positively associated with prevention of cardiovascular diseases and cancer, the margarine produced in this research can be a healthy alternative to commercial margarines.

ACKNOWLEDGMENTS

We would like to thank Dr. Alejandro G. Marangoni (University of Guelph, Canada) for his assistance with the solid fat content analysis. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2009-65503-05734 from the USDA National Institute of Food and Agriculture.

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Table 4.1 Total and positional fatty acid profile of structured lipid and commercial margarine fat (mol%)

Fatty acid	Total		*sn*-2		*sn*-1,3	
	SL[a]	EF[b]	SL	EF	SL	EF
14:0	nd	1.0±1.1	nd[c]	nd	nd	1.5±1.1
16:0	14.1±1.1a	24.8±1.9b	12.8±1.3a	23.1±2.0b	14.8±1.3a	25.7±2.3b
16:1n-7	0.1±0.0a	0.1±0.0a	nd	nd	0.2±0.0a	0.2±0.0a
18:0	15.1±1.2a	4.8±1.0b	8.2±0.9a	4.6±1.1b	18.6±1.9a	4.9±1.2b
18:1n-9*c*	25.8±2.3a	42.1±3.2b	15.0±1.6a	28.2±2.2b	31.2±2.5a	49.1±4.1b
18:2n-6*c*	18.9±1.4a	20.3±2.0b	45.8±3.8a	41.3±3.4b	5.5±1.7a	9.8±0.8b
20:0	0.9±0.0a	0.3±0.0b	nd	nd	1.4±1.0a	0.5±0.0b
18:3n-6	2.6±1.1a	1.8±0.6b	3.8±0.9	nd	2.0±1.2a	2.7±1.0b
20:1n-9	0.6±0.0a	0.5±0.0a	nd	nd	0.9±0.0a	0.8±0.0a
18:3n-3	9.9±0.6a	3.8±0.6b	5.5±1.2a	2.8±1.0b	12.1±1.7a	4.3±1.4b
21:0	0.1±0.0a	0.1±0.0a	nd	nd	0.2±0.0a	0.2±0.0a
18:4n-3	10.5±1.0	nd	9.8±1.6	nd	10.9±1.3	nd
22:0	0.7±0.0a	0.4±0.0b	nd	nd	1.1±0.1a	0.6±0.0b
20:3n-3	nd	0.1±0.0	nd	nd	nd	0.2±0.0
20:5n-3	nd	0.2±0.0	nd	nd	nd	0.3±0.0
n-6/n-3	1.1	5.8	3.2	14.8	0.3	2.9

[a]SL, large-scale Lipozyme TLIM-catalyzed structured lipid. [b]EF, extracted fat from commercial margarine. [c]nd, not determined. Each value is the mean of triplicates ± standard deviation. Values with the same letter in each row within total, *sn*-2, and *sn*-1,3 columns separately are not significantly different at $P < 0.05$.

Table 4.2. Relative percent of triacylglycerol (TAG) molecular species of structured lipid and commercial margarine fat (%)

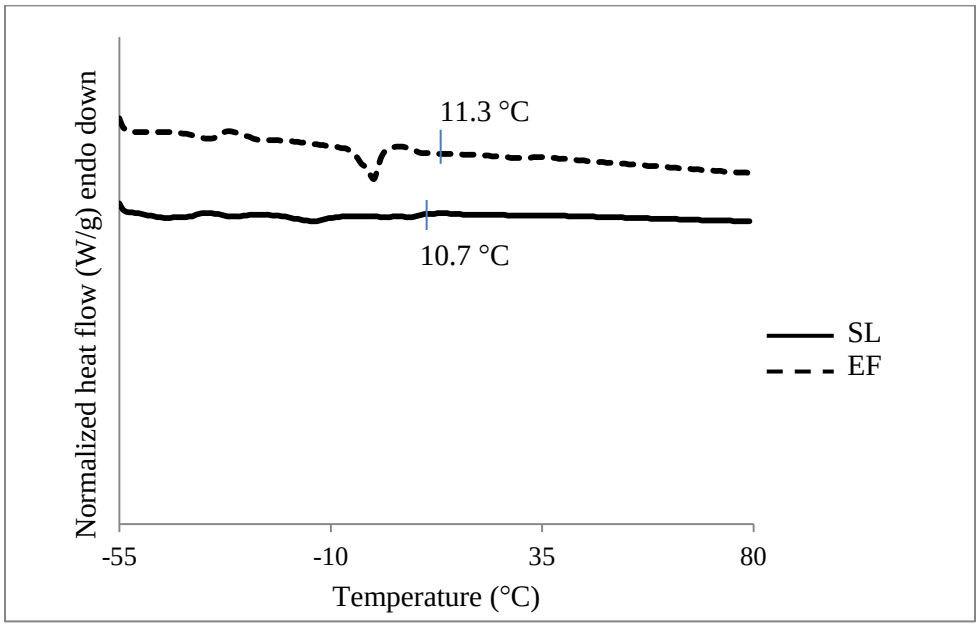
TAG species	SL[a]	EF[b]
StStSt	1.1±0.4	nd[c]
StLnSt	2.6±0.9	nd
StLnLn	2.4±0.5	nd
StOSt/LnGSt	2.6±0.2	nd
LnLnLn	1.7±0.3a	0.4±0.0b
LnLnL	7.7±1.1a	1.4±0.1b
LnLnO/LLLn	5.0±1.0a	3.8±0.9b
StOO	3.9±1.2	nd
LnOO	nd	6.2±1.3
OLLn	2.5±0.7	nd
LLL	12.4±1.9a	7.4±1.1b
LnGS	2.6±0.7a	1.2±0.0b
LnLP	3.4±0.9a	5.6±1.2b
OLL	10.7±1.6a	7.1±1.4b
PLL	12.2±1.7a	4.4±0.5b
OOL	1.1±0.0a	4.6±0.8b
SLL	1.6±0.1a	5.2±0.4b
POL	14.4±1.3a	8.0±0.9b
PPL	2.4±0.0a	2.3±0.0a
OOO	nd	10.3±1.3
POO	nd	14.4±1.2
POP	nd	12.5±1.0
PPP	nd	1.8±0.0
SOL	5.5±0.7	nd
PSL	2.4±1.2	nd
SOO	0.1±0.0a	2.3±0.3b
SOS	1.6±0.1a	1.5±0.4a

[a]SL, large-scale Lipozyme TLIM-catalyzed structured lipid. [b]EF, extracted fat from commercial margarine. [c]nd, not determined. P is palmitic, S is stearic, O is oleic, L is linoleic, Ln is linolenic, G is γ-linolenic, St is stearidonic acid. Each value is the mean of triplicates ± standard deviation. Values with the same letter in each row are not significantly different at $P < 0.05$.

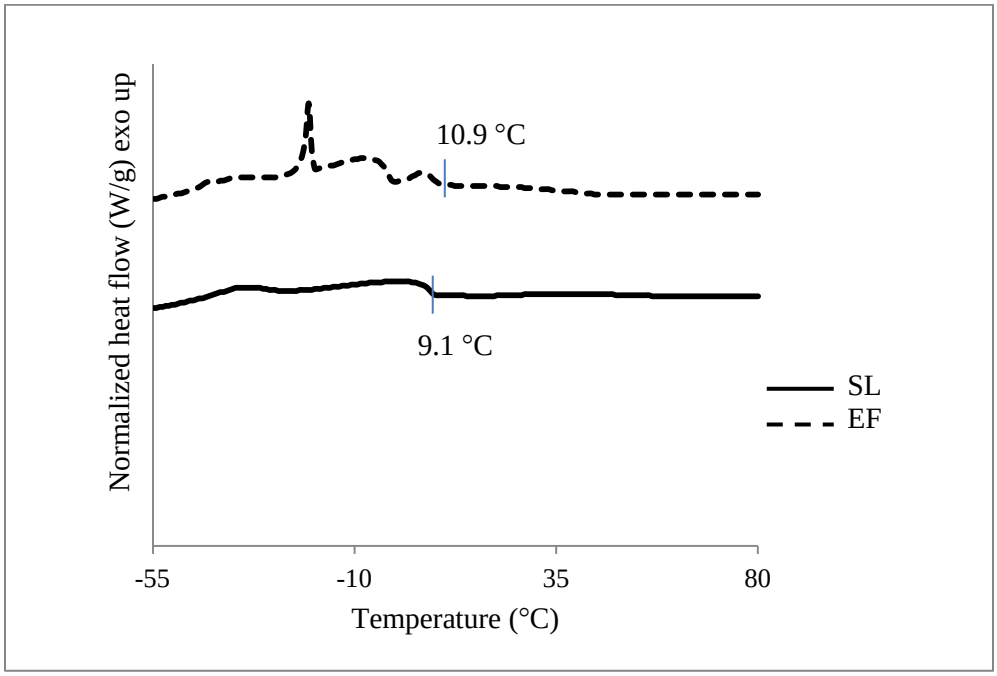
Table 4.3 Tocopherol content (ppm) and oxidative stability index (OSI) (h) of substrates, large-scale SL, and extracted commercial fat

	Tocopherol content							OSI at 110 °C
	α-T[a]	α-T3[b]	β-T	γ-T	γ-T3	δ-T	δ-T3	
SDASO[c]	82.6±4.9	nd[d]	1.1±0.9	867.4±8.3	nd	286.9±7.3	nd	6.5±1.1
HSSO[e]	74.8±3.1	nd	nd	755.8±5.2	nd	194.7±9.4	nd	44.2±2.3
Before SPD[f]	230.2±12.6	nd	1.1±0.4	2376.3±11.7	nd	67.3±9.8	nd	
SL[g](after SPD)	98.3±9.1	nd	0.4±0.0	1126.3±10.8	nd	286.4±9.7	nd	13.8±1.3
SPD waste	126.5±3.5	nd	0.7±0.0	1220.7±5.7	nd	375.9±4.	nd	
EF[h]	130.1±6.8	82.6±3.3	8.3±1.2	931.6±8.7	106.8±7.4	346.4±8.8	26.4±3.2	15.4±1.0

Each value is the mean of triplicates ± standard deviation
[a]T, tocopherols. [b]T3, tocotrienols. [c] SDASO, stearidonic acid soybean oil. [d]nd, not detected. [e]HSSO, high stearate soybean oil. [f]SPD, short-path distillation. [g]SL, large-scale Lipozyme TLIM-catalyzed structured lipid. [h]EF, extracted fat from commercial margarine



(a)



(b)

Fig. 4.1 Melting (a) and crystallization (b) thermograms of large-scale structured lipid (SL) and extracted fat from commercial margarine (EF).

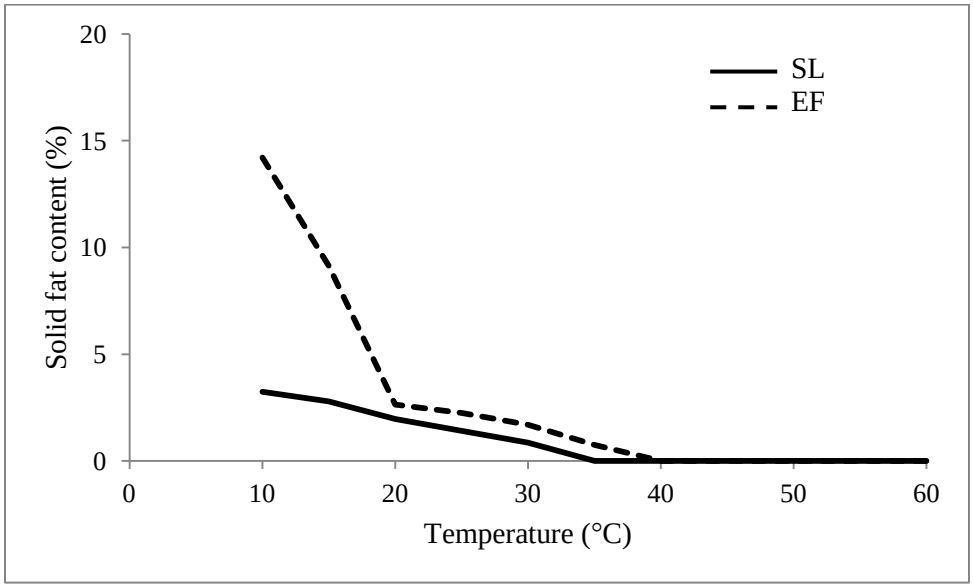


Fig. 4.2 Solid fat content (%) of large-scale structured lipid (SL), and extracted fat from commercial margarine (EF).

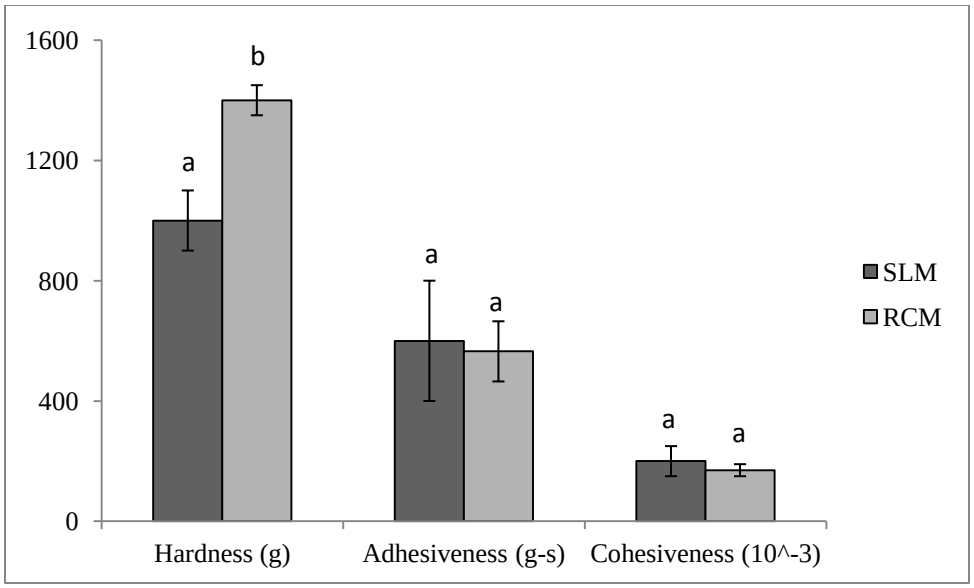
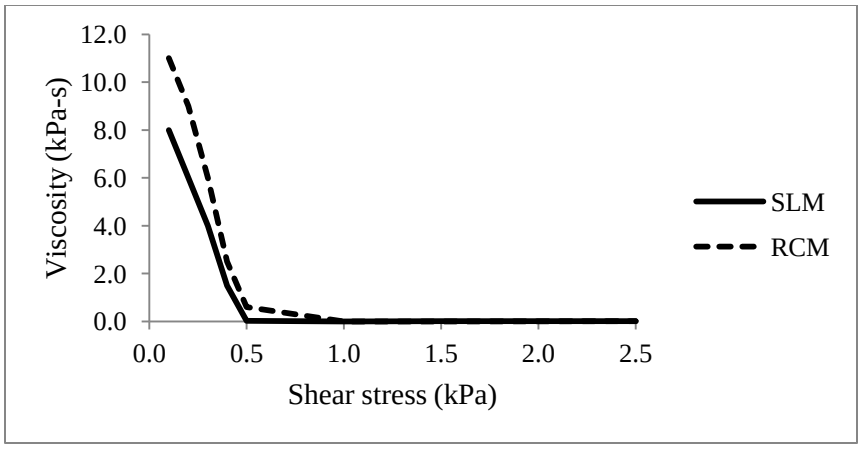
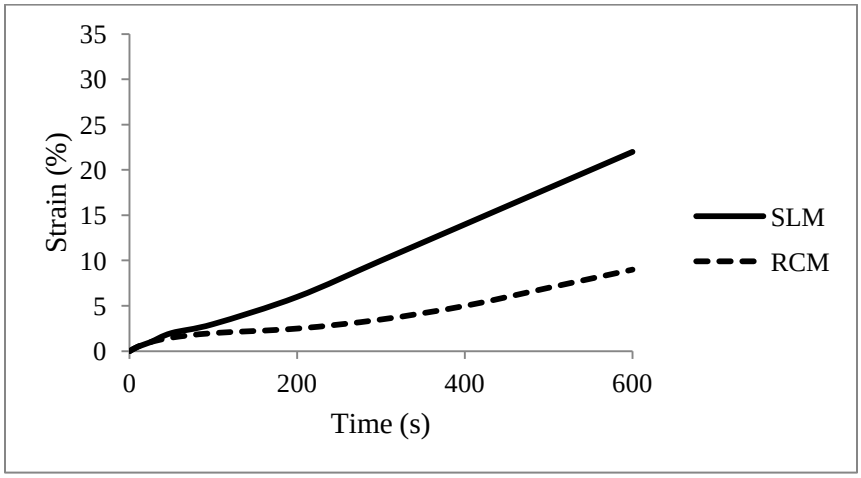


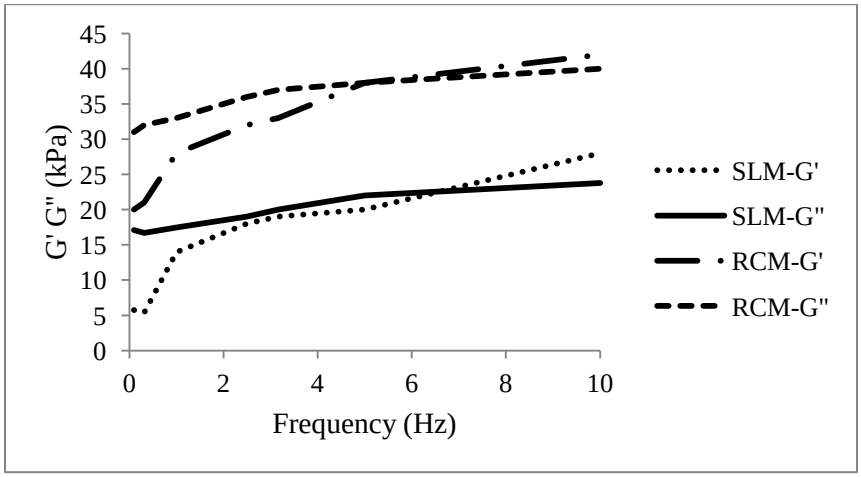
Fig. 4.3 Texture profile analysis of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM). Each value is the mean of triplicates ± standard deviation. Columns with the same letter within each texture attribute are not significantly different at $P < 0.05$.



(a)



(b)



(c)

Fig. 4.4. Stress viscometry (a), creep analysis (b), and dynamic analysis (c) of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM).

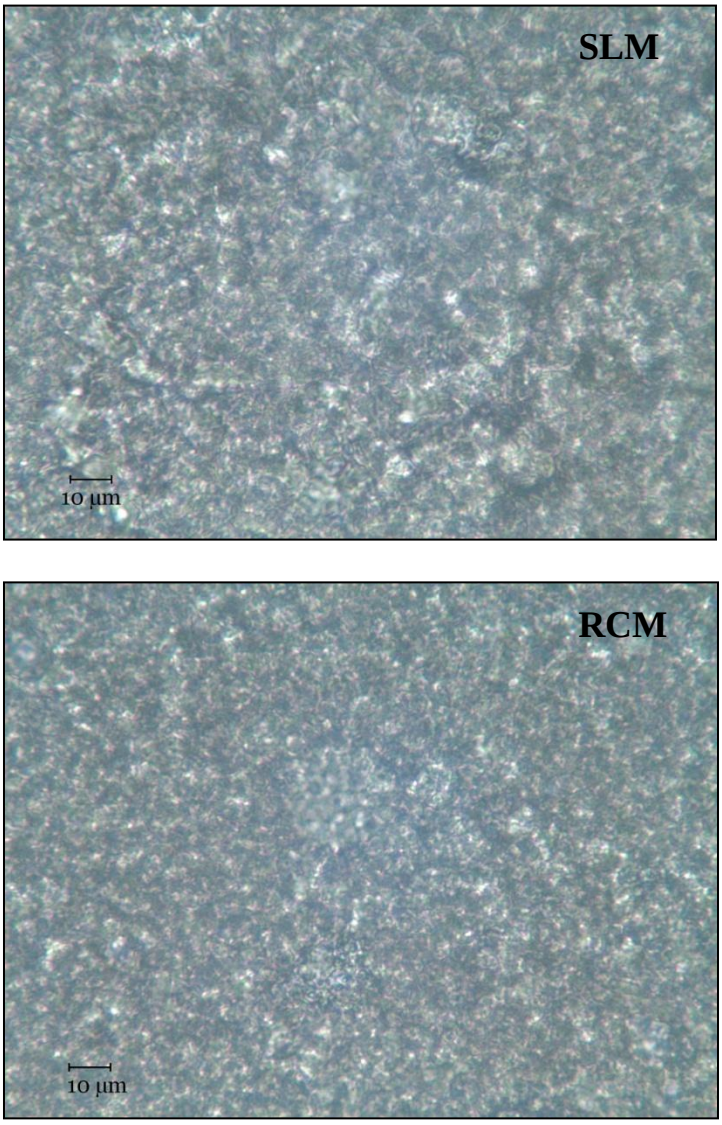


Fig. 4.5 Morphology of fat crystals of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM).

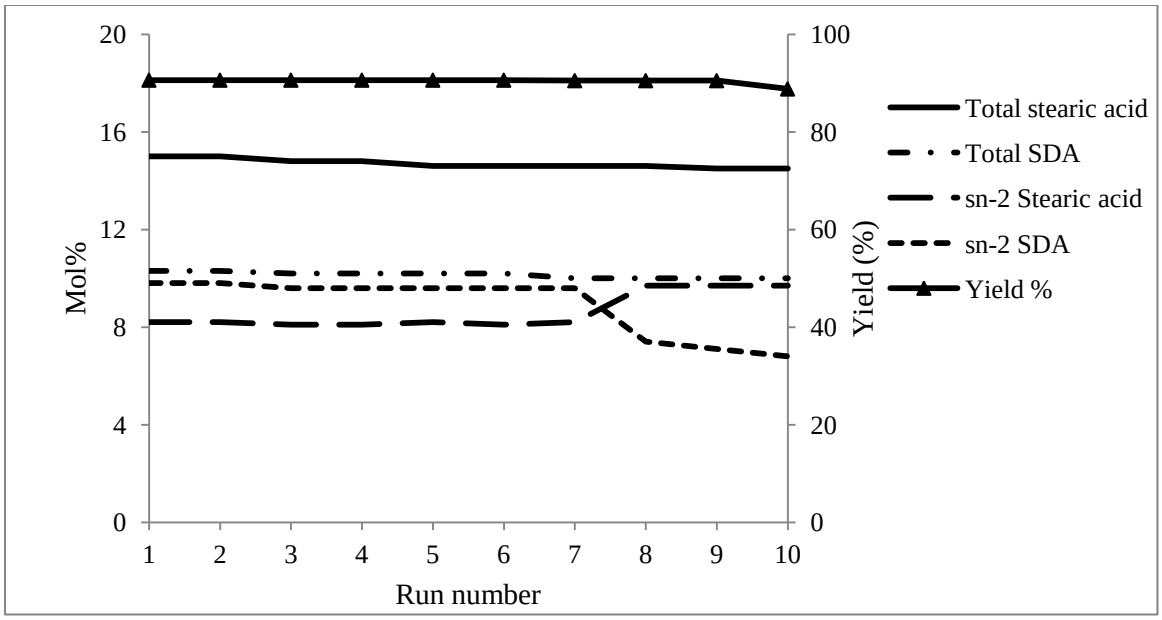


Fig. 4.6 Mol% stearic acid incorporation and SDA content (primary y-axis) and yield% (secondary y-axis) of structured lipid as determining factor of Lipozyme TLIM lipase reusability.

CHAPTER 5

ENZYMATIC SYNTHESIS OF *TRANS*-FREE STRUCTURED MARGARINE FAT ANALOGUES WITH HIGH STEARATE SOYBEAN OIL AND PALM STEARIN AND THEIR CHARACTERIZATION[1]

[1]Pande, G. and Casimir C. Akoh. Submitted to *LWT-Food Science and Technology*, 8th February 2012

ABSTRACT

High intake of *trans* fat is associated with several chronic diseases such as cardiovascular disease and cancer. Enzymatic synthesis of *trans*-free structured margarine fat analogues from high stearate soybean oil (HSSO) and palm stearin (PS) was optimized using response surface methodology (RSM). The independent variables considered for the design were substrate molar ratio (SR, PS:HSSO, 2-5), temperature (50-65 °C), time (6-22 h), and enzymes (Lipozyme® TLIM and Novozym® 435). The response was stearic acid incorporation. All linear parameters had a negative effect on stearic acid incorporation except Novozym® 435. Time was not significant but its interaction terms with temperature and SR had significant effect on the response. Desirable structured lipids (SL) containing 11.2 and 8.9 g/100 g stearic acid were obtained at 50 °C, 20 h, 2:1 SR with Novozym 435 (SL1) and 57 °C, 6.5 h, 2:1 SR with Lipozyme TLIM (SL2), respectively. Using optimal conditions, SLs were synthesized in 1 L stir-batch reactor and characterized for fatty acid profile, triacylglycerol species, polymorphism, thermal behavior, and solid fat content. The yield for SL1 and SL2 were 87.3 and 94.8%, respectively. Novozym 435-catalyzed SL had desirable fatty acid profile, physical properties, and suitable β' polymorph for margarine formulation.

KEYWORDS

Enzymatic synthesis; high stearate soybean oil; margarine fat analogues; palm stearin; response surface methodology; structured lipid

INTRODUCTION

trans Fatty acids (TFAs) are unsaturated fatty acids with at least one double bond in a *trans* configuration. This results in a greater bond angle than that of *cis*-unsaturated double-bond-containing fatty acids and a more extended fatty acid carbon chain similar to saturated fatty acids (Filip, Fink, Hribar, & Vidrih, 2010). Partial hydrogenation, a common industrial process for solidifying oils, results in isomerization and migration of double bonds leading to formation of TFA. Intake of high amounts of TFA has been positively correlated with increased risk of coronary heart disease (Hu *et al*., 1997), inflammation (Mozaffarian *et al*., 2004), and cancer (Astorg, 2005). Studies have shown that TFA increases LDL, decreases HDL, and increases total: HDL cholesterol (Mozaffarian & Clarke, 2009). The mean daily intake of TFA per person in the United States is 3–4 g (Filip, Fink, Hribar, & Vidrih, 2010). Dietary Guidelines for Americans 2010 recommends dietary TFA intake should be as low as possible (Dietary Guidelines for Americans, 2010). As of January 1, 2006, food manufacturers have been required by the United States Food and Drug Administration (FDA) to list *trans* fats on food labels (FDA Federal Register, 2003). This has led the food industry to seek alternative processes to produce cost effective zero or reduced *trans* fat foods with acceptable functional properties. One such potential alternative is interesterification.

Interesterification is a chemically or enzymatically catalyzed ester exchange reaction between two acyl groups, thereby altering the overall chemical composition and physical properties of the interesterified fats. Although chemical interesterification involves lower cost and is easily scalable, it lacks specificity. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) from various organisms have been successfully used as

biocatalysts in the presence or absence of organic solvents for lipid modification (Mukherjee, 2000). Lipases are preferred over chemical interesterification because they are regio-and stereo-specific and offer a better control over the final product (Marangoni & Rousseau, 1995). Structured lipids (SLs) are defined as triacylglycerols (TAGs) that have been structurally modified either by incorporation of new fatty acids or changing the position of existing fatty acids to yield novel TAGs of desired physical, chemical, and nutritional properties (Fomuso & Akoh, 1996).

High stearate soybean oil (HSSO) was developed to contain elevated levels of stearic acid for increased stability when used in many types of foods that require solid fat functionality. Palm stearin (PS) is an important co-product obtained during palm oil fractionation. It is the harder fraction of palm oil containing higher proportion of saturated fatty acids and a natural source of solid fat. It is extensively used in the production of *trans*-free margarines and shortenings. The first objective of this research was to optimize the reaction conditions for enzymatic syntheses of *trans*-free margarine fat analogues using response surface methodology (RSM). The second objective was to scale-up the syntheses of the SLs, characterize and compare them with physical blends (PBs).

MATERIALS AND METHODS

Materials

High stearate soybean oil was kindly provided by Monsanto Company (St. Louis, MO). Palm stearin was kindly provided by Cargill Inc. (Minneapolis, MN). The immobilized enzymes, Lipozyme® TLIM (*Thermomyces lanuginosus* lipase, *sn*-1,3 specific, specific activity 250 IUN/g: IUN=Interesterification Unit) and Novozym® 435 (*Candida*

antarctica lipase, non-specific, specific activity 10,000 PLU/g: PLU=Propyl Laurate Unit) were purchased from Novozymes North America Inc. (Franklinton, NC). Supelco 37 FAME mix and 14% boron trifluoride in methanol were purchased from Sigma Chemical Co. (St. Louis, MO). Nonadecanoic acid and its methyl ester were purchased from TCI America (Portland, OR). Stearidonic acid and its methyl ester were purchased from Cayman Chemical Company (Ann Arbor, MI). Organic solvents and chemicals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA).

Experimental design for response surface methodology (RSM)

RSM is an important statistical technique for the development, improvement and optimization of processes. It is an efficient method to evaluate the effects of multiple variables and their interaction effects on the response using a reduced number of experiments (Huang & Akoh, 1996). Four independent variables were studied namely: time (6, 10, 14, 18, 22 h), temperature (50, 55, 60, 65 °C), substrate molar ratio (PS:HSSO, 2, 3, 4, 5), and two enzymes (Lipozyme TLIM and Novozym 435), for stearic acid incorporation as response. RSM was done using Modde 9.0 software (Umetrics, Umeå, Sweden) to obtain the relationship between the response and the independent variables. Twenty six runs were generated and experiments at each design point were randomly performed in triplicate. The data obtained from the design in Table 5.1 were used to fit a second-order polynomial function as follows:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i \; + \; \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i \; X_j \; + \; \varepsilon_{ij} \qquad (1)$$

where Y = the response; β_0 = constant; β_i = linear (first-order model); β_{ii} = quadratic (second-order model); β_{ij} = interaction term coefficients; X_i and X_j = independent variables; and ε_{ij} = error term.

Small-scale SL synthesis

SL synthesis was performed in screw-cap test tubes as per the conditions generated by RSM. Briefly, 100 mg of the HSSO was weighed into the test tube and PS was added according to the determined respective molar ratios. 2 mL hexane was added and the substrates were thoroughly mixed while gently warming the test tubes. Specific enzymes were added at 10% total substrate weight. Test tubes were then incubated in water bath shaker at 200 rpm at time-temperature combinations generated by RSM. Reaction was stopped and the product filtered through anhydrous sodium sulfate column to remove the enzymes. Pure TAG bands were collected after silica gel TLC using petroleum ether:ethyl ether:acetic acid (80:20:0.5, v/v/v) as developing solvent system. TAG bands were analyzed for FA profile after conversion to methyl esters. All reactions were performed and analyzed in triplicates and average values reported.

Fatty acid profile

Lipid samples were converted to fatty acid methyl esters following the AOAC Official Method 996.01 (AOAC, 1998) and analyzed with Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) using Supelco SP-2560, 100 m x 25 mm x 0.2 μm column. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. Injection volume was 1 μL and a spilt ratio of 20:1 was used. The column was initially held at 140 ºC for 5 min, then increased to 240 ºC at 4 ºC/min, and finally held at

240 ºC for 25 min. Detection was with flame ionization detector at 300 ºC. All samples were analyzed in triplicates and average values reported.

Large-scale SL synthesis

The scaled-up reactions using the optimal conditions achieved from the small-scale reactions were performed in a stir-batch reactor. Two SL were prepared: SL1 at 50 °C, 20 h, 2:1, using Novozym 435 and SL2 at 57 °C, 6.5 h, 2:1, using Lipozyme TLIM. Corresponding physical blends (PB1 and PB2) were also prepared at the same reaction conditions without the respective enzymes. Free fatty acids (FFAs) were removed by short-path distillation KDL-4 unit (UIC, Joliet, IL) at <0.2 mmHg and flow rate 10 mL/min. FFAs were determined by AOCS Official Method Ac 5-41 (AOCS, 2009).

Fatty acid and positional analysis

Fatty acids were determined as described earlier. *sn*-2 Positional fatty acid composition was determined following the AOCS Official Method Ch 3-9 (AOCS, 2009). All samples were analyzed in triplicates and average values reported.

Triacylglycerol (TAG) molecular species

The TAG composition was determined with a nonaqueous reverse phase HPLC (Agilent Technologies 1260 Infinity, Santa Clara, CA) equipped with a Sedex 85 ELSD (Richard Scientific, Novato, CA). The column was Beckman Ultrasphere® C18, 5 μm, 4.6 x 250 mm with temperature set at 30 °C. The injection volume was 20 μL. The mobile phase at a flow rate of 1 mL/min consisted of solvent A, acetonitrile and solvent B, acetone. A gradient elution was used starting with 35% solvent A to 5% solvent A at 45 min and then returning to the original composition in 5 min. Drift tube temperature was set at 70 °C, pressure at 3.0 bar and gain at 8. The samples were dissolved in chloroform with

final concentration of 5 mg/mL. The TAG peaks were identified by comparison of retention times with those of the standards and also by equivalent carbon number (ECN). ECN is defined as CN – 2n, where CN is the number of carbons in the TAG (excluding the three in the glycerol backbone) and n is the number of double bonds. Triplicate determinations were made and averaged.

Differential scanning calorimetry (DSC)

Melting and crystallization profiles of lipid samples were determined with Perkin-Elmer model DSC7 (Norwalk, CT) following AOCS Official Method Cj 1-94 (AOCS, 2009). 8-12 mg samples were weighed into aluminum pans and sealed. Normal standardization was performed with n-decane and indium. Samples were rapidly heated to 80˚C at 50˚C /min, and held for 10 min. The samples were then cooled to -55˚C at 10˚C/min, and held for 30 min and finally heated to 80˚C at 5˚C/min. All samples were analyzed in triplicates and average values reported.

Polymorphism

The polymorphic forms of the samples were determined with Bruker D8 Advance diffractometer (Bruker AXS, Inc., Madison, WI). The diffractometer had a 2θ configuration (2θ range used was 20-32°), a solid state detector, and cobalt tube as the X-ray source. Corundum was used as standard. The scan rate was 4.2°/min with a step increment of 0.01°. The samples were rotated at 15 rev/min. Samples were melted and poured into rectangular aluminum molds. They were held at room temperature for 2-3 h, at 4 °C for 4-5 h, and then stored at -20 °C overnight. The sample molds were placed over custom-designed aluminum holders containing dry ice to prevent melting of the sample during analysis. Short spacings of the major polymorphs were identified as

follows: α, a single spacing at 4.15 Å; β', two strong spacings at 3.8 and 4.2 Å; and β, a very strong spacing at 4.6 and another one usually at 3.85 Å (Ribeiro, Basso, Grimaldi, Giovelli, & Gonçalves, 2009). All samples were analyzed in triplicates and average values reported.

Solid fat content (SFC)

SFC was determined according to the AOCS Official Method Cd 16-81 (AOCS, 2009) on a BrukerPC/20 Series NMR analyzer, Minispec (Bruker Optics, Milton, On, Canada). Samples were tempered at 100˚C for 15 min and then kept at 60˚C for 10 min, followed by 0˚C for 60 min and finally for 30 min at each temperature of measurement. SFC was measured at intervals of 5˚C from 5 up to 60 ˚C. All samples were analyzed in duplicates and average values reported.

Statistical analysis

Regression analysis, response surfaces, and statistical significance were performed using Modde 9.0 software (Umetrics, Umea, Sweden) and backward elimination by Statistical Analysis System software (SAS 9.2, Cary, NC). SAS was also used to determine significant difference ($P \leq 0.05$.) between SLs and PBs. All samples, except SFC, were analyzed in triplicates and average values reported.

RESULTS AND DISCUSSION

Model fitting and verification

The incorporation amounts of stearic acid in the *trans*-free margarine fat analogues ranged from 8.1 – 11.3 g/100 g (Table 5.1). Quadratic model was obtained for the incorporation of stearic acid of SLs by multiple linear regression and backward elimination. The regression coefficients (β) and significance (P) values were calculated

based on the results in Table 5.1. The R^2 value, the fraction of the variation of the response explained by the model, was 0.982 and Q^2, the fraction of the variation of the response that can be predicted by the model, was 0.906. The observed vs. prediction plots had linear distributions for both responses (data not shown). The model for stearic acid incorporation showed a lack of fit ($P < 0.05$) but the *P*-value for multiple regression was <0.001 (data not shown). The model equation after backward elimination at $\alpha = 0.05$ can therefore be written as:

Stearic acid incorporation = 8.81 - 0.15Temp - 0.70SR ± 0.25Enz + 0.30Temp*Temp + 0.10Temp*Time + 0.25Temp*SR - 0.36Time*SR ± 0.25SR*Enz (2)

where, Temp is temperature in °C, Time in hours, SR is substrate molar ratio, Enz is enzyme.

To verify the model, five regions from the contour plot were randomly chosen to perform experiments using the conditions specified for these regions. A chi-squared value of 1.136 was obtained which is much smaller than the cutoff point (9.488) at α=0.05 and DF 4 indicating no significant difference between the observed and predicted values.

Effect of parameters

The reaction temperature has a dual effect on the rate of reaction. As temperature is increased the rate of collisions between reactants and the enzyme also accelerates, thus resulting in increased acyl exchange. The elevated temperature is one of the most common causes of enzyme inactivation, thereby producing less incorporation. The overall effect of temperature is also affected by other factors such as the amount and type of substrate used, and presence of water in the reaction. Also, substrate molar ratio is an important factor to obtain the highest product yield and the lowest side reaction (Kim *et*

al., 2004). Temperature, substrate molar ratio (SR), and Lipozyme TLIM had a negative effect whereas Novozym 435 had a positive effect on stearic acid incorporation. Time was not significant as a linear parameter but its interaction term with temperature had a positive effect and with SR it had a negative effect. The effect of interaction terms are shown in Fig.5.1. As shown in Fig.5.1a, at low temperature, low time (6 h) had higher incorporation but as temperature was increased high time (22 h) had higher incorporation. Significant difference in incorporation was found between low SR (2) and high SR (5) at low temperature but as temperature increased this difference decreased (Fig.5.1b). Fig. 5.1d shows the effect of interaction of time and SR on the response. As time increased, incorporation increased with low SR (2) and decreased with high SR (5). At low SR (2) Novozym 435 resulted in higher incorporation whereas at high SR (5) no difference was found between the two enzymes (Fig. 5.1f). Novozym 435 always had higher incorporation than Lipozyme TLIM at all temperatures and times (Figs. 5.1c and 1e).

Optimization of the reaction

Contour plots help in the optimization of reactions by identifying parameter combinations that will produce a desired response. Contour plots were generated by Modde 9 software and shown in Fig. 5.2. The reaction parameter with the greatest effect was kept on the y-axis (substrate molar ratio), the second was placed on the x-axis (temperature), and the one with the least effect was held constant (time). As the response is a factor of a complex relationship of first order, second order variables, and interactions among them, several combinations of the factors are possible to obtain desired results (Mu, Xu, Adler-Nissen, & Høy, 1999). Economic consideration such as cost of enzymes is an important factor in optimization of the reaction parameters. Based on the predicted desirable

composition of 11.2 and 8.9 g/100 g stearic acid, SL1 was synthesized at 50 °C, 20 h, 2:1, using Novozym 435 and SL2 at 57 °C, 6.5 h, 2:1, using Lipozyme TLIM, respectively. Corresponding physical blends (PB1 and PB2) were also prepared.

Product yield

Lipase-catalyzed interesterification reactions result in desired SL and other by-products such as FFA, partial acylglycerols, or any undesired product that needs to be removed. Removal of FFA is important as it may cause rancidity and off-flavor in the final product. After purification by short-path distillation, the SLs contained 0.1% FFA which is an acceptable level to be used as ingredients for food products. Yield % was calculated as:

Yield % = (Final product weight)*100/(Total substrate weight) (3)

The yield % for SL1 and SL2 were 87.3 and 94.8%, respectively. The difference in the yield can be due to the different enzymes used and also due to their different carrier material. Novozym 435 which was immobilized on macroporous acrylic resin absorbed more product resulting in lower product recovery. On the other hand, product recovery was higher when using Lipozyme TLIM which was immobilized on granulated silica.

Total and positional fatty acid profile

Fatty acid (FA) compositions of HSSO and PS are given in Table 5.2 and those of SLs and PBs are given in Table 5.3. The type and positional distribution of FA on the glycerol backbone greatly influences the physical behavior and metabolism of dietary fats. Unsaturated FAs (UFAs) are better metabolized and utilized in our body when present at *sn*-2 position (Karupaiah & Sundram, 2007). The major FAs in HSSO were linoleic (50.2 g/100 g), oleic (16.0 g/100 g), and stearic (16.8 g/100 g) acids. The major FAs in PS were palmitic (58.1 g/100 g) and oleic acids (26.9 g/100 g). HSSO and PS contained

0.6 and 0.4 g/100 g TFA, respectively. The major FA at *sn*-2 position of HSSO was linoleic (84.2 g/100 g). No saturated FA was found at *sn*-2 position of HSSO. On the other hand, *sn*-2 position of PS consisted of approximately equal amount of saturated and UFAs. The main FA at *sn*-2 position of PS were palmitic (48.9 g/100 g) and oleic (38.3 g/100 g). Novozym 435 catalyzed SL (SL1) had 12.3 g/100 g stearic acid whereas the physical blend (PB1) had 8.3 g/100 g stearic acid (Table 5.3). For Lipozyme TLIM, the SL (SL2) had 9.5 g/100 g stearic acid while the physical blend (PB2) produced under similar conditions had 8.6 g/100 g stearic acid. The SLs had a wide range of FAs and no *trans* fat. The predominant FAs in SL1 were linoleic (34.6 g/100 g) and palmitic (31.0 g/100 g) and in SL2 were palmitic (38.5 g/100 g) and oleic (25.5 g/100 g). The SLs had higher saturated fat at *sn*-2 position compared to the physical blends because enzymes change the type and position of FAs on the glycerol backbone. Although SL2 was catalyzed by *sn*-1,3 specific Lipozyme TLIM, it had 6.0 g/100 g stearic acid at *sn*-2 position. This may be attributed to acyl migration that can occur during short-path distillation and/or during pancreatic lipase reaction (Xu, Skands, & Alder-Nissen, 2001). It is also possible that temperature, reaction time, and substrates can cause migration of FA from *sn*-1,3 into *sn*-2 positions during interesterification reaction and also affect the specificity of Lipozyme TLIM.

TAG molecular species

Fats containing highly diverse TAG profiles or greater FA chain-length diversity tend to crystallize in the β′ form, the desirable polymorph in margarines, whereas oils with little TAG diversity crystallize in the β form (Ribeiro, Basso, Grimaldi, Giovelli, & Gonçalves, 2009). The TAG molecular species are shown in Fig. 5.3. The major TAG species in

HSSO were LLL (20.3 g/100 g) and SLL (16.6 g/100 g) and in PS were POP (47.3 g/100 g) and PPP (27.0 g/100 g). The main TAG species for SL1 were POP (28.5 g/100 g) and PLP (16.3 g/100 g) whereas in PB1 it was POP (42.8 g/100 g) and PPP (24.6 g/100 g). The predominant TAG species for SL2 were POP (26.6 g/100 g) and POL (17.5 g/100 g) and in PB2 were POP (45.9 g/100 g) and PPP (19.0 g/100 g). The major TAG of HSSO, SLL, decreased to 1.8, 2.0, and 0 g/100 g in SL1, SL2, and PBs, respectively. HSSO was composed of only UUU (triunsaturated) (42.8 g/100 g), SUU (monosaturated-diunsaturated) (43.2 g/100 g), and SUS (disaturated-monounsaturated) (14.1 g/100 g) type TAGs whereas UUU, SUU, SUS, and SSS (trisaturated) type TAGs were present in PS, SLs, and PBs. Relative to HSSO, UUU type TAG decreased 87.8, 94.8, 87.6, and 95.7% in SL1, PB1, SL2, PB2, respectively. An increase of 230.7, 244.3, 220.9, and 281.4% was observed in SL1, PB1, SL2, and PB2, respectively, in SUS type TAGs compared to HSSO. This change in TAG species influences the thermal behavior of the products. Both PBs had higher proportion of SSS and SUS type TAGs and lower proportion of SUU and UUU type TAGs than the SLs. This difference in the type of TAGs between the SLs and PBs affects the physical properties of product. The TAG molecular species of the PBs resembled those of PS whereas the SLs consisted of more diverse TAGs which enhanced crystallization in the β′ form, the desirable polymorph in margarines and spreads.

Thermal behavior

Melting and crystallization profiles of the substrates and products are shown in Figs. 5.4a and 5.4b, respectively. The melting completion temperature (T_{mc}) depends on the amount and type of the TAG molecular species. As UUU type TAGs decrease and SSS type

TAGs increase, T_{mc} also increases. Also, the T_{mc} is inversely proportional to the amount of UFAs present. Among all samples, PS had the least UFAs (34.4 g/100 g) and mainly SSU and SSS type TAGs. Therefore, it had the highest T_{mc} (55.6 °C). HSSO had the most UFAs (71.5 g/100 g) and comprised of mainly SUU and UUU type TAGs, and therefore had the lowest T_{mc} (14.4 °C). The T_{mc} of SL1, SL2, and PBs were 45.4, 47.5, and 55.1 °C, respectively. The T_{mc} of the SLs was lower than the PBs because both SLs had more UUU and SUU type TAGs and less SSS and SSU type TAGs than PBs. Since both SLs were not completely melted at room temperature they can be used in the production of stick margarines. Compared to HSSO, the SLs showed broader peaks indicating a better plastic range which may be desirable for margarine. The crystallization onset temperature (T_{co}) of HSSO and PS were 33.1 and 29.6 °C, respectively. T_{co} also depends on the TAG molecular species as more than 30 g/100 g POP results in slow crystallization which increases susceptibility to post hardening (deMan, deMan, & Blackman, 1989). PS and both PBs have more than 40 g/100 g POP and therefore may have higher tendency for post hardening. T_{co} of SL1, SL2, and PBs were 30.7, 30.3, and 29.2 °C, respectively and were completely crystallized at -10 °C , well below the freezer temperature (-20 °C). SLs have smaller differences between their T_{mc} and T_{co} than PBs which shortens their crystallizing time and may eventually help in margarine production.

Solid fat content

One of the important parameters of margarine is solid fat content (SFC) which is a measure of solid/liquid ratio of a fat at various temperatures. Three useful measurement temperatures are refrigeration, room, and body temperatures which are related to the spreadibility, product stability, and texture and mouthfeel, respectively, of margarines

(Ribeiro, Basso, Grimaldi, Giovelli, & Gonçalves, 2009). The SFC of SLs and PBs are given in Table 5.4. Overall, the PBs exhibited higher SFC than their respective SLs at all temperatures. Since interesterification involves redistribution of fatty acids on TAG structure, it results in compositional changes in SLs. It has been reported that at 25 °C, SFC should be 15-35% for desired spreadibility and texture and more than 10% at 20 °C to avoid oil separation (Rao, Sankar, Sambaiah, & Lokesh, 2001). At 25 °C the SLs had 23.6-25.7% SFC while the PBs had 28.6-30.5% SFC. At 20 °C SLs had 30.9-33.0% SFC and PBs had higher SFC of 35.1-35.5%. Both SLs and PBs met these criteria suggesting that they were suitable for use as stick margarine stock. The SFC of SLs will help facilitate the packaging of such margarine by retaining their shape and maintaining shape at room temperature for a reasonable time.

Polymorphism

The tendency of fatty acids residues to occur in differentiated crystalline forms in a periodical three-dimensional pattern having the same composition but different structure is called polymorphism. In lipids there are three dominant crystal forms namely, α, β′, and β polymorphs which greatly influences the physical properties and processing of the final product (Ribeiro, Basso, Grimaldi, Giovelli, & Gonçalves, 2009). The type of crystal formed depends on the acyl groups of TAGs. Fats containing highly diverse TAG profiles or greater FA chain-length diversity tend to crystallize in the β′ form, whereas fats with little TAG diversity crystallize in the β form. Interesterification alters the TAG composition, leading to modifications in the crystalline morphology of the fats. Fats containing predominantly β′ TAG crystals impart smooth texture or mouth-feel to margarine since they are softer and allow for good aeration and creaming properties,

whereas those with predominantly β TAG crystals impart grainy texture (Ribeiro, Basso, Grimaldi, Giovelli, & Gonçalves, 2009). The polymorphic forms are shown in Table 5.5. Palm stearin is high in C48 TAG, or tripalmitin (PPP) which is β-crystal tending (Miskander, Man, Yusoff, & Rahman, 2005). Both SLs showed the most dominant β' crystal form as they have low PPP (~9 g/100 g) whereas the PBs had higher PPP and therefore less β' crystal.

Both SLs from this study can be utilized in the production of *trans*-free margarine. Novozym 435 synthesized SL (SL1) may be more suitable as margarine fat stock because of its desirable fatty acid content, TAG species, melting temperature, dominant β' polymorph, and solid fat content. The second-order polynomial model developed in this study has strong predictability and reproducibility power. Therefore, our results suggested that the interesterified product (SL1) could be used as an alternative to partially hydrogenated fat but without *trans* fat to formulate *trans*-free foods by the food industry.

ACKNOWLEDGMENTS

We would like to thank Dr. Alejandro G. Marangoni (University of Guelph, Canada) for his assistance with the solid fat content analysis. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2009-65503-05734 from the USDA National Institute of Food and Agriculture.

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FIGURE CAPTIONS

Fig. 5.1a. Effect of interaction of temperature and time on stearic acid incorporation.

Fig. 5.1b. Effect of interaction of temperature and substrate molar ratio (SR) on stearic acid incorporation.

Fig. 5.1c. Effect of interaction of temperature and enzymes (E) (N435, Novozym 435; TLIM, Lipozyme TLIM) on stearic acid incorporation.

Fig. 5.1d. Effect of interaction of time and substrate molar ratio (SR) on stearic acid incorporation.

Fig. 5.1e. Effect of interaction of time and enzymes (E) (N435, Novozym 435; TLIM, Lipozyme TLIM) on stearic acid incorporation.

Fig. 5.1f. Effect of interaction of substrate molar ratio (SR) and enzymes (E) (N435, Novozym 435; TLIM, Lipozyme TLIM) on stearic acid incorporation.

Fig. 5.2a. Contour plots showing the effect of substrate molar ratio and temperature on stearic acid incorporation while keeping the time constant at 6 h using Novozym 435 lipase.

Fig. 5.2b. Contour plots showing the effect of substrate molar ratio and temperature on stearic acid incorporation while keeping the time constant at 22 h using Novozym 435 lipase.

Fig. 5.2c. Contour plots showing the effect of substrate molar ratio and temperature on stearic acid incorporation while keeping the time constant at 6 h using Lipozyme TLIM lipase.

Fig. 5.2d. Contour plots showing the effect of substrate molar ratio and temperature on stearic acid incorporation while keeping the time constant at 22 h using Lipozyme TLIM lipase.

Fig. 5.3a. TAG molecular species of high stearate soybean oil (HSSO). P is palmitic, S is stearic, O is oleic, L is linoleic, Ln is linolenic.

Fig. 5.3b. TAG molecular species of palm stearin (PS).

Fig. 5.3c. TAG molecular species of Novozym 435 catalyzed structured lipid (SL1) and its corresponding physical blend (PB1).

Fig. 5.3d. TAG molecular species of Lipozyme TLIM catalyzed structured lipid (SL2) and its corresponding physical blend (PB2).

Fig. 5.4a. Melting thermograms of high stearate soybean oil (HSSO), palm stearin (PS), Novozym 435 catalyzed structured lipid (SL1), its corresponding physical blend (PB1), Lipozyme TLIM catalyzed structured lipid (SL2), and its corresponding physical blend (PB2).

Fig. 5.4b. Crystallization thermograms of high stearate soybean oil (HSSO), palm stearin (PS), Novozym 435 catalyzed structured lipid (SL1), its corresponding physical blend (PB1), Lipozyme TLIM catalyzed structured lipid (SL2), and its corresponding physical blend (PB2).

Table 5.1 Experimental settings of the factors and the responses used for optimization by response surface methodology

Exp	Temp[a] (°C)	Time (h)	SR[b]	Enz[c]	Stearic acid inc[d] (g/100 g)
N1	55	6	2	TLIM[e]	8.9±1.1
N2	65	10	2	TLIM	8.9±0.4
N3	50	18	2	TLIM	10.1±0.6
N4	60	22	2	TLIM	9.7±0.9
N5	50	6	3	TLIM	9.3±0.7
N6	65	6	3	TLIM	8.8±0.2
N7	55	22	3	TLIM	8.8±1.0
N8	50	10	4	TLIM	8.7±0.7
N9	65	22	4	TLIM	8.7±0.2
N10	55	6	5	TLIM	8.9±0.5
N11	60	10	5	TLIM	8.3±0.1
N12	65	18	5	TLIM	8.7±0.3
N13	50	22	5	TLIM	8.1±0.8
N14	50	6	2	N435[f]	10.7±1.2
N15	65	6	2	N435	9.6±0.9
N16	50	22	2	N435	11.3±1.1
N17	65	22	2	N435	10.5±0.9
N18	55	14	3	N435	9.6±0.5
N19	60	18	4	N435	8.9±0.8
N20	50	6	5	N435	9.0±0.2
N21	65	6	5	N435	8.8±0.5
N22	50	22	5	N435	8.1±0.6
N23	65	22	5	N435	8.5±0.3
N24	65	22	5	N435	8.4±0.4
N25	65	22	5	N435	8.5±0.3
N26	65	22	5	N435	8.5±0.2

[a] Temp, temperature. [b] SR, substrate molar ratio (PS:HSSO). [c] Enz, enzyme. [d] Inc, incorporation. [e] TLIM, Lipozyme TLIM (*Thermomyces lanuginosus* lipase). [f] N435, Novozym 435 (*Candida antarctica* lipase). Each value is the mean of triplicates ± standard deviation.

Table 5.2 Total and positional fatty acid composition (g/100 g) of high stearate cotton seed oil and palm stearin

	HSSO[a]		PS[b]	
fatty acid	total	*sn*-2	total	*sn*-2
C12:0	nd[c]	nd	0.4±0.0	nd
C14:0	0.1±0.0	nd	1.4±0.9	nd
C16:0	10.1±1.5	nd	58.1±4.9	48.9±4.3
C16:1n-7*c*	0.1±0.0	nd	0.1±0.0	nd
C18:0	16.8±0.9	nd	5.2±1.1	2.9±0.9
C18:1n-9*c*	16.0±1.8	12.6±1.0	26.9±2.7	38.3±2.2
C18:2n-6*t*	0.6±0.0	0.0±0.0	0.4±0.1	nd
C18:2n-6*c*	50.2±3.2	84.2±5.9	7.6±1.1	11.8±1.0
C20:0	1.0±0.0	nd	0.3±0.0	nd
C18:3n-6*c*	0.4±0.0	nd	nd	nd
C20:1n-9	0.5±0.0	nd	nd	nd
C18:3n-3*c*	4.3±1.6	3.0±0.7	0.2±0.0	nd
C22:0	0.6±0.0	nd	nd	nd
∑SFA[d]	28.5	0.0	65.3	51.8
∑UFA[e]	71.5	99.8	34.4	49.1
∑TFA[f]	0.6	0.0	0.4	0.0

[a]HSSO, high stearate soybean oil. [b]PS, palm stearin. [c]nd, not determined. [d]SFA, saturated fatty acids. [e]UFA, unsaturated fatty acids. [f]TFA, *trans* fatty acids. Each value is the mean of triplicates ± standard deviation.

Table 5.3 Total and positional fatty acid composition (g/100 g) of structured lipids and physical blends

	SL1[a]		PB1[b]		SL2[c]		PB2[d]	
fatty acid	total	*sn*-2	total	*sn*-2	total	*sn*-2	total	*sn*-2
C12:0	0.1±0.0a	nd[e]	0.1±0.0a	nd	0.2±0.0b	nd	0.1±0.0a	nd
C14:0	0.7±0.0a	nd	0.8±0.0ab	nd	0.9±0.0b	nd	0.8±0.3ab	nd
C16:0	31.0±3.3a	38.8±4.0a	38.1±3.8b	27.9±2.5b	38.5±4.2c	41.6±3.7c	39.3±3.3bc	21.3±2.1d
C16:1n-7*c*	0.1±0.0a	nd	0.1±0.0a	nd	0.1±0.0a	nd	0.1±0.0a	nd
C18:0	12.3±1.8a	9.4±1.9a	8.3±1.2b	nd	9.5±0.6c	6.0±1.0b	8.6±1.0b	nd
C18:1n-9*c*	20.1±2.1a	28.5±2.6a	21.2±3.0b	37.9±3.1b	25.5±2.9c	26.9±3.1c	22.6±3.2d	42.4±3.8d
C18:2n-6*t*	nd	nd	nd	nd	nd	nd	nd	nd
C18:2n-6*c*	34.6±2.9a	25.5±2.4a	29.4±3.2b	34.1±4.0b	23.8±1.8c	22.2±2.6c	26.7±3.2d	36.3±3.5d
C20:0	nd	nd	0.4±0.0a	nd	0.5±0.0b	nd	nd	nd
C18:3n-6*c*	0.1±0.0a	nd	0.1±0.0a	nd	0.1±0.0a	nd	0.1±0.0a	nd
C20:1n-9	0.2±0.0a	nd	0.2±0.0a	nd	0.2±0.0a	nd	0.2±0.0a	nd
C18:3n-3*c*	2.1±0.7a	nd	1.3±0.1b	nd	1.5±0.1c	nd	1.3±0.0b	nd
∑SFA	44.1	48.2	47.6	27.9	49.6	47.6	48.9	21.3
∑UFA	57.2	54.0	52.2	72.1	51.2	49.1	50.9	78.7
∑TFA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]SL1, Novozym 435-catalyzed structured lipid. [b]PB1, corresponding physical blend of SL1. [c]SL2, Lipozyme TLIM-catalyzed structured lipid.[d]PB2, corresponding physical blend of SL2. [e]nd, not determined. Each value is the mean of triplicates ± standard deviation. Values with the same letter in each row within total and *sn*-2 columns separately are not significantly different at $P \leq 0.05$.

Table 5.4 Solid fat content (%) of structured lipids and physical blends

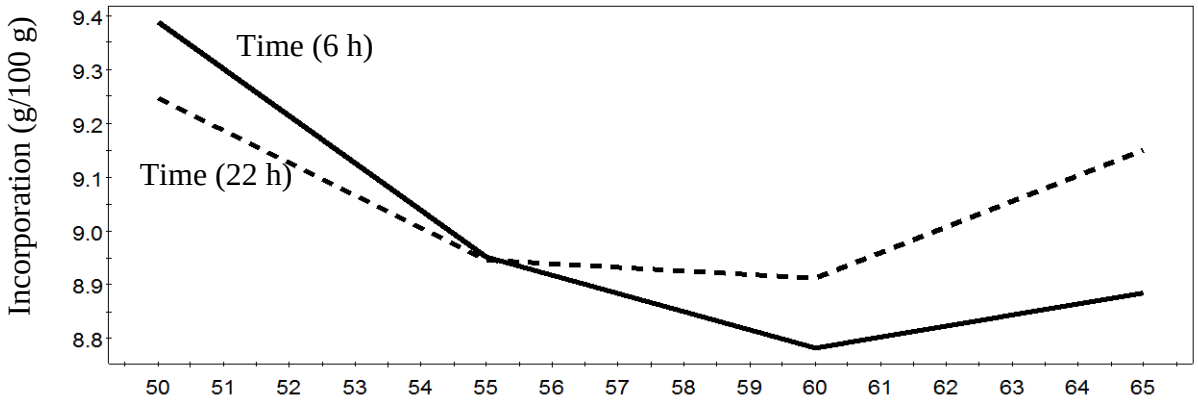
Temperature (°C)	Solid fat content (%)			
	SL1[a]	PB1[b]	SL2[c]	PB2[d]
5	52.8±2.4a	60.3±3.9b	56.9±2.2c	62.2±3.2d
10	43.9±1.1a	52.3±1.9b	43.8±1.8a	51.0±2.3b
15	43.4±1.5a	45.7±1.3b	42.6±1.4a	44.8±1.8b
20	33.0±1.8a	35.5±1.2b	30.9±1.1c	35.1±1.4b
25	25.7±0.9a	30.5±1.6b	23.6±1.0c	28.6±1.0d
30	16.5±0.8a	24.0±1.0b	15.8±0.9c	22.0±1.1d
35	13.3±0.9a	20.1±1.1b	11.9±0.6c	18.2±0.8d
40	7.4±0.3a	16.9±0.9b	7.2±0.2ab	14.2±0.8c
45	0.5±0.0a	12.3±0.9b	0.5±0.0a	10.3±0.7d
50	0.0±0.0	3.4±0.1a	0.0±0.0	2.1±0.1b
55	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
60	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

[a]SL1, Novozym 435-catalyzed structured lipid. [b]PB1, corresponding physical blend of SL1. [c]SL2, Lipozyme TLIM-catalyzed structured lipid.[d]PB2, corresponding physical blend of SL2. Each value is the mean of duplicates ± standard deviation. Values with the same letter in each row are not significantly different at $P \leq 0.05$.

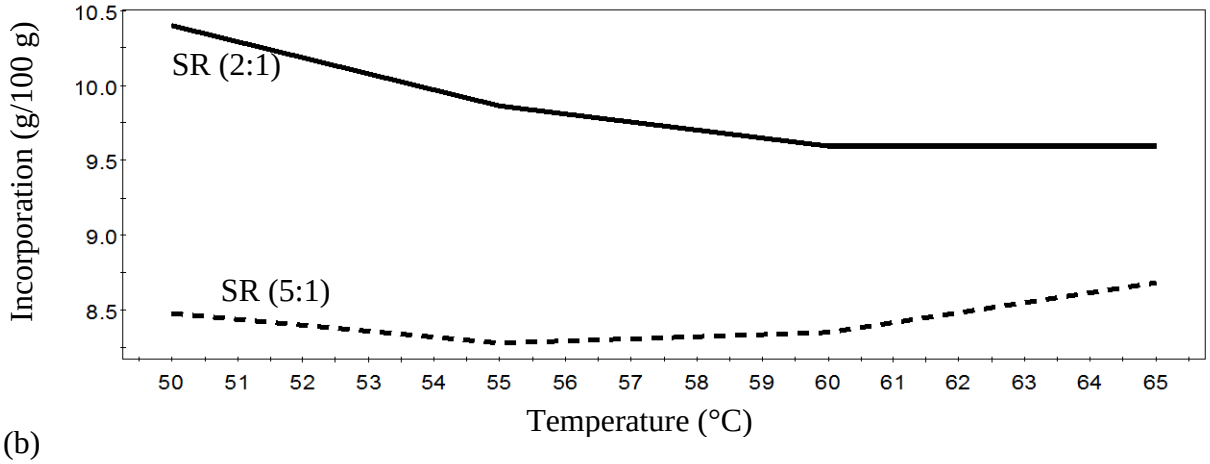
Table 5.5 Polymorphic forms of high stearate soybean oil, palm stearin, structured lipids, and physical blends

Sample	Polymorphic form
HSSO[a]	β′ > β
PS[b]	β′ + β
SL1[c]	β′ >> β
PB1[d]	β′ + β
SL2[e]	β′ >> β
PB2[f]	β′ > β

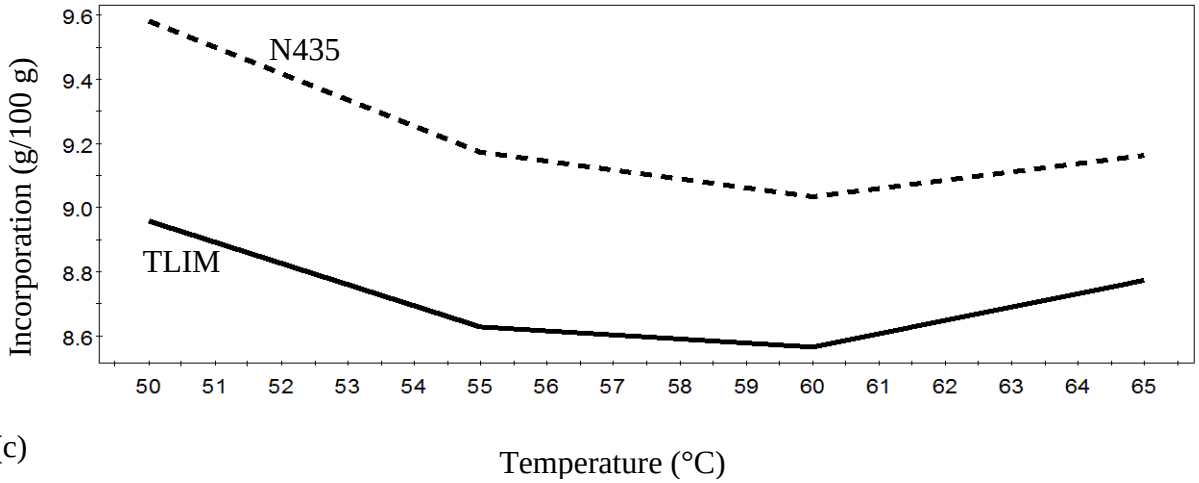
[a]HSSO, high stearate soybean oil. [b]PS, palm stearin, [c]SL1, Novozym 435-catalyzed structured lipid. [d]PB1, corresponding physical blend of SL1. [e]SL2, Lipozyme TLIM-catalyzed structured lipid. [f]PB2, corresponding physical blend of SL2.


Incorporation (g/100 g)
9.4
9.3
9.2
9.1
9.0
8.9
8.8
Time (6 h)
Time (22 h)
50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65
Temperature (°C)

(a)

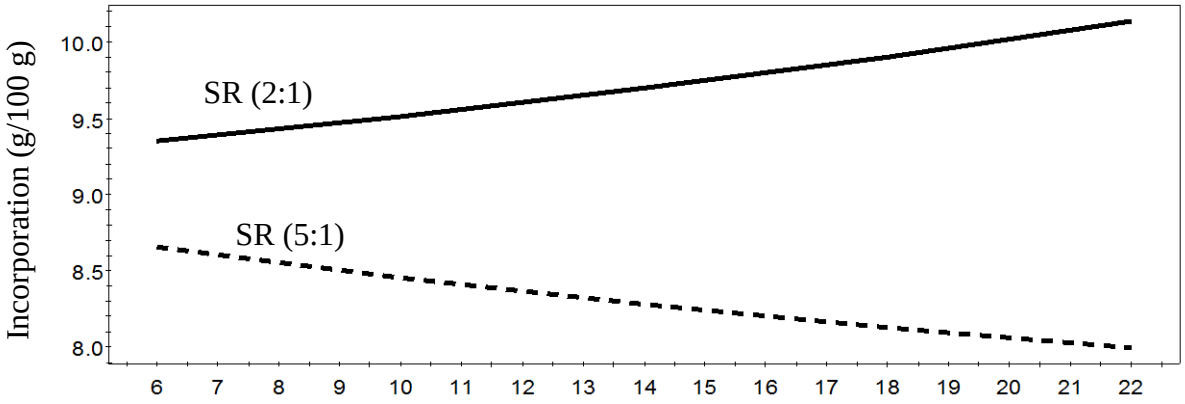

Incorporation (g/100 g)
10.5
10.0
9.5
9.0
8.5
SR (2:1)
SR (5:1)
50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65
Temperature (°C)

(b)

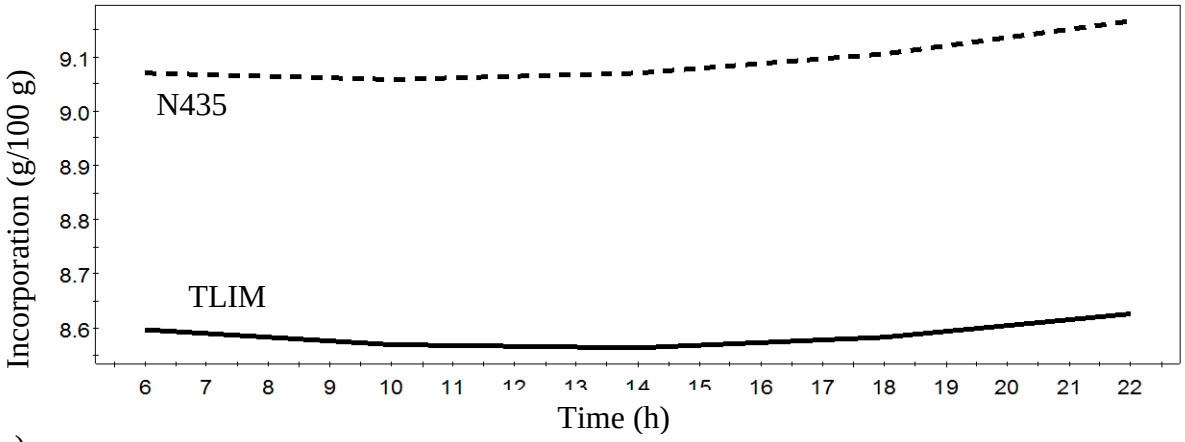

Incorporation (g/100 g)
9.6
9.4
9.2
9.0
8.8
8.6
N435
TLIM
50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65
Temperature (°C)

(c)

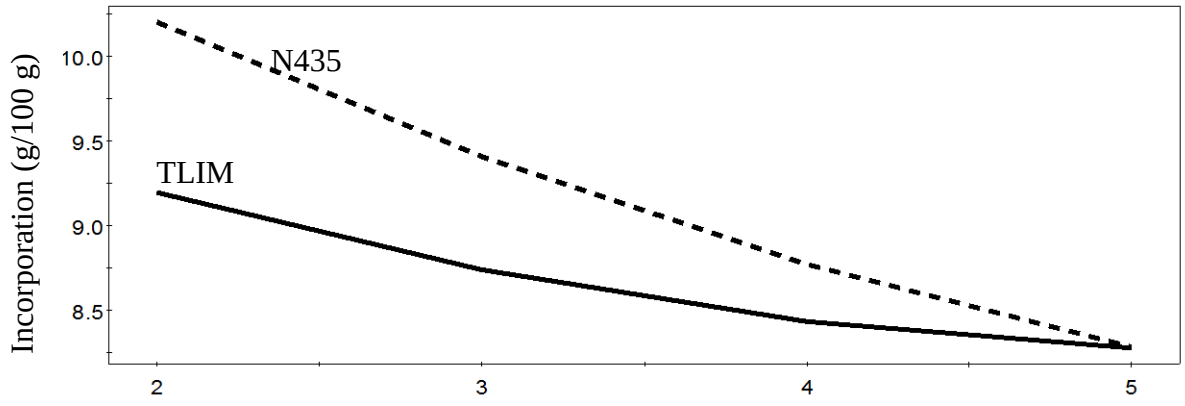
Fig. 5.1


Incorporation (g/100 g)
10.0
9.5
9.0
8.5
8.0
SR (2:1)
SR (5:1)
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Time (h)

(d)

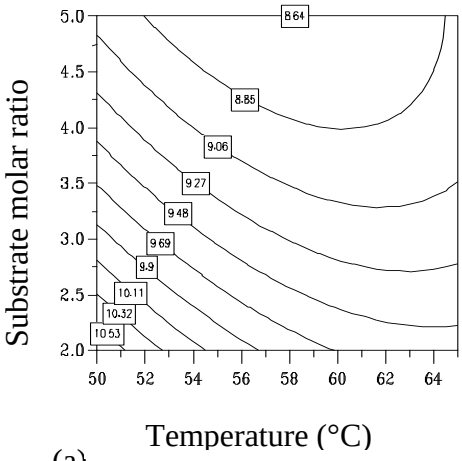

Incorporation (g/100 g)
9.1
9.0
8.9
8.8
8.7
8.6
N435
TLIM
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Time (h)

(e)

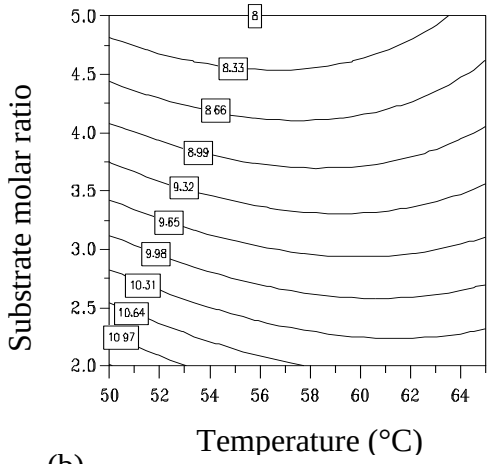

Incorporation (g/100 g)
10.0
9.5
9.0
8.5
N435
TLIM
2 3 4 5
Substrate molar ratio

(f)

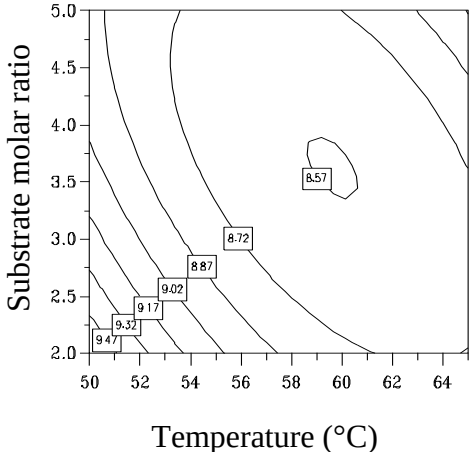
Fig. 5.1


Substrate molar ratio
Temperature (°C)
8.64
8.85
9.06
9.27
9.48
9.69
9.9
10.11
10.32
10.53

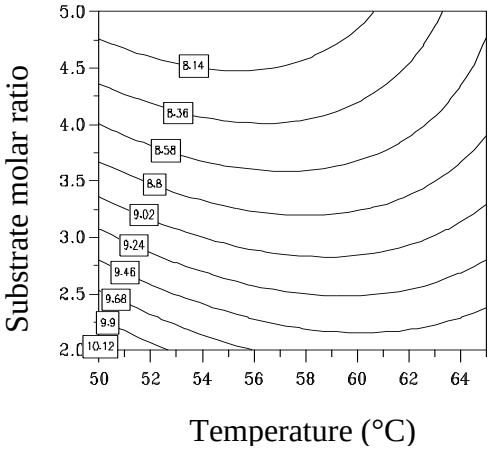
(a)


Substrate molar ratio
Temperature (°C)
8
8.33
8.66
8.99
9.32
9.65
9.98
10.31
10.64
10.97

(b)

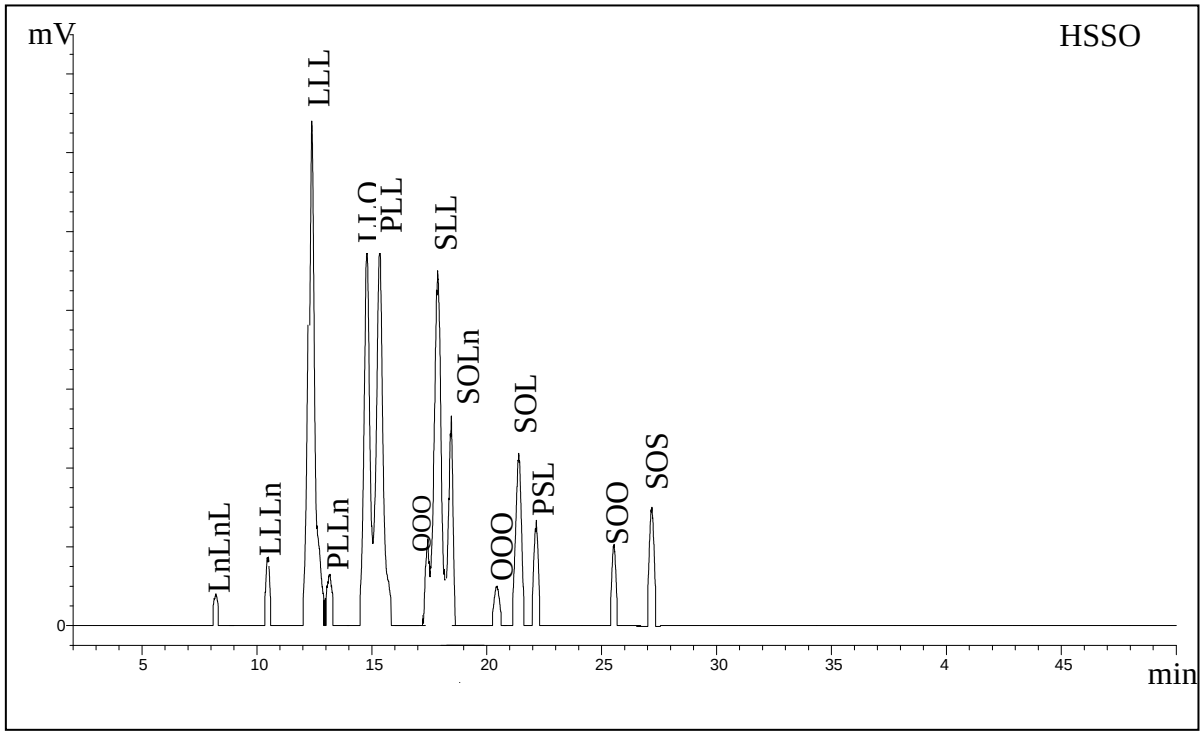

Substrate molar ratio
Temperature (°C)
8.57
8.72
8.87
9.02
9.17
9.32
9.47

(c)

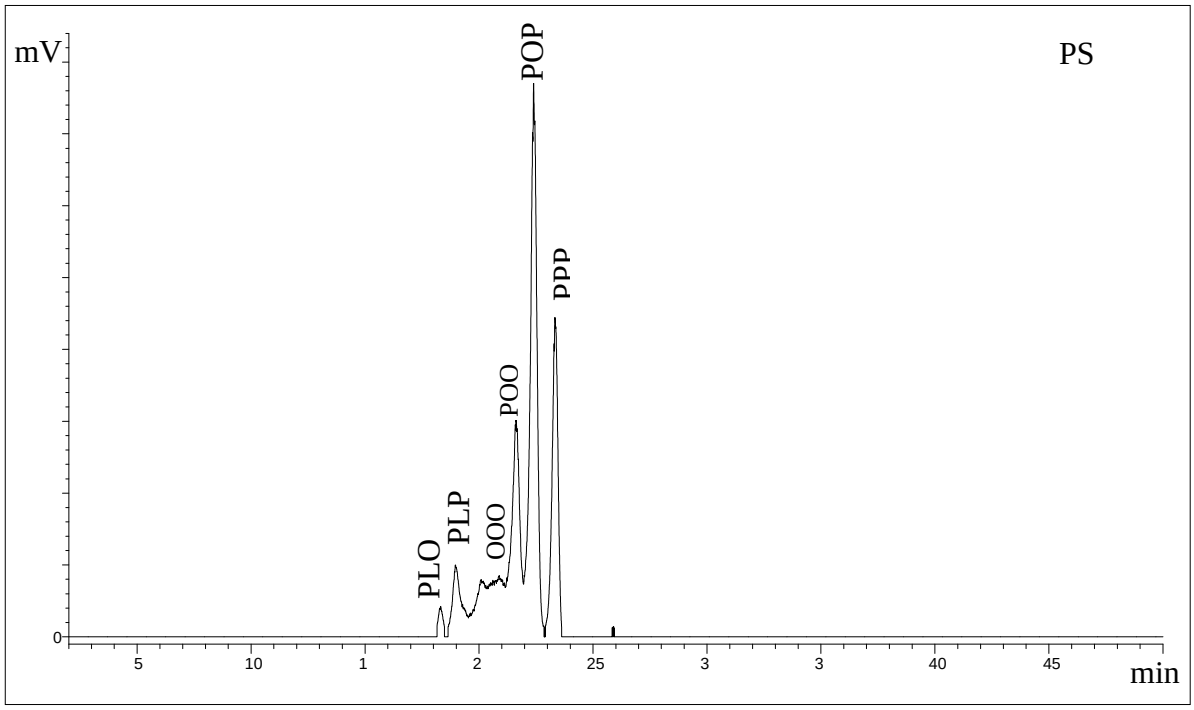

Substrate molar ratio
Temperature (°C)
8.14
8.36
8.58
8.8
9.02
9.24
9.46
9.68
9.9
10.12

(d)

Fig. 5.2

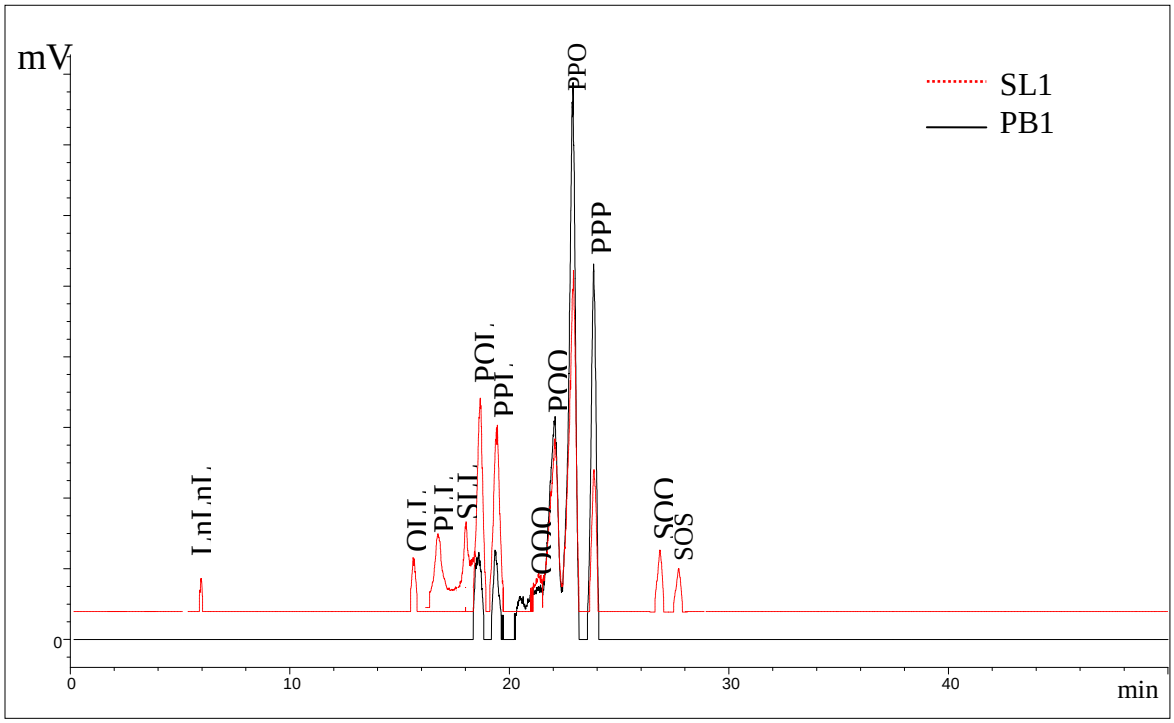

mV
HSSO
LnLnL
LLLn
LLL
PLLn
OLL
PLL
OOO
SLL
SOLn
OOO
SOL
PSL
SOO
SOS
0
5
10
15
20
25
30
35
4
45
min

(a)

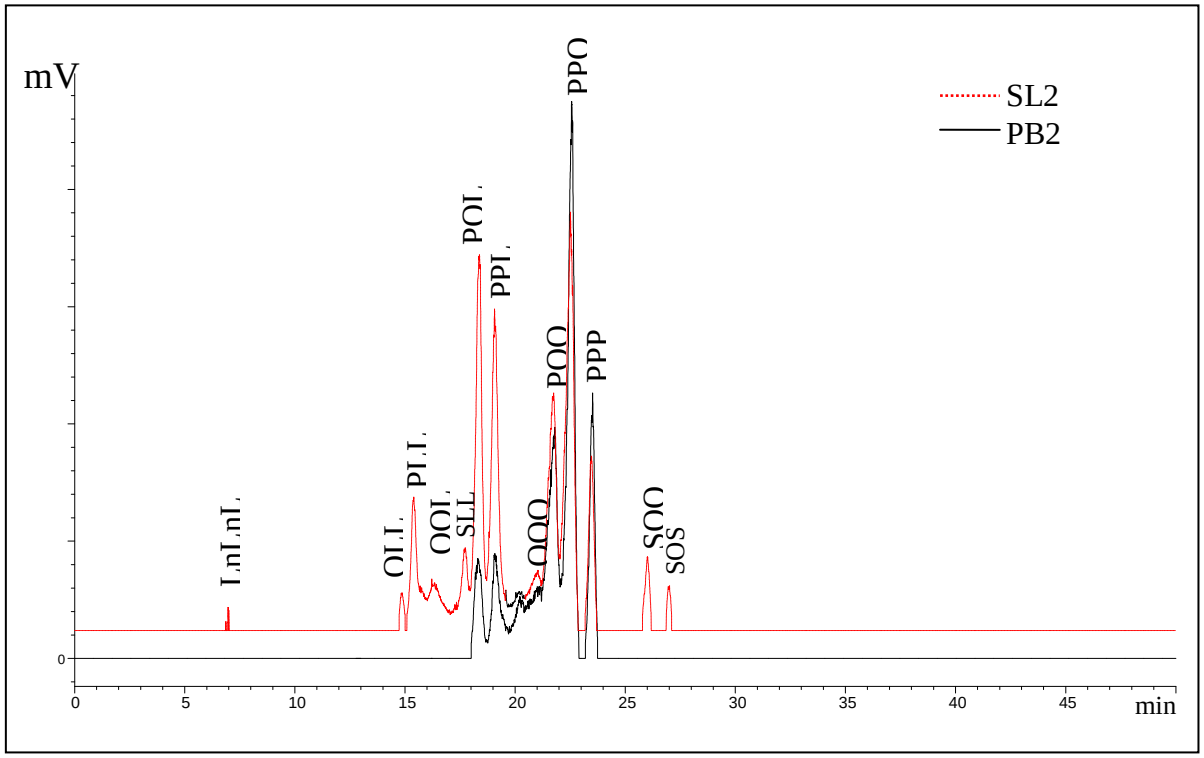

mV
PS
POP
PPP
POO
PLO
PLP
OOO
0
5
10
1
2
25
3
3
40
45
min

(b)

Fig. 5.3

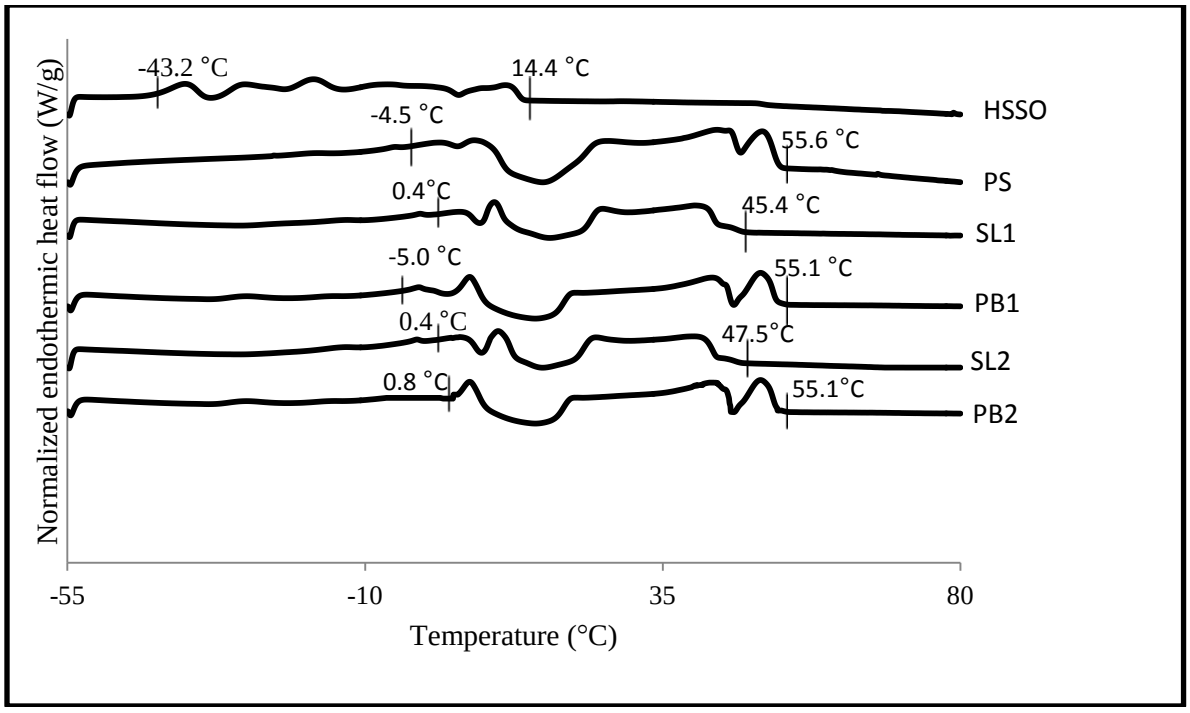

mV
SL1
PB1
PPO
PPP
POO
PPL
PLO
SLL
PLL
OLO
OOO
OOS
SOS
0
0
10
20
30
40
min

(c)

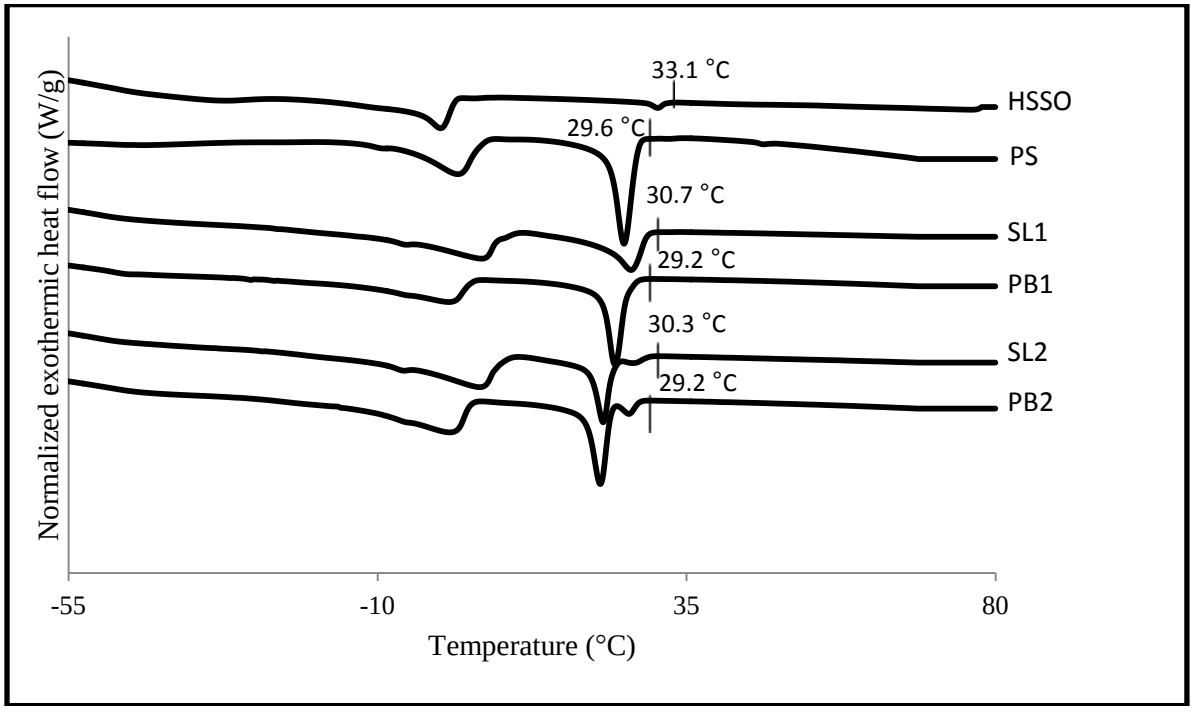

mV
SL2
PB2
PPO
PPP
POO
PPL
PLO
SLL
PLL
OLO
OOO
OOS
SOS
0
0
5
10
15
20
25
30
35
40
45
min

(d)

Fig. 5.3


Normalized endothermic heat flow (W/g)
-43.2 °C
14.4 °C
HSSO
-4.5 °C
55.6 °C
PS
0.4°C
45.4 °C
SL1
-5.0 °C
55.1 °C
PB1
0.4 °C
47.5°C
SL2
0.8 °C
55.1°C
PB2
-55
-10
35
80
Temperature (°C)

(a)


Normalized exothermic heat flow (W/g)
33.1 °C
HSSO
29.6 °C
PS
30.7 °C
SL1
29.2 °C
PB1
30.3 °C
SL2
29.2 °C
PB2
-55
-10
35
80
Temperature (°C)

(b)

Fig. 5.4

CHAPTER 6

UTILIZATION OF ENZYMATICALLY INTERESTERIFIED COTTONSEED OIL AND PALM STEARIN-BASED STRUCTURED LIPID IN THE PRODUCTION OF *TRANS*-FREE MARGARINE[1]

[1]Pande, G.; Casimir C. Akoh and Robert L. Shewfelt. To be submitted to *European Journal of Lipid Science and Technology*.

ABSTRACT

trans-Free margarine fat was enzymatically synthesized from palm stearin (PS) and regular cottonseed oil (CO). Response surface methodology (RSM) was used for optimization. The independent variables were substrate molar ratio (PS:CO, 2-5), temperature (50-65 °C), time (6-22 h), and lipases (Lipozyme® TLIM and Novozym® 435). Incorporation of stearic acid (mol %) was the dependent variable. Desirable products composition were achieved at 57 °C, 14 h, 4:1, using Lipozyme TLIM, with 5.2 mol% stearic acid and at 56 °C, 6 h, 4:1, using Novozym 435, with 5.9 mol% stearic acid. Using optimal conditions, structured lipids (SLs) were synthesized in a 1 L stir-batch reactor. Solid fat contents at 25 °C were lower for SLs (24.8 - 30.8%) than the corresponding physical blends (34.7 - 39.3%). Novozym 435-catalyzed SL product had desirable fatty acid profile, physical properties, β′ polymorph, and was further formulated into margarine. Compared to commercial margarine (19.1 mol% *trans* fatty acids (TFA), the SL containing margarine had no *trans* fat. However, it was harder and less spreadable than commercial margarine but no difference was observed in their taste. In this study, we were able to formulate the margarine suitable for possible use as hard/industrial margarine with high oxidative stability and no TFA.

PRACTICAL APPLICATION

Intake of high amounts of *trans* fat has been positively correlated with increased risk of several chronic diseases such as cardiovascular, inflammatory, autoimmune diseases, and cancer. The levels of *trans* fat in the American diet is of concern. Interesterification is a possible alternative to partial hydrogenation to obtain desirable physical and functional properties of lipids. In this study, we were able to enzymatically synthesize SL which was used to formulate *trans*-free margarine as firm industrial margarine. This research resulted in production of *trans*-free margarine as an alternative to partially hydrogenated fat containing margarines.

INTRODUCTION

Cottonseed oil (CO) is one of the main vegetable oils produced in the US and is used primarily as salad oil, cooking oil, shortening, and margarine. In 2010-2011, the estimated total production of cottonseed oil in the US was 835 million pounds [1]. Refined cottonseed oil has light golden color and bland flavor which is favorable for its use in food products. Cottonseed oil requires less hydrogenation to achieve the same degree of hardness compared to other linoleic oils due to its fatty acid (FA) composition [2]. Palm stearin (PS) is an important co-product obtained during palm oil fractionation. It is the harder fraction of palm oil containing higher proportion of saturated fatty acids (SFA) and a natural source of solid fat. Palm stearin has also been used with several other oils such as rice bran oil and coconut oil [3], pine nut oil [4], canola oil and palm kernel oil [5] in enzymatic interesterification to produce *trans*-free margarine fat analogues.

Margarine is a water-in-oil (w/o) emulsion comprising of at least 80% lipid phase [6]. Based on their applications, margarines can be classified into hard and medium margarine for baking, and medium and soft for table margarine [7]. Table margarine is further divided into tub and brick margarine. Tub margarine has low solid fat content (SFC) at low temperature, thus enabling it to be spreadable direct from the refrigerator. Brick margarine should have properties similar to tub margarine, but with higher SFC. Many vegetable oils have been modified to produce margarine with required SFC and melting behavior. Partial hydrogenation is the main process to convert liquid oils to solid fats for use in margarines and shortenings. However, solidification is accompanied by the formation of *trans* fatty acids (TFA). Partial hydrogenation is responsible for 80% of

TFA in the US diet [8]. High intake of TFA is associated with increased LDL, decreased HDL, and increased total: HDL cholesterol [9], and cancer [10]. Chemical and/or enzymatic interesterification is used for improving the physical and functional properties of fats and oils by changing either the distribution or type of FAs on the glycerol backbone. The restructured fats and oils with desired physical, chemical, and nutritional properties are called structured lipids (SL) [11]. Interesterification does not change the degree of unsaturation of the FAs but a redistribution of these FAs on the triacylglycerol (TAG) molecule occurs resulting in a *trans*-free product with desirable properties [12]. Lipases are preferred over chemical catalyst because they are regio-and stereo-specific and offer a better control over the final product [13].

The main objective of this research was to produce *trans*-free SL for possible use in margarine. This was accomplished by optimization at small-scale using response surface methodology (RSM) followed by scale-up synthesis of the SLs and their comparison with physical blends (PBs). Finally, margarine was formulated using SL and compared with a commercial margarine.

MATERIALS AND METHODS

Materials

CO and PS were kindly provided by Cargill Inc. (Minneapolis, MN). The immobilized enzymes, Lipozyme® TLIM (*Thermomyces lanuginosus* lipase, *sn*-1,3 specific, specific activity 250 IUN/g: IUN=Interesterification Unit) and Novozym® 435 (*Candida antarctica* lipase, non-specific, specific activity 10,000 PLU/g: PLU=Propyl Laurate Unit) were purchased from Novozymes North America Inc. (Franklinton, NC). Commercial stick margarine and food grade soy lecithin fluid were purchased from a

local grocery store. Lipid standards, Supelco 37 Component FAME mix, 14% boron trifluoride in methanol, triolein, 2-oleoylglycerol, tripalmitin, tristearin, 1,2-dioleoyl-3-palmitoyl-rac-glycerol, 1-palmitoyl-2-oleoyl-3-linoleoyl, and tocopherol standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), and the internal standard, C19:0-nonadecanoic acid and its methyl ester, were purchased from TCI America (Portland, OR). The TAG standard mix (GLC reference standard) was purchased from Nu-chek Prep, Inc. (Elysian, MN). Organic solvents and chemicals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA).

Experimental design for response surface methodology (RSM)

Response surface methodology (RSM) is an effective and a powerful statistical process optimization method. It is a faster and more efficient method to evaluate the effects of multiple variables and their interaction effects on the response using a reduced number of experiments than a full-factorial design [14]. Four independent variables were studied namely: time (6, 10, 14, 18, 22 h), temperature (50, 55, 60, 65 °C), substrate molar ratio (PS:CO, 2, 3, 4, 5), and two enzymes (Lipozyme TLIM and Novozym 435 lipases), for stearic acid incorporation as response. RSM was done using Modde 9.0 software (Umetrics, Umeå, Sweden) to obtain the relationship between the response and the independent variables. Twenty six runs were generated and experiments at each design point were randomly performed in triplicate. SL synthesis was performed in screw-cap test tubes in 2 mL hexane as per the conditions generated by RSM. Specific enzymes were added at 10% total substrate weight. Test tubes were then incubated in water bath shaker at 200 rpm. After the reaction was over, the product was filtered through anhydrous sodium sulfate column to remove the enzymes. TAG bands were collected

using TLC and analyzed for FA profile after conversion to methyl esters. All reactions were performed and analyzed in triplicates and average values reported.

Fatty acid profile

Fat and oil samples were converted to fatty acid methyl esters following the AOAC Official Method 996.01 [15] and analyzed with Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) using Supelco SP-2560, 100 m x 25 mm x 0.2 μm column. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. Injection volume was 1 μL and a spilt ratio of 20:1 was used. The column was initially held at 140 ºC for 5 min, then increased to 240 ºC at 4 ºC/min, and finally held at 240 ºC for 25 min. Detection was with flame ionization detector at 300 ºC. All samples were analyzed in triplicates and average values reported.

Large-scale SL synthesis

The scaled-up reactions using the optimal conditions achieved from the small-scale reactions were performed in 1L stir-batch reactor. Corresponding physical blends (PB1 and PB2) were also prepared at the same reaction conditions but without the respective enzymes. The SL selected for margarine formulation was synthesized in 4L stir-batch reactor and designated as SL_{ls}. Free fatty acids (FFAs) were removed by short-path distillation (SPD) KDL-4 unit (UIC, Joliet, IL). The reaction products were passed through the unit under high vacuum (<0.2 mmHg absolute pressure) at the flow rate of ca. 10 mL/min. The evaporator and condenser temperatures were maintained at 185 and 25 °C, respectively. FFAs were determined by AOCS Official Method Ac 5-41[16].

Fatty acid and positional analysis

Fatty acids were determined as described above. *sn*-2 Positional fatty acid composition was determined following the AOCS Official Method Ch 3-9 [17]. All samples were analyzed in triplicates and average values reported.

TAG molecular species

The TAG composition was determined with a nonaqueous reverse phase HPLC (Agilent Technologies 1260 Infinity, Santa Clara, CA) equipped with a Sedex 85 ELSD (Richard Scientific, Novato, CA) [18]. Triplicate determinations were made and averaged.

Differential scanning calorimetry (DSC)

Melting and crystallization profiles of lipid samples were determined following AOCS Official Method Cj 1-94 [19]. SL1, PB1, SL2, AND PB2 were analyzed with Perkin-Elmer model DSC7 (Norwalk, CT) and SL_{ls} and extracted fat from commercial margarine (EF_{cm}) on Mettler Toledo model DSC 1 STARe System (Columbus, OH). All samples were analyzed in triplicates and average values reported.

Polymorphism

The polymorphic forms of the samples were determined with Bruker D8 Advance diffractometer (Bruker AXS, Inc., Madison, WI). The diffractometer had a 2θ configuration (2θ range used was 20-32°), a solid state detector, and cobalt tube as the X-ray source. Corundum was used as standard. The scan rate was 4.2°/min with a step increment of 0.01°. The samples were rotated at 15 rev/min. Samples were melted and poured into rectangular aluminum molds. They were held at room temperature for 2-3 h, at 4 °C for 4-5 h, and then stored at -20 °C overnight. Short spacings of the major polymorphs were identified as follows: α, a single spacing at 4.15 Å; β', two strong

spacings at 3.8 and 4.2 Å; and β, a very strong spacing at 4.6 and another one usually at 3.85 Å [12]. All samples were analyzed in triplicates and average values reported.

SFC

SFC was determined according to the AOCS Official Method Cd 16-93b [20] on a BrukerPC/20 Series NMR analyzer, Minispec (Bruker Optics, Milton, On, Canada). All samples were analyzed in duplicates and average values reported.

Tocopherol analysis

Normal phase high-performance liquid chromatography (HPLC) system (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 column was a LiChrosorb Si 60 column (4 mm, 250 mm, 5 μm particle size, Hiber Fertigs€aule RT, Merck, Darmstadt, Germany). The sample concentration was 20 mg/mL in HPLC-grade hexane. The samples were vortexed for 1 min and then centrifuged at 1000 rpm (104.72 rad/s) for 5 min at room temperature (23 °C). The top hexane layer was transferred into HPLC vials for analysis. Injection volume was 20 μL. The tocopherols were identified by comparing their retention times with those of authentic standards (α-, β-, δ-, and γ-tocopherol, 1.25-20 μg/mL in hexane containing 0.01% butylated hydroxytoluene). Tocopherols were quantified based on the standard calibration curves and reported as parts per million (ppm) from the average of triplicate determinations.

Oxidative stability index (OSI)

The OSI was determined according to AOCS Method Cd 12b-92 [21] using an Oil Stability Instrument (Omnion, Rockland, MA) at 110 °C. All samples were analyzed in triplicates and average values reported.

Margarine formulation

The commercial margarine was melted at 80 °C. The top fat layer was decanted into a separatory funnel, washed five times with the same volume of warm water, and filtered through an anhydrous sodium sulfate layer with a Whatman filter paper (pore size = 0.45 μm) under vacuum. This extracted fat from commercial margarine was designated EF_{cm}. The margarines were formulated as described by Kim *et al* [5]. The ingredients (w/w %) were lipid phase, 80.5% (EF_{cm} or SL_{ls}, 80%; soy lecithin fluid, 0.5%; and TBHQ, 0.01%) and aqueous phase, 19.5% (distilled water, 18%; and table salt, 1.5%). The lipids and water were heated to 60 °C and poured into a tabletop blender. The mixture was emulsified by vigorously mixing for 5 min. Commercial artificial butter flavor was added to mask any difference in the aroma of the samples. The resulting liquid emulsion was crystallized for 15 min using an ice cream maker (Krups North America, Peoria, IL) and then refrigerated overnight. The margarines were tempered at room temperature for 4 h and vigorously mixed with a hand mixer to obtain a smooth product. The margarine sample were placed into plastic tubs and stored at 4 °C. The margarines formulated with SL_{ls} and EF_{cm} were designated SLM and RCM (reformulated commercial margarine), respectively.

Texture profile analysis (TPA)

Margarine samples were taken out from refrigerator and measured at room temperature (23 °C). A double compression test was performed using a TA-X2 texture analyzer (Stable Micro Systems, London, United Kingdom) [5]. A 45º conical probe attached to a 25 kg compression load cell was used to penetrate the samples at 1.0 mm/s to a depth of 10 mm from the sample surface, and withdrawn at the same speed. Textural properties (hardness, adhesiveness, and cohesiveness) of margarines were evaluated at 23 °C. All samples were analyzed in triplicates and average values reported.

Rheological properties

A series of tests were performed on a dynamic stress-controlled rheometer SR5000 (Rheometric Scientific, Piscataway, NJ) at 23 °C to determine the rheological properties of margarine samples. A 25 mm parallel plate (0.6 mm gap) was used. In dynamic analysis, margarine samples were subjected to an oscillatory varying stress (0.4 kPa) and storage/elastic (G') and loss/viscous (G'') moduli were calculated. In creep analysis, a constant low stress (0.4 kPa) was applied to samples and deformation per unit time measured as an indicator of emulsion stability. In stress analysis, viscosity was measured across different stress (0.1-2.5 kPa). All samples were analyzed in triplicates and average values reported.

Microstructural morphology

Microstructure of margarines was observed with a polarized light microscope (Leica Microsystem Inc., Allendale, NJ) attached to an SPOT Idea™ digital camera (SPOT™ Imaging Solutions, Sterling Heights, MI). Margarine samples were melted at 80 °C and using a preheated capillary a small drop was placed on a preheated microslide. The

sample was carefully covered with a preheated coverslip to obtain a uniform film. The glass slides were kept in the refrigerator overnight and were measured at room temperature (23 °C). Microstructure was observed using 50x lens. All samples were observed in triplicates.

Sensory evaluation

All methods and procedures for sensory studies were approved by the University of Georgia (UGA) Institutional Review Boards on Human Subjects. Thirty three UGA staff and students above 18 yrs of age participated in the sensory evaluation. A triangle test was conducted in individual booths under fluorescent light to study the difference between experimental and commercial margarines. During textural and rheological analyses significant difference in spreadibility of RCM and SLM was observed. Therefore, in order to obtain data for flavor and mouthfeel of the samples rather than just spreadibility, the margarines were spread on toast and then given to panelists. Each participant was provided with 3 coded toasts with margarine samples, two of which were the same and one different. Subjects were asked to chew the samples but were not required to swallow them. They had to identify the odd sample and mention the difference observed. They were provided with water, non-salted crackers (to cleanse the mouth during the test), and empty cups for expectoration.

Statistical analysis

Regression analysis, response surfaces, and statistical significance were performed using Modde 9.0 software (Umetrics, Umea, Sweden) and backward elimination by Statistical Analysis System software (SAS 9.2, Cary, NC). Duncan's multiple-range test was performed using SAS to determine significant difference ($P < 0.05$) between SLs and

PBs, SL_{ls} and EF_{cm}, and between SLM and RCM. All samples, except SFC, were analyzed in triplicates and average values reported.

RESULTS AND DISCUSSION

Model fitting and optimization

The incorporation amounts of stearic acid in the *trans*-free margarine fat analogues ranged from 4.3-5.9 mol% (Table 6.1). Quadratic model was obtained for the incorporation of stearic acid in SLs by multiple linear regression and backward elimination. R^2 (explained variability) and the Q^2 coefficients (predicted variability) are often used to evaluate the goodness of a statistical model. Both coefficients should be near unity for a good model with strong prediction power. The R^2 and Q^2 values were 0.961 and 0.734, respectively. The model for stearic acid incorporation also showed no lack of fit ($P > 0.05$) (data not shown). The model equation after backward elimination at α=0.05 can therefore be written as:

Stearic acid incorporation = 5.65 - 0.15Temp + 0.31SR – 0.21Temp*Temp – 0.16Time*Time – 0.13SR*SR + 0.11Temp*SR ± 0.18Sr*Enz

where, Temp is temperature in °C, Time in hours, SR is substrate molar ratio, Enz is enzyme. To verify the model, five regions from the contour plot were randomly chosen to perform experiments using the conditions specified for these regions. A chi-squared value of 1.246 was obtained which is much smaller than the cutoff point (9.488) at α=0.05 and DF 4 indicating no significant difference between the observed and predicted values.

Temperature had a negative effect on stearic acid incorporation whereas substrate molar ratio (SR) had a positive effect on the response. The type of enzyme used was not significant as linear variable but in its interaction term with SR, Lipozyme TLIM had a

negative effect and Novozym 435 had a positive effect on stearic acid incorporation. Time had no significant effect on the response. Contour plots were generated by Modde 9 software and used for optimization of reactions by identifying parameter combinations that will produce a desired response. Contour plots are shown in Fig. 6.1. When Novozym 435 was used, keeping time constant at 14 h, higher incorporation was achieved at lower temperature and higher SR (Fig. 6.1a). On the other hand, using Lipozyme TLIM at 6 h, high incorporation was achieved at mid values of temperature and SR (Fig. 6.1b). Based on the predicted desirable composition of 5.9 and 5.2 mol% stearic acid, SL1 was synthesized at 56 °C, 6 h, 4:1, using Novozym 435 and SL2 at 57 °C, 14 h, 4:1, using Lipozyme TLIM, respectively. Corresponding physical blends (PB1 and PB2) were also prepared.

Product yield

During interesterification reactions FFA, partial acylglycerols, or any undesired product are also produced with desired SL. To obtain a high quality product, these side-products should be removed. After purification by short-path distillation, the SLs contained 0.1% FFA which is an acceptable level to be used as ingredients for food products. Yield % was calculated as:

Yield % = (Final product weight)*100/(Total substrate weight)

The yield % for SL1, SL2, and SL_{ls} were 88.5, 94.8, and 91.2%, respectively. This difference in the yield of SL1 and SL2 is due to the different enzymes and their different carrier material. Novozym 435 which was immobilized on macroporous acrylic resin, which absorbs more product resulting in lower product recovery. On the other hand,

product recovery was higher when using Lipozyme TLIM immobilized on granulated silica because silica gel facilitates easy removal of lipid product.

Total and positional fatty acid profile

Fatty acid (FA) compositions of CO, PS, interesterified fats, and physical blends are given in Table 6.2. The type and positional distribution of FA on the glycerol backbone greatly influences the physical behavior and metabolism of dietary fats. From a nutritional point of view, unsaturated FAs (UFA) are better metabolized and absorbed in our body when present at *sn*-2 position [22]. Therefore, SLs containing SFA at *sn*-1,3 positions and UFA at *sn*-2 position have different metabolic effects compared to lipids containing SFA at *sn*-2 position. The major FAs in CO were linoleic (54.9 mol%), palmitic (23.3 mol%), and oleic (16.8 mol%) acids. The major FAs in PS were palmitic (58.1 mol%) and oleic acids (26.9 mol%). CO and PS contained 0.4 mol% TFA. The major FA at *sn*-2 position of CO was linoleic (80.7 mol%). CO had 97.4 and 3.3 mol% UFA and SFA, respectively, at *sn*-2 position. On the other hand, *sn*-2 position of PS consisted of approximately equal amount of saturated fatty acids (SFAs) and UFAs. The main FA at *sn*-2 position of PS were palmitic (48.9 mol%) and oleic (38.3 mol%). Compared to CO, interesterified products had higher palmitic and stearic acids. Similarly, oleic acid also increased whereas linoleic acid decreased in the SLs indicating that SFA increased at the expense of FA having higher degree of unsaturation. Novozym 435-catalyzed SL (SL1) had 5.7 mol% stearic acid whereas the physical blend (PB1) had 4.3 mol% stearic acid (Table 6.2). For Lipozyme TLIM, the SL (SL2) had 5.1 mol% stearic acid while the physical blend (PB2) produced under similar conditions had 4.6 mol% stearic acid. No *trans* fat was found in SLs and PBs. Palmitic acid was the predominant

FA at *sn*-2 position of both SLs whereas oleic acid was the major FA at *sn*-2 position of both PBs. Although SL1 had higher total SFA (52.8 mol%) than SL2 (48.5 mol%) but it had lower SFAs (42.8 mol%) than SL2 (58.6 mol%) at *sn*-2 position. Therefore, it may be more suitable for margarine formulation. On the other hand, SL2 contains 53.6 mol% palmitic acid at *sn*-2 position thereby, making it suitable for possible use in human milk fat substitutes where higher palmitic acid is required at *sn*-2 position for better nutritional purposes. The major FA in the EF_{cm} were oleic (31.6 mol%) and linoleic (29.4 mol%). It had 19.1 mol% total TFA of which 15.6 mol% was present at *sn*-2 position. EF_{cm} had 72.4 and 12.1 mol% UFA and SFA, respectively, at *sn*-2 position.

TAG molecular species

The relative concentration (%) of TAG molecular species are given in Table 6.3. The major TAG species in CO were PLL (36.5%) and LLL (22.7%) and in PS were POP (47.3 %) and PPP (26.9 %). CO was composed of only UUU (triunsaturated) (36.2 %), SUU (monosaturated-diunsaturated) (54.1 %), and SUS (disaturated-monounsaturated) (9.6 %) type TAGs whereas UUU, SUU, SUS, and SSS (trisaturated) type TAGs were present in PS, SLs, PBs, and SL_{ls}. EF_{cm} had only UUU (30.6 %) and SUU (68.9 %) TAGs. A highly diverse TAG profile or greater FA chain-length diversity favors the formation of β′ crystal, the desirable polymorph in margarines [12]. After interesterification, UUU and SUU type TAGs increased whereas SUS and SSS type TAGs decreased compared to PS. This change in TAG species influenced the thermal behavior of the products. Both PBs had higher proportion of SSS and SUS type TAGs and lower proportion of SUU and UUU type TAGs than the SLs. This difference in the type of TAGs between the SLs and PBs affected the physical properties of product. The

predominant TAG species in SL_{ls} were POP (38.8%) and PPP (17.4%) whereas POO (54.3%) and LLL (14.4%) were the main TAG species in EF_{cm}.

Thermal behavior

Melting and crystallization profiles of the substrates and products are shown in Figs. 6.2a and 6.2b, respectively. Melting characteristics of margarine fat analogues are important for flavor release and consumer acceptance. Fats and oils do not have a distinct melting point but rather a melting range because of the different FAs present. The melting completion temperature (T_{mc}) depends on the amount and type of the TAG molecular species and also on the type of FAs. T_{mc} of CO, PS, SL1, PB1, SL2, and PB2 were 6.2, 54.2, 40.5, 51.6, 46.2, and 54.8 °C which is proportional to the decrease in UUU type TAGs and increase in SSS type TAGs. The T_{mc} of the SLs was lower than the PBs because both SLs had more UUU and SUU type TAGs and less SSS and SSU type TAGs than PBs. Therefore, melting properties of fats and oils can be changed by enzymatic interesterification. After interesterification, T_{mc} decreased from 54.2 °C in PS to 40.5-46.2 °C in SLs. Both SLs were not completely melted at room temperature but SL1 had lower T_{mc} than SL2 and therefore, can be used in the production of stick margarines. Melting and crystallization profiles of the SL_{ls} and EF_{cm} are shown in Fig. 6.2c. The higher proportion of saturated TAGs in SL_{ls} resulted in higher melting point for SL_{ls} (41.1 °C) than EF_{cm} (38.6 °C). The crystallization onset temperature (T_{co}) of CO and PS were -5.8 and 30.5 °C, respectively. Significant difference ($P < 0.05$) was found between T_{co} of SL1 (31.3 °C) and PB1 (26.0 °C); SL2 (25.8 °C) and PB2 (31.5 °C); and SL_{ls} (31.3 °C) and EF_{cm} (17.1 °C).

Solid fat content

One of the important parameters of margarine is solid fat content (SFC) which is a measure of solid/liquid ratio of a fat at various temperatures. Three useful measurement temperatures are refrigeration, room, and body temperatures which are related to the spreadibility, product stability, and texture and mouthfeel, respectively, of margarines [12]. The SFC of SLs, PBs, SL_{ls}, and EF_{cm} are shown in Fig 6.3. To prevent any waxy sensation in the mouth, the margarine should have SFC below 3.5% at 33.3 °C [23]. Only EF_{cm} fulfilled this criterion. It has been reported that at 25 °C, SFC should be 15-35% for desired spreadibility and texture and more than 10% at 20 °C to avoid oil separation [24]. At 25 °C, SFC of SL1, SL2, PB1, PB2, SL_{ls}, and EF_{cm} were 24.8, 30.8, 34.7, 39.3, 22.7, and 8.5%. At 20 °C, all samples except EF_{cm} had >10% SFC. The SLs met these criteria suggesting that they were suitable for use as hard margarine stock.

Polymorphism

In lipids there are three dominant crystal forms namely, α, β′, and β polymorphs which greatly influence the physical properties and processing of the final product [12]. The type of crystal formed depends on the acyl groups of TAGs. A wide range of TAG profiles and FA tend to crystallize in the β′ form. Since interesterification alters the TAG composition, crystalline morphology of the fats can be modified. Fats containing predominantly β′ crystals impart smooth texture and mouthfeel to margarine whereas those with predominantly β crystals impart grainy texture [12]. CO is β′-crystal tending whereas PS is high in C48 TAG, or tripalmitin (PPP) which is β-crystal [25]. All samples had β′ crystal as the dominant polymorph type. SL_{ls} had higher intensity of β′ crystal than EF_{cm} (data not shown).

Oxidative stability and tocopherols

The tocopherol content and oxidative stability of the samples are given in Table 6.4. Oxidative stability is an important characteristic of the product which determines its shelf life and flavor. The oxidative stability of SLs depends on the processing condition, type and positional distribution of FAs, and amount of indigenous antioxidant present in the starting oils. Tocopherols (T) and tocotrienols (T3), collectively known as vitamin E, are important natural antioxidant present in fats and oils. PS had the lowest total vitamin E content (275.5 ppm) with γ-T3 (142.6 ppm) as the major homologue. CO had 749.4 ppm total vitamin E, the major one being γ- and α-T. The predominant vitamin E in SL_{ls} were α-T (310.9 ppm) and γ-T3 (310.7 ppm) and whereas in EF_{cm} it was γ-T (810.6 ppm). The higher OSI value of SL_{ls} (20.2 h) than EF_{cm} (17.3 h) can be attributed to its higher SFA content and also due to higher antioxidant power of α-T and γ-T3 compared to γ-T. [26]. After SPD, α-, γ-, and δ-T3 were decreased by 50.6, 45.3, and 45.4%, respectively. On the other hand, α- and γ-T were reduced by 38.5 and 35.7%, respectively. After SPD, a loss of 38.7% was observed in the total Vitamin E content of SL. The main vitamin E in SPD waste were γ-T3 (236.6 ppm) and α-T (170.4 ppm). The mass balance of tocopherols was calculated and the total percent of tocopherols in the waste was 96%. The difference of 4% could be attributed to loss of tocopherols as tocopheryl esters during SL production [27]. For confirmation, TLC was done to separate the tocopheryl esters and further analyze them. Silica gel TLC was performed for separation and identification of tocopheryl esters. The developing solvent was hexane/diethyl ether/formic acid (90:10;1, v/v/v). A mix of tocopheryl linoleate and tocopheryl oleate (Tri-K Industries, Inc., Northvale, NJ) was used as standard. No tocopheryl ester band

was observed in the waste sample. This suggests that during interesterification reaction between CO and PS, the tocopherols were lost mainly as free tocopherols and not as esters as was the case in acidolysis [27]. This is because their FFA content were very low (product before interesterification and after SPD had 0.3 and 0.01% FFA, respectively). Therefore, the remaining 4% could be the tocopherols that were left on the short-path distillator that were not fully recovered in the waste. Also, some tocopherols may be lost due to short-path distillation heat.

Margarine characteristics

Based on the above mentioned analyses, Novozym 435 synthesized SL (SL1) was found to be more suitable than SL2 as margarine fat analogue and was scaled up (SL_{ls}) for margarine formulation. The margarine formulated with SL_{ls} was designated SLM and compared with reformulated commercial margarine (RCM).Texture profile analysis of SLM and RCM is shown in Fig. 6.4. The texture of margarine depends on the FA composition, crystal morphology, and the processing and storage conditions [28]. Hardness was measured as the maximum force during the first compression. The higher the hardness value of a margarine, the more difficult it is to spread. Significant difference ($P < 0.05$) was found between SLM and RCM in terms of hardness. SLM was much harder than RCM implying that it will be difficult to spread. This is because SL_{ls} had more SFA (53.1 mol%) than EF_{cm} (16.7%). The negative force area for the first compression was reported as adhesiveness which is related to the stickiness of food samples. Cohesiveness is the ratio of the positive force area during the second compression to that of the first compression and relates to how crumbly or brittle a food product is. Both adhesiveness and cohesiveness affects the texture and mouthfeel of

margarine. No significant difference ($P > 0.05$) was found between SLM and RCM for adhesiveness and cohesiveness suggesting that these two margarines will have similar mouthfeel.

Rheology is the study of deformation and flow of matter under the influence of applied stress and strain [29]. The rheological properties of SLM and RCM are shown in Fig. 6.5. In creep analysis, the lower the degree of deformation, the less likely the tendency of margarine to show syneresis. Although both SLM and RCM showed lower deformation along time, SLM had lower % strain than RCM (Fig. 6.5a). This means that SLM is least likely to show syneresis. During dynamic analysis, the frequency (Hz) at which G' and G" cross over is calculated as a measure of spreadibility. The higher the crossover frequency, the more spreadable the margarine is [30]. In Fig. 6.5b, SLM has lower cross over frequency (0.4 Hz) than RCM (2.5 Hz) meaning that SLM will be more difficult to spread. In stress viscometry, stress is applied stepwise to margarine and viscosity is calculated. Samples showing high viscosities at low stresses would be more difficult to spread. Again, SLM had higher viscosity at low stress than RCM explaining its difficult to spread behavior (Fig. 6.5c).

Fig 6.6 shows the microstructural morphology of SLM and ECM. The main structural component of plastic fats is the crystalline network which can be studied by PLM. Attributes such as spreadibility and the melting sensation depend on the mechanical strength of the crystalline network [12]. PLM can distinguish between the solid (brighter) and the liquid phase (darker) based on how they refract the light. Crystal morphology affects product consistency and mouthfeel. Smaller crystals lead to firmer fats with smooth texture or mouthfeel, whereas larger crystals or crystal aggregates

produce softer fats and may impart grainy texture or mouthfeel to the final product. Both, individual crystals and crystal aggregates were observed in the samples. RCM had more uniformly dispersed, sharp, and finer crystals whereas SLM had larger diffused crystals. This difference in distribution pattern and size of fat crystals influences the spreadibility, texture, and mouthfeel of margarines. RCM is likely to be smoother and easily spreadable than SLM due to its microstructural morphology.

A triangle test was used to evaluate the difference between SLM and RCM. Margarine samples were spread on toast and then given to the panelist. This was done to eliminate bias due to spreadibility difference and so that information on overall flavor and mouthfeel could be obtained. No significant difference ($P > 0.05$) was observed between SLM and RCM (data not shown).

In this study, we were able to synthesize SL suitable for margarine formulation. The second-order polynomial model developed in this study has strong predictability and reproducibility power. Novozym 435 synthesized SL (SL1) was suitable as hard margarine fat stock because of its desirable fatty acid content, TAG species, melting temperature, and solid fat content. It had no *trans* fat compared to 19.1 mol% TFA in commercial margarine fat. It was also more oxidatively stable than commercial margarine fat. Although the margarine formulated with SL (SLM) and reformulated commercial margarine (RCM) had different hardness, solid fat content, and spreadibility, there was no difference in their taste. Therefore, our results suggested that the interesterified product (SL1) could be used as an alternative to partially hydrogenated fat to formulate *trans*-free foods by the food industry. The margarine formulated with this SL did not contain any *trans* fat and had properties suitable for its use as hard margarine.

ACKNOWLEDGMENTS

We would like to thank Dr. Alejandro G. Marangoni (University of Guelph, Canada) for his assistance with the solid fat content analysis. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2009-65503-05734 from the USDA National Institute of Food and Agriculture.

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FIGURE CAPTIONS

Fig. 6.1a Contour plot showing the effect of substrate molar ratio and temperature on stearic acid incorporation while keeping the time constant at 6 h using Novozym 435 lipase.

Fig. 6.1b Contour plot showing the effect of substrate molar ratio and temperature on stearic acid incorporation while keeping the time constant at 14 h using Lipozyme TLIM lipase.

Fig. 6.2a. Melting thermograms of cottonseed oil (CO), palm stearin (PS), Novozym 435-catalyzed structured lipid (SL1), its corresponding physical blend (PB1), Lipozyme TLIM-catalyzed structured lipid (SL2), and its corresponding physical blend (PB2).

Fig. 6.2b. Crystallization thermograms of cottonseed oil (CO), palm stearin (PS), Novozym 435-catalyzed structured lipid (SL1), its corresponding physical blend (PB1), Lipozyme TLIM-catalyzed structured lipid (SL2), and its corresponding physical blend (PB2).

Fig. 6.2c. Melting and crystallization thermograms of large-scale structured lipid (SL_{ls}) and extracted fat from commercial margarine (EF_{cm}).

Fig. 6.3. Solid fat content (%) of Novozym 435-catalyzed structured lipid (SL1), its corresponding physical blend (PB1), Lipozyme TLIM-catalyzed structured lipid (SL2), its corresponding physical blend (PB2), large-scale structured lipid (SL_{ls}), and extracted fat from commercial margarine (EF_{cm}).

Fig. 6.4. Texture profile analysis of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM). Each value is the mean of triplicates ±

standard deviation. Columns with the same letter within each texture attribute are not significantly different at $P < 0.05$.

Fig. 6.5a. Creep analysis of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM).

Fig. 6.5b. Dynamic analysis of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM).

Fig. 6.5c. Stress viscometry of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM).

Fig. 6.6. Morphology of fat crystals of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM).

Table 6.1 Experimental settings of the factors and the responses used for optimization by response surface methodology

Exp	Temp[a)] [°C]	Time [h]	SR[b)]	Enz[c)]	Stearic acid inc[d)] [mol%]
N1	55	6	2	TLIM[e)]	5.3±1.1
N2	65	10	2	TLIM	4.8±0.4
N3	50	18	2	TLIM	5.4±0.6
N4	60	22	2	TLIM	5.1±0.9
N5	50	6	3	TLIM	5.1±0.7
N6	65	6	3	TLIM	5.2±0.2
N7	55	22	3	TLIM	5.5±1.0
N8	50	10	4	TLIM	5.3±0.7
N9	65	22	4	TLIM	5.3±0.2
N10	55	6	5	TLIM	5.3±0.5
N11	60	10	5	TLIM	5.5±0.1
N12	65	18	5	TLIM	5.5±0.3
N13	50	22	5	TLIM	5.5±0.8
N14	50	6	2	N435[f)]	5.1±1.2
N15	65	6	2	N435	4.4±0.9
N16	50	22	2	N435	4.9±1.1
N17	65	22	2	N435	4.3±0.9
N18	55	14	3	N435	5.7±0.5
N19	60	18	4	N435	5.6±0.8
N20	50	6	5	N435	5.9±0.2
N21	65	6	5	N435	5.6±0.5
N22	50	22	5	N435	5.7±0.6
N23	65	22	5	N435	5.5±0.3
N24	65	22	5	N435	5.5±0.4
N25	65	22	5	N435	5.6±0.3
N26	65	22	5	N435	5.4±0.2

Each value is the mean of triplicates ± standard deviation.
[a)] Temp, temperature
[b)] SR, substrate molar ratio (PS:CO)
[c)] Enz, enzyme
[d)] Inc, incorporation
[e)] TLIM, Lipozyme TLIM (*Thermomyces lanuginosus* lipase)
[f)] N435, Novozym 435 (*Candida antarctica* lipase)

Table 6.2 Total and *sn*-2 positional fatty acid [mol%] of the substrates, gram-scale, and large-scale products

Fatty acid			C14:0	C16:0	C16:1*c*	C18:0	C18:1*t*	C18:1*c*	C18:2n6*t*	C18:2n6*c*	C18:3n3	Minor[a)]
Substrate	CO[b)]	total	0.7±0.0	23.3±1.8	0.6±0.0	2.6±0.8	nd[c)]	16.8±1.0	0.4±0.0	54.9±3.4	0.2±0.0	0.7
		sn-2	nd	3.3 ± 0.4	nd	nd	nd	16.7±1.1	nd	80.7±4.2	nd	nd
	PS[d)]	total	1.4±0.9	58.1±4.9	0.1±0.0	5.2±1.1	nd	26.9±2.7	0.4±0.1	7.6±1.1	0.2±0.0	0.9
		sn-2	nd	48.9±4.3	nd	2.9±0.9	nd	38.3±2.2	nd	11.8±1.0	nd	nd
Gram scale	SL1[e)]	total	1.1±0.0a	46.0±3.9a	0.2±0.0a	5.7±1.0a	nd	24.9±2.1a	nd	20.6±1.9a	0.2±0.0a	0.5
		sn-2	nd	39.4±2.9a	nd	3.4±1.1a	nd	36.6±2.9a	nd	19.8±1.8a	nd	nd
	PB1[f)]	total	1.0±0.0a	47.8±3.2b	0.2±0.0a	4.3±0.6b	nd	21.5±1.7b	nd	25.8±2.4b	0.1±0.0a	0.3
		sn-2	nd	30.9±3.1b	nd	nd	nd	44.3±2.9b	nd	24.8±2.0b	nd	nd
	SL2[g)]	total	1.0±0.2a	42.4±4.1c	0.2±0.0a	5.1±1.1a	nd	22.7±2.5bc	nd	29.0±3.8c	0.2±0.0a	0.4
		sn-2	nd	53.6±4.2c	nd	5.0±0.8b	nd	25.1±1.8c	nd	16.3±1.9c	nd	nd
	PB2[h)]	total	1.1±0.0a	48.8±2.7a	0.2±0.0a	4.6±1.3b	nd	23.8±2.8ac	nd	21.1±2.1d	0.2±0.0a	0.4
		sn-2	nd	29.5±3.4b	nd	nd	nd	43.1±4.7b	nd	27.4±3.2d	nd	nd
Large scale	SL_{ls}[i)]	total	1.1±0.1a	46.1±3.3a	0.2±0.0a	5.5±0.6a	nd	24.3±2.1a	nd	23.1±1.3a	0.2±0.0a	0.4
		sn-2	nd	38.2±3.8a	nd	3.3±0.5a	nd	37.5±3.6a	nd	21.4±1.7a	nd	nd
	EF_{cm}[j)]	total	0.1±0.0b	10.2±1.5b	0.1±0.0a	6.1±0.7a	18.7±1.9	31.6±3.2b	0.4±0.0	29.4±1.9b	3.2±0.2b	0.3
		sn-2	nd	6.5± 1.4b	nd	5.6±1.5b	15.6±2.7	32.1±3.6b	nd	40.3±4.8b	nd	nd

Each value is the mean of triplicates ± standard deviation. Values with the same letter in each column within total and *sn*-2 rows separately are not significantly different at $P < 0.05$. Comparisons were done separately within gram-scale and large-scale but not between them.

a) Minor, sum of C12:0, C20:0, C21:0, C22:0, C24:0

*b)*CO, cottonseed oil

*c)*nd, not detected

*d)*PS, palm stearin

*e)*SL1, Novozym 435-catalyzed structured lipid

*f)*PB1, corresponding physical blend of SL1

*g)*SL2, Lipozyme TLIM-catalyzed structured lipid

h) PB2, corresponding physical blend of SL2

i)SL_{ls}, large-scale SL1

j)EF_{cm}, extracted fat from commercial margarine

Table 6.3 Relative concentration [%] of triacylglycerol (TAG) species in substrates and products

	CO[a)]	PS[b)]	SL1[c)]	PB1[d)]	SL2[e)]	PB2[f)]	SL_{ls}[g)]	EF_{cm}[h)]
LnOLn	nd[i)]	nd	1.1±0.9	nd	0.1±0.0	nd	1.1±0.0	5.1±1.2
LLLn	nd	nd	1.4±1.0	nd	1.7±0.0	nd	1.4±0.0	1.7±0.9
LLL	22.7±3.1	nd	nd	nd	nd	nd	nd	14.4±1.1
LOL	11.8±1.6	nd	nd	nd	nd	nd	nd	7.2±0.8
PLL	36.5±3.7	nd	2.0±0.8	1.8±0.9	2.2±0.0	1.5±0.1	2.0±0.1	6.3±0.5
SLL	nd	nd	0.7±0.0	nd	0.8±0.0	nd	0.6±0.0	0.3±0.0
OOL	1.7±0.9	nd	nd	nd	nd	nd	nd	nd
POL	17.6±2.1	2.0±0.6	9.0±1.0	7.3±0.9	15.5±1.1	6.4±0.2	8.8±0.4	5.8±0.3
PLP	9.6±1.1	6.5±0.3	11.2±1.1	10.2±1.1	18.0±1.2	9.9±0.7	10.9±0.3	nd
OOO	nd	1.8±0.9	1.5±0.2	0.2±0.0	1.8±0.7	1.3±0.2	1.8±0.0	2.2±0.8
POO	nd	14.8±1.2	15.5±0.9	14.7±1.0	13.0±1.0	12.8±1.7	16.2±1.8	54.3±4.3
POP	nd	47.3±3.2	36.8±2.7	49.2±3.5	39.4±2.3	46.8±4.2	37.8±3.2	nd
PPP	nd	26.9±2.9	16.0±2.1	19.7±1.3	15.4±0.8	20.4±2.7	16.4±1.6	nd
PSO	nd	nd	nd	nd	nd	nd	0.2±0.0	nd
SOO	nd	nd	nd	nd	nd	nd	0.9±0.0	2.2±0.2

Each value is the mean of triplicates ± standard deviation. Ln, linolenic, O, oleic, L, linoleic, P, palmitic, S, stearic

[a)] CO, cottonseed oil

[b)] PS, palm stearin

[c)] SL1, Novozym 435-catalyzed structured lipid

[d)] PB1, corresponding physical blend of SL1

[e)] SL2, Lipozyme TLIM-catalyzed structured lipid

[f)] PB2, corresponding physical blend of SL2

[g)] SL_{ls}, large-scale SL1

[h)] EF_{cm}, extracted fat from commercial margarine

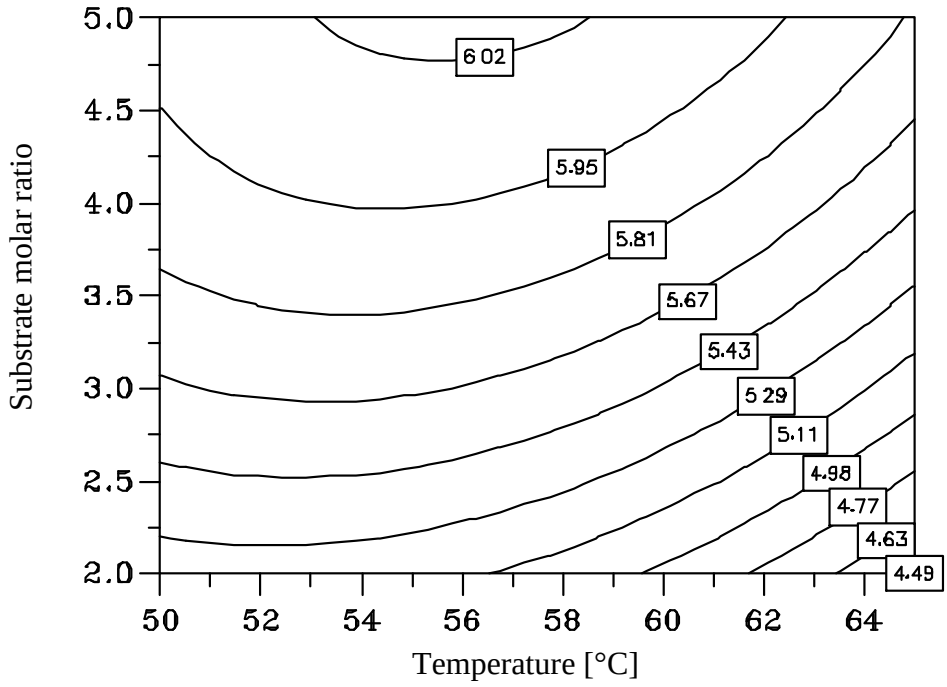
[i)] nd, not detected

Table 6.4 Tocopherol content and oxidative stability index (OSI) of substrates and large-scale products

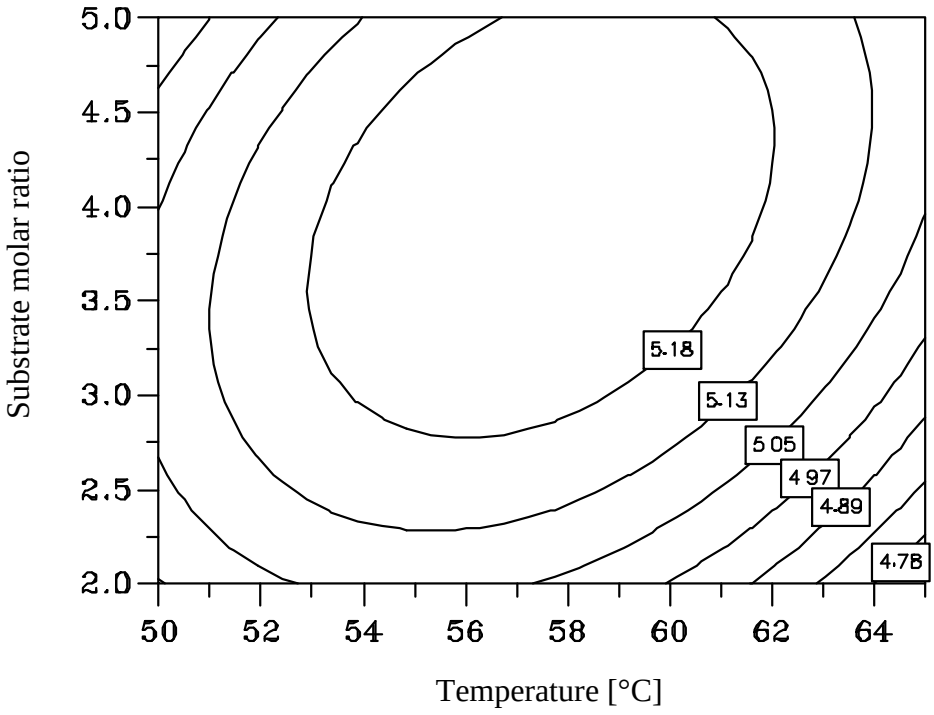
	Tocopherol content [ppm]							OSI at 110 °C [h]
	α-T[a)]	α-T3[b)]	β-T	γ-T	γ-T3	δ-T	δ-T3	
PS[c)]	48.6±4.3	52.3±2.3	nd[d)]	nd	142.6±1.3	nd	32.0±1.1	12.3±1.4
CO[e)]	316.5±4.7	nd	nd	432.9±8.2	nd	nd	nd	7.1±1.8
Before SPD[f)]	505.9±7.3	204.2±6.6	nd	428.9±5.8	568.4±3.5	nd	125.0±2.7	-
SL_{ls}[g)]	310.9±3.9	100.8±4.4	nd	275.7±3.8	310.7±4.3	nd	68.2±1.9	20.2±0.6
SPD waste	170.4±2.5	85.6±1.7	nd	140.5±2.2	236.6±3.7	nd	47.5±1.9	-
EF_{cm}[h)]	56.3±1.2	nd	2.3±1.1	810.6±7.7	nd	283.1±8.9	nd	17.3±0.8

Each value is the mean of triplicates ± standard deviation

*a)*T, tocopherol
*b)*T3, tocotrienol
c) PS, palm stearin
*d)*nd, not detected
e) CO, cottonseed oil
*f)*SPD, short-path distillation
g)SL_{ls}, large-scale SL1
h)EF_{cm}, extracted fat from commercial margarine

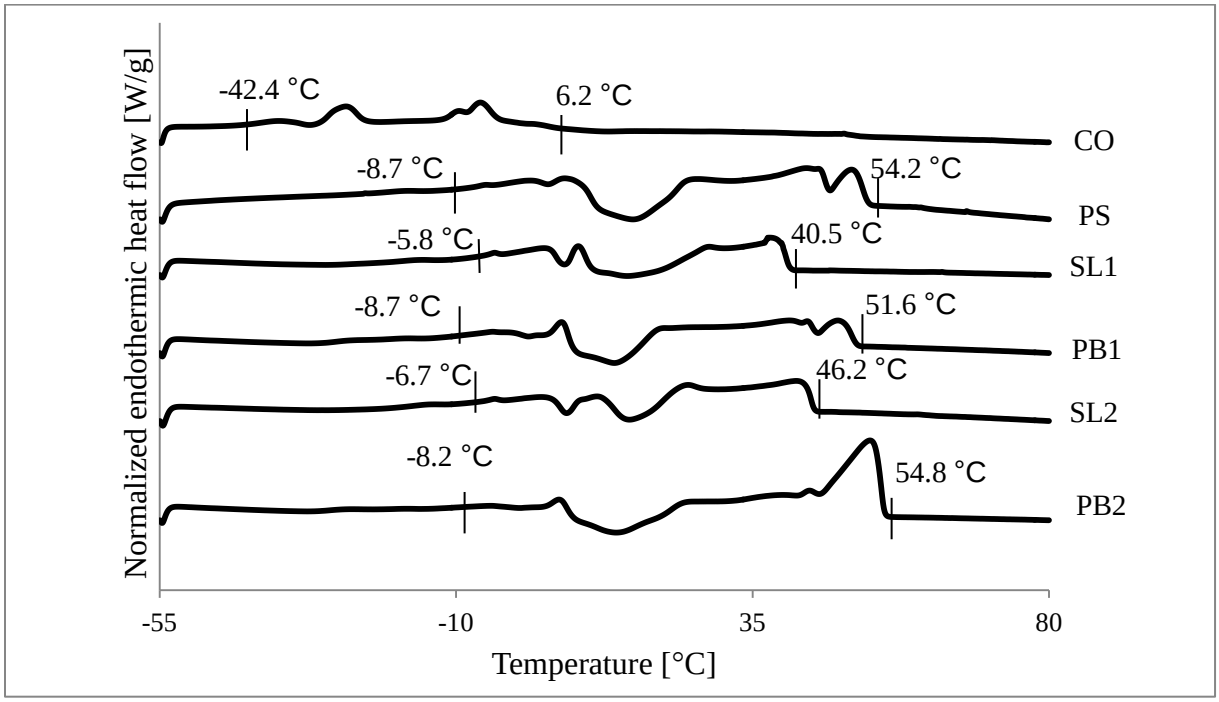

Substrate molar ratio
5.0
4.5
4.0
3.5
3.0
2.5
2.0
6.02
5.95
5.81
5.67
5.43
5.29
5.11
4.98
4.77
4.63
4.49
50
52
54
56
58
60
62
64
Temperature [°C]

(a)

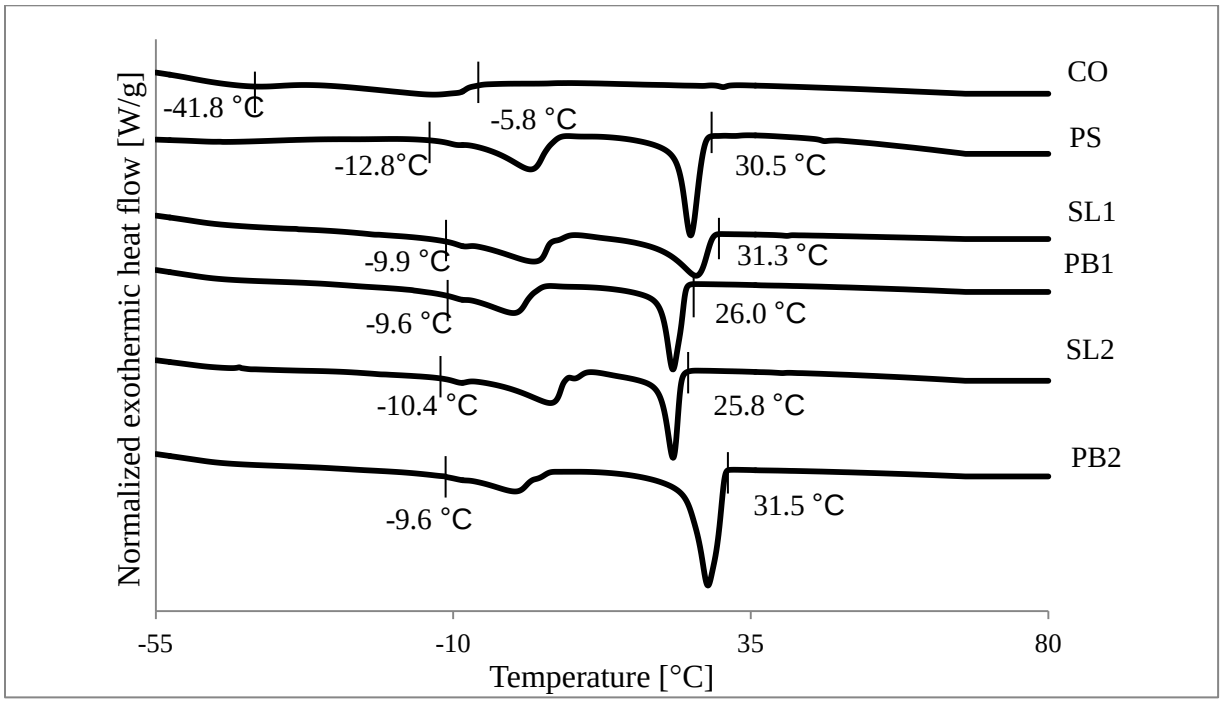

Substrate molar ratio
5.0
4.5
4.0
3.5
3.0
2.5
2.0
5.18
5.13
5.05
4.97
4.89
4.78
50
52
54
56
58
60
62
64
Temperature [°C]

(b)

Fig. 6.1

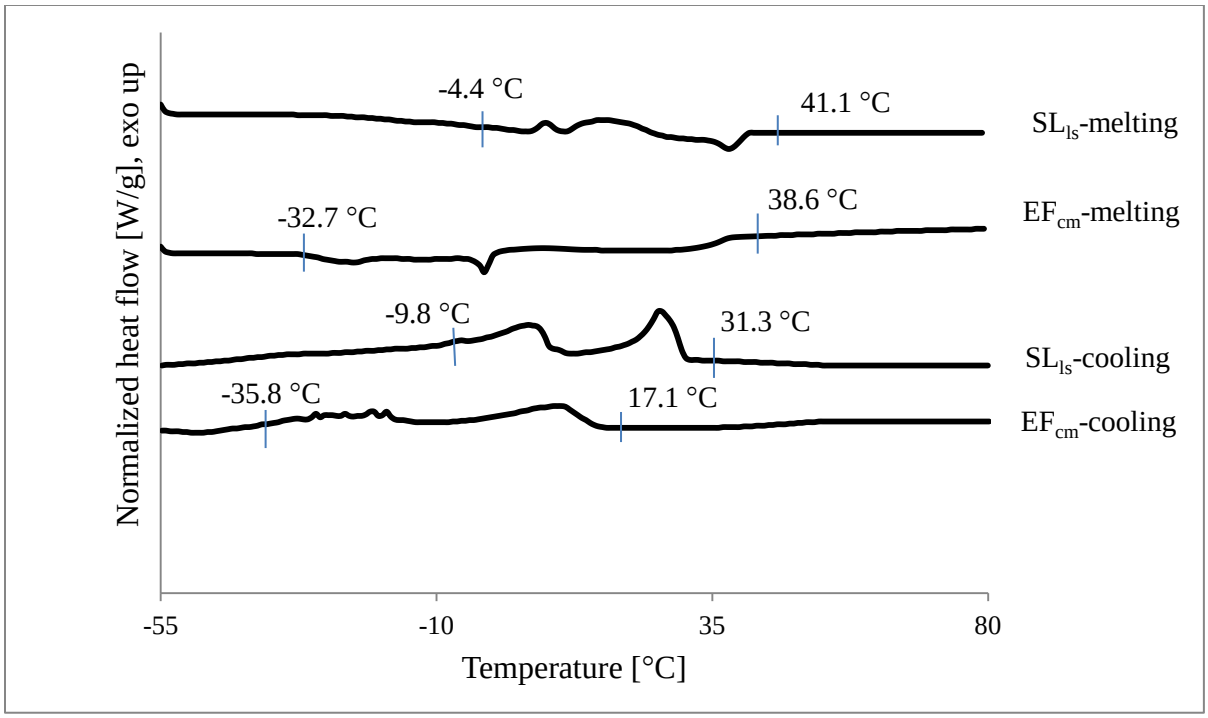

Normalized endothermic heat flow [W/g]
-42.4 °C
6.2 °C
CO
-8.7 °C
54.2 °C
PS
-5.8 °C
40.5 °C
SL1
-8.7 °C
51.6 °C
PB1
-6.7 °C
46.2 °C
SL2
-8.2 °C
54.8 °C
PB2
-55
-10
35
80
Temperature [°C]

(a)


Normalized exothermic heat flow [W/g]
CO
-41.8 °C
-5.8 °C
PS
-12.8°C
30.5 °C
SL1
-9.9 °C
31.3 °C
PB1
-9.6 °C
26.0 °C
SL2
-10.4 °C
25.8 °C
PB2
-9.6 °C
31.5 °C
-55
-10
35
80
Temperature [°C]

(b)

Fig. 6.2


Normalized heat flow [W/g], exo up
-4.4 °C
41.1 °C
SLls-melting
-32.7 °C
38.6 °C
EFcm-melting
-9.8 °C
31.3 °C
SLls-cooling
-35.8 °C
17.1 °C
EFcm-cooling
-55
-10
35
80
Temperature [°C]

(c)

Fig. 6.2

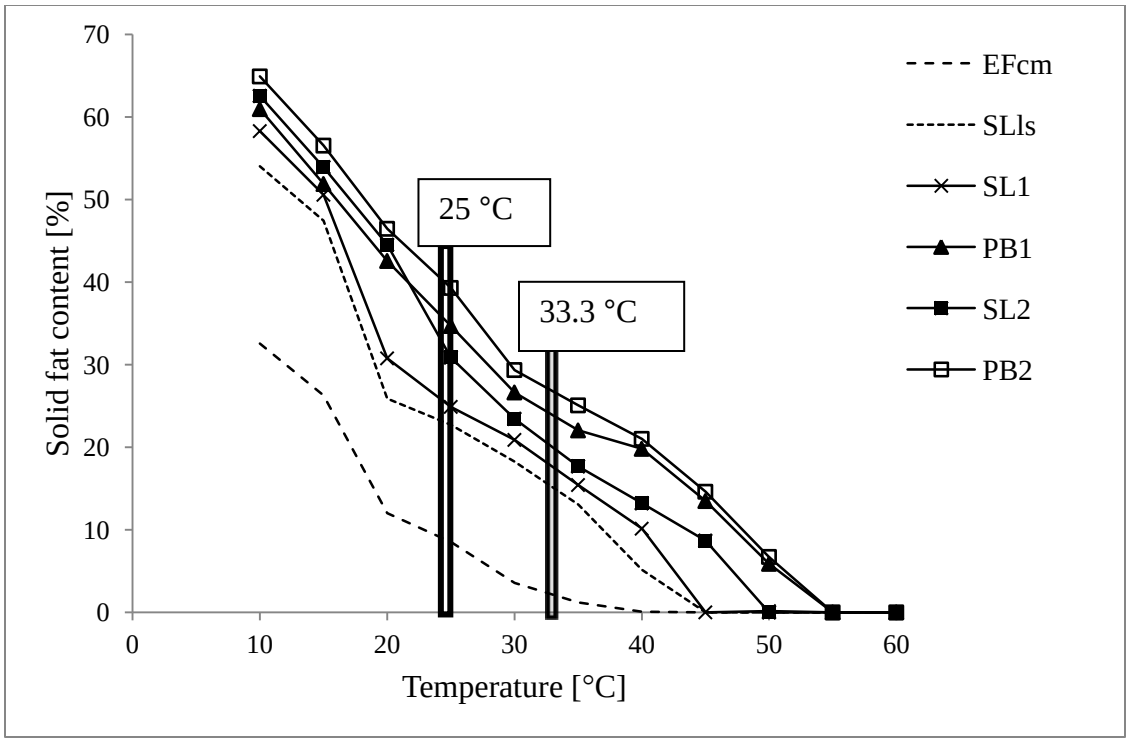

70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Solid fat content [%]
Temperature [°C]
25 °C
33.3 °C
EFcm
SLls
SL1
PB1
SL2
PB2

Fig. 6.3

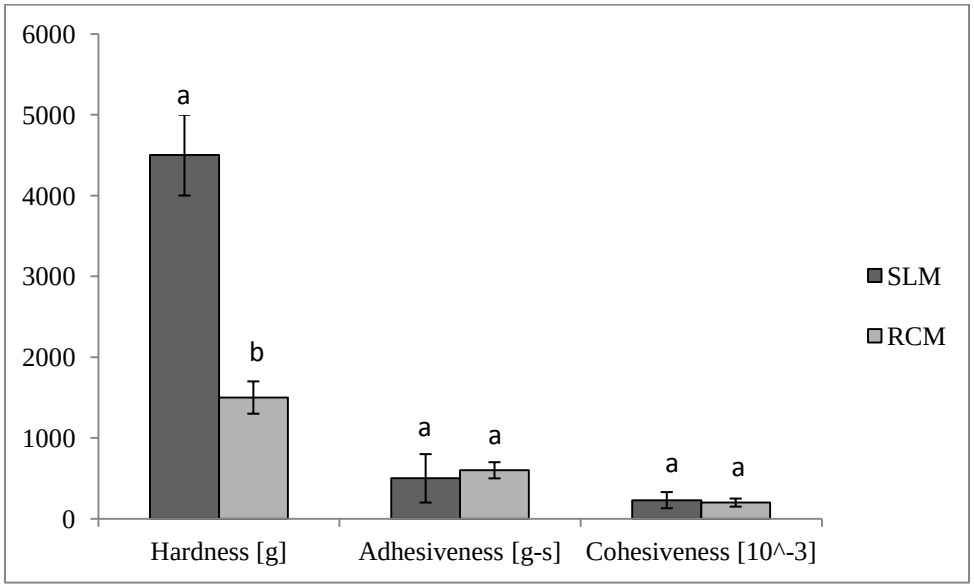
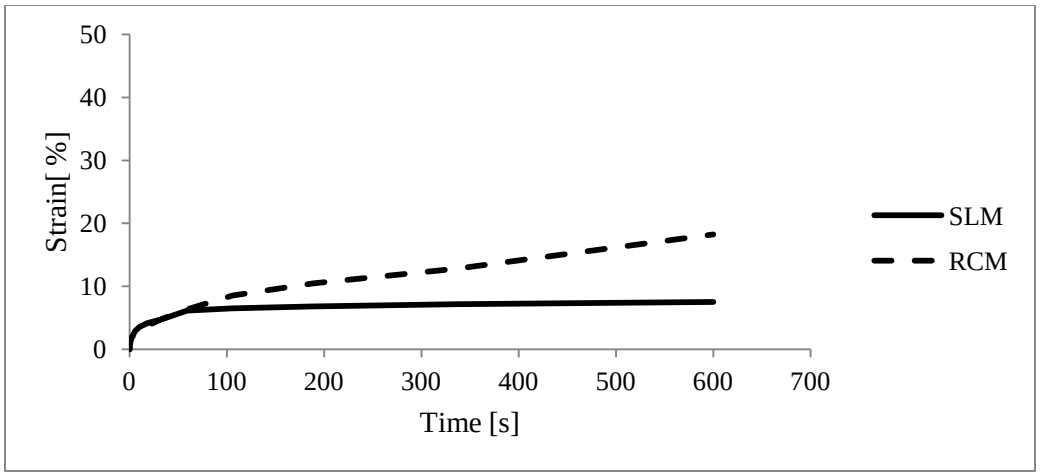
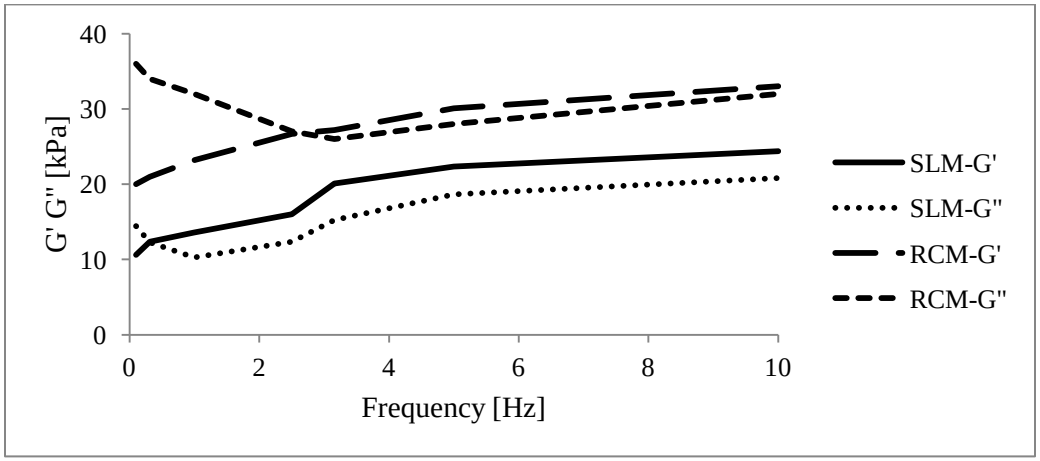

6000
5000
4000
3000
2000
1000
0
a
b
a
a
a
a
SLM
RCM
Hardness [g]
Adhesiveness [g-s]
Cohesiveness [10^-3]

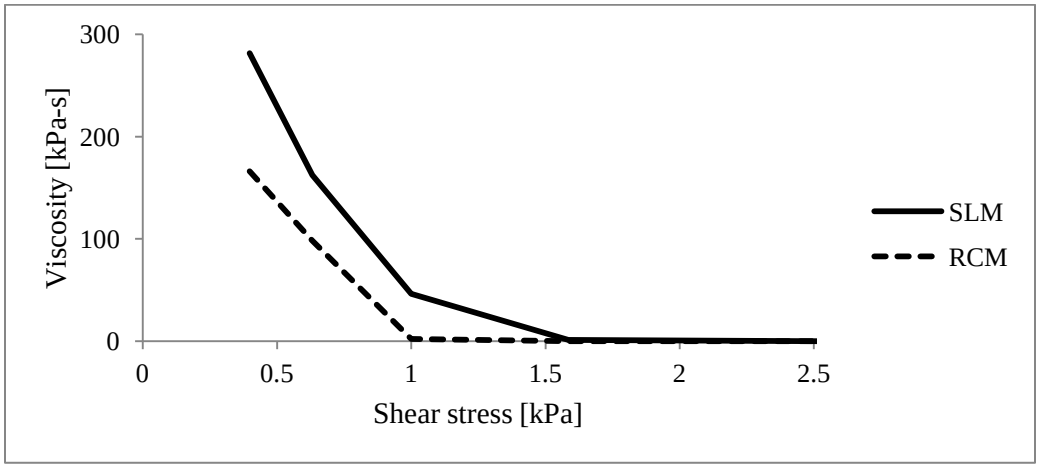
Fig. 6.4


50
40
30
20
10
0
Strain[%]
0
100
200
300
400
500
600
700
Time [s]
SLM
RCM

(a)


40
30
20
10
0
G' G'' [kPa]
0
2
4
6
8
10
Frequency [Hz]
SLM-G'
SLM-G''
RCM-G'
RCM-G''

(b)


300
200
100
0
Viscosity [kPa-s]
0
0.5
1
1.5
2
2.5
Shear stress [kPa]
SLM
RCM

(c)

Fig. 6.5

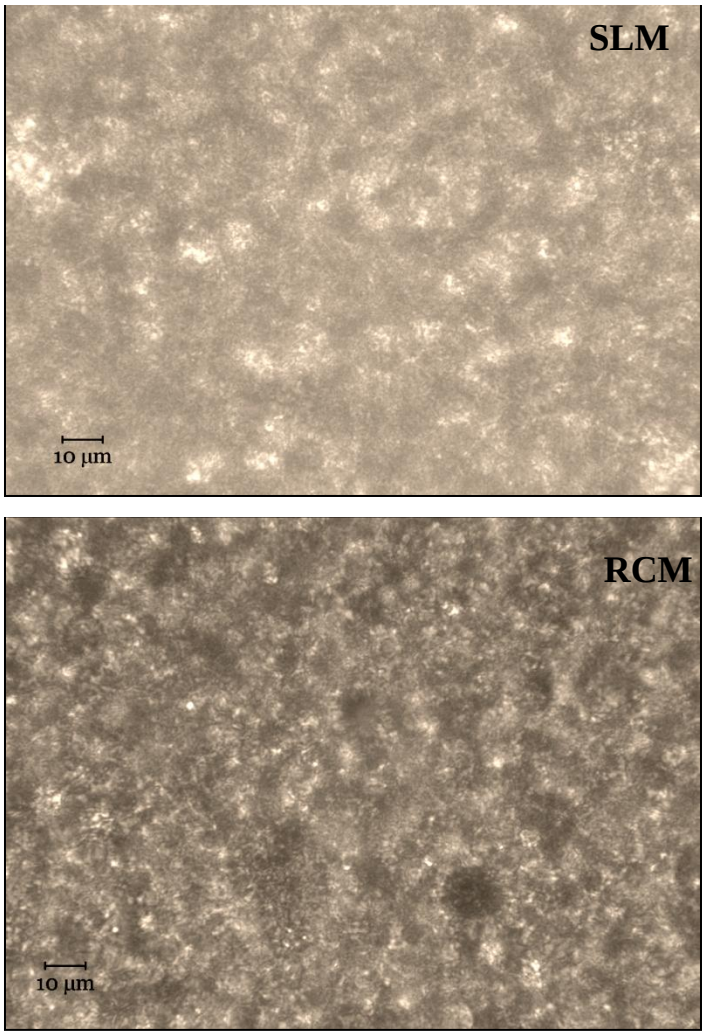


Fig. 6.6

CHAPTER 7

PRODUCTION OF *TRANS*-FREE MARGARINE WITH ENZYMATICALLY INTERESTERIFIED FULLY HYDROGENATED COTTONSEED OIL AND PALM STEARIN[1]

[1]Pande, G. and Casimir C. Akoh. To be submitted to *Journal of the Science of Food and Agriculture.*

ABSTRACT

Background: Enzymatic synthesis of *trans*-free structured margarine fat analogs from fully hydrogenated cottonseed oil and palm stearin was optimized using response surface methodology. *trans*-Free hard margarine was produced with the enzymatically interesterified structured lipid (SL)

Results: The yield % for Lipozyme TLIM-catalyzed SL (SL1) and Novozym 435-catalyzed SL (SL2) were 89.3 and 84.8 %, respectively. Desirable product composition (233.0 g kg^{-1} stearic acid) was achieved at 65 °C, 16.5 h, and 2:1 (PS:HCO) substrate molar ratio using Lipozyme TLIM lipase. SL1 was used to formulate *trans*-free margarine with β'-polymorph and high solid fat content. The higher proportion of saturated triacylglycerols in large-scale SL ($SL_{ls)}$ resulted in higher melting point for SL_{ls} (50.1 °C) than extracted fat from commercial margarine (EF_{cm}) (38.6 °C).

Conclusions: The SL showed desirable physical properties and suitable crystal form (β'-polymorph) for possible use as a hard margarine stock. Therefore, our result suggested that the interesterified fat without *trans* fatty acid could be used as an alternative to partially hydrogenated fat.

INTRODUCTION

Margarine is a water-in-oil (w/o) emulsion with specific standards of identity regulated by FDA 21CFR166.110[1]. These include specific fat content (80% lipid phase) and limitations on ingredient usage. The lipid phase consists of fat, antioxidant, and emulsifier whereas the aqueous phase contains water, preservatives and salt. There are three main types of margarines, table margarine which is softer and easily spreadable, a firmer industrial margarine, and puff pastry margarine with higher solid fat content (SFC) for required plasticity[2]. Table margarine can be further classified as tub margarine which are spreadable at refrigeration temperature and stick margarine which are spreadable at ambient temperature. Three basic steps in margarine production are: i) emulsification of the oil and aqueous phases, ii) crystallization of the fat phase, and iii) plasticization of the crystallized emulsion. Many vegetable oils have been modified to produce margarine with required SFC and melting behavior.

Oxidation can be a major problem when using vegetable oils. Saturated fatty acids (SFA) are less prone to oxidative rancidity than unsaturated fatty acids (UFA)[3]. Oxidation leads to rancidity resulting in undesirable flavors and short shelf life of the final product. Therefore, most vegetable oils containing a large percentage of UFAs need to be partially hydrogenated in order to increase shelf life. Partial hydrogenation is the main process to convert liquid oils to solid fats for use in margarines and shortenings. As the degree of hydrogenation increases, the amount of SFA increases and UFA decrease. Along with saturation of double bonds, isomerization and migration of double bonds also occur. This gives rise to *trans* fatty acids (TFA) in partially hydrogenated fats/oils. A fully hydrogenated fat has no TFA as all FA have been converted to SFA.

Partial hydrogenation is responsible for 80% of *trans* fatty acids (TFA) in the US diet[4]. The *trans* conformation results in a straight chain similar to that of a SFA. Therefore, *trans* form have a higher melting point than their corresponding *cis* form[5]. High intake of TFA is associated with increased LDL and cholesterol[6], cancer[7], and coronary heart disease[8]. Dietary Guidelines for Americans 2010 suggest that TFA consumption should be as low as possible[9]. Interesterification is an alternative to partial hydrogenation in which ester exchange occurs between two acyl groups, in the presence of a catalyst, thereby altering the overall chemical composition and physical properties of the interesterified fats. During interesterification reaction there is no double bond isomerization or change in unsaturation as in partial hydrogenation process[10]. Lipases are preferred over chemical interesterification because they are regio-and stereo-specific and offer a better control over the final product[11].

The first objective of this research was to optimize the reaction conditions for enzymatic syntheses of *trans*-free margarine fat analogues using fully hydrogenated cottonseed oil (HCO) and palm stearin (PS) by response surface methodology (RSM). The second objective was to scale-up the syntheses of the SLs, characterize and compare them with physical blends (PBs). The third objective was to formulate margarine using SL and compare it with a commercial margarine.

MATERIALS AND METHODS

Materials

Fully hydrogenated cottonseed oil (HCO) and palm stearin (PS) were kindly provided by Cargill Inc. (Minneapolis, MN). The immobilized enzymes, Lipozyme® TLIM (*Thermomyces lanuginosus* lipase, *sn*-1,3 specific, specific activity 250 IUN/g: IUN=Interesterification Unit) and Novozym® 435 (*Candida antarctica* lipase, non-

specific, specific activity 10,000 PLU/g: PLU=Propyl Laurate Unit) were purchased from Novozymes North America Inc. (Franklinton, NC). Lipid standards, Supelco 37 Component FAME mix, 14% boron trifluoride in methanol, triolein, 2-oleoylglycerol, tripalmitin, and tristearin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Nonadecanoic acid and its methyl ester, were purchased from TCI America (Portland, OR). Commercial stick margarine and food grade soy lecithin fluid were purchased from a local grocery store. Organic solvents and chemicals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA).

Experimental design for response surface methodology (RSM)

RSM is an important statistical technique for the development, improvement and optimization of processes. The independent variables used in this study were time (6, 10, 14, 18, 22 h), temperature (50, 55, 60, 65 °C), substrate molar ratio (PS:HCO, 2, 3, 4, 5), and two enzymes (Lipozyme TLIM and Novozym 435 lipases). The dependent variable/response was stearic acid content. RSM was performed using Modde 9.0 software (Umetrics, Umeå, Sweden) to obtain the relationship between the response and the independent variables. Twenty six runs were generated and experiments at each design point were randomly performed in triplicate. SL synthesis was performed in screw-cap test tubes in 3 mL hexane to maintain the substrates in a liquid form. Specific enzymes were added at 10% total substrate weight. Test tubes were then incubated in water bath shaker at 200 rpm for different time-temperature combinations. After the reaction was over, the product was filtered through anhydrous sodium sulfate column twice to remove the enzymes. TAG bands were collected using TLC and analyzed for FA profile after conversion to methyl esters. All reactions were performed and analyzed in

triplicates and average values reported. The data obtained from the design in Table 7.1 were used to fit a second-order polynomial function as follows:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i \; + \; \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i \; X_j \; + \; \varepsilon_{ij}$$

where Y = the response; β_0 = constant; β_i = linear (first-order model); β_{ii} = quadratic (second-order model); β_{ij} = interaction term coefficients; X_i and X_j = independent variables; and ε_{ij} = error term.

Fatty acid profile

Lipid samples were converted to fatty acid methyl esters following the AOAC Official Method 996.01[12] and analyzed with Hewlett-Packard 6890 series II gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) using Supelco SP-2560, 100 m x 25 mm x 0.2 μm column. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. Injection volume was 1 μL and a spilt ratio of 20:1 was used. The column was initially held at 140 ºC for 5 min, then increased to 240 ºC at 4 ºC/min, and finally held at 240 ºC for 25 min. Detection was with flame ionization detector at 300 ºC. All samples were analyzed in triplicates and average values reported.

Large - scale SL synthesis

The scaled-up reactions using the optimal conditions achieved from the small-scale reactions were performed in 1L stir-batch reactor. Corresponding physical blends (PB1 and PB2) were also prepared at the same reaction conditions without the respective enzymes. The SL selected for margarine formulation was synthesized in 4L stir-batch reactor. Free fatty acids (FFAs) were removed by short-path distillation (SPD) KDL-4 unit (UIC, Joliet, IL). The reaction products were passed through the unit under high vacuum (<0.2 mmHg absolute pressure) at the flow rate of ca. 10 mL/min. The

evaporator and condenser temperatures were maintained at 185 and 25 °C, respectively. FFAs were determined by AOCS Official Method Ac 5-41[13]. This SL was designated as SL_{ls}.

Fatty acid and positional analysis

Fatty acids were determined as described above. *sn*-2 Positional fatty acid composition was determined following the AOCS Official Method Ch 3-91[14]. All samples were analyzed in triplicates and average values reported.

Triacylglycerol (TAG) molecular species

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

Differential scanning calorimetry (DSC)

Melting and crystallization profiles of lipid samples were determined following AOCS Official Method Cj 1-94[15]. SL1, PB1, SL2, AND PB2 were analyzed with Perkin-Elmer model DSC7 (Norwalk, CT) and SL_{ls} and extracted fat from commercial margarine (EF_{cm}) on Mettler Toledo model DSC 1 STARe System (Columbus, OH). All samples were analyzed in triplicates and average values reported.

Polymorphism

The polymorphic forms of the samples were determined with Bruker D8 Advance diffractometer (Bruker AXS, Inc., Madison, WI). The diffractometer had a 2θ configuration (2θ range used was 20-32°), a solid state detector, and cobalt tube as the X-ray source. The scan rate was 4.2°/min with a step increment of 0.01°. The samples were rotated at 15 rev/min. Samples were melted and poured into rectangular aluminum molds. They were held at room temperature for 2-3 h, at 4 °C for 4-5 h, and then stored at -20 °C overnight. All samples were analyzed in triplicates and average values reported.

Solid fat content (SFC)

SFC was determined according to the AOCS Official Method Cd 16-93b[16] on a BrukerPC/20 Series NMR analyzer, Minispec (Bruker Optics, Milton, On, Canada). All samples were analyzed in duplicates and average values reported.

Margarine formulation

The fat from the commercial margarine was extracted by melting at 80 °C. The top fat layer was decanted into a separatory funnel, washed five times with the same volume of warm water, and filtered through an anhydrous sodium sulfate layer with a Whatman

filter paper (pore size = 0.45 μm) under vacuum. This extracted fat from commercial margarine was designated EF_{cm}.

Margarines were formulated using lipid phase, 80.5% (EF_{cm} or SL_{ls}, 80%; soy lecithin fluid, 0.5%; and TBHQ, 0.01%) and aqueous phase, 19.5% (distilled water, 18%; and table salt, 1.5%). The fat and water were heated to 80 °C. Both phases were poured into a tabletop blender and vigorously mixed for 10 min to emulsify them. Artificial butter flavor was added to mask any difference in the aroma of the samples. The resulting liquid emulsion was then crystallized for 15 min using an ice cream maker (Krups North America, Peoria, IL). The crystallized emulsion was refrigerated overnight and then tempered at room temperature for 4 h. The margarines were vigorously mixed with a hand mixer to obtain a smooth product. The margarine sample were placed into plastic tubs and stored at 4 °C. The margarines formulated with SL_{ls} and EF_{cm} were designated SLM and RCM (reformulated commercial margarine), respectively.

Texture profile analysis (TPA)

Margarine samples were taken out from refrigerator and measured at room temperature (23 °C). A double compression test was performed using a TA-X2 texture analyzer (Stable Micro Systems, London, United Kingdom). A 45º conical probe attached to a 25 kg compression load cell was used to penetrate the samples at 1.0 mm/s to a depth of 10 mm from the sample surface, and withdrawn at the same speed. Textural properties (hardness, adhesiveness, and cohesiveness) of margarines were evaluated at 23 °C. All samples were analyzed in triplicates and average values reported.

Rheological properties

A series of tests were performed on a dynamic stress-controlled rheometer SR5000 (Rheometric Scientific, Piscataway, NJ) at 23 °C to determine the rheological properties of margarine samples. A 25 mm parallel plate (0.6 mm gap) was used for analysis. In dynamic analysis, margarine samples were subjected to a sinusoidally varying stress (0.4 kPa) and the strain output and phase difference between the input and output signals were measured. Storage/elastic (G') and loss/viscous (G'') moduli were calculated from this information. In creep analysis, a constant low stress (0.4 kPa) was applied to samples and deformation per unit time measured as an indicator of emulsion stability. In stress analysis, viscosity was measured across different stress (0.1-2.5 kPa). All samples were analyzed in triplicates and average values reported.

Sensory evaluation

All methods and procedures for sensory studies were approved by the University of Georgia (UGA) Institutional Review Boards on Human Subjects. Thirty three UGA staff and students above 18 yrs of age participated in the sensory evaluation. A triangle test was conducted in individual booths under fluorescent light to study the difference between experimental and commercial margarines. As a significant difference in spreadibility between RCM and SLM was observed in textural and rheological analyses, the margarines were spread on toast and then given to panelists. This was done in order to obtain data for flavor and mouthfeel of the samples rather than just spreadibility. Each participant was provided with 3 coded toasts with margarine samples, two of which were the same and one different. Subjects were to chew the samples but not required to swallow them. They had to identify the odd sample and explain the difference observed.

They were provided with an evaluation sheet with all instructions, water, non-salted crackers (to cleanse the mouth during the test), and empty cups for expectoration.

Statistical analysis

Regression analysis, response surfaces, and statistical significance were performed using Modde 9.0 software (Umetrics, Umea, Sweden) and backward elimination by Statistical Analysis System software (SAS 9.2, Cary, NC). Duncan's multiple-range test was performed using SAS to determine significant difference ($P < 0.05$) between SLs and PBs, SL_{ls} and EF_{cm}, and between SLM and RCM. All samples, except SFC, were analyzed in triplicates and average values reported.

RESULTS AND DISCUSSION

Model fitting and process optimization

After interesterification reaction stearic acid content in the *trans*-free margarine fat analogues ranged from 61.0-310.0 g kg^{-1} (Table 7.1). RSM was used to obtain a quadratic model for the effect of independent variables on the response. The R^2 value (explained variability) was 0.987 and Q^2 (predicted variability) was 0.884 indicating that the model obtained was good with strong prediction ability. The model showed no lack of fit ($P > 0.05$) (data not shown). The model equation after backward elimination at $\alpha=0.05$ can therefore be written as:

Stearic acid incorporation = 22.98 + 0.51Time – 4.63SR ± 1.07Enz – 3.02Time*time ± 0.94Temp*Enz ± 0.99Time*Enz + 0.39Time*SR

where, Temp is temperature in °C, Time in hours, SR is substrate molar ratio, Enz is enzyme. The model was verified by a chi-square test. No significant difference between the observed and predicted values.

The effect of linear and quadratic variables on the response is shown in Fig. 7.1 SR and Novozym 435 had a negative effect on stearic acid incorporation whereas time and Lipozyme TLIM had a positive effect on the response. Temperature was not significant as linear variable but in its interaction term with Lipozyme TLIM, it had a positive effect. Using contour plots, desirable product composition (202.0 g kg^{-1} stearic acid) was achieved at 65 °C, 16.5 h, and 2:1 substrate molar ratio using Lipozyme TLIM (SL1). Novozym 435 gave 250.0 g kg^{-1} stearic acid at 65 °C, 6 h, and 2:1 substrate molar ratio (SL2). Corresponding physical blends (PB1 and PB2) were also prepared.

Product yield

After purification by SPD, the SLs contained less than 0.1% FFA which is an acceptable level to be used as ingredients for food products. Yield % was calculated as:

Yield % = (Final product weight)*100/(Total substrate weight)

The yield % for SL1, SL2, and SL_{ls} were 89.3, 84.8, and 90.2%, respectively. Lipozyme TLIM was immobilized on granulated silica. Silica being highly polar does not absorb the fat tightly and therefore results in higher product recovery. On the other hand, Novozym 435 which was immobilized on macroporous acrylic resin which due to its structure absorbed more product resulting in lower yield.

Total and positional fatty acid profile

Fatty acid (FA) compositions of HCO, PS, interesterified fats, and physical blends are given in Table 7.2.1. The major FAs in HCO were stearic (847.0 g kg^{-1}) and palmitic (30.0 g kg^{-1}). The major FAs in PS were palmitic (581.0 g kg^{-1}) and oleic acids (269.0 g kg^{-1}). Being fully hydrogenated, HCO had no TFA whereas PS contained 4.0 g kg^{-1} TFA. SLs are lipids (usually TAG, but can also include monoacylglycerols (MAG),

diacylglycerols (DAG), and glycerophospholipids (GPL)) that have been structurally modified by changing the positions of FAs, or the FA profile, or synthesized to yield novel TAGs through chemical or enzymatic processes[17]. Compared to PS, palmitic acid decreased by 29.9 and 27.0% in SL1 and SL2, respectively, whereas stearic acid increased by 348.1 and 344.2% in SL1 and SL2, respectively. SL1 contained 407.0 g kg^{-1} palmitic acid and 233.0 g kg^{-1} stearic acid and 345.0 g kg^{-1} UFA. *sn*-2 Positional fatty acid of the substrates and products are given in Table 7.2.2. The major FA at *sn*-2 position of HCO was stearic (436.0 g kg^{-1}). HCO had more than 70% SFA at *sn*-2 position. On the other hand, *sn*-2 position of PS consisted of approximately equal amount of SFA and UFA. The main FA at *sn*-2 position of PS was palmitic acid (489.0 g kg^{-1}) followed by oleic acid (383.0 g kg^{1}). SL2 had higher SFA (695.0 g kg^{-1}) at *sn*-2 position compared to SL1 (628.0 g kg^{-1}). The composition and positional distribution of FA are important determinants of the chemical and physical properties of the SL. The type of FA and its position on the glycerol backbone greatly influences the physical, functional, and nutritional behavior of dietary fats in food products. The saturated fatty acids are better hydrolyzed and absorbed when present at *sn*-1, 3 positions while unsaturated fatty acids are better absorbed at *sn*-2 position[18]. The major FA in the EF_{cm} were oleic (316.0 g kg^{-1}) and linoleic (294.0 g kg^{-1}). It had 191.0 g kg^{-1} total TFA of which 156.0 g kg^{-1} was present at *sn*-2 position. EF_{cm} had 724.0 and 121.0 g kg^{-1} UFA and SFA, respectively, at *sn*-2 position.

TAG molecular species

TAG molecular species influences the melting, crystallization, and texture properties of the final product. Fats consisting of wide range of TAG molecular species tend to

crystallize in the β′ form, the desirable polymorph in margarines[10]. β form is obtained when there is little diversity in TAG species. TAG molecular species are analyzed using reverse phase HPLC-ELSD. The concentration (g kg^{-1}) of TAG molecular species is given in Table 7.3. The major TAG species in HCO were PSS (506.0 g kg^{-1}) and SSS (352.0 g kg^{-1}). Stearic acid was the main acyl moiety in the TAGs of HCO since it has 847 g kg^{-1} stearic acid. In PS, since palmitic is the predominant FA, the major TAG species were POP (473.0 g kg^{-1}) and PPP (269.0 g kg^{-1}). However, in all SLs and PBs, POO was the major TAG. EF_{cm} had the highest content of POO (543.0 g kg^{-1}) followed by PBs (~420.0 g kg^{-1}) and then SLs (~300.0 g kg^{-1}). The levels of saturated and unsaturated TAGs affect the physical and thermal properties of lipids. Both PBs had higher proportion of SSS (trisaturated) and SUS (disaturated-monounsaturated) type TAGs and lower proportion of SUU (diunsaturated-monsaturated) and UUU (triunsaturated) type TAGs than the SLs. This affected the melting profiles of SLs and PBs as explained below.

Thermal behavior

Melting and crystallization profiles of the substrates and products are shown in Figs. 7.2a and 7.2b, respectively. The melting completion temperature (T_{mc}) depends on the amount and type of the TAG molecular species. T_{mc} of HCO, PS, SL1, PB1, SL2, and PB2 were 63.8, 54.2, 50.9, 56.3, 52.1, and 56.2 °C, respectively. The T_{mc} of the SLs was lower than the PBs because both SLs had more UUU and SUU type TAGs and less SSS and SUS type TAGs than PBs. Both SLs were not completely melted at room temperature but SL1 had lower T_{mc} (50.9 °C) than SL2 (52.1 °C) and therefore, can be used in the production of hard margarines. Melting and crystallization profiles of the SL_{ls} and EF_{cm} are shown in

Fig. 7.2c. The higher proportion of saturated TAGs in SL_{ls} resulted in higher melting point for SL_{ls} (50.1 °C) than EF_{cm} (38.6 °C). Therefore, it suggests that SL_{ls} will be more suitable for hard/industrial margarine compared to EF_{cm} which is exracted from spreadable stick margarine. The crystallization onset temperature (T_{co}) of HCO and PS were 47.8 and 31.5 °C, respectively. T_{co} of EF_{cm} was 17.1 °C whereas SLs and PBs had higher T_{co} (38.3 °C)

Solid fat content

SFC is one of the important parameters of margarine functionality. It is a measure of solid/liquid ratio of a fat at various temperatures and helps in classifying margarines for different applications. Three useful measurement temperatures are refrigeration, room, and body temperatures which are related to the spreadibility, product stability, and texture and mouthfeel, respectively, of margarines[10]. The SFC of SLs, PBs, SL_{ls}, and EF_{cm} are shown in Fig 7.3. To prevent any waxy sensation in the mouth, the margarine should have SFC below 3.5% at 33.3 °C[19]. Only EF_{cm} fulfilled this criterion. SLs had higher SFC than EF_{cm} at all temperatures suggesting that they may be suitable for use as hard margarine stock but not as spreadable stick margarine. It can also be used as a blend with other vegetable oils to achieve desired SFC without the use of partial hydrogenation

Polymorphism

The tendency of FAs residues to occur in differentiated crystalline forms in a periodical three-dimensional pattern having the same composition but different structure is called polymorphism. In lipids there are three dominant crystal forms namely, α, β′, and β polymorphs which greatly influence the physical properties and processing of the final product. Short spacings of the major polymorphs were identified as follows: α, a single

spacing at 4.15 Å; β', two strong spacings at 3.8 and 4.2 Å; and β, a very strong spacing at 4.6 and another one usually at 3.85 Å[10]. Interesterification alters the TAG composition, leading to modifications in the crystalline morphology of the fats. Fats containing predominantly β′ TAG crystals impart smooth texture or mouthfeel to margarine since they are softer and allow for good aeration and creaming properties, whereas those with predominantly β TAG crystals impart grainy texture. Both SLs and EF_{cm} had β′ crystal as the dominant polymorph type.

Margarine characteristics

Based on the above mentioned analyses, Lipozyme TLIM synthesized SL (SL1) was found to be more suitable than SL2 as margarine fat stock and was scaled up (SL_{ls}) for margarine formulation. The margarine formulated with it was designated SLM and compared with reformulated commercial margarine (RCM) produced using EF_{cm}. Textural attributes of SLM and RCM are shown in Fig. 7.4. Temperature during processing and storage of margarine greatly influences the texture of the product. It alters the distribution of solid and liquid TAGs and size of fat crystals. Larger fat crystals and high SFC results in a harder, grainy and more brittle product that tend to be more adhesive and less cohesive[20]. Hardness was measured as the maximum force during the first compression. Hardness is directly proportional to the spreadibility of margarines and spreads. Significant difference ($P < 0.05$) was found between SLM and RCM in terms of hardness. SLM was much harder than RCM implying that it will be difficult to spread. This is because SL_{ls} had more SFA (883.0 g kg^{-1}) than EF_{cm} (167.0 g kg^{-1}). The negative force area for the first compression was reported as adhesiveness which relates to the product's stickiness. Cohesiveness is the ratio of the positive force area during the second

compression to that of the first compression. Cohesiveness relates to how crumbly or brittle a food product is. Significant difference ($P < 0.05$) was found between SLM and RCM for adhesiveness and cohesiveness.

The rheological properties of SLM and RCM are shown in Fig. 7.5. In creep analysis, when a constant low stress is applied to samples deformation per unit time is measured as an indicator of emulsion stability[21]. The lower the degree of deformation, the less likely the margarine will show syneresis i.e separation of water from fat phase. Although both SLM and RCM showed lower deformation along time, SLM had lower % strain than RCM (Fig. 7.5a). This means that SLM is least likely to show syneresis and is harder than RCM. In dynamic analysis, margarine samples are subjected to a sinusoidally varying stress and storage modulus (G′) and loss modulus (G′′) are measured. The higher the G' and G" crossover frequency, the more spreadable the margarine is[21]. In Fig. 7.5b, SLM has much lower cross over frequency (0.2 Hz) than RCM (2.5 Hz) meaning that SLM will be very difficult to spread. During stress viscometry, stress is applied stepwise to margarine and viscosity is calculated. Samples showing high viscosities at low stresses would be more difficult to spread[21]. Again, SLM had higher viscosity at low stress than RCM explaining its lack of spreadibility (Fig. 7.5c).

A triangle test was used to discriminate between SLM and RCM. Margarine samples were spread on toast and then given to the panelist so that they do not base their decision just on spreadibility and information on overall flavor could be obtained. Significant difference ($P < 0.05$) was noted between SLM and RCM even after spreading the margarines on the toast.

In this study, we were able to synthesize SL suitable for hard margarine that may be used in puff pastries, cooking, or baking purposes. This SL can also be used for blending with other vegetable oils for cooking purposes or in a low fat spread. The second-order polynomial model developed in this study has strong predictability and reproducibility power. Lipozyme TLIM-catalyzed SL (SL1) was suitable as hard margarine fat stock because of its FA content, TAG species, melting temperature, and SFC. It had no *trans* fat compared to 191.0 g kg-1 TFA in commercial margarine fat. Therefore, it can be used solely or as a blend with vegetable oils to obtain desired SFC without the use of partial hydrogenation. The margarine formulated with this SL did not contain any *trans* fat and had properties suitable for use as hard margarine.

ACKNOWLEDGMENTS

We would like to thank Dr. Alejandro G. Marangoni (University of Guelph, Canada) for his assistance with the solid fat content analysis. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2009-65503-05734 from the USDA National Institute of Food and Agriculture.

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FIGURE CAPTIONS

Fig. 7.1 Effect of independent variables on the response

Fig. 7.2a. Melting thermograms of fully hydrogenated cottonseed oil (HCO), palm stearin (PS), Lipozyme TLIM-catalyzed structured lipid (SL1), its corresponding physical blend (PB1), Novozym 435-catalyzed structured lipid (SL2), and its corresponding physical blend (PB2).

Fig. 7.2b. Crystallization thermograms of fully hydrogenated cottonseed oil (HCO), palm stearin (PS), Lipozyme TLIM-catalyzed structured lipid (SL1), its corresponding physical blend (PB1), Novozym 435-catalyzed structured lipid (SL2), and its corresponding physical blend (PB2).

Fig. 7.2c. Melting and crystallization thermograms of large-scale structured lipid (SL_{ls}) and extracted fat from commercial margarine (EF_{cm}).

Fig. 7.3. Solid fat content (%) of Lipozyme TLIM-catalyzed structured lipid (SL1), its corresponding physical blend (PB1), Novozym 435-catalyzed structured lipid (SL2), its corresponding physical blend (PB2), large-scale structured lipid (SL_{ls}), and extracted fat from commercial margarine (EF_{cm}).

Fig. 7.4. Texture profile analysis of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM). Each value is the mean of triplicates ± standard deviation. Columns with the same letter within each texture attribute are not significantly different at $P < 0.05$.

Fig. 7.5a. Creep analysis of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM).

Fig. 7.5b. Dynamic analysis of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM).

Fig. 7.5c. Stress viscometry of margarine formulated with structured lipid (SLM) and reformulated commercial margarine (RCM).

Table 7.1 Experimental settings of the factors and the responses used for optimization by response surface methodology

Exp	Temp[a] (°C)	Time (h)	SR[b]	Enz[c]	Stearic acid inc[d] (g kg^{-1})
N1	55	6	2	TLIM[e]	28.1±1.1
N2	65	10	2	TLIM	29.5±0.4
N3	50	18	2	TLIM	31.0±0.6
N4	60	22	2	TLIM	29.6±0.9
N5	50	6	3	TLIM	20.9±0.7
N6	65	6	3	TLIM	19.7±0.2
N7	55	22	3	TLIM	24.4±1.0
N8	50	10	4	TLIM	20.8±0.7
N9	65	22	4	TLIM	18.8±0.2
N10	55	6	5	TLIM	18.6±0.5
N11	60	10	5	TLIM	18.9±0.1
N12	65	18	5	TLIM	18.8±0.3
N13	50	22	5	TLIM	22.3±0.8
N14	50	6	2	N435[f]	24.7±1.2
N15	65	6	2	N435	26.4±0.9
N16	50	22	2	N435	21.5±1.1
N17	65	22	2	N435	26.7±0.9
N18	55	14	3	N435	24.6±0.5
N19	60	18	4	N435	19.0±0.8
N20	50	6	5	N435	16.4±0.2
N21	65	6	5	N435	16.7±0.5
N22	50	22	5	N435	16.6±0.6
N23	65	22	5	N435	16.1±0.3
N24	65	22	5	N435	15.8±0.4
N25	65	22	5	N435	16.3±0.3
N26	65	22	5	N435	16.1±0.2

Each value is the mean of triplicates ± standard deviation.
[a]Temp, temperature. [b]SR, substrate molar ratio (PS:HCO). [c]Enz, enzyme. [d]Inc, incorporation. [e]TLIM, Lipozyme TLIM (*Thermomyces lanuginosus* lipase). [f]N435, Novozym 435 (*Candida antarctica* lipase)

Table 7.2.1 Total fatty acid (g kg^{-1}) content of the substrates, gram-scale, and large-scale products

Fatty acid	C16:0	C16:1*c*	C18:0	C18:1*t*	C18:1*c*	C18:2n6*t*	C18:2n6*c*	C18:3n3	Minor[a]
HCO[b]	32.0±1.8a	2.0±0.0a	847.0±0.8a	nd[c]	22.0±1.0a	nd	126.0±3.4a	nd	14.0
PS[d]	581.0±4.9b	1.0±0.0a	52.0±1.1b	nd	269.0±2.7b	4.0±0.1a	76.0±1.1b	2.0±0.0a	23.0
SL1[e]	407.0±3.9c	2.0±0.0a	233.0±1.0c	nd	162.0±2.1c	nd	182.0±1.9c	1.0±0.0a	5.0
PB1[f]	448.0±3.2d	2.0±0.0a	252.0±0.6d	nd	155.0±1.7d	nd	158.0±2.4d	1.0±0.0a	3.0
SL2[g]	424.0±4.1e	2.0±0.0a	231.0±1.1c	nd	167.0±2.5c	nd	170.0±3.8e	1.0±0.0a	4.0
PB2[h]	428.0±2.7e	1.0±0.0a	246.0±1.3d	nd	178.0±2.8e	nd	141.0±2.1f	1.0±0.0a	4.0
SL_{ls}[i]	401.0±3.3c	2.0±0.0a	235.0±0.6c	nd	163.0±2.1c	nd	181.0±1.3c	2.0±0.0a	4.0
EF_{cm}[j]	102.0±1.5f	1.0±0.0a	61.0±0.7e	187.0±1.9	316.0±3.2f	4.0±0.0a	294.0±1.9g	32.0±0.2b	4.0

Each value is the mean of triplicates ± standard deviation. Values with the same letter in each column not significantly different at $P < 0.05$.
[a]Minor, sum of C12:0, C14:0, C20:0, C21:0, C22:0, C24:0. [b]HCO, fully hydrogenated cottonseed oil. [c]nd, not detected. [d]PS, palm stearin. [e]SL1, Lipozyme TLIM-catalyzed structured lipid. [f]PB1, corresponding physical blend of SL1. [g]SL2, Novozym 435-catalyzed structured lipid. [h]PB2, corresponding physical blend of SL2. [i]SL_{ls}, large-scale SL1. [j]EF_{cm}, extracted fat from commercial margarine

Table 7.2.2 *sn*-2 Positional fatty acid (g kg^{-1}) of the substrates, gram-scale, and large-scale products

Fatty acid	C16:0	C18:0	C18:1*t*	C18:1*c*	C18:2n6*c*
HCO[a]	313.0±1.4a	436.0±2.8a	nd[b]	27.0±1.1a	127.0±1.2a
PS[c]	489.0±4.3b	29.0±0.9b	nd	383.0±2.2b	118.0±1.0b
SL1[d]	426.0±2.9c	202.0±1.1c	nd	253.0±2.9c	108.0±1.8c
PB1[e]	303.0±3.1a	265.0±1.2d	nd	343.0±2.9d	78.0±1.0d
SL2[f]	446.0±4.2d	249.0±0.8e	nd	215.0±1.8e	83.0±1.9e
PB2[g]	295.0±3.4a	247.0±1.2e	nd	331.0±4.7f	74.0±3.2d
SL_{ls}[h]	422.0±3.8c	203.0±0.5c	nd	275.0±3.6g	104.0±1.7c
EF_{cm}[i]	65.0± 1.4f	56.0±1.5f	156.0±2.7	321.0±3.6h	403.0±4.8f

Each value is the mean of triplicates ± standard deviation. Values with the same letter in each column not significantly different at $P < 0.05$.
[a]HCO, fully hydrogenated cottonseed oil. [b]nd, not detected. [c]PS, palm stearin. [d]SL1, Lipozyme TLIM-catalyzed structured lipid. [e]PB1, corresponding physical blend of SL1. [f]SL2, Novozym 435-catalyzed structured lipid. [g]PB2, corresponding physical blend of SL2. [h]SL_{ls}, large-scale SL1. [i]EF_{cm}, extracted fat from commercial margarin

Table 7.3 Relative concentration (g kg^{-1}) of triacylglycerol (TAG) species in substrates and products

Tag Species	HCO[a]	PS[b]	SL1[c]	PB1[d]	SL2[e]	PB2[f]	SL_{ls}[g]	EF_{cm}[h]
LnOLn	nd[i]	nd	nd	nd	nd	nd	nd	51.0±1.2
LLLn	nd	nd	12.0±0.2a	nd	nd	nd	11.0±0.0a	17.0±0.9b
LLL	nd	nd	nd	nd	nd	nd	nd	144.0±1.1
LOL	nd	nd	nd	nd	nd	nd	nd	72.0±0.8
PLL	nd	nd	nd	nd	nd	nd	nd	63.0±0.5
SLL	nd	nd	nd	nd	nd	nd	nd	3.0±0.0
OOL	nd	nd	nd	nd	nd	nd	nd	nd
POL	nd	20.0±0.3a	19.0±0.8a	nd	21.0±0.7a	21.0±0.2a	18.0±0.1a	58.0±0.3b
PLP	nd	65.0±1.0	nd	nd	nd	nd	nd	nd
OOO	nd	18.0±0.7a	50.0±0.2b	116.0±1.2c	68.0±1.1d	155.0±1.1e	52.0±1.1b	22.0±0.8a
POO	nd	148.0±1.2a	297.0±1.8b	410.0±3.4c	311.0±1.6b	434.0±3.2c	295.0±2.2b	543.0±4.3d
POP	nd	473.0±2.4a	202.0±2.0b	177.0±1.6c	188.0±1.0bc	129.0±1.0d	197.0±2.0b	nd
PPP	nd	269.0±1.7a	147.0±1.1b	nd	126.0±1.0c	nd	157.0±1.0b	nd
PSO	nd	nd	242.0±3.1a	30.0±0.8b	224.0±2.1c	33.0±0.0b	237.0±1.6a	22.0±0.8d
PSP	132.0±1.2	nd	nd	nd	nd	nd	nd	nd
SSL	9.0±0.1	nd	nd	nd	nd	nd	nd	nd
PSS	506.0±2.2a	nd	31.0±0.5b	171.0±1.6c	63.0±0.3d	144.0±0.9e	30.0±1.2b	nd
SSS	352.0±1.7a	nd	nd	96.0±1.1b	nd	84.0±0.7b	nd	nd

Each value is the mean of triplicates ± standard deviation. Values with the same letter in each row not significantly different at $P < 0.05$. Ln, linolenic, O, oleic, L, linoleic, P, palmitic, S, stearic

[a]HCO, fully hydrogenated cottonseed oil. [b]PS, palm stearin. [c]SL1, Lipozyme TLIM-catalyzed structured lipid . [d]PB1, corresponding physical blend of SL1. [e]SL2, Novozym 435-catalyzed structured lipid. [f] PB2, corresponding physical blend of SL2. [g]SL_{ls}, large-scale SL1. [h]EF_{cm}, extracted fat from commercial margarine. [i]nd, not detected

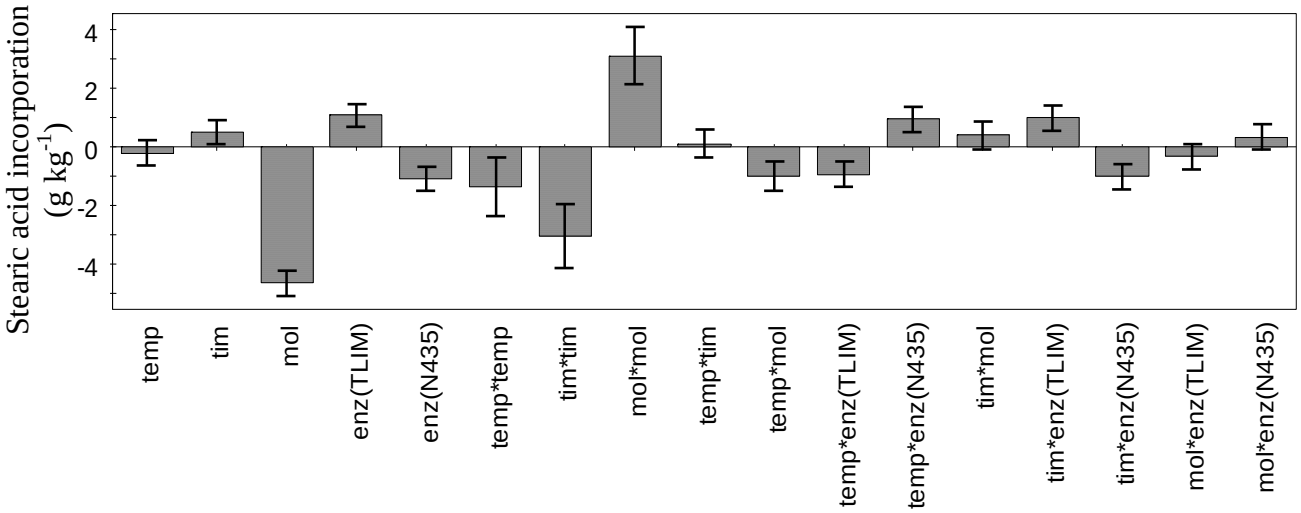
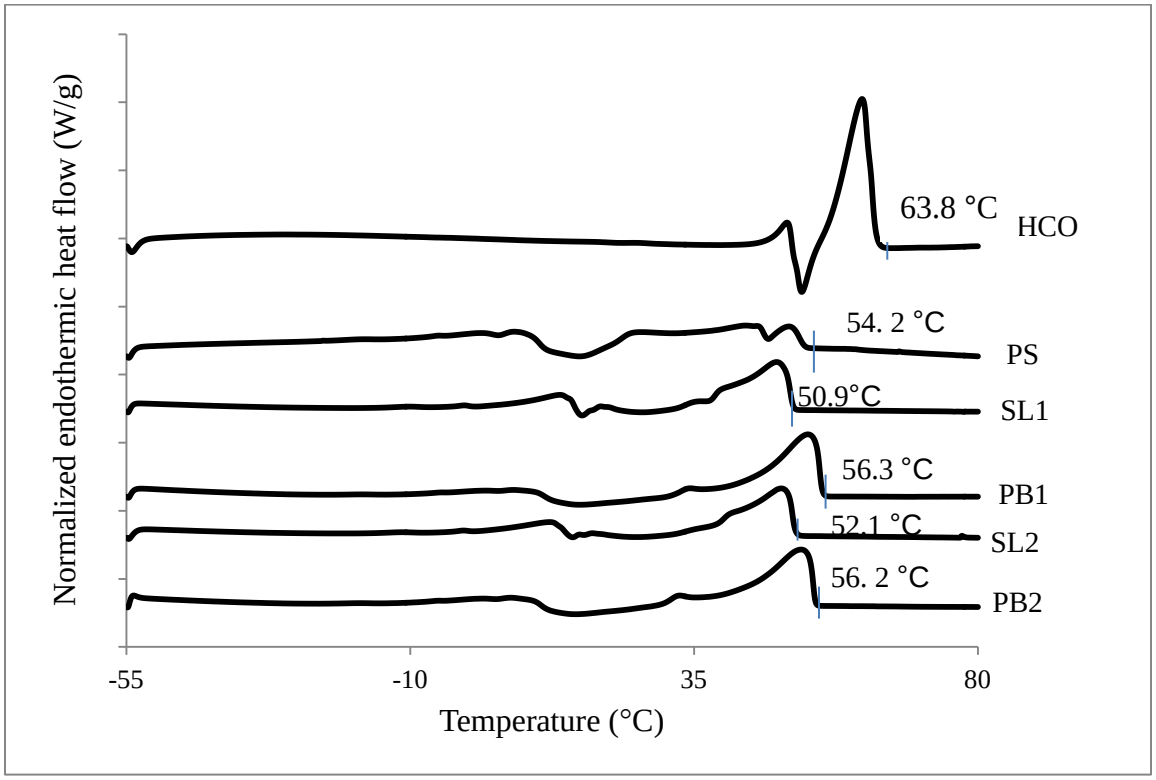
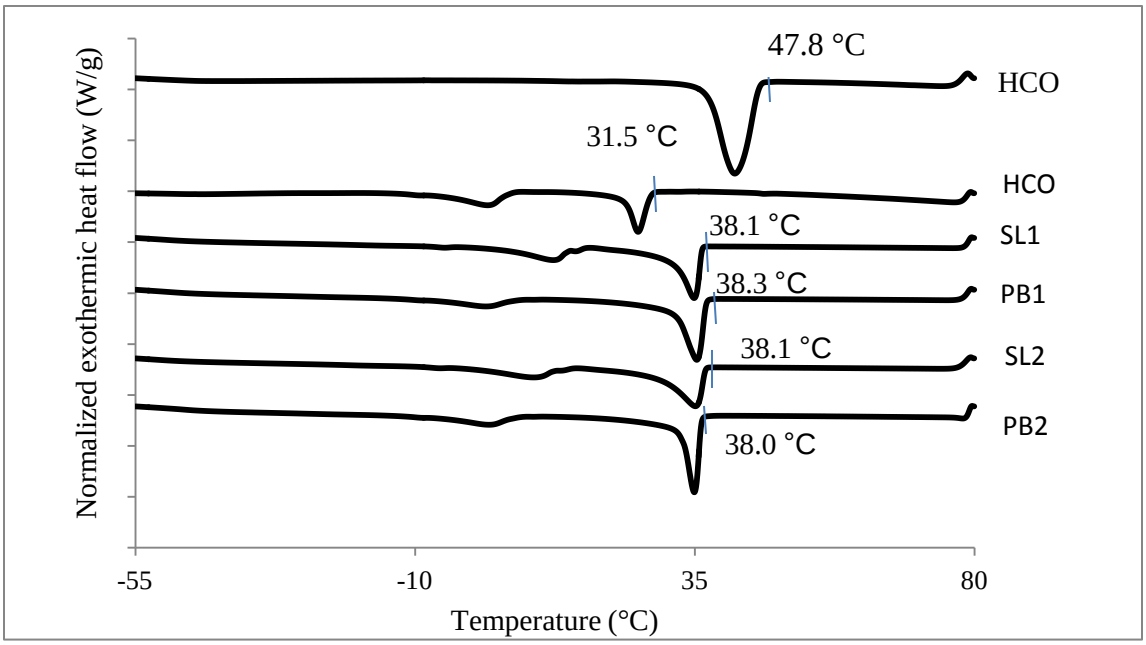

Stearic acid incorporation
(g kg-1)
4
2
0
-2
-4
temp
tim
mol
enz(TLIM)
enz(N435)
temp*temp
tim*tim
mol*mol
temp*tim
temp*mol
temp*enz(TLIM)
temp*enz(N435)
tim*mol
tim*enz(TLIM)
tim*enz(N435)
mol*enz(TLIM)
mol*enz(N435)

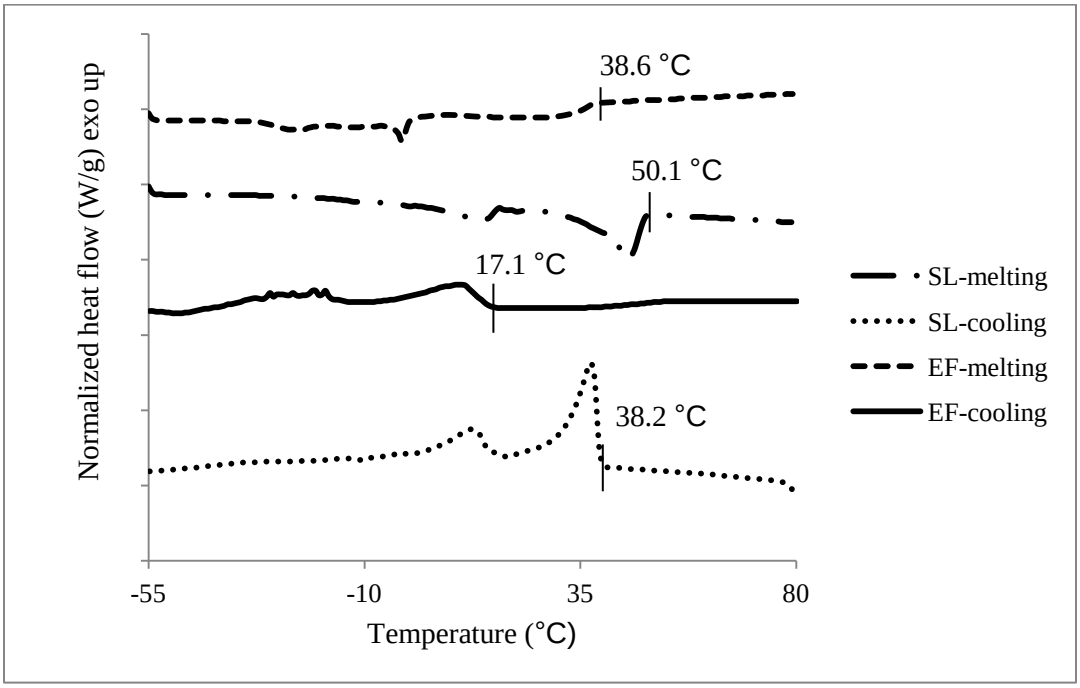
Fig. 7.1


Normalized endothermic heat flow (W/g)
63.8 °C
HCO
54. 2 °C
PS
50.9°C
SL1
56.3 °C
PB1
52.1 °C
SL2
56. 2 °C
PB2
-55
-10
35
80
Temperature (°C)

(a)


Normalized exothermic heat flow (W/g)
47.8 °C
HCO
31.5 °C
HCO
38.1 °C
SL1
38.3 °C
PB1
38.1 °C
SL2
PB2
38.0 °C
-55
-10
35
80
Temperature (°C)

(b)
Fig. 7.2


38.6 °C
50.1 °C
17.1 °C
38.2 °C
Normalized heat flow (W/g) exo up
-55
-10
35
80
Temperature (°C)
SL-melting
SL-cooling
EF-melting
EF-cooling

(c)

Fig. 7.2

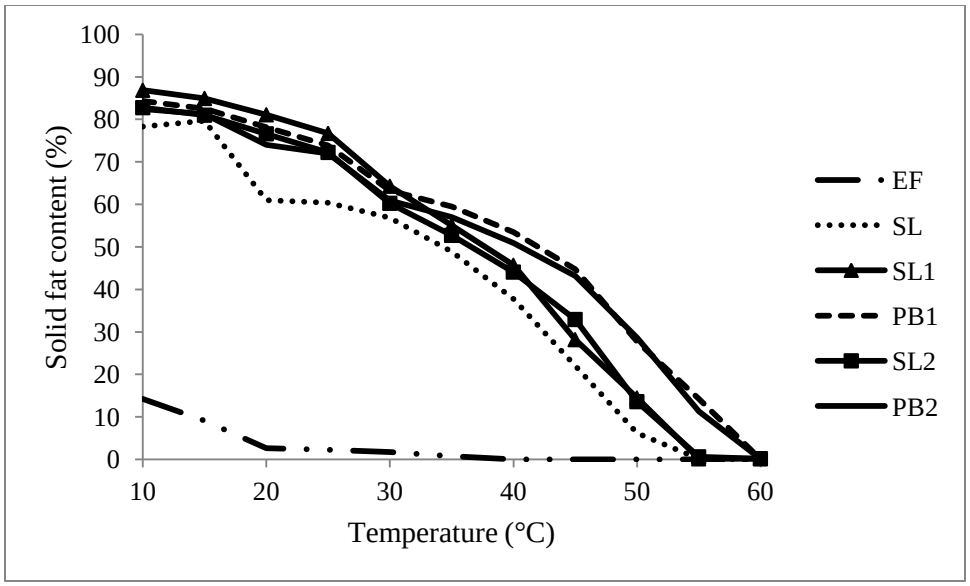

100
90
80
70
60
50
40
30
20
10
0
Solid fat content (%)
10
20
30
40
50
60
Temperature (°C)
EF
SL
SL1
PB1
SL2
PB2

Fig. 7.3

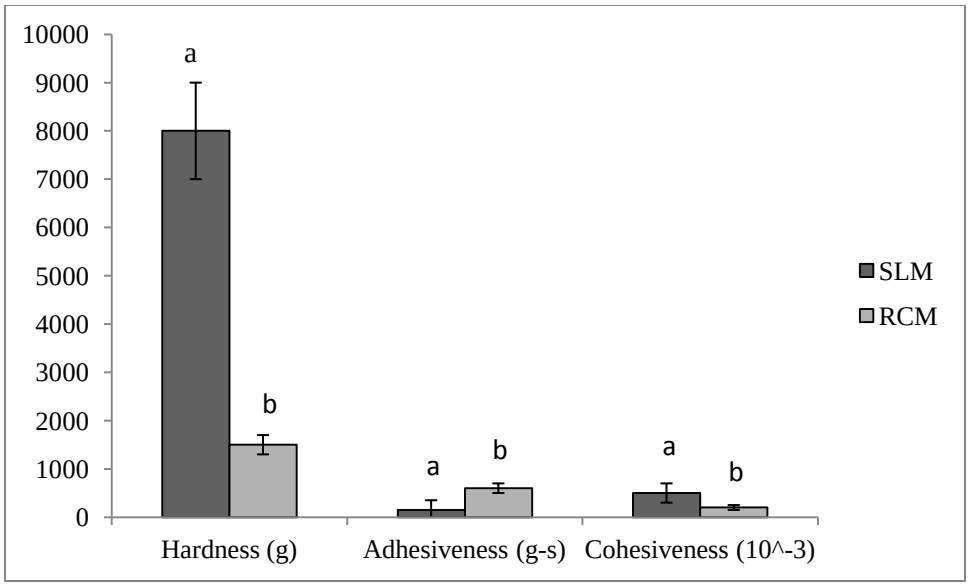
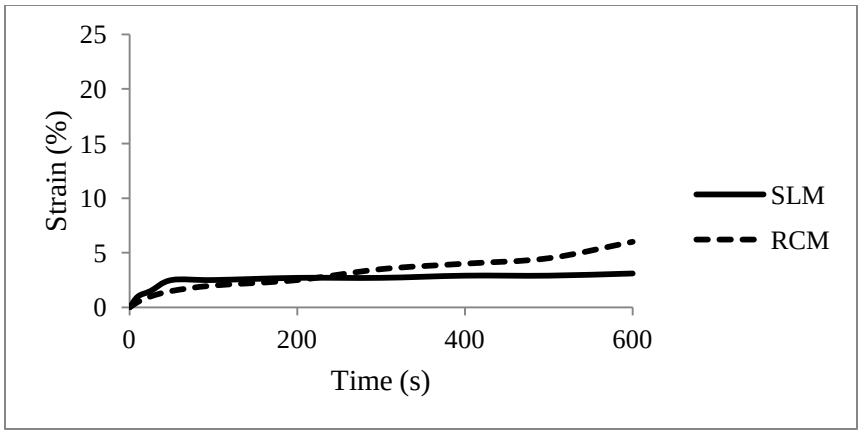
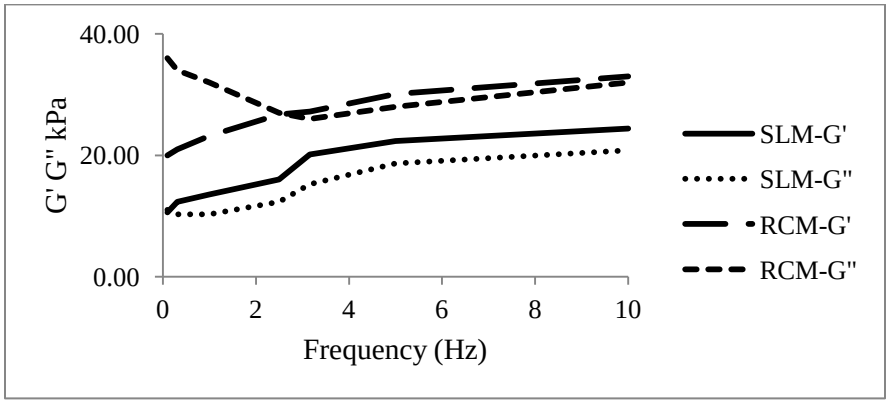

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
a
b
a
b
a
b
SLM
RCM
Hardness (g)
Adhesiveness (g-s)
Cohesiveness (10^-3)

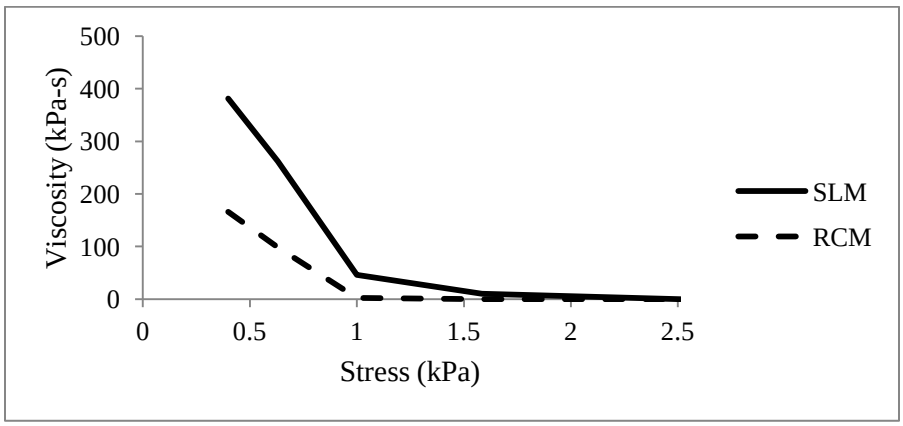
Fig. 7.4


25
20
15
10
5
0
Strain (%)
0
200
400
600
Time (s)
SLM
RCM

(a)


40.00
20.00
0.00
G' G" kPa
0
2
4
6
8
10
Frequency (Hz)
SLM-G'
SLM-G"
RCM-G'
RCM-G"

(b)


500
400
300
200
100
0
Viscosity (kPa-s)
0
0.5
1
1.5
2
2.5
Stress (kPa)
SLM
RCM

(c)

Fig. 7.5

CHAPTER 8

CONCLUSIONS

In this study we were able to synthesize different SLs suitable for different types of margarines. The margarines formulated had different fatty acid profiles, solid fat contents (SFC), melting temperatures, triacylglycerol (TAG) molecular species, and textures, but they were all *trans*-free. The oils/fats used were stearidonic acid (SDA) soybean (SDASO), high stearate soybean (HSSO), cottonseed (CO), fully hydrogenated cottonseed (HCO) oils, and palm stearin (PS). For the first combination, optimal products composition were achieved at 50 °C, 18 h, 2:1 (HSSO:SDASO) using Lipozyme TLIM, containing 15.1 mol% stearic acid and 10.5 mol% SDA. It had desirable polymorphism (β'), thermal properties, and SFC for formulation of soft margarine. The margarine formulated with this SL was *trans*-free and n-3 FA enriched. For the second combination, desirable SL containing 12.3 mol% stearic acid and no TFA was obtained at 50 °C, 20 h, 2:1 (PS:HSSO) using Novozym 435 lipase. This SL was more suitable for stick/hard margarine because of its higher melting completion temperature (45.4 °C). Using PS and CO, SL was synthesized at 56 °C, 6 h, 4:1 (PS:CO) catalyzed by Novozym 435. The margarine formulated with this SL had high oxidative stability and no TFA and may be suitable as hard/industrial margarine. In the last combination, SL synthesized at 65 °C, 16.5 h, 2:1 (PS:HCO) using Lipozyme TLIM, was used to formulate a *trans*-free hard margarine with possible use for puff pastries, cooking, or baking purposes. The following specific aims of this study were met.

(1) Optimization of the reaction conditions and blending ratios of substrates for *trans*-free structured margarine fat synthesis. *It is hypothesized that optimization of reaction conditions and blending ratios will result in a model that can be adapted in large-scale synthesis of trans-free SL*. **This hypothesis was accepted. All the models developed using response surface methodology had high predictive power and were used in large-scale syntheses of SLs.**

(2) Characterization of physical and chemical properties of the SLs and comparison with physical blends (PBs). *It is hypothesized that enzymatically produced SLs will have better physical and chemical properties than the physical blend and would be more suitable for margarine formulation.* **This hypothesis was accepted. The SLs synthesized had fatty acid profile, solid fat content, crystal type, and thermal behavior better suited for margarine formulation than those of PBs**.

(3) Characterization of textural and sensory properties of margarines prepared with selected SL and comparison with commercial brands. *It is hypothesized that the margarines prepared with trans-free SL will have similar or superior textural and sensory properties when compared to commercial brands.* **This hypothesis was accepted for the first combination (HSSO+SDASO) in which the margarine prepared with SL had better spreadibility and fatty acid profile, and comparable taste as that of commercial margarine (spreadable tub). In the second (PS+HSSO) combination the margarine produced was harder than commercial margarine (spreadable stick) with significant difference in taste. In the third combination (PS+CO), the margarine prepared with SL had**

lower spreadibility but similar taste as the commercial stick margarine and in the fourth combination (PS+HCO), the margarines produced were much harder and different in taste than commercial stick margarine. However, all the margarines were *trans*-free compared to 19.1 mol% TFA present in commercial margarine. These margarines were more suitable for possible use as hard industrial margarine for baking and puff pastry rather than as spreadable margarine.

This research resulted in the production of *trans*-free SLs as alternatives to partially hydrogenated fat and can be used by the food industry to formulate *trans*-free foods.

Suggestions for future work:

(1) To study the performance of these margarines, especially the SDA-containing margarines, in baking/cooking applications in terms of FA profile, texture, shelf-life, and sensory attributes of the final product.

(2) To study the additions of phytosterols/phytostanols to these margarines.

(3) Animal study involving feeding of SDA-enriched margarine and analyzing the lipid profile, weight gain, and inflammation biomarkers in the animals.

(4) To study the effect of different types of emulsifiers and antioxidants on the emulsion and oxidative stability of the margarines.