ENZYMATIC INCORPORATION OF STEARIC ACID INTO CANOLA OIL TO PRODUCE *TRANS*-FREE STRUCTURED LIPIDS FOR POSSIBLE MARGARINE FORMULATION

by

STEPHEN ENYAM LUMOR

(Under the Direction of CASIMIR C. AKOH)

ABSTRACT

Incorporation of stearic acid into canola oil to produce a *trans*-free structured lipid (SL) as a healthy alternative to partially hydrogenated fats for margarine formulation was investigated. This study showed that Lipozyme RM IM from *Rhizomucor miehei* was more suitable for the process than *Candida rugosa* lipase isoform 1 (LIP1). The activity of LIP1 was not enhanced after immobilization on Celite 545, Duolite A7, and Sephadex G-25. Regiospecific analysis using ^{13}C NMR spectrometry showed that stearic acid was incorporated into canola oil, mainly at the *sn*-1,3 positions. However, most SL products did not have adequate solid fat content or β' crystal forms for tub margarine.

Solid fat content (SFC) and crystal properties of the *trans*-free SLs were optimized by blending with palm mid-fraction (PMF). Addition of sucrose stearate (S-170), sorbitan tristearate (STS), and distilled monoglycerides (DMG) to one of the blends, SL40:PMF (70:30, w/w), did not improve crystal polymorphism, but had significant effects on crystal morphology. The emulsifiers significantly delayed crystal growth, resulting in smaller crystal sizes compared to the control. However, they were unable to inhibit the formation of granular crystals (30 to 140 μm), which are undesirable in margarine after 4 weeks of storage at 0˚C. These crystals

aggregates were not observed upon visual and physical examination, and may therefore not impart the sensory properties of the finished products negatively.

Consequently, a *trans*-free margarine (MG-X) was formulated with a blend of a SL synthesized by reacting canola oil with 40% stearic acid (w/w), PMF, and cottonseed oil (CTO). Physical and sensory attributes of MG-X and two commercial margarines (MG-A and MG-B) were studied. MG-X was considerably harder than MG-A and MG-B, least cohesive, and its adhesiveness was intermediate between those of MG-A and MG-B. Stability of MG-X to syneresis was also intermediate between those of MG-A and MG-B. Sensory evaluation showed that MG-X was comparable to MG-A in terms of spreadability and texture only, but was significantly different from MG-B in all attributes. Also, more subjects were willing to accept MG-X in spite of its sensory properties if it carried a health claim.

INDEX WORDS: *Candida rugosa* lipase isoform 1, canola oil, Lipozyme RM IM, margarine, palm mid-fraction, stearic acid, *trans*-free structured lipids

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DEDICATION

Dedicated to my mom, Mrs. Grace Aku Alamu-Lumor, and my siblings for their continued support and prayers.

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CHAPTER 1

INTRODUCTION

Concerns about significant consumption of *trans* fatty acids (TFA) have been a major health and policy issue over the past decade. *Trans* fatty acids occur naturally in dairy products as a result of bio-hydrogenation of *cis*-unsaturated fatty acids by rumen microorganisms. These naturally occurring TFA are found in small amounts in tissues of ruminants and products derived from them. Examples include milk, cheese, tallow and butter. The majority of TFA consumed, however, comes from products formulated with partially hydrogenated fats. These products include frying oils, margarines, spreads, shortenings, bakery products and fast foods. The amount of TFA present in these foods is estimated to be between 0 and 35% of total fatty acids (Hunter, 2005), and the mean daily intake of TFA per person in the United States population is 2.6% energy or 5.3 g (Allison and others, 1999).

Intake of high amounts of TFA has been correlated with increased risk of cardiovascular diseases, primarily due to their effects on plasma lipid profile (Wahle and James, 1993; Willet and others, 1993). These effects include elevated LDL-cholesterol and lipoprotein[a] levels, and decreased HDL-cholesterol level. The dietary levels of *trans*-fatty acids required to increase LDL-cholesterol is estimated to be approximately 4% energy or higher, and approximately 5 - 6% energy or higher to decrease HDL-cholesterol (Hunter, 2005). These and other studies have heightened health concerns among consumers and regulatory agencies in Europe and USA. As a result, the United States Food and Drug Administration (FDA) issued a final ruling requiring foods containing TFA to be labeled accordingly, effective from January 2006 (Federal Register, 2003). The food industry is responding to these concerns by developing processes that will produce foods with zero or reduced *trans* fat contents. These alternatives include modification of

the hydrogenation process to reduce the level of TFA in the products, use of transesterification to make structured lipids (SL), use of fractions high in solids from natural oils, such as palm stearin and palm kernel stearin, and use of trait-enhanced oils such as laurate canola and high-stearate soybean oils.

Structured lipids (SL) are defined as triacylglycerols or phospholipids that have been modified to change the natural composition or positions of fatty acids on the glycerol backbone to achieve desired nutritional, physical or chemical properties. This can be accomplished by the process of transesterification, which involves the transfer of acyl groups between lipid molecules - acylglycerols, glycerophospholipids, and fatty acids. This process can be catalyzed by sodium methoxide (chemical transesterification), or by commercial lipases such as Lipozyme RM IM from *Rhizomucor miehei* and *Candida rugosa* lipase isoform 1 (LIP1). As an alternative to partially hydrogenated fats, structured lipids are synthesized by incorporating high melting fatty acids into oils or by reacting high melting fractions of natural oils or fully hydrogenated fats with liquid oils. This process (transesterification) increases the solid fat content and oxidative stability of the product, and does not result in the formation of *trans* fatty acids, as is the case with partial hydrogenation.

The goal of this study was to enzymatically incorporate stearic acid into canola oil to produce a *trans*-free structured lipid for possible margarine formulation. Lipozyme RM IM, an immobilized lipase from *Rhizomucor miehei* and *Candida rugosa* lipase isoform 1 (LIP1) were used as biocatalysts. Stearic acid was chosen because it has a high melting point and has no known adverse effects on plasma cholesterol levels (Keys and others, 1965; Hegsted and, 1965; Grande and others, 1970; Bonanome and Grundy, 1988). Besides oleic and linoleic acids, canola oil also contains α- and γ-linolenic acids which are important in reducing coronary heart disease

(CHD) risk factors. This study is of significance because death from degenerative diseases such as cancer, cardiovascular disease, and diabetes can be reduced significantly by the consumption of healthy diets. We believe that *trans*-free SLs made by incorporating stearic acid into canola oil will not only impart functional benefits to margarine, but will also provide nutritional or health benefits as well. Besides the absence of TFA, which have been implicated in atherogenesis, our SLs are expected to have the following positive health indicators: (1) Low ratios of ω-6 to ω-3 fatty acids, and (2) reduced palmitic acid contents. The hypothesis for this study is that *trans*-free SLs synthesized by incorporating stearic acid into canola oil will be suitable for the formulation of tub margarines having similar or superior physicochemical and sensory properties as selected commercial margarine samples. The specific objectives of this study were to:

1. Determine the effects of acyl donor and lipase types on incorporation of stearic acid into canola oil using response surface methodology, and characterize the structured lipid products for possible margarine formulation.
2. Immobilize LIP1 on Celite 545, Sephadex G-25, and Duolite®A7, and determine the activities of the immobilized enzymes.
3. Optimize solid fat content and crystal properties of structured lipids by blending with palm mid-fraction.
4. Formulate margarine and compare its physical and sensory properties to those of re-crystallized commercial margarine samples.

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CHAPTER 2

LITERATURE REVIEW

The health implications of significant consumption of *trans* fatty acids (TFA) have been well established (Wahle and James, 1993; Willet and others, 1993; Pietinen and others, 1997; Kholsa and Hayes, 1996). TFA significantly increase the risk of cardiovascular diseases by increasing plasma total cholesterol, LDL-cholesterol and lipoprotein[a] levels, and by decreasing HDL-cholesterol level. One of the first clinical cohort studies that linked consumption of TFA with coronary heart disease was the Finnish Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study conducted by Pietinen and others (1997). The study recruited 21,930 nonsmoking men between the ages of 50-69 years who were initially free of diagnosed cardiovascular disease. After 6.1 years of follow-up, 1,399 major coronary events and 635 coronary deaths were reported. After controlling for variables such as age, supplement group, several coronary risk factors, total energy, and fiber intake, a strong positive association was found between the consumption of TFA and the risk of coronary death. Other long term (6 – 20 years) studies, comprising about 667 to 80,082 men and women in different age groups, that have provided compelling data linking TFA consumption to coronary heart disease include the US Health Professional's Follow-Up Study (Ascherio and others, 1996), the US Nurse's Health Study (Hu and others, 1997; Oh and others, 2005), and the Dutch Zutphen Elderly Study (Oomen and others, 2001). In an intervention study (Judd and others, 1994) comprising 29 men and 29 women, a significant increase in LDL-cholesterol and a decrease in HDL-cholesterol was noted after consumption of diets high in TFA. The mechanism by which TFA affect plasma lipoprotein levels is reported (Kholsa and Hayes, 1996) to be analogous to that of the C12 – C16 saturated fatty acids, resulting in increased LDL production or delayed LDL clearance. By

contrast, HDL suppression by TFA is believed to be due to the increased activity of serum cholestryl ester transferase, which results in the transfer of cholestryl esters from HDL to LDL.

In light of convincing evidence regarding the possitive association between TFA and the risk of cardiovasular disease, varaible detrimental levels of intake have been reported. Kholsa and Hayes (1996) reported that adverse effects of TFA on plasma lipoproteins were measurable above 3% of energy as TFA. In a feeding study (Zock and Katan, 1992) with diets containing *trans* fat levels between 0.1 and 7.7% of energy, the 7.7% energy diet significantly lowered HDL-cholesterol and raised LDL-cholesterol. Hunter (2005) argued that the different values reported in various studies were due to the level of linoleic acid in these diets. It seemed that higher levels of linoleic acid were associated with lower TFA levels and vice versa. In analyzing nine studies that controlled for non-*trans* components, Hunter (2005) estimated that the dietary level of TFA needed to significantly affect plasma LDL-cholesterol was 4% energy or higher; whereas, approximately 5 to 6% energy or higher was needed to significantly affect plasma HDL-cholesterol.

Trans fatty acids are positively associated with certain medical conditions besides cardiovascular disease. A study (Kohlmeier and others, 1997) comprising 698 postmenopausal incident cases of primarily breast cancer and controls randomly drawn from a local population and patient registries revealed a positive association between the levels of adipose tissue TFA and postmenopausal breast cancer in European women. In a related study (King and others, 2005), the relationship between serum phopholipid TFA and prostate cancer incidence in 272 cases and 426 control men was studied. Results indicated consistent trends for increased prostate cancer risk with higher levels of C18 TFA. A recent study (Chavarro and others, 2007) has suggested that TFA may increase the risk of ovulatory infertility when consumed instead of

carbohydrates and unsaturated fatty acids. The study reported that each 2% energy increase in the intake of TFA was associated with a 73% greater risk of ovulatory infertility after adjustment for known and suspected risk factors for this condition. TFA have also been reported to be positively associated with symptoms of asthma, allergic rhinoconjunctivis, and atopic eczema in European children (Weiland and others, 1999), and systemic inflammation in women (Mozaffarian and others, 2004).

The main sources of TFA are products formulated with partially hydrogenated fats. These include frying oils, margarines, spreads, shortenings, bakery products and fast foods. With regards to levels of consumption, a study (Hulshof and others, 1999) in Western Europe reported that TFA intake ranged from 0.5% (Greece, Italy) to 2.1% (Iceland) of energy intake among men and from 0.8% (Greece) to 1.9% (Iceland) among women. In the United States, the mean daily intake per person was estimated to be 2.6% of energy (Allison, 1999). This was based on 24 h recollections and 2-day food records of over 11200 subjects who took part in the United States Deparment of Agriculture's Continuing Survey of Food Intakes by Individuals.

In view of the adverse effects of TFA on health and the levels of consumption in the developed world, governmental and non-governmental organizations have sought to regulate the levels of *trans* fatty acids in food, and to educate the general public about the harmful effects of TFA. The Joint WHO/FAO Expert Consultation on Diet, Nutrition and the Prevention of Chronic Diseases (2002) advised that intakes of fats should be limited to 15 – 30% of total daily energy, with saturated fats providing less than 10% energy and *trans* fat less than 1% energy. Besides global efforts to regulate the levels of *trans* fat in food, some developed countries have enacted regulations to limit the amounts of *trans* fat in food. In 2003, Canada became the first country to regulate TFA in food. The regulation called for mandatory labeling of prepackaged

foods to declare the levels of *trans* fat by December 2005. The regulation also requires food labels to carry the absolute amount of *trans* fat in grams per serving of the food and the percent daily value (DV) for *trans* and saturated fats combined. In the United States, the FDA issued a ruling requiring most prepackaged foods and dietary supplements to be labeled adequately with respect to the absolute amount of *trans* fat per serving of the food (Federal Register, 2003). Unlike the Canadian regulation, no %DV was established. The European Union and countries such as New Zealand, Australia and South African also have some forms of regulations aimed at controlling the amount of *trans* fat present in food.

The food industry is responding to these regulatory mandates by developing processes that will produce foods with zero or reduced *trans* fat contents. Novel hydrogenation processes such as electrocatalytic hydrogenation, precious metal catalyst hydrogenation, and supercritical fluid state hydrogenation have been reported to reduce the level of *trans* fat in vegetable oils (Jung and Min, 2005). In electrocatalytic hydrogenation, an electrically conducting catalyst such as Raney nickel or platinum black is used as the cathode. Hydrogen is produced at the cathode as a result of the reduction of protons or water molecules. The hydrogen produced is absorbed by the cathode and used to hydrogenate (reduce) the double bonds of unsaturated fatty acids. Precious metal catalyst hydrogenation uses catalysts such as palladium, platinum, and ruthenium that are active at low temperatures. Low-temperature hydrogenation with these precious metals produces less TFA than the conventional high-temperature hydrogenation using nickel. Supercritical fluid state hydrogenation is an improvement over the conventional nickel hydrogenation, which takes place in a three phase system made up of hydrogen gas phase, liquid oil phase, and solid catalyst phase. Supercritical fluid hydrogenation provides a homogeneous phase which improves the transfer of hydrogen to the catalyst surface during the process, which

is the rate-limiting step in conventional hydrogenation. Examples of fluids that have been used for this process include a mixture of carbon dioxide and hydrogen (King and others, 2001), and propane (Marcher and Holmqvist, 2001).

STRUCTURED LIPIDS

Structured lipids (SL) are defined as triacylglycerols or phospholipids that have been modified to change the natural composition or positions of fatty acids on the glycerol backbone to achieve desired nutritional, physical or chemical properties. This can be accomplished by the process of transesterification, which involves the transfer of acyl groups between lipid molecules - acylglycerols, glycerophospholipids, and fatty acids. This process can be catalyzed by a chemical catalyst such as sodium methoxide or by commercial lipases such as Lipozyme RM IM from *Rhizomucor miehei* and *Candida rugosa* lipase isoform 1 (LIP1).

Structured lipids impart functional properties to products such as cocoa butter equivalents, shortenings, margarine and high-oleic frying oils where properties such as melting, textural, organoleptic and oxidative stability are desired. Low-calorie fats, human milkfat substitutes, oils enriched with physiologically important fatty acids (conjugated-linoleic, alpha-linolenic, gamma-linolenic, eicosapentaenoic and docosahexaenoic) and structured triacylglycerols containing both long-chain and short-chain fatty acids are examples of structured lipids that have been used to impart physiological or nutritional benefits. Triacylglycerols (TAGs) containing medium chain fatty acids are easily metabolized and absorbed in the small intestine and therefore provide quick energy. Caprenin, containing medium and long chain fatty acids (Proctor & Gamble, OH), Bohenin, containing behenic and oleic acids (Fuji Vegetable Oil Inc., GA), Salatrim, containing short and long chain fatty acids (Cultor Food Science, NY) and

Betapol™ (Loders Croklaan Company, Holland), a human milkfat substitute, are some examples of commercially available structured lipids.

As an alternative to partially hydrogenated fats, structured lipids are synthesized by incorporating high melting fatty acids into oils, or by reacting high melting fractions of natural oils or fully hydrogenated fats with liquid oils. This process (transesterification) increases the solid fat content and oxidative stability of the product, and prevents post-hardening effects of margarine when stored (Young, 1985). Moreover, this process does not result in the formation of *trans* fatty acids, as is the case with partial hydrogenation, and therefore has no adverse effects on plasma lipoprotein profile. A number of studies have reported the use of structured lipids for formulating *trans*-free or reduced *trans* margarines. A recent study (Fattahi-far and others, 2006) reported the use of the interesterified product of hydrogenated and non-hydrogenated tea seed oil to formulate margarines having physical and sensory properties comparable to those of commercial margarines. The TFA contents of the experimental margarines ranged from 1.8 to 2.2%. In another study (Zhang and others, 2005), SLs synthesized by transesterifying palm stearin and coconut oil (70:30, w/w) were blended with 50% sunflower oil and used to formulate *trans*-free margarines. The margarines produced were reported to be similar to selected commercial margarine samples in terms of physical and sensory attributes. United States Patent 6808737 (2004) described the synthesis of a *trans*-free structured lipid for margarine formulation. The structural fat was synthesized by transesterifying a non-hydrogenated high-melting palm oil fraction with a non-hydrogenated palm kernel oil fraction. Other studies worth mentioning are those of Pal and others (2001), Kok and others (2001), Rousseau and Marangoni (1999), Fomuso and Akoh (2001), Seriburi and Akoh (1998) and Lai and others (1999). Unilever

has a patent (1997) for a SL made with interesterified lauric rapeseed oil (65%) and fully hydrogenated soybean oil (35%). SL can be synthesized by using one of the following methods:

Direct esterification: Glycerol + Fatty acid → SL + Water

Acidolysis: TAG (oil) + Fatty $acid_1$ → SL + Fatty $acid_2$

Alcoholysis: TAG (oil) + $Alcohol_1$ → SL + $Alcohol_2$

Interesterification: $TAG_1 + TAG_2 \rightarrow TAG_3 (SL_1) + TAG_4 (SL_2)$

Chemical versus Enzymatic Transesterifications

The types of TAGs formed during the transesterification process are dependent on the nature of the reaction. Naturally, vegetable oils consist of acylglycerols having saturated fatty acids exclusively at the *sn*-1,3 positions and unsaturated fatty acids at the *sn*-2 position (Gunstone, 1961; Rozenaal, 1992; Desnuelle and others, 1959). Animal fats, on the other hand, are quite saturated at the *sn*-2 position (Nawar 1996). Chemical transesterification results in the random redistribution of fatty acids on the triacylglycerol backbone to produce new acylglycerols species. This may not be desirable when SLs with certain physiological and nutritional benefits are to be synthesized. For example, some fatty acids are better absorbed at the *sn*-2 position (Lien, 1994) and significant amounts of certain fatty acids such as palmitic at the *sn*-2 position may be atherogenic (Kritchevsky, 2000). More selective interesterification can be achieved with lipases as catalysts. Selectivity could be regiospecific or preference for certain fatty acids as a result of chain length, saturation or degree of unsaturation. Enzymatic interesterification can therefore be used to synthesize SLs so as to maximize the absorption of certain beneficial fatty acids in addition to other benefits. Betapol^{TM}, a SL with palmitic acid at the *sn*-2 position is used in infant formula to enhance absorption of palmitic acid and to prevent

intestinal loss of calcium. Bohenin, a SL with oleic acid exclusively at the *sn*-2 position is used to inhibit fat bloom in chocolate.

Immobilized Lipases

Immobilized lipases are preferred for transesterification because they are stable and reusable, and also offer easy separation of the enzyme from reactants and products. Immobilization of lipases can also affect their selectivity and chemical and physical properties (Willis and Marangoni, 2002), and creates non-aqueous conditions around the enzyme which is necessary for ester synthesis or esterification (Christensen and others, 2003). Different immobilization methods have been developed and can be broadly classified as follows (Carr and Bowers, 1980):

1. Covalent bonding of the enzyme to a derivatized, water-insoluble matrix
2. Intermolecular cross-linking of enzyme molecules using multi-functional reagents
3. Adsorption of enzyme onto a water-insoluble support material
4. Encapsulation of the enzyme, for example in alginate or carrageenan beads
5. Entrapment of the enzyme inside a water-insoluble polymer matrix

Adsorption is the most commonly used method because of its simplicity. It is accomplished by mixing an aqueous solution of the lipase with the solid support or by precipitating the aqueous lipase solution onto the support using acetone, ethanol, or methanol. The mixture is filtered and the immobilized enzyme freeze-dried to reduce its moisture content. The success of the immobilization process depends on factors such as pH, temperature, solvent type, ionic strength, and protein and adsorbent concentration (Willis and Marangoni, 2002).

The stability of the immobilized lipase depends on a number of factors including the type of support material used. Enzymes immobilized on hydrophobic supports such as polyethylene,

polypropylene, and acrylic polymers are more stable, retain higher degree of activity, and are less desorbed compared to enzymes immobilized on hydrophilic supports such as doulite, celite, silica gel, activated carbon, clay, and sepharose (Malcata and others, 1990; Malcata and othes, 1992). The success of immobilization is also dependent on the pore size of the support material. For support materials with very narrow pores, most of the enzyme is immobilized on the surface of the support resulting in higher activity. The use of supports with larger pores causes some of the lipase to be immobilized inside the pores resulting in reduced accessibility of the substrate to the enzyme (Willis and Marangoni, 2002). The effectiveness value, which is a measure of the relative amount of activity lost upon immobilization, is determined by dividing the activity of the immobilized lipase by the activity of an equal amount of the free lipase determined under the same operating conditions (Willis and Marangoni, 2002). Values close to 1 indicate little enzyme activity has been lost upon immobilization, whereas values much lower than 1 indicate significant loss of enzyme activity.

Candida rugosa lipase isoform 1 (LIP1) is one of the seven forms of *Candida rugosa* lipase (CRL). Its use in the synthesis of structured lipids has become attractive because of its ability to significantly incorporate acyl chains at the *sn*-1,2,3 positions of the TAG backbone (Srivastava and others, 2006). The non-specific incorporation of acyl chains results in increased TAG diversity, which improves the preponderance of β' crystals that impart smooth mouthfeel to margarine. Immobilization of CRL to enhance its activity has been reported by a number of studies. One such study was conducted in our laboratory using clay, Celite 545, DEAE-Sephadex and CM-Sephadex as support materials (Lee and Akoh, 1998). Results showed that interesterification activity was highest when the lipase was immobilized on Celite 545. In another study, the lipase was immobilized on eleven different support materials, and used for the

resolution of *dl*-menthol (Wang and others, 2002). *Candida rugosa* lipase immobilized on DEAE-Sephadex A-25 produced the highest yield and optical purity of the product and retained its activity after 34 days of repeated use.

Production of SL

Structured lipids are typically synthesized (in milligram quantities) in screw-cap test tubes contained in a shaking water-bath for the purpose of monitoring the progress of the reaction or the evaluation of the effects of reaction parameters on the process. The reaction is stopped by removing the enzyme by passage through a column (5 mm di.) of anhydrous sodium sulfate. The lipid bands are separated by thin-layer chromatography (TLC) and their fatty acid profiles determined by gas chromatography (GC). For bench-scale synthesis, four types of bioreactors commonly used are: stirred batch reactors, continuous stirred tank reactors, fixed bed reactors, and membrane reactors.

Stirred batch reactors are commonly used in laboratory experiments because they are simple to make and use, and do not cost much. The substrates and the enzymes are fed manually into the reactor and the reaction takes place for a definite period of time. No addition of reactants or removal of products is performed except at the initial and the final stages of the reaction. Since there is no flow in or out of the system, substrate to product ratio decreases over time with a corresponding decrease in the rate of conversion. The rate of conversion can be maintained by adding more enzymes (Cheetham, 1988) or by increasing the speed of the stirrer (Lee and Akoh, 1998). The temperature of the bioreactor is maintained by circulating water from a water batch in between the double-layered wall of the bioreactor. The continuous stirred tank reactor is an improvement over the stirred batch reactor. Substrates are fed by means of a pump into the bioreactor containing the enzyme and the product removed at the same rate, while providing

continuous stirring to eliminate mass transfer limitations encountered in fixed bed reactors (Willis and Marangoni, 2002). The rate of conversion is dependent on the residence time of the substrates in the bioreactor, and is controlled by adjusting the flow rate.

Membrane bioreactors consist of two-phase systems, where the interface of the two phases is a semi-permeable membrane onto which enzymes have been immobilized. Unlike the stirred tank bioreactors, reaction and separation of substrate and products can be accomplished in one unit. The movement of solutes across the membrane is made possible by the action of a driving force, which could be a chemical potential, a pressure drop or an electric field (Prazers and Cabral, 1994). The separation of substrates and products is especially helpful in glycerolysis and acidolysis where the formation of water as a by-product, if not removed, will reduce the rate of substrate conversion or promote hydrolysis.

Fixed bed bioreactors consist of a column or a flat bed packed with an immobilized enzyme through which the substrate and product streams are pumped in and out at the same rate. The fixed bed column bioreactor typically consists of two columns in series: the reaction takes place in the inner column and the outer column serves as a precolumn in which water is circulated to control reaction temperature. Fixed bed bioreactors can be used for large-scale synthesis. They are very efficient and easy to operate. The ratio of substrate to enzyme is much lower in fixed bed bioreactors compared to batch reactors resulting in shorter reaction times, and reduction in unwanted side reactions such as acyl migration (Mu and others, 1998). The rate of substrate conversion is mainly dependent on the residence time, which is controlled by the flow rate.

Downstream Processing of SL

The method of choice for separating and purifying SL depends on the amount of SL produced. SL products from small scale synthesis are usually separated by thin-layer chromatography. The lipid band of interest (e.g., TAG) is scraped off and its fatty acid profile determined by gas chromatography or its TAG profile analyzed by reverse-phase high performance liquid chromatography (RPHPLC). Normal-phase HPLC can also be used to determine the different classes of lipids that may be present in the reaction product. For large scale synthesis, short-path distillation and alkaline deacidification have been used to remove free fatty acids from 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 vessels – one for the purified product and the other for waste. The temperature of the distillator is controlled by circulation heating oil in between the two walls of the vessel. Fractionation is usually performed at high temperatures and low pressures (< 1 mm Hg) in order to protect heat sensitive compounds, and to prevent autooxidation of oils. To separate the free fatty acids from oils, the incoming sample stream is smeared against the hot inner wall of the vessel by rotating wipers. This causes free fatty acids and any compounds having boiling points lower than the operating temperature to vaporize. The vaporized components precipitate on a condenser and drip into the waste receiving vessel, whereas the non-volatile component (purified oil) remains on the inner wall of the vessel and drips into the product receiving vessel. Short-path distillation has been used to remove free fatty acids from SL products (Lumor and others, 2007; Moussata and Akoh, 1998). Alkaline deacidification is accomplished by reacting lipid samples with an alkali (sodium hydroxide or

potassium hydroxide) in 20% ethanol solution to form soap. The soap is then separated from the neutral oil.

PROPERTIES OF SL

Fatty Acid Profile

The fatty acid composition of a structured lipid is an important determinant of its chemical and physical properties. Fats and oils with higher proportions of unsaturated fatty acids are softer and may be more prone to oxidation than fats and oils with higher proportions of saturated fatty acids. Lauric, myristic, palmitic, and *trans* fatty acids are considered atherogenic, whereas oleic, linoleic, alpha- and gamma-linolenic, eicosapentaenoic, and docosahexaenoic acids are protective against cardiovascular and other degenerative diseases. In addition to fatty acid composition, the position of fatty acids on the TAG backbone is very important. Saturated fatty acids are better hydrolyzed and absorbed when present at the *sn*-1,3 positions of the TAG, whereas unsaturated fatty acids are better absorbed at the *sn*-2 position (Gebauer and others, 2005).

Gas chromatography is commonly used to determine fatty acid composition. New methods based on infra-red (IR) spectroscopy have been developed for the determination of *trans* fatty acids (Kim and others, 2007; Ratnayake, 1995). *Sn*-2 fatty acid composition is determined by cleaving fatty acids at the *sn*-1,3 positions by pancreactic lipase digestion or by Grignard degradation. The resulting *sn*-2 monoacylglycerol (MAG) is converted to fatty acid methyl esters and analyzed by GC. Another method that is currently gaining attention in this area is high resolution proton-decoupled ^{13}C nuclear magnetic resonance (NMR) spectroscopy. This is a rapid and nondestructive method used to determine fatty acid composition and positional distribution on the glycerol backbone (Ng, 1985; Wollenberg, 1995; Gunstone, 1990;

Sacchi and others, 1997; Mannina and others, 1999). This is because the acyl carbonyl, C-2, and olefinic carbons on the triacylglycerol backbone show slightly different ^{13}C chemical shifts depending on their positional distribution (*sn*-1,3 or *sn*-2) and degree of unsaturation. Lumor and others (2007) have used this method to determine the positional distribution of fatty acids in a structured lipid synthesized by reacting stearic acid with canola oil.

TAG Profile

TAG profile of fats and oil is very important in predicting physical properites such as solid fat content and crystal habits. TAGs with higher amounts of saturated fats contribute to higher solid fat content, and vice versa. Lumor and others (2007) observed that solid fat content was not only dependent on the saturated fatty acid content of a fat or oil, but also on the degree of saturation of the TAGs. In the said study, a SL which had comparable saturated fat content as a commercial margarine fat, showed significant lower solid fat content at 10˚C. A closer examination of TAG profiles of the two fats showed that the margarine fat contained higher proportions of TAGs with at least two saturated fatty acids in their structures. Fats containing highly diverse TAG profiles or greater fatty acid chain-length diversity tend to crystalize in the β' form, whereas oils with little TAG diversity crystalize in the β form. 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. Fats and oils will show any of the crystal forms depending on the presence of specific TAGs and emulsifiers, the level of liquid oil present, and temperature fluctuations during storage (deMan and deMan, 2001).

Reverse-phase high performance liquid chromatography (RP-HPLC) equipped with an evaporative light scattering detector (ELSD) is commonly used to determine TAG profile. TAGs are identified by comparing their retention times to those of TAG standards determined under the same operating conditions. Alternatively, the HPLC can be interfaced with a mass detector for the identification of TAG species (Lumor and others, 2007).

Crystal Properties

The consistency of fats depend on the number, size and types of crystals (Nawar, 1996), solid fat content, temperature fluctuations and mechanical working. TAG crystals exist in one of three major polymorphic forms known as α, β, and β'. The α form is the least stable and the β form the most stable. Fats containing predominantly β' TAG crystals impart smooth texture or mouthfeel to margarine, whereas those with predominantly β TAG crystals impart grainy texture. Oils such as canola, soybean, sunflower, and olive crystallize in the β form, whereas oils such as palm, palm kernel and cottonseed crystallize in the β' form. Oils that crystallize in the β' form usually have increased TAG or fatty acid chain-length diversity (Babayan, 1988). For most oils and fats used in the food industry, chain-length diversity refers to the ratio of 16 and 18 carbon fatty acids (deMan and deMan, 2001). The higher the level of palmitic acid, the more likely the fat will be stable in the β' form. Transesterification of β-tending oils or fats with β'-tending fats to increase fatty acid chain-length diversity has been reported to increase the proportions of β' crystals (Rousseau and Marangoni, 1999).

The polymorphic forms of fats can be studied by X-ray diffraction. Beta-prime polymorphs show two strong signals at d-spacings of 3.9 and 4.3 Å, whereas β polymorphs show three strong signals at 4.6, 3.9, and 3.8 Å. The relative proportions of β and β' crystals in the blends are calculated by dividing the peak intensity at 4.6Å (β) by the peak intensity at 4.3Å (β').

Polymorphic forms can also be determined by differential scanning calorimetry (DSC) due to their characteristic melting points. An instrument that allows for simultaneous X-ray diffraction and DSC to be carried out has been reported (Keller and others, 1998).

Crystal morphology is of great importance in the manufacture of margarine because it affects product consistency and acceptability (Rousseau and others, 1996). Smaller crystals lead to firmer fats and impart smooth texture or mouthfeel to margarine, whereas larger crystals or crystal aggregates produce softer fats and may impart grainy texture or mouthfeel to margarine. It is thought that samples which have more homogeneously distributed crystals in their fat networks provide higher resistance to penetration by a cone than samples with regions of high and low crystal densities (Campos and others, 2002). Crystal morphology is investigated by means of polarized light microscopy.

Solid Fat Content

Solid fat content (SFC) is a good indicator of the spreadability of a margarine or spread. The most commonly used method to estimate SFC is low-resolution NMR spectroscopy, which calculates the percentage of solids in fats. SFC is dependent on fatty acid and TAG compositions of the fat. Fats or TAGs with higher proportions of saturated fatty acids have higher solid fat contents compared to fats higher in unsaturated fatty acids. A desirable margarine is one that has at least 7.6% SFC at 10˚C needed to maintain good crystal structure (Nor and others, 1996), easily spreadable once taken out of the refrigerator, and melts completely in the mouth. Complete melting in the mouth ensures the release of flavor, and also imparts smooth mouthfeel to margarine. Fats with not more than 32% SFC at 10˚C are spreadable at non-refrigeration temperatures (Lida and Ali, 1998). However, fats with moderate SFC (7.6 to 13%) at 10˚C and a steep SFC curve at non-refrigeration temperatures are more spreadable.

Rheology

Rheology is the study of deformation and flow of matter under the influence of an applied force or stress, such as what happens in the spreading of margarine. With regards to margarine, stress viscometry, creep and dynamic analyses are used to evaluate rheological properties (Bohlin Application Notes). 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/elastic (G') and loss/viscous (G'') moduli are calculated from this information. The frequency (Hz) at which G' and G" crossover is a measure of spreadability. The higher the crossover frequency, the more spreadable the margarine is.

Rheology can also be used to estimate the melting profile of fat by conducting a temperature sweep test in which the viscosity of the fat is measured at different temperatures. This test has been used to predict the perceived melting of spreads (Chronakis and Kasapis, 1995; Berwanker and others, 1992). In a related study, Fomuso and Akoh (2001) observed that G', an indicator of elastic or solid behavior, decreased exponentially with decreasing solid fat content of a SL-based margarine. In another study, a direct relationship was observed between G' of a fat and its hardness index as measured by cone penetrometry (Rousseau and others, 1996).

Texture Evaluation

Texture is one of the factors that greatly affect consumers' preference or liking for a product. The texture of margarine is dependent on the fatty acid composition of the fat, the types and morphologies of crystals, the strength of the crystal network, the working of the margarine, and temperature fluctuations during storage. Fats made up of very small homogeneous crystals tend to be firmer than fats that contain crystals that are of irregular shapes and sizes (Campos and others, 2002). Also, the relative proportions of β' and β crystals affect margarine texture. The texture of margarine can be studied using cone penetrometry, a texture analyzer, or an Instron universal testing machine.

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 (A_2) for the first compression is reported as adhesiveness. The ratio of the positive force area during the second compression (A_3) to that of the first compression (A_1) 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.

Sensory Evaluation

Sensory evaluation is an important tool that links product attributes with consumer preferences. The type of sensory evaluation performed is dictated by the intent of the test. The

three main forms of sensory evaluation are preference test, difference test and descriptive analysis. Preference and difference tests are used to compare products, whereas descriptive analysis is used to evaluate specific attributes of products. The preference study draws inference on the general population's preference using an untrained panel of at least 50 and preferably more than 100 subjects. The 9-point hedonic scale (Peryam and Giraridot, 1952) is used by most sensory scientists to evaluate preference. The scale ranges from "like extremely" to "dislike extremely." A panel average is generated to express consumer preference. This method is reported to have some shortcomings. It suffers from end effects because panelists are less willing to use the ranks, 1, 2, 8 and 9 (Pangborn, 1980; O'Mahony, 1991; Faller and Faller, 2000). Also, the scale uses unequal intervals (O' Mahony, 1991; MosKowitz, 1994) making the reporting of means rankings inappropriate. For example, the distance between 5 and 6 is not necessarily the same as between 6 and 7, or 7 and 8, etc. Moreover, the 9-point hedonic scale measures liking which is not the same as acceptability (Land, 1998; Kramer and Twig, 1970; Shewfelt and others, 1997), and therefore may not be suitable for evaluating consumers' acceptance or willingness to purchase a food product. To make up for these shortcomings, the 5-point willingness-to-purchase scale, ranging from "will definitely buy" to "will definitely not buy" and the 3-point acceptability scale, ranging from "tastes great" to "unacceptable", are used (Dubost and others, 2003; Shewfelt and others; 1997).

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 identity 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.

In order to understand why samples are different, descriptive analysis using highly trained panelists to evaluate specific attributes of products is performed. The three main methods used for descriptive analysis are the flavor profile method (FPM), quantitative descriptive analysis (QDA), and Spectrum™. QDA and Spectrum™ methods are different from FPM in the sense that measurement is taken from individual panelists and a panel average generated, rather than the generation of a group consensus profile as with FPM (Piggott and others, 1998). In QDA, the panel is made up of 8 to 12 prescreened individuals who are trained to generate descriptors for the product. Panelists are asked to indicate their response using a dash on an unstructured line. The marks are converted to a numerical scale with a ruler and the data analyzed statistically. In Spectrum™, the panel size is the same as QDA, but the panel uses an already established descriptor list. Panelists score the intensities of the attributes using a dash on a line scale that is anchored on either end, and can be a 15 cm line scale, or a line with 15 equal intervals. A panel average is then generated for each attribute and statistical analysis performed. Most sensory professionals combine QDA and Spectrum™.

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CHAPTER 3

SYNTHESIS AND CHARACTERIZATION OF CANOLA OIL-STEARIC ACID-BASED *TRANS*-FREE STRUCTURED LIPIDS FOR POSSIBLE MARGARINE FORMULATION[1]

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ABSTRACT

Incorporation of stearic acid into canola oil to produce *trans*-free structured lipid (SL) as a healthy alternative to partially hydrogenated fats for margarine formulation was investigated. Response surface methodology was used to study the effects of Lipozyme RM IM from *Rhizomucor miehei* and *Candida rugosa* lipase isoform 1 (LIP1) and two acyl donors, stearic acid and ethyl stearate, on the incorporation. Lipozyme RM IM and ethyl stearate gave the best result. Gram quantities of SLs were synthesized using Lipozyme RM IM and the products compared to SL made by chemical catalysis, and fat from commercial margarines. After short-path distillation, the products were characterized by GC and RPHPLC-MS to obtain fatty acid and triacylglycerol profiles, ^{13}C NMR spectrometry for regiospecific analysis, X-ray diffraction for crystal forms, and DSC for melting profile. Stearic acid was incorporated into canola oil, mainly at the *sn*-1, 3 positions for the lipase reaction and no new *trans* fatty acids formed. Most SL products did not have adequate solid fat content or β' crystal forms for tub margarine, although these may be suitable for light margarine formulation.

Keywords: Candida rugosa lipase isoform 1, Canola oil, Interesterification, Lipozyme RM IM, Response surface methodology, Sodium methoxide, Stearic acid

INTRODUCTION

Concerns over significant consumption of *trans* fatty acids (TFA) has been a major health and policy issue since the past decade. *Trans* fatty acids occur naturally in small amounts in dairy products as a result of bio-hydrogenation of *cis*-unsaturated fatty acids by rumen microorganisms. The majority of TFA consumed, however, comes from products formulated with partially hydrogenated fats such as frying oils, margarines, spreads, shortenings, bakery products and fast foods. The amount of TFA present in these foods is estimated to be between

0% and 35% of total fatty acids (*1*), and the mean daily intake of TFA per person in the US population is 2.6% energy or 5.3 g (*2*).

Intake of high amounts of TFA has been correlated with increased risk of cardiovascular diseases, primarily due to their adverse effects on plasma lipid profile (*3, 4*). These and other studies have heightened health concerns among consumers and regulatory agencies in Europe and the United States. As a result, the US Food and Drug Administration (FDA) issued a final ruling requiring foods containing TFA to be labeled accordingly, effective from January 2006 (*5*). The food industry is responding to these concerns by developing processes that will produce foods with zero or reduced *trans* fat contents. These alternatives, among others, include the use of transesterification to make structured lipids (SL). Structured lipids are synthesized by incorporating high-melting fatty acids into oils or by blending high-melting fractions of natural oils or fully hydrogenated fats with liquid oils. This process (transesterification) therefore increases the solid fat content and oxidative stability of the product and prevents post-hardening effects of margarine when stored (6). Moreover, this process does not result in the production of *trans* fatty acids, as is the case with partial hydrogenation (1*, 4*), and therefore has no adverse effects on plasma lipoprotein profile.

The suitability of fats for margarine formulation depends on their physical, crystal and melting properties, which in turn is dependent on the amount and type of triacylglycerols (TAGs), and to a lesser extent, the amount of diacylglycerols (DAGs) present. The aim of this study was to incorporate stearic acid into canola oil by the process of transesterification. The effects of lipase (lipozyme RM IM and *Candida rugosa* lipase isoform 1) and acyl donor (stearic acid and ethyl stearate) types, as well as temperature, substrate ratio and time on the process, were investigated using response surface methodology (RSM). SLs produced by lipase- and sodium methoxide-

catalyzed reactions were characterized for possible margarine formulation. Stearic acid was our choice of fatty acid because of its high melting point. Besides, it has no known adverse effects on plasma cholesterol levels (*7-10*). Canola oil contains α- and γ-linolenic acids which are important in reducing coronary heart disease (CHD) risk factors (*11, 12*). Our SL products were therefore expected to have the following positive health indicators: (1) low ratios of ω-6:ω-3 fatty acids, (2) reduced contents of atherogenic fatty acids, and (3) zero *trans* fatty acid contents.

MATERIALS AND METHODS

Materials. Stearic acid, sodium methoxide and citric acid were purchased from Sigma Chemical Co. (St. Louis, MO). Canola oil (peroxide value 0.0, acid value 0.28) was bought from a local grocery store. Immobilized Lipozyme RM IM was purchased from Novo Nordisk A/S (Bagsværd, Denmark) and unimmobilized LIP1 (genetically engineered and expressed in *P. pastoris*) was donated by Dr. Jei-Fu Shaw of Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei, Taiwan. Organic solvents and chemicals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA). All other chemicals used were of analytical or HPLC grade.

Experimental Design for RSM. The design consisted of five variables. These were enzyme (Enz), acyl donor (Acyl), temperature (Te), substrate molar ratio (Sr) and time (t). Enzyme and acyl donor had two factor levels while the others had five. The worksheet is shown in **Table 1**. For creating response surfaces, the data obtained based on the above design were fitted to a second-order polynomial equation of the form:

$$y = \beta_o + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1}^{5} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{ij} X_i X_j + \varepsilon_{ij}$$

Where y = % incorporation of stearic acid; β_o = constant; β_i = linear term coefficients; β_{ii} = quadratic term coefficients; β_{ij} = interaction term coefficients; X_i and X_j = independent variables; and $\boldsymbol{\varepsilon_{ij}}$ = error term. Regression analyses, statistical significance and response surfaces were done using MODDE 7.0 software (Umetrics, Umeå, Sweden) to obtain the relationship between the response and the independent variables.

RSM Study of Structured Lipid Synthesis. SL synthesis was performed in screw-cap test tubes incubated in an orbital shaking water bath at 200 rpm using the conditions specified in **Table 1**. The reaction mixture typically contained 100 mg of canola oil and milligrams of stearic acid or ethyl stearate corresponding to the mole ratios. The reactants were dissolved in 1.5 mL hexane. The amount of enzyme used was 10% of the total weight of the substrates. After the reaction was stopped, 2 mL of hexane was added to the reaction products, and the enzymes filtered off by passage through a column of anhydrous sodium sulfate. Fatty acid profiles of the products were determined as described below. All reactions were performed in triplicate and average values reported.

Determination of Stearic Acid Incorporation. About 50 μL of the reaction product was spotted onto silica gel G TLC plates. A mixture of petroleum ether, ethyl ether and acetic acid was used to develop the plates [80:20:0.5, (v/v/v), combination for SL made with stearic acid; and 90:10:1, (v/v/v), for SL made with ethyl stearate]. Bands were visualized under UV light after spraying with 0.2% 2, 7-dichlorofluorescein in methanol, and those corresponding to TAGs were scraped off and methylated in 3 mL methanol (containing 6% HCl) at 75˚C for 2 h to form fatty acid methyl esters (FAME). The methyl esters were extracted twice with 2 mL hexane and dried by passing through a column of anhydrous sodium sulfate. The methyl esters were separated on an AT-225, 30 m x 0.25 mm, 0.25 μm film column using an Agilent 6890N gas chromatograph

equipped with a flame ionization detector (FID). Injection (1 μL) was performed in the split mode, at a split ratio of 50:1. Helium was the carrier gas, and the linear velocity was 18 cm/sec and the flow rate was 1.7 mL/min. The oven temperature was initially held at 80˚C for 3 min, then programmed to 215˚C at 10˚C/min, and held isothermally for 20 min. The injection port temperature was 250˚C while that of the detector was 260˚C. The different amounts of FAME were analyzed and integrated by an online computer with C17:0 as internal standard. The analyses of FAME were performed in triplicate for each SL and average values for stearic acid reported (Table 1).

Gram Scale SL Synthesis. Structured lipid synthesis was performed in a stir-batch reactor at 50˚C for 12 h. The reaction mixture typically contained 300 g of canola oil, 10 - 40% stearic acid (by weight of canola oil), and 10% Lipozyme RM IM (by total weight of reactants). For the anhydrous sodium methoxide-catalyzed reaction, reactants (70 g canola oil and 7 g ethyl stearate) were dried under nitrogen gas at 110˚C for 15 min. After lowering the temperature to 80˚C, 0.3% sodium methoxide powder was added, and the mixture heated to 100˚C with vigorous stirring under nitrogen gas for 1 hr. The temperature was lowered to 70˚C, and 20 mL of 20% (w/v) citric acid added to stop the reaction. Excess catalyst and citric acid were removed by warm water washes. The SL was separated and dried using anhydrous sodium sulfate and free fatty acids were removed by short-path distillation.

Short-Path Distillation. Short-path distillation was carried out with a KDL-4 (UIC Inc., Joliet, IL) unit under the following conditions: heating oil temperature, 185˚C; cooling water temperature, 15˚C; pump vacuum, <1 mm Hg; feed rate, maintained at 100 mL/h. The reaction product was passed through the system twice to reduce the free fatty acid percentage to an

acceptable level. Free fatty acid content (0.13 – 1.08% oleic acid) was determined according to AOCS Official Method, Ca 5a-40 (*13*).

Determination of Fatty Acid Profiles. Between 0.1 – 0.2 g of each lipid sample (in duplicate) was converted to fatty acid methyl esters (FAME) using AOAC Official method 996.01, Section E (*14*). Briefly, 1 mL of 20 mg/mL C13:0 (internal standard) dissolved in chloroform was added to each sample in flat-bottom flasks. Methanolic NaOH (10 mL) was added, and the mixture was refluxed for 10 min, after which 10 mL BF_3 reagent was added. Reflux continued for an additional 5 min. n-Heptane (10 mL) was then added, followed by an additional minute of reflux, after which the reaction mixture was allowed to cool and then transferred to a measuring cylinder/centrifuge tube. The flat-bottom flask was rinsed with 10 mL saturated NaCl solution, and the wash transferred to the centrifuge tube. The contents of the centrifuge tube were mixed thoroughly and kept for 10 min to allow for phase separation. The organic phase containing the FAME was transferred to a GC vial and used for analysis. The FAME were analyzed in parallel with a FAME standard (Supelco 37 component FAME mix, Supelco®, Bellafonte, PA) using an Agilent Technology 6890N gas chromatograph equipped with a flame-ionization detector (FID). An SP-2560, 100 m x 0.25 mm ID, 0.20 μm film column was used for separation. One injection (1 μL) per sample duplicate was performed in the split mode, at a split ratio of 50:1. Helium was the carrier gas, and the linear velocity was 18 cm/sec and the flow rate was 1mL/min. The oven temperature was initially held at 120˚C for 5 min and then programmed to 240˚C at 30˚C/min, and held isothermally for 20 min. The injection port temperature was 200˚C while that of the detector was 250˚C. The different amounts of FAME were analyzed and integrated by an online computer, and values for duplicate samples averaged to give fatty acid profile of each sample (Table 4).

HPLC Analyses. SL products, canola oil and margarine fats (TAG species) were analyzed by reverse-phase HPLC (RPHPLC) using an HP1050 (Agilent Technologies, Little Falls, DE) equipped with a quaternary pump, autosampler, and a Varex MKIII (Burtonville, MD) evaporative light-scattering detector (ELSD). Separation was performed on a Waters Symmetry C18 (150 x 2.1 mm i.d.) column attached to a guard column (50 x 2.1 mm i.d.). Aliquots (20 μL) of each sample were dissolved in 300 μL of methylene chloride (CH_2Cl_2) and 700 μL of acetonitrile (ACN). Ten microliters of the final solution was injected into the HPLC. A binary gradient system of ACN and CH_2Cl_2 was used at 0.2 mL/min. Separation was performed using a solvent gradient profile starting with ACN: CH_2Cl_2 [65:35, (v/v)] for 10 min, followed by a linear increase of the CH_2Cl_2 concentration to 50:50, v/v over a period of 40 min, an isocratic period of 5 min, a linear decrease to initial conditions over a 5 min period, and a final isocratic period of 5 min.

HPLC-MS Analyses. HPLC-MS was performed on Waters 2690 separations module (Waters Corp., Milford, MA) coupled to a Micromass ZMD MS (Micromass, Manchester, UK) with an atmospheric pressure chemical ionization (APCI) probe. Column and separation conditions were the same as HPLC-ELSD. Acylglycerol species were identified using a database (www.byrdwell.com/Tryacylglycerols) for protonated TAGs and DAG-like fragment ions formed from TAGs in APCI-MS. Chromatograms from the HPLC-MS (APCI) and HPLC-ELSD analyses were compared and peaks with similar retention times were analyzed to obtain qualitative and quantitative information.

^{13}C NMR Analyses. A proton-decoupled ^{13}C NMR was used to analyze the positional distribution of fatty acids on the TAG backbone. Lipid samples (50 μL) were dissolved in $CDCl_3$ (500 μL) in 5 mm NMR tubes and NMR spectra were recorded on a 9.2 Tesla Varian INOVA

spectrometer operating at 100 MHz. The ^{13}C spectra of both the lipid samples and the standard samples were acquired with a spectral width of 25000 Hz, pulse angle of 45˚, a relaxation delay 3 s, 60K data points, and 512 transients. In addition, the spectra of the lipid samples were re-acquired with a 30 s relaxation delay, 128K or 256K data points, and 1800 – 2400 transients. Since these ^{13}C spectra used a relaxation time that was at least 5 times the relaxation time of the carbonyls, it was possible to perform a quantitative integration of the carbonyl region to determine the relative concentrations, and hence the data were used for analyses. Prior to Fourier transformation, a TRAF apodization function with a line-broadening factor of 0.5 Hz was applied. Chemical shifts were referenced to tetramethylsilane (TMS) at 0 ppm. Tripalmitin, tristearin, triolein, trilinolien and trilinolenin were used as standard TAGs.

Differential Scanning Calorimetry (DSC). Melting profile of lipid samples were determined by DSC on a Perkin-Elmer model DSC1 (Norwalk, CT). The melting profiles of purified products were compared to those of fat extracted from four commercial margarine samples. Analysis was performed using a modification of AOCS recommended procedure Cj 1-94 (*15*). Briefly, samples were held for 2 min at 25˚C, followed by rapid heating to 80˚C at 10˚C /min, and held for 10 min. The samples were then cooled to -80˚C at 5˚C/min, and held for another 10 min. In the final step, samples were heated to 80˚C at 5˚C/min. Normal standardization was performed with cyclohexane (2 thermal transitions; 1 at -87.06 ˚C and 1 at 6.54 ˚C) and indium (thermal transition at 156.6 ˚C). Liquid nitrogen (-196˚C) was used as the coolant.

Solid Fat Content Analyses. Solid fat content (SFC) was determined according to the AOCS official methods Cd 16-81 (*16*) on a MARAN-20 pulsed NMR spectrometer (Resonance Instruments Ltd., Oxon, UK). 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 at 30 min at each temperature of measurement. SFC was measured at intervals of 5˚C from 5 up to 45˚C.

RESULTS AND DISCUSSION

Model Fitting. The amount (mole percent) of stearic acid incorporated into canola oil at different experimental conditions are shown in **Table 1**. Generally, reactions catalyzed by LIP1 produced little or no stearic incorporation, while those catalyzed by lipozyme RM IM produced appreciable levels of stearic acid incorporation: as low as 22.3 mol%, and as high as 59.10 mol%. The results obtained (**Table 1**) were fitted to a second-order polynomial model by multiple linear regression and backward elimination using MODDE 7.0 software (Umetrics, Umeå, Sweden). The squared term, t*t and the interaction terms Enz*Acyl, Enz*Te, Enz*t, Acyl*t, Te*Sr, and Sr*t were deleted from the model because they were not significant at $\alpha_{0.05}$. R^2, the fraction of the variation of the response explained by the model was, 0.99 and Q^2, the fraction of the variation of the response that can be predicted by the model, was 0.97. R^2_{adj} was 0.99. The reproducibility and validity of the model were 0.99 and 0.74, respectively. The normal probability plot (**Figure 1a**) showed a linear distribution, indicating that our assumption of normality of model errors was not violated. Likewise, the residual plot (**Figure 1b**) showed no patterns, indicating that our model assumption of constant error variance was not violated either. Furthermore, the model showed no lack of fit ($P > 0.05$), and the multiple regression *P*-value was < 0.001 (**Table 2**). The model equation can therefore be written as:

Inc = 27.56 ± 17.95 Enz ± 4.26 Acyl + 2.41 Te + 7.86 Sr – 0.95 t – 3.74 Te*Te – 3.23 Sr*Sr ± 4.72 Enz*Sr ± 1.98 Acyl*Te ± 2.57 Acyl*Sr – Te*t

Except for time (**Table 3**), all coefficients were highly significant ($P < 0.05$). Time (t) was kept in the model because of its significant ($P < 0.05$) interaction term with temperature (Te*t).

Effect of Parameters. Figure 2 shows the effect of reaction parameters on stearic acid incorporation. All linear parameters, except time, had positive effect on incorporation. The squared terms of temperature and substrate ratio (Te*Te and Sr*Sr) had negative effects while the interaction terms, Enz (RM IM)*Sr, Acyl (Ethyl stearate)*Sr, and Acyl (Ethyl stearate)*Te had positive effects. Only the interaction term of Te*t had a negative effect on incorporation. The projected response for substrate molar ratio, time and temperature when all, but the parameter of interest were held constant is shown in **Figure 3.** These were for lipozyme RM IM-catalyzed reactions. Stearic acid incorporation was higher (**Figure 3a**) when ethyl stearate was used as acyl donor than when stearic acid was used (**Figure 3b**). Substrate ratio produced the highest change in incorporation per change in factor level in both cases. The effect of time on incorporation implies that prolonging the reaction would result in a steady but nevertheless insignificant decline in the level of stearic acid incorporation ($P > 0.05$, **Table 3**). The effect of temperature on incorporation (**Figures 3a** and **3b)** did not follow the same pattern as did the effects of substrate ratio and time. The highest point of stearic acid incorporation was at 60˚C (**Figure 3a)** and at 50˚C (**Figure 3b)**. This temperature-dependent relationship has been previously discussed by Lumor and Akoh (*17*).

Increasing temperature has two effects on reaction rate: First, it increases the rate of productive collisions between reactants and the enzyme, resulting in increased acyl incorporation. Second, it can also accelerate the rate of enzyme inactivation (*18*), thereby producing less incorporation (*19, 20*). However, whether incorporation will increase or decrease with increasing temperature will depend on a number of factors such as concentration and type of substrate used, and other

reaction conditions such as the presence of water. Although most lipase-catalyzed reactions require water content of less than 1% w/v (*21*) for effective transesterification, accumulation of water during the course of reaction, as is the case for acidolysis reactions involving oils containing partial acylglycerols (*17*), causes equilibrium to shift in favor of hydrolysis. This phenomenon, coupled with the denaturing effect of increasing temperatures on the lipase are mostly responsible for the disparity in the levels of stearic acid incorporated between the interesterification (**Figure 3a**) and the acidolysis (**Figure 3b**) reactions. Ester-interchange or interesterification reactions on the other hand do not involve accumulation of water.

Optimization of Reaction. As is the case for quadratic models, the response is not only affected by the first-order variables, but also by second-order and interaction terms and may have more than one solution (*22*). Evaluation of the relationship between the response and parameters is best done by means of contour plots. This is done by placing the reaction parameter with the greatest effect 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 allow the researcher to identify parameter combinations that will produce a desired response, which in this case, is the level of stearic acid incorporation that will produce SLs with similar melting and crystal properties as commercial margarine samples.

The contour plots are shown in **Figure 4**. Time, being the least significant variable (**Table 3**) was held constant at 12 h, while the other parameters were varied. In general, incorporation increased with increasing temperature and substrate ratio. It can be seen that both enzymes favored stearic acid incorporation more when ethyl stearate, rather than stearic acid was used as acyl donor. This observation is consistent with other studies (*23-26*) which showed that (1) incorporation was more favored with the ethyl ester form and (2) lipozyme RM IM proved to be a better biocatalyst for the process than LIP1. The ineffectiveness of LIP 1 for incorporation of

certain long chain fatty acids into TAGs has previously been reported (*27*). In a nutshell, the contour plots show that a combination of ethyl stearate and Lipozyme RM IM, at any experimental conditions will produce the highest incorporation. This is followed by the pairs, RM IM: stearic acid, LIP1: ethyl stearate, and LIP1: stearic acid, in that order.

Fatty Acid Profile of Samples. The fatty acid profiles of canola oil, SL products and margarine samples were determined (**Table 4**). There was significant incorporation of stearic acid in both the lipase (EZ-A2 to EZ-D2) and sodium methoxide (CI-A) catalyzed reactions. These values ranged from 10.71 to 26.86% (in samples EZ-A2 to EZ-D2) as substrate mole ratio increased from 10% to 40% stearic acid by weight of canola oil. Stearic acid content of CI-A was 6.27%. For the margarine samples, stearic acid content ranged from 4.31 (MG-E) to 12.53% (MG-B). The increased stearic acid content of the SL products is not expected to pose any health risks since the neutrality of stearic acid with regard to plasma cholesterol levels has been established (*7-10*). The amounts of atherogenic fatty acids such as palmitic and myristic were less than 5% in all SL samples but ranged from 10.53 to 26.6% in the margarine samples. Dietary intake of saturated fatty acids below 10% energy is recommended in order not to significantly alter plasma cholesterol levels (*28*). Oleic acid was the main fatty acid in canola oil and the SL products. Its content decreased from 59.72 in canola oil to between 41.65 and 54.58% in the SL products as stearic acid incorporation increased. Oleic acid, the main fatty acid in Mediterranean diets has been correlated with low incidence of coronary heart disease (*29*), and is more stable to oxidation compared to polyunsaturated fatty acids.

Table 4 also gives the *trans* fatty acid contents of canola oil, SL products and commercial margarine samples. Elaidic acid (18:1*t*) was detected in all samples while 18:2*t* was detected only in margarine samples (MG-B and MG-E). The *trans* fat content of the SL products were

considerably lower than those in the margarine samples. The source of 18:1*t* in the SL products was most definitely canola oil since there was no significant difference between the amounts present in the SLs (0.07-0.09%) and canola oil (0.09%). However, the level of *trans* fatty acids in these samples may not be enough to pose any health risks. The dietary levels of *trans*-fatty acids required to increase LDL-cholesterol is said to be approximately 4.3% energy or higher, and approximately 5-6% energy or higher to decrease HDL-cholesterol (*1*).

Triacylglycerol Profile. Significant amounts of stearic acid-containing TAGs were found in the SL samples (**Figure 5**). These were OSO/OOS, SSL, SOL, LLS, SOLn, SSLn, PSO, and SOS for the lipase-catalyzed reactions. Stearic acid-containing acylglycerols species increased in amount (approximately from 30.4 to 57.7%) as the amount of stearic acid used in the reactions increased from 10 to 40% (**Table 5**). For the sodium methoxide catalyzed reaction, OSO/OOS, SOL, PoSA and SSP were the only stearic acid-containing TAGs found. They constituted approximately 13% of the total TAGs in the product. Small amounts (3-10%) of DAGs were also found in all SL products. These were smaller (<1%) in canola oil. Major canola oil TAGs that decreased significantly with stearic acid incorporation were OOO, OLO, OOLn, LLO, and OOP. OOO and OLO decreased between 40-70% and 18-60% of their initial amounts in lipase-catalyzed reaction products, respectively. In the sodium methoxide reaction, OOO decreased by 22% and OOLn by 27% of their original amounts. TAGs containing high melting fatty acids found in the margarine samples were PPL, POL, PPO, OOP, OOS, PSO and POL (**Table 6**).

Positional Distribution of Fatty Acids. High resolution proton-decoupled ^{13}C nuclear magnetic resonance spectroscopy is a rapid and non-destructive method used to determine fatty acid composition and positional distribution on the glycerol backbone (*30-34*). This is because the acyl carbonyl, C-2 and olefinic carbons on the triacylglycerol backbone show slightly different

^{13}C chemical shifts depending on their positional distribution (*sn*-1,3 or *sn*-2) and degree of unsaturation (*33*). Since the relaxation delay for these experiments was used consistently for all samples, and since all of the carbonyls are in similar chemical environments, the integrated intensities of these peaks could be used semi-quantitatively to determine the relative concentrations of the components. The carbonyl ^{13}C chemical shifts occur usually between 172-174 ppm. **Figure 6** shows the carbonyls regions of canola oil and the SL products. Using the published resonance assignments, our ^{13}C NMR data showed that stearic acid was mainly incorporated at the *sn*-1,3 positions (173.30-173.55 ppm) in the lipase-catalyzed reaction products (**Figures 6c-f**). No significant stearic acid peaks were observed at the *sn*-2 position (172.95-173.10 ppm). On the other hand, stearic acid peaks were observed at the *sn*-1,3 and 2 positions of the sodium methoxide-catalyzed reaction product (**Figure 6b**). This observation confirms the *sn*-1,3-specificity of lipozyme RM IM and the non-specificity of chemical interesterification. It is also significant because saturated fatty acids are better hydrolyzed and absorbed when present at the *sn*-1, 3 positions while unsaturated fatty acids are better absorbed at the sn-2 position (*11*).

Physical Properties. The melting profiles of our products were compared to those of canola oil and commercial margarine fats. DSC thermograms (**Figure 7**) showed the emergence of high melting endotherms (C and D) in the lipase-catalyzed structured lipid products as a result of increased stearic acid incorporation into acylglycerols, while the native peaks of canola oil (A and B) decreased. For CI-A, a broadening of peaks A and B and a small peak (E) were observed, signifying the formation of triacylglycerol species with close melting points. The melting ranges of the SLs were narrower and within those of the commercial margarine samples, indicating their suitability for formulating softer margarines. SFC data (**Figure 8**) showed that only one sample,

i.e., the one containing the highest amounts of stearic acid (EZ-D2), had more than the minimum solid fat content (7.6% at 0˚C) needed to maintain a good crystal structure desirable for soft margarine formulation (*35*). The others would be suitable for liquid or light margarine formulation. The X-ray crystallography data (not shown) indicated that the crystal structures of the SLs were predominantly β. Ongoing studies in our laboratory include increasing solid fat content and regulating the crystal-forming habits of the SLs by blending with hydrogenated palm oil mid-fraction and/or by using emulsifiers.

We also observed that even though EZ-D2 and MG-E had comparable saturated fatty acid contents (**Table 4**), there was a noticeable difference in their SFC curves (**Figure 8**). This was most probably due to the fact that MG-E contained more TAGs (**Table 6**) with at least two saturated fatty acids than was found in EZ-D2 (**Table 5**). This indicates that the saturated fatty acid content of a fat is not enough to predict its melting behavior, but much depends on the saturated fatty acid content of the TAG species.

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Table 1. Experimental Settings of the Factors and the Responses Used for Optimization of the Reaction by RSM

expt	enzyme	acyl donor	temperature (˚C)	substrate ratio	time (h)	Inc[a] (Mol%)
N1	LIP1	Stearic acid	35	1	14	0.01 ± 0.00
N2	LIP1	Stearic acid	35	5	14	0.52 ± 0.91
N3	LIP1	Stearic acid	35	3.7	6	0.58 ± 1.01
N4	LIP1	Stearic acid	35	2.3	18	0.00 ± 0.01
N5	LIP1	Stearic acid	65	1	10	0.01 ± 0.01
N6	LIP1	Stearic acid	65	5	10	0.42 ± 0.73
N7	LIP1	Stearic acid	65	2.3	6	0.00 ± 0.00
N8	LIP1	Stearic acid	65	3.7	18	0.00 ± 0.00
N9	LIP1	Stearic acid	45	1	6	0.00 ± 0.00
N10	LIP1	Stearic acid	55	1	18	0.00 ± 0.00
N11	LIP1	Stearic acid	45	5	6	0.00 ± 0.00
N12	LIP1	Stearic acid	55	5	18	0.00 ± 0.00
N13	RM IM	Stearic acid	35	1	6	22.68 ± 8.72
N14	RM IM	Stearic acid	65	5	6	49.93 ± 3.70
N15	RM IM	Stearic acid	65	1	18	23.45 ± 2.89
N16	RM IM	Stearic acid	35	5	18	44.70 ± 1.16
N17	RM IM	Stearic acid	50	3	12	41.37 ± 4.77
N18	LIP1	Ethyl stearate	35	1	6	0.00 ± 0.00
N19	LIP1	Ethyl stearate	65	5	6	23.39 ± 0.68
N20	LIP1	Ethyl stearate	65	1	18	0.00 ± 0.00
N21	LIP1	Ethyl stearate	35	5	18	7.92 ± 13.72
N22	RM IM	Ethyl stearate	65	1	6	32.54 ± 0.92
N23	RM IM	Ethyl stearate	35	5	6	48.80 ± 4.23
N24	RM IM	Ethyl stearate	35	1	18	23.21 ± 1.17
N25	RM IM	Ethyl stearate	65	5	18	59.10 ± 1.28
N26	RM IM	Ethyl stearate	50	3	12	48.47 ± 2.44
N27	RM IM	Ethyl stearate	50	3	12	52.80 ± 2.14
N28	RM IM	Ethyl stearate	50	3	12	49.33 ± 2.62
N29	RM IM	Ethyl stearate	50	3	12	52.29 ± 3.74

[a]Abbreviation: Inc., incorporation of stearic acid into canola oil

Table 2. ANOVA Table for Incorporation of Stearic Acid

Inc. of stearic acid	DF[a]	SS	MS (variance)	F-value	*P*-value	SD
total	29	25654.9	884.653			
constant	1	11662.1	11662.1			
total corrected	28	13992.8	499.743			22.3549
regression	11	13867.5	1260.68	170.986	0.000	35.506
residual	17	125.341	7.37301			2.71533
lack of fit (model error)	14	111.57	7.9693	1.7361	0.360	2.823
pure error (replicate error)	3	13.771	4.59034			2.14251

N = 29; Q^2 = 0.965; $R^2_{Adj.}$ = 0.985

DF = 17; R^2 = 0.991; RSD = 2.7153

[a]Abbreviations: DF, degree of freedom; SS, sum of squares; MS, mean square; RSD, relative standard deviation; SD, standard deviation; Q^2, R^2, and R^2 Adj, explained in text.

Table 3. Coefficient List for Incorporation of Stearic Acid

Inc. of stearic acid	coeff[a]	std error	*P*-value[b]	conf int (±)
constant	27.5609	1.09177	6.46136e-015	2.30346
Enz	DF = 1			
Enz(LIP1)	-17.9538	0.556608	1.08675e-016	1.17435
Enz(RM IM)	17.9538	0.556608	1.08675e-016	1.17435
Acyl	DF = 1			
Acyl(Stearic acid)	-4.25799	0.550759	5.79499e-007	1.16201
Acyl(Ethyl stearate)	4.25799	0.550759	5.79499e-007	1.16201
Te	2.40803	0.615244	0.0011174	1.29806
Sr	7.86007	0.628361	5.317e-010	1.32574
T	-0.954043	0.60053	0.130559	1.26702
Te*Te	-3.74231	1.29444	0.0101529	2.73105
Sr*Sr	-3.23097	1.28407	0.0221988	2.70917
Enz*Sr	DF = 1			
Enz(LIP1)*Sr	-4.71995	0.628361	8.51351e-007	1.32574
Enz(RM IM)*Sr	4.71995	0.628361	8.51351e-007	1.32574
Acyl*Te	DF = 1			
Acyl(Stearic acid)*Te	-1.98042	0.615244	0.00503818	1.29806
Acyl(Ethyl stearate)*Te	1.98042	0.615244	0.00503818	1.29806
Acyl*Sr	DF = 1			
Acyl(Stearic acid)*Sr	-2.57206	0.628361	0.000757643	1.32574
Acyl(Ethyl stearate)*Sr	2.57206	0.628361	0.000757643	1.32574
Te*t	-1.79607	0.682067	0.0174358	1.43905

N = 29; Q^2 = 0.965; $R^2_{Adj.}$ = 0.985;
DF = 17; R2 = 0.991; RSD = 2.7153; conf lev = 0.95

[a]Abbreviations: Coef, multiple regression coefficients; Std Err, standard error; Conf int, confidence interval; Te, temperature (˚C); Sr, substrate molar ratio; t, time (h); Sr*Sr, quadratic term of Sr; Te*Sr, interaction term of Sr and Te; Sr*t, interaction term of Sr and t; RSD, relative standard deviation; SD, standard deviation; Q^2, R^2, and R^2 Adj, explained in text.
[b]Coefficients with *P*-value less than 0.05 are significant.

Tables 4. Fatty Acid Profile of Samples

fatty acid	canola oil	EZ-A2[a]	EZ-B2	EZ-C2	EZ-D2	CI-A	MG-B	MG-E
16:0	4.8 ± 0.00	4.17 ± 0.00	3.88 ± 0.01	3.64 ± 0.01	3.44 ± 0.00	4.55 ± 0.14	10.44 ± 0.17	26.01 ± 0.2
18:0	2.06 ± 0.00	10.71 ± 0.00	17.44 ± 0.06	22.80 ± 0.06	26.86 ± 0.00	6.27 ± 0.20	12.53 ± 0.16	4.31 ± 0.03
18:1 trans	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.01	0.07 ± 0.00	3.2 ± 0.06	0.28 ± 0.00
18:1 cis	59.72 ± 0.01	52.19 ± 0.00	47.78 ± 0.03	44.29 ± 0.03	41.65 ± 0.00	54.58 ± 1.44	24.48 ± 1.12	37.17 ± 0.13
18:2 trans							0.06 ± 0.00	0.01 ± 0.01
18:2 cis	21.29 ± 0.01	19.39 ± 0.70	18.68 ± 0.02	17.72 ± 0.02	17.04 ± 0.01	20.77 ± 0.66	40.89 ± 0.65	25.96 ± 0.2
20:0	0.67 ± 0.03	0.62 ± 0.00	0.60 ± 0.01	0.59 ± 0.00	0.58 ± 0.00	0.66 ± 0.02	0.42 ± 0.00	0.37 ± 0.00
18:3 n6	1..33 ± 0.00	1.25 ± 0.00	1.19 ± 0.00	1.12 ± 0.00	1.08 ± 0.01	1.31 ± 0.04	0.13 ± 0.00	0.43 ± 0.00
20:1	2.8 ± 0.00	2.45 ± 0.00	2.25 ± 0.00	2.10 ± 0.00	1.97 ± 0.00	2.67 ± 0.08	0.36 ± 0.01	0.35 ± 0.00
18:3 n3	7.72 ± 0.01	7.34 ± 0.01	6.92 ± 0.01	6.57 ± 0.01	6.29 ± 0.00	7.69 ± 0.24	6.07 ± 0.09	3.2 ± 0.01
Other[b]	2.05 ± 0.00	1.89 ± 0.00	1.76 ± 0.00	1.64 ± 0.00	1.56 ± 0.00	2.04 ± 0.01	1.89 ± 0.00	2.25 ± 0.00
ω-6:ω-3 ratio	2.76	2.64	2.70	2.70	2.71	2.70	6.74	8.11
satd fat	8.21 ± 0.02	16.28 ± 0.11	22.57 ± 0.04	27.62 ± 0.08	31.45 ± 0.00	12.24 ± 0.39	24.02 ± 0.16	32.35 ± 0.36
unsatd fat	91.79 ± 0.02	83.72 ± 0.11	77.43 ± 0.04	72.38 ± 0.08	68.55 ± 0.00	87.76 ± 0.39	75.98 ± 0.16	67.65 ± 0.36
monounsat. fat	63.16 ± 0.02	55.17 ± 0.01	50.52 ± 0.02	46.85 ± 0.03	44.04 ± 0.01	57.82 ± 1.33	28.17 ± 1.08	37.97 ± 0.14
polyunsat. fat	28.73 ± 0.00	28.55 ± 0.12	26.91 ± 0.02	25.53 ± 0.05	24.51 ± 0.02	29.93 ± 0.94	47.8 ± 0.93	29.68 ± 0.22
% trans fat	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.00	0.08 ± 0.01	0.07 ± 0.00	3.72 ± 0.06	0.29 ± 0.00

[a]EZ-A2, EZ-B2, EZ-C2 and EZ-D2 are SL samples made from Lipozyme RM IM-catalyzed reaction set-ups containing 10, 20, 30, and 40% (by weight of canola oil) stearic acid, respectively; CI-A is SL made from the sodium methoxide-catalyzed reaction set-up containing 10% stearic acid (by weight of canola oil). MG-B is a soft margarine made with a blend of soybean oil, hydrogenated soybean oil, partially hydrogenated soybean oil and canola oil; MG-E is a soft margarine made from a blend of palm, soybean, canola and olive oils.
[b]Others refer to sum of 12:0, 14:0, 16:1, 20:0, 21:0, 20:2, 22:0, 20:3 n6, 22:1 n9.

Table 5. Acylglycerol Species of Canola Oil and SLs Identified by HPLC-MS

				area%					
peak	acylglycerol species	diacylglycerol ions $[M-RCO]^+$	molecular ions $[M+H]^+$	canola oil	CI-A	EZ-A2	EZ-B2	EZ-C2	EZ-D2
1	OL	601.52		-	1.0	1.4	1.1	1.3	1.1
2	OO	603.52		0.3	0.9	2.1	1.8	1.6	1.0
3	PO, OO	577, 603.52		-	-	1.7	1.8	1.4	1.3
4	SO	605.55		-	-	1.3	1.6	1.7	1.8
5	LnLnO	599.50, 595.47	878.34	0.9	0.7	1.4	-	-	-
6	LLL	599.50	880.30	1.8	0.7	0.4	-	-	-
7	OLLn	601.52, 597.49, 599.5	880.30	5.9	5.9	4.8	4.7	3.8	3.6
8	PLLn	575.5, 597.5, 573.5	854.28	0.8	2.0	-	-	-	-
9	LLO	599.5, 601.52	882.26	8.6	7.8	6.6	5.1	3.8	3.0
10	OOLn	603.54, 599.54	883.26	11.9	8.7	9.0	6.9	5.2	4.1
11	LLP	575.5, 599.5	856	1.9	1.7	-	-	-	-
12	POLn	577.52, 599.5, 573.49	856.24, 882.02	1.9	-	1.5	1.4	1.3	1.3
13	OLO	601.52	884.23	21.0	20.5	17.6	14.3	10.8	8.6
14	LLS	599.50, 603.54	884.23	-	-	1.3	2.1	2.4	2.6
15	SOLn, POL	601.52, 599.50, 605.55, 577.52, 575.52	884.23, 858.21	5.4	6.5	6.4	8.3	8.6	8.7
16	OLG, OLnA	599.50, 601.52 631.57, 629.55	912.46	1.5	1.1	1.1	-	0.9	0.9
17	OOO	603.54	886.19	24.0	18.5	14.9	11.8	9.1	7.3
18	SOL	601.54, 603.54, 605.55	886.19	1.7	4.9	8.1	11.8	13.1	13.5
19	OOP	603.54, 577.52	860	4.7	4.5	3.5	2.9	2.4	2.1
20	SSLn, PSL	601.52, 607.57, 575.50, 579.54, 603.54	860.17, 886.19	-	-	0.7	1.4	2.2	2.8
21	PPO, OOG	551.5, 577.52, 603.54	885.95	1.4	1.5	-	-	-	-
22	SOL, OOG	601.52, 603.54, 605.55	886.44, 912.18	-	-	0.9	0.7	1.0	1.1
23	OSO/OOS	605.55, 603.54	888.40	1.9	6.3	8.5	12.5	13.8	14.3
24	SSP	579.54, 605.55	862.38	-	<1.5	-	-	-	-
25	SSL	603.54, 607.57	888.40	-	-	0.6	1.8	3.3	4.7
26	PSO	577.52, 579.54, 605.55	862.38	-	-	0.8	1.3	1.8	2.1
27	OOA	603.54, 633.58	915.90	0.5	<1.0	-	-	-	-
28	PoSA	577.52, 607.57, 633.58	890.12	-	1.0	-	-	-	-
29	SOS	605.54	890.37	-	-	1.0	3.1	5.5	7.5
30	AAO	663.63, 633.58	945.89	<0.5	<1.0	-	-	-	-
% TAGs containing at least one saturated fatty acid						36.4	50.9	59.1	64.6
% TAGs containing at least two saturated fatty acid						2.9	7.6	13.4	17.7

[a]Abbreviations: La, lauric acid; M, myristic; P, palmitic; Po, palmitoleic; S, stearic; O, oleic; L, linoleic; Ln, linolenic; A, arachidic; G, godoleic.

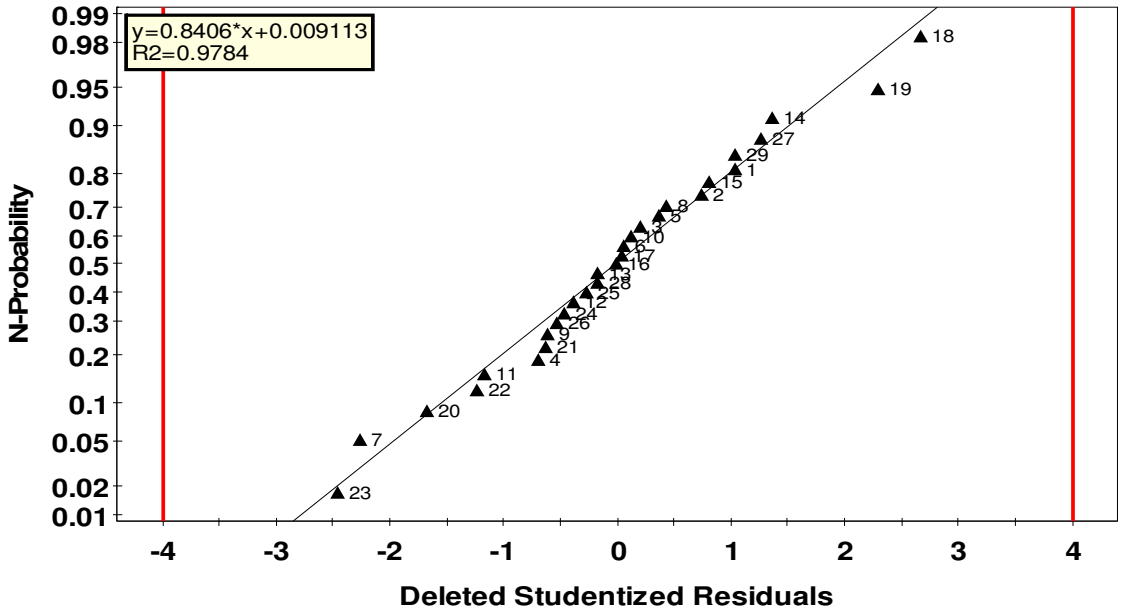
Table 6. Acylglycerol Species of MG-B and MG-E Identified by HPLC-MS

peak	acylglycerol species	diacylglycerol ions $[M-RCO]^+$	molecular ions $[M+H]^+$	area %	
				MG-B	MG-E
1	OO, PO[a]	603.54, 577.52		-	1.0
2	PP	551.50		-	0.4
3	LnLnL	595.47, 597.49	876.13	0.7	0.2
4	LLLn	599.50, 597.49	878.09	5.4	3.1
5	LLL	599.50	880.30	13.4	5.8
6	OLLn	601.52, 597.49, 599.50	880.06	3.7	2.9
7	PLLn	575.50, 579.49, 573.49	854.03	2.3	1.1
8	LLO	599.50, 601.52	882.26	13.7	7.0
9	OOLn	603.54, 5999.50	882.26	-	1.9
10	LLP	599.50, 575.50	856.24	10.1	5.4
11	POLn	577.52, 599.5, 573.49	856.24	-	1.1
12	OLO	601.52	884.26	7.3	7.1
13	LLS	599.50, 603.54	883.74	3.8	1.6
14	POL	577.53, 601.52, 575.50	858.21	7.9	8.4
15	PPL	551.50, 575.50	831.7	1.8	5.1
16	OOO	603.54	886.19	2.6	7.3
17	SOL	605.55, 601.52, 603.54	886.19	6.2	2.2
18	OOP	603.54, 577.52	860.17	3.5	13.2
19	PSL	579.54, 603.54, 575.50	859.68	2.5	1.2
20	PPO	551.50, 577.52	833.90	-	15.0
21	OOS	603.54, 605.55	888.40	3.0	3.6
22	PSO	579.54, 605.55, 575.50	862.14	1.8	2.9
23	OOA	603.54, 633.58	916.14	-	0.5
24	SSO	607.57, 605.55	890.37	4.2	<0.5
% TAGs containing at least one saturated fatty acid				47	62.7
% TAGS containing at least two saturated fatty acids				10.3	25.1

[a]Abbreviations: La, lauric acid; M, myristic; P, palmitic; Po, palmitoleic; S, stearic; O, oleic; L, linoleic; Ln, linolenic; A, arachidic; G, godoleic.

Figure 1. (a) Normal probability plot of residuals for incorporation of stearic acid; and (b) Residual plot. Numbers inside both graphs represent experimental numbers. The linear (a) and random (b) distribution of the experimental numbers are indicative of a good model.

Figure 1a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R2=0.9784
N-Probability
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b

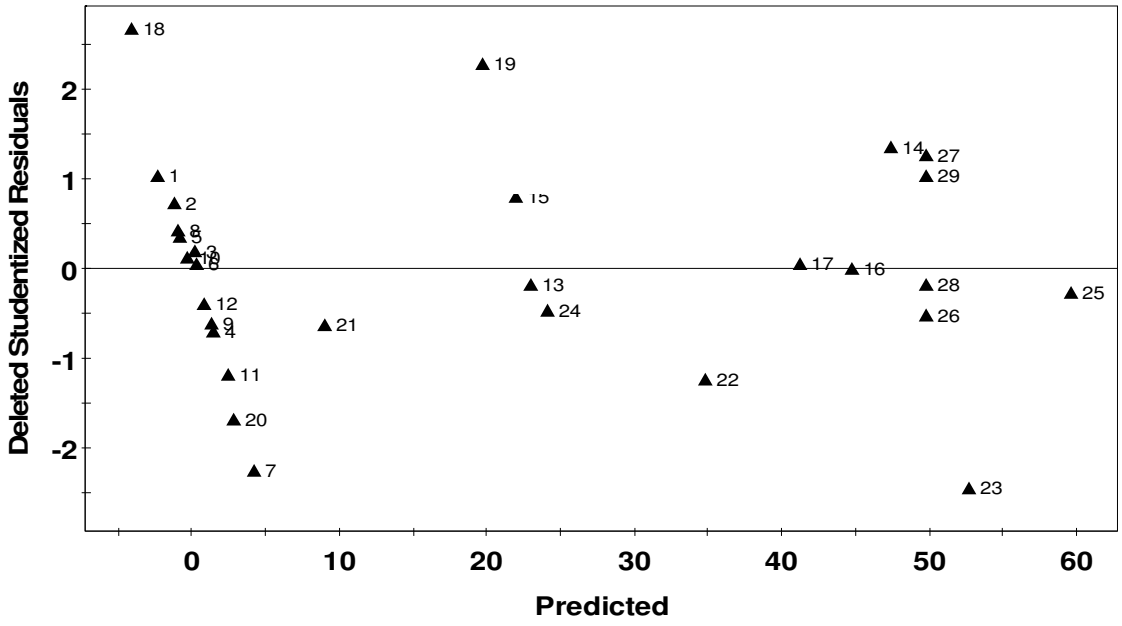
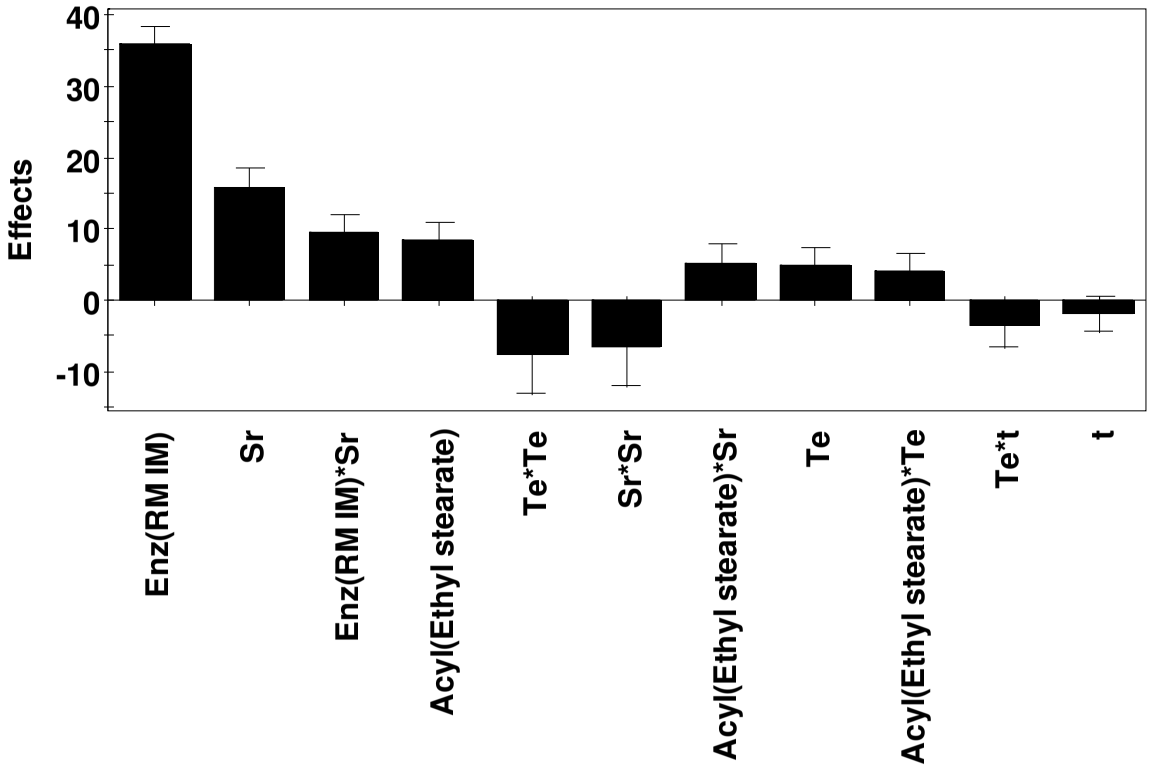

Deleted Studentized Residuals
Predicted

Figure 2. (a) Effect of parameters and their significance on incorporation of stearic acid. Sr, substrate molar ratio; Te, temperature (˚C); t, time (h); Sr*Sr, quadratic term of Sr; Te*t, interaction term of Te and t; Enz(RM IM), Lipozyme RM IM; Acyl (Ethyl stearate)*Sr, interaction term of ethyl stearate; Acyl (Ethyl stearate)*Te, interaction term of ethyl stearate and Te.

Figure 2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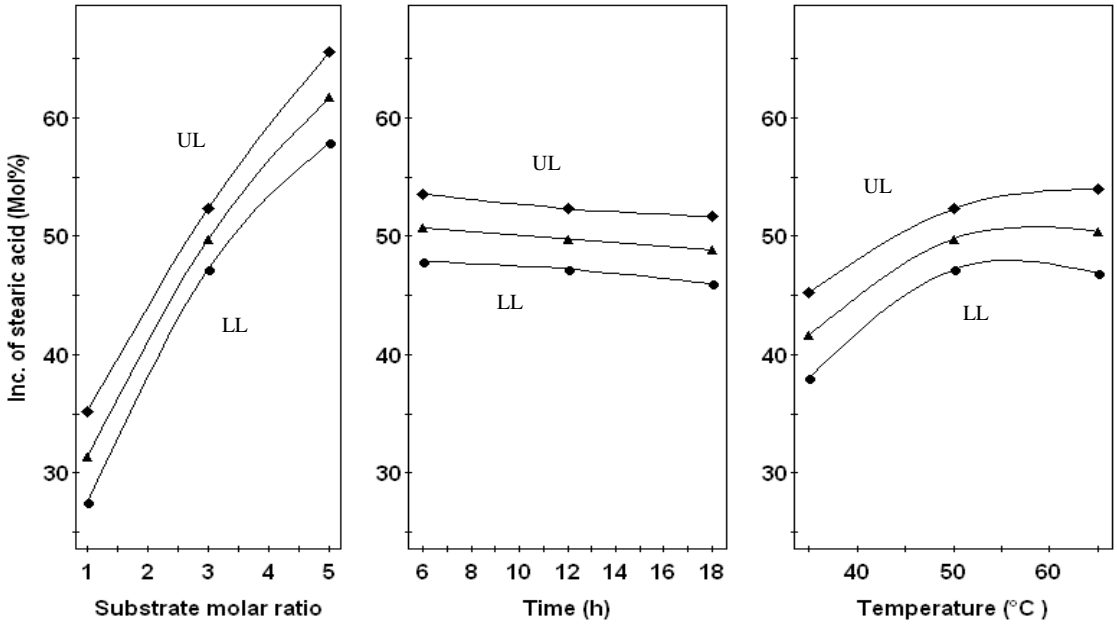
30
20
10
0
-10
Enz(RM IM)
Sr
Enz(RM IM)*Sr
Acyl(Ethyl stearate)
Te*Te
Sr*Sr
Acyl(Ethyl stearate)*Sr
Te
Acyl(Ethyl stearate)*Te
Te*t
t

Figure 3. Projected response for substrate molar ratio, time and temperature when all but the parameter of interest were held constant at 50˚C, 12 h and/or substrate molar ratio 3 in both Lipozyme RM IM-catalyzed interesterification (**3a**) and acidolysis (**3b**) reactions. UL and LL refer to upper and lower confidence limits.

Figure 3a


Inc. of stearic acid (Mol%)
UL
LL
Substrate molar ratio
Time (h)
Temperature (°C)

b

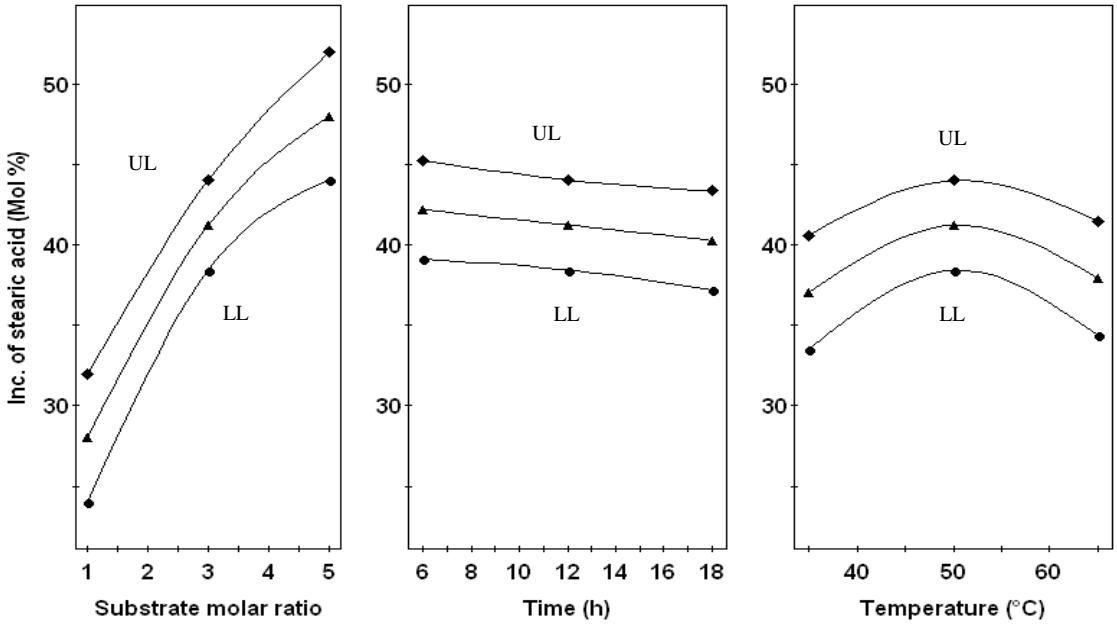

Inc. of stearic acid (Mol %)
UL
LL
Substrate molar ratio
Time (h)
Temperature (°C)

Figure 4. Contour plots showing (a) effects of Lipozyme RM IM and ethyl stearate, (b) Lipozyme RM IM and stearic acid, (c) LIP1 and ethyl stearate, and (d) LIP1 and stearic acid, on incorporation of stearic acid at 12 h. The numbers inside the contour plots indicate the level of stearic acid incorporation (Mol%).

Figure 4

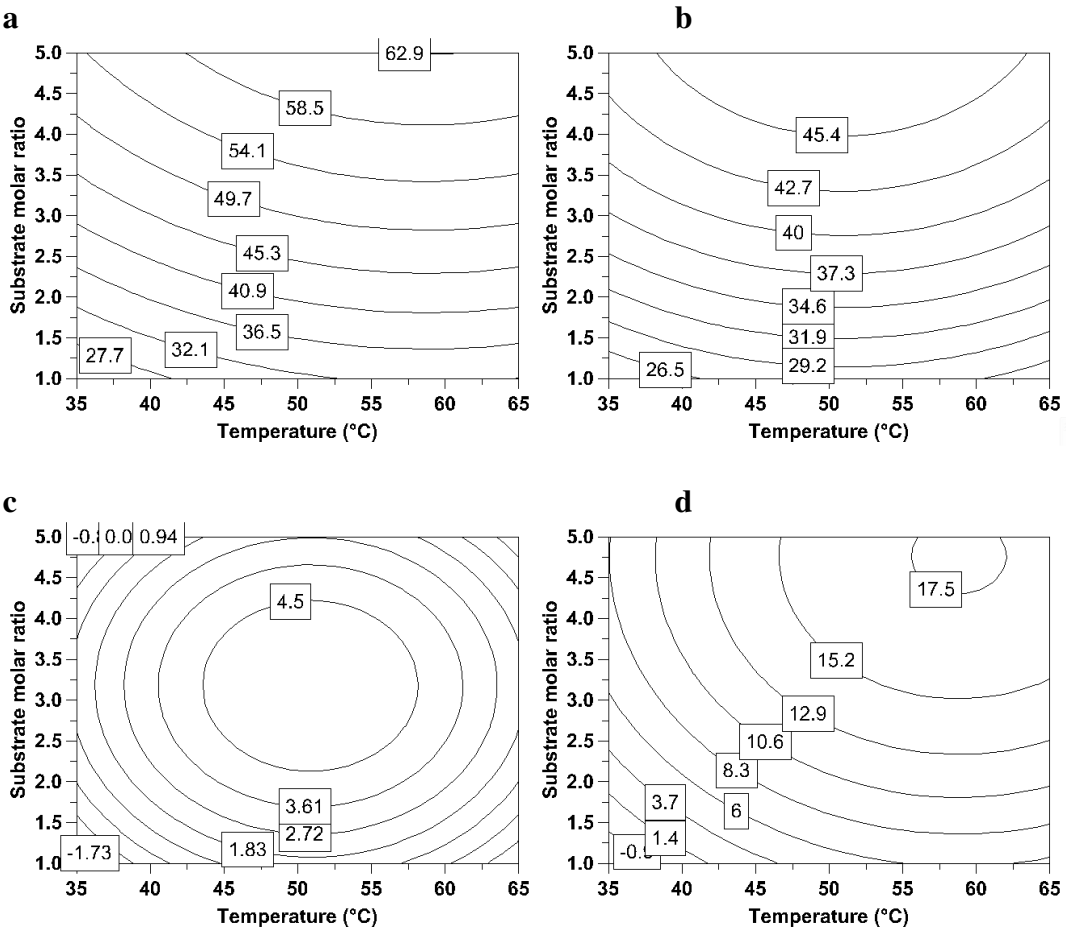

a
b
c
d
Substrate molar ratio
Temperature (°C)
62.9
58.5
54.1
49.7
45.3
40.9
36.5
32.1
27.7
45.4
42.7
40
37.3
34.6
31.9
29.2
26.5
4.5
3.61
2.72
1.83
-1.73
0.94
17.5
15.2
12.9
10.6
8.3
6
3.7
1.4

Figure 5. HPLC-ELSD chromatograms of canola oil, and structured lipids produced by Lipozyme RM IM-catalyzed (EZ-A2) and sodium methoxide-catalyzed (CI-A) transesterification.

Figure 5

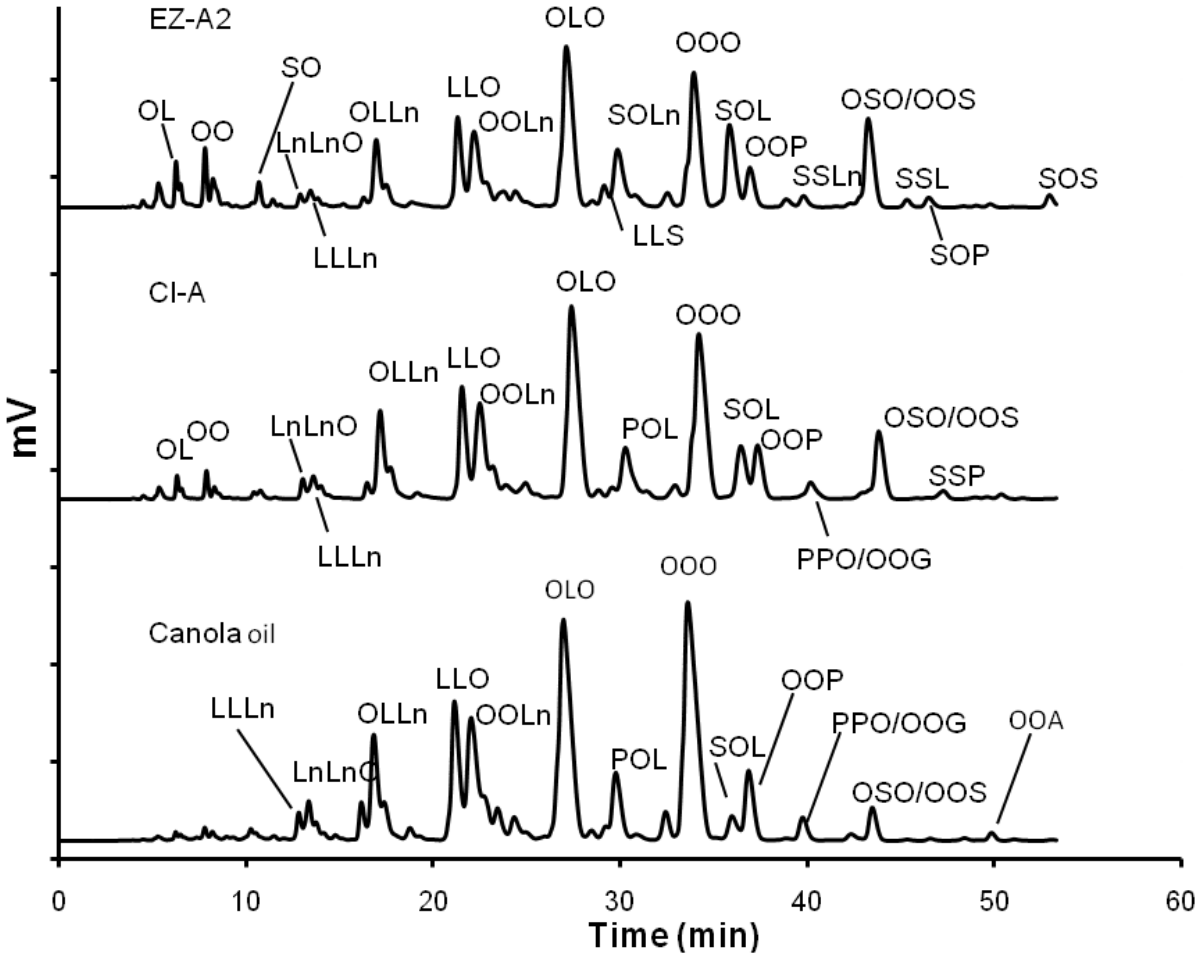

EZ-A2
OL
OO
SO
LnLnO
LLLn
OLLn
LLO
OOLn
OLO
SOLn
LLS
OOO
SOL
OOP
SSLn
OSO/OOS
SSL
SOP
SOS
CI-A
OL
OO
LnLnO
LLLn
OLLn
LLO
OOLn
OLO
POL
OOO
SOL
OOP
OSO/OOS
SSP
PPO/OOG
Canola oil
LLLn
LnLnO
OLLn
LLO
OOLn
OLO
POL
OOO
SOL
OOP
PPO/OOG
OOA
OSO/OOS
mV
0
10
20
30
40
50
60
Time (min)

Figure 6. 100 MHz ^{13}C NMR carbonyl spectra of canola oil (a), structured lipid products CI-A (b), EZ-A2 (c), EZ-B2 (d), EZ-C2 (e), and EZ-D2 (f). The acyl groups are palmitic (P), stearic (S), oleic (O), linoleic (L) and linolenic (Ln).

Figure 6a

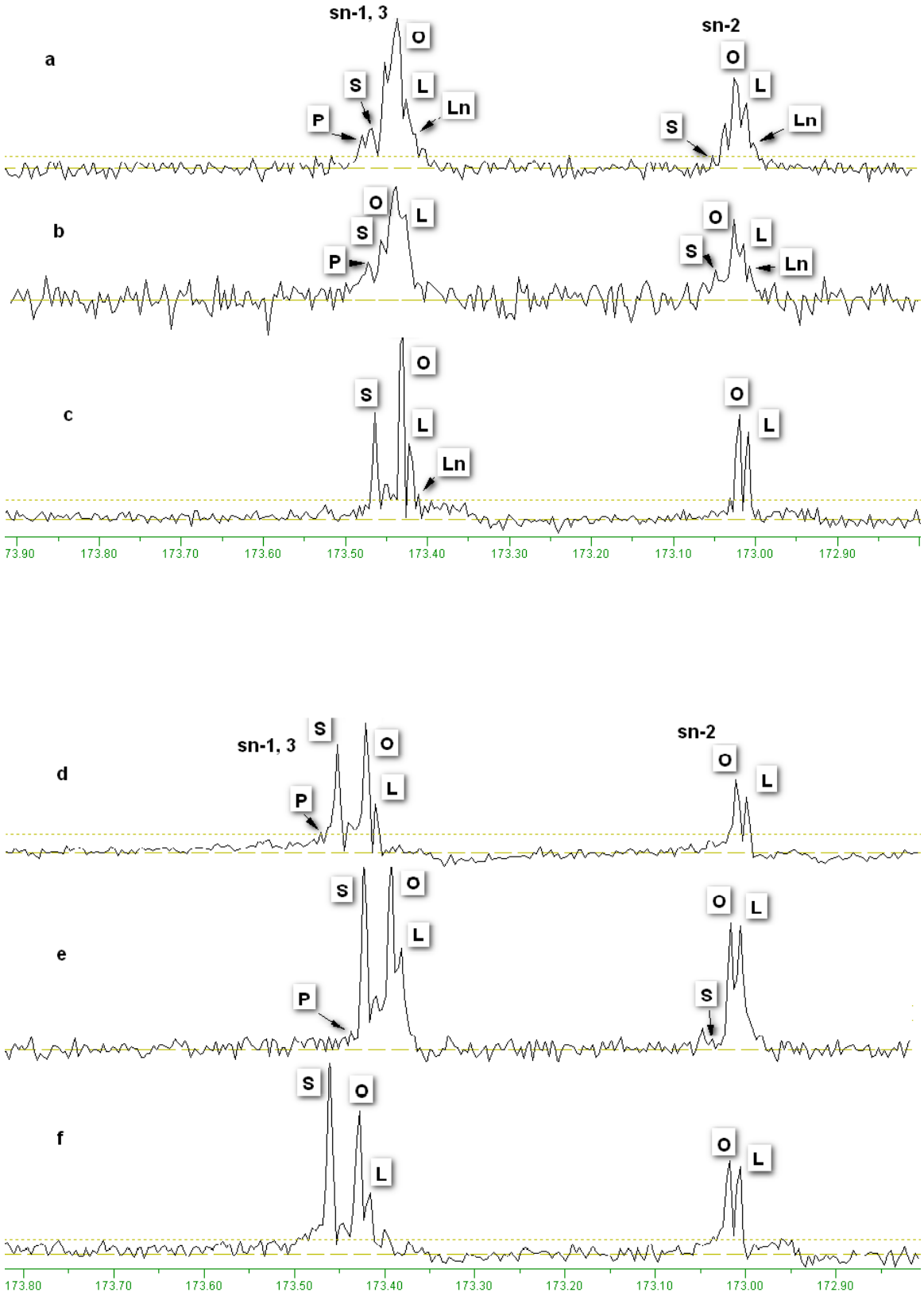

a
sn-1, 3
sn-2
O
S
L
Ln
P
b
c
d
e
f
173.90
173.80
173.70
173.60
173.50
173.40
173.30
173.20
173.10
173.00
172.90

Figure 7. DSC thermograms of canola oil, structured lipid products and margarine samples.

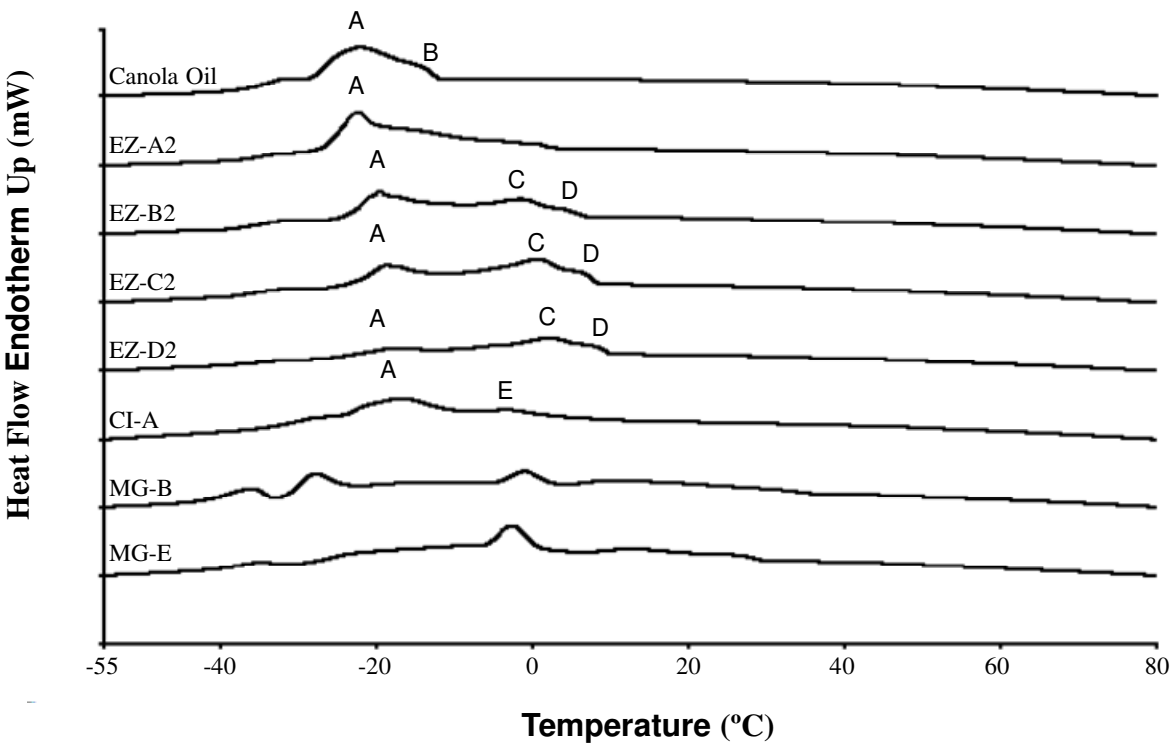
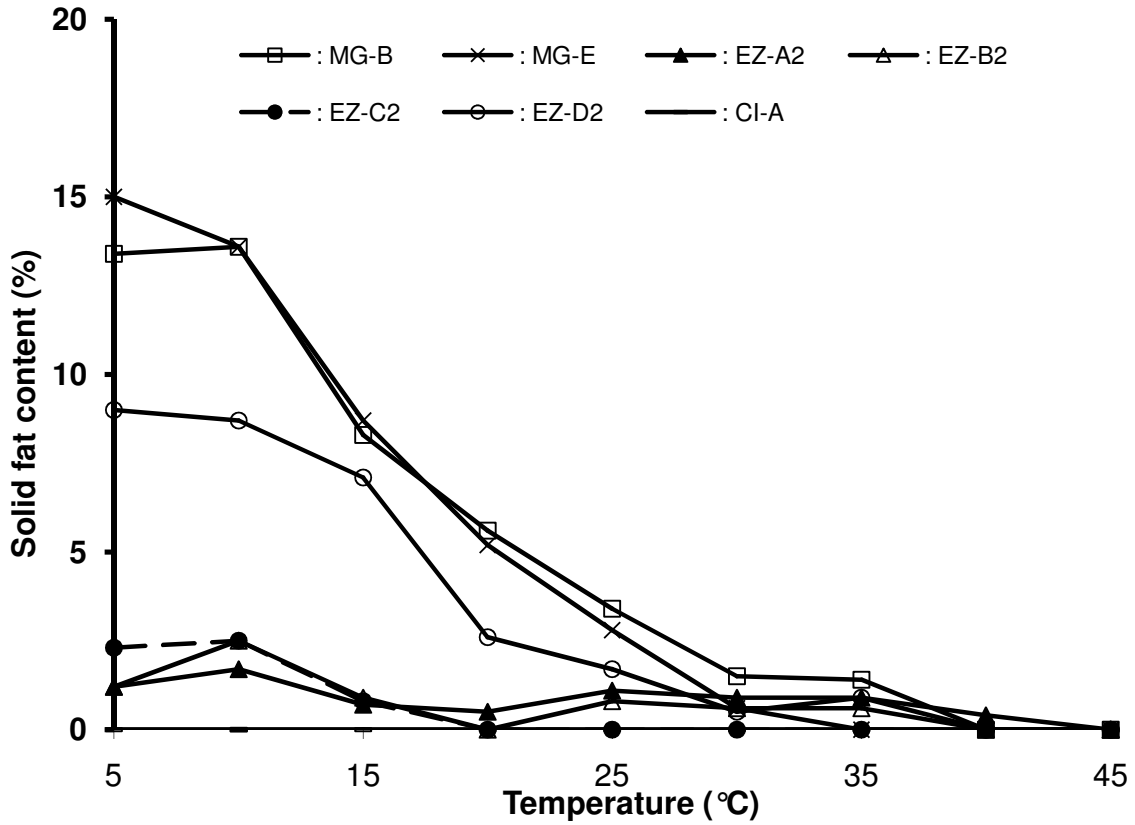

Heat Flow Endotherm Up (mW)
Canola Oil
EZ-A2
EZ-B2
EZ-C2
EZ-D2
CI-A
MG-B
MG-E
A
B
C
D
E
-55
-40
-20
0
20
40
60
80
Temperature (ºC)

Figure 8. Curves showing solid fat content (SFC) of canola oil, structured lipid products (CI-A, EZ-A2, EZ-B2, EZ-C2 and EZ-D2), and commercial margarine samples (MG-B and MG-E).

Figure 8


20
15
10
5
0
Solid fat content (%)
: MG-B
: MG-E
: EZ-A2
: EZ-B2
: EZ-C2
: EZ-D2
: CI-A
5
15
25
35
45
Temperature (℃)

CHAPTER 4

ESTERIFICATION AND HYDROLYTIC ACTIVITIES OF *CANDIDA RUGOSA* LIPASE ISOFORM 1 (LIP1) IMMOBILIZED ON CELITE 545, DUOLITE A7, AND SEPHADEX G-25[1]

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[1]Stephen E. Lumor and Casimir C. Akoh. Submitted to Journal of Agricultural and Food Chemistry, 07/14/2008.

ABSTRACT

The esterification and hydrolytic activities of free and immobilized *Candida rugosa* lipase isoform 1 (LIP1) were investigated. Esterification activity was determined by reacting caprylic acid with glycerol in the presence of molecular sieves (30% w/w), and the volume of 1.0 M NaOH consumed by the reaction products upon titration was used to calculate esterification activity. Caprylic acid was also reacted with cottonseed oil and the amount of caprylic acid incorporated after 12 h of reaction was determined. Results indicated that LIP1 had little esterification activity, which was not significantly improved upon immobilization. Hydrolytic activity was determined by incubating tricaprylin emulsion (15% w/w) with the respective lipases for 60 min, and the reaction products titrated against 0.5 M NaOH. LIP1 showed hydrolytic activity comparable to Lipozyme RM IM. Hydrolytic activity improved significantly upon immobilization. Immobilization on Celite 545 produced the highest increase in hydrolytic activity.

Keywords: Celite 545, Duolite A7, Immobilization,, LIP1, Lipozyme RM IM, Sephadex G-25

INTRODUCTION

Immobilized lipases are preferred for transesterification because they are stable and reusable, and also offer easy separation of the enzyme from reactants and products. Immobilization of lipases can also affect their selectivity, and chemical and physical properties (*1*), and creates non-aqueous conditions around the enzyme which is necessary for ester synthesis or esterification (*2*). Commonly used immobilization methods include simple adsorption of the lipase to the surface of a solid support, covalent bonding of the enzyme to a solid support, encapsulation, and entrapment. Adsorption is the most commonly used method because of its simplicity. It is accomplished by mixing an aqueous solution of the lipase with the solid support or by

precipitating the aqueous lipase solution onto the support using acetone, ethanol, or methanol. The mixture is filtered and the immobilized enzyme freeze-dried to reduce its moisture content.

Candida rugosa lipase isoform 1 (LIP1) is one of the seven isoforms of *Candida rugosa* lipase (CRL). Its use in the synthesis of structured lipids has become attractive because of its ability to significantly incorporate acyl chains at the *sn*-1,2,3 positions of the triacylglycerol (TAG) backbone (*3*). The non-specific incorporation of acyl chains results in increased TAG diversity, which improves the preponderance of β' crystals that impart smooth mouthfeel to margarine. A previous study in our laboratory (*4*) reported the inability of LIP1 to significantly incorporate stearic acid into canola oil. The aim of this study, therefore, was to immobilize LIP1 on Celite 545, Duolite A7, and Sephadex-G25 to enhance its activity for possible application in the synthesis of *trans*-free structured lipids. The activities of the immobilized lipases were compared to those of the free lipase (LIP1) and Lipozyme RM IM.

MATERIALS AND METHODS

Materials. Caprylic acid, tricaprylin, glycerol, Celite 545, Sephadex G-25, and Duolite A7 were purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized Lipozyme RM IM was purchased from Novo Nordisk A/S (Bagsværd, Denmark) and unimmobilized LIP1 (genetically engineered and expressed in *P. pastoris*) was donated by Dr. Jei-Fu Shaw of Institute of Plant and Microbial Biology, Academia Sinica, Nankang, Taipei, Taiwan. Cottonseed oil was donated by Archer Daniels Midland Co. (Valdosta, Georgia). Organic solvents and chemicals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA). All other chemicals used were of analytical or HPLC grade.

Immobilization Procedure. LIP1 was immobilized on three different carriers (Celite 545, Sephadex G-25 and Duolite A7) as described (*5*). Briefly, LIP1 (3 g) was dissolved in 60 mL of

10 mM sodium phosphate buffer (pH 6) and mixed with 12 g of carrier. A volume (240 mL) of cold acetone (-20˚C) was added and the mixture stirred for 30 min at room temperature (20˚C). The mixture was filtered (suction) and washed with 60 mL of cold acetone (10˚C), dried in an oven (25˚C) for 72 h, and stored at 4˚C. Protein content was determined by means of a bicinchoninic acid (BCA) protein assay kit (Sigma Chemical Co., St. Louis, MO), and water content was measured on a Mettler Toledo moisture analyzer (Columbia, MD).

Esterification Activity. Glycerol (1 g) and caprylic acid (1.27 g) were incubated with each lipase (10%, w/w) for 1 h in screw-cap test tubes placed in an orbital shaking (200 rpm) water-bath. The reaction temperature was 60˚C. Thirty percent (w/w) of molecular sieves (4 Å dia, 8-12 mesh) was added to increase the rate of reaction by absorbing water produced as reaction by-product. After the reaction was stopped, 1 mL methanol was added and the content of the screw-cap test tubes titrated against 1.0 M sodium hydroxide (NaOH) with 1% phenolphthalein as indicator. The esterification activity was determined by the following expression:

Activity = (V x M)/(W x t) ---------- equation (1),

where the product of *V* (the difference in titre values between the blank and samples) and *M* (molarity) is the amount (moles) of caprylic acid consumed due to esterification after reaction time *t* (min). *W* is the amount of lipase (mg) used in the reaction. Esterification activity is defined as the amount of caprylic acid (μmol) consumed per min, per mg lipase in the reaction. All reactions were performed in duplicates.

Hydrolytic Activity. Lipase hydrolytic activity was assayed by preparing 200 mL of 0.33M (or 15% w/v) tricaprylin emulsion using 10 mM sodium phosphate buffer (pH 6). Gum Arabic (5%, w/v) was used as emulsifier. An aliquot (10 mL) of the emulsion was incubated with 500 mg of each lipase at 60˚C for 1 h. The reaction products were titrated against 0.5 M NaOH with 1%

phenolphthalein as indicator. The difference in titer values between samples and the blank was used to calculate the amount of caprylic acid released. Hydrolytic activity was defined as the amount (μmol) of caprylic acid released per min, per mg of lipase, and was calculated from equation (1) above. All reactions were performed in duplicates.

Acidolysis Reaction. Structured lipids were synthesized in duplicates by reacting 1 g of cottonseed oil with 0.3 g caprylic acid in screw-cap test tubes contained in an orbital shaking (200 rpm) water-bath. The amount of each lipase used was 10% by total weight of reactants. The reactions were carried out for 12 h at 40, 50, and 60˚C. Triacylglycerol (TAG) bands were separated from other lipid classes by means of thin-layer chromatography as previously described (*4*). The TAG bands were scrapped off, and converted to fatty acid methyl esters (FAME) using the boron trifluoride (BF_3) method. Briefly, the TAG bands were hydrolyzed at 90˚C in screw-cap test tubes by reacting with 2 mL 0.5 M NaOH in methanol for 10 min, followed by 14% BF_3 in methanol for another 10 min. FAME were extracted with 2 mL hexane and analyzed in parallel with a FAME standard (Supelco 37 component FAME mix, Supelco®, Bellefonte, PA) using an Agilent Technology 6890N gas chromatograph as previously described (*4*). The different amounts of FAME were analyzed and integrated by an online computer, and the amount of caprylic acid incorporated recorded.

Statistical Analyses. SAS Statistical Software Version 9.1 (SAS Institute, Cary, NC) was used for statistical analyses. Duncan's multiple comparison test was used to determine significant differences between treatments.

RESULTS AND DISCUSSIONS

Protein contents of the lipases were determined and expressed as percentage weight of the respective lipase preparations. Since the same amount of free LIP 1 was used for immobilization on the solid supports, the effectiveness of the immobilization process can be evaluated by comparing the protein contents of the immobilized lipases to that of the free lipase (LIP1). From **Table 1**, it can be seen that immobilization on Sephadex retained the most protein, and Celite 545 retained the least. Since this study was limited with regards to the determination of specific proteins present in the free and immobilized LIP1 preparations, it was assumed that protein content was fairly representative of the LIP1 content in these lipase preparations. The water contents of the lipases are also given in **Table 1**.

The hydrolytic activities of the lipases were determined by titrating reaction products against 0.5 M NaOH, and the amount (μmol) of caprylic acid released per min per mg lipase preparation calculated. LIP1 lipase preparations showed significantly higher hydrolytic activities than Lipozyme RM IM (**Table 1**). In terms of specific activity (determined by dividing lipase activity by the protein content of the lipase used), the value for free LIP1 (4.32 μmol/min/mg protein) was comparable to, but significantly different from that of Lipozyme RM IM (4.02 μmol/min/mg protein). Our results also show that the specific activity of LIP1 was significantly improved upon immobilization. The specific activities for the immobilized lipase preparations were 22.84 for LIP1[Celite 545], 13.75 for LIP1[Duolite A7], and 5.79 μmol/min/mg protein for LIP1[Sephadex G-25].

With regards to esterification activity, the free and immobilized LIP1 preparations showed no activity, whereas Lipozyme RM IM showed significant activity (0.09 μmol/min/mg lipase). A similar pattern was observed in the acidolysis reaction involving caprylic acid and cottonseed oil

(**Table 2**). The amount of caprylic acid incorporated after 12 h of reaction was significantly higher for reactions catalyzed by Lipozyme RM IM (15.01 – 21.8 wt%) compared to reactions catalyzed by the free and immobilized LIP1 lipases (0 – 1.94 wt%). Our results also indicate that the immobilized LIP1 preparations showed higher specific incorporation (Inc/h/mg protein) than the free LIP1, even though these were not significantly different. It was highest for LIP1[Celite 545] (0.14 Inc/h/mg protein) at 50˚C. The effect of reaction temperature was significant in this study. Specific incorporation, the amount (mg%) of caprylic acid incorporated into cottonseed oil per hour per mg protein, increased between 40 and 50˚C, and decrease between 50 and 60˚C for all lipases. Reaction at 50˚C may therefore be suitable for the activities of these lipases under the reaction conditions used in this study.

This study has shown that LIP1, whether free or immobilized, had no significant effects on the esterification process compared to Lipozyme RM IM. It however showed significant hydrolytic activity. LIP1 has been reported (*6*) to show preference for medium-chain fatty acids (C_8-C_{10}), but its esterification activity with regards to caprylic acid (C8:0) was not significant in this study. This study determined that immobilization of LIP1 would not improve its suitability for the synthesis of structured lipids.

ACKNOWLEDGEMENT

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(6) Akoh, C. C.; Lee, G.-C.; Shaw, J.-F. Protein engineering and applications of *Candida rugosa* lipase isoforms. *Lipids* **2004,** 39, 513-526.

Table 1. Hydrolytic Activity of Lipases

lipase	protein (wt%)	moisture (wt%)	activity (μmol/min/mg)	specific activity (μmol/min/mg protein)
Lipozyme RM IM	0.87	4.34	0.04 ± 0.00d [a]	4.02 ± 0.14e
LIP1	5.89	3.38	0.26 ± 0.00a	4.32 ± 0.00d
LIP1[Celite 545]	0.58	1.12	0.13 ± 0.00c	22.84 ± 0.20a
LIP1[Duolite A7]	1.03	7.94	0.14 ± 0.00b	13.75 ± 0.00b
LIP1[Sephadex G-25]	2.29	8.70	0.13 ± 0.00c	5.79 ± 0.05c

[a]Values having the same letter in each column are not significantly different at $\alpha_{0.05}$.

Table 2. Incorporation of Caprylic Acid into Cottonseed Oil

lipase	reaction temperature					
	40˚C		50˚C		60˚C	
	Inc[a] (mg%)	spec Inc (Inc/h/mg protein)	Inc (mg%)	spec Inc (Inc/h/mg protein)	Inc (mg%)	spec Inc (Inc/h/mg protein)
Lipozyme RM IM	15.01 ± 1.07a[b]	1.11 ± 0.08a	21.8 ± 2.22a	1.61 ± 0.16a	17.11 ± 0.72a	1.26 ± 0.03b
LIP1	1.83 ± 0.33b	0.02 ± 0.00b	1.76 ± 0.37b	0.02 ± 0.00b	0b	0b
LIP1[Celite 545]	0.75 ± 0.33b,c	0.08 ± 0.04b	1.27 ± 0.11b	0.14 ± 0.01b	0b	0b
LIP1[Duolite A7]	1.69 ± 0.09b,c	0.10 ± 0.01b	1.94 ± 0.04b	0.12 ± 0.00b	0.82 ± 0.86b	0.05 ± 0.03b
LIP1[Sephadex G-25]	0.40 ± 0.28c	0.01 ± 0.01b	0.76 ± 0.34b	0.02 ± 0.01b	0.97 ± 0.77b	0.03 ± 0.01b

[a]Abbreviations: Inc, incorporation of caprylic acid into cottonseed oil; spec Inc, specific incorporation (incorporation per hour per mg protein).
[b]Values having the same letter in each column are not significantly different at $\alpha_{0.05}$.

CHAPTER 5

OPTIMIZATION OF SOLID FAT CONTENT AND CRYSTAL PROPERTIES OF A *TRANS*-FREE STRUCTURED LIPID BY BLENDING WITH PALM MID-FRACTION[1]

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[1]Stephen E. Lumor, Byung Hee Kim and Casimir C. Akoh. Submitted to Journal of Agricultural and Food Chemistry, 06/10/2008.

ABSTRACT

Optimization of solid fat content (SFC) and crystal properties of *trans*-free structured lipids (SL) synthesized by incorporating stearic acid into canola oil was investigated. The SLs were blended with varying amounts of palm mid-fraction (PMF). SFC and crystal polymorphism were improved. Addition of sucrose stearate (S-170), sorbitan tristearate (STS), and distilled monoglycerides (DMG) to one of the blends, SL40:PMF (70:30, w/w), did not improve crystal polymorphism, but had significant effects on crystal morphology. The emulsifiers significantly delayed crystal growth, resulting in smaller crystal sizes compared to the control. They were unable to inhibit the formation of granular crystals (30 to 140 μm), which are undesirable in margarine, after 4 weeks of storage at 0˚C. Blends treated with S-170 and STS showed many small evenly distributed crystals interspersed with large crystal aggregates (after 4 weeks of storage); whereas, the blend treated with DMG and the control showed irregularly shaped globular crystals, also interspersed with large crystal aggregates. However, these crystals aggregates were not observed upon visual and physical examination, and may therefore not impart the sensory properties of the finished products negatively.

Keywords: Distilled monoglycerides, palm mid-fraction, trans-free structured lipid, sorbitan tristearate, sucrose stearate

INTRODUCTION

Physical properties such as consistency, solid fat content, melting profile, crystal polymorphism and morphology are related to sensory properties, and subsequently, consumer acceptance of margarines. These physical properties depend on the triacylglycerol (TAG) composition, the level of liquid oil present, and temperature fluctuations during storage (*1*). In designing *trans*-free structured lipids (SLs) as alternatives to conventional fats used for

margarine formulation, it is imperative that these *trans*-free alternatives possess desirable physical and sensory properties. Previous studies in our laboratory (*2*) have shown that *trans*-free SLs made by incorporating stearic acid into canola oil did not have desirable solid fat content (SFC) and crystal types for tub margarine formulation.

Palm oil and its fractions, including palm mid-fraction (PMF), have become a major source of fat for the margarine industry because of the number of desirable properties they impart to the finished product, including high oxidative stability, and plasticity at room temperature. PMF is the two-stage fractionation product of palm oil. It is characterized by a high disaturated triacylglycerol content (> 60%) and a low monosaturated triacylglycerol content (< 30%), and has a short melting range which makes it suitable for use in cocoa butter equivalents and margarines (*3*). However, the use of palm oil fractions in blends for the manufacture of margarine has some problems due to poor crystallization properties such as low rate of nucleation and the formation of granular crystals during storage (*4*). These granular crystals are responsible for sandy mouthfeel of margarine products, and are formed by the segregation of 1,3-dipalmitoyl-2-oleoyl glycerol (POP) from other TAG crystals and their eventual polymorphic transition from β' to β form (*1*). This phenomenon is also dependent on a number of factors such as the level of liquid oil present, the presence of specific TAG – 1,3-dipalmitoyl-2-stearoyl glycerol (PSP) and 1,3-dipalmitoyl-2-elaidoyl glycerol (PEP), and temperature fluctuations during storage (*1*). Maintenance of β' polymorphic form is therefore important in preserving smooth texture and easy spreadability of margarine. This is accomplished by the inclusion of β'-tending fats in blends and/or by using emulsifiers such as distilled monoglycerides (DMG), sucrose stearate (S-170), and sorbitan tristearate (STS), that hinder or retard polymorphic transition (*5*). The aim of this study, therefore, was to optimize the solid fat content, crystal form

and morphology of the *trans*-free SLs synthesized in our previous study (*2*), by blending with palm mid-fraction (PMF) and by using emulsifiers. The inclusion of the three emulsifiers in the structured lipid:palm mid-fraction blends was expected to hinder the growth of undesirable crystal aggregates.

MATERIALS AND METHODS

Stearic acid was purchased from Sigma Chemical Co. (St. Louis, MO). Canola oil (peroxide value 0.0 meq/Kg, acid value 0.28%) was bought from a local grocery store. Palm mid-fraction (peroxide value 1.0 meq/Kg, acid value 0.1%, iodine value 43-48) was donated by Fuji Vegetable Oil Inc. (Savannah, Georgia). Immobilized Lipozyme RM IM was purchased from Novo Nordisk A/S (Bagsværd, Denmark). Organic solvents and chemicals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA). All other chemicals used were of analytical or HPLC grade. Sorbitan tristearate (STS) with hydrophile/lipophile balance (HLB) of 2.1, and distilled monoglycerides (DMG) with HLB of 3.8 were donated by Danisco A/S (Copenhagen, Denmark), and sucrose stearate (S-170) with HLB of 1 was supplied by Mitsubishi-Kasei Food Corporation (Tokyo, Japan).

Structured lipid synthesis was performed in a stir-batch reactor at 65˚C for 12 h. The reaction mixture typically contained 300 g of canola oil, 40 - 50% stearic acid (by weight of canola oil), and 10% Lipozyme RM IM (by total weight of reactants). The product was separated from the enzyme by suction filtration, and free fatty acids removed by short-path distillation with a KDL-4 (UIC Inc., Joliet, IL) unit under the following conditions: heating oil temperature, 185˚C; cooling water temperature, 55˚C; pump vacuum, <1 mm Hg; feed rate, maintained at 100 mL/h. The reaction product was passed through the system twice to reduce the free fatty acid percentage to an acceptable level. Free fatty acid content (0.12 – 1.05% oleic acid) was

determined according to AOCS Official Method, Ca 5a-40 (*6*). A series of blends of the two structured lipids (SL40 and SL50) containing from 20 to 50% PMF were prepared and analyzed to determine their suitability for margarine formulation in terms of solid fat content. The composition of the blends is given in **Table 1**.

Solid fat content (SFC) of samples were determined according to AOCS Official Method Cd 16-81(*7*) on a MARAN-20 pulsed NMR spectrometer (Resonance Instruments Ltd., Oxon, UK). 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 at 30 min at each temperature of measurement. SFC was measured at intervals of 5˚C, from 5 to 45˚C.

Powder X-ray diffraction (XRD) was used to study TAG crystal polymorphism using an ARL Scintag® XDS 2000 automated diffractometer (Ecublens, Switzerland). The diffractometer had 2θ configuration and the generation power was set at 30 KV and 25 mA. The scan range was from 21 to 29˚, and the scan rate was 4˚/min. The samples were kept at 80˚C for 30 min, then poured into plastic molds and kept at 0˚C for 12 h, after which they were analyzed.

Crystal morphology of the samples was investigated with a Leica DMLB compound microscope (Wetzlar, Germany) equipped with a light polarizer. The microscope was equipped with a digital camera, and controlled by a SPOT Basic software. The objective lens was 20x, and the ocular lens 1x. The temperature of the stage was maintained at 0 - 5˚C by means of dry ice pellets. Prior to analyses, the samples (5 g) were treated with 2% of S-170, STS and DMG, kept at 80˚C for 1 h, followed by -20˚C for another 1 h, and finally stored at 0˚C for analysis at 24 h and 4 weeks. The control was a blend of SL40 and PMF (70:30, w/w).

RESULTS AND DISCUSSION

The solid fat content (SFC) curve of a fat is a good measure of the spreadability of the finished product. A desirable margarine is one that has at least 7.6% SFC at 10˚C needed to maintain good crystal structure (*8*), easily spreadable once taken out of the refrigerator, and melts completely in the mouth. Complete melting in the mouth ensures the release of flavor, and also imparts smooth mouthfeel to margarine. Fats with moderate SFC (7.6 to 13%) at 10˚C and a steep SFC curve at non-refrigeration temperatures are easily spreadable. The SFC curves of blends containing structured lipids (SL40 and SL50) and PMF, and two commercial margarine fats (MG-A and MG-B) are shown in **Figure 1**. All but one (Blend A) of the samples, had the minimum SFC (7.6%) at 10˚C needed to maintain good crystal structure, and most of these samples, with the exception of MG-B, were totally melted at 35˚C. Of all the blends, sample B, a blend of SL40 and PMF (70:30, w/w), had the most desirable SFC curve for tub margarine formulation. It showed moderate SFC (12.4%) at 10˚C and a steep SFC curve at non-refrigeration temperatures, and was completely melted at 30˚C. This blend is expected to impart good spreadability and mouthfeel to the margarine product. Samples D (SL40:PMF, 50:50, w/w) and H (SL50:PMF, 50:50, w/w) had the highest SFC values at 10˚C, showed steep SFC curves at non-refrigeration temperatures, and were completely melted at 30 and 35˚C, respectively. These two would be suitable for stick margarine formulation. Margarines formulated with samples C (SL40:PMF, 60:40, w/w) and G (SL50:PMF, 60:40, w/w), by the nature of their SFC curves, would have intermediate consistencies relative to margarines formulated with samples B, D and H.

Equally important, besides SFC, is the crystal habit of the TAG. Fats containing predominantly β' TAG crystals impart smooth texture to margarine, whereas those with predominantly β TAG

crystals impart grainy texture. Crystal polymorphs are identified by their characteristic d-spacings. Beta-prime polymorphs show two strong signals at d-spacings of 3.9 and 4.3 Å, whereas β polymorphs show three strong signals at 4.6, 3.9, and 3.8 Å. The relative proportions of β and β' crystals in the blends were calculated by dividing the peak intensity at 4.6Å (β) by the peak intensity at 4.3Å (β'). The crystal habits of the samples are given in **Table 2**. The TAG crystals present in SL40 and SL50 were mainly the β polymorphs but became predominantly β' upon blending with PMF. Addition of sucrose stearate (S-170), distilled monoglycerides (DMG) and sorbitan tristearate (STS) to the SL40:PMF (70:30, w/w) blend did not improve crystal polymorphism further after 24 h of crystallization. The relative proportions of β to β' crystals were slightly higher in blends treated with S-170 and STS than in the blend treated with DMG, and the control (**Table 2**). The β to β' ratio for the blend treated with DMG was very close to that of SL40:PMF (70:30, w/w), the control. These findings suggest that emulsifiers may not always have significant effects on crystal polymorphism. A study by Cerdeira *et al.* (*9*) showed that TAG composition played a big role in the polymorphic behavior of fats treated with emulsifiers. In that study, sucrose palmitate (P-170) and sucrose stearate (S-170) had significant effects on the polymorphic behavior of a high melting fraction of milk fat (HMF), but showed no significant effects when HMF was blended with 60% (w/w) sunflower oil (SFO). Chemical analysis of the TAGs revealed that TAGs with acyl carbon numbers between 36 and 50 decreased upon blending HMF with SFO, while TAGs with acyl carbon number of 54 increased from 3.2% in HMF to 28.3% in the 60% SFO blend.

Figures 2 and **3** show polarized light microscopy (PLM) images of crystal morphologies of blends treated with or without emulsifiers after 24 h and 4 weeks of storage at 0˚C, respectively. Addition of the emulsifiers to the fat blends had significant influence on crystal morphology. The

blend treated with S-170 did not show any crystals (**Figure 2a**), whereas small homogeneous crystals were observed in the blend treated with STS (**Figure 2b**). The blend treated with DMG (**Figure 2c**) showed small globular crystals evenly interspersed with needle-like crystal structures. Larger and irregularly shaped crystals were observed in the control (**Figure 2d**), which was a blend of SL40 and PMF (70:30, w/w). The absence of crystals (at the magnification used) in the blend treated with sucrose stearate (S-170) was due to the tendency of sucrose stearate to lengthen nucleation time and inhibit crystal growth (*9, 10*). S-170 is believed to do this by co-crystallizing with TAG crystals because of their similar acyl chains, but structural dissimilarities between TAGs and the emulsifier results in the delay of nucleation and inhibition of crystal growth. Other emulsifiers or crystal regulators behave in the same manner, but to different extents. It can be seen from **Figure 2** that the effect of STS on TAG crystal growth was less pronounced compared to that of S-170. Two possible theories have been proposed by Garbolino *et al.* (*10*) to explain this. First, steric hindrance may be a factor during interactions between acyl chains of TAGs and emulsifiers. A bulkier emulsifier (S-170) will most likely disrupt TAG crystallization more than a less bulky one such as STS. Second, the higher solubility of STS in the oil phase should result in smaller disruptive forces that would delay nucleation and hinder crystal growth. The first theory would explain why the blend treated with DMG showed larger crystal growth, since DMG is less bulky than S-170 and STS.

Figure 3 shows the crystal morphologies of the blends after 4 weeks of storage at 0˚C. All blends showed small or globular crystals interspersed with crystal aggregates with sizes ranging between 30 and 140 μm, larger than the range (20 to 50 μm) reported to be responsible for sandy texture in margarine (*4*). These crystal aggregates were slightly more pronounced in the control (**Figure 3d**). Another study (*11*) reported that crystal aggregates become visible to the naked eye

and can be perceived in the mouth when they reach sizes between 100 and 3000 μm. Visual and physical (by rubbing in between the fingers) examination of the treated and non-treated fat blends did not show the presence of these bothersome crystal aggregates, even after 5 months of storage. **Figures 3a** (blend treated with S-170) and **3b** (blend treated with STS) show very small evenly distributed crystals interspersed with large crystal aggregates, whereas Figures **3c** (blend treated with DMG) and **3d** (control) show irregular shapes and sizes of crystals interspersed with very large crystal aggregates. These similarities are accounted for by structural similarities and properties within these two groups. S-170 and STS are non-glycerol-based, bulky with at least 3 acyl chains, and therefore have more disruptive forces that will delay nucleation and hinder crystal growth compared to DMG, which is a monoacylglycerol (MAG). The structural similarity between DMG and TAG, and the less bulkiness of DMG were responsible for the moderate delay of nucleation and inhibition of crystal growth, compared to the two non-glycerol-based emulsifiers.

Crystal morphology is of great importance in the manufacture of margarine because it affects product consistency and acceptability (*12*). Smaller crystals lead to firmer fats, while larger crystals produce softer fats. Gabolino *et al.* (*10*) reported that blends that showed many small crystals upon treatment with sucrose palmitate (P-170) and stearate (S-160), had higher hardness values than blends that showed the presence of large and irregularly shaped crystals. It is thought that samples which have more homogeneously distributed crystals in their fat networks provide higher resistance to penetration by a cone than samples with regions of high and low crystal densities (*13*). In our study, S-170 and STS promoted the formation of homogenously distributed small crystals, while DMG promoted the formation of larger and irregularly shaped crystals. It can therefore be concluded that margarine products formulated with blends treated with 2% S-

170 and STS will be firmer and less spreadable than margarine formulated with the blend treated with DMG and the control.

This study has shown that blending of PMF with SL40 improved solid fat content as well as crystal polymorphism. The addition of S-170, STS and DMG to the SL40:PMF blend did little to improve crystal polymorphism, but had significant effects on morphology. S-170, STS and DMG restricted the growth of TAG crystals relative to the control, and may have significantly reduced the extent to which large crystal aggregates were formed, but were unable to totally prevent their growth after 4 weeks of storage at 0˚C. However, these crystal aggregates were not detected upon visual and physical (by rubbing in between the fingers) examination, and may therefore not impact the sensory properties of the finished products negatively. The choice of emulsifier for use in the formulation of tub margarine will depend on the desired consistency of the margarine product. Use of emulsifiers such as S-170 and STS, that promote the formation of very small evenly distributed crystals will result in firmer fats, whereas use of emulsifiers such as DMG, that promote the formation of irregularly shaped crystals will result in softer fats.

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Table 1. Composition of SL:PMF Blends

blend	composition (wt%)
A	[a]SL40:PMF (80:20)
B	SL40:PMF (70:30)
C	SL40:PMF (60:40)
D	SL40:PMF (50:50)
E	SL50:PMF (80:20)
F	SL50:PMF (70:30)
G	SL50:PMF (60:40)
H	SL50:PMF (50:50)

[a]Abbreviations: SL40, SL made by reacting 40% stearic acid with canola oil; SL50, SL made by reacting 50% stearic acid with canola oil; PMF, palm mid-fraction.

Table 2. Relative Proportion of β and β' Crystals in Each Blend

sample	ratio of intensity at 4.6 Å to 4.3 Å	designation
[a]SL50	1.00	β
SL50:PMF (50:50, w/w)	0.51	β' >> β
SL50:PMF (60:40, w/w)	0.52	β' >> β
SL40	1.00	β
[b]SL40:PMF (70:30, w/w)	0.56	β' >> β
2% S-170 in SL40:PMF (70:30, w/w)	0.70	β' > β
2% STS in SL40:PMF (70:30, w/w)	0.66	β' > β
2% DMG in SL40:PMF (70:30, w/w)	0.58	β' >> β

[a]Abbreviations: SL40, SL made by reacting 40% stearic acid with canola oil; SL50, SL made by reacting 50% stearic acid with canola oil; PMF, palm mid-fraction; S-170, sucrose stearate; STS, sorbitan tristearate; DMG, distilled monoglycerides.

[b]SL40:PMF (70:30, w/w) is the control for the emulsifier treatments.

Figure 1. Curves showing solid fat content (SFC) of structured lipid and palm mid-fraction blends (see **Table 1** for compositions of blends), and fat from two commercial margarine products (MG-A and MG-B).

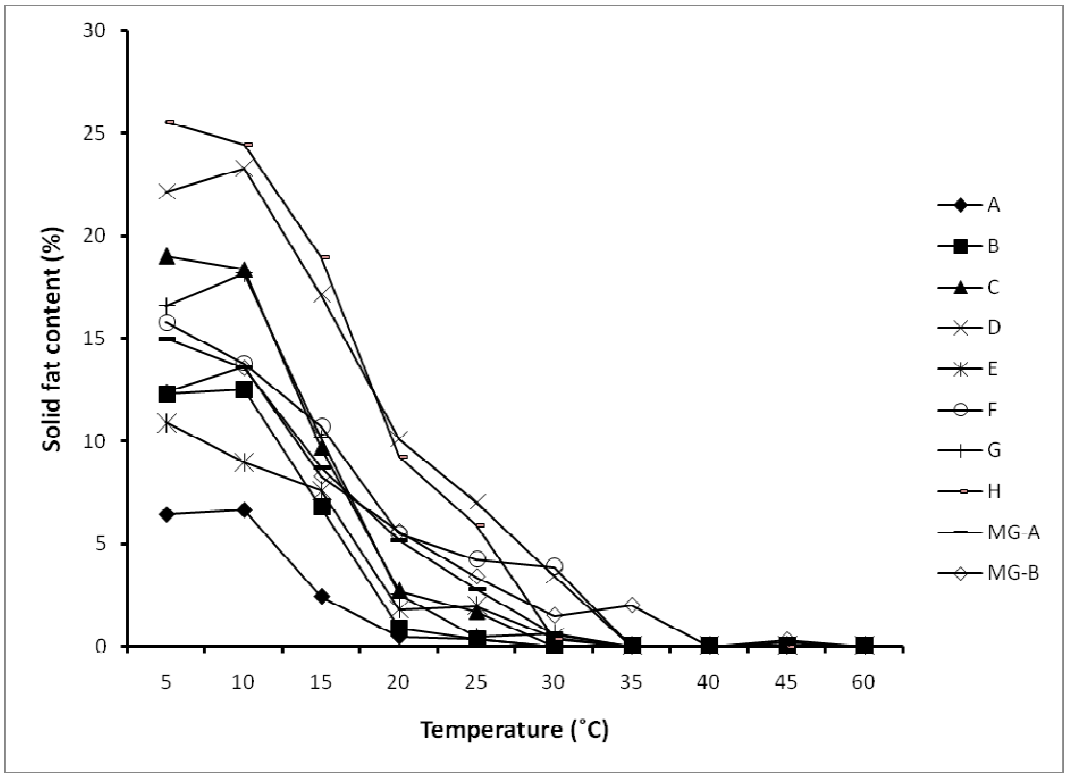
30
25
20
15
10
5
0
Solid fat content (%)
5
10
15
20
25
30
35
40
45
60
Temperature (˚C)
A
B
C
D
E
F
G
H
MG-A
MG-B

Figure 1

Figure 2. Polarized light microscopy images of SL40:PMF (70:30, w/w) blend treated with (a) 2% S-170, (b) 2% STS and (c) 2% DMG, and (d) control – SL40:PMF (70:30, w/w), after 24 h at 0˚C.

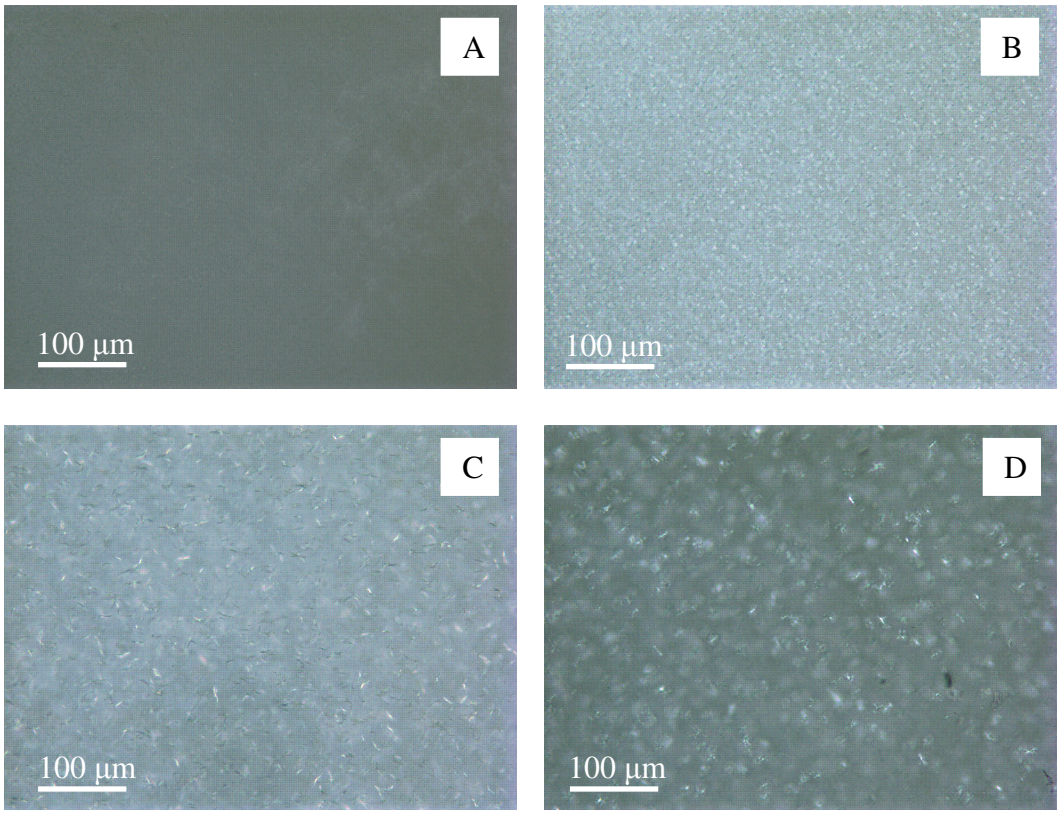

Figure 2

Figure 3. Polarized light microscopy images of SL40:PMF (70:30, w/w) blend treated with (a) 2% S-170, (b) 2% STS and (c) 2% DMG, and (d) control – SL40:PMF (70:30, w/w), after 4 weeks at 0˚C.

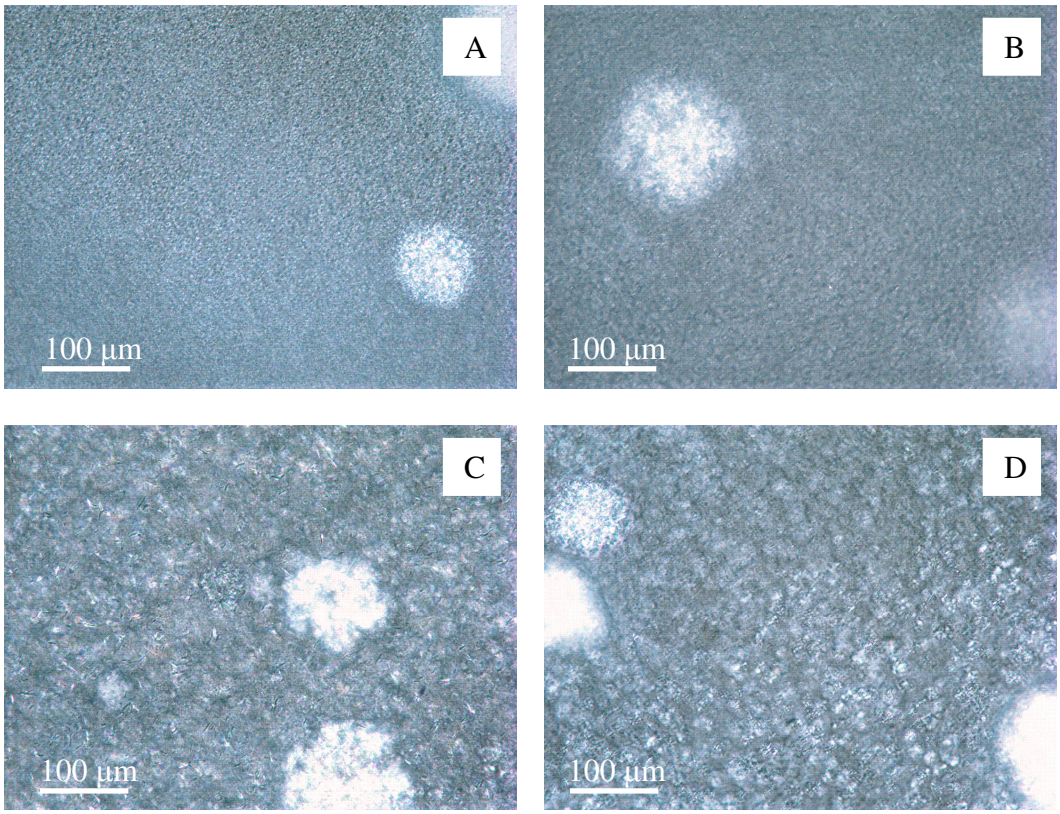

Figure 3

CHAPTER 6

PHYSICAL AND SENSORY ATTRIBUTES OF A *TRANS*-FREE MARGARINE FORMULATED WITH A BLEND OF A STRUCTURED LIPID, PALM MID-FRACTION, AND COTTONSEED OIL[1]

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[1]Stephen E. Lumor, Robert L. Shewfelt and Casimir C. Akoh. Submitted to Journal of Food Science, 07/14/2008.

ABSTRACT

Physical and sensory attributes of an experimental *trans*-free margarine (MG-X) and two commercial margarines (MG-A and MG-B) were studied. The *trans*-free margarine was formulated with a blend of a structured lipid (SL) synthesized by reacting canola oil with 40% stearic acid (w/w), palm mid-fraction (PMF), and cottonseed oil (CTO). No t*rans* fatty acids were detected in MG-X, whereas the *trans* fatty acid contents of MG-A and MG-B were 0.3 and 3.7% (w/w), respectively. MG-X was considerably firmer than MG-A and MG-B, less cohesive, and its adhesiveness was intermediate between those of MG-A and MG-B. MG-X's stability to syneresis was also intermediate to those of MG-A and MG-B. Sensory evaluation showed that MG-X was comparable to MG-A in terms of spreadability and texture only, but was significantly different from MG-B in all attributes. The study also found that more subjects would be willing to accept MG-X in spite of its sensory properties if it carried a health claim.

Keywords: Canola oil, cottonseed oil, palm mid-fraction, stearic acid, structured lipid, *trans*-free margarine

Introduction

Consumption of significant amounts of *trans* fatty acids (TFA) has been a major health concern for consumers and regulatory agencies over the past decade. The major dietary sources of *trans* fatty acids are products formulated with partially hydrogenated fats. Examples include margarines, shortenings, bakery products, and fast foods. The amount of TFA present in these foods is estimated to be between 0 - 35% of total fatty acids (Hunter 2005), and the mean daily intake of TFA per person in the US population is 2.6% energy (Allison and others 1999). Intake of high amounts of TFA has been correlated with increased risk for cardiovascular diseases, primarily due to their adverse effects on plasma lipid profile (Wahle and James 1993; Willet and others 1993). These health concerns have led to a near global regulation of the levels of TFA in

food. In the United States, the Food and Drug Administration (FDA) issued a final ruling requiring foods containing TFA to be labeled accordingly, effective from January 2006 (Federal Register 2003). This regulatory mandate and consumer concerns have led to the development of alternative processes that will produce foods with zero or reduced TFA contents. The synthesis of structured lipids (SL) by the process of transesterification is one of such alternatives. This process does not result in the production of TFA, as is the case with partial hydrogenation (Hunter 2005).

The aim of this study was to evaluate physical and sensory attributes of a *trans*-free margarine produced from a blend of a structured lipid synthesized by enzymatically incorporating stearic acid into canola oil, and addition of palm mid-fraction (PMF) and cottonseed oil (CTO). PMF was added to increase solid fat content, and together with CTO, promote β' crystal formation to impart smooth texture to margarine. This study is of significance because death from degenerative diseases such as cancer, cardiovascular disease, obesity and diabetes can be reduced significantly by consumption of healthy diets. The production of margarine with zero *trans* fat content will therefore be beneficial.

Materials and Methods

Materials

Stearic acid was purchased from Sigma Chemical Co. (St. Louis, MO). Canola oil (peroxide value 0.0 meq/Kg, acid value 0.28%) was purchased from a local grocery store. Palm mid-fraction (peroxide value 1.0 meq/Kg, acid value 0.1%, iodine value 43-48) was donated by Fuji Vegetable Oil Inc. (Savannah, Georgia), and cottonseed oil (peroxide value 0.0 meq/Kg, acid value 0.15%) by Archer Daniels Midland Co. (Valdosta, Georgia). Immobilized Lipozyme RM IM was purchased from Novo Nordisk A/S (Bagsværd, Denmark). Sorbitan tristearate (STS)

with hydrophile/lipophile balance (HLB) of 2.1, and distilled monoglycerides (DMG) with HLB of 3.8 were donated by Danisco A/S (Copenhagen, Denmark), Annatto OS 15 food color was donated by D. D. Williamson Colors, LLC (Port Washington, WI). ButterBuds Food Ingredients (Racine, WI) supplied margarine flavors. Organic solvents and chemicals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA). All other chemicals used were of analytical or HPLC grade.

SL synthesis

Structured lipid synthesis was performed in a stir-batch reactor at 65˚C for 12 h. The reaction mixture typically contained 300 g of canola oil, 40% stearic acid (by weight of canola oil), and 10% Lipozyme RM IM (by total weight of reactants). The product was separated from the enzyme by suction filtration, and free fatty acids removed by short-path distillation with a KDL-4 (UIC Inc., Joliet, IL) unit under the following conditions: heating oil temperature, 185˚C; cooling water temperature, 55˚C; pump vacuum, <1 mm Hg; feed rate, maintained at 100 mL/h. The reaction product was passed through the system twice to reduce the free fatty acid content to an acceptable level. Free fatty acid content (0.12 – 1.05% oleic acid) was determined according to AOCS Official Method, Ca 5a-40 (1989).

Tub margarine production

The structured lipid product (SL40) was blended with palm mid-fraction (PMF) and cottonseed oil (CTO) in the ratio of 11:7:3 (w/w/w). The compositions of the lipid and water phases are given in Table 1**.** The lipid phase (70%, w/w) was kept at 80˚C for 1 h to melt components, after which the aqueous phase (30%, w/w) was added and the mixture homogenized at 1000 rpm for 5 min using a Gifford-Wood's Rotor-Stator Homogenizer Model 2L (Hudson, NH). The emulsion was crystallized in a batch ice cream freezer model 104 (The Taylor Company, Rockton, IL) for

10 min. The output temperature was -10˚C. The product was tempered at room temperature (22˚C) for 2 h, after which the margarine was worked with a hand whip for 10 min. The product was then poured into containers and stored at 0˚C. For comparison, two commercial margarine (MG-A and MG-B) samples were melted and crystallized as described above.

Fatty acid profile analysis

Between 0.1 – 0.2 g of fat (in duplicate) from each margarine sample was converted to fatty acid methyl esters (FAME) using AOAC Official method 996.01, Section E (1998) as previously described (Lumor and others 2007). The FAME were analyzed in parallel with a FAME standard (Supelco 37 component FAME mix, Supelco®, Bellefonte, PA) using an Agilent Technology 6890N gas chromatograph equipped with a flame-ionization detector (FID). An SP-2560, 100 m x 0.25 mm ID, 0.20 μm film column was used for separation. One injection (1 μL) per sample in duplicate was performed in the split mode, at a split ratio of 50:1. Helium was the carrier gas, the linear velocity was 20 cm/sec, and the flow rate was 1mL/min. The oven temperature was initially held at 140˚C for 5 min and then programmed to 240˚C at 4˚C/min, and held isothermally for 20 min. The injection port temperature was 250˚C while that of the detector was 260˚C. The different amounts of FAME were analyzed and integrated by an online computer, and values for duplicate samples averaged to give fatty acid profile of each sample (Table 2).

Texture profile analysis (TPA)

Textural properties (hardness, adhesiveness, and cohesiveness) of margarine were evaluated at 22 ºC using the TPA procedure (Bourne 1978). 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 5 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. The maximum force

(N) during the first compression was reported as hardness. The negative force area in N·s (A_2) for the first compression was reported as adhesiveness. The ratio of the positive force area during the second compression (A_3) to that of the first compression (A_1) was reported as cohesiveness. Each sample was analyzed in triplicate.

Rheological properties

A series of tests were performed on a dynamic stress-controlled rheometer SR5000 (Rheometric Scientific, Piscataway, NJ) at 22˚C to determine the rheological properties of margarine samples. A 25 mm parallel plate (0.6 mm gap) was used for analysis. 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. The lower the degree of deformation, the less likely the margarine will show syneresis (Bohlin Application Note). In dynamic analysis, margarine samples were subjected to a sinusoidally varying stress (0.4 MPa) 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. The frequency (Hz) at which G' and G" crossover is a measure of spreadability. The higher the crossover frequency, the more spreadable the margarine is (Bohlin Application Note).

Sensory evaluation

Sensory evaluation was performed with panelists in individual booths under fluorescent light. Subjects were students/staff of the University of Georgia (UGA). Coded margarine samples were presented simultaneously to the subjects together with slices of bread, non-salted crackers, and water. Evaluation was performed by spreading the margarine samples on slices of bread, followed by chewing. Subjects were not required to swallow samples. Subjects ate the crackers, and rinsed their mouths with water before, between and after each sample evaluation.

Consumer acceptance was determined by 97 subjects using a 5-point willingness-to-purchase scale, ranging from would definitely buy (1) to would definitely not buy (5). Subjects evaluated the experimental margarine (MG-X) and the two re-crystallized commercial margarines (MG-A & MG-B). A ranking (difference) test using 37 untrained panelists was used to rank the margarine products in terms of the attributes, appearance, taste, aroma, spreadability, and texture (mouthfeel). No ties were allowed. The average responses for the consumer acceptance and ranking tests were calculated and analysis of variance (ANOVA) performed by means of SAS Statistical Software Version 9.1 (SAS Institute, Cary, NC). Significant differences between samples in terms of each attribute were determined using the Duncan's multiple comparison test.

Consumer behavior

After sensory evaluation of margarine samples, subjects (120) ranked, from 1 to 5, the following factors in the order in which they affect their decision to purchase margarine: price, health claim, fat content, sensory, and brand. Participants were asked to indicate how their evaluation of MG-X would have been affected if it carried the health claim, "contains no *trans*-fat, which is a risk factor for cardiovascular disease." They were also asked to indicate how much extra they would be willing to pay for MG-X over a popular brand, assuming MG-X carried the health claim mentioned above. The responses were presented as percentage distributions.

Results and Discussion

The fatty acid profiles of the experimental margarine (MG-X) and commercial margarines, MG-A (70% fat, w/w) and MG-B (60% fat, w/w), are given in Table 2. No *trans* fatty acids (TFA) were detected in MG-X, whereas C18:1t and C18:2t were detected in MG-B, and C18:1t in MG-A. Even though the levels of TFA detected in the two commercial margarines would

amount to less than the 4.6% energy as TFA required to adversely affect plasma lipoprotein levels (Hunter 2005), a product containing absolutely zero TFA content would be more desirable. Among the three margarines, MG-A and MG-B contained the lowest levels of C18:0 and C16:0, respectively. The saturated fatty acid (SFA) contents of MG-X and MG-A were comparable. While majority of this was C16:0 in the case of MG-A, the contents of C16:0 and C18:0 were about equal in MG-X and MG-B. This is of significance because unlike C16:0, stearic acid contributes to plasticity without having adverse effects on plasma lipoprotein levels (Keys and others 1965; Hegsted and others 1965; Grande and others 1970; Bonanome and Grundy 1988), and can therefore substitute for a fraction of C16:0. This cannot be a full substitution because the level of C16:0 in a fat is critical for the preponderance of β' triacylglycerol (TAG) crystals (deMan and deMan 2001), which impart smooth mouthfeel to margarine.

The textural properties (hardness, adhesiveness and cohesiveness) of the margarine products are given in Figure 1. All three margarines were significantly different in terms of each attribute. MG-X had the highest hardness value (N) and will therefore be more difficult to spread compared to the other two. MG-B had the least hardness value most probably because it was formulated with less fat (60%, w/w) and the content of SFA was 24% compared to 32.9 and 32.4% for MG-X and MG-A, respectively. Even though MG-X and MG-A contained comparable amounts of SFA (Table 2) and total fat (70%, w/w), the disparity in hardness values between them can be attributed to their different TAG compositions. Lumor and others (2007) observed that solid fat content or hardness was mostly dependent on how saturated the TAGs present in the fat or oil were. For two fats having comparable SFA contents, the one having higher levels of highly saturated TAGs will most likely be harder than the one having lower

levels of highly saturated TAGs. Solid fat content or hardness can also be affected by the presence of minor components such as emulsifiers. These emulsifiers, together with the types of TAGs present, can influence crystal properties (polymorphism and morphology) and solid fat content, which in turn impact rheological properties of margarines (Marangoni and Narine 2002). Adhesiveness (N.s) of MG-X was intermediate between those of MG-A and MG-B. MG-B was the least adhesive and most cohesive. MG-X was the least cohesive while MG-A was the most adhesive. The more adhesive a margarine is, the more likely it will stick to utensil such as spreading knife. Cohesiveness is a measure of intermolecular strength. The spreadability of margarine is affected to different extents by adhesiveness and cohesiveness.

Figure 2 shows the rheological properties of the margarine products. Creep analysis (Figure 2) is a measure of emulsion stability. A low constant stress (0.4 MPa) was applied to samples and deformation (strain) per unit time measured. The sample that showed the least deformation with time would be the least likely to show syneresis. The order of margarine emulsion stability was MG-B > MG-X > MG-A. In dynamic analysis (Figure 3), a sinusoidally varying stress (0.4 MPa) was applied to samples. Storage/elastic (G') and loss/viscous (G'') moduli were calculated and plotted as a function of frequency (Hz). The frequency at which G' and G" crossover was indicative of spreadability. The higher the crossover frequency, the more spreadable the margarine was. The order of spreadability was MG-B > MG-A > MG-X.

Sensory evaluation is an important tool that links product attributes with consumer preferences. Figure 4 shows consumer acceptance (intent-to-purchase) of the experimental margarine (MG-X) and the two re-crystallized commercial margarine samples (MG-A and MG-B). Acceptance of MG-X was not comparable to MG-A and MG-B. Only a little over 10% of panelists would definitely buy MG-X, and about 35% would definitely not buy. In the ranking (difference) test

(Figure 5), subjects actively differentiated between margarine samples by ranking them (rank 1 was highest and rank 5 was lowest), and no ties were allowed. MG-A and MG-B were not significantly different with respect to all attributes except for spreadability where MG-B had a higher mean ranking. MG-A was not significantly different from MG-X in terms of spreadability and texture. Results of the sensory evaluation showed that MG-X was comparable to MG-A in terms of spreadability and texture only. These are the two attributes that are directly related to the kind of fat used for margarine formulation. The poor performance of MG-X with respect to overall acceptance, appearance, taste, and aroma was due to formulation challenges encountered. The PMF and short-path distilled structured lipid used were not deodorized, and therefore had characteristic aroma and taste, which persisted in MG-X. This study has shown that a *trans*-free margarine formulated from a blend of SL40, PMF, and CTO can compete with commercial margarines in terms of all attributes if the formulation challenges encountered in this study are taken care of by using deodorized PMF and SL.

This study also evaluated factors that influence consumers' decision to purchase margarine (Figure 6). About 40% of respondents (120) indicated sensory was the most important factor affecting their decision to purchase, while about 10% indicated brand was the most important factor (Figure 6a). The mean ranking of these factors (Figure 6b) showed that sensory was most important, followed by health claim, price, fat content and brand, in that order. It has been reported (Jaeger 2006) that consumer behavior or preference is affected by a number of interacting factors, and there are usually trade-offs between two or more of these factors in the decision making process. When asked if their evaluation of MG-X would have been affected had the sample carried the health claim, "contains no trans fat, which is a risk factor for cardiovascular disease (CVD)", about 10% of those who indicated health claim was the most

important factor said no (Figure 7). About 35% of those who indicated sensory was most important and 30% of those who indicated brand was most important said no. When asked how much extra they would be willing to pay for MG-X over a popular brand if MG-X carried the above health claim, about 15% of those who indicated price was the most important factor affecting their decision to purchase would pay $0.35-0.50, whereas about 37% of those who chose health content as the most important factor would pay $0.35-0.50 (Figure 8). The above situations show how consumer behavior/preference is affected by information (advertisement). A little less than two-thirds of those who indicated sensory was most important in their decision making would purchase MG-X (with health claim) in spite of its sensory properties. Unlike sensory-sensitive subjects, only about 40% of price-sensitive subjects would be willing to pay more than $0.15 for MG-X (with health claim) over a popular brand. This study shows that *trans*-free margarines formulated with structured lipids or blends containing structured lipids would compete with popular brands, especially with advertisement.

Conclusions

Foods with zero or reduced *trans* fat are becoming more desirable because of health implications associated with significant consumption of *trans* fatty acids. The experimental margarine (MG-X) formulated in this study did not contain *trans* fatty acids and contained less palmitic acid compared to MG-A. Sensory evaluation showed that MG-X was not significantly different from MG-A with respect to spreadability and texture, but was significantly different from MG-B in all attributes. The study also showed that more subjects would purchase or be willing to pay more for MG-X if it carried a health claim.

Acknowledgement

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Table 1. Formula for experimental margarine (MG-X)

Ingredient	wt%
Lipid phase	
SL40:PMF:CTO[a] (5.5:3.5:1.5. w/w)	67.17
Lecithin	0.20
DMG:STS (3:2, w/w)	0.50
Tenox 20A antioxidant	0.10
Annatto food color	0.03
Butter buds (flavor)	0.50
Aqueous phase	
Water	27.25
Skim milk powder	1.25
Salt	1.50
Buttermilk buds (flavor)	1.50

[a]Abbreviations:SL40, structured lipid made by reacting canola oil with 40% stearic acid; PMF, palm mid-fraction; CTO, cottonseed oil; DMG, distilled monoglycerides; STS, sorbitan tristearate.

Table 2. Fatty acid profile of experimental (MG-X) and commercial (MG-A & MG-B) margarines

Fatty acid	MG-X	MG-A	MG-B
C16:0	17.2 ± 0.3	26.0 ± 0.2	10.4 ± 0.2
C18:0	14.9 ± 0.0	4.3 ± 0.0	12.5 ± 0.2
C18:1 cis	40.4 ± 0.2	37.2 ± 0.1	24.5 ± 1.2
C18:1 trans		0.3 ± 0.0	3.2 ± 0.1
C18:2 cis	22.7 ± 0.2	25.9 ± 0.2	40.9 ± 0.7
C18:2 trans			0.5 ± 0.0
C18:3 ω6	0.3 ± 0.0	0.4 ± 0.0	0.1 ± 0.0
C18:3 ω3	2.7 ± 0.0	3.2 ± 0.0	6.1 ± 0.1
C20:0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
C20:1 ω9	0.9 ± 0.0	0.4 ± 0.0	0.4 ± 0.1
Others[a]	0.7 ± 0.0	2.3 ± 0.0	1.9 ± 0.0
ω6:ω3	8.5	8.1	6.7
Satd fat	32.9± 0.2	32.4 ± 0.4	24.0 ± 0.2
Unsatd fat	62.1± 0.2	67.6 ± 0.4	75.9 ± 0.2
% trans fat		0.3 ± 0.0	3.7 ± 0.1

[a]Others refer to the sum of C12:0, C14:0, C16:1, C20:0, C21:0, C20:2, C22:0, C20:3 ω6, and C22:1 ω9.

Figure 1. Texture profile analysis of experimental (MG-X) and commercial (MG-A & MG-B) margarines. The margarines differed significantly (p-value < 0.001 at $\alpha_{0.05}$) from one another with respect to the three attributes. The different letters signify significant difference.

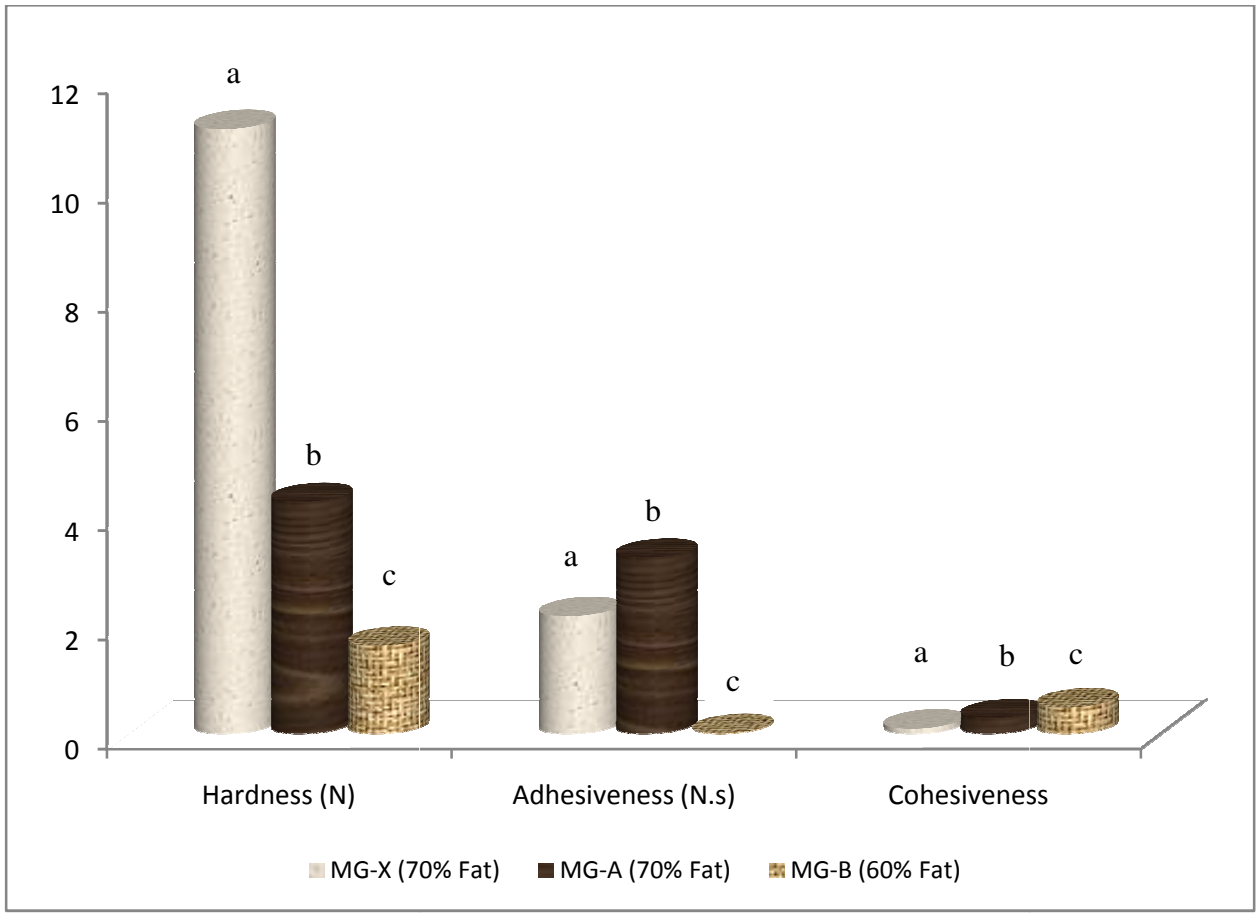
12
10
8
6
4
2
0
a
b
c
a
b
c
a
b
c
Hardness (N)
Adhesiveness (N.s)
Cohesiveness
MG-X (70% Fat)
MG-A (70% Fat)
MG-B (60% Fat)

Figure 1

Figure 2. Creep analysis of experimental (MG-X) and commercial (MG-A & MG-B) margarines.

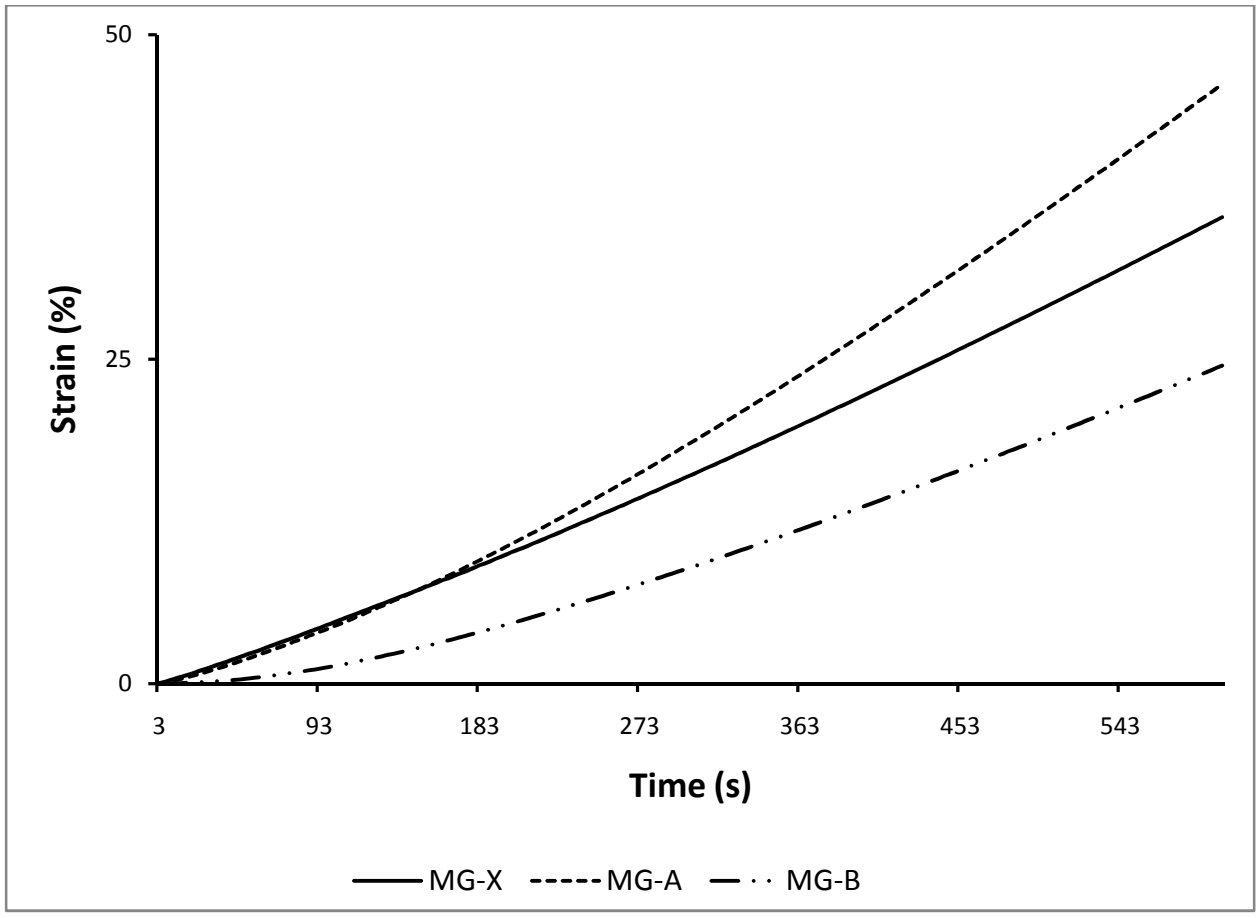

Figure 2

Figure 3. Dynamic analysis of experimental (MG-X) and commercial (MG-A & MG-B) margarines.

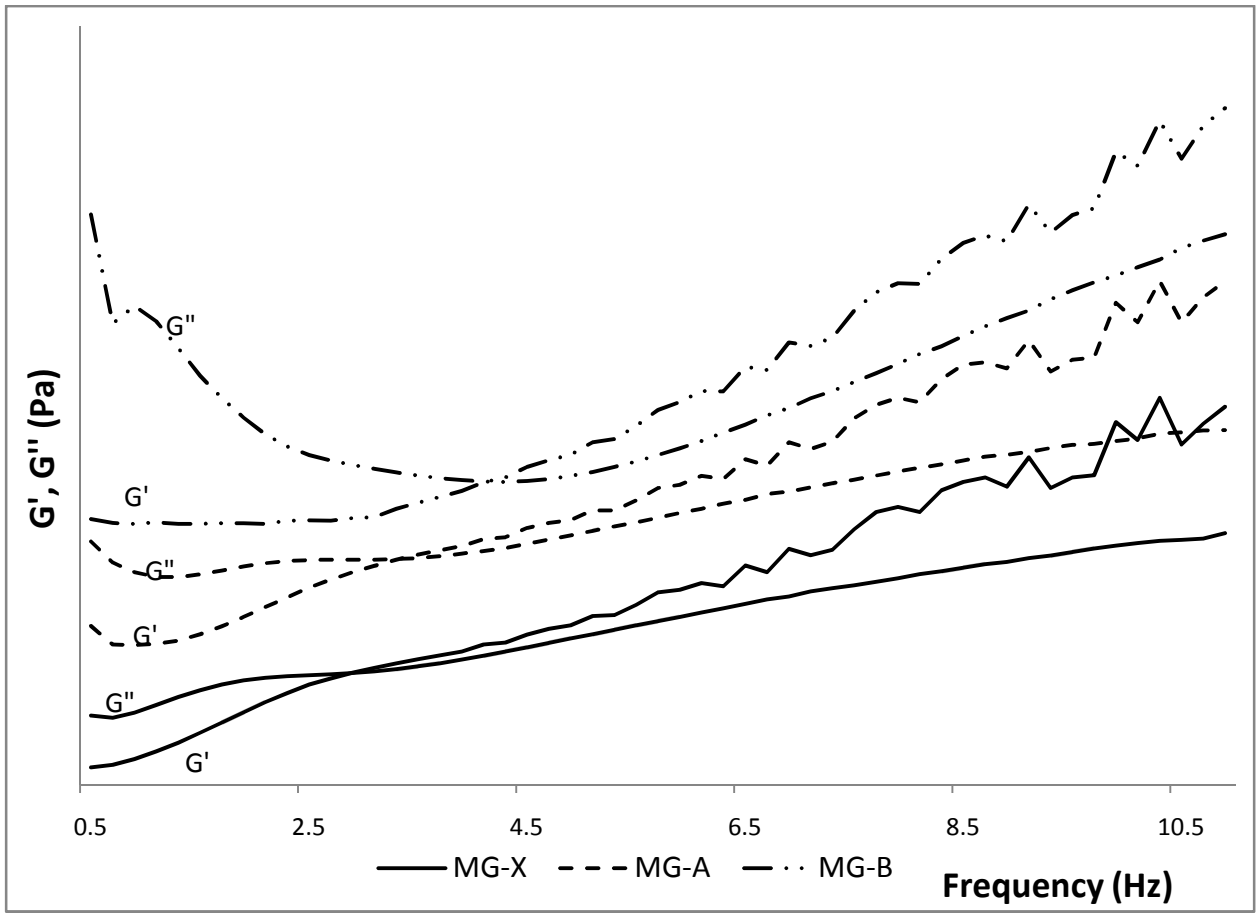

Figure 3

Figure 4. . Plot showing consumer acceptance of experimental (MG-X) and commercial (MG-A & MG-B) margarines.

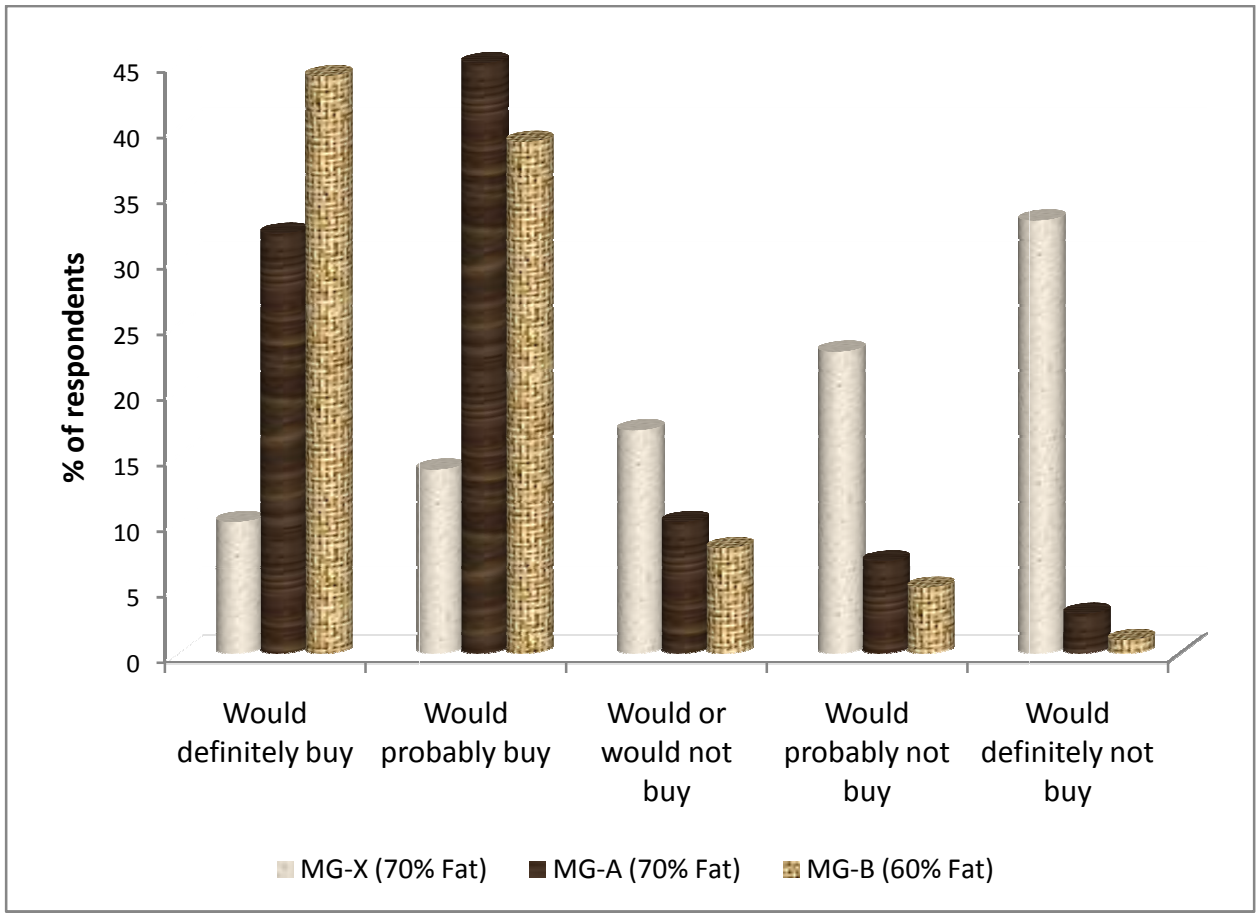
45
40
35
30
25
20
15
10
5
0
% of respondents
Would definitely buy
Would probably buy
Would or would not buy
Would probably not buy
Would definitely not buy
MG-X (70% Fat)
MG-A (70% Fat)
MG-B (60% Fat)

Figure 4

Figure 5. Mean ranking of attributes of experimental (MG-X) and commercial (MG-A & MG-B) margarines in the ranking (difference) test. The circles represent the points where no significant difference was observed between MG-A and the other margarine. Rank 1 is highest and 3 is lowest.

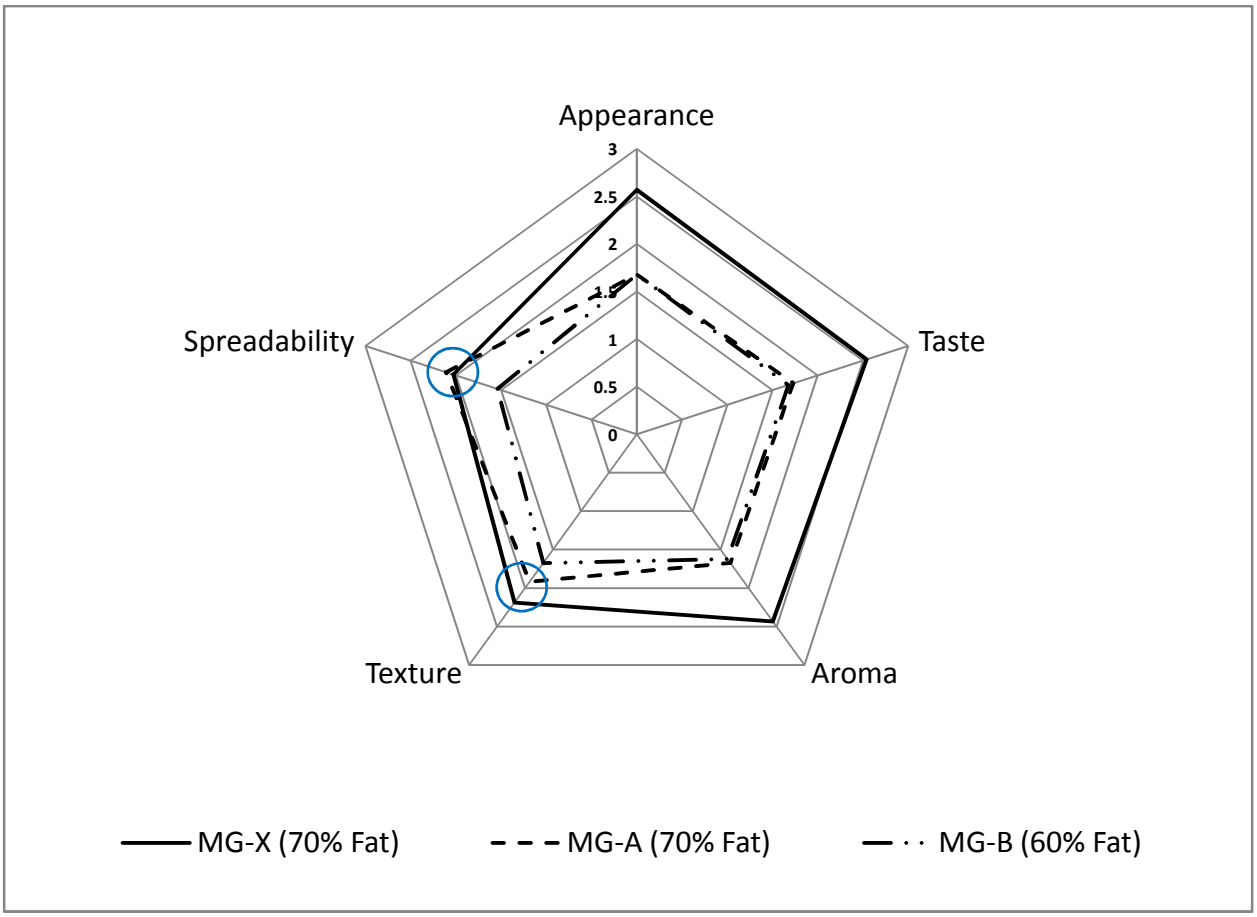
Appearance
3
2.5
2
1.5
1
0.5
0
Taste
Aroma
Texture
Spreadability
MG-X (70% Fat)
MG-A (70% Fat)
MG-B (60% Fat)

Figure 5

Figure 6. Chart showing (a) the most important factors and (b) mean ranking of factors that affect the decision to purchase margarine.

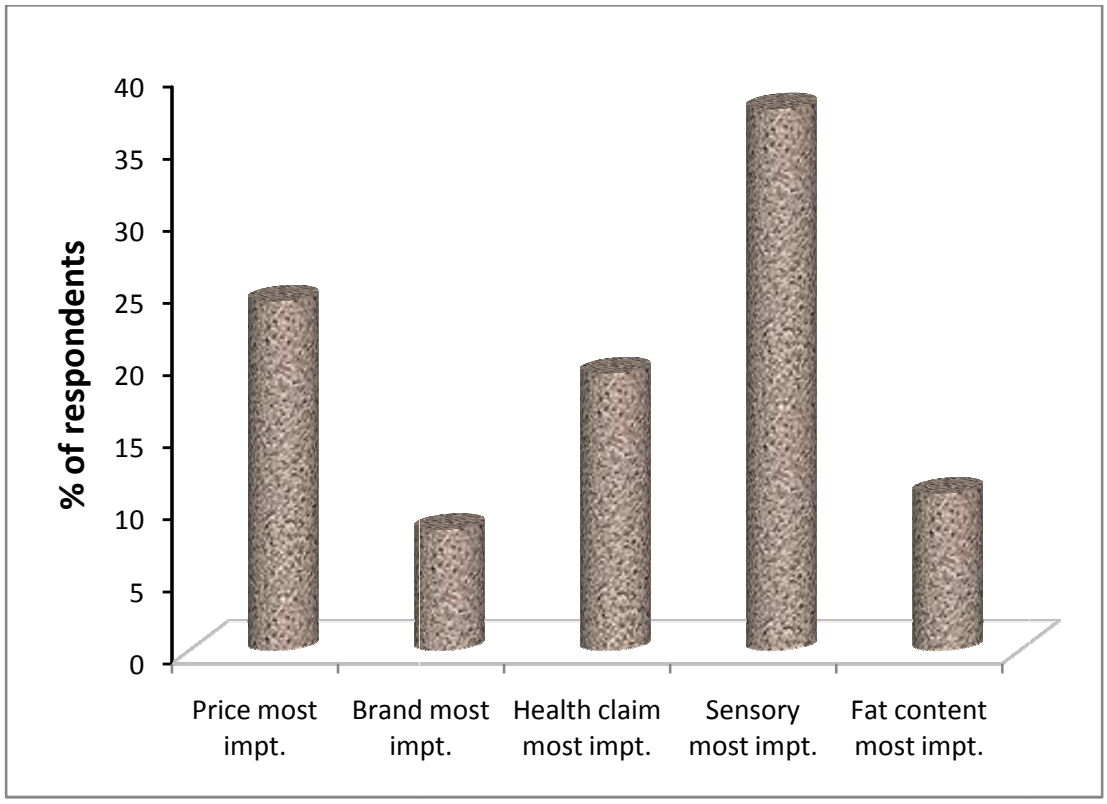

Figure 6a

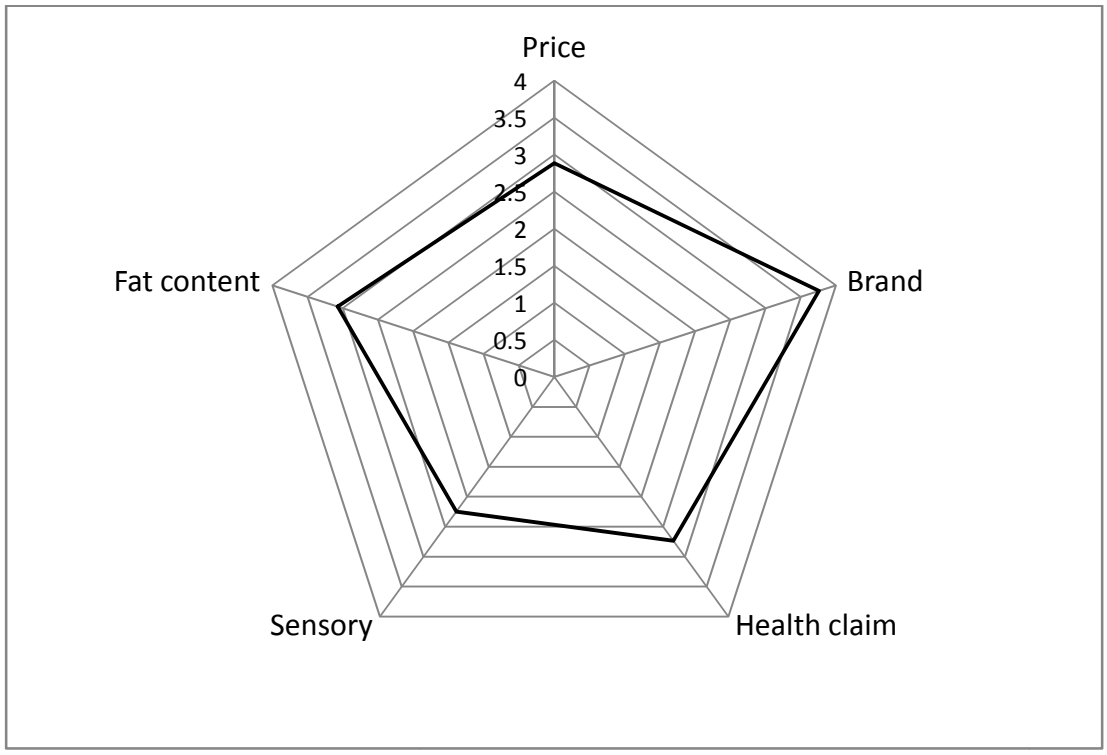

Figure 6b

Figure 7. How evaluation of the experimental margarine (MG-X) would have been affected among the different subject groups, had it carried the health claim, “contains no trans fat, which is a risk factor for cardiovascular disease.” Rank 1 is highest and 4 is lowest.

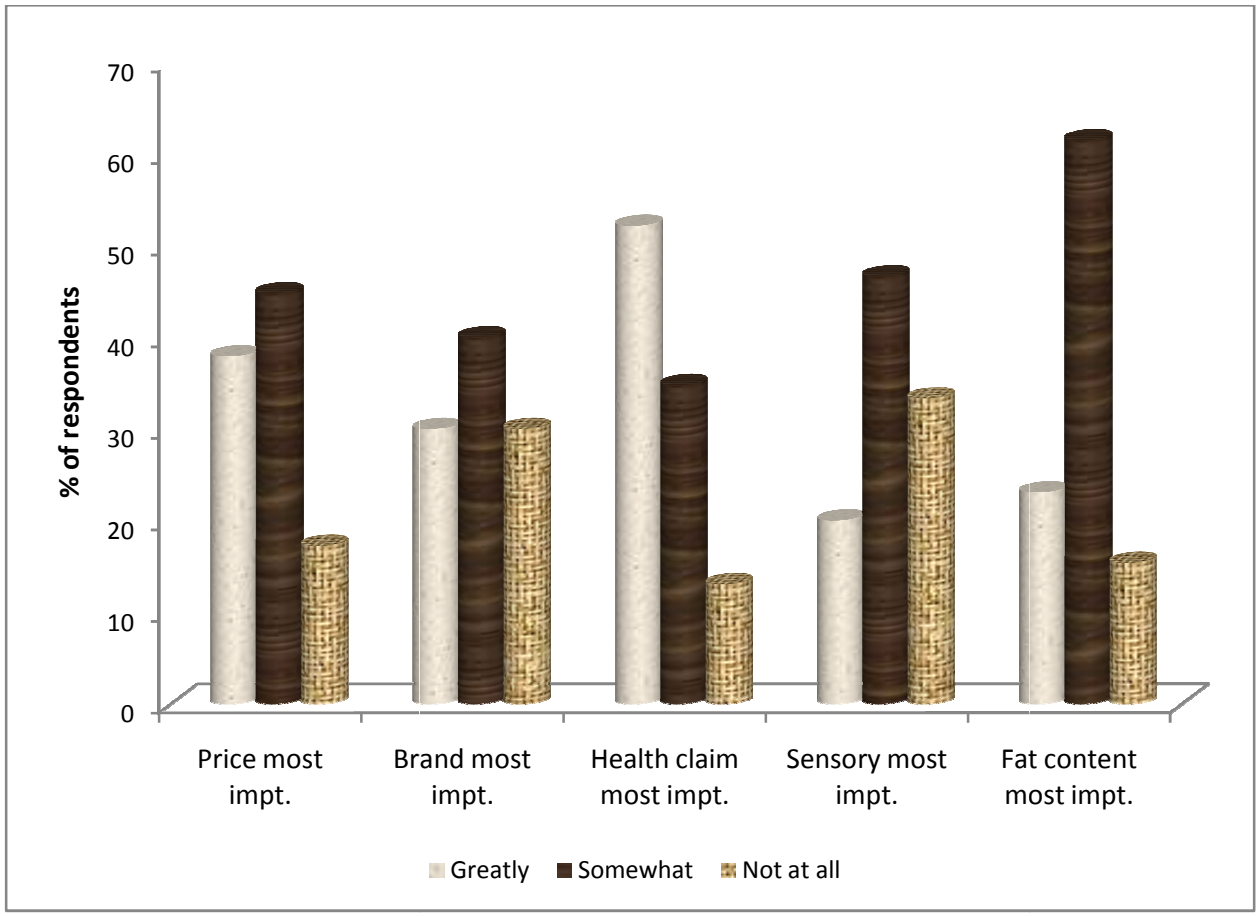
70
60
50
40
30
20
10
0
% of respondents
Price most impt.
Brand most impt.
Health claim most impt.
Sensory most impt.
Fat content most impt.
Greatly
Somewhat
Not at all

Figure 7

Figure 8. How much extra the different groups of subjects would want to pay for the experimental margarine (MG-X) over a popular brand if MG-X carried the health claim, "contains no trans fat, which is a risk factor for cardiovascular disease."

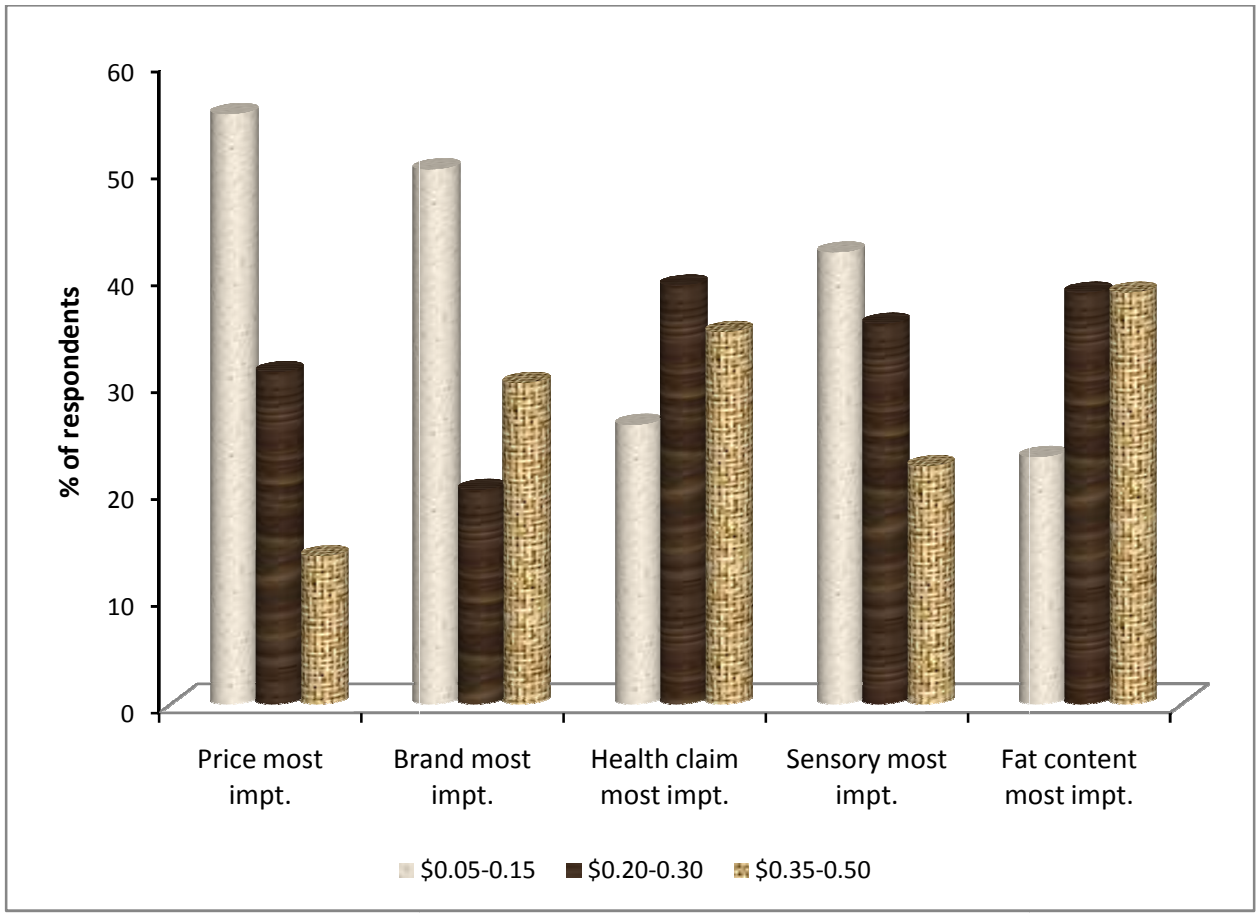
60
50
40
30
20
10
0
% of respondents
Price most impt.
Brand most impt.
Health claim most impt.
Sensory most impt.
Fat content most impt.
$0.05-0.15
$0.20-0.30
$0.35-0.50

Figure 8

CHAPTER 7

CONCLUSIONS

Reducing the amount of *trans* fatty acids (TFA) present in diet is very important in light of the adverse effects of TFA on health. It is therefore important that alternatives to partial hydrogenation be sought to significantly reduce or eliminate the presence of these harmful fatty acids from food. This study has shown that it is possible to synthesize *trans*-free structured lipids for possible applications in margarines.

In this study, Lipozyme RM IM was more favorable as a catalyst for the incorporation of stearic acid into canola oil than *Candida rugosa* lipase isoform 1 (LIP1), and ethyl stearate was a better acyl donor compared to stearic acid. This study also showed that stearic acid was mostly incorporated at the *sn*-1,3 positions of triacylglycerols (TAG), but the structured lipids (SL) did not have adequate solid fat contents or crystal types desirable in margarine. Solid fat content and crystal properties of the SL were improved by blending with palm mid-fraction (PMF). Emulsifiers such sorbitan tristearate, distilled monoglycerides and sucrose stearate were used to improve the morphologies of TAG crystals.

Consequently, a *trans*-free margarine was formulated with a blend of the SL40, PMF and cottonseed oil. The margarine product was evaluated with respect to its physical and sensory properties. Results showed that the *trans*-free margarine was only comparable to one of the two commercial margarine samples evaluated in terms of spreadability and texture only. Consumer acceptance of the experimental margarine was low. Our study also showed that subjects were most likely to purchase the experimental margarine if it carried the health claim, “contains no *trans* fat, which is a risk factor for cardiovascular disease.”

Clearly, this study indicates that the use of structured lipids for *trans*-free margarine formulation to replace margarines formulated with partially hydrogenated fats is possible. For future studies, oils that contain moderate amounts of palmitic acid should be used as starting materials for the synthesis of *trans*-free SLs because they result in increased fatty acid chain-length diversity, which promotes the stability of TAG crystals in the β' form. Cottonseed oil should be a more suitable starting material compared to canola oil because it contains moderate amounts of palmitic acid, and crystallizes in the β' form. Fully hydrogenated palm mid-fraction may be included in the blend for margarine formulation because of the presence of β' crystal stabilizing TAGs such as 1,3-dipalmitoyl-2-elaidoyl glycerol (PEP), and 1,3-dipalmitoyl-2-stearoyl glycerol (PSP). The use of deodorized oils is very critical in the formulation of margarine because the oils used are not expected to impart taste or aroma to the margarine. The low consumer acceptance of the experimental margarine in this study can be attributed to the use of a non-deodorized oil blend, which imparted undesirable taste and aroma to the margarine.