PRODUCTION OF MENHADEN OIL-BASED STRUCTURED LIPIDS: ORGANOGELATION, MICROENCAPSULATION, AND APPLICATION IN YELLOW CAKE

by

SARAH ALEXANDRA WILLETT

(Under the Direction of Casimir C. Akoh)

ABSTRACT

The aim of this research was to enzymatically modify menhaden oil with various acyl donors to produce structured lipids (SL). Using the Taguchi method, optimal reaction parameters for incorporating 30 mol% of C10:0 were 1:3 menhaden oil:ethyl caprate substrate molar ratio, 10% (w/w of total substrates) Lipozyme® 435 lipase (from *Candida antarctica*), 60°C temperature, and 24h reaction time. Linear interpolation determined substrate molar ratios that incorporated 20 or 30 mol% of caprylic or stearic acid. The 1:3.03 and 1:4.58 molar ratios resulted in incorporation of 20 and 30 mol% C8:0, respectively. The 1:1.32 and 1:2.41 molar ratios led to incorporation of 20 and 30 mol% C18:0, respectively. A 1:3:1 molar ratio (menhaden oil:C8:0:C18:0) led to incorporation of 14 mol% each of caprylic and stearic acid. The SL containing caprylic and/or stearic acid all had a melting point of 25-35°C. For all SL, higher amounts of medium-long-medium (MLM)-type triacylglycerols (TAG) were formed using

Lipozyme® 435 as the biocatalyst. Organogels (also known as oleogels) of menhaden oil and SL were formed using two different organogelator blends (β-sitosterol/γ-oryzanol or sucrose stearate/ascorbyl palmitate (SSAP)) at a 1:1 molar ratio. The organogels (SSAP: 72°C, phytosterol: 68°C) had higher melting completion temperatures than the menhaden oil (13°C) and SL (14-35°C). Physicochemical properties of the organogels depended on lipid and organogelator type. Encapsulation of these organogels in alginate microparticles led to a significant reduction in the amount of leaching (from 16-19% to 3-12%). Both organogels were also acceptable zero *trans*-fat substitutes for shortening in yellow cake by having similar physicochemical properties such as specific gravity (0.85), cake specific volume (0.99cm^3/g), and rheological properties (Power-Law Model values of n:0.78 and k:31 Pa s^n) to shortening. Organogelation and microencapsulation were both good methods for improving the oxidative stability of menhaden oil and SL (Oil Stability Index values from 4-18 h to 18-29 h when measured at 80°C). These lipids may find use in a wide range of food and nutraceutical applications.

INDEX WORDS: Structured lipid, Lipase, Menhaden oil, Organogel, Oleogel, Microencapsulation, Cake, Capric acid, Caprylic acid, Stearic acid, Sucrose stearate, Ascorbyl palmitate, Phytosterols

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DEDICATION

To my family, boyfriend, and puppy

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INTRODUCTION

There is increasing interest in the use of omega-3 polyunsaturated fatty acids (PUFA) in food products based on their potential health benefits such as lowering risk of cardiovascular disease, reducing inflammation, and aiding in brain health and development (He, 2009). Fish oil, such as menhaden oil, contain significant amounts of the omega-3 PUFA eicosapentaenoic (EPA, 20:5n3) and docosahexaenoic (DHA, 22:6n3) acids. However, it is challenging to add fish oil into food products because it has a low oxidative stability, low melting point, and fishy off-flavors/odors (Anbudhasan, Surendraraj, Karkuzhali, & Ramasamy, 2014). Because of these challenges, addition of fish oil/omega-3 PUFA is limited to a small range of foods.

Several efforts have been made to increase the use of high PUFA-containing oils in food products. Addition of antioxidants, formation of organogels, microencapsulation, and production of structured lipids (SL), are some of the methods for altering the oxidative stability, thermal behavior, triacylglycerol molecular species, and reducing fishy flavors of high PUFA oils (Anbudhasan et al., 2014; Co & Marangoni, 2012; Kim & Akoh, 2015). Based on these methods, successful alteration of high PUFA oils for use in food applications can depend on the type of antioxidant, organogelator(s), microencapsulation method, encapsulating agent, and acyl donor.

Structured lipids (SL) are defined as lipids that have been chemically or enzymatically altered from their natural form (Kim & Akoh, 2015). Some SL of interest in food applications include medium-long-medium (MLM) chain type, and zero *trans*-fat SL. Because menhaden oil contains significant amounts of the long chain fatty acids, EPA and DHA, at the *sn*-2 position, it is a desirable substrate for producing health beneficial MLM-type SL. During digestion of MLM-type SL, the medium chain fatty acids are rapidly hydrolyzed by *sn*-1,3 specific pancreatic lipase and enter the liver directly via the portal vein as a source of quick energy (Kim & Akoh, 2015). The long chain fatty acid, in 2-monoacylglycerol (MAG) form, is absorbed. Formation of an MLM-type SL containing menhaden oil may allow for increased absorption of DHA (Bandarra et al., 2016). Production of zero *trans*-fat SL that contain EPA and DHA as substitutes to highly saturated fats may allow for lower total saturated fat consumption and add the additional health benefits of consuming omega-3 PUFA. Stearic acid is of increasing interest as an acyl donor in production of zero *trans*-fat SL due to its high melting point and research has found that stearic acid may have a neutral effect on cholesterol levels in the body (Grundy & Denke, 1990). While the production of MLM-type SL by incorporation of capric and/or caprylic acid into fish oil has been explored previously, incorporation of stearic acid, or blend of caprylic and stearic acid into menhaden oil has not yet been explored.

Production of SL enzymatically depend on factors such as substrate type, enzyme type and load, temperature, reaction time, reaction vessel, and presence of solvent and type of solvent (Zam, 2015). Optimization of these factors in a full factorial designed experiment is time consuming and expensive. Use of experimental design to optimize

enzymatic reactions for production of SL can save time and resources and reduce the number of experiments required by statistically determining optimal reactions parameters (Chandrasekar, 2014). The Taguchi method is one such experimental design method that may allow for optimal reaction parameters to be determined statistically through use of orthogonal arrays (Savaghebi, Safari, Rezaei, Ashtari, & Farmani, 2012). The use of the Taguchi method to incorporate an acyl donor into menhaden oil has not yet been explored.

Another method for potential alteration of menhaden oil and SL is the formation of organogels. Organogels (also known as oleogels) are lipid gels that show promise as a non-TAG structuring method for use as low saturated, zero *trans*-fat substitutes (Chaves, Barrera-Arellano, & Badan Ribeiro, 2018). Current food grade organogelators include phytosterols, lecithin, sorbitan esters, MAG, fatty acid alcohols, ceramides, proteins, and waxes (Co & Marangoni, 2012). However, some of these food grade organogelators are expensive to produce, are indigestible, or do not have the desired physicochemical properties. The physicochemical properties of the organogel depends on the lipid phase and organogelator type (Chaves et al., 2018). The use of sucrose stearate and ascorbyl palmitate as organogelators has not yet been explored. Both sucrose stearate and ascorbyl palmitate are relatively cheap and readily available. There may likely be intermolecular interactions between the long chain fatty acid tails, polar functional groups, and lipid phase (Dassanayake, Kodali, Ueno, & Sato, 2012). Use of SL as the lipid phase to form organogels has also not yet been explored.

Microencapsulation of fish oil may improve oxidative stability and mask fishy off-flavors/odors. Several studies have encapsulated fish oil using a variety of methods

and encapsulating agents (Encina, Vergara, Gimenez, Oyarzun-Ampuero, & Robert, 2016). One concern with microencapsulation is leaching of the internal phase during storage. Some studies have successfully microencapsulated organogels to improve microcapsule stability (Sagiri, Pal, Basak, Rana, Shakir, & Anis, 2014; Sagiri, Sethy, Pal, Banerjee, Pramanik, & Maiti, 2012). Microencapsulation of organogels with phytosterols or sucrose stearate/ascorbyl palmitate has not yet been explored.

A main challenge of adding fish oil such as menhaden oil in food products is that is has a low melting point, limiting use of menhaden oil in products such as baked goods that require fats with a higher melting point. In yellow cake, shortening plays an important role in the final texture and taste (Matthews & Dawson, 1966). However, shortening contains significant amounts of saturated and *trans*-fat, both of which have been found to raise LDL cholesterol (He, 2009). It is of interest to produce low saturated fat alternatives that can be substituted for shortening in the formulation of yellow cake. Organogels and SL are both promising alternatives. However, it has been found that SL are often lower in oxidative stability than the unmodified oil due to the removal of endogenous antioxidants during purification by short-path distillation, limiting its use in baked good applications (Zou & Akoh, 2013). Organogels have been found to improve the oxidative stability of the lipid being gelled (Co & Marangoni, 2012). Preparation of yellow cake using the sucrose stearate/ascorbyl palmitate blend, or menhaden oil SL have not yet been explored.

There are eight chapters in this research work. The first chapter introduces the topic rationales, hypotheses, and objectives of this research. The second chapter is a literature review of topics related to structured lipids, omega-3 PUFA, and food

fortification utilizing organogels and microencapsulation. The third chapter reports the incorporation of capric acid into menhaden oil using immobilized lipases and optimization of the enzymatic reactions using the Taguchi method to produce structured lipids (SL) having MCFA at the *sn*-1,3 positions and EPA and DHA at the *sn*-2 position. The fourth chapter compares physicochemical properties of different organogels using menhaden oil or SL as lipid phase and organogelator blends of β-sitosterol and γ-oryzanol, or sucrose stearate and ascorbyl palmitate. The fifth chapter reports the incorporation of caprylic and/or stearic acid into menhaden oil using immobilized lipases, and utilization of linear interpolation to produce SL with desired acyl donor incorporation and melting point. The sixth chapter determines the physicochemical characteristics of microencapsulated organogels to improve oxidative stability and reduce the amount of leaching during storage. The seventh chapter looks at organogels as low saturated fat substitutes to shortening in the production of yellow cake. The eighth chapter highlights the significance of this work and suggests ideas for future research. The specific objectives and hypotheses for this research are as follows:

Objective 1: To use the Taguchi method to optimize incorporation of capric acid into menhaden oil to produce medium-long-medium (MLM)-type triacylglycerol (TAG) structured lipids (SL). **Hypothesis:** Different levels of incorporation of acyl donor and TAG molecular species will vary based on acyl donor (capric acid versus ethyl caprate) and lipase (Lipozyme® 435 (food grade, weakly specific in non-polar solvents) and Lipozyme® RM IM (*sn*-1,3 specific)).

Objective 2: To form different organogels using different lipid phase (menhaden oil or SL) and organogelator blend (β-sitosterol and γ-oryzanol blend or sucrose stearate and

ascorbyl palmitate blend) and compare their physicochemical properties. **Hypothesis:** Physicochemical properties of the organogels will differ based on lipid phase and organogelator type.

Objective 3: To use linear interpolation to incorporate caprylic and/or stearic acid into menhaden oil to produce SL that have melting point between 25-35°C. **Hypothesis:** Enzymatic reactions (in terms of substrate ratio and acyl donor incorporation) will have a strong linear correlation and a linear regression equation can determine optimal substrate molar ratios to incorporate desired amount of acyl donor.

Objective 4: To encapsulate oleogels in alginate microparticles. **Hypothesis:** Encapsulation of oleogels will reduce amount of leaching during storage of the microcapsules and improve oxidative stability.

Objective 5: To replace shortening in yellow cake with oleogels and determine the physicochemical properties of the batter and cake. **Hypothesis:** Acceptability of the oleogels as low saturated fat alternatives to shortening will depend on lipid phase (menhaden oil or SL) and oleogelator type (β-sitosterol and γ-oryzanol blend or sucrose stearate and ascorbyl palmitate blend).

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CHAPTER 2

LITERATURE REVIEW

Structured Lipids

Structured lipids (SL) are defined as lipids that have been chemically or enzymatically modified from their natural biosynthetic form (Kim & Akoh, 2015). Lipids are typically altered by changing the fatty acid (FA) composition and/or the positions of the FA in the glycerol backbone (Willis, Ghazani, & Marangoni, 2017). Lipid substrates may include acylglycerols (triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG)), glycerol, free fatty acids (FFA), ethyl esters, or phospholipids. There are a few different types of enzymatic reactions to produce SL, including acidolysis, interesterification, alcoholysis, and direct esterification reactions. Acidolysis reactions involve the exchange of acyl groups from FFA and TAG (Willis et al., 2017). Interesterification reactions typically take place with two or more TAG molecules as the substrates and involve the exchange of acyl groups (Willis et al., 2017). The transferring of acyl groups from a TAG molecule to an alcohol such as glycerol (glycerolysis) or ethanol (ethanolysis) is termed an alcoholysis reaction. Direct esterification reactions take place with the substrates of glycerol and FA, producing TAG and water.

In the industry, chemical modification is commonly used for producing margarine and other fats. The typical reaction is chemical interesterification using sodium

methoxide as the catalyst (Willis et al., 2017). Although this method is common, there are some disadvantages. Only randomized products can be made, and the reaction process often occurs under harsh conditions. The use of enzymes as biocatalysts to modify lipids have several advantages. Products from enzymatic modification are specific, allowing for production of SL that have desirable physicochemical properties and/or nutritional benefits (Lai, Phuah, Lee, Akoh, & Weete, 2017). Enzymatic reactions typically occur under milder conditions and are considered an environmentally green process due to the use of less energy and no harsh chemicals (Kim & Akoh, 2015). Enzymatic modification also produces fewer byproducts, allowing for increased product yield. Although enzymatic modification is typically higher in cost than chemical modification, the use of immobilized enzymes allows for recovery and reuse of the enzyme, which lowers production costs (Willis et al., 2017).

There are numerous factors that affect lipase-catalyzed reaction product composition, purity, and yield. These factors include lipase type, lipase activity and load, substrates, reaction temperature, water activity, solvent or solvent-less conditions, time, and reactor type (Zam, 2015). For example, some lipases may prefer (or yield higher incorporation) the ethyl ester over the FFA of the same carbon chain length (Bloomer, Adlercreutz, & Mattiasson, 1992). These factors must be accounted for when conducting lipase-catalyzed synthesis reactions in order to produce the desired product. The different reactions allow for production of products with specific physicochemical properties or nutritional benefits.

Lipases

Specific and non-specific lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are the two groups of lipases (along with phospholipases A1 (PLA; EC 3.1.1.32) and A2 (PLA2; EC 3.1.1.4)) that can be used as biocatalysts in the enzymatic modification of lipids. Many commercial lipases are available, and come from microbial, plant, and animal sources. Of the different sources, microbial lipases are often preferred because they are thermostable and usually do not require cofactors (Xu, 2000). Common lipases that are used in production of SL include lipases from *Candida rugosa*, *Rhizomucor miehei*, *Candida antarctica*, and *Thermomyces lanuginosus* (Xu, 2000). Lipases such as these may differ in activity and display FA and/or positional specificity. Specific lipases are *sn*-1,3 specific and show preference in altering the acyl ester bonds in the first and third position of the glycerol backbone (Kim & Akoh, 2015). Common lipases that exhibit *sn*-1,3 specificity include pancreatic lipase, *Rhizomucor miehei, Thermomyces lanuginosus, Aspergillus niger,* and *Rhizopus delemar* (*oryzae*) (Xu, 2000). Non-specific enzymes differ in that there is usually not a distinct preference in acyl alteration. Lipases that fall into this group include *Candida rugosa* and *Candida antarctica*. Lipases can also show preference to certain FA or range of FA chain lengths. For example, *G. candidum* shows specificity for long chain FA containing a *cis* double bond in the C9 position such as oleic acid (Jensen, 1983). Lipases may also discriminate against certain FA. It has been shown that pancreatic lipase, commonly used for the *sn*-2 positional analysis assay for TAG, may hydrolyze long chain polyunsaturated fatty acids (PUFA) slower than other FA (Solaesa, Bucio, Sanz, Beltran, & Rebolleda, 2014). Pancreatic lipase has also been shown to selectively hydrolyze docosapentaenoic acid (DPA) over eicosapentaenoic

acid (EPA) and docosahexaenoic acid (DHA) due to its FA specificity (Akanbi, Sinclair, & Barrow, 2014).

Despite the many advantages of utilizing lipases as biocatalysts in the production of SL, there has so far been limited use in industrial applications. One of the main concerns is the stability and high costs associated with lipase use. Lipase load affects the reaction rate by accelerating the reaction (lowering activation energy) and increasing incorporation of acyl donors (Xu, 2000). Using a lot of lipase increases costs and acyl migration, so for optimal acyl donor incorporation and lowest cost, enzyme load at <20% of total substrates should be used (Xu, 2000). Another method to lower costs, improve stability, and allow for enzyme reuse is immobilization of the lipase. Immobilization often affects the lipase selectivity and physicochemical properties; however, the enzymes can undergo reactions in a wider temperature and pH range (Mohamad, Marzuki, Buang, Huyop, & Wahab, 2015). Commercial immobilized lipases of use in the production of SL include Lipozyme® RM IM (*Rhizomucor miehei* lipase immobilized on a resin carrier with specific activity of 442 IUN/g [interesterification unit per gram] as specified by manufacturer, *sn*-1,3 specific), Lipozyme® TL IM (*Thermomyces lanuginosus* lipase immobilized on silica gel with specific activity of 275 IUN/g as specified by manufacturer, *sn*-1,3 specific), Novozyme® 435 (*Candida antarctica* lipase B immobilized on an acrylic resin with specific activity of 10000 PLU/g [propyl laurate unit per gram] as specified by manufacturer, non-specific), and Lipozyme® 435 (recombinant lipase from *Candida antarctica*, expressed on *Aspergillus niger*, and immobilized on a macroporous hydrophobic resin with specific activity of 10000 PLU/g as specified by the manufacturer, *sn*-1,3 specific in non-polar media). One IUN is the

amount of enzyme activity that liberates 1 μmol of butyric acid from tributyrin per minute under defined standard conditions. One PLU is the amount of enzyme activity that produces 1 μmol of propyl laurate per minute from esterification of lauric acid with *n*-propyl alcohol under defined standard conditions. Food grade enzymes are a step towards increasing enzyme use in the food industry. Lipozyme® 435 is a commercial immobilized food grade lipase that has higher catalytic activity than other *sn*-1,3 specific enzymes and lower in cost (Wang, Chen, Ma, Jin, & Wang, 2015). Because of the lower cost and food grade status, Lipozyme® 435 has potential for use in enzymatic reactions to produce SL on an industrial scale within the food industry.

Structured Lipid Products

Specific SL products of current research interest include medium-long-medium (MLM) chain-type TAG, human milk fat analogues, cocoa butter alternatives, reduced calorie fats, *trans*-free fats, and structured phospholipids (Kim & Akoh, 2015). This research focuses on the production of MLM-type TAG and *trans*-free fats.

MLM-type SL

The structure of an MLM-type TAG includes having a glycerol backbone, with medium chain fatty acids (MCFA) at the *sn*-1,3 positions of the TAG and a long chain FA at the *sn*-2 position. MLM-type TAG are nutritionally beneficial, and have been reported useful in parenteral and enteral feeding, treatment of lipid malabsorption, and treatment of metabolic syndromes (Nagao & Yanagita, 2010; DeMichele, Karlstad, Bistrian, Istfan, Babayan, & Blackburn, 1989). During digestion, as shown in the schematic in Figure 2.1, it has been found that the MCFA located at the *sn*-1,3 positions

are hydrolyzed readily by pancreatic lipase and can be transported to the liver directly via the portal vein. The long chain FA, in 2-MAG form are absorbed more efficiently, and assimilates via the lymphatic system and then accumulates as TAG in adipose tissue or as membrane phospholipids where they can be released when needed for various physiological functions (Christensen, Høy, Becker, & Redgrave, 1995; Jensen, Christensen, & Høy, 1994).

Several approaches have been developed for producing MLM-type TAG. Most commonly, an acidolysis reaction is conducted using an *sn*-1,3 specific lipase with the substrates of a TAG containing significant amounts of desired long chain FA at the *sn*-2 position and medium chain FFA (Kim & Akoh, 2015). These SL can be produced in various reactors, such as a shaking water bath, heating block with stir bar, jacketed reactor, and packed bed reactor. Table 2.1 shows a summary of some of the MLM-type TAG produced in recent years, from 2014-2019. A comprehensive list of MLM-type TAG from 2010-2014 is found elsewhere (Kim & Akoh, 2015). Most MLM-type TAG were produced via acidolysis reactions. Of these, only some were produced with fish oil. It is of interest to produce SL containing fish oil with different enzymes and/or acyl donors to determine changes in FA composition, TAG molecular species, and other physicochemical properties.

Trans-free and Low Saturated Fats

The typical Western diet contains significant amounts of saturated and *trans*-fat. As of June 2018, partially hydrogenated oils (PHO), a source of *trans*-fat, was no longer included in food products since the FDA removed its Generally Recognized as Safe (GRAS) status (Food and Drug Administration, 2015). Food products that commonly

contain *trans*-fat and high amounts of saturated fat include shortening, margarine, fast food, frosting, spreads, snack foods, and fried/baked products. PHO are produced when a vegetable oil is hydrogenated, meaning it is exposed to hydrogen gas and a catalyst (often a metal such as nickel), converting the oil to solid fat by saturation of the double bonds (Freeman & Melnikov, 2015). When unsaturated FA (in cis form), found in vegetable oils, are partially hydrogenated, *trans*-FA are produced. *Trans*-fats are cheaper than normal saturated fats and more suitable for industrial scale baking in terms of thermal behavior and have a longer shelf life than vegetable oils (Lucca & Tepper, 1994).

Significant consumption of *trans*-fat has been associated with increased risk of cardiovascular disease, coronary heart disease, stroke, type-II diabetes, obesity, increased LDL and VLDL cholesterol, and neurodegenerative diseases (Sacks et al., 2017). Saturated fat has been found to raise both LDL and HDL cholesterol (Sacks et al., 2017; DiNicolantonio & O'Keefe, 2018). While HDL cholesterol is typically classified as the "good cholesterol", not all HDL cholesterol is considered good. There are some types of HDL that are antiatherogenic, while others, sometimes called "non-functioning" may do almost nothing. The main purpose of HDL is to carry away bad cholesterol toward excretion and inhibit LDL oxidation (Eren, Yilmaz, & Aydin, 2012). However, the non-functioning HDL cholesterol is practically indifferent towards cholesterol, or can convert HDL cholesterol into LDL cholesterol. Non-functioning HDL is usually found in lower amounts in the body than good HDL cholesterol naturally (Eren, Yilmaz, & Aydin, 2012).

There is a need for low saturated, zero *trans*-fat alternatives for fat-based applications in foods. Consumption of unsaturated FA is seen as more health beneficial

than replacing fat with carbs and proteins. A review of 15 clinical trials, covering 59,000 people, found that there was a 27% drop in heart disease risk when saturated fats were replaced with unsaturated fats (Hooper, Martin, Abdelhamid, & Davey Smith, 2015). The same study found there was no change in heart disease risk when saturated fat was replaced with carbs and proteins. A current commercial SL low *trans*-fat product is NovaLipid (from ADM, Decatur, IL, USA), and is produced through lipase-catalyzed interesterification of soybean oil (high in unsaturated FA) with fully hydrogenated soybean and cottonseed oils. Still, NovaLipid is not *trans*-free and other alternatives that have been produced in literature still contain significant amounts of saturated fat (Kim & Akoh, 2015). This research proposes two options, production of a SL and formation of organogels. Organogels will be discussed in further detail in later sections. SL that have been the most successful alternatives to *trans*-fat are produced through reacting a highly saturated fat and highly unsaturated plant oil in the presence of lipase and heat (Kim & Akoh, 2015). There is need for *trans*-free fats that are low in saturated fats and/or contain extra health benefits such as having MLM-type TAG or omega-3 FA such as EPA and DHA.

Omega-3 FA

Omega-3 FA are PUFA characterized by having a double bond between the third and fourth carbon from the omega or methyl end of the FA chain. Common conventional naming designates “n” based on the position of the first double bond from the methyl terminus. Important omega-3 FA for human physiology include α-linolenic acid (ALA) (C18:3n3), stearidonic acid (SDA) (C18:4n3), EPA (C20:5n3), and DHA (C22:6n3). The

main source of ALA and SDA in the diet are from plant oils. EPA and DHA are found in fish and algae.

ALA and SDA

A common omega-3 FA found in many plants is ALA, which makes up a larger proportion of omega-3 FA consumed through diet versus EPA and DHA (Simopoulos, 2002). ALA is found in significant quantities in plant oils such as flaxseed, walnut, *echium*, canola, soybean, and pecan oils (Surette, Edens, Chilton, & Tramposch, 2004). ALA serves as a substrate for the synthesis of EPA and DHA, both of which are necessary in the body for tissue function. While it is possible for the body to convert ALA to EPA and DHA by elongase and desaturase enzymes, previous studies have found that this process is inefficient (Swanson, Block, & Mousa, 2012; Simopoulos, 2002). It is believed that only about 5-10% of the essential FA ALA can be converted to EPA, and only about 2-5% can additionally be converted to DHA (Swanson et al., 2012). SDA is a metabolic intermediate after the rate limiting step during the conversion of ALA to EPA. By bypassing the rate limiting step, SDA is more efficiently converted to EPA than ALA. However, SDA is not found in high amounts in commonly consumed foods. Seed oils such as *echium*, borage, and blackcurrant contain up to 10% of SDA, and a genetically engineered soybean crop, transgenic SDA soybean, contains 15-30% SDA (Monsanto Co., 2009). Many consumers are currently looking for non-genetically engineered foods and many may not want to consume the transgenic SDA soybean oil. Thus, optimal EPA and DHA levels necessary for human health are best obtained through diet from marine sources.

EPA and DHA

EPA and DHA are found in marine oils such as fish, squid, and krill oils. Salmon, mackerel, herring, sardine, anchovy, trout, cod, and menhaden are all oily fish that contain significant amounts of the omega-3 PUFA EPA and DHA (Ackman, 1967). Fish also provide certain proteins, vitamin D, selenium, and minerals that have potential cardiovascular benefits (Fox et al., 2004; Roos, Wahab, Chamnan, & Thilsted, 2007). The amount of EPA and DHA, as well as total and positional FA compositions in the fish oil, can differ based on fish species, diet, and environmental factors such as water temperature and salinity (Ackman, 1967). Based on the amount and positional distribution of PUFA and other FA, marine oils can be used as a substrate in reactions to synthesize desirable SL such as MLM-type TAG. EPA and DHA have been found to be very beneficial to human health, especially in reducing risk of cardiovascular and neurodegenerative diseases, blood TAG levels, and inflammation (He, 2009). It has also been reported that EPA and DHA are important in cognitive function and fetal development (Swanson et al., 2012). Consuming significant amounts of EPA and DHA may be beneficial for ensuring brain growth and neural development, which is particularly important during the fetal stage (Swanson et al., 2012; Simopoulos, 2002). DHA is also the predominant structural FA in the central nervous system and retina (Swanson et al., 2012). EPA and DHA may also be more biologically active in metabolic processes, particularly cardiovascular processes. Both EPA and DHA can serve as substrates for lipid mediators, can be part of the structural components in lipid bilayers, and may contribute to the prevention of chronic diseases (Swanson et al., 2012; Simopoulos, 2002). The challenge is getting adequate intake of EPA and DHA through the diet. The current Western diet is high in saturated fat, as well

as having a higher proportion of omega-6 FA consumed over omega-3 FA (Simopoulos, 2002). While both omega-3 and omega-6 FA have been found necessary for nutrition, the optimum dose is having a higher ratio of omega-3 to omega-6 for preventing chronic disease and inflammation (Simopoulos, 2002). EPA and DHA are found in significant quantities in many fish and shellfish. Another product that could be added to the diet are marine oils, which are produced from fatty fish and are high in EPA and DHA.

Effects of Omega-3 FA on Cardiovascular Disease (CVD)

Cardiovascular disease (CVD) is the leading cause of death for both men and women in the United States, and 1 in every 4 deaths are caused by heart disease (CDC & NCHS, 2005). There are many risk factors known to increase the risk of heart disease, including high blood pressure, high cholesterol, smoking, diabetes, obesity, poor diet, excessive alcohol use, and physical inactivity (Stewart, 2017). Diet plays a major role in the risk of developing CVD. Poor diet and low amounts of exercise can lead to obesity and developing type-II diabetes (Stewart, 2017). There are a wide range of dietary factors that are known to influence CVD risk, including certain FA, intake of nuts, fruits, vegetables, and legumes, alcohol consumption, micronutrients, and food processing (Hu & Willett, 2002; Mente, de Koning, Shannon, & Anand, 2009). There are more cardiovascular related deaths in the United States due to low intake of EPA and DHA than deaths due to low intake of fruits and vegetables (Simopoulos, 2016). This is likely due to increase in total fat, *trans*, and saturated fat consumption over time, as shown in Figure 2.2. Currently there is also excess consumption of pro-inflammatory omega-6 FA in the Western diet, and excess omega-6 FA consumption has been linked to obesity, atherosclerosis, and diabetes (Let, Jacobsen, & Meyer, 2007).

There are other intermediary risk factors that are influenced by diet. These intermediary risk factors include circulating triacylglycerols, inflammation, endothelial function, coagulation and thrombosis, and arrhythmic risk (Mozaffarian & Wu, 2011). Consumption of omega-3 PUFA has been shown to lower plasma TAG, reduce resting heart rate, reduce both systolic and diastolic blood pressure, and improve endothelial function (Harris & Bulchandani, 2006; Geleijnse, Giltray, Grobbee, Donders, & Kok, 2002; Mozaffarian, Geelen, Brouwer, Gelenijnse, Zock, & Katan, 2005). These health benefits of consuming omega-3 PUFA may be partly responsible for the protective effects of omega-3 PUFA against CVD (Stewart, 2017).

Both the diet and intermediary risk factors affect clinical heart disease outcomes, and may vary depending on the sex, age, insulin sensitivity, genetic variation, and physical activity of the patient (Go et al., 2013). The effects of omega-3 PUFA EPA and DHA intake on CVD has been assessed by evidence from many studies, and many findings indicate that consumption of fish oil significantly reduces the chance of a heart disease related death (Go et al., 2013). A widely recognized physiological effect of omega-3 PUFA consumption is anti-arrhythmia, where even modest consumption of PUFA (~250 mg EPA & DHA) reduced risk of sudden cardiac death by 36% when compared to no consumption over time (Lemaitre, King, Mozaffarian, Kuller, Tracy, & Siscovick, 2003). However, a recent Cochrane review of 79 trials of over 112,000 people found that taking omega-3 capsules containing EPA & DHA may not reduce heart disease, stroke, or death, but did reduce serum TAG and raised HDL (Abdelhamid et al., 2018). Previous studies also indicated that up to 80% of omega-3 supplements are oxidized, potentially explaining why the health benefits were not seen in the Cochrane

review (Abdelhamid et al., 2018; Cameron-Smith, Albert, & Cutfield, 2015; Ismail, Bannenberg, Rice, Schutt, & MacKay, 2016; Wasowicz et al., 2004; Albert, Cameron-Smith, Hofman, & Cutfield, 2013).

Effects of Omega-3 FA on Inflammation

The omega-3 FA, EPA and DHA, have been shown to possess anti-inflammatory properties. One study on 250 patients who experienced neck or back pain, showed that consumption of 1200mg per day of EPA and DHA led to reduced arthritic pain and lower use of prescription nonsteroidal anti-inflammatory drugs (NSAID) medications (Maroon & Bost, 2006). Part of the anti-inflammatory properties of EPA and DHA are due to their use in production of eicosanoids (thromboxanes, leukotrienes, and prostaglandins), which regulate important bodily functions including blood pressure, blood viscosity, vasoconstriction, and immune and inflammatory response (Maroon & Bost, 2006). Omega-3 PUFA have been found to compete with arachidonic acid in the conversion to eicosanoids, preventing formation of pro-inflammatory eicosanoids by serving as an alternative substrate (Endo & Arita, 2016). EPA and DHA are also precursors to the potentially potent anti-inflammatory compounds of resolvins, protectins, and other resolving mediators (Serhan, 2007, 2010).

Molecular Mechanisms of Omega-3 PUFA

In 2004, researchers proposed a biomarker for CVD, known as the omega-3 index, which is the sum of EPA and DHA found in red blood cell membranes expressed as a percentage of total erythrocyte FA (Harris & von Schacky, 2004). The omega-3 index was proposed since it correlated well to the risk of a variety of CVD endpoints, as

well as cholesterol ratio levels. Omega-3 FA influence on various molecular pathways may influence the observed physiological effects of CVD risk factors and endpoints such as heart disease and stroke.

Omega- 3 PUFA have been found to have several important biological effects on a wide range of cellular functions such as cell and organelle membrane structure and function, ion channels and electrophysiology, nuclear receptors, and transcription factors (Jenkins & Josse, 2008). In the body, EPA is primarily found in cholesterol esters, TAG, and phospholipids, while DHA is primarily in phospholipids (Simopoulos, 1991). Incorporation of EPA & DHA into membrane phospholipids altered membrane associated protein function and signaling, which may contribute to potential anti-inflammatory and anti-arrhythmic effects (Ma et al., 2004). Altering the physicochemical properties of the cell membrane due to omega-3 PUFA incorporation was also found to affect membrane fluidity, lipid microdomain formation (rafts and caveolae), and signaling across membranes (Endo & Arita, 2016). Omega-3 PUFA were also found to alter function of membrane ion channels such as the sodium channel, L-type calcium channel, and sodium-calcium exchanger to prevent lethal arrhytmias (Ferrier, Redondo, Zhu, & Murphy, 2002; Xiao, Ke, Chen, Morgan, & Leaf, 2004; Endo & Arita, 2016). EPA and DHA are also natural ligands of many nuclear receptors and transcription factors that regulate gene expression in multiple tissues (Adkins & Kelley, 2010; Jump, 2008). Specifically, EPA and DHA have been found to downregulate inflammation-related genes by inhibition of nuclear factor (NF)- κB signaling by blocking IκB phosphorylation or through nuclear receptor peroxisome proliferator-activated receptor (PPAR)α/γ (Zhao, Joshi-Barve, Barve, & Chen, 2004; Gani & Sylte, 2008). Omega-3

PUFA have also been shown to augment GLUT4 expression to promote glucose uptake in adipocytes, which may play an important part in whole-body glucose homeostasis (Endo & Arita, 2016).

Dietary Guidelines for Omega-3 FA

The American Heart Association (AHA) recommends those with coronary heart disease to consume 1,000 mg of EPA and DHA per day (Dietary Guidelines for Americans, 2010). The World Health Organization recommends consumption of 300-500 mg of EPA and DHA daily for the general population, but the typical Western diet lacks this amount of omega-3 FA (Simopoulos, 2016). It has been found that a higher omega-3 to omega-6 ratio is beneficial at reducing risk of cardiovascular disease, cancer and inflammation (Swanson et al., 2012; Simopoulos, 2002; Dietary Guidelines for Americans, 2010). However, the typical Western diet is much higher in omega-6 FA than omega-3, and excessive amounts of omega-6 FA may promote pathogenesis of many diseases (Simopoulos, 2002).

Adding Omega-3 PUFA into Food Products

Because the Western diet lacks in omega-3 PUFA, it is necessary to incorporate omega-3 FA into more food products that consumers would want to buy. Consumers are interested in the health benefits associated with consumption of products containing omega-3's, such as seafood, supplements, and fortified foods. The global fish market is expected to grow about 5.2% from 2016-2021 and is largely due to the large growth in aquaculture (fish oil is a main ingredient in aqua feed), and desires of human consumers to increase consumption of omega-3 FA and reduce consumption of *trans* and saturated fat

(Newswire, 2016). Current issues concerning the consumption of fish are that they are higher in cost compared to beef or chicken, and consumer preference (Mason, 2000). The same is true for marine oils. Anchovy, mackerel, sardines, cod, herring, and menhaden are the main fish species used for production of fish meal and fish oil (Bibus, 2016). Fish oil supplements are a popular choice for consumers. Over a third of American adults consume fish oil in the form of supplements and fish oil supplements are now the most popular supplement in the United States (Albert et al., 2013). However, often these supplements may be oxidized, which may negate the health benefits (Albert et al., 2013; Ismail et al., 2016; Wasowicz et al., 2004). Consumers also often experience "fishy burps" after consumption of these supplements, often leading to discontinued consumption of the supplements. Table 2.2 shows the percent of consumers in different countries who do not take omega-3 supplements due to the fishy taste. The GOED surveyed 9 countries on consumer acceptance/consumption of EPA/DHA products and found that the main barrier for not consuming omega-3 products was the fishy taste/burp (Ismail et al., 2016). Fortification of food products with marine oils is another option, however there are challenges associated with adding omega-3's to food products. Marine oils, which are high in omega-3 PUFA EPA and DHA are lower in oxidative stability compared to vegetable oils, making incorporation of marine oils in products very challenging. Direct incorporation of omega-3 PUFA into food can compromise quality and consumer acceptability due to development of undesirable odors and flavors present in the oil and oxidation of the oil during food processing/storage (Jacobsen, 2015). Several studies, including this research, have focused on developing strategies to improve omega-3 oxidative stability in food products.

MLM-type SL Containing Omega-3 PUFA

Production of MLM-type SL containing omega-3 PUFA at the *sn*-2 position is of interest in terms of human nutrition. During digestion of an MLM-type SL, *sn*-1,3 specific pancreatic lipase rapidly hydrolyzes the MCFA at the *sn*-1,3 positions, while the long chain FA at the *sn*-2 position in MAG form is absorbed (Salentinig, Yepuri, Hawley, Boyd, Gilbert, & Darwish, 2015). It has been found that DHA has higher bioavailability when present at the *sn*-2 in significant quantity, therefore increasing the health benefits of the MLM-type SL. This is particularly necessary for expectant mothers or those who have or are at risk of developing cardiovascular disease (Bibus, 2016).

There have been studies on the production of omega-3 FA containing MLM-type TAG. Shimada et al. conducted acidolysis reactions using tuna oil, caprylic acid, and immobilized *sn*-1,3 specific *Rhizopus delemar* lipase to produce MLM-type TAG with significant amounts of DHA at the *sn*-2 position (Shimada et al., 1996; Shimada, Sugihara, & Tominaga, 2000). Menhaden oil has also been modified to produce MLM-type SL, however all the studies lack TAG species analysis (Xu, Fomuso, & Akoh, 2000; Jennings & Akoh, 1999; Jennings & Akoh, 2001).

One key component of MLM-type TAG analysis that is necessary is the TAG molecular species, particularly in a sample such as fish oil that contains many different FA and TAG. Regiospecific characteristics of the PUFA in TAG significantly affect the physicochemical and physiological properties of marine oils (Zhang, Shen, Zhang, Li, & Wang, 2018). In production of MLM-type TAG, is important to determine the TAG species to see which TAG the MCFA esterify to, as this may also affect the physicochemical and physiological properties of the SL. The FA composition may reveal

significant amounts of PUFA at the *sn*-2 position and MCFA at the *sn*-1,3 position, however analysis of the TAG species may show that all the MCFA are found in TAG with saturated LCFA at the *sn*-2 position. Certain lipases, such as *Rhizomucor miehei*, may discriminate against PUFA, leading to a lower amount of MLM-type TAG containing PUFA at the *sn*-2 position (Sellappan & Akoh, 2001).

There is need for the TAG molecular species to be determined for SL products, particularly products containing fish oil, as it has a complex mixture of TAG species. There have been a few studies that have determined the TAG molecular species of fish oil, and to the best of our knowledge, only one study that analyzed the TAG species of a SL produced from the substrates of fish oil and hydrogenated soybean oil (Solaesa et al., 2014; Lopez-Hernandez, Torres, Garcia, & Hill, 2004; Perona & Ruiz-Gutierrez, 1999; Zeng, Araujo, Du, Nguyen, Froyland, & Grung, 2010; Zhang et al., 2018). However, the studies show incomplete separation of many TAG species, and with varied amounts of TAG peaks. There is need for a method to identify more TAG species in fish oil, since over 553 TAG species have been identified in separate studies, without including the regioisomers and enantiomers (Zeng et al., 2010). Some studies analyzed the TAG species using a modified equivalent carbon number (ECN) equation to account for MUFA and PUFA (Solaesa et al., 2014). The difficulty with TAG identification is that there are many TAG in fish oil with the same traditional ECN number (Perona & Ruiz-Gutierrez, 1999). The modified equation helps improve peak identity accuracy, yet each peak can still have multiple different TAG (Solaesa et al., 2014). When fish oil is modified and other acyl donors are incorporated, the TAG species significantly alters, so there is need to determine the different TAG formed in these fish oil SL.

Menhaden Oil

The menhaden fish reside primarily in the Atlantic Ocean along the eastern United States and Gulf of Mexico, and are genera *Brevoortia* and *Ethmidium*, though primarily the genera *Brevoortia* (International Union for Conservation of Nature, 2015). Menhaden are silver colored fish with some black spotting behind the gill. During their lifetime, they feed on phytoplankton and zooplankton, which are rich in omega-3 FA. Menhaden are considered oily fish and are harvested primarily for fish oil, fish meal, and bait (International Union for Conservation of Nature, 2015). Fishers use a technique known as seine fishing to capture menhaden fish. Two fishing boats surround a school of menhaden with a large net which captures many menhaden fish. This technique is efficient for catching a lot of menhaden with low levels of bycatch (other fish species or juvenile fish) (International Union for Conservation of Nature, 2015). To produce fish oil, fish come in off boats almost immediately after catching, and enters a processing facility. Here the fish are cooked by steam, pressed, and centrifuged to separate solids from the liquid oil (Pike & Jackson, 2010). The solids are further processed for use as fish meal. Water and other impurities are removed from the fish oil, and antioxidants are added before packaging the oil in containers.

As of 2016, the average yearly production of menhaden oil was 74,700 metric tons (MT) (Bibus, 2016). A recent study found that the United States has enough supply of menhaden oil to meet the nutritional needs of several at-risk demographics and could save up to $1.7 billion in healthcare costs (Bibus, 2016). The major at-risk demographics are those who have or are at risk of developing cardiovascular disease, as well as expectant mothers. The FDA reports adequate consumption of fish by expectant mothers

may increase children's IQ by 5.5 points as well as improve verbal development at an early age. Expectant mothers, lactating mothers, or those who may become pregnant should consume 8-12 oz of fish per week, or 2-3 servings of oily fish per week (Bibus, 2016).

Menhaden oil is a rich and sustainable source of EPA and DHA and is also the best fish source of DPA (Bibus, 2016; NOAA, 2016). Literature indicates that menhaden contains a high omega-3/omega-6 ratio as well as a significant amount of EPA and DHA at the *sn*-2 position (He, 2009; Joseph, 1985). The sample received from Omega Protein Inc. (Reedville, VA) and used in this research has a Certificate of Analysis (COA) showing EPA and DHA content to be 12.77 and 10.27 w/w%, respectively. Menhaden oil also may be a desirable oil substrate for enzymatic modification to produce MLM-type SL because of the high EPA and DHA content that is found at the *sn*-2 position (Joseph, 1985).

Lipid Oxidation

Lipids oxidation occurs when lipids interact with reactive oxygen species and subsequently breakdown into rancid and potentially toxic products (Frankel, Huang, Kanner, & German, 1994). There are three stages in lipid oxidation: initiation, propagation, and termination. The initiation stage occurs when lipids are exposed to irradiation from visible light or catalyzed by lipoxygenases or by metal ions. Lipid radicals, lipid peroxyl radicals, and hydroperoxides (primary lipid oxidation product) are produced during the initiation stage. The propagation step occurs as lipid radicals react with other lipids and hydroperoxides, and in a chain reaction, eventually forming secondary lipid oxidation products such as aldehydes and ketones. Lipid oxidation is

accelerated by high temperature and pressure during initiation. Other factors that affect the rate of lipid oxidation include degree of unsaturation/FA composition and presence of oxygen or impurities such as protein or heavy metals, pigments, phospholipids, FFA, MAG and DAG, oxidized compounds, and antioxidants (Choe & Min, 2006). Because SL are often lower in oxidative stability compared to unmodified oil, there is concern the menhaden oil SL will not be oxidatively stable enough for food and nutraceutical applications (Zou & Akoh, 2013). This research investigates some methods for improving the oxidative stability of the menhaden oil and SL to allow for usage of these health beneficial lipids in a wider range of applications.

Oxidative Stability of SL

After enzymatic modification, SL products are typically purified by short-path distillation to remove excess FFA and/or FA ethyl esters. Short-path distillation is a desirable method for purifying SL because there is short residence time (1-10s), and lower temperatures (due to the system being under vacuum) (Lutisan, & Cvengros, 1995; Lutisan, Cvengros, & Micov, 2002). Short-path distillation separates compounds based on volatility, and samples are split into two fractions, residue (heavy fraction), and distillate/waste (light fraction) (Zou & Akoh, 2013). Vitamin E derivatives (tocopherols and tocotrienols) are naturally occurring minor components in many vegetable oils that have known antioxidant ability (Zou & Akoh, 2013). It has been found that short-path distillation also removes endogenous antioxidants, such as Vitamin E derivatives, and reduces its oxidative stability (Zou & Akoh, 2013). There is need for adding back some of the antioxidants lost after short-path distillation or use of another method to improve the oxidative stability of the purified SL.

Omega-3 PUFA Oxidation

Unsaturated FA are more susceptible to lipid oxidation than saturated FA due to the presence of one or more double bonds, and susceptibility to oxidation increases with increasing number of double bonds. Fish oil contains a significant amount of PUFA such as EPA and DHA and is highly susceptible to lipid oxidation. Processing of fish oil typical requires steps involving exposure to heat, light, and oxygen, so caution is necessary when processing these marine oils for commercial use (Pike & Jackson, 2010). Ways to slow oxidation during omega-3 oil processing include the addition of antioxidants, refining oils under vacuum, and flushing storage containers with nitrogen to limit oxygen and light exposure (Ismail et al., 2016). Other options include formation of organogels or microencapsulating the lipid, and these are the two methods proposed in this research to improve oxidative stability of the menhaden oil and SL.

Many omega-3 products on the market may contain antioxidants such as natural tocopherols or TBHQ to help improve the oxidative stability of the oil. Antioxidants can be classified as either primary or secondary. Primary antioxidants can delay initiation or stop the propagation step (Choe & Min, 2006). Secondary antioxidants slow lipid oxidation through mechanisms such as oxygen scavenging, metal chelation, and working synergistically with primary antioxidants by providing hydrogen (Choe & Min, 2006). The Global Organization for EPA and DHA omega-3s (GOED) has voluntarily set lower limits for omega-3 oxidation than when comparing with other edible oils (Ismail et al., 2016). 19 commercially available brands of fish oils using antioxidants were found to be stable and having minimal changes to oxidation when studied over 22 days at room temperature (Ismail et al., 2016).

A variety of oxidative products are formed during the oxidation of fish oil, such as peroxides, alcohols, ketones, and aldehydes. Peroxidation products such as prostaglandin-like F3-isoprostanes and F4-neuroprostanes are formed from EPA and DHA, respectively. Figure 2.3 shows the oxidative metabolism of EPA and arachidonic acid (another PUFA). Because of the instability of peroxides, they further degrade to form secondary oxidation products such as the aldehydes 4-hydroxy-2-hexenal (4-HHE) and malonaldehyde (MDA) (Albert et al., 2013). Specifically, for highly unsaturated FA, oxidation products include 4-HHE, 4-hydroxy-2-nonenal (4-HNE), a wide variety of isoprostanes, and many others. A study found that 4-HHE, an oxidized product from fish oil may protect vascular function. Isoprostanes, MDA, and 4-HNE are potentially atherogenic and genotoxic compounds in animals, however not likely toxic to humans (Ismail et al., 2016). 35 human studies on isoprostane levels were found to have no effect or reduced markers, and a couple found increases in isoprostane levels when consuming large quantities or when the body was put under stress. 20 human clinic studies found that there was no effect on MDA or 4-HNE levels when EPA/DHA oils were consumed (Albert et al., 2013).

Consumption of Oxidized Omega-3 Oil

Marine oils have beneficial effects on plasma triacylglycerols, blood pressure, and inflammation. So, oxidation of marine oils may reduce these beneficial health effects. However, much of the health effects are still under current debate. Potential harm from these oxidation products is relative to concentration, type of oxidation product, and the antioxidant defenses in the body (Ismail et al., 2016). There have only been a few studies on the health effects of oxidized omega-3 oils. In 2012, GOED did two studies on

oxidized EPA and DHA oils and found they are not biomarkers for disease or reduction in health when looking at effects on specific lipid oxidation or oxidative stress parameters (Albert et al., 2013). The human clinical trials conducted with oxidized EPA and DHA found no negative health effects when looking at oxidative stress that may be seen by several markers (4-HHE, 4-HNE, 8-iso-$PGF_2\alpha$, alpha-tocopherol, total GP_x, CAT, C-Reactive-Protein, slCAM-2, sVCAM-1, IL-6, and oxidized LDL cholesterol) in the blood and urine (Ismail et al., 2016). Another study found oxidized EPA inhibited the inflammatory NF-κB pathway and affected vascular and platelet function, which may be mediated by n-3 isoprostanes (Albert et al., 2013). Consumption of oxidized oil may also lead to membrane peroxidation (decrease in membrane fluidity, transport, and cell signaling), production of proinflammatory cytokines, and cell damage, which are all known to be mechanisms for disease. Looking at the nutritional and sensory implications of lipid oxidation, it is possible that the oxidized oil is so unpleasant that it is unwanted for consumption, and thus avoiding the potential negative effects of consuming highly oxidized oils that have significantly lower nutritive value or potential toxicity (Wasowicz et al., 2004). More studies on these oxidized oils and their potential health effects are necessary.

Measuring Lipid Oxidation in Fish Oil

Measuring lipid oxidation of omega-3 oils is complex due to the differences in their physicochemical characteristics. Not all methods for determining quality and lipid oxidation are suitable for all types of oils. The two typical tests for measuring omega-3 oil oxidation (and oxidation of other oils) are peroxide value (PV) and *p*-Anisidine value (*p*-AV). The PV measures hydroperoxides, a primary oxidation product, and is an

iodometric titration-based method (Halvorsen & Blomhoff, 2011). The *p*-AV measures amount of aldehydes (secondary oxidation products) present, and is a spectrophotometric method that produces a yellow color in the presence of aldehydes, and the color intensity depends on the structure of the aldehydes. The conjugated dienes test is another useful test that correlates well to PV to measure lipid oxidation as it is proportional to the uptake of oxygen and hydroperoxide formation. The benefit of the conjugated dienes method is that it is quicker and does not depend on chemical reactions or color development, however oils containing carotenoids are not suitable for this test (Wasowicz et al., 2004). TOTOX is another way for measuring lipid oxidation as it is an equation based on PV and *p*-AV with the goal of giving an overall picture of both the primary and secondary lipid oxidation products (Halvorsen & Blomhoff, 2011). The PV and *p*-AV are cheaper and simpler than other methods such as GC-MS or other chromatographic techniques that measure specific lipid peroxides or secondary oxidation products.

For flavored oils or oils such as krill or virgin salmon oils that contain natural colors, the *p*-AV is not a valid method as they interfere with the *p*-AV and give invalid data. The *p*-AV test is also not valid for phospholipid sources and oils that contain aldehyde type flavors (as *p*-AV measures the presence of aldehydes) (Ismail et al., 2016). Other methods for measuring lipid oxidation include: HPLC, ESR to measure free radicals, thiobarbituric acid reactive substances (TBARS), dynamic headspace sampling/solid phase microextraction (DHS/SPME), GC-MS, and potential novel ways for measuring lipid oxidation such as chemometry and electronical sensing. Methods for assessing lipid stability include the Schaal oven test, oxygen uptake, light or metal catalysts, Rancimat, and Oxidative Stability Instrument (OSI) (Wasowicz et al., 2004).

Each method has both advantages and drawbacks, so it is important to be aware of the type of oil being tested and the limitations of each test.

Volatile Lipid Oxidation Products of Fish Oil

Peroxides decompose to secondary oxidation products such as aldehydes and other volatiles that produce off-flavors (Wada et al., 2007). Volatile oxidation products in fish oils include short and medium chain saturated, mono and polyunsaturated aldehydes, ketones, and alcohols, and some of those products are shown in Table 2.3 and Figure 2.4. There are many ways to analyze/measure the volatile products. GC-MS is a common instrument used for analysis, often coupled with SPME and dynamic headspace sampling (Jacobsen, 2015; Let et al., 2007). Other methods for measuring volatile oxidation products include HPLC and C-13 NMR (Frankel, 1987). GC chromatographic analysis shows presence and concentrations of aldehydes, ketones, alcohols, furanones, and lactones. The volatiles that have been found to contribute to fishy off-flavors of oxidized fish oil include (but are not limited to): 2-ethylfuran, 1-penten-3-one, pentanal, 2-*E*-pentenal, hexanal, heptanal, 2-pentylfuran, 2-*E*-heptenal, and 2,4-heptadienal (Jacobsen & Timm, 2001).

Lean Six Sigma

Recently there has been implementation of Lean Six Sigma and experimental design in both research and industry applications (Dora & Gellynck, 2015). Lean Six Sigma is a combination of lean manufacturing and Six Sigma. The goal behind Six Sigma is to improve processing through variation elimination while the goal behind lean manufacturing is waste elimination, and the combination of both allows for identification

of the most critical processes to save costs (Dora & Gellynck, 2015). Many optimization projects include full factorial experiments with multiple variables at multiple levels that are both costly and time consuming to complete (Chandrasekar, 2014). Experimental design methods such as the Taguchi Method are a great option for reducing time and costs for a factorial type optimization experiment (Dora & Gellynck, 2015).

Taguchi Method

The Taguchi method is a type of robust parameter fractional factorial experimental design that allows for reducing the number of experiments needed to optimize a process (Savaghebi, Safari, Rezaei, Ashtari, & Farmani, 2012). The Taguchi method is part of Lean Six Sigma and has been utilized mostly in the engineering field to reduce costs and wastes associated with production and operations (Shahavi, Hosseini, Jahanshahi, & Najafpour, 2016). Few applications have been done outside the engineering field, so it is of interest to see if the Taguchi method is applicable to fields such as the food industry (Farbodi, 2017). The Taguchi Method aims at using parameter design with orthogonal arrays, ANOVA, and signal/noise (S/N) ratio to reduce the number of experiments but also provide a balanced effect of all factors on each experiment (Chandrasekar, 2024). Orthogonal arrays allow for many variables to be studied while conducting a small number of experiments (Farbodi, 2017). An orthogonal array is set-up, so each column of independent variables is orthogonal, meaning each parameter is represented in an equal amount (Savaghebi et al., 2012; Shahavi et al., 2016; Farbodi, 2017).

For example, to achieve the highest incorporation of a substrate in an enzymatic reaction, the reaction conditions of substrate ratio, enzyme load, temperature and time need to be optimized. A full factorial experiment would be time consuming and costly, so experimental

design is one way to determine optimal processing conditions in less time and cost (Dora & Gellynck, 2015). Many studies have used response surface methodology (RSM) to optimize processes, however there are disadvantages to use of this experimental design. The factors in RSM must be continuous while in the Taguchi method they can be continuous or discrete (Shahavi et al., 2016). RSM has also been viewed as an outdated/overused method that may not provide accurate results in optimization studies (Dora & Gellynck, 2015). While RSM is easy to apply, the analysis is often difficult. The Taguchi method allows for simpler analysis, requires less experiments, and is easy to apply to many different processes, such as optimization of an enzymatic reaction (Savaghebi et al., 2012).

Organogels

Fats serve an important function in many foods, particularly in confections, baked goods, dairy products, and margarines/spreads. However, fats contain significant amounts of saturated FA. High consumption of saturated fat has been associated with numerous health risks such as raising LDL (or bad) cholesterol and increase risk for CVD (Sacks et al., 2017; DiNicolantonio & O'Keefe, 2018). Replacing saturated fat with monounsaturated FA (MUFA) or PUFA minimizes the negative impact on health (Sacks et al., 2017). However, due to its unique fat crystal network, it is difficult to find alternatives to highly saturated fats (Rogers, 2009). Concerns for replacing saturated fats include finding alternatives with similar physicochemical properties, oxidative stability, ease of availability, cost, and no adverse health effects.

Organogels, also known as oleogels, are lipid gels that could potentially be used as an alternative to highly saturated fats (Co & Marangoni, 2012). Organogels are a relatively recent discovery in the past decade and have a wide range of applications (Co & Marangoni, 2012;

Lu, Cao, Ho, & Huang, 2016; Dassanayake, Kodali, Ueno, & Sato, 2013; Sagiri, Sethy, Pal, Banerjee, & Maiti, 2012; Hughes, Marangoni, Wright, Rogers, & Rush, 2009; Patel et al., 2014; Banerjee & Bhattacharya, 2012; Almdal, Dyre, Hyidt, & Kramer; Wijarnprecha, Aryusuk, Santiwattana, Sonwai, & Rousseau, 2018; Patel & Dewettinck, 2015). Studies have shown these organogels (or oleogels for edible purposes) may inhibit oil migration in chocolate, improve oxidative stability, be acceptable low saturated fat alternatives to shortening/margarines, and control the release of sensitive compounds such as antioxidants, bioactive compounds, and essential PUFA that are susceptible to oxidation (Co & Marangoni, 2012). Because this research is relatively new, the formation of organogels, the types of gelators, gel mechanism, and functionality of these gels are of great interest.

Organogelation

The definition of a gel has changed multiple times as new research has progressed. The most widely accepted definition of a gel is: a material that "has a continuous structure with macroscopic dimensions that is permanent on the time scale of an analytical experiment and is solid-like in its rheological properties" (Flory, 1974). Gels are can be classified into four general categories: well-ordered lamellar structures such as lyotropic phases, covalently linked polymer networks, entangled polymer networks held together by transient physical interactions, and disordered particulate structures (Flory, 1974). Gels can be also be classified based on the material being gelled. Gels can either be hydrogels, where water is gelled, or an organogel, where a hydrophobic liquid (such as crude or vegetable oil) is gelled. Examples of hydrogels include gelatin, gummy bears (starch gel), cheese curds (casein proteins), and pimentos in olives (alginate gel) (Co & Marangoni, 2012). Examples of organogels include automotive grease and art conservation cleaners (Co & Marangoni, 2012). Organogels can also be further

classified into two categories: polymeric organogels and low-molecular weight organogels. Most organogels that have been studied in literature are of the low-molecular weight type organogel and are often crystalline dispersions of two phases: dispersed structurant (organogelator) and solvent being structured (oil).

Traditionally, most gels are polymeric in nature, however most organogels self-assemble into a gel structure by non-covalent interactions (Co & Marangoni, 2012). Since there are no covalent bonds formed, the gels are thermoreversible and temperature dependent. Organogel formation is also dependent upon the type of solvent that is going to be gelled (Co & Marangoni, 2012). There needs to be a balance between the solubility and insolubility of the gelator when it is in solvent (Banerjee & Bhattacharya, 2012; Almdal et al., 1993). The insolubility of the gelator allows for it to crystallize and self-assemble into the 3D gel structure network while the solubility of the gelator in the solvent allows for the gelator to interact with the solvent molecules (Banerjee & Bhattacharya, 2012; Almdal et al., 1993). Too much insolubility results in a precipitate while too much solubility results in a solution, that is, not a gel. Finding gelators that have the right balance between solubility and insolubility can be difficult, however there has been some success in finding these gelators.

Organogelators

There are many known organogelators that have a wide range of physiochemical properties (Co & Marangoni, 2012; Lu et al., 2016; Dassanayake et al., 2013; Sagiri et al., 2012; Hughes et al., 2009; Patel et al., 2014; Banerjee & Bhattacharya, 2012; Almdal et al., 2015). Organogels are typically formed using low molecular weight molecules for gelation, however some polymers and proteins are also used to form organogels (Co & Marangoni, 2012). The best gelators have been found to form gels at low concentrations and form networks

that will have similar physiochemical properties to highly saturated fats (Rogers, 2009). Organogelators that have been studied in the literature include: *n*-alkanes, waxes (candelilla, rice bran, carnauba), FA and fatty alcohols, hydroxylated FA (12-hydroxystearic acid and ricinelaidic acid), MAG, phytosterols, ceramides, sorbitan esters, lecithins, polymers, and proteins (Co & Marangoni, 2012). The physicochemical properties of the organogels differ based on the type of organogelator. While many organogelators have been reported in the literature, there is potential for other organogelators to be studied.

While many are used successfully in non-food industries, there are many requirements that the organogelators must meet to be an ideal fat structurant for food. The most important is that the gelator needs to be food-grade. Many organogelators that have been discovered are toxic or not suitable for food applications (Co & Marangoni, 2012; Rogers, 2009). The gelator should be economical and efficient at producing a gel, meaning it is inexpensive, readily available, and can produce gels at relatively small concentrations (< 20% w/w) (Banerjee & Bhattacharya, 2012; Almdal et al., 1993). Also, the gelator needs to meet the desired physical properties of the fat that it is replacing at similar temperatures as well as a similar melting point and melting profile (Rogers, 2009). Finally, the organogelators must be versatile, meaning that it can be used in a wide range of different applications.

Based upon the requirements necessary for a successful organogelator, only a few meets these requirements (Co & Marangoni, 2012). Ceramides and phytosterols are two gelators that show the most promise, however they are somewhat expensive due to the steps needed to isolate or produce these compounds (Rogers, 2009). Lecithin, plant waxes, and MAG are relatively cheaper than ceramides or phytosterols, though some are indigestible.

There are also many steps required to produce the phytosterols (Rogers, 2009). This proposal introduces the possibility of another blend that will form an organogel, a blend of sucrose stearate and ascorbyl palmitate (SSAP). This blend is significantly cheaper than the phytosterol blend and is food-grade. Sucrose stearate is well-known for its emulsification properties and is a sucrose FA ester of stearic acid and sucrose (Lu et al., 2016). Sucrose stearate is a versatile emulsifier, in that it can have different hydrophile-lipophile balance (HLB) values based on the number of stearic acids that are esterified to the free hydroxyl groups of sucrose. A lower HLB value corresponds to more stearic acid that is esterified, and a more lipophilic molecule. Ascorbyl palmitate is the fat-soluble counterpart to ascorbic acid (Vitamin C). Ascorbyl palmitate is known for its antioxidant capabilities and is a FA ester of ascorbic acid and palmitic acid (Cort, 1974). Both sucrose stearate and ascorbyl palmitate contain long chain FA tails that are esterified to cyclic polar compounds. There may likely be intermolecular interactions between the long hydrocarbon chains, polar functional groups, and oil phase (Dassanayake et al., 2012). It is of interest to investigate the physicochemical properties of this type of organogel since literature has shown that several different structures may form based on the organogelator(s) and the oil phase (Co & Marangoni).

Microencapsulation

Another method that may improve the oxidative stability of menhaden oil, omega-3 PUFA, and SL is microencapsulation. While addition of antioxidants to the bulk fish oil is sufficient in improving oxidative stability, microencapsulation also masks some of the undesirable fishy off flavors and odors associated with fish oil (Encina et al., 2016). Microencapsulation is the process of coating tiny particles/droplets to form small (micro-

sized) capsules. The core material, or internal phase is typically an oil in food applications, however the internal phase can be solid, liquid, or gas (Bakry et al., 2016). When these microcapsules are utilized, the internal phase gradually diffuses through the coating and releases outside the microcapsule. This gradual release allows for controlled release of the internal phase which is beneficial in many applications such as in drug delivery or controlling release of bioactive or health beneficial compounds (Bakry et al., 2016; Sagiri et al., 2014; Sagiri et al., 2012). The wall coating is typically made up of carbohydrates (glucose syrup, maltodextrin, n-OSA starch, pectin, chitosan, lecithin, methylcellulose, trehalose, lactose, or corn syrup) or proteins (whey protein, caseins) in food applications. (Encina et al., 2016; Bakry et al., 2016). These coatings offer a barrier that protects the internal phase from outside exposure. When lipids such as fish oil (that are highly susceptible to lipid oxidation) are microencapsulated, their stability and shelf life increase due to the decrease in exposure to factors that cause lipid oxidation such as oxygen, light, heat, and moisture (Bakry et al., 2016).

Fish Oil Microencapsulation Methods

Wall composition, type of internal phase, and method/technique for encapsulation determine the physicochemical properties and potential applications. Common methods for microencapsulation include: emulsification, spray-drying, biopolymer gelation, freeze-drying, complex coacervation, *in situ* polymerization, extrusion, fluidized bed coating, and supercritical fluid technology (Bakry et al., 2016; Drusch & Mannino, 2009; Gouin, 2004). Many methods have been found to protect fish oil from oxidative deterioration, improve shelf life, and mask fishy off flavors (Bakry et al., 2016; Sagiri et al., 2014; Sagiri et al., 2012). Encapsulation of fish oil by spray drying is the most used

method (Encina et al., 2016). Spray drying turns the encapsulation solution into a dry powder when the solution is atomized in the form of small droplets and comes in contact with the drying medium (air or nitrogen) (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007).

Many studies have found that microencapsulated fish oil can be used to fortify food products to increase the nutrition of the food products due to the addition of health beneficial PUFA (Bakry et al., 2016). This study focuses on the microencapsulation of menhaden fish oil and SL containing menhaden oil using a double emulsion technique, as shown in Figure 2.5. In this method, an o/w emulsion is first formed with the lipid (oil) and water (containing encapsulating agent and divalent cation). Then a double emulsion (o/w/o) is formed by addition of more oil. Acidified oil is then added to induce ionic crosslinking between the encapsulating agent (such as sodium alginate) and the divalent cation (such as calcium) (Sagiri et al., 2014). Microcapsules can then be washed and isolated. Formation of a stable emulsion is the key step in this method and other methods such as spray drying. It influences the physicochemical properties of the final microcapsules (Encina et al., 2016).

Problems with Microencapsulation

There are a few downsides to microencapsulation. One consequence of microencapsulation is the stability of the microcapsules over time. Leaching of the internal phase out of the microcapsule is a big concern as this reduces encapsulation efficiency (Sagiri et al., 2012). This research proposes to microencapsulate organogels to improve stability of the microcapsules. One study encapsulated sorbitan monostearate organogels in alginate microparticles and showed improved microcapsule stability (Sagiri

et al., 2014). It is of interest to determine if other organogelator blends can improve microcapsule stability when encapsulated.

Another consequence of microencapsulation is that some have reported lower stability of the microencapsulated fish oil than the bulk fish oil (Encina et al., 2016). The oxidative stability of the microencapsulated fish oil largely depended on storage conditions (light, temperature, oxygen) and microparticle components (coating, oil, and method type) (Bakry et al., 2016). Organogels have been shown to improve oxidative stability of the lipid, so encapsulation of organogels may allow for higher oxidative stability of the microcapsules. Previous literature that encapsulated organogels did not study the oxidative stability of the microcapsules, as these studies were focused on pharmaceutical applications such as drug loading (Sagiri et al., 2012, 2014). For food applications, it is necessary to study the oxidative stability of the microcapsules, particularly when encapsulating lipids such as fish oil that are highly susceptible to oxidation.

Function of Fat in Yellow Cake

Solid fats influence both taste and texture of cakes. Solid fats also contribute to the perception of mouthfeel, aroma/odor, creaminess, appearance, palatability, lubricity of foods, and overall feeling of satiety during meals (Lucca & Tepper, 1994; Jennings & Akoh, 2010; Psimouli & Oreopoulou, 2013). This is primarily due to the physicochemical properties of solid fats. Solid fats form a colloidal network primarily composed of saturated FA that physically traps oil within the structure (Lee & Akoh, 2008). The solid fat in cake is usually shortening, which functions to incorporate air into the batter during mixing, shortens the gluten network, helps leaven the product, tenderizes the crumb, produces a homogenous crumb, and enhances

mouthfeel (Psimouli & Oreopoulou, 2013; Lee & Akoh, 2008; Khalil, 1998). Without shortening/solid fat in the cake formulation, the volume of the cake decreases, the crumb is less tender, and there is a non-uniform cell structure (Matthews & Dawson, 1966). Even when oil is used in place of solid fat, cakes were found to be harder, compact (less air incorporation) and have uneven cell structure (Matthews & Dawson, 1966; Patel et al., 2014).

While shortening helps to provide the desired taste and texture of cake, it is composed of significant amounts of saturated and *trans*-fat. It has been found that consuming significant amounts of saturated and *trans*-fat may increase LDL cholesterol levels, risk of cardiovascular disease, stroke, inflammation, and other chronic diseases (He, 2009). The current dietary guidelines recommend limiting total fat intake to 20-35% of calories, and saturated fats to <10% of total energy intake (HHS & USDA, 2015). So, there is need for producing low saturated, zero-*trans*-fat alternatives to shortening without affecting cake quality.

Oxidation of Lipids in a Cake System

One of the contributing factors for determining shelf-life of a cake is lipid oxidation during batter preparation, baking, and storage. Type of fat plays an important part in determining the shelf-life of cake, as use of highly unsaturated lipids in cake lowers the shelf-life (Matthews & Dawson, 1966). There have been many studies on addition of antioxidants to help slow lipid oxidation in cakes, and these antioxidants may offer other health benefits (Park, Lim, & Hwang, 2012). For example, one study looked at use of turmeric powder (contains biologically active curcuminoids) in yellow cakes as an antioxidant with other health benefits such as anti-inflammatory properties (Park et al., 2012). Preparation of the batter also affects lipid oxidation. One study investigated effects of batter beating and baking on the oxidation of PUFA contained in formulas by

measuring primary oxidation products (conjugated dienes) and secondary oxidation markers (volatile lipid oxidation products) (Maire, Rega, Cuvelier, Soto, & Giampaoli, 2013). It was found that in sponge cake, lipid oxidation occurred during the batter preparation stage, and only minor oxidation during the baking process (Maire et al., 2013). So, there is need for ways to help slow lipid oxidation during cake making, particularly if it is desired for the formulation to contain unsaturated FA.

Fat Replacers for use in Yellow Cake

There have been several studies that reported adequate alternatives to shortening for use in baked goods (Lucca & Tepper, 1994; Patel et al., 2014; Stankov, Baeva, Goranova, & Marudova, 2016; Banerjee & Bhattacharya, 2012; Rogers, 2009; Patel & Dewettinck, 2015; Jennings & Akoh, 2010; Psimouli & Oreopoulou, 2013; Lee & Akoh, 2008; Khalil, 1998). The goal behind fat replacers is to have a low saturated, zero *trans*-fat product that mimics characteristics of highly saturated fats in terms of their physicochemical properties (Lucca & Tepper, 1994; Rogers, 2009). These fat replacers may have the potential to replace highly saturated fats in a wide range of food products such as margarines/spreads, baked goods, and confections. These fat replacers are typically placed into two categories, fat mimetics and fat substitutes (Rogers, 2009). Fat mimetics are usually protein or carbohydrate based and have very different chemical structures than fat. Fat substitutes typically resemble TAG physically and chemically and can be produced either by chemical or enzymatic modification, or physical blending.

Some fat substitutes that have been produced for use in baked goods include SL. An MLM-type SL of canola oil and caprylic acid has been substituted for PHO shortening in cookies, up to 75% successfully (Agyare, Addo, Xiong, & Akoh, 2005).

Another example is a low-calorie SL of sunflower oil and ethyl behenate that was successfully substituted for a commercial bakery fat in biscuits, though it needed addition of an emulsifier to have similar characteristics (Kanjilal et al., 2016). Physical blending is another alternative, though the TAG molecular species is typically different than when it is enzymatically modified (Ifeduba, Martini, & Akoh, 2016). These fat substitutes can successfully replace highly saturated fats because they have desirable physicochemical properties, such as certain thermal behavior, FA/TAG composition, SFC, and polymorphism.

A potential option that has seen recent interest is non-TAG structuring of oils, including the formation of organogels, which has been discussed in detail above. There have been some studies on use of organogels in the production of cake. For example, Patel and others (2014), produced sunflower organogels with the organogelators as methylcellulose and xanthan gum. It was found that the organogel batters had rheological properties that matched margarine and shortening batters versus the batters produced with sunflower oil. The organogel cakes also showed similar hardness to the margarine cake during storage, while the sunflower oil cake had a much higher hardness (Patel et al., 2014). These results show that organogels can be suitable alternatives to shortening and margarine in baked good applications such as cake. More research is necessary on the suitability of organogels in baked goods (and other food products) when formed with different lipid phase(s) and organogelator(s).

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Tables

Table 2.1 Summary of the MLM-type TAG produced in recent years (2014-2019)

Study	Reaction Scheme	Reaction Type	Enzyme*	Reactor Type
Bassan et al., 2019	Grapeseed Oil + C8:0 or C10:0	Acidolysis	TL IM, RM IM, N 435	Jacketed Cylindrical Glass Reactor
He, et al., 2018	*Isochrysis galbana* (microalgae) + C8:0	2-step, Ethanolysis then Acidolysis	TL IM	250-mL Stirred Tank Reactor
Balieiro et al., 2018	Olive Oil + C8:0 or C10:0	Acidolysis	*Rhizopus oryzae*	Cylindrical Glass Vessel
Sousa, Campos, Nunes, & Pires-Cabral, 2018	Pumpkin Seed Oil + C10:0	Acidolysis	TL IM	Cylindrical Glass Vessel
Sitompul et al., 2018	Canarium Oil + C8:0	2-step, Ethanolysis then Acidolysis	RM IM	Orbital Shaker
Willett & Akoh, 2018	Menhaden Oil + C8:0 (FFA or Ethyl Ester)	Acidolysis and Interesterification	L 435 and RM IM	Jacketed Cylindrical Glass Reactor
Yuksel & Yesilcubuk, 2018	*Echium* FFA + Tricaprylin	Acidolysis	RM IM	Orbital Shaking Air-Bath
Costa et al., 2017	Grapeseed Oil + C8:0 or C10:0	Acidolysis	*Rhizopus oryzae* and *Carica papaya*	20 mL Cylindrical Glass Reactor
Sanchez, Tonetto, & Ferreira, 2017	Dicaprin + C16:0	Acidolysis	*Pseudomonas fluorescens*, *Burkholderia cepacian*, RM IM, and N 435	10-mL Vial
Todorova et al., 2015	Walnut Oil + C8:0	Acidolysis	RM IM and *Rhizopus delemar*	Screw-Capped Vial
Sousa et al., 2014	Fish Oil + C8:0	Acidolysis	TL IM	Cylindrical Glass Vessel
Caballero et al., 2014	Avocado Oil + C8:0	Acidolysis	TL IM	Cylindrical Glass Vessel
Ifeduba & Akoh, 2014	High-Stearidonic Soybean Oil + C8:0	Acidolysis	*Rhizomucor miehei*	Jacketed Cylindrical Glass Reactor
Qin et al., 2014	Soybean Oil + C8:0	Acidolysis	*Geobacillus* sp. T1 (free)	Batch (undefined)
Silroy et al., 2014	Mustard Oil + C10:0	Acidolysis	TL IM	Batch (undefined)

*N 435: Novozyme® 435 (from *Candida antarctica*), RM IM: Lipozyme® RM IM (from *Rhizomucor miehei*), TL IM: Lipozyme® TL IM (from *Thermomyces lanuginosus*), L 435: Lipozyme® 435 (recombinant lipase from *Candida antarctica*)

Table 2.2 Percent of consumers who do not take omega-3 supplements due to the fishy taste (Ismail et al., 2016)

	Australia	Brazil	China	France	UK	USA	Russia	Germany	Japan
% of Omega-3 Non-Users	23.2	14.1	15.5	12.9	18.8	11.5	14.7	20.3	6.5
% of Total Population	10.6	4.4	6.0	6.6	8.5	1.8	3.4	7.5	4.6

Table 2.3 Some of the volatile oxidation products of fish oil (Jacobsen, 2015)

Saturated Aldehydes	Mono-unsaturated Aldehydes	Polyunsaturated Aldehydes	Ketones	Alcohols
Butanal	2-Butenal	2,4-Hexadienal	1-Penten-3-one	1-Penten-3-ol
Pentanal	2-Pentenal	2,4-Heptadienal	3,5-Octadione	2-Penten-1-ol
Hexanal	2-Hexenal	2,4-Octadienal		
Heptanal	4-Heptenal	2,6-Nonadienal		
Octanal	2-Heptenal	2,4-Decadienal		
Nonanal	2-Octenal			
Decanal	2-Decenal			

Figures

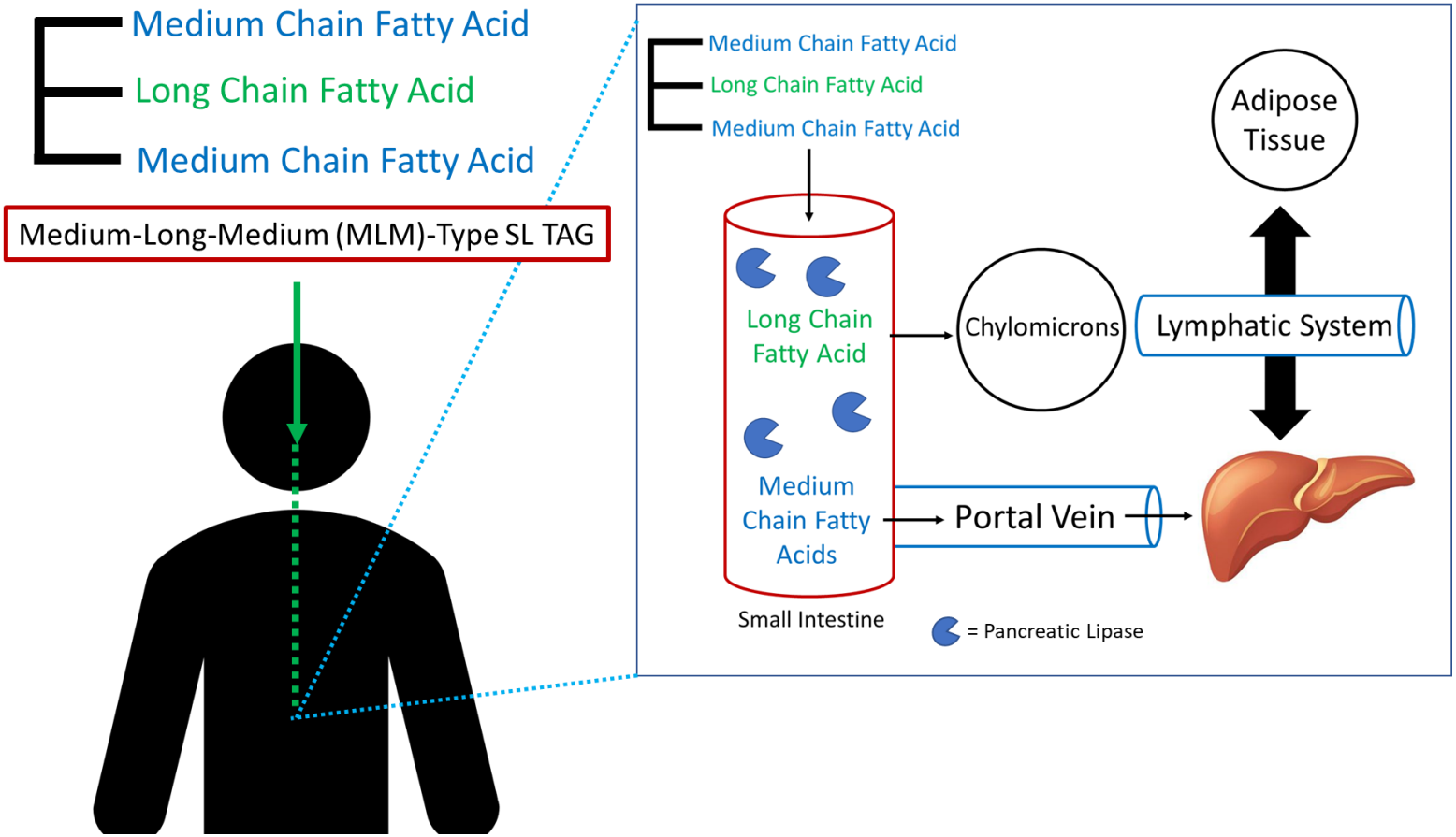

Figure 2.1 Schematic of MLM-type TAG digestion

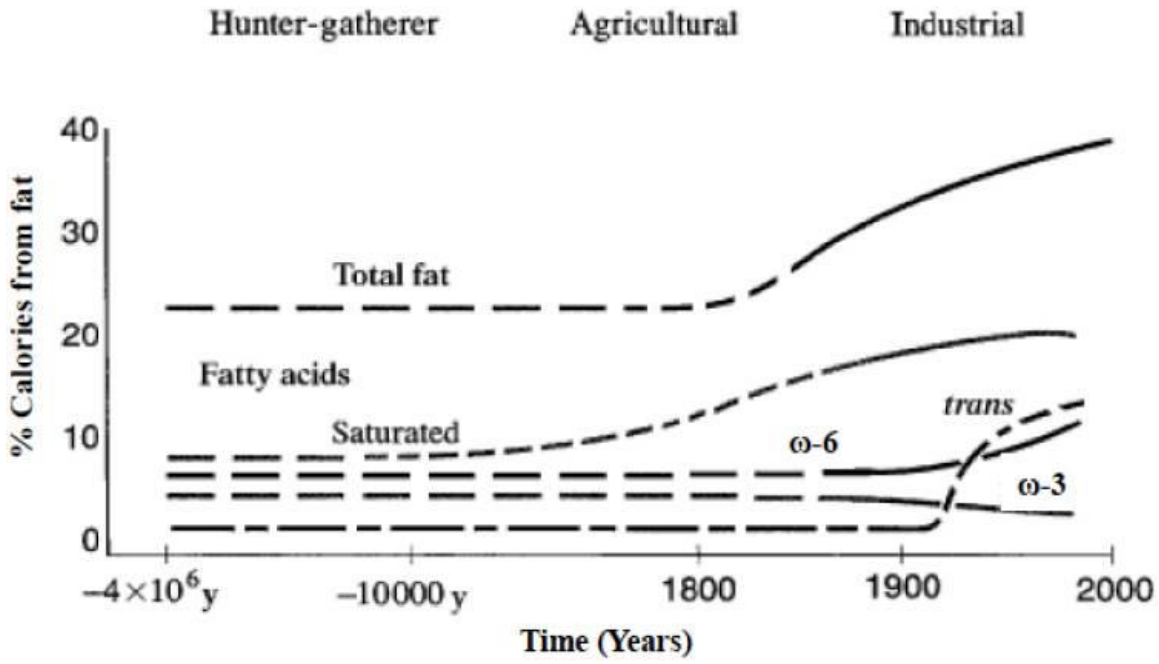

Figure 2.2 Fat calorie % intake over time (Simopoulos, 2016)

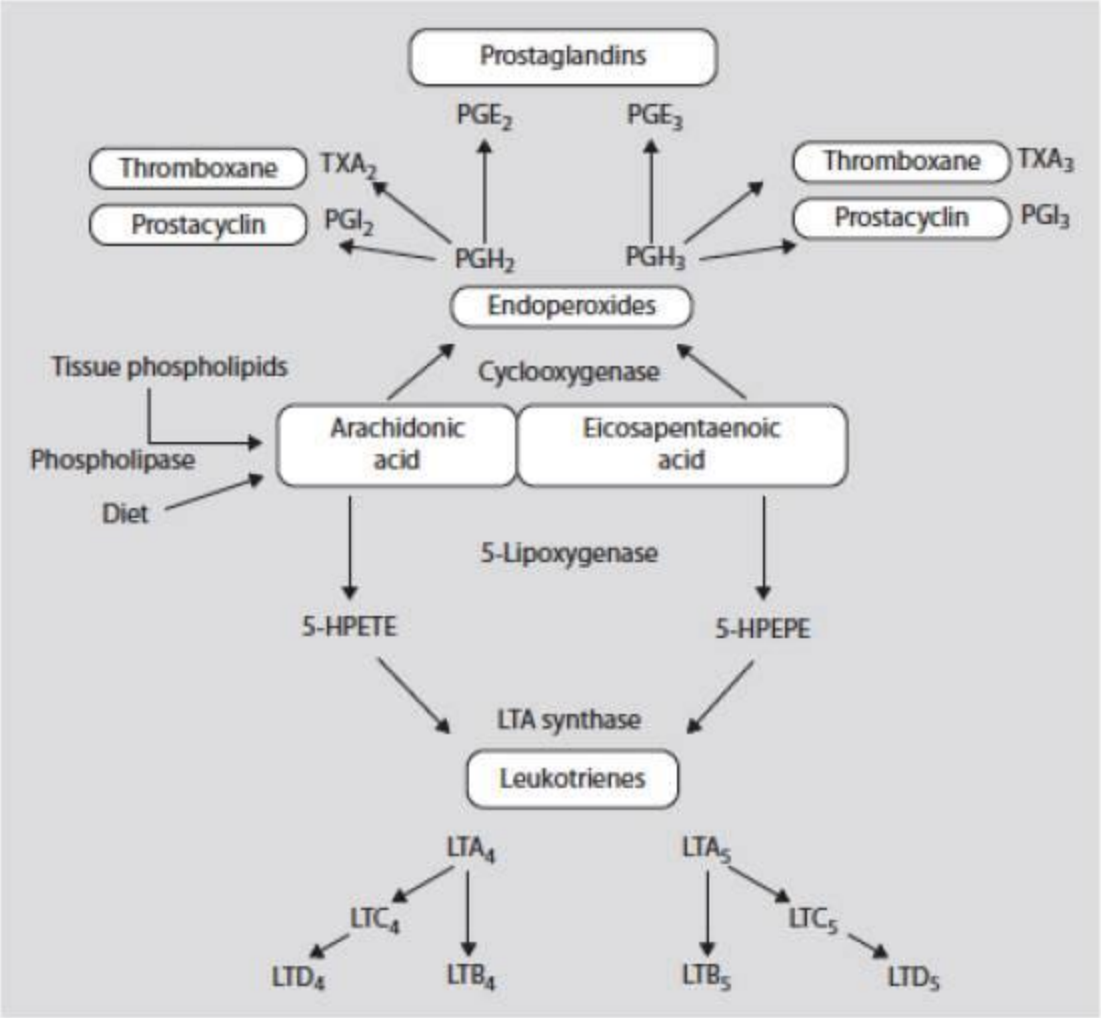

Figure 2.3 Oxidative metabolism of arachidonic acid and EPA (Simopoulos, 2016)

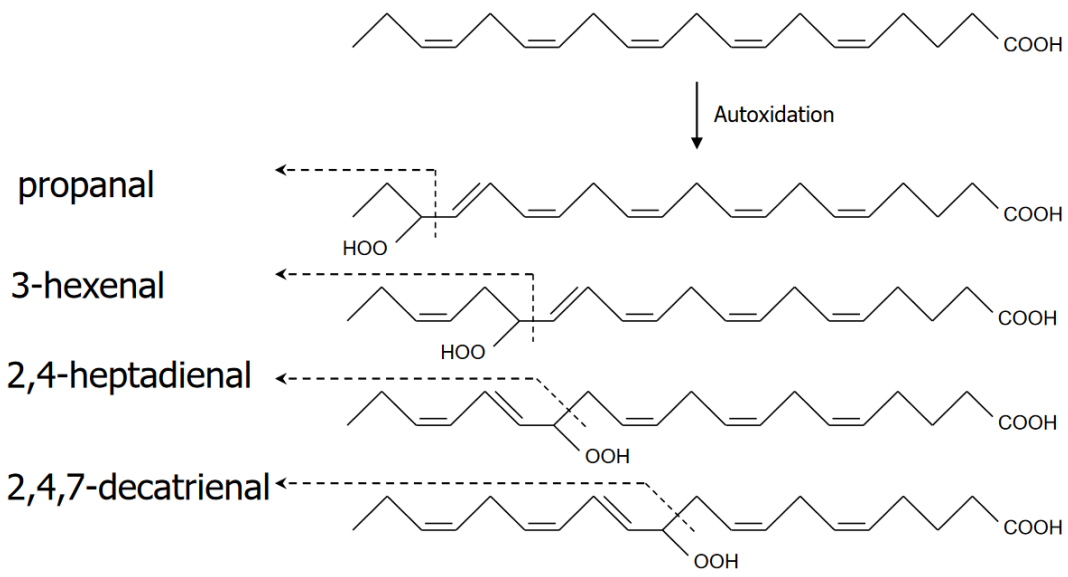

Figure 2.4 Secondary volatile oxidation products of omega-3 fatty acids (Jacobsen, 2015)

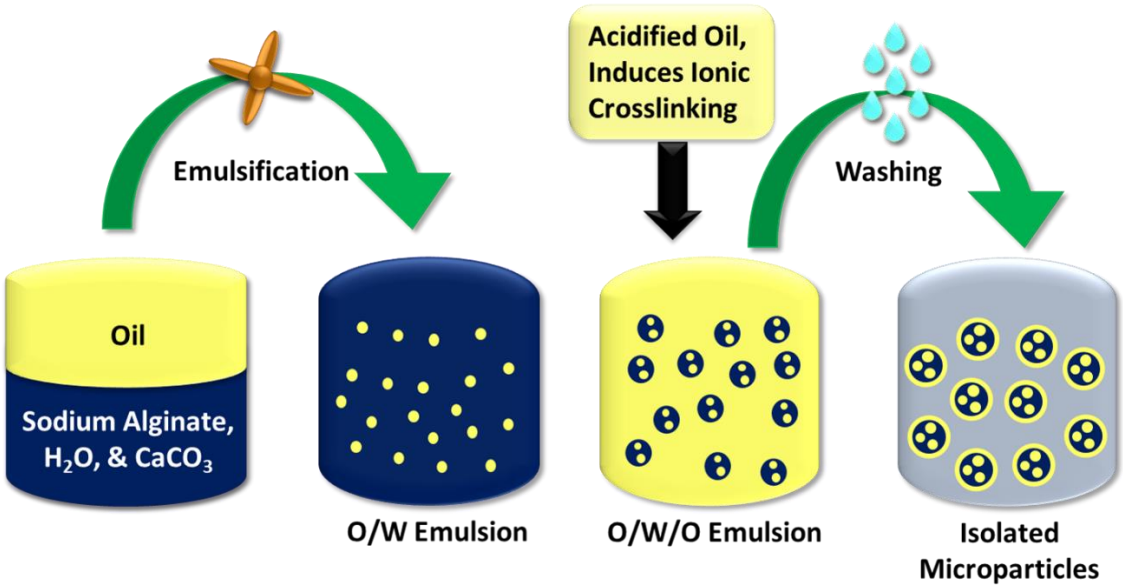

Figure 2.6 Microencapsulation double emulsion method

CHAPTER 3

APPLICATION OF THE TAGUCHI METHOD IN THE ENZYMATIC MODIFICATION OF MENHADEN OIL TO INCORPORATE CAPRIC ACID[1]

[1]Willett, S. A., and Akoh, C. C. 2018. Accepted by the Journal of the American Oil Chemists' Society. Reproduced here with permission from John Wiley & Sons, Inc.

Abstract

Structured lipids (SLs) were produced using menhaden oil and capric acid or ethyl caprate as the substrate. Enzymatic reactions conditions were optimized using the Taguchi method L9 orthogonal array with three substrate molar ratio levels of capric acid or ethyl caprate to menhaden oil (1:1, 2:1, and 3:1), three enzyme load levels (5, 10, and 15% [w/w]), three temperature levels (40, 50, and 60˚C), and three reaction times (12, 24, 36 h). Recombinant lipase from *Candida antarctica*, Lipozyme® 435, and *sn*-1,3 specific *Rhizomucor miehei* lipase, Lipozyme® RM IM (Novozymes North America, Inc., Franklinton, NC, USA), were used as biocatalysts in both acidolysis and interesterification reactions. Total and *sn*-2 fatty acid compositions, triacylglycerol (TAG) molecular species, thermal behavior, and oxidative stability were compared. Optimal conditions for all reactions were 3:1 substrate molar ratio, 10% [w/w] enzyme load, 60˚C, and 16 h reaction time. Reactions with ethyl caprate incorporated significantly more C10:0, at 30.76±1.15 and 28.63±2.37 mol% versus 19.50±1.06 and 9.81±1.51 mol%, respectively, for both Lipozyme® 435 and Lipozyme® RM IM, respectively. Reactions with ethyl caprate as substrate and Lipozyme® 435 as biocatalyst produced more of the desired medium-long-medium (MLM)-type TAGs with polyunsaturated fatty acids (PUFA) at *sn*-2 and C10:0 at *sn*-1,3 positions.

Keywords: Structured lipids, menhaden oil, Taguchi method, TAG molecular species, Lipozyme® 435, capric acid

Introduction

The omega-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are beneficial to human health, especially in promoting cardiovascular health, as well as reducing blood triacylglycerol (TAG) levels, inflammation, and risk of neurodegenerative diseases (He, 2009). EPA and DHA are found naturally in marine oils. The amount of EPA and DHA, as well as total and positional fatty acid compositions, can differ based on fish species, diet, and environmental factors such as water temperature and salinity (Ackman, 1967). Based on the amount and positional distribution of PUFA and other FA, marine oils can be used as a substrate in reactions to synthesize desirable structured lipids (SL).

SL are products of chemical or enzymatic modification of an oil to alter its TAG composition; they have a wide range of applications in nutraceuticals, infant formula, cocoa butter equivalents, and food products (Kim & Akoh, 2015). Enzymatic modification is of interest as reactions are typically under milder conditions and have higher specificity to produce the desired medium-long-medium (MLM) chain-type TAG product than chemical modification (Hamam & Budge, 2010). MLM-type TAGs are desired because there is a reduction in saturated long chain fatty acids (LCFA) that are metabolized slowly when present at the *sn*-1,3 positions (Kim & Akoh, 2015). Medium chain fatty acids (MCFA) such as capric acid (C10:0) are metabolized faster and allow the FA at the *sn*-2 position to be more readily absorbed. Because the FA at the *sn*-2 position is favored during digestion and intestinal absorption, having EPA or DHA present at the *sn*-2 position increases the health benefits of the MLM-type TAG (Salentinig et al., 2015).

Menhaden oil used in this research contained a significant amount of EPA and DHA at the *sn*-2 position. However, menhaden oil does not contain any MLM-type TAG and contains many different saturated LCFA at the *sn*-1,3 positions. So, it is desirable to enzymatically modify menhaden oil with a MCFA such as C10:0 to produce a SL of MLM-type TAG to combine benefits of LCFA (EPA and DHA) and MCFA (capric acid) (Jennings & Akoh, 2001).

The purpose of this study was to enzymatically produce an MLM-type SL of menhaden oil (as main substrate) with capric acid or ethyl caprate as the acyl donor. Two biocatalysts were compared: recombinant lipase from *Candida antarctica*, Lipozyme® 435, and *sn*-1,3 specific *Rhizomucor miehei* lipase, Lipozyme® RM IM, for their ability to synthesize the desired MLM-type SL product that incorporates the highest amount of C10:0 and yields the desired stereochemical MLM configuration with PUFA at *sn*-2 and C10:0 at *sn*-1,3 positions.

Materials and Methods

Materials

Menhaden oil was obtained from Omega Protein, Inc. (Reedville, VA, USA). Capric acid (≥98 % purity), ethyl caprate (≥98 % purity), lauric acid (C12:0) standard, and 2-oleoylglycerol standard were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Lipozyme® RM IM (*Rhizomucor miehei* lipase immobilized on a resin carrier with specific activity of 442 IUN g^{-1} (interesterification unit per gram) as specified by manufacturer) and Lipozyme® 435 (recombinant lipase from *Candida antarctica*, expressed on *Aspergillus niger*, and immobilized on a macroporous hydrophobic resin

with specific activity of 10000 PLU g^{-1} (propyl laurate unit per gram) as specified by the manufacturer) were obtained from Novozymes North America, Inc. (Franklinton, NC, USA). 1 IUN is the amount of enzyme activity that liberates 1 μmol of butyric acid from tributyrin per minute under defined standard conditions. 1 PLU is the amount of enzyme activity that produces 1 μmol of propyl laurate per minute from esterification of lauric acid with *n*-propyl alcohol under defined standard conditions. TAG reference standard mixture (GLC-437 and GLC 570) and GLC-461 fatty acid methyl ester (FAME) mix were purchased from Nu-check Prep, Inc. (Elysian, MN, USA). All other reagents and solvents were of analytical or HPLC grade and purchased from Fisher Chemical (Fair Lawn, NJ, USA), Sigma-Aldrich, and J. T. Baker Chemical Co. (Phillipsburg, NJ, USA).

Taguchi Method

To achieve the highest incorporation of C10:0, the reaction conditions, namely the substrate ratio, enzyme load, temperature, and time, were optimized using the Taguchi method. The Taguchi method is a type of robust parameter fractional factorial experimental design that allows for reducing the number of experiments needed to optimize a process (Shahavi et al., 2016). The Taguchi method is part of Lean Six Sigma and has been utilized mostly in the engineering field to reduce cost and wastage associated with production and operations (Chandrasekar, 2014). Few applications have been done outside the engineering field, so it is of interest to see whether the Taguchi method is applicable to fields such as the food industry (Farbodi, 2017).

The first step in using the Taguchi method is orthogonal array selection based upon the desired conditions that are being optimized. Orthogonal arrays allow many variables to be studied while conducting a small number of experiments (Farbodi, 2017). The L9 orthogonal

array was selected to optimize four reaction parameters at three different levels. The parameters were substrate molar ratio of capric acid or ethyl caprate to menhaden oil (1:1, 2:1, and 3:1), enzyme load (5, 10, and 15% [w/w]), temperature (40, 50, and 60˚C), and reaction time (12, 24, 36 h). Enzyme load was selected on the basis of the total weight of the substrates, and other parameters were based on previous literature (Jennings & Akoh, 2001; Kim & Akoh, 2015). There were nine runs in the experiment, as shown in Table 3.1. The orthogonal array is set up such that each column of independent variables is orthogonal, meaning each parameter is represented in an equal amount (Farbodi, 2017). Under the L9 orthogonal array, two acidolysis reactions with capric acid and two interesterification reactions with ethyl caprate were performed to compare the two biocatalysts of recombinant lipase from *Candida antarctica*, Lipozyme® 435 and *sn*-1,3 specific *Rhizomucor miehei* lipase, Lipozyme® RM IM. Experiments were conducted in triplicate using 5 g of menhaden oil, where the menhaden oil was mixed with capric acid or ethyl caprate, either Lipozyme® 435 or Lipozyme® RM IM, and reactions were conducted in test tubes at specified temperature and time in a shaking water bath set at 250 rpm. Reactions were stopped by filtering the product through an anhydrous sodium sulfate column. Free fatty acids (FFA) were removed as described below, and total fatty acid composition was determined for all products as described below for FAME analysis. Average mol% and standard deviations were determined.

Statistical Analysis

Statistical analysis of the experimental results obtained by the Taguchi method was performed using JMP® software (Version 10, SAS Institute Inc., Cary, NC, USA). The signal/noise (S/N) ratio is a measure of robustness that can be used to identify the control factor settings for minimum impact of noise on a response (Savaghebi et al., 2012). The S/N ratio was

calculated for C10:0 incorporation into menhaden oil using the equation of "larger is better" to maximize C10:0 incorporation amount, where *n* is the number of experiments and *y* is the response variable (Savaghebi et al., 2012).

Eq. (1) $$S/N = -10\log\left(\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y^2}\right)$$

The S/N ratios for each variable (substrate ratio, enzyme load, temperature, and time) was used to find the optimum reaction parameters and then analyzed by ANOVA. The F-ratio was calculated to determine the statistical significance. The percent contributions of each parameter were also determined to see how much each parameter affected the reaction in terms of C10:0 incorporation.

Time-Course Reactions

Once the optimal reaction conditions were found using the Taguchi method, time-course reactions were conducted to validate the method. In triplicate, 5 g of menhaden oil was used for each sample, and individual samples were pulled at 1, 4, 8, 16, 24, and 36 h using the optimum parameters for the substrate molar ratio (3:1), enzyme load (10% [w/w]), and temperature (60˚C). Reactions were stopped by filtering through an anhydrous sodium sulfate column and analyzed as described below for TAG molecular species, FFA removal, FAME, and ethanolysis reaction methods. Average mol% and standard deviations of TAG molecular species, total, and *sn*-2 fatty acid composition of the products were determined.

FFA Removal

FFA were removed from the reaction products through alkaline deacidification by modifying the AOCS method Ca 5a-40, where, after titration, the neutralized mixture was

mixed with 15 mL of hexane in a separatory funnel (American Oil Chemists' Society, 2011). The mixture was shaken vigorously and allowed to separate into two layers. The lower layer containing FFA soaps was discarded. The upper layer was collected and the hexane was evaporated in a rotary evaporator at 60˚C. Products were passed through an anhydrous sodium sulfate column, placed in a vial, flushed with nitrogen, and stored at -20˚C until further analysis.

Fatty Acid Composition and Positional Analysis

For total fatty acid composition, the reaction products were converted to FAME using AOAC Official Method 996.01 and analyzed using an Agilent 6890 N GC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and Supelco SP-2560 capillary column (100 m x 0.25 mm ID, 0.20 μm film) (Sigma-Aldrich Co.) (Satchithanandam et al., 2001). C12:0 was the internal standard and GLC-461 FAME mix was the external standard. GC analysis and procedures were conducted as described elsewhere (Ifeduba & Akoh, 2013). One microliter of the sample was injected at a split ratio of 50:1 for total FA composition analysis and 5:1 for *sn*-2 FA composition analysis. The carrier gas (He) flow was 1.1 mL min^{-1}. The detector temperature was 250°C. The oven was held at 140°C for 5 min, then increased to 240°C at a rate of 4°C/min and held isothermally for 15 min. The average relative FAME content was calculated as mol% and standard deviations were determined.

To carry out the *sn*-2 positional analysis, an ethanolysis reaction was conducted as described by Solaesa et al. (2014). This method was used over the pancreatic lipase method because it has been found that pancreatic lipase may not hydrolyze all FA in the same way,

particularly very long chain PUFA (Solaesa et al., 2014). When Lipozyme® 435 is mixed with excess ethanol, it behaves as an *sn*-1,3 specific lipase and the reaction has higher 2-monoacylglycerol (MAG) yield and lower acyl migration when compared to the method using pancreatic lipase (Solaesa et al., 2014). The oil product in excess ethanol (1:3 [w/w]) was mixed with 4% Lipozyme® 435 [w/w] of total substrate in a 30˚C shaking water bath at 250 rpm for 4 h. Before the reaction, menhaden oil and ethanol are not miscible, however due to the polarity of the 2-MAG and ethyl esters formed after the 4 h reaction, the mixture becomes homogenous (Solaesa et al., 2014). Samples were filtered through an anhydrous sodium sulfate column and concentrated to one-third their original volume with nitrogen. The samples were spotted on a thin-layer chromatography (TLC) plate and developed in hexane/diethyl ether/formic acid (60:40:1.6, v/v/v) with 2-monoolein as standard. Plates were sprayed with 0.2% 2',7'-dichlorofluorescein in methanol to detect bands, and the corresponding MAG band was scraped off and converted to FAME as described above. The average relative FAME content was calculated as mol% and standard deviations were determined.

TAG Molecular Species

TAG molecular species were analyzed for all reaction products using an Agilent 1100 HPLC system (Agilent Technologies, Inc.) equipped with a Sedex Model 55 evaporative light scattering detector (ELSD) (Sedere, Alfortville, France) and a 4 mm x 250 mm, 5 μm particle size, Ultrasphere C18 reverse-phase analytical column. Separation was achieved by a modification of methods from the literature using a gradient mobile phase of acetonitrile, acetone: MTBE (methyl *tert*-butyl ether) (90:10, v/v), and chloroform with a flow rate of 1mL min^{-1} (Lopez-Hernandez et al., 2004; Perona & Ruiz-Gutierrez, 1999; Solaesa et al., 2014). The column temperature was 25˚C, ELSD drift tube temperature was set at 70°C, the nebulizer gas

pressure at 3.5 bar, and gain at 8. Twenty microliters of sample was injected with the sample concentration of 3 mg mL^{-1} in acetone. MTBE was used with acetone in the mobile phase to ensure sharper peaks. Chloroform was used to ensure better solubility of the sample in the mobile phase (Lopez-Hernandez et al., 2004). The difficulty with analyzing TAG of menhaden oil is that it contains many fatty acids, and there have been 553 TAG species identified without including the regioisomers and enantiomers (Zeng et al., 2010). Samples were analyzed using a modified equivalent carbon number (ECN) equation to account for MUFA and PUFA (Solaesa et al., 2014). The difficulty with TAG identification is that there are many TAG in fish oil with the same traditional ECN number (Perona & Ruiz-Gutierrez, 1999). The modified equation helps improve peak identity accuracy, yet each peak can still have multiple different TAG (Solaesa et al., 2014). The average mol % and standard deviations were determined.

Large-Scale Synthesis of SL under Optimized Reaction Conditions

After total and *sn*-2 fatty acid compositions and TAG species analysis by the Taguchi method and time course reactions, the selected reaction was 3:1 ethyl caprate/menhaden oil substrate molar ratio and 10% [w/w] Lipozyme® 435, 16 h reaction time, and conducted at 60°C. A large-scale reaction was performed using those reaction parameters in a 1 L stirred batch reactor. In the batch reactor, 500 g of menhaden oil and ethyl caprate (at 3:1 substrate molar ratio) were combined, and Lipozyme® 435 (10% total substrate weight) was added. Substrates and enzyme were mixed at 250 rpm using a SL 2400 StedFast stirrer (Fisher Scientific Co., Fair Lawn, NJ, USA), fitted with a 4-blade propeller for 16 h at 60°C. Reaction was stopped by filtering through a Büchner funnel equipped with filter paper and anhydrous sodium sulfate. Excess FFA and esters were removed from the product by modifying the temperature to 50°C from 185°C using the short-path distillation method as described

elsewhere (Zou & Akoh, 2013). FFA content was determined as described above. Percent yield was calculated based upon FFA content and product weight before and after short-path distillation. Product was placed in an opaque Nalgene bottle, flushed with nitrogen, and stored at -80˚C until further analysis.

Lipid Class Analysis

An Agilent 1260 Infinity Quaternary LC (Agilent Technologies, Inc.) HPLC system equipped with a Sedex Model 85 ELSD (Sedere, Alfortville, France) was used to analyze the lipid class composition of menhaden oil and the large-scale reaction before and after short-path distillation. The normal-phase column used was an Agilent Zorbax RX-SIL (4.6 mm x 250 mm, 5 μm particle size) and analysis was performed using the AOCS Official Method Cd 11d-96 (American Oil Chemists' Society, 2011). Peaks corresponding to TAG, DAG, MAG, and ethyl ester (EE), were identified by comparing retention times to lipid standards. To quantify each lipid class, response calibration curves were constructed using triolein, 1,3-diolein, 1-monoolein, and ethyl oleate (Sigma-Aldrich). Samples were analyzed in triplicate and average mol% was determined.

Thermal Behavior

Thermal behavior of menhaden oil and large-scale reaction product was analyzed by differential scanning calorimetry (DSC) using a 204 F-1 Phoenix DSC instrument (Netzsch-Gerätebau GmbH, Selb, Germany). DSC analysis was performed using the AOCS Official Method Cj 1-94 (American Oil Chemists' Society, 2011). Crystallization onset and melting completion temperatures were determined by analyzing the data with the Proteus thermal

analysis software (Netzsch-Gerätebau GmbH, Selb, Germany). Samples of menhaden oil and final modified oil were analyzed in triplicate and average values were determined.

Oxidative Stability

Oxidative stability was determined by measuring peroxide (PV) and *p*-Anisidine (*p*-AV) values over 12 days. 5 g of each sample was weighed into glass vials, closed, and placed in a Reacti-Therm™ Heating and Stirring Module (Thermo Fisher Scientific, Waltham, MA, USA) at 65°C. PV was measured using a method from the International Dairy foundation (Shantha & Decker, 1994). *p*-AV was determined using AOCS Official Method Cd 18-90 (American Oil Chemists' Society, 2011). Using PV and *p*-AV, the total oxidation (TOTOX) value was calculated. Oil stability index (OSI) of menhaden oil and modified oil were determined using an Oxidative Stability Instrument (Omnion Inc., Rockland, MA, USA) and AOCS Official Method Cd 12b-92 (American Oil Chemists' Society, 2011). Samples of menhaden oil and final modified oil were analyzed in triplicate and average values were determined.

Results and Discussion

Taguchi Method

Table 3.2 shows the total C10:0, EPA, and DHA composition of the unmodified menhaden oil and each Taguchi method run product. The total mol % EPA and DHA decreased from 40 mol% to between 30-36 mol%. C10:0 incorporation ranged from 2 to 29 mol%, and the results of these runs were used to find the optimum parameter conditions. Since the goal of the Taguchi method was to maximize the amount of C10:0 incorporated into menhaden oil, the equation "larger is better" was used to determine the optimum reaction

conditions to incorporate the most C10:0. Optimal conditions for all Taguchi method reactions were found to be as follows: substrate molar ratio of 3:1, enzyme load of 10% [w/w], 60˚C, and 24 h reaction time. The percent contributions of each parameter (substrate ratio, enzyme load, temperature, and time) was calculated to determine which parameters contributed the most to incorporating C10:0 into the menhaden oil. Substrate ratio had a significantly larger impact on reactions with ethyl caprate than with capric acid, and overall substrate ratio affected C10:0 incorporation the most. Temperature and enzyme load were the next most contributing factors, and time contributed the least in affecting C10:0 incorporation. ANOVA was then used to determine significant difference between the parameter levels and percent contribution. From the ANOVA results, the high F-ratio and low *p*-value showed that substrate ratio was the only significantly different parameter ($p < 0.05$) and had the largest percent contribution on experimental results, meaning it had the largest impact on the C10:0 incorporation amount.

Time-Course Reactions

Figure 3.1 shows the time-course results for C10:0 incorporation for all four reactions. Reactions with ethyl caprate as the acyl donor incorporated significantly more C10:0 than reactions with capric acid. When capric acid was the substrate, Lipozyme® 435 incorporated significantly more C10:0 than Lipozyme® RM IM when the reaction was performed for 8 h or more. There was no significant difference in C10:0 incorporation between 16, 24, and 36 h reactions when ethyl caprate was the substrate, which confirms the Taguchi method selection of 24 h as the optimum reaction time. Since there was no significant difference in C10:0 incorporation between 16, 24, and 36 h, the optimum time was selected as 16 h. The positional analysis of the reaction times

showed that the 16 h reaction also had lower mol% of C10:0 at the *sn*-2 position than the 24 h reaction.

Table 3.3 shows the total and positional analysis for the time-course reactions at 16 h. Based on the results of the Taguchi method, the expected total amount of C10:0 was to be around 30 mol% for the ethyl caprate reactions, 25 mol% for the Lipozyme® 435 and capric acid reaction, and 15% for the Lipozyme® RM IM and capric acid reaction. The reactions with ethyl caprate resulted in C10:0 incorporation of 29 and 31 mol % while reactions with capric acid incorporated 10 and 20 mol%. The total amount of EPA and DHA was reduced from 23 and 17 mol% to a range of 16-20 mol % and 10-14 mol%, respectively. There was 34-41 mol% of EPA and DHA at the *sn*-2 position, and significantly more DHA than EPA at the *sn*-2 position. Because the C10:0 incorporation was significantly lower for the acidolysis reactions and the Taguchi method analysis found that substrate ratio affected the C10:0 incorporation the most, the substrate molar ratio may need to be increased to achieve a higher C10:0 incorporation. There was significantly more C10:0 at the *sn*-1,3 positions when ethyl caprate was the substrate, which makes ethyl caprate the more desirable acyl donor. While there was significantly more C10:0 at the *sn*-2 position when Lipozyme® 435 was the biocatalyst, the majority was in the tricaprin TAG (see Table 4 and TAG molecular species analysis). There was significantly more C10:0 incorporation at the *sn*-1,3 positions (~2%) when Lipozyme® RM IM was the biocatalyst and ethyl caprate the acyl donor. However, to select the optimal reaction for scale-up, the TAG molecular species of each reaction also needs to be analyzed.

TAG Molecular Species

Figure 3.2(a) shows 46 peaks that were identified in menhaden oil before modification. After modification, 51-57 peaks were identified in Figs. 2(b-e). The TAG mol% for each reaction is reported in Table 3.4. There were no MLM-type TAG in the unmodified menhaden oil, and the chromatographs of the enzymatic reactions show the formation of many new TAG species. The predominant TAG of the unmodified menhaden oil were MMEp, PEpPo, PoDhP, PEpDh, MDhM, DpDpDp, PEpDh, PoPoEp, PDhDh, PPoEp, MEpP, MPDh, PPDh, and PPoPo. After modification with Lipozyme® 435 as the biocatalyst, the major TAGs were CCC, StStSt, CEpC, CDhC, CMC, CPoC, PoPoEp, CStC, and StEpC. After modification with Lipozyme® RM IM, the major TAGs were CPoC, DhDhDh, CDpC, MMEp, PEpPo, PoDhP, PPoEp, MEpP, MPDh, CMC, PoEpO, PoPoDh, CPC, and MMP.

Reactions with Lipozyme® 435 as biocatalyst showed that there was significantly higher mol% of MLM-type TAG, particularly TAG with C10:0 at *sn*-1,3 positions and PUFA such as stearidonic acid, EPA, DPA, and DHA at the *sn*-2 position. Reactions with ethyl caprate as the acyl donor also showed higher mol% of MLM-type TAG. The reaction with Lipozyme® 435 and ethyl caprate as the acyl donor had 38 mol% of MLM-type TAG, of which 21 mol% comprised the desired TAG with PUFA at the *sn*-2 position and C10:0 at the *sn*-1,3. Because there was a significantly higher amount of the desired MLM-type TAG and a significant amount of C10:0 incorporated, the reaction with Lipozyme® 435 and ethyl caprate was chosen over the reaction of Lipozyme® RM IM and ethyl caprate. Reactions with Lipozyme® 435 also produced a significantly higher amount of CCC TAG. Despite not being an MLM-type TAG, this medium-chain TAG (CCC) is still beneficial for human health, as

medium-chain TAG (MCTs) are metabolized quickly and are a good source of energy (Salentinig et al., 2015).

Large-Scale Total and Positional Fatty Acid Composition

When selecting the optimum reaction for large-scale production after conducting the time-course reactions, there were many factors to consider. For acyl donor substrate selection, ethyl caprate was chosen since there was significantly more C10:0 incorporation when ethyl caprate was the acyl donor substrate over capric acid. Based on the TAG analysis, Lipozyme® 435 was the selected enzyme as this biocatalyst produced more MLM-type TAG, especially those that contained PUFA at *sn*-2 and C10:0 at *sn*-1,3 positions. Lipozyme® RM IM was not chosen as it produced less MLM-type TAG. Lipases can show preference towards certain FA (or ranges of FA) and the FA degree of unsaturation. Previous studies have shown that Lipozyme® RM IM can discriminate against DHA, further explaining the results in this study (Halldorsson et al., 2003).

After large-scale reaction and short-path distillation, the calculated percent FFA was 0.062%, and the yield was 90.05%. Table 3.5 shows the relative total and *sn*-2 fatty acid composition of unmodified menhaden oil, small-scale, and large-scale reactions. The major FA in menhaden oil before and after modification were C14:0, C16:0, C16:1, C20:5, C22:5, and C22:6. After modification, C10:0 was the FA present in highest concentration at 31 mol% for small-scale and 28 mol% for large-scale total fatty acid composition. EPA and DHA were reduced from 23 and 17 mol% to 16 and 10 mol% for small-scale reaction, respectively, and 17 and 11 mol% for large-scale reaction total fatty acid composition, respectively. The difference in mol% from small-scale to large-scale was not significantly different at α=0.05.

Lipid Class Analysis

Table 3.6 shows the lipid composition of the unmodified menhaden oil and the large-scale SL before and after short-path distillation. The TAG content of the menhaden oil was 99.38 mol%. The menhaden oil also contained small amounts of DAG and MAG (0.38 and 0.24 mol%, respectively). After the enzymatic modification in the batch reactor under optimal conditions, the TAG content of the SL before short-path distillation was 94.91 mol%. There was also an increase in amounts of DAG, MAG, and EE (0.94, 2.09, and 2.06 mol%, respectively). After short-path distillation, the SL TAG content increased to 97.94 mol%, and there was a decrease in amounts of DAG, MAG, and EE (0.89, 1.15, and 0.02 mol%, respectively). The loss of DAG and MAG after short-path distillation may account for the decrease in the amount of C10:0 present at the *sn*-1,3. There was almost complete removal of FFA and EE from the SL. To increase the removal of the EE, a longer residence time in the distillator or higher temperature may be necessary, however there is concern for increased oxidation of the SL (Hamam & Budge, 2010; Zou & Akoh, 2013).

Thermal Behavior

Figure 3.3 shows DSC thermograms of the crystallization (a) and melting (b) profiles of the menhaden oil and the purified large-scale reaction product. The large-scale reaction had higher crystallization onset (C_0) and melting completion (M_c) temperatures than the unmodified menhaden oil at -5.3 and 10.3°C versus -6.5 and -2.7°C, respectively. This is likely due to the introduction of new TAG species with C10:0, specifically the tricaprin TAG being present in a significant amount contributed to the increase in melting temperature. While the melting temperature of the oil is significantly

raised, it may be useful to modify menhaden oil with FA that have a higher melting point than capric acid for fat-based applications such as margarine spreads or for baked goods.

Oxidative Stability

Figure 3.4 shows the OSI and changes in the TOTOX of the menhaden oil and large-scale SL reaction product. The menhaden oil remained relatively stable over the 12 d storage, while oxidation of the large-scale reaction product took place earlier at 3-6 d. The OSI values were significantly lower in the SL than the unmodified menhaden oil. Both the OSI and the storage stability results showed that the SL had lower oxidative stability than the menhaden oil. This is likely due to the enzymatic reaction process and short-path distillation that has been shown in other studies to reduce the tocopherols or antioxidants present in the modified oil (Zou & Akoh, 2013). Replenishing the lost antioxidants may improve the oxidative stability (Hamam & Budge, 2010).

Conclusion

The Taguchi method was successful in optimizing the reaction conditions for incorporating capric acid into menhaden oil. The method was validated by the time-course reactions. The reactions with ethyl caprate as the acyl donor incorporated significantly more C10:0 than with capric acid. There were significantly higher amounts of MLM-type TAG in reactions with Lipozyme® 435 and ethyl caprate as the acyl donor substrate where C10:0 was at the *sn*-1,3 positions and PUFA were at the *sn*-2 position. The enzymatic modification of menhaden oil to incorporate C10:0 has the potential for use as a nutraceutical lipid due to the conservation of EPA and DHA at the *sn*-2 position. Successful use of Lipozyme® 435, a

commercial, food grade, immobilized lipase improves the viability of enzymatic interesterification at an industrial scale and potential use of the product in food applications.

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Conflict of Interest: The authors declare that they have no conflicts of interest.

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Table 3.1 Reaction conditions under Taguchi method L9 orthogonal array to be applied to all four reactions in triplicate using either capric acid or ethyl caprate with menhaden oil as substrates and either Lipozyme® 435 or Lipozyme® RM IM as enzyme

Run Number	Substrate Molar Ratio C10:0: menhaden oil	Enzyme Load (w/w %)	Temperature (˚C)	Time (h)
1	1:1	5	40	12
2		10	50	24
3		15	60	36
4	2:1	5	50	36
5		10	60	12
6		15	40	24
7	3:1	5	60	24
8		10	40	36
9		15	50	12

Table 3.2 Total C10:0, EPA, and DHA from all four of the Taguchi method reactions

Sample*	Total Fatty Acid Composition (mol%)		
	C10:0	C20:5n3	C22:6n3
Menhaden Oil	ND	22.82±1.85	16.97±1.83
L1	2.05±0.13	21.57±0.24	13.59±0.38
LE1	6.47±0.54	21.00±0.31	13.00±0.22
R1	1.84±0.61	22.12±0.08	14.02±0.15
RE1	5.98±0.70	21.20±0.27	13.11±0.18
L2	7.06±2.82	20.62±1.14	13.42±1.17
LE2	7.06±0.15	21.57±0.07	13.72±0.25
R2	6.06±1.26	21.28±0.63	13.89±0.59
RE2	7.46±0.93	21.37±0.16	13.47±0.09
L3	8.01±2.23	20.34±1.47	13.07±1.04
LE3	7.17±0.84	20.82±0.62	13.29±0.38
R3	9.24±0.40	20.64±0.28	15.32±0.30
RE3	6.99±0.02	21.26±0.04	14.85±0.02
L4	8.59±0.98	19.91±0.32	12.75±0.13
LE4	19.08±1.21	18.54±0.29	11.66±0.19
R4	6.69±1.78	21.27±0.02	14.40±0.77
RE4	18.77±1.07	18.33±0.17	11.52±0.14
L5	15.55±1.32	17.63±0.10	12.15±0.06
LE5	17.11±2.24	18.44±1.21	12.51±0.71
R5	13.56±0.02	20.03±0.07	15.08±0.15
RE5	19.69±0.01	18.28±0.003	12.29±0.004
L6	12.48±0.86	18.33±0.16	11.98±0.11
LE6	18.83±0.41	18.02±0.32	11.28±0.30
R6	8.46±2.59	21.55±0.08	14.68±0.26
RE6	16.98±1.57	19.68±1.09	12.56±0.78
L7	14.65±0.01	17.87±0.03	12.14±0.02
LE7	27.43±1.63	16.80±0.87	10.79±0.68

R7	12.59±1.37	20.42±0.13	14.85±0.40
RE7	27.93±0.05	16.81±0.04	10.90±0.02
L8	14.00±1.92	17.74±0.56	12.09±0.23
LE8	28.54±0.42	17.48±0.16	11.03±0.07
R8	8.46±0.25	21.29±0.51	14.37±0.50
RE8	25.63±1.74	17.18±0.35	11.74±0.51
L9	9.12±0.77	19.28±0.29	12.52±0.05
LE9	24.13±1.15	17.59±0.37	10.90±0.23
R9	9.60±2.50	20.69±0.47	13.22±0.55
RE9	21.84±2.78	17.92±0.62	11.30±0.50

Mean ± STD, *n*=3, ND= not detected

* Numbers correspond to run number in L9 Taguchi Array on Table 1, L= Lipozyme® 435 + capric acid substrate, LE= Lipozyme® 435 + ethyl caprate substrate, R= Lipozyme® RM IM + capric acid substrate, RE= Lipozyme® RM IM + ethyl caprate substrate

Table 3.3 Relative total and positional fatty acid compositions for all four reactions taken from the time course reaction data at 16 h

Reactions*	TAG Position	Fatty Acid (mol%)***					
		C10:0	C14:0	C16:0	C16:1	C20:5n3	C22:6n3
Menhaden Oil	Total	ND	7.71±0.65	13.87±1.16	10.21±0.85	22.82±1.85	16.97±1.83
	sn-2	ND	10.43±0.61	14.86±0.74	12.88±0.94	15.32±0.74	29.34±3.08
	sn-1,3**	ND	6.35±0.67	13.38±1.37	8.88±0.81	26.57±2.41	10.79±1.21
Lipozyme 435® & Capric Acid	Total	19.50± 1.06	7.42±0.17	13.63±0.28	10.64±0.25	16.74±0.20	11.69±0.36
	sn-2	8.29±0.37	8.80±0.10	12.58±0.11	11.71±0.10	13.58±0.14	25.06±0.19
	sn-1,3	25.11±1.41	6.73±0.21	14.16±0.37	10.11±0.33	18.32±0.23	5.01±0.45
Lipozyme 435® & Ethyl Caprate	Total	30.76±1.15	6.19±0.07	11.44±0.11	8.62±0.25	15.42±0.17	9.83±0.37
	sn-2	15.11±0.51	8.65±0.39	13.36±0.55	11.64±0.34	13.55±0.37	20.79±0.64
	sn-1,3	38.59±1.47	4.96±0.09	10.48±0.11	7.11±0.21	16.36±0.07	4.35±0.24
Lipozyme® RM IM & Capric Acid	Total	9.81±1.51	7.80±0.14	14.08±0.24	10.78±0.18	20.70±0.36	14.13±0.50
	sn-2	3.18±0.26	9.44±0.66	13.44±0.63	12.14±0.18	14.44±1.25	26.96±2.50
	sn-1,3	13.13±2.14	6.98±0.12	14.40±0.05	10.10±0.18	23.83±0.09	7.72±0.50
Lipozyme® RM IM & Ethyl Caprate	Total	28.63±2.37	6.29±0.24	11.46±0.51	8.54±0.52	16.25±0.30	10.61±0.27
	sn-2	4.61±0.80	10.02±0.17	14.17±0.12	12.16±0.12	15.88±0.18	23.36±0.29
	sn-1,3	40.64±3.16	4.43±0.28	10.11±0.71	6.73±0.72	16.44±0.36	4.24±0.26

Mean ± STD, *n*=3, ND= not detected

*Reaction conditions: substrate molar ratio of 3:1, enzyme load of 10% w/w, temperature of 60˚C, and time of 16 h

** *sn*-1,3 mol% determined by equation: *sn*-1,3 (mol%)= [3xtotal mol%- *sn*-2 mol %]/2

***Other fatty acids found at >1mol%: C17:1n7, C18:0, C18:1n9, C18:2n6, C20:0, C18:4n3, C22:5n3, and <1 mol%: C13:0, C14:1n7, C15:0, C17:0, C19:0, C18:3n6, C20:1n, C18:3n3, C20:2n6, C22:0, C20:3n6, C20:3n3, C23:0, C22:2n6, C24:1n9, C22:4n6

Table 3.4 Relative TAG molecular species of all reactions from time course data taken at 16 h*

TAG****	TAG (mol%)					Peak Number**				
	M***	L	LE	R	RE	M	L	LE	R	RE
CCC	ND	9.64±0.27	13.35±0.46	0.22±0.02	0.42±0.06	ND	6	6	10	12
CStC/StEpC	ND	3.25±0.11	5.39±0.32	0.75±0.06	1.89±0.30	ND	9	7	11	15
StDhC	ND	4.48±0.12	5.88±0.08	1.39±0.05	3.59±0.44	ND	10	8	12	16
StStSt/CEpC	0.21±0.02	5.64±0.22	8.68±0.29	1.45±0.19	2.67±0.32	1	15	13	16	20
CDhC	ND	3.51±0.10	4.44±0.08	1.41±0.14	1.66±0.12	ND	16	14	17	21
EpEpEp/DhDhC	0.10±0.01	0.83±0.03	1.79±0.13	1.02±0.09	1.97±0.15	4	18	16	19	23
DhDhDh/CDpC	1.26±0.25	0.89±0.03	2.73±0.18	1.58±0.25	6.46±0.05	8	22	20	24	28
CPoC	ND	7.23±0.41	10.50±0.31	3.21±0.45	4.25±0.34	ND	23	21	25	32
CMC	ND	2.91±0.08	3.67±0.16	1.91±0.32	3.92±0.26	ND	27	24	29	33
PEpEp	4.82±0.56	3.53±0.44	3.84±0.50	1.92±0.38	4.13±0.34	19	28	25	33	35
DpDpDp/MDhM/ PEpDh	9.26±1.10	1.12±0.11	1.10±0.07	1.30±0.06	1.09±0.18	20	30	27	34	36
PoPoEp/PDhDh	8.51±2.26	4.10±0.40	4.24±0.11	1.90±0.11	2.83±0.18	26	31	28	35	37
PoEpO/PoPoDh/ CPC	4.82±1.37	3.27±0.32	2.03±0.14	4.95±0.20	2.69±0.07	ND	35	32	40	40
MMEp/PEpPo/ PoDhP	14.96±1.46	5.77±0.82	2.25±0.21	13.50±0.32	5.54±1.29	27	36	33	41	42
PPoEp/MEpP/ MPDh	9.45±0.31	3.88±0.63	1.61±0.05	8.31±0.45	3.60±0.91	28	38	35	42	43
PEpP/PPDh	4.56±0.41	2.76±0.16	2.48±0.27	2.70±0.17	2.41±0.34	33	41	38	45	46
PPDh/PPoPo	7.45±0.73	1.48±0.13	1.67±0.06	0.88±0.16	1.23±0.04	34	44	41	48	49
MMP	3.66±1.37	3.19±0.12	1.44±0.20	5.25±0.38	3.46±0.89	36	45	42	49	50
SDpDp/MPPo	4.27±0.25	2.20±0.09	1.06±0.10	2.94±0.12	1.76±0.54	37	47	44	51	52
PSLo/SSLo/ PPoS	2.73±0.33	1.40±0.04	0.71±0.02	2.08±0.14	1.31±0.23	45	52	49	56	57
Total MLM-type TAGs	ND	26.70±0.18	37.44±0.21	15.26±0.23	23.54±0.21	-	-	-	-	-
Total C-PUFA-C TAGs	ND	13.29±0.12	21.24±0.22	5.19±0.16	12.68±0.20	-	-	-	-	-

Mean ± STD, *n*=3, ND= not detected
*Reaction conditions: substrate molar ratio of 3:1, enzyme load of 10% w/w, temperature of 60˚C, and time of 16 h
**Numbers correspond to peak on chromatographs in Figure 2
*** Where M=unmodified menhaden oil, L= Lipozyme® 435 + capric acid substrate, LE= Lipozyme® 435 + ethyl caprate substrate, R= Lipozyme® RM IM + capric acid substrate, RE= Lipozyme® RM IM + ethyl caprate substrate
**** Letters correspond to the following FA: C= Capric, M= Myristic, P= Palmitic, Po= Palmitoleic, S= Stearic, O= Oleic, Lo= Linoleic, St= Stearidonic, Ep= EPA, Dp= DPA, and Dh= DHA

Table 3.5 Relative total and positional fatty acid distribution comparing unmodified menhaden oil with small scale time course and large-scale batch produced SL

Product	TAG Position	Fatty Acid*		
		C10:0	C20:5n3	C22:6n3
Menhaden Oil	Total	ND	22.82±1.85	16.97±1.83
	sn-2	ND	15.32±0.74	29.34±3.08
	sn-1,3**	ND	26.57±2.41	10.79±1.21
SL: Small-Scale***	Total	30.76±1.15	15.42±0.17	9.83±0.37
	sn-2	15.11±0.51	13.55±0.37	20.79±0.64
	sn-1,3	38.59±1.47	16.36±0.07	4.35±0.24
SL: Large-Scale****	Total	26.73±0.05	17.09±0.39	10.57±0.23
	sn-2	20.33±0.66	12.95±0.36	19.53±0.45
	sn-1,3	29.93±0.26	19.16±0.41	6.09±0.12

Mean ± STD, *n*=3, ND= not detected

*Other fatty acids found at >1mol%: C14:0, C16:0, C16:1n7, C17:1n7, C18:0, C18:1n9, C18:2n6, C20:0, C18:4n3, C22:5n3, and <1 mol%: C13:0, C14:1n7, C15:0, C17:0, C19:0, C18:3n6, C20:1n11, C18:3n3, C20:2n6, C22:0, C20:3n6, C20:3n3, C23:0, C22:2n6, C24:1n9, C22:4n6

** *sn*-1,3 mol% determined by equation: *sn*-1,3 (mol%)= [3xtotal mol%- *sn*-2 mol %]/2

***Small Scale= 5 g reaction done in test tube with conditions: ethyl caprate substrate molar ratio of 3:1, Lipozyme® 435 enzyme load of 10% w/w, temperature of 60˚C, and time of 16 h

****Large-Scale= 500 g reaction done in batch reactor with conditions: ethyl caprate substrate molar ratio of 3:1, Lipozyme® 435 enzyme load of 10% w/w, and temperature of 60˚C for 16 h

Table 3.6 Lipid composition of menhaden oil and large-scale* SL before and after short-path distillation

Product	Lipid Class	Lipid Composition (mol%)
Menhaden Oil	TAG	99.38±1.86
	DAG	0.38±0.15
	MAG	0.24±0.05
	EE	ND
SL Before Short-Path	TAG	94.91±3.66
	DAG	0.94±0.86
	MAG	2.09±0.95
	EE	2.06±2.35
SL After Short-Path	TAG	97.94±2.17
	DAG	0.89±0.49
	MAG	1.15±0.69
	EE	0.02±2.23

Mean ± STD, *n*=3, ND= not detected

*Large-Scale= 500 g reaction performed in batch reactor with reaction conditions: ethyl caprate substrate molar ratio of 3:1, Lipozyme® 435 enzyme load of 10% w/w, and temperature of 60˚C for 16 h

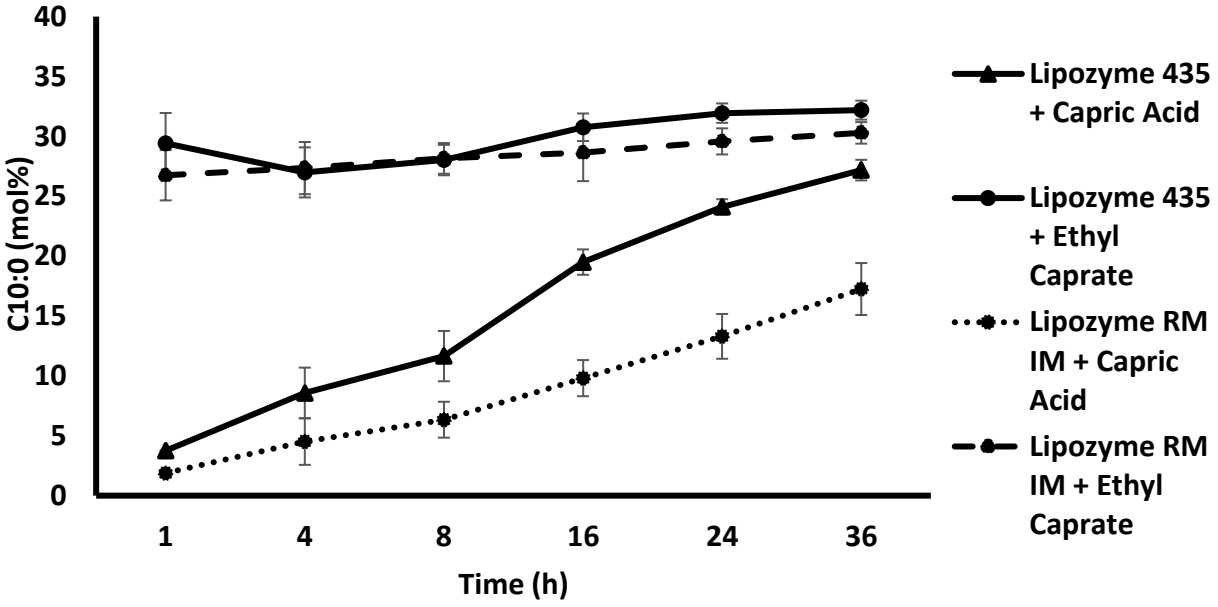
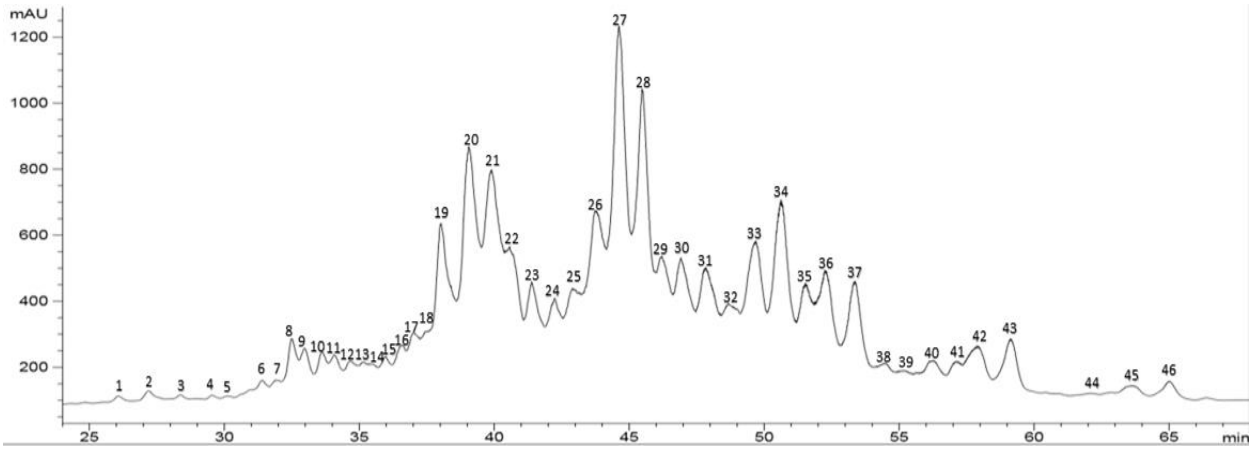
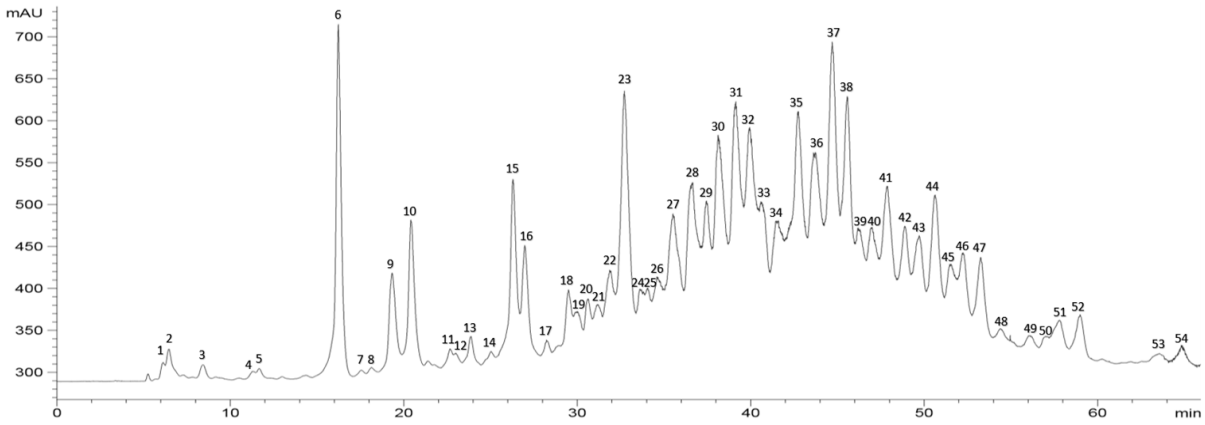


Figure 3.1 Time course reactions showing C10:0 incorporation into menhaden oil using optimized reaction conditions from the Taguchi method results. Substrate molar ratio was 3:1, 10% enzyme load, and water bath temperature was 60˚C

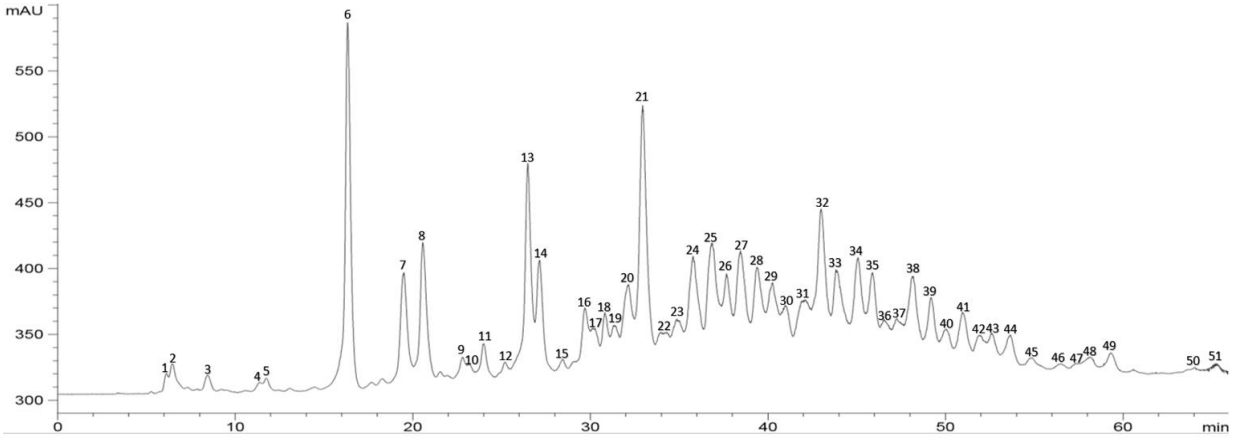
a) Unmodified menhaden oil



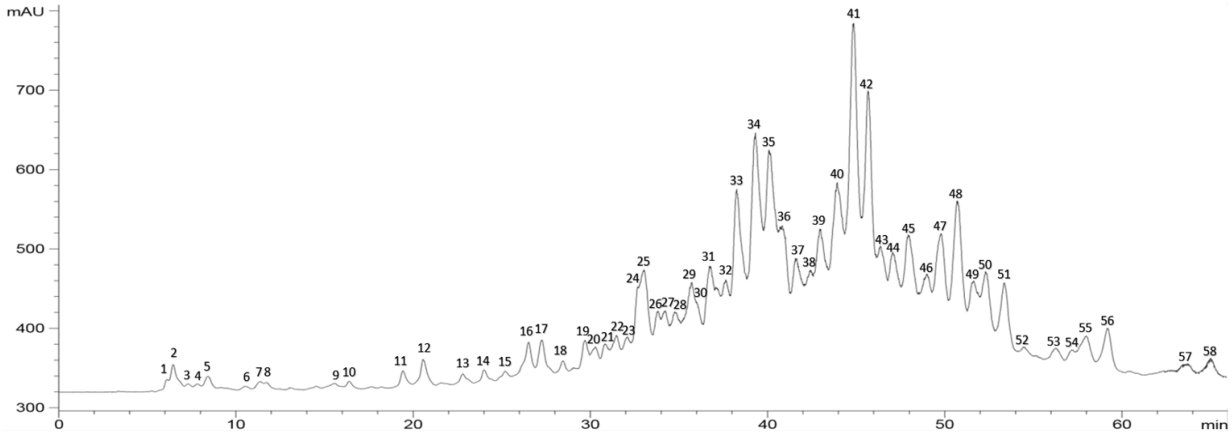
b) Lipozyme® 435 + capric acid substrate



c) Lipozyme® 435 + ethyl caprate substrate



d) Lipozyme® RM IM + capric acid substrate



e) Lipozyme® RM IM + ethyl caprate substrate

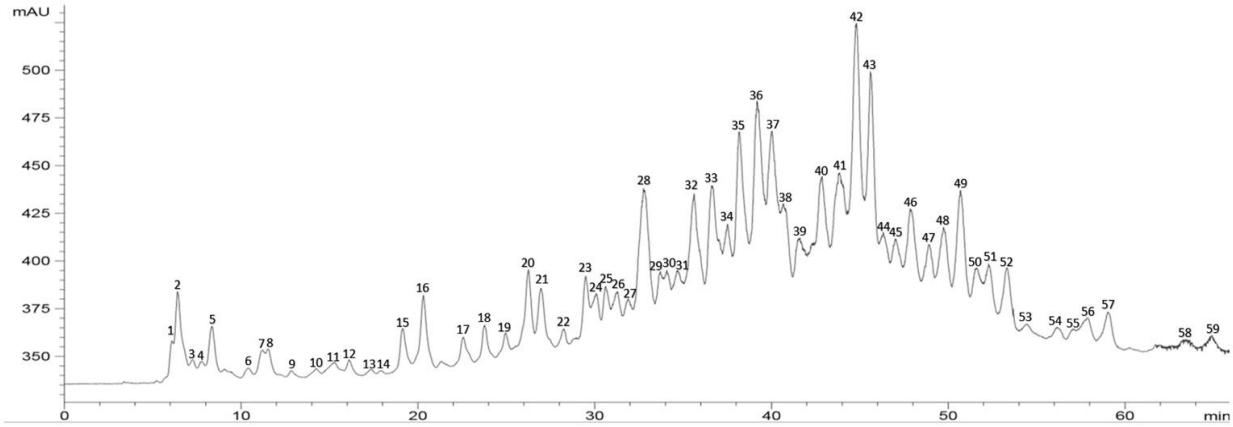


Figure 3.2 HPLC chromatographs for TAG molecular species of all reactions from time course data taken at 16 h

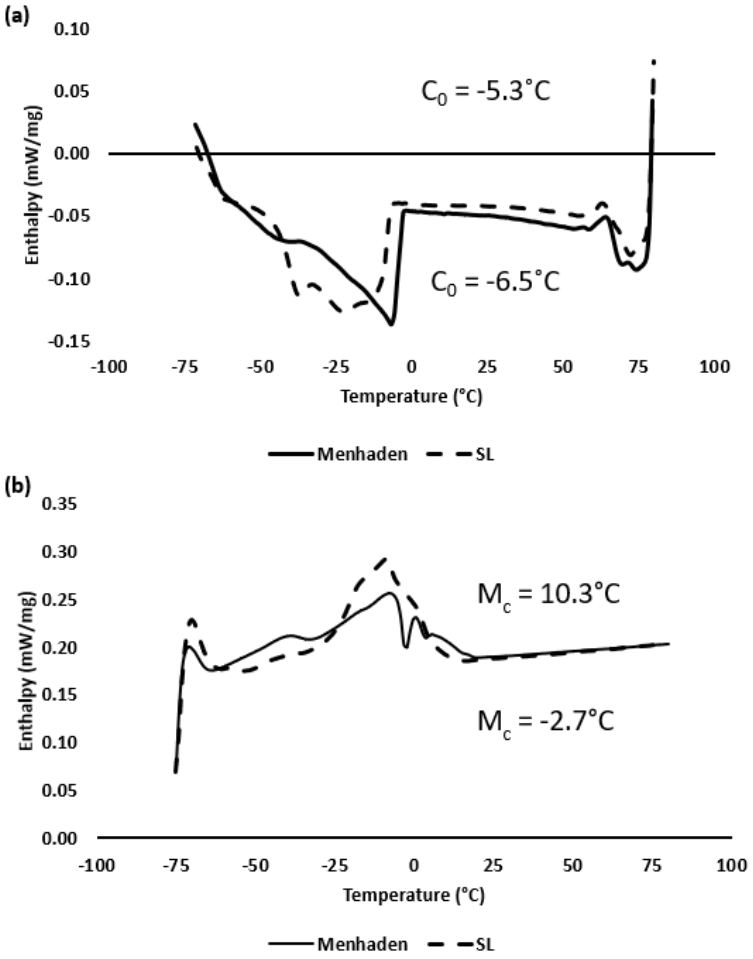


Figure 3.3 Differential Scanning Calorimetry (DSC) thermograms: (a) crystallization and (b) melting profiles of menhaden oil and large-scale SL (3:1 menhaden oil: ethyl caprate substrate molar ratio, 10% Lipozyme® 435, 60˚C, and 16 h). C_0 and M_c are crystallization onset and melting completion temperatures, respectively. For both (a) and (b), the top temperature value is for the SL while the bottom temperature is for the menhaden oil. For both (a) and (b), the solid line is the menhaden oil and the dashed line is the SL

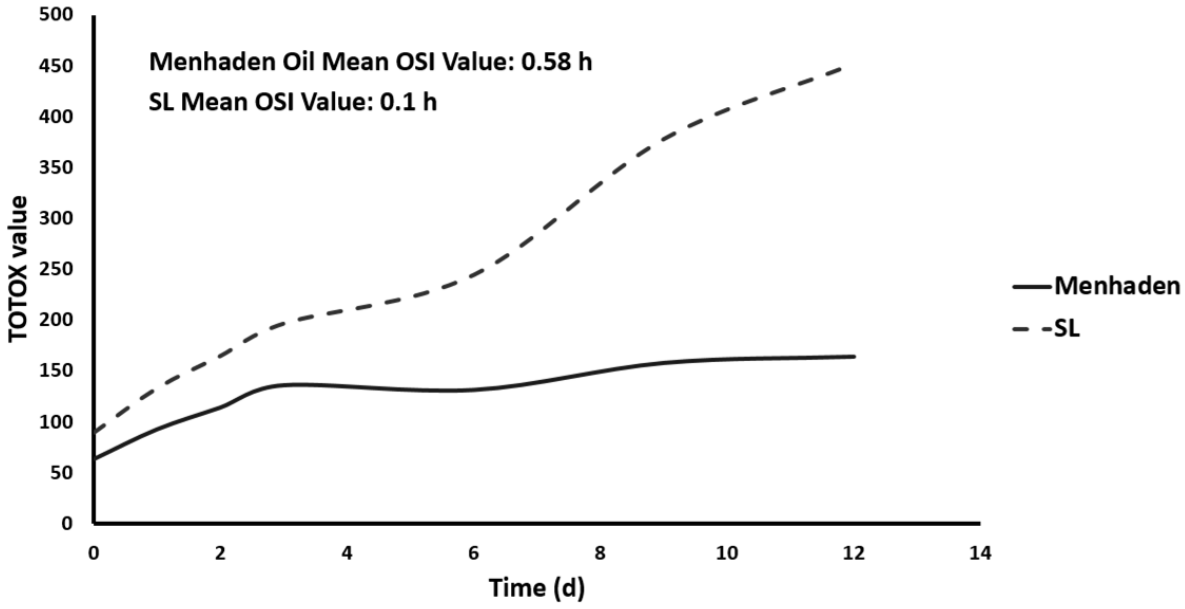


Figure 3.4 TOTOX and OSI values for menhaden and large-scale SL (reaction conditions of 3:1 ethyl caprate: menhaden oil substrate molar ratio, 10% Lipozyme® 435, 60˚C, and 16 h). TOTOX value $= 2(\mathrm{PV}) + p\mathrm{AV}$, where PV= peroxide value, *p*-AV= *p*-Anisidine value

CHAPTER 4

PHYSICOCHEMICAL CHARACTERIZATION OF ORGANOGELS PREPARED FROM MENHADEN OIL OR STRUCTURED LIPID WITH PHYTOSTEROL BLEND OR SUCROSE STEARATE/ASCORBYL PALMITATE BLEND[1]

[1]Willett, S. A., and Akoh, C. C. 2018. Accepted by Food & Function. Reproduced here with permission from The Royal Society of Chemistry.

Abstract

The purpose of this research was to produce organogels using two different organogelator blends and two different oil substrates and compare their physicochemical properties. A medium-long-medium (MLM)-type structured lipid (SL) containing 37.44 mol% MLM-type triacylglycerols (TAG) was produced by enzymatic modification of menhaden oil with ethyl caprate. Menhaden oil and SL were used as oil substrates to form organogels. One organogel was formed using a phytosterol blend of β-sitosterol and γ-oryzanol (molar ratios of 2:1, 1:1, and 1:2 at 4, 6, and 8% (w/w) in oil), and another was formed with a blend of sucrose stearate (HLB value: 2) and ascorbyl palmitate (SSAP) (molar ratios of 3:1, 1:1, and 1:3 at 8, 10, and 12% (w/w) in oil). Organogels were formed for all ratios except 2:1 and 3:1 for the phytosterol and SSAP blend, respectively. For both organogels, the 1:1 molar ratio was optimal. This ratio produced organogels with higher melting completion temperatures than menhaden oil and SL (13.6 and 14.3 °C). The SSAP blend had higher melting completion temperatures (72.3 and 72.4 °C) than the phytosterol blend (69.2 and 68.4 °C) for organogels formed using menhaden oil and SL, respectively. All 1:1 molar ratio blends exhibited β′ polymorphic form with short spacing peaks at 4.20, 3.97, and 3.71 Å. All organogels improved the oxidative stability of the menhaden oil and SL. These organogels have the potential for use as nutraceuticals or health beneficial low saturated fat alternatives to saturated and/or *trans*-fats.

Keywords: organogels; structured lipids; menhaden oil; sucrose stearate; ascorbyl palmitate; oleogels; phytosterols

Abbreviations

CCD, charge coupled device; DSC, differential scanning calorimeter; FA, fatty acid; HLB, hydrophile-lipophile balance; M, menhaden oil; MLM-type TAG, medium-long-medium-type triacylglycerol; MUFA, monounsaturated fatty acid; OSI, oil stability index; P, phytosterol organogel; *p*-AV, *p*-anisidine value; PM6, phytosterol organogel produced with menhaden oil at organogelator blend 1:1 molar ratio; PSL6, phytosterol organogel produced with structured lipid (SL) at organogelator blend 1:1 molar ratio; PUFA, polyunsaturated fatty acid; PV, peroxide value; S, sucrose stearate/ascorbyl palmitate (SSAP) organogel; SFC, solid fat content; SL, structured lipid; SM6, sucrose stearate/ascorbyl palmitate (SSAP) organogel produced with menhaden oil at organogelator blend 1:1 molar ratio; SSAP, sucrose stearate/ascorbyl palmitate; SSL6, sucrose stearate/ascorbyl palmitate (SSAP) organogel produced with SL at organogelator blend 1:1 molar ratio; TAG, triacylglycerol; TOTOX, total oxidation value

Introduction

It has been reported that consuming significant amounts of saturated fats will raise LDL cholesterol in the body, which increases the risk of heart disease and stroke (He, 2009). It is necessary to reduce the consumption of highly saturated fats by consuming more monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA). Many fish oils and plant oils contain significant amounts of MUFA or PUFA (Ackman, 1967). However, fats and oils do not have similar applications in food products (Lucca & Tepper, 1994). So, there is a need to produce a low saturated, zero *trans*-fat that behaves similarly to highly saturated fats in terms of their physicochemical properties.

Organogels (also known as oleogels for edible purposes) are a promising alternative for high saturated and *trans*-fats. Most oils contain saturated fat naturally, but in significantly lower amounts than in highly saturated fats (Co & Marangoni, 2012). Organogels, a lipid gel, have the potential for many applications within and outside the food industry. Studies have shown that these organogels may inhibit oil migration in chocolate and control the release of health beneficial sensitive compounds such as antioxidants, bioactive compounds, and PUFA (Co & Marangoni). Organogels have been shown to increase oxidative stability of the oil by trapping the oil in a gel network so there is decreased exposure to oxygen (Kim et al., 2014). Certain celluloses, waxes, and phytosterols such as a blend of β-sitosterol and γ-oryzanol have been shown to be good organogelators for food applications (Co & Marangoni, 2014; Chaves et al., 2018).

A blend of sucrose stearate/ascorbyl palmitate (SSAP) is proposed in this research to form an organogel. This blend is significantly cheaper than the phytosterol blend and the gelators are food-grade as well. Sucrose stearate is a sucrose fatty acid ester of stearic acid

and sucrose and is a known food emulsifier (Lu et al., 2016). Sucrose stearate has different hydrophile-lipophile balance (HLB) values based on the number of stearic acids that are esterified to the free hydroxyl groups of sucrose. The more stearic acid that is esterified, the lower the HLB number and the more lipophilic the molecule is. Ascorbyl palmitate is a fatty acid ester of ascorbic acid and palmitic acid with known antioxidant capability (Cort, 1974). Both sucrose stearate and ascorbyl palmitate have long chain fatty acid tails that are ester linked to cyclic polar compounds. There are currently no publications on the use of this blend to form an organogel. There may likely be intermolecular interactions between the long hydrocarbon chains, polar functional groups, and oil phase (Dassanayake et al., 2012). It is of interest to investigate the type of structure and physicochemical properties that this blend will have since literature has shown that several different structures may form based on the organogelator(s) and the oil phase (Co & Marangoni, 2014).

The objective of this study was to form different organogels with menhaden oil or a structured lipid (SL) as the oil phase. Oils such as menhaden oil that are lower in saturated fat and contain significant amounts of health beneficial PUFA, could be used to form organogels with additional nutritional benefits such as promoting cardiovascular health, reducing blood triacylglycerol (TAG) levels, inflammation, and risk of neurodegenerative diseases (He, 2009). To the best of our knowledge, there is no research on the formation of an organogel using a SL as the oil phase. Specifically, medium-long-medium (MLM)-type SL are of interest for use in forming organogels because of the desirable nutritional benefits (Kim & Akoh, 2015). The medium chain fatty acids (FA) in an MLM-type SL are metabolized quickly in the body and provide quick energy. The

long chain FA, in 2-monoacylglycerol form, is more readily absorbed. These organogels may also improve the oxidative stability and alter the physicochemical properties of the menhaden oil and SL, allowing for their use in a wider range of applications.

Materials and Methods

Materials

Menhaden oil, obtained from Omega Protein Inc. (Reedville, VA, USA), and the acyl donor ethyl caprate (≥98 % purity), purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), were used as substrates in an interesterification reaction to produce an MLM-type SL. The SL was prepared according to the method outlined previously (Willett & Akoh, 2018). Briefly, menhaden oil and ethyl caprate at a 1:3 substrate molar ratio were mixed with Lipozyme® 435 lipase (Novozymes North America, Inc., Franklinton, NC, USA) at 10% (w/w) of total substrates. The reaction took place in a 1L batch reactor at 60°C, with stirring at 250 rpm using a SL 2400 StedFast stirrer (Fisher Scientific Co., Fair Lawn, NJ, USA) for 16 h. Table 4.1 shows the relative fatty acid composition of the menhaden oil and SL. The SL contained 29.93±0.26 mol% capric acid at the *sn*-1,3 positions, and 12.95±0.36 mol% EPA and 19.53±0.45 mol% DHA at the *sn*-2 position. The SL contained 37.44 mol% MLM-type TAG. The detailed fatty acid and TAG composition, oxidative stability, and thermal behavior of the SL have been discussed in further detail elsewhere (Willett & Akoh, 2018). Ryoto Sugar Ester S-270 (melting point 61°C), a sucrose fatty acid ester, was obtained from Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan). The “S” corresponds to stearic acid as the primary fatty acid esterified to sucrose. The designation of “270” corresponds to the HLB value, ester composition, and purity of the stearic acid. The “2” indicates the product has

an HLB value of 2, and the "70" indicates that the purity of stearic acid is 70%. Per manufacturer details, the Ryoto Sugar Ester S-270 contains 10% sucrose monostearate and 90% sucrose di-, tri-, and poly-stearate. Ascorbyl palmitate (melting point 115°C) and β-sitosterol (melting point 136°C) were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The γ-oryzanol (melting point 151°C) was obtained from TCI America (Portland, OR, USA). All other reagents and solvents were of analytical or HPLC grade and were purchased from Fisher Chemical (Fair Lawn, NJ, USA), Sigma-Aldrich Chemical Co (St. Louis, MO, USA), and J. T. Baker Chemical Co. (Phillipsburg, NJ, USA).

Organogel Preparation

The phytosterol organogels were formed in triplicate by dissolving the blend of β-sitosterol and γ-oryzanol at various concentrations in 10 g of oil (menhaden oil or SL) at 90˚C. The varying blend molar ratios and phytosterol: oil (w/w%) were based on previous literature and are shown in Table 4.2 (Bot & Agterof, 2006). The mixture was stirred constantly for 10 min until fully dissolved and were then poured into a vial. Samples were flushed with nitrogen and cooled at 4˚C to set the gel and stored until further analysis.

Selection of sucrose stearate with HLB value of 2 was based upon previous literature that formed organogels with sucrose stearate (HLB value of 3) and Tween 80 (Lu et al., 2016). There is a need for a balance between solubility and insolubility of the gelators within the solvent (Co & Marangoni, 2014). Individually, it was found that neither sucrose stearate (HLB values 1-15) or ascorbyl palmitate formed organogels at

concentrations between 5-25% with menhaden oil or SL but formed viscous solutions or precipitates. Too much insolubility resulted in a precipitate (HLB values > 8) while too much solubility resulted in a solution, that is, not a gel (HLB values < 8). Sucrose stearate with HLB value 2 resulted in a more viscous appearing solution than sucrose stearate of other HLB values less than 8. The extensive hydrogen bonding between polar hydroxyl groups of both the sucrose stearate and ascorbyl palmitate may contribute to the stabilization of the organogel (Samateh et al., 2011). In triplicate, the SSAP organogels were formed by dissolving the blend of sucrose stearate (HLB value 2) and ascorbyl palmitate at various concentrations in 10 g of oil (menhaden oil or SL) at 110˚C. The varying blend weight ratios and blend: oil (w/w%) are shown in Table 4.3. The solution was stirred constantly for 10 min until fully dissolved and were then poured into a vial. Samples were flushed with nitrogen and cooled at 4˚C to set the gel and stored until further analysis.

Thermal Behavior

Thermal behavior of products was analyzed using a 204 F-1 Phoenix differential scanning calorimeter (DSC) (Netzsch-Gerätebau GmbH, Selb, Germany). DSC analysis was performed using the AOCS Official Method Cj 1-94 (American Oil Chemists' Society, 2017). Crystallization onset and melting completion temperatures were determined using Proteus thermal analysis software (Netzsch-Gerätebau GmbH, Selb, Germany). Samples were analyzed in triplicate and average values were reported.

Rheological Properties

Rheological analyses were performed using an HR-3 Discovery Hybrid Rheometer (TA Instruments, New Castle, DE, USA). A parallel plate (P50 Ti L, diameter

50 mm, gap= 100 ± 0.2 μm) was used for measurements. Temperature was controlled with a Peltier Plate Temperature System (TA Instruments, New Castle, DE, USA). All experiments were conducted in triplicate. Data was analyzed using Trios software (TA Instruments, New Castle, DE, USA).

Heating-cooling sweeps were performed between 5 and 90˚C at 1˚C/min with a frequency fixed at 1 Hz to evaluate the gel formation process. Changes in the slope of complex modulus (G*) as a function of temperature were evaluated using the Savitzky and Golay filter to better visualize the thermal transitions (Savitzky & Golay, 1964). All measurements were done within the linear viscoelastic region.

Flow curves were obtained using a shear rate ranging from 5 to 200 s^{-1} at 5 and 25˚C. The organogels underwent three shear rate sweeps (up-down-up) to eliminate thixotropy and data was obtained in steady state. Apparent viscosity was also evaluated at 5 and 25˚C at shear rates of 10, 50, and 100 s^{-1} to mimic changes during common food processing conditions. The Power-law model was used to fit the non-Newtonian fluid behavior and is defined by the equation: $\sigma = k(\gamma)^n$, σ is shear stress (Pa) and (γ) is shear rate (s^{-1}) (Rocha et al., 2013).

Polarized Light Microscopy

The morphology of the organogels was characterized and imaged by a Leica DMRXA2 Microscope (Leica Micro-systems Canada Inc., Richmond Hill, Canada). To prepare slides, samples were heated to 60˚C and 1 drop of melted sample was placed between a stationary and moving glass plate. The samples were then crystallized by

storing at 4˚C overnight (Da Pieve et al., 2010). Images were acquired using a charged coupled device (CCD) camera (QImaging Retiga, Burnaby, BC, Canada).

X-Ray Diffraction

Samples were first annealed before analysis. Samples and sample pans were placed at 60°C for 1 h in the oven. Melted samples were transferred to the warm sample pans, then held at 25°C for 4 h. Cooled samples were then placed at 4°C for an additional 12 h. Finally, samples were stored at -80°C until analysis. A Bruker D8 Advance x-ray powder diffractometer (Billerica, MA, USA) was used to analyze the polymorphism. The operating conditions were Co Kα radiation (λ=1.79037 Å), voltage 35 kV, amperage 40mA, scanning rate of 0.2°/sec, and a diffraction angle (2θ) range from 10-40°. Samples were analyzed in triplicate and short d-spacings (Å) of the crystalline structures were determined using EVA-diffraction software (Billerica, MA, USA).

Solid Fat Content

Solid fat content (SFC) was analyzed using a Benchtop NMR MQC Analyzer (Oxford Instruments, Oxfordshire, UK) following the AOCS Official Method Cd 16b-93 for non-stabilizing fats (American Oil Chemists' Society, 2017). Calibration standards (Oxford Instruments, Oxfordshire, UK) had SFC values of 0, 32.6, and 70.5%. Samples were melted at 100˚C and held for 15 min then filled in NMR tube to 1.5 cm height. Samples in NMR tubes were tempered at 60˚C for 5 min, cooled to 0˚C for 60 min, and then held at each measuring temperature for 30 min. Measuring temperatures were at 5˚C intervals from 0-40˚C. Samples were analyzed in triplicate and average values were reported.

Oxidative Stability

Oxidative stability was conducted by measuring peroxide (PV) and *p*-anisidine (*p*-AV) values over 12 days. 5 g of sample was weighed into glass vials, closed, and placed in a Reacti-Therm™ Heating and Stirring Module (Thermo Fisher Scientific, Waltham, MA, USA) at 65°C. PV was determined using a method from the International Dairy foundation (Shantha & Decker, 1994). The *p*-AV was determined using AOCS Official Method Cd 18-90 (American Oil Chemists' Society, 2017). Using PV and *p*-AV data, the total oxidation (TOTOX) value was calculated. Oil stability index (OSI) of products was determined using an Oxidative Stability Instrument (Omnion Inc., Rockland, MA, USA) and AOCS Official Method Cd 12b-92(American Oil Chemists' Society, 2017).

Statistical Analysis

Statistical analysis was performed on the data using JMP® software (Version 10, SAS Institute Inc., Cary, NC, USA). Data was analyzed using ANOVA and significant differences ($p < 0.05$) were determined by the Tukey test.

Results and Discussion

Organogel Formation

Figure 4.1 shows the phytosterol and SSAP organogels at various organogelator blend ratios. Organogels were formed for all molar ratios except 2:1 and 3:1 for the phytosterol and SSAP blend, respectively. Because these two ratios did not form organogels, they were not considered for further analysis.

The phytosterol blend formed organogels that appeared translucent with both oil phases at organogelator concentrations of 6 and 8% (w/w) for the 1:1 molar ratio and only at 8% (w/w) for the 1:2 molar ratio. However, when the phytosterol organogels were stored over a 1-month period at 4°C, it was noted that only the 8% (w/w) for the 1:1 molar ratio showed no phase separation. Similar results were seen in previous literature that the 1:1 molar ratio was optimal for the phytosterol blend (Bot & Agterof, 2006). Due to the higher stability over time, this blend was chosen for further analysis, labeled as PM6 and PSL6 for the phytosterol blend organogels that were formed with menhaden oil or SL, respectively.

The SSAP blend formed organogels that were opaque with both oil phases at organogelator concentrations of 10 and 12% (w/w) for the 1:1 molar ratio, and only at 12% for the 1:3 molar ratio. However, when the SSAP organogels were stored over a 1-month period, it was noted that only the 12% (w/w) for the 1:1 molar ratio showed no phase separation. A previous study found that the stability of organogels produced with sorbitan monostearate, a hydrophobic surfactant, was stabilized by addition of polysorbate 20, a hydrophilic surfactant (Murdan et al., 1999). The hydroxyl groups of the ascorbic acid in ascorbyl palmitate may function to help stabilize the organogel. Due to the higher stability over time, this blend was chosen for further analysis, labeled as SM6 and SSL6, for the SSAP blend organogels that were formed with menhaden oil or SL, respectively.

Thermal Behavior

Figure 4.2 shows DSC thermograms of the crystallization (a) and melting (b) profiles of menhaden oil, SL, and organogels (PM6, PSL6, SM6, and SSL6). The SL did

not have significantly different ($p > 0.05$) crystallization onset (C_0) and melting completion (M_c) temperatures than the unmodified menhaden oil at -6.5 and 14.3°C versus -5.3 and 13.6°C, respectively. Menhaden oil had a higher melting peak, and this is likely a TAG molecular species containing stearic acid. Menhaden oil was found to contain a little less than 1 mol% tristearin TAG (Willett & Akoh, 2018). PSL6 and PM6 C_0 and M_c temperatures were not significantly different ($p > 0.05$), at 64.2 and 68.4°C versus 64.9 and 69.2°C, respectively. The phytosterol organogels had higher C_0 and M_c temperatures ($p < 0.05$) when compared to the original oils. A previous study on the phytosterol organogel showed that C_0 and M_c temperatures were between 40-60°C and 60-80°C, respectively, depending on the organogelator ratio and concentration in sunflower oil (Bot et al., 2008). The SSAP organogels had significantly higher ($p < 0.05$) C_0 and M_c temperatures than the phytosterol blend organogels, at 66.5 and 72.4°C, and 66.4 and 72.3°C, for SM6 and SSL6, respectively. There was no significant difference in C_0 and M_c temperatures between SM6 and SSL6 ($p > 0.05$). The SSAP organogels likely have higher M_c temperatures due to the higher melting stearic and palmitic acid found in sucrose stearate and ascorbyl palmitate, respectively, that interact with the non-polar oil phases.

Looking at the DSC curves in Figure 4.2 there was a lower enthalpy peak, with a broad melting and crystallization range at higher temperatures for the phytosterol and SSAP organogels. This suggests that there are likely two different events occurring. The reason why the second peaks have a lower enthalpy is likely due to the small quantity of sample that is melting at the higher temperature, which may not release enough heat to be resolved by DSC (Aquilera & Lillford, 2008). The bulk of the organogel is menhaden oil

or SL, which have relatively low melting points compared to the organogelators that are present in small quantities. Another reason is that the organogel network may have low thermal resistance (Rocha et al., 2013). The thermal behavior results were further discussed when comparing the DSC values to the rheological data.

Rheological Properties

Figure 4.3 shows rheological properties during heating and cooling sweeps for the phytosterol and SSAP organogels (PM6, PSL6, SM6, and SSL6). These results showed that the organogels exhibited thermoreversibility. During heating, shown in Figure 4.3a, complex modulus (G*) values decreased after 30°C. The organogels formed with the SL generally exhibited higher G* values than organogels formed with menhaden oil. This is likely due to the higher amount of saturated fatty acids in the SL. A higher SFC typically results in higher rheological values (Campos et al., 2002). At temperatures above 40°C, the G* values of the SSAP organogels were higher than the phytosterol organogels. At temperatures above 50°C, the behavior of the phytosterol and SSAP organogels were similar, likely due to the complete melting of the organogel network, similarly observed by the thermal behavior. Melting temperatures were estimated from the rheological measurements using Savitzky and Golay filter on inflection point, presenting values of 42.0°C, 45.2°C, 52.1°C, and 53.1°C for PM6, PSL6, SM6, and SSL6, respectively (Savitzky & Golay, 1964). These values correspond to the higher temperature peak on the DSC results (Figure 4.2) and is related to the breakage of the organogel network.

Figure 4.3b shows the G* values during the cooling temperature sweep. A similar rheological pattern was observed for both the heating and cooling sweeps. Only SM6 had G* values that increased dramatically at a lower temperature (44.1°C) than the heating

sweep (52.1°C). There was also no significant difference in G* values between organogels formed with menhaden oil or the SL for the SSAP organogels. Crystallization temperatures were also estimated as 40.1°C, 43.1°C, 44.1°C, 53.1°C, for PM6, PSL6, SM6, and SSL6, respectively. In this temperature range the point at which tan(δ)=1, or gelation point, was also observed. These values correspond to the second peak on the DSC thermograms (Figure 4.2) and are related to the formation of the organogel network.

Figure 4.3c and d show the loss tangent curves for the phytosterol and SSAP organogels (PM6, PSL6, SM6, and SSL6). At temperatures lower than 25.1°C or 38.1-42.1°C for phytosterol or SSAP organogels, respectively, tan(δ) < 1, indicating that the values of G′ were higher than G″. At higher temperatures, the tangent values returned to be lower than 1. The point at which tan(δ) = 1 (G′ to G″ crossover) may serve as a simple indicator of gelation point. When comparing the heating and cooling sweeps, there was no significant difference in gelation point for the phytosterol organogels ($p > 0.05$). However, for the SSAP organogels there was a decrease ($p < 0.05$) in gelation points of SM6 (38.1 and 34.3°C) and SSL6 (42.1 and 39.9°C) for heating and cooling sweeps, respectively.

Figure 4.4 shows the flow curves for the phytosterol (PM6 and PSL6) and SSAP organogels (SM6 and SSL6) at (a) 5 and (b) 25°C. Table 4.4 shows the estimated apparent viscosity and Power-law parameters for the organogels. The Power-law model for S2 curves (steady state) was fitted to all organogels and rheological parameters (flow index **n** and consistency index **k**) were estimated. The flow index values for the phytosterol organogels were slightly higher than the SSAP organogels. The SSAP organogel showed higher pseudoplasticity (lower **n**), consistency index, and viscosity

than the phytosterol organogels, which is characteristic of a more complex and dense gel network (Rocha et al., 2013). The apparent viscosities show that the SSAP organogels had higher apparent viscosities than the phytosterol organogels, and the organogels formed with the SL had higher apparent viscosities than organogels formed with menhaden oil.

Polarized Light Microscopy

Polarized light micrographs of phytosterol and SSAP organogels are shown in Figure 4.5. The micrographs show that the crystal morphology of the organogels were influenced by both oil and organogelator type. The crystalline structures of all organogels exhibited birefringence. The crystal morphology of the organogels produced with menhaden oil (PM6 and SM6) were fibrous or plate-like while the crystal morphology of the organogels produced with the SL (PSL6 and SSL6) were needle-like. The needle-like morphology was found in previous studies to have a higher propensity for oil binding (Dassanayake et al., 2012; Fayaz et al., 2017). Previous studies have found that larger particle size and needle-like morphology correspond to higher viscosity (Bot et al., 2008). The SSAP organogels were shown to exhibit higher apparent viscosities (Table 4.4). The SSAP organogel networks were also denser and filled with many small crystals, which typically corresponds to a harder, more viscous fat.

The differences in morphology based on oil type are likely due to the differences in oil composition. The menhaden oil and SL have different fatty acid compositions, TAG molecular species, and thermal behavior (Willett & Akoh, 2018). When comparing organogelator type, the SSAP blend organogels had a crystal matrix that was more densely packed with smaller crystals. The phytosterol blend had fewer, more sporadic

nuclei, despite both types of organogels being cooled under the same conditions. The SSAP blend may form gel structure more rapidly than the phytosterol blend upon cooling. Samples that form a gel more rapidly have been shown to result in smaller and more numerous crystals (Aquilera & Lillford, 2008). In Figure 4.3b, the SSAP blend organogel generally exhibited higher G* values than the phytosterol organogels. This result, along with the smaller crystals, may indicate the SSAP blend organogels have stronger intermolecular interactions. The phytosterol blend may form gel structure slowly and may form larger crystal structures over time.

Another factor that influences crystallization rate is HLB value, as a lower HLB value typically results in a faster crystallization rate (Yuki et al., 1990). The sucrose stearate used had an HLB value of 2, which is likely a contributing factor to the growth of numerous small crystals. The formation of organogels from the SSAP blend showed that there were intermolecular interactions between the organogelators and oil phase (Dassanayake et al., 2012). Since crystal size, shape, and density all affect the final physicochemical properties, it may be of interest in the future to evaluate the effects of cooling rate and storage temperature on the formation of these organogels to determine the effects on the physicochemical properties.

X-Ray Diffraction

Figure 4.6 shows the x-ray diffraction patterns of menhaden oil, SL, and all organogels (PM6, PSL6, SM6, and SSL6). All the samples were β′ polymorphs with strong short spacing peaks at 4.20, 3.97, and 3.71 Å. The β′ polymorphic form is characteristic of having small to moderate crystal sizes and a smooth soft texture. However, the samples differed in peak intensity. Differences in peak intensity may

correspond to differences in morphology. It has been shown that stronger short spacing peaks correspond to more needle-like morphology (Dassanayake et al., 2012). Figure 4.6 shows that the organogels produced with the SL had stronger short spacing peaks (particularly at 4.20Å) and a needle-like morphology (Figure 4.5). On the other hand, organogels produced with menhaden oil had lower intensity short spacing peaks and exhibited spherulitic crystal morphology (Figure 4.5). There were two additional higher intensity peaks, at 3.50 and 2.70 Å, likely due to the presence of minor components in the menhaden oil such as small amounts of monoacylglycerols, certain TAG species such as POP, cholesterol, or small amounts of crystalline organogelators (Willett & Akoh, 2018; Sawalha et al., 2015; D'Souza et al., 2015; Takeuchi et al., 2002).

Solid Fat Content

Figure 4.7 shows the SFC of the phytosterol (PM6 and PSL6) and SSAP organogels (SM6 and SSL6). The phytosterol organogels had SFC values that started around 2% at 0°C while the SSAP organogels had SFC values that started between 8-11%. The phytosterol had significantly lower SFC values for all temperatures but 35 and 40°C. PSL6 had significantly higher ($p < 0.05$) SFC values between 0-10°C. SSL6 had significantly higher SFC values than SM6 for all temperatures but 35 and 40°C. The SSAP organogels likely have higher SFC values, particularly at low temperatures, because they contained higher melting stearic and palmitic acids from the sucrose stearate and ascorbyl palmitate, respectively. The organogels formed with the SL had higher SFC values than the organogels formed with menhaden oil. This is likely due to the SL having altered TAG molecular species (Willett & Akoh, 2018). During enzymatic modification with ethyl caprate, significant amounts of capric acids were esterified to the *sn*-1,3

positions, which increased the melting point. Melting point can be correlated to both the saturated fatty acid content and SFC. The SL has a higher melting point and higher amounts of saturated fatty acids, which correlated to having higher SFC values. SFC can also be used to predict firmness/hardness of fats, as a higher SFC typically results in a firmer fat (Aquilera & Lillford, 2008). These results align well with the rheological properties and morphology described above. The SSAP organogels had higher SFC values and exhibited a denser gel network of small crystals, which typically indicates a harder fat due to the larger number of crystal-crystal interactions (Aquilera & Lillford, 2008). In terms of rheological properties, the SSAP organogels had higher apparent viscosities.

Oxidative Stability

Table 4.5 shows the OSI values and Figure 4.8 shows the changes in TOTOX values of the organogels (PM6, PSL6, SM6, and SSL6), menhaden oil, and SL. The menhaden oil was relatively stable over the 12 days of storage while the TOTOX value of the SL increased more pronouncedly after day 3 (Figure 4.8). The OSI values (Table 4.5) were significantly lower for the SL than the unmodified menhaden oil ($p < 0.05$). Both the OSI and TOTOX results showed that the SL had lower oxidative stability than the menhaden oil. Though production of SL enzymatically occurs under milder conditions, the reduced oxidative stability is likely due to both the enzymatic reaction process and short-path distillation. Menhaden oil is highly sensitive to oxidation due to the high amounts of polyunsaturated fatty acids. Purification (by removal of free fatty acids or fatty acid ethyl esters) of SL by short-path distillation has been shown in other studies to reduce the tocopherols or antioxidants present in the modified oil (Zou & Akoh, 2013).

Also, the unmodified menhaden oil contained 200ppm TBHQ and 1000ppm mixed tocopherols from the manufacturer (Omega Protein Inc., Reedville, VA, USA) which helped improve its oxidative stability.

The SSAP organogels exhibited significantly improved ($p < 0.05$) oxidative stability when compared to the phytosterol organogels and the original oils. The SSAP organogels were relatively stable over the 12 days of storage (Figure 4.8) and had significantly higher OSI values (Table 4.5) than the respective phytosterol organogels and original oils ($p < 0.05$). This is likely due to the antioxidant contribution of ascorbyl palmitate in the SSAP blend. The ascorbyl palmitate functioned as both an organogelator and antioxidant in this study. The TOTOX values were lower for PSL6 and SSL6 than the original SL for all days indicating PSL6 and SSL6 have a higher oxidative stability than the SL. The OSI values were also higher in the phytosterol organogels than the respective original oils. These results show that the organogels did improve the oxidative stability of the menhaden oil and SL. Therefore, organogelation of SL may be a very good process to improve the oxidative stability of purified SL produced through enzymatic modification.

Conclusions

Both organogels have potential for use as nutraceuticals by utilizing menhaden oil and an MLM-type SL containing health beneficial PUFAs and medium chain fatty acids that provide a quick energy source. Due to their desirable physicochemical properties, these organogels may also find use as low saturated, zero *trans*-fats in the formulation of margarines, shortenings, baked goods, confections, and other food products. Both organogel networks had a relatively low thermal resistance, though the rheological

properties indicated that the SSAP organogel presented higher resistance under shear, likely due to the different morphology and higher SFC values. The differences in physicochemical properties are likely due to the different interactions of the organogelators and oils during the formation of the organogels and may be attributed to the differences in their structures and TAG compositions, respectively.

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Conflicts of Interest: There are no conflicts of interest to declare.

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Table 4.1 Relative fatty acid composition (mol%) of menhaden oil and structured lipid, SL

Sample	Fatty Acid (mol%) ***			
	Position	C10:0	C20:5n3	C22:6n3
Menhaden Oil	Total	ND	22.82±1.85	16.97±1.83
	sn-2	ND	15.32±0.74	29.34±3.08
	sn-1,3**	ND	26.57±2.41	10.79±1.21
SL*	Total	26.73±0.05	17.09±0.39	10.57±0.23
	sn-2	20.33±0.66	12.95±0.36	19.53±0.45
	sn-1,3	29.93±0.26	19.16±0.41	6.09±0.12

Mean ± STD, *n*=3, ND= not detected

*SL reaction conditions: menhaden oil: ethyl caprate substrate molar ratio 1:3, 10% Lipozyme® 435 lipase (w/w), 60°C, stirred at 250 rpm for 16 h in a 1L batch reactor

** *sn*-1,3 mol% determined using equation: *sn*-1,3 (mol%)= [3xtotal mol%- *sn*-2 mol %]/2

***Other major fatty acids were C14:0, C16:0, & C16:1n-7

Table 4.2 Parameters for producing the phytosterol organogels under varying phytosterol blend molar ratios and phytosterol blend: oil (w/w%) ratios

Gel Number	β-Sitosterol/γ-Oryzanol Molar Ratio	Blend to Oil Ratio (w/w%)
1	2:1	4
2		6
3		8
4	1:1	4
5		6
6		8
7	1:2	4
8		6
9		8

Table 4.3 Parameters for producing the sucrose stearate/ascorbyl palmitate (SSAP) organogels under varying SSAP blend molar ratios and SSAP blend: oil (w/w%) ratios

Gel Number	Sucrose Stearate/Ascorbyl Palmitate Molar Ratio	Blend to Oil Ratio (w/w%)
1	3:1	8
2		10
3		12
4	1:1	8
5		10
6		12
7	1:3	8
8		10
9		12

Table 4.4 Estimated Power-law parameters and apparent viscosity of phytosterol organogels (PM6 and PSL6), and sucrose stearate/ascorbyl palmitate (SSAP) organogels (SM6 and SSL6)

Sample*	PM6		PSL6		SM6		SSL6	
	5°C	25°C	5°C	25°C	5°C	25°C	5°C	25°C
n	0.891±0.01	0.740±0.02	0.739±0.03	0.700±0.02	0.529±0.41	0.501±0.05	0.508±0.01	0.467±0.02
k (Pa s^n)	0.153±0.02	0.141±0.01	0.345±0.01	0.330±0.25	0.823±0.18	0.166±0.73	1.043±0.02	0.838± 0.15
R^2	0.999	0.998	0.998	0.999	0.999	0.999	0.997	0.999
η_{10} (Pa s)	0.0861	0.0592	0.127	0.104	0.240	0.0690	0.252	0.183
η_{50} (Pa s)	0.0707	0.0272	0.0825	0.0606	0.132	0.0494	0.134	0.0971
η_{100} (Pa s)	0.0662	0.0226	0.0703	0.0503	0.0916	0.0432	0.0967	0.0658

*P: phytosterol organogel; S: SSAP organogel; M: menhaden oil; SL: structured lipid, reaction conditions and relative fatty acid composition as described in Table 1; 6: 1:1 molar ratio of organogelators, corresponds to gel number from Table 2 and 3 for phytosterol and SSAP organogels, respectively

Table 4.5 Oil stability index (OSI) values for menhaden oil, SL, phytosterol organogels (PM6 and PSL6), and SSAP organogels (SM6 and SSL6)

Sample*	Oxidative Stability Index (OSI) (h)**
M	17.55±1.21[a]
SL	5.52±0.29[b]
PM6	20.88±1.03[c]
PSL6	18.03±0.08[a]
SM6	24.92±3.70[d]
SSL6	23.33±2.06[c]

Mean ± STD, *n*=3. Different letters in the same column are significantly different.

*P: phytosterol organogel; S: SSAP organogel; M: menhaden oil; SL: structured lipid, reaction conditions and relative fatty acid composition as described in Table 1; 6: 1:1 molar ratio of organogelators, corresponds to gel number from Table 2 and 3 for phytosterol and SSAP organogels, respectively

**OSI conducted at 80°C

a) b)

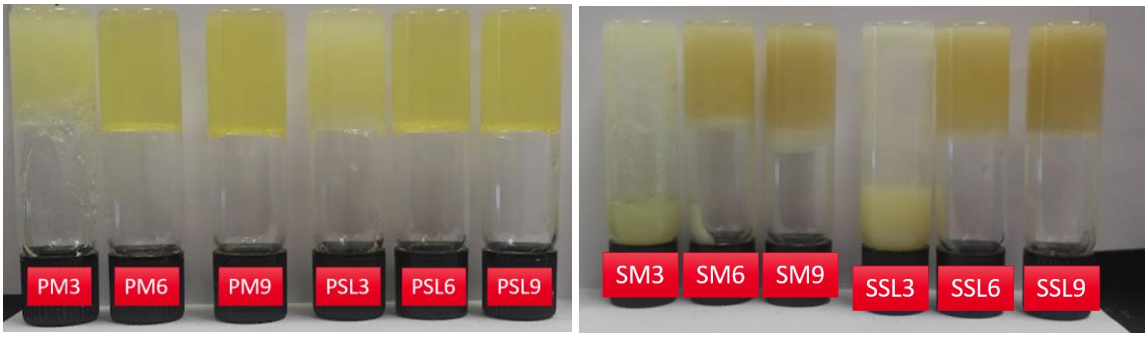


Figure 4.1 Phytosterol (a) and SSAP (b) organogels*

*P: phytosterol organogel; M: menhaden oil; SL: structured lipid, S: SSAP organogel; 6: 1:1 molar ratio of organogelators, corresponds to gel number from Table 2 and 3 for phytosterol and SSAP organogels, respectively

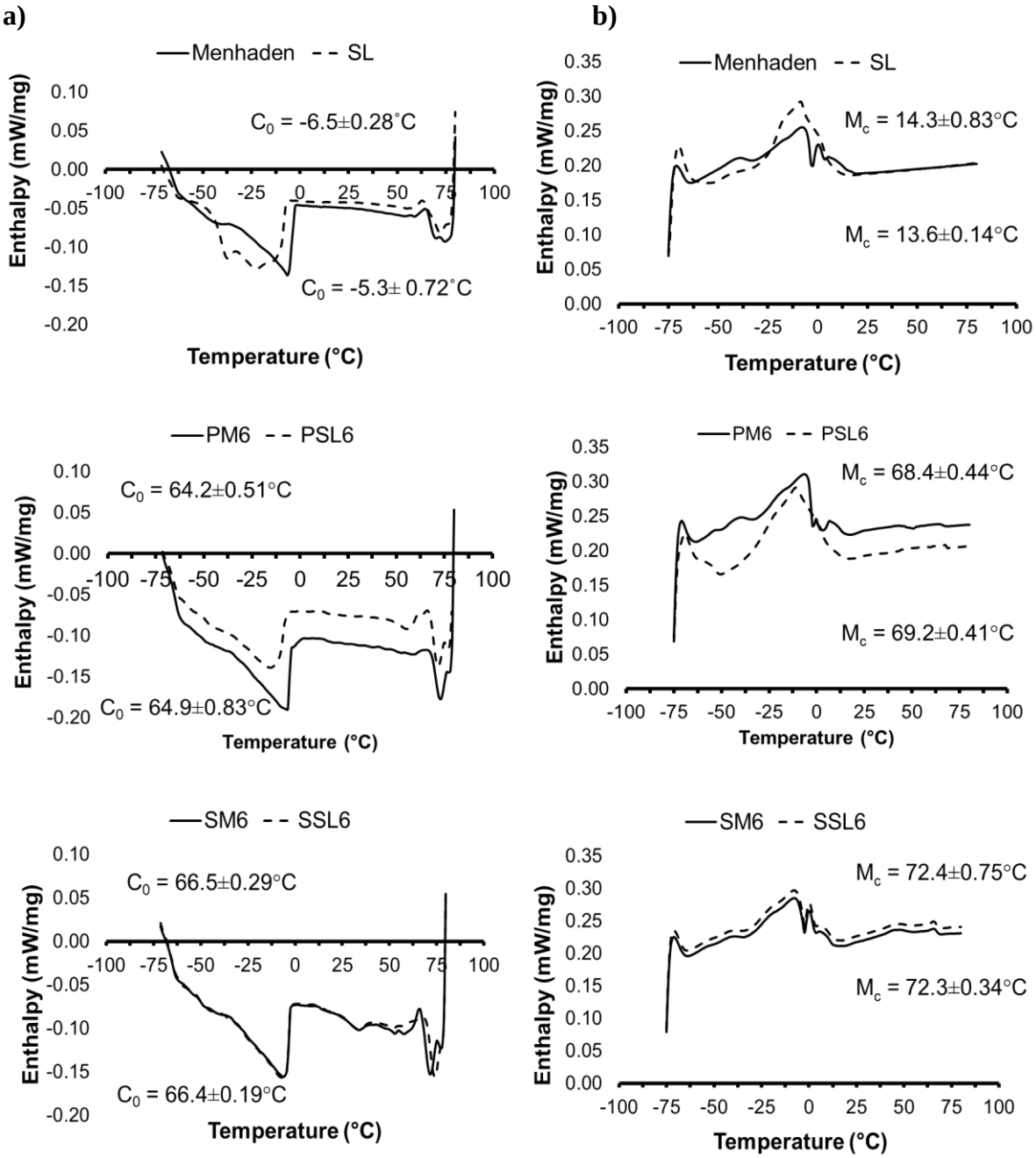


Figure 4.2 Differential scanning calorimetry (DSC) thermograms: (a) crystallization and (b) melting profiles of menhaden oil, SL, and organogels (PM6, PSL6, SM6, and SSL6) *. C_0 and M_c are crystallization onset and melting completion temperatures, respectively. For both (a) and (b), the top temperature value is for SL, PSL6, and SSL6, while the bottom temperature is for menhaden oil, PM6, and SM6

*P: phytosterol organogel; M: menhaden oil; SL: structured lipid, S: SSAP organogel; 6: 1:1 molar ratio of organogelators, corresponds to gel number from Table 2 and 3 for phytosterol and SSAP organogels, respectively

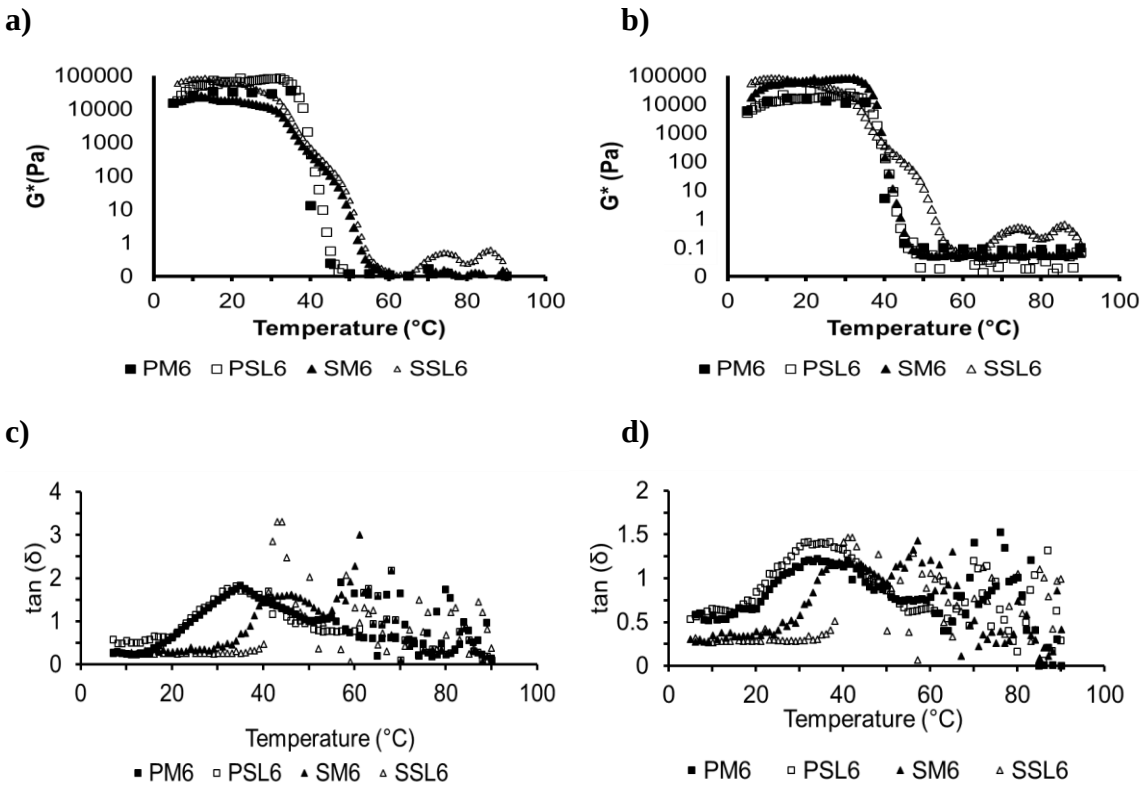


Figure 4.3 Temperature sweep of phytosterol organogels (PM6 and PSL6), and SSAP organogels (SM6 and SSL6) *, a) complex modulus of heating sweep, b) complex modulus of cooling sweep, c) tan delta of heating sweep, and d) tan delta of cooling sweep

*P: phytosterol organogel; S: SSAP organogel; M: menhaden oil; SL: structured lipid, 6: 1:1 molar ratio of organogelators, corresponds to gel number from Table 2 and 3 for phytosterol and SSAP organogels, respectively

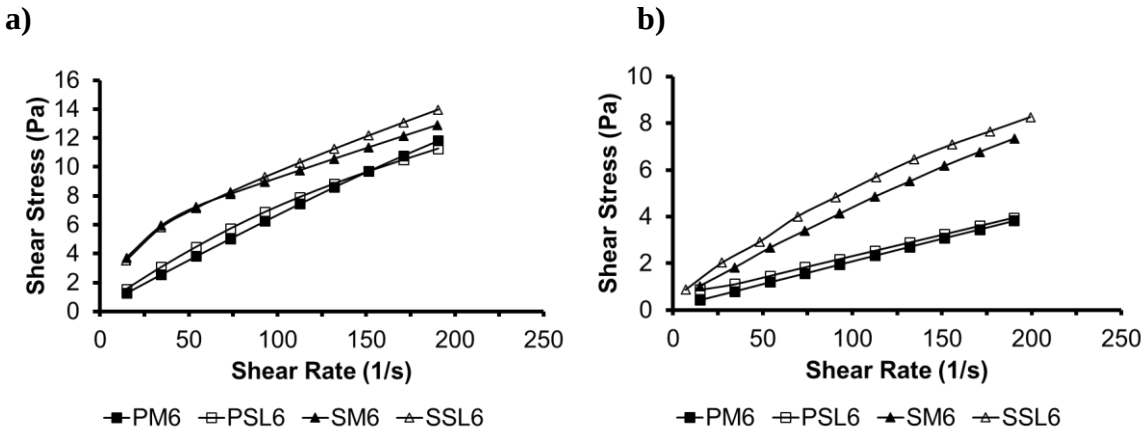


Figure 4.4 Flow curves of phytosterol organogels (PM6 and PSL6), and SSAP organogels (SM6 and SSL6) * at a) 5°C and b) 25°C obtained at steady state

*P: phytosterol organogel; S: SSAP organogel; M: menhaden oil; SL: structured lipid, 6: 1:1 molar ratio of organogelators, corresponds to gel number from Table 2 and 3 for phytosterol and SSAP organogels, respectively

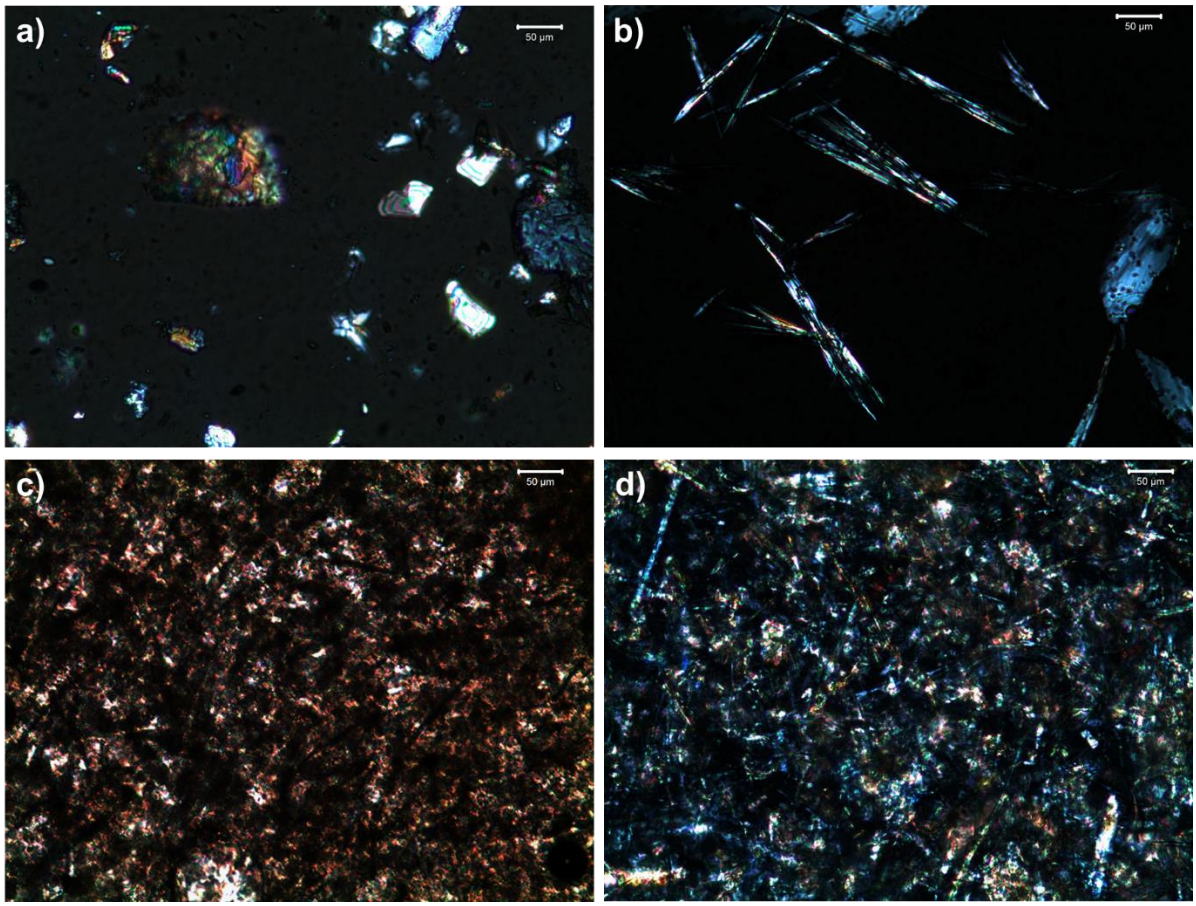
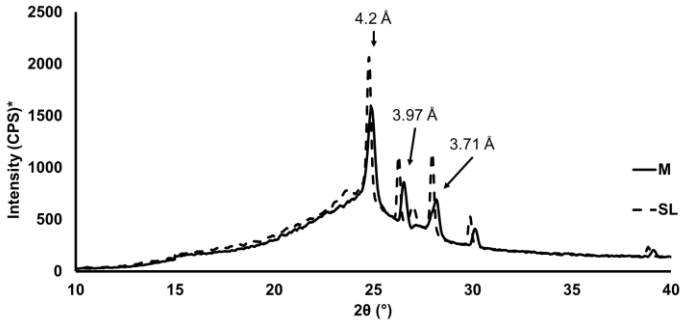


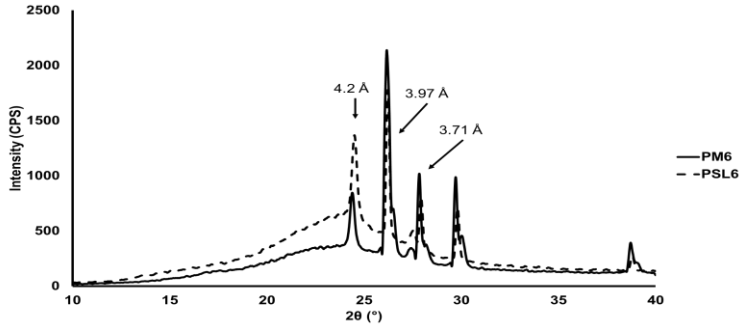
Figure 4.5 Polarized light micrographs of a) PM6, b) PSL6, c) SM6, and d) SSL6*

*P: phytosterol organogel; S: SSAP organogel; M: menhaden oil; SL: structured lipid, 6: 1:1 molar ratio of organogelators, corresponds to gel number from Table 2 and 3 for phytosterol and SSAP organogels, respectively

a)



b)



c)

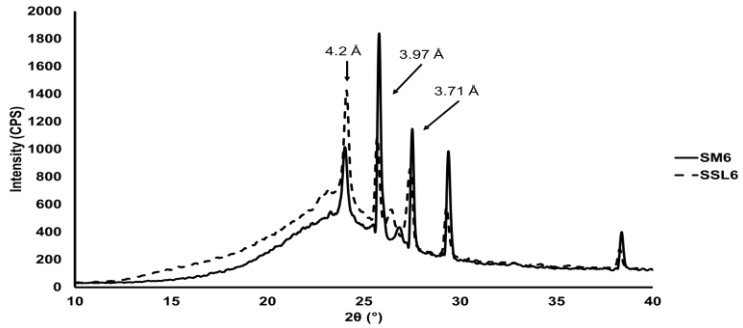


Figure 4.6 Crystalline structures of (a) menhaden oil and SL, (b) phytosterol organogels (PM6 and PSL6), and SSAP organogels (SM6 and SSL6) **

*CPS: counts per second.

**P: phytosterol organogel; S: SSAP organogel; M: menhaden oil; SL: structured lipid, 6: 1:1 molar ratio of organogelators, corresponds to gel number from Table 2 and 3 for phytosterol and SSAP organogels, respectively

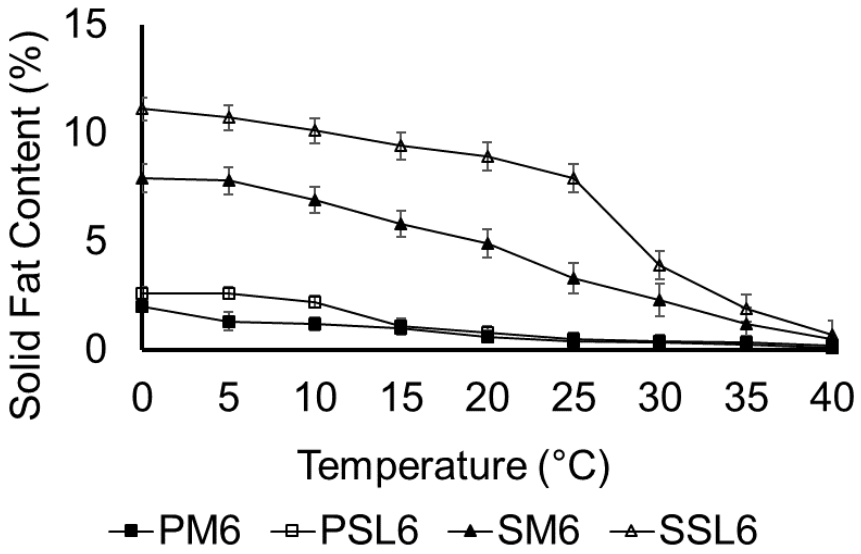


Figure 4.7 Solid fat content of phytosterol organogels (PM6 and PSL6), and SSAP organogels (SM6 and SSL6) *

*P: phytosterol organogel; S: SSAP organogel; M: menhaden oil; SL: structured lipid, 6: 1:1 molar ratio of organogelators, corresponds to gel number from Table 2 and 3 for phytosterol and SSAP organogels, respectively

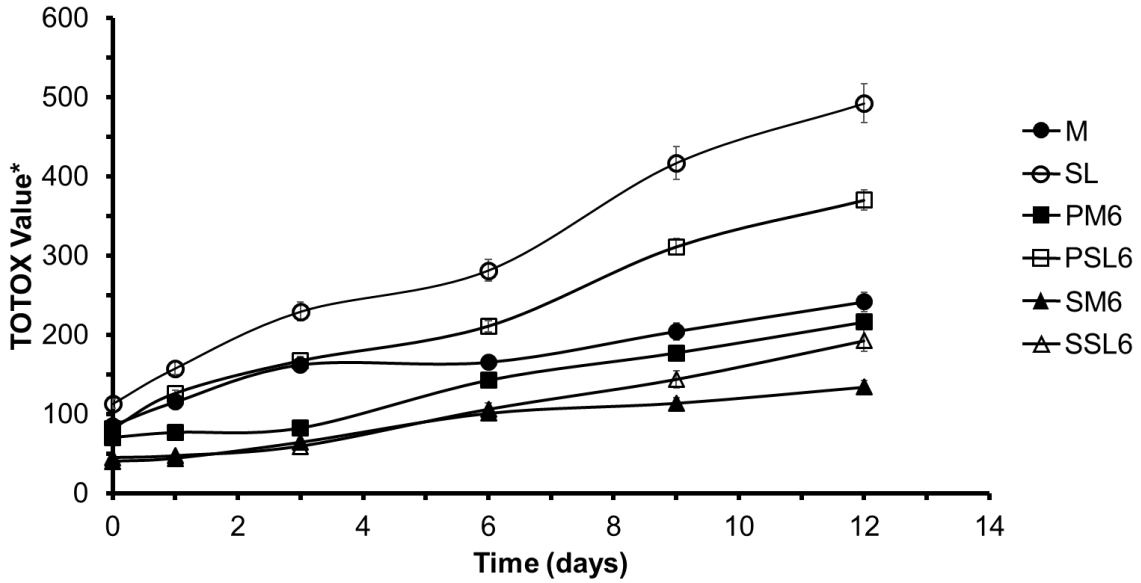


Figure 4.8 TOTOX values for menhaden oil, SL, phytosterol organogels (PM6 and PSL6), and SSAP organogels (SM6 and SSL6) **

*TOTOX value $= 2(\text{PV}) + p\text{AV}$, PV= peroxide value, *p*-AV= *p*-Anisidine value.

*P: phytosterol organogel; S: SSAP organogel; M: menhaden oil; SL: structured lipid, 6: 1:1 molar ratio of organogelators, corresponds to gel number from Table 2 and 3 for phytosterol and SSAP organogels, respectively

CHAPTER 5

ENZYMATIC MODIFICATION OF MENHADEN OIL TO INCORPORATE CAPRYLIC AND/OR STEARIC ACID[1]

[1]Willett, S. A., Martini, S., & Akoh, C. C. 2019. Submitted to the Journal of the American Oil Chemists' Society on 02/12/19.

Abstract

Menhaden oil was enzymatically modified with caprylic (C8:0) and/or stearic acid (C18:0) to produce structured lipids (SL). The goal was to produce SL with high amounts of polyunsaturated fatty acids (PUFA), low level of saturation, and melting point of 25-35°C. Substrate (menhaden oil to acyl donor) molar ratios were 1:1, 1:3, and 1:5 for C8:0, and 1:1, 1:2, and 1:3 for C18:0. Enzyme load was 10% of the total weight of substrates. Time course study determined optimal time for maximum acyl donor incorporation. Linear interpolation estimated molar ratios that yielded SL with 20 or 30 mol% incorporation of C8:0 or C18:0. Enzymatic reactions were also conducted with molar ratios of menhaden oil to acyl donors:C8:0:C18:0 (1:1:3, 1:2:2, and 1:3:1). Lipases from *Candida antarctica*, Lipozyme® 435, and *Rhizomucor miehei*, Lipozyme® RM IM (Novozymes North America, Inc., Franklinton, NC, USA), were compared for all reactions. Total and *sn*-2 fatty acid compositions, TAG molecular species, thermal behavior, volatile lipid oxidation products, solid fat contents, and oxidative stability were compared. When C8:0 was the acyl donor, the 1:3.03 and 1:4.58 molar ratios resulted in incorporation of 20 and 30 mol% C8:0, respectively. With C18:0 as the acyl donor, the 1:1.32 and 1:2.41 molar ratios led to incorporation of 20 and 30 mol% C18:0, respectively. The 1:3:1 molar ratio SL had a crystallization onset (C_0) of 15.3°C and melting completion (M_c) of 33.1°C. The physicochemical properties of these SL suggest that some may be useful in formulating food products such as margarines and spreads.

Keywords: structured lipid, menhaden oil, Lipozyme® 435, Lipozyme® RM IM, caprylic acid, stearic acid

Introduction

Consuming significant amounts of saturated fatty acids (SFA) has been associated with an increase in LDL cholesterol, which increases the risk of heart disease and stroke (He, 2009). It is necessary to reduce the amount of saturated fats and increase the consumption of monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA). Many plant and fish oils are low in saturated fat and contain significant amounts of MUFA and/or PUFA. The issue is that they do not have similar physicochemical properties to highly saturated fats and therefore are not an acceptable replacement for highly saturated fats in food products such as baked goods and confections (Lucca & Tepper, 1994). There is need to find a way to produce low saturated, zero *trans*-fats that mimic similar characteristics of highly saturated fats in terms of desired crystalline structure, thermal behavior, and other physicochemical properties (Lucca & Tepper, 1994). These low saturated fat alternatives may have potential to replace highly saturated fats in a wide range of food products such as margarines/spreads, baked goods, and confections.

One method of producing a low saturated fat is the production of a structured lipid (SL). SL are lipids that have been chemically or enzymatically modified from their natural form (Kim & Akoh, 2015). Enzymatic modification is gaining interest over chemical modification because enzymatic modification typically occurs under milder conditions such as lower temperatures, uses fewer toxic chemicals or occur under solvent-free conditions, and produce more specific products (Kim & Akoh, 2015). Additionally, enzymatic modification produces higher product yields, fewer byproducts than chemical modification, and products are more easily purified. Though enzymes can be more costly, recent advancements in producing immobilized and/or food grade enzymes allow for increasing industry use. Immobilized

enzymes allow for reuse of enzyme and use of food grade enzymes may allow for production of desirable products for use in food and nutraceutical applications.

Specifically, one desirable SL product is a medium-long-medium chain fatty acids (MLM)-type SL, which have been found to have desirable health benefits (Kim & Akoh, 2015). During digestion of an MLM-type SL, *sn*-1,3 specific pancreatic lipase rapidly hydrolyzes the medium chain FA at the *sn*-1,3 positions, while the long chain FA at the *sn*-2 position in monoacylglycerol (MAG) form is conserved and absorbed (Kim & Akoh, 2015). Menhaden oil is of interest as a substrate for enzymatic modification because it contains significant amounts of the omega-3 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) at the *sn*-2 position of the triacylglycerol (TAG), and could be used in the production of MLM-type SL. It has been found that DHA has higher bioavailability when present at the *sn*-2 in significant quantity, therefore increasing the potential health benefits of the MLM-type SL. High amounts of DHA are particularly necessary for expectant mothers or those who have or are at risk of developing cardiovascular disease (Bibus, 2016). Previous studies have modified menhaden oil; however, the TAG molecular species was not determined for the products (Jennings & Akoh, 1999; Xu, Fomuso, & Akoh, 2000; Jennings & Akoh, 2001). A previous study in our lab produced an MLM-type SL of menhaden oil and capric acid using ethyl caprate as the acyl donor, however the melting point was less than 25°C (Willett & Akoh, 2018). Our current interest was to produce MLM-type SL with higher melting point (25-35°C) for use in a wider range of applications.

Because menhaden oil contains high amounts of PUFA, it has a relatively low melting point and does not have the desired thermal behavior compared to highly saturated fats, for use in food products such as baked goods, confections, margarines, and spreads. So, modification

of menhaden oil with SFA may produce a SL with desirable physicochemical properties and added health benefits (Kim & Akoh, 2015). There is increasing interest in using stearic acid (C18:0) in the production of SL because it has been found to be neutral in ability to raise cholesterol levels (Grundy, 1990). Stearic acid is a long chain SFA and has a higher melting point than other SFA such as palmitic, myristic, and caprylic acids (Vieira, McClements, & Decker, 2015). Modification of menhaden oil with stearic acid may produce a SL with a higher melting point and still retain the health benefits of menhaden oil PUFA.

The purpose of this research was to enzymatically modify menhaden oil (which contains no MLM-type TAG and is low in saturated fat) with caprylic and/or stearic acid to produce SL low in saturated fat contents (20 or 30 mol % as C8:0 and/or C18:0) with melting points of 25-35°C. Two biocatalysts were compared, a food grade recombinant lipase from *Candida antarctica*, Lipozyme® 435, and an *sn*-1,3 specific *Rhizomucor miehei* lipase, Lipozyme® RM IM, for their ability to synthesize the specific SL.

Materials and Methods

Materials

Menhaden oil was obtained from Omega Protein Inc. (Reedville, VA, USA). Caprylic acid (C8:0) (≥98 % purity), stearic acid (C18:0) (≥98 % purity), heneicosanoic acid (C21:0) standard, and 2-oleoylglycerol standard were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Lipozyme® RM IM (*Rhizomucor miehei* lipase immobilized on a resin carrier with specific activity of 442 IUN/g (Interesterification Unit per gram) as specified by manufacturer) and Lipozyme® 435 (recombinant lipase from *Candida antarctica*, expressed on *Aspergillus niger*, and immobilized on a

macroporous hydrophobic resin with specific activity of 10000 PLU/g (Propyl Laurate Unit per gram) as specified by the manufacturer) were obtained from Novozymes North America, Inc. (Franklinton, NC, USA). 1 IUN is the amount of enzyme activity which liberates 1 μmol of butyric acid from tributyrin per minute under defined standard conditions. 1 PLU is the amount of enzyme activity which produces 1 μmol of propyl laurate per minute from esterification of lauric acid with *n*-propyl alcohol under defined standard conditions. TAG reference standard mixture (GLC-437 and GLC 570) and GLC-461 FAME mix were purchased from Nu-check Prep, Inc. (Elysian, MN, USA). All other reagents and solvents were of analytical or HPLC grade and purchased from Fisher Chemical (Fair Lawn, NJ, USA), Sigma-Aldrich Chemical Co., and J. T. Baker Chemical Co. (Phillipsburg, NJ, USA).

Acidolysis Reactions

Time course reactions were conducted to determine the optimal time for maximum C8:0 or C18:0 mol % incorporation. Reactions were conducted in triplicate. Five grams of menhaden oil was mixed in a test tube with C8:0 at molar ratios of 1:1, 1:3, and 1:5, or C18:0 at molar ratios of 1:1, 1:2, and 1:3. Substrate molar ratios were selected based on previous studies (Jennings & Akoh, 2001; Yamaguchi, Akoh, & Lai, 2007). Lipase, Lipozyme® 435 or Lipozyme® RM IM, was added at 10% total weight of the substrates, and was based on a previous study (Willett & Akoh, 2018). Reactions were conducted in a 250 rpm shaking water bath at 65°C or 75°C for C8:0 or C18:0 acyl donor substrates, respectively. Individual samples were pulled at 1, 4, 8, 16, 24, and 36 h.

Enzymatic reactions were also conducted with blends of C8:0 and C18:0 in triplicate. Five grams of menhaden oil was mixed with C8:0 and C18:0 at molar ratios of

1:1:3, 1:2:2, and 1:3:1 as described above. These substrate blends were labeled Blend A, B, and C, respectively. Lipase, Lipozyme® 435 or Lipozyme® RM IM, was added at 10% total weight of the substrates. Reactions were conducted in a shaking water bath at 75°C for 24 h with mixing at 250 rpm. Reactions were stopped by filtering through anhydrous sodium sulfate column. Products were placed in vials, flushed with nitrogen, and stored at -20˚C until further analysis.

Linear Interpolation

The maximum mol % incorporation for each substrate ratio was plotted against substrate molar ratios. Linear interpolation was used to estimate molar ratios that would yield SL with 20 or 30 mol % incorporation of C8:0 or C18:0. Target C8:0 or C18:0 level (y) was plugged into respective linear equations and the required C8:0 or C18:0 ratio (x) was determined.

To validate this method, acidolysis reactions were conducted as described above. Validation was confirmed if there was 20 or 30 mol% incorporation for each optimal substrate ratio as determined by linear interpolation. In a 1L stirred batch reactor, 500g of menhaden oil was mixed with either C8:0 or C18:0 at the substrate ratios determined by linear interpolation to incorporate 20 or 30 mol% of C8:0 or C18:0. Lipozyme® 435 (10% total substrate weight) was added. Substrates and enzyme were mixed at 250 rpm using a SL 2400 StedFast stirrer (Fisher Scientific Co., Fair Lawn, NJ, USA), fitted with a 4-blade propeller for 24 h at 65 or 75°C. Reactions were stopped by filtering through a Büchner funnel containing filter paper and anhydrous sodium sulfate (to remove moisture). Excess free fatty acids (FFA) were removed from the product by short-path distillation, with modification of the temperature from 185°C to 100°C (Willett & Akoh, 2018). FFA content

was determined using AOCS method Ca 5a-40 (American Oil Chemists' Society, 2017). Percent yield was calculated based upon FFA content and product weight pre-and post-short-path distillation. Products were placed in opaque Nalgene bottles, flushed with nitrogen, and stored at -80˚C until further analysis.

Large-Scale Production of SL Under Optimal Reaction Conditions

Using optimal enzymatic reaction parameters, a large-scale production was performed in a 5L stirred batch reactor. 1 kg of menhaden oil and optimal acyl donor substrate molar ratios of C8:0 (1:3.03 or 1:4.58), C18:0 (1:1.37 or 1:2.46), or a blend of C8:0 and C18:0 (at a molar ratio of 1:3:1, Blend C) were added to a 5L batch reactor. Lipozyme® 435 (10% total substrate weight) was added. Substrates and enzyme were mixed at 250 rpm using a SL 2400 StedFast stirrer (Fisher Scientific Co., Fair Lawn, NJ, USA), fitted with a 4-blade propeller for 24 h at 65°C or 75°C. Reactions were stopped by filtering through a Büchner funnel as described above. Excess FFA were removed from the product by short-path distillation, and FFA content and percent yield were determined as described above. Products were placed in opaque Nalgene bottles, flushed with nitrogen, and stored at -80˚C until further analysis.

Fatty Acid Composition Analysis

To isolate TAG of small-scale reaction products, samples were spotted on TLC plates and developed in hexane: diethyl ether: formic acid (60:40:1.6, v/v/v) with triolein TAG standard. TLC plates were sprayed with 0.2% 2',7'-dichlorofluorescein in methanol to detect bands and the corresponding TAG band was scraped off. Scraped off bands or purified large-scale SL products (post short-path distillation) were converted to fatty acid

methyl esters (FAME) using AOAC Official Method 996.01 (Satchithanandam, Fritsche, & Rader, 2001). A known amount of internal standard C21:0 (100 µL of 20 mg/ml was added to 100mg of purified large-scale product, while 50 µL was added to scraped off TLC bands) was added. Samples were saponified by adding 2mL of 0.5 N NaOH and incubated at 100˚C for 5 min. After cooling, 2 mL of 14% BF_3 in methanol was added and samples were incubated at 100˚C for 5 min. Then, 2 mL of hexane and 2mL of saturated NaCl solution were added and vortexed for 2 min to extract the FAME. Samples were centrifuged at 1,000 rpm for 5 min and then the organic upper layer containing hexane and FAME was passed through an anhydrous sodium sulfate column to remove moisture before GC analysis. GLC-461 FAME mix was used as the external standard.

For the *sn*-2 positional analysis, an ethanolysis reaction was conducted as described by Solaesa et al. (2014). The lipid sample was mixed with ethanol (1:3 [w/w]) and 4% Lipozyme® 435 [w/w] of total substrate in a 30˚C shaking water bath at 250 rpm for 4 h. While menhaden oil and ethanol were not miscible before the reaction, after the 4 h reaction the mixture became homogenous due to the polarity of the 2-MAG and ethyl esters that were formed (Solaesa et al., 2014). Samples were filtered through anhydrous sodium sulfate column and concentrated with nitrogen to one-third of their original volume. Samples were spotted on a TLC plate with 2-monoolein as standard, developed, and converted to FAME as described above. The average relative FAME content was calculated as mol% and standard deviations were determined.

GC Analysis

FAME were analyzed using an Agilent 6890 N GC system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and Supelco SP-2560 capillary column (100 m x 0.25 mm ID, 0.20 µm film) (Sigma-Aldrich Co. St.

Louis, MO, USA). GC analysis and procedures were conducted as described elsewhere (Willett & Akoh, 2018; Ifeduba & Akoh, 2013). Briefly, 1 μL of the sample was injected at a split ratio of 50:1 and 5:1 respectively, for total FA composition and *sn*-2 FA composition analysis. The carrier gas (He) flow was 1.1 mL/min and the detector temperature was 250˚C. The oven was held at 140˚C for 5 min, then increased to 240˚C at a rate of 4˚C per minute, and held isothermally for 15 min. The average relative FAME content was calculated as mol % and standard deviations were reported.

Triacylglycerol Molecular Species

The TAG molecular species of products were analyzed using an Agilent 1260 Infinity Quaternary LC HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a Sedex Model 85 evaporative light scattering detector (ELSD) (Sedere, Alfortville, France) and a 4 mm x 250 mm, 5 μm particle size, Ultrasphere C18 reverse-phase analytical column (Beckman Coulter Inc., Pasadena, CA, USA). HPLC analysis and procedures were conducted as described elsewhere (Willett & Akoh, 2018). Peak separation was achieved using a gradient mobile phase of acetonitrile, acetone: methyl *tert*-butyl ether (MTBE) (90:10, v/v), and chloroform with a flow rate of 1 mL/min (Willett & Akoh, 2018; Solaesa et al., 2014). The column temperature was 25˚C, the ELSD drift tube temperature was set to 70°C, the nebulizer gas pressure was at 3.5 bar, and gain was set to 8. 20 μL of sample was injected and sample concentration was 10 mg/mL in acetone. For sharper peaks, MTBE was mixed with acetone in the mobile phase. Chloroform was used in the mobile phase to have better solubility of sample in the mobile phase (Willett & Akoh, 2018; Solaesa et al., 2014). Menhaden oil contains many different TAG species because it contains so many different fatty acids. This makes analysis of the TAG species difficult. There have been 553 TAG species of

menhaden oil identified without including the regioisomers and enantiomers (Solaesa et al., 2014). Because menhaden oil contains many different TAG molecular species with the same equivalent carbon number (ECN), a modified ECN equation was used to improve peak identity accuracy (Solaesa et al., 2014). While the modified ECN equation accounts for high amounts of MUFA and PUFA, each peak can still have multiple different TAG (Solaesa et al., 2014). The average mol % and standard deviations were determined.

Solid Fat Content

Solid fat content (SFC) was analyzed using a Benchtop NMR MQC Analyzer (Oxford Instruments, Oxfordshire, UK) following the AOCS Official Method Cd 16b-93 for non-stabilizing fats (American Oil Chemists' Society, 2017). Samples were melted at 100°C for 15 min and filled in NMR tube to 1.5 cm height. Samples in NMR tube were tempered at 60°C for 5 min, cooled to 0°C for 60 min, and then held at each measuring temperature for 30 min. Measuring temperatures were at 5°C intervals from 10-40°C. Samples were analyzed in triplicate and average values (%) were determined.

Thermal Behavior

Thermal behavior of all products was analyzed by differential scanning calorimetry (DSC) using a 204 F-1 Phoenix differential scanning calorimeter (Netzsch-Gerätebau GmbH, Selb, Germany). The AOCS Official Method Cj 1-94 was used for DSC analysis (American Oil Chemists' Society, 2017). Crystallization onset and melting completion temperatures were determined by Proteus thermal analysis software (Netzsch-Gerätebau GmbH, Selb, Germany). Samples were analyzed in triplicate and average values were determined.

Oxidative Stability

Oxidative stability was analyzed by measuring peroxide (PV) and *p*-Anisidine (*p*-AV) values over 12 days of storage. 5 g of sample was weighed into glass vials, closed, and placed in a Reacti-Therm™ Heating and Stirring Module (Thermo Fisher Scientific, Waltham, MA, USA) at 65°C. PV was determined using a method from the International Dairy foundation (Shantha & Decker, 1994). The AOCS Official Method Cd 18-90 was used to determine *p*-AV (American Oil Chemists' Society, 2017). Using PV and *p*-AV data, the total oxidation (TOTOX) value was calculated over the 12 days. Oil stability index (OSI) of products was determined using an Oxidative Stability Instrument (Omnion Inc., Rockland, MA, USA) and AOCS Official Method Cd 12b-92 (American Oil Chemists' Society, 2017). Samples were analyzed in triplicate, and average values were determined.

Volatile Oxidation Products

Volatile lipid oxidation products of the samples were analyzed in triplicate using a combination of methods (Qiu et al., 2017; Jiang, Wu, Zhou, & Akoh, 2016). 5 g of each sample was placed in 40 mL head-space vials sealed with polytetrafluoroethylene/silica septa and polypropylene opentop caps. Samples were equilibrated with magnetic stirring (500 rpm) at 65°C for 30 min. Then a pre-conditioned (250°C for 30 min) 65 μm thickness polydimethylsiloxane/divinylbenzene (PDMS/DVB) solid-phase microextraction (SPME) fiber (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was manually injected into the vial headspace at a fixed position and exposed for an additional 30 min at 65°C. After sampling, the fiber was desorbed thermally at 250°C for 5 min in the GC injection port (5890 Series II GC system, Agilent Technologies Inc., Santa Clara, CA, USA). A DB5 column (30 m, 0.32 mm x

0.75 mm film thickness, Agilent Technologies, Santa Clara, CA, USA) was used to separate the volatiles. The oven temperature was kept at 45°C for 5 min, increased to 55°C at a rate of 1°C/min, increased from 55 to 90°C at a rate of 5°C/min, increased to 225°C at a rate of 15°C/min, and finally kept at 225°C for 4 min. The carrier gas was helium with a flow rate of 1 ml/min. Analysis was carried out in splitless mode. MS detection was performed on a single-quadrupole mass spectrometer (HP 5972 MSD, Agilent Technologies, Inc., Santa Clara, CA, USA) in full scan electron ionization mode, and conditions were: ion source, 230°C; electron energy, 70 eV; transfer line, 280°C; quadrupole, 150°C; *m/z* range, 35-350; solvent delay, 0 min. Volatile compounds were tentatively identified using the NIST 02 and Wiley 11 libraries. The relative peak area percentages of the volatile compounds were determined, and standard deviations were reported. Identifications were further confirmed by calculating and comparing retention time indices with those previously reported in literature (Qiu et al., 2017; Van den Dool & Kratz, 1963; Hsleh, Williams, Vejaphan, & Meyers, 1989; Horiuchi, Umano, & Shibamoto, 1998; Shahidi, 1998).

Statistical Analysis

Statistical analysis was performed on the data using JMP® software (Version 10, SAS Institute Inc., Cary, NC, USA). Data was analyzed using ANOVA and significant differences ($p \leq 0.05$) were determined by the Tukey test.

Results and Discussion

Time Course of Acidolysis Reactions

Time course reactions determined the optimal time for maximum mol % incorporation of C8:0 and C18:0. Figure 5.1 shows that there was no significant difference ($p > 0.05$) between total mol % incorporation for 24 and 36 h for all reactions. Therefore, the reaction time selected for the large-scale reactions was 24 h. For all reactions, the higher the substrate molar ratio, the higher the incorporation of either C8:0 or C18:0. Approximately 35 mol% of either C8:0 or C18:0 was incorporated after 24 h when the substrate molar ratio was 1:5 or 1:3, for menhaden oil:C8:0 or menhaden oil:C18:0, respectively. Approximately 5-15 mol% of either C8:0 or C18:0 was incorporated after 24 h when the substrate molar ratio was 1:1, for menhaden oil:C8:0 or menhaden oil:C18:0, respectively. Total mol% incorporation values were below 20 mol% and higher than 30 mol%. These results indicate that the molar ratios selected were ideal for using linear interpolation to determine optimal substrate molar ratios to incorporate 20 or 30 mol% of either C8:0 or C18:0.

Linear Interpolation Validation

Previous studies used different experimental design methods for optimizing enzymatic reactions to produce SL, including response surface methodology (RSM), Taguchi method, and linear interpolation (Ifeduba & Akoh, 2013; Willett & Akoh, 2018; Ifeduba, Martini, & Akoh, 2016). Linear interpolation is a curve fitting method that uses the linear regression equation of a data set to determine specific values that lie within the data set. Linear interpolation is of interest in the production of tailor-made SL because it

may offer a relatively simple way to determine optimal substrate molar ratios to produce a specific SL (certain mol% incorporation), thus increasing its use in conducting enzymatic reactions at an industrial scale.

Figure 5.2 shows total mol % incorporation of C8:0 or C18:0 plotted against substrate molar ratios. Linear interpolation was used to estimate molar ratios that would yield targeted SL with 20 or 30 mol % incorporation of C8:0 or C18:0. For all reactions, there were strong linear correlations, with R^2 values between 0.988 and 0.998. These results align well with a previous study that found that enzymatic reactions had strong linear correlation between substrate ratio and fatty acid incorporation (Ifeduba et al., 2016). For reactions with Lipozyme® 435, the molar ratios of 1:3.03 and 1:4.58 were determined to be optimal for incorporating 20 and 30 mol % C8:0, and the molar ratios of 1:1.37 and 1:2.46 were determined to be optimal for incorporating 20 and 30 mol % of C18:0. For reactions with Lipozyme® RM IM, 1:3.06 and 1:4.52 were determined to be optimal for incorporating 20 and 30 mol % C8:0 and the molar ratios of 1:1.26 and 1:2.35 were determined to be optimal for incorporating 20 and 30 mol % of C18:0.

To validate the linear interpolation results, acidolysis reactions were conducted using the optimal substrate ratios. Table 5.1 shows that each reaction under the optimal ratios confirm that the linear interpolation is valid for both enzymes. The major FA in menhaden oil were: C14:0, C16:0, C16:1, C20:5, C22:5, and C22:6. Table 5.1 shows that after modification, the majority of C8:0 and C18:0 were found at the *sn*-1,3 positions. EPA and DHA total amounts were reduced to approximately 12 mol% of EPA and 11 mol% DHA in the SL. Most of the PUFA were present in higher amounts at the *sn*-2 position for menhaden oil and SL, with higher amounts of DHA (21.6-29.4 mol%) than

EPA (11.1-16.4 mol%). For reactions with Lipozyme® 435, the molar ratios of 1:3.03 and 1:4.58 incorporated 20 and 30 mol % C8:0; the molar ratios of 1:1.37 and 1:2.46 incorporated 20 and 30 mol % of C18:0. For reactions with Lipozyme® RM IM, the molar ratios of 1:3.06 and 1:4.52 incorporated 20 and 30 mol % C8:0; the molar ratios of 1:1.26 and 1:2.35 incorporated 20 and 30 mol % of C18:0. These results showed that linear interpolation is a relatively simple and accurate way to determine optimal molar ratios of substrates when producing tailor-made SL with desired incorporation of specific fatty acids.

Caprylic/Stearic Blend Acidolysis Reactions

The enzymatic reactions with blends of C8:0 and C18:0 produced SL with varying mol % incorporation of C8:0 and C18:0. Table 5.1 shows that the 1:1:3 ratio (Blend A) incorporated the highest amount of C18:0, at 23.04 and 16.60 mol% when Lipozyme® 435 and Lipozyme® RM IM were the biocatalysts, respectively. The 1:3:1 ratio (Blend C) incorporated the highest amount of C8:0 (13.46 and 9.02 mol%), and about equal amounts of C8:0 and C18:0. All SL produced with the C8:0 and C18:0 blends incorporated between 27-30 mol % total of C8:0 and C18:0. When comparing each blend (A, B, and C), Lipozyme® 435 incorporated significantly more ($p < 0.05$) C8:0 and C18:0 than Lipozyme® RM IM. Total C8:0 and C18:0 mol % incorporation decreased as C8:0 increased within the starting substrate ratios. The low incorporation of C8:0 and C18:0 by Lipozyme® RM IM has been reported in a previous study (Sellappan & Akoh, 2001). This may be due to the acidification of the lipase environment by increased C8:0, which may cause possible reduction in lipase activity (Sellappan & Akoh, 2001).

TAG Molecular Species

It has been reported that pancreatic lipase may not hydrolyze all FA in the same way, particularly very long chain PUFA, so the ethanolysis method was chosen (Solaesa et al., 2014). Lipozyme® 435 behaves as an *sn*-1,3 specific lipase when mixed with excess ethanol, and yields higher 2-MAG and lower acyl migration compared to the pancreatic lipase method (Solaesa et al., 2014).

Before modification, the major TAG species of menhaden oil were: MDhM/PEpDh, PoPoEp/PDhDh, MMEp/PoEpPo/PoDhP, MPoEp/MDhP, PPDh/PPoPo, as shown in Table 5.2. The letters correspond to the following FA: M= C14:0, P= C16:0, Po= C16:1, S= C18:0, St= C18:4, Ep= C20:5, Dp= C22:5, and Dh= C22:6. The TAG molecular species designations do not specify stereochemical configuration, however the positional distribution of the fatty acids were predicted based on the total and *sn*-2 fatty acid compositions as determined by GC.

After modification with C8:0 as the acyl donor, the major TAG were: CyCyCy, CyStCy/CyDpCy, StEpCy/StDhCy, StStSt/CyEpCy, CyDhCy, CyPoCy, CyMCy, MDhM/PEpDh, and MPoEp/MDhP. The letters correspond to the FA as described above, and Cy: C8:0. Table 5.2 shows the SL with 30 mol% incorporation of C8:0 when Lipozyme® 435 was the biocatalyst (LC30) had the highest amount (32.6 mol%) of MLM-type TAG, and 23.8 mol% of the specific MLM-type TAG with C8:0 at the *sn*-1,3 and PUFA at the *sn*-2. On the other hand, the SL of C8:0 when Lipozyme® RM IM was the biocatalyst (RC30) had significantly ($p < 0.05$) lower amounts of specific MLM-type TAG (31.1 total and 15.5 mol% specific MLM-type TAG). These results agree with previous studies that found Lipozyme® RM IM may be selective against DHA, and

Lipozyme® 435 was found to produce more of the specific MLM-type TAG when menhaden oil and ethyl caprate were the reaction substrates (Willett & Akoh, 2018; Sellappan & Akoh, 2001). Because more of the specific TAG were formed when Lipozyme® 435 was the biocatalyst, Lipozyme® 435 was chosen as the enzyme for the large-scale reactions.

When C18:0 was the acyl donor substrate, the major TAG for the SL were: PEpPo/PEpEp, MDhM/PEpDh, MPoEp/MDhP, SDpDp/MPPo/StDhS, SStS/SDpS, SEpS, SDhS, SPoS, SMS, and SSS. Table 5.2 shows the SL with 30 mol% incorporation of C18:0 when Lipozyme® 435 was the biocatalyst (LS30). Many of the new TAG molecular species that were formed were TAG with C18:0 at the *sn*-1,3 positions, at 34.4 mol%. Also, 17.4 mol% of the new TAG formed were PUFA at the *sn*-2 and C18:0 at the *sn*-1,3 positions. 12.1 mol% of TAG that were formed when Lipozyme® RM IM was the biocatalyst were TAG with C18:0 at the *sn*-1,3 and myristic or palmitoleic acid at the *sn*-2 positions. Because more of the specific TAG were formed when Lipozyme® 435 was the biocatalyst, Lipozyme® 435 was chosen as the enzyme for the large-scale reactions also. Successful use of Lipozyme® 435, a commercial, food grade, immobilized lipase improves the viability of enzymatic interesterification at an industrial scale for food and nutraceutical applications.

When a blend of C8:0 and C18:0 were the acyl donor substrates, the major TAG were: CyCyCy, CyStCy/CyDpCy, StEpCy/StDhCy, StStSt/CyEpCy, CyDhCy, CyPoCy, CyMCy, PoPoEp/PDhDh, PoEpO/PoPoDh/CyPCy, MMEp/PoEpPo/PoDhP, PPDh/PPoPo, SDpDp/MPPo/StDhS, SStS/SDpS, SEpS, and SDhS. Table 5.2 shows that of the new TAG molecular species formed, Lipozyme® 435 Blend C contained a

significantly higher ($p < 0.05$) amount (31.9 mol%) of MLM-type TAG, and 18.8 mol% were the specific MLM-type TAG with C8:0 at the *sn*-1,3 and PUFA at the *sn*-2 positions. These SL contained both MLM-type TAG and higher stearic acid content. Because more of the specific TAG were formed, Lipozyme® 435 and Blend C was chosen as the enzyme and substrate molar ratio, respectively, for the large-scale reactions.

Fatty Acid Composition of Large-Scale Products

Table 5.3 shows the fatty acid composition of menhaden oil and each SL. Almost all large-scale produced SL were statistically similar ($p > 0.05$) to the fatty acid composition of the small-scale reactions in Table 5.1. LC 20 contained 19.34 mol% of C8:0 and LS 20 contained 19.50 mol% of C18:0. LC 30 contained 29.03 mol% of C8:0 and LS 30 contained 29.71 mol% of C18:0. The Lipozyme® 435 Blend C SL contained 16.24 mol% of C8:0 and 13.04 mol% of C18:0. Large-scale produced Blend C with Lipozyme® 435 in Table 3 had about 3 mol% higher incorporation of C8:0 than the small-scale reaction in Table 5.1. After short-path distillation, the calculated percent FFA was less than 0.1% for all SL. Percent yield after short-path distillation was between 86.0 and 96.5% for all SL. Table 5.3 showed that all the SL contained high amounts of PUFA, with approximately 24-28 mol% of EPA and DHA. DHA was found in a higher amount than EPA at the *sn*-2, having between 17-25 mol% DHA at the *sn*-2 for all SL. Similar fatty acid incorporation between small and large-scale SL show that these SL can be successfully produced on a large-scale.

Solid Fat Content

Figure 5.3 shows the SFC values of menhaden oil and each SL at 0-40°C. At 0°C, LS 30 had an SFC value of approximately 30%. LS 30 had a significantly higher ($p < 0.05$) SFC value from 0-30°C. LS 20 and Lipozyme® 435 Blend C had SFC values of approximately 15% at 0°C and had statistically similar SFC values from 0-40°C. LS 20 and Lipozyme® 435 Blend C had significantly higher ($p < 0.05$) SFC values than LC 20, LC 30, and menhaden oil from 0-30°C. LC 20 and LC 30 had SFC values of approximately 5% at 0°C. LC 30 had a significantly higher ($p < 0.05$) SFC value at 5 and 10°C, though LC 20 and LC 30 did not have significantly different ($p > 0.05$) SFC values from 15-40°C. Menhaden oil had an SFC value of approximately 2% at 0°C. Menhaden oil had significantly lower ($p < 0.05$) SFC values for 5 and 10°C, and from 15-40°C, there was no significant difference in SFC values for LC 20, LC 30, and menhaden oil. SFC is an important parameter in applications such as margarines for determining spreadability, texture, and mouthfeel. It has been reported that at 20-22°C, SFC should have values between 15-35% for desired spreadability and texture (Vieira et al., 2015). At 20°C, SFC should be at least 10% to avoid potential oil separation. At 35-37°C, SFC should be around 1-3% for desirable thickness and mouthfeel (Vieira et al., 2015). At 20°C, LS 30 had an SFC value of around 17%, indicating LS 30 may be suitable for use in the formation of margarines. LS 20 and Lipozyme® 435 Blend C had SFC values of around 10% at 20°C, indicating that these SL may not be suitable in providing desired spreadability and texture, but may still have acceptable stability. LC 20, LC 30, and

menhaden oil all had SFC values near 0% at 20°C, indicating these lipids would likely not be suitable for the formation of margarines without further modification.

Thermal Behavior

Enzymatic modification significantly altered the thermal behavior of the menhaden oil. Table 5.4 and Figure 5.4 shows that menhaden oil has crystallization onset (C_0) and melting completion (M_c) temperatures of -5.3 and 13.6°C. When C8:0 was the acyl donor, the C_0 and M_c were 4.0 and 22.5°C, and 8.5 and 24.5°C for 20 and 30 mol% incorporation, respectively. The thermal behavior of the SL with 20 or 30 mol% incorporation of caprylic acid showed there was an increase ($p < 0.05$) in C_0 and M_c temperatures from the 20 to 30 mol% incorporation. The melting peak was very broad for both samples, as shown in Figure 4. The SL with 30 mol% incorporation (LC 30) had the desired thermal behavior of melting between 25-35°C, though on the low end at 24.5°C for the M_c temperature.

When C18:0 was the acyl donor, the C_0 and M_c were 16.9 and 34.1°C, and 21.9 and 43.9 °C, for 20 and 30 mol% incorporation, respectively. The thermal behavior of the SL with 20 or 30 mol% incorporation of stearic acid showed there was a statistically significant ($p < 0.05$) increase in C_0 and M_c temperatures from the 20 to 30 mol% incorporation ($p < 0.05$). The melting peak was very broad for both samples, as shown in Figure 4. The SL with 20 mol% incorporation of stearic (LS 20) had the desired thermal behavior of melting between 25-35°C, with it being on the high end at 34.1°C for the M_c temperature.

For the C8:0 and C18:0 blends, the C_0 and M_c were 27.4 and 50.0°C, 22.1 and 42.5°C, and 15.3 and 33.1°C, respectively for Blend A, B, and C. The SL produced by incorporating caprylic and stearic acid significantly increased ($p < 0.05$) the C_0 and M_c temperatures from the unmodified menhaden oil. From Blend A to Blend C there was a decrease in incorporation of stearic acid, but an increase in the incorporation of caprylic acid. The DSC results in Table 5.4 and Figure 5.4 show there was a decrease in C_0 and M_c temperatures from Blend A to C. Only Blend C had the desired melting point of 25-35°C and also incorporated the highest amount of MLM-type TAG. This SL had a M_c temperature that was lower ($p < 0.05$) than the SL with 20 mol% incorporation of stearic acid (LS20), at 33.1 and 34.1°C, respectively, and the potential health benefit is that this SL (Lipozyme® 435 Blend C) also contained the MLM-type TAG due to the incorporation of caprylic acid.

Oxidative Stability

Figure 5.5 results showed the TOTOX values of menhaden oil and each SL over 12 d. Differences were seen in the oxidative stability of the SL and menhaden oil. All SL saw a more rapid increase in TOTOX values after day 3, while the menhaden oil was more stable up to day 6. From day 9 to day 12, LC 20 had significantly higher TOTOX values than the other SL and menhaden oil. Menhaden oil had a significantly lower TOTOX value on day 12. These results show the oxidative stability was lower in all the SL than the menhaden oil. This is likely due to the processes after enzymatic modification, such as the short-path distillation, which has been found to reduce the amount of antioxidants such as tocopherols in the purified SL (Ifeduba et al., 2016).

Figure 5.5 showed the OSI values for menhaden oil and each SL. Menhaden oil had the highest OSI value (17.55 h) compared to the SL. The menhaden oil contained 200 ppm TBHQ and 1000 ppm mixed tocopherols added by the manufacturer (Omega Protein Inc., Reedville, VA, USA), which helped improve its oxidative stability. The SL with caprylic acid incorporation (LC 20 & LC 30) were statistically lower ($p < 0.05$) in oxidative stability than the SL that incorporated stearic acid (LS20 & LS30). When comparing 20 and 30 mol% incorporation of each SFA, higher incorporation of either SFA increased the OSI. Finally, Lipozyme® 435 Blend C had an OSI value that was not significantly different ($p > 0.05$) from LS 30. Higher amounts of stearic acid may help to increase the oxidative stability of the SL. This is likely due to the increase in saturation that helps make the SL more stable (Vieira et al., 2015). However, addition of lost antioxidants during the reaction and short-path distillation is encouraged before food application of these SL (Ifeduba et al., 2016).

Volatile Oxidation Products

Table 5.5 shows the volatile oxidation products of menhaden oil and each SL. There were 35 peaks identified from the mass spectra. Compounds that were identified from these peaks were classified as alcohols, aldehydes, aromatics, furans, hydrocarbons, and ketones. It has been reported that common oxidation products from fish oils are 1-octen-3-ol, (E)-2-heptenal, (E,E)-2,4-heptadienal, (E,Z)-2,6-nonadienal, and 2-nonenal (Qiu et al., 2017; Hsleh et al., 1989; Horiuchi et al., 1998; Shahidi, 1998). All of these compounds were identified for menhaden oil and each SL, and the volatile oxidation products found were similar to compounds detected in other studies (Qiu et al., 2017; Hsleh et al., 1989; Horiuchi et al., 1998; Shahidi, 1998). (*E*,*E*)-2,4-heptadienal. (*E*,*E*)-2,4-

octadienal, and 3,6-nonadienal (a decomposition product from EPA) were found in menhaden oil and the SL. A previous study suggested that these compounds, which impart fishy, green, rancid, and fatty flavors, may impact the overall sensory acceptability (Horiuchi et al., 1998). Acrolein is another known major compound formed in the headspace of heated fish oil that may indicate early stages of oxidation (Shahidi, 1998). Acrolein was found to have a high peak area % for all samples and was significantly higher ($p < 0.05$) for LC 30 and Lipozyme® 435 Blend C.

Hexanal, 1-penten-3-one, 1-penten-3-ol, and 2,4-heptadienal were all identified in the menhaden oil and SL and were previously reported to be important lipid oxidation markers for fish oil (Qiu et al., 2017). There was no significant difference ($p > 0.05$) in peak area % of hexenal and 1-penten-3-one for all samples. Menhaden oil had significantly lower peak area % of 1-penten-3-ol and (*E*,*E*)-2,4-heptadienal, suggesting the menhaden oil may have a lower degree of oxidation. Menhaden oil had a significantly higher ($p < 0.05$) peak area % of 2-butanone than the SL. 2-butanone, which has a buttery flavor, was previously reported to positively influence the sensory quality of fish oil when present in higher amounts (Horiuchi et al., 1998). Propanal is also another compound used for assessing lipid oxidation (Shahidi, 1998). There was a significantly higher peak area % of propanal for LC 30, LS 20, and Lipozyme® 435 Blend C, indicating these samples may be more oxidized than the menhaden oil. These results align well with the OSI and TOTOX results, which found the SL to be generally lower in oxidative stability. To improve the quality of the SL, antioxidants may be added after purification by short-path distillation to improve oxidative stability (Ifeduba et al., 2016).

Conclusion

Linear interpolation was successful at estimating the molar ratios that would incorporate targeted 20 or 30 mol % of C8:0 or C18:0. Linear interpolation is a relatively simple method that could be applied to future research and industrial applications. Enzymatic reactions with menhaden oil, C8:0, and C18:0 (molar ratio of 1:3:1) successfully produced SL that contained MLM-type TAG and had a melting point between 25-35°C. These SL have the potential for use as nutraceuticals due to the conservation of PUFA at the *sn*-2 position. Successful use of Lipozyme® 435, a commercial, food grade, immobilized lipase improves the viability of enzymatic interesterification at an industrial scale and use of SL. The reformulation of food products with relatively low saturated fat SL that contain health beneficial omega-3 PUFA may be helpful in reducing the risk of certain chronic diseases.

Abbreviations Used

DHA, docosahexaenoic acid; DSC, differential scanning calorimeter; ECN, equivalent carbon number; ELSD, evaporative light scattering detector; EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl esters; FFA, free fatty acids; FID, flame ionization detector; MAG, monoacylglycerol; MLM, medium-long-medium; MTBE, methyl *tert*-butyl ether; MUFA, monounsaturated fatty acids; OSI, oil stability index; PDMS/DVB, polydimethylsiloxane/divinylbenzene; PUFA, polyunsaturated fatty acids; *p*-AV, *p*-Anisidine; PV, peroxide value; RSM, response surface methodology; SFA, saturated fatty acids; SFC, solid fat content; SL, structured lipid; SPME, solid-phase microextraction; TAG, triacylglycerol; TOTOX, total oxidation

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Conflicts of Interest

There are no conflicts to declare.

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Tables

Table 5.1 Relative fatty acid composition of C8:0, C18:0, and C8:0/C18:0 blend reaction products

Samples [a]	TAG Position	Fatty Acid (mol%) [b]			
		C8:0	C18:0	C20:5n3	C22:6n3
Menhaden Oil	Total	ND	3.08±0.05	16.90±0.10	15.40±0.16
	sn-2	ND	0.21±0.24	16.42±0.64	29.37±0.95
	sn-1,3 [c]	ND	4.52±0.13	17.14±0.45	8.42±0.37
LC20	Total	20.27±1.53	3.18±0.70	11.25±1.58	10.67±1.37
	sn-2	17.65±0.33	0.34±0.19	10.96±1.68	22.08±0.38
	sn-1,3	21.58±0.13	4.60±0.95	11.25±1.42	4.97±0.57
RC20	Total	19.79±0.35	3.11±0.35	11.35±0.56	10.57±0.46
	sn-2	6.80±0.44	0.26±0.47	13.37±0.73	23.75±0.25
	sn-1,3	26.29±0.37	4.54±0.29	10.34±0.76	3.98±0.16
LC30	Total	30.29±1.10	3.76±0.78	11.56±2.55	10.29±0.64
	sn-2	10.69±0.35	0.28±0.69	12.19±0.53	22.32±1.71
	sn-1,3	40.09±0.56	5.50±0.83	11.25±0.98	4.28±0.87
RC30	Total	28.90±0.76	3.57±0.52	12.66±0.16	10.75±0.42
	sn-2	1.66±1.90	0.21±0.26	12.68±0.19	28.17±0.63
	sn-1,3	42.52±0.63	5.25±0.65	12.65±0.21	2.04±0.55
LS20	Total	ND	20.58±1.25	12.88±0.66	10.78±0.56
	sn-2	ND	13.29±0.66	11.31±0.56	21.62±0.80
	sn-1,3	ND	24.23±0.84	13.67±0.52	5.36±0.68
RS20	Total	ND	19.32±0.84	8.94±0.64	8.69±0.58
	sn-2	ND	7.40±0.30	12.30±0.42	26.61±0.76
	sn-1,3	ND	25.28±0.46	7.26±0.37	0.27±0.31
LS30	Total	ND	30.02±0.98	11.15±0.59	10.14±0.34
	sn-2	ND	14.92±0.66	11.06±0.67	23.35±0.06
	sn-1,3	ND	37.57±1.04	11.20±0.85	3.54±0.22
RS30	Total	ND	29.25±1.12	7.84±0.13	7.62±0.76
	sn-2	ND	4.52±0.95	13.41±0.51	25.05±0.26
	sn-1,3	ND	41.62±0.76	5.06±0.32	4.91±0.59
Lipozyme® 435 Blend A	Total	6.40±0.50	23.04±1.31	10.04±0.25	10.65±0.29
	sn-2	5.35±0.63	14.02±1.02	10.20±0.14	22.47±0.46
	sn-1,3	6.93±0.45	27.55±0.87	9.96±0.21	4.74±0.71
Lipozyme® 435 Blend B	Total	10.54±1.25	17.80±2.81	10.09±0.82	10.70±0.72
	sn-2	6.82±0.47	10.95±0.46	10.96±0.52	22.43±0.13
	sn-1,3	12.40±1.04	21.23±1.54	9.66±0.84	4.84±0.23
Lipozyme® 435 Blend C	Total	13.46±0.41	13.80±1.42	10.66±0.29	11.79±0.37
	sn-2	11.14±0.91	8.55±0.67	10.09±0.76	24.17±0.21
	sn-1,3	14.62±0.73	16.43±0.93	10.95±0.43	5.60±0.03
Lipozyme® RM IM Blend A	Total	4.40±0.53	16.60±1.11	14.02±0.45	13.29±0.44
	sn-2	4.20±0.11	8.01±0.64	12.11±0.52	24.35±0.02
	sn-1,3	4.50±0.72	20.90±0.28	14.98±0.83	7.76±0.05
Lipozyme® RM IM Blend B	Total	6.85±0.54	13.38±0.38	14.10±0.08	13.45±0.11
	sn-2	4.00±1.20	6.28±0.48	12.05±0.31	24.54±0.45
	sn-1,3	8.28±0.32	16.93±0.26	15.13±0.24	7.91±0.16
Lipozyme® RM IM Blend C	Total	9.02±1.10	10.04±1.22	14.40±0.21	13.95±0.23
	sn-2	3.71±0.42	5.03±0.31	11.91±0.72	24.62±0.93
	sn-1,3	11.68±0.32	12.55±0.12	15.65±0.66	8.62±0.23

Mean ± STD, *n*=3, ND= not detected

[a] LC20: Lipozyme® 435 as biocatalyst, 20 mol % incorporation C8:0; RC20: Lipozyme® RM IM as biocatalyst, 20 mol% incorporation C8:0; LC30: Lipozyme® 435 as biocatalyst, 30 mol % incorporation C8:0; RC30: Lipozyme® RM IM as biocatalyst, 30 mol% incorporation C8:0;

LS20: Lipozyme® 435 as biocatalyst, 20 mol % incorporation C18:0; RS20: Lipozyme® RM IM as biocatalyst, 20 mol% incorporation C18:0; LS30: Lipozyme® 435 as biocatalyst, 30 mol % incorporation C18:0; RS30: Lipozyme® RM IM as biocatalyst, 30 mol% incorporation C18:0

Blends (A: 1:1:3, Blend B: 1:2:2, Blend C: 1:3:1) are molar substrate ratios of menhaden oil, C8:0, and C18:0

[b] Other fatty acids found at >1mol%: C14:0, C16:0, C16:1n7, C17:1n7, C18:1n9, C18:2n6, C20:0, C18:4n3, C22:5n3, and <1 mol%: C13:0, C14:1n7, C15:0, C17:0, C19:0, C18:3n6, C20:1n11, C18:3n3, C20:2n6, C22:0, C20:3n6, C20:3n3, C23:0, C22:2n6, C24:1n9, C22:4n6

[c] *sn*-1,3 mol% determined by equation: *sn*-1,3 (mol%) = [3xtotal mol%- *sn*-2 mol %]/2

Table 5.2 Relative TAG molecular species of menhaden oil and each SL

TAG [a]	TAG (mol%)														
	M [b]	LC 20	RC 20	LC 30	RC 30	LS 20	RS 20	LS 30	RS 30	Lipozyme® 435 Blend A	Lipozyme® RM IM Blend A	Lipozyme® 435 Blend B	Lipozyme® RM IM Blend B	Lipozyme® 435 Blend C	Lipozyme® RM IM Blend C
CyCyCy	ND	1.2±0.25	0.9±0.77	4.4±0.73	2.3±0.36	ND	ND	ND	ND	1.6±0.17	0.8±0.23	1.4±0.58	1.1±0.92	2.9±0.54	1.5±0.11
CyStCy/CyDpCy	ND	4.1±0.36	3.4±0.53	5.2±0.78	4.6±0.08	ND	ND	ND	ND	1.9±0.80	1.6±0.64	1.1±0.58	0.8±0.05	3.1±0.93	1.4±0.26
StEpCy/StDhCy	ND	4.4±0.63	2.9±0.69	7.2±0.90	3.7±0.84	ND	ND	ND	ND	2.8±0.73	1.8±0.83	2.6±0.76	2.4±0.28	6.3±0.67	5.7±0.16
StStSt/CyEpCy	0.2±0.02	4.6±0.55	3.0±0.48	9.3±0.95	5.1±0.46	ND	ND	ND	ND	2.3±0.64	1.2±0.24	5.6±1.73	4.2±0.50	6.7±0.48	5.9±0.83
CyDhCy	ND	7.2±0.43	4.5±0.06	9.3±0.67	5.8±0.33	ND	ND	ND	ND	3.4±0.24	1.4±0.75	5.5±0.45	3.6±0.58	9.0±0.18	4.3±0.84
CyPoCy	ND	4.0±0.03	6.6±0.05	3.7±0.47	7.1±0.57	ND	ND	ND	ND	3.1±0.85	4.2±0.74	3.5±0.28	5.1±0.53	2.9±0.41	5.8±0.57
CyMCy	ND	3.9±0.92	4.7±0.05	3.5±0.58	4.6±0.65	ND	ND	ND	ND	2.4±0.68	2.7±0.66	2.7±0.69	4.6±0.75	3.0±0.28	5.2±0.14
PEpPo/PEpEp	4.8±0.56	2.6±0.58	2.5±0.04	2.4±0.83	2.3±0.94	5.6±1.12	3.9±0.87	5.2±0.12	2.5±0.62	4.4±0.25	2.6±0.39	3.4±0.68	4.5±0.32	3.8±0.03	4.2±0.47
MDhM/PEpDh	9.3±1.10	6.3±0.84	5.8±0.94	5.5±1.52	5.3±0.94	5.8±0.25	8.9±0.59	5.7±0.11	1.3±0.06	6.7±1.85	4.3±0.52	4.3±0.57	2.2±0.23	2.2±0.28	1.5±0.83
PoPoEp/PDhDh	8.5±2.26	4.3±0.79	4.8±0.10	2.0±0.58	2.6±0.37	4.2±0.56	6.5±0.45	2.4±0.35	3.9±0.93	4.8±0.04	3.3±0.68	5.1±0.74	5.0±0.51	4.9±0.78	4.6±0.58
PoEpO/PoPoDh/CyPCy	4.8±1.37	2.7±1.59	4.2±0.49	1.6±0.22	3.9±0.38	1.3±0.89	5.5±0.37	2.0±0.73	5.3±0.88	8.4±0.83	7.4±0.24	8.3±0.24	7.9±0.13	7.2±0.55	7.7±1.57
MMEp/PoEpPo/PoDhP	15.0±1.46	2.8±0.24	2.2±0.88	2.3±0.80	1.9±0.60	1.7±0.58	1.7±0.55	1.4±0.05	1.3±0.55	7.5±0.25	7.2±0.58	7.3±0.43	7.3±0.53	5.9±2.73	7.4±0.24
MPoEp/MDhP	9.5±0.31	4.0±0.33	7.9±0.47	3.1±0.30	7.4±0.03	4.9±0.50	4.4±0.88	3.2±0.99	3.2±0.63	4.8±0.84	4.2±0.89	4.3±0.40	4.4±0.67	3.1±0.24	5.9±0.78
PEpP/PPDh/StEpS	4.6±0.41	3.1±0.06	4.0±0.67	2.6±0.74	3.7±0.17	8.3±0.83	8.6±0.62	2.4±0.53	2.5±0.58	5.4±0.09	5.9±0.59	3.8±0.48	3.6±0.85	3.6±0.07	3.9±0.94
PPDh/PPoPo	7.5±0.73	2.6±0.97	3.8±0.58	2.1±0.83	3.9±1.47	6.9±0.26	8.5±0.58	1.4±0.73	5.8±0.44	3.4±0.27	6.4±0.04	4.3±0.03	5.9±0.68	4.3±0.59	5.6±0.85
SDpDp/MPPoS/StDhS	4.3±0.25	2.1±0.92	3.3±0.73	1.9±0.11	3.4±0.63	7.7±0.47	9.2±0.73	7.4±1.22	6.3±0.68	8.3±0.15	8.9±0.56	8.1±0.96	5.8±1.03	8.5±0.88	5.6±0.67
SStS/SDpS	1.9±0.12	1.0±0.35	1.4±0.26	0.9±0.18	1.2±0.84	8.4±0.44	7.8±0.57	5.2±0.46	4.5±0.74	4.2±0.63	3.9±0.97	4.4±0.83	3.3±0.69	2.7±0.72	3.6±0.45
SEpS	ND	ND	ND	ND	ND	5.1±0.73	4.1±0.23	5.8±0.86	4.5±0.52	6.2±0.76	5.2±0.25	2.8±0.35	2.1±0.07	5.1±0.24	4.3±0.74
SDhS	ND	ND	ND	ND	ND	4.2±0.71	1.0±1.55	9.4±0.89	5.6±0.47	2.0±0.68	5.9±0.85	1.2±0.15	3.3±0.20	1.0±0.59	2.1±0.56
SPoS	ND	ND	ND	ND	ND	2.9±0.64	1.2±0.47	4.8±0.02	5.8±0.36	3.9±0.27	2.6±0.86	2.1±0.44	1.4±0.35	1.8±0.77	1.1±0.34
SMS	ND	ND	ND	ND	ND	2.2±0.98	1.5±0.36	4.8±0.64	6.3±1.58	3.5±0.56	1.6±0.33	1.9±0.73	1.1±0.72	1.5±0.62	0.9±0.35
SSS	0.9±0.06	0.8±0.52	0.6±0.05	0.7±0.03	0.6±0.18	1.7±0.37	1.3±0.14	4.4±0.21	2.6±0.36	3.50±0.68	1.7±0.27	1.2±0.67	0.8±0.12	0.9±0.61	0.7±0.75
Total MLM-type TAGs	ND	26.5±0.65 a	26.4±0.28 a	32.6±0.61 b	31.1±0.41 b	ND	ND	ND	ND	21.5±0.67 c	18.5±0.55 d	26.7±0.50 a	26.2±0.42 a	31.9±0.47b	30.3±0.70b
Total Cy-PUFA-Cy TAGs	ND	15.9±0.45 a	10.9±0.36 b	23.8±0.80 c	15.5±0.29 a	ND	ND	ND	ND	7.6±0.56b	4.2±0.54d	12.2±0.59 e	8.6±0.38b	18.8±0.53c	11.6±0.64e

Mean ± STD, *n*=3, ND= not detected. Different letters in same row indicate significant difference ($p < 0.05$).

[a] Letters correspond to the following FA: Cy= Caprylic, M= Myristic, P= Palmitic, Po= Palmitoleic, S= Stearic, O= Oleic, Lo= Linoleic, St= Stearidonic, Ep= EPA, Dp= DPA, and Dh= DHA

[b] Abbreviations and reaction conditions are same as described in Table 1, 2, and 3.

Table 5.3 Relative major total and positional fatty acid compositions of menhaden oil and large-scale SL products

Samples [a]	TAG Position	Fatty Acid (mol%) [b]			
		C8:0	C18:0	C20:5n3	C22:6n3
Menhaden Oil	Total	ND	3.08±0.05	16.90±0.10	15.40±0.16
	sn-2	ND	3.21±0.37	16.42±0.64	29.37±0.95
	sn-1,3 [c]	ND	3.02±0.21	17.14±0.45	8.42±0.37
LC 20	Total	19.34± 2.06	2.81±0.14	15.84±0.54	11.04±0.13
	sn-2	11.37±0.32	2.53±0.21	12.06±1.61	24.13±0.82
	sn-1,3	23.33±1.93	2.95±0.18	17.73±1.11	4.50±0.48
LC 30	Total	29.03±1.37	2.74±0.10	13.61±0.38	10.42±0.03
	sn-2	11.72±0.13	2.97±0.09	12.22±0.03	25.17±0.46
	sn-1,3	37.69±0.75	2.63±0.10	14.31±0.21	3.05±0.25
LS 20	Total	ND	19.50±0.23	14.16±0.11	14.62±0.39
	sn-2	ND	10.07±0.78	11.85±0.26	22.98±0.18
	sn-1,3	ND	24.22±0.51	16.21±0.19	10.44±0.29
LS 30	Total	ND	29.71±0.58	14.04±1.66	12.46±0.41
	sn-2	ND	11.00±1.30	14.90±0.86	17.49±0.24
	sn-1,3	ND	39.07±0.22	13.61±1.06	9.95±0.50
Lipozyme® 435 Blend C	Total	16.24±0.56	13.04±0.10	13.87±0.26	12.50±0.20
	sn-2	7.31±1.01	8.82±0.55	10.69±0.32	16.66±1.39
	sn-1,3	20.71±0.34	15.14±0.13	15.46±0.23	10.42±0.40

Mean ± STD, *n*=3, ND= not detected

[a] Abbreviations and reaction conditions are same as described in Table 1, 2, and 3.

[b] Other fatty acids found at >1mol%: C14:0, C16:0, C16:1n7, C17:1n7, C18:1n9, C18:2n6, C20:0, C18:4n3, C22:5n3, and <1 mol%: C13:0, C14:1n7, C15:0, C17:0, C19:0, C18:3n6, C20:1n22, C18:3n3, C20:2n6, C22:0, C20:3n6, C20:3n3, C23:0, C22:2n6, C24:1n9, C22:4n6

[c] *sn*-1,3 mol% determined by equation: *sn*-1,3 (mol%) = [3xtotal mol%- *sn*-2 mol %]/2

Table 5.4 DSC crystallization onset (C_0) and melting completion (M_c) temperatures of menhaden oil and each SL

Sample [a]	C_o (°C)	M_c (°C)
Menhaden Oil	-5.3±0.72a	13.6±0.14a
LC 20	4.0±0.15b	22.5±0.41b
LC 30	8.5±0.24c	24.5±0.23c
LS 20	16.9±0.75e	34.1±0.16e
LS 30	21.9±0.37f	43.9±0.94f
Lipozyme® 435 Blend A	27.4±0.38g	50.0±0.05g
Lipozyme® 435 Blend B	22.1±0.47f	42.5±0.77f
Lipozyme® 435 Blend C	15.3±0.22d	33.1±0.38d

Mean ± STD, *n*=3. Different letters in the same column are significantly different (α = 0.05).

[a] Abbreviations and reaction conditions are same as describe in Table 1, 2, and 3.

Table 5.5 Volatile oxidation products of menhaden oil and each SL

Compound	Retention Time Index (RI) [a]	Odor Description	GC Peak Area %			
			Menhaden Oil	LC 30 [b]	LS 20	Lipozyme® 435 Blend C
Alcohols						
(*Z*)-2-pentenol	917	fruity	2.57±1.11a	0.08±0.06b	0.31±0.25b	0.20±0.06b
1-penten-3-ol	947	milky/butter	3.31±1.06a	11.26±0.55b	9.74±1.79b	10.21±0.58b
1-octen-3-ol	1040	mushroom/musty	0.57±0.54a	0.50±0.46a	2.74±1.73a	0.30±0.15a
hexanol	1155	green/herbal	0.11±0.10a	0.12±0.09a	1.34±1.09a	0.68±0.13a
(*E*)-2-pentenol	1308	mushroom	0.09±0.08a	0.02±0.01a	0.42±0.40a	2.40±1.12b
1-hexen-3-ol	1364	green/fruity	0.54±0.50a	1.25±1.00a	0.54±0.49a	0.09±0.04a
Aldehydes						
propanal	726	plastic	16.11±0.82a	20.25±0.07b	20.84±1.04b	20.07±0.03b
acrolein	762	burnt/cooked	20.01±0.44a	48.56±0.19c	20.33±1.81a	38.38±0.30b
(*E*)-2-butenal	855	plastic	1.20±0.78a	0.19±0.08b	2.23±1.20a	0.17±0.12b
butanal	889	fermented/musty	0.45±0.28a	0.12±0.08a	1.06±0.81a	0.10±0.09a
pentanal	948	woody/fruity	0.32±0.29a	0.02±0.01a	0.75±0.39a	0.08±0.07a
(*E*)-2-pentenal	956	oily/soapy	4.37±1.50a	2.72±0.93a	3.95±1.76a	2.72±1.90a
(*E*,*E*)-2,4-hexadienal	958	green/vegetable	0.36±0.30a	0.06±0.05a	0.59±0.53a	0.17±0.14a
(*E*,*E*)-2,4-heptadienal	1041	rancid/fatty	0.15±0.11a	0.18±0.16a	6.38±2.00b	4.13±1.26b
hexanal	1045	grassy/fatty	2.94±1.34a	2.67±1.37a	2.27±1.05a	2.64±0.14a
(*E*)-2-hexenal	1056	green/fatty	0.34±0.33a	0.82±0.32a	0.82±0.73a	0.61±0.44a
(*E*)-2-heptenal	1089	fatty/almond	1.37±1.01a	2.18±0.77a	3.30±2.86a	0.67±0.35a
(*E*,*Z*)-2,6-nonadienal	1136	cucumber	1.77±1.69a	0.34±0.15a	1.21±1.85a	1.40±1.17a
heptanal	1145	burnt	0.83±0.70a	0.73±0.04a	5.02±1.51b	0.15±0.05a
(*Z*)-4-heptenal	1179	sweet	0.24±0.16a	0.18±0.09a	0.21±0.12a	0.08±0.02a
3,6-nonadienal	1179	green	2.21±0.07a	1.54±1.17a	2.03±1.11a	3.11±1.65a
octanal	1234	citrus	0.24±0.19a	0.68±0.55a	0.06±0.02a	0.12±0.06a
(*E*,*E*)-2,4-octadienal	1246	green/fatty	1.36±1.07a	0.34±0.19a	0.35±0.28a	2.56±2.03a
2-nonenal	1361	tallow/cucumber	4.65±0.85a	3.31±0.12b,c	1.29±0.32b	1.34±0.60b
Aromatics						
benzene	1072	sweet	0.18±0.16a	0.38±0.31a	0.87±0.69a	0.13±0.10a
Furans						
2-ethylfuran	1059	sweet/coffee	1.97±1.00a	2.44±1.13a	0.16±0.06b	0.09±0.01b
2-pentylfuran	1358	floral/fruity	0.85±0.41a	0.36±0.32a	0.61±0.23a	0.94±0.60a
Hydrocarbons						
heptane	1111	petroleum	0.38±0.44a	0.57±0.49a	0.48±0.26a	0.12±0.10a
octane	1262	petroleum	0.51±0.40a	0.16±0.09a	0.91±0.65a	0.17±0.11a
decane	1462	petroleum	0.02±0.01a	0.12±0.03a	0.20±0.06b	0.03±0.01a
dodecane	1645	petroleum	0.02±0.01a	0.06±0.05a	0.11±0.08a	0.21±0.19a
Ketones						
2-butanone	817	cheese/butter	10.58±0.30a	2.22±0.08b	2.05±0.78b	3.19±1.15b
3-penten-2-one	937	fruity/fishy	1.74±0.99a	0.08±0.02b	0.96±0.27a	0.08±0.06b
1-penten-3-one	955	plastic/leather	1.01±0.01a	1.73±0.90a	0.97±0.72a	1.56±1.02a
3-hexen-2-one	1069	fatty	0.84±0.40a	0.26±0.04a	2.80±0.41b	0.30±0.08a

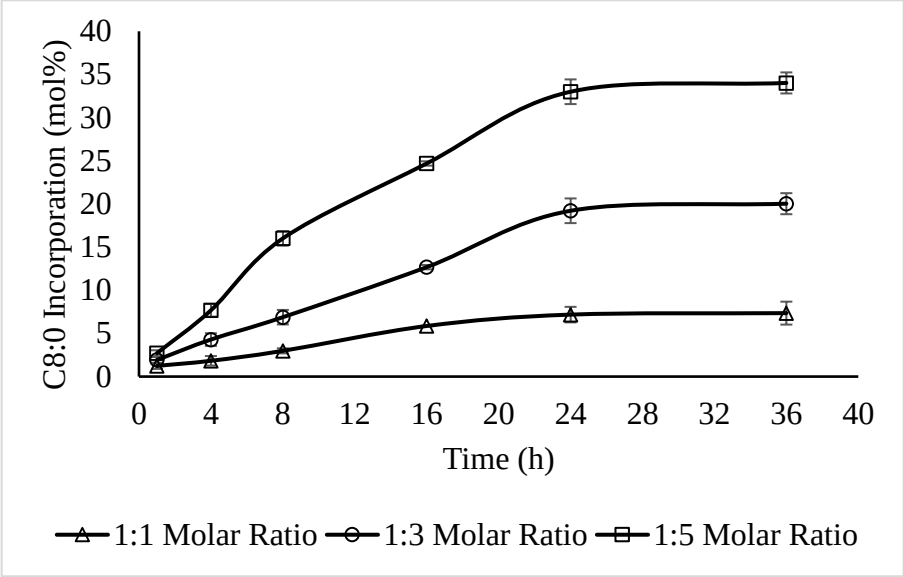
Mean ± STD, *n*=3. Different letters in the same row are significantly different (α = 0.05).

[a] RI determined according to Van den Dool and Kratz[23]

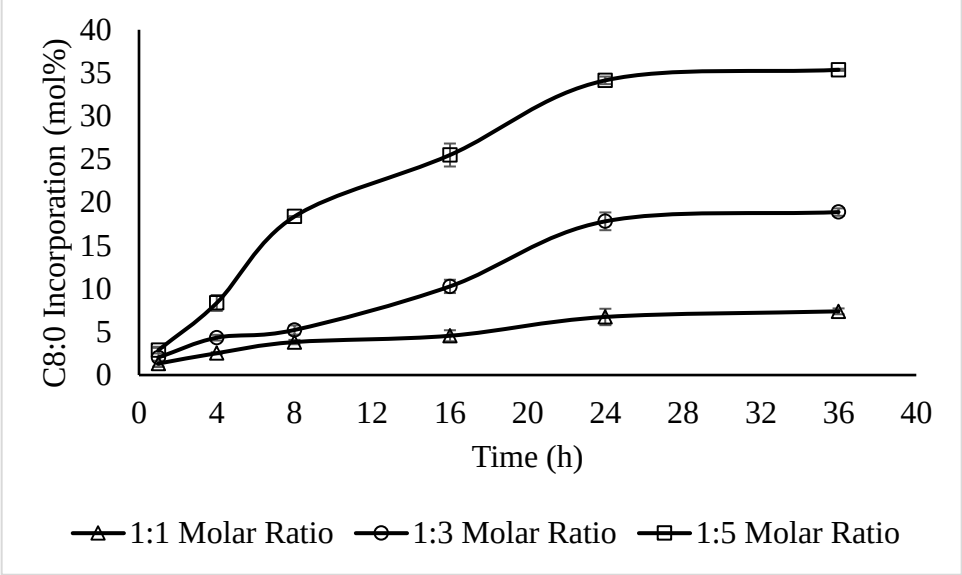
[b] Abbreviations and reaction conditions are same as described in Table 1, 2, and 3.

Figures

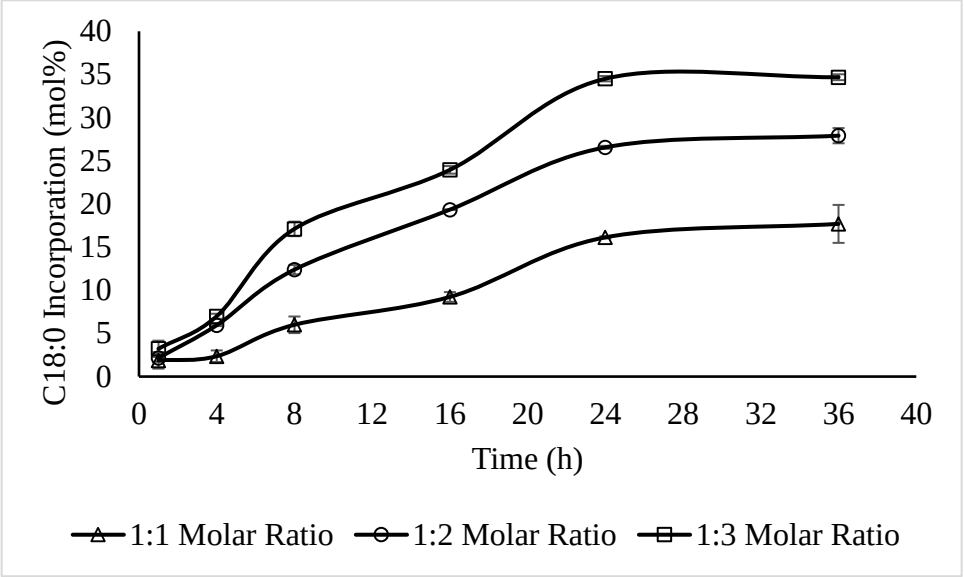
a) Lipozyme® 435



b) Lipozyme® RM IM



c) Lipozyme® 435



d) Lipozyme® RM IM

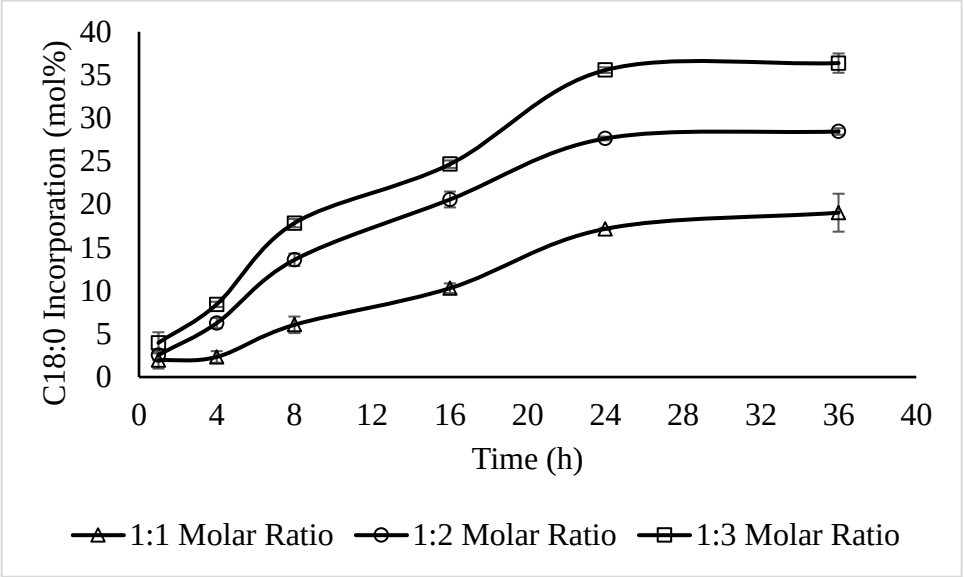
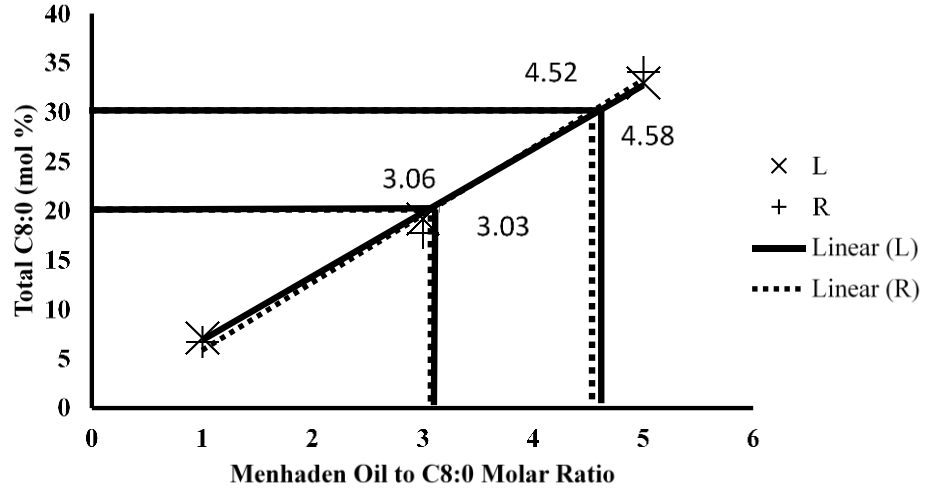


Figure 5.1 Time course reactions for all C8:0[a] and C18:0[b] enzymatic reactions, where a) incorporation of C8:0 using Lipozyme® 435 as biocatalyst, b) incorporation of C8:0 using Lipozyme® RM IM as biocatalyst c) incorporation of C18:0 using Lipozyme® 435 as biocatalyst, and d) incorporation of C18:0 using Lipozyme® RM IM as biocatalyst

[a] Conditions for C8:0 reactions: substrate molar ratios of menhaden oil to C8:0 of 1:1, 1:3, and 1:5, at 60°C in a 250 rpm shaking water bath, enzyme load 10% (w/w) of total substrates

[b] Conditions for C18:0 reactions: substrate molar ratios of menhaden oil to C18:0 of 1:1, 1:2, and 1:3, at 75°C in a 250 rpm shaking water bath, enzyme load 10% (w/w) of total substrates

a)



b)

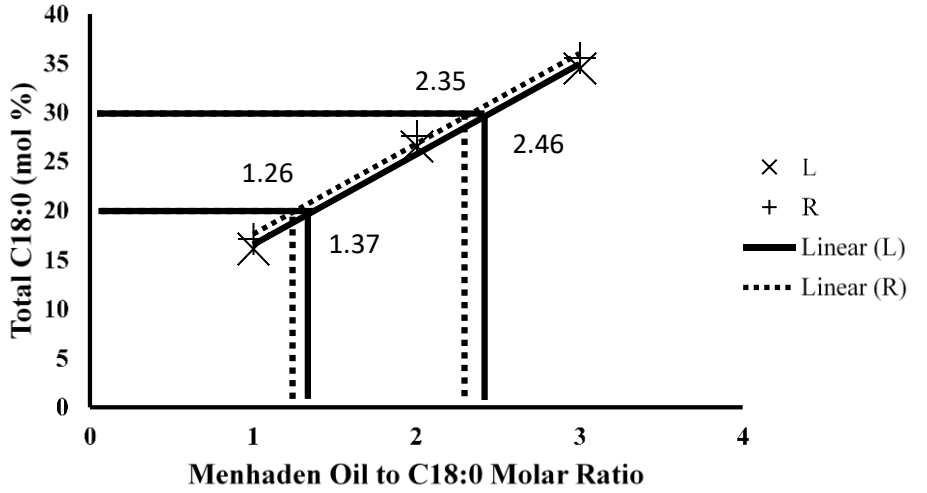


Figure 5.2 Linear interpolation for 20 and 30 mol % of **a)** C8:0 incorporation and **b)** C18:0 incorporation

Numbers on top of trendlines are optimal substrate molar ratios for reactions with Lipozyme® RM IM (R), numbers below lines are optimal substrate molar ratios for reactions with Lipozyme® 435 (L). Linear regression equations for each figure are: **a)** L: $y = 6.4555x + 0.4294$, $R^2 = 0.9985$, R: $y = 6.8525x - 0.9975$, $R^2 = 0.9879$, and **b)** L: $y = 9.19x + 7.3533$, $R^2 = 0.994$, R: $y = 9.195x + 8.4$, $R^2 = 0.9936$. Reactions were performed at 60 or 75°C for C8:0 and C18:0 reactions, respectively in a shaking water bath at 250 rpm for 24h with enzyme load of 10% (w/w) of total substrates and substrate molar ratios as described in Figure 1

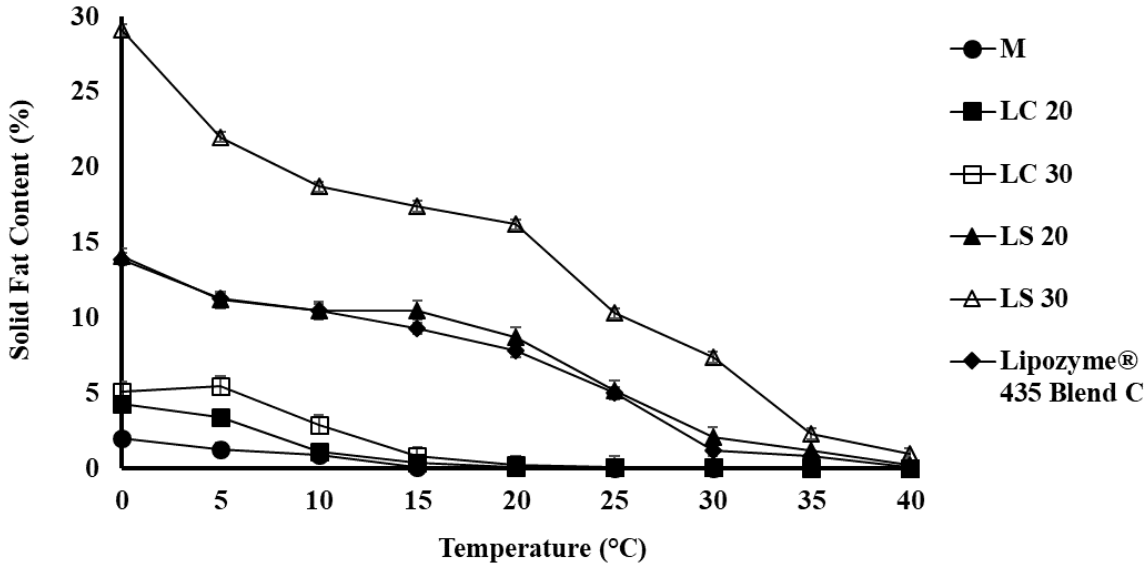
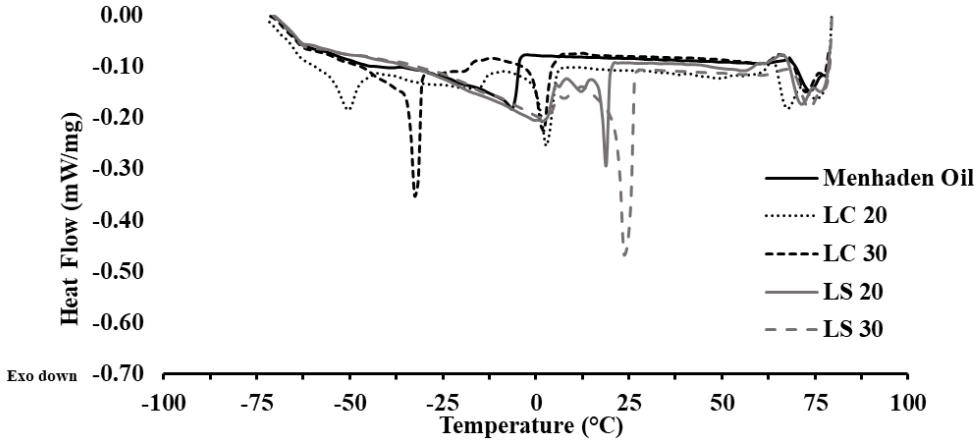
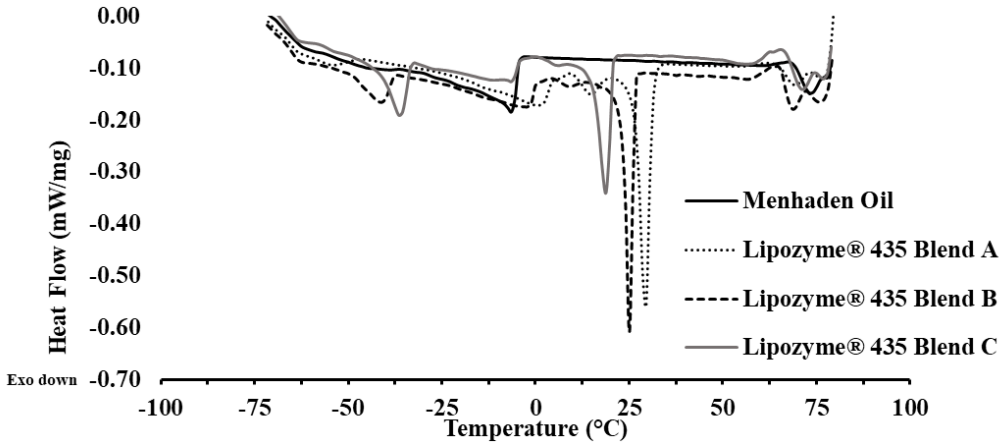


Figure 5.3 SFC of menhaden oil and each SL. Abbreviations and reaction conditions are same as described in Table 1, 2, and 3

a)


0.00
-0.10
-0.20
-0.30
-0.40
-0.50
-0.60
-0.70
Heat Flow (mW/mg)
Exo down
-100
-75
-50
-25
0
25
50
75
100
Temperature (°C)
Menhaden Oil
LC 20
LC 30
LS 20
LS 30


0.00
-0.10
-0.20
-0.30
-0.40
-0.50
-0.60
-0.70
Heat Flow (mW/mg)
Exo down
-100
-75
-50
-25
0
25
50
75
100
Temperature (°C)
Menhaden Oil
Lipozyme® 435 Blend A
Lipozyme® 435 Blend B
Lipozyme® 435 Blend C

b)

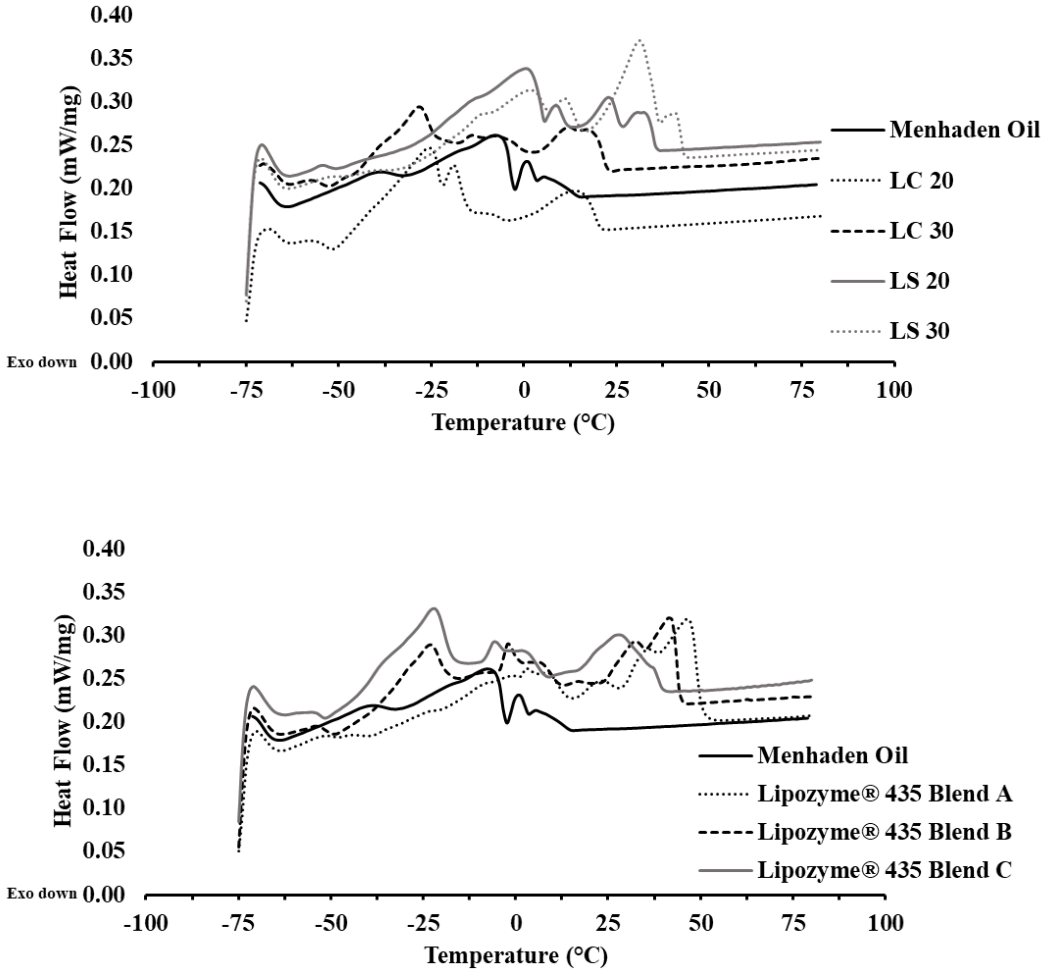


Figure 5.4 DSC thermograms (a) crystallization and (b) melting profiles of menhaden oil and each SL
Abbreviations and reaction conditions are same as described in Table 1, 2, and 3

Sample [a]	Oxidative Stability Index (OSI) (h) [b]
Menhaden Oil	17.55±1.21a
LC 20	3.03±0.28b
LC 30	4.37±0.88b,c
LS 20	9.13±1.07c
LS 30	12.90±1.65d
Lipozyme® 435 Blend C	11.70±1.31d

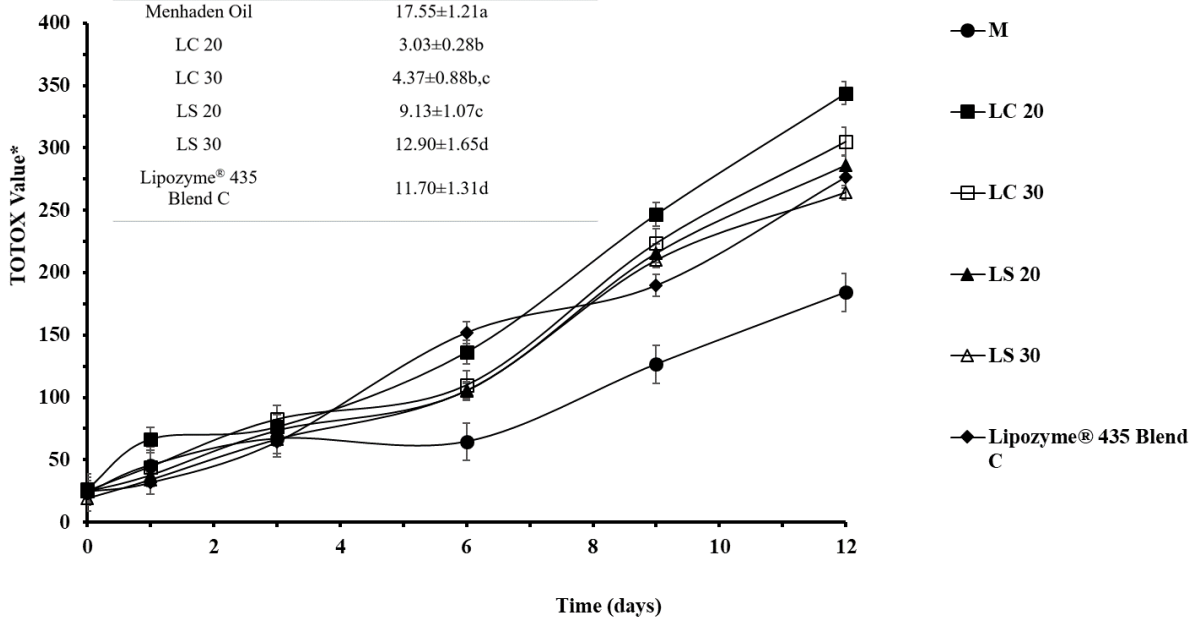


Figure 5.5 TOTOX and OSI values of menhaden oil and each SL

Mean ± STD, *n*=3. Different letters in the same column are significantly different (α = 0.05)

$\text{TOTOX value} = 2(\text{PV}) + p\text{AV}$, PV= peroxide value, *p*-AV= *p*-anisidine value.

[a] Abbreviations and reaction conditions are same as described in Table 1, 2, and 3.

[b] OSI conducted at 80°C

CHAPTER 6

ENCAPSULATION OF MENHADEN OIL STRUCTURED LIPID OLEOGELS IN ALGINATE MICROPARTICLES[1]

[1]Willett, S. A., and Akoh, C. C. 2019. Submitted to LWT-Food Science and Technology on 03/26/19.

Abstract

Leaching of the internal phase during storage is a concern of microencapsulated products. This research proposes encapsulation of oleogels to reduce leaching and improve oxidative stability of the lipids. Oleogels were produced using a phytosterol blend of β-sitosterol/γ-oryzanol or a blend of sucrose stearate/ascorbyl palmitate (SSAP) as organogelators, and menhaden oil or structured lipid (SL) prepared from menhaden oil and caprylic and/or stearic acid as the lipid phase. The SL were produced enzymatically using the biocatalyst Lipozyme® 435, a recombinant lipase from *Candida antarctica*. Menhaden oil, SL, or respective phytosterol or SSAP oleogels, were encapsulated in alginate microparticles using a double emulsion method. Encapsulation efficiency (EE), morphology, oxidative stability, percent leaching, and other physicochemical properties were determined. Encapsulation of phytosterol or SSAP oleogels increased the EE for all lipids, from 89.6-91.3% for microcapsules containing only the lipid phases and from 95.7-99.2% for microcapsules containing the oleogels. Encapsulation of oleogels significantly reduced leaching, from 16.3-18.5% to 3.3-11.9%. Microencapsulated products had higher Oil Stability Index values (19.75-29.18 h) than the non-microencapsulated lipids (4.37-17.55 h), when measured at 80°C. These microcapsules may find use as stable nutraceuticals or in fortification of food products with stabilized omega-3 fatty acids.

Keywords: menhaden oil, oleogel, structured lipid, microencapsulation, oxidative stability

Abbreviations

DSC: differential scanning calorimeter; EE: encapsulation efficiency; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; GOED: Global Organization for EPA and DHA omega-3; M: menhaden oil; OSI: Oil Stability Index; *p*-AV: *p*-Anisidine value; PV: peroxide value; P-M: menhaden oil phytosterol oleogel; P-SL-C: SL-C phytosterol oleogel; P-SL-CS: SL-CS phytosterol oleogel; P-SL-S: SL-S phytosterol oleogel; PUFA: polyunsaturated fatty acids; SL: structured lipid; SL-S: SL-S SSAP oleogel; SL-C: structured lipid of menhaden oil and caprylic acid; SL-CS: structured lipid of menhaden oil and a blend of caprylic and stearic acids; SL-S: structured lipid of menhaden oil and stearic acid; SSAP: sucrose stearate/ascorbyl palmitate; SSAP-M: menhaden oil SSAP oleogel; SSAP-SL-C: SL-C SSAP oleogel; SSAP-SL-CS: SL-CS SSAP oleogel; SSAP-SL-S: SL-S SSAP oleogel

Introduction

A structured lipid (SL) is any lipid that has undergone chemical or enzymatic modification from its natural biosynthetic form (Kim & Akoh, 2015). SL have a wide range of applications, though one main concern is their oxidative stability. Although enzymatic production of SL typically occurs under milder conditions than chemical modification, previous studies have found that purified SL have lower oxidative stability than the unmodified fat or oil (Zou & Akoh, 2013; Ifeduba, Martini, & Akoh, 2016; Decker, Warner, Richards, & Shahidi, 2005; Pacheco, Crapiste, & Carrin, 2015). Short-path distillation, a purification process necessary to remove free fatty acids and/or fatty acid ethyl esters from the SL, has been shown to reduce the tocopherols or antioxidants naturally present in the fat or oil (Zou & Akoh, 2013).

Polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in menhaden fish oil, are highly susceptible to oxidation due to the high amount of unsaturation. Further modification of menhaden oil to produce SL in previous studies was found to further reduce the oxidative stability (Jennings & Akoh, 2001; Willett & Akoh, 2018a). One way of improving oxidative stability of SL is by forming oleogels. Oleogels (also known as organogels), a lipid gel, have been shown to inhibit oil migration in chocolate, control the release of health beneficial sensitive compounds such as antioxidants, bioactive compounds, and PUFA, and increase oxidative stability of the oil (Co & Marangoni, 2012). However, organogelation may not reduce the fishy off-flavors present in menhaden fish oil, limiting its use in food products. Another method that has been shown to improve the oxidative stability is by encapsulation of oil within polymeric microparticles. Microencapsulation is a method of

coating tiny droplets (such as high PUFA-containing oils) to form small capsules and has been found to mask fishy off-flavors (Bakry et al., 2016). Encapsulation of oil reduces its exposure to light, moisture, oxygen, and heat, all of which decrease the quality of the oil through oxidative deterioration, formation of undesirable flavor compounds, and production of free radicals (Sagiri et al., 2014).

One disadvantage of microencapsulation is leaching of the internal oil phase during storage, which undoes the encapsulation and therefore reduces its efficiency (Sagiri et al., 2014). To reduce leaching, there are many approaches such as using blended polymers to encapsulate the oil or using a more complex method of producing the microcapsules such as coacervation (Sagiri et al., 2012; Ifeduba & Akoh, 2015). There is need for a simple method to reduce oil leaching. Previous literature has reported success at reducing leaching by encapsulating a sorbitan ester-based sunflower oil oleogel in alginate microparticles for drug delivery (Sagiri et al., 2014). However, the studies on encapsulation of oleogels did not report oxidative stability (Sagiri et al., 2012, 2014). It is of interest to determine the physicochemical properties, encapsulation efficiency, amount of leaching during storage, and oxidative stability of the alginate microparticles when different organogelators and oil phases are used.

The purpose of this research was to microencapsulate menhaden oil or SL oleogels in alginate microparticles to potentially reduce the amount of leaching during storage and improve the oxidative stability of the lipids. Two different organogelator blends were compared in forming the microcapsules: a phytosterol blend of β-sitosterol and γ-oryzanol, and a blend of sucrose stearate/ascorbyl palmitate (SSAP). Oxidative stability, amount of leaching during storage, and other physicochemical properties of the

microcapsules were studied. Physicochemical properties of the microcapsules will depend on the lipid phase encapsulated. These microencapsulated menhaden oil oleogels may have the potential for use in a wide range of food and nutraceutical applications.

Materials and Methods

Materials

Menhaden oil was obtained from Omega Protein Inc. (Reedville, VA, USA). Acyl donors, caprylic acid (≥98 % purity) and stearic acid (≥98 % purity), were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). These were used as substrates in acidolysis reactions to produce the different SL according to the method outlined previously (under review). All acidolysis reactions were conducted in a 1L batch reactor for 24 h, with biocatalyst with Lipozyme® 435 lipase (Novozymes North America, Inc., Franklinton, NC, USA) at 10% (w/w) of total substrates and stirring at 250 rpm using a SL 2400 StedFast stirrer (Fisher Scientific Co., Fair Lawn, NJ, USA). For the SL containing 30 mol% of caprylic acid (SL-C), menhaden oil and caprylic acid at a 1:4.58 substrate molar ratio were incubated at 65°C. For the SL containing 20 mol% of stearic acid (SL-S), menhaden oil and stearic acid at a 1:1.37 substrate molar ratio were incubated at 75°C. For the SL containing about 14 mol% each of caprylic and stearic acid (SL-CS), menhaden oil, caprylic acid, and stearic acid, at a 1:3:1 substrate molar ratio were incubated at 75°C. Table 6.1 shows the relative fatty acid composition of the menhaden oil and all SL (under review). All SL had melting points between 25-35°C. The detailed fatty acid and triacylglycerol (TAG) composition, oxidative stability, thermal behavior, and other physicochemical properties of the SL have been discussed in further detail elsewhere (under review). Ryoto Sugar Ester (sucrose stearate) S-270 was

obtained from Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan). Ascorbyl palmitate, Tween 80, sodium alginate, Span 80, and β-sitosterol were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). γ-Oryzanol was obtained from TCI America (Portland, OR, USA). Calcium carbonate was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Calcium chloride was obtained from VWR International, LLC (West Chester, PA, USA). All other reagents and solvents were of analytical or HPLC grade and were purchased from Fisher Chemical (Fair Lawn, NJ, USA), Sigma-Aldrich Chemical Co (St. Louis, MO, USA), and J. T. Baker Chemical Co. (Phillipsburg, NJ, USA).

Microencapsulation of Lipids and Oleogels

Menhaden oil (M), SL-C, SL-S, SL-CS, or respective phytosterol or SSAP oleogels, were microencapsulated in alginate microparticles. Table 6.2 shows the internal phase composition of each of the microcapsules studied. The formation and physicochemical characterization of the phytosterol and SSAP oleogels have been discussed in detail elsewhere (Willett & Akoh, 2018b). Phytosterol oleogels were formed for each lipid, and labeled P-M, P-SL-C, P-SL-CS, and P-SL-S. Briefly, a 1:1 molar ratio blend of β-sitosterol and γ-oryzanol was dissolved at 8% (w/w) in 10 g of lipid (menhaden oil, SL-C, SL-CS, or SL-S). The mixture was stirred for 10 min at 90°C and cooled at 4°C to set the gel. SSAP oleogels were formed for each lipid, and labeled SSAP-M, SSAP-SL-C, SSAP-SL-CS, and SSAP-SL-S. A 1:1 molar ratio blend of sucrose stearate (HLB value of 2) and ascorbyl palmitate was dissolved at 12% (w/w) in 10 g of lipid (menhaden oil, SL-C, SL-CS, or SL-S). The mixture was stirred for 10 min at 110°C and cooled at 4°C to set the gel.

The microcapsules were prepared by a modified double emulsion method, also known as an internal gelation method (Sagiri et al., 2014). First, an oil-in-water emulsion was prepared by mixing 0.5 g of sodium alginate and 20 g of water at 25°C and 250rpm. Then 0.4 g calcium carbonate was added, stirred at 250rpm, and homogenized. After homogenization, 0.5 g of Span 80 and 5 g of internal phase (either menhaden oil, SL, or oleogel) was added and homogenized for 15 min. The resulting emulsion was used to form a double emulsion. The emulsion was homogenized further in an ice bath for 10 min to form a thick emulsion. This emulsion was added to 60 mL of ice-cold menhaden oil (external phase) and homogenized for 5 min at 250 rpm. 5 mL of acidified oil (4.5 mL of menhaden oil mixed with 0.5 mL glacial acetic acid) was added to the external phase while stirring to induce ionic crosslinking and gelation of the alginate layer to form the microcapsules. The formed microcapsules were washed with 0.5 M calcium chloride containing 1% tween 80, and then washed with water. Microcapsules were stored at 4°C until further analysis.

Leaching of the Internal Phase

Leaching of the internal phase was studied to determine the stability of the microcapsules. The higher the amount of leaching, the lower the stability of the microcapsules, as leaching of the internal phase into the coating and to the outside undoes the encapsulation. Microcapsules were wiped with filter paper to remove any traces of oil or moisture on the surface of microcapsules. 0.5 g of microcapsule was weighed and placed on clean filter paper. The microcapsules were then placed in the oven at 37°C, and leakage was visually monitored for 2 h.

To quantify the amount of leaching, another method was used (Sagiri et al., 2014; Brodenave, Janaswamy, & Yao, 2014). Briefly, 0.1 g of microcapsule was soaked in 1 mL of deionized (DI) water for 1 h at 37°C. The mixture was centrifuged at 2000 rpm for 5 min. The supernatant was collected and dried at 55°C for 48 h. The dried supernatant was weighed and leaching (%) was calculated as:

$$\%\ leaching = \frac{dried\ supernatant\ wt\ (g)}{microcapsule\ initial\ wt\ (g)}\ x\ 100$$

Samples were analyzed in triplicate and average values were determined.

Encapsulation Efficiency

Encapsulation efficiency (EE) was determined as previously described (Ifeduba & Akoh, 2015). Briefly, both the solvent extractable and total oil of the microcapsules were determined and EE (%) was calculated as follows:

$$EE\ (\%) = 100 - [(\%Solvent\ extractable\ /\ \%\ Total\ oil) \times 100]$$

To determine solvent extractable oil, 0.25 g of microcapsule was added to 2.5 mL of hexane and vortexed for 15 min. The mixture was centrifuged for 5 min at 1000 rpm. The organic layer was collected, filtered through an anhydrous sodium sulfate, and then transferred to a pre-weighed round bottom flask. Solvent was removed at 60°C using a rotary evaporator. Solvent extractable oil was calculated as w/w % of suspension. Samples were analyzed in triplicate and average values were determined.

To determine total oil, 5 mL of 5 M HCl was added to 0.5g of microcapsule and the mixture was agitated at 60°C for 3 h and then cooled to room temperature. The mixture was transferred to a separatory funnel and extracted twice with 5 mL hexane.

The extracted organic layer was filtered through an anhydrous sodium sulfate column and transferred to a pre-weighed round bottom flask. Solvent was removed at 60°C using a rotary evaporator. Total oil was calculated at w/w % of suspension. Samples were analyzed in triplicate and average values were reported.

Texture Analysis

Cohesiveness of the primary emulsions was determined by a compressive analysis test *via* backward extrusion studies using a TA-XTi2 texture analyzer (Stable Microsystems, Surrey, UK). Analysis was performed by moving the probe at a speed of 1 mm s^{-1} to a 10-mm distance within the emulsion and returned to the original position at the same speed. The experiment was performed in auto-force mode with a trigger force of 3 g. Samples were analyzed in triplicate and average values were determined.

Rheological Properties

Rheological analyses were performed using an HR-3 Discovery Hybrid Rheometer (TA Instruments, New Castle, DE, USA). A rotational cone and plate (gap of 0.15mm, cone angle 5.4°, plate diameter of 30 mm) was used for measurements. Analysis of the samples was conducted by varying the shear rate from 15-95s^{-1} at 25°C. Apparent viscosity of the primary emulsions were evaluated at 25°C, at shear rates of 10, 50, and 100s^{-1} to mimic changes during common food processing conditions. Temperature was controlled with a Peltier Plate Temperature System (TA Instruments, New Castle, DE, USA). All experiments were conducted in triplicate. Data was analyzed using Trios software (TA Instruments, New Castle, DE, USA).

Microscopy

The microstructure of the microcapsules was observed under an upright bright-field microscope (Leica DMRXA2 Microscope, Leica Micro-systems Canada Inc., Richmond Hill, Canada). Slides were prepared by placing the microcapsules on a slide with a small amount of DI water. Images were acquired using a charged coupled device (CCD) camera (QImaging Retiga, Burnaby, BC, Canada).

Thermal Behavior

Thermal behavior of microcapsules was analyzed using a 204 F-1 Phoenix differential scanning calorimeter (DSC) (Netzsch-Gerätebau GmbH, Selb, Germany). Microcapsule samples were heated from 22 to 120°C at a rate of 20°C/min. DSC analysis of peaks was determined using Proteus thermal analysis software (Netzsch-Gerätebau GmbH, Selb, Germany). Samples were analyzed in triplicate and average values were reported.

Oxidative Stability

Oxidative stability of the microcapsules was determined by measuring peroxide (PV) and *p*-anisidine (*p*-AV) values over 1 month of storage at 4°C. 5 g of sample was weighed into glass vials, closed, and placed in the refrigerator, held at 4°C for 30 days. PV was determined using a method from the International Dairy foundation (Shantha & Decker, 1994). The *p*-AV was determined using AOCS Official Method Cd 18-90 (American Oil Chemists' Society, 2017). The lipid portion of the microcapsule was extracted with diethyl ether under the conditions specified by ICC Standard No. 136, with some modification (International Association for Cereal Science and Technology, 1984). 5 g of microcapsule was mixed with 4 mL of 95% ethanol and 1mL of 7M NaOH. Then, 5 mL of

diethyl ether was added and shaken. After phase separation, 5mL of hexane was added, mixed vigorously, and phase separation was allowed. The organic layer was collected, washed with 5 mL of 1 M HCl, followed with 5 mL of 0.5 M Na_2SO_4. The organic layer was transferred to a pre-weighed round bottom flask and solvent was evaporated under nitrogen. PV and *p*-AV were then measured. Oil Stability Index (OSI) of the microcapsules was also determined using an Oxidative Stability Instrument (Omnion Inc., Rockland, MA, USA) and AOCS Official Method Cd 12b-92 (American Oil Chemists' Society, 2017). Samples were analyzed in triplicate and average values were reported.

Statistical Analysis

Statistical analysis was performed on the data using JMP® software (Version 10, SAS Institute Inc., Cary, NC, USA). Data was analyzed using ANOVA and significant differences ($p < 0.05$) were determined using the Tukey test.

Results and Discussion

Leaching of the Internal Phase

Figure 6.1 shows a visual representation of internal phase leaching after 2 h in a 37°C oven for M, P-M, and SSAP-M. The menhaden oil (M) microcapsule showed visual leakage compared to the P-M and SSAP-M. Both P-M and SSAP-M showed some visual leakage of the internal phase, but it was significantly less compared to the menhaden oil microcapsule. This is likely due to higher apparent viscosity and stabilization of the emulsion by the oleogels, which will be discussed in the rheological properties section.

Table 6.3 shows the quantitative percent leaching for all microcapsule samples. The microcapsules that contained either the phytosterol oleogel or the SSAP oleogel all

had significantly ($p < 0.05$) lower percentages of leaching compared to the microcapsules containing a non-gellated internal phase. Leaching of the internal phase was reduced from 16.3-18.5% to 3.3-11.9%. A previous study that compared encapsulating sunflower oil and sorbitan monostearate oleogel found 46.1 and 9.4% leaching, respectively (Sagiri et al., 2014). Amount of leaching can be attributed to internal phase composition (Sagiri et al., 2012). The SSAP and phytosterol oleogels showed a lower amount of leaching compared to the sorbitan monostearate oleogel and may be better organogelators than sorbitan monostearate in terms of forming a stronger gel network that aids in preventing leaching.

Encapsulation Efficiency

Table 6.4 shows the EE for all microcapsule samples. A higher EE is preferred as that meant more lipid was encapsulated. The EE for all samples ranged from 89.8-99.2%. The microcapsules that contained either the phytosterol oleogel or the SSAP oleogel had significantly ($p < 0.05$) higher EE compared to the M, SL-C, SL-CS, and SL-S microcapsules. The strong gel network of the phytosterol and SSAP oleogels were able to stabilize the o/w emulsions, allowing for a higher proportion of lipid to be encapsulated (Willett & Akoh, 2018b). These results show that organogelation may be a suitable method for improving the stability of the microcapsules by reducing amount of leaching and improving EE.

Texture Analysis

Table 6.5 shows the cohesiveness of the primary emulsions for all samples. Generally, all phytosterol and SSAP oleogel samples had significantly ($p < 0.05$) higher cohesiveness values. The menhaden oil emulsion had a lower cohesiveness. SL-C and SL-CS primary emulsions had statistically similar ($p > 0.05$) cohesiveness compared to the menhaden oil emulsion. SL-S had a significantly higher cohesiveness than the menhaden oil, SL-C, and SL-CS. This was likely due to the higher stearic acid content and melting point, which aided in a stronger fat crystal network (Rogers, 2009). Addition of the oleogel enhanced the cohesiveness of the emulsion. Higher cohesiveness typically corresponds to a higher apparent viscosity and will be discussed in further detail in the rheological properties section.

Rheological Properties

Figure 6.2 shows the apparent viscosity of the primary emulsions for all samples. Almost all the phytosterol and SSAP oleogel samples had a higher apparent viscosity. These results correspond well to the texture analysis. The menhaden oil microcapsule had a significantly lower apparent viscosity, lower cohesiveness, and lower EE (sections 3.2 and 3.3). The phytosterol and SSAP oleogel samples had higher cohesiveness and higher apparent viscosity, which led to the higher EE. The higher EE and reduced amount of leaching may be attributed to the gelation of the menhaden oil and SL. Increase in apparent viscosity has been shown to reduce leaching (Sagiri et al., 2014; Sagiri et al., 2012; Sharma, Madan, & Senshang, 2016). Previous studies have shown that fatty acyl oleogels, similar to the SSAP oleogels, have a tendency to accommodate water within the gel network, and may have resulted in the increase in viscosity of the emulsion (Behera,

Sagiri, Pal, & Srivastava, 2013). There was no gel network in the menhaden oil sample, likely accounting for it having a lower apparent viscosity. Both the cohesiveness and apparent viscosity of the primary emulsions followed a similar trend, where a higher cohesiveness corresponds to a higher apparent viscosity. The increase in apparent viscosity for the phytosterol and SSAP oleogel samples was likely due to an increase in cohesiveness among the different components. These results were comparable to similar studies (Sagiri et al, 2014; Jeyakumari, Zynudheen, Parvathy, & Binsi, 2018). The results suggest that having an oleogel as the internal phase enhanced the viscosity and cohesiveness, which may have accounted for the reduction in leaching and increase in EE.

Microscopy

Figure 6.3 shows the microcapsules under the light microscope. The microcapsules all exhibited a spherical shape, though varied in size. Apart from P-M and SSAP-SL-C, the oleogel microcapsules had a larger particle size than M, SL-C, SL-CS, and SL-S (non-oleogel microcapsules). EE can be influenced by partition coefficient of target molecule in the solvents used in formulation, method used, and particle size (Piacentini, Poerio, Bazzarelli, & Giorno, 2016). The non-oleogel microcapsules (M, SL-C, SL-CS, and SL-S) all had mostly smaller particle sizes, and lower EE. The oleogel microcapsules also seemed to aggregate together more often than the non-oleogel microcapsules. This may be partly due to the oleogel primary emulsions having higher cohesiveness, as shown in Figure 6.2 and Table 6.5. A previous study found that higher aggregation and particle size was seen with emulsions that exhibited higher cohesiveness and apparent viscosity (Jeyakumari et al., 2018; Sagiri et al., 2012).

Thermal Analysis

For all samples there was an endothermic peak between 35-110°C (data not shown). Table 6.6 shows the values for peak temperature onset, completion, and enthalpy of all samples. The non-oleogel microcapsules (M, SL-C, and SL-CS) had a peak onset temperature at around 75°C and peak completion temperature at around 92°C. P-SL-CS also had a statistically similar ($p > 0.05$) peak onset temperature (77.68°C) but higher completion temperature (98.88°C). In other samples, the oleogel microcapsules, had peak onset temperatures between 36.48-56.20°C, and peak completion temperatures between 92.37-107.52°C. In a previous study, we reported the gel to sol transition temperature of the phytosterol and SSAP oleogels to be between 40.1-53.1°C. The broad peaks for the oleogel microcapsules may be explained by the simultaneous transition of the oleogel from gel to sol, and evaporation of water present in the oleogel. Previous studies have also shown this, where although the microcapsule samples were dried, it was difficult to remove bound water present (Sagiri et al., 2014). The shift in the endothermic peak suggests the lipids and respective phytosterol and SSAP oleogels were successfully encapsulated within the alginate microparticles.

Oxidative Stability

Table 6.7 shows the OSI values for the microcapsules. All the microencapsulated products had significantly higher ($p < 0.05$) OSI values (19.75-29.18 h) than the non-microencapsulated samples (4.37-22.31 h). Also, the oleogel samples (either microencapsulated or non-microencapsulated) had significantly higher OSI values than the corresponding non-gelled lipid. These results show that organogelation and formation of microcapsules with oleogels improved the oxidative stability of the lipids.

Table 6.8 shows the PV and *p*-AV of the microcapsules after 30 days of storage. The Global Organization for EPA and DHA omega-3 (GOED) sets voluntary limits for PV (5 meq/kg) and *p*-AV (20) of fish oil, which are much lower than the limits set for refined vegetable oils in most countries (5-10 meq/kg, and 15-30, respectively) (Ismail, Bannenberg, Rice, Schutt, & Mackay, 2016). Of the two values, *p*-AV is more indicative of the overall quality of the oil.

The results in this study show that at day 0 of storage, all microencapsulated samples, except for SL-C, SL-CS, and SL-S, had PV of less than 10 meg/kg. During purification of SL by short-path distillation, endogenous antioxidants are lost, explaining the lower oxidative stability of the SL (Zou & Akoh, 2013). Only SSAP-SL-C had a PV less than 5 meq/kg, following the GOED guidelines. The oleogel microcapsules, except for P-M, all had significantly lower ($p < 0.05$) PV than the microcapsules containing the non-gelled lipids (M, SL-C, SL-CS, and SL-S). After 30 days of storage, P-M, SSAP-M, SSAP-SL-C, P-SL-S, and SSAP-SL-S had PV less than 10 meq/kg. Both SSAP-M and SSAP-SL-C had PV less than 5 meq/kg. Interestingly there was a decrease in PV for P-M and SSAP-M after 30 days of storage, which is likely due to the formation of secondary oxidation products from the hydroperoxides, as shown in the increase in *p*-AV from 0 to 30 days of storage.

For the *p*-AV, all microcapsules, apart from SL-C, SL-CS, and SL-S, had *p*-AV less than 20, per GOED guidelines. The oleogel microcapsules also had significantly lower ($p < 0.05$) *p*-AV than the non-gelled lipid (M, SL-C, SL-CS, and SL-S) microcapsules. After 30 days of storage, all microcapsules, except for SL-C, SL-CS, and SL-S, had *p*-AV less than 20, showing that the quality of the oils was still acceptable after

30 days of storage. Previous studies have shown that microencapsulation may lead to a lower oxidative stability than the bulk lipid, due to the encapsulation process (Encina, Vergara, Gimenez, Oyarzun-Ampuero, & Robert, 2016). Our results showed that microencapsulation of oleogels is a good method for improving oxidative stability of SL and oils high in omega-3 fatty acids. The oils were protected from oxidation during the microencapsulation process.

Conclusion

We showed that the leakage of menhaden oil or SL from the alginate microparticles were reduced by use of respective phytosterol or SSAP oleogels as internal phase. Encapsulation of the oleogels also led to increased EE, likely due to higher primary emulsion cohesiveness and apparent viscosity. Almost all microcapsules had acceptable oxidative stability, within the typical limits set by GOED and many countries. Because of the higher stability, these microcapsules should be acceptable for use in the fortification of food products with health beneficial omega-3 fatty acids and/or MLM-type SL.

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Tables

Table 6.1 Relative fatty acid composition of menhaden oil and SL (Willett, Akoh, & Martini, under review)

Samples[a]	TAG Position	Fatty Acid (mol%)[b]				
		C8:0	C16:0	C18:0	C20:5n3	C22:6n3
Menhaden Oil	Total	ND	10.20±0.85	3.08±0.05	16.90±0.10	15.40±0.16
	sn-2	ND	14.86±0.74	3.21±0.37	16.42±0.64	29.37±0.95
	sn-1,3[c]	ND	7.87±0.91	3.02±0.21	17.14±0.45	8.42±0.37
SL-C	Total	29.03±1.37	5.55±0.06	2.74±0.10	13.61±0.38	10.42±0.03
	sn-2	11.72±0.13	16.94±0.07	2.97±0.09	12.22±0.03	25.17±0.46
	sn-1,3	37.69±0.75	0.15±0.05	2.63±0.10	14.31±0.21	3.05±0.25
SL-CS	Total	16.24±0.56	4.73±0.21	13.04±0.10	13.87±0.26	12.50±0.20
	sn-2	7.31±1.01	15.11±0.06	8.82±0.55	10.69±0.32	16.66±1.39
	sn-1,3	20.71±0.34	0.46±0.29	15.14±0.13	15.46±0.23	10.42±0.40
SL-S	Total	ND	7.50±0.05	19.50±0.23	14.16±0.11	14.62±0.39
	sn-2	ND	4.29±0.11	10.07±0.78	11.85±0.26	22.98±0.18
	sn-1,3	ND	9.11±0.02	24.22±0.51	16.21±0.19	10.44±0.29

Mean ± STD, *n*=3, ND= not detected

[a]SL-C: structured lipid (SL) of menhaden oil and 30 mol% caprylic acid, SL-S: structured lipid (SL) of menhaden oil and 20 mol% stearic acid, SL-CS: structured lipid (SL) of menhaden oil and 16 mol% caprylic acid and 13 mol% stearic acid

[b]Other fatty acids found at >1mol%: C14:0, C16:1n7, C17:1n7, C18:1n9, C18:2n6, C18:3n3, C20:0, C18:4n3, C22:5n3, and <1 mol%: C13:0, C14:1n7, C15:0, C17:0, C19:0, C18:3n6, C20:1n7, C20:2n6, C22:0, C20:3n6, C20:3n3, C23:0, C22:2n6, C24:1n9, C22:4n6

[c]*sn*-1,3 mol% determined by equation: *sn*-1,3 (mol%) = [3xtotal mol%- *sn*-2 mol %]/2

Table 6.2 Internal phase composition of the microcapsules

Sample	Internal Phase
M	Menhaden oil
P-M	Phytosterol oleogel containing menhaden oil
SSAP-M	SSAP oleogel containing menhaden oil
SL-C	SL of menhaden oil containing 30 mol% caprylic acid
P-SL-C	Phytosterol oleogel containing SL-C
SSAP-SL-C	SSAP oleogel containing SL-C
SL-CS	SL of menhaden oil containing ~14 mol% each caprylic & stearic acid
P-SL-CS	Phytosterol oleogel containing SL-CS
SSAP-SL-CS	SSAP oleogel containing SL-CS
SL-S	SL of menhaden oil containing 20 mol% stearic acid
P-SL-S	Phytosterol oleogel containing SL-S
SSAP-SL-S	SSAP oleogel containing SL-S

Table 6.3 Percent leaching of menhaden oil, SL, or oleogel microcapsules

Sample[a]	Leaching (%)
M	18.54±1.13[a]
P-M	9.02±0.60[b]
SSAP-M	11.93±0.78[b]
SL-C	20.30±1.08[a]
P-SL-C	8.42±1.71[b]
SSAP-SL-C	9.88±0.11[b]
SL-CS	18.22±1.03[a]
P-SL-CS	9.14±0.74[b]
SSAP-SL-CS	4.38±0.33[c]
SL-S	16.31±0.85[a]
P-SL-S	7.27±0.56[b]
SSAP-SL-S	3.28±0.34[c]

Mean ± STD, n=3. Different letters in the same column are significantly different (α=0.05)

[a] Abbreviations are the same as described in Table 1 & 2

Table 6.4 Encapsulation efficiency of menhaden oil, SL, or oleogel microcapsules

Sample[a]	Encapsulation Efficiency (%)
M	89.79±0.77[a]
PM	96.87±0.61[b]
SM	95.69±0.72[b]
SL-C	89.57±0.32[a]
P-SL-C	98.09±0.77[c]
S-SL-C	96.13±0.67[b]
SL-CS	90.64±0.95[a]
P-SL-CS	96.24±0.76[b]
S-SL-CS	98.92±0.45[c]
SL-S	91.26±0.89[a]
P-SL-S	97.08±0.96[c]
S-SL-S	99.17±0.59[c]

Mean ± STD, n=3. Different letters in the same column are significantly different (α=0.05)

[a]Abbreviations are the same as described in Table 1 & 2

Table 6.5 Cohesiveness of the primary o/w emulsions

Sample[a]	Cohesiveness ($kg\ s^{-1}$)
M	0.01±0.04[a]
P-M	0.32±0.06[b]
SSAP-M	0.21±0.05[b]
SL-C	0.07±0.01[a]
P-SL-C	0.18±0.07[b]
SSAP-SL-C	0.18±0.02[b]
SL-CS	0.10±0.01[a]
P-SL-CS	0.15±0.02[a,b]
SSAP-SL-CS	0.21±0.02[b]
SL-S	0.19±0.04[b]
P-SL-S	0.26±0.03[b]
SSAP-SL-S	0.31±0.07[b]

Mean ± STD, n=3. Different letters in the same column are significantly different (α=0.05)

[a]Abbreviations are the same as described in Table 1 & 2

Table 6.6 Thermal behavior of the menhaden oil, SL, and oleogel microcapsules

Sample[a]	Onset (°C)	Completion (°C)	Peak Enthalpy (mW/mg)
M	75.57±0.51[a]	92.03±0.62[a]	1.08±0.15[a]
P-M	42.50±0.64[b]	107.52±0.31[b]	4.05±0.03[b]
SSAP-M	36.48±0.32[c]	92.37±0.05[a]	1.57±1.68[a]
SL-C	75.71±0.95[a]	92.12±0.97[a]	1.12±0.16[a]
P-SL-C	42.90±0.04[b]	95.01±0.27[c]	1.09±0.99[a]
SSAP-SL-C	53.10±0.30[d]	93.87±0.31[c]	3.28±0.17[b]
SL-CS	74.98±0.18[a]	97.17±1.02[d]	7.65±0.33[c]
P-SL-CS	77.68±0.16[a]	98.88±0.23[d]	1.23±0.61[a]
SSAP-SL-CS	55.11±0.42[d]	105.53±0.62[b]	5.81±1.49[b]
SL-S	75.65±0.37[a]	94.01±0.34[c]	1.83±0.37[a]
P-SL-S	37.81±0.02[c]	96.81±0.21[d]	4.36±0.52[b]
SSAP-SL-S	56.20±0.15[d]	106.51±0.55[b]	6.63±0.19[c]

Mean ± STD, n=3. Different letters in the same column are significantly different (α=0.05)

[a]Abbreviations are the same as described in Table 1 & 2

Table 6.7 Oil stability index (OSI) values for menhaden oil, SL, or oleogel microcapsules

Sample[a]	Non-Microencapsulated	Microencapsulated
	OSI (h)	OSI (h)
M	17.55±1.21[a]	21.13±0.21[a]
P-M	20.88±1.03[b]	28.80±0.22[b]
SSAP-M	24.92±3.70[c]	29.18±0.08[b]
SL-C	4.37±0.88[d]	19.75±0.22[a]
P-SL-C	7.24±0.82[e]	25.67±0.23[c]
SSAP-SL-C	12.72±1.04[f]	26.15±0.05[c]
SL-CS	11.70±1.31[f]	20.23±0.10[a]
P-SL-CS	15.48±0.91[a]	26.72±0.36[c]
SSAP-SL-CS	22.31±0.74[b]	26.78±0.15[c]
SL-S	9.13±1.07[e]	20.35±0.10[a]
P-SL-S	13.66±0.37[f]	28.53±0.21[b]
SSAP-SL-S	18.42±0.04[a]	27.63±0.08[b]

Mean ± STD, n=3. Different letters in the same column are significantly different (α=0.05)

[a]Abbreviations are the same as described in Table 1 & 2. OSI conducted at 80°C

Table 6.8 Peroxide and *p*-Anisidine values of menhaden oil, SL, or oleogel microcapsules after 30 days of storage at 4°C

Sample[a]	Peroxide Value (meq/kg)		*p*-Anisidine Value	
	Day 0	Day 30	Day 0	Day 30
M	7.59±1.53[a]	14.61±2.53[a]	9.16±1.56[a]	13.34±1.01[a]
P-M	8.81±1.56[b]	5.73±1.77[b]	5.16±0.30[b]	6.54±0.22[b]
SSAP-M	7.85±1.77[a]	3.01±0.72[c]	2.00±0.15[c]	6.20±0.35[b]
SL-C	19.20±3.25[c]	24.37±1.92[d]	52.36±0.75[d]	56.20±1.42[c]
P-SL-C	8.62±1.21[b]	11.03±0.89[e]	7.48±0.66[b]	19.78±0.82[d]
SSAP-SL-C	2.79±0.48[d]	3.01±0.26[c]	2.80±0.52[c]	15.06±0.75[d]
SL-CS	11.51±1.34[b]	38.61±2.84[e]	21.70±0.87[e]	28.84±1.11[e]
P-SL-CS	5.42±0.63[a]	15.73±0.68[a]	12.92±0.35[a]	13.74±0.26[a]
SSAP-SL-CS	2.63±0.51[d]	13.08±0.35[a]	5.80±0.12[b]	12.66±0.60[a]
SL-S	18.48±1.45[c]	22.27±0.84[d]	26.56±1.14[e]	40.02±0.85[c]
P-SL-S	8.18±0.93[b]	9.69±0.18[e]	19.98±1.24[f]	24.62±1.32[e]
SSAP-SL-S	6.07±0.65[a]	8.58±1.53[e]	15.16±1.24[f]	16.40±0.64[d]

Mean ± STD, n=3. Different letters in the same column are significantly different (α=0.05)

[a]Abbreviations are the same as described in Table 1 & 2

Figures

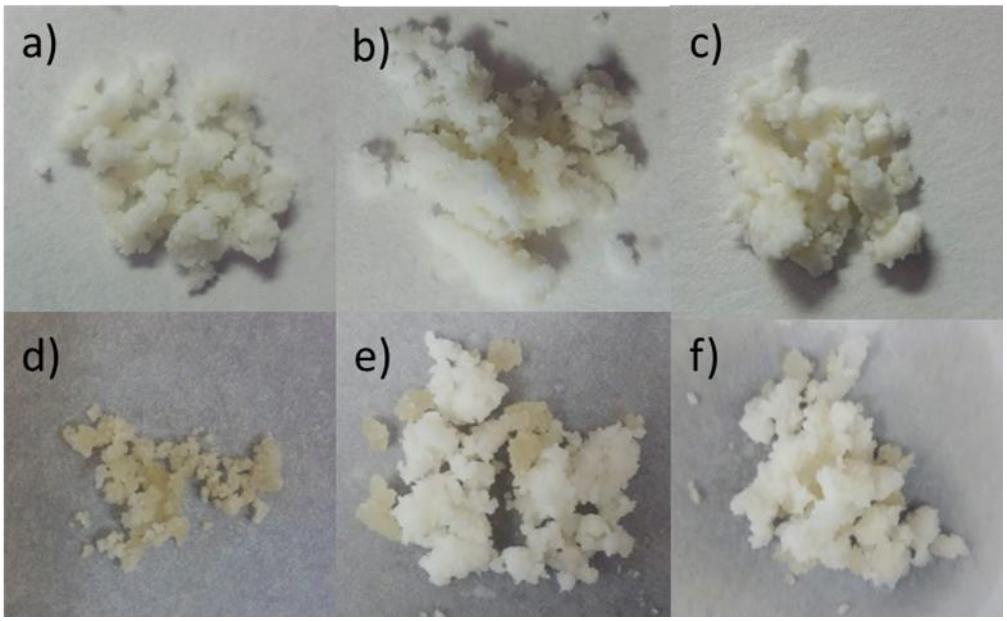

Figure 6.1 Visualization of microcapsules before and after 2h in 37°C oven. Menhaden oil microcapsules before (a) and after (d) 2h, P-M microcapsules before (b) and after (e) 2 h, and SSAP-M microcapsules before (c) and after (f) 2h

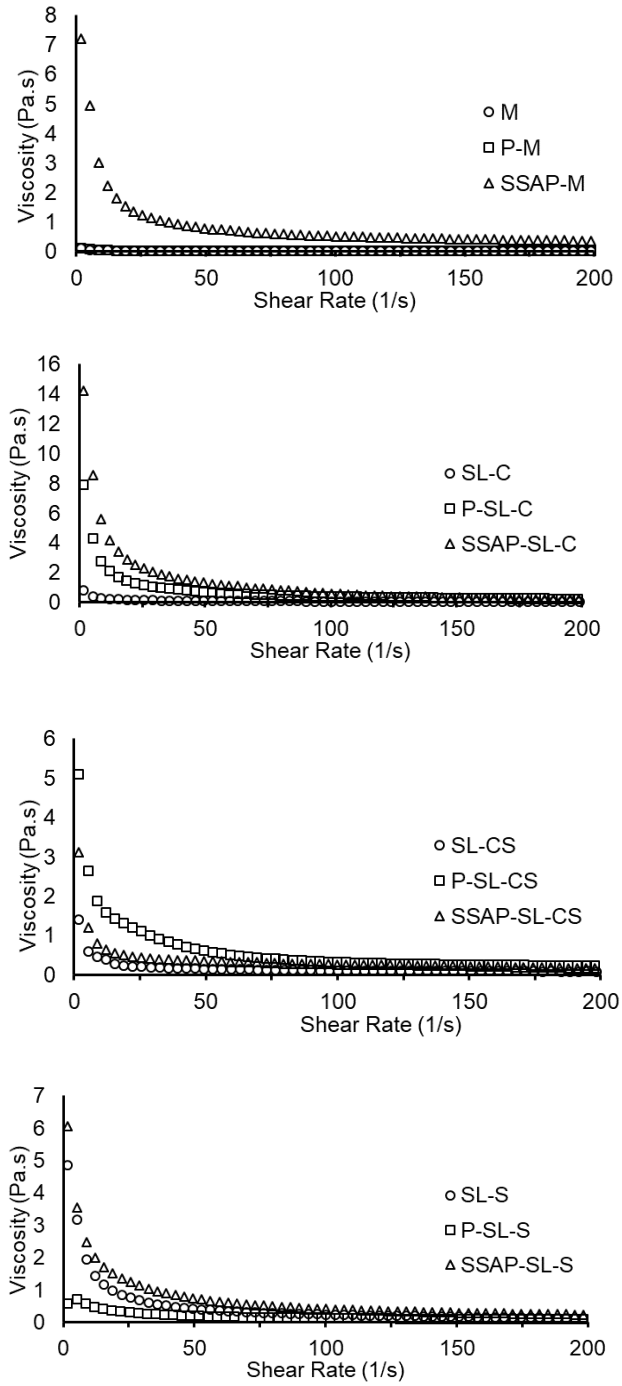

Figure 6.2 Apparent viscosity of primary emulsions. Abbreviations are the same as described in Table 1 & 2

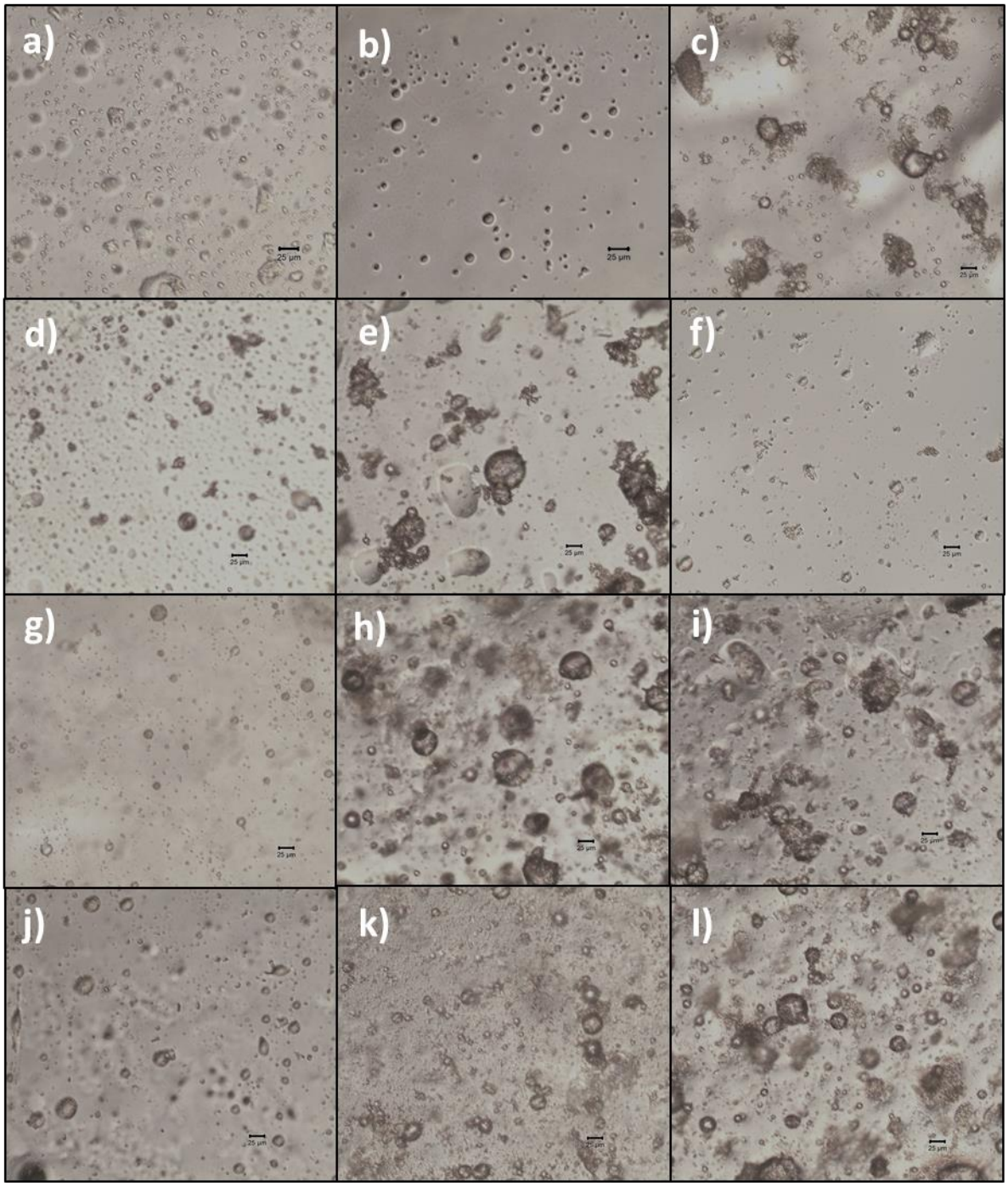

Figure 6.3 Light microscopy of microcapsules produced with different lipid portion, where a) menhaden oil, b) P-M, c) SSAP-M, d) SL-C, e) P-SL-C, f) SSAP-SL-C, g) SL-CS, h) P-SL-CS, i) SSAP-SL-CS, j) SL-S, k) P-SL-S, and l) SSAP-SL-S. Abbreviations for the different lipid portions are the same as described in Table 1&2. Scale bars = 25µm

CHAPTER 7

PHYSICOCHEMICAL PROPERTIES OF YELLOW CAKE PREPARED WITH STRUCTURED LIPID OLEOGELS[1]

[1]Willett, S. A., and Akoh, C. C. 2019. Accepted by the Journal of Food Science on 04/06/19.

Abstract

Oleogels were produced using a phytosterol blend of β-sitosterol/γ-oryzanol or a blend of sucrose stearate/ascorbyl palmitate (SSAP) as oleogelators. Four lipid phases were compared in oleogel formation for each oleogelator blend: menhaden oil, structured lipid (SL) of menhaden oil and 30 mol% caprylic acid (SL-C), SL of menhaden oil and 20 mol% stearic acid (SL-S), and SL of menhaden oil and 14 mol% each of caprylic and stearic acid (SL-CS). All SL were produced enzymatically using a recombinant lipase from *Candida antarctica* as the biocatalyst. Menhaden oil, SL, phytosterol, or SSAP oleogels were evaluated as alternatives to shortening in the preparation of yellow cake in terms of batter and cake physicochemical properties. The shortening, phytosterol, and SSAP oleogel batters, exhibited statistically similar specific gravities (0.85). The shortening, and menhaden oil phytosterol and SSAP oleogel batters, exhibited similar Power-Law values (n: 0.78, k: 31 Pa s^n), while all SL (and respective oleogels) batters typically had lower flow index values (n: 0.68-0.72), and higher consistency index values (k: 45-79 Pa s^n). All SL (and respective oleogels) cakes exhibited lower hardness (5-8 N) and chewiness (4-6 N) than the shortening cake (12 N, 9 N). Menhaden oil and SL-S phytosterol oleogel cakes, and SL-CS SSAP oleogel cake, showed similar textural properties to the shortening cake. Both phytosterol and SSAP oleogels were acceptable as zero *trans*-fat substitutes for shortening in yellow cake.

Keywords: menhaden oil, cake, oleogels, structured lipids, lipase

Practical Application: The oleogels in this study may be a suitable replacement for shortening in yellow cake. These oleogels, which contain health beneficial omega-3 fatty

acids, EPA and DHA, have the potential to lower consumer consumption of total saturated fat when used in foods.

Introduction

Solid fats are one of the main components that influence both taste and texture of cakes. This is primarily due to the ability of solid fats to form a colloidal network that physically traps oil within the structure, aiding in air bubble incorporation and stabilization in cakes (Matthews & Dawson, 1966). Solid fats, however, are mostly composed of saturated fatty acids (SFA). It has been found that consuming significant amounts of certain SFA may increase LDL cholesterol levels and risk of cardiovascular disease and stroke (He, 2009). There are many plant and fish oils that are low in total saturated fat and contain significant amounts of monounsaturated fatty acids (MUFA) and/or polyunsaturated fatty acids (PUFA) (Ackman, 1967). However, oils high in MUFA and/or PUFA do not have the same physicochemical properties as solid fats that are necessary in applications such as baked goods. One alternative to SFA is structuring liquid oils through the formation of oleogels (also known as organogels).

Oleogels are lipid gels that have promise for use as low saturated fat substitutes in food applications (Co & Marangoni, 2012). Studies have shown the use of oleogels as a replacement for solid fats in ice creams, frankfurters, cookies, cakes, and chocolate spreads (Co & Marangoni, 2012; Hughes, Marangoni, Wright, Rogers, & Rush, 2009; Patel et al., 2014). A previous study in our lab successfully identified another oleogelator blend, sucrose stearate/ascorbyl palmitate (SSAP), that has potential for food applications based on its physicochemical properties (Willett & Akoh, 2018). Previous literature has found that the use of sucrose stearate as an emulsifier in baked goods lead to desirable cake qualities such as uniform texture and crumb, increased mechanical resistance, and increased volume after baking

(Hughes et al., 2009; Patel et al., 2014; Stankov, Baeva, Goranova, & Marudova, 2016). The SSAP blend is also an inexpensive and cheap alternative to other oleogelators such as phytosterols or ceramides. In this current study, the objective is to determine if this oleogelator blend (SSAP) can be a suitable low saturated fat substitute for shortening in yellow cake. Due to its gel network structure and higher oxidative stability, the oleogels may have similar batter and cake physicochemical properties when compared to shortening.

In this research, two oleogelator blends were compared: a phytosterol blend of β-sitosterol/γ-oryzanol or a blend of SSAP. Four lipid phases were compared in oleogel formation for each oleogelator blend: menhaden oil, structured lipid (SL) of menhaden oil and 30 mol% caprylic acid (SL-C), SL of menhaden oil and around 14 mol% each of caprylic and stearic acid (SL-CS), and SL of menhaden oil and 20 mol% stearic acid (SL-S). Menhaden oil contains significant amounts of health beneficial omega-3 PUFA, particularly high amounts of DHA at the *sn*-2 position, and is a sustainable fish oil source (Bibus, 2016). The menhaden oil-based SL all exhibited different physicochemical properties (such as higher melting point) than the menhaden oil but were lower in oxidative stability. Use of oleogelation may improve the oxidative stability and physicochemical properties of the menhaden oil and SL for use in food applications such as baked goods. The menhaden oil, SL and respective oleogels were compared to commercial shortening (Crisco®) in the formulation of yellow cake. Successful use of these lipids in formulation of yellow cake may allow for increased consumption of healthful omega-3 PUFA.

Materials and Methods

Materials

Menhaden oil, obtained from Omega Protein Inc. (Reedville, VA, USA), and the acyl donors, caprylic acid (≥98 % purity) and stearic acid (≥98 % purity), purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), were used as substrates in acidolysis reactions to produce SL. The SL were produced according to the method outlined previously (Willett, Martini, & Akoh, under review). Briefly, all acidolysis reactions were conducted in a 1L batch reactor for 24 h, with Lipozyme® 435 lipase (Novozymes North America, Inc., Franklinton, NC, USA) at 10% (w/w) of total substrates and stirring at 250 rpm using a SL 2400 StedFast stirrer (Fisher Scientific Co., Fair Lawn, NJ, USA). For SL-C, menhaden oil and caprylic acid at a 1:4.58 substrate molar ratio were reacted at 65°C. For SL-S, menhaden oil and stearic acid at a 1:1.37 substrate molar ratio were reacted at 75°C. For SL-CS, menhaden oil, caprylic acid, and stearic acid, at a 1:3:1 substrate molar ratio were reacted at 75°C. Table 7.1 shows the relative fatty acid composition of the menhaden oil and all SL (Willett, Martini, & Akoh, under review). SL-C contained 30 mol% caprylic acid, SL-S contained 20 mol% stearic acid, and SL-CS contained about 14 mol% each of caprylic and stearic acid. All SL had melting points between 25-35°C. The detailed fatty acid and TAG composition, oxidative stability, thermal behavior, and other physicochemical properties of the SL have been discussed in further detail elsewhere (Willett, Martini, & Akoh, under review). Ryoto Sugar Ester S-270 (melting point 61°C), a sucrose fatty acid ester, was obtained from Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan). The “S” corresponds to stearic acid as the primary fatty acid esterified to sucrose. The designation of “270” corresponds to the HLB

value, ester composition, and purity of the stearic acid. The "2" indicates the product has an HLB value of 2, and the "70" indicates that the purity of stearic acid is 70%. Per manufacturer details, the Ryoto Sugar Ester S-270 contains 10% sucrose monostearate and 90% sucrose di-, tri-, and poly-stearate. Ascorbyl palmitate (melting point 115°C) and β-sitosterol (melting point 136°C) were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The γ-oryzanol (melting point 151°C) was obtained from TCI America (Portland, OR, USA). Commercial shortening (Crisco®, fatty acid profile shown in Table 1), cake flour, sugar, nonfat dry milk, salt, whole eggs, baking powder, and vanilla extract were obtained from a local Kroger store in Athens, GA, USA (Kroger, Cincinnati, Ohio, USA). All other reagents and solvents were of analytical or HPLC grade and were purchased from Fisher Chemical (Fair Lawn, NJ, USA), Sigma-Aldrich Chemical Co (St. Louis, MO, USA), and J. T. Baker Chemical Co. (Phillipsburg, NJ, USA).

Cake Preparation

In triplicate, cakes were prepared by a previous method in 100g batches. 23.82g of cake flour, 28.59g sugar, 1.79g nonfat dry milk, 0.71g salt, 9.56g egg, and 1.49g baking powder were blended for 1 min on low speed using a mixer with paddles (Rogers, Malouf, Langemeier, Gelroth, & Ranhotra, 1993). Then, 0.11g vanilla extract, 7.15g fat portion, and 14.89g (of 33.36g total) water, were added and mixed for 1 min on low speed and 3 min on medium speed. More water (7.15g) was added. The batter was mixed for 1 min on low speed and 2 min on medium speed. Finally, the remaining water (11.32g) was added. The batter was mixed again for 2 min on low speed. The batter was

then poured into a 4-in, round, cake pan and baked for 17 min at 182.2°C. Baked cakes were stored at 25°C until further analysis.

The fat portion of the cake was either shortening (Crisco®), menhaden oil (M), SL-C, SL-S, SL-CS, or respective phytosterol or SSAP oleogels. The abbreviations for all fat portions are shown in Table 7.2. The formation (oleogelator blend molar ratio and w/w% in oil selection) and physicochemical characterization of the phytosterol and SSAP oleogels have been discussed in detail elsewhere (Willett & Akoh, 2018). Phytosterol oleogels were formed for each lipid, and labeled P-M, P-SL-C, P-SL-S, and P-SL-CS. Briefly, a 1:1 molar ratio blend of β-sitosterol and γ-oryzanol was dissolved at 8% (w/w) in 10 g of lipid (menhaden oil, SL-C, SL-S, or SL-CS). The mixture was stirred for 10 min at 90°C and cooled at 4°C to set the gel. SSAP oleogels were formed for each lipid, and labeled SSAP-M, SSAP-SL-C, SSAP-SL-S, and SSAP-SL-CS. A 1:1 molar ratio blend of sucrose stearate (HLB value of 2) and ascorbyl palmitate was dissolved at 12% (w/w) in 10 g of lipid (menhaden oil, SL-C, SL-S, or SL-CS). The mixture was stirred for 10 min at 110°C and cooled at 4°C to set the gel.

Rheological Properties of Cake Batter

Rheological measurements were carried out using an HR-3 Discovery Hybrid Rheometer (TA Instruments, New Castle, DE, USA). A parallel plate (P50 Ti L, diameter 50 mm, gap= 100 ± 0.2 μm) was used for measurements. Temperature was controlled with a Peltier Plate Temperature System (TA Instruments, New Castle, DE, USA). Batters were prepared right before conducting rheological measurements and stored in closed containers until analyzed. All experiments were conducted in triplicate. Data was analyzed using Trios software (TA Instruments, New Castle, DE, USA).

Flow curves of the batter were obtained using a shear rate ranging from 5 to 200 1/s at 25˚C. The cake batter samples underwent three shear rate sweeps (up-down-up) to eliminate thixotropy and data was obtained in steady state. Apparent viscosity of the batter was also evaluated at 25˚C at shear rates of 10, 50, and 100 s^{-1} to mimic changes during typical food processing. The Power-law model was used to fit the non-Newtonian fluid behavior of the batters and is defined by the equation: $\sigma = k (\gamma)^n$, σ is shear stress (Pa) and (γ) is shear rate (s^{-1}) (Da Pieve, Calligaris, Co, Nicoli, & Marangoni, 2010).

Light Microscopy

The cake batters were imaged by a Leica DMRXA2 Microscope (Leica Micro-systems Canada Inc., Richmond Hill, Canada). To prepare slides, 1 drop of sample was placed between a stationary and moving glass plate. Images were acquired using a charged coupled device (CCD) camera (QImaging Retiga, Burnaby, BC, Canada).

Differential Scanning Calorimetry

Thermal measurements of 35-40 mg of batter were carried out using a 204 F-1 Phoenix differential scanning calorimeter (DSC) (Netzsch-Gerätebau GmbH, Selb, Germany). Samples in hermetically sealed aluminum pans were heated from 22 to 120°C at a rate of 20°C/min. The endothermic peak obtained was considered as starch gelatinization during the heating of the cake batter (Ndife, Sumnu, & Bayindirli, 1998). The peak onset and completion temperatures were determined using Proteus thermal analysis software (Netzsch-Gerätebau GmbH, Selb, Germany). Samples were analyzed in triplicate and average values were reported.

Physical Measurements

To determine air incorporation, batter specific gravity was measured by taking the difference in weight of batter over weight of distilled water in the same standard container (Psimouli & Oreopoulou, 2013). Specific volume of cake was determined as the ratio of the cake volume by weight. The volume of the cake was determined by the rapeseed displacement method according to the AACC approved method 10-05 (American Association of Cereal Chemists, 1995). Measurements were conducted in triplicate and average values were reported.

Crumb and crust color of the cakes were measured using a tristimulus chromatometer (CR200, Minolta Co., Osaka, Japan). Measurements at 9 different regions were conducted and results were expressed as average L (darkness to lightness), a (redness to greenness), and b (blueness to yellowness) values of the CIELAB system. L values range from 0 to 100, a values range from -100 to 100, and b values range from -100 to 100. Measurements were conducted in triplicate and average values reported.

Crumb texture was determined by TA-XT plus Texture Analyzer (Stable Microsystems, Surrey, UK). Samples of cake were sliced in pieces sized 40x40x20 mm and underwent a 2-cycle compression test (Texture Profile Analysis) using a 36 mm diameter cylindrical probe (test speed 1mm/s, penetration distance 8mm, time between 2 cycles will be 15 s). From the force-time diagram, hardness, springiness, cohesiveness, resilience, and chewiness were calculated and average values were reported.

Oxidative Stability

Oxidative stability was conducted by measuring peroxide (PV) and *p*-Anisidine (*p*-AV) values over 7 days. 10 g of cake was weighed into glass vials, closed, and stored at 22°C. PV was determined using a method from the International Dairy foundation (Shantha & Decker, 1994). The *p*-AV was determined using AOCS Official Method Cd 18-90 (American Oil Chemists' Society, 2017). The lipid portion of the cakes was extracted with diethyl ether under the conditions specified by ICC Standard No. 136 (International Association for Cereal Science and Technology, 1984). After the diethyl ether was removed using a rotary evaporator, PV and *p*-AV values were measured. Oil stability index (OSI) of the cake was determined using an Oxidative Stability Instrument (Omnion Inc., Rockland, MA, USA) and AOCS Official Method Cd 12b-92 (American Oil Chemists' Society, 2017). Samples were analyzed in triplicate and average values were reported.

Statistical Analysis

Statistical analysis was performed on the cake data using JMP® software (Version 10, SAS Institute Inc., Cary, NC, USA). Data was analyzed using ANOVA and significant differences ($p < 0.05$) were determined using the Tukey test.

Results and Discussion

Cake Preparation

Fig. 7.1 shows pictures of all the cakes. The overall cake quality largely depends on crumb texture and final volume (Patel, Cludts, Sintang, Lesaffer, & Dewettinck, 2014). In an ideal yellow cake, having a fine texture for most desirable sensory characteristics, the crumb should contain small, evenly distributed gas cells (Rutkowska

& Zbikowska, 2010). Tunnels, or uneven air cells throughout the crumb, are not desirable. The shortening cake showed evenly distributed, relatively small gas cells with some slight tunneling at the bottom of the crumb. Generally, all the SSAP cake samples had similar size gas cells as the shortening and larger gas cells than the phytosterol cake samples. Height is an important visual characteristic of baked goods, and the menhaden oil (M) and SL-C cakes had lower height than the shortening cake. Lower height may mean lower stabilization of the air bubbles as the cake expands during baking (Hedayati & Tehrani, 2018). Fat plays an important part in the incorporation and subsequent stabilization of air cells (Bath, Shelke, & Hoseney, 1992). Different fats may allow for more air bubble incorporation into the batter and better stabilization of these air bubbles during baking. This will be further discussed in later sections.

Batter Rheology

Table 7.3 shows the apparent viscosity and Power-law parameters for the cake batters. The Power-law model was fitted to all steady state curves for batters and rheological parameters (flow index **n** and consistency index **k**) were estimated. All R^2 correlation coefficients were greater than 0.99. All batters had flow index values of less than 1.0, demonstrating that they were shear-thinning. Batters produced with SL-CS or SL-S, and their respective phytosterol or SSAP oleogels, had significantly ($p < 0.05$) higher viscosities, lower n (0.68-0.72), and higher k (45-79 Pa s^n) than the shortening batter. The higher k values for all the SL may be a result of having higher solid fat content (SFC) values than the menhaden oil samples (Willett, Martini, & Akoh, under review). A previous study on replacing shortening with canola oil-carnauba wax oleogels found that higher replacement of shortening with oleogel (low in saturated fat) in cake

batter resulted in lower consistency index values (Kim, Lim, Lee, Hwang, & Lee, 2017). Formation of cake batter with phytosterol or SSAP oleogels may allow for higher air incorporation and retention. Air retention in batters is a function of batter viscosity, which may correspond to having higher cake specific volumes (Bath et al., 1992). Menhaden oil (M) had a significantly lower ($p < 0.05$) consistency index (22 Pa s^n) and higher flow index (0.91) than the shortening batter. Batters produced with P-M and SSAP-M exhibited Power-law values that were most similar to the shortening batter. These results show that the phytosterol and SSAP oleogels can structure menhaden oil and SL to have comparable rheological parameters to shortening in a cake batter system.

Light Microscopy

Fig. 7.2 shows the cake batters under the light microscope. The amount of air incorporated to form bubbles during mixing and changes in the bubbles during baking have a significant effect on cake texture (Patel, Cludts, Sintang, Lesaffer, & Dewettinck, 2014). Amount of air incorporated depends on batter viscosity, as discussed above, and how well the batter can retain air bubbles. Differences were seen among the samples in terms of air bubble incorporation. Generally, the batters made with the SSAP oleogel incorporated more air bubbles than the phytosterol oleogel batters. The least amount of air bubble incorporation was seen in the batters without either of the oleogels (M, SL-C, SL-S, and SL-CS). While the SL have higher SFA contents, there is still a large percentage of PUFA in the lipids which may hinder air incorporation because of a smaller amount of crystalline fat network. Air incorporation may be aided by the oleogels, as emulsifiers have been found to increase air bubble incorporation and stabilize the air bubbles formed (Patel, Cludts, Sintang, Lesaffer, & Dewettinck, 2014). Sucrose stearate

is a known emulsifier and may aid in the air bubble stabilization as there were higher amounts of air bubbles present in the samples with the SSAP oleogel than the phytosterol oleogel samples. The phytosterol oleogel may aid in the stabilization of the air bubbles by the ability of the gel network to trap liquid oil, allowing for the gel network structure to aid in air incorporation (Willett & Akoh, 2018; Co & Marangoni, 2012). The highest air bubble incorporation was seen in the SSAP-SL-S cake batter sample, likely due to containing both the SSAP oleogel (which contains high melting stearic and palmitic acids) and the SL having high stearic acid content (with high SFC and melting point) which aided in the fat crystal network formation (Willett & Akoh, 2018; Willett, Martini, & Akoh, under review). In the menhaden oil (M) sample, large liquid oil droplets were seen. This is a negative quality in baking, as without the fat crystal structure at room temperature, the lipid is unable to retain or stabilize air bubbles that are incorporated into the batter (Zhou, Faubion, & Walker, 2011).

Differential Scanning Calorimetry

Table 7.4 and Fig. 7.3 show the starch gelatinization onset and completion temperatures for each of the samples. Onset temperatures ranged from 74.7-93.5°C and completion temperatures ranged from 89.0-106.5°C. Only the batter produced with SL-CS had onset and completion temperatures that were statistically similar to the shortening batter. High onset temperatures for all samples were likely seen because of the high sugar content, as sugar is known to raise starch gelatinization temperature (Lin, Czuchajowska, & Pomeranz, 1994). For M, SL-C, SL-CS, and SL-S, generally the higher the melting point of the lipid, the higher the starch gelatinization onset and completion temperatures. The one exception is SL-CS, which had a higher melting point than SL-C, and this is

likely due to SL-CS having a lower stearic acid content than SL-S, as seen in Table 1. Almost all batter samples produced with the SSAP oleogel exhibited lower onset temperatures than the phytosterol or non-gellated oil samples. A previous study found similar temperature onset and completion values when sucrose stearate was added to cake batter at a concentration of 1% (Stankov et al., 2015). Generally, the gelatinization completion temperatures for all samples were higher than that of the shortening sample. The higher the temperature of completion, the longer it takes for the starch to gelatinize. Delay of starch gelatinization has been shown to delay solidification of the cake, increases the period of gas expansion, and therefore affect the final structure of the cake (Stankov et al., 2015). Almost all samples had higher completion temperatures than the shortening, and this likely impacted the final structure and texture of the cakes.

Physical Measurements

Specific gravity is useful in determining aeration of the batter. A lower specific gravity typically means higher air incorporation, better ability to retain air bubbles during beating, and overall greater final cake volume and height (Khalil, 1998). Table 7.5 shows the specific gravity of the batters having a different fat portion. The batter produced with shortening had a specific gravity of 0.85. Typical yellow cake batter has been found to have a specific gravity between 0.70-0.90 (Suas, 2009). The cake batters produced with menhaden oil (M), SL-C, and SL-CS had significantly higher ($p < 0.05$) specific gravities than the shortening batter, at 0.97, 0.90, and 0.90, respectively. This is likely due to the inability of these lipids to stabilize and hold air bubbles in the batter while mixing (Zhou, Jon, & Chuck, 2011). The light microscopy results were directly correlated to the batter specific gravity. Fig. 7.2 shows similar results with low amounts of air bubbles seen in

the M, SL-C, SL-CS, and SL-S batter samples. Plastic fats such as shortening have solid crystals that can adsorb to air bubbles and stabilize them, allowing for a lower specific gravity (Zhou, Faubion, & Walker, 2011). Addition of an emulsifier is another way to aid in air incorporation (Lakshminarayan, Rathinam, & KrishnaRau, 2006). The batters produced with the respective phytosterol and SSAP oleogels for M, SL-C, and SL-CS, did not have significantly different ($p > 0.05$) specific gravities from the shortening batter. This shows that P-M, SSAP-M, P-SL-C, SSAP-SL-C, P-SL-CS, and SSAP-SL-CS all had similar air incorporation into the batter compared to the shortening batter and may be suitable alternatives to shortening in cake preparation. All batters produced with SL-S (SL-S, P-SL-S, and SSAP-SL-S) had significantly lower specific gravities than the shortening batter, at 0.77, 0.75, and 0.72, respectively. This meant that SL-S, P-SL-S, and SSAP-SL-S batters had higher air incorporation into the batter than the shortening batter. Fig. 2 also shows high amounts of air bubbles for these samples compared to the other samples. The higher specific gravities of batters with SL-S, P-SL-S, and SSAP-SL-S may be due to the higher saturated fatty acid content. It has been found that a balance between saturated and unsaturated fatty acids, as seen in palm oil/palm oil fractions, allow for desired physicochemical properties in edible applications (Mamat & Hill, 2014). These SL have a desirable balance between the unsaturated fatty acids provided from the menhaden oil and the saturated fatty acids incorporated during enzymatic modification.

Table 7.5 shows the specific volume of the cake samples, which ranged from 0.96 to 1.01. Cakes prepared with P-M, SSAP-M, SL-C, SL-S, or SSAP-SL-S all had specific volumes that were statistically similar ($p > 0.05$) to the shortening cake, with values of 0.98-0.99 cm^3/g. Only the cake produced with menhaden oil had a significantly lower (p

< 0.05) specific volume (0.96 cm^3/g) compared to the shortening cake. Low viscosity batters have been associated with low cake volumes (Bath et al.,1992; Lakshminarayan et al., 2006; Lee, Kim, & Inglett, 2005). The cake produced with menhaden oil had both a low viscosity and low specific volume compared to all the other cakes. This is likely due to low air bubble incorporation and stabilization. It has been found that final cake volume depends on initial air incorporation and stability of the air bubbles during baking (Zhou, Faubion, & Walker, 2011). All other samples had specific volumes that were higher than the shortening cake, with values of 1.00-1.01 cm^3/g. These samples likely were able to better stabilize the air bubbles formed during the mixing of the batter and air bubble expansion during baking (Lakshminarayan et al., 2006).

Table 7.6 shows the L*a*b color values for the crust and crumb of the cakes. There was no significant difference in the crust color for all cakes. The crumb of cakes made with the SSAP oleogels generally had significantly higher a and b values, which corresponds to being slightly more orange in color. This was likely due to the opaquer, slightly orange color of the SSAP oleogels, which likely impacted the final color of the cakes (Willett & Akoh, 2018).

Textural Analysis of Cakes

Table 7.7 shows the results from the texture profile analysis (TPA). Hardness, springiness, cohesiveness, resilience, and chewiness were the properties chosen for analysis of the cakes. Hardness is defined as a maximum force during the first compression (Brandt, Skinner, & Coleman, 1963). The cake produced with menhaden oil (M) had significantly higher hardness than the shortening cake, with values of 21 N and 12 N, respectively. This is likely due to the M cake having low volume and less air

incorporation. Springiness reveals the ability of the cake to recover its height between the two compression cycles of the TPA test and is associated with the number of air bubbles (Sanz, Salvador, Baixauli, & Fiszman, 2009). A product with a higher springiness value is a more aerated product, though there was no significant difference ($p > 0.05$) in springiness for all cakes. Cohesiveness is the energy needed for second compression in TPA test and in sensory studies it often relates to crumbliness or perceptions of denseness (Brandt, Skinner, & Coleman, 1963). There was no significant difference ($p > 0.05$) in cohesiveness for all cake samples. Resilience is determined by dividing the upstroke energy of the first compression by the downstroke energy of the first compression (Brandt, Skinner, & Coleman, 1963). There was no significant difference ($p > 0.05$) in resilience for all cake samples. Chewiness is a function of hardness, cohesiveness, and springiness (Brandt, Skinner, & Coleman, 1963). Generally, cakes made with SL-C, SL-CS, and SL-S (and respective phytosterol or SSAP oleogels) exhibited lower hardness (5-8 N) and chewiness (4-6 N) when compared to the cake made with shortening (12 N, 9 N). Cakes made with P-M and SSAP-SL-CS had similar texture to the shortening cake in terms of hardness, resilience, cohesiveness, springiness, and chewiness. These results show that both the phytosterol and SSAP oleogels have potential to replace shortening in the production of cakes, but further adjustments to the cake formulation may be necessary to achieve more similar textural properties. Further research could investigate lowering the concentration of the fat portion when using the phytosterol or SSAP oleogels.

Oxidative Stability

Table 7.8 shows the OSI, PV, and *p*-AV values for each cake sample. The shortening cake sample had the highest OSI value, meaning it had the highest oxidative

stability. Generally, the phytosterol and SSAP oleogels increased the oxidative stability of the cakes compared to the non-gellated lipids, but all samples had significantly lower ($p < 0.05$) OSI values than the shortening. SL-C, SL-CS, and SL-S had significantly lower OSI values than the menhaden oil cake. This was due to the loss of endogenous antioxidants during short-path distillation to purify the SL (Zou & Akoh, 2013). P-SL-CS, SSAP-SL-CS, P-SL-S, and SSAP-SL-S had the highest OSI values of all cake samples, apart from the shortening cake. This was likely due to the oleogelation and/or higher saturated fatty acid content, particularly the higher stearic acid content (Table 1), that contributed to the higher OSI values. Oleogelation with the phytosterol or SSAP blends have significantly improved the oxidative stability of the menhaden oil and SL in a cake system, showing that oleogelation of highly unsaturated oils can be acceptable replacements for *trans*-fats.

Table 7.8 also shows the PV and *p*-AV values of all cake samples at day 0 and day 7 of storage. PV is a measurement of primary lipid oxidation products, or hydroperoxides (Shahidi, 1998). Table 7.8 shows that the shortening cake had the lowest PV at day 0 and day 7 of storage, with values of 2.73 and 6.84 meq/kg, respectively. PV for all samples increased from day 0 to day 7 of storage. The phytosterol and the SSAP oleogel cakes had lower PV than the non-gelled lipid cake samples. These results agree with the OSI results. Also, the SSAP oleogel cake samples generally had lower PV than the phytosterol oleogel cakes. The *p*-AV measures secondary products of lipid oxidation that are formed due to breakdown of the hydroperoxides (Shahidi, 1998). Table 7.8 shows that the shortening cake had the lowest *p*-AV value at day 0 and day 7 of storage, with values of 8.08 and 13.61, respectively. The *p*-AV value for all samples increased

from day 0 to day 7 of storage. The phytosterol and the SSAP oleogel cakes also had lower *p*-AV value than the non-gellated lipid cake samples. Also, the SSAP oleogel cake samples generally had lower *p*-AV values than the phytosterol oleogel cakes. The Global Organization for EPA and DHA omega-3 (GOED) sets voluntary limits for PV (5 meq/kg) and *p*-AV (20) of fish oil, which are lower limits than those set for refined vegetable oils in most countries (5-10 meq/kg, and 15-30, respectively) (Ismail, Bannenberg, Rice, Schutt, & Mackay, 2016). The *p*-AV is more indicative of the overall quality of the oil. The results from Table 7.8 shows that only the shortening and SSAP-M were within the PV limit at day 0 based on GOED requirements. For *p*-AV, shortening, M, P-M, and SSAP-M were all within the GOED limit for day 0 and day 7. Apart from SSAP-SL-S, all cake samples fell well outside the *p*-AV limit. The unmodified menhaden oil (M) contained 200 ppm TBHQ and 1000 ppm mixed tocopherols from the manufacturer (Omega Protein Inc., Reedville, VA, USA) which likely resulted in lower PV and *p*-AV values when compared to the non-gellated SL. While the use of the phytosterol or SSAP oleogels improved the oxidative stability of the cake during storage, additional antioxidant is necessary for the SL cakes containing high content of PUFA to achieve similar oxidative stability to the shortening cake.

Conclusion

Both phytosterol and SSAP oleogels show promise as acceptable alternatives to shortening in the production of yellow cake. Because higher air incorporation and stabilization were obtained for the oleogel cakes, further modification of the cake formulation could be done, specifically reducing the concentration of the fat portion. Oleogelation seemed to be a suitable way to produce low saturated fat alternative to

highly saturated and/or *trans*-fats. Further research should focus on addition of an antioxidant, use of other health beneficial (and more oxidatively stable) lipids in the formation of the oleogels for a cake system, or utilization of these oleogels in another food system that requires less exposure to heat.

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Author Contributions

S. A. Willett designed the study, collected the data, interpreted the results, and drafted the manuscript. C. C. Akoh advised on the study design and edited the manuscript.

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Tables

Table 7.1 Relative fatty acid composition of menhaden oil, SL, and shortening (Willett, Martini, & Akoh, under review).

Samples[a]	TAG Position	Fatty Acid (mol%)[b]								
		C8:0	C16:0	C18:0	C18:1n9c	18:2n6t	18:2n6c	18:3n3	C20:5n3	C22:6n3
Shortening (Crisco®)	Total	ND	13.42±0.03	17.08±0.26	37.71±0.32	0.02±0.01	24.57±0.03	6.94±0.05	ND	ND
	sn-2	ND	6.48±0.05	15.59±0.25	26.36±0.43	0.01±0.02	40.03±0.07	8.13±0.23	ND	ND
	sn-1,3[c]	ND	16.89±0.02	17.83±0.27	43.39±0.27	0.03±0.01	16.84±0.01	6.35±0.04	ND	ND
Menhaden Oil	Total	ND	10.20±0.85	3.08±0.05	2.98±0.46	ND	2.01±0.21	0.43±0.30	16.90±0.10	15.40±0.16
	sn-2	ND	14.86±0.74	3.21±0.37	0.09±0.16	ND	0.95±0.85	0.87±0.76	16.42±0.64	29.37±0.95
	sn-1,3	ND	7.87±0.91	3.02±0.21	4.43±0.61	ND	2.54±0.11	0.21±0.07	17.14±0.45	8.42±0.37
SL-C	Total	29.03±1.37	5.55±0.06	2.74±0.10	5.37±0.17	ND	2.36±0.16	0.75±0.03	13.61±0.38	10.42±0.03
	sn-2	11.72±0.13	16.94±0.07	2.97±0.09	1.24±0.08	ND	3.74±0.03	1.20±0.16	12.22±0.03	25.17±0.46
	sn-1,3	37.69±0.75	0.15±0.05	2.63±0.10	7.44±0.22	ND	1.67±0.23	0.53±0.04	14.31±0.21	3.05±0.25
SL-CS	Total	16.24±0.56	4.73±0.21	13.04±0.10	7.63±0.11	ND	1.83±0.04	0.36±0.08	13.87±0.26	12.50±0.20
	sn-2	7.31±1.01	15.11±0.06	8.82±0.55	1.11±0.11	ND	2.89±0.07	0.98±0.04	10.69±0.32	16.66±1.39
	sn-1,3	20.71±0.34	0.46±0.29	15.14±0.13	10.89±0.11	ND	1.30±0.03	0.05±0.10	15.46±0.23	10.42±0.40
SL-S	Total	ND	7.50±0.05	19.50±0.23	6.70±0.11	ND	1.57±0.07	0.76±0.17	14.16±0.11	14.62±0.39
	sn-2	ND	4.29±0.11	10.07±0.78	0.96±0.14	ND	1.41±0.06	0.95±0.42	11.85±0.26	22.98±0.18
	sn-1,3	ND	9.11±0.02	24.22±0.51	9.57±0.10	ND	1.65±0.08	0.67±0.05	16.21±0.19	10.44±0.29

Mean ± STD, *n*=3, ND= not detected

[a]SL-C: structured lipid (SL) of menhaden oil and 30 mol% caprylic acid, SL-S: structured lipid (SL) of menhaden oil and 20 mol% stearic acid, SL-CS: structured lipid (SL) of menhaden oil and 16 mol% caprylic acid and 13 mol% stearic acid

[b]Other fatty acids found at >1mol%: C14:0, C16:1n7, C17:1n7, C20:0, C18:4n3, C22:5n3, and <1 mol%: C13:0, C14:1n7, C15:0, C17:0, C19:0, C18:3n6, C20:1n7, C20:2n6, C22:0, C20:3n6, C20:3n3, C23:0, C22:2n6, C24:1n9, C22:4n6

[c]*sn*-1,3 mol% determined by equation: *sn*-1,3 (mol%) = [3xtotal mol%- *sn*-2 mol %]/2

Table 7.2 Abbreviations for all the fat portions used in the formulation of yellow cake.

Sample	Internal Phase
Shortening	Shortening (Crisco®)
M	Menhaden oil
P-M	Phytosterol oleogel containing menhaden oil
SSAP-M	SSAP oleogel containing menhaden oil
SL-C	SL of menhaden oil containing 30 mol% caprylic acid
P-SL-C	Phytosterol oleogel containing SL-C
SSAP-SL-C	SSAP oleogel containing SL-C
SL-CS	SL of menhaden oil containing ~14 mol% each caprylic & stearic acid
P-SL-CS	Phytosterol oleogel containing SL-CS
SSAP-SL-CS	SSAP oleogel containing SL-CS
SL-S	SL of menhaden oil containing 20 mol% stearic acid
P-SL-S	Phytosterol oleogel containing SL-S
SSAP-SL-S	SSAP oleogel containing SL-S

Table 7.3 Power-Law Model parameter values of batters produced with different fat portions.

Sample[a]	n[b]	k (Pa s^n)	R^2	η_{10} (Pa s)	η_{50} (Pa s)	η_{100} (Pa s)
Shortening	0.78±0.03[a]	30.79±0.13[a]	0.99949	0.51±0.03[a]	0.31±0.03[a]	0.27±0.03[a]
M	0.91±0.06[b]	22.17±0.22[b,d]	0.99913	0.48±0.04[b]	0.36±0.04[a]	0.34±0.03[b]
P-M	0.88±0.06[a]	33.59±0.46[a]	0.99951	0.63±0.20[a]	0.50±0.17[a]	0.47±0.14[b]
SSAP-M	0.87±0.02[a]	30.43±0.15[a]	0.99968	0.72±0.02[c]	0.58±0.02[b]	0.56±0.02[b,c]
SL-C	0.86±0.02[a]	60.35±0.78[b,c]	0.99922	1.05±0.18[c,d]	0.68±0.11[b]	0.60±0.06[b,c]
P-SL-C	0.85±0.06[a]	66.52±0.35[b,c]	0.99939	1.12±0.08[c,d]	0.83±0.07[b,c]	0.75±0.06[b,d]
SSAP-SL-C	0.68±0.01[b,c]	79.40±0.14[b,c]	0.99568	1.33±0.10[c,e]	0.88±0.01[b,c]	0.72±0.02[b,d]
SL-CS	0.72±0.01[b]	77.90±0.08[b,c]	0.99723	1.34±0.15[c,e]	0.71±0.37[b]	0.67±0.30[b]
P-SL-CS	0.65±0.02[b,c]	51.56±0.06[b]	0.99556	1.33±0.08[c,e]	0.75±0.02[b]	0.67±0.01[b]
SSAP-SL-CS	0.66±0.03[b,c]	61.35±0.65[b,c]	0.99866	1.33±0.15[c,e]	0.83±0.13[b,c]	0.74±0.08[b,d]
SL-S	0.85±0.01[a]	45.54±0.15[b]	0.99388	1.63±0.01[c,f]	0.84±0.01[b,c]	0.70±0.07[b,d]
P-SL-S	0.78±0.01[a]	62.40±0.34[b]	0.99448	1.64±0.07[c,f]	0.87±0.01[b,c]	0.68±0.02[b,c]
SSAP-SL-S	0.76±0.01[a]	68.17±0.12[b]	0.99453	1.75±0.01[c,f]	0.91±0.01[b,c]	0.63±0.01[b,c]

Mean ± STD, n=3. Different letters in the same column are significantly different (α=0.05)

[a]Abbreviations for different fat portions used in batter or cake production are the same as described in Table 1 and Table 2

[b]n: Power-Law/flow index, k: consistency index, η: viscosity

Table 7.4 Starch gelatinization onset and completion temperatures of cake batters with different fat portions.

Sample[a]	Onset (°C)	Completion (°C)
Shortening	88.2±0.06[a]	99.6±0.30[a]
M	85.4±0.36[b]	101.4±0.35[b]
P-M	93.1±0.06[c]	104.5±0.26[c]
SSAP-M	74.8±0.35[d]	95.3±0.40[d]
SL-C	89.4±0.32[e]	103.7±0.25[e]
P-SL-C	74.7±0.20[d]	106.5±0.45[f]
SSAP-SL-C	82.5±0.36[f]	89.0±0.51[g]
SL-CS	88.4±0.32[a]	99.3±0.15[a]
P-SL-CS	85.5±0.32[b]	97.6±0.44[h]
SSAP-SL-CS	76.4±0.44[g]	101.1±0.45[b]
SL-S	93.5±0.50[c]	105.2±0.21[i]
P-SL-S	93.4±0.40[c]	103.8±0.15[e]
SSAP-SL-S	91.7±0.25[h]	104.0±0.31[c]

Mean ± STD, n=3. Different letters in the same column are significantly different (α=0.05)

[a]Abbreviations for different fat portions used in batter or cake production are the same as described in Table 1 and Table 2

Table 7.5 Specific gravities of batters and specific volume of cakes with different fat portions.

Sample[a]	Batter Specific Gravity	Cake Specific Volume (cm^3/g)
Shortening	0.85±0.02[a]	0.98±0.05[a]
M	0.97±0.01[b]	0.96±0.02[b]
P-M	0.84±0.01[a]	0.98±0.01[a]
SSAP-M	0.83±0.04[a]	0.99±0.06[a]
SL-C	0.90±0.01[b]	0.99±0.04[a]
P-SL-C	0.86±0.01[a]	1.00±0.04[c]
SSAP-SL-C	0.85±0.01[a]	1.00±0.02[c]
SL-CS	0.90±0.01[b]	1.00±0.01[c]
P-SL-CS	0.83±0.01[a]	1.01±0.01[c]
SSAP-SL-CS	0.82±0.04[a]	1.01±0.01[c]
SL-S	0.77±0.02[b,c]	0.99±0.08[a]
P-SL-S	0.75±0.01[b,c]	1.00±0.07[c]
SSAP-SL-S	0.72±0.01[b,c]	0.99±0.01[a]

Mean ± STD, n=3. Different letters in the same column are significantly different (α=0.05)

[a]Abbreviations for different fat portions used in batter or cake production are the same as described in Table 1 and Table 2

Table 7.6 Crumb and crust color of cakes produced with different fat portions.

Sample[a]	Crumb			Crust		
	L	a	b	L	a	b
Shortening	75.31±0.39[a]	-3.58±0.73[a]	18.32±0.72 [a]	67.13±0.36[a]	-0.81±0.08[a]	28.14±0.47[a]
M	70.57±0.57[b]	-3.69±0.31[a]	17.22±0.98 [a]	67.39±0.19[a]	-2.67±0.09[a]	23.69±0.19[a]
P-M	68.48±0.25[b]	-3.02±0.20[a]	19.35±0.35 [a]	65.44±0.25[a]	-1.30±1.03[a]	25.34±0.23[a]
SSAP-M	74.43±0.46[a]	-1.77±0.60[b]	17.78±0.91 [a]	68.70±1.50[a]	1.08±0.29[a]	29.17±1.34[a]
SL-C	78.92±0.32[b,c]	-3.18±0.09[a]	19.18±1.02 [a]	59.65±0.49[a]	8.14±0.23[a]	33.39±0.25[a]
P-SL-C	76.87±0.46[a]	-3.00±0.11[a]	18.53±0.52 [a]	58.88±0.72[a]	7.64±0.41[a]	32.85±0.40[a]
SSAP-SL-C	73.60±0.34[a]	-1.47±0.50[b]	21.13±1.07 [b]	64.01±0.66[a]	3.61±0.38[a]	28.38±0.22[a]
SL-CS	79.21±1.97[b,c]	-3.07±0.11[a]	18.62±0.53 [a]	67.82±0.53[a]	2.20±1.38[a]	31.81±2.41[a]
P-SL-CS	77.39±0.32[b,c]	-3.03±0.17[a]	18.98±0.28 [a]	69.67±0.39[a]	1.12±0.28[a]	30.78±0.41[a]
SSAP-SL-CS	73.17±1.69[a]	-1.02±0.83[b]	21.24±0.97 [b]	60.81±0.43[a]	5.76±0.26[a]	33.46±0.26[a]
SL-S	74.22±1.93[a]	-3.35±0.29[a]	17.27±1.11 [a]	61.82±0.67[a]	5.09±0.33[a]	31.64±0.33[a]
P-SL-S	71.84±0.81[a]	-3.24±0.53[a]	17.98±0.84 [a]	62.31±0.65[a]	3.55±0.42[a]	30.53±0.33[a]
SSAP-SL-S	73.95±2.09[a]	-2.18±0.20[b]	19.12±0.48 [a]	60.14±0.42[a]	6.01±0.32[a]	33.55±0.30[a]

Mean ± STD, n=3. Different letters in same column are significantly different (α=0.05)

[a]Abbreviations for different fat portions used in cake production are the same as described in Table 1 and Table 2

Table 7.7 Texture characteristics of cake with different fat portions.

Sample[a]	Hardness (N)	Resilience (%)	Cohesiveness	Springiness (%)	Chewiness (N)
Shortening	12.24±0.82[a]	42.24±0.04[a]	0.77±0.05[a]	95.32±0.02[a]	8.67±0.99[a]
M	20.91±0.52[b]	42.06±0.07[a]	0.71±0.07[a]	97.38±0.02[a]	7.63±0.49[a]
P-M	16.21±2.84[a]	39.45±0.01[a]	0.71±0.01[a]	96.13±0.02[a]	10.98±1.74[a]
SSAP-M	4.32±0.87[b,c]	38.19±0.03[a]	0.73±0.05[a]	90.44±0.06[a]	2.86±0.74[b]
SL-C	15.20±0.38[a]	40.88±0.02[a]	0.78±0.02[a]	98.42±0.01[a]	5.89±0.60[b]
P-SL-C	5.20±0.84[b,c]	43.35±0.01[a]	0.80±0.01[a]	95.43±0.05[a]	4.01±0.78[b]
SSAP-SL-C	5.87±0.66[b,c]	41.85±0.05[a]	0.79±0.07[a]	95.75±0.03[a]	4.46±0.58[b]
SL-CS	8.28±1.01[b,c]	39.81±0.02[a]	0.74±0.02[a]	93.40±0.05[a]	5.74±1.00[b]
P-SL-CS	7.16±2.83[b,c]	41.83±0.01[a]	0.78±0.01[a]	93.78±0.06[a]	5.31±2.15[b]
SSAP-SL-CS	10.54±0.76[a]	40.92±0.03[a]	0.76±0.02[a]	97.21±0.03[a]	8.04±0.93[a]
SL-S	7.77±2.00[b,c]	39.17±0.03[a]	0.74±0.05[a]	96.40±0.03[a]	5.47±1.19[b]
P-SL-S	15.15±0.73[a]	39.59±0.02[a]	0.74±0.02[a]	94.65±0.06[a]	6.12±1.58[b]
SSAP-SL-S	5.74±3.14[b,c]	36.62±0.02[a]	0.71±0.02[a]	98.23±0.02[a]	4.02±2.17[b]

Mean ± STD, n=3. Different letters in same column are significantly different (α=0.05)

[a]Abbreviations for different fat portions used in cake production are the same as described in Table 1 and Table 2

Table 8 Oil stability index (OSI), peroxide, and *p*-Anisidine values for each cake sample.

Sample[a]	OSI (h)[b]	Peroxide Value (meq/kg)		*p*-Anisidine Value	
		Day 0	Day 7	Day 0	Day 7
Shortening	25.57±0.69[a]	2.73±0.72[a]	6.84±1.18[a]	8.08±0.39[a]	13.61±1.16[a]
M	11.03±0.22[b]	5.93±0.20[b]	26.34±0.77[b]	14.87±0.48[b]	22.30±0.46[b]
P-M	10.95±0.16[b]	5.41±0.35[b]	24.50±1.53[b]	11.62±0.79[c]	14.45±0.39[c]
SSAP-M	14.54±0.62[c]	4.50±0.41[b]	14.90±1.28[c]	11.17±0.48[c]	13.37±0.29[c]
SL-C	3.59±0.11[d]	43.63±0.12[c]	68.25±2.41[d]	50.94±0.41[d]	97.46±1.14[d]
P-SL-C	5.94±0.21[e]	24.19±1.58[d]	47.41±2.80[e]	45.46±1.44[d]	61.35±1.12[e]
SSAP-SL-C	13.88±0.12[e]	10.90±1.13[e]	17.17±2.67[c]	33.16±0.62[e]	54.97±0.70[f]
SL-CS	3.74±0.15[d]	26.20±1.15[d]	49.06±2.72[e]	45.00±0.18[d]	95.25±0.40[d]
P-SL-CS	19.29±0.36[f]	18.40±2.04[d,e]	35.54±0.96[b,e]	33.46±1.62[e]	65.84±0.85[e]
SSAP-SL-CS	20.79±0.24[f]	6.57±1.24[b]	14.36±1.98[c]	28.70±0.40[f]	58.82±0.93[f]
SL-S	4.04±0.33[d]	23.27±1.26[d]	40.03±2.33[b,e]	31.18±0.26[e]	71.63±0.58[d,e]
P-SL-S	20.70±0.15[f]	10.42±0.32[e]	28.54±1.13[b]	32.98±0.69[e]	59.37±0.48[f]
SSAP-SL-S	21.08±0.48[f]	7.63±0.26[b]	15.52±2.61[c]	21.31±1.03[f]	27.74±0.74[g]

Mean ± STD, n=3. Different letters in same column are significantly different (α=0.05)

[a]Abbreviations for different fat portions used in cake production are the same as described in Table 1 and Table 2

[b]OSI conducted at 110°C

Figures

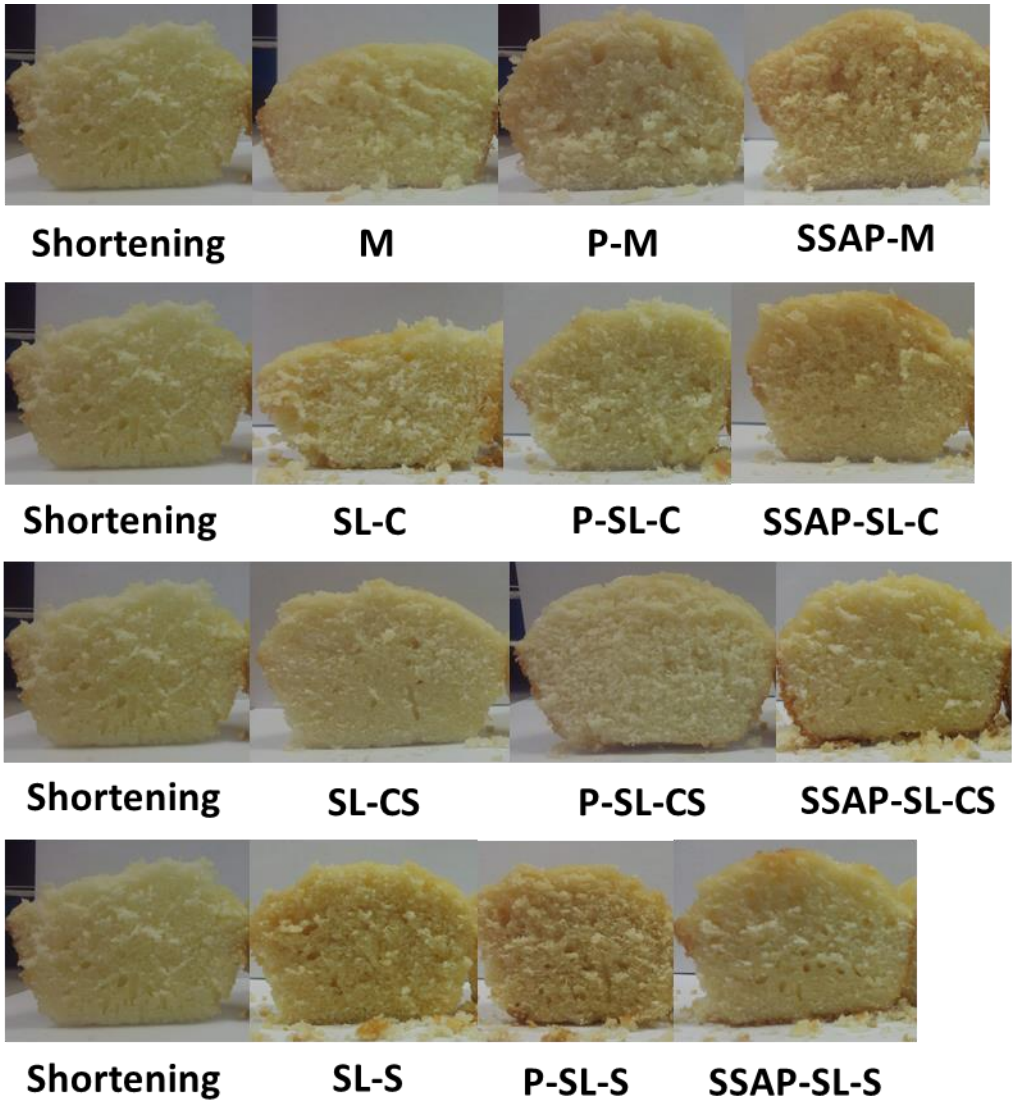


Fig. 7.1 Yellow cakes produced using different fat portions. Abbreviations for different fat portions used in cake production are the same as described in Table 1 and Table 2

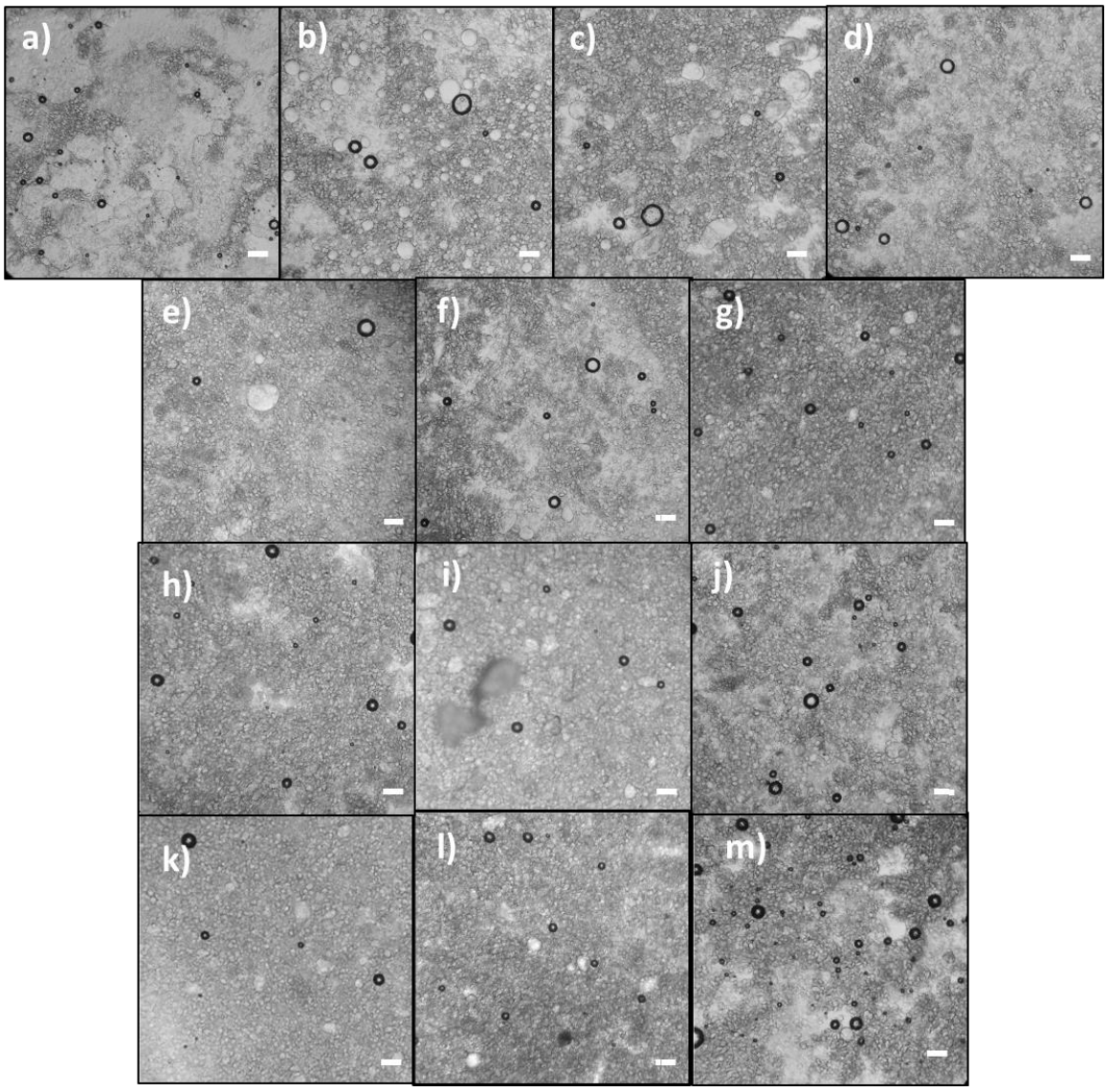
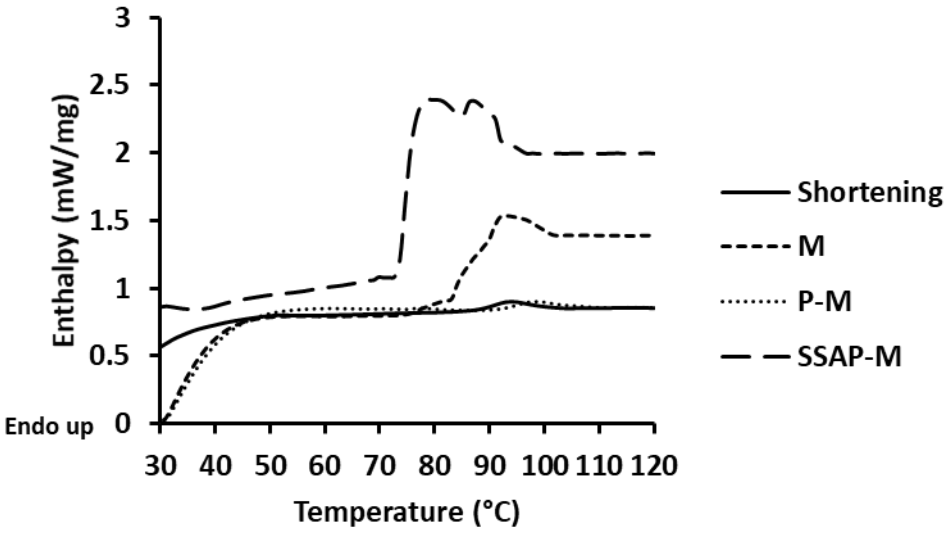
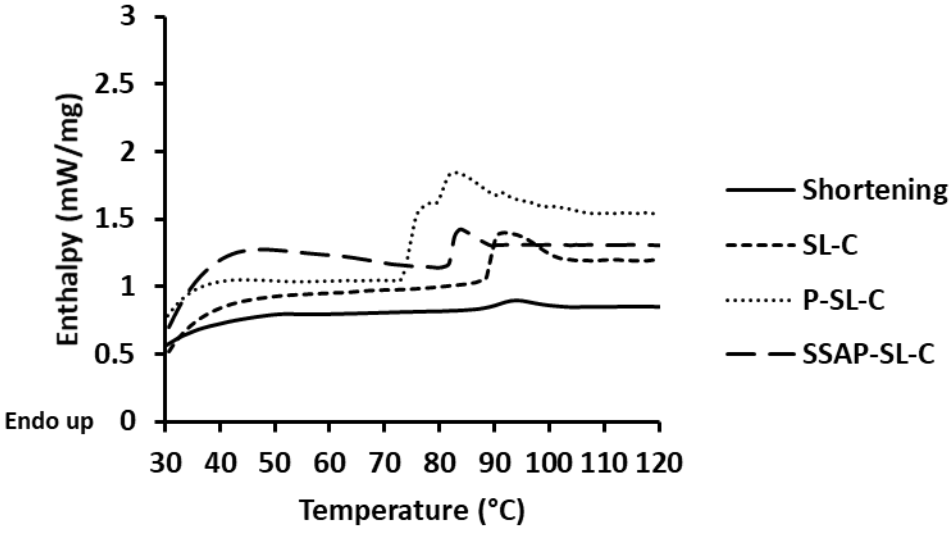


Fig. 7.2 Light microscopy of cake batters produced with different fat portion, where a) shortening, b) menhaden oil, c) P-M, d) SSAP-M, e) SL-C, f) P-SL-C, g) SSAP-SL-C, h) SL-S, i) P-SL-S, j) SSAP-SL-S, k) SL-CS, l) P-SL-CS, and m) SSAP-SL-CS. Abbreviations for different fat portions used in batter production are the same as described in Table 1 and Table 2. Scale bars = 100μm


Enthalpy (mW/mg)
3
2.5
2
1.5
1
0.5
0
Endo up
30 40 50 60 70 80 90 100 110 120
Temperature (°C)
Shortening
M
P-M
SSAP-M


Enthalpy (mW/mg)
3
2.5
2
1.5
1
0.5
0
Endo up
30 40 50 60 70 80 90 100 110 120
Temperature (°C)
Shortening
SL-C
P-SL-C
SSAP-SL-C

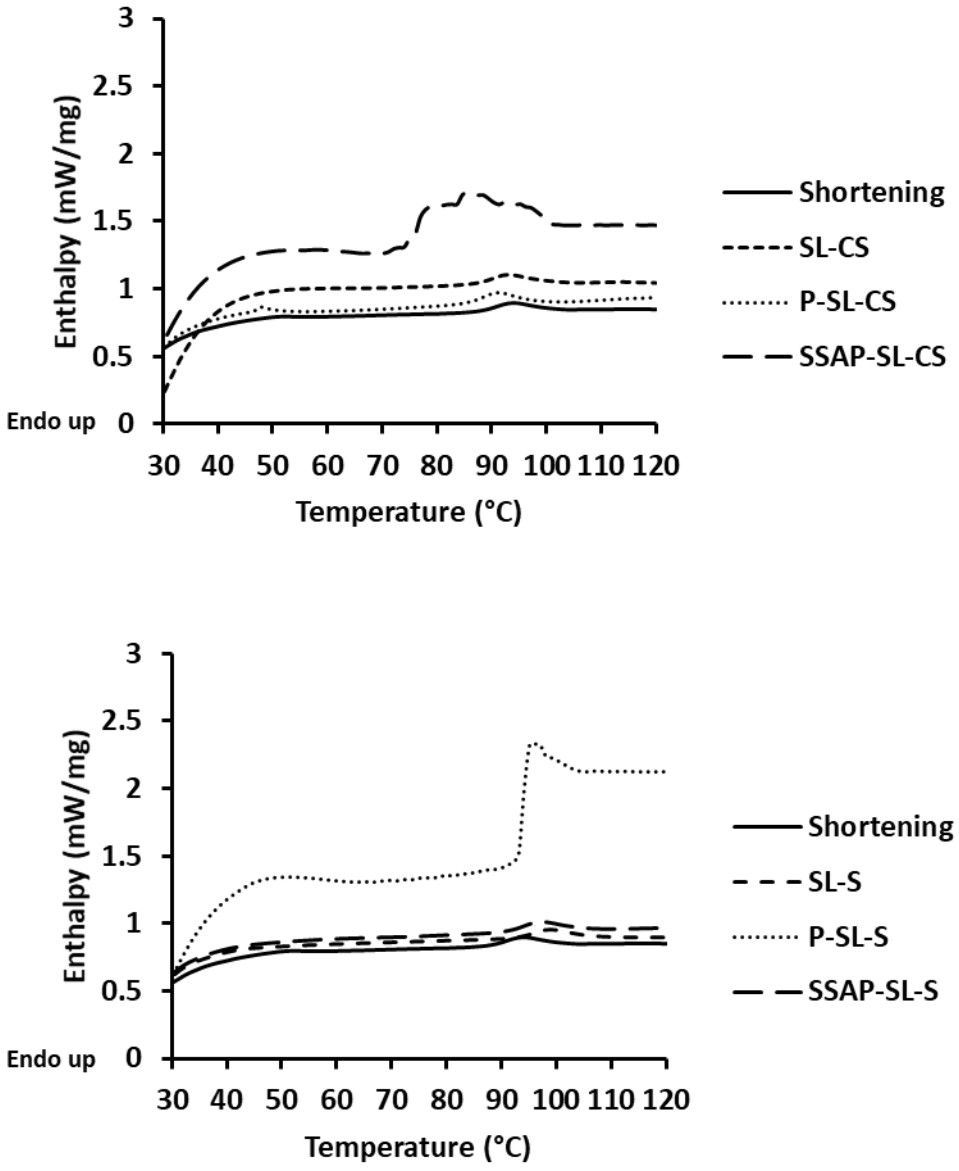


Fig. 7.3 DSC analysis of cake batter. Abbreviations are the same as described in Table 1 and Table 2.

CHAPTER 8

CONCLUSIONS

All the objectives set in Chapter 1 were achieved. The Taguchi method was a successful method for optimizing the enzymatic reactions based on time, temperature, enzyme load, and substrate molar ratio for incorporating 30 mol% capric acid into menhaden oil. Linear interpolation was also a good method for estimating the substrate molar ratios required to incorporate the desired quantity (20 or 30 mol%) of acyl donor (caprylic or stearic acid) during the production of menhaden oil SL. Both methods are relatively simpler than many other methods and could be easily applied to future research and industrial applications. The MLM-type SL produced with menhaden oil may be used as 'heart healthy' nutraceuticals or in the formulation of functional foods. The lipase, Lipozyme® 435, from *Candida antarctica,* showed preference for ethyl caprate over capric acid, as well as preference for incorporating capric, caprylic, and stearic acids at the *sn*-1,3 positions. Use of this immobilized, food grade enzyme, in the production of SL may improve the viability of using SL in products by the food industry.

Both the phytosterol and SSAP oleogels gave desirable physicochemical properties for use as low saturated, zero *trans*-fats in the formulation of cakes and have the potential for use in a wide range of food products. The oleogelation of health beneficial lipids such as menhaden oil and MLM-type SL should allow for their future use in fortification of functional foods such as margarines, spreads, baked goods, dairy

products, and others. The physicochemical properties of the phytosterol and SSAP oleogels differed in terms of rheological properties, morphology, and SFC. The differences are likely attributed to different interactions of the oleogelators and lipids during oleogelation and may be due to differences in their structures and TAG composition, respectively. When used in the formulation of yellow cake, both the phytosterol and SSAP oleogels were acceptable substitutes for shortening. The difference in terms of potential health benefits of the oleogels and shortening are that the organogels contain a lower amount of saturated fat and no *trans*-fat. The oleogels were high in the omega-3 fatty acids, EPA and DHA. However, while the oxidative stability of the menhaden oil and SL were improved when they were oleogelated, in a food application such as cake, the oxidative stability was still lower than that of shortening due to the high PUFA-containing lipids, so further modification of the formulation is necessary.

Finally, menhaden oil, SL, and oleogels were successfully encapsulated in alginate microparticles. The leakage of microcapsules during storage was reduced by use of oleogels as the internal phase. Oleogelation may also protect the lipid from oxidation during the encapsulation process and may allow for encapsulation of heat sensitive lipids such as menhaden oil. Because the oleogel microcapsules had higher EE and stability, these microcapsules have the potential for use in the fortification of food products with health beneficial lipids.

Suggestions for future work:

1. Applying the Taguchi method to other enzymatic reactions, such as in the production of cocoa butter substitutes or human milk fat analogues.
2. Utilization of the SSAP oleogel in other food applications, particularly in dairy products or frozen foods, and use of this blend in oleogelation of other health beneficial and/or more oxidatively stable lipids.
3. Use of the microencapsulated oleogels in food applications such as yogurt or other dairy products, or baked goods, and study of the acceptability in terms of their sensory properties.