ENZYMATIC MODIFICATION OF STEARIDONIC ACID SOYBEAN OIL AND IMPROVEMENT OF OXIDATIVE STABILITY

by

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(Under the Direction of Casimir C. Akoh)

ABSTRACT

The aim of this study was to design enzymatic processes for producing 'heart-healthy' stearidonic acid (SDA)-enriched triacylglycerol (TAG) concentrates from SDA soybean oil (SDASO) and to develop microencapsulation systems for improving the oxidative stability of SDASO. SDA concentrates were prepared from chemical and enzymatic hydrolysis products of SDASO. A 62% SDA free fatty acid fraction (SDA-FFA) was obtained by low temperature crystallization of the chemical hydrolysis product. Partial hydrolysis of SDASO by *Candida rugosa* lipase yielded a 51% SDA acylglycerol fraction (SDA-GLY). Using response surface methodology (RSM), reaction conditions for enzymatic acidolysis between SDA-FFA and SDA-GLY fractions were optimized to produce ~60% SDA TAG concentrates. Structured lipids (SL) were produced by Celite-immobilized, *Rhizomucor miehei* lipase-catalyzed incorporation of caprylic acid (C8:0) into SDASO Substrate molar ratio, reaction time, and enzyme load were optimized. The acidolysis of SDASO or an SDA-enriched TAG concentrate resulted in SLs with 17-33% C8:0 and 20-42% SDA. The microencapsulation of SDASO in gelatin (GE)-gum arabic (GA) coacervates crosslinked by transglutaminase (TG) or the Maillard reaction (MR)

was investigated. The total oxidation (TOTOX) value of microencapsulated SDASO was determined after 28-30 days of storage. TOTOX values were highest in the TG-modified microcapsules (25.9-56.6), but lower in the control (19.9-47.6) and MR-modified microcapsules (10.2-18.4). When microcapsules were used to formulate SDA-fortified yogurt, oil release during heat treatment was lowest for the MR-modified microcapsules (1.1-8.6%), but higher for TG-modified (1.8-12.3%) and control (2.0-26.4%) microcapsules. MR-modified microcapsules displayed best oxidative and thermal stability. The antioxidant capacity of the MR-modified encapsulant was then optimized by varying the biopolymer composition and dry-heating time. GE and GA were crosslinked, with or without maltodextrin (MD), to produce anti-oxidative Maillard reaction products (MRP) which were subsequently used as encapsulants. The optimal Trolox equivalent antioxidant capacity (TEAC) for MRPs derived from the [GE-GA] and [GE-GA-MD] blend was ~ 26 and 29 mg Trolox eq/10 g, respectively. TOTOX values ranged from 24.5-33.1for the optimized [GE-GA] blend and from 7.1-14.3 for the optimized [GE-GA-MD] blend. Microencapsulation of SDASO using the optimal MR-modified [GE-GA-MD] blend may be suitable for making microcapsules with high oxidative stability and formulating SDA-fortified food products.

INDEX WORDS: Stearidonic acid soybean oil, Lipase, Acidolysis, Hydrolysis, Structured lipid, Lipid oxidation, Microencapsulation, Complex coacervation, Maillard reaction, Antioxidant, Oxidative stability

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DEDICATION

To my dear fiancée, parents, and siblings

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CHAPTER 1

INTRODUCTION

The consumption of marine long chain omega-3 polyunsaturated fatty acids (PUFA), specifically eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids, has been reported to confer numerous health benefits including cardioprotection (Lavie, Milani, Mehra, & Ventura, 2009). Evidence from various epidemiological studies indicate that dietary omega-3 PUFA significantly reduces the risk of death from coronary heart disease (CHD) (Mozaffarian, 2008; Mozaffarian & Wu, 2011). The American Heart Association and other health organizations have recommended an increased consumption of oily fish and/or omega-3 PUFA supplements by the general population to reduce cardiovascular disease (CVD) risk (Go et al., 2013; USDA, 2010). Transgenic stearidonic acid (SDA, C18:4n-3) soybean was developed for formulating omega-3 PUFA enriched food products to provide more dietary omega-3 options for consumers who wish to supplement fish intake or do not consume fish (Decker, Akoh, & Wilkes, 2012). When ingested, SDA may be metabolized for energy by β-oxidation or converted to EPA and incorporated into tissue phospholipids. Results from animal models and human studies demonstrate that supplementing diet with SDA can increase tissue levels of EPA as well as the omega-3 index (Harris et al., 2007; Lemke et al., 2010). Therefore, SDA soybean oil can serve as a dietary source for EPA and provide cardioprotective benefits similar to fish oil. Furthermore, SDA soybean oil has a bland (non-fishy) flavor and better oxidative stability compared to fish oil. SDA

soybean oil has been deemed suitable for formulating omega-3 PUFA fortified functional foods such as yogurts, frankfurters, strawberry beverages, granola bars, bagels, spreads, and mayonnaises (Decker et al., 2012).

Omega-3 PUFA concentrates enable nutritionally significant doses of omega-3 PUFA to be obtained with minimal total lipid intake and are gaining attraction from the health food industry as food supplements and ingredients for functional foods. Using different fractionation methods, various forms of fish oil concentrates containing 30-95 % EPA and DHA have been produced in the form of esters, free fatty acids (FFA), triacylglycerols (TAG), and structured TAG containing medium chain fatty acids (MCFA) (Haraldsson & Hjaltason, 2006). SDA soybean oil has been successfully used to prepare SDA concentrates in the form of FFA (60%), TAG (38-54%), acylglycerols (41-63%), ethyl (96%) and lauryl (58%) esters using a variety of fractionation methods (Kleiner, 2012; Vázquez , Kleiner, & Akoh, 2012; Vázquez & Akoh, 2011, 2012). The TAG form of PUFA concentrates has been determined to be more bioavailable than the ester forms and is generally perceived to be 'natural' (Shahidi & Wanasundara, 1998). So, there is a need to further enrich SDA TAG concentrates up to 60% as this level of enrichment has never been achieved. Furthermore, there is need to develop SDA concentrates as structured TAG containing MCFAs which may be applied in parenteral and enteral nutrition for meeting the energy and essential fatty acid requirements of patients suffering from nutrient malabsorption.

Microencapsulation of omega-3 PUFA in food-grade biopolymers is a well-suited approach to facilitate their incorporation into food products because the microencapsulated oil can be conveniently incorporated into aqueous-based food

products where the direct addition of the oil may be problematic (Augustin & Sanguansri, 2015). Microencapsulation also offers a number of functions such as masking of unpleasant flavors, controlled-release, and extended oxidative stability due to physical/antioxidant protection from lipid oxidation. Several microencapsulation techniques are available for food and pharmaceutical applications. However, there is significant interest in the complex coacervation technique because of impressively high loading and controlled release capacities (Gouin, 2004). Complex coacervation typically involves the use of a protein + polysaccharide blend, for example gelatin + gum arabic, as encapsulant. It is often necessary to harden the walls of microcapsules produced by complex coacervation in order to improve their resilience to a broad range of food processing conditions. Coacervate-based microcapsule walls are typically hardened by adding chemical or enzymatic crosslinking agents (Gouin, 2004). However, currently available crosslinking agents, although effective, are limited for food applications due to potential toxicity and/or negative consumer perceptions. Therefore, food-grade biopolymer-based microencapsulation systems that would not require the addition of crosslinking agent are desirable. When proteins and polysaccharides are heated under controlled conditions, the crosslinking of biopolymers occurs spontaneously due to the Maillard reaction, which may result in the formation of Maillard reaction products (MRP) with enhanced antioxidant and emulsifying capacities (Nursten, 2005). However, the potential of using the Maillard reaction to crosslink protein + polysaccharide before microencapsulating omega-3 oils by complex coacervation has not yet been explored.

There are seven chapters in this research work. The first chapter is an introduction that specifies the rationales, hypotheses and objectives of this research. The second

chapter is a literature review of topics related to omega-3 PUFA and cardiovascular health, fractionation techniques, and food fortification. The third chapter reports the development and optimization of a chemoenzymatic method for producing SDA-enriched TAG concentrates from SDA soybean oil. The fourth chapter reports the incorporation of caprylic acid into SDA soybean oil using *sn*-1,3-specific immobilized lipases to produce structured lipids (SL) having MCFA at the *sn*-1 and -3 positions and omega-3 PUFA at the *sn*-2 position. In the fifth chapter, we compared the characteristics and performance of microcapsules based on non-crosslinked (control), transglutaminase- or Maillard reaction-crosslinked gelatin-gum arabic coacervates. The sixth chapter reports the optimization of antioxidant capacity of Maillard reaction-crosslinked biopolymers to maximize the oxidative stability of microencapsulated SDA soybean oil. The seventh chapter highlights the research significance of each study and suggests ideas for future work. The specific objectives and hypotheses are as follows:

Objective 1: To develop 60% SDA TAG concentrates from SDA soybean oil using a combination of low temperature crystallization and lipase-catalyzed hydrolysis/esterification. **Hypothesis:** The extent of *Candida rugosa* lipase-catalyzed hydrolysis of SDA soybean oil will determine the level of SDA enrichment.

Objective 2: To synthesize structured lipids (SL) by incorporating caprylic acid into SDASO using immobilized *sn*-1, 3-specific *Rhizomucor miehei* lipase. **Hypothesis:** The type of immobilization support and reaction temperature used for SL synthesis has an effect on the extents of caprylic acid incorporation and acyl migration.

Objective 3: To investigate the effects of Maillard reaction on the characteristics and performance (thermal and oxidative stability) of gelatin-gum arabic coacervate-based

microcapsules containing SDA soybean oil. **Hypothesis:** Microcapsules based on Maillard reaction-modified coacervates have improved thermal and oxidative stability compared to those based on classical coacervates.

Objective 4: To optimize the antioxidant capacity of Maillard reaction-modified coacervates in order to produce microcapsules that would confer superior oxidative stability to SDA soybean oil compared to non-crosslinked controls. **Hypothesis:** The extent of Maillard browning in protein-polysaccharide blends has an effect on antioxidant capacity as well as the oxidative stability of resulting microcapsules.

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CHAPTER 2

LITERATURE REVIEW

Omega-3 Fatty Acids and Cardiovascular Disease

The traditional diet-heart paradigm purports that total dietary fat and cholesterol increases the risk of cardiovascular disease (CVD) by elevating blood total and LDL cholesterol levels (Siscovick, Lemaitre, & Mozaffarian, 2003). However, this paradigm has proven to be an oversimplified view of the role of diet in CVD. Rather, a wide range of dietary factors are now known to influence CVD risk, including specific fatty acids (omega-3s, *trans*-, and other specific saturated, mono-, and polyunsaturated fatty acids), carbohydrate quantity, type, and quality; intakes of legumes, nuts, fruits, and vegetables, alcohol, micronutrients, food processing, and food preparation methods (Hu & Willett, 2002; Mente, de Koning, Shannon, & Anand, 2009). Furthermore, blood total and LDL cholesterol levels are not the only risk factors influenced by dietary habits. Intermediary coronary heart disease (CHD) risk factors such as circulating triacylglycerols, inflammation, endothelial function, coagulation and thrombosis, and arrhythmic risk are also influenced by diet (Mozaffarian & Wu, 2011). Dietary habits and intermediary risk factors also do not cause CHD as a single distinct event, but impact a broad range of CHD outcomes such as chronic progression of atherosclerosis, plaque instability and acute rupture, and cardiac arrhythmias (Rao & Kiranmayi, 2012). The emergence of clinical outcomes given dietary and intermediary risk factors may vary depending on individual susceptibility because of potential modifying factors such as sex, age, physical

activity, underlying insulin sensitivity, and genetic variation (Go et al., 2013). These complex interrelationships need to be fully integrated to understand the effects of dietary habits on CVD risk.

The effects of long-chain omega-3 polyunsaturated fatty acid (PUFA) intake, specifically eicosapentaenoic acid (EPA) (20:5n-3) and docosahexaenoic acid (DHA) (22:6n-3), on CVD has been studied from careful assessment of evidence from observational studies, randomized clinical trials (RCTs), animal experiments, and *in vitro* studies (Mente et al., 2009; Mozaffarian, 2008; Mozaffarian & Wu, 2011). The strength of this evidence has been established by agreement of results obtained from these studies considering the complementary strengths and limitations of each type of study design. Overall, the findings indicate that consumption of fish or fish oil significantly reduces CHD mortality, including fatal myocardial infarction and sudden cardiac death, in populations with and without established CVD (Go et al., 2013). The final common pathway for most cardiac deaths is arrhythmia. In *in vitro* and animal models, omega-3 PUFA stabilize partially depolarized ischemic myocytes, therefore reducing susceptibility to triggered ventricular arrhythmias (Leaf, Kang, Xiao, & Billman, 2003; McLennan, 2001). These findings are consistent with clinical reductions in cardiac death.

Effects of omega-3 fatty acids on cardiovascular risk factors:

Omega-3 PUFAs have different physiological effects in multiple tissues, including the heart, liver, vasculature, and circulating cells. Some of these effects are dose-dependent.

Plasma triacylglycerols

The lowering of plasma triacylglycerols (TAG) by omega-3 PUFA is well recognized (Harris & Bulchandani, 2006). Reduced hepatic very low-density lipoprotein (VLDL) synthesis contributes to this effect, including reduced fatty acid availability for TAG synthesis due to decreased *de novo* lipogenesis (DNL) (the process of converting carbohydrates into fat), increased fatty acid oxidation, and decreased heapatic lipid uptake (Clarke, 2001; Jump, 2008; Jump et al., 2005). TAG-lowering is linearly dose-dependent across a wide range of consumption but with variable individual responses. The accrued modest benefits of reduced hepatic DNL, sustained over time from habitual omega-3 PUFA consumption, could partly contribute to lower CVD risk by mitigating development of hepatic steatosis (abnormal retention of lipids) and hepatic insulin resistance (González-Périz et al., 2009; Masterton, Plevris, & Hayes, 2010).

Heart rate and blood pressure

Omega-3 PUFA consumption has been reported to reduce resting heart rate (HR) and systolic and diastolic blood pressure (Geleijnse, Giltay, Grobbee, Donders, & Kok, 2002; Mozaffarian et al., 2005). Experimental studies suggest that HR lowering could result from direct effects on cardiac electrophysiological pathways (McLennan, 2001). In short-term trials, omega-3 PUFA consumption increased nitric oxide production, mitigated vasoconstrictive responses to norepinephrine, angiotensin II, and enhanced vasodilatory responses (Chin, Gust, Nestel, & Dart, 1993; Harris, Rambjør, Windsor, & Diederich, 1997; Kenny et al., 1992). Such effects could contribute to lowering of systemic vascular resistance and blood pressure.

Endothelial function

Several studies have demonstrated that omega-3 PUFA supplementation improved flow mediated arterial dilation, a measure of endothelial function and health (Dangardt et al., 2010; Engler et al., 2005). Because endothelial health is strongly associated with nitric oxide synthesis (Doshi et al., 2001), experimental effects of omega-3 PUFA on related biomarkers provide plausible biological mechanisms for such effects (Okuda et al., 1997). Some studies have found that omega-3 PUFA consumption lowers circulating biomarkers of endothelial dysfunction, such as E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 (Kris-Etherton, Harris, & Appel, 2002; Robinson & Stone, 2006). Therefore, normalization of endothelial function could partly mediate protective effects of omega-3 PUFA against CVD.

Cardiac filling and myocardial efficiency

Animal experiments and growing evidence in human studies suggest that omega-3 PUFA consumption improves cardiac filling and myocardial efficiency. In animal models and short-term experimental trials in healthy adults and patients with chronic heart failure, omega-3 PUFA consumption was found to enhance both early (energy-dependent) and late (compliance-dependent) left ventricular diastolic filling (Charnock, McLennan, & Abeywardena, 1992; Ghio et al., 2010). Such effects could partly relate to long-term improvements in ventricular compliance due to reduced systemic vascular resistance. In animal experiments and at least 1 RCT in humans, fish oil consumption was also reported to improve myocardial efficiency and reduce workload-specific myocardial oxygen demand without reducing peak performance (Ghio et al., 2010; Nodari et al., 2011).

Inflammation

Omega-3 PUFA is known to alter several inflammatory pathways. However, it remains unclear whether such anti-inflammatory effects are clinically meaningful at typical dietary doses. In several trials, omega-3 PUFA supplementation reduced plasma and urine levels of inflammatory eicosanoids such as leukotriene E4 (Nakamura et al., 2005; von Schacky, Kiefl, Jendraschak, & Kaminski, 1993), while findings for other circulating inflammatory biomarkers, such as interleukin-1-beta and tumor necrosis factor-alpha, are inconclusive. Omega-3 PUFA, particularly DHA, have been shown to reduce adhesion and migration of monocytes and influence leucocyte-endothelial cell interactions in atherosclerosis and inflammation (Jung, Torrejon, Tighe, & Deckelbaum, 2008). Fish oil was proposed for adjunctive therapy for inflammatory diseases such as rheumatoid arthritis (James, Proudman, & Cleland, 2010). Meta-analyses of placebo-controlled trials found that high-dose omega-3 PUFA supplementation (1.7 to 9.6 g/day) reduced morning stiffness and joint pain in patients with rheumatoid arthritis (Goldberg & Katz, 2007). Furthermore, omega-3 PUFA are precursors to resolvins, protectins, and other inflammation resolving mediators that might have potent anti-inflammatory properties and assist in the resolution of inflammation (Serhan, 2007, 2010).

Arrhythmia

One of the most recognized physiological effects of omega-3 PUFA in humans is anti-arrhythmia. *In vitro* and animal experiments suggest that omega-3 PUFA directly influence atrial and ventricular myocyte electrophysiology, potentially mediated by effects on membrane ion channels (McLennan, 2001). Pooled analysis of prospective cohort studies and randomized clinical trials demonstrated that modest omega-3 PUFA

consumption (~250 mg EPA+DHA, or 2.25 calories/d) reduced risk of sudden cardiac death by 36% compared with no consumption (Lemaitre et al., 2003). This suggests that the anti-arrhythmic effects seen in experimental studies could extend to humans. Overall, evidence from *in vitro* studies, animal-experiments, and some human studies remains compelling. However, it is unclear whether such benefits are due to direct effects on myocyte electrophysiology or more of indirect influences such as improvements in myocardial efficiency and local inflammatory responses (Mozaffarian & Wu, 2011).

Omega-3 index

The omega-3 index is the sum of EPA and DHA in red blood cell membranes expressed as a percentage of total erythrocyte fatty acids (Harris & von Schacky, 2004). The omega-3 index was proposed as a new biomarker for CVD because it correlated well with the risk for a variety of CVD endpoints and was comparable to traditional risk factors for sudden cardiac death, such as C-reactive protein, LDL cholesterol, and total:HDL cholesterol ratio (Harris, 2007, 2008). The cardioprotective target level for the omega-3 index appeared to be about 8% and the level associated with increased risk for CHD death was <4% (Harris, 2007).

Molecular mechanisms of omega-3 PUFA

Fatty acids play important and diverse roles in cellular and organelle membrane structure and function, tissue metabolism, and gene regulation. Omega-3 PUFA influence multiple relevant molecular pathways which, individually or in sum, might contribute to the observed effects on physiological CVD risk factors and clinical outcomes such as CHD, sudden cardiac death, and stroke.

Cell and organelle membrane structure and function

Cellular and organelle functions are strongly influenced by membrane lipid environments. Lipid microdomains, such as membrane rafts and caveolae, function as operational platforms to modulate numerous cellular functions, including signal transduction, protein and membrane trafficking, and ion channel kinetics (Coskun & Simons, 2010; Dart, 2010). In cell culture and animal studies, the incorporation of omega-3 PUFA into membrane phospholipids alters the physicochemical properties of membrane rafts and caveolae, thereby influencing membrane associated protein localization and function (Ma et al., 2004). Membrane-bound omega-3 PUFA might also enhance protein signaling efficiency as exemplified by the interaction between DHA and rhodopsin, a G-protein–coupled receptor critical in the visual system (Grossfield, Feller, & Pitman, 2006). Incorporation of omega-3 PUFA into cellular membranes with subsequent alteration of protein function and signaling might contribute to potential anti-inflammatory and anti-arrhythmic effects.

Ion channels and electrophysiology

In animal models and *in vitro* studies, omega-3 PUFA has been reported to influence myocyte electrophysiology by altering the function of membrane ion channels such as the sodium channel, L-type calcium channel, and sodium–calcium exchanger (Ferrier, Redondo, Zhu, & Murphy, 2002; Xiao, Ke, Chen, Morgan, & Leaf, 2004). Additionally, some evidence suggests that omega-3 PUFA might also directly interact with membrane ion channels and proteins when modulating their function (Talukdar et al., 2010; Xiao et al., 2001). The incorporation of omega-3 PUFA into and resultant changes in lipid membranes could contribute to effects on ion channels. Such effects might contribute to reduced myocyte excitability and cytosolic calcium fluctuations,

particularly in ischemic or damaged cells susceptible to partial depolarization and triggered arrhythmia (Leaf et al., 2003).

Nuclear receptors and transcription factors

Omega-3 PUFA are natural ligands of several nuclear receptors and transcription factors that regulate gene expression in multiple tissues (Adkins & Kelley, 2010; Jump, 2008). Non-esterified omega-3 PUFA or their acyl-CoA thioesters can bind and directly modulate activities of such receptors (Hertz, Magenheim, Berman, & Bar-Tana, 1998). These receptors are central regulators of vital cellular functions related to CVD, including lipid metabolism, glucose-insulin homeostasis, and inflammation. For example, effects of omega-3 PUFA on these pathways likely contribute to triacylglycerol-lowering (Deckelbaum, Worgall, & Seo, 2006) and increased production of beneficial adipocytokines (Banga et al., 2009).

Arachidonic acid (AA)-derived eicosanoids

Eicosanoids are bioactive lipid mediators derived from metabolism of PUFA by cyclooxygenases, lipoxygenases, and cytochrome P450. Competition between omega-3 and omega-6 fatty acids for cyclooxygenases and lipoxygenases results in production of different types and amounts of eicosanoids. Omega-3 PUFA has been considered anti-inflammatory because they lower the production of pro-inflammatory or pre-thrombotic AA derived 2-series prostaglandins, thromboxanes, and 4-series leukotrienes in humans (Trebble et al., 2003; Vedin et al., 2010; von Schacky et al., 1993). The anti-inflammatory effects of omega-3 PUFA may sometimes be independent of AA, for example; via direct interactions with G-protein-coupled receptors (Talukdar et al., 2010). However, several AA-derived eicosanoids, such as epoxyeicosatrienoic acid (EET) and

lipoxins, have been associated with lower systemic inflammation and lower CHD risk in some prospective observational studies (Ferrucci et al., 2006; Harris, Poston, & Haddock, 2007). Thus, the significance and consequences of altered AA-derived metabolites following omega-3 PUFA consumption appears complex and needs to be resolved.

Omega-3 PUFA-derived eicosanoids

Omega-3 PUFA may be metabolized into eicosanoids such as specialized pro-resolving mediators (SPMs) (Serhan, 2010) and cytochrome P450-generated mono-epoxides (MEFAs) (Arnold, Konkel, Fischer, & Schunck, 2010) which possess unique bioactivities that might reduce CVD risk. In addition, anti-inflammatory eicosanoids such as 3-series prostaglandins (E_3 or $F_{3\alpha}$), thromboxanes (A_3), and 5-series leukotrienes (A_5, B_5, and C_5) are derived from EPA (Klurfeld, 2008). Traditionally, it was thought that the breakdown of local pro-inflammatory mediators was sufficient to end the inflammatory response (Serhan et al., 2007). However, specific cellular "resolution programs" have been identified to be essential to ensure timely inflammation resolution and return of tissue homeostasis (Serhan, 2010). Omega-3 derived SPMs, such as resolvins, protectins, and maresins are key drivers of these resolution programs. MEFAs are potent vasodialators, modulate several ion channels, and reduce inflammation *in vitro* (Morin, Sirois, Échavé, Albadine, & Rousseau, 2010). Robust effects of SPMs and MEFAs in multiple tissues and animal models suggest that they could play a key role in the cardioprotective effects of omega-3 PUFA.

Dietary guidelines and sources of omega-3 fatty acids

Fish is the major food source of the long-chain omega-3 PUFAs, EPA and DHA. The richest dietary sources include cold water oily fish, such as salmon, herring,

anchovies, bluefish, and sardines (Mozaffarian & Wu, 2011). Fish do not synthesize omega-3 PUFA, but accumulate them by consuming either microalgae or other prey that have accumulated omega-3 fatty acids along with antioxidants. In addition to long-chain omega-3 PUFA, fish provide specific proteins, vitamin D, selenium, and minerals with potential cardiovascular benefits (Fox et al., 2004; Roos, Wahab, Chamnan, & Thilsted, 2007). Concerns have been raised over potential harm from marine contaminants, such as methylmercury, dioxins, and polychlorinated biphenyls, present in some fish species (Gandhi et al., 2014; Mergler et al., 2007). However, for the general population, risk-benefit analysis concludes that the health benefits of modest fish consumption significantly outweigh the potential risk, while specific guidance is available for sensitive subpopulations such as women of childbearing age and young children (USDA, 2010). Several national and international organizations have recommended minimum intake levels of fish or omega-3 PUFA for the general population (Mozaffarian & Wu, 2011). Recommendations for EPA+DHA are typically based on the prevention of CHD mortality and there is strong agreement between these guidelines in recommending at least 250 mg/day EPA+DHA or two servings/wk of oily fish. For women who are or might become pregnant, nursing mothers, and young children, additional specific recommendations are available for minimum DHA consumption to optimize brain development in their children (USDA, 2010). Data are insufficient on the relative importance of EPA versus DHA or any specific ratio of their intakes for CVD benefits, and thus guidelines are based on their combined consumption.

Alpha-linolenic acid (ALA) (18:3n-3) is the plant-derived omega-3 fatty acid found in oils extracted from a limited set of seeds and nuts including soybean, flaxseed,

canola, walnuts, and peacans. Alpha-linolenic acid is essential in the human diet because it is the substrate for the synthesis of EPA and DHA which are required for tissue function. Biochemical pathways exist to convert ALA to EPA and EPA to DHA (Figure 2.1), but such endogenous conversion is limited in humans; between 0.2 - 8% of ALA is converted to EPA and 0 - 4% of ALA to DHA (Burdge, 2006; Pawlosky, Hibbeln, Novotny, & Salem, 2001). Thus, tissue and circulating EPA and DHA levels are primarily determined by direct dietary consumption. Some effects of ALA on physiological risk factors suggest that ALA might have cardiovascular benefits, but overall evidence remains inconclusive (Mozaffarian, 2004; Wang et al., 2006). Recommendations for ALA or total omega-3 consumption are based only on prevention of essential fatty acid deficiency as available evidence does not allow more specific recommendations for CVD or other chronic diseases (USDA, 2010). Thus, ALA cannot be considered as an alternative to seafood-derived omega-3 PUFA for cardioprotective benefits.

Stearidonic acid (SDA) (18:4n-3) is a metabolic intermediate in the conversion of ALA to EPA (Figure 2.1). SDA is a minor omega-3 fatty acid in seafood, contributing 0.5–2% of total fatty acids (Passi, Cataudella, Di Marco, De Simone, & Rastrelli, 2002), and seaweed (such as *Undaria pinnatifida* and *Ulva pertusa*) which contains 0.7–1.9 mg SDA/g dry weight (Khan et al., 2007). SDA is rarely found in commonly consumed vegetables, fruits, seeds, nuts, or commercial oils. However, plants from the Boraginaceae and Grossulariaceae families are unique because they contain significant amounts of SDA. Seed oils of echium and borage which belong to the Boraginaceae family are by far the most common dietary sources of SDA. By weight, echium oil is the

richest commercially available plant source of SDA (3.5–9.0%) (Guil-Guerrero, Gómez-Mercado, Garcıa-Maroto, & Campra-Madrid, 2000), followed by species of the Grossulariaceae family (for example, blackcurrant seed oils) at 2–6% (Ruiz del Castillo, Dobson, Brennan, & Gordon, 2004). EPA is slowly formed from ALA because the Δ6 desaturase (EC 1.14.99.25) step, the first reaction of the omega-3 metabolic pathway, is rate limiting in mammals (Yamazaki, Fujikawa, Hamazaki, Yano, & Shono, 1992). However, dietary SDA enters the metabolic pathway bypassing the Δ6-desaturase-catalyzed step and is more efficiently converted to EPA compared to ALA. Following consumption of SDA, tissue EPA levels (in plasma, neutrophil, heart, and erythrocyte phospholipids) have been reported to increase significantly while tissue SDA levels remain negligible (Harris, DiRienzo, et al., 2007; James, Ursin, & Cleland, 2003; Miles, Banerjee, & Calder, 2004). Therefore, SDA has been targeted as a potential metabolic proxy for EPA. However, most naturally occurring plant sources of SDA are yet to be adapted for large-scale production because yields are low and variable (Ursin, 2003). Genetic engineering has provided the capability to modify common vegetable oils to contain SDA by introducing genes for appropriate desaturases into the parent plant. The modification of conventional soybean (*Glycine max*) to transgenic SDA soybean was achieved by introducing two desaturase genes that encode for the proteins, *Primula juliae* Δ6 desaturase and *Neurospora crassa* Δ15 desaturase (Monsanto Co., 2009). The extracted SDA soybean oil contains 15 – 30% SDA by weight. The use of this plant-based omega-3 source is considered more sustainable than marine sources due to concerns of over-fishing. Therefore, the substitution of SDA soybean oil for fish oil may alleviate some of the commercial pressures on fish reserves. Furthermore, the palatability

and shelf-life issues with fish oil are not associated with SDA soybean oil because it has a bland flavor and less susceptible to oxidation than EPA and DHA (Decker, Akoh, & Wilkes, 2012; Whelan, 2009).

As the current per capita fish and omega-3 PUFA consumption in most countries fall below recommended levels, the consumption of food products fortified with omega-3 fatty acids has been recognized to be a viable option for individuals who wish to supplement fish consumption or who do not consume fish altogether (Patch et al., 2005). SDA soybean oil, due to its bland flavor and good oxidative stability, is well-suited for formulating a broad range of omega-3 fortified functional foods (Decker et al., 2012). Food products formulated with SDA soybean oil may be labeled as "heart-healthy" based on evidence from clinical studies which demonstrate the efficacy of SDA soybean oil in raising blood levels of EPA and the omega-3 index (Harris et al., 2008; James et al., 2003). Based on a conversion efficacy of SDA to EPA of 6:1, a target fortification level of 375 mg SDA per serving was proposed so that a daily minimum of 250 mg EPA/d can be achieved from consuming 1.5 SDA/d (Monsanto Co., 2009). Consumer acceptance tests on a range of food products, such as yogurt, spreads, mayonnaise, and strawberry-flavored beverage, showed no significant difference in mean consumer acceptance scores between SDA-fortified and non-fortified prototypes (Decker et al., 2012).

Production of Omega-3 PUFA Concentrates for Food Applications

There is high interest in producing omega-3 PUFA concentrates for the health food and pharmaceutical industries as concentrated forms of omega-3 PUFA enable the consumption of nutritionally significant doses with minimal total lipid intake. For example, the dosage of cod liver oil required to achieve desired biological effects has

been reported to carry the risk of vitamin A and D overdose as well as excessive cholesterol and saturated fatty acid (SFA) intake (Davidson, Burns, Subbaiah, Conn, & Drennan, 1991). So, the use of concentrated omega-3 oils in formulating functional foods, dietary supplements, and pharmaceuticals provides new opportunities to improve omega-3 PUFA intake by the human population and contribute to reducing CVD risk (Shahidi & Wanasundara, 1998). Omega-3 PUFA concentrates may be roughly divided into three classes (Haraldsson & Hjaltason, 2001). First, free fatty acids or ethyl esters with various enrichment levels have been developed into health supplements and drugs. The free fatty acids or ethyl esters may also be subsequently esterified to glycerol. Second, omega-3 PUFA concentrates in TAG form or of high TAG content that are available at various enrichment levels but where the fatty acid distribution is not of much concern. Finally, structured TAGs enriched with omega-3 PUFA at the *sn*-2 position and medium-chain fatty acids (MCFA) at the *sn*-1 and -3 positions (also known as MLM-type structured TAG) are considered to be a desirable form of omega-3 concentrates (Haraldsson & Hjaltason, 2006).

Marine oils have traditionally been used as the raw material for preparing EPA and DHA concentrates. There has also been considerable interest in producing other PUFA concentrates from plant-based oils, including SDA soybean (Kleiner, 2012), echium (Baik, Kim, Oh, & Kim, 2015), and *Mortierella* (Yokochi, Usita, Kamisaka, Nakahara, & Suzuki, 1990) oils. The methods for concentrating omega-3 PUFA can be broadly categorized into physical/non-enzymatic and enzymatic fractionation methods (Haraldsson & Hjaltason, 2006; Shahidi & Wanasundara, 1998). Sometimes, a combination of fractionation techniques is required to obtain omega-3 PUFA in highly

purified forms especially when the starting oil contains a wide variety of fatty acids with varying chain lengths and degrees of unsaturation. The PUFA concentrate obtained may be more susceptible to oxidation than the non-concentrated starting material due to higher level of unsaturation and loss of antioxidants during the fractionation process. Therefore, the major challenge for food scientists engaged in this area of research is to develop cost-effective methods to produce high quality PUFA concentrates with reasonable oxidative stability.

Production of SDA concentrates from SDA soybean oil

The recommended intake level for SDA is 750-1500 mg/d because this amount has been reported to be biologically equivalent to 250 mg EPA/d based on SDA to EPA conversion efficiencies of ~3:1 and 6:1 (James et al., 2003; Lemke et al., 2010). The total SDA soybean oil intake required to provide 1500 mg of dietary SDA would be dependent on the concentration of SDA in the oil. For example, 1500 mg of dietary SDA could be obtained from 10 g of a 15% SDA soybean oil or 5 g of a 30% SDA soybean oil. So, there is interest in producing SDA concentrates from which biologically significant amounts of dietary SDA may be obtained while minimizing total lipid intake. Table 1 provides a literature review of fractionation methods that have been adapted for producing SDA concentrates from SDA soybean oil.

Physical/Non-enzymatic Methods

Adsorption Chromatography

High-performance liquid chromatography (HPLC) and silver resin chromatography have been used for preparing omega-3 concentrates. Using appropriate adsorbents and solvents, fatty acids can be separated according to carbon number, degree

of unsaturation, configuration, and position of double bonds (Beebe, Brown, & Turcotte, 1988). Reversed-phase HPLC has been used to produce long-chain omega-3 PUFA concentrates on a preparative scale (Beebe et al., 1988). However, the use of silver ion-impregnated silica gel column as adsorbent has been reported to be effective for improving the purity of PUFA concentrates (Guil-Guerrero & Belarbi, 2001). Silver ions in the stationary phase are able to form reversible polar complexes with unsaturated regions of fatty acids, which can then be eluted subsequently using an appropriate solvent (Dobson, Christie, & Nikolova-Damyanova, 1995). Silver ion chromatography has been used to isolate EPA and DHA methyl esters derived from squid-liver oil to obtain 85-96% EPA and 95-98% DHA concentrates (Teshima, Kanazawa, & Tokiwa, 1978). SDA soybean oil was derivatized to ethyl esters, loaded on a silver ion-silica gel open column, and fractionated by stepwise elution using hexane or hexane/acetone (Kleiner-Shuhler, Vázquez, & Akoh, 2011). Sample loading amounts and elution volumes were optimized to maximize the purity and yield of SDA ethyl esters. The fractionation of SDA ethyl esters by silver-ion chromatography was demonstrated to be reproducible and scalable. However, although chromatographic methods can be used to produce omega-3 concentrates of high purity, large amounts of organic solvents are needed and the production capacity is relatively low compared to other fractionation methods (Haraldsson & Hjaltason, 2006).

Low Temperature Crystallization

Low temperature crystallization (LTC) or solvent winterization was originally developed to separate TAGs, fatty acids, and other lipids based on variations in their solubility in organic solvents at different temperatures (Brown & Kolb, 1955). The

process consists of cooling the oil or fatty acids in a solvent, holding for a specific period of time, and separating the crystallized (insoluble) fraction from the solvent. A rule of thumb for fractionating lipids by LTC is that the solubility of lipids in organic solvent decreases with increasing molecular weight, but increases with increasing degree of unsaturation (or polarity) and branching. Therefore, when fatty acids are saturated, long chains are less soluble than short chains; SFAs are less soluble than mono-, di-, and polyunsaturated fatty acids of equal chain length; *trans* isomers are less soluble than *cis* isomers, and straight-chain fatty acids are less soluble than branched-chain counterparts (Shahidi & Wanasundara, 1998). For fish oil- derived FFA mixture, most of the saturated, mono-, and dienoic fatty acids can be separated by LTC while the PUFA would remain dissolved in the organic solvent. The concentration of PUFA in the solvent phase generally increases with increasing holding time and reducing crystallization temperature. EPA and DHA concentrates in TAG and FFA forms have been prepared from sea blubber oil by LTC using hexane and acetone as solvents (Wanasundara, 1996). SDA concentrates in the form of TAG and FFA were also prepared from SDA soybean oil by LTC (Vázquez & Akoh, 2011, 2012). Process variables such as crystallization time and temperature, type of solvent, and oil to solvent ratio were optimized to maximize product purity and yield.

Enzymatic Methods

Lipases (EC 3.1.1.3) can be used to produce omega-3 PUFA concentrates from oils because of their selectivity towards or against omega-3 PUFA (Haraldsson & Hjaltason, 2001). Compared with more traditional physical methods, the lipase-catalyzed methods offer some advantages. First, the high catalytic efficiency of lipases allows for

relatively small amounts of lipase to be used for production on large scale, and re-use is possible with immobilized lipases. Second, lipase selectivity allows for discrimination between different PUFA which may be difficult to separate by physical means. Third, the lipase-catalyzed reactions are performed at mild conditions in terms of temperature, pH, and pressure, which are well suited for omega-3 PUFA, since they are prone to oxidation. Fourth, lipases are able to retain high activity under low water conditions and thermodynamic equilibrium can be shifted from hydrolysis towards esterification (synthesis). A number of microbial lipases have been successfully applied for the enrichment of omega-3 PUFA from fish oils. They include *Aspergillus niger, Candida rugosa, Geotrichum candidum, Pseudomonas cepacia, Pseudomonas fluorescens, Rhizomucor miehei, Rhizopus delemar,* and *Rhizopus oryzae* lipases (Haraldsson & Hjaltason, 2006). Lipase-mediated enrichment of omega-3 PUFA may occur by hydrolysis, esterification, or exchange of fatty acids (acidolysis). The direction and efficiency of the reaction is influenced by experimental conditions such as substrate molar ratio, temperature, and enzyme-load.

Selective esterification

There are several reports on esterification of fish oil-derived FFA with simple monohydric alcohols using lipases. In one of the first reports using this approach, Takagi (1989) used methanol and an EPA+DHA FFA concentrate obtained by urea precipitation of sardine oil FFA, comprising 30-40% EPA and 25-30% DHA. Using *Rhizomucor miehei* lipase as biocatalyst, the methyl ester product was enriched with EPA (>50%) while the residual FFA concentrate was enriched with DHA (~50%). Using a similar

approach, the concentration of SDA as lauryl esters was achieved by *Candida rugosa* lipase-catalyzed selective esterification of SDA soybean oil-derived FFA mixture (Vázquez, Kleiner, & Akoh, 2012). Process variables such as the type of biocatalyst (*Candida rugosa, Mucor javanicus,* and *Rhizomucor miehei* lipases), substrate molar ratio, time, and enzyme load were optimized to optimize SDA purity and yield.

Acidolysis

This involves lipase-catalyzed exchange of acyl group between a free fatty acid and TAG. This reaction would readily occur in organic solvent or solvent-free media with low water content. In principle, PUFA enrichment can be achieved by exchanging saturated, monoenoic, and dienoic fatty acids on TAGs with free PUFAs until chemical equilibrium is established (Figure 2.2A). In practice, however, preferential incorporation of PUFA into triacylglycerols is more likely to occur when the free PUFA is in excess. Using immobilized *Rhizomucor miehei* as biocatalyst, the EPA and DHA content of cod liver oil (initially 8.6 and 12.7%, respectively) was increased to 25 and 40%, respectively (Yamane, Suzuki, Sahashi, Vikersveen, & Hoshino, 1992). Similarly, the production of SDA concentrates was achieved by lipase-catalyzed acidolysis between SDA-enriched FFA mixture and SDA soybean oil or an SDA-enriched TAG (Kleiner, Vázquez, & Akoh, 2012a). Two different biocatalysts, Novozyme 435 and Lipozyme TL IM, were compared under varied levels of substrate molar ratio, incubation time, enzyme load, and type of media. Lipozyme TL IM was reported to display better performance than Novozyme 435 in incorporating SDA into the SDA soybean oil by acidolysis.

Structured TAG comprising MCFA at the *sn*-1 and -3 positions and long chain omega-3 PUFA at the *sn*-2 position have gained increasing attention as dietary and health

supplement. Omega-3 concentrates with this MLM configuration are of particular interest from human nutritional point of view. The MCFA located at the end positions are hydrolyzed by pancreatic lipase and rapidly assimilated via the portal vein to the liver where they can serve as an immediate source of energy. The residual 2-monoacylglycerols (2-MAG) are assimilated via the lymphatic system and accumulated as TAG in adipose tissues or as membrane phospholipids where they can be released upon demand for various physiological functions (Christensen, Høy, Becker, & Redgrave, 1995; Jensen, Christensen, & Høy, 1994). Various approaches have been developed for preparing MLM-type structured TAG comprising long chain omega-3 PUFA and MCFA (Haraldsson & Hjaltason, 2006). The simplest approach is by acidolysis, in which fish oil with relatively high *sn*-2 omega-3 PUFA content is reacted with MCFA as free fatty acids in the presence of an *sn*-1,3 specific lipase (Figure 2.2B). When MLM-type structured TAG concentrates are synthesized, it is important to curtail acyl migration (the spontaneous transfer of omega-3 PUFA from the *sn*-2 to the *sn*-1 or -3 positions) in order to minimize the incorporation of MCFA at the sn-2 position. Using acidolysis, Shimada and coworkers produced MLM-type structured TAG enriched with DHA at the *sn*-2 position by exchanging fatty acids at the *sn*-1 and -3 positions of tuna oil for caprylic acid using immobilized *sn*-1,3 specific *Rhizopus delemar* lipase (Shimada et al., 1996; Shimada, Sugihara, & Tominaga, 2000). However, there is yet need to investigate enzymatic synthesis of MLM-type structured TAG from SDA soybean oil.

Selective hydrolysis

Lipase-catalyzed partial hydrolysis of TAG results in the release of fatty acids and formation of a mixture of monoacylglycerols (MAG), diacylglycerols (DAG), TAG, and

glycerol depending on the extent of hydrolysis. Partial hydrolysis using a microbial lipase that selectively hydrolyzes saturated, mono-, and dienoic fatty acids and discriminate against glycerol-bound long-chain PUFA would result in a PUFA-enriched acylglycerol fraction (Figure 2.2C). Lipase discrimination against glycerol-bound long-chain PUFA is thought to occur because the terminal methyl group of these fatty acids lies close enough to the ester bond to sterically hinder lipase-catalyzed hydrolysis (Bottino, Vandenburg, & Reiser, 1967). Hoshino and coworkers examined several microbial lipases for selective hydrolysis of cod liver and sardine oil to concentrate EPA and DHA and reported that the lipases from *Candida cylindracea* and *Aspergillus niger* yielded acylglycerol fractions with two-fold higher PUFA content than the starting oil (Hoshino, Yamane, & Shimizu, 1990). The ability of microbial lipases from *Candida rugosa, Penicillum camembertii, Candida cylindracea*, *Rhizopus oryzae*, and *Rhizomucor miehei* to yield SDA-enriched acylglycerol fractions by selective hydrolysis of SDA soybean oil has also been studied (Kleiner, Vázquez, & Akoh, 2012b). *Candida rugosa* lipase was found to be the most effective at concentrating SDA soybean oil by selective hydrolysis.

Incorporation of Omega-3 PUFA into Food

As consumers become more aware of the health benefits of omega-3 PUFA, there is increasing interest in consumption of seafood, omega-3 PUFA supplements, and fortified functional foods for enhancing health and preventing CVD. Health-conscious consumers seek out functional food and beverage options because maintaining a healthy diet and lifestyle can prevent chronic disease. Although foods containing supplement-level dose of omega-3 PUFA has captured the interest of consumers, the process of fortifying an existing food product with it can be very challenging because omega-3

PUFA are particularly prone to lipid oxidation. Therefore, direct incorporation of omega-3 PUFA into food may lead to the development of undesirable odors and flavors which can compromise quality and consumer acceptability. Therefore, food formulators and researchers have recognized the need to develop strategies to improve the oxidative stability of omega-3 PUFA in food systems. A great deal of research has been carried out to elucidate lipid oxidation mechanisms in bulk and emulsified fats and oils (Frankel, 1984; McClements & Decker, 2000). This has provided important insights into the factors that influence lipid oxidation in food systems as well as strategies to manage it (Berdahl, Nahas, & Barren, 2010).

Lipid Oxidation

Lipid oxidation is a complex sequence of chemical changes that occur when lipids interact with reactive oxygen species and lead to rancid and potentially toxic end products (Frankel, 1984; Hyun & Min, 2008). Lipid oxidation can be divided into three distinct stages: initiation, propagation, and termination. Lipid oxidation is initiated by irradiation including exposure to visible light or catalyzed by enzymes (specifically lipoxygenases) and metal ions. High temperature and pressure accelerate lipid oxidation when initiated. From Figure 2.3, the three major reaction pathways involved in the initiation of lipid oxidation can be identified:

(I) Oxygen activation by metal catalysis to yield the hydroxyl radical which abstracts an allylic or bis-allylic hydrogen from an unsaturated lipid (LH) to form a lipid radical ($L^{\bullet}$) that can in turn react with ground state oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$). Other radicals ($R^{\bullet}$) like the chlorine radical from disinfectants or

generated by myeloperoxidases may also initiate the chain reaction through formation of $L^{\bullet}$.

(II) Lipoxygenases incorporate oxygen in the unsaturated lipid to yield lipid hydroperoxides (LOOH) which are recognized to be the primary products of lipid oxidation

(III) Photosensitized oxidation to yield singlet oxygen ($^1O_2$), an electrophile which adds to the double bond of unsaturated lipids to yield lipid hydroperoxides directly.

Lipid oxidation propagates as lipid radicals react with other lipids and lipid hydroperoxides in their immediate vicinity. The lipid peroxyl radical reacts with another unsaturated lipid to generate a lipid hydroperoxide and new lipid radical. Lipid hydroperoxides are cleaved reductively by metal ion catalysis to yield lipid alkoxyl radicals ($LO^{\bullet}$) which initiate new chain reactions by reacting with lipid hydroperoxides or breakdown to secondary lipid oxidation products such as aldehydes and ketones which are responsible for the rancidity of oxidized lipids. Finally, some of the lipid radicals are terminated when they react with other radicals.

The precise mechanism of lipid oxidation in a particular food depends on the nature of the reactive species present and their physicochemical environment (McClements & Decker, 2000). The rate of lipid oxidation in bulk lipids depends primarily on the degree of unsaturation with fish oils being more prone than plant oils, and with animal fats being most stable. In dispersed systems, surface area is increased and lipids become more vulnerable to oxidation, especially in the presence of metal ions which can bind to negatively charged surfactants in the oil/water interface and react with

lipid hydroperoxides (McClements & Decker, 2000). One of the most effective means of retarding lipid oxidation is to incorporate antioxidants. Antioxidants function by different mechanisms, including inactivation of lipid radicals, scavenging of oxygen, and chelation of pro-oxidant metal ions (Choe & Min, 2009; Decker, 1998). They may be classified according to their mechanism of action as primary or secondary antioxidants. A primary antioxidant, also known as a 'chain-breaking' antioxidant is able to delay the initiation step or terminate the propagation step by reacting with lipid radicals and converting them to more stable products (Reische, Lillard, & Eitenmiller, 2008). The antioxidant radicals formed by this process are less reactive and less likely to promote oxidation. Secondary antioxidants retard lipid oxidation through a variety of mechanisms, including chelation of metal ions, replenishing hydrogen to primary antioxidants, oxygen scavenging, and deactivation of reactive oxygen species. The spatial location of antioxidants has been shown to be very important for their effectiveness in bulk and emulsion systems (Laguerre et al., 2009; Pekkarinen, Stöckmann, Schwarz, Heinonen, & Hopia, 1999). The polar paradox, that is, the observation that hydrophilic antioxidants are efficient in bulk oil while lipophilic antioxidants are more efficient in emulsions, has been used as a guiding principle for optimizing the oxidative stability of food lipids for long term storage (Frankel, Huang, Kanner, & German, 1994; Porter, Black, & Drolet, 1989).

Microencapsulation of Omega-3 PUFA

In addition to incorporating antioxidants, encapsulation of omega-3 PUFA in food grade biopolymers also improves their resistance to oxidation in the ingredient state as well as in the final food product and facilitates omega-3 PUFA fortification in a wider range of food products (Augustin & Sanguansri, 2015; Taneja & Singh, 2012).

Proprietary methods for encapsulating omega-3 PUFA include spray drying emulsions, fluidized bed coating, extrusion, complex coacervation, and biopolymer gelation (Drusch & Mannino, 2009; Gouin, 2004). Most encapsulation processes have their research roots in the pharmaceutical industry and were developed for enhancing the stability of formulations and for controlled- release of drugs. Significant advances in pharmaceutical applications and in the development of cost effective preparation techniques and materials have allowed a significant increase in the use of encapsulation processes in the food industry. However, a significant challenge in designing food-grade delivery systems is finding biopolymers or biopolymer combinations that would provide a good balance between resilience (physical and thermal stability) and bioavailability (Haraldsson & Hjaltason, 2006; Velikov & Pelan, 2008). Biopolymers are convenient for delivering lipids because of their amphiphilic nature which provides high solubility in aqueous media as well as hydrophobic groups for engaging interactions with lipophilic substances. This characteristic enables biopolymers to adsorb to the oil-water interface and form adsorbed layers which are capable of stabilizing oil-in-water emulsions. It is worth noting that the formation of an emulsion is one of the key steps in encapsulation processes such as spray drying and complex coacervation. The microcapsules formed can have a variety of external and internal structures. Mononuclear microcapsules are made up of a single oil droplet surrounded by a continuous wall of biopolymer whereas, in multinuclear microcapsules, the core material is dispersed within multiple small compartments embedded within a continuous polymer matrix (See Figure 2.4).

The use of protein-polysaccharide blends as delivery systems for omega-3 PUFA and other lipophilic bioactive materials is very attractive because of the variety of

biopolymers combinations that could be adapted for different delivery requirements, such as hydrophile-lipophile balance, mechanical properties, and permeability (Schmitt & Turgeon, 2011). Proteins in a solution at pH value below their isoelectric point carry a net positive charge and would readily form complexes with one or more polysaccharides that carry a negative ionic charge. This interaction may either result in complex coacervation, or soluble complex formation, depending on various factors such as the type of polysaccharide used (anionic/cationic), the solution pH, ionic strength, and the ratio of polysaccharide to protein (De Kruif, Weinbreck, & de Vries, 2004; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998).

The electrostatic interaction of oppositely charged protein and polysaccharide can be utilized for the encapsulation and delivery of lipophilic substances (De Kruif & Tuinier, 2001). Microencapsulation by complex coacervation is a unique and promising technique because very high pay loads can be achieved as well as controlled and targeted release in response to environmental conditions (temperature, pH, pressure, or chemical reactions). The term 'complex coacervation' is defined as the spontaneous phase separation of a solution of oppositely charged protein + polysaccharide into a solvent-rich phase and a solvent-depleted coacervate phase (Bungenberg de Jong, 1965b). This phase separation is driven by attractive electrostatic interactions between the protein and polysaccharide, resulting in the formation of electrostatic protein-polysaccharide soluble complexes. The rearrangement of such complexes leads to the formation of liquid droplets, also called coacervates, which ultimately coalesce and sediment to form the coacervate phase (De Kruif et al., 2004). However, in the presence of dispersed oil droplets, the coacervates deposit on the surface of oil droplets to form a continuous shell

around oil droplets. Complex coacervation involving hydrocolloids like gelatin and gum arabic has long been applied in the area of microencapsulation (Green & Schleicher, 1957). Classical studies on complex coacervation were performed using the gelatin-gum arabic system (Bungenberg de Jong, 1965a, 1965b) but a considerable number of other hydrocolloid systems have been evaluated for complex coacervation (Thies, 2012; Xiao, Liu, Zhu, Zhou, & Niu, 2014). Complex coacervation is typically used to encapsulate flavor oils (Xiao et al., 2014), but it has been adapted for other core materials such as fish oils, vitamins, enzymes, and preservatives (Gouin, 2004). Aqueous suspensions or powdered forms of coacervate-based microcapsules can be conveniently incorporated into aqueous-based food products in which the direct addition of the neat oil may be problematic. Human trials have shown that omega-3 fish oils delivered as microencapsulated complex coacervates are bioequivalent to oils consumed as soft gel capsules (Barrow, Nolan, & Holub, 2009). From Figure 2.5, there are four basic steps involved in the microencapsulation of oils by complex coacervation:

1. Emulsification: Form an oil-in-water (O/W) emulsion by homogenizing the oil in a solution containing dissolved biopolymers.

2. Coacervation: Adjust pH of the O/W emulsion formed in step 1 to a value deemed appropriate for complex coacervation to occur. Dispersed oil droplets become engulfed in a coating of liquid coacervate.

3. Gelation: Gel the coacervate coating formed in step 2 to stabilize the shell and form a suspension of microcapsules. Gelation may be made to occur by cooling the system. The shell is typically strengthened by adding a crosslinking agent to make microcapsules resilient to a wide range of food processing conditions.

4. Drying (optional): The microcapsules are dried by spray, fluidized bed, or freeze drying to yield a free-flowing powder.

Table 2.2 shows the types of crosslinking agent used for various applications of gelatin-gum arabic coacervate-based microcapsules. Some investigators used gluteraldehyde or formaldehyde in accordance with the traditional approach of chemical crosslinking of gelatin-based microcapsule shells. However, due to toxicity concerns with gluteraldehyde and formaldehyde, a safer approach for food applications is to crosslink coacervate shells with transglutaminase (TG; amine γ-glutamyl transferase, EC 2.3.2.13). TG is an enzyme that crosslinks proteins or peptides by catalyzing the formation of an isopeptide bond between the free amine group of a lysine residue and the amide group of a glutamine residue (Figure 2.6). However, TG-catalyzed crosslinking requires long processing times (12-16 h) which may tie up resources and increase production cost (Gouin, 2004). Moreover, TG has been controversially dubbed "meat glue" and has received significant negative publicity for its use in commercial meat processing as a binding agent. So, the presence of TG-crosslinked microcapsules in a food product may not be as label-friendly as previously thought. Maintaining appropriate microcapsule size and size distribution is an important aspect of all encapsulation processes. To use the complex coacervation approach successfully, the system composition and preparation conditions must be controlled to form stable colloidal particles, otherwise flocculation may occur and result in the formation of large microcapsule aggregates which is undesirable in most food applications (Thies, 2012).

Covalently crosslinked protein-polysaccharide conjugates produced by the Maillard reaction has potential applications for effective encapsulation and delivery of

omega-3 PUFA and other lipiphilic bioactive ingredients (Augustin, Sanguansri, & Bode, 2006; Oliver, Augustin, & Sanguansri, 2009; Rusli, Sanguansri, & Augustin, 2006). The Maillard browning reaction occurs spontaneously under controlled conditions of dry heating (60-90 °C at a relative humidity of 45-80%) for any binary system of protein + polysaccharide, for example, β-lactoglobulin + dextran, whey protein isolate (WPI) + dextran, and WPI + maltodextrin (Akhtar & Dickinson, 2003, 2007; Dickinson & Galazka, 1991). The emulsion-stabilizing properties of Maillard-type covalent conjugates have been found to be much better than those of the equivalent simple mixtures of protein + polysaccharide or the protein alone (Akhtar & Dickinson, 2007; Wooster & Augustin, 2006, 2007). The excellent emulsion stabilizing properties of covalent conjugates are associated with the adsorbed polysaccharide chains on the oil-water interface which restricts inter-particle contact and confers colloidal stability to emulsified droplets (Dickinson, 2008). Furthermore, Maillard reaction products (MRP) of protein + polysaccharide blends have been found to have enhanced antioxidant capabilities compared to simple mixtures of protein + polysaccharide (Augustin et al., 2006; Nursten, 2005). Microcapsules have been successfully produced by spray drying oil-in-water emulsions stabilized by Maillard-type protein-polysaccharide conjugates (Augustin et al., 2006). Therefore, it would be interesting to explore the use of Maillard reaction for crosslinking protein + polysaccharide mixtures for the microencapsulation of omega-3 oils by complex coacervation.

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Table 2.1 Methods for producing stearidonic acid (SDA) concentrates from SDA soybean oil

Method	Type of concentrate	SDA concentration (mol%)	References
Physical/Non-enzymatic			
Chromatography	Ethyl esters	96.0	Kleiner-Shuhler et al. (2011)
Low temperature	Free fatty acids	59.8	Vázquez and Akoh (2011)
crystallization	Triacylglycerols	45.2	Vázquez and Akoh (2012)
Enzymatic			
Selective esterification	Lauryl esters	58.0	Vázquez et al. (2012)
Acidolysis	Triacylglycerols	37.6, 53.5	Kleiner et al. (2012a)
Selective hydrolysis	Acylglycerols [a]	40.9, 62.7	Kleiner et al. (2012b)

[a] Composed of mono-, di-, and triacylglycerols

Table 2.2 Selected applications of gelatin-gum arabic complex coacervates in the encapsulation of lipophilic materials

Core material	Crosslinking agent	Application	Reference
Chlorinated diphenyl	Formaldehyde	Printing	Green and Schleicher (1957)
Peppermint oil	Glutaraldehyde or transglutaminase	Food	Dong, Toure, Jia, Zhang, and Xu, (2007); Dong et al. (2008)
Fish oil	NC	Food	Tamjidi, Nasirpour, and Shahedi (2012)
Fish oil ethyl esters	Ethanol or dehydroascorbic acid	Pharmaceutical	Lamprecht, Schäfer, and Lehr (2000)
Flaxseed oil	NC	Food	Liu, Low, and Nickerson (2010)
Sunflower oil	Glutaraldehyde	Food	Piacentini, Giorno, Dragosavac, Vladisavljević, and Holdich (2013)
Allyl isothiocyanate	Tannic acid	Food	Zhang, Pan, and Chung (2011)
MTC	Glutaraldehyde	Food	Leclercq, Milo, and Reineccius (2009)
Dodecanol	Formaldehyde or glutaraldehyde	Pest control	Gu, Zhu, Kong, and Tan (2010) Kong, Gu, Zhu, and Zhang (2009)
Patchouli oil	Glutaraldehyde	Textiles	Yang et al. (2011)
Lemon oil	Glutaraldehyde or formaldehyde	Textiles	Specos, Escobar, et al. (2010)
Citronella oil	Gluteraldehyde	Textiles	Specos, García, et al. (2010)
Vetiver oil	Glutaraldehyde or transglutaminase	Pharmaceutical	Prata, Zanin, Ré, and Grosso (2008)
Garlic oil	Formaldehyde	Pharmaceutical	Siow and Ong (2013)
Jasmine oil	Transglutaminase	Food	Lv, Zhang, Abbas, and Karangwa (2012)
Camphor oil	Glutaraldehyde	Pharmaceutical	Chang, Leung, Lin, and Hsu (2006)

NC, crosslinking agent not used; MTC, medium chain triacylglycerol

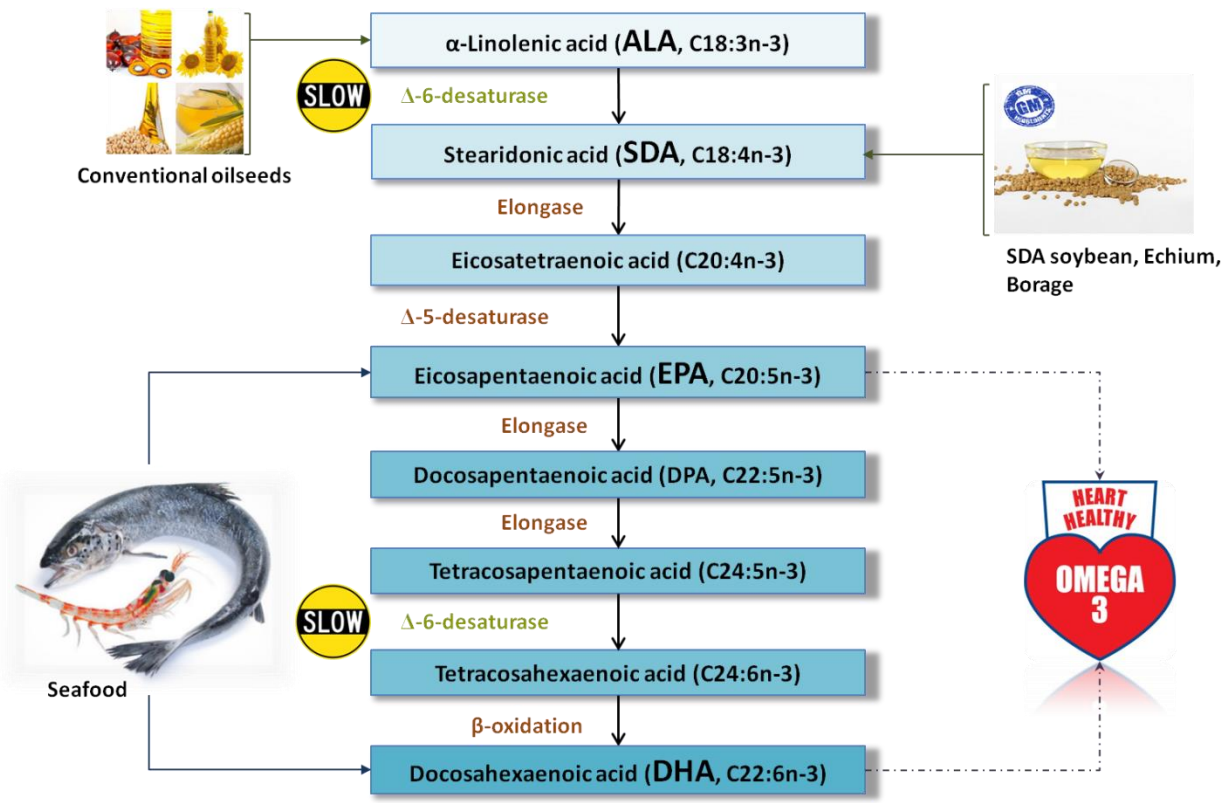

Figure 2.1 Metabolism of omega-3 fatty acids from different dietary sources

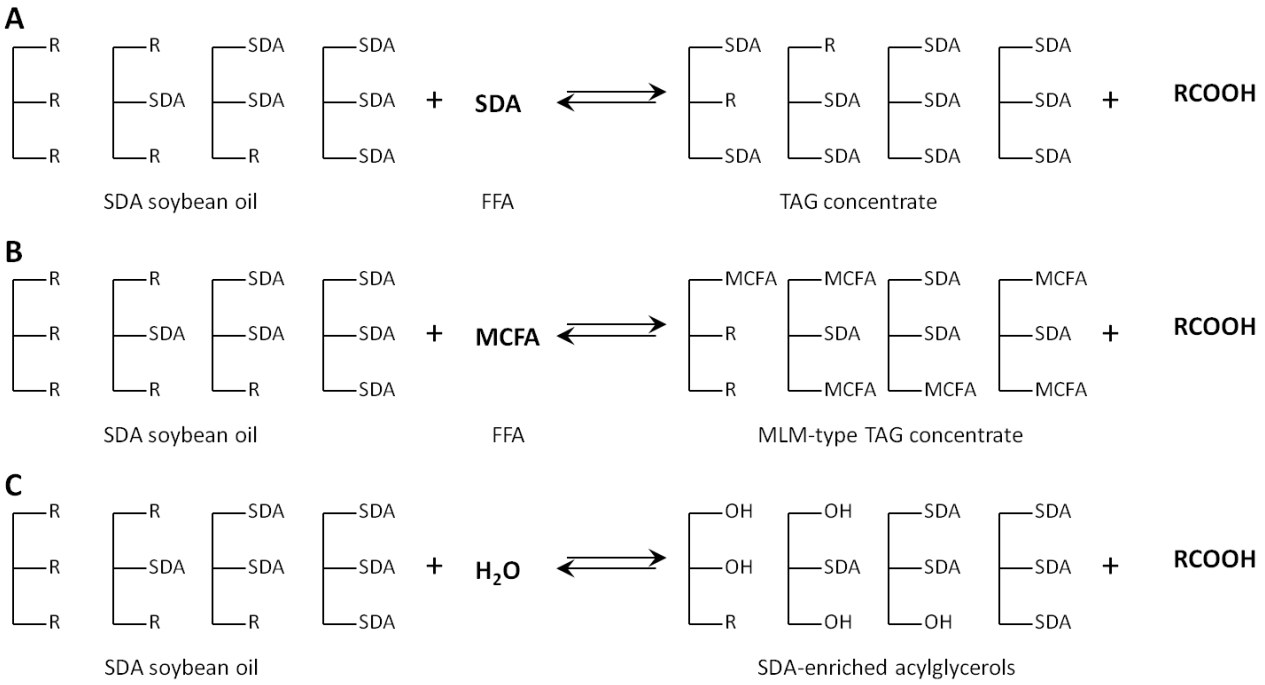

Figure 2.2 Schematic representations of lipase-catalyzed **A:** acidolysis of stearidonic acid (SDA) soybean oil with SDA-enriched free fatty acids (FFA) to yield SDA-enriched TAG concentrate, **B:** acidolysis of SDA soybean oil with medium chain fatty acids (MCFA) to yield MLM-type structured TAG concentrate, and **C:** selective (partial) hydrolysis of SDA soybean oil to yield an SDA-enriched acylglycerol fraction (R represents any fatty acyl chain)

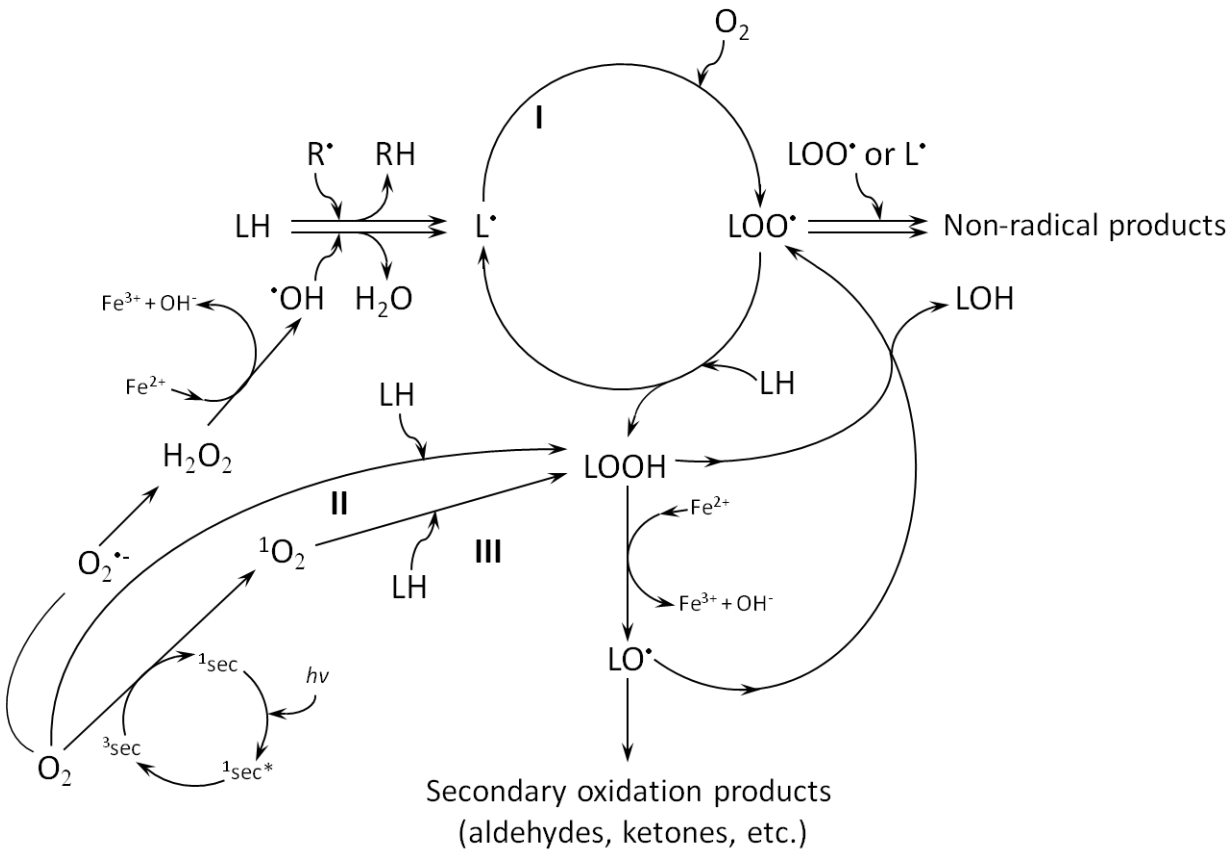

Figure 2.3 Three major reaction pathways are responsible for lipid oxidation in food: (I) Free radical chain reaction initiated by oxygen activation; (II) Enzymatic formation of lipid hydroperoxides through lipoxygenase activity; (III) Photosensitized formation of lipid hydroperoxides. Modified from Skibsted (2010)

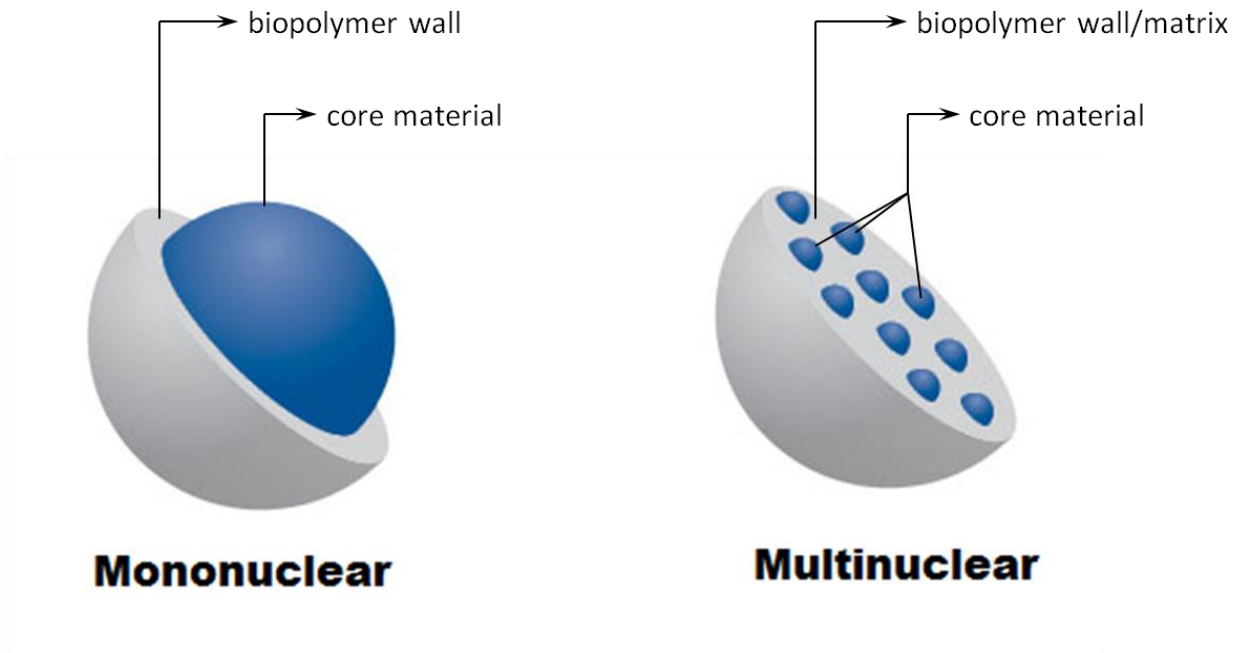

Figure 2.4 Schematic representations of mononuclear and multinuclear microcapsules

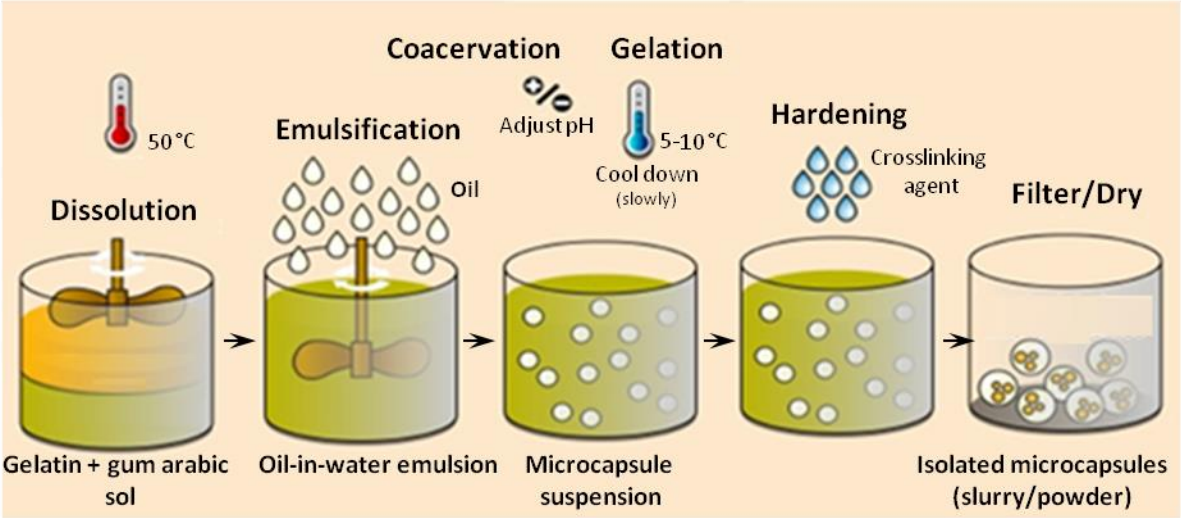

Figure 2.5 Basic steps of microencapsulation by complex coacervation

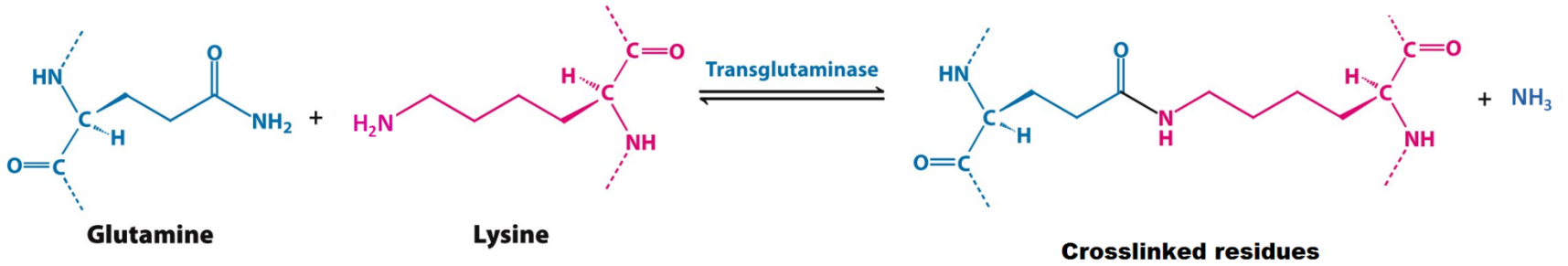

Figure 2.6 Transglutaminase-catalyzed crosslinking between lysine and glutamine residues

CHAPTER 3

CHEMOENZYMATIC METHOD FOR PRODUCING STEARIDONIC ACID CONCENTRATES FROM STEARIDONIC ACID SOYBEAN OIL[1]

[1] Ifeduba, E. A., and Akoh, C. C. 2013. *Journal of the American Oil Chemists' Society, 90*(7), 1011-1022. Reprinted here with permission from Springer.

Abstract

The aim of this study was to produce stearidonic acid (SDA, 18:4 n-3) omega-3 concentrates from 25% SDA soybean oil (SDASO) by enzymatic acidolysis. Substrates were prepared by chemical and enzymatic hydrolysis of SDASO. A 62% SDA free fatty acid mixture (SDA-FFA) was obtained by low temperature crystallization of the chemical hydrolyzate while partial hydrolysis of SDASO by non-immobilized lipase AY 30 (*Candida rugosa*) yielded a 51% SDA acylglycerol mixture (SDA-GLY). Reaction conditions for acidolysis between SDA-FFA and SDA-GLY were optimized using response surface methodology (RSM). Incorporation of SDA into SDA-GLY by immobilized Lipozyme RM IM (*Rhizomucor miehei*) and non-immobilized Lipomod 34P-LO34P (*C. cylindracea [rugosa]*) lipases were modeled under varying levels of substrate molar ratio (S_r), temperature (T), and time (t). Optimal conditions for production of a 60% SDA concentrate were predicted to be S_r = 4.8, T = 65 ^oC and t = 8 h for Lipozyme RM IM and S_r = 5.0, T = 43 ^oC and t = 48 h for Lipomod 34P-L034P. The model was verified experimentally by gram scale synthesis under optimal conditions which resulted in production of 59.98 and 58.98% SDA concentrates (≥ 96% triacylglycerols) by Lipozyme RM IM and Lipomod 34P-L034P, respectively.

Keywords: stearidonic acid; soybean oil; omega-3 concentrate; hydrolysis, acidolysis; lipase; response surface methodology

Introduction

Dietary intake of omega-3 long chain polyunsaturated fatty acids (LCPUFAs), particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), have been reported to confer a number of health benefits, notably cardioprotection which reduces risk of cardiovascular disease (Lavie, Milani, Mehra, & Ventura, 2009). In addition, these fatty acids are essential for visual acuity and cognitive function because they are required for growth and development of cells of the retina and brain (Farquharson et al., 1995; Makrides, Neumann, Simmer, Gibson, & Pater, 1995). The cardioprotective mechanism of omega-3 fatty acids begins with incorporation of DHA and EPA into tissue membrane phospholipids. The details of this mechanism have been discussed elsewhere (Harris, 2007). Blood levels of these LCPUFAs, expressed as the omega-3 index (percentage sum of EPA and DHA on erythrocyte membrane) reflects dietary intake and serves as a biomarker for cardiovascular disease risk (Harris, 2007). In principle, EPA and DHA may be synthesized from the parent omega-3 α-linolenic acid (ALA) through a series of elongation and desaturation reactions, however this conversion is inefficient in humans (Gerster, 1997), thus EPA and DHA must be obtained directly from diet. Because the 'Western diet' is characteristically poor in omega-3 fatty acids and this deficiency has been linked to a variety of chronic disease conditions, a host of health agencies and professional organizations issued recommendations to increase intake of omega-3 LCPUFAs (Harris, 2007). Mainstream dietary sources of omega-3 LCPUFAs include seafood products and fish oil supplements, however, chemical safety, shelf life stability, sustainability, and consumer acceptability issues have been raised with the use of these resources (Naylor et al., 2000; Whelan, 2009). The need for alternative and sustainable sources of omega-3 fatty acids led to the development of the novel plant

derived stearidonic acid (SDA, 18:4n-3) soybean oil. SDA soybean oil compares favorably with fish oils in terms of sustainability, consistency of supply, oxidative stability and palatability (Froman, Duong, & Listello, 2011). In addition, the dietary intake of SDA is more advantageous than ALA for raising omega-3 index because while the major pathway for ALA metabolism is via *β*-oxidation (Brenna, 2002), dietary SDA is rapidly converted to EPA *in vivo* (James, Ursin, & Cleland, 2003), and incorporated into erythrocyte and myocardial membranes where it raises omega-3 index and confers cardioprotection. Since SDA functions as a metabolic proxy for EPA, SDA soybean oil bears the 'heart healthy' claim associated with the intake of EPA (Monsanto Co., 2009).

There has been a lot of interest in the production of omega-3 fatty acid concentrates for health foods and pharmaceutical applications. As consumers become more informed on the health benefits of omega-3 fatty acids, deployment of double and triple strength omega-3 supplements and products have been envisaged (Nutra-Ingredients, 2012). Furthermore, omega-3 concentrates are required in smaller quantities than traditional fish oils since they provide higher amounts of omega-3 fatty acids per serving. Therefore, omega-3 concentrates may be advantageous for formulating nutrient dense low calorie foods since they allow for low total lipid intake. Shahidi and Wanasundara (1998) reviewed technologies available for production of omega-3 fatty acid concentrates from fish oils, namely: adsorption chromatography, molecular distillation, enzymatic hydrolysis/acidolysis, low-temperature crystallization, supercritical fluid extraction and urea complexation. Some of these technologies have been successfully used for the production of SDA concentrates from SDA soybean oil; namely, adsorption chromatography (Kleiner-Shuhler, Vázquez, & Akoh, 2011), low temperature crystallization (Vázquez & Akoh, 2011), and enzymatic

hydrolysis/acidolysis (Kleiner, Vázquez, & Akoh, 2012a, 2012b). Of these technologies, the enzymatic approach is believed to be the most attractive from a marketing point of view because it leads to production of PUFA concentrates in the form of triacylglycerols (TAGs) which are often endorsed as more 'natural' than the fatty acid derivatives from the other processes (Shahidi & Wanasundara, 1998). However, lipase-catalyzed production of SDA concentrates as TAGs from SDA soybean oil has not been performed on a large scale. In the present study, we report a chemoenzymatic method for producing valuable SDA omega-3 concentrates as triacylglycerols from SDA soybean oil. A scheme for the process is shown in Figure 3.1. First, SDA soybean oil was completely hydrolyzed to free fatty acids (FFA) by chemical hydrolysis. The hydrolyzate obtained by chemical hydrolysis was then subjected to low temperature crystallization to yield a low-melting SDA-rich FFA fraction. Second, SDA soybean oil was partially hydrolyzed by lipase AY 30 to yield an SDA-rich mixture of acylglycerols. Third, Lipozyme RM IM and Lipomod 34P-L034P were used to catalyze acidolysis between the SDA-rich FFA fraction and acylglycerol mixture to yield SDA concentrates as triacylglycerols. Reaction conditions for lipase-catalyzed acidolysis were optimized by response surface methodology (RSM).

Materials and Methods

Materials

SDA soybean oil (SDASO) was supplied by Monsanto Company (St. Louis, MO). The non-immobilized, non-specific lipase AY 30, isolated from *Candida rugosa*, was donated by Amano Enzyme Inc. (Nagoya, Japan). Lipomod 34P-L034P, a non-immobilized, non-specific, *C. cylindracea [rugosa]* lipase (CCL), was donated by Biocatalysts (Wales, UK). Lipozyme RM IM, an *sn*-1,3 specific *Rhizomucor miehei* lipase (RML) immobilized on an anion exchanger, was obtained from Novozymes North

America Inc. (Franklinton, NC, USA). The specific activities of lipase AY 30, CCL and RML were 2400, 4023, and 2845 U/g, respectively. Specific activities of these enzymes were determined by the titrimetric method using triolein as substrate (Pinsirodom & Parkin, 2001). Supelco 37 Component fatty acid methyl ester (FAME) mix, triolein, 1-monoolein, 2-monoolein, oleic acid, and pancreatic lipase were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 1,3-Diolein standard was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). Stearidonic acid methyl ester standard was purchased from Cayman Chemical Company (Ann Arbor, MI, USA) while nonadecanoic acid (C19:0) standard was purchased from TCI America (Portland, OR, USA). ACS or HPLC grade organic solvents and chemicals were purchased from Fisher Scientific (Norcross, GA, USA) and Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Production of SDA-Enriched Free Fatty Acids

Complete alkaline hydrolysis of SDASO followed by low temperature crystallization of the chemical hydrolyzate was performed as described by (Vázquez & Akoh, 2011). This produced a low melting free fatty acid fraction that was designated SDA-FFA.

Lipase-Catalyzed Partial Hydrolysis of SDASO

The method for enzymatic hydrolysis of SDASO was modified from that described by (Kleiner et al., 2012b). SDASO (100 g) and phosphate buffer (150 mL of a 0.1 M solution; pH 7.0) were placed in a stirred tank glass reactor (1L) and heated to 40 ^{o}C with 200 rpm (3.58 × *g*) stirring. After substrates had reached the incubation temperature, 4032 units (40.32 U/g oil) of lipase AY 30 was added to initiate hydrolysis and stirring was continued. Aliquots (10 mL) of hydrolyzate were collected at 4, 6, 8 10, 12, 24, and 48 h of incubation for analysis. Lipase activity was halted by adding 2 mL

methanol. Hexane (5 mL) was added and mixed by vortex for 2 min. The mixture was centrifuged at 2000 rpm (357.76 × *g*) for 2 min and the upper organic layer was filtered through anhydrous sodium sulfate column. The filtrates constituted the SDASO hydrolyzate (SDASO-H) and were stored in capped, UV-light-protected vials at -80 ^{o}C until further use.

Determination of Hydrolysis Percentage

The saponification value of SDASO was determined according to AOCS Official Method Cd 3a-94 (AOCS, 2009). The acid values of SDASO and SDASO-H were determined from percentage FFA according to AOCS Official Method Ca 5a-40 (AOCS, 2009). After determination of acid value, hexane (50 mL) was added to the neutralized SDASO-H mixture and transferred into a separatory funnel to allow phase separation. The upper hexane layer was collected and passed through a bed of anhydrous sodium sulfate. Hexane was removed with a rotary evaporator at 40 ^{o}C to recover total acylglycerol fraction of SDASO hydrolyzate (SDA-GLY). Percentage hydrolysis was calculated using Eq. 1.

$$\text{Hydrolysis (\%)} = [(\text{Acid value}_{(\text{hydrolyzate})} - \text{Acid value}_{(\text{original oil})}) - (\text{Saponification value}_{(\text{original oil})} - \text{Acid value}_{(\text{original oil})})] \times 100 \quad [1]$$

Preparation of SDA-Enriched Acylglycerol Mixture

Lipase AY 30-catalyzed hydrolysis of SDA soybean oil was carried out as previously described but the reaction was stopped after 24 h. Free fatty acids (FFAs) were separated from acylglycerols by neutralization with 1.0 M NaOH. Fifty milliliters (50 mL) of 95% ethanol and 3 drops of 1% phenolphthalein solution were added to the reaction mixture and titrated with 1.0 M NaOH to a permanent faint pink endpoint. The mixture was transferred into a separatory funnel and hexane (50 mL) was added. The

separatory funnel was swirled and allowed to phase separate. The upper organic phase was passed through a bed of anhydrous sodium sulfate and hexane was removed with a rotary evaporator at 40 ^{o}C to recover a mixture of acylglycerols (SDA-GLY) which was stored in UV light protected vials at – 80^{o}C till further use.

Lipase-catalyzed Acidolysis of SDA-GLY and SDA-FFA

Acidolysis reaction mixture was made up of 4 mL *n*-hexane, 200 mg SDA-GLY and SDA-FFA blended to different substrate molar ratios. Variables considered in the experimental design were molar ratio of SDA-FFA to SDA-GLY (S_r) (3-6), incubation temperature (T) (40-65 ^{o}C for RML and 40-55 ^{o}C for CCL), and time (t) (8-48 h). To maintain a low water activity in the reaction medium, 50 mg of molecular sieve (4Å) was added. RML (10% by wt of substrates) or normalized amount of CCL was added to initiate the reaction. Equivalent weight of CCL was determined using Eq. 2.

$$W_{CCL} \equiv W_{RML} \times (SA_{RML}/SA_{CCL}) \quad [2]$$

Where W_{CCL} = weight of CCL (g), W_{RML} = weight of RML (g), SA_{RML} = specific activity of RML (U/g), SA_{CCL} = specific activity of CCL (U/g).

Test tubes containing the reaction mixture were flushed with nitrogen and incubated in an orbital shaking water bath at 200 rpm (3.58 × *g*). After incubation, the biocatalyst was separated from the reaction products by filtration through an anhydrous sodium sulfate column. All reactions were performed in duplicate and average results were reported.

Experimental Design for Lipase-Catalyzed Acidolysis

RSM was used to model the effect of different reaction parameters on the incorporation of SDA into SDA-GLY by RML and CCL-catalyzed acidolysis. Central Composite Face (CCF) design with 3 factors and 3 levels was used to generate factor

combinations (Table 3.1). A total of 17 experimental runs with 3 center points were generated per biocatalyst investigated. Results were fitted into a second-order polynomial function of the form:

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

Where Y = % incorporation of SDA; β_o = constant; β_i = linear term coefficients; β_{ii} = quadratic term coefficients; β_{ij} = interaction term coefficient, and X_i and X_j are the independent variables. MODDE 5.0 software (Umetrics, Sweden) was used to fit the second order polynomial equation to the experimental data.

Gram Scale Synthesis

Lipase-catalyzed acidolysis was carried out under optimum levels of substrate molar ratio, incubation temperature, and time as predicted by RSM. For RML-catalyzed acidolysis, 1.27 g of biocatalyst was added to a substrate mixture made up of 5.0 g of SDA-GLY, 7.7 g SDA-FFA, 100 mL *n*-hexane and 1.0 g molecular sieve, and incubated at 65 ^{o}C for 8 h. For CCL-catalyzed acidolysis, 0.92 g of biocatalyst (see Eq. 2) was added to a substrate mixture made up of 5.0 g of SDA-GLY, 8.0 g SDA-FFA, 100 mL *n*-hexane and 1.0 g molecular sieve, and incubated at 43 ^{o}C for 48 h. After incubation, reaction mixtures were filtered through anhydrous sodium sulfate to remove enzymes. FFAs were separated from acidolysis products by neutralization as described earlier. The isolated SDA concentrates were stored in UV light protected vials and stored at – 80^{o}C until further analysis.

Analysis of Hydrolysis and Acidolysis Products

SDASO-H and acidolysis products were spotted along with triolein, 1,3-diolein, 1-monoolein, and oleic acid standards on activated silica gel G TLC plates. Plates were

developed with petroleum ether/diethyl ether/acetic acid (70:30:2, v/v/v) and bands were visualized under UV light after spraying with 0.2% 2′,7′-dichlorofluorescein in methanol. For SDASO-H, bands corresponding to triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), and FFA were scraped off for fatty acid composition analysis while for acidolysis products, TAG and FFA bands were collected for analysis.

Lipid Class Analysis by HPLC-ELSD

The HPLC system used to analyze the lipid class composition of SDASO, SDA-GLY and SDA concentrates was an Agilent 1260 Infinity Quaternary LC (Agilent Technologies, Santa Clara, CA) equipped with a Sedex Model 85 evaporative light scattering detector (Sedere, Alfortville Cedex, France). The column employed was an Agilent Zorbax RX-SIL (4.6 × 250 mm, 5 μm particle size) and analysis was carried as per the AOCS Official method Cd 11d-96 (AOCS, 2009). Peaks were identified by comparing retention times to those of authentic lipid standards. For quantification, response calibration curves were constructed using triolein, 1,3-diolein, and 1-monoolein.

Positional Analysis of Triacylglycerols

Positional analysis of SDASO and SDA concentrates was performed according to a modified version of the method described by (Luddy, Barford, Herb, Magidman, & Riemenschneider, 1964). Two milliliters of 1.0 M Tris-HCl buffer (pH 8), 0.5 mL of 0.05% sodium cholate solution, and 0.2 mL 2.2% $CaCl_2$ solution was added to 100 mg of oil substrate. The mixture was vortexed for 2 min and 40 mg of pancreatic lipase was added. Test tubes were incubated in an orbital shaking water bath at 40 ^{o}C for 3 min at 200 rpm (38.5 × g). After incubation, tubes were vortexed for another 2 min and 1 mL of 6 N HCl was added to stop the reaction. Lipids were extracted by adding 4 mL of diethyl ether and centrifugation at 1000 rpm (89.44 × g) for 3min. The upper layer was collected,

filtered through an anhydrous sodium sulfate column and concentrated under nitrogen to one-third of the original volume. The concentrated organic layer was spotted on silica gel G plates alongside triolein, 1,3-diolein, 2-monoolein, and oleic acid standards. Plates were developed with hexane/diethyl ether/formic acid (60:40:1.6, v/v/v) and bands were detected under UV light after spraying with 0.2% 2′,7′-dichlorofluorescein in methanol. Bands corresponding to MAG were scraped off and methylated directly. The fatty acid content at the *sn*-1, 3 positions was calculated (see Table 3.4, footnote).

Fatty Acid Methyl Ester (FAME) Preparation

SDASO, SDASO-H, SDA-GLY and acylglycerol species isolated by TLC were converted to FAME following the AOAC Official Method 996.01, Section E (AOAC, 2005) with minor modifications. A known amount of nonadecanoic acid (C19:0) was added as internal standard (100 µL of 20 mg/mL internal standard was added to 100 mg of SDASO, SDASO-H, and SDA-GLY while 50 µL was added to scrapped TLC bands). Samples were saponified with 2 mL of 0.5 N NaOH and incubated at 100 ^{o}C for 5 min. Subsequently, 2 mL 14% BF_3 in methanol was added and samples incubated again at 100 ^{o}C for 5 min. Hexane (2 mL) and 2 mL saturated NaCl solution were added to extract FAMEs. After centrifugation at 1000 rpm (89.44 × *g*) for 5 min, the upper hexane layer was passed through anhydrous sodium sulfate column before GC analysis. FFAs were converted to FAME following the method described by (Ichihara & Fukubayashi, 2010).

GC Analysis

FAMEs were analyzed on an Agilent Technology 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a Supelco SP-2560 capillary GC column (100 m × 0.25 mm ID, 0.20 µm film, Sigma-Aldrich Co. St. Louis, MO). Injection of 1 µL of sample was made at a split ratio of 5:1. Helium carrier gas flow was

1.1 mL/min. The detector temperature was 250 ^{o}C. The oven was held at 140 ^{o}C for 5 min, then increased to 240 ^{o}C at 4 ^{o}C/min and held isothermally for 15 min. The relative FAME content was calculated using GC ChemStation Revision B.04.03 software (Agilent Technologies, Santa Clara, CA). The average and standard deviations were reported.

Results and Discussion

Fatty acids identified by GC analysis of SDASO included SDA (24.8%) and γ-linolenic acid (GLA) (7.2%), both of which are not present in conventional soybean oil. The 12.5% palmitic and 11% ALA found in SDASO was marginally higher than that of conventional soybean oil. The introduction of Δ-6 and Δ-15 desaturase genes promoted the conversion of linoleic acid to GLA, increased ALA levels and promoted metabolic flux to SDA (Froman et al., 2011). Oleic (14.9%) and linoleic (24.6%) acid contents were lower than those of conventional soybean oil. A 62% SDA-FFA fraction was obtained by low temperature crystallization of SDASO chemical hydrolyzate (roughly 1.5 times higher than that of the feed mixture). The ALA (5.5%), linoleic (8.3%), oleic (4.5%), palmitic (0.5%) and stearic (0.1%) acid contents of this fraction were lower than those of SDASO. The GLA content (17.5%) was higher in the concentrated FFA fraction than in SDASO because GLA co-precipitates with SDA. The fatty acid composition of the SDA-FFA fraction produced by low temperature crystallization was similar to the high SDA FFA concentrate obtained by (Vázquez & Akoh, 2011).

Figure 3.2 shows changes in lipid composition of SDASO hydrolyzate with time. The reaction was catalyzed by lipase AY 30. Major hydrolysis products were FFAs and glycerol. FFA levels rose rapidly within the first 6 h of incubation from an initial < 0.1 to 55%. Subsequent increments in FFA levels were less drastic as hydrolysis approached

equilibrium. Partial acylglycerols were formed at lower levels than FFAs. After 4 h of hydrolysis, MAG and DAG levels rose to a maximum of 4.8 and 12.6%, respectively and thereafter dropped slightly before attaining equilibrium. Within the first 6 h of hydrolysis, triacylglycerol levels dropped from ~ 99 to 35.6% and tended towards equilibrium. Table 3.2 shows changes in SDA content of acylglycerol species and free fatty acid fractions isolated from the SDASO hydrolyzate over a time course of 48 h. The SDA contents of TAGs, DAGs and MAGs isolated from the hydrolyzate were consistently higher than that of the original oil. The GLA contents of TAGs, DAGs and MAGs were also higher than that of the original oil (data not shown). SDA content of total acylglycerols increased as hydrolysis progressed, but reached a peak value of 51% after 24 h (degree of hydrolysis ~ 67%). Therefore, 24 h of lipase AY 30-catalyzed hydrolysis was considered as optimum incubation time for producing an SDA-enriched total acylglycerol fraction from SDASO. The TAG content of this fraction was ~ 82% by weight while DAG and MAGs made up ~15 and 3%, respectively. The SDA content of FFAs released during enzymatic hydrolysis was consistently lower than the SDA content of acylglycerols, hence the good recoveries of SDA (Table 3.2). However, FFAs liberated during enzymatic hydrolysis consistently contained higher amounts of ALA, palmitic, oleic, and linoleic acids than was retained in the acylglycerol fraction (data not shown). These observations suggest that ALA, palmitic, oleic, and linoleic acids were preferentially hydrolyzed from SDASO by lipase AY 30 over SDA and GLA. This pattern was also observed by Kleiner et al. (2012b) and thus guided our choice of non-immobilized lipase AY 30 for the lipase-mediated SDA enrichment step. We made no further attempt to immobilize the *C. rugosa* lipase since this may alter the catalytic behavior of the lipase. Several investigators have used lipase from *C. rugosa* for enrichment of DHA and EPA in fish oils based on

selective hydrolysis (Kahveci & Xu, 2011; McNeill, Ackman, & Moore, 1996; Sun, Pigott, & Herwig, 2002). However, a major limitation with this approach is that product yield decreases with increasing levels of PUFA enrichment. So, for the process to be feasible, a viable compromise must be made between the desired level of enrichment and production yield. In other words, hydrolysis should not be allowed to proceed to a point where yield loss may not be justified by the gain in PUFA enrichment. However, the enriched product can be sold at a higher price.

Bottino, Vandenburg, and Reiser (1967) theorized that the resistance of certain long chain PUFA to enzymatic hydrolysis could be attributed to the inhibitory effect of a double bond located close to the carboxylic group or to the presence of multiple *cis* double bonds which force the terminal methyl group of the fatty acid to lie close to the ester bond and sterically hinder lipase action. In addition, Jachmanian, Schulte, and Mukherjee (1996) observed that *C. rugosa* lipase strongly discriminates against unsaturated fatty acids having the first double from the carboxyl end at an even numbered carbon. The presence of a double bond on C-6 of SDA and GLA distinguishes these fatty acids from other 18 carbon unsaturated fatty acids in SDASO and may therefore serve as a structural basis for their resistance to *C. rugosa* lipase-catalyzed hydrolysis. Although the hypothesis put forth by Bottino et al. (1967) explains the resistance of certain PUFAs to specific lipases, it is, however, limited when the diversity in fatty acid selectivity of lipases is considered. For example, cod gut lipases which preferentially hydrolyze PUFA over shorter chain fatty acids (Lie & Lambertsen, 1985) offer an interesting exception to the hypothesis.

The 51% SDA acylglycerol (SDA-GLY) product of lipase AY 30-catalysed hydrolysis in Table 3.2 was further enriched in SDA by RML and CCL-catalyzed

acidolysis. The 62% SDA-FFA mixture obtained by low temperature crystallization was used as acyl group donor. The use of this SDA rich acyl group donor was expected to increase the likelihood of SDA incorporation into acylglycerols during enzymatic acidolysis. The effect of substrate molar ratio (moles of SDA-FFA to SDA-GLY), incubation temperature, and time, on SDA incorporation by RML and CCL were mathematically modeled using response surface methodology.

Within the limits of factor level combinations tested, enzymatic acidolysis brought about the formation of SDA concentrates (SDACOs) with SDA levels that ranged from 54–60% (Table 3.1). Regression analysis was used to obtain the best fitting quadratic model that explained the relationship between process factors and SDA incorporation. ANOVA (Table 3.3) was used to determine whether reliable predictions or conclusions could be made based on the model. For CCL-catalyzed acidolysis, all first order model terms were statistically significant ($p < 0.05$) while for RML, only S_r and T were significant. Furthermore, all second order and interaction terms except $S_r \times t$ and $T \times t$ were not significant ($p > 0.05$) for RML. For CCL, all second order model terms where statistically significant whereas interaction terms were not significant ($p > 0.05$). Results of ANOVA (Table 3.3) indicated that both models were statistically adequate for predicting SDA incorporation. The F_{model} values (20.06 and 111.91 for RML and CCL, respectively) were much higher than the $F_{24, 9\ (table)}$ value (2.90) ($\alpha = 0.05$). Both models showed no lack of fit ($p > 0.05$). The correlation coefficient (R^2) which is the fraction of the variation of response accounted for by the model was 0.883 for RML and 0.977 for CCL. The R^2 adjusted for the number of independent variables in each model (R^2_{adj}) was 0.839 and 0.968 for RML and CCL. Q^2 which is the fraction of the variation of response that can be predicted by the model was 0.761 for RML and 0.977 for CCL. There was a

good correlation between predicted and actual responses. The R^2, R^2_{adj}, Q^2 values as well as regression *p* values < 0.001 and lack of fit *p* values > 0.05 all indicated that both models were well fitted to experimental data. Therefore, the model equation for the SDA incorporation by RML-catalyzed acidolysis could be expressed as:

$$Y_{RML} = 58.61 + 0.55Sr + 0.81T - 0.11t - 0.19Sr^2 - 0.07T^2 - 0.20t^2 - 0.12Sr \times T + 0.35Sr \times t - 0.65T \times t \quad [4]$$

While the model equation for the SDA incorporation by CCL-catalyzed acidolysis could be expressed as:

$$Y_{CCL} = 59.08 + 0.55Sr - 0.76T + 0.77t - 0.42Sr^2 - 0.37T^2 - 0.22t^2 + 0.05SrT - 0.01Sr \times t + 0.06T \times t \quad [5]$$

Where Y_{RML} = incorporation of SDA (mol%) by RML-catalyzed acidolysis, Y_{CCL} = incorporation SDA (mol%) by CCL-catalyzed acidolysis, Sr = substrate molar ratio, T = incubation temperature (^oC), and t = incubation time (h).

Prediction plots for SDA incorporation were generated based on the quadratic models to illustrate the main effects of process factors on SDA incorporation (Figure 3.3). Each plot shows predicted changes in SDA incorporation when a single factor was varied from its low to high level while the other two factors in the design were set on their midpoint. For both enzymes, SDA incorporation increased with increasing substrate molar ratio (Figures 3.3A and 3.2B). It can also be seen from Figure 3.3C that SDA incorporation by RML increased with increasing incubation temperature while for CCL, SDA incorporation decreased with increasing temperature (Figure 3.3D). Based on the Arrhenius equation, the rate of a chemical reaction is temperature dependent provided that diffusion of substrate or enzyme is not restricted. So in RML-catalyzed acidolysis, elevated temperature increased the rate of productive collisions between substrates and

enzyme hence raising the chances for SDA incorporation. However, in CCL-catalyzed acidolysis the rate of enzyme denaturation may have surpassed that of productive collisions, leading to decreased SDA incorporation. Therefore, RML maintained operational stability at high temperature while CCL did not. Figure 3.3E shows that SDA incorporation by RML increased only marginally with time until 28 h and then decreased thereafter, while Figure 3.3F shows that SDA incorporation by CCL increased with time. The most obvious difference between both enzymes was that RML required high incubation temperature and short incubation time to maximize SDA incorporation, whereas CCL required low incubation temperature and long incubation time to achieve the same result.

Using the optimizer function of MODDE 5.0 software, reaction conditions under which SDA incorporation could be maximized were generated. Figures 3.4A and B are contour plots for SDA incorporation by RML and CCL, respectively. In each plot, temperature was set constant at the optimal value. For RML, 60% SDA incorporation was predicted at S_r = 4.8, T = 65 ^oC and t = 8 h, while for CCL, 60.2% SDA incorporation was predicted at S_r = 5.0, T = 43 ^oC and t = 48 h. Lipase-catalyzed acidolysis was carried out in gram scale under these optimal conditions to verify the models. RML-catalyzed acidolysis led to the production of a 59.98% SDA concentrate (RM-SDACO) while that of CCL produced a 58.98% SDA concentrate (CC-SDACO). These results were satisfactorily close to those predicted by each model. HPLC analysis of acylglycerol acylglycerol distribution in SDA concentrates revealed higher amounts of TAGs (≥ 96% by weight) and lower amounts DAGs (2%) and MAGs (< 1%) compared to the 51% SDA acylglycerol substrate (SDA-GLY). This indicates that partial acylglycerols (MAG and DAG) in the substrate were esterified *in situ* during enzymatic

acidolysis. This reduction in DAG and MAG content was advantageous since partial acylglycerols are known to act as prooxidants in soybean oil (Mistry & Min, 1988).

The fatty acid profile of SDASO, RM-SDACO and CC-SDACO are compared in Table 3.4. The composition and positional distribution of fatty acids in SDASO was substantially modified by the chemoenzymatic process. The total SDA content of both concentrates were approximately 2.4 times that of the original SDASO. SDA enrichment was concurrent with a significant increase in GLA and decreases in ALA, palmitic, stearic, oleic, and linoleic acid contents. The total SDA contents of RM-SDACO and CC-SDACO were not different at the 5% level of significance but the SDA content at the *sn*-2 position was significantly higher in CC-SDACO (52.68%) than in RM-SDACO (46.67%). This may be attributed to differences in positional specificity of the lipases. CCL being a non-specific lipase was able to incorporate more SDA at the *sn*-2 position compared to the *sn*-1,3 specific RML. Increased SDA content at the *sn*-2 position of SDACOs compared to SDASO suggests increased absorption efficiency of this omega-3 PUFA. Furthermore, the ratio of omega-3 to omega-6 fatty acids increased from 1.1 in SDASO to ~2.3 in SDACOs.

Conclusion

Optimized enzymatic acidolysis led to the production of two SDACOs with similar total SDA contents. The preferred enzymatic route would be that which proves cost-effective. First, RML offers the cost saving benefits associated with the use of immobilized enzymes which include easy separation of the enzyme from product and reuse of the enzyme (Tischer & Wedekind, 1999). Second, the optimal incubation time for RML was 6 times shorter than that of CCL which translates into lower energy requirement. Third, as shown in Table 3.5, the 61.24% SDA-FFA fraction recovered

from RML-catalyzed acidolysis should be better suited for recycling through the acidolysis step than the 55.49% SDA-FFA fraction recovered from CCL-catalyzed acidolysis. Therefore, RML seemed more advantageous than CCL from a commercial feasibility standpoint. However it is possible that immobilized CCL will perform as good as RML in terms of product yield and cost effectiveness.

The chemoenzymatic scheme outlined in Figure 3.1 led to the production of ~60% SDA concentrates which, compared to the original SDASO, were lower in saturated and monounsaturated fatty acid levels but contained higher levels of omega-3 PUFA. The process involved chemical hydrolysis, low temperature crystallization, and enzymatic hydrolysis and acidolysis. In previous studies (Kleiner et al., 2012a, 2012b; Vázquez & Akoh, 2011), these unit operations were studied independently while the enzymatic steps were performed on a small scale. In the present study, however, these operations were successfully combined for gram scale production of SDA concentrates from SDASO. Evidently, the SDA omega-3 concentrates produced constitute value-added products derived from SDASO which may be used for formulating nutritionally functional foods with the 'heart healthy' claim. This omega-3 concentrate is desirable because it enables nutritionally significant amounts of omega-3 PUFAs to be conveniently ingested with minimal total lipid intake.

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Table 3.1 Experimental factors and observed responses in central composite face design

Enzyme	Exp No	Factors			Response
		Sr [a]	T	t	(SDA Inc)[b]
Lipozyme	1	3	40	8	56.30 ± 0.00
RM IM	2	6	40	8	57.27 ± 0.00
lipase	3	3	65	8	60.02 ± 0.14
	4	6	65	8	59.58 ± 0.13
	5	3	40	48	57.06 ± 0.04
	6	6	40	48	58.51 ± 0.05
	7	3	65	48	57.26 ± 0.27
	8	6	65	48	59.14 ± 0.00
	9	3	52.5	28	57.58 ± 0.09
	10	6	52.5	28	59.23 ± 0.07
	11	4.5	40	28	57.89 ± 0.13
	12	4.5	65	28	59.16 ± 0.08
	13	4.5	52.5	8	58.33 ± 1.41
	14	4.5	52.5	48	58.47 ± 0.12
	15	4.5	52.5	28	58.87 ± 0.10
	16	4.5	52.5	28	58.49 ± 0.22
	17	4.5	52.5	28	58.50 ± 0.09
Lipomod	1	3	40	8	56.82 ± 0.22
34P-L034P	2	6	40	8	58.20 ± 0.11
lipase	3	3	55	8	54.66 ± 0.11
	4	6	55	8	56.12 ± 0.04
	5	3	40	48	58.75 ± 0.84
	6	6	40	48	59.78 ± 0.10
	7	3	55	48	56.71 ± 0.11
	8	6	55	48	58.38 ± 0.05
	9	3	47.5	28	57.39 ± 0.02
	10	6	47.5	28	58.92 ± 0.07
	11	4.5	40	28	59.31 ± 0.05
	12	4.5	55	28	57.28 ± 0.05
	13	4.5	47.5	8	57.71 ± 0.17
	14	4.5	47.5	48	59.69 ± 0.00
	15	4.5	47.5	28	59.22 ± 0.15
	16	4.5	47.5	28	59.32 ± 0.09
	17	4.5	47.5	28	59.24 ± 0.16

[a] Abbreviations: Exp No, experiment numbers; Sr, substrate molar ratio if SDA-FFA to SDA-GLY (mol/mol); T, incubation temperature (^{o}C); t, incubation time (h); SDA Inc, incorporation of stearidonic acid (mol%).

[b] Each value is a mean of duplicates ± standard deviation

Table 3.2 SDA content of acylglycerols and free fatty acids obtained by lipase AY 30-catalyzed hydrolysis of SDA soybean oil (SDASO) at various incubation times

			SDA content (mol%)[a] of SDASO hydrolyzate (SDASO-H)					
Time (h)	Hydrolysis (%)	Acid value[b]	TAG	DAG	MAG	SDA-GLY[c]	FFA	SDA recovery (%)[d]
4	48.3	94	43.10 ± 2.00	51.51 ± 0.15	60.76 ± 0.77	45.87 ± 0.11	6.42 ± 0.21	96.6
6	56.6	110	44.94 ± 0.51	51.83 ± 0.93	52.87 ± 1.93	46.57 ± 0.09	6.48 ± 0.78	82.7
8	57.6	112	45.60 ± 1.46	52.37 ± 0.70	52.64 ± 0.59	47.34 ± 0.40	8.00 ± 0.57	81.7
10	59.7	116	45.93 ± 0.99	54.22 ± 0.39	59.65 ± 2.81	47.52 ± 1.06	10.38 ± 0.06	78.2
12	59.2	115	45.72 ± 0.33	54.93 ± 0.40	59.87 ± 0.71	49.23 ± 0.77	11.81 ± 0.13	81.6
24	67.4	131	47.09 ± 1.01	55.59 ± 0.33	55.87 ± 0.83	51.08 ± 0.18	15.16 ± 0.71	67.6
48	76.7	149	41.12 ± 0.90	46.31 ± 0.87	49.08 ± 3.02	46.74 ± 0.28	18.48 ± 1.20	48.3

[a] Each value is a mean of triplicates ± standard deviation

[b] Acid and saponification values of the original SDASO was 0.6 and 194, respectively.

[c] Total acylglycerol fraction composed of TAG, DAG and MAG

[d] SDA recovery (%) = $[(\text{SDA content}_{(\text{acylglycerol})}) \times (100 - \text{hydrolysis (\%)})] \div \text{SDA content}_{(\text{original oil})}$

Table 3.3 ANOVA table for incorporation of SDA by enzymatic acidolysis [a]

Enzyme	SDA Inc	DF	SS	MS (variance)	*F*-value	*P*-value	SD
Lipozyme RM IM lipase	Total	34	115725.586	3403.694			
	Constant	1	115691.930	115691.930			
	Total corrected	33	33.656	1.020			1.010
	Regression	9	29.707	3.301	20.057	0.000	1.817
	Residual	24	3.950	0.165			0.406
	Lack of fit	5	1.541	0.308	2.432	0.073	0.555
	Pure error	19	2.408	0.127			1.817
	N = 34		R^2= 0.883	R^2 Adj = 0.839			
	DF = 24		Q^2= 0.761	RSD = 0.403			
Lipomod 34P-L034P lipase	Total	34	114788.883	3376.144			
	Constant	1	114722.633	114722.633			
	Total corrected	33	66.250	2.008			1.417
	Regression	9	64.708	7.190	111.911	0.000	2.681
	Residual	24	1.542	0.064			0.253
	Lack of fit	5	0.637	0.127	2.674	0.054	0.357
	Pure error	19	0.905	0.048			0.218
	N = 34		R^2 = 0.977	R^2 Adj = 0.968			
	DF = 24		Q^2= 0.926	RSD = 0.2535			

[a]Abbreviations: SDA, stearidonic acid; DF, degree of freedom; SS, sum of squares; MS, mean square; RSD, relative standard deviation; N, number of experiments; SD, standard deviation; R^2, correlation coefficient; R^2 Adj, correlation coefficient adjusted for the number of independent factors in the model; Q^2, prediction power of the model.

Table 3.4 Fatty acid composition of SDA soybean oil and SDA concentrates produced by lipase-catalyzed acidolysis (mol%)[a]

Fatty acid	SDA soybean oil [b]			SDA concentrates					
				RM-SDACO[c]			CC-SDACO [d]		
	Total	*sn*-2	*sn*-1,3 [e]	Total	*sn*-2	*sn*-1,3	Total	*sn*-2	*sn*-1,3
C16:0	12.72 ± 0.02 a	3.27± 0.18 d	17.45 ± 0.05 g	2.15 ± 0.07 b	5.94 ± 0.33 e	0.81 ± 0.00 h	1.97 ± 0.00 c	4.84 ± 0.57 f	0.54 ± 0.03 i
C18:0	4.10 ± 0.00 a	1.23 ± 0.09d	5.54 ± 0.01g	0.73 ± 0.01 b	1.74 ± 0.20 e	0.23 ± 0.04 f	0.77 ± 0.01 c	1.99 ± 0.26 e	0.16 ± 0.01 g
C18:1n-9	15.12 ± 0.01 a	13.27 ± 0.24 d	16.05 ± 0.01 g	5.52 ± 0.02 b	11.53 ± 0.21 e	2.52 ± 0.01 h	5.20 ± 0.02 c	6.33 ± 0.04 f	4.64 ± 0.01 i
C18:2n-6	24.90 ± 0.01a	29.63 ± 0.52 d	22.54 ± 0.14 f	10.13 ± 0.06 b	11.60 ± 0.25 e	9.40 ± 0.04 g	10.34 ± 0.02 c	12.42 ± 0.70 e	9.30 ± 0.03 h
C18:3n-6	7.17 ± 0.00 a	10.43 ± 0.17d	5.54 ± 0.07 f	17.54 ± 0.28 b	16.95 ± 0.40 g	17.84 ± 0.20 bc	17.26 ± 0.00 b	15.67 ± 0.05 e	18.06 ± 0.04 c
C18:3n-3	11.12 ± 0.02 a	7.36 ± 0.07 d	13.00 ± 0.03 f	3.95 ± 1.66 b	5.57 ± 0.10 c	3.14 ± 0.13 g	5.48 ± 0.01 c	6.07 ± 0.26 e	5.19 ± 0.14 h
C18:4n-3	24.84 ± 0.03 a	32.61 ± 0.82 c	20.96 ± 0.12 f	59.98 ± 1.09 b	46.67 ± 1.13 d	66.64 ± 0.31 g	58.98 ± 0.01 b	52.68 ± 0.11 e	62.13 ± 0.08 h
∑SFA	16.82 ± 0.01 a			2.88 ± 0.05 b			2.74 ± 0.03 c		
∑MUFA	15.12 ± 0.01 a			5.52 ± 0.03 b			5.20 ± 0.02 c		
∑PUFA	68.03 ± 0.04 a			91.60 ± 1.02 b			92.06 ± 0.02 b		
n-3/n-6	1.12			2.31			2.34		

[a] Each value is a mean of triplicates ± standard deviation

[b] Contained trace amounts of C14:0, C16:1, C17:0, C20:0, C20:1n-9 and C22:0

[c] SDA concentrates produced by Lipozyme RM IM lipase at substrate molar ratio of 4.8, incubation temperature of 65 ^{o}C and 8 h incubation time

[d] SDA concentrates produced by Lipomod 34P-L034P lipase at substrate molar ratio of 5.0, incubation temperature of 43 ^{o}C and 48 h incubation time

[e] *sn*-1,3 (mol%) = [3 × total (mol%) −*sn*-2 (mol%)]/2

Values with the same letter in each row are not significantly different ($p > 0.05$)

Table 3.5 Fatty acid composition of initial SDA-enriched free fatty acids (FFA) and FFA recovered after production of SDA concentrates (mol%) [a]

Fatty acid	SDA-FFA [b]	RM-FFA [c]	CC-FFA [d]
C16:0	0.48 ± 0.14 a	1.48 ± 0.04 b	2.29 ± 0.01 c
C18:0	0.10 ± 0.00 a	0.56 ± 0.02 b	1.11 ± 0.01 c
C18:1n-9	4.47 ± 0.03 a	4.20 ± 0.05 b	6.16 ± 0.10 c
C18:2n-6	8.27 ± 0.39 a	9.85 ± 0.01 b	11.63 ± 0.05 c
C18:3n-6	17.73 ± 0.35 a	17.11 ± 0.11 b	17.71 ± 0.17 c
C18:3n-3	5.55 ± 0.31 a	5.57 ± 0.01a	5.61 ± 0.04 a
C18:4n-3	63.40 ± 0.38 a	61.24 ± 0.03 b	55.49 ± 0.39 c
∑SFA	0.58 ± 0.01 a	2.04 ± 0.04 b	3.4 ± 0.03 c
∑MUFA	4.47 ± 0.02 a	4.20 ± 0.06 b	6.16 ± 0.06 c
∑PUFA	94.95 ± 0.25a	93.77 ± 0.23b	90.44 ± 0.21c

[a] Each value is a mean of triplicates ± standard deviation
[b] Liquid fraction of low temperature crystallization product of SDA soybean oil free fatty acids
[c] Free fatty acids recovered from Lipozyme RM IM lipase-catalyzed acidolysis
[d] Free fatty acids recovered from Lipomod 34P-L034P lipase-catalyzed acidolysis
Values with the same letter in each row are not significantly different ($p > 0.05$)

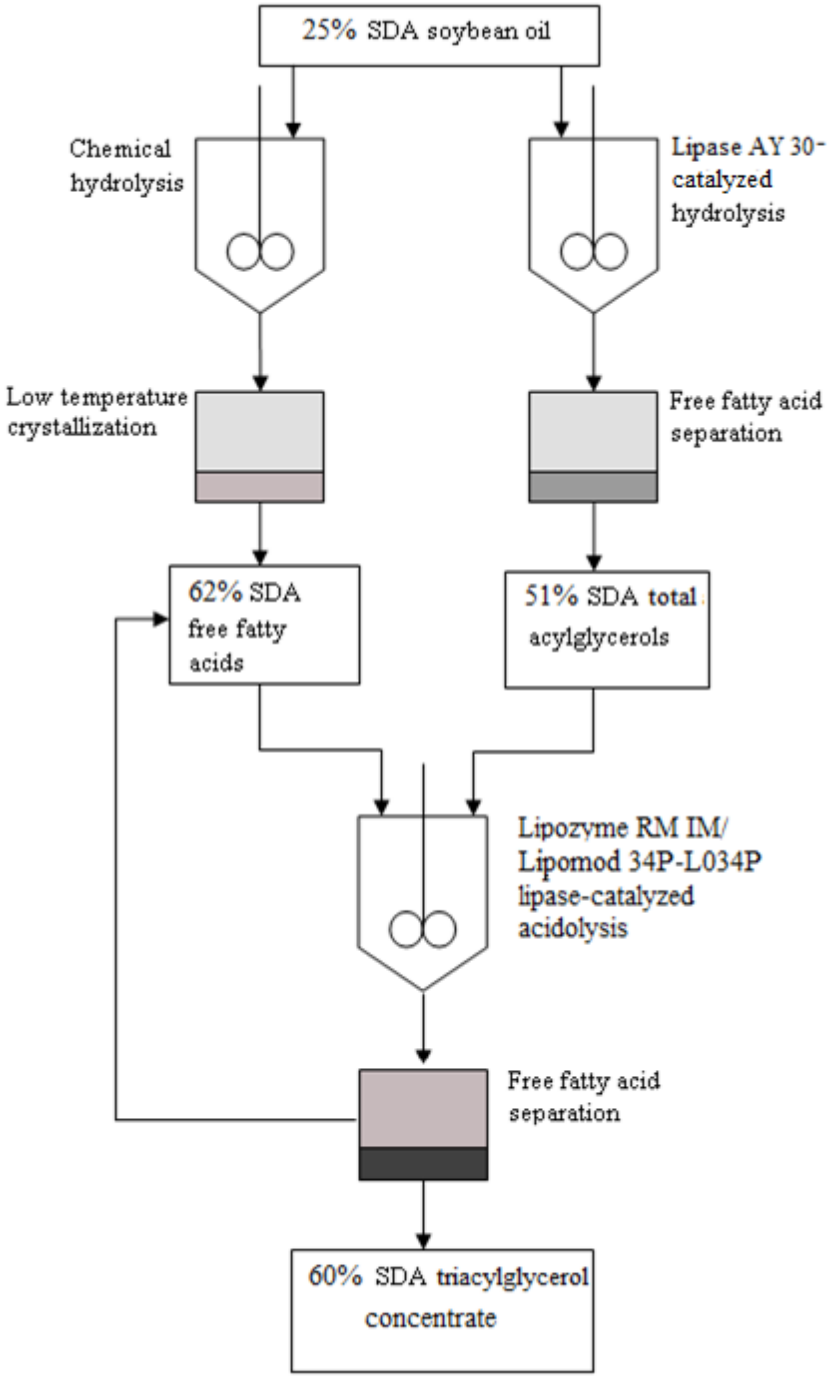

Figure 3.1 Chemoenzymatic process for producing SDA concentrate from SDA soybean oil

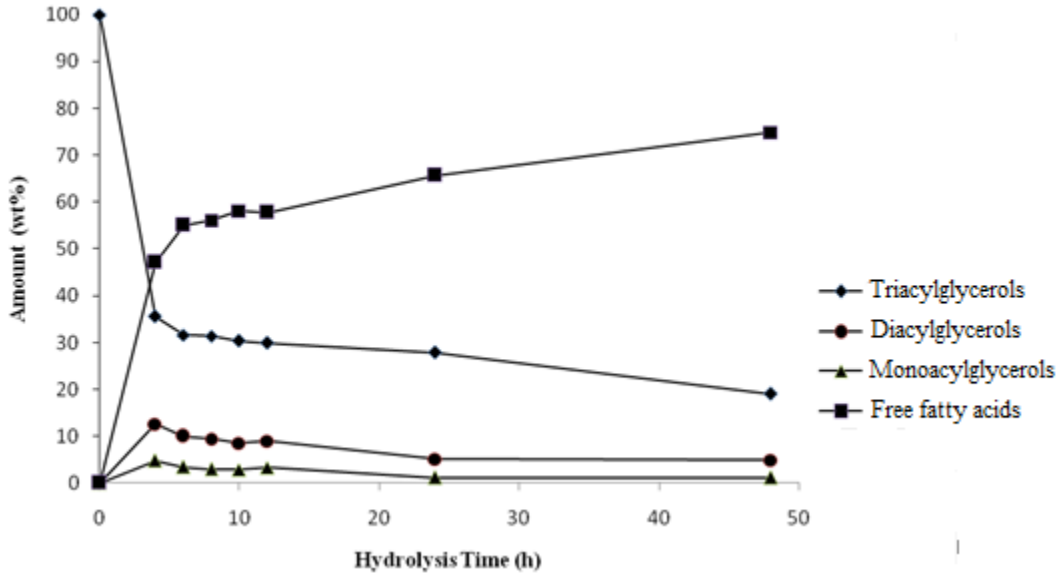

Figure 3.2 Time course of hydrolysis of SDA soybean oil by lipase AY 30

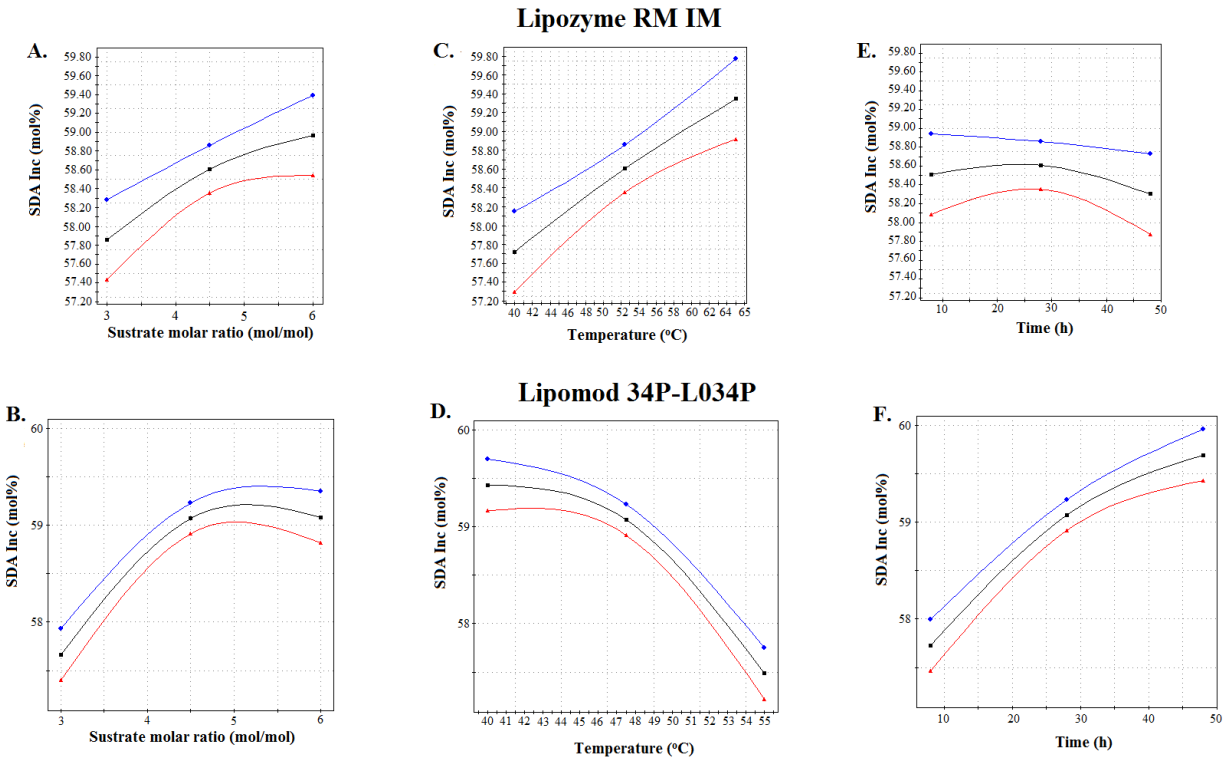

Figure 3.3 Response prediction plots showing main effects of substrate molar ratio, incubation temperature, and time on Lipozyme RM IM (**A, C** and **E**, respectively) and Lipomod 34P-L034P lipase (**B, D** and **F**, respectively) catalyzed acidolysis.

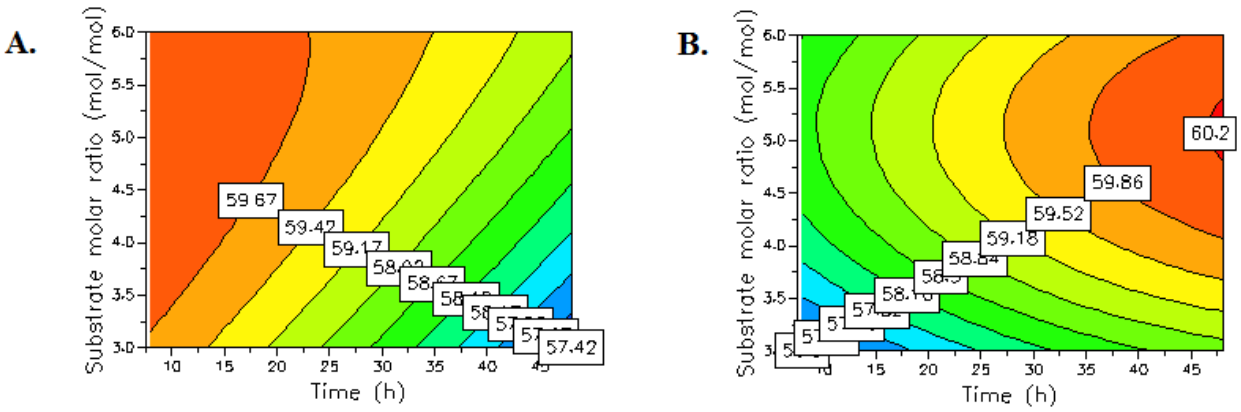

Figure 3.4 Contour plots predicting incorporation of SDA into acylglycerols at optimal incubation temperatures for **A**, Lipozyme RM IM (65 ^{o}C), and **B**, Lipomod 34P-L034P lipase (43 ^{o}C).

CHAPTER 4

MODIFICATION OF STEARIDONIC ACID SOYBEAN OIL BY IMMOBILIZED RHIZOMUCOR MIEHEI LIPASE TO INCORPORATE CAPRYLIC ACID[1]

[1] Ifeduba, E. A., and Akoh, C. C. 2014. *Journal of the American Oil Chemists' Society, 91*(6), 953-965. Reprinted here with permission from Springer.

Abstract

Immobilized *sn*-1,3 specific *Rhizomucor miehei* lipase (RML) was used to catalyze the incorporation of caprylic acid (C8:0) into high stearidonic acid (SDA, C18:4ω3) soybean oil (SDASO) to form structured lipids (SLs). Effects of the type of biocatalyst (Celite-, octyl-sepharose-, and Duolite-immobilized RML) and incubation temperature (30, 40, 50, and 60 °C) on acidolysis and acyl migration were studied. Celite-immobilized RML (C-RML) at 50 °C maximized C8:0 incorporation and minimized acyl migration. The optimal substrate molar ratio (C8:0 to SDASO), incubation time, and enzyme load for SL synthesis by C-RML at 50 °C was determined by response surface methodology to be 6:1, 24 h, and 20% weight of substrates, respectively. This optimum treatment was scaled-up in hexane or solvent-free reaction media using SDASO or an SDA-enriched concentrate as initial triacylglycerol substrates. This yielded various SLs with C8:0 contents ranging from 17.0 - 32.5 mol% and SDA contents ranging from 20.6 - 42.3 mol%. When digested, these SLs may deliver C8:0 for quick energy and SDA for heart health making them potentially valuable for medical and nutraceutical applications.

Keywords: structured lipids; stearidonic acid soybean oil; response surface methodology; immobilization; *Rhizomucor miehei* lipase; nutraceutical; acidolysis; acyl migration

Introduction

Structured lipids (SLs) are tailor-made fats and oils in which the fatty acid composition and/or distribution has been chemically, enzymatically or genetically modified to suit specific nutritional and functional applications (Gunstone, 1998; Osborn & Akoh, 2002). One such application is in parenteral and enteral nutrition which involves the use of lipid emulsions based on medium-chain triacylglycerol (MCT) and long-chain triacylglycerol (LCT) mixtures to meet the energy and essential fatty acid requirements of preterm infants and patients suffering from pancreatic insufficiency or recovering from surgery (Calder, 2009; Lehner et al., 2006). The efficacy of SLs designed to contain both medium-chain fatty acids (MCFA) and long-chain polyunsaturated fatty acids (LC-PUFA) on the same triacylglycerol molecule and simultaneously deliver MCFA and LC-PUFA through parenteral formulations has also been explored (Akoh & Kim, 2008; Staarup & Høy, 2000; Zhu & Li, 2013). SLs with the MLM stereochemical configuration, that is, MCFA at the *sn*-1 and 3 positions and LC-PUFA at the *sn*-2 position of the glycerol backbone, are metabolized differently from normal fats and oils. During digestion, the MCFAs are rapidly hydrolyzed from SLs by the *sn*-1,3 specific pancreatic lipase and absorbed through the portal vein to the liver where they are oxidized for energy. The monoacylglycerols (MAG) with LC-PUFA at the *sn*-2 position are then re-esterified into triacylglycerols (TAG), incorporated into chylomicrons, and delivered through the lymphatic system to peripheral tissues (Akoh & Kim, 2008) where LC-PUFA serve as structural constituents of cell membranes and biological mediators for various physiological functions.

Results of clinical studies and meta-analyses show that omega-3 fish-oil based parenteral emulsions may help alleviate clinical conditions characterized by hyper-inflammation such as sepsis, pancreatitis, and acute respiratory distress (Calder, 2013; de Meijer, Gura, Le, Meisel, & Puder, 2009; Pradelli, Mayer, Muscaritoli, & Heller, 2012; X. Wang, Li, Li, & Li, 2008). This may be due to the anti-inflammatory effects of eicosapantaenoic (EPA, 20:5ω3) and docosahexaenoic (DHA, 22:6ω3) acids, particularly by the reduction of tissue concentrations of arachidonic acid (ARA, 20:4ω6) and inflammatory eicosanoids. Furthermore, evidence from observational, clinical, and experimental studies indicates that dietary intake of these omega-3 LC-PUFAs is beneficial for the prevention and management of cardiovascular disease. Stearidonic acid soybean oil (SDASO) has recently been developed as a renewable source of the omega-3 PUFA known as stearidonic acid (SDA, C18:4ω3). The nutritional benefit of consuming SDA in the diet has been demonstrated in clinical studies by its conversion *in vivo* to EPA which results in elevation of the Omega-3 Index and reduction in the risk of death from coronary heart disease (Harris et al., 2008; James, Ursin, & Cleland, 2003; Lemke et al., 2010). Therefore, SDASO has been recommended as a sustainable plant source of omega-3 PUFA for heart-healthy food applications (Decker, Akoh, & Wilkes, 2012).

The incorporation of MCFAs into fish oil by transesterification is known to yield SLs which combine the rapid assimilation and energy release properties of MCFAs with the therapeutic value of omega-3 PUFA (Chambrier, Lauverjat, & Bouletreau, 2006; Christensen et al., 1998; K. T. Lee, Akoh, & Dawe, 1999). Several reports have investigated lipase catalyzed-synthesis of these SL (Akoh & Kim, 2008; Camacho et al., 2007; Chen, Zhang, Cheong, Tan, & Xu, 2012; K.-T. Lee & Akoh, 1998; Osborn &

Akoh, 2002; Rodríguez et al., 2012). The aforementioned effect may be obtainable in SL produced by the incorporation of MFCAs into the plant-based SDASO. However, the enzymatic synthesis of SL by incorporation of caprylic acid into SDASO has never been reported in the literature. The purpose of this study was to synthesize SLs by incorporating caprylic acid into SDASO using immobilized *sn*-1,3-specific *Rhizomucor miehei* lipase (RML). Reaction conditions were optimized to maximize caprylic acid incorporation and minimize acyl-migration to yield SL with the desired stereochemical configuration, MLM, in this case. First, the effects of three types of immobilized enzymes, namely; Celite-, octyl-sepharose-, and Duolite-immobilized RML, on caprylic acid incorporation and acyl migration were studied at different incubation temperatures (30, 40, 50, and 60 °C). RML was non-covalently immobilized onto Celite and octyl-sepharose and compared with Lipozyme RM IM (RML commercially immobilized onto Duolite). Enzyme immobilization is known to be driven by different types of interactions that depend on the surface properties of the enzyme and support (Hanefeld, Gardossi, & Magner, 2009). The immobilization of RML onto Celite, octyl-sepharose, and Duolite exemplified different types of non-covalent binding interactions, namely: hydrogen bonding, hydrophobic and ionic interactions, respectively. The combination of biocatalyst and incubation temperature that gave the best response was further optimized by response surface methodology (RSM) with respect to substrate molar ratio, incubation time, and enzyme load. Finally, SL production was scaled-up in hexane or solvent-free media using SDASO or SDA-enriched concentrate (SDACO) as substrate.

Materials and Methods

Materials

Stearidonic acid soybean oil (SDASO) was generously donated by Monsanto Co. (St. Louis, MO). Caprylic acid (C8:0, 99% pure), Celite 545, octyl-sepaharose CL-4B, Supelco 37 Component FAME mix, 2-oleoylglycerol, and porcine pancreatic lipase were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Palatase and Lipozyme RM IM with declared activities of 22118.30 LU/g and 5 - 6 BAUN/g respectively, were from Novozymes North America Inc. (Franklinton, NC). One lipase unit (LU) is defined as the amount of enzyme that releases 1 mmol of butyric acid per minute at 30 °C and pH 7.0 from tributyrin emulsified with Gum Arabic while Batch Acidolysis Units Novo (BAUN) is a measure of the initial rate of decanoic acid incorporation into high oleic sunflower oil at 70 °C. Palatase is the free form of RML while Lipozyme RM IM is the same lipase immobilized onto Duolite A568, a weak anion exchange resin. TAG reference standard mixture was from Nu-check Prep, Inc. (Elysian, MN). Methyl stearidonate was purchased from Cayman Chemical Company (Ann Arbor, MI). Triolein and nonadecanoic acid were from TCI America (Portland, OR). Bicinchoninic acid (BCA) protein assay kit was from Thermo Fisher Scientific (Rockford, IL). All reagents and solvents were of analytical or HPLC grade and purchased from either Fisher Scientific (Norcross, GA) or J. T. Baker Chemical Co. (Philipsburg, NJ).

Lipase Immobilization

RML was immobilized onto Celite powder by deposition and onto octyl-sepharose by adsorption. Celite-immobilized RML (C-RML) was prepared by precipitation of the soluble lipase in the presence of cold acetone and deposition of the

precipitate onto Celite powder. Deposition is facilitated by the formation of hydrogen bonds between hydrophilic groups on the surface of RML and the support while adsorption of RML onto octyl-sepharose is largely driven by entropic changes and van der Waals forces that result from interactions between hydrophobic regions of the lipase and on the carrier (Hanefeld et al., 2009). Ten milliliters of Palatase (protein concentration ~50 mg/mL) was diluted with 10 mL of sodium phosphate buffer (pH 7.0, 20 mM). The diluted lipase solution (20 mL) was added to 1 g of Celite and stirred at 4 °C for 2 h. Next, 5 mL of cold acetone (-20 °C) was added and the mixture was stirred for 1 h. The mixture was then filtered and the residue (C-RML) washed with 10 mL phosphate buffer (pH 7.0, 20 mM). C-RML was collected and dried over silica gel in vacuum for 24 h at 25 °C. The filtrate and wash fractions were combined and the protein concentration determined by BCA protein assay. The protein content of C-RML was estimated from the difference between the amount of protein in the diluted lipase solution and combined filtrate-wash fraction. Octyl-sepharose gel (50 mL) was first washed with 500 mL of distilled water to remove ethanol from the medium. The carrier was decanted, equilibrated in 15 mL phosphate buffer (pH 7.0, 20 mM), and filtered. The diluted lipase solution (20 mL) was added to the damp octyl-sepharose resin and stirred continuously for 3 h at 25 °C. Octyl-sepharose-immobilized RML (O-RML) was separated from the mixture by centrifugation at 2000 × g for 5 min. Next, the pellet (O-RML) was washed with 10 mL of the same phosphate buffer, filtered, and dried over silica gel in vacuum for 24 h at 25 °C. The supernatant and wash fractions were combined and the protein concentration determined by BCA protein assay. The protein content of O-RML was

estimated from the difference between the amount of protein in the diluted lipase solution and combined supernatant-wash fraction.

Protein and Water Activity Measurement

Colorimetric detection and quantitation of total protein in diluted lipase solution and residual fractions from immobilization was performed using the BCA protein assay kit according to the manufacturer's instructions. Lipozyme RM IM (250 mg) was stirred overnight in 10 mL of 4.5% sodium dodecyl sulfate solution to desorb the enzyme from the carrier surface. Next, the carrier was filtered off and the total protein content of the filtrate was determined. Bovine serum albumin was used as standard. Water activities of free and immobilized RML samples were measured using an Aqua Lab 3TE water activity meter (Decagon Devices Inc., Pullman, WA).

Lipase Assay

The activities of free and immobilized RML samples were determined using a modified version of the titrimetric method described by Pinsirodom and Parkin (2001). Olive oil (5 g) and 1 g of gum arabic were added to 100 mL of sodium phosphate buffer (pH 8.0, 50 mM) and homogenized with a Polytron high-speed batch disperser (Kinematica Inc., Bohemia, NY). A known amount of lipase was added to 50 mL of the olive oil-gum arabic emulsion and incubated at 37 °C in a water bath shaker at 200 rpm ($3.58 \times g$). Five milliliter aliquots of the hydrolyzate were removed at 2, 5, 10, 15, and 20 min and each transferred to a flask containing 10 mL of 95% (v/v) ethanol and 2 drops of 1% thymolphthalein. Subsamples and a blank were titrated with 0.02 N NaOH to a light blue end point. The quantity of free fatty acids (FFAs) released by lipase was calculated using the equation:

$$\text{FFA released } \left(\frac{\mu\text{mol}}{\text{mL}}\right) = \frac{[(\text{ml NaOH for sample} - \text{ml NaOH for blank}) \times 0.02\ N \times 1000]}{5\ \text{mL}} \quad (1)$$

The quantity of FFAs released was plotted against incubation time and hydrolytic activity was determined from the slope of the linear portion of the curve. One unit (U) of activity was defined as the amount of lipase that produced 1 μmol of FFA per minute under the specified assay conditions. Specific activities of free and immobilized RML were calculated by dividing lipase activity by protein content.

Small-Scale Synthesis of SL

Caprylic acid (MW = 144.2 g/mol) was blended with 500 mg of SDASO (MW = 867.4 g/mol) at different molar ratios (1:1, 3.5:1, or 6:1) followed by addition of 4 mL of hexane and 50 mg of molecular sieve (4 Å). Acidolysis was initiated by adding biocatalyst at 10, 15, or 20% weight of substrates. The reaction mixture was incubated at different temperatures (30, 40, 50, or 60 °C) for different times (12, 18, or 24 h) in a water bath shaker at 200 rpm (3.58 × *g*). Acidolysis was stopped by filtering off the lipase. FFAs were removed by alkaline deacidification. Ten milliliters of 95% (v/v) ethanol and one drop of 1% phenolphthalein in ethanol were added to the product mixture. Next, FFAs in the mixture was neutralized with 0.5 M NaOH to a pink endpoint. The deacidified mixture was transferred to a separatory funnel, mixed vigorously with 15 mL of hexane, and allowed to undergo phase separation. The lower hydroethanolic layer containing FFA soaps was discarded while the upper hexane layer was collected and passed through a column of anhydrous sodium sulfate. SLs were recovered by evaporating hexane under vacuum at 40 °C in a rotary evaporator. SL products were transferred to amber sample vials, flushed with nitrogen, and stored at −80 °C.

Experimental Design and Data Analysis

Effects of the type of biocatalyst and incubation temperature on acidolysis and acyl migration were studied (Table 4.1). Acidolysis extent was measured by caprylic acid content of SLs while the caprylic acid content at the *sn*-2 position of SLs was a measure of the extent of acyl migration. For the set of treatments shown in Table 4.1, substrate molar ratio (S_R) and incubation time (t) were fixed at their center points, 3.5:1 and 18 h, respectively, while enzyme load (E_L) was fixed at 10% weight of substrates (or 13.6 U) for Lipozyme RM IM. The added amounts of C-RML (194 mg) and O-RML (13 mg) were equivalent to 13.6 U of Lipozyme RM IM as determined by the equation:

$$\mathrm{W_x} \equiv \frac{\mathrm{U_{Lipozyme\ RM\ IM}}}{\mathrm{A_x}} \tag{2}$$

Where W_x = equivalent weight of C-RML or O-RML (g), $U_{Lipozyme\ RM\ IM}$ = units of Lipozyme RM IM (U) used for biocatalysis, and A_x = activity of C-RML or O-RML (U/g) (see Table 4.2).

Treatments were run in triplicate and completely randomized. The relationship between the independent variables (A: biocatalyst, and B: incubation temperature) and response variables (caprylic acid content of SL and caprylic acid content at the *sn*-2 position of SL) was modeled by two-way analysis of variance (ANOVA). The statistical model for this relationship is given by:

$$y_{ijk} = \mu + \tau_i + \beta_j + (\tau\beta)_{ij} + \varepsilon_{ijk} \begin{cases} i = 1,2,\dots,a \\ j = 1,2,\dots,b \\ k = 1,2,\dots,n \end{cases} \tag{3}$$

Where y_{ijk} is the response variable, μ is the overall mean response, τ_i is the effect of the *i*th level of factor A, β_j is the effect of the *j*th level of factor B and $(\tau\beta)_{ij}$ is the interaction between τ_i and β_j.

The numerical optimization function of Design Expert 8.0.7.1 (Stat-Ease, Inc., Minneapolis, MN) was used to select which treatment gave the best combined response. Optimization goals were to maximize total caprylic acid content of SLs and minimize caprylic acid content at the *sn*-2 position of SLs. These goals were assigned the same weights and rated equally in relative importance. Based on these goals, treatments and observed responses were ranked according to desirability, ranging from 0 to 1 (least to most desirable, respectively). This desirability value may be used as an overall indicator of success when multiple response variables are optimized simultaneously and a more detailed explanation of the mathematical basis of desirability is given elsewhere (Anderson & Whitcomb, 2005). The treatment with the highest desirability value was deemed to produce the best combined response and was optimized further by RSM with respect to S_R, *t*, and E_L.

Experimental design and data analysis for RSM were executed in MODDE 5.0 (Umetrics, Umeå, Sweden). Low and high levels of the independent variables were as follows; S_R (1:1 and 6:1), *t* (12 and 24 h), and E_L (10 and 20% weight of substrates). The response modeled was the caprylic acid content of synthesized SLs. Three replicates of the center point were included in the design to detect curvature in the response. The Central Composite Face design, shown in Table 4.3, was adopted for this study. Experiment runs were performed in triplicate and completely randomized. Multiple linear

regression was used to fit the data into a second-order model that approximated the relationship between the independent variables and response.

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

Where Y is the response (caprylic acid content of SL). β_o, β_i, β_{ii}, and β_{ij} are the constant, linear, quadratic, and interaction regression coefficients, respectively. X_i and X_j are the independent variables while ε_{ij} is the model error.

The significance of the model and model terms were determined by ANOVA. Statistical significance was judged by the F-test at $\alpha = 0.05$. The model goodness-of-fit was assessed by R^2, R^2_{adj}, and lack of fit test. The predictive power of the model was given by Q^2. After model fitting, residual analysis was conducted to identify outliers and validate the assumptions used in ANOVA. Main and interaction effects of process parameters were visualized on effects plots. The optimizer function of the software was used to determine optimal levels of S_R, t, and E_L for immobilized RML-catalyzed SL synthesis based on the response surface.

Scaled-up Synthesis of SL

Acidolysis was performed at optimal levels of S_R, t, and E_L determined by the model. Caprylic acid (50 g) was blended with 50 g of SDASO (S_R = 6:1) in a 1 L stirred batch reactor and 0.5 g of molecular sieve was added. Solvent (addition of 400 mL of *n*-hexane) and solvent-free conditions were explored. Acidolysis was initiated by adding C-RML at 20% weight of substrates. The reaction was incubated at 50 °C for 24 h with continuous stirring at 200 rpm. Scale-up synthesis of SL at optimal condition was repeated with 20 g of stearidonic acid concentrate (SDACO) produced by *Candida*

rugosa lipase-catalyzed partial hydrolysis of SDASO (Ifeduba & Akoh, 2013). Solvent and solvent-free conditions were also explored for this substrate. After incubation, the reactions were stopped by filtering the biocatalyst. FFA in the product mixture was removed by alkaline deacidification and SL products were recovered as described previously.

Fatty Acid Composition

SDASO and SDACO samples were converted to fatty acid methyl esters (FAME) and analyzed with an Agilent 6890N GC system (Agilent Technologies Inc., Santa Clara, CA). SL samples were separated into acylglycerol classes by thin-layer chromatography (TLC). TAGs were isolated from SL, converted to FAME, and analyzed by GC. Protocols for TLC and fatty acid analysis are described elsewhere (Ifeduba & Akoh, 2013). Fatty acid compositions of SDASO, SDACO, and their respective SL were compared by Duncan's multiple-range test ($\alpha = 0.05$) using SAS software, version 9.2 (SAS Institute Inc., Cary, NC).

Triacylglycerol Molecular Species

Characterization of TAG molecular species in SDASO, SDACO, and their respective SL was performed by reversed-phase HPLC on an Agilent 1260 Infinity HPLC system (Agilent Technologies Inc., Santa Clara, CA) equipped with a Sedex Model 85 evaporative light scanning detector (ELSD) (Sedere, Alfortville, France) and a 4 mm × 250 mm, 5 μm particle size, Ultrasphere C18 analytical column (Beckman Coulter Inc., Pasadena, CA). Oil and SL samples were diluted in chloroform to a concentration of ~5 mg/mL before analysis. Sample injection volume was 20 μL. The column temperature was set at 30 °C. The mobile phase was acetonitrile (A) and acetone (B). The flow rate

was set at 1 mL/min. Gradient elution began with 65% B at 0 min to 95% B at 55 min, and was followed by a 10 min post-run at 65 % B. The drift tube temperature for ELSD was set at 70 °C, and the nebulizer gas pressure was 3.0 bars. The retention times of TAG species depended on polarity and equivalent carbon number (ECN). ECN is defined as $C_N - 2n$, where C_N is the number of carbon atoms in the TAG excluding the three carbon atoms of glycerol, and n is the number of double bonds. For peak identification, the retention times of sample TAG species were compared with TAG standards of known ECN, namely, tricaprylin (24), tricaprin (30), trilaurin (36), trimyristin (42), tripalmitin (48), triolein (48), tristearin (54), and triarachidin (60).

Melting and Crystallization Profile

Thermal properties of SDASO, SDACO, and their respective SLs were studied by differential scanning calorimetry (DSC) on a DSC 204 F1 Phoenix system (Netzsch-Gerätebau GmbH, Selb, Germany). DSC analysis was performed to assess the effect of caprylic acid incorporation on the thermal properties of SDASO and SDACO, specifically their melting completion and crystallization onset temperatures. DSC analysis was performed according to AOCS Official Method Cj 1-94 (AOCS, 2009). The instrument was standardized using indium as reference standard. Samples were completely melted and 8−12 mg of each were weighed in an aluminum DSC crucible and sealed hermetically. The sample crucible was placed in the DSC along with a blank reference at room temperature. Next, the sample was heated to 80 °C at 20 °C /min and held for 10 min, then cooled to −80 °C at 5 °C/min and held for 30 min, and then heated again to 80 °C at 5 °C/min. DSC data were analyzed by Proteus thermal analysis software (Netzsch-Gerätebau GmbH, Selb, Germany).

Operational Stability of C-RML and Lipozyme RM IM

C-RML and Lipozyme RM IM recovered from the small-scale study was used to catalyze six subsequent acidolysis reactions. S_R, t, and E_L were fixed at 3.5:1, 18 h, and 10% weight of substrates for Lipozyme RM IM or the equivalent of C-RML. Temperatures for C-RML and Lipozyme RM IM-catalyzed acidolysis were 50 and 60 °C, respectively. After each reaction cycle, the immobilized lipase was filtered from the product mixture, washed with hexane, and dried over silica gel in a vacuum before reuse.

Results and Discussion

Free RML, C-RML, O-RML, and Lipozyme RM IM are characterized in terms of protein content, hydrolytic, specific, and water activity in Table 4.2. The highest hydrolytic activity was observed in O-RML (1080 U/g) while the lowest activity was observed in C-RML (70 U/g). The specific activity of free RML increased by 174% after immobilization onto octyl-sepharose but decreased by 79% after immobilization onto Celite. The immobilization of an enzyme is known to change its native conformation, spatial orientation, and micro-environment in a way that influences its activity and performance. Previous studies have demonstrated that lipases immobilized on hydrophobic carriers tend to be more active than free lipases because while the former mimics the open conformation on the carrier, the latter needs to undergo interfacial activation to become activated (Rodríguez et al., 2012). The apparent hyperactivation of RML by immobilization onto octyl-sepharose is in agreement with previous findings in the literature (Nieto et al., 2005; Rodrigues & Fernandez-Lafuente, 2010). On the other hand, the reduced hydrolytic activity of C-RML compared to free RML may be a result

of mass transfer effects, that is, external and internal diffusional restrictions on the surface and within the pores, respectively, of the carrier (Tischer & Wedekind, 1999).

Immobilized RML preparations were used to catalyze the incorporation of caprylic acid into SDASO at 30, 40, 50, and 60 ^{o}C. Response values for caprylic acid content of SL ranged from 0.33–25.83 mol%, while *sn*-2 caprylic acid content of SLs ranged from 0.19–15.42 mol% (Table 4.1). The two-way ANOVA models for both responses were significant ($P < 0.01$). The model terms, A (biocatalyst), B (incubation temperature), and AB interaction, were highly significant for both responses ($P < 0.001$). The highest level of caprylic acid incorporation (25.83 mol%) was achieved by C-RML at 50 °C, followed by Lipozyme RM IM (23.51 mol%) at 60 °C. Interestingly, O-RML which had the highest hydrolytic activity performed worst during acidolysis. Given that acidolysis requires an appropriate balance between hydrolysis rate of TAG substrates and esterification rate of the diacylglycerol (DAG) and MAG intermediates, an explanation for the low incorporation of caprylic acid by O-RML is that the high water activity of O-RML (0.871) may have favored hydrolysis events over esterification. The water activities of C-RML, O-RML, and Lipozyme RM IM are shown in Table 4.2. Previous studies have demonstrated that a low water activity medium is required to maintain an appropriate balance between these events (Akoh & Kim, 2008; Camacho et al., 2007). The low water activities of C-RML and Lipozyme RM IM, 0.331 and 0.337, respectively, suggest that they were better suited for acidolysis than O-RML. Positional analysis of SL products revealed that caprylic acid was esterified at the *sn*-2 position during acidolysis. The level of caprylic acid at the *sn*-2 position of SLs increased with temperature for C-RML and Lipozyme RM IM-catalyzed acidolysis (Table 4.1). This may be because acyl

migration increased with temperature. Other factors known to influence acyl migration include the type of immobilization support, solvent type, water activity, incubation time, and pH (Kapoor & Gupta, 2012). Under conditions favorable to acyl migration, fatty acyl chains at the *sn*-2 positions of *sn*-1,2 and 2,3-DAG intermediates formed by hydrolysis of the TAG substrate may spontaneously transfer to the primary positions to form *sn*-1,3-DAG species which may then be further hydrolyzed at the *sn*-1 and 3 positions or esterified with fatty acid at the *sn*-2 position. The *sn*-2 caprylic acid content in SL was therefore a good measure of acyl migration. In the present study, the highest acyl migration was observed in Lipozyme RM IM-catalyzed acidolysis at 60 °C. The best combination of immobilization-support and temperature was selected by numerical optimization. Reaction optimization goals were to maximize the incorporation of caprylic acid into SDASO and minimize acyl migration. The assumption made here was that minimizing acyl migration would reduce the likelihood of losing omega-3 PUFAs from the *sn*-2 position and increase the chances of forming SL with the MLM configuration during acidolysis. C-RML at 50 °C gave the highest overall desirability (0.781) based on these criteria and was deemed most suitable for catalyzing the production of MLM-type SLs from SDASO and caprylic acid.

The caprylic acid contents of the SLs synthesized by C-RML-catalyzed acidolysis at 50 °C under varying levels of S_R, t, and E_L ranged from 7.18–35.02 mol% (Table 4.3). The regression model obtained by ANOVA was statistically significant ($P < 0.05$). The R^2 value, the fraction of the total variation of the response that could be explained by the model was 0.918. Furthermore, the plot of observed versus predicted response values was approximately linear (data not shown). The R^2_{adj} value of 0.887 was close enough to R^2

to indicate that non-explanatory variables were excluded from the model. The Q^2 value, the fraction of the total variation of the response that could be predicted by the model, was 0.848. The test for lack of fit yielded non-significant results ($P > 0.05$) showing that the model was well fitted to the data. Significant terms of the model ($P < 0.05$) were the linear coefficients of S_R, t, and E_L, the quadratic coefficient of S_R, and the interaction coefficients $S_R t$ and $S_R E_L$. Therefore, the second order model after eliminating non-significant coefficients could be expressed as:

$$Y = 22.416 + 7.110S_R + 3.485t + 2.713E_L - 5.919S_R^2 + 2.856S_R t + 2.86S_R E_L \quad (5)$$

This model was deemed adequate for explaining the relationship between the reaction variables (S_R, t, and E_L) and the caprylic acid content of resulting SLs. All the main effects were significant ($P < 0.05$). S_R had the highest and most significant effect on the caprylic acid content of SLs, followed by t and E_L. The main effect of S_R was estimated at 14.22. This implies that SLs produced when substrate molar ratio was 6:1 had, on average, 14.22 mol% more caprylic acid than SLs produced when substrate molar ratio was 1:1. The main effect of t was estimated at 6.97 which imply that SLs produced after 24 h of incubation had, on average, 6.97 mol% more caprylic acid than SLs produced after 12 h of incubation. The main effect of E_L was 5.43 meaning that SLs produced with an enzyme load of 20% the weight of substrates had, on average, 5.43 mol% more caprylic acid than SLs produced with an enzyme load of 10% the weight of substrates. Therefore, within the limits of factor levels studied, S_R, t, and E_L had positive effects on acidolysis. This is generally in agreement with other reports on lipase-catalyzed modification of oils by acidolysis (Akoh & Kim, 2008; Camacho et al., 2007; Chen et al., 2012; K.-T. Lee & Akoh, 1998; Rodríguez et al., 2012). Significant

interaction effects were observed between S_R and t as well as S_R and E_L (Figure 4.1). The interaction effect S_Rt was estimated at 5.71 meaning that the main effect of S_R at 24 h of incubation was, on average, 8.51 units ($S_R - S_Rt$) higher than the main effect of S_R at 12 h of incubation. This is visualized by the interaction plot of S_R and t in Figure 4.1a. The interaction effect S_RE_L was estimated at 4.78 which means that the main effect of S_R at enzyme load of 20% of the weight of substrates was, on average, 9.44 units ($S_R - S_RE_L$) higher than the main effect of S_R at enzyme load of 10% of the weight of substrates. This is visualized by the interaction plot of S_R and E_L in Figure 4.1b. This interactive effect may be due to the greater number of active sites available for biocatalysis at E_L of 20% than at 10% the weight of substrates or just due to substrate concentration effect.

The optimal condition for SL production by C-RML-catalyzed acidolysis of SDASO with caprylic acid determined by RSM was S_R = 6:1, t = 24 h, and E_L = 20% weight of substrates. This treatment was deemed optimal because increasing enzyme load beyond 20% seemed unfeasible while increasing the levels of substrate molar ratio and incubation time beyond 6:1 and 24 h, respectively, did not lead to appreciable increases in caprylic acid content of SLs (data not shown). This plateau in the extent of fatty acid incorporation with increasing levels of reaction variables such as substrate molar ratio, enzyme loading, and time is a fairly common observation with lipase-catalyzed acidolysis (Kim, Kim, Lee, Chung, & Ko, 2002; Y. Wang, Xia, Xu, Xie, & Duan, 2012; Zhao, Lu, Bie, Lu, & Liu, 2007). The caprylic acid content of SLs produced under this treatment was predicted to be 35.05 $\pm$ 3.32 mol% [95% prediction interval (PI)]. Fatty acid compositions of SDASO, SDACO and their respective SLs produced in solvent and solvent-free conditions are shown in Table 4.4. The mean caprylic acid content of SLs

produced from SDASO in solvent was 32.50 mol% while that of SLs produced in solvent-free media was 21.23 mol%. The response obtained for solvent media was within the PI whereas that obtained in solvent-free media was below the PI. This is not surprising given that optimal conditions for SL production were based on response data obtained from experiments performed in solvent. The lower incorporation of caprylic acid in solvent-free media, compared to solvent media, may be due to hindered C-RML activity by high free caprylic acid concentration in solvent-free media. The palmitic, stearic, oleic, linoleic, and α-linolenic acid contents of SDASO were reduced to a greater extent compared to γ-linolenic and stearidonic acids after acidolysis. This may indicate that caprylic acid was incorporated mostly at the expense of palmitic, stearic, oleic, linoleic, and α-linolenic acids. Positional distribution of fatty acids in SL produced from SDASO and SDACO are shown in Table 4.5. The mean caprylic acid contents at the *sn*-2 position of SLs produced from SDASO under solvent and solvent-free conditions were 6.26 and 5.17 mol%, respectively. This shows that although acyl migration was not completely eliminated, it was minimized. The combined caprylic acid contents at the primary (*sn*-1,3) positions of SL produced in solvent and solvent-free media were 45.27 and 29.26 mol%, respectively, while SDA contents at the *sn*-2 position of SL produced in solvent and solvent-free conditions were 28.21 and 25.83 mol%, respectively. The mean caprylic acid content of SLs produced from SDACO in solvent was 20.82 mol% while that of SLs produced in solvent-free media was 17.04 mol%. The caprylic acid contents of SLs produced from SDACO were significantly lower than those of SL produced from SDASO. It was previously noted that C-RML may have incorporated caprylic acid at the expense of palmitic, stearic, oleic, linoleic, and α-linolenic acids. This implies that the

incorporation of caprylic acid into SDACO may have been limited by the fatty acid composition of SDACO given that it was relatively lower in palmitic, stearic, oleic, linoleic, and α-linolenic acids compared to SDASO. The incorporation of caprylic acid into SDACO was higher in solvent than in solvent-free media. Again, this may be due to C-RML inhibition by high free caprylic acid concentration in solvent-free media compared to solvent media. The mean caprylic acid contents at the *sn*-2 position of SLs produced from SDACO in solvent and solvent-free media were 1.88 and 3.52 mol%, respectively, while the combined caprylic acid contents at the primary (*sn*-1,3) positions of SLs produced in solvent and solvent-free media were 31.23 and 29.30 mol%, respectively (Table 4.5). SDA contents at the *sn*-2 position of SL produced in solvent and solvent-free media were 62.60 and 62.42 mol%, respectively. The ratio of omega-3/omega-6 fatty acids of the SL products ranged from 1.2-2.2 (Table 4.4).

TAG molecular species in SDASO, SDACO, and their respective SLs produced in solvent were characterized by RP-HPLC as shown in Figure 4.2. SDASO was resolved into 12 groups of TAG species with ECN ranging from 30-48 (Figure 4.2a) while SDACO were resolved into 8 groups of TAG species with ECN ranging from 30-44 (Figure 4.2b). The first and second chromatographic peaks in SDASO and SDACO were determined to be solely composed of tristearidonoyl glycerol (StStSt) and distearidonoyl linolenoyl glycerol (LnStSt), respectively, while subsequent chromatographic peaks were composed of at least two TAG molecular species with identical ECN and polarities. SLs produced from SDASO were resolved into 8 groups of TAG species with ECN ranging from 26-40 (Figure 4.2c and Table 4.6) while SLs produced from SDACO were resolved into 6 groups of TAG species with ECN ranging from 26-36 (Figure 4.2d and Table 4.6).

The possible identities of mono- and di-substituted TAG molecular species are shown in Table 4.6. The di-substituted TAG species were located in peaks 1-4 of SLs, namely: dicapryloyl stearidonoyl glycerol (CCSt) in peak 1, dicapryloyl linolenoyl glycerol (CCLn) in peak 2, dicapryloyl linoleoyl glycerol (CCL) in peak 3, and dicapryloyl oleoyl glycerol (CCO) in peak 4. It should be noted that these TAG descriptions do not reflect the stereochemical configuration of TAG species. So CCSt, for example, could be a mixture of *sn*-CCSt, *sn*-CStC, and *sn*-StCC in any proportion. However, the TAG species having the MLM stereochemical configuration, such as *sn*-CStC and *sn*-CLnC, may predominate in the aforementioned peaks given that the reaction was optimized to minimize caprylic acid at the *sn*-2 position.

Figure 4.3 shows the melting and crystallization profiles of SDASO, SDACO, and their respective SL produced in solvent. Thermal properties of fats and oils are influenced by the diversity of TAG species, fatty acid composition and degree of saturation (Marikkar, Ghazali, Man, & Lai, 2003). Melting completion temperatures for SDASO and the SL produced from SDASO were -4 and -20 °C, respectively, while those of SDACO and the SL produced from SDACO were -8 and -34 °C, respectively. Crystallization onset temperatures for SDASO and the SL produced from SDASO were -4 and -10 °C, respectively, while those of SDACO and the SL produced from SDACO were -1 and -20 °C, respectively. Evidently, the incorporation of caprylic acid into SDASO and SDACO led to lower melting completion and crystallization onset temperatures. The melting peak temperature of the SLs produced from SDASO and SDACO was -42 °C. SDASO showed two sharp crystallization peaks at -8 and -34 °C while SDACO had rather broad peaks at -13 and -47 °C.

The operational stability of C-RML was compared to that of the commercially available Lipozyme RM IM. As can be seen in Figure 4.4, the activity of C-RML decreased slightly after the first 3 cycles but rapidly deteriorated during the next 4 consecutive cycles while Lipozyme RM IM activity reduced only slightly after 7 cycles. The gradual loss of C-RML activity may be due to leaching away of lipase from the carrier. The operational stability of C-RML may be improved by optimizing the immobilization method, reducing incubation time and mixing speed or by use of a different support material.

Conclusion

SLs were successfully synthesized by incorporating caprylic acid into SDASO and SDACO using C-RML as biocatalyst. Although C-RML was less stable than Lipozyme RM IM, it seemed more advantageous for producing MLM-type SLs from SDASO due to lower acyl migration rates. Acyl migration was undesirable because it promoted the incorporation of caprylic acid at the *sn*-2 position. The SLs synthesized contained 17.0–32.5 mol% of caprylic acid and 20.6-42.3 mol% of SDA depending on the fatty acid composition of the original substrate and whether the reaction was performed with or without solvent (Table 4.4). When digested by pancreatic lipase, these SL may deliver the medium chain caprylic acid which can be rapidly assimilated for energy while SDA can be converted, *in vivo*, to EPA and incorporated into heart tissue for cardioprotective benefits.

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	Factors [a]		Responses (mol%) [b]	
Exp no.	Biocatalyst	T (°C)	C8:0	*sn*-2 C8:0
1	C-RML	30	17.98 ± 0.09	1.93 ± 0.56
2	C-RML	40	21.91 ± 0.33	4.23 ± 0.28
3	C-RML	50	25.83 ± 0.83	5.95 ± 0.30
4	C-RML	60	22.72 ± 0.59	10.33 ± 0.73
5	O-RML	30	3.43 ± 0.24	0.27 ± 0.02
6	O-RML	40	7.07 ± 1.39	2.36 ± 1.23
7	O-RML	50	0.33 ± 0.03	0.30 ± 0.05
8	O-RML	60	0.43 ± 0.05	0.19 ± 0.07
9	Lipozyme RM IM	30	16.70 ± 0.97	2.77 ± 0.99
10	Lipozyme RM IM	40	15.66 ± 0.12	4.86 ± 0.33
11	Lipozyme RM IM	50	16.52 ± 0.66	7.39 ± 1.06
12	Lipozyme RM IM	60	23.51 ± 0.65	15.42 ± 0.39

Table 4.1 Effects of type of biocatalyst and incubation temperature on acidolysis and acyl migration: treatments and observed responses

Exp no. experiment number, T incubation temperature, C8:0 caprylic acid content of SL, *sn*-2 C8:0 caprylic acid content at the *sn*-2 position of SL, C-RML Celite-immobilized *R. miehei* lipase, O-RML octyl-sepharose-immobilized *R. miehei* lipase

[a] All reactions were carried out for 18 h at substrate molar ratio (caprylic acid to stearidonic acid soybean oil) of 3.5:1 with 13.6 U of each biocatalyst

[b] Mean ± SD, n = 3

Table 4.2 Properties of free and immobilized *Rhizomucor miehei* lipase (RML)

Lipase	Protein content (mg of protein/g)	Lipase activity (U/g) [a]	Specific activity (U/mg of protein)	a_w
Free RML	49.7	543	10.92	0.981
C-RML	30.0	70	2.33	0.331
O-RML	36.1	1080	29.88	0.870
Lipozyme RM IM	47.5	170	3.58	0.337

w

a_w, water activity, C-RML Celite-immobilized *R. miehei* lipase, O-RML octyl-sepharose-immobilized *R. miehei* lipase

[a] One unit (U) of activity was defined as the amount of lipase preparation that produced 1 μmol of FFA per min from a 5% (w/v) olive oil-gum arabic emulsion at pH 8.0 and temperature of 37 °C

Table 4.3 Effect of substrate molar ratio, incubation time, and enzyme load on C-RML-catalyzed acidolysis: treatments and observed responses

	Factors [a]			Response (mol%) [b]
Exp no.	S_R	t (h)	E_L (%)	C8:0
1	1:1	12	10	7.18 ± 0.29
2	1:1	18	15	14.75 ± 2.55
3	1:1	24	10	9.68 ± 1.10
4	1:1	24	20	8.37 ± 0.07
5	1:1	24	20	9.47 ± 0.37
6	3.5:1	12	15	25.87 ± 1.54
7	3.5:1	18	10	18.28 ± 3.54
8	3.5:1	18	15	23.15 ± 0.76
9	3.5:1	18	15	24.58 ± 2.31
10	3.5:1	18	15	26.06 ± 1.43
11	3.5:1	18	20	24.32 ± 1.17
12	3.5:1	24	15	28.67 ± 1.03
13	6:1	12	10	11.71 ± 2.89
14	6:1	12	20	22.20 ± 1.16
15	6:1	18	15	23.76 ± 0.71
16	6:1	24	10	25.37 ± 0.86
17	6:1	24	20	35.02 ± 1.52

C-RML Celite-immobilized *R. miehei* lipase, Exp no. experiment number, S_R substrate molar ratio of caprylic acid to stearidonic acid soybean oil, t incubation time, E_L enzyme load (% weight of substrates), C8:0 caprylic acid content of SL

[a] All reactions were carried out at 50 °C

[b] Mean ± SD, n = 3

Table 4.4 Total fatty acid compositions of SDA soybean oil, SDA concentrate and their respective structured lipids produced by C-RML-catalyzed acidolysis (mol%) [a]

	SDA soybean oil (SDASO)			SDA concentrate (SDACO)		
	Unmodified [b]	Structured lipid [c]		Unmodified	Structured lipid [d]	
Fatty acid		Solvent	Solvent-free		Solvent	Solvent-free
C8:0	ND	32.50 ± 1.05 a	21.23 ± 0.17 b	ND	20.82 ± 0.25 b	17.04 ± 1.18 c
C16:0	12.32 ± 0.22 a	6.66 ± 0.10 c	7.54 ± 0.08 b	6.85 ± 0.03 c	5.88 ± 0.23 d	5.54 ± 0.77 d
C18:0	4.10 ± 0.04 a	2.08 ± 0.02 d	2.79 ± 0.02 c	1.56 ± 0.10 b	0.91 ± 0.08 c	0.84 ± 0.06 c
C18:1ω9	15.12 ± 0.02 a	9.16 ± 0.37 c	11.35 ± 0.08 b	8.21 ± 0.12 d	6.14 ± 0.20 e	5.95 ± 0.89 e
C18:2ω6	24.60 ± 0.01 a	15.02 ± 0.22 c	17.55 ± 0.18 b	11.63 ± 0.12 d	10.54 ± 0.74 e	10.46 ± 1.27 f
C18:3ω6	7.17 ± 0.10 d	6.55 ± 0.07 d	5.06 ± 0.06 e	13.72 ± 0.40 a	11.69 ± 0.44 b	12.31 ± 1.56 c
C18:3ω3	10.15 ± 0.02 a	6.06 ± 0.04 c	7.83 ± 0.06 b	5.90 ± 0.20 c	3.75 ± 0.37 d	3.52 ± 0.42 d
C18:4ω3	24.40 ± 0.03 d	20.56 ± 0.04 f	23.65 ± 0.29 e	48.56 ± 0.29 a	36.97 ± 0.56 c	42.34 ± 0.08 b
∑SFA	16.4	41.2	31.6	8.4	29.6	25.4
∑MUFA	15.1	9.2	11.4	8.2	6.1	6.0
∑PUFA	66.3	48.2	54.1	79.8	60.9	67.6
ω3/ω6	1.1	1.2	1.4	2.1	2.0	2.2

SDA stearidonic acid, C-RML Celite-immobilized *R. miehei* lipase, ND not detected, SFA saturated fatty acid, MUFA monounsaturated fatty acid, PUFA polyunsaturated fatty acid

[a] Mean ± SD, n = 3

[b] Low levels of C14:0, C16:1, C17:0, C20:0, C20:1ω9, and C22:0 were detected

[c] Acidolysis reactions were carried out for 24 h at 50°C with substrate molar ratio (caprylic acid to SDASO) of 6:1, and enzyme load of 20% weight of substrates

[d] Acidolysis reactions were carried out for 24 h at 50°C with substrate molar ratio (caprylic acid to SDACO) of 6:1, and enzyme load of 20% weight of substrates

Means with the same lower case letter in each row are not significantly different ($P > 0.05$)

Table 4.5 Positional distributions of fatty acids in structured lipids produced from SDA soybean oil (SDASO) and SDA concentrate (SDACO) by C-RML-catalyzed acidolysis (mol%) [a]

	Structured lipid from SDA soybean oil [b]				Structured lipid from SDA concentrate [c]			
	Solvent		Solvent-free		Solvent		Solvent-free	
Fatty acid	*sn*-2	*sn*-1,3 [d]	*sn*-2	*sn*-1,3	*sn*-2	*sn*-1,3	*sn*-2	*sn*-1,3
C8:0	6.96 ± 0.41 d	45.27 ± 0.30 h	5.17 ± 0.46 c	29.26 ± 0.14 f	1.88 ± 0.05 a	30.29 ± 0.30 g	3.52 ± 0.58 b	23.80 ± 0.14 e
C16:0	6.26 ± 0.33 d	6.86 ± 0.50 d	1.54 ± 0.09 a	10.54 ± 0.13 e	5.69 ± 0.69 c	5.98 ± 0.50 c	4.89 ± 1.14 b	5.87 ± 0.13 c
C18:0	2.15 ± 0.04 e	2.05 ± 0.14 e	ND	4.19 ± 0.11 f	1.79 ± 0.41 d	0.47 ± 0.04 a	1.34 ± 0.61 c	0.59 ± 0.01 b
C18:1ω9	13.12 ± 0.85 f	7.18 ± 0.11 c	16.69 ± 1.65 g	8.68 ± 0.20 e	2.86 ± 0.09 b	7.78 ± 0.11 d	2.40 ± 0.15 a	7.73 ± 0.20 c
C18:2ω6	26.49 ± 0.53 e	9.29 ± 0.44 c	34.18 ± 0.50 f	9.24 ± 0.35 c	5.60 ± 0.10 b	13.01 ± 0.44 d	4.66 ± 0.18 a	13.36 ± 0.35 d
C18:3ω6	8.95 ± 0.22 d	5.35 ± 0.10 b	8.99 ± 0.64 d	3.09 ± 0.45 a	18.43 ± 1.02 e	8.32 ± 0.10 c	18.93 ± 0.30 e	9.00 ± 0.04 d
C18:3ω3	7.86 ± 0.13 f	5.16 ± 0.13 d	7.60 ± 0.49 e	7.95 ± 0.19 f	3.03 ± 1.11 b	4.11 ± 0.13 c	1.84 ± 0.10 a	4.36 ± 0.19 c
C18:4ω3	28.21 ± 0.39 d	16.74 ± 0.10 a	25.83 ± 0.58c	22.56 ± 0.04 b	62.60 ± 1.39 f	24.16 ± 0.10 c	62.42 ± 1.11 f	32.30 ± 0.04 e

SDA stearidonic acid, C-RML Celite-immobilized *R. miehei* lipase, ND not detected

[a] Mean ± SD, n = 3

[b] Acidolysis reactions were carried out for 24 h at 50°C with substrate molar ratio (caprylic acid to SDASO) of 6:1, and enzyme load of 20% weight of substrates

[c] Acidolysis reactions were carried out for 24 h at 50°C with substrate molar ratio (caprylic acid to SDACO) of 6:1, and enzyme load of 20% weight of substrates

[d] *sn*-1,3 (mol%) = [3 × total (mol%) −*sn*-2 (mol%)]/2

Means with the same lower case letter in each row are not significantly different ($P > 0.05$)

Table 4.6 Identification of possible triacylglycerol species in structured lipids produced from SDA soybean oil and SDA concentrate by C-RML-catalyzed acidolysis

Structured lipid [a]	Peak no [b]	ECN	TAG species [c]	Area %
SL-SDASO	1	26	CCSt	18.5
	2	28	CStSt, CCLn	18.9
	3	30	CLnSt, CCL	21.7
	4	32	CCP, CCO, CLSt	16.9
	5	34	CCS, CPSt, COSt, CLLn	11.4
	6	36	CSSt, CLL, COLn, CPLn	6.5
	7	38	CSLn, COL, CPL	3.9
	8	40	CSL, COO, CPP, COP	2.1
SL-SDACO	1	26	CCSt	27.8
	2	28	CStSt, CCLn	30.5
	3	30	CLnSt, CCL	20.3
	4	32	CCP, CCO, CLSt	15.0
	5	34	CCS, CPSt, COSt, CLLn	10.4
	6	36	CSSt, CLL, COLn, CPLn	4.9

C-RML Celite-immobilized *R. miehei* lipase, ECN equivalent carbon number, SL-SDASO structured lipid produced from stearidonic acid soybean oil, SL-SDACO structured lipid produced from stearidonic acid concentrate, C caprylic acid, P palmitic acid, O oleic acid, S stearic acid, L linoleic acid, Ln α- or γ-linolenic acid, St stearidonic acid

[a] Acidolysis reactions were carried out in hexane for 24 h at 50°C with substrate molar ratio (caprylic acid to SDASO or SDACO) of 6:1, and enzyme load of 20% weight of substrates

[b] Chromatographic peak numbers for SL-SDASO and SL-SDASO in Figures 2c and d, respectively

[c] TAG species description does not specify stereochemical configuration of structured lipids

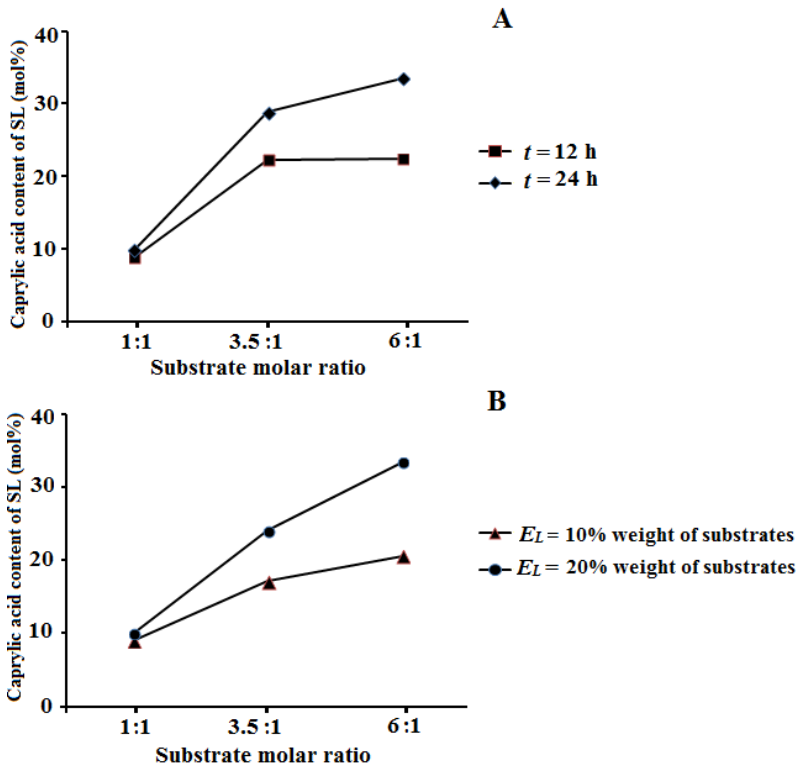

Figure 4.1 Interaction plots for substrate molar ratio and incubation time (*t*) (**a**), and substrate molar ratio and enzyme load (E_L) (**b**) in Celite-immobilized *R. miehei* lipase (C-RML)-catalyzed acidolysis of SDA soybean oil with caprylic acid

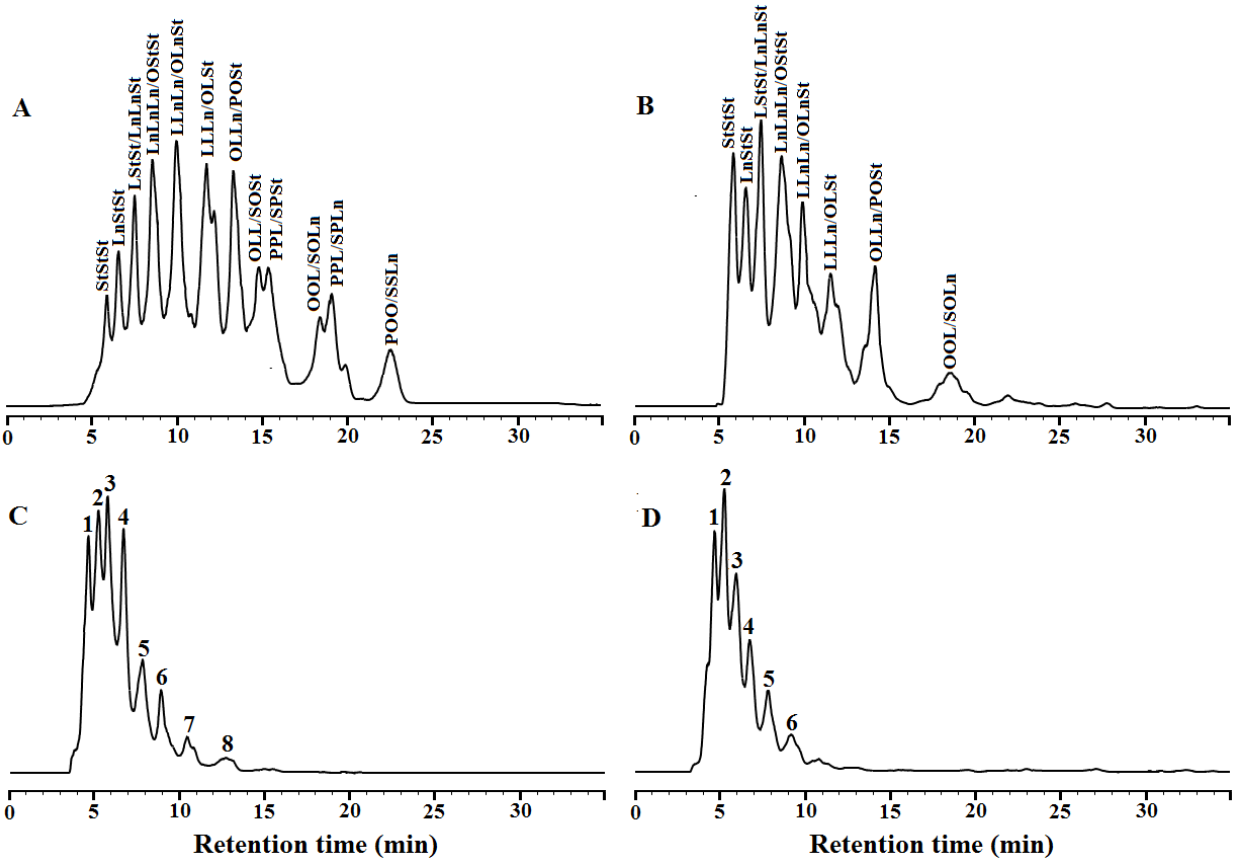

Figure 4.2 HPLC chromatograms showing TAG species in SDA soybean oil (SDASO) (**a**), SDA concentrate (SDACO) (**b**), SL produced from SDASO (**c**), and SL produced from SDACO (**d**). Annotations in A and C do not imply stereochemical configuration of TAG species. P, palmitic acid; O, oleic acid; S, stearic acid; L, linoleic acid; Ln, α- or γ-linolenic acid; St, stearidonic acid. Peak numbers 1-6 are identified in Table 4.6

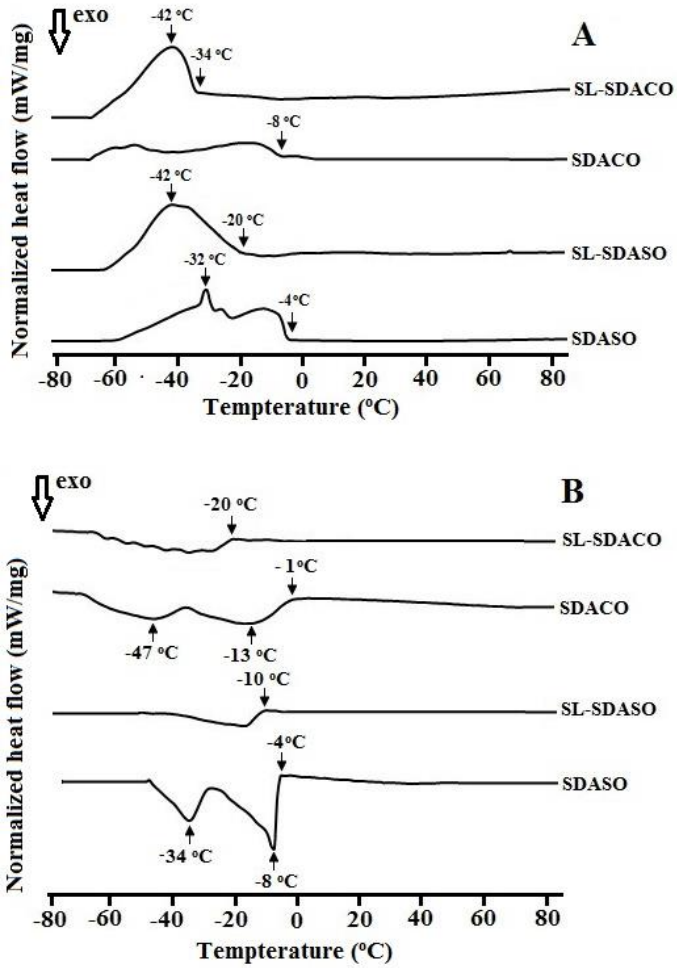

Figure 4.3 DSC thermograms for melting (**a**) and crystallization (**b**) of SDASO, SL produced from SDASO, SDACO, and SL produced from SDACO. Solid arrows indicate melting completion and peak temperatures in (**a**) and crystallization onset and peak temperatures in (**b**). See Figure 4.2 for meanings of abbreviations

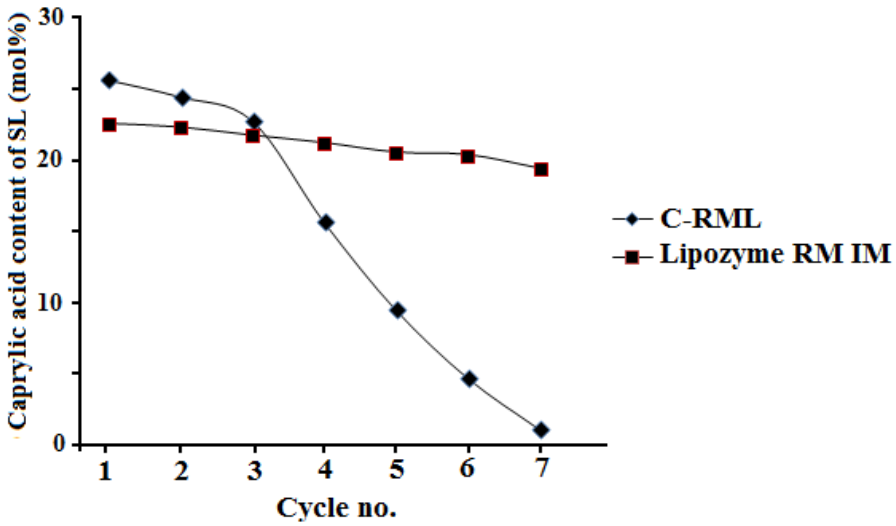

Figure 4.4 Operational stability of Celite-immobilized *R. miehei* lipase (C-RML) and Lipozyme RM IM

CHAPTER 5

MICROENCAPSULATION OF STEARIDONIC ACID SOYBEAN OIL IN COMPLEX COACERVATES MODIFIED FOR ENHANCED STABILITY[1]

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Abstract

The aim of this work was to compare encapsulation of stearidonic acid soybean oil (SDASO) by complex coacervation in the classical gelatin (GE)-gum arabic (GA) system with that of a Maillard reaction product (MRP). A portion of the control (microcapsules based on the GE-GA system) was crosslinked with transglutaminase (TG) after encapsulation. In the Maillard reaction (MR)-modified system, the gelatin-gum arabic mixture was crosslinked under controlled dry-heating conditions before use as encapsulating agent. The applicability of control, TG-, and MR-modified microcapsules in formulating SDASO-fortified yogurt was assessed for thermal and oxidative stability. SDS-polyacrylamide gel electrophoresis was used to confirm the covalent modification of encapsulants in TG- and MR-modified microcapsules. The MR-modified microcapsules displayed colloidal stability at conditions where the non-modified and TG-modified microcapsules were highly flocculated. ABTS free radical scavenging activity of control and modified GA-GE sols showed that antioxidant capacity was enhanced by MR but reduced by TG. The MR-modified microcapsules displayed superior oxidative stability during 28 days of storage at 4°C compared to control, while the oxidative stability of TG-modified microcapsules was lowest. Based on the amount of oil released from microcapsules during heat treatment (85 °C for 30 min) in yogurt milk base, MR-modified microcapsules displayed the highest thermal stability. Furthermore, yogurts formulated with MR-modified microcapsules had the best oxidative stability during 14 days of storage at 4 °C demonstrating that the antioxidant components of MR-modified microcapsules had good carry-through properties.

Keywords: microencapsulation; stearidonic acid soybean oil; complex coacervation; Maillard reaction; transglutaminase; oxidative stability

Introduction

Scientific evidence from multiple epidemiological studies provides strong support for the cardioprotective benefits of seafood-derived long-chain omega-3 fatty acids, namely; eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Mozaffarian & Wu, 2011). Since per capita fish consumption in most countries falls below recommended levels, the consumption of food products fortified with omega-3 fatty acids has been recognized to be a viable option for individuals who wish to supplement fish consumption or who do not consume fish altogether (Patch et al., 2005). However, the direct fortification of food products with omega-3 fatty acids can lower consumer acceptability because such food products could easily acquire the characteristic flavor of fish. Furthermore, the relatively high susceptibility of DHA and EPA to oxidation can compromise the quality and shelf life of omega-3 fortified foods if measures for controlling lipid oxidation are inadequate or lacking. Stearidonic acid (SDA, C18:4n-3) soybean oil is a plant based source of omega-3 fatty acids that was developed to serve as a sustainable alternative to fish oils for formulating omega-3 fortified foods. Food products formulated with SDA soybean oil (SDASO) may be labeled as "heart-healthy" based on evidence from clinical studies which demonstrate the efficacy of SDA in raising blood levels of EPA and the Omega 3 index (Decker, Akoh, & Wilkes, 2012). Other attributes of SDASO such as bland flavor and good oxidative stability make it attractive for formulating omega-3 fortified food products.

Microencapsulation is a well-suited approach to facilitate the incorporation of omega-3 fatty acids into foods. When implemented efficiently, microencapsulating omega-3 fatty acids within food-grade biopolymers can offer a number of advantages,

including: masking of unpleasant flavors, controlled-release, and extended shelf-life due to protection from oxidation and/or undesirable interactions with other ingredients. Several microencapsulation techniques are available for food and pharmaceutical applications. However, there is significant interest in the complex coacervation technique because of impressively high loading and controlled release capacities (Gouin, 2004). Complex coacervation occurs when dilution or pH adjustment of a sol containing oppositely charged colloids elicits the formation of an electro-neutral complex that separates out in a new "colloid-rich" phase known as a coacervate (Bungenberg de Jong, 1965a). When complex coacervation is elicited in the presence of dispersed oil droplets, microencapsulation occurs by deposition of coacervate film around oil droplets, provided the system is kept above the gel point of the hydrocolloids (Green and Schleicher, 1957). Subsequent cooling below the gel point stabilizes the coacervate film surrounding one or more oil droplets to form microcapsules. These microcapsules may be used as a suspension or dried further and used in powdered form. Microencapsulation by complex coacervation has been extensively investigated using different protein-polysaccharide pairs although fundamental studies were based on the gelatin-gum arabic (GE-GA) system (Xiao et al., 2014).

The hardening of microcapsules produced by complex coacervation is crucial to improve their mechanical and thermal stability to a broad range of food processing conditions. This step usually involves the covalent modification of the encapsulant, for example, by crosslinking. Chemical crosslinking agents such as formaldehyde and glutaraldehyde are effective but cannot be used in food and pharmaceutical applications due to concerns related to toxicity. Safer crosslinking agents such as tannic acid, genipin,

and iridoids have been explored (Xiao et al., 2014; Gouin, 2004). Enzymatic crosslinking is another interesting and safe alternative for microcapsule hardening. For example, microcapsules were treated with transglutaminase (TG; amine γ-glutamyl transferase, EC 2.3.2.13) which crosslinks proteins by catalyzing the formation of isopeptide bonds between lysine and glutamine residues (Soper & Thomas, 2000). However, long treatment times are required for TG-catalyzed crosslinking of microcapsules which may limit industrial scale applications. Also, measures must be taken to deactivate TG in the TG-modified preparation but otherwise their use may be limited to applications where TG activity is desirable or inconsequential.

Studies have shown that the functional properties of food proteins may be improved by conjugation with polysaccharides. The Maillard reaction is one of the most promising approaches for producing protein-polysaccharide conjugates for food applications at the industrial scale (Kato, 2002). When solid or aqueous mixtures of protein and polysaccharide are heated, Maillard reaction is initiated by a condensation reaction between amino groups of protein and carbonyl groups on the reducing-ends of polysaccharides. Protein-polysaccharide conjugates are formed at this initial stage but a very wide variety of break-down and polymerization products are formed at the intermediate and final stages (Nursten, 2005a). Altogether, these products contribute to the enhanced emulsifying, antioxidant, and antimicrobial properties, as well as improved thermal stability and reduced allergenic properties reported for Maillard-type protein-polysaccharide conjugates (Kato, 2002). Microcapsules have been successfully produced by spray drying oil-in-water emulsions stabilized by Maillard-type protein-polysaccharide conjugates (Augustin, Sanguansri, & Bode, 2006). However, little has

been done to explore the potential of using Maillard-type conjugates as encapsulants for microencapsulation by complex coacervation. Therefore, the objective of this work was to compare the microencapsulation of SDASO in the classical gelatin-gum arabic system with a Maillard reaction-modified gelatin-gum arabic system. Microcapsules produced using either system were characterized in terms of particle size, morphology, *in-vitro* release, and oxidative stability. Correlations between the antioxidant capacities of the encapsulants in sol state and oxidative stability in corresponding microcapsules were also examined. Furthermore, the applicability of microcapsules in formulating an SDA omega-3 fortified yogurt was assessed for thermal and oxidative stability.

Materials and methods

Materials

Stearidonic acid soybean oil (SDASO) was donated by Monsanto Co. (St. Louis, MO). Porcine gelatin (Type A, ~300g bloom), gum arabic, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, ABTS), butylated hydroxytoluene (BHT), bile salts (sodium cholate: sodium deoxycholate, 50:50 [w/w])**,** horseradish peroxidase (HRP, 950-2000 units/mg solid, based on ABTS substrate), pepsin from porcine gastric mucosa (≥ 250 units/mg solid, based on hemoglobin substrate), pancreatin (8 × US Pharmacopeia [USP] specifications; lipase, amylase, and protease activities of 33, 200, and 200 USP units/mg, respectively), and lipase (type II, 100-500 units/mg protein, based on olive oil substrate) from porcine pancreas were purchased from Sigma-Aldrich (St. Louis, MO). Transglutaminase (90 U/g) was from C&P additives (Boca Raton, FL). Criterion XT 3-8% tris-acetate precast gels, XT sample (Laemmli) and tricine buffer, Bio-Safe Coomassie blue stain, and Precision Plus protein standards were purchased from Bio-Rad

Laboratories, Inc. (Hercules, CA). Pierce glycoprotein staining kit was from Thermo Fischer Scientific, Inc. (Rockford, IL). Pasteurized skimmed milk and non-fat dry milk were purchased from a local store. Freeze dried yogurt starter culture was obtained from Euro-Cuisine, Inc. (Los Angeles, CA). The starter culture contained a blend of *Lactobacillus delbrueckii* subsp *bulgaricus*, *Streptococcus thermophilus,* and *Lactobacillus acidophilus*.

Microencapsulation in the Classical Gelatin-Gum Arabic System

Microcapsules were prepared as described by Green and Schleicher (1957) with some modifications. Five grams each of gelatin and gum arabic were completely dissolved in 150 g of deionized water at 50 °C to yield a 6.7% gelatin-gun arabic (1:1, w/w) sol. SDASO (30g) was dispersed in the sol using one of three levels of homogenization. At the low homogenization (LH) level, a StedFast stirrer model SL 2400 fitted with a 60mm, 4-blade general purpose propeller (Fisher Scientific, Co., Fair Lawn, NJ) was used at setting 2.5 (~ 600 rpm). At the high homogenization (HH) level, a Polytron PT 10-35 homogenizer fitted with a PTA 7 dispersion aggregate (Kinematica, Inc., Bohemia, NY) was used at setting 5 (~ 19,400 rpm) and at a higher homogenization (HH′) level, setting 6 (~ 21,600 rpm) was used. Homogenizations were performed at 50 °C for 15 min. The resulting emulsion which now contained gelatin, gum arabic, and SDASO at a ratio of 1:1:6 (w/w/w) was transferred to a temperature-controlled reaction vessel at 50 °C and continuously stirred at 200 rpm. Next, 55 g of deionized water was added to bring the total biopolymer concentration to 4.9% (w/w). The pH of the emulsion was adjusted to 4.4 with 10% (w/w) citric acid. The batch was cooled at ~1 °C/min to 10 °C to yield a suspension of microcapsules which was weighed, transferred to opaque

Nalgene containers, flushed with nitrogen, and stored at 4 °C. These microcapsules served as controls (C) and three types were produced, namely, CLH, CHH, and CHH′, corresponding to different homogenization treatments.

A portion of each control preparation was treated with TG to yield corresponding TG-modified microcapsules, namely, TLH, THH, and THH′. TG powder was added to the microcapsule suspension at 0.5% (w/w) and incubated at 25 °C for 12 h with continuous stirring at 100 rpm. TG-modified microcapsules were weighed, transferred to opaque Nalgene containers, flushed with nitrogen, and stored at 4 °C.

Microencapsulation in a Maillard Reaction-Modified Gelatin-Gum Arabic System

Maillard reaction between gelatin and gum arabic was initiated by controlled dry heating using the method described by Kato et al. (1992) with some modifications. A gelatin and gum arabic (1:1, w/w) sol was prepared and subsequently freeze-dried with a Unitop 600L, Freezemobile 25SL unit (The Virtis Company, Gardiner, NY). The sample was pre-frozen at -40 °C and then dried for 24 h at 0.13 mbar to a final temperature of 25 °C. The freeze-dried cake was pulverized using a Kitchen Aid coffee grinder (Kitchen Aid, St. Joseph, MI) and then incubated at 80 °C for 5 h under ~ 79% relative humidity in a closed desiccator containing saturated KI solution at the bottom. After incubation, the Maillard reaction product (MRP) cake formed was pulverized with a coffee grinder. The color of the freeze-dried powder, before and after Maillard reaction, was analyzed using a CR-410 Chroma Meter (Minolta Co., Ltd, Japan). Color values were expressed in polar coordinates, where L^* defines lightness, C^* specifies chroma, and $h°$ denotes hue angle. Next, 10 g of MRP powder was completely dissolved in 150 g of deionized water at 70 °C to give a 6.7% MRP sol. SDASO (30g) was dispersed in the MRP sol with each of the

three homogenization treatments specified previously. The resulting MRP/SDASO (1:3, w/w) emulsion was transferred to a temperature-controlled reaction vessel at 50 °C and continuously stirred at 200 rpm. This was followed by addition of 55 g of deionized water to bring total biopolymer concentration to 4.9% (w/w). Adjustment of pH, cooling and storage was performed exactly as described for the control. Three types of Maillard reaction (MR)-modified microcapsules were obtained, namely, MLH, MHH, and MHH′, corresponding to different homogenization levels. Overall, nine different microcapsules were prepared by varying the encapsulant (control, TG-, and MR-modified) and level of homogenization during microcapsule preparation (LH, HH, and HH′)

SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm the covalent modification of encapsulants by TG and Maillard reaction. Encapsulants were isolated from LH-type microcapsules. One milliliter of LH-type microcapsule suspension was added to 5 mL of 2% SDS solution and subjected to 30 min sonication. This was followed by 5 min centrifugation at 2000 rpm using a Dynac II benchtop centrifuge (Becton, Dickinson & Co., Franklin Lakes, NY). The supernatant was discarded while the pellet which contained mostly oil-free coacervates was washed twice with 10 mL hexane to remove traces of oil, filtered, and dried over silica gel in vacuum for 24 h at 25 °C. Samples were then solubilized in Laemmli sample buffer by heating at 70 °C for 10 min. The final biopolymer concentration for the gelatin and gum arabic sample was 4 µg/µL while that of dried coacervates was 8 µg/µL. After heating, samples were centrifuged at 2000 rpm for 15 min and 5 - 10 µL of clear supernatant was loaded alongside protein standards onto two tris-acetate gels in a Criterion

electrophoresis cell (Bio-Rad Laboratories, Inc., Hercules, CA). Electrophoresis was performed at constant voltage (200 V) for 45 min in tricine buffer. After electrophoresis, one gel was stained for protein with Coomassie blue while the other gel was stained for glycoprotein with periodic acid-Schiff reagent as per manufacturers' instructions.

Antioxidant Activity

ABTS free radical scavenging activities of gelatin, gum arabic, control, TG-, and MR-modified sols were measured using the ABTS/H_2O_2/ HRP decoloration method (Arnao, Cano, and Acosta, 2001). Gelatin and gum arabic were separately dissolved in deionized water to a final concentration of 4.9% (w/w). The gelatin and gum arabic sols were then blended at 1:1 (v/v) ratio to yield the control sol. A portion of the control was then treated with 0.5% (w/w) TG at 25 °C for 12 h to yield the TG-modified sol. The MR-modified sol was prepared by dissolving the MRP powder in deionized water to a final concentration of 4.9% (w/w). The assay mixture contained 2mM ABTS, 15 µM H_2O_2, and 0.25 µM HRP in 50 mM sodium phosphate buffer (pH 7.5) in a total volume of 1mL. After the reaction mixture gave a stable absorbance reading at 730 nm, 10 µL of sol (sample) or deionized water (blank) was added. Absorbance at 730 nm was measured after 10 min of incubation at 25 °C. The loss of absorbance was determined from the difference between the sample and blank. This difference is proportional to the amount of ABTS radical cation quenched by the sol. Antioxidant activity was expressed as mg trolox equivalents per dry weight of biopolymer (mg/10 g). Calculations were based on a dose-response curve of loss of absorbance against varying amounts (0-200 nmol) of trolox.

Microcapsule Morphology

One drop of microcapsule suspension was applied onto a glass slide and diluted with a few drops of deionized water. Microcapsules were directly viewed through the low power (10×) objective of a Leitz Laborlux K binocular microscope (Ernst Leitz, Wetzlar Germany). Images of microcapsules were captured from the 10× eyepiece on a handheld smart phone.

Particle Size Distribution

Particle size distribution was measured with an LS 13 320 MW particle size analyzer (Beckman Coulter, Inc., Miami, FL) using the Fraunhofer optical model. Samples were added drop-wise to deionized water in the dispersion cell until an obscuration value of 9-11% was achieved. The real and imaginary refractive indexes (RI) of particle were 1.471 and 0, respectively, while the RI of the suspension fluid was 1.332. Particle size distribution measurements were carried out under low sonication and volume mean diameters ($D_{4,3}$) were reported.

Encapsulation Efficiency

Encapsulation efficiency (EE) was calculated by estimating solvent extractable and total oil in microcapsule suspensions. To estimate solvent extractable oil, 5 g of suspension was agitated thoroughly with 50 mL petroleum ether for 15 min using a vortex mixer. The mixture was transferred to a separatory funnel and left to stand for 2 h to allow phase separation. The organic layer was carefully collected, washed with saturated NaCl solution, and transferred to a pre-weighed round-bottom flask. Solvent was removed under reduced pressure at 60 °C to a constant mass using a rotary

evaporator. Solvent extractable oil was then determined gravimetrically and expressed as % (w/w) of suspension.

To estimate total oil, 1 mL of 10 M HCl was added to 10 g of suspension and agitated at 60 °C for 3 h. After cooling to room temperature, mixture was transferred to a separatory funnel and extracted twice with 20 mL petroleum ether/diethyl ether (1:1, v/v). Organic layers were combined, washed with saturated NaCl solution, and transferred to a pre-weighed round bottom flask. Solvent was removed under reduced pressure at 60 °C to a constant mass using a rotary evaporator. Total oil was then determined gravimetrically and expressed as % (w/w) of suspension. EE (%) was calculated as follows:

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

In Vitro Digestion

Simulated digestion of microcapsules was performed using a standardized static *in vitro* digestion method (Minekus et al., 2014). Although the static *in vitro* digestion model is based on current understanding of *in vivo* digestion conditions, it does not take the dynamic aspects of digestion into account. So, the model was used to compare the accessibility of different microcapsules to digestive enzymes under simulated physiological conditions but the results do not reflect what would be obtained *in vivo*. Oil release was determined after simulated digestion in the gastric phase while the extent of lipolysis was determined after sequential digestion in gastric and intestinal phases. For the gastric phase, 10 g of microcapsule suspension was mixed with 7.5 mL of simulated gastric fluid (SGF) stock solution, 1.6 mL porcine pepsin stock solution (25 000 U mL^{-1}) made up in SGF stock solution, 5μL of 0.3 M $CaCl_2$, and pH was adjusted to 3.0 with 1

M HCl. The mixture was stirred in a shaking water bath set at 37 °C and 120 rpm for 2 h. After incubation, pH in the gastric phase was neutralized by adding 0.5 M sodium bicarbonate. The neutralized digesta was transferred to a separatory funnel and extracted twice with 50 mL petroleum ether/diethyl ether (1:1, v/v). The amount of oil released during the simulated gastric digestion was determined gravimetrically as was solvent extractable oil.

For the intestinal phase, the gastric chyme (~20 mL) was mixed with 11 mL of simulated intestinal fluid (SIF) stock solution, 5.0 mL of pancreatin stock solution with additional porcine pancreatic lipase (16 000 U mL^{-1} based on lipase activity), 2.5 mL of bile salt solution (80mM sodium cholate and sodium deoxycholate), 40 μL of 0.3 M $CaCl_2$, 1.31 mL of water, and pH was adjusted to 7.0 with 1 M NaOH. The mixture was stirred in a shaking water bath set at 37 °C and 120 rpm for 2 h. Ten milliliter aliquots of digesta were collected at zero time (blank) and after the 2 h incubation. Aliquots (10 mL) were added to 20 mL of 96% (v/v) ethanol containing 2 drops of 1% (w/v) thymolphthalein indicator titrated with 0.1 N NaOH to a light blue end point. The concentration of FFA per aliquot was determined using the following equation:

$$\text{FFA } (\mu\text{mol/ml}) = ([\text{Vs} - \text{Vb}] \times \text{N} \times 1000)/\text{Volume of aliquot (ml)}$$

Where, N is normality of NaOH and Vs and Vb represent the volumes (ml) of NaOH at sample and blank end points, respectively.

To determine the extent of lipolysis, the total amount of FFA released per assay was expressed as a percentage of two-thirds the amount of fatty acids present in 1.2 g of SDASO based on estimated total oil content in 10 g of microcapsule suspension. The

average molecular weight of SDASO was calculated to be 867.4 g/mol based on fatty acid composition.

Oxidative Stability of Microcapsules

Lipid oxidation was studied as a function of encapsulant type and particle size. Peroxide (PV) and *p*-anisidine (*p*-AV) values of microcapsules produced at LH and HH levels were measured weekly over 28 days of storage at 4 °C. Microcapsules produced at the HH′ level were excluded because particle size did not correlate properly with homogenization at this level, particularly for the control and TG-modified categories. A modified version of the International Dairy Foundation (IDF, 1995) standard method 172:1995 for extracting lipids and lipid-soluble compounds from milk was used to extract a portion of microencapsulated oil from suspensions. Twenty grams of suspension was mixed with 16 mL of 96% (v/v) ethanol and 4 mL of 7 M NaOH in a separatory funnel. Next, 20 mL of diethyl ether containing 0.01% (w/v) BHT was added followed by vigorous shaking for 1 min. After phase separation, 20 mL of hexane was added, mixed carefully, and allowed to undergo phase separation. The aqueous layer was discarded while the organic layer was washed with 20 mL of 1 M HCl followed by 20 mL of 0.4 M Na_2SO_4 solution. The organic layer was then transferred to a pre-weighed conical flask and the solvent evaporated by flushing with nitrogen. Primary lipid oxidation products in extracted SDASO samples were measured by PV based on the IDF standard method described by Shantha & Decker (1994). Secondary lipid oxidation products, specifically aldehydes produced from the breakdown of lipid hydroperoxides, were measured by *p*-AV according to the American Oil Chemists' Society (AOCS, 2011) Official Method Cd

18-90. The total oxidation (TOTOX) value on the 28th day was calculated using the following equation:

$$\text{TOTOX value} = 2(\text{PV}) + p\text{AV}$$

Formulation of SDA Fortified Omega-3 Yogurt

SDA fortified yogurts were formulated using control, TG-, and MR-modified microcapsules produced at LH- and HH-levels. Microcapsules had been in storage for 7 days at 4 °C before use in formulating yogurts. The solids content of skimmed milk was adjusted to ~ 12% by dispersing 100 g non-fat dry milk in 3825 g skimmed milk using a batch stirrer. Microcapsule suspension (12.5 g) was mixed with 157.5 g of milk base to yield ~ 1.5 g SDASO or 375 mg SDA per serving (170 g) of fortified milk base. This level of fortification was chosen based on a conversion efficacy of SDA to EPA of 6:1, so that four daily servings of fortified yogurt would be equivalent to 250 mg EPA per day (Decker, Akoh, & Wilkes, 2012). Six replicates of fortified milk bases were formulated for each type of microcapsule. Non-fortified and fortified milk bases (170 g) were heated at 80-85 °C for 30 min on a hot plate stirrer. The percentage of total oil released to the milk surface after heating was determined for 3 replicates of fortified samples as solvent extractable oil. The percentage of total oil released was used as a measure of microcapsule thermal stability. Non-extracted, heat treated milk bases were transferred to pre-weighed glass jars, and cooled to 43 °C in a water bath. Yogurt starter culture was then added at ~ 0.42% (w/w) according to the manufacturer's instructions. Samples were incubated until a pH of 4.5 ± 0.1 was achieved. The liquid whey on the yogurt surface,

after the incubation period, was carefully removed and weighed before samples were stored at 4 °C.

Physical Properties of Yogurts

Spontaneous separation of whey from yogurt gels during fermentation was measured as described by Lee and Lucey (2010). The degree of whey separation was calculated with the following equation:

$$\text{Whey separation (\%)} = (\text{Weight of whey}/\text{Total weight of yogurt}) \times 100$$

The water holding capacity (WHC) of cooled yogurt samples was determined using the method described by Tamjidi, Nasirpour, & Shahedi (2012). Twenty grams of yogurt was centrifuged for 10 min at 3200 rpm. After centrifugation, the supernatant (whey) was removed and weighed. WHC was calculated using the following equation:

$$\text{WHC (g/kg)} = 1000 - ([\text{Weight of whey}/\text{Total weight of yogurt}] \times 1000)$$

Color analysis of yogurts was carried out using a CR-410 Chroma Meter. Color values were expressed in polar coordinates, where L* defines lightness, C* specifies chroma, and h° denotes hue angle. Using the non-fortified yogurt as reference, ΔE values for fortified yogurts were determined to assess total color difference.

Oxidative Stability of SDA Fortified Yogurts

Lipid oxidation in yogurts containing control, TG-, and MR-modified microcapsules produced at LH and HH levels was monitoring by measuring PV and *p*-AV after 14 days of storage at 4 °C. Fourteen days was chosen because this is the expected shelf-life of yogurt under typical refrigeration conditions. SDASO was extracted from fortified yogurts using the IDF standard method 172:1995. PV was determined based on the IDF standard method described by Shantha & Decker (1994)

while, *p*-AV was determined according to the AOCS Official Method Cd 18-90. PV and *p*-AV values at 0 and 14 days of storage were determined to estimate primary and secondary lipid oxidation, respectively, in yogurt during the storage period. The change PV and *p*-AV after storage was determined by difference and reported.

Statistical Analysis

All measurements were performed in triplicate. Results were expressed as mean ± SD. ANOVA was carried out and mean comparisons were performed using Duncan's multiple range tests. Significant differences were determined at $p < 0.05$. Statistical analysis was performed on Microsoft Excel using the DSAASTAT version 1.101 macro (DSAA, Perugia, Italy).

Results and discussion

Properties of Encapsulants

Color measurement

The freeze-dried gelatin-gum arabic powder turned from white to brownish yellow after heating. The odor of MRP cake was reminiscent of baked pastry while that of the gelatin-gum arabic powder was relatively bland. Comparing color values before and after Maillard reaction, the MRP powder was darker (ΔL^* = -11.86), more saturated (ΔC^* = +18.22), and more yellow in hue ($\Delta h°$ = +28.22). The development of flavor and color was due to products formed at intermediate and final stages of the Maillard reaction (Nursten, 2005a).

SDS-PAGE analysis

Electrophoresis profiles of gelatin and gum arabic along with the control, TG- and MR-modified encapsulants are shown in Figure 5.1. The profile of the control (lane 4)

was similar to that of gelatin (lane 2) when visualized in protein stain (Figure 5.1A). This is in agreement with the consensus that complex coacervation is driven only by non-covalent interactions which do not alter the molecular weight distribution of gelatin (Xiao et al., 2014). However, the profile for the TG-modified encapsulant (lane 5) showed increased band intensity above 250 kDa and reduced band intensity between 37 - 150 kDa. This indicates that TG-induced crosslinking of gelatin polypeptides occurred which resulted in the formation of higher molecular weight polypeptides with concomitant depletion of lower molecular weight peptides. Similar electrophoretic profiles have been reported for TG-modified gelatin (Yi et al., 2006; Bae et al., 2008). Conversely, the profile for the MR-modified encapsulant (lane 6) showed markedly reduced band intensity above 250 kDa and increased band intensity between 37 – 100 kDa. This was possibly due to heat-induced fragmentation of large (>250 kDa) gelatin polypeptide fractions into lower molecular weight fractions. In addition, the smearing of bands that were otherwise distinct in the control is indicative of heat-induced crosslinking of polypeptides (Guo et al., 1995; Chung, Sanguansri, & Augustin, 2011). Polypeptide crosslinking is known to occur along side with fragmentation during thermal treatment of gelatin (Kozlov, 1983).

Electrophoretic profiles for gelatin, gum arabic, and encapsulants observed in glycoprotein stain are shown in Figure 5.1B. Staining intensities for the control (lane 4) and TG-modified encapsulant (lane 5) were similar to that of gum arabic (lane 3). However, the MR-modified encapsulant (lane 6) retained more of the staining reagent which was indicative of a higher glycoprotein concentration and may be accounted for by the formation of new protein-polysaccharide conjugates as a result Maillard reaction.

Overall, the control contained unmodified gelatin while TG-modified encapsulants contained polymerized gelatin fractions formed by protein-protein crosslinks. On the other hand, the MR-modified encapsulant contained a combination of protein-protein and protein-polysaccharide crosslinks in addition to low molecular weight polypeptide fragments.

Free radical scavenging activity

Table 5.1 shows the ABTS free radical-scavenging activities for the gelatin and gum arabic sols, as well as the control (C-[GE-GA]), TG-modified (T-[GE-GA]), and MR-modified (M-[GE-GA]) sols. Assuming that activities of gelatin and gum arabic sols were additive, C-[GE-GA] would be expected to have a value close to 8.17 mg Trolox eq/10 g. However, the observed activity for C-[GE-GA] was lower than this expected value suggesting that the association between gelatin and gum arabic in solution had an antagonistic effect on overall antioxidant activity. Interestingly, the treatment of C-[GE-GA] with TG resulted in a complete loss of antioxidant activity. Instead, pro-oxidant activity was observed as indicated by the negative sign because T-[GE-GA] samples gave higher absorbance readings than the assay blank. Since partial proteolysis of gelatin to lower molecular peptides is known to increase free radical scavenging activity (Chang et al. 2013), it follows that polymerization of gelatin would yield the opposite effect. So, the loss of antioxidant activity in T-[GE-GA] may be associated with the depletion of the low molecular weight peptide fractions of gelatin. However, the basis for the pro-oxidant activity is unclear. Conversely, the observed free radical scavenging activity for M-[GE-GA] was about three times the expected additive value which is indicative of a synergistic antioxidant effect in M-[GE-GA] relative to C-[GE-GA]. Heat-induced

fragmentation of gelatin to lower molecular weight peptides as well as other potentially reducing MRP such as reductones and melanoidins may have contributed to the overall antioxidant activity of M-[GE-GA] (Eichner, 1979; Nursten, 2005b).

Properties of Microcapsules

Particle morphology and size distribution

The morphologies of different microcapsule types can be observed from photomicrographs in Figure 5.2. Microcapsules produced at the LH level were mononuclear, in other words, they had one oil droplet in the core. These LH-type microcapsules had similar mean diameters (Table 5.2) and size distributions (Figure 5.3A). The geometry of control and TG-modified microcapsules (Figures 5.2A & B, respectively) could be best described as prolate spheroid. A similar geometry has been reported for mononuclear microcapsules made by complex coacervation (Soper and Thomas, 2000; Gouin, 2004). MR-modified microcapsules were roughly spherical (Figure 5.2C) with capsular walls which appeared to be less hydrated compared to the control. Bungenberg de Jong (1965b) pointed out that the amount of unbound water in gelatin-gum arabic coacervates is generally very large compared to the true hydration water. However, since dry heated gelatin exhibits markedly reduced water solubility (Kozlov and Burdygina, 1983), it is reasonable to perceive that MR-modified coacervates had a reduced tendency to hydration. Given that the magnitude of effective attraction between oppositely charged colloids in a coacervate is inversely associated with the tendency to hydration (Bungenberg de Jong, 1965b), capsular walls formed by MR-modified coacervates may have been more compact than those of the control.

At the HH level, combinations of mono- and multi-nucleated microcapsules could be observed for control, TG-, and MR-modified microcapsules (Figures 5.2D, E, & F, respectively). These microcapsules had similar size distributions (Figure 5.3B) and, as expected, smaller mean diameters than LH-type microcapsules (Table 5.2). However, the HH-type control and TG-modified (Figure 5.2E) microcapsules showed a tendency to flocculate over time, whereas, the MR-modified microcapsules (Figure 5.2F) seemed more stable to flocculation. At the HH′ level, control and TG-modified microcapsules were irreversibly agglomerated clusters of mono- and multi-nucleated microcapsules (Figures 5.2G & H, respectively). As a result of particle agglomeration, HH′-type control and TG-modified microcapsules had higher mean particle size than observed at the HH level (Table 5.2). A similar observation was made by Liu, Low, and Nickerson (2010) who reported a transition from spherical mononuclear to irregular-shaped multinuclear microcapsules with increasing homogenization speed during microencapsulation of flaxseed oil in gelatin-gum arabic coacervates. However, for the MR-modified category, microcapsules produced at the HH′ level were a combination of mono- and multi-nucleated microcapsules stable to agglomeration (Figure 5.2I). Consequently, MR-modified HH′-type microcapsules had a lower mean particle size than was observed at the HH level (Table 5.2). Particle size distributions of HH′-type microcapsules are compared in Figure 5.3C and the effect of particle agglomeration is evident in the control and TG-modified categories. With respect to odor, MR-modified microcapsule suspensions had very slight roasty and earthy odor notes while the control and TG-modified preparations were relatively bland.

Gelatin-gum arabic coacervates are recognized for their tendency to flocculate when present in a highly dispersed state (Bungenberg de Jong, 1965a), and this may be partly responsible for the agglomeration of control microcapsules produced at the HH′ level. Evidently, particle agglomeration disrupted the expected correlation between particle size and the level of shear used for the HH′ treatment. However, it is remarkable that MR-modified coacervates were more resistant to agglomeration so that microcapsule size could be controlled as a function of homogenization shear rate. A previously reported approach to preventing the agglomeration of gelatin-gum arabic coacervate microcapsules required maintaining a critical level of turbulence (Reynolds number > 15000) in the suspension during the gelation (cooling) phase (Lemetter, Meeuse, & Zuidam, 2009). Equilibrium agglomerate size decreased with increasing share rates due to reduced inter-particle contact time but accompanying increases in particle collision frequency may have disrupted the proper formation of capsular walls.

MR-modified gelatin-gum arabic coacervates provided a more convenient means to minimize particle agglomeration so that size reduction could be achieved without losing colloidal stability. One possible explanation for the enhanced colloidal stability of MR-modified microcapsules may be derived from principles for the stabilization of protein-polysaccharide complexes at oil-water interfaces (Dickinson, 2008). Hence, in the control where gelatin and gum arabic were fully intermixed at the interface in a single electro-neutral layer, particle surfaces tended to have net attraction at short range, leading to a potentially unstable colloidal system. Whereas in the MR-modified system, the presence of Maillard-type gelatin-gum arabic conjugates at the interface resulted in a secondary polysaccharide layer which imparted steric repulsion between particle

surfaces, leading to improved colloidal stability. Lemetter, Meeuse, & Zuidam (2009) pointed to the wide gelation range of the gelatin-gum arabic coacervate as a contributing influence that allowed particle agglomeration to occur during the cooling phase. In view of this hypothesis, it is also possible that thermal modification of gelatin favorably altered the gelling behavior of MR-modified coacervate.

Encapsulation efficiency and in vitro digestibility

Encapsulation efficiency and *in vitro* digestibility data are shown in Table 5.2. Microcapsules in suspension exhibited impressively high encapsulation efficiencies, averaging 99.3% across LH-type and 99.8% across HH- and HH′ microcapsules. Among LH-type microcapsules, oil release in the gastric phase was highest for the control, followed by TG-modified category, but lowest for the MR-modified category. Lower oil release from TG- and MR-modified microcapsules was due to the presence of covalent crosslinks within their capsular walls. Electrophoretic patterns (Figure 5.1) suggested protein-protein crosslinks in the TG-modified encapsulant and a combination of protein-protein and protein-carbohydrate crosslinks in the MR-modified encapsulant. Previous studies that compared *in vitro* gastric release of fish oil encapsulated in protein-polysaccharide physical blends versus corresponding heat-treated MRP blends reported reduced oil release in the latter (Chung, Sanguansri, & Augustin, 2011; Kosaraju, Weerakkody, & Augustin, 2009). The presence of protein-protein and/or protein-carbohydrate crosslinks in the encapsulant wall may reduce *in vitro* digestibility when the bond involved in the crosslink is resistant to enzyme-catalyzed proteolysis (Buchert et al., 2010). Oil release generally decreased with increasing homogenization level across different types of encapsulants. This is because increasing homogenization speed

promoted the formation of multinucleated microcapsules which release their contents more slowly over time compared to mononuclear microcapsules which would release their contents in a single burst (Liu, Low, & Nickerson, 2010). Among HH- and HH′-type microcapsules, more oil was released from MR-modified categories than control and TG-modified counterparts. However, there was no significant difference in oil release between HH- and HH′-type microcapsules in the control and TG-modified categories. This effect may be a direct consequence of particle agglomeration in simulated gastric conditions. On the other hand, there was progressive decrease in oil release between LH-, HH-, and HH′-type MR-modified microcapsules. This implied a strong correlation between particle size and oil release in the MR-modified system. Therefore, it may be possible, with the MR-modified system, to fine-tune the release properties of microcapsules by varying homogenization levels.

Among control and TG-modified microcapsules, there was a direct association between the amount of oil released during the gastric phase and the extent of lipolysis. However, within the MR-modified category there was an inverse relationship between gastric oil release and the extent of lipolysis. Based on these observations, the extent of lipolysis in control and TG-modified microcapsules was mainly influenced by particle agglomeration while in MR-modified microcapsules, particle size was more important. Specifically, in control and TG-modified microcapsules, the extent of lipolysis reduced with increasing particle agglomeration while in MR-modified microcapsules, where particle agglomeration was curtailed, the extent of lipolysis increased with reducing particle size.

Oxidative stability

Oxidative stability was assessed based on the ability of the microcapsules to minimize the formation of fatty acid hydroperoxides (Figure 5.4A) and aldehyde compounds as secondary products of lipid oxidation (Figure 5.4B). In addition, 28th day TOTOX values for control, TG-, and MR-modified microcapsules are compared in Figure 5.4C. From the results shown in Figure 5.4, lipid oxidation of SDASO in microcapsules of the MR-modified category was maintained at lower levels compared to the control and TG-modified categories. Lipid oxidation was highest in the latter. Independent of the type of encapsulant, HH-type microcapsules generally had higher PV and *p*-AV compared to the LH-type microcapsules. This implied an inverse relationship between particle size and lipid oxidation rate which is plausible since reduction in oil droplet size increases surface area to volume ratio (Laurent, Métro, & Genot, 2002). However, it is noteworthy that differences between PV of HH- and LH-type microcapsules of the MR-modified category was small compared to what was observed for the control and TG-modified categories. From Figure 5.4A, it can be seen that HH- and LH-type microcapsules of the MR-modified category had nearly equal PV for the first 14 days, whereas in control and TG-modified categories, differences between PV of LH- and HH-type microcapsules were relatively large and increased gradually over time. Furthermore, the difference between 28th day TOTOX values of HH- and LH-type microcapsules was 15 units for the MR-modified category but ~ 67 units for the control and TG-modified categories (Figure 5.4C). These observations suggest that the potential for lipid oxidation rates to increase with reduction in particle size was buffered by the MR-modified system. Additionally, from Figure 5.4C, there was an inverse relationship

between the free radical scavenging activity of encapsulant in sol state and the 28th day TOTOX values of SDASO in corresponding microcapsule. This implies that the superior oxidative stability of MR-modified microcapsules could be associated with the enhanced antioxidant properties of the MR-modified sol, while the inferior oxidative stability of TG-modified microcapsules could be related to the pro-oxidative activity of the TG-modified sol. Antioxidant mechanisms in MR-modified coacervates are likely to include the inactivation of fatty acyl free radicals, oxygen scavenging, and reduction of hydroperoxides at the interfacial layer (Eichner, 1979). Also, MR-modified coacervates may form a more compact capsular wall that provides physical protection from oxygen and other factors that could promote lipid oxidation. Another antioxidant mechanism of MRP is the chelation of pro-oxidant metal ions which may be relevant but additional studies would be required to provide confirmation.

Thermal stability in yogurt base

Microencapsulated oil release during heat treatment (85 °C for 30 min) of fortified yogurt bases are shown in Table 5.3. Oil release during heat treatment in fortified milk base followed the same general trend as observed for oil release in gastric phase. Among LH-type microcapsules, heat stability was highest for the MR-modified category, followed by the TG-modified, and lowest for the control. Although the same trend was observed between HH-type microcapsules, it was not statistically significant ($p > 0.05$). Again, the increased heat stability of MR- and TG-modified microcapsules may be accounted for by crosslinking of the biopolymers that make up their capsular walls. HH-type microcapsules seemed to be more heat stable than LH-type microcapsules suggesting an inverse relationship between particle size and heat stability. However,

since small oil droplets could have been easily re-emulsified by milk proteins, the solvent extraction method may have underestimated how much oil was released from HH-type microcapsules by heat treatment.

Properties of Non-Fortified and SDASO-Fortified Yogurts

Physical properties

Physical attributes of non-fortified and fortified yogurts namely, color, whey separation, and water holding capacity are also shown in Table 5.3. Since a ΔE value > 3 indicates a color difference obvious to an untrained observer, fortified yogurts were comparable to the non-fortified yogurt in terms of appearance. This is in agreement with the results obtained by Tamjidi, Nasirpour, & Shahedi (2012) for yogurts fortified with fish oil encapsulated in gelatin-gum arabic coacervates.

Whey separation and WHC were measured to determine how fortification influenced yogurt gel network stability. Spontaneous whey separation is related to the configuration of protein-protein bonds of the gel network while WHC is associated with the resistance of the gel network to external compaction (Lee & Lucey, 2010). Vegetable oil-based yogurts have been reported to exhibit higher whey separation and less gel firmness than conventional yogurts (Tamime & Robinson, 1999). In the present study, however, whey separation and WHC in non-fortified and fortified yogurts were comparable. This was possibly because of the presence of gelatin in fortified yogurts which is used by some manufacturers to stabilize the yogurt gel against whey separation (Hutkins, 2006). Comparing within fortified yogurts types, the extent of whey separation was less in yogurts containing HH-type microcapsules compared to those containing LH-type microcapsules. Also, WHC was higher for yogurts containing HH-type

microcapsules compared to those containing LH-type microcapsules. These observations suggest that the size of microcapsules used for fortification influences yogurt gel network stability. Simply put, small microcapsules were less destabilizing to the gel network than the large microcapsules. Overall, yogurts fortified with TG-modified microcapsules showed greatest gel stability because they exhibited lowest whey separation and highest WHC. This may be due to the action of residual active TG from TG-modified microcapsules. TG has been reported to decrease whey separation, improve gel firmness, and WHC of yogurt by inducing the crosslinking of milk proteins (Buchert et al., 2010), which results in a more compact microstructure with reduced gel permeability so that more water can be retained within the gel network.

Oxidative stability of fortified yogurts

Oxidative stability was based on the ability of microcapsules to minimize increases in PV and *p*-AV of microencapsulated SDASO in yogurt after 14 days at 4 °C (Table 5.3). The expected shelf life for yogurt at typical refrigeration (0 – 4 °C) is 14 days. Based on changes in PV and *p*-AV after 14 days, yogurts fortified with MR-modified microcapsules exhibited greater oxidative stability than yogurts fortified with control and TG-modified microcapsules. As was observed with microcapsule suspensions, lipid oxidations rates were generally lower in yogurts fortified with LH- than HH-type microcapsules. It is worth mentioning that the PV of the yogurt fortified with MR–modified LH-type microcapsules remained virtually unchanged over the 14 day storage period, whereas those of the control and TG-modified counterparts increased by ~10 units. Antioxidant mechanisms in yogurts fortified using MR-modified microcapsules may include the reduction of hydroperoxides as well as radical chain

breaking, and oxygen scavenging activities. These results demonstrate that anti-oxidative components of MR-modified coacervates have good carry-through properties and this is in agreement with various studies that have demonstrated the ability of MRP to delay the development of rancidity and improve storage and oxidative stability in a wide variety of foods (Nursten, 2005b).

Conclusions

The classical gelatin-gum arabic system for microencapsulating oils by complex coacervation was improved upon thermal pre-treatment of the gelatin-gum arabic mixture by taking advantage of the Maillard reaction. This was done to initiate the crosslinking of gelatin and gum arabic prior to the complex coacervation process, potentially eliminating the need for the use of crosslinking agents after coacervation. The anti-oxidative products of Maillard reaction helped to minimize lipid oxidation in the MR-modified microcapsules during storage. Using omega-3 fortified yogurt as a model, the thermal and oxidative stability of the MR-modified system outperformed those based on the classical gelatin-gum arabic system. Other attributes such as enhanced capsule wall rigidity and colloidal stability indicate that MR-modified microcapsules could have a wide range of potential food applications. This makes the MR-modified gelatin-gum arabic system an attractive solution for the microencapsulation of oils by complex coacervation.

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Table 5.1 ABTS free radical scavenging activities of hydrocolloids and encapsulants as sols (4.9 wt%)

Hydrocolloid/ Encapsulant	Antioxidant activity (mg Trolox equivalents/10g)
Gelatin (GE)	10.57 ± 0.49b
Gum Arabic (GA)	5.78 ± 0.28c
C-[GE-GA][a]	4.65 ± 0.28c
T-[GE-GA]	-(1.46) ± 0.59d
M-[GE-GA]	23.73 ± 1.4a

[a] Abbreviations: C-[GE-GA], control gelatin-gum arabic (1:1, w/w) ; T-[GE-GA], transglutaminase-crosslinked gelatin-gum arabic (1:1, w/w); M-[GE-GA], Maillard reaction product of gelatin-gum arabic (1:1, w/w)

Means followed by same letters in a column are not significantly different ($p > 0.05$) according to Duncan's multiple range test

Table 5.2 Mean particle size, encapsulation efficiency, and *in vitro* digestion of control and modified microcapsules

Factors and levels				*In vitro* digestion	
Encapsulant	Homogenization	Particle size ($D_{4,3}$ [μm]) [a]	Encapsulation efficiency (%)	*Gastric phase* Oil released (%)	*Intestinal phase* Lipolysis (%)
C-[GE-GA]	LH	296.5 ± 75.2	99.3 ± 0.1b	53.1 ± 2.6a	55.7 ± 1.9a
	HH	56.4 ± 30.9	99.7 ± 0.1a	1.8 ± 0.1f	43.9 ± 0.8cd
	HH′	251.2 ± 138.9	99.8 ± 0.1a	1.7 ± 0.1f	38.1 ± 2.2e
T-[GE-GA]	LH	287.4 ± 98.9	99.4 ± 0.2b	45.9 ± 2.6b	52.5 ± 0.8a
	HH	55.4 ± 29.7	99.8 ± 0.0a	2.1 ± 0.1f	46.8 ± 3.0bc
	HH′	274.7 ± 156.7	99.8 ± 0.2a	0.8 ± 0.2f	41.0 ± 2.2de
M-[GE-GA]	LH	286.6 ± 135.9	99.3 ± 0.2b	24.8 ± 0.5c	39.0 ± 3.8de
	HH	45.3 ± 23.7	99.8 ± 0.1a	12.2 ± 0.2d	46.8± 6.0bc
	HH′	37.9 ± 22.7	99.9 ± 0.1a	3.8 ± 0.1e	50.6 ± 1.5ab

[a]Abbreviations: $D_{4,3}$, volume mean diameter, LH, low homogenization (batch stirrer at setting 2.5); HH, high homogenization (batch disperser at setting 5); HH′, higher homogenization (batch disperser at setting 6). See Table 5.1 for meanings of C-[GE-GA], T-[GE-GA], and M-[GE-GA]

Means followed by same letters in a column are not significantly different ($p > 0.05$) according to Duncan's multiple range test

Table 5.3 Properties of non-fortified and stearidonic acid soybean oil-fortified yogurts (Y): thermal and oxidative stability of control and modified microcapsules in fortified yogurts

Yogurt	Oil released during heat treatment (%)[a]	Whey separation (g/100g)	Water holding capacity (g/kg)	Color difference (ΔE)	Lipid oxidation after storage (14 days at 4 °C)	
					ΔPV [b]	Δ*p*-AV
Non-fortified	NA	0.59 ± 0.05a	699.39 ± 38.39b	Ref.	NA	NA
Y-CLH	26.4 ± 0.4a	0.59 ± 0.01a	699.39 ± 38.88b	0.66	10.61	4.07
Y-CHH	2.0 ± 0.2d	0.35 ± 0.07c	758.04 ± 8.77a	2.19	10.87	15.62
Y-TLH	12.3 ± 1.7b	0.46 ± 0.05bc	748.58 ± 9.30a	1.89	10.19	8.11
Y-THH	1.8 ± 0.3d	0.22 ± 0.06d	776.25 ± 14.30a	2.41	11.10	16.13
Y-MLH	8.6 ± 1.1c	0.57 ± 0.04ab	686.10 ± 26.36b	2.30	0.04	5.01
Y-MHH	1.1 ± 0.2d	0.46 ± 0.02bc	706.66 ± 10.82b	2.98	6.62	7.00

[a] 85 °C for 30 min

[b] Abbreviations: ΔPV, change in peroxide value; Δ*p*-AV, change in *p*- anisidine value; NA, not applicable; Ref; reference.
See Figure 5.4 for meanings of CLH, CHH, TLH, THH, MLH, and MHH

Means followed by same letters in a column are not significantly different ($p > 0.05$) according to Duncan's multiple range test

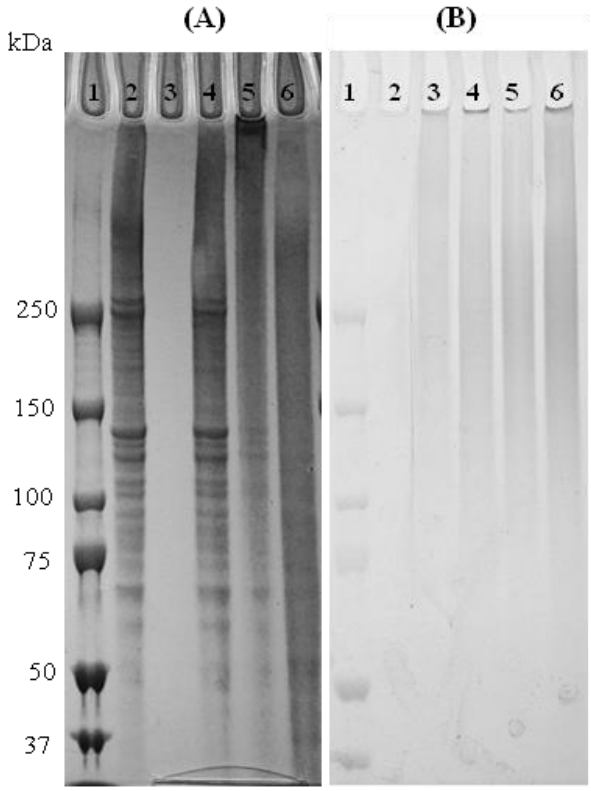

Figure 5.1 SDS-PAGE profiles of control and modified complex coacervates. A: protein stain; B: glycoprotein stain. The labeled lanes are: (1) molecular weight marker; (2) gelatin (GE); (3) gum arabic (GA); (4) control (GE-GA) coacervate; (5) transglutaminase-modified coacervate; (6) Maillard reaction-modified coacervate.

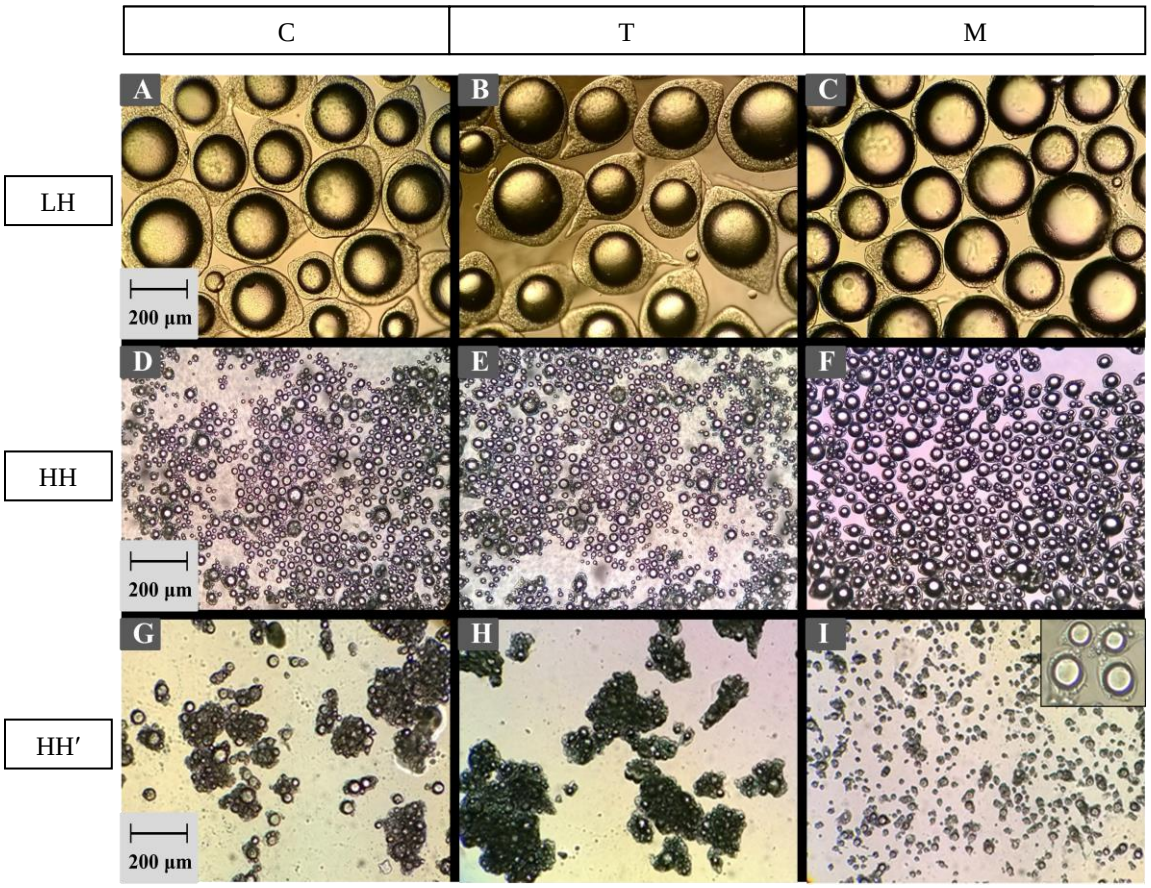

Figure 5.2 Photomicrographs of control (C), transglutaminase- (T), and Maillard reaction (M)- modified microcapsules produced at low (LH), high (HH), and higher (HH′) homogenization levels.

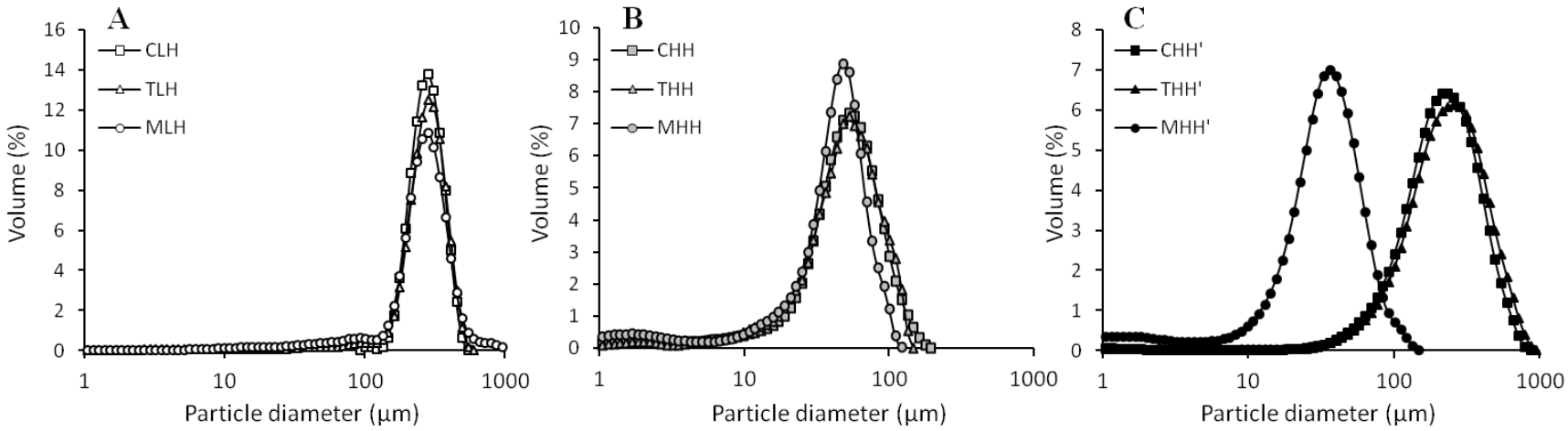

Figure 5.3 Particle size distributions of control (C), transglutaminase- (T), and Maillard reaction (M)- modified microcapsules produced at, A: low (LH); B: high (HH), and C: higher (HH′) homogenization levels.

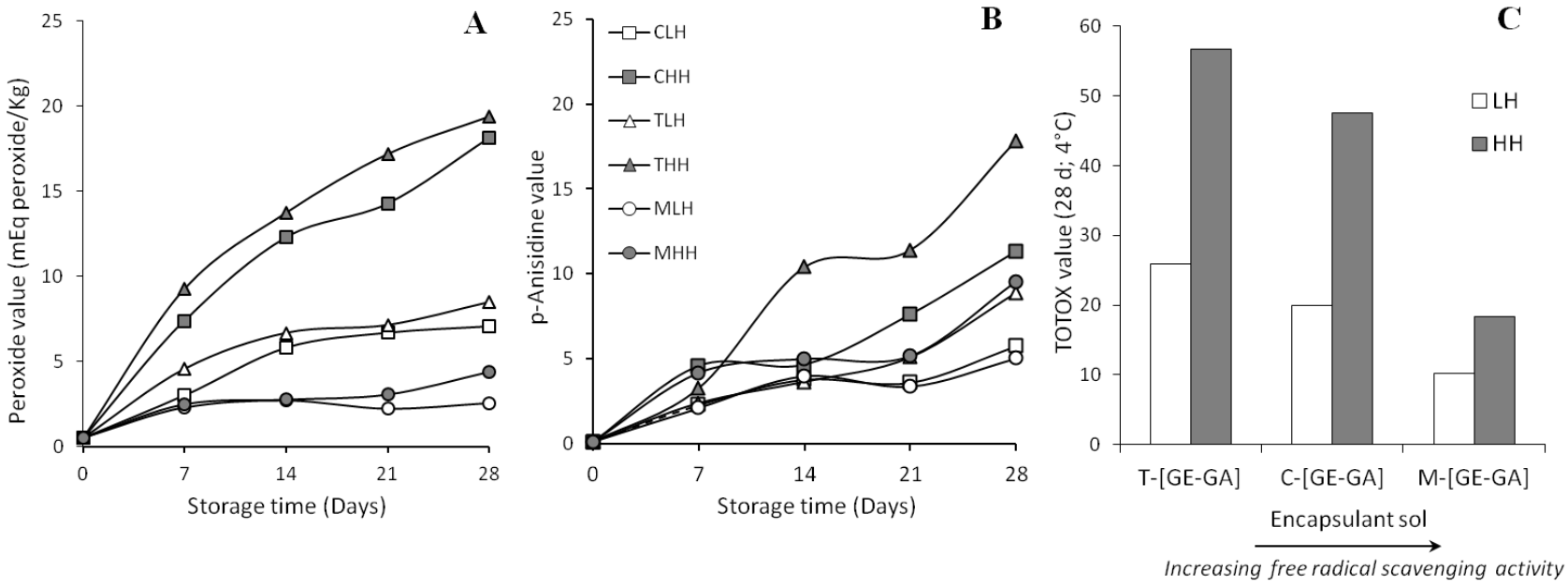

Figure 5.4 Oxidation of encapsulated stearidonic acid soybean oil during storage for 28 days at 4 °C in control (C), transglutaminase- (T), and Maillard reaction (M)- modified microcapsules produced at low (LH) and high (HH) homogenization levels. A: Peroxide value; B: *p*-Anisidine value; C: total oxidation (TOTOX) value after 28 days. See Table 5.1 for meanings of C-[GE-GA], T-[GE-GA], and M-[GE-GA].

CHAPTER 6

MICROENCAPSULATION OF STEARIDONIC ACID SOYBEAN OIL IN MAILLARD REACTION-MODIFIED COMPLEX COACERVATES[1]

[1] Ifeduba, E. A., & Akoh, C. C. Submitted to *Food Chemistry* 06/23/15.

Abstract

The antioxidant capacity of Maillard reaction (MR)-modified gelatin (GE)-gum arabic (GA) coacervates was optimized to produce microcapsules with superior oxidative stability compared to the unmodified control. MR was used to crosslink GE and GA, with or without maltodextrin (MD), to produce anti-oxidative Maillard reaction products (MRP) which was used to encapsulate stearidonic acid soybean oil (SDASO) by complex coacervation. Biopolymer blends (GE-GA [1:1, w/w] or GE-GA-MD [2:2:1, w/w/w]) were crosslinked by dry-heating at 80 °C for 4, 8, or 16 h. Relationships between the extent of browning, Trolox equivalent antioxidant capacity (TEAC), and the total oxidation (TOTOX) of encapsulated SDASO were fitted to quadratic models. The extent of browning and TEAC of MRPs derived from [GE-GA-MD] was significantly higher than corresponding [GE-GA] blends. Maximum TEAC was observed for MRPs derived from [GE-GA-MD] at 8 h, but the 4 h product was optimal for minimizing the TOTOX value of encapsulated SDASO.

Keywords: microencapsulation; complex coacervation; stearidonic acid soybean oil; omega-3 oils, Maillard browning; antioxidant capacity, oxidative stability

Introduction

Complex coacervation is a phase separation phenomena that occurs when oppositely-charged hydrocolloids interact non-covalently to form a liquid electro-neutral complex known as a coacervate (Bungenberg de Jong, 1965). The formation of coacervates may be initiated by adjusting the pH, temperature, or by dilution of the bulk sol. When complex coacervation is elicited in the presence of dispersed oil droplets, encapsulation occurs by deposition of a coacervate film around oil droplet(s). Subsequent cooling transforms the coacervate film to a hydrogel and forms microcapsules with high payloads and controlled-release capabilities (Gouin, 2004). Since encapsulation by coacervation is driven by non-covalent interactions, a crosslinking agent must be added to stabilize the capsular wall. Microencapsulation of lipophilic substances by complex coacervation was first described by Green and Schleicher (1957) who used porcine gelatin (GE) and gum arabic (GA) as the encapsulating biopolymers. Since then many encapsulation studies based on complex coacervation have been reported, most of which focused on improving the acceptability and performance of encapsulating biopolymers for food applications. Consequently, a wide collection of proteins, polysaccharides, and blends thereof, have been identified as potential candidates for encapsulation of food ingredients by coacervation on commercial scale (Thies, 2012). Although complex coacervation is a promising encapsulation technique, application by the food industry was initially limited by perceptions of high process cost and toxicity associated with the use of chemical crosslinking agents such as gluteraldehyde or formaldehyde as process aids. Food industry interest has increased due to reports demonstrating that potentially harmful chemical crosslinking agents can be replaced by enzyme-induced crosslinking with

transglutaminase (TG; amine γ-glutamyl transferase, EC 2.3.2.13) (Thies, 2012), but long processing times and negative consumer perception of TG (controversially dubbed "meat glue") are issues that need to be addressed.

Research interest in complex coacervation technique continue to be high as studies demonstrating that stable microcapsules loaded with high-value core materials such as omega-3 oils can be produced. In a recent study, we found that the encapsulation of stearidonic acid soybean oil (SDASO) in complex coacervates based on a Maillard reaction product (MRP) of GE and GA yielded microcapsules that displayed higher colloidal, thermal, and oxidative stabilities compared to microcapsules based on non- or TG-crosslinked [GE-GA] complex coacervates (Ifeduba & Akoh, 2015). The use of Maillard reaction (MR) to crosslink GE and GA and produce anti-oxidative MRPs prior to encapsulation of omega-3 oil by coacervation was demonstrated. However, there is need to improve the antioxidant capacity of the MR-modified [GE-GA] complex coacervate since the antioxidant effect of MRPs could vary with the substrates used, reaction conditions, and extent of browning (Nursten, 2005). Therefore, the objective of this work was to optimize the antioxidant capacity of MR-modified [GE-GA] coacervates in order to produce microcapsules that would confer superior oxidative stability to SDASO compared to non-crosslinked controls. Maltodextrin (MD) was added to the GE-GA blend to study its effect on Maillard browning rate and antioxidant capacity of the resulting MRP. Six different types of MRPs were made by varying the biopolymer blends (GE-GA [1:1, w/w] versus GE-GA-MD [2:2:1, w/w/w]), and dry-heating time (4, 8, or 16 h). We checked for correlations between the extent of browning, antioxidant capacity, and ability to minimize the oxidation of encapsulated SDASO over 30-day storage. This

study gave us some insight into how MR-modified coacervates can be developed to optimize the oxidative stability of encapsulated high-value omega-3 oils such as SDASO.

Materials and Methods

Materials

Stearidonic acid soybean oil (SDASO) was donated by Monsanto Co. (St. Louis, MO). Porcine gelatin (GE: Type A, ~300 g bloom), gum arabic (GA), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, ABTS), butylated hydroxytoluene (BHT), and horseradish peroxidase (HRP, 950-2000 units/mg solid, based on ABTS substrate) were purchased from Sigma-Aldrich (St. Louis, MO). Maltodextrin (MD: dextrose equivalent [DE] 18) was from Tate and Lyle (Decatur, IL). Criterion TGX (Any KD) precast gels, Laemmli and Tris/glycine/SDS buffers, Bio-Safe Coomassie blue stain, and Precision Plus protein standards were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Pierce glycoprotein staining kit was from Thermo Fischer Scientific, Inc. (Rockford, IL).

Crosslinking of Biopolymers by Maillard Reaction

MRPs of [GE-GA] (1:1, w/w) and [GE-GA-MD] (2:2:1, w/w) blends were prepared using the controlled dry-heating method described by Kato, Mifuru, Matsudomi, and Kobayashi (1992) but at different reaction temperature and time. The [GE-GA] and [GE-GA-MD] blends were completely dissolved in deionized water and subsequently freeze-dried using a Unitop 600L, Freezemobile 25SL unit (The Virtis Company, Gardiner, NY). Samples were initially frozen to -40 °C and then dried for 24 h at 0.13 mbar to a final temperature of 25 °C. Freeze-dried cakes were pulverized using a Kitchen Aid coffee grinder (Kitchen Aid, St. Joseph, MI) and then crosslinked by incubating at 80 °C for 4, 8, or 16 h under ~ 79% relative humidity in a closed desiccator containing

saturated KI solution at the bottom. MRPs obtained at 4, 8, and 16 h were designated M1, M2, and M3, respectively. After incubation, MRP cakes were pulverized with a coffee grinder.

Extent of Maillard Browning

The color of unheated and dry-heated [GE-GA] and [GE-GA-MD] blends was measured using a CR-410 Chroma Meter (Minolta Co., Ltd, Japan). Color was expressed as CIE $L^*a^*b^*$ values where L^* represents lightness ($L^* = 0$ yields black and $L^* = 100$ indicates diffuse white), a^*indicates a position between green and red, and b^* indicates a position between blue and yellow. Unheated and dry-heated blends (1.47 g) were completely dissolved in 30 g deionized water to yield a 4.9% (w/w) sol which was centrifuged for 10 min at 2000 rpm. Absorbance of the supernatant was measured at 420 nm.

Microencapsulation of SDASO by Complex Coacervation

Microcapsules were prepared as described by Green and Schleicher (1957) with slight modifications. Ten grams each of M1, M2, and M3 products derived from [GE-GA] and [GE-GA-MD] blends were dissolved in 150 g of deionized water at 70 °C to yield six different 6.7% (w/w) MRP sols. The MRP blends required a higher temperature than the control to dissolve completely. For the control, 10 g of the unheated [GE-GA] blend was dissolved in 150 g of deionized water at 50 °C to yield a 6.7% (w/w) control sol. SDASO (30g) was dispersed in each sol using a VDI 25 S41 homogenizer (VWR International, Randor, PA) fitted with a 25 G ST dispersion element at a speed setting of '4' (17, 500 rpm). Homogenizations were performed at ~50 °C for 3 min. The resulting emulsion was transferred to a temperature-controlled reaction vessel at 50 °C and

continuously stirred at 200 rpm with a StedFast SL 2400 stirrer fitted with a 60mm, 4-blade general purpose propeller (Fisher Scientific, Co., Fair Lawn, NJ). Next, 55 g of deionized water was added to the emulsion to bring the total biopolymer concentration to 4.9% (w/w). At this point an aliquot (10 mL) of each emulsion was collected to measure particle size and creaming rate. To induce complex coacervation, the pH of the emulsion was adjusted to 4.4 with 10% (w/w) citric acid. The emulsion was then cooled at ~1 °C/min to 10 °C to yield a suspension of microcapsules which was weighed, transferred to opaque Nalgene containers, and stored at 4 °C. One half of each microcapsule preparation was freeze-dried using a Unitop 600L, Freezemobile 25SL unit. Samples were pre-frozen at -40 °C and then dried for 24 h at 0.13 mbar to a final temperature of 25 °C. Freeze dried cakes were ground using a mortar and pestle, transferred to transparent plastic bags, and stored at room temperature.

SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm covalent modification of biopolymers by Maillard reaction. Unheated and dry-heated [GE-GA] and [GE-GA-MD] samples were solubilized in Laemmli sample buffer to a final biopolymer concentration of 8 µg/µL by heating at 70 °C for 10 min. Samples were centrifuged at 2000 rpm for 15 min. Ten microliters of clear supernatant was loaded alongside protein standards onto two gels and placed in a Criterion electrophoresis cell (Bio-Rad Laboratories, Inc., Hercules, CA). Electrophoresis was performed at constant voltage (200 V) for 60 min in Tris/glycine/SDS buffer. After electrophoresis, one gel was stained for protein with Coomassie blue while the other gel

was stained for glycoprotein with periodic acid-Schiff reagent as per manufacturers' instructions.

Antioxidant Capacity

ABTS free radical scavenging capacities of unheated and dry-heated [GE-GA] and [GE-GA-MD] blends were measured using the ABTS/H_2O_2/ HRP decoloration method (Arnao, Cano, & Acosta, 2001). Samples were dissolved in deionized water to a final biopolymer concentration of 4.9% (w/w). The assay mixture contained 2 mM ABTS, 15 µM H_2O_2, and 0.25 µM HRP in 50 mM sodium phosphate buffer (pH 7.5) in a total volume of 1mL. After the reaction mixture gave a stable absorbance reading at 730 nm, 10 µL of sample or deionized water (blank) was added. Absorbance at 730 nm was measured after 10 min of incubation at 25 °C. Loss of absorbance was determined by difference between the sample and blank. This difference was proportional to the amount of ABTS radical cation quenched by the sample. Antioxidant capacity was expressed as Trolox equivalent antioxidant capacity (TEAC) (mg Trolox per 10 g dry weight of blend). Calculations were based on a dose-response curve of loss of absorbance against varying amounts (0-200 nmol) of Trolox. Scatter plots of the extent of browning and TEAC of blends were obtained and fitted to a quadratic model using Microsoft Excel.

Microcapsule Morphology

One drop of microcapsule suspension was applied onto a glass slide and diluted with few drops of deionized water. Microcapsules were directly viewed through the 10× or 40× objective lens of a Leitz Laborlux K binocular microscope (Ernst Leitz, Wetzlar Germany). Images of microcapsules were captured from the 10× eyepiece on a handheld smart phone.

Particle Size Distribution

Particle size distribution was measured with an LS 13 320 MW particle size analyzer (Beckman Coulter, Inc., Miami, FL) using the Fraunhofer optical model. Emulsions or microcapsules were added drop-wise to the dispersion cell, which contained deionized water, until an obscuration value of 9-11% was achieved. The real and imaginary refractive indexes (RI) of particles were 1.471 and 0, respectively, while the RI of the suspension fluid was 1.332. Particle size distribution measurements were carried out under low sonication and volume mean diameters ($D_{4,3}$) were reported.

Emulsion Stability

Creaming rates of emulsions stabilized by the control and MR-modified biopolymer blends were measured using Turbiscan Classic MA2000 (Formulaction Inc., Davie, FL). The light source was an electro luminescent diode in the near infrared (λ_{air} = 850 nm). Seven milliliters of emulsion sample was carefully dispensed into a flat bottom glass cell to ensure a good meniscus and placed in the measurement chamber of the instrument. Automatic measurement of transmitted and backscattered light across sample height (~50 mm) was acquired over the course of 1 h with a 2 min delay between scans. Using the first scan (0 min) as reference, peak thickness kinetics was computed from changes in % backscattering within the creaming zone as a function of time. The migration rate software was used to calculate creaming rate (mm/min) from the slope on the linear part of the peak thickness kinetics.

Encapsulation Efficiency

Encapsulation efficiency (EE) was calculated by estimating hexane extractable (surface) and total oil in microcapsule preparations. To estimate hexane extractable oil,

2.5 g of microcapsule suspension or powder was agitated thoroughly with 25 mL hexane for 5 min using a vortex mixer. The mixture was then centrifuged for 5 min at 2000 rpm. The organic layer (supernatant) was carefully collected, filtered through anhydrous Na_2SO_4, and transferred to a pre-weighed round-bottom flask. Solvent was removed under reduced pressure at 60 °C to a constant mass using a rotary evaporator. Solvent extractable oil was then determined gravimetrically and expressed as % (w/w) of microcapsule preparation.

To estimate total oil, 10 mL of 5 M HCl was added to 2.5 g of microcapsule suspension or powder and agitated at 60 °C for 3 h. After cooling to room temperature, the mixture was transferred to a separatory funnel and extracted twice with 25 mL hexane. Organic layers were combined, filtered through anhydrous Na_2SO_4, and transferred to a pre-weighed round bottom flask. Solvent was removed using a rotary evaporator under reduced pressure at 60 °C to a constant mass. Total oil was then determined gravimetrically and expressed as % (w/w) of suspension. EE (%) was calculated as follows:

$$\text{EE (\%)} = 100 - ([\%\ \text{Hexane extractable (surface) oil} / \%\ \text{Total oil}] \times 100)$$

Oxidative Stability of Microcapsules

Peroxide (PV) and *p*-anisidine (*p*-AV) values of microencapsulated SDASO was measured after 30 days of storage. Microcapsules suspensions were stored at 4 °C while powders were stored at ambient temperature ~ 25 °C. PV and *p*-AV of bulk SDASO before microencapsulation were measured. The International Dairy Foundation (IDF) lipid extraction method 172:1995 (IDF, 1995) was adapted to extract a portion of

microencapsulated oil from suspensions and powders. Microcapsule suspension (10 g) or powder (2.5 g) was mixed with 8 mL 96% (v/v) ethanol and 2 mL 7 M NaOH in a separatory funnel. Powdered samples were dispersed in 10 mL deionized water before adding ethanol and NaOH. Next, 10 mL of diethyl ether containing 0.01% (w/v) BHT was added followed by vigorous shaking for 1 min. After phase separation, 10 mL of hexane was added, mixed carefully, and allowed to undergo phase separation. The aqueous layer was discarded while the organic layer was washed with 10 mL of 1 M HCl followed by 10 mL of 0.4 M Na_2SO_4 solution. The organic layer was then transferred to a pre-weighed conical flask and the solvent evaporated by flushing with nitrogen. Primary lipid oxidation products were measured by PV based on the IDF standard method described by Shantha and Decker (1994). Secondary lipid oxidation products, specifically unsaturated aldehydes, were measured by *p*-AV according to the American Oil Chemists' Society Official Method Cd 18-90 (AOCS, 2011). The total oxidation (TOTOX) value on the 30th storage day was calculated using the following equation:

$$\text{TOTOX value} = 2(\text{PV}) + p\text{AV}$$

Scatter plots of the TEAC of control and MR-modified blends and 30th day TOTOX values of corresponding microcapsule preparations were obtained and fitted to a quadratic model using Microsoft Excel. Oxidation induction temperature (OIT_{temp}) of freeze-dried microcapsule powders was determined using a differential scanning calorimeter (DSC) 204 F1 Phoenix system (Netzsch-Gerätebau GmbH, Selb, Germany). Microcapsule powder (8-12 mg) was weighed in an aluminum crucible and sealed with a pierced lid. The sample crucible was placed in the DSC along with a blank reference at room temperature. Next, the sample was heated up continuously to 250 °C at 10 °C /min

under pure oxygen gas flow (20 mL/min). OIT_{temp} was determined as the onset of the decomposition signal. DSC data were analyzed by Proteus thermal analysis software (Netzsch-Gerätebau GmbH, Selb, Germany).

Statistical Analysis

Measurements were performed in duplicate or triplicate. Results were expressed as mean ± SD. ANOVA was carried out and mean comparisons were performed using Duncan's multiple range tests. Significant differences were determined at $P < 0.05$. Statistical analysis was performed on Microsoft Excel using the DSAASTAT version 1.101 macro (DSAA, Perugia, Italy).

Results and Discussion

Heating Time and Maillard Browning

Colorless compounds are formed at the initial stage of the Maillard reaction. However, as the reaction progress through intermediate to final stages, brown color gradually develops due to formation of nitrogeneous, high molecular weight (HMW) polymers known as melanoidins (Nursten, 2005). Color measurements revealed that the level of browning of dry-heated biopolymer blends generally increased with heating time. *L** values of the [GE-GA] and [GE-GA-MD] blends decreased while *a** and *b** values increased progressively over the course of dry-heating (Figure 6.1). Similar trends in *L**, *a**, and *b** have been reported for the progress of color development in heated protein-carbohydrate mixtures (Augustin, Sanguansri, & Bode, 2006; MacDoougall & Granov, 1998). Interestingly, the [GE-GA-MD] blend showed more intense brown color formation than the [GE-GA] blend. The browning rate, based on changes in *a**values, was higher in the [GE-GA-MD] blend compared to the [GE-GA] blend (Figure 6.1B).

This was possibly due to a higher number of available carbonyl reducing ends in the [GE-GA-MD] blend. This observation is in agreement with that of Lingnert and Eriksson (1981) who studied Maillard browning in a histidine/glucose model and reported increased browning when glucose concentration was increased. Furthermore, different combinations of reducing sugars are known to produce different types of melanoidins (Bedinghaus & Ockerman, 1995). It is possible that the addition of MD resulted in more intensely colored types of melanoidins. There was a positive correlation (r = 0.993) between a^* values of [GE-GA] blends and the A_{420} of their respective 4.9% (w/w) sols indicating that the concentration of water-soluble melanoidins increased with heating time. For the [GE-GA-MD] blends, a^* values highly correlated (r = 0.999) to the A_{420} of their respective sols only between 0 - 8 h. However, between 8 - 16 h, a^* value increased while A_{420} reduced. This decrease was due to the presence of insoluble MRPs in the 16 h [GE-GA-MD] product which were removed before absorbance measurements. The removal of these insoluble products was necessary because the measurement of Maillard browning by absorbance is only valid for non-turbid solutions (Fayle & Gerrard, 2002). After long reaction periods, melanoidins formed at the final stage of the Maillard reaction may polymerize further to form water insoluble, higher molecular mass products (Davies & Labuza, 1998; Nursten, 2005). This may account for the insoluble MRPs observed in the 16 h [GE-GA-MD] product. However, it is interesting that these insoluble MRPs were observed only in the 16 h [GE-GA-MD] product and not in the 16 h [GE-GA] product. The higher reaction rate of the [GE-GA-MD] blend may account for this discrepancy.

SDS-PAGE Profiles

Electrophoresis profiles of unheated and dry-heated biopolymer blends are shown in Figure 6.2. The gel stained for protein (Figure 6.2A) showed that high molecular weight (HMW) (>250 kDa) protein aggregates were present in dry-heated [GE-GA] and [GE-GA-MD] blends specifically at 8 and 16 h. These HMW MRPs were lodged close to the origins of lanes 3, 4, 7, and 8. Also, protein bands below 25 kDa in the unheated blends were observed to fade gradually in dry-heated blends suggesting heat-induced polymerization of gelatin polypeptides. Similar SDS-PAGE patterns have been reported for dry-heated whey protein-maltodextrin (Akhtar & Dickinson, 2007) and wheat protein-glyceraldehyde (Fayle & Gerrard, 2002) blends. The formation of HMW MRPs may also be by conjugation between polypeptides and polysaccharides or by polymerization of LMW MRPs. The gel stained for glycoproteins (Figure 6.2B) provided confirmation that the HMW products close to the origins of lanes 3, 4, 7, and 8 were protein-polysaccharide conjugates and may include HMW melanoidins formed at the final stage of the Maillard reaction by aldehyde-amine condensation reactions (Nursten, 2005).

Antioxidant Capacity

From Figure 6.3A, antioxidant capacities of [GE-GA] and [GE-GA-MD] blends were enhanced by dry-heating, possibly due to formation of anti-oxidative MRPs such as reductones and melanoidins. The antioxidant capacities of dry-heated [GE-GA-MD] blends were significantly higher than those of the corresponding [GE-GA] blends. Furthermore, there was a curvilinear relationship between the extent of browning (a^*) and antioxidant capacity. A similar trend was reported for the relationship between color and antioxidant activity of brews made from coffee beans roasted to varying degrees (del

Castillo, Ames, & Gordon, 2002). Comparing between quadratic curves in Figure 6.3A, it is interesting to note that if [GE-GA] and [GE-GA-MD] blends were heated to the same *a** level, a higher antioxidant capacity could always be expected from the [GE-GA-MD] blend. For example, although an *a** value of ~ 4 was obtained for the 16 h [GE-GA] and 8 h [GE-GA-MD] products (see Figure 6.1B), the antioxidant capacity of the latter was significantly higher (Figure 6.3A). This implies that antioxidant capacities of samples were not simply dependent on the extent of browning but also on the type and concentration of anti-oxidative MRPs present. By solving for the vertices of the quadratic equations in Figure 6.3A, it was found that the theoretical (expected) maximum antioxidant capacity attainable for [GE-GA] and [GE-GA-MD] were 30.08 and 47.02 mg Trolox eq/10 g, respectively, under the given dry-heating conditions. Furthermore, this maximum antioxidant activity was expected to be attained at *a** values of 4.75 and 3.52 for [GE-GA] and [GE-GA-MD], respectively. From Figure 6.1B, it is evident that this theoretical maximum antioxidant activity would be attained at a shorter dry-heating time with [GE-GA-MD] than with [GE-GA]. The antioxidant capacity of MRPs of protein-polysaccharide blends eventually reached a plateau or peak with continued heating due to pyrolysis and/or polymerization of anti-oxidative MRPs (Brand & Eichner, 2002; del Castillo et al., 2002).

Emulsion Stability and Microcapsule Morphology

Table 6.1 shows mean particle sizes and creaming rates of emulsions stabilized by control (C-[GE-GA]) and MR-modified blends before coacervation. Similar particle size distributions were obtained across different formulations as they were prepared using identical homogenization treatments. The observed creaming rate of the emulsion

formulated with C-[GE-GA] blend was much higher than those formulated with MR-modified [GE-GA] and [GE-GA-MD] blends. With the MR-modified blends, there was increased emulsion stability (less creaming) with increasing dry-heating time from M1-M3. In all encapsulation systems, gelation after complex coacervation was accompanied by increases in particle size due to agglomeration. However, the degree or index of agglomeration of microcapsules was positively correlated (r = 0.999) with the creaming rates of the pre-existing emulsions. In other words, more stable emulsions yielded less agglomerated microcapsules and vice versa. The agglomeration index of microcapsules encapsulated in C-[GE-GA] was considerably higher than those of MR-modified systems while agglomeration indices of microcapsules based on MR-modified [GE-GA] and [GE-GA-MD] systems decreased with increasing dry-heating times from M1-M3.

Variations in emulsion stability among different encapsulation systems may be explained on the basis of interfacial layer architecture (Dickinson, 2008; Wooster & Augustin, 2007). For the control, complexation of GE and GA at the oil-water interface is expected to yield a single intermixed layer with an overall thickness not much greater than that of gelatin alone (Dickinson, 2008). The interaction potential between a pair of such surfaces has been shown to be attractive at short range (Ettelaie, Dickinson, & Murray, 2005). Moreover, at the prevailing emulsion pH (5.2 ± 0.1), interfacial complexation of GE and GA would result in charge neutralization (Schmitt & Turgeon, 2011) which would lower the electrostatic repulsion needed to counter van der Waals-London (VDWL) attractive forces between droplet surfaces (Shi, 2002). This may account for the relatively high creaming rate of C-[GE-GA] emulsion. Subsequent encapsulation of oil droplets in electro-neutral coacervates coupled with dominating

VDWL attractive forces promoted flocculation in the C-[GE-GA] system and upon gelation by cooling, microcapsules readily agglomerated to form large irregular shaped aggregates of GE-GA coacervates (Figure 6.4A). This observation is in agreement with Weinbreck, Minor, and De Kruif (2004) who reported that emulsions stabilized by whey protein-gum arabic blends exhibited a high creaming rate at the coacervation pH and formed microcapsules that aggregated into clusters similar to those shown in Figure 6.4A.

The presence of protein-polysaccharide conjugates in MR-modified system implies that interfacial layers formed by MR-modified [GE-GA] or [GE-GA-MD] included a secondary (steric) layer of polysaccharide extends well beyond the protein layer into the bulk solution (Dickinson, 2008). The interaction potential between a pair of such surfaces has been determined to be repulsive at long range (Ettelaie et al., 2005). The steric layers are mutually repulsive and confer colloidal stability by effectively keeping the particles at a distance from each other (steric stabilization) (Shi, 2002). According to one generally accepted entropic stabilization theory, the basis for steric stabilization is that polysaccharide chains on the surface of one particle must lose configurational entropy to interact with the surface of another such particle which makes inter-particle interaction thermodynamically unfavorable (Sato & Ruch, 1980). The overlap of steric layers from two or more colliding particles would increase ΔG and produce a repulsive force to counter VDWL attractive forces. Furthermore, the strength of repulsion increases with the thickness or density of the steric layer (Sato & Ruch, 1980). Steric stabilization may account for lower creaming rates of emulsions stabilized by MR-modified systems compared to the control. This observation is in agreement with those of Wooster and Augustin (2006 & 2007) who showed that the conjugation of

carbohydrates to β-lactoglobulin by Maillard reaction resulted in increased emulsion stability against electrolyte or heat-induced flocculation, compared to emulsions stabilized by β-lactoglobulin alone. They also reported that the colloidal stability of MR-conjugate emulsions was improved because the attached carbohydrate moieties formed a steric layer around oil droplets which stabilized the emulsions against flocculation. It is possible that the presence of Maillard-type conjugates in electro-neutral coacervate layers provided steric repulsion between particles that conferred some degree of flocculation stability. Upon gelation by cooling, agglomeration indices of MR-modified microcapsules were considerably lower compared to the control. Therefore, MR-modified coacervates systems yielded smaller multinuclear microcapsules and finer suspensions than the control as shown in Figure 6.4B. The observed trends in emulsion stability and agglomeration indices within MR-modified categories may be accounted for by increases in the extent of protein-polysaccharide conjugation with dry-heating time from M1-M3. It is possible that steric layer thickness or density increased with the extent of protein-polysaccharide conjugation and VDWL attraction forces weakened as the interaction potential between particles progressively became more repulsive from M1 to M3.

Oxidative Stability of Microencapsulated SDASO

Table 6.2 shows PV, *p*-AV, and TOTOX values of SDASO extracted from the different encapsulation systems after 30 days of storage. The initial PV, *p*-AV, and TOTOX values of the bulk SDASO before microencapsulation were 1.71 mEq/kg, 0.56, and 3.98 respectively. Homogenization of the bulk SDASO in sols would increase surface area and may incorporate oxygen which could increase susceptibility to

oxidation. So, oxidative stability of different encapsulations systems was assessed based on their ability to minimize increases in PV and *p*-AV of microencapsulated SDASO. TOTOX values were calculated to account for both primary and secondary lipid oxidation. In addition, OIT_{temp} of freeze-dried microcapsules was determined to estimate the stability of the encapsulant under accelerated oxidation conditions. Oxidation induction temperature or time may be used but the former was preferred due to better reproducibility and easier measurement set up (Schmid, Ritter, & Affolter, 2006). There was a strong correlation (r = -0.903) between TOTOX and OIT_{temp} of freeze-dried microcapsule powders showing reasonable agreement between these measures of oxidative stability. From Table 6.2, PV, *p*-AV, and TOTOX values of SDASO extracted from C-[GE-GA] microcapsules after storage were significantly higher than those of the MR-modified microcapsules. This was true for both microcapsule suspensions and freeze-dried powders and shows that the MR-modified [GE-GA] and [GE-GA-MD] blends conferred higher oxidative stability to the encapsulated SDASO compared to C-[GE-GA]. This may be attributed to the presence of anti-oxidative MRPs, such as melanoidins and reductones, in MR-modified blends which reduced the rate of lipid oxidation during microcapsule preparation and storage. The antioxidant mechanisms involved in MR-modified encapsulation systems may include inactivation of lipid-derived free radicals, oxygen scavenging, and reduction of hydroperoxides to more stable hydroxy fatty acids (Eichner, 1979). The presence of various MRPs with different antioxidant mechanisms may provide additional benefit as synergistic interactions would maximize protection (Decker, Chen, Panya, & Elias, 2010).

Within the MR-modified [GE-GA] category, PV, *p*-AV and TOTOX values reduced with increasing dry-heating time from M1 to M3 for both microcapsule suspensions and powders. This implied that the oxidative stability of encapsulated SDASO increased with dry-heating time for MR-modified [GE-GA] blends. This observation was in agreement with that reported by Augustin et al. (2006) for heat treated protein-polysaccharide blends used for encapsulating fish oil by spray-drying. Within the MR-modified [GE-GA-MD] category, a non-linear trend was observed between the oxidative stability of encapsulated SDASO and dry-heating time for the blend. The M1-[GE-GA-MD] microcapsules, for which the 4 h [GE-GA-MD] product was encapsulating agent, conferred the highest oxidative stability to SDASO since their PV, *p*-AV, and TOTOX values were lowest. However, the M2-[GE-GA-MD] microcapsules, for which the 8 h [GE-GA-MD] product was encapsulating agent, yielded the lowest oxidative stability since their PV, *p*-AV, and TOTOX values were highest. This was surprising because the 8 h [GE-GA-MD] product had the highest TEAC overall (Figure 6.3A). This implied that maximizing the free-radical scavenging activity of MR-modified blends did not lead to optimal protection of encapsulated SDASO. It is not clear why the M2-[GE-GA-MD] microcapsules showed the lowest oxidative stability. However, some studies have suggested that HMW fractions of certain melanoidins may exhibit pro-oxidant activity in oil (Brand & Eichner, 2001; Cammerer, Fuch, & Kroh, 2001). Further investigation would be required to ascertain if the HMW fraction of M2-[GE-GA-MD] had pro-oxidant activity.

Scatter plots in Figures 6.3B and C show a curvilinear relationship between the antioxidant capacity of control and MR-modified biopolymer blends and the 30 day

TOTOX values of SDASO in corresponding microcapsules. The data was fitted to a quadratic model to gain insight into how the antioxidant capacity of biopolymer blends may be optimized for better oxidative stability of encapsulated SDASO. It is worth noting that microcapsules stored as freeze-dried powder would be expected to display higher oxidative stability than aqueous suspensions for any given TEAC value. The higher oxidative stability of freeze-dried microcapsules may be attributed to a reduction in water content in agreement with Karel (1979) who pointed out that the oxidation of food lipids tend to be accelerated at high water activity and vice versa. The theoretical optimal TEAC to minimize TOTOX value ($TOTOX_{min)}$ may be calculated from the vertices of polynomial equations in Figures 6.3B and C. For powdered preparations, a $TOTOX_{min}$ of 24.48 would be expected for microcapsules prepared from MR-modified [GE-GA] blend with TEAC of 26.65 mg Trolox eq/10 g, while a $TOTOX_{min}$ of 7.11 would be expected for microcapsules prepared from a MR-modified [GE-GA-MD] blend with TEAC of 29.24 mg Trolox eq/10 g. For suspensions, a $TOTOX_{min}$ of 33.09 would be expected for microcapsules prepared from a MR-modified [GE-GA] blend with TAEC of 25.16 mg Trolox eq/10 g, while a $TOTOX_{min}$ of 14.28 would be expected for microcapsules prepared from a MR-modified [GE-GA-MD] blend with TEAC of 27.81 mg Trolox eq/10 g. The extent of Maillard browning (*a** value) required to obtain [GE-GA] and [GE-GA-MD] blends with theoretical optimal TEAC may then be calculated from quadratic equations in Figure 6.3A.

Encapsulation Efficiency

The encapsulation efficiency of aqueous microcapsule suspensions was > 96% for all encapsulation systems. However, it can be seen from Table 6.2 that encapsulation

efficiencies decreased considerably after aqueous microcapsule suspensions were freeze-dried to powder form. This is possibly because the surface of freeze-dried microcapsules can become porous as a result of sublimation of frozen unbound water during the primary drying stage (Sadikoglu, 2010). The encapsulation efficiency of the control microcapsule powder was, on average, higher than those of MR-modified microcapsules. Within the MR-modified [GE-GA] category, encapsulation efficiency increased with the extent of Maillard browning (M3 > M2 > M1), suggesting that more compact coacervates were formed with increasing [GE-GA] blend dry-heating time. This effect was possibly brought about by increasing extents of polymerization and/or GE-GA conjugation. Within the MR-modified [GE-GA-MD] category, encapsulation efficiency increased with the extent of Maillard browning from M1 to M2 but fell drastically at M3. This drastic reduction of encapsulation efficiency for the M3-[GE-GA-MD] system was probably due to lower effective wall to oil ratio caused by formation of water-insoluble MRPs which were unable to function as encapsulants.

Some studies have suggested that there is a positive correlation between encapsulation efficiency and the oxidative stability of microencapsulated oil (Eratte, Wang, Dowling, Barrow, & Adhikari, 2014; Wang, Adhikari, & Barrow, 2014). This is because a high proportion of surface oil and/or highly porous microcapsule surface would promote contact with oxygen and increase susceptibility to oxidation. In the present study, however, antioxidant capacity of the encapsulating agent seemed to have a stronger effect on oxidative stability than encapsulation efficiency considering that microcapsules based on C-[GE-GA] and M2-[GE-GA-MD] blends had high encapsulation efficiencies but relatively poor oxidative stability while microcapsules

based on the M3-[GE-GA-MD] blend had low encapsulation efficiency but better oxidative stability. This finding is in agreement with the observations reported by Carneiro, Tonon, Grosso, and Hubinger (2013) for flaxseed oil microencapsulated using different combinations of wall materials.

In conclusion, the antioxidant capacity of the [GE-GA] blend was significantly improved by dry-heating due to the formation of anti-oxidative MRPs. Interestingly, addition of MD to the [GE-GA] blend resulted in MRPs with TEAC values significantly higher than the corresponding blends without MD. The use of complex coacervates derived from MRPs of [GE-GA-MD] blends in microencapsulation of omega-3 oil was demonstrated. The oxidative stability of microencapsulated SDASO was dependent on the antioxidant capacity of dry-heated blends which, in turn, varied with the extent of Maillard browning. Since oxidation of SDASO was minimal when encapsulated in the 4 h [GE-GA-MD] product, it may be best-suited for producing microcapsules for fortifying food products with SDASO. Based on quadratic models shown in Figures 6.3B and C, the [GE-GA-MD] blend was deemed more advantageous than the [GE-GA] blend for preparing MR-modified coacervates for microencapsulating SDASO since the optimized [GE-GA-MD] blend was expected to confer higher oxidative stability than the optimized [GE-GA] blend. Furthermore, the optimal [GE-GA-MD] blend would be produced in less than half the heating time required to produce the optimal [GE-GA] blend (Figure 6.3A). Maintenance of colloidal stability during the encapsulation process is also important in order to obtain microcapsules with appropriate size and size distributions. Unrestricted particle-particle contact during microcapsule formation may cause extensive agglomeration which is undesirable in most food applications (Thies, 2012). Therefore,

inter-particle contact during the gelation step must be minimized to control microcapsule agglomeration in coacervate-based encapsulation systems. Taking a mechanical approach, Lemetter, Meeuse, and Zuidam (2009) showed that microcapsule agglomeration could be controlled by maintaining high mixing speeds (Reynolds number > 15000) during the gelation step. However, in the present study, results show that agglomeration could also be controlled at the macromolecular level on the basis of steric repulsion, possibly brought about by the presence of Maillard-type protein-polysaccharide conjugates in the coacervate layer surrounding oil droplets.

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Table 6.1 Emulsion stability and changes in particle size after microencapsulation

Encapsulation system	Emulsion		Microcapsule	
	$D_{4,3}$ (μm) [a]	Creaming rate (mm/min)	$D_{4,3}$ (μm)	Agglomeration index [b]
C-[GE-GA]	7.6 ± 4.7	1.8180	627.6 ± 344.7	81.57
M1-[GE-GA]	7.7 ± 5.0	0.0434	38.7 ± 20.2	4.04
M2-[GE-GA]	7.1 ± 4.7	0.0242	23.0 ± 10.7	2.26
M3-[GE-GA]	7.5 ± 4.0	0.0238	17.9 ± 9.9	1.40
M1-[GE-GA-MD]	8.2 ± 4.8	0.0399	53.0 ± 23.2	5.48
M2-[GE-GA-MD]	8.5 ± 2.8	0.0361	38.9 ± 17.9	3.57
M3-[GE-GA-MD]	8.9 ± 4.5	0.0204	14.4 ± 6.9	0.62

[a]Abbreviations: $D_{4,3}$, volume mean diameter; [GE-GA], gelatin-gum arabic (1:1, w/w) blend; [GE-GA-MD], gelatin-gum arabic-maltodextrin (2:2:1, w/w/w) blend; C, unheated control; M1, heated for 4 h; M2, heated for 8 h; M3, heated for 16 h

[b] $\text{Agglomeration index} = \frac{(D_{4,3[\text{microcapsule}]} - D_{4,3[\text{emulsion}]})}{D_{4,3[\text{emulsion}]}}$

Table 6.2 Oxidative stability and encapsulation efficiency of microcapsules containing stearidonic acid soybean oil [a]

Encapsulation system	Aqueous suspension [b]				Freeze-dried powder [c]				
	PV (mEq/kg) [d]	*p*-AV	TOTOX	EE (%)	PV (mEq/kg)	*p*-AV	TOTOX	OIT_{temp} (°C)	EE (%)
C-[GE-GA]	41.5 ± 1.4 a	12.8 ± 0.3 a	95.8	99.7 ± 0.3 a	36.7 ± 0.7 a	12.1 ± 0.3 a	85.5	176.0 ± 1.0 e	77.2 ± 1.0 a
M1-[GE-GA]	27.3 ± 0.6 c	4.2 ± 0.1 c	58.8	98.6 ± 0.1 b	25.1 ± 0.7 b	2.7 ± 0.1 b	52.9	179.6 ± 0.8 d	62.9 ± 0.7 d
M2-[GE-GA]	17.7 ± 0.7 d	2.7 ± 0.2 d	38.1	98.7 ± 0.2 b	13.7 ± 0.6 d	0.8 ± 0.0 d	28.2	182.2 ± 0.6 c	68.2 ± 0.9 c
M3-[GE-GA]	14.9 ± 0.5 e	3.0 ± 0.1 d	32.8	99.1 ± 0.5 a	11.4 ± 0.4 e	0.8 ± 0.1 d	23.6	183.8 ± 0.4 b	74.3 ± 2.0 b
M1-[GE-GA-MD]	6.3 ± 0.1 f	1.3 ± 0.1 e	13.9	99.2 ± 0.5 a	5.4 ± 0.0 f	0.5 ± 0.3 e	11.3	185.8 ± 0.8 a	73.9 ± 0.4 b
M2-[GE-GA-MD]	30.6 ± 0.2 b	6.8 ± 0.1 b	68.0	99.6 ± 0.3 a	23.6 ± 0.3 c	2.2 ± 0.1 c	49.4	179.5 ± 0.7 d	76.5 ± 1.8 a
M3-[GE-GA-MD]	15.1 ± 0.2 e	2.7 ± 0.1 d	32.9	96.6 ± 0.7 c	5.8 ± 0.3 f	0.1 ± 0.0 f	11.7	181.5 ± 0.9 c	24.9 ± 1.0 e

[a] Initial PV, *p*-AV, and TOTOX values of 1.71 mEq/Kg, 0.56, and 3.98 respectively

[b] Stored for 30 days at 4 °C

[c] Stored for 30 days at 25 °C

[d] Abbreviations: PV, peroxide value; *p*-AV, *p*-anisidine value; TOTOX, total oxidation value; EE, encapsulation efficiency; OIT_{temp}, oxidation induction temperature; [GE-GA], gelatin-gum arabic (1:1, w/w) blend; [GE-GA-MD], gelatin-gum arabic-maltodextrin (2:2:1, w/w/w) blend; C, unheated control; M1, heated for 4 h; M2, heated for 8 h; M3, heated for 16 h

Means followed by same letters in a column are not significantly different ($p > 0.05$) according to Duncan's multiple range test

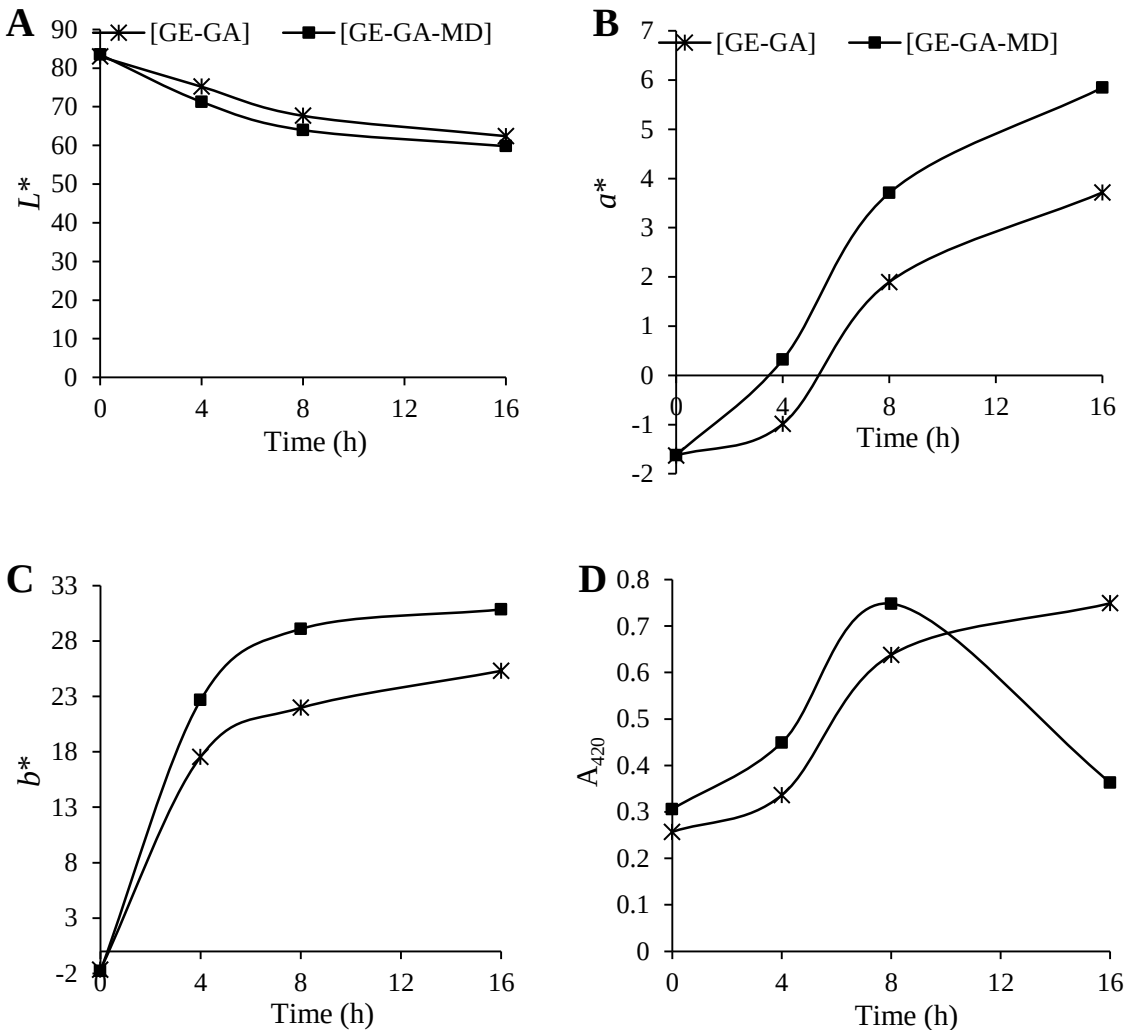

Figure 6.1 Time course of color development for dry-heated gelatin-gum arabic ([GE-GA]; 1:1, w/w) and gelatin-gum arabic-maltodexdtrin ([GE-GA-MD]; 2:2:1, w/w/w) blends: Changes in A: *L**; B: *a**; C: *b**; and D: absorbance of a 4.9 % (w/w) sol at 420 nm.

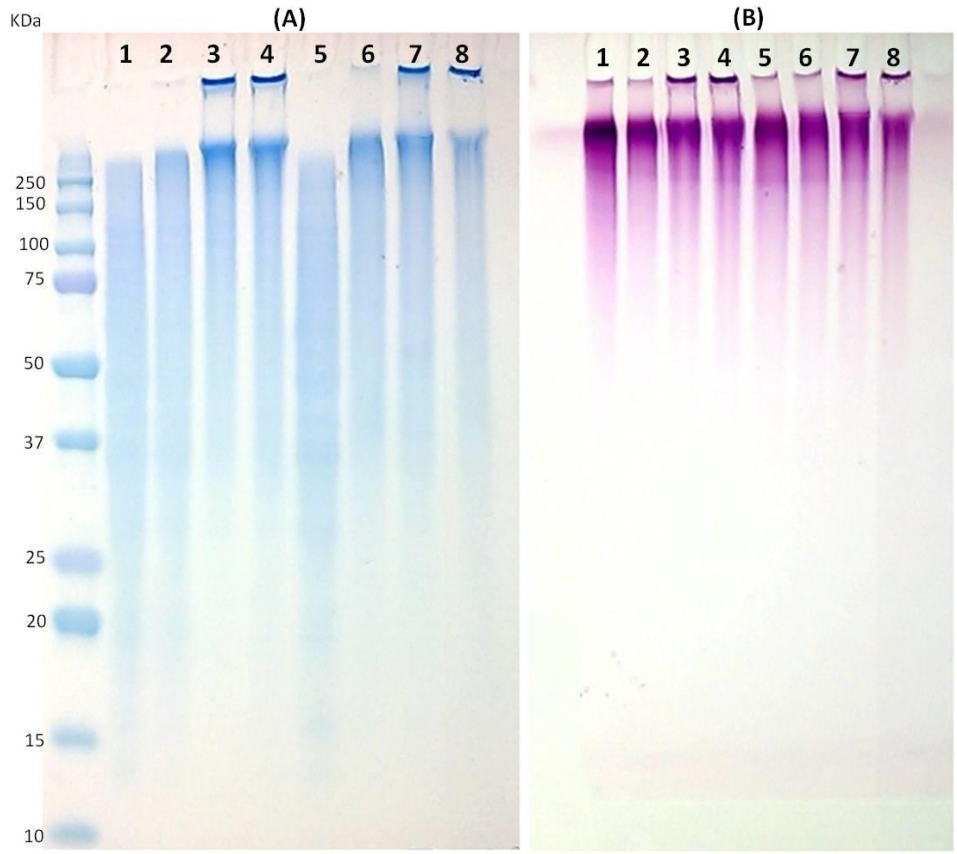

Figure 6.2 SDS-PAGE profiles of unheated and dry-heated biopolymer blends. A: protein stain (blue); B: glycoprotein stain (pink). Lanes (from left to right): 1, gelatin-gum arabic ([GE-GA]; 1:1, w/w); 2-4, heated at 80 °C for 4, 8, and 16 h, respectively; 5, gelatin-gum arabic-maltodextrin ([GE-GA-MD]; 2:2:1, w/w/w) blend; 6-8, heated at 80 °C for 4, 8, and 16 h, respectively.

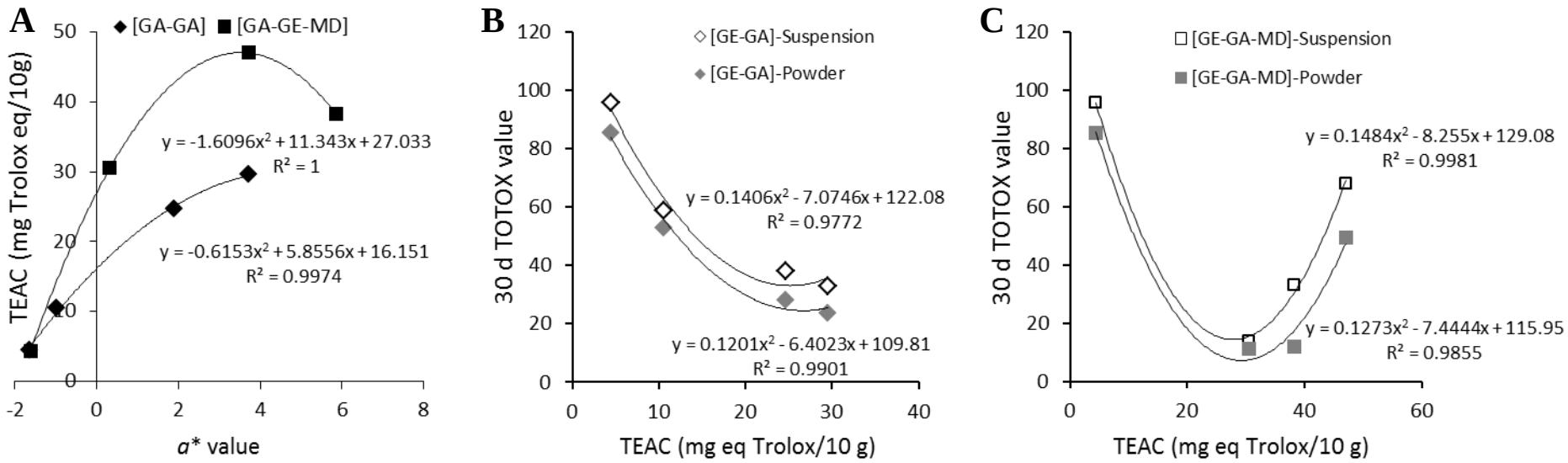

Figure 6.3 Relationships between, A: the extent of Maillard browning (*a** value) and Trolox equivalent antioxidant capacity (TAEC) for gelatin-gum arabic ([GE-GA]; 1:1, w/w) and gelatin-gum arabic-maltodexdtrin ([GE-GA-MD]; 2:2:1, w/w/w) blends; B: TEAC of [GE-GA] blends and 30 day total oxidation (30 d TOTOX) value of microencapsulated stearidonic acid soybean oil (SDASO); C: TEAC of [GE-GA-MD] blends and 30 d TOTOX value of microencapsulated SDASO.

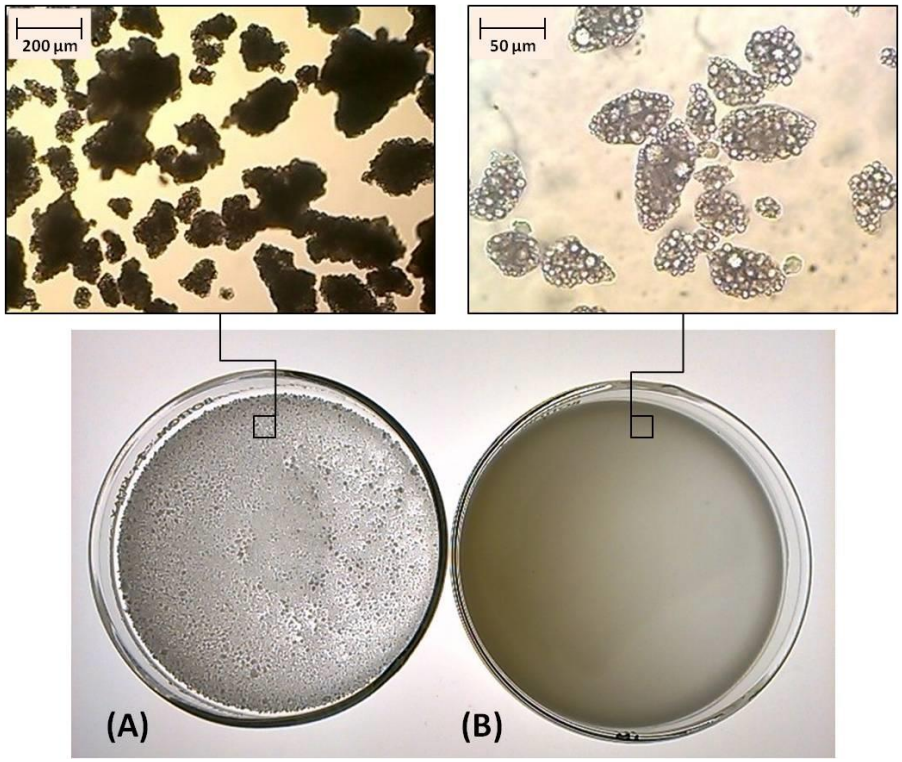

Figure 6.4 Photographs and photomicrographs of microcapsules in suspension. A: Control; B: Maillard reaction-modified microcapsules

CHAPTER 7

CONCLUSIONS

A chemoenzymatic method for producing stearidonic acid (SDA) TAG concentrates from SDA soybean oil was developed. The method used a combination of SDA enrichment steps that involved the use low temperature crystallization and lipase-catalyzed hydrolysis and acidolysis. The ~60% SDA TAG concentrates produced are value-added fractions from SDA soybean oil and may be used as 'heart-healthy' food supplement or for formulating functional foods. Caprylic acid was successfully incorporated into SDA soybean oil by immobilized *sn*-1,3 specific *Rhizomucor miehei* lipase (RML) to yield structured lipids (SL). Enzymatic synthesis of SL by incorporation of caprylic acid into SDA soybean oil was demonstrated for the first time. The type of immobilization support and reaction temperature was found to influence the extent of caprylic acid incorporation and the rate of acyl-migration. Using the optimal immobilization support (Celite) and reaction conditions, SL products with caprylic acid contents ranging from 17.0-32.5 mol% and SDA contents ranging from 20.6-42.3 mol% were synthesized. These SLs may combine the rapid assimilation and energy release properties of caprylic acid with the heart-health benefits of SDA when consumed.

The classical gelatin-gum arabic system for encapsulating oils by complex coacervation was modified by thermal pre-treatment of the gelatin-gum arabic blend. Heat treatment of the blend initiated the Maillard reaction which resulted in crosslinking of these biopolymers and the formation of other Maillard reaction products (MRPs). The heat-

treated gelatin-gum arabic blend showed enhanced antioxidant capability compared to the unheated blend. Furthermore, microencapsulation of SDA soybean oil within coacervates formed from the MR-modified gelatin-gum arabic blend was successful. The microcapsules formed using the MR-modified coacervates exhibited enhanced oxidative, thermal, and colloidal stability compared to microcapsules based on unmodified or transglutaminase-crosslinked coacervates.

When gelatin-gum arabic [GE-GA] or gelatin-gum arabic-maltodextrin [GE-GA-MD] blends were crosslinked by Maillard reaction, the MRP from both types of blends were found to contain protein-polysaccharide conjugates. They also had enhanced antioxidant and emulsifying capabilities compared to non-crosslinked blends. During heat treatment, the [GE-GA-MD] blends browned faster and had significantly higher antioxidant capacities than [GE-GA] blends. The microencapsulation of SDASO in complex coacervates formed from MR-modified [GE-GA] and [GE-GA-MD] blends was successfully demonstrated. Microcapsules produced from MR-modified blends had improved colloidal and oxidative stability than the controls. The antioxidant capacity of MR-modified blends was optimized to produce coavervate-based microcapsules that minimized the oxidation of microencapsulated SDASO. The optimized MR-modified [GE-GA-MD] blend was more effective than the optimized [GE-GA] blend because it conferred higher oxidative stability to the microencapsulated SDA soybean oil. Overall, MR-modified coacervate-based microcapsules are well-suited for incorporating SDA soybean oil into foods and may facilitate the formulation of omega-3 PUFA-fortified functional food products at the commercial scale.

Suggestions for future work:

1. Replace porcine gelatin with fish gelatin in MR-modified blends for kosher and halal food applications.
2. Microencapsulation of other omega-3 oils including fish oil in MR-modified gelatin-gum arabic coacervates and study the effect on oxidative stability.
3. Sensory analysis to evaluate the implications of adding MR-modified coacervate-based microcapsules on acceptability of a food product.