ENRICHMENT OF MODIFIED SOYBEAN OIL AND ITS DERIVATIVES IN STEARIDONIC ACID BY CHEMICAL AND ENZYMATIC TECHNOLOGY

by

LESLIE KLEINER

(Under the Direction of Casimir C. Akoh)

ABSTRACT

Stearidonic acid (SDA, 18:4 ω-3) is a polyunsaturated fatty acid that is a metabolic intermediate in the conversion of α-linolenic acid (ALA, 18:3 ω-3) to eicosapentaenoic acid (EPA, 20:5 ω-3). The objective of this research was to explore methods to obtain SDA enriched triacylglycerols (TAGs) and/or their derivatives, from modified soybean oil (MSO) containing ~25% SDA. Argentation silica gel column chromatography led to the isolation of SDA ethyl esters of 97% purity and 71% yield, in a scaled-up process. Enzymatic transesterification (acidolysis) was used to produce a structured lipid with 53.46 ± 1.85% SDA (36.37 ± 3.14% at the *sn*-2 position). Lipase-catalyzed concentration of SDA by partial hydrolysis of MSO resulted in unhydrolyzed TAG, 1,3-diacylglycerols, 2,3(1)-diacylglycerols, and monoacylglycerols with 58.66 (65.71 at the *sn*-2 position), 71.22, 70.22 (52.90 at the *sn*-2 position), and 59.43% SDA, respectively. All the SDA enriched products could have potential use as nutraceutical compounds.

INDEX WORDS: Acidolysis, Argentation silica gel column chromatography, Hydrolysis, Stearidonic acid, Structured lipids, Winterization

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DEDICATION

I dedicate this work to those who inspired me to keep looking forward.

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CHAPTER 1

INTRODUCTION

The dietary consumption of polyunsaturated fatty acids (PUFAs) has long been associated with health benefits to humans. In particular, dietary α-linolenic acid (ALA, 18:3 ω-3), eicosapentaenoic acid (EPA, 20:5 ω-3), and docosahexaenoic acid (DHA, 22:6 ω-3) have been linked to a decrease in cardiovascular disease [1], inflammation [2], cancer [3], and neurological disorders [4]. Recently, FDA approved Lovaza®, the first ω-3 prescription drug targeted to reduce the hepatic production of triacylglycerols. This drug is comprised of 90% or more of ethyl ester derivatives of EPA and DHA [5]. Although EPA and DHA are metabolic products in the ω-3 PUFA conversion pathway of ALA, the involvement of the Δ6-desaturase enzyme presents a rate limiting step that leads to a poor overall conversion [5, 6]. Furthermore, due to the genetic absence of Δ12 and Δ15- desaturases in animals, it is not possible to metabolize oleic acid (18:1 ω-9) into ALA as it is seen in plants [7], therefore ALA, EPA and DHA must be incorporated into the diet for their nutritional uptake.

EPA and DHA are present in all fish, but are found in higher concentrations in fatty fish such as salmon, rainbow trout, mackerel, tuna, and herring [5]. As a consequence of over-fishing, a severe decline of marine stocks has been observed in the last decades [7]. This decline in the fish population, developed into the "fishing down of marine food webs", which is the fishing of species lower in the food chain than more

desirable fish populations [8]. Therefore, it is of interest to develop alternative methods for the production of fish oils. Aquaculture, single cell oil, and plant modification are three different approaches employed to relieve pressures on marine environments. Aquaculture requires fish oil as a dietary input for the production of fish, therefore becoming the largest consumer of fish oils [6, 9] and making this approach unsustainable. The single cell oil production of EPA and DHA is financially viable for specific high value markets, such as infant formula and related products [10], but costly to scale-up for other applications [7]. However, the modification of the biosynthetic capabilities of plants is commercially viable, applicable to various markets, reduces pressure on marine ecosystems, and has been successful in the production of EPA and other PUFAs [7].

Recently, much attention has been given to stearidonic acid (SDA, 18:4 ω-3); a PUFA that is a metabolic intermediate in the conversion of ALA to EPA [11]. In this conversion, SDA has been shown to increase the total red blood cell- EPA with approximately 17% of the efficiency of EPA, while the efficiency of ALA in the same process was 0.1% [12]. SDA biosynthesis bypasses the rate limiting Δ6-desaturase step in the metabolic pathway of (ω-3) PUFA, becoming nutritionally desirable due to its direct conversion to EPA [7]. Structurally, this fatty acid has a shorter carbon chain than EPA and DHA and it has 4 double bonds instead of 5 and 6, as are present in EPA and DHA, respectively. These characteristics make SDA more stable than fatty acids with higher unsaturation indices, which lead to a more rapid oxidation, the development of off-flavors, and a shorter shelf-life [6]. Since there are reported health benefits after dietary consumption of SDA, and this fatty acid is less prone to lipid oxidation than EPA and DHA, it is of interest to explore this PUFA as a source of ω-3 in the diet.

SDA is found in low quantities in fish and some other marine life. While EPA and DHA constitute 15-20% of the total fatty acid profile of fish, SDA comprises 0.5-2% of the fatty acids [13]. Low levels of SDA (0.7-1.9 mg/ g dry weight) are found in seaweed (*Undaria pinnatifida*); however, with the exception of the Boraginaceae and Grossulariaceae family, SDA is rarely found in terrestrial plants [7]. Echium oil (*Echium plantagineum*), from the Boraginaceae family, represents the richest commercially available source of SDA (3.5-9.0%, by weight) and this is followed by currant seed oil (*Ribes nigrum*) from the Grossulariaceae family, which contains 2-6% SDA [7]. By genetically modifying soybean oil, it is possible to increase the SDA content of modified soybean oil (MSO) to ~25% of the total fatty acid profile. However, a further SDA enrichment of MSO is of interest for its applications as a functional food component; therefore we have studied various approaches for the SDA-enrichment of MSO and its derivatives (ethyl esters, mono- and diacylglycerols).

This thesis includes six chapters concerning the SDA enrichment of MSO and its derivatives. The first chapter is an introduction with the objectives of this research. This is followed by a literature review concerning current approaches to purify or enrich SDA containing lipids. In the third chapter, argentation silica gel column chromatography was explored in order to develop a method to purify ethyl esters of SDA (SDA-EE) from ethyl ester derivatives of MSO. This method was later scaled up from analytical to semi-preparative scale. In the fourth chapter, an enzymatic interesterification approach was employed to produce SDA enriched structured lipids under different reaction conditions. Novozym 435 and Lipozyme TL IM immobilized enzymes, as well as substrate molar ratio, solvent, and enzyme load conditions were studied for the total incorporation of

SDA, as well as for the incorporation of SDA at the *sn*-2 position. The fifth chapter concerns the lipase-catalyzed concentration of SDA in MSO by hydrolysis reactions, as well as a study on lipase specificity toward SDA. In this work, immobilized and non-immobilized powdered enzymes were explored for their ability to both hydrolyze MSO and concentrate SDA in the hydrolysis products. The specific objectives of this thesis are:

1. To isolate SDA-EE from a mixture of over 20 fatty acid ethyl esters (FAEE) by argentation silica gel column chromatography.
2. To study viable conditions for the enzyme-catalyzed synthesis of structured lipids enriched in SDA.
3. To concentrate SDA in the acylglycerol products obtained by the lipase-catalyzed hydrolysis of MSO, and to study lipase specificity for SDA and/or its ability to discriminate against SDA.

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CHAPTER 2

LITERATURE REVIEW

Stearidonic Acid

Dietary ω-3 PUFAs, such as EPA and DHA, have long been recognized for their preventive benefits in cardiovascular disease (CVD) and rheumatoid arthritis, among other benefits [1]. However, not enough fish is consumed in the Western diet, and growing concerns over the bioaccumulation of methlymercury in some fish [2] can only discourage an increase in fish consumption. For healthy individuals, the American Heart Association (AHA) recommends a dietary intake of fish that is equivalent to ~500 mg EPA+DHA/d; this dosage increases to 1 and 2-4 g/d for patients with CVD and hypertriglyceridemia, respectively [3]. The potential mechanisms for the reduction of CVD are linked to the antithrombogenic, hypotriglyceridemic, and antiinflammatory properties of ω-3 fatty acids [4]. In addition, ω-3 PUFAs reduce the susceptibility of the heart to ventricular arrhythmia and retard the growth of atherosclerotic plaque [4].

Current approaches to increase the availability of fish oil in the Western diet involve the use of oils rich in plant derived ω-3 (ALA), the reformulation of conventional foods to incorporate fish oils, and/or the use of ω-3 supplementation. As discussed in Chapter 1, the first approach leads to a poor in vivo conversion of ALA into EPA, reducing the benefits associated with ω-3 fatty acid consumption. The second approach, incorporation of fish oils into foods, is problematic for two particular reasons: availability and stability of fish oils. Concerns over the sustainability of global fish stocks indicate

that marine ecosystems need to be repaired and are no longer ideal sources for the production of fish oil. Furthermore, once the fish oil has been extracted, its incorporation into reformulated food products is hindered due to the poor stability and cost associated with this oil [2]. Therefore, it is desirable to find a terrestrial source of ω-3 PUFA that would improve the bioconversion deficiencies of ALA and reduce environmental pressure on marine ecosystems, while providing similar health benefits to those associated with EPA and DHA.

Stearidonic acid (SDA, C18:4 ω-3) is an ω-3 PUFA that is primarily found in oily fish, seaweed, and in few terrestrial plants, such as those of the Boraginaceae and Grossulariaceae family. Currently, the richest commercially available source of SDA is Echium oil (3.5-9.0% of SDA, by weight); however interest in the health benefits and structural properties of this fatty acid led to the development of other terrestrial sources of SDA [1,2]. SDA is a metabolic intermediate in the biosynthetic pathway of ω-3 PUFA, and it has the metabolic advantage of bypassing the Δ6-desaturase step which is rate limiting in the conversion of ALA to EPA. From Figure 2.1, it is seen that the essential fatty acid ALA is the precursor to the ω-3 family of PUFA, and SDA is the second metabolite in its conversion to EPA and DHA. This conversion is catalyzed by a series of steps involving desaturase and elongase enzymes. The first step in this pathway involves the Δ6-desaturase enzyme, which is rate limiting in humans [5] and thought to limit further conversion down the pathway.

Several studies have focused on the health benefits associated with the dietary intake of SDA and its comparison to that of other ω-3 PUFAs [5]. James et al. [3] examined the ability of SDA to increase the tissue concentration of EPA and DHA in

healthy human volunteers, and compared its efficacy to that of dietary ALA and EPA. In this study, recruited subjects were screened for normolipidemia, and were randomly assigned in blocks of 3 to one of three groups in which ALA, SDA, or EPA was administered on a double-blind basis. The ω-3 PUFAs were supplied as ethyl esters in capsules for a three week period; subjects were instructed to avoid ω-6 PUFAs and were provided with food products high in ω-9 fatty acids to replace those usually containing high levels of ω-6 fatty acids. The avoidance of ω-6 fatty acids was of interest due to the competition for the same desaturase and elongase enzymes seen in the metabolism of ω-3 PUFAs. These enzymes are also involved in the metabolic conversion of ω-6 fatty acids to eicosanoids and other hormone-like compounds [5], see Figure 2.1.

The main outcome of this study was the finding that dietary SDA was metabolized to EPA in humans, and that SDA was more effective than ALA in its conversion to EPA [3]. The effectiveness of SDA in comparison to ALA and EPA was quantified as ~1:0.3:0.07, indicating that 1 g of dietary SDA was approximately equivalent in its ability to increase EPA concentrations in tissue, to 300 mg of dietary EPA [3]. Other studies on SDA have elucidated potent inhibitory characteristics in cancer cell growth, as well as an inhibitory effect on platelet aggregation, and increased efficiency over ALA in the reduction of the ω-6/ω-3 PUFA ratio in cells [5]. Furthermore, Lemke et al. [2] studied the ability of SDA from stearidonic acid-enriched soybean oil to increase the ω-3 index, which is defined as the percentage of erythrocyte EPA + DHA over the total fatty acids. The dietary effect of stearidonic acid-enriched soybean oil (~28% SDA, by weight) was also compared to that of conventional soybean oil (<0.1% SDA, by weight) in a randomized, double-blind clinical study. The authors

reported that in agreement with previous studies using ethyl esters, SDA-enriched soybean oil is approximately one third to one fifth as effective as EPA at increasing tissue content of EPA in humans [2].

In addition to the health benefits associated with SDA, its physical structure leads to stability advantages over EPA and DHA. From Figure 2.2 it is seen that SDA is comprised of an 18-carbon chain with four double bonds. EPA and DHA are composed of 20- and 22-carbon chains with 5 and 6 double bonds, respectively. Since SDA has a shorter acyl chain and less double bonds than EPA and DHA, it is physically more stable and resistant to lipid oxidation (off-flavors production and shorter shelf-life), than PUFA of longer acyl chain and higher degree of unsaturation [5]. Based on its nutritional value, stability, and land-based production, we have utilized stearidonic-acid enriched soybean oil (modified soybean oil (MSO)) containing ~23-25% SDA, to explore strategies to further increase its SDA content in its triacylglycerol form, as well as that of some of its derivatives. Table 2.1 illustrates the fatty acid profile of MSO. The major fatty acids in MSO are SDA (~25%), linoleic, oleic, palmitic acid (~24, 14, 13%, respectively), ALA (~11%), γ-linolenic acid (GLA, ~7%), and stearic acid (~4%).

Modified Soybean Oil

The bioengineering of soybean and canola oilseeds has been a topic of special interest as a sustainable plant-based source for the synthesis of ω-3 fatty acids [6]. In this sense, MSO is produced in an effort to obtain stearidonic acid from a sustainable plant source. For the production of MSO, the Monsanto Company (St. Louis, MO, USA) has modified soybean MON 87769 by introducing two different desaturase genes that encode for the

proteins *Primula juliae* Δ6 desaturase and *Neurospora crassa* Δ15 desaturase, respectively [7]. The Δ6 desaturase gene is not naturally present in soybean and it is required for the synthesis of SDA; however, the presence of this gene may also convert linoleic acid to GLA [7,8]. Therefore, it is necessary to introduce the Δ15 desaturase gene, which has a temporal expression and its presence is intended to reduce the levels of linoleic acid, as well as to increase the flux of ALA to SDA. The resulting phenotype has been reported to be stable over several generations [7].

The methods used to process this oil are standard processing methods, and the resulting fatty acid profile of the refined MSO is different than the fatty acid profile of conventional soybean oil. Conventional soybean oil contains neither SDA nor GLA; however, MSO contains 15 to 30% SDA and 5 to 8% GLA [7]. In addition, MSO has a slightly higher content of ALA (9-12%) and palmitic acid (9-13%) than that observed in conventional soybean oil (5-10%, 7-12%, respectively). The contents of oleic (10-20%) and linoleic acid (15-30%) are lower than those observed in conventional soybean oil (19-30%, 48-65%, respectively) [7]. This oil received GRAS (Generally Regarded as Safe) status from The Food and Drug Administration in 2009 [8], but it is not commercially available at this time. MSO has an intended application as a food ingredient in the United States in many different products aimed to deliver 375 mg SDA/serving [7,8]. These products are oils and fats, baked goods, cereals, grains, dairy products and their analogs, sauces, snacks, candies, meats, poultry, processed fruits and vegetables, and others [7,8].

Argentation Silica Gel Column Chromatography

Argentation (Ag) silica gel chromatography is a technique that has been utilized to perform chromatographic separations of various types of lipids and PUFAs [9,10]. Interest in this type of chromatography arises from the ability of the silver ions to form reversible polar complexes with unsaturated locations in organic molecules [11]. Based on the degree of unsaturation and the geometrical configuration and position of the double bonds, the strength of the polar complexes formed vary, therefore allowing for the differentiation of lipids based on their polarity. This principle has been applied to thin layer chromatography (TLC), column chromatography, high pressure chromatography (HPLC), and supercritical fluid chromatography [11].

In Ag-silica gel column chromatography, the silica comprising the stationary phase is coupled to silver ions (Ag^+) by means of a silver nitrate solution. The concentration of this solution influences the amount of Ag^+ coupled to the silica gel, therefore affecting the retention time of the solute loaded into the column containing this stationary phase [12]. Different polar compounds can then be separated based on the polar complexes formed with the stationary phase and the polarity of the solvent used for elution (the mobile phase). This technique presents several advantages over winterization [13], urea complexation [14], and HPLC [15], which are other methods commonly used for the fractionation of PUFAs. Unlike winterization and urea methods, argentation silica gel column chromatography allows for the isolation of PUFAs with purities higher than 90%, without the additional coupling to HPLC. This additional step further increases the cost and time associated with purification. Regarding urea complexation methods, it has

been observed that these can lead to the formation of ethyl carbamate (urethane), which is a known carcinogen in animals [16]. Therefore, Ag-silica gel column chromatography is a suitable method for the isolation of PUFAs.

Different types of PUFAs have been fractionated by Ag- silica gel column chromatography. Belarbi et al. [17] employed this technique to isolate EPA esters from the biomass of the diatom *Phaeodactylum tricornutum* and the green alga *Monodus subterraneus*. Initially, a simultaneous extraction and transesterification of microalgal fatty acids with methanol was performed. The recovered methyl ester products were later fractionated by Ag-silica gel column chromatography, leading to the isolation of EPA methyl esters of over 90% purity and 70% yield. In the same study, the authors also reported the isolation of EPA from the ethyl esters of Cod liver oil (82.7% purity, 68% yield). For both microalgal and fish oil esters, the optimal loading of the esters on the stationary phase was 3% (w/w, weight of sample/ weight of Ag-silica gel). The solvents used for elution of the esters were combinations of hexane and acetone, with the acetone content increasing in the final fractions in order to isolate the more polar esters.

In a related work, Guil-Guerrero and Belarbi [18] further explored purification processes for PUFAs from Cod liver oil. In this case, EPA and DHA were fractionated by a combined four-step process. Saponification was performed to remove unsaponifiable material and acidify the remaining soaps to free the fatty acids. These free fatty acids (FFAs) were subjected to urea concentration for the isolation of PUFAs, and further derivatized to fatty acid methyl esters (FAME). The resulting FAMEs were separated by Ag-silica chromatography at a maximum fatty ester loading into the stationary phase of 4% (w/w). As reported by Belarbi et al. [17], the elution solvents were hexane and

acetone solutions, with increased acetone content in the final fractions. DHA methyl esters (ME) of 100% purity and 64% yield, as well as EPA-ME of 90.6% purity and 29.6% yield were obtained in the combined process.

Ag-silica gel chromatography has also been employed to purify PUFAs from seed oil sources. In a similar, four-step approach, Guil-Guerrero et al. [10] isolated GLA-ME from the seed oils of *Anchusa azurea*, *Scrophularia sciophila* and *Echium fastuosum*, respectively. The combined process led to the recovery of GLA-ME of 97% purity, 72.7% yield and 97% purity, 64.4% yield from A. *azurea* and *S. sciophila*, respectively. For *E. fastuosum* the recovery was lower (86% purity, 60% yield) due to the co-elution of ALA- and GLA-ME in the same fraction. Similar to other studies by the same group [17, 18], the methyl ester load was 3.5% (w/w) and the mobile phase was comprised of hexane and acetone solutions. A related approach was followed by Ryu et al. [19] to isolate ALA-ME from a hydrolyzate of perilla oil which was concentrated by winterization (low temperature crystallization). By pulling three fractions, the authors obtained ALA-ME of ~90% purity and 90% yield.

Few studies have been reported on the use of Ag-silica gel column chromatography for the isolation of SDA derivatives. In a study concerning various types of PUFAs, Guil-Guerrero et al. [9] isolated EPA, DHA, and SDA-ME from Shortfin Mako (*Isurus oxyrinchus*) liver oil. From a 30 mg methylated crude oil sample, the authors were able to isolate a fraction of SDA of 83.2% purity and 96.3% yield. Conditions were as previously employed by this group, and consisted of a maximum load into the column of 4% (w/w) and a mobile phase comprised of hexane and acetone solutions. Furthermore, Rincon-Cervera and Guil-Guerrero [15] have also isolated SDA

from *Echium plantagineum* by means of Ag-silica gel column chromatography (30.8 % purity). Based on the aforementioned studies it was of interest in our work to study Ag-silica gel column chromatography as an approach to isolate SDA from the ethyl ester derivatives of MSO.

Structured Lipids and Enzymatic Transesterification (Acidolysis)

Structured lipids (SLs) are triacylglycerols (TAGs) that have been chemically or enzymatically modified to restructure and/or incorporate fatty acids in the acylglycerol backbone [20], therefore changing the fatty acid profile of the original TAG. This type of modification is intended to achieve desired nutritional and/ or physical characteristics in the lipid. From a nutritional aspect, it is desirable to redirect and/or incorporate PUFAs to the *sn*-2 position of the acylglycerol backbone where they are better absorbed [15], see Figure 2.3. In this sense, Teichert and Akoh [21] have enriched SDA containing soybean oil with palmitic acid (PA) at the *sn*-2 position, for use as human milk fat analogues. Human milk has over 60% of PA located at the *sn*-2 position, and this serves to improve the absorption of calcium and fat in infants [21]. By synthesizing a SL with both SDA and PA, ω-3 PUFAs could also be added to the diet of the infant.

TAGs are also modified in order to impart different physical characteristics to a lipid and therefore affect its functionality. After the restructuring of a lipid, the melting point, oxidative stability, iodine value, and saponification value are properties that usually change, therefore reflecting differences from the original TAGs. For example, with the aim of producing low-calorie fats, Yankah and Akoh [22] have synthesized SLs containing both medium and long chain fatty acids. This is of interest because the

addition of medium-chain TAGs has been linked to physiological benefits, due to their rapid absorption and low tendency to remain in the body as stored fat [23]. The authors produced two types of SLs by incorporating caprylic acid and oleic acid into tristearin, respectively [22]. Furthermore, in contrast to the physical properties of tristerin, these structured lipids had melting profiles that indicated plasticity. This is of particular interest for the development of spreads.

SLs can have applications ranging from nutraceutical components in formulated products, to fat substitutes and parenteral nutrition support. Some commercially available SLs are Caprenin (Procter & Gamble Co., Cincinnati, OH), Benefat^{TM} (Salatrim) (Cultor Food Science, New York), and Intralipid (Kabi Vitrum, Berkeley, CA) [20]. Caprenin is a low calorie SL that is liquid or semi-solid at room temperature, can be used as a cocoa butter substitute, and has applications in soft candies and confectionary products [20]. Benefat^{TM} is a low-calorie fat that is intended for use in baking and other products [20]. Intralipid is a 20% soybean oil emulsion that constitutes the main source of calories in total parenteral nutrition, which is administrated to patients with severe intestinal failure [24].

One method to produce SLs is that of enzymatic transesterification. In this process, acyl groups are exchanged between two ester containing molecules. Transesterification can be performed between two different acylglycerols, an acylglycerol and a fatty acid (acidolysis), an acylglycerol and an alcohol (alcoholysis), and glycerol and a triacylglycerol (glycerolysis) [25]. Although transesterification can be accomplished by chemical methods, the use of enzymes introduces several advantages to the process. While chemical transesterification produces a complete positional

randomization of the acyl groups in the TAGs [25], enzymatic transesterification allows for positional and/or specificity toward particular fatty acids. In addition, the use of enzymes reduces the amount of solvents and other chemicals needed in the reaction, as well as the cost associated with solvent removal and its further treatment. Therefore, enzymatic transesterification can be considered a green chemistry approach to the synthesis of SLs.

Of the reactions described above, enzymatic acidolysis (lipase-catalyzed acidolysis) allows incorporation of novel fatty acids into the acylglycerol backbone, as shown in Figure 2.4. This approach has been employed by Teichert and Akoh [26] to produce a SL with possible applications in infant formula. This SL was based on the enrichment of previously synthesized SLs with high palmitic acid content at the *sn*-2 position, and GLA or DHA. To catalyze the reactions, the authors utilized the immobilized lipase, Lipozyme TL IM (*Thermomyces lanuginosus*). The SLs obtained by the lipase-catalyzed acidolysis reaction retained PA at the *sn*-2 position and incorporated GLA or DHA at the *sn*-1,3 position of the TAG [26]. Similarly, Senanayake and Shahidi [27] studied the incorporation of DHA into evening primrose oil (*Oenothera biennis* L.) by Novozym 435 lipase-catalyzed acidolysis, among other lipases. Novozym 435 is a lipase extracted from *Candida antarctica*. The authors were able to incorporate 37.4% DHA into evening primrose oil; this oil had no detectable DHA previous to the enzymatic modification.

The mechanism for acidolysis is commonly considered a two-step reaction in which diacylglycerols (DAGs) are the reaction intermediates [28] that lead to the structured TAGs, see Figure 2.5. Triacylglycerol lipases (triacylglycerol acylhydrolases

EC 3.1.1.3) catalyze the hydrolysis and transesterification of lipids, with hydrolysis being predominant in reaction media with high contents of water, and esterification occurring when the water content is low [29]. Since enzymes inherently need some moisture content to be active, enzymatic acidolysis is a reversible reaction. Once the reaction reaches equilibrium, it yields a variety of products. The products obtained depend on the substrate molar ratio between esters and fatty acids (TAG: FFA), and reaction conditions such as temperature, incubation time, solvents, lipase, and enzyme load employed [27, 28, 30].

An example of the role of these parameters in lipase-catalyzed acidolysis is given by the work of Akoh and Mousatta [31], which studied substrate molar ratio, incubation time, solvents, and enzyme load in the SP435- and IM60 lipase-catalyzed acidolysis of Borago oil (*Borago officinalis* L.) and capric and eicosapentaenoic acids. SP435 is a non-specific lipase from *Candida antarctica*, and IM60 is a *sn*-1,3-specific lipase from *Rhizomucor miehei*. The authors observed that by increasing the substrate molar ratio, incubation time, and enzyme load, the incorporation of capric acid and EPA into the structured lipid also increased [31]. The authors also reported hexane and isooctane as the best solvents for these reactions. In agreement with the work by Akoh and Mousatta [31], Senanayake and Shahidi [27] noted that in the Novozym 435-catalyzed incorporation of DHA into evening primrose oil, as substrate molar ratio, incubation time, and enzyme concentration increased, so did the DHA content incorporated into the SL. In addition, the DHA incorporation was better in hexane than in solvent-free media.

Regarding the use of solvents, Klibanov [30] has previously noted that enzymes retain their activity in certain organic solvents, particularly hydrophobic ones. This is of

importance because many enzymatic processes are not possible in water (in the case of acidolysis, this would mean hydrolysis versus esterification). In addition, the solvent changes the enzyme specificity [30]; therefore, novel outcomes can be explored by performing the same lipase-catalyzed reactions under different solvent conditions. An example of the role of solvent is illustrated by the work of Jennings and Akoh [32], which studied the *Rhizomucor miehei* lipase-catalyzed acidolysis between menhaden fish oil concentrate and capric acid in hexane and solvent-free media. The authors noted that hexane, led to a higher incorporation of capric acid into the SL than the solvent-free reaction did. Although, for food applications it is preferred to perform lipid modification in solvent-free media, it is possible that this would affect the overall outcome of the SL produced.

Lipase-catalyzed acidolysis has been studied for the incorporation of ω-3 PUFAs into various types of oils. Nagachinta and Akoh [33] studied the Novozym 435 lipase-catalyzed acidolysis between palm olein and a free fatty acid mixture obtained from DHASCO® and ARASCO®; substrate molar ratio, reaction temperature, and incubation time were also studied. The authors successfully enriched palm olein with DHA and ARA, for its potential use as functional food component. Similarly, in the aforementioned work by Teichert and Akoh [21] lipase-catalyzed acidolysis was employed to produce a human milk fat analog from SDA-enriched soybean oil and palmitic acid. The authors employed Novozym 435 (non-specific) and Lipozyme TL IM (*sn*-1,3 specific) lipases under various conditions of substrate molar ratio, temperature, and incubation time.

Based on the many benefits associated with the enzymatic production of SLs, and on the successful synthesis of SLs containing ω-3 PUFAs, acidolysis was chosen as an

approach to increase the SDA content of MSO. The type of lipase, substrate molar ratio, incubation time, enzyme load, and solvent conditions were studied in order to determine the best conditions that would lead to a high SDA incorporation in the SL, as well as to an economically viable process.

Lipase-Catalyzed Hydrolysis

The hydrolysis of oils is an important component in the concentration of ω-3 PUFAs from marine oils. Although the consumption of fish oils is valuable for its health benefits, PUFA concentrates may be more beneficial due to lower saturated fatty acids contents [34]. As previously discussed, there are many different methods for the enrichment of oils with PUFAs. Some of these methods include urea complexation, chromatographic isolation, and low temperature crystallization (winterization). One of the advantages of the hydrolysis of oils is that the resulting products are in the acylglycerol form; this form is nutritionally more favorable than the methyl or ethyl ester form of the fatty acids [34].

Hydrolysis is usually achieved by high pressure steam splitting ($250^{o}C$, $7x10^{6}$ Pa), alkaline hydrolysis, or enzymatic hydrolysis [29]. The high temperature and pressure needed for steam splitting may lead to the thermal degradation and/ or polymerization of ω-3 PUFA, therefore becoming unsuitable for the concentration of PUFAs in fish oil [35]. Alkaline hydrolysis is associated with the use of extreme pH for the production of soaps and their further acidification and removal; this translates to longer processes and high costs. However, lipase-catalyzed hydrolysis of oils can be carried out at ambient conditions (approximately 35 ^{o}C) and depending on the lipase, for a short incubation

time. In addition, there are several advantages associated with this enzymatic process. For example, there is no involvement of extreme temperatures and pressures, and the use of lipases can lead to the selective cleavage of undesirable fatty acids. This has been particularly useful for the concentration of EPA and DHA in oils. Due to the high degree of unsaturation of these fatty acids, and the non-linearity of their respective molecules, many lipases are unable to hydrolyze EPA and DHA from its proximate location to the ester bond of the TAG [34].

In enzymatic hydrolysis, lipases catalyze the hydrolysis of oils into free fatty acids and glycerol, yielding 1 mol of glycerol and 3 moles of fatty acids per mole of TAG, at completion of the reaction [36]. If the reaction is arrested before its completion, a variety of acylglycerol forms are obtained in the product. The reaction products are therefore comprised of monoacylglycerols (MAGs), diacylglycerols (DAGs), unhydrolyzed triacylglycerols, glycerol and FFAs. The acylglycerols are extracted in organic solvent (usually hexane) and the glycerol and lipase remain in the aqueous phase [28]. The steps of enzymatic hydrolysis of oils and fats are shown in Figure 2.6. Depending on the lipase used for the hydrolysis reaction, the kinetics of the reaction varies. In consequence, so does the percentage of hydrolysis at a specific incubation time, and the individual fatty acid profiles of each of the acylglycerol products obtained.

The products from the partial hydrolysis of oil have a diverse commercial value. MAGs and DAGs are important emulsifiers that are widely used in the food industry [36]. In addition to emulsifying applications, the Kao Corporation of Japan introduced DAG oils (~80% DAGs) into the cooking oil market in 1999 [37]. The rationale for the use of these oils is that contrary to TAG oils, the long-term ingestion of dietary DAG oils

reduces the accumulation of body fat in humans [38]. This is probably due to DAG oils having higher contents of 1,3-DAGs instead of 1,2-DAGs [37]. These 1,3-DAGs are metabolized in a different fashion than TAGs, and is therefore suspected that their metabolism leads to various health benefits in humans [38]. Some of these benefits include the maintenance of lower body weight, improved blood sugar in diabetic patients, and the control of post-meal blood lipids [37]. Other products derived from the hydrolysis of oils, such as FFAs, can be recovered for their further use in acidolysis, among other reactions.

Several lipases have been studied for the hydrolysis of many different types of oils and ethyl esters. Wanasundara and Shahidi [39] studied the hydrolysis of seal blubber and menhaden oil (SBO and MHO, respectively) by different lipases. The objective of their work was to hydrolyze the marine oils while concentrating EPA and DHA in the acylglycerol products. The authors noted that *Candida cylindracea* (CC) lipase led to the highest concentration of ω-3 PUFAs in both oils. From ~6.4% EPA and ~7.6% DHA originally present in SBO, the CC lipase-catalyzed hydrolysis led to a total EPA and DHA content in the acylglycerol products of 9.75 and 24%, respectively. Similarly, from 13.2% EPA and 10.1% DHA originally present in MHO, CC-lipase led to a total EPA and DHA content in the reaction products of 18.5 and 17.3%, respectively. In the same work, it was noted that *Rhyzopus oryzae* lipase led to a better degree of hydrolysis of MHO than that achieved with CC lipase; however, the EPA content of the products decreased with time and extent of hydrolysis. This illustrates that in the concentration of ω-3 PUFA via lipase-catalyzed hydrolysis, a balance needs to be found between the hydrolysis of the oil and the ability to discriminate against ω-3 PUFAs.

Hoshino et al. [40] have also studied the selective hydrolysis of fish oil. The authors studied the ability of six different lipases to hydrolyze cod liver oil and refined sardine oil. Under the conditions studied, *Candida cylindracea* led to more than two-fold increase of ω-3 PUFA in the acylglycerol products. Similarly, Okada and Morrissey [41] studied lipase-catalyzed hydrolysis reactions for the concentration of ω-3 PUFAs in oil extracted from Pacific sardines (*Sardinops sagax*). The authors noted that, of the lipases studied, *Candida rugosa* lipase was the most effective enzyme for this process, since EPA and DHA contents were increased by 25 and 120%, respectively. The same *Candida rugosa* lipase, was the most effective lipase used by Sun et al. [34] in the concentration of ω-3 PUFA in farmed Atlantic salmon (*Salmo salar* L.) viscera oil. With this enzyme, the original concentration of EPA in viscera oil (64 mg/g) was increased to 91 mg/g).

Regarding fatty acid selectivity of lipases, Halldorsson et al. [42] studied numerous commercially available lipases for their ability to discriminate EPA from DHA. The authors utilized *Candida rugosa* and *Rhizopus oryzae* lipase, among others, to study the hydrolysis of fatty acid ethyl esters from sardine oil. The lipases assayed were categorized based on their ability to promote more than 50% hydrolysis of the ethyl esters in a short time (4 h), ability to discriminate against ω-3 PUFA, and/ or the ability to differentiate between EPA and DHA. Some of these high-activity lipases were *Candida rugosa* and *Rhizopus oryzae*, which showed a moderate to strong ability to differentiate EPA from DHA. As previously mentioned for the *Rhizopus oryzae*-catalyzed hydrolysis of SBO and MHO, this lipase was specific toward EPA (as seen by the reduction of EPA content with an increase of hydrolysis time) [39].

Although many studies have been performed on the concentration of ω-3 PUFAs in marine oils, most studies have focused on the concentration of EPA and/ or DHA. To the best of our knowledge, no studies have been performed on either the concentration of SDA in oils via lipase-catalyzed hydrolysis reactions. Furthermore, a literature search showed no studies concerning the ability of lipases to either discriminate against SDA or be selective toward it. Therefore, it was of interest to study the lipase-catalyzed concentration of SDA in MSO, and also the behavior of the lipases in their selectivity toward SDA.

Low-Temperature Crystallization (Winterization)

Low temperature crystallization (LTC), also known as winterization, is a method that is commonly used to concentrate PUFAs in large scale. Although urea complexation is a technique that is also available, as previously mentioned, it has been associated with the formation of ethyl carbamates [16]. In LTC, the lipids are subjected to a partial crystallization in order to separate by filtration a solid (crystallized) fraction from a liquid fraction [13]. Since the degree of unsaturation of fatty acids highly influences their melting point, it is possible to use this characteristic to separate saturated from unsaturated fatty acids. Therefore, the crystallized fraction is mostly comprised of long chain saturated fatty acids, which have higher melting points than unsaturated ones [43]. This allows collecting the remaining liquid fraction, which is mostly comprised of PUFA.

Since it has been reported that the organic solvent and temperature affect the concentration of PUFAs [13], it is crucial to determine suitable temperature and solvent

conditions. In a generic LTC process, the oil is slowly chilled to approximately 68 ^{o}C during a 24-h period, then cooling is stopped and the solid/liquid mixture is allowed to rest for 6-8 h. This is followed by filtration to collect either one or both fractions [44]. In solvent winterization however, LTC is performed in oil that has been previously dissolved in a desirable solvent. This allows for the selective crystallization of TAG or FFA components at different temperatures, which can be affected by the solubility of the TAG or the FFA in the solvent. Lopez-Martinez et al. [13] reported the concentration of GLA from FFA from *Borago officinalis* and *Echium fastuosum* by LTC. The authors studied various winterization solvents, oil:solvent ratios, and temperatures, and reported that hexane at 10% (w/w) and -70 ^{o}C led to the best GLA purity and yield for both types of FFAs.

Although several studies have been performed on the LTC of PUFAs, few have focused on the concentration of SDA. With the aim of increasing SDA content in the TAG form, Vazquez and Akoh [45] studied the LTC of MSO. At a constant temperature of -80 ^{o}C, the authors explored variables such as winterization time, solvent-type, and oil:solvent ratio leading to the best SDA purity and yield in the concentrated fraction. At best conditions (10% oil:solvent ratio, with hexane:acetone (10:90, v/v) solvent, and winterization time of 24 h), it was possible to collect a fraction containing SDA of 44.5% purity and 35.1% yield. The authors also noted an improvement of this method by producing fractions with higher SDA yield (57.4%); however, the purity of SDA in the fraction was remarkably lower (39.4%).

In an additional work by the same authors, the LTC of SDA in FFA and FAEE form from MSO was also explored [46]. In this work, chemical hydrolysis and

ethanolysis were performed to transform the TAGs from MSO into FFA and FAEE, respectively. The variables studied were, as in the previous work, winterization time, solvent-type, and oil:solvent ratio. The best SDA purity and yield for FFAs was attained at the same conditions than for TAGs (10% oil:solvent ratio, with hexane:acetone (10:90, v/v) solvent, and winterization time of 24 h at -80 ^{o}C). In a scaled-up process, the SDA purity and yield obtained for the FFAs were 59.6% and 82.6%, respectively. Although the concentration of SDA in the FFA form was quite successful, the winterization of FAEE was not efficient. This was due to the low melting point of the mixture (the melting point of SDA is -57^{o}C in the fatty acid form). The products obtained by the authors provide a source of SDA-enriched TAGs and FFAs that can be used in additional methods intended to further increase the SDA content in MSO and some of its derivatives.

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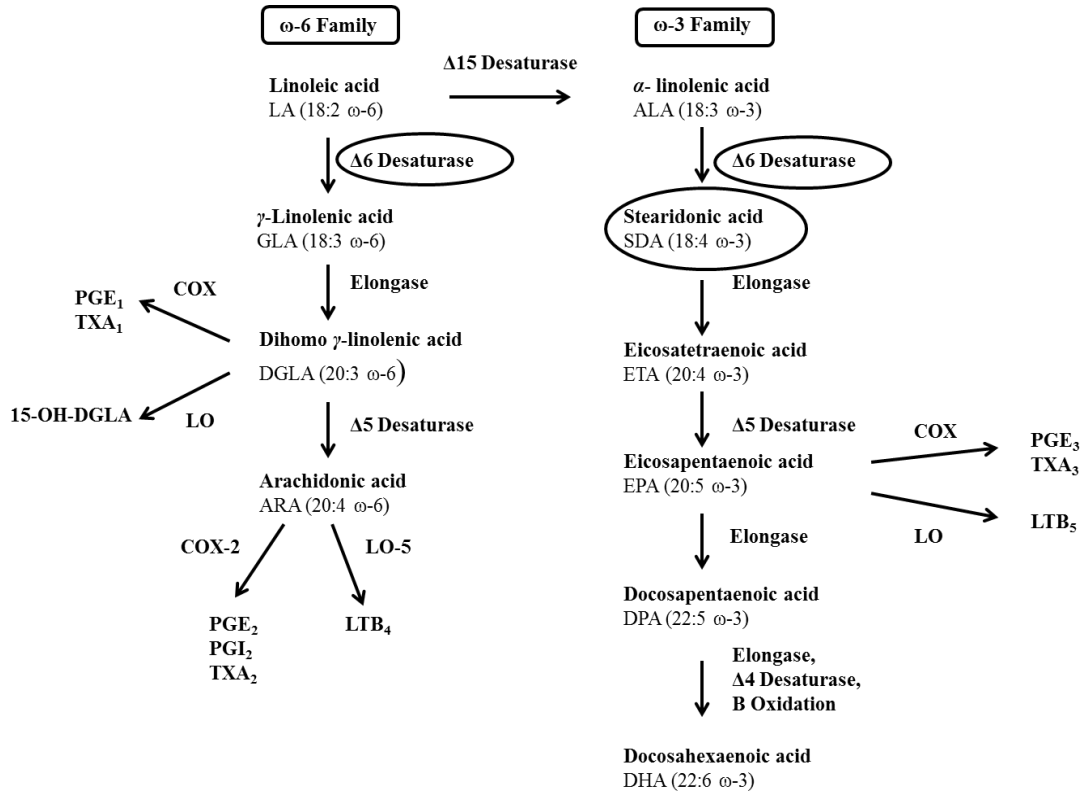
Table 2.1 Composition of modified soybean oil[a]

	Modified Soybean Oil (MSO)[b]		
		Positional distribution	
Fatty acid	**Total (mol%)[c]**	***sn*-2 (mol%)**	***sn*-1,3[d] (mol%)**
Palmitic C16:0	12.71 ± 0.05	3.82 ± 3.07	17.16 ± 1.54
Stearic C18:0	4.30 ± 0.00	2.05 ± 1.57	5.42 ± 0.79
Oleic C18:1*c* (ω-9)	14.48 ± 0.05	14.78 ± 2.85	14.34 ± 1.43
Oleic C18:1*t*	1.62 ± 0.07	ND	2.43 ± 0.10
Linoleic C18:2 (ω-6)	23.69 ± 0.05	41.75 ± 1.06	14.66 ± 0.53
γ-Linolenic C18:3 (ω-6)	7.35 ± 0.01	8.28 ± 0.65	6.89 ± 0.33
α-Linolenic C18:3 (ω-3)	10.85 ± 0.01	6.29 ± 0.44	13.13 ± 0.22
Stearidonic C18:4 (ω-3)	25.00 ± 0.06	23.01 ± 0.42	25.99 ± 0.23

[a] All analysis were performed in triplicate and average values ± SD are reported
[b] Fatty acids found in trace amounts were: C14:0, C16:1, C17:0, C18:2 (trans), C18:4 (trans), C20:0, C20:1 (ω-9) and C22:0
[c] Mean ± SD, n = 3
[d] *sn*-1,3 (mol %) = [3 x total (mol%) - *sn*-2 (mol%)] / 2



Metabolism of ω-6 PUFA leads to the cyclooxygenase (COX) and lipoxygenase (LO)-catalyzed synthesis of eicosanoids (prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT), while the metabolism of ω-3 PUFA leads to the synthesis of EPA and DHA.

Figure 2.1 Biosynthetic pathways of ω-6 and ω-3 PUFAs. Redrawn from [5]

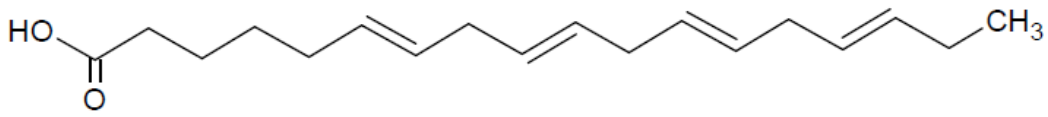
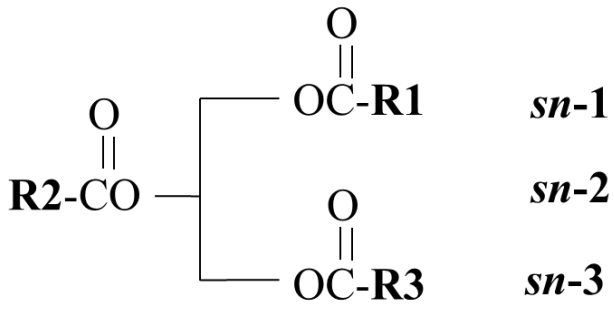


Figure 2.2 Stearidonic acid: (6Z,9Z,12Z,15Z)-octadecatetraenoic acid (SDA, 18:4 ω-3)



R1, R2, and R3 represent fatty acids

Figure 2.3 Generic triacylglycerol structure

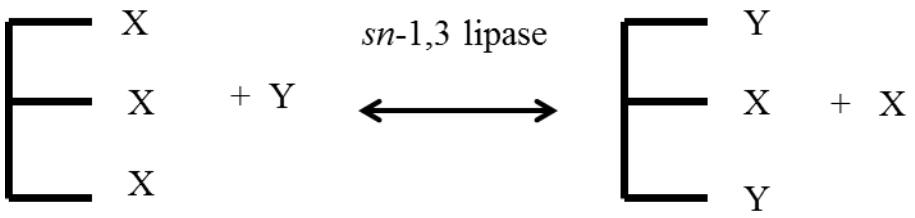
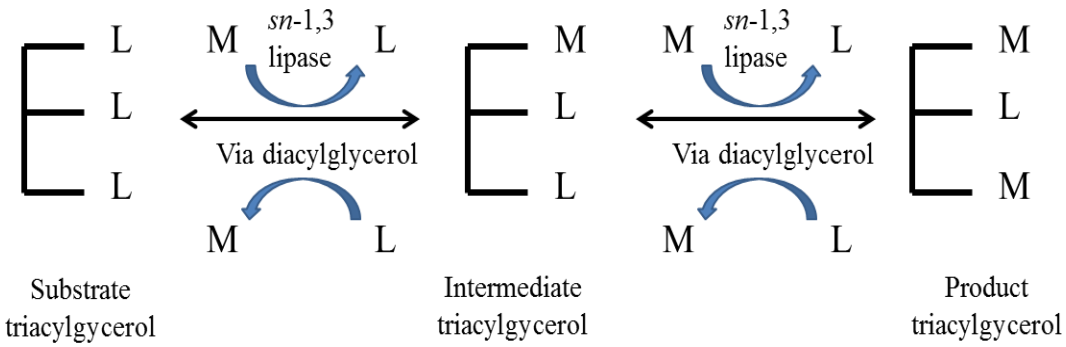


Figure 2.4 Generic *sn-1,3* lipase-catalyzed acidolysis between TAG (XXX) and a fatty acid (Y)



L and M are long chain and medium chain fatty acids, respectively

Figure 2.5 *sn*-1,3 Lipase-catalyzed acidolysis reaction between TAG (LLL) and M fatty acid. Redrawn from [28]

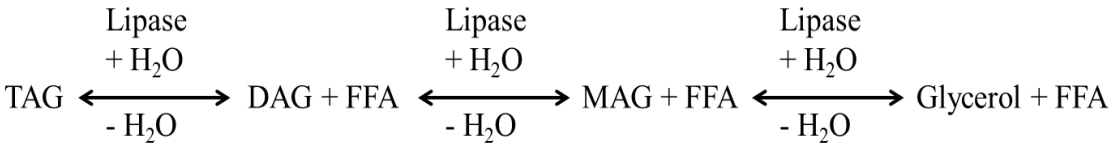


Figure 2.6 Generic enzymatic hydrolysis of oils and fats (TAG: triacylglycerols, DAG: diacylglycerols, MAG: monoacylglycerols, FFA: free fatty acids). Redrawn from [28]

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CHAPTER 3

PURIFICATION OF STEARIDONIC ACID FROM MODIFIED SOYBEAN OIL BY ARGENTATION SILICA GEL COLUMN CHROMATOGRAPHY[1]

[1]Kleiner-Shuhler L, Vazquez L, Akoh CC (2011) Purification of stearidonic acid from modified soybean oil by argentation silica gel column chromatography. J Am Oil Chem Soc 88:1161-1171

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ABSTRACT

The objective of this study was to purify stearidonic acid (SDA, 18:4 ω-3) from modified soybean oil containing a mixture of over 20 fatty acids (23% SDA). Interest in obtaining purified fractions of SDA arises from reported health benefits associated with polyunsaturated fatty acids (PUFA), such as cardiovascular disease prevention. In addition, SDA may also provide improved stability characteristics since its unsaturation index is less than longer PUFA, such as eicosapentaenoic acid (EPA, 20:5 ω-3). First, a chemical ethanolysis of modified soybean oil was performed to transform the triacylglycerols into fatty acid ethyl esters (FAEE). Then, the FAEE were fractionated and SDA-EE was purified by argentation silica gel (10% $AgNO_3$) open column chromatography, which allows selectivity based on degree of unsaturation. Different FAEE sample loads and mobile phases were explored until the best purification of SDA-EE was achieved. The solvents used were hexane and hexane:acetone mixtures (99 and 95%). Under the optimal conditions, a fraction with high SDA-EE purity (96%) was obtained with 77% yield. Besides, it was possible to obtain another fraction enriched in α-linolenic acid-EE (37% purity and 68% yield) and γ-linolenic acid-EE (22% purity and 61% yield). A scaled-up process resulted in 840 mg of final product composed of 97% SDA-EE with 71% yield.

INTRODUCTION

The dietary consumption of polyunsaturated fatty acids (PUFA) has been associated with a variety of health benefits. In particular, highly unsaturated PUFA such as α-linolenic acid (ALA, 18:3 ω-3), eicosapentaenoic acid (EPA, 20:5 ω-3), and docosahexaenoic acid (DHA, 22:6 ω-3), have been found to have effects on the reduction of cardiovascular disease [1], inflammation [2], cancer [3], and neurological disorders [4]. Accordingly, EPA and DHA ethyl esters are the main components (more than 90%) of Lovaza®, the first prescription omega-3 product approved by the FDA. Lovaza is used to reduce the hepatic production of triacylglycerols [5]. More recently, there has been increasing interest in stearidonic acid (SDA, 18:4 ω-3), a PUFA that is a metabolic intermediate in the conversion of ALA to EPA [6]. SDA has less degree of unsaturation than EPA and DHA and is therefore, less susceptible to lipid oxidation, the formation of undesirable free radicals, aldehydes, and off-flavors [7]. Because of its physical characteristics and its role on the metabolism of other PUFA, SDA became of particular interest to the food industry.

SDA is found naturally in low quantities in fish and some other marine life. EPA and DHA constitute 15–20% of the total fatty acid profile of fish, while SDA typically constitutes 0.5–2% of the fatty acids [8]. The increased interest in incorporating PUFA into the diet and their low bioavailability have led to overfishing and a severe decline in some fish populations [9]. For these reasons there is a current interest in the development of alternative methods to obtain PUFA. Thus, current genetic engineering technologies have been used to produce vegetable oils containing SDA.

PUFA have been traditionally purified by discriminating the fatty acids present based on their polarity and/or degree of unsaturation. Some methods that use these principles are winterization (low temperature crystallization) [10], urea complexation methods [11], and argentation (Ag) silica gel column chromatography [12]. In order to obtain individual PUFA with purities higher than 90%, winterization and urea complexation methods need to be coupled to HPLC [13]. This extra step further increases the cost associated with the purification techniques, as well as the time needed to obtain a purified fraction.

Ag-silica gel chromatography is performed under mild conditions (no extreme temperatures or pressures are needed), and it has been shown to provide high purity fractions (>90%) of fatty acid methyl esters (FAME), without further purification by HPLC [12, 13]. Different compounds can be fractionated by their specific interactions between the stationary phase (usually silica gel) and the mobile phase (elution solvents). For Ag-silica gel chromatography, the silica gel is coupled to silver ions (Ag^+) that form reversible polar complexes with unsaturated locations in organic molecules [14]. Due to that reversible complexation, it is possible to use this technique to separate PUFA based on the number, position, and geometric configuration of double bonds. The extent and the strength of complexation control the mobility of a solute, as does the polarity of the mobile phase. The concentration of silver nitrate loaded per unit mass of silica gel influences the surface density of Ag^+ ions on the solid matrix, hence, affecting the retention time of the solute [15]. This principle has also been applied to thin layer chromatography (TLC) [16] and HPLC [17].

Ag-silica gel chromatography has been used to purify ALA, γ- linolenic acid (GLA, 18:3 ω-6), EPA, DHA, and SDA from a variety of free fatty acids (FFA) [18] and FAME [12, 13, 15] mixtures. For example, Ryu et al. [18] obtained 95% ALA from a hydrolysate of perilla oil. Sajilata et al. [15] and Guerrero et al. [12] used this technology to purify GLA methyl ester from *Spirulina platensis* and several plant sources, respectively. In a different study, Guil-Guerrero et al. [13] obtained ALA methyl ester from linseed oil, GLA methyl ester from Borage oil, and EPA, DHA, and SDA methyl esters from Shortfin Mako (*Isurus oxyrinchus*) liver oil. The elution solvents employed were solutions of hexane:acetone, and the maximum sample load on the silver ion silica gel did not exceed 4% (w/w) (weight of sample/weight of stationary phase) [12, 13, 15, 18].

The main goal of the current work was to isolate SDA ethyl ester (SDA-EE) with high purity and yield by Ag-silica gel open column chromatography from high SDA modified soybean oil. Different variables were investigated, such as the maximum sample load, the optimal polarity of mobile phases and volumes of the elution solvents. In particular, this work focused on the FAEE load optimization to enhance the amount of final product obtained in a single process.

MATERIALS AND METHODS

Materials

High SDA modified soybean oil was provided by the Monsanto Company (St. Louis, MO, USA). Modified soybean oil is not yet commercially available. SDA-enriched soybean oil was produced through the overexpression of lipid biosynthetic genes in

normal soybean oil (Monsanto Co., St. Louis, MO, USA) resulting in the presence and increased levels of SDA in soybean oil. This modified soybean oil can provide a sustainable and inexpensive plant source of omega-3 fatty acids. Palmitic acid ethyl ester (purity, >99%) and stearic acid ethyl ester (purity, 99%) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Sodium ethoxide, 21% (w/w) in ethanol, was obtained from Alfa Aesar, a Johnson Matthey Company (Ward Hill, MA, USA). Stearidonic acid methyl ester was purchased from the Cayman Chemical Company (Ann Arbor, MI, USA). Silica gel (63–200 μm particle size, Catalog No: 196724) was purchased from Selecto Scientific (Suwanee, GA, USA). Silver nitrate (ACS grade crystals) was obtained from EMD Biochemicals (Darmstadt, Germany). Pasteur pipettes (5 ¾ inch length), crystalline sodium chloride, anhydrous sodium sulfate, acetone, hexane and ethanol (96%, v/v) were purchased from Fisher Scientific (Pittsburgh, PA, USA). All the solvents and compounds were ACS grade.

Ethanolysis of High SDA ω-3 Oil

A transesterification reaction (ethanolysis) of the high SDA ω-3 oil (modified soybean oil) was carried out in order to transform all the TAG into their corresponding FAEE. The methodology employed was based on the method described by Vazquez and Akoh [19]. The reaction was performed in a 1-L cylindrical vessel. 200 mL of the oil was mixed with sodium ethoxide (2.625%, w/v) in absolute ethanol at a ratio of 4:2 (v/v) (2.25 fold molar excess of ethanol). The mixture was heated at 60 ^{o}C with mechanical shaking for 40 min, under a nitrogen atmosphere. The product was washed twice in order to completely remove the remaining ethanol, glycerin or any other polar compounds. First washing was

done with a saturated NaCl solution, and the second with distilled water. Separation of two phases was done using a separatory funnel (2 L capacity) and centrifugation was not necessary. The volume used in each washing was half of the volume of oil utilized. Finally, the product of the ethanolysis reaction was dried over anhydrous sodium sulfate and vacuum filtered. Using this methodology, the FAEE content in the final product was ~99%, with yield over 95%. This product was used as the starting material for subsequent fractionation of FAEE.

Argentation of Silica Gel (10% $AgNO_3$)

The argentation of the silica gel was based on the method described by Ryu et al. [18] and Guil-Guerrero et al. [13] as follows: 100 g of silica gel (63–200 μm particle size) were placed in a beaker containing 200 mL ethanol (96%, v/v). This slurry was agitated with a mechanical stirrer for 10 min. Then, 10 g $AgNO_3$ were dissolved in 35 mL of ethanol:water (70:30,v/v), and the solution obtained was added to the slurry. The mixture was agitated for another 10 min and the solvent evaporated in a rotary evaporator at 60 ^{o}C. $AgNO_3$ silica gel was dried at 100 ^{o}C for 2 h, cooled at room temperature and kept in the dark under dry atmosphere until further use.

Fractionation of FAEE by Ag-silica Gel Open Column Chromatography (analytical scale)

The chromatography columns employed for all analytical scale experiments were Pasteur pipettes (146 mm length and approximately 5.7 mm diameter), with the ends plugged with glass wool. Prior to packing the column, the Ag-silica gel was activated for 2 h at

100 ^{o}C. Approximately 1 g of the Ag-silica gel was dry packed into the column as the stationary phase. For all experiments the packed height was 70 mm measured from the top of the glass wool. The column was tapped while packing in order to ensure compact packing of the gel. Conditioning of the column was by gravity after adding 4 mL hexane as elution solvent.

At this time, the volumetric flow rate was measured to be 0.286 mL/min (1 mL/3.5 min). Sample of FAEE was then loaded in the column. For the optimization of FAEE sample loads, samples of 5, 10, 15, 20, 25, 30 and 50% w/w (weight of sample/weight of stationary phase) were explored. The samples were prepared by dissolving, respectively, 50, 100, 150, 200, 250, 300, and 500 mg of FAEE in 1 mL of the solvent of the first fraction (hexane).

The solvents used in this work were: hexane; hexane:acetone (99:1, v/v); hexane:acetone (95:5, v/v) and hexane:acetone (90:10, v/v). In order to simplify the nomenclature, these solvents were designated as 100, 99, 95 and 90% hexane, respectively. These solvents were introduced into the column in order of increasing polarity and were allowed to elute by gravity. The number of fractions and the volumes of the solvents were investigated and discussed in ‘‘Results.’’

After the elution, each fraction was then washed with NaCl solution (1%, w/v). The volume of the NaCl solution employed was half of the volume of each fraction. The organic layer was collected, dried with anhydrous sodium sulfate, and the solvent evaporated under nitrogen. It was crucial to wash the anhydrous sodium sulfate with another 2 mL of hexane to effectively recover all the FAEE of each fraction. The residue obtained was then analyzed by GC. Experiments under optimal conditions were

performed in triplicate. The mean values of purity, yield, total weights and their corresponding standard deviations are shown in tables.

Scale-up

A glass column of 140 mm length and 27.8 mm diameter was employed for the scale-up process. Based on the diameter of the column, the following parameters were calculated: volumetric flow rate, sample load, and volume of the elution solvents. The packed height of the column was maintained at 70 mm, as measured from the top of the glass wool plugging it. The scale up was performed by using Eqs. (1) and (2) in order to calculate the new sample load and volumetric flow rate, respectively. In these equations, ‘‘s-u’’ refers to scaleup process, and ‘‘an’’ to analytical or small scale process.

$$\text{Sample Load}_{s\text{-}u} = (\text{Radius}_{s\text{-}u} / \text{Radius}_{an})^2 \times \text{Sample Load}_{an} \quad [20] \qquad (1)$$

$$\text{Volumetric Flow Rate}_{s\text{-}u} = (\text{Sample}_{s\text{-}u} / \text{Sample}_{an}) \times \text{Volumetric Flow Rate}_{an} \quad [20] \qquad (2)$$

Once the sample load and volumetric flow rate for the scaled-up process were determined, Eq. (3) was used to calculate the volume of elution solvent for each fraction.

$$\text{Volume}_{s\text{-}u} = (\text{Volume}_{an} / \text{Volumetric Flow Rate}_{an}) \times \text{Volumetric Flow Rate}_{s\text{-}u} \qquad (3)$$

Equation (3) was deduced from the relationship:

$$\text{Volume}_{s\_u} / \text{Volumetric flow rate}_{s\_u} = \text{Volume}_{an} / \text{Volumetric flow rate}_{an}$$

The values of the different variables are reported in ‘‘Results’’ (scale-up). As in the analytical scale processes, prior to the packing of the column, the Ag-silica gel was activated for 2 h at 100 ^{o}C and allowed to cool down to room temperature. In the scaled-up processes, a similar procedure to that of analytical scale was performed, with slight variations. In this case, a wet packing method was performed. Thus, after plugging the exit of the column with glass wool, hexane was added to the column until approximately 50 mm height. Then, 21 g of Ag-silica gel was sequentially and homogeneously loaded. After that, more hexane was added and the stopcock was opened. The Ag-silica gel was packed while hexane flowed and if necessary more was added to reach the 70 mm height in the column. The final amount of Ag-silica gel was 21.4 g. The FAEE sample was loaded after dissolving it in 24 mL of the solvent of the first fraction (hexane).

Four fractions were sequentially collected in a separatory funnel for their subsequent washings with NaCl solution (1%, w/v) and the separation of phases. The organic layer was collected, dried with anhydrous sodium sulfate, and the solvent removed in a rotary evaporator at 50 ^{o}C. The residue obtained was then analyzed by GC. Scale-up processes were performed in duplicate. The mean values of purity, yield, total weights and their corresponding standard deviations are shown in tables.

GC Analysis

FAEE were analyzed using an Agilent Technology (Santa Clara, CA, USA) 6890N gas chromatograph equipped with a flame ionization detector. Separation was achieved with an SP-2560 column, 100 m 0.25 mm i.d., and 0.20 μm film (Supelco Inc., Bellefonte, PA, USA). Injection (1 μL) was performed at a split ratio of 20:1. Helium was the carrier gas

at a constant flow rate of 1.1 mL/min. The injector temperature was 250 ^{o}C, and the FID set point was 300 ^{o}C. The temperature was held at 140 ^{o}C for 5 min, and then increased up to 240 ^{o}C with ramping at 4 ^{o}C/min and held isothermally for 15 min. FAEE relative content was calculated by integration using a GC Chemstation software. Identification of the various FAEE was based on the retention times and relative area percentages of a Supelco 37 Component FAME mix (Supelco Inc. Bellefonte, PA, USA). Quantification was via an external standard of palmitic acid ethyl ester, stearic acid ethyl ester and stearidonic methyl ester. These standards were selected to calculate the response factors of different fatty acids according to their chain length. FAEE samples were dissolved in hexane for GC analyses at 20–25 mg/mL.

Statistical Analysis

Software package ORIGIN 8.0 (OriginLab, Northampton, MA, USA) was used to calculate averages, standard deviations and to perform the analysis of variance (ANOVA). The significance level was $p < 0.05$.

RESULTS AND DISCUSSION

Two main responses were evaluated:

a. Purity or composition (%) =

[weight of a fatty acid in a fraction (mg) /weight of an entire fraction (mg)] x 100

b. Yield (%) = [weight of a fatty acid in a fraction (mg)/ weight of this fatty acid present in the starting material (mg)] x 100

The behavior of different FAEE was investigated. Nevertheless, the study was mainly focused on the isolation of SDA ethyl ester (SDA-EE).

Method Development-Preliminary Experiments

Two preliminary experiments were performed to determine the optimal solvents to fractionate FAEE by Ag-silica gel chromatography and obtaining high purity and yield of SDA-EE. According to previous works [12, 13, 15, 18], pure hexane, and hexane:acetone solutions were used as elution solvents. It had been previously shown that by increasing the solvent polarity, the composition of the eluents followed this order: saturated, monounsaturated, and PUFA [13].

In the current study the FAEE load was higher than that reported in the literature (4%, w/w) [12, 13, 18], with the aim of processing a larger amount of FAEE in a single step. At the end of the separation, solutions with remarkably increased acetone content were added to force the elution of all polyunsaturated FAEE from the column. Hence, all FAEE loaded in the column were eluted with approximately 100% recovery of FAEE.

Table 3.1 shows the conditions investigated and SDA-EE purity and yield obtained in the preliminary studies (small scale). Purification of SDA-EE was not achieved under conditions of the first experiment, since SDA-EE was distributed in several fractions, and the highest SDA-EE yield (53.3%) was low in purity (48.6%).

In the second experiment, fractionation of FAEE was improved and thus, most of the SDA-EE was recovered between 99% hexane and 90% hexane. However, the SDA-EE yield was distributed in several fractions and thus, SDA-EE isolation was also inadequate. The reason could be that in these experiments, an overload of the chromatographic column may occur with 300 mg of sample. Because of that, optimization of the FAEE load was carried out.

Optimization of FAEE Sample Load

Different FAEE sample loads were investigated: 5% (w/w), 10% (w/w), 15% (w/w), 20% (w/w), 25% (w/w), 30% (w/w), and 50% (w/w). In this optimization, the volume of 100% hexane was higher than that of preliminary experiments. The aim was to avoid fast changes in the polarity by delaying the introduction of more polar solvents and to slow down the separation of FAEE inside the column. Besides, the number of fractions collected was higher than in previous works [12, 13, 15, 18] in order to control and improve the fractionation of FAEE. Thus, part of 100% hexane and all 99% hexane were collected in several fractions of a reduced volume (3 mL). Table 3.2 shows the conditions investigated and SDA-EE purity and yield obtained in these experiments.

Poor separation of SDA-EE was achieved with 5% load. In this case, no FAEE were obtained in the intermediate fractions (100% F2 to 99% F3), and most of the FAEE coeluted with SDA-EE in the 90% hexane first fraction. Because of that, although this fraction gave the highest SDA-EE yield (70%), it was very low in SDA-EE purity (24.4%). The low amount of sample loaded could increase the retention of FAEE, and most of them could elute together when the most polar solvent (90% hexane) was added

to the column. It should be noted that good SDA-EE separation was achieved at 10% load. Under these conditions, a single fraction with high SDA-EE purity (83.0%) and yield (74.3%) was obtained in the first fraction eluted with 90% hexane.

Although no single fraction was isolated with high SDA-EE purity and yield with increasing load, under these conditions some of the fractions can be pooled to obtain a good balance between purity and yield. This possibility may be valuable on an industrial scale, since a higher load significantly enhances the amount of final product obtained in a single process with a reduced amount of solvents. Thus, a cost/benefit analysis would be needed to evaluate the best option.

Equations (4) and (5) define total purity and yield of pooled fractions.

$$\text{Purity}_{(F1+F2+\ldots+Fn)} = \{[(\text{Purity}_{F1} \times \text{Weight}_{F1})/100] + [(\text{Purity}_{F2} \times \text{Weight}_{F2})/100] + \ldots[\text{Purity}_{Fn} \times \text{Weight}_{Fn})/100] / (\text{Weight}_{F1} + \text{Weight}_{F2} + \ldots + \text{Weight}_{Fn})\} \times 100 \quad (4)$$

$$\text{Yield}_{(F1+F2+\ldots+Fn)} = \text{Yield}_{F1} + \text{Yield}_{F2} + \ldots + \text{Yield}_{Fn} \quad (5)$$

where F1, F2,…, Fn = pooled fractions.

For example, by pooling 99% F4 and 90% F1 fractions at 20% load, SDA-EE purity and yield were 86.2 and 42.8%, respectively (Table 3.3). Similarly, by pooling 99% F3, 99% F4 and 90% F1 fractions at 20% load, 72.5% SDA-EE purity with 71.5% yield were achieved. Table 3.3 summarizes the results of SDA-EE purity and yield obtained of selected pooled fractions. As observed in this table, at 30% load, by pooling the fractions 99% F4 and 90% F1, 73.7% SDA-EE purity with 55.9% yield were attained, whereas by pooling the fractions 99% F3, 99% F4 and 90% F1, SDA-EE purity and yield

were 64.5 and 64.6%, respectively. Loads of 15, 20, 25 and 30% may lead to acceptable separation of SDA-EE by pooling fractions. However, SDA-EE purity and yield obtained under these conditions were lower than those obtained with 10% load.

Furthermore, as reported in Table 3.2, with loads higher than 20%, an initial decrease in SDA-EE yield was found with the 100% hexane fractions following this order: 25% (7.6%) < 30% (18.9%) < 50% (48.1%). This suggests an overload of the column, since under these conditions, the Ag-silica gel was saturated with FAEE, and there is an amount of SDA-EE that elutes without retention inside the column. Figure 3.1 shows the increase in SDA-EE yield in 100% F1 at different FAEE loads. At 50% load, there was a loss of 48.14% in SDA-EE yield in the first 100% hexane fraction collected, indicating very poor separation and column overload.

Table 3.2 proved that the 90% F1 was the fraction with the highest SDA-EE purity and yield in all experiments. Figure 3.1 shows how these responses varied in 90% F1, based on FAEE sample load. It was observed that for all sample loads, with the exception of 5%, similar SDA-EE purity was obtained. However, SDA-EE yield significantly decreased with increasing FAEE load. As observed in Fig. 3.1 and Table 3.2, 10% load was optimal to collect the highest purity and yield of SDA EE in one single fraction. It should be remarked that this load is 2.5 times higher than the maximum acceptable sample load (4%, w/w) previously reported in the literature [12, 13, 15, 18].

Optimization of Fractions, Solvents and Volumes-Study of Reproducibility

In this part, some fractions obtained with the same solvents were pooled to obtain one single fraction. Therefore, the first two fractions were composed of 29 mL hexane and 12

mL 99 % hexane, respectively. In addition, some variations were introduced in the fractionation method with the objective of improving the isolation of SDA-EE.

In the previous experiments, SDA-EE was the last FAEE to be eluted and hence, solvents with high polarity were added in the last fractions to ensure complete elution of FAEE through the column. In these experiments, it was found that, SDA-EE purity was lower than 90% in all cases. However, in fractions with 90% hexane (highest polarity), almost only SDA-EE were detected as FAEE in the GC analyses. In other words, in these fractions approximately 5–15% of the mass fraction was composed of compounds not detected by GC. This suggests that other relatively polar compounds co-eluted with SDA-EE, and this led to a little drop in purity. Likewise, when the acetone content was higher than 10% in the preliminary experiments, we observed that some fractions contained a white residue after solvent evaporation. A possible explanation is that part of the stationary phase may have been dissolved by the high polar solvents. The main objective of the next optimization was to determine the most adequate polarity of the final solvent that will ensure total recovery of FAEE and not dissolve part of the stationary phase. This polarity was modulated by the acetone content. Guil-Guerrero et al. [13] reported the highest SDA methyl ester purity and yield with an intermediate fraction composed of hexane:acetone (95:5, v/v). For that reason, in our work, 90% hexane was replaced with 95% hexane as solvent for the last fraction.

Furthermore, the volume of this fraction was optimized and hence, 95% hexane was added to several fractions until the FAEE obtained was less than 1 mg. Although 20 mL gave similar results, 24 mL was established as optimal, since after the elution of this volume no FAEE remained in the column. Therefore, the last fraction was composed of

24 mL 95% hexane. Under these conditions, the fraction was free of unknown compounds and led to remarkable SDA-EE purity improvements (Table 3.4) with a recovery of FAEE over 95%.

In a further optimization, another 2 mL of 99% hexane were added in order to keep eluting FAEE different from SDA-EE and thus, improving the SDA-EE purity in the final fraction. In other words, the goal of this optimization was to obtain a purer SDA-EE, free of other FAEE, in the final fraction. For this purpose, after the first 12 mL, four fractions of 1 mL 99% hexane were collected. Table 3.4 reports the conditions and results of this optimization. As observed in this table, after addition of 12 mL + 1 mL + 1 mL fractions of 99% hexane, SDA-EE purity were significantly increased to 83.3 and 90.0% in the last 1 mL fractions. For that reason, a final volume of 14 mL was established for the 99% hexane fraction.

After these last optimizations, the process under optimal conditions was repeated three times to study the reproducibility of the separation. Table 3.5 reports the mean values of purity and yield of main fatty acids, the weights of fractions and their corresponding standard deviations. It should be noted that the fraction obtained with 95% hexane was remarkably improved in SDA-EE purity (96.3%), with 77.0% yield. The table also showed the high reproducibility of this process.

A reduction from 29 to 20 mL in the volume of the 100% hexane fraction was also attempted (data not shown in tables). However, although SDA-EE purity in 95% fraction was high (92.5%), SDA-EE yield decreased to 62.1 and 18.0% of total SDA-EE was obtained in the 99% (12 mL) fraction. For that reason the volume of 100% hexane

was maintained at 29 mL, since it was proved that this volume played an essential role in the FAEE fractionation.

Enrichment of ALA and GLA Ethyl Ester

A further experiment was performed to optimize the isolation of ALA ethyl ester (ALA-EE) and GLA ethyl ester (GLA-EE) in different fractions from that of SDA-EE. Since most of total GLA-EE and ALA-EE co-eluted with other FAEE in the 12 mL 99% hexane fraction, in this experiment this volume was collected in several 1 mL fractions. Table 6 reports the purity and yield of ALA-EE and GLA-EE obtained in the different fractions. The yield of these compounds significantly increased when fractions from 99% F4–F6 were pooled with 99% F7–F9 fractions. However, a decreased ALA-EE and GLA-EE purity was also observed, mainly due to the presence of linoleic acid (18:2 ω-6) ethyl ester. Therefore, the elution volume obtained with 99% hexane was divided into two fractions, consisting of 10 and 4 mL. The last 4–5 mL of 99% hexane gave a good balance between purity (31.1–36.6% for ALA-EE and 19.7–22.2% for GLA-EE) and yield (67.9–89.3% for ALA-EE and 61.2–84.2% for GLA-EE) (Table 3.6). Therefore, the optimal fractions used for the scale-up were: 29 mL 100%, 10 mL 99%, 4 mL 99% (ALA-EE and GLA-EE), and 24 mL 95% (SDA-EE).

Scale-up

Fractionation of FAEE by Ag-silica gel chromatography was scaled-up to 2,380 mg of the starting FAEE in a single step. The best conditions obtained on an analytical scale were used for the scale-up processes.

From Eqs. (1), (2), and (3), described in ‘‘Methods’’ (scale-up), values of FAEE sample load (2,380 mg), volume flow rate (6.7 mL/min), and volumes of elution solvents were calculated. Table 3.7 reports mean values of purity and yield of main fatty acids, the weights of fractions and their corresponding standard deviations, obtained in the scaled-up processes.

It should be noted that experimental conditions were successfully scaled-up to obtain 420 mg of 96.9% SDA-EE with 71.3% yield (Table 3.7). The scale-up process was repeated twice, yielding 840 mg of the purified SDA-EE, as product for further applications. Regarding the isolation of SDA-EE, it should be noted that good reproducibility was achieved in both scaled-up processes (Table 3.7) and on an analytical scale (Table 3.5). ANOVA was performed for the comparison of SDA purity and yield on an analytical scale and scaled-up processes. No significant differences ($p > 0.05$) were found in SDA purity in all fractions obtained. On the contrary, significant differences ($p < 0.05$) were found in SDA yield in several fractions. Thus, SDA yield was significantly higher ($p < 0.05$) on an analytical scale (77.0% ± 1.7) than in the scaled-up processes (71.3% ± 0.2) in the 95% hexane fraction. However, these differences were not pronounced. Due to this high reproducibility, in our opinion, the process should be readily scaled-up with high SDA-EE purity and yield.

In addition, it was possible to obtain another fraction enriched in ALA-EE (31.6% purity and 47.9% yield) and GLA-EE (18.1% purity and 41.0% yield) (Table 3.7). It was found that, for these FAEE, the reproducibility between the analytical scale and the scaled-up process was lower than with SDA-EE. The reason could be that fractionation of ALA-EE and GLA-EE occurred between two fractions with the same

composition (99% hexane), whereas SDA-EE eluted after changing the polarity of solvent (95% hexane).

We attempted to reutilize the Ag-silica gel used in the scaled-up processes after its activation at 100 ^{o}C for 2 h. However, the separation was significantly worse than that achieved in the previous study. Therefore, we do not believe that reutilization of the Ag-silica gel is feasible.

Ag-silica gel open column chromatography could be used to effectively isolate SDA-EE from high SDA modified soybean oil FAEE with high purity (> 96%) and yields (> 70%). Due to its high purity, the SDA-EE obtained can compete with fish oil-EE (EPA-EE) and can be used for the production of structured lipids, nutraceuticals, functional lipids for clinical studies, or as fine ingredient. The process described in the paper can be scaled-up with high reproducibility, selectivity, efficiency and low cost, since no expensive equipment is needed. Besides, the optimal sample load was established at 10% (w/w), which is 2.5 times higher than the maximum acceptable sample load previously reported in the literature [12, 13, 15, 18]. In addition, in this process, no urea crystallization methods that may lead to carcinogenic carbamates were involved. For these reasons, this process and products may be useful and valuable for clinical trials and food or pharmaceutical applications.

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Table 3.1 SDA-EE purity and yield in the preliminary experiments of Ag-silica gel column chromatography of FAEE

Fraction (%)[a]	Volume (mL)	SDA-EE purity (%)	SDA-EE yield (%)	Weight (mg)
Experiment 1				
100% F1	10	6.2	15.4	180.9
99% F1	18	48.6	53.3	79.4
98% F1	6	94.2	4.3	3.3
98% F2	4	98.4	2.3	1.7
70% F1	10	66.8	16.0	17.4
70% F2	10	14.7	0.5	2.6
70% F3	10	3.1	0.1	1.2
Experiment 2				
100% F1	5	1.4	2.7	139.7
100% F2	5	20.4	10.0	36.0
100% F3	5	27.9	3.7	9.8
100% F4	5	30.3	2.6	6.2
99% F1	5	31.8	2.0	4.6
99% F2	5	32.7	7.0	15.7
99% F3	5	59.0	29.2	36.1
99% F4	5	88.2	5.5	4.6
90% F1	10	88.3	24.5	18.4
90% F2	10	73.3	5.5	5.5
80% F1	10	0.1	0.5	5.9

The experiments were performed under these conditions: FAEE load: 300 mg (30%, w/w), column length 70 mm, flow rate 0.286 mL/min, argentation silica gel 1 g

[a]100%: pure hexane, 99%: hexane:acetone (99:1, v/v), 98%: hexane:acetone (98:2, v/v), 90%: hexane:acetone (90:10, v/v), 80%: hexane:acetone (80:20, v/v), 70%: hexane:acetone (70:30, v/v)

Table 3.2 SDA-EE purity and yield in the optimization of FAEE load

		50 mg (5%, w/w)			100 mg (10%, w/w)			150 mg (15%, w/w)			200 mg (20%, w/w)		
Fraction [a]	V[b] (mL)	P (%)	Y (%)	W (mg)	P (%)	Y (%)	W (mg)	P (%)	Y (%)	W (mg)	P (%)	Y (%)	W (mg)
100% F1	20	0.4[c]	0.1[c]	1.8[c]	0.2	0.1	10.4	0.0	0.1	58.4	0.2	0.5	120.9
100% F2	3				0.1	0.0	1.8	0.1	0.0	3.2	3.0	0.5	7.6
100% F3	3				0.3	0.0	0.7	0.1	0.0	2.6	4.6	0.2	2.6
100% F4	3				0.2	0.0	1.4	0.1	0.0	2.2	6.6	0.4	2.6
99% F1	3				0.2	0.0	1.3	0.2	0.0	2.0	8.9	0.3	1.8
99% F2	3				0.2	0.0	1.7	0.1	0.0	4.9	39.8	17.6	21.6
99% F3	3				0.1	0.1	20.3	19.0	20.7	39.1	58.6	28.6	23.9
99% F4	3	0.1	0.1	10.9	6.3	8.6	32.9	76.8	22.5	10.5	78.9	9.2	5.7
90% F1	10	25.4	70.0	33.0	83.0	74.3	21.4	85.9	40.5	16.9	88.4	33.6	18.6
90% F2	10	79.8	14.0	2.1	61.9	10.4	4.0	78.8	3.1	1.4	63.3	2.2	2.3
90% F3	10	67.8	5.1	0.9	14.3	0.5	0.9	14.7	0.8	2.0	14.9	0.7	1.7

		250 mg (25%, w/w)			300 mg (30%, w/w)			500 mg (50%, w/w)		
Fraction	V (mL)	P (%)	Y (%)	W (mg)	P (%)	Y (%)	W (mg)	P (%)	Y (%)	W (mg)
100% F1	20	2.8	7.6	164.0	7.0	18.9	194.5	15.2	48.1	376.5
100% F2	3	27.8	1.1	2.4	31.9	1.2	2.7	33.3	0.3	0.9
100% F3	3	34.0	1.3	2.3	32.5	0.9	1.9	34.0	0.7	2.3
100% F4	3	31.5	0.8	1.6	33.2	1.1	2.4	34.6	0.5	1.7
99% F1	3	32.8	0.8	1.5	33.6	1.4	3.0	31.5	0.6	2.1
99% F2	3	33.9	0.9	1.6	34.8	1.3	2.6	33.1	0.4	1.6
99% F3	3	48.9	28.5	34.8	35.7	8.7	17.5	49.7	16.0	38.3
99% F4	3	82.6	19.9	14.4	64.5	27.9	31.1	80.5	12.0	17.8
90% F1	10	84.2	29.6	21.0	86.0	28.0	23.4	87.6	13.2	18.0
90% F2	10	26.2	0.7	1.6	13.1	0.7	3.7	55.7	1.6	3.4
90% F3	10	0.1	0.0	1.7	5.6	0.1	3.7	0.1	0.0	2.2

All the experiments were performed under these conditions: column length 70 mm, flow rate 0.286 mL/min, Ag-silica gel: 1 g

[a] 100% pure hexane, 99%: hexane:acetone (99:1, v/v), 90%: hexane:acetone (90:10, v/v)

[b] *V* volume (mL), *P* SDA-EE purity (%), *Y* SDA-EE yield (%), *W* weight (mg)

[c] Fractions from 100% F1 to 99% F3 were pooled before analysis due to the very low weight recovered in each fraction

Table 3.3 SDA-EE purity and yield obtained by pooling fractions in the study of optimization of FAEE load

FAEE load (%) (w/w)	Pooled fractions [a]			
	99% F3 + 99% F4 + 90% F1		99% F4 + 90% F1	
	Purity (%)	Yield (%)	Purity (%)	Yield (%)
15	-	-	82.4	62.9
20	72.5	71.5	86.2	42.8
25	66.3	77.9	83.5	49.5
30	64.5	64.6	73.7	55.9
50	66.3	41.2	84.1	25.2

All the experiments were performed at these conditions: FAEE load: 100 mg (10%, w/w); column length: 70 mm; flow rate: 0.286 mL/min; argentation silica gel: 1 g

[a] 99%: hexane:acetone (99:1, v/v); 90%: hexane:acetone (90:10, v/v)

Table 3.4 SDA-EE purity and yield in the optimization of fractions, solvents and volumes

Separated fractions						Pooled fractions				
Fraction (%) [a]	V [b] (mL)	*P* (%)	*Y* (%)	*W* (mg)		Fraction	*V* [b](mL)	*P* (%)	*Y* (%)	*W* (mg)
100 F1	29	0.1	0.0	18.4	→	100% F1	29	0.1	0.0	18.4
99 F1	12	3.7	8.1	51.5	→	99% F1	12	3.7	8.1	51.5
99F2	1	23.9	3.4	3.4	→	99% F2	2	32.0	5.8	4.3
99F3	1	62.5	2.4	0.9						
99 F4	1	83.3	3.5	1.0						
99 F5	1	90.0	2.1	0.7						
95 F1	12	98.4	54.8	12.7	→	95% F1	22	94.2	75.7	18.7
95 F2	4	81.4	9.2	2.7						
95 F3	4	91.3	6.1	1.6						

All the experiments were performed under these conditions: FAEE load 100 mg (10%, w/w), column length 70 mm, flow rate 0.286 mL/min, argentation silica gel 1 g

[a] 100% pure hexane, 99% hexane:acetone (99:1, v/v), 95%: hexane:acetone (95:5, v/v)

[b] *V* volume (mL), *P* SDA-EE purity (%), *Y* SDA-EE yield (%),*W* weight (mg)

Table 3.5 Purity, yield and standard deviation of main FAEE in the study of reproducibility

	Raw FAEE [b]	Fractions [a]			
		100% F1 (29 mL)	99% F1 (12 mL)	99% F2 (2 mL)	95% F1 (24 mL)
Fatty acid (purity, %)					
Palmitic C16:0	12.1	61.0 ± 3.7	0.6 ± 0.4	N D [d]	0.1 ± 0.0
Stearic C18:0	4.1	20.8 ± 1.2	0.1 ± 0.1	N D	0.0 ± 0.1
Oleic C18:1 (ω-9)	15.5	12.7 ± 4.8	23.5 ± 0.5	0.3 ± 0.0	0.1 ± 0.0
Linoleic C18:2 (ω-6)	24.5	N D	44.5 ± 2.6	4.1 ± 2.6	0.1 ± 0.1
γ-Linolenic C18:3 (ω-6)	7.2	1.8 ± 0.1	10.2 ± 0.6	23.2 ± 1.5	0.6 ± 0.1
α-Linolenic C18:3 (ω-3)	10.7	1.4 ± 0.0	15.2 ± 1.1	42.5 ± 1.7	1.3 ± 0.3
Stearidonic C18:4 (ω-3)	**23.8**	**1.1 ± 0.0**	**3.5 ± 0.7**	**28.5 ± 3.2**	**96.3 ± 1.9**
Fatty acid (yield, %)					
Palmitic C16:0	-	98.2 ± 3.5	2.5 ± 1.7	N D	0.1 ± 0.1
Stearic C18:0	-	99.7 ± 3.7	1.7 ± 1.3	N D	0.2 ± 0.4
Oleic C18:1 (ω-9)	-	16.4 ± 7.9	76.3 ± 4.8	0.1 ± 0.0	0.1 ± 0.1
Linoleic C18:2 (ω-6)	-	N D	91.3 ± 1.6	0.9 ± 0.5	N D
γ-Linolenic C18:3 (ω-6)	-	4.7 ± 0.1	71.3 ± 6.3	17.5 ± 6.2	1.5 ± 0.4
α-Linolenic C18:3 (ω-3)	-	0.7 ± 0.1	71.3 ± 7.7	21.4 ± 7.0	2.3 ± 0.6
Stearidonic C18:4 (ω-3)	-	**0.1 ± 0.0**	**7.4 ± 1.7**	**6.3 ± 1.3**	**77.0 ± 1.7**
Total weight (mg)	100	19.6 ± 2.0	50.5 ± 2.0	5.4 ± 1.6	19.3 ± 0.7

All the experiments were performed in triplicate under these conditions: FAEE load: 100 mg (10%, w/w),
column length 70 mm, flow rate 0.286 mL/min, argentation silica gel 1 g; *ND* Not Detected

[a] 100% pure hexane, 99%: hexane:acetone (99:1, v/v), 95%: hexane:acetone (95:5, v/v)

[b] Raw FAEE product obtained from ethanolysis of high SDA ω-3 modified soybean oil

Table 3.6 Purity and yield of ALA-EE and GLA-EE in the optimization of fractions, solvents and volumes

Fraction (%) [a]	*V* [b] (mL)	*W* (mg)	*P* (%) ALA-EE	*Y* (%) ALA-EE	*P* (%) GLA-EE	*Y* (%) GLA-EE
100 F1	29	22.0	0.1	0.9	0.3	4.7
99 F1	6	4.2	0.1	0.6	0.0	0.1
99 F2	1	1.9	0.0	0.1	0.0	0.0
99 F3	1	9.0	0.1	0.6	0.0	0.4
99 F4	1	12.4	0.6	5.9	0.6	8.3
99 F5	1	11.0	2.3	21.4	1.7	23.0
99 F6	1	10.7	3.7	34.6	2.4	32.8
99 F7	1	6.0	2.5	23.6	1.5	20.8
99 F8	1	2.3	0.9	8.2	0.5	6.5
99 F9	1	1.0	0.2	1.4	0.1	1.0
95 F1	24	18.7	0.1	1.3	0.1	0.7

Pooled fractions (%)	*P* (%) ALA-EE	*Y* (%) ALA-EE	*P* (%) GLA-EE	*Y* (%) GLA-EE
(99) F4+F5+F6+F7+F8+F9	23.7	95.2	15.4	92.4
(99) F5+F6+F7+F8+F9	31.1	89.3	19.7	84.2
(99) F6+F7+F8+F9	36.6	67.9	22.2	61.2
(99) F7+F8+F9	38.6	33.2	22.1	28.4

The experiments were performed under these conditions: FAEE load: 100 mg (10%,w/w), column length 70 mm, flow rate: 0.286 mL/min, argentation silica gel 1 g

[a] 100% pure hexane, 99% hexane:acetone (99:1, v/v), 95% hexane:acetone (95:5, v/v)

[b] *V* volume (mL), *P* purity (%), *Y* yield (%), *W* weight (mg)

Table 3.7 Purity, yield and standard deviation of main FAEE in the scaled-up processes

	Fractions [a]			
	100% F1 (680 mL)	99% F1 (234 mL)	99% F2 (94 mL)	95% F1 (563 mL)
Fatty acid (purity, %)				
Palmitic C16:0	40.7 ± 0.6	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
Stearic C18:0	13.8 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
Oleic C18:1 (ω-9)	34.3 ± 2.8	14.0 ± 3.0	0.8 ± 1.1	0.1 ± 0.0
Linoleic C18:2 (ω-6)	4.8 ± 2.3	57.2 ± 2.4	21.4 ± 2.8	0.3 ± 0.1
γ-Linolenic C18:3 (ω-6)	1.1 ± 0.0	4.8 ± 6.9	18.1 ± 0.9	0.6 ± 0.2
α-Linolenic C18:3 (ω-3)	0.6 ± 0.0	19.7 ± 9.1	31.6 ± 0.1	1.4 ± 0.3
Stearidonic C18:4 (ω-3)	**0.1 ± 0.0**	**2.4 ± 1.4**	**25.9 ± 4.1**	**96.9 ± 0.5**
Fatty acid (yield, %)				
Palmitic C16:0	97.7 ± 0.7	0.4 ± 0.0	0.1 ± 0.1	0.2 ± 0.2
Stearic C18:0	98.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.2	0.5 ± 0.5
Oleic C18:1 (ω-9)	64.2 ± 5.7	27.5 ± 4.0	0.8 ± 1.1	0.1 ± 0.0
Linoleic C18:2 (ω-6)	5.7 ± 2.7	71.7 ± 7.7	14.3 ± 2.9	0.2 ± 0.1
γ-Linolenic C18:3 (ω-6)	4.6 ± 0.2	44.3 ± 6.9	41.0 ± 5.1	1.5 ± 0.5
α-Linolenic C18:3 (ω-3)	1.7 ± 0.1	40.7 ± 6.4	47.9 ± 3.5	2.2 ± 0.6
Stearidonic C18:4 (ω-3)	**0.1 ± 0.0**	**3.0 ± 1.7**	**17.6 ± 1.5**	**71.3 ± 0.2**
Total weight (mg)	695 ± 7.1	735 ± 49.5	390 ± 28.3	420 ± 0.0 [b]

The experiments were performed in duplicate under these conditions: FAEE load 2,380 mg (~10%,w/w), column length 70 mm, flow rate 6.7 mL/min, argentation silica gel 21.4 g

[a] 100% pure hexane, 99% hexane:acetone (99:1, v/v); 95% hexane:acetone (95:5, v/v)

[b] The scaled-up process was repeated twice to yield 840 mg SDA-EE

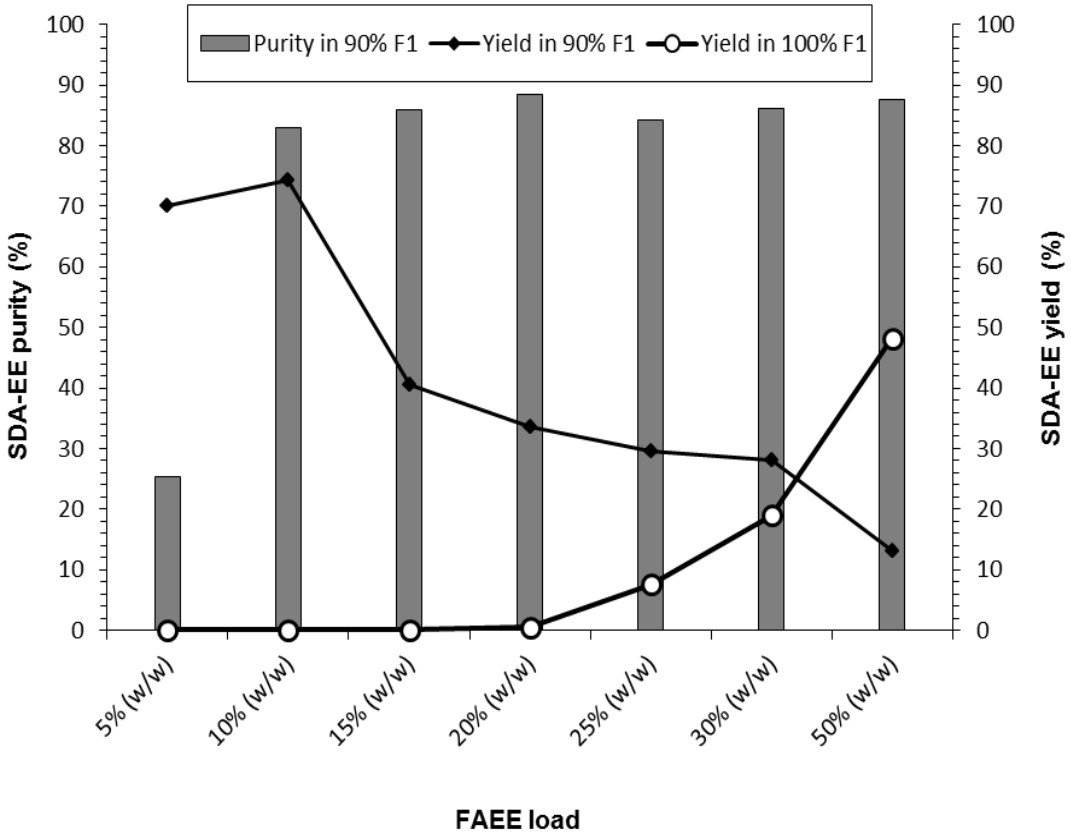

Figure 3.1 Values of SDA-EE purity in 90% F1, SDA-EE yield in 90% F1 and SDA-EE yield in 100% F1, after optimization of FAEE load. F1, First fraction; 100%, pure hexane; 90% hexane:acetone (90:10, v/v)

CHAPTER 4

INCREASING STEARIDONIC ACID (SDA) IN MODIFIED SOYBEAN OIL BY LIPASE-MEDIATED ACIDOLYSIS[1]

[1]Kleiner L, Vazquez L, Akoh, CC (2011) Increasing stearidonic acid (SDA) in modified soybean oil by lipase-mediated acidolysis. J Am Oil Chem Soc DOI 10.1007/s11746-012-2022-1

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ABSTRACT

The objective of this work was to synthesize a structured lipid (SL) enriched in stearidonic acid (SDA, C18:4 ω-3), from modified soybean oil (MSO) originally containing ~25% SDA. Low temperature crystallization (LTC) of MSO triacylglycerols (TAG) and free fatty acids (FFA) was performed. The TAG and FFA crystallization products (LTC-TAG and LTC-FFA, respectively) had SDA contents of 48.72 and 60.78%, respectively. Enzymatic acidolysis between MSO and LTC-FFA was studied utilizing Novozym 435 and Lipozyme TL IM as biocatalysts. Substrate molar ratio, incubation time, solvent, and enzyme load were explored. Equilibrium was reached at 96 and 48 h for Novozym 435 and Lipozyme TL IM-catalyzed reactions, respectively. The best conditions from these studies were also applied to the acidolysis of LTC-TAG and LTC-FFA. Utilizing Lipozyme TL IM and solvent free conditions, SLs with SDA contents of 37.61 ± 1.00% (20.86 ± 6.48% at *sn*-2 position) and 53.46 ± 1.85% SDA (36.37 ± 3.14% at *sn*-2 position) were obtained from the acidolysis reaction between MSO and LTC-FFA, and LTC-TAG and LTC-FFA, respectively. Compared to the original SDA content of MSO, this process leads to a 52 and 116% increase in SDA content, respectively.

INTRODUCTION

The long chain polyunsaturated fatty acids (LCPUFA), eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3), have long been associated with many health benefits. These LCPUFA have been found to modify the expression of inflammatory genes, thus becoming potentially powerful antiinflammatory agents [1], able to reduce the incidence of cardiovascular disease in epidemiological and clinical trials [2], and inhibit carcinogenesis [3].

Stearidonic acid (SDA) is another LCPUFA, and it is a metabolic intermediate in the conversion of α-linolenic acid (ALA, 18:3 ω-3) to EPA and DHA [4]. The conversion from ALA to SDA occurs via the rate limiting enzymatic activity of Δ6 desaturase. However, most ALA appears to undergo oxidation before the conversion to SDA can take place [4]. In contrast to ALA, when SDA enters the metabolic pathway no conversion via Δ6 desaturase is needed. Therefore, SDA works as a ‘‘pro-EPA’’ fatty acid that can be rapidly converted to EPA [4]. In addition to its health benefits, when used as a functional food component, SDA is less susceptible to lipid oxidation, which leads to the formation of undesirable free radicals, aldehydes and off-flavors, than the more unsaturated EPA and DHA [5]. Based on its ease of conversion to EPA and its physical properties, producing a structured lipid (SL) enriched in SDA would be of interest to nutritionist and food industry in order to increase the availability of EPA in the western diet, as well as to improve the shelf life and sensory characteristics of functional foods enriched in LCPUFA.

Sources of LCPUFA include fish and other marine life; however, the quantities present are quite low. EPA and DHA comprise 15–20% of the total fatty acid profile of

fish, while SDA represents 0.5–2% of the fatty acids [6]. A secondary source of SDA is Echium oil (extracted from the *Echium plantagineum* plant), a commercially available plant source containing approximately 3.5–9% SDA by weight [4]. Due to pollution of aquatic systems and the effects of over-fishing, concerns over the sustainability of global fish stocks has increased, leading to the development of transgenic plants able to produce LCPUFA [7].

Previous work on SDA enriched lipids focused mainly on the purification of SDA fatty acid methyl esters (FAME), fatty acid ethyl esters (FAEE), and triacylglycerols (TAG). Guil-Guerrero et al. [8] obtained SDA methyl ester of 83.2% purity by argentation silica gel column chromatography from Shortfin Mako liver oil (*I. oxyrinchus*). This technique has been also used by Kleiner-Shuhler et al. [9] to obtain FAEE with SDA purity of 97% in a scaled-up process. In addition, Vazquez and Akoh [10,11] have purified SDA enriched FFA and TAG from modified soybean oil by low temperature crystallization. The FFA and TAG had an SDA content of 59.6 and 45.2%, respectively.

In a different study, Rincon-Cervera et al. [12] isolated a fraction of *E. plantagineum* seed oil with 30.8% SDA content using a gravimetric chromatographic column with silica gel and silver nitrate. In the same work, reversed phase HPLC was used to isolate a fraction of TAG, with 44.4% SDA content [12]. With the exception of Teichert and Akoh [13], who incorporated SDA into a human milk fat analogue SL, to the best of our knowledge, there are no other studies concerning SLs enriched in SDA. Therefore, it is of interest to produce a SL with higher SDA content than originally present in MSO (~25%), as well as with a higher SDA content than previously achieved by purification techniques.

Structured lipids are chemically and/or enzymatically modified TAG, in which fatty acids have been incorporated and/or restructured, therefore modifying the FA profile and/or physicochemical properties. In this work, modified soybean oil containing ~25% SDA was used to produce a SL enriched in SDA. SLs can be produced by lipase-catalyzed interesterification reactions. Lipozyme TL IM (*Thermomyces lanuginosus* immobilized on silica gel, an *sn*-1,3 specific lipase, specific activity 250 IUN/g) and Novozym 435 (*Candida antarctica* lipase immobilized on macroporous acrylic resin beads, a nonspecific lipase, specific activity 10,000 PLU/g) were evaluated for their ability to incorporate SDA into a SL. IUN and PLU refer to interesterification units Novo, and propyl laurate units, respectively. The effects of substrate molar ratio, enzyme load, incubation time, and solvent conditions on the incorporation of SDA were also explored.

The SL was produced by the acidolysis reaction between MSO and FFA (60.8% SDA content) obtained by low temperature crystallization (winterization) of MSO. Since LCPUFA esterified at the *sn*-2 position of TAG are more easily absorbed than those esterified at *sn*-1,3 positions [12], positional analysis were performed. The conditions yielding the best total SDA incorporation and highest content of SDA at the *sn*-2 position were then applied to the acidolysis reaction of TAG and FFA, both obtained from low-temperature crystallization of MSO.

MATERIALS AND METHODS

Materials

Modified soybean oil (~25% SDA) was donated by the Monsanto Company (St. Louis, MO). This oil is not yet commercially available, and it was produced by the

overexpression of lipid biosynthetic genes in normal soybean oil, leading to increased SDA levels. Immobilized lipases, Novozym 435 (nonspecific lipase) and Lipozyme TL IM (*sn*-1,3 specific lipase), were obtained from Novozymes North America Inc. (Franklinton, NC). A C76 shaking water bath from New Brunswick Scientific (Edison, NJ) was employed for incubation and mixing the reactants. The lipid standards Supelco 37 Component FAME mix, triolein, and 2-oleoylglycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Stearidonic acid methyl ester was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The internal standard nonadecanoic acid (C19:0) was purchased from TCI America (Portland, OR). Other solvents and chemicals were purchased from Fisher Scientific (Norcross, GA) and Sigma-Aldrich Chemical Co. (St. Louis, MO). All the solvents and chemical compounds were ACS grade.

Low Temperature Crystallization of MSO

For the production of SDA enriched FFA and TAG, low temperature crystallization of MSO was performed as described by Vazquez and Akoh [10, 11]. Hereafter, the FFA and TAG obtained by low temperature crystallization will be referred to as LTC-FFA and LTC-TAG, respectively. LTC-FFA was initially used for an acidolysis reaction with MSO. Later, the acidolysis reaction between LTC-FFA and LTC-TAG for the production of an SDA enriched SL was explored.

Acidolysis Reactions Between MSO and LTC-FFA

In a test tube, 100 mg of MSO and 96 mg of LTC-FFA (160 mg for 1:5 substrate molar ratio) were weighed and the cap sealed with a Teflon tape to prevent solvent evaporation.

Novozym 435 or Lipozyme TL IM was added at 10% by weight of the substrates, respectively. After addition of the pertinent enzymatic catalyst and 2 mL n-hexane, the tubes were incubated at 60 ^{o}C in a shaking water bath at 200 rpm (3.589 x g). On completion of the specified incubation time, the reaction products were filtered through an anhydrous sodium sulfate column. An additional 1 mL of n-hexane was used to wash the sodium sulfate column. The product was stored in capped, UV light protected vials at -10 ^{o}C until further use. For the increased enzyme load (IEL) acidolysis reactions, the procedure was followed as described above, with the exception that Novozym 435 or Lipozyme TL IM were added at 20% by weight of the substrates, respectively. For the solvent-free (SF) acidolysis reactions, catalyst addition was at 10% by weight of the substrates, respectively. For these reactions, the procedure described above was modified as follows: no hexane was used in the reaction medium, after the reaction time, 2 mL n-hexane was added to the test tubes, the samples were then vortexed to extract the reaction product into the solvent as previously described. All experiments were performed in triplicate, and average results and standard deviations are reported.

Acidolysis Reaction Between LTC-TAG and LTC-FFA

Two experiments, one under SF and the other under IEL conditions, were performed utilizing Lipozyme TL IM at 1:3 molar substrate ratio and incubation time of 48 h. One hundred milligrams of LTC-TAG and 96 mg of LTC-FFA were weighed into Teflon tape sealed test tubes. For these SF and IEL experiments, the procedures were followed as described above, respectively. The results reported are the average and standard deviation of triplicate analysis.

Recovery of TAG as SL Product

The purpose of this step was to isolate the SL as TAG from FFA. Silica gel G TLC plates were activated in an oven at 100 ^{o}C for 1 h and allowed to cool down to room temperature in a desiccator. The mobile phase used to separate TAG was a mixture of petroleum ether and diethyl ether (70:30, v/v) equilibrated in a TLC tank for 30 min. Then, 100 μL of the reaction product were spotted on the TLC plates along with triolein standard used for TAG identification. The plate was sprayed with 0.2% 2,7-dichlorofluorescein in methanol to identify the lipid bands under UV light. The TAG band was recovered by scraping off into test tubes and extracting with 2 mL diethyl ether. The tubes were centrifuged for 3 min at 1,000 rpm (89.449 x g) and the product filtered through an anhydrous sodium sulfate column. This step was performed to separate the silica from the TAG before FAME and positional analysis.

Determination of Fatty Acid Profiles (MSO, LTC-FFA, LTC-TAG, and SL)

The AOAC Official Method 996.01, Section E [14] with minor modifications [15] was used to convert the TAG samples to FAME. For analysis of the MSO, LTC-FFA, and LTC-TAG, 100 mg of the sample was weighed into a test tube, 100 μL of the internal standard, C19:0 in hexane (20 mg/mL) was added, and the mixture was dried under nitrogen to remove solvent. For analysis of the SL, 100 μL of the internal standard was added to the recovered TAG band, and the solvent was evaporated under nitrogen. Two mL of 0.5 N NaOH in methanol solution was added to the mixture, vortexed, and incubated for 5 min at 100 ^{o}C to saponify the lipid. After incubation, 2 mL of 14% BF_3 in methanol was added. The sample was vortexed for 1 min and further incubated at 100 ^{o}C for 5 min for methylation. To quench the reaction and extract the FAME, 2 mL each of

hexane and saturated NaCl solution were added. The sample was vortexed for 2 min and then centrifuged for 5 min at 1,000 rpm (89.449 x g). The upper organic layer was removed and recovered in a GC vial for analysis. The FAME external standard used was the Supelco 37 component FAME mix and was run parallel with the samples.

Pancreatic Lipase-Catalyzed *sn*-2 Positional Analysis

The TAG recovery procedure was repeated for this analysis, followed by complete solvent evaporation under nitrogen. A modified version of the Luddy et al. [16] method was used to perform the pancreatic lipase-catalyzed *sn*-2 positional analysis. For MSO and LTC-TAG, 100 mg of oil were used. For the SL, the extracted and dried TAG was used. The TAG were placed into test tubes and 2 mL of 1.0 M Tris–HCl buffer (pH 8), 0.5 mL of 0.05% sodium cholate solution, and 0.2 mL 2.2% calcium chloride solution were added. The mixture was vortexed for 2 min to emulsify, and 40 mg of pancreatic lipase was added. After further vortexing for 1 min, the tubes were incubated in a water bath at 40 ^{o}C for 3 min while shaking at 200 rpm. The samples were vortexed again for 2 min and the reaction was quenched by adding 1 mL of 6 N HCl. The hydrolyzed TAG were extracted by adding 4 mL of diethyl ether, vortexing, and collecting the organic layer after 3 min of centrifugation at 1,000 rpm (89.449 x g). This layer containing the solubilized lipids was filtered through an anhydrous sodium sulfate column. The samples were concentrated under nitrogen until approximately 100 μL was left.

A mixture of hexane, diethyl ether, and formic acid (60:40:1.6, v/v/v) was used as the TLC mobile phase for the separation of 2-monoacylglycerol (2-MAG) bands. The concentrated reaction product was spotted on the activated and dried silica gel G TLC plates and placed into the tanks. The standard used for identification of the 2-MAG band

was 2-oleoylglycerol, and it was run in parallel with the samples. To recover the 2-MAG band, the plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The scraped 2-MAG band was extracted in ether as described for TAG recovery. FAME preparation was performed by adding 50 μL of C19:0 in hexane as an internal standard (20 mg/mL). The fatty acid content at the *sn*-2 position was quantified by GC.

GC Analysis

Fatty acid methyl esters were analyzed using an Agilent Technology 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) with a flame ionization detector. A Supelco SP-2560 column (Supelco, Bellefonte, PA), 100m x 250 μm, 0.20 μm film was used to attain separation. Injection of 1 μL of sample was made at a split ratio of 20:1. Helium was the carrier gas and its flow rate at constant pressure was of 1.1 mL/min. The injector temperature and the FID set point were 300 ^{o}C. The oven was held at 140 ^{o}C for 5 min, then increased up to 240 ^{o}C at a rate of 4 ^{o}C/min, and held isothermally for 15 min. The relative FAME content was calculated using GC Chem- Station Revision A.08.03 (Agilent Technologies, Santa Clara, CA). The average and standard deviation of triplicate analyses are reported.

Statistical Analysis

The analysis of variance (ANOVA) was performed using IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL). Two-way and General Linear Model ANOVAs were performed with a significance level of $p < 0.05$. The main effects were compared by Tukey's test. As

previously stated, all experiments were performed in triplicate and average and standard deviations were calculated and reported in the tables and figures.

RESULTS AND DISCUSSION

The fatty acid profiles and positional distribution of MSO, LTC-TAG and LTC-FFA, were comparable to that previously reported for MSO [9, 13], LTC-TAG [11] and LTCFFA [10], and are shown in Table 4.1. For MSO, SDA (25.0%) was the major fatty acid (FA), and this was followed by linoleic (23.7%), oleic (14.5%), palmitic (12.7%), α-linolenic (ALA) and γ-linolenic (GLA) acids (10.8, 7.4%, respectively). Linoleic acid (41.8%) was the major FA at the *sn*-2 position, followed by SDA (23.0%), and oleic (14.8%) acids. The fatty acid profile of LTC-TAG was mostly comprised of SDA (48.7%), ALA (15.8%), GLA (12.4%) and linoleic (11.0%) acids. In this case, the major FAs at the *sn*-2 positions were SDA (46.0%), linoleic acid (24.2%) and GLA (14.5%). For LTC-FFA, SDA (60.8%) was the major FA followed by GLA (17.2%) and linoleic acid (9.5%). The molecular weights of MSO (867.6 ± 0.04 g/mol), LTC-TAG (870.9 ± 0.24 g/mol) and LTC-FFA (277.6 ± 0.0015 g/mol) were calculated from Table 4.1. The calculated molecular weights were used to determine the corresponding amounts of each substrate for the various acidolysis reactions.

Substrate Molar Ratios and Incubation Time

One of the objectives of this study was to investigate the effect of the substrate molar ratio on the incorporation of SDA into the SL. SL refers to those structured lipids obtained by the acidolysis reaction between MSO and LTC-FFA. In this study, 1:3 and 1:5 substrate molar ratios of MSO to LTC-FFA were explored. The 1:3 substrate molar

ratio was selected based on the work of Senanayake and Shahidi [17], which found this ratio to be optimal for the Novozym 435-catalyzed incorporation of DHA into evening primrose oil. For both molar ratios, incubation times of 12, 18, 24, and 48 h were investigated.

Table 4.2 compares the results obtained utilizing Novozym 435 and Lipozyme TL IM enzymes. For the reactions catalyzed by Novozym 435, the substrate molar ratio was not statistically significant ($p > 0.05$) for the incorporation of SDA into MSO to form SL, until 48 h of incubation. For the reactions catalyzed by Lipozyme TL IM, no statistical difference ($p > 0.05$) was found between 1:3 and 1:5 substrate molar ratios. Since there is no economic advantage to using increased substrate molar ratios, and/or prolonged incubation times, we used 1:3 substrate molar ratio for further investigation.

Pina-Rodriguez and Akoh [15] had previously shown that in the enzymatic interesterification of amaranth oil with ethyl palmitate, there was no major difference in the overall fatty acid profile of the SL obtained utilizing a low (1:4) and a high (1:6) substrate molar ratio. This might be explained by the findings of Senanayake and Shahidi [17], which showed that in the enzymatic incorporation of DHA (free acid) to evening primrose oil, a higher concentration of DHA in the reaction medium may result in substrate inhibition of the lipase. This observation was also noted by Jennings and Akoh [18] for the enzymatic incorporation of capric acid into fish oil. In this case, the capric acid (a medium chain fatty acid) concentration present in the reaction medium was found to possibly lead to substrate inhibition.

An additional objective of this study was to establish the equilibrium point at which a prolonged reaction time would no longer lead to further SDA incorporation. Table 4.2 compares the time course of SDA incorporation into the SL utilizing both

enzymes. It has been previously reported that increasing reaction times leads to increased incorporation of the fatty acids into the SL. Yankah and Akoh [19] observed an increase in the enzymatic incorporation of oleic acid into tristearin, with 72 h giving the highest incorporation of oleic acid. Similarly, Lee and Foglia [20] observed that increasing reaction time increased the caprylic acid incorporation in the enzymatic transesterification of chicken fat. Therefore, additional incubation times of 96 and 120 h were explored.

In our study, the best SDA incorporation obtained with Novozym 435 (40.24 ± 1.33%) and Lipozyme TL IM (40.11 ± 0.98%) was achieved at 96 and 48 h, respectively (Table 4.2). A possible explanation for the effect of increased incorporation of fatty acid with increased incubation time is that, longer residence times allow for prolonged contact between the enzyme and the substrates [20]. For Novozym 435 and Lipozyme TL IM catalysts, no further incorporation of SDA into the SL was observed after 96 and 48 h, respectively. Therefore, the equilibrium point was established at these times.

Solvent Conditions

Enzymatic reactions in organic solvents, as opposed to aqueous solvents, are desirable due to the enzyme's thermostability, increased stability against proteolysis, pH independence, and the ability to shift the reaction towards synthesis instead of hydrolysis [21]. Organic solvents are also reported to lead to enzymatic conformational changes, therefore affecting their substrate specificity and affinity [21]. In this sense, different substrate specificities can be achieved by varying the solvent of choice for the reaction medium. Although exploring different solvents is of interest to modify lipase selectivity [22], a solvent-free reaction medium is preferred for food applications. This is due to

concerns over hexane toxicity, as well as the increased cost and time associated with its use and further product isolation [18].

In this study, the acidolysis reaction between MSO and LTC-FFA was explored in hexane and SF conditions. Table 4.2 shows the SDA incorporation obtained with Novozym 435 and Lipozyme TL IM under control conditions (C) in hexane, IEL, and SF conditions. For the reactions catalyzed by Novozym 435, significant differences were observed ($p < 0.05$) between the SDA incorporation achieved in hexane and SF conditions at all incubation times except for that of 48 h. For the reactions catalyzed by Lipozyme TL IM, no significant differences ($p > 0.05$) on SDA incorporation into the SL were observed between C and SF conditions, except at 18 h of incubation.

Our observations for the Novozym 435-catalyzed acidolysis reactions are in agreement with those of Lubary et al. [23], who reported on the Novozym 435-catalyzed ethanolysis of milk fat triacylglycerols in hexane, solvent free, and CO_2-expanded liquid conditions, that the use of solvent affected the rate of the reaction but not the final conversion. The authors noted that the solvent could possibly dilute the reactants leading to a decreased reaction rate. In our work, we have produced a SL with 39.84% SDA under 48 h and SF conditions, and an SL with 40.24% SDA at 96 h and C conditions. Therefore, it is possible that SF conditions might increase the rate of the reaction without affecting the overall incorporation of SDA at equilibrium.

In contrast, the SDA incorporation achieved by Lipozyme TL IM under C and SF conditions was not significantly affected ($p > 0.05$) by the use of solvent. The exception was at 18 h, at which the SDA incorporation catalyzed by Lipozyme TL IM was higher ($p < 0.05$) for the reaction in hexane (35.76 mol%) than for the SF reaction (29.54 mol%). Zhang et al. [24] reported that for Lipozyme TL IM, the substrate and hydrophobicity of

the carrier medium can induce changes in lipolytic activity. This interaction was observed by Lubary et al. [23] in the Lipozyme TL IM-catalyzed ethanolysis reactions of milk fat, in which alcohol was bound to the hydrophilic carrier causing ethanol inhibition of the lipase. Therefore, the lower SDA incorporation achieved under solvent-free conditions at 18 h could be explained by the interaction of the substrate and the carrier used for enzyme immobilization, which was hydrophilic granulated silica for Lipozyme TL IM [25].

Enzyme Load and Positional Analysis

Regarding enzyme load, Yang et al. [26] indicated that IEL led to an increased reaction rate, improved incorporation of the acyl donor in acidolysis reactions, and increased acyl migration. However, the improved incorporation of the acyl donor seems to be substrate specific. Zhao et al. [22] studied the enzymatic incorporation of capric acid into lard utilizing various enzymes and enzyme loads ranging from 5–30%, by weight of the substrate. In their work, the incorporation of capric acid at 5% enzyme load was not significantly different than other enzyme loads. In a different study, Senanayake and Shahidi [17] explored the incorporation of DHA into evening primrose (*Oenothera biennis* L.) via lipase-catalyzed transesterification. The authors observed that with an increase in lipase concentration, the extent of DHA incorporation had increased. However, after reaching a particular enzyme concentration, the authors noted that the incorporation of DHA remained constant [17].

In our work, the effect of IEL was studied only in hexane due to the inability to achieve thorough mixing of the enzymes with the substrates in a solvent-free environment. From Table 4.2, it is seen that for the Novozym 435-catalyzed acidolysis

reactions, there was no significant difference ($p > 0.05$) between the use of 10 and 20% enzyme per weight of the substrate (C and IEL, respectively). In this case, it is possible that at 10% enzyme load, the incorporation of SDA had already reached the best incorporation possible under the conditions employed. However, for the Lipozyme TL-IM catalyzed reactions, a statistical difference was found ($p < 0.05$) between the reactions performed under C and IEL conditions at 12 and 18 h of incubation. At longer incubation times no significant differences ($p < 0.05$) were found in the SDA incorporation into the SL. This suggests that IEL conditions might lead to an increased reaction rate [26] for the incorporation of SDA into the SL.

Enzyme load has also been positively correlated with acyl migration [23, 24]. Acyl migration is commonly observed in acidolysis reactions, and it is thought to occur between the steps of TAG hydrolysis into diacylglycerols (DAG) and MAG, and the subsequent esterification of new fatty acids into the TAG [24]. However, it has been shown that in certain enzymatic interesterification reactions, the enzyme load also has a strong positive influence on acyl migration. Xu et al. [25] showed that in the Lipozyme IM (*Rhizomucor miehei*)-catalyzed interesterification between rapeseed oil and capric acid under solvent-free conditions, enzyme load was secondary to temperature in significant factors that contributed to acyl migration. In this case, the support utilized for enzyme immobilization (hydrophilic, Duolite anion-exchange resin) was found to contribute to acyl migration [26].

A similar observation was reported by Du et al. [27], who studied acyl migration in the immobilized Lipozyme TL (*T. lanuginosus*)-catalyzed transesterification of soybean oil with methanol. In this work, it was found that the silica gel used for enzyme immobilization was the main factor contributing to acyl migration [27]. Although in

many cases acyl migration is seen as an undesirable side reaction in the enzymatic production of SLs, in our study it was of interest to explore enzyme load increase as a way to induce SDA acyl migration to the *sn*-2 position.

Table 4.3 shows the positional analysis of SLs produced with Novozym 435 under C, IEL and SF conditions. For each reaction condition, the SDA incorporation at the *sn*-2 and *sn*-1,3 positions were shown at the shortest incubation (12 h) and at 96 h of incubation, at which the total SDA incorporation was the best with this enzyme (40.24 mol%). Significant differences ($p < 0.05$) were found between the SDA incorporated at the sn-2 position under C and IEL, as well as C and SF conditions, respectively. Increasing the enzyme load led to a ~3.5 times increase in SDA (37.11 ± 3.07) at the *sn*-2 position than that obtained utilizing 10% enzyme (10.14 ± 0.98%). Also, carrying the reaction under SF conditions, led to a ~three times increase in SDA (29.6 ± 3.96%) at the *sn*-2 position than that obtained in hexane. Although, no significant difference ($p > 0.05$) was found between IEL and SF conditions for the incorporation of SDA at the *sn*-2 position, IEL conditions led to a SL with higher ($p < 0.05$) total SDA content (38.83%) than achieved under SF conditions (31.83%).

The positional analysis of SLs produced with Lipozyme TL IM under C, IEL, and SF conditions is also shown in Table 4.3. For each reaction condition, the *sn*-2 and *sn*-1,3 SDA incorporation into the SL is shown at the shortest incubation (12 h) and at 48 h of incubation, which led to the best SDA incorporation (40.11 mol%) into the SL. With Lipozyme TL IM in hexane, the SDA incorporated at the *sn*-2 position (3.62 ± 0.89%), was statistically different ($p < 0.05$) than that incorporated under IEL (22.08 ± 3.43%) and SF conditions (20.86 ± 6.48%), respectively. Therefore, it can be concluded that an increase in enzyme load lead to acyl migration of SDA in the SL. However, no

significant difference ($p > 0.05$) was found between the SDA incorporation at the sn-2 position under IEL and SF conditions. Since increasing the enzyme load can be costly, for Lipozyme TL IM catalyzed reactions, the use of a solvent- free medium yielded the best conditions for the production of a SL with high SDA content at the *sn*-2 position.

Acidolysis Reaction of LTC-TAG and LTC-FFA

Based on the studies on the acidolysis reaction between MSO and LTC-FFA, and with the aim of further increasing total SDA incorporation in the SL and at the *sn*-2 position, two experiments were performed with LTC-TAG (~49% SDA) and LTC-FFA substrates. For the best SDA incorporation achieved under C conditions, both Novozym 435 (96 h) and Lipozyme TL IM (48 h) enzymes led to the synthesis of SL with lower SDA content at the *sn*-2 position, than originally present in MSO. In addition to the cost associated with the use of hexane, the loss of SDA located at the *sn*-2 position makes this reaction condition less desirable than IEL and SF conditions. Lipozyme TL IM was selected over Novozym 435, because under IEL conditions higher ($p < 0.05$) incorporation of SDA into the SL was obtained with shorter incubation (48 h instead of 96, see Table 4.2). Therefore, for the acidolysis reaction between LTC-TAG and LTC-FFA, Lipozyme TL IM was explored at 48 h of incubation, IEL, and SF conditions.

From Table 4.4 it is seen that under SF conditions, it was possible to produce a SL with ~54% total SDA content and ~36% SDA content at the *sn*-2 position. When the conditions were those of IEL, the total SDA content of the SL and *sn*-2 position were ~52 and 40%, respectively. No statistical difference ($p > 0.05$) was found between the SDA (for either total SDA or at *sn*-2 position) incorporated into the SL under SF and IEL conditions. The acidolysis reactions between MSO and LTC-TAG under IEL and SF

conditions (48 h) led to a larger increase in SDA content of the SL (60 and 50%, respectively), than that achieved by the respective acidolysis reactions between LTC-TAG and LTC-FFA (10 and 7%, respectively). Since the only difference between the acidolysis of MSO and LTC-FFA and that of LTC-TAG and LTC-FFA, is the fatty acid profile of the TAG substrate, it is possible that the specificity of Lipozyme TL IM changed with the change of fatty acid profile of one of the substrates.

CONCLUSION

In this work, we have studied the acidolysis reaction of MSO (~25% SDA) and LTC-FFA (~61%) under different conditions of substrate molar ratio, incubation time, solvent, and enzyme load. By utilizing a Lipozyme TL IM biocatalyst under SF conditions, it was possible to synthesize a SL with ~38% total SDA content and ~21% SDA at the *sn*-2 position. Comparing the SDA content in this SL to that in MSO, a 52% increase in total SDA content was achieved. The same reaction conditions applied to the acidolysis reaction between LTC-TAG and LTC-FFA yielded a SL with ~54% total SDA content and ~36% SDA in the *sn*-2 position. Comparing the total SDA content of this SL to that of MSO, the process led to a 116% increase in SDA content. Therefore, we have developed a SF method that could be used to produce SLs with an SDA content of over 50%. This SL could have commercial applications as a ‘‘pro-EPA’’ functional food ingredient and aid in the efforts to develop food products rich in omega-3 fatty acids.

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Table 4.1 Composition of modified soybean oil, LTC-TAG and LTC-FFA

Fatty acid	**Total (mol %)[b]**	**Positional distribution**	
		***sn*-2 (mol%)**	***sn*-1,3[c] (mol%)**
Modified Soybean Oil (MSO)[a]			
Palmitic C16:0	12.71 ± 0.05	3.82 ± 3.07	17.16 ± 1.54
Stearic C18:0	4.30 ± 0.00	2.05 ± 1.57	5.42 ± 0.79
Oleic C18:1*c* (ω-9)	14.48 ± 0.05	14.78 ± 2.85	14.34 ± 1.43
Oleic C18:1*t*	1.62 ± 0.07	ND	2.43 ± 0.10
Linoleic C18:2 (ω-6)	23.69 ± 0.05	41.75 ± 1.06	14.66 ± 0.53
γ-Linolenic C18:3 (ω-6)	7.35 ± 0.01	8.28 ± 0.65	6.89 ± 0.33
α-Linolenic C18:3 (ω-3)	10.85 ± 0.01	6.29 ± 0.44	13.13 ± 0.22
Stearidonic C18:4 (ω-3)	25.00 ± 0.06	23.01 ± 0.42	25.99 ± 0.23
LTC-TAG[d]			
Palmitic C16:0	2.10 ± 0.05	0.49 ± 0.07	2.91 ± 0.08
Stearic C18:0	0.36 ± 0.02	0.19 ± 0.01	0.45 ± 0.03
Oleic C18:1*c* (ω-9)	7.07 ± 0.35	7.45 ± 0.03	6.88 ± 0.42
Oleic C18:1*t*	2.55 ± 0.25	ND	3.82 ± 0.30
Linoleic C18:2 (ω-6)	11.02 ± 2.80	24.23 ± 0.13	4.42 ± 3.42
γ-Linolenic C18:3 (ω-6)	12.36 ± 0.45	14.52 ± 0.14	16.47± 0.60
α-Linolenic C18:3 (ω-3)	15.82 ± 0.49	7.15 ± 0.09	14.97 ± 0.54
Stearidonic C18:4 (ω-3)	48.72 ± 1.38	45.98 ± 0.06	50.09 ± 1.70
LTC-FFA[e]			
Palmitic C16:0	0.44 ± 0.00		
Stearic C18:0	0.13 ± 0.00		
Oleic C18:1*c* (ω-9)	3.58 ± 0.57		
Oleic C18:1*t*	2.36 ± 0.52		
Linoleic C18:2 (ω-6)	9.51 ± 0.08		
γ-Linolenic C18:3 (ω-6)	17.16 ± 0.04		
α-Linolenic C18:3 (ω-3)	6.05 ± 0.04		
Stearidonic C18:4 (ω-3)	60.78 ± 0.05		

All analysis were performed in triplicate and average values ± SD are reported

[a] Fatty acids found in trace amounts were: C14:0, C16:1, C17:0, C18:2 (trans), C18:4 (trans), C20:0, C20:1 (ω-9) and C22:0

[b] Mean ± SD, n = 3

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

[d] LTC-TAG: TAG obtained from low temperature crystallization of modified soybean oil (MSO)

[e] LTC-FFA: FFA obtained from low temperature crystallization of modified soybean oil (MSO)

Table 4.2 Incorporation (mol %) of SDA into SL produced by acidolysis of MSO and LTC-FFA at various reaction conditions[a]

Time (h)	Novozym 435				Lipozyme TL IM			
	$C^{b}_{1:5}$	$C_{1:3}{}^{c}$	$IEL_{1:3}{}^{d}$	$SF_{1:3}{}^{e}$	$C^{b}_{1:5}$	$C_{1:3}{}^{c}$	$IEL_{1:3}{}^{d}$	$SF_{1:3}{}^{e}$
0	25.00 ± 0.06	25.00 ± 0.06	25.00 ± 0.06	25.00 ± 0.06	25.00 ± 0.06	25.00 ± 0.06	25.00 ± 0.06	25.00 ± 0.06
12	32.41 ± 1.36A[a]	30.28 ± 1.49A[a]	31.21 ± 1.30A[a]	36.96 ± 1.08B[a]	38.90 ± 5.63A[a]	32.09 ± 1.37A[a]	38.20 ± 1.50B[b]	32.25 ± 1.97A[b]
18	35.68 ± 4.30A[a]	33.48 ± 0.43A[a]	36.11 ± 0.40AB[a]	38.31 ± 2.03B[a]	37.69 ± 1.44A[a]	35.76± 1.32A[b]	40.03 ± 0.64B[b]	29.54 ± 2.22C[b]
24	40.48 ± 5.01A[a]	33.01 ± 2.20A[a]	34.99 ± 1.58AB[a]	38.45 ± 0.50B[a]	34.22 ± 4.15A[a]	35.80 ± 1.60A[a]	39.23 ± 0.53A[b]	35.83 ± 2.30A[a]
48	42.18 ± 1.93A[a]	36.89 ± 1.77BC[a]	34.78 ± 2.39B[a]	39.84 ± 1.84C[a]	36.17 ± 5.62A[a]	38.82 ± 4.16A[a]	40.11 ± 0.98A[b]	37.61 ± 1.00A[a]
96	-	40.24 ± 1.33B[a]	38.83 ± 0.67B[a]	31.83 ± 3.50C[a]	-	38.70 ± 1.44A[a]	39.23 ± 1.59A[a]	34.75 ± 4.75A[a]
120	-	38.98 ± 0.93	-	-	-	37.56 ± 0.81	-	-

[a] All experiments were performed in triplicate and average values ± SD reported. Means with different capital letters in the same row are significantly different ($p < 0.05$) within the same enzyme. Means with different lower case superscript letters in the same row are significantly different ($p < 0.05$) between both enzymes, for the same time and reaction condition.

[b] $C_{1:5}$: Control (2 mL hexane, 10% enzymatic catalyst, 1:5 molar ratio, incubated at 60^{o}C in a shaking water bath at 200 rpm)

[c] $C_{1:3}$: Control (2 mL hexane, 10% enzymatic catalyst, 1:3 molar ratio, incubated at 60^{o}C in a shaking water bath at 200 rpm)

[d] $IEL_{1:3}$: Increased Enzyme Load (2 mL hexane, 20% enzymatic catalyst, 1:3 molar ratio, incubated at 60^{o}C in a shaking water bath at 200 rpm)

[e] $SF_{1:3}$: Solvent-free (10% enzymatic catalyst, 1:3 molar ratio, incubated at 60^{o}C in a shaking water bath at 200 rpm)

Table 4.3 The *Sn*-2, and *sn*-1,3 SDA incorporation (mol %) into selected SLs produced by acidolysis of MSO and LTC-FFA with Novozym 435 or Lipozyme TL IM biocatalysts under different conditions

Positional distribution

T (h) and C[a]	*sn*-2	*sn*-1,3[b]	T (h) and C[a]	*sn*-2	*sn*-1,3[b]	T (h) and C[a]	*sn*-2	*sn*-1,3[b]
Novozym 435								
12-$C_{1:3}$[c]	17.82 ± 4.81A	36.51 ± 3.27	12-$IEL_{1:3}$[d]	19.30 ± 7.13A	37.17 ± 4.06	12-$SF_{1:3}$[e]	2.57 ± 0.55B	54.16 ± 1.64
96-$C_{1:3}$	10.14 ± 0.98A	55.29 ± 2.05	96-$IEL_{1:3}$	37.11 ± 3.07B	39.69 ± 1.83	96-$SF_{1:3}$	29.60 ± 3.96B	32.95 ± 5.61
Lipozyme TL IM								
12-$C_{1:3}$[c]	10.47 ± 2.76A	42.90 ± 2.48	12-$IEL_{1:3}$[d]	8.68 ± 2.22A	52.96 ± 2.51	12-$SF_{1:3}$[e]	9.45 ± 1.38A	43.65 ± 3.03
48-$C_{1:3}$	3.62 ± 0.89A	56.42 ± 6.26	48-$IEL_{1:3}$	22.08 ± 3.43B	49.13 ± 2.26	48-$SF_{1:3}$	20.86 ± 6.48B	45.99 ± 3.57

All experiments were performed in triplicate and average values ± SD reported. Means with different capital letter in the same row are significantly different ($p < 0.05$)

[a] T time; C condition

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

[c] $C_{1:3}$: Control (2 mL hexane, 10% enzymatic catalyst, 1:3 molar ratio, incubated at 60^{o}C in a shaking water bath at 200 rpm)

[d] $IEL_{1:3}$: Increased enzyme load (2 mL hexane, 20% enzymatic catalyst, 1:3 molar ratio, incubated at 60^{o}C in a shaking water bath at 200 rpm)

[e] $SF_{1:3}$: Solvent-free (10% enzymatic catalyst, 1:3 molar ratio, incubated at 60^{o}C in water bath shaking at 200 rpm)

Table 4.4 Fatty acid profile (mol %) of SL produced by acidolysis of LTC-TAG and LTC-FFA

	48-SF$_{1:3}$[a]			**48-IEL$_{1:3}$**[b]		
		Positional distribution			**Positional distribution**	
Fatty acid	**Total**[c]	***sn*-2**	***sn*-1,3**[d]	**Total**[c]	***sn*-2**	***sn*-1,3**[d]
Palmitic C16:0	1.39 ± 0.28	3.89 ± 0.24	0.15 ± 0.45	1.22 ± 0.06	3.30 ± 0.18	0.18 ± 0.13
Stearic C18:0	0.57 ± 0.07	1.51 ± 0.23	0.10 ± 0.33	0.56 ± 0.07	1.36 ± 0.12	0.15 ± 0.13
Oleic C18:1*c* (ω-9)	4.59 ± 0.42	6.29 ± 0.95	3.74 ± 0.79	4.47 ± 0.08	5.06 ± 0.21	4.17 ± 0.16
Oleic C18:1*t*	1.57 ± 0.20	1.36 ± 0.27	1.68 ± 0.19	1.62 ± 0.04	1.57 ± 0.19	1.64 ± 0.11
Linoleic C18:2 (ω-6)	15.93 ± 1.01	33.32 ± 3.25	7.24 ± 1.67	17.78 ± 2.19	30.82 ± 7.73	11.26 ± 5.07
γ-Linolenic C18:3 (ω-6)	8.82 ± 0.63	5.68 ± 0.32	10.40 ± 1.17	8.94 ± 0.24	6.14 ± 1.20	10.33 ± 0.70
α-Linolenic C18:3 (ω-3)	13.66 ± 0.16	11.60 ± 0.81	14.69 ± 0.94	13.36 ± 0.39	11.85 ± 1.40	14.11 ± 0.91
Stearidonic C18:4 (ω-3)	53.46 ± 1.85	36.37 ± 3.14	62.01 ± 3.35	52.07 ± 1.68	39.72 ± 5.13	58.24 ± 3.60

All analysis were performed in triplicate and average values ± SD reported

[a] $SF_{1:3}$: Solvent-free (10% Lipozyme TL IM, 1:3 molar ratio, incubated at 60^{o}C in a shaking water bath at 200 rpm)

[b] $IEL_{1:3}$: Increased enzyme load (2 mL hexane, 20% Lipozyme TL IM, 1:3 molar ratio, incubated at 60^{o}C in a shaking water bath at 200 rpm)

[c] Total content of SDA in the SL

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

CHAPTER 5

LIPASE-CATALYZED CONCENTRATION OF STEARIDONIC ACID IN MODIFIED SOYBEAN OIL BY PARTIAL HYDROLYSIS[1]

[1]Kleiner L, Vazquez L, Akoh CC. Reviewed, revised, and resubmitted for publication to J Am Oil Chem Soc on 04/24/2012.

ABSTRACT

Stearidonic acid (SDA, 18:4 ω-3) content of modified soybean oil (MSO) containing ~25% SDA, was increased by lipase-catalyzed hydrolysis. Four non-immobilized powdered lipases, Lipase AY 30 (*Candida rugosa*), Lipase G 50 (*Penicillium camembertii*), Lipomod^{TM} 34P-L034P (*Candida cylindracea [rugosa]*), Lipomod^{TM} 36P-L036P (*Rhizopus oryzae*), and an immobilized lipase, Lipozyme RM IM (*Rhizomucor miehei*) were assessed, at various incubation times, for their ability to hydrolyze MSO and specificity toward SDA. The SDA enriched products contained triacylglycerols (TAG), diacylglycerols (DAG) and monoacylglycerols (MAG). Lipase 34P-L034P exhibited specificity towards SDA, while Lipase AY was able to discriminate against it. The highest total SDA content (40.90 mol%) was obtained with Amano AY lipase at 4 h incubation (66.19% hydrolysis). Unhydrolyzed TAG, 1,3-DAG, 2,3(1)-DAG, and MAG contained 37.66 (56.37 at the *sn*-2 position), 41.64, 51.51 (54.92 at the *sn*-2 position), and 49.89% SDA, respectively. Amano AY lipase was also used to hydrolyze previously SDA-enriched TAG (48.72% SDA) obtained from low temperature crystallization of MSO. The highest total SDA content (62.69 mol%) was obtained at 12 h incubation (85.92% hydrolysis). The SDA contents of unhydrolyzed TAG, 1,3-DAG, 2,3(1)-DAG, and MAG were 58.66 (65.71 at the *sn*-2 position), 71.22, 70.22 (52.90 at the *sn*-2 position), and 59.43%, respectively.

INTRODUCTION

Eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3), two types of long chain polyunsaturated fatty acids (LCPUFA), have long been associated with many health benefits. These LCPUFA have been found to be potentially powerful antiinflammatory agents due to their ability to modify the expression of inflammatory genes [1]. In addition, it has been reported that consumption of EPA and DHA leads to a reduced incidence of cardiovascular disease in epidemiological and clinical trials [2], as well as to the inhibition of carcinogenesis [3].

Stearidonic acid (SDA, 18:4 ω-3) is another type of LCPUFA, and it is a metabolic intermediate in the conversion of α-linolenic acid (ALA, 18:3 ω-3) to EPA and DHA [4]. ALA is converted to SDA via the rate limiting enzymatic activity of Δ6 desaturase; however, most ALA appears to undergo oxidation before this conversion can take place [4]. In contrast, no conversion via Δ6 desaturase is needed for SDA to be rapidly converted to EPA, therefore becoming a "pro-EPA" fatty acid [4]. In addition to its health benefits, when used as a functional food component, SDA is less susceptible to lipid oxidation, which leads to the formation of undesirable free radicals, aldehydes and off-flavors, than the more unsaturated EPA and DHA [5].

Sources of LCPUFA include fish and other marine life. However, the quantities present are quite low. EPA and DHA comprise 15-20% of the total fatty acid profile of fish, while SDA represents 0.5-2% of the fatty acids [6]. A secondary source of SDA is Echium oil (extracted from the *Echium plantagineum* plant), a commercially available plant source containing approximately 3.5-9% SDA by weight [4]. Since the effects of pollution and over-fishing led to increased concerns over the sustainability of global fish stocks, transgenic plants able to produce LCPUFA have been developed [7].

Previous work on SDA enriched lipids focused mainly on the purification of SDA fatty acid methyl esters (FAME), fatty acid ethyl esters (FAEE), and triacylglycerols (TAG). Guil-Guerrero et al. [8] obtained SDA methyl ester of 83.2% purity by argentation silica gel column chromatography from Shortfin Mako liver oil (*I. oxyrinchus*). This technique was also used in a scaled-up process by Kleiner-Shuhler et al. [9] to obtain FAEE with SDA purity of 97% from modified soybean oil. Vazquez and Akoh have purified SDA enriched FFA and TAG by low temperature crystallization of modified soybean oil (MSO) [10, 11]. The FFA and TAG had an SDA content of 59.6% and 45.2%, respectively. From Echium plantagineum seed oil, and by gravimetric chromatographic column with silica gel and silver nitrate, Rincon-Cervera et al. [12] isolated a fraction of oil with 30.8% SDA content. In the same work, reversed-phase HPLC was used to isolate a fraction of TAG, with 44.4% SDA content [12].

A few studies have been reported on the production of structured lipids (SL) enriched in SDA. Teichert and Akoh [13] incorporated SDA (~6% of total fatty acids) into a human milk fat analog by the enzymatic interesterification reaction between MSO and tripalmitin. Similarly, Yüksel et al. [14] incorporated SDA (2.0% of total fatty acids) into a human milk fat analogue by enzymatic acidolysis reactions between tripalmitin and a mixture of FFA from hazelnut and Echium oil. We have previously reported the production of SLs with SDA contents of 37.61%. These were synthesized by the acidolysis reaction between MSO and FFA obtained from the low temperature crystallization of MSO (LTC-FFA) [15]. We also reported the production of SLs containing 53.46% SDA by the acidolysis reaction between TAG obtained from low temperature crystallization of MSO (LTC-TAG) and LTC-FFA [15].

A different approach to enrich oils with LCPUFAs is through partial hydrolysis of marine oils. This process leads to controlled production of unhydrolyzed TAG, diacylglycerols (DAG), and monoacylglycerols (MAG). These reaction products are nutritionally more advantageous than n-3 PUFA enriched methyl or ethyl esters of fatty acids, which have been shown to have impaired intestinal absorption in laboratory animals [16]. Hydrolysis reactions can be achieved by high pressure steam splitting, alkaline, and enzymatic approaches [17]. Of these three routes, lipase-catalyzed reactions offer biotechnologists the ability to work with LCPUFAs while avoiding high temperatures and pressures, as well as strong alkaline conditions [17]. Wanasundara and Shahidi [16] studied the concentration of n-3 PUFA in seal blubber oil and menhaden oil utilizing various lipases. Kahveci and Xu [18] produced n-3 PUFA enriched salmon oil by *Candida rugosa* lipase-catalyzed repeated hydrolysis.

Although there are many reports regarding the concentration of n-3 PUFA by enzymatic hydrolysis of oil, to the best of our knowledge, there are no studies to date focusing on the concentration of SDA by this method. Therefore, we have assessed various lipases for their hydrolytic activity on MSO and for their specificity toward SDA. The acylglycerol products of hydrolysis reactions, TAG, DAG, and MAG have various applications in the food, pharmaceutical, and cosmetic industries [19]. DAG are of interest as DAG oils, with 1,3-DAG being particularly important for their beneficial effects on the prevention of obesity and lipemia [20]. MAG are widely used in the food industry as emulsifiers and food additives [21]. Therefore, it is of interest to produce SDA enriched TAG, DAG, and MAG that would have diverse applications as functional food components.

MATERIALS AND METHODS

Materials

Modified soybean oil (~25% SDA) was donated by Monsanto Company (St. Louis, MO, USA). This oil is not yet commercially available. It was produced by the overexpression of lipid biosynthetic genes in normal soybean oil, leading to increased SDA levels. Lipase AY 30 (*Candida rugosa*, non-immobilized lipase) and Lipase G 50 (*Penicillium camembertii*, non-immobilized lipase) were kindly donated by Amano Enzyme Inc. (Nagoya, Japan). Lipases Lipomod™ 34P-L034P (*Candida cylindracea* [*rugosa*], non-immobilized lipase) and Lipomod™ 36P-L036P (*Rhizopus oryzae*, non-immobilized lipase, formerly *Rhizopus javanicus*) were generously donated by Biocatalysts (Wales, UK). Lipase Lipozyme RM IM (*Rhizomucor miehei*, immobilized on an anion-exchange resin) was obtained from Novozymes North America Inc. (Franklinton, NC, USA). A C-76 shaking water bath from New Brunswick Scientific (Edison, NJ, USA) was employed for incubation and mixing of the reactants.

The lipid standards Supelco 37 Component FAME mix, triolein, 1, 2-dipalmitoyl-sn-glycerol, and 2-oleoylglycerol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The standard 1,3-diolein was purchased from MP Biomedicals, LLC (Santa Ana, CA, USA). Stearidonic acid methyl ester was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The internal standard nonadecanoic acid (C19:0) was purchased from TCI America (Portland, OR, USA). Other solvents and chemicals were purchased from Fisher Scientific (Norcross, GA, USA) and Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All the solvents and chemical compounds were ACS grade.

Lipase Activity Assay

Hydrolytic activity of lipases was measured at 37 ^{o}C according to the titrimetric method described by Pinsirodom and Parkin [22], with slight modifications. The substrate was comprised of a triolein and gum arabic mixture (50%, w/w) emulsified in sodium phosphate buffer at pH 8.0 (5%, wt/vol). One unit (U) of lipase activity was defined as the amount of lipase that liberates 1 μmol fatty acid/min under assay conditions. All experiments were performed in triplicate and average values reported.

Low Temperature Crystallization of TAG from MSO (LTC-TAG)

TAG from MSO was SDA enriched by low temperature crystallization, as described by Vazquez and Akoh [11].

Lipase-catalyzed Partial Hydrolysis of MSO and LTC-TAG

Hydrolysis of MSO and LTC-TAG was based on a modified version of the method described by Wanasundara and Shahidi [16]. MSO or LTC-TAG (1 g), 0.1M phosphate buffer (1.5 mL, pH 5 for Amano G lipase, pH 7.0 for all other lipases) and 3218 U of lipase, were placed in test tubes which were nitrogen flushed prior to capping. The tubes were incubated at 40^{o}C in a shaking water bath at 200 rpm. The incubation times were 2, 4, 6, and 12 h. At completion of the specified incubation time, the reaction was quenched by the addition of 0.5 mL methanol. Hexane (3 mL) was added to extract unhydrolyzed oil, hydrolyzed acylglycerols, and free fatty acids produced by the hydrolysis of MSO. After centrifugation at 2000 rpm (357.76 x g), the organic layer containing the reaction products was filtered through an anhydrous sodium sulfate column and this filtrate was

stored in capped, UV light protected vials at -10^{o}C until further use. All experiments were performed in triplicate and average values and standard deviations reported.

Determination of Hydrolysis Percentage

After enzymatic treatment, the hydrolysis percentages of MSO and LTC-TAG were determined as described by Wanasundara and Shahidi [16], see Eq. (1). The reaction products were used to determine the acid value according to the AOCS Official Method Cd 3d-63. The calculated saponification value of unhydrolyzed MSO and LTC-TAG were obtained per the AOCS Recommended Practice Cd 3a-94, respectively. All experiments were performed in triplicate and average values and standard deviations reported.

$$\text{Hydrolysis (\%)} = [(\text{Acid value}_{(\text{hydrolyzed oil})} - \text{Acid value}_{(\text{unhydrolyzed oil})}) / (\text{Saponification value}_{(\text{unhydrolyzed oil})} - \text{Acid value}_{(\text{unhydrolyzed oil})})] \times 100 \quad [16] \qquad (1)$$

Separation of the Acylglycerol Products

This step was performed to isolate the unhydrolyzed TAG and partial acylglycerols from the hydrolyzed FFAs in order to determine the overall SDA content of the acylglycerol products. Silica gel G TLC plates were activated in an oven at 100 ^{o}C for 1 h and allowed to cool down to room temperature in a desiccator. The mobile phase used to separate the reaction products was a mixture of petroleum ether: diethyl ether: acetic acid (70:30:2, v/v/v), and this was equilibrated in a TLC tank for 30 min. 100 μL of the reaction product were spotted on the TLC plates along with triolein, 1,3-diolein, 1,2-dipalmitoyl-sn-glycerol, and 2-oleoylglycerol as standards for TAG, DAG, and MAG identifications.

The plates were sprayed with 0.2% 2,7-dicholorofluorescein in methanol to identify the lipid bands under UV light. The TAG, DAG, and MAG bands were recovered by scrapping off into one test tube and extracting with 2 mL diethyl ether. The test tubes were centrifuged for 3 min at 1000 rpm (89.44 x g) and the products were filtered through an anhydrous sodium sulfate column. This step was performed to separate the silica from the recovered acylglycerols before FAME derivatization.

Regiospecific Analysis by Boric Acid TLC

For MSO, the products of the enzymatic hydrolysis leading to the highest total SDA content were further analyzed by TLC plates impregnated with boric acid. The boric acid TLC plates were prepared by following a modified method from that described by Storch et al. [23] and Muño et al. [24]. Silica gel G TLC plates were wetted by immersion in a hydroethanolic solution (50%, v/v) of boric acid (1.2%, w/v). The plates were dried overnight at room temperature, baked at 100 ^{o}C for 1 h and allowed to cool down to room temperature in a desiccator before use. This procedure was also followed for the hydrolysis reactions performed with LTC-TAG as substrate. TAG, 1,3 DAG, 2,3(1)-DAG, and MAG were extracted as described above with the exception that each band was collected into a separate tube for its respective FAME derivatization.

Determination of Fatty Acid Profiles

The AOAC Official Method 996.01, Section E [25] with minor modifications [26] was used to convert all acylglycerols to FAME. For analysis of MSO and LTC-TAG, 100 mg of each sample was weighed into a test tube, 100 μL of the internal standard, C19:0 in hexane (20 mg/mL) was added. For analysis of the acylglycerols recovered from the

enzymatic hydrolysis, 100 μL of the internal standard was added to the TAG, DAG, and MAG bands which were recovered in the same tube. For analysis of the individual types of acylglycerols recovered by boric acid TLC, 25 μL of the internal standard was added to the respective acylglycerol. For all mixtures, the solvent was then evaporated under nitrogen. Two mL of 0.5 N NaOH in methanol solution was added to the mixture, vortexed, and incubated for 5 min at 100 ^{o}C to saponify the lipid. After incubation, 2 mL of 14% BF_3 in methanol was added. The sample was vortexed for 1 min and further incubated at 100 ^{o}C for 5 min for methylation. To quench the reaction and extract the FAME, 2 mL each of hexane and saturated NaCl solution were added. The sample was vortexed for 2 min and then centrifuged for 5 min at 1000 rpm (89.44 x g). The upper organic layer was removed and recovered in a GC vial for analysis. The FAME external standard used was the Supelco 37 component FAME mix and was ran parallel with the samples.

GC Analysis

FAME were analyzed using an Agilent Technology 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector. A Supelco SP-2560 column, 100m x 250 μm, 0.20 μm film was used for the separation. Injection of 1 μL of sample was made at a split ratio of 5:1. Helium was the carrier gas and its flow rate at constant pressure was 1.2 mL/min. The injector temperature and the FID set point were 300 ^{o}C. The oven was held at 140 ^{o}C for 5 min, then increased up to 240 ^{o}C at a rate of 4 ^{o}C/min, and held at this temperature for 15 min. The relative FAME content was calculated using GC ChemStation Rev.B.04.03 software (Agilent Technologies, Santa Clara, CA, USA). The average and standard deviation of triplicate analyses are reported.

Pancreatic Lipase-Catalyzed *sn*-2 Positional Analysis

The procedure for regiospecific analysis by boric acid TLC was repeated to collect enough TAG and 2,3(1)-DAG for this analysis, and it was followed by complete solvent evaporation. A modified version of the Luddy et al. [27] method was used to perform the pancreatic lipase-catalyzed *sn*-2 positional analysis. For MSO and LTC-TAG, 100 mg of each oil was used. The TAG or 2,3(1)-DAGs were placed into test tubes and 2 mL of 1.0 M Tris-HCl buffer (pH = 8), 0.5 mL of 0.05% sodium cholate solution, and 0.2 mL 2.2% calcium chloride solution were added. The mixture was vortexed for 2 min to emulsify, and 40 mg of pancreatic lipase was added. After further vortexing for 1 min, the tubes were incubated in a water bath at 40 ^{o}C for 3 min while shaking at 200 rpm. The samples were vortexed again for 2 min and the reaction was quenched by adding 1 mL of 6 N HCl. The hydrolyzed TAG or 2,3(1)-DAG were extracted by adding 4 mL of diethyl ether, vortexing, and collecting the organic layer after 3 min of centrifugation at 1000 rpm (89.44 x g). This layer containing the solubilized lipids was filtered through an anhydrous sodium sulfate column. The samples were concentrated under nitrogen until approximately 100 μL was left.

A mixture of hexane, diethyl ether, and formic acid (60:40:1.6, v/v/v) was used as the TLC mobile phase for the separation of 2-monoacylglycerol (2-MAG) bands. The concentrated reaction product was spotted on activated and dried silica gel G TLC plates and placed into previously equilibrated TLC tanks. The standard used for identification of the 2-MAG band was 2-oleoylglycerol, and it was ran in parallel with the samples. To recover the 2-MAG band, the plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The scraped 2-MAG band was extracted in ether as previously described for acylglycerols recovery. FAME preparation was performed by

adding 25 μL of C19:0 (20 mg/mL) in hexane as an internal standard. The fatty acid content at the *sn*-2 position was quantified by GC. The average and standard deviation of triplicate analyses are reported.

RESULTS AND DISCUSION

Fatty Acid Composition of MSO and LTC-TAG

The fatty acid profiles and positional distribution of MSO and LTC-TAG are shown in Table 5.1. For MSO, SDA (25.00%) was the major fatty acid (FA), and this was followed by linoleic (23.69%), oleic (14.48%), palmitic (12.71%), α-linolenic (ALA, 10.85%) and γ-linolenic (GLA) acids (7.35%), respectively. Linoleic acid (41.75%) was the major FA at the *sn*-2 position, followed by SDA (23.01%), and oleic (14.78%) acids. The fatty acid profile of LTC-TAG was mostly comprised of SDA (48.72%), ALA (15.82%), GLA (12.36%), and linoleic (11.02%) acids. In this case, the major FAs at the *sn*-2 positions were SDA (45.98%), linoleic acid (24.23%) and GLA (14.52%). The fatty acid profiles of MSO and LTC-TAG were in agreement with those reported previously [11,15].

Selection of Lipases and Lipase Activity

All lipases used in this work were selected based on their reported ability to hydrolyze oils containing n-3 PUFA, and/or on their reported ability to discriminate against LCPUFA. *Candida cylindracea* (CC), *Candida rugosa* (CR), *Rhizopus oryzae* (RO), and *Rhizomucor miehei* (RM) lipases have been previously used to successfully catalyze the hydrolysis of marine oils and/ or marine oil ethyl esters. CC lipase, has been extensively used in hydrolysis reactions due to its high hydrolytic activity [16, 28]. CR lipase was

reported by Halldorson et al. [29] to be a high activity lipase leading to 50% hydrolysis of fish oil ethyl esters in less than 4 h. RO lipase was also classified as a high activity lipase [29], and this enzyme led to the best degree of hydrolysis of menhaden oil, when compared to that achieved with other lipases [16]. Similarly, a high degree of hydrolysis (~50%) of seal blubber and menhaden oil was achieved with *Mucor miehei* lipase [16]. This lipase has been renamed *Rhizomucor miehei* [30].

In addition to CC lipase, which was shown to concentrate n-3 PUFA in hydrolyzed seal blubber oil [16], CR and *Penicillium camembertii* (PC) lipases have also been reported to concentrate EPA and DHA in fish oils. Moore and McNeill [31] successfully concentrated DHA and EPA in Chilean fish oil by CR lipase-catalyzed hydrolysis. Similarly, the EPA and DHA content of sardine oil ethyl ester was increased by the PC lipase-catalyzed hydrolysis of sardine oil ethyl esters [29]. Therefore, in our work it was of interest to explore the lipolytic activity, as well as the selectivity toward SDA, of CC, CR, RM, RO, and PC lipases on MSO as substrate.

The results of the lipase activity assay are shown in Table 5.2. For the conditions assessed, the highest activity on a triolein substrate (4023 U/g) was achieved by CC lipase. This was followed by PC (3111 U/g), RM (2845 U/g), RO (2823 U/g), and CR lipase (2400 U/g). Although in our work we were interested in correcting for lipase activity, our group has previously determined that a 10% (w/w) enzyme load was an effective enzyme concentration for the production of various types of structured lipids by lipase-catalyzed interesterification reactions [13, 26]. Since the concentration of water present in the reaction regulates the degree of hydrolysis relative to esterification [32], for the hydrolysis of MSO and LTC-TAG we have established an enzyme load of 3218 U/g of oil, which is equivalent to 10% (w/w) of CC lipase. CC was used as a reference due to

the high activity observed in our assay, and to the reported ability to hydrolyze various types of marine oils [16, 33]. The enzyme load of 3218 U/g is equivalent to 100, 142, 141, 129, and 168 mg/g of CC, RO, RM, PC, and CR lipase, respectively.

Lipase-Catalyzed Hydrolysis of MSO

Figure 5.1 illustrates the hydrolysis percentages of MSO as a function of incubation time. All the lipases assayed were able to hydrolyze MSO, but at different rates and extents of hydrolysis. At 12 h of incubation, CC lipase achieved the best hydrolysis percentage (73.56%) followed by CR lipase (67.11%). The best hydrolysis percentages achieved by RM, RO, and PC lipases (35.84, 26.84, and 7.45%, respectively) were much lower than that obtained with CR and CC lipases.

The results obtained with CC lipase were in general agreement with the high percent hydrolysis (>60%) achieved in the CC lipase-catalyzed hydrolysis of seal blubber oil [16]. Similarly, our results for the CR lipase-catalyzed hydrolysis of MSO were comparable to the hydrolysis percentage reported for sardine oil ethyl esters, with the same lipase [29]. RO and RM lipases provided a moderate (<40%) degree of hydrolysis, while PC lipase weakly (<10%) hydrolyzed MSO in the times studied. These observations are in agreement with the findings of Okada and Morrissey [33], which reported the hydrolysis of sardine oil by CR and CC lipases (non-specific lipases) in comparison to other *sn*-1,3 specific lipases. In their work, the non-specific lipases led to a higher percent of hydrolysis of sardine oil than that achieved by the *sn*-1,3 specific lipases.

The hydrolysis percentages achieved by RO and RM lipases on MSO were approximately half the amount observed on seal blubber and menhaden oil [16] and/or

sardine oil ethyl esters [29]. Similarly, PC lipase exhibited a 5 fold decrease in the hydrolysis of MSO from that reported for the hydrolysis of sardine oil ethyl esters by the same lipase [29]. A possible explanation for this behavior is that the type of fish oil can affect lipase specificity [34]. However, it has been noted by Hoshino et al. [35] that specificity alone cannot explain the diversity observed in the hydrolysis of fish oil. Other factors, such as the fatty acid profile of the oil and the reactivity of the lipase toward partial acylglycerols are also consequential [35]. In addition, in the hydrolysis of fatty acid ethyl esters, lipases might experience less steric hindrance than in the hydrolysis of fatty acids from TAG.

Lipase Selectivity Toward SDA in MSO Substrate

Figure 5.2 shows the total SDA content in the acylglycerol fraction of the reaction products, as a function of time. The highest total SDA content (40.90%) was achieved with CR lipase at 4 h. For all other lipases, the best total SDA content was ~28%. It is worth noting that although CC lipase provided the best degree of hydrolysis of MSO (73.56%), this lipase exhibited selectivity toward SDA. At most incubation times, this enzyme led to hydrolysis products containing a lower total SDA content in the acylglycerol fraction than originally present in MSO.

Okada and Morrissey [29] studied both CC and CR lipases for their ability to hydrolyze and concentrate n-3 PUFA in Pacific sardine (*Sardinops sagax*) oil. The authors observed that the percentages of hydrolysis achieved by CC and CR lipase were ~60 and 70%, respectively. Although CC lipase was able to increase the concentration of EPA and DHA in the hydrolyzed products, the increase was 37 and 6% lower than that achieved with CR lipase, respectively. In our work, the percentage of hydrolysis of MSO

obtained with CC lipase was as expected based on the literature [29]. However, this lipase was unable to discriminate against SDA and exhibited specificity toward this particular fatty acid. From Figure 5.2, it is seen that at 2 and 4 h of incubation, the SDA contents were 16.84 and 15.36%, respectively. This was followed by an increase in SDA at 6 h (25.58%), probably due to difference in the reactivity of the lipase toward partial acylglycerols [35]. A subsequent decrease in SDA content (14.83%) followed at 12 h of incubation.

CR lipase was successful at discriminating against SDA, and was also the next best in the hydrolysis of MSO, following CC lipase. CR lipase has been shown to increase its ability to discriminate against fatty acids in the range of C18 to C22, as the acyl chain increases [31]. In addition, this lipase has been reported to have substrate specificity toward saturated and monounsaturated fatty acids leading to the concentration of n-3 PUFA in the acylglycerol fraction of hydrolyzed oils [18]. From Figure 5.2, it is seen that at 4 h, CR lipase achieved the best concentration of SDA in the acylglycerol fraction. However, after 4h of incubation the total SDA content decreased from 40.90% to 22.45% (55% decrease after 8 h).

A similar observation has been previously reported by Sun et al. [34] for the CR lipase-catalyzed concentration of EPA and DHA in Atlantic salmon (*Salmo salar L.*) viscera. The authors reported that the best concentration of EPA and DHA was achieved at 12 h of incubation, followed by a slight decrease of these LCPUFAs as the reaction progressed. They suggested that TAG molecules free of EPA and DHA were hydrolyzed before molecules containing EPA and/or DHA [31]. Similarly, Kahveci and Xu [18] described the CR lipase-catalyzed hydrolysis of salmon oil as a two-step reaction. In this reaction, TAG molecules without DHA were hydrolyzed first, and then hydrolysis of

DHA rich TAG molecules followed. It is possible that a similar mechanism of hydrolysis could have taken place in MSO. An initial hydrolysis of SDA free TAG molecules followed by the hydrolysis of SDA enriched ones, would explain the decrease of SDA in the acylglycerol products that was observed after 4 h.

The best concentrations of SDA achieved by the remaining lipases were 27.19, 26.93, and 25.58% for RO, RM and PC lipase, respectively. These enzymes were inefficient at concentrating SDA in MSO at the conditions studied. When compared to other lipases, RO has been reported to achieve the best concentration of total n-3 PUFA in menhaden oil but not in seal blubber oil [16]. RO selectively hydrolyzed EPA in both oils by exhibiting acyl-chain specificity toward this fatty acid. In this sense, the total EPA content in the acylglycerol products was reduced from that originally present in the oils, while the DHA content was considerably increased. The same lipase acting on a sardine oil ethyl ester substrate was able to approximately double the contents of both EPA and DHA [32].

A possible explanation for the discrepancies on the selectivity of RO lipase toward EPA lies in the substrate and the positional distribution of this fatty acid. Wanasundara and Shahidi noted that the *sn*-1,3 specificity of the lipase affected the rate of hydrolysis of EPA in seal blubber and menhaden oil, but not its overall hydrolysis [16]. EPA was located mostly at the *sn*-1,3 positions of the seal blubber oil TAG, but randomly distributed in menhaden oil. In MSO, RO led to a low increase (~9%) in the SDA content of the acylglycerol products (27.19% at 6 h) followed by a decrease in SDA content (20.50% at 12 h). MSO has approximately an equal distribution of SDA (~23 and 26% SDA at *sn*-2, and *sn*-1,3 positions, respectively), therefore it is possible that in this

case and for a certain time, the lipase exhibited the ability to discriminate against SDA located at the *sn*-1,3 position.

As in the outcome obtained with RO lipase and at best conditions, RM lipase increased the SDA content of the acylglycerol products by ~8% (26.93% SDA at 4 h). This behavior was in agreement with the Mucor miehei-lipase catalyzed hydrolysis of seal blubber oil, which led to an overall increase in EPA content of 9% [16]. The same lipase acting on a menhaden oil substrate, led to ~20% increase in EPA content [16]. Therefore, it is possible that the low increase in SDA content achieved with this lipase was due to the specific fatty acid profile of MSO. Finally, PC lipase exhibited a slight specificity toward SDA, as shown by the lower SDA content achieved in the acylglycerol products at 2 and 12 h (23.80 and 23.85%, respectively).

Of all lipases assayed, CR led to the best combination of percent hydrolysis of MSO and total SDA content under the conditions studied. With this lipase, we were able to increase the overall SDA content by ~64% in a 4 h period (Figure 5.2). Table 5.3 shows the SDA content of the unhydrolyzed TAG, DAG and MAG present in the reaction products catalyzed by this lipase, at all incubation times studied. At 4 h of incubation, the SDA content in unhydrolyzed TAG, 1,3-DAG, 2,3(1)-DAG, and MAG were 37.66 (56.37 at the *sn*-2 position), 41.64, 51.51 (54.92 at the *sn*-2 position), and 49.89%, respectively. It is of particular interest to note, that at this incubation time the TAG and 2,3(1)-DAG SDA content at the *sn*-2 position was ~55% and the best observed for all incubation times.

The fatty acid profile of the acylglycerol products obtained with CR at 4 h incubation are shown in Table 5.4. The major constituents of unhydrolyzed TAG were SDA (~38%), linoleic and oleic acids (~17 and 11%, respectively), GLA (~11%), ALA

(~9%) and palmitic acid (~9%). SDA comprised ~56% of the fatty acids located at the *sn*-2 position. For 1,3-DAG products, the major fatty acids were SDA (~42%), GLA (~15%), linoleic, oleic, palmitic acids, and ALA (~14, 10, 10 and 5%, respectively). 2,3(1)-DAG products contained SDA (~52%), GLA (~16%), linoleic, palmitic, oleic acids, and ALA (~11, 7, 7 and 5%, respectively). The SDA content at the *sn*-2 position of the 2,3(1) DAG was ~55%. MAG products were composed of SDA (~50%), GLA (~19%), linoleic, palmitic and oleic acids (~8, 8 and 7%, respectively).

From Tables 5.1 and 5.4, it is seen that at the reaction conditions studied, CR lipase exhibited an overall specificity toward linoleic, ALA, oleic, and palmitic acids. Comparing the original contents of these fatty acids in MSO (Table 5.1) with that in the MAG reaction products, a decrease in linoleic (~64.7%), ALA (~66.6%), oleic (~53.1%), and palmitic acids (~37.1%) was observed. In addition, CR lipase exhibited the ability to discriminate against both GLA and SDA. This is of particular interest due to the novelty of studies on SDA, and because previous studies have reported difficulties in separating ALA from GLA. ALA and GLA are fatty acids of similar polarities, and this factor is thought to be the reason for difficulties observed in the chromatographic separation of these fatty acids [36, 37].

Candida rugosa Lipase-Catalyzed Hydrolysis of LTC-TAG

Based on the ability of CR lipase to increase the total SDA content in the MSO acylglycerol products by approximately two-fold (40.90% at 4 h), this lipase was selected over the other lipases to concentrate SDA in LTC-TAG (~49% SDA, initially). As shown in Figure 5.2, the highest total SDA content (62.68 %) achieved for LTC-TAG was at 12 h, at which the best hydrolysis percentage (85.92%) was also achieved, see

Table 5.3. From Figure 5.2, it is seen that the ability of CR lipase to discriminate against SDA in LTC-TAG as a function of time, differed from that observed in MSO substrate. This is indicative of the role of the fatty acids present in the substrate. From Table 5.1 it is seen that, when compared to MSO, the content of palmitic, stearic, oleic, and linoleic acids in LTC-TAG was reduced by ~83.47, 91.63, 51.17, and 53.48%, respectively. However, GLA, ALA, and SDA contents in LTC-TAG increased from that in MSO by ~68.16, 45.80, and 94.88%, respectively.

Table 5.3 shows the SDA content of each LTC-TAG acylglycerol product at the incubation times previously explored for MSO. For the best hydrolysis percentage (85.92%), the SDA content of unhydrolyzed TAG, 1,3-DAG, 2,3(1)-DAG, and MAG were 58.66 (65.71% at the *sn*-2 position), 71.22, 70.22 (52.90% at the *sn*-2 position), and 59.43%, respectively. The fatty acid profile of these acylglycerol products is shown in Table 5.4. For unhydrolyzed TAG, the major fatty acids present were SDA (~59%), GLA (~13%), ALA (~8%) and oleic acid (~5%); the SDA content at the *sn*-2 position was ~66%. 1,3-DAG was mostly comprised of SDA (~71%), GLA (~19%) and linoleic acid (~5%). SDA (~70%), GLA (~18%) and linoleic acid (~5%) were also the major constituents of 2,3(1)-DAG, which contained ~53% SDA at the *sn*-2 position. Finally, MAG was mostly comprised of SDA (~59%), GLA (~18%), palmitic (~5%) and linoleic acids (~5%).

As observed for MSO, CR lipase exhibited specificity towards oleic, linoleic and ALA. Comparing LTC-TAG to the MAG product achieved by hydrolysis at 12 h, the reduction in oleic, linoleic, and ALA were 74.52, 81.17, and 45.53%, respectively. However, unlike the observations on MSO substrate, CR lipase did not exhibit specificity toward palmitic acid in the hydrolysis of LTC-TAG at the conditions studied. This is

probably due to the overall low palmitic acid content of the substrate (2.10%). As in the hydrolysis of MSO, GLA and SDA contents were increased in the CR-lipase catalyzed hydrolysis of LTC-TAG, indicating the ability of this lipase to discriminate against both GLA and SDA under the conditions assayed.

CONCLUSIONS

We reported the ability of various lipases to hydrolyze MSO and LTC-TAG, as well as their specificity toward SDA, and their ability to concentrate this LCPUFA in the acylglycerol products. CC lipase exhibited specificity toward SDA, while CR lipase exhibited the capability to discriminate against it. Furthermore, CR lipase was able to discriminate against GLA from ALA, while showing specificity toward ALA, oleic, linoleic and palmitic acids. With this lipase, it was possible to obtain high percentages of hydrolysis of MSO and LTC-TAG (~66 and 86%, respectively), while increasing the SDA content of all acylglycerol products. Comparing the total SDA content of the acylglycerol products of the hydrolysis of MSO (4 h) and LTC-TAG (12 h) with that of original MSO, the process led to an increase in SDA of ~64% and ~151%, respectively. The reaction conditions needed to obtain these SDA-enriched products were mild (mild temperatures, no pressure changes, and no organic solvents were needed), therefore making this process desirable for industries seeking a green chemistry approach to omega-3 enriched products. In order to separate these reaction products for further applications in different products, low temperature crystallization (isolation of MAG), short-path distillation (removal of FFA), and further crystallization or solvent extraction (separation of unhydrolyzed TAG from DAG) are suggested for a scaled-up process.

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Table 5.1 Composition of Modified Soybean Oil and LTC-TAG

Modified Soybean Oil[a]

		Positional distribution	
Fatty acid	**Total (mol%)**[b]	***sn*-2 (mol%)**	***sn*-1,3**[c] **(mol%)**
Palmitic C16:0	12.71 ± 0.05	3.82 ± 3.07	17.16 ± 1.54
Stearic C18:0	4.30 ± 0.00	2.05 ± 1.57	5.42 ± 0.79
Oleic C18:1*c* (ω-9)	14.48 ± 0.05	14.78 ± 2.85	14.34 ± 1.43
Oleic C18:1*t*	1.62 ± 0.07	ND	2.43 ± 0.10
Linoleic C18:2 (ω-6)	23.69 ± 0.05	41.75 ± 1.06	14.66 ± 0.53
γ-Linolenic C18:3 (ω-6)	7.35 ± 0.01	8.28 ± 0.65	6.89 ± 0.33
α-Linolenic C18:3 (ω-3)	10.85 ± 0.01	6.29 ± 0.44	13.13 ± 0.22
Stearidonic C18:4 (ω-3)	25.00 ± 0.06	23.01 ± 0.42	25.99 ± 0.23

LTC-TAG[d]

		Positional distribution	
Fatty acid	**Total (mol %)**[b]	***sn*-2 (mol%)**	***sn*-1,3**[c] **(mol%)**
Palmitic C16:0	2.10 ± 0.05	0.49 ± 0.07	2.91 ± 0.08
Stearic C18:0	0.36 ± 0.01	0.19 ± 0.01	0.45 ± 0.02
Oleic C18:1*c* (ω-9)	7.07 ± 0.28	7.45 ± 0.03	6.88 ± 0.42
Oleic C18:1*t*	2.55 ± 0.20	ND	3.83 ± 0.30
Linoleic C18:2 (ω-6)	11.02 ± 2.28	24.23 ± 0.13	4.42 ± 3.42
γ-Linolenic C18:3 (ω-6)	12.36 ± 0.36	7.15 ± 0.09	14.97 ± 0.54
α-Linolenic C18:3 (ω-3)	15.82 ± 0.40	14.52 ± 0.14	16.47 ± 0.60
Stearidonic C18:4 (ω-3)	48.72 ± 1.13	45.98 ± 0.06	50.09 ± 1.70

All analysis were performed in triplicate and average values ± SD are reported
[a] Fatty acids found in trace amounts were: C14:0, C16:1, C17:0, C18:2 (trans), C18:4 (trans), C20:0, C20:1 (ω-9) and C22:0
[b] Mean ± SD, n = 3
[c] *sn*-1,3 (mol%) = [3 x total (mol%) - *sn*-2 (mol%)] / 2
[d] LTC-TAG: TAG obtained from low temperature crystallization of modified soybean oil (MSO)

Table 5.2 Hydrolytic Activity and Characteristics of Microbial Lipases Assessed

Enzyme	Source	Optimum Temperature (^{o}C)	Optimum pH	Specificity	Activity (U/g)[a]
Amano AY	*Candida rugosa*	30-50	7.0	non-specific	2400
Amano G	*Penicillium camembertii*	45	5.0	1,3 >>> 2-	3111
Lipomod™ 34P-L034P	*Candida cylindracea [rugosa]*	40-55	5.0-8.0	non-specific	4023
Lipomod™ 36P-L036P	*Rhizopus oryzae*	40	6.5-7.5	1,3 >>> 2-	2823
Lipozyme RM IM	*Rhizomucor miehei*	30-70	6.0-7.0	1,3 >>> 2-	2845

[a] Enzyme activity was determined on a triolein/ gum Arabic substrate. U/g refers to units of lipase activity per gram of substrate

Table 5.3 SDA Content of the Acylglycerol Products Obtained by CR Lipase-Catalyzed Hydrolysis of MSO[a] and LTC-TAG at Various Times

Time (h)	Hydrolysis (%)	SDA content (mol%)[b] MSO Substrate							
		TAG	***sn*-2**	***sn*-1,3[c]**	**1,3-DAG**	**2,3(1)-DAG**	***sn*-2**	***sn*-1,3[c]**	**MAG**
2	61.97	27.41 ± 1.68	46.91 ± 1.35	17.66 ± 2.61	39.89 ± 2.06	51.07 ± 1.59	24.15 ± 1.15	64.53 ± 2.45	40.98 ± 4.66
4	66.19	37.66 ± 0.36	56.37 ± 1.39	28.31 ± 0.88	41.64 ± 1.65	51.51 ± 2.95	54.92 ± 1.75	49.81 ± 4.51	49.89 ± 3.14
6	60.52	34.59 ± 1.79	43.03 ± 5.37	30.37 ± 3.80	35.88 ± 4.66	51.79 ± 3.31	31.27 ± 0.53	62.05 ± 4.57	51.03 ± 5.42
12	67.11	18.53 ± 0.27	27.88 ± 3.91	13.85 ± 1.20	41.76 ± 2.08	49.45 ± 4.24	53.50 ± 0.08	47.43 ± 6.36	35.59 ± 5.21

Time (h)	Hydrolysis (%)	SDA content (mol%)[b] LTC-TAG[d] substrate							
		TAG	***sn*-2**	***sn*-1,3**	**1,3-DAG**	**2,3(1)-DAG**	***sn*-2**	***sn*-1,3**	**MAG**
2	51.52	58.58 ± 0.53	62.94 ± 2.56	56.4 ± 1.51	70.68 ± 3.39	70.15 ± 1.43	58.96 ± 3.94	75.75 ± 2.91	69.06 ± 1.49
4	77.58	59.50 ± 1.32	58.46 ± 2.51	60.02 ± 2.34	62.64 ± 0.64	67.31 ± 0.15	33.59 ± 0.71	84.17 ± 0.42	47.33 ± 3.58
6	79.42	59.16 ± 0.98	58.88 ± 6.64	59.30 ± 3.63	68.72 ± 1.78	69.29 ± 0.63	52.81 ± 5.19	77.53 ± 2.76	61.65 ± 2.24
12	85.92	58.66 ± 1.05	65.71 ± 0.31	55.14 ± 1.58	71.22 ± 1.91	70.22 ± 2.72	52.90 ± 4.07	78.88 ± 4.56	59.43 ± 2.78

All analysis were performed in triplicate and average values ± SD are reported

[a] Fatty acids found in trace amounts were: C14:0, C16:1, C17:0, C18:2 (trans), C18:4 (trans), C20:0, C20:1 (ω-9) and C22:0

[b] Mean ± SD, n = 3

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

[d] LTC-TAG: TAG obtained from low temperature crystallization of modified soybean oil (MSO)

Table 5.4 Fatty Acid Profiles of Acylglycerol Products Obtained by CR Lipase-Catalyzed Hydrolysis of MSO (4 h) and LTC-TAG (12 h)

Modified Soybean Oil[a]

	Unhydrolyzed TAG			1,3-DAG	2,3(1)-DAG			MAG
		Positional distribution				Positional distribution		
Fatty acid	**Total (mol %)**[b]	***sn*-2 (mol%)**	***sn*-1,3 (mol%)**[c]	**Total (mol %)**[b]	**Total (mol %)**[b]	***sn*-2 (mol%)**	***sn*-1,3 (mol%)**[c]	**Total (mol %)**[b]
Palmitic C16:0	8.93 ± 0.21	7.06 ± 0.99	9.87 ± 0.59	9.42 ± 0.24	7.23 ± 1.13	10.08 ± 1.27	5.81 ± 1.81	7.99 ± 0.72
Stearic C18:0	3.30 ± 0.09	2.95 ± 0.02	3.48 ± 0.14	4.02 ± 0.16	2.66 ± 0.61	4.57 ± 0.45	1.71 ± 0.94	3.86 ± 0.14
Oleic C18:1*c* (ω-9)	11.25 ± 0.46	4.64 ± 0.04	14.56 ± 0.69	10.23 ± 0.50	7.12 ± 1.15	4.42 ± 0.68	8.47 ± 1.76	6.79 ± 1.29
Oleic C18:1*t*	0.92 ± 0.18	ND	1.38 ± 0.27	1.06 ± 0.07	ND	ND	ND	ND
Linoleic C18:2 (ω-6)	17.36 ± 0.05	4.86 ± 0.70	23.61 ± 0.36	13.60 ± 0.79	10.73 ± 0.48	3.74 ± 0.13	14.23 ± 0.72	8.37 ± 2.68
γ-Linolenic C18:3 (ω-6)	11.53 ± 0.22	22.57 ± 0.31	6.01 ± 0.36	14.92 ± 0.32	15.45 ± 0.33	0.73 ± 0.63	6.46 ± 0.39	19.24 ± 3.06
α-Linolenic C18:3 (ω-3)	9.04 ± 0.33	1.53 ± 0.02	12.80 ± 0.50	5.12 ± 0.32	4.55 ± 0.15	21.55 ± 0.67	12.40 ± 0.60	3.63 ± 1.59
Stearidonic C18:4 (ω-3)	37.66 ± 0.36	56.37 ± 1.39	28.31 ± 0.88	41.64 ± 1.65	51.51 ± 2.95	54.92 ± 1.75	49.81 ± 4.51	49.89 ± 3.14
LTC-TAG[d]								
Palmitic C16:0	1.26 ± 0.09	3.58 ± 1.00	0.10 ± 0.51	ND	0.84 ± 0.14	2.19 ± 0.30	0.17 ± 0.26	4.98 ± 1.10
Stearic C18:0	0.22 ± 0.30	0.65 ± 0.12	0.01 ± 0.45	ND	ND	ND	ND	3.04 ± 0.78
Oleic C18:1*c* (ω-9)	5.00 ± 0.29	2.31 ± 0.43	6.35 ± 0.49	2.17 ± 0.49	1.76 ± 0.34	3.81 ± 1.20	0.74 ± 0.79	3.69 ± 0.62
Oleic C18:1*t*	0.66 ± 0.01	ND	0.99 ± 0.02	ND	ND	ND	ND	ND
Linoleic C18:2 (ω-6)	11.26 ± 0.68	3.57 ±0.30	15.11 ± 1.03	4.75 ± 0.54	5.08 ± 0.71	8.61 ± 5.36	3.32 ± 2.88	4.46 ± 1.33
γ-Linolenic C18:3 (ω-6)	13.31 ± 2.42	23.15 ± 0.15	8.39 ± 3.64	18.59 ± 0.93	18.23 ± 1.42	18.27 ± 0.33	18.21 ± 2.14	18.49 ± 0.96
α-Linolenic C18:3 (ω-3)	7.84 ± 0.18	1.03 ± 1.46	11.25 ± 0.78	3.28 ± 0.05	3.88 ± 0.11	4.87 ± 0.53	3.39 ± 0.31	5.91 ± 1.16
Stearidonic C18:4 (ω-3)	58.66 ± 1.05	65.71 ± 0.31	55.14 ± 1.58	71.22 ± 1.91	70.22 ± 2.72	52.90 ± 4.07	78.88 ± 4.56	59.43 ± 2.78

All analysis were performed in triplicate and average values ± SD are reported

[a] Fatty acids found in trace amounts were: C14:0, C16:1, C17:0, C18:2 (trans), C18:4 (trans), C20:0, C20:1 (ω-9) and C22:0

[b] Mean ± SD, n = 3

[c] sn-1,3 (mol%) = [3 x total (mol%) - sn-2 (mol%)] / 2

[d] LTC-TAG: TAG obtained from low temperature crystallization of modified soybean oil (MSO)

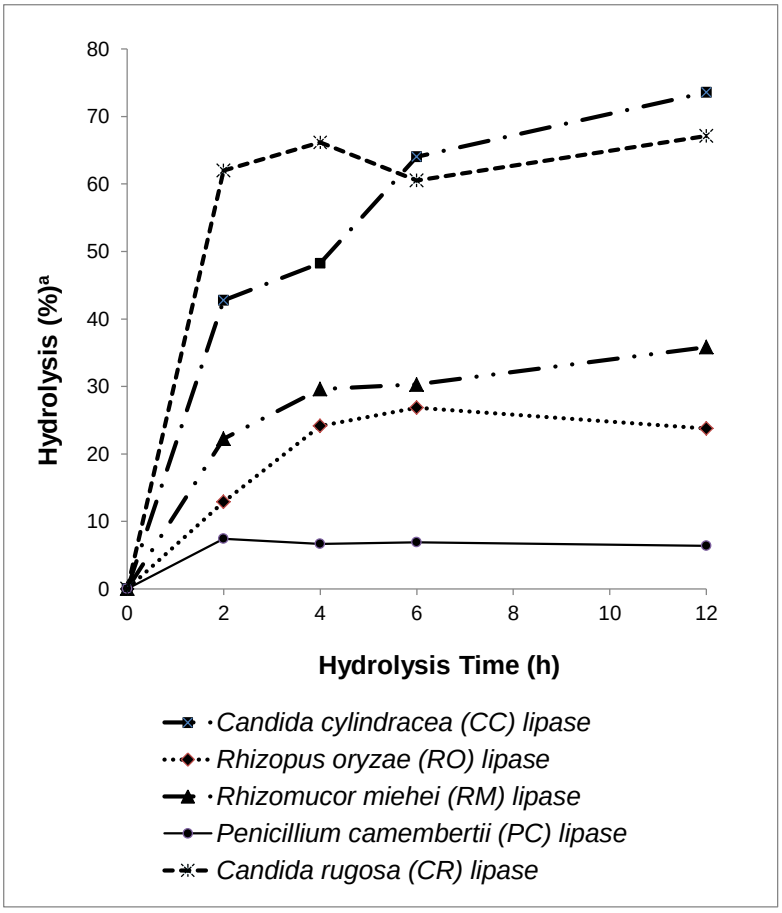

Figure 5.1 Time course for the hydrolysis of MSO by different lipases
[a] Experiments were performed in triplicate and all SD were below 5%

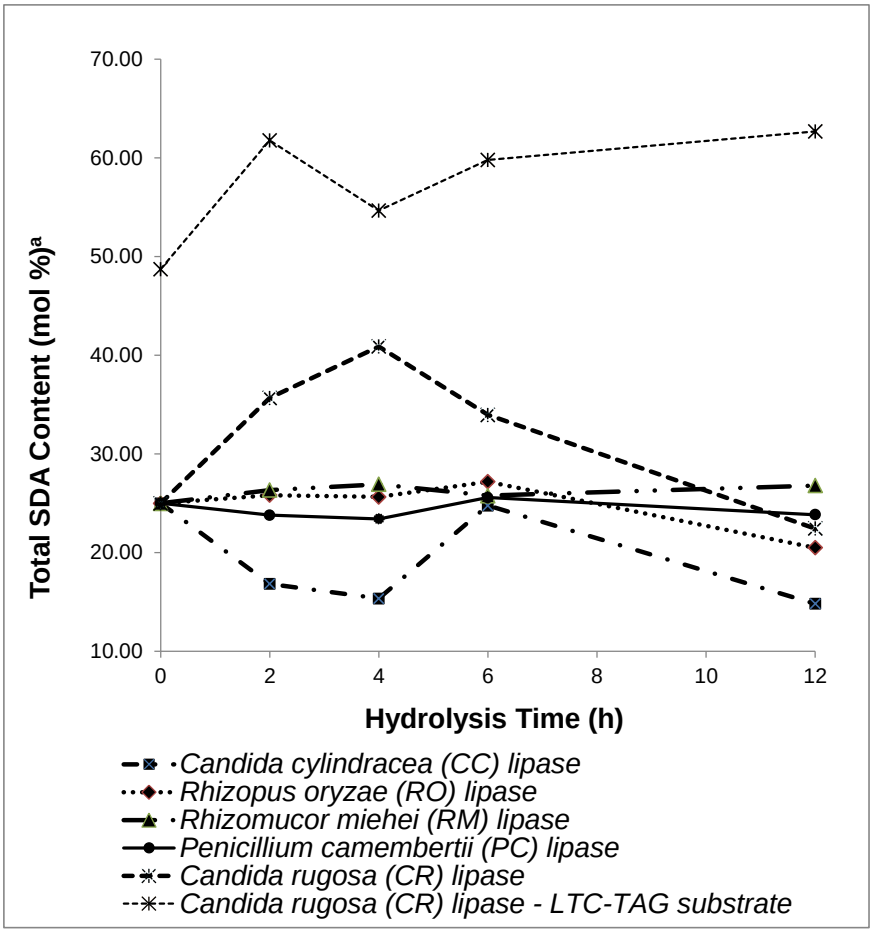

Figure 5.2 Time course showing SDA content during the hydrolysis of MSO by various lipases, and LTC-TAG by *Candida rugosa* lipase

[a] Experiments were performed in triplicate and all SD were below 5%

CHAPTER 6

CONCLUSIONS

From fatty acid ethyl esters (FAEE) derived from modified soybean oil (MSO) containing ~23% stearidonic acid (SDA), a fraction containing ~96% SDA-EE (77% yield) was succesfully isolated by argentation silica gel column chromatography. In a scaled-up process, the same method was used to isolate 840 mg of 97% SDA-EE (71% yield). The conditions that led to the recovery of SDA-EE of high purity (> 95%) and yield (>70%) were a stationary phase comprized of silica (10% $AgNO_3$) and a mobile phase composed of hexane and hexane:acetone mixtures (99 and 95%). Therefore, a method has been developed to succesfully isolate SDA in the ethyl ester form.

Since triagylglycerols (TAGs) are better absorbed than FAEE, it was of interest to also develop a method to produce structured lipids (SLs) enriched in SDA. Low temperature crystallization of MSO containing ~25% SDA was performed. The TAG and FFA crystallization products (LTC-TAG and LTC-FFA, respectively) had SDA contents of 48.72 and 60.78%, respectively. The enzymatic acidolysis between MSO and LTC-FFA was studied with Novozym 435 and Lipozyme TL IM as biocatalysts. Substrate molar ratio, incubation time, solvent, and enzyme load were explored. At best conditions, a SL with SDA contents of 37.61 ± 1.00% (20.86 ± 6.48% at *sn*-2 position) was produced by catalyzing the reaction with Lipozyme TL IM. These conditions were also applied to the acidolysis of LTC-TAG and LTC-FFA, to produce a SL containing 53.46 ± 1.85% SDA (36.37 ± 3.14% at *sn*-2 position). These SLs were synthesized under solvent-free

conditions. This is of importance due to the toxicity risk associated with the use of solvents, and the cost associated with their removal. In addition to having developed a lower cost process for the production of SDA enriched SLs, the SLs synthesized have high contents of SDA located at the *sn*-2 position, in which they are most nutritionally favorable. Therefore, these lipids could have many applications as healthier oils and/or nutraceutical components.

In a further attempt to concentrate SDA in the acylglycerol form, four free lipases and one immobilized lipase were assessed at various incubation times for their ability to hydrolyze MSO. The lipases were also assessed for their specificity toward SDA. The SDA enriched hydrolysis products contained TAG, diacylglycerols (DAG) and monoacylglycerols (MAG). TAG have applications as oils and nutraceutical components, DAG have applications as novel oils and as emulsifiers, and MAG are commonly used in food and personal care applications, as emulsifiers. Of the lipases studied, Amano AY lipase at 4 h incubation (66.19% hydrolysis) gave the best total SDA content (40.90 mol %). The SDA content of unhydrolyzed TAG, 1,3-DAG, 2,3(1)-DAG, and MAG were 37.66 (56.37 at the *sn*-2 position), 41.64, 51.51 (54.92 at the *sn*-2 position), and 49.89 % SDA, respectively.

Amano AY lipase was also used to hydrolyze previously SDA-enriched TAG (48.72 % SDA) obtained from low temperature crystallization of MSO. The best total SDA content (62.69 mol %) was obtained at 12 h incubation (85.92% hydrolysis). The SDA contents of unhydrolyzed TAG, 1,3-DAG, 2,3(1)-DAG, and MAG were 58.66 (65.71 at the *sn*-2 position), 71.22, 70.22 (52.90 at the *sn*-2 position), and 59.43%, respectively. In addition to having concentrated SDA in the acylglycerol form, this study

found an enzyme that showed specificity toward SDA (lipase 34P-L034P), and one that exhibited the ability to discriminate against SDA (lipase AY). This is of importance for future experimental design for the lipase-catalyzed synthesis of SDA-enriched lipids, since the type of enzymatic reactions performed depends on the specificity of the lipase used.

Many different avenues could be explored based on the work described above. The FAEE separated by argentation silica gel column chromatography could find applications as pharmaceutical grade fish oil, or could be used as a substrate to further enrich other oils with SDA. As to the SLs produced, it would be of interest to scale-up the production and test the stability of the oil and its physical characteristics. Studies on iodine value, melting and crystallization profiles, tocopherol content, and oxidative stability would be valuable to characterize the SL synthesized. It would also be of interest to perform sensory analysis on this oil (fried and baked products), to increase possible applications. Furthermore, comparing the shelf life of a formulated product containing this SL with that of the same formulated product containing other commercially available oils, would provide valuable information on the applicability of this oil to diverse food products by the food industry. Finally, nutritional and health realted studies would be essential to commercialize this SL.

Regarding the products of the lipase-catalyzed hydrolysis of MSO, these can be scaled-up and separated for further use as oils and/ or emulsifiers. The separation can be performed by low-temperature crystallization to isolate MAG, short-path destillation for the removal of FFA, and solvent extraction for the separation of DAG from TAG. The individual acylglycerol forms can be further characterized and studies of the

emulsification properties of MAG and DAG performed. In addition, the reaction products could be used as starting substrates for further enzymatic modification to produce desirable products. These SLs would have high contents of SDA and could be designed to have a variety of functional or nutraceutical applications.