LIPASE-CATALYZED ACIDOLYSIS OF ALGAL OILS WITH A MEDIUM-CHAIN FATTY ACID, CAPRIC ACID

FAYEZ HAMAM

Your file Votre référence
ISBN: 0-612-89629-3
Our file Notre référence
ISBN: 0-612-89629-3

Lipase-catalyzed acidolysis of algal oils with a medium-chain fatty acid, capric acid

BY

FAYEZ HAMAM

A thesis submitted to the School of Graduate Studies

In partial fulfillment of the requirements for the degree of the Master of Science

Department of Biochemistry

Memorial University of Newfoundland

2003

St. John's Newfoundland and Labrador Canada

ABSTRACT

Lipase-assisted acidolysis of algal oils with a medium-chain fatty acid (capric acid) was studied. Five commercially available lipase preparations from *Candida antarctica, Mucor miehei, Pseudomonas sp., Aspergillus niger* and *Candida rugosa* were initially used as biocatalysts for the incorporation of capric acid (CA) into selected algal oils. The algal oils of interest were arachidoinc acid single cell oil (ARASCO), docosahexaenoic acid single cell oil (DHASCO) and single cell oil rich in docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) (OMEGA-GOLD). Among the lipases examined, *Pseudomonas sp.* was the most efficient.

Effects of various reaction variables, namely the mole ratio of substrates, enzyme amount, time course, temperature and the amount of added water were examined for *Pseudomonas sp.* Response surface methodology was used to obtain a maximum incorporation of CA into algal oils. The process variables studied were the amount of enzyme (2-12 %), reaction temperature (25-55°C) and incubation time (12-48h). All experiments were conducted according to a face-centred cube design. Under optimum conditions (12.3 % of enzyme; 45°C; 29.4 h), the incorporation of CA was 20.0 % into ARASCO. Optimization of acidolysis of DHASCO with CA gave rise to a maximum of 22.6 % at 4.2 % enzyme amount, and a reaction temperature of 43.3°C and reaction time of 27.1 h. Similarly, the maximum incorporation of CA into the OMEGA-GOLD oil was obtained when enzyme amounts, reaction temperature and time were 2.5 %, 46.6°C and 25.2 h, respectively.

Stereospecific analysis was performed to establish positional distribution of fatty acids on the glycerol backbone of modified ARASCO, DHASCO and the OMEGA-GOLD

oil. In all oils examined CA was mainly located at the sn-1 and sn-3 positions of the triacylglycerol (TAG) molecules. In ARASCO-based SL, arachidonic acid (ARA) was mostly esterified at the sn-2 position of the glycerol backbone. In DHASCO-based SL, DHA was concentrated in the sn-2 position, but also present in the sn-1,3 positions. DHA or DPA were preferentially esterified at the sn-2 position of the modified OMEGA-GOLD oil.

The oxidative stabilities of enzymatically modified oils as well as their unmodified counterparts were assessed under Schaal oven conditions at 60°C over a 72 h storage period. Conjugated dienes (CD), and 2-thiobarbituric acid reactive substances (TBARS) were used to follow the primary and secondary products of oxidation, respectively. Among the oils tested, the structured lipids (SL) of ARASCO, DHASCO, and the OMEGA-GOLD oil had higher conjugated dienes than those of their unmodified counterparts. TBARS values of modified DHASCO were significantly higher than their origin oil at all times. TBARS values of both modified and unmodified ARASCO increased gradually over the entire storage period. TBARS values of the OMEGA-GOLD-based SL increased steadily with increasing storage time. The results presented in this study suggest that enzymatically modified oils are more susceptible to oxidation than their unmodified counterparts. However, when the three oils (ARASCO, DHASCO and the OMEGA-GOLD) were subjected to the same procedure steps, the stabilities of the oils were adversely affected to a large extent. This observation might possibly be due to the loss of natural antioxidants during the reaction work up.

ACKNOWLEDGEMENTS

I am grateful to Dr. Fereidoon Shahidi for providing me with financial support to conduct graduate studies under his supervision. I greatly appreciate his valuable suggestions and encouragement throughout the course of this study. I wish to convey my thanks to Drs. R. Hoover and A.M Martin for serving as supervisory committee members and also their constructive suggestions. Thanks are also extended to the School of Graduate Studies and the Department of Biochemistry, Memorial University of Newfoundland for providing me with a graduate fellowship during the course of my M.Sc. studies. Thanks are conveyed to all fellows in Dr. Shahidi's lab for their assistance and helpful suggestions, particularly Mr. Ahmad Khan. My sincere appreciation is conveyed to my loving parents, sisters, brothers, and parents-in-law in my homeland. My thanks are extended to my best friends in St. John's. I also gratefully appreciate my loving Reem for her patience, suggestions, and encouragements throughout this programme.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ARA	- Arachidonic acid
ARASCO	- Arachidonic acid single cell oil
AOCS	- American Oil Chemists' Society
ALA	- α-linolenic acid
CA	- Capric acid
CD	- Conjugated dienes
DHA	- Docosahexaenoic acid
DHASCO	- Docosahexaenoic acid single cell oil
DPA	- Docosapentaenoic acid
EPA	- Eicosapentaenoic acid
FAMEs	- Fatty acid methyl esters
FFA	- Free fatty acids
GC	- Gas chromatography
GLA	-γ-linolenic acid
LA	- Linoleic acid
LCFA	- Long-chain fatty acids
LCT	- Long-chain triacylglycerols
MAG	- Monoacylglycerols
MCFA	- Medium-chain fatty acids
MCT	- Medium-chain triacylglycerols
SD	- Standard deviation
SL	- Structured lipids

TAG - Triacylglycerols

TBA - Thiobarbituric acid

TBARS - Thiobarbituric acid reactive substances

CHAPTER 1

INTRODUCTION

Structured lipids (SL) are triacylglycerols modified to change the fatty acid composition and /or their location in the glycerol backbone via chemical or enzymatic means (Lee and Akoh, 1998). Recently, structured lipids have attracted much attention due to their potential biological functions and nutritional perspectives, including reduction in serum triacylglycerol (TAG), low-density lipoprotein (LDL) cholesterol and total cholesterol (Ikeda *et al.*, 1991), improvement of immune function, protection against thrombosis (Kennedy, 1991), reduction of protein breakdown (Babayan, 1987; DeMichele, 1988), improvement of absorption of other fats (Ikeda *et al.*, 1991), reduction of calories, preservation of reticuloendothelial system function (Sandstorm *et al.*, 1993), as well as improvement of nitrogen balance (Akoh, 1995), and reduction of risk of cancer (Crosby *et al.*, 1990; Ling *et al.*, 1991).

Strategies for lipid modification include genetic engineering of oilseed crops, production of concentrated oil containing high polyunsaturated fatty acids, and lipase-assisted or chemically-assisted interesterification reactions (Willis and Maragoni, 1999). Lipase-catalyzed interesterification offers many advantages over chemically-assisted interesterification. It produces fats or oils with a defined structure because it incorporates a specific fatty acid at a specific position of the glycerol moiety. It requires mild experimental conditions without potential for side reactions, reduction of energy consumption, reduced heat damage to reactants, and easy purification of products (Akoh, 1997; and Gandi, 1997). These structured lipids may be produced via direct esterification, acidolysis, alcoholysis or interesterification reactions. However, the

ordinary methods cited in the literature for production of SL are based on reactions between two triacylglycerol molecules (interesterification) or between a triacylglycerol and an acid (acidolysis).

Docosahexaenoic acid single cell oil (DHASCO) is a concentrated source of DHA, which is derived from marine microalgae known as *Crypthecodinium cohnii* (Arterburn *et al.*, 2000). DHASCO contains approximately 40 % (w/w) DHA, but no eicosapentaenoic acid (EPA) or other long-chain polyunsaturated fatty acids (LCPUFA) (Arterburn *et al.*, 2000). DHASCO represents the first concentrated well-defined, vegetarian source of DHA for infant formulas and food supplementaion. DHA and EPA belonging to the ω-3 family have many health benefits related to cardiovascular disease, immune and renal disorders, allergies, diabetes, inflammation, and cancer (Haumann, 1997b; Illingworth, 1990). DHA is also a physiological-crucial nutrient for early human development and functioning, including normal brain function and retina development (Holub, 2001).

Arachidonic acid single cell oil (ARASCO) is a concentrated source of arachidonic acid (ARA), in the amount of 40-50 % and only small amounts of other long-chain PUFA. ARASCO is prepared from the microfungus *Morticrella alpina* (Arterburn *et al.*, 2000b).

OMEGA-GOLD oil is a commercial name for an oil derived from microalgae *Schizochytrium sp.* via a fermentation process. The OMEGA-GOLD oil is a rich source of DHA (41 %) and DPA (docosapentaenoic acid, ω-6, 18 %). Myristic acid (9%) and palmitic acid (22%) are the other major fatty acids in the OMEGA-GOLD oil (Zeller *et al.*, 2001).

Medium-chain fatty acids (MCFA) are saturated fatty acids with a carbon chain length ranging from 6 to 12 and are prepared mostly from tropical fruits oils, such as coconut and palm kernel oils (Akoh, 1995; 1997). Medium chain triacylglycerols (MCT) exhibit unique structural and physiological features; they are easily absorbed and metabolised as quickly as glucose. MCT are known to rapidly clear from the blood (Babayan, 1987). Structured lipids containing medium-chain fatty acids (MCFA) and long-chain fatty acids (LCFA) have modified absorption rates because MCFA are quickly oxidized for energy and LCFA are oxidized very slowly. These structured lipids are structurally and metabolically different from simple physical mixtures of medium-chain triacylglycerols (MCT) and long-chain triacylglycerols (LCT).

In order to examine the oxidative stability of modified and unmodified ARASCO, DHASCO, and the OMEGA-GOLD oil, a number of stability tests are usually employed. These tests include chemical and instrumental techniques (Rossell, 1991; Shahidi and Wanasundra, 1998). The oxidative stability tests detect either the primary or secondary products of lipid oxidation. The conjugated dienes test is a simple and rapid method to evaluate primary products of lipid oxidation (hydroperoxide), whereas the 2-thiobarbituric acid (TBA) test is used to examine secondary oxidation products of lipid oxidation.

Many researchers have successfully incorporated MCFA (caprylic or capric acids) into fish oil containing PUFA via lipase-assisted acidolysis (Akoh and Moussata, 2001; Jenning and Akoh, 1999; Kawashima *et al.*, 2001,2002; Senanyake and Shahidi, 2002; Shimada *et al.*, 1996) and into borage oil rich in γ-linolenic acid (Akoh and Moussata, 1998; Shimada *et al.*, 1999; Kawashima *et al.*, 2002).

So far little attention has been paid to incorporate capric acid (10:0) into triacylglycerols rich in polyunsaturated fatty acids (PUFA) such as ARASCO, DHASCO, and the OMEGA-GOLD oil. The objectives of this study were: (1) to incorporate medium-chain fatty acids (capric acid), as a rapid source of energy, into glycerol backbones rich in PUFA, such as ARASCO, DHASCO, and the OMEGA-GOLD oil; (2) to optimise the reaction conditions for ARASCO-, DHASCO-, and the OMEGA-GOLD-based SLs;(3) to determine the positional distribution of fatty acids in the enzymatically-modified ARASCO, DHASCO, and the OMEGA-GOLD oil; and (4) to evaluate the oxidative stability of SL produced via acidolysis of ARASCO, DHASCO, and the OMEGA-GOLD oils with capric acid (10:0).

CHAPTER 2

LITERATURE REVIEW

2.1.1 Structured lipids-definitions

The concept of structured lipid (SL) for nutritional and medical uses in patients was first introduced in 1987 by Babayan. Lee and Akoh (1998) defined SL as triacylglycerols (TAG) modified to alter the fatty acid composition and/or their location in the glycerol backbone via chemical or enzymatic means. SL are triacylglycerols in which the glycerol moiety is esterified to a mixture of short-, medium-, and long-chain fatty acids for functional purposes (Haumann, 1997). SL are sometimes defined as "nutraceutical" lipids (Scott and Lee, 1996) and may be produced by lipase-assisted transesterification reactions to incorporate target fatty acids into the glycerol backbone (Ikeda *et al.*, 1991; Jensen *et al.*, 1994).

A broad definition of SL includes fats or oils that have been modified to change their fatty acid compositions and/or the positions of fatty acids from their original state (Akoh, 1998). Lipids can be restructured to meet essential fatty acid requirements or to incorporate specific fatty acids of interest (Akoh, 1997). SL may offer the most efficient means of delivering target fatty acids for nutritive or therapeutic purposes as well as targeting specific disease and metabolic conditions (Lee and Akoh, 1998). Structured lipids can also be produced to modify or change the physical and/or chemical features of TAG, including melting point, iodine and saponification values (Babayan, 1987).

2.2 Structured lipids applications

Structured lipids provide the most efficient means of delivering desired fatty acids for nutritional and medical uses (Table 2.1), including reduction in serum

Table 2.1 Potential benefits of structured lipids

Benefits of structured lipids	Reference
Improvement of immune function	Akoh *et al.*(1997), Haw *et al.* (1991), Haumann (1997)
Prevention of thrombosis	Gottenbos (1988)
Reduction risk of cancer	Akoh (1995), Cohen and Thompson (1987), Cohen *et al.* (1984), Ling *et al.* (1991), Mendez *et al.* (1992)
Reduced calorie lipid	Artz and Hansen (1996), Heydinger and Nakhasi (1996), Babayan *et al.* (1990)
Improved absorption of other fats	Ikeda *et al.*(1991), Jandacek *et al.* (1987), Sandstorm *et al.*(1993), Mckenna *et al.* (1985), Mascioli *et al.* (1987)
Reduction in serum triacylglycerols, LDL-cholesterol and cholesterol	Ikeda *et al.*(1991), Jandacek *et al.* (1987), Lee and Akoh (1997)
Improvement of nitrogen balance	Akoh (19950, Mascioli *et al.* (1987), Daly *et al.*(1991), Gollar *et al.* (1992)
Preservation of reticuloendothelial system	Mascioli *et al.* (1987), Haw *et al.* (1991)
Lipid emulsion for enteral and parenteral feeding	Jensen and Jensen (1992), Mascioli and Babayan (1988)

triacylglycerol (TAG), low-density lipoprotein (LDL) cholesterol and total cholesterol (Ikeda *et al.*, 1991), improvement of immune function (Akoh *et al.*, 1997; Haumann, 1997), protection against thrombosis (Kennedy, 1991), reduction of protein breakdown (Babayan, 1987; DeMichele, 1988), improvement of absorption of other fats (Ikeda *et al.*, 1991), reduction of calories, preservation of reticuloendothelial system function (Mascioli *et al.*, 1988; Sandstorm *et al.*, 1993), improvement of nitrogen balance (Akoh, 1995; Daly *et al.*, 1991; Mascioli *et al.*, 1988), and reduction of risk of cancer (Cohen and Thompson, 1987; Cohen *et al.*, 1984;Crosby *et al.*, 1990; Ling *et al.*, 1991).

2.2.1 Improvement of immune function

SL containing a mixture of medium-, and long-chain triacylglycerols will not block the reticuloendothelial macrophages and maintain their ability to phagocytose microorganism from the circulation, thus avoiding the immune suppressive effects of long-chain triacylglycerols (Haw *et al.*, 1991). The uptake of high amounts of foods rich in ω-6 PUFA such as linoleic acid, will cause an increase in the amount of arachidoinc acid, resulting in an imbalance of immune function through the production of proinflammatory lipids (Haw *et al.*, 1991). Akoh and colleagues (1997) have found that there was a higher ratio of T-helper cells to T-cytotoxic suppressor cells in female mice fed a modified oil containing both EPA and DHA and a medium-chain fatty acid (caprylic acid), as compared with mice fed soybean oil (control group). Their results indicated that there was increased reactivity in the immune system. Haumann (1997), using a SL containing EPA, demonstrated decreased inflammatory response and improved immune function in postsurgical cancer patients.

2.2.2 Reduction in serum TAG, LDL-cholesterol and total cholesterol

Ikeda *et al.* (1991) have reported the effects of different SL containing both medium-chain (caprylic or capric acid) and long-chain fatty acids on fatty acid and cholesterol absorption in lymph-cannulated rats. Cholesterol was absorbed to a large extent when SL was administrated, compared to the control group, a fat-free emulsion. The lymphatic absorption of linoleic acid (LA) in the case of 10:0/18:2/10:0 seemed to be higher than that from trilinolein (18:2/18:2/18:2) and 18:2/10:0/18:2. The absorption of capric and caprylic acids was considerably lower than linoleic acid (LA). Moreover, lymphatic absorption of caprylic acid was lower than that of LA, and was also lower than that of capric acid. Jandacek *et al.* (1987) showed that SL with caprylic acid at positions 1 and 3 and a long-chain fatty acid, such as linoleic or oleic acid, at position 2 can be rapidly hydrolysed when compared to that of typical long-chain fatty acid TAG in an animal model of pancreatic insufficiency. A SL containing caprylic acid and n-3 fatty acids such as EPA and DHA, was produced via enzymatic-assisted acidolysis. The results showed that the concentration of total cholesterol, LDL- cholesterol and triacylglycerol had declined considerably in the SL-fed as compared with those fed soybean oil (Lee and Akoh, 1997).

2.2.3 Improved absorption of other fats

Many researchers have reported that SL improves the absorption of other fats (Ikeda *et al.*, 1991; Jandacek *et al.*, 1987; Sandstorm *et al.*, 1993). Jandacek and his colleagues (1987) reported that TAG with caprylic acid in the sn-1 and sn-3 positions and LCFA in the sn-2 position are hydrolysed and absorbed more effectively than long-chain triacylglycerols (LCT). Sandstorm *et al.* (1993) described safety and tolerance for

structured triacylglycerol fat emulsion compared with a traditional LCT fat emulsion (intralipid 20%) when given to patients after a major surgery. There were no allergic reactions or any symptoms of nausea or chills associated with administration of structured triacylgylcerols. Sandstorm *et al.*(1993) concluded that structured triacylglycerols were safe, tolerated and appeared to be quickly metabolized when given to patients postoperative.

Jensen *et al.* (1994) compared the lymphatic absorption of enteraly fed modified TAG containing MCT and fish oil with their equivalent physical mixture in a canine model. It was also found that MCT can be absorbed through the lymphatic route if interesterified with LCT, providing a more effective means to deliver MCFA as a source of energy as well as deliver LCFA as a source of essential fatty acids. The amount of MCFA absorbed in the lymph at any given time during feeding of the animal was 2.6 times higher for SL than a physical mixture diet (Jensen *et al.*, 1994).

Mascioli *et al.* (1987) have developed a new lipid emulsion for enteral and parenteral use. Three groups of rats were fed 300 calories/day, with one-third of the non-protein calories as LCT, MCT or SL containing MCT. The results showed that SL containing MCT increased the heat production process compared with LCT. McKenna *et al.* (1984;1985) noticed improved absorption of linoleic acid in cystic fibrosis patients fed SL containing long-chain fatty acids and medium-chain fatty acids.

Jandacek *et al.* (1989) showed that a SL containing caprylic acid at the sn-1,3 positions and a long-chain fatty acid at the sn-2 position was more quickly hydrolysed and more effectively absorbed than a typical LCT. They suggested that SL may be

produced to give the most desired characteristics of MCFA and LCFA for use as nutrients in cases of pancreatic deficiency.

IMPACT is a medical product containing SL and is made via interesterification of high-lauric acid (12:0) and high-linoleic acid oil. IMPACT is produced by the Novartis Nutrition Company, based in Menneapolis, MN, and is used for patients suffering from trauma surgery, sepsis or cancer (Haumann, 1997). A SL containing MCFA and linoeic acid is more efficient in cystic fibrosis patients than safflower oil, which has about twice as much linoleic acid (McKenna *et al.*, 1985). The SL diet, IMPACT, containing low amounts of linoleic acid resulted in decreased infection and shorter stays at the hospital as compared to other enteral formulas. Bower and colleagues (1995) also showed a decreased length of hospital time as well as rate of infection when utilizing diets with a low level of linoeic acid and added fish oil.

2.2.4 Reduction of cancer risk

The SL containing ω3 fatty acids and MCFA produced via chemical hydrolysis and random interesterification of fish oil and MCT inhibited cancer growth in Yoshida sarcoma bearing rats (Akoh, 1995). Cohen and Thompson (1987) used a rat mammary cancer model to compare the cancer-stimulating effects of a high-fat diet containing a mixture of MCT and corn oil with the effects of a high-fat and a low-fat corn oil diet. Their results showed that a high-fat diet containing high levels of MCT did not stimulate the development of mammary cancers, whereas a high-fat diet containing the common LCFA did. In another study, Cohen and colleagues (1984) showed that a high fat diet containing high levels of MCFA did not stimulate the development of mammary cancer in rats compared with the diet containing LCFA. Rats were given diets in powdered

form and tap water *ad labitum* and were used to compare the cancer-stimulating impacts of diets containing low (5 %) and high (23 %) levels of corn oil to a high fat diet (23 %) containing MCT to corn oil in the ratio three to one. No difference in cancer multiplicity, tumor size or body weight increase was observed in any of the treatment groups.

Ling *et al.* (1991) produced a SL from fish oil and medium-chain TAG. They compared the SL produced with conventional LCT and found that it lowered cancer protein synthesis, and decreased tumor growth in Yoshida sarcoma-bearing rats. Mendez and colleagues (1992) showed that the rate of tumor proliferation in rats was decreased when they were fed SL containing fish oil and MCFA.

2.2.5 Improvement of nitrogen balance

The SL containing ω-3 fatty acids and MCFA produced via chemical hydrolysis and random esterification of fish oil and MCT caused better nitrogen balance in Yoshida sarcoma bearing rats (Akoh, 1995). Mascioli and colleagues (1987) reported the improvement of nitrogen balance in rats fed with SL containing MCT compared with those fed LCT. Daly *et al.* (1991) reported that structured lipids have a protein sparing impact, therefore enhancing the nitrogen balance. This led to a better weight increase and an improvement in a crucially ill individual, whereas MCT, LCT, or their physical mixtures were not as efficient as SL. Gollar *et al.* (1992) cited that protein-sparing action linked with SL administration were not observed when SL supplies fifty percent of protein calories and proposed that the protein-sparing action of SL may be dependent on the ratio of MCT to LCT used to produce the SL.

Mendez *et al.* (1992) compared the impact of SL containing fish oil and MCFA with a physical mixture of fish oil and MCT and found that SL enhanced the nitrogen balance in animals, possibly due to modification of absorption rates of SL.

2.2.6 Prevention of thrombosis

Thrombosis refers to the formation of blood clots. Blood clotting involves the clustering of platelets into large aggregates and is provoked when endothelial cells lining the artery walls are destroyed, leading to blocked vessels in the heart, brain and elsewhere in the body. Gottenbos (1988) reported that consumption of SL synthesized from fish oil resulted in a reduction of the arachidonic acid level (an excessive level of AA in platelets can cause blood clots in vessels), and can therefore help in preventing thrombosis.

2.2.7 Preservation of reticuloendothelial system (RES)

The reticuloendothelial system (RES) is responsible for the clearing of fat droplets as well as bacteria from the blood circulation. The ability of the macrophages of RES to phagocytose microorganism is decreased as they get filled with fat droplets. Mascioli *et al.* (1988) reported that MCT and SL did not interfere with the RES and thus protected its function. The use of medium-chain triacylglycerols and SL together provides means of caloric administration that will not block the macrophages or result in liver malfunction, therefore avoiding the suppresion impact on immune system by long-chain triacylglycerols alone (Haw *et al.*, 1991).

2.2.8 Calorie-reduced structured lipids

The production of low-calorie lipids, that are characterized by a mixture of SCFA and/or MCFA and LCFA in the same glycerol moiety, has recently attracted the attention of food manufacturers. Interest in such products stemmed from the fact that they contain

5-7 kCal/g energy compared to the 9 kCal/g for usual fats and oils because of the lower caloric content of SCFA or MCFA compared to LCFA. Reduced-calorie specialty lipids are intended for use in baking chips, dips, coatings, bakery and dairy products, or as cocoa butter replacer. Examples of commercially available low-calorie lipids containing medium-chain fatty acids include Caprenin® and Neobee®.

2.2.8.1 Caprenin

Caprenin is a commercially available reduced calorie SL, consisting of one molecule of very long-chain saturated fatty acid, behenic acid ($C_{22:0}$), and two molecules of medium-chain saturated fatty acids, caprylic acid ($C_{8:0}$) and capric acid ($C_{10:0}$). It provides 5 kCal/g and was initially produced by the Cincinnatti-based Procter and Gamble Company (Akoh, 2002).

Caprenin behaves similarly to cocoa butter and can be used as a cocoa butter substitute in selected sweet products. It is digested, absorbed, and metabolised by the same pathway as other TAG (Artz and Hansen, 1996). Caprenin is a liquid or semi-solid compound at room temperature, has a mild taste and is reasonably heat stable.

2.2.8.2 Neobee

Neobee consists of capric and caprylic acids and made by Stepan Company (Maywood, NJ). These structured lipids comprise different products: Neobee® 1053 and Neobee® M-5 contain both capric and caprylic acids, while Neobee® 1095 contains only capric acid (Heydinger and Nakhasi, 1996). Neobee® 1095 is a solid product, therefore, in applications requiring solid fats, this product may be appropriate. Neobee® 1814 is a MCT derivative produced by interesterification MCT with butter oil (Babayan *et al.*, 1990). This product contains half of the long-chain saturated fatty acid found in usual

butter oil and is suitable for replacing butter oil in a variety of applications. Neobee® 1814 may act as a flavour carrier and serves as a texture constituent for low-fat food products (Heydinger and Nakhasi, 1996).

2.3 Synthesis of structured lipids

Depending on the types of substrates available, chemical or enzymatic reactions can be used for synthesis of SL, including direct esterification (reaction of fatty acids and glycerol), acidolysis (transfer of acyl group between an acid and ester), and alcoholysis (exchange of alkoxy group between an alcohol and an ester) (Lee and Akoh, 1998).

2.3.1 Chemically-catalyzed interesterification

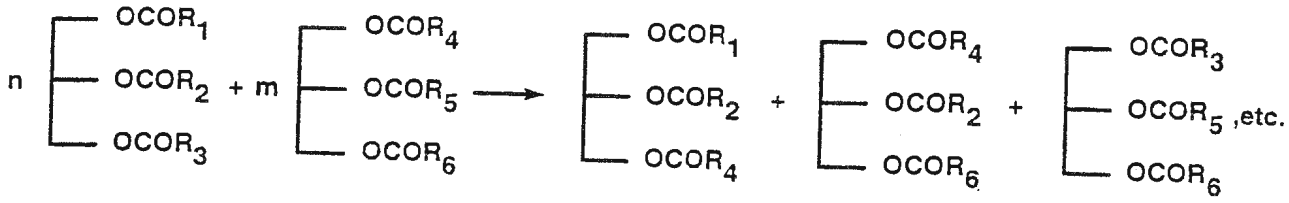
Chemically-catalyzed interesterification, using alkali such as sodium methoxide, is cheap and easy to scale up. However, the reaction lacks specificity and offers little or no control over the positional distribution of fatty acids in the final product (Willis and Marangoni, 1999). It is also carried out under harsh conditions, such as high temperatures (80-90°C) and produces side products which are difficult to eliminate. It requires long synthesis time and fails to produce specific products with desired physico-chemical features (Akoh, 1997).

Figure 2.1 Schematic diagram of lipase-assisted lipid modification strategies for the synthesis of structured lipids

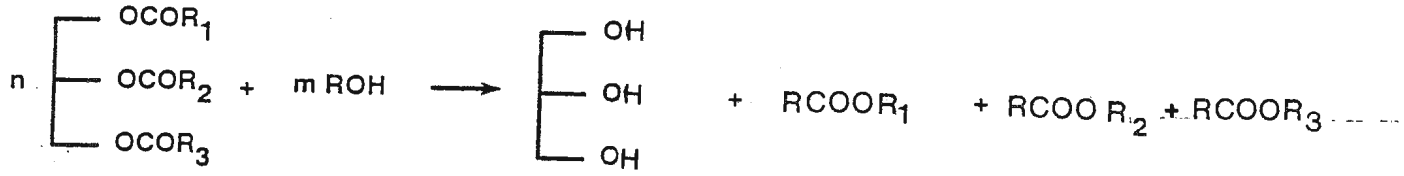
(i) Esterification

(ii) Transesterification

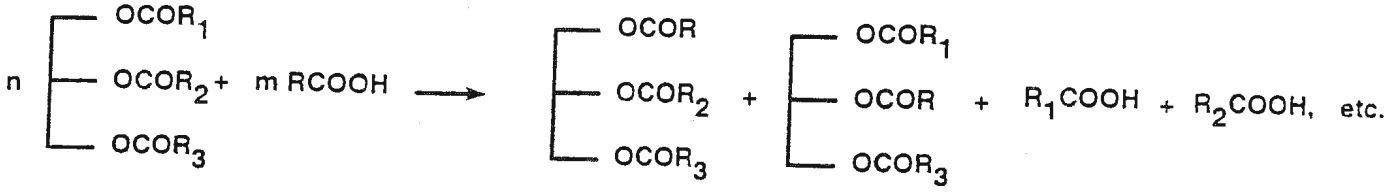
(a) Interesterification



(b) Alcoholysis



(c) Acidolysis



2.3.2 Enzymatically-catalyzed interesterification

An alternative to the chemical synthesis of structured lipids is the use of lipases. Potential advantages of lipase-assisted interesterification may be found in the enzymes regioselectivity, chemoselectivity, and fatty acid chain length specificity. Lipases can be used to design SL, on a case-by-case basis and to target specific food or medical uses (Akoh, 1995; 1997; 2002). It produces fats or oils with a defined structure because it incorporates desired fatty acids at a specific position. It adds value to fats or oils and enhances their functionality and desirable characteristics. It requires mild experimental conditions with little or no side compounds, and the whole process can be controlled. It reduces energy consumption and heat damage to reactants. The end products are easily recovered (Akoh, 1997).

2.4 Acidolysis

Acidolysis is referred to as the exchange of an acyl group between a fatty acid and an ester. Acidolysis is an efficient method for incorporating specific fatty acids into triacylglycerols to achieve desired functionality. SL are also produced via acidolysis in order to enhance or change the physical and/or chemical properties of TAG.

Oils from fish and other marine creatures can be restructured or altered to incorporate medium-chain fatty acids (MCFA) for certain desired nutritional characteristics. Structured lipids with PUFA residues at the *sn*-2 position and MCFA at the *sn*-1, 3 positions have a variety of applications in biomedical and nutraceutical fields. In this form, PUFA residues may be shielded from oxidation by the two saturated MCFA residues. They are also better absorbed in the intestinal tract as 2-monoacylglycerol (2-MAG) upon hydrolysis of the TAG by the pancreatic lipase. MCFA are absorbed

effectively and are a fast source of energy without being accumulated in the adipose tissues (Akoh, 2002).

2.4.1 Acidolysis of fish oils with MCFA

Incorporation of CA into the fish oil triacylglycerols (containing 40.9 % EPA and 33.0 % DHA) using immobilized lipase (IM 60) from *Rhizomucor miehei* as a biocatalyst via acidolysis was successfully carried out by Jennings and Akoh (1999). They found that there was an average of 43 % CA incorporation into fish oil, while EPA and DHA declined to 27.8 and 23.5 %, respectively. In another study, Akoh (2001) modified fish oil using immobilized sn-1,3 specific lipase (IM -60) from *Rhizomucor miehei* as the biocatalyst. The produced SL contained EPA (25.0 mol %), DHA (22.6 mol%), and 40.8 mol % CA. Nutritionists consider a level of 2-5 % of n-3 highly unsaturated fatty acids in SL as the maximum for enhanced immune function, reduced blood clotting, decreased serum TAG, and a lower risk of coronary heart disease (Kenndy, 1991).

Akoh and Moussata (2001) modified fish oil as well as canola oil with caprylic acid using lipozyme IM from *Rhizomucor miehei*. Their results showed that Lipozyme IM incorporated a higher level of caprylic acid (40.1 %) into canola oil than into fish oil (29.5 %). Moreover, incorporation of caprylic acid into fish oil did not change the total PUFA content of the original fish oil. Monoenes, particularly oleic acid (OA), were totally substituted by caprylic acid in fish oil, and their level was reduced from 61.9 to 34.7 % in canola oil.

Shimada *et al.* (1996) Reported that caprylic acid (8:0) was successfully incorporated into tuna oil containing DHA, via acidolysis using immobilized lipase from *Rhizomucor delemar*. This specific lipase catalysed the exchange of almost 65 % of the

fatty acids at the sn-1,3 positions with caprylic acid. However, DHA esterified at the sn-1 and sn-3 positions of tuna oil triacylglycerols (TAG) was exchanged due to the low activity of the *Rhizomucor delemar* on DHA. Senanayake and Shahidi (2002) used acidolysis to incorporate CA into seal blubber oil. They used immobilized lipase, Lipozyme-IM from *Mucor miehei* and found, after 24 h of incubation, the highest CA incorporation (25.4 %) into seal blubber oil at a temperature of 45°C and a 1% water level. Lipase-assisted interesterification between fish oil and medium-chain TAG (containing 60 mol % caprylic acid and 40 % mol capric acid) has been studied in a packed-bed reactor using a commercially immobilized lipase from *Thermomyces lanuginose* (Xu *et al.*, 2002). The effects of various parameters such as flow rate, temperature and lipase stability were investigated to show the promising features of using this biocatalyst. The results showed that the degree of reaction was significantly correlated to the flow rate and reached equilibrium at 30-40 min at a temperature of 60°C. Moreover, the results indicated that lipase was stable for at least 2 weeks without adjustment of water content. Fatty acids distribution analysis showed that PUFA, particularly EPA and DHA, remained in the sn-2 position in the enzymatically interesterified product compared to one that was produced chemically (Xu *et al.*, 2002).

Lee and Akoh (1998b) reported that the synthesis of SL containing n-3 (EPA and DHA) and medium-chain (caprylic) fatty acids using immobilized lipase (SP 435) from *Candida antarctica* via acidolysis of tricaprylin with n-3 fatty acids in a 1:2 mole ratio (tricaprylin / n-3 fatty acids) at 55°C in a bioreactor. Their results showed that 64.3 mol % of caprylic, 17.8 mol % of EPA, and 15.0 mol % of DHA were esterified to the sn-2 position of the TAG molecules of SL. In another study, tricaprin and trilinolein were

interestrified to produce two types of SL; the first, SL1, contained one linoleic acid and two capric acids and the second, SL2, contained two linoleic acids and one capric acid. Two immobilized lipase, IM 60 from *Rhizomucor miehei* and SP 435 from *Candida antarctica* were used as biocatalysts. The fatty acids at the sn-2 position with IM 60 lipase were 57.7 mol % capric acid and 42.3 mol % linoleic acid for SL1 and 43.3 mol % capric acid and 56.7 mol % linoleic acid for SL2. With SP 435 lipase 43.6 mol % capric acid and 56.4 mol % linoleic acid were observed at the sn-2 position for SL1 and 56.6 mol % capric acid and 43.4 mol % linoleic acid at the sn-2 position for SL2 (Lee and Akoh, 1997).

Zhou *et al.* (2000) examined six lipases for their ability to incorporate caprylic acid into fish oil containing EPA and DHA. Lipase AK from *Pseudomonas fluoresescens* catalysed the highest percent incorporation of caprylic acid into the fish oil. Furthermore, this enzyme had the highest activity toward the saturated and monounsaturated fatty acids in fish oil and much lower activity toward EPA and DHA.

Lipozyme IM catalyzed incorporation of caprylic acid, up to 70 %, in the sn-1,3 positions of the modified fish oil (Xu *et al.*, 1998). In another study, Xu *et al.* (2000) produced SL containing 40 % caprylic acid and 35 % EPA and DHA, with less than 3 % caprylic acid at the sn-2 position via acidolysis of menhaden oil with caprylic acid using Lipozyme IM in a solvent-free system. Shimada and colleagues (1996c) also found that 65 % of caprylic acid was located in the sn-1 and sn-3 positions of modified tuna oil upon modification with caprylic acid in a solvent-free medium.

2.4.2 Acidolysis of borage oil with MCFA

Akoh and Moussata (1998) used acidolysis to incorporate CA and EPA into borage oil using lipases from *Candida antarctica* and *Rhizomucor miehei.* The latter enzyme catalysed a higher incorporation of CA (26.3 %) and EPA (10.2 %) into borage oil, while *Candida antarctica* incorporated a lesser amount of CA (15.5 %) and EPA (8.8 %) into borage oil. In another study, Shimada *et al.* (1999) reported the production of a structured lipid containing GLA by continuous acidolysis of borage oil with caprylic acid using a 1,3-specific *Rhizopus delemar* biocatalyst in a solvent free system. The content of caprylic acid in the triacylglycerols was 50-55 mol %, when the mixture of borage oil and caprylic acid was fed at 30°C and a flow rate of 4.5 ml/h into a column packed with immobilized *Rhizopus delemar.* Furthermore, it was shown that caprylic acid in the transesterified borage oil was located only at the sn-1 and sn-3 positions.

Kawashima *et al.* (2000) produced structured TAG that was rich in caprylic acid at the sn-1,3 positions and γ-linolenic acid (GLA) at the sn-2 position. A two-step process comprising selective hydrolysis of borage oil (GLA content 45.4 w%) using *C. rugosa* and its acidolysis with caprlic acid using 5 % immobilized *Rhizomucor. oryzae* lipase. Acidolysis of borage oil with caprlic acid revealed that the contents of palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2) declined quickly and reached a plateau after 20-30 h at 30°C. Meanwhile, the content of GLA decreased progressively, along with a decrease in other fatty acids while that of caprylic acid increased.

2.4.3 Acidolysis of single-cell oils with MCFA

Iwasaki and colleagues (1999) reported lipase-assisted acidolysis of a single-cell oil (produced by a marine microorganism, *Schizochytrium sp.*) containing DHA (41 %)

and DPA (18 %) with caprylic acid. Two lipases from *Rhizomucor miehei* and *Pseudomonas sp.* were used as biocatalysts. The end products were modified oils containing caprylic acid in the sn-1,3 positions and DHA and DPA at the sn-2 position of the glycerol backbone. *Pseudomonas sp.* lipase catalysed the exchange of more than 60 % of fatty acids in the single-cell oil with caprylic acid, while *Rhizomucor miehei* lipase catalysed incorporation of 23 % of caprylic acid into the single-cell oil.

Sellappan and Akoh (2001) reported the synthesis of a modified oil via acidolysis of trilinolein with caprylic acid. Lipozyme IM-60 was used as a biocatalyst. The highest incorporation of caprylic acid (23.73 %) into trilinolein was achieved after 32 h of incubation at 55°C, with a mole ratio of 1:4 (trilinolein /caprylic acid), water content of 1 % (w/w), and an enzyme load of 10 % (w/w). Moreover, Lipozyme IM-60 was found to be more active toward long-chain fatty acids than medium-chain fatty acids. In another study, Huang and Akoh (1996) successfully produced a SL via transestrification of caprylic acid ethyl ester and triolein using eight lipases. Among the enzymes tested, immobilized lipase IM 60 from *Rhizomucor miehei* converted most of the triolein into SL dicaprylooleín (41.7 %) and monocaprylooleín (46.0 %). However, lipase SP 435 from *Candida antarctica* catalysed the conversion of triolein into dicaprylooleín (62.0%) and monocaprylooleín (33.5 %) at 55°C. Huang and Akoh (1996) proposed that the use of MCFA ethyl ester as an acyl donor had some benefits, indicating high rate of reaction, no inhibition at a high mole ratio and a high concentration of substrates.

Lee and Akoh (1998c) reported the synthesis of a SL by solvent-free enzymatic acidolysis of peanut oil (containing 58 % oleic acid) and caprylic acid in a stirred batch reactor. The reaction was conducted for 72 h at 50°C using lipase IM 60 from

Rhizomucor miehei. The highest incorporation of caprylic acid was reached at a substrate (peanut /caprylic acid) mole ratio of 1:2 after 72 h. Moreover, a high incorporation of caprylic acid (14.3 mol %) was achieved at a mixing speed of 640 rmp, indicating that good mixing is essential in a stirred batch reactor.

Kawashima *et al.* (2001) synthesized a high-purity SL with PUFA at the sn-2 position and caprylic acid at the sn-1,3 positions by a two-step enzymatic method. The first step involved the production of TAG by esterification of glycerol with PUFA using 5 % immobilized lipase from *Candida anatrctica.* The second step included changing TAG to the target SL by acidolysis with caprylic acid, using 7 % immobilized lipase from *Rhizopus delemar.* The caprylic acid content reached 66 mol % after 48 h at 30°C, whereas the content of dicapryloyl-eicosapentaenoyl-glycerol reached 86 mol % of triacylglycerols.

Kim *et al.* (2002) modified perilla oil (contains 60 % α-linolenic) with caprylic acid using two lipases; Lipozyme RM IM from *Rhizomucor miehei* and Lipozyme TL IM from *Thermomyces lanuginose* . After 24 h of incubation in n-hexane and a one to ten mole ratio of oil to caprylic acid, caprylic acid was incorporated at a level of 48.5 mol % with lipase from *Rhizomucor miehei* and 51.4 mol % with lipase from *Thermomyces lanuginose.* However, α-linolenic (ALA) content was reduced from 61.4 to 31.5 mol % with *R. miehei* and to 28.4 mol % with *T. lanuginose.*

When PUFA are present in the sn-2 position and MCFA are located in the sn-1 and sn-3 positions, MCFA are quickly hydrolysed by pancreatic lipase, absorbed and oxidized for energy, while essential fatty acids are absorbed as 2-monoacylglycerol. Therefore, modifying TAG with MCFA and PUFA via acidolysis may considerably

enhance the nutritional characteristics of TAG (Quinlan and Moore, 1993). McNeil and Sonnet (1995) have developed a calorie reduced SL via acidolysis of long-chain monoacylglycerols containing behenic acid with capric acid. Since the behenic acid is incompletely absorbed during digestion, the TAG produced contains about half the calories when compared to natural TAG.

2.5 Sources of fatty acids for structured lipid synthesis

The constituent fatty acids and their location in the glycerol backbone determine the functional and physical features, the metabolic fate, and the health benefits of the SL. Therefore, it is appropriate to go over the function and metabolism of the component fatty acids.

2.5.1 Saturated fatty acids

2.5.1.1 Short-chain fatty acids (SCFA)

Short-chain fatty acids (SCFA) range from C2:0 to C4:0 and include acetic (2:0), propionic (3:0) and butyric acids (4:0). They are the end products of carbohydrate fermentation in the human gastrointestinal tract (Wolin, 1980). SCFA are more quickly absorbed in the stomach than MCFA because of their higher solubility in water, smaller molecular size, and shorter chain length (Bezard and Bugaut, 1986) and provide fewer calories than MCFA or LCFA (acetic acid, 3.5 kCal; propionic acid, 5.0 kCal; butyric acid, 6.0 kCal; caproic acid, 7.5 kCal). SCFA are mostly located on the sn-3 position in the milk of cows, goats, and sheep (Brekenridge and Kuksis, 1967) and are likely to be totally degraded in the lumen of the stomach and small intestine.

In addressing nutritional implications, there has been a growing interest in the use of SCFA as an alternative or additional source of energy to the MCFA and LCFA

counterparts. SCFA are useful constituents in the production of low-calorie SL, such as Benefat (a brand name for Salatrium, contains C2:0-C4:0, and C18:0 and developed by Nabisco Food Group), because SCFA are lower in caloric value than MCFA and LCFA (Smith *et al.*, 1994).

2.5.1.2 Medium-chain fatty acids (MCFA)

Medium-chain fatty acids (MCFA) comprise 6-12 carbon saturated fatty acids that are obtained from hydroysis of tropical plant oils such as those of coconut and palm kernel (Akoh, 1995, 1997). MCT is a good source of MCFA for production of structured lipids. Pure MCT have a caloric value of 8.3 kCal /g and do not supply essential fatty acids (Heird *et al.*, 1986; Lee and Hastilow, 1999). MCFA are more hydrophilic than their long-chain fatty acid counterparts. Medium-chain triacylglycerols (MCT) were first synthesized by Babayan in 1950 from coconut-oil fatty acids released by steam hydrolysis (Babayan,1987). MCFA have many distinctive features such as high oxidative stability, low viscosity and low melting point (Kim *et al.*, 2002)

MCT exhibit unique structural and physiological characteristics; they are different from other fats and oils because they can be absorbed via the portal system without hydrolysis and reesterification because they are relatively soluble in water (Akoh, 1995). MCT do not require chylomicron formation to transfer from blood stream to the cells and have a more rapid β-oxidation to form acetyl CoA end products which are further oxidized to yield CO_2 in the Kreb's cycle (Lee and Akoh, 1998). Absorption and metabolism of MCT is as quick as glucose and have approximately twice the caloric concentration than proteins and carbohydrates. They have little affinity to accumulate as body fat. Medium-chain triacylglycerols are not dependent on carnitine (an enzyme

necessary for transport of fatty acids across the inner mitochondria membrane) to enter mitochondria. The higher solubility, and smaller molecular size of MCFA make their absorption, transport and metabolism much easier than long-chain fatty acids. Finally, MCT rapidly clear from the blood (Babayan, 1987). While MCFA are used to produce SL, they are also used as flavours, and carrier of dyes, pigments and vitamins. They are frequently used in the diet of patients with maldigestion and malabsorption disorders (Kennedy, 1991).

MCT are used together with LCT in order to offer a balanced nutrition in enteral and parenteral products (Roediger and Rae, 1982; Ulrich *et al.*, 1996). In numerous medical foods, a combination of MCT and LCT is used to offer both speedy and slowly metabolized sources of energy as well as essential fatty acids. Clinical nutritionists have taken advantage of MCT's simpler digestion to feed individuals who can not make use of LCT. Any irregularity in the many enzymes or processes implicated in the digestion of LCT can cause symptoms of fat malabsorption. Therefore, patients with certain diseases such as cystic fibrosis, Crohn's disease, colitis and enteritis, have shown progress when MCT is integrated in their food (Kennedy, 1991). On the other hand, pure MCT in high doses may be toxic, leading to the accumulation of ketone bodies (a condition known as metabolic acidosis or ketonemia) and they may suffer from lack of essential polyunsaturated fatty acids (Akoh, 1996).

From the previous discussion we can conclude that developing new fats and oils that combine MCFA as a rapid source of energy and LCT as a source of functional fatty acids is attractive in order to meet the nutritional requirement of patients and those with special food needs.

2.5.1.3 Long-chain fatty acids (LCFA)

Saturated fatty acids are generally believed to raise plasma and serum cholesterol levels, but fatty acids with chain length ranging from 4 to 10 do not increase cholesterol levels (Akoh, 2002). Stearic acid (18:0) has also been cited not to increase cholesterol levels in plasma (Bonanone and Grundy, 1988). Triacylglycerols containing high amounts of long chain saturated fatty acids, especially stearic acid, are poorly absorbed in the human body partly because stearic acid has a higher melting point than body temperature and they also display poor emulsion properties (Hashim and Babayan, 1978). The poor absorption of long chain saturated fatty acids makes them possible reactants for synthesis of low-calorie SL. For example, Nabisco Food Group used this feature of C18:0 to produce the group of low-calorie SL called Salatrium, which consist of SCFA and LCSFA, mainly stearic acid (Finley, 1994).

2.5.2 Polyunsaturated triacylglycerols

Most lipids consist of long-chain fatty acids (>C12) and are referred to as long-chain triacylglycerols (LCT). The bulk of our food uptake consists of TAG with LCFA. Lipids from diary, meat and vegetables serve as major sources of LCT. Long-chain triacylglycerols that are slowly absorbed via lymphatic system, depend on carnitine in their entry to mitochondria and tend to deposit as body fat because they are slowly oxidized to provide fuel and energy, they are slowly clearing from the blood. However, they provide essential fatty acids (Babayan, 1987). Algal oils provide a good source of long-chain polyunsaturated triacylglycerols. Among commercially available algal oils are arachidonic acid single cell oil (ARASCO), docosahexaenoic acid single cell oil (DHASCO), and a single cell oil rich in docosahexaenoic acid (DHA) and

docosapentaenoic acid (DPA) (OMEGA-GOLD). In this study, three long-chain triacylglycerols, namely ARASCO, DHASCO, and the OMEGA-GOLD were used for SL synthesis.

2.5.2.1 DHASCO

Fish and fish oils afforded a good source of DHA. However, fish reserves are limited and intensive aquaculture practices are producing fish with lesser amounts of DHA. DHASCO oil from microalgae has recently been produced by a single cell alga *Crypthecodinium cohnii* (a member of the Dinophyta, a phylum of unicellular eucaryotic microalgae); grow under strictly controlled fermentation conditions. DHASCO is the first concentrated, well-defined, vegetarian source of DHA for infant formulas and food fortification. DHASCO is a rich source of DHA, (40-50 % DHA), but contains no EPA or other long-chain polyunsaturated fatty acids (Arterburn *et al.*, 2000). DHASCO is yellow-orange oil with > 95 % triacylglycerols with some diacylglycerols and nonsaponifiable matter (about 1.5 % by weight). Myher *et al.* (1996) reported that about 45 % of the DHA found in DHASCO was located in the sn-2 position. Since, positional distribution of DHA in DHASCO triacylglycerols is almost the same as that of human milk DHA digestion and absorption from DHASCO should be the same as that from human milk.

2.5.2.1.1 Safety studies of DHASCO

DHASCO is a macronutrient, vitamin, or drug. It is non-toxic and of much interest for use in infant formulas. In the last ten years, many safety investigations have been carried out and these included *in vitro* assays of mutagenicity and genotoxicity, traditional rat studies, including acute, subchronic, development, and multigenerational

reproductive studies (Kyle, 2001). Arterburn *et al.* (2000a) reported that DHASCO and ARASCO oils did not have any genotoxic potential in three different *in vitro* mutagenesis assays (Ames reverse mutation, mouse lymphoma TK $^{+/-}$ forward mutation, and chromosomal aberration assays). In another study, Arterburn *et al.* (2000b) showed that DHASCO and ARASCO oils did not have any unfavourable developmental effects on pregnant rats. When DHASCO and ARASCO oils were administered orally at a dose of up to 1.25 and 2.50 g /kg body weight, respectively. Neither oil resulted in any fatal malformation such as soft tissues or skeletal variations.

To further establish the safety of DHASCO oil, a 90-day subchronic investigation was conducted in pregnant rats using doses of DHASCO oil of 0.50 and 1.25 g/kg body weight/day. The following parameters were investigated: haematology, clinical chemistry, pathology, and neurotoxicity. There were no unfavourable impacts on all parameters examined upon the administration of the test material. DHASCO oil did not lead to any mutation in developing fetus at doses that represented a 100-fold safety factor higher than expected use levels (Aretburn *et al.*, 2000c).

2.5.2.1.2 Clinical studies using a DHASCO

Many studies on feeding of DHASCO supplemented formulas to infants for various lengths of time have been reported. All of these studies showed that DHASCO-supplemented formulas increased infants DHA status to that of breast-fed infants (Zeller *et al.*, 2001). Two studies using DHASCO-supplemented formulas, which also included ARASCO oil, reported considerable improvements in the growth rates of the babies fed the supplemented formulas compared to regular formulas (Carlson *et al.*, 1999; Diersen

et al., 1999). When infants were fed DHASCO-supplemented formulas, there was a significant improvement in their visual and mental acuity (Birch *et al.*, 1998).

2.5.2.2 Arachidonic acid single-cell oil (ARASCO)

Arachidonic acid single-cell oil (ARASCO) is a concentrated source of arachidonic acid (40-50 %) and is produced by the microfungus *Mortierella alpina* using fermentation technology. ARASCO contains small levels of other polyunsaturated fatty acids. Oleic acid (14.0 %) and linoleic acid (7.1 %) are other major fatty acids present in ARASCO. Furthermore, it also contains saturated fatty acids such as stearic (10.5 %) and palmitic (7.8 %) acids (Arterburn *et al.*, 2000b).

2.5.2.3 Single-cell oil rich in DHA and DPA (OMEGA-GOLD)

OMEGA-GOLD oil is a commercial oil produced by the microalgae *Schizochytrium sp.* via a fermentation process. OMEGA-GOLD oil is a rich source of DHA (41 %) and DPA (docosapentaenoic acid, the omega-6 isomer), which accounts for 18 % of the crude oil fraction. Myristic acid (9 %) and Palmitic acid (22 %) are the other major fatty acids in OMEGA-GOLD crude oil fraction. The crude oil from algae *Schizochytrium sp* comprises triacylglycerols (90-92 %), polar lipids (5 %), minor amounts of fatty acyl sterol esters (0.4 %), diacylglycerols (1 %), free sterols (1 %) and free fatty acids (0.1 %) (Zeller *et al.*, 2001). ^{13}C NMR analysis indicated that 23 mol % of combined DPA and DHA were present in the sn-2 position and 27 mol % located in the sn-1 and sn-3 positions of the glycerol backbone of the OMEGA-GOLD oil (Zeller *et al.*, 2001).

2.6 Oxidative stability of oils containing highly unsaturated fatty acids

Lipid oxidation is a chemical reaction that occurs between fats or oils containing unsaturated fatty acids and molecular oxygen via a free radical chain mechanism that is mostly initiated by exposing fats and oils to light, heat, ionizing radiation, metal ions, or metalloprotein catalysts. The enzyme lipooxygenase can also initiate oxidation (Shahidi *et al.*, 1992). The autoxidation process consists of three steps of initiation, propagation and termination. Formation of a lipid radical ($R^{\bullet}$) is the key event in the initiation step. Lipid radicals are highly reactive and can quickly undergo propagation reactions. The propagation step normally starts with the addition of molecular oxygen to alkyl free radical ($R^{\bullet}$) to form an unstable peroxy free radical ($ROO^{\bullet}$), which may in turn abstract a hydrogen atom from another unsaturated fatty acid to form a hydroperoxide (ROOH) and a new alkyl free radical. The oxygenation reaction is very quick, having a reaction energy of zero (Shahidi *et al.*, 1992). The propagation step may be terminted by the formation of nonradical products resulting from the reaction of two radical species (Shahidi and Wanasundra, 1998).

The oxidation of lipids is one of the major changes that takes place during processing, distribution, and final preparation of food. Oxidation of fats and oils initiates other alterations in the food system that influences its nutritional quality, wholesomeness, flavour, colour, safety, and texture (Shahidi *et al.*, 1992). The oxidation of fats and oils containing unsaturated fatty acids has been of the most widely studied areas of research in nutrition and food science because it is linked to the deterioration of foods related to off-flavour development as well as nutrition, safety and storage of products (Arouma, 1998). Furthermore, oxidation of fats and oils via free radical reactions in the human

body may cause oxidative damage to cells, tissues and may in turn lead to various diseases and clinical disorders (Halliwell and Gutteridge, 1999). Products of lipid oxidation are involved in the disruption of biological membranes, inactivation of enzymes, damage to proteins, and formation of age pigments in damaged membranes (Halliwell and Gutteridge, 1999). Oxidized lipids have also been reported to cause health problems such as diarrhea, growth depression, and damage to tissues in living organisms (Chow, 1992).

Hydroperoxides (or primary products of oxidation of unsaturated fatty acids) are colourless, odourless and tasteless (King *et al.*, 1995). Hydroperoxides are very unstable and decompose into various secondary products. Hydroperoxides are decomposed via hemolytic cleavage of oxygen-oxygen bond to produce hydroxyl and alkoxy free radicals; the latter in turn decomposes by carbon-carbon cleavage to form aldehydes, ketones, alcohols and/or hydrocarbons, among others (Frankel, 1998). Secondary oxidation products are believed to be responsible for off-flavour, off-colour, and off-odour development in foods (Shahidi and Wanasundara, 1998).

2.6.1 Methodologies for assessing lipid oxidation

There are several methods for the measurement of fats and oils oxidation in foods. Changes in chemical, physical, or organoleptic characteristics of lipids during storage, processing and modification may be monitored to evaluate the degree of lipid oxidation. However, there is no standard method for identifying all oxidative changes in all food systems (Shahidi and Wanasundra, 1998). The available procedures to examine lipid oxidation in foods and biological systems may be categorized into two groups; the first

group determines primary oxidative changes and the second measures secondary changes that take place in each system.

2.6.1.1 Primary changes

2.6.1.1.1 Changes in reactants

Changes in substrates (unsaturated fatty acids) is a useful technique for identifying class of fats or oils and fatty acids that are involved in the oxidative change (Coxon, 1987; Gray and Monahan, 1992) and also for evaluating lipid oxidation provoked by different metal complexes which give different products (Gutterigde and Hallwelli, 1999). However, this method is only useful when highly unsaturated lipids are present.

2.6.1.1.2 Weight gain

It is widely accepted that formation of hydroperoxides during early stages of autoxidation can be quantified. Thus, the induction period can be determined from mass increase data. The weight gain method offers many advantages such as unlimited capacity, speedy sample processing, and low cost of equipment. Ke and Ackman (1976) used mass gain method to compare lipid oxidation from various parts of fish.

2.6.1.1.3 Peroxide value (PV)

This is one of the most commonly used methods for measuring oxidative stability of lipids in foods. It measures the amount of peroxides as an index of the degree of lipid oxidation. The hydroperoxides content can be quantified by means of an iodometric titration and referred to as PV. The basis of PV determination is the reduction of hydroperoxides (ROOH) with iodide (I^-). The resultant iodine is titrated against standardized solution of sodium thiosulphate ($Na_2S_2O_3$) (Gray, 1978). PV has many

disadvantages and a low PV may be a result of rapid breakdown of peroxides rather than slow formation. The structure and reactivity of peroxides and reaction time and temperature are factors that may affect PV. Moreover, this method fails to measure low PV due to problems encountered in identifying the titration end point. Another problem associated with PV is the need for careful sampling and handling.

2.6.1.1.4 Conjugated dienes (CD)

During oxidation of lipids containing polyunsaturated fatty acids there is an increase in the absorption of oils in the ultraviolet region. Lipids containing dienes or polyenes show a shift in the position of their double-bond position upon oxidation due to isomerisation and formation of conjugated hydroperoxides (Logani and Davies, 1980). The resulting conjugated dienes display maximum absorption at 234 nm, whereas conjugated trienes absorb at 268. Shahidi *et al.* (1994) and Wanasundara *et al.* (1995) have shown a good correlation between conjugated dienes and peroxide values (PV) during oxidation of edible oils. Therefore, CD and/or peroxide values (PV) may be used as an indicator of lipid stability under oxidation conditions. However, CD test suffer from a major drawback as coloured oils containing conjugated carotenoids afford high absorbance values at 234 nm which may interfere with such determinations (Shahidi and Wanasundara, 1998). Nontheless, this method uses a minimum amount of material and thus is used extensively in studies of lipid oxidation.

2.6.1.2 Secondary changes

Secondary products of lipid oxidation are generally responsible for undesirable odours associated with lipid oxidation, therefore measurement of these products as an index of lipid oxidation is more suitable, as far as off-odours are concerned, than

measurement of primary products which are colourless and odourless. However, it is essential to monitor both primary and secondary oxidation products in all oxidative stability studies. The following sections highlight common methods used for determining secondary products of lipid oxidation.

2.6.1.2.1 Thiobarbituric acid (TBA) test

2-Thiobarbituric acid reactive substances (TBARS) are determined by the TBA test and these provide the oldest and most widely used test for evaluating the extent of lipid oxidation in food and other biological systems. TBA test measures the total amount of aldehydes in the oxidized lipids. Malonaldehyde (MA) constitutes a relatively small portion of oxidation products of polyunsaturated fatty acids. During the TBA test one molecule of MA interacts with two molecules of TBA reagent to yield a pink coloured complex with an absorption maximum at 532 nm (Shahidi and Wanasundara, 1998).

TBA test has two major drawbacks as MA is only produced by fats possessing three or more double bonds (Benzie, 1996). The precision of TBA test has also been questioned because TBA reacts with other molecules such as sugars and oxidized proteins (Rossell, 1994).

2.6.1.2.2 Anisidine Value (p-AnV)

Anisidine value is defined as 100 times the absorbance of a solution resulting from the reaction of 1 g of lipid in 100 mL of a mixture of solvent and p-anisidine measured at 350 nm in a 1-cm cell (AOCS, 1990). The molar absorbance at 350 nm increases if the aldehyde contains a double bond, therefore this method determines the amount of aldehydes (particularly 2-alkenals and 2-4-alkadienals). This method is useful

for oils with low peroxide values such as frying oil. In general, for good oils the anisidine value should be less than 10 (Rossell, 1994).

2.6.1.2.3 Total oxidation (TOTOX) value

Total oxidation (TOTOX) value is referred to as 2 PV + p-AnV, while Wanasundara and Shahidi (1995) have defined TOTOX $_{TBA}$ as 2PV + TBA because determination of p-anisidine value may not be always possible. The TOTOX value is useful because of combining evidence about the past history of an oil as indicated in p-anisidine value with its current status as reflected in the PV. Thus, measurement of TOTOX value has been widely used to evaluate oxidative deterioration of food lipids (Rossell, 1993). However, TOTOX value dose not attract attention of the scientists because it combines variables with different of dimensions.

2.6.1.2.4 Headspace analysis of volatiles

The oil is heated at 40-60°C in closed vials. The volatiles, from decomposition of hydroperoxides and those present prior to heating are collected above the oil and then analyzed using gas chromatography. In this method, the total area under a peak of the volatiles is generally increased with the length of the storage period of an oil (Frankel, 1998). Headspace analysis of volatiles offers many advantages and is particularly appropriate for highly volatile compounds because they have a favourable equilibrium between the sample and its headspace. This method is quick and appropriate for routine analysis of several samples. Because only the volatile components are injected into the gas chromatography, headspace analysis of volatiles does not need any cleaning between sample injections (Frankel, 1998). This method suffers one major drawback, which is the

difficulty of reaching complete equilibrium with semi-solid and viscous samples as well as with polyunsaturated lipid samples that may readily oxidize during heating.

2.7 Optimisation procedure for production of SL via acidolysis

Response surface methodology (RSM) has been a popular and efficient statistical procedure for the study of complex processes. Hill and Hunter (1966) first reported the origin of RSM and its application in food research. RSM consists of a group of mathematical and statistical procedures that can be used to investigate correlations between one or more responses and a number of independent factors. RSM identifies the effect of the independent variables, alone or in combination, on the process. In addition to analysing the effects of independent variables, this experimental methodology produces a mathematical model that precisely describes the overall method.

RSM is an optimisation procedure that determines optimum process conditions by combining particular experimental designs with modelling using first or second order polynomial equations in a sequential testing procedure. RSM examines a number of variables at a time, uses special experimental designs to reduce the number of required determinations, and measures many effects by objective tests (Mason *et al.*, 1989).

The results of traditional one-variable-at-a-time do not indicate real changes in the environment as they disregard interactions among variables, which are in effect concomitant. More complicated designs, such as RSM, can describe simultaneous influences more fully and help in a more precise optimisation of variables that affect the process and permit concomitant solution of multivariate which specify the optimum product for a specific set of parameters through mathematical models (Mason *et al.*, 1989).

Enzyme amount and incubation time are main variables influencing the cost of preparation of SL. Moreover, reaction temperature and time can be considered significant as they affect the oxidative status of prepared oils as well as the economy of the process.

A face-centred cube design was employed (Figure 2.2) in this study; the actual levels of factors used in each experimental run are shown in Table 2.2. This design was selected over others, such as a rotatable design, because it uses only three levels of each variable whereas, other central composite designs would need five levels of each (Mason *et al.*, 1989). Having three levels instead of five is attractive because it decreases the time of preparation.

The production of SL via acidolysis of triolein and capric acid was reported by Shieh and colleagues (1995). Incubation time, amount of enzyme, and mole ratio were optimised using RSM. A total product of mixed mono-and dicaproolein of up to 100 % was obtained. In the meantime, Huang and Akoh (1996) reported SL production by transesterification of ethyl caprylate and soybean or sunflower oil. Under optimum conditions, 67.6 % caprylic acid was incorporated into TAG of sunflower oil.

The synthesis of SL from menhaden oil and caprylic acid in a packed bed reactor was reported by Xu *et al.* (2000) using response surface methodology. Effects of variables including residence time, mole ratio of substrates and reaction temperature were studied. The residence time was considered to be the main significant parameter. Under optimum conditions, the SL contained 38.8 % caprylic acid and 29.0 % EPA and DHA. RSM was also employed to optimise conditions for the incorporation of n-3 or n-6 PUFA into coconut oil TAG. Under optimum conditions, a maximum incorporation (13.65 %) of n-3 PUFA was predicted at a 1:4 mole ratio of TAG to FFA at 54^0C over 34 h. Similarly, maximum

incorporation (45.5 %) of n-6 PUFA was observed at a 1:3 mole ratio of TAG to n-3 PUFA at 39^0C for 48.5 h (Rao *et al.*, 2002).

Figure 2.2 Graphical representation of the face-centred cube design (adapted from Mason *et al.*, 1989)

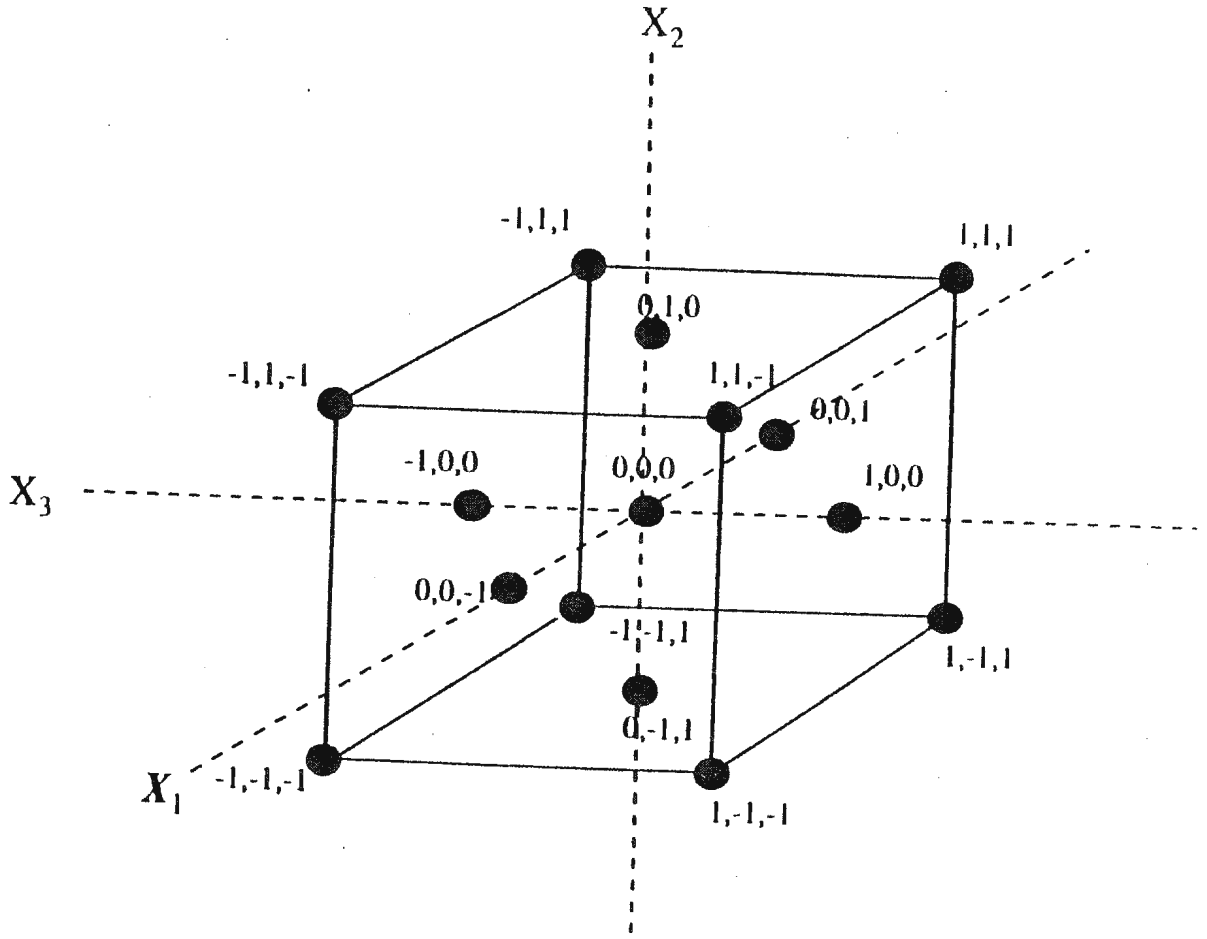

X2
X3
X1
-1,1,1
1,1,1
0,1,0
-1,1,-1
1,1,-1
0,0,1
-1,0,0
0,0,0
1,0,0
0,0,-1
-1,-1,1
1,-1,1
0,-1,1
-1,-1,-1
1,-1,-1

Table 2.2 Face-centred cube design arrangement

Design point	Independent variables		
	Amount of enzyme (w %)	Temperature (^{0}C)	Time (h)
1	-1[a]	-1	-1
2	-1	-1	+1
3	-1	+1	-1
4	-1	+1	+1
5	+1	-1	-1
6	+1	-1	+1
7	+1	+1	-1
8	+1	+1	+1
9	-1	0	0
10	+1	0	0
11	0	-1	0
12	0	+1	0
13	0	0	-1
14	0	0	+1
15	0	0	0
16	0	0	0
17	0	0	0

[a] Coded variable levels. Coded value=(Original value –M) /S, where, M and S are the mean of the highest and lowest parameter levels, and half their difference, respectively.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Two lipases from *Candida anatrctica* (Novozyme-435) and *Mucor miehei* (Lipozyme-1M) were acquired from Novo Nordisk (Franklinton, NC). Other lipases, namely *Pseudomonas sp.* (PS-30), *Aspergillus niger* (AP-12), and *Candida rugosa* (AY-30) were obtained from Amano Enzyme (Troy, VA). Algal oils, namely docosahexaenoic acid (DHA) single cell oil (DHASCO) containing 47.4 % DHA and arachidonic acid (ARA) single-cell oil (ARASCO) containing 40-50 % ARA were obtained from Martek Bioscience Corporation (Columbia, MD). OMEGA-GOLD is the commerical name for another oil from single-cell microalgae and was obtained from Monsanto (St. Louis, MO). Fatty acid methyl esters (FAMEs; GLC-461) were purchased from Nu-Check (Elysian, MN). Reagents, butylated hydroxytoluene (BHT), anhydrous sodium sulphate, monoolein, diolein, triolein, capric acid (CA), hydroquinone, phenolphthalein, sodium hydroxide, boric acid, porcine pancreatic lipase (EC 3.11.3), sodium taurocholate, silica gel thin layer chromotographic plates (TLC; 20× 20 cm; 60-Å mean pore diameter, 2-25 μm mean particle size, 500 – μm thickness, with dichlorofluorescein) were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol, hexane, methanol, sulphuric acid, hydrochloric acid, isooctane, isobutanol, chloroform, acetone, acetic acid, diethyl ether, carbon disulphide were purchased from Fisher Scientific Company (Nepean, ON). Helium, hydrogen, nitrogen and compressed air were purchased from Canadian Liquid Air Ltd. (St. John's, NL).

3.2 Methods

3.2.1 Acidolysis reaction

In general, ARASCO, DHASCO, or the OMEGA-GOLD oil 500 mg each was mixed with CA at different moles ratios of oil to CA ranging from 1 to 3, in a screw-capped test tube, and then lipase (2-10 % by weight of substrates) and water (1-2.5 % by weight of substrates and enzyme) were added in hexane (3.0 mL). The mixture was incubated for different time periods (12 to 48 h) in an orbital shaker at 250 rpm at temperatures ranging from 25 to 55°C.

3.2.2 Separation of acylglycerols after acidolysis

After a given time period a mixture of acetone and ethanol (20 ml; 1:1, v/v), was added to the reaction mixture to stop the reaction. In order to neutralize free fatty acids, the reaction mixture was titrated against a 0.5 N NaOH solution (using a phenolphathalein indicator) until the colour of the solution turned pink. Hexane (25 mL) was added to the mixture to extract the acylglycerol. The mixture was transferred into a separatory funnel and thoroughly mixed. The two layers (aqueous, hexane) were allowed to separate, and the lower aqueous layer was discarded. The hexane layer was passed through a bed of anhydrous sodium sulphate to remove any residual water. The hexane was evaporated using a rotary evaporator at 45°C and the acylglycerol fraction was recovered and transferred to special transmethylation vials.

3.2.3 Fatty acid composition of products

3.2.3.1 Preparation of fatty acid methyl esters (FAMEs)

Fatty acid profiles of products were determined following their conversion to methyl esters. Transmethylation reagent (2.0 mL, freshly prepared 6.0 mL of

concentrated sulphuric acid made up to 100 mL with methanol and 15 mg of hydroquinone as an antioxidant) was added to the sample vial, followed by vortexing. The mixture was incubated at 60°C for 24 h and subsequently cooled to room temperature. Distilled water (1mL) was added to the mixture and after thorough mixing, few crystals of hydroquinone were added to each vial to prevent oxidation; lipids were extracted three times, each with 1.5 mL of pesticide-grade hexane. The hexane layers were separated, combined and transferred to a clean test tube and then washed two times, each with 1.5 mL of distilled water. The hexane layer (the upper layer) was separated from the aqueous layer and evaporated under a stream of nitrogen. FAMEs were then dissolved in 1.0 mL of carbon disulphide and used for subsequent gas chromatographic analysis.

3.2.4 Analysis of FAMEs by gas chromatography

The FAMEs were analysed using a Hewlett Packard 5890 Series II gas chromatography (Hewlett Packard, Toronto, ON) equipped with a Supelcowax-10 column(30m length, 0.25 mm diameter, 0.25 μ m film thickness; Supelco Canada Ltd., Oakville, ON). The oven temperature was first set at 220°C for 10.25 min and then raised to 240°C at 30°C /min and held there for 15 min. The injector (flame ionization) and detector (FID) temperatures were both set at 250°C. UHP helium was used as a carrier gas at a flow rate of 15 mL /min. The data were treated using Hewlett Packard 3365 Series II Chem Station Software (Hewlett Packard, Toronto,ON). The FAMEs were identified by comparing their retention times with those of authentic standard mixtures (GLC-461; Nu-Check) and the results were presented as % w/w.

3.3 Hydrolysis by pancreatic lipase

Hydrolysis of modified oil by pancreatic lipase was performed according to the method described by Christie (1982, 1988) with a slight modification. Tris-HCl buffer (5.0 mL;1.0 M, pH 8.0), 0.5 mL of calcium chloride (2.2 %, w/v) and 1.25 mL of sodium taurocholate (0.05) were added to 25 mg of modified oil in a glass test tube. The whole mixture was allowed to stand at 40° C in a water bath for 1.0 min, followed by addition of 5.0 mg of porcine pancreatic lipase (EC 3.11.3; Sigma). The mixture was subsequently placed in a Gyrotory water bath shaker at 250 rmp under nitrogen for 1 h at 40 ° C. Ethanol (5.0 mL) was added to the mixture to stop the enzymatic reaction, followed by addition of 5.0 mL of HCl (6.0 N). The hydrolytic products were extracted three times with 50.0 mL of methanol/chloroform (1:1, v/v) and the upper layer removed and washed twice with distilled water and passed through a bed of anhydrous sodium sulphate. The solvent containing hydrolytic products was evaporated under a stream of nitrogen. TLC plates (20× 20 cm; 60-Å mean pore diameter, 2-25 μm mean particle size, 500 – μm thickness , with dichlorofluorescein, Sigma) were evenly sprayed with 5 % (w/v) boric acid and dried at 100° C for 1h. The hydrolytic products were separated on a silica gel TLC. The plates were developed using hexane/diethyl ether/ acetic acid (70:30:1, v/v/v) for 40-50 min and then allowed to dry in air. The bands were located by viewing under a short (254 nm) and a long (365 nm) wavelength light (Spectroline, model ENF-240C; Spectronics Co., Westbury, NY). The bands were scraped off and their lipids extracted into methanol/chloroform (1:1, v/v). Fatty acid profile of lipids were analysed by the GC method as described in section 3.2.4.

3.4 Oxidative Stability Tests

Structured lipids produced from ARASCO, DHASCO, and OMEGA-GOLD oils via acidolysis with CA as well as their original oils were kept under accellerated oxidation conditions at 60°C in a Schaal-oven for a period of 3 days. It is widely accepted that each day (24 h) of storage of oils under Schaal-oven conditions at 60°C is equal to one month storage at room temperature (Abou-Gharbia *et al.*, 1996). Oils (0.4 - 0.5 g) were placed in loosely capped test tubes (10 mm diameter and 4.0 cm height) and stored at 60°C in a forced air oven (Thelco, Model, Prcision Scientific Co., Chicago, IL). Samples were removed at 0, 6, 12, 24, 36, 48, and 72 h from the oven, cooled to room temperature, flushed with nitrogen, capped and stored at -20°C until analyzed. The experiments were carried out in triplicate.

3.4.1 Conjugated Dienes (CD)

Conjugated dienes in the oils were determined according to the IUPAC method 20505 (IUPAC ,1987). Oils samples (0.02-0.04 g) were weighted into 50 mL volumetric flasks, dissolved in iso-octane (2,2,4- trimethylpentane) and made up to the mark with the same solvent. The contents were thoroughly mixed and the absorbance was read at 234 nm using a Hewlett Packard Co. (Missisauga,ON). Pure iso-ocatne was used as the blank. Conjugated dienes were calculated using the following formula:

$CD = A/(c \times d)$

Where, A = absorbance of the solution at 234 nm, c = concentration of the solution in g/mL, and d = length of the cell in cm.

3.4.2 Thiobarbituric acid reactive substances (TBARS) determination

The determination of TBARS was carried out as described by the AOCS (method Cd 19-90, AOCS, 1990). Oil samples (0.05-0.10 g) were precisely weighed into 25 mL volumetric flasks and dissolved in a small volume of 1-butanol and made up to the mark with the same solvent. Five millilitres of the mixture (oil and solvent) were transferred into a dry screw capped test tube, and then 5 mL of freshly prepared TBA reagent (0.5 g TBA in 250 mL 1-butanol) were added. The constituents of the mixture were thoroughly mixed and placed in a water bath at 95°C for 2 h. Heated samples were cooled in an ice bath and the absorbance of the resulting coloured complex was read at 532 nm using a Hewlett Packard diode array Model 8452 A spectrophotometer (Hewlett Packard Co., Missisauga, ON). The number of μmol of malonaldehyde (MA) equivalent per gram of oil, expressed as TBARS values, was calculated using the formula:

$C=(0.415*A_{532})/ w$

Where 0.415 is a factor obtained from a standard regression line produced using 1,1,3,3-tetramethoxypropane as a precursor of malonaldehyde (Khan, 1999). In this formula C is the concentration of MA, A the absorbance of the coloured complex at 532 nm and w is the mass of oil.

3.5 Experimental design for response surface analysis

Before response surface methodology (RSM) was employed, approximate conditions for CA incorporation, enzyme load, reaction temperature and reaction time were determined by changing one factor at a time while keeping the others constant (Figure 2.3). A proper range for each factor was determined for RSM.

A three-factor and three-level face-centred cube design with 17 individual design points was selected in this study (Mason et al., 1989; Gao and Mazza, 1996). The independent parameters (X_i) and their levels are shown in Table 2.3. To avoid bias, 17 runs were performed in a totally random order. The independent variables or factors investigated were enzyme amount (w %; X_1), reaction temperature (°C; X_2) and reaction time (h; X_3) (Table 2.3). Response or dependent variable (Y) studied was CA incorporation (%). Triplicate experiments were conducted at all design points.

The second order polynomial model used for optimisation of CA incorporation in oil (Y) was:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum\sum_{i<j=1}^{3} \beta_{ij} X_i X_j$$

Where, β_o, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are the independent variables. Data were analysed using the response surface regression (RSREG) procedure (SAS Institute Inc., 1990) and fitted to the second order polynomial equation after logarithmic transformation (Thompson, 1982). Response surfaces and contour plots were attained using the fitted model, by keeping the least effective independent variable at a constant value while changing the other two variables. Confirmation experiments were performed using mixtures of variables at different levels (within the experimental range).

The independent factors are coded for an experimental design (Table 2.3). The centre point for each independent variable level is given a code of zero. The highest and lowest levels of concern for each independent factor are coded plus or minus one, respectively, for this three level design (Table 2.3). The major benefit of the design is that it

enables researchers to study one or more parameters at the same time in a single experimental design of workable size (Montgomery, 1991; Senanayake & Shahidi, 1999a).

The most efficient lipase for the acidolysis of ARASCO, DHASCO, and the OMEGA-GOLD oil with CA was *Pseudomonas sp.* Lipase. Nonetheless, there are several other variables that affect the product yields (CA incorporation) of lipase-assisted acidolysis of acylglycerols. These include reaction temperature, time of incubation, and amounts of enzyme and reactant, among others. Thus, it is essential to study these factors together to find the optimum reaction conditions to attain maximum incorporation of CA by the most efficient enzyme. In this study, reaction parameters such as enzyme load (X1), reaction temperature (X2), and reaction time (X3) were chosen for opitimzation. The substrate mole ratio of 1:3 (oil /CA) was kept constant because CA incorporation into ARASCO, DHASCO, and OMEGA-GOLD oils was reasonable at this mole ratio.

3.6 Statistical analysis

All experiments were performed in triplicate. Data are reported as mean ± standard deviation (SD). Normality was tested using Sigma Stat version 2.01, SSPS Inc. Analysis of variance and Tukeys test were carried out at a level of $p < 0.05$ to assess the significance of differences between mean values.

CHAPTER 4

RESULTS AND DISSCUSIONS

4.1 Fatty acid composition of ARASCO, DHASCO, and OMEGA-GOLD oils

The fatty acid profile of ARASCO (arachidoinc acid single cell oil) before and after modification with CA (capric acid) by *Pseudomonas sp.* is shown in Table 4.1. The major fatty acids found in ARASCO before enzymatic incorporation were arachidonic (39.03 %), oleic (34.09 %), and linoleic (5.07 %) acids. Furthermore, this oil also contained saturated fatty acids such as stearic (7.27 %) and palmitic (4.28 %) acids. The proportion of different fatty acids in ARASCO in this work were different from those reported by Arterburn *et al.* (2000b). These researchers found that ARASCO derived from microfungus *Mortiello alpina* contained arachidonic (51.4 %), oleic (8.3 %), linoleic (6.3 %), γ-linolenic (3.6 %), palmitic (9.4 %), and stearic (10.3 %) acids. The differences between the fatty acid profile presented here and those reported by Arterburn *et al.* (2000b) might be due to variation in the source and batch of the oil, method of preparation, and fermentation conditions employed.

DHASCO (docosahexaenoic acid single cell oil) is another oil produced by the marine microalgae via fermentation technology. The fatty acid composition of DHASCO before and after modification with CA is shown in Table 4.2. The results presented in Table 4.2 indicate that the original DHASCO, before modification with CA, contained 37.14 % DHA. It also contained small amounts of other polyunsaturated fatty acids. Lauric (3.46 %), myristic (12.92 %), and palmitic (10.52 %) acids were the saturated fatty acids in DHASCO. Kyle (2001) reported that DHASCO oil prepared from microalgae *Cryptoecodinium cohnii* contained 43.53 % DHA. The present study

Table 4.1 Fatty acid composition of ARASCO triacylglycerols before and after modification with CA *

Fatty acid (wt %)	Before modification	After modification**
C10:0	0.03 ± 0.01	19.27 ± 0.58
C14:0	0.18 ± 0.01	0.16 ± 0.01
C16:0	4.28 ± 0.04	2.72 ± 0.03
C18:0	7.27 ± 0.17	5.01 ± 0.07
C18:1 n-9	34.09 ± 0.59	23.41 ± 0.29
C18:2 n-6	5.07 ± 0.09	3.96 ± 0.04
C18:3 n-6	2.05 ± 0.19	1.54 ± 0.02
C20:3 n-6	1.27 ± 0.02	1.08 ± 0.02
C20:4 n-6	39.03 ± 0.56	36.50 ± 0.46
C20:5 n-3	1.71 ± 0.03	1.25 ± 0.01
C22:5 n-6	2.01 ± 0.21	2.34 ± 0.17
C24:0	0.19 ± 0.02	ND
others	2.82	1.68

* Values are mean values of triplicate determinations ± standard deviation.

** The reaction mixture contained 500 mg ARASCO, 315 mg CA, 4% (w/w) enzyme for *Pseudomonas sp.* and 3.0 mL hexane. The reaction mixture was incubated at 45°C for 24 h in an orbital shaking water bath at 250 rmp.

Table 4.2 Fatty acid composition of DHASCO triacylglycerols before and after modification with CA *

Fatty acid (wt %)	Before modification	After modification **
C10:0	0.46 ± 0.01	10.17 ± 0.11
C12:0	3.46 ± 0.05	3.03 ± 0.04
C14:0	12.92 ± 0.08	10.01 ± 0.02
C16:0	10.52 ± 0.05	7.93 ± 0.07
C18:0	0.86 ± 0.02	0.79 ± .01
C18 :1 n-9	26.95 ± 1.54	26.26 ± 1.19
C18:2 n-6	1.43 ± 0.02	1.21 ± 0.00
C20:5 n-3	0.28 ± 0.01	ND
C22:5 n-6	0.75 ± 0.05	1.66 ± .0.05
C22:6 n-3	37.14 ± 0.39	37.13 ± 0.29
Others	2.86	-

* Values are mean values of triplicate determinations ± standard deviation.

** The reaction mixture contained 500 mg DHASCO, 315 mg CA, 4% (w/w) enzyme for *Pseudomonas sp.* and 3.0 mL hexane. The reaction mixture was incubated at 45°C for 24 h in an orbital shaking water bath at 250 rmp.

Table 4.3 Fatty acid composition of the OMEGA-GOLD oil triacylglycerols before and after modification with CA *

Fatty acid (wt %)	Before modification	After modification**
C10:0	0.07 ± 0.01	20.33 ± 0.12
C14:0	8.27± 0.03	5.64 ± 0.06
C15:0	0.46 ± 0.01	0.29 ± 0.01
C16:0	23.44 ± 0.23	12.93 ± 0.07
C16:1	1.59 ± 0.03	1.2 ± 0.01
C18:0	0.55 ± 0.01	0.37 ± 0.01
C18:1 n-9	0.72 ± 0.2	0.45 ± 0.01
C20:3 n-6	3.21 ± .11	1.46 ± 0.23
C20:4 n-6	0.29 ± 0.5	0.20 ± 0.03
C20:5 n-3	3.10 ± .12	3.20 ± 0.03
C22:5 n-6	14.14 ± .15	13.05 ± 0.01
C22:6 n-6	36.82 ± 0.39	34.11 ± 0.37
Others	7.33	6.77

* Values are mean values of triplicate determinations ± standard deviation.

** The reaction mixture contained 500 mg OMEGA-GOLD oil, 315 mg CA, 4% (w/w) enzyme for *Pseudomonas sp.* and 3.0 mL hexane. The reaction mixture was incubated at 45°C for 24 h in an orbital shaking water bath at 250 rmp.

indicates that DHASCO contained a relatively high level of oleic acid (26.95 %) as compared with 14.67 % reported by Kyle (2001).

Table 4.3 shows fatty acid composition of the OMEGA-GOLD oil, another single cell oil, before and after modification with CA. OMEGA-GOLD oil is a concentrated source of DHA (36.82 %) and omega-6 DPA (14.14 %). The oil also contained myristic (8.27 %) and palmitic (23.44 %) acids. Other fatty acids in OMEGA-GOLD oil were EPA (C20: 5n-3, 3.10 %) and eicosatrienoic acid (C20: 3n-6, 3.21 %). The results reported here some what agree with those of Zeller *et al.* (2001) who reported that the OMEGA-GOLD oil prepared from *Schizochtrium sp.* contained 41 % DHA, 18 % DPA, 9 % myristic acid, and 22 % palmitic acid.

4.2 Enzymatic incorporation of capric acid (CA) into ARASCO, DHASCO and the OMEGA-GOLD oil

4.2.1 Enzyme screening

Five lipases from *Candida antarctica, Mucor miehei, Pseudomonas sp., Candida rugosa, and Aspergillus niger* were screened for their ability to incorporate capric acid (CA) into ARASCO, DHASCO, and the OMEGA-GOLD oil (Table 4.4). These lipases catalysed incorporation of CA into the oils tested to varying degrees. The degree of CA incorporation into ARASCO and DHASCO with various lipases was in the order of *Pseudomonas sp.* > *Mucor miehei* > *Candida rugosa* > *Aspergillus niger*> *Candida antarctica.* The incorporation of CA into the three oils was effectively catalysed by all five lipases tested. However, there was no significant difference ($p>0.05$) in the CA incorporation into ARASCO oil when lipases from *Candida antarctica* and *Aspergillus niger* were employed.

Table 4.4 Effect of enzyme type on the incorporation (%) of capric acid (CA) into ARASCO, DHASCO, and the OMEGA-GOLD oil

Source of enzyme	ARASCO	DHASCO	OMEGA-GOLD
Candida antarctica	2.39 ± 0.21^{a}	3.32 ± 0.09^{a}	2.17 ± 0.10^{b}
Mucoe miehei	21.68 ± 0.33^{d}	21.02 ± 0.56^{d}	19.05 ± 0.24^{d}
Pseudomonas sp.	23.96 ± 0.47^{e}	31.32 ± 0.54^{e}	27.92 ± 0.85^{e}
Candida rugosa	3.92 ± 0.19^{c}	10.91 ± 0.43^{c}	0.10 ± 0.03^{a}
Aspergillus niger	2.97 ± 0.21^{ba}	6.56 ± 0.84^{b}	4.45 ± 0.18^{c}

* Values are mean values of triplicate determinations ± standard deviation. Values with each column with different superscripts are different ($p<0.05$) from one another.

Pseudomonas sp. gave the highest degree of incorporation of CA into ARASCO (23.96 %, after 24 h), DHASCO (31.32 %, after 24 h), and the OMEGA-GOLD oil (27.92 %, after 24 h). The results reported here agree with the findings of Zhou *et al.* (2000) who reported that lipase from *Pseudomonas fluoresescens* gave the highest incorporation of caprylic acid (C8:0) into fish oil. Because acidolysis of the three oils with CA was best with lipase from *Pseudomonas sp.*, this enzyme was selected for subsequent experiments.

4.2.2 Mole ratio effect

The effect of mole ratio of substrates on the incorporation of CA into ARASCO, DHASCO, and the OMEGA-GOLD oil is shown in Table 4.5. As the number of moles of CA increased from 1 to 3, its incorporation into the three oils increased steadily. The optimum incorporation of CA into ARASCO (23.32 %), DHASCO (31.32 %), and the OMEGA-GOLD oil (27.92 %) may be obtained at an oil to CA mole ratio of 1:3 because TAG molecules can incorporate a maximum of three fatty acids in their backbone. Shimada and colleagues (1996) reported that as the weight ratio of caprylic acid to tuna oil increased from 2 to 8, the incorporation of caprylic acid into tuna oil did not change significantly (from 41 to 42 mol %). Therefore, the weight ratio of 2:1 of caprylic acid / tuna oil gave the optimum incorporation. On the other hand, the present results agree with those of Soumannou *et al.* (1998) who observed that the stoichiometric ratio of 1:3 was optimal for the production of SL via esterification of 2-monoolein and caprylic acid with Lipozyme in the presence of hexane.

4.2.3 Enzyme load effect

The effect of enzyme load (%) on the incorporation of CA into ARASCO, DHASCO, and the OMEGA-GOLD oil is shown in Figure 4.1 (A, B, and C). As the

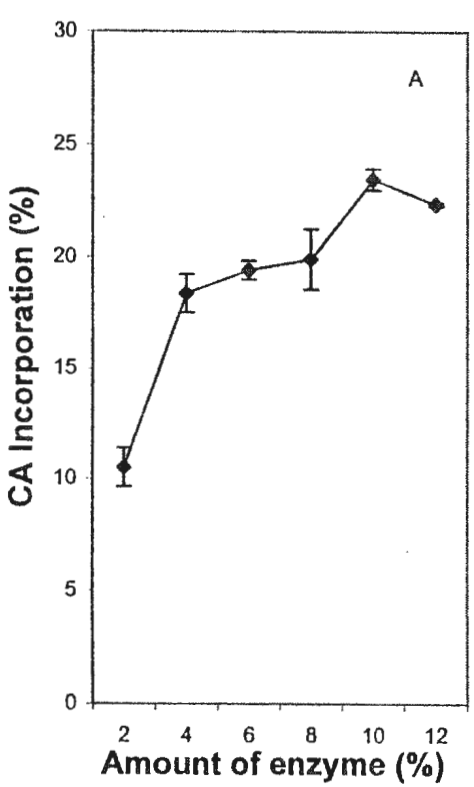
Table 4.5 Effect of mole ratio of substrates on CA incorporation into ARASCO, DHASCO, and the OMEGA-GOLD oil*

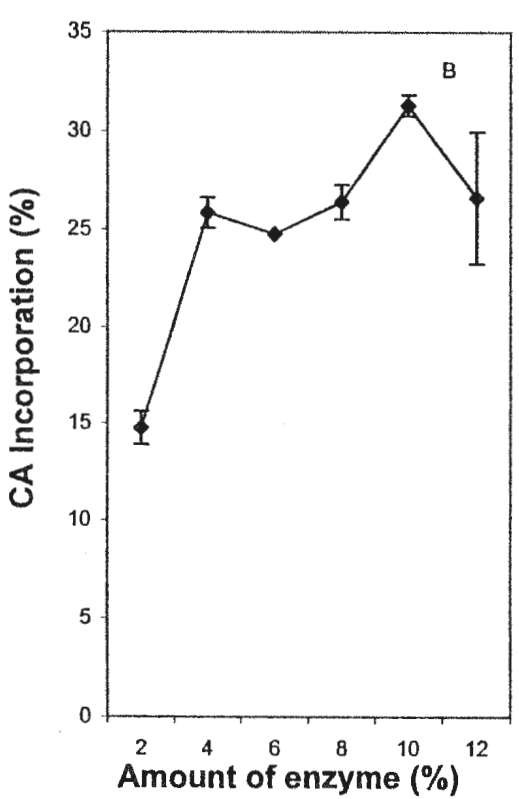
Oil	Mole ratio CA /oil**		
	1:1	1:2	1:3
ARASCO	12.57 ± 0.65^{a}	20.50 ± 1.24^{b}	23.96 ± 0.47^{c}
DHASCO	13.83 ± 0.34^{a}	22.56 ± 2.08^{b}	31.32 ± 0.54^{c}
OMEGA-GOLD	12.71 ± 0.07^{a}	24.47 ± 1.88^{b}	27.92 ± 0.85^{c}

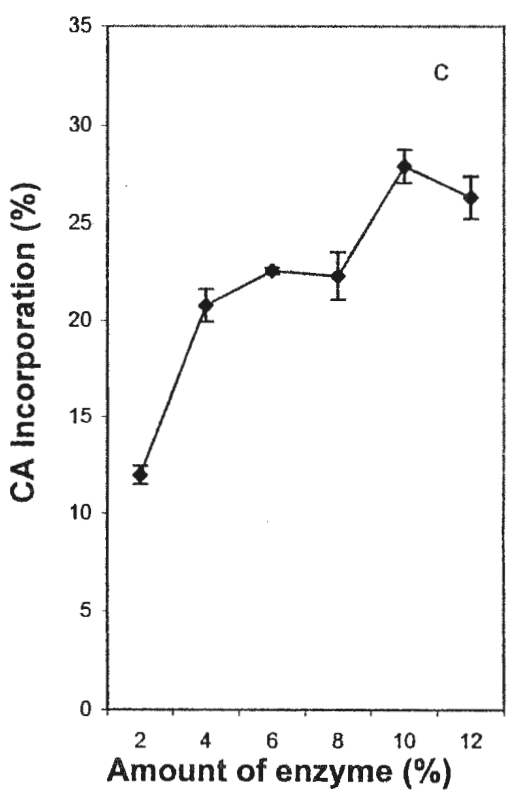
*Values are mean values of triplicate determinations ± standard deviation. Values with each row with different superscripts are different ($p<0.05$) from one another.

** Mole ratios of ARASCO, DHASCO, and the OMEGA-GOLD oil to CA were 1:1, 1:2, and 1:3. The reaction mixture contained 500 mg of oil, 105-315 mg of CA, 10 % *Pseudomonas sp*. Lipase preparation, distilled water (2 % by weight of enzyme and substrates) and 3.0 mL hexane. The reaction mixture was incubated at 45°C for 24 h in an orbital water bath at 250 rpm

Figure 4.1 The effect of enzyme load on the incorporation of CA into ARASCO (A), DHASCO (B), and the OMEGA-GOLD oil (C). The reaction mixture contained 500 mg oil, 315 mg CA, 2-12 % *Pseudomonas sp.* lipase preparation, distilled water (2% by weight of enzyme and substrates) and 3.0 ml hexane. The reaction mixture was incubated at 45°C for 24 h in an orbital water bath at 250 rpm.


A
CA Incorporation (%)
0
5
10
15
20
25
30
2
4
6
8
10
12
Amount of enzyme (%)


B
CA Incorporation (%)
0
5
10
15
20
25
30
35
2
4
6
8
10
12
Amount of enzyme (%)


C
CA Incorporation (%)
0
5
10
15
20
25
30
35
2
4
6
8
10
12
Amount of enzyme (%)

enzyme load (%) increased from 2 to 10 %, the incorporation of CA into the three oils increased gradually. Above the 10 % enzyme load, there was a slight decrease in the incorporation of CA into the three oils, possibly due to factors such as deficiency of the available water for hydration of the enzyme. As the amount of lipase in the reaction mixture was raised the amount of added water remained constant at a 2 % (w/w) level. The highest CA incorporation into ARASCO (23.46 %), DHASCO (26.4 %), and the OMEGA-GOLD oil (22.39 %) was achieved at an enzyme load of 10 %. Results of this work agree with those of Jennings and Akoh (1999) who reported that the maximum level of CA incorporation (41.4 %) into fish oil was achieved with an enzyme load of 10 %. Furthermore, the present results agree with the findings of Akoh and Huang (1995) who reported that an enzyme load of 10 % was adequate for acidolysis of triolein with caprylic acid. Recently, Senanayake and Shahidi (2002) observed similar results when incorporating CA into seal blubber oil; the incorporation of CA was increased up to 10 % enzyme load. Shimada *et al.* (1996) found that 2, 4 and 8 % enzyme from *Rhizopus delemar* resulted in the incorporation of 40, 44, and 45 mol % caprylic acid into tuna oil, respectively.

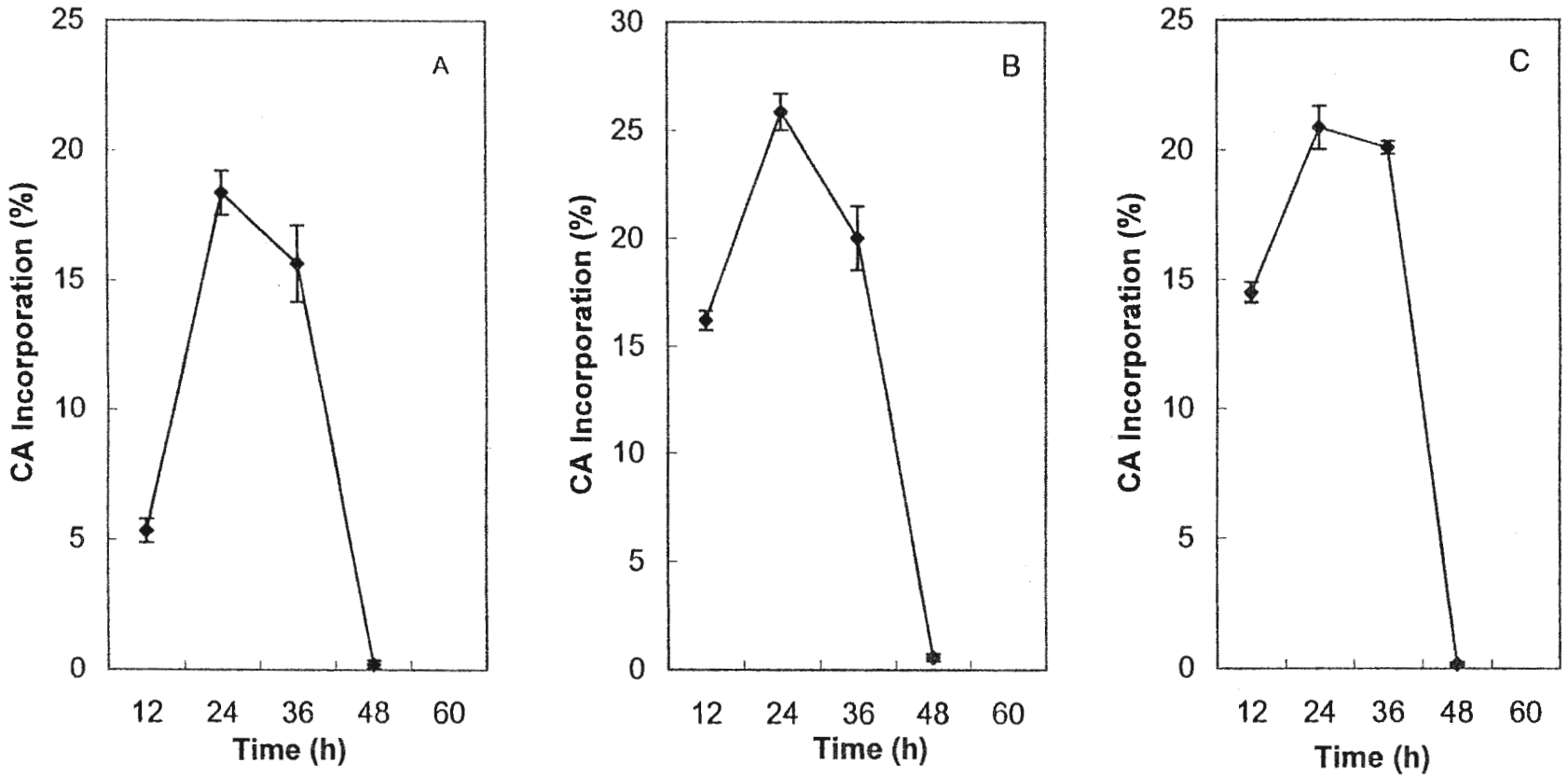
Although a better incorporation of CA into the three oils was obtained with a 10 % enzyme load, we chose an enzyme load of 4 % for subsequent experiments because this amount of enzyme was appropriate when considering the marginal increase in the yield at a higher enzyme level and hence the overall economy of the process.

4.1.4 Time course

The time course of the reaction is useful in determining the shortest period needed to obtain the highest incorporation of CA into ARASCO, DHASCO, and the OMEGA-

GOLD oil as it affects production costs. Figure 4.2 (A-C) shows the time course of lipase-assisted acidolysis of CA into ARASCO, DHASCO, and the OMEGA-GOLD oil using PS-30 from *Pseudomonas sp.* As the time progressed from 12 to 24 h, the percent CA incorporation into the three oils increased significantly, and reached a maximum at 24 h. Above 24 h there was a dramatic decrease in CA incorporation into ARASCO (0.21 %), DHASCO (0.57 %), and the OMEGA-GOLD (0.16 %) oil, reaching approximately zero at 48 h. The dramatic decrease of CA incorporation into the three oils after 48 h may be due to the prolonged incubation time at high temperatures, resulting in denaturation of the lipase. Another possible explanation for the decreased incorporation of CA into the three oils may be due to the occurrence of the reverse reaction. Mu and colleagues (1998) made a laboratory scale continuous reactor for the synthesis of SL containing linoleic acid (18:2 n-6) located on the sn-2 position and capric acid in the sn-1 and sn-3 positions. Their results showed that incorporation of capric acid (48 %) into the triacylglycerol containing linoleic acid was obtained after 7 h of reaction. Jennings and Akoh (2000) successfully incorporated capric acid (up to 27 %) into rice bran oil in 72 h using immobilized lipase from *Rhizomucor miehei.* The results reported here are in contrast with the findings of Jennings and Akoh (1999) who reported that the highest incorporation (41.2 mol %) into fish oil occurred at 48 h and those of Senanayake and Shahidi (2002) who found that as the reaction time of acidolysis of seal blubber oil with CA increased from 24 to 72 h, the CA incorporation was increased from 25.4 to 29.6 %.

Figure 4.2 The effect of time course on the incorporation of CA into ARASCO (A), DHASCO (B), and the OMEGA-GOLD oil (C). The reaction mixture contained 500 mg oil, 315 mg CA, 4 % *Pseudomonas sp.* lipase preparation, distilled water (2% by weight of enzyme and substrates) and 3.0 ml hexane. The reaction mixture was incubated at 45°C for 12– 48 h in an orbital water bath at 250 rpm.

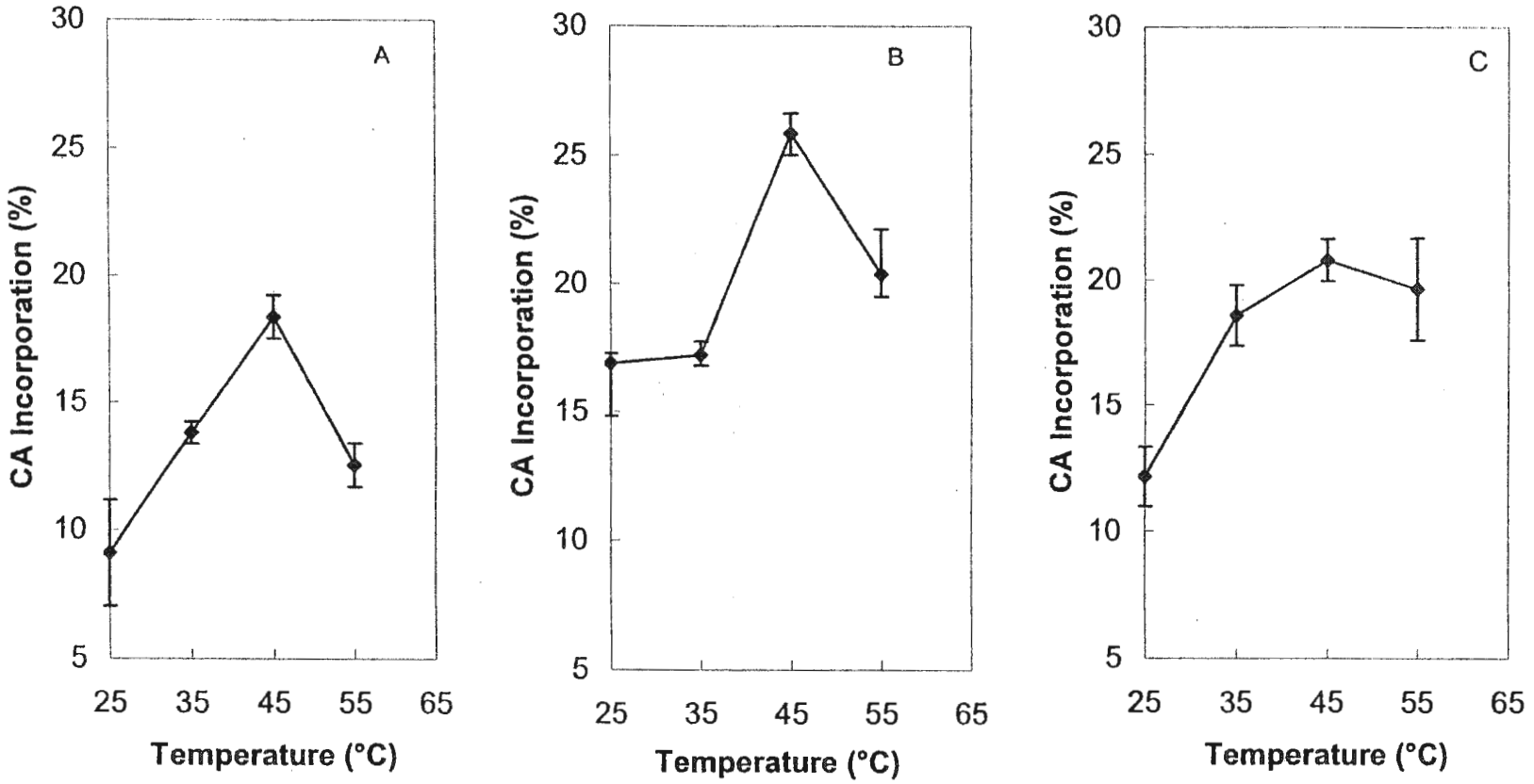

A
B
C
CA Incorporation (%)
Time (h)
0
5
10
15
20
25
30
12
24
36
48
60

4.2.5 Reaction temperature effect

The effect of reaction temperature on enzyme activity is a well-established phenomenon. As the surrounding temperature heats up, the rate of enzyme activity increases until an optimum rate is reached. This reaction parameter was investigated to determine the optimum temperature for PS-30 from *Pseudomonas sp.* lipase for incorporation of CA into ARASCO, DHASCO, and the OMEGA-GOLD oil as shown in Figure 4.3 (A-C). The incorporation of CA into the three oils gradually increased as the reaction temperature increased from 25 to 45°C. The optimum incorporation of CA into ARASCO (18.3 %), DHASCO (25.86 %), and the OMEGA-GOLD oil (20.80 %) was obtained at 45°C. At lower temperatures (25-35°C), CA incorporation into the three oils has observed at. When temperature increased above 45°C, CA incorporation into the three oils declined. In general, a 10°C increase in temperature results in doubling of the reaction rate and hence a higher incorporation of CA. Senanayake and Shahidi (2002) observed that as the reaction temperature increased from 30 to 50°C, incorporation of CA into seal blubber oil was increased and reached the highest (26.9 %) at 45°C thus lending support to the findings in this study. On the other hand, Shimada *et al.* (1996) observed that incorporation of caprylic acid into tuna oil, rich in DHA and EPA, was almost the same (43-45 mol %) in the temperature range of 30 to 45°C.

The results presented here showed that *Pseudomonas sp.* lipase is more reactive at 45°C than at 25-35°C. Therefore, reaction temperature of 45°C was used for the rest of the experiments.

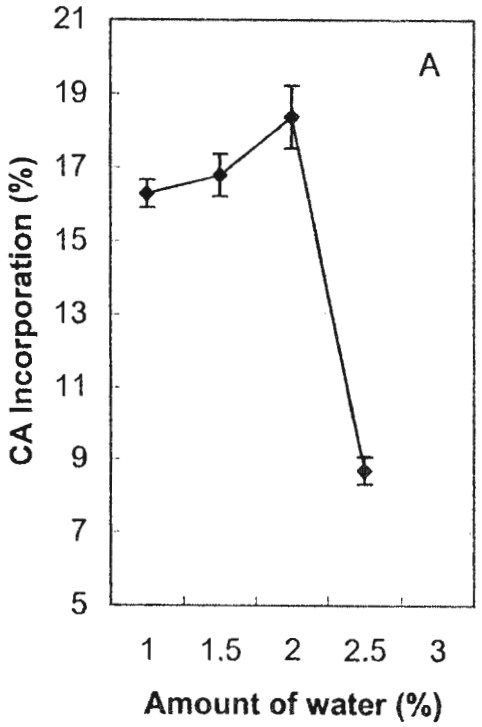
Figure 4.3 The effect of reaction temperature on the incorporation of CA into ARASCO (A), DHASCO (B), and the OMEGA-GOLD oil (C). The reaction mixture contained 500 mg oil, 315 mg CA, 4 % enzyme load from *Pseudomonas sp.*, distilled water (2% by weight of enzyme and substrates) and 3.0 mL hexane. The reaction mixture was incubated at different temperatures (25-55°C) for 24 h in an orbital water bath at 250 rpm.

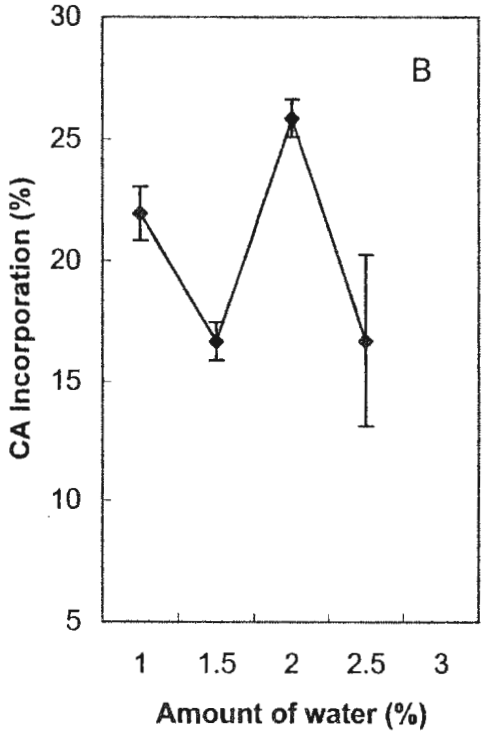

A
B
C
CA Incorporation (%)
Temperature (°C)
30
25
20
15
10
5
25
35
45
55
65

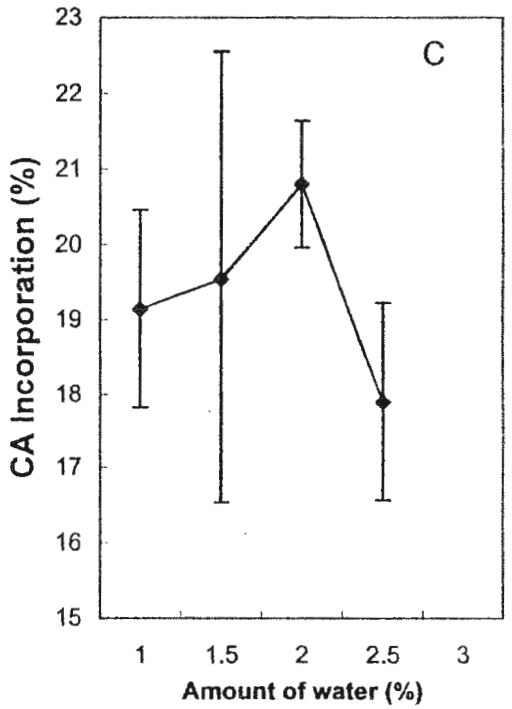
4.2.6 Effect of added water

Controlling the amount of water is very important in enzymic lipid modification. Presence of small amount of water on the surface of the enzyme is required for maintaining its three-dimensional structure. However, excess water usually leads to hydrolysis. Figure 4.4 (A-C) shows the effect of water (1-2.5 %) on the incorporation of CA into ARASCO, DHASCO, and the OMEGA-GOLD oil. In general, as the amount of water increased from 1 to 2 %, incorporation of CA into ARASCO increased significantly ($p<0.05$). Meanwhile CA incorporation (%) into the OMEGA-GOLD oil did not change significantly ($p>0.05$) as the amount of water increased from 1 to 2.5 %. A highest incorporation of capric acid into ARASCO (18.38 %), DHASCO (25.86 %) and the OMEGA-GOLD oil (20.8 %) was noticed at a 2 % (w/w) water content. Further increase in the content of water, above 2 %, led to a decline in CA incorporation into the three oils. The drop in CA incorporation into the three oils might be attributed to the presence of excess water, which usually leads to hydrolysis. The present results agree with the findings of Jennings *et al.* (1999) who reported that the incorporation of CA into fish oil declined as the amount of water added increased. Moreover, Jennings *et al.* (1999) reported that the optimal CA incorporation occurred at 1 % added water (47.9 %). Furthermore, Senanayake and Shahidi (2002) noticed that the highest CA incorporation (25.4 %) into seal blubber oil was obtained at a 1 % (w/w) water content. Added water at 2 % gave the highest CA incorporation into ARASCO (18.38 %), DHASCO (25.86 %), and the OMEGA-GOLD oil (20.8 %). Huang and Akoh (1996) reported that the amount of added water had little impact on incorporation of caprylic acid into triolein when the

Figure 4.4 The effect of added water on the incorporation of CA into ARASCO (A), DHASCO (B), and the OMEGA-GOLD oil (C). The reaction mixture contained 500 mg oil, 315 mg CA, 4 % *Pseudomonas sp.* lipase preparation, distilled water (1 -2.5 % by weight of enzyme and substrates) and 3.0 mL hexane. The reaction mixture was incubated at 45°C for 24 h in an orbital water bath at 250 rpm.


A
CA Incorporation (%)
21
19
17
15
13
11
9
7
5
1
1.5
2
2.5
3
Amount of water (%)


B
CA Incorporation (%)
30
25
20
15
10
5
1
1.5
2
2.5
3
Amount of water (%)


C
CA Incorporation (%)
23
22
21
20
19
18
17
16
15
1
1.5
2
2.5
3
Amount of water (%)

water content was altered from 0.0 to 0.06 %. Based on the above, a 2 % (w/w) water was used for subsequent experiments.

4.3 Positional Distribution of Structured Lipids

The positional distribution of fatty acids on the glycerol backbone of triacylglycerols is of great significance in relation to the physical and functional properties as well as the metabolism of fats and oils. During digestion specific enzymes, such as pancreatic lipase, release fatty acids from the sn-1 and sn-3 positions of the triacylglycerols. The fatty acids in the sn-2 remain as monoacylglycerols, which are absorbed and distributed in the form of chylomicron (Akoh, 1995; Sellappan and Akoh, 2001). Pancreatic lipase from mammals hydrolyses the ester bond at the sn-1 and sn-3 positions with a preference for medium-chain fatty acids over long-chain fatty acids (Iwaski and Yamane, 2000). The positional distribution of fatty acids on the glycerol backbone of unmodified ARASCO is summarized in Table 4.6. Arachidonic acid (ARA) was mainly located at the sn-2 position (22.06 %) of the TAG molecules. Table 4.7 shows the fatty acid constituents at the sn-2 and sn-1 + sn-3 of the modified ARASCO with CA performed by pancreatic lipase hydrolysis. CA was mostly esterified to the sn-1,3 positions. Thus, lipase from *Pseudomonas sp.* shows some regiospecificity and may preferentially incorporate CA at the sn-1 + sn-3 positions of TAG. Iwaski and Yamane (2000) reported that the use of non-specific *Pseudomonas sp.* lipase might be useful for the synthesis of SL when the starting materials are oils rich in TAG having two or three fatty acid residues which are resistant against fungal lipases such as *R. miehei* and *R. delemar*. Akoh and Moussata (2001) showed that caprylic acid was mostly incorporated at the sn-1,3 positions and it replaced a high proportion of the n-3 PUFA in modified fish

Table 4.6 Positional distribution (sn-2 and sn-1+ sn-3) of fatty acids in unmodified ARASCO

Fatty acid	Position	
	sn-2[a]	sn-1 +sn-3
C10:0	0.93 ± 0.53 (31.0)	0.79 ± 0.99 (69.0[c])
C14:0	1.98 ± 0.92 (3.37)	0.44 ± 0.08 (96.33)
C16:0	3.25±0.66 (27.26)	5.59± 0.47 (72.74 [c])
C18:0	4.77± 1.44 (21.87)	8.81± 0.94 (78.13[c])
C18:1	18.31± 5.56 (17.90)	29.85± 5.99 (82.09[c])
C18: 2n-6	3.55± 0.43 (23.34)	4.96± 1.17 (76.66[c])
C18: 3 n-6	1.25± 0.90 (20.33)	2.01± 0.73 (79.67[c])
C20:4 n-6	22.06± 8.67 (18.84)	23.14± 3.44 (81.16[c])

Values in parenthesis indicate percent fatty acid distribution of total triacylglycerols present at the sn-1 +sn-3, and sn-2 positions. [a] Values in parenthesis are (% fatty acid at the sn-2 position / % fatty acid in triacylglycerols X 3) X 100; for sn-1 + sn-3 = 100 - sn-2.

Table 4.7 Positional distribution (sn-2 and sn-1+ sn-3) of fatty acids in lipase-assisted acidolysis of ARASCO with CA

Fatty acid	Position	
	sn-2[a]	sn-1 +sn-3
C10:0	4.74 ± 0.53 (6.55)	4.32 ± 0.72 (93.45 [c])
C16:0	4.97 ± 0.31 (75.94 [c])	4.62 ± 0.88 (24.01)
C18:0	4.17 ± 0.34 (6.93)	4.59 ± 0.75(93.07[c])
C18:1	1.59 ± 0.65 (15.17)	0.86 ± 0.45 (84.83[c])
C20:4 n-6	31.16 ± 2.13 (27.52)	29.74 ± 1.59 (72.45[c])

Values in parenthesis indicate percent fatty acid distribution of total triacylglycerols present at the sn-1 + sn-3, and sn-2 positions. [a] Values in parenthesis are (% fatty acid at the sn-2 position / % fatty acid in triacylglycerols X 3) X 100; for sn-1 + sn-3 = 100- sn-2 Fatty acids (C12:0, C14:0, C18: 2 n-6, and C18: 3 n-6) were present at 0- 0.5 % (not shown)

oil. This suggested that most of the EPA and DHA were present at the sn-2 position and were retained during Lipozyme IM (*Rhizomucor miehei*) catalysed acidolysis. However, Kim *et al.* (2002) found that about 7.1 to 10.7 mol % of caprylic acid was located at the sn-2 position in the TAG molecules produced via acidolysis of perilla oil containing 60 % α-linolenic acid with caprylic acid despite using 1,3-specific lipases (*Rhizomucor miehei* and *Thermomyces lanuginose).* This result might reflect acyl migration occurring upon pancreatic hydrolysis or lipase-assisted acidolysis.

Positional distribution analysis of unmodified DHASCO revealed that 29.69 % of DHA was located at the sn-2 position of TAG molecules (Table 4.8). However, Myher *et al.* (1996) reported that 45 % of DHA in DHASCO was located at the sn-2 position.

The positional distribution of fatty acids in DHASCO modified with CA is shown in Table 4.9. The results showed that DHA is located to a higher extent in the sn-2 position, as compared to the sn-1 + sn-3 positions. However, CA was mainly located at the sn-1 and sn-3 positions confirming the preference of pancreatic lipase for medium-chain fatty acids over long-chain fatty acids during hydrolysis. The present results agree with those of Senanayake and Shahidi (2002) who reported that CA was mainly located at sn-1 and sn-3 positions (35.9 %) of SL produced via acidolysis of seal blubber oil with CA. A proximately 12 % of CA was in the sn-2 position, possibly as a result of acyl migration. Furthermore, Shimada *et al.* (1996) reported a similar fatty acid composition for the sn-2 position of tuna oil before and after acidolysis with caprylic acid, thus suggesting that caprylic acid was incorporated in the sn-1 and sn-3 positions of tuna oil.

Table 4.10 shows the positional distribution of fatty acids in the unmodified OMEGA-GOLD oil. DHA was preferentially located at sn-2 position (34.83 %),

Table 4.8 Positional distribution (sn-2 and sn-1+ sn-3) of fatty acids in unmodified DHASCO

Fatty acid	sn-2[a]	sn-1 +sn-3
C10:0	1.49 ± 0.13 (41.3)	0.61 ± 0.28 (58.70)
C12:0	3.81 ± 0.73 (36.70[c])	3.88 ± 0.52 (63.30)
C14:0	9.11 ± 1.06 (23.50)	11.29 ± 2.86 (76.50[c])
C16:0	7.73 ± 1.98 (24.49)	10.77 ± 3.37 (75.51[c])
C18: 1n-9	11.77 ± 2.44 (14.56)	15.37 ± 3.06 (85.44[c])
C18: 2n-6	1.51 ± 0.94 (35.19[c])	4.52 ± 2.80 (64.81)
C22: 6 n-3	29.68 ± 6.00 (26.64)	22.94 ± 3.75 (73.36[c])

Values in parenthesis indicate percent fatty acid distribution of total triacylglycerols present at the sn-1 + sn-3, and sn-2 positions. [a] Values in parenthesis are (% fatty acid at the sn-2 position / % fatty acid in triacylglycerols X 3) X 100; for sn-1+ sn-3 =100 – sn-2

Table 4.9 Positional distribution (sn-2 and sn-1+ sn-3) of fatty acids in lipase-assisted acidolysis of DHASCO with capric acid

Fatty acid	sn-2[a]	sn-1 +sn-3
C10:0	5.61 ± 2.08 (5.97)	16.86 ± 7.11(94.03 [c])
C12:0	1.78 ± 0.16 (26.73)	2.72 ± 0.81(73.27[c])
C14:0	5.69±1.29 (25.25)	7.06 ± 1.28 (74.75[c])
C16:0	8.67 ± 1.62 (45.23[c])	9.58 ± 1.17(54.77)
C22: 6 n-3	39.97 ± 4.79 (41.44[c])	35.68 ± 6.91(58.56)

Values in parenthesis indicate percent fatty acid distribution of total triacylglycerols present at the sn-1 +sn-3, and sn-2 positions. [a] Values in parenthesis are (% fatty acid at the sn-2 position / % fatty acid in triacylglycerols X 3) X 100; for sn-1 + sn-3 =100– sn-2 Fatty acids (C18: 1 n-9, and C18: 2 n-6) were present at 0-0.5 % (not shown)

followed by the sn-1 and sn-3 positions (25.35 %). The same trend was observed for DPA. Zeller *et al.* (2001) reported that 23 % of DPA + DHA was located at the sn-2 position and 27 % present in the sn-1,3 positions of the glycerol backbone using ^{13}C NMR .

Table 4.11 shows the positional distribution of fatty acids in the modified OMEGA-GOLD oil upon acidolysis with CA. CA was located mainly at the sn-1,3 positions (14.09 % at sn-1 and sn-3), whereas DHA and DPA were mainly located at the sn-2 position of the glycerol moiety. The present results agree with the findings of Iwaski and Yamane (2000) who suggested that the use of non-specific *Pseudomonas sp.* might be advantageous in the incorporation of caprylic acid into oils rich in DHA and DPA.

4.4 Oxidative stability of enzymatic modified ARASCO, DHASCO and the OMEGA-GOLA oil

4.4.1 Conjugated Dienes (CD)

A conjugated dienes test is a simple and rapid physical procedure to evaluate primary products of lipid oxidation. Determination of CD does not require chemical reactions and requires only a small amount of sample of milligrams. Moreover, CD can be used to estimate the initial rate of lipid oxidation (Gray, 1978). The conjugated diene values of the OMEGA-GOLD modified in the presence of lipase and without lipase (the reactants were subjected to the same reaction process) as well as the control unmodified oil are shown in Figure 4.5 (C). The enzyme-catalyzed structured lipid of the OMEGA-GOLD oil had a higher conjugated diene values than the original oil

Table 4.10 Positional distribution (sn-2 and sn-1+ sn-3) of fatty acids in the unmodified OMEGA-GOLD oil

Fatty acid	Position	
	sn-2[a]	sn-1 + sn-3
C10:0	ND	ND
C14:0	6.46 ±1.38 (26.04)	7.44 ± 0.81 (73.96[c])
C16:0	20.49 ± 2.69 (29.14)	21.18 ± 2.33 (70.86[c])
C16:1	1.46 ± 0.28 (30.61)	1.34 ± 0.65 (69.39)
C18:0	0.36 ± 0.01 (21.82)	1.56 ± 1.05 (78.18[c])
C18:1n-9	0.69 ± 0.01 (31.94)	1.35 ± 0.54 (68.06[c])
C20:4n-6	ND	1.85 ± 0.87 (-)
C20:5n-3	2.83 ± 0.01(30.43)	1.89 ± 0.51(69.57[c])
C22:5 n-6	9.87 ± 2.66 (23.27)	10.44 ± 5.06 (76.73[c])
C22:6 n-3	34.83 ± 7.97 (31.53)	25.35 ± 7.24 (68.47[c])

Values in parenthesis indicate percent fatty acid distribution of total triacylglycerols present at the sn-1 +sn-3, and sn-2 positions. [a] Values in parenthesis are(% fatty acid at the sn-2 position / % fatty acid in triacylglycerols X 3) X 100; for sn1 + sn-3 = 100 – sn-2

Table 4.11 Positional distribution (sn-2 and sn-1+ sn-3) of fatty acids in lipase-assisted acidolysis of the OMEGA-GOLD oil with capric acid

Fatty acid	Position	
	sn-2[a]	sn-1 + sn-3
C10:0	4.25 ±1.31 (5.05)	14.09 ± 2.70 (94.94 [c])
C14:0	3.70 ± 0.47 (24.42)	6.21 ± 1.91 (75.58[c])
C16:0	12.95 ± 3.90 (32.73)	13.75 ± 3.45 (67.27[c])
C20:4n-6	2.95 ± 0.01 (80.60 [c])	3.69 ± 0.52 (19.40)
C22:5 n-6	13.40 ± 1.91 (42.09[c])	11.86 ± 1.02 (57.90)
C22:6 n-3	30.07 ± 3.30 (38.96[c])	33.86 ± 6.83 (61.04)

Values in parenthesis indicate percent fatty acid distribution of total triacylglycerols present at the sn-1 + sn-3, and sn-2 positions. [a] Values in parenthesis are(% fatty acid at the sn-2 position /% fatty acid in triacylglycerols X 3) X 100; for sn-1 + sn-3 = 100 –sn-2 Fatty acids (C16: 1 and C20: 5 n-3) were present at 0-0.5 % (not shown)

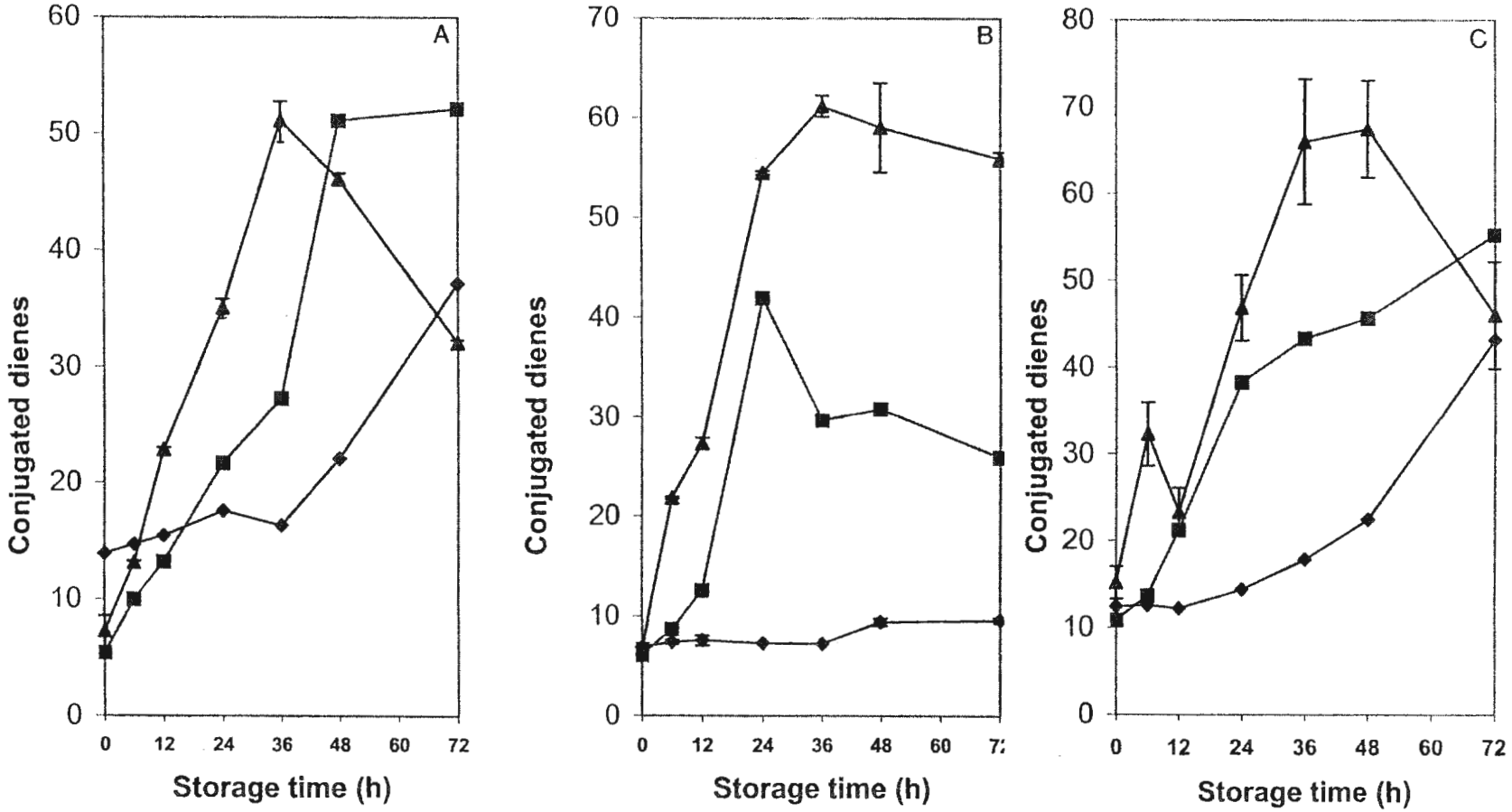
over the storage period from 6 to 72 h. As the storage time was extended from 6 to 72 h, the CD values of the modified OMEGA-GOLD and the original oils increased gradually, thus reflecting the formation of more primary products of lipid oxidation. The CD of the modified OMEGA-GOLD oil increased to 55.24 after 72 h of storage under Schaal oven conditions at 60°C, whereas the corresponding CD value of the unmodified oil was 37.11. This is in contrast to the expectation as incorporation of capric acid (saturated) into the OMEGA-GOLD oil should increase its resistance to oxidation. The present results indicate that enzymatically modified OMEGA-GOLD oil was less stable than the original oil. This observation might possibly be due to the loss of natural antioxidants present in the oil during preparation of SL. This possibility was validated when starting materials were subjected to the same reaction process in the absence of enzymes. The oils so treated were indeed considerably less stable than the unmodified control oil (Figure 4.5). In addition, access of oxygen to the unsaturated sites might be easier in the modified oils containing medium-chain CA as compared to long-chain fatty acids in the unmodified counterparts.

The CD values of SL produced via acidolysis of ARASCO with CA as well as the original oil are shown in Figure 4.5 (A). Results are also shown for subjecting the reactants, in the absence of enzyme, to the same reaction process. The control unmodified (original) oil was stable under oxidative conditions from 6 to 36 h of storage. As the storage time increased from 36 to 72 h, the CD values of the original ARASCO increased steadily and reached its maximum value (37.11) at 72 h. The CD of modified ARASCO oil increased progressively with the time of storage (6 to 48 h) under Schaal oven conditions at 60°C. The highest CD value (51.10) was obtained at 48 h, after which the

CD value reached a plateau. The values of conjugated dienes were higher in ARASCO subjected to the same reaction process in the absence of any enzyme over the entire storage period. The values of conjugated dienes were higher in modified ARASCO than in the original oil from 24-72 h. The results show that modified ARASCO was less stable than the original oil. This was demonstrated to be, in part, due to the loss of natural antioxidants during the preparation of SL, as shown here and also explained earlier for the OMEGA-GOLD oil and as supported by the findings of Akoh and Moussata (2001) who reported a significant loss of tocopherols in fish-, and canola-based SL containing caprylic acid. Considerable loss of tocopherols occurs during operations involved in the process. Theoretically, incorporation of CA (saturated fatty acid) into highly unsaturated oils should have enhanced their oxidative stability.

Figure 4.5 (B) shows the conjugated diene values of DHASCO modified in the presence of lipase and without lipase as well as the control unmodified oil. The CD values of unmodified oil did not change, during storage from 6 to 72 h, indicating the stability of the unmodified DHASCO. As the storage time was extended to 24 h, the CD values of DHASCO-based SL sharply increased and peaked (41.92) at 24 h. The sharp increase in the CD might be accounted for by the formation of more and more primary products of oxidation such as hydroperoxides. After 24 h of storage, the CD values decreased to 27.31, possibly due to the breakdown of unstable hydroperoxides. The oxidative stability of modified DHASCO oil reached a plateau. The present results indicate that a modification of DHASCO oil resulted in its lower stability, similar to other oils examined here.

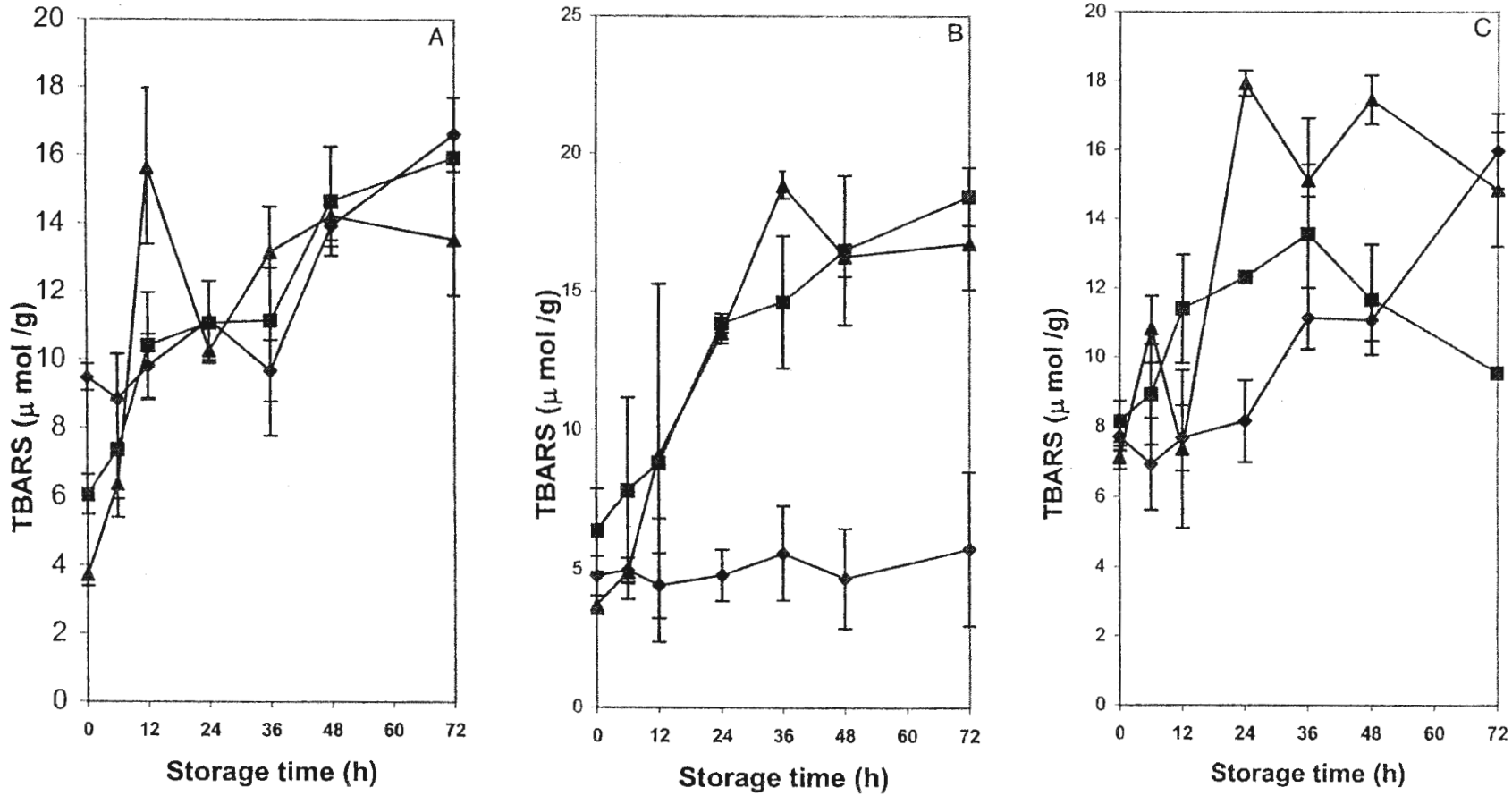
Figure 4.5 Conjugated diene values of (♦) unmodified, (■) modified, and (▲) the corresponding oils subjected to the same reaction conditions in the absence of enzyme of ARASCO (A), DHASCO (B), and the OMEGA-GOLD oil (C) stored under Schaal oven conditions at 60°C


A
B
C
Conjugated dienes
Storage time (h)
0
10
20
30
40
50
60
70
80
0
12
24
36
48
60
72

4.4.2 Thiobarbituric acid reactive substances (TBARS)

The 2-thiobarbituric acid (TBA) test is widely used for evaluating the secondary products of lipid oxidation related to the amount of total aldehydes in the oxidized lipids. TBARS values of modified DHASCO in the presence of lipase and without lipase as well as the control unmodified oils are shown in Figure 4.6 (B). As the accelerated storage period was extended up to 72 h, TBARS values of enzymatically modified DHASCO increase gradually. The TBARS values of the modified oil were considerably higher than the original oil over the entire storage period. There was no difference ($P > 0.05$) between enzymatically modified DHASCO and the mixture subjected to the same reaction condition, but in the absence of any enzyme. Therefore, removal of antioxidants during the process might be responsible for the compromised stability of the modified product. However, TBARS values of the unmodified control DHASCO remained nearly constant during the entire storage period (6 to 72 h), indicating that the unmodified control oil was stable under Schaal oven conditions at 60°C. The general increase in TBARS values of modified DHASCO during the storage time may be due to the break down of lipid hydroperoxides to produce secondary oxidation products. These results agree with those of Akoh and Moussata (2001) who reported that SL produced via acidolysis of canola and fish oils with caprylic acid had higher TBA values than their unmodified counterparts. The compromised oxidative stability of DHASCO based-structured lipid may be attributed to the loss of natural antioxidants during preparation of SL. Figure 4.6 (A) shows TBARS values of ARASCO modified in the presence of lipase and without

Figure 4.6 TBARS values of (♦) unmodified, (■) modified, and (▲) the corresponding oils subjected to the same reaction conditions in the absence of enzyme of ARASCO (A), DHASCO (B), and the OMEGA-GOLD (C) oil stored under Schaal oven conditions at 60°C


A
B
C
TBARS (μ mol /g)
Storage time (h)
0
12
24
36
48
60
72

lipase as well as the unmodified control oils. The TBARS values of both oils increased steadily over the entire storage period. There was no difference ($P > 0.05$) between modified and unmodified oils. The results reported here indicate that the modification of ARASCO with capric acid led to destabilization of the oil.

TBARS results for the OMEGA-GOLD based-structured lipid and the origin oil are shown in Figure 4.6 (C). Results are also shown for subjecting the reactants, in the absence of enzyme, to the same reaction process. Again, the TBARS values of the unmodified control oil increased progressively as the storage time increased from 6 to72h However, the TBARS values of the modified OMEGA-GOLD oil increased gradually with increasing storage time up to 36 h. After 48 h, TBARS values dramatically decreased.

4.5 Response surface methodology

Response surface methodology is a statistical design that enables one to determine optimal conditions for enzymatically-assisted reactions by conducting a minimum number of experiments. Tables 4.12, 4.13, and 4.14 summarize the experimental data for response variable Y_1, Y_2, and Y_3 (% CA incorporation into ARASCO, DHASCO, and the OMEGA-GOLD oil, respectively). Multiple regression coefficients, obtained by employing a least squares procedure to predict the second–order polynomial model for the CA incorporation into the three oils, are summarized in Table 4.15. Testing of these coefficients with the t-test indicated that in modified ARASCO with CA, linear and quadratic terms of reaction temperature (X_2) were highly significant ($p \leq 0.0001$There was a significant interaction ($p \leq 0.01$) between the amount of enzyme (X_1) used and reaction time (X_3).

Table 4.12 Face-centred cube design arrangement and responses for the acidolysis of ARASCO with capric acid[a]

Run	Independent variables			Response [b]
	Enzyme (%)	Temperature (^{O}C)	Time (h)	(Y)
1	2* (-1)**	35(-1)	12(-1)	8.98
2	2 (-1)	35(-1)	36(+1)	8.37
3	2 (-1)	45(0)	24(0)	10.52
4	2 (-1)	55(+1)	12(-1)	8.93
5	2 (-1)	55(+1)	36(+1)	8.86
6	4(0)	35(-1)	24(0)	13.80
7	4(0)	45(0)	12(-1)	15.30
8	4(0)	45(0)	24(0)	18.38
9	4(0)	45(0)	24(0)	18.38
10	4(0)	45(0)	24(0)	18.38
11	4(0)	45(0)	36(+1)	15.65
12	4(0)	55(+1)	24(0)	12.54
13	6(+1)	35(-1)	12(-1)	8.43
14	6(+1)	35(-1)	36(+1)	11.16
15	6(+1)	45(0)	24(0)	19.42
16	6(+1)	55(+1)	12(-1)	9.10
17	6(+1)	55(+1)	36(+1)	10.38

[a] Nonrandomized

[b] Average of triplicate determinations from different experiments

Y = % CA incorporation into ARASCO oil

* Uncoded variable levels

** Coded variable levels

Table 4.13 Face-centred cube design arrangement and responses for the acidolysis of DHASCO with capric acid[a]

Run	Independent variables			Response [b]
	Enzyme (%)	Temperature (^{O}C)	Time (h)	(Y)
1	2* (-1)**	35(-1)	12(-1)	11.20
2	2 (-1)	35(-1)	36(+1)	12.35
3	2 (-1)	45(0)	24(0)	14.76
4	2 (-1)	55(+1)	12(-1)	7.85
5	2 (-1)	55(+1)	36(+1)	16.48
6	4(0)	35(-1)	24(0)	17.22
7	4(0)	45(0)	12(-1)	16.19
8	4(0)	45(0)	24(0)	25.86
9	4(0)	45(0)	24(0)	25.86
10	4(0)	45(0)	24(0)	25.86
11	4(0)	45(0)	36(+1)	20.01
12	4(0)	55(+1)	24(0)	20.37
13	6(+1)	35(-1)	12(-1)	17.55
14	6(+1)	35(-1)	36(+1)	18.32
15	6(+1)	45(0)	24(0)	14.79
16	6(+1)	55(+1)	12(-1)	16.31
17	6(+1)	55(+1)	36(+1)	12.50

[a] Nonrandomized

[b] Average of triplicate determinations from different experiments

Y = % CA incorporation into DHASCO oil

* Uncoded variable levels

** Coded variable levels

Table 4.14 Face-centred cube design arrangement and responses for the acidolysis of the OMEGA-GOLD oil with capric acid[a]

Run	Independent variables			Response [b]
	Enzyme (%)	Temperature (^{O}C)	Time (h)	(Y)
1	2* (-1)**	35(-1)	12(-1)	10.25
2	2 (-1)	35(-1)	36(+1)	11.16
3	2 (-1)	45(0)	24(0)	11.99
4	2 (-1)	55(+1)	12(-1)	13.00
5	2 (-1)	55(+1)	36(+1)	13.20
6	4(0)	35(-1)	24(0)	12.59
7	4(0)	45(0)	12(-1)	14.50
8	4(0)	45(0)	24(0)	20.84
9	4(0)	45(0)	24(0)	20.84
10	4(0)	45(0)	24(0)	20.84
11	4(0)	45(0)	36(+1)	20.09
12	4(0)	55(+1)	24(0)	19.63
13	6(+1)	35(-1)	12(-1)	12.55
14	6(+1)	35(-1)	36(+1)	15.51
15	6(+1)	45(0)	24(0)	22.59
16	6(+1)	55(+1)	12(-1)	13.30
17	6(+1)	55(+1)	36(+1)	13. 93

[a] Nonrandomized

[b] Average of triplicate determinations from different experiments

Y = % CA incorporation into OMEGA-GOLD oil

* Uncoded variable levels

** Coded variable levels

Table 4.15 Regression coefficients of predicted quadratic polynomial model for response (Y)

Coefficients[a]	ARASCO, CA (%) (Y_1)	DHASCO, CA (%) (Y_2)	OMEGA-GOLD, CA (%) (Y_3)
β_0	-111.58***	-44.1407	-108.6707**
Linear			
β_1	-0.1158	17.2919	0.5932
β_2	5.1781***	0.9322	4.7512**
β_3	1.0884***	0.7619	1.4204*
Quadratic			
β_{11}	-0.0275	-1.5956	0.1459
β_{22}	-0.0575***	-0.0093	-0.0492***
β_{33}	-0.0238	-0.0111	-0.0259*
Interactions			
β_{12}	-0.001	-0.0490	-0.0351
β_{13}	0.0271*	-0.0668	0.0129
β_{23}	-0.004	-0.0030	-0.0032
β_{123}	-	-	-
R^2	0.99	0.73	0.92
CV%	3.97	23.38	11.07

[a] Coefficients refer to the general model

R^2 = coefficient of determination

CV%= coefficient of variation

* Significant at ($p \leq 0.01$); ** Significant at ($p \leq 0.001$); *** Significant at ($p \leq 0.0001$)

However, interactions of the amount of enzyme (X_1) and reaction temperature (X_2) and also reaction time (X_3) and temperature (X_2) were not significant ($p>0.1$). In modified DHASCO with CA, all linear, second order, and interaction were not significant ($p> 0.1$). The coefficients obtained for OMEGA-GOLD-based SL showed that linear and quadratic terms of reaction temperature (X_2) were highly significant ($p\leq 0.0001$), while linear and quadratic terms of reaction time (X_3) were significant ($p\leq 0.01$). However, interactions of amount of enzyme (X_1) and reaction temperature (X_2), or reaction time (X_3) were not significant ($p> 0.1$). Thus, these results indicate that linear and quadratic interaction effects of the amount of enzyme, and reaction temperature are the primary determining parameters for CA incorporation into ARASCO and the OMEGA-GOLD oil.

The coefficient of determinations for Y_1 (ARASCO, CA%) (R^2 =0.99) indicate that the fitted model could explain 99 % of the variations. The coefficient of determinations for Y_2 (DHASCO, CA %), and Y_3 (OMEGA-GOLD, CA %) were 0.73, and 0.92, respectively. Coefficient of variations (CV) for ARASCO model was less than 5 %, thus indicating that this model is reproducible. However, coefficient of variations for DHASCO and the OMEGA-GOLD oil models were more than 5 % pointing out these models were not reproducible.

Canonical analysis is a mathematical method used to locate the stationary point of the response surface and to determine whether it represents a maximum, minimum or saddle point (Montgomery, 1991). Therefore, to characterize the nature of the stationary points, canonical analysis was performed on the second-order polynomial models to examine the overall shape of the response surface curves. The canonical forms of the

equations for incorporation of CA (%) into ARASCO (Y_1), DHASCO (Y_2), and the OMEGA-GOLD oil (Y_3) were:

$Y_1 = 20.05 - 0.09\ W_1^2 - 3.46\ W_2^2 - 5.75\ W_3^2$

$Y_2 = 22.590 - 0.08\ W_1^2 - 1.59\ W_2^2 - 6.55\ W_3^2$

$Y_3 = 26.71 + 0.61\ W_1^2 - 3.71\ W_2^2 - 4.96\ W_3^2$

Where W_1, W_2, and W_3 are the axes of the response surface. All the eigenvalues were negative for Y_1 and Y_2 indicating that the stationary point was a maximum for both models. However, the eigenvalues were positive for CA incorporation into the OMEGA-GOLD oil (Y_3) model pointing that the stationary point was a saddle.

Table 4.16 shows critical values for the three factors (enzyme load, reaction time and temperature) examined. The stationary point for the degree of CA incorporation (%) into ARASCO by acidolysis reaction reached a maximum of 20.0 % at 12.3 % enzyme, at 45° and reaction time of 29.4 h. The maximum incorporation of 22.6 % into DHASCO was predicted at 4.2 % of enzyme load at 43.3°C over 27.1 h. Meanwhile, the stationary point for the CA incorporation into the OMEGA-GOLD oil via acidolysis predicted a maximum of 20.7 % at 2.5 % enzyme, 46.6°C for 25.2 h.

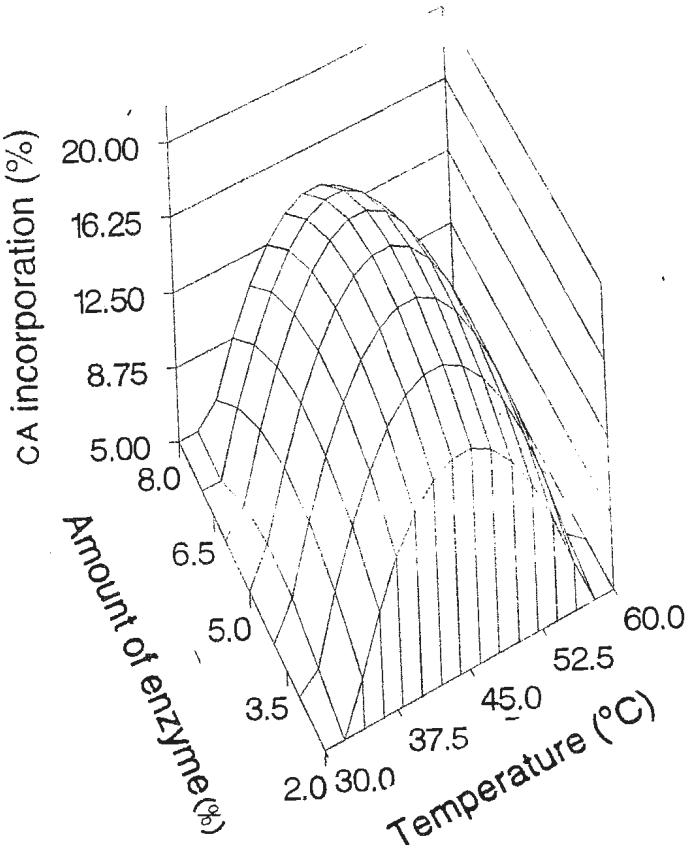
Table 4.16 Canonical analysis of response surface

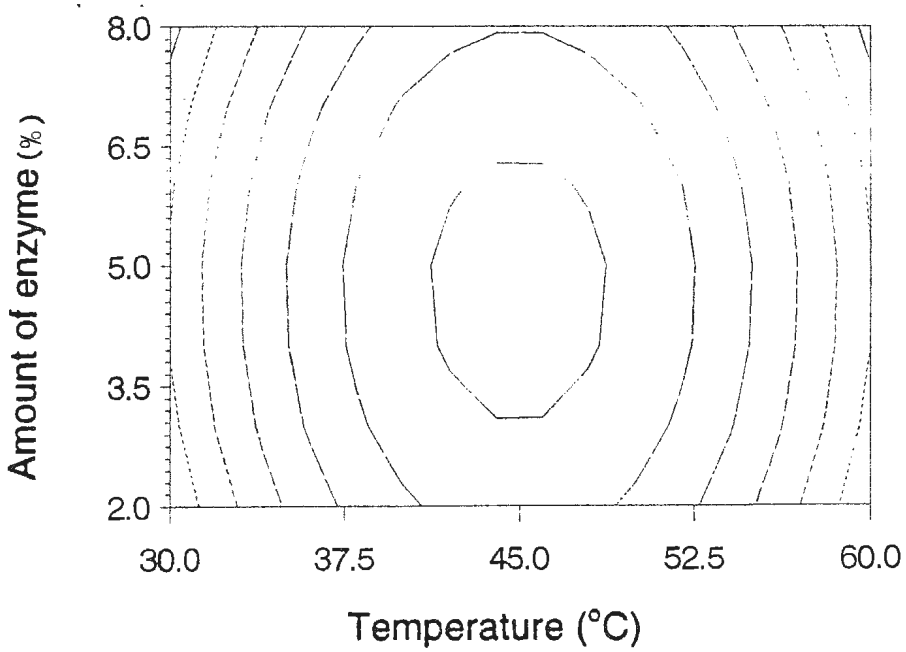
Factor	ARASCO, CA (%)	DHASCO, CA (%)	OMEGA-GOLD, CA (%)
Amount of enzyme (w %, X1)	12.3	4.2	2.5
Reaction temperature (^{0}C, X2)	45.0	43.3	46.6
Reaction time (h, X3)	29.4	27.1	25.2
Stationary point	Maximum	Maximum	Saddle
Predicted value [a]	20.0	22.6	20.7
Observed value [b]	21.45 ± 3.56	22.19 ± 0.99	18.37 ± 1.92

[a] Predicted using the polynomial model

[b] Mean of triplicate determinations from different experiments ± SD

Figure 4.7 Three-dimensional response surface and two-dimensional contour plot demonstrating the effects of enzyme amount and reaction temperature on the predicted CA incorporation (%) in ARASCO

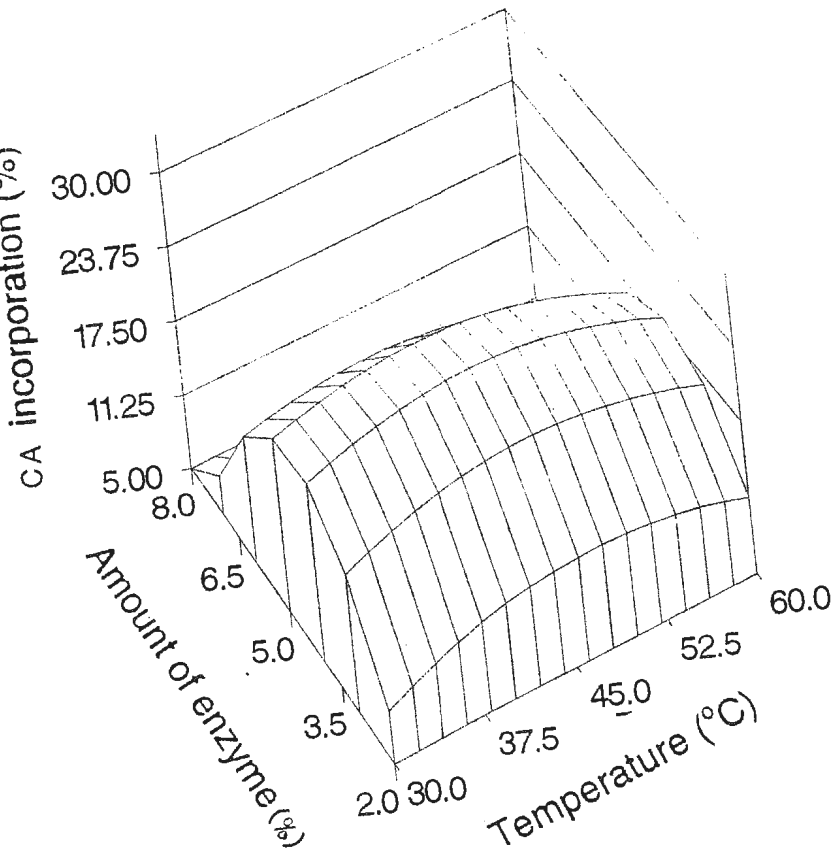




CA incorporation (%)

——	−0.03	·····	2.83
- - - -	5.68	— — —	8.54
— — —	11.40	—— —	14.25
——	17.11		

Figure 4.8 Three-dimensional response surface and two-dimensional contour plot demonstrating the effects of enzyme amount and reaction temperature on the predicted CA incorporation (%) in DHASCO


CA incorporation (%)
30.00
23.75
17.50
11.25
5.00
8.0
6.5
5.0
3.5
2.0
Amount of enzyme (%)
30.0
37.5
45.0
52.5
60.0
Temperature (°C)

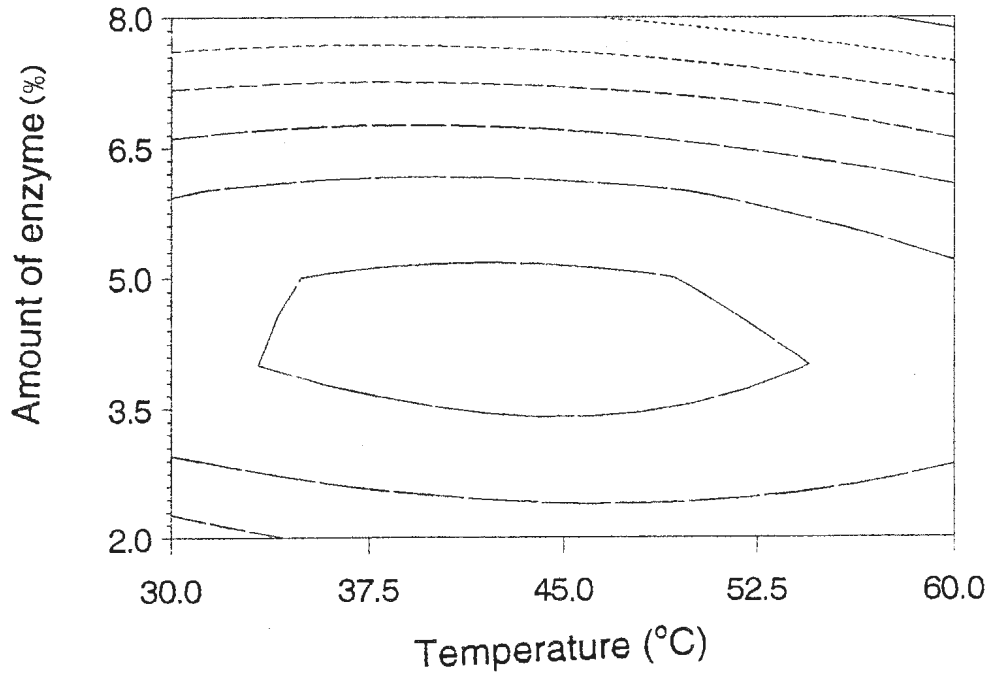
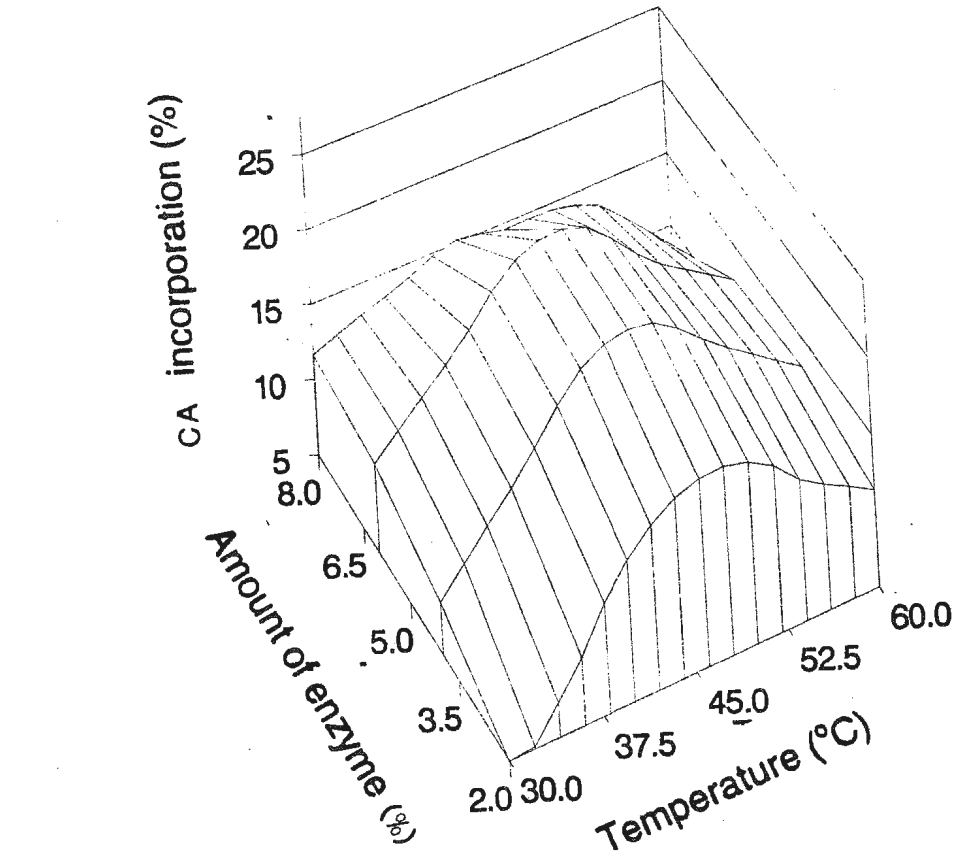
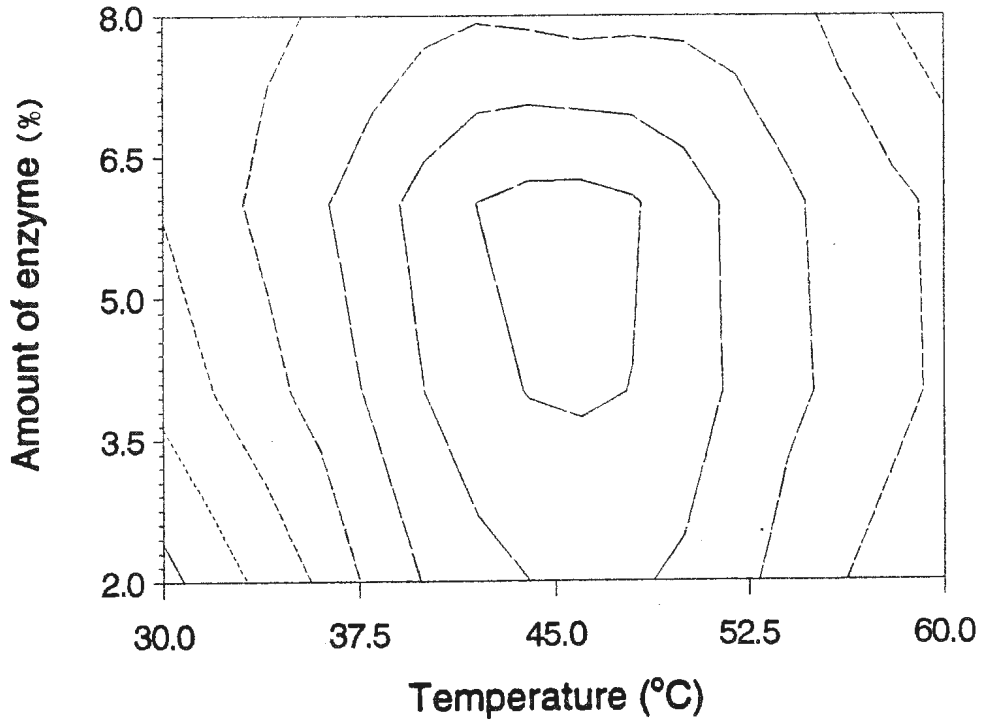

Amount of enzyme (%)
8.0
6.5
5.0
3.5
2.0
30.0
37.5
45.0
52.5
60.0
Temperature (°C)
% CA
−7.76
−3.06
1.65
6.35
11.06
15.76
20.47

Figure 4.9 Three-dimensional response surface and two-dimensional contour plot demonstrating the effects of enzyme amount and reaction temperature on the predicted CA incorporation (%) in the OMEGA-GOLD oil





% CA — 6.14 — 8.44 — 10.74 — 13.04 — 15.34 — 17.65 — 19.95

CONCLUSIONS AND FUTURE WORK

Screening of five commercially-available lipase for incorporation of capric acid (CA) into algal oil indicated that lipase PS-30 from *Pseudomonas sp.* was most effective. Of the various reaction parameters examined, namely the mole ratio of substrates, enzyme amount, time of incubation, reaction temperature, and the amount of added water, for CA incorporation into the oils, the optimum conditions were oil to CA mole ratio of 1:3 at a temperature of 45°C and incubation time of 24 h. Response surface methodology (RSM) was employed in subsequent experiments. In ARASCO-based SL, maximum incorporation of CA (20.0 %) was obtained at a 12.3 % enzyme, a reaction temperature of 45°C and reaction time of 29.4 h. The maximum incorporation of CA into DHASCO (22.6 %) was obtained when enzyme amount, reaction temperature and time were 4.2 %, 43.3°C and 27.1 h, respectively. Similarly, optimization of reaction variables gave a maximum of 20.7 % CA incorporation into the OMEGA-GOLD oil at 2.5 % enzyme, a reaction temperature of 46.6°C for 25.2 h.

Examination of positional distribution of fatty acids on the glycerol backbone of modified ARASCO, DHASCO and the OMEGA-GOLD oil with CA showed that for all oils tested, CA was located in the sn-1,3 positions of the TAG molecules. Arachidoinc acid (ARA) was mainly located at the sn-2 position of the TAG molecule of modified ARASCO. Positional analysis of DHASCO-based structured lipids also showed that DHA was favorably present in the sn-2, but also located in the sn-1 and sn-3 positions. DHA or DPA was mainly esterified to the sn-2 position of the TAG molecule of the modified OMEGA-GOLD oil. Modified oils so prepared are expected to serve better

than their physical mixtures in terms of their digestion and absorption in the body. However, clinical studies should be performed to test this hypothesis.

The oxidative stabilities of modified ARASCO, DHASCO, the OMEGA-GOLD oils were compared with those of the original oils. Among the oils examined, enzymatically modified oils had a higher conjugated dienes (CD) values than their unmodified counterparts. In general, 2-thiobarbituric acid reactive substances (TBARS) of modified DHASCO and the OMEGA-GOLD oil were significantly higher than their original oils. TBARS values of both modified and unmodified ARASCO oils increased progressively over the whole storage period. Furthermore, there was no significant difference between TBARS values of both modified and unmodified ARASCO oils. The results presented here suggest that enzymatically modified oils were more susceptible to oxidation than their unmodified counterparts. Removal of natural antioxidants during oil modification might play a significant role in rapid oxidative deterioration of enzymatically modified oils. This possibility was confirmed when starting materials were subjected to the same reaction process in the absence of enzymes. The oils so treated were indeed significantly less stable than the control unmodified oils. Therefore, it is recommended to verify the loss of natural antioxidants during preparation of SL. Furthermore, replacement of bulky acyl groups with medium-chain fatty acid (CA) in the modified oils might enhance access of oxygen to the unsaturated sites as compared to the unmodified counterparts. Consequently, it is recommended to stabilize the modified ARASCO, DHASCO, and the OMEGA-GOLD oils by addition of adequate amounts of appropriate antioxidants to protect them against oxidation. Furthermore, it is recommended that solvent-free systems be developed for modification of ARASCO,

DHASCO, and the OMEGA-GOLD oils in order to eliminate the possibility of contamination of products with solvent residues and other unwanted chemicals.

References

Abou-Gharbia, H.A., A.A.Y. Shehata, M. Youssef, and F. Shahidi, Oxidative stability of sesame paste (Tehinal. J. Food Lipids, **3**: 129-137 (1996).

Akoh, C.C., Structured lipids-enzymatic approach. Inform **6**: 1055-1061 (1995).

Akoh, C.C., Enzymatic modification of lipids, In: Food Lipids and Health(R. E. McDonald and D. B. Min, Eds.) Marcel Dekker Inc., New York, NY, pp117-137 (1996).

Akoh, C.C., Making new structured fats by chemical reaction and enzymatic modification. Lipid Tech., **9**: 61-66 (1997).

Akoh, C.C., and L.N. Yee, Enzymatic synthesis of position-specific low-calorie structured lipids. J. Am. Oil Chem. Soc., **74**: 1409-1413 (1997).

Akoh, C.C., Structured lipids containing omega-3 highly unsaturated fatty acids. In: Omega-3 Fatty Acids: Chemistry, Nutrition, and Health Effects(F. Shahidi and J.W. Finley, Eds.) Am.Chem. Soc., Washington, DC, pp151-161 (2001).

Akoh, C.C., Structured lipids. In: Food Lipids: Chemistry, Nutrition, and Biotechnology (C.C. Akoh and D.B. Min, Eds.) Marcel Dekker Inc., New York, NY, pp 877-908 (2002).

Akoh, C.C., and K.H. Huang, Enzymatic synthesis of structured lipids: transesterification of triolein and caprylic acid. J. Food Lipids, **2**: 219-230 (1995).

Akoh, C.C., and B.H. Jennings, and D.A. Lillard, Enzymatic modification of evening primrose oil: incorporation of ω-3 polyunsaturated fatty acids. J. Am. Oil Chem. Soc., **73**: 1059-1062 (1996).

Akoh, C.C., and C.O. Moussata, Lipase-catalyzed modification of borage oil: incorporation of capric and eicosapentaenoic acids to form structured lipids. J. Am. Oil Chem. Soc., **75**: 697-701 (1998).

Akoh, C.C., and C.O. Moussata, Characterization and oxidative stability of enzymatically produced fish and canola oil-based structured lipids. J. Am. Oil Chem. Soc., **78**: 25-30 (2001).

AOCS, Official Methods and Recommended Practices of the American Oil Chemists Society. 4th edn., AOCS Press, Champaign, IL (1990).

Arouma, O.I., Free radicals, oxidative stress, and antioxidants in human health and disease. J. Am. Oil Chem. Soc.,**75**: 149-153 (1998).

Arterburn, L.M., K.D. Boswell, T. Lawlor, M.H. Cifone, H. Murli, and D.J. Kyle, *In vitro* genotoxicity testing of ARASCO and DHASCO oils. Food and Chemical Toxicology, **38**: 971-976 (2000a).

Arterburn, L.M., K.D. Boswell, S.M. Henwood, and D.J. Kyle, Adevelopmental safetystudy using DHA- and ARA-rich single-cell oils. Food and Chemical Toxicology, **38**: 763-771(2000b).

Arterburn, L.M., K.D. Boswell, E-K. Koskelo, S.L. Kassner, C. Kelly, and D.J. Kyle, Acombined subchronic (90-days) toxicity and neurotoxicity study of a single-cell source of DHA triglyceride (DHASCO oil). Food and Chemical Toxicology, **38**: 35-49 (2000c).

Artz, W.E. and S.L Hansen, Current developments in fat replacers. In: Food Lipids and Health (R.E. McDonald, and D.B. Min, Eds.) Marcel Dekker, Inc., NY, pp 385-415 (1996).

Babayan, V.K., Medium-chain triglycerides and structured lipids. Lipids **22**: 417-420(1987).

Babayan, V.K., G.L Blackburn, and B.R. Bistrian, Structured lipid containing dairy fat. United States Patent # 4, 1990, 952, 606.

Bell, S.J., E.A. Mascioli, B.R. Bistrian, V.K. Babayan, and G.L. Blackburn, Alternative lipid sources for enteral and parenteral nutrition: long- and medium-chain triglycerides, structured triglycerides, and fish oils. J. Am. Diet. Assoc., **91**: 74-78(1991).

Benzi, I.F.F., Lipid peroxidation: a review of causes, consequences, measurement and dietary influences. Int. J. Food Sci. Nutr., **47**: 233-261(1996).

Bezard, J. and M. Bugaut, Absorption of glycerides containing short, medium and long chain fatty acids. In: Fat Absorption (A. Kuksis, Ed.) CRC Press, Boca Raton, FL, pp 119-158 (1986).

Birch, E.E., D.R. Hoffman, R. Uauy, D.G. Birch, C. Prestidge. Pediatr. Res., **44**:201-209 (1998).

Bonanone, A., and S.M. Grundy, Effect of dietary stearic acid on plasma cholesterol and lipoprotein levels. N. Engl. J. Med., **318**: 1244-1249 (1988).

Bower, R.H., J.M. Daly, M.D. Lieberman, J. Goldfine, J. Shon, F. Weintraub, E.F. Rosata, and P. Lavin, Early enteral administration of a formula (Impact^{R}) supplemented with arginine, nucleotides, and fish oil in intensive care unit patient: results of multicenter prospective, randomised clinical trial. Crit. Care Med., **23**: 436-449(1995).

Brekenridge, W.C., and A. Kuksis, Molecular weight distribution of milk fat triglycerdes from seven species. J. Lipid Res., **8:** 473-478(1967).

Carlson, S.E., S. Mehra, W.J. Kagey, K.L. Merkel, D.A. Diersen-Schade, C.L. harris, and J.W. Hansen. Pediatr. Res., **45**: 278A-281A(1999).

Chin, S.F., W. Liu, J.M. Storkson, Y.L. Ha, and M.W. Pariza, Dietary sources of conjugated dienoic isomers of linleic acid, a newly recognized class of anticarcinogens. J. Food Comp. Anal., **5**: 185-197 (1992).

Chow, C.K., Biological effects of oxidized fatty acids. In: Fatty Acids in Foods and their Health Implications (C.K. Chow, Ed.) Marcel Dekker Inc., pp 689-705 (1992).

Christie, W.W., Structural analysis of lipids by means of enzymatic hydrolysis. In: Lipid Analysis. (W.W. Christie, Ed.) Pergamon Press, new York, NY, pp 155-166 (1982).

Cohen, L.A., and D.O. Thompson, The influence of dietary medium chain triglycerides on rat mammary tumor development. Lipids, **22**: 455-461 (1987).

Cohen, L.A., and D.O. Thompson, Y. Maeura, and J.H. Weisburger,The influence of dietary medium chain triglycerides on the development of N-methylnitrosamine- rat mammary tumors. Lipids, **44**: 5023-5028 (1984).

Crosby, L.E., E.S. Swenson, V.K. Babayan, N. Istfan, G.L. Blackburn, and B.R. Bistrain, Effect of structured lipid-enriched total parental nutrition in rats bearing Yoshida Sarcoma. J. Nutr., **1:** 41-47 **(**1990).

Daly, J.M., M. Lieberman, J. Goldine, J. Shon, F.N. Weintraub, F.F. Rosato, and P. Lavin, Enteral nutrition with supplemental arginine, RNA and omega-3 fatty

acids: a prospective clinical trial. J. Parenteral and Enteral Nutr., **15:** supplement (1991).

DeMichele, S.J., M.D. Karlstad, V.K. Babayan, N. Istfan, G.L. Blackburn, and B.R. Bristrain, Enhanced skeletal muscle and liver protein synthesis with structured lipid in enterally fed burned rats. Metabolism, **8:** 788-795 (1988).

Diersen-Schade, D.A., J.W. Hansen, C.L. Harris, K.L.. Merkel, K.D. Wisont, and J.A. Boettcher, In: Essential fatty Acids and Eicosanoids (R.A. Riemersona, R. Armstroma, R.W. Kelly, R. Wilson, Eds.) AOCS Press Champaign, USA, (1999).

Finley, J.W., L.P. Klemann, G.A. Levelle, M.S. Otterburn, and C.G. Walchak, Caloric availability of Salatrium in rats and humans. J. Agric. Food Chem., **42**:495-499 (1994).

Frankel, E.N., Lipid oxidation: mechanisms, products and biological significance. J. Am. Oil Chem. Soc., **61**: 1908-1917 (1984).

Gandhi, N.N., Application of lipase. J. Am. Oil Chem. Soc., **74**: 621-634 (1997).

Gao, L. and G. Mazza, Extraction of anthocyanin pigments from purple sunflower. J. Food Sci., **61:** 600-603 (1996).

Ghosh, S., and D.K. Bhattacharyya, Medium-chain fatty acid rich glycerides by chemical and lipase-catalyzed polyester-monoester interchange reaction. J. Am. Oil Chem. Soc., **74**: 593-595 (1997).

Gollaher, C.J., E.S. Swenson, E.A. Mascioli, V.K. Babayan, G.L. Blackburn, and B.R. Bistrain, Dietary fat level as determinant of protien-sparing actions of structured triglycerides. Nutrition, **8**: 348-352(1992).

Gottenbos, J.J., Nutritional evaluation of n-6 and n-3 polyunsaturated fatty acids. In: Dietary Fat Requirement in Health and Development(J. Beare-Rogers, Ed.) J. Am Oil Chem. Soc., pp 107 (1988).

Gray, J.I., and F.J. Monahan, Measurement of lipid oxidation in meat and meat products. Trends Food Sci. Technol., **3:** 320-324 (1992).

Halliwell, B., and J.M.C. Gutteridge, In: Free Radical in Biology and Medicine. Oxford University Press Inc., NY, pp 1-104 (1999).

Hashim, A., and V.K. Babayan, Studies in man of partially absorbed dietary fats. Am. J. Clin. Nutri., **31**: S273(1987).

Haumann, B.F., Structured lipids allow fat. Inform **8**: 1004-1011 (1997a).

Haumann, B.F., Nutritional aspects of n-3 fatty acids. Inform., **8**: 428-447 (1997b).

Haw, M.P., S.J. Bell, and G.L. Blackburn, Potential of parenteral and enteral nutrition in inflammation and immune dysfunction: A new challenge for dietitians. Am. J. Diet. Assoc., **91**: 701-709(1991).

Heird, W.C., S.M. Grundy, and V.S. Hubbard, Structured lipids and their use in clinical nutrition. Am. J. Clin. Nutr., **43**: 320-324(1986).

Heydinger, J.A. and D.K. Nakhasi, Medium chain triacylglycerols. J. Food Lipids, **3**: 251-257(1996).

Hill, W.J., and W.G. Hunter, A review of response surface methodology: A literature survey. Technometrics, **8**: 571-575 (1966).

Holub, B.J., Docosahexaenoic acid in human health. In: Omega-3 Fatty Acids: Chemistry, Nutrition, and Health Effects(F. Shahidi and J. W. Finley, Eds.) American Chemical Society, Washington, DC, pp 54-65 (2001).

Huang, K., and C.C Akoh, Enzymatic synthesis of structured lipids: transesterification of triolein and caprylic ethyl ester. J. Am. Oil Chem. Soc., **73**: 245-250 (1996a).

Huang, K., and C.C. Akoh, Optimization and scale-up of enzymatic synthesis of structured lipids using RSM. J. Food Sci, **61**: 137-141 (1996b).

Huang, F.C., Y.H. Ju, and C.W. Huang, γ-linolenic acid rich triacylglycerol derived from borage oil via lipase-catalyzed reactions. J Am. Oil Chem. Soc., **76**: 833-837 (1999).

Huang, F.C., Y.H. Ju, and J.C. Chiang, Enrichment of γ-linolenic acid from borage oil via lipase-catalyzed reactions. J Am. Oil Chem. Soc., **74**: 977-981 (1997).

Ikeda, I., Y. Tomari, M. M. Sugano, S. Watanabe, and J. Nagata, Lymphatic absorption of structured glycerides containing medium-chain fatty acids and linoleic acid, and their effect on cholestrol absorption in rats. Lipids, **26**: 369-373 (1991).

Illingworth, D.R., and D. Ullmann, Effects of omega-3fatty acids on risks factors for cardiovascular disease, In Omega-3 Fatty Acids in Health and Disease, (R.S. Lees and M. Karel, Eds.) Marcel Dekker, Inc., NY, pp 39-69 (1990).

Irimescu, R., M. Yasui, Y. Iwasaki, N. Shimidzu, and T. Yamane, Enzymatic synthesis of 1,3-dicaprylol-2-eicosapentaenoylglycerol. J. Am. Oil Chem. Soc., **77**: 501-505(2000).

Iwasaki, Y., J.J. Han, M. Narita, R. Rosu, and T. Yamane, Enzymatic synthesis of structured lipids from single cell oil of high docosahexaenoic acid content. J. Am. Oil Chem. Soc., **76**: 563-569.

Jandacek, R.J., J.A. Whiteside, B.N. Holcombe, R.A. Volpenhein, and J.D. Taalbee, The rapid hydrolysis and efficient absorption of triglycerides with octaenoic acid in the 1 and 3 positions and long-chain fatty acid in the 2 position. Am. J. Clin. Nutr., **45**: 940-945 (1987).

Jennings, B.H., and C.C. Akoh, Enzymatic modification of triacylglycerols of high eicosapentaenoic and docosahexaenoic acids content to produce structured lipids. J. Am. Oil Chem. Soc., **76**: 1133-1137 (1999).

Jennings, B.H., and C.C. Akoh, Lipase-catalyzed modification of rice bran oil to incorporate capric acid. J. Agric. Food Chem., **48**: 4439-4443(2000).

Jensen, G.L., and R.G. Jensen, Speciality lipids for infant nutrition II concernsd, new developments, and future applications. J. Pediatr. Gastroenterol. Nutr., **15**: 382-394 (1992).

Jensen, G.L., N. McGarvey, R. Taraszewsk, S.K. Wixson, D.L. Seidner, T. Pai, Y.Y. Yeh, T.W. Lee, and S.J. DeMichele, Lymphatic absorption of enterally fed structured triacylglycerol vs physical mix in a canine model. Am. J. Clin. Nutr., **60** : 518-524 (1994).

Kawashima, A., Y. Shimada, M. Yamamoto, A. Sugihara, T. Nagao, S. Komemushi, and Y. Tominaga, Enzymatic synthesis of high-purity structured lipids with caprylic acid at 1,3-postions and polyunsaturated fatty acid at 2-position. J. Am. Oil Chem. Soc., **78**: 611-616 (2001).

Kawashima, A., Y. Shimada, T. Nagao, A. Ohara, T. Matsuhisa, A. Sugihara, and Y. Tominaga, Production of structured TAG rich in 1,3-dicapryloyl-2-γ-linolenoyl glycerol from borage oil. J. Am. Oil Chem. Soc., **79**: 871-877 (2002).

Ke, P.J., and R.G. Ackman, Metal-catalyzed oxidation in mackerel skin and meat lipids. J. Am. Oil Chem. Soc., **53**: 336-640 (1976).

Kennedy, J.P., Structured lipids: fats of the future. Food Technol., **38**: 76-83 (1991).

Kim, I.-H., H. Kim, K.-T. Lee, S.-H. Chung, and S.-N. Ko, lipase-catalyzed acidolysis of perilla oil with caprylic acid to produce structured lipids. J. Am. Oil Chem. Soc., **79**: 363-367 (2002).

King, D.L., T.S. Hahm, and D.B. Min, Chemistry of antioxidants in relation to shelflife of foods, In: Shelf Life Status of Foods and Beverages, Chemical, Biological, Physical and Nutritional Aspects (G. Charalambous, Ed.) Elsevier Applied Science, NY, pp 629-705 (1995).

Kyle, D.J., The large-scale production and use of a single-cell oil highly enriched in docosahexaenoic acid. In: Omega-3 Fatty Acids: Chemistry, Nutrition, and Health Effects (F. Shahidi and J.W. Finley, Eds.) American Chemical Society, Washington, DC, pp 92-105 (2001).

Lee, K.-T., and C.C. Akoh, Effects of selected substrate forms on the synthesis of structured lipids by two immobilized lipases. J. Am. Oil Chem. Soc., **74**: 579-584 (1997).

Lee, K-T., and C.C. Akoh, Structured lipids: synthesis and application. Food Res. Inter., **14**: 17-34 (1998).

Lee, T.W. and C.I. Hastilow, Quantitative determination of triacylglycerol profile of structured lipid by capillary supercritical fluid chromatography and high-temperature gas chromatogaphy. J. Am. Oil Chem. Soc., **76**:1405-1413(1999).

Ling, P.R., N.W. Istfan, S.M. Lopes, V.K. Babayan, G.L. Blackburn, and B.R. Bistrian, Structured lipids made from fish oil and medium-chain triglycerides alters tumor and host metabolism in Yoshida Sarcoma-bearing rats. Am. J. Clin. Nutr., **53**: 1177-1184(1991).

Logani, M.K., and R.E. Davies, Lipids oxidation: Biological effects and antioxidants. Lipids, **15**: 485-495 (1980).

Mascioli, E.A., B.R. Bistrian, V.K. Babayan, and G.L. Blackbum, Medium chain triglycerides and structured lipids as unique nonglucose energy source in hyperalimentation, Lipids, **22**: 421-423 (1987).

Mason, R.L., R.F. Gunst, and J.L. Hess, Response-surface designs. In: Statistical Design and Analysis of Experiments, John Wily and Sons, NY, pp 204-233 (1989).

McKenna, M.C., V.S. Hubbard, and J.G. Aeri, Linoleic acid absorption for lipid supplements in patients with cystic fibrosis with pancreatic insufficiency and in control subjects. J. Pediatr. Gastrenterol. Nutr., **4**: 45-48(1985).

McNeill, G.P., R.G. Ackman, and S.R. Moore. Lipase-catalyzed enrichment of long-chain polyunsaturated fatty acids. J. Am. Oil Chem. Soc. **73**: 1403-1407 (1996).

McNeill, G.P., and P.E. Sonnet, Low-calorie triglyceride synthesis by lipase-catalyzed esterification of monoglycerides. J. Am. Oil Chem. Soc.,**72**: 1301-1306 (1995).

Mendez, B., P.R. Ling, N.W. Istfan, V.K. Babyan, and B.R. Bistrain, Effects of different lipid sources in total parenteral nutrition on whole body protein kinetics and tumor growth. J. Parenteral Enteral Nutr., **16**: 545-551(1992).

Montgomery, D.C. Response surface methods and designs. In: Design and analysis of experiments. John Wiley & Sons,NY, pp 521-568 (1991).

Moussata, C.O. and C.C. Akoh, Lipase-catalyzed incorporation of oleic acid into melon seed oil. J. Am. Oil Chem. Soc. **74**: 177-179 (1997).

Mu, H., X. Xu, and C.-E.. Hoy, Production of specific-structured triacylglycerols by lipase-catalyzed interesterification in a laboratory-scale continous reactor. J. Am. Oil Chem. Soc., **75**: 1187-1193(1998).

Myher, J.J., A. Kuksis, K. Geher, P.W. Park, and D.A. Diersen-Schade. Lipids, **31**:207-215(1996).

Quinlan, P., and S. Moore, Modification of triglycerides by lipase: process technology and its application to the production of nutritionally improved fats. INFORM, **4**: 580-585(1993).

Rao, N.M., and V.M. Shanmugam, Lipase-mediated synthesis of dodecyl oleate and oleyl oleate in aqueous foams. J. Am. Oil Chem. Soc., **77**: 605-608 (2000).

Rao, R., B. Manohar, K. Sambaiah, and B.R. Lokesh, Enzymatic acidolysis in hexane to produce n-3 or n-6 FA-enriched structured lipids from cocnut oil: optimisation of reactions by Response Surface Methodology. J. Am. Oil Chem. Soc., **79**: 885-890 (2002).

Roediger, W.E.W., and D.A. Rae, Trophic effect of short-chain fatty acids on mucosal handling of ions by the defunctioned colon. Brit. J. Surg., **69**: 23-25 (1982).

Rossell, J.B., Measurement of rancidity. In: Rancidity in Foods (J.C. Allen and R.J. Hailton, Eds.) Blackie Academic and Professional, Bishopbriggs, Glasgow, pp. 22-53 (1994).

Sandstorm, R., A. Hyltande, U. Korner, and K. Lundholm, Structured triglycerides to postoperative patients: a safety and tolerance study. J. Parent. Ent. Nutrit., **17**: 153-157 (1993).

SAS Institute, Inc. Statistical analytical systems user's guide, Vol. 2. SAS Institute Inc., Cary, NC, (1990).

Scott, FW., and N.S. Lee, Recommendations for defining and dealing with functional foods. Report of the Bureau of Nutritional Science Committee on Functional Foods. Food Directorate, Health Protection Branch, Ottawa, 1996.

Sellappan, S., C. Akoh, Synthesis of structured lipids by transesterification of trilinolein catalyzed by lipozyme IM60. J. Agric. Food Chem., **49**: 1-9 (2001).

Senanayake, S.P.J.N., and F. Shahidi, Enzyme-assisted acidolysis of borage(*Borage officinalis* L.) and evening primrose (*Oenothera biennis* L.) oils: incorporation of omega-3 polyunsaturated fatty acids. J. Agric. Food Chem., **47**: 3105-3112 (1999).

Senanayake, S.P.J.N., and F. Shahidi, Chemical and stability of structured lipids from borage(*Borage officinalis* L.) and evening primrose (*Oenothera biennis* L.) oils. J. Food Sci., **67**: 2038-2045 (2002).

Senanayake, S.P.J.N., and F. Shahidi, Enzyme-catalyzed synthesis of structured lipids via acidolysis of seal (*Phoca groenlandica*) blubber oil with capric acid. Food Res. Inter., **35**: 745-752 (2002).

Shahidi, F., P.K. Janitha, and U.N. Wanasundara, Phenolic antioxidants. Crit. Rev. Food Sci., **328**: 67-103 (1992).

Shahidi, F., and U.N. Wanasundara, Methods of measuring oxidative rancidity in fats and oils. Food Lipids, Nutrition, and Biotecnology. (Akoh, C.C., and D.B. Min, Eds.). Marcel Dekker Inc., Shahidi, F., and U.N. Wanasundra, pp. 377-396 (1998).

Shahidi, F., and U.N. Wanasundara, and N. Brunet, Oxidative stability of oil from blubber of harp seal (Phoca groenlandica) as assessed by NMR and standard procedures. Food Res. Int., **27**:555-562 (1994).

Shieh, C.-J., C.C. Akoh, and P.E. Koehler, Four-factor response surface optimisation of the enzymatic modification of triolein to structured lipids. J. Am. Oil Chem. Soc., **72**: 619-623 (1995).

Shimada, Y., A. Sugihara, H. Nakano, T. Yokota, T. Nagao, S. Komemushi, and Y. Tominaga, Production of structured lipids containing essential fatty acids by immobilized *Rhizopus delemar* lipase. J. Am. Oil Chem. Soc., **73**: 1415-1419 (1996a).

Shimada, Y., M. Suenaga, A. Sugihara, S. Seiichi, and Y. Tominaga, Continuos production of structured lipids containing γ-linolenic and caprylic acids by immobilized *Rhizopus delemar* lipase. J. Am. Oil Chem. Soc., **76**: 189-193 (1996b).

Shimada, Y., A. Sugihara, K. Maruyama, T. Nagao, S. Nakayama, H. Nakano, Y. Tominaga, Production of structured lipids containing docosahexaenoic and caprylic acids using immobilized *Rhizopus delemar*. J. Ferment. Bioeng., **81**: 299-303 (1996c).

Smith, R.E., J.W. Finley, and G.A. Leveille, Overview of Salatrium, a family of low-calorie fats. J. Agric. Food Chem., **42**: 432-435 (1994).

Soumanou, M.M., U.T., Bornscheuer, U., Menge, and R.D. Schmid, Synthesis of structured triglycerides from peanut oil with immobilized lipase. J. Am. Oil Chem. Soc., **74**: 427-433 (1997).

Soumanou, M.M., U.T., Bornscheuer, U. Schmid, and R.D. Schmid, Synthesis of structured triglycerides lipase catalysis. Fett Lipid, **100**: 156-160 (1998).

Thompson, D., Response surface experimentation. J. Food Proc. and Preser., **6** :155-188 (1982).

Ulrich, H., S.M. Pastores, D.P. Katz, and V. Kvetan, Parenteral use of medium-chain triglycerides: a reappraisal. Nutrition, **12**: 231-238(1996).

Vanderhoof, J., S. Gross, T. Hegyi, T. Clandinin, P. Porcelli, J. DeCristolaro, T. Rhodes, R. Tsang, K. Shattuck, R. Cowett, D. Adamkin, C. McCarton, W. Heird, B. Hook-Morris, G. Pereira, G. Chan, J. VanAerde, F. Boyle, K. Pramuk, A. Euler, E. Lien. J. Pediatr. Gastroenterol, 29:318-326 (1999).

Wanasundara, U.N., and F. Shahidi, and C.R. Jablonski, Comparison of standard and NMR methodologies for assessment of oxidative stability of canola and soybean oils. Food Chem., **52**:249-253 (1995).

Wanasundara, U.N., and F. Shahidi, Lipase-assisted concentration of omega-3 polyunsaturated fatty acids in acylglycerol from marine oils. J. Am. Oil Chem. Soc., **75**: 945-951 (1998).

Wanasundara, U.N., and F. Shahidi, Concentration of omega-3 polyunsaturated fatty acids of seal blubber oil by urea complexation: optimization of reaction conditions. Food Chem., **65**: 41-49 (1999).

Willis, W.M., and A.G. Maragoni, Assessment of lipase and chemically catalyzed lipid modification strategies for the production of structured lipids. J. Am. Oil Chem. Soc., **76**: 443-450 (1999).

Wolin, M.J., Fermentation in the rumen and large intestine. Science, **213**: 1463-1467 (1981).

Wong, M.J., Fermentation in the rumen and large intestine. Science, **213**: 1463-1467(1981).

Wong, W.C., M. Bari, C.N.A. Razak, and A.B. Salleh, Synthesis of medium-chain glycerides using lipase from *Candida rugosa*. J. Am. Oil Chem. Soc., **77**: 85-88 (2000).

Xu, X., Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review. Eur. J. Lipid Sci. Technol., **75** : 287-303 (2000).

Xu, X., L.B. Fomuso, and C.C Akoh, Modification of menhaden oil enzymatic acidolysis to produce structured lipids : optimization by response surface design in a packed bed reactor. J. Am. Oil Chem. Soc., **77**: 171-176 (2000).

Xu, X., T. Porsgaard, H. Zhang, J. Adler-Nissen, and C.-E. Hoy, Production of structured lipids in a packed-bed reactor with *Thermomyces lanuginsoa* lipase. J. Am. Oil Chem. Soc., **79**: 561-565(2002).

Xu, X., S. Balchen, C.-E. Hoy, and J. Adler-Nissen, Pilot batch production of specific-structured lipids by lipase-catalyzed interesterification: primary study on

incorporation and acyl migeration. J. Am. Oil Chem. Soc., **75**: 301-308 (1998).

Yankah, V.V., and C.C. Akoh, Lipase-catalyzed acidolysis of tristearin with oleic or caprylic acids to produce structured lipids. J. Am. Oil Chem. Soc., **77** : 495-500 (2000).

Yang, T.H., Y. Jang, J.J. Han, and J.S. Rhee, Enzymatic synthesis of low-calorie structured lipids in a solvent- free system. J. Am. Oil Chem. Soc., **78**: 291-296 (2001).

Zeller, S., W. Barclay, and R. Abril, Production of docosahexaenoic acid, In Omega-3 Fatty Acids: Chemistry, Nutrition, and Health Effects (F. Shahidi and J.W. Finley, Eds.) American Chemical Society, Washington, DC, pp 108-125 (2001).

Zhou, D., X. Mu, C. H. , and J. Adler-Nissen, Lipase-catalyzed production of structured lipids via acidolysis of fish oil with caprylic acid. J. Food Lipids, **7**: 263-274(2000).