BIOLOGY AND BIOTECHNOLOGY OF MODIFIED OILS

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Biology and Biotechnology of Modified Oils

BY

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Dedicated to My Loving:

Wife, Reem

Sons: Ghassan and Adnan

Mother and Father

Free Homeland

Abstract

The objectives of this study were three fold and these are described in three parts. In the first part incorporation of long-chain n-3 fatty acids (FA) into three types of high-laurate canola oils was examined. Incorporation of the n-3 FA, namely eicosapentaenoic acid (EPA, C20: 5 n-3), docosapentaenoic acid (DPA, C22: 5 n-3), and docosahexaenoic acid (DHA, C22: 6 n-3) into three types of high-laurate canola oils, known as Laurical 15, 25, and 35 with 15, 25, and 35% oleic acid content, respectively, was carried out.

Production of SL via acidolysis of Laurical 15 with EPA, DPA, and DHA was carried out using five lipases from *Candida antarctica*, *Mucor miehei*, *Pseudomonas sp.*, *Aspergillus niger*, and *Candida rugosa*. *Pseudomonas sp.* lipase gave the best incorporation of EPA, DPA, or DHA into Laurical 15. Optimum reaction conditions for EPA incorporation into Laurical 15 were 4% enzyme load, and an oil to EPA mole ratio of 1:3 at 45°C over 36 h. For DPA incorporation into Laurical 15, the optimum conditions were 6% lipase amount, and an oil to DPA mole ratio of 1:2 at 35°C over 48 h. Similarly, incorporation of DHA into Laurical 15 was best achieved at a mole ratio of oil to DHA of 1:3, 10% lipase concentration, at 35°C over 48 h. Lauric acid remained mostly esterified to the *sn*-1,3 positions while EPA, DPA or DHA was also located mainly in the *sn*-1,3 positions of the modified oils. The modified oils were more prone to oxidation than their unmodified counterparts, as evidenced by the 2-thiobarbituric acid reactive substances (TBARS) test.

In another study, response surface methodology (RSM) was employed to obtain a maximum incorporation of EPA or DHA into Laurical 25. Under optimum conditions incorporation of EPA (61.6%) into Laurical 25 was achieved using 4.6% enzyme from

Pseudomonas sp. at 39.9°C over 26.2 h. The corresponding maximum incorporation of DHA into Laurical 25 was 37.3% using 4.79% enzyme from *Pseudomonas sp.*, 46.1°C, and 30.1 h. For EPA-modified Laurical 25, lauric acid was present mainly in the *sn*-1,3 positions while EPA was randomly distributed over the three positions. Similarly, DHA as well as lauric acid were primarily located at the *sn*-1,3 positions of the modified oil. The unmodified oil remained unchanged during storage for 72 h as indicated by the conjugated diene (CD) values, but EPA- or DHA-modified Laurical 25 SL were oxidized to a much higher level than the original oil. The CD values were higher in DHA-modified than EPA-modified oil. The modified oils also attained considerably higher TBARS values than the original oil over the entire storage period.

Lipases from *Mucor miehei* (Lipozyme-IM), *Pseudomonas sp.* (PS-30), and *Candida rugosa* (AY-30) catalyzed optimum incorporation of EPA, DPA, and DHA, into Laurical 35, respectively. The maximum incorporation of EPA (62.2%) into Laurical 35 using RSM was predicted at 4.36% of enzyme load and 43.2°C over 23.9 h. Under optimum conditions (5.41% enzyme; 38.7°C; 33.5 h), incorporation of DPA into Laurical 35 was 50.8%. The corresponding maximum incorporation of DHA (34.1%) into Laurical 35 was obtained using 5.25% enzyme, at 43.7°C, and over 44.7 h. EPA and DHA were mainly esterified to the *sn*-1,3 positions of the modified oils while DPA was randomly distributed over the three positions of the TAG molecules. Meanwhile, lauric acid remained primarily esterified to the *sn*-1 and *sn*-3 positions of the modified oils. The modified oils were more susceptible to oxidation than the unmodified oil, when considering both CD and TBARS values.

In the second part, the effect of chain length, number of double bonds, the location and geometry of double bonds, the reaction conditions, and reactivity of different lipases on the incorporation of selected long-chain FA (LCFA) into triacylglcerols, such as tristearin, triolein, trilinolein, and trilinolinin, was studied. Five lipases were screened for their effect on catalyzing the acidolysis of tristearin with selected LCFA. *Candida antarctica* lipase catalyzed a higher incorporation of oleic acid (OA), γ-linolenic acid (GLA), EPA, and DHA into tristearin. *Candida rugosa* lipase catalyzed higher incorporation of linoleic acid (LA), α- linolenic acid (ALA), and conjugated linoleic acid (CLA) into tristearin. Thus, these two lipases might be considered as promising biocatalysts for acidolysis of tristearin with selected LCFA. EPA was better incorporated into tristearin than DHA using the enzymes tested. LA incorporation was better than CLA, and ALA was more reactive than GLA. In another set of experiments, a mixture of equimole amounts of C18 FA were used for acidolysis of tristearin with C18 FA at mole ratios of 1:1, 1:2, and 1:3. As the mole ratio of C18 FA increased from 1:1 to 1:3, incorporation of OA and LA increased except for the reaction catalyzed by *A. niger* and *C. rugosa*. In general, the highest incorporation of n-3 FA into tristearin was obtained at a mole ratio of tristearin to n-3 FA of 1:3.

The same enzymes were used to examine the acidolysis of triolein (tri C18:1), trilinolein (tri C18:2), and trilinolinin (tri C18:3) with selected LCFA. Lipases from *Mucor miehei* and *Pseudomonas sp.* were the most effective enzymes for acidolysis of triolein with selected LCFA. In general, incorporation of LCFA into triolein (tri C18:1) may be affected by chain length, number of double bonds, and the location and geometry of the double bonds as well as reaction conditions and reactivity and specificity of lipases used.

As the number of moles of triolein to a mixture of equimole quantities of C18 FA changed from 1 to 3, incorporation of C18 FA into triolein using lipase from *Mucor miehei* increased accordingly. Incorporation of n-3 FA into triolein increased, as the mole ratio of a combination of equimole amounts of n-3 FA increased from 1:1 to 1:3. Similarly, as the number of moles of a mixture of n-6 FA was changed from 1 to 3, incorporation of n-6 FA increased accordingly.

Incorporation (%) of a mixture of C18 FA into trilinolein using the most effective lipase from *Pseudomonas sp.* was in the order of SA>OA>GLA>ALA>CLA. The order of incorporation of n-3 FA into trilinolein using lipases from *C. antarctica and M. miehei* was ALA>EPA>DPA>DHA. Meanwhile, the degree of n-6 FA incorporation into trilinolein with *Pseudomonas sp.* lipase was in the order of GLA>AA>CLA.

Lipases from *Mucor miehei* and *Pseudomonas sp.* better catalyzed the incorporation of a mixture of C18 FA into trilinolinin while the remaining lipases catalyzed slight incorporation (≤1%) of these FA into this oil. SA was most reactive among C18 FA examined while CLA was the least reactive one. EPA was better incorporated into trilinolinin than DPA or DHA using the enzymes examined. Lipases from *Pseudomonas sp., Candida rugosa,* and *Mucor miehei* better catalyzed the incorporation of a mixture of equimole quantities of n-6 FA into trilinolinin than other lipases tested. The degree of incorporation of EPA into tristearin, triolein, trilinolein, and trilinolinin decreased as the number of double bonds increased from zero in tristearin to nine in trilinolinin. The same trends were observed for ALA and DHA.

The unmodified triolein remained unchanged during storage for 72 h as indicated in both CD and TBARS values. The modified oils with n-3 FA were more

susceptible to oxidation than the oils modified with n-6 FA, when considering both CD and TBARS values.

In the third and final part, reasons behind compromised stability of the SL so produced were explored. For the first time, the removal of endogenous antioxidants from modified oil, at least partially, was demonstrated to occur through the formation of tocopheryl esters during the acidolysis reaction. Tocopherols in the oils were found to react with carboxylic acids present in the medium, thus leading to the formation of tocopheryl esters that do not render any stability to the resultant modified oils. Therefore, compromised stability of the structured lipids could, at least be partially, be explained on a molecular basis because of the formation tocopheryl esters that do not exhibit any *in vitro* antioxidant activity.

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List of abbreviations

AA	- Arachidonic acid
AOCS	- American Oil Chemists' Society
ALA	- α-linolenic acid
CA	- Capric acid
CD	- Conjugated dienes
CLA	- Conjugated linoleic acid
DHA	- Docosahexaenoic acid
DHASCO	- Docosahexaenoic acid single cell oil
DPA	- Docosapentaenoic acid
EPA	- Eicosapentaenoic acid
FA	- Fatty acid
FFA	- Free fatty acids
EFA	- Essential fatty acids
FAMEs	- Fatty acid methyl esters
GC	- Gas chromatography
HPLC-MS	- High performance liquid chromatography-mass spectrometry
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
R	- Acyl group
SD	- Standard deviation
SL	- Structured lipids
TAG	- Triacylglycerols
TBA	- Thiobarbituric acid
TBARS	- Thiobarbituric acid reactive substances

List of publications from this thesis

1. Shahidi, F., and **F. Hamam,** Improving Life and Health with structured Lipids, *INFORM* 17: 178-180 (2006).

2. **Hamam, F.**, and F. Shahidi, Synthesis of Structured Lipids Containing Medium-Chain and Omega-3 Fatty Acids, *J. Agric. Food Chem.* 54: 4390-4396 (2006).

3. **Hamam, F.**, J. Daun, and F. Shahidi, Lipase-Catalyzed Acidolysis of High-Laurate Canola Oil with Eicosapentaenoic Acid, *J. Am. Oil Chem. Soc.* 82: 875-879 (2005).

4. **Hamam, F.**, and F. Shahidi, Structured Lipids from High-Laurate Canola Oil and Long-Chain Omega 3 Fatty Acids, *J. Am. Oil Chem. Soc.* 82: 731-736 (2005).

Chapter 1

General Introduction

1.1 Background

Lipids are a chemically heterogeneous group of compounds that are insoluble or sparingly soluble in water, but soluble in non-polar solvents. They serve several important biological functions including: (a) acting as structural components of all membranes; (b) serving as storage form and transport medium of metabolic fuel; (c) serving as a protective cover on the surface of several organs; and (d) being involved as cell-surface components concerned with cell recognition, species specificity and tissue immunity. Lipids also constitute a major component of the daily diet of human, and provide both energy and essential fatty acids. In addition, lipids act as carriers of fat-soluble vitamins A, D, E and K and help in their absorption. Finally, they act as a heating medium for food processing and affect the texture, mouthfeel and flavour of foods.

1.2 SL-definition and medicinal applications

Specialty lipids include a wide range of products amongst which structured lipids are a main class. Structured lipids (SL) are triacylglycerols (TAG) or phospholipids (PL) in which fatty acids are placed in specific locations in the glycerol backbone and are produced using a chemical or enzymatic process.

Much attention is being paid to SL, containing selected fatty acids, due to their potential biological functions and nutritional perspectives, including reduction in serum 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).

1.3 Synthesis of structured lipids

Strategies for lipid modification include genetic engineering of oilseed crops, production of concentrated oil containing high levels of polyunsaturated or other types of fatty acids (FA), and lipase- 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; Gandi, 1997). Depending on the type of substrate available, chemical or enzymatic reactions can be used for the synthesis of SL, including direct esterification (reaction of FA and glycerol), acidolysis (transfer of acyl group between an acid and ester), and alcoholysis (exchange of alkoxy group between an alcohol and an ester). However, the usual methods cited in the literature for production of SL are based on reactions between two TAG molecules (interesterification) or between a TAG and a fatty acid (acidolysis).

Lipases are important biocatalysts when conducting novel reactions. This is primarily due to their ability to use a wide spectrum of substrates, high stability towards

extremes of temperature, pH and organic solvents, and chemo-, regio- and enantioselectivity (Saxena *et al.*, 1999). The determination of their three-dimensional structures has also shed light into their unique structure–function relationship. Lipases can be obtained from different sources, such as plants, animals, and microbes (bacteria, fungus, and yeast); it is the microbial lipases that find vast application. This is because microbes can be easily cultivated and their lipases can assist a wide variety of hydrolytic and synthetic reactions. Lipases have many applications in biotechnological fields, including food and especially dairy, detergent, pharmaceutical, fat and oil hydrolysis, and biosurfactant synthesis industries (Saxena *et al.*, 1999). A relatively smaller number of bacterial lipases have been well studied compared to plant and fungal lipases (Saxena *et al.*, 1999). Most of the bacterial lipases reported so far are nonspecific in their substrate specificity, and a few bacterial lipases are thermostable. Among bacteria, *Achromobacter sp.*, *Alcaligenes sp.*, *Arthrobacter sp.*, *Pseudomonas sp.*, *Staphylococcus sp.*, and *Chromobacterium sp.* have been used for the production of lipases (Godfredson, 1990). Fungal lipases are being exploited due to their low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents. The main fungal producers of commercial lipases are *Aspergillus niger*, *Candida cylindracea*, *Humicola lanuginosa*, *Mucor miehei*, *Rhizopus arrhizus*, *R. delemar*, *R. japonicus*, *R. niveus* and *R. oryzae* (Godfredson, 1990).

1.4 Positional distribution of FA

The type of fatty acids involved in dietary fats and oils and their location within the TAG molecules are important factors affecting their absorption, nutritional, and health effects. Therefore, designing SL with selected fatty acids at specific locations of the TAG

for medicinal application has attracted much attention. The position of FA in the TAG molecules (*sn*-1, *sn*-2, and *sn*-3) would have a significant impact on their metabolism in the body. In general, FA at the terminal positions of TAG (*sn*-1 and *sn*-3) are hydrolyzed by pancreatic lipase and absorbed while those at the middle position of TAG (*sn*-2) remain unchanged and are used in the synthesis of new TAG. For example, it may be desirable to develop a SL containing PUFA at the *sn*-2 position with medium-chain fatty acids (MCFA) at the *sn*-1,3 positions for patients with mal-digestion as well as cystic fibrosis. These SL are quickly hydrolyzed by pancreatic lipase, absorbed into the intestine and rapidly transported to the liver where they are consumed as a rapid source of energy. A SL containing a mixture of MCFA, for rapid energy release, and LCFA, as a source of essential FA, would be useful in alleviation of specific disease and metabolic conditions.

1.5 Optimization procedure for SL production

In order to optimize process conditions for production of SL, response surface methodology (RSM) was employed. RSM is an optimization procedure that determines optimal process conditions by combining particular experimental designs with modeling using first or second order polynomial equations. RSM examines a number of variables at a time, and uses special experimental designs to reduce the number of required determinations (Mason *et al.*, 1989). The results of traditional one-variable-at-a-time do not indicate real changes in the environment as they ignore interactions among variables, which are in effect concomitant.

1.6 Oxidative stability tests

In order to examine the shelf-life and oxidative stability of modified and unmodified oils, a number of stability tests are usually employed. These tests include

chemical and instrumental techniques (Rossell, 1991; Shahidi and Wanasundara, 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 products of lipid oxidation.

1.7 Sources of fatty acids for SL synthesis

The constituent fatty acids (FA) 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 FA.

1.7.1 The n-3 fatty acids

All organisms can introduce one or more double bonds into a chain. The issue is where they can introduce them. Animal systems cannot introduce double bonds beyond the Δ^9 (the delta describes a bond position relative to the carboxylic acid end group) position and hence second and subsequent double bonds are always inserted at Δ^9, Δ^6, Δ^5, and Δ^4 positions, and in plants, at the Δ^9, Δ^{12}, Δ^{15} (Cook, 1985). There are two groups of PUFA, the n-3 and the n-6 FA. They are defined by the location of double bond in the molecule nearest to the methyl end of the chain. In the n-3 group of FA, the first double bond occurs between the third and fourth carbon atoms and in the n-6 group of FA it is situated between the sixth and seventh carbon atoms.

The n-3 or omega-3 FA have many health benefits related to cardiovascular disease (Conquer and Holub, 1997), inflammation (Mori and Beilin, 2004), cancer (Cave, 1991), immune response (Puertollo *et al.*, 2004), diabetes (Stene and Joner, 2003), hypertension

(Aguilera *et al.*, 2004) and renal disorders (Plotnick, 1996). Epidemiological studies have linked the low incidence of coronary heart disease in Greenland Eskimos with their high dietary intake of n-3 PUFA (Bang and Dyerberg, 1972, 1986).

1.7.2 Saturated fatty acids

Saturated fatty acids (SFA) contain only single carbon-carbon bonds in the aliphatic chain and hydrogen atoms occupy all other available sites. SFA fall into three classes, based on the number of carbon atoms in the aliphatic chain. Short-chain fatty acids (SCFA) range from C2:0 to C4:0, medium-chain fatty acids (MCFA) comprise 6-12 carbon atoms, and long-chain fatty acids (LCFA) comprise > 12 carbon atoms.

1.7.2.1 Medium-chain fatty acids (MCFA)

MCFA are saturated FA from C6 to C12 and are prepared mostly from tropical fruit oils such as coconut and palm kernel oils (Akoh, 1995; 1997). Medium chain triacylglycerols (MCT) exhibit unique structural and physiological features. Structured lipids containing 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 SL are structurally and metabolically different from simple physical mixtures of medium-chain triacylglycerols (MCT) and long-chain triacylglycerols (LCT). High-laurate canola oil is a product from genetically engineered seed containing a MCFA (lauric acid; C12:0). This oil was produced by Calgene Inc. (Davis, CA) to provide an alternative source of oil to those produced by several palm kernel oil fractions (Del Vecchio, 1996).

1.8 Objectives

The objectives of this study were three fold and these are described in three parts. The first objective was to study the incorporation of long-chain n-3 fatty acids (EPA,

DPA, and DHA) into three types of high-laurate canola oils, namely Laurical 15, 25, and 35 with oleic acid content of 15, 25, and 35%, respectively. Another objective was to optimise the reaction conditions for high-laurate canola oil based SL. Determination of the positional distribution of FA in the enzymatically-modified and unmodified oils was also intended. Finally, evaluation of the oxidative stability of SL so produced was also carried out.

It was proposed that the factors, including chain length, degree of unsaturation represented by the number of double bonds, the location and geometry of double bonds, the reaction conditions, and reactivity of different lipases would have a marked effect on the incorporation of selected long-chain fatty acids (LCFA) into different oils. The objectives for the second part were to incorporate selected LCFA into triacylglycerols such as tristearin, triolein, trilinolein, and trilinolinin using different lipases, namely Novozyme-435 from *Candida antarctica*, AY-30 from *Candida rugosa*, PS-30 from *Pseudomonas sp.*, AP-12 from *Aspergillus niger*, and Lipozyme-1M from *Mucor miehei*. It was also designed to verify the effect of chain length, number of double bonds, and the location and geometry of double bonds on the incorporation of these FA into tristearin, triolein, trilinolein, and trilinolinin.

It was hypothesized that tocopherols present in the oils might react with carboxylic acids present in the reaction medium, thus leading to the formation of tocopheryl esters that do not render any stability to the resultant modified oils. The third part was to explore reasons behind compromised stability of the SL so produced, particularly possible formation of tocopheryl esters from the reaction of carboxylic acids with tocopherols present in the oils.

This thesis consists of ten chapters; each chapter has its own references at the end. The first two chapters (introduction and literature review) describe some general terms related to the definition of structured lipids (SL), medicinal applications, laboratory scale production, positional distribution of fatty acids (FA) in the resultant SL, and optimization procedure for SL production. Oxidative stability of the oils and the general objectives of the research reported are also discussed. Chapter 3 describes materials and methods, while results and discussion are given in chapters 4-9. Chapters 4-6 report on the incorporation of the n-3 FA (EPA, DPA, and DHA) into Laurical oils (Laurical 15, 25, and 35). Incorporation of n-3 FA into these oils was compared using a number of lipases; positional distribution of FA in the TAG was also determined along with the oxidative stability of the SL so produced.

Chapters 7 and 8 present the effect of chain length, number of double bonds, and the location and geometry of double bonds on the incorporation of selected long-chain FA into tristearin, triolein, trilinolein, and trilinolinin. These chapters also discuss reasons behind different degrees of incorporation of selected LCFA into these oils on a molecular basis which can fill an important gap in the existing scientific literature regarding the differences in the reactivity of different fatty acids.

Chapter 9 describes, for the first time, a possible mechanism for the removal of endogenous antioxidants through the formation of tocopheryl esters from the reaction of carboxylic acids and tocopherols in the oils during the acidolysis process in the presence of enzymes. Finally, a summary and recommendation section is given in chapter 10.

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Chapter 2

Literature review

2.1 Structured lipids-definitions

The concept of structured lipid (SL) for nutritional and medical uses in patients was first introduced in 1987 by Babayan. SL are triacylglycerols (TAG) or phospholipids (PL) modified to change the fatty acid composition and/or their location in the glycerol backbone using chemical or enzymatic process (Lee and Akoh, 1998). SL are TAG or PL in which the glycerol moiety is esterified to a mixture of short-, medium-, and long-chain fatty acids for functional purposes (Haumann, 1997a).

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, 2002). Lipids can be restructured to meet essential fatty acid requirements or to incorporate specific fatty acids of interest (Akoh, 1997). SL may offer efficient means of delivering target fatty acids for nutritive or therapeutic purposes (Lee and Akoh, 1998). SL can also be produced to obtain TAG with modified physical and/or chemical features, including melting point, iodine and saponification values (Babayan, 1987).

2.2 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) (Figure 2.1) (Lee and Akoh, 1998). However, the usual methods cited in the literature for production of structured

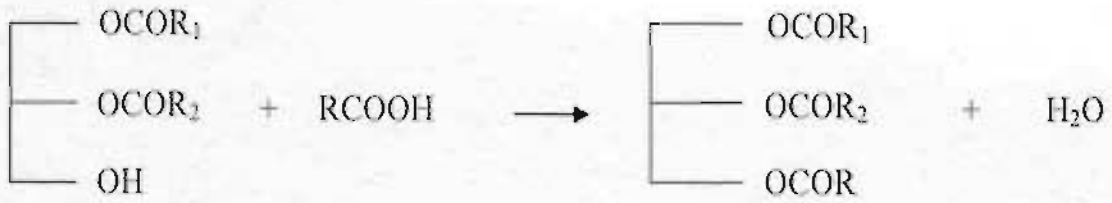
triacylglycerols are based on reactions between two TAG molecules (interesterification) or between a TAG and a fatty acid (acidolysis) (Figure 2.1).

2.2.1 Chemically-catalyzed interesterification

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

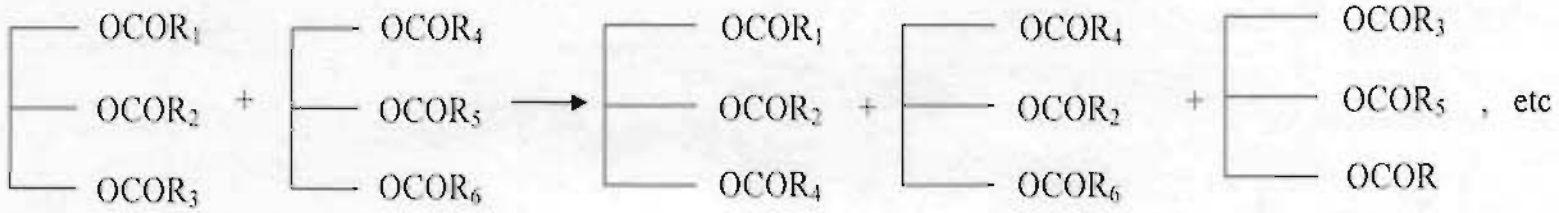
2.2.2 Enzymatically-catalyzed interesterification

An alternative to the chemical synthesis of SL is the enzymatic process which may use a variety of lipases. Potential advantages of lipase-assisted interesterification may be found in the enzyme's regioselectivity, chemoselectivity, and fatty acid chain length specificity. Lipases can be used to produce and design SL on a case-by-case basis to target for application in specific food or medical uses (Akoh, 1995; 1997; 2002). Lipase-assisted interesterification produces fats or oils with a defined structure because it incorporates desired fatty acids at a specific position. It requires mild experimental conditions without potential for side reactions, reduced energy expenditure and reduced heat damage to reactants, and easy purification of products (Akoh, 1997).

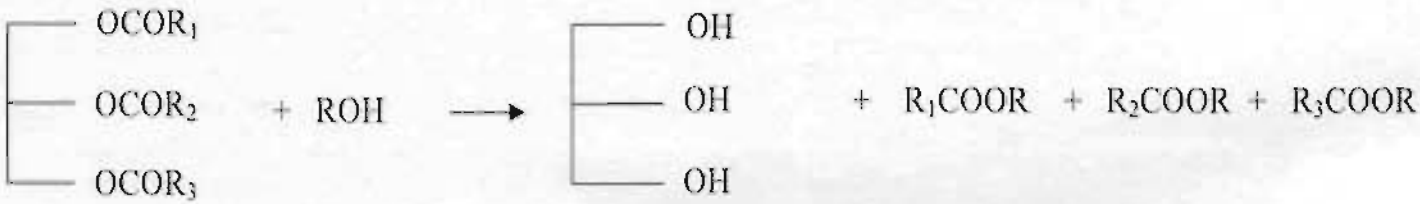


(b) Transesterification

(1) Interesterification



(2) Alcoholysis



(3) Acidolysis

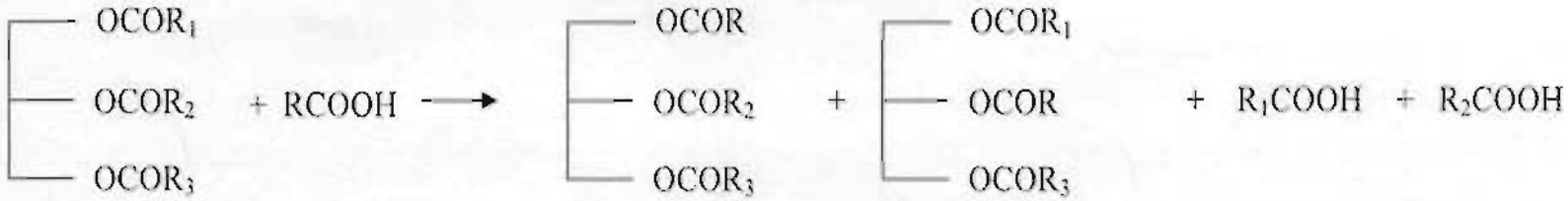


Figure 2.1 Schematic diagram of lipase-assisted lipid modification strategies for the synthesis of structured lipids

2.3 Acidolysis

Acidolysis refers to the exchange of acyl group between an acid (fatty acid) and an ester. Acidolysis is an efficient method for incorporating specific fatty acids into TAG 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. SL 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. In addition, they are better absorbed in the intestinal tract as 2-monoacylglycerol (2-MAG) upon hydrolysis of the TAG by pancreatic lipase. MCFA are absorbed effectively and are a fast source of energy without being accumulated in the adipose tissues (Akoh, 2002).

Incorporation of capric acid (CA, C10:0) into arachidonic acid (AA, C20:4n-6) single cell oil, using five commercial lipases, indicated that lipase PS-30 from *Pseudomonas sp.* was most effective (Hamam and Shahidi, 2004a). The optimum conditions were oil to CA mole ratio of 1:3 at a temperature of 45°C, incubation time of 24 h, 4% of lipase from *Pseudomonas sp.* and a 2% (w/w) water content. Examination of positional distribution of fatty acids on the glycerol backbone of modified AA single cell oil with CA showed that 89.7% of CA was placed in the *sn*-1,3 positions of the TAG molecules. AA was mainly located at the *sn*-2 position of the modified AA single cell oil. The enzymatically modified oil was more susceptible to oxidation than its unmodified counterpart, when considering both conjugated dienes (CD) and thiobarbituric acid

reactive substances (TBARS) values (Hamam and Shahidi, 2004a). In another study, Hamam and Shahidi (2004b) reported that PS-30 from *Pseudomonas sp.* was most effective among the five commercially available lipases examined for incorporation of capric acid (CA) into docosahexaenoic acid single cell oil (DHASCO). The optimum conditions were mole ratio of 1:3 (DHASCO:CA) at a temperature of 45°C, a reaction time of 24 h in the presence of 4% enzyme and 2% water content. Examination of the positional distribution of fatty acids on the glycerol backbone of the modified DHASCO with CA showed that CA was present mainly in the *sn*-1,3 positions of the TAG molecules. Meanwhile, DHA was favorably present in the *sn*-2 position, but also located in the *sn*-1 and *sn*-3 positions.

Screening of five commercially available lipases for incorporation of capric acid (CA) into a single cell oil rich in DHA and docosapentaenoic acid (DPA, C22:5n-6), commercially known as the OMEGA-GOLD oil, indicated that lipase PS-30 from *Pseudomonas sp.* was most effective. The optimum reaction mixture and conditions were mole ratio of 1:3 (OMEGA-GOLD:CA) at a temperature of 45°C a reaction time of 24 h in the presence of 4% (w/w of substrates) PS-30 lipase from *Pseudomonas sp.* and 2% (w/w of substrates and enzyme) water content. The results showed that CA was present mainly in the *sn*-1,3 positions of the TAG molecules. Meanwhile, DHA or DPA (n-6) were mainly esterified to the *sn*-2 position. The enzymatically modified oil had higher conjugated dienes (CD) values than their unmodified counterpart. In general, TBARS values of the modified OMEGA-GOLD oil were much higher than the original oil over the storage period up to 36 h. However, when the oil was subjected to the same reaction conditions in the absence of any enzyme, there was a significant difference ($P<0.05$) in its

oxidative stability when compared with enzymatically modified OMEGA-GOLD. Therefore, removal of antioxidants during the process is primarily responsible for the compromised stability of the modified oil (Hamam and Shahidi, 2005). In addition, tocopherols may be converted to tocopheryl esters by their esterification with free fatty acids present; this newly formed compound will be discussed in this study and it will be the first time that we report the formation of tocopherol esters.

Senanayake and Shahidi (2002) used acidolysis to incorporate capric acid (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. Incorporation of CA into the fish oil (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 *Rhizpmucor miehei* as the biocatalyst. The SL produced contained EPA (25.0 mol%), DHA (22.6 mol%), and 40.8 mol% CA).

SL containing palmitic, oleic, stearic, and linoleic acids, resembling human milk fat (HMF), were produced by enzymatic acidolysis reactions between tripalmitin, hazelnut oil fatty acids, and stearic acid using immobilized *sn*-1,3-specific lipase, Lipozyme RM IM, obtained from *Rhizomucor miehei* was used as a biocatalyst (Sahin *et al.*, 2005). The results showed that the highest C18:1 incorporation (47.1%) and highest C18:1/C16:0 ratio were produced at 65°C and 24 h of incubation with the highest substrate mole ratio of

tripalmitin/hazelnut oil fatty acids/stearic acid of 1:12:1.5. The highest incorporation of stearic acid was obtained at a 1:3:0.75 (tripalmitin/hazelnut oil fatty acids/stearic acid) substrate mole ratio at 60°C and 24 h. For both oleic and stearic acids, the incorporation level increased with reaction time. The SL produced in this study have potential use in infant formulas (Sahin *et al.*, 2005). Lipase-catalyzed acidolysis of coconut oil and/or tricaprylin, and a mixture containing 73% CLA was performed to produce SL containing CLA. An immobilized lipase from *Mucor miehei* (Lipozyme IM) was used as the biocatalyst in a solvent-free system. CLA was incorporated effectively into coconut oil or tricaprylin using 5% lipase at 65°C over 48 h (Rocha-Uribe and Hernandez, 2004).

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%). The total PUFA (EPA and DHA) of fish oil remained unchanged after the modification while PUFA of canola oil were reduced from 29.6 to 21.2%. Monoenes, particularly 18:1n-9 and 16:1n-7, were remarkably reduced by caprylic acid in fish oil. Indeed, oleic acid was completely replaced by caprylic acid in fish oil whereas it was reduced to 34.7% in canola oil.

Shimada *et al.* (1996a) reported that caprylic acid 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 TAG was exchanged due to the low activity of the *Rhizomucor delemar* on DHA. 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 and showed the promising features of using this biocatalyst. The results showed that the degree of reaction progress 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 this 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 that produced chemically (Xu *et al.*, 2002).

Lee and Akoh (1998) reported 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 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 one (SL1) contained one linoleic acid and two capric acids and the second one (SL2) contained two linoleic acids and one capric acid. Two immobilized lipases, 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).

Enzymatic acidolysis of borage oil (BO) or evening primrose oil (EPO) with EPA showed that nonspecific lipase PS-30 from *Pseudomonas sp.* gave the highest incorporation of EPA into both oils at 45-55°C and at 150-250 enzyme activity units over 24 h. Among the solvents examined, n-hexane served best for the acidolysis of EPA with both oils. The highest EPA incorporations of 39.9 and 37.4% in BO and EPO, respectively, occurred at the stoichiometric mole ratio of 1:3 for an oil to EPA (Senanayake and Shahidi, 2002). In another study conducted by Senanayake and Shahidi (1999), Novozyme 435 from *Candida antarctica,* among seven lipases tested, catalyzed the highest degree of DHA incorporation into borage oil during acidolysis of borage oil with DHA in organic solvents. Incorporation of DHA increased with increasing the amount of enzyme, reaching 27.4% at an enzyme concentration of 150 lipase activity units. As incubation time progressed, DHA incorporation also increased. After a reaction time of 24 h, the contents of total n-6 and n-3 PUFA in acylglycerols were 44.0 and 27.6%, respectively. The highest degree of DHA incorporation was achieved when hexane was used as the reaction medium. DHA was randomly distributed over the *sn*-1, *sn*-2, and *sn*-3 positions of the TAG. Thus, preparation of modified borage oil acylglycerols containing both DHA (27.4%) and γ-linolenic acid (17.0%) was successfully achieved. The final oil had a ratio of n-3 to n-6 of 0.42-0.62 which is nutritionally more suitable than the original unaltered borage oil (Senanayake and Shahidi, 1999).

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*

catalyzed 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 *et al.* (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.

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 production of TAG by esterification of glycerol with PUFA using a 5% immobilized lipase from *C. antarctica*. The second step included changing of 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 TAG.

Kim *et al.* (2002) modified perilla oil (containing 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 at a mole ratio of oil to caprylic acid of 1:10, 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 esterification may considerably enhance the nutritional characteristics of TAG (Quinlan and Moore, 1993). McNeil and Sonnet (1995) have developed calorie reduced SL via esterification of long-chain monoacylglycerols containing behenic acid with capric acid. Since behenic acid is incompletely absorbed during digestion, the TAG produced contains about half the calories when compared with natural TAG.

2.4. Sources of fatty acids for structured lipid synthesis

The constituent fatty acids (FA) 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 FA.

2.4.1 Saturated fatty acids

Saturated fatty acids contain only single carbon-carbon bonds in the aliphatic chain and hydrogen atoms occupy all other available bonds. The most abundant saturated fatty acids in animal and plant tissues are usually straight chain compounds with 10, 12, 14, 16 and 18 carbon atoms. In general, saturated fats are solid at room temperature. They are found mainly in margarine, shortening, coconut and palm oils as well as foods of animal origin. For a series of saturated fatty acids the melting point increases as the length of the

chain increases. Typically, adding double bonds to a saturated fatty acid will lower its melting point.

2.4.1.1 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 (4:0) acids. 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 kCa). 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. Tsuzki (2005) reported the production of SL containing SCFA and triolein. *Aspergillus oryaze* lipase catalyzed incorporation of 80% of butyric acid into triolein at a mole ratio of triolein to butyric acid of 1 to 10 at 52°C for 72 h.

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).

2.4.1.2 Medium-chain fatty acids (MCFA)

Medium-chain fatty acids (MCFA) comprise 6-12 carbon saturated fatty acids that are produced from hydrolysis of tropical plant oils such as those of coconut and palm kernel (Akoh, 1995, 1997). Oils from tropical plants, such as those from coconut and palm kernel, contain very high amounts (approximately 50%) of lauric acid (C12:0). They also contain considerable amounts of caprylic (C8:0), capric (C10:0), and myristic (C14:0) acids. Medium-chain triacylglycerols (MCT) is a good source of MCFA for production of structured lipids. Pure MCT have a caloric value of 8.3 kCal/g. However, they do not supply essential fatty acids (Heird *et al.*, 1986; Lee and Hastilow, 1999). MCFA are more hydrophilic than their long-chain FA counterparts. 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 (Kin *et al.*, 2002).

MCT exhibit unique structural and physiological characteristics. MCT do not require chylomicron formation to transfer from the 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 (Figure 2.2) (Lee and Akoh, 1998). They are easily absorbed, metabolized as quickly as glucose, have approximately two fold the caloric concentration of proteins and carbohydrates, and have little affinity to accumulate as body fat. They are not dependent on carnitine (an enzyme necessary for transport of FA 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 LCFA. Finally, MCT rapidly clear from the blood (Babayan, 1987).

MCT are used together with LCT in order to offer a balanced nutrition in enteral (tube feeding) and parenteral products (Roediger and Rae, 1982; Ulrich *et al.*, 1996). Clinical nutritionists have taken advantage of MCT's simpler digestion to feed individuals who can not make use of LCT (Kennedy, 1991). 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 and can lead 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 FA is attractive in order to meet the nutritional requirement of patients and those with special food needs.

2.4.1.3 Long-chain fatty acids (LCFA)

Saturtaed FA are generally believed to raise plasma and serum cholesterol levels, but fatty acids with chain length ranging from 4 to 10 do not increase the cholesterol levels (Akoh, 2002). Palmitic acid (16:0) is a widely occurring SFA and is found in almost all vegetable oils, as well as in fish oils and body fat of land animals. Palmitic acid is found abundantly in palm oil, cottonseed oil, lard and tallow, among others. Stearic acid (18:0) has also been cited not to increase cholesterol levels in plasma (Bonanone and Grundy, 1988). TAG containing high amounts of long chain saturated fatty acids (LCSFA), 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 LCSFA 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 Salatrim, which consist of SCFA and LCSFA, mainly stearic acid (Finley, 1994).

2.4.2 Unsaturated fatty acids

Unsaturated FA contain carbon-carbon double bonds in their aliphatic chain. In general, these fats are soft at room temperature. Monounsaturated fatty acids (MUFA) contain one carbon-carbon double bond. On the other hand, PUFA contain two or more carbon-carbon double bonds. The PUFA are liquid at room temperature due to the fact that the double bonds are rigid, thus preventing the fatty acids from packing close together. In general, they have low melting points and are susceptible to oxidation. Because most PUFA are liquid at room temperature, they are generally referred to as oils. The common sources of PUFA include grains, nuts, vegetables and seafood.

2.4.2.1 The n-9 fatty acids

The double bond in n-9 FA is located between the ninth and tenth carbon atoms from the methyl end group. They are found in vegetable oils such as olive, almond, hazelnut, canola, peanut and high-oleic sunflower as oleic acid (18:1n-9). Oleic acid is the most widely distributed and the most extensively produced of all FA. Olive oil (60-80%), hazelnut oil (60-70%) and almond oil (60-70%) are rich sources of this fatty acid (Gunstone, 1997). The human body can synthesize oleic acid, therefore it is not considered as an essential fatty acid. It plays a moderate role in lowering plasma cholesterol in the body (Gottenbos, 1988); increasing the uptake of oleic acid in young

healthy humans is known to increase plasma high density lipoprotein (HDL) and decrease TAG (Mensink and Katan, 1987).

2.4.2.2 Polyunsaturtaed fatty acids (PUFA)

There are two groups of PUFA, the n-3 and the n-6 FA. They are defined by the location of double bond in the molecule nearest to the methyl end of the chain. In the n-3 group of FA, the first double bond occurs between the third and fourth carbon atoms and in the n-6 group of FA it is situated between the sixth and seventh carbon atoms. The parent compounds of the n-6 and n-3 groups of fatty acids are linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3 n-3), respectively. These parent compounds are metabolized in the body via a series of alternating desaturation (in which an extra double bond is inserted by removing two hydrogen atoms) and elongation (in which two carbon atoms are added) steps as shown in Figure 2.3.

2.4.2.2.1 The n-3 fatty acids

The n-3 FA, such as α-linolenic acid (ALA), eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3), and docosahexaenoic acid (DHA; 22:6n-3) have many health benefits related to cardiovascular disease, inflammation, allergies, cancer, immune response, diabetes, hypertension and renal disorders (Kyle, 2001). Docosahexaenoic acid single cell oil (DHASCO) is an important source of commercial DHA, which is derived from *Crypthecodinium cohnii* (a member of the Dinophyta, a phylum of unicellular eukaryotic microalgae); this microalgae grows under strictly controlled fermentation conditions (Arterburn *et al.*, 2000). DHASCO contains approximately 40% (w/w) DHA, but no EPA or other LCPUFA are present in its oil (Arterburn *et al.*, 2000).

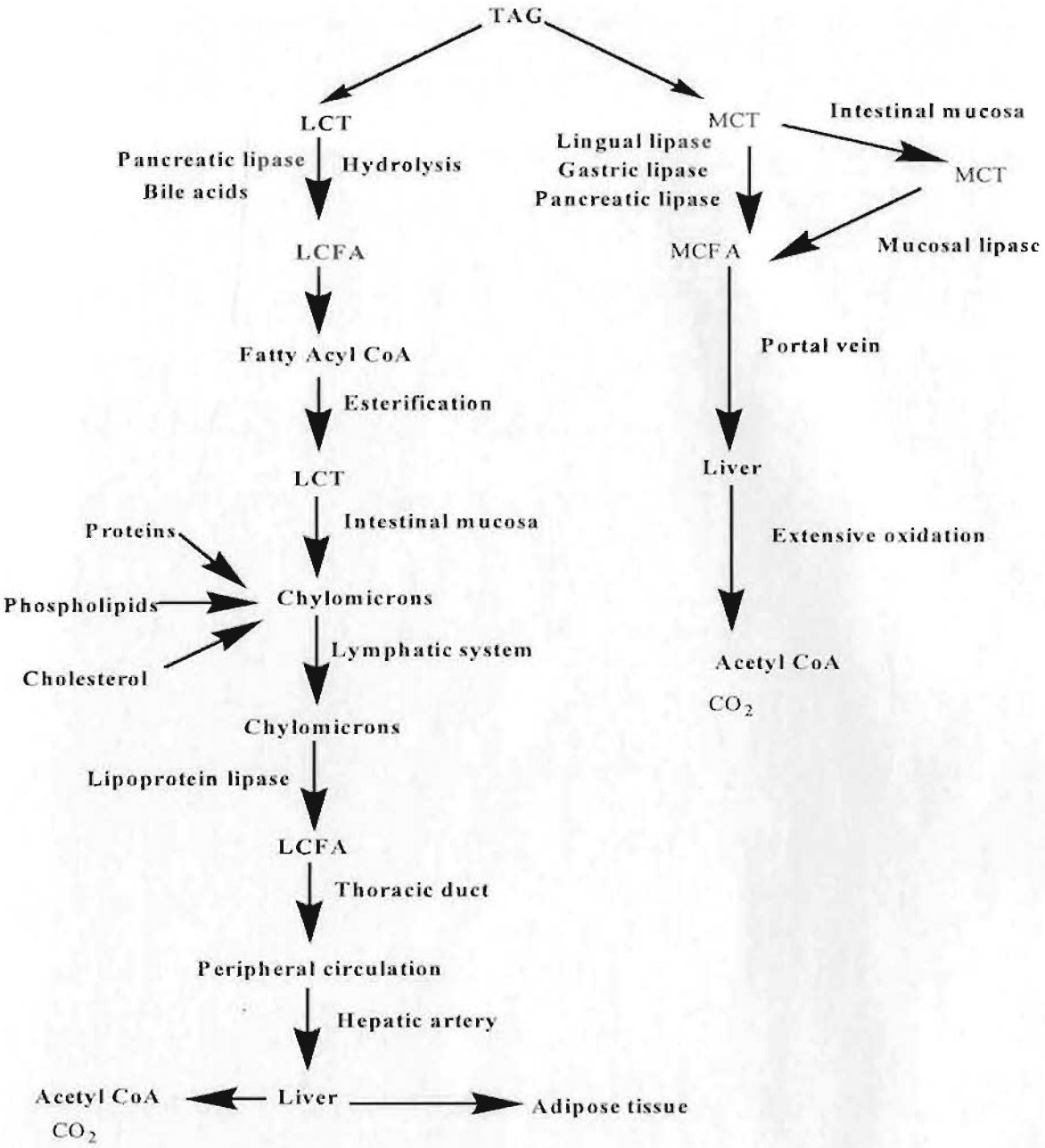


Figure 2.2 Digestion, absorption and transport of medium-chain and long-chain fatty acids. Symbols are: MCFA, medium-chain fatty acids; MCT, medium-chain triacylglycerols LCFA, long-chain fatty acids; LCT, long-chain triacylglycerols; TAG, triacylglycerols (adapted from Akoh, 2002).

Epidemiological studies have linked the low incidence of coronary heart disease in Greenland Eskimos with their high dietary intake of n-3 PUFA (Bang and Dyerberg, 1972, 1986). Research studies have shown that DHA is essential for appropriate functioning of the central nervous system and visual acuity of infants (Kyle, 2001). The n-3 FA are essential for normal growth and development throughout the life cycle of humans and therefore should be included in the diet. The n-3 FA have been extensively studied for their influence on cardiovascular disease (CVD). However, the exact mechanism by which these effects are rendered remains unknown, but research results have shown that these FA in marine oils may prevent CVD by decreasing serum TAG and acting as anti-atherogenetic and antithrombotic agents (Newton, 2001). DHA deficiency has been associated with depression and may be the underlying reason for the positive correlation between depression and myocardial infraction. Neurological disorders associated with decreased levels of DHA have been reported in schizophrenia, Alzheimer's disease, and depression, among others (Conquer and Holub, 1997).

Marine oils are rich sources of n-3 FA, especially EPA and DHA. Cod liver, menhaden, and sardine oils contain approximately 30% EPA and DHA (Kyle, 2001). Alpha-linolenic acid (ALA; 18:3n-3), the parent of n-3 FA, can be metabolically converted to DHA via desaturation and elongation reactions (Figure 2.3). However, the efficiency of conversion of ALA to DHA in human adults is very restricted (approximately 4%) and even more restricted in infants (< 1%) (Holub, 2001). In certain disease conditions, the rate of conversion of ALA to DHA and/or EPA is much lower, therefore long-chain polyunsaturated fatty acids (LC PUFA) such as DHA and EPA are considered conditionally essentials and must be obtained from dietary sources (Holub, 2001). ALA is

a main constituent of flaxseed oil (50-60%). When ALA is absorbed into the animal body through the diet, it forms long-chain PUFA with an n-3 terminal structure. EPA and DHA are also important n-3 FA. DHA is a major constituent of the gray matter of the brain and the retina of the eye. Infants fed on mother's milk show a higher IQ and intelligence level than infants fed on formula that lack any DHA (Shahidi and Finley, 2001). In addition, EPA is a precursor of a series of eicosanoids and is important in protecting against heart attacks primarily due to its antithrombotic effect (Bang *et al.*, 1978). EPA was also shown to increase bleeding time and to decrease serum cholesterol levels (Bang *et al.*, 1978).

In conclusion, LC PUFA exhibit multifunctional roles in promotion of health and prevention of disease in the human body. However, they are highly susceptible to oxidation when stored and are known, upon consumption, to increase the body's load on natural antioxidants such as α-tocopherol. Therefore, it is very important to stabilize oils rich in LC PUFA during storage by incorporation of appropriate antioxidants and adequate packaging technologies.

2.4.2.2.2 The n-6 fatty acids

The n-6 FA display a variety of physiological functions in the human body. The main functions of these FA are related to their roles in the membrane structure and in the biosynthesis of short-lived derivatives (eicosanoids) which regulate several aspects of cellular activity. The n-6 FA are responsible for maintaining the integrity of the water impermeability barrier of the skin. They are also involved in the regulation of cholesterol transport in the body. Linoleic acid (LA; 18:2n-6) is the most common FA of this type. LA is found in all vegetable oils and is essential for normal growth, reproduction and health. LA serves as a precursor of n-6 family that are formed by desaturation and chain

elongation, in which the terminal (n-6) structure is retained (Figure 2.3). Of these, arachidonic acid (AA; 20:4n-6) is principally important as a fundamental constituent of the membrane phospholipids and as a precursor of eicosanoids. On the other hand, γ-linolenic acid (GLA; 18:3n-6), an important intermediate in the biosynthesis of AA from LA, is a component of certain seed oils, such as borage, evening primrose, and black currant, and has been a subject of intensive studies (Senanayake and Shahidi, 1999, 2002). It is proposed that the uptake of 1-2% LA in the diet is adequate to protect against chemical and clinical disorders in infants. The absence of LA in the diet is associated with manifestation of several disorders, including impaired growth and reproduction, excessive water loss via the skin, scaly dermatitis and poor wound healing (Akoh, 2002).

2.5 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 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.

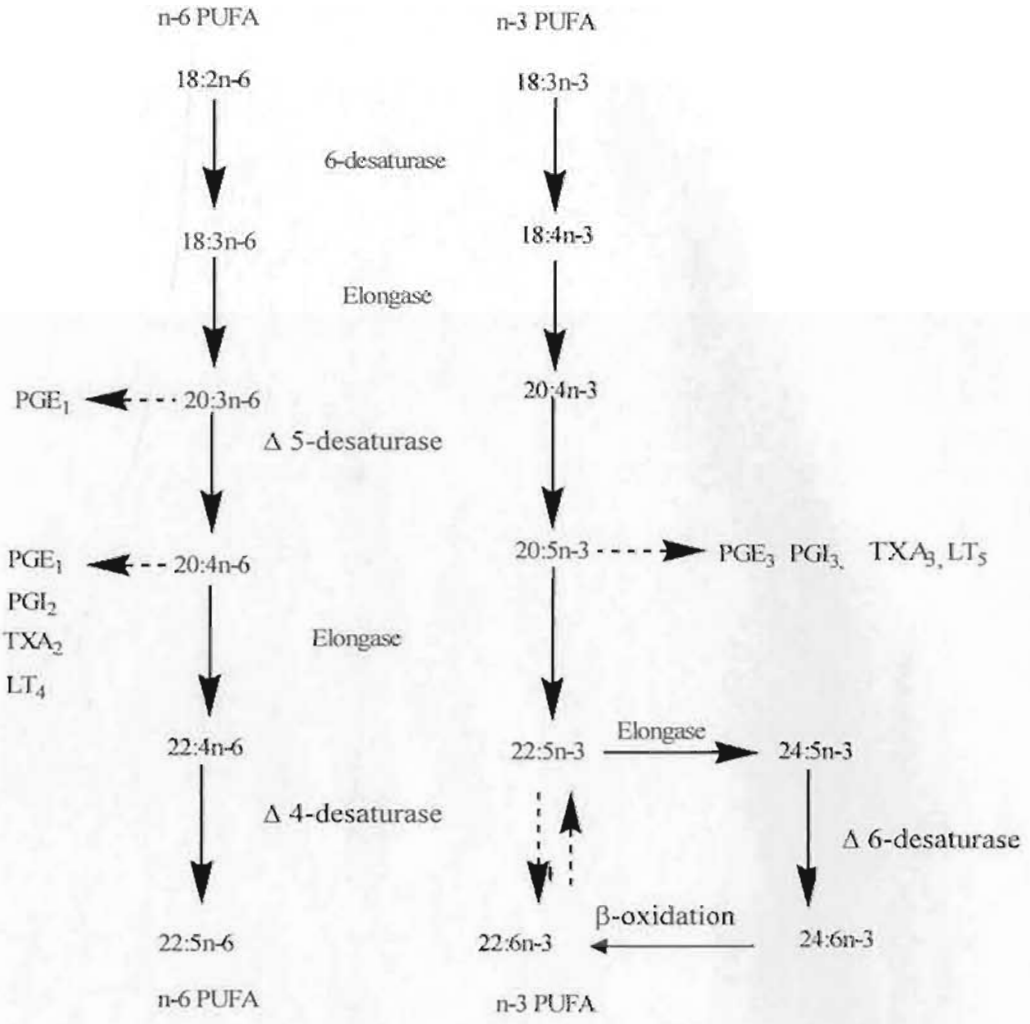


Figure 2.3 Metabolic pathways of n-3 versus n-6 fatty acids. Symbols are: PGE, prostaglandin E; PGI, prostaglandin I; TXA, thromboxanes A; LT, leukotrienes (adapted from Akoh, 2002).

The propagation step normally starts with the addition of molecular oxygen to alkyl free radical ($R^{\bullet}$) to form an unstable peroxyl 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 zero activation energy (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 Wanasundara, 1998).

A simplified scheme explaining the mechanism of autoxidation is given below:

Initiation:

$$RH \longrightarrow R^{\bullet} + H^{\bullet}$$

Propagation:

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \rightleftharpoons ROOH + R^{\bullet}$$

Termination:

$$2\ ROO^{\bullet} \longrightarrow \text{Non-radical products}$$

$$ROO^{\bullet} + R^{\bullet} \longrightarrow \text{Non-radical products}$$

$$R^{\bullet} + R^{\bullet} \longrightarrow \text{Non-radical products}$$

The free radical chain reaction can be terminated either by self-quenching or polymerization of free radicals to non-radical dimers, trimers and polymers or by antioxidants which react competitively with alkyl free radicals and/ or peroxyl radicals and remove them from the system (King *et al.*, 1995). As oxidation normally proceeds very

slowly at the initial stage, the time to reach a sudden increase in oxidation rate is referred to as the induction period.

Oxidation of lipids is one of the major changes that occurs during processing, distribution, and final preparation of food. Furthermore, 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 FA has been most widely studied 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).

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 FA) are colourless, odourless, and tasteless (King *et al.*, 1995). Hydroperoxides are very unstable and decompose into various secondary products. Hydroperoxides are decomposed via homolytic cleavage of oxygen-oxygen bond to produce hydroxyl and alkoxyl 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).

2.5.1 Methodologies for assessing lipid oxidation

There are several methods for the measurement of fats and oils oxidation in foods and biological systems. 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 of the oxidative changes in all food systems (Shahidi and Wanasundara, 1998). It is necessary to select a proper and adequate method for a particular application. 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.

2.5.1.1 Primary changes

2.5.1.1.1 Measurement of reactant change

Changes in reactants (unsaturated fatty acids) is a useful technique for identifying class of fats or oils and fatty acids that are involved in the oxidative change (Gray and Monahan, 1992) and also for evaluating lipid oxidation provoked by different metal complexes which give different products (Gutterigde and Hallwelli, 1999). In this method, lipids are extracted from food or biological systems and subsequently converted into derivatives appropriate for chromatographic analysis (Shahidi and Wanasundara, 2002). Fatty acid methyl esters (FAME) are the derivatives commonly used for determination of fatty acid composition, usually by gas chromatography (GC) (Fruhwirth *et al.*, 2003). This method is an insensitive way of evaluating oxidative deterioration, and

the application of this technique is restricted due to its inability to serve as a marker of oxidation of more saturated lipids (Shahidi and Wanasundara, 2002). This method is useful when highly unsaturated lipids are present.

2.5.1.1.2 Weight gain

It is widely accepted that formation of hydroperoxides during early stages of autoxidation can be quantified. Thus, induction period can be determined from mass increase data. Heating an oil and periodically testing for weight gain is one of the oldest methods for assessing oxidative stability (Antolovich *et al.*, 2002). This method requires simple equipment and points out directly oxygen absorption through mass change. Oil samples are weighed, and stored in an oven at a set temperature with no air circulation. The weight gain method offers many advantages such as unlimited capacity, speedy sample processing, and low cost of equipment. Ke and Ackman (1976) used the mass gain method to compare lipid oxidation from various parts of fish. However, the weight gain method has several drawbacks such as discontinuous heating of the sample, which may give rise to nonreproducible results, and the need of long analysis time and intensive human involvement (Shahidi and Wanasundara, 2002).

2.5.1.1.3 Peroxide value (PV)

Lipid oxidation involves the continuous formation of hydroperoxides as primary oxidation products which may break down to a range of non-volatile and volatile secondary products (Dobarganes and Velasco, 2002). Analytical methods for hydroperoxides in fats and oils can be catagorized as those measuring the total amount of hydroperoxides and those based on chromatographic methods giving detailed information on the structure and amount of specific hydroperoxides present in a certain oil sample

(Dobarganes and Velasco, 2002). The PV represents the total hydroperoxide content and is one of the most common quality indicators of fats and oils during production and storage (Antolovich *et al.*, 2002). A number of techniques have been used for measuring of PV, among which the iodometric titration, ferric ion complexes spectrophotometry, and infrared spectroscopy are mainly commonly used (Dobarganes and Velasco, 2002).

PV 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.

2.5.1.1.4 Conjugated dienes (CD)

The formation of conjugated dienes in fats or oils gives rise to an absorption peak at 230-235 nm in the ultraviolet (UV) region. In the 1960s, monitoring diene conjugation emerged as a useful technique for the study of lipid oxidation (Antolovich *et al.*, 2002). During oxidation of lipids containing PUFA 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 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. Ultraviolet detection of conjugated dienes is simple, fast, and requires no chemical reagents and only small amounts of samples are needed. However, this method has less specificity and sensitivity than PV measurement (Antolovich *et al.*, 2002). Furthermore, the result may be affected by the presence of compounds absorbing in the same region, such as carotenoids (Shahidi and Wanasundara, 1998). To avoid these interferences, an alternate spectroscopic method measuring conjugable oxidation products (COPs) has been suggested. In this method, hydroperoxides and some decomposition products are changed to more conjugated chromophores by reduction and subsequent dehydration. The concentrations of the resultant conjugated trienes and tetraenes are determined from their respective absorption at 268 and 301 nm and expressed as COP values (Gordon *et al.*, 2001; Shahidi and Wanasundara, 2002).

2.5. 1.2 Secondary changes

The primary oxidation products (hydroperoxides) are unstable and prone to decomposition. A complex mixture of volatile, nonvolatile, and polymeric secondary oxidation products is produced through decomposition reactions, providing a range of indicators of lipid oxidation (Kamal-Eldin *et al.*, 2003). Secondary oxidation products include aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids and epoxy compounds, among others. 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.

2.5.1.2.1 Thiobarbituric acid (TBA) test

During lipid oxidation, malonaldehyde (MA), a minor constituent of FA with 3 or more double bonds, is formed as a result of the break down of PUFA. It is usually used as a marker of the lipid oxidation process, both for the early appearance as oxidation occurs and for the sensitivity of the analytical method (Cesa, 2004). 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. The TBA test measures the total amount of aldehydes in the oxidized lipids. During the TBA test one molecule of MA interacts with two molecules of TBA reagent to produce a pink coloured complex with an absorption maximum at 532 nm (Figure 2.4) (Shahidi and Wanasundara, 1998). The degree of oxidation is reported as the TBA value and is expressed as milligrams of MA equivalents per kilogram of sample or as micromoles of MA equivalents per gram of sample. It must, however, be noted that alkenals and alkadienals also react with the TBA reagent and yield a pink color. Thus the term thiobarbituric acid reactive substances (TBARS) is now used instead of MA.

The TBA test is used frequently to evaluate the oxidation of a variety of food systems, despite its limitations such as lack of specificity and sensitivity (Delas-Heras *et*

al., 2003). As already noted, many other substances may react with the TBA reagent and contribute to absorption, causing an overestimation of the intensity of colour complex (Delas-Heras *et al.*, 2003). The TBA test has two major drawbacks as MA is only produced by fats possessing three or more double bonds (Benzie, 1996). The accuracy and/or specificity of TBA test has also been questioned because the TBA reacts with other molecules such as sugars and oxidized proteins (Rossell, 1994).

In order to improve the specificity and sensitivity of the TBA test, a number of modifications to the original TBA procedures have been suggested, such as lowering the heating temperature to stabilize the yellow colour of aldehyde-TBA complex, addition of antioxidants to the sample in an attempt to minimize oxidation during the test (Gomes *et al.*, 2003), extraction of the MA prior to the formation of the chromogen (Jardine *et al.*, 2002), use of HPLC to separate the complex before measurement or to characterize the individual species of TBARS (Antolovich *et al.*, 2002; Jardine *et al.*, 2002).

2.5.1.2.2 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.

2.5.1.2.3 Total oxidation (TOTOX) value

Total oxidation (TOTOX) value is referred to as 2 PV + *p*-AnV, while Wanasundara *et al.* (1995) have defined TOTOX $_{TBA}$ as 2PV + TBA value 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, the TOTOX value dose not attract attention of the scientists because it combines variables with different dimensions (units).

2.5.1.2.4 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 chromatograph, headspace analysis of volatiles does not need any cleaning between sample injections (Frankel, 1998). This method suffers one major drawback that 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.

In conclusion, lipid oxidation may be evaluated in many ways, among which changes in the initial reactants and formation of oxidation products are commonly

evaluated. Each method displays both advantages and disadvantages, therefore it is significant to choose the most adequate method, depending on the system under investigation and the state of oxidation itself. The use of two or more methods for evaluating both primary and secondary oxidation products is highly recommended.

2.6 Optimisation procedure for production of SL via acidolysis

Response surface methodology (RSM) has been a popular and efficient statistical procedure for 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 accurately describes the overall method.

RSM is an optimisation method that determines optimum process conditions by combining particular experimental designs with modelling using first- or second-order polynomial equations. RSM examines a number of variables at a time, reduces the number of required determinations, and measures many effects by objective tests.

The results of traditional one-variable-at-a-time do not indicate real changes in the environment as they ignore interactions among variables that are, in effect, concomitant. RSM can describe simultaneous influences more fully, and help in a more accurate optimisation of variables that affect the process, and allow concomitant solution of multivariate which specify the optimum product for a specific set of parameters through mathematical models.

Enzyme amount and incubation time are the main variables influencing the cost of preparation of SL. Moreover, reaction temperature and time can be considered important as they affect the oxidative status of prepared oils as well as the economy of the process. This design, a three-factor and three-level face-centered cube, 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 considered attractive because it decreases the time of preparation.

RSM was used to determine optimal conditions for the lipase-catalyzed enrichment of hazelnut oil by incorporating n-3 PUFA from menhaden oil. The optimal conditions for incorporating n-3 PUFA (19.6%) into hazelnut oil were: 45-60°C, over 30-40 h, 1:1-2:1 (mol hazelnut oil /mol menhaden oil concentrate), and 3-5% (w/w) for water content (Can and Ozcelik, 2005).

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 RSM. 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 (13.7%) of n-3 PUFA was predicted at a 1:4 mole ratio of TAG to FFA at 54°C 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 °C for 48.5 h (Rao *et al.*, 2002).

2.7 Tocopherols

Edible oils consist mainly of TAG (95%). Non-TAG (also known as minor components or unsaponifiable matter) make up the remaining 5%. These minor components are naturally-occurring compounds with antioxidative properties that give these oils the capability to protect themselves from oxidation and play a significant role in the stability of vegetable oils (Shahidi and Shukla, 1996; Espin *et al.*, 2000). The minor components of vegetable oils are mainly composed of phospholipids, tocopherols, tocotrienols, flavonoids, such as flavones, and other phenolic compounds, pigments (carotenoids, chlorophylls), sterols, and free fatty acids, as well as mono- and diacylglycerols (Hamilton, 1994; Shahidi and Shukla, 1996). Several classes of these components might be found in each oil and contribute to its oxidative stability (Shahidi and Shukla, 1996).

Tocols include tocopherols and tocotrienols and each series has four compounds. These are designated α, β, γ, and δ based on the number and location of methyl groups on the chromane ring (Shahidi and Shukla, 1996). The main biochemical function of tocols is believed to be protection of PUFA against peroxidation. The antioxidant activity of tocopherols is mainly due to their ability to give phenolic hydrogens to lipid free radicals produced during free radical chain reactions (Yamauchi *et al.*, 1995). The chemical

structure of the various tocopherols supports a hydrogen donating power and *in vivo* antioxidant activity is in the order $\alpha>\beta>\gamma>\delta$. However, *in vitro* activities of tocopherols are not only dependent on their chemical reactivity toward hydroperoxy and other free radicals, but also on other factors such as tocopherol concentration, temperature, light, and type of substrate and solvent (Kamal-Eldin and Appelqvist, 1996). Meanwhile, tocopherols are known to be strong singlet oxygen scavengers and thus are able to prevent or control lipid photooxidation (Bruun-Jensen *et al.*, 1996).

Among the tocopherol homologues, α-tocopherol has the highest vitamin E activity and occurs most abundantly in natural sources. Although α-tocopherol has been the centre of vitamin E studies, γ- and δ-tocopherol are known to have stronger antioxidant activity *in vitro*. Results from an *in vitro* test showed that γ-tocopherol was 1.4 times as efficient as α-tocopherol in inhibiting oxidation of PUFA (Wu *et al.*, 1979). The difference among tocopherol homologues in their transportation, deposition, distribution and retention in animal tissues may possibly account for the varied biological antioxidant efficiency *in vivo* (Kamal-Eldin and Appelqvist, 1996). The differences in the antioxidant potency of the tocopherols *in vitro* and *in vivo* may be due to the major structural differences between the two systems or to differences in bioavailability which is due to differences in solubility and affinities to tissues and transporting proteins than to differences in chemical reactivity (Kamal-Eldin and Appelqvist, 1996). While the *in vitro* antioxidant activity is highly dependent on the varying physical and chemical compositions of the system, the *in vivo* vitamin E activity seems to be highly related to the lipophilicity of the vitamin (Kamal-Eldin and Appelqvist, 1996). Tocopherols (vitamin E) are used widely as antioxidants to increase the oxidative stability of foods.

Other than its vitamin E activity, α-tocopherol is a powerful biological antioxidant that can protect biological membranes and lipid components containing unsaturated fatty acids against attack by reactive oxygen species (ROS) (Huang and Huang, 2004).

In order to measure the effect of processing and the contribution of minor constituents to the stability of edible oils, it is possible to strip the oil from its non-TAG components. To achieve this, the oil is subjected to a multilayered column separation. The stripping of borage and evening primrose oils indicated that oxidation products and most of the minor components were removed. The oils were more stable, as such, than their stripped counterparts when the oils were subjected to accelerated oxidation under Schaal oven condition at 60°C, possibly due to the removal of minor components (Khan and Shahidi, 2000; 2001; 2002).

During processing of oils and as a result of reactions in production of structured and other new lipids many of these components are removed and thus the relative stability of the preparation is compromised. In enzymic acidolysis of algal oils with capric acid we found that the resultant oils were much less stable than their unmodified counterparts despite a decrease in the degree of unsaturation of the products (Hamam and Shahidi, 2004 a, b, 2005). Experiments carried out in the absence of any enzyme demonstrated the removal of the endogenous antioxidants and this might primarily be responsible for this phenomenon, thus lending further support to the findings of Senanayake and Shahidi (2002) who found that removal of natural antioxidants, such as tocopherols, during modification of borage and evening primrose oils with n-3 fatty acids might play an important role in enhancing the oxidative deterioration of the modified oils, and those of Akoh and Moussata (2001) who reported a significant loss of tocopherols in fish-, and

canola-based SL containing caprylic acid. However, these studies did not investigate the mechanism of the removal of natural antioxidants during processing and synthesis of specialty lipids. Another possible explanation of compromised stability of the modified oils that has not been investigated so far would be conversion of tocopherols to tocopheryl esters through the reaction of carboxylic acids and tocopherols. This mechanism would be explored, for the first time, in this study and details are shown in chapter 9 of this thesis.

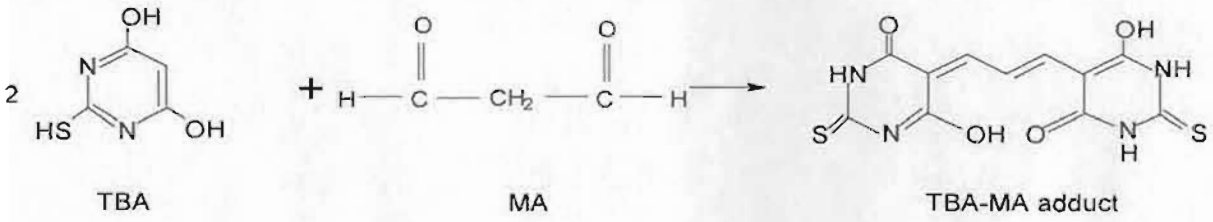
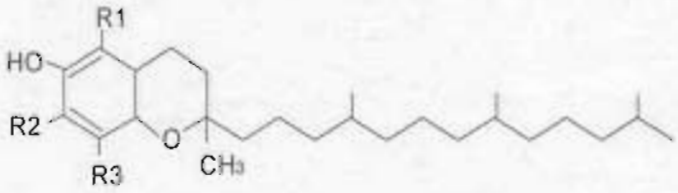
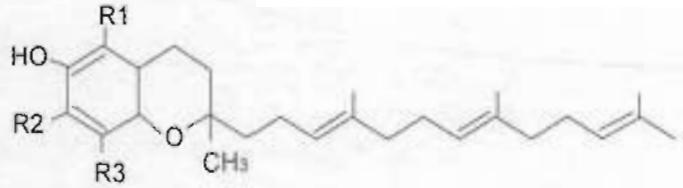


Figure 2.4 Reaction of 2-thiobarbituric acid (TBA) and malonaldehyde (MA).



R1	R2	R3	
CH_3	CH_3	CH_3	α-tocopherol
CH_3	H	CH_3	β-tocopherol
H	CH_3	CH_3	γ-tocopherol
H	H	CH_3	δ-tocopherol



R1	R2	R3	
CH_3	CH_3	CH_3	α-tocotrienol
CH_3	H	CH_3	β-tocotrienol
H	CH_3	CH_3	γ-tocotrienol
H	H	CH_3	δ-tocotrienol

Figure 2.5 Chemical structures of tocopherols and tocotrienols

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Chapter 3

Materials and Methods

3.1 Materials

Two lipases from *Candida antarctica* (Novozyme-435) and *Mucor miehei* (Lipozyme-1M) were acquired from Novo Nordisk (Bagsvaerd, Denmark). Other lipases, namely *Pseudomonas sp.* (PS-30), *Aspergillus niger* (AP-12), and *Candida rugosa* (AY-30) were obtained from Amano Enzyme (Troy, VA). Algal oil, docosahexaenoic acid (DHA) single-cell oil (DHASCO) containing approximately 40% DHA, was obtained from Martek Bioscience Corporation (Columbia, MD). Reagents, butylated hydroxytoluene (BHT), anhydrous sodium sulphate, monoolein, diolein, triolein, hydroquinone, phenolphthalein, sodium hydroxide, boric acid, porcine pancreatic lipase (EC 3.1.1.3), 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), tocopherols (α-, β-, γ-, and δ-tocopherol) as well as α-tocopherol acetate and stearic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol, hexane, methanol, sulphuric acid, hydrochloric acid, isooctane, isobutanol, chloroform, acetone, acetic acid, diethyl ether, and 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). All solvents used in this work were of analytical grade and were purchased from Fisher Scientific (Nepean, ON). Eicosapentaenoic acid (EPA, >99% pure), was from Fuso Pharmaceutical Industries LTD. (Osaka, Japan) and kindly provided by Dr. K. Miyashita. Samples of high-laurate canola oil, known as Laurical 15, 25, and 35 were products of Calgene's Oils Division

(Davis, CA) and were provided by Dr. James Daun from the Canadian Grain Commission (Winnipeg, MB). Arachidonic acid (AA; >90%), α-linolenic acid (ALA; >95%), conjugated linoleic acid (CLA; >95%), fatty acid methyl esters (FAMEs; GLC-461), triolein (tri C18:1), trilinolein (tri C18:2), and trilinolinin (tri C18:3) were obtained from Nu-Chek (Elysian, MN). Docosapentaenoic acid (DPA, C22:5 n-3) was prepared as a concentrate using a proprietary procedure.

3.2 Methods

3.2.1 Preparation of free fatty acids from algal oil

Preparation of free fatty acids from algal oil was conducted according to the method described by Wanasundara and Shahidi (1999). Algal oil (25 g, treated with 200 ppm butylated hydroxytoluene) was saponified using a mixture of KOH (5.75 g), water (11 mL) and 95% (v/v) aqueous ethanol (66 mL) by refluxing for 1 h at 60°C under an atmosphere of nitrogen. Distilled water (60 mL) was added to the saponified mixture and the unsaponifiable matter was extracted into hexane (2x 100 mL) and discarded. The aqueous layer containing saponifiable matter was acidified (pH =1.0) with 3.0 N HCl. The mixture was transferred to a separatory funnel, and the liberated fatty acids were extracted into hexane (50 mL). The hexane layer containing free fatty acids was then dried over anhydrous sodium sulphate and the solvent removed in a rotary evaporator at 40°C to recover the free fatty acids which were then stored at – 20°C until use.

3.2.2 Preparation of DHA concentrate from algal oil by urea complexation

The separation of DHA from the hydrolyzed fatty acid mixture of algal oil was performed using urea-fatty acid adduct formation according to Wanasundara and Shahidi (1999). Free fatty acids (10 g) were mixed with urea (20%, w/v) in 150 mL 95% aqueous

ethanol and heated at 60°C with stirring until the whole mixture turned into a clear homogeneous solution. The urea-fatty acid adduct was allowed to crystallize at room temperature but was later placed in a cold room where the temperature was maintained at 4°C for 24 h. The crystals formed were separated from the liquid (nonurea complexing fraction, NUCF) by suction filtration through a thin layer of glass wool. The filtrate (NUCF) was diluted with an equal volume of water and acidified to pH 4-5 with 6 N HCl; an equal volume of hexane was subsequently added and the mixture stirred thoroughly for 1 h, then transferred to a separatory funnel. The hexane layer containing liberated FA was separated from the aqueous layer containing urea and washed with distilled water to remove any remaining urea and then dried over anhydrous sodium sulphate. The solvent was subsequently removed at 40°C using a rotary evaporator. The NUCF was weighed and percentage recovery calculated.

3.2.3 Acidolysis reaction

3.2.3.1 Acidolysis of high-laurate canola oil and n-3 fatty acids (EPA or DPA or DHA)

High-laurate canola oil (100 mg) was mixed with n-3 FA (EPA or DPA or DHA) at different mole ratios of oil to n-3 fatty acids ranging from 1 to 3, in a screw-capped test tube, and then lipase (2-10% by weight of substrates) and water (2% by weight of substrates and enzyme) were added in hexane (3.0 mL). Samples were flushed with nitrogen, the containers capped and incubated for different time periods (12 to 48 h) in an orbital shaker at 250 rpm at 25- 55°C.

3.2.3.2 Acidolysis of tristearin (tri C18:0) and selected long-chain fatty acids

Tristearin (100 mg) was mixed with different fatty acids (OA, GLA, LA, CLA, and ALA) at a mole ratio of acid to tristearin of 3:1 in a screw-capped test tube, then lipase (10% by weight of substrates) and water (2% by weight of substrates and enzyme) were added in hexane (3.0 mL). The mixture was incubated at 45 ± 2°C for 24 h in a shaking water bath at 250 rpm. In another set of experiments, a combination of equimole quantities of unsaturated C18 FA (OA+LA+CLA+GLA+ALA) at tristearin to C18 FA ratios of 1:1, 1:2, and 1:3 was used to investigate the effect of substrate mole ratio on incorporation of these FA into tristearin. The experimental conditions were the same as these mentioned earlier.

A mixture of equimole quantities of n-3 FA (ALA+EPA+DHA) at tristearin to n-3 FA ratios ranging from 1:1 to 1:3 was mixed in a screw-capped test tube then lipase (10%) and water (2%) were added in hexane (3.0 mL). The mixture was incubated at 45 ± 2°C for 24 h in a shaking water bath at 250 rpm.

3.2.3.3 Acidolysis of triolein (tri C18:1) and selected long-chain fatty acids

Triolein (100 mg) was mixed with different fatty acids (SA, GLA, LA, CLA, ALA, AA, EPA, DPA, and DHA) at a mole ratio of acid to triolein of 3:1 in a screw-capped test tube, then lipase (4% by weight of substrates) and water (2% by weight of substrates and enzyme) were added in hexane (3.0 mL). The mixture was incubated at 45 ± 2°C for 24 h in a shaking water bath at 250 rpm. In another set of experiments, a combination of equimole quantities of C18 FA (SA+LA+CLA+GLA+ALA) at triolein to C18 FA ratios of 1:1, 1:2, and 1:3 was used to investigate the effect of substrate mole ratio on incorporation of these fatty acids into triolein. The experimental conditions were the same

as these mentioned earlier. Similarly, a mixture of equimole quantities of unsaturated C18 FA (LA+CLA+GLA+ALA) at triolein to C18 FA ratios ranging from 1:1 to 1:3 was mixed with in a screw-capped test tube. The enzyme amount, reaction temperature, and incubation time were 4%, 45 ± 2°C, and 24 h, respectively.

The same was done for a combination of equimole quantities of n-3 FA (ALA+EPA+DHA+DPA) or n-6 FA (LA+GLA+AA).

3.2.3.4 Acidolysis of trilinolein (tri C18:2) or trilinolinin (tri C18:3) and selected long-chain fatty acids

A combination of equimole quantities of C18 FA (SA+OA +CLA+GLA+ALA) at trilinolein (tri C18:2) or trilinolinin (tri C18:3) to C18 FA mole ratio of 1:3 was mixed with in a screw-capped test tube. The enzyme amount, reaction temperature, and incubation time were 4%, 45 ± 2°C, and 24 h, respectively. The same was done for a mixture of equimole quantities of n-3 FA (ALA+EPA+DHA+DPA) or n-6 FA (LA+GLA+AA).

3.2.3.5 Acidolysis of oleic acid and tocopherols

In general, oleic acid (100 mg) was mixed with α-, γ-, and δ- tocopherols at different mole ratios of oil to tocopherol ranging from 1 to 3, in a screw-capped test tube, and then lipase (4% by weight of substrates) and water (2% by weight of substrates and enzyme) were added in n-hexane (3.0 mL). The mixture was flushed with stream of nitrogen, stirred, and then incubated for 24 h in an orbital shaker at 250 rpm at 45°C. In another set of experiments, triolein was mixed with α-, γ-, and δ- tocopherols at different mole ratios of oil to tocopherol ranging from 1 to 3. The mixture was subjected to the same experimental conditions as mentioned earlier.

3.2.4 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 FFA, 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 acylglycerols. The mixture was thoroughly mixed and transferred into a separatory funnel and 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. A portion of the fraction (5-10 mg) was transferred to special transmethylation vials.

3.2.5 Fatty acid composition of products

3.2.5.1 Preparation of fatty acid methyl esters (FAMEs)

FA 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 overnight and subsequently cooled to room temperature. Distilled water (1 mL) was added to the mixture, after thorough mixing, few crystals of hydroquinone were added to each vial to prevent oxidation. The FAMEs 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 (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 GC analysis.

3.2.5.1 Analysis of FAMEs by gas chromatography

The FAMEs were analysed using a Hewlett-Packard 5890 Series II gas chromatograph (Agilent, Palo Alto, CA) equipped with a SUPELCOWAX-10 column (30 m length, 0.25 mm diameter, 0.25 μm 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. Ultra-high purity (UHP) helium was used as a carrier gas at a flow rate of 15 mL/min. Data were treated using Hewlett Packard 3365 Series II Chem Station Software (Agilent). The FAMEs were identified by comparing their retention times with those of authentic standard mixture (GLC-461; Nu-Check-Prep, Elysian, MN), and the results were presented as weight percentages.

3.3 Hydrolysis by pancreatic lipase

Hydrolysis of the 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), calcium chloride (0.5 mL, 2.2%, w/v), and sodium taurocholate (1.25 mL; 0.05%) were added to 25 mg of modified oil in a glass test tube. The entire 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 3.1.1.3; Sigma). The mixture was subsequently placed in a gyrotory water bath shaker at 250 rpm under nitrogen for 10-15 min 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 25.0 mL of hexane and the upper layer removed and washed twice with distilled water and subsequently 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 1 h. The hydrolytic products were separated on a silica gel TLC plates. The plates were developed using hexane/diethyl ether/acetic acid (70:30:1, v/v/v) for 40-50 min and then allowed to air dry. 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). The FA profile of lipids extracted was analysed as described in the section on analysis of FAMEs by GC.

3.4 Oxidative stability tests

Structured lipids as well as the original oils were kept under accelerated oxidation conditions at 60°C in a Schaal-oven for a period of 3 days. Each day (24 h) of storage of oils under Schaal-oven conditions at 60°C is equivalent to one month of 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, Precision 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 analysed.

3.4.1 Conjugated Dienes (CD)

Conjugated dienes in the oils were determined according to the IUPAC (1987) method 20505. Oil samples (0.02-0.04 g) were weighed 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 diode array Model 8452 A spectrophotometer (Agilent Palo Alto, CA). Pure iso-octane 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 AOCS (1990) method Cd 19-90. 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. An aliquot of 5.0 mL of the mixture (oil and solvent) was transferred into a dry screw capped test tube, and then 5.0 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 the test tube was 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 (Agilent). The number of micromoles of malonaldehyde (MA)

equivalents 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

The approximate conditions for n-3 FA incorporation, enzyme load, reaction temperature and reaction time were determined by changing one factor at a time while keeping the others constant. Thus, a proper range for each factor was determined for RSM.

A three-factor and three-level face-centered cube design with 17 individual design points was selected in this study (Hamam and Shahidi, 2004 a, b; Senanayake and Shahidi, 2002; Mason *et al.*, 1989; Gao and Mazza, 1996). 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). The response or dependent variables (Y) studied were n-3 FA incorporation (%). Triplicate experiments were conducted at all design points.

The second-order polynomial model predicted for optimisation of n-3 FA incorporation into the 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 analyzed 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. 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 were coded plus or minus one, respectively, for this three-level design. 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 and Shahidi, 1999; Hamam and Shahidi, 2004).

3.6 Synthesis of tocopheryl ester

3.6.1 Acyl chloride syntheses

Acyl chloride was synthesized according to the method described by Taylor *et al.* (1988) with some modifications. A 50 mL, three-necked flask equipped with a dropping funnel and a reflux condenser fitted with an inert gas (nitrogen) inlet tube that was attached to a mineral oil bubbler. The system was flushed with nitrogen, flame dried, cooled to room temperature, and maintained under a positive pressure of nitrogen. The mixture contained oleic acid (1.0 g) and 0.422 g (0.257 mL, 0.004 mol) distilled thionyl chloride ($SOCl_2$) was added dropwise over 30 min at a mole ratio of acid to thionyl

chloride of 1:1. Pyridine was added to the mixture. The mixture was heated under reflux over seven hours. The tocopheryl esters were extracted three times, each with diethyl ether and HCl (10%, v/v). The mixture was thoroughly mixed and transferred into a separatory funnel. Two layers (aqueous and diethyl ether) were allowed to separate, and the lower aqueous layer was discarded. The diethyl ether layer was then passed through a bed of anhydrous sodium sulfate to remove any residual water. The diethyl ether was evaporated using a rotary evaporator at 45°C and the presumed tocopheryl oleate was recovered.

The mixture was separated on activated silica gel TLC plates. The plates were developed using hexane/diethyl ether/ acetic acid (70:30:1, v/v/v) for 40-50 min and then allowed to air dry. The bands were located by viewing them under a short- (254 nm) and a long- (365 nm) wavelength light (Spectroline, Co., Westbury, NY). The bands `were scraped off and their contents extracted into methanol/chloroform (1:1, v/v).

3.7 Normal phase HPLC-MS analysis of tocopherols

Tocopherol contents as well as tocopheryl esters in samples were determined by normal phase high performance liquid chromatography (HPLC) - mass spectrometry (MS). The analysis was performed using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA) with a UV-diode array detector (UV-DAD). Separation was achieved on a Supelcosil LC-Si column (250 mm length, 4.6 mm i.d., 5μm particle size, Sigma-Aldrich Canada Ltd., Oakville, ON) coupled with a Supelcosil LC-Si guard column. Tocopherols and tocopheryl esters were eluted using an isocratic solvent system containing hexane/2-propanol (95:5, v/v) at a flow rate of 1.0 ml/min. Fifty microliters of each tocopherol standard and sample was injected. Tocopherols were detected at 290 nm by the UV

detector. LC flow was analyzed on-line by a mass spectrometric detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA) with a positive ion APCI (Atmospheric pressure chemical ionization). The operating conditions used were 121V for the fragmentor voltage, 350°C for drying temperature, 400°C for APCI temperature, 60 psi for the nebulizer pressure, and 7 L/min for the drying gas flow.

3.8 Statistical analysis

All experiments were performed in triplicate. Data are reported as mean ± standard deviation (SD). Normality was tested using SigmaStat (Richmond, CA). Analysis of variance (ANOVA) and Tukey's test were carried out at a level of $p < 0.05$ to assess the significance of differences among mean values.

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Chapter 4

Lipase-assisted acidolysis of high-laurate canola oil (Laurical 15) with long-chain omega 3 fatty acids

4.1 Introduction

Medium-chain fatty acids (MCFA) comprise 6-12 carbon saturated fatty acids, obtained from hydrolysis of tropical plant oils such as those of coconut and palm kernel (Akoh and Huang, 1995; Akoh, 1997). Pure medium-chain triacylglycerols (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 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. MCT do not require chylomicron formation to transfer from the 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 MCT have approximately twice the caloric value of proteins and carbohydrates and have little propensity to accumulate as body fat. The higher solubility and smaller molecular size of MCFA make their absorption, transport and metabolism much easier than long-chain fatty acids. MCT are hydrolyzed more quickly and completely by pancreatic lipase than long-chain triacylglycerols (LCT).

Epidemiological studies have linked the low incidence of coronary heart disease in Greenland Eskimos with their high dietary intake of n-3 PUFA (Bang and Dyerberg, 1972, 1986). The n-3 FA are essential for normal growth and development throughout the life

cycle of humans and therefore should be included in the diet. The n-3 fatty acids have been extensively studied for their influence on cardiovascular disease (CVD). However, the exact mechanism by which these effects are rendered remains unknown, but research results have shown that these FA in marine oils may prevent CVD by decreasing serum TAG and acting as anti-atherogenetic and antithrombotic agents (Newton, 2001).

In many foods for medical application, a mixture of MCT and LCT is used to provide both rapidly and slowly metabolizing fuel as well as essential fatty acids. Any abnormality in the many enzymes or processes involved in the digestion of LCT can cause symptoms of fat malabsorption. Thus, patients with certain diseases (Crohn's disease, cystic fibrosis, colitis and enteritis, etc.) have shown improvement when MCT are incorporated into their diet (Kennedy, 1991).

Structured lipids (SL) provide the most efficient means of delivering desired FA for nutritional and/or medical purposes (Akoh, 1995; 1996; 1997). Strategies for lipid modification include genetic engineering of oilseed crops, production of oils containing high levels of PUFA, and lipase- or chemically-assisted interesterification reactions. Depending on the type of substrate available, chemical or enzymatic reactions can be used for the synthesis of modified lipids, including direct esterification (reaction of fatty acids and glycerol), acidolysis (transfer of the acyl group between an acid and ester), and alcoholysis (exchange of the alkoxy group between an alcohol and an ester) (Lee and Akoh, 1998). However, the common methods cited in the literature for production of SL are based on reactions between two TAG molecules (interesterification) or between a TAG and an acid (acidolysis). Chemically-catalyzed interesterification, using alkali such as sodium methoxide, is cheap and easy to scale up. However, such reactions lack

specificity and offer little or no control over the positional distribution of FA in the final product (Lee and Akoh, 1998). An alternative to the chemical synthesis of SL is the enzymatic process using a variety of lipases. Lipase-assisted interesterification offers many advantages over the chemical process. 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, with a reduction of energy consumption, and with reduced heat damage to reactants, and easy purification of products (Akoh and Huang, 1995; Akoh, 1997). Another approach is to produce structured lipids through bioengineering. An early attempt was the production of a high-laurate canola oil containing 40% lauric acid (C12:0) (DeVecchio, 1996). It would be useful to use this oil as a model oil for incorporating LCFA into MCT. Recently, incorporation of n-3 PUFA, especially EPA and DHA, into melon seed oil (Huang and Akoh, 1994a), vegetable oils (Huang and Akoh, 1994b), borage oil (Akoh and Sista, 1995; Ju *et al.*, 1998; Akoh and Moussata, 1998), evening primrose oil (Senanayake and Shahidi, 1999; Akoh *et al.*, 1996), and trilinolein (Akoh *et al.*, 1995) has been reported. Synthesis of SL containing MCFA in the *sn*-1,3 positions and an unsaturated LCFA in the *sn*-2 position of the glycerol backbone using 1,3-regiospecific lipases was recently reported by Soumano et al. (1998). The final product contained more than 94% caprylic acid in the *sn*-1 and *sn*-3 positions, whereas more than 98% of the unsaturated FA (oleic acid 20.1%, linoleic acid 78.0%) were present in the *sn*-2 position. Shimada *et al.* (1996) described an effective method of producing SL that contain linoleic acid (LA) and α-linolenic acid (ALA) by acidolysis of safflower and linseed oils with caprylic acid (CA, C8:0), respectively, using immobilized *Rhizopus*

delemar lipase. Acidolysis of safflower oil with CA produced only 1,3-capryloyl-2-linoleoylglycerol and 1,3-capryloyl-2-oleoylglycerol, with a ratio of 86:14 (w/w). Additionally, all TAG in linseed oil were converted to 1,3-capryloyl-2-α-linolenoylglycerol, 1,3-capryloyl-2-linoleoylglycerol, and 1,3-capryloyl-2-oleoylglycerol, at a ratio of 60:22:18 (w/w/w). Kosugi and Azuma (1994) described a simple method to prepare and isolate high yields of pure TAG from PUFA using immobilized lipase from *Candida antarctica* and *Rhizomucor miehei*. The esterification of glycerol and FFA or ethyl esters of the FA was performed at 50-60°C over 24 h. More than 95% of PUFA was converted to TAG. Fomuso and Akoh (2001) synthesized SL from high-laurate canola oil and stearic acid using lipase from *Candida antarctica*. The resulting SL was then used to prepare trans fatty acid-free margarine. Addition of canola oil to the SL improved spreadability at refrigeration temperatures and reduced the hardening effect of lauric acid in the SL.

The aim of this study was to (1) produce SL via acidolysis of high-laurate canola oil with EPA, DPA, and DHA; (2) determine the effect of reaction variables such as the type of enzyme, enzyme concentration, incubation time, reaction temperature, and substrate mole ratio on the extent of incorporation of n-3 fatty acids into high-laurate canola oil; (3) determine the positional distribution of FA in the modified and unmodified oils; and (4) assess the oxidative stability of the resultant SL.

4.2 Materials and methods

Materials and methods are detailed in Chapter 3.

4.3 Results and Discussion

4.3.1 Fatty acid composition of Laurical 15 before and after enzymatic modification

The lauric acid content of Laurical 15 was 37.6 % (Table 4.1). Furthermore, this oil also contained other saturated fatty acids, namely stearic (36.0%), palmitic (3.21%), and myristic (3.82%) acids, and its oleic acid content was almost 15%.

4.3.2 Enzyme screening

Five lipases, one each from *C. antarctica, M. miehei, Pseudomonas sp., C. rugosa, and A. niger*, at 4% enzyme amount at 45°C over 24 h, were screened for their ability to incorporate EPA, DHA, and DPA into Laurical 15 (Table 4.2). The order of incorporation of EPA into Laurical 15 was *Pseudomonas sp.* > *Candida rugosa* ≥ *Aspergillus nige r*≥ *Candida antarctica* > *Mucor miehei*. It was found that lipase from *Pseudomonas sp.* was most effective in its ability to incorporate EPA into Laurical 15. This is despite the activity of enzymes tested (*C. antarctica,* 554 U; *M. miehei,* 13613 U; *Pseudomonas sp.,* 11936 U; *A. niger,* 8142 U; and *C. rugosa,* 38707 U). *Pseudomonas sp.* lipase gave the highest degree of incorporation of DHA (23.7%, after 24 h) and DPA (6.58%, after 24 h) into Laurical 15. Because acidolysis of Laurical 15 with long-chain n-3 FA was best achieved using lipase from *Pseudomonas sp.*, this enzyme was selected for subsequent experiments.

4.3.3 Mole ratio effect

When the mole ratio of substrates increased from 1:1 to 1:3, incorporation of EPA into Laurical 15 increased accordingly (Table 4.3). The highest incorporation of EPA into Laurical 15 (66.9%) was obtained at an oil to EPA mole ratio of 1:3. As the number of moles of DHA increased from 1 to 3, its incorporation into Laurical 15 increased steadily. The optimum incorporation of DHA into Laurical 15 (23.7%) was obtained at oil to DHA mole ratio of 1:3 because TAG molecules can incorporate a maximum of three fatty acids

in their backbone. However, in enzymatic reactions, this ratio is not always limited to 1:3. When the mole ratio of substrates increased from 1:1 to 1:2, the incorporation of DPA increased accordingly. The optimum incorporation of DPA into Laurical 15 (7.19 %) was obtained at oil to DPA mole ratio of 1:2.

4.3.4 Positional distribution

The positional distribution of fatty acids in modified and unmodified high-laurate canola oil (Laurical 15) was determined (Tables 4.4 and 4.5). Lauric acid was mainly located at the *sn*-1,3 positions of the original, unmodified oil. Lauric acid remained mostly esterified to the *sn*-1,3 positions of the TAG molecules while EPA, DPA or DHA was primarily located in the *sn*-1,3 positions of the TAG molecules of modified oils. Although EPA, DPA, and DHA were incorporated mostly at the *sn*-1,3 positions of the glycerol backbone of modified Laurical 15, incorporation into the *sn*-2 position was also observed.

4.3.5 Time course

The time course of lipase-assisted acidolysis of EPA, DHA, and DPA into Laurical 15 using PS-30 from *Pseudomonas sp.* is shown in Figure 4.1. As the time progressed from 24 to 36 h, incorporation of EPA into Laurical 15 increased significantly, reaching an optimum at 36 h, followed by an insignificant ($p > 0.05$) decrease (38.5%) at 48 h, possibly due to the occurrence of the reverse hydrolysis, or reverse acidolysis reaction. As the time progressed from 12 to 48 h, the percent DHA incorporation into Laurical 15 increased accordingly and reached an optimum at 48 h. The same trend was observed for DPA incorporation into Laurical 15. The highest incorporation of EPA (40.6%) may be

obtained over 36 h, DHA (35.5%) over 48 h, and DPA (43.2%) over 48 h into Laurical 15.

4.3.6 Reaction temperature effect

The effect of temperature on the reaction rate of enzyme-assisted reaction, prior to the denaturation temperature (above 56 °C), is a well-established phenomenon. Thus, to determine the optimum temperature for incorporation of n-3 fatty acids into Laurical 15, the reaction was carried out at temperatures ranging from 25 to 55°C as shown in Figure 4.2; reaction rates gradually increased from 35 to 45°C. The optimum incorporation of EPA into Laurical 15 (66.9%) was obtained at 45°C. When temperature increased above 45°C, EPA incorporation into Laurical 15 declined. Therefore, a reaction temperature of 45°C was used for the subsequent experiments when considering acidolysis reactions of Laurical 15 and EPA. The optimum incorporation of DHA into Laurical 15 (27.6%) was obtained at 35°C. When temperature increased above 35°C, DHA incorporation into Laurical 15 declined. However, the percent incorporation of DHA was not significantly different ($p>0.05$) when the reaction temperature changed from 35 to 45°C. Similarly, as the reaction temperature altered from 25 to 35°C, the DPA incorporation (%) increased accordingly and reached a highest value (34.8%) at 35°C after which the percent incorporation of DPA was not significantly different ($p>0.05$). Thus, a reaction temperature of 35°C was selected for the remaining experiments of Laurical 15 with DHA or DPA.

4.3.7 Enzyme load effect

As the enzyme load (%) increased from 2 to 4%, incorporation of EPA into Laurical 15 increased gradually reaching a maximum (66.9%) at 4% (Figure 4.3), but at above 4% enzyme load, the percent incorporation of EPA decreased but was not significantly different ($p>0.05$). This may be due to a deficiency of the available water for hydration of the enzyme because the amount of added water was held constant at a 2% (w/w) level, regardless of the amount of enzyme added. As the enzyme load (%) increased from 2 to 10%, incorporation of DHA into Laurical 15 increased gradually and reached a maximum (28.9%) at 10%. As the enzyme concentration (%) changed from 2 to 10%, incorporation of DPA into Laurical 15 increased gradually reaching a maximum (36.6%) at 6%. The percent incorporation of EPA into Laurical 15 was significantly ($p<0.05$) higher than both DHA or DPA incorporation into Laurical 15. Furthermore, the percent incorporation of DPA was significantly ($p<0.05$) higher than those of DHA, as the enzyme load was altered from 2 to 10%. EPA is more reactive than DPA or DHA. EPA has five double bonds while DHA has six. The more double bonds the chain has in the cis configuration, the more bent it is. Since DHA has six cis double bonds, it becomes quite curved compared to EPA and hence DHA has more steric hindrance than EPA. DPA and DHA have the same chain length and both belong to the n-3 family. However, DPA has one less double bond than DHA and hence less steric hindrance Therefore, the structural differences (chain length, number and location double bonds) between these three molecules as well as specificity of the enzymes used had a marked effect on their incorporation into Laurical 15.

4.3.8 Conjugated dienes (CD)

The CD values of the modified Laurical 15 with EPA, DHA, and DPA and the original oil, are shown in Figure 4.4. The control (original) oil was stable under oxidative conditions over the entire storage period (0 to 72 h) because Laurical 15 contained almost 80% saturated fatty acids as well as 15% oleic acid that resist oxidation. All modified oils containing EPA, DPA, and DHA oxidized during storage, albeit to different extents. Then reached a maximum at 12, 24, and 6 h, respectively. The sharp increase in the CD might be accounted for by the formation of more and more hydroperoxides as primary products of oxidation, then decreased, possibly due to the breakdown of unstable hydroperoxides. The results showed that modified Laurical 15 oils with EPA, DPA, and DHA were less stable than the original oil due to incorporation of long-chain omega 3 FA with five to six double bonds and hence become more susceptible to oxidation.

4.3.9 Thiobarbituric acid reactive substances (TBARS)

Figure 4.5 shows TBARS values of Laurical 15 modified with n-3 FA as well as the control unmodified oil. As expected, the control (original) oil was stable under oxidative conditions over the entire storage period. The TBARS values of modified Laurical 15 with EPA oil increased progressively with the storage time (0 to 48 h) under Schaal oven conditions at 60°C. The highest TBARS value (18.1 μmol/g) was obtained at 48 h, after which the TBARS values decreased, possibly due to the breakdown of the secondary products. The highest TBARS value (12.2 μmol/g) for DHA-modified oil was found at 36 h then decreased. For DPA-modified Laurical 15, the same trend was noted, reaching a maximum (10.1 μmol/g) after 12 h. In general, TBARS values of EPA-modified oil were higher than those of DHA-or DPA-modified Laurical 15. The order of

oxidative stability was EPA-modified Laurical 15 < DHA-modified Laurical 15 < DPA-modified Laurical 15. There was a significant difference ($P<0.05$) between the modified and unmodified oils. The results reported here indicate that modification of Laurical 15 with n-3 FA had significant effect on TBARS values of the oil. TBARS represent secondary oxidation products resulting from degradation of hydroperoxides. Hence their appearance depends on the rate of breakdown of the hydroperoxides involved. However, this may be influenced by the presence of antioxidants in these systems.

4.3.10 Conclusions

Lipase assisted production of SL via acidolysis of Laurical 15 with EPA was achieved using 4% lipase from *Pseudomonas sp.* (possibly due to the experimental conditions employed in this study which were suitable for this enzyme) at a mole ratio of EPA to Laurical 15 of 3:1 at 45°C over a 36 h. Similarly, incorporation of DHA into Laurical 15 was better obtained at a mole ratio of oil to DHA of 1:3, 10% lipase, at 35°C over 48 h. For DPA incorporation into Laurical 15, the optimum conditions were mole ratio of 1:2 (oil: DPA) at 35°C, over 48 h in the presence of 6% enzyme and 2% water content. Lauric acid was mainly located at the *sn*-1,3 positions of the original, unmodified oil; it remained mostly esterified to the *sn*-1,3 positions of the TAG molecules of the modified oils while EPA, DPA or DHA was also primarily located in the *sn*-1,3 positions of the TAG molecules of modified oils. Although EPA, DHA, or DPA was incorporated mainly at the *sn*-1, 3 positions, incorporation into the *sn*-2 position was also noted. Modified Laurical 15 oils were more prone to oxidation than their unmodified counterpart.

Table 4.1. Fatty acid composition (wt%) of modified and unmodified Laurical 15 oils [a]

Fatty acid	Unmodified	EPA-Modified	DHA-Modified	DPA-Modified
12:0	37.6 ±1.33	9.81 ± 0.08	18.1 ± 0.14	17.4 ± 0.38
14:0	3.82 ± 0.07	1.16 ± 0.01	2.15 ± 0.03	1.97 ± 0.02
16:0	3.21 ± 0.08	1.14 ± 0.01	1.92 ± 0.02	1.88 ± 0.02
18:0	36.0 ± 1.04	15.2 ± 0.33	16.8 ± 0.57	28.9 ± 0.66
18:1 n-9	14.7 ± 0.71	5.94 ± 0.03	13.6 ± 0.57	9.17 ± 0.29
20:5 n-3	-	64.9 ± 0.26	-	-
22:5 n-3	-	-	-	33.3 ± 0.09
22:6 n-3	-	-	35.5 ±1.11	-
Others	4.56	1.85	11.9	7.38

[a] Results are mean values of triplicate determinations ± standard deviation.

The reaction mixture contained Laurical 15 (100 mg), fatty acid to Laurical 15 mole ratio of 3:1, enzyme at 4% by weight of substrates, water at 2% by weight of enzyme and substrates, and 3.0 mL of hexane. The mixture was kept at 45 °C for 24 h in a shaking water bath at 250 rpm

Table 4.2. Effect of enzyme type on the incorporation (%) of long-chain n-3 fatty acids into Laurical 15[a]

Enzyme source	EPA	DHA	DPA
Candida antarctica	55.5 ± 0.70^{b}	16.3 ± 0.63^{b}	3.33 ± 0.35^{b}
Mucor miehei	50.8 ± 1.02^{a}	20.5 ± 2.19^{c}	3.52 ± 0.27^{b}
Pseudomonas sp.	64.6 ± 0.43^{d}	23.7 ± 3.35^{c}	6.58 ± 3.57^{b}
Candida rugosa	58.1 ± 1.23^{c}	14.2 ± 0.22^{a}	1.68 ± 0.17^{a}
Aspergillus niger	56.1 ± 0.48^{c}	16.8 ± 1.49^{b}	1.78 ± 0.39^{a}

[a] Results are mean values of triplicate determinations ± standard deviation. Values within each column with different superscripts are different ($p<0.05$) from one another. [a, b, c, d, e] the order of EPA or DPA or DHA incorporation into Laurical 15, from lowest to highest, was as follows: a < b< c< d < e.

The reaction mixture contained Laurical 15 (100 mg), fatty acid to Laurical 15 mole ratio of 3:1, enzyme at 4% by weight of substrates, water at 2% by weight of enzyme and substrates, and 3.0 mL of hexane. The mixture was kept at 45 °C for 24 h in a shaking water bath at 250 rpm.

Table 4.3. Effect of mole ratio of substrates on incorporation of long-chain n-3 fatty acids into Laurical 15[a]

	Mole ratio n-3 fatty acids /oil		
Oil	1:1	1:2	1:3
EPA-modified	34.1 ± 0.46[a]	53.4 ± 2.36[b]	66.9 ± 3.54[c]
DHA-modified	1.14 ± 0.16[a]	18.8 ± 4.97[b]	23.7 ± 3.35[b]
DPA-modified	1.04 ± 0.17[a]	7.19 ± 2.27[b]	6.58 ± 3.57[b]

[a]Results are mean values of triplicate determinations ± standard deviation. Values within each row with different superscripts are different ($p<0.05$) from one another. [a, b, c] the order of EPA or DPA or DHA incorporation into Laurical 15, from lowest to highest, was as follows: a < b< c.

Table 4.4. Positional distribution [a] (wt%, *sn*-2 and *sn*-1+ *sn*-3) of FA in modified and un-modified Laurical 15

	unmodified		modified with EPA		modified with DPA	
Fatty acid	*sn*-2	*sn*-1 + *sn*-3	*sn*-2	*sn*-1 + *sn*-3	*sn*-2	*sn*-1+ *sn*-3
C12:0	32.3 ± 13.7 (28.5)	41.7 ± 5.39 (71.5)	9.87 ± 3.39 (8.72)	19.9 ± 7.55 (91.3)	20.1 ± 1.10 (38.5)	11.6 ± 4.72 (61.5)
C14:0	3.49 ± 0.86 (30.5)	3.98 ± 0.44 (69.5)	2.03 ± 0.51 (17.7)	2.11 ± 0.92 (82.3)	2.53 ± 0.22 (42.8)	1.80 ± 0.01 (57.2)
C16:0	3.88 ± 0.97 (40.3)	3.09 ± 0.34 (59.7)	6.49 ± 4.82 (67.4)	3.73 ± 3.43 (32.6)	3.12 ± 0.13 (55.3)	2.95 ± 0.12 (44.7)
C18: 0	34.8 ± 6.50 (32.2)	27.5 ± 9.13 (67.8)	16.9 ± 3.59 (15.7)	15.2 ± 4.83 (84.3)	23.2 ± 0.89 (26.7)	25.2 ± 1.15 (73.3)
C18:1 n-9	15.3 ± 1.29 (34.7)	13.9± 0.94 (65.3)	10.0 ± 7.98 (22.7)	8.42 ± 3.46 (77.3)	7.68 ± 0.82 (27.9)	10.6 ± 0.25 (72.1)
C20:5 n-3	-	-	37.2 ± 19.4 (19.2)	44.8 ± 22.8 (80.2)	25.8 ± 1.06 (25.8)	27.1 ± 2.30 (74.2)
C22:5 n-3	-	-	-	-		

[a] Results are mean values ± SD. Values in parenthesis indicate percentage of each FA located at the *sn*-1 + *sn*-3 and the *sn*-2 positions, calculated as (% FA at the *sn*-2 position / (% FA in TAG x 3) X 100 for *sn*-2 position; and as 100 – *sn*-2 for (*sn*-1 + *sn*-3). Sample calculation for EPA; EPA from column 4 of this table and EPA in the modified oil as such from Table 4.1. FA at the *sn*-2 position = (37.2/ (64.9 x 3) X 100 = 19.2; for (*sn*-1 + *sn*-3) = 100 – 19.2 = 80.8

Table 4.5. Positional distribution [a] (wt%, *sn*-2 and *sn*-1+ *sn*-3) of fatty acids in modified and un-modified Laurical15

Fatty acid	Unmodified		Modified with DHA	
	sn-2	*sn*-1 + *sn*-3	*sn*-2	*sn*-1 + *sn*-3
C12:0	32.3 ± 13.7 (28.5)	41.7 ± 5.39 (71.5)	11.8 ± 3.70 (21.7)	9.29 ± 4.72 (78.3)
C14:0	3.49 ± 0.86 (30.5)	3.98 ± 0.44 (69.5)	1.86 ± 0.66 (28.8)	2.11 ± 0.33 (71.2)
C16:0	3.88 ± 0.97 (40.3)	3.09 ± 0.34 (59.7)	1.98 ± 0.50 (34.8)	3.02 ± 0.53 (65.2)
C18: 0	34.8 ± 6.50 (32.2)	27.5 ± 9.13 (67.8)	19.1± 4.30 (37.9)	22.6 ± 4.05 (62.1)
C18:1n-9	15.3 ± 1.29 (34.7)	13.9± 0.94 (65.3)	6.24 ± 3.24 (15.3)	7.49 ± 1.88 (84.7)
C20:5 n-3	-	-		
C22:6 n-3	-	-	17.7 ± 1.01 (16.6)	24.1 ± 7.25 (83.4)

[a] Results are mean values ± SD. Values in parenthesis indicate percentage of each FA located at the *sn*-1 + *sn*-3 and the *sn*-2 positions, calculated as (% FA at the *sn*-2 position / (% FA in TAG x 3) X 100 for *sn*-2 position; and as 100 – *sn*-2 for (*sn*-1 + *sn*-3).

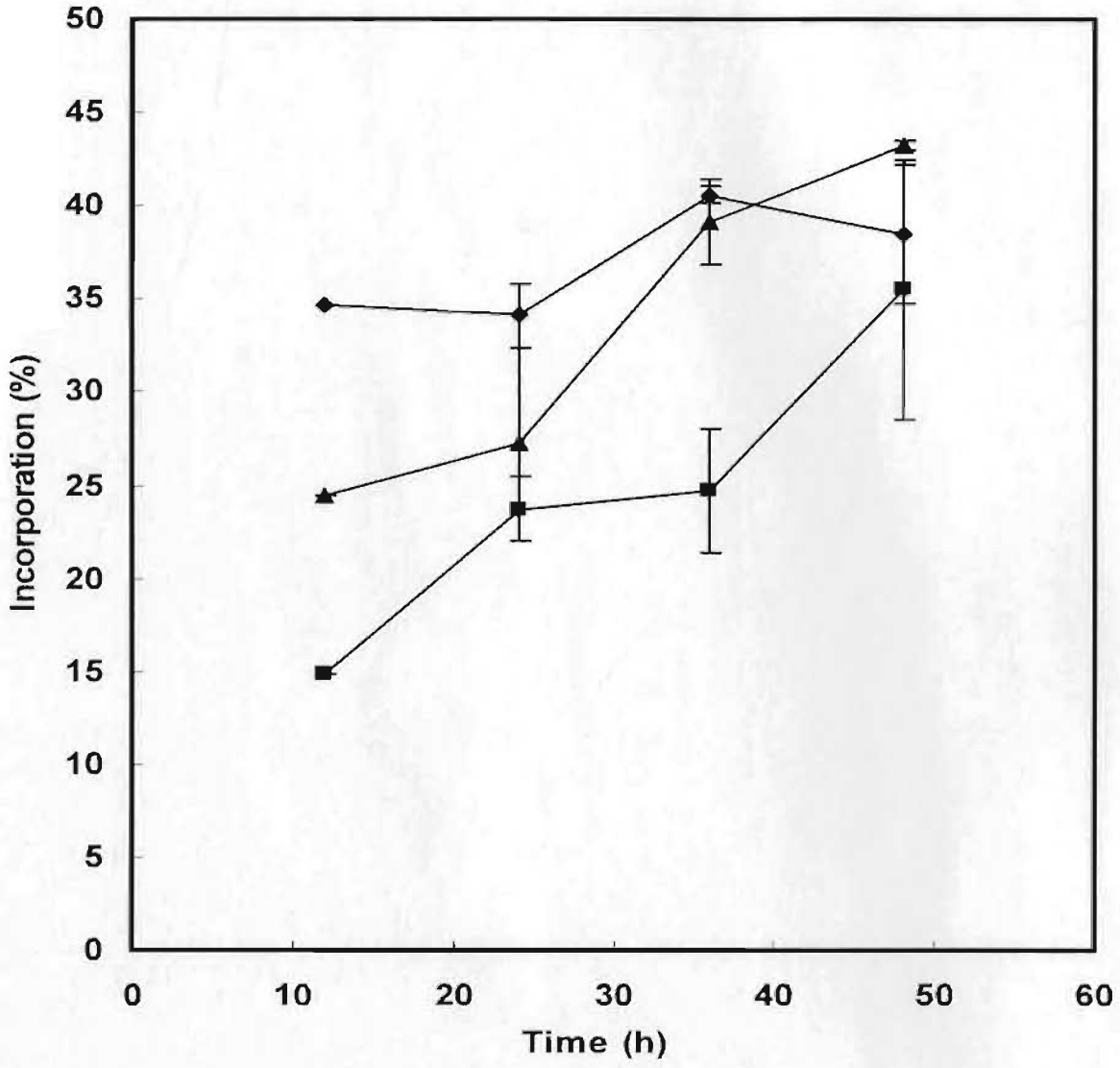

Figure 4.1. The effect of time course on the incorporation of EPA (◆), DHA (■), and DPA (▲) into Laurical 15 using lipase from *Pseudomonas sp.* (PS-30).

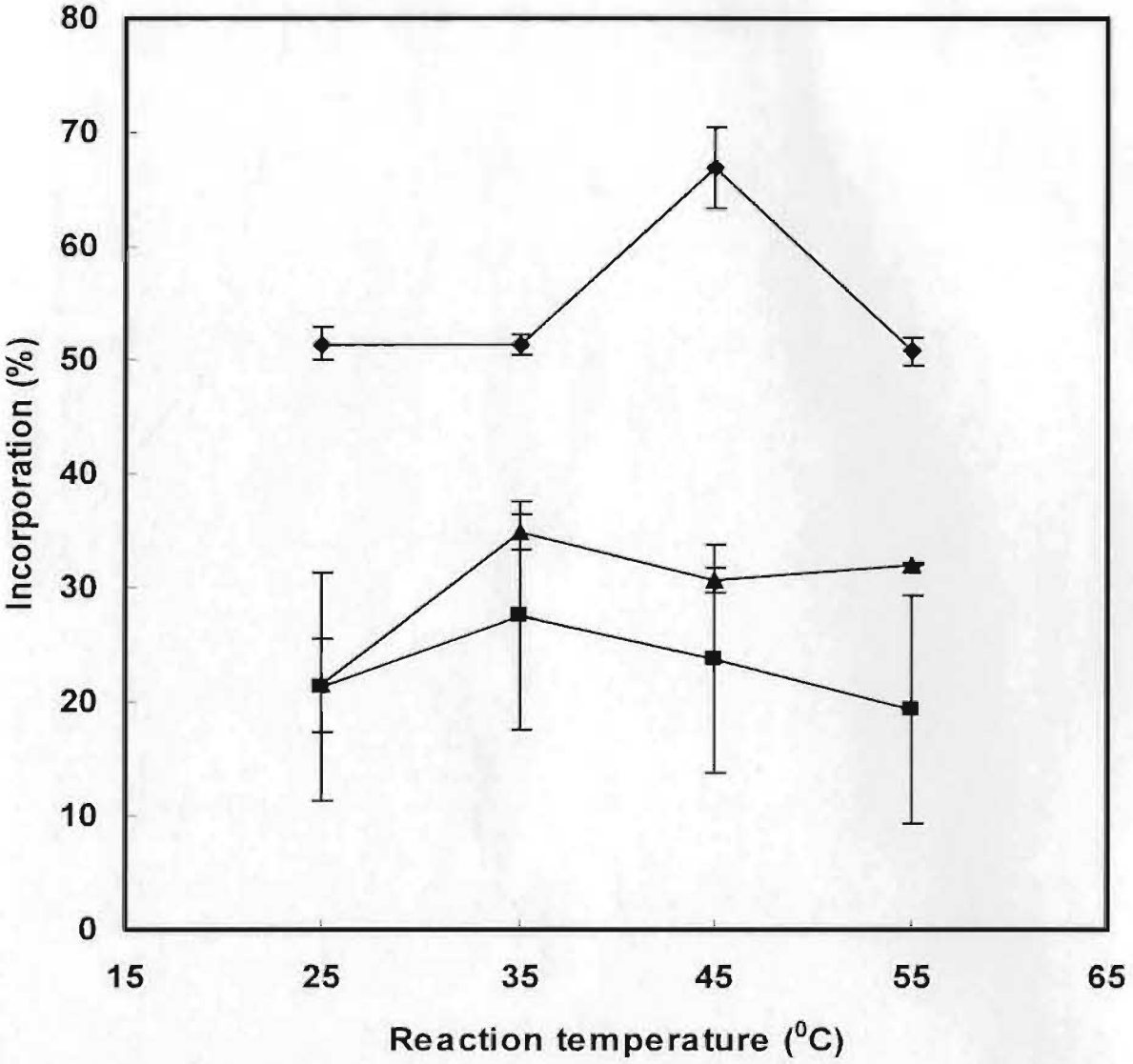

Figure 4.2. The effect of reaction temperature on the percent incorporation of EPA (◆), DHA (■), and DPA (▲) into Laurical 15 using lipase from *Pseudomonas sp.* (PS-30).

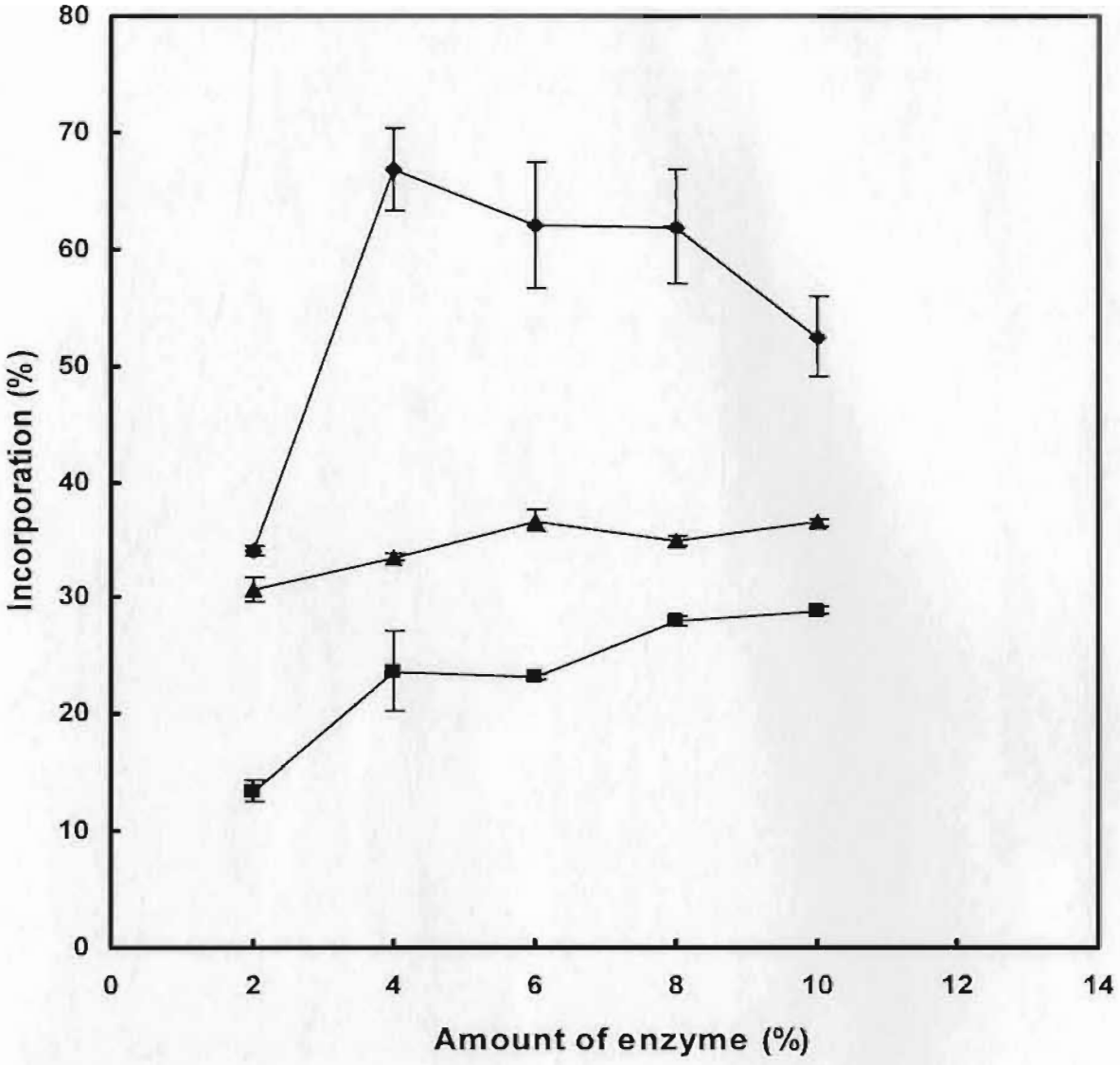

Figure 4.3. Effect of enzyme concentration on the percent incorporation of EPA (♦), DHA (■), and DPA (▲) into Laurical 15 using lipase from *Pseudomonas sp.* (PS-30).

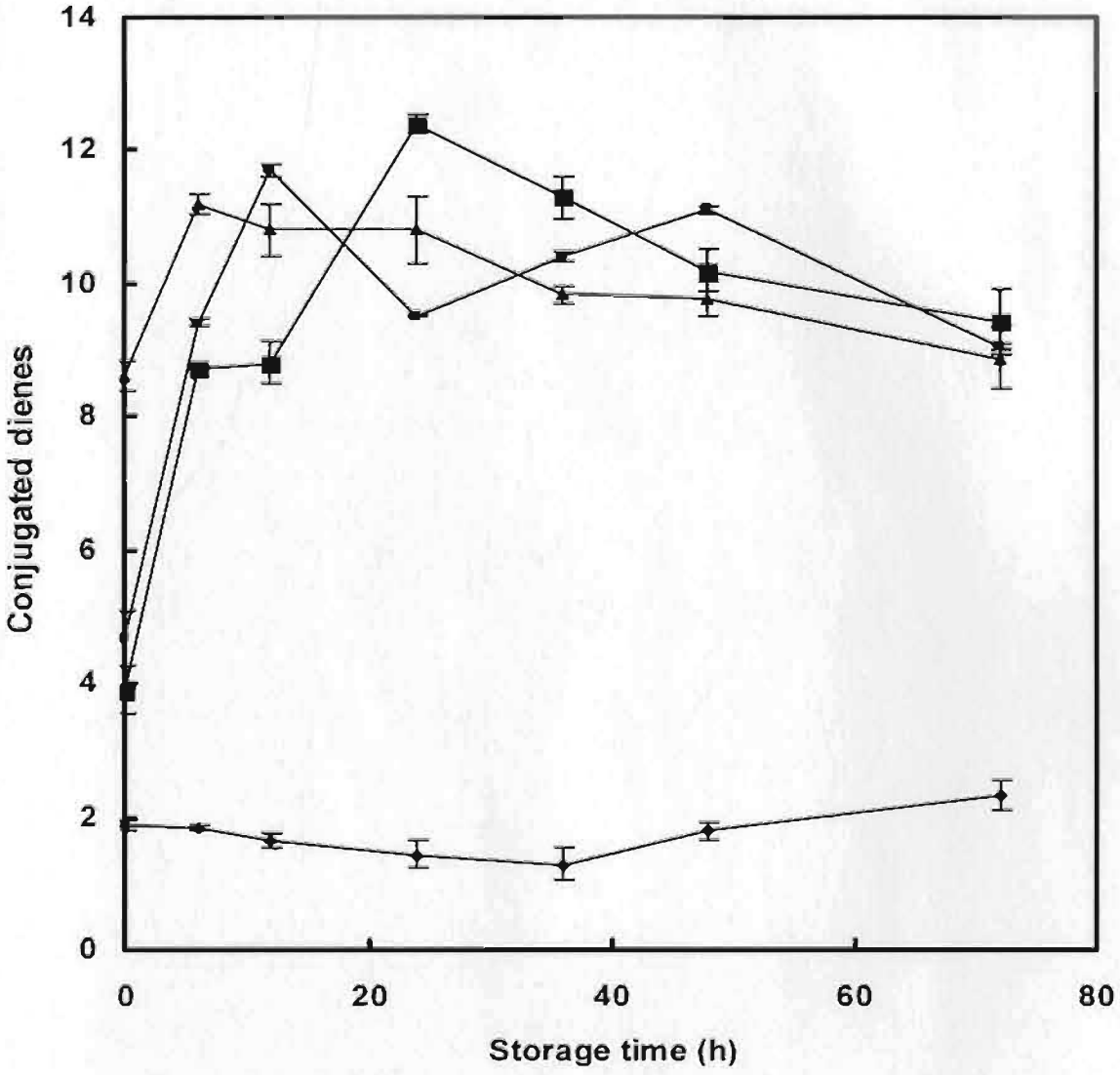

Figure 4.4. Conjugated diene values of (■) modified Laurical 15 with DPA, (▲) modified Laurical 15 with DHA, (*) modified Laurical 15 with EPA, and (♦) the control unmodified oil stored under Schaal oven conditions at 60°C.

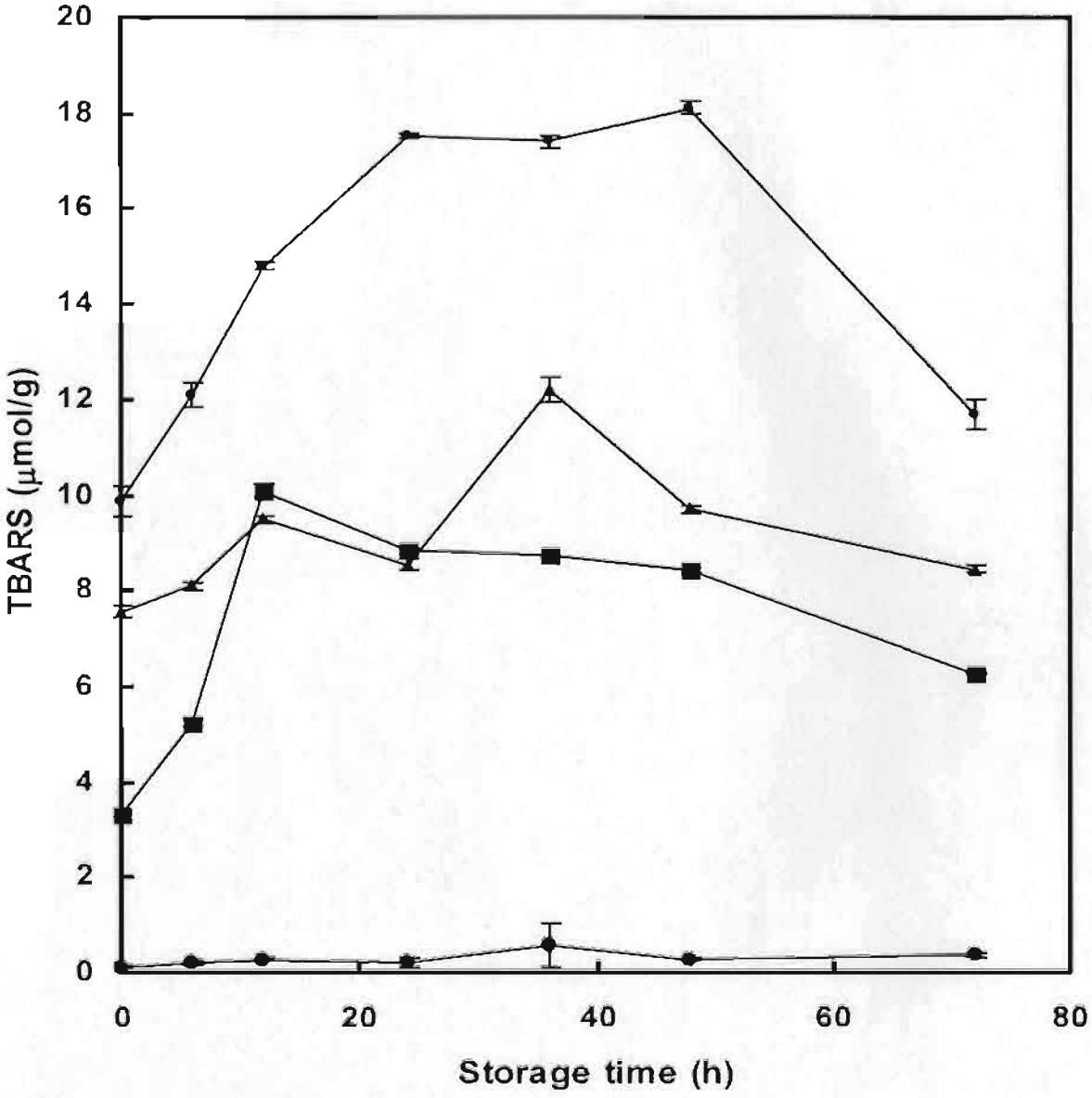

Figure 4.5. TBARS values (µmol/g) of (■) modified Laurical 15 with DPA, (▲) modified Laurical with DHA, (*) modified Laurical with EPA, and (♦) the control unmodified oil stored under Schaal oven conditions at 60°C.

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Chapter 5

Structured lipids from high-laurate canola oil (Laurical 25) and long-chain omega-fatty acids

5.1 Introduction

Structured lipids (SL) are triacylglycerols (TAG) or phospholipids (PL) in which fatty acids are placed in specific locations in the glycerol backbone using a chemical or enzymatic process. Much attention has been paid to SL due to their potential biological functions and nutritional perspectives. Designing SL with selected fatty acids at specific locations of the TAG has attracted attention. For example, it may be desirable to develop a SL for patients with mal digestion as well as cystic fibrosis, which contains polyunsaturated fatty acids (PUFA) at the *sn*-2 position with medium-chain fatty acids (MCFA) at the *sn*-1,3 positions because MCFA are quickly hydrolyzed by pancreatic lipase, absorbed into the intestine and rapidly transported to the liver where they are consumed as a rapid source of energy.

Epidemiological studies have indicated that dietary DHA may protect against cardiovascular disease (both in terms of morbidity and mortality), including the risk of primary cardiac arrest and sudden cardiac death (Albert *et al.*, 1998). Furthermore, Horrocks and Yeo (1999) reported a strong link between fish consumption and a decrease in sudden death from myocardial infraction. The decline was about 50% with 200 mg per day of DHA from fish. DHA deficiency has been associated with depression and may be the underlying reason for the positive correlation between depression and myocardial infraction.

High-laurate canola oil (Laurical 25) is a high Laurate (C12:0) obtained from genetically engineered canola. This oil was produced by Calgenes's Inc. (Davis, CA) to

provide an alternative source of oil that are generally produced by palm kernel oil upon fractionation (Del Vecchio, 1996).

Incorporation of DHA or EPA into canola oil rich in lauric acid (40%), mostly esterified to the *sn*-1,3 positions, would provide a novel specialty oil for specific nutritional and clinical needs. In this study, the ability of different lipases to catalyze the acidolysis of high-laurate canola oil with DHA or EPA was explored. The effects of enzyme load, incubation time, and reaction temperature on the extent of DHA or EPA incorporation into canola oil rich in lauric acid using response surface methodology (RSM) were also investigated. In addition, this study intended to assess the oxidative stability of the resultant SL.

RSM is an optimization procedure that determines optimal process conditions by combining particular experimental designs with modeling using first- or second-order polynomial equations. 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 methods do not indicate real changes in the environment because they disregard interactions among variables, which are in effect, concomitant.

5.2 Materials and Methods

Materials and methods are detailed in Chapter 3.

5.2.1 Acidolysis reaction

In general, Laurical 25 (100 mg each) was mixed with DHA or EPA at respective mole ratio of 1:3 (oil/DHA or oil/ EPA), in a screw-capped test tube, and then lipase from

Pseudomonas sp. (2-6% by weight of substrates) and water (2% by weight of substrates and enzyme) were added in hexane (3 mL). The mixture was incubated for different time periods (12 to 36 h) in an orbital shaker at 250 rpm with temperatures ranging from 35 to 55°C.

5.3 Results and discussion

5.3.1 The fatty acid profile of Laurical 25

The fatty acid (FA) profile of high-laurate canola oil (Laurical 25) before and after enzymatic modification is shown in Table 5.1. The lauric acid content of Laurical 25 was 40.7%. This oil also contained other saturated FA such as stearic (21.9%), palmitic (2.93%), and myristic (3.82%) acids. The major unsaturated FA found in Laurical 25 before enzymatic modification was oleic acid (25.9%).

5.3.2 Enzymatic incorporation of DHA or EPA into Laurical 25

Five lipases were screened for their ability to incorporate DHA or EPA into Laurical 25 (Table 5.2). These lipases catalyzed incorporation of DHA into Laurical 25 to varying degrees. The degree of DHA incorporation into high-laurate canola oil with various lipases was in the following order: *Pseudomonas sp.* > *M. miehei* > *C. rugosa* ≥ *C. antarctica* > *A. niger*. The incorporation of DHA into Laurical 25 was effectively catalyzed by only three of the five lipases tested, possibly due to the experimental conditions which might be appropriate for these three lipases. However, there was no significant difference ($p>0.05$) in the DHA incorporation into Laurical 25 when lipases from *C. antarctica* and *C. rugosa* were employed. It was found that lipase from *Pseudomonas sp.* was most effective in its ability to incorporate EPA into Laurical 25 (Table 5.2). The order of incorporation of EPA into Laurical 25 was: *Pseudomonas sp.* >

Candida rugosa > *Aspergillus niger* ≥ *Mucor miehei* ≥ *Candida antarctica*. However, there was no significant difference ($p>0.05$) in the EPA incorporation into Laurical 25 when lipases from *C. antarctica*, *A. niger*, and *M. miehei* were employed, probably due the experimental conditions selected in this study that were not suitable for these lipases. *Pseudomonas sp.* gave the highest degree of incorporation of DHA (34.8%, after 24 h), or EPA (61.0%, after 24) into high-laurate canola oil. Because the acidolysis of DHA or EPA with Laurical 25 was best achieved using the *Pseudomonas sp.* lipase, this enzyme was selected in subsequent experiments.

5.3.3 Positional distribution of SL

Table 5.3 summarizes the results of studies on positional distribution of FA in the unmodified and modified Laurical 25. Lauric acid was mostly esterified to the *sn*-1,3 positions in the unmodified oil. Lauric acid remained esterified mainly to the *sn*-1,3 positions of TAG molecules. Meanwhile, DHA was mostly incorporated into the *sn*-1,3 positions of the glycerol backbone of the DHA-modified high-laurate canola oil. Examination of positional distribution of FA on the glycerol backbone of modified high-laurate canola oil with EPA showed that EPA was randomly distributed over the three positions of the TAG molecules. Lauric acid remained esterified mainly to the *sn*-1 and *sn*-3 positions of the modified oil.

5.3.4 Response surface methodology

Table 5.4 summarizes the experimental data for response variable Y_1 (DHA% incorporation into Laurical 25), or Y_2 (EPA% incorporation into Laurical 25). Multiple regression coefficients, obtained by employing a least squares procedure to predict the second–order polynomial model for the DHA or EPA incorporation into Laurical 25 are

summarized in Table 5.5. Testing of these coefficients with the t-test indicated that in modified Laurical 25 with DHA, linear and quadratic terms of the amount of enzyme (X_1) were highly significant ($p \leq 0.001$). Interactions of the amount of enzyme (X_1) and reaction temperature (X_2), reaction time (X_3) and temperature (X_2), as well as the amount of enzyme (X_1) and the reaction time (X_3) were not significant ($p>0.1$). In modified Laurical 25 with EPA, all linear, second order and interaction terms were insignificant ($p> 0.1$). Similarly, interactions of the amount of enzyme (X_1) and reaction temperature (X_2), or reaction time (X_3) were insignificant ($p> 0.1$).

The coefficient of determinations for Y_1 (Laurical 25, DHA%) ($R^2=0.94$) indicates that the fitted model could explain 94% of the variations, while the coefficient of variation (CV) was 9%. The coefficient of determinations and coefficient of variation (CV) for Y_2 (Laurical 25, EPA %) were 0.49 and 5.28%, respectively.

In order to characterize the nature of the stationary points, a canonical analysis was performed. This is a mathematical method used for locating the stationary point of the response surface, and to see if it is a maximum, minimum or saddle point (Montogmery, 1991). It 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 Y_1 (Laurical 25, DHA%) or Y_2 (Laurical 25, EPA%) were:

$$Y_1 = 37.3 + 0.27\ W_1^2 - 1.95\ W_2^2 - 8.47\ W_3^2$$

$$Y_2 = 61.6 + 0.41\ W_1^2 - 1.28\ W_2^2 - 2.71\ W_3^2$$

where W_1, W_2, and W_3 are the axes of the response surface for the respective oil examined. The eigenvalue was positive for DHA and EPA incorporation into high-laurate canola oil model, pointing out that the stationary points were saddles points.

Table 5.6 and Figures 5.1-5.4 show critical values for the three factors (enzyme load, reaction time, and temperature) examined. The stationary point for the degree of DHA incorporation (%) into Laurical 25 reached a maximum of 37.3% at 4.79% enzyme concentration, at 46.1°C in 30.1 h. Similarly, the maximum incorporation of EPA (61.6%) into high-laurate canola oil was obtained when the enzyme amount, reaction temperature and time were 4.6%, 39.9°C, and 26.2 h, respectively.

5.3.5 Conjugated dienes (CD)

Figure 5.5 shows the CD values of modified Laurical 25 with EPA or DHA as well as the control unmodified oil. The CD values of the unmodified oil did not change during storage, from 6 to 72 h, reflecting good stability of the unmodified oil. As the storage time was extended to 24 h, the CD values of DHA-modified Laurical 25 increased significantly ($p < 0.05$) and peaked (19.5) at 24 h, possibly due to the formation of more and more hydroperoxides as primary products of oxidation. After 24 h of storage, the CD values decreased to 13.8, possibly due to the breakdown of unstable hydroperoxides. For EPA-modified Laurical 25, the CD values increased significantly ($p < 0.05$) with storage time (12 to 48 h), and reached a maximum value (14.5) at 48 h, after which the CD value decreased sharply. The values of CD were significantly ($p < 0.05$) higher in the modified oils than the original oil over the entire storage period. The results showed that the modified oils were less stable than the original oil. This was demonstrated to be due to incorporating of highly susceptible FA, EPA and DHA, to oxidation. Theoretically, incorporation of PUFA into highly saturated oil should lower their oxidative stability. The present results indicate that modification of Laurical 25 with EPA and DHA resulted in lower stability, not only due to an increase in the degree of unsaturation, but also possibly

due to the loss of natural antioxidants, particularly formation of tocopheryl esters from reaction of carboxylic acids and tocopherols present in the oil during the process of SL preparation. This possibility will be detailed in Chapter 9. In agreement with expectation, DHA-modified oil was less stable than EPA-modified oil as reflected in the CD values.

5.3.6 Thiobarbituric acid reactive substances (TBARS)

The 2-thiobarbituric acid (TBA) test is widely used for assessing the secondary products of lipid oxidation related to the amount of aldehydes and dialdehydes in the oxidized lipids. TBARS values of modified Laurical 25 with EPA or DHA as well as the control unmodified oils are shown in Figure 5.6. As the accelerated storage period was extended up to 72 h, TBARS values of EPA-modified oil increased gradually and reached its maximum (17.1 μmol/g) after 24 h, then decreased to 11.3 μmol/g. As the storage time increased from 0 to 12 h, the TBARS values of DHA-modified oil increased steadily and reached its maximum (15.6 μmol/g) at 12 h, then decreased gradually and reached its lowest value (12.9 μmol/g) at 48 h. TBARS of the modified oil were considerably higher than those of the original oil over the entire storage period.

5.3.7 Conclusions

Among the five lipases tested, lipase PS-30 from *Pseudomonas sp.* was most effective for incorporating DHA or EPA into Laurical 25. RSM showed that in Laurical 25-based SL, the maximum incorporation of DHA (37.3%) was reached at 4.79% enzyme, a reaction temperature of 46.1°C, and reaction time of 30.1 h. The maximum incorporation of EPA into Laurical 25 (61.6%) was obtained when enzyme amount, reaction temperature and time were 4.61%, 39.9°C and 26.3 h, respectively. Thus, the number of double bonds and the chain length of fatty acids had a marked effect on the

incorporation of EPA or DHA into Laurical 25. For EPA-modified Laurical 25, lauric acid was present mainly in the *sn*-1,3 positions while EPA was randomly distributed over the three positions. Similarly, DHA was primarily located at the *sn*-1,3 positions of the TAG molecules, but lauric acid also remained mainly at the *sn*-1,3 positions of the modified oil. The oxidative stability of the modified oil in comparison with the original unmodified oil, as indicated in the conjugated diene (CD) values, showed that the unmodified oil remained unchanged during storage for 72 h, but EPA- or DHA-modified Laurical 25 SL were oxidized to a much higher level than the original oil. The values of CD were higher in the modified oil with DHA than EPA-modified oil. The modified oil also attained considerably higher TBARS values than the original oil over the entire storage period.

Table 5.1. Fatty acid composition (wt%) of high-laurate canola oil (Laurical 25)*

Fatty acid	Unmodified	Modified with DHA	Modified with EPA
12:0	40.7 ± 1.24	14.4 ± 0.49	10.7 ± 0.49
14:0	3.82 ± 0.04	1.75 ± 0.04	1.25 ± 0.03
16:0	2.93 ± 0.06	1.64 ± 0.06	1.16 ± 0.01
18:0	21.9 ± 0.63	17.2 ± 0.53	10.3 ± 0.29
18:1 n-9	25.9 ± 0.49	18.5 ± 0.19	9.08 ± 0.44
20:5 n-3	-	-	62.5 ± 0.96
22:6 n-3	-	42.0 ± 1.68	-
Others	4.66	5.51	5.01

* Results are mean values of triplicate determinations ± standard deviation. The reaction mixture contains Laurical 25 (100 mg), at a mole ratio of 1:3 (oil/DHA or oil/EPA), lipase from *Pseudomonas sp.* amount (4%), water (2%) in 3.0 mL of hexane. The mixture was kept at 45°C for 24 h in an orbital water bath at 250 rpm.

Table 5.2. Effect of enzyme type on the incorporation (%) of DHA or EPA into Laurical 25[a]

Source of enzyme	DHA	EPA
Candida antarctica	17.2 ± 0.10^{b}	48.7 ± 2.3^{a}
Mucor miehei	29.3 ± 2.54^{c}	48.9 ± 1.55^{a}
Pseudomonas sp.	34.8 ± 2.76^{d}	61.0 ± 1.40^{c}
Candida rugosa	17.5 ± 0.54^{b}	55.2 ± 2.60^{b}
Aspergillus niger	9.79 ± 3.19^{a}	51.5 ± 0.45^{a}

[a] Results are mean values of triplicate determinations ± standard deviation. Values in each column with different roman superscript letters are different ($p<0.05$). [a, b, c, d, e] the order of EPA or DHA incorporation into Laurical 25, from lowest to highest, was as follows: a < b< c< d < e.

The reaction mixture contained Laurical 25 (100 mg), fatty acid to Laurical 25 mole ratio of 3:1, enzyme at 4% by weight of substrates, water at 2% by weight of enzyme and substrates, and 3.0 mL of hexane. The mixture was kept at 45 °C for 24 h in a shaking water bath at 250 rpm.

Table 5.3. Positional distribution[a] (wt%, *sn*-2 and *sn*-1+ *sn*-3) of fatty acids in modified high-laurate canola oil (Laurical 25)

	Unmodified		Modified with DHA		Modified with EPA	
Fatty acid	*sn*-2	*sn*-1 + *sn*-3	*sn*-2	*sn*-1 + *sn*-3	*sn*-2	*sn*-1 + *sn*-3
12:0	28.5 ± 5.99 (23.3)	46.1 ± 8.21 (76.7)	7.07 ± 1.04 (16.4)	14.4 ± 1.14 (83.6)	7.39 ± 1.85 (16.4)	2.31 ± 0.19 (83
14:0	4.03 ± 0.44 (35.2)	4.33 ± 0.72 (64.8)	2.34 ± 0.59 (44.6)	1.84 ± 0.28 (55.6)	1.29 ± 0.35 (34.4)	0.25 ± 0.03 (65
16:0	4.36 ± 0.82 (49.6)	3.20 ± 1.01 (50.4)	2.45 ± 0.38 (49.8)	2.59 ± 0.79 (50.2)	1.56 ± 0.44 (44.8)	0.24 ± .0.05 (5
18: 0	26.6 ± 1.25 (40.4)	19.2 ± 1.98 (59.6)	12.0 ± 1.96 (23.3)	10.9 ± 0.65 (76.7)	9.91 ± 3.13 (32.1)	1.32 ± 0.34 (67
18:1n-9	28.8 ± 2.29 (37.0)	22.4 ± 5.59 (63.0)	12.3 ± 1.90 (22.2)	21.2 ± 1.96 (77.8)	7.28 ± 1.59 (26.7)	1.58 ± 0.32 (73
20:5n-3	-	-	-	-	63.4 ± 1.60 (33.8)	89.8 ± 0.94 (66
22:6 n-3	-	-	9.84 ± 1.01 (9.37)	26.7 ± 1.63 (90.6)	-	-

[a] Results are mean values ± SD. Values in parenthesis indicate percentage of each FA located at the *sn*-1 + *sn*-3 and the *sn*-2 positions.

Values in parenthesis are, for *sn*-2 position (% FA at the *sn*-2 position / (% FA in TAG x 3) X 100; for (*sn*-1 + *sn*-3) = 100 – *sn*-2.

Table 5.4. Face-centred cube design arrangement and response for the analysis of high-laurate canola oil (Laurical 25) with DHA or EPA[a]

Run	Independent variables			Response (Y_1) [b]	Response (Y_2)
	Enzyme (%)	Temperature (C)	Time (h)		
1	2[c] (-1)[d]	35(-1)	12(-1)	19.5	59.1
2	2 (-1)	35(-1)	36(+1)	22.2	57.7
3	2 (-1)	45(0)	24(0)	18.2	58.8
4	2 (-1)	55(+1)	12(-1)	18.9	56.2
5	2 (-1)	55(+1)	36(+1)	18.5	54.4
6	4(0)	35(-1)	24(0)	35.3	57.3
7	4(0)	45(0)	12(-1)	27.9	56.4
8	4(0)	45(0)	24(0)	34.8	61.0
9	4(0)	45(0)	24(0)	37.1	61.0
10	4(0)	45(0)	24(0)	36.1	61.0
11	4(0)	45(0)	36(+1)	38.9	61.9
12	4(0)	55(+1)	24(0)	35.6	66.8
13	6(+1)	35(-1)	12(-1)	28.1	57.1
14	6(+1)	35(-1)	36(+1)	32.5	60.6
15	6(+1)	45(0)	24(0)	35.5	62.0
16	6(+1)	55(+1)	12(-1)	31.7	59.2
17	6(+1)	55(+1)	36(+1)	32.7	57.7

[a] Nonrandomized.

[b] Average of triplicate determinations from different experiments. Y_1, % DHA incorporation into Laurical 25; Y_2, % EPA incorporation into Laurical 25.

[c] Uncoded variable levels.

[d] Coded variable levels.

Table 5.5. Regression coefficients of predicted quadratic polynomial model for response (Y)

Coefficients[a]	Laurical 25, DHA (%) (Y_1)	Laurical 25, EPA (%) (Y_2)
β_0	-15.293	50.804
Linear		
β_1	17.365**	1.0104
β_2	-0.1903	-0.2634
β_3	1.0243	1.0405
Quadratic		
β_{11}	-2.1088**	-0.3323
β_{22}	0.0016	0.0032
β_{33}	-0.0131	-0.0179
Interactions		
β_{12}	0.0506	0.0337
β_{13}	0.0161	0.0271
β_{23}	-0.0068	-0.0056
R^2	0.94	0.49
CV%	9.06	5.28

[a] Coefficients refer to the general model. R^2, coefficient of determination; CV%, coefficient of variation; **, Significant at ($p \leq 0.001$).

Table 5.6. Canonical analysis of response surface for acidolysis of Laurical 25 with DHA or EPA

Factor	Laurical 25, DHA (%)	Laurical 25, EPA (%)
Amount of lipase from *Pseudomonas sp.* (wt%, X_1)	4.79	4.61
Reaction temperature (°C, X_2)	46.1	39.9
Reaction time (h, X_3)	30.1	26.3
Stationary point	Saddle	Saddle
Predicted value [a]	37.3	61.6
Observed value [b]	41.9 ± 1.67	62.5 ± 0.96

[a] Predicted using the polynomial model.

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

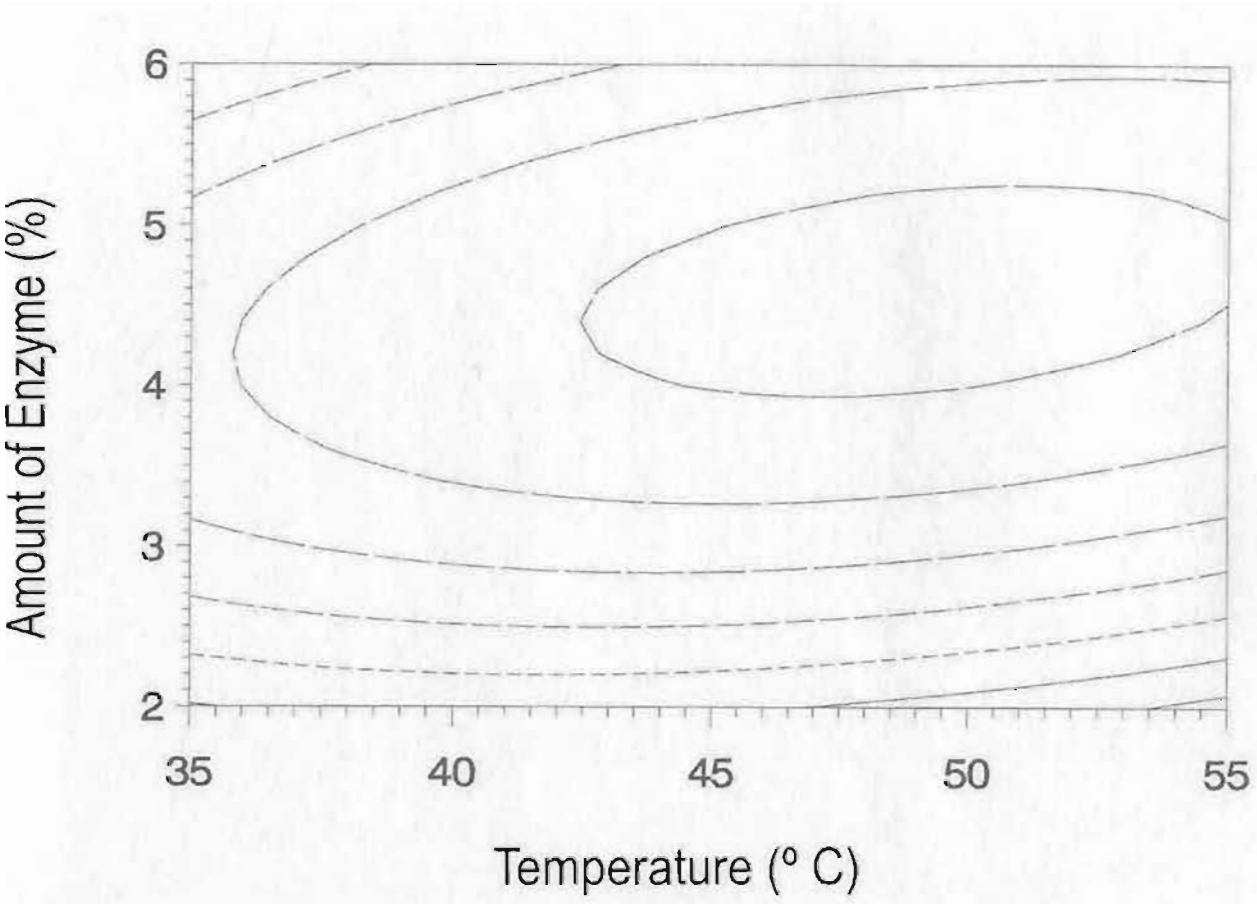

Figure 5.1. Two-dimensional contour plot demonstrating the effects of lipase from *Pseudomonas sp.* amount and reaction temperature on the predicted EPA incorporation (%) into Laurical 25. EPA incorporation (%) in the contour plot, defined by lines from inside to outside, are: 60.9, 60.3, 59.6, 58.9, 58.3, 57.6, and 56.9, respectively.

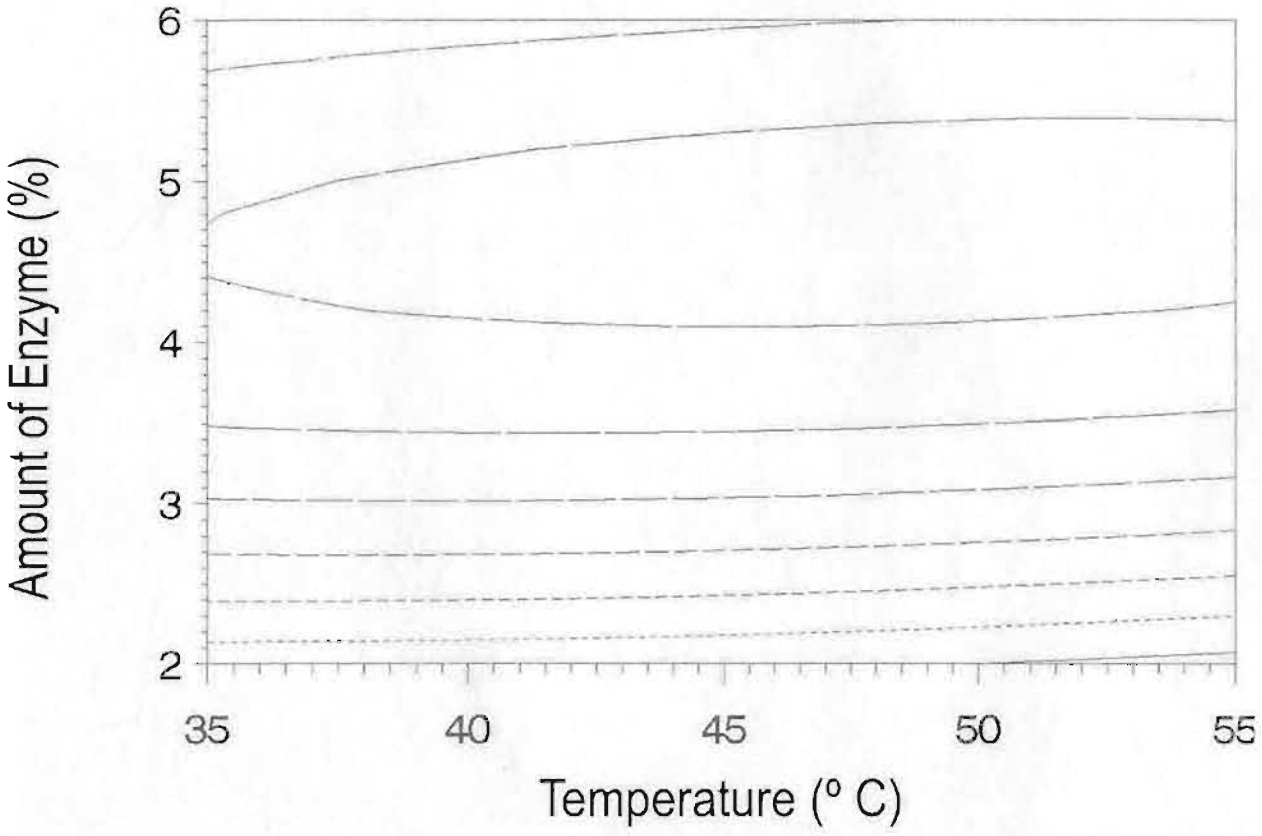

Figure 5.2. Two-dimensional contour plot demonstrating the effects of lipase from *Pseudomonas sp.* amount and reaction temperature on the predicted DHA incorporation (%) into Laurical 25. DHA incorporation (%) in the contour plot, defined by lines from inside to outside, are: 35.5, 32.8, 30.1, 27.4, 24.6, 21.9, and 19.2, respectively.

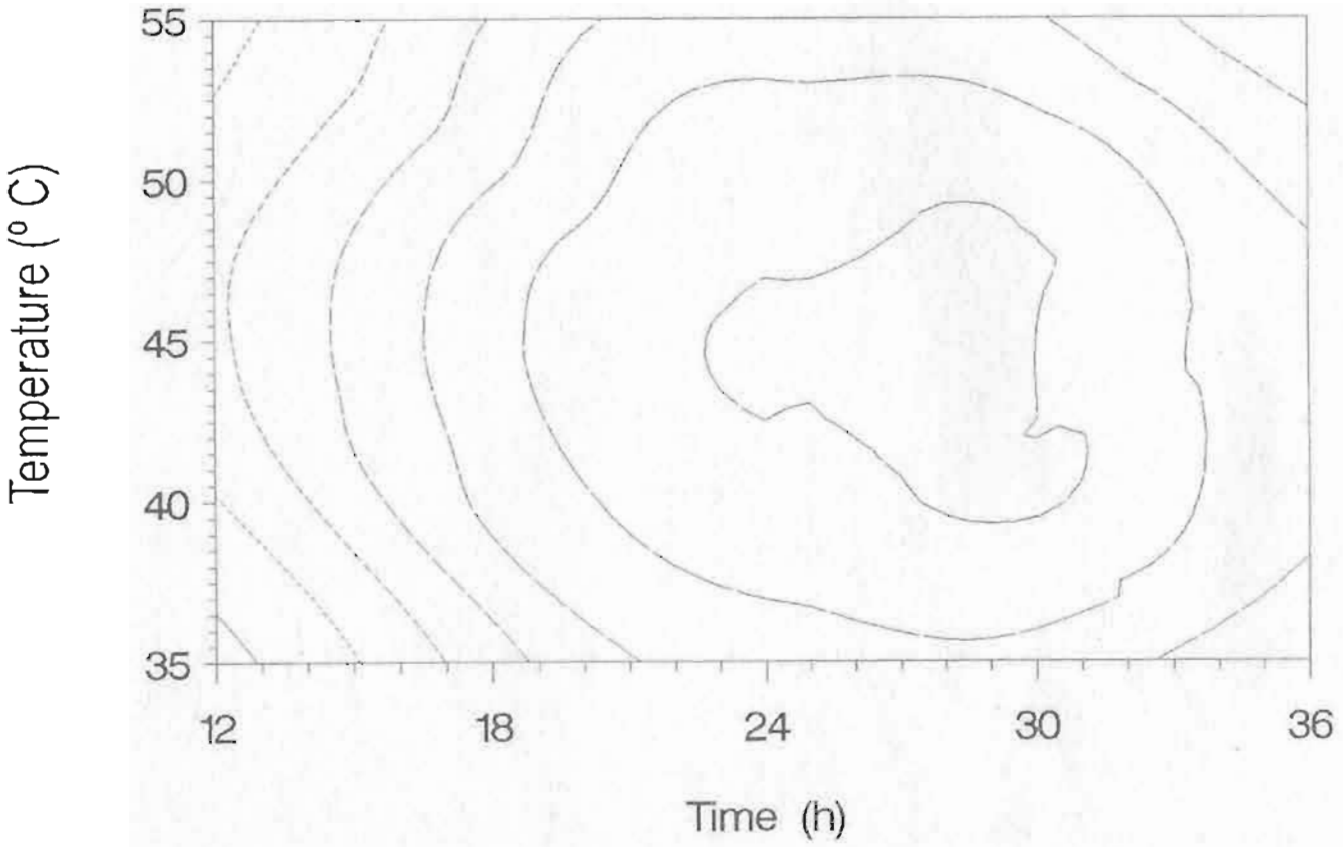

Figure 5.3. Two-dimensional contour plot demonstrating the effects of incubation time and reaction temperature on the predicted DHA incorporation (%) into Laurical 25. DHA incorporation (%) in the contour plot, defined by lines from inside to outside, are: 33.5, 32.0, 30.5, 28.95, 27.45, 25.95, and 24.45, respectively.

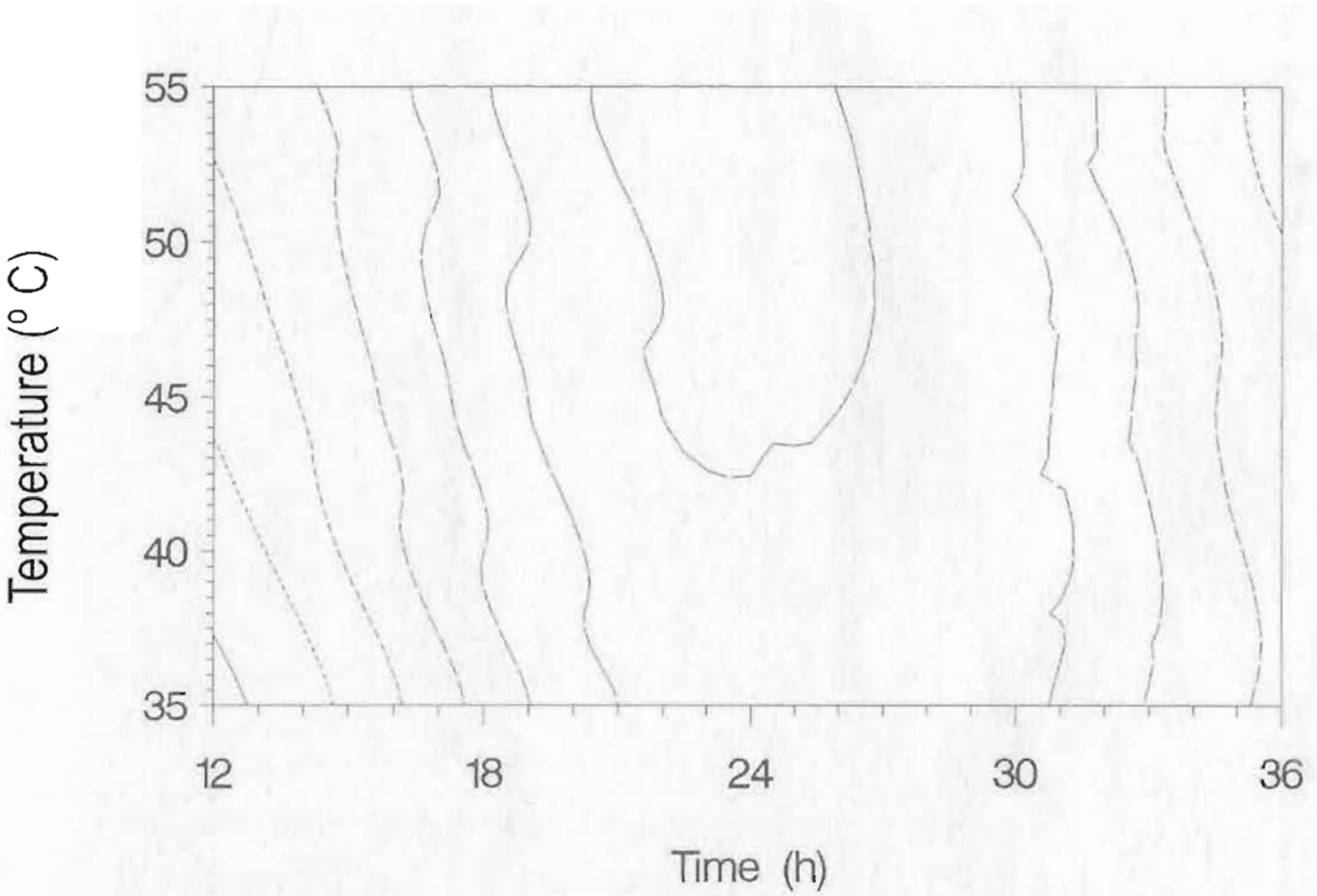

Figure 5.4. Two-dimensional contour plot demonstrating the effects of incubation time and reaction temperature on the predicted EPA incorporation (%) into Laurical 25. EPA incorporation (%) in the contour plot, defined by lines from inside to outside, are: 61.2, 60.4, 59.7, 59.0, 58.3, 57.6, and 56.9, respectively.

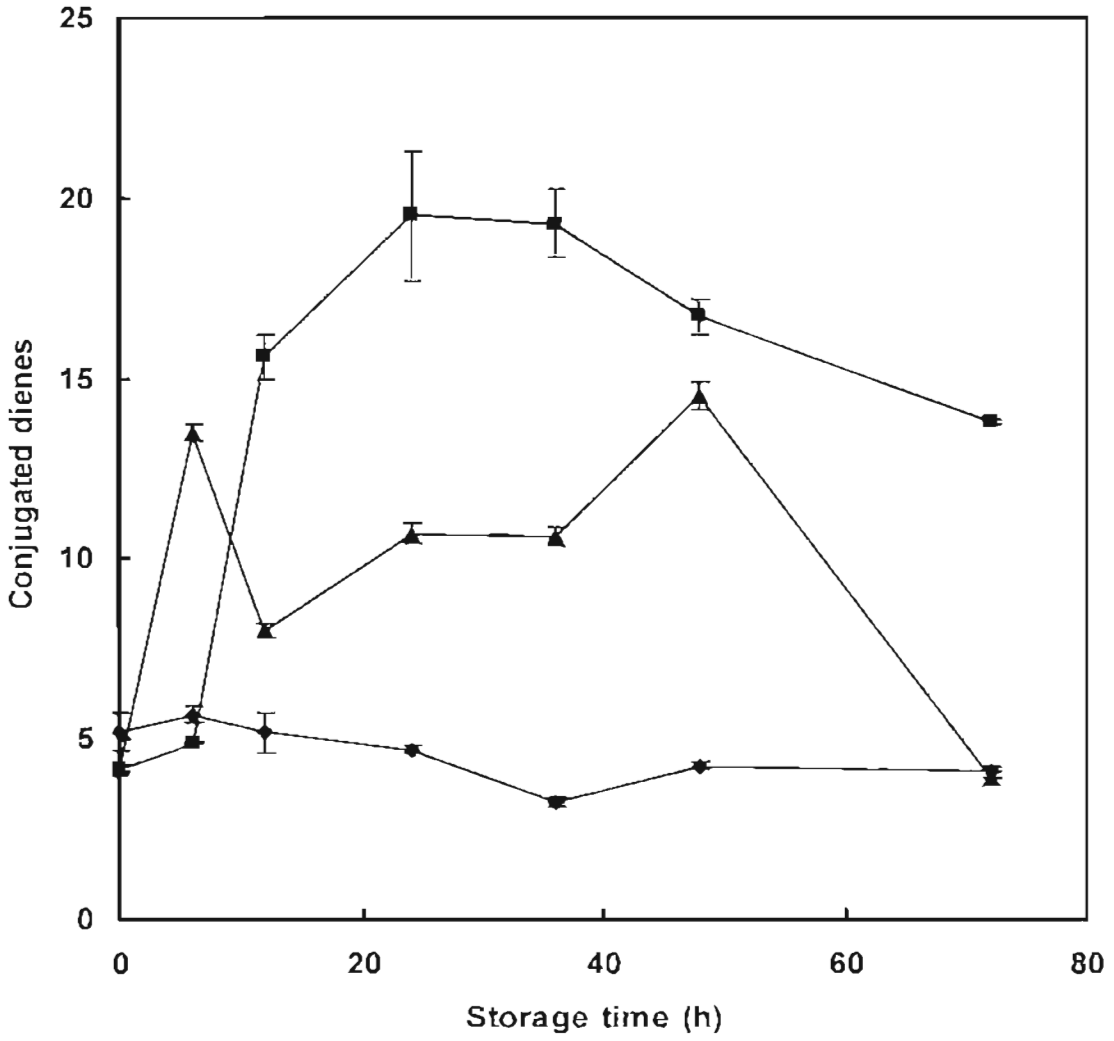

Figure 5.5. Conjugated diene values of (■) modified Laurical 25 with DHA, (▲) modified Laurical with EPA, and (♦) the control unmodified oil stored under Schaal oven conditions at 60°C.

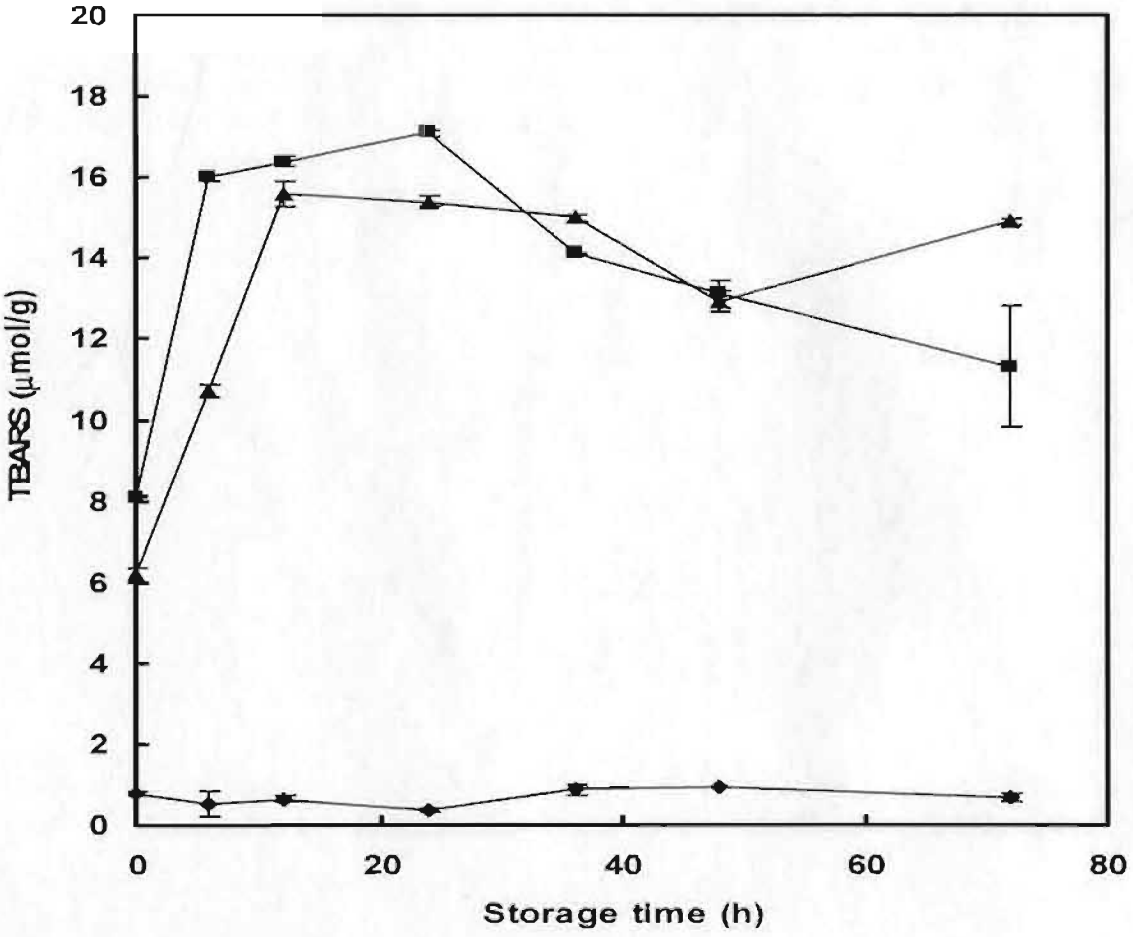

Figure 5.6. TBARS values (µmol/g) of (▲) modified Laurical 25 with DHA, (■) modified Laurical 25 with EPA, and (♦) the control unmodified oil stored under Schaal oven conditions at 60°C.

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Chapter 6

Synthesis of structured lipids containing medium-chain and omega-3 fatty acids

6.1 Introduction

Recognition of the health benefits associated with consumption of seafoods is one of the most promising developments in human nutrition and disease prevention research in the past three decades. The n-3 fatty acids have many health benefits related to cardiovascular disease (Conquer and Holub, 1997), inflammation (Mori and Beilin, 2004), cancer (Cave, 1991), immune response (Puertollano *et al.*, 2004), diabetes (Stene and Joner, 2003), hypertension (Aguilera *et al.*, 2004) and renal disorders (Plotnick, 1996). Deficiency in n-3 fatty acids (FA), particularly docosahexaenoic acid (DHA), has been associated with depression and may be the underlying reason for the positive correlation between depression and myocardial infraction.

Docosapentaenoic acid (DPA, C22:5 n-3) is an elongation metabolite of EPA. DPA has not been studied in any detail like other n-3 FA (Shahidi and Zhong, 2005). This is because of its limited availability; DPA is present in a much lower concentration compared to that of EPA and DHA in marine oils, and also because of the difficulty in purifying it from mixtures containing EPA and DHA with similar physico-chemical properties (Yazawa, 2001). While most fish oils contain less than 1% DPA, harp seal oil contains 4-6% of DPA. DPA would be as important or even more important than either EPA or DHA. About one third of the long-chain n-3 FA circulating in human blood are attributed to DPA as the effective agent (Ho, 2003). DPA may have pharmacological impacts different from those of EPA and DHA, and has recently appeared as a focus topic

(Yazawa, 2001). In animals, DHA deficiency is accompanied by increased DPA, which suggests that the ratio of DHA to DPA could be a useful biochemical marker of low n-3 fatty acid status. In contrast to the findings in animals, DPA concentration is not a useful measure of low n-3 fatty acid uptake in preschool children, possibly due to physiological differences of n-3 fatty acids deficiency between humans and animals (Innis *et al.*, 2004). It has been demonstrated that angiogenic activity in endothelial cells, induced by vascular endothelial growth factor (VEGF), can be suppressed by n-3 PUFA treatment. Among long-chain polyunsaturated fatty acids (LC PUFA), DPA was the most powerful inhibitor of angiogenesis (the growth and proliferation of blood vessels); the inhibitory activity by DPA pre-treatment was almost 6 times that of EPA and DHA, pointing out that DPA could be developed as a new drug or supplement against angiogenesis-related diseases (Tsuji *et al.*, 2003). Angiogenesis plays a significant function in tumor growth and metastasis, and blocking angiogenesis can limit tumor growth. In addition, the stimulative effect of EPA on endothelial cell migration (an important process in the control of wound healing responses of blood vessels) may be due to DPA. *In-vitro* studies have shown that the activity of DPA to stimulate endothelial cell migration is 10-fold that of EPA (Kanayasu-Toyoda *et al.*, 1996). Hence, it is possible that the antiarteriosclerotic function of seal oil is mainly due to DPA rather than EPA and DHA (Yazawa, 2001). Evidence proposed that DPA is the most significant fatty acid in keeping artery walls soft and plaque-free (Ho, 2003). Moreover, arachidonic acid-stimulated blood platelet aggregation was inhibited by n-3 fatty acids in a dose-dependent manner, among which DPA was the most effective inhibitor (Kanayasu-Toyoda *et al.*, 1996). DPA displays significant activity for interfering with the cyclooxygenase pathways, and accelerating the lipoxygenase

pathway, thus inhibiting platelet aggregation most efficiently (Akiba *et al.*, 2000). Eldho *et al.* (2003) reported that insufficient supply of DHA or its n-3 precursors during infancy resulted in replacement of DHA by n-6 DPA (22:5 n-6, an elongation product of arachidonic acid). This replacement is generally nonfatal, but is associated with some loss of brain activity.

In this study, the ability of different lipases to catalyze the acidolysis of high-laurate canola oil (Laurical 35) with n-3 DPA, DHA, and EPA was explored. Effects of enzyme amount, incubation time, and reaction temperature on the percent of DPA, DHA, and EPA incorporation into canola oil rich in lauric acid using response surface methodology (RSM) were also investigated. The oxidative stability of the resultant SL was assessed.

6.2 Materials and methods

Materials and methods are the same as those detailed in Chapter 3.

6.2.1 Acidolysis reaction

In general, high-laurate canola oil (Laurical 35) (70 mg each) was mixed with EPA, n-3 DPA, and DHA at respective mole ratio of 1:3 (oil/EPA, oil/DPA, and oil/DHA), in a screw-capped test tube, and then lipase (2-6% by weight of substrates), and water (2% by weight of substrates and enzyme) were added in hexane (3.0 mL). The mixture was incubated for different time periods (12 to 36 h) in an orbital shaker at 250 rpm at temperatures ranging from 35 to 55°C.

6.3 Results and discussion

6.3.1 The fatty acid profile of Laurical 35

Table 6.1 shows the fatty acid composition of high-laurate canola oil, commercially known as Laurical 35 before and after modification with EPA, DPA, and DHA. The original oil contained 37.0% lauric acid as well as saturated FA such as stearic (12.7%), palmitic (3.05%), and myristic (3.81%) acids. The major unsaturated FA found in Laurical 35 before enzymatic incorporation were oleic (33.9%), and linoleic (3.35%) acids.

6.3.2 Enzymatic incorporation of n-3 FA into Laurical 35

Table 6.2 shows the effect of enzyme type on percent incorporation of DPA, DHA, and EPA into Laurical 35. Lipase from *Mucor miehei* catalyzed significantly ($p < 0.05$) higher incorporation of DPA into Laurical 35. However, there was no significant difference incorporation ($p > 0.05$) of DPA into Laurical 35 when lipases from *Candida antarctica, Pseudomonas sp.*, and *Candida rugosa* were employed. This is despite the activity of enzymes tested (*C. antarctica,* 554 U; *M. miehei,* 13613 U; *Pseudomonas sp.,* 11936 U; *A. niger,* 8142 U; and *C. rugosa,* 38707 U). The order of incorporation of DHA into Laurical 35 was: *Pseudomonas sp.* > *Candida antarctica* > *Mucor miehei* > *Candida rugosa* > *Aspergillus niger.* *Pseudomonas sp.* gave the highest degree of incorporation of DHA (42.5%, after 24 h) into high-laurate canola oil. The incorporation of EPA into Laurical 35 was most effectively catalyzed by two of five lipases tested. However, there was no significant difference ($p>0.05$) in the EPA incorporation into Laurical 35 when lipases from *C. antarctica, A. niger,* and *Mucor miehei* were employed. *Candida rugosa* gave the highest degree of incorporation of EPA into Laurical 35 (64.0%,

after 24 h). Results indicated that lipase from *Candida rugosa* was most effective in incorporating EPA into Laurical 35. Acidolysis of DHA with Laurical 35 was best achieved with *Pseudomonas sp.* lipase, while acidolysis of Laurical 35 with DPA was better attained with lipase from *Mucor miehei*. *Candida rugosa* gave the highest incorporation of EPA into Laurical 35.

6.3.3 Positional distribution of SL

Positional distribution analysis of unmodified Laurical 35 revealed that 71.9% of lauric acid was located at the *sn*-1 + *sn*-3 positions of TAG molecules (Table 6.3). The positional distribution of fatty acids in Laurical 35 modified with DPA showed that DPA was randomly distributed over the three positions of the glycerol backbone. However, lauric acid was mainly attached to the *sn*-1 and *sn*-3 positions (Table 6.3). For DHA-modified Laurical 35, lauric acid as well as DHA were esterified mostly to the *sn*-1,3 positions of the modified oil (Table 6.3). Although DPA or DHA were esterified mainly to the *sn*-1,3 positions of the TAG molecule of modified Laurical 35, significant incorporation into the *sn*-2 position was also observed. The same trend was noted for EPA-modified Laurical 35, lauric acid as well as EPA were esterified mostly to the *sn*-1,3 positions of the modified oil (Table 6.4).

6.3.4 Response surface methodology

Table 6.5 summarizes the experimental data for response variable Y_1 (DPA% incorporation into Laurical 35), Y_2 (DHA% incorporation into Laurical 35), and Y_3 (EPA % incorporation into Laurical 35). Multiple regression coefficients, obtained by employing a least squares procedure to predict the second–order polynomial model for the DPA or DHA or EPA incorporation into Laurical 35 are summarized in Table 6.6. Testing

of these coefficients with the t-test indicated that in modified Laurical 35 with DPA, linear and quadratic terms of amount of enzyme (X_1) were significant ($p \leq 0.01$). Interactions of the amount of enzyme (X_1) and reaction temperature (X_2), reaction time (X_3) and temperature (X_2), as well as the amount of enzyme (X_1) and the reaction time (X_3) were insignificant ($p > 0.1$). In modified Laurical 35 with DHA, all linear, second order and interaction terms were insignificant ($p > 0.1$). Similarly, interactions of the amount of enzyme (X_1) and reaction temperature (X_2), or reaction time (X_3) were insignificant ($p > 0.1$). In modified Laurical 35 with EPA, all linear, second order and interaction terms were insignificant ($p > 0.1$) except the amount of enzyme (X_1) which is significant ($p < 0.001$). Similarly, interactions of the amount of enzyme (X_1) and reaction temperature (X_2), or reaction time (X_3) were insignificant ($p > 0.1$).

The coefficient of determinations for Y_1 (Laurical 35, DPA%) ($R^2 = 0.94$) indicates that the fitted model could explain 94% of the variance. The coefficient of variation (CV) was 5%. The coefficient of determinations and variation (CV) for Y_2 (Laurical 35, DHA%) were 0.57, and 20%, respectively. The coefficient of determinations for Y_3 (Laurical 35, EPA%) ($R^2 = 0.88$) indicates that the fitted model could explain 88% of the variance, while the coefficient of variance (CV) was 3.73%.

A 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, a canonical analysis was conducted on the second-order polynomial models to examine the overall shape of the response surface curves. The canonical forms of the

equations for Y_1 (Laurical 35, DPA%) or Y_2 (Laurical 35, DHA%) or Y_3 (Laurical 35, EPA%) were:

$$Y_1 = 50.8 + 0.48\ W_1^2 - 1.32\ W_2^2 - 4.70\ W_3^2$$

$$Y_2 = 34.1 + 6.61\ W_1^2 - 0.94\ W_2^2 - 4.89\ W_3^2$$

$$Y_3 = 62.2 - 0.86\ W_1^2 - 2.29\ W_2^2 - 4.84 W_3^2$$

Where W_1, W_2, and W_3 are the axes of the response surface for the oil examined. The eigenvalue was positive for DPA or DHA incorporation into Laurical 35 model pointing out that the stationary points were saddles. All eigenvalues were negative for Y_3 indicating that the stationary point was a maximum for Y_3 (Laurical 35, EPA %) model.

Table 6.7 shows critical values for the three factors (enzyme load, reaction time and temperature) examined. The stationary point for the degree of DPA incorporation (%) into Laurical 35 reached a maximum of 50.8% at 5.41% enzyme concentration, at 38.7°C in 33.5 h. Similarly, the maximum incorporation of DHA (34.1%) into high-laurate canola oil was obtained when enzyme amounts, reaction temperature and time were 5.25%, 43.7°C, and 44.7 h, respectively. The maximum incorporation of EPA (62.2%) into Laurical 35 was predicted at 4.36% of enzyme load at 43.2° C over 23.9 h.

6.3.5 Conjugated dienes (CD)

The CD values of the modified Laurical 35 with DHA or DPA or EPA, and the original oil are shown in Figure 6.1. As the storage time increased from 0 to 24 h, the CD values of the original oil increased steadily and reached its maximum value (6.33) at 24 h. After 24 h of storage, the CD values decreased to 4.04, possibly due to the breakdown of unstable hydroperoxides. The oxidative stability of unmodified oil reached a plateau over the period 48-72 h. As the storage time was increased from 0 to 48 h, the CD values of

modified Laurical 35 with DPA increased accordingly and peaked (14.8) at 48 h, hence reflecting a longer induction period for modified Laurical 35 with DPA. The sharp increase in the CD might be accounted for by the formation of more and more hydroperoxides as primary products of oxidation. After 48 h of storage, the CD values decreased to 11.3. As the storage time was extended to 12 h, the CD values of modified Laurical 35 with DHA increased sharply and peaked (14.1) at 12 h, hence reflecting a shorter induction period. After 24 h of storage, the CD values decreased to 10.9. The highest CD values of modified Laurical 35 with EPA was (11.4) at 48 h. After 48 h of storage, the CD values decreased to 10.5. In general, the CD values of both modified Laurical 35 with DPA or DHA were higher than those of EPA-modified oil. The values of conjugated dienes were higher in modified Laurical 35 with DHA or DPA or EPA than the original oil over the entire storage period. The present results indicate that modification of Laurical 35 with DHA, DPA or EPA resulted in its lower stability, due to the incorporation of highly susceptible FA, DHA or DPA or EPA, to oxidation. Another possible explanation could be attributed to the formation of tocopheryl esters from reaction of carboxylic acids and tocopherols present in the oil during the process of preparation of SL, and thus the loss of natural antioxidants. This possibility was confirmed through a series of experiments and will be detailed in Chapter 9.

6.3.6 Thiobarbituric acid reactive substances (TBARS)

TBARS are secondary oxidation products resulting from degradation of hydroperoxides. Hence their appearance depends on the rate of breakdown of the hydroperoxides involved. Figure 6.2 shows TBARS values (μmol/g) of modified Laurical 35 with DHA, DPA or EPA as well as the control unmodified oils. The TBARS values of

the unmodified oil did not change during the entire storage time, indicating good stability of the unmodified oil, in agreement with the expectation. The TBARS values of the modified oil with DHA increased steadily as storage time increased from 0 to 12 h. After 12 h of storage, the TBARS values reached a plateau over the period 12-36 h. After 36 h, the TBARS values decreased to 11.9 μmol/g. As the storage time was increased from 0 to 12 h, the TBARS values of modified Laurical 35 with DPA increased sharply. As storage time was extended up to 36 h, the TBARS values increased accordingly and reached its maximum value (20.5 μmol/g) at 48 h. The highest TBARS values of modified Laurical 35 with EPA was (15.6 μ mol/g) at 36 h. After 36 h of storage, the TBARS values decreased to 10.6 μmol/g. There was a significant difference ($P<0.05$) between the modified and unmodified oils. Furthermore, the TBARS values of modified Laurical 35 with DPA were significantly higher than those of modified Laurical 35 with DHA or EPA over entire storage time, possibly due to low stability of DPA.

Finally, we examined each lipase and its action on incorporation of EPA, DPA, and DHA into Laurical 35. *Candida rugosa* that was found best for EPA (58.2 ± 2.31%) under optimized conditions, affording 2.97 ± 0.57% and 10.1 ± 1.44% incorporation of DPA, and DHA, respectively, into Laurical 35 under these conditions. Meanwhile, *Pseudomonas sp.* that proved to be the best enzyme for DHA incorporation (31.8 ± 0.22%), afforded 61.9 ± 1.68% and 25.0 ± 6.79% of EPA and DPA incorporation, respectively. For DPA, *Mucor miehei* afforded 29.6 ± 0.31% incorporation into Laurical 35 when tested while EPA and DHA were incorporated at 53.2 ± 1.07% and 33.3 ± 0.81%, respectively. In this set of experiment, incorporation of DPA was less than that found in our optimization work. However, the enzyme which was used from different batches.

Using three batches of the same enzyme, we found results were always nearly the same for EPA and DHA while variation was observed for DPA incorporation. However, stability of the enzyme and DPA might have affected the results. Thus, caution must be exercised in this regard.

6.3.7 Conclusions

Lipases from *Pseudomonas sp. Mucor miehei,* and *Candida rugosa* were most effective, for incorporating DHA, DPA, and EPA into Laurical 35, respectively. RSM showed that in Laurical 35-based SL, maximum incorporation of DHA (34.3%) was obtained at 5.25% enzyme, 43.7°C, and 44.7 h. The maximum incorporation of DPA into Laurical 35 (50.8%) was obtained when the enzyme amount, reaction temperature, and time were 5.41%, 38.7°C and 33.5 h, respectively. Lauric acid remained mainly esterified to the *sn*-1,3 in both DHA- or DPA-modified Laurical 35. DPA was randomly distributed over the three positions in modified Laurical 35 with DPA. DHA was present mainly in the *sn*-1,3 positions of the modified oil. For EPA-modified Laurical 35, lauric acid as well as EPA were esterified mostly to the *sn*-1,3 positions of the modified oil. The modified oils were highly prone to oxidation as evidenced by higher CD and TBARS values when compared to their unmodified counterpart.

Table 6.1. Fatty acid composition (wt%) of Laurical 35 before and after modification with DPA, DHA, or EPA using lipase from *Pseudomonas sp.**

Fatty acid	Unmodified	Modified with DPA	Modified with DHA	Modified with EPA
12:0	37.0 ± 0.20	14.3 ± 0.36	18.6 ± 0.16	10.5 ± 2.20
14:0	3.81 ± 0.03	1.62 ± 0.04	2.24 ± 0.04	1.35 ± 0.22
16:0	3.05 ± 0.05	1.48 ± 0.06	2.08 ± 0.06	1.09 ± 0.17
18:0	12.7 ± 0.29	8.02 ± 0.21	9.93 ± 0.45	5.47 ± 0.49
18:1 n-9	33.9 ± 0.30	22.1 ± 0.33	28.9 ± 1.39	14.1 ± 1.23
18:2 n-6	3.35 ± 0.04	2.22 ± 0.03	0.76 ± 0.22	1.37 ± 0.18
20:5 n-3	-	-	-	64.0 ± 5.30
22:5 n-3	-	48.7 ± 0.62	-	-
22:6 n-3	-	-	38.8 ± 3.11	-

*Results are mean values of triplicate determinations ± standard deviation. The reaction mixture contained Laurical 35 (70 mg), fatty acid to Laurical 35 mole ratio of 3:1, enzyme at 4% by weight of substrates, water at 2% by weight of enzyme and substrates, and 3.0 mL of hexane. The mixture was kept at 45 °C for 24 h in a shaking water bath at 250 rpm.

Table 6.2. Effect of enzyme type on the incorporation (%) of DPA, DHA, and EPA into Laurical 35[a]

Source of enzyme	DPA	DHA	EPA
Candida antarctica	28.0 ± 1.29^{b}	39.8 ± 0.95^{d}	52.4 ± 1.35^{a}
Mucor miehei	40.5 ± 0.77^{c}	29.6 ± 2.20^{c}	52.0 ± 0.91^{a}
Pseudomonas sp.	30.5 ± 4.29^{b}	42.5 ± 0.75^{e}	62.5 ± 1.58^{b}
Candida rugosa	21.5 ± 5.26^{ab}	10.8 ± 0.75^{b}	64.0 ± 5.30^{b}
Aspergillus niger	17.5 ± 1.82^{a}	7.73 ± 0.50^{a}	53.9 ± 2.11^{a}

Results are mean values of triplicate determinations ± standard deviation. Values in each column with different roman superscript letters are different ($p<0.05$). [a, b, c, d, e] the order of EPA or DPA or DHA incorporation into Laurical 35, from lowest to highest, was as follows: a < b< c< d < e. The reaction mixture contained Laurical 35 (70 mg), fatty acid to Laurical 35 mole ratio of 3:1, enzyme at 4% by weight of substrates, water at 2% by weight of enzyme and substrates, and 3.0 mL of hexane. The mixture was kept at 45 °C for 24 h in a shaking water bath at 250 rpm.

Table 6.3. Positional distribution [a] (wt%, *sn*-2 and *sn*-1+ *sn*-3) of fatty acids in modified and unmodified Laurical 35[a]

	Unmodified		Modified with DPA		Modified with DHA	
Fatty acid	*sn*-2	*sn*-1 + *sn*-3	*sn*-2	*sn*-1+*sn*-3	*sn*-2	*sn*-1+*sn*-3
12:0	31.2 ± 6.87 (28.1)	49.4 ± 10.7 (71.9)	14.3 ± 0.40 (33.3)	11.9 ± 1.35 (66.7)	16.9 ± 1.52 (30.3)	15.2 ± 3.82 (69.7)
14:0	4.15 ± 0.15 (36.3)	4.42 ± 0.76 (63.7)	1.70 ± 0.40 (34.9)	1.93 ± 0.11 (65.1)	2.90 ± 0.35 (43.2)	1.96 ± 0.50 (56.8)
16:0	4.12 ± 0.87 (45.0)	3.30 ± 0.75 (54.5)	1.64 ± 0.13 (36.9)	2.52 ± 0.39 (63.1)	3.61 ± 0.91 (57.9)	1.62 ± 0.06 (42.1)
18:0	14.5 ± 2.49 (37.8)	8.70 ± 1.09 (62.2)	7.23 ± 0.59 (30.0)	9.64 ± 0.86 (70.0)	12.6 ± 2.37 (42.3)	6.36 ± 0.28 (57.7)
18:1n-9	41.0 ± 3.45 (40.2)	29.1 ± 9.81 (59.7)	18.8 ± 2.31 (28.4)	22.2 ± 0.55 (71.6)	30.9 ± 0.45 (35.6)	17.8 ± 3.23 (64.4)
22:5 n-3	-	-	48.2 ± 0.89 (32.9)	42.3 ± 0.50 (67.1)	-	-
22:6 n-3	-	-	-	-	24.6 ± 4.25 (22.4)	36.5 ± 4.51 (77.6)

[a] Results are mean values ± SD. Values in parenthesis indicate percentage of each FA located at the *sn*-1 + *sn*-3 and the *sn*-2 positions.

Values in parenthesis are, for the *sn*-2 position (% FA at the *sn*-2 position / (% FA in TAG x 3) X 100; for (*sn*-1 + *sn*-3) = 100 – *sn*-2

Table 6.4. Positional distribution [a] (wt%, *sn*-2 and *sn*-1+ *sn*-3) of fatty acids in modified and unmodified Laurical 35[a]

	Unmodified		Modified with EPA	
Fatty acid	*sn*-2	*sn*-1 + *sn*-3	*sn*-2	*sn*-1+*sn*-3
12:0	31.2 ± 6.87 (28.1)	49.4 ± 10.7 (71.9)	35.3 ± 4.48 (31.8)	2.47 ± 0.93 (68.2)
14:0	4.15 ± 0.15 (36.3)	4.42 ± 0.76 (63.7)	3.41 ± 0.29 (84.2)	0.31 ± 0.10 (15.8)
16:0	4.12 ± 0.87 (45.0)	3.30 ± 0.75 (54.5)	2.92 ± 0.123 (89.3)	0.32 ± 0.11 (10.7)
18: 0	14.5 ± 2.49 (37.8)	8.70 ± 1.09 (62.2)	10.3 ± 0.55 (62.8)	1.22 ± 0.39 (37.2)
18:1 n-9	41.0 ± 3.45 (40.2)	29.1 ± 9.81 (59.7)	26.5 ± 0.99 (62.6)	3.12 ± 1.15 (37.4)
20:5 n-3	-	-	6.43 ± 2.16 (3.35)	87.9 ± 2.65 (96.7)

[a] Results are mean values ± SD. Values in parenthesis indicate percentage of the subjected FA present at the *sn*-1 + *sn*-3, and the *sn*-2 positions. Values in parenthesis are, for the *sn*-2 position (% FA at the *sn*-2 position / (% FA in TAG x 3) X 100; for (*sn*-1 + *sn*-3) = 100 – *sn*-2

Table 6.5. Face-centred cube design arrangement and response for the analysis of Laurical 35 with DPA or DHA or EPA[a]

Run	Independent variables			Response (Y_1) [b]	Response (Y_2)	Response (Y_3)
	Enzyme (%)	Temperature (C)	Time (h)			
1	2[c] (-1)[d]	35(-1)	12(-1)	37.5	29.1	50.4
2	2 (-1)	35(-1)	36(+1)	37.2	35.1	53.9
3	2 (-1)	45(0)	24(0)	34.9	17.2	55.5
4	2 (-1)	55(+1)	12(-1)	33.5	36.6	53.9
5	2 (-1)	55(+1)	36(+1)	35.4	32.6	53.6
6	4(0)	35(-1)	24(0)	50.8	35.6	57.8
7	4(0)	45(0)	12(-1)	42.3	33.7	59.5
8	4(0)	45(0)	24(0)	48.5	37.5	64.0
9	4(0)	45(0)	24(0)	49.4	37.5	64.0
10	4(0)	45(0)	24(0)	45.5	37.5	64.0
11	4(0)	45(0)	36(+1)	49.7	23.5	59.1
12	4(0)	55(+1)	24(0)	44.4	35.6	58.9
13	6(+1)	35(-1)	12(-1)	45.9	24.1	57.2
14	6(+1)	35(-1)	36(+1)	48.8	35.1	59.4
15	6(+1)	45(0)	24(0)	50.5	32.4	56.8
16	6(+1)	55(+1)	12(-1)	45.6	40.4	56.7
17	6(+1)	55(+1)	36(+1)	51.9	46.2	50.3

[a] Nonrandomized

[b] Average of triplicate determinations from different experiments. Y_1, % DPA incorporation into Laurical 35; Y_2, % DHA incorporation into Laurical 35; Y_3, % EPA incorporation into Laurical 35

[c] Uncoded variable levels

[d] Coded variable levels

Table 6.6. Regression coefficients of predicted quadratic polynomial model for response (Y)

Coefficients[a]	Laurical 35, DPA (%) (Y_1)	Laurical 35, DHA (%) (Y_2)	Laurical 35, EPA (%) (Y_3)
β_0	39.082	134.43	-38.471
Linear			
β_1	8.8692*	2.1454	14.077**
β_2	-0.7528	-5.5640	2.6007
β_3	0.1355	0.6950	1.1566
Quadratic			
β_{11}	-1.1456*	-1.1144	-1.1121*
β_{22}	0.0032	0.0634	-0.0225
β_{33}	-0.0089	-0.0046	-0.0090
Interactions			
β_{12}	0.0556	0.1400	-0.0800
β_{13}	0.0411	0.0770	-0.0385
β_{23}	0.0061	-0.0158	-0.0129
R^2	0.94	0.57	0.88
CV%	5.09	20.3	3.73

[a] Coefficients refer to the general model. R^2, coefficient of determination; CV%, coefficient of variation; *, Significant at ($p \leq 0.01$); **, Significant at ($p \leq 0.001$); and β_0, intercept

Table 6.7. Canonical analysis of response surface for acidolysis of Laurical 35 with DPA or DHA or EPA

Factor	Laurical 35, DPA (%)	Laurical 35, DHA (%)	Laurical 35, EPA (%)
Amount of enzyme (wt%, X_1)	5.41	5.25	4.36
Reaction temperature (^{o}C, X_2)	38.7	43.7	43.2
Reaction time (h, X_3)	33.5	44.7	23.9
Stationary point	saddle	saddle	maximum
Predicted value [a]	50.8	34.2	62.2
Observed value [b]	48.7 ± 0.62	38.8 ± 3.11	61.1 ± 0.95

[a] Predicted using the polynomial model

[b] Mean of triplicate determinations from different experiments ± standard deviation

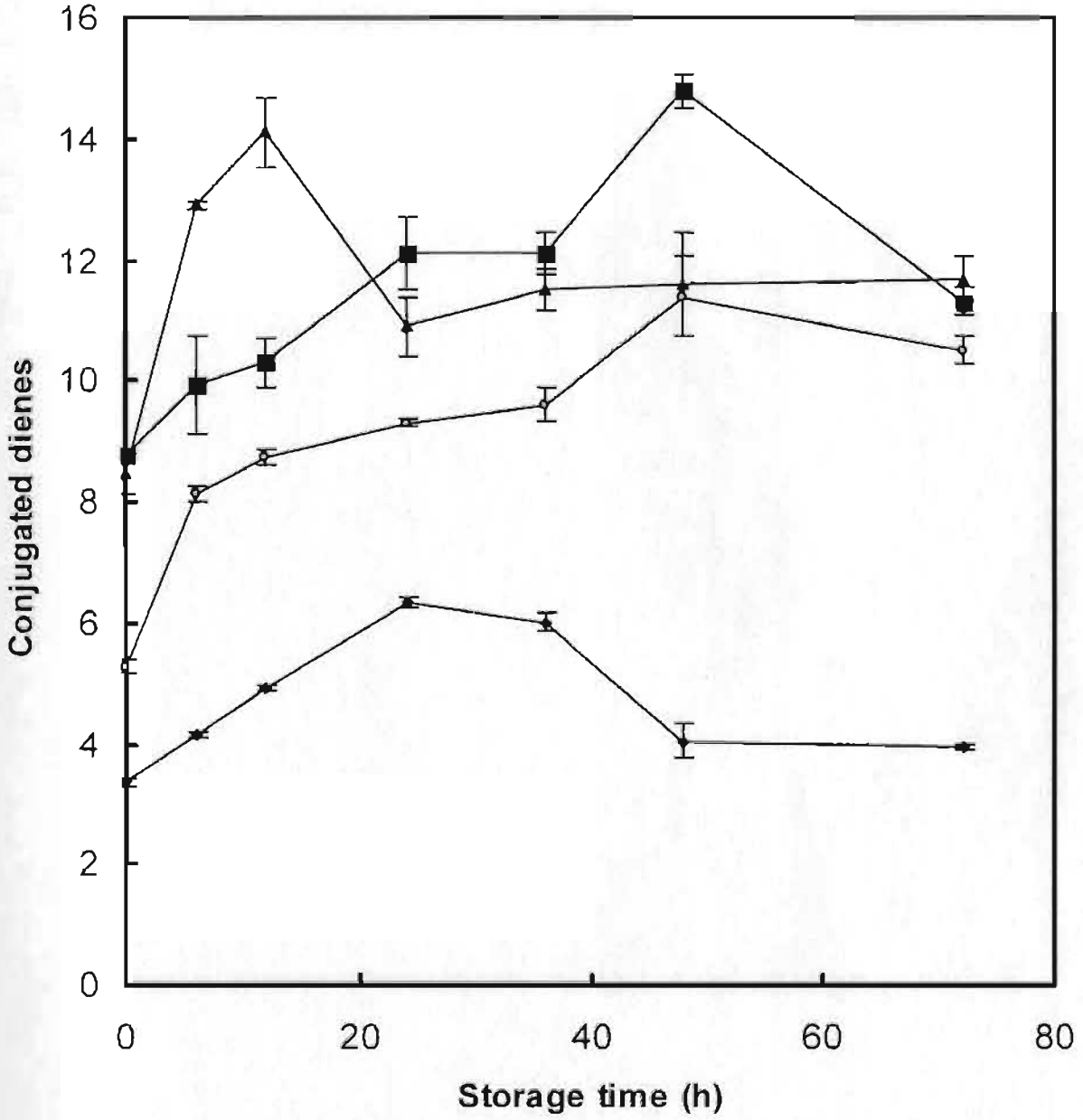

Figure 6.1. Conjugated diene values of (■) modified Laurical 35 with DPA, (▲) modified Laurical with DHA, (*) modified Laurical with EPA, and (♦) the control unmodified oil stored under Schaal oven conditions at 60°C.

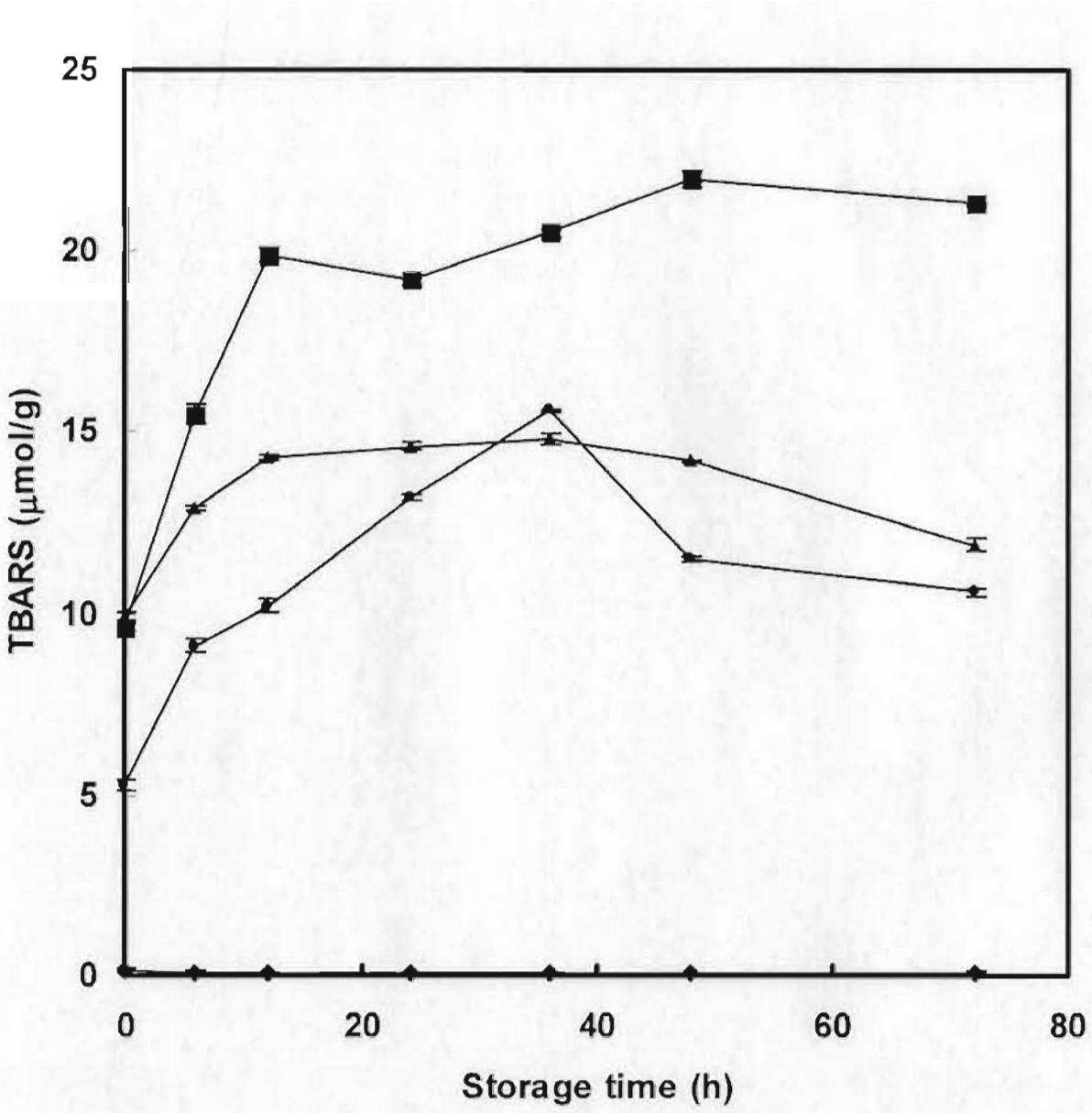

Figure 6.2. TBARS values (µmol/g) of (■) modified Laurical 35 with DPA (▲), modified Laurical 35 with DHA, (●) modified Laurical 35 with EPA, and (♦) the control unmodified oil stored under Schaal oven conditions at 60°C.

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Chapter 7

Lipase-catalyzed acidolysis of tristearin with selected long chain fatty acids

7.1 Introduction

Acidolysis is one type of interesterification, defined as the exchange of acyl groups between an ester (triacylglycerol, TAG) and an acid (fatty acid). Yang *et al.* (2000) compared the acyl incorporation into tristearin (SSS) of linoleic (LA) and conjugated linoleic (CLA) acids in a solvent-free system at 60°C using 5% Lipozyme RM IM from *Rhizomucor miehei*. At 5 h reaction time, LA was more easily incorporated (up to 50 mol%) into tristearin than CLA (only 28 mol%). Formation of trilinolein (LALALA) was five times higher than triconjugated-linolein (CLACLACLA), suggesting that LA was more reactive than CLA probably due to the rigidity of the latter (Yang *et al.*, 2000). Yankah and Akoh (2000) reported the highest incorporation (55.2%) of oleic acid (OA) into tristearin after 72 h at 45°C using 10% immobilized IM-60 from *Mucor miehei* in the presence of hexane.

Senanayake and Shahidi (1999) reported that lipase Novozyme-435 from *Candida antarctica* exhibited the highest degree of DHA incorporation (25.8-28.7%, after 24 h) into borage and evening primrose oils. On the other hand, the highest degree of incorporation of (28.7-30.7%, after 24 h) of EPA in both oils was obtained with lipase PS-30 from *Pseudomonas sp.* In addition, Akoh and Yee (1997) produced SL upon enzymic-catalyzed transesterification of tristearin and tricaprylin. The specific enzyme from *Rhizomucor miehei* produced SL with a total carbon number C_{41} (44.2%) and C_{49} (40.5%). In another study, Huang *et al.* (1999) produced a concentrate containing 52.0% GLA in acylglycerols derived from borage oil via

acidolysis using the enzyme from *Candida rugosa*. Akoh and Huang (1995) screened 12 lipases for their capacity to produce SL via acidolysis of triolein and caprylic acid. Monocaprylоolein was the major constituent with a 57.4 mol% and IM-60 lipase from *Rhizomucor miehei* served as the best biocatalyst tested.

Little attention has been paid to incorporating different FA (OA; LA; CLA; GLA, γ-linolenic acid; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid) into tristearin using lipases: Novozyme-435 from *Candida antarctica*, AY-30 from *Candida rugosa,* PS-30 from *Pseudomonas sp.*, AP-12 from *Aspergillus niger*, and Lipozyme-IM from *Mucor miehei* to catalyze the acidolysis of tristearin with selected LCFA. Also, this study aimed to verify the effect of chain length, number of double bonds, and the location and geometry of double bonds, the reaction conditions, and reactivity of different lipases on the incorporation of these FA (OA, LA, CLA, GLA, ALA, EPA, and DHA) into tristearin. This work is expected to expand the existing knowledge, both basic and applied, in the area of lipid biotechnology. The information obtained in this work would allow the scientists and manufacturers to design and/or predict reaction results for incorporating different fatty acids of interest into triacylglycerols.

7.2 Materials and methods

Materials and methods are the same as those detailed in Chapter 3.

7.2.1 Methods

7.2.1.1 Preparation of conjugated linoleic acid by alkali isomerization

Conjugated linoleic acid was prepared according to the method described by Chin *et al.* (1992). Ethylene glycol (100 g) was placed in a 500 mL round bottom

flask which was placed in an oil bath at 180°C for 10 min and then removed from the oil bath to allow the temperature to decrease to 160°C, and 26 g of potassium hydroxide were subsequently added to its content. The flask was placed again in the oil bath and the temperature increased to 180°C and held there for 10 min. Linoleic acid (50 g) was added to the mixture in the flask after it was removed from the oil bath, and then it was stirred for few seconds. The flask was returned to the oil bath and the temperature maintained at 180°C for 2 h under a stream of nitrogen to prevent autoxidation; the flask was then cooled to room temperature using tap water. Methanol (200 mL) was subsequently added to the mixture, which was then transferred to a one-liter separatory funnel and acidified (pH<2.0) with 250 mL of 6 M HCl. Distilled water (200 mL) was added to the mixture for dilution. CLA was extracted from the mixture with 200 mL hexane. A washing step was conducted by 30% methanol in water (3x200 mL), then with double distilled water (3x200 mL). The extract was passed through an anhydrous sodium sulphate bed to remove any residual water. The hexane was removed at 45°C using a rotary evaporator.

7.2.1.2 Acidolysis reaction

Tristearin (100 mg) was mixed with different FA (OA, GLA, LA, CLA, and ALA) at a mole ratio of acid to tristearin of 3:1 in a screw-capped test tube, then lipase (10%) and water (2%) were added in hexane (3.0 mL). The mixture was incubated at 45 ± 2°C for 24 h in a shaking water bath at 250 rpm. In another set of experiments, a combination of equimole quantities of unsaturated C18 FA (OA+LA+CLA+GLA+ALA) at tristearin to C18 FA ratios of 1:1, 1:2, and 1:3 was used to investigate the effect of substrate mole ratio on incorporation of these fatty

acids into tristearin. The experimental conditions were the same as these mentioned earlier. One mole of tristearin was mixed with 0.2 mole of each of the five selected C18 FA at tristearin to C18 FA ratio of 1:1. For a mole ratio of tristearin to C18 FA of 1:2, one mole of tristearin was mixed with 0.4 mole of each of the five C18 FA, and for mole ratio of 1:3, this was 0.6 for each fatty acid to one mole of tristearin

In another set of experiments, a mixture of equimole amounts of n-3 fatty acids (ALA+EPA+DHA) at tristearin to n-3 fatty acids ratios ranging from 1:1, 1:2, and 1:3 was mixed in a screw-capped test tube then lipase (10%) and water (2%) were added in hexane (3.0 mL). The mixture was incubated at 45 ± 2°C for 24 h in a shaking water bath at 250 rpm.

7.2.1.3 Statistical analysis. All experiments were performed in triplicate. Data reported are mean ± standard deviation (SD). Analysis of variance and Tukey's test were carried out at a level of $p < 0.05$ to assess the significance of differences among mean values.

7.3 Results and discussion

Table 7.1 shows the degree of incorporation of C18 FA (OA, GLA, LA, CLA, and ALA) into tristearin. LA was more easily incorporated into tristearin (46.7%) than CLA (31.8%) using Novozyme-435 from *Candida antarctica*. The acidolysis of tristearin (SSS) and LA and CLA acids can be written into the following equations for the initial reactions, respectively:

$$\text{LA} + \text{SSS} \rightleftharpoons \text{LASS} + \text{S} \qquad \text{(eq.1)}$$

$$\text{CLA} + \text{SSS} \rightleftharpoons \text{CLASS} + \text{S} \qquad \text{(eq.2)}$$

According to thermodynamic laws, the free energy change ΔG can be calculated at a particular instant in time (Campbell and Smith, 1994). The more extended shape of CLA (Figure 7.1), due to the conjugation of the double bonds, results in less stability of CLASS compared to LASS (Yang *et al.*, 2001). Therefore, the free energy of LASS (G LASS; eq.1) is less than that of CLASS (G CLASS; eq.2). When the free energies on both sides of the reaction are equal, equilibrium is reached (Campbell and Smith, 1994). The lesser ΔG for a reaction, the more complete a reaction will be (Campbell and Smith, 1994; Yang *et al.*, 2001). Since G LASS is less than G CLASS, the reaction between LA and tristearin was more favoured than that between CLA and tristearin. The results reported from this study are consistent with those of Yang *et al.* (2001) who reported that LA incorpration into tristearin was up to 50 mol%, whereas CLA incorporation was only 28 mol% in the same acidolysis reaction catalyzed by the Lipozyme RM IM from *Rhizomucor miehei*. Furthermore,Yang *et al.* (2001) suggested that LA was more reactive than CLA, probably due to the rigid structure of the latter arising from conjugation of its double bonds. The rigidity and hindrance of CLA could produce obstacles for its access to the active site of the lipase and hence lead to low percent incorporation into tristearin.

ALA incorporation (65.2%) into tristearin was significantly ($p<0.05$) higher than GLA (55.9%). While ALA and GLA share the same chain length and number of double bonds, the first double bond in ALA is located between C3 and C4 from the methyl end of the molecule while that in GLA is located between C6 and C7 (Figure 7.2). From the carboxylic acid end group, the first double bond in ALA is located at C9 while in GLA it is positioned at C6. Therefore, ALA might have a less bent shape

structure because its three double bonds are positioned on one side of the molecule (Figure 7.2), while GLA might have a more bent shape since the three double bonds are loacted in the middle of it (Figure 7.2). This was confirmed when chemical models were constructed for ALA and GLA as shown in Figure 7.3, demonstrating that GLA has a more bent shape than that of ALA. Therefore, the structural differences between ALA and GLA related to the location of double bonds as well as specificity of the enzymes used might lead to variation in their reactivity.

The highest incorporation (50.0%) of C18 FA examined, catalyzed by Lipozyme-IM from *Mucor miehei,* was for OA. This was also confirmed by Yankah and Akoh (2000) who reported the highest incorporation (55.2%) of OA into tristearin after 72 h at 45°C using immobilized IM-60 from *Mucor miehei.* Moreover, Soumanou *et al.* (1997) reported that lipase from *Mucor miehei* gave the highest yield of structured lipids (73% at 40°C) using peanut oil and tricaprylin, while the lowest incorporation was reported for CLA. The percent incorporation (43.4) of ALA was higher than that of GLA (39.6).

The results presented in this study showed that lipase from *Pseudomonas sp.* catalyzed significant ($p < 0.05$) incorporation of LA (46.2%) compared to that of CLA (31.8%). Incorporation of GLA into tristearin (44.8%) was higher than that of ALA (41.4%). This result contrasts with those from the reactions catalyzed by lipases from *Candida antarctica* and *Mucor miehei.* Incorporation (35.3%) of OA into tristearin corresponded with the findings of Moussata and Akoh (1997) who reported 53.0% incorporation of OA into melon seed oil at 55°C after 24 h using lipase from *Pseudomonas sp.*

The incorporation of C18 FA into tristearin using lipase from *Candida rugosa* as a biocatalyst is also shown in Table 7.1. Although, the highest incorporation (74.8%) was observed for ALA followed by LA (75.8%) there was no significant ($p > 0.05$) difference between these two molecules, while the lowest incorporation (38.3%) was for GLA. These results are contrary to those of Huang *et al.* (1997) who reported that the IM-60 lipase from *Candida rugosa* catalyzed acidolysis reaction between the GLA-rich free FA and the unhydrolyzed acylglycerols, increasing the GLA content from 52.1 to 75% at 50°C, after 4 h (Huang *et al.*, 1997). Incorporation of ALA (74.8%) was almost double that of GLA (38.3%). Meanwhile, OA was incorporated at 47.2% into tristearin, whereas a higher percentage of conversion (>90%) of OA was obtained with esterification of dodecyl alcohol with OA using *Candida rugosa* at 40°C, after 4 h in solvent free foams (Rao and Shanmugam, 2000).

Table 7.2 shows the results for incorporation of EPA and DHA into tristearin. The reactions were catalyzed by lipases from *Candida antarctica, Mucor miehei, Pseudomonas sp., Candida rugosa, and Aspergillus niger.* EPA incorporation (58.2%) was significantly ($p<0.05$) higher than that of DHA (47.7%) using *Candida antarctica* as a biocatalyst. EPA has a shorter chain length and one less double bond than DHA, therefore EPA has a less bent shape compared to DHA (Figure 7.3). These results lend further support to the findings of Senanayake and Shahidi (1999) who reported the maximum incorporation of EPA (28.7-30.7%) into borage oil and evening primrose was obtained with lipase from *Pseudomonas sp.*, and those of Akoh *et al.* (1995) who found that EPA was more easily incorporated into trilinolein than DHA. In general, the extent of incorporation of EPA, using the five selected lipases,

was higher than that of DHA. These results suggest that EPA was more reactive than DHA.

A combination of equimole quantities of unsaturated C18 FA (OA+LA+CLA+GLA+ALA), at a tristearin to C18 FA ratios of 1:1, 1:2, and 1:3, was used to investigate the effect of substrate mole ratio on the incorporation of these fatty acids into tristearin; results are shown in Table 7.3. As the mole ratio of tristearin to C18 FA increased from 1:1 to 1:3, the incorporation of OA and LA increased except for the reaction catalyzed by *A. niger* and *C. rugosa*. All lipases tested failed to allow GLA or CLA to participate in the acidolysis reaction and ALA was only slightly incorporated into tristearin when *Mucor miehei* was used. All five lipases examined assisted incorporation of OA and LA into tristearin except for *Mucor miehei* which only incorportaed OA. *Candida rugosa* lipase better catalyzed incorporation of OA and LA into tristearin than other lipases tested while lowest incorporation was observed for *Pseudomonas sp.*

The effect of mole ratio of substrates on the incorporation of a combination of equimole amounts of n-3 FA into tristearin is shown in Table 7.4. The mole ratio of tristeasrin to a mixture of n-3 FA (ALA, EPA, and DHA) was varied from 1:1 to 1:3. When the mole ratio of substrates increased from 1:1 to 1:3, incorporation of n-3 FA increased accordingly. In general, the highest incorporation of n-3 FA into tristearin may be obtained at a mole ratio of tristearin to n-3 FA of 1:3 because triacylglycerol (TAG) molecules can incorporate a maximum of three FA in their backbone. EPA was significantly ($p<0.05$) more reactive than ALA and DHA during acidolysis using the five selected enzymes employed in this study. In general, the order of

incorporation of a mixture of n-3 FA into tristearin was: EPA>ALA>DHA. Theortically, the order of degree incorporation of these acids should be in the order of ALA>EPA>DHA when chain length and number of double bonds are taken into consideration. Although, EPA has a longer chain length (20 in EPA compared to 18 in ALA) and more double bonds (five in EPA to three in ALA) than ALA, its percent incorporation into tristearin was significantly ($p<0.05$) higher than ALA. Reasons behind higher reactivity of EPA compared to ALA remain unclear, but might be influenced by the specificity of the enzymes in the acidolysis reaction.

In general, incorporation of se;ected long-chain FA into tristearin may be affected by chain length, number of double bonds, and the location and geometry of the double bonds as well as reaction conditions and reactivity and specificity of lipases used. LA was more reactive than CLA due to the rigidity of the latter. EPA was more reactive than DHA, due to the structural differences between them (number of double bonds, chain length). Novozyme-435 enzyme from *Candida antarctica* catalyzed higher incorporation percentage of OA (58.2), GLA (55.9), EPA (81.6), and DHA (47.7) into tristearin. Lipase from *Candida rugosa* catalyzed higher percent incorporation of LA (75.8), ALA (74.8), and CLA (53.5) into tristearin. Thus, these two lipases might be considered as promising biocatalysts for acidolysis of tristearin and selected LCFA.

The high percent incorporation of fatty acids into tristearin using lipase from *Candida antarctica* or *Candida rugosa* might be due to the experimental conditions employed in this study which were suitable for these two enzymes. Thus, further studies should be conducted to verify the optimum conditions for each enzyme examined.

Table 7.1. Effect of different lipases on percent incorporation of C18 fatty acids into tristearin[a]

Enzyme source	OA	LA	CLA	ALA	GLA
Candida antarctica	58.2 ± 1.24[c]	46.7 ± 0.52[b]	31.8 ± 1.44[a]	65.2 ± 3.20[c]	55.9 ± 2.34[c]
Mucor miehei	50.0 ± 3.79[d]	47.7 ± 1.94[c]	30.3 ± 1.52[a]	43.4 ± 2.10[b]	39.6 ± 1.15[b]
Pseudomonas sp.	35.3 ± 2.07[a]	46.2 ± 2.35[c]	31.8 ± 1.02[a]	41.4 ± 2.71[b]	44.8 ± 2.46[b]
Candida rugosa	47.2 ± 3.24[b]	75.8 ± 2.82[d]	53.5 ± 1.89[c]	74.8 ± 0.95[d]	38.3 ± 1.34[a]
Aspergillus niger	51.4± 0.74[b]	66.9 ± 0.90[c]	38.32 ± 0.82[a]	53.7 ± 1.93[b]	38.6 ± 1.48[a]

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

[a, b, c, d, e] The order of incorporation of C18 fatty acids into tristearin, from lowest to highest, was as follows: a < b< c< d < e. Symbols are: OA, oleic acid; LA, linoleic acid; CLA, conjugated linoleic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; and ND, not detected. The reaction mixture contained tristearin (100 mg), fatty acid to tristearin at a mole ratio 3:1, enzyme amount (10% by weight of substrates), water (2% by weight of enzyme and substrates), and 3.0 mL of hexane. The mixture was kept at 45 ±1°C for 24 h in a shaking water bath at 250 rpm.

Table 7.2. Effect of different lipases on percent incorporation of n-3 fatty acids into tristearin[a]

Enzyme source	EPA	DHA
Candida antarctica	58.2 ± 2.05[b]	47.7 ± 2.45[a]
Mucor miehei	47.7 ± 1.86[b]	33.1 ± 2.42[a]
Pseudomonas sp.	68.8 ± 1.84[b]	30.8 ± 1.84[a]
Candida rugosa	66.8 ± 1.59[b]	47.7 ± 1.54[a]
Aspergillus niger	69.9 ± 3.56[b]	38.8 ± 1.61[a]

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

[a, b] The order of incorporation of n-3 fatty acids into tristearin, from lowest to highest, was as follows: a < b. Symbols are: EPA, eicosapentaenoic acid; DHA, and docosahexaenoic acid. The reaction mixture contained tristearin (100 mg), fatty acid to tristearin at a mole ratio of 3:1, enzyme amount (10% by weight of substrates), water (2% by weight of enzyme and substrates), and 3.0 mL of hexane. The mixture was kept at 45 ±1°C for 24 h in a shaking water bath at 250 rpm.

Table7.3. Effect of mole ratio of substrates on percent incorporation of C 18 fatty acids into tristearin[a]

Mole ratio Tristearin: C18 FA	Enzyme source	OA	LA	CLA	ALA	GLA
1:1	*Candida antarctica*	10.9 ± 0.74[b]	8.29 ± 1.58[a]	ND	ND	ND
	Mucor miehei	5.87 ± 0.38[b]	ND	ND	0.25 ± 0.01[a]	ND
	Pseudomonas sp.	5.74 ± 0.75[a]	4.86 ± 0.17[a]	ND	ND	ND
	Candida rugosa	16.7 ± 0.72[b]	14.4 ± 0.81[a]	ND	ND	ND
	Aspergillus niger	14.1 ± 0.46[a]	13.0 ± 0.96[a]	ND	ND	ND
1:2	*Candida antarctica*	11.0 ± 0.97[a]	10.9 ± 0.73[a]	ND	ND	ND
	Mucor miehei	7.74 ± 0.51[a]	ND	ND	0.33 ± 0.14[a]	ND
	Pseudomonas sp.	9.80 ± 1.01[b]	7.05 ± 0.49[a]	ND	ND	ND
	Candida rugosa	18.1 ± 0.49[b]	14.5 ± 0.98[a]	ND	ND	ND
	Aspergillus niger	12.8 ± 0.74[b]	10.0 ± 0.46[a]	ND	ND	ND
1:3	*Candida antarctica*	14.4 ± 0.38[b]	11.5 ± 0.73[a]	ND	ND	ND
	Mucor miehei	8.22 ± 0.12[b]	ND	ND	0.24 ± 0.01[a]	ND
	Pseudomonas sp.	8.09 ± 0.55[b]	6.85 ± 0.01[a]	ND	ND	ND
	Candida rugosa	17.9 ± 0.86[b]	14.9 ± 0.19[a]	ND	ND	ND
	Aspergillus niger	10.2 ± 0.26[a]	9.66 ± 0.31[a]	ND	ND	ND

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

[a, b, c, d, e] The order of C18 fatty acids incorporation into tristearin, from lowest to highest, was as follows: a < b< c< d < e. Symbols are: FA, fatty acids; OA, oleic acid; LA, linoleic acid; CLA, conjugated linoleic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; and ND, not detected.

Table 7.4. Effect of mole ratio of substrates on percent incorporation of n-3 fatty acids into tristearin[a]

Mole ratio Tristearin: n-3 FA	Enzyme source	ALA	EPA	DHA
1:1	*Candida antarctica*	17.5 ± 0.74^{c}	15.9 ± 0.27^{b}	6.62 ± 0.21^{a}
	Mucor miehei	10.3 ± 0.67^{b}	13.1 ± 0.06^{c}	4.70 ± 0.30^{a}
	Pseudomonas sp.	11.5 ± 0.47^{b}	25.3 ± 1.30^{c}	4.69 ± 0.33^{a}
	Candida rugosa	15.3 ± 1.09^{b}	29.2 ± 0.84^{c}	7.59 ± 0.89^{a}
	Aspergillus niger	15.9 ± 0.42^{b}	41.2 ± 0.92^{c}	7.11 ± 0.43^{a}
1:2	*Candida antarctica*	19.8 ± 0.50^{b}	25.7 ± 0.27^{c}	10.9 ± 0.58^{a}
	Mucor miehei	13.7 ± 0.25^{b}	17.6 ± 0.83^{c}	5.72 ± 0.58^{a}
	Pseudomonas sp.	11.5 ± 1.08^{b}	28.7 ± 0.38^{c}	6.65 ± 0.17^{a}
	Candida rugosa	20.4 ± 1.19^{b}	58.1 ± 0.55^{c}	7.80 ± 0.39^{a}
	Aspergillus niger	15.8 ± 2.50^{b}	50.0 ± 0.85^{c}	8.23 ± 0.64^{a}
1:3	*Candida antarctica*	28.3 ± 0.84^{b}	33.0 ± 2.04^{c}	14.4 ± 0.47^{a}
	Mucor miehei	20.1 ± 1.18^{b}	19.2 ± 0.02^{b}	5.89 ± 0.04^{a}
	Pseudomonas sp.	13.7 ± 0.92^{b}	33.4 ± 0.46^{c}	7.26 ± 0.71^{a}
	Candida rugosa	12.5 ± 0.32^{b}	60.6 ± 0.57^{c}	6.37 ± 0.57^{a}
	Aspergillus niger	11.7 ± 0.69^{b}	59.9 ± 2.00^{c}	5.22 ± 0.58^{a}

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

[a, b, c] The order of n-3 FA (ALA+EPA+DHA) incorporation into tristearin, from lowest to highest, was as follows: a < b< c. Symbols are: FA, fatty acids; ALA, α-linolenic acid; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid.

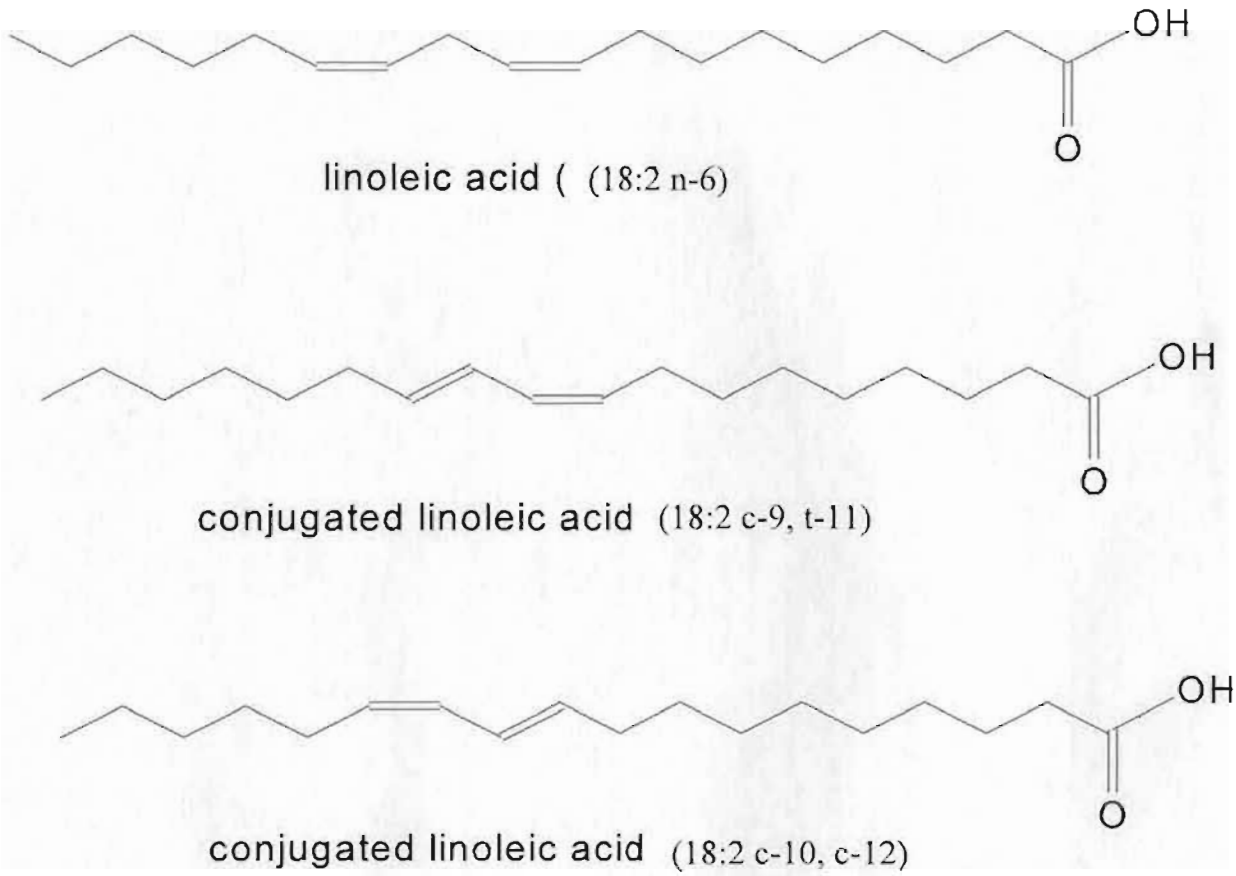

Figure 7.1 Chemical structures of linoleic and isomers of conjugated linoleic acids.

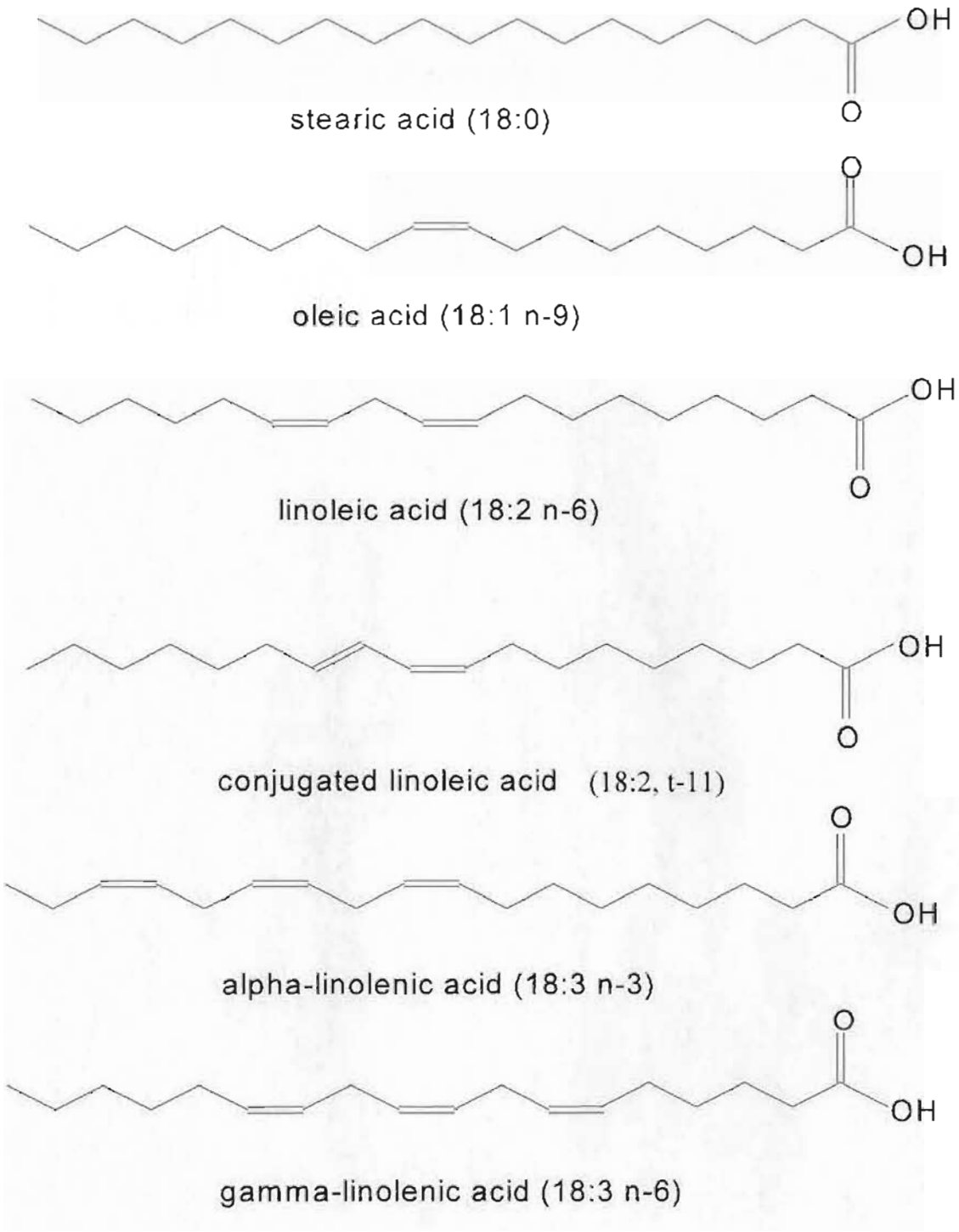

Figure 7.2 Chemical structures of C18 fatty acids: stearic (SA), oleic (OA), linoleic (LA), conjugated linoleic (CLA), α-linolenic (ALA), and, γ-linolenic (GLA) acids.

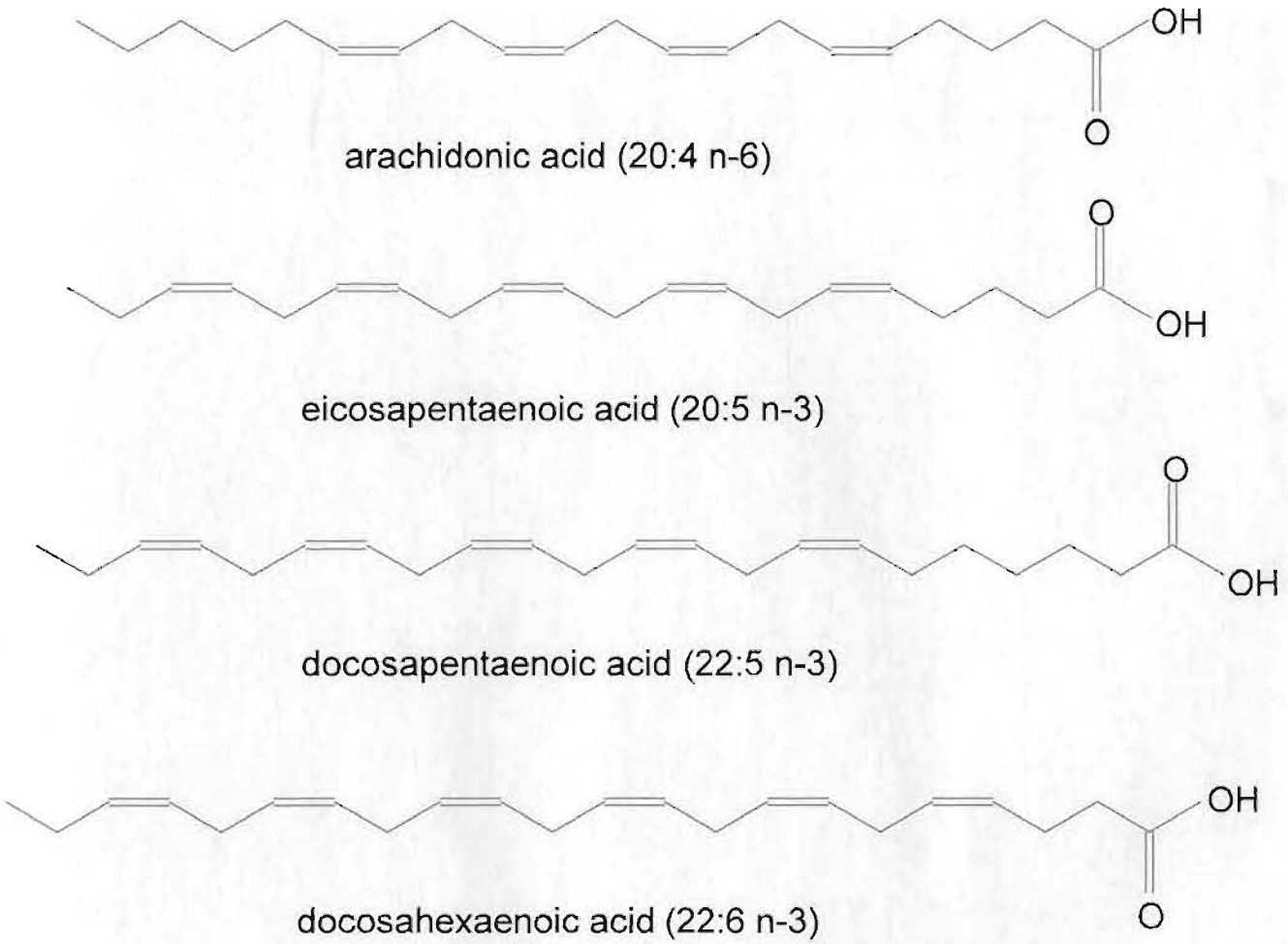

Figure 7.3. Chemical structures of arachidonic (AA), eicosapentaenoic (EPA), docosapentaenoic (DPA), and docosahexaenoic (DHA) acids.

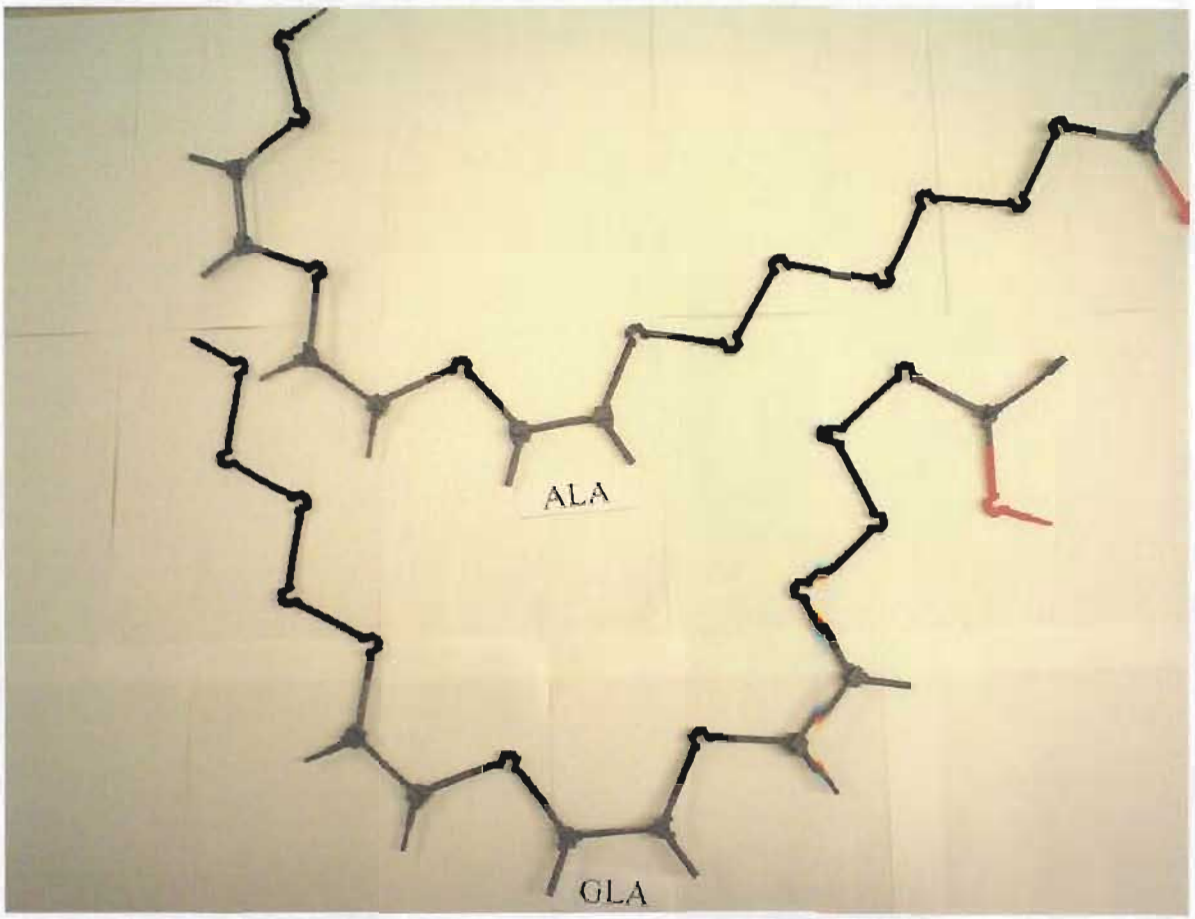

Figure 7.4 Chemical models of α-linolenic acid (ALA), and γ-linolenic acid (GLA).

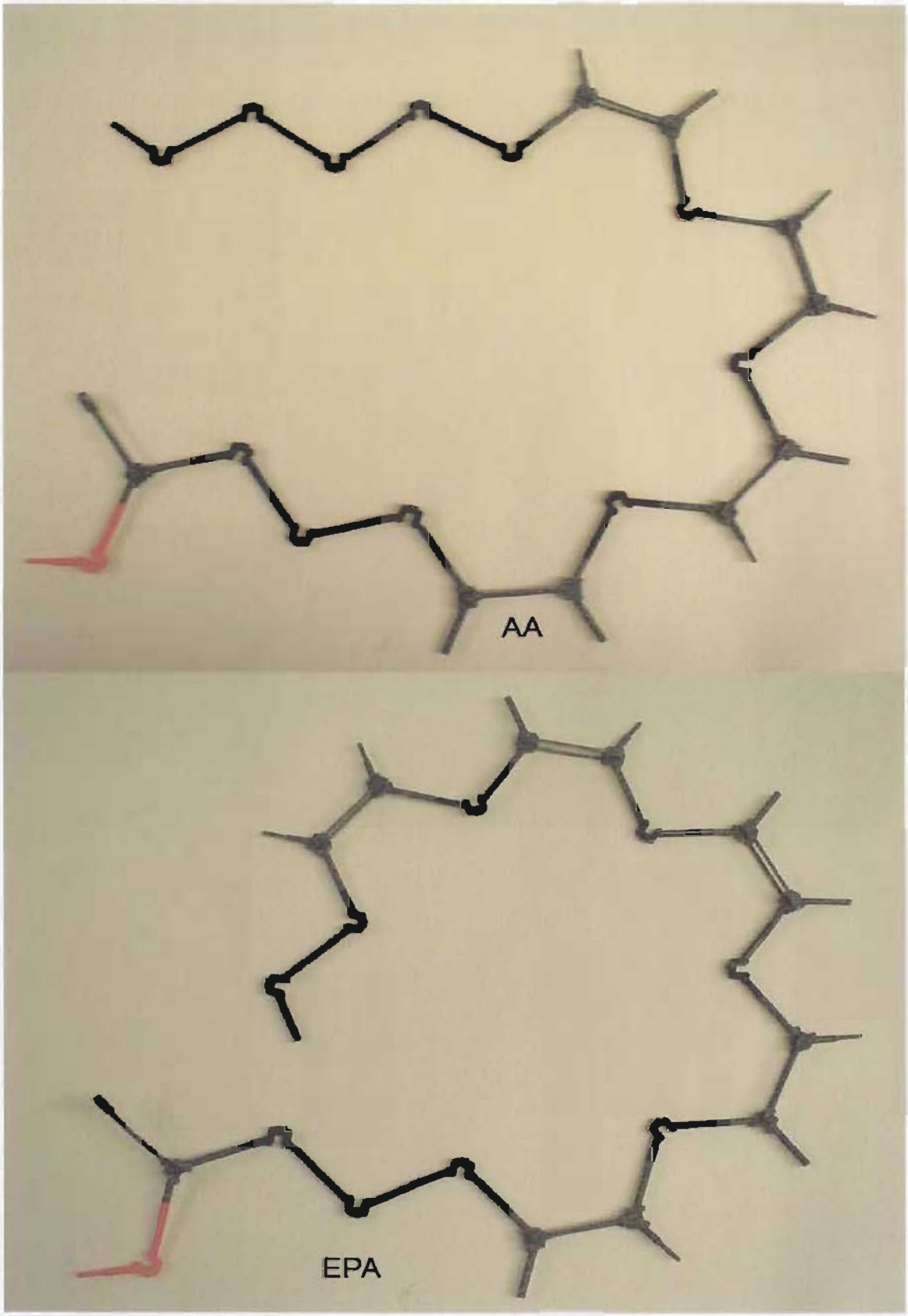

Figure 7.5 Chemical models of arachidonic (AA), and eicosapentaenoic (EPA) acids.

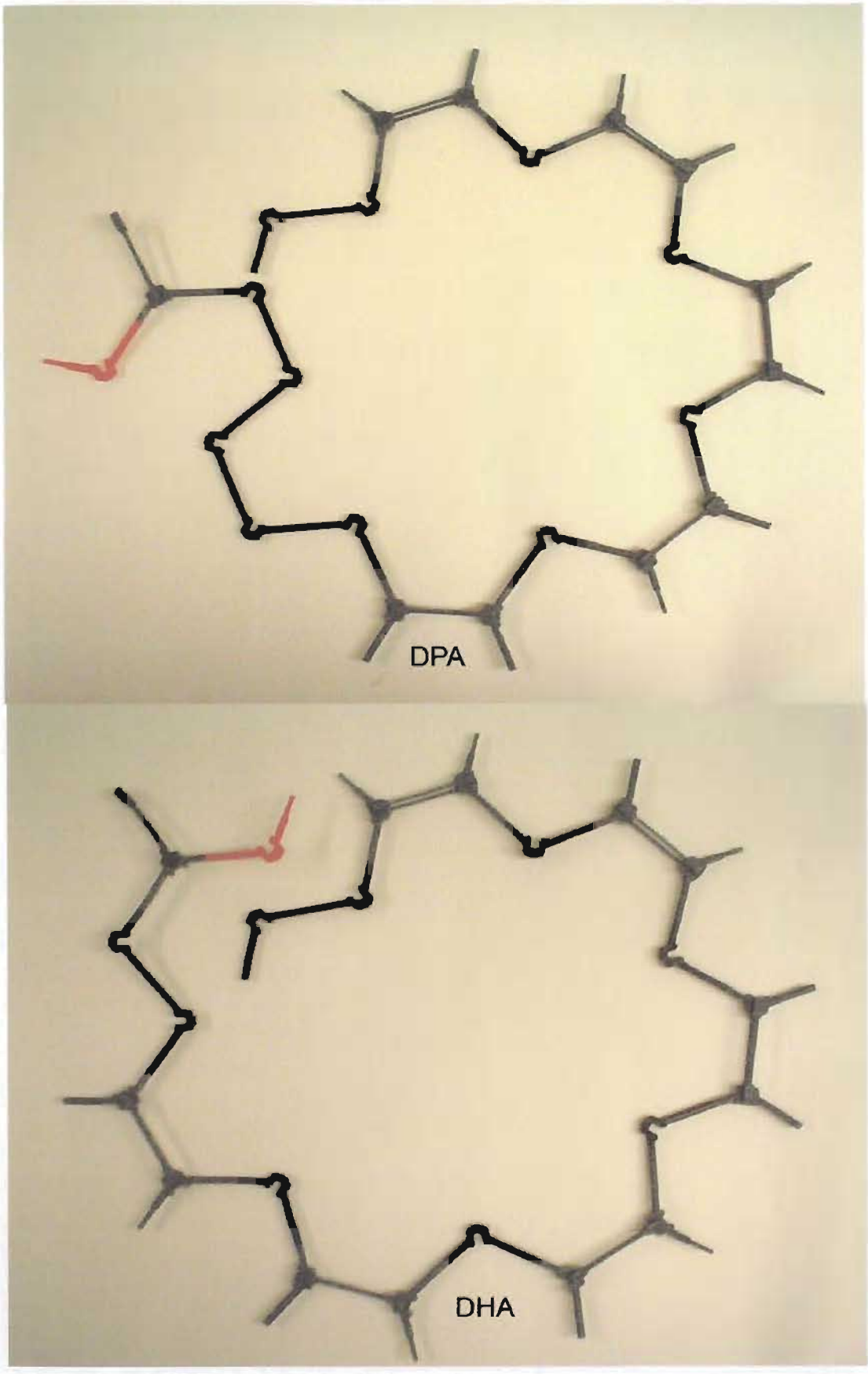

Figure 7.6 Chemical models of docosapentaenoic (DPA), and docosahexaenoic (DHA) acids.

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Chapter 8

Incorporation of selected long-chain fatty acids into triolein, trilinolein, and trilinolinin via lipase-assisted acidolysis

8.1 Introduction

Different fatty acids under the same conditions have different enzyme-assisted percent incorpration into the same oils. The degree of reactivity of different fatty acids also varies in different systems due to the existing differences of lipases employed, and water activity, among others (Yang *et al.*, 2001). Many lipases have been shown to be more selective toward C18 fatty acids with higher degrees of unsaturation in esterification and interesterification reactions (C18:0<C18:1<C18:2). These lipases include those from *Penicillium cyclopium, Candida cylindracea, Mucor miehei, Rhizopus arrhizus, Penicillium sp., Chromobacterium viscosum, Candida rugosa, Pseudomonas fluorescens,* and *Candida lipolytica* (Ronne *et al.*, 2005).

Yang *et al.* (2000) compared acyl incorporation and migeration into tristearin (SSS) of linoleic (LA) and conjugated linoleic (CLA) acids in a solvent-free system at 60°C using 5% Lipozyme RM IM from *Rhizomucor miehei*. Formation of trilinolein (LALALA) was five times higher than triconjugated-linolein (CLACLACLA) (Yang *et al.*, 2000).

Tsuzuki (2005) screened ten lipases for their ability to catalyze acidolysis of triolein and short-chain FA (SCFA; C2:0, C3:0, and C4:0). Lipase from *Aspergillus oryzae* afforded the highest yields of products in the reaction of triolein and C2:0, C3:0, and C4:0 which were 86, 71, 60%, respectively. The results of this study indicated that as the chain length decreased, the degree of incorporation of SCFA into

triolein increased. Paez and coworkers (2003) reported that incorporation of caprylic acid (C8:0) into triolein was favoured compared with that of oleic acid. Again chain length of FA might play a role in the trends observed. Furthermore, it was found that the acidolysis reaction had a maximal value at caprylic acid to triolein mole ratio of 4-6:1. Lipase IM60 from *Rhizomucor miehei* was most effective in SL production from acidolysis of caprylic acid and triolein. The products contained 57.4 mol% monocaprylоolein with a total carbon number of (C_{47}), 35.4 mol% dicaprylоolein (C_{57}), and 5.3 mol% unreacted triolein (C_{57}). The optimal conditions included an oil-to-caprylic acid mole ratio of 1:4, at 55°C, over 24 h, and 10% enzyme concentration (Akoh and Huang, 1995). Lipase IM60 from *Rhizomucor miehei* was most effective in catalyzing acidolysis of triolein and short-chain FA (caporic acid, C6:0; butyric acid, C4:0) to produce low calorie SL. The SL so produced contained 49 mol% disubsitituted (two short- and one long-chain fatty acids, SLS), 38 mol% monosubsitituted (one short- and two long-chain fatty acids, SLL), and 13 mol% unreacted triolein (LLL), at triolein to caproic or butyric acids mole ratio of 1:4:4 mole ratio, at 55°C over 24 h (Fomuso and Akoh, 1997). However, the authors did not comment on any incorporation variation into triolein between caporic acid and butyric acid. 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 dicaprylоolein (41.7%) and monocaprylоolein (46.0%). However, lipase SP 435 from *Candida antarctica* catalysed the conversion of triolein into dicaprylоolein (62.0%) and monocaprylоolein (33.5%) at 55°C.

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, mole ratio of 1:4 (trilinolein/caprylic acid), water content of 1% (w/w), and 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.

Structured lipids (SL) were synthesized by interesterification of trilinolein and tricaproin with the *sn*-1,3-specific (IM 60) and nonspecific (SP 435) lipases. The interesterification reaction was performed by incubating trilinolein and tricaproin at a mole ratio of 1:2 (trilinolein : tricaproin) in 3 mL hexane at 45°C for the IM 60 lipase from *Rhizomucor miehei*, at 55°C, for the SP 435 lipase from *Candida antarctica*. IM 60 lipase produced 53.5 mol% dicaproyllinolein with a total carbon number of 33 (C_{33}) and 22.2% monocaproyldilinolein (C_{45}). SP 435 lipase produced 41% C_{33} and 18% C_{45}. When caproic acid was used in place of tricaproin as the acyl donor, the IM 60 lipase produced 62.9% C_{33} (Fomusa and Akoh, 1998). Akoh and coworkers (1995) used two immobilized lipases, IM60 from *Mucor miehei* and SP435 from *Candida antarctica*, to modify trilinolein (tri C18:2) with EPA and DHA, by using their ethyl esters as acyl donors. The total EPA product yields with *Mucor miehei* and *Candida antarctica* lipases were 79.6 and 81.4%, respectively. The optimal conditions included an oil-to-EPA mole ratio of 1:4, 10% enzyme load over 24 h. Meanwhile, the total DHA product yields with *Mucor miehei* and *Candida antarctica* lipases were 70.5 and 79.7%, respectively, at an oil-to-DHA mole ratio of 1:5 over 12 h. However,

they did not give any explanation regarding higher EPA reactivity compared to DHA (Akoh *et al.*, 1995).

Little attention has been paid to incorporating selected FA into triacylglycerols such as triolein (tri C18:1), trilinolein (tri C18:2), and trilinolinin (tri C18:3) using different lipases. Thus, this study aimed to demonstrate the effect of the following factors, notably, chain length, number of double bonds, the location and geometry of double bonds, the reaction conditions, and reactivity of different lipases on the incorporation of these FA into triolein, trilinolein, and trilinolinin. It also discusses reasons behind different degrees of incorporation of selected LCFA into triolein, trilinolein, and trilinolinin on a molecular basis in order to fill an important gap in the existing scientific literature regarding the differences in the reactivity of different fatty acids. The oxidative stability of SL so produced was also assessed.

8.2 Materials and methods

Materials and methods are the same as those detailed in Chapter 3.

8.2.1 Methods

8.2.1.1 Acidolysis of triolein (tri C18:1) and selected long-chain FA

Triolein (100 mg) was mixed with different FA (SA, GLA, LA, CLA, ALA, AA, EPA, DPA, and DHA) at a mole ratio of acid to triolein of 3:1 in a screw-capped test tube, then lipase (4% by weight of substrates) and water (2% by weight of substrates and enzyme) were added in hexane (3.0 mL). The mixture was incubated at 45 ± 2°C for 24 h in a shaking water bath at 250 rpm. In another set of experiments, a mixture of equimole amounts of C18 FA (SA+LA+CLA+GLA+ALA) at C18 FA to

triolein ratios of 1:1, 2:1, and 3:1 was used to investigate the effect of substrate mole ratio on incorporation of C18 FA into triolein. The experimental conditions were the same as these mentioned earlier. Similarly, a combination of equimole quantities of unsaturated C18 FA (LA+CLA+GLA+ALA) at triolein to C18 FA ratios ranging from 1:1 to 1:3 was mixed in a screw-capped test tube. The enzyme amount, reaction temperature, and incubation time were 4%, 45 ± 2°C, and 24 h, respectively.

A similar experiment was carried out using a mixture of equimole amounts of n-3 FA (ALA+EPA+DHA+DPA) or n-6 FA (LA+GLA+AA) in order to examine their reactivity in the acidolysis reaction.

8.2.1.2 Acidolysis of trilinolein (tri C18:2) or trilinolinin (tri C18:3) and selected long-chain FA

A combination of equimole quantities of C18 FA (SA+OA +CLA+GLA+ALA) at trilinolein (tri C18:2) or trilinolinin (tri C18:3) to C18 FA mole ratio of 1:3 was mixed in a screw-capped test tube. The enzyme amount, reaction temperature, and incubation time were 4%, 45 ± 2°C, and 24 h, respectively. The same was done for a mixture of equimole amounts of n-3 FA (ALA+EPA+DHA+DPA) or n-6 FA (LA+GLA+AA).

8.3 Results and discussion

8.3.1 Acidolysis of triolein (tri C18:1) and selected LCFA

8.3.1.1 Acidolysis of triolein (tri C18:1) and C18 FA

Table 8.1 shows the degree of incorporation of five C18 FA, namely SA, LA, CLA, ALA, and GLA, into triolein. Novozyme-435 from *Candida antarctica* catalyzed the highest LA incorporation (33.8%) into triolein, while GLA showed the

lowest incorporation (13.4%). ALA was more easily incorporated (16.7%) than GLA (13.4%). This observation might be attributed to the structural differences related to the location of double bonds in the two molecules examined. Meanwhile, LA was more easily incorporated into triolein (33.8%) than CLA (13.4%). Although, CLA and LA have the same carbon and double bond numbers, the different positions of double bonds and difference in their geometrical configurations result in their different chemical, physical, and biological properties (Figure 7.1) (Yang *et al.*, 2001).

The acidolysis of triolein (OOO) with linoleic (LA) and conjugated linoleic (CLA) acids may be represented by the following equations for the initial reactions (the initial rate of an enzyme reaction refers to the rate at the earliest time that a reaction can be measured after mixing the reactants), respectively:

$$LA + OOO \rightleftharpoons LAOO + O \qquad \text{(eq.1)}$$

$$CLA + OOO \rightleftharpoons CLAOO + O \qquad \text{(eq.2)}$$

G can be calculated at a particular instant in time (Campbell and Smith, 1994). Therefore, at any instant in time:

$$G\,LA \rightleftharpoons G\,LAOO + G\,O - G\,OOO - G\,LA \qquad \text{(eq.3)}$$

$$G\,CLA \rightleftharpoons G\,CLAOO + G\,O - G\,OO - G\,CLA \qquad \text{(eq.4)}$$

where G LA and G CLA are free energy changes for the acidolysis of linoleic and conjugated linoleic acids with triolein, respectively, and G LAOO, G OOO, G O, G LA, and G CLA are the free energies of LAOO, OOO, O, LA, CLAOO, and CLA, respectively. March (1985) and Barrow (1988) described the relationship between a chemical structure and the free energy; the more extended shape of CLA, due to the

conjugation of the double bonds, results in less stability of CLAOO compared to LAOO. Therefore, G LAOO is less than G CLAOO. The magnitude and sign of the free energy determine the direction in which the reaction will proceed. When the free energies on both sides of the reaction are equal, equilibrium is reached (Campbell and Smith, 1994). The lower ΔG for a reaction, the higher will be the completion of that reaction (Campbell and Smith, 1994; Yang *et al.*, 2001). Since G LAOO is less than G CLAOO, the reaction between LA and triolein occurs more favourably than that between CLA and triolein. The results reported in this study agree with those of Yang *et al.* (2001) who reported LA incorporation up to 50 mol% into tristearin, whereas CLA incorporation into tristearin was only 28 mol%; the acidolysis reactions were catalyzed by Lipozyme RM IM from *Rhizomucor miehei*. Furthermore, these authors suggested that LA was more reactive than CLA, probably due to the rigid structure of the latter and because of the conjugation of double bonds. The rigidity and hindrance of CLA could produce obstacles to the access of CLA to the active site of the lipase, and hence lead to its low incorporation into triolein. The results presented in this study demonstrated that incorporation of CLA into glycerol backbone of triolein was more difficult than that of linoleic acid and this lends further support to the finding of Yang *et al.* (2001).

The highest incorporation (41.6%) of FA, assisted by Lipozyme-IM from *Mucor miehei,* was for ALA. On the other hand, the lowest incorporation (17.3%) was observed for CLA. Incorporation (41.6%) of ALA was higher than that of GLA (25.4%). Meanwhile, LA was more easily incorporated into triolein (33.8%) than CLA (16.1%). The results of this study demonstrate that lipase PS-30 from

Pseudomonas sp. catalyzes the highest incorporation (40.2%) of LA and the lowest incorporation (18.9%) of CLA. The incorporation of FA into triolein, using lipase AY-30 from *Candida rugosa* as a biocatalyst, is also shown in Table 8.1. The highest incorporation (21.4%) was observed for LA followed by SA (14.9%), while the lowest incorporation (8.19%) was for GLA. Incorporation (8.39%) of ALA into triolein was higher than that of GLA (8.19%). This result agrees with those from the reactions catalyzed by lipases from *Candida antarctica, Pseudomonas sp.*, and *Mucor miehei.* The lipase AP-12 from *Aspergillus niger* catalyzed the highest incorporation (23.6%) of LA, whereas CLA showed the lowest incorporation (5.03%) into triolein.

8.3.1.2 Acidolysis of triolein (tri C18:1) and C20 FA

Table 8.2 shows the effect of different lipases on percent incorporation of C20 (AA and EPA) fatty acids into triolein. Among the lipases tested, PS-30 lipase from *Pseudomonas sp.* catalyzed a highest incorporation of EPA (59.3%) into triolein, whereas the lowest incorporation (44.5%) was observed for acidolysis reaction catalyzed by Novozyme-435 from *Candida antarctica.* Lipozyme-IM from *Mucor miehei* catalyzed a highest incorporation of AA (34.1%) into triolein, while lipase AY-30 from *Candida rugosa* catalyzed the lowest incorporation of AA (10.4%). EPA was more easily incorporated into triolein than AA using the five selected lipases. EPA and AA have the same chain length (C20) and the first double bond is located on carbon number 5 near the carboxylic end group, but EPA has five double bonds while AA has four. The more double bonds the chain has in the cis configuration, the more bent it is (Figures 7.3 and 8.3). Since EPA has five cis double bonds, it becomes quite curved compared to AA and hence EPA has more steric hinderance than AA.

Therefore, structural differences between AA and EPA related to the location of double bonds as well as specificity of the enzymes used might lead to variation in their reactivity. Nontheless, EPA was more reactive than AA when the five lipases were examined. The exact reason behind the higher reactivity of EPA compared to that of AA remains unclear and needs further investigation.

8.3.1.3 Acidolysis of triolein (tri C18:1) and C22 FA

Table 8.3 shows the effect of enzyme type on percent incorporation of C22 (DPA and DHA) FA into triolein. The highest incorporation of DPA (28.2%) into triolein was assisted by *Mucor miehei,* while the lowest DPA incorporation (5.16%) into triolein was obtained with lipase from *Aspergillus niger*. Lipase from *Pseudomonas sp.* catalyzed a higher incorporation of DHA (21.4%) into triolein, whereas the lowest incorporation (4.16%) was observed for acidolysis reaction catalyzed by *Aspergillus niger* lipase. DPA was more reactive than DHA except for the reaction catalyzed by *Pseudomonas sp.* and *Candida antarctica*, possibly due to the fact that DPA has one less double bond and hence a less bent structure than that of DHA as well as specificity of the enzymes used might lead to variation in their reactivity (Figures 7.3 and 8.3). Reasons behind higher incorporation of DHA into triolein using *Pseudomonas sp.* and *Candida antarctica* are unclear, but might be attributed to the selectivity of this enzyme toward DHA compared to DPA. Therefore, further research should be conducted to verify this assumption.

8.3.1.4 Acidolysis of triolein and a combination of equimole quantities of C18 FA

A mixture of equimole quantities of C18 FA (SA+LA+CLA+GLA+ALA) at triolein to C18 FA ratios of 1:1, 1:2, and 1:3 was used to investigate the effect of

substrate mole ratio on the incorporation of these FA into triolein (Table 8.4). As the mole ratio of triolein to C18 FA changed from 1:1 to 1:3, incorporation of C18 FA into triolein increased accordingly for the reaction catalyzed by *Mucor miehei*. In contrast, incorporation of C18 FA into triolein decreased when the mole ratio of triolein to C18 FA was altered from 1:1 to 1:3 when the remaining lipases were used. One possible explanation for a decrease in the incorporation of C18 FA into triolein at high FA content could be related to the water activity. The solubility of water in the FA decreases as the chain length of the FA increases (Paez *et al.*, 2003). Therefore, as the amount of FA increases, the overall water activity is expected to decrease, and hence may reduce the degree of incorporation of these FA into triolein. These results are consistent with the data obtained by Paez *et al.* (2003) who reported that increased caprylic acid content leads to decreased water activity and thus a decreased rate of incorporation of caprylic acid into triolein. Similarily, the extent of capric acid (10:0) incorporation (26.3%) into borage oil was much higher than that of EPA (10.2%) using IM 60 from *Rhizomucor miehei* (Akoh and moussata, 1998). It was demonstrated that FA chain length had a role in FA reactivity. In contrast, incorporation of stearic acid (18:0) was higher than that of caprylic acid (8:0) during transesterification of trilinolein and stearic or caprylic acid using 10% lipase from IM 60 from *Rhizomucor miehei*, at 55°C over 32 h, and a mole ratio of 1:4:4 (trilinolein/stearic acid/caprylic acid). They suggested that the chain length, relative polarity of stearic acid and/or selectivity of this lipase (*Rhizomucor miehei*) might be responsible for differences in incorporation of these two molecules (Sellappan and Akoh, 2001).

Lipases from *A. niger* and *C. rugosa* failed to allow CLA participation in the acidolysis reaction, while the remaining FA (SA, LA, GLA, and ALA) were only slightly incorporated into triolein. *Pseudomonas sp.* and *Mucor miehei* lipases catalyzed better incorporation of C18 fatty acids into triolein at each mole ratio examined. Thus, these two lipases might be considered as promising biocatalysts for acidolysis of triolein and C18 FA. *Candida antarctica* lipase catalyzed slight incorporation of C18 fatty acids into triolein except at a mole ratio of triolein to C18 fatty acids of 1 to 1.

In general, the order of C18 FA incorporation into triolein for the most effective biocatalysts (*Pseudomonas sp.* and *Mucor miehei)* was obtained at a mole ratio of triolein to C18 FA of 1 to 3: SA ≥ LA > ALA ≥ GLA> CLA. These results are consistent with expectation because stearic acid is a saturated FA with a straight chain (Figure 7.2) and thus with less sterically hindered. LA with two double bonds causes the chain to bend which would then produce a hindrance for the access of LA to the active site of the lipase. ALA and GLA, with three double bonds, form a hooked shape (Figures 7.2 and 8.2) which make their incorporation into triolein more difficult than that of SA or LA. ALA and GLA shared the same chain length and number of double bonds, but the first double bond in ALA is located at C9 while in GLA it is positioned at C6 from the carboxylic end group. Therefore, location of these three double bonds in ALA on one side of the molecule, while their location in GLA in the middle of it may result in less bent shape of ALA compared to GLA (Figures 7.2 and 8.2). This explanation was supported when chemical models for ALA or GLA were constructed Figure (7.4). Although these structural differences might lead to

variations in the incorporation of ALA and GLA into triolein, further work needs to be carried out to further shed light into these findings.

Table 8.5 shows the effect of incorporation of a mixture of equimole amounts of unsaturated C18 FA (LA+CLA+GLA+ALA) into triolein at triolein to C18 FA ratios of 1:1, 1:2, and 1:3. As the mole ratio of triolein to C18 FA increased from 1:1 to 1:3, incorporation of LA increased except for the reaction catalyzed by *A. niger* and *C. rugosa*. As the number of moles of C18 FA varied from 1 to 3, percent incorporation of GLA into triolein increased accordingly when *C. rugosa* lipase was employed as a biocatalyst. Meanwhile GLA incorporation into triolein decreased when the number of moles of C18 FA was increased from 1 to 3 using lipase from *Mucor miehei*. As the mole ratio of triolein to unsaturated C18 FA increased from 1 to 2, GLA incorporation into triolein increased accordingly. As the mole ratio of triolein to C18 FA increased to 1:3, incorporation of GLA into triolein decreased for the reaction catalyzed by *Pseudomonas sp.*, *Aspergillus niger*, and *Candida antarctica*. Incorporation of ALA into triolein was increased as the number of moles of C18 FA was changed from 1 to 2 for the acidolysis reaction assisted by all five lipases examined. At a higher mole ratio of triolein to C18 FA of 1:2, ALA incorporation into triolein decreased. In general, all five lipases tested catalyzed the lowest incorporation of CLA into triolein compared to other C18 FA used in this set of experiments. Lipases from *Pseudomonas sp.* and *Mucor miehei* catalyzed a gradual increase of CLA incorporation into triolein, as the mole ratio of triolein to C18 FA increased from 1:1 to 1:3.

8.3.1.5 Acidolysis of triolein and a combination of equimole quantities of n-3 FA

The effect of mole ratio of substrates on the incorporation of a mixture of equimole amounts of n-3 FA (ALA, EPA, DPA, and DHA) into triolein is shown in Table 8.6. When the mole ratio of substrates increased from 1:1 to 1:3, incorporation of n-3 FA increased accordingly. In general, highest incorporation of n-3 FA into triolein was obtained at mole ratio of triolein to n-3 FA of 1:3 because TAG molecules can incorporate a maximum of three FA in their backbone. EPA was more reactive than ALA, DPA, and DHA during acidolysis using the enzymes tested. *Aspergillus niger* and *Candida rugosa* lipases catalyzed slight incorporation (almost 1%) of ALA, DPA, and DHA into triolein, possibly due to the experimental conditions employed in this study which might not be adequate for these two enzymes. Thus, further studies should be conducted to verify the optimum conditions for each enzyme examined, perhaps using response surface methodology. DPA was more reactive than DHA except for the acidolysis reaction catalyzed by *Pseudomonas sp.* DPA and DHA have the same chain length and both belong to the n-3 family. However, DPA has one less double bond than DHA and hence less steric hindrance (Figures 7.3 and 8.3). In general, incorporation of n-3 FA into triolein was in the order of EPA>ALA>DPA>DHA. Theortically, ALA incorporation into triolein is expected to be higher than that of EPA when considering factors such as chain length, number of double bonds, and steric hindrance (Figures 7.3 and 8.3). However, at this stage, we are unable to offer any explanation for the observed trend.

8.3.1.6 Acidolysis of triolein and a combination of equimole quantities of n-6 FA

Table 8.7 shows the effect of mole ratio of substrates on the incorporation of a mixture of equimole amounts of n-6 FA into triolein. As the number of moles of a mixture of n-6 FA (LA+ GLA + AA) was changed from 1 to 3, incorporation of n-6 FA increased accordingly except for the reaction assisted by *Candida Antarctica* lipase. LA was more reactive than GLA or AA during acidolysis using the enzymes examined in this study. This result might be attributed to structural differences related to the number of double bonds in these three molecules, together with specificity of the lipases examined for different FA. Lipozyme-IM from *Mucor miehei* catalyzed the highest incorporation of LA (7.12%) and AA (6.54%) into triolein at a mole ratio of triolein to a mixture of n-6 FA (LA, GLA, and AA) of 1:1 and 1:2. Meanwhile, lipases from *Pseudomonas sp.* assisted the highest incorporation of LA (15.1%) and GLA (13.7%) into triolein at a mole ratio of triolein to a mixture of n-6 FA of 1:3.

Incoporation of n-6 FA into triolein for the reactions catalyzed by *Pseudomonas sp.* at all mole ratios employed was in the order of LA>GLA>AA. However, the order of incorporation of these FA into triolein using the remaining lipases (*Candida antarctica*, *Mucor miehei*, *Aspergillus niger*, and *Candida rugosa*) was: LA>AA>GLA. In both groups, LA was the most reactive FA among n-6 FA. The reasons for the higher incorporation of AA than GLA are not clear, but GLA has a shorter chain length than AA and fewer double bonds (3 versus 4).

8.3.2 Acidolysis of trilinolein (tri 18:2) and LCFA

8.3.2.1 Acidolysis of trilinolein (tri 18:2) and a combination of equimole amounts of C18 FA

Incorporation (%) of a mixture of equimole quantities of C18 FA into trilinolein for the most effective lipase (*Pseudomonas sp.*) was in the order of GLA≥OA≥SA>ALA>CLA (Table 8.8). On the other hand, the remaining enzymes catalyzed slight incorporation (<1%) of these FA into trilinolein and the order was: OA>SA>GLA>ALA>CLA. Stearic acid is expected to be more reactive than oleic acid when considering its lower steric hindrance. Percent incorporation of C18 FA into trilinolein was lower (Table 8.8) than their incorporation into triolein (Table 8.4) because trilinolein has higher steric hindrance than that of triolein (Figure 8.1).

8.3.2.2 Acidolysis of trilinolein (tri 18:2) and a mixture of equimole amounts of n-3 FA

Table 8.9 shows the effect of enzyme type on the incorporation of a combination of equimole quantities of n-3 FA into trilinolein. The order of incorporation of n-3 FA into trilinolein using lipases from *C. antarctica and M. miehei* was ALA>EPA>DPA>DHA. Meanwhile, the rest of the lipases (*Candida rugosa*, *Pseudomonas sp.*, and *Aspergillus niger*) catalyzed incorporation (%) of these FA into trilinolein at different order: EPA>ALA>DPA≥DHA. Reasons behind higher EPA reactivity compared to ALA remain unclear, but factors such as interactions between these FA, and selectivity of lipases used might lead to this result. Incorporation of n-3 FA into trilinolein, as shown in Table 8.9, was lower than their incorporation into triolein (Table 8.6). Higher reactivity of EPA among n-3 FA is

consistent with the findings of Akoh *et al.* (1995) who found that EPA was more easily incorporated into trilinolein than DHA, and those of Senanayake and Shahidi (1999; 2002) who reported that EPA incorporation (%) into borage and evening primrose oils was higher than that of DHA. However, reasons behind different degrees of incorporation between EPA and DHA into these oils were not provided.

8.3.2.3 Acidolysis of trilinolein (tri 18:2) and a combination of equimole quantities of n-6 FA

The degree of n-6 FA incorporation into trilinolein with *Pseudomonas sp.* was in the order of GLA>AA>CLA. Meanwhile, the remaining enzymes catalyzed a marginal degree of incorporation (<1%) of these FA into trilinolein and a highest value was observed for AA while the lowest percent incorporation was for CLA (Table 8.10). Incorporation of n-6 FA into trilinolein (Table 8.10) was lower than their incorporation into triolein (Table 8.7).

8.3.3 Acidolysis of trilinolinin (tri 18:3) and LCFA

8.3.3.1 Acidolysis of trilinolinin (tri 18:3) and a combination of equimole quantities of C18 FA

The extent of C18 FA incorporation into trilinolinin with *Mucor miehei* was in the order of SA≥LA>OA>GLA>CLA (Table 8.11). The rest of enzymes (*Candida antarctica, Candida rugosa*, *Pseudomonas sp.*, and *Aspergillus niger*) catalyzed incorporation of C18 FA into trilinolinin in the order of LA>OA>SA >GLA>CLA. Lipases from *Mucor miehei* and *Pseudomonas sp.* catalyzed better incorporation of a mixture of C18 FA into trilinolinin while the remaining lipases (*Candida antarctica, Candida rugosa*, and *Aspergillus niger*) catalyzed slight incorporation (≤1%) of these FA into the oil.

8.3.3.2 Acidolysis of trilinolinin (tri 18:3) and a combination of equimole quantities of n-3 FA (EPA, DPA, and DHA)

EPA was better incorporated into trilinolinin than DPA or DHA using the enzymes tested (Table 8.12). This difference might be due to the existing differences in the chain length and the number of double bonds in the respective moleclues; thus incorporation of DPA or DHA into trilinolinin was more difficult than EPA. These results lend further support to the findings of Senanayake and Shahidi (1999; 2002) who reported that incorporation of EPA into borage and evening primrose oils was better than that of DHA with lipase from *Pseudomonas sp.*

8.3.3.3 Acidolysis of trilinolinin (tri 18:3) and a combination of equimole quantities of n-6 FA

Lipases from *Pseudomonas sp., Candida rugosa,* and *Mucor miehei* catalyzed better incorporation of a mixture of equimole quantities of n-6 FA into trilinolinin than other lipases tested while lowest incorporation (≤1%) was observed using *Candida antarctica* and *Aspergillus niger* (Table 8.13), possibly due to the experimental conditions employed in this study which were unsuitable for these enzymes. Thus, further studies should be performed to verify the optimum conditions for each enzyme examined, perhaps using response surface methodology.

8.3.4 The effect of number and location of double bonds on n-3 FA incorporation into tristearin, triolein, trilinolein, and trilinolinin

Table 8.14 shows the effect of the number and location of double bonds on n-3 FA incorporation into tristearin, triolein, trilinolein, and trilinolinin. As the number of double bonds increased from zero in tristearin to six in trilinolein, the degree of

incorporation of ALA into tristearin, triolein, and trilinolein decreased from 13.7 to 5.79%. Tristearin is expected to have less steric hindrance than that of triolein or trilinolein (Figure 8.1) hence the high incorporation of n-3 FA into tristearin. The degree of incorporation of EPA into tristearin, triolein, and trilinolein declined from 33.8 to 20.2% as the number of double bonds increased from zero in tristearin to nine in trilinolinin. The steric hindrance effect was in the order of trilinolinin>trilinolein >triolein >tristearin.

8.3.5 Oxidative stability tests

8.3.5.1 Conjugated dienes (CD)

The CD values of the modified triolein with n-3 FA or with n-6 FA, and the original oil, are shown in Figure 8.4. The control (original) oil was stable under Schaal-oven conditions at 60°C during the entire storage time (72 h). As the storage time was extended to 48 h, the CD values of modified triolein with n-3 FA increased sharply and peaked (11.6) at 48 h. The sharp increase in the CD might be accounted for by the formation of more and more hydroperoxides as primary products of oxidation. After 48 h of storage, the CD values decreased to 10.4, possibly due to the breakdown of unstable hydroperoxides. As the accelerated storage period was extended to 72 h, the CD values of enzymatically modified triolein with n-6 FA increased gradually and reached its maximum (6.68). The CD values of the modified oils were considerably higher than the original oil over the entire storage period. Furthermore, the CD values of modified triolein with n-3 FA were significantly ($p<0.05$) higher than those of the corresponding triolein with n-6 FA, over the entire storage period. The n-3 FA (ALA, EPA, DPA, and DHA) have more double bonds

than their counterparts in n-6 FA (LA, GLA, and AA), and thus they are more prone to oxidation than n-6 fatty acids.

8.3.5.2 Thiobarbituric acid reactive substances (TBARS) determination

TBARS are secondary oxidation products resulting from degradation of hydroperoxides. Hence their appearance depends on the rate of breakdown of the hydroperoxides involved. TBARS values of modified triolein with n-3 or n-6 FA as well as the control unmodified oils are shown in Figure 8.5. As the accelerated storage period was extended up to 36 h, TBARS values of enzymatically modified triolein with n-3 FA increased gradually and peaked (8.34 μmol/g) at 36 h. After 36 h of storage, TBARS of the modified triolein with n-3 FA decreased to 5.53 μmol/g. As the storage time increased up to 6 h, TBARS values of modified triolein with n-6 FA increased accordingly. After 6 h of storage, TBARS values of modified triolein with n-6 FA reached a plateau. After 24 h of storage, TBARS values decreased and reached its mimmum (1.29 μmol/g) at 72 h. Meanwhile, TBARS values of the control unmodified triolein remained constant during the entire storage period (0 to 72 h), indicating its good stability under Schaal oven conditions at 60°C. The general increase in TBARS values of modified triolein with n-3 or n-6 FA during the storage time may be due to the break down of lipid hydroperoxides and production of secondary oxidation products. TBARS of the modified triolein with n-3 FA were higher than those of the modified triolein with n-6 FA over the entire storage period. Thus, as expected for their degree of unsaturation, modified trioleins with n-3 FA were more prone to oxidation than modified triolein with n-6 FA.

8.3.6 Conclusions

Five lipases were used to assist the acidolysis of triolein (tri C18:1), trilinolein (tri C18:2), and trilinoinin (tri C18:3) with selected LCFA. *Mucor miehei* lipase catalyzed a highest incorporation of SA, ALA, GLA, AA, and DPA, while lipase from *Pseudomonas sp.* catalyzed a highest incorporation of LA, EPA, and DHA into triolein. Thus, lipases from *Mucor miehei* and *Pseudomonas sp.* might be considered the most effective enzymes for acidolysis of triolein with selected LCFA. LA incorporation was better than CLA. ALA was more reactive than GLA. LA incorporation into triolein was the highest among C18 FA for all reactions except for the reaction catalyzed by *Mucor miehei.* EPA was more easily incorporated into triolein than AA using the enzymes tested in this study. Thus, the number and location of double bonds as well as chain length in these two molecules, together with specificity of the lipases examined for different FA might had a marked effect on the incorporation of AA or EPA into triolein. DPA was more easily incorporated into triolein than DHA except for acidolysis reactions catalyzed by *Pseudomonas sp.* This result might be attributed to the existing structural differences related to the number of double bonds in these two molecules.

As the number of moles of triolein to a mixture of equimole quantities of C18 FA (SA+OA+LA+CLA+GLA+ALA) changed from 1 to 3, incorporation of C18 FA into triolein increased accordingly with *Mucor miehei.* Incorporation of n-3 FA into triolein increased, as the mole ratio of a combination of equimole amounts of n-3 FA (ALA, DPA, DHA, and EPA) increased from 1:1 to 1:3. In general, the incorporation of n-3 FA into triolein may be obtained at a mole ratio of 1:3. Similarly, as the

number of moles of a mixture of n-6 FA (LA+ GLA+ AA) was changed from 1 to 3, incorporation of n-6 FA increased accordingly except for the reaction assisted by *Candida antarctica.* In general, incorporation of n-3 FA into triolein was in the order of EPA>ALA>DPA>DHA, while incorporation of n-6 FA into triolein using the most effective enzyme (*Pseudomonas sp.*) at all mole ratios employed was in the order of LA>GLA>AA.

Incorporation (%) of a mixture of C18 FA into trilinolein for the most effective lipase (*Pseudomonas sp.*) was in the order of SA>OA>GLA>ALA>CLA. This is in agreement with expectation when factors, such as number, location and geometry of the double bonds were taken into consideration. The order of incorporation of n-3 FA into trilinolein using lipases from *C. antarctica* and *M. miehei* was ALA>EPA>DPA>DHA. Meanwhile, the degree of n-6 FA incorporation into trilinolein with *Pseudomonas sp.* was in the order of GLA>AA>CLA.

Lipases from *Mucor miehei* and *Pseudomonas sp.* catalyzed better incorporation of a mixture of C18 FA into trilinolinin while the remaining lipases catalyzed slight incorporation (≤1%) of these FA into the oil. SA was most reactive among C18 FA examined while CLA was the least reactive. EPA was better incorporated into trilinolinin than DPA or DHA using the five selected enzymes. Lipases from *Pseudomonas sp., Candida rugosa,* and *Mucor miehei* catalyzed better incorporation of a mixture of equimole quantities of n-6 FA into trilinolinin than other lipases tested while the lowest incorporation (≤1%) was observed using *Candida antarctica* and *Aspergillus niger*, possibly due to the experimental conditions employed in this study which were suitable for these enzymes. The degree of

incorporation of EPA into tristearin, triolein, and trilinolein decreased as the number of double bonds increased from zero in tristearin to 9 in trilinolinin. The same trends were observed for ALA and DHA.

The oxidative stability of the modified triolein in comparison with the original oil, as indicated in both CD and TBARS values, showed that the unmodified oil remained unchanged during storage for 72 h. The modified oils with n-3 FA were more susceptible to oxidation than their the oils modified with n-6 FA, when considering both CD and TBARS values.

Table 8.1. Effect of different lipases on incorporation of (wt%) C18 FA into triolein

Enzyme source	SA	LA	CLA	ALA	GLA
Candida antarctica	14.6 ± 1.00	33.8 ± 3.70	11.9 ± 0.00	19.2 ± 0.25	12.9 ± 0.41
Mucor miehei	31.8 ± 2.40	36.4 ± 1.75	17.3 ± 2.65	41.6 ± 2.85	25.4 ± 0.55
Pseudomonas sp.	31.8 ± 0.20	40.2 ± 1.75	18.9 ±1.35	27.2 ± 1.75	19.2 ± 0.75
Candida rugosa	15.9 ± 0.95	21.4 ± 2.6	14.9 ± 1.35	8.39 ± 0.11	8.19 ± 0.39
Aspergillus niger	8.64 ± 0.35	23.6 ± 2.08	5.03 ± 0.62	9.83 ± 3.07	8.65 ± 0.15

Symbols are: SA, stearic acid; LA, linoleic acid; CLA, conjugated linoleic acid; ALA, α-linolenic acid; GLA, and γ-linolenic acid. The reaction mixture contains triolein (100 mg), fatty acid at mole ratio 3:1, enzyme at 4% by weight of substrates, water at 2% by weight of enzyme and substrates, and 3.0 mL of hexane. The mixture was kept at 45±1°C for 24 h in an orbital water bath at 250 rpm.

Table 8.2. Effect of different lipases on incorporation of (wt %) C20 FA into triolein

Enzyme source	AA	EPA
Candida antarctica	15.5 ± 0.90	44.5 ± 3.35
Mucor miehei	34.1 ± 0.15	47.5 ± 0.10
Pseudomonas sp.	20.3 ± 2.20	59.3 ± 0.65
Candida rugosa	10.4 ± 1.23	56.9 ± 1.00
Aspergillus niger	11.2 ± 0.70	52.0 ± 0.70

Symbols are: AA, arachidonic acid; EPA, eicosapentaenoic acid. The reaction mixture contains triolein (100 mg), fatty acid at mole ratio of an acid to triolein of 3:1, enzyme at 4%by weight of substrates, water at 2% by weight of enzyme and substrates, and 3.0 mL of hexane. The mixture was kept at 45 ±1°C for 24 h in an orbital water bath at 250 rpm.

Table 8.3. Effect of different lipases on incorporation (wt%) of C22 n-3 FA into triolein

Enzyme source	DPA	DHA
Candida antarctica	6.25 ± 0.09	8.64 ± 1.96
Mucor miehei	28.2 ± 1.45	22.4 ± 0.70
Pseudomonas sp.	11.8 ± 0.55	21.4 ± 0.12
Candida rugosa	5.72 ± 0.96	4.31 ± 0.09
Aspergillus niger	5.16 ± 0.36	4.16 ± 0.05

Symbols are: DPA, docosapentaenoic acid; DHA, and docosahexaenoic acid. The reaction mixture contains triolein (100 mg), fatty acid at mole ratio of an acid to triolein of 3:1, enzyme at 4% by weight of substrates, water at 2% by weight of enzyme and substrates, and 3.0 mL of hexane. The mixture was kept at 45±1°C for 24 h in an orbital water bath at 250 rpm.

Table 8.4. Effect of mole ratio of substrates on incorporation (wt%) of C18 FA into triolein

Mole ratio (Triolein/FA)	Enzyme source	SA	LA	CLA	GLA	ALA
1:1	*Candida antarctica*	2.45 ± 0.03	2.50 ± 0.23	0.95 ± 0.06	2.43 ± 0.16	2.69 ± 0.32
	Mucor miehei	4.42 ± 1.05	4.88 ± 0.44	2.34 ± 0.04	1.40 ± 0.37	4.88 ± 0.59
	Pseudomonas sp.	4.33 ± 0.35	6.09 ± 0.54	2.11 ± 0.26	4.68 ± 0.49	5.42 ± 0.44
	Candida rugosa	0.83 ± 0.26	0.91 ± 0.44	ND	0.97 ± 0.71	ND
	Aspergillus niger	1.34 ± 0.43	1.51 ± 0.61	ND	0.79 ± 0.43	1.74 ± 0.89
1:2	*Candida antarctica*	1.06 ± 0.30	0.73 ± 0.21	ND	0.62 ± 0.17	0.64 ± 0.24
	Mucor miehei	5.17 ± 0.46	5.86 ± 0.23	1.54 ± 0.05	2.34 ± 0.28	5.02 ± 0.47
	Pseudomonas sp.	2.29 ± 0.07	3.09 ± 0.04	0.70 ± 0.04	2.64 ± 0.18	2.76 ± 0.08
	Candida rugosa	0.56 ± 0.14	0.39 ± 0.07	ND	0.23 ± 0.04	0.24 ± 0.07
	Aspergillus niger	0.44 ± 0.03	0.36 ± 0.03	ND	0.30 ± 0.03	0.24 ± 0.05
1:3	*Candida antarctica*	0.55 ± 0.08	0.62 ± 0.10	ND	0.60 ± 0.11	0.51 ± 0.09
	Mucor miehei	8.65 ± 0.37	6.75 ± 0.14	3.12 ± 0.09	2.27 ± 0.03	6.29 ± 0.05
	Pseudomonas sp.	1.13 ± 0.01	0.92 ± 0.01	0.35 ± 0.02	1.07 ± 0.01	0.73 ± 0.01
	Candida rugosa	0.37 ± 0.10	0.46 ± 0.07	ND	0.32 ± 0.03	0.25 ± 0.04
	Aspergillus niger	0.39 ± 0.09	0.49 ± 0.03	ND	0.32 ± 0.04	0.29 ± 0.05

Symbols are: SA, stearic acid; LA, linoleic acid; CLA, conjugated linoleic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; FA, fatty acids; and ND, not detected.

Table 8.5. Effect of mole ratio of substrates on incorporation (wt %) of C 18 FA into triolein

Mole ratio (Triolein/FA)	Enzyme source	LA	CLA	GLA	ALA
1:1	*Candida antarctica*	2.91 ± 0.42	0.67± 0.15	2.15 ± 0.54	2.06 ± 0.39
	Mucor miehei	8.70 ± 0.17	1.88 ± 0.04	3.98 ± 0.09	6.64 ± 0.05
	Pseudomonas sp.	4.60 ± 0.09	1.08 ± 0.17	3.44 ± 0.02	3.01 ± 0.67
	Candida rugosa	2.04 ± 0.25	0.57 ± 0.14	1.18 ± 0.24	1.36 ± 0.13
	Aspergillus niger	1.66 ± 0.10	0.45 ± 0.12	0.89 ± 0.07	0.97 ± 0.07
1:2	*Candida antarctica*	3.61 ± 1.22	0.67 ± 0.27	2.92 ± 1.18	2.94 ± 1.22
	Mucor miehei	9.10 ± 0.77	1.59 ± 0.38	3.68 ± 0.27	7.56 ± 0.61
	Pseudomonas sp.	6.56 ± 1.11	1.12 ± 0.26	5.57 ± 0.75	6.27 ± 1.05
	Candida rugosa	4.59 ± 2.18	0.12 ± 0.07	1.19 ± 0.03	1.90 ± 0.87
	Aspergillus niger	5.24 ± 1.82	0.55 ± 0.13	2.70 ± 1.13	1.22 ± 0.35
1:3	*Candida antarctica*	3.67 ± 0.78	0.46 ± 0.01	1.01 ± 0.16	0.69 ± 0.04
	Mucor miehei	9.82 ± 0.01	2.91 ± 0.32	2.73 ± 0.44	6.23 ± 0.64
	Pseudomonas sp.	6.45 ± 0.61	1.54 ± 0.12	2.96 ± 0.18	2.48 ± 0.02
	Candida rugosa	4.17 ± 0.03	0.36 ± 0.01	1.54 ± 0.01	1.66 ± 0.01
	Aspergillus niger	1.04 ± 0.11	0.36 ± 0.07	0.63 ± 0.07	0.68 ± 0.08

Symbols are: LA, linoleic acid; CLA, conjugated linoleic acid; ALA, α-linolenic acid; FA, fatty acids; and GLA, γ-linolenic acid.

Table 8.6. Effect of mole ratio of substrates on incorporation (wt%) of n-3 FA into triolein

Mole ratio (Triolein/FA)	Enzyme source	ALA	EPA	DPA	DHA
1:1	*Candida antarctica*	5.12 ± 0.02	4.23 ± 0.03	5.37 ± 0.11	4.86 ± 0.47
	Mucor miehei	7.36 ± 0.41	7.61 ± 0.67	5.19 ± 0.49	2.83 ± 0.04
	Pseudomonas sp.	6.36 ± 0.86	8.88 ± 0.92	3.07 ± 0.51	3.86 ± 0.53
	Candida rugosa	2.55 ± 0.12	10.7 ± 1.22	1.18 ± 0.14	1.09 ± 0.16
	Aspergillus niger	1.26 ± 0.20	11.6 ± 2.22	1.16 ± 0.37	0.91 ± 0.32
1:2	*Candida antarctica*	5.24 ± 0.08	4.24 ± 0.69	4.39 ± 0.47	4.04 ± 0.39
	Mucor miehei	10.0 ± 0.47	9.56 ± 0.94	8.73 ± 0.33	4.72 ± 0.17
	Pseudomonas sp.	6.57 ± 0.43	14.6 ± 1.50	4.16 ± 1.00	4.21 ± 0.64
	Candida rugosa	1.54 ± 0.51	16.0 ± 0.70	1.51 ± 0.38	1.34 ± 0.38
	Aspergillus niger	0.95 ± 0.02	17.8 ± 1.95	1.06 ± 0.03	0.82 ± 0.01
1:3	*Candida antarctica*	8.52 ± 0.64	8.70 ± 0.14	8.08 ± 0.62	6.57 ± 0.30
	Mucor miehei	11.5 ± 0.80	14.5 ± 4.25	9.77 ± 0.19	3.95 ± 0.21
	Pseudomonas sp.	10.3 ± 0.37	22.2 ± 0.05	4.68 ± 0.09	5.06 ± 0.28
	Candida rugosa	1.81 ± 0.17	25.7 ± 0.05	1.74 ± 0.03	1.24 ± 0.19
	Aspergillus niger	1.28 ± 0.12	25.9 ± 1.75	1.47 ± 0.12	0.98 ± 0.09

Symbols are: ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; and DHA, docosahexaenoic acid.

Table 8.7. Effect of mole ratio of substrates on incorporation (wt%) of n-6 FA into triolein

Mole ratio (Triolein/FA)	Enzyme source	LA	GLA	AA
1:1	*Candida antarctica*	2.68 ± 0.41	1.99 ± 0.13	2.81 ± 0.01
	Mucor miehei	7.12 ± 1.09	4.09 ± 0.66	6.54 ± 0.75
	Pseudomonas sp.	6.35 ± 1.35	4.84 ± 1.04	3.12 ± 1.12
	Candida rugosa	3.85 ± 0.14	1.31 ± 0.18	1.52 ± 0.15
	Aspergillus niger	2.39 ± 0.30	1.36 ± 0.05	1.87 ± 0.09
1:2	*Candida antarctica*	2.49 ± 0.03	1.61 ± 0.25	2.54 ± 0.02
	Mucor miehei	9.76 ± 1.45`	4.83 ± 0.58	8.51 ± 0.97
	Pseudomonas sp.	9.45 ± 0.49	7.58± 0.80	3.86 ± 0.08
	Candida rugosa	5.87 ± 1.59	1.64 ± 0.08	1.86 ± 0.08
	Aspergillus niger	3.62 ± 0.07	1.39 ± 0.02	2.22 ± 0.31
1:3	*Candida antarctica*	1.42 ± 0.10	0.77 ± 0.02	1.36 ± 0.13
	Mucor miehei	14.2 ± 0.15	4.77 ± 0.12	13.2 ± 0.50
	Pseudomonas sp.	15.1 ± 0.25	13.7 ± 0.40	6.23 ± 0.27
	Candida rugosa	8.96 ± 0.46	2.03 ± 0.36	1.67 ± 0.18
	Aspergillus niger	2.83 ± 0.40	1.14 ± 0.15	1.67 ± 0.09

Symbols are: LA, linoleic acid; GLA, γ-linolenic acid; and AA, arachidonic acid.

Table 8.8. Effect of enzyme type on incorporation (wt%) of C 18 FA into trinlinolein

Enzyme source	SA	OA	CLA	GLA	ALA
Candida antarctica	0.40 ± 0.22	0.81± 0.17	ND	0.43 ± 0.07	0.32 ± 0.05
Mucor miehei	1.39 ± 0.10	1.68 ± 0.09	0.28 ± 0.02	0.60 ± 0.05	1.02 ± 0.05
Pseudomonas sp.	1.65 ± 0.01	1.66 ± 0.04	0.33 ± 0.01	1.69 ± 0.07	1.21 ± 0.05
Candida rugosa	0.55 ± 0.02	0.81± 0.04	ND	0.31 ± 0.02	0.29 ± 0.01
Aspergillus niger	0.42 ± 0.07	0.85 ± 0.01	ND	0.32 ± 0.02	0.30 ± 0.01

Symbols are: SA, stearic acid; LA, linoleic acid; CLA, conjugated linoleic acid; ALA, α-linolenic acid; and GLA, γ-linolenic acid.

Table 8.9. Effect of enzyme type on incorporation of (wt%) n-3 FA into trilinolein

Enzyme source	ALA	EPA	DPA	DHA
Candida antarctica	6.31 ± 0.24	5.79 ± 0.23	5.65 ± 0.31	4.22 ± 0.20
Mucor miehei	10.6 ± 0.48	10.3 ± 0.37	8.73 ± 0.39	6.85 ± 0.26
Pseudomonas sp.	5.79 ± 0.11	21.2 ± 1.75	2.91 ± 0.24	3.55 ± 0.25
Candida rugosa	2.36 ± 0.02	15.3 ± 0.29	1.95 ± 0.03	1.90 ± 0.02
Aspergillus niger	1.05 ± 0.03	17.7 ± 0.48	1.08 ± 0.01	1.27 ± 0.02

Symbols are: ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; and DHA, docosahexaenoic acid.

Table 8.10. Effect of enzyme type on incorporation (wt%) of n-6 FA into trilinolein

Enzyme source	CLA	GLA	AA
Candida antarctica	0.24 ± 0.01	0.56 ± 0.23	0.63 ± 0.03
Mucor miehei	0.62 ± 0.12	1.08 ± 0.02	1.77 ± 0.02
Pseudomonas sp.	1.23 ± 0.00	5.09 ± 0.01	2.37 ± 0.02
Candida rugosa	0.27 ± 0.01	0.50 ± 0.03	0.58 ± 0.03
Aspergillus niger	0.36 ± 0.02	0.79 ± 0.04	0.94 ± 0.06

Symbols are: GLA, γ-linolenic acid; CLA, conjugated linoleic acid; and AA, arachidonic acid.

Table 8.11. Effect of enzyme type on incorporation (wt%) of C18 FA into trinlinolinin

Enzyme source	SA	OA	CLA	LA	GLA
Candida antarctica	1.22 ± 0.01	1.45 ± 0.07	ND	1.48 ± 0.02	0.81 ± 0.01
Mucor miehei	3.85 ± 0.14	3.76 ± 0.11	0.62 ± 0.01	3.84 ± 0.13	1.63 ± 0.04
Pseudomonas sp.	4.65 ± 0.03	4.85 ± 0.04	0.93 ± 0.02	5.48 ± 0.05	4.57 ± 0.04
Candida rugosa	1.06 ± 0.01	1.25± 0.01	ND	1.29 ± 0.01	0.76 ± 0.01
Aspergillus niger	0.69 ± 0.02	0.78 ± 0.03	ND	0.97 ± 0.02	0.46 ± 0.03

Symbols are: SA, stearic acid; OA, oleic acid; LA, linoleic acid; CLA, conjugated linoleic acid; and GLA, γ-linolenic acid.

Table 8.12. Effect of enzyme type on incorporation (wt%) of n-3 FA into trilinolinin

Enzyme source	EPA	DPA	DHA
Candida antarctica	11.3 ± 0.89	8.31 ± 0.03	8.44 ± 0.09
Mucor miehei	16.0 ± 0.08	14.8 ± 0.08	8.39 ± 0.26
Pseudomonas sp.	20.2 ± 0.33	8.06 ± 0.07	8.40 ± 0.00
Candida rugosa	24.3 ± 1.44	5.99 ± 0.12	7.30 ± 0.11
Aspergillus niger	23.7 ± 0.33	2.47 ± 0.07	1.63 ± 0.06

Symbols are: EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; and DHA, docosahexaenoic acid.

Table 8.13. Effect of enzyme type on incorporation (wt%) of n-6 FA into trilinolinin

Enzyme source	CLA	LA	GLA	AA
Candida antarctica	ND	0.63 ± 0.35	0.59 ± 0.01	0.75 ± 0.01
Mucor miehei	1.97 ± 0.16	6.58 ± 0.11	5.21 ± 0.33	6.98 ± 0.24
Pseudomonas sp.	2.30 ± 0.11	7.70 ± 0.05	9.47 ± 0.11	4.98 ± 0.04
Candida rugosa	1.69 ± 0.02	5.98 ± 0.05	7.61 ± 0.05	3.91 ± 0.07
Aspergillus niger	0.31 ± 0.01	1.33 ± 0.01	0.98 ± 0.02	1.12 ± 0.01

Symbols are: LA, linoleic acid; GLA, γ-linolenic acid; CLA, conjugated linoleic acid; and AA, arachidonic acid.

Table 8.14. Effect of double bonds number and location on incorporation (wt%) of equimole amounts of n-3 FA into tristearin, triolein, trilinolein, and trilinolinin

Oil type	ALA	EPA	DHA
Tristearin	13.7 ± 0.92	33.8 ± 2.53	26.0 ± 0.04
Triolein	10.3 ± 0.37	22.2 ± 0.05	5.06 ± 0.28
Trilinolein	5.79 ± 0.11	21.2 ± 1.75	3.55 ± 0.25
Trilinolinin	-	20.2 ± 0.33	1.63 ± 0.06

A combination of equimole quantities of n-3 fatty acids (ALA+EPA+DHA) at triacylglycerol to n-3 FA ratio of 1:3 was mixed in a screw-capped test tube then *Pseudomonas sp.* lipase (4%) and water (2%) were added in hexane (3.0 mL). The mixture was incubated at 45 ± 1°C for 24 h in a shaking water bath at 250 rpm.

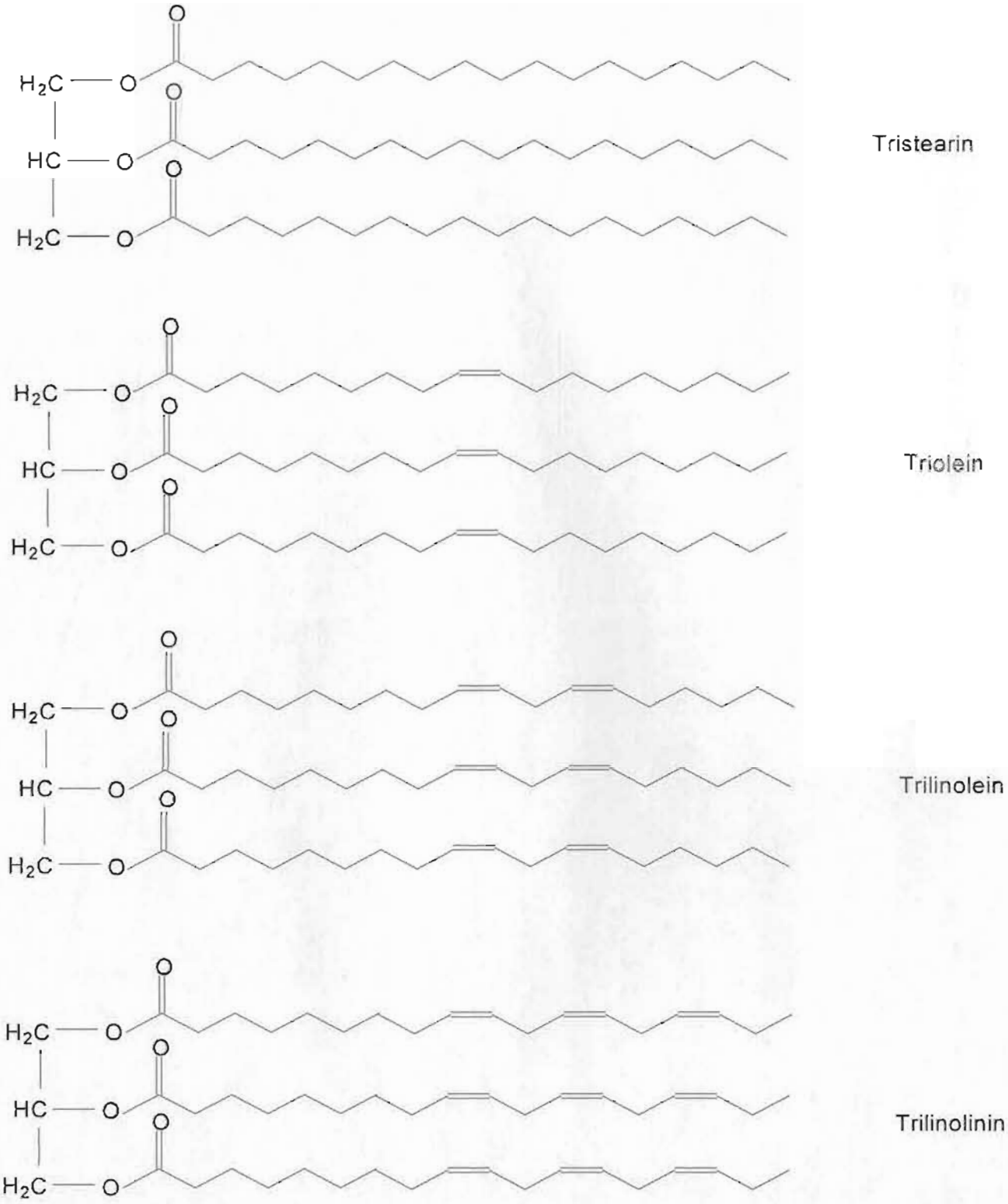
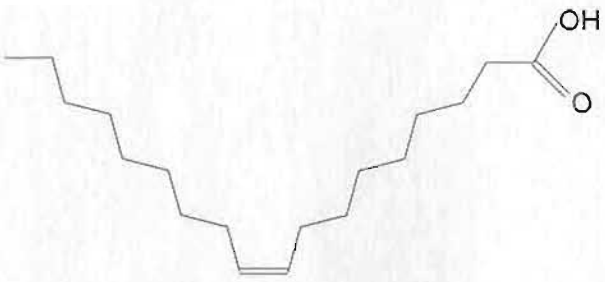
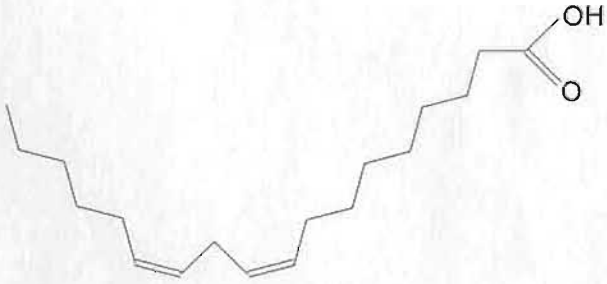


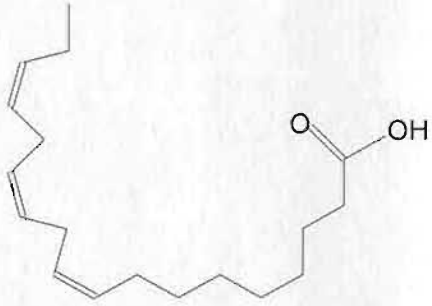
Figure 8.1 Chemical structures of tristearin, triolein, trilinolein, and trilinolinin.



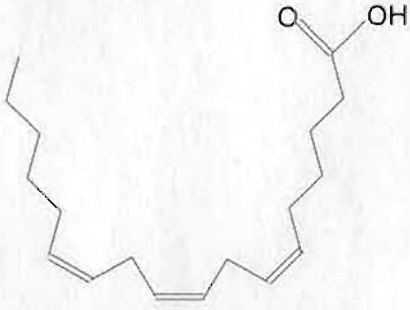
oleic acid (18:1 n-9)



linoleic acid (18:2 n-6)

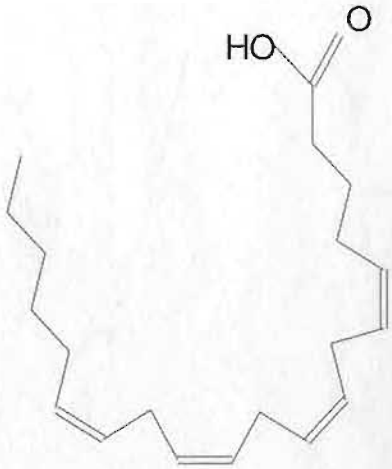


alpha-linolenic acid (18:3 n-3)

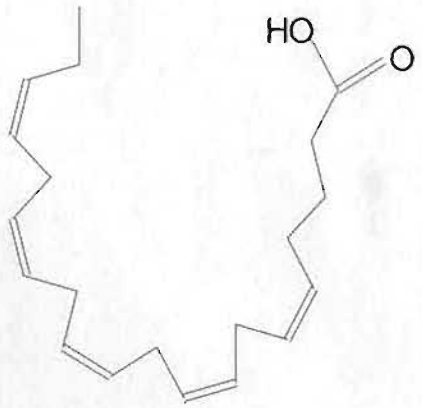


gamma-linolenic acid (18:3 n-6)

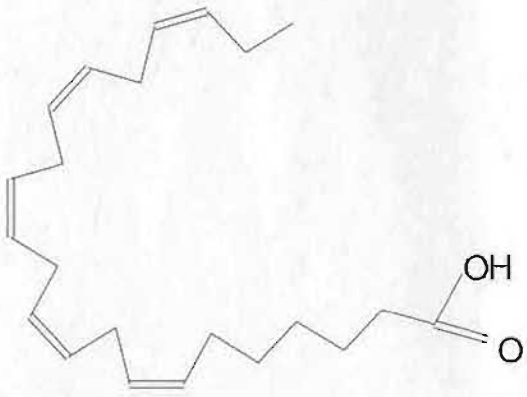
Figure 8.2 Chemical structures of C18 fatty acids: oleic (OA), linoleic (LA), α-linolenic (ALA), and, γ-linolenic (GLA) acids.



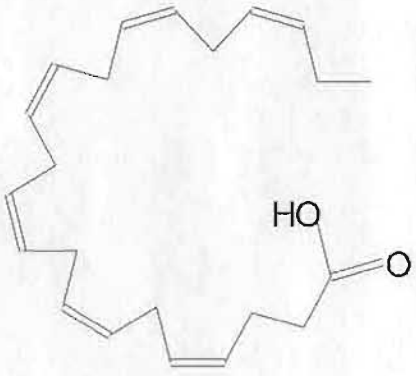
arachidonic acid (20:4 n-6)



eicosapentaenoic acid (20:5 n-3)



docosapentaenoic acid (22:5 n-3)



docosahexaenoic acid (22:6 n-3)

Figure 8.3 Chemical structures of arachidonic (AA), eicosapentaenoic (EPA), docosapentaenoic (DPA), and docosahexaenoic (DHA) acids.

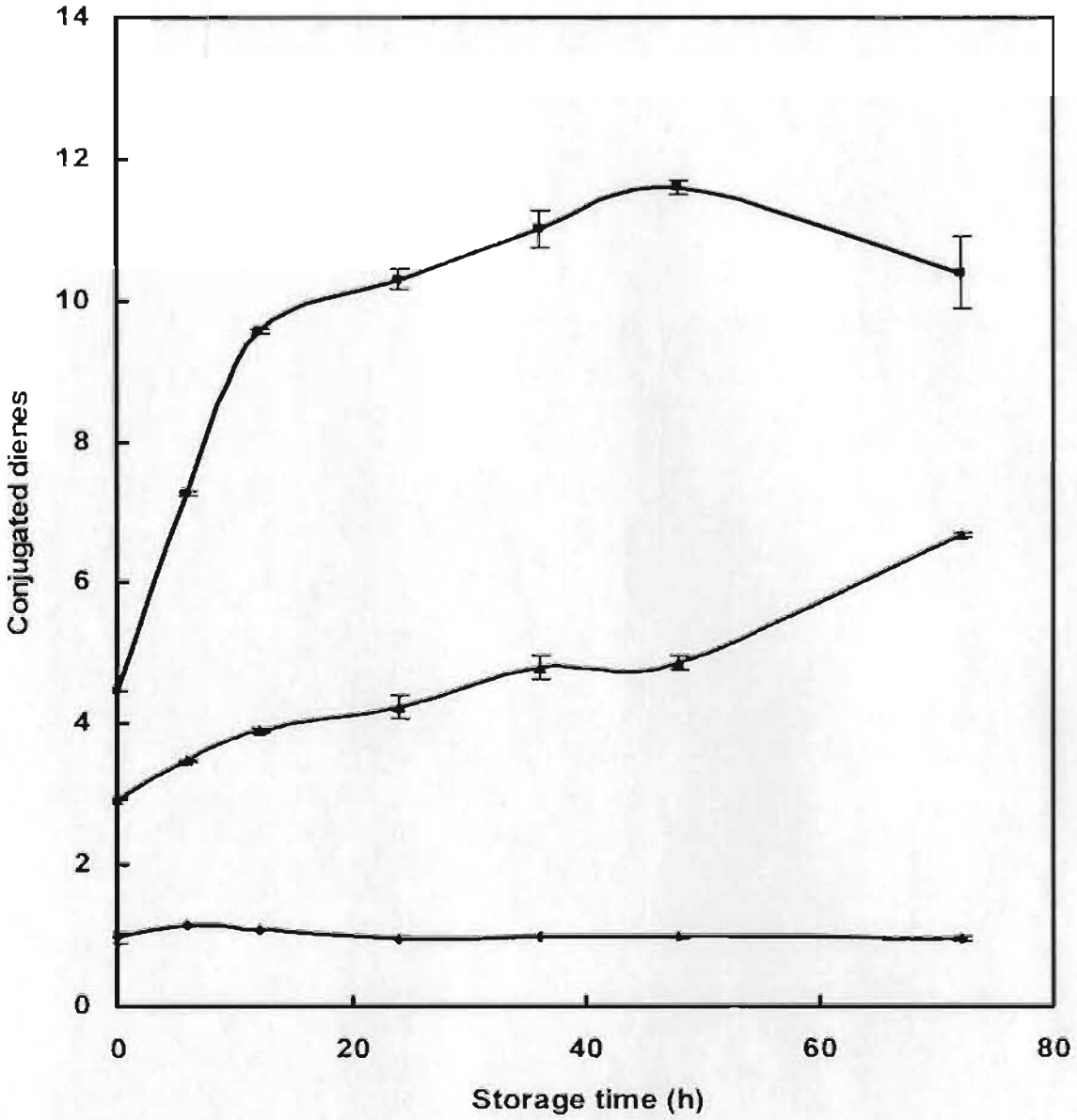


Figure 8.4. Conjugated diene values of (■) modified triolein with n-3 fatty acids, (▲) modified triolein with n-6 fatty acids, and (♦) the control unmodified oil.

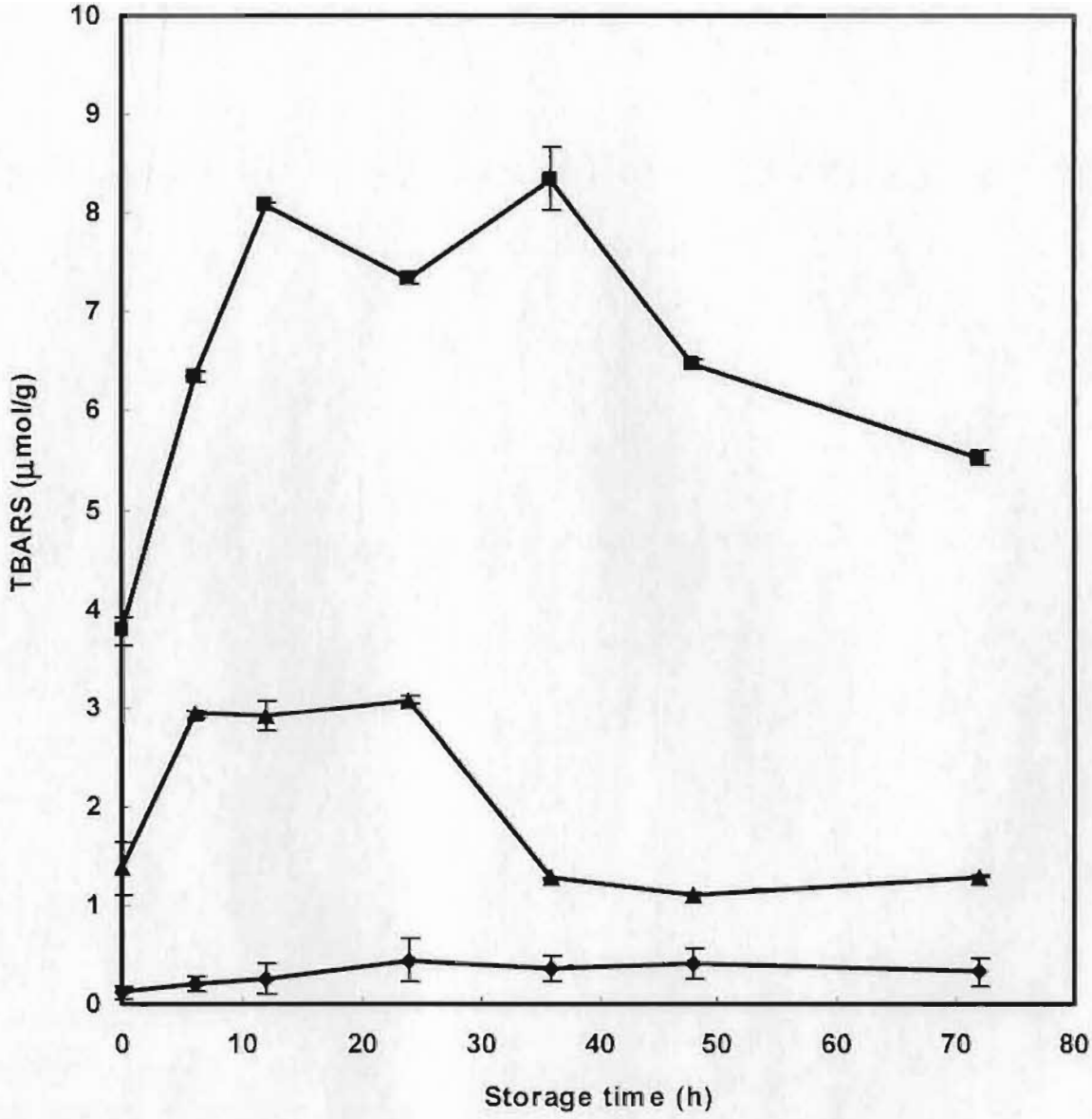


Figure 8.5. TBARS values (µmol/g) of (■) modified triolein with n-3 fatty acids, (▲) modified triolein with n-6 fatty acids, and (♦) the control unmodified oil.

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Chapter 9

Acidolysis reactions lead to esterification of endogenous tocopherols and compromised oxidative stability of modified oils

9.1 Introduction

Stability of fats and oils is dictated mainly by their fatty acid composition, governed by the degree of unsaturation, as well as the content of endogenous antioxidants, presence of oxygen and storage and/or packaging conditions (Shahidi *et al.*, 2005). Edible oils consist mainly of triacylglycerols (TAG, 95%). Non-triacylglecerols (also known as minor components or unsaponifiable matter) make up the remaining 5%. These minor components are naturally-occurring compounds, some of which have antioxidative properties that give the oils the ability to protect themselves against oxidation (Shahidi and Shukla, 1996; Espin *et al.*, 2000). The minor components of vegetable oils are primarily composed of phospholipids, tocopherols, tocotrienols, flavonoids and other phenolic compounds, pigments (carotenoids, chlorophylls), sterols, hydrocarbons, free fatty acids, as well as mono- and diacylglycerols (Shahidi and Shukla, 1996; Hamilton, 1994). Several classes of minor components might be present in each oil and these contribute to its oxidative stability (Shahidi and Shukla, 1992).

Tocols, both tocopherols and tocotrienols, include four naturally occurring homologues (α-, β-, γ- and δ-), that are present in various oils in different proportions and amounts and are known to increase the oxidative stability of foods. Among tocopherol homologues, α-tocopherol has the highest vitamin E activity and occurs most abundantly in natural sources. Although α-tocopherol has been the center of vitamin E studies, γ- and δ-tocopherols are known to have stronger antioxidant activity *in vitro*. Generally, the

antioxidant activity of tocopherols is in the order of δ- > γ- > β- > α-tocopherol (Shahidi, 2000). Results from *in vitro* studies have demonstrated that γ-tocopherol is 1.4 times as efficient as α-tocopherol in inhibiting oxidation of polyunsaturated fatty acids (Wu *et al.*, 1979). Nevertheless, α-tocopherol serves as a more powerful antioxidant than γ-tocopherol *in vivo*.

During processing of oils and in the production of structured and specialty lipids many of the minor constituents are eliminated, leading to faster oxidative deterioration of the modified oils. In enzymic acidolysis of algal oils with capric acid, we found that the resultant oils were less stable than their unmodified counterparts despite an increase in the degree of saturation of the products due to the incorporation of capric acid in the glycerol backbone of the molecules (Hamam and Shahidi, 2004 a, b, 2005). Senanayake and Shahidi (2002) reported that removal of the minor components, such as tocopherols, during modification of borage and evening primrose oils with n-3 fatty acids might play a significant role in the compromised oxidative stability of the resultant products as much lower amounts of tocopherols were present in the modified oils. Akoh and Moussata (2001) reported a considerable loss of tocopherols in fish-, and canola-based structured lipids (SL) containing caprylic acid. However, these studies did not investigate the exact mechanism by which the removal and loss of natural antioxidants during processing and synthesis of specialty lipids took place. In chapters 4-6, we found that EPA-, DPA, or DHA-modified Laurical oils were oxidized to a much higher level than the original, unmodified oil. The present results indicate that modification of Laurical oils with EPA, DPA, and DHA resulted in lower stability of products, due to the incorporation of highly oxidatively susceptible fatty acids, EPA, DPA, and DHA. Another possibility could be

the formation of tocopheryl esters from reaction of carboxylic acids in the medium and tocopherols present in the oils during the production process of SL, hence loss of natural antioxidants.

This study aimed to investigate reasons behind compromised stability of stuctured lipids during acidolysis reaction, particularly the formation of tocopheryl esters from the reaction of free carboxylic acids in the medium and tocopherols present in the oils. Model systems were also employed to demonstrate this viewpoint.

9.2 Materials and methods

9.2.1 Materials

Pseudomonas sp. (PS-30) was obtained from Amano Enzyme (Troy, VA). Oleic acid was purchased from Nu-Check (Elysian, MN). Tocopherols (α-, γ-, and δ- tocopherols) were purchased from Sigma Chemical Company (St. Louis, MO). Eicosapentaenoic acid (EPA, >99% pure), was from Fuso Pharmaceutical Industries Ltdm (Osaka, Japan) and kindly provided by Dr. K. Miyashita. Samples of high-laurate canola oil, commerically known as Laurical 15, 25, and 35, were products of Calgene's Oils Division (Davis, CA) and were provided by Dr. James Daun from Canadian Grain Commission (Winnipeg, MB). Mixed tocopherols, used as standards containing α-, 94.0 mg/g; β, 11.0 mg/g; γ-, 441.0 mg/g, and δ-, 167.0 mg/g were acquired from Archer Daniels Midland Company, Decatur, IL.

All solvents used in these experiments were of analytical grade and were purchased from Fisher Scientific (Nepean, ON).

9.2.2 Methods

9.2.2.1 Acidolysis reactions

In general, oleic acid (100 mg) was mixed with α-, γ-, and δ- tocopherols at a mole ratio of oleic acid to tocopherol of 1:1, in a screw-capped test tube, and then 10% by weight of substrates of the most effective lipase (PS-30 from *Pseudomonas sp.*) and water (2% by weight of substrates and enzyme) were added in n-hexane (3.0 mL). The mixture was flushed with a stream of nitrogen, stirred, and then incubated for 48 h in an orbital shaker at 250 rpm at 45°C. In another set of experiments, high-laurate canola oil, namely Laurical 15, 25, and 35 were mixed with EPA at a mole ratio of 1:3 (oil/EPA), at 45°C, over 24 h in the presence of 4% *Pseudomonas sp.* and 2% water content. The mixture was flushed with a stream of nitrogen, stirred, and then incubated for 24 h in an orbital shaker at 250 rpm at 45°C. Laurical 35-based structured lipids were produced from the reaction of Laurical 35 and EPA, DPA, and DHA at a mole ratio of 1:3 (Laurical 35/EPA, DPA, and DHA), in the presence of 4% lipases from *Pseudomonas sp.* and 2% water. The mixture was flushed with a stream of nitrogen, stirred, and then incubated in an orbital shaker at 250 rpm at 45°C, over 24 h.

9.2.2.2 Separation of tocopheryl esters after acidolysis.

After a given time period the reaction was stopped by the addition of a mixture of acetone and ethanol (20 mL, 1:1, v/v). In order to neutralize the released and unused free fatty acids, the reaction mixture was titrated with a 0.5 M NaOH solution (using a phenolphthalein indicator) until the colour of the solution turned pink. The tocopheryl esters were then extracted into n-hexane (25 mL). The two layers (aqueous, n-hexane) were allowed to separate in a separatory funnel, and the lower aqueous layer was

discarded. The n-hexane layer was passed through a bed of anhydrous sodium sulphate to remove any residual water. The solvent was evaporated using a rotary evaporator at 45°C and the residue stored at -20°C until further analysis.

9.2.2.3 Synthesis of α-tocopheryl ester. Acyl chloride was synthesized according to the method described by Taylor *et al.* (1988) with some modifications (Figure 9.1). A 50 mL, three-necked flask equipped with a dropping funnel and a reflux condenser fitted with an inert gas (nitrogen) inlet tube that was attached to a mineral oil bubbler was used. The system was flushed with nitrogen, flame dried, cooled to room temperature, and maintained under a positive nitrogen pressure. The mixture contained oleic acid (1.0g), and 0.422g α-tocopherol. Distilled thionyl chloride ($SOCl_2$) (0.257 mL, 4.0 mmol) was added dropwise over a 30 min period. Pyridine (5-10 mL) was added to the mixture. The mixture was heated under reflux condition for 7 h. The resultant tocopheryl esters were extracted three times, each with diethyl ether (20-25 mL). The mixture was thoroughly mixed and transferred into a separatory funnel. The two layers (aqueous and diethyl ether) were allowed to separate, and the lower aqueous layer was discarded. The diethyl ether layer was then passed through a bed of anhydrous sodium sulphate to remove any residual water. The diethyl ether was evaporated using a rotary evaporator at 45°C and the residue was recovered.

Silica gel TLC plates were evenly sprayed with 5% (w/v) boric acid and dried at 100°C for 1 h. The mixture was separated on activated TLC plates which were then developed using hexane/diethyl ether/acetic acid (70:30:1, v/v/v) for 40-50 min and then allowed to air dry. The bands were located by viewing under a short- (254 nm) and a long- (365 nm) wavelength light (Spectroline, Co., Westbury, NY). The bands were scraped off and

extracted with methanol/chloroform (1:1, v/v). The solvent (methanol/chloroform) was evaporated using a rotary evaporator at 45°C and the residue was recovered and stored at -20 °C until used for further analysis as a standard.

9.2.2.4 Normal phase HPLC-MS analysis of tocopherols

Tocopherol standards were prepared by dissolving a certain amount of known standards in hexane and then diluting into desired concentrations (0.1, 0.5, 1.0, and 5.0 mg/mL). Both sample and standard solutions were filtered using a 0.45 μm syringe-filter and then used for HPLC analysis. Care was taken throughout the procedure to avoid exposure of tocopherols to light. Tocopherol presence as well as tocopheryl esters in samples were determined by normal phase high performance liquid chromatography (HPLC)-mass spectrometry (MS). The analysis was performed using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA) with a UV-diode array detector (UV-DAD). Separation was achieved on a Supelcosil LC-Si column (250 mm length, 4.6 mm i.d., 5 μm particle size, Sigma-Aldrich Canada Ltd., Oakville, ON) coupled with a Supelcosil LC-Si guard column. Tocopherols and tocopheryl esters were eluted using an isocratic solvent system containing hexane/2-propanol (95:5, v/v) at a flow rate of 1.0 mL/min. Fifty microliters of each sample were injected. Tocopherols were detected at 290 nm by UV detection. LC flow was analyzed on-line by a mass selective detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA) with a positive ion APCI (atmospheric pressure chemical ionization). The operating conditions used were 121V for the fragmentor voltage, 350°C for drying temperature, 400°C for APCI temperature, 60 psi for the nebulizer pressure, and 7 L/min for the drying gas flow.

9.3 Results and discussion

It was thought that tocopherols present in the oils might react with carboxylic acids in the reaction medium during acidolysis reaction, thus leading to the formation of tocopheryl esters that are not analyzed as free tocopherols and do not render any stability to the resultant modified oils. In order to examine this possibility, α-tocopheryl oleate was synthesized through the reaction of oleic acid and α-tocopherol following the formation of the corresponding acyl chloride in the presence of thionyl chloride and pyridine. The mass spectrum obtained from the mixture, using normal phase HPLC-MS, showed the presence of a peak of α-tocopheryl oleate with 695.5 m/z (Figure 9.2). In another set of experiments, reaction products of tocopheryl esters from model systems that used oleic acid and α-, γ-, and δ-tocopherols in the presence of enzymes were subjected to HPLC-MS analysis. Results obtained showed the presence of the corrresponding esters as evidenced by 695.5, 681.3, and 667.4 m/z data, respectively, for each isolated compound (Figures 9.3-9.5).

The third step was designed to detect the molecular species of tocopheryl esters in a real acidolysis reaction system involving Laurical oils (Laurical 15, 25, and 35) and EPA.

The main tocopherols, as determined by HPLC, are summarized in Table 9.1. Laurical 35 had the highest total tocopherols (250 ppm) compared to Laurical 25 (193 ppm) and Laurical 15 (186 ppm). Two hundred and five parts per million of α-tocopherol, and 45 ppm of γ-tocopherol were detected in Laurical 35; corresponding amounts in Laurical 25 were 165, and 28, respectively. Meanwhile, Laurical 15 contained 160 ppm of α-tocopherol and 26 ppm of γ- tocopherol.

The mass spectrum obtained from HPLC-MS analysis of tocopheryl esters of the modified Laurical 15, 25, or 35 with EPA are shown in Figures 9.4 and 9.5; major peaks are specified and summarized in Table 9.2. A number of tocopheryl esters were identified in the reaction products and these included those of lauric acid esterified to tocopherols, namely α-tocopheryl laurate, 599 m/z, and γ-tocopheryl laurate, 613.1 m/z; myristic acid and tocopherols, notably α-tocopheryl myristate, 641.1 m/z; and γ-tocopheryl myristate, 627.1 m/z; as well as palmitic acid and tocopherols homologues, namely α-tocopheryl palmitate, 669.1 m/z; and γ- tocopheryl palmitate, 655.1 m/z. The mixture also contained different species of tocopheryl oleates; these were α-tocopheryl oleate, 695.5 m/z; and γ-tocopheryl oleate, 681.1 m/z. Esters produced from reactions of EPA and different tocopherols were also detected, namely α-tocopheryl eicosapentaenoate, 715.3 m/z; and γ-tocopheryl eicosapentaenoate, 701.3 m/z. Furthermore, modified Laurical 35 contained various species of tocopheryl linoleate; these were α-tocopheryl linoleate, 692.8 m/z; and γ-tocopheryl linoleate, 679.2 m/z. Thus, many species of tocopheryl esters were present in the modified Laurical oils with EPA. This confirms the assumption that formation of tocopheryl esters from the reaction of carboxylic acids and tocopherols present in the oils is a possible route by which endogenous tocopherols are removed from the oils and a contributing factor responsible for rapid oxidation of structured lipids.

9.4 Conclusions

For the first time, a possible mechanism for the removal of endogenous antioxidants through the formation of tocopheryl esters during acidolysis reactions is proposed and confirmed. Tocopherols in the oils were found to react with carboxylic acids present in

the medium, thus leading to the formation of tocopheryl esters that do not render any stability to the resultant modified oils. Tocopheryl oleate, used as a standard, was synthesized through the reaction of acyl chloride of oleic acid with α-tocopherol (695.5 m/z as evidenced by mass spectrometry). Subsequently, lipase-assisted esterification of α-, γ-, and δ-tocopherols with oleic acid was carried out and corresponding tocopheryl esters were isolated. In a real acidolysis reaction system involving Laurical oils and EPA, HPLC-MS analysis demonstrated the presence of tocopheryl esters corresponding to the dominant fatty acids and both α- and γ-tocopherols.

Table 9.1 Tocopherol contents (mg/kg oil) of Laurical oils (Laurical 15, 25, and 35)

Tocopherol	Laurical 15	Laurical 25	Laurical 35
α	160	165	205
γ	26	28	45
δ	ND	ND	ND
Total	186	193	250

Table 9.2 Major tocopheryl esters identified in the acidolysis reaction of modified high laurate canola oils (Laurical 15, 25, and 35) with eicosapentaenoic acid (EPA)

Tocoheryl ester	(m/z)	Laurical15	Laurical25	Laurical25
α- tocopheryl laurate	613.1	+	+	+
γ- tocopheryl laurate	599.0	+	+	+
α- tocopheryl myristate	641.5	+	+	+
γ- tocopheryl myristate	627.5	+	+	+
α- tocopheryl palmitate	669.1	+	+	+
γ- tocopheryl palmitate	655.1	+	+	+
α- tocopheryl oleate	695.5	+	+	+
γ- tocopheryl oleate	681.2	+	+	+
α- tocopheryl linoleate	692.8	–	–	+
γ- tocopheryl linoleate	679.2	–	–	+
α- tocopheryl eicosapentaenoate	715.3	+	+	+
γ- tocopheryl eicosapentaenoate	701.3	+	+	+

Symbols are: LAA, lauric acid; MA, myristic acid; PA, palmitic acid; OA, oleic acid; LA, linoleic acid; EPA, eicosapentaenoic acid; +, tocopheryl ester present; –, tocopheryl ester absent; m/z, mass to charge ratio.

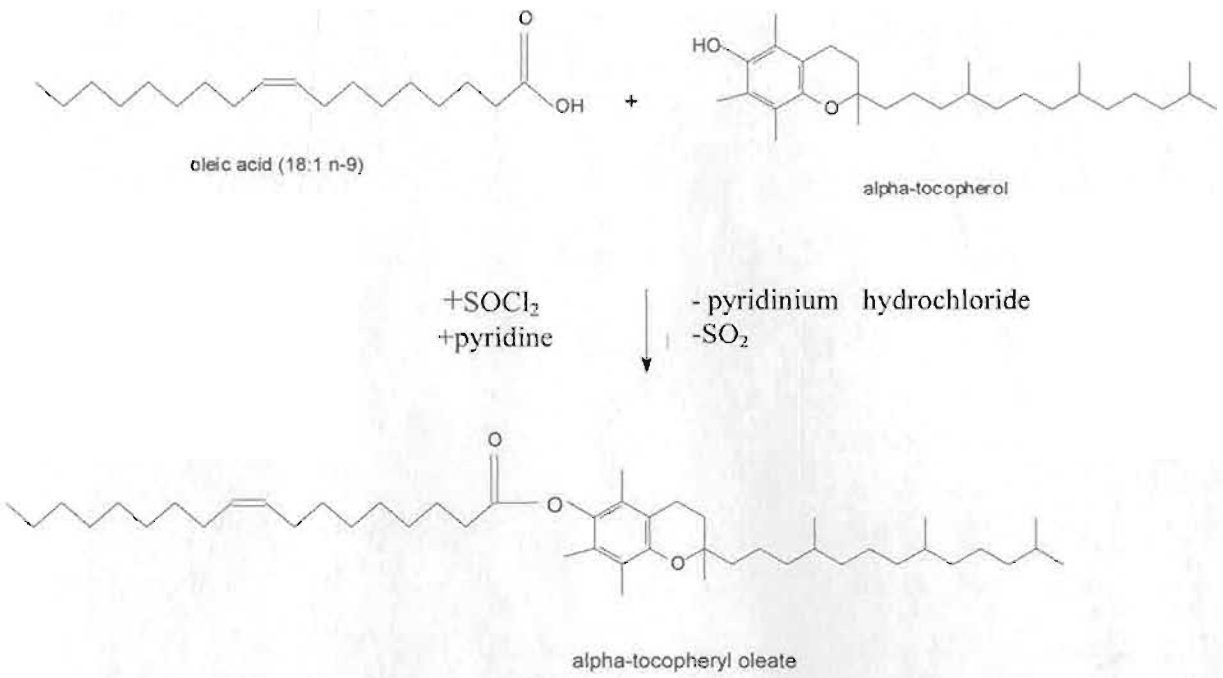

Figure 9.1 Synthesis of α- tocopheryl oleate from reaction of oleic acid and α-tocopherol.

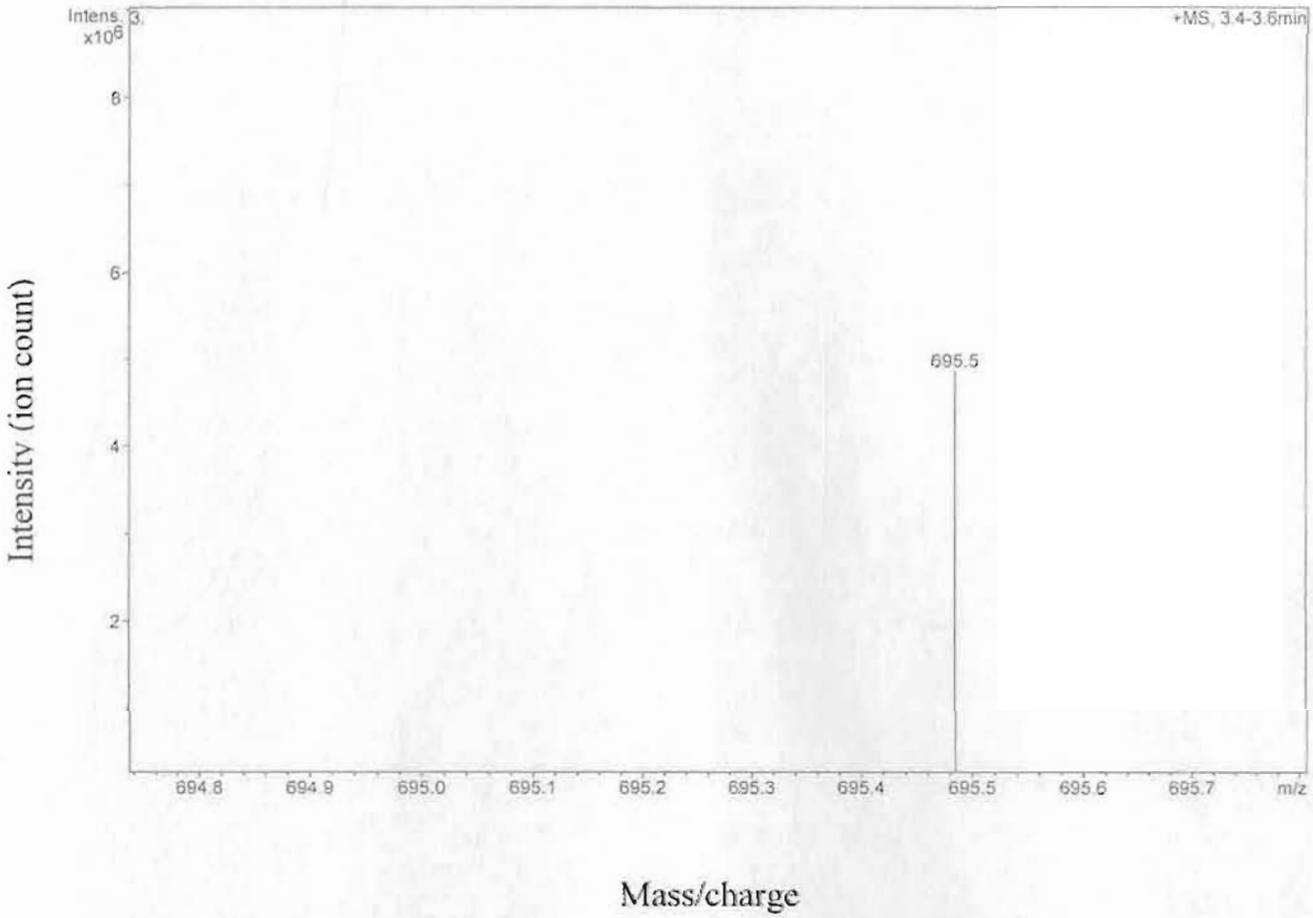

Figure 9.2 Mass spectrum obtained from HPLC-MS analysis of synthetic α-tocopheryl oleate from reactions of oleic acid and α-tocopherol.

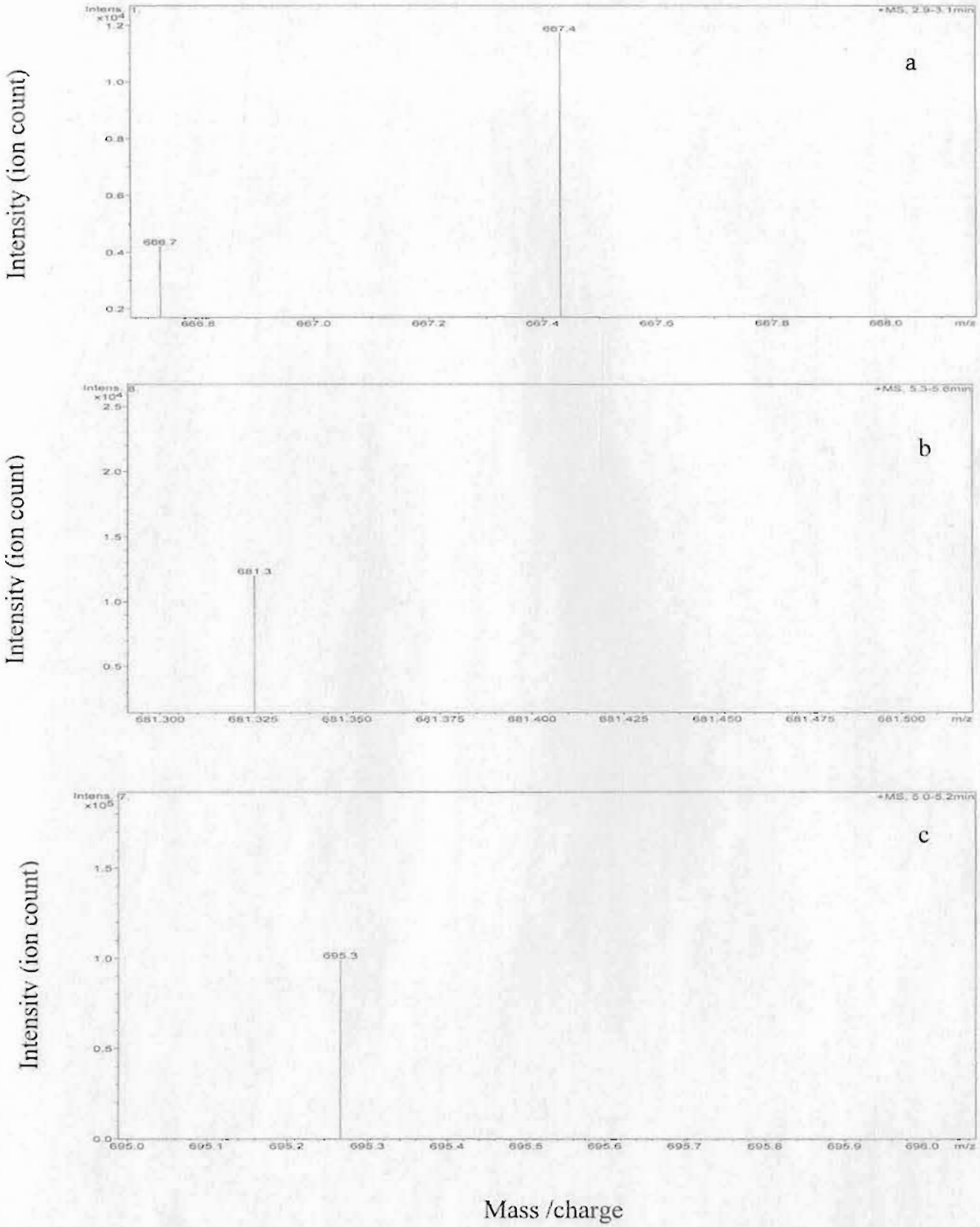

Figure 9.3 Mass spectrum obtained from HPLC-MS analysis of (a) δ-, (b) γ-, and (c) α-tocopheryl oleate from reactions of oleic acid and α-, γ-, and δ-tocopherols in the presence of lipase PS-30 from *Pseudomonas sp.*

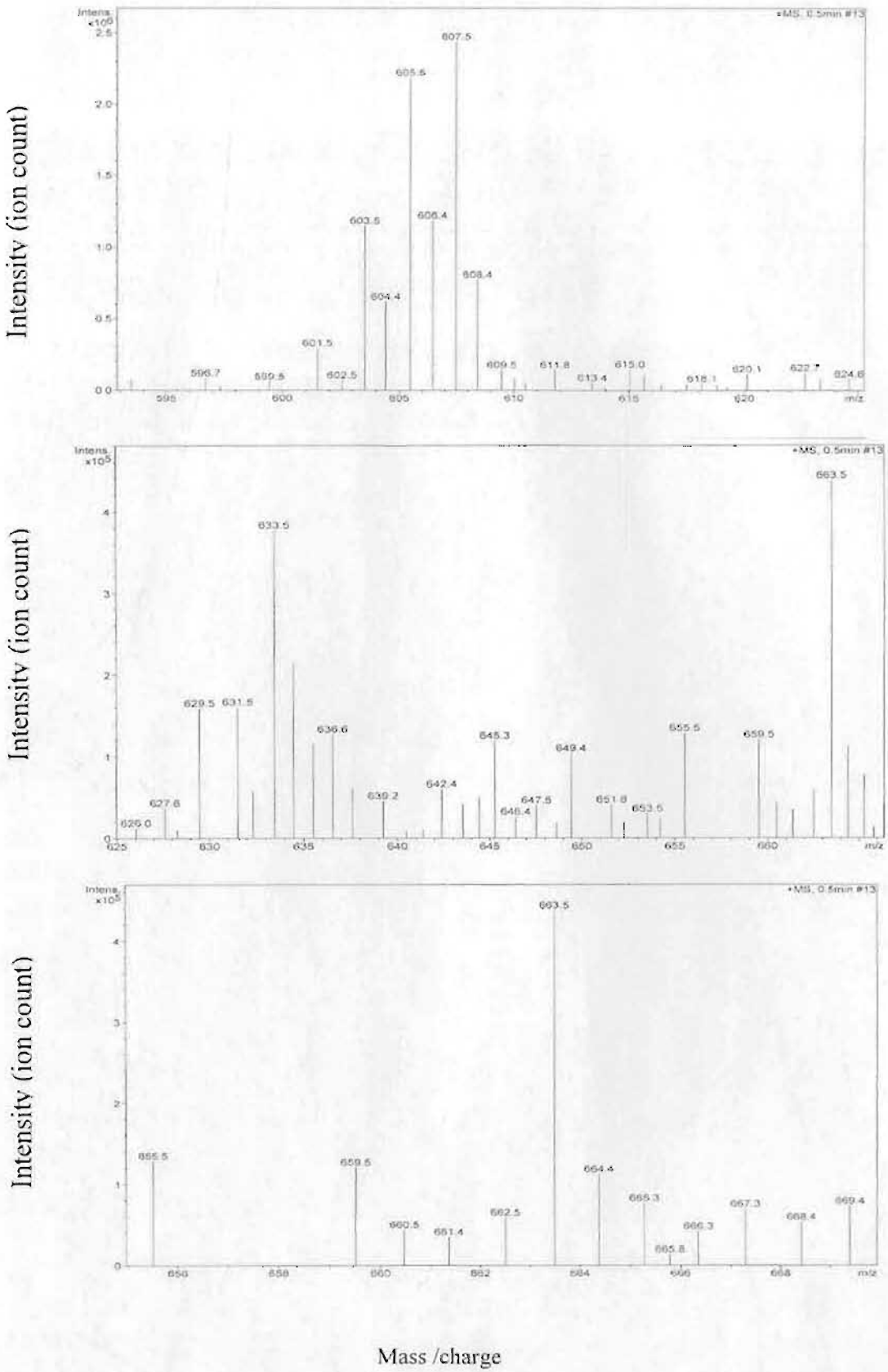

Figure 9.4 Mass spectrum obtained from HPLC-MS analysis of tocopheryl esters present in Laurical 35-based structured lipids.

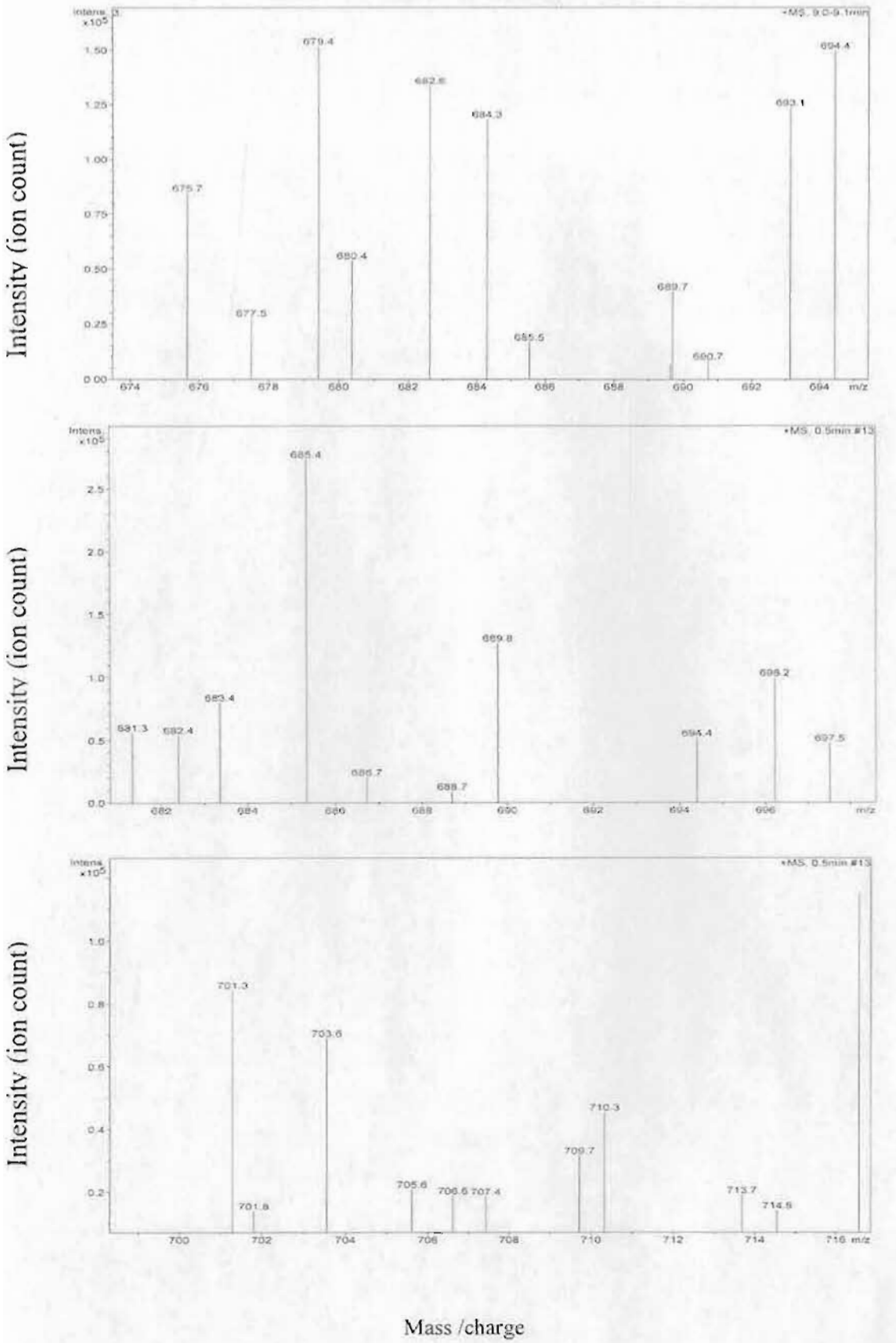

Figure 9.5 Mass spectrum obtained from HPLC-MS analysis of tocopheryl esters present in Laurical 35-based structured lipids.

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Chapter 10

Conclusions and recommendations for future studies

There were three objectives to the work reported in this thesis and these are summarized in three parts. The first part was the incorporation of n-3 fatty acids (FA), namely eicosapentaenoic acid (EPA), DPA (docosapentaenoic acid), and docosahexaenoic acid (DHA) into three types of high-laurate canola oils, namely Laurical 15, 25, and 35. Production of structured lipids via acidolysis of Laurical 15 with EPA, DPA, and DHA was carried out using five lipases, namely *Candida antarctica*, *Mucor miehei*, *Pseudomonas sp.*, *Aspergillus niger*, and *Candida rugosa*. *Pseudomonas sp.* gave best incorporation of EPA, DPA, or DHA into Laurical 15. Optimum reaction conditions for EPA incorporation into Laurical 15 were 4% lipase, at an oil to EPA mole ratio of 1:3 at 45°C over 36 h. For DPA incorporation into Laurical 15, the optimum conditions were 6% lipase, at an oil to DPA mole ratio of 1:2 at 35°C over 48 h. Similarly, incorporation of DHA into Laurical 15 was better achieved at an oil to DHA mole ratio of 1:3, 10% lipase concentration, at 35°C over 48 h. Lauric acid remained esterified mainly to the *sn*-1,3 positions of the triacylglycerol (TAG) molecules while EPA, DPA or DHA was also located primarily in the *sn*-1,3 positions of modified oils. The modified oils were more prone to oxidation than their unmodified counterpart, albeit to different extents as evidenced by the 2-thiobarbituric acid reactive substances (TBARS). Therefore, further studies should be conducted to stabilize the resultant modified oils by addition of appropriate antioxidants.

In another study we used response surface methodology (RSM) to achieve a maximum incorporation of EPA or DHA into Laurical 25. Under optimum conditions

incorporation of EPA (61.6%) into Laurical 25 was obtained using 4.6% enzyme, reaction temperature of 39.9°C, over a period of 26.2 h. The corresponding maximum incorporation of DHA into Laurical 25 was 37.3% using 4.79% enzyme, 46.1°C, and 30.1 h. Thus, the number of double bonds and the chain length of fatty acids, as well as specificity of the enzymes used might lead to variation in their reactivity. For EPA-modified Laurical 25, lauric acid was present mainly in the *sn*-1,3 positions while EPA was randomly distributed over the three positions. Similarly, DHA was primarily esterified at the *sn*-1,3 positions of the TAG molecules, and lauric acid also remained primarily to the *sn*-1,3 positions of the modified oil. The oxidative stability of the modified oil in comparison with the original unmodified oil, as indicated by the conjugated diene (CD) values, showed that the unmodified oil remained unchanged during storage for 72 h, but EPA- or DHA-modified Laurical 25 SL were oxidized to a much higher level than the original oil. The values of CD were higher in the modified oil with DHA than EPA-modified oil. The modified oil also attained considerably higher TBARS values than the original oil over the entire storage period.

Incorporation of EPA, DPA or DHA into Laurical 35 was also attempted. Lipases from *Mucor miehei, Pseudomonas sp.*, and *Candida rugosa* catalyzed best incorporation of DPA, DHA, and EPA into Laurical 35, respectively. RSM showed that incorporation of n-3 FA into Laurical 35 was in the order of EPA>DPA>DHA, reflecting the influence of the number of double bonds and chain length in their incorporation into the TAG molecules. In the modified oils, EPA and DHA were mainly esterified to the *sn*-1 and *sn*-3 positions, while DPA was randomly distributed over the three positions of the triacylglycerols. Again, lauric acid remained esterified, primarily to the *sn*-1,3 positions

of the modified oil and the modified oils were highly prone to oxidation as evidenced by higher CD and TBARS values when compared to their unmodified counterpart.

The second part was designed to incorporate selected long-chain fatty acids (LCFA) into triacylglcerols, such as tristearin, triolein, trilinolein, and trilinolinin. In general, incorporation of LCFA into tristearin may be affected by chain length, number of double bonds, the location and geometry of the double bonds, as well as reaction conditions, and reactivity and specificity of lipases used. Linoleic acid was more reactive than conjugated linoleic acid due to the rigidity of the latter. EPA was more reactive than DHA, possibly due to the structural differences between them (number of double bonds and chain length) together with specificity of the enzymes for different FA. The high percent incorporation of FA into tristearin using lipase from *Candida antarctica* or *Candida rugosa* might be due to the experimental conditions employed in this study which were suitable for these two enzymes. Thus, further studies should be conducted to verify the optimum conditions for each enzyme examined, perhaps using response surface methodology.

The results of this study demonstrated that incorporation of CLA into glycerol backbone of oils (tristearin, triolein, trilinolein, and trilinolinin) was more difficult than that of LA, probably due to the rigid structure, and the extended shape of the CLA. In general, ALA was more easily incorporated into the oils examined than GLA. The structural differences between ALA and GLA related to the location of double bonds might lead to variation in their reactivity together with specificity of the enzymes for different fatty acids. Therefore, further research should be carried out to investigate this assumption. EPA was more reactive than arachidonic acid (AA) when the lipases tested in

this study for acidolysis reactions of triolein (tri C18:1) and C20 FA. The exact reason behind the higher reactivity of EPA compared to that of AA needs further investigation. DPA was more reactive than DHA except for the reaction catalyzed by *Pseudomonas sp.* Reasons behind higher incorporation of DHA into triolein, trilinolein or trilinolinin using *Pseudomonas sp.* are unclear. Therefore, further research should be conducted to verify this assumption.

When the mole ratio of substrates increased from 1:1 to 1:3, incorporation of n-3 FA into tristearin or triolein increased accordingly. In general, the highest incorporation of n-3 FA into tristearin or triolein was obtained at an oil to n-3 FA mole ratio of 1:3. The same mole ratio effect was observed for acidolysis reactions of n-6 FA and triolein. As the mole ratio of triolein to C18 FA changed from 1:1 to 1:3, incorporation of C18 FA into triolein increased accordingly for the reaction catalyzed by *Mucor miehei.* In contrast, incorporation of C18 FA into tri C18:1 decreased when the mole ratio of triolein to C18 FA was altered from 1:1 to 1:3 when the remaining lipases were used. Similarly, as the mole ratio of tristearin to C18 FA changed from 1:1 to 1:3, the incorporation of OA and LA increased for the reactions catalyzed by *Pseudomonas sp.*, *Mucor miehei,* and *Candida antarctica.* Further studies should be carried out at higher mole ratios of 1:6, 1:9, and 1:12 (tristearin or triolein: C18, n-3, or n-6 FA). Further research should also be performed to investigate reasons behind low percent incorporation of a mixture of C18 FA into triolein at 1:3 mole ratio of triolein to C18 FA.

As the number of double bonds increased from zero in tristearin (tri C18:0) to nine in trilinolinin (tri C18:3), the degree of incorporation of ALA or EPA or DHA into tristearin, triolein, and trilinolein decreased. Therefore, this study showed that the number

of double bonds in TAG molecules had a clear impact on the incorporation of n-3 PUFA into the oils examined.

The oxidative stability of the modified triolein in comparison with the original oil, as indicated in both CD and TBARS values, showed that the unmodified oil remained unchanged during storage for 72 h. The modified oils with n-3 FA were more susceptible to oxidation than the oils modified with n-6 FA, when considering both CD and TBARS values.

The third part highlights reasons behind compromised stability of the SL so produced. For the first time, the removal of endogenous antioxidants from modified oil, at least partially, was demonstrated to occur through the formation of tocopheryl esters during the acidolysis reaction. Tocopherols in the oils were found to react with carboxylic acids present in the medium, thus leading to the formation of tocopheryl esters that do not render any stability to the resultant modified oils as they lack any free hydroxyl groups on the phenolic ring of the molecule. In a real acidolysis reaction system involving Laurical oils and EPA, HPLC-MS analysis demonstrated the presence of tocopheryl esters corresponding to the dominant FA and both α- and γ-tocopherols. Compromised stability of the structured lipids could then be explained on a molecular basis because tocopheryl esters so produced do not exhibit any antioxidant activity *in vitro*. However, these esters could resume their antioxidant activity *in vivo* upon hydrolysis by different lipases. Thus, tocopheryl esters present in the system are expected to render their beneficial effects in the body upon consumption of such preparations.

Despite their health benefits, as expected, SL containing PUFA are highly prone to oxidative deterioration and thus require adequate protection to deter their oxidation.

Therefore, further research is needed to optimize their stabilization and storage by use of appropriate antioxidants and packaging technologies. Incorporation of SL containing n-3 PUFA into foods needs to be justified using evidence collected from animal studies and clinical trials. Therefore, future research should focus on the metabolism and medicinal importance and economic feasibility of large-scale production of SL containing MCFA and n-3 FA, among others.