PRODUCTION OF ACYLGLYCEROLS CONTAINING HIGH PROPORTIONS OF DOCOSAPENTAENOIC ACID FROM SEAL BLUBBER OIL AND THEIR OXIDATIVE STABILITY

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Production of Acylglycerols Containing High Proportions of Docosapentaenoic Acid from Seal Blubber Oil and Their Oxidative Stability

BY

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Abstract

The objective of this study was to concentrate polyunsaturated fatty acids (PUFA) from seal blubber oil and to investigate the use of lipases as catalysts for synthesizing acylglycerols from glycerol and polyunsaturated fatty acid concentrates. Additionally, the study of the oxidative stability of acylglycerols synthesized by lipases was intended. Urea complexation was used to concentrate polyunsaturated fatty acids from seal blubber oil. The concentration of docosapentaenoic acid in final products reached as high as 24%. Enzymatic synthesis of acyglycerols directly from glycerol and fatty acid concentrates in organic solvents was studied. Three lipases (Lipase SP435 from Candida Antactica, lipase IM60 from *Mucor miehei,* lipase PS30 from *Pseudomonas sp.*) were used as biocatalysts for esterification. Lipase SP435 from *Candida Antarctica* showed the highest activity for esterification. Effects of reaction parameters, namely temperature, time course and mole ratio of glycerol to fatty acids were followed with all three lipases. The optimal reaction time was 24 h at 30 °C and the mole ratio of glycerol to fatty acid was 14 for lipase SP435. The maximum degree of acylglycerol synthesis exceeded 90%. The effect of time course and mole ratio of glycerol to fatty acids on acylglycerols distribution was also determined. The proportion of monoacylglycerols in samples was low and remained almost constant as the reaction proceeded. However, the proportion of diacylglycerol was increased rapidly in a short time period and then decreased gradually to some extent, depending on the lipase, and then plateaued. The proportion of triacylglycerol was increased gradually to some extent depending on the lipase, than it was maintained constant. The effect of mole ratio of glycerol to fatty acids on the

acylglycerols distribution was studied. The higher mole ratio of glycerol to fatty acids, the higher was the proportion of monoacylglycerols and the lower the proportion of triacylglycerols. The oxidative stability of different samples was tested under Schaal-oven conditions. Progression of oxidation was monitored by determination of conjugated dienes (CD), propanal content and TBARS (thiobarbituric acid reactive substances) values. In this study, three samples were used – original seal blubber oil, and concentrate of fatty acid esters and triacylglycerols synthesized by lipase SP435. The results demonstrated that the oxidative stability of synthesized acylglycerols was superior to that of the fatty acid esters. At the beginning, the CD and TBARS values of the three samples were similar. However, fatty acid esters gave rise to higher CD and TBARS values compared to the other two samples as oxidation reaction proceeded.

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List of Abbreviations

α-linolenic acid (ALA)

Arachidonic acid (AA)

Conjugaged diene(CD)

Diacylglycerol (DAG)

Docosahexaenoic acid (DHA)

Docosapentaenoic acid (DPA)

Eicosapentaenoic acid (EPA)

Free fatty acid (FFA)

Gas chromatography

Highly unsaturated fatty acids (HUFA)

Linolic acid (LA)

long chain fatty acid (LCFA)

Monoacylglycerol (MAG)

Phosphatidylcholine (PC)

Phosphatidylethanolamine (PE)

Polyunsaturated fatty acid (PUFA)

Polyunsaturated fatty acids (PUFAs)

Peroxide value (PV)

Seal blubber oil (SBO)

Supercritical fluid extraction (SFE)

Thiobarbituric acid reactive substances (TBARS)

Triacylglycerol(TAG)

CHAPTER 1

INTRODUCTION

The ω-3 fatty acids, including eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), have several health benefits related to cardiovascular disease (Schmidt and Dyerberg, 1999), hypertension (Deferne and Leeds, 1992), autoimmune and renal disorders (Calder, 1999; Bechoua *et al.*, 1999), inflammation (Boissonneault and Hayek, 1992), allergies (Illingworth and Ullmann, 1990), diabetes (Bhathena, 1992) and cancer (Carroll, 1990; Haumann, 1997a). The ω3 fatty acids are suggested to participate in the biosynthesis of prostaglandins, thromboxanes and leukotrienes, which possess high physiological activity, thus influencing blood platelet aggregation and contraction and dialation of vascular glands. Arachidonic acid (AA)-stimulated blood platelet aggregation was inhibited by ω3 fatty acids in a dose-dependent manner, among which DPA was the most potent inhibitor (Kanayasu-Toyoda et al., 1996). However, very few studies have been carried out to examine different reaction, properties of health effects of DPA.

Marine oils, such as fish oil and seal oil, are important natural sources of ω3 fatty acids. However, the concentration of ω3 fatty acids in raw marine oils is not always high enough for therapeutic applications, especially for DPA, which is present in much lower concentration compared to that of EPA and DHA in marine oils. In order to obtain a high concentration of ω3 fatty acids from marine

oils, several procedures are generally used. Of the several practical methods available for concentration of PUFA from oils, urea complexation is one of the most efficient and simplest techniques used for preparation of polyunsaturated fatty acid (PUFA) concentrates from seal blubber oil (Shahidi and Wanasundara, 1998a). Most studies have been focused on concentrating EPA and DHA and no studies have yet been carried out on concentrating DPA from seal blubber oil.

It is generally accepted that the positional distribution of fatty acids within the triacylglycerol(TAG) (*sn*-1, *sn*-2 and *sn*-3) molecules might affect the metabolic fate of fatty acids. Hence, it is thought that knowledge of the stereospecific structure of PUFA-enriched triacylglycerols is important because it influences their absorption and assimilation in the body. Use of enzymes to produce triacylglycerols has an advantage over the traditional methods. For example, chemical interesterification involves use of high temperature which may partially destroy the natural all-*cis* ω3 PUFA *via* oxidation and result in *cis-trans* isomerization. Therefore, the mild conditions used in enzymatic reactions provide a promising alternative that could also save energy and increase product selectivity.

Despite their health benefits, oils containing highly unsaturated fatty acids (HUFA) are susceptible to rapid oxidative deterioration and thus experience stability problems. It is important to prevent oxidation of such oils in order to maintain their quality and safety. Oxidation of fats and oils may be initiated by light, heat and presence of metal ions *via* a free-radical chain reaction involving

initiation, propagation and termination steps (Shahidi and Wanasundara, 1996; 1997). Oxidative deterioration of edible oils involves autoxidation accompanied by various reactions having oxidative and nonoxidative characteristics (Gray, 1978). Hydroperoxides are the primary products of autoxidation which in themselves are tasteless and odorless. Their decomposition, however, leads to the formation of a variety of volatile compounds which result in the development of undesirable flavors and off-odors (Frankel, 1987). Oxidized fats and oils have also been reported to cause biological problems, such as diarrhea, growth depression and tissue damage in living organisms (Chow, 1992).

In order to determine the oxidative state and quality of edible oils, a number of stability tests are routinely employed. Methods reported in the literature include chemical and instrumental techniques (Rossell, 1991; Shahidi and Wanasundara, 1998b). These methods detect either the primary or secondary products of lipid oxidation and have been found to correlate well with descriptive sensory analysis (Rossell, 1994). However, it is desirable to develop methods that could simultaneously determine both the primary and secondary products of lipid oxidation.

The objectives of this study were: (1) to concentrate DPA from seal blubber oil using the urea complexation technique, (2) to synthesize DPA- enriched triacylglycerols from seal blubber oil, (3) to optimize the reaction conditions for synthesis of DPA-enriched triacylglycerols, (4) to determine the positional distribution of fatty acids in the

DPA-enriched triacylglycerols, and (6) to assess the oxidative stability of DPA-enriched triacylglycerols.

CHAPTER 2

LITERATURE REVIEW

2.1 The Omega-3 fatty acids

Fatty acids perform a wide variety of metabolic functions which are critical to all forms of life. They are a rich source of energy and carbon, and well designed as a convenient unit of energy storage. They are also essential components of every living cell, being especially important for the integrity of bilayer structures of cell membranes. In addition, fatty acids serve as precursors for numerous biologically active compounds such as eicosanoids (Figure 2.1). Polyunsaturated fatty acids (PUFA) have two or more double bonds in their backbone structures. There are two groups of PUFA, the ω6 (or n-6) and the ω3 (or n-3) families defined by the position of the first double bond in the molecule starting from the methyl end of the chain (Holman, 1988). A simple shorthand notation is used to define PUFA structure. For example, DPA is 22:5ω3 (or 22:5n-3) (Figure 2.2), while EPA is 20:5ω3 (or 20:5n-3). The first number defines the number of carbon atoms in the chain while the second one after the colon specifies the number of double bonds. The ω3 fatty acids have been the subject of considerable nutritional studies as some of them are considered to be essential fatty acids (EFA) and must be provided in the food because they cannot

Figure 2.1 Formation of Eicosanoids from ω3 and ω6 PUFA; Their effects on platelet and blood vessels (*From Groom, 1993*).

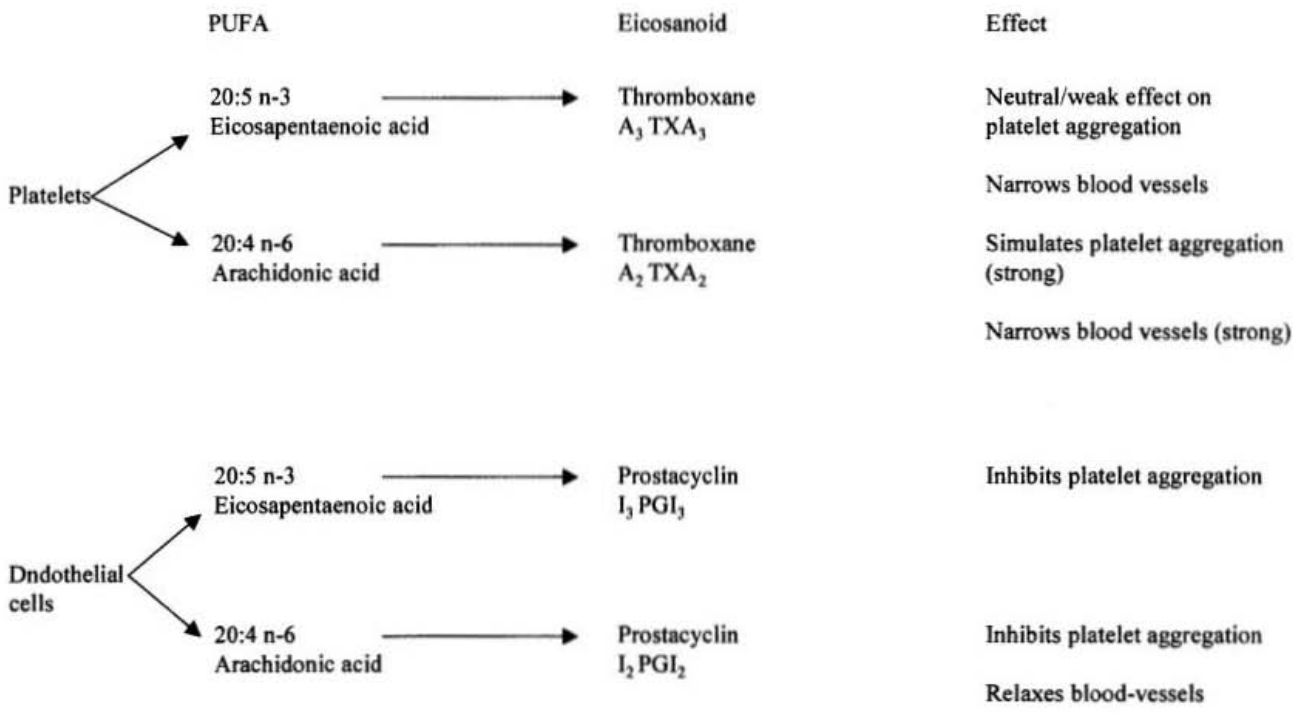
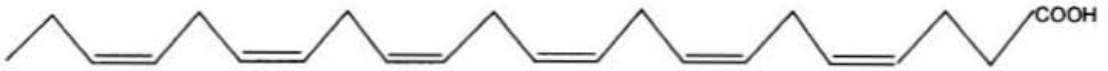

PUFA
Eicosanoid
Effect
20:5 n-3
Eicosapentaenoic acid
Thromboxane
A_3 TXA_3
Neutral/weak effect on platelet aggregation
Narrows blood vessels
Platelets
20:4 n-6
Arachidonic acid
Thromboxane
A_2 TXA_2
Simulates platelet aggregation (strong)
Narrows blood vessels (strong)
20:5 n-3
Eicosapentaenoic acid
Prostacyclin
I_3 PGI_3
Inhibits platelet aggregation
Dndothelial cells
20:4 n-6
Arachidonic acid
Prostacyclin
I_2 PGI_2
Inhibits platelet aggregation
Relaxes blood-vessels

Figure 2.2 The chemical structure of EPA, DPA and DHA.

Eicosapentaenoic acid (EPA 20:5 ω3)

Docosapentaenoic acid (DPA 22:5 ω3)



Docosahaxanenoic acid (DHA 22:6 ω3)

be easily manufactured within the body. In order to obtain ω3 and ω6 families of long chain fatty acids (LCFA), dietary source is important. Members of the ω6 family of LCFA are more prevalent in foods than the ω3 fatty acids. Examples of ω3 fatty acids include α-linolenic acid (ALA), EPA DPA and DHA. Sources of ω3 PUFA include seafood and certain plants. Marine oils contain moderately high levels of omega-3 fatty acids, namely EPA, DPA and DHA (Shahidi and Wanasundara, 1998a), while plant-based ω3 fatty acids are present in flaxseed, canola and soybean oils, in the form of α-linolenic acid (ALA) (Beare-Rogers, 1988; Rice, 1991).

2.1.1 Metabolism of PUFA

The parent compounds of the ω6 and ω3 series are linoleic acid (LA) (18:2ω6) and α-linolenic acid (ALA) (18:3ω3), respectively. LA and ALA are metabolized by a series of alternating desaturation (in which a further double bond is introduced) and elongation (in which two carbon atoms are added) steps. `The enzyme systems involved in the metabolism of the two types of PUFA are identical and both ω3 and ω6 PUFA compete with one another in the metabolic pathway (Sprecher, 1982). On the whole the enzyme systems seem to have a higher affinity for the ω3 PUFA so that, other things being equal, the ω3 PUFA will be preferentially metabolized.

In the metabolism chain, the conversions among EPA, DPA and DHA need to be emphasized. EPA can be metabolised to DPA and DHA. However, there is evidence for retro-conversion of DHA to docosapentaenoic acid EPA and DPA in humans (Ackman and Ratnayake, 1989). After ingestion of ethyl ester of DHA, the content of both EPA and DHA in plasma phospholipids was increased, but DPA remained essentially unchanged (Kinsella, 1990). However, ingestion of ethyl ester DHA increased the levels of EPA, DPA and DHA in the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions of the platelets. Blood platelet aggregation was significantly decreased by ingestion of DHA, thus supporting the view that dietary ω3 PUFAs may alleviate certain forms of cardiovascular dysfunction.

2.1.2 The importance of ω3 PUFA

The importance of marine oils in human nutrition and disease prevention was scientifically recognized three decades ago. Epidemiological studies in the early 1970s postulated that the low incidence of coronary heart disease of Greenland Eskimos might be related to their distinctive dietary habit and use of marine lipids rich in PUFA (Dyerberg et al., 1975). A large number of studies have been carried out on the potential benefits of the ω3 fatty acids in human diseases since 1970's. The beneficial effects of PUFA have been ascribed to their ability to lower serum triacylglycerol and possibly cholesterol levels and enhance their

excretion, to increase membrane fluidity and by conversion to eicosanoids to reduce thrombosis.

Many of the physiological effects attributed to ω3 fatty acids relate to their role in eicosanoid production. Eicosanoids are short-lived, locally acting hormone-like substances and exert diverse actions on the cardiovascular, reproductive, respiratory, renal, endocrine, skin, nervous and immune systems. Eicosanoids include the prostanoids (prostaglandins, prostacyclins and thromboxanes), leukotrienes and hydroxyl fatty acids (Wardlaw, 1996). They are synthesized from 20-carbon PUFA with three, four or five double bonds of either the ω3 or the ω6 families.

Physiological effects of ω3 fatty acids have been documented in the areas of heart and circulatory diseases, immune response and cancer. The first category includes prevention or treatment of atherosclerosis, thrombosis, hyperglyceridemia and high blood pressure. The second area relates to the treatment of asthma, arthritis. The third category includes treatment of cancer of breast, prostate and colon (Shahidi, 1998c).

2.1.2.1 Cardiovascular disease

Cardiovascular disease is the leading cause of death in industrialized countries. Recent research indicates that long chain ω3 PUFA may be effective in reducing the clinical risk of cardiovascular disease by favorably altering lipid and

haemostatic factors such as bleeding time and platelet aggregation (Groom, 1993). Dietary supplementation of EPA, DHA and other ω3 PUFA has also been recommended to lower the risk of cardiovascular disease and to improve the overall health of human beings (Nettleton, 1994).

Thrombosis is the formation of blood clots. Blood clotting involves the clumping together of platelets into large aggregates and is triggered when endothelial cells lining the artery walls are damaged. If the platelet membranes are rich in long-chain ω3 PUFA, formation of certain eicosanoids such as prostacyclin I_3 and thromboxane A_3 is promoted. These do not trigger platelet aggregation as much as the corresponding eicosanoids, prostacyclin I_2 and thromboxane A_2 that are formed from ω6 PUFA. Therefore, long-chain ω3 PUFA may help to reduce the tendency for blood to clot and hence reducing the chance of heart attach.

2.1.2.2 Arthritis

Arthritis is a chronic inflammatory disease of the joints. Beneficial effects of diets high in ω3 and ω6 fatty acids have been reported in arthritic patients (Kremer et al., 1985, 1987, 1991). However, EPA increased the incidence of collagen-induced arthritis in mice (Prickett et al., 1984). In another study, arthritic patients showed significant improvement in morning stiffness and

number of tender joints when consuming EPA supplements compared to placebo in a double blinded, crossover study (Kremer et al., 1987).

2.1.2.3 Cancer

A lipid made from fish oil and medium chain TAG was found to decrease tumor growth in mice (Ling *et al.*, 1991). In another study, the tumor growth rate was slowed in rats fed with lipid containing medium chain fatty acids and fish oil. In contrast to tumor promoting effects of diets high in fat, diets high in fish oil failed to promote the development of tumors in rats (Braden and Carroll, 1986). Reddy and Maruyama (1986) also pointed out that diets containing high levels of fish oil inhibited or suppressed tumor growth in animal models. Dietary intake of fish oils was effective in destroying some cancer cells, but it is not known whether such results are reproducible with humans or potential side effects might exist. It is known that ω3 fatty acids play an important role in the growth of certain cells in the human body, but the mechanisms involved in their effect on cancer treatment remain somewhat elusive.

2.2 Production of ω3 PUFA

It has been suggested that PUFA concentrate is much better than marine oils themselves since it avoids intake of more saturated fatty acid. Therefore it allows keeping the daily intake of total lipids as low as possible (Hassgsma et al, 1982). Several methods are available for production of concentrates, including

adsorption chromatography, molecular distillation, enzymatic splitting, low-temperature crystallization, supercritical fluid extraction and urea complexation. Each technique has its own advantages and drawbacks (Shahidi and Wanasundara, 1998a).

2.2.1 Adsorption chromatography

High performance liquid chromatography (HPLC) and silver resin chromatography have been used for preparation of ω3 fatty acid concentrates. Breivik et al. (1997) have used a silver nitrate impregnated silica gel column to separate EPA and DHA from squid liver oil after their conversion to the corresponding methyl esters. These authors were able to isolate 85-96% EPA and DHA with a yield of 39 and 48%, respectively. However, the starting materials for these studies were PUFA concentrates. The other drawback is that a large amount of solvent is needed. HPLC is known to have speedy and relatively high resolution abilities in the separation of materials. Yamamura and Shimomura (1997) were able to purify DHA and DPA ethyl esters from a single cell oil into a 99% concentrate with 23.2 and 79.6% recovery, respectively, using an industrial HPLC procedure.

2.2.2 Distillation

Distillation has been used for partial separation of mixtures of fatty acid esters. This method takes advantage of differences in the boiling point and

molecular weight of fatty acids under reduced pressure. This is a rather old technique and requires high temperature of approximately 250°C. The most widely used distillation procedure is fractional distillation of methyl esters under reduced pressure. Even under these conditions, moderately high temperatures are required. However, fractionation of marine oil esters is difficult since separation of these components becomes less effective with increasing molecular weight and also exposure of long chain ω3 PUFA to high temperatures during distillation which may induce hydrolysis, thermal oxidation, polymerization and isomerization.

2.2.3 Low-temperature crystallization

Low temperature crystallization was originally developed to separate certain triacylglycerols (TAG), fatty acids, esters and other lipids which are highly soluble in organic solvents at temperatures above 0 °C, but become insoluble at temperatures down to -80 °C (Brown and Kolb, 1955). The solubility of fats in organic solvents decreases with increasing mean molecular weight and increases with increasing unsaturation (Chawla and Deman, 1990). Singletion (1960) and Stout et al. (1990) have determined the solubility of numerous fatty acids and their corresponding esters in a variety of solvents and revealed the following rules: when the acids are saturated, long chains are less soluble than short chains; saturated acids are less soluble than monoenoic and dienoic acids of equal chain length; trans isomers are less soluble than cis isomers, and normal acids are less

soluble than branched acids. For example, the melting points of fatty acids are dependent on their degree of unsaturation. EPA and DHA melt at –54 and -44.5°C compared to 13.4 and 69.6°C for 18:1 and 18:0, respectively (Merck Index, 1983). At low temperatures, long chain saturated fatty acids which have higher melting points crystallize out and PUFA remain in the liquid form. However, as the number and type of fatty acid components in the mixture increases, the crystallisation process becomes more complex and repeated crystallisation and separation of fractions may be necessary in order to obtain purified fractions. In the case of marine oils, not only there is a very wide spectrum of fatty acids but the fatty acids exist, not in the FFA form, but esterified in the TAG form. However, the principle of low temperature crystallisation can still be applied to marine oils to concentrate TAG rich in ω3 PUFA (Shahidi and Wanasundara, 1998a).

Preparation of ω3 PUFA concentrates from seal blubber oil (SBO) by low temperature fractional crystallization was recently reported (Wanasundara, 1996). At -60 and -70 °C in hexane, the total ω3 PUFA content reached up to 58.3 and 66.7% with recoveries of 39 and 24.8%, respectively. However, this method cannot adequately separate PUFA such as EPA, DPA and DHA. The recovery is also much lower than enzymatic methods and urea complexation procedures.

2.2.4 Urea complexation

The simplest and most efficient technique for obtaining ω3 PUFA concentrates in the form of free fatty acids is urea complexation. The readiness of straight-chain saturated fatty acids to form inclusion complexes with urea in comparison with PUFA is well established and conventional urea complexation techniques using ethanol or methanol as a solvent can be applied to the fatty acids of oils or their methyl or ethyl esters to produce a fraction rich in PUFA. Urea alone crystallizes in a tightly packed tetragonal structure with channels of 5.67 Å diameter (Figure 2.3). However, in the presence of long straight chain molecules it crystallizes in a hexagonal structure with channels of 8-12 Å diameter within the hexagonal crystals. The channels formed, in the presence of long chain unbranched molecules, are sufficiently large to accommodate aliphatic chains. While straight chain saturated fatty acids with six carbon atoms or more are readily adducted, the presence of double bonds in the carbon chain increases the bulk of the molecule and reduces the likelihood of its complexation with urea. The saturated and monounsaturated fatty acids easily complex with urea and crystallize out on cooling and may subsequently be removed by filtration. The carbon chains occupy the free space inside the hexagonal channels and are held *via* van der Waals forces, London dispersion forces or induced electrostatic attractions. The liquid or non-urea complexed fraction is enriched in ω3 PUFA. Urea complex formation of fatty acids has been extensively used for enriching

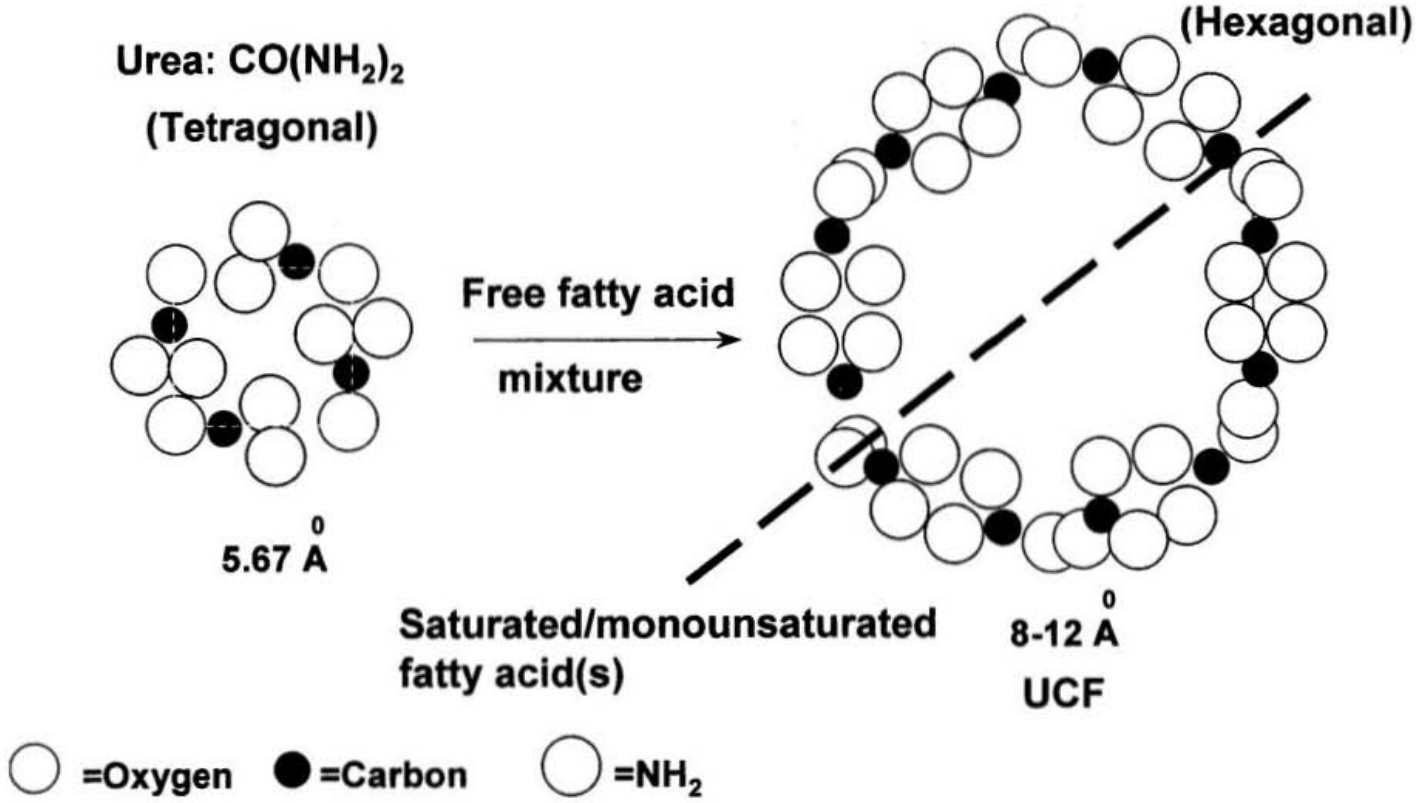
fish oils in ω3 PUFA (Ratnayake *et al.*, 1988; Breivik *et al.*, 1997; Hayes *et al.*, 2000). Many publications have described the application of urea complexation to concentrate PUFA. Haagsma *et al.* (1982) described a urea complexation method for enriching the EPA and DHA levels of cod liver oil from 12 to 28% and 11 to 45%, respectively. Recently, this method was used to concentrate ω3 PUFA from SBO (Shahidi, et al., 1994; Wanasundara and Shahidi, 1999). Under optimum conditions, the maximum concentration of ω3 PUFAs (88.2%) was obtained. Among the major ω3 PUFA, more than 80% of DHA and EPA was recovered. Similarly, Senanayake and Shahidi (2001) were able to produce a 97% DHA concentrate from an algal oil using the urea complexation procedure. Urea complexation has the advantage that complexed crystals are extremely stable and filtration does not necessarily have to be carried out at the very low temperatures which solvent crystallization of fatty acids would require.

2.2.5 Supercritical fluid extraction (SFE)

Supercritical fluid extraction is a relatively novel technique, which has found use in the food and pharmaceutical applications. The process makes use of the fact that at a combined temperature and pressure above a critical point, a gas such as CO_2 has a liquid-like density and possesses a high solvation capacity (Shahidi and Wanasundara, 1998a). It is possible to vary the solvation capacity of the fluid and consequently achieve extraction and separation of solutes within a narrow

Figure 2.3 Tetragonal and hexagonal structures of urea molecules.

Urea Complexation



range of temperature and pressure. Physical principles underlying SFE technology have been reviewed (Mishra et al., 1993). This technology has been used in fish processing for extraction of oil rich in ω3 fatty acids from fish and seaweeds (Yamagouchi et al., 1986; Choi et al., 1987; Hardardottir and kinsella, 1988) and for concentration of ω3 fatty acids or their esters (Eisenbach, 1984; Krukonis, 1988; Nilsson et al., 1988, 1989; Rizvi et al., 1988). This method is mild and, because it uses CO_2, minimizes autoxidation. It separates fatty acids most effectively on the basis of chain length; hence the method works best for oils with low levels of long-chain fatty acids. Fish oils in the form of free fatty acids and fatty acid esters have been extracted with supercritical gaseous CO_2 to yield concentrates of EPA and DHA (Nilsson et al., 1988).

2.2.6 Lipase

For the concentration of PUFA on a large scale, each of the above physical and chemical separation methods has some disadvantages either in terms of low yield, a requirement for large volumes of solvent or sophisticated equipment, a risk of structural changes in the fatty acid product, or high operational costs. Lipases can catalyze esterification, hydrolysis or exchange of fatty acids in esters under mild conditions of temperature and pH (Gandhi, 1997). The direction and efficiency of the reaction can be influenced by the choice of experimental conditions, including the concentration of fatty acids, esters, water and alcohol. There are numerous reports in the literature involving biotransfromation of ω3

PUFAs from marine oil by lipases (Haraldsson and Kristinsson, 1998; Moore and McNeill, 1996). There are two approaches to concentrate the ω3 PUFAs from marine oils using lipases. The first method is based on the positional distribution of fatty acids in the marine oils. In many fish oils, the ω3 fatty acids are placed, primarily, in the 2-position of the triacylglycerol surrounded by saturated and monounsaturated acids (Haraldsson and Kristinsson, 1998). Using a 1,3-specific lipase, a specific fatty acid can be incorporated into the outer positions (Sn-1 and Sn-3) without changing the fatty acid residues in Sn-2 position by a process known as acidolysis. In addition to specificity towards certain positions in triacylglycerols, lipases may show specificity towards certain fatty acids. Lipases from two strains of bacteria (*Pseudomonas fluorescens* and *Pseudomonas species lipases* from Amano (Milton Keynes, England) were observed to convert the bulk of the unwanted saturated and monounsaturated fatty acids of the fish oil triacylglycerols into ethyl esters (McNeill et al, 1996). The ω3 PUFAs, including both EPA and DHA, on the other hand, remained attached to the residual acylglycerols, mainly as mono- and diacylglycerols, but also triacylglycerols, depending upon the extent of conversion. By using this method, it is easy to obtain concentration levels of 50% EPA+DHA in the residual acylglycerol mixture with a high EPA and DHA recovery. Many of the commercially available lipases discriminate against ω3 PUFAs and that lipases displaying any significant

activity toward ω3 fatty acids usually prefer EPA to DHA as a substrate (Haraldsson and Kristinsson, 1998).

2.3 Esterification reactions

Physiological studies on metabolism of ω3 fatty acids from various sources have indicated that triacylglycerols are more easily absorbed than either methyl or ethyl esters (Lawson and Hughes, 1988a; El-Boustani et al., 1987). Nordoy et al (1991), however, demonstrated that fish oils taken as ethyl esters or triacylglycerols were equally absorbed. Nonetheless, acylglycerols are preferred for delivery of PUFA to human subjects in order to avoid complications due to the release of toxic methanol or less toxic ethanol in the body (Lawson and Hughes, 1988b).

In recent years, the use of lipases as biocatalysts for interesterification has become of great industrial interest for the production of useful TAG mixtures. This is mainly because of the specificity of these enzymes with respect to acylglycerol positions and fatty acid types. The high temperatures (220-250 °C) used in current chemical technologies for acylglycerol synthesis and hydrolysis of fats and oils cause discoloration and degradation of some fatty acids (McNeill et al., 1991), hence compromising product quality and yield, particularly when highly unsaturated and hydroxylated fatty acids are involved. Such disadvantages may be overcome by using lipases, which react under mild conditions and allow production of desired products without leaving harmful residues.

2.3.1 Mechanism of esterification

Esterification reactions between polyhydric alcohols and free fatty acids are, in essence, the reverse of the hydrolysis reaction of the corresponding acylglycerols. The equilibrium between the forward and the reverse reactions is usually controlled by the water content of the reaction mixture.

In general terms, lipases contain several distinct sites, each responsible for a specific function. The hydrolysis of the ester bond is accomplished by the catalytic triad, responsible for nucleophilic attack on the carbonyl carbon of the ester bond, assisted by the oxyanion hole, which stabilizes the tetrahedral intermediates involved. The fatty acid recognition pocket defines the specificity of the leaving acid. There is also one or more interface activation sites, responsible for the conformational change in the enzyme (Derewenda, 1994).

For the lipase to be active, the active site must become accessible to the substrate. The substrate, an ester such as TAG or fatty acid ester of a monohydric alcohol, binds to the active site, the carboxyl carbon is positioned in close proximity to the hydroxyl oxygen in the serine side chain. This oxygen makes a nucleophilic attack on the carbonyl carbon of the substrate and a tetrahedral transition state, an acyl enzyme intermediate, is formed (Derewenda, 1994). The serine is made a stronger nucleophile by the presence of histidine and aspartic acid residues. The hydrogen from the hydroxyl group of serine is transferred temporarily to the histidine residue close to the serine residue. The intermediate

rearranges and the hydrogen is transferred from the histidine to the alcohol moiety of the substrate ester and an alcohol is formed, which then leaves the lipase. The fatty acid moiety is now covalently linked to the enzyme *via* an ester bond, this is called the acyl enzyme. In the next step, the reversal of the acylation of the lipase, the acyl enzyme is attacked by an alcohol or a water molecule. Again, an acyl enzyme intermediate is formed, which rearranges and a new ester is released. This type of mechanism is referred to as a Ping-Pong Bi-Bi mechanism (Reyes and Hill, 1994).

2.3.2 The effects of reaction variables on esterification reaction

2.3.2.1 Solvent

Most organic compounds of commercial interest are sparingly soluble in water and are often unstable in aqueous solutions. Chemists have realized these limitations of aqueous-based catalysis and have long ago replaced water with more suitable organic solvents. Unlike chemical processes, conventional biocatalysts have been thought to perform well in aqueous solutions. This is mainly due to the preconceived notion that nature intended enzymes to be catalytically active in water and that organic solvents serve only to destroy the catalytic power of enzymes. However, many enzymes or multienzyme complexes, including lipases, esterases and dehydrogenases, function in natural hydrophobic environments, usually immobilized to a membrane (Borgstrom and Brockman, 1984). It should not be surprising, then, that enzymes are catalytically active in

organic solvent systems. So use of appropriate organic solvents may be beneficial for construction of homogeneous reaction systems containing lipophilic and water-insoluble substrates and to facilitate continuous reactor processes. For the synthesis of useful compounds by means of hydrolytic enzymes, such as lipases and esterases, it is essential to reduce the water content in the reaction mixture by replacing water with appropriate organic solvents, if necessary, or to use neat substrates. Many studies have shown that the most suitable solvent for acylglycerols synthetic reaction systems is dependent on the type of enzyme used. Li and Ward (1993) reported that isooctane and hexane were better solvents compared to several other solvents examined for lipase PS30. On the other hand, heptane, hexane, pentane and isooctane were suitable solvent for lipase IM60. However, studies have shown that less polar solvents were better than polar solvents, such as benzene and acetone (Dordick, 1989).

2.3.2.2 Water

Enzymes have been described to function in non-aqueous media, although, in at least the majority of cases, a finite level of water associated with the protein (enzyme) must be present to retain conformational integrity, and thereby, activity (Miller, et al., 1988). On the other hand, lipase-catalyzed reactions are reversible and governed by the water content of the reaction mixture because water is one of the reaction products. Presence of large amounts of water, however, favors the hydrolysis reaction. Studies have shown that the degree of synthesis gradually

declines at a higher initial water content (Li and Ward, 1993). With a moisture content of 5% (v/v) in the reaction mixture, lipase PS30 showed maximum acylglycerol synthesis of about 82%. Lipase IM60 is an immobilized lipase of Mucor miehei containing about 5% water. With a moisture content of 1% (v/v) in the reaction mixture, a maximum degree of synthesis of 92% was obtained.

2.3.2.3 Glycerol

In the esterification reaction of acylglycerols, glycerol is one of the substrates. The effect of quantity of glycerol in the reaction mixture on acylglycerol synthesis by lipases has been reported (He and Shahidi, 1997; Li and Ward, 1993). At a low glycerol content, acylglycerol synthesis increased with increasing the amount of glycerol present. At a high glycerol content, the degree of synthesis did not increase (Li and Ward, 1993).

2.3.2.4 Reaction temperature

Temperature plays a key role for any reaction. In general, chemical reactions, including enzymatic reactions, obey what is known as the Q_{10} rule which states that the rate of reactions increases by a factor of about two for every 10 °C increase in temperature. This is true for many enzymatic reactions, including those using commercial enzyme preparations for the food industry, until the temperature reaches about 60-70 °C. At about this temperature range, the energy introduced to the system may alter the enzyme from its required conformation

and hence its activity. After a sufficient period of time, the enzyme will unfold and be inactivated, usually permanently. The higher the temperature, the more quickly denaturation takes place. Some enzymes derived form a specific bacterium are able to function in boiling water. Even in frozen foods, enzymes can continue to function. For the majority of commercial enzymes, the optimal temperature range is usually about 40-60 °C. Based on this point, the optimal temperature for specific enzyme is different. Li and Ward (1993) reported that 30 °C was best for lipase PS-30 and 50 °C was optimum for lipase IM-60. The higher temperature optimum for the latter enzyme was probably partly due to the fact that immobilization conferred greater thermostability on this enzyme. In another study, lipase LP-401-AS showed a maximum esterification at 50 °C. In a specific reaction, selection of temperature also relies on the amount of enzyme in the reaction mixture, reaction time, the oxidative stability of substrates and stability of products.

2.4 Lipid oxidation

Fish oils are much more susceptible to oxidation than other fats and oils because of their high concentration of PUFAs. Lipid oxidation may compromise the safety, nutritional quality and shelf life of edible oils. Lipid oxidation products are toxic and implicated in the disruption of biological membranes, formation of age pigments, inactivation of enzymes and damage to proteins. Proper understanding of lipid oxidation mechanisms and measurement techniques

as well as use of synthetic and natural antioxidants my help to control lipid oxidation and therefore prevent or minimize oxidative deterioration of foods as well as oxidative damage in the human body (Frankel, 1993b).

There are many catalytic systems that can oxidize lipids. Among these are light, temperature, enzymes, metals, metalloproteins and microorganisms. Most of these reactions involve some type of free radical and/or oxygen species. Since enzymes and metalloproteins are liable to heat denaturation, lipoxygenase activity is normally absent in a refined oil. Therefore, oxidation catalyzed by enzymes does not create a problem in the storage and use of processed fats and oils. Oxidative reactions of lipids, especially autoxidation, are greatly accelerated at higher temperatures. The oxidation of lipids at higher temperatures is referred to as thermal oxidation. Autoxidation and thermal oxidation are more directly involved in the oxidation of fatty acids during storage and processing (Vercellotti et al., 1992)

2.4.1 Autoxidation

Oxidation in the presence of air at room temperature is referred to as autoxidation. Generally, this is a slow process. This may require time, but eventually leads to the development of objectionable flavours and odours. Products containing a higher proportion of unsaturated fatty acids are more prone to oxidation than those containing lesser amounts. Autoxidation of unsaturated fatty acids occurs via a free radical chain mechanism. These processes often consist

of a complex series of reactions involving initiation, propagation and termination steps (Figure 2.4). In the first step, oxidation of lipids may be initiated by endogenous species (H_2O_2, ROOH) and radicals ($O_2^{\bullet-}$, $ROO^{\bullet}$, $HO^{\bullet}$) or by exogenous species ($^1O_2$, O_3), radicals (NO_X, SO_3), and environmental factors (UV, ionizing radiation, heat) (Simic, et al., 1988). The targets of attack of these agents are diverse and are specific to each agent and conditions. The formation of free radicals in this step is catalyzed by light, heat, high energy radiation, metal catalysts, metalloporphyrins (haem) and other radical compounds (Hamilton, 1994). The resultant alkyl free radical ($R^{\bullet}$) reacts with atmospheric oxygen to form an unstable peroxy free radical ($ROO^{\bullet}$) which in turn abstracts a hydrogen atom from another unsaturated fatty acid to form a hydroperoxide (ROOH) and another free radical, $R^{\bullet}$. This reaction is referred to as propagation. The free radicals formed can initiate and promote oxidation of large amounts of lipid (Porter *et al.*, 1995). The free radical chain reaction may be terminated by self-quenching or polymerization of free radicals to form non-radical products (RR, ROOR, etc.) or by abstraction of hydrogen atom from antioxidants (King *et al.*, 1995).

Figure 2.4 Initiation, propagation and termination steps of Autoxidation

Initiation

$LH \longrightarrow L^{\bullet}$ (1)

$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$ (2)

Propagation

$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$ (3)

$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet}$ (4)

$L^{\bullet} + AH \longrightarrow LH + A^{\bullet}$ (5)

Termination

$A^{\bullet} + LOO^{\bullet} \longrightarrow$ Non-radical products (6)

$A^{\bullet} + A^{\bullet} \longrightarrow$ Non-radical products (7)

2.4.2 Decomposition of hydroperoxides

Hydroperoxides (or primary oxidation products) are flavourless and odourless. However, they are very unstable and regardless of the mechanism of their formation, they are decomposed further to secondary oxidation products. Depending on the mode of this reaction, the products so formed may be carbonyl compounds (i.e., aldehydes and ketones), alcohols, esters and hydrocarbons, among others, which are believed to be responsible for the development of off-flavour and oxidative rancidity in foods. The aldehydes, which are powerful flavour compounds with very low flavour thresholds, are to a large extent responsible for the rancid flavour of fats and oils (Civille and Dus, 1992) The decomposition of hydroperoxides occurs *via* homolytic cleavage of the weak oxygen-oxygen bond to yield hydroxyl and alkoxy free radicals. The alkoxy radical may then undergo further cleavage to form an aldehyde and a new free radical. This reaction involves carbon-carbon bond scission which can occur on either side of the radical. The aldehyde which is formed due to scission, can either be a short-chain volatile compound or it can remain attached to the acylglycerol part of the molecule as a non-volatile product. Abstraction of a hydrogen atom from another molecule can yield an alcohol and a new free radical. Free radicals formed during these reactions may participate in propagation of the chain reactions while interaction of two free radicals can yield non-radical products and thus, terminate the chain reaction, leading to the formation of a ketone (Simic et al., 1992)

2.4.3 Measurement of lipid oxidation

Lipid oxidation involves a set of complicated processes. There is no universal technique which can be applied to monitor the progress of lipid oxidation (Prior and Loliger, 1994). Several chemical, instrumental and sensory techniques are used to monitor the oxidation of foods and thus predict their shelf life. While some techniques measure the loss of reactants such as oxygen or the substrates such as fatty acids, others measure primary oxidation products (free radicals, hydroperoxides and conjugated dienes) or secondary oxidation products (alcohols, aldehydes, hydrocarbons and ketones)(Lampi et al. 1997). Determination of peroxide value (PV) and conjugated dienes (CD) is frequently carried out to measure primary oxidation products. Meanwhile thiobarbituric acid reactive substances (TBARS) and headspace volatiles are determined as indices for monitoring secondary oxidation products (Shahidi and Wanasundara, 1996).

2.4.3.1 Conjugated dienes

It has been observed that the hydroperoxides formed during oxidation of linoleic and linolenic acids at the initial stages contain conjugated diene groups which absorb UV light strongly at 234 nm. This observation forms the basis of their spectrophotometric determination. Edible oil oxidation has been followed by measuring the absorbance at 234 nm by the IUPAC method 2.505 (Wanasundara et al, 1995; Abou-Gharbia et al, 1997). This method is very useful

in evaluating the oxidative stability of products including those of the refined or stripped edible oils because dienes, trienes and tetraenes, which absorb at 234, 274 and 392 nm, respectively, are not normally present in the system (Prior and Loliger, 1994)

2.4.3.2 2 Thiobarbituric acid reactive substances (TBARS)

Undesirable flavours associated with lipid oxidation arise from further decomposition of lipid hydroperoxides to yield aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones (Hamilton, 1994). Some analytical techniques used to measure these products relate to the total amount of aldehydes in the oxidized lipids such as TBARS, while others can precisely determine individual secondary oxidation products such as determination of headspace volatiles.

The 2-thiobarbituric acid reactive substances (TBARS) or the TBA test is frequently used for the measurement of lipid oxidation in foods. One molecule of malonaldehyde (MA), which is a secondary product of lipid oxidation, reacts with two molecules of 2-thiobarbituric acid (TBA) to form TBA-MA complex which has a maximum absorbance at 532 nm (King et al., 1995). The accuracy of the TBA test has been questioned due to the reaction of TBA with other molecules, including sugars and oxidized proteins (Rossell, 1994). However, these products are normally absent in edible oils, thus determination of TBARS is frequently used to assess their oxidation (Kaitaranta, 1992; Ganthavorn and Hughes, 1997).

Meanwhile, TBA might also react with other oxidation products such as 2-alkenals and 2,4-alkadienals present in edible oil, which might reflect the total amount of aldehydes rather than MA alone (Rossell, 1994; Shahidi and Wanasundara, 1998).

2.4.3.3 Headspace analysis of volatiles

Hydroperoxides present in oils can decompose to volatiles. Gas chromatographic analyses of oils have revealed that hexanal and pentane are the major volatiles of oxidation of n-6 PUFA, while propanal is the predominant volatile derived from n-3 PUFA (Frankel, 1993; Shahidi and Wanasundara, 1998). In most of these techniques, the oil is heated at 180 °C in closed vials. Under this condition, the volatiles are evaporated and collected in the headspace above the oil, then the volatiles are analyzed by gas chromatography. The total peak area of the volatiles in this technique increases with the length of storage of an oil. Therefore, gas chromatography can provide useful data about the origin of flavour volatiles and their precursors (Rossell, 1994). Moreover, King et al. (1995) have observed that an excellent correlation existed between GC data and sensory scores in photooxidized soybean oil, while pentanal and hexanal correlated well with flavour scores of autoxidized soybean oil.

2.4.4 Accelerated oxidation tests

In order to estimate the stability and susceptibility of fats and oils towards oxidation and thus their quality and shelf life, a number of accelerated oxidation tests are used, including the Schaal oven test, the Sylvester test, the Swift test and test by Oxidative Stability Instrument (Rossel, 1994). Heating is the most commonly used and effective means of accelerating oxidation, although fluorescent light can also be used to study the role of the sensitizers on the stability of oils. Furthermore, all tests have limitations (Rossel, 1994). The Schaal oven test (65 °C) suffers from few limitations as well. During the stability tests, induction period (IP) is measured as the time required to reach the end point of oxidation that is corresponding to detectable activity or a sudden change in the rate of oxidation (Frankel 1993a; Hamilton 1994). The induction period (IP) represents a lower degree of oxidation and results correlate well with evaluation of actual shelf lives. Using higher temperatures, however, might interfere with the proper prediction of shelf life. Therefore, the conditions of accelerated oxidation tests should be maintained as close as possible to those under which the food is stored (Frankel, 1993b).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Seal blubber oil was obtained from local sources in Newfoundland. Fatty acid methyl esters standard were purchased from Nu-Check (Elysian, MN) company. The lipases Novozym-435 from *Candida antarctica* and IM60 from *Mucor miehei* were provided by Novo Nordisk (Bagsvaerd, Denmark). The lipase PS30 from *Pseudomonas sp.* was provided by Amano enzyme, USA Co., Ltd (Troy, VA). Reagents, sodium sulphate, triolein, diolein, monoolein, calcium chloride, sodium bicarbonate, potassium hydroxide, sodium hydroxide, boric acid, porcine pancreatic lipase, propanal, butanal, pentanal, hexanal, heptanal, nonanal, silicic acid and silica gel TLC plates (20 x 20 cm; 60**C** mean pore diameter, 2-25 μm mean particle size, 500 μm thickness, with dichlorofluorescein) were purchased from Sigma Chemical Co. (St. Louis, MO). Hexane, ethanol, hydrochloric acid, isooctane, acetone, acetic acid, diethyl ether were purchased from Fisher Scientific (Nepean, ON). Hydrogen, nitrogen and compressed air were obtained form Canadian Liquid Air Ltd. (St. John's, NF). All other chemicals used in this study were of American Chemical Society (ACS) grade or better.

3.2 Methods

3.2.1 Preparation of ω3 PUFA concentrates from seal blubber oil

3.2.1.1 Preparation of free fatty acids from seal blubber oil

The method used for preparing free fatty acids from seal blubber oil was the same as that described by Wanasundara (1999) with some modification. The procedure is outlined in Figure 3.1. To 100 g seal blubber oil, 23 g KOH, 44 mL deionized water and 264 mL ethanol (95%, v/v) were added. The concentration of tertiary-butylhydroquinone (TBHQ) in mixture was 200 ppm. The mixture was refluxed under nitrogen at 62 °C for 1 hr. To the saponified mixture, 50 mL ethanol (95% v/v) and 250 mL deionized water were added. The mixture was extracted with 200 mL hexane twice and the hexane layer was discarded. The aqueous layer was acidified with 6N HCl to pH 1.0, then 200 mL hexane were added to the resultant free fatty acids. The hexane layer was dried over anhydrous sodium sulphate and the solvent removed using a rotary evaporator at 40 °C.

3.2.1.2 Preparation of ω3 PUFA concentrate by urea complexation procedure

For the purpose of concentrating the omega-3 fatty acids, the urea complexation procedure was used. The outline for production of concentrate is shown in Figure 3.2. In the 350 mL 20% (w/v) urea solution which was dissolved in 60 °C ethanol (95%), 20 g free fatty acids were added. The mixture was allowed to crystallize at room temperature until it cooled down to room

temperature but colder temperature was maintained (in a cold room at 4 °C) for 8 hr. The urea complexed fraction was subsequently separated from the liquid by filtration. The crystals were then dissolved in water and acidified with 6 N HCl to pH 4 - 5. An equal volume of hexane was subsequently added and the mixture was stirred thoroughly for 1 hr. The hexane layer containing liberated fatty acids was separated from the aqueous layer. The hexane layer was washed with distilled water to remove any of the remaining urea and then dried over anhydrous sodium sulphate. The solvent was removed at 40 °C using a rotary evaporator. The free fatty acids (10 g) were subsequently mixed with urea (20% w/v) in 95% ethanol (100 mL) heated at 60 °C . The mixture was maintained in a freezer at -18 °C for 24 hr. Then the non-urea complexed and urea complexed fractions were separated. The filtrate 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 was stirred thoroughly for 1 hr and subsequently transferred to a separatory funnel. The hexane layer was washed with distilled water to remove any remaining urea and then dried over anhydrous sodium sulphate and the solvent was removed at 40 °C using a rotary evaporator.

Figure 3.1 The procedure of hydrolysis of seal blubber oil

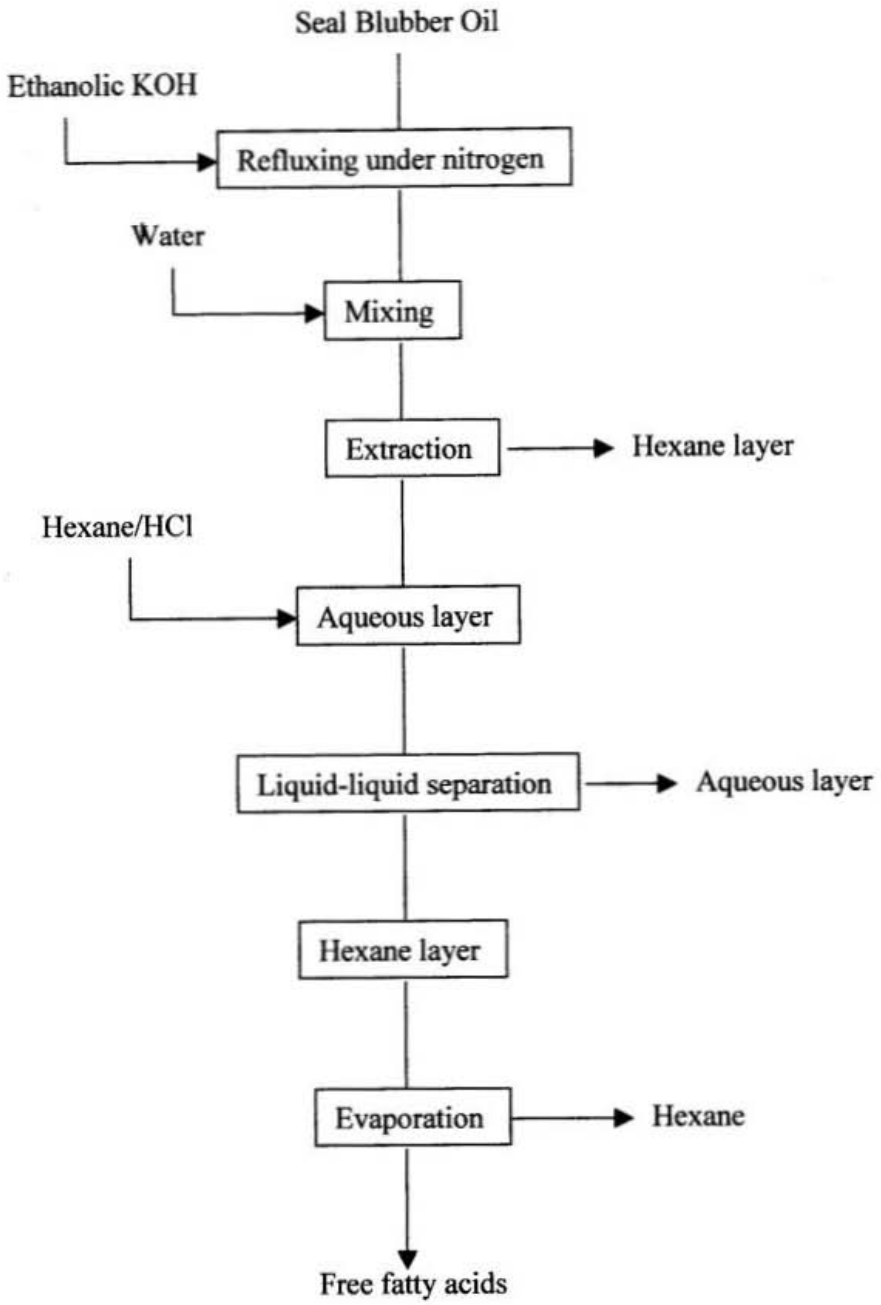
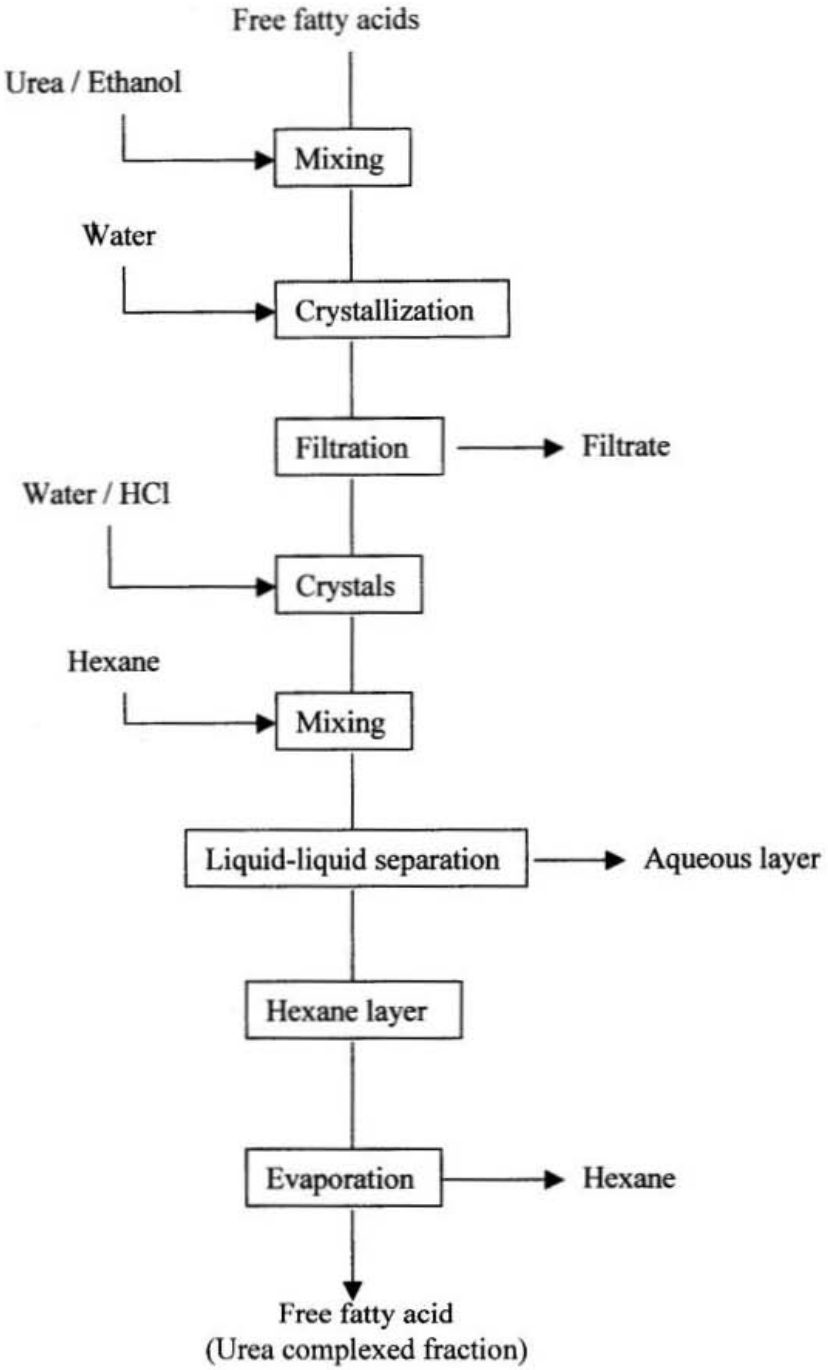
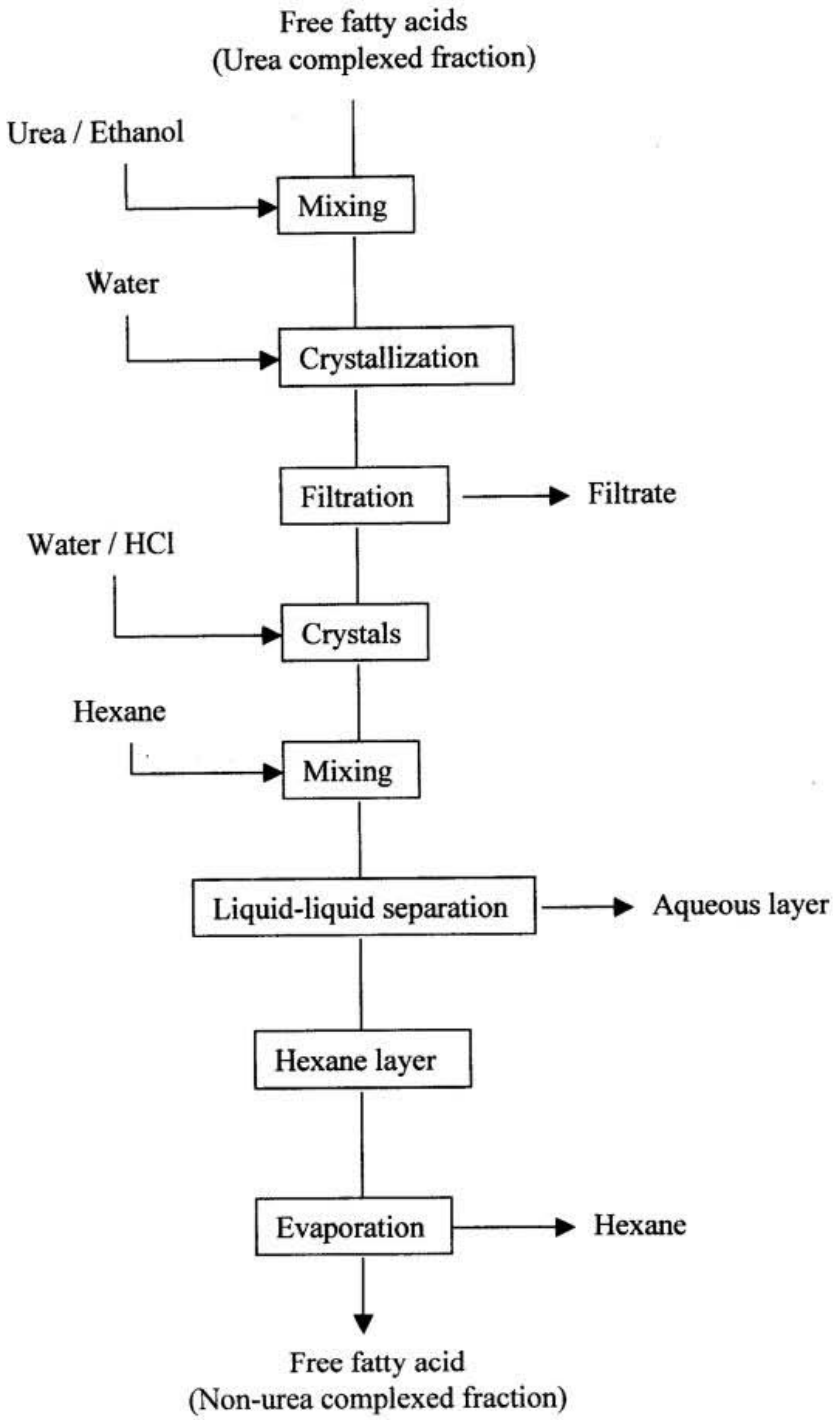

Seal Blubber Oil
Ethanolic KOH
Refluxing under nitrogen
Water
Mixing
Extraction
Hexane layer
Hexane/HCl
Aqueous layer
Liquid-liquid separation
Aqueous layer
Hexane layer
Evaporation
Hexane
Free fatty acids

Figure 3.2 Two steps urea complexation procedure.


Free fatty acids
Urea / Ethanol
Mixing
Water
Crystallization
Filtration
Filtrate
Water / HCl
Crystals
Hexane
Mixing
Liquid-liquid separation
Aqueous layer
Hexane layer
Evaporation
Hexane
Free fatty acid
(Urea complexed fraction)


Free fatty acids
(Urea complexed fraction)
Urea / Ethanol
Mixing
Water
Crystallization
Filtration
Filtrate
Water / HCl
Crystals
Hexane
Mixing
Liquid-liquid separation
Aqueous layer
Hexane layer
Evaporation
Hexane
Free fatty acid
(Non-urea complexed fraction)

3.2.2 Esterification reactions

3.2.2.1 Esterification method

Acylglycerols were synthesized from glycerol and the ω3 fatty acid concentrate. In a typical synthesis, 2 g of glycerol was combined with 0.4 g ω3 PUFAs concentrate and 50 mg lipase of PS30, IM60 or SP435. Then 100 μL of water and 2 mL of hexane were added to the mixture. The reaction mixture was placed in a 50-mL Erlenmeyer flask, and then flashed with nitrogen for 30 s. The mixture was stirred in an orbital shaker at 180 rpm and at different temperatures (20 , 30, 40 and 50 °C) for various time periods (3-48 hr).

3.2.2.2 Estimation of degree of synthesis

The esterification reaction was stopped by addition of 20 mL of acetone/ethanol mixture (1:1, v/v), and free fatty acids were titrated with 0.1 N NaOH to a phenolphthalein endpoint. A similar mixture without free fatty acids was used as the blank. The degree of synthesis (%) represents the mole percent of initial fatty acids consumed in the reaction mixture. The degree of synthesis was calculated as: $D = 100*\{[W / M - 0.1*(V-V_o)] / (W /M)\}$

Where D = the degree of synthesis (%); W = the weight of oil concentrate (g); M = the average molecular weight of oil concentrate; V = the volume of NaOH consumed by the sample (L); V_o = the volume of NaOH consumed by the blank (L).

3.2.2.3 Extraction of acylglycerols from mixture

The products, TAG (triacylglycerols), DAG (diacylglycerols) and MAG (monoacylglycerols) were extracted by diethyl ether three times. Subsequently, the free fatty acids were titrated. After titration, 4 mL of 0.1 N NaOH and 50 mL of deionized water were added to the mixture. The mixture was then transferred to a separatory funnel and thoroughly mixed with 25 mL diethyl ether. The lower aqueous layer was discarded. The same procedure was repeated twice. The diethyl ether layers were combined and dried over anhydrous sodium sulphate. Solvent was removed by rotary evaporator at 40 °C. The components were identified and quantified by thin layer chromatography-flame ionization detector (TLC-FID). The mixture was developed using a solvent system of hexane/diethyl ether/formic acid (80:30:1, v/v/v) (Senanayake and Shahidi, 1999).

3.2.2.4 Determination of fatty acid composition

TAG, DAG and MAG in products were separated by thin layer chromatography (TLC). The products extracted from reaction mixture were dissolved in the hexane and applied to several TLC plates (20 x 20 cm; Silica gel, 2-25 μm mean particle size, 500 μm thickness, with dichlorofluorescein (Sigma, St. Louis, MO). Thirty milligrams of acylglycerols were loaded onto each plate. The plates were developed in solvent systems of hexane/diethyl ether/formic acid (70:30:1, v/v/v). After drying, the bands were located by viewing under short (254 nm) UV light (Spectraline, Model ENF-240C,

Spectronics Co. Westbury, NY). The three bands were scraped and then extracted three times with diethyl ether as described previously (Section 3.2.3.3) with minor modification. The scraped silicic acid with acylglycerols was collected in 50 mL centrifuge tubes and 30 mL diethyl ether were added and vortexed for 2 min. The mixtures were centrifuged at 3000 rpm (2000g) for 3 min. The ether layer was collected and the silicic acid was extracted twice more. The ether layers were combined and evaporated under a stream of nitrogen to obtain TAG, DAG and MAG. The fatty acid composition of each acylglycerol was analyzed using gas chromatography (GC).

3.2.3 GC analysis

3.2.3.1 Preparation of fatty acid methyl esters (FAMEs)

Fatty acid composition of lipids was determined following their conversion to corresponding methyl esters. Approximately 30 mg of each sample were weighed into a 6 mL well-cleaned Teflon-lined, screw capped conical vial. Two milliliters of freshly prepared transmethylation reagent (6% concentrated sulphuric acid in HPLC grade methanol with 200 ppm t-butylhydroquinone) were added to the sample vial and mixed by vortex. The mixture was incubated overnight at 60°C and subsequently cooled, and 1 mL distilled water then added to it. The mixture was mixed thoroughly and extracted two times with 1mL of HPLC grade hexane. The hexane layers were separated, combined and transferred to a tube and then washed with distilled water twice. After washing, the hexane layer was separated

and evaporated under a stream of nitrogen. Fatty acid methyl esters were then dissolved in 1mL of carbon disulphide and used for gas chromatographic analysis.

3.2.3.2 Gas chromatography (GC)

A Hewlett Packard 5890 Series II gas chromatograph (Palo Alto, CA) equipped with a Supelcowax –10 column (0.25 mm diameter, 30 m length, 0.25 um film thickness; Supelco Canada Ltd., Oakville, ON) was used for analyzing FAMEs. The oven temperature was initially 220°C for 10.25 min and then raised to 240°C at 30 °C/min and then held there for 13 min. The temperature of both injector and detector was 270 °C. Ultra high purity (UHP) helium was used as a carrier gas (15mL/min). HP 3365 Series II ChemStation software (Hewlett Packard, USA was used for data analysis. The FAMEs were tentatively identified by comparison of their retention times with those of an authentic standard mixture (PUFA 1; Supelco Canada Ltd. And GLC-416; Nu-Check).

3.2.4 Oxidative stability of lipids

3.2.4.1 Preparation of samples for oxidative stability test

Three samples were prepared for oxidative stability test. The samples were purified by column chromatography. One hundred twenty grams of silicic acid (100 mesh) were washed with one litter of deionized water three times and the silicic acid was dehydrated with 200 mL of methanol three times. The dehydrated

silicic acid was dried in an oven at 100 °C for 6 hr and then dried in a furnace at 200 °C overnight. The dried silicic acid was mixed with 400 mL *n*-hexane and degassed by sonification for 15 min. The silicic acid in hexane was then loaded into a column (diameter: 2 x 40 cm) and equilibrated with hexane (3 ml/min) for 5 hr. The acylglycerols were loaded and the column was washed with hexane (3 ml/min). At the same time, the collection was monitored by TLC. The collections for TAG, DAG and MAG were combined and the solvent was evaporated at 40 °C.

3.2.4.2 Oxidative Stability Test

Comparison of the oxidative stability of different samples was carried out under Schaal-oven condition at 60 °C. It is generally accepted that each day of storage of lipid under Schaal-oven test condition at 60 °C is equivalent to one month of storage at ambient temperatures (Robards et al., 1988).

The specifications of the experiments carried out under Schaal-oven test conditions were as follows. Each sample (2 g) in triplicate, was placed in test tubes (10mm x 12cm) and stored in a forced air oven (Thelco, Model 2, Precision Scientific Co., Chicago, IL) at 60 °C. The oxidative stability was determined by chemical methods -- conjugated dienes; 2-thiobarbituric acid reactive substances (TBARS) value; headspace volatiles. The samples (0.3 g) were removed periodically at 0, 6, 12, 24, 48 and 72 hr from the oven, cooled to room temperature, flushed with nitrogen, capped and stored at -20°C until analysis.

3.2.4.3 Determination of conjugated dienes (CD)

Conjugated diene values of oil samples were measured using a procedure described previously by Shahidi and Spurvey (1996). Samples (0.03-0.06 g) were weighed into 25 mL volumetric flasks, dissolved in isooctane(2,2,4-trimethylpentane) and made up to the mark with the same solvent. The solution was thoroughly mixed and the absorbance read at 234 nm using a Hewlett-Packard 8452A diode spectrophotometer (Palo Alto, CA). Pure isooctane was used as the reference. Conjugated diene value was calculated as CD =a/(c*d)

where a = absorbance of the solution at 234 nm, c = concentration of the solution in g/100 ml of solution and d = length of the cell (cm)

3.2.4.4 Analysis of headspace volatiles of oxidized oils

A Perkin-Elmer 8500 gas chromatograph and HS-6 headspace sampler (Perkin-Elmer Corp., Montreal, PQ) were used for analysis of volatiles produced during oxidation of oil samples. The volatiles in the headspace of oxidized oils (obtained from the accelerated oxidation method) were separated using a high polarity Supelcowax-10 fused silica capillary column (0.32 mm internal diameter, 30 m length, 0.1 μm film thickness, Supelco Canada Ltd. Toronto, ON) UHP helium was used as the carrier gas. The inlet column pressure was 17.5 psig and the split ratio was 7:1. The oven temperature was maintained at 40 °C for 5

min and then raised to 200 °C at 20 °C/min and held there for 5 min. The injector and flame ionization detector (FID) temperatures were set at 280 °C.

For headspace analysis, 0.10 g of each sample was transferred to a 5 mL headspace vial (Chromatographic Specialties Inc., Brockville, ON). The vials were capped with Teflon-lined septa and crimped. The vial was then preheated in the headspace magazine assembly at 40 °C for a 45 min equilibration period. Pressurization time of the vial was 6 s, and the volume of the vapor phase drawn was approximately 1.5 mL. The area under each peak was expressed as integrator count units. Volatile compounds were identified by comparison of their retention times with those of authentic compounds. Quantitative determination of dominant volatiles (mainly hexanal and propanal) was accomplished using 2-heptanone as an internal standard.

3.2.4.5 Determination of TBARS in oil samples

A standard solution of malonaldehyde was prepared by hydrolyzing the malonaldehyde precursor 1, 1, 3, 3-tetramethoxypropane in a 0.1N HCl solution at a concentration of 1 mg/mL. Serial dilutions, in duplicate, were made by diluting 1, 2, 3, 4, and 5 mL aliquots to 100 mL with an acid solution in a volumetric flask. One hundred microliters of each serial dilution were transferred into a clean 16 x 150 mm test tube with a Teflon cap. To each tube, 4.9 mm 1-butanol and 5 mL of a 2 mg/mL TBA solution in 1-butanol were added with a pipet. The tubes were heat at 95°C in a thermostated water bath for 120 min and

subsequently cooled under running tap water for about 10 min until reaching room temperature. The absorbance of the solutions was read at 532 nm using the HCl / butanol / TBA solvent system (1:49:50, v/v/v) as the reagent blank.

An oil sample (20 to 50 mg) was weighed accurately into a 25 mL volumetric flask. The sample was dissolved in a small volume of 1-butanol and then made to the mark. Five milliliters of the samples were pipetted to dry test tubes and then to each 5 mL of the TBA reagent were added. The tubes were capped and the contents mixed thoroughly. Prepared tubes were heated in a thermostated water bath at 95 °C for 120 min. Afterwards, tubes were cooled under running tap water for about 10 min until reaching room temperature. The absorbance of the solutions was read at 532 nm using a butanol / TBA solvent system (1:1, v/v)as the reagent blank.

The TBA value was expressed as the total mmol of malonaldehyde equivalents per g of sample and calculated according to the following equation.

$C = 0.355\ A/W$

Where C=concentration of MA; W=weight of oil (g); and A=absorbance of solution at 532 nm.

3.2.5 Positional Distribution of Triacylglycerols

3.2.5.1 Preparation of Pure Triacylglycerols

Acylglycerols were prepared by lipase SP405 as described previously (Section 3.2.2.1). Triacylglycerols in the acylglycerols mixture were purified by column

chromatography. Two hundred grams silicic acid (100 mesh) was washed with 1 L deionized water three times, and then dehydrated with 200 mL methanol three times. The washed silicic acid was dried in an oven at 100 °C for 6 hr and then put in a furnace at 200 °C overnight. The dried silicic acid was mixed with 400 mL *n*-hexane and degassed by sonication for 15 min. Afterwards, silicic acid in hexane was loaded into a column (diameter: 2 x 40 cm), which was subsequently equilibrated with balanced hexane (3 mL/min) for 5 hr. The acylglycerols were loaded and the column was washed with hexane (3 mL/min), hexane/diethyl ether (1:1, v/v), and diethyl ether. At the same time, the collection was monitored by TLC. The first peak was triacylglycerols. The collections in different tubes were combined and the solvent was then evaporated at 40 °C using a rotary evaporator.

3.2.5.2 Hydrolysis of triacylglycerols by pancreatic lipase

Hydrolysis of purified triacylglycerols by pancreatic lipase was carried out according to the following method. Tris-hydrochloric acid buffer (5 mL, 1.0 M, pH=8.0), 0.5 mL of calcium chloride (2.2%, w/v) and 1.25 mL of sodium taurocholate (0.05%, w/v) were added to 25 mg of oil in a glass test tube. The whole mixture was allowed to equilibrate at 40°C in a water bath for 1 min and subsequently 5.0 mg of porcine pancreatic lipase (EC. 3.1.1.3, Sigma) were added to it. The mixture was then placed in a Gyrotory water bath shaker (Model G76, New Brunswick Scientific Co. Inc. NB) at 180rpm under nitrogen for 1hr at 40°C. Ethanol (5 mL) was added to stop the enzymatic hydrolysis followed by

addition of 5.0 mL of 6.0N HCl. The hydrolytic products were extracted three times with 50 mL of diethyl ether and ether layer was washed twice with distilled water and dried over anhydrous sodium sulphate. After removal of the solvent under vacuum at 40°C, the hydrolytic products were separated on TLC plates (20 × 20cm, silica gel, 2-25um mean particle size, 500um thickness, Sigma, St. Louis, MO). The plates were developed using hexane/diethyl ether/ acetic acid (70:30:1, v/v/v). After drying, the bands were located by viewing under short (254nm) UV light (Spectraline, Model ENF-240C, Spectronics Co.). The bands were scraped off and their lipids extracted into diethyl ether three times and subsequently used for fatty acid analysis by the gas chromatographic procedure described in Section 3.2.3.2.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Preparation of docosapentaenoic acid (DPA) rich concentrate from seal blubber oil via urea complexation

4.1.1 Seal blubber oil hydrolysis

A concentrate of docosapentaenoic acid (DPA) was obtained by urea complexation of fatty acids of seal blubber oil. Seal blubber oil may be concentrated in the form of TAG, as free fatty acids, or as the simple alkyl ester. Most of the seal blubber oil products sold are in the TAG form. Seal blubber oil is abundant in DHA, DPA and EPA, especially for DPA which may be present at up to 5%. The urea complexation could be performed either on the free fatty acids or their alkyl esters. In this investigation, the free fatty acids were preferred due to their better solubility in the urea/ethanol solution as compared to that of their corresponding methyl or ethyl esters. This is important as it minimizes the amount of solvent (ethanol) required for the process.

In order to prepare a PUFA concentrate rich in DPA, seal blubber oil was used as the starting material. Initially, the triacylglycerols of the seal blubber oil were hydrolyzed into their constituent fatty acids and glycerol by alkaline hydrolysis in ethanol (Wanasundara and Shahidi, 1999). In this step, more than 95% of seal blubber oil was hydrolyzed. Less than 5% of other components, such as sterols, vitamins A and D, could not be hydrolysed. This non-hydrolysable

components as well as monoacylglycerols and diacylglycerols remaining in the oil were removed by hexane. Table 4.1 shows the concentration of different fatty acids in the oil following hydrolysis. In the original oil after hydrolysis, around 15% of fatty acids were saturated in nature. About 60% of fatty acids in seal blubber oil are mono-, di-, or triunsaturated fatty acids. Another about 20% of seal blubber oil was DHA, DPA and EPA. The DPA concentration in the oil sample examined was about 4% of total oil. The concentration of both DHA and EPA was about 8% (Table 4.1).

4.1.2 Concentration of polyunsaturated fatty acids

In order to concentrate PUFA from seal blubber oil containing high percentage of DPA, in this study a new processing procedure was approached using urea complexation method. As described earlier, the formation of fatty acid-urea adduct depends on the degree of unsaturation of fatty acids. DHA has the highest degree of unsaturation compared to DPA and EPA while DPA has the lowest degree of unsaturation. In this study, the differences of unsaturation of the three fatty acids were used to eliminate most of DHA and EPA present in the original seal blubber oil.

In this new process, there were two urea complexation steps. In the first step, high ratio of urea to fatty acids was used because DPA was easier to form urea-fatty acid complex at high ratio of urea to fatty acids than EPA and DHA. Based

Table 4.1. Fatty acid composition of hydrolyzed seal blubber oil.

Fatty acid	Weight (%)
14:0	4.49 ± 0.13
14:1	0.88 ±0.05
15:0	0.26 ±0.02
16:0	7.34 ± 0.38
16:1	17.55 ± 1.21
17:0	0.83 ± 0.05
17:1	0.51 ± 0.04
18:0	0.93 ± 0.06
18:1 ω9	21.62 ± 0.83
18:1 ω11	4.72 ± 0.21
18:2 ω6	1.98 ± 0.11
18:3 ω3	0.60 ± 0.13
20:1 ω9	12.30 ± 0.32
20:5 ω3	7.48 ± 0.22
22:1 ω11	1.72 ± 0.10
22:5 ω3	4.03 ± 0.15
22:6 ω3	8.32 ± 0.17
Total	95.56

on this hypothesis, part of EPA and DHA can be eliminated using urea complex method. However, saturated, monounsaturated, diunsaturated and triunsaturated fatty acids also formed urea-complex as DPA form urea-complex. In order to eliminate the saturated, monounsaturated, diunsaturated and triunsturated fatty acids, a second urea complexation step was employed. In the second step, low ratio of urea to fatty acids was used because DPA was harder to form urea-fatty acid complex at low ratio of urea to fatty acids than saturated, and other unsaturated fatty acids present in sample.

4.1.2.1 First step urea complexation

In this study, a set of experiments were carried out in which the weight of fatty acids was kept constant at 20 g, but the weight of urea was varied. A high urea to fatty acid ratio was used in this step. The ratio of urea to fatty acids varied from 3.0 to 4.5. The urea complex was allowed to crystallize at 4°C for 8 h. The EPA, DPA and DHA concentration of the urea complexing fraction (UCF) and non-urea complexing fraction (NUCF) produced are given in Table 4.2. DPA concentration of urea complexed fraction was increased from 3.06 to 4.18 as the ratio of urea to fatty acid increased from 3.0 to 4.5. Table 4.2 shows that the urea to fatty acid ratio of 3.3 was the best. Under this condition, a minimum EPA and DHA in the urea complexed fraction was obtained and most of the DPA was in the urea complexed fraction. The urea complexed fraction in

this step was used for the next step. Therefore conditions leading to higher proportions of DPA in the UCF are preferred. However, when the concentration of DPA in UCF increased, the concentration of DHA and EPA also increased (Figure 4.1). For example, as the urea to fatty acid ratio increased from 3.5 to 4.5, the concentration of DPA increased insignificantly from 4.16 to 4.18% (Table 4.2). At the same time, the concentration of DHA increased from 0.85 to 1.95% (Table 4.2). Similarly, as the urea to fatty acid ratio increased from 3.3 to 3.5, the concentration of DPA increased from 3.78 to 4.16%, and the concentration of EPA increased from 3.10 to 3.91%. In both cases, DHA or EPA increase was more than that of DPA (Figure 4.1). In order to increase the DPA concentration in the final product, the ratio of urea to fatty acid for producing the UCF in this step was 3.3.

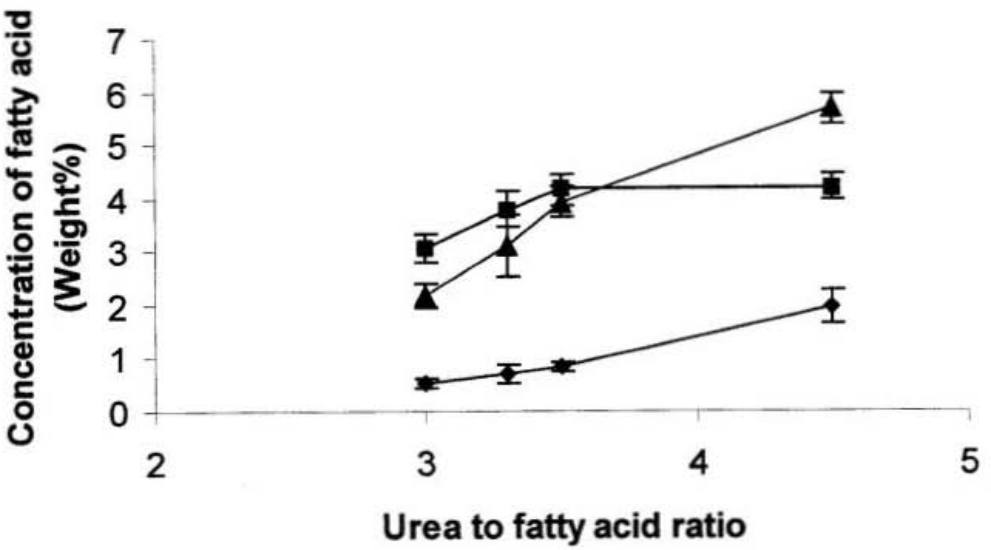
The yield of the NUCF is varied for different urea to fatty acid ratios. The yield increased slightly from 72 to 75% as the urea to fatty acid ratio increased from 3.0 to 4.5. The recovery efficiency of DPA in UCF was 67.8% of that present in the original seal blubber oil. The recovery efficiency of DHA and EPA in UCF was 6.27 and 30.2% respectively. These results show that most of the DHA and more than half of EPA were in the non-urea complexed fraction under conditions employed. At the same time, most of DPA is remained in the product.

Not only the degree of fatty acid unsaturation can influence the urea-fatty acid complex, but also many other factors have the similar affect, such as the ratio of the urea to fatty acid, reaction time, and reaction temperature. In a

Table 4.2. The contents of EPA, DPA and DHA in the urea complexed and non- urea complexed fractions under different conditions.

Urea to Fatty acid ratio	Urea complexed fraction			
	EPA (%)	DPA (%)	DHA (%)	Yield (%)
3.0	2.18 ± 0.22	3.06 ± 0.25	0.53 ± 0.09	72.0 ± 4.25
3.3	3.10 ± 0.56	3.78 ± 0.32	0.71 ± 0.16	72.3 ± 4.98
3.5	3.91 ± 0.28	4.16 ± 0.29	0.85 ± 0.10	75.0 ± 3.88
4.5	5.65 ± 0.30	4.18 ± 0.23	1.95 ± 0.29	75.0 ± 4.79
Urea to fatty acid ratio	Non-urea complexed fraction			
	EPA (%)	DPA (%)	DHA (%)	Yield (%)
3.0	26.3 ± 1.89	7.48 ± 0.75	37.4 ± 4.20	20.0 ± 1.56
3.3	25.2 ± 2.22	4.75 ± 0.66	40.1 ± 5.25	18.7 ± 2.22
3.5	23.9 ± 2.10	3.08 ± 0.28	44.7 ± 5.87	16.5 ± 2.64
4.5	18.9 ± 3.10	2.82 ± 0.30	50.2 ± 4.63	12.0 ± 1.88

Figure 4.1. The relationship between fatty acid concentration and the ratio of urea to fatty acid in first step. The Y value represents the concentration of fatty acid (Weight %) in urea complexed fraction. Symbols are: ▲, EPA; ■, DPA; and ♦, DHA.


Concentration of fatty acid (Weight%)
7
6
5
4
3
2
1
0
2
3
4
5
Urea to fatty acid ratio

previous study in our lab, influences of all these factors on the concentrations of fatty acids in the product and its yield were reported (Wanasundara and Shahidi, 1999). The ratio of urea to fatty acid was the most important factor as opposed to the reaction time and temperature. As the ratio of urea to fatty acid changed, the composition of fatty acids in the PUFA concentrate was also changed. So in this study, only the ratio of urea to fatty acid was varied.

4.1.2.2 Second urea complexation step

In the second step, a low ratio of urea to fatty acid was used to concentrate the DPA and EPA from the urea complexed fraction from products of the first step of the process. As described earlier, compared to the ratio of urea to fatty acids, reaction time and temperature were minor factors which influenced the fatty acid concentration in PUFA concentrates. In a previous study (Wanasundara and Shahidi, 1999), the highest DPA content was achieved with a urea to fatty acid ratio of 2, a crystallization time of 12 h and a crystallization temperature of −18 °C. In this study, in order to get rid of saturated and monounsaturated fatty acids as much as possible, the crystallization time was increased to 24 h.

Details of the urea complexation procedure in the second step was similar to that of the first step. In this set of experiments, however, the weight of fatty acids was kept constant at 10 g while the weight of urea was varied. Compared to the first step, low urea to fatty acid ratio was used because NUCF was retained as the final product. If a high

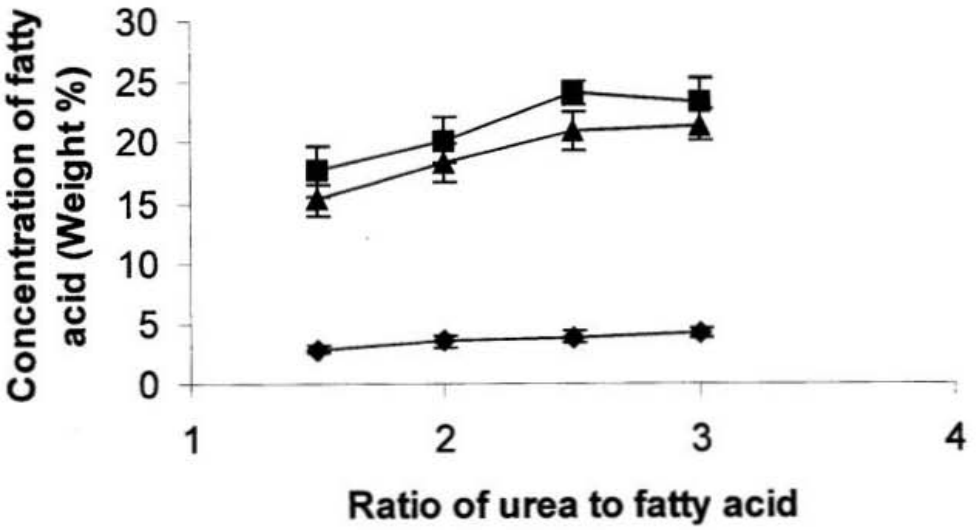
Table 4.3. The contents of EPA, DPA and DHA in urea complexed and non- urea complexed fractions under different conditions in the second step.

Urea to Fatty acid ratio	Urea complexed fraction			
	EPA (%)	DPA(%)	DHA(%)	Yield (%)
1.5	0.38 ± 0.12	0.53 ± 0.11	0.23 ± 0.08	74.3 ± 4.89
2.0	0.43 ± 0.21	0.61 ± 0.28	0.20 ± 0.06	75.2 ± 3.68
2.5	0.51 ± 0.16	0.65 ± 0.12	0.18 ± 0.10	75.8 ± 7.21
3.0	0.63 ± 0.18	0.98 ± 0.19	0.21 ± 0.03	77.9 ± 6.23
Urea to fatty acid ratio	Non-urea complexed fraction			
	EPA(%)	DPA(%)	DHA(%)	Yield (%)
1.5	15.2 ± 1.23	17.6 ± 2.01	2.86 ± 0.36	15.6 ± 1.98
2.0	18.3 ± 1.56	20.1 ± 1.88	3.49 ± 0.52	14.0 ± 1.64
2.5	20.8 ± 1.58	24.0 ± 1.05	3.85 ± 0.45	12.4 ± 1.53
3.0	21.3 ± 1.25	23.2 ± 2.06	4.23 ± 0.45	11.5 ± 2.11

ratio of urea to fatty acid is used, the DPA may complex with urea and precipitate out. The ratio of urea to fatty acids investigated was 1.5-3.0. The urea complex was allowed to crystallize at – 18 °C for 24 h. The EPA, DPA and DHA concentration of the urea complexing and non-urea complexing fractions produced from it are given in Table 4.3. DPA concentration in the urea complexed fraction was increased from 17.6 to 23.2% as the ratio of urea to fatty acids was increased from 1.5 to 3.0. In this step, non-urea complexed fraction (NUCF) was the final product, thus a high concentration of DPA in the NUCF is achieved best. However, as the concentration of DPA increased in NUCF, the concentration of DHA and EPA also increased (Figure. 4.2). Therefore, as the urea to fatty acid ratio increased from 1.5 to 2.0, the concentration of DPA increased from 17.6 to 20.1%, with a parallel increase in the concentration of DHA from 2.86 to 3.49% and EPA from 15.2 to 18.3% (Table 4.3). However, the concentration of DPA in NUCF did not increase with the ratio of urea to fatty acid from 2.5 to 3.0. At the same time, the concentration of DPA in UCF increased from 0.65 to 0.98%. This result demonstrated that DPA is more readily complexed with urea as the ratio of urea to fatty acid increased form 2.5 to 3.0. The concentration of EPA and DHA still increased as the ratio of urea to fatty acid increased from 2.5 to 3.0 (Table 4.3). The results demonstrate that the DPA concentration increased up to 24%. In order to have a high DPA concentration in the final product, the ratio of urea to fatty acid for producing the UCF in this step was 2.5.

The yield of the NUCF is varied for different urea to fatty acid ratios. The

Figure 4.2 The relationship between fatty acid concentration and ratio of urea to fatty acids in the second step. The Y value represents the concentration of fatty acids (weight %) in non-urea complexed fraction. Symbols are: ▲, EPA; ■, DPA; and ♦, DHA.


Concentration of fatty acid (Weight %)
30
25
20
15
10
5
0
1
2
3
4
Ratio of urea to fatty acid

yield decreased from 15.6 to 11.5% as the urea to fatty acid ratio increased from 1.5 to 3.0. The recovery efficiency of DPA in NUCF was up to 78% of that present in the UCF in the first step. The recovery efficiency of EPA and DHA in UCF was 83 and 67 %, respectively.

The main fatty acid compositions of the original seal blubber oil and that of the concentrate produced from seal blubber oil are given in Table 4.4 and Table 4.5. The major fatty acids present in the seal blubber oil were 16:1 (17.55%), 18:1 (21.62%) and 20:1 (12.30%) (Table 4.5). However, the major fatty acids present in the concentrates were 16:1, EPA and DPA. DPA was enriched from 4.03% to 24.02% in the concentrate. EPA was also enriched from 7.48% to 20.77%. However, DHA was decreased from 8.32% to 3.85% (Table 4.4).

Monounsaturated fatty acids (MUFA) found in the original seal blubber oil were 16:1, 18:1 and 20:1. The total content of MUFA in the product was decreased from 50% to 20% following the two steps urea crystallization process. The monounsaturated fatty acids present in concentrate were dominated by 16:1. These results demonstrated that, among the saturated and monounsaturated fatty acids, the longer-chain fatty acids complexed with urea more readily than shorter chain fatty acids. Ackman *et al.* (1988) observed a similar behaviour with fatty acids of redfish oil.

However, it was difficult to remove all of the saturated fatty acids and monounsaturated fatty acids in order to obtain a 100% PUFA concentrate. Ratnayake *et al.* (1988) have also reported that complete removal of saturated fatty acids by urea

complexation may be impossible since some of the shorter chain saturated fatty acids do not complex with urea during the crystallization process. The present results also confirm that some of the shorter chain saturated fatty acids (10:0, 12:0 and 14:0) could not be removed completely from seal blubber oil by this process (Table 4.5). Furthermore, the content of monounsaturated fatty acid in the concentrate produced was still high (e.g. 16:1 at 19%), similar to that in the original seal blubber oil.

In earlier studies, urea complexation method was used to concentrate all PUFA present in the samples (Wanasundara and Shahidi, 1999). As a consequence, the concentration of DPA in the resultant preparation was not higher than that of EPA and DHA. For example, urea complexation was used to prepare ω3 PUFA concentrates from seal blubber oil (Wanasundara and Shahidi, 1999). Among the major ω3 PUFAs present, DHA was found almost exclusively (65.2%) in the non urea complexed fraction (NUCF) of seal blubber oil under selected experimental conditions. Urea complexation of seal blubber oil, under optimum process conditions, gave a total PUFA content of 92.3% in the NUCF. Under certain conditions, the concentration of DPA reached as high as 12%. However, DHA still was the main fatty acid under all experimental conditions tested.

Table 4.4. The contents of EPA, DPA and DHA in the original free fatty acids, first and second steps of urea complexation process for production of concentrates.

Material	EPA (%)	DPA (%)	DHA (%)
Original fatty acids	7.48±0.22	4.03±0.15	8.32±0.17
First step concentrates (UCF)	3.10±0.56	3.78±0.32	0.71±0.16
Second step concentrates (NUCF)	20.77±1.58	24.02±1.05	3.85±0.45

Table 4.5. Fatty acid composition of the original oil and the concentrate.

Fatty acid	Original oil (%)	Concentrates (%)
16:0	7.34 ± 0.38	0.15 ± 0.05
16:1 ω7	17.55 ± 1.21	18.86 ± 0.90
18:1 ω9	21.62 ± 0.83	0.36 ± 0.11
18:3 ω3	0.6 ± 0.13	2.93 ± 0.21
20:1 ω9	12.30 ± 0.32	0.1 ± 0.04
20:5 ω3	7.48 ± 0.22	20.77 ± 1.58
22:5 ω3	4.03 ± 0.15	24.02 ± 1.05
22:6 ω3	8.32 ± 0.17	3.85 ± 0.45

4.2 Enzymatic synthesis of acylglycerols

4.2.1 Enzyme activity

Three commercial enzymes from *Candida antarctica*, *Mucor miehei* and *Pseudomonas sp.* were screened for their ability to synthesize acylglycerols from glycerol and free fatty acids at 30 °C in hexane. These lipases catalyzed esterification reaction to various extent. The degree of synthesis attained with various lipases was in the order of *Candida antarctica* > *Mucor miehei* > *Pseudomonas sp.* The lipase from *Pseudomonas sp.* were less effective in this reaction. The lipase from *Candida antarctica* gave the highest degree of synthesis (89.0% after 24 h). Thus, this lipase was selected for subsequent experiments to determine optimal synthesis conditions. Since lipases from *Candida antarctica* and *Mucor miehei* were used in the immobilized form, it should be noted that their activity might have been affected by the immobilization process.

4.2.2 Effect of temperature on esterification reaction

Table 4.6 illustrates the effect of temperature on lipase-catalyzed synthesis by three lipases examined. The temperature range tested was 20-50 °C. The degree of synthesis increased as the temperature increased for all three enzymes. However, response of three enzymes to temperature was varied. For enzyme PS30 from *Pseudomonas sp.*, the degree of synthesis was 26.21% at 20 °C. At 30

°C, the degree of synthesis rapidly increased to 51.56% (Table 4.6). As temperature increased from 30 °C to 40 °C or 50 °C, the degree of synthesis did not increase very fast. The other two enzymes gave similar results. However, compared to enzyme PS30 from *Pseudomonas sp.*, the degree of synthesis of enzyme IM60 from *Mucor miehei* was 30% higher at the same temperature. The degree of synthesis enzyme SP435 from *Candida antarctica* was 40% higher than PS30 from *Pseudomonas sp.*.

For all three enzymes, when the temperature was higher than 30 °C, the degree of synthesis did not change much. For enzyme PS30 from *Pseudomonas sp.*, the degree of synthesis was 51.87% at 30 °C and 62.14% at 50 °C. The degree of synthesis increased about 20% as the temperature increased from 30 to 50 °C. On the other hand, the enzyme IM60 from *Mucor miehei* and enzyme SP435 from *Candida antarctica* did not afford similar results. The increase in the degree of synthesis was less than 2% as the temperature increased from 30 to 50 °C. These results are also supported by other studies. Li and Ward (1993) reported that the degree of synthesis increased from 87.5 to 91.4% as the temperature increased from 30 to 50 °C for lipase IM60, reflecting an increase of about 4%. Thus, increasing the temperature is not an efficient way to move the reaction forward to products for the two immobilized enzymes, IM60 from *Mucor miehei* and SP435 from *Candida antarctica*.

Table 4.6. The effect of temperature on the degree of synthesis of acylglycerols from glycerol and free fatty acids with different enzymes.

Temperature (^{0}C)	Degree of Synthesis (%)		
	PS30	IM60	SP435
20	26.21 ± 2.83	59.10±2.83	68.35±1.06
30	51.56 ± 5.39	81.12±4.52	88.98±1.40
40	52.87 ± 10.79	80.86±1.32	89.29±0.38
50	62.14 ± 0.81	81.89±2.48	90.05±1.57

It is interesting to note that enzyme activity is still very high even at 50 °C for all three enzymes. Table 4.5 shows that the highest degree of synthesis occurred at 50 °C for all three enzymes. This finding lends further support to those reported by Akoh and Huang (1995). The higher temperature optimum for *Candida antarctica* enzyme was probably partly due to the fact that immobilization conferred greater thermostability to this enzyme. Kosugi and Azuma (1994) used an immobilized lipase from *Candida antarctica* for production of pure TAG from EPA or DHA with glycerol. The rate of TAG formation was reported to be faster at 60 °C even though the TAG yield was the same at 40 and 60 °C. Li and Ward (1993) reported that the degree of esterification increased with temperature in the range of 0 to 50 °C for lipase IM60 from *Mucor miehei.* However, the optimum temperature for acylglycerol synthesis by lipase PS30 from *Pseudomonas sp.* was 30 °C. As the temperature increased from 30 to 50 °C, the degree of synthesis decreased from 85.5 to 25.6%. In this study, the optimum temperature for acylglycerol synthesis by lipase PS30 from *Pseudomonas sp.* was also 50 °C. This difference may have been caused by the different source of enzymes or different reaction conditions. Temperature effects on lipase-catalysed ester synthesis were reported to be dependent on the reaction medium, enzyme source and substrate (Welsh *et al.*, 1989). Lipase PS30 from *Pseudomonas sp* is not an immobilized lipase. These

results may demonstrate that this enzyme can tolerate temperature as high as 50 °C under conditions employed in this study.

4.2.3 Time course

Time course experiments are useful in monitoring the progress of enzyme-catalyzed reactions. They are also used to determine the shortest time necessary to obtain the highest degree of synthesis and to minimize the production costs. These sets of experiments were conducted at 30 °C for different time periods.

Figure 4.3 shows the changes in the degree of synthesis with time for different lipases. Acylglycerols were successfully synthesized using all three enzymes as the biocatalyst. However, the degree of synthesis was not the same for different lipases at same time point. Lipase SP435 from *Candida antarctica* gave the highest degree of synthesis for every time point from the beginning to the end. Lipase IM60 from *Mucor miehei* gave lower degree of synthesis at a given time point. Lipase PS 30 from *Pseudomonas sp.* gave the lowest degree of synthesis. Time course results also demonstrated that lipase SP435 from *Candida antarctica* was a better enzyme for synthesis of acylglycerols using this particular substrate.

For lipases SP435 and IM60, the degree of synthesis was more than 80% after 12 h of incubation in hexane (Figure 4.3). For lipase PS30, the degree of synthesis depended more on the reaction time. Based on these results, the lipase SP435 was selected for production of acylglycerols from PUFA concentrates.

4.2.4 Effect of mole ratio of glycerol to free fatty acids on the degree of synthesis

The effects of mole ratio of glycerol to free fatty acids on the degree of synthesis as catalyzed by the three lipases were investigated. The experiments were conducted at 30 °C for 24 h. Glycerol to fatty acids mole ratios were studied. The ratios of glycerol to fatty acids were 27, 13.5, 7, 3.5, 2, and 1. The results are shown in Figure 4.4. As the number of moles of glycerol increased, the degree of synthesis was also increased. For lipase SP435, the degree of synthesis increased very fast as the mole ratio of increased from 1:1 to 13.5:1. At a mole ratio of 1:1, the degree of synthesis was about 20%. When the mole ratio was 13.5:1, the degree of synthesis was over 90%. At mole ratios lager than 13.5, the degree of synthesis remained relatively constant. For lipase IM60 from *Mucor miehei,* the degree of synthesis also increased very fast as the mole ratio increased from 1:1 to 7:1. At a mole ratio of 1:1, the degree of synthesis was less than 20%, but it increased to >65% at a mole ratio of 7:1. However, the degree of synthesis increased much slower when the mole ratio was

Figure 4.3 Time course of esterification by lipases. The reaction was conducted at 30 °C. Symbols are: ▼, lipase IM60; ●, lipase SP435; and ○, lipase PS30.

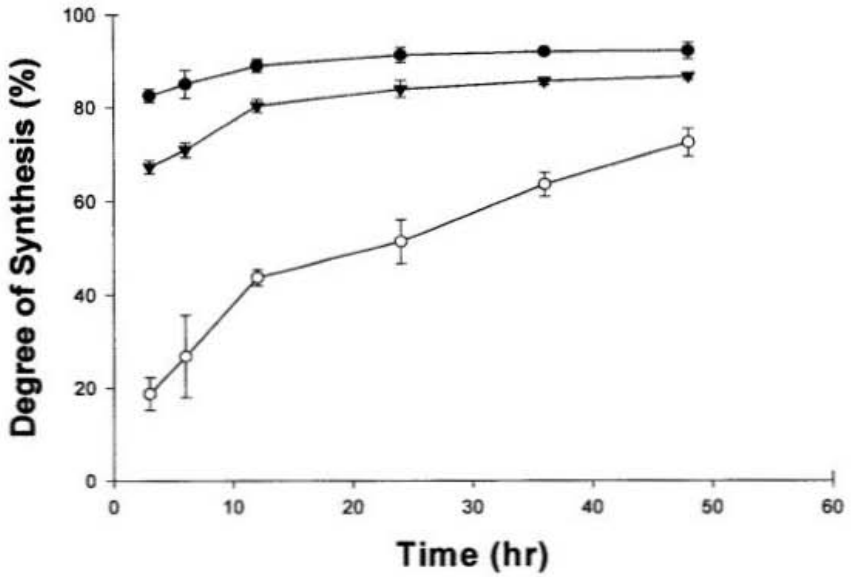
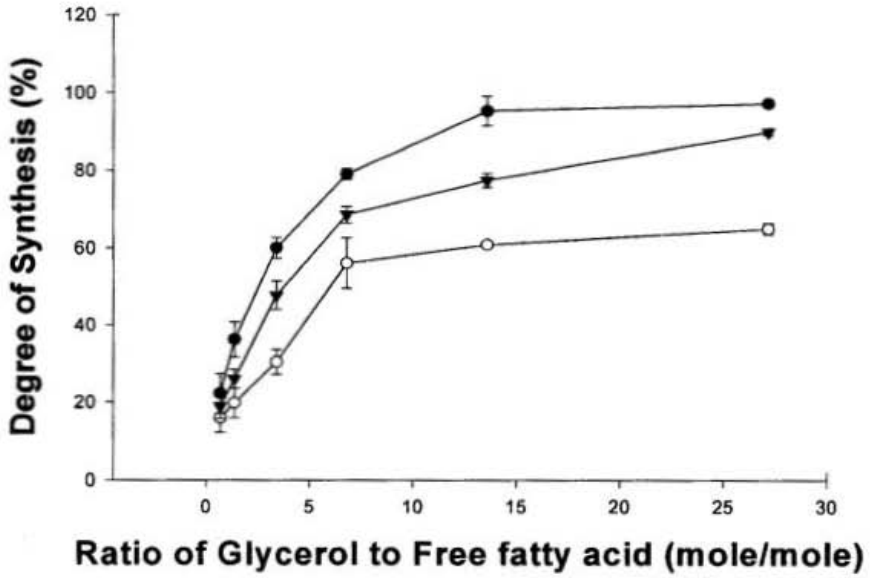

Degree of Synthesis (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (hr)

Figure 4.4 The effects of glycerol content on the degree of synthesis of acylglycerols by lipases. The reaction was conducted at 30 °C for 24 h. Symbols are: ▼, lipase IM60; ●, lipase SP435; and ○, lipase PS30.


Degree of Synthesis (%)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Ratio of Glycerol to Free fatty acid (mole/mole)

larger than 7:1. The degree of synthesis was more than 65% when the mole ratio was 7:1. The degree of synthesis was approximately 80% when the mole ratio was increased to 27:1. Unlike the lipase SP435, the degree of synthesis of which remained almost constant at mole ratios of greater than 13.5:1, the degree of synthesis still increased for lipase IM60. For lipase PS30, the effect of mole ratio on the degree of synthesis was similar to that of lipase IM60. At low mole ratios, the degree of synthesis increased faster, and at high mole ratios, the degree of synthesis increased much slower. Compared to a mole ratio of 1:1, the degree of synthesis increased by more than 4 times at a glycerol to fatty acids ratio of 13.5:1 for lipases SP435 and IM60. However, this resulted in the formation of more mono- and diacylglycerols as compared to triacylglycerols. In cases when the formation of triacylglycerols is intended, the ratio of free fatty acids to glycerol should be in the opposite direction.

Li and Ward (1993) have reported that the glycerol content affects the degree of synthesis. The results of both lipases PS30 and IM60 were similar to those obtained in this study. At low mole ratios of glycerol to fatty acids (about 2:1), the degree of synthesis was 14.5 and 29.2% for lipase PS30 and lipase IM60, respectively. At high mole ratios (7:1), the degree of synthesis was higher in this study and reached 89.2% for lipase PS30.

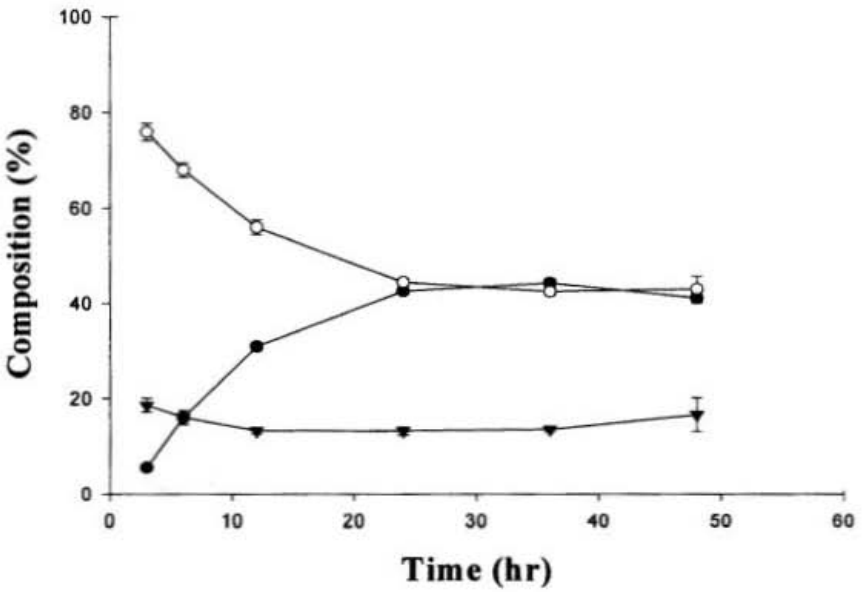
4.2.5 Distribution of acylglycerols synthesized by lipases

4.2.5.1 Distribution of acylglycerols synthesized by lipase SP435

4.2.5.1.1 Effect of reaction time on the distribution of acylglycerols

In the esterification reaction, the products formed were mixtures of triacylglycerol, diacylglycerols and monoacylglycerols. The proportions of TAG, DAG and MAG in the product may vary according at different reaction time periods. Therefore, time course experiments are not only useful in monitoring the progress of enzyme-catalyzed reactions, but are also useful in monitoring the component in the product. The detailed changes the composition of lipids in the product mixture during the time course of esterification reaction by lipases were investigated in this study. As described previously (Section 4.2.3), the esterification reactions were carried out at 30 °C for various reaction time periods. The reaction mixture contained 2.0 g glycerol, 0.4 g of fatty acid concentrate, 2.0 mL n-hexane, water and lipase (50 mg). The acylglycerols synthesized by lipase 435 were extracted by diethyl ether. The components of acylglycerols were identified and quantified by TLC-FID in this study. The results showed that the relative content of triacylglycerols was dependent on the reaction time (Figure 4.5). At a short time period, diacylglycerols were much higher than triacylglycerols and monoacylglycerols. At all reaction periods tested, monoacylglycerols were always at a low level (less than 20%) and nearly constant. For diacylglycerols, the proportion went down as the reaction

Figure 4.5 Distribution of acylglycerols synthesized by lipase SP435 over different time periods. The reaction was conducted at 30 °C. Symbols are: ●, TAG; ○, DAG; and ▼, MAG.


100
80
60
40
20
0
Composition (%)
0
10
20
30
40
50
60
Time (hr)

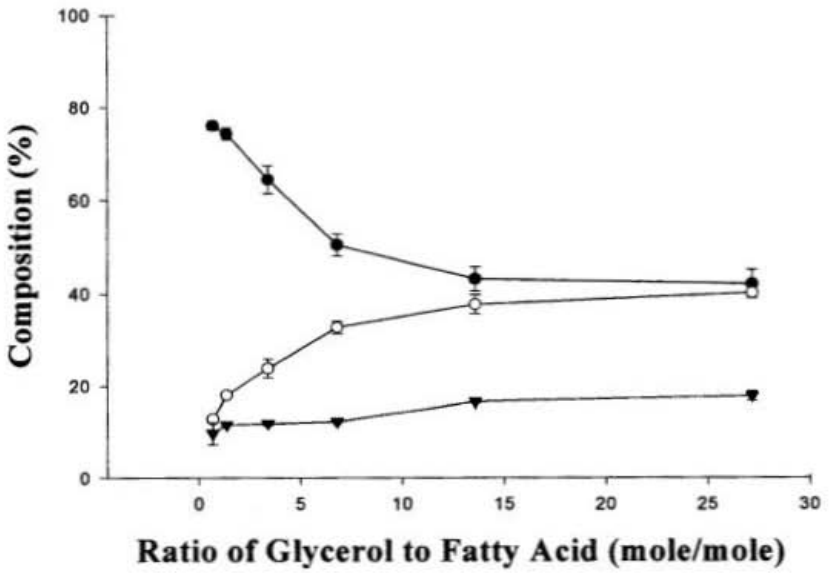
progressed. At 3 h time point, the proportion of diacylglycerols in the product was more than 75%. As the reaction progressed to 24 h, the proportion of diacylglycerols decreased to nearly 40%. From 24 h to 48 h, the proportion of diacylglycerols remained relatively constant. On the other hand, the proportion of triacylglycerols increased as the reaction continued. At 3 h time point, the proportion of triacylglycerols in the product was less than 5%. After 24 h, the proportion of triacylglycerols reached 40%. Similarly, the proportion of triacylglycerols reached a plateau from 24 h to 48 h. The results show that the relative content of triacylglycerols will also reach a maximum value. For synthesis of acylglycerols, the more triacylglycerols in the product is considered better.

4.2.5.1.2 Effect of the ratio of glycerol to fatty acids on the distribution of acylglycerols

The ratio of glycerol to fatty acids also affected the distribution of acylglycerols synthesized by lipases. Theoretically the mole ratio of glycerol to fatty acids in esterification reaction would be one to three. However, in order to move the reaction equilibrium to product, increasing relative concentration of one of the reactants is the best way. In this study, because glycerol is much cheaper than ω3 fatty acids concentrate, the way to increase the degree of medium. The results shown in Section 4.2.4 already demonstrated that the

concentration of glycerol did enhance the degree of synthesis dramatically. As described previously (Section 4.2.4), the esterification reactions were carried out at 30 °C for 24 h. The reaction mixture contained 0.4 g of n-3 fatty acids concentrate, 2.0 mL n-hexane, water, lipase and various amounts of glycerol. The components of acylglycerols were also identified and quantified by TLC-FID. Figure 4.6 shows that the proportion of triacylglycerols decreased as the ratio of glycerol to fatty acids increased. At a mole ratio of glycerol to fatty acids of 1:1, the proportion of triacylglycerols in the resultant product was more than 75%. When the mole ratio of glycerol to fatty acids increased to 13.5:1, the proportion of triacylglycerols in the product decreased dramatically to around 40%. Further increasing of the mole ratio of glycerol to fatty acids to 27:1, the proportion of the resultant triacylglycerols reached a plateau. However, the proportion of diacylglycerols increased as the ratio of glycerol to fatty acids increased. At a mole ratio of glycerol to fatty acids of 1:1, the proportion of diacylglycerols in the resultant was less than 15%. When the ratio of glycerol to fatty acids was increased to 13.5:1, the proportion of diacylglycerols was increased very fast and reached nearly 38%. When the mole ratio of glycerol to fatty acids was further increased to 27:1, the proportion of diacylglycerols in the product increased only to a very small degree, similar to that observed for triacylglycerols. Theoretically, when a large amount of

Figure 4.6 Distribution of acylglycerols synthesized with different amounts of glycerol using lipase SP435. The reaction was conducted at 30 °C. Symbols are: ●, TAG; ○, DAG; and ▼, MAG.


Composition (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
Ratio of Glycerol to Fatty Acid (mole/mole)

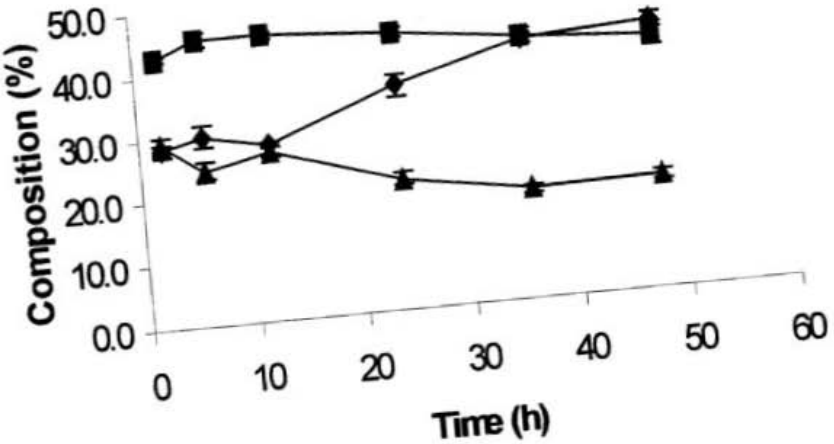
excessive glycerol is present in the reaction medium, monoglycerols should have a large proportion. Interestingly, the proportion of monoglycerols in the product increased very slowly as the mole ratio increased from 1:1 to 27:1. At a mole ratio of glycerol to fatty acids of 12 1:1, the proportion of monoacylglycerol was about 10% and at 27:1, this was increased to 16%. Formation of low levels of monoacylglycerols in the product may be dictated by the properties of the lipase in use.

4.2.5.2 Distribution of acylglycerols synthesized by lipase PS30

4.2.5.2.1 Effect of reaction time on the distribution of acylglycerols

The detailed changes in the composition of the product mixture during the time course of esterification reaction by lipase PS30 were investigated. The esterification reactions were carried out under the same condition as described in Section 4.2.5.1.1, except that lipase PS30 was used instead. The results showed that the relative content of triacylglycerols was dependent on the reaction time (Figure 4.7). Over a short period, diacylglycerols were present in higher amounts than triacylglycerols and monoacylglycerols, similar to that observed for lipase SP435. At all reaction periods tested, monoacylglycerols were always at a low level and their content decreased slightly as time progressed. However, compared to lipase SP435, the proportion of triacylglycerols and monoacylglycerols was high in short reaction times. For diacylglycerols, the

Figure 4.7 Distribution of acylglycerols synthesized by lipase PS30 over different time periods. The reaction was conducted at 30 °C. Symbols are: ♦, TAG; ■, DAG; and ▲, MAG.

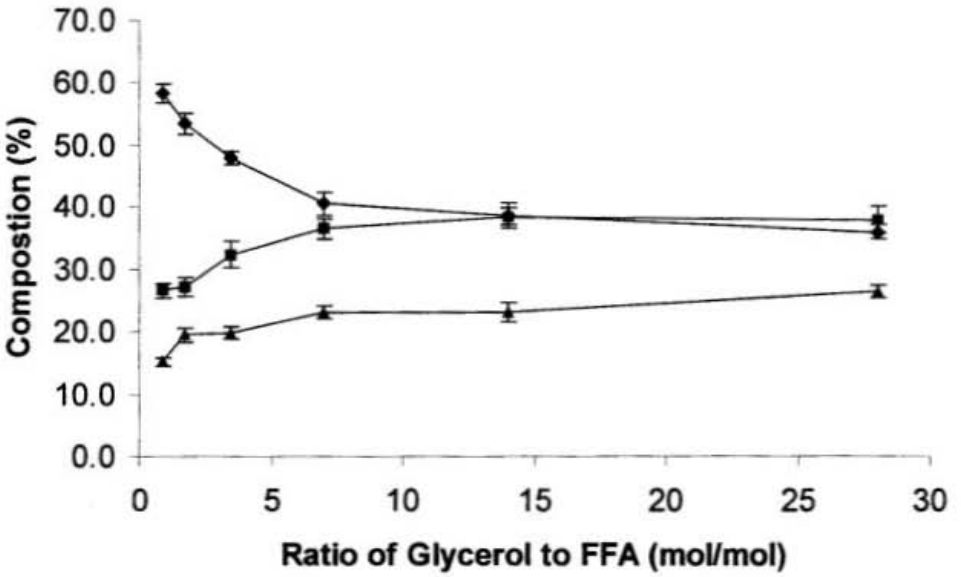

Composition (%)
50.0
40.0
30.0
20.0
10.0
0.0
0
10
20
30
40
50
60
Time (h)

proportion went down slightly as the reaction progressed. At 3 h time point, the proportion of diacylglycerol in the product was more than 45%. As the reaction progressed to 48 h, the proportion of diacylglycerol decreased to less than 40%. On the other hand, the proportion of triacylglycerols increased as the reaction continued. At 3 h time point, the proportion of triacylglycerols in the product was about 28%, which is much higher than that of lipase SP435 (5%). After 48 h, the proportion of triacylglycerols reached 40%, which is, relatively speaking, a maximum value.

4.2.5.2.2 Effect of the ratio of glycerol to fatty acids on the distribution of acylglycerols

The influence of the ratio of glycerol to free fatty acids on the changes in the proportion of acylglycerols synthesized by lipase PS30 was investigated. The esterification reactions were carried out under the same condition as described in Section 4.2.5.1.2 except that the enzyme used was lipase PS30. Figure 4.8 shows that the proportion of triacylglycerols decreased as the ratio of glycerol to fatty acids increased, similar to that observed for lipase SP435. However, the proportion of triacylglycerols in resultant product was different. At a mole ratio of glycerol to free fatty acids of 1:1, the proportion of triacylglycerols in the reaction product was less than 60%, which is less than that in the lipids

Figure 4.8 Distribution of acylglycerols synthesized with different amounts of glycerol using lipase PS30. The reaction was conducted at 30 °C. Symbols are: ♦, TAG; ■, DAG; and ▲, MAG.


70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Compostion (%)
0
5
10
15
20
25
30
Ratio of Glycerol to FFA (mol/mol)

synthesized by lipase SP435. When the mole ratio of glycerol to fatty acids increased to 13.5:1, the proportion of triacylglycerols in the product decreased dramatically to around 40%. Further increasing the mole ratio of glycerol to fatty acids to 27:1, brought about a small decrease in the proportion of triacylglycerols in the product. However, the proportion of diacylglycerols increased as the ratio of glycerol to fatty acids increased. At a mole ratio of glycerol to fatty acids of 1:1, the proportion of diacylglycerols in the resultant product was less than 30%. Upon increase of the ratio of glycerol to fatty acids to 13.5:1, the proportion of diacylglycerols was increased very fast, reaching nearly 38%. When the mole ratio of glycerol to fatty acids was further increased to 27:1, the proportion of diacylglycerols reached a plateau. The proportion of monoacylglycerols in the product increased faster than that of lipids synthesized by lipase SP435 as the mole ratio increased from 1:1 to 27:1. At a mole ratio of glycerol to fatty acids of 1:1, the proportion of monoacylglycerol was about 15%, but at a ratio of 27:1 the proportion of monoacylglycerols was more than 25%.

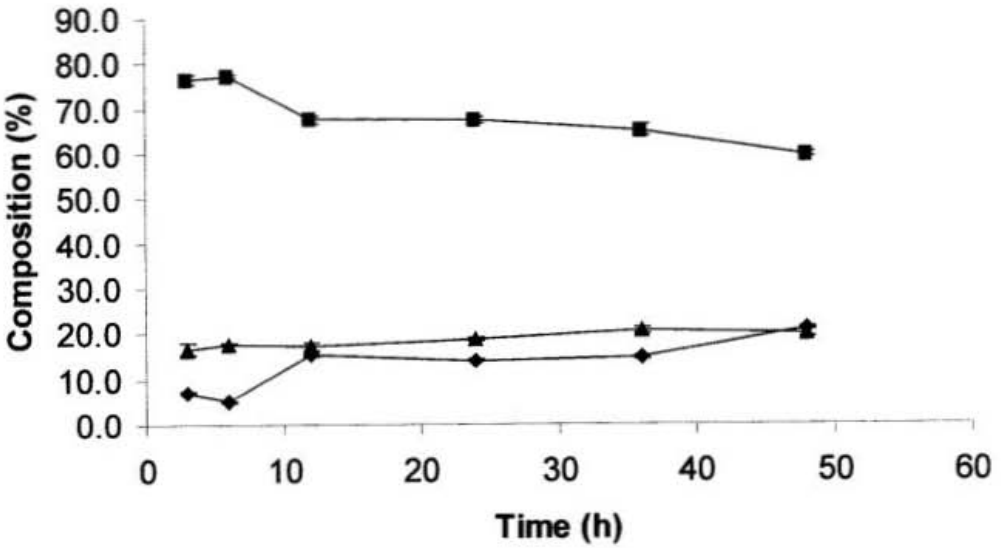
4.2.5.3 Distribution of acylglycerols synthesized by lipase IM60

4.2.5.3.1 Effect of reaction time on the distribution of acylglycerols

The changes in the composition of lipids in the product mixture during the time course of esterification reaction by lipase IM60 were investigated. The esterification reactions were carried out under the same experimental conditions

as described in Section 4.2.5.1.1, but instead using lipase IM60. The relative content of triacylglycerols was also dependent on the reaction time (Figure 4.9). However, this dependence was quite different from the esterification reaction by lipases PS30 and SP435. At a short time period, the concentration of diacylglycerols in the product mixture was also higher than those of triacylglycerols and monoacylglycerols, similar to that of lipases SP435 and PS30. However, their proportion went down slightly as the reaction progressed. At 3 h time point, the proportion of diacylglycerols in the product was more than 75%, which is much higher when compared with lipases SP435 and PS30. As the reaction progressed to 48 h, the proportion of diacylglycerols in the product decreased to about 60%, which is still higher than those for lipases SP435 and PS30. On the other hand, the proportion of triacylglycerols increased as the reaction continued. At 3 h time point, the proportion of triacylglycerols in the product was about 10%. Compared to that of lipases SP435 and PS30, it was in the middle. After 48 h, the proportion of triacylglycerols reached 22%. Among products synthesized by the three lipases, the proportion of triacylglycerols in products synthesized by lipase IM60 was the lowest. At all reaction periods tested, monoacylglycerols were always at a low level and remained relatively constant.

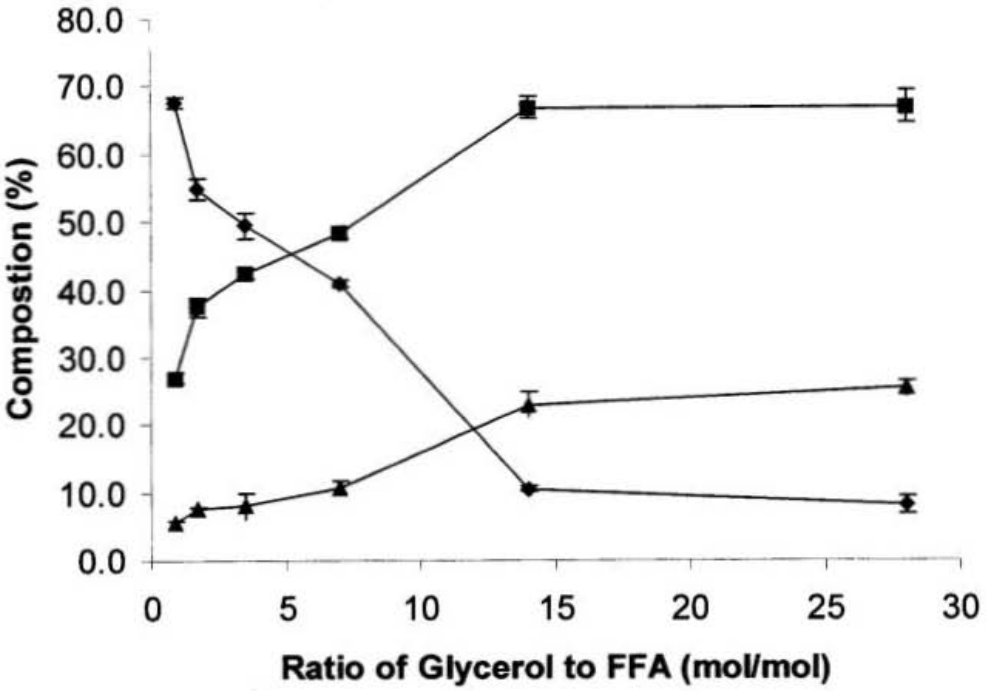
Figure 4.9 Distribution of acylglycerols synthesized by lipase IM 60 over different time periods. The reaction was conducted at 30 °C. Symbols are: ♦, TAG; ■, DAG; and ▲, MAG.


90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Composition (%)
0
10
20
30
40
50
60
Time (h)

4.2.5.3.2 Effect of the ratio of glycerol to free fatty acids on the distribution of acylglycerols

The influence of the ratio of glycerol to free fatty acid on the changes in the proportion of acylglycerols produced by lipase IM60 was investigated. The esterification reactions were carried out under the same conditions as those described in Section 4.2.5.1.2, but using lipase IM60 instead. Figure 4.10 shows that the relative proposition of triacylglycerols decreased as the ratio of glycerol to fatty acids increased, similar to that observed for lipases SP435 and PS30. However, the proportion of triacylglycerols in the product was different. At a mole ratio of glycerol to free fatty acids of 1:1, the proportion of triacylglycerols in the resultant product was more than 65% and in between that for lipases SP435 and PS30. When the mole ratio of glycerol to free fatty acids increase to 13.5:1, the proportion of triacylglycerols in the product decreased dramatically to around 12%. Compared to other two lipases (lipase PS30 and lipase SP435), the proportion of triacylglycerols in acylglycerols synthesized by lipase IM60 was much lower. Increasing the mole ratio of glycerol to fatty acid further to 27:1, resulted in a minor decrease in the proportion of triacylglycerols in the product. However, the proportion of diacylglycerols increased as the ratio of glycerol to fatty acids increased. At a mole ratio of glycerol to free fatty acids of 1:1 the proportion of diacylglycerols in the product was less than 30% and at a ratio of 13.5:1, the proportion of diacylglycerols was increased very fast to nearly 70%, much higher than that for the other two lipases. When the mole

Figure 4.10 Distribution of acylglycerols synthesized with different amounts of glycerol using lipase IM60. The reaction was conducted at 30 °C. Symbols are: ♦, TAG; ■, DAG; and ▲, MAG.


80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Compostion (%)
0
5
10
15
20
25
30
Ratio of Glycerol to FFA (mol/mol)

ratio of glycerol to free fatty acids was further increased to 27:1, the proportion of diacylglycerols in the resultant product also reached a plateau. At a mole ratio of glycerol to fatty acids of 1:1, the proportion of monoacylglycerol was about 5%, but it increased to more than 25% when the ratio of glycerol to fatty acids reached to 27:1.

4.2.6 Fatty acid composition of acylglycerols synthesized by different lipases

The fatty acid composition of acylglycerols synthesized by different lipases, using a GC procedure, is shown in Table 4.7. For acylglycerols synthesized by lipase SP435, the difference of fatty acid composition between synthesized acylglycerols and concentrate was insignificant. The EPA, DPA and DHA concentration was 21.03, 24.69, and 3.81%, respectively. For acylglycerols synthesized by lipase PS30, the fatty acid composition was slightly different from that of the concentrate and those using the other two enzymes. The EPA concentration in acylglycerols synthesized by lipase PS30 was 16.31%, somewhat lower than that of the concentrate (20.77%).

Table 4.7 Fatty acid composition of acylglycerols synthesized by lipases.

Fatty acids	Concentrate (%)	Acylglycerols synthesized by SP435 (%)	Acylglycerols synthesized by PS30 (%)	Acylglycerols synthesized by IM60 (%)
16:0	0.15±0.05	0.26±0.08	0.19±0.05	0.28±0.09
16:1ω7	18.86±0.90	19.22±0.72	18.68±1.01	19.21±1.32
18:1ω9	0.36±0.11	0.30±0.03	0.35±0.09	0.25±0.12
18:3ω3	2.83±0.21	2.93±0.15	3.05±0.17	2.97±0.10
20:5ω3	20.77±1.58	21.03±0.89	16.31±0.92	21.55±0.95
22:5ω3	24.02±1.05	24.69±1.02	25.22±0.87	24.40±0.93
22:6ω3	3.83±0.45	3.81±0.26	4.58±0.36	2.27±0.22

4.2.7 Positional distributions of fatty acids of triacylglycerols

The fatty acid composition of the Sn-2 and Sn-1 + Sn-3 positions of the triacylglycerols performed by pancreatic lipase hydrolysis are shown in Table 4.8. Pancreatic lipase quite specifically hydrolyzes the fatty acids esterified to the primary positions of the triacylglycerols, thus yielding 2-monoacylglycerols, the fatty acid composition of which accurately reflects that of position 2 in the original triacylglycerols. The results of this study showed that DPA, DHA and EPA were fairly evenly distributed over all three positions of TAG molecules. For example, at the Sn-1 and Sn-3 positions, the DPA content was about 23.66% and it was 24.36% at position 2. The concentration of DPA in these position is similar to the concentration of DPA in the TAG synthesized by lipase SP435 (23.65%) (Table 4.8). The positional specificity of *Candida antarctica* depends on the type of reactants. In some reactions, this enzyme functions as a nonspecific lipase whereas in others it shows *sn*-1,3 positional specificity (Novo Nordisk, 1999). The results of this study showed that under assay conditions employed, this enzyme acts as a nonspecific lipase.

Table 4.8 Positional distribution of fatty acids in triacylglycerols (TAG) synthesized by lipase SP435.

Fatty Acid	TAG (%)	Sn-1 + Sn-3 (%)	Sn-2 (%)
16:0	0.28±0.10	0.26±0.06	0.29±0.08
16:1ω7	19.52±1.21	19.28±0.98	19.46±1.13
18:1ω9	0.54±0.08	0.50±0.07	0.51±0.06
18:3ω3	3.35±0.24	3.28±0.14	3.31±0.20
20:5ω3	22.19±1.56	22.32±1.05	22.25±0.99
22:5ω3	23.65±1.25	23.66±1.58	24.36±1.43
22:6ω3	3.32±0.36	3.26±0.06	3.48±0.17

4.3 Oxidative stability of acylglycerols synthesized by lipase SP435

The enzymatically synthesized oils procured under optimum conditions were assessed for their oxidative stability by accelerated oxidation under Schaal oven conditions at 60 °C and over 96 hour period. The samples were removed periodically at 0, 6, 12, 24, 48 and 72 h from the oven, cooled to room temperature, flushed with nitrogen, capped and stored at -20°C until analysed. Progression of oxidation was monitored by determination of conjugated dienes (CD), propanal content and TBARS (thiobarbituric acid reactive substances) values. In this study, three samples were used – original seal blubber oil, and concentrate of fatty acid esters and triacylglycerols synthesized by lipase SP435.

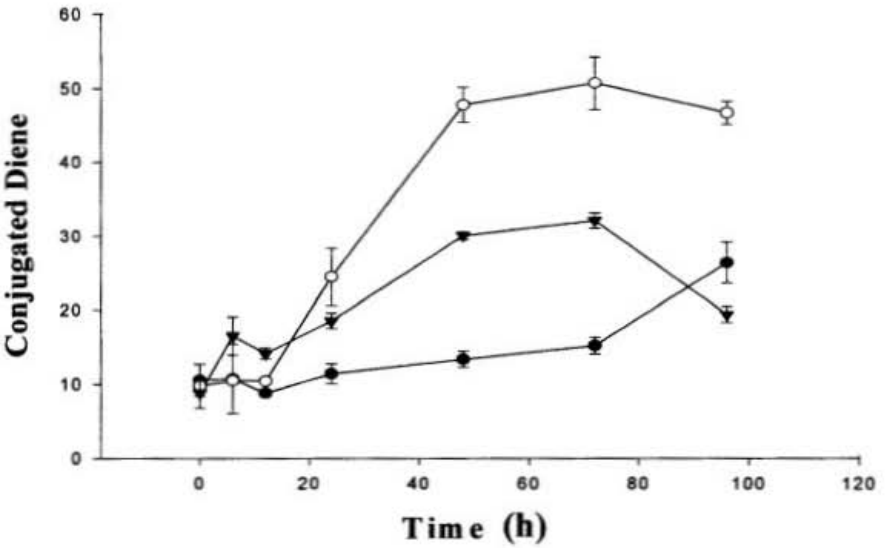
4.3.1 CD value

The formation of conjugated dienes (CD), as reflected in the absorption readings at 234 nm, during storage of original seal blubber oil (SBO), fatty acid ethyl esters (FAEEs) and acylglycerols synthesized by lipase SP435 is shown in Figure 4.11. All samples, followed an increasing trend in their CD content throughout the experimentation, but their rates of formation of CD were different. The fatty acid esters and synthesized acylglycerols had higher conjugated diene values than the original seal blubber oil. A similar pattern was reported by St. Angelo *et al.* (1975) and Khatoon and Krishna (1998) for decomposition products of peanut butter and safflower oil, respectively. As lipid peroxidation proceeded, more primary products such as hydroperoxides and

conjugated dienes were formed. Initially, the CD of original seal blubber oil, fatty acid esters and synthesized acylglycerols were 9.8, 8.7, and 9.2, respectively. However, the corresponding CD values increased to 14.0, 50.2 and 31.5, respectively, after 3 days of storage under Schaal oven conditions at 60 °C. However, the CD value decreased to a lower value after 3 days of storage. The high content of CD in the concentrated preparations may arise from their high proportions of readily oxidizable ω3 PUFA as compared to the original seal blubber oil. CD value of fatty acid esters was higher than that of the synthesized acylglycerols. The higher probability of double bonds in fatty acid esters for attack by O_2 than acylglycerols, especially triacylglycerols and diacylglycerols, may be responsible for this observation.

The formation of lipid hydroperoxides normally coincides with the formation of CD upon autoxidation (Farmer and Sutton, 1943; Jackson, 1981). Since hydroperoxides, the primary products of lipid oxidation (Labuza, 1971), are unstable, thus measurement of peroxide value (PV) provides information only about the initial oxidation potential of the oil. The CD assay is faster than peroxide value determination and does not depend on chemical reactions such as colour development for its determination. The conjugated dienes are formed due to the shift in the double bond position upon oxidation of lipids

Figure 4.11 Conjugaged diene value of seal blubber oil, ●; fatty acid esters (ethyl ester); ○; and synthesized acylglycerols, ▼. Experiments were carried out at 60 ^{o}C for 96h.


Conjugated Diene
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Time (h)

containing dienes or polyenes (Logani and Davies, 1980). Thus, conjugated diene values can be used as an index of stability for lipid-containing foods after extraction of these lipids (St. Angelo et al., 1975).

When the storage time exceed 3 days under Schaal oven conditions at 60 °C, the CD values of fatty acid esters and synthesized acylglycerols decreased. The lesser generation of primary products than their degradation to secondary products may be responsible for this observation.

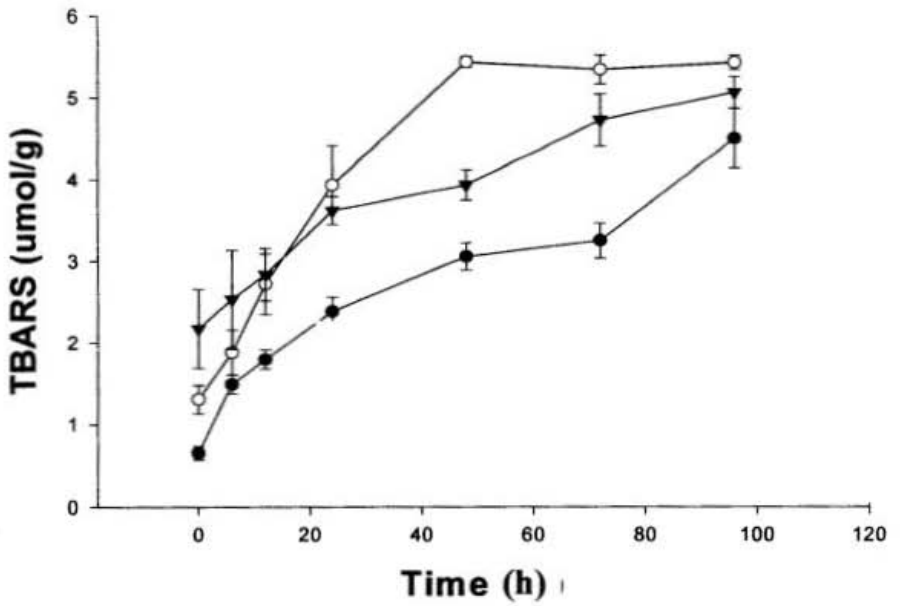
4.3.2 TBARS values

The TBARS test, which measures secondary products of lipid oxidation, is the most frequently used indicator for monitoring stability of edible oils. Production of TBARS of the original seal blubber oil and fatty acid esters and synthesized acylgycerols are given in Figure 4.12. The TBARS values, expressed as μmol malonaldehyde equivalents per g oil, increased progressively over the entire storage period. Furthermore, the products of ω3 fatty acid concentrates had significantly higher ($p \leq 0.05$) TBARS values than those of their original seal blubber oil. The observed changes in TBARS (Figure 4.12) were similar to the trends observed for changes in the conjugated dienes (Figure 4.11). The general increase in TBARS values during the storage period is due to the fact that as oxidation proceeds, lipid hydroperoxides break down to produce secondary oxidation products, as supported by the findings of Park *et al.* (1996) and Strange *et al.* (1997). The main compounds in the oils reacting with the 2-thiobarbituric

acid (TBA) reagent are malonaldehyde as well as alkenals and alkadienals. The high content of TBARS in fatty acid esters and synthesized acylglycerols is due to the high proportions of PUFA in the oils examined.

The TBA method is nonspecific and subject to interference by many substances (Hoyland and Taylor, 1991). Bucknall *et al.* (1978) studied the reaction of hydroxyl radicals with D-glucose at C-5 and C-6 and showed the formation of malonaldehyde. Also, the reaction of TBA with saturated aldehydes, i.e., butanal, hexanal and heptanal produced pigments with absorption maxima at 455 nm (yellow) and 532 nm (red) (Kosugi and Kikugawa, 1986). Nonetheless, TBA test remains to serve a useful purpose when relative values and trends are required rather than absolute values for oxidation.

Figure 4.12 TBARS value of the original seal blubber oil ●; fatty acid esters (ethyl ester); ○; and synthesized acylglycerols, ▼. Experiments were carried out at 60 °C for 96h.


TBARS (umol/g)
0
1
2
3
4
5
6
0
20
40
60
80
100
120
Time (h)

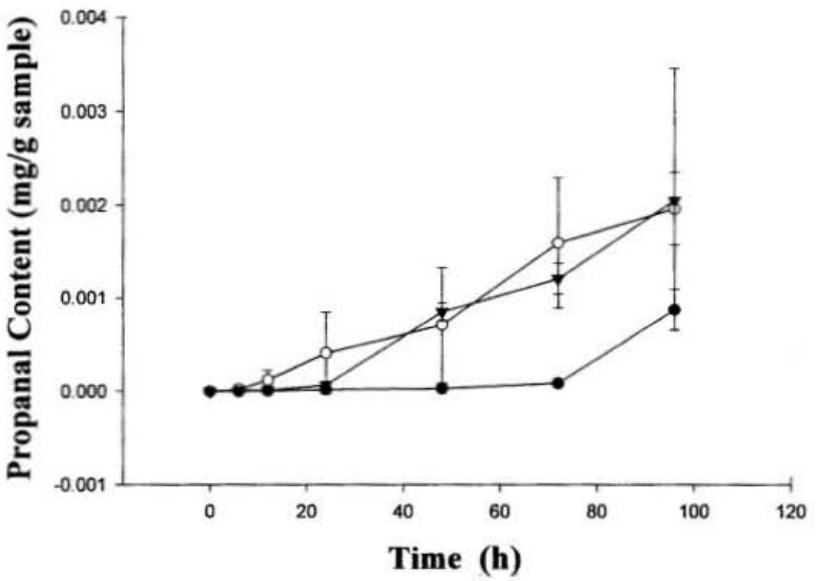
4.3.3 Headspace value

Hydroperoxides present in the oil may be decomposed to volatiles. Thus, an alternative approach for assessing lipid oxidation in edible oils is to measure the headspace carbonyl compounds formed upon degradation of fatty acid hydroperoxides. PUFA in vegetable and fish oils produce a complex array of low- and high-molecular weight secondary products that provide rich sources of volatile compounds (Frankel, 1993b). The volatile carbonyl compounds have been implicated as being significant contributors to off-flavour development in fats and oils. The concentration of hexanal and propanal, in particular, has been suggested as being the primary markers of oxidative deterioration of vegetable and fish oils, respectively (Frankel *et al.*, 1994; Shahidi and Spurvey, 1996). Gas chromatographic analyses of oils have revealed that propanal is the predominant volatile derived from ω3 PUFA (Frankel, 1993; Shahidi and Wanasundara, 1998). The individual volatile compounds were tentatively identified by comparing the relative retention times of GC peaks with those of commercially available standards. Quantitative determination of dominant aldehyde, namely propanal, was accomplished using 2-heptanone as an internal standard.

The amount of propanal produced in modified oils increased with storage time (Figure 4.13). Under similar conditions, original seal blubber oil showed no significant formation of propanal in three days. In the fourth day, the content of propanal increased

dramatically. Compared to the original seal blubber oil, the content of propanal in fatty acid esters and synthesized acylglycerol increased immediately from the beginning of storage.

Figure 4.13 Propanal content original seal blubber oil, •; fatty acid esters (ethyl ester); ○; and synthesized acylglycerols, ▼. Experiments were carried out at 60 °C for 96h.


Propanal Content (mg/g sample)
0.004
0.003
0.002
0.001
0.000
-0.001
0
20
40
60
80
100
120
Time (h)

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

To date, most of the published literature on production of PUFA has focused on docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). However, in this study, a facile two-step urea complexation process was introduced which produced PUFA with a high proportion of DPA using seal blubber oil as the starting material. Depending on the composition of the oil to be concentrated, different ratios of urea to fatty acid were used. In the first urea complexation step, a high urea to fatty acid ratio was employed because DPA complexed with urea better than DHA and EPA. In the second urea complexation step, a low ratio of urea to fatty acid was used because it was more difficult for DPA to complex with urea under conditions employed.

At each step, the effect of the ratio of urea to fatty acids was investigated. In the first step, a ratio of urea to fatty acids of 3.5 gave the best results. The recovery efficiency of DPA in UCF (urea complexed fraction) was 67.8% of that present in the original seal blubber oil. The concentration of DPA in UCF was up to 4.16%. The recovery efficiency of DHA and EPA in UCF was 6.27 and 30.2%, respectively. In the second step, a ratio of urea to fatty acid of 2.5 gave the best results. The concentration of DPA in NUCF (non-urea complexed fraction) was 24%. The recovery efficiency of DPA in NUCF was up to 78% of that present in the UCF

in the first step. The recovery efficiency of DHA and EPA in UCF was 67 and 83%, respectively. These results demonstrate that the two step complexation method was a feasible technique to concentrate DPA or other fatty acids, depending on the composition of the original oil. While in this study the ratio of urea to fatty acids was investigated, other factors such as reaction time and reaction temperature could be examined in order to possibly improve the concentration and recovery efficiency of DPA. Use of this procedure in conjunction with other techniques for concentration and purification of DPA might prove beneficial.

As a second phase in this study, acylglycerols were synthesized from free fatty acids and glycerol using an enzymatic method. Three commercial enzymes from Candida antarctica, Mucor miehei and Pseudomonas sp. were screened for their ability to synthesize acylglycerols from glycerol and free fatty acids at 30 °C in hexane. The lipase from Candida antarctica gave the highest degree of synthesis (89.0% after 24 h). Comparing to chemical means, mild conditions of enzymatic synthesis may better serve for production of specialty lipids containing PUFA (polyunsaturated fatty acids).

The effect of various reaction parameters such as the amount of glycerol, reaction temperature and reaction time on the properties of synthesized oil were studied. The degree of synthesis increased as the temperature increased for all three enzymes. As temperature increased from 30 to 40 or 50 °C, the degree of synthesis increased slightly. Thus, increasing the

temperature was not an efficient way to move the reaction forward to products at temperatures above 30 °C. After 24 h, the degree of synthesis reached the highest level for all three lipases. However, small differences between lipases were noticed. Time course results demonstrated that the lipase SP435 from Candida antarctica was a better enzyme for the synthesis of acylglycerols using the particular substrate under investigation. Reaction time also influenced the composition of acylglycerols. At all reaction periods tested, monoacylglycerols were always at a low and nearly constant level (less than 20%). For diacylglycerols, their proportion decreased as the reaction progressed. On the other hand, the proportion of triacylglycerols increased as the reaction continued. The proportion of triacylglycerols reached a plateau at 24 h. Formation of low levels of monoacylglycerols in the product may be dictated by the properties of the lipase employed. As the number of moles of glycerol increased, the degree of synthesis was also increased. At mole ratios lager than 13.5, the degree of synthesis remained relatively constant. At a mole ratio of glycerol to fatty acids of 1:1, the proportion of triacylglycerols in the resultant product was more than 75%. Further increase of the mole ratio of glycerol to fatty acids from 13.5:1 to 27:1 did not change the amount of the resultant triacylglycerols to any marked degree.

The oxidative stability of acylglycerols synthesized by lipases employed in this study was assessed. Compared to the free fatty acid esters,

synthesized acylglycerols had significantly lower conjugated dienes, 2-thiobarbituric acid reactive substances and propanal contents. The results of this study showed that the oxidative stability of acylglycerols was better than fatty acid esters, but worse than the original seal blubber oil. Because of the high concentration of PUFA in the synthesized acylglycerols, the products were more prone to oxidation than the original oil. In order to protect the synthesized oil from oxidation, it is recommended that appropriate antioxidants be added to the synthesized acylglycerols in order to prevent their oxidation and to extend their shelf-life. Studies on the absorption and metabolism of structured lipids so produced may also prove beneficial.

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