ENZYME-ASSISTED SYNTHESIS OF STRUCTURED LIPIDS CONTAINING LONG-CHAIN OMEGA-3 AND OMEGA-6 POLYUNSATURATED FATTY ACIDS

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BY

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THIS WORK IS DEDICATED

TO

MY LOVING WIFE, CHARMANIE

ABSTRACT

Lipase-catalyzed acidolysis of borage (*Borago officinalis* L.) and evening primrose (*Oenothera biennis* L.) oils with long-chain ω3-polyunsaturated fatty acids (LCω3-PUFA), namely docosahexaenoic acid (DHA) and/or eicosapentaenoic acid (EPA), in organic solvents was studied. Six microbial lipases from *Candida antarctica*, *Mucor miehei*, *Pseudomonas sp.*, *Aspergillus niger*, *Candida rugosa* and *Thermomyces lanuginosus* were initially used as biocatalysts for the acidolysis reaction. Among the enzymes tested, an immobilized lipase, Novozym-435 from *Candida antarctica* showed the highest degree of DHA incorporation (25.8-28.7%, after 24 h) in borage oil (BO) and evening primrose oil (EPO). However, the maximum incorporation of EPA (28.7-30.7%, after 24 h) in both oils was achieved with lipase PS-30 from *Pseudomonas sp.* In another study, incorporation of EPA+DHA into BO and EPO was carried out by first screening of lipases listed above; lipase PS-30 from *Pseudomonas sp.* was the most efficient enzyme examined (31.7-32.7% EPA+DHA incorporation, after 24 h).

Effects of variation of reaction parameters, namely enzyme load, temperature, time course, mole ratio of substrates and type of organic solvents were monitored for the most effective enzymes, those from *Candida antarctica* and *Pseudomonas sp.*, as the biocatalysts of choice. Incorporation of DHA and/or EPA increased significantly ($p \leq 0.05$) with increasing the amount of enzyme. As the incubation time progressed, incorporation of these fatty acids was also increased, similar to that observed when the

temperature and mole ratio of substrates increased. The highest DHA (37.4-39.7%) or EPA (37.4-39.9%) incorporation occurred at a mole ratio of 1:3 (oil/ DHA or EPA). However, maximum incorporation of EPA+DHA (54-57.5%) occurred at a mole ratio of 1:2:2 (oil/EPA/DHA) and then remained constant between mole ratios of 1:2:2 and 1:3:3. Among solvents examined, *n*-hexane served best in giving rise to 25.5-27.4% DHA, 25.2-26.8% EPA and 27.8-33.3% EPA+DHA incorporation in the oils. However, solvent-free reactions also gave satisfactory incorporation of 18.1-20.5% DHA, 18.6-20.4% EPA and 23.4-28.8% EPA+DHA in the oils tested.

Response surface methodology (RSM) was used to obtain a maximum yield of DHA, EPA and EPA+DHA incorporation while using the minimum amount of enzyme possible. Process parameters studied were the amount of enzyme (100-350 units), reaction temperature (20-60°C) and reaction time (6-30 h). All experiments were carried out according to a face-centred cube design. Under optimum conditions (162-165 units of *Candida antarctica* enzyme; 43-50°C; 25-27 h), incorporation of DHA was 35.6% in BO and 33.5% in EPO. Optimization of acidolysis of oils with EPA, gave rise to a maximum of 35.4 and 33.9% EPA incorporation in BO and EPO, respectively, at 299-309 units of *Pseudomonas sp*. enzyme, reaction temperature of 40-44 °C and reaction time of 25-27 h. Similarly, maximum incorporation of EPA+DHA in BO (35.5%) and EPO (33.6%) was attained at 278-299 units of *Pseudomonas sp* enzyme, at 42-43 °C after 24-26 h.

In another study, enzymatically modified oils, produced under optimum reaction conditions, were classified using thin-layer chromatography-flame ionization detection (TLC-FID). The results showed that the content of TAG (85.1-95.1%) was much higher than that of the DAG (2.4-11.2%) and MAG (0.3-9.7%) in the structured lipids so produced. Because free fatty acids were removed by NaOH after the acidolysis reaction, they were not detected by TLC-FID. The products were also separated by TLC and the fatty acid compositions of their corresponding isolated bands analyzed by gas chromatography. Results showed that DHA and/or EPA were mainly located in TAG fractions of enzymatically modified oils (33.2-35.4% DHA; 32.5-33.2% EPA and 33.6-35.5% EPA+DHA in DHA, EPA and EPA+DHA-enriched oils, respectively). The TAG fractions also contained appreciable proportions of γ-linolenic acid (GLA) (11.0-17.1 and 7.5-7.6% in DHA, EPA and EPA+DHA-enriched BO and EPO, respectively).

Stereospecific analysis was carried out to establish positional distribution of fatty acids in the TAG of DHA, EPA and EPA+DHA-enriched oils. In DHA-enriched BO, DHA was randomly distributed over the three positions of TAG while GLA was mainly esterified at the *sn*-2 and *sn*-3 positions. In DHA-enriched EPO, however, DHA and GLA were concentrated in the *sn*-2 position. In EPA-enriched BO, EPA was randomly distributed over the three positions of TAG, similar to that observed for DHA. In EPA-enriched EPO, however, this fatty acid was mainly located at the primary positions (*sn*-1 and *sn*-3) of TAG. In both oils, GLA was preferentially esterified at the *sn*-2 position. In

EPA+DHA-enriched BO. EPA and DHA were mainly esterified at the *sn*-1 and *sn*-3 positions of TAG while GLA was mainly located at the *sn*-2 position. In EPA+DHA-enriched EPO. GLA was mainly located at the *sn*-2 and *sn*-3 positions; EPA was preferentially esterified at the *sn*-1 and *sn*-3 positions, while DHA was found mainly at the *sn*-3 position.

The oxidative stability of enzymatically modified oils as well as their unmodified counterparts was evaluated under Schaal oven conditions at 60°C over a 96 h storage period. Conjugated dienes (CD), 2-thiobarbituric acid reactive substances (TBARS) and headspace volatiles were determined. In addition, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was used to monitor relative changes in the proton absorption pattern of the fatty acids of oils during storage. Among the oils examined, enzymatically modified products gave rise to higher CD and TBARS as compared to those of their unmodified counterparts. The main volatile compounds identified in enzymatically modified oils were acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal and nonanal. However, the main volatile compound found in unmodified oils was hexanal. The contents of propanal and hexanal produced by enzymatically modified oils were significantly higher ($p \leq 0.05$) than those produced by their unmodified counterparts. These results suggested that the modified oils were more prone to oxidation than their unmodified counterparts.

The double bond index (DBI) and methylene bridge index (MBI), represent the number of double bonds and *bis* allylic methylene bridge positions in PUFA, respectively, were calculated. DBI and MBI of enzymatically modified oils were significantly ($p \leq 0.05$) higher than those of their unmodified counterparts. During oxidation of oils, DBI and MBI were decreased. Regression analysis was carried out to correlate various parameters of oxidation (CD, TBARS, hexanal and propanal contents) with DBI and MBI of oils; a negative correlation ($r = 0.574$-0.975; $p \leq 0.1$-0.05) existed between these variables.

Relative changes of aliphatic to olefinic (R_{ao}) and aliphatic to diallylmethylene (R_{ad}) protons ratios, during oil oxidation, were determined by ^{1}H NMR spectroscopy. An increase in R_{ao} and R_{ad} values was obtained over the entire storage period. A highly significant correlation ($r = 0.930$-0.992; $p \leq 0.005$) existed between the CD values and changes in R_{ao} and R_{ad} during oxidation of all oils. The correlation coefficient between TBARS and changes in R_{ao} and R_{ad} values was in the range of 0.779 -0.983 ($p \leq 0.05$). A high correlation ($r = 0.948$-0.996; $p \leq 0.005$) was found between hexanal content and R_{ao} and R_{ad} of oils. Propanal content was also highly correlated ($r = 0.950$-0.990; $p \leq 0.005$) with R_{ao} and R_{ad}. This suggests that ^{1}H NMR could be used to simultaneously estimate both primary and secondary oxidation changes in native and enzymatically modified oils.

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AA	- Arachidonic acid
AOCS	- American Oil Chemists' Society
ALA	- α-linolenic acid
ANOVA	- Analysis of variance
AV	- Acid value
BHA	- Butylated hydroxy anisole
BHT	- Butylated hydroxy toluene
BCO	- Blackcurrant oil
BO	- Borage oil
CA	- Capric acid
CD	- Conjugated dienes
CPL	- *Carica papaya* latex
DAG	- Diacylglycerol
DBI	- Double bond index
DGLA	- Dihomo-γ-linolenic acid
DHA	- Docosahexaenoic acid
DPA	- Docosapentaenoic acid
EDHA	- Ethyl ester of docosahexaenoic acid
EDTA	- Ethylenediaminetetraacetic acid

EEPA	- Ethyl ester of eicosapentaenoic acid
EPA	- Eicosapentaenoic acid
EPO	- Evening primrose oil
FAMEs	- Fatty acid methyl esters
FDA	- Food and Drug Administration
FFA	- Free fatty acids
FID	- Flame ionization detector
FTIR	- Fourier transform infrared
GC	- Gas chromatography
GLA	- γ-Linolenic acid
GLM	- General linear model
HDL	- High-density lipoprotein
HUFA	- Highly unsaturated fatty acids
IV	- Iodine value
LA	- Linoleic acid
LCFA	- Long-chain fatty acids
LCT	- Long-chain triacylglycerols
LDL	- Low-density lipoprotein
MAG	- Monoacylglycerols
MA	- Malonaldehyde

MBI - Methylene bridge index

MCFA - Medium-chain fatty acids

MCT - Medium-chain triacylglycerols

NMR - Nuclear magnetic resonance

NUCF - Non-urea complexed fraction

OSI - Oil stability instrument

PC - Phosphatidylcholine

PE - Phosphatidylethanolamine

PG - Propyl gallate

PGE - Prostaglandin

PGI - Prostacyclin

POSt - 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol

PUFA - Polyunsaturated fatty acids

PV - Peroxide value

RSM - Response surface methodology

RSREG - Response surface regression

SAS - Statistical analysis system

SCFA - Short-chain fatty acids

SCT - Short-chain triacylglycerols

SD - Standard deviation

SL	- Structured lipids
StOSt	- 1,3-distearoyl-2-monolein
SV	- Saponification value
TAG	- Triacylglycerols
TBA	- Thiobarbituric acid
TBARS	- Thiobarbituric acid reactive substances
TLC-FID	- Thin layer chromatography-flame ionization detection
TOTOX	- Total oxidation
TPN	- Total parenteral nutrition
TX	- Thromboxane
UCF	- Urea complexed fraction

CHAPTER 1

INTRODUCTION

Structured lipids (SL), sometimes referred to as "nutraceutical lipids, functional lipids, or designer lipids" (Akoh, 1996a; 1998), may be produced from short-, medium- or long-chain triacylglycerols (TAG), any animal or vegetable oils, using lipase-assisted reactions with specific fatty acids or their alkyl esters, for potential use in food and nutritional preparations (Lee and Akoh, 1998a). There is a need for enzymatic synthesis of these specialty lipids which are currently receiving research and industry attention.

Modification of lipids, using lipases, provides a useful means to improve the characteristics of TAG. Through enzyme-catalyzed reactions, it is possible to incorporate desired fatty acids or their alkyl esters onto a specific position of the TAG. However, chemically catalyzed reactions do not possess this regiospecificity due to the random nature of the reaction (Akoh, 1995). Thus, lipase-catalyzed reactions may provide regio- or stereospecific SL for nutritional, medical and food applications (Akoh, 1995; 1998).

Borage (*Borago officinalis* L.) and evening primrose (*Oenothera biennis* L.) have been the subject of increasing agricultural interest because of the potential market for their seed oils, which contain γ-linolenic acid (GLA; 18:3ω6). Borage seeds contain 28-38% oil, of which 20-25% is GLA (Beaubaire and Simon, 1987), whereas seeds of evening primrose contain 17-25% oil (Wolf *et al.*, 1983) with 8-10% GLA (Fieldsend, 1996). GLA is one of the essential ω6 polyunsaturated fatty acids (PUFA) and must be

provided in food because it cannot be easily synthesized within the body (Horrobin, 1990). In recent years, much research has been directed toward the production of GLA (Mukherjee and Kiewitt, 1991; Rahmatullah *et al.*, 1994a,b; Shimada *et al.*, 1998; Huang *et al.*, 1999) for applications such as curing or treating of certain skin-related diseases as well as diabetes, hypertension, cancer, premenstrual syndrome, inflammatory and cardiovascular disorders (Horrobin, 1990; 1992a; 1994; Gurr, 1997). In humans and in other mammals, GLA is the first metabolite formed during the bioconversion of linoleic acid (LA; 18:2ω6) to prostaglandins by the desaturation at the C-6 position *via* the enzyme Δ6-desaturase (Horrobin, 1990). Individuals who lack Δ6-desaturase suffer from a number of diseases due to an imbalance in GLA production and formation of successive metabolites that lead to prostaglandins. The physical and chemical properties as well as nutritional and medicinal importance of GLA have recently been reviewed (Gunstone, 1992; Horrobin, 1992b)

On the other hand, the ω3 fatty acids, including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), also 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). Apart from having a favourable effect on several disorders, DHA is also considered important for proper

function of the nervous system and visual acuity (Crawford *et al.*, 1999; Rotstein *et al.*, 1999; Kim and Edsall, 1999) in humans and serves as one of the most abundant components of structural lipids of the brain (Salem *et al.*, 1986; Carlson, 1995; Hamazaki *et al.*, 1999).

Several sources of information suggest that humans originally consumed a diet with a ratio of ω6 to ω3 fatty acids of about 1:1 whereas today this ratio is ranged from 10:1 to 20:1 in the affluent western societies (Simopoulos, 1999). Therefore, western diets are deficient in their ω3 fatty acids content as compared with the diet on which humans evolved.

The beneficial effects of both ω6 (GLA) and ω3 (EPA and DHA) fatty acids are attributed to eicosanoid synthesis. Incorporation of EPA and DHA from marine sources into GLA-rich borage or evening primrose oil may provide unique specialty oils for specific nutritional and clinical applications. This would be possible *via* lipase-catalyzed acidolysis reaction. Enzyme-mediated reactions have been successfully used for restructuring lipids by interesterification of ω3 fatty acids with either plant or marine oils (Lee and Akoh, 1998a,b). Use of enzymes to produce SL has an advantage over the traditional methods (chemical hydrolysis, chemical interesterification, physical blending, etc.,) since such methods involve high temperature processes which may partially destroy the natural all-*cis* ω3 PUFA *via* oxidation and *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 edible oils and of foods that contain them in order to maintain their quality and safety. Oxidation of fats and oils may be initiated by light, heat and presence of metal ions. Oxidation of oils occurs *via* a free-radical chain reaction mechanism 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 odourless. Their decomposition, however, leads to the formation of a variety of volatile compounds which result in the development of undesirable flavours and off-odours (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 analyses (Rossell, 1994). However, it is desirable to develop methods that could simultaneously determine both the primary and secondary products of lipid oxidation. Nuclear magnetic resonance (NMR) spectroscopy is considered to have the potential for quantification of the overall extent of lipid oxidation (Shahidi *et al.*, 1994; Shahidi and Wanasundara, 1997). This method measures the relative changes that occur in the NMR absorption pattern of lipid fatty acids during oxidation.

Of the several procedures considered for concentration of PUFA from oils, urea complexation is one of the most efficient and simplest techniques which may lend itself for preparation of DHA concentrates from algal oils. Based on literature evidence for enzymatic production of SL from plant oils with PUFA, it is hypothesized that SL containing GLA, EPA and DHA in the same molecule may be successfully produced from borage (BO) or evening primrose oils (EPO) with ω3 PUFA (EPA and DHA). It is generally accepted that the positional distribution of fatty acids within the TAG (*sn*-1, *sn*-2 and *sn*-3) might affect the metabolic fate of fatty acids. Hence, it is thought that knowledge of the stereospecific structure of PUFA-enriched BO and EPO is important since it influences their absorption and assimilation in the body. Since oxidative stability of oils is dictated by their degree of unsaturation, it was thought that enzyme-mediated PUFA-enriched BO and EPO might also act in a similar fashion. Furthermore, little is known about the oxidative stability of BO and EPO, and virtually nothing is known about

the oxidative stability of these oils when PUFA are incorporated into them *via* enzyme-catalyzed acidolysis reactions. To examine these hypotheses and to fill an important gap in the scientific literature regarding the synthesis, characteristics and stability of PUFA-enriched BO and EPO, several approaches were considered. The objectives of this study were: (1) to concentrate DHA from algal oil using the urea complexation technique, (2) to synthesize DHA, EPA and EPA+DHA-enriched oils from BO and EPO with ω3 PUFA (EPA and DHA), (3) to optimize the reaction conditions for synthesis of DHA, EPA and EPA+DHA-enriched oils, (4) to determine chemical characteristics of DHA, EPA and EPA+DHA-enriched oils and unmodified oils, (5) to determine the positional distribution of fatty acids in the DHA, EPA and EPA+DHA-enriched oils, and (6) to assess oxidative stability of DHA, EPA and EPA+DHA-enriched oils as well as their unmodified counterparts.

CHAPTER 2

LITERATURE REVIEW

2.1 Structured lipids and their significance

Structured lipids (SL) may be defined as triacylglycerols (TAG) restructured or modified to change the fatty acid composition and/or their positional distribution in glycerol molecules by a chemical or enzymatic process (Lee and Akoh, 1998a,b). These specialty lipids have been developed to fully optimize the benefit of various fatty acid moieties. SL have been reported to have beneficial effects on a range of metabolic parameters including immune function, nitrogen balance, and improved lipid clearance from the bloodstream (Quinlan and Moore, 1993). SL are also synthesized to improve or change the physical and/or chemical properties of TAG such as melting point, solid fat content, iodine and saponification values.

Structured lipids are often referred to as a new generation of lipid that can be considered as "nutraceutical" (Kennedy, 1991). Nutraceutical is a term used to describe foods that provide health benefits beyond those ascribed to their nutritional effects (Scott and Lee, 1996). These products may be referred to as functional foods or functional lipids if they are incorporated into products that have the usual appearance of food, but to which they may be added and provide specific health benefits (Scott and Lee, 1996). The nomenclature is still confusing and needs to be worked out by the scientists in this field.

Thus, SL can be designed for use as medical or functional foods as well as nutraceuticals (Akoh, 1996a), depending on their form of application.

One way of preparing SL may be *via* the hydrolysis of fatty acyl groups from a mixture of TAG followed by random reesterification onto the glycerol backbone (Babayan, 1987). Typically, a variety of fatty acids are used in this process, including different classes of saturated, monounsaturated and polyunsaturated fatty acids (PUFA), depending on the desired metabolic effect. Thus, a mixture of fatty acids is incorporated onto the same glycerol molecule. These manufactured lipids are structurally and metabolically quite different from the more simple, random physical mixtures of medium-chain triacylglycerols (MCT) and long-chain triacylglycerols (LCT) (Figure 2.1).

2.1.1 Synthesis of structured lipids

Chemical or enzyme-catalyzed reactions, namely esterification, interesterification, alcoholysis and acidolysis may be used to produce SL (Figure 2.2). However, the method of choice depends on the type of substrates available (Lee and Akoh, 1998a) and that of the end products required. The following sections will focus on various lipase-catalyzed reactions.

Figure 2.1 The difference between a simple physical mixture of medium-chain triacylglycerol (MCT) and long-chain triacylglycerol (LCT) and a structured lipid (SL) containing randomized medium- and long-chain fatty acids in the same glycerol molecule

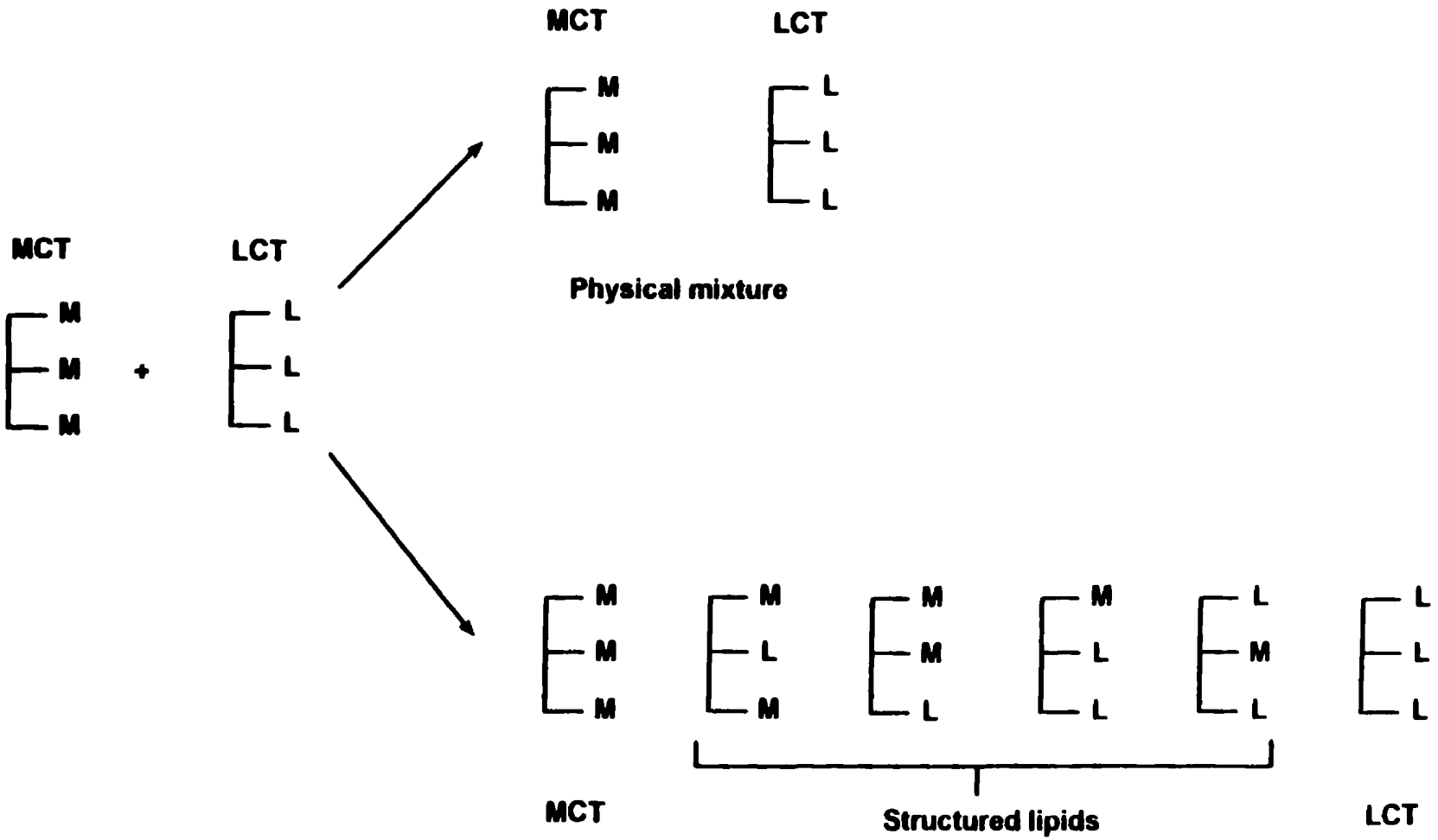
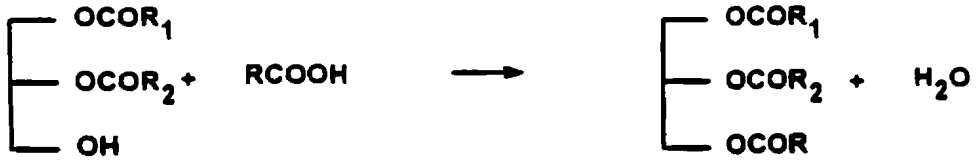

MCT
LCT
M
M
M
L
L
L
Physical mixture
MCT
LCT
M
M
M
M
L
M
M
M
L
M
L
L
L
M
L
L
L
L
MCT
Structured lipids
LCT

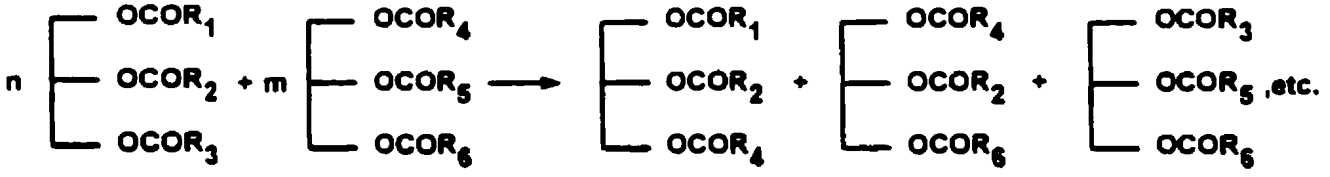
Figure 2.2 Schematic diagrams of lipase-catalyzed lipid modification strategies for the production of structured lipids (SL)

(i) Esterification

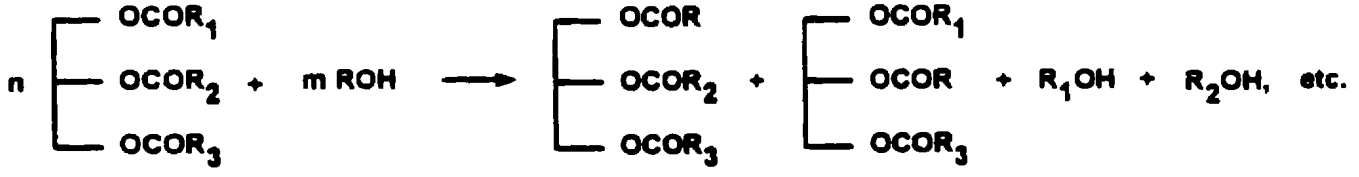


(ii) Transesterification

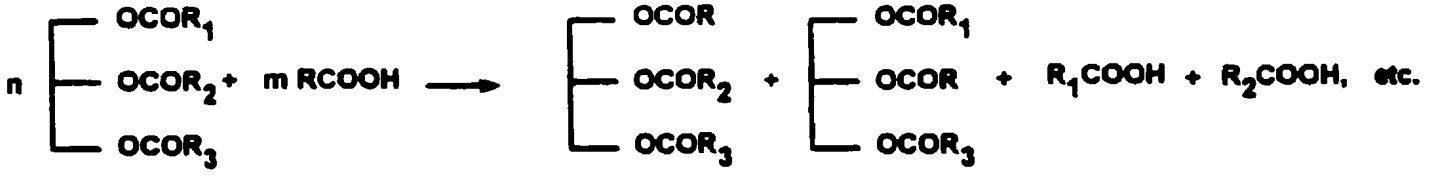
(a) Interesterification



(b) Alcoholysis



(c) Acidolysis



2.1.1.1 Esterification

Esterification is the reverse of the hydrolysis process. It is carried out by reacting fatty acids with glycerol. The reaction is reversible and proceeds to completion only if water is removed from the medium. The equilibrium between the forward reaction (hydrolysis) and the reverse reaction (esterification) is controlled by water content of the reaction mixture (Akoh, 1996a). In the presence of excess water, hydrolysis predominates, whereas under water-limiting conditions esterification is favoured (Marangoni and Rousseau, 1995; Willis and Marangoni, 1999). The accumulation of water during esterification is a concern because it may inhibit the activity of lipase and possibly enhance the hydrolysis of the formed esters. The water, which is produced during this reaction, may be continuously removed and this is usually accomplished by carrying the reaction in the presence of molecular sieves (Ergan *et al.*, 1988). However, a small amount of water is needed in the reaction to maintain the activity of the enzyme.

When the esterification reactants are properly adjusted, monoacylglycerols (MAG), diacylglycerols (DAG) and possibly TAG may be produced *via* esterification with glycerol. The products of these reactions are always a mixture of MAG and DAG and contain variable quantities of unreacted glycerol. These products have many applications, among which food, cosmetic and pharmaceutical emulsifiers and stabilizers are prominent examples (Arcos and Otero, 1996; Rosu *et al.*, 1999).

Several investigators have used selective esterification reactions to concentrate γ-linolenic acid (GLA) from borage, evening primrose and fungal oils. Schmitt-Rozieres *et al.* (1999) used selective esterification to enrich GLA (up to 68.4%) from borage oil with immobilized *Candida rugosa* lipase as the biocatalyst. GLA present in borage oil was also concentrated (up to 70%) in esterification reactions that were catalyzed by lipase from *Geotrichum candidum* (Foglia and Sonnet, 1995). The ability of the lipase from rapeseed (*Brassica napus*) to discriminate against GLA has been utilized for the enrichment of this fatty acid from the mixture of fatty acids derived from those of evening primrose oil *via* kinetic resolution during lipase-catalyzed esterification with n-butanol (Hills *et al.*, 1989). Similarly, the observed selectivity of the lipase from *Mucor miehei* against GLA has been used for concentrating this fatty acid from fatty acids of evening primrose oil (Hills *et al.*, 1990) and fungal oil (Mukherjee and Kiewitt, 1991) during lipase-catalyzed reactions.

In another study, structured TAG containing two molecules of caprylic acid (8:0) and one molecule of erucic acid (22:1ω9) were prepared by lipase-catalyzed esterification of caprylic acid and monoerucin; upon hydrogenation of the erucoyl moieties to behenoyl moieties, the resulting TAG yielded products resembling caprenin, a commercially available low-calorie SL (McNeill and Sonnet, 1995). Cerdan *et al.* (1998) carried out an in-depth study of the best reaction conditions for esterification of glycerol and ω3 PUFA concentrate using *Candida antarctica* lipase in organic solvents. Under optimum

conditions, an enriched TAG with a yield of 85% containing 27.4% eicosapentaenoic acid (EPA; 20:5ω3) and 45.1% docosahexaenoic acid (DHA; 22:6ω3) was obtained.

Wong *et al.* (2000) described enzymatic synthesis of medium-chain acylglycerols from capric acid (CA; 10:0) and glycerol using *Candida rugosa* lipase. The amount of CA converted to acylglycerols was 33.2% which included 15% dicaprin and 17.3% tricaprin. Osada and co-workers (1992) have employed lipases from *Chromobacterium viscosum* and *Candida cylindracea* for direct esterification of glycerol with individual free fatty acids (FFA) including EPA and DHA. The *Chromobacterium viscosum* lipase was superior to that of *Candida cylindracea* lipase affording 89-95% incorporation. Akoh *et al.* (1992) described the synthesis of MAG in organic solvents by lipase G from *Penicillium sp.* as the biocatalyst. This enzyme successfully catalyzed esterification of glycerol with oleic acid or EPA in hexane. Esterification at 40°C for 24 h resulted in 86.3 and 64.3 mol% incorporation of oleic acid and EPA, respectively. Esterification of glycerol with ω3 PUFA, obtained from seal blubber oil, was achieved by He and Shahidi (1997a) using *Chromobacterium viscosum* lipase. The degree of synthesis reached was up to 94%.

2.1.1.2 Transesterification

Transesterification is one of the major reactions used by the industry for modification of natural fats and oils. In its simplest form, transesterification corresponds

to an exchange of acyl groups between two TAG, resulting in the formation of new TAG that have chemical and physical properties deemed superior to those of the starting TAG (Willis and Marangoni , 1998).

Lipase-catalyzed transesterification has been applied particularly to the production of cocoa-butter-type TAG and these processes exploit the *sn*-1,3-specificity which is common among microbial lipases (Macrae and Hammond, 1985; Chong *et al.*, 1992). The types of TAG products obtainable by these reactions depend on the specificity of the lipase used.

Jang *et al.* (1998) synthesized a low-calorie SL by transesterification of triacetin with stearic acid in a solvent-free system using lipase from *Candida antarctica* (Novozym 435) as the biocatalyst. These researchers were able to obtain the highest yield (approximately 70%) of SL at a mole ratio of 1:1.4. Foglia and Villeneuve (1997) also described the synthesis of low-calorie TAG analogs based on a *Carica papaya* latex (CPL) lipase-catalyzed transesterification reaction. In a solvent-free system, an equimolar mixture of tributyrin and hydrogenated soybean oil was reacted in the presence of 10% (w/w) CPL and SL containing short- and long-chain fatty acids were obtained. Mangos *et al.* (1999) also reported the CPL-catalyzed synthesis of low-calorie SL by transesterification of hydrogenated soybean oil with triacetin.

Structured TAG of the monoacylglycerol diacetate and diacylglycerol monoacetate types are prepared using *sn*-1,3-specific lipases by transesterification of

plant oils with triacetin (Kuo and Parkin, 1995) or an alkyl acetate (Mukherjee and Kiewitt, 1996).

SL resembling TAG of human milk have been produced by transesterification of tripalmitin, derived from plant oil, with oleic acid or PUFA, obtained from plant oils, using *sn*-1,3-specific lipases as biocatalysts (Quinlan and Moore, 1993). Such TAG were found to closely mimic the fatty acid distribution of human milk and may be used in infant food formulations.

In the literature, the term 'transesterification' is often used to describe reactions that involve the exchange of acyl residues between an ester and an acid (acidolysis), an ester and an alcohol (alcoholysis), or an ester with another ester (interesterification). The following sections will describe each of these processes.

2.1.1.3 Interesterification

Interesterification is a process which is used in fats and oils to modify the physical and functional properties of TAG mixtures (Sreenivasan, 1978). Interesterification alone and in combination with other processes extends the utility of edible oils, and such modified oils are extensively employed in a wide variety of foods. There are two types of interesterification reactions presently in use- chemical and enzymatic. Enzymatic modifications rely on the use of random or regiospecific (*sn*-1,3- or *sn*-2 specific) and fatty acid-specific lipases as catalysts.

Lipase-assisted interesterification offers possibilities for transformation of lipids beyond those possible using chemical interesterification (Macrae, 1983). Enzymatic interesterification has many advantages such as mild reaction conditions, the possibility of lipase specificity and high catalytic efficiency.

Irimescu *et al.* (2000) used an interesterification reaction for synthesis of 1,3-dicapryloyl-2-eicosapentaenoylglycerol (CEC) from trieicosapentaenoylglycerol (EEE) and ethyl caprylate (EtC) using Lipozyme IM from *Rhizomucor miehei*. After some of the reaction conditions were optimized, the maximum mole content of CEC in the TAG of the reaction mixture was 91.0%.

2.1.1.4 Alcoholysis

Alcoholysis is the esterification reaction between an alcohol and an ester (Figure 2.2). During alcoholysis, hydrolysis of TAG to produce DAG and MAG can occur (Millqvist *et al.*, 1994), although the presence of small amounts of alcohol can inhibit hydrolysis.

Glycerolysis is the exchange of acyl groups between glycerol and a TAG to produce MAG, DAG and TAG (Willis and Marangoni, 1998). Enzymatic glycerolysis of fats and oils under atmospheric pressure and at nearly ambient temperatures has been investigated as an alternative method for the conventional chemical procedures used for industrial production of MAG and DAG (Noureddini and Harmeier, 1998). There are

several ways to produce MAG which are of great importance in the food industry as surface-active agents and emulsifiers. MAG may be produced by ester exchange between TAG and glycerol, or by reaction of FFA and glycerol, although only the former reaction is termed glycerolysis.

Alcoholysis has been used in the production of methyl esters from esterification of TAG and methanol with yields of up to 53% (Briand *et al.*, 1994). Alcoholysis has also been used in the production of MAG enriched with ω3 PUFA (Zuyi and Ward, 1993). Zuyi and Ward (1993) used lipase-catalyzed alcoholysis to concentrate EPA and DHA from cod liver oil. The substrates used in the reaction were cod liver oil and isopropanol. The lipase from *Pseudomonas sp.* was used as the biocatalyst. These authors were able to produce MAG containing 40% ω3 PUFA. Millqvist *et al.* (1994) also prepared MAG from TAG by an alcoholysis reaction using an immobilized *sn*-1,3 -specific *Rhizopus arrhizus* lipase.

2.1.1.5 Acidolysis

Acidolysis, the transfer of an acyl group between an acid and an ester, is an effective means of incorporating novel fatty acids into TAG (Figure 2.2). It may be used to incorporate FFA or ester forms of EPA and DHA into vegetable and fish oils to improve their nutritional properties.

Performing an acidolysis between GLA-rich FFA and borage oil acylglycerols, Huang *et al.* (1999) used an immobilized lipase from *Mucor miehei* to increase the GLA content in the acylglycerol from 52.1 to 75%. Acidolysis is also a common method for production of cocoa butter substitutes. Chong *et al.* (1992) used acidolysis to incorporate stearic acid into palmolein to produce cocoa butter-like TAG.

Jennings and Akoh (1999) used acidolysis to incorporate capric acid (CA) into the fish oil TAG using immobilized lipase, IM 60, from *Rhizomucor miehei* as a biocatalyst. The fish oil (produced by Pronova Biocare Inc., Sandefjord, Norway) originally contained 40.9% EPA and 33.0% DHA. After a 24-h incubation in hexane, there was an average of 43% incorporation of CA into fish oil, while EPA and DHA decreased to 27.8 and 23.5%, respectively. Acidolysis reaction has also been used by Akoh and Moussata (1998) to incorporate CA and EPA into borage oil using lipase from *Candida antarctica* and *Rhizomucor miehei* as biocatalysts. Higher incorporation of EPA (10.2%) and CA (26.3%) was obtained with *Rhizomucor miehei* lipase, compared to 8.8 and 15.5%, respectively, with *Candida antarctica* lipase.

The lipase-catalyzed acidolysis of a single-cell oil (produced by a marine microorganism, *Schizochytrium sp.*) containing docosapentaenoic acid (DPA; 22:5ω6) and DHA with caprylic acid (8:0) was investigated by Iwasaki *et al.* (1999). Lipases from *Rhizomucor miehei* and *Pseudomonas sp.* were used as the biocatalysts. The targeted products were SL containing caprylic acid at the *sn*-1 and *sn*-3 positions and DHA or

DPA at the *sn*-2 position of glycerol. When *Pseudomonas sp.* was used, more than 60% of fatty acids in the single-cell oil were exchanged with caprylic acid. With *Rhizomucor miehei* lipase, incorporation of caprylic acid was only 23%. The results of this study suggested that the difference in the degree of acidolysis by the two enzymes was due to their different selectivity toward DPA and DHA as well as the difference in their positional specificities.

Lipase-catalyzed acidolysis was also utilized by Haraldsson and Thorarensen (1999) to synthesize phospholipids highly enriched with ω3 PUFA using an immobilized Lipozyme from *Rhizomucor miehei*. Furthermore, Miura *et al.* (1999) investigated a rapid method for preparation of SL with a purity over 95% using an immobilized Lipozyme-IM from *Rhizomucor miehei*. In this study, acidolysis of triolein and lauric acid resulted in a TAG fraction enriched with 70% lauric acid.

2.2 Lipases in lipid modification

Lipases occur widely in nature and are active at the oil/water interface in heterogeneous reaction systems and are used for modification of lipids. Lipases, or TAG acylhydrolases (E.C. 3.1.1.3), are enzymes that preferentially catalyze the hydrolysis and synthesis of esters and TAG. The physiological function of lipases is to hydrolyze lipids for conversion to accessible energy for the cells or organisms. Lipases are ubiquitous in nature, being present in the animal, microbial and plant kingdoms. The most well known

and well-studied lipases are human pancreatic lipase, pig pancreatic lipase and several microbial lipases. The common feature that distinguishes lipases from esterases, which also catalyze the hydrolysis of ester bonds, is that lipases are activated by an interface (Verger and de Haas, 1976). In contrast to esterases, lipases show low activity towards monomeric substrates, but as soon as the substrate can form micelles or a separate phase, the activity of lipase increases dramatically; this phenomenon is called interfacial activation (Verger, 1984).

The major advantage of lipase-catalyzed reactions over those carried out with chemical catalysts lies in the fact that a wide variety of products having different composition and properties can be prepared, depending on substrate specificity or stereospecificity of the lipase used. Further advantages of lipases-catalyzed reactions include mild reaction conditions leading to reduced energy consumption and less thermal damage of reactants and products.

Although enzymes have been used for many years to modify the structure and composition of foods, they have only recently become available for large-scale use in the industry. According to enzyme manufacturers, progress in genetics and in process technology may now enable the enzyme industry to offer products with improved properties at a reduced cost (Vulfson, 1993). For industrial exploitation of enzyme-catalyzed reactions, reuse of the enzyme is certainly necessary. To this end, enzymes have been immobilized in a variety of ways. Immobilization of enzymes can simply be

accomplished by mixing an aqueous solution of the enzyme with a suitable support material and removing the water under reduced pressure; for activation of the enzyme, a small amount of water is added to the reaction mixture. Suitable support materials for enzyme immobilization include celite, acrylic resin and glass beads, among others.

Lipases may be used to catalyze interesterification reactions by restricting the amount of water in the medium whereupon the interesterification reaction predominates over hydrolysis. This is generally achieved by performing the reaction in organic solvents which also help dissolving the reactants. However, it is insufficient to only lower the water concentration; reversal of the hydrolysis reaction requires operation at a water activity (A_w) below 1 (Halling, 1984). Thus, attempts for at interesterification in a predominantly aqueous environment have resulted in a poor yield of products (Stevenson *et al.*, 1979).

2.2.1 Structure

While lipases may be derived from different sources, they all tend to have similar three-dimensional structures. Recent research, and success in crystallizing proteins, has led to the determination of structures of several lipases by X-ray crystallography. These include human pancreatic lipase (Winkler *et al.*, 1990), lipase from *Mucor miehei* (Brady *et al.*, 1990; Brzozowski *et al.*, 1991), lipase from *Geotrichum candidum* (Schrag *et al.*, 1991), lipase B from *Candida antarctica* (Uppenberg *et al.*, 1994) and lipase from

Candida rugosa (Groshulski *et al.*, 1993). In general, a lipase is a polypeptide chain folded into two domains, the C-terminal domain and the N-terminal domain. The N-terminal domain contains the active site with a hydrophobic tunnel from the catalytic serine to the surface that can accommodate a long fatty acid chain. A common structural feature among lipases is the α/β structures; alternating β-pleated sheets and α-helices (Brady *et al.*, 1990; Uppenberg *et al.*, 1994). Lipase sequences from several prokaryotic and eukaryotic organisms are known (Brady *et al.*, 1990). It has been shown that they all contain a Gly-X-Ser-X-Gly/Ala sequence, where X can be any amino acid (Boel *et al.*, 1988). This polypeptide sequence has also been found in serine proteases (Schrag *et al.*, 1991). The active site of lipases is composed of serine, aspartate/glutamate and histidine, which is in the opposite order compared to serine proteases. All lipases characterized to date have a serine as their nucleophilic residue (Uppenberg *et al.*, 1994). The catalytic site (Ser-His-Asp) is buried under surface loops folded onto the triad and stabilized by extensive hydrophobic and electrostatic interactions (Brady *et al.*, 1990). The mechanism, discussed below, is suggested to be equivalent to the mechanism of serine proteases.

An outstanding feature among these lipases is a 'lid'-region, a part of the polypeptide chain, that moves upon interaction with a hydrophobic surface. The opening of this lid is believed to be one of the key features of interfacial activation. The lid may be small, as in lipase B from *C. antarctica* (Uppenberg *et al.*, 1994), large as in lipase from *M. miehei* (Brzozowski *et al.*, 1991), or composed of two lids as in lipase from *G.*

candidum (Schrag *et al.*, 1991). The movement of the lid changes the overall surface at the entrance of the active site, making it more hydrophobic and thereby changing the lipid-binding properties (Uppenberg *et al.*, 1994). The lipase is not active unless the lid is open since the substrate cannot reach the buried active site in the closed conformation. In lipase from *G. candidum* the active site is more deeply buried, thus exhibiting a strong preference for long-chain fatty acids (Schrag *et al.*, 1991).

2.2.2 Mechanism of action

For the lipase to be active, the lid has to be open so that the active site is accessible to the substrate. The substrate, as noted earlier, an ester such as TAG, fatty ester of a monohydric alcohol or fatty acid (with H replacing in the hydroxyl group as R_2 in Figure 2.3), binds to the active site, the carboxyl carbon is positioned in close proximity to the hydroxyl oxygen in the serine side chain (Figure 2.3). This oxygen makes a nucleophilic attack on the carbonyl carbon of the substrate and a tetrahedral transition state, an acyl enzyme intermediate, is formed. 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

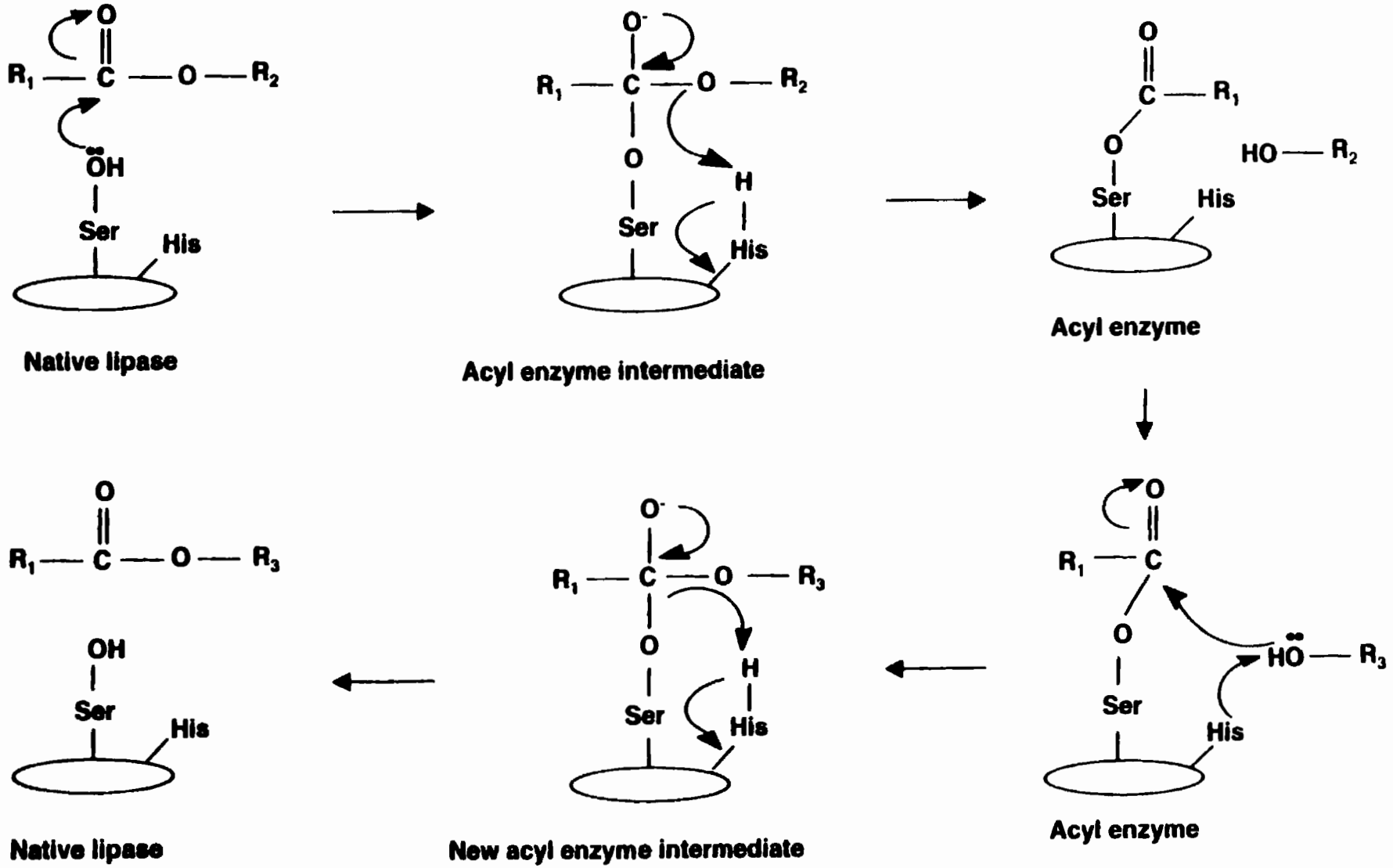
ester bond, this is called the acyl enzyme. In the next step, the reversal of the acylation of the lipase, the acyl enzyme is attacked by an alcohol or a water molecule. Again, an acyl enzyme intermediate is formed, which rearranges and a new ester is released. This type of mechanism is referred to as a Ping-Pong Bi-Bi mechanism (Reyes and Hill, 1994).

2.2.3 Lipase specificity

Specificity is a comparative difference in rates of catalysis of certain reactions. After an enzyme is identified as a lipase, several specificities within the class are identified or can be expected to occur. The main advantage of lipases, which differentiate enzymatic reactions from chemically-catalyzed reactions, is lipase specificity. Lipases have turned out to be very useful enzymes for catalyzing various types of reactions with a rather wide substrate specificity. The fatty acid specificity of lipases has been exploited to produce SL and to enrich lipids with specific fatty acids to improve the nutritional characteristics of lipids. Certain lipases display positional specificity (regiospecificity) towards fatty acids in a TAG molecule as well as fatty acid selectivity.

Usually lipases are classified into three categories in terms of their substrate specificity: random (non-regiospecific) or *sn*-1,3-specific (regiospecific) towards TAG or specific for a particular fatty acid or, more generally, a class of fatty acids. Examples of non-specific lipases include those from *Candida cylindraceae*, *Geotrichum candidum*,

Figure 2.3 The reaction mechanism for lipase-mediated transesterification


Native lipase
Acyl enzyme intermediate
Acyl enzyme
Acyl enzyme
New acyl enzyme intermediate
Native lipase

Corynebacterium acnes, *Staphylococcus aureus*, *Penicillium expansum* and *Pseudomonas cepacia* (Macrae, 1983; Villeneuve and Foglia, 1997). They catalyze complete breakdown of TAG to glycerol and FFA. DAG and MAG are intermediates in the reaction, but do not normally accumulate to a high level during the reaction, presumably because they are hydrolyzed more rapidly than TAG.

Lipases that are *sn*-1,3 specific include those from *Mucor miehei*, *Mucor javanicus*, *Aspergillus niger*, *Pseudomonas fluorescens*, *Rhizopus delemar*, *Rhizopus arrhizus* and pancreatic lipase (Macrae, 1983; Mukherjee, 1990; Villeneuve and Foglia, 1997; Gandhi, 1997). With these lipases, TAG are hydrolyzed to afford FFA, 1,2 (2,3)-DAG and 2-MAG. The rate of hydrolysis of TAG is normally faster than that of DAG, and consequently substantial quantities of both DAG and MAG accumulate during the reaction.

The lipase from *Geotrichum candidum* is an example of a lipase possessing fatty acid specificity towards long-chain fatty acids containing a *cis*-double bond in the 9-position (Jensen *et al.*, 1983; Villeneuve and Foglia, 1997). Such lipase will then hydrolyze acylglycerol esters of these fatty acids regardless of their position on the glycerol backbone. Moreover, most lipases possess enantioselectivity towards various ester, alcohol and acid substrates. This has been utilized for carrying out resolution of enantiomers from racemic mixtures of the substrates, and is highly important in organic synthesis (Sonnet, 1988). Thus, the unique specificity of *Geotrichum candidum* lipase

may be useful for production of special fatty-acid fractions. For example, γ-linolenic acid (GLA) in borage oil has been concentrated in esterification reactions catalyzed by this lipase (Foglia and Sonnet, 1995).

Short-chain fatty acid preference is also a lipase specificity that can be exploited on an industrial scale. Such lipases may be used in the production of low-calorie SL (Akoh, 1995) or in the dairy industry to obtain specific flavour components by the release of short- or medium- chain fatty acids from milk fat (Villeneuve and Foglia, 1997).

2.2.4 Applications

To date, enzyme-catalyzed reactions have been employed for production of TAG used for confectionery fat formulations and nutritional applications. In the area of confectionery fats, interesterification of high oleate sunflower oil and stearic acid using immobilized *Rhizomucor miehei* lipase produces mainly 1,3-distearoyl-2-monolein (StOSt). Other reactants may also be used for production of TAG useful as confectionery fats. In particular, there are many reports on enzymatic interesterification of mixtures of palm oil fractions and stearic acid or stearic acid esters to produce fats containing high concentrations of StOSt and POSt (Macrae, 1983). These TAG are the main components of cocoa butter (Lipp and Anklam, 1998a,b), and enzymatic interesterification processes

can produce fats with compositions and physical properties very similar to cocoa butter (Macrae, 1985).

In the area of nutritional fats, enzyme-catalyzed reactions are used to produce a human milk fat substitute for use in infant formula (Quinlan and Moore, 1993; Christensen and Holmer, 1993; Mukherjee, 1998). Acidolysis reaction of a mixture of tripalmitin and unsaturated fatty acids using a *sn*-1,3-specific lipase as a biocatalyst afforded TAG derived entirely from vegetable oils rich in 2-position palmitate with unsaturated fatty acyl groups in the *sn*-1 and *sn*-3 positions (Mukherjee, 1998). These TAG closely mimic the fatty acid distribution found in human milk fat, and when they are used in infant formula instead of conventional fats the presence of palmitate in the *sn*-2 position of the TAG has been shown to improve digestibility of the fat and absorption of other important nutrients such as calcium (Quinlan and Moore, 1993; Lucas *et al.*, 1997).

The possible application of enzyme-assisted reactions for production of lower value nonspecialty lipids such as margarine hardstocks and cooking oils has been investigated (Zainal and Yusoff, 1999). When nonspecific lipases such as *Candida cylindraceae* and *C. antarctica* are used as biocatalysts for interesterification of oil blends, the TAG products are very similar to those obtained by chemical interesterification (Macrae, 1983). Therefore, replacement of chemical interesterification by an enzyme process giving similar products is technically feasible, although it has not

yet been adopted on a commercial scale, largely because of the comparatively high process and catalyst costs.

Enzymatic interesterification can also be used for production of fats and oils containing nutritionally important PUFA, such as EPA and DHA. For example, various vegetable and fish oils have been enriched with EPA and DHA using enzyme-catalyzed reactions (Akoh, 1996a). Use of this technique to produce SL with MCFA and PUFA located specifically in either the *sn*-2 or *sn*-1,3 positions of the TAG has been described. Enzymatic processes are particularly suitable for the production and modification of lipids containing PUFA, because these unstable fatty acids are susceptible to damage under the more severe conditions used for chemical processing.

If regio- or stereospecific lipases are used to interesterify oil blends, the products formed are different from those obtained by chemical interesterification, and may exhibit better functional properties. For example, interesterification of blends of canola and palm oils, using the *sn*-1,3-specific *Rhizopus delemar* lipase as catalyst, gave oils with improved fluidity compared with the original blends or chemically interesterified products.

Interesterification of blends of palm and hydrogenated canola oils and cottonseed and hydrogenated soybean oils using *sn*-1,3-specific lipases as catalysts gave fats with a low trans fatty acid content that were effective as margarine hardstocks (Mohamed and Larsson, 1994). Reaction of mixtures of palm stearine and lauric fats using immobilized

Rhizomucor miehei as a catalyst also produced fats that were functional as margarine hardstocks (Posorske *et al.*, 1988). With these enzymatically interesterified fats, margarine could be formulated without using hydrogenated fats.

2.3 Sources of fatty acids for structured lipid synthesis

The unsaturated fatty acids belonging to the ω3, ω6 and ω9 families may be included in SL to promote health and nutrition (Kennedy, 1991). The clinical advantages of SL are derived from the short-, medium- and long-chain fatty acids and the uniqueness of the SL molecule itself. Many of these effects are due to the differences in metabolic fate of the various fatty acids involved. It is important to consider the metabolism of fatty acids to understand their physiological effects. In general, the metabolism of ω3 and ω6 PUFA that facilitates prevention and treatment of different diseases has been addressed by considering changes in the eicosanoids in the circulatory system. Since eicosanoids are ultimately derived from PUFA provided by the diet, it is clear that qualitative and quantitative changes in the supply of dietary PUFA will have a profound effect on the production of eicosanoids.

2.3.1 Short-chain fatty acids (SCFA)

Short-chain fatty acids (SCFA) are saturated aliphatic monocarboxylic acids and include acetic (2:0), propionic (3:0) and butyric acids (4:0). They are produced by

fermentation of dietary carbohydrates in the human gastrointestinal tract (Stein, 1999). SCFA are present in the diet in small amounts, for example acetic acid in vinegar and butyric acid in bovine milk and butter. They may also be present in fermented foods. In humans, it is estimated that SCFA contribute to 3% of total energy expenditure (Hashim and Babayan, 1978).

In nutritional applications, there has been a growing interest in the use of SCFA as an alternative or additional source of energy to their MCFA and LCFA counterparts. The SCFA, namely acetate, propionate and butyrate, are rapidly absorbed by the intestinal mucosa (Ruppin *et al.*, 1980) and provide an important source of calories. Short-chain triacylglycerols (SCT) such as triacetin and tributyrin are neutral, chemically stable and rapidly hydrolyzed by gastric and pancreatic lipases to glycerol and their respective SCFA (Lairon *et al.*, 1980). Parenterally administered SCT are readily hydrolyzed to glycerol and FFA in the bloodstream. The fact that triacetin, in contrast to tributyrin, is water-soluble and does not require emulsification makes it a very versatile alternative energy source to be incorporated into total parenteral nutrition (TPN) and enteral nutrition regimens (Bailey *et al.*, 1991).

The use of SCT in enteral nutritional formulas is also encouraging. Recently, Kripke *et al.* (1991) demonstrated that a chemically synthesized diet containing 40% (w/w) of nonprotein as SCT (1:1, triacetin and tributyrin) maintained body weight, improved nitrogen balance and liver function and enhanced jejunal and colonic mucosal

adaptation in rats after 60% distal small-intestine resection with cecectomy, when compared to short-intestine animals receiving a diet without supplemental lipid calories with MCT. Thus, enteral administration of SCT to either the small or large intestine may provide a useful alternative therapy in patients with intestinal loss due to injury (e.g. short bowel syndrome). The use of SCT, in combination with LCT, in regimens of TPN, has been shown to decrease the incidence of complications, such as leakage from colonic anastomoses (Lynch *et al.*, 1994) and intestinal atrophy during prolonged TPN (Linseisen and Wolfram, 1997).

2.3.2 Medium-chain fatty acids (MCFA)

Continued reports of inefficiencies in intravenous lipid metabolism and the accumulation of new findings that parenteral and enteral lipids can also suppress immune function (Sorbrado *et al.*, 1985; Hamawy *et al.*, 1985; Seidner *et al.*, 1989) have prompted a search for alternative lipid sources. A number of novel lipid moieties such as MCT, SL, and ω3 fatty acids provide new opportunities for improving fat utilization and immunologic responsiveness.

Medium-chain fatty acids (MCFA) are saturated fatty acids with 6-12 carbon atoms (Bach and Babayan, 1982) and are derived primarily from tropical fruit oils such as those of coconut and palm kernel (Akoh, 1995, 1997; Bell *et al.*, 1991). For example, coconut oil naturally contains some 65% MCFA (Young, 1983). One of the first medical

foods developed as an alternative to conventional lipids was MCT. Pure MCT have a caloric value of 8.3 calories per gram. However, they do not provide essential fatty acids (Heird *et al.*, 1986; Lee and Hastilow, 1999). MCFA, which are often used to produce SL (Haumann, 1977a), are more hydrophilic than their long-chain counterparts, and hence solubilization as micelles is not a prerequisite for their absorption (Ikeda *et al.*, 1991). MCT can also be incorporated into mucosal cells without hydrolysis and may readily be oxidized in the cell. Unlike other fatty acids, MCT pass directly into the portal vein and are readily oxidized in the liver to serve as an energy source. Thus, they are less likely to be deposited in the adipose tissues (Megremis, 1991) and are more prone to oxidation in the tissues (Mascioli *et al.*, 1987). MCT are well tolerated by the enteral route and are frequently used in patients with maldigestion and malabsorption.

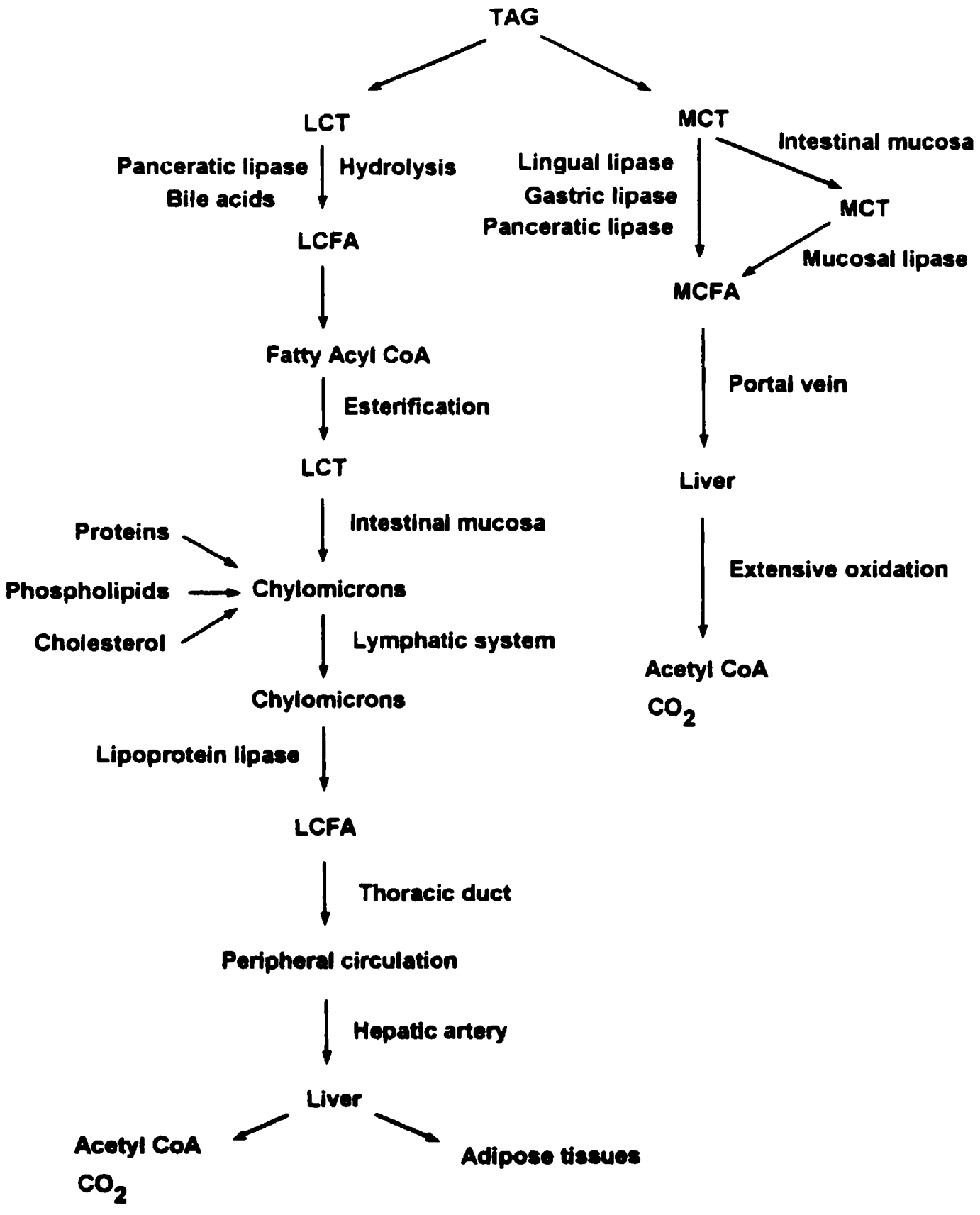
MCT may offer several advantages over LCT. Their lower melting point, greater solubility and smaller molecular size accounts for their easy absorption, transport and metabolism compared to LCT (Babayan, 1987). MCT are hydrolyzed by pancreatic lipase more rapidly and completely than are LCT (Bell *et al.*, 1991). They may be directly absorbed by the intestinal mucosa with minimum pancreatic or biliary function. They are transported predominantly by the portal vein to the liver for oxidation (Heydinger and Nakhasi, 1996) rather than through the intestinal lymphatics (Figure 2.4). In addition, MCFA are more rapidly oxidized to produce acetyl-CoA and ketone bodies and are independent of carnitine for entry into the mitochondria.

Since MCT alone do not contain essential fatty acids, they need to be used with LCT containing essential fatty acids to provide a balanced nutrition in enteral and parenteral products (Haumann, 1997a; Ulrich *et al.*, 1996). Thus, in many medical foods, a mixture of MCT/LCT is used to provide both rapidly metabolized and slowly metabolized fuel as well as essential fatty acids. Clinical nutritionists have taken advantage of MCT's simpler digestion to nourish individuals who cannot utilize LCT. Any abnormality in the numerous enzymes or processes involved in the digestion of LCT can cause symptoms of fat malabsorption. Thus, patients with diseases like Crohn's disease, cystic fibrosis, colitis and enteritis have shown improvement when MCT is included in their diets (Kennedy, 1991). MCT are also increasingly utilized in the feeding of critically ill or septic patients who presumably gain benefits in the setting of associated intestinal dysfunction. Further investigation should clarify potential roles for MCT in patients with lipid disorders associated with lipoprotein lipase and carnitine deficiencies. MCT may be used in confectioneries and in other functional foods as flavour carriers (Megremis, 1991). MCT have clinical applications in the treatment of lipid malabsorption and obesity (Bach and Babayan, 1982).

2.3.3 Long-chain fatty acids (LCFA)

Dietary lipids in medical foods are commonly composed of LCT. These lipids supply a source of noncarbohydrate calories. LCT are generally recognized as useful

Figure 2.4 Digestion, absorption and transport of medium-chain and long-chain fatty acids


TAG
LCT
Panceratic lipase
Bile acids
Hydrolysis
LCFA
Fatty Acyl CoA
Esterification
LCT
Intestinal mucosa
Proteins
Phospholipids
Cholesterol
Chylomicrons
Lymphatic system
Chylomicrons
Lipoprotein lipase
LCFA
Thoracic duct
Peripheral circulation
Hepatic artery
Liver
Acetyl CoA
CO_2
Adipose tissues
MCT
Lingual lipase
Gastric lipase
Panceratic lipase
Intestinal mucosa
MCT
Mucosal lipase
MCFA
Portal vein
Liver
Extensive oxidation
Acetyl CoA
CO_2

nutrient substrates. However, there are some concerns regarding their clinical application. The digestion and absorption of LCT require a normal functioning gastrointestinal tract, which may be compromised in patients with maldigestion, malabsorption and critical illness. These lipids are slowly cleared from the systemic circulation and may not be readily oxidized.

There has been some concern that excessive linoleic acid (LA) may result in excessive production of eicosanoids, which may affect immune competence and vascular integrity during the stress response to acute injury. In a study of several burned patients who were given enteral products with a variety of lipid-based formulas, the intravenous solution highest in linoleic acid (LA) content was associated with increased length of hospital stay and the highest mortality rate (Gottschlich *et al.*, 1990). These findings were confirmed by Daly *et al.* (1992) who studied postsurgical patients in which two diets, one high LA and the other with only 2% of the total calories from LA, were compared. The SL diet, Impact®, which contained low levels of LA resulted in decreased infections and decreased length of hospital stay as compared to the other enteral formula. Bower *et al.* (1995) also demonstrated a decreased length of hospital stay and decreased infection rate using diets with a low level of LA and added fish oil.

2.3.4 Omega-3 fatty acids

Omega-3 fatty acids have been the subject of considerable nutritional studies as they are considered to be essential fatty acids (EFA) and must be provided in the food because they cannot be easily manufactured within the body. Examples of ω3 fatty acids include α-linolenic acid (ALA), EPA and DHA. The parent, and simplest, member of the ω3 family is ALA. Sources of ω3 PUFA include seafood and certain plants. Marine oils contain moderately high levels of EPA and DHA (Shahidi and Wanasundara, 1998a), while plant sources including flaxseed, canola and soybean oils contain ALA (Beare-Rogers, 1988; Rice, 1991). EPA and DHA are synthesized by algae and hence enter the food web through the animals such as fish that feed on algae (Groom, 1993).

PUFA have two or more double bonds in their backbone structure. 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, GLA is 18:3ω6 (or 18:3n-6) (Figure 2.5) while LA is 18:2ω6 (or 18:2n-6). 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 parent compounds of the ω6 and ω3 series are LA (18:2ω6) and ALA (18:3ω3). LA and ALA are metabolised by a series of alternating desaturation (in which a further double bond is introduced) and elongation (in which two carbon atoms are added) as outlined in Figure 2.6.

Figure 2.5 Chemical structures of selected long-chain fatty acids (LCFA)

Oleic acid (18:1ω9)

Linoleic acid (18:2ω6)

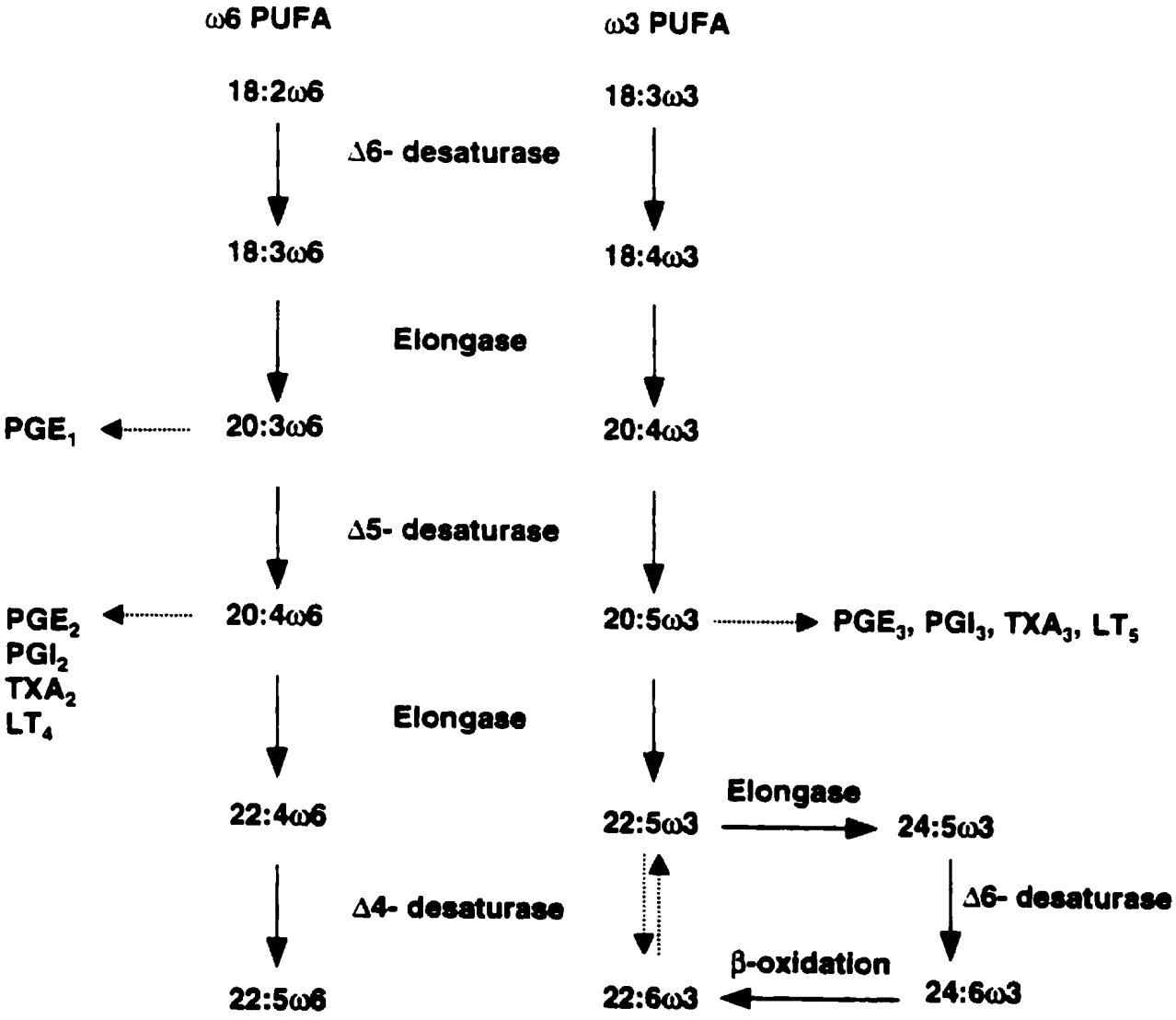
α-Linolenic acid (18:3ω3)

γ-Linolenic acid; GLA (18:3ω6)

Eicosapentaenoic acid; EPA (20:5ω3)

Docosahexaenoic acid; DHA (22:6ω3)

Figure 2.6 Metabolic pathways of ω3 *versus* ω6 fatty acids


ω6 PUFA
ω3 PUFA
18:2ω6
18:3ω3
Δ6- desaturase
18:3ω6
18:4ω3
Elongase
PGE1
20:3ω6
20:4ω3
Δ5- desaturase
PGE2
PGI2
TXA2
LT4
20:4ω6
20:5ω3
PGE3, PGI3, TXA3, LT5
Elongase
Elongase
22:4ω6
22:5ω3
24:5ω3
Δ4- desaturase
Δ6- desaturase
β-oxidation
22:5ω6
22:6ω3
24:6ω3

It is generally believed that the enzyme systems involved in the metabolism of the two types of PUFA are identical. It has also been established that the ω3 and the 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 metabolised.

In the cell there is a competition between the two precursors and high amounts of ω6 (i.e. linoleic acid) inhibit the conversion of ALA to EPA. Because of this, the key factor in regards to the consumption of these classes of fatty acids is the ratio in which they are consumed. Typical western diets contain 10-30 g/day of LA and 0.5-1.0 g/day of ALA (the primary ω3 FA in Western diets). With current dietary patterns, the observation that ALA supplementation fails to increase blood EPA concentration may be easily understood (Rice, 1991).

DHA has also gained considerable attention by itself since a number of studies have shown its importance in early neurological and visual developments (Kim and Edsall, 1999). DHA is one of the major components of the gray matter of the brain (Ward, 1995), the phospholipids of the retina (Akoh, 1995), the testes and the sperm (Langholz *et al.*, 1989). DHA deficiency is a problem in preterm infants that have inadequate fat stores. The presence of sufficient DHA in mother's milk is required for normal visual development, otherwise supplementation is necessary (Crawford, 1997; Crawford *et al.*, 1999; Haumann, 1997b).

There is evidence for retro-conversion of DHA to docosapentaenoic acid (DPA) and EPA in humans (Ackman and Ratnayake, 1989). After ingestion of ethyl esters of DHA, the DHA and EPA in plasma phospholipids was increased, but DPA remained essentially unchanged (Kinsella, 1990). However, ingestion of DHA increased the levels of DHA, DPA and EPA 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.

The optimal intake of ω3 and ω6 fatty acids has been studied and suggestions made that the dietary ratio of the ω6 to ω3 PUFA should be in the range of 4:1 to 10:1 (Caston and Leeson, 1990). Today, the ratio of ω6/ω3 is between 10-20:1 in Western Europe and the United States, whereas during evolution it was 1:1 (Simopoulos, 1999). The western diet currently contains an average of 1.7g ω3 PUFA per day of which 90% is ALA (Van Elswyk, 1993). It has been recommended that the daily intake of ω3 PUFA should be increased to 3.0 g/day of which 1 g should include EPA and DHA. The recommended daily intake of ω3 PUFA in Canada is a minimum of 0.55 g/1000 kcal or 0.5% of energy (Ajuyah *et al.*, 1991).

The therapeutic benefits of ω3 PUFA are quite varied, involving a broad range of metabolic, cardiovascular and immunological conditions. Epidemiological studies have attributed the low incidence of cardiovascular disease in Eskimos to their relatively high

dietary intake of ω3 PUFA (Bang and Dyerberg, 1972, 1986). Fish oil has been shown to lower systemic blood pressure in patients with mild hypertension in a controlled, double-blind, crossover study (Levinson *et al.*, 1990). Fish oil supplementation has also been shown to decrease serum TAG and cholesterol levels while increasing concentrations of high-density lipoprotein (HDL) cholesterol (Kinsella, 1986). Chronic immunologically mediated diseases including atopic dermatitis and psoriasis have been shown to improve with short-term use of fish oil supplementation (Bittiner *et al.*, 1988).

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 hydroxy fatty acids (Wardlaw, 1996). They are synthesized from 20-carbon PUFA with three, four or five double bonds of either ω3 or ω6 families. The three important fatty acids involved in eisosanoid production are dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA) and EPA (Braden and Carroll, 1986). As they have different numbers of double bonds, they give rise to different series of eicosanoids. Thus, prostanoids of 1-series and leukotrienes of 3-series are formed from DGLA. AA produces prostanoids of 2-series and leukotrienes of 4-series, while EPA is converted to prostanoids of 3-series and leukotrienes of 5-series. The two families of fatty acids, ω3 and ω6, compete for the same enzymes, and hence,

depending on the availability of fatty acids, different series of eicosanoids are formed (Figure 2.6).

The eicosanoids formed from AA and EPA are biologically more active and more important than those of DGLA (Alexander, 1998). These fatty acids are usually derived from phospholipids by the action of phospholipase A_2. Prostanoids are produced in most tissues, whereas leukotrienes are generally formed in different blood cells. EPA can serve as a precursor of thromboxane A_3 (TXA_3) and prostacyclin I_3 (PGI_3) (Figure 2.6). In platelets, AA forms TXA_2 whereas EPA forms TXA_3. In endothelial cells of blood vessels, the major product of AA is prostacyclin I_2 (PGI_2) and that of EPA is PGI_3. TXA_3 differs from TXA_2 in that it does not induce aggregation of platelets. However, PGI_3 is as effective as PGI_2 in inhibiting platelet aggregation (Rice, 1991). Moreover, both PGI_3 and PGI_2 are vasodilators. For these reasons, dietary EPA is implicated in reducing the risk of thrombosis. Prostaglandin E_1 (PGE_1) derived from DGLA also has antiaggregatory and anti-inflammatory activities (Fan and Chapkin, 1998). Leukotrienes, on the other hand, promote smooth muscle contraction.

2.3.5 Omega 6 fatty acids

The ω6 fatty acids have at least four roles: (a) modulation of membrane structure; (b) formation of short-lived local regulating molecules such as eicosanoids; (c) control of water impermeability of the skin; and (d) regulation of cholesterol transport and

cholesterol synthesis (Horrobin, 1992b). The most important ω6 fatty acids are LA, GLA, DGLA and AA.

2.4 Significance of γ-linolenic acid (GLA)

GLA (*cis,cis,cis*-6,9,12-octadecatrienoic acid), the first metabolite of LA, is formed from it by Δ6-desaturation. It is an essential PUFA (Horrobin, 1992a) and must be provided in the food because it cannot be easily manufactured within the body. In the human body, GLA is merely an intermediate in the pathway from LA to AA and does not accumulate in mammalian cells.

GLA is important biologically, being an indispensable precursor for the synthesis of long-chain PUFA having structural functions in cellular membranes and of eicosanoids. It also affects cholesterol metabolism by reducing total cholesterol (more efficiently than LA) and low-density lipoprotein (LDL) cholesterol as well as increasing HDL cholesterol. Like other ω6 fatty acids, GLA has an antiaggregating effect on platelets (Schmitt-Rozieres *et al.*, 1999).

The rate limitation of the Δ6-desaturase in the ω6 pathway is important in understanding the claims for health benefits of GLA. Δ6-Desaturase activity, required in GLA synthesis from LA, decreases with age and is also depressed by stress-related hormones, adrenalin and cortisol, high cholesterol levels, high alcohol intake, fatty acids of the ω3 family, and in the condition of diabetes.

Many investigators have actively investigated the concentration of GLA from borage, evening primrose and fungal oils for pharmaceutical and dietetic purposes. Available methods for enrichment of GLA include urea complexation (Traitler *et al.*, 1988), low-temperature crystallization (Yokochi *et al.*, 1990), separation on Y-zeolite (Arai *et al.*, 1987), selective enzymatic hydrolysis (Rahmatullah *et al.*, 1994b) or esterification (Mukherjee and Kiewitt, 1991; Rahmatullah *et al.*, 1994a; Schmitt-Rozieres *et al.*, 1999).

2.4.1 Nutritional and medicinal uses of GLA

The literature is replete with claims for therapeutic benefits of GLA. Disorders for which GLA efficacy has been tested, under controlled clinical trials, include atopic eczema, hypertension, multiple sclerosis, diabetic neuropathy, premenstrual syndrome and several types of cancer. In some categories the results have been inconsistent.

Several groups of researchers have argued that GLA, DGLA and AA are present in human milk for a very specific purpose (Clandinin *et al.*, 1982). They contend that the conversion of LA to GLA in humans is well established as a rate-limiting metabolic step (Brenner, 1982), with only a small proportion of dietary LA being converted to GLA and beyond (Manku *et al.*, 1988). The PUFA beyond the rate-limiting step are critical for the development of many tissues and especially those of the brain, which by weight contain about 20% of Δ-6-desaturated PUFA. According to these investigators, infants may have

difficulty in producing adequate amounts of all of the EFAs if LA is the only dietary source of ω6 PUFA, and this is perhaps why preformed GLA, DGLA and AA are present in human milk.

GLA has been tested by oral administration of evening primrose oil (EPO) in order to inhibit the growth of a variety of animal tumours, particularly those of the mammary glands (Horrobin, 1994). Administration of GLA to animals with tumours, including nude mice with implanted human tumours, substantially limited cancer growth (Horrobin, 1993). These animal studies encouraged the development of a clinical research program.

For most human cancers, GLA has been found to be one of the most effective selective agents. Various routes and forms of administration of GLA have been tried in attempts to treat human cancers. High doses of oral GLA in the form of EPO have produced evidence of responses and prolongation of life without side effects in patients with liver, breast, brain, and oesophageal cancer (Horrobin, 1994).

There has been an extensive study on EPO in the treatment of atopic eczema (Burton, 1990, Marshall and Evans, 1990). In young adults with atopic eczema, it has been found that plasma levels of LA are high, whereas those of GLA are low, thus supporting the theory of a block in PUFA conversion at the commencement of the ω6 pathway (Horrobin and Manku, 1990). Trials have shown significant improvements in all features of the disease, but especially in itch. Studies have found that GLA can

significantly reduce skin roughness in atopic eczema (Marshall and Evans, 1990). Moreover, the need for other medications such as steroids that may have toxic effects in long term use was reduced.

Clinical studies of the use of GLA in breast pain and premenstrual syndrome have shown, in most cases, that GLA is effective in relieving such symptoms. As a result the British Department of Health has licensed EPO (Efamast) for the treatment of breast pain (Burton, 1990; Horrobin, 1992a).

Diabetes impairs the conversion of LA to its Δ6-desaturated metabolites in both animals and humans (Brenner, 1982). It has been suggested that this reduced ability to desaturate LA may account for many of the long term renal, retinal and neurological complications of diabetes (Horrobin, 1992a). In diabetics, GLA has been found to reverse neurological damage (Julu, 1990) and to lower plasma cholesterol and TAG. In alcoholics, GLA has been found to accelerate recovery of liver function and to reduce the severity of withdrawal symptoms (Glen *et al.*, 1990).

2.4.2 Potential sources of GLA

GLA itself is not present in high concentrations in most foodstuffs and there is no simple, commercially viable method for its synthesis at present. It does occur, however, in relatively high concentrations in borage, evening primrose, blackcurrant and fungal oils

(Phillips and Huang, 1996). These sources of GLA are discussed in the following sections.

2.4.2.1 Borage (*Borago officinalis* L.)

Borage is an annual herbaceous plant belonging to the family of Boragenaceae and is commercially grown in North America. The *Borago* genus includes three species including *Borago officinalis*, commonly known as borage (Helme, 1996). Large-scale commercial production of borage presents unique challenges because of the plant's indeterminate vegetative growth, lack of concentrated flowering and seed set and non-uniform seed maturation (Simpson, 1993a,b). Borage oil (BO), extracted from seeds of the blue, star shaped borage flower, is gaining much attention by alternative health practitioners and mainstream medicine alike for its profound medicinal properties. Although the oil is getting all of the credit, it is actually the oil's active component, GLA that has drawn the interest of researchers. The seeds of borage contain approximately 38% of oil with a GLA content of 20-25% (Beaubaire and Simon, 1987). The level of GLA in the seeds is at around 7% and this is about three times that in evening primrose seed. The oil is made up of 95.7% neutral lipids, 2.0% glycolipids and 2.3% phospholipids (Senanayake and Shahidi, 2000b). Neutral lipids of BO are composed of TAG (99.1%), DAG (0.06%), MAG (0.02%), FFA (0.91%) and sterols (0.02%) (Senanayake and Shahidi, 2000b).

2.4.2.2 Evening primrose (*Oenothera biennis* L.)

Evening primrose is a biennial plant belonging to the family of Onagraceae and is a common weed that is native to North America. Oil obtained from *Oenothera biennis* is designated as evening primrose oil (EPO). Interest in this oil has intensified in recent years because of its GLA content. Although the evening primrose plant does not produce a high yield of seeds compared to the well-known commercial oilseeds, it is preferred to other sources of GLA because it is easy to produce and does not contain any ALA. At present, EPO is the most important source of GLA, which is in growing demand for its clinical and pharmaceutical applications (Hudson, 1984).

EPO is currently available in over 30 countries as a nutritional supplement or as a constituent of specialty foods. The seeds are cultivated in at least 15 countries, including Canada, United States, France, Holland, Hungary, United Kingdom, Yugoslavia, Australia and New Zealand. The U.S. and Canada currently produce 300–400 tons of the seeds annually. In a number of countries, certain nutritional products require governmental registration before they can be marketed. EPO has received such registration in Australia, Canada, Cyprus, Denmark, Finland, France, Italy, Phillipines, Spain, Sweden, Switzerland and the United Arab Emirates. Several large organisations have been able to establish moderately large-scale extraction of the oils. The EPO capsules contain 10 to 12% GLA and in Canada are marketed by "Efamol" company.

The oil content of seeds is 17-25% (Wolf *et al.*, 1983; Beaubaire and Simon, 1987), of which 7-10% is GLA (Gibson *et al.*, 1992; Fieldsend, 1996). The total GLA content of the seeds is approximately 2.5% (Wolf *et al.*, 1983). The oil, as marketed, is made up of 97-98% TAG, 1.5-2.0% unsaponifiable matter and 0.5-1.0% polar lipids (Hudson, 1984). The EPO is generally obtained by mechanical pressure followed by extraction with hexane (Helme, 1996). The seed oils from various *Oenothera* species have been characterized; the highest amount of GLA was found in *O. acerviphilla nova* (16%) and *O. paradoxa* (14%). There are preliminary indications that EPO may be more effective in some of its physiological effects than other oils in which GLA occurs. One possible explanation is that GLA is present in EPO almost entirely as molecular species of TAG in which one GLA is combined with two LA molecules (Fieldsend, 1996). Another possibility is that minor components of EPO, not GLA, are responsible for some of the effects. GLA from other oils (borage, blackcurrant and fungal) may also be biologically less effective than that from EPO, partly because of the other fatty acids present and partly because of the different TAG structure of the oils (Horrobin, 1990). The TAG stereospecific structure of EPO is distinct, with GLA being concentrated in the *sn*-3 position (Lawson and Hughes, 1988).

2.4.2.3 Blackcurrant (*Ribes nigrum* L.)

Blackcurrant is a perennial berry crop belonging to the family of Saxifragaceae. It is mainly cultivated in Europe and Asia. Blackcurrant is a round, dull black berry (Helme, 1996) and its seeds contain about 30% oil (Traitler *et al.*, 1984) which may be extracted by hexane (Helme, 1996). The oil differs from that of BO and EPO in that it contains two ω-3 fatty acids, namely ALA (18:3ω3) and stearidonic acid (18:4ω3). Blackcurrant oil (BCO), having a GLA content of 15 to 19% (Traitler *et al.*, 1984; Walker, 1991), also contains a potent GLA inhibitor, erucic acid (22:1ω9) which reduces its advantage as a medicinal oil (Walker, 1991). LA is the major PUFA found in the BCO (Lawson and Hughes, 1988). The main uses of BCO, as in the case of borage and evening primrose, are generally based on claims concerning pharmacological properties of GLA (Helme, 1996).

2.4.2.4 Other sources of GLA

GLA also occurs in unicellular organisms such as blue-green algae, *Spirulina* (Ciferri and Tiboni, 1985; Ward, 1995) as well as fungi such as phycomycetes and protozoa (Carter, 1988). The *Spirulina* has historically been utilized as food by people of both Central America and Central Africa. Today, *Spirulina* is widely sold in specialty nutrition stores throughout Europe and North America. Around 10% of dried *Spirulina* are lipid and of which 20-25% is GLA. At its commonly recommended daily dose of 10g, *Spirulina* provides 200-250 mg GLA (Carter, 1988). Certain fungi, including species of

Rhizopus, *Mucor* and *Mortierella*, also produce oils containing GLA (Phillips and Huang, 1996). Particular interest is paid to the observation that GLA is present in human milk (Clandinin *et al.*, 1982; Carter, 1988).

2.5 Enzymatic modification of fats and oils to produce structured lipids

Several research groups have successfully incorporated ω3 PUFA into plant oils using enzyme-catalyzed reactions (Sridhar and Lakshminarayana, 1992; Huang *et al.*, 1994; Huang and Akoh, 1994; Akoh and Sista, 1995; Akoh *et al.*, 1996; Ju *et al.*, 1998; Akoh and Moussata, 1998). Huang and Akoh (1994) studied the ability of immobilized lipases IM60 from *Mucor miehei* and SP435 from *Candida antarctica* to modify the fatty acid composition of soybean oil by incorporation of ω3 PUFA. The transesterification reaction was carried out with free fatty acid and ethyl esters of EPA and DHA as acyl donors. With free EPA as acyl donor, IM60 gave a higher incorporation of EPA than SP435. However, when ethyl esters of EPA and DHA were the acyl donors, SP435 gave a higher incorporation of EPA and DHA than IM60. Moussata and Akoh (1997) investigated the ability of lipase PS-30 from *Pseudomonas sp.* to modify the fatty acid profile of melon seed oil by incorporation of oleic acid (18:1ω9). Oleic acid content increased from 13.5 to 53%, while linoleic acid (18:2ω6) content decreased from 65 to 33%. Huang *et al.* (1994) incorporated EPA into crude melon seed oil by two immobilized lipases, IM60 from *Mucor miehei* and SP435 from *Candida antarctica* as

biocatalysts. Higher EPA incorporation was obtained using EPA ethyl ester than using EPA free fatty acid for both enzyme-catalyzed reactions.

Akoh *et al.* (1995) used two immobilized lipases, nonspecific SP435 from *Candida antarctica* and sn-1,3 specific IM 60 from *Mucor miehei*, as biocatalysts for restructuring of trilinolein to incorporate EPA and DHA with ethyl esters (EEPA and EDHA, respectively) as acyl donors. With EEPA as acyl donor, the total EPA product yields with IM60 and SP435 were 79.6 and 81.4%, respectively. However, with EDHA as acyl donor and IM60 and SP435 as biocatalysts, the total DHA product yields were 70.5 and 79.7%, respectively.

Recently, EPA and capric acid (10:0) have been incorporated into borage oil using two immobilized lipases, SP435 from *Candida antarctica* and IM60 from *Rhizomucor miehei* as biocatalysts (Akoh and Moussata, 1998). Higher incorporation of EPA (10.2%) and 10:0 (26.3%) was obtained with IM60 lipase, compared to 8.8 and 15.5%, respectively, with SP435 lipase.

Ju *et al.* (1998) incorporated ω3 PUFA into the acylglycerols of borage oil. They have selectively hydrolyzed borage oil using immobilized *Candida rugosa* lipase and then used this product with ω3 PUFA for the acidolysis reaction. The total content of ω3 and ω6 PUFA in acylglycerols was 72.8% following acidolysis. The contents of GLA, EPA and DHA in the structured lipid so prepared were 26.5, 19.8 and 18.1%,

respectively. The corresponding ω3/ω6 ratio changed from 0 to 1.09 after the modification.

The incorporation of GLA into the acylglycerols of borage oil, in an acidolysis reaction catalyzed by *Mucor miehei* lipase in an organic solvent, was systematically studied by Huang *et al.* (1999). In this work, GLA-rich fatty acids derived from the urea complexation of borage oil were used as a substitute for pure GLA. They selectively hydrolyzed borage oil using an immobilized *Candida rugosa* lipase and increased the GLA content in the unhydrolyzed acylglycerols from 23.6 to 52.1% and then used this product with GLA for acidolysis. After acidolysis reaction, the GLA content in the acylglycerols of borage oil was increased from 52.1 to 75%.

Akoh and Sista (1995) have previously reported the modification of fatty acid composition of borage oil using EPA ethyl ester with an immobilized nonspecific SP435 lipase from *Candida antarctica* as a biocatalyst. The highest incorporation (31%) was obtained with 20% SP435 lipase. At a substrate mole ratio of 1:3, the corresponding ratio of ω3 to ω6 PUFA was 0.64. Under similar conditions, Akoh *et al.* (1996) were able to increase the ω3 PUFA content (up to 43%) of evening primrose oil with a corresponding increase in the ω3/ω6 ratio from 0.01 to 0.6. Sridhar and Lakshminarayana (1992) were able to effectively modify the fatty acid composition of groundnut oil by incorporating EPA and DHA using a *sn*-1,3 specific lipase from *Mucor miehei* as the biocatalyst. The

contents of EPA and DHA incorporated into groundnut oil were 9.5 and 8.0%, respectively.

Yankah and Akoh (2000) synthesized two different SL by transesterifying tristearin with caprylic acid (8:0) or oleic acid (18:1). The reaction was catalyzed by IM-60 lipase from *Rhizomucor miehei* in hexane. The effects of reaction parameters affecting the incorporation of caprylic acid into tristearin were compared with those for incorporating oleic acid into tristearin. For all parameters studied, oleic acid incorporation was higher than that of caprylic acid.

The production of SL by lipase-catalyzed reaction, at present, is a promising method that has not yet been optimized or investigated in detail, in particular for solvent-free systems. Shieh *et al.* (1995) reported a four-factor response surface optimization of the enzymatic modification of triolein to SL with hexane as a solvent. Huang and Akoh (1996a) also reported the optimization and scale-up of enzymatic synthesis of SL using response surface methodology (RSM) in a similar system using a non-specific enzyme.

Xu *et al.* (1998a) also used response surface design in order to optimize production of SL from rapeseed oil and capric acid in a solvent-free medium catalyzed by Lipozyme IM from *Rhizomucor miehei*. The effects of parameters such as reaction time, temperature and water content on acyl migration and the net incorporation of capric acid into rapeseed oil were studied. Xu *et al.* (1998b) reported large scale synthesis and

production (1 kg and 40 kg) of SL in batch reactors and noted the effects of acyl migration on the purity of SL.

Huang and Akoh (1996b) reported transesterification of triolein and caprylic acid ethyl ester in hexane using different lipases. Their results showed that an immobilized lipase IM60 from *Rhizomucor miehei* converted most of triolein into SL (41.7% dicaprylolein, 46% monocaprylolein and 12.3% unreacted triolein). However, lipase SP435 from *Candida antarctica* had a higher activity at a higher temperature. The reaction catalyzed by lipase SP435 yielded 62% dicaprylolein, 33.5% monocaprylolein and 4.5% unreacted triolein at 55°C. Lee and Akoh (1996) used Lipozyme IM 60 to synthesize a SL with EPA at specific positions from medium-chain TAG and EPA ethyl ester and reported a very high specificity of the enzyme. Shimada *et al.* (1996 a,b) investigated the production of SL containing DHA or other essential fatty acids and caprylic acid by immobilized *Rhizopus delemar* lipase in a solvent-free system. The effects of water and other parameters were researched and the absolute *sn*-1,3 specificity of the enzyme used was claimed.

2.6 Low calorie structured lipids

Another area of interest in the field of SL is the synthesis of low-calorie TAG that are characterized by the presence of SCFA and/or MCFA and LCFA into a single TAG structure. Interest in these types of SL stems from the fact that they contain 5-7 kcal/g

caloric value compared with the 9 kcal/g of natural fats and oils because of the lower caloric content of SCFA compared to that of their long-chain counterparts. Reduced-calorie SL are intended for use in baking chips, coatings, dips, bakery and dairy products, or as cocoa butter substitutes. Currently, such SL are produced by random chemical interesterification between a short-chain TAG (SCT) and LCT, typically a hydrogenated vegetable oil such as soybean or canola oil (Smith *et al.*, 1994). The most familiar classes of low-calorie fats are Caprenin®, Salatrim® and MCT preparations. The following sections will describe each of these products.

2.6.1 Caprenin

Caprenin is a reduced calorie SL contributing about 5 kcal/g compared to 9 kcal/g of conventional fats and oils (Akoh, 1997). It has a defined structure of caprocaprylobehenin ($C_{8:0}$-$C_{10:0}$-$C_{22:0}$). It is a TAG formed by esterification of glycerol with the medium-chain saturated fatty acids caprylic acid ($C_{8:0}$) and capric acid ($C_{10:0}$) and the very long-chain saturated fatty acid behenic acid ($C_{22:0}$). All of these fatty acids are derived from natural food sources. Caprylic and capric acids are obtained by fractionation of palm kernel and coconut oils. Behenic acid is produced from rapeseed oil (Finley *et al.*, 1997) and is also found in peanuts and marine oils. Behenic acid is poorly absorbed (Akoh, 1998) and the MCFA provide fewer calories than absorbable LCFA. It was originally produced by Procter and Gamble Company (Cincinnati, OH) from coconut

and palm kernel oils fatty acids (Finley *et al.*, 1997). Caprenin has functional properties similar to cocoa butter and is intended to replace some of the cocoa butter in selected confectionery products (Akoh, 1996b). It is digested, absorbed, and metabolized by the same pathway as other TAG (Artz and Hansen, 1996). US Food and Drug Administration (FDA) has received a Generally Recognised As Safe (GRAS) petition for caprenin for use in soft candy bars and in confectionery coatings for nuts, fruits and cookies (Akoh, 1996b).

2.6.2 Salatrim (Benefat)

Salatrim, similar to caprenin, is also a reduced calorie SL with a caloric content of 5 kcal/g. It is composed of a mixture of SCFA ($C_{2:0}$-$C_{4:0}$) and LCFA (predominantly $C_{18:0}$) (Smith *et al.*, 1994). The SCFA are chemically transesterified with vegetable oils such as highly hydrogenated canola or soybean oil (Klemann *et al.*, 1994). Again, the saturated stearic acid is said to be partially absorbed with the SCFA contributing little calories to the overall molecule. Salatrim was developed by Nabisco Foods Group (East Hanover, NJ) (Finley *et al.*, 1997) and is now marketed by Cultor Food Science (New York, NY) under the brand name, Benefat (Akoh, 1998). It has the taste, texture and functional properties of conventional fats and oils. It can be produced to have different melting profiles by adjusting the amounts of SCFA and LCFA used in their chemical synthesis. One product in the market that contains Benefat is the reduced fat baking chips

introduced in 1995 by Hershey Food Corporation. Salatrim has the FDA GRAS status as of 1994 (Akoh, 1996b) and can also be used as a cocoa butter substitute. Salatrim is intended for use in chocolate-flavoured coatings, chips, caramel, fillings for confectionery and baked goods, peanut spreads, savoury dressings, dips and sauces and in dairy products (Kosmark, 1996).

2.6.3 Medium-chain triacylglycerols (MCT)

Medium-chain triacylglycerols (MCT) were originally developed for therapeutic purposes to provide a source of energy for individuals with compromised gastrointestinal systems. MCT have been used extensively in parenteral nutrition formulations. MCT are structured TAG composed of fatty acids with chain lengths between 8 and 10 carbon atoms obtained from coconut oil. Caprylic and capric acids comprise more than 96% of the fatty acids in MCT preparations. MCT are readily hydrolyzed by digestive enzymes, and the fatty acid end products are rapidly absorbed into the bloodstream (Babayan, 1974). MCFA are used as immediate sources of energy by the liver, yielding fewer calories per gram than LCFA. The caloric value of MCT preparation is 7 kcal/g (Finley *et al.*, 1997).

2.7 Digestion and absorption of structured lipids

The metabolic advantages of SL over simple physical mixtures are likely due to the position, or stereospecificity, of certain fatty acids on the glycerol backbone. One theory is that SL with MCFA in the *sn*-2 position of the TAG molecule are more slowly removed from circulation than if they are given as standard MCT. This stereospecificity is maintained by enhanced lymphatic absorption of MCFA in the *sn*-2 position of enterally administered SL, which has been shown in humans (Jensen *et al.*, 1989) and animals (Jensen and Jensen, 1992). The enhanced absorption is believed to be the result of the action of pancreatic lipase which preferentially hydrolyzes fatty acid moieties from the *sn*-1 and *sn*-3 positions of TAG, that are transported *via* the intestinal lymphatics to the systemic circulation. Further investigation is required to completely explain the nutritional effects of SL.

SL containing medium-chain and long-chain essential fatty acids meet the nutritional needs of patients and those with special dietary requirements. When medium chain fatty acids such as caproic (6:0) and capric (10:0) acids are consumed, they are not incorporated into the chylomicrons and are therefore not likely to be stored, but will be used for energy. They are readily oxidized in the liver and constitute a highly concentrated source of energy for premature babies and patients with fat malabsorption. *In vitro* lipase digestions and absorption of isolated intestinal loop studies revealed that a SL containing MCFA (caprylic acid; 8:0) at the *sn*-1 and *sn*-3 positions and a LCFA (LA)

at the *sn*-2 position is more rapidly hydrolyzed and effectively absorbed than a typical long-chain TAG (Jandacek *et al.*, 1987). Ikeda *et al.* (1991) have also confirmed that SL are well absorbed.

Jensen *et al.* (1990) were among the first to confirm that fatty acids in the *sn*-2 position are preferentially absorbed. They administered a bolus of either SL or randomized oil to lymph-cannulated rats. The SL contained medium-chain fatty acids in the *sn*-1 and *sn*-3 positions of the TAG and LA in the *sn*-2 position whereas in the randomized oil the same fatty acids were distributed randomly between the three positions. Absorption of MCFA was the highest from a randomized oil in which approximately 33% medium-chain fatty acids were located in the *sn*-2 position. The absorption of LA was highest from SL where LA was located in the *sn*-2 position, indicating that their intestinal absorption is affected by TAG structure and that the absorption is enhanced for fatty acids located in the *sn*-2 position.

Christensen *et al.* (1995 a,b) showed that SL with EPA and DHA, predominantly in the *sn*-2 position of the TAG, were a more readily absorbable source of EPA and DHA. Jensen *et al.* (1995) suggested that the structure of dietary TAG affects the distribution between lymphatic and intestinal absorption of fats. The intravenous applications of SL were also examined by Hultin *et al.* (1994).

In a randomized trial of preterm infants fed one of the three infant formulas, Lucas *et al.* (1997) demonstrated that a synthetic SL (Betapol®) containing palmitic acid (16:0)

predominantly in the *sn*-2 position (74%) had a significant impact on palmitic acid absorption, reduced the formation of insoluble calcium soaps in the intestine and improved calcium absorption from the diet. These findings suggested a potentially important role for such SL in preterm infant formulas since they can mimic the stereoisomeric structure of those in human milk.

McKenna *et al.* (1985) and Hubbard and McKenna (1987) observed enhanced absorption of LA in cystic fibrosis patients fed SL containing LCFA and MCFA. Work by Babayan and colleagues (Bach and Babayan, 1982; Mascioli *et al.*, 1987) have demonstrated that SL, *via* both the enteral and parenteral route, can decrease infection and improve survival by serving as a more efficient fuel and by producing fewer inflammatory and immunosuppressive eicosanoids when compared with conventional TAG. Investigators continue to pursue the potential clinical utility of SL emulsions. Whether the distinct metabolic advantages of SL outweigh the lower cost of a physical mixture of LCT and MCT remains to be determined.

SL have been designed to modify their effective energy content while maintaining their taste, mouthfeel, and flavour enhancement characteristics. The mixture of varying lengths of fatty acids affects the behaviour of the fat in the absorption and digestion process, thereby reducing the energy content. The Nabisco Foods Group (East Hanover, NJ) and the Procter and Gamble company (Cincinnati, OH) have developed two such SL for commercial applications (Finley *et al.*, 1994a; Wardlaw *et al.*, 1995). Nabisco

developed a lipid containing short-chain fatty acids (Salatrim); Procter and Gamble made a SL predominantly with medium-chain fatty acids (Caprenin), as discussed earlier. Both of these SL contribute less energy than other TAG containing mainly long-chain fatty acids. In addition, the saturated long-chain fatty acid components, namely stearic acid (in salatrim) and behenic acid (in caprenin) further reduce the effective energy of these SL (Finley *et al.*, 1994a; Wardlaw *et al.*, 1995). Stearic acid is less absorbed as a fatty acid in the *sn*-1 or *sn*-3 position because of reduced solubility in the intestinal lumen (Finley *et al.*, 1994a). Behenic acid is a very-long-chain fatty acid that is a solid at body temperature and poorly absorbed regardless of its position on the glycerol backbone (Wardlaw *et al.*, 1995).

Salatrim is a SL made from TAG containing short chain fatty acids (e.g., tributyrin) and long-chain saturated TAG (e.g., hydrogenated rapeseed oil). This product has the potential to substantially reduce the energy value of foods into which it is incorporated while still retaining desirable textural properties. In a human clinical trial in which 17 individuals received a diet containing 22% of energy from Salatrim for 7 days, a metabolizable energy value of 4.9 kcal/g and absorption of stearic acid ranging from 63 to 70% were reported for this product (Finley *et al.*, 1994b).

Nordenstrom *et al.* (1995) infused low (0.38 g TAG/ kg body weight) and high (1 g TAG/ kg body weight) equimolar doses of SL (Structolipid®) and LCFA (Intralipid®) for 6 h in eight healthy volunteers. The SL consisted of a mixture of LCT and MCT in a

ratio of 64:36 by weight (50:50 by mole basis). The subjects served as their own controls. After SL infusion, plasma concentrations of the MCFA/LCFA ratio were similar to the mole ratio of the infused lipid emulsion, indicating that MCFA and LCFA were released at the same time.

Numerous experimental studies have reported that high levels of MCFA cause toxic effects and should therefore be avoided. Even during infusion of a physical mixture of MCT/LCT, MCFA are released into the bloodstream more rapidly than LCFA, resulting in high blood concentrations of MCFA. Due to the simultaneous generation of LCFA and MCFA, MCFA should be provided more slowly with SL-containing emulsions. In a preliminary study, Flaaten *et al.* (1995) showed that the production of MCFA is significantly lower when SL (Structolipid®) is administered than when a MCT/LCT physical mixture is used. In addition, plasma fatty acid profiles during SL infusion were similar to the fatty acid composition of the infused emulsion (Nordenstrom *et al.*, 1995).

2.8 Metabolism of structured lipids

Early studies have shown that SL synthesized from a mixture of MCT and LCT conferred several unique advantages over simple physical mixtures of the same fatty acids, including the ability to improve nitrogen retention while preserving reticuloendothelial function (Sorbrado *et al.*, 1985). The reason for this is not entirely

understood but may relate to a more rapid oxidation and clearance of the LCFA components of the SL.

The investigation of SL emulsions has shown interesting properties with regard to nitrogen metabolism and immune function in experimental animals. Mendez *et al.* (1992) compared the effects of a SL (produced by interesterifying fish oil with MCFA) with a physical mixture of fish oil and MCT and found that the SL improved the nitrogen balance in mice, possibly because of the modified absorption rates of SL. Their results also revealed that tumour growth rate was slowed in SL-fed mice with significant increases in rates of tumour protein synthesis and tumour protein breakdown.

In another study, Mok *et al.* (1984) studied the effects of various lipid sources on protein metabolism. The following fat sources were used: (a) LCT, (b) MCT, (c) 1:1 physical mixture of LCT and MCT, and (d) SL (made from 40% LCT and 60% MCT). Again, rats receiving SL had gained more body weight and had significantly higher nitrogen retention and serum albumin concentrations. This study confirmed the benefits of SL over MCT and LCT, whether each lipid is given alone or as a physical mixture. Improved nitrogen balance, hepatic protein synthesis and decreased leucine oxidation have been found when SL were given as a component of total parenteral nutrition to burned rats (Maiz *et al.*, 1984). Hence, SL were shown to have unique properties that may be beneficial during critical illness; however, these findings could not be reproduced

when SL emulsions were given as the sole nonprotein energy source during hypocaloric feedings in septic animals (Yamazaki *et al.*, 1984).

DeMichele *et al.* (1988) also studied rats that were burned >25-30% of their body surface and were enterally fed a diet with 40% of nonprotein energy as fat. Four lipid sources were used: (a) LCT, (b) MCT, (c) a SL of modified dairy fat (50% butter oil, which contained SCFA, 35% MCT, and 15% safflower oil), and (d) another SL (64% MCT, 36% safflower oil). Both SL diets were shown to reduce postburn catabolism and resulted in a higher cumulative nitrogen balance, whereas the group given modified dairy fat showed a higher liver fractional synthetic rate and a higher whole-body protein synthetic rate than groups given either LCT or MCT. Groups given SL and MCT had significantly higher albumin concentrations than groups given LCT.

In a similar study, burned animals received fat as LCT, MCT, a physical mixture of 85% palm kernel and 15% sunflower oil (43% MCT, 57% LCT), and a SL composed of 85% palm kernel and 15% sunflower oil (12% MCT, 7% LCT, 81% of the SL). Again, the groups receiving SL and the physical mixture exhibited better nitrogen balance and an increased rate of protein synthesis. Higher serum albumin levels were observed in the SL and MCT fed groups. Again, the SL appeared to better attenuate the protein catabolic effect after injury (DeMichele *et al.*, 1989).

A SL with a high percentage of PUFA provided as fish oil has been shown to inhibit tumour growth while improving body weight and nitrogen retention in sarcoma-

bearing rats (Ling *et al.*, 1991). These studies compared SL composed of MCT and fish oil (with only enough LA to meet essential fatty acid requirements) with similar physical mixtures and lipid emulsions with excessive amounts of LA. In a similar study, Teo *et al.* (1989) compared the effect of enteral feeding with LCT (sunflower oil, which is rich in linoleic acid) and with SL (60% MCT, 40% fish oil). Burned rats were fed *via* a gastrostomy tube; 40% nonprotein energy was obtained from one of the two fats. The SL fed group had a higher cumulative nitrogen balance and higher muscle and liver fractional synthetic rates; metabolic rate was 7% lower in the SL group. The influence on metabolism observed in this study (i.e., reduced protein catabolism) was similar to that seen with other SL. However, the resultant reduced energy expenditure is unique to this SL and may be related to the ability of ω3 PUFA to diminish the injury response.

The effect of various levels of a SL (made from MCT and fish oil) on protein and energy metabolism has also been investigated in enterally fed hypermetabolic rats (Gollaher *et al.*, 1993). Rats were infused continuously with diets providing 200 kcal/ kg /day and 2 g amino acid nitrogen/ kg /day. The proportion of nonnitrogen calories as SL was varied: 0, 5, 15 or 30% fat. A 30% LCT diet was also provided as a control to compare the protein-sparing abilities of these two types of fat. Nitrogen excretion, plasma albumin, plasma TAG and whole-body and liver and muscle protein kinetics were determined after 3 days of feeding. Whole-body protein breakdown, flux, and oxidation were similar in all groups. The 15% SL diet maximized whole-body protein synthesis.

The liver fractional synthetic rate was significantly higher in animals receiving 5% of their nonprotein calories as SL. Muscle fractional synthetic rate was unchanged. Plasma TAG were markedly elevated in the 30% SL-fed rats. The 30% SL diet maintained plasma albumin levels better than diets containing no fat, 5% SL or 30% LCT. Nitrogen excretion was lower in animals receiving 30% nonnitrogen calories as a SL than in those receiving 30% as LCT (Gollaher *et al.*, 1993).

The effects of a SL, Captex 810D (Abitec Corporation, Columbus, OH; produced by chemical interesterification of MCFA and LCFA), on energy metabolism have been investigated in lean and obese zucker rats (Akoh *et al.*, 1998). Consumption of the SL diet resulted in increased energy expenditure or heat production in the obese rats. Thus, these results supported the idea that the Captex may be useful in limiting obesity.

Kenler *et al.* (1996) conducted a randomized trial comparing safety, gastrointestinal tolerance, and clinical efficacy of feeding an enteral diet containing a SL (made from fish oil and MCT) versus an isonitrogenous, isocaloric formula (Osmolite HN; Ross Laboratories, Columbus, OH) in patients undergoing major abdominal surgery for upper gastrointestinal malignancies. Patients receiving SL experienced no adverse side effects, significant incorporation of EPA in plasma and erythrocyte phospholipids, and 50% fewer gastrointestinal complications and infections than patients given Osmolite HN formula. These data strongly suggest improved liver and renal function during postoperative period in the SL fed group. The authors concluded that early enteral feeding

with SL was safe and well tolerated. Their results suggest that the use of such a formula during the postoperative period reduces the number of infections and gastrointestinal complications and improves renal and liver function by modulation of urinary prostaglandin levels. Additional clinical trials should be undertaken to further assess the clinical benefits of this SL.

When the SL caprocaprylobehenin (Caprenin) at 40% of total calories was fed to healthy men as formula diet for 6 days along with diets containing LCT (18:1ω9 and 18:2ω6) or MCT (8:0-10:0), there was no effect on plasma cholesterol (Swift *et al.*, 1992). However, the HDL-cholesterol was reduced by 14% by the SL, 15% by the MCT, but was unchanged by the LCT. Plasma TAG were increased by 42% by the MCT, but were unaffected by either diets containing LCT or the SL (Swift *et al.*, 1992).

Data from human studies, using intravenous administration of SL, have been scarce so far. In two preliminary studies SL administered intravenously have been shown to be safe and to have beneficial effects over conventional lipids given intravenously (Sandstrom *et al.*, 1993; Nordenstrom *et al.*, 1995). Sandstrom *et al.* (1993) evaluated the safety and tolerance of SL emulsion 73403 (Kabi Pharmacia Parenterals, Stockholm, Sweden), an interesterified mixture of MCFA and LCFA in the same glyceryl moiety, administered to postoperative patients requiring total parenteral nutrition. No difference in safety and tolerance of the SL emulsion (73403) compared with the standard LCT emulsion, which contained only LCFA in a TAG molecule (Intralipid® 20%, Kabi

Pharmacia Parenterals), was observed. This study underscores the need for more studies on metabolic efficiencies of SL in postoperative patients. There is also need for determining the optimum dosage of these lipids for specific treatments. A review of the optimum lipid sources in enteral and parenteral nutrition was published by Gottschlich (1992).

In a study in human patients after major surgery, Sandstrom *et al.* (1995) for the first time observed that the provision of SL is associated with a higher whole-body lipid oxidation rate than LCT in metabolically stressed patients. The lipid oxidation rate was significantly higher with SL than with LCT.

2.9 Structured lipids in disease prevention

The ω3 and ω6 fatty acids, especially EPA, DHA and GLA, may be incorporated into SL to promote health and nutrition. These fatty acids have several health benefits to combat arthritis, thrombosis, cardiovascular disease, diabetes and cancer (Horrobin, 1990; Vartak *et al.*, 1997), as explained below.

2.9.1 Arthritis

Arthritis is a chronic inflammatory disease in joints (Das and Huang, 2000). Beneficial effects of diets high in ω3 and ω6 fatty acids have been reported in arthritic patients. 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).

2.9.2 Thrombosis

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 (Groom, 1993).

2.9.3 Cardiovascular disease

Interest in fish oils has stemmed from the observations made in the 1960's of the almost total absence of heart disease among Greenland Eskimos despite their consumption of a diet very high in fat and cholesterol (Bang and Dyerberg, 1972; Rice, 1991). It was later found that the blood of Eskimos contained a high concentration of EPA and DHA from a diet rich in fish and seal meat (Bang and Dyerberg, 1972; Ahmad, 1998). Recent work of Mori *et al.* (1997) suggests that ω3 intake from fish consumption

in conjunction with a low fat diet is most beneficial in terms of reducing cardiovascular disease.

Cardiovascular disease is the leading cause of death in industrialized countries (Newton, 1996). Recent research indicates that the long chain ω3 PUFA, especially EPA and DHA, may be effective in reducing the clinical risk of cardiovascular disease by favourably altering lipid and haemostatic factors such as bleeding time and platelet aggregation (Hornstra, 1989; Lauy-Dagach and Valenzuela, 1996). 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 humans.

2.9.4 Diabetes

In diabetic subjects, dietary supplementation of long-chain PUFA from fish oils has been studied more extensively than that of other fatty acids. The two predominant PUFA in fish oil are EPA and DHA. Possible effects of ω3 fatty acids on individuals with diabetes have been studied. Recently, the development of insulin resistance in normal rats fed a high-fat, safflower oil, diet was found to be prevented by partial replacement of linoleic acid with EPA and DHA from fish oil (Storlien *et al.*, 1987). From human studies, it is clear that in diabetic subjects, ω3 fatty acids exert beneficial effects on lipid metabolism and may decrease the severity of cardiac disorder and hence lower the incidence of coronary artery disease (Bhathena, 1992).

Mitsuyoshi *et al.* (1992) studied the effect of SL (synthesized as $C_{8:0}$-$C_{18:2}$-$C_{8:0}$) as an energy substrate after hepatic resection in diabetic rats. The lipid sources used in this study were MCT, LCT, a simple physical mixture of MCT/LCT and SL. The blood ketone body ratio (acetoacetate/β-hydroxybutyrate) and the cumulative excretion of $^{14}CO_2$ in expired breath after [^{14}C] glucose administration were significantly higher in the SL group than in the other groups. These findings suggest that SL may be a superior energy substrate to other TAG preparations during the critical period after hepatectomy in diabetic patients.

2.9.5 Cancer

A SL made from fish oil and medium-chain TAG was found to decrease tumour growth in mice (Ling *et al.*, 1991). In another study, the tumour growth rate was slowed in rats fed with SL containing medium-chain fatty acids and fish oil (Mendez *et al.*, 1992). In contrast to tumour-promoting effects of diets high in fat, diets high in fish oil failed to promote the development of tumours in rats (Braden and Carroll, 1986). Reddy and Maruyama (1986) also pointed out that diets containing high levels of fish oil inhibit or suppress tumour 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 what potential side effects might exist (Haumann, 1997b). 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 remains somewhat elusive.

Impact® (produced by Novartis Nutrition, Minneapolis, MN) is a medical product containing SL. It is produced by interesterifying high-lauric oil with high-linoleic acid oil. This product is used for patients suffering from trauma or surgery, sepsis or cancer (Haumann, 1997a).

2.10 Structural analysis of oils

If the TAG has two different fatty acids esterified to the primary hydroxyl groups of glycerol then the molecule is optically active. In order to specify the position of the fatty acid on the glyceryl moiety, stereospecific numbering (*sn*) is used. In an optically active molecule drawn in the Fischer projection with the secondary hydroxyl group to left of the middle carbon atom (*sn*-2), the carbon atom above this is *sn*-1 and the one below it is *sn*-3 (Laakso, 1996).

The structure of TAG molecule has an impact on its nutritional and biochemical properties. Intestinal absorption of fatty acids has been reported to be dependent on their arrangement in the TAG molecules. Investigation of the absorption of fatty acids in a canine model suggested that the positional distribution of fatty acids within the TAG molecules might affect the metabolic fate of fatty acids (Jensen *et al.*, 1994). During digestion, lipases hydrolyze TAG into FFA, predominantly from the *sn*-1 and *sn*-3

positions and 2-MAG (Mattson and Volpenhein, 1962) which will be absorbed into the intestinal mucosal cells of the small intestine. Although most dietary lipids are in TAG form, relatively little is known about the importance of stereospecific composition of TAG on the biological activity of dietary fatty acids (Kubow, 1996). During the biosynthesis of TAG, all glycerol positions are known to be important. The major routes of TAG synthesis include *sn*-glycerol-3-phosphate, dihydroxyacetone phosphate and MAG pathways (Laakso, 1996). In most cases, the *sn*-3 position is the last to be esterified (Laakso, 1996). Differences in the positional distribution of fatty acids have also been shown to have specific effects on the profile, structure and composition of lipoproteins (Kubow, 1996).

2.10.1 Stereospecific analysis of triacylglycerols (TAG) of oils

Stereospecific analysis determines how the fatty acids of TAG are distributed over the three different positions of the glycerol (Brockerhoff, 1971). Differences in the distributions of fatty acids among the positions of the glyceryl moiety in TAG from natural fats and oils were first demonstrated systematically by enzymatic hydrolysis procedures, especially pancreatic lipase hydrolysis for the analysis of the fatty acids of position *sn*-2, before complex stereospecific hydrolysis procedures were developed that permitted complete positional distributions of fatty acids to be determined. Because of this historical development of the analytical procedures, there has been a tendency to assume that the composition of fatty acids esterified to the sole secondary hydroxyl group must have greater

importance than those of the two primary positions. It is certainly true that the composition of position *sn*-2 is of great importance when TAG are consumed and digested by animals, since *sn*-2-MAG are then formed which can be absorbed by the intestines and utilized as such (Carey *et al.*, 1983). On the other hand, the results of stereospecific analyses have shown that the compositions of all three positions in certain fats can be distinctive and can highlight important aspects of the biosynthetic processes. Position *sn*-3, for example, is the last position to be acylated during TAG biosynthesis (Laakso, 1996) and this step is potentially important in the cellular control mechanism.

Positional distribution of fatty acids in TAG can also be determined either by high-resolution ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy (Aursand *et al.*, 1995; Bergana and Lee, 1996) on the basis of the chemical shift of esterified fatty acids in all three positions, and by HPLC of the diastereomeric derivatives (urethane) of partial acylglycerols (Redden *et al.*, 1995; Myher *et al.*, 1996).

As previously discussed, the differentiation of fatty acids between primary and secondary positions might be essential from a nutritional point of view. The methods for complete stereospecific analysis of TAG are almost always based on the formation and separation of DAG. The traditional methods include phosphorylation of DAG and their hydrolysis with phospholipases (Brockerhoff, 1965). More recently, however, methods for separation of enantiomeric acylglycerols have been developed (Takagi and Itabashi, 1986, 1987; Takagi and Ando, 1990). There are two main ways to separate enantiomers. First, by

reaction with an achiral reagent; enantiomers form enantiomeric derivatives that may be separated by a chiral stationary phase. Reaction with a chiral reagent may also be carried out, upon which enantiomers form diastereomeric derivatives that are separated by achiral stationary phases (Takagi, 1990).

The traditional methods of stereospecific analysis were pioneered by Brockerhoff (1965). The first step was partial deacylation of TAG with pancreatic lipase or Grignard reagent. Currently, Grignard reaction is preferred because it has no fatty acid specificity and causes less acyl migration than other available methods. Since Grignard reagent reacts in a random manner with ester linkages, *sn*-1,2 DAG, *sn*-1,3 DAG, MAG and tertiary alcohols are produced.

The *sn*-1,2 DAG can be separated from the *sn*-1,3 DAG and other fractions by TLC (Wanasundara and Shahidi, 1997). These enantiomeric acylglycerols can also be separated by HPLC using silica gel as the stationary phase (Damiani *et al.*, 1994) or using a chiral column (Itabashi *et al.*, 1993). The purified DAG are then phosphorylated to their phosphatidylphenols by reacting with phenyldichlorophosphate. The fatty acid in the *sn*-2 position is hydrolyzed using phospholipase A_2 leaving a lysophosphatidylglycerol with the fatty acid in position *sn*-1 and unreacted *sn*-2,3-DAG phosphatidylglycerol. The fatty acid composition of position *sn*-2 of TAG can therefore be determined from either the FFA or from 2-MAG. The fatty acid composition of *sn*-1 is determined by analysis of lysophosphatidylphenols and *sn*-3 is determined indirectly by calculation (Laakso, 1996).

The determination of fatty acyl residues esterified to the primary and secondary hydroxyl groups of TAG is most often accomplished with pancreatic lipase. The composition of position *sn*-2 of TAG can be determined by reacting them with the enzyme pancreatic lipase, the properties of which have been reviewed by Brockerhoff and Jensen (1974), and Verger (1984). However, the pancreatic lipase technique does not distinguish between *sn*-1 and *sn*-3 positions of TAG.

2.10.2 Positional distributions of fatty acids in plant oils

In general, seed oils containing common fatty acids show preferential placement of unsaturated fatty acids at the *sn*-2 position (Arcos *et al.*, 2000). Linoleic acid is especially concentrated at this position. The saturated fatty acids occur almost exclusively at the *sn*-1,3 positions. In most cases, the individual saturated or unsaturated fatty acids are distributed in approximately equal quantities between the *sn*-1 and *sn*-3 positions. For example, in maize, soybean, linseed, olive and wheat germ oils, the *sn*-2 position is almost exclusively occupied by unsaturated fatty acids, while saturated as well as unsaturated fatty acids occur in approximately similar quantities in positions 1 and 3 (Brockerhoff and Yurkowski, 1966; Arunga and Morrison, 1971).

The more saturated fatty acids of plant origin show a different distribution pattern. Approximately 80% of the TAG in cocoa butter are disaturated with oleic acid concentrated in the *sn*-2 position and saturated fatty acids almost exclusively located in

the *sn*-1 and *sn*-3 positions. There is approximately 2 times more oleic in the *sn*-1 than in the *sn*-2 position (Takagi and Ando, 1995). Approximately 84% of the TAG in coconut oil are trisaturated (Young, 1983) with lauric acid concentrated at the *sn*-2 position, caprylic acid at the *sn*-3, and myristic and palmitic acids at the *sn*-1 position. Plant oils containing erucic acid (22:1ω9) also exhibit considerable positional selectivity in the placement of fatty acids. In rapeseed oil, much greater quantities of erucic acid are preferentially located at the *sn*-1,3 positions, but more of it is present at the *sn*-3 position than at the *sn*-1 position (Brockerhoff and Yurkowski, 1966). In peanut oil, arachidic (20:0) and behenic (22:0) acids are found mainly in position *sn*-3, while twice as much LA is esterified to position *sn*-1 compared to position *sn*-3 (Myher *et al.*, 1977).

2.11 Concentration of highly unsaturated fatty acids from oils

Several techniques have been explored for the concentration of PUFA from various oils. Methods traditionally employed for the concentration of PUFA in oils make use of differences in physical and chemical properties between saturated and unsaturated fatty 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 and 69.6°C for 18:1 and 18:0, respectively (Merck Index, 1983). As the temperature of a mixture of a saturated and unsaturated fatty acid decreases, the saturated fatty acid, having a higher melting point, starts to crystallise out first and the liquid phase becomes enriched in the

unsaturated fatty acids. 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 must be carried out to obtain purified fractions. In the case of marine oils, not only is there a very wide spectrum of fatty acids but the fatty acids exist, not in the FFA form, but esterified in TAG. However, the principle of low temperature crystallisation can still be applied to marine oils partially to concentrate TAG rich in ω3 PUFA (Shahidi and Wanasundara, 1998a).

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 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). Urea complexation of fatty acids of borage oil, using methanol, can increase the GLA content from 23.6 to 94% (Huang *et al.*, 1999). 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.

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).

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.

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 work under mild conditions of temperature and pH (Gandhi, 1997), a factor which favours their potential use for the enrichment of PUFA in oils. Lipases which act on neutral lipids generally hydrolyze the esters of PUFA at a slower rate than those of more saturated fatty acids (Villeneuve and Foglia, 1997). Use has been made of this relative substrate specificity to increase the concentration of ω3 PUFA in seal blubber and menhaden oils by subjecting them to hydrolysis by a number of microbial lipases (Wanasundara and Shahidi, 1998).

2.12 Oxidative stability of oils containing highly unsaturated fatty acids

The oxidation of unsaturated lipids of foods has been one of the most extensively studied areas of research in food science and nutrition as it relates to the deterioration of foods. Numerous chemical and biochemical reactions affect the quality attributes (colour,

odour, flavour, texture), nutritional value and safety of food components and none epitomizes this more than the oxidation of unsaturated fatty acids (Hsieh and Kinsella, 1989). Oxidation of unsaturated fats and oils is a major concern because it is directly related to cost, nutrition, flavour, safety and storage of products.

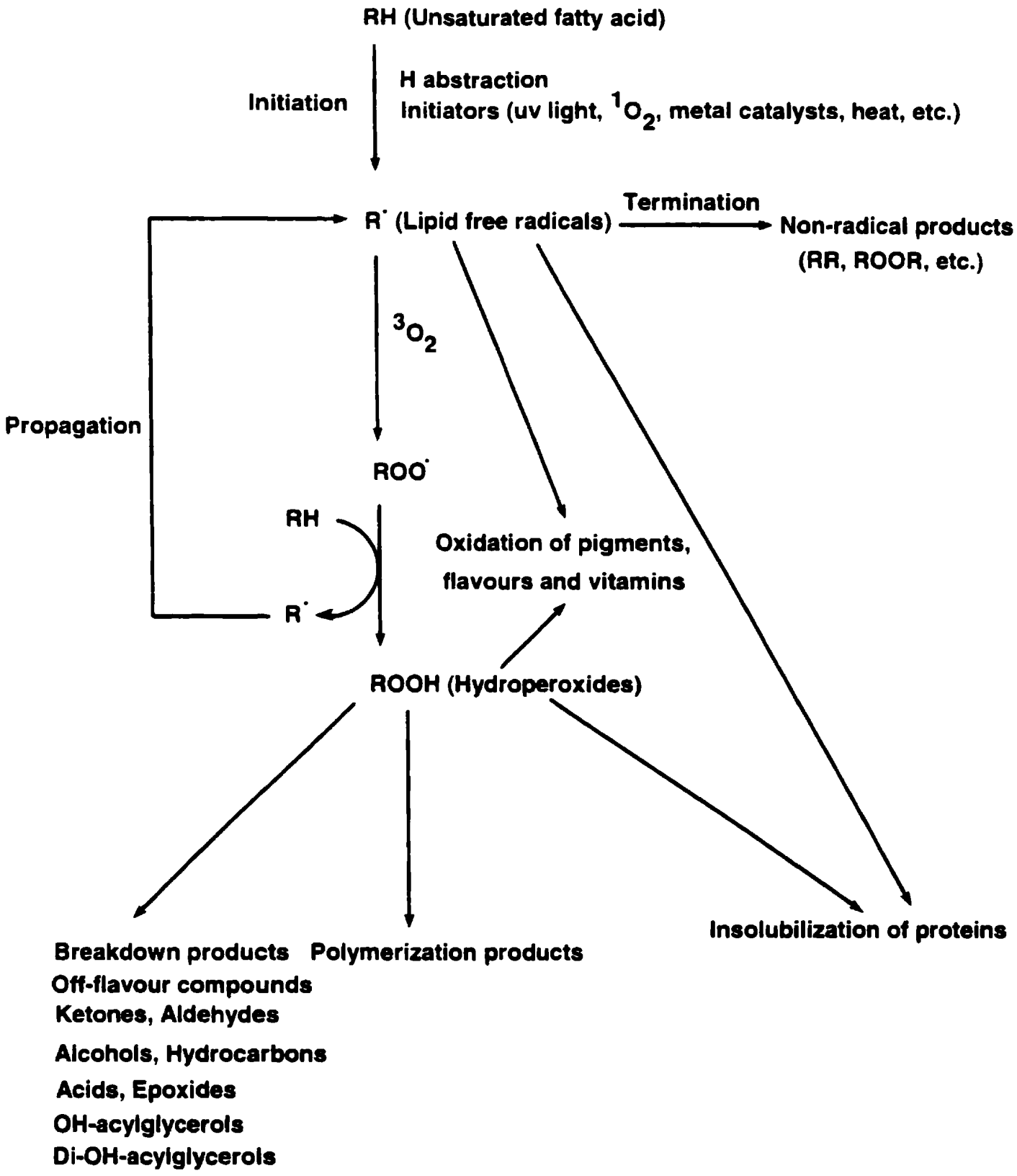
Oxidation of food lipids may proceed *via* enzymatic oxidation, photooxidation, autoxidation and thermal oxidation. Since enzymes are liable to heat denaturation, lipoxygenase activity is normally absent in a refined oil. Therefore, oxidation catalyzed by enzymes does not create a problem in the storage and use of fats and oils. Formation of hydroperoxides from unsaturated fatty acids by photooxidation involves the generation of highly reactive singlet oxygen. Autoxidation is the process of oxidation induced by air. Oxidative reactions of lipids are greatly accelerated at higher temperatures. The oxidation of lipids at higher temperatures is referred to as thermal oxidation.

Lipid oxidation is due to a combination of triplet oxygen and singlet oxygen reactions (Ho and Chen, 1994). Triplet oxygen lipid oxidation has been extensively studied in the 20^{th} century in order to improve oxidative stability of foods (Labuza, 1971). However, it does not fully explain the initiation step of lipid oxidation (Lee and Min, 1990). Rawls and VanSanten (1970) suggested that singlet oxygen is involved in the initiation of lipid oxidation because singlet oxygen can react directly with double bonds of fatty acids without the formation of free radicals.

2.12.1 Chemistry and mechanism of autoxidation

One of the primary pathways of lipid degradation is that of autoxidation. Autoxidation of unsaturated fatty acids occurs *via* a free-radical mechanism (Crapiste *et al.*, 1999) in which oxygen is added to the unsaturated fatty acid chains of lipid molecules. Autoxidation involves initiation, propagation and termination steps (Figure 2.7). These processes often consist of a complex series of reactions. The initiation process may occur due to the abstraction of a hydrogen atom adjacent to the double bond in a fatty acid (RH) and a free radical ($R^{\bullet}$) is formed. 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 antioxidants which react completely with alkyl free radicals and/or peroxy radicals and remove them from the system (King *et al.*, 1995).

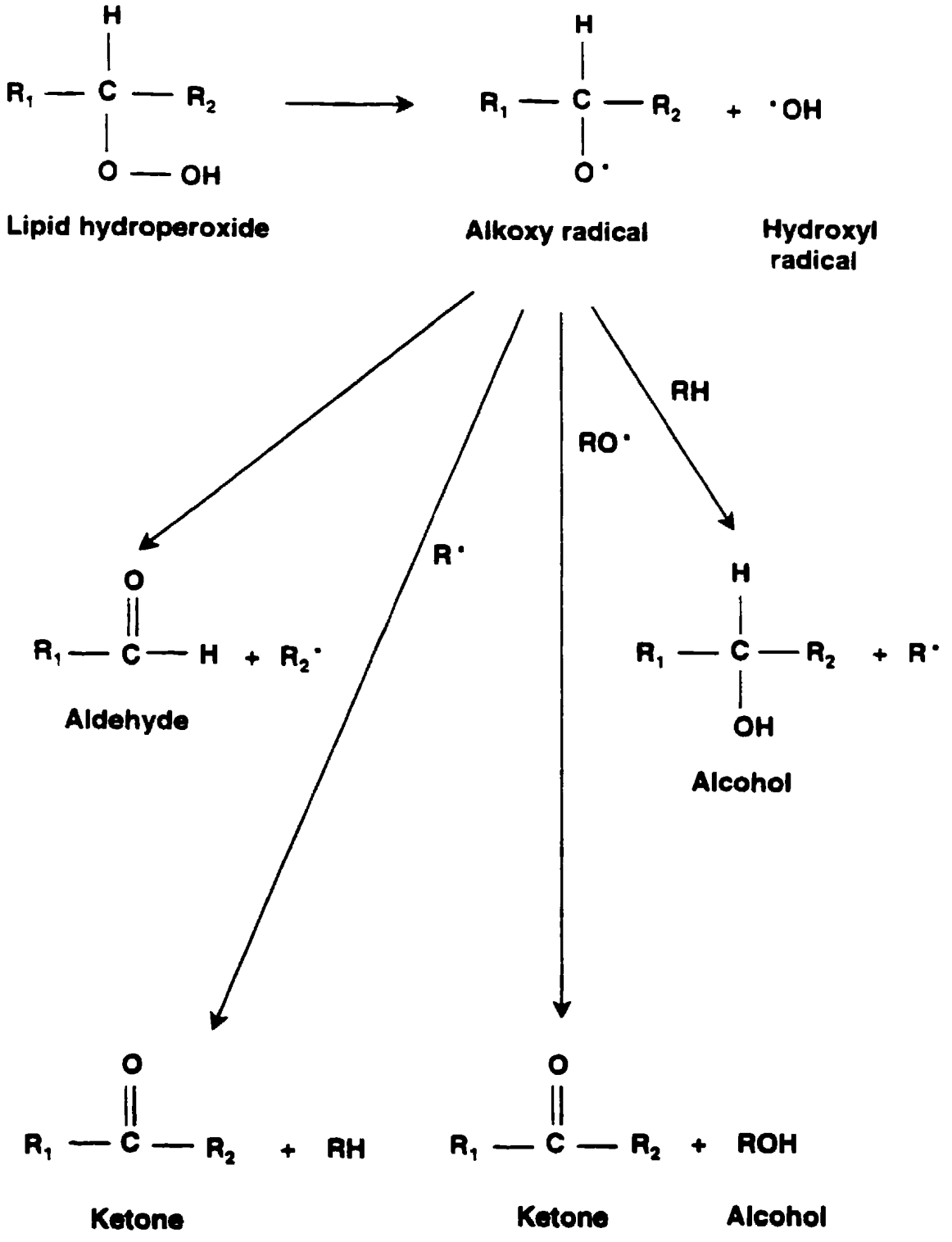
Figure 2.7 Generalized scheme for autoxidation of lipids


RH (Unsaturated fatty acid)
Initiation
H abstraction
Initiators (uv light, $^1O_2$, metal catalysts, heat, etc.)
R· (Lipid free radicals)
Termination
Non-radical products
(RR, ROOR, etc.)
$^3O_2$
Propagation
ROO·
RH
R·
Oxidation of pigments,
flavours and vitamins
ROOH (Hydroperoxides)
Insolubilization of proteins
Breakdown products
Off-flavour compounds
Ketones, Aldehydes
Alcohols, Hydrocarbons
Acids, Epoxides
OH-acylglycerols
Di-OH-acylglycerols
Polymerization products

2.12.2 Decomposition of hydroperoxides

Hydroperoxides (or primary oxidation products) do not have any off-flavour or off-odour. However, they are very unstable and regardless of the mechanism of 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 or hydrocarbons which are believed to be responsible for the development of off-flavour and oxidative rancidity in foods (Figure 2.8). 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. The decomposition of hydroperoxides occurs *via* homolytic cleavage of the oxygen-oxygen bond to yield hydroxyl and alkoxy free radicals. The alkoxy radical can then undergo 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 ketones.

Figure 2.8 Decomposition of hydroperoxides and subsequent termination of chain reactions


H
R1 — C — R2
O — OH
Lipid hydroperoxide
H
R1 — C — R2
O·
+ ·OH
Alkoxy radical
Hydroxyl radical
RH
RO·
R·
O
R1 — C — H + R2·
Aldehyde
H
R1 — C — R2
OH
+ R·
Alcohol
O
R1 — C — R2 + RH
Ketone
O
R1 — C — R2 + ROH
Ketone
Alcohol

2.12.3 Autoxidation of fatty acids

The rate of autoxidation of fatty acids depends greatly on the degree of unsaturation. In general, the rate of oxidation increases rapidly with greater unsaturation in the fatty acid moiety of the TAG. For example, the relative rate of autoxidation of oleate, linoleate and linolenate was reported to be in the order of 1:40-50:100 on the basis of oxygen uptake and in the order of 1:12:25 on the basis of peroxide formation (Hsieh and Kinsella, 1989).

Possible autoxidation products of oleic, linoleic (LA) and α-linolenic (ALA) acids are shown in Figures 2.9, 2.10 and 2.11, respectively. Autoxidation of oleic acid involves hydrogen abstraction from carbon-8 and carbon-11 with formation of two allylic radicals (Figure 2.9). These intermediates react with oxygen to produce a mixture of 8-, 9-, 10- and 11-allylic hydroperoxides (Frankel, 1985). Hydrogen abstraction on the double allylic carbon-11 of LA produces a pentadienyl radical (Ho and Chen, 1994) which reacts at both ends with oxygen to produce a mixture of conjugated 9- and 13-diene hydroperoxides (Figure 2.10) (Frankel *et al.*, 1982). Hydrogen abstraction of the ALA occurs on the two active methylenes on carbon-11 and carbon-14 and produces two pentadienyl radicals which react with oxygen at the end carbon to form a mixture of conjugated diene-triene 9-, 12-, 13- and 16-hydroperoxides (Figure 2.11) (Chan and Levett, 1977).

Figure 2.9 Mechanism of autoxidation of oleic acid and formation of possible primary and secondary products

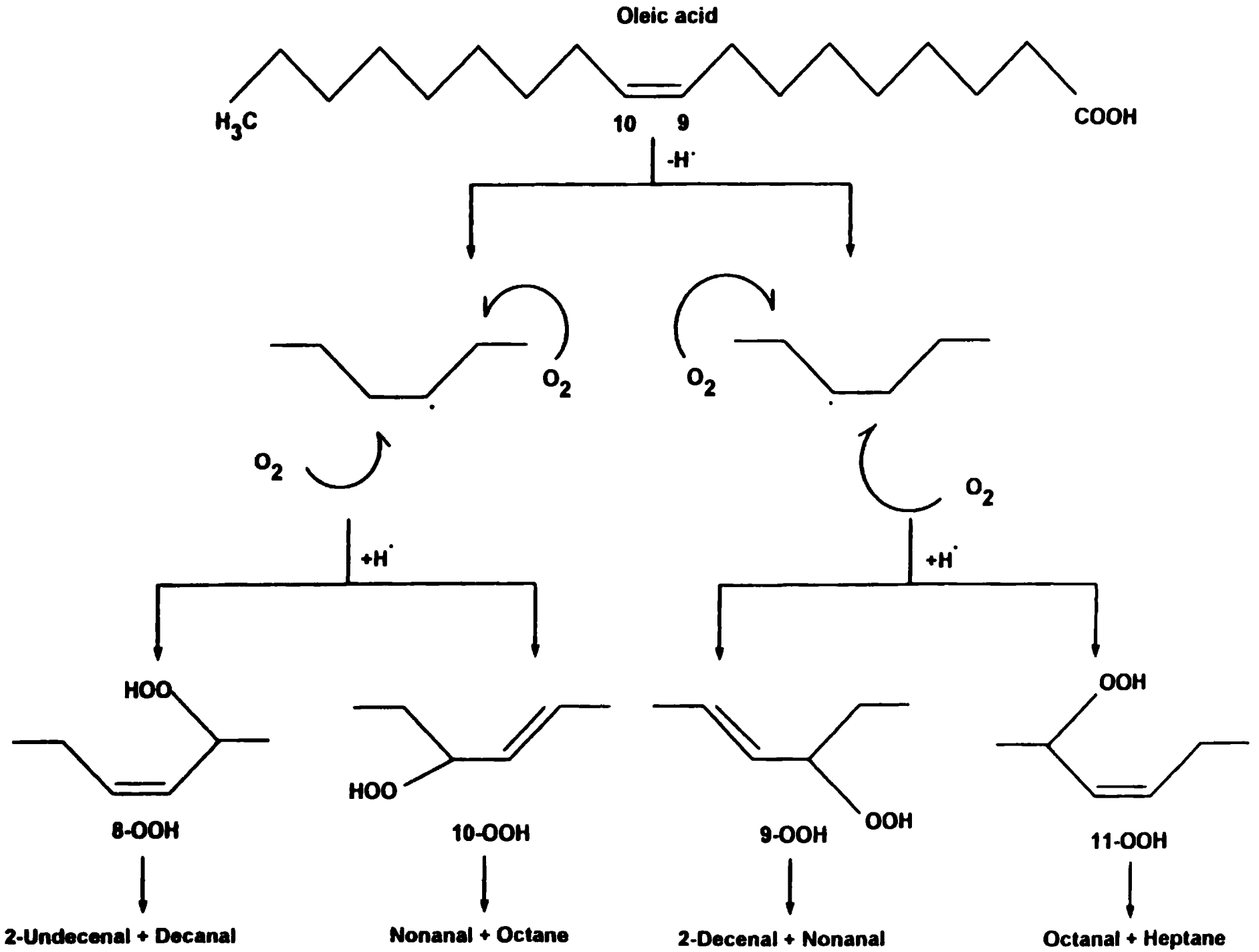

Oleic acid
H_3C
10
9
COOH
-H·
O_2
O_2
O_2
O_2
+H·
+H·
HOO
HOO
OOH
OOH
8-OOH
10-OOH
9-OOH
11-OOH
2-Undecenal + Decanal
Nonanal + Octane
2-Decenal + Nonanal
Octanal + Heptane

Figure 2.10 Mechanism of autoxidation of linoleic acid and formation of possible primary and secondary products

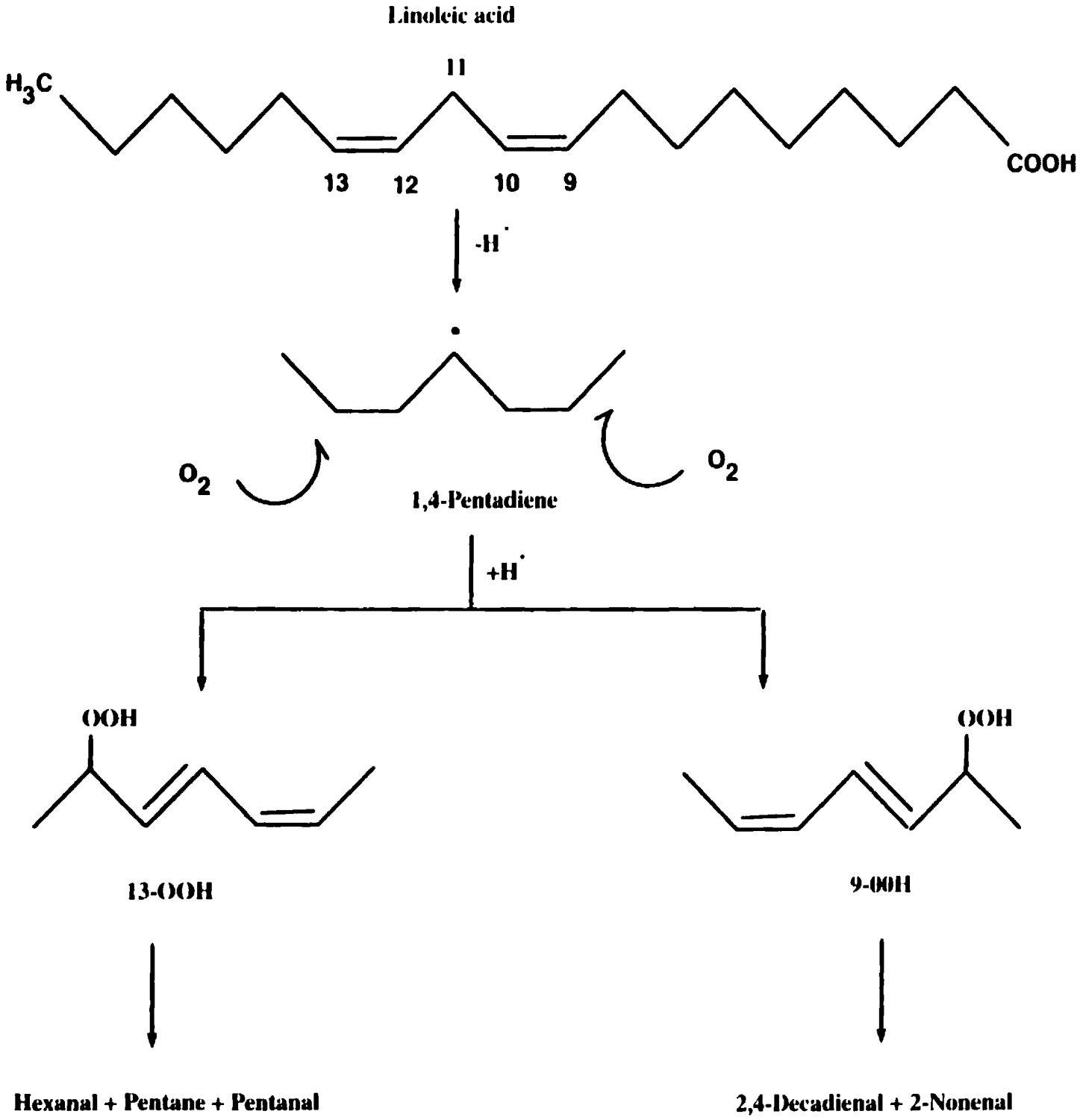
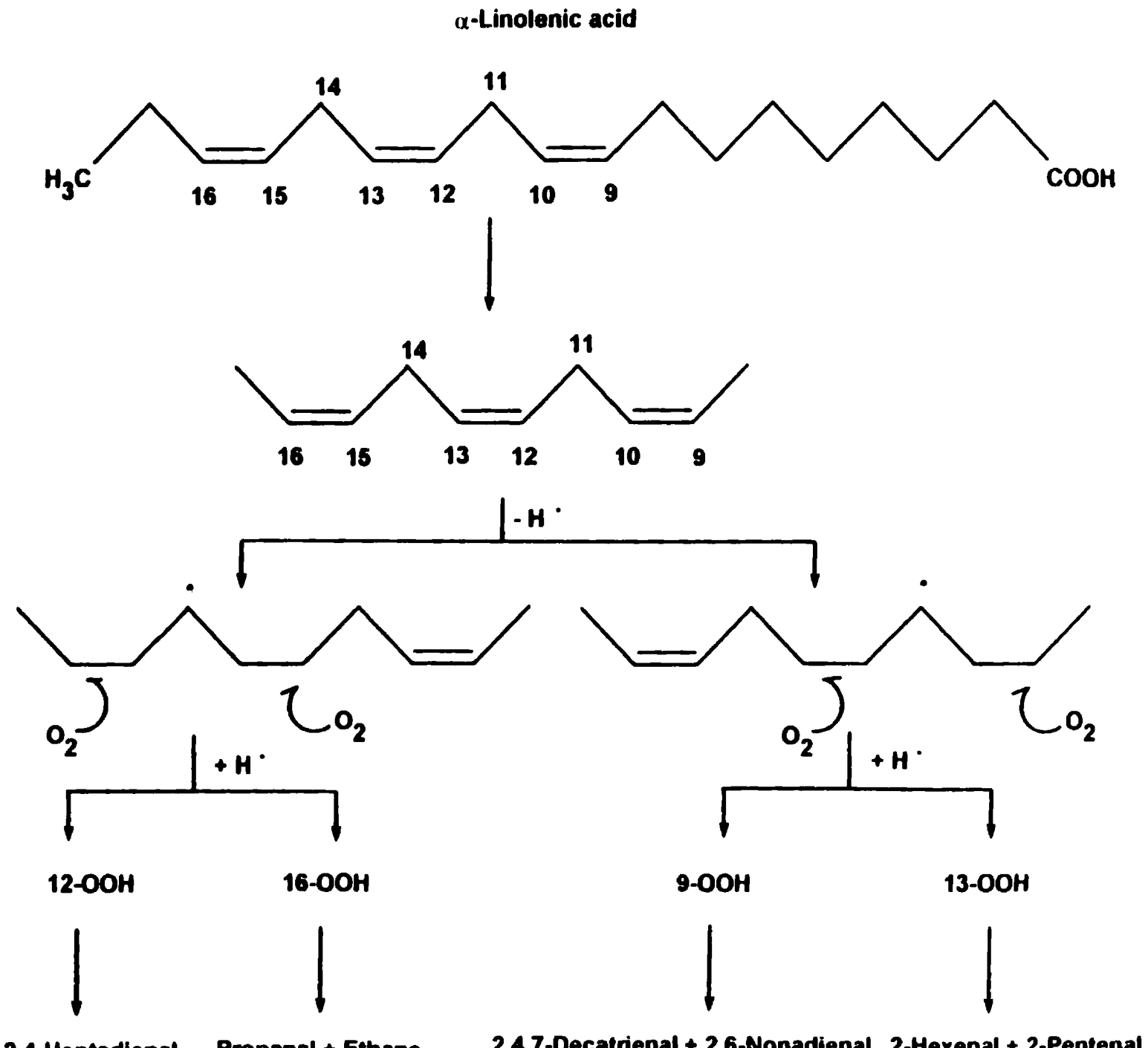

Linoleic acid
H_3C
11
13
12
10
9
COOH
-H·
O_2
O_2
1,4-Pentadiene
+H·
OOH
OOH
13-OOH
9-OOH
Hexanal + Pentane + Pentanal
2,4-Decadienal + 2-Nonenal

Figure 2.11 Mechanism of autoxidation of α-linolenic acid and formation of possible primary and secondary products

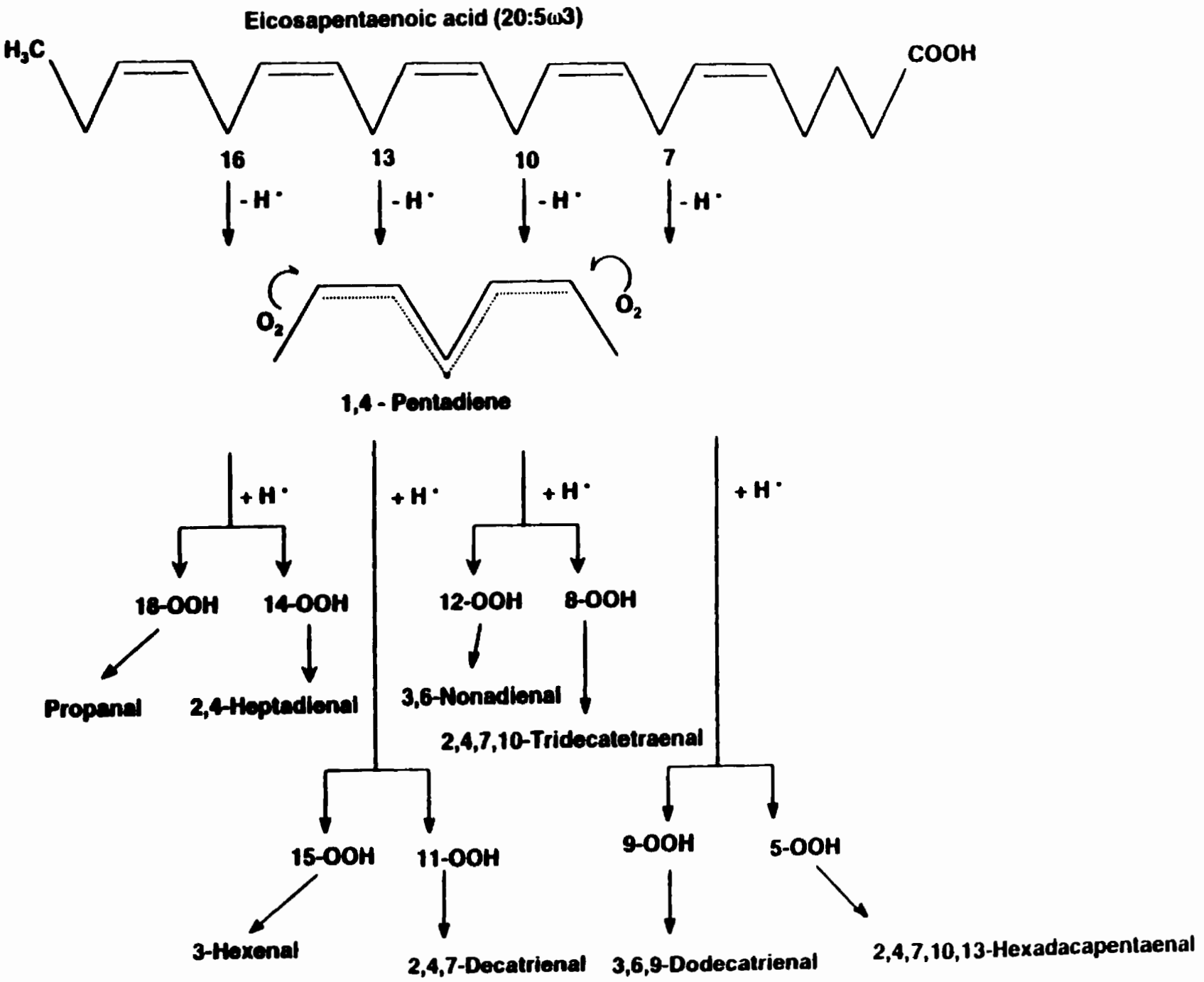

α-Linolenic acid
14
11
H_3C
16
15
13
12
10
9
COOH
14
11
16
15
13
12
10
9
- H ·
O_2
O_2
+ H ·
O_2
O_2
+ H ·
12-OOH
16-OOH
9-OOH
13-OOH
2,4-Heptadienal
Propanal + Ethane
2,4,7-Decatrienal + 2,6-Nonadienal
2-Hexenal + 2-Pentenal

Linoleic acid oxidizes much faster than oleic acid (Rossell, 1991) because it has an active *bis*-allylic methylene group on carbon-11 that can lose a hydrogen atom very easily. The greater reactivity of LA to autoxidation is due to the formation of a pentadienyl radical intermediate which is more effectively stabilized by resonance, and the resulting dienoic hydroperoxides produced are stabilized by conjugation (Frankel, 1998a). ALA has two *bis*-allylic methylene groups and reacts twice as fast with oxygen as does LA.

PUFA such as AA, EPA and DHA, containing 4,5 and 6 double bonds, respectively, are much more liable to oxidation than linoleic and linolenic acids (Hsieh and Kinsella, 1989). AA was reported to oxidize 2.9 times faster than LA (Porter *et al.*, 1981). Hydrogen abstraction at the double allylic carbon-7, carbon-10, and carbon-13 positions of AA produces three pentadienyl radicals, which then react with oxygen at the end-carbons, carbon-5 and carbon-9, carbon-8 and carbon-12, as well as carbon-11 and carbon-15, respectively (Terao and Matsushita, 1981).

As the number of double bonds increases in PUFA, they produce more complex mixtures of hydroperoxides which are easily decomposed and become very difficult to analyse quantitatively. The most important ω3 PUFA found in fish and marine oils include EPA and DHA. The hydroperoxides produced from EPA and DHA have been identified but not quantified (Frankel, 1998a). By the same mechanism established for linolenic acid, EPA produced 5-, 8-, 9-, 11-, 12-, 14-, 15- and 18-hydroperoxides (Figure 2.12) (Yamaguchi *et*

Figure 2.12 Mechanism of autoxidation of eicosapentaenoic acid (EPA) and formation of possible primary and secondary products


Eicosapentaenoic acid (20:5ω3)
H3C
COOH
16
13
10
7
- H·
- H·
- H·
- H·
O2
O2
1,4 - Pentadiene
+ H·
+ H·
+ H·
+ H·
18-OOH
14-OOH
12-OOH
8-OOH
Propanal
2,4-Heptadienal
3,6-Nonadienal
2,4,7,10-Tridecatetraenal
15-OOH
11-OOH
9-OOH
5-OOH
3-Hexenal
2,4,7-Decatrienal
3,6,9-Dodecatrienal
2,4,7,10,13-Hexadacapentaenal

al., 1985) while DHA produces 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17- and 20-hydroperoxides (Van Rollins and Murphy, 1984).

2.12.4 Autoxidation *versus* photooxidation

The reaction mechanism and reactivity rates of autoxidation are different from those of photooxidation. Therefore, autoxidation of unsaturated fatty acids produces different hydroperoxides from those formed by photooxidation. In the autoxidation, the initiation stage, represented by the lag phase of a measured reaction, includes the formation of the first hydroperoxides. The ensuing propagation phase involves the breakdown of these hydroperoxides to form free radicals which further generate their own formation in an autocatalytic chain reaction. The initiation stage of autoxidation has been considered to be of principal importance in determining the onset of rancidity in lipid containing foods (Labuza, 1971), because it is during the early stages of autoxidation that small molecular weight compounds causing off-flavours are formed.

In the photooxidation of lipids, the reaction is apparently not autocatalytic. The quantity of hydroperoxides formed is proportional to the total amount of light absorbed. Furthermore, in contrast to autoxidation, the hydroperoxides formed during photooxidation may be non-conjugated (Terao and Matsushita, 1977). As hydroperoxides are formed, they undergo further oxidation by light to form free radicals (Neff *et al.*, 1983), yet this does not give autocatalytic character to photooxidation. Pigments present in foods such as

chlorophyll, porphyrins and riboflavin (Usuki *et al.*, 1984) may act as photooxidation sensitizers by transferring their absorbed light energy to either molecular oxygen or a substrate (lipid) which then becomes a reactive intermediate. If an excited sensitizer reacts with molecular oxygen (relatively unreactive in its ground triplet state), singlet oxygen may be formed. Singlet oxygen is a reactive intermediate in the photooxidation of lipids and further, its participation as the primary source for the original hydroperoxide formation in the initiation of fatty acid autoxidation has been suggested (Rawls and Van Santen, 1970).

2.13 Methodologies for assessing lipid oxidation

Lipid oxidation can be measured by objective and subjective methods. Although widely used, subjective methods of assessing the oxidative stability of prepared foods are time consuming, and taste panels are difficult to maintain. Several techniques for measuring the extent of lipid oxidation has been extensively reviewed recently (Gray, 1978; Hoyland and Taylor, 1991; Frankel, 1993a; Shahidi and Wanasundara, 1996). These include conjugated dienes, peroxide value, TBA test, measurement of volatile carbonyl compounds, nuclear magnetic resonance (NMR) and fourier tansform infrared (FTIR) methods.

Most of the analytical methods to follow lipid oxidation have limitations which will be discussed in the following sections. Furthermore, the methodologies used to evaluate oxidative stability of edible oils must be carefully interpreted based on the analytical procedure used to determine the extent and the end point of the oxidation. Therefore, it is

recommended that progress of oxidation be followed by more than one method by measuring different types of products, including primary and secondary products of lipid oxidation (Shahidi and Wanasundara, 1996). It is also possible to determine the extent to which the various methods agree with one another using carefully controlled model systems. This can be achieved by calculating the correlation coefficients (Hudson and Gordon, 1994) or by employing linear regression analysis (Shahidi and Wanasundara, 1996). The following sections will focus on widely used methods and analytical techniques employed for assessing lipid oxidation.

2.13.1 Conjugated dienes

Oxidation of PUFA is accompanied by an increase in the ultraviolet absorption of the product. Lipids containing methylene-interrupted dienes or polyenes show a shift in their double-bond position during oxidation that is due to isomerization and conjugate formation (Logani and Davies, 1980). The resulting conjugated dienes can be determined quantitatively by their strong absorption maximum (λ_{max}) at 234 nm; similarly conjugated trienes can be determined by their absorption maximum (λ_{max}) at 268 nm. A weighed oil is diluted in isooctane and the absorbance at 234 nm measured spectrophotometrically.

Edible oil oxidation has been followed by measuring the absorbance at 234 nm by the IUPAC method (IUPAC, 1987) or by calculating the conjugated dienes as mmol of methyl linoleate hydroperoxide per kg of oil using molar absorptivity of 26,000. The

presence of molecules containing double bonds, such as carotenoids that can absorb ultraviolet light at 234 nm, may interfere with CD determination (Shahidi and Wanasundara, 1998b). However, this method is faster than the peroxide value determination, is much simpler, does not depend on chemical or colour reactions, and requires a smaller sample size.

2.13.2 Peroxide value (PV)

The peroxide value is the result of chemical analysis of the hydroperoxide content of the oil. This is one of the most commonly used methods for measuring oxidative stability of oils and food lipids. The primary products of lipid oxidation are hydroperoxides which are generally referred to as peroxides. Therefore, it seems reasonable to determine the content of peroxides as a measure of the extent of lipid oxidation.

The various procedures available for determining peroxide value are highly empirical and their accuracy depends on standardization of the experimental conditions. Methods based on iodometric titration are most commonly employed. The content of hydroperoxides can be determined quantitatively by means of an iodometric titration and expressed as PV. The determination of PV is based on the reduction of hydroperoxides (ROOH) with iodide (I^-). The liberated iodine is titrated with a standardized sodium thiosulphate ($Na_2S_2O_3$) solution. Therefore, the amount of released iodine is proportional to

that of peroxides present. The peroxide value is expressed as milliequivalents of active oxygen (i.e., peroxide) per kg of lipid (meq/kg).

Errors in the iodometric procedures have been attributed to addition of iodine to double bonds of unsaturated fatty acids and liberation of iodine by air oxidation of the iodide. The result is also affected by the structure and reactivity of the peroxides and the reaction temperature and time. However, iodometric titration method in Section Cd 8-53 of the AOCS (1990) eliminates most of these problems and is the method recommended by the author.

In practice, often only the PV is measured, but this can be misleading. It can be seen that (a) a low PV may be the result of fast breakdown of hydroperoxides rather than slow formation and (b) a high PV during processing is not good, but the effect on final taste and stability may be limited by selective breakdown to secondary products with less taste impact. Another problem with the PV is the need for careful sampling and handling.

The PV is a valuable measure of the early stages of lipid oxidation occurring at ambient temperatures (Shen *et al.*, 1999). However, it is less useful in assessing frying oils wherein peroxides decompose rapidly at typical frying temperatures (170-250°C) (Robards *et al.*, 1988).

Other reactions based on the reduction of hydroperoxides have been utilized for analytical purposes but are less popular at present. Thus, a spectrophotometric method

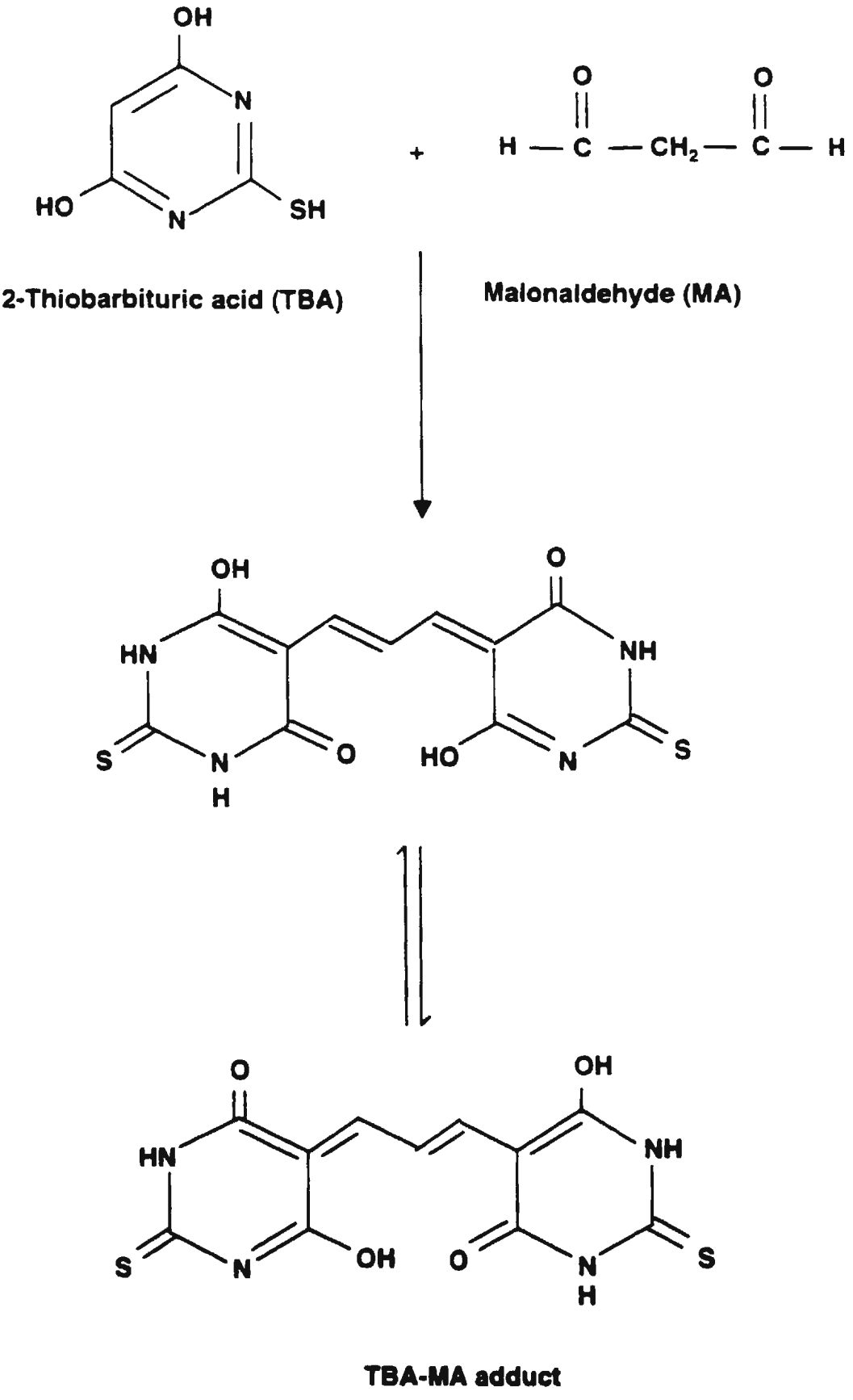
based on the peroxide oxidation of Fe^{2+} to Fe^{3+} and determination of the latter as the Fe^{3+}-triiodide complex has been proposed (Lovaas, 1992).

Good correlations have been observed between PV and sensory scores of several vegetable oils (Frankel, 1993a). Nonetheless, oils with low PV value have not always received high flavour scores. Therefore, it has been recommended that additional analytical methods be used to assess the stability of edible oils (King *et al.*, 1995).

2.13.3 Thiobarbituric acid (TBA) test

The 2-thiobarbituric acid (TBA) test is a convenient method for measurement of the content of secondary oxidation products, referred to as TBA reactive substances (TBARS). This method is frequently used for quantitation of lipid oxidation in foods. One of the carbonyl decomposition products of lipids in foods is malonaldehyde (MA), which is a three-carbon dialdehyde (Pearson *et al.*, 1983). MA is an important aldehyde resulting from lipid oxidation of unsaturated fatty acids with three or more double bonds (Melton, 1983). The content of MA and other TBARS in products may be assessed by first isolating and then reacting them with the TBA reagent. During the TBA test, one molecule of MA reacts with two molecules of TBA at high temperatures to form a pink-coloured 'TBA-MA adduct' (Figure 2.13). The absorption intensity of this coloured chromogen is measured at 532 nm.

Figure 2.13 Steps involved in the formation of thiobarbituric acid-malonaldehyde (TBA-MA) adduct



TBA-MA adduct

A major disadvantage of the TBA test is that MA is only formed by fatty acids which contain three or more double bonds (Benzie, 1996). Contents of other products of lipid oxidation such as pentane, n-propanal, n-hexanal, etc. may also be determined and related to the degree of autoxidation in foods. Furthermore, the accuracy of TBA test has been questioned due to the reaction of TBA with other molecules such as sugars and oxidized proteins (Rossell, 1994). However, these products are normally not present in edible oils, thus TBA may be used to assess their oxidation (Ganthavorn and Hughes, 1997). The TBA test is unsuitable for following lipid oxidation in some shelf life studies where TBA values increase and then decrease while sensory analysis shows a steady increase (Hoyland and Taylor, 1991). Meanwhile, other substances such as 2-alkenals and 2,4-alkadienals present in edible oil, give a positive test with the TBA reagent and contribute to the absorption intensity at 532 nm which may reflect the total amount of aldehydes present rather than that of MA alone (Rossell, 1994). However, despite its limitations, the TBA test is frequently used to measure lipid oxidation in foods, especially on a comparative basis.

2.13.4 Anisidine value

The anisidine value is empirically defined as 100 times the absorbance of a solution resulting from the reaction of 1 g of oil or fat in 100 mL of a mixture of solvent and *p*-anisidine, measured at 350 nm in a 1-cm cell under the conditions of the test (AOCS, 1990).

The anisidine test, standardized by the American Oil Chemists' Society (AOCS, 1990), involves a condensation reaction between the conjugated dienals or 2-alkenals in the sample and *p*-anisidine reagent in isooctane followed by absorbance reading at 350 nm.

It is important that the sample and reagents are dry. In particular, glacial acetic acid used in the preparation of the reagent solution must be anhydrous. In the presence of acetic acid, *p*-anisidine reacts with aldehydes producing a yellowish colour. The molar absorbance at 350 nm increases if the aldehyde contains a double bond. Thus, the anisidine value is mainly a measure of 2-alkenals and 2,4-alkadienals (Tompkins and Perkins, 1999). This test is particularly useful for abused oils with low PVs such as frying oils. As a rule of thumb, for good oils the anisidine value should be less than about 10 (Rossell, 1994). An expression termed the Totox value (a measure of the total oxidation), which is equivalent to anisidine value plus twice the peroxide value, has been suggested for the assessment of oxidation of oils. The totox value is considered useful in that it combines evidence about the past history of the oil, in the anisidine value, with that of the present state of the oil, in the PV (Rossell, 1994). However, totox value is considered as a poor index of oxidation because it represents a sum of two quantities with incompatible units and provides less information than the anisidine and peroxide values reported separately (Robards *et al.*, 1988a).

2.13.5 Active oxygen and Oil stability Instrument (OSI)/ Rancimat methods

The active oxygen method, also known as the Swift test, is a commonly used accelerated method for assessing oxidative stability of fats and oils. This method is based on the principle that oxidation of lipids is accelerated by aeration in a tube held at a constant elevated temperature. The peroxide value reached by the active oxygen method at which an oil will be rancid by organoleptic evaluation varies with the nature of the lipid. Even though this method has been used extensively over the years, its inherent deficiencies are determined by the amount of peroxides in the oxidized oil; peroxides are unstable and decompose readily to more stable secondary products and during the rapid oxidation phase, the reaction is extremely susceptible to variations in the oxygen supply.

Automated versions of the active-oxygen apparatus, known as the Oil Stability Instrument (OSI), Rancimat and Oxidograph are now available for monitoring the oxidative stability of oils. These methods may be considered as automated active-oxygen methods because they employ the principle of accelerated oxidation. However, the OSI and Rancimat tests measure the changes in conductivity caused by ionic volatile organic acids, mainly formic acid, automatically and continuously, whereas in the active-oxygen method, peroxide values are determined. Rancimat tests proceed slowly at first because during the induction period little acid is released. The end point is selected as the point at which the rapid rise in conductance begins. The Rancimat is capable of running eight samples

simultaneously, however, OSI is capable of running up to 24 samples at a time (Shahidi and Wanasundara, 1997; 1998b).

2.13.6 Headspace analysis of volatiles

Various types of headspace analyses, using gas chromatography, have been used to assess the oxidation of edible oils. In most of these techniques, the oil is heated at 40-60°C in closed vials (Frankel, 1998b). The volatiles, from decomposition of hydroperoxides as well as those present before heating, collected in the headspace above the oil, are analyzed by gas chromatography. The total peak area of the volatiles in this technique increases with the length of the storage period of an oil. Therefore, this method can provide useful information about the origin of flavour volatiles and their precursors (Frankel, 1993a; Rossell, 1994). This method is particularly suitable for highly volatile compounds because they have a favourable equilibrium between a sample and its headspace, producing a higher concentration of volatile compounds in the headspace. This method is rapid and suitable for routine analysis of many samples and does not require any cleaning between sample injection because only the volatile components are injected into the gas chromatograph, but the nonvolatile portion of the sample is being retained in the vial (Frankel, 1998). The main disadvantage of this method is the difficulty of reaching complete equilibrium with viscous and semi-solid samples and with oxidized polyunsaturated lipid samples that can be easily decomposed during the equilibration heating step.

Gas chromatographic (GC) analyses of edible oils have revealed that hexanal and pentane are the major volatiles of oxidation of ω6 PUFA, while propanal is the predominant volatile derived from ω3 PUFA (Shahidi and Wanasundara, 1998). Moreover, King *et al* (1995) reported an excellent correlation between GC results and sensory scores in photooxidized soybean oil, while pentanal and hexanal correlated well with flavour scores of autoxidized soybean oil.

2.13.7 Nuclear Magnetic Resonance Spectroscopy

Hydrogen atoms (protons, ^{1}H) of various types in triacylglycerol (TAG) molecules could be determined using high resolution nuclear magnetic resonance (NMR) spectroscopy because hydrogen atoms in a strong magnetic field absorb energy from radio frequency region of the electromagnetic spectrum, depending on the environment in which they are placed in a molecule. During oxidation of lipids, changes occur in the environment in which protons in an oxidizing TAG molecule are located. These changes may be monitored by employing ^{1}H NMR spectroscopy. ^{1}H NMR spectroscopy offers major advantages over alternative laboratory methods since it permits the rapid, simultaneous study of many primary and secondary lipid oxidation products (Silwood and Grootveld, 1999). Use of NMR methodology for evaluation of oxidative stability of vegetable and marine oils has been reported (Saito and Nakamura, 1990; Saito and Udagawa, 1992; Wanasundara and Shahidi, 1993; Shahidi *et al.*, 1994; Senanayake and Shahidi, 1999b).

The ratios of aliphatic to olefinic (R_{ao}) and aliphatic to diallylmethylene (R_{ad}) protons can be easily calculated. These ratios increase steadily during the oxidation of oils. Numerical values of R_{ao} and R_{ad} may be plotted against corresponding TOTOX values for edible oils. Shahidi *et al.* (1994) have shown a significant ($p < 0.05$) linear correlation between R_{ao} and R_{ad} and TOTOX values for oxidized seal blubber and cod liver oils. Therefore, NMR methodology can be used to monitor oxidation of oils rich in PUFA. This method is particularly useful for assessing oxidation of lipids during later stages of storage when peroxide values start to decline due to the decomposition of hydroperoxides.

Saito and Nakamura (1990) have shown that the ratio of olefinic to aliphatic protons, measured by NMR spectroscopy, decreases continuously as the oxidation proceeds. They suggested that the NMR technique may be useful for measuring oxidation of PUFA-containing oils, even at stages beyond the point at which the peroxide value reached its maximum. Saito and Udagawa (1992) used this method to evaluate oxidative stability of brown fish meal. They suggested that the NMR method is suitable for comparing the storage conditions of fish meal as well as estimating the effect of antioxidants in fish meals and oils. These authors reported good correlations between peroxide values and NMR data. However, Shahidi *et al.* (1994) found that linear relationships between peroxide values and NMR data were not as suitable as those of TOTOX values and NMR data.

The NMR method is at least as useful as PV, however, accurate measurement of NMR is required because decreases in R_{ao} and R_{ad} are quite small as compared to large

increases in PV (Saito, 1997). Nonetheless, NMR methodology is useful for measuring oxidative deterioration of vegetable and marine oils.

2.14 Control of lipid oxidation

The oxidation of edible oils can be controlled by proper application of natural antioxidants, synthetic antioxidants, use of partially hydrogenated polyunsaturated fats, minimizing the loss of natural tocopherols, inactivation of prooxidant metals, blending of polyunsaturated fats with more stable monounsaturated fats, minimizing exposure to oxygen, light and high temperatures, use of inert gas or vacuum packaging and genetic modification of fatty acid composition. Ideal natural and synthetic antioxidants for food applications, should meet certain criteria such as safety, ease of incorporation, effectiveness at low concentration, absence of undesirable odour, flavour and colour, resistance to high temperature experienced during frying as well as availability at low cost (Coppen, 1994).

2.14.1 Removal of oxygen

Oxygen is an essential reactant in oxidation of unsaturated fatty acids. Therefore, control of oxygen availability is a critical factor in minimizing oxidation of lipids. The level of available oxygen may be controlled by vacuum and modified atmosphere packaging (Josephson *et al.*, 1985) and by using oxygen scavengers such as ascorbic acid oxidase and glucose oxidase (Hsieh and Kinsella, 1989). These precautions reduce the rate and extent of

lipid oxidation, especially when combined with antioxidants and low temperature storage in the dark.

2.14.2 Use of antioxidants

Antioxidants are added to oils to retard oxidation and to reduce development of rancidity. However, antioxidants cannot improve the quality of already oxidized food products (Dziezak, 1986). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary-butylhydroquinone (TBHQ) have been commonly used to inhibit lipid oxidation and to retard the rancidity development in foods. However, their use is increasingly contested for a variety of reasons (health concerns, legal issues, etc.) and there is a considerable commercial interest in the use of natural antioxidants.

Antioxidants can be divided into two broad classes, referred to as chain-breaking antioxidants, which interfere with one or more of the propagation steps, and preventative antioxidants, which reduce the rate of initiation. Chain-breaking antioxidants inhibit or retard lipid oxidation by interfering with either chain propagation or initiation by readily donating hydrogen atoms to lipid peroxy radicals.

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

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

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

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

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

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

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

Phenolic compounds with bulky alkyl groups such as BHA, BHT, TBHQ and tocopherols are effective chain-breaking antioxidants (AH) because they produce stable and relatively unreactive antioxidant radicals $A^{\bullet}$, by reactions (4) and (5), that are too unreactive to propagate the chain. They are also able to compete with the lipid substrate (LH), present in much higher concentration in reaction (3), for the chain-carrying peroxy radicals, $LOO^{\bullet}$.

Preventative antioxidants prevent or delay lipid oxidation by decreasing oxygen active compounds in the medium. Chelating agents are the most important compounds of this type. These compounds deactivate metal ions, which promote the initiation and decomposition of hydroperoxides, and thus retard the formation of secondary aldehydes. Common chelating compounds include citric acid, phosphoric acid and ethylenediaminetetraacetic acid (EDTA).

Natural antioxidants acting as radical scavengers are generally phenolic compounds. Tocopherols are one of the important natural antioxidants and serve as free radical scavengers and singlet oxygen quenchers (Shahidi and Wanasundara, 1992; Cuppett *et al.*, 1997). They act in the mode of inhibition of free radicals produced by singlet oxygen

oxidation of FFA and in the mode of direct quenching of singlet oxygen (Yamauchi and Matsushita, 1977). These compounds not only quench singlet oxygen by physical quenching mechanism, but also react with singlet oxygen by chemical quenching (Yang and Min, 1994). However, physical quenching is the major mechanism in the tocopherols. α-Tocopherol has been reported to be the major antioxidant defence against lipid oxidation (Janero, 1991) α-Tocopherol slows or stops propagation of oxidation although it does not appear to be able to prevent initiation of the process (Frei *et al.*, 1989). Other types of natural antioxidants include ascorbic acid, flavonoids and green tea catechins and rosemary, sage and other spice extracts. For example, ascorbic acid is the natural compound most commonly used as an oxygen scavenger (Lee *et al.*, 1997) and carotenoids are the most common singlet oxygen quenchers employed (Decker, 1998).

2.14.3 Packaging

Since photooxidation occurs in the presence of light, it can be prevented if the proper packaging material is used. Ideal containers for oils should be impermeable to air and moisture and should be opaque to light to prevent further oxidation during prolonged storage under ambient conditions. Polyvinyl chloride is preferred because it is less permeable to oxygen and superior to polyethylene, which is permeable to oxygen. Generally, coloured or opaque plastic containers are preferable to protect oils from photooxidation.

Although the following materials are not all used practically, their protection against photooxidation is as follows: polyester > wax paper > polyethylene > nylon (Faria and Mukai, 1983). This protection is due to the absorption of light by packaging materials causing a spectral transmission.

2.14.4 Microencapsulation

PUFA containing highly oxidizable oils such as fish and marine oils can be protected by a process known as microencapsulation which coats the oil with a thin matrix of carbohydrate (starch, dextran, sucrose), protein (casein, albumin, gelatin, gluten) or lecithin, among others. This process provides protection against oxidation and imparts oxidative stability. The use of cylcodextrins as a coating is claimed to provide better protection of oils by improved oxygen barrier properties (Wanasundara and Shahidi, 1995). For special applications as nutritional supplements, fish and marine oils enriched in ω3 PUFA are microencapsulated into a powder product that is relatively stable for storage at ambient temperatures.

2.15 Response surface methodology (RSM) and process optimization

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analyzing problems in which several independent variables influence a dependent variable or response, with the goal being to optimize this

response. In most RSM problems, the form of the relationship between the response and the independent variables is unknown. Thus, the first step in RSM is to find a suitable approximation for the true functional relationship between the response and the set of independent variables. Usually, a low-order polynomial in some region of the independent variables is employed. If the response is well-modeled by a linear function of the independent variables, then the approximating function is the first-order model. If there is curvature in the system, then a polynomial of higher degree, such as the second-order model must be used. Almost all RSM problems utilize one or both of these approximating polynomials. Of course, it is unlikely that a polynomial model will be a reasonable approximation of the true functional relationship over the entire space of the independent variables, but in a relatively small region they usually work quite well.

Polynomial models provide sufficient flexibility to adequately approximate many complicated, but unknown, relationships between a dependent variable or response and one or more independent variables. In many important problems in science the underlying mechanism that generates the data is not well understood, due to the complexity of the problem and lack of sufficient theory. In these cases polynomial models can provide adequate approximations to the unknown functional relationship (Mason *et al.*, 1989). One should start with the simplest model warranted by what is known about the response under investigation. If a lack-of-fit test indicates that the proposed model is an inadequate approximation to the observed responses, one can either add the next higher-order terms

into the model or investigate nonlinear models. In many experimental situations, a first- or second-order polynomial is adequate to describe a response (Mason *et al.*, 1989).

Some attractive features of RSM are that (a) it is a sequential approach, the results at each stage guiding the experimentation to be conducted at the next (at each step of the iteration only a limited number of experimental trials are run), thereby ensuring that ones resources are not squandered on unproductive trials; (b) it casts the experimental problem in readily understood geometric terms; and (c) it is applicable to any number of variables.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Borage oil was obtained from Bioriginal Food and Science Corporation (Saskatoon, SK), and evening primrose oil was provided by Efamol, Inc. (Kentville, NS). Algal oil containing DHA (47.4%) was from Martek Biosciences Corporation (Columbia, MD). EPA concentrate was provided through Dr. T. Ohshima (Tokyo, Japan). Three 1 kg containers of each type of oil were received and stored at -20°C until use.

Fatty acid methyl esters (GLC-461 and methyl tricosanoate) were purchased from Nu-Check (Elysian, MN) company. Reagents, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, butylated hydroxytoluene (BHT), hydroquinone, sodium sulphate, triolein, diolein, monoolein, oleic acid, cupric acetate, pyridine, gum arabic, starch, phenolphthalein, phenyl dichlorophosphate, calcium chloride, sodium bicarbonate, potassium hydroxide, sodium hydroxide, boric acid, sodium thiosulphate, potassium iodide, tris base, methyl magnesium bromide, Hanus iodine solution, phospholipase A_2, porcine pancreatic lipase, sodium taurocholate, acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, nonanal, decanal, 2,4-decadienal, silicic acid and silica gel TLC plates (20 x 20 cm; 60Å mean pore diameter, 2-25 µm mean particle size, 500 µm thickness, with dichlorofluorescein) were purchased from Sigma Chemical Co. (St. Louis, MO).

Deuterated chloroform was obtained from Cambridge Isotope Laboratories (Andover, MA). Hexane, ethanol, methanol, hydrochloric acid, sulphuric acid, chloroform, isooctane, isobutanol, petroleum ether (boiling point range 35-60°C), acetone, benzene, toluene, acetic acid, diethyl ether, ethyl acetate, triethylamine, triethylammonium bicarbonate, carbon disulphide and ammonium hydroxide were purchased from Fisher Scientific (Nepean, ON). Helium, hydrogen, nitrogen and compressed air were obtained form Canadian Liquid Air Ltd. (St. John's, NF). Six types of microbial lipases used were provided by different manufacturers as listed in Table 3.1. Lipozym-IM is a 1,3-specific lipase from *Mucor miehei* immobilized on a macroporous anoin exchange resin. Novozym-435 from *Candida antarctica* was immobilized on a macroporous acylic resin. However, lipases from *Pseudomonas sp.*, *Aspergillus niger*, *Candida rugosa* and *Thermomyces lanuginosus* used were not immobilized.

3.2 Methods

3.2.1 Preparation of DHA concentrate from algal oil

3.2.1.1 Preparation of free fatty acids from algal oil

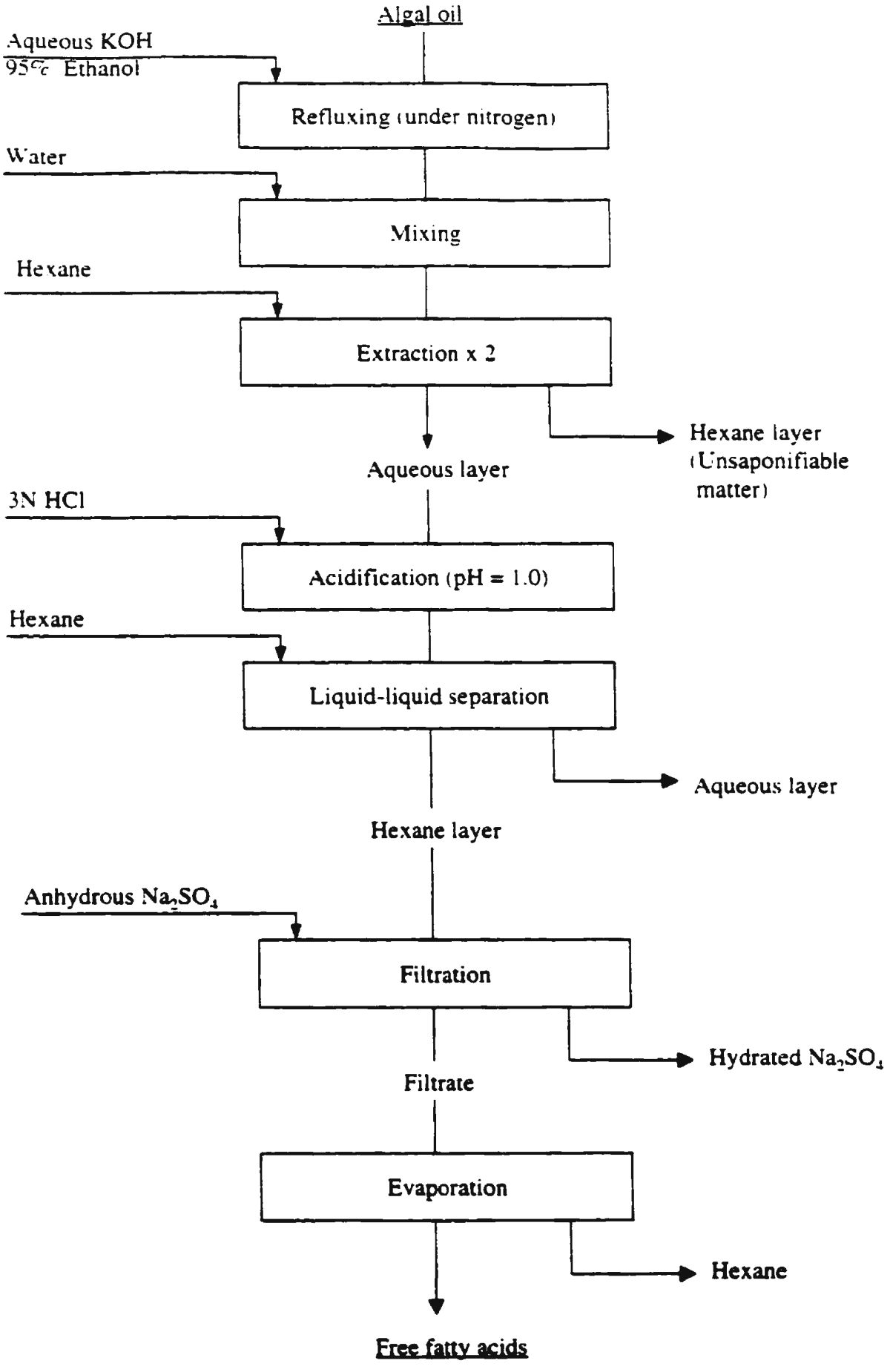
Preparation of free fatty acids from algal oil was carried out according to the scheme given in Figure 3.1. Algal oil (25 g, treated with 200 ppm BHT) was saponified by refluxing in 95% (v/v) aqueous ethanol (66 mL) with water (11 mL) and KOH (5.75

Table 3.1 Microbial lipases employed, their suppliers and characteristics

Enzyme	Commercial code	Manufacturer[a]	Positional specificity	Enzyme activity (U)[b]
Candida antarctica	Novozym-435	Novo	*sn*-1,3/ Nonspecific	554
Mucor miehei	Lipozyme-IM	Novo	*sn*-1,3 specific	13,613
Pseudomonas sp.	PS-30	Amano enzyme	Nonspecific	11,936
Aspergillus niger	AP-12	Amano enzyme	*sn*-1,3 specific	8,142
Candida rugosa	AY-30	Amano enzyme	Nonspecific	38,707
Thermomyces lanuginosus	Novozym-677BG	Novo	*sn*-1,3 specific	7,658

[a]Manufacturer locations: Amano Enzymes U.S.A Co., Ltd., Troy, VA; Novo Nordisk Biochem North America, Inc., Franklinton, NC.
[b]Enzyme activity was determined as given in Section 3.2.2.

Figure 3.1 Flowsheet for preparation of free fatty acids (FFA) from algal oil

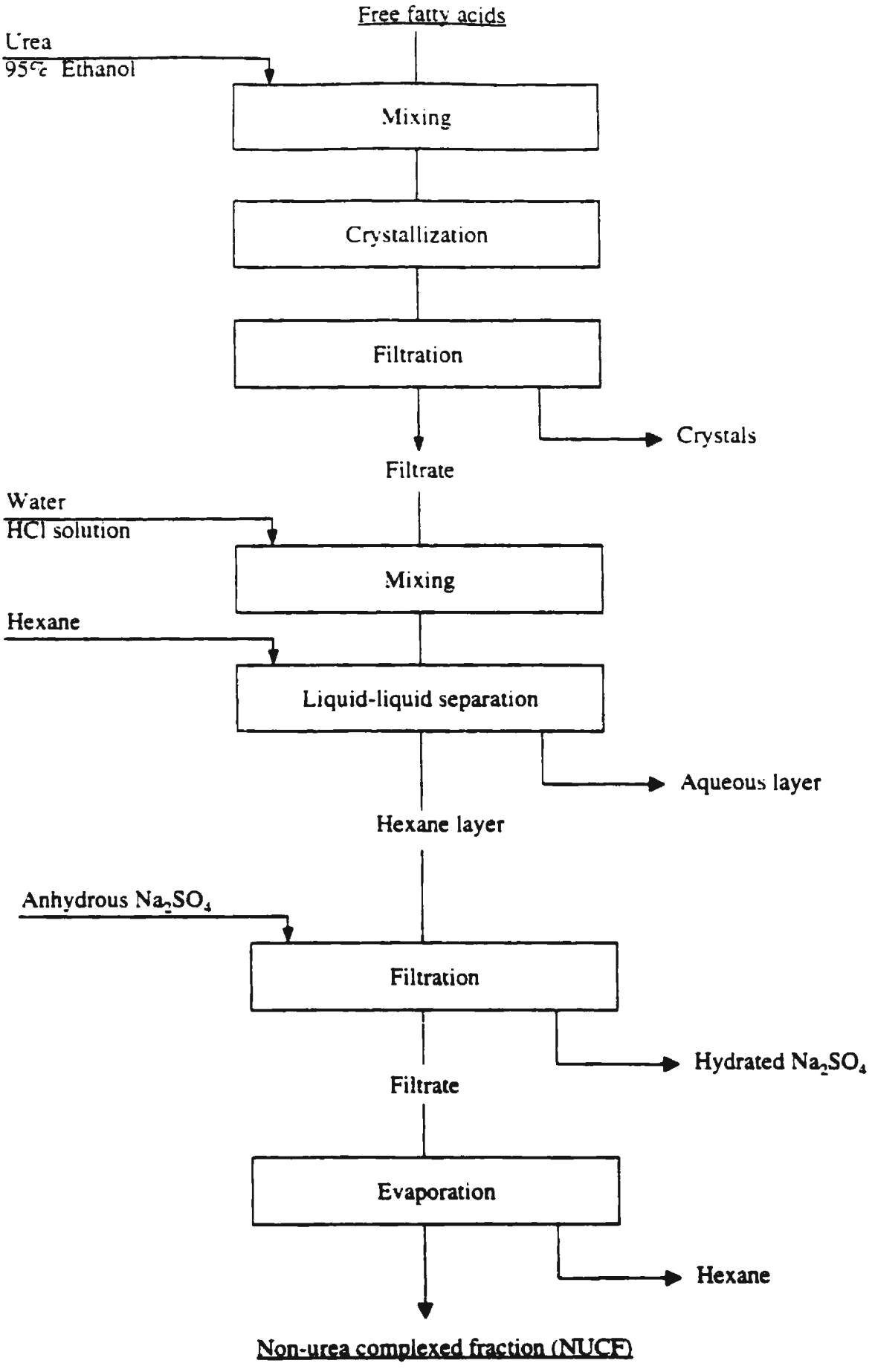

Algal oil
Aqueous KOH
95% Ethanol
Refluxing (under nitrogen)
Water
Mixing
Hexane
Extraction x 2
Hexane layer
(Unsaponifiable
matter)
Aqueous layer
3N HCl
Acidification (pH = 1.0)
Hexane
Liquid-liquid separation
Aqueous layer
Hexane layer
Anhydrous Na_2SO_4
Filtration
Hydrated Na_2SO_4
Filtrate
Evaporation
Hexane
Free fatty acids

g) for 1 h at the boiling temperature of the mixture (62 ± 2°C) under a blanket of nitrogen. Following saponification, distilled water (50 mL) was added to the mixture and the unsaponifiable matter was extracted into hexane (2 x 100 mL) and discarded. The aqueous layer containing saponifiable matter was acidified (pH = 1.0) with 3N HCl. The mixture was transferred to a separatory funnel and the liberated fatty acids were extracted into 50 mL of hexane. The hexane layer containing free fatty acids was then dried over anhydrous sodium sulphate and the solvent removed at 40°C to recover free fatty acids which were then stored at –20°C until use.

3.2.1.2 Preparation of DHA concentrate from algal oil by urea complexation

The separation of DHA from the hydrolyzed fatty acid mixture (Section 3.2.1.1) of algal oil was carried out by urea-fatty acid adduct formation according to the scheme given in Figure 3.2. Free fatty acids (10 g) were mixed with urea (20%, w/v) in 95% aqueous ethanol and heated at 60°C while stirring until the whole mixture turned into a clear homogenous solution. Initially, the urea-fatty acid adduct was allowed to crystallize at room temperature, but was later placed in a cold room at 4°C for 24 h for further crystallization. The crystals (urea-fatty acid adducts, also referred to as the urea complexing fraction; UCF) formed were separated from the liquid (non-urea complexing fraction; NUCF) by suction filtration through a thin layer of glass wool. The filtrate (NUCF) was diluted with an equal volume of water and acidified to pH 4-5 with 6N HCl;

Figure 3.2 Flowsheet for preparation of DHA concentrate by urea complexation

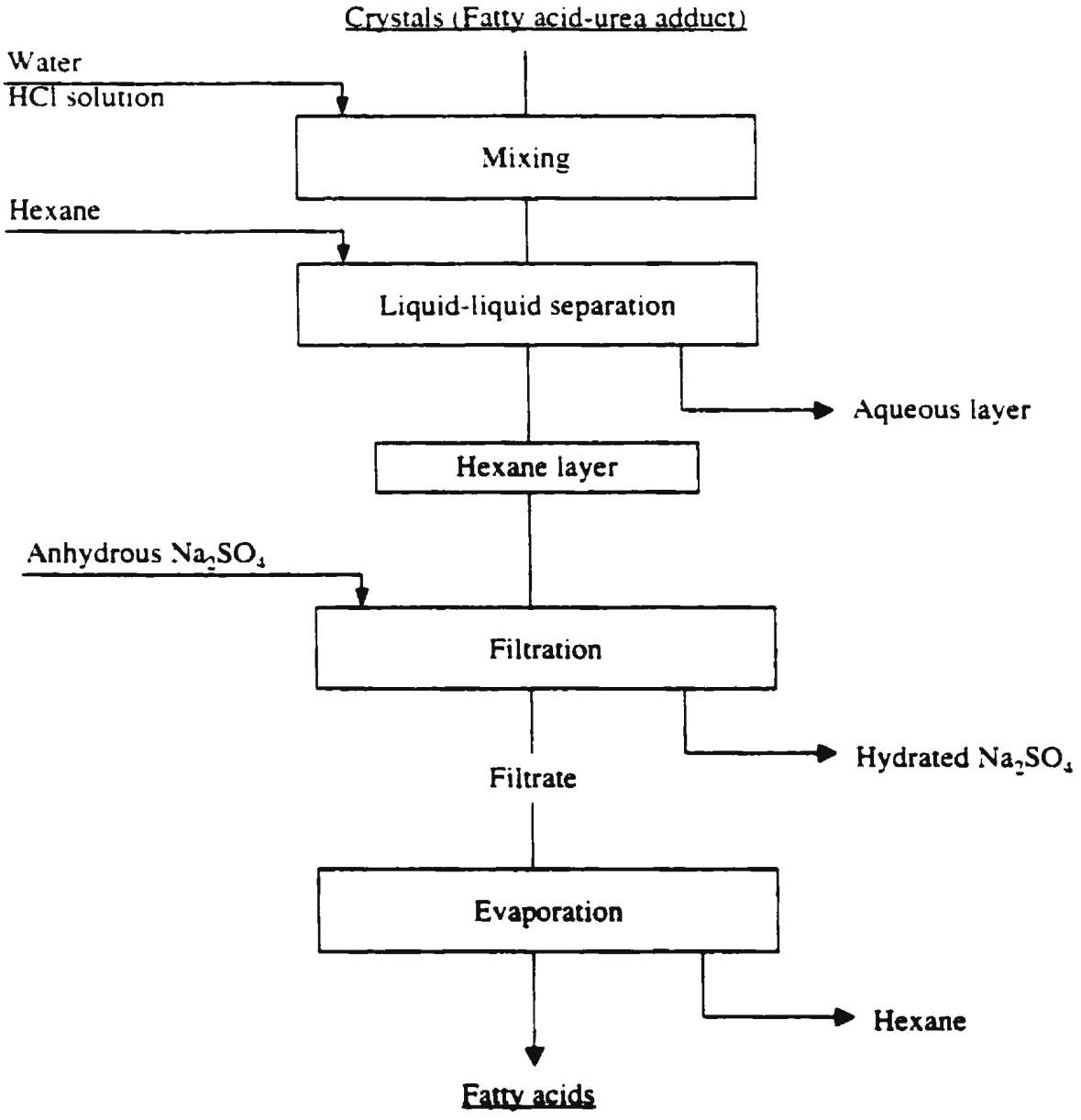

Free fatty acids
Urea
95°c Ethanol
Mixing
Crystallization
Filtration
Crystals
Filtrate
Water
HCl solution
Mixing
Hexane
Liquid-liquid separation
Aqueous layer
Hexane layer
Anhydrous Na_2SO_4
Filtration
Hydrated Na_2SO_4
Filtrate
Evaporation
Hexane
Non-urea complexed fraction (NUCF)

an equal volume of hexane was subsequently added and the mixture stirred thoroughly for 1 h, then transferred to a separatory funnel. The hexane layer containing liberated fatty acids was separated from the aqueous layer containing urea and washed with distilled water to remove any remaining urea and then dried over anhydrous sodium sulphate. The solvent was subsequently removed at 40°C using a rotary evaporator. Fatty acids from UCF were recovered after addition of water/6N HCl and hexane in a similar manner (Figure 3.3). The two fractions were weighed separately and percentage recovery calculated.

3.2.2 Determination of enzyme activity of microbial lipases

Lipase activity was measured by assaying fatty acids produced from the hydrolysis of triacylglycerols. All experiments were carried out in screw-capped test tubes in triplicate. Triolein was used as the substrate. It was emulsified at a concentration of 50 mM in 5% (w/v) gum arabic for 1 min using a Polytron homogenizer (Model PT-3000; Brinkmann, Littau-Switzerland) at 8000 rpm. The assay mixture contained 1 mL of substrate emulsion and the enzyme (10-100 mg). Reactions were carried out for up to 1 h in a shaking water bath at 250 rpm and 35°C. Fatty acid release varied linearly with time for more than 1 h. The released fatty acids were assayed colorimetrically as copper soaps using cupric acetate-pyridine reagent (Lowry and Tinsley, 1976; Kwon and Rhee, 1986).

Figure 3.3 Recovery of fatty acids from urea complexed fraction (UCF)


Crystals (Fatty acid-urea adduct)
Water
HCl solution
Mixing
Hexane
Liquid-liquid separation
Aqueous layer
Hexane layer
Anhydrous Na_2SO_4
Filtration
Hydrated Na_2SO_4
Filtrate
Evaporation
Hexane
Fatty acids

The purity of triolein was verified by thin-layer chromatography-flame ionization detection (TLC-FID); no mono- or diacylglycerols were present.

The enzyme reaction in the emulsion system was stopped by adding 6 N HCl (1 mL) and isooctane (5 mL) followed by mixing for 1 min. Cupric acetate (1 mL, 5% w/v, pH 6.1) solution was then added to the mixture and stirred for 90 s using a vortex mixer; the absorbance of the upper isooctane layer was read at 715 nm (Arribère *et al.*, 1994). One unit of lipase activity was defined as nanomoles of fatty acids (oleic acid equivalents) produced per minute per gram of enzyme.

3.2.3 Acidolysis

In general, borage (300 mg) or evening primrose oil (297 mg) was mixed with EPA and/or DHA, in a screw-capped test tube, and then lipase (150-350 enzyme activity units) and water (2% by weight of substrates plus enzyme) were added in hexane (3 mL). The mixture was stirred in an orbital shaker at 250 rpm and temperatures ranging from 20 to 60°C. Individual sample vials were removed and analyzed at different time periods (6-30 h).

3.2.3.1 Analysis of products

The enzymes were removed by passing the reaction mixture through a bed of anhydrous sodium sulphate. Samples were placed in 250-mL conical flasks and 20 mL of

a mixture of acetone/ethanol (1:1, v/v) were added. The reaction mixture was titrated with 0.5 N NaOH to a phenolphthalein endpoint. The mixture was transferred to a separatory funnel and thoroughly mixed with 25 mL hexane. The lower aqueous layer was separated and discarded. The upper hexane layer containing acylglycerols was passed through a bed of anhydrous sodium sulphate. The acylglycerol fraction was subsequently recovered following hexane removal at 45°C using a rotary evaporator. The fatty acid composition of the acylglycerols was analyzed by gas chromatography as described in Section 3.2.8.

The products (TAG, DAG and MAG), obtained under optimum reaction conditions, were quantified by thin-layer chromatography-flame ionization detection (TLC-FID) using benzene/chloroform/acetic acid (70:30:4, v/v/v) as the developing solvent (Angelo and James, 1993). The reaction products were also fractionated on TLC plates (20 x 20 cm; Silica gel, 60 Å mean pore diameter, 500 µm thickness, with dichlorofluorescein, Sigma) impregnated with a 5% (w/v) boric acid solution. 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 (254 nm) and long (365 nm) UV lights (Spectroline, Model ENF-240C, Spectronics Co., Westbury, NY). The bands were scraped off and their lipids extracted into diethyl ether and subsequently used for fatty acid analysis by gas chromatography as described in Section 3.2.8.

3.2.4 Thin Layer Chromatography-Flame Ionization Detection (TLC-FID)

The products obtained under optimum reaction conditions after acidolysis were chromatographed separately on silica gel coated Chromarods S-III and then analyzed on an Iatroscan MK-5 (Iatron Laboratories Inc., Tokyo, Japan) analyzer equipped with a flame ionization detector (FID) connected to a computer loaded with T data scan software (Scientific Products and Equipment, Concord, ON) for data handling. The FID was operated using a hydrogen flow rate of 160 mL/min and an air flow rate of 2000 mL/min. The rods were scanned at a speed of 30 s/rod.

3.2.4.1 Preparation of Chromarods

The Chromarods were cleaned by soaking in concentrated nitric acid overnight and then thoroughly washed with distilled water and acetone. To improve separation, Chromarods were subsequently impregnated with boric acid by dipping in a 3% (w/v) boric acid solution for 5 min followed by drying at 120°C for 5 min. The Chromarods were scanned twice to burn off any remaining impurities.

3.2.4.2 Calibration of Chromarods

A composite stock solution of lipid standards containing free fatty acids (oleic acid), monoacylglycerol (monoolein), diacylglycerol (diolein) and triacylglycerol (triolein) were prepared in chloroform/methanol (2:1, v/v) and stored under nitrogen at -20°C. Different

dilutions of the stock solution (10 mg/mL), ranging from 0.1 to 10 µg/µL of lipid mixture, were used as working standards. Before making the composite standard mixture, each compound was developed individually and run on the Iatroscan-FID to determine its purity and R_f value. The samples dissolved in appropriate solvents were spotted on rods using Drummond microcap disposable pipettes (Drummond Scientific Co., Broomall, PA). As soon as the samples were spotted, solvents were dried off using a stream of cold air supplied by a blow dryer. Prior to development, the Chromarods were conditioned in a humidity chamber containing saturated calcium chloride for 10 min and then immediately transferred to the developing tank.

3.2.4.3 Chromarod development

The lipids were dissolved in chloroform/methanol (2:1, v/v) in order to obtain a concentration of 1µg lipid/µL. The sample (1 µL/rod) was applied on nine out of ten rods and a randomly selected rod was used for the standard mixture.

The development of rods was carried out for 45 min in benzene/chloroform/acetic acid (70:30:4, v/v/v) (Angelo and James, 1993). The chromarods were then dried at 110°C for 3 min and scanned using the Iatroscan TLC-FID Analyzer. This procedure was repeated three times for each sample. Peaks in each chromatogram were integrated with TSCAN data software. The identity of each peak was determined by comparison with a chromatogram of standards ran concurrently with the samples. The determination of the weight of the

individual compounds was achieved by construction of standard curves using a known amount of each standard on Chromarods developed under identical conditions described earlier.

3.2.5 Optimization procedure for production of structured lipids *via* acidolysis

3.2.5.1 Experimental design and data analysis

The experimental design adapted for response surface methodology (RSM) was a three-factor and three-level face-centred cube design with 17 individual design points (Table 3.2 and Figure 3.4) (Mason *et al.*, 1989; Gao and Mazza, 1996). The independent variables or factors studied were the amount of enzyme (units; X_1), reaction temperature (°C; X_2) and reaction time (h; X_3) (Table 3.2). Responses or dependent variables (Y) studied were DHA, EPA and EPA+DHA incorporation (%). To avoid bias, 17 runs were performed in a randomized order. Duplicate experiments were carried out at all design points except at the centre point (0,0,0) where three replications were performed to allow the estimation of the "pure error".

The second-order quadratic polynomial regression model was assumed for predicting response variable. The generalized model propose for response (Y) was:

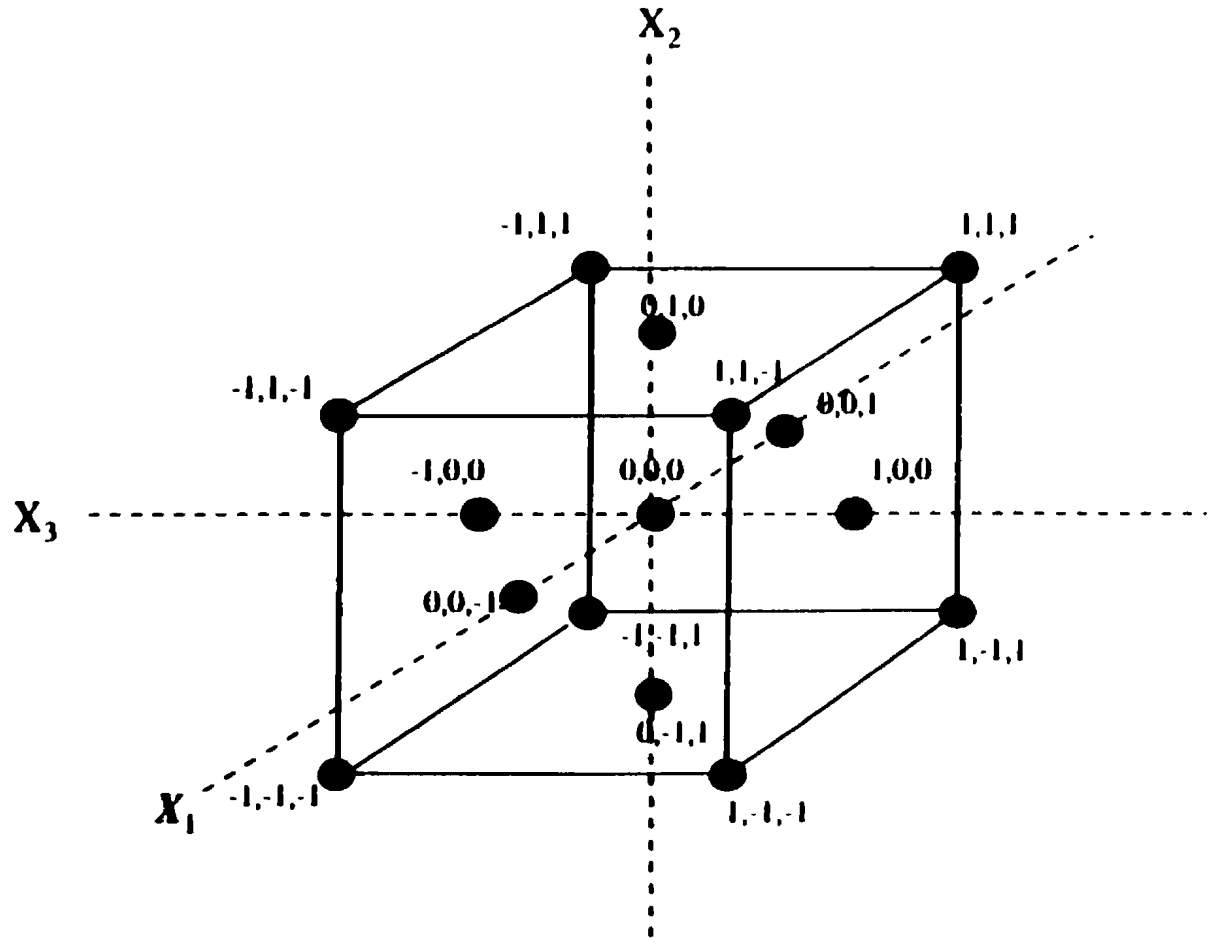
$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<j=1}^{3} \sum \beta_{ij} X_i X_j$$

Table 3.2 Face-centred cube design

Design point	X_1 (Enzyme units)	X_2 (°C)	X_3 (h)
1	(-1)[a]	(-1)	(-1)
2	(-1)	(-1)	(+1)
3	(-1)	(+1)	(-1)
4	(-1)	(+1)	(+1)
5	(+1)	(-1)	(-1)
6	(+1)	(-1)	(+1)
7	(+1)	(+1)	(-1)
8	(+1)	(+1)	(+1)
9	(-1)	(0)	(0)
10	(+1)	(0)	(0)
11	(0)	(-1)	(0)
12	(0)	(+1)	(0)
13	(0)	(0)	(+1)
14	(0)	(0)	(-1)
15	(0)	(0)	(0)
16	(0)	(0)	(0)
17	(0)	(0)	(0)

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

Figure 3.4 Graphical representation of the face-centred cube design


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

where β_0, β_i, β_{ii}, and β_{ij} are intercept, linear, quadratic and interaction regression coefficient terms, respectively, and X_i and X_j are independent variables. The statistical analysis system (SAS Institute Inc., 1990) was used for multiple regression analysis, analysis of variance (ANOVA) and canonical analysis. Response surfaces and contour plots were developed using the fitted quadratic polynomial equations obtained from response surface regression (RSREG) analysis and holding the independent variable with the least effect on the response at a constant value and changing the levels of the other two variables. Verification experiments were carried out using combinations of variables at different levels (within the experimental range).

3.2.6 Chemical and instrumental analyses

3.2.6.1 Determination of iodine value (IV)

The iodine value of oil samples was determined according to the Official Method of the American Oil Chemists' Society (AOCS, 1990; Method Cd 1-25). Samples (0.1-0.2 g of oil) were weighed into 250 mL glass-stoppered Erlenmeyer flasks and dissolved in 10 mL of chloroform. After thorough mixing, the flask was wrapped with aluminum foil and 25 mL of Hanus iodine solution were added to it and the mixture was allowed to stand for 30 min in the dark. Afterwards 10 mL of 15% (w/v) potassium iodide (KI) solution and 100 mL distilled water were added to the sample. The mixture was subsequently titrated against a standardized 0.1 N solution of sodium thiosulphate

($Na_2S_2O_3$) with constant shaking until the yellow colour of the mixture disappeared. Subsequently, 0.5 mL of starch indicator solution (1%, w/v) was added to the mixture and titration continued until the blue colour of starch-iodine complex had disappeared. A blank titration was conducted each time. The IV was expressed as the uptake of iodine in grams by 100 g of oil.

$IV = (V_{Blank} - V_{Sample}) \times N\ Na_2S_2O_3 \times 12.692 /$ Mass of sample (g)

Where V is the volume (mL) and N is the normality of sodium thiosulphate solution.

3.2.6.2 Determination of peroxide value (PV)

The official Method of the American Oil Chemists' Society (AOCS, 1990; method Cd 8-53) was used to determine PV of each oil. Samples (2.0-4.0 g of oil) were weighed into 250 mL glass-stoppered Erlenmeyer flasks and dissolved in 30 mL of acetic acid/chloroform (3:2, v/v). The contents were mixed until the oil had completely dissolved, upon which 0.5 mL of a saturated solution of potassium iodide (KI) was added. The mixture was allowed to stand in the stoppered flasks with occasional shaking for exactly 1 min and then mixed with 30 mL of distilled water. The liberated iodine was titrated against a standardized solution of 0.01 N sodium thiosulphate ($Na_2S_2O_3$) while shaking constantly until the yellow colour disappeared. About 0.5 mL of starch indicator solution (1%, w/v) was then added to the content of the flask and titration continued with vigorous shaking until the blue colour of the solution disappeared. A blank titration was

conducted each time. Peroxide value was expressed as the uptake of milliequivalents of active oxygen (i.e. peroxide) per kg of oil.

$PV = (V_{Sample} - V_{Blank}) \times N\ Na_2S_2O_3 \times 1000 /$ Mass of sample (g)

Where V is the volume (mL) and N is the normality of the sodium thiosulphate solution.

3.2.6.3 Determination of saponification value (SV)

The saponification value of oil samples was determined according to the Official Method of the American Oil Chemists' Society (AOCS, 1990; Method Cd 3-25). Samples (2-3 g of oil) were weighed into a 100 mL round bottom flask and mixed with 25 mL of a 0.5 N alcoholic KOH solution while stirring. The flask was attached to a condenser and the mixture was refluxed in a hot water bath (60 ± 5°C) for 60 min. Refluxing was continued until oil droplets in the mixture disappeared. Phenolphthalein indicator (1 mL, 1%, w/v) was added to the flask and the mixture titrated with 0.5 N HCl solution until the pink colour of the solution had disappeared. A blank titration was conducted in a manner similar to that of the sample but in the absence of any oil. The SV was expressed as the number of mg of KOH required to saponify 1 g of oil as given below:

$SV = (V_{Blank} - V_{Sample}) \times N_{HCl} \times 56.1 /$ Mass of sample (g)

Where V is the volume (mL) and N is the normality of hydrochloric acid solution.

3.2.6.4 Determination of acid value (AV)

The acid value of oil samples was determined according to the Official Method of the American Oil Chemists' Society (AOCS, 1990; Method Cd 3a-63). Samples (2-10 g of oil) were weighed into a 250 mL glass Erlenmeyer flask and mixed with 50 mL of 95% (v/v) aqueous ethanol (neutralized with 0.5 N KOH) and 2 mL of 1% phenolphthalein indicator. The mixture was heated to 70°C while stirring and then titrated against a standardized 0.1 N alcoholic potassium hydroxide (KOH) solution to attain a permanent pink colour. A blank titration was conducted each time. The AV was expressed as the amount of KOH (in mg) required to neutralize free fatty acids present in 1 g of oil.

$$AV = (V_{Sample} - V_{Blank}) \times N_{KOH} \times 56.1 / \text{Mass of sample (g)}$$

Where V is the volume (mL) and N is the normality of potassium hydroxide solution.

3.2.6.5 Determination of 2-thiobarbituric acid-reactive substances (TBARS)

The direct TBARS value determination of the American Oil Chemists' Society (AOCS, 1990; Method Cd 19-90) was employed. Oil (50-200 mg) was accurately weighed into a 25 mL volumetric flask and dissolved in a small volume of 1-butanol and made up to the mark with the same solvent. Five mL of this solution was transferred into a dry test tube to which 5 mL of fresh TBA reagent (200 mg 2-thiobarbituric acid in 100 mL 1-butanol) was added. The contents were thoroughly mixed and heated in a water bath at 95°C for 2 h. Heated samples were cooled in an ice bath and the absorbance of the

resulting coloured complex was read at 532 nm using a Hewlett Packard diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Missisauga, ON). A standard curve was prepared using 1,1,3,3-tetramethoxypropane (TMP) as a malonaldehyde (MA) precursor (Figure A.1) (Yu and Sinnhuber, 1967). The number of µmol of MA equivalents in each gram of oil, expressed as TBARS value, was calculated using the equation $C = (0.335A_{532})/W$ ($r = 0.996$). Where C represents the concentration of MA, A is the absorbance of the coloured complex at 532 nm and W is the mass of oil.

3.2.7 Stereospecific analysis of enzymatically modified oils

3.2.7.1 Removal of constituents other than triacylglycerol in enzymatically modified oils

Removal of constituents other than triacylglycerols from enzymatically modified borage and evening primrose oils was carried out using column chromatography (1.25 cm internal diameter and 10 cm height) on silicic acid (100-200 mesh size, Sigma). The column was first washed with hexane and then 1.25 g oil was introduced onto it. Hexane (50 mL) was added to the column, which was then eluted with 10% (v/v) diethyl ether in hexane (250 mL). The solvent was removed under vacuum at 40°C using a rotary evaporator. The recovered oil was then passed through a layer of anhydrous sodium sulphate. In order to prevent oxidation of purified oils, a few crystals of BHT were added to the mixture.

3.2.7.2 Grignard reaction on enzymatically modified borage and evening primrose oils

Grignard reaction was performed on purified modified oils (Section 3.2.7.1) according to the method described by Brockerhoff *et al.* (1963) and Brockerhoff (1971) with some modifications. The purified oil (1.0 g) was dissolved in anhydrous diethyl ether (50 mL) and mixed with methyl magnesium bromide (3.5 mL, 3.0 M CH_3MgBr, Sigma). The Grignard reaction was allowed to proceed with continuous stirring until a clear solution was obtained. To stop the reaction, glacial acetic acid (1.0 mL) was slowly added to the mixture followed by 10% (w/v) boric acid solution (10 mL; to minimize acyl migration). Stirring of the reaction mixture was continued for another 2 to 3 min. The whole mixture was then transferred to a separatory funnel and allowed to separate into two layers. The top ether layer was removed and the lower aqueous layer was washed twice with diethyl ether. The combined ether layers were washed successively with 10 mL of water, 10 mL of 2% (w/v) aqueous sodium bicarbonate and 10 mL of water and then dried over anhydrous sodium sulphate.

3.2.7.3 Separation of individual lipids after Grignard reaction

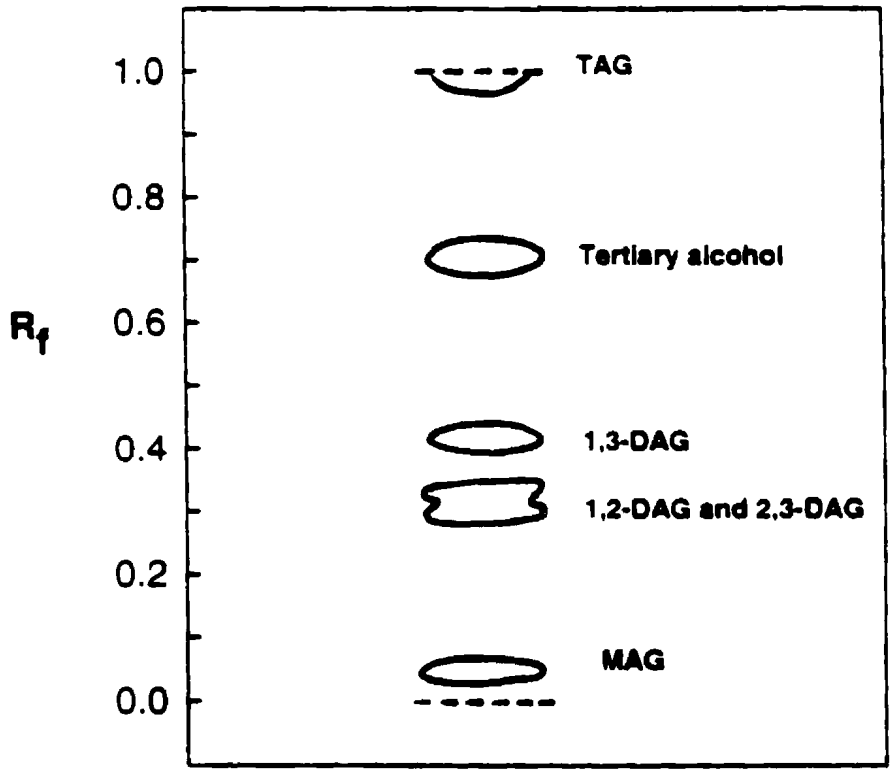
Products of the Grignard reaction from Section 3.2.7.2 were dissolved in a minimum amount of chloroform and applied to several TLC plates (20 x 20 cm; Silica gel, 60 Å mean pore diameter, 2-25 µm mean particle size, 500 µm thickness, with

dichlorofluorescein, Sigma) impregnated with 5% (w/v) boric acid. The plates were developed in two different solvent systems of diethyl ether/petroleum ether (boiling point 30-60°C) (8:92, v/v) and diethyl ether/petroleum ether (40:60, v/v), respectively. After drying, the bands were located by viewing under short (254 nm) and long (356 nm) wavelength UV lights (Spectraline, Model ENF-240C, Spectronics Co., Westbury, NY). From the separated bands of triacylglycerol (TAG; R_f = 0.99), tertiary alcohol (R_f = 0.72), 1,2- (1,2-DAG) and 2,3-diacylglycerols (2,3-DAG; R_f = 0.32), 1,3-diacylglycerol (1,3-DAG; R_f = 0.41) and monoacylglycerols (MAG; R_f = 0.05) (Figure 3.5), 1,2- and 2,3-diacylglycerol bands were scraped and then extracted with diethyl ether. The ether layer was evaporated under nitrogen to obtain 1,2- and 2,3-diacylglycerols. After removing a small sample for fatty acid analysis, the diacylglycerol fractions were used to prepare synthetic phospholipids.

3.2.7.4 Preparation of synthetic phospholipids from diacylglycerol fraction

The 1,2- and 2,3-diacylglycerols, obtained as described in Section 3.2.7.3, were dissolved in 1.0 mL of diethyl ether and mixed with 2.5 mL pyridine/diethyl ether/phenyl dichlorophosphate (1:1:0.5, v/v/v). The reaction mixture was then allowed to stand at room temperature for 1 h, after which 5 mL of pyridine, 3.0 mL of diethyl ether and a few drops of water were added while cooling in an ice bath. The content of flask was subsequently mixed with 86 mL of methanol/water/chloroform/triethylamine (30:25:30:1,

Figure 3.5 TLC chromatogram of Grignard deacylation products


1.0
0.8
0.6
0.4
0.2
0.0
R_f
TAG
Tertiary alcohol
1,3-DAG
1,2-DAG and 2,3-DAG
MAG

v/v/v/v). After standing, the lower chloroform layer containing synthetic phospholipids (1,2-diacyl-3-phosphatide and 2,3 –diacyl-1-phosphatide) was separated, and the solvent removed at 40°C using a rotary evaporator. The recovered synthetic phospholipids were used for stereospecific hydrolysis by the phospholipase A_2 enzyme.

3.2.7.5 Stereospecific hydrolysis of synthetic phospholipids by phospholipase A_2

The synthetic phospholipids (1,2-diacyl-3-phosphatide and 2,3-diacyl-1-phosphatide) obtained in Section 3.2.7.4 were dissolved in 3.0 mL of diethyl ether and transferred to a solution containing 15 mL of 0.1 M triethylammonium bicarbonate (pH 7.5), 100 µl of 0.1 M calcium chloride and 2.0 mg of phospholipase A_2 (EC. 3.1.1.4; Sigma) obtained from snake venom (*Crotalus adamantus*). The mixture was then shaken gently overnight in a Gyrotary water bath shaker (Model G76, New Brunswick Scientific Co. Inc., New Brunswick, NJ) and water in the mixture was evaporated at 40°C using a rotary evaporator. In order to prevent foaming during evaporation, 15 mL of isobutanol was added to the mixture.

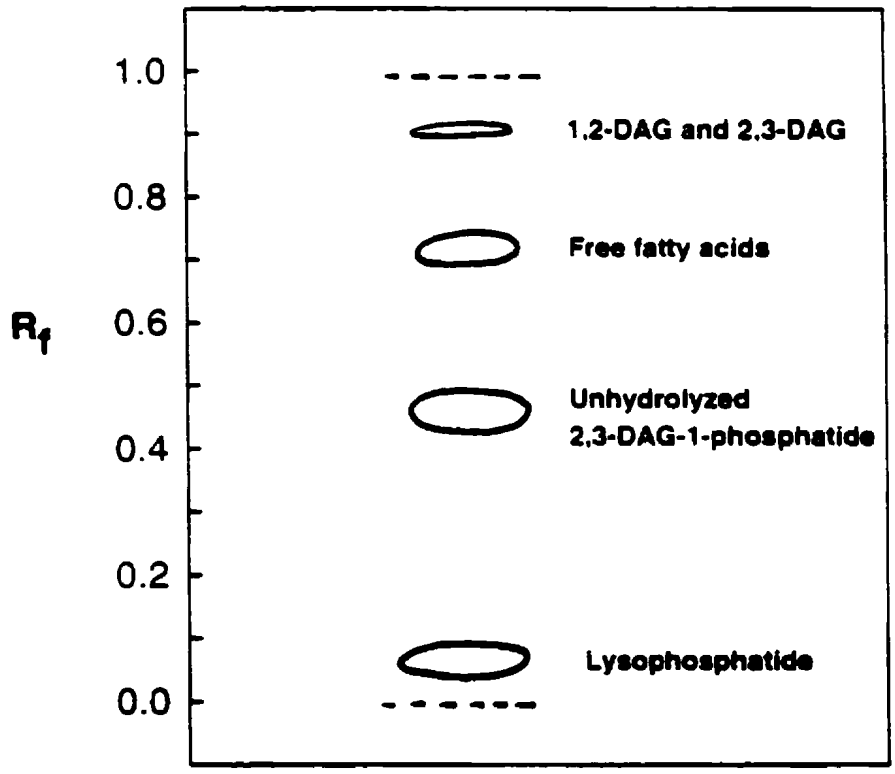
The hydrolyzed products were dissolved in 1.0 mL of chloroform/methanol (1:1, v/v) containing one drop of glacial acetic acid. The dissolved hydrolytic products were applied to silica gel TLC plates (20 x 20 cm; 60 Å mean pore diameter, 2-25 µm mean particle size, 500 µm thickness, with dicholofluorescein, Sigma) impregnated with 5% (w/v) boric acid. The plates were developed in diethyl ether/petroleum ether (40:60, v/v)

dried in a fume hood and then kept over concentrated aqueous ammonia for 10 min and subsequently redeveloped in aqueous ammonia/methanol/diethyl ether (2:15:83, v/v/v). After drying, the bands were located by viewing under short (254 nm) and long (356 nm) wavelength UV light (Spectraline, Model ENF-240C, Spectronics Co., Westbury, NY). The separated bands: free fatty acids (hydrolyzed from *sn*-2 position of 1,2-diacyl-3-phosphatide: R_f = 0.72), unhydrolyzed 2,3-diacyl-1-phosphatide (R_f = 0.49), lysophosphatide (R_f = 0.08), and small amounts of 1,2- and 2,3-diacylglycerols (R_f = 0.91) (Figure 3.6) were scraped off and extracted into chloroform/methanol (1:1, v/v). After removing a small sample for fatty acid analysis, the unhydrolyzed 2,3-diacyl-1-phosphatide fraction was subjected to porcine pancreatic lipase hydrolysis using the procedure described in Section 3.2.7.6 in order to obtain 2-monoacyl-1-phosphatide and free fatty acids (hydrolyzed from *sn*-3 position). All separated lipid fractions were analyzed for their fatty acid composition by employing the gas chromatographic procedure described in Section 3.2.8

3.2.7.6 Hydrolysis of enzymatically modified oils by pancreatic lipase

Hydrolysis of purified oils as well as separated 2,3-diacyl-1-phosphatide by pancreatic lipase was carried out according to the method described by Christie (1982). Tris-hydrochloric buffer (5 mL; 1.0 M, pH 8.0), 0.5 mL of calcium chloride (2.2%, w/v)

Figure 3.6 TLC chromatogram of products of phospholipase A_2 hydrolysis


1.0
0.8
0.6
0.4
0.2
0.0
R_f
1,2-DAG and 2,3-DAG
Free fatty acids
Unhydrolyzed
2,3-DAG-1-phosphatide
Lysophosphatide

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 equilibriate 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.) at 200 rpm under nitrogen for 8 to 10 min at 40°C. Ethanol (5 mL) was added to stop the enzymatic hydrolysis followed by addition of 5.0 mL of 6.0 N 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 30°C, the hydrolytic products were separated on silica gel TLC plates (20 x 20 cm; 60 Å mean pore diameter, 2-25 µm mean particle size, 500 µm thickness, with dichlorofluorescein, Sigma) impregnated with 5% (w/v) boric acid. 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 (254 nm) and long (356 nm) wavelength UV lights (Spectraline, Model ENF-240C, Spectronics Co., Westbury, NY). The bands were scraped off and their lipids extracted into chloroform/methanol (1:1, v/v) or diethyl ether and subsequently used for fatty acid analysis by the gas chromatographic procedure described in Section 3.2.8.

3.2.8 Analysis of fatty acid composition of lipids

3.2.8.1 Preparation of fatty acid methyl esters (FAMEs)

Fatty acid composition of lipids was determined following conversion to methyl esters. About 15 mg of each oil was weighed into a 6 mL well-cleaned Teflon-lined, screw capped conical vial. The internal standard (250 ng/ 100µl chloroform, methyl tricosanoate, $C_{23:0}$) was added to the vial and the solvent in the oil-internal standard mixture was evaporated under a stream of nitrogen. Transmethylation reagent (2 mL, freshly prepared 6 mL of concentrated sulphuric acid made up to 100 mL with spectral grade methanol and 15 mg of hydroquinone as an antioxidant) was added to the sample vial and mixed by vortexing. The mixture was incubated overnight at 60°C and subsequently cooled (Wanasundara and Shahidi, 1995). Distilled water (1 mL) was added to the mixture after thorough mixing, and it was extracted three times with 1.5 mL of pesticide-grade hexane. A few crystals of hydroquinone were added to each vial prior to extraction with hexane. Hexane layers were separated, combined and transferred to a clean tube and then washed two times with 1.5 mL of distilled water. In the first wash, the aqueous layer was removed and in the second wash, the hexane layer was separated and evaporated under a stream of nitrogen. Fatty acid methyl esters were then dissolved in 1 mL of carbon disulphide and used for gas chromatographic analysis (see below).

3.2.8.2 Analysis of fatty acid methyl esters (FAMEs) by gas chromatography (GC)

A Hewlett Packard 5890 Series II gas chromatograph (Hewlett Packard, Toronto, ON) equipped with a SUPELCOWAX-10 column (0.25 mm diameter, 30 m length, 0.25 µm film thickness; Supelco Canada Ltd., Oakville, ON) was used for analyzing FAMEs. The oven temperature was initially set at 220°C for 10.25 min and then ramped to 240°C at 30 °C/min and then held there for 9 min. The injector and detector (flame ionization, FID) temperatures were both at 270°C. UHP helium was used as a carrier gas (15 mL/ min). HP 3365 Series II ChemStation software (Hewlett Packard, Toronto, ON) was used for data handling. The FAMEs were tentatively identified by comparison of their retention times with those of authentic standard mixtures (GLC-461; Nu-Check). The area under each peak was calculated on a weight percentage basis using methyl tricosanoate ($C_{23:0}$) as an internal standard.

3.2.9 Assessment of oxidative stability of oils by accelerated oxidation methods

Comparison of the oxidative stability of enzymatically modified oils as well as unmodified oils was carried out under Schaal-oven test conditions at 60°C. It is generally accepted that each day (24 h) of storage of oils under Schaal-oven test conditions at 60°C is equivalent to one month of storage at ambient temperatures (Evans *et al.*, 1973; Abou-Gharbia *et al.*, 1996).

The specifications of the experiments carried out under Schaal oven test conditions were as follows. Each oil (1.8 g), in triplicate, was placed in test tubes (10 mm diameter and 12 cm height) and stored in a forced-air oven (Thelco, Model 2, Precision Scientific Co., Chicago, IL) at 60°C. To estimate oxidative stability by various methods (conjugated dienes: Section 3.2.9.1, 2-thiobarbituric acid reactive substances value: Section 3.2.6.5, headspace volatiles: Section 3.2.9.3 and ^{1}H NMR: Section 3.2.10), samples were removed periodically at 0, 6, 12, 24, 48, 72 and 96 h from the oven, cooled to room temperature, flushed with nitrogen for 30 s, capped and stored at -20°C until analyzed (usually within 15 days).

3.2.9.1 Determination of conjugated dienes (CD)

Conjugated diene values of oil samples was measured by the method of IUPAC (1987). Oil samples (0.02-0.04 g) were weighed into 25 mL volumetric flask, 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 diode array spectrophotometer (Model 8452A, Hewlett Packard Co., Missisauga, ON). Pure isooctane was used as the reference. Conjugated diene value was calculated as:

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

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).

3.2.9.2 Determination of the 2-thiobarbituric acid reactive substances (TBARS)

TBARS of oil samples was measured by the method of American Oil Chemists' Society (AOCS, 1990; Method Cd 19-90) as described in Section 3.2.6.5.

3.2.9.3 Static headspace gas chromatographic analysis

A Perkin-Elmer 8500 gas chromatograph and HS-6 headspace sampler (Perkin-Elmer Corp., Montreal, PQ) were used for analysis of volatiles produced during storage 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, 0.10 μm film thickness; Supelco Canada Ltd., Oakville, ON). UHP helium was the carrier gas employed at an inlet column pressure of 17.5 psig with a split ratio of 7:1. The oven temperature was maintained at 40°C for 5 min and then ramped to 200°C at 20°C/min and held there for 5 min. The injector and flame ionization detector (FID) temperatures were adjusted to 280°C and held at this temperature throughout the analysis.

For headspace (HS) analysis, 0.20 g of each oil was transferred to a 5 mL headspace vial (Chromatographic Specialties Inc., Brockville, ON). The vials were capped with Teflon-lined septa, crimped and then frozen and kept at -20°C until used. To avoid heat shock after removal of sample vials from storage, frozen vials were tempered at room temperature for 10 min and then preheated in the HS-6 magazine assembly at

90°C for 10 min equilibration period. Pressurization time of the vial was 6 s, and the volume of the vapour phase drawn was approximately 1.5 mL. Chromatograph peak areas were expressed as integrator count units. Individual volatile compounds were tentatively identified by comparison of their retention times with those of commercially available standards. Quantitative determination of dominant volatiles (mainly hexanal and propanal) was accomplished using 2-heptanone as an internal standard (Shahidi and Pegg, 1994).

3.2.10 Proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR spectra of the oil samples, subjected to accelerated oxidation, were recorded using a 300 MHz nuclear magnetic resonance spectrometer (GE-GN 300; General Electric Inc., Fremont, CA) in $CDCl_3$ solvent. Tetramethylsilane (TMS) was used as the internal standard. Solutions containing 15 mg oil in $CDCl_3$ (0.3 mL) were placed in NMR tubes (No. 509-UP; 5 mm outer diameter, 178 mm length; Norell, Inc., Landisville, NJ) and the spectra were recorded. Chemical shifts (δ, ppm) were reported relative to TMS. Data were analyzed using NUTS software (NMR Data Processing Program, Acron NMR Inc., Fremont, CA). The total number of protons under each peak was calculated on the basis of integration of methylene protons of the TAG backbone.

3.2.11 Determination of double bond index (DBI)

The fatty acid composition of oxidized and unoxidized oils was determined by gas chromatography as described in Section 3.2.8. For determination of double bond index (DBI), the number of double bonds contained in each fatty acid was multiplied by its respective mole percentage and summed for all fatty acids detected. The mean number of double bonds per fatty acyl chain was then expressed as the DBI (Wagner *et al.*, 1994).

3.2.12 Determination of methylene bridge index (MBI)

The mean number of *bis*-allylic methylene bridge positions of fatty acid constituents, expressed as methylene bridge index (MBI), was calculated by multiplying the number of *bis*-allylic methylene bridge positions contained in each fatty acid species by its respective mole percentage and summed for all fatty acids present (Vartak *et al.*, 1997).

3.2.13 Statistical analyses

All experiments, except RSM, in this study were replicated at least three times. Data are reported as mean ± standard deviation (SD). RSM used a 3-factor, 3-level, face-centred cube design with triplicate determinations at the centre point. Section 3.2.5 explains data analysis carried out for the optimization study using RSM. Analysis of variance (ANOVA) was performed and significant differences among mean values were

determined using Tukey's studentized test at $P < 0.005$, 0.05 or 0.1 and employing ANOVA and Tukey's procedures of statistical analysis system (SAS Institute Inc., 1990). Simple linear and multiple regression analyses were also performed using the same software in the general linear model (GLM) and response surface regression (RSREG) procedures, respectively.

CHAPTER 4

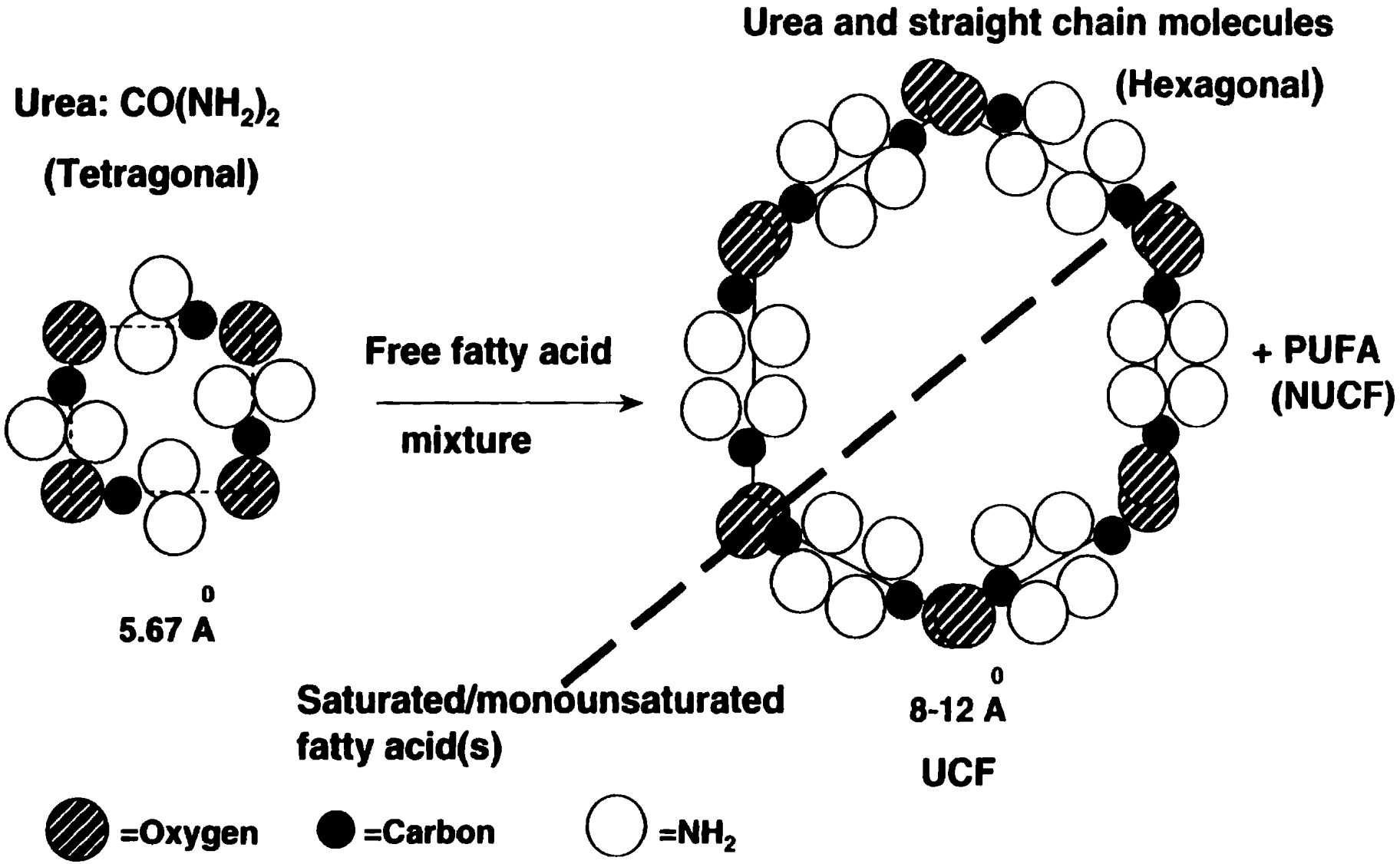
RESULTS AND DISCUSSION

4.1 Preparation of docosahexaenoic acid (DHA) concentrate from algal oil *via* urea complexation

A docosahexaenoic acid (DHA) concentrate was obtained by urea complexation of algal oil fatty acids. Urea complexation is a well-established technique for elimination of saturated and monounsaturated fatty acids (Ratnayake *et al.*, 1988). The simplest and most efficient technique for obtaining polyunsaturated fatty acid (PUFA) concentrates in the form of free fatty acids is urea complexation (Wanasundara and Shahidi, 1999). Initially, the triacylglycerols (TAG) of the algal oil are split into their constituent fatty acids by alkaline hydrolysis using alcoholic KOH or NaOH and unsaponifiables such as sterols, vitamins A and D as well as other non-TAG components are removed from the mixture. The free fatty acids (FFA) are then mixed with an alcoholic (ethanol or methanol) solution of urea. The saturated and monounsaturated fatty acids are easily complexed with urea and crystallize out on cooling and may subsequently be removed by filtration. The liquid or non-urea complexed fraction (NUCF) is enriched with PUFA.

Although urea alone crystallizes in a tightly packed tetragonal form (Figure 4.1), X-ray studies of urea complexes have shown them to be hexagonal in shape (Swern, 1964). In forming the complex, urea molecules build up the structural framework in a helical manner. Urea molecules bond together *via* hydrogen bonding (Hayes *et al.*, 2000). The included compounds occupy the free space inside the hexagonal channels and are held there *via* van

Figure 4.1 Formation of urea crystals in the presence of long-chain fatty acids


Urea and straight chain molecules
(Hexagonal)
Urea: CO(NH2)2
(Tetragonal)
Free fatty acid
mixture
+ PUFA
(NUCF)
5.67 A
0
Saturated/monounsaturated
fatty acid(s)
8-12 A
0
UCF
=Oxygen
=Carbon
=NH2

der Waals' forces, London dispersion forces or induced electrostatic attractions. While straight-chain saturated fatty acids with six carbon atoms or more are readily complexed, the presence of double bonds in the carbon chain increases the bulk of the molecule and reduces the likelihood of its complexation with urea (Swern, 1964). Monounsaturated fatty acids are more readily complexed as compared to diunsaturated fatty acids, which, in turn, are more easily complexed than triunsaturated fatty acids. Therefore, the formation of fatty acid-urea adducts depends on the degree of unsaturation of fatty acids involved.

Algal oil in this work was extracted from the marine microalga *Crypthecodinium cohnii*, and contained approximately 40% (w/w) DHA (Haumann, 1998; Jiang and Chen, 2000). It is a yellow-orange, free flowing oil which contains about 95% TAG (Haumann, 1998). Algal oil may be concentrated in the form of TAG, as free fatty acids, or as the simple alkyl esters. Most of the algal oil products sold are in the TAG form (Haumann, 1998). Algal oils are abundant in DHA and may be used as a raw material for preparation of DHA concentrates. Of the several procedures considered for concentration of PUFA, urea complexation is one of the promising methods as it allows handling of large quantities of material. In this study, urea complexation of algal oil was carried out in order to produce a DHA concentrate.

The urea complexation could be performed either on the free 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.

The influence of the ratio (w/w) of urea to fatty acid on the yield (%) of DHA in the concentrate was studied. In this particular set of experiments the weight of fatty acids was kept constant at 10 g, but the weight of urea was varied. The urea complex was allowed to crystallize at 4°C for 24 h. The concentration of DHA reached a maximum at an approximate urea to fatty acid ratio of 3:1 (Figure 4.2). When an excess urea was used, more of the DHA was complexed and found its way into the UCF. Thus, in this study the urea/fatty acid weight ratio was kept at 3:1 (w/w). Haagsma *et al.* (1982) reported that the efficiency of urea-adduct formation reached a maximum when the urea/fatty acid ratio was about 3:1 for cod liver oil fatty acid methyl esters. A similar conclusion was reached by Ratnayake *et al.* (1988) using menhaden oil fatty acids.

The fatty acid compositions of the original algal oil and that of the urea complexing and nonurea complexing fractions produced from it are given in Table 4.1. The major saturated fatty acids present in the algal oil were 14:0 (15.0%) and 16:0 (9.0%). However, small amounts of 10:0, 12:0 and 18:0 were also present. As a result of urea adduct formation, total saturated fatty acids were decreased from 25.9 to 1.1%. Thus, most of the saturated fatty acids, except small amounts of 10:0, 12:0 and 14:0, were removed (Table 4.1). Monounsaturated fatty acids (MUFA) found in the original algal oil were 14:1, 16:1 and 18:1. The total content of MUFA in the product was decreased from 21.3 to 0.6% following the urea crystallization process. PUFA present in algal oil were 18:2, 22:5 and 22:6. Total PUFA was increased from 48.9 to 98.2% in the NUCF. DHA was enriched from

Figure 4.2 Effect of urea/fatty acid ratio on percentage (%) of DHA in the concentrate after urea complexation

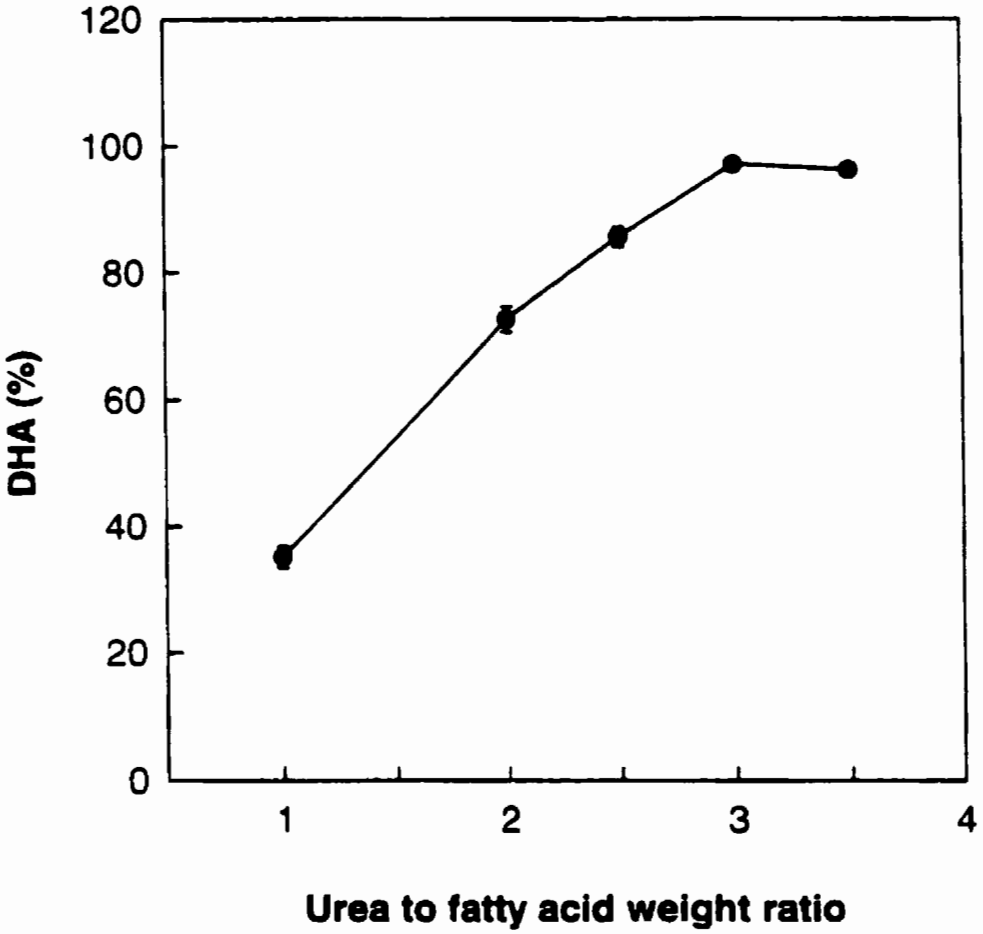

120
100
80
60
40
20
0
DHA (%)
1
2
3
4
Urea to fatty acid weight ratio

Table 4.1 Fatty acid profile of algal oil and its urea complexing (UCF) and nonurea complexing (NUCF) fractions obtained by urea complexation

Fatty acid	Algal oil	NUCF[a]	UCF[b]
10:0	0.58 ± 0.06	0.47 ± 0.04	1.15 ± 0.02
12:0	1.12 ± 0.05	0.51 ± 0.02	7.67 ± 0.22
14:0	14.9 ± 0.07	0.13 ± 0.02	17.9 ± 0.21
14:1	0.20 ± 0.03	0.16 ± 0.10	0.30 ± 0.04
16:0	9.05 ± 0.12	ND[c]	6.85 ± 0.05
16:1	2.20 ± 0.08	0.25 ± 0.01	3.71 ± 0.02
18:0	0.20 ± 0.01	ND[c]	0.17 ± 0.02
18:1	18.9 ± 0.32	0.22 ± 0.01	36.9 ± 0.09
18:2	1.01 ± 0.02	0.65 ± 0.03	1.14 ± 0.01
22:5	0.51 ± 0.05	0.41 ± 0.01	0.67 ± 0.07
22:6ω3	47.4 ± 0.15	97.1 ± 0.02	23.1 ± 0.35
Saturated fatty acids	25.87	1.11	26.02
MUFA[d]	21.32	0.63	40.92
PUFA[e]	48.94	98.16	32.92
Yield (wt %)	-	32.5	52.0
DHA recovery (%)	-	66.5	25.3

Results are mean of triplicate determinations from different experiments.
[a]Non-urea complexing fraction
[b]Urea complexing fraction
[c]Not detected
[d]Monounsaturated fatty acids
[e]Polyunsaturated fatty acids

47.4 to 97.1% and the process yield was 32.5% with respect to the weight of the original oil. Haagsma *et al.* (1982) have reported 100% recovery of DHA from NUCF. 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. Although NUCF contained 97.1% DHA, a considerable proportion of it (23.1%) was invariably complexed with urea and ended up in the UCF. The recovery efficiency of DHA in NUCF was 66.5% of that present in the original algal oil (Table 4.1).

Recently, urea complexation was used to prepare ω-3 PUFA concentrates from seal blubber oil (Wanasundara and Shahidi, 1999). Among the major ω-3 PUFAs, DHA was found almost exclusively (65.2%) in the 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. However, it was difficult to remove all of the saturated 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 algal oil by this process (Table 4.1).

In urea complexation, the complexed crystals are very stable and hence filtration at the very low temperatures used for solvent crystallization of fatty acids is not required

(Anonymous, 1986). This method is also favoured by many researchers because complexation depends upon the configuration of fatty acid moieties governed by the presence of multiple double bonds rather than pure physical properties such as solubility or melting point.

4.2 Enzymatic incorporation of ω3 fatty acids (EPA and DHA) into borage oil (BO) and evening primrose oil (EPO)

4.2.1 Enzyme screening

Six commercial enzymes from *Candida antarctica*, *Mucor miehei*, *Pseudomonas sp.*, *Candida rugosa*, *Aspergillus niger* and *Thermomyces lanuginosus* were screened for their ability to incorporate DHA into borage oil (BO) and evening primrose oil (EPO) at 37°C in hexane (Table 4.2). These lipases catalyzed DHA incorporation into BO to various extents. The degree of DHA incorporation attained with various lipases was in the order of *Candida antarctica* > *Pseudomonas sp.* > *Mucor miehei* > *Aspergillus niger* > *Candida rugosa* > *Thermomyces lanuginosus*. The lipases from *Aspergillus niger*, *Candida rugosa* and *Thermomyces lanuginosus* were less effective in this regard. The lipase from *Candida antarctica* gave the highest degree of DHA incorporation into BO (25.8%, after 24 h). Thus, this lipase was selected for subsequent experiments to determine optimal acidolysis conditions. In EPO, the degree of DHA incorporation with various lipases was in the order of *Candida antarctica* > *Pseudomonas sp.* > *Mucor miehei* > *Aspergillus niger* > *Candida rugosa* > *Thermomyces lanuginosus*. The lipases form *Candida antarctica*, *Mucor miehei*

Table 4.2 Effect of different lipases on DHA incorporation (%) into borage (BO) and evening primrose oils (EPO)

Enzyme	Commercial code	Enzyme activity (U)	DHA incorporation (%) in BO[a]	DHA incorporation (%) in EPO[a]
Candida antarctica	Novozym-435	554	25.8 ± 0.1	28.7 ± 0.5
Mucor miehei	Lipozyme-IM	13,613	13.1 ± 1.2	20.1 ± 0.4
Pseudomonas sp.	PS-30	11,936	16.8 ± 0.6	24.2 ± 1.3
Aspergillus niger	AP-12	8142	2.73 ± 0.5	14.0 ± 0.8
Candida rugosa	AY-30	38,707	2.04 ± 0.2	7.53 ± 0.2
Thermomyces lanuginosus	Novozym-677BG	7658	1.72 ± 0.4	2.62 ± 0.4

[a]The reaction mixture contained 500 mg oil, 194 mg DHA, 500 units of enzyme and 3 mL hexane. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm. Results are mean of triplicate determinations from different experiments.

and *Pseudomonas sp.* and, to a limited extent, lipases from *Aspergillus niger* and *Candida rugosa* incorporated DHA into EPO. However, lipase from *Thermomyces lanuginosus* was not efficient in incorporating DHA into EPO. Lipase from *Candida antarctica* was used further in this study because it afforded the highest DHA incorporation into EPO (28.7%, after 24 h). 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.

In another study, EPA was incorporated into BO and EPO using a variety of commercial lipases. The degree of EPA incorporation in both oils was in the order of *Pseudomonas sp.* > *Mucor miehei* > *Candida antarctica* > *Aspergillus niger* > *Candida rugosa* > *Thermomyces lanuginosus* (Table 4.3). The results showed that lipases from *Pseudomonas sp.*, *Mucor miehei* and *Candida antarctica* were highly effective in incorporating EPA into the oils examined. Since the acidolysis of both oils with EPA was best performed with lipase from *Pseudomonas sp.*, this enzyme was chosen for subsequent experiments. In another study, incorporation of EPA+DHA in BO and EPO was also studied using the above lipases. The degree of incorporation of EPA+DHA followed the same order as that observed for EPA-enriched oils (Table 4.3). The lipase from *Pseudomonas sp.* was found to be highly effective in incorporating EPA+DHA into BO and EPO. Therefore, this lipase was selected for further experimentation.

Table 4.3 Effect of different lipases on EPA incorporation (%) and EPA+DHA incorporation (%) into borage (BO) and evening primrose oils (EPO)

Enzyme	EPA incorporation (%) in BO[a]	EPA incorporation (%) in EPO[a]	EPA + DHA incorporation (%) in BO[b]	EPA + DHA incorporation (%) in EPO[b]
Candida antarctica	21.0 ± 0.8	22.9 ± 1.9	18.2 ± 0.3	16.9 ± 0.5
Mucor miehei	23.3 ± 2.1	25.6 ± 0.7	21.9 ± 1.7	22.4 ± 1.0
Pseudomonas sp.	28.7 ± 0.6	30.7 ± 0.2	31.7 ± 0.9	32.7 ± 0.5
Aspergillus niger	14.1 ± 0.6	13.0 ± 2.4	14.6 ± 0.3	15.3 ± 0.7
Candida rugosa	12.0 ± 1.5	10.7 ± 1.1	12.6 ± 0.9	13.8 ± 0.3
Thermomyces lanuginosus	6.81 ± 2.1	8.51 ± 2.9	10.8 ± 0.8	11.3 ± 1.0

[a]The reaction mixture contained 500 mg oil, 178 mg EPA, 500 units of enzyme and 3 mL hexane. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm. Results are mean of triplicate determinations from different experiments

[b]The reaction mixture contained 500 mg oil, 89 mg EPA, 97 mg DHA, 500 units of enzyme and 3 mL hexane. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm. Results are mean of triplicate determinations from different experiments

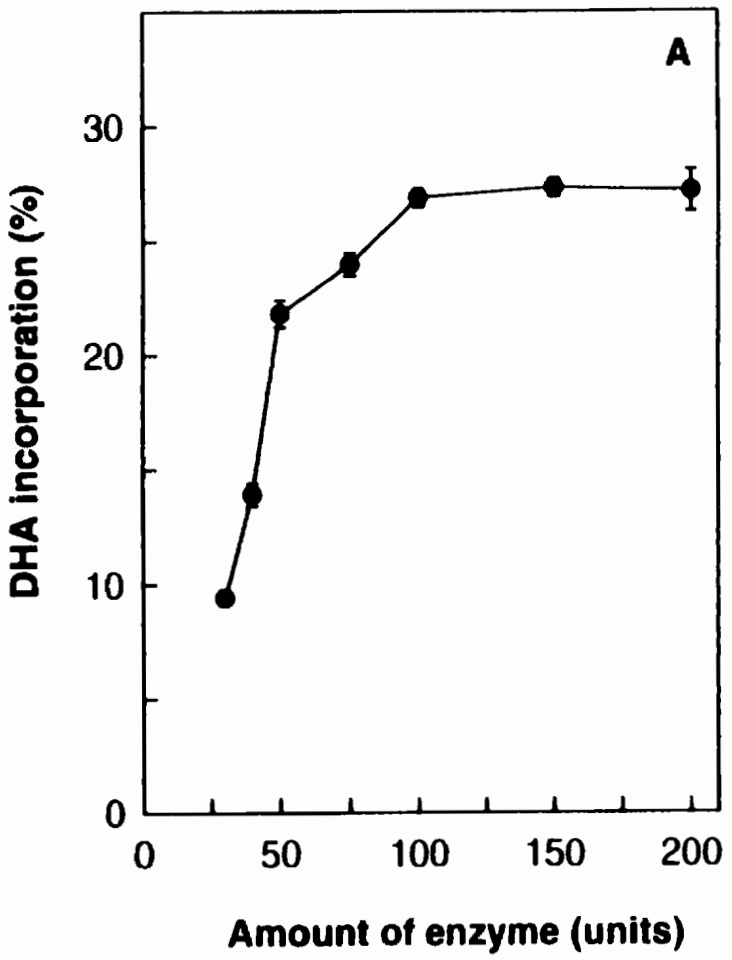
4.2.2 Effect of enzyme load

The effect of enzyme load (lipase from *Candida antarctica*) on the incorporation of DHA (%) into acylglycerols of BO and EPO is shown in Figure 4.3. Increasing the amount of enzyme in the mixture increased the extent of DHA incorporation, but a significant increase was not observed ($p > 0.05$) when the enzyme was present at a level greater than 100-150 units. Thus, 100-150 units of enzyme were sufficient to saturate the reaction system in terms of the enzyme load. Figure 4.4 shows the effect of enzyme content (lipase from *Pseudomonas sp.*) on EPA incorporation into BO and EPO. As the amount of enzyme was increased, incorporation of EPA was increased. The highest incorporation (28.1% EPA in BO and 27.4% EPA in EPO) was obtained with 150-250 units of enzyme. Akoh *et al.* (1996) have also reported increased incorporation of EPA into EPO with increasing enzyme load (SP435 from *Candida antarctica*). Akoh and Sista (1995) used an immobilized nonspecific lipase from *Candida antarctica* to incorporate EPA into BO. Their results showed that the highest incorporation (31% EPA) was obtained with 20% (w/w of substrates) lipase. The effect of enzyme load (lipase from *Pseudomonas sp.*) on EPA+DHA incorporation in oils is depicted in Figure 4.5. A high incorporation of EPA+DHA was observed at an early stage of the reaction. Also increasing the enzyme load increased the degree of incorporation of EPA+DHA into the oils tested.

4.2.3 Effect of temperature

Temperature is a well known parameter that affects enzyme activity. The rate of

Figure 4.3 Effect of enzyme load on the incorporation of DHA into borage (A) and evening primrose oils (B). The reaction mixture contained 297-300 mg oil, 120 mg DHA, 30-200 units of *Candida antarctica* lipase preparation and 3 mL hexane. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm.


A
30
20
10
0
0
50
100
150
200
DHA incorporation (%)
Amount of enzyme (units)

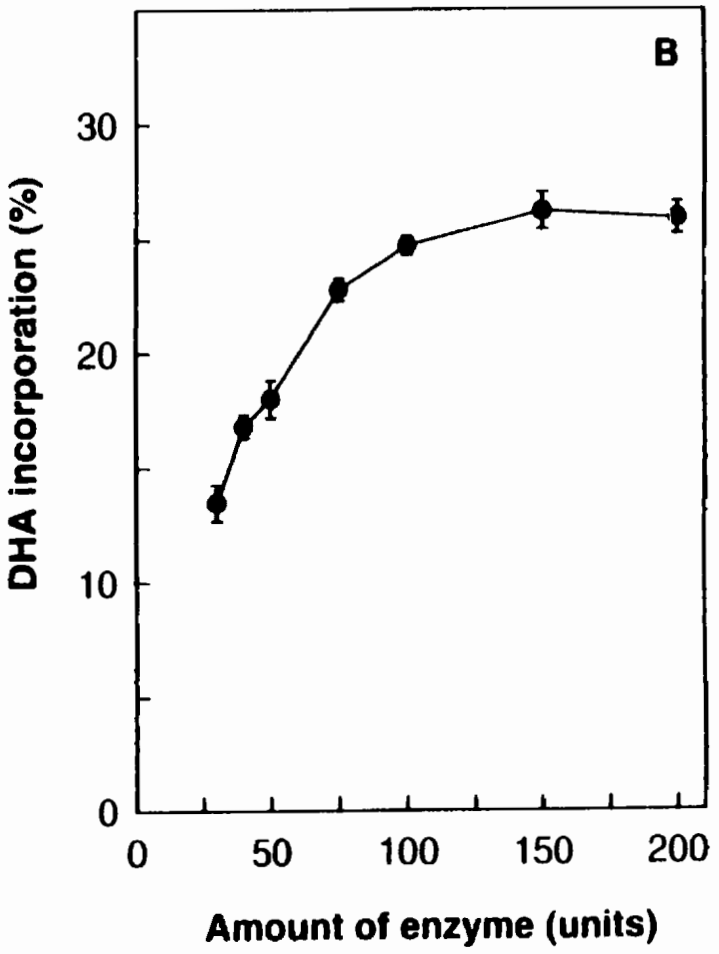

B
30
20
10
0
0
50
100
150
200
DHA incorporation (%)
Amount of enzyme (units)

Figure 4.4 Effect of enzyme load on the incorporation of EPA into borage (A) and evening primrose oils (B). The reaction mixture contained 297-300 mg oil, 115 mg EPA, 50-450 units of *Pseudomonas sp.* lipase preparation and 3 mL hexane. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm.

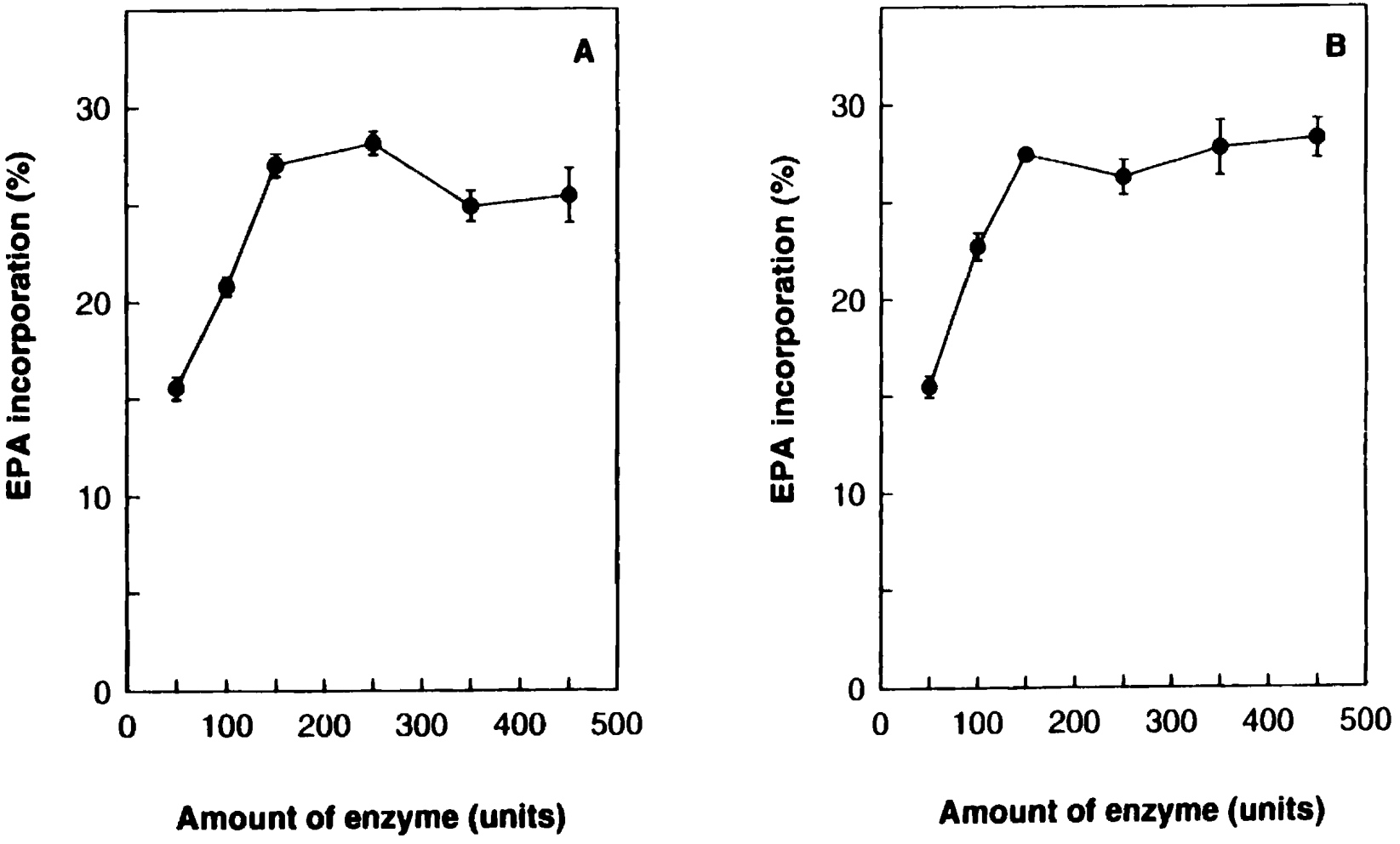
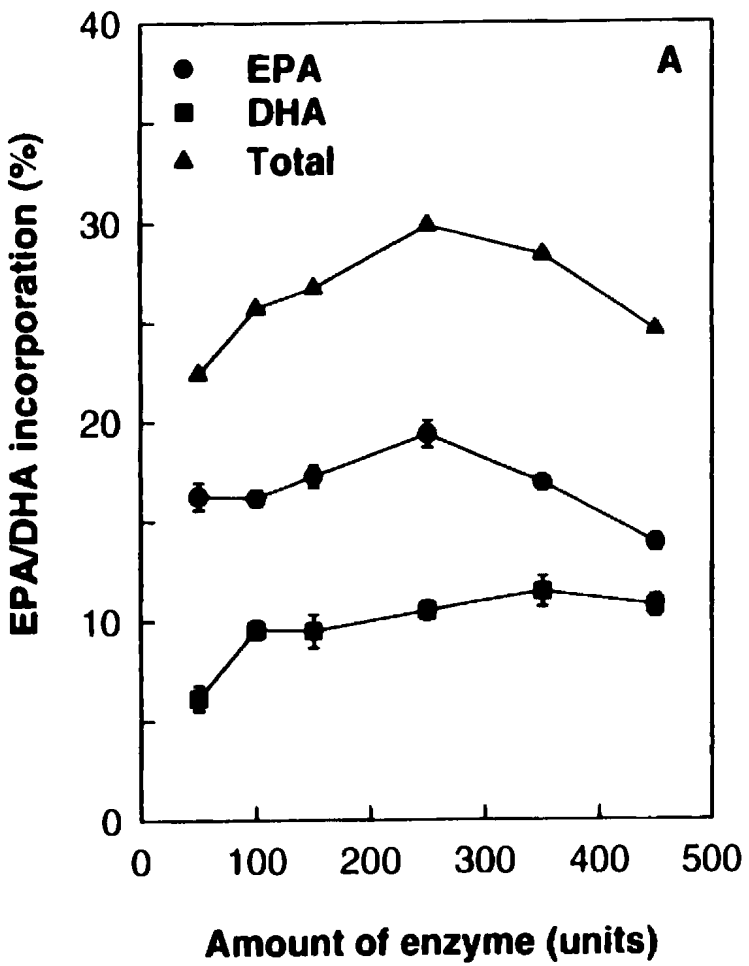
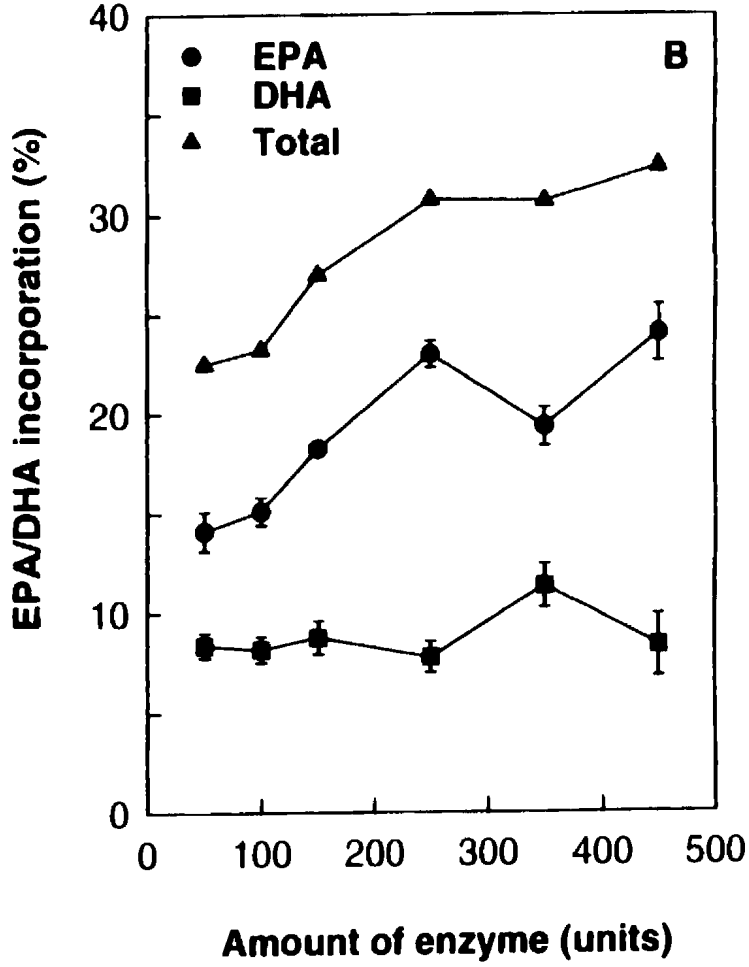

A
B
EPA incorporation (%)
0
10
20
30
0
100
200
300
400
500
Amount of enzyme (units)

Figure 4.5 Effect of enzyme load on the incorporation of EPA+DHA into borage (A) and evening primrose oils (B). The reaction mixture contained 297-300 mg oil, 54 mg EPA, 58 mg DHA, 50-450 units of *Pseudomonas sp.* lipase preparation and 3 mL hexane. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm.

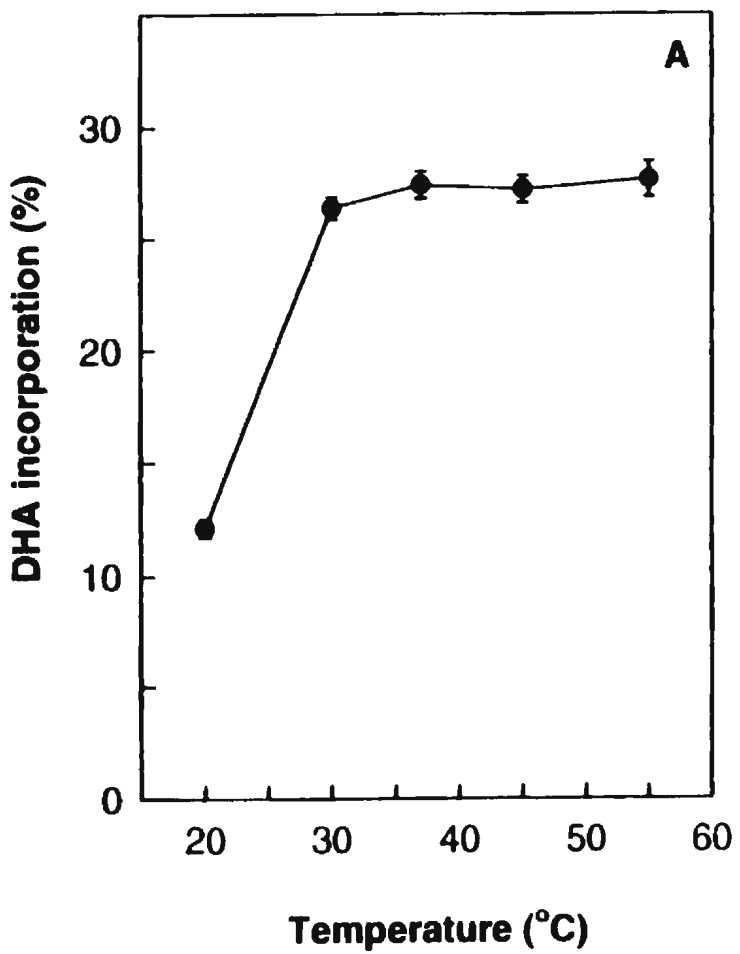

A
EPA
DHA
Total
EPA/DHA incorporation (%)
0
10
20
30
40
0
100
200
300
400
500
Amount of enzyme (units)

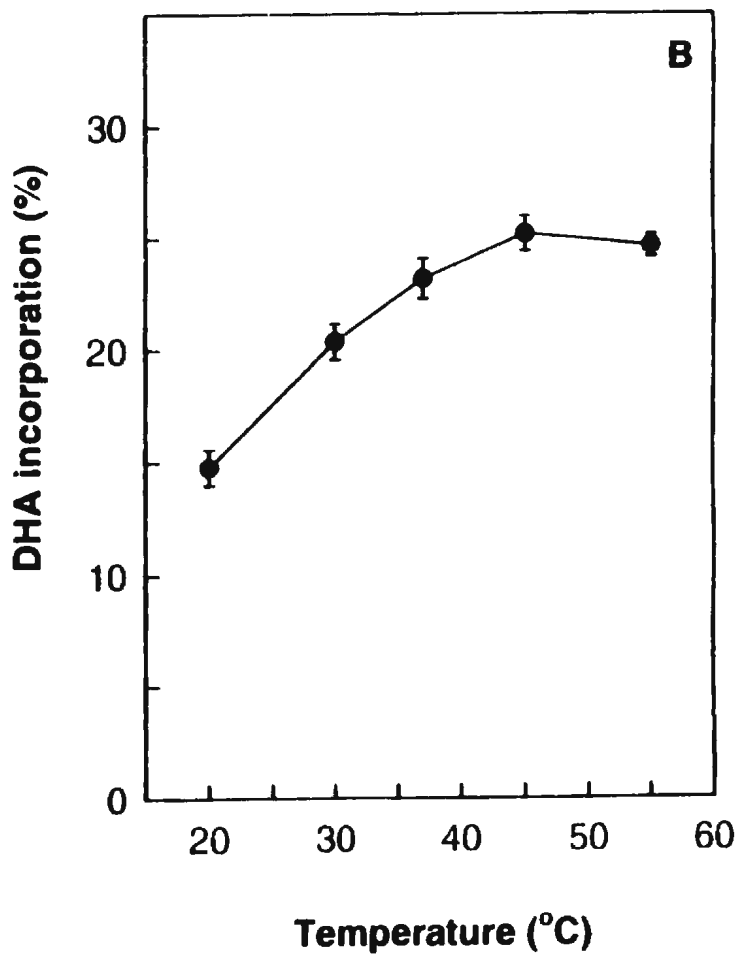

B
EPA
DHA
Total
EPA/DHA incorporation (%)
0
10
20
30
40
0
100
200
300
400
500
Amount of enzyme (units)

enzyme activity does increase as the environment heats up, until a maximum rate is reached. That rate is the optimum temperature for a given enzyme. When temperature increases further, the reaction rate plummets. The reason is that hydrogen bonds and other weak attractions holding the enzyme in its three-dimensional shape are sensitive to temperature changes in its surroundings. Increased temperature increases the kinetic energy of the enzyme's molecular framework; and molecules in the surroundings also collide more frequently with the enzyme itself. At some point, the disturbances are so great that denaturation occurs. The effect of temperature on lipase-mediated acidolysis of oils with fatty acids has been investigated (Akoh and Huang, 1995; Ju *et al.*, 1998). Temperature effects on lipase-catalysed ester synthesis were reported to be dependent on the reaction medium, enzyme source and substrate (Welsh *et al.*, 1989).

Figure 4.6 illustrates the effect of temperature on lipase-catalyzed acidolysis of BO and EPO with DHA by an immobilized *Candida antarctica* lipase. The temperature range tested was 20 to 55°C. DHA incorporation increased as the temperature was increased up to 37°C and 45°C in BO and EPO, respectively. When the temperature increased further, the degree of DHA incorporation remained constant. The optimum temperature range for this reaction was 37-55°C. Thus, higher temperatures, up to 55°C, seemed more suitable for better performance of *Candida antarctica* lipase. 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 on this enzyme. Kosugi and Azuma (1994) used an immobilized lipase from

Figure 4.6 Effect of temperature on DHA incorporation into borage (A) and evening primrose oils (B). The reaction mixture contained 297-300 mg oil, 120 mg DHA, 150 units of *Candida antarctica* lipase and 3 mL hexane. The reaction mixture was incubated at different temperatures (20-55°C) for 24 h in an orbital shaking water bath at 250 rpm.


A
DHA incorporation (%)
0
10
20
30
20
30
40
50
60
Temperature (°C)


B
DHA incorporation (%)
0
10
20
30
20
30
40
50
60
Temperature (°C)

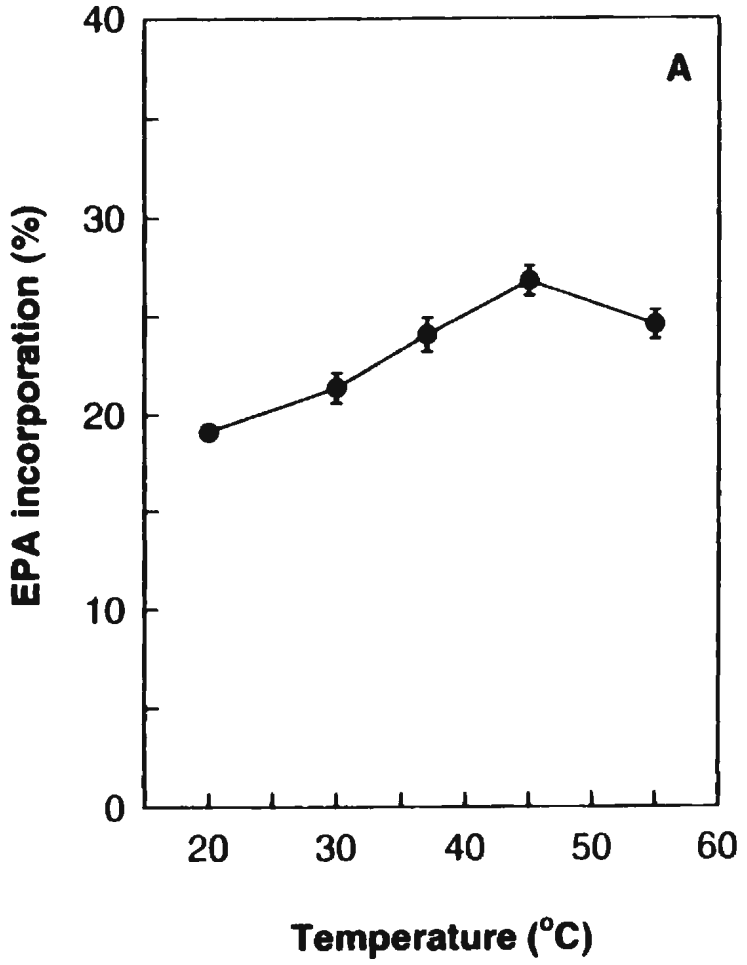
Candida antarctica for production of pure TAG from EPA or DHA with glycerol. The rate of TAG formation was reported to be faster at 60 °C even though the TAG yield was the same at 40 and 60 °C.

The effect of temperature on acidolysis of BO and EPO with EPA by lipase from *Pseudomonas sp.* was also studied (Figure 4.7). Reaction temperatures were varied from 20 to 55°C. It was found that higher temperatures (45-55°C) were more suitable for the reaction. In another experiment, the effect of temperature on EPA+DHA incorporation in the oils by *Pseudomonas sp.* lipase was examined and the results are shown in Figure 4.8. The EPA+DHA incorporation by *Pseudomonas sp.* lipase was monitored at temperatures ranging from 20 to 55°C. The *Pseudomonas* lipase-catalyzed acidolysis reactions exhibited a maximum reaction rate at 45-50°C. Ju *et al.* (1998) studied the effect of temperature on reaction rate (μmole ω3 PUFA/ time) of acidolysis of BO with ω3 PUFA. The IM-60 (from *Mucor miehei*) lipase-catalysed acidolysis reaction showed a maximum rate at 50°C.

4.2.4 Time course

Table 4.4 shows the changes in fatty acid composition of BO with time during the course of acidolysis with DHA. DHA was successfully incorporated into BO using *Candida antarctica* lipase as the biocatalyst. After 24 h of incubation in hexane, 27.4% DHA was incorporated (Table 4.4). DHA incorporation increased as incubation time increased, up to 24 h. Figure 4.9A shows the changes in fatty acid composition of BO following *Candida antarctica* lipase-catalyzed acidolysis with DHA for up to 48 h. Predominant fatty acids

Figure 4.7 Effect of temperature on EPA incorporation into borage (A) and evening primrose oils (B). The reaction mixture contained 297-300 mg oil, 115 mg EPA, 150 units of *Pseudomonas sp.* lipase and 3 mL hexane. The reaction mixture was incubated at different temperatures (20-55°C) for 24 h in an orbital shaking water bath at 250 rpm.


A
EPA incorporation (%)
0
10
20
30
40
20
30
40
50
60
Temperature (°C)

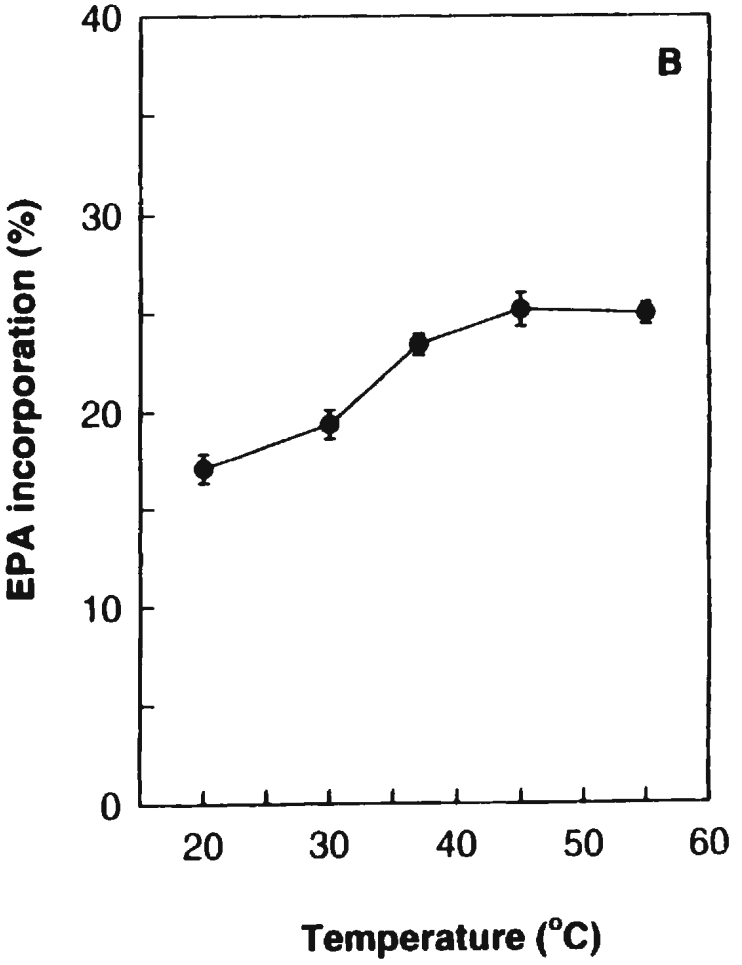
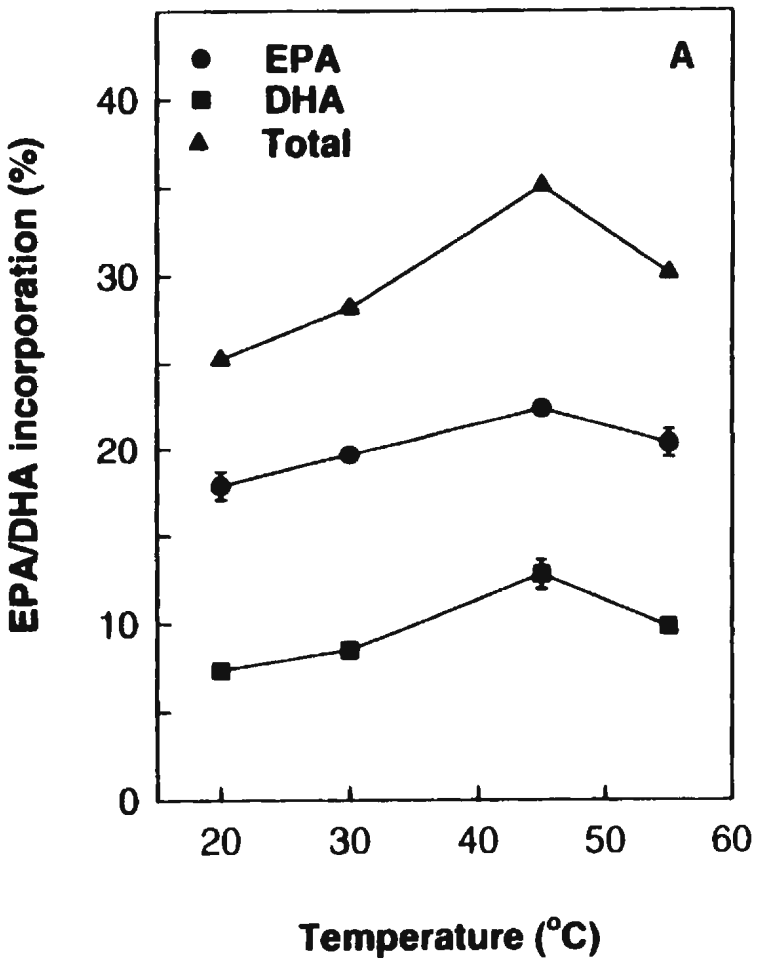

B
EPA incorporation (%)
0
10
20
30
40
20
30
40
50
60
Temperature (°C)

Figure 4.8 Effect of temperature on EPA+DHA incorporation into borage (A) and evening primrose oils (B). The reaction mixture contained 297-300 mg oil, 54 mg EPA, 58 mg DHA, 150 units of *Pseudomonas sp.* lipase and 3 mL hexane. The reaction mixture was incubated at different temperatures (20-55°C) for 24 h in an orbital shaking water bath at 250 rpm.


EPA
DHA
Total
A
EPA/DHA incorporation (%)
0
10
20
30
40
20
30
40
50
60
Temperature (°C)

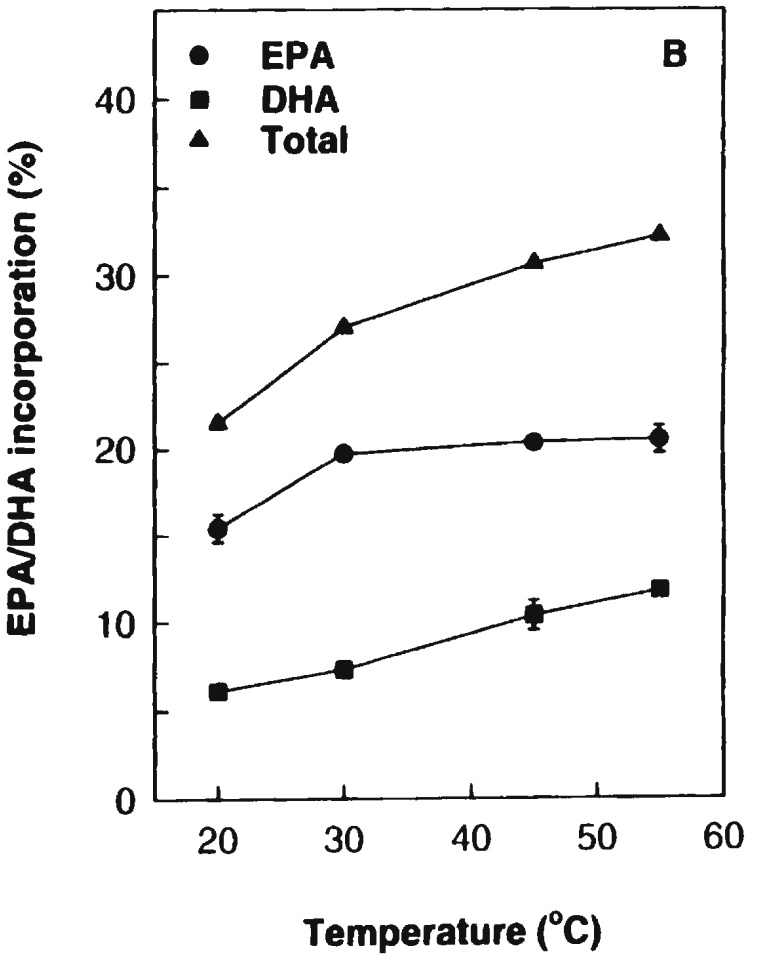

EPA
DHA
Total
B
EPA/DHA incorporation (%)
0
10
20
30
40
20
30
40
50
60
Temperature (°C)

Table 4.4 Fatty acid composition of borage oil (BO) before and after lipase-catalyzed acidolysis with DHA[a]

Fatty acid	Duration of acidolysis (h)			
	0	12	18	24
14:0	0.07 ± 0.02	0.07 ± 0.04	0.07 ± 0.02	ND[b]
16:0	9.60 ± 0.50	7.10 ± 0.20	7.07 ± 0.20	6.90 ± 0.33
16:1	0.20 ± 0.05	0.16 ± 0.05	0.16 ± 0.05	0.17 ± 0.23
17:0	0.10 ± 0.01	ND[b]	ND[b]	ND[b]
18:0	3.50 ± 0.03	2.62 ± 0.02	2.62 ± 0.02	2.55 ± 0.50
18:1	15.5 ± 0.70	13.4 ± 0.42	11.5 ± 0.30	11.3 ± 0.21
18:2ω6	37.8 ± 1.10	30.8 ± 0.92	27.6 ± 0.55	27.0 ± 0.82
18:3ω6	23.5 ± 0.85	18.7 ± 0.56	17.1 ± 0.70	17.0 ± 0.50
18:3ω3	0.21 ± 0.05	0.18 ± 0.07	0.17 ± 0.08	0.17 ± 0.06
20:0	0.22 ± 0.08	0.20 ± 0.01	0.17 ± 0.02	0.17 ± 0.02
20:1	4.20 ± 0.10	3.00 ± 0.01	3.00 ± 0.12	3.09 ± 0.01
20:2	0.21 ± 0.05	ND[b]	0.15 ± 0.02	0.15 ± 0.06
22:0	0.15 ± 0.07	ND[b]	ND[b]	0.12 ± 0.05
22:1	2.35 ± 0.12	1.90 ± 0.10	1.86 ± 0.05	1.81 ± 0.22
24:1	1.50 ± 0.10	1.00 ± 0.06	0.53 ± 0.16	ND[b]
22:6ω3	ND[b]	20.7 ± 0.57	26.5 ± 0.20	27.4 ± 0.10
ω3/ω6 Ratio	0.003	0.42	0.59	0.62

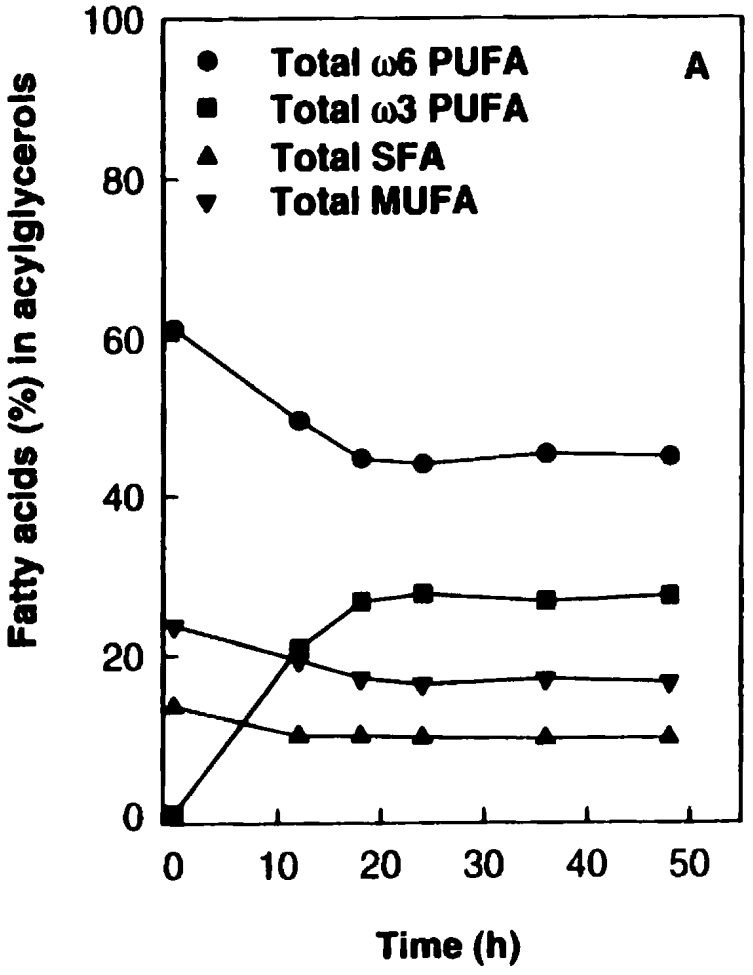
[a]The reaction mixture contained 300 mg borage oil, 120 mg DHA, 150 units of *Candida antarctica* and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm. Experimental results are means of triplicate determinations.

[b]Not detected

found in BO prior to the acidolysis reaction were linoleic acid (37.8%) and GLA (23.5%), in agreement with values reported in the literature (Gunstone, 1992; Akoh and Sista, 1995; Horrobin, 1992). As the DHA incorporation increased, the proportion of monounsaturated, saturated and total ω6 fatty acids (LA and GLA) decreased (Figure 4.9A). However, the amount of GLA was decreased to a lesser extent (from 23.5 to 17.0% after 24 h incubation). The maximum amount of DHA incorporation was 27.4% (after 24 h). The modified oil had an ω3/ω6 ratio of 0.42-0.62. This oil may prove to be nutritionally more favourable than unmodified BO. Similar results from the reaction of BO and EPA ethyl ester were obtained with an immobilized *Candida antarctica* at a substrate mole ratio of 1:3 (ω3/ω6 of 0.64) (Akoh and Sista, 1995). The changes in fatty acid profile of EPO after acidolysis with DHA are given in Table 4.5. DHA was incorporated into EPO using the same enzyme. After 24 h reaction, the content of DHA incorporated into this oil was 25.2%. The main fatty acid found in EPO before enzymatic modification was LA (72.6%). The content of GLA found in this oil was 9.12%. The amounts of monounsaturated, saturated and total ω6 fatty acids decreased upon DHA incorporation into the oil (Figure 4.9B). The modified EPO had an ω3/ω6 ratio of 0.16-0.4.

Similarly, EPA was successfully incorporated into BO and EPO using nonspecific *Pseudomonas sp.* lipase and the changes in fatty acid composition with time are given in Tables 4.6 and 4.7, respectively. After 24 h reaction, the amounts of EPA incorporated into BO and EPO were 26.8 and 25.2%, respectively. The modified BO and EPO had an ω3/ω6 ratios of 0.18-0.66 and 0.14-0.40, respectively. Previously, Akoh and Sista (1995) modified

Figure 4.9 Changes in total contents of ω3, ω6, saturated and monounsaturated fatty acids of borage (A) and evening primrose oils (B) during lipase-catalysed acidolysis with DHA. The reaction mixture contained 297-300 mg oil, 120 mg DHA, 150 units of *Candida antarctica* lipase and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm.


Total ω6 PUFA
Total ω3 PUFA
Total SFA
Total MUFA
A
Fatty acids (%) in acylglycerols
100
80
60
40
20
0
0
10
20
30
40
50
Time (h)

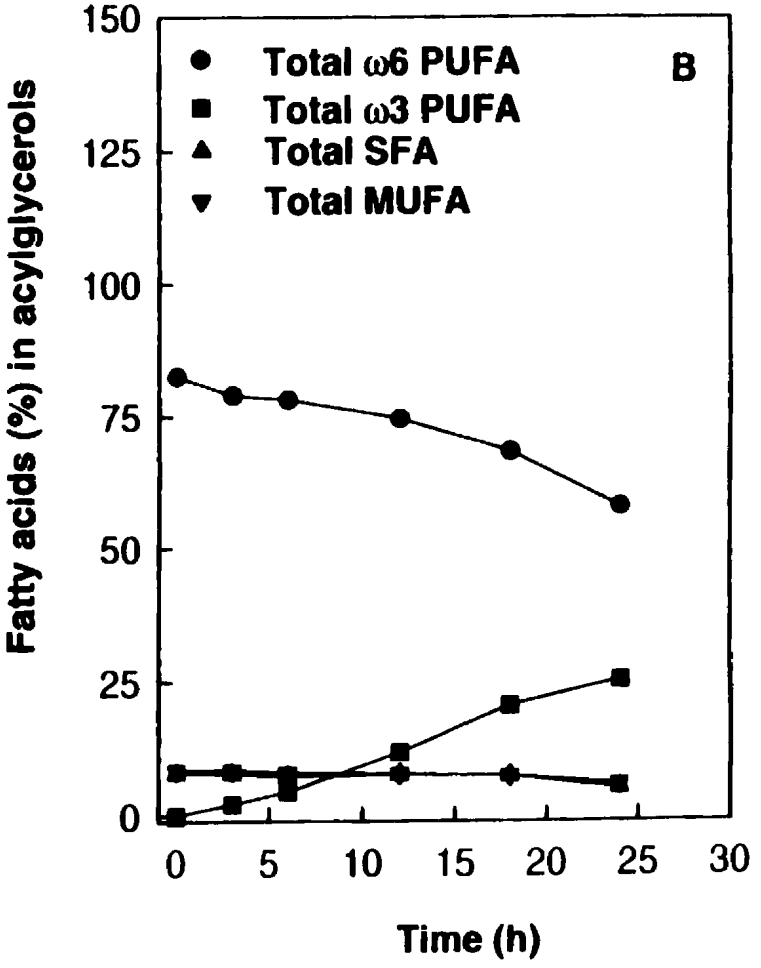

Total ω6 PUFA
Total ω3 PUFA
Total SFA
Total MUFA
B
Fatty acids (%) in acylglycerols
150
125
100
75
50
25
0
0
5
10
15
20
25
30
Time (h)

Table 4.5 Fatty acid composition of evening primrose oil (EPO) before and after lipase-catalyzed acidolysis with DHA[a]

Fatty acid	Duration of acidolysis (h)			
	0	12	18	24
14:0	0.04 ± 0.01	0.04 ± 0.02	ND[b]	ND[b]
16:0	6.17 ± 0.09	5.03 ± 0.10	4.73 ± 0.20	4.70 ± 0.50
16:1	0.04 ± 0.02	ND[b]	ND[b]	ND[b]
17:0	0.08 ± 0.01	ND[b]	ND[b]	ND[b]
18:0	1.75 ± 0.12	1.31 ± 0.51	0.74 ± 0.21	0.69 ± 0.07
18:1	8.65 ± 0.56	7.89 ± 0.83	5.12 ± 0.61	4.21 ± 0.50
18:2ω6	72.6 ± 0.91	63.7 ± 1.72	60.3 ± 1.52	54.3 ± 1.26
18:3ω6	9.12 ± 0.38	8.95 ± 0.65	7.92 ± 1.00	7.6 ± 0.45
18:3ω3	0.16 ± 0.03	0.15 ± 0.04	0.11 ± 0.01	ND[b]
20:0	0.34 ± 0.05	0.25 ± 0.03	ND[b]	ND[b]
20:1	0.29 ± 0.07	0.22 ± 0.02	0.21 ± 0.03	ND[b]
20:2	0.05 ± 0.05	ND[b]	ND[b]	ND[b]
22:0	0.14 ± 0.05	ND[b]	ND[b]	0.11 ± 0.04
22:1	0.12 ± 0.01	ND[b]	ND[b]	ND[b]
22:6ω3	ND[b]	11.7 ± 0.82	20.5 ± 0.75	25.2 ± 0.10
ω3/ω6 Ratio	0.001	0.16	0.30	0.40

[a]The reaction mixture contained 297 mg evening primrose oil, 120 mg DHA, 150 units of *Candida antarctica* and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm. Experimental results are means of triplicate determinations.

[b]Not detected

Table 4.6 Fatty acid composition of borage oil (BO) before and after lipase-catalyzed acidolysis with EPA[a]

Fatty acid	Duration of acidolysis (h)			
	0	12	18	24
14:0	0.07 ± 0.02	0.04 ± 0.01	0.05 ± 0.03	0.04 ± 0.01
16:0	9.60 ± 0.50	7.07 ± 0.52	6.49 ± 0.36	6.42 ± 0.56
16:1	0.20 ± 0.05	0.16 ± 0.05	0.12 ± 0.05	0.10 ± 0.08
17:0	0.10 ± 0.01	ND[b]	ND[b]	ND[b]
18:0	3.50 ± 0.03	3.12 ± 0.14	2.98 ± 0.54	2.59 ± 0.07
18:1	15.5 ± 0.70	14.0 ± 0.53	12.3 ± 0.25	12.0 ± 0.63
18:2ω6	37.8 ± 1.10	27.0 ± 0.92	26.6 ± 1.27	25.3 ± 0.52
18:3ω6	23.5 ± 0.85	17.0 ± 0.34	15.3 ± 0.55	15.2 ± 0.84
18:3ω3	0.21 ± 0.05	0.19 ± 0.07	0.17 ± 0.05	0.15 ± 0.02
20:0	0.22 ± 0.08	0.24 ± 0.08	0.23 ± 0.06	0.21 ± 0.05
20:1	4.20 ± 0.10	3.19 ± 0.21	3.11 ± 0.42	3.00 ± 0.53
20:2	0.21 ± 0.05	ND[b]	0.15 ± 0.08	0.14 ± 0.03
20:5ω3	ND[b]	7.98 ± 0.25	25.5 ± 1.40	26.8 ± 0.96
22:0	0.15 ± 0.07	0.10 ± 0.02	0.16 ± 0.07	0.15 ± 0.06
22:1	2.35 ± 0.12	2.19 ± 0.58	2.10 ± 0.23	2.10 ± 0.54
24:1	1.50 ± 0.10	1.37 ± 0.63	1.21 ± 0.11	1.10 ± 0.07
ω3/ω6 Ratio	0.003	0.18	0.61	0.66

[a]The reaction mixture contained 300 mg borage oil, 115 mg EPA, 150 units of *Pseudomonas sp.* and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm. Experimental results are means of triplicate determinations.

[b]Not detected

Table 4.7 Fatty acid composition of evening primrose oil (EPO) before and after lipase-catalyzed acidolysis with EPA[a]

Fatty acid	Duration of acidolysis (h)			
	0	12	18	24
14:0	0.04 ± 0.01	0.04 ± 0.03	0.02 ± 0.03	ND[b]
16:0	6.17 ± 0.09	5.82 ± 0.25	4.14 ± 0.51	4.02 ± 0.50
16:1	0.04 ± 0.02	0.04 ± 0.03	0.02 ± 0.01	ND[b]
17:0	0.08 ± 0.01	0.08 ± 0.04	ND[b]	ND[b]
18:0	1.75 ± 0.12	1.70 ± 0.38	1.16 ± 0.09	1.11 ± 0.08
18:1	8.65 ± 0.56	8.20 ± 0.54	5.62 ± 0.61	5.56 ± 0.41
18:2ω6	72.6 ± 0.91	65.3 ± 1.55	56.9 ± 1.71	55.6 ± 0.94
18:3ω6	9.12 ± 0.38	8.82 ± 0.82	7.34 ± 0.26	7.12 ± 0.55
18:3ω3	0.16 ± 0.03	0.10 ± 0.05	0.06 ± 0.04	ND[b]
20:0	0.34 ± 0.05	0.33 ± 0.08	0.21 ± 0.05	ND[b]
20:1	0.29 ± 0.07	0.22 ± 0.06	0.21 ± 0.04	ND[b]
20:2	0.05 ± 0.05	0.04 ± 0.07	0.04 ± 0.05	ND[b]
20:5ω3	ND[b]	10.3 ± 1.34	21.6 ± 1.47	25.2 ± 1.45
22:0	0.14 ± 0.05	0.11 ± 0.03	ND[b]	ND[b]
22:1	0.12 ± 0.01	0.10 ± 0.04	ND[b]	ND[b]
ω3/ω6 Ratio	0.001	0.14	0.34	0.40

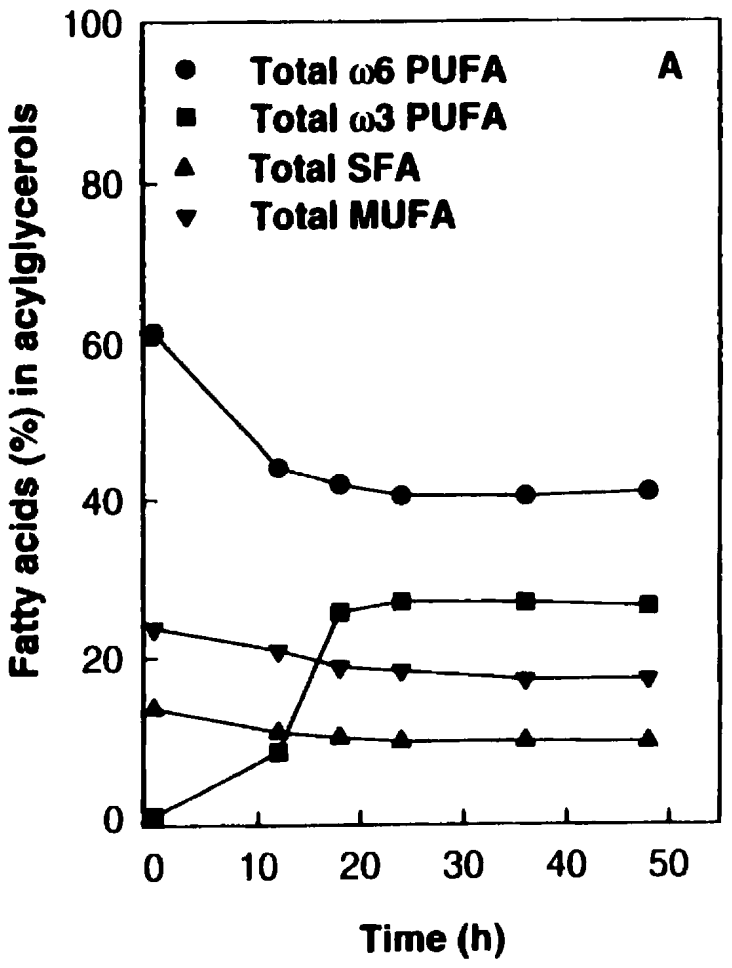
[a]The reaction mixture contained 297 mg evening primrose oil, 115 mg EPA, 150 units of *Pseudomonas sp.* and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm. Experimental results are means of triplicate determinations.

[b]Not detected

the fatty acid composition of BO using EPA ethyl ester in the presence of an immobilized nonspecific SP435 lipase from *Candida antarctica* as a biocatalyst. The highest incorporation (31%) of EPA was achieved with 20% (w/w) SP435 lipase. At a substrate mole ratio of 1:3, the corresponding ratio of ω3 to ω6 PUFA was 0.64. Huang *et al.* (1994) incorporated EPA ethyl ester into melon seed oil using two immobilized lipases, IM60 from *Mucor miehei* and SP435 from *Candida antarctica*. IM60 showed a higher incorporation of EPA (31.2% in 24 h) than SP435 lipase (24.0% in 24 h). Time courses of acidolysis reaction of BO and EPO with EPA by lipase from *Pseudomonas sp.* are illustrated in Figures 4.10A and 4.10B, respectively. The total content of monounsaturated, saturated and ω6 fatty acids (LA and GLA) decreased up to 24 h and then reached a plateau. On the other hand, the content of EPA incorporation increased up to 24 h. In BO, the amount of GLA was decreased from 23.5 to 15.2% in a 24 h period. Similarly, in EPO, the content of GLA was decreased from 9.12 to 7.12% in 24 h. After 24 h reaction with EPA, the content of LA in BO and EPO was decreased by 12.5 and 17.0%, respectively. The content of EPA incorporated into BO and EPO was 26.8 and 25.2%, respectively (in a 24 h period).

The lipase from *Pseudomonas sp.* also effectively incorporated EPA+DHA into BO and EPO. Tables 4.8 and 4.9 show the fatty acid composition of BO and EPO, before and after acidolysis with EPA and DHA by the nonspecific lipase from *Pseudomonas sp.* The amounts of monounsaturated, saturated and total ω6 fatty acids decreased due to EPA and DHA incorporation into the oils (Figure 4.11). After enzymatic modification of BO, LA and GLA decreased by 11.3 and 5.1%, respectively. The amounts of EPA and DHA

Figure 4.10 Changes in total contents of ω3, ω6, saturated and monounsaturated fatty acids of borage (A) and evening primrose oils (B) during lipase-catalysed acidolysis with EPA. The reaction mixture contained 297-300 mg oil, 115 mg EPA, 150 units of *Pseudomonas sp.* lipase preparation and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm.


Total ω6 PUFA
Total ω3 PUFA
Total SFA
Total MUFA
A
Fatty acids (%) in acylglycerols
Time (h)
0
20
40
60
80
100
0
10
20
30
40
50

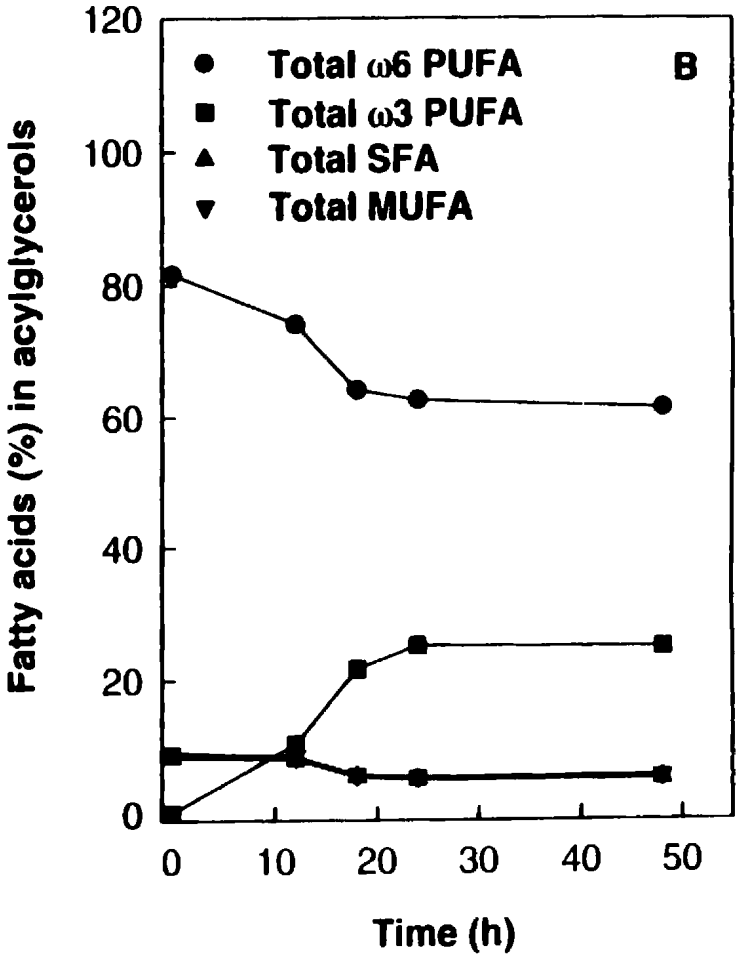

Total ω6 PUFA
Total ω3 PUFA
Total SFA
Total MUFA
B
Fatty acids (%) in acylglycerols
Time (h)
0
20
40
60
80
100
120
0
10
20
30
40
50

Table 4.8 Fatty acid composition of borage oil (BO) before and after lipase-catalyzed acidolysis with EPA + DHA[a]

Fatty acid	Duration of acidolysis (h)			
	0	12	18	24
14:0	0.07 ± 0.02	ND[b]	ND[b]	ND[b]
16:0	9.60 ± 0.50	4.86 ± 0.15	4.55 ± 0.57	4.00 ± 0.26
16:1	0.20 ± 0.05	ND[b]	ND[b]	ND[b]
17:0	0.10 ± 0.01	ND[b]	ND[b]	ND[b]
18:0	3.50 ± 0.03	2.21 ± 0.14	2.06 ± 0.15	2.00 ± 0.14
18:1	15.5 ± 0.70	11.0 ± 0.26	10.9 ± 0.20	10.7 ± 0.54
18:2ω6	37.8 ± 1.10	27.4 ± 1.20	26.6 ± 0.31	26.5 ± 0.26
18:3ω6	23.5 ± 0.85	18.9 ± 0.38	18.2 ± 0.20	18.4 ± 0.38
18:3ω3	0.21 ± 0.05	ND[b]	ND[b]	ND[b]
20:0	0.22 ± 0.08	ND[b]	ND[b]	ND[b]
20:1	4.20 ± 0.10	2.71 ± 0.52	2.62 ± 0.25	2.60 ± 0.07
20:2	0.21 ± 0.05	ND[b]	ND[b]	ND[b]
20:5ω3	ND[b]	21.5 ± 0.41	23.1 ± 0.42	23.1± 0.28
22:0	0.15 ± 0.07	ND[b]	ND[b]	ND[b]
22:1	2.35 ± 0.12	1.88 ± 0.27	1.50 ± 0.11	1.42 ± 0.58
24:1	1.50 ± 0.10	0.98 ± 0.13	0.24 ± 0.04	ND[b]
22:6ω3	ND[b]	8.45 ± 0.32	8.42 ± 0.36	8.66 ± 0.35
ω3/ω6 Ratio	0.003	0.65	0.70	0.71

[a]The reaction mixture contained 300 mg borage oil, 54 mg EPA, 58 mg DHA, 150 units of *Pseudomonas sp.* and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm. Experimental results are means of triplicate determinations.

[b]Not detected

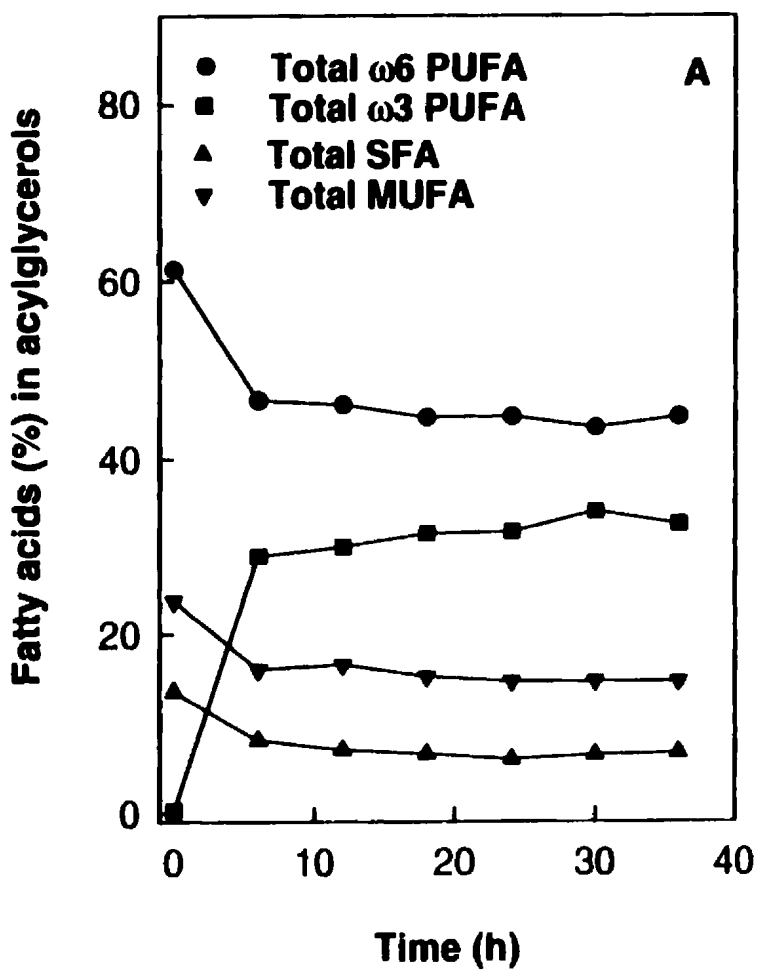
Table 4.9 Fatty acid composition of evening primrose oil (EPO) before and after lipase-catalyzed acidolysis with EPA + DHA[a]

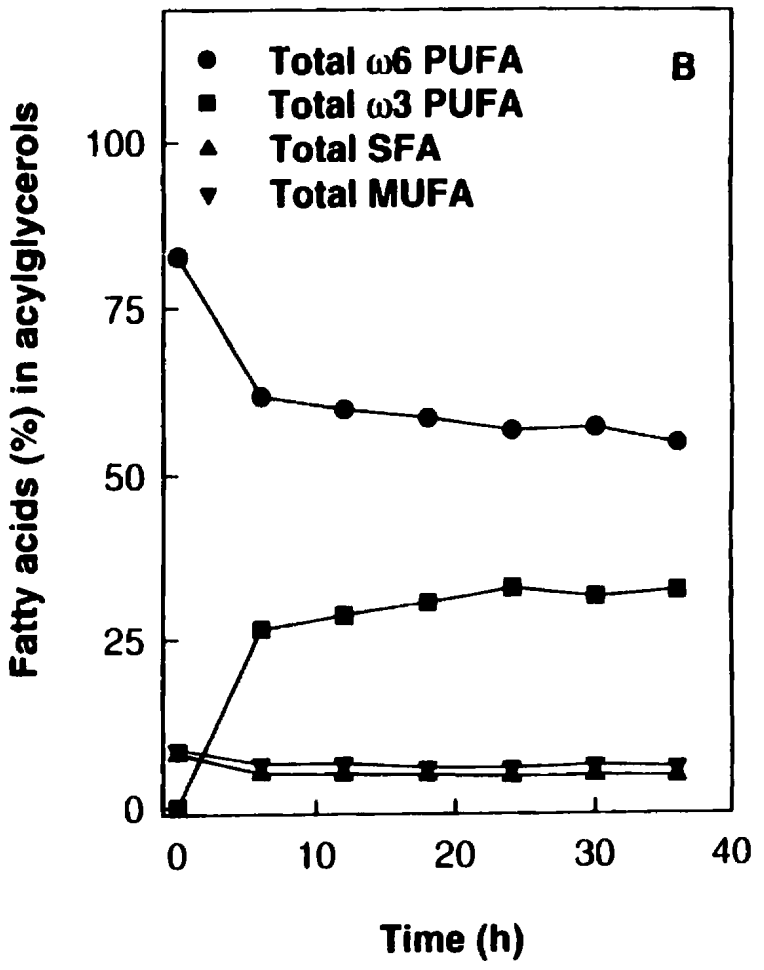
Fatty acid	Duration of acidolysis (h)			
	0	12	18	24
14:0	0.04 ± 0.01	ND[b]	ND[b]	ND[b]
16:0	6.17 ± 0.09	3.72 ± 0.24	3.70 ± 0.42	3.51 ± 0.27
16:1	0.04 ± 0.02	ND[b]	ND[b]	ND[b]
17:0	0.08 ± 0.01	ND[b]	ND[b]	ND[b]
18:0	1.75 ± 0.12	1.12 ± 0.04	1.07 ± 0.16	1.00 ± 0.05
18:1	8.65 ± 0.56	6.53 ± 0.27	5.84 ± 0.32	5.86 ± 0.41
18:2ω6	72.6 ± 0.91	52.0 ± 0.93	50.6 ± 1.07	49.4 ± 0.96
18:3ω6	9.12 ± 0.38	7.93 ± 0.43	8.06 ± 0.40	7.44 ± 0.35
18:3ω3	0.16 ± 0.03	ND[b]	ND[b]	ND[b]
20:0	0.34 ± 0.05	ND[b]	ND[b]	ND[b]
20:1	0.29 ± 0.07	ND[b]	ND[b]	ND[b]
20:2	0.05 ± 0.05	ND[b]	ND[b]	ND[b]
20:5ω3	ND[b]	21.7 ± 0.35	21.9 ± 0.58	23.5 ± 0.59
22:0	0.14 ± 0.05	ND[b]	ND[b]	ND[b]
22:1	0.12 ± 0.01	ND[b]	ND[b]	ND[b]
22:6ω3	ND[b]	6.92 ± 0.26	8.64 ± 0.42	9.21 ± 0.56
ω3/ω6 Ratio	0.001	0.48	0.52	0.58

[a]The reaction mixture contained 297 mg evening primrose oil, 54 mg EPA, 58 mg DHA, 150 units of *Pseudomonas sp.* and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm. Experimental results are means of triplicate determinations.

[b]Not detected

Figure 4.11 Changes in total contents of ω3, ω6, saturated and monounsaturated fatty acids of borage (A) and evening primrose oils (B) during lipase-catalysed acidolysis with EPA+DHA. The reaction mixture contained 297-300 mg oil, 54 mg EPA, 58 mg DHA, 150 units of *Pseudomonas sp.* lipase and 3 mL hexane. The reaction mixture was incubated at 37°C in an orbital shaking water bath at 250 rpm.


Total ω6 PUFA
Total ω3 PUFA
Total SFA
Total MUFA
A
Fatty acids (%) in acylglycerols
0
20
40
60
80
0
10
20
30
40
Time (h)


Total ω6 PUFA
Total ω3 PUFA
Total SFA
Total MUFA
B
Fatty acids (%) in acylglycerols
0
25
50
75
100
0
10
20
30
40
Time (h)

incorporated into BO were 23.1 and 8.66%, respectively. The ratio of ω3 PUFA/ω6 PUFA increased from 0.00 to 0.71 (after 24 h reaction). In the case of EPO, the content of LA was decreased drastically by 23.2%. However, the content of GLA was reduced only by 1.7%. The contents of EPA and DHA of the resultant modified EPO was 23.5 and 9.2%, respectively. The corresponding ω3 PUFA/ω6 PUFA increased from 0 to 0.58. Akoh *et al.* (1996) were also able to increase the ω3 PUFA (up to 43%) of EPO with a corresponding increase in the ω3/ω6 ratio from 0.01 to 0.60. Sridhar and Lakshminarayana (1992) were able to effectively modify groundnut oil by incorporating EPA and DHA using a *sn*-1,3-specific lipase from *Mucor miehei* as the biocatalyst. The resultant contents of EPA and DHA of the modified oil were 9.5 and 8.0%, respectively. Haraldsson *et al.* (1989) succeeded in preparing EPA-enriched TAG (40% EPA and 25% DHA) as well as DHA-enriched TAG of 48% DHA and 12% EPA using appropriate EPA or DHA concentrates, respectively. *Mucor miehei* lipase-catalysed interesterification of cod liver oil with ω3 PUFA concentrates was used in the latter study.

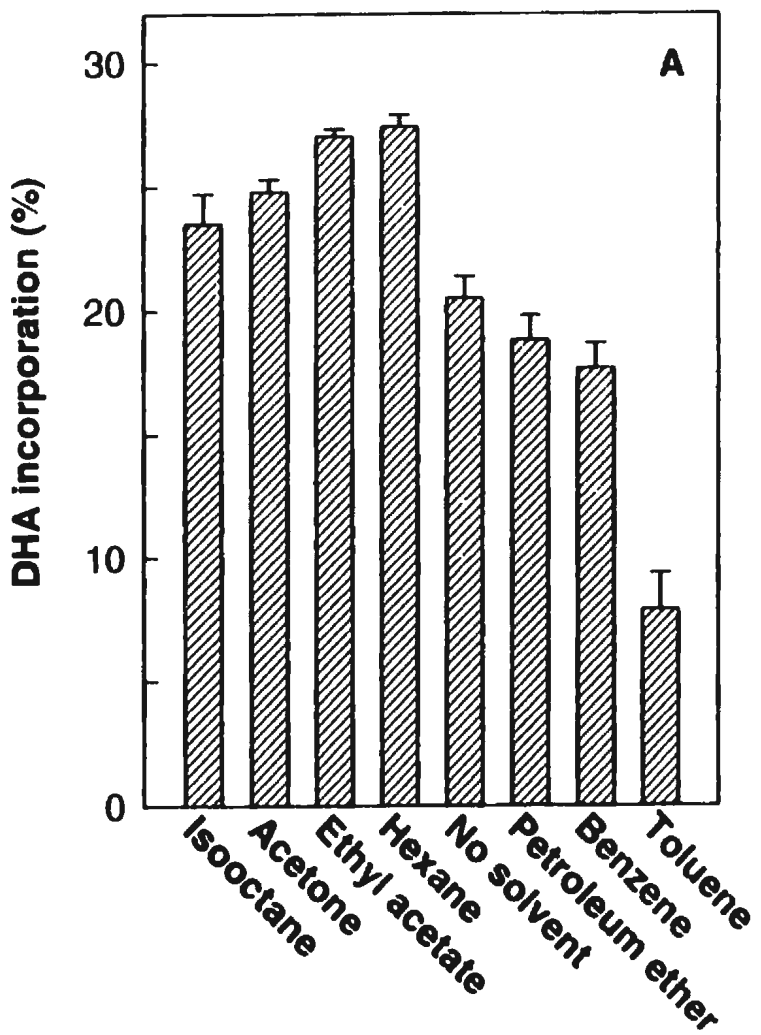
4.2.5 Effect of organic solvents

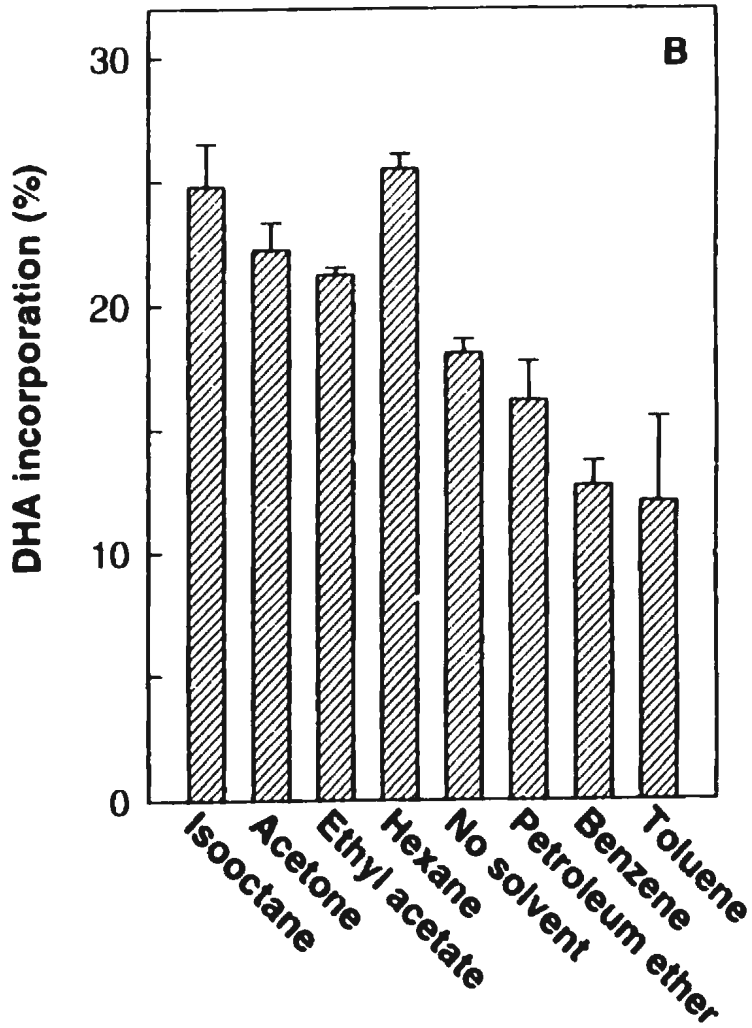
The use of organic solvents is necessary to carry out bioconversion of lipophilic compounds effectively (Kang and Rhee, 1989; He and Shahidi, 1997a). The ability of hydrophobic solvents to sustain and enhance enzyme catalysis has been demonstrated (Laane *et al.*, 1987; Kilbanov, 1989; Welsh *et al.*, 1989; Hirata *et al.*, 1990). From a mechanistic standpoint, the effect of organic solvents on enzyme catalysis is still debated

(Laane *et al.*, 1987; Hirata *et al.*, 1990). Organic solvents induce various physicochemical effects on enzyme molecules, and these effects differ depending upon the type of organic solvent and enzyme used (Li and ward, 1993). Conformational changes in enzymes, when suspended in organic solvents, have been reported to result in alteration of substrate specificity and affinity of substrates for enzymes (Dordick, 1989). The polarity of organic solvents affects lipase-catalysed reactions. Several variables are critical to enzyme activity in organic media. The nature of solvent is crucial for maintaining a layer of essential water around the enzyme molecules. The most hydrophobic solvents are best for this purpose as there is no incentive for the essential water to partition into the solvent, thus it remains on and around the enzyme (Laane *et al.*, 1987). It has been proposed that the log *P* (the logarithm of partition coefficient between water and octanol as a measure of polarity) parameter be used as a means of predicting the denaturing effect of a solvent on a biocatalytic system (Laane *et al.*,1985). Laane *et al.* (1985) concluded that solvents with log *P* values in the range of 2-4 may be used in an aqueous/organic solvent system. It has been reported that a certain level of water is necessary for the lipase-catalyzed reaction in organic media (Yamane, 1987), but when the amount of water reaches a critical level it promotes hydrolysis (Dordick, 1989). However, complete depletion of water from the system results in no biochemical reaction (Liu and Chi, 1997).

To select the most suitable solvent for acidolysis of BO and EPO with DHA by lipase from *Candida antarctica*, the effect of the presence of various organic solvents (isooctane; log *P* = 4.5, hexane; log *P* = 3.5, toluene; log *P* = 2.5, benzene; log *P* = 2.0,

Figure 4.12 Effect of different organic solvents on the incorporation of DHA into borage (A) and evening primrose oils (B). The reaction mixture contained 297-300 mg oil, 120 mg DHA, 150 units of *Candida antarctica* lipase preparation and 3 mL hexane. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm.


A
DHA incorporation (%)
30
20
10
0
Isooctane
Acetone
Ethyl acetate
Hexane
No solvent
Petroleum ether
Benzene
Toluene

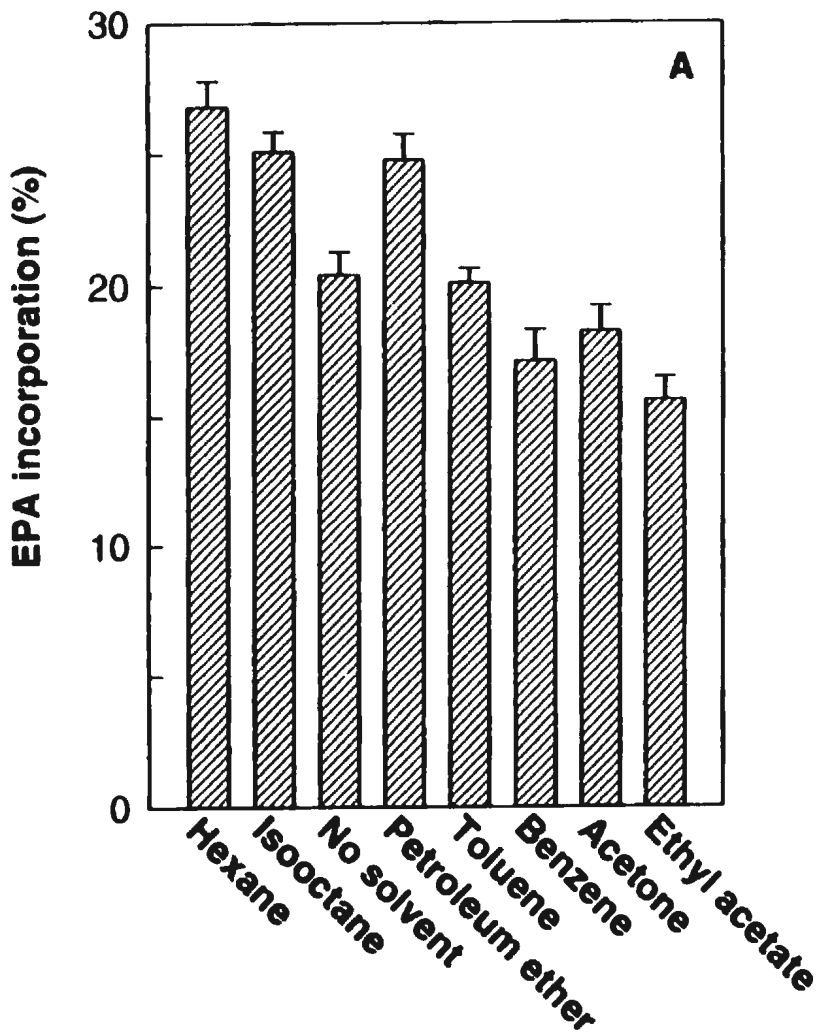

B
DHA incorporation (%)
30
20
10
0
Isooctane
Acetone
Ethyl acetate
Hexane
No solvent
Petroleum ether
Benzene
Toluene

acetone: log P = -0.23, ethyl acetate: log P = 0.68 and petroleum ether: no log P value was reported) in the reaction medium was examined (Figure 4.12). *n*-Hexane with a log P value of 3.5 was found to be the best solvent, affording a DHA incorporation of 27.4 and 25.5% in BO and EPO, respectively; thus the present results lend further support to the findings of Akoh *et al.* (1995,1996) who reported that *n*-hexane was highly effective in incorporating EPA and DHA into oils. Organic solvents such as *n*-hexane have several functions, including increasing the solubility of nonpolar substrates and shifting the reaction towards synthesis rather than hydrolysis (Klibanov, 1986). However, satisfactory incorporation of 20.5 and 18.1% of DHA in BO and EPO, respectively, was achieved in solvent-free systems. For food applications, the solvent-free reaction may be the method of choice. Claon and Akoh (1994) have demonstrated that this enzyme worked well in the solvent-free synthesis of primary terpene acetates.

Various solvents were also tested as reaction media to determine their effects on EPA incorporation into BO and EPO in the presence of *Pseudomonas sp.* lipase (Figure 4.13). The highest incorporation was achieved in *n*-hexane (26.8% in BO and 25.2% in EPO) while isooctane produced the second highest EPA incorporation. The solvent-free reactions gave acceptable incorporation of 20.4 and 18.6% of EPA into BO and EPO, respectively. It may then be concluded that *n*-hexane serves best for the acidolysis reaction of oils with EPA.

Figure 4.14 shows the effect of different organic solvents and no solvent as the reaction media for acidolysis reaction of oils with EPA and DHA catalyzed by

Figure 4.13 Effect of different organic solvents on the incorporation of EPA into borage (A) and evening primrose oils (B). The reaction mixture contained 297-300 mg oil, 115 mg EPA, 150 units of *Pseudomonas sp.* lipase preparation and 3 mL hexane. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm.


A
EPA incorporation (%)
30
20
10
0
Hexane
Isooctane
No solvent
Petroleum ether
Toluene
Benzene
Acetone
Ethyl acetate

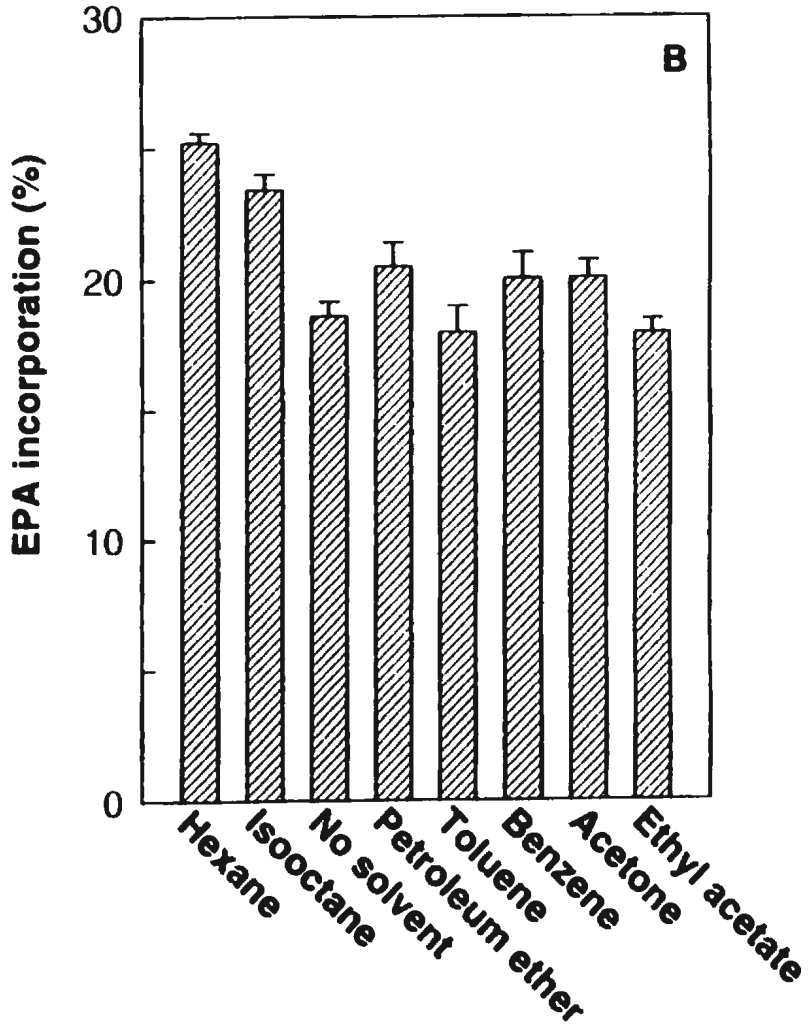
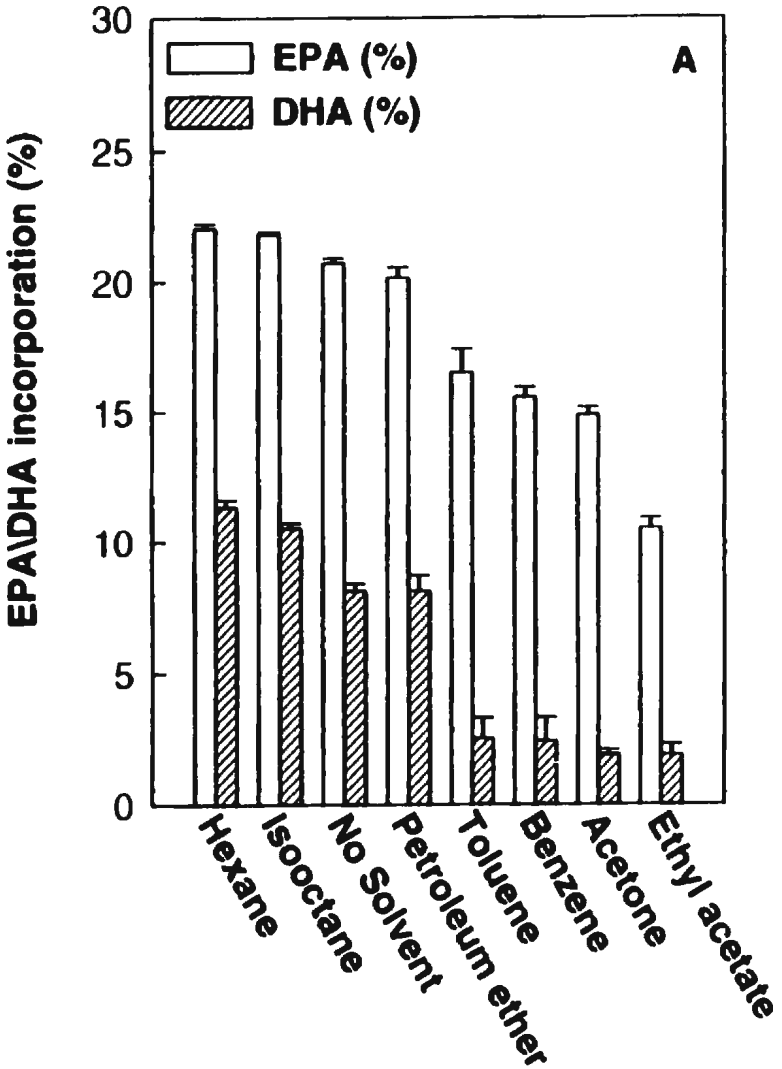
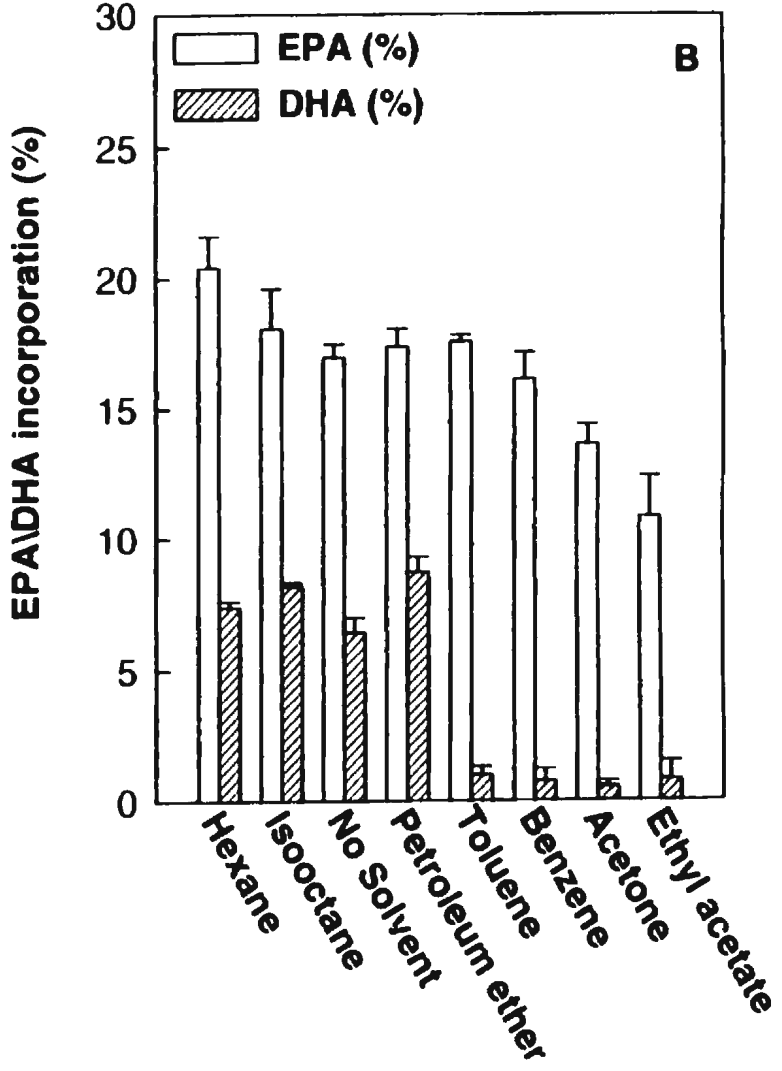

B
EPA incorporation (%)
30
20
10
0
Hexane
Isooctane
No solvent
Petroleum ether
Toluene
Benzene
Acetone
Ethyl acetate

Figure 4.14 Effect of different organic solvents on the incorporation of EPA+DHA into borage (A) and evening primrose oils (B). The reaction mixture contained 297-300 mg oil, 54 mg EPA, 58 mg DHA, 150 units of *Pseudomonas sp.* lipase preparation and 3 mL hexane. The reaction mixture was incubated at 37°C for 24 h in an orbital shaking water bath at 250 rpm.


EPA (%)
DHA (%)
A
EPA\DHA incorporation (%)
30
25
20
15
10
5
0
Hexane
Isooctane
No Solvent
Petroleum ether
Toluene
Benzene
Acetone
Ethyl acetate


EPA (%)
DHA (%)
B
EPA\DHA incorporation (%)
30
25
20
15
10
5
0
Hexane
Isooctane
No Solvent
Petroleum ether
Toluene
Benzene
Acetone
Ethyl acetate

Pseudomonas sp. lipase. The best solvent was *n*-hexane, which gave a total EPA+DHA incorporation of 33.3 and 27.8% in BO and EPO, respectively. Lipase from *Pseudomonas sp.* also performed well when the reaction was carried out in the absence of any organic solvent, giving rise to 28.8 and 23.4% EPA+DHA incorporation in BO and EPO, respectively. Akoh *et al.* (1995) tested five organic solvents with *Candida antarctica* lipase on EPA and DHA incorporation in trilinolein. They reported that the best solvents were *n*-hexane and isooctane, which gave total yields ranging from 63.2 to 76.2%.

4.2.6 Effect of mole ratio of substrates

The effect of BO/DHA mole ratio on DHA incorporation by *Candida antarctica* lipase is shown in Table 4.10. As the number of moles of DHA increased, its incorporation into BO was also increased, reaching a 1.4 fold increase at a BO/DHA mole ratio of 1:2 and remained constant up to a ratio of 1:3 which is the stoichiometric ratio of the TAG to FFA involved. As the mole ratio increased, the amount of GLA was decreased, but to a lesser extent than the amount of DHA incorporation (Table 4.10). Incorporation of DHA in BO was increased up to 39.7% at a mole ratio of 1:3 (Table 4.10).

Table 4.11 shows the effect of increasing substrate mole ratio (from 1:1 to 1:3) on DHA incorporation into EPO. As the number of moles of DHA was increased, incorporation increased. Incorporation of DHA in EPO was increased up to 37.4% at a mole ratio of 1:3. Successful incorporation of oleic acid into melon seed oil has been reported by Moussata and Akoh (1997) who showed that the use of non-specific lipase PS30 from

Table 4.10 Effect of mole ratio of substrates on DHA incorporation into borage oil (BO)[a]

Major Fatty acids	Mole ratio		
	1:1	1:2	1:3
16:0	7.20 ± 0.16	5.84 ± 0.23	5.41 ± 0.15
18:0	3.04 ± 0.13	2.44 ± 0.11	2.24 ± 0.14
18:1	12.3 ± 0.29	10.3 ± 0.35	10.0 ± 0.12
18:2ω6	26.4 ± 0.20	22.4 ± 0.63	21.8 ± 0.26
18:3ω6	16.3 ± 0.27	14.0 ± 0.16	13.4 ± 0.38
22:6ω3	27.0 ± 0.33	37.4 ± 0.84	39.7 ± 1.21

[a]Mole ratios of borage oil to DHA were varied from 1:1 to 1:3. Reactions were carried out at 37^{0}C for 24 h in an orbital shaking water bath at 250 rpm.

Table 4.11 Effect of mole ratio of substrates on DHA incorporation into evening primrose oil (EPO)[a]

Major Fatty acids	Mole ratio		
	1:1	1:2	1:3
16:0	4.53 ± 0.50	3.72 ± 0.17	2.81 ± 0.23
18:0	0.22 ± 0.07	0.21 ± 0.05	0.20 ± 0.03
18:1	4.81 ± 0.50	4.33 ± 0.24	3.84 ± 0.52
18:2ω6	53.3 ± 1.26	50.6 ± 1.60	47.3 ± 1.34
18:3ω6	7.61 ± 0.45	7.40 ± 0.21	6.21 ± 0.25
22:6ω3	27.2 ± 0.10	31.1 ± 1.80	37.4 ± 0.82

[a]Mole ratios of evening primrose oil to DHA were varied from 1:1 to 1:3. Reactions were carried out at 37^{0}C for 24 h in an orbital shaking water bath at 250 rpm.

Pseudomonas sp. resulted in a 53.4% incorporation of oleic acid into the oil at a mole ratio of 1:5.

The mole ratio of substrates to fatty acid also affected EPA incorporation (%) into BO and EPO (Tables 4.12 and 4.13), respectively. The EPA incorporation increased as its mole ratio in the reaction medium increased. The largest increase (by 10.6 and 9.9% in BO and EPO, respectively) occurred between the mole ratios of 1:1 and 1:2 and the smallest increase (by 2.5 and 2.3% in BO and EPO, respectively) occurred between the mole ratios of 1:2 and 1:3. However, EPA incorporation was maximum at the stoichiometric oil/EPA ratio of 1:3. At the mole ratio of 1:3, incorporation of EPA into BO and EPO was 39.9 and 37.4%, respectively. Similar results from the reaction of EPO with EPA ethyl ester was obtained with an immobilised lipase SP435 from *Candida antarctica* (Akoh *et al.*, 1996). The results so obtained indicated that between mole ratios of 1:1 and 1:2, the largest increase of 16% occurred, and that the smallest increase of 6% took place between mole ratios of 1:2 and 1:3. Previously, Akoh and Sista (1995) modified the fatty acid composition of BO with EPA using an immobilized nonspecific lipase from *Candida antarctica* as the biocatalyst. These authors were able to increase the EPA incorporation up to 28.1% at a mole ratio of 1:3.

Tables 4.14 and 4.15 show the effect of increasing the amount of substrates on EPA+DHA incorporation into BO and EPO, respectively. This study was performed by varying the mole ratio of oils to EPA and DHA, respectively, from 1:0.5:0.5 to 1:3:3. Initially, the incorporation of EPA+DHA increased with increasing mole ratio, up to a mole

Table 4.12 Effect of mole ratio of substrates on EPA incorporation into borage oil (BO)[a]

Major Fatty acids	Mole ratio		
	1:1	1:2	1:3
16:0	7.22 ± 0.17	5.84 ± 0.28	5.42 ± 0.15
18:0	3.00 ± 0.15	2.40 ± 0.16	2.23 ± 0.18
18:1	12.3 ± 0.24	10.3 ± 0.34	10.0 ± 0.14
18:2ω6	26.4 ± 0.28	22.4 ± 0.66	21.8 ± 0.25
18:3ω6	16.3 ± 0.29	14.0 ± 0.19	13.4 ± 0.33
20:5ω3	26.8 ± 0.30	37.4 ± 0.87	39.9 ± 1.20

[a]Mole ratios of borage oil to EPA were varied from 1:1 to 1:3. Reactions were carried out at 37^{0}C for 24 h in an orbital shaking water bath at 250 rpm.

Table 4.13 Effect of mole ratio of substrates on EPA incorporation into evening primrose oil (EPO)[a]

Major Fatty acids	Mole ratio		
	1:1	1:2	1:3
16:0	4.52 ± 0.50	3.72 ± 0.17	2.80 ± 0.23
18:0	0.22 ± 0.07	0.23 ± 0.05	0.20 ± 0.03
18:1	4.81 ± 0.50	4.3 ± 0.24	3.83 ± 0.52
18:2ω6	53.3 ± 1.26	50.6 ± 1.60	47.3 ± 1.34
18:3ω6	7.61 ± 0.45	7.44 ± 0.21	6.21 ± 0.25
20:5ω3	25.2 ± 0.10	35.1 ± 1.80	37.4 ± 0.82

[a]Mole ratios of evening primrose oil to EPA were varied from 1:1 to 1:3. Reactions were carried out at 37^{0}C for 24 h in an orbital shaking water bath at 250 rpm.

Table 4.14 Effect of mole ratio of substrates on EPA + DHA incorporation into borage oil (BO)[a]

Major fatty acids	Mole ratio			
	1:0.5:0.5	1:1:1	1:2:2	1:3:3
16:0	5.67 ± 0.51	4.41 ± 0.48	2.58 ± 0.22	3.20 ± 0.54
18:0	2.55 ± 0.26	2.20 ± 0.57	2.00 ± 0.86	1.63 ± 0.31
18:1	12.1 ± 0.57	10.4 ± 0.33	6.67 ± 1.00	7.23 ± 0.43
18:2ω6	24.3 ± 1.20	20.0 ± 1.59	11.9 ± 0.63	14.1 ± 0.92
18:3ω6	15.9 ± 1.11	13.4 ± 0.42	8.16 ± 0.14	9.40 ± 0.55
20:0	2.71 ± 0.51	2.50 ± 0.51	1.54 ± 0.81	1.04 ± 0.57
20:5ω3	21.8 ± 0.83	32.3 ± 0.83	48.5 ± 1.20	43.2 ± 1.10
22:1	2.04 ± 0.62	2.00 ± 0.09	1.62 ± 0.39	1.55 ± 0.09
22:6ω3	6.88 ± 0.69	9.17 ± 0.54	9.00 ± 1.42	9.99 ± 0.98

[a]Mole ratios of borage oil to EPA to DHA were varied from 1:0.5:0.5 to 1:3:3. Reactions were carried out at 37^{0}C for 24 h in an orbital shaking water bath at 250 rpm.

Table 4.15 Effect of mole ratio of substrates on EPA + DHA incorporation into evening primrose oil (EPO)[a]

Major fatty acids	Mole ratio			
	1:0.5:0.5	1:1:1	1:2:2	1:3:3
16:0	3.71 ± 0.25	3.03 ± 0.23	2.21 ± 0.31	2.03 ± 0.42
18:0	1.12 ± 0.33	1.01 ± 0.12	0.76 ± 0.24	0.68 ± 0.44
18:1	6.54 ± 0.98	5.78 ± 0.29	4.30 ± 0.51	3.60 ± 0.28
18:2ω6	47.9 ± 1.09	41.1 ± 1.00	29.9 ± 0.82	26.9 ± 1.27
18:3ω6	7.26 ± 1.07	6.10 ± 1.20	4.28 ± 0.32	3.91 ± 0.13
20:5ω3	23.7 ± 0.27	32.9 ± 0.97	47.9 ± 1.45	49.3 ± 1.07
22:6ω3	6.54 ± 0.99	5.99 ± 0.11	6.11 ± 0.98	3.30 ± 0.26

[a]Mole ratios of evening primrose oil to EPA to DHA were varied from 1:0.5:0.5 to 1:3:3. Reactions were carried out at 37^{0}C for 24 h in an orbital shaking water bath at 250 rpm.

ratio of 1:2:2, then remained constant between mole ratios of 1:2:2 and 1:3:3. The total incorporation of EPA and DHA of 57.5 and 54.0% was obtained at a mole ratio of 1:2:2 for BO and EPO, respectively. There is no economic advantage in using high substrate mole ratios, especially EPA and DHA. Depending on the level of EPA and DHA incorporation desired in the final product, the substrate mole ratio can be manipulated to achieve it. High EPA and DHA concentrations in the medium may indeed lead to reaction inhibition. Similar observations have been reported by Akoh and Yee (1997) in a reaction involving lipase IM 60 (*Rhizomucor miehei*)-catalysed synthesis of SL from high mole ratios of caprylic acid and triolein. Kuo and Parkin (1993) have also reported a concentration-dependent substrate preference for lipase-mediated acidolysis reactions. At concentrations above 300-500 μmol/g, reaction inhibition was observed for fatty acid substrates, and inhibition took place at lower concentrations for shorter-chain length fatty acids. Inhibition was primarily attributed to acidification of the microaqueous environment of the lipase. Desorption of water by the fatty acid substrate may be a secondary mode of inhibition (Kuo and Parkin, 1993).

4.3 Optimization of enzymatic acidolysis of borage (BO) and evening primrose oils (EPO) with ω3 PUFA (EPA and DHA)

4.3.1 Locating an appropriate experimental region for response surface methodology (RSM)

As mentioned in the previous section, the most important parameters that affect

incorporation of DHA, EPA and EPA+DHA into the oils are the amount of enzyme, reaction temperature and reaction time. Figures 4.15A and 4.16A show the effect of the amount of enzyme on DHA incorporation (%) into BO and EPO, respectively, while corresponding results for EPA incorporation in these oils are illustrated in Figures 4.17A and 4.18A. Similarly, Figures 4.19A and 4.20A display the effect of amount of enzyme on EPA+DHA incorporation (%) in BO and EPO, respectively. The response behaved as second order function of the independent variable (amount of enzyme) with higher correlation coefficients as compared with those of the linear models. As the amount of enzyme increased, the DHA, EPA and EPA+DHA incorporation also increased. In DHA-enriched oils, incorporation of DHA was maximum at a 100-150 enzyme (lipase from *Candida antarctica*) unit concentration. The design points selected for optimization of DHA-enriched oil production were 100, 150 and 200. On the other hand, in EPA-enriched oils, incorporation of EPA was maximum at 150-250 enzyme (lipase from *Pseudomonas sp.*) units. Therefore, the three design points selected for optimization of EPA-enriched oils were 150, 250 and 350. In EPA+DHA-enriched oils, incorporation of EPA+DHA was maximum at 250-350 enzyme (lipase from *Pseudomonas sp.*) units. Therefore, variable levels of 150, 250 and 350 were selected as the lower, middle and upper points, respectively.

The effects of incubation temperature and reaction time on DHA, EPA and EPA+DHA incorporation in oils were second order functions (Figures 4.15 - 4.20). In DHA-enriched oils, the DHA incorporation at 20°C was low, but increased with

Figure 4.15 Effects of amount of enzyme (A), incubation temperature (B) and reaction time (C) on DHA incorporation into borage oil. Each data point represents the average of three determinations. LM and QM denote linear and quadratic models, respectively.

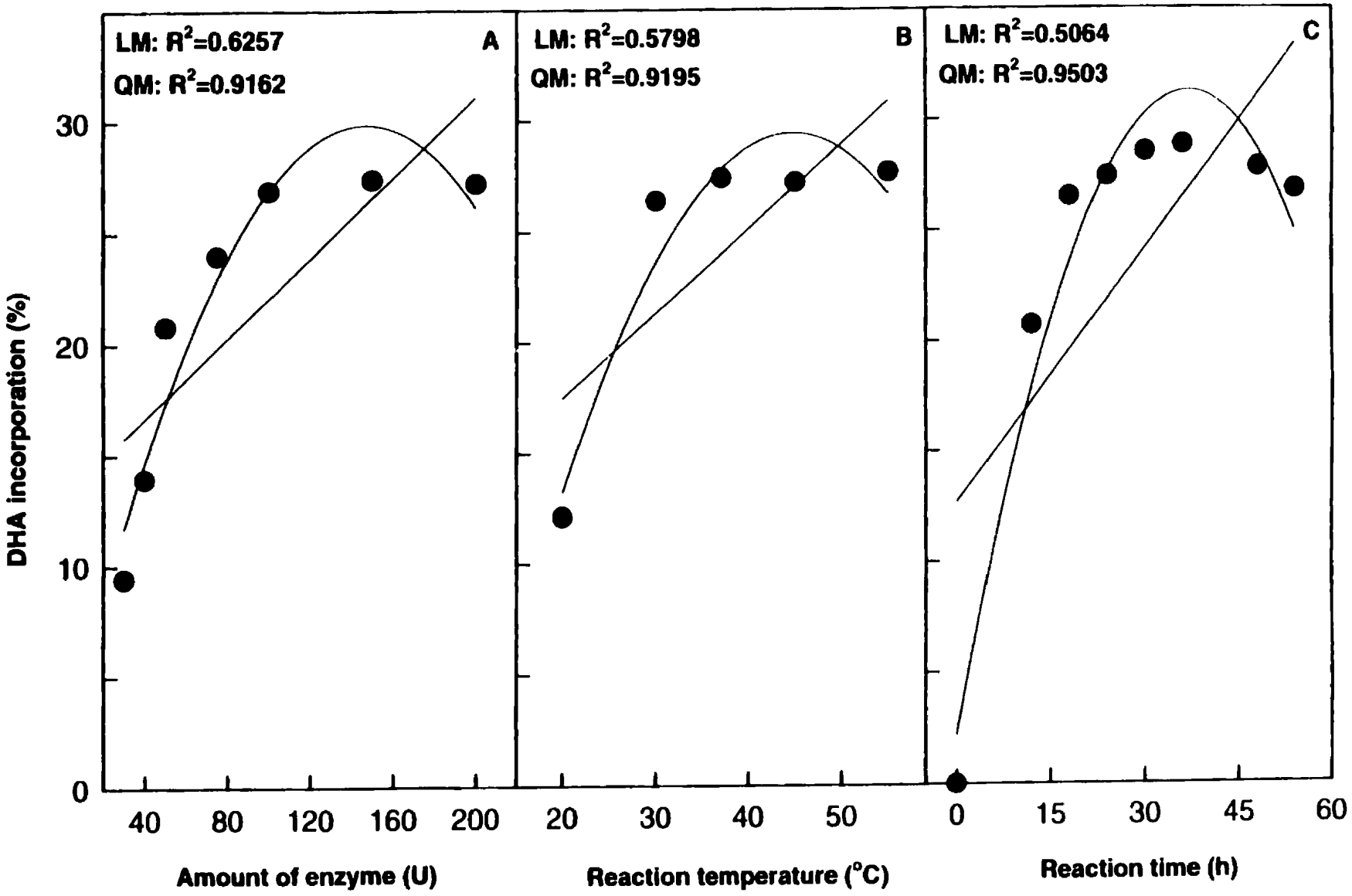

LM: R²=0.6257
QM: R²=0.9162
A
LM: R²=0.5798
QM: R²=0.9195
B
LM: R²=0.5064
QM: R²=0.9503
C
DHA incorporation (%)
0
10
20
30
40
80
120
160
200
Amount of enzyme (U)
20
30
40
50
Reaction temperature (°C)
0
15
30
45
60
Reaction time (h)

Figure 4.16 Effects of amount of enzyme (A), incubation temperature (B) and reaction time (C) on DHA incorporation into evening primrose oil. Each data point represents the average of three determinations. LM and QM denote linear and quadratic models, respectively.

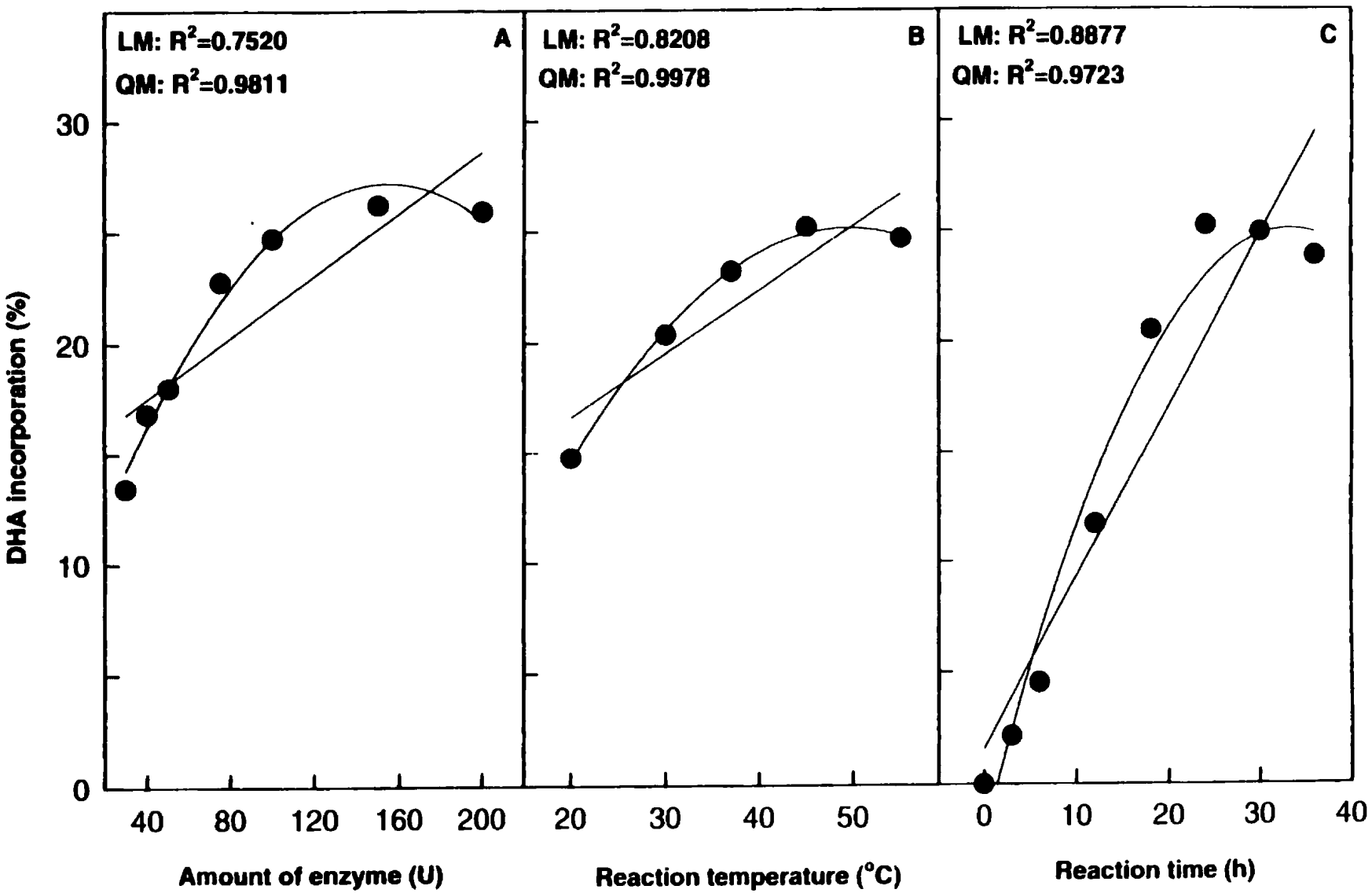

LM: R²=0.7520
QM: R²=0.9811
A
LM: R²=0.8208
QM: R²=0.9978
B
LM: R²=0.8877
QM: R²=0.9723
C
DHA incorporation (%)
0
10
20
30
40
80
120
160
200
20
30
40
50
0
10
20
30
40
Amount of enzyme (U)
Reaction temperature (°C)
Reaction time (h)

Figure 4.17 Effects of amount of enzyme (A), incubation temperature (B) and reaction time (C) on EPA incorporation into borage oil. Each data point represents the average of three determinations. LM and QM denote linear and quadratic models, respectively.

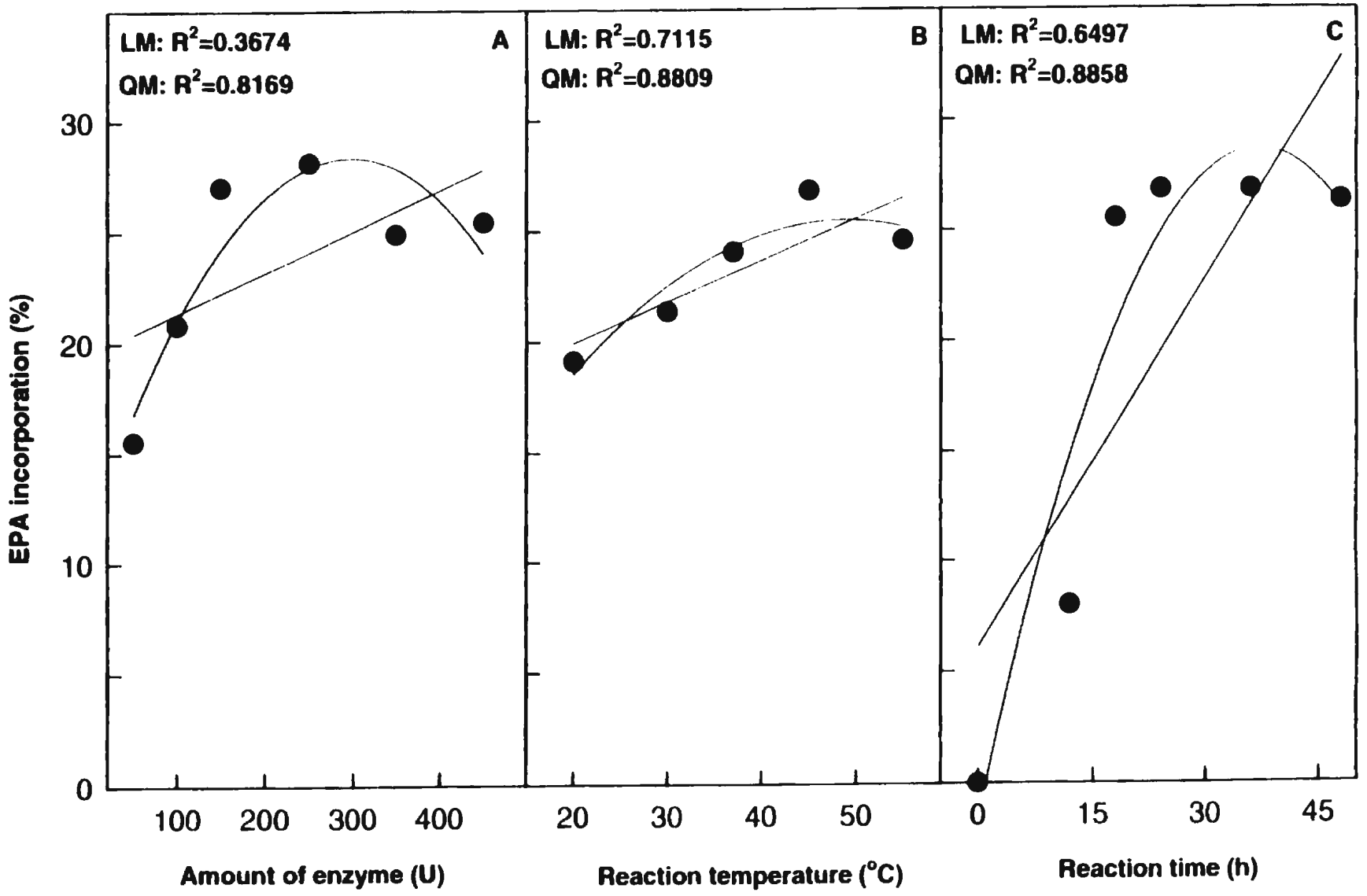

LM: R²=0.3674
QM: R²=0.8169
A
LM: R²=0.7115
QM: R²=0.8809
B
LM: R²=0.6497
QM: R²=0.8858
C
EPA incorporation (%)
0
10
20
30
100
200
300
400
Amount of enzyme (U)
20
30
40
50
Reaction temperature (°C)
0
15
30
45
Reaction time (h)

Figure 4.18 Effects of amount of enzyme (A), incubation temperature (B) and reaction time (C) on EPA incorporation into evening primrose oil. Each data point represents the average of three determinations. LM and QM denote linear and quadratic models, respectively.

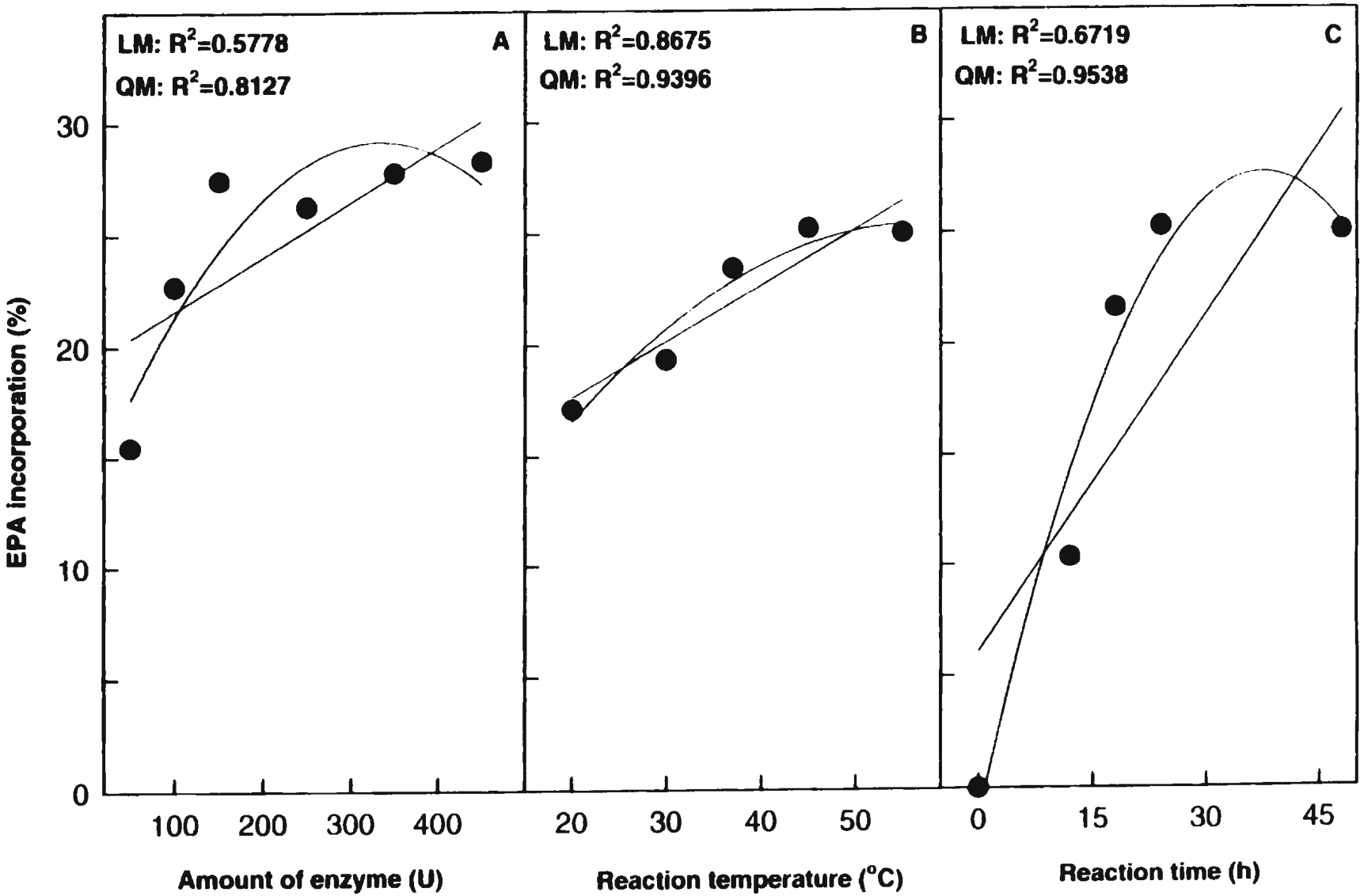

LM: R2=0.5778
QM: R2=0.8127
A
LM: R2=0.8675
QM: R2=0.9396
B
LM: R2=0.6719
QM: R2=0.9538
C
EPA incorporation (%)
0
10
20
30
100
200
300
400
Amount of enzyme (U)
20
30
40
50
Reaction temperature (°C)
0
15
30
45
Reaction time (h)

Figure 4.19 Effects of amount of enzyme (A), incubation temperature (B) and reaction time (C) on EPA+DHA incorporation into borage oil (BO). Each data point represents the average of three determinations. LM and QM denote linear and quadratic models, respectively.

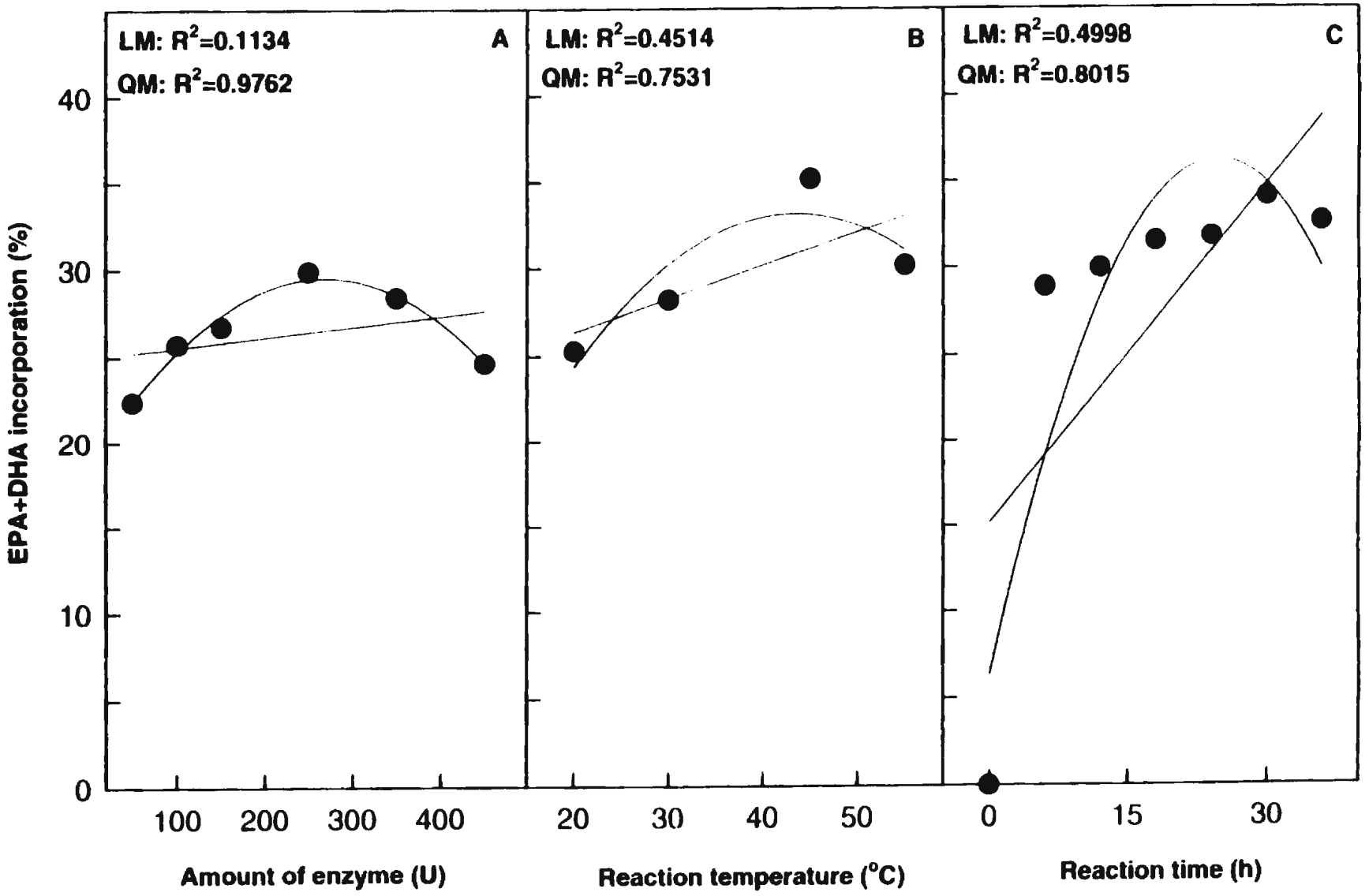
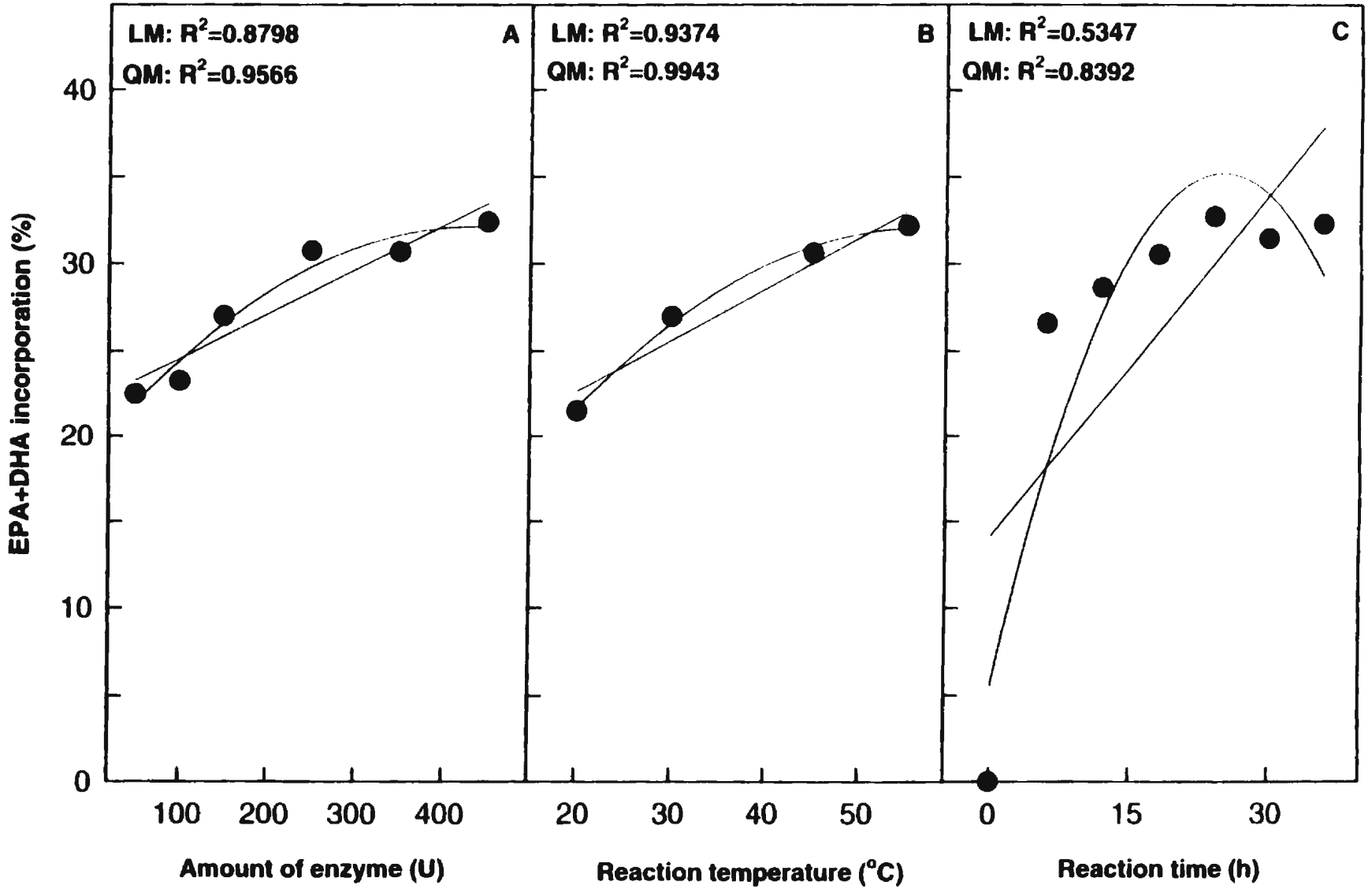

LM: R2=0.1134
QM: R2=0.9762
A
LM: R2=0.4514
QM: R2=0.7531
B
LM: R2=0.4998
QM: R2=0.8015
C
EPA+DHA incorporation (%)
0
10
20
30
40
100
200
300
400
Amount of enzyme (U)
20
30
40
50
Reaction temperature (°C)
0
15
30
Reaction time (h)

Figure 4.20 Effects of amount of enzyme (A), incubation temperature (B) and reaction time (C) on EPA+DHA incorporation into evening primrose oil (EPO). Each data point represents the average of three determinations. LM and QM denote linear and quadratic models, respectively.


LM: R²=0.8798
QM: R²=0.9566
A
LM: R²=0.9374
QM: R²=0.9943
B
LM: R²=0.5347
QM: R²=0.8392
C
EPA+DHA incorporation (%)
0
10
20
30
40
100
200
300
400
Amount of enzyme (U)
20
30
40
50
Reaction temperature (°C)
0
15
30
Reaction time (h)

increasing temperatures up to 50-60°C. Therefore, variable levels of 30, 45 and 60°C were chosen as the lower, middle and upper points, respectively. In EPA-enriched oils, incorporation of EPA was satisfactory at 20°C. However, higher temperatures (up to 60°C) were more suitable for the reaction. The variable levels of 20, 40 and 60 were chosen as design points. Incorporation of EPA+DHA in oils was low at 20°C, but increased with increasing temperatures up to 55°C. Therefore, design points of 30, 45 and 60 were selected for optimization experiments.

Results for time course study showed that as the reaction time increased, DHA incorporation increased, reaching a maximum and then declining with increasing reaction time. The three design points selected for the time course were 18, 24 and 30 h. In EPA-enriched oils and EPA+DHA-enriched oils, incorporation of PUFA was low at 6 h, but increased with time up to 24-30 h. Therefore, the three design points chosen were 6, 18 and 30 h.

4.3.2 Experimental design for response surface analysis

Response surface methodology (RSM) is an optimization technique which determines optimum process conditions by combining special experimental designs with modelling by first or second order polynomial equations in a sequential testing procedure. RSM tests several variables at one time, uses special experimental designs to cut the number of required determinations, and measures several effects by objective tests. The results of classical one-variable-at-a-time experiments do not reflect actual changes in the

environment as they ignore interactions between factors, which are in effect simultaneous. More sophisticated designs such as RSM can describe concomitant effects more fully and help in more accurate optimization of factors that affect the process and allow simultaneous solution of multivariate equations which specify the optimum yield for a specific set of factors through mathematical models.

The most effective lipase for the acidolysis of BO and EPO with DHA was *Candida antarctica* lipase. On the other hand, the most effective lipase for acidolysis of BO and EPO with EPA and EPA+DHA was lipase from *Pseudomonas sp.* However, there are many other factors that affect the product yields (incorporation of DHA, EPA and EPA+DHA) of lipase-catalyzed acidolysis of acylglycerols. These include temperature of the reaction medium, reaction time, substrate and enzyme concentrations, among others. Therefore, it is necessary to study these factors collectively to find the optimum reaction conditions to obtain maximum incorporation of DHA, EPA and EPA+DHA by the most effective enzymes mentioned above. For this study, reaction parameters such as the amount of enzyme (X_1), reaction temperature (X_2) and reaction time (X_3) were selected for optimization. The substrate mole ratios of 1:1 (oil/DHA and oil/EPA) and 1:0.5:0.5 (oil/EPA/DHA) were kept constant because incorporation of DHA, EPA and EPA+DHA was satisfactory at these mole ratios. Therefore, these ratios were selected for optimization by RSM. Enzyme concentration and reaction time are major factors that affect the cost of preparation of DHA, EPA and EPA+DHA-enriched oils *via* lipase catalyzed acidolysis. Furthermore, temperature of the reaction medium and also reaction time can be considered

important as they influence the oxidative status of prepared oils.

In this study, a face-centred cube design was employed (Tables 4.16, 4.17 and 4.18); the actual levels of variables used in each experimental run are shown in Tables 4.16, 4.17 and 4.18. This design was chosen over alternatives such as a rotatable design because it uses only three levels of each factor, whereas other central composite designs would require five levels of each (Mason *et al.*, 1989). Having three levels instead of five is considered desirable because it reduces the preparation time. Three replicates were taken at the design centre (0,0,0) so that total number of observations was n = 8 + 6 + 3 = 17. The main advantage of the design is that it enables one to study one or more variables simultaneously in a single experimental design of practicable size (Montgomery, 1991).

The data obtained for DHA, EPA and EPA+DHA incorporation (%) from the seventeen experimental points were used for statistical analysis to optimize the process variables: the amount of enzyme, reaction temperature and reaction time. Tables 4.16, 4.17 and 4.18 summarize the experimental data for response variables Y_1 and Y_2 (% DHA incorporation in BO and EPO, respectively), Y_3 and Y_4 (% EPA incorporation in BO and EPO, respectively), and Y_5 and Y_6 (% EPA+DHA incorporation in BO and EPO, respectively).

Multiple regression coefficients, obtained by employing a least squares technique to predict quadratic polynomial model for incorporation of DHA, EPA and EPA+DHA (%) in oils, are summarized in Table 4.19. Examination of these coefficients with the t-test indicated that in DHA-enriched BO, linear and quadratic terms of reaction time (X_3)

Table 4.16 Face-centred cube design arrangement and responses for the acidolysis of oils with DHA[a]

Run	Independent variables			Response[b]	
	Amount of enzyme	Temperature	Time	Y_1	Y_2
1	100* (-1)**	30 (-1)	18 (-1)	25.7	26.5
2	100 (-1)	30 (-1)	30 (+1)	29.6	29.5
3	100 (-1)	45 (0)	24 (0)	32.4	31.2
4	100 (-1)	60 (+1)	18 (-1)	28.0	28.1
5	100 (-1)	60 (+1)	30 (+1)	30.9	29.5
6	150 (0)	30 (-1)	24 (0)	32.2	30.8
7	150 (0)	45 (0)	18 (-1)	29.2	30.5
8	150 (0)	45 (0)	24 (0)	34.1	31.3
9	150 (0)	45 (0)	24 (0)	34.7	31.5
10	150 (0)	45 (0)	24 (0)	34.5	32.0
11	150 (0)	45 (0)	30 (+1)	32.4	31.6
12	150 (0)	60 (+1)	24 (0)	32.1	30.4
13	200 (+1)	30 (-1)	18 (-1)	28.6	29.5
14	200 (+1)	30 (-1)	30 (+1)	29.7	30.6
15	200 (+1)	45 (0)	24 (0)	32.5	30.9
16	200 (+1)	60 (+1)	18 (-1)	30.8	29.1
17	200 (+1)	60 (+1)	30 (+1)	31.4	29.4

[a]Nonrandomized; [b]Averages of duplicate determinations from different experiments: Y_1 = % DHA in BO; Y_2 = % DHA in EPO; *Uncoded variable levels; **Coded variable levels

Table 4.17 Face-centred cube design arrangement and responses for the acidolysis of oils with EPA [a]

Run	Independent variables			Response[b]	
	Amount of enzyme	Temperature	Time	Y_3	Y_4
1	150* (-1)**	20 (-1)	6 (-1)	10.8	7.27
2	150 (-1)	20 (-1)	30 (+1)	26.4	24.6
3	150 (-1)	40 (0)	18 (0)	28.7	26.2
4	150 (-1)	60 (+1)	6 (-1)	13.9	16.3
5	150 (-1)	60 (+1)	30 (+1)	22.8	25.3
6	250 (0)	20 (-1)	18 (0)	26.3	24.4
7	250 (0)	40 (0)	6 (-1)	23.7	21.9
8	250 (0)	40 (0)	18 (0)	32.9	32.5
9	250 (0)	40 (0)	18 (0)	32.6	31.0
10	250 (0)	40 (0)	18 (0)	33.6	31.8
11	250 (0)	40 (0)	30 (+1)	36.4	34.4
12	250 (0)	60 (+1)	18 (0)	27.7	32.4
13	350 (+1)	20 (-1)	6 (-1)	17.0	12.2
14	350 (+1)	20 (-1)	30 (+1)	29.2	28.4
15	350 (+1)	40 (0)	18 (0)	33.7	33.9
16	350 (+1)	60 (+1)	6 (-1)	24.9	23.4
17	350 (+1)	60 (+1)	30 (+1)	29.0	29.6

[a]Nonrandomized; [b]Averages of duplicate determinations from different experiments: Y_3 = % EPA in BO; Y_4 = % EPA in EPO; *Uncoded variable levels; **Coded variable levels

Table 4.18 Face-centred cube design arrangement and responses for the acidolysis of oils with EPA+DHA[a]

Run	Independent variables			Response[b]	
	Amount of enzyme	Temperature	Time	Y_5	Y_6
1	150* (-1)**	30 (-1)	6 (-1)	21.7	23.2
2	150 (-1)	30 (-1)	30 (+1)	28.9	28.8
3	150 (-1)	45 (0)	18 (0)	30.1	30.2
4	150 (-1)	60 (+1)	6 (-1)	20.5	23.1
5	150 (-1)	60 (+1)	30 (+1)	27.9	28.3
6	250 (0)	30 (-1)	18 (0)	32.0	30.2
7	250 (0)	45 (0)	6 (-1)	29.8	29.5
8	250 (0)	45 (0)	18 (0)	35.3	33.3
9	250 (0)	45 (0)	18 (0)	34.0	33.7
10	250 (0)	45 (0)	18 (0)	33.8	34.6
11	250 (0)	45 (0)	30 (+1)	36.4	33.6
12	250 (0)	60 (+1)	18 (0)	29.3	28.1
13	350 (+1)	30 (-1)	6 (-1)	29.5	29.0
14	350 (+1)	30 (-1)	30 (+1)	31.7	30.3
15	350 (+1)	45 (0)	18 (0)	34.8	33.8
16	350 (+1)	60 (+1)	6 (-1)	25.3	25.4
17	350 (+1)	60 (+1)	30 (+1)	27.7	28.6

[a]Nonrandomized; [b]Averages of duplicate determinations from different experiments; Y_5 = % EPA+DHA in BO; Y_6 = % EPA+DHA in EPO; *Uncoded variable levels; **Coded variable levels

Table 4.19 Regression coefficients of predicted quadratic polynomial model for response (Y)

Coefficients[a]	BO, DHA (%) (Y_1)	EPO, DHA (%) (Y_2)	BO, EPA (%) (Y_3)	EPO, EPA (%) (Y_4)	BO, EPA+DHA (%) (Y_5)	EPO,EPA+DHA (%) (Y_6)
β_0	-43.5149***	-13.1619**	-33.192***	-39.9979***	-34.3671***	-21.7043***
Linear						
β_1	0.1668*	0.1519***	0.1262***	0.1572***	0.1731***	0.0945***
β_2	0.5628*	0.6322***	1.2941***	1.1224***	1.6482***	1.5779***
β_3	3.9774***	1.4356***	1.7631***	2.1170***	0.8601***	0.6489***
Quadratic						
β_{11}	-0.0004	-0.0003**	-0.0002***	-0.0003***	-0.0002***	-0.0001
β_{22}	-0.0052*	-0.0053***	-0.0155***	-0.0105***	-0.0179***	-0.0172***
β_{33}	-0.0702***	-0.0205**	-0.0219***	-0.0311***	-0.0110***	-0.0103**
Interactions						
β_{12}	0.00005	-0.0005**	0.0005***	0.0002***	-0.0005**	-0.0004
β_{13}	-0.0021*	-0.0013*	-0.0008***	-0.0004***	-0.0010***	-0.0007**
β_{23}	-0.0020	-0.0033	-0.0077***	-0.0095***	-0.0003	-0.0010
β_{123}	–	–	–	–	–	–
R^2	0.94	0.95	0.99	0.99	0.99	0.98
CV%	2.92	1.53	2.94	4.29	2.25	2.47

[a]Coefficients refer to the general model; R^2 = coefficient of determination; CV%= coefficient of variation
*Significant at 10% level; **Significant 5 % level; ***Significant 1 % level

were highly significant ($p \leq 0.01$). However, linear terms of the amount of enzyme (X_1) and reaction temperature (X_2) and quadratic term of reaction temperature (X_2) were significant at $p \leq 0.1$ level. There was also a significant interaction ($p \leq 0.1$) between the amount of enzyme (X_1) and reaction time (X_3). However, interactions of the amount of enzyme (X_1) and reaction temperature (X_2) and also reaction temperature (X_2) and time (X_3) were not significant ($p > 0.1$). In DHA-enriched EPO, all linear and quadratic terms were significant at $p \leq 0.05$. There were significant interactions ($p \leq 0.1$) between the amount of enzyme (X_1) and reaction temperature (X_2) and also between the amount of enzyme (X_1) and reaction time (X_3). The coefficients obtained for EPA-enriched BO and EPO showed that all linear, quadratic and interaction terms were highly significant at $p \leq 0.01$. In EPA+DHA-enriched BO, all linear and quadratic terms were highly significant ($p \leq 0.01$). There were significant interactions ($p \leq 0.05$) between the amount of enzyme (X_1) and incubation temperature (X_2) and also between the amount of enzyme (X_1) and incubation time (X_3). On the other hand, in EPA+DHA-enriched EPO, all linear terms had the greatest effect on EPA+DHA incorporation as they were highly significant ($p \leq 0.01$). Among the quadratic terms, reaction temperature and time were significant at $p \leq 0.05$. There was also a significant interaction ($p \leq 0.05$) between the amount of enzyme (X_1) and incubation time (X_3). Therefore, these results suggest that linear, quadratic and interaction effects of the amount of enzyme, reaction temperature and reaction time are the primary determining factors for EPA and DHA incorporation into BO and EPO.

The contribution of linear and quadratic terms to the models in DHA-enriched oils

was 22-25 and 63-65%, respectively. Corresponding values in EPA-enriched oils were 49-57 and 37-46%, respectively. The linear and quadratic terms for Y_5 (EPA+DHA-enriched BO) contributed 38.0 and 55.0% to the model, respectively. Contribution of linear and quadratic terms of Y_6 (EPA+DHA-enriched EPO) to the model were 32.0 and 62.0%, respectively. The coefficient of determinations for Y_1 and Y_2 (R^2 = 0.94-0.95) imply that 94-95% of the variations could be explained by the fitted model. The coefficient of determinations for Y_3, Y_4, Y_5 and Y_6 were 0.99, 0.99, 0.99 and 0.98, respectively. Coefficient of variations (CV) for all models of less than 5% indicated that the models were reproducible (Table 4.19).

The coefficients of independent variables, the amount of enzyme (X_1), reaction temperature (X_2) and reaction time (X_3), determined for quadratic polynomial models for DHA incorporation in BO (Y_1) and EPO (Y_2) are given below:

$$Y_1 = -43.515 + 0.167X_1 + 0.563X_2 + 3.98X_3 - 0.00035X_1^2 - 0.0052X_2^2 - 0.0702X_3^2 + 0.00005X_1X_2 - 0.0021X_2X_3 - 0.0021X_1X_3$$

$$Y_2 = -13.162 + 0.152X_1 + 0.632X_2 + 1.436X_3 - 0.0003X_1^2 - 0.0053X_2^2 - 0.0204X_3^2 - 0.0005X_1X_2 - 0.0033X_2X_3 - 0.0013X_1X_3$$

The quadratic polynomial models for EPA incorporation in BO (Y_3) and EPO (Y_4) were:

$$Y_3 = -33.192 + 0.126X_1 + 1.294X_2 + 1.763X_3 - 0.0002X_1^2 - 0.016X_2^2 - 0.022X_3^2 + 0.0005X_1X_2 - 0.0078X_2X_3 - 0.0009X_1X_3$$

$$Y_4 = -39.998 + 0.157X_1 + 1.122X_2 + 2.117X_3 - 0.0003X_1^2 - 0.011X_2^2 - 0.031X_3^2 + 0.0002X_1X_2 - 0.0096X_2X_3 - 0.0004X_1X_3$$

The quadratic polynomial models for EPA+DHA incorporation in BO (Y_5) and EPO (Y_6) are given below:

$$Y_5 = -34.3671 + 0.1731X_1 + 1.6482X_2 + 0.8601X_3 - 0.0002X_1^2 - 0.0179X_2^2 - 0.0110X_3^2 - 0.0005X_1X_2 - 0.0003X_2X_3 + 0.0010X_1X_3$$

$$Y_6 = -21.7043 + 0.0945X_1 + 1.5779X_2 + 0.6489X_3 - 0.0001X_1^2 - 0.0172X_2^2 - 0.0103X_3^2 - 0.0004X_1X_2 - 0.0010X_2X_3 - 0.0007X_1X_3$$

Canonical analysis was performed on the predicted quadratic polynomial models to examine the overall shape of the response surface curves and used to characterize the nature of the stationary points. This is a mathematical approach used to locate the stationary point of the response surfaces and to determine whether it represents a maximum, minimum or a saddle point (Montgomery, 1991). Thus, to determine the nature of the stationary points, canonical analysis was carried out on the second order polynomial

models. The canonical forms of the equations for DHA-enriched BO (Y_1), DHA-enriched EPO (Y_2), EPA-enriched BO (Y_3), EPA-enriched EPO (Y_4), EPA+DHA-enriched BO (Y_5) and EPA+DHA-enriched EPO (Y_6) were:

$Y_1 = 34.12 - 0.81W_1^2 - 1.18W_2^2 - 2.59W_3^2$

$Y_2 = 31.91 - 0.55W_1^2 - 0.78W_2^2 - 1.34W_3^2$

$Y_3 = 36.10 - 1.65W_1^2 - 3.22W_2^2 - 6.49W_3^2$

$Y_4 = 35.11 - 2.46W_1^2 - 3.31W_2^2 - 5.50W_3^2$

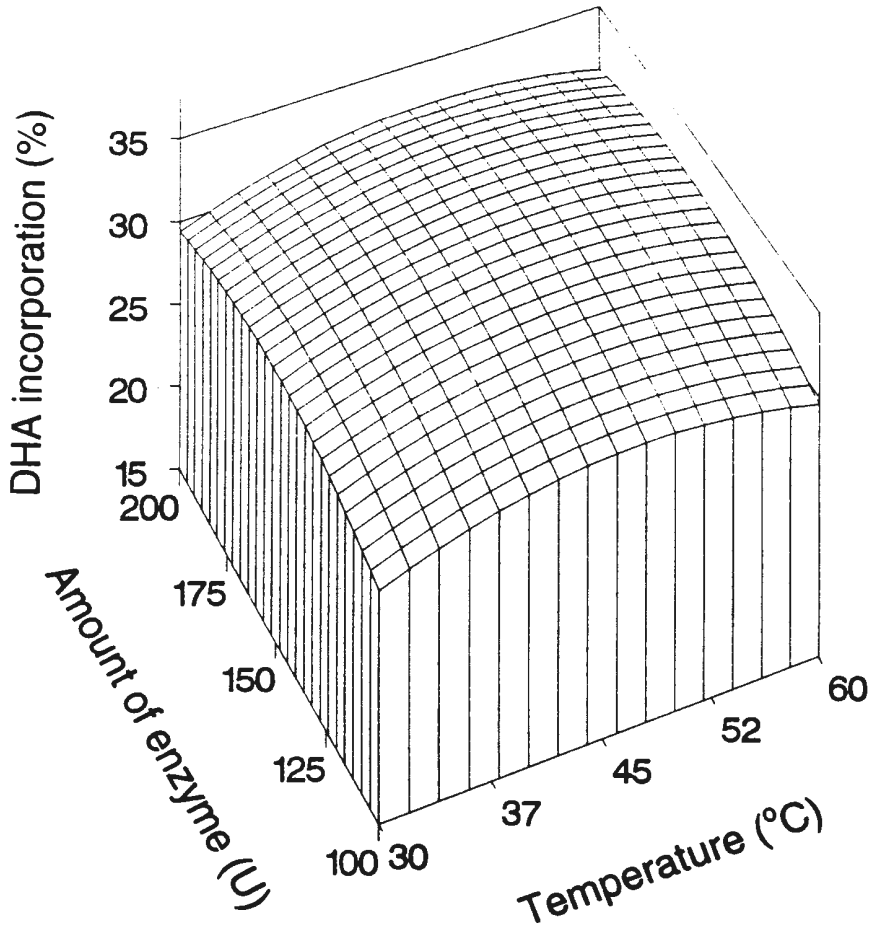
$Y_5 = 35.85 - 1.19W_1^2 - 2.56W_2^2 - 4.12W_3^2$

$Y_6 = 34.30 - 0.77W_1^2 - 1.71W_2^2 - 3.91W_3^2$

where W_1, W_2 and W_3 are the axes of the response surface. It is evident that all the eigenvalues were negative indicating that the stationary point was a maximum (Montgomery, 1991) for Y_1, Y_2, Y_3, Y_4, Y_5 and Y_6.

The linear, quadratic and cross product terms in the second order polynomials were used to generate three dimensional response surface graphs and two-dimensional contour plots (Figures 4.21 - 4.26) of DHA, EPA and EPA+DHA incorporation into the oils. While these three dimensional graphs can assist the researcher to determine the direction to take to increase a desired response and graphically show the nature of the fitted surface as maximum, minimum or a saddle point, it is difficult to determine levels of variables to afford a specific DHA, EPA or EPA+DHA incorporation (%) from such graphs. This can be more readily achieved from contour plots of the same variables,

Figure 4.21 Three-dimensional response surface and two-dimensional contour plot illustrating the effects of amount of enzyme and reaction temperature on the predicted DHA incorporation (%) in borage oil (BO)


DHA incorporation (%)
35
30
25
20
15
200
175
150
125
100
Amount of enzyme (U)
30
37
45
52
60
Temperature (°C)

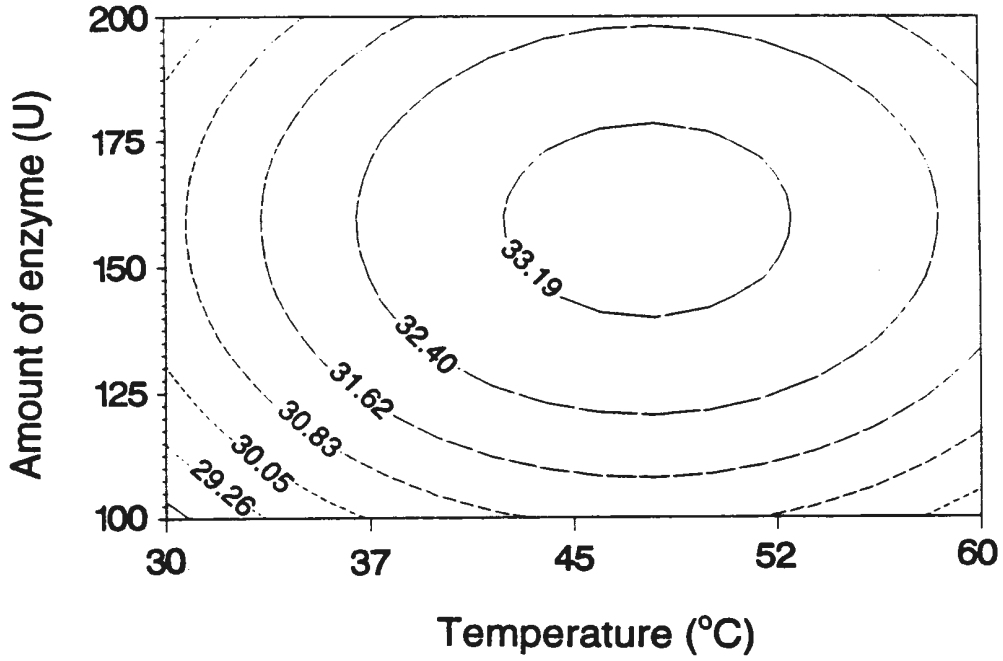
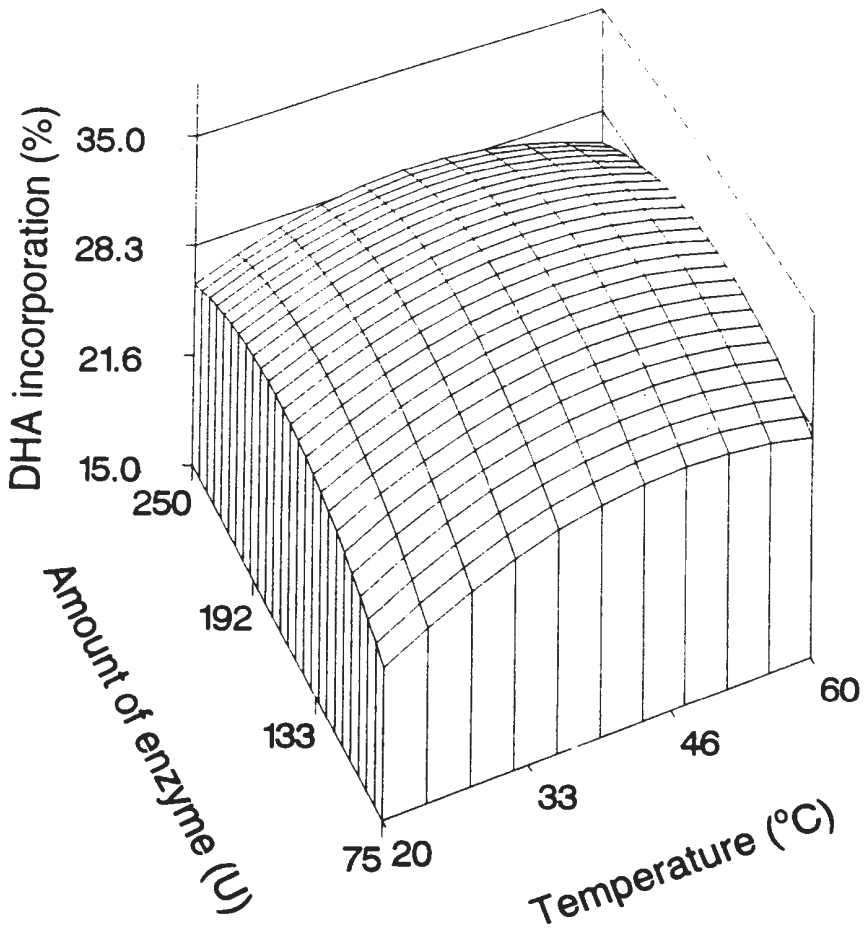

Amount of enzyme (U)
200
175
150
125
100
33.19
32.40
31.62
30.83
30.05
29.26
30
37
45
52
60
Temperature (°C)

Figure 4.22 Three-dimensional response surface and two-dimensional contour plot illustrating the effects of amount of enzyme and reaction temperature on the predicted DHA incorporation (%) in evening primrose oil (EPO)


DHA incorporation (%)
35.0
28.3
21.6
15.0
250
192
133
75
Amount of enzyme (U)
20
33
46
60
Temperature (°C)

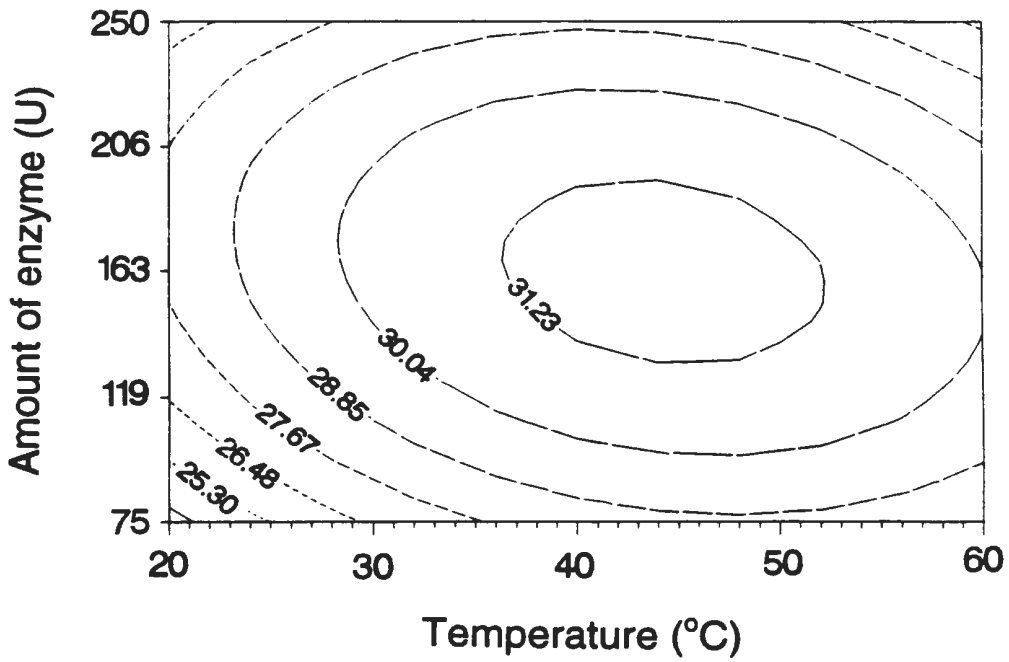
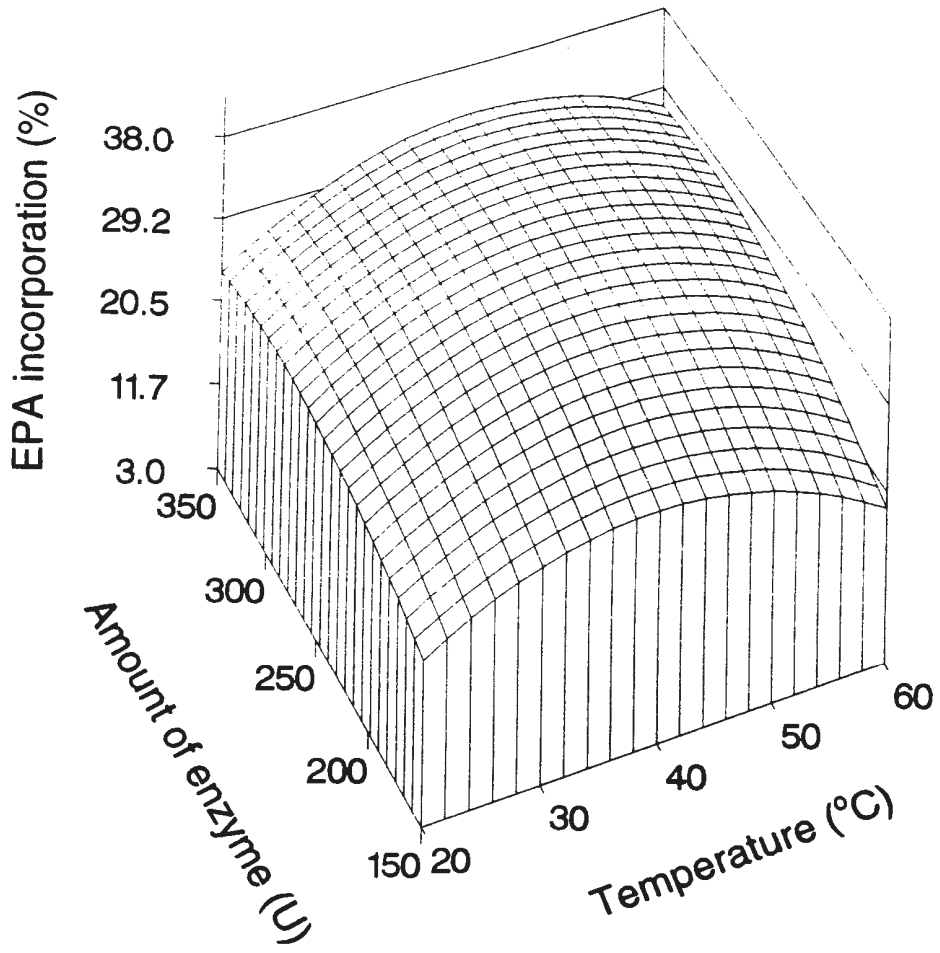

Amount of enzyme (U)
250
206
163
119
75
31.23
30.04
28.85
27.67
26.48
25.30
20
30
40
50
60
Temperature (°C)

Figure 4.23 Three-dimensional response surface and two-dimensional contour plot illustrating the effects of amount of enzyme and reaction temperature on the predicted EPA incorporation (%) in borage oil (BO)


EPA incorporation (%)
38.0
29.2
20.5
11.7
3.0
350
300
250
200
150
Amount of enzyme (U)
20
30
40
50
60
Temperature (°C)

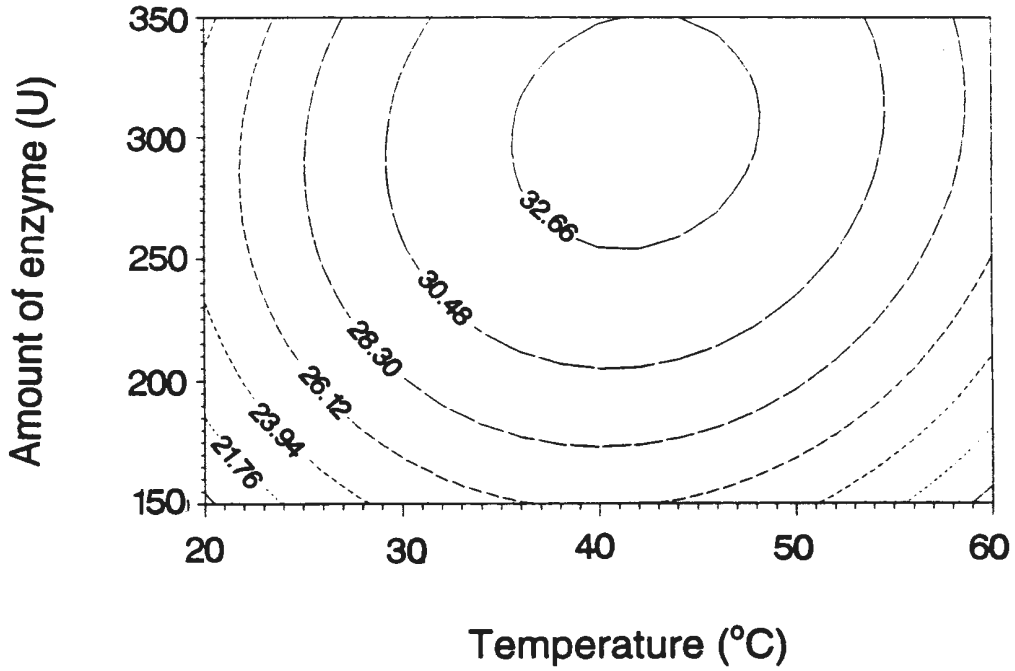
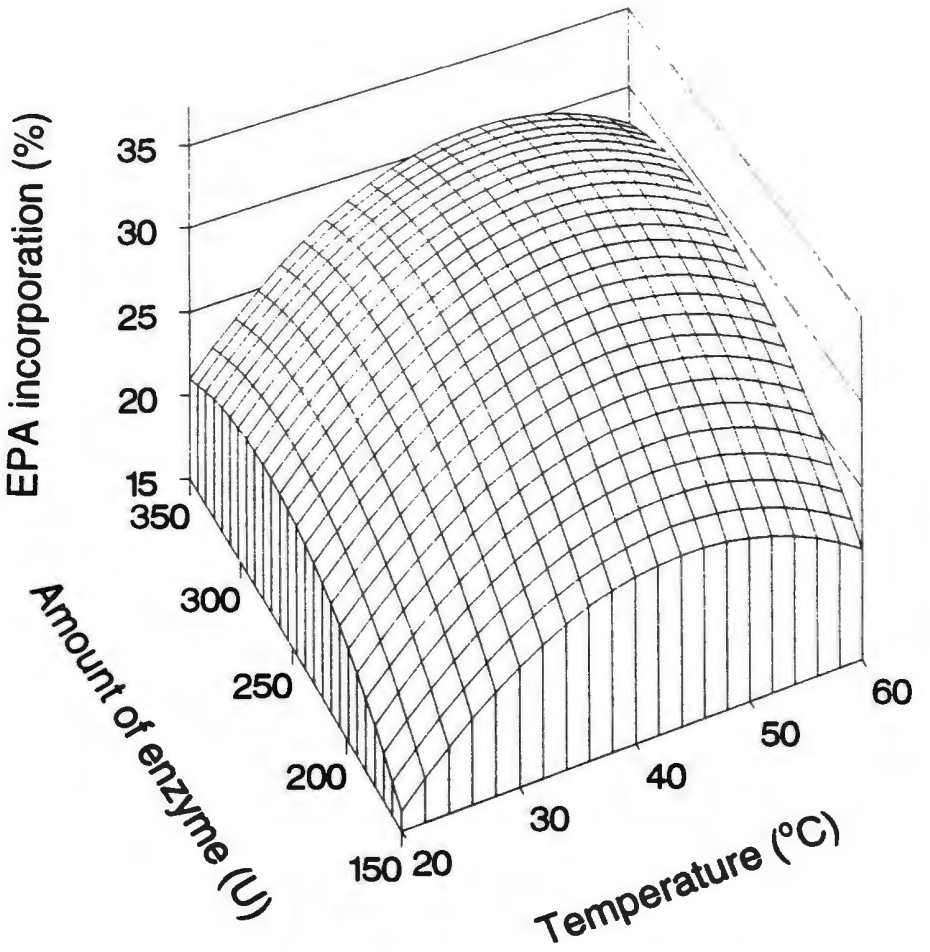

Amount of enzyme (U)
350
300
250
200
150
32.66
30.48
28.30
26.12
23.94
21.76
20
30
40
50
60
Temperature (°C)

Figure 4.24 Three-dimensional response surface and two-dimensional contour plot illustrating the effects of amount of enzyme and reaction temperature on the predicted EPA incorporation (%) in evening primrose oil (EPO)


EPA incorporation (%)
35
30
25
20
15
350
300
250
200
150
Amount of enzyme (U)
20
30
40
50
60
Temperature (°C)

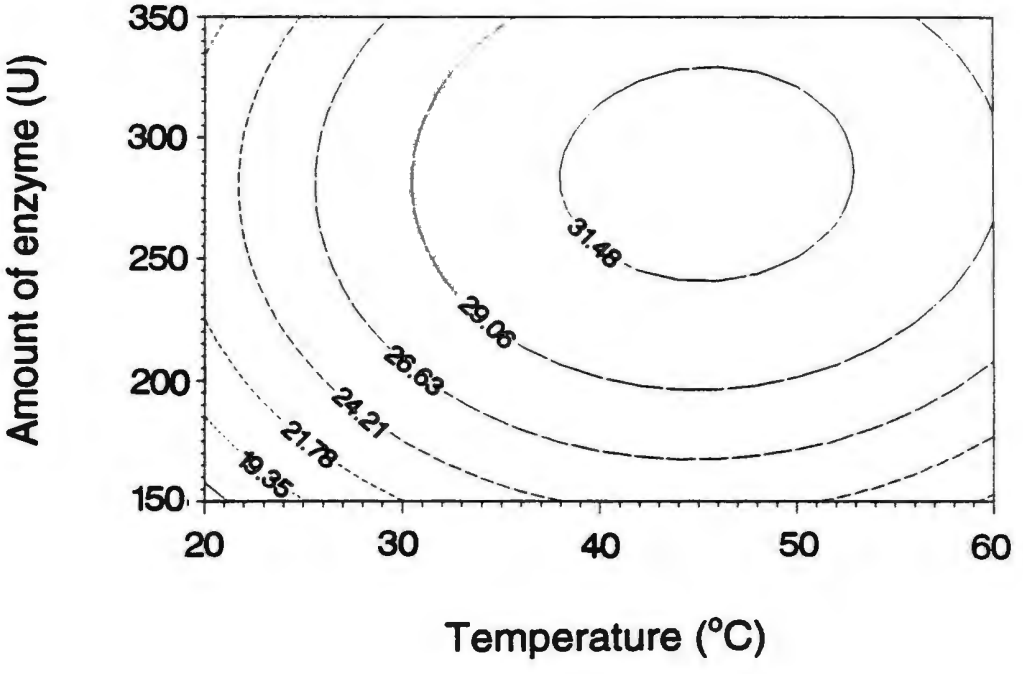
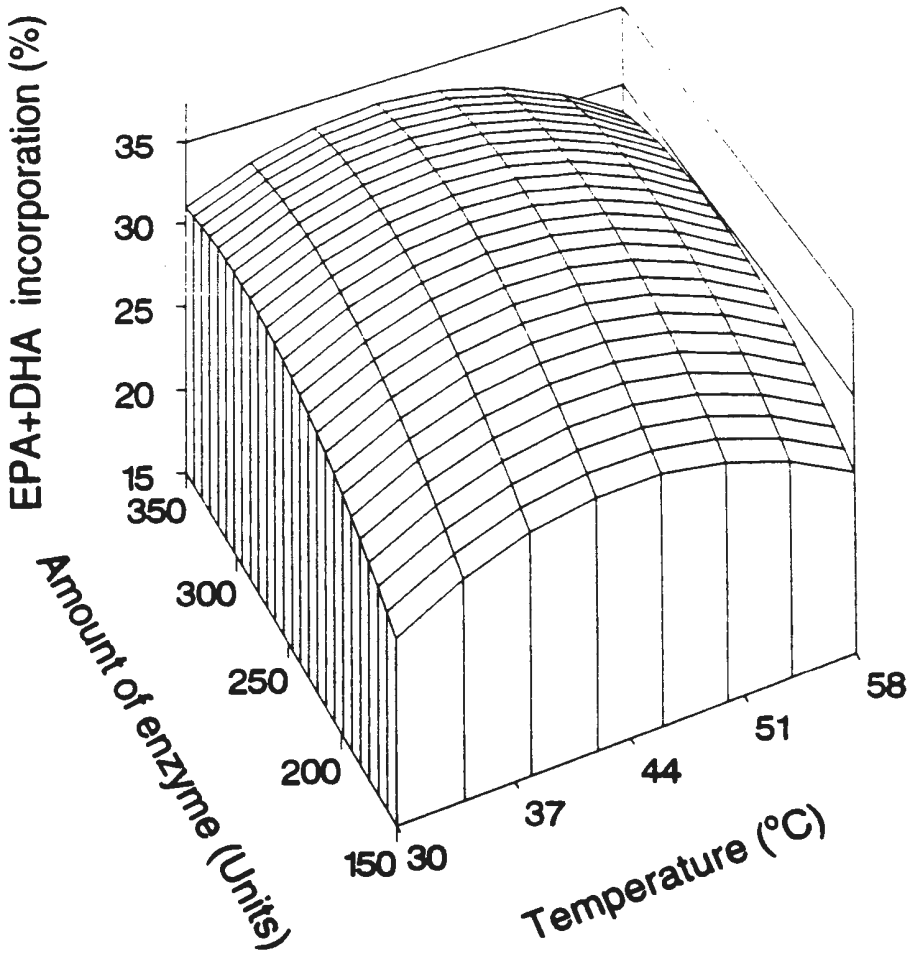

Amount of enzyme (U)
350
300
250
200
150
31.48
29.06
26.63
24.21
21.78
19.35
20
30
40
50
60
Temperature (°C)

Figure 4.25 Three-dimensional response surface and two-dimensional contour plot illustrating the effects of amount of enzyme and reaction temperature on the predicted EPA+DHA incorporation (%) in borage oil (BO)


EPA+DHA incorporation (%)
35
30
25
20
15
350
300
250
200
150
Amount of enzyme (Units)
30
37
44
51
58
Temperature (°C)

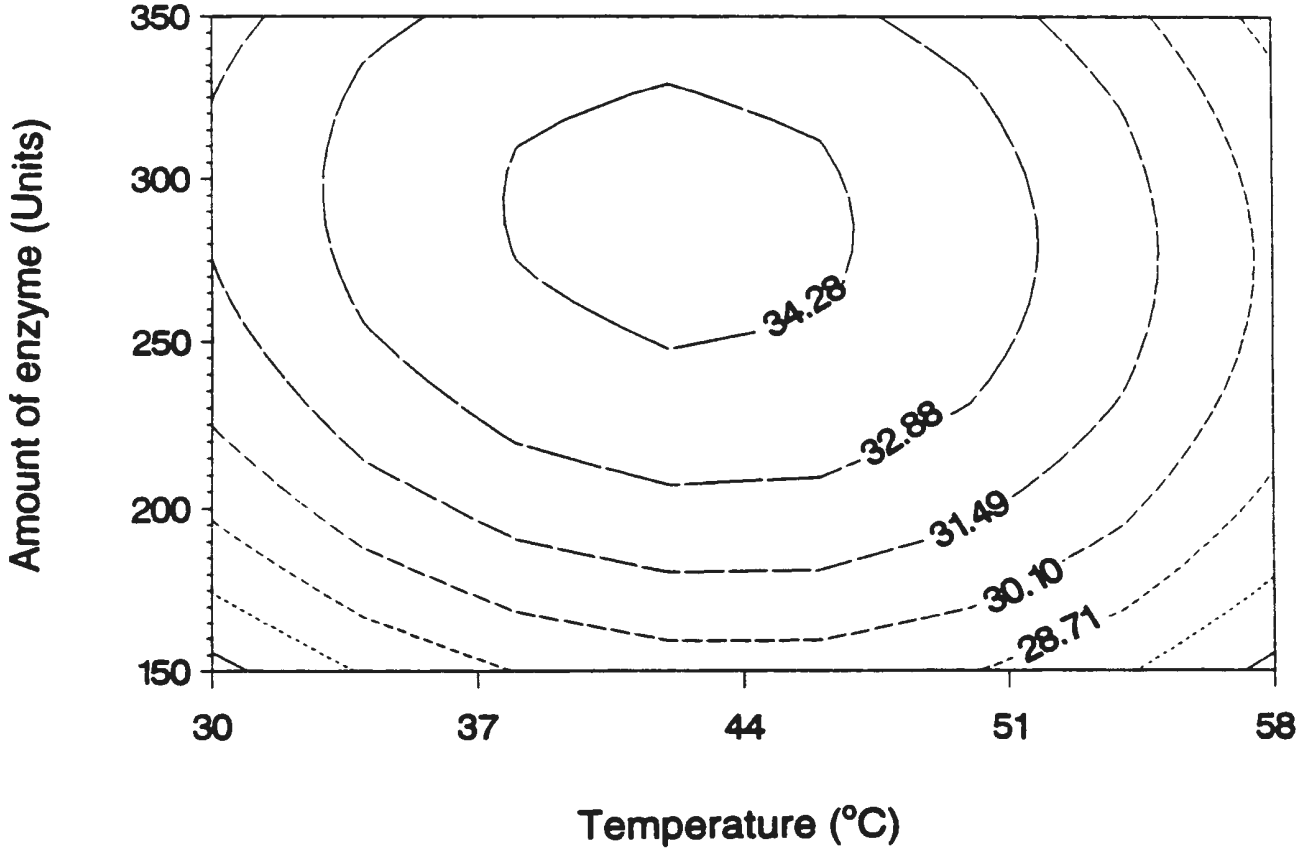
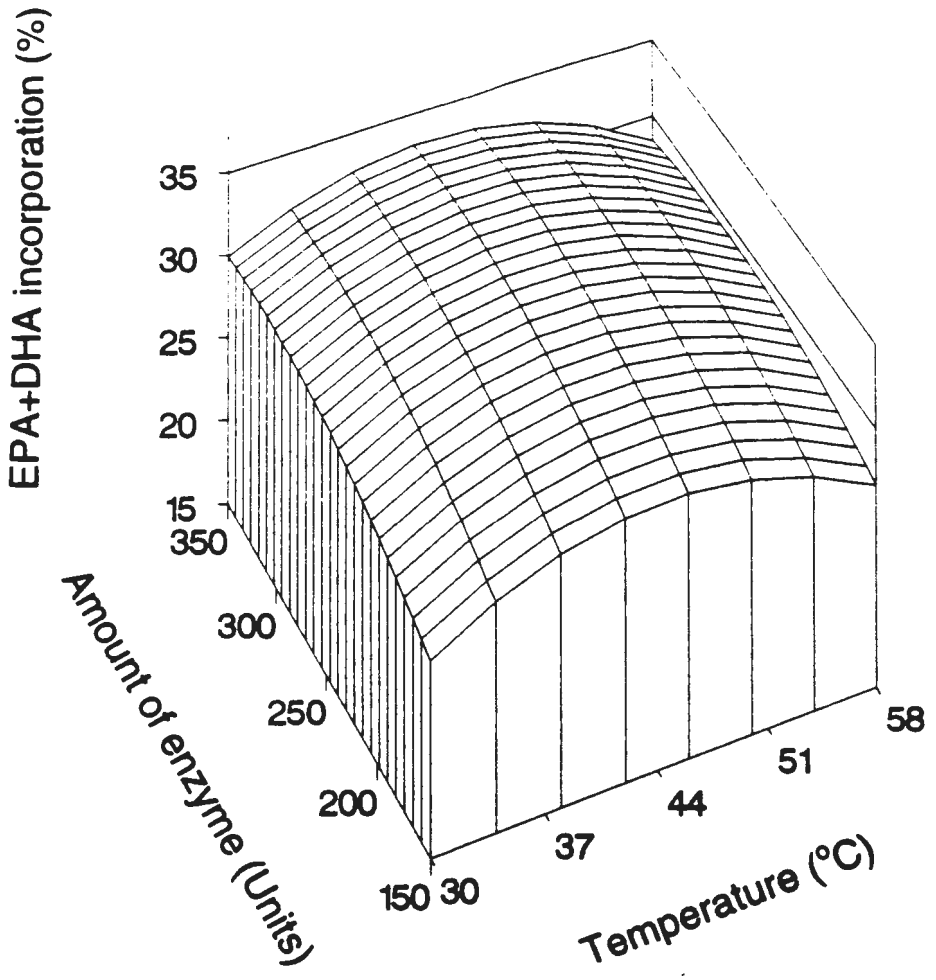
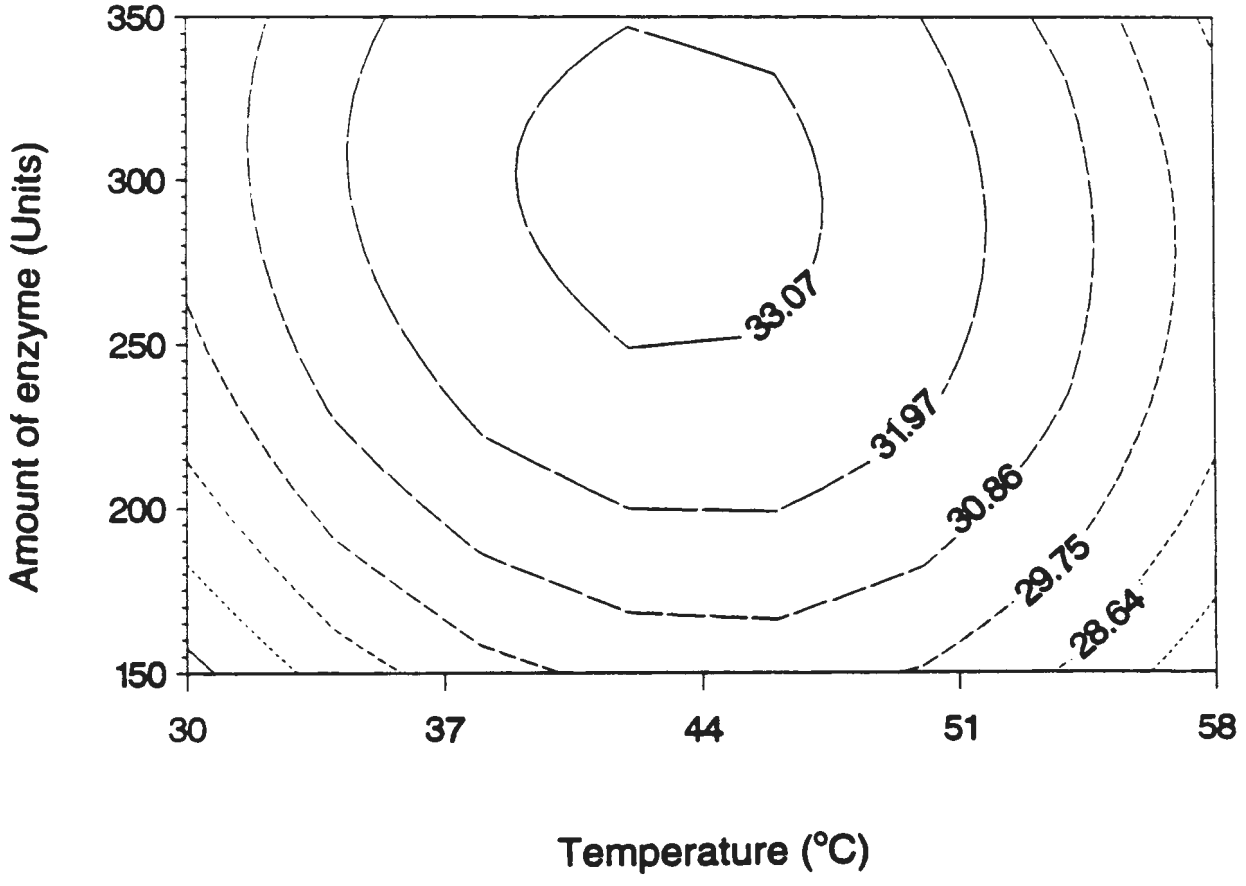

Amount of enzyme (Units)
350
300
250
200
150
34.28
32.88
31.49
30.10
28.71
30
37
44
51
58
Temperature (°C)

Figure 4.26 Three-dimensional response surface and two-dimensional contour plot illustrating the effects of amount of enzyme and reaction temperature on the predicted EPA+DHA incorporation (%) in evening primrose oil (EPO)


EPA+DHA incorporation (%)
35
30
25
20
15
350
300
250
200
150
Amount of enzyme (Units)
30
37
44
51
58
Temperature (°C)


Amount of enzyme (Units)
350
300
250
200
150
33.07
31.97
30.86
29.75
28.64
30
37
44
51
58
Temperature (°C)

examples of which are shown in Figures 4.21 - 4.26. In a contour plot, curves of equal response values are drawn on a plane whose coordinates represent the levels of the independent variables. Each contour represents a specific value for the height of the surface, above the plane defined for combination of the levels of the independent variables. Therefore, different surface height values enable one to focus attention on the levels of independent variables at which changes in the surface height occur. The contour plots illustrate the combination of levels of enzyme, reaction temperature and reaction time that can afford the same amount of DHA, EPA and EPA+DHA incorporation (Figures 4.21 - 4.26).

Critical values for the three variables (enzyme level, reaction temperature and time) were within the experimental region (Table 4.20). The stationary point for the extent of DHA incorporation (%) into BO by acidolysis reaction predicted a maximum of 34.1% at 165 units of *Candida antarctica* enzyme at 50°C over a period of 25 h (Table 4.20). On the other hand, in EPO, the maximum DHA incorporation of 32.0% was predicted at 162 units of *Candida antarctica* enzyme at 43°C over 27 h. The stationary point for EPA incorporation in BO predicted a maximum of 36.1% at 309 units of *Pseudomonas sp.* enzyme at 40°C over 27 h. However, in EPO, the maximum EPA incorporation of 35.1% was predicted at 299 units of *Pseudomonas sp.* enzyme, incubation temperature of 44°C and reaction time of 25 h; for total EPA+DHA incorporation (%) into BO a maximum of 36% was predicted at corresponding values of 278 units, 42°C and 26 h, respectively. In EPO, values for maximum EPA+DHA

Table 4.20 Canonical analysis of response surfaces

Factor	BO, DHA (%)	EPO, DHA (%)	BO, EPA (%)	EPO, EPA (%)	BO, EPA+DHA (%)	EPO, EPA+DHA (%)
X_1	165	162	309	299	278	299
X_2	50	43	40	44	42	43
X_3	25	27	27	25	26	24
Stationary point	Maximum	Maximum	Maximum	Maximum	Maximum	Maximum
Predicted value[a]	34.1	32.0	36.1	35.1	36.0	34.3
Observed value[b]	35.6 ± 1.7	33.5 ± 0.8	35.4 ± 0.5	33.9 ± 1.7	35.5 ± 2.6	33.6 ± 1.8

X_1 = Amount of enzyme (units); X_2 = Reaction temperature (°C); X_3 = Reaction time (h); [a]Predicted using the polynomial model;

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

incorporation of 34.3% were predicted at 299 units of *Pseudomonas sp.* enzyme, 43°C and 24 h.

The contour plot derived from the result of canonical analysis showed ellipsoidal contours at the maximum point (Figures 4.21 - 4.26). Results of independent experiments carried out to examine the adequacy of the predicted values by the models were very close to those observed for responses (Table 4.20). These verification results revealed that the predicted values from the models were reasonable and reproducible. Therefore, acidolysis of BO and EPO with DHA by *Candida antarctica* lipase can increase the incorporation of DHA up to 35.6 and 33.5%, with process yields of 80.5 and 85.1%, respectively. Similarly, acidolysis of BO and EPO with EPA by *Pseudomonas sp.* lipase may increase the incorporation of EPA up to 35.4 and 33.9%, respectively. The process yields of EPA-enriched BO and EPO were 89.4 and 82.3 %, respectively. On the other hand, acidolysis of BO and EPO with EPA+DHA by *Pseudomonas sp.* lipase can increase the total EPA+DHA incorporation to 35.5 and 33.6%, with process yields of 78.5 and 71.1%, respectively.

Xu *et al.* (2000) used RSM to optimize the production of SL from menhaden oil and caprylic acid (8:0) in a packed bed reactor. Effects of parameters such as residence time, substrate mole ratio and reaction temperature were investigated. The residence time was considered the most important factor. Under optimum conditions, the SL contained 38.8% caprylic acid and 29.0% EPA and DHA. Previously, Shieh *et al.* (1995) had reported the synthesis of SL by acidolysis of triolein and capric acid (10:0). Reaction

time, temperature, mole ratio and enzyme load were optimized. A total yield of combined mono- and dicaproolein of up to 100% was obtained. Meanwhile, optimization of synthesis of SL by transesterification of ethyl caprylate and soybean or sunflower oil was reported by Huang and Akoh (1996a). Under optimum conditions, 67.6% caprylic acid was incorporated into TAG of oils.

4.4 Separation of acylglycerols of enzymatically modified oils

DHA-enriched oils, produced under optimum reaction conditions, were separated and quantified by thin layer chromatography-flame ionization detection (TLC-FID). The results showed that the relative content of triacylglycerols (TAG; 87.9-88.5%) was much higher than that of diacylglycerols (DAG; 2.4-8.8%) and monoacylglycerols (MAG; 2.6-9.7%). No free fatty acids (FFA) were found since these were removed by the NaOH after acidolysis reaction (Tables 4.21 and 4.22). The fatty acid composition of the isolated bands was analyzed by gas chromatography (Table 4.21 and 4.22). In DHA-enriched BO, the content of DHA in the TAG, DAG and MAG fractions was 35.4, 26.0 and 18.7%, respectively, with that of GLA was 16.1, 15.0 and 8.3%, respectively. Linoleic acid (LA) was mainly found in the TAG (25.2%) and DAG fractions (28.8%) (Table 4.21). On the other hand, in DHA-enriched EPO, the content of DHA in TAG, DAG and MAG was 33.2, 30.6 and 24.2%, respectively with corresponding GLA contents in TAG, DAG and MAG of 7.6, 4.3 and 12.5%, respectively. LA predominanted in all acylglycerol fractions (Table 4.22).

Table 4.21 Fatty acid composition of acylglycerol components of DHA-enriched borage oil (BO) separated after acidolysis by *Candida antarctica* lipase[a]

Fatty acid	Lipid component (%)		
	TAG[b] (88.5 ± 0.67)	DAG (8.80 ± 0.82)	MAG (2.60 ± 1.20)
14:0	0.10 ± 0.05	0.12 ± 0.01	1.17 ± 0.12
16:0	5.33 ± 0.04	8.47 ± 0.02	9.69 ± 0.06
16:1	0.20 ± 0.02	0.23 ± 0.06	2.13 ± 0.09
18:0	2.10 ± 0.20	3.86 ± 0.21	6.34 ± 0.07
18:1	11.3 ± 0.52	14.8 ± 0.50	16.8 ± 0.67
18:2ω6	25.2 ± 1.20	28.8 ± 0.89	19.7 ± 0.55
18:3ω6	16.1 ± 0.97	15.0 ± 0.20	8.33 ± 0.92
18:3ω3	0.20 ± 0.05	0.20 ± 0.06	0.99 ± 0.10
20:1	2.52 ± 0.20	3.71 ± 0.06	3.53 ± 0.24
20:2	0.13 ± 0.07	0.22 ± 0.05	1.02 ± 0.39
22:0	0.12 ± 0.04	0.21 ± 0.08	0.22 ± 0.10
22:1	1.24 ± 0.10	2.45 ± 0.06	4.35 ± 0.59
22:6ω3	35.4 ± 1.24	26.0 ± 0.78	18.7 ± 0.75

[a]DHA-enriched borage oil was prepared under optimum reaction conditions (165 enzyme units, 50°C, 25 h)
[b]TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols

Table 4.22 Fatty acid composition of acylglycerol components of DHA-enriched evening primrose oil (EPO) separated after acidolysis by *Candida antarctica* lipase[a]

Fatty acid	Lipid component (%)		
	TAG[b] (87.9 ± 2.71)	DAG (2.40 ± 0.55)	MAG (9.70 ± 1.80)
14:0	0.12 ± 0.03	0.31 ± 0.01	0.13 ± 0.02
16:0	3.52 ± 0.41	2.69 ± 0.02	3.78 ± 0.61
16:1	0.06 ± 0.02	0.23 ± 0.06	0.19 ± 0.04
18:0	1.31 ± 0.55	0.75 ± 0.21	1.49 ± 0.23
18:1	4.53 ± 0.25	4.16 ± 0.50	5.45 ± 0.35
18:2ω6	48.5 ± 1.18	55.1 ± 0.91	46.8 ± 1.41
18:3ω6	7.60 ± 0.57	4.32 ± 0.61	12.5 ± 0.62
18:3ω3	0.21 ± 0.02	0.23 ± 0.04	0.22 ± 0.02
20:1	0.21 ± 0.21	0.15 ± 0.07	0.31 ± 0.05
22:1	0.13 ± 0.05	ND[c]	ND[c]
22:6ω3	33.2 ± 0.65	30.6 ± 0.78	24.2 ± 0.54

[a]DHA-enriched evening primrose oil was prepared under optimum reaction conditions (162 enzyme units, 43°C, 27 h)
[b]TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols
[c]Not detectable

In another study, EPA-enriched oils were separated and quantified by TLC-FID (Tables 4.23 and 4.24). The TAG fraction (85.1-95.1%) was again much higher compared to DAG (3.2-11.2%) and MAG (1.7-3.7%) fractions, similar to that observed for DHA-enriched oils; FFA were not detected in modified oils as these were removed by NaOH after the acidolysis reaction. In EPA-enriched BO, TAG, DAG and MAG fractions contained 32.5, 28.2 and 30.8% EPA, respectively. LA was the major fatty acid found in all three acylglycerol fractions (TAG, DAG and MAG) at 26.2, 29.1 and 28.2%, respectively. The content of GLA found in TAG, DAG and MAG fractions was 11.0, 15.1 and 18.7%, respectively. In EPA-enriched EPO, the amount of EPA found in TAG, DAG and MAG were 33.2, 25.6 and 28.9%, respectively. LA was the predominant fatty acid found in all three fractions (44.5% in TAG; 49.1% in DAG; 47.4% in MAG). The amount of GLA present in TAG, DAG and MAG was 7.5, 8.7 and 8.8%, respectively.

The EPA+DHA-enriched oils, produced under optimum reaction conditions, were also analysed by TLC-FID. The TAG (89-91%) were dominant and present at a much higher amount than DAG (8.5-9.0%) and MAG (0.3-1.8%) (Tables 4.25 and 4.26). The fatty acid composition of the isolated TLC bands were analyzed by GC (Tables 4.25 and 4.26). The results showed that EPA (26%) was mainly located in TAG fractions of EPA+DHA-enriched BO and EPO. The contents of GLA and DHA in TAG fractions of these oils were 7.6-17.1 and 7.9-9.6%, respectively. The contents of GLA and DHA in DAG fractions were 9.0-22.7 and 11%, respectively. However, the content of EPA in DAG fractions was negligible. The contents of GLA, EPA and DHA in the MAG fraction were 9.4-19.6, 4.2-7.3 and 8.4-9.8%, respectively.

Table 4.23 Fatty acid composition of acylglycerol components of EPA-enriched borage oil (BO) separated after acidolysis by *Pseudomonas sp.* lipase[a]

Fatty acid	Lipid component (%)		
	TAG[b] (95.1 ± 0.53)	DAG (3.20 ± 0.85)	MAG (1.70 ± 1.06)
12:0	0.16 ± 0.02	0.19 ± 0.06	0.04 ± 0.01
14:0	0.14 ± 0.07	0.11 ± 0.03	0.05 ± 0.02
16:0	6.50 ± 0.41	5.21 ± 0.25	3.85 ± 0.64
16:1	0.47 ± 0.22	0.35 ± 0.13	0.07 ± 0.04
18:0	2.86 ± 0.40	2.14 ± 0.16	1.57 ± 0.72
18:1	12.0 ± 0.38	11.5 ± 0.63	10.8 ± 0.42
18:2ω6	26.2 ± 0.85	29.1 ± 1.34	28.2 ± 0.39
18:3ω6	11.0 ± 0.42	15.1 ± 0.10	18.7 ± 1.07
18:3ω3	0.17 ± 0.07	0.12 ± 0.05	0.15 ± 0.06
20:0	0.25 ± 0.03	0.10 ± 0.05	0.15 ± 0.07
20:1	3.02 ± 0.52	2.60 ± 0.60	1.50 ± 0.43
20:2	0.19 ± 0.05	0.17 ± 0.06	0.12 ± 0.09
20:5ω3	32.5 ± 1.04	28.2 ± 0.84	30.8 ± 0.89
22:1	2.08 ± 0.40	1.24 ± 0.67	1.19 ± 0.26
24:1	1.25 ± 0.31	1.05 ± 0.52	0.61 ± 0.53

[a]EPA-enriched borage oil was prepared under optimum reaction conditions (309 enzyme units, 40°C, 27 h)

[b]TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols

Table 4.24 Fatty acid composition of acylglycerol components of EPA-enriched evening primrose oil (EPO) separated after acidolysis by *Pseudomonas sp.* lipase[a]

Fatty acid	Lipid component (%)		
	TAG[b] (85.1 ± 0.88)	DAG (11.2 ± 0.71)	MAG (3.70 ± 0.50)
12:0	ND[c]	0.08 ± 0.04	0.40 ± 0.02
14:0	0.07 ± 0.02	0.15 ± 0.06	0.26 ± 0.12
16:0	4.60 ± 0.25	3.54 ± 0.25	2.95 ± 0.17
16:1	0.12 ± 0.06	0.13 ± 0.08	0.15 ± 0.09
18:0	1.70 ± 0.41	2.84 ± 0.46	3.70 ± 0.64
18:1	6.91 ± 0.52	6.83 ± 0.90	6.57 ± 0.59
18:2ω6	44.5 ± 1.84	49.1 ± 1.46	47.4 ± 1.08
18:3ω6	7.50 ± 0.47	8.71 ± 0.59	8.75 ± 0.92
18:3ω3	0.17 ± 0.10	0.15 ± 0.07	0.10 ± 0.07
20:0	0.31 ± 0.07	0.15 ± 0.04	0.20 ± 0.03
20:1	0.28 ± 0.04	0.18 ± 0.06	0.20 ± 0.05
20:5ω3	33.2 ± 1.45	25.6 ± 1.80	28.9 ± 0.72
22:1	0.11 ± 0.05	ND[c]	ND[c]

[a]EPA-enriched evening primrose oil was prepared under optimum reaction conditions (299 enzyme units, 44°C, 25 h)
[b]TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols
[c]Not detectable

Table 4.25 Fatty acid profile of acylglycerol components of EPA+DHA enriched borage oil (BO) separated after acidolysis by *Pseudomonas sp.* lipase[a]

Fatty acid	Lipid component (%)		
	TAG[b] (91.2 ± 0.68)	DAG (8.50 ± 0.64)	MAG (0.30 ± 0.07)
10:0	0.05 ± 0.01	0.05 ± 0.01	ND[c]
12:0	0.04 ± 0.02	0.06 ± 0.05	ND[c]
14:0	0.04 ± 0.01	0.06 ± 0.02	0.37 ± 0.02
16:0	5.06 ±0.56	6.92 ± 0.22	6.84 ± 0.27
16:1	0.38 ± 0.32	0.15 ± 0.04	ND[c]
18:0	2.05 ± 0.01	2.60 ± 0.56	3.52 ± 0.50
18:1	10.9 ± 0.09	14.6 ± 0.10	11.7 ± 0.35
18:2ω6	22.5 ± 0.33	34.3 ± 0.52	27.7 ± 0.87
18:3ω6	17.1 ± 0.19	22.7 ± 0.20	19.6 ± 0.90
18:3ω3	0.04 ± 0.02	0.17 ± 0.03	0.91 ± 0.07
20:0	0.14 ± 0.01	ND[c]	ND[c]
20:1	2.61 ± 0.02	3.43 ± 0.12	2.55 ± 0.52
20:2	0.13 ± 0.02	0.17 ± 0.04	ND[c]
20:4	0.56 ± 0.01	ND[c]	ND[c]
20:5ω3	25.9 ± 0.20	0.50 ± 0.07	4.23 ± 0.55
22:1	1.24 ± 0.20	2.09 ± 0.13	2.66 ± 0.62
24:1	0.83 ± 0.01	1.15 ± 0.25	4.11 ± 0.33
22:6ω3	9.60 ± 0.02	11.1 ± 0.89	9.83 ± 0.51

[a]EPA+DHA enriched borage oil was prepared under optimum reaction conditions (278 enzyme units, 42°C, 26 h)

[b]TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol

[c]Not detected

Table 4.26 Fatty acid profile of acylglycerol components of EPA+DHA enriched evening primrose oil (EPO) separated after acidolysis by *Pseudomonas sp.* lipase[a]

Fatty acid	Lipid component (%)		
	TAG[b] (89.2 ± 0.51)	DAG (9.00 ± 0.95)	MAG (1.80 ± 0.64)
10:0	0.04 ± 0.04	0.50 ± 0.02	0.14 ± 0.02
12:0	0.04 ± 0.01	0.07 ± 0.03	1.51 ±0.03
14:0	0.02 ± 0.01	0.05 ± 0.01	0.23 ± 0.03
16:0	3.25 ± 0.06	4.62 ± 0.46	4.46 ± 0.50
16:1	0.07 ± 0.02	0.06 ± 0.02	ND[c]
18:0	1.15 ± 0.02	1.32 ± 0.23	1.32 ± 0.04
18:1	6.74 ± 0.05	8.96 ± 0.53	7.44 ± 0.07
18:2ω6	45.7 ± 0.10	62.1 ± 1.21	54.6 ± 0.55
18:3ω6	7.63 ± 0.01	8.96 ± 0.46	9.38 ± 0.26
18:3ω3	0.13 ± 0.05	0.16 ± 0.02	ND[c]
20:0	0.21 ± 0.06	0.28 ± 0.08	ND[c]
20:1	0.29 ± 0.02	0.21 ± 0.06	ND[c]
20:4	0.58 ± 0.02	ND[c]	ND[c]
20:5ω3	25.7 ± 0.90	0.59 ± 0.06	7.27 ± 0.28
22:1	0.33 ± 0.06	0.16 ± 0.21	0.65 ± 0.30
22:6ω3	7.91 ± 0.50	11.3 ± 0.53	8.40 ± 0.76

[a]EPA+DHA enriched evening primrose oil was prepared under optimum reaction conditions (299 enzyme units, 43°C, 24 h)

[b]TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol

[c]Not detected

4.5 Stereospecific analysis of triacylglycerols (TAG) of DHA, EPA and EPA+DHA-enriched oils

Stereospecific analyses of DHA, EPA and EPA+DHA-enriched BO and EPO and the intermediates involved are shown in Figure 4.27. In the first step, TAG of both oils were hydrolysed by porcine pancreatic lipase in order to split fatty acids at the *sn*-1 and *sn*-3 positions, yielding 2-MAG, which accurately provides the fatty acid composition of the *sn*-2 position of TAG. In the second step, TAG of both oils were modified by Grignard degradation using methyl magnesium bromide (CH_3MgBr). Laakso and Christie (1990) and Nwosu and Boyd (1997) used Grignard reaction to obtain partially deacylated acylglycerols. In this reaction, the electrons in the C=O bond (sigma and pi) of the carbonyl groups in TAG are drawn towards the electronegative oxygen atom (Figure 4.28) and the carbon atom of the carbonyl group with a partial positive charge can be attacked by the nucleophilic carbon of the Grignard reagent. The products of the reaction are a ketone and a magnesium salt of acylglycerol. In the presence of an aqueous acid, the magnesium salt of acylglycerols yields acylglycerols and an inorganic magnesium bromide (Figure 4.28). The resulting ketone reacts further with the Grignard reagent to yield a tertiary alcohol and an inorganic magnesium bromide. The products of the Grignard degradation of TAG may be separated on TLC plates (Figure 3.5) to afford MAG (R_f = 0.05), 1,2- and 2,3-DAG (R_f = 0.32), 1,3-DAG (R_f = 0.41) and a tertiary alcohol (R_f = 0.72). Among these, the band with an R_f value of 0.32 (1,2- and 2,3-DAG) was isolated and used to prepare synthetic racemic phosphatides *via* reaction with

Figure 4.27 Procedure for the stereospecific analysis of triacylglycerols (TAG) of DHA, EPA and EPA+DHA-enriched borage (BO) and evening primrose oils (EPO)

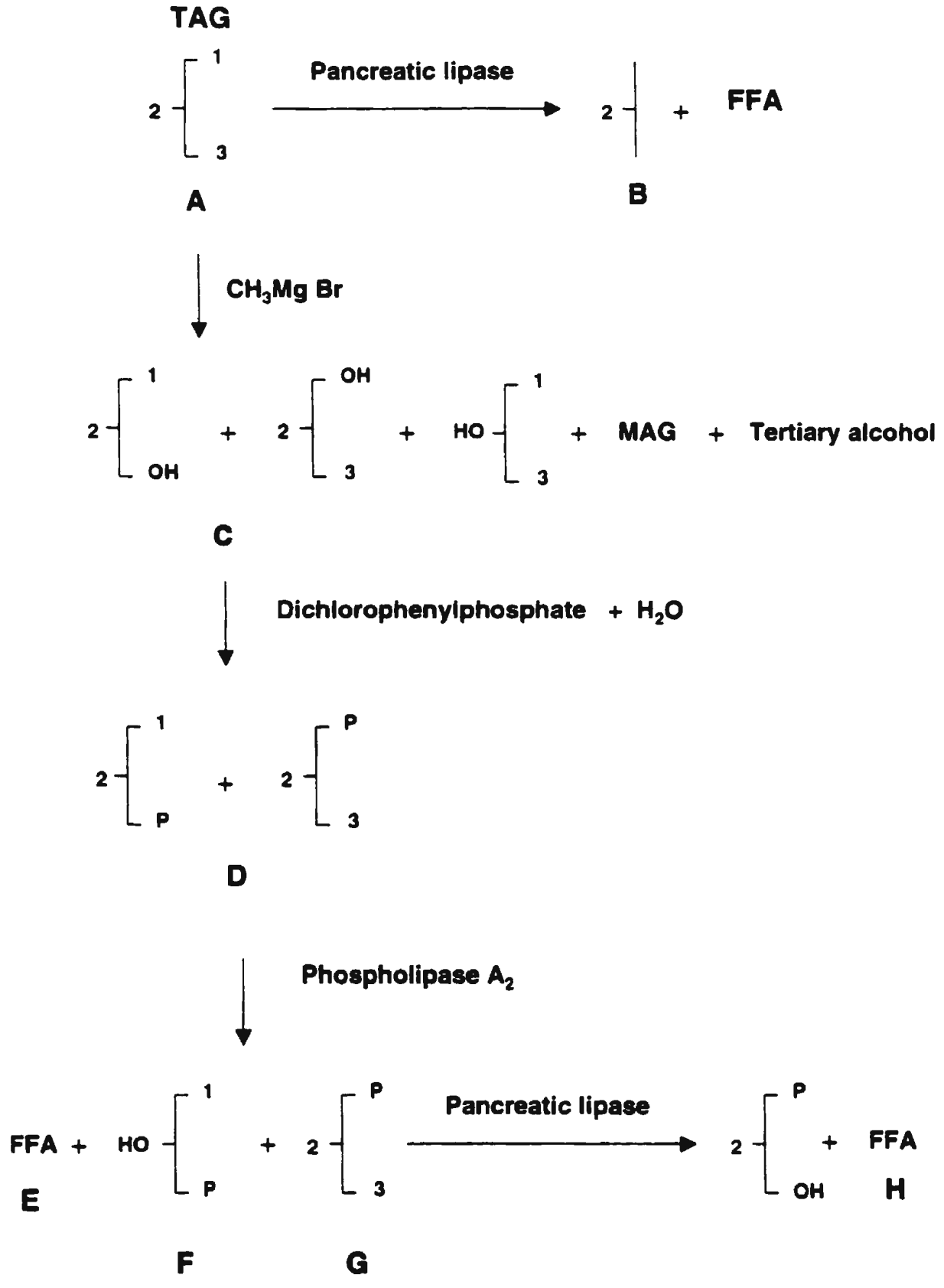
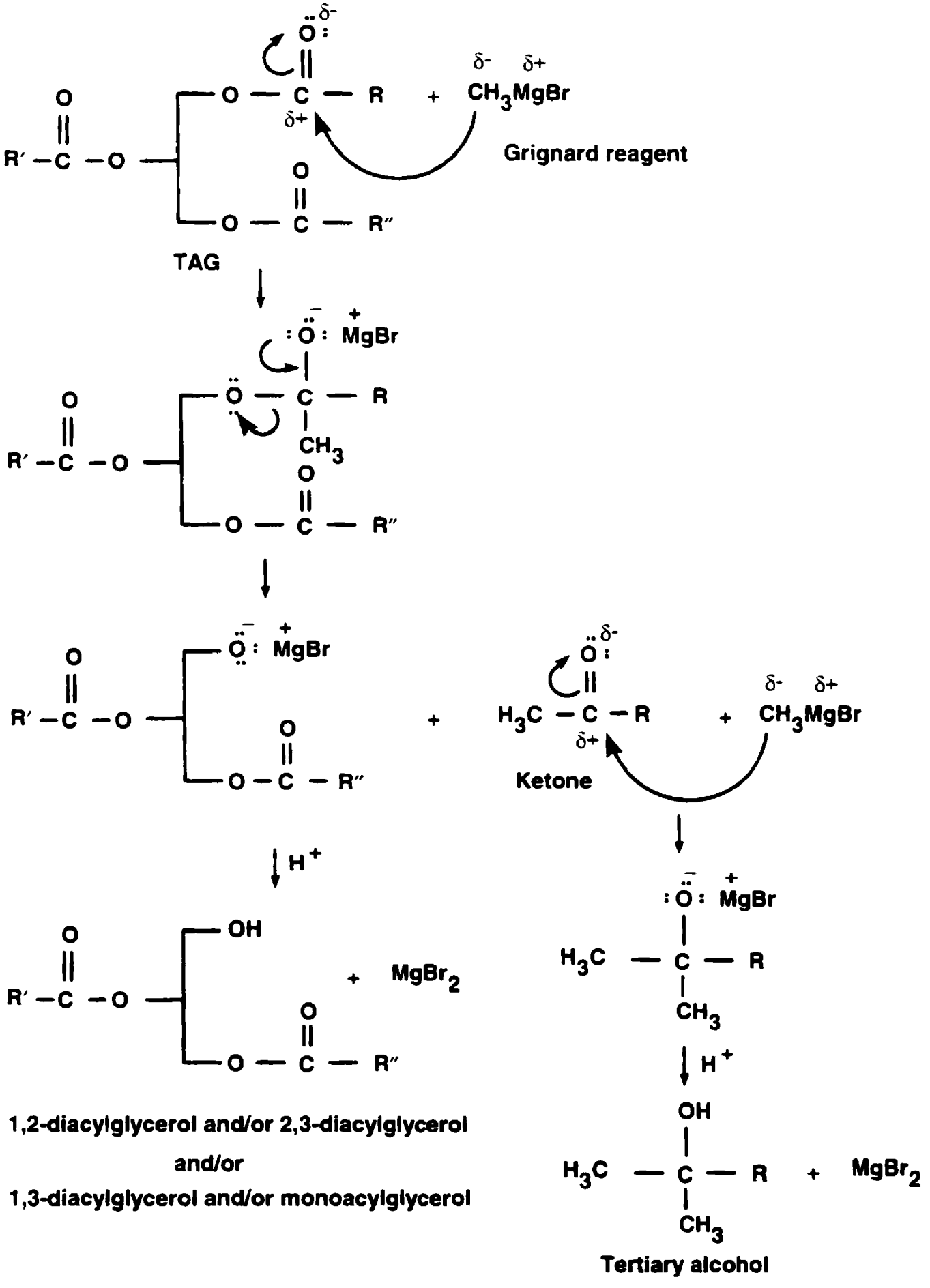

TAG
Pancreatic lipase
FFA
A
B
CH_3Mg Br
OH
MAG
Tertiary alcohol
C
Dichlorophenylphosphate + H_2O
D
Phospholipase A_2
FFA
E
Pancreatic lipase
FFA
H
F
G

Figure 4.28 Chemical reactions involved during Grignard degradation of triacylglycerols (TAG) of DHA, EPA and EPA+DHA-enriched borage (BO) and evening primrose oils (EPO)


Grignard reagent
TAG
Ketone
1,2-diacylglycerol and/or 2,3-diacylglycerol
and/or
1,3-diacylglycerol and/or monoacylglycerol
Tertiary alcohol

dichlorophenylphosphate (Figure 4.27). This reaction produced 1,2-diacylglycero-3-phosphatide (L-isomer) and 2,3-diacylglycero-1-phosphatide (D-isomer) which were subsequently hydrolysed by stereospecific phospholipase A_2. This enzyme reacts only with 1,2-diacylglycero-3-phosphatide (L-isomer; naturally present) and releases FFA from the *sn*-2 position (Verheij and Dijkstra, 1994). The products of phospholipase A_2 hydrolysis (FFA from the *sn*-2 position and L-lysophosphatide) and the unchanged 2,3-diacylglycero-1-phosphatide (D-isomer; unnatural) are subsequently separated on TLC plates (Figure 3.6). The separated bands are those of FFA from the *sn*-2 position of 1,2-diacyl-3-phosphatide (R_f = 0.78), unchanged 2,3-diacylglycero-1-phosphatide (R_f =0.51) and L-lysophosphatide (R_f = 0.06). The band with an R_f value of 0.06 (L-lysophosphatide) was isolated, extracted into chloroform/methanol (1:1, v/v) and then used for fatty acid analysis. These allowed identification of the fatty acid composition at the *sn*-1 position of TAG of DHA, EPA and EPA+DHA-enriched BO and EPO.

In order to determine the fatty acid composition at the *sn*-3 position of TAG, a band with an R_f value of 0.51 (unchanged 2,3-diacylglycero-1-phosphatide) was isolated and extracted into chloroform/methanol (1:1, v/v). The latter compound was subjected to porcine pancreatic lipase hydrolysis (Figure 4.27), the products of which were 2-monoacylglycero-1-phosphatide and the FFA released from the *sn*-3 position of TAG of DHA, EPA and EPA+DHA-enriched BO and EPO.

4.5.1 Positional distributions of fatty acids of DHA, EPA and EPA+DHA-enriched oils

The positional distribution of fatty acids in TAG of DHA-enriched oils were determined (Tables 4.27 and 4.28). The results of this study showed that DHA was fairly evenly distributed over all three positions (34.6% at *sn*-1, 33.5% at *sn*-2 and 35.9% at *sn*-3) of TAG molecules of DHA-enriched BO (Table 4.27). In DHA-enriched EPO, however, this fatty acid was mainly esterified at the *sn*-2 position (38.2%) (Table 4.28). The positional specificity of *Candida antarctica* depends on the type of reactants. In some reactions, this enzyme functions as a nonspecific lipase whereas in other reactions it shows *sn*-1,3 positional specificity (Novo Nordisk, 1999). The results showed that under assay conditions employed in this study, this enzyme acts as a nonspecific lipase. In DHA-enriched BO, the saturated fatty acids 16:0 and 18:0 favoured the *sn*-2 position. However, in DHA-enriched EPO, these fatty acids were concentrated in the *sn*-1 and *sn*-3 positions. The stereospecific distribution of fatty acids in the native BO and EPO have previously been determined (Lawson and Hughes, 1988; Redden *et al.*, 1995). In BO, GLA was distributed asymmetrically and preferentially esterified at the *sn*-2 and *sn*-3 positions (Redden *et al.*, 1995; Lawson and Hughes, 1988). In EPO, GLA was found to be concentrated in the *sn*-3 position. LA was nearly evenly distributed in all positions of EPO, but was concentrated in the *sn*-1 position of BO (Lawson and Hughes, 1988). In DHA-enriched BO, GLA was mainly located in the *sn*-2 (18.4%) and *sn*-3 (19.2%) positions of TAG (Tables 4.27). In DHA-enriched EPO, however, GLA was concentrated in the *sn*-2

Table 4.27 Positional distribution of fatty acids in DHA-enriched borage oil (BO)[a]

Fatty acids (w/w %)	TAG	*sn*-1	*sn*-2	*sn*-3
8:0	0.04 ± 0.01	0.08 ± 0.03	ND[b]	0.05 ± 0.02
10:0	0.15 ± 0.02	0.17 ± 0.02	0.07 ± 0.02	0.30 ± 0.02
12:0	0.16 ± 0.03	0.12 ± 0.06	0.10 ± 0.05	0.19 ± 0.08
14:0	0.09 ± 0.01	0.05 ± 0.03	0.08 ± 0.04	0.11 ± 0.03
14:1	0.04 ± 0.01	0.02 ± 0.01	ND[b]	0.06 ± 0.02
16:0	4.33 ± 0.57	3.90 ± 0.33	4.52 ± 0.52	3.50 ± 0.05
16:1	0.19 ± 0.03	0.12 ± 0.08	0.17 ± 0.05	0.23 ± 0.10
17:0	ND[b]	0.02 ± 0.01	ND[b]	0.04 ± 0.02
17:1	0.21 ± 0.07	0.45 ± 0.08	ND[b]	0.15 ± 0.01
18:0	2.09 ± 0.15	1.12 ± 0.21	2.92 ± 0.07	1.95 ± 0.30
18:1	12.3 ± 0.50	12.3 ± 0.56	11.7 ± 0.82	8.24 ± 0.40
18:2ω6	24.7 ± 0.90	23.9 ± 1.08	22.8 ± 1.57	25.3 ± 0.37
18:3ω6	16.1 ± 0.26	13.2 ± 0.38	18.4 ± 0.85	19.2 ± 0.51
18:3ω3	0.19 ± 0.09	0.27 ± 0.08	0.17 ± 0.06	0.20 ± 0.07
20:0	ND[b]	0.11 ± 0.04	0.22 ± 0.05	ND[b]
20:1	2.12 ± 0.20	2.79 ± 0.46	2.70 ± 0.10	1.09 ± 0.31
20:2	0.13 ± 0.07	0.23 ± 0.10	0.12 ± 0.30	0.08 ± 0.04
22:1	1.24 ± 0.08	1.77 ± 0.14	1.90 ± 0.09	0.72 ± 0.07
22:6ω3	35.3 ± 1.50	34.6 ± 0.85	33.5 ± 0.25	35.9 ± 0.74
24:1	0.47 ± 0.09	0.89 ± 0.12	0.11 ± 0.06	0.41 ± 0.09

[a]Mean ± SD of triplicate determinations from different experiments; [b]Not detected

Table 4.28 Positional distribution of fatty acids in DHA-enriched evening primrose oil (EPO)[a]

Fatty acids (w/w %)	TAG	*sn*-1	*sn*-2	*sn*-3
12:0	0.12 ± 0.08	0.15 ± 0.02	0.13 ± 0.02	0.08 ± 0.04
14:0	0.12 ± 0.05	0.09 ± 0.02	0.13 ± 0.04	0.17 ± 0.04
14:1	0.08 ± 0.02	0.12 ± 0.01	0.08 ± 0.05	0.07 ± 0.03
16:0	7.55 ± 0.17	7.81 ± 0.35	3.74 ± 0.41	10.6 ± 0.73
16:1	0.06 ± 0.02	0.08 ± 0.04	0.17 ± 0.04	ND[b]
18:0	2.31 ± 0.42	2.90 ± 0.51	1.47 ± 0.26	3.97 ± 0.59
18:1	4.63 ± 0.35	7.81 ± 0.16	1.49 ± 0.54	3.20 ± 0.61
18:2ω6	43.4 ± 0.64	45.1 ± 0.97	44.9 ± 0.59	41.5 ± 0.88
18:3ω6	7.05 ± 0.15	5.48 ± 0.56	7.49 ± 0.82	4.79 ± 0.51
18:3ω3	0.21 ± 0.06	0.29 ± 0.03	0.23 ± 0.05	0.08 ± 0.04
20:0	0.50 ± 0.12	0.42 ± 0.08	0.32 ± 0.04	0.59 ± 0.11
20:1	0.29 ± 0.16	0.14 ± 0.05	0.36 ± 0.06	0.36 ± 0.17
22:1	0.13 ± 0.04	0.15 ± 0.05	0.17 ± 0.03	0.12 ± 0.06
22:6ω3	33.1 ± 0.70	24.5 ± 0.72	38.2 ± 0.52	33.1 ± 0.87

[a]Mean ± SD of triplicate determinations from different experiments; [b]Not detected

(7.5%) position (Table 4.28). LA was randomly distributed over the three positions of TAG of both oils.

Lee and Akoh (1996) performed pancreatic lipase hydrolysis on a SL synthesized *via* interesterification reaction between medium chain triacylglycerol (MCT) and EPA ethyl ester using *Candida antarctica* lipase as the biocatalyst. Their results showed that EPA was mainly incorporated in the *sn*-2 position of the TAG. This demonstrated that *Candida antarctica* lipase has a high specificity for *sn*-2 position under experimental conditions employed by these researchers.

In another study, the positional distribution of fatty acids in TAG of EPA-enriched oils was determined and the results are shown in Tables 4.29 and 4.30. The saturated fatty acids 16:0 and 18:0 were concentrated at the *sn*-2 position of TAG of EPA-enriched oils. The EPA of EPA-enriched BO was randomly distributed in the TAG (33.4% at *sn*-1; 32.5% at *sn*-2; 30.9% at *sn*-3) (Table 4.29). In EPA-enriched EPO, however, this fatty acid was mainly esterified at the primary positions (39.5% at *sn*-1 and 42.1% at *sn*-3) of TAG (Table 4.30) and was also present in appreciable amounts (23.2%) at the *sn*-2 position. Therefore, it is assumed that *Pseudomonas sp.* lipase shows no specificity and may incorporate EPA in all three positions of TAG of the oils. In both oils, GLA was esterified preferentially at the *sn*-2 position (18.6 and 7.2% in EPA-enriched BO and EPO, respectively). In EPA-enriched BO, LA in TAG was distributed randomly while in EPA-enriched EPO it was mainly located at the *sn*-2 position (Tables 4.29 and 4.30).

The positional distribution of fatty acids in TAG of EPA+DHA-enriched oils

Table 4.29 Positional distribution of fatty acids in EPA-enriched borage oil (BO)[a]

Fatty acids (w/w %)	TAG	*sn*-1	*sn*-2	*sn*-3
8:0	0.03 ± 0.01	0.09 ± 0.04	ND[b]	ND[b]
10:0	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.02	0.04 ± 0.02
12:0	0.16 ± 0.05	0.19 ± 0.06	ND[b]	0.28 ± 0.09
14:0	0.13 ± 0.07	0.05 ± 0.02	0.05 ± 0.03	0.33 ± 0.14
14:1	0.02 ± 0.01	0.04 ± 0.02	ND[b]	0.06 ± 0.02
16:0	6.45 ± 0.21	4.31 ± 0.15	8.82 ± 0.18	4.75 ± 0.46
16:1	0.47 ± 0.07	0.58 ± 0.13	0.08 ± 0.05	0.69 ± 0.11
18:0	1.85 ± 0.23	0.84 ± 0.15	1.95 ± 0.43	0.69 ± 0.21
18:1	11.6 ± 0.52	10.3 ± 0.52	10.8 ± 0.35	14.5 ± 0.74
18:2ω6	22.2 ± 0.91	23.6 ± 0.91	21.2 ± 0.42	20.2 ± 0.83
18:3ω6	15.1 ± 0.82	11.5 ± 0.34	18.6 ± 0.63	13.4 ± 0.12
18:3ω3	0.17 ± 0.05	0.14 ± 0.09	ND[b]	0.30 ± 0.15
20:0	0.27 ± 0.06	0.54 ± 0.16	ND[b]	0.13 ± 0.06
20:1	3.00 ± 0.09	2.15 ± 0.38	3.80 ± 0.04	3.18 ± 0.38
20:2	0.18 ± 0.02	0.39 ± 0.16	ND[b]	0.17 ± 0.09
20:4	0.14 ± 0.05	0.08 ± 0.03	ND[b]	0.45 ± 0.05
20:5ω3	32.7 ± 1.28	33.4 ± 0.86	32.5 ± 0.79	30.9 ± 1.05
22:0	0.17 ± 0.09	0.04 ± 0.02	0.11 ± 0.06	0.38 ± 0.06
22:1	2.05 ± 0.11	3.58 ± 0.15	1.20 ± 0.28	1.04 ± 0.34
22:5	0.07 ± 0.01	0.07 ± 0.04	ND[b]	0.19 ± 0.06
24:0	0.05 ± 0.01	ND[b]	ND[b]	0.29 ± 0.13
24:1	1.22 ± 0.08	0.24 ± 0.05	0.58 ± 0.24	2.77 ± 0.47

[a]Mean ± SD of triplicate determinations from different experiments; [b]Not detected

Table 4.30 Positional distribution of fatty acids in EPA-enriched evening primrose oil (EPO)[a]

Fatty acids (w/w %)	TAG	*sn*-1	*sn*-2	*sn*-3
8:0	ND[b]	0.08 ± 0.03	ND[b]	0.10± 0.04
10:0	0.03 ± 0.01	0.09 ± 0.02	0.03 ± 0.02	0.04 ± 0.03
12:0	ND[b]	ND[b]	0.40 ± 0.08	ND[b]
14:0	0.06 ± 0.02	0.09 ± 0.04	0.05 ± 0.05	0.08 ± 0.04
14:1	ND[b]	0.04 ± 0.03	0.03 ± 0.01	ND[b]
16:0	4.55 ± 0.36	2.94 ± 0.14	5.98 ± 0.15	3.18 ± 0.52
16:1	0.12 ± 0.05	0.11 ± 0.08	0.12 ± 0.04	0.08 ± 0.05
18:0	1.65 ± 0.49	0.08 ± 0.04	3.60 ± 0.27	1.43 ± 0.28
18:1	6.92 ± 0.58	6.48 ± 0.67	8.54 ± 0.44	5.31 ± 0.40
18:2ω6	43.7 ± 1.71	39.1 ± 0.59	48.4 ± 0.61	37.5 ± 0.75
18:3ω6	5.43 ± 0.29	4.39 ± 0.54	7.16 ± 0.37	4.84 ± 0.46
18:3ω3	0.16 ± 0.07	0.08 ± 0.04	0.10 ± 0.04	0.28 ± 0.08
20:0	0.31 ± 0.05	0.49 ± 0.17	0.20 ± 0.04	0.26 ± 0.12
20:1	0.27 ± 0.04	0.22 ± 0.08	0.19 ± 0.06	0.34 ± 0.07
20:2	ND[b]	ND[b]	0.03 ± 0.02	0.05 ± 0.02
20:4	0.11 ± 0.02	0.21 ± 0.04	0.10 ± 0.04	0.27 ± 0.05
20:5ω3	35.1 ± 0.78	39.5 ± 0.86	23.2 ± 0.58	42.1 ± 1.55
22:0	ND[b]	0.04 ± 0.03	ND[b]	0.03 ± 0.02
22:1	0.11 ± 0.04	0.20 ± 0.06	ND[b]	0.24 ± 0.07
22:4	ND[b]	ND[b]	0.08 ± 0.05	ND[b]

[a]Mean ± SD of triplicate determinations from different experiments: [b]Not detected

were also determined and the results are given in Tables 4.31 and 4.32. In EPA+DHA-enriched BO and EPO, saturated fatty acids such as 16:0 and 18:0 occurred mainly at the *sn*-1 and *sn*-3 positions of TAG. However, these modified oils were different in the dominancy and distribution of ω3 and ω6 PUFA in their TAG molecules. In EPA+DHA enriched BO, GLA was mainly located at the *sn*-2 position (32.9%). However, EPA and DHA were preferentially esterified at *sn*-1 and *sn*-3 positions of TAG molecules (Table 4.31) and their quantities were EPA, 26.1 and 30.8%; and DHA, 8.3 and 9.8%, respectively. In EPA+DHA enriched EPO, GLA was located mainly at the *sn*-2 (10.8%) and *sn*-3 (9.0%) positions of TAG. EPA was preferentially esterified at the *sn*-1 (31.5%) and *sn*-3 (24.1%) positions while approximately half of DHA was located in the *sn*-3 position (10.5%) of TAG (Table 4.32). Therefore, lipase from *Pseudomonas sp.*, under the conditions used in this study, has the ability to incorporate ω3 fatty acids (EPA and DHA) preferentially at the *sn*-1 and *sn*-3 positions of TAG.

Brockerhoff *et al.* (1968) reported that in fish oils the long-chain PUFA tend to be concentrated in the *sn*-2 position whereas in marine mammals they favour the *sn*-1 and *sn*-3 positions of TAG. Recently, Wanasundara and Shahidi (1997) reported similar results for seal blubber and menhaden oils. Aursand *et al.* (1995) investigated the positional distribution of ω3 PUFA in fish and marine mammal oils using high resolution ^{13}C nuclear magnetic resonance (^{13}C NMR) spectroscopy. These authors found that in fish oils DHA was concentrated in the *sn*-2 position while EPA was more randomly distributed over the three positions of TAG. In seal oil, DHA was predominantly present

Table 4.31 Fatty acid distribution in different positions of triacylglycerols of EPA+DHA enriched borage oil (BO)[a]

Fatty acids (w/w %)	TAG	*sn*-1	*sn*-2	*sn*-3
8:0	0.02 ± 0.01	ND[b]	0.03 ± 0.02	0.40 ± 0.04
10:0	0.02 ± 0.01	ND[b]	0.05 ± 0.03	1.41 ± 0.20
12:0	0.05 ± 0.02	0.59 ± 0.10	0.08 ± 0.03	1.38 ± 0.51
14:0	0.07 ± 0.05	0.69 ± 0.20	0.09 ± 0.06	0.89 ± 0.20
16:0	5.06 ± 0.50	7.15 ± 0.80	1.05 ± 0.06	5.99 ± 0.80
16:1	ND[b]	ND[b]	0.21 ± 0.02	1.99 ± 0.30
17:0	0.02 ± 0.01	ND[b]	0.05 ± 0.02	0.22 ± 0.05
17:1	0.20 ± 0.05	ND[b]	0.30 ± 0.06	ND[b]
18:0	2.21 ± 0.05	5.15 ± 0.70	1.02 ± 0.50	2.76 ± 0.50
18:1	11.5 ± 0.40	12.8 ± 0.80	14.1 ± 1.20	8.85 ± 1.00
18:2ω6	20.5 ± 2.00	22.9 ± 1.00	26.2 ± 2.60	15.3 ± 1.25
18:3ω6	16.9 ± 0.70	4.12 ± 1.00	32.9 ± 1.50	15.1 ± 0.80
18:3ω3	ND[b]	3.71 ± 0.85	0.45 ± 0.04	0.64 ± 0.50
20:1	2.94 ± 0.50	3.06 ± 0.60	1.40 ± 0.08	2.43 ± 0.60
20:2	0.20 ± 0.04	ND[b]	0.12 ± 0.06	ND[b]
20:4	0.63 ± 0.05	ND[b]	1.43 ± 0.50	0.14 ± 0.05
20:5ω3	25.9 ± 2.30	26.1 ± 1.50	15.2 ± 0.30	30.8 ± 2.50
22:1	1.24 ± 0.40	3.49 ± 0.80	0.77 ± 0.05	1.07 ± 0.50
22:6ω3	8.50 ± 0.85	8.33 ± 1.01	3.77 ± 0.80	9.81 ± 0.80

[a]Mean ± SD of triplicate determinations from different experiments; [b]Not detected

Table 4.32 Fatty acid distribution in different positions of triacylglycerols of EPA+DHA enriched evening primrose oil (EPO)[a]

Fatty acids (w/w %)	TAG	*sn*-1	*sn*-2	*sn*-3
8:0	0.02 ± 0.01	0.35 ± 0.10	ND[b]	0.89 ± 0.04
10:0	0.04 ± 0.01	0.81 ± 0.03	0.03 ± 0.01	0.44 ± 0.20
12:0	0.04 ± 0.01	0.49 ± 0.06	0.03 ± 0.01	0.18 ± 0.51
16:0	3.75 ± 0.40	3.43 ± 0.30	1.61 ± 0.05	4.10 ± 0.80
16:1	ND[b]	1.06 ± 0.20	0.07 ± 0.01	0.32 ± 0.30
18:0	1.15 ± 0.50	1.49 ± 0.40	1.28 ± 0.40	2.00 ± 0.05
18:1	6.22 ± 0.80	2.14 ± 0.70	2.53 ± 0.60	14.2 ± 0.70
18:2ω6	45.6 ± 1.50	40.1 ± 2.40	61.0 ± 2.80	32.6 ± 1.50
18:3ω6	8.59 ± 0.50	4.22 ± 0.70	10.8 ± 0.90	9.04 ± 1.10
18:3ω3	0.13 ± 0.05	2.71 ± 0.50	0.33 ± 0.02	1.02 ± 0.50
20:0	ND[b]	0.82 ± 0.50	ND[b]	ND[b]
20:1	0.40 ± 0.20	1.38 ± 0.41	0.23 ± 0.08	0.32 ± 0.20
20:2	0.20 ± 0.03	ND[b]	ND[b]	ND[b]
20:4	0.58 ± 0.02	ND[b]	ND[b]	ND[b]
20:5ω3	25.0 ± 1.00	31.5 ± 2.00	17.2 ± 0.30	24.1 ± 0.60
22:1	0.37 ± 0.03	0.92 ± 0.06	ND[b]	ND[b]
22:6ω3	7.91 ± 0.91	5.80 ± 0.70	4.82 ± 0.55	10.5 ± 1.00

[a]Mean ± SD of triplicate determinations from different experiments; [b]Not detected

in the *sn*-1 and *sn*-3 positions (Wanasundara and Shahidi, 1997; Ikeda *et al.*, 1998). Ando *et al.* (1992) have also determined the positional distribution of fatty acids in the TAG of fish oils (capelin, herring, menhaden, sardine and saury) by high-performance liquid chromatography using a chiral stationary phase and found that in these oils DHA was present predominantly at the *sn*-2 position. However, Ota *et al.* (1994) found that in flounder liver and flesh lipids there was no preference for the *sn*-2 position in contrast to the general tendency for distribution of long-chain PUFA of fish oils in this position. Analysis of DHA-rich fish oils (bonito head and tuna orbital) was reported by Ando *et al.* (1996). The TAG showed a preferential occupation of the *sn*-1 and *sn*-3 positions by the DHA, especially of the *sn*-3 position.

Shimada *et al.* (2000) synthesized a SL by acidolysis of tripalmitin with arachidonic acid (AA; 20:4ω6) using 1,3-specific *Rhizopus delemar* lipase. They reported that in SL, AA was predominantly present at *sn*-1,3 positions (56.9%) of TAG.

Stereospecific analysis of TAG and hydrolysis products provides some useful information about the mechanism of gastric digestion. Intestinal absorption of fatty acids has been reported to be dependent on their arrangement in the TAG molecules. The influence of fatty acid specificity on intestinal absorption in adults has recently been reviewed and positively correlated with the TAG structure in chylomicrons (Small, 1991). During digestion, fatty acids in the *sn*-1 and *sn*-3 positions of the TAG are liberated by a positional specific enzyme such as pancreatic lipase, but the fatty acids attached to the *sn*-2 position of the TAG are absorbed and distributed in the body in the chylomicron form

(Nelson and Innis, 1999). However, clinical studies need to be carried out to verify this latter assumption. The enzymatically modified oils prepared in this study would be potentially useful as they contain desirable functional fatty acids in the same molecule.

4.6 Chemical characteristics of oils

After preparation of enzymatically modified (DHA-enriched, EPA-enriched and EPA+DHA-enriched) oils, their chemical properties, mainly peroxide value, conjugated dienes, thiobarbituric acid reactive substances (TBARS), iodine value, saponification value and acid value, were determined using standard methodologies described by the American Oil Chemists' Society (1990). Tables 4.33 and 4.34 summarize the characteristics of unmodified BO and EPO and their enzymatically modified-counterparts. These oils had good initial qualities with peroxide values of 1.7-3.2 (meq/kg of oil) for native oils and 0.2-5.2 (meq/kg of oil) for their enzymatically modified counterparts (Tables 4.33 and 4.34). The higher peroxide value of modified oils may be a consequence of their higher content of more readily oxidizable PUFA as compared to their unmodified counterparts. Since the peroxide value of these oils was below 10, the maximum acceptable level for edible oils (Frankel, 1985), it is assumed that oxidation has not occurred to any appreciable extent. Similarly, enzymatically modified oils had higher conjugated dienes and TBARS values compared to unmodified oils. Spectrophotometric determination of conjugated dienes determines the diene conjugation of unsaturated linkages present. The conjugated diene values of unmodified and modified oils were 1.5-9.8 and 3.4-10.1, respectively, while

Table 4.33 Chemical characteristics of unmodified and enzymatically modified borage oils (BO)[a]

Characteristic	Unmodified BO	DHA-enriched BO	EPA-enriched BO	EPA+DHA-enriched BO
Peroxide value (meq/kg)	1.7 ± 0.4	4.0 ± 0.9	0.2 ± 0.9	3.7 ± 0.5
Conjugated dienes	1.5 ± 0.7	4.7 ± 0.5	3.4 ± 0.4	6.1 ± 0.1
TBARS (µmol/g)	2.0 ± 0.1	5.2 ± 0.3	4.1 ± 0.5	6.0 ± 0.1
Iodine value	141.7 ± 0.7	198.4 ± 0.7	250.8 ± 1.1	247.1 ± 0.9
Saponification value	269.4 ± 0.9	118.7 ± 0.5	103.0 ± 0.6	142.3 ± 0.5
Acid value	0.6 ± 0.1	0.5 ± 0.3	0.7 ± 0.4	0.4 ± 0.2

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

Table 4.34 Chemical characteristics of unmodified and enzymatically modified evening primrose oils (EPO)[a]

Characteristic	Unmodified EPO	DHA-enriched EPO	EPA-enriched EPO	EPA+DHA-enriched EPO
Peroxide value (meq/kg)	3.2 ± 0.5	5.2 ± 0.5	0.4 ± 0.9	4.1 ± 0.7
Conjugated dienes	9.8 ± 0.2	9.9 ± 0.4	10.1± 0.1	9.7 ± 0.3
TBARS (μmol/g)	0.4 ± 0.2	3.1 ± 0.5	5.8 ± 0.6	6.0 ± 0.2
Iodine value	153.9 ± 0.8	207.7 ± 0.6	259.2 ± 1.0	232.6 ± 0.7
Saponification value	273.5 ± 0.2	142.5 ± 0.4	120.7 ± 0.7	118.4 ± 0.9
Acid value	0.9 ± 0.2	1.1 ± 0.4	1.0 ± 0.3	0.8 ± 0.3

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

corresponding TBARS values were 0.4-2.0 and 3.1-6.0 (μmol malonaldehyde equivalents/ g of oil) (Tables 4.33 and 4.34). The iodine values of enzymatically modified oils were higher than those of their unmodified counterparts. This could be attributed to a higher degree of unsaturation in the modified oils. The iodine values of unmodified and modified oils were 141.7-153.9 and 198.4-259.2, respectively (Tables 4.33 and 4.34). The saponification values of enzymatically modified oils were lower than those of their modified counterparts. The unmodified and modified oils had saponification values of 269.4-273.5 and 103.0-142.5, respectively (Tables 4.33 and 4.34). However, the acid values were similar for both modified (0.4-1.1) and unmodified (0.6-0.9) oils (Tables 4.33 and 4.34). The quantity of FFA present in oils is expressed as the acid value which serves as an important quality indicator for edible oils.

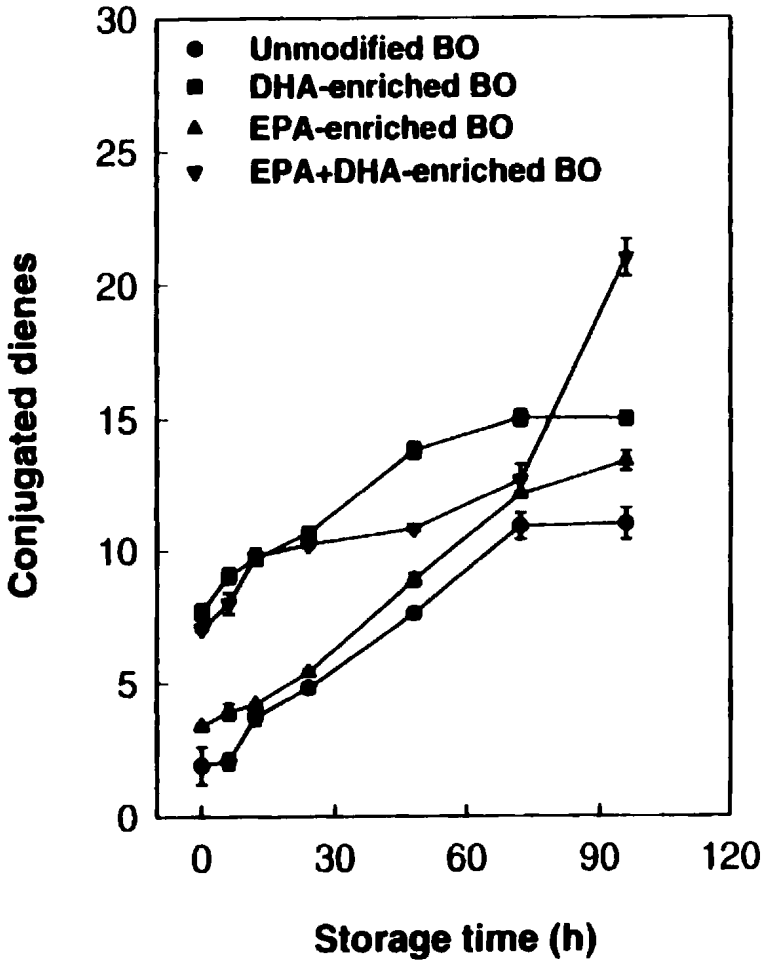
4.7 Oxidative stability of enzymatically modified oils

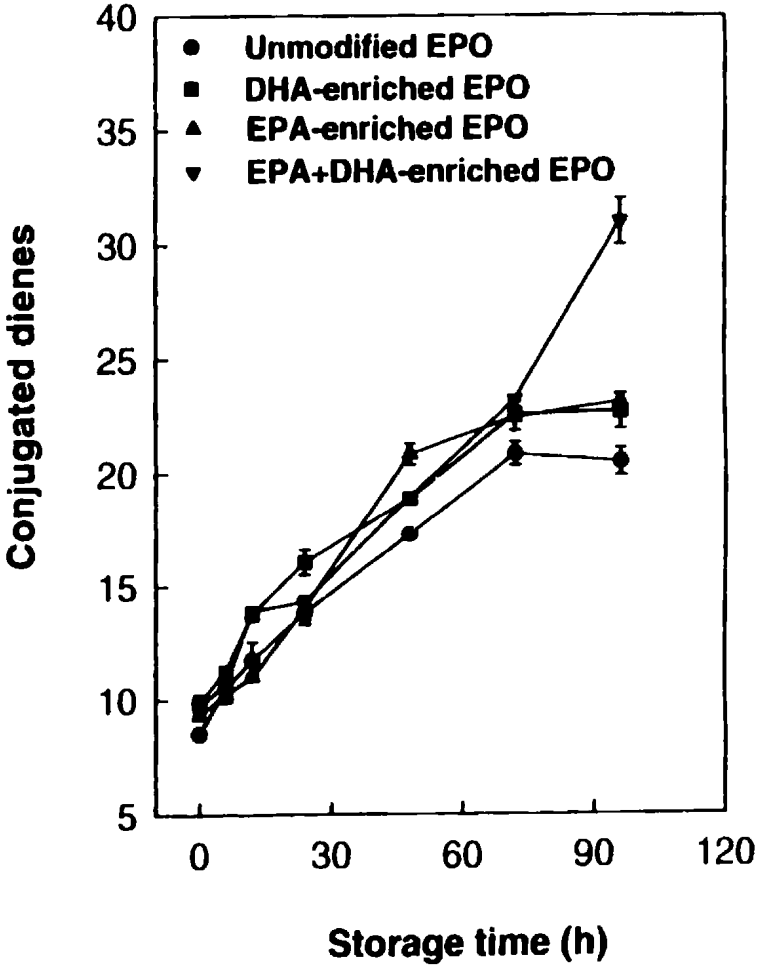
The enzymatically modified oils, namely DHA, EPA and EPA+DHA-enriched oils, produced under optimum reaction conditions, were assessed for their oxidative stability under Schaal oven conditions at 60°C over a 96 h period. The progression of oxidation was monitored by employing conjugated diene determination, thiobarbituric acid reactive substances (TBARS) test, headspace analysis of volatiles and proton nuclear magnetic resonance (H^1 NMR) spectroscopy.

4.7.1 Conjugated dienes

The formation of conjugated dienes (CD), as reflected in the absorption readings at 234 nm, during storage of DHA, EPA and EPA+DHA-enriched oils is shown in Figure 4.29. Conjugated dienes reflect the content of primary products of oxidation. The enzymatically-modified oils had higher conjugated diene values than unmodified oils. All samples, both unmodified and enzymatically modified, followed an increasing trend in their CD content throughout the experimentation, but the rates of formation of CD were different for each. 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, CD of DHA, EPA and EPA+DHA-enriched BO were 7.7, 3.4 and 7.1, respectively. The initial CD values of DHA, EPA and EPA+DHA-enriched EPO were within the range of 9.3-9.9. Unmodified BO and EPO had corresponding values of 1.9 and 8.5, respectively. The CD of DHA, EPA and EPA+DHA-enriched BO increased to 15.0, 13.4 and 21.0, respectively, after 4 days of storage under Schaal oven conditions at 60°C. However, corresponding CD values for unmodified BO did not exceed 11.0. Similarly, the CD of DHA, EPA and EPA+DHA-enriched EPO reached 22.7, 23.1 and 31.0, respectively, as compared to that of 20.5 for unmodified EPO. The high content of CD in enzymatically modified oils may arise from their high proportions of readily oxidizable ω3 PUFA as compared to those of their unmodified counterparts. Lipid radicals formed during the initial oxidation step may undergo rearrangement; thus the

Figure 4.29 Conjugated diene values of DHA, EPA and EPA+DHA-enriched borage (BO) and evening primrose oils (EPO) as well as their unmodified counterparts stored under Schaal oven conditions at 60°C


Unmodified BO
DHA-enriched BO
EPA-enriched BO
EPA+DHA-enriched BO
Conjugated dienes
Storage time (h)
0
5
10
15
20
25
30
0
30
60
90
120


Unmodified EPO
DHA-enriched EPO
EPA-enriched EPO
EPA+DHA-enriched EPO
Conjugated dienes
Storage time (h)
5
10
15
20
25
30
35
40
0
30
60
90
120

methylene-interrupted feature of PUFA is lost in favour of formation of CD, including those of hydroperoxides. CD values may be used to determine the initial rate of lipid oxidation and formation of primary oxidation products (Gray, 1978). Formation of lipid hydroperoxides normally coincides with the CD upon autoxidation (Farmer and Sutton, 1943; Jackson, 1981). Since hydroperoxides, the primary products of lipid oxidation (Labuza, 1971), are unstable, measurement of peroxide value provides only information about the initial oxidation potential of the oil. The CD assay is faster than PV determination and does not depend on chemical reactions such as colour development for its determination. It has been observed that conjugated dienes are formed due to the shift in the double bond position upon oxidation of lipids containing dienes or polyenes (Logani and Davies, 1980). St. Angelo *et al.* (1975) have suggested that conjugated diene values can be used as an index of stability for lipid-containing foods.

Moussata and Akoh (1998) measured the oxidative stability (using oxidation tests such as conjugated dienes, peroxide value and oxidative stability index) of melon seed oil interesterified with high oleic sunflower oil, and found that oleic acid could enhance the stability of melon seed oil. This observation is in accordance with previous studies of sardine oil, interesterified with oleic acid (Endo *et al.*, 1993) and reflects incorporation of a less unsaturated fatty acid in the oils examined.

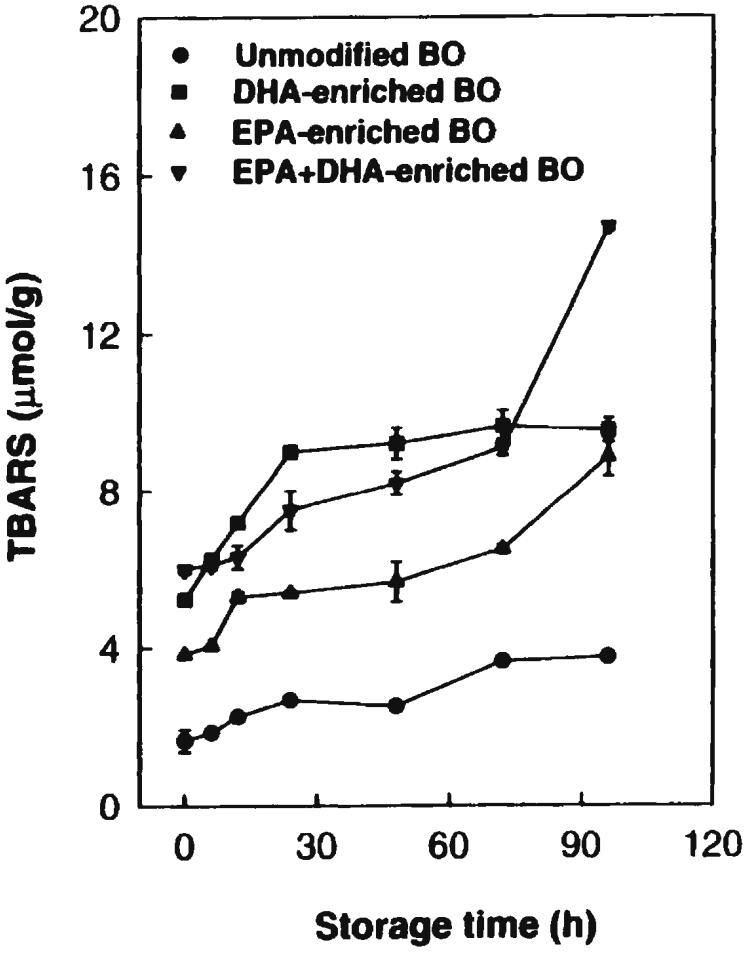
4.7.2 Thiobarbituric acid reactive substances (TBARS)

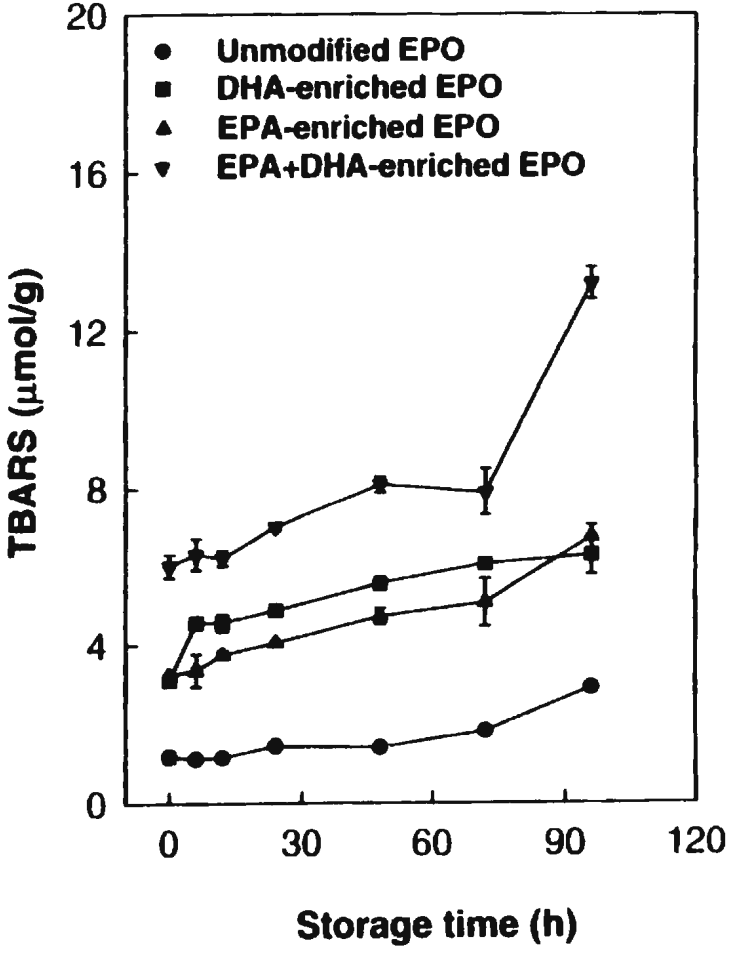
The TBARS test, which measures secondary products of lipid oxidation, is the most

frequently used indicator for monitoring stability of edible oils. Production of TBARS of both enzymatically modified and unmodified oils are given in Figure 4.30. The TBARS values, expressed as μmol malonaldehyde equivalents per g oil, increased progressively over the entire storage period. Furthermore, enzymatically modified oils had significantly higher ($p \leq 0.05$) TBARS values than those of their unmodified counterparts. The observed changes in TBARS (Figure 4.30) are similar to the trends observed for changes in conjugated dienes (Figure 4.29). 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 enzymatically modified oils is due to the incorporation of high proportions of PUFA in the oils examined. Therefore, oils modified with highly unsaturated fatty acids were more susceptible to oxidation than their unmodified counterparts.

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 that resulted in 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).

Figure 4.30 TBARS values of DHA, EPA and EPA+DHA-enriched borage (BO) and evening primrose oils (EPO) as well as their unmodified counterparts stored under Schaal oven conditions at 60°C


Unmodified BO
DHA-enriched BO
EPA-enriched BO
EPA+DHA-enriched BO
TBARS (μmol/g)
Storage time (h)
0
4
8
12
16
20
0
30
60
90
120


Unmodified EPO
DHA-enriched EPO
EPA-enriched EPO
EPA+DHA-enriched EPO
TBARS (μmol/g)
Storage time (h)
0
4
8
12
16
20
0
30
60
90
120

4.7.3 Headspace volatile analysis

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 primary markers of oxidative deterioration of vegetable and fish oils, respectively (Frankel *et al.*, 1994; Shahidi and Spurvey, 1996).

The oxidative stability of enzymatically modified oils, namely DHA, EPA and EPA+DHA-enriched oils, was compared with that of unmodified oils by measuring volatile products formed during storage using static headspace gas chromatographic (GC) analysis. 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 aldehydes, mainly hexanal and propanal, was accomplished using 2-heptanone as an internal standard.

The chromatograms of headspace volatiles of oils (both modified and unmodified) after 48, 72 and 96 hours of storage under Schaal oven conditions at 60°C are shown in Figures 4.31 - 4.34. Headspace analysis of these oils revealed a striking increase in the number and intensity of peaks (Figures 4.31 - 4.34). Similar to other sources of ω6 fatty

Figure 4.31 Chromatograms of the headspace volatiles of unmodified borage oil (BO) after 48, 72 and 96 h of storage under Schaal oven conditions at 60°C

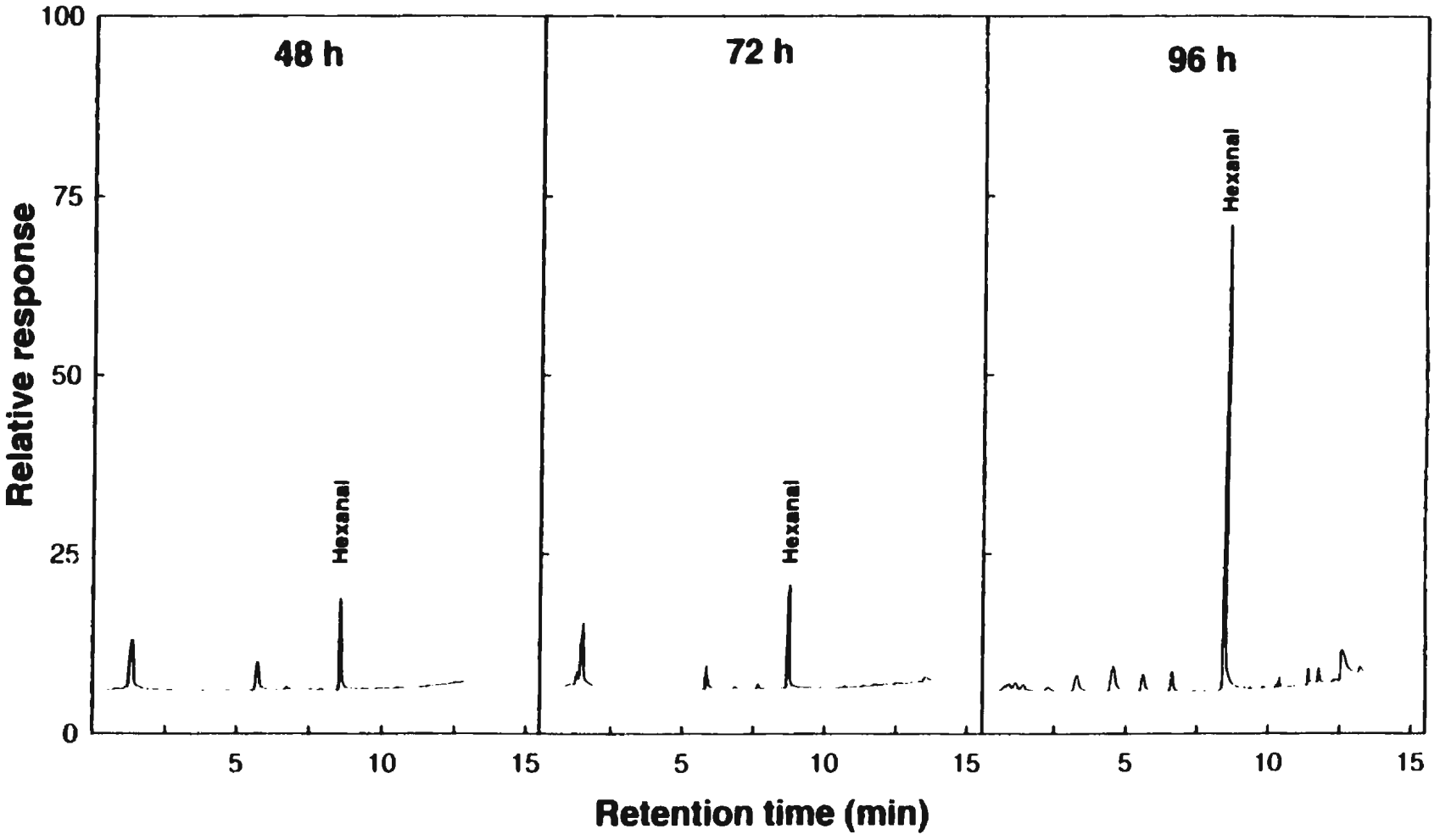

48 h
72 h
96 h
Hexanal
Hexanal
Hexanal
Relative response
100
75
50
25
0
5
10
15
Retention time (min)

Figure 4.32 Chromatograms of the headspace volatiles of unmodified evening primrose oil (EPO) after 48, 72 and 96 h of storage under Schaal oven conditions at 60°C

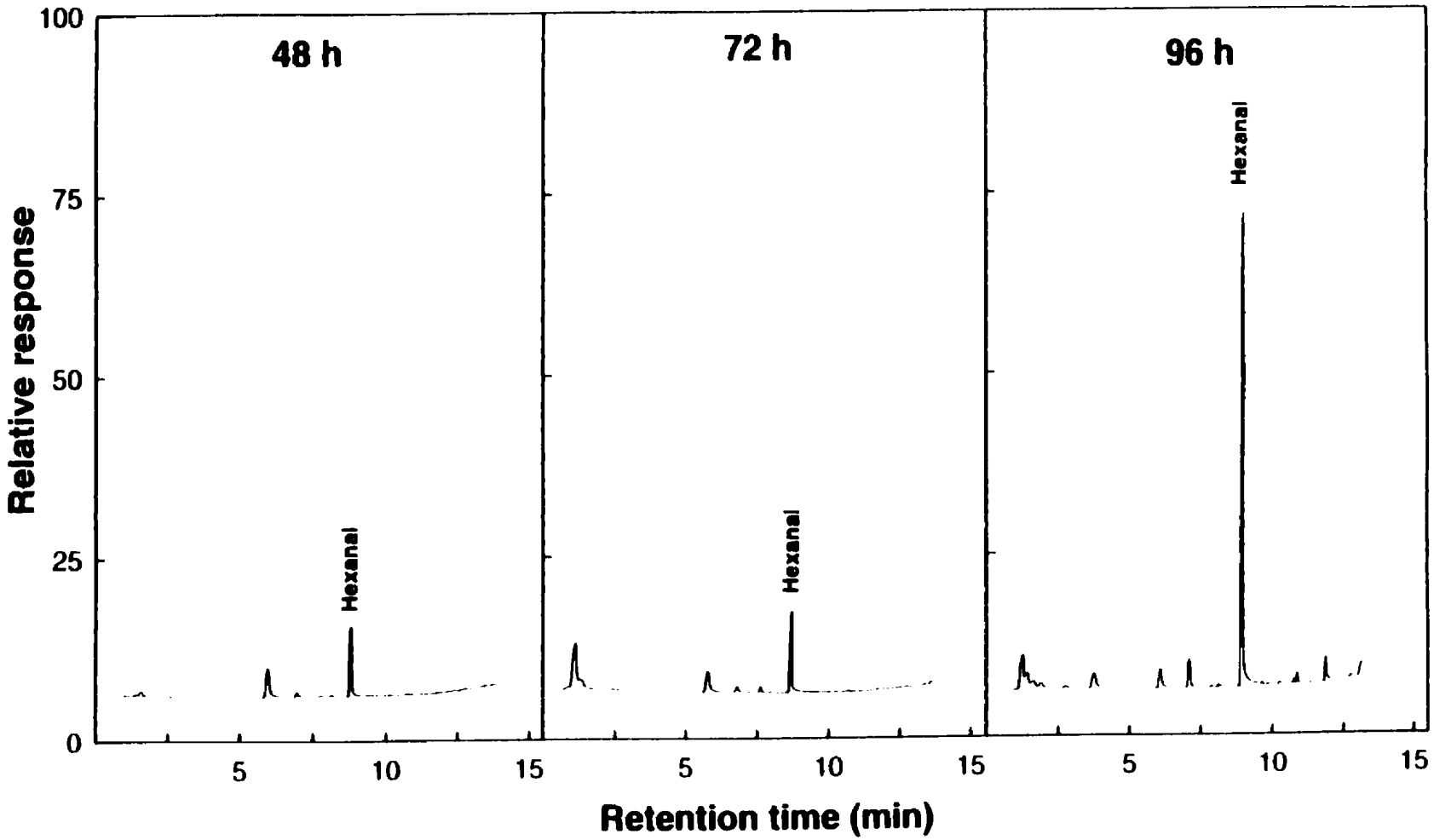

48 h
72 h
96 h
Hexanal
Hexanal
Hexanal
Relative response
100
75
50
25
0
5
10
15
Retention time (min)

Figure 4.33 Chromatograms of the headspace volatiles of EPA+DHA-enriched borage oil (BO) after 48, 72 and 96 h of storage under Schaal oven conditions at 60°C

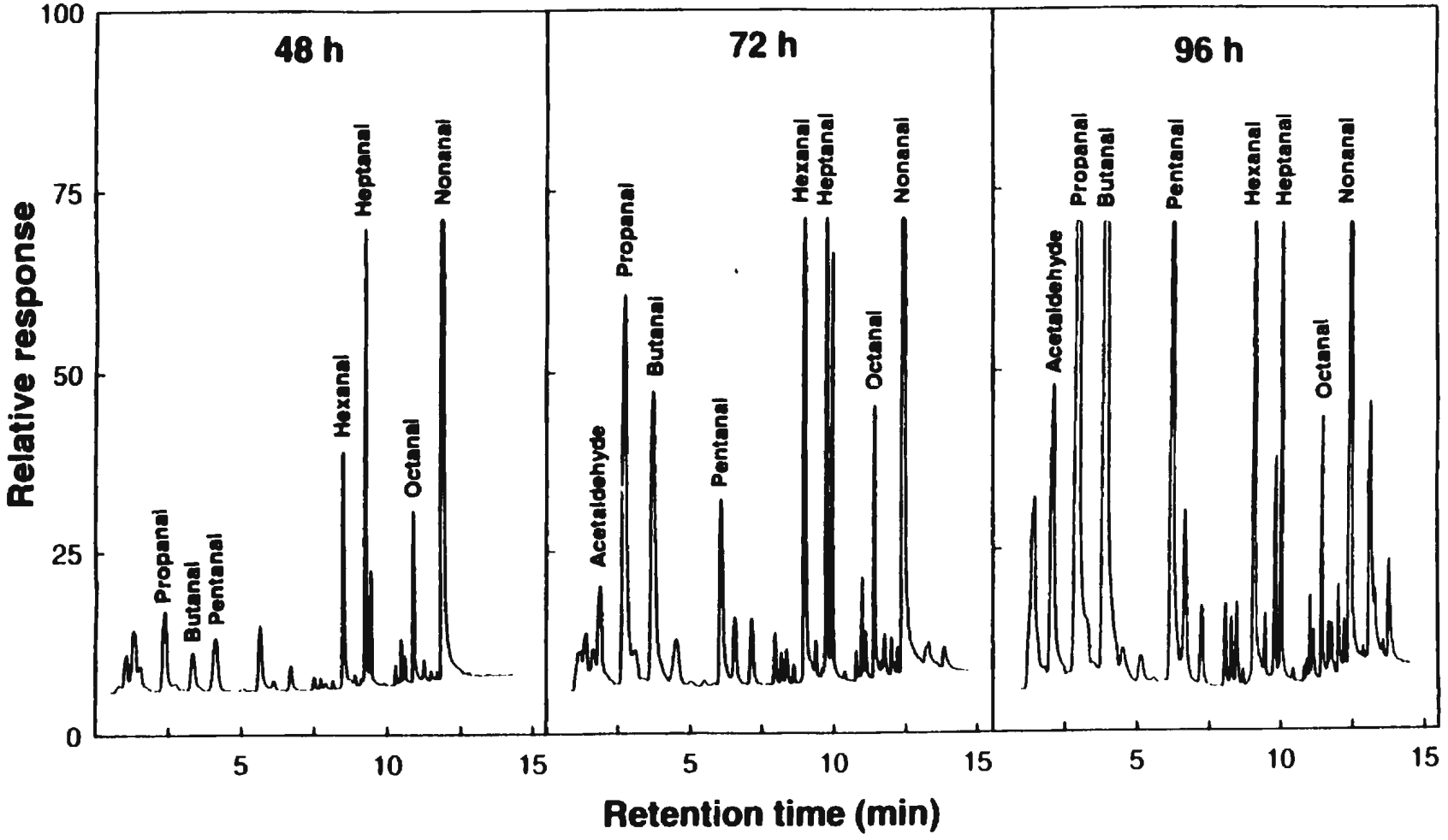
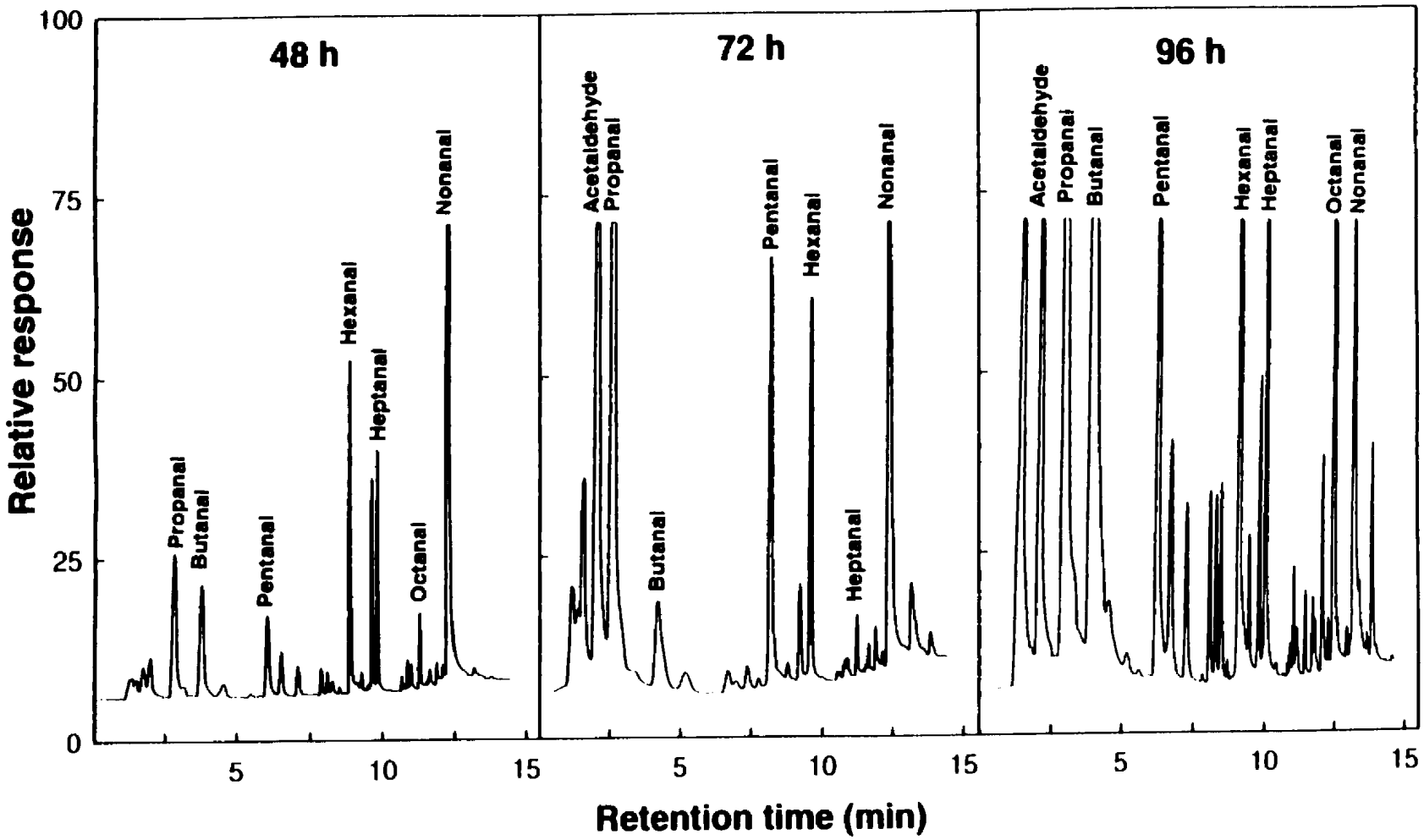

48 h
72 h
96 h
Relative response
Retention time (min)
100
75
50
25
0
5
10
15
Propanal
Butanal
Pentanal
Hexanal
Heptanal
Octanal
Nonanal
Acetaldehyde

Figure 4.34 Chromatograms of the headspace volatiles of EPA+DHA-enriched evening primrose oil (EPO) after 48, 72 and 96 h of storage under Schaal oven conditions at 60°C


48 h
72 h
96 h
Relative response
Retention time (min)
100
75
50
25
0
5
10
15
Propanal
Butanal
Pentanal
Hexanal
Heptanal
Octanal
Nonanal
Acetaldehyde
Propanal
Butanal
Pentanal
Hexanal
Heptanal
Nonanal
Acetaldehyde
Propanal
Butanal
Pentanal
Hexanal
Heptanal
Octanal
Nonanal

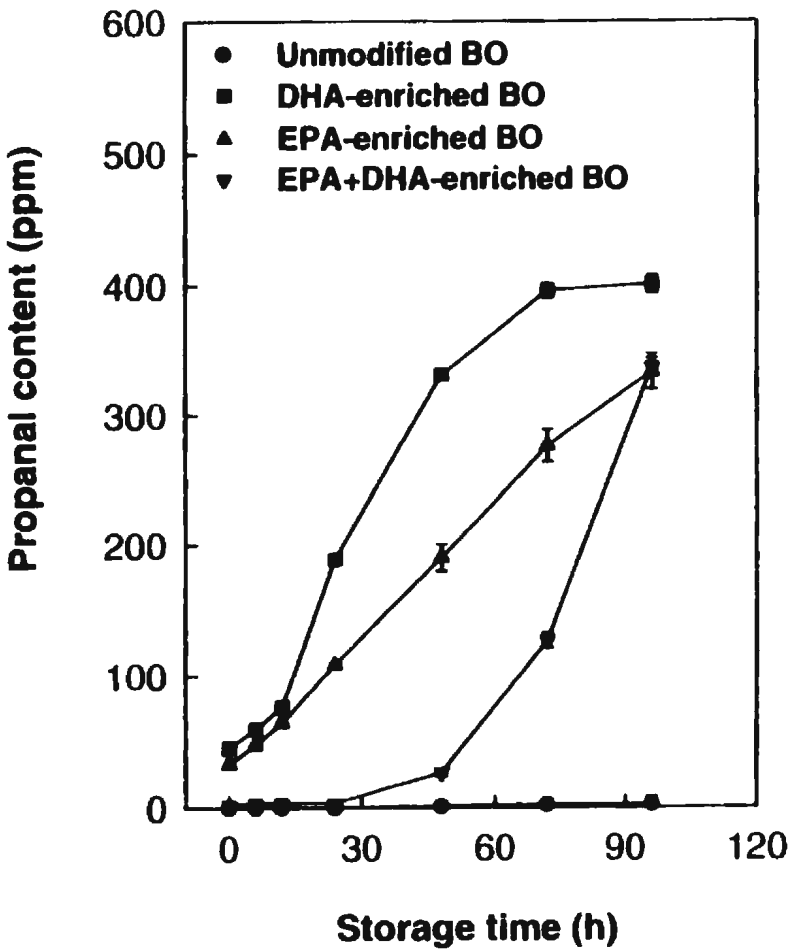
acids, unmodified BO and EPO degrade and produce hexanal as their dominant volatile (Figures 4.31 and 4.32). This is not surprising since BO and EPO contain substantial amounts of ω6 fatty acids, mainly LA. Upon enrichment of these oils with EPA and DHA *via* enzyme-catalysed acidolysis, they produced both hexanal and propanal as their main volatiles during degradation; propanal being a major breakdown product of ω3 fatty acids such as EPA and DHA (Frankel *et al.*, 1992; Frankel and Huang, 1994). The other volatile compounds identified (by comparing relative retention times with those of authentic standards) in enzymatically modified (EPA, DHA and EPA+DHA-enriched) oils were acetaldehyde, butanal, pentanal, heptanal, octanal and nonanal (Figures 4.33 and 4.34). The formation of these volatiles appears to be consistent with autoxidation of EPA, DHA and LA which are present in appreciable amounts in modified oils. The absence of 2,4-decadienal, a major autoxidation product of LA, in the oxidized samples may be attributed to its further oxidation to hexanal (Schieberle and Grosch, 1981; Robards *et al.*, 1988b). Konishi *et al.* (1995) and Neff and List (1999) reported that LA-derived volatiles, predominantly pentane, pentanal, hexanal and heptenal, were formed following oxidation of soybean oil stored at 60°C. Grun *et al.* (1996) were able to identify some volatiles, mainly 2,4-heptadienal and 2,4-decadienal, during autoxidation of menhaden oil stored at 30°C. On the other hand, Neff *et al.* (1994) reported a number of compounds (propanal, 2,4-heptadienal, 2-heptenal, pentanal, pentane, hexanal and nonanal) as major volatiles generated during oxidation of canola oil. Shahidi and Spurvey (1996) identified formaldehyde, acetaldehyde, propanal, pentanal and hexanal as the most dominant

aldehydes formed during oxidation of fish muscle lipids. Medina *et al.* (1999) noted that in fish muscle lipids, the levels of acetaldehyde, propanal, heptane, 2-ethylfuran, pentanal and hexanal increased appreciably during oxidation. Maloba *et al.* (1996) reported that the production of volatiles in sunflower oil was related to the extent of oil deterioration.

The amount of propanal produced in modified oils increased with the storage time (Figure 4.35). Under similar conditions, unmodified oils showed no significant formation of propanal due to their low content of ω3 PUFA as compared to the amounts present in the modified oils. Similar increases in propanal levels during storage of fish oils (Frankel, 1993b) and fish muscle lipids (Shahidi and Spurvey, 1996; Medina *et al.*, 1999) were reported. The initial oxidation products of α-linolenic acid (ALA) are dominated by 9-, 12-, 13- and 16-hydroperoxides because the diallylic radicals formed favour the attack of oxygen on these specified positions (Frankel, 1984). The hydroperoxides so formed degrade to a variety of products, including propanal, ethane and 2,4,7-decatrienal (Ho *et al.*, 1996). Propanal is a predominant oxidation product of ALA and lipids containing a large proportion of this fatty acid or those containing long-chain PUFA such as EPA and DHA (Shahidi and Spurvey, 1996; He and Shahidi, 1997b).

Surprisingly, the content of hexanal produced in enzymatically modified oils was significantly increased ($p \leq 0.05$) despite a great reduction in the proportion of LA in the products (Figure 4.36). Propanal and other oxidation products generated in modified oils may act as pro-oxidants. Various pro-oxidants can accelerate lipid oxidation and it seemed feasible that secondary degradation products of hydroperoxides (volatiles) act as pro-

Figure 4.35 Propanal contents of DHA, EPA and EPA+DHA-enriched borage (BO) and evening primrose oils (EPO) as well as their unmodified counterparts stored under Schaal oven conditions at 60°C


Unmodified BO
DHA-enriched BO
EPA-enriched BO
EPA+DHA-enriched BO
Propanal content (ppm)
Storage time (h)
0
100
200
300
400
500
600
0
30
60
90
120

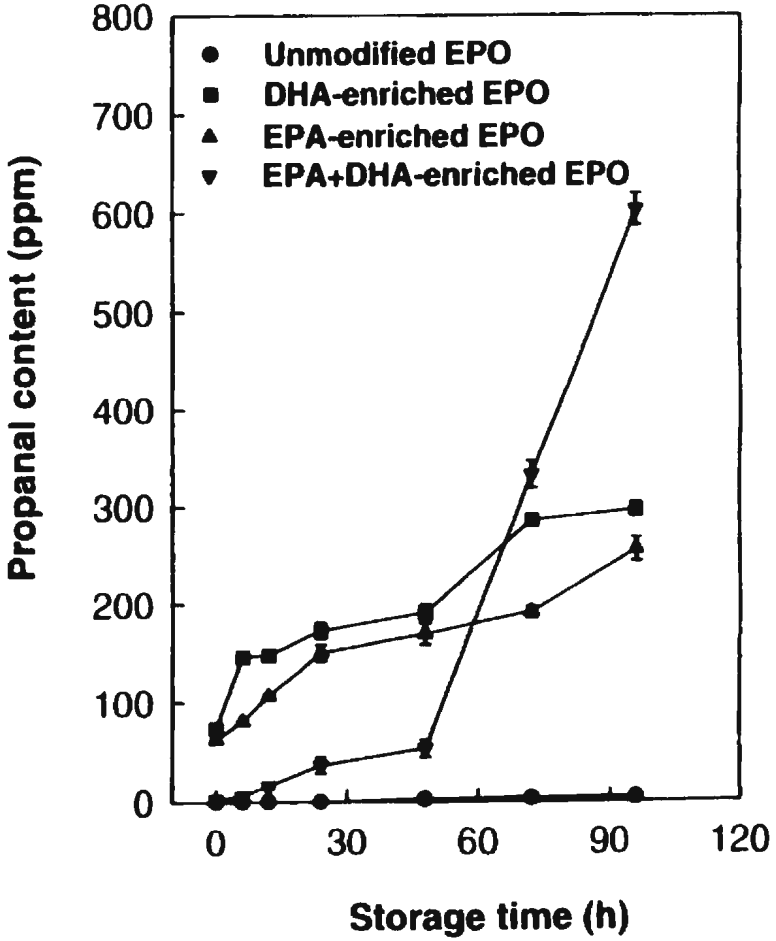
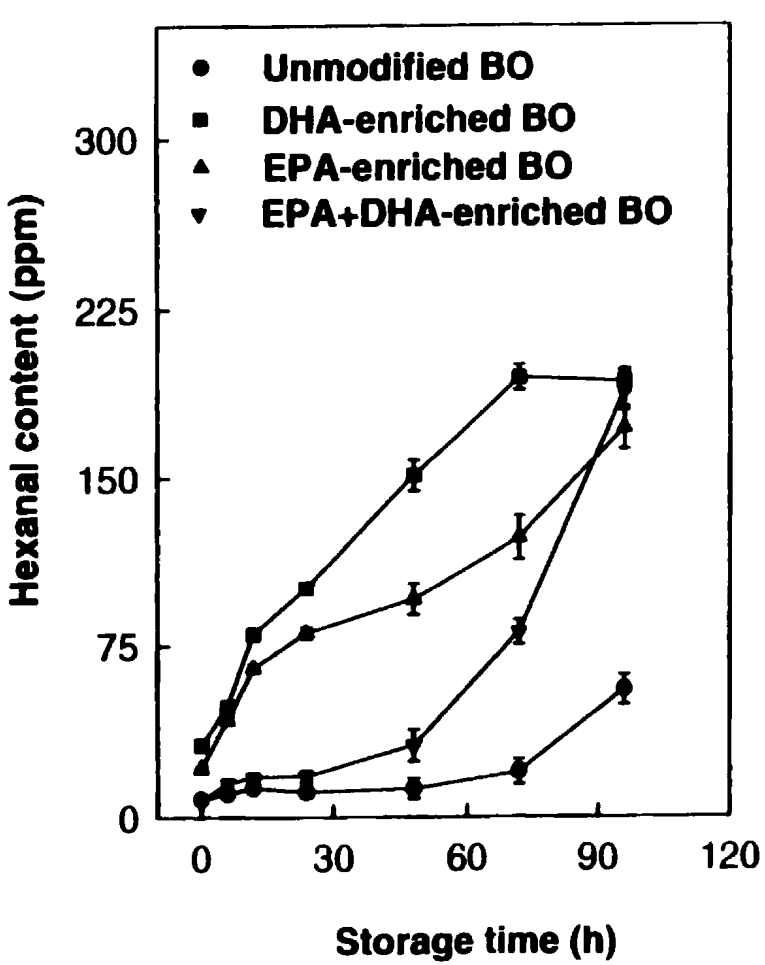
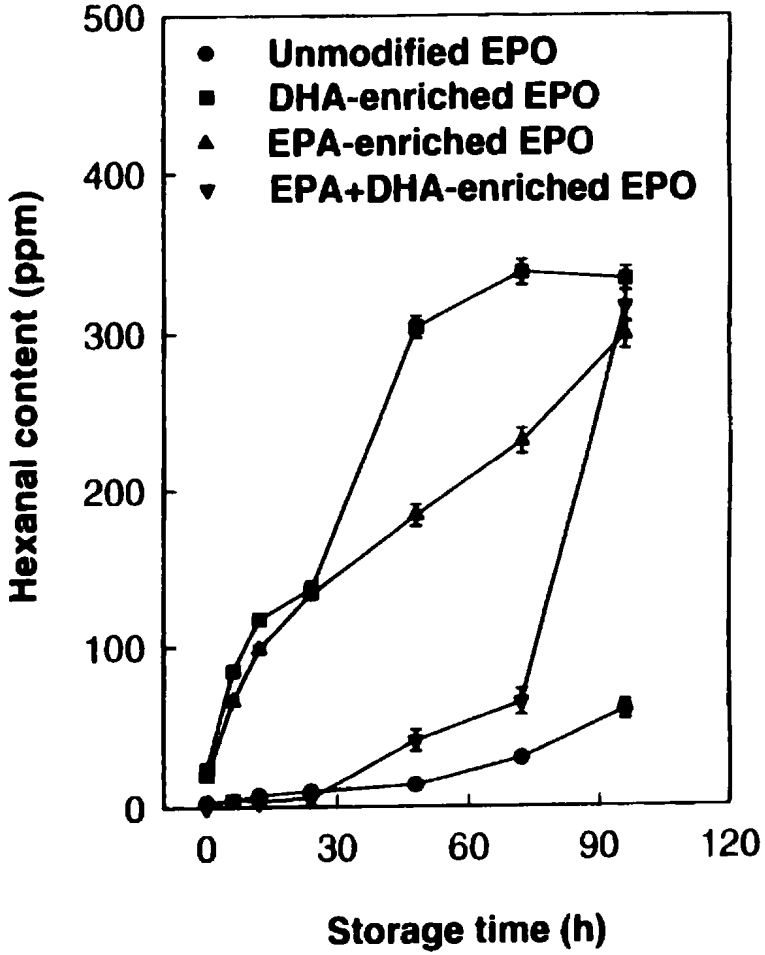

Unmodified EPO
DHA-enriched EPO
EPA-enriched EPO
EPA+DHA-enriched EPO
Propanal content (ppm)
Storage time (h)
0
100
200
300
400
500
600
700
800
0
30
60
90
120

Figure 4.36 Hexanal contents of DHA, EPA and EPA+DHA-enriched borage (BO) and evening primrose oils (EPO) as well as their unmodified counterparts stored under Schaal oven conditions at 60°C


Unmodified BO
DHA-enriched BO
EPA-enriched BO
EPA+DHA-enriched BO
Hexanal content (ppm)
0
75
150
225
300
0
30
60
90
120
Storage time (h)


Unmodified EPO
DHA-enriched EPO
EPA-enriched EPO
EPA+DHA-enriched EPO
Hexanal content (ppm)
0
100
200
300
400
500
0
30
60
90
120
Storage time (h)

oxidants.This might explain why a higher concentration of hexanal was observed in modified oils. This confirms the findings of El-Magoli *et al.* (1979) who reported that presence of lipid degradation products (volatile compounds resulting from PUFA oxidation) in the oils catalyzed their oxidation. Furthermore, Jung *et al.* (1989) reported that deodourization of oils may increase oxidative stability because prooxidants such as oxidation products, moisture, monoacylglycerols and free fatty acids remaining in the bleached oil are removed during this process. Based on hexanal contents (Figure 4.36), DHA, EPA and EPA+DHA- enriched BO and EPO were more susceptible to oxidation ($p \leq 0.05$) than their unmodified counterparts. This trend is similar to that obtained when considering TBARS values. Increase in hexanal contents during oxidation of high-oleic sunflower oil, blended with polyunsaturated vegetable oils, was also reported by Frankel and Huang (1994).

During the oxidation process, ω3 PUFA, especially EPA and DHA, produce more 18- and 20- hydroperoxides than other hydroperoxides which may afford propanal upon homolytic cleavage, respectively (Figure 2.12). Similarly, ω6 fatty acids, especially LA, produce more of the 13-hydroperoxides as their primary product of oxidation and produce hexanal upon homolytic cleavage (Figure 2.10) (Frankel, 1982). LA (ω6) can be expected to produce pentane, hexanal, malondialdehyde, octanal and 2,4-decadienal upon oxidation (Vega and Brewer, 1994). Shahidi *et al.* (1987) found a direct relationship between hexanal content and sensory scores of cooked ground pork which contains a high amount of LA. Medina *et al.* (1999) reported that headspace volatiles generated from oxidized fish muscle

lipids correlated highly with TBARS values. Warner *et al.* (1978) showed that pentanal and hexanal serve as good quality indicators in soybean oil since their content correlates well with flavour scores. Therefore, it is important to consider dominant fatty acids of oils before selecting a specific aldehyde as an indicator for oxidative stability determination.

Miyashita *et al.* (1994, 1995) investigated the oxidative stability of highly unsaturated fatty acids (HUFA) and HUFA-containing lipids in an aqueous system and reported that EPA and DHA, which were highly oxidizable in the air, were more stable than LA and ALA in water. However, Endo *et al.* (1997) showed that HUFA were very unstable in both nonaqueous and aqueous systems when they were highly concentrated in a single TAG molecule.

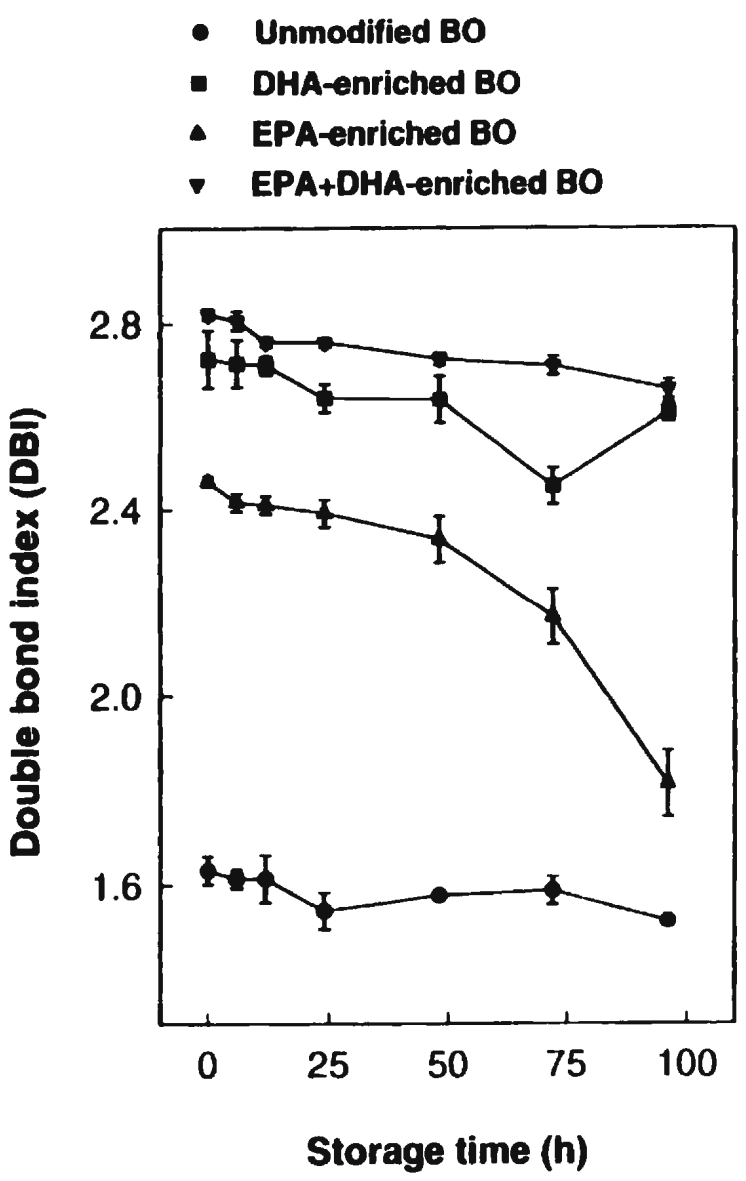
Song and Miyazawa (1997) compared the oxidative stability of DHA-enriched oils, in the form of phospholipids, triacylglycerols and ethyl esters, with those of a control oil (palm oil supplemented with 20% soybean oil; containing no DHA). Their results indicated that DHA-enriched oils in the form of phospholipids were oxidatively more stable than those in the form of triacylglycerols and ethyl esters.

4.7.4 Changes in double bond index (DBI) and methylene bridge index (MBI) during oxidation of oils

The changes in double bond index (DBI) and methylene bridge index (MBI) of DHA, EPA and EPA+DHA-enriched BO as well as unmodified BO were plotted against storage period (Figure 4.37). There was a decline in the DBI and MBI of modified and

unmodified oils during oxidation. The extent of decrease produced was greater ($p \leq 0.05$) in the modified oils, which are more unsaturated, but this decrease was minimum for unmodified BO. The DBI decreased from 2.73 to 2.61 in DHA-enriched BO, from 2.46 to 1.81 in EPA-enriched BO, from 2.82 to 2.66 in EPA+DHA-enriched BO and from 1.63 to 1.52 in unmodified BO (Figure 4.37). Similarly, the MBI was also decreased from 1.84 to 1.75 in DHA-enriched BO, from 1.59 to 1.39 in EPA-enriched BO, from 1.92 to 1.77 in EPA+DHA-enriched BO and from 0.80 to 0.73 in unmodified BO (Figure 4.37). Similar results were obtained for enzymatically modified EPO and unmodified EPO. The DBI decreased from 2.53 to 2.47 in DHA-enriched EPO, from 2.49 to 2.32 in EPA-enriched EPO, from 2.84 to 2.64 in EPA+DHA-enriched EPO and from 1.81 to 1.76 in unmodified EPO (Figure 4.38). The MBI decreased from 1.63 to 1.57 in DHA-enriched EPO, from 1.58 to 1.43 in EPA-enriched EPO, from 1.92 to 1.74 in EPA+DHA-enriched EPO and from 0.91 to 0.89 in unmodified EPO (Figure 4.38). As expected from their higher degrees of unsaturation, the DBI and MBI values were significantly higher ($p \leq 0.05$) in the enzymatically modified oils as compared with those of their unmodified counterparts. The DBI represents the number of double bonds while MBI represents the number of *bis*-allylic methylene bridge positions in PUFA (Vartak *et al.*, 1997). Wagner *et al.* (1994) demonstrated that the number of *bis*-allylic positions from which hydrogen can be abstracted by free radical processes is the major determinant for oxidizability of cellular lipids. In studies using homogeneous solutions of purified lipids, a linear correlation existed between the number of *bis*-allylic positions and the oxidizability of the lipids (Cosgrove *et*

Figure 4.37 Changes in double bond index (DBI) and methylene bridge index (MBI) of DHA, EPA and EPA+DHA-enriched borage oils (BO) and unmodified BO during storage at 60°C


Unmodified BO
DHA-enriched BO
EPA-enriched BO
EPA+DHA-enriched BO
Double bond index (DBI)
2.8
2.4
2.0
1.6
0
25
50
75
100
Storage time (h)

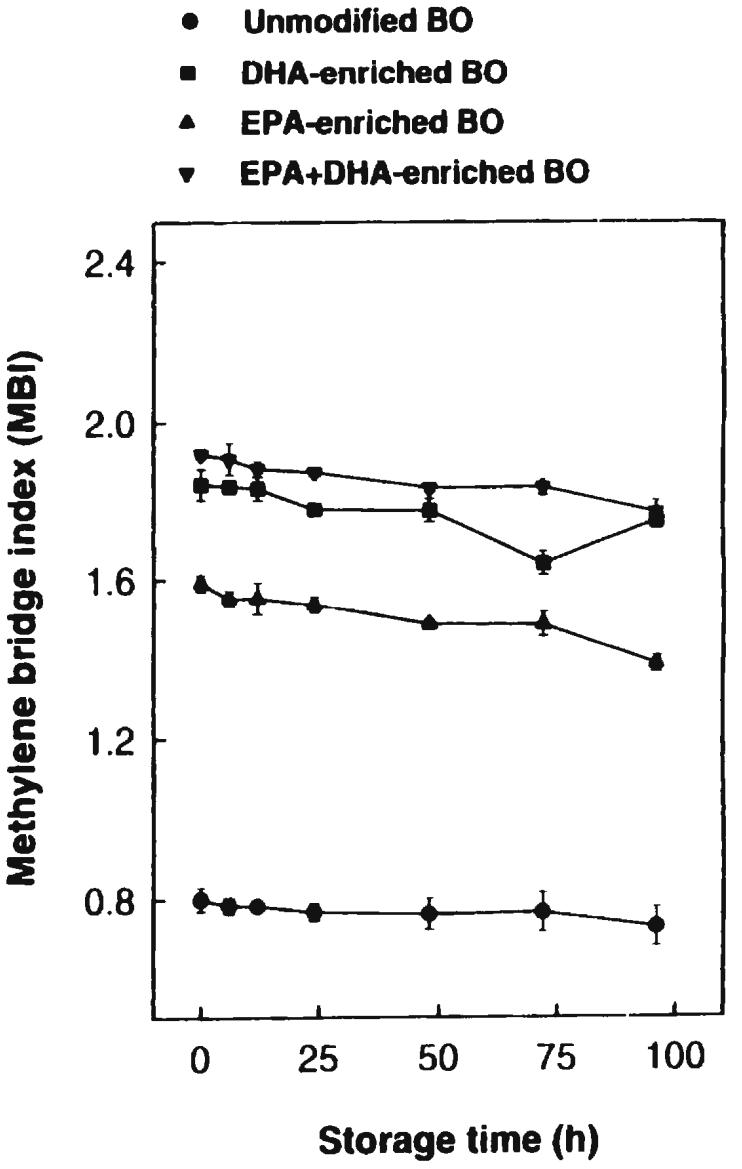
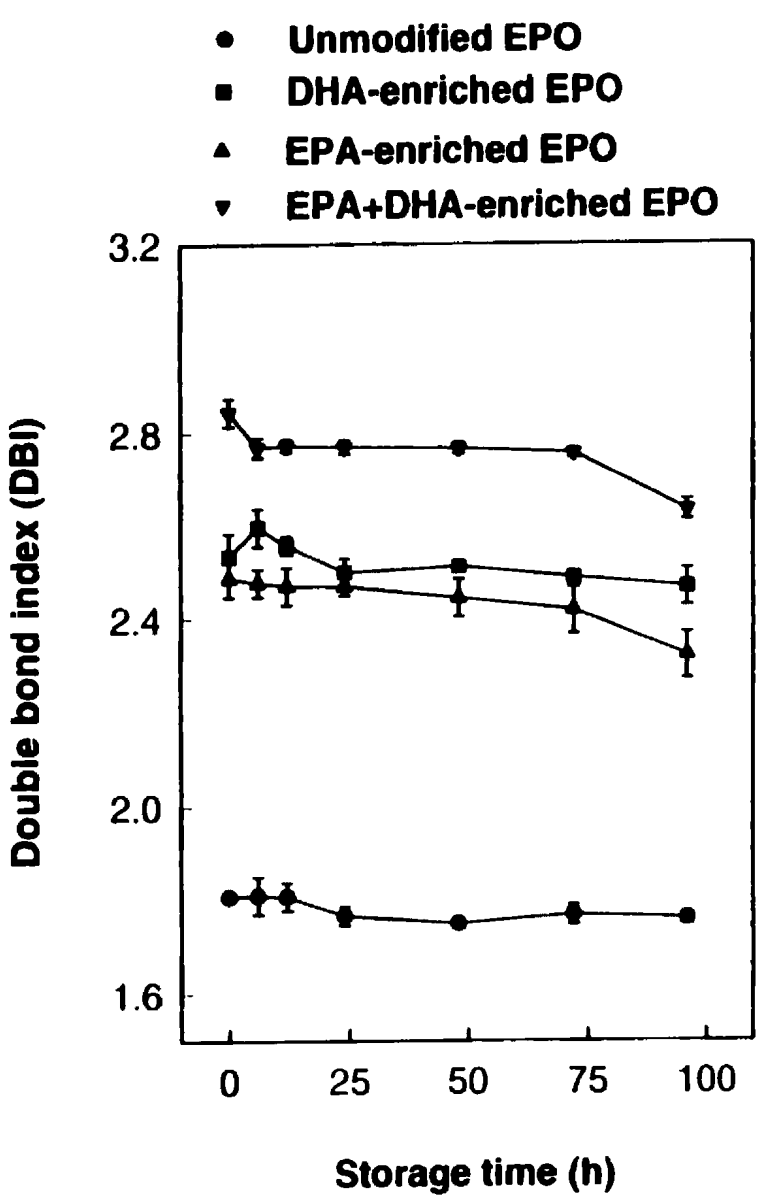
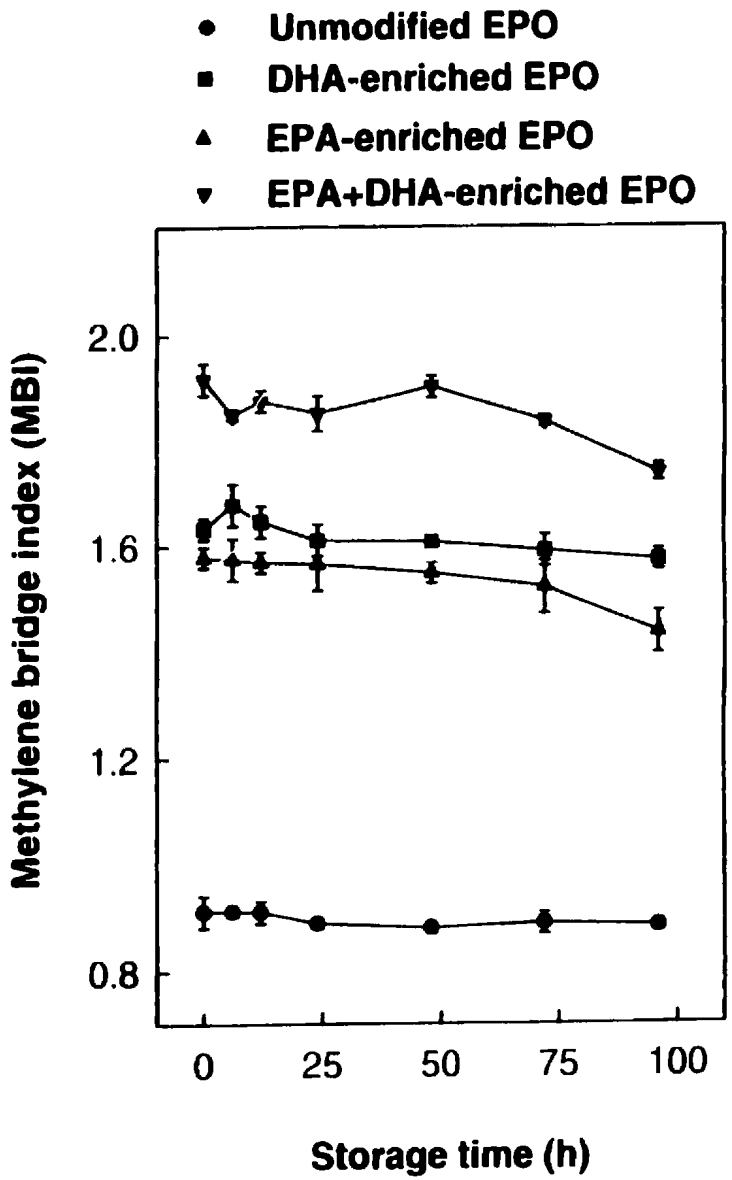

Unmodified BO
DHA-enriched BO
EPA-enriched BO
EPA+DHA-enriched BO
Methylene bridge index (MBI)
2.4
2.0
1.6
1.2
0.8
0
25
50
75
100
Storage time (h)

Figure 4.38 Changes in double bond index (DBI) and methylene bridge index (MBI) of DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO) and unmodified EPO during storage at 60°C


Unmodified EPO
DHA-enriched EPO
EPA-enriched EPO
EPA+DHA-enriched EPO
Double bond index (DBI)
3.2
2.8
2.4
2.0
1.6
0
25
50
75
100
Storage time (h)


Unmodified EPO
DHA-enriched EPO
EPA-enriched EPO
EPA+DHA-enriched EPO
Methylene bridge index (MBI)
2.0
1.6
1.2
0.8
0
25
50
75
100
Storage time (h)

al., 1987; Wagner *et al.*, 1994).

To test how DBI and MBI can be used to evaluate oxidative stability of oils, regression analyses were carried out between each index and conjugated dienes, TBARS, hexanal and propanal contents of oils. Since DBI and MBI decrease while other parameters increase during oxidation, negative correlations ($p \leq 0.05$) existed between these variables. Significant negative correlations ($p \leq 0.05$) between data for DBI and MBI and conjugated diene values of modified oils were present; correlation coefficients were 0.802-0.922 for DBI and 0.792-0.939 for MBI. However, corresponding values for unmodified oils were 0.741-0.752 and 0.742-0.910 ($p \leq 0.05$), respectively (Table 4.35; Figures A.2 and A.3). Good negative correlations were obtained between DBI or MBI and TBARS values of oils; correlation coefficients were 0.621-0.975 for DBI and 0.677-0.971 ($p \leq 0.1$) for MBI for modified oils and the corresponding values for their unmodified counterparts were 0.754-0.811 and 0.760-0.973 ($p \leq 0.05$), respectively (Table 4.35; Figures A.4 and A.5). In addition, negative correlations existed between DBI and MBI with hexanal content of oils; the correlation coefficients (r) were 0.728-0.932 for DBI and 0.793-0.971 ($p \leq 0.05$) for MBI for modified oils and 0.576-0.718 for DBI and 0.574-0.877 ($p \leq 0.1$) for MBI for their unmodified counterparts (Table 4.36; Figures A.6 and A.7). Propanal contents were also negatively correlated with those for DBI and MBI of modified oils with respective r = 0.703-0.912 and 0.751-0.942 ($p \leq 0.05$) (Table 4.36; Figures A.8 and A.9). Therefore, these results suggest that indicators such as DBI and MBI, representing structural characteristics of lipid molecules involved, have a great influence on oxidative stability of both modified

Table 4.35 Correlation coefficients (r) between conjugated dienes (CD) and double bond index (DBI) and methylene bridge index (MBI) as well as between thiobarbituric acid reactive substances (TBARS) and DBI and MBI of oxidized oils

Sample	CD *Vs* DBI	CD *Vs* MBI	TBARS *Vs* DBI	TBARS *Vs* MBI
Unmodified BO	-0.741[b]	-0.910[a]	-0.811[b]	-0.973[a]
DHA-enriched BO	-0.821[b]	-0.843[b]	-0.771[b]	-0.791[b]
EPA-enriched BO	-0.894[b]	-0.923[a]	-0.956[a]	-0.962[a]
EPA+DHA-enriched BO	-0.922[a]	-0.939[a]	-0.904[a]	-0.932[a]
Unmodified EPO	-0.752[b]	-0.742[b]	-0.754[b]	-0.760[b]
DHA-enriched EPO	-0.821[b]	-0.866[b]	-0.621[c]	-0.677[c]
EPA-enriched EPO	-0.802[b]	-0.792[b]	-0.975[a]	-0.971[a]
EPA+DHA-enriched EPO	-0.872[b]	-0.796[b]	-0.916[a]	-0.860[b]

[a]Significant at $p < 0.005$ level; [b]Significant at $p < 0.05$ level; [c]Significant at $p < 0.1$ level; BO, borage oil; EPO, evening primrose oil

Table 4.36 Correlation coefficients (r) between hexanal content and double bond index (DBI) and methylene bridge index (MBI) as well as between propanal content and DBI and MBI of oxidized oils

Sample	Hexanal content *Vs* DBI	Hexanal content *Vs* MBI	Propanal content *Vs* DBI	Propanal content *Vs* MBI
Unmodified BO	-0.718[b]	-0.877[b]	-	-
DHA-enriched BO	-0.842[b]	-0.865[b]	-0.831[b]	-0.853[b]
EPA-enriched BO	-0.932[a]	-0.971[a]	-0.912[a]	-0.942[a]
EPA+DHA-enriched BO	-0.865[b]	-0.896[b]	-0.839[b]	-0.868[b]
Unmodified EPO	-0.576[c]	-0.574[c]	-	-
DHA-enriched EPO	-0.728[b]	-0.793[b]	-0.703[b]	-0.751[b]
EPA-enriched EPO	-0.904[a]	-0.895[b]	-0.908[a]	-0.902[a]
EPA+DHA-enriched EPO	-0.911[a]	-0.877[b]	-0.853[b]	-0.876[b]

[a]Significant at $p < 0.005$ level; [b]Significant at $p < 0.05$ level; [c]Significant at $p < 0.1$ level; BO, borage oil; EPO, evening primrose oil

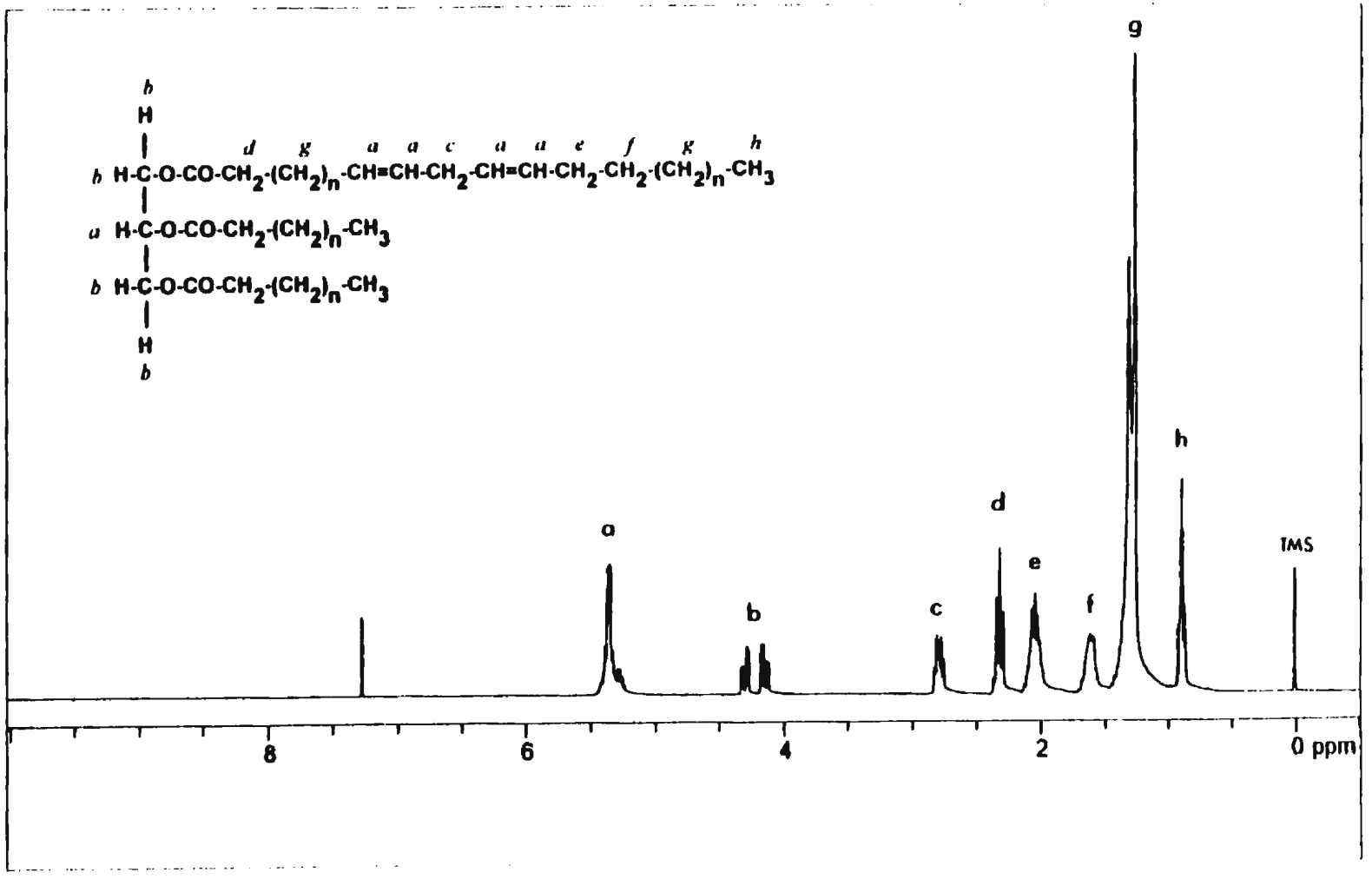
and unmodified oils.

4.7.5 Proton NMR study of enzymatically modified and unmodified oils

The use of NMR spectroscopy for evaluation of oxidative stability of oils was also considered. ^{1}H NMR makes it possible to determine hydrogen atoms of various types in TAG molecules of lipids. This is because in a strong magnetic field, hydrogen atoms absorb energy from radio frequency depending on their molecular environment. During oxidation of food lipids, changes occur in the protons (^{1}H) in oxidizing molecules that may be monitored by employing ^{1}H NMR spectroscopy.

The ^{1}H NMR spectrum of BO is shown in Figure 4.39. The spectrum signals are annotated from a to h. These groups are assigned as follows: a, olefinic protons and one methine proton in the glyceryl group (δ 5.2-5.5 ppm); b, four methylene protons in the glyceryl group (δ 4.0–4.4 ppm); c, diallylmethylene protons (=CH-CH_2-HC=; δ 2.6-2.9 ppm); d, six α-methylene protons adjacent to carbonyl carbon (α-CH_2; δ 2.2-2.4 ppm); e, protons in the CH_2 groups attached to saturated carbons and double-bonded carbon atoms (CH_2-C =; δ 1.7-2.2 ppm); f, protons in the CH_2 groups attached to the saturated carbon atoms ($C\underline{H}_2$-CH_2-C =; δ 1.5-1.7 ppm); g, protons in the CH_2 groups bonded to two saturated carbon atoms ($[CH_2]_n$; δ 1.0-1.5 ppm); h, the nine terminal methyl protons (δ 0.6-1.0 ppm) (Wanasundara and Shahidi, 1993; Miyake *et al.*, 1998a,b). The sharp signal at the high applied field is due to the internal standard, tetramethylsilane (TMS; δ 0.0 ppm). The relative number of protons in each group was calculated based on the integration of

Figure 4.39 ^{1}H NMR spectrum of borage oil (peaks at 0 ppm and 7.26 ppm for TMS and residual $CHCl_3$ protons, respectively)


h
H
d g a a c a a e f g h
b H-C-O-CO-CH2-(CH2)n-CH=CH-CH2-CH=CH-CH2-CH2-(CH2)n-CH3
a H-C-O-CO-CH2-(CH2)n-CH3
b H-C-O-CO-CH2-(CH2)n-CH3
H
b
g
h
d
a
e
TMS
b
c
f
8
6
4
2
0 ppm

methylene protons (δ 4.0–4.4 ppm) of the glyceryl moiety of the TAG.

Relative changes in the total number of aliphatic (CH_3 + $[CH_2]_n$ + CH_2-CH_2-C = + CH_2-C = + α-CH_2: δ 0.6-2.5 ppm), olefinic (-HC = CH-: δ 5.2-5.5 ppm) and diallylmethylene (=CH-CH_2-HC=: δ 2.6-2.9 ppm) protons of enzymatically modified oils and unmodified oils during oxidation are shown in Figures 4.40 and 4.41. It was found that during a 96 h storage, the relative number of olefinic and diallylmethylene protons decreased and the total number of aliphatic protons increased (Figures 4.40 and 4.41). This is in agreement with the results reported by Wanasundara and Shahidi (1993) for canola and soybean oils. In DHA-enriched BO and EPO, the number of olefinic protons decreased from 20.8 to 13.9 and from 15.8 to 10.2, respectively. The number of olefinic protons of EPA-enriched BO and EPO decreased from 17.7 to 13.4 and from 15.7 to 10.5, respectively. In EPA+DHA-enriched BO and EPO, the number of olefinic protons decreased from 18.3 to 7.7 and from 20.3 to 11.7, respectively. Similarly, the number of olefinic protons of unmodified BO and EPO decreased from 12.2 to 8.9 and from 12.6 to 9.1 respectively (Figures 4.40 and 4.41).

During oxidation of DHA-enriched BO and EPO, the number of diallylmethylene protons decreased from 13.4 to 9.2 and from 14.4 to 7.3, respectively. The number of diallylmethylene protons of EPA-enriched BO and EPO decreased from 11.3 to 8.6 and from 11.3 to 8.1, respectively. In EPA+DHA-enriched BO and EPO, the number of diallylmethyelene protons decreased from 12.2 to 7.1 and from 14.3 to 7.2, respectively. Similarly, the number of diallylmethylene protons of unmodified BO and EPO decreased

Figure 4.40 Total olefinic, diallylmethylene and aliphatic protons of DHA, EPA and EPA+DHA-enriched borage oils (BO) and unmodified BO during accelerated oxidation at 60°C

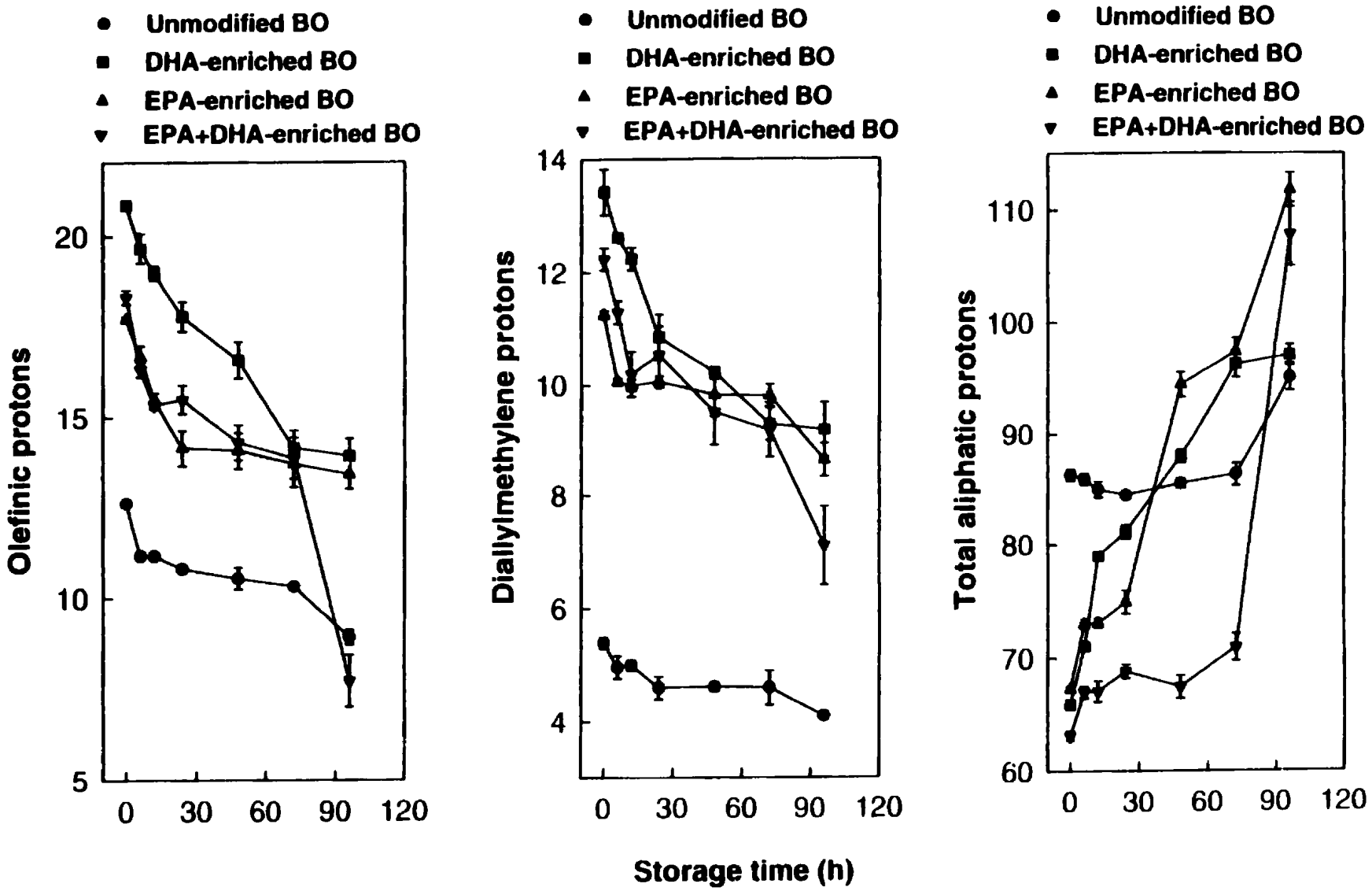
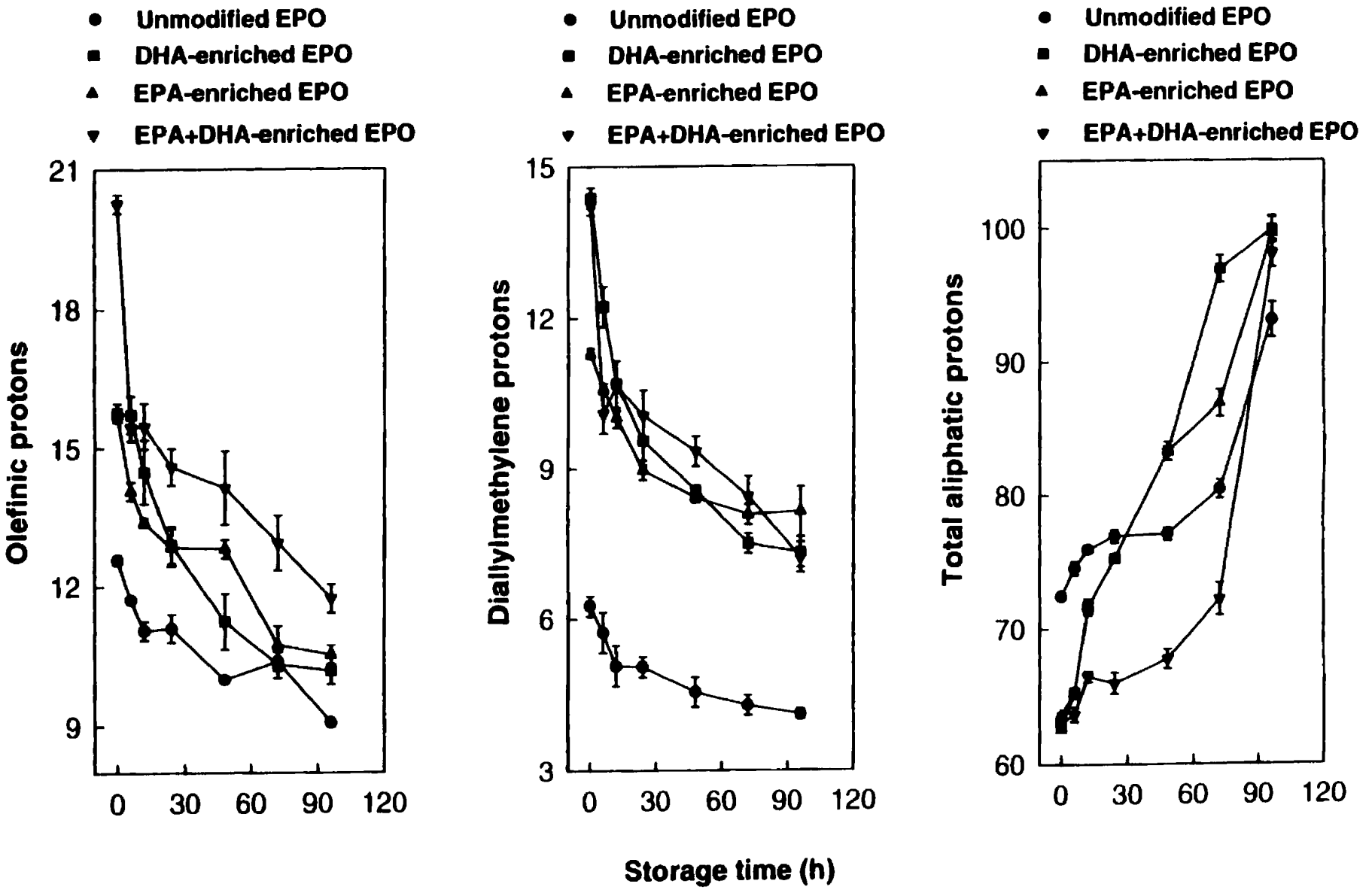

Unmodified BO
DHA-enriched BO
EPA-enriched BO
EPA+DHA-enriched BO
Olefinic protons
Diallylmethylene protons
Total aliphatic protons
Storage time (h)
0
30
60
90
120
5
10
15
20
4
6
8
10
12
14
60
70
80
90
100
110

Figure 4.41 Total olefinic, diallylmethylene and aliphatic protons of DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO) and unmodified EPO during accelerated oxidation at 60°C

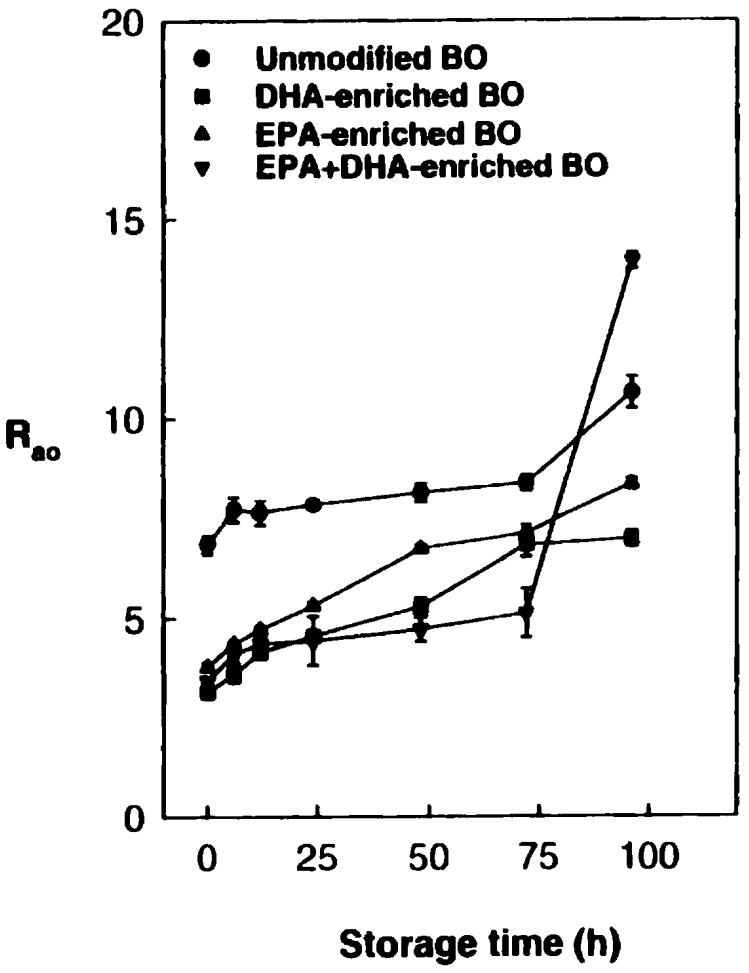

Unmodified EPO
DHA-enriched EPO
EPA-enriched EPO
EPA+DHA-enriched EPO
Olefinic protons
21
18
15
12
9
0
30
60
90
120
Diallylmethylene protons
15
12
9
6
3
Total aliphatic protons
100
90
80
70
60
Storage time (h)

from 5.4 to 4.1 and from 6.2 to 4.1 respectively (Figures 4.40 and 4.41). Considering olefinic and diallylmethylene protons of modified and unmodified oils, modified oils had a relatively higher number of both types of protons than their unmodified counterparts. This might be due to a higher degree of unsaturation in modified oils as compared to unmodified oils. Meanwhile, there was a corresponding increase in total number of aliphatic protons in all oils examined. For example, in DHA-enriched oils the total number of aliphatic protons increased from 63-66 to 97-100 as a result of oxidation. The total number of aliphatic protons of EPA-enriched oils increased from 64-67 to 99-112. In EPA+DHA-enriched oils, the corresponding increase was from 63 to 98-109 and that in unmodified BO and EPO was from 72-86 to 93-95 (Figures 4.40 and 4.41).

During autoxidation, diallylmethylene protons are attacked by free radicals followed by intramolecular rearrangement of the olefinic protons. This is a consequence of the fact that the behaviour of the olefinic proton group is closely related to the diallylmethylene proton group because of the interchangeability of allyl radicals (Saito and Udagawa, 1992). Consequently, during oxidation the relative number of diallylmethylene and olefinic protons decreases. Khatoon and Krishna (1998) assessed the oxidation of heated safflower oil by ^{1}H NMR technique and reported the disappearance of allylic and olefinic protons during extensive oxidation.

The ratio of aliphatic to olefinic protons (R_{ao}) and that of aliphatic to diallylmethylene protons (R_{ad}) were determined. It was found that R_{ao} and R_{ad} increased gradually and significantly ($p \leq 0.05$) during the storage of enzymatically modified oils and

Figure 4.42 Changes in aliphatic proton to olefinic proton ratio (R_{ao}) and aliphatic to diallylmethylene proton ratio (R_{ad}) of DHA, EPA and EPA+DHA-enriched borage oils (BO) and unmodified BO stored at 60°C


Unmodified BO
DHA-enriched BO
EPA-enriched BO
EPA+DHA-enriched BO
R_{ao}
20
15
10
5
0
0
25
50
75
100
Storage time (h)

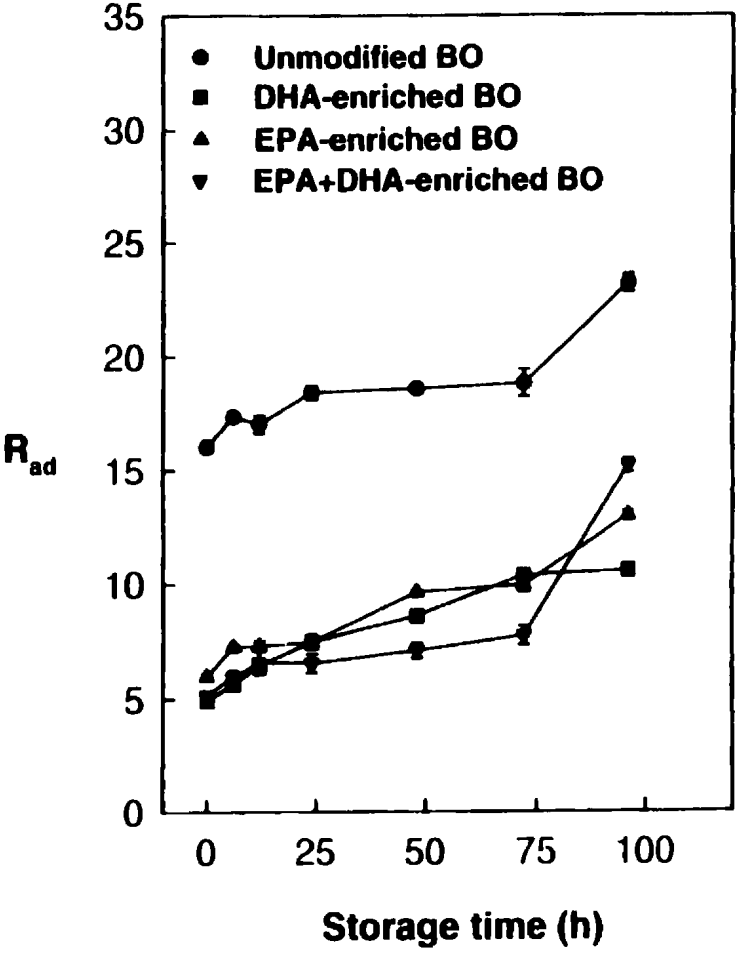
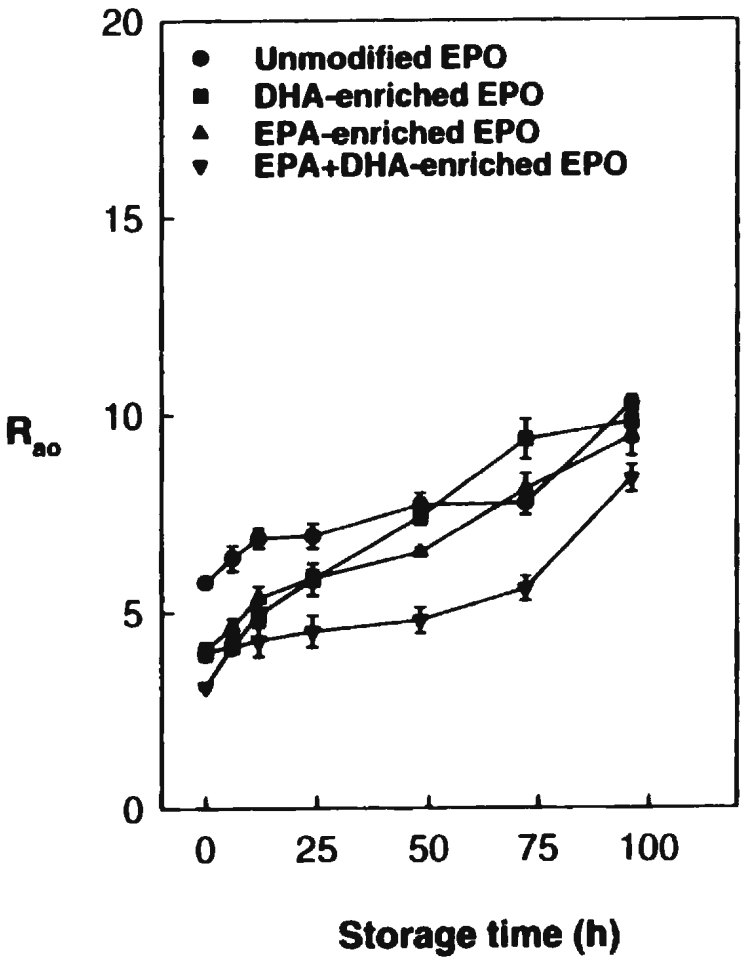
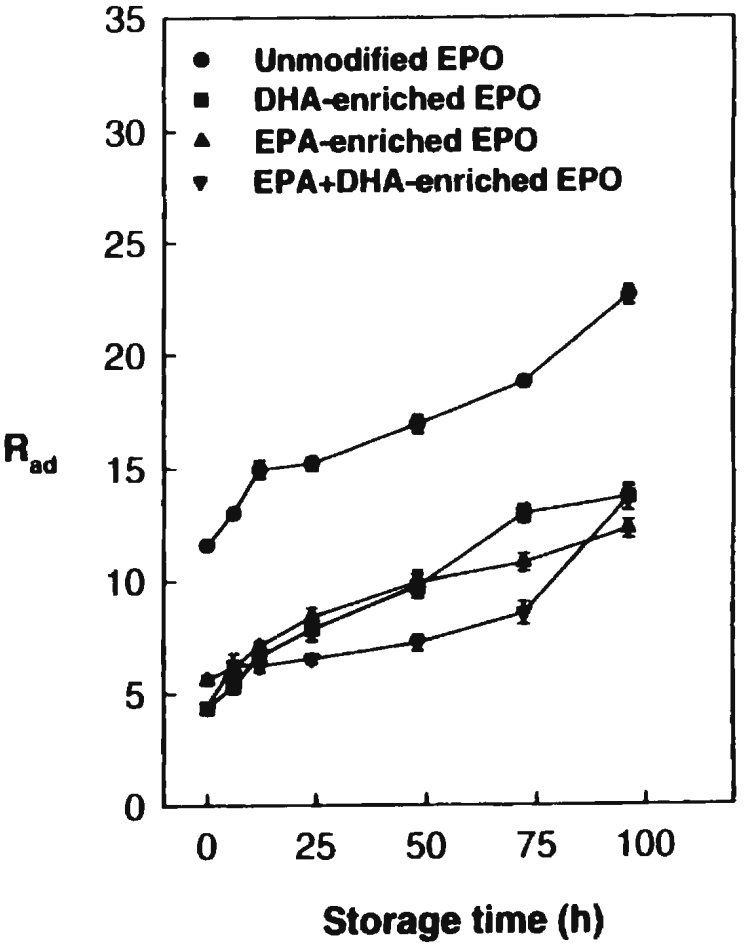

Unmodified BO
DHA-enriched BO
EPA-enriched BO
EPA+DHA-enriched BO
R_{ad}
35
30
25
20
15
10
5
0
0
25
50
75
100
Storage time (h)

Figure 4.43 Changes in aliphatic proton to olefinic proton ratio (R_{ao}) and aliphatic to diallylmethylene proton ratio (R_{ad}) of DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO) and unmodified EPO stored at 60°C


Unmodified EPO
DHA-enriched EPO
EPA-enriched EPO
EPA+DHA-enriched EPO
R_{ao}
0
5
10
15
20
0
25
50
75
100
Storage time (h)


Unmodified EPO
DHA-enriched EPO
EPA-enriched EPO
EPA+DHA-enriched EPO
R_{ad}
0
5
10
15
20
25
30
35
0
25
50
75
100
Storage time (h)

their unmodified counterparts (Figures 4.42 and 4.43). Similar findings have been reported by others (Wanasundara and Shahidi, 1993; Shahidi *et al.*, 1994; Shahidi and Spurvey, 1996). R_{ao} and R_{ad} values of modified BO increased from 3.2-3.8 to 7.0-14.0 and from 4.9-6.0 to 10.6-15.2, respectively. Corresponding increases in the R_{ao} and R_{ad} values of modified EPO were from 3.1-4.1 to 8.4-9.8 and from 4.4-5.6 to 12.3-13.7. Similarly, in unmodified BO and EPO, R_{ao} and R_{ad} increased, respectively, from 5.8-6.8 to 10.3-10.7 and from 11.6-16.0 to 22.7-23.2. However, modified oils initially showed a lower R_{ao} and R_{ad} values than those of their unmodified counterparts. This is a consequence of the higher number of total olefinic and diallylmethylene protons in modified oils than those in unmodified oils.

The numerical values of R_{ao} and R_{ad} were plotted against the corresponding conjugated diene values of enzymatically modified BO and EPO as well as unmodified oils (Figures A.10 and A.11). A highly significant correlation ($p \leq 0.005$) existed between both R_{ao} and R_{ad} and conjugated dienes; correlation coefficients (r) were 0.930-0.988 for R_{ao} and 0.947-0.992 for R_{ad} for modified oils and 0.980-0.982 for R_{ao} and 0.985-0.987 for R_{ad} for their unmodified counterparts (Table 4.37).

Strong correlation coefficients were evident for relationships between both R_{ao} and R_{ad} and TBARS values of modified and unmodified oils (Figures A.12 and A.13). The correlation coefficients (r) were 0.885-0.976 ($p \leq 0.05$) for R_{ao} and 0.926-0.980 ($p \leq 0.005$) for R_{ad} for modified oils and 0.881-0.978 for R_{ao} and 0.779-0.983 ($p \leq 0.05$) for R_{ad} for their unmodified counterparts (Table 4.37).

Table 4.37 Correlation coefficients (r) between conjugated dienes (CD) and the ratios of aliphatic to olefinic (R_{ao}) and aliphatic to diallylmethylene protons (R_{ad}) as well as between thiobarbituric acid reactive substances (TBARS) and R_{ao} and R_{ad} of oxidized oils

Sample	CD *Vs* R_{ao}	CD *Vs* R_{ad}	TBARS *Vs* R_{ao}	TBARS *Vs* R_{ad}
Unmodified BO	0.982[a]	0.987[a]	0.978[a]	0.983[a]
DHA-enriched BO	0.971[a]	0.983[a]	0.885[b]	0.926[a]
EPA-enriched BO	0.977[a]	0.947[a]	0.936[a]	0.954[a]
EPA+DHA-enriched BO	0.964[a]	0.985[a]	0.963[a]	0.980[a]
Unmodified EPO	0.980[a]	0.985[a]	0.881[b]	0.779[b]
DHA-enriched EPO	0.988[a]	0.992[a]	0.915[a]	0.937[a]
EPA-enriched EPO	0.930[a]	0.974[a]	0.976[a]	0.960[a]
EPA+DHA-enriched EPO	0.960[a]	0.953[a]	0.967[a]	0.972[a]

[a]Significant at $p < 0.005$ level; [b]Significant at $p < 0.05$ level; BO, borage oil; EPO, evening primrose oil

As depicted in Figures A.14 and A.15, both R_{ao} and R_{ad} values of modified as well as unmodified oils strongly correlated with hexanal contents of the oils. The correlation coefficients (r) were 0.954-0.991 for R_{ao} and 0.961-0.996 for R_{ad} for modified oils and 0.960-0.965 for R_{ao} and 0.948 ($p \leq 0.005$) for R_{ad} for their unmodified counterparts (Table 4.38).

Figures A.16 and A.17 depict the relationships between both R_{ao} and R_{ad} values and propanal contents of enzymatically modified oils. A highly significant correlation ($p \leq 0.005$) existed between both R_{ao} and R_{ad} and propanal content of oils; correlation coefficients (r) were 0.961-0.986 for R_{ao} and 0.959-0.978 for R_{ad} for enzymatically modified BO and 0.950-0.981 for R_{ao} and 0.952-0.990 for R_{ad} for enzymatically modified EPO (Table 4.38). These results suggest that parameters such as R_{ao} and R_{ad} may be used as indicators of oxidative stability of oils.

The NMR methodology, as demonstrated above, offers a rapid, non-destructive procedure for evaluation of the oxidative deterioration of enzymatically modified oils as well as their unmodified counterparts. It also provides an alternative method to estimate the overall changes in the primary and secondary oxidation products of lipids.

Wang and Tao (1998) monitored hypochlorite-assisted oxidation of soy fatty acids using NMR spectroscopy. They reported that the ratio of the double bond protons to methyl protons may be used to monitor the oxidation of fatty acids. The use of ^{1}H NMR has proven to be useful for monitoring fatty acid double-bond cleavage. Silwood and Grootveld (1999) compared ^{1}H NMR spectra of oxidized PUFA-rich culinary oil with that of a corresponding

Table 4.38 Correlation coefficients (r) between hexanal content and ratios of aliphatic to olefinic (R_{ao}) and aliphatic to diallylmethylene protons (R_{ad}) as well as between propanal content and R_{ao} and R_{ad} of oxidized oils

Sample	Hexanal content *Vs* R_{ao}	Hexanal content *Vs* R_{ad}	Propanal content *Vs* R_{ao}	Propanal content *Vs* R_{ad}
Unmodified BO	0.965[a]	0.948[a]	-	-
DHA-enriched BO	0.987[a]	0.996[a]	0.963[a]	0.978[a]
EPA-enriched BO	0.978[a]	0.969[a]	0.986[a]	0.959[a]
EPA+DHA-enriched BO	0.962[a]	0.976[a]	0.961[a]	0.971[a]
Unmodified EPO	0.960[a]	0.948[a]	-	-
DHA-enriched EPO	0.965[a]	0.961[a]	0.958[a]	0.975[a]
EPA-enriched EPO	0.991[a]	0.994[a]	0.981[a]	0.990[a]
EPA+DHA-enriched EPO	0.954[a]	0.963[a]	0.950[a]	0.952[a]

[a]Significant at $p < 0.005$ level; BO, borage oil; EPO, evening primrose oil

control (unoxidized oil) and reported that the ratios of the intensities of *bis*-allylic protons and the total olefinic protons to that of the acyl chain terminal $C\underline{H}_3$ protons were markedly lower in the oxidized oil.

PUFA are among the most easily oxidizable component of foods and cell membrane lipids; many of the oxidized products of lipids, including hydroperoxides, free radicals and aldehydes are toxic and mutagenic (Pearson *et al.*, 1983; Hageman *et al.*, 1990). A high percentage of humans are frequently and continually exposed to lipid oxidation products in the diet (arising, for example, from deep-frying of PUFA-rich oils), and the possibility that regular consumption of oxidized lipids may be deleterious to human health has recently attracted much interest (Chow, 1992). The short-term feeding of heated and/or oxidized oils and fats to experimental animals gave rise to loss of appetite, diarrhoea, haemolytic anaemia, growth retardation and apparent accumulation of peroxides in adipose tissues (Sanders, 1983). Moreover, cellular damage in various organs, elevated liver and kidney weights, and a modified fatty acid composition of tissue lipids in rats were shown to result from the short-term feeding of oils and fats subjected to the heat and oxidation associated with normal usage (Yoshida and Kujimoto, 1989).

The ease of autoxidation of unsaturated fatty acids is proportional to the number of allylic methylene groups; thus modified oils with a high content of PUFA are more prone to oxidation than unmodified oils. Due to the presence of high proportions of PUFA in modified oils, protection of these fatty acids is necessary in order to counterbalance any harmful effects which may arise as a result of their oxidation and to take full advantage of

their nutritional and health related benefits associated with their $\omega 3$ and $\omega 6$ components.

SUMMARY AND CONCLUSIONS

Enzyme-assisted acidolysis of γ-linolenic acid (GLA)-rich oils, namely borage (BO) and evening primrose oils (EPO), with docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) and their combination were studied. Six commercially available lipases were screened for their ability to incorporate the above fatty acids into GLA-rich oils. Among the enzymes tested, the highest incorporation of DHA into BO and EPO was obtained when lipase Novozym-435 from *Candida antarctica* used for the acidolysis reaction. However, the lipase PS-30 from *Pseudomonas sp.* was found to be most effective in incorporating EPA or EPA+DHA into both oils. These differences might be of interest when enrichment/incorporation with a particular PUFA or PUFA mixture is required.

The effects of various reaction parameters such as amount of lipase, reaction temperature, reaction time, mole ratio of substrates and type of organic solvents on DHA and/or EPA incorporation were studied. In general, as the amount of enzyme, temperature, reaction time and mole ratio increased, incorporation of DHA and/or EPA into BO and EPO was also increased. Among the organic solvents tested, *n*-hexane served best for incorporation of DHA and/or EPA into the oils examined. However, solvent-free reactions also produced a satisfactory incorporation of the above fatty acids into the oils tested.

Incorporation of DHA, EPA and EPA+DHA into BO and EPO was optimized, under laboratory conditions, for process parameters such as the amount of enzyme, reaction

temperature and reaction time using response surface methodology (RSM). In DHA-enriched oils, maximum incorporation of DHA (33.5-35.6%) was obtained using *Candida antarctica* enzyme at a 162-165 units, a reaction temperature of 43-50°C and reaction time of 25-27 h. On the other hand, in EPA-enriched oils, the maximum incorporation of EPA was obtained when *Pseudomonas sp.* enzyme was used at enzyme units, reaction temperature and reaction time of 299-309 U, 40-44°C and 25-27 h, respectively. Similarly, optimization of reaction parameters gave maxima of 35.5 and 33.6% EPA+DHA into BO and EPO, respectively. Conditions for optimum EPA+DHA incorporation were closely related for both oils and were achieved at 278-299 units of *Pseudomonas sp.* enzyme at 42-43°C and 24-26 h. Therefore, optimization for incorporation of DHA and/or EPA into BO and EPO *via* acidolysis by lipases from *Candida antarctica* or *Pseudomonas sp.* was found possible.

The positional distribution of fatty acids in the TAG molecules of DHA, EPA and EPA+DHA-enriched oils was determined; DHA was randomly distributed over the three positions of TAG of DHA-enriched BO while in DHA-enriched EPO this fatty acid was mainly located at the *sn*-2 position. In EPA-enriched BO, EPA was randomly distributed in the TAG molecules. However, in EPA-enriched EPO and in EPA+DHA-enriched BO and EPO, EPA and DHA favoured the *sn*-1 and *sn*-3 positions of TAG. In all oils examined, GLA was concentrated in the *sn*-2 and/or *sn*-3 positions of TAG. Therefore, enzymatically modified oils are expected to be superior to simple physical mixtures of the

oils in terms of their absorption and assimilation in the body. However, clinical studies should be carried out to verify this assumption.

It is anticipated that production of TAG rich in GLA and DHA and/or EPA would provide the maximum health benefit. Long-chain PUFA, with a balance between ω3 and ω6 components, are required by the body as their deficiency has been associated with a number of clinical disorders. Thus, structured lipids obtained enzymatically from BO or EPO may be useful in certain nutritional applications.

The oxidative stability of enzymatically modified oils and their unmodified counterparts were assessed. Among the oils examined, enzymatically modified oils had significantly ($p \leq 0.05$) higher conjugated dienes (CD), 2-thiobarbituric acid reactive substances (TBARS), hexanal and propanal contents than those of their unmodified counterparts. Therefore, enzymatically modified oils were more susceptible to oxidation than their unmodified counterparts.

The double bond index (DBI) and methylene bridge index (MBI) of oils were decreased during oxidation. The extent of decrease was significantly ($P \leq 0.05$) higher in the modified oils, which are more unsaturated. An attempt was made to correlate various parameters of oxidation (CD, TBARS, hexanal and propanal contents) with DBI and MBI of oils; correlation coefficients (-r) were within the range of 0.574 - 0.975. This suggests that indicators such as DBI and MBI have an influence on oxidative stability of oils.

The ratio of aliphatic to olefinic protons (R_{ao}) and that of aliphatic to diallylmethylene protons (R_{ad}) in all oils, determined as NMR spectroscopy, increased steadily over the entire length of storage period, indicating progressive oxidation of unsaturated fatty acids in both modified oils and unmodified oils. A highly significant correlation existed between these ratios and other parameters of oxidation (CD, TBARS, hexanal and propanal contents) for almost all oils, thus suggesting that NMR methodology can be used as an effective means to simultaneously estimate both primary and secondary oxidation changes in both modified and unmodified oils.

The results presented in this study have provided simple and reliable analytical methods to follow the oxidation of modified and unmodified BO and EPO, and enhanced our understanding of the parameters involved in the oxidation of nutritional and medicinal oils. Based on the results obtained in this study, it is recommended that the applicability of DBI and MBI as well as ^{1}H NMR as indicators of oxidation of lipids in other food systems be evaluated. Furthermore, the relationships of these indicators with sensory properties need to be evaluated and confirmed.

The ease of autoxidation of fatty acids is proportional to the number of methylene groups between double bonds, thus modified BO and EPO with a higher content of EPA and DHA were more prone to oxidation than their unmodified counterparts. Thus, modified oils rich in PUFA compared to unmodified oils must be protected against oxidation in order to counterbalance any harmful effects from production of oxidation

products and to take advantage of their nutritional and health benefits. It is recommended that the addition of appropriate antioxidants (natural or synthetic) to the modified BO and EPO up to the level that would prevent oxidation of PUFA before incorporation into food, used as nutraceuticals or for other applications be examined. The possibility of using microencapsulation for oils should also be investigated as this could improve the oxidative stability of oils and preserve the integrity of nutritionally important PUFA. Incorporation of ω3 PUFA-enriched BO and EPO into foods may reduce the risk of developing certain disease conditions in humans, but such uses need to be justified using evidence gathered from animal studies and clinical trials. Further research on ω3 PUFA-enriched BO and EPO should therefore focus on the metabolism and clinical benefits as well as safety issues and unravelling of their nutritional and medicinal importance and economic feasibility of large-scale production.

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APPENDIX 1

Figure A.1 Standard line of concentration dependence of TBARS as reflected in the absorbance of the TBA-malonaldehyde complex at 532 nm

Correlation coefficient (r) = 0.996
Equation of the line was $Y = aX + b$ where,
Y = absorbance at 532 nm ($A_{532\ nm}$)
X = concentration of malonaldehyde (MA) in 5 mL solution, µmol (C)
a = 15.0158
b = 0
Therefore, $C = 0.067\ A_{532\ nm}$
Since the W grams of oil dissolved in 25 mL solution, the MA concentration is:
C (µmol of MA/ g oil) = $(0.335A_{532\ nm})/W$

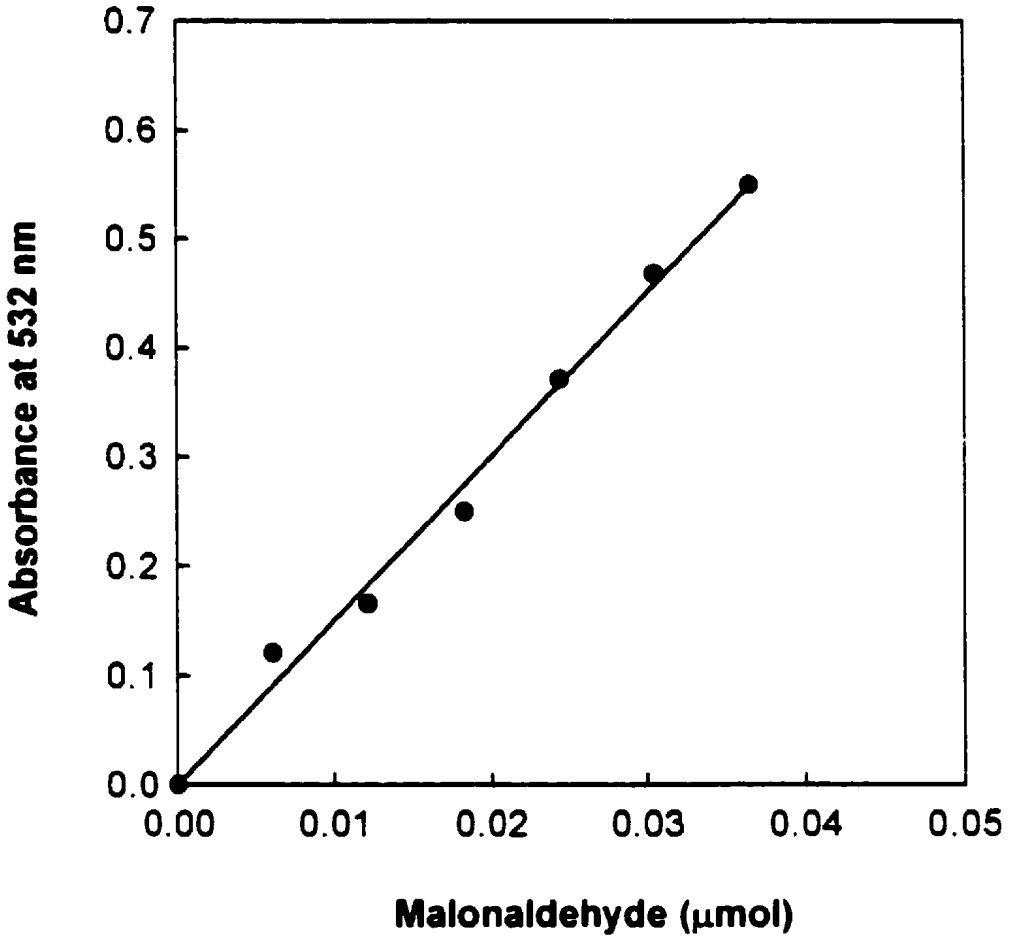
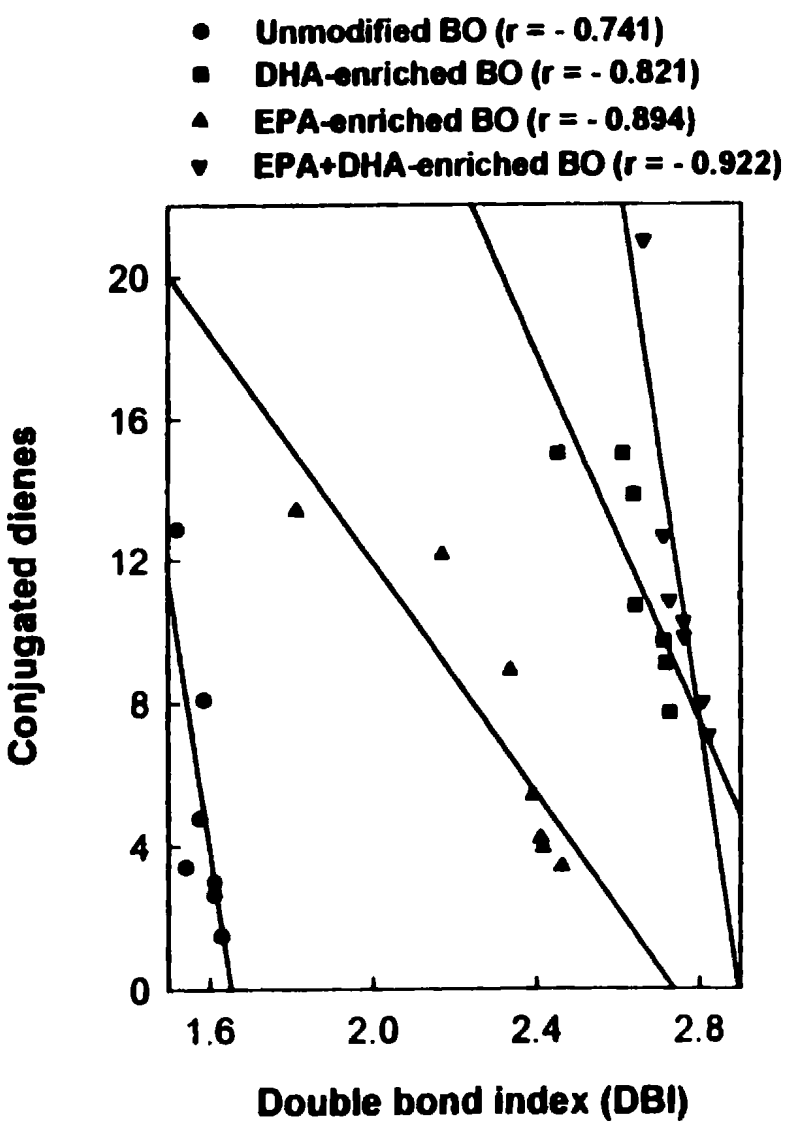

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 532 nm
0.00
0.01
0.02
0.03
0.04
0.05
Malonaldehyde (μmol)

Figure A.2 Relationships between conjugated dienes and double bond index (DBI) and methylene bridge index (MBI) of oxidized DHA, EPA and EPA+DHA-enriched borage oils (BO) and unmodified BO


Unmodified BO (r = - 0.741)
DHA-enriched BO (r = - 0.821)
EPA-enriched BO (r = - 0.894)
EPA+DHA-enriched BO (r = - 0.922)
Conjugated dienes
0
4
8
12
16
20
1.6
2.0
2.4
2.8
Double bond index (DBI)

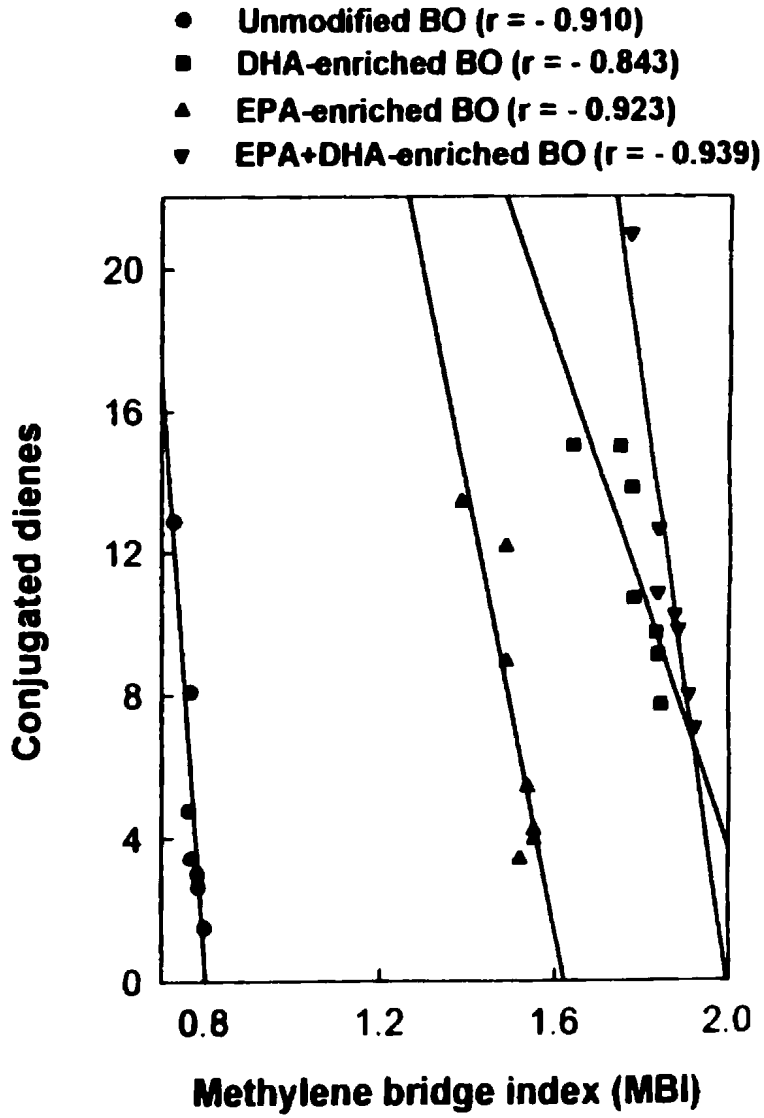
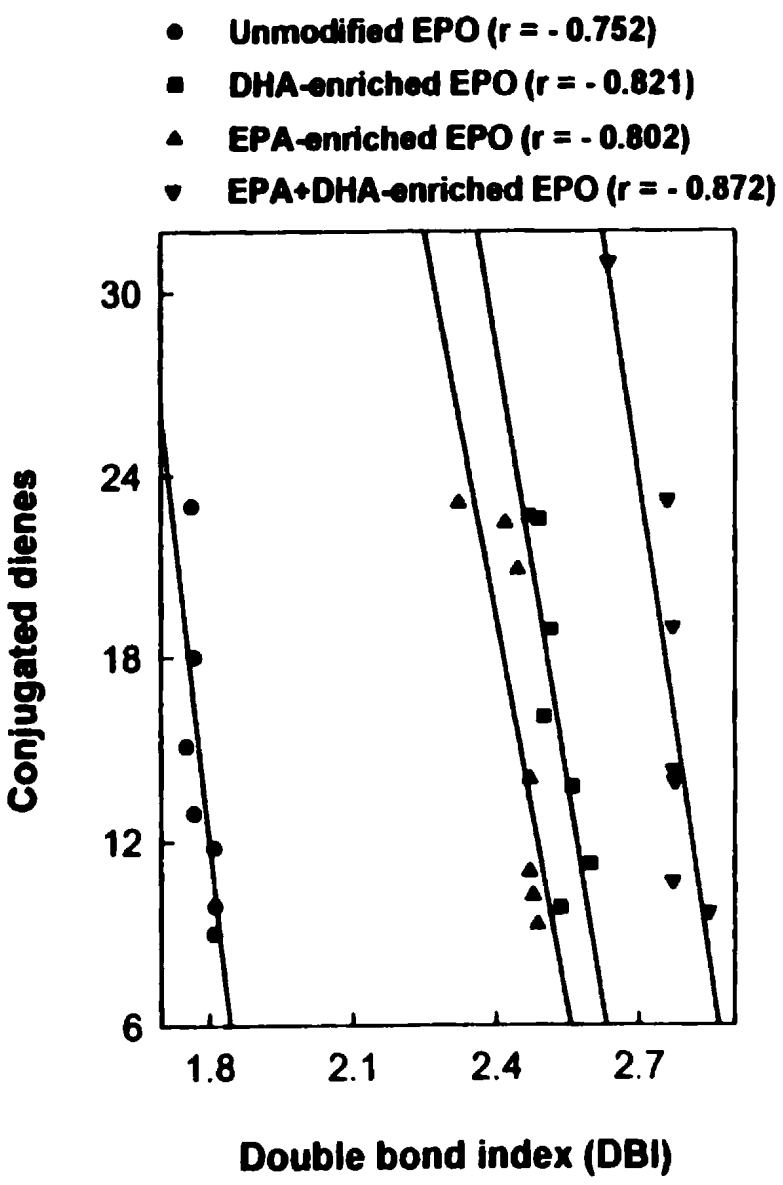

Unmodified BO (r = - 0.910)
DHA-enriched BO (r = - 0.843)
EPA-enriched BO (r = - 0.923)
EPA+DHA-enriched BO (r = - 0.939)
Conjugated dienes
0
4
8
12
16
20
0.8
1.2
1.6
2.0
Methylene bridge index (MBI)

Figure A.3 Relationships between conjugated dienes and double bond index (DBI) and methylene bridge index (MBI) of oxidized DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO) and unmodified EPO


Unmodified EPO (r = - 0.752)
DHA-enriched EPO (r = - 0.821)
EPA-enriched EPO (r = - 0.802)
EPA+DHA-enriched EPO (r = - 0.872)
Conjugated dienes
30
24
18
12
6
1.8
2.1
2.4
2.7
Double bond index (DBI)

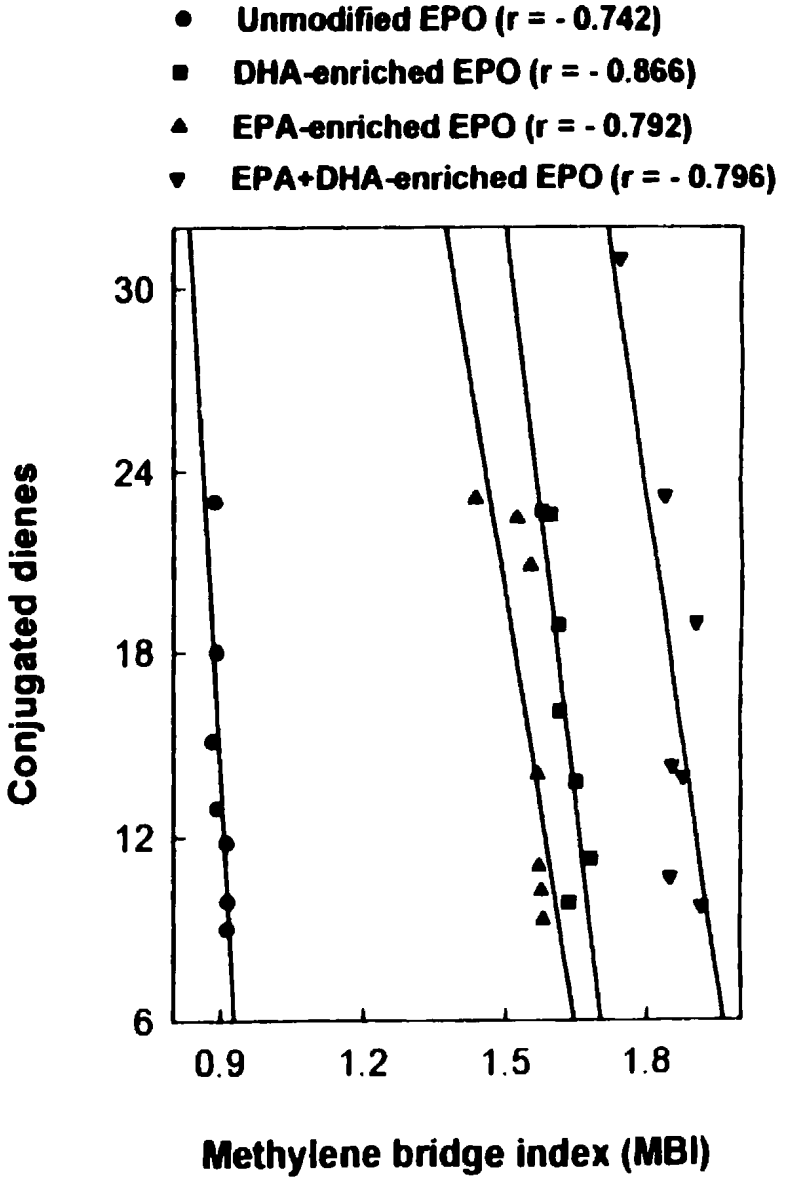
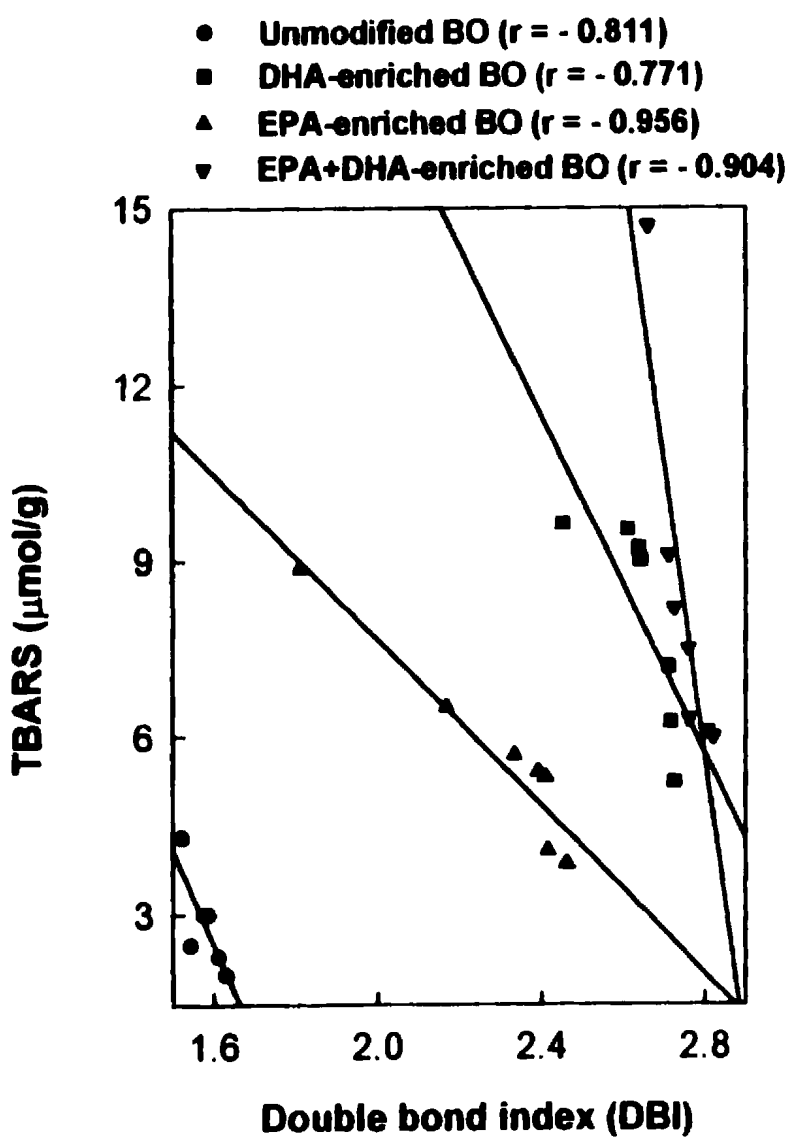

Unmodified EPO (r = - 0.742)
DHA-enriched EPO (r = - 0.866)
EPA-enriched EPO (r = - 0.792)
EPA+DHA-enriched EPO (r = - 0.796)
Conjugated dienes
30
24
18
12
6
0.9
1.2
1.5
1.8
Methylene bridge index (MBI)

Figure A.4 Relationships between TBARS values and double bond index (DBI) and methylene bridge index (MBI) of oxidized DHA, EPA and EPA+DHA-enriched borage oils (BO) and unmodified BO


Unmodified BO (r = - 0.811)
DHA-enriched BO (r = - 0.771)
EPA-enriched BO (r = - 0.956)
EPA+DHA-enriched BO (r = - 0.904)
TBARS (μmol/g)
15
12
9
6
3
1.6
2.0
2.4
2.8
Double bond index (DBI)

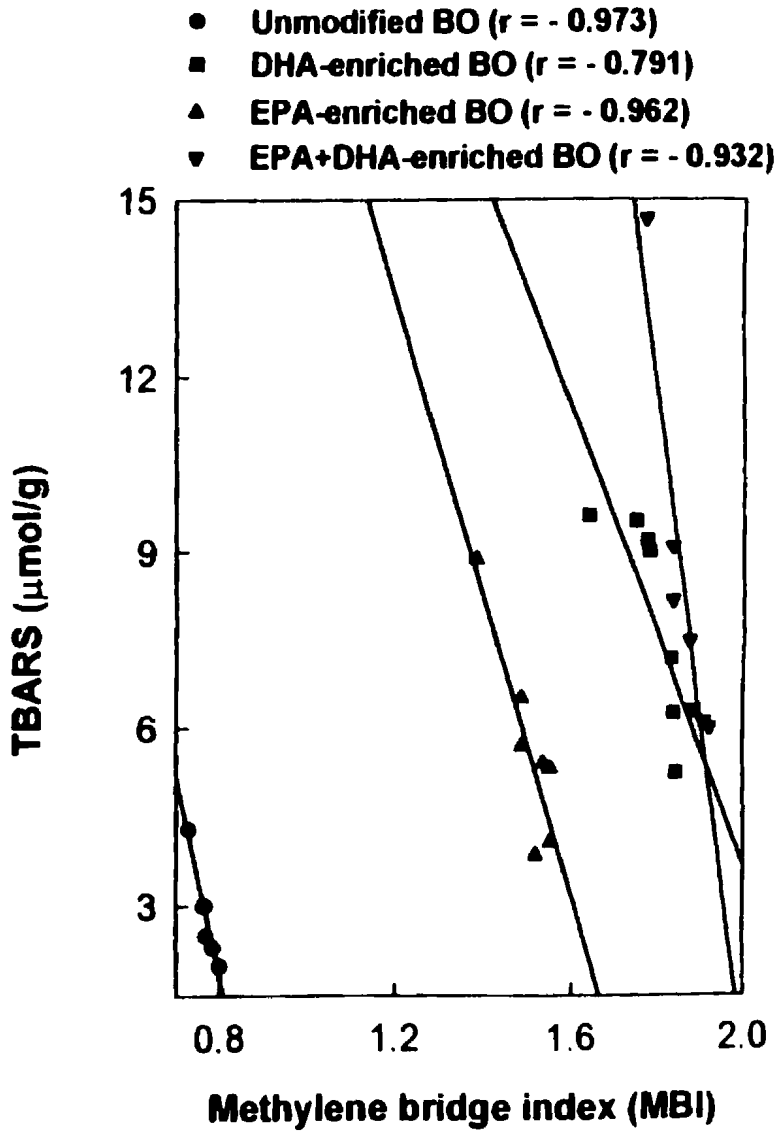
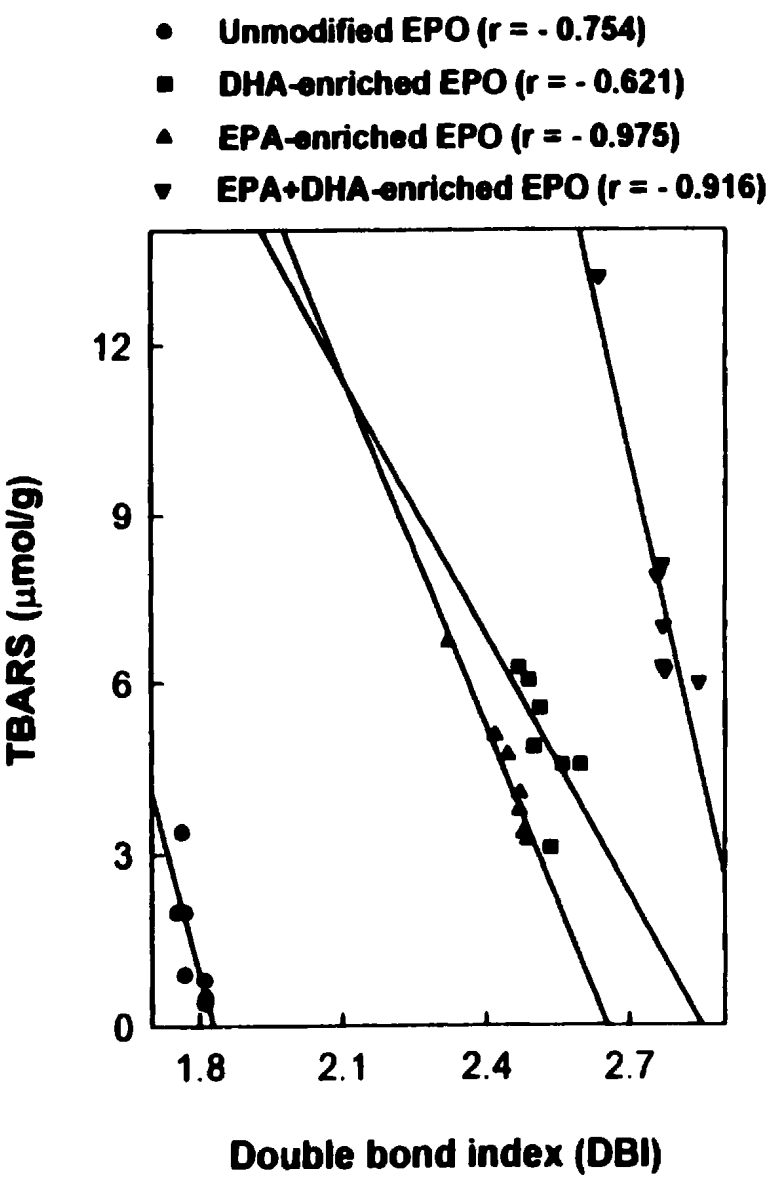

Unmodified BO (r = - 0.973)
DHA-enriched BO (r = - 0.791)
EPA-enriched BO (r = - 0.962)
EPA+DHA-enriched BO (r = - 0.932)
TBARS (μmol/g)
15
12
9
6
3
0.8
1.2
1.6
2.0
Methylene bridge index (MBI)

Figure A.5 Relationships between TBARS values and double bond index (DBI) and methylene bridge index (MBI) of oxidized DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO) and unmodified EPO


Unmodified EPO (r = - 0.754)
DHA-enriched EPO (r = - 0.621)
EPA-enriched EPO (r = - 0.975)
EPA+DHA-enriched EPO (r = - 0.916)
TBARS (μmol/g)
0
3
6
9
12
1.8
2.1
2.4
2.7
Double bond index (DBI)

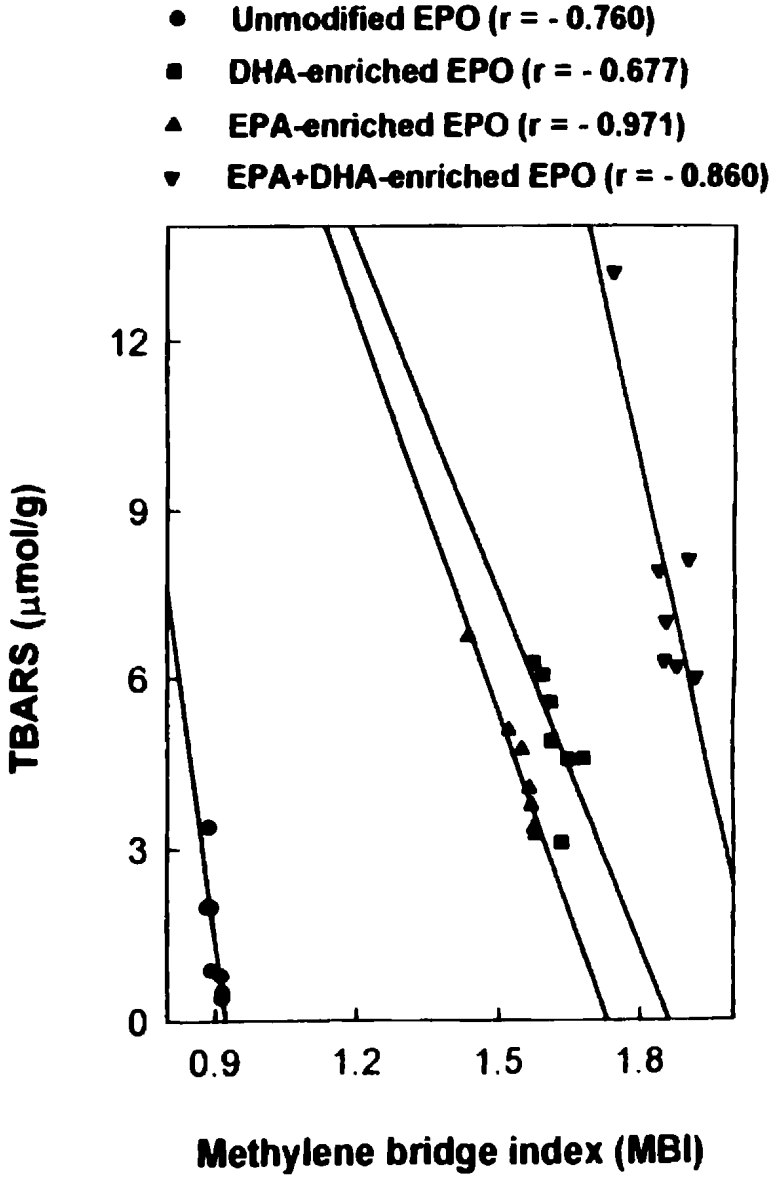
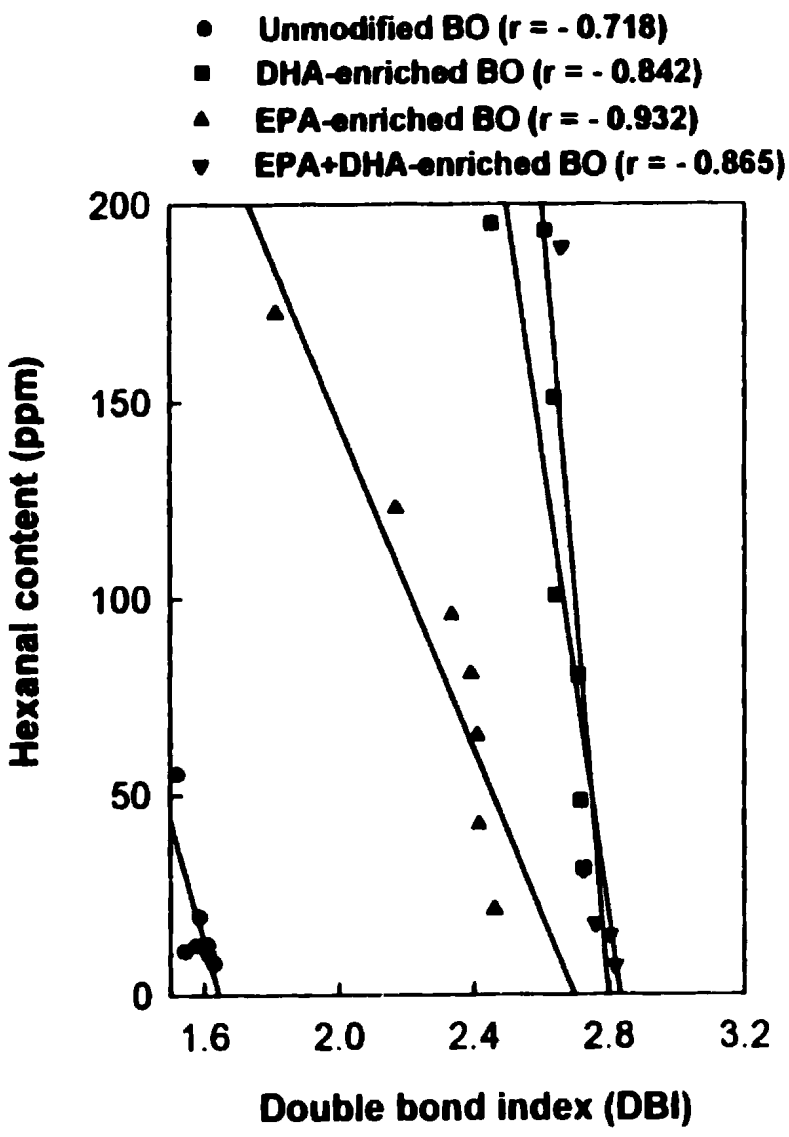

Unmodified EPO (r = - 0.760)
DHA-enriched EPO (r = - 0.677)
EPA-enriched EPO (r = - 0.971)
EPA+DHA-enriched EPO (r = - 0.860)
TBARS (μmol/g)
0
3
6
9
12
0.9
1.2
1.5
1.8
Methylene bridge index (MBI)

Figure A.6 Relationships between hexanal contents and double bond index (DBI) and methylene bridge index (MBI) of oxidized DHA, EPA and EPA+DHA-enriched borage oils (BO) and unmodified BO


Unmodified BO (r = - 0.718)
DHA-enriched BO (r = - 0.842)
EPA-enriched BO (r = - 0.932)
EPA+DHA-enriched BO (r = - 0.865)
Hexanal content (ppm)
200
150
100
50
0
1.6
2.0
2.4
2.8
3.2
Double bond index (DBI)

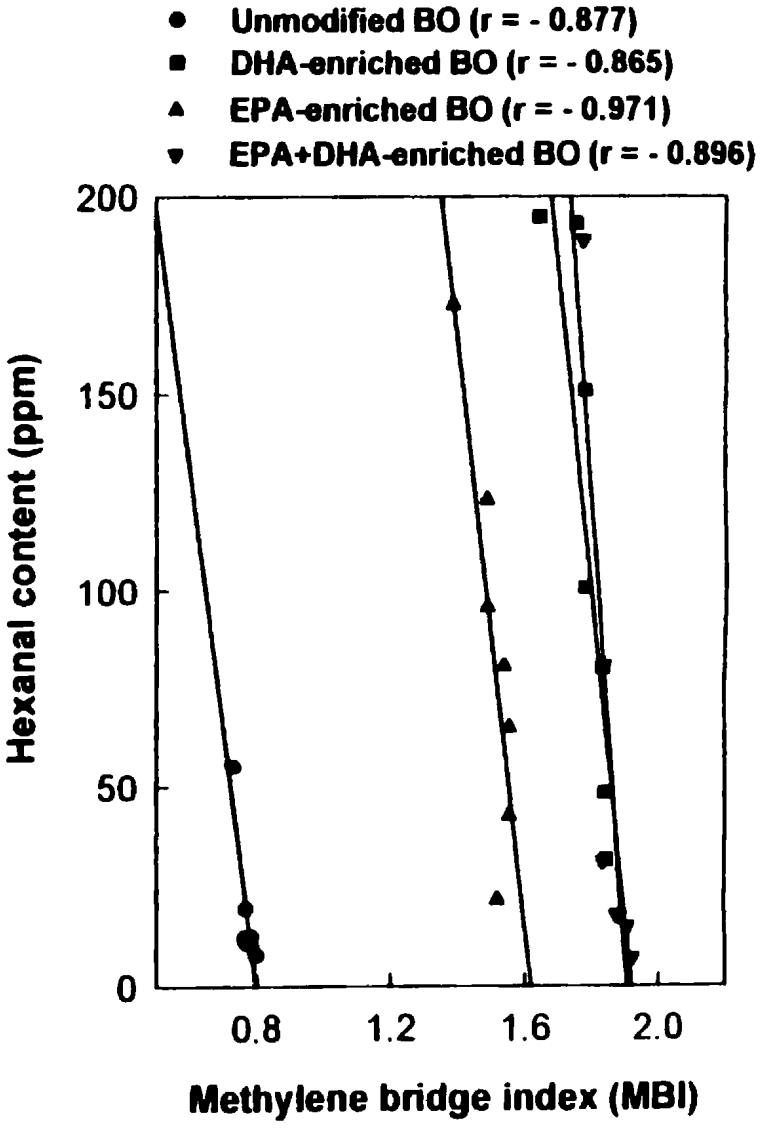
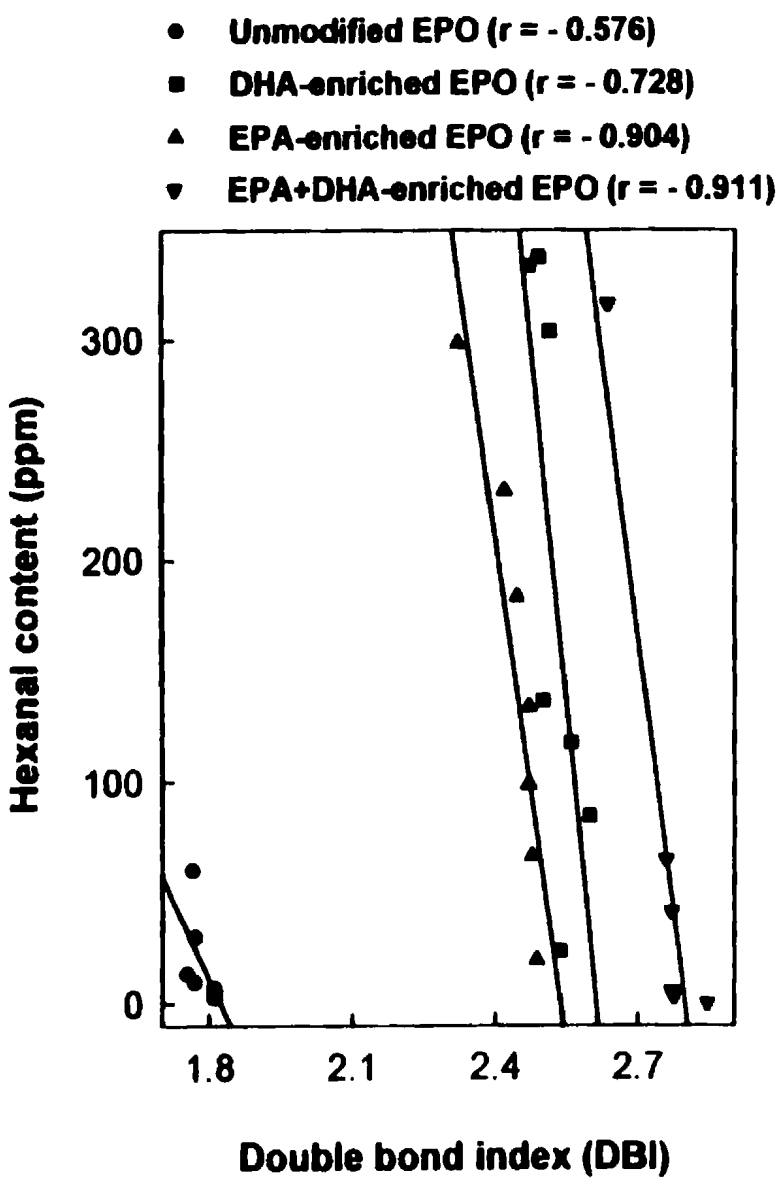

Unmodified BO (r = - 0.877)
DHA-enriched BO (r = - 0.865)
EPA-enriched BO (r = - 0.971)
EPA+DHA-enriched BO (r = - 0.896)
Hexanal content (ppm)
200
150
100
50
0
0.8
1.2
1.6
2.0
Methylene bridge index (MBI)

Figure A.7 Relationships between hexanal contents and double bond index (DBI) and methylene bridge index (MBI) of oxidized DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO) and unmodified EPO


Unmodified EPO (r = - 0.576)
DHA-enriched EPO (r = - 0.728)
EPA-enriched EPO (r = - 0.904)
EPA+DHA-enriched EPO (r = - 0.911)
Hexanal content (ppm)
300
200
100
0
1.8
2.1
2.4
2.7
Double bond index (DBI)

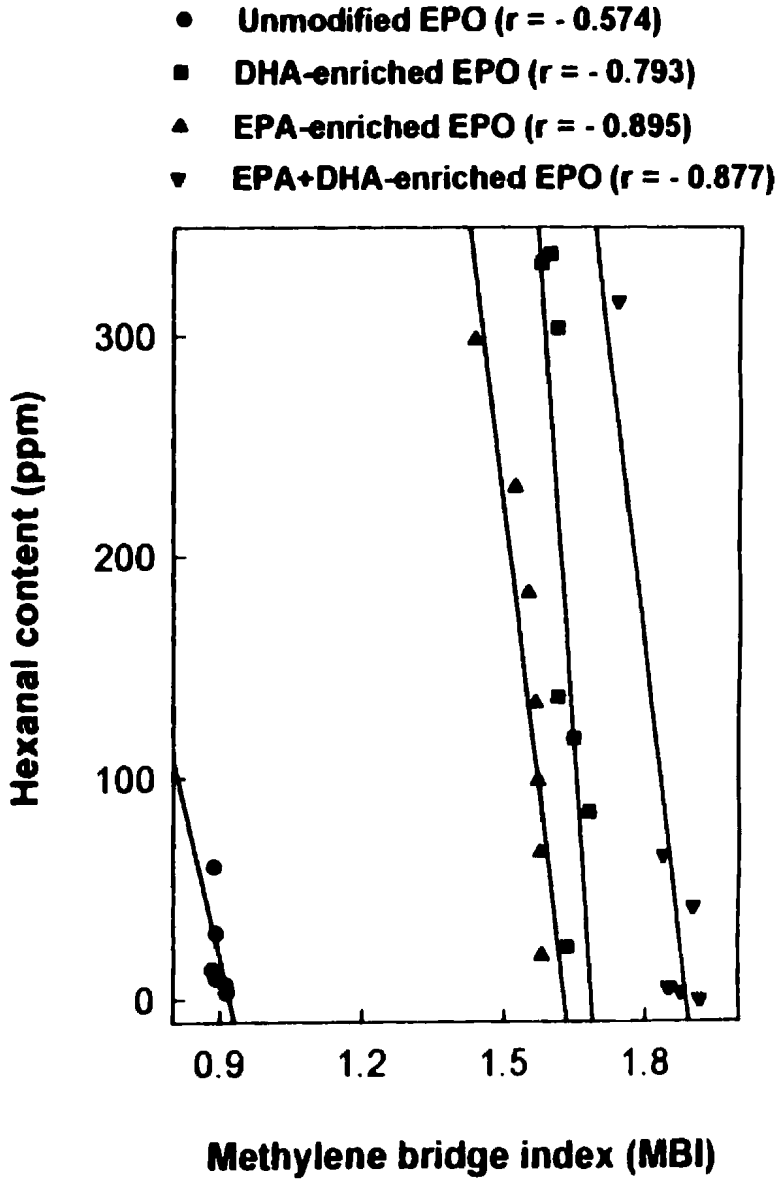
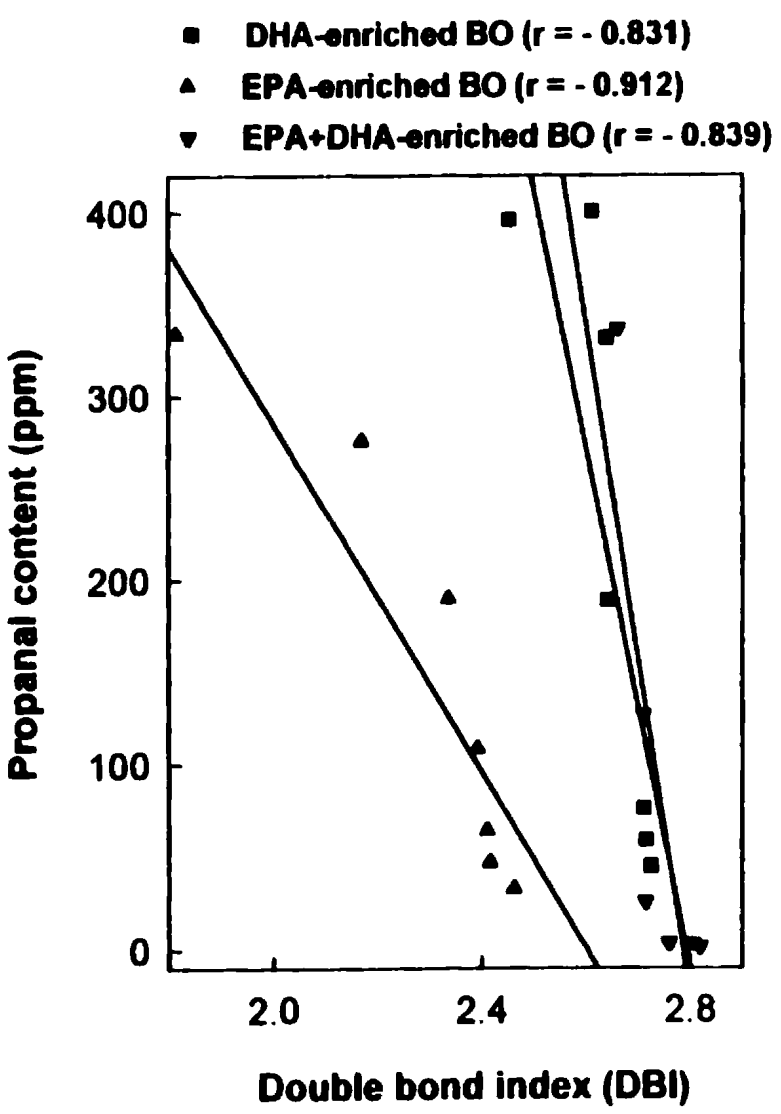

Unmodified EPO (r = - 0.574)
DHA-enriched EPO (r = - 0.793)
EPA-enriched EPO (r = - 0.895)
EPA+DHA-enriched EPO (r = - 0.877)
Hexanal content (ppm)
300
200
100
0
0.9
1.2
1.5
1.8
Methylene bridge index (MBI)

Figure A.8 Relationships between propanal contents and double bond index (DBI) and methylene bridge index (MBI) of oxidized DHA, EPA and EPA+DHA-enriched borage oils (BO)


DHA-enriched BO (r = - 0.831)
EPA-enriched BO (r = - 0.912)
EPA+DHA-enriched BO (r = - 0.839)
Propanal content (ppm)
400
300
200
100
0
2.0
2.4
2.8
Double bond index (DBI)

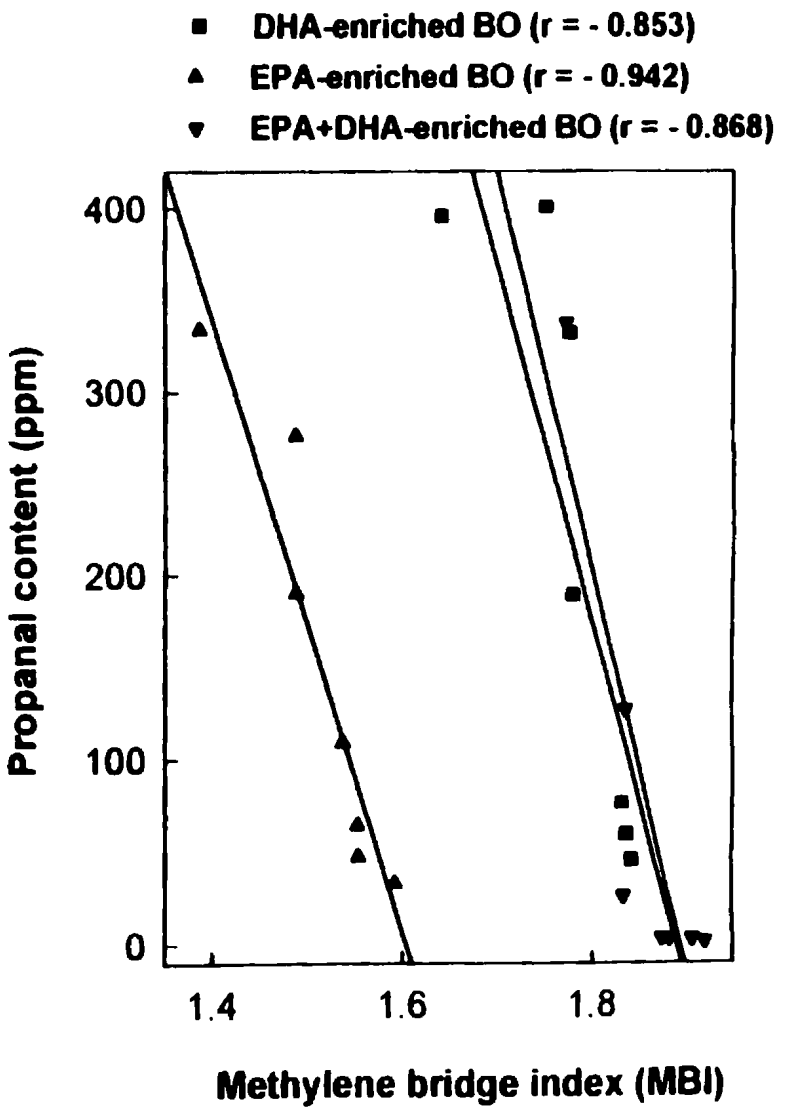
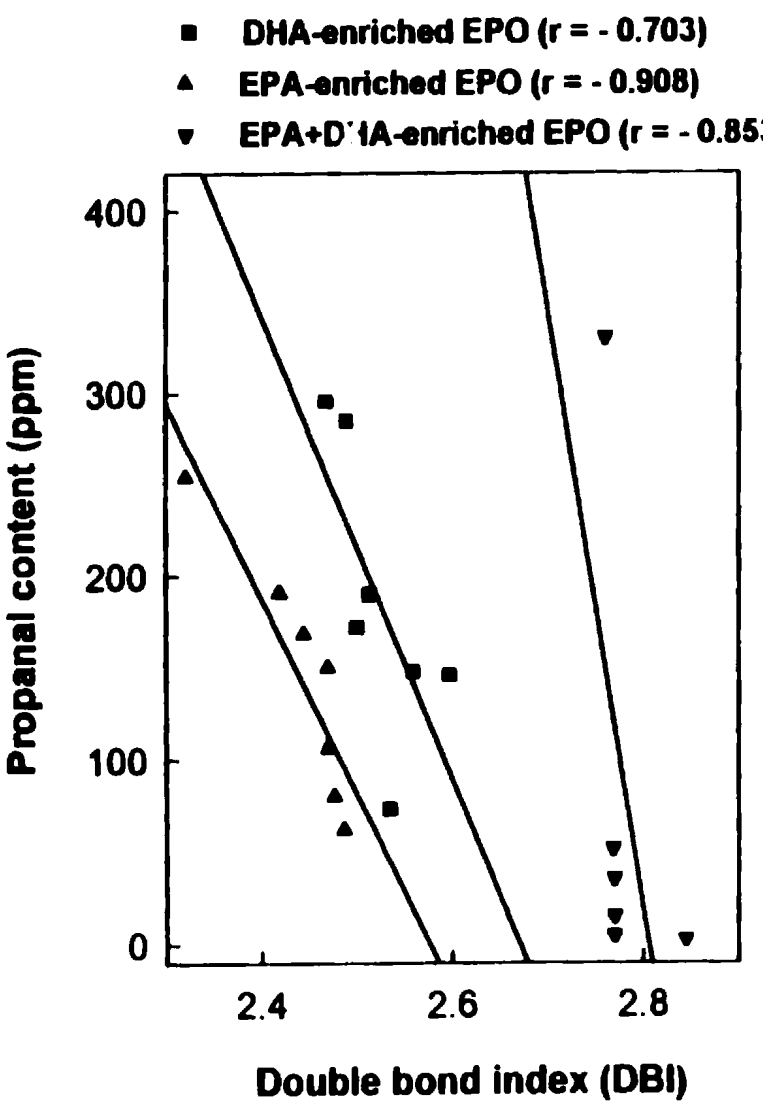

DHA-enriched BO (r = - 0.853)
EPA-enriched BO (r = - 0.942)
EPA+DHA-enriched BO (r = - 0.868)
Propanal content (ppm)
400
300
200
100
0
1.4
1.6
1.8
Methylene bridge index (MBI)

Figure A.9 Relationships between propanal contents and double bond index (DBI) and methylene bridge index (MBI) of oxidized DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO)


DHA-enriched EPO (r = - 0.703)
EPA-enriched EPO (r = - 0.908)
EPA+DHA-enriched EPO (r = - 0.853)
Propanal content (ppm)
400
300
200
100
0
2.4
2.6
2.8
Double bond index (DBI)

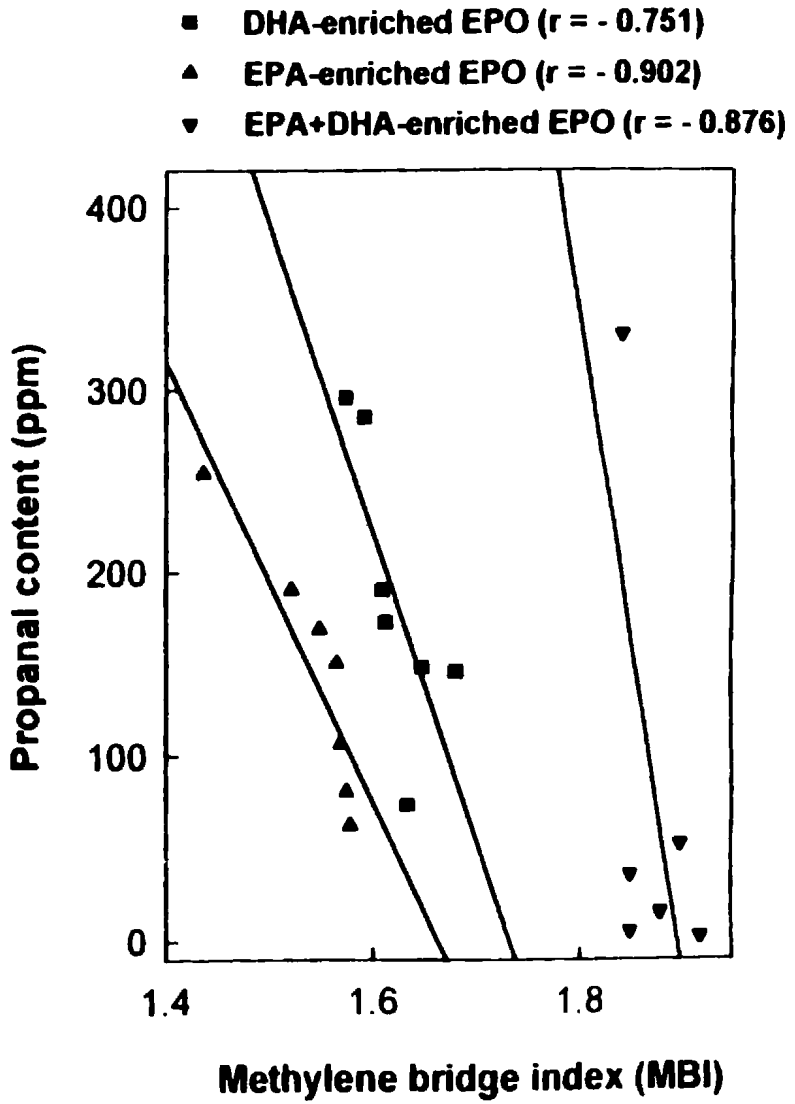
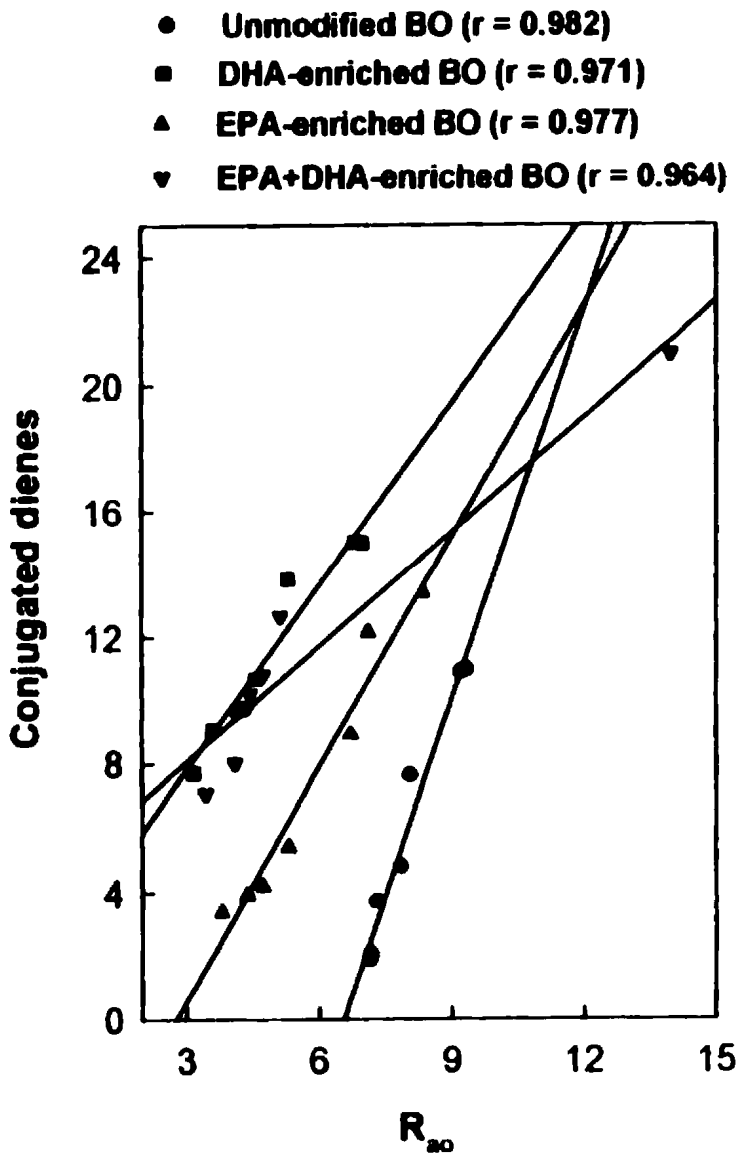

DHA-enriched EPO (r = - 0.751)
EPA-enriched EPO (r = - 0.902)
EPA+DHA-enriched EPO (r = - 0.876)
Propanal content (ppm)
400
300
200
100
0
1.4
1.6
1.8
Methylene bridge index (MBI)

Figure A.10 Relationships between conjugated dienes and the ratio of aliphatic to olefinic protons (R_{ao}) and ratio of aliphatic to diallylmethylene protons (R_{ad}) of oxidized DHA, EPA and EPA+DHA-enriched borage oils (BO) and unmodified BO


Unmodified BO (r = 0.982)
DHA-enriched BO (r = 0.971)
EPA-enriched BO (r = 0.977)
EPA+DHA-enriched BO (r = 0.964)
Conjugated dienes
0
4
8
12
16
20
24
3
6
9
12
15
R_{ao}

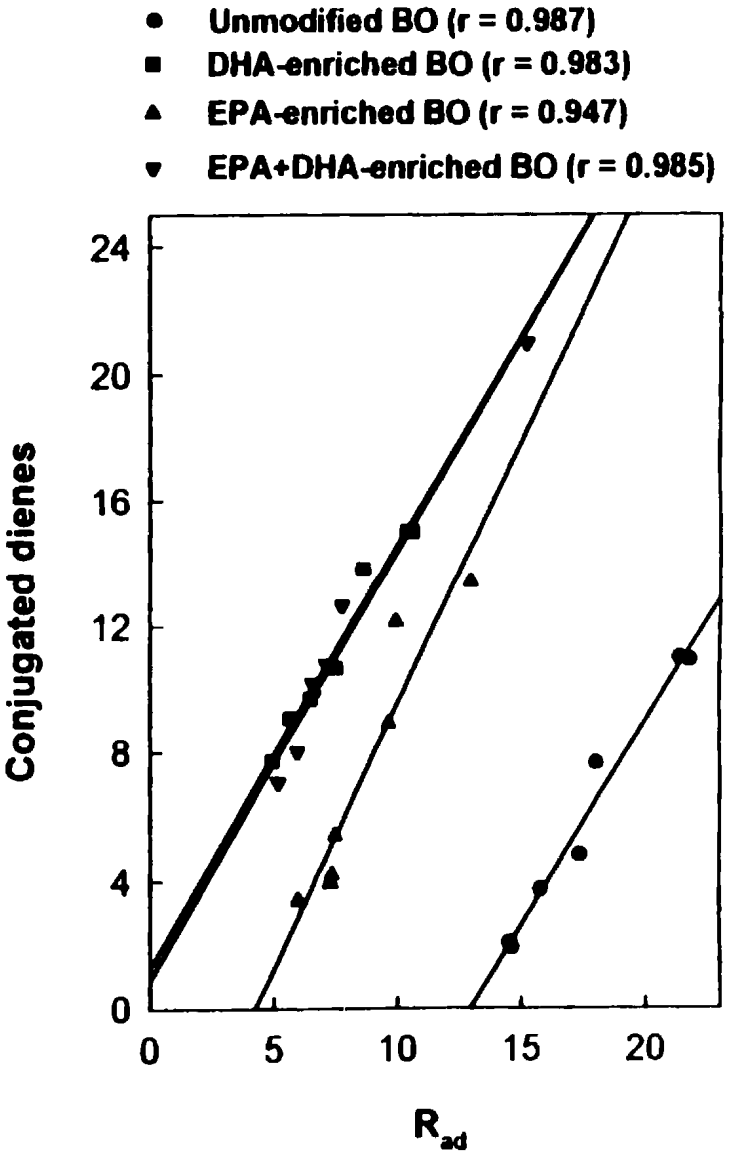
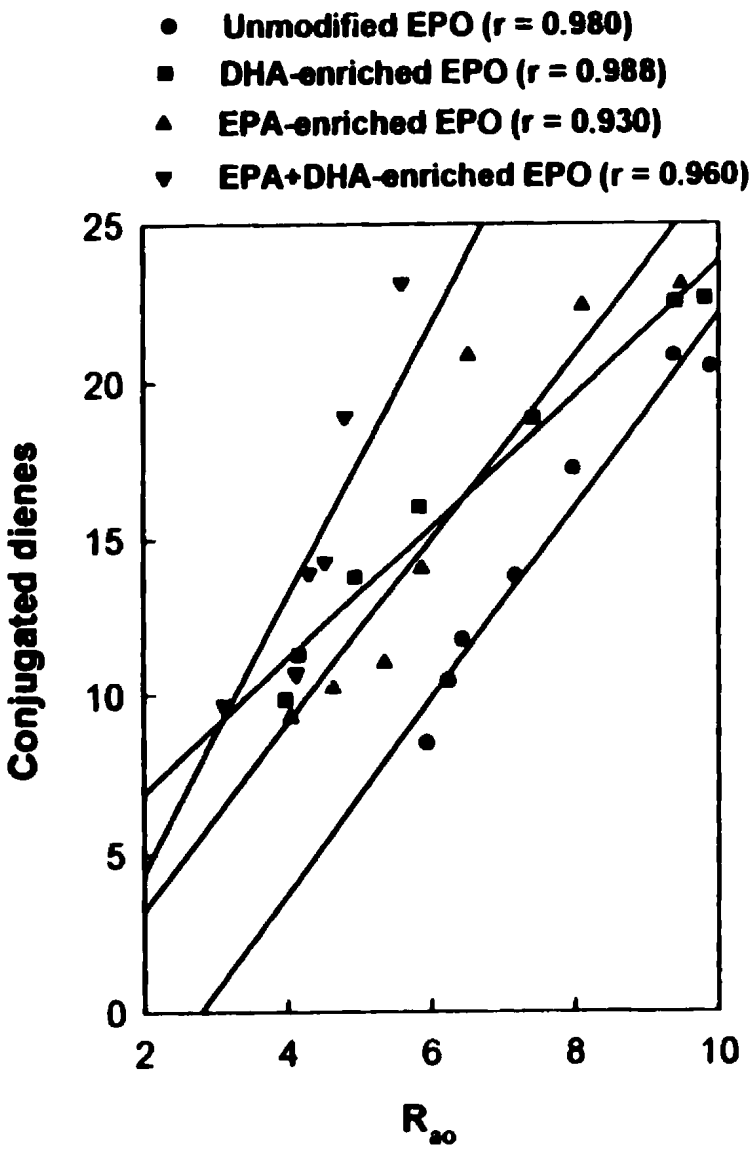

Unmodified BO (r = 0.987)
DHA-enriched BO (r = 0.983)
EPA-enriched BO (r = 0.947)
EPA+DHA-enriched BO (r = 0.985)
Conjugated dienes
0
4
8
12
16
20
24
0
5
10
15
20
R_{ad}

Figure A.11 Relationships between conjugated dienes and the ratio of aliphatic to olefinic protons (R_{ao}) and ratio of aliphatic to diallylmethylene protons (R_{ad}) of oxidized DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO) and unmodified EPO


Unmodified EPO (r = 0.980)
DHA-enriched EPO (r = 0.988)
EPA-enriched EPO (r = 0.930)
EPA+DHA-enriched EPO (r = 0.960)
Conjugated dienes
0
5
10
15
20
25
2
4
6
8
10
R_{ao}

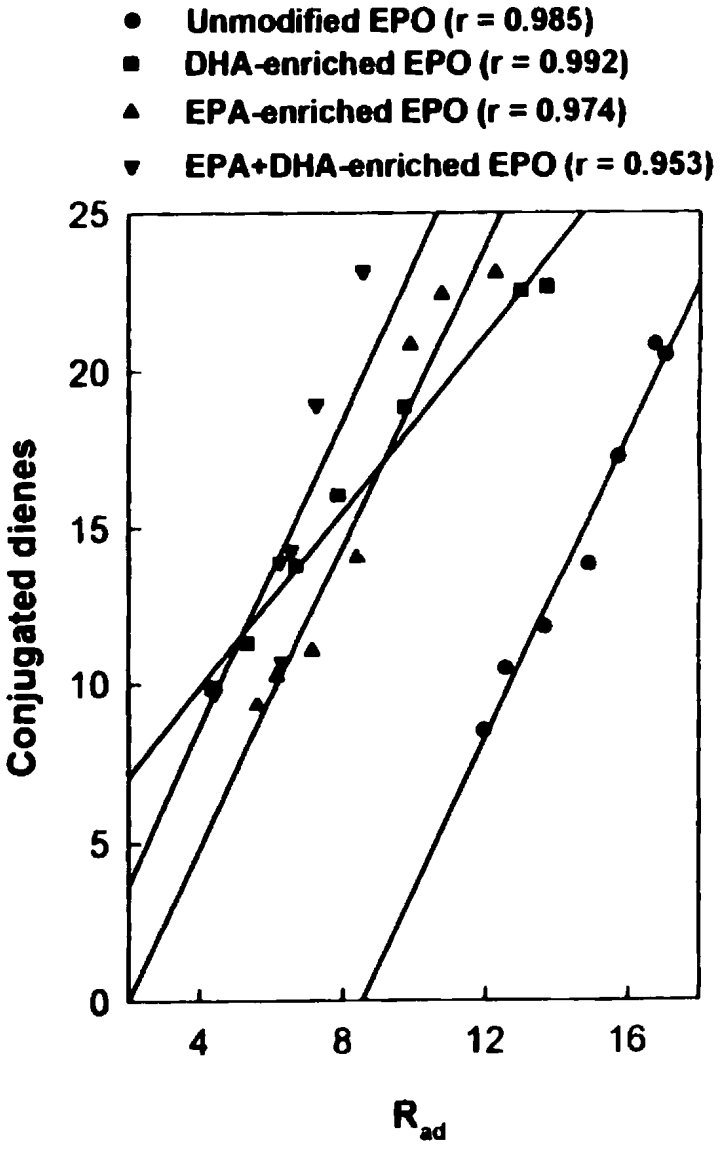
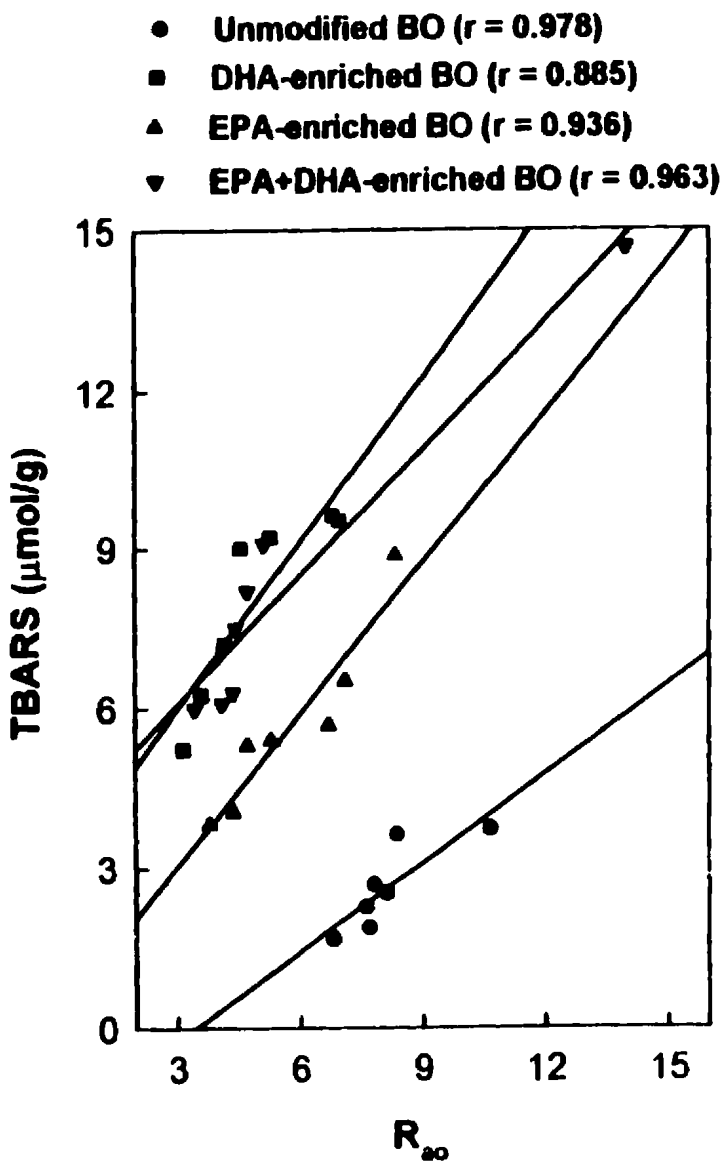

Unmodified EPO (r = 0.985)
DHA-enriched EPO (r = 0.992)
EPA-enriched EPO (r = 0.974)
EPA+DHA-enriched EPO (r = 0.953)
Conjugated dienes
0
5
10
15
20
25
4
8
12
16
R_{ad}

Figure A.12 Relationships between TBARS values and the ratio of aliphatic to olefinic protons (R_{ao}) and ratio of aliphatic to diallylmethylene protons (R_{ad}) of oxidized DHA, EPA and EPA+DHA-enriched borage oils (BO) and unmodified BO


Unmodified BO (r = 0.978)
DHA-enriched BO (r = 0.885)
EPA-enriched BO (r = 0.936)
EPA+DHA-enriched BO (r = 0.963)
TBARS (μmol/g)
0
3
6
9
12
15
R_{ao}

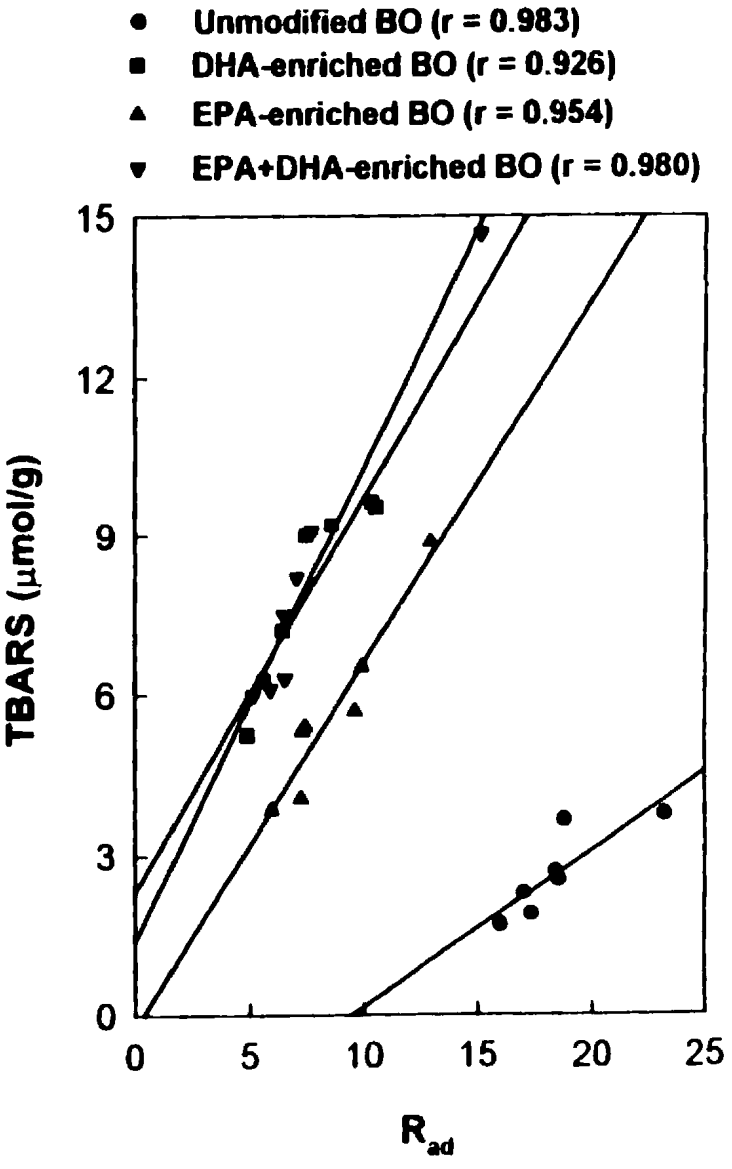
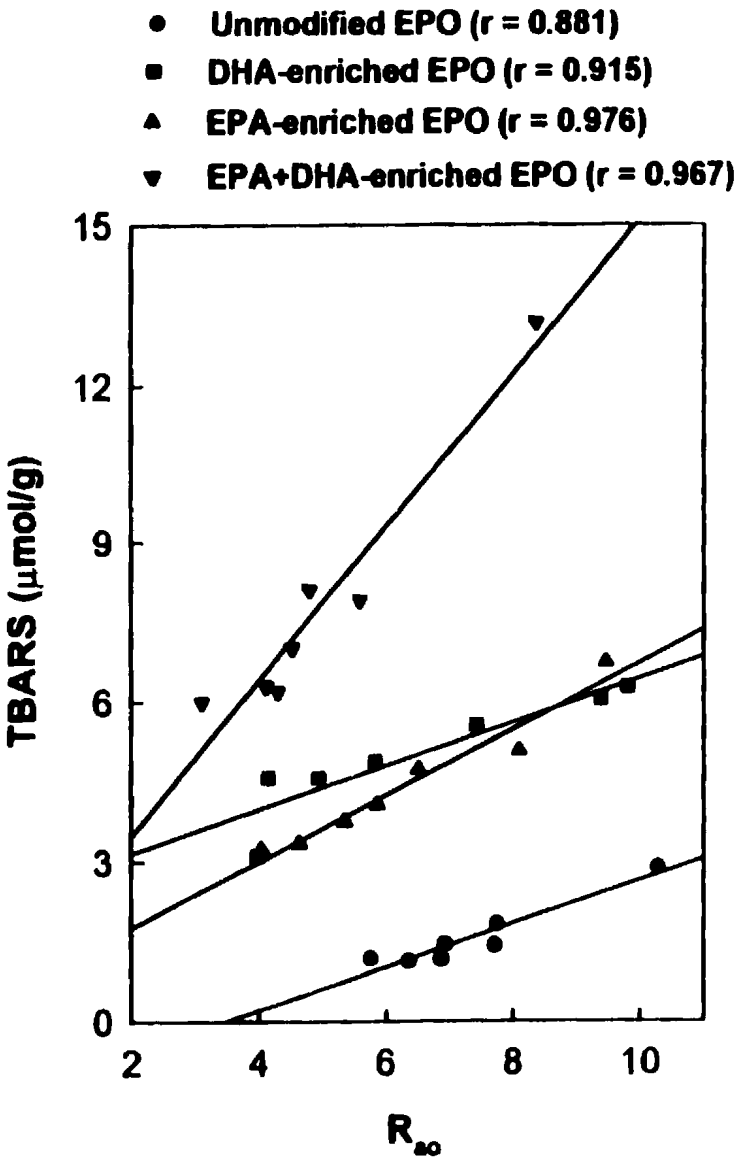

Unmodified BO (r = 0.983)
DHA-enriched BO (r = 0.926)
EPA-enriched BO (r = 0.954)
EPA+DHA-enriched BO (r = 0.980)
TBARS (μmol/g)
0
3
6
9
12
15
0
5
10
15
20
25
R_{ad}

Figure A.13 Relationships between TBARS values and the ratio of aliphatic to olefinic protons (R_{ao}) and ratio of aliphatic to diallylmethylene protons (R_{ad}) of oxidized DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO) and unmodified EPO


Unmodified EPO (r = 0.881)
DHA-enriched EPO (r = 0.915)
EPA-enriched EPO (r = 0.976)
EPA+DHA-enriched EPO (r = 0.967)
TBARS (μmol/g)
0
3
6
9
12
15
2
4
6
8
10
R_{ao}

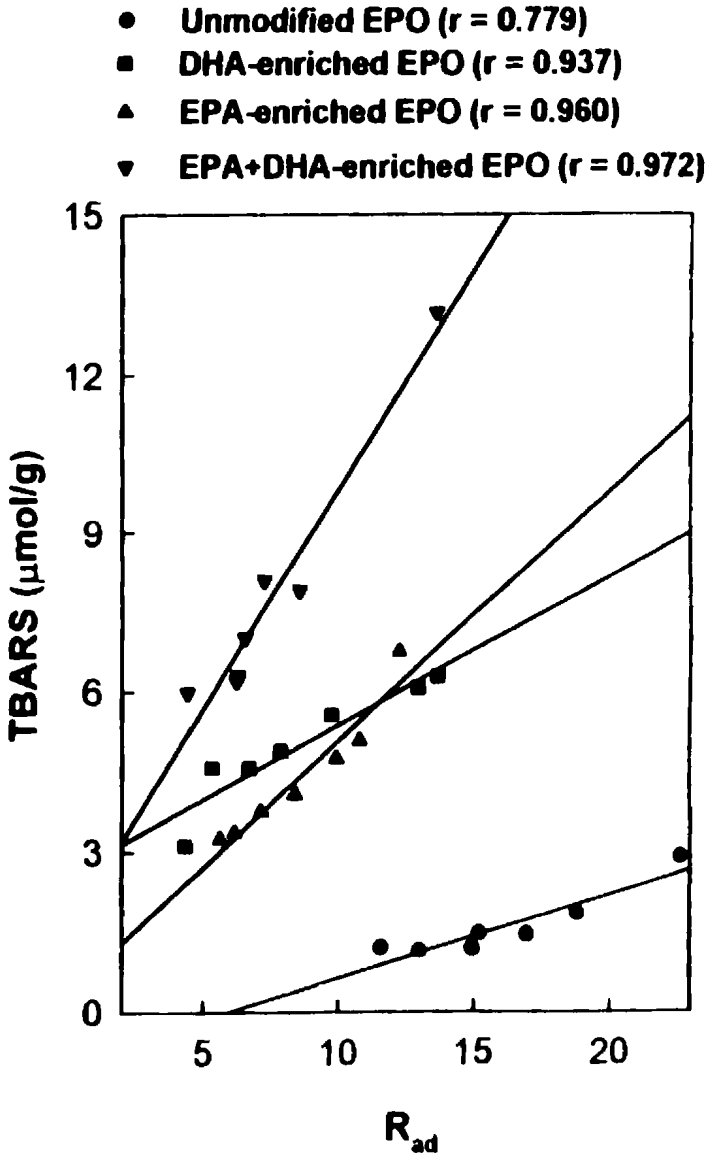
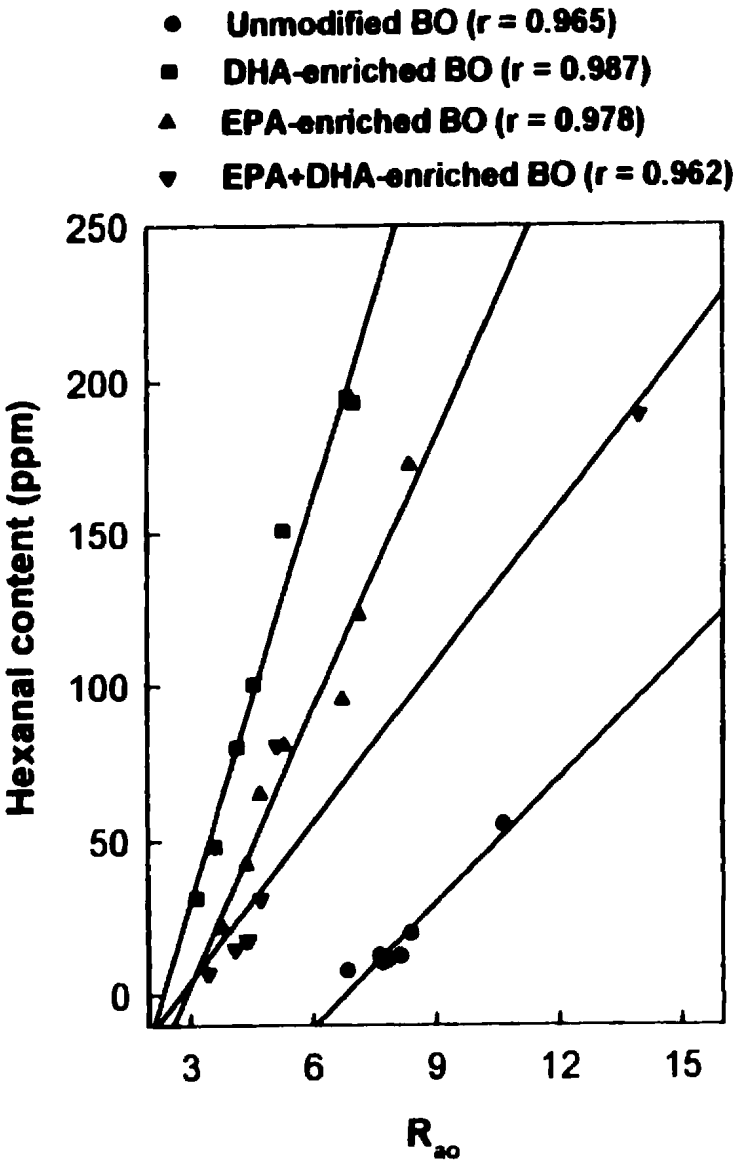

Unmodified EPO (r = 0.779)
DHA-enriched EPO (r = 0.937)
EPA-enriched EPO (r = 0.960)
EPA+DHA-enriched EPO (r = 0.972)
TBARS (μmol/g)
0
3
6
9
12
15
5
10
15
20
R_{ad}

Figure A.14 Relationships between hexanal contents and the ratio of aliphatic to olefinic protons (R_{ao}) and ratio of aliphatic to diallylmethylene protons (R_{ad}) of oxidized DHA, EPA and EPA+DHA-enriched borage oils (BO) and unmodified BO


Unmodified BO (r = 0.965)
DHA-enriched BO (r = 0.987)
EPA-enriched BO (r = 0.978)
EPA+DHA-enriched BO (r = 0.962)
Hexanal content (ppm)
250
200
150
100
50
0
3
6
9
12
15
R_{ao}

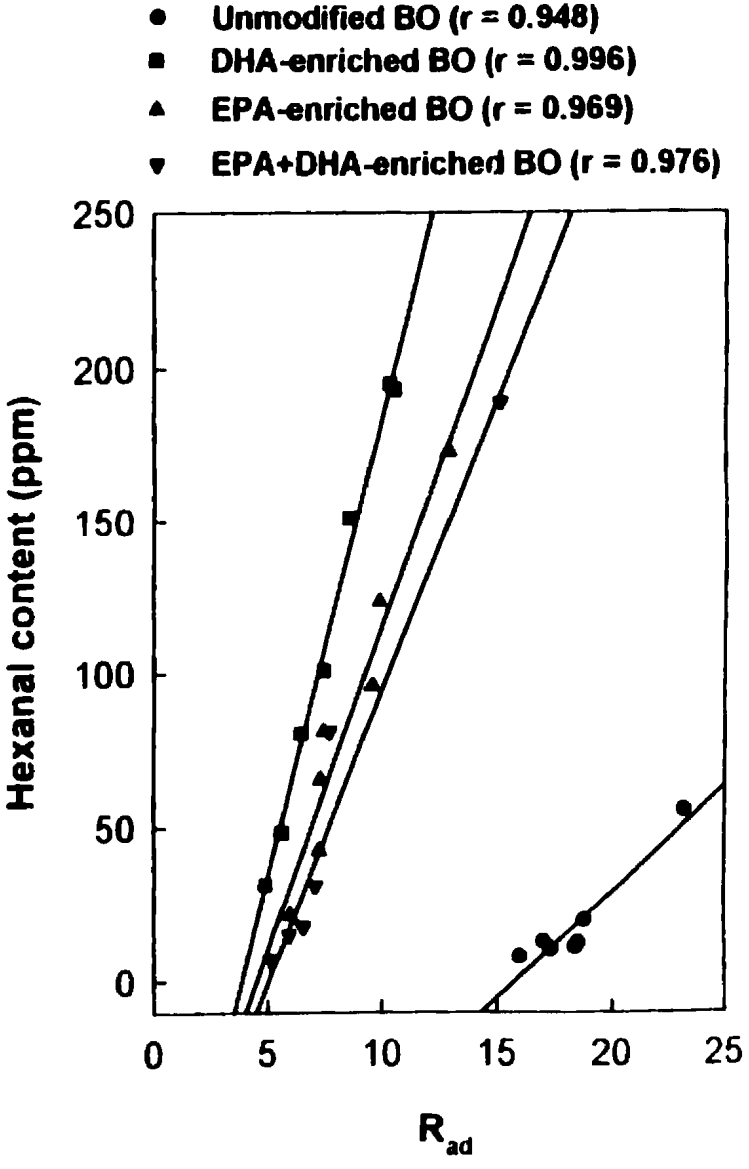
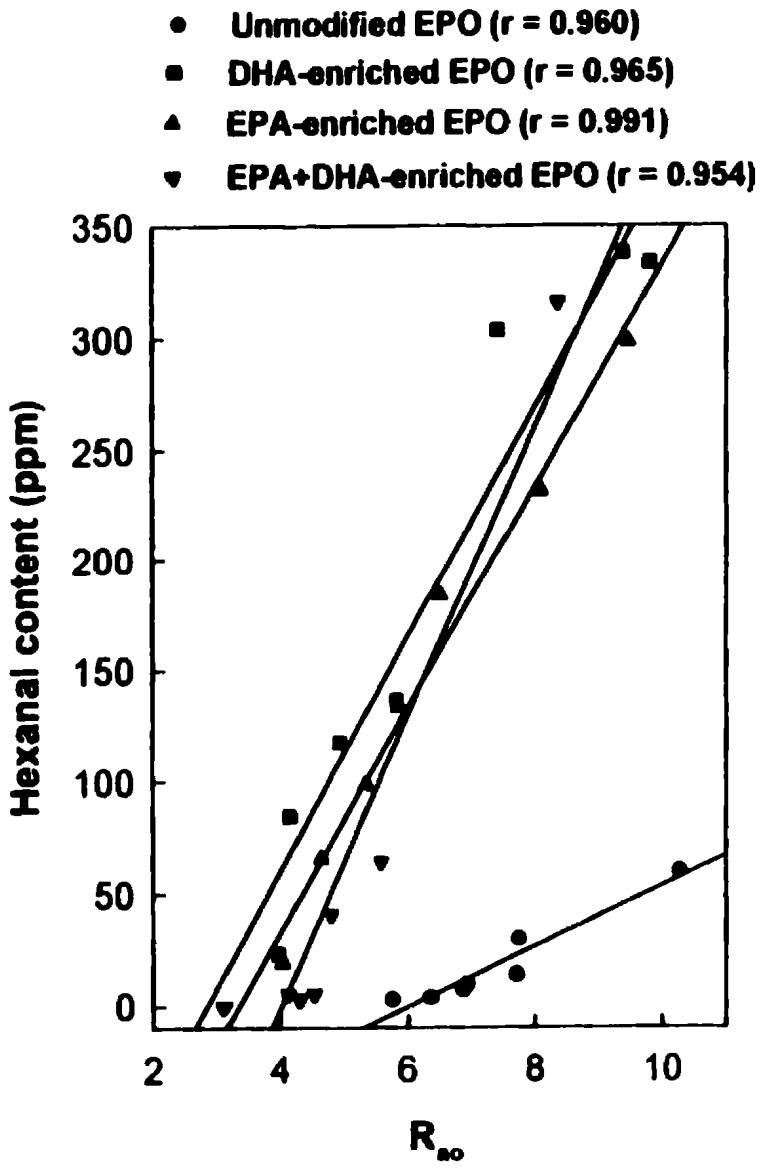

Unmodified BO (r = 0.948)
DHA-enriched BO (r = 0.996)
EPA-enriched BO (r = 0.969)
EPA+DHA-enriched BO (r = 0.976)
Hexanal content (ppm)
250
200
150
100
50
0
0
5
10
15
20
25
R_{ad}

Figure A.15 Relationships between hexanal contents and the ratio of aliphatic to olefinic protons (R_{ao}) and ratio of aliphatic to diallylmethylene protons (R_{ad}) of oxidized DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO) and unmodified EPO


Unmodified EPO (r = 0.960)
DHA-enriched EPO (r = 0.965)
EPA-enriched EPO (r = 0.991)
EPA+DHA-enriched EPO (r = 0.954)
Hexanal content (ppm)
350
300
250
200
150
100
50
0
2
4
6
8
10
R_{ao}

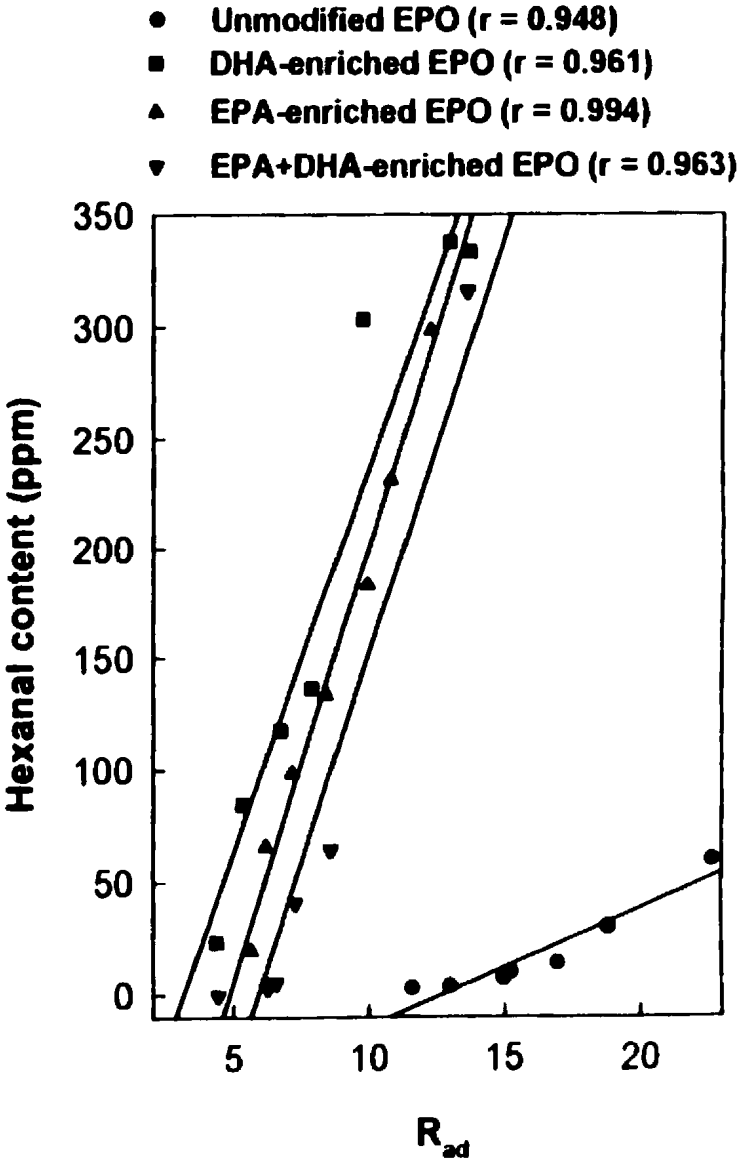
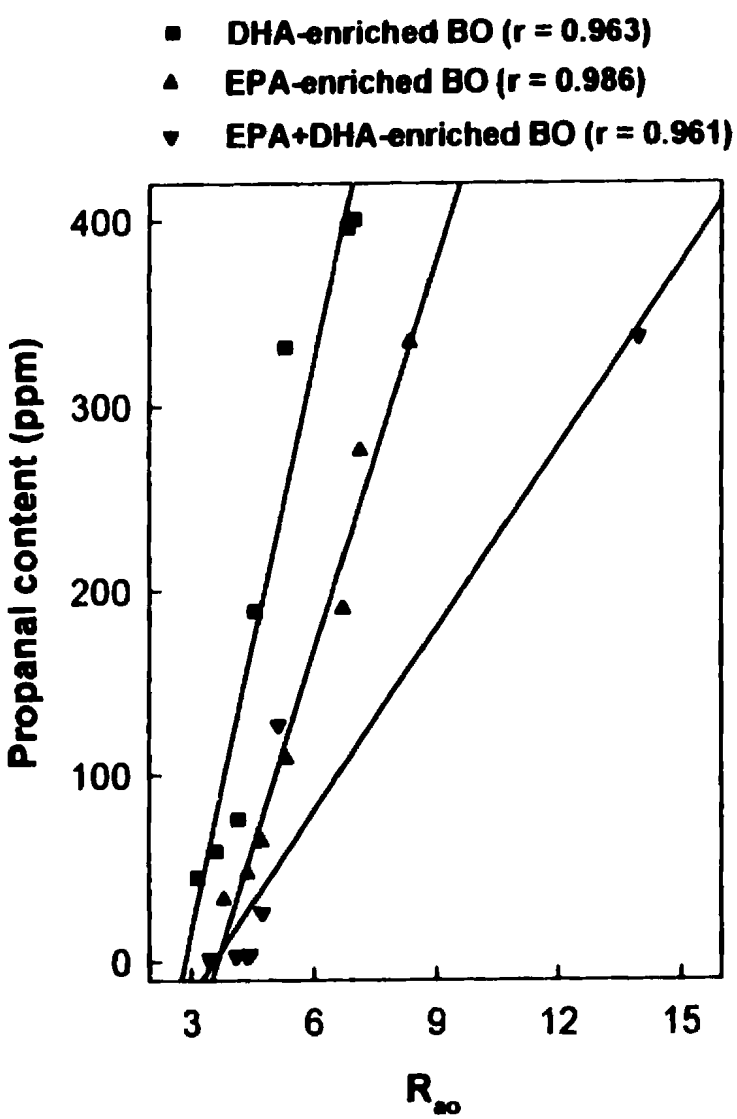

Unmodified EPO (r = 0.948)
DHA-enriched EPO (r = 0.961)
EPA-enriched EPO (r = 0.994)
EPA+DHA-enriched EPO (r = 0.963)
Hexanal content (ppm)
350
300
250
200
150
100
50
0
5
10
15
20
R_{ad}

Figure A.16 Relationships between propanal contents and the ratio of aliphatic to olefinic protons (R_{ao}) and ratio of aliphatic to diallylmethylene protons (R_{ad}) of oxidized DHA, EPA and EPA+DHA-enriched borage oils (BO)


DHA-enriched BO (r = 0.963)
EPA-enriched BO (r = 0.986)
EPA+DHA-enriched BO (r = 0.961)
Propanal content (ppm)
400
300
200
100
0
3
6
9
12
15
R_{so}

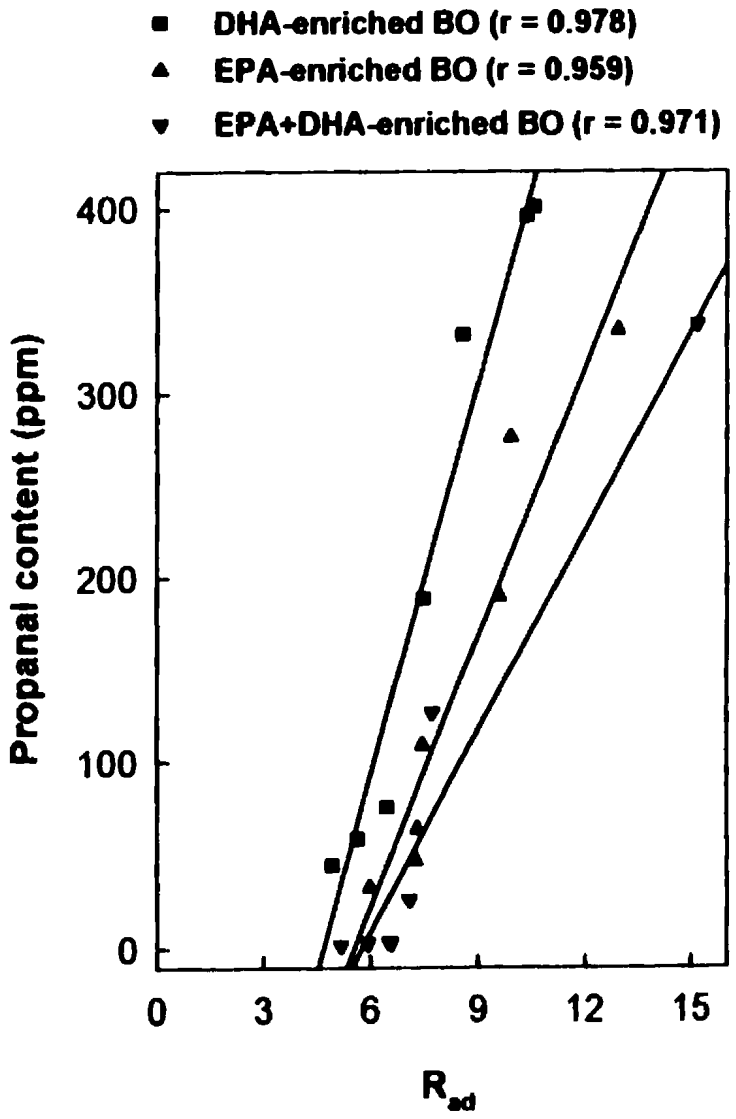
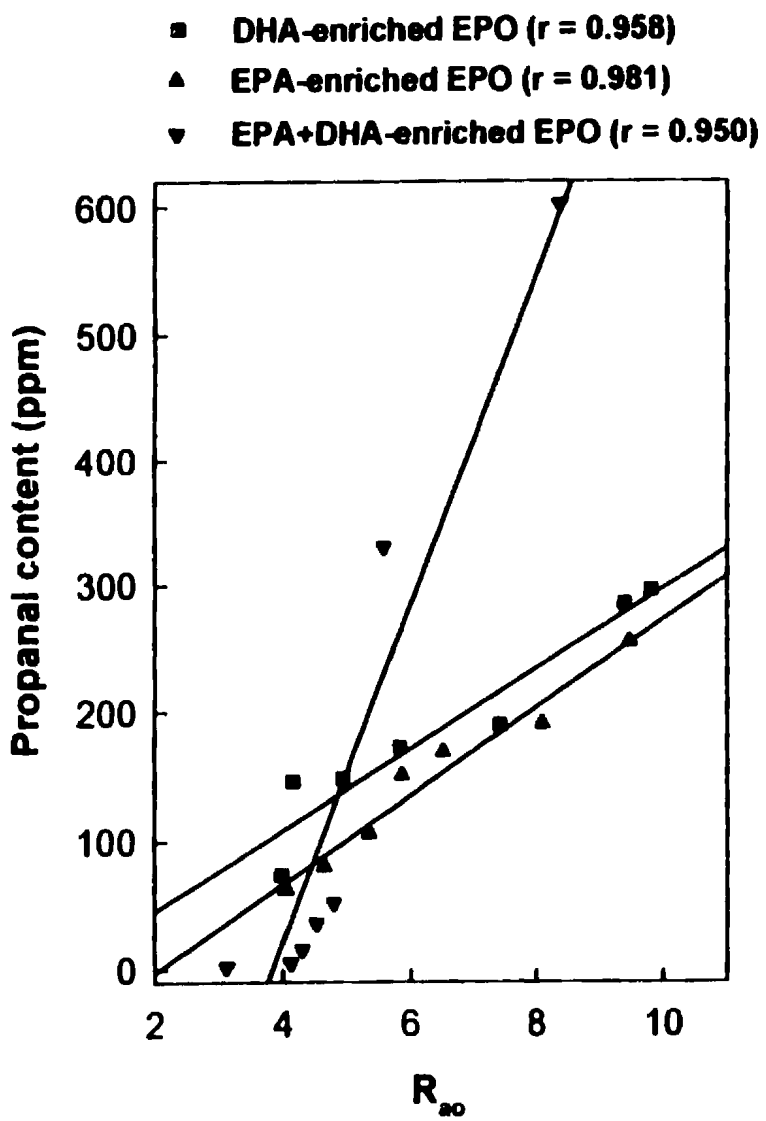
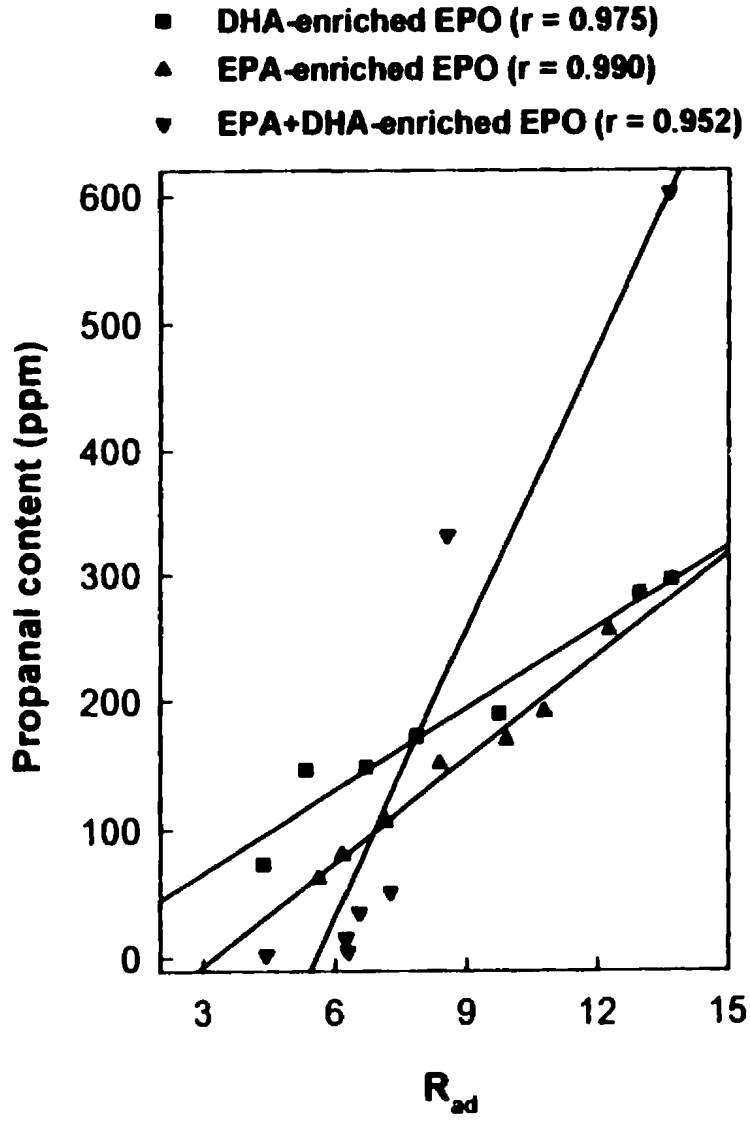

DHA-enriched BO (r = 0.978)
EPA-enriched BO (r = 0.959)
EPA+DHA-enriched BO (r = 0.971)
Propanal content (ppm)
400
300
200
100
0
0
3
6
9
12
15
R_{sd}

Figure A.17 Relationships between propanal contents and the ratio of aliphatic to olefinic protons (R_{ao}) and ratio of aliphatic to diallylmethylene protons (R_{ad}) of oxidized DHA, EPA and EPA+DHA-enriched evening primrose oils (EPO)


DHA-enriched EPO (r = 0.958)
EPA-enriched EPO (r = 0.981)
EPA+DHA-enriched EPO (r = 0.950)
Propanal content (ppm)
600
500
400
300
200
100
0
2
4
6
8
10
R_{ao}


DHA-enriched EPO (r = 0.975)
EPA-enriched EPO (r = 0.990)
EPA+DHA-enriched EPO (r = 0.952)
Propanal content (ppm)
600
500
400
300
200
100
0
3
6
9
12
15
R_{ad}

APPENDIX 2

Details of data analysis obtained by SAS programme for optimization of incorporation of EPA+DHA (%) into borage oil

Y = EPA+DHA incorporation (%)

X_1 = Amount of enzyme (units)

X_2 = Reaction temperature (°C)

X_3 = Reaction time (h)

Coding Coefficients for the Independent Variables

Factor	Subtracted off	Divided by
X1	250.000000	100.000000
X2	45.000000	15.000000
X3	18.000000	12.000000

Response Surface for Variable Y: % TOTAL DHA+EPA

Response Mean	29.923529
Root MSE	0.673595
R-Square	0.9901
Coef. of Variation	2.2511

Regression	Degrees of Freedom	Type I Sum of Squares	R-Square	F-Ratio	Prob > F
Linear	3	123.326000	0.3827	90.602	0.0000
Quadratic	3	178.728476	0.5546	131.3	0.0000
Crossproduct	3	17.020000	0.0528	12.504	0.0034
Total Regress	9	319.074476	0.9901	78.136	0.0000

Residual	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F
Lack of Fit	5	1.849446	0.369889	0.558	0.7413
Pure Error	2	1.326667	0.663333		
Total Error	7	3.176113	0.453730		

Parameter	Degrees of Freedom	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEPT	1	-34.367113	3.958235	-8.682	0.0001
X1	1	0.173087	0.022175	7.806	0.0001
X2	1	1.648160	0.171582	9.606	0.0000
X3	1	0.860100	0.130023	6.615	0.0003
X1*X1	1	-0.000224	0.000041152	-5.440	0.0010
X2*X1	1	-0.000500	0.000159	-3.149	0.0162
X2*X2	1	-0.017950	0.001829	-9.814	0.0000
X3*X1	1	-0.001042	0.000198	-5.249	0.0012
X3*X2	1	0.000278	0.001323	0.210	0.8397
X3*X3	1	-0.011033	0.002858	-3.861	0.0062

Parameter	Parameter Estimate from Coded Data
INTERCEPT	34.550704
X1	1.990000
X2	-1.310000
X3	2.580000
X1*X1	-2.238732
X2*X1	-0.750000
X2*X2	-4.038732
X3*X1	-1.250000
X3*X2	0.050000
X3*X3	-1.588732

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F
X1	4	70.029170	17.507293	38.585	0.0001
X2	4	65.383133	16.345783	36.025	0.0001
X3	4	85.846604	21.461651	47.300	0.0000

Canonical Analysis of Response Surface
(based on coded data)

Factor	Critical Value Coded	Uncoded	
X1	0.280139	278.013881	ENZYME UNITS
X2	-0.183865	42.242030	TEMP
X3	0.698870	26.386434	TIME

Predicted value at stationary point 35.851415

Eigenvalues	Eigenvectors X1	X2	X3
-1.192502	-0.531615	0.077450	0.843437
-2.555843	0.820811	-0.198541	0.535585
-4.117852	0.208938	0.977028	0.041975

Stationary point is a maximum.

Estimated Ridge of Maximum Response for Variable Y: % TOTAL DHA+EPA

Coded Radius	Estimated Response	Standard Error	Uncoded Factor Values X1	X2	X3
0.0	34.550704	0.288231	250.000000	45.000000	18.000000
0.1	34.876896	0.287920	255.553676	44.470911	18.903713
0.2	35.153744	0.287112	260.799307	43.997763	19.854112
0.3	35.382268	0.286258	265.605589	43.575561	20.855613
0.4	35.563652	0.286282	269.828162	43.202090	21.912770
0.5	35.699311	0.288809	273.313384	42.877219	23.028860
0.6	35.790939	0.296368	275.912988	42.601997	24.204061
0.7	35.840514	0.312332	277.510143	42.377429	25.433715
0.8	35.850235	0.340266	278.049199	42.203157	26.707721
0.9	35.822393	0.382823	277.553197	42.076576	28.011899
1.0	35.759217	0.440948	276.116789	41.992854	29.330952