

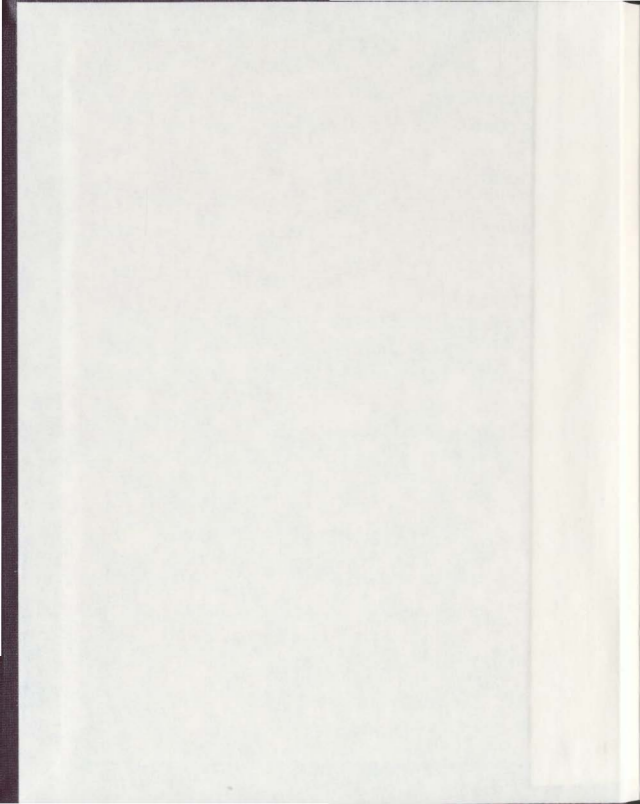
PRODUCTION OF STRUCTURED LIPIDS VIA
ENZYMATIC INTERESTERIFICATION OF
GAMMA-LINOLENIC ACID (GLA) AND
MARINE OILS

CENTRE FOR NEWFOUNDLAND STUDIES

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**PRODUCTION OF STRUCTURED LIPIDS
VIA ENZYMATIC INTERESTERIFICATION OF
GAMMA-LINOLENIC ACID (GLA) AND MARINE OILS.**

BY

©**SHARON A. SPURVEY, B.Sc. (Hon)**

A thesis submitted to the School of Graduate

Studies in partial fulfillment of the
requirements for the degree of

Master of Science

Department of Biochemistry

Memorial University of Newfoundland

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ABSTRACT

The importance of polyunsaturated fatty acids (PUFA) in human nutrition and disease prevention has long been recognised. Both ω 3 and ω 6 PUFA serve as precursors of eicosanoids, which are involved in many important biochemical processes in the human body.

Omega-3 fatty acids, which are present in marine oils, play an important role in the prevention and treatment of coronary heart disease, hypertension, arthritis, and other inflammatory and autoimmune disorders as well as cancer. Gamma-linolenic acid (GLA), an ω 6 fatty acid, is present in oils from borage and evening primrose seeds at 17-25% and 8-10%, respectively. GLA has been reported to be important for the prevention and/or treatment of skin diseases, pre-menstrual syndrome, diabetes, inflammatory and autoimmune disorders, and cancer.

Urea complexation of borage oil resulted in the concentration of gamma-linolenic acid (GLA) in the non-urea complexed fraction thus allowing easy separation of GLA from the hydrolysed borage oil. The process parameters such as the mole ratio of urea-to-fatty acid, reaction temperature and reaction time were optimised by response surface methodology (RSM) using a 3-factor-3-level face-centred cube design to achieve the maximum amount of GLA in the borage oil concentrate. The optimum conditions for production of GLA concentrate were: urea-to-fatty acid ratio of 3.7, reaction temperature of -7°C and reaction time of 16 h, which yielded a 91% GLA concentrate.

The GLA was subsequently enzymatically reacted with seal blubber oil and menhaden oil to produce a structured lipid. The process variables studied for the lipase-esterified reaction were the concentration of enzyme (100-700 units/g of oil), reaction temperature (30-60°C), reaction time (0-48h) and the mole ratio of GLA to triacylglycerols (TAG) (1:1-5:1). Two lipases chosen for the interesterification reaction were from *Pseudomonas sp.* and *Mucor miehei*. The lipase from *Pseudomonas sp.* was chosen over that from *Mucor miehei* to catalyse the interesterification reaction due to higher incorporation of GLA. For the interesterification reaction, the best conditions were 3:1 mole ratio of GLA to TAG, reaction temperature of 40 °C, reaction time of 24 h and an enzyme concentration of 500 units/g of oil. Under these conditions, incorporation of GLA was 37.1% for seal blubber oil (SBO) and 39.6% for menhaden oil (MO). The resultant oils containing both ω3 and ω6 fatty acids are considered important for clinical as well as nutritional purposes.

Stereospecific analysis was carried out to establish the positional distribution of fatty acids in the triacylglycerols (TAG) of the modified seal blubber oil (MSBO) and modified menhaden oil (MMO). In MSBO, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) were located mainly in the *sn*-1 and *sn*-3 positions of the TAG molecules. In MMO, EPA, DHA and DPA were equally distributed amongst the *sn*-1, *sn*-2 and *sn*-3 positions of the TAG. GLA was also present in all positions of the TAG of MMO and MSBO. This indicates that lipase from *Pseudomonas sp.* (PS-30) was able to involve the middle position of the TAG in the acidolysis process.

Thus, structured lipids containing EPA, DHA, DPA and GLA were successfully produced; consumption of these products is expected to render health benefits. The structured lipids (MSBO and MMO) produced were assessed for their autooxidative and photooxidative stability toward accelerated oxidation under Schaal oven conditions at 60°C, or storage at room temperature under fluorescent lighting. Conjugated diene (CD), 2-thiobarbituric acid reactive substances (TBARS) and nuclear magnetic resonance (NMR) determinations were employed to monitor progression of the oxidation of the oils. During autooxidation, the modified oils were least stable due to their high content of polyunsaturated fatty acids (PUFA) and loss of natural antioxidants during the acidolysis process. In the case of photooxidation, both the modified and unmodified seal blubber oils (SBO and MSBO) were more stable than their MO counterparts. The presence of highly polyunsaturated fatty acids (PUFA) in the latter oils as well as photosensitizers, such as chlorophyll in MO, might be responsible for this observation.

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TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	viii
LIST OF TABLES	ix
LIST OF ABBREVIATIONS	xi
CHAPTER 1. INTRODUCTION	1
CHAPTER 2. LITERATURE REVIEW	4
2.1 Importance of fats and oils in the marketplace	4
2.2 Essential fatty acids in fats and oils and their metabolism	5
2.3 Significance of marine oils	8
2.3.1 Sources of marine lipids	8
2.3.2 Chemistry and composition of marine lipids	9
2.3.2.1 Triacylglycerols and free fatty acids	11
2.3.2.2 Phospholipids	16
2.3.3 Industrial uses of marine lipids	17
2.3.4 Food uses of marine lipids	18
2.3.5 Marine lipids in nutrition and disease prevention	19
2.4 Significance of gamma linolenic acid (GLA)	25
2.4.1 Sources of GLA	26
2.4.2 Uses of GLA	26
2.4.3 GLA in health promotion and disease prevention	27
2.5 Production of ω 6 fatty acid concentrate	29
2.5.1 Methods of concentration of GLA	30
2.5.1.1 Urea adduct formation	30
2.5.1.2 Solvent winterization	32

2.5.1.3 Enzymatic selective hydrolysis or esterification	37
2.5.2 Process optimization	38
2.6 Importance of structured lipids	39
2.6.1 Synthesis of structured lipids	40
2.6.1.1 Transesterification	43
2.6.1.2 Acidolysis	45
2.6.1.3 Alcoholysis	47
2.6.2 Importance of positional distribution in triacylglycerols (TAG)	48
2.7 Oxidation of lipids	49
2.7.1 Autooxidation	49
2.7.2 Photooxidation	55
2.7.3 Decomposition of hydroperoxides	60
2.7.4 Factors affecting lipid oxidation	62
2.8 Control of lipid oxidation	64
2.8.1 Use of antioxidants	65
2.8.2 Mechanism of action of antioxidants	66
2.8.3 Synthetic antioxidants	67
2.8.4 Natural antioxidants	68
2.9 Measurement of lipid oxidation	70
2.9.1 Primary changes	71
2.9.1.1 Measurement of hydroperoxides	71
2.9.1.2 Measurement of conjugated dienes (CD)	72
2.9.2 Secondary oxidative changes and their measurements	73
2.9.2.1 2-Thiobarbituric acid (TBARS)	73
2.9.2.2 Nuclear magnetic resonance (NMR)	74
CHAPTER 3. MATERIALS AND METHODS	76
3.1 Materials	76
3.1.1 Samples	76
3.1.2 Chemicals	76
3.2 Production of gamma linolenic acid (GLA) concentrates from borage oil (BO)	76
3.2.1 Preparation of free fatty acids from borage oil (BO)	77
3.2.2 Preparation of gamma linolenic acid (GLA) concentrates from borage oil (BO) by urea complexation	77

3.3 Optimization procedure for production of gamma linolenic acid concentrate via urea complexation of borage oil (BO)	83
3.3.1 Experimental design and data analysis	83
3.4 Preparation of structured lipids containing ω 3 and gamma linolenic acid (GLA) by enzymatic acidolysis	84
3.4.1 Determination of lipase activity	84
3.4.2 Acidolysis of seal blubber oil (SBO) and menhaden oil (MO) with the gamma linolenic acid (GLA) concentrate	85
3.4.3 Separation of acylglycerols and free fatty acids after interesterification	85
3.5 Analysis of fatty acid composition	87
3.5.1 Preparation of fatty acid methyl esters	87
3.5.2 Analysis of fatty acid methyl esters (FAMES) by gas chromatography (GC)	88
3.6 Positional distribution of structured lipids	88
3.7 Chemical and instrumental analysis	89
3.7.1 Assessment of oil stability by accelerated oxidation and photooxidation	90
3.7.2 Analysis of conjugated dienes	92
3.7.3 Determination of 2-thiobarbituric acid reactive substances (TBARS)	92
3.7.4 Nuclear magnetic resonance (NMR) spectrometric analysis	93
3.8 Statistical analysis	93
CHAPTER 4. RESULTS AND DISCUSSION	95
4.1 Preparation of gamma linolenic acid (GLA) concentrates from borage oil by urea complexation	95
4.1.1 Optimization of process conditions to maximize the content of gamma linolenic acid (GLA) in borage oil concentrates	98
4.1.2 Diagnostic checking of fitted model	101
4.1.3 Response surface plotting and optimization based on canonical analysis	102
4.2 Preparation of structured lipids by enzyme-assisted acidolysis of menhaden oil (MO) and seal blubber oil (SBO) with gamma linolenic acid (GLA)	106
4.2.1 Determination of reaction conditions for acidolysis	106
4.2.2 Effect of individual variables on the degree of incorporation of gamma linolenic acid (GLA) into seal blubber oil (SBO) and menhaden oil (MO)	110

4.2.2.1	Effect of enzyme concentration on the incorporation of gamma linolenic acid (GLA)	110
4.2.2.2	Effect of mole ratio on the incorporation of gamma linolenic acid (GLA)	113
4.2.2.3	Effect of reaction time on the incorporation of gamma linolenic acid (GLA)	116
4.2.2.4	Effect of reaction temperature on the incorporation of gamma linolenic acid (GLA)	119
4.2.3	Conditions chosen as optimum for the production of structured lipids	119
4.3	Determination of the positional distribution of fatty acids in triacylglycerols of the enzymatically modified seal blubber oil (MSBO) and menhaden oil (MMO) ..	124
4.4	Comparison of the oxidative stability of modified and unmodified seal blubber oil and menhaden oil	127
4.4.1	Conjugated dienes	127
4.4.2	Thiobarbituric acid reactive substances (TBARS)	128
4.4.3	Nuclear magnetic resonance (NMR)	134
4.4.4	Summary of oxidation results	141
CONCLUSION AND RECOMMENDATIONS		142
REFERENCES		144
PUBLICATIONS		174

LIST OF FIGURES

Figure 2.1	Metabolism of $\omega 3$ and $\omega 6$ fatty acids	6
Figure 2.2	Chemical structures of saturated, monounsaturated and polyunsaturated fatty acids	12
Figure 2.3	Structures of triacylglycerols and phospholipids	14
Figure 2.4	Biosynthesis of eicosanoids from the metabolism of $\omega 3$ and $\omega 6$ fatty acids (adapted from Branden and Carroll, 1986)	23
Figure 2.5	Solubility of fatty acids at different temperatures in acetone (adapted from Stout <i>et al.</i> , 1990)	33
Figure 2.6	Enzymatic hydrolysis and esterification reactions	36
Figure 2.7	Interesterification of structured lipids	44
Figure 2.8	Mechanisms of autooxidation of oleic (a) linoleic acid (b) and formation of possible primary and secondary products (adapted from Frankel, 1985)	53
Figure 2.9	Mechanisms of autooxidation of linolenic and formation of possible primary and secondary products (adapted from Frankel, 1985)	54
Figure 2.10	Mechanism of autooxidation of eicosapentaenoic acid and formation of possible primary and secondary products (adapted from Frankel, 1985)	56
Figure 2.11	Mechanism of autooxidation of docosahexaenoic acid and formation of possible primary and secondary products (adapted from Frankel, 1985)	57
Figure 2.12	Photosensitized oxidation of lipids (RH) adapted from Min (1998)	59
Figure 2.13	Structures of some synthetic antioxidants and α -tocopherols	69
Figure 3.1	Flowsheet for the preparation of free fatty acids from borage oil (BO)	78
Figure 3.2	Flowsheet for the preparation of γ -linolenic acid concentrates by urea complexation	79

Figure 3.3	Flowsheet for the preparation of structured lipids containing GLA and $\omega 3$ fatty acids by microbial lipase	86
Figure 4.1	Response surface for the effect of urea-to-fatty acid ratio and crystallisation temperature on the concentration of γ -linolenic acid (GLA) from borage oil by urea complexation	104
Figure 4.2	Contour plot for the effect of urea-to-fatty acid ratio and crystallisation temperature on the concentration of γ -linolenic acid (GLA) from borage oil by urea complexation	105
Figure 4.3	The effect of varying enzyme concentration on the incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase PS-30 from <i>Pseudomonas species</i> . The reaction mixture contained 300 mg oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 100-700 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C for 24 h. Some standard deviations are within the domain of the symbols	111
Figure 4.4	The effect of varying enzyme concentration on the incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase Lipozyme-IM from <i>Mucor miehei</i> . The reaction mixture contained 300 mg oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 100-700 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C for 24 h. Some standard deviations are within the domain of the symbols	112
Figure 4.5	The effect of varying the mole ratio (GLA:TAG) on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase PS-30 from <i>Pseudomonas species</i> . Mole ratios of GLA to TAG were varied from 1:1 to 5:1. The reaction mixture contained enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C for 24 h. Some standard deviations are within the domain of the symbols	114
Figure 4.6	The effect of varying the mole ratio (GLA:TAG) on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase Lipozyme-IM from <i>Mucor miehei</i> . Mole ratios of GLA to TAG were varied from 1:1 to 5:1. The reaction mixture contained enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were	

	carried out at 37°C for 24 h. Some standard deviations are within the domain of the symbols.....	115
Figure 4.7	The effect of varying the reaction time on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase PS-30 from <i>Pseudomonas species</i> . The reaction mixture contained 300 mg of oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C for 24 h. Some standard deviations are within the domain of the symbols.....	117
Figure 4.8	The effect of varying the reaction time on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase Lipozyme-IM <i>Mucor miehei</i> . The reaction mixture contained 300 mg of oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C for 24 h. Some standard deviations are within the domain of the symbols.....	118
Figure 4.9	The effect of varying the reaction temperature on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase PS-30 from <i>Pseudomonas species</i> . The reaction mixture contained 300 mg of oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 30-60°C for 24 h. Some standard deviations are within the domain of the symbols.....	120
Figure 4.10	The effect of varying the reaction temperature on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase Lipozyme-IM from <i>Mucor miehei</i> . The reaction mixture contained 300 mg of oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 30-60°C for 24 h. Some standard deviations are within the domain of the symbols.....	121
Figure 4.11	Conjugated dienes values of menhaden oil and seal blubber oil-based structured oils and unmodified oils stored under Schaal oven conditions at 60°C.....	129

Figure 4.12 Conjugated diene values of menhaden oil and seal blubber oil-based structured lipids and unmodified oils stored under fluorescent lights at room temperature	130
Figure 4.13 TBARS values of menhaden and seal blubber oil-based structured lipids stored under Schaal oven conditions at 60°C	132
Figure 4.14 TBARS values of menhaden and seal blubber oil-based structured lipids stored under fluorescent lights at room temperature	135
Figure 4.15 Relationship between storage time at 60°C and the ratio of aliphatic to olefinic (Rao) protons of modified and unmodified seal blubber oil (SBO) and menhaden oil (MO).	136
Figure 4.16 Relationship between storage time at 60°C and the ratio of aliphatic to diallylmethylenic (Rad) protons of modified and unmodified seal blubber oil (SBO) and menhaden oil (MO).	137
Figure 4.17 Relationship between storage time at 25°C under fluorescent light and the ratio of aliphatic to olefinic (Rao) protons of modified and unmodified seal blubber oil (SBO) and menhaden oil (MO).	139
Figure 4.18 Relationship between storage time at 25°C under fluorescent light and the ratio of aliphatic to diallylmethylenic (Rad) protons of modified and unmodified seal blubber oil (SBO) and menhaden oil (MO).	140

LIST OF TABLES

Table 2.1	Total content and fatty acid composition of some marine and vegetable oils (w/w %)	10
Table 2.2	Melting points of some fatty acids present in lipids	34
Table 2.3	Potential food applications of structured lipids	41
Table 2.4	Benefits of structured lipids	42
Table 3.1	Independent variables and experimental design levels used for face-centred cube design in the optimization of GLA concentrate production by urea complexation	81
Table 3.2	Coded and uncoded variables of face-centred cube design (FCCD) in optimization of GLA concentration from borage oil by urea	82
Table 4.1	Independent variables and experimental design levels used for face-centred cube design	96
Table 4.2	Face-centred cube design arrangement and response	97
Table 4.3	Fatty acid composition (%) of borage oil and its gamma linolenic acid concentrate	99
Table 4.4	Regression coefficients of the predicted quadratic polynomial model	100
Table 4.5	Predicted and observed values for the response variable (concentration of GLA) in urea complexation experiment of borage oil	103
Table 4.6	Effect of water content on the acidolysis of seal blubber oil or menhaden oil with gamma linolenic acid (GLA) using a non-specific lipase, <i>Pseudomonas species</i> (PS-30)	108
Table 4.7	Effect of solvent volume on the degree of gamma-linolenic acid (GLA) incorporation upon acidolysis of seal blubber oil or menhaden oil using a non-specific lipase <i>Pseudomonas sp.</i> (PS-30)	109

Table 4.8 Fatty acid composition (w/w, %) of refined-bleached deodorised SBO and MO triacylglycerols (TAG) before and after enzymatic interesterification with γ -linolenic acid (GLA)	123
Table 4.9 Positional distribution of structured lipids produced using lipase PS-30	126

LIST OF ABBREVIATIONS

ANOVA	- Analysis of variance
CD	- Conjugated dienes
FAME	- Fatty acid methyl ester
FFA	- Free fatty acids
FID	- Flame ionization detector
GC	- Gas chromatography
GLA	- Gamma linolenic acid
HS	- Headspace
HUFA	- Highly unsaturated fatty acids
LC	- Light cooked
LU	- Light uncooked
MA	- Malonaldehyde
NMR	- Nuclear magnetic resonance
PUFA	- Polyunsaturated fatty acid
Rad	- Ratio of aliphatic to diallylmethylenic protons
Rao	- Ratio of aliphatic to olefinic protons
rpm	- Revolutions per minute
TAG	- Triacylglyceride

TBA	- 2-Thiobarbituric acid
TBARS	- 2-Thiobarbituric acid reactive substances
TCA	- Trichloroacetic acid
TMAO	- Trimethylamine <i>N</i> -oxide
TMP	- 1,1,3,3-Tetramethoxypropane
TMS	- Tetramethylsilane

CHAPTER 1

INTRODUCTION

The importance of marine oils in human nutrition and disease prevention was scientifically recognized three decades ago. Epidemiological studies in the early 1970s postulated that the low incidence of coronary heart disease among Greenland Eskimos might be related to their distinctive dietary habits and use of marine lipids rich in ω 3 polyunsaturated fatty acids (PUFA) (Dyerberg, 1986). Effects associated with ω 3 PUFA, especially eicosapentaenoic acid (EPA; 20:5 ω 3) and docosahexaenoic acid (DHA; 22:6 ω 3), have since been well established. EPA and DHA are considered essential for normal growth and development (Simopoulos, 1990), and may play an important role in the treatment and prevention of cardiovascular disease (Bruckner, 1992), hypertension (Meland *et al.*, 1989; Deferme and Leeds, 1992), inflammation, autoimmune disorders (Boissonneault and Hayek, 1992), diabetes (Bhatena, 1992) and cancer (Carroll, 1990). The beneficial effects of ω 3 PUFA have been ascribed to their ability to lower serum cholesterol and triacylglycerol (TAG) levels and enhance their excretion, to increase membrane fluidity, and by conversion to eicosanoids, to reduce thrombosis (Kinsella, 1986; Simopoulos, 1997).

Another important group of PUFA is the ω 6 family, which also includes γ -linolenic acid (GLA; 18:3 ω 6). GLA is an intermediate in the normal bioconversion of oleic acid (LA; 18:2 ω 6) to arachidonic acid (AA; 20:4 ω 6) which is the eicosanoid precursor. The bioconversion includes a series of alternating desaturation and elongation

steps. The desaturation of LA is catalyzed by the liver $\Delta 6$ desaturase, the rate-limiting step. Delta 6-desaturase may be impaired by aging, high levels of alcohol or cholesterol, and in certain cancer and virally infected cells (Horrobin, 1990). Presently, GLA has been used in the treatment of rheumatoid arthritis, diabetic neuropathy, hypertension, premenstrual syndrome, asthma, atopic dermatitis, multiple sclerosis, migraine and cancer (Horrobin, 1992; Gurr, 1997). Therefore, many investigators have actively participated in research on GLA; this includes preparation of concentrates for dietetic and pharmaceutical purposes.

Until recently, $\omega 3$ PUFA- and GLA-rich oils have been used individually or as physical mixtures in feeding trails (Chapkin *et al.*, 1988; Miller *et al.*, 1990; Fan and Chapkin, 1992). Incorporation of GLA from borage oil into marine oils, by enzyme catalyzed reactions, would provide a unique specialty oil with the potential for enhanced physiological effects and health benefits.

The objectives of this research include three parts. First, the production of GLA concentrates from borage oil using the urea complexation process was studied. Factors such as urea-to-fatty acid ratio, crystallization time and crystallization temperature were studied collectively in order to optimize the conditions to obtain a maximum concentration of GLA. The second goal of the study was to produce an oil containing both $\omega 3$ and $\omega 6$ fatty acids on the same triacylglycerol molecules. Lipases from *Mucor miehei* and *Pseudomonas* species were used to catalyze the interesterification reaction of seal blubber or menhaden oil with the GLA concentrate. The factors considered for interesterification were reactions time, reaction temperature, concentration of enzyme and

ratio of GLA to triacylglycerols (TAG). Finally, the stability of the modified oils produced was tested to determine if these structured lipids were stable under high temperatures and fluorescent light conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 Importance of fats and oils in the marketplace

The annual production of oils and fats was over 88.5 million tonnes (mt) in the 1994 harvest year and it rose to over 114 mt by the year 2000 (Oil World Weekly, 2001). Eighty percent of the oil was used for human food, 6% for animal feed and the other 14% for the production of soaps and detergents (Gunstone, 1996).

Oil and fat come from plant, including algal, and animal sources. Some major plant sources are soyabean, canola/rapeseed, and sunflower which constituted 43.1% of the oil products in 2000 whereas, tree crop sources such as palm and olive oils, made up 23.4%. The animal sources contributed 19.7% from land animals (cows, sheep, pigs) and marine animals (fish) (Oil World Weekly, 2001).

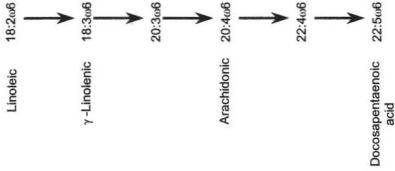
Dietary fats of animal and plant origin are classified as "visible" (adipose tissue, milk fat, seed oils) or "invisible" (derived from animal or vegetable membranes). They are mainly triacylglycerols (TAG) along with minor amounts of phospholipids, glycolipids, sterol esters and vitamins (Gunstone, 1996). Dietary fats are considered essential for several reasons: they constitute the building blocks and energy required for rapid growth (especially of the central nervous system), for the supply of fat-soluble vitamins and essential fatty acids, for the transport and metabolism of cholesterol and for the production of an impermeable barrier of the skin and other tissues (Horrobin, 1990; Bengmark, 1998).

2.2 Essential fatty acids in fats and oils and their metabolism

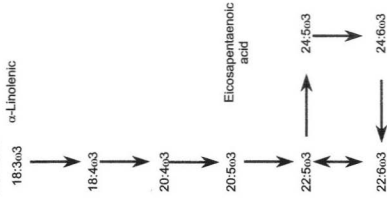
Essential fatty acids (EFA) are defined as nutrients which must be provided in the food because they can not be manufactured within the body (Gurr and Harwood, 1991; Horrobin, 1992). EFAs have two or more double bonds in their carbon chain. There are two groups of EFAs, the $\omega 6$ family based on linoleic acid and the $\omega 3$ family based on alpha-linolenic acid. These differ depending on the position of the double bond from the methyl end of the chain. These fatty acids are biosynthesized via an extension of the saturated fatty acid pathway, in which stearate is converted to oleate (18:1 $\omega 9$) and then linoleate (Figure 2.1) (Gill and Rao, 1997). The parent compounds of the two series are linoleic (LA; 18:2 $\omega 6$) and alpha-linolenic acid (ALA; 18:3 $\omega 3$). LA and ALA are metabolized by a series of desaturation (a new double bond is introduced) and elongation (2 carbon atoms are added) reactions (Figure 2.1). This sequence of desaturation and elongation allows tissues to produce a variety of polyunsaturated fatty acids (PUFA) tailored to fit their needs (Gurr and Harwood, 1991). The elongase and desaturase enzymes required for the *de novo* production of PUFA are present in algae, bacteria, fungi, insects and some invertebrates. However, some plants and animals have lost their ability to produce fatty acids exceeding 18 C, due to the absence of these enzymes. It appears that humans cannot synthesize LA and ALA or other longer PUFA due to the lack of the Δ -4 desaturase. However, they can elongate LA and ALA, therefore, these PUFA are essential nutrients for the human body (Gill and Rao, 1997) whereas, γ -linolenic acid (GLA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are not always essential in the human diet as long as

Figure 2.1 Metabolism of $\omega 3$ and $\omega 6$ Fatty Acids

ω 6 EFAs



ω 3 EFAs



linoleic and linolenic acids are supplied and the enzyme system in the body functions appropriately. These PUFA are known as essential metabolites (Gurr and Harwood, 1991).

LA and ALA compete with one another for the Δ -6 desaturase enzyme. The metabolic pathway that predominates depends on the affinity of the enzyme for the substrate and the amount of substrate available. The affinity of the substrate for the Δ 6 desaturase is in the order $18:3 > 18:2 > 18:1$. Normally, the ω 6 pathway beginning with LA is preferred by the Δ -6 desaturase (Gurr and Harwood, 1991), however, as a whole the enzyme systems seem to have a higher affinity for the ω 3 EFA's (Horrobin, 1992). This means that the ω 3 EFA are effective in displacing the ω 6 EFA. Since LA is abundant in the Western diet, the ω 6 pathway is utilized more frequently. LA, the parent of the ω 6 EFA, is desaturated to GLA, which is subsequently converted to dihomo- γ -linolenic acid (DGLA). The main end product is AA, which is formed by the desaturation of DGLA. AA is the precursor for eicosanoids such as 2-prostaglandin, 2-thromboxanes and 4-leukotrienes. However, there are circumstances when the ω 3 pathway dominates; that is when the amount of linoleic acid in the diet is low or when the diet is swamped by large amounts of ALA. In this case, the ALA undergoes a series of desaturation and elongation to produce the eicosanoid precursor, EPA. EPA is the precursor of 3-prostaglandin, 5-leukotrienes and 3-thromboxanes. These eicosanoids are important for their biological activity (Gurr and Harwood, 1991).

2.3 Significance of marine lipids

Since the times of early civilization, fish has been a major source of protein. Lipids extracted from the body and organs of fish may have been used as food, medicine and fuel. Recently, there has been an increased interest in marine oils due to the recognition of the nutritional benefits of polyunsaturated fatty acids in preventing and possibly curing certain human diseases. The use of marine oils as food ingredients is being expanded. These oils are now being supplemented into infant milk formulas, shortenings in bakery products, margarines, low calorie spreads and salad oils (Bimbo, 1990). The main marine oil producers are Peru, Chile, Denmark, USA, Norway, Iceland, Japan and South Africa (Valenzuela and Uauy, 1999). Canada contributes 1.1×10^4 metric tonnes (mt) per year of marine oil as compared with a total annual global production of 2.0×10^6 mt (Fishery Statistics Commodities, FAO Year Book, 1993). The estimates for the 1998 global fish oil production are really low at 0.832×10^6 mt. This was attributed to the impact of the El Niño affecting catches in South America (Anonymous, 1999).

2.3.1 Sources of marine lipids

Lipids are major components of adipose tissue, and together with proteins and carbohydrates they constitute the principal structural components of living cells. In addition to serving as structural components of cells and tissues, lipids function as a source of energy, provide bouyancy to marine animals, act as chemical messengers and are involved in the control of metabolism (Gurr and Harwood, 1991). Depot fats

comprise the largest part of total lipids of marine organisms (Pigott and Tucker, 1987). In lean fish, such as cod, halibut and shark, storage fat is carried in the liver whereas, in fatty fish, such as mackerel, menhaden, herring, anchovy and capelin, depot fat occurs as extracellular droplets in the muscle tissue (Ke *et. al.*, 1977). The blubber (subcutaneous depot fat layer) of marine mammals, such as seal, whale and walrus is exceptionally rich in lipids.

The muscle tissues of fatty fish show large seasonal variations in lipid, primarily during the reproductive cycle of fish. For example the lipid content of mackerel is known to vary between 5.1 and 22.6% during the course of a year (Leu *et. al.*, 1981). Other factors that affect the content and composition of fish and marine mammals are their diets, phylogen, physiological conditions (e.g. age), geographical location, season of the year, water temperature and availability of food. Table 2 provides the average content and fatty acid composition of selected fish and marine mammal species and compares them with vegetable oils.

2.3.2 Chemistry of marine lipids

Marine oils are generally characterized by a rather large group of saturated and unsaturated fatty acids, which are commonly associated with mixed triacylglycerols. In addition to triacylglycerols, marine oils are accompanied by very low levels of free fatty acids, phospholipids, wax esters, carotenoids, glycerol esters and sterols. These oils typically contain large amounts of long chain polyunsaturated fatty acids, with five, six or more double bonds (Singh and Chandra, 1988).

Table 2.1 Total content and fatty acid composition of some marine and vegetable oils (w/w%)

Component	Atlantic herring ¹	Menhaden ²	Cod liver ³	Blubber (Gray seal) ⁴	Blubber (Harp seal) ²	Canola ¹	Soybean ⁵
Total lipid Content	12-14	ND	55-64	85-95	ND	45-48	18-24
14:0	6.8	8.3	7.3	3.7	4.4	0.1	0.2
16:0	14.8	17.1	12.7	6.0	7.9	3.6	11.6
18:0	1.1	3.3	2.2	0.9	1.2	1.4	3.5
20:0	-	0.2	-	0.1	0.1	0.4	-
22:0	-	0.1	-	-	-	0.2	-
14:1	-	0.4	-	-	1.1	-	0.1
16:1	7.8	11.4	9.8	12.7	18.0	0.3	0.1
18:1 ⁶	16.6	10.1	18.4	16.5	26.0	55.6	21.9
20:1	0.4	1.4	16.1	0.3	12.1	1.8	-
22:1	15.9	0.1	9.7	2.6	2.0	1.6	-
18:2 ⁶	0.8	1.4	1.1	22.6	1.5	21.9	53.9
18:3 ⁶	13.4	1.8	-	0.7	0.6	13.0	8.7
18:4 ω 3	-	2.9	1.1	2.1	1.0	-	-
20:4 ω 6	-	0.8	-	0.3	0.5	-	-
20:5 ω 3	4.5	13.2	7.6	7.0	6.4	-	-
22:5 ω 3	-	2.4	1.4	5.9	4.7	-	-
22:6 ω 3	2.9	10.1	4.7	14.5	7.6	-	-

¹Ackman (1982, 1990), ²Wanasundara and Shahidi (1998), ³Shahidi and Dunajski (1994), ⁴Piggot and Tucker (1987), ⁵Huang and Akoh (1994), ⁶All isomers included

2.3.2.1 Triacylglycerols and fatty acids

Triacylglycerols (TAG) are esters of glycerol with three fatty acids. They usually contain a mixture of two or three different fatty acids rather than three identical ones. The fatty acids attached to the glycerol molecules differ according to two important chemical characteristics: (1) the length of the carbon chain, which affects its solubility; the short and medium chain triacylglycerols are more easily absorbed, and (2) the degree of the chain's saturation with hydrogen (Dudek, 1993).

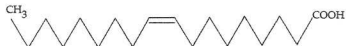
Fatty acids are made up of chains of carbon atoms of varying lengths, generally from 2 to 22, with hydrogen atoms attached along the chain, with a methyl group (CH_3) on one end and a carboxyl group (COOH) on the other end. Based on the length of the carbon chain, fatty acids may be classified as short-chain fatty acids, which contain 6 or less carbon atoms, medium chain fatty acids, which contain 8 to 10 carbon atoms, or long chain fatty acids, which contain 12 or more carbon atoms. Most foods contain predominantly long chain fatty acids.

The majority of fatty acids have an even number of carbon atoms because they are synthesized from two carbon fatty acid units (acetic acid) in the process of lipogenesis (Williams and Anderson, 1993). Fatty acids are usually unbranched and are saturated or unsaturated, however, in marine lipids small quantities of branched and odd carbon numbered fatty acids may be present (Patterson, 1989). When the carbon atoms in the hydrogen chain of a fatty acid hold their full complement of hydrogen they are defined as saturated fatty acids (Figure 2.2). Saturated fatty acids are most stable either in the free fatty acid form or in the TAG form and pack together more easily in the solid state

Figure 2.2 Chemical structures of saturated, monounsaturated and polyunsaturated fatty acids



Stearic acid (C18:0)



Oleic acid (C18:1)



Linoleic acid (C18:2)



Linolenic acid (C18:3)



Eicosapentaenoic acid; EPA (C20:5)



Docosapentaenoic acid; DPA (C22:5)



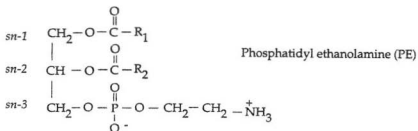
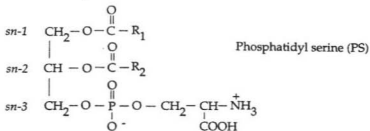
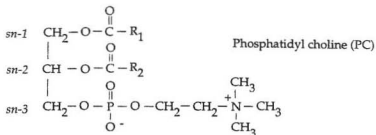
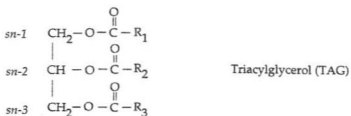
Docosahexaenoic acid; DHA (C22:6)

due to their contour arrangement. This behaviour generally favours the lipid to be a solid at room temperature and have higher melting points. As the chain length increases the melting point of the material increases steadily (Dudek, 1993).

Fatty acids are classified as unsaturated when one or more double bonds exist between carbon atoms; the carbon atoms are not saturated because all four potential binding sites are not used. Monounsaturated fatty acids contain only one double bond between carbon atoms. The most prevalent monounsaturated fatty acid is oleic acid (18:1 ω 9, Figure 2.2). Polyunsaturated fatty acids (PUFA) have two or more double bonds along the carbon chain of an unsaturated fatty acid (Dudek, 1993). The double bonds in natural unsaturated fatty acids exist in the *cis* (Z) form; this means that at the double bond the molecule turns back on itself. *Cis* isomers cannot be packed together closely like *trans* or saturated fatty acids (Anderson *et. al*, 1982). PUFA tend to be soft or liquid at room temperature and have low melting points. The double bonds and the methylene (-CH₂.) group immediately adjoining them (α -methylene group) are very reactive. When the methylene group lies between two double bonds (i.e. diallyl methylene group such as that of linoleic acid, 18:2 ω 6) the reactivity of the α -hydrogen is further enhanced (Patterson, 1989).

In a TAG molecule, the primary ester groups previously known as α and the secondary ester group, known as β , are now identified as *sn*-1 or *sn*-3 and *sn*-2, respectively (Figure 2.3). The International Union of Pure and Applied Chemistry (IUPAC) Commission on Biochemical Nomenclature developed this nomenclature.

Figure 2.3 Structures of triacylglycerols and phospholipids



There is current interest in the positioning of the fatty acids in the triacylglycerol molecule because the position, as well as the chain length and degree of unsaturation of the fatty acids, affect the melting point and, thereby, the digestibility and absorption of the fat. When a triacylglycerol molecule is formed on a glycerol base, a saturated fatty acid is usually present at the first carbon atom position (*sn*-1), and an unsaturated fatty acid at *sn*-2. Either form may be present at position *sn*-3 (Williams and Anderson, 1993).

The fatty acid composition of marine lipids varies considerably, especially when compared to vegetable oils (Table 2.1). Marine oils generally contain a wide range of fatty acids ranging in chain length from C_{14} to C_{26} with zero to six double bonds. Their major fatty acids are generally saturated (14:0 and 16:0), monoenic (16:1, 18:1, 20:1, and 22:1), and ω 3 polyenoic acids (18:4, 20:5 and 22:6) (Gunstone, 1996; 1997). Also, some species of fish contain small amounts of C_{17} and C_{19} saturated fatty acids (Piggot and Tucker, 1987). These unique features differentiate marine species from land animals. The two major ω 3 fatty acids in marine species are eicosapentaenoic acid (EPA, 20:5 ω 3) and docosahexaenoic acid (DHA, 22:6 ω 3), which are both from phytoplankton and macro algae. They are eventually transferred through the food web and are incorporated into lipids of aquatic species such as fish and marine mammals. The higher content of ω 3 fatty acids in marine lipids is suggested to be a consequence of cold temperature adaptation, because at lower habitat temperatures ω 3-PUFA remain liquid and oppose any tendency to crystallize (Ackman, 1988).

The Bligh and Dyer (1959) extraction procedure may be used to isolate total lipids. Separation of the TAG fraction from total lipids can be achieved by thin layer

chromatography (TLC) or column chromatography (CC) by using non-polar solvents. The amount of TAG in the total lipid fraction may be decided by densitometry on TLC plates, gas liquid chromatography (GLC), TLC-FID IATROSCAN®, spectrophotometry and radioisotopic derivatisation techniques (Holmer, 1989).

2.3.2.2 Phospholipids

Phospholipids are a group of compound lipids that are quite similar to triacylglycerols in that they contain a glycerol molecule and two fatty acids. However, in place of the third fatty acid, phospholipids have a phosphate group and a nitrogen-containing base (Figure 2.3). As in triacylglycerols, the fatty acids are in an ester linkage to the glycerol molecule. The phosphate is in an ester linkage with both the glycerol and nitrogenous base. Phospholipids, structural compounds found in the cell membrane, are essential components of certain enzyme systems, are involved in the transport of lipids in the plasma and serve as a source of energy (Anderson *et. al*, 1982). The major types of phospholipids in marine lipids are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl inositol (PI) with minor amounts of sphingomyelin, lysophosphatidylcholine (LPC) and cardiolipin (Thomas and Patton, 1972). Weihrauch and Son (1983) have reported that in menhaden oil, phospholipids accounted for 5.5% of the total lipid content and consisted of PC, PE + PS, sphingomyelin and LPC at 62.9, 17.0, 3.1 and 8.2%, respectively. Lovren (1964) has reported that phospholipids present in marine species are generally unsaturated and esterified mainly with EPA and DHA. Menzel and Olcott (1964) have studied PC and PE

constituents of menhaden oil and found that their PUFA of the menhaden oil are predominantly located on the *sn*-2 position of the TAG molecule.

Phospholipids are usually extracted with total lipids during the Bligh and Dyer (1959) extraction procedure. Phospholipids can be recovered by column chromatography with methanol after eluting neutral lipids. TLC-FID IATROSCAN® (Parrish, 1987) and two dimensional TLC (Christie, 1982) can be used to separate individual phospholipids.

Other minor components of marine oils are fat-soluble vitamins, wax esters and cholesterol. The main vitamins in marine oil are vitamins A, D and E. Their concentrations vary not only among different species, but also among fish within the same species (Nettleton, 1985).

2.3.3 Industrial uses of marine lipids

Marine oils are highly unsaturated triacylglycerols, which oxidize and polymerize readily. This enables marine oils to produce elastic durable polymers or diverse long chain fatty acids that add to the plasticity, lubricity and detergency functionalities (Fineberg and Johnson, 1967). Industrial uses today are mainly in paints, varnishes, chalks and sealants, printing inks, lubricants, greases, buffing agents, insecticides, animal feeds, in the production of linoleum, and as a sealant for concrete, wood, metal and fibre (Gunstone, 1999). In addition, marine oils are used in cutting oils, glazings, core oils, polyethylene foams, and ceramics (Bimbo and Crowther, 1992).

2.3.4 Food uses of marine lipids

Nearly one million metric tonnes of marine oil are produced annually that may be used as food ingredients (Bimbo, 1989; 1990). These oils may be supplemented into infant milk formulas, shortenings in bakery products such as cakes, pastries, bread, cookies and biscuits, margarine, low calorie spreads, cooking oils, mayonnaise and salad oils (Bimbo, 1990; 1997). Other foods that are considered for fortification with marine oils are chocolate bars, meats and salad dressings. Marine oils are also used in the canning of fish and production of food emulsifiers.

Barlow and Young (1988) published a report on the novel uses of fish oils. They reported that the optimum incorporation of fish oil into meat products (e.g. salami, frankfurters) was 9 to 16.5% of the total lipids. Schnepf *et al.* (1991) showed that the overall acceptability of French salad dressing prepared with soybean/menhaden oil blends (maximum 30% menhaden oil) was similar to those prepared with soybean oil alone. Li and Regenstien (1990) found that mayonnaise prepared with 100% menhaden oil can be stored under a blanket of nitrogen at 30°C for 8 weeks without changes in its physical appearance. Jafar *et al.* (1994) reported that mayonnaise produced from menhaden oil had a shelf-life of one day without any added antioxidants. Nevertheless, the shelf-life increased by 49 days at room temperature if citric acid or sodium citrate and propyl gallate were added to the oil phase and ethylenediaminetetraacetic acid (EDTA) and ascorbic acid were included in the aqueous phase. A study by Kolanowski *et al.* (1999) examined the enrichment of food products with fish oils. They found that the highest inclusion level was obtained with the instant-powder-milk-based formulae concentrates,

fats and products of high sweetness and elevated flavour intensity foods that mask unpleasant fishy odour and taste. The addition of 0.1% fish oil to liquid products of low flavour and sweetness intensity like milk and vegetable juices were highly unpalatable. However, products with excellent acceptability have now been produced (Hoffman-La-Roche, IFT, 2001). Concentrates of EPA and DHA can be produced from marine oils and added to food products as a PUFA supplement. Some food products that may be supplemented with PUFA concentrates are dairy products, mayonnaise, shortenings, margarine, table spreads, vegetable oils, pastry fats, biscuits and bread doughs (Bimbo, 1989).

2.3.5 Marine lipids in nutrition and disease prevention

The discovery of the essentiality of PUFA was first introduced by Burr and Burr (1929). At that time essentiality meant promotion of growth and prevention of dermatitis when a fat-free diet was fed to rats. It was not until the late 1960's that the nutritional importance of EFA was recognised, when clinical signs of essential fatty acid deficiency became apparent in infants fed skim milk-based formula (Uaiy-Dagach and Valenzuela, 1996; Holman, 1998). The structural role of the long chain PUFA derived from EFA are increasingly recognised (Sprecher, 1981; Lee *et al.*, 1986). The long chain PUFA, namely arachidonic acid (AA; 20:4 ω 6) and eicosapentaenoic acid (EPA; 20:5 ω 3) are important membrane components and precursors to potent bioactive oxygenated products, i.e. eicosanoids (Takahata *et al.*, 1998).

Epidemiological evidence on the benefits of marine oils ω 3 PUFA consumption on health has accumulated over the past few decades (Simopoulos, 1991; Galli and Simopoulos, 1994; Katan; 1995). For example, during the second world war, Norway experienced a marked drop in cardiovascular mortality. During this time fish became the main source of fat and protein as a result of food shortage. Another example, which is of more relevance to the role of marine oil in health, is the low incidence of coronary heart disease in Greenland Eskimos. Eskimos living in Greenland consume a diet rich in seafood (in which seal meat and blubber made an important contribution) compared to their counterparts living in Denmark who consumed limited amounts of seafood (Bang *et al.*, 1976). Subsequent analysis revealed that Greenland Eskimos had significantly lower levels of serum cholesterol, triacylglycerols, very low density lipoprotein (VLDL), low density lipoproteins (LDL) and higher concentration of high density lipoproteins (HDL) compared to the Danish population who consumed a typical European diet.

Other studies in Japan, New Guinea and the Netherlands support the claim of beneficial effects of marine foods on health. The low death rate from coronary heart disease (CHD) in the Japanese population was attributed to their higher consumption of fish, particularly in Okinawa where the lowest death rate was noted and the consumption of fish exceeds 200 grams per day (Hirai *et al.* 1984; Ahmad, 1998). On the Trobriand Islands in Papua, New Guinea, where the lifestyle is uninfluenced by Western diet (tubers, fruit, coconut and fish are the main staples), Lindeberg (1994) found no signs of stroke, ischemic heart disease or cancer. However, on other Micronesian Islands these

diseases were observed to increase in parallel to the adoption of a Western lifestyle (Lindeberg, 1994; Bengmark, 1998).

Several mechanisms have been proposed to explain the beneficial effects of dietary marine oils. Illingworth and Ullmann (1990) proposed that the decrease in plasma lipids by reduced hepatic synthesis of fatty acids and VLDL could result from the consumption of marine oils. It has been suggested that ω 3 fatty acids have a direct effect on the heart muscle itself, decrease blood viscosity, reduce blood platelet adhesion, increase blood flow, decrease blood triacylglycerols, decrease arrhythmia, decrease the size of infarct and reduce several chemical and cellular processes that comprise heart function (Nestel, 1990; Fatima and Qadri, 1993; Hasulo, 1998). It has also been suggested that marine oils retard atherogenesis through their effect on platelet function, platelet-endothelial interactions and on the inflammatory response. It is generally agreed that the principal mechanism of action of ω 3 PUFA is in their down regulation of AA metabolism and modulation of eicosanoid synthesis (Simopoulos *et al.*, 1997).

Omega-3 fatty acids with 20 carbon atoms (i.e. EPA and AA) are precursors of eicosanoids, such as prostaglandins, leukotrienes, thromboxanes, prostacyclins, lipoxins and hydroxy-derivatives of fatty acids. These molecules are important for several cellular functions like platelet aggregability, endothelial cell mobility, growth of smooth muscle cells, and synthesis of white blood cells, endothelial cells and leukotrienes which are important for chemotactic factors (Drevon, 1993). It is important to recognize that ω 3 fatty acids are precursors of eicosanoids that are in general less potent than eicosanoids derived from the ω 6 family and have a different structure, function and biological activity

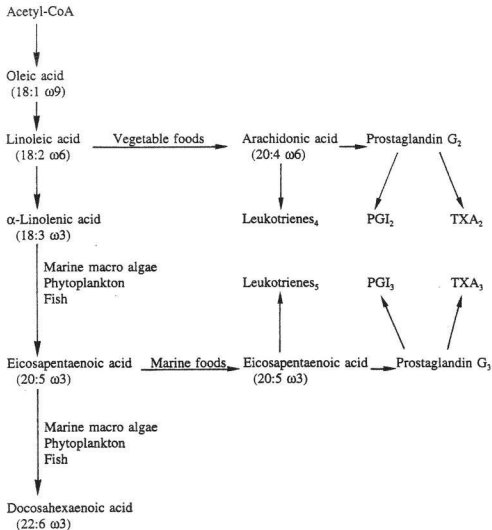
(Fisher, 1989; Drevon, 1993). Figure 2.4 provides a summary of eicosanoids produced from dietary fats. Some positive and negative physiological effects of ω 3 PUFA have been observed in the areas of the heart and circulatory, immune response and cancer. The first category involves prevention or treatment of hypertriacylglyceridemia (Sanders, 1991; Innis, *et al.*, 1996; Tinker *et al.*, 1998), atherosclerosis (Dyerberg, 1986; Nordoy, 1999); thrombosis (Kinsella, 1986; Weber and Leaf, 1991; Weksler, 1994), cardiac rhythm (Leifert *et al.*, 1999) and high blood pressure (Weber and Leaf, 1991; Knapp, 1993; Engler *et al.*, 2000). The second area relates to the treatment of asthma (Thein *et al.*, 1993; Peat, 1996), arthritis (Singh and Chandra, 1988), inflammation (Tomobe *et al.*, 2000) migraine headache, psoriasis and nephritis (Robinson *et al.*, 1994). The third category involves cancer of the breast (Gonzalez, 1995; Bougnoux *et al.*, 1999; Chajes *et al.*, 1999), prostate and colon (Singh and Chandra, 1988).

Prevention and treatment of disorders and diseases have been addressed by changes in the eicosanoids in the circulatory system. Whenever a disorder occurs in a particular tissue due to excessive production of particular eicosanoids, the mechanistic action of the dietary ω 3 PUFA is to reduce conversion of LA to AA from the agonist phospholipid tissue pools and to competitively inhibit cyclooxygenase or lipoxygenase and thereby reduce the synthesis of bioactive eicosanoids in a particular tissue. In addition, EPA may be converted to eicosanoid analogs with low activity which displace the normal eicosanoids by binding to the active sites thereby reducing platelet aggregation, blood viscosity and blood triacylglyceride levels (Fatima and Qadri, 1993). In this way ω 3 PUFA's regulate the balance of eicosanoids which in turn control the specific

Figure 2.4 Biosynthesis of eicosanoids from the metabolism of $\omega 3$ and $\omega 6$ fatty acids
(adapted from Branden and Carroll, 1986)

Natural food chain

Mammalian metabolism



pathological conditions of tissue. Since eicosanoids are derived from PUFA provided from the diet, it is clear that quantitative and qualitative changes in the supply of dietary PUFA have a profound effect on the production of eicosanoids. Recent studies have indicated that during pregnancy and lactation, mothers become deficient in $\omega 3$ fatty acids (Al *et al.*, 1990; Hamilton *et al.*, 2000). This may suggest that mothers were mobilizing membrane DHA to meet the high fetal requirement for this nutrient and in many cases the mothers' supply was working to its limit or was even inadequate. Ghebremeskel *et al.*, (2000) compared the concentration of DHA in plasma and red blood cells of women who were pregnant or lactating and those who were non-pregnant. Pregnant or lactating women showed a decline in their DHA concentration through pregnancy and lactation. Carlson *et al.* (1986) showed that premature babies have lower levels of DHA in their tissues than full-term babies, but when fed formula supplemented with marine oils the premature babies accumulated as much DHA as full-term breast fed babies. Studies have shown that DHA is necessary for the growth and development of infants' neural and visual tissues especially during the first 6 months of their life. A study by Cunnane *et al.* (2000) showed that the brain of infants fed formula devoid of DHA accumulates half the DHA of the brain of the breast-fed infants while the rest of the body actually loses DHA over the first 6 months of life. These studies lend evidence that enrichment of foods with $\omega 3$ fatty acids is beneficial and necessary.

Adequate intakes for PUFA have been suggested by the International Society for the Study of Fatty Acids and Lipids (ISSFAL) based on age and energy needs. Based on a 2000 kCals diet, an adult should take in a maximum of 0.65 grams per day of $\omega 3$ fatty

acids. Additional amounts of DHA fatty acids are recommended for pregnant and lactating women with increasing amounts from first to second trimester. The International Society for the Study of Fatty Acids and Lipids recommended a minimum intake of 300 mg/day of DHA for pregnant and lactating women (Simopoulos *et al.*, 1999). British Nutrition Foundation Task Force (1992) on unsaturated fatty acids has recommended that 5% of the total daily energy supply come from $\omega 3$ fatty acids.

2.4 Significance of γ -linolenic acid (GLA)

As explained earlier, GLA (18:3 $\omega 6$) belongs to the $\omega 6$ family of EFA. In animals and humans, it is synthesized from the dietary fatty acid LA (18:2 $\omega 6$), with the introduction of a third double bond in the molecule by the $\Delta 6$ desaturase enzyme. GLA is then further metabolized by chain elongation to DGLA and finally desaturated by $\Delta 5$ desaturase to AA. DGLA and AA are very important to the normal physiological processes for the fetus and infants since they are required for cell structures and are also the precursors of eicosanoids (Hassam, 1985; Horrobin, 1990; Huang *et al.*, 1997, 1999). Studies by Hassam *et al.* (1975) have shown that the desaturation steps tend to be very slow but the elongation steps are rapid. Their results also suggest that the conversion of LA to GLA by $\Delta 6$ desaturase is the rate-limiting step in the conversion of LA to AA *in vivo*.

Normal synthesis of GLA from LA via the $\Delta 6$ desaturase can be decreased or blocked in humans by various factors such as diet, stress, aging, hormones, diabetes, high alcohol intake and viral infection (Brenner, 1974, 1982; Horrobin, 1992; Haro and Rao,

1998). Supplementation of the diet with GLA has been shown to reverse and correct health disorders related to the deficiency of EFA and prostaglandins (Table 2.1).

2.4.1 Sources of GLA

GLA is found in the seed oil of many plants (Haro and Rio, 1998). Some of the plant families include: Onagraceae (Wolfe *et al.*, 1983; Hudson, 1984; Cisoski *et al.*, 1993), Boraginaceae (Hudson, 1984; Whipkey *et al.*, 1988; Sewon and Tyystjarvi, 1993; Tsevegsuren and Aitzetmuller, 1996; Rio *et al.*, 1998; Huang *et al.*, 1997, 1999), Scrophulariaceae (Wolfe *et al.*, 1983), Aceraceae (Bohannon and Kleiman, 1976), Moraceae (Roberts and Stevens, 1963), Liliaceae (Morice, 19667), Ranunculaceae (Tsevegsuren and Aitzetmuller, 1993) and Saxifragaceae (Ribes) (Wolfe, 1983; Trailter *et al.*, 1984). However, only seeds of borage (*Borago officinalis* L.), evening primrose (*Oenothera biennis* L.) and blackcurrant (*Ribes nigrum* L.) are commercially available. Lately, microorganisms such as *Mortierella sp.* (Hanson and Dostalek, 1988; Shimuzu *et al.*, 1989) and *Mucor ambigua* (Fukuda and Morikawa, 1987) have also been explored for production of GLA.

2.4.2 Uses of GLA

Oils from the seeds of evening primrose (*Oenothera spp.*) and borage (*Borago officinalis*) are currently available in over 30 countries as a nutritional supplement or as a constituent of specialty foods. These seeds are cultivated in at least 15 countries, including Australia, Canada, France, Holland, Hungary, New Zealand, United Kingdom,

United States of America and Yugoslavia. The U.S. and Canada produce 400 tonnes of the seeds annually (Carter, 1988).

As with other seed oils, evening primrose and borage are composed almost entirely of triacylglycerols. The maximum label-recommended daily dosage of GLA is 300-360 mg (Carter, 1988).

Several pharmaceutical and nutraceutical companies are developing a variety of GLA-containing nutrition-oriented supplements and specialty foods for infants, the elderly and people with health problems. Companies involved include Kabi Vitrum AB (Stockholm, Sweden) and Nestle S.A. (Vevry, Switzerland) which are introducing products for infant nutrition. Roussel Uclaf S.A. (Paris, France) is marketing nutritional products targeted for geriatric patients.

Furthermore, GLA is used for industrial purposes: Due to its highly unsaturated nature, GLA oxidizes and polymerizes readily and is used in paints, varnishes, inks, linoleum production, and as a sealant for concrete (Gunstone, 1999). GLA may also be incorporated into cosmetics and personal care products such as skin cleaners, bath preparations, lotions, creams, toothpaste, shaving preparations, shampoos and soaps (Gunstone, 1996; Lichtenstein, 1999; Shimada et al, 1999).

2.4.3 GLA in health promotion and disease prevention

The wild evening primrose (*Oenothera biennis*) and borage (*Borago officinalis*), (also known as tree primrose, scurvish, scabbish, king's cure and nightwillow herb) have long been used by herbalists worldwide. Various parts of the plants have been reputed in

folk medicine to be beneficial in treating colds, mental depression, and complaints of the liver, spleen and digestive tract (Kies, 1989).

Current research interest in these oils are centered on the GLA component. GLA is an important intermediate in the normal bioconversion of linoleic acid (LA; 18:2 ω 6) to arachidonic acid (AA; 20:4 ω 6), as explained earlier. In humans only a few grams of LA is converted to GLA each day (Kies, 1989). In North America, 10-20% of the people cannot maintain normal blood levels of GLA (Carter, 1988). Evidence indicates that Δ 6 desaturase may be impaired by aging, diabetes, high levels of cholesterol and alcohol consumption, and in certain cancers and virally infected cells (Horrobin, 1992). Other possible inhibitors are a high supply of linoleic acid, starvation, carbohydrates, α -linoleic acid and stress related hormones such as epinephrine, corticoids and glucagon (Speilman *et al.*, 1988).

Some important functions of GLA play a role in human nutrition are: 1) modulation of membrane structure; 2) formation of regulating molecules such as prostaglandins and leukotrienes; 3) control of water impermeability of the skin and permeability of other membranes; and 4) regulation of cholesterol transport and synthesis (Horrobin, 1992; Gurr, 1997). It is due to these processes that GLA is currently being used in the treatment and prevention of diabetes (Barcelli *et al.*, 1990), hypertension (Deferne and Leeds, 1992; Kremer, 1996), thromboembolic disease (Kernoff *et al.*, 1977), atopic eczema (Wright and Burton, 1982; Manku *et al.*, 1984; Scott, 1989; Lindskov and Holmer, 1992; Fiocchi *et al.*, 1994), rheumatoid arthritis (Kunkel *et al.*, 1981; Jantti *et al.*, 1989; Zurier, 1996), multiple sclerosis (Barber, 1988), premenstrual syndrome, asthma,

migraines and cancer (Horrobin, 1992;1994; Gurr, 1997). In some clinical trials, dietary supplementation with GLA increased epidermal levels of eicosanoid precursors (Miller and Ziboh, 1988) and produced significant decreases in itching and antihistamine use (Fiocchi *et al.*, 1994; Bordoni *et al.*, 1988). Furthermore, a GLA-containing oil was effective for curing rheumatoid arthritis (Zurier *et al.*, 1996; Jantti *et al.*, 1989) and multiple sclerosis (Barber, 1988). GLA supplementation has also been proven to favourably lower blood pressure (Engler and Engler, 1998), exert an anti-inflammatory effect (Johnson *et al.*, 1996) and enhance platelet aggregation (Barre *et al.*, 1993).

2.5 Production of an $\omega 6$ fatty acid concentrate

The $\omega 6$ fatty acid that has received the most recognition in the past decade is GLA. As an alternative to the substitution of generally used dietary lipids by rich sources of GLA, supplementation has been recommended. Oils from these seeds are currently available in many countries, including Canada, as nutritional supplements, and constituents of specialty foods and ingredient in cosmetics (Carter, 1988; Crozier and Secretin, 1996). Thus, a highly purified GLA preparation is desired for use in medicine and an ingredient in cosmetics. For some pharmaceutical purposes, GLA concentrations of up to 90% or more are required.

Several investigations have been carried out to examine the ability of GLA to reduce blood pressure (Engler and Engler, 1992; 1998). It has been concluded that GLA lowers blood pressure, but that concentrates of GLA devoid of saturated and monounsaturated fatty acids are much better than seed oils themselves because they keep

the quantity of total lipids down. Therefore, concentrated forms of GLA are preferred for pharmaceutical purposes as well as enrichment of foods.

2.5.1 Methods of concentration of GLA

Methods for the concentration of GLA include urea adduct formation, solvent winterization and enzyme selective hydrolysis or esterification. All of these techniques have their own advantages and drawbacks. The following provides a background to each of these methods.

2.5.1.1 Urea adduct formation

The simplest and most efficient technique for obtaining a GLA concentrate in the free fatty acid form is urea complexation. Urea complexation is one of the most promising procedures as it requires no organic solvent, except ethanol, and allows handling of large quantities of material. In addition, the mild condition of urea complexation should not affect the structural integrity of this PUFA (Ratnayake *et al.*, 1988).

Urea usually crystallizes in a tetragonal structure; however, an x-ray study by Traitter *et al.* (1988) has shown urea inclusion compounds consist of hexagonal crystal forming channels which are capable of including long-chain molecules. The formation of inclusion compounds with fatty acids depends on their degree of unsaturation; the more unsaturated, the less will be the likelihood of their inclusion into the urea crystals (Gunstone, 1996).

Initially, the TAG of the oil are split into their constituent fatty acids by alkaline hydrolysis using alcoholic KOH and the unsaponifiable compounds such as sterols, vitamins and other components are removed. These fatty acids are then mixed with an ethanolic solution of urea and allowed to cool to a specified temperature depending on the degree of concentration desired. The unsaturated fatty acids, monoenes and to a lesser extent dienes, easily complex with urea and crystallize out on cooling, which may subsequently be removed by filtration (Wanasundara and Shahidi 1999). The liquid or non-urea complexing fraction (NUCF) is enriched in GLA. This procedure may be performed on free fatty acids or methyl and ethyl esters, however, there are advantages to each of these options. More alcohol is required for this procedure when using esters because they are less soluble in alcohol than free fatty acids. However, if esters are used then the step of re-esterifying the concentrates is eliminated.

Many papers have been published describing the use of urea complexation in fatty acid chemistry. Iverson and Weik (1967) and Strocchi and Bonaga (1975) have correlated fatty acid structures with their preferential order of urea complexation formation. Other publications have described this application for concentrating specific fatty acids such as isoprenoid acids (Ackman *et al.*, 1977), *cis-trans* isomers (Piconneaux *et al.*, 1985), ω 3 fatty acids (Ratnayake *et al.*, 1988; Wanasundara and Shahidi, 1999) and preparation of a GLA concentrate from blackcurrant (Trautler *et al.*, 1988).

Urea complexation is often selected because complexation depends upon the configuration of the fatty acid moieties due to the presence of double bonds, rather than pure physical properties such as melting point (Wanasundara, 1996). This method also

has the advantage of extremely stable complexed crystals. Thus, filtration does not have to be carried out at very low temperatures which solvent crystallization would require (Anon, 1986).

2.5.1.2 Solvent Winterization

The concentration of fatty acids from natural mixtures by crystallization using appropriate organic solvents is known as solvent winterization. Solvent winterization was developed to separate TAG, diacylglycerols, fatty acids, esters and other lipids that are soluble in organic solvents above 0°C, but only slightly soluble at temperature down to -80°C (Brown and Kolb, 1955). Saturated fatty acids are solid at room temperature and can crystallised out at room or cold-room temperatures. However, PUFA have lower melting points and higher solubilities; therefore, PUFA must be cooled to a temperature between 0 and -80°C for crystallization (Gunstone, 1984). The solubility of an oil is dependent upon the degree of unsaturation and the mean molecular weight. As the degree of unsaturation increases and the mean molecular weight decreases, the solubility of the oil in organic solvents increases (Chawla and deMan, 1990). Singleton (1960) and Stout *et al.* (1990) have reported the solubilities of many fatty acids (Figure 2.5) and esters and have developed rules as to how these crystallize out of solution. When the acids are saturated, long chains fatty acids are less soluble than short chains; saturated are less soluble than monoenoic or dienoic acids of equal length; *trans* isomers are less soluble than *cis* isomers and normal acids are less soluble than branched acids. The melting point of fatty acids change greatly with the type and degree of unsaturation (Table 2.2), thus the

Figure 2.5 Solubility of fatty acids at different temperatures in acetone
(adapted from Stout *et al.*, 1990)

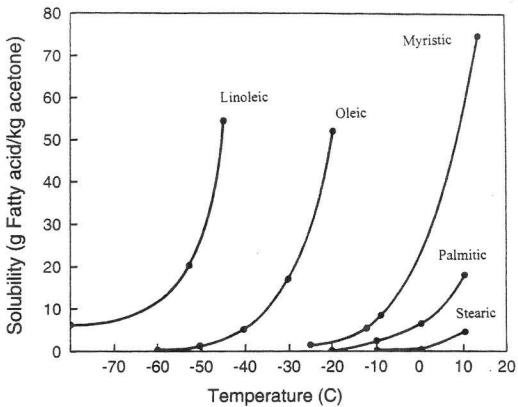


Table 2.2 Melting points of some fatty acids present in lipids^a

Systematic names	Trivial name	Numerical Symbol	Melting point (°C)
Saturates			
Tetradecanoic acid	Myristic acid	14:0	54.4
Hexadecanoic acid	Palmitic acid	16:0	62.9
Heptadecanoic acid	Margaric acid	17:0	61.3
Octadecanoic acid	Stearic acid	18:0	69.6
Eicosanoic acid	Arachidic acid	20:0	74 to 76
Docosanoic acid	Behenic acid	22:0	80
Monounsaturates			
<i>cis</i> -9-Tetradecenoic acid	Myristoleic acid	14:1 ω 5	-4.5 to -4
<i>cis</i> -9-Hexadecenoic acid	Palmitoleic acid	16:1 ω 7	-0.5
<i>cis</i> -9-Octadecenoic acid	Oleic acid	18:1 ω 9	13.4
<i>trans</i> -9-Octadecenoic acid	Elaidic acid	18:1	43 to 45
<i>cis</i> -13-Docosenoic acid	Eruric acid	22:1 ω 9	33 to 35
Polyunsaturates			
<i>cis</i> -9,12-Octadecadienoic acid	Linoleic acid	18:2 ω 6	-6.5
<i>cis</i> -6,9,12-Octadecatrienoic acid	γ -Linolenic acid	18:3 ω 6	-11
<i>cis</i> -9,12,15-Octadecatrienoic acid	α -Linolenic acid	18:3 ω 3	-12.8
<i>cis</i> -5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4 ω 6	-49.5
<i>cis</i> -5,8,11,14,17-Eicosapentaenoic acid	-	20:5 ω 3	-54 to -53
<i>cis</i> -4,7,10,13,16,19-Docosahexaenoic acid	-	22:6 ω 3	-44.5 to -44.1

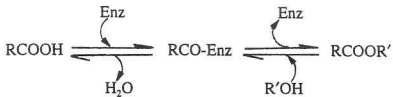
^aAdapted from Merck Index (1983)

separation of fatty acids is made possible (Haraldsson, 1984). Different organic solvents and temperature have been reported to affect the concentration of PUFA (Brown and Kolb, 1955; Yokochi *et al.*, 1990) such as GLA. Fungal oil extracted from *Mortierella* genus was used for the concentration of GLA by solvent crystallization (Kreulen, 1976; Yokochi *et al.*, 1990). The concentration of GLA in different solvents was in the order of acetone (-20°C) > n-hexane (-20°C) > acetone (4°C) > petroleum ether (-20°C) at a solvent to oil ratio of 5:1 (v/v). Timms (1997) examined the effect of solvent on separation of lipids, and found it to have little effect on the separation of TAG. However, the selectivity of separation between lipid classes, particularly TAG from diacylglycerols, free fatty acids and other lipids affected more the polar components than the TAG. Timms (1997) also found that the polar acetone was more suitable for the separation of lipid classes than the nonpolar hexane.

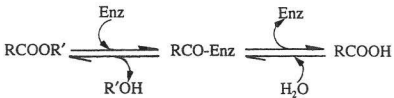
Solvent winterization is a mild procedure suitable for polyenoic acids that are easily oxidized at elevated temperatures (Gunstone, 1984). This method is essential for preparing pure fatty acids because it requires the least number of steps and simplest equipment (Schlenk, 1961; Markley, 1964). In brief, the process consists of cooling the oil or fatty acids in a solvent, holding for a period of time and removing the crystals by filtration. However, low process yields might have a negative impact on the economy of the process.

Figure 2.6 Enzymatic hydrolysis and esterification reactions

Enzymatic ester synthesis
 $[R'OH] \gg [H_2O]$



Enzymatic ester hydrolysis
 $[H_2O] \gg [R'OH]$



2.5.1.3 Selective enzymatic hydrolysis or esterification

Lipase can catalyse esterification, hydrolysis or exchange of fatty acids in esters (Marangoni and Rousseau, 1995). The direction and efficiency of the reaction can be influenced by the choice of experimental conditions (Yadwad *et al.*, 1991). The generally accepted mechanism of lipase catalysed hydrolysis and/or esterification is shown in Figure 2.6. The reaction is reversible and under low water conditions the enzyme functions "in reverse", that is the synthesis of an ester bond rather than its hydrolysis (Miller *et al.*, 1988).

Huang *et al.* (1995) found that during *in vitro* hydrolysis of natural and synthetic GLA-containing TAG, the stereospecific position of GLA in TAG molecules and the constituent of its neighbouring fatty acids modulate GLA availability from GLA-containing TAG or GLA-rich oils. The release of GLA from the TAG molecule is hindered by the co-presence of another GLA molecule or a long chain ($C_{20} + C_{22}$) monounsaturated fatty acid, which limits the bioavailability of GLA. Several lipases have been shown to discriminate against GLA or GLA moieties in lipase catalysed hydrolysis (Hills *et al.*, 1989, 1990; Mukherjee and Kiewitt, 1991; Rahmattullah *et al.*, 1994b; Shimada *et al.*, 1997; Huang *et al.*, 1997, 1999), esterification (Hills *et al.*, 1989; Mukherjee and Kiewitt, 1991; Hills *et al.*, 1990; Rangheard and Langrand, 1989; Rahmatullah *et al.*, 1994; Foglia and Sonnet, 1995; Shimada *et al.*, 1997, 1998; Huang *et al.*, 1997, 1999) and transesterification (Osterberg *et al.*, 1989; Huang *et al.*, 1997, 1999) reactions. Specifically, the inability of lipases to catalyze the hydrolysis of GLA

moieties of TAG, as compared to other fatty acids, has been used to concentrate GLA in GLA-containing oils during lipase catalyzed hydrolysis. According to Rahmattullah *et al.* (1994), 1,3-specific lipase from *Candida cylindracea* under suitable conditions was able to produce unhydrolyzed acylglycerols containing 46.5% GLA (w/w) from an evening primrose oil with an original GLA content of 9.4% (w/w). Huang *et al.* (1997) reported a two-fold increase in the concentration of GLA in the unhydrolyzed acylglycerols portion of borage oil using lipase from *Candida rugosa*.

During enzyme catalyzed esterification reactions, the GLA-containing oil is first hydrolyzed into its free fatty acid (FFA) form and then esterified with an alcohol to form esters. Shimada *et al.* (1997) initially used lipase from *Psuedomonas sp.* to hydrolyze borage oil into FFA, followed by esterification with lauryl alcohol using *Rhizopus delemar* lipase. Lipases from *Rhizopus delemar* discriminate against the esterification of GLA, therefore, GLA concentrates in the FFA fraction. Shimada *et al.* (1997) produced a GLA concentrate of 93.7% in the FFA form with a recovery of 67.5%. A three-step procedure involving hydrolysis, esterification and acidolysis was performed by Huang *et al.* (1997) in order to produce a 65% (w/w) GLA concentrate in the acylglycerol form. Lipases from *Candida rugosa* were employed for the hydrolysis reaction, while lipase from *Mucor miehei* was utilized for both the esterification and acidolysis reactions.

2.5.2 Process optimization in food product development

Experimental design is a systematic approach that enables several variables and their interactions to be studied simultaneously. This allows for the acquisition of large

amounts of data from a minimum number of experiments and at the lowest cost. Experimental designs also allow the prediction of the effects or changes that occur in any of the variables found to be critical for giving the user a competitive edge. Good design can reduce time, wastage and costs and rework during production.

When many factors and interactions affect the desired outcome, response surface methodology (RSM) serves as an effective tool for optimizing the process conditions. Process optimization by RSM is a rapid and economical method for gathering research results than a one-variable-at-a-time or full factorial experimentation (Lee and Hosney, 1982). This method has been adopted in many optimization studies (Lee and Hosney, 1982; Chen, 1994; Shieh *et al.*, 1995; Chung *et al.*, 1996; Wanasundara and Shahidi, 1996a; Senanayake and Shahidi, 1999b).

2.6 Structured lipids

Nowadays food processors are quite interested in developing methods to improve the nutritional and functional properties of fats and oils. Changing the positional distribution, molecular weight or unsaturation of the acids adjacent to the TAG molecule may alter their physical and nutritional properties (Goderis *et al.*, 1987). The alternation of lipid structure at a molecular level is a reality that has lead to the production of "structured lipids". Structured lipids (SL) are defined as "TAG modified by incorporation of new fatty acids, restructured to change the position of the fatty acids, or the fatty acid profile, from the natural state, or synthesized to yield novel TAG (Akoh, 1998).

There are many reasons for developing structured lipids: 1) to improve the organoleptic properties of foods; 2) to reduce the total saturated fat in food; 3) to improve the processing or storage properties of a product; 4) to target specific diseases and metabolic conditions; 5) to improve digestability; and 6) to change the physical characteristics of oils such as melting points (O'Carroll, 1995; Lee and Akoh, 1998; Akoh, 1995; 1998). Some applications of structured lipids and their reported benefits are summarized in Tables 2.3 and Table 2.4, respectively.

2.6.1 Synthesis of structured lipids

The process of interesterification has been used for many years in the production of SL. There are two ways of interesterification in use, chemical and enzymatic. During chemical or enzymatic interesterification, acyl groups are redistributed first intramolecularly, then intermolecularly, to achieve a random distribution (Marangoni and Rousseau, 1995). The catalysts available for these rearrangements are alkali metal derivatives (such as sodium methoxide) and enzymes. One disadvantage of chemical interesterification is that the reaction is carried out at a very high temperature and may degrade the oils, whereas with enzymatic interesterification, the conditions are mild (Akoh, 1998). With enzymatic interesterification it is possible to control the final product composition and produce acylglycerol mixtures that are not possible with chemical interesterification (Macrae, 1983; Gunstone, 1994). Other advantages of enzymatic interesterification are that the reaction: a) requires less severe conditions and produces less waste (Willis and Marangoni, 1998); b) produces less thermal damage of reactants

Table 2.3 Potential food applications of structured lipids^a

-
1. Production of margarine, butter, spreads, shortenings, dressings, dips and sauces
 2. Production of fats with improved melting points of fats
 3. Confectioneries
 4. Dairy products
 5. Snack foods
 6. Baked goods
 7. Soft candies
 8. Reduced or low calorie fats
 9. Cocoa butter substitutes
 10. Infant formula
-

^a Adapted from Akoh (1998)

Table 2.4 Benefits of structured lipids^a

Benefit	Reference
1. Superior nitrogen retention	Mok <i>et al.</i> (1984)
2. Preservation of reticuloendothelial system (RES) function	Sobrado <i>et al.</i> (1985)
3. Reduced protein-catabolic effect after thermal injury	DeMichele <i>et al.</i> (1989); Teo <i>et al.</i> (1989, 1991)
4. Enhanced absorption of fatty acids in the <i>sn</i> -2 position	McKenna <i>et al.</i> (1985); Hubbard and McKenna (1987)
5. Reduction in serum TAG, LDL-cholesterol and cholesterol	Lee <i>et al.</i> (1997); Ikeda <i>et al.</i> (1991)
6. Improved immune function	Kennedy (1991); Mascioli <i>et al.</i> (1988)
7. Prevention of thrombosis	Kennedy (1991)
8. Lipid emulsion for enteral and parental feeding	Mascioli <i>et al.</i> (1988); Jensen and Jensen (1992)
9. Calorie reduction	Miller (1995)
10. Improved absorption of other fats	Jandacek <i>et al.</i> (1987); Ikeda <i>et al.</i> (1991); Jensen <i>et al.</i> (1994)

^aAdapted from Akoh (1995)

and products (Mukherjee, 1990; 1995); c) produces a wide variety of products; d) produces a high catalytic efficiency (Haraldsson, 1992); f) enzyme catalyst are selective; g) produces few or no unwanted side reactions; and h) improves functionality and properties of fats (Akoh, 1998).

There are several variations of the interesterification reactions worthy of consideration when producing SL. Transesterification is one, in which ester-ester interchanges occur between TAG, and TAG and monoesters, among others. Acidolysis is another way in which fatty acyl exchange reactions occur between TAG and fatty acids. Alcoholysis is the third one in which TAG react with a simple alcohol or glycerol (glycerolysis) leading to a mixture of mono- and diacylglycerols. Figure 2.7 shows each of these reactions.

Schuch and Mukherjee (1988) studied the rate of interesterification reactions with medium-chain TAG catalyzed by 1,3-specific immobilized lipase from *Mucor meihei* in hexane at 45°C. These reactions were acidolysis with heptadecanoic acid, transesterification with methyl heptadecanoate and trioleate, alcoholysis with octadecyl alcohol and glycerolysis with glycerol. The rates of interesterification were in the general order of long chain alcohols > TAG > fatty acids > methyl esters > polyhydric alcohols.

2.6.1.1 Transesterification

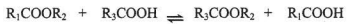
Transesterification is the exchange of an acyl group between two esters, namely two TAG. It is used primarily to change the physical properties of individual fats and oils or fat-oil blends by altering the positional distribution of the fatty acid in the TAG (Willis

Figure 2.7 Interesterification reactions of lipids

- TRANSESTERIFICATION



- ACIDOLYSIS



- ALCOHOLYSIS



and Marangoni, 1998). Transesterification has been used in the industry to improve the plasticity of fat (Kalo *et al.*, 1986;1990), the textural properties of tallow and rapeseed oil mixtures (Forssell *et al.*, 1992), as well as to develop cocoa butter equivalents. Transesterification has also been used to produce a SL that contains both medium and long chain fatty acids in order to meet the energy requirements of specific individuals. Peanut oil and tricaprylin were transesterified with lipase from *Rhizomucor miehei*, *Candida sp.* and *Chromobacterium viscosum*. It was found that *Rhizomucor miehei* gave the highest yield (73%) of structured lipids that contained both long- and medium-chain fatty acids (Soumanou *et al.*, 1997). These structured lipids are nutritionally beneficial because they are easily absorbed and provide essential fatty acids. Transesterification has also been used extensively in the production of low-calorie structured lipids. Tristearin has been successfully reacted with tricaprin and tricaprylin using lipase from *Rhizomucor miehei* to produce a low calorie SL (Akoh and Yee, 1997). Mangos *et al.* (1999) transesterified triacetin and hydrogenated soybean oil using a plant derived 1,3-specific lipase from *Carica papaya* in order to produce another low-calorie TAG.

2.6.1.2 Acidolysis

Acidolysis is a method used for the development of novel TAG by performing the interesterification of a TAG with a free fatty acid (Tanaka *et al.*, 1981). This technology has been used extensively in the food industry to modify the physical properties or improve the nutritional value of oils. Structured lipids resembling TAG of human milk are derived from the acidolysis of tripalmitin, derived from palm oil, with oleic acid or

other PUFA, obtained from plant oils, using a 1,3-specific lipase as a biocatalyst (Quinlan and Moore, 1993). Acidolysis has also been used to incorporate EPA and DHA into vegetable and fish oils to improve their nutritional properties. Yamane *et al.* (1992) performed an acidolysis reaction on cod liver oil and free EPA and DHA in order to increase the amount of EPA and DHA in the oil. They used an immobilized lipase from *Mucor miehei* to increase the content of EPA in the oil from 8.6 to 25% and the DHA content from 12.7 to 40%. Lipase from *Pseudomonas sp.* was chosen by Adachi *et al.* (1993) for the acidolysis of sardine oil with free EPA and DHA. The content of EPA and DHA in the acylglycerols were increased from 29 to 65%. Senanayake and Shahidi (1999) studied the incorporation of EPA and DHA into borage and evening primrose oils using lipases from *Candida antarctica*, *Mucor miehei* and *Pseudomonas sp.* They found that the lipase from *Pseudomonas sp.* showed the highest incorporation of EPA and DHA into both oils. Under optimum conditions, the incorporation of EPA+DHA was 35.5% in borage oil and 33.6% in the evening primrose oil. Acidolysis has also been used industrially to improve the physical properties of lard (Meusel *et al.*, 1990), to produce cocoa butter substitutes from cheaper oils and to produce fats containing acetic acid. The preparation of products resembling cocoa butter in their TAG structure and physical properties from inexpensive starting materials is of great interest to the confectionary, cosmetic and pharmaceutical industries. These products have been prepared by several methods including: a) acidolysis of olive oil with stearic acid catalysed by an immobilized 1,3-specific lipase from *Rhizopus delemar* (Yokozeki *et al.*, 1982; Tanaka *et al.*, 1988); b) reaction of a palm oil fraction with myristic acid using lipase from

Aspergillus sp. (Wisdom *et al.*, 1984); and c) interesterification of olive oil with palmitic acid using Lipozyme from *Mucor Miehei* (Neilsen, 1985).

2.6.1.3 Alcoholysis

Alcoholysis is the reaction of a TAG or an ester with a simple alcohol. Methyl esters have been produced by the reaction of TAG with methanol. The main use of alcoholysis is in the performance of glycerolysis reactions. Glycerolysis is the exchange of acyl groups between glycerol and TAG to produce monoacylglycerols, diacylglycerols and new TAG. The main problem with lipase-catalysed glycerolysis is the long reaction time (4-5 days) required to produce high yields (McNeill *et al.*, 1991). Monoacylglycerols are very important in the food industry because they act as surface-active agents and emulsifiers. TAG, from common oils, as well as waxy esters of jojoba oil have been transesterified with sugar alcohols in pyridine using lipases from *Chromobacterium viscosum* or porcine pancreas to yield monoesters of sugar alcohols having excellent surfactant properties (Chopineau *et al.*, 1988). Alcoholysis has also been used in the production of esters of terpene alcohols which are important flavour and fragrance compounds. Yee *et al.* (1997) produced the fragrance compounds citronellyl butyrate and geranoyl caproate with lipase from *Pseudomonas sp.* by reacting terpene alcohols (citronellol and geraniol) with tributyrin and tricaproin. Chatterjee and Bhattacharyya (1998) also produced fragrance compounds by using lipase from *Mucor miehei* to perform alcoholysis of terpene alcohols and ethyl acetate.

2.6.2 Importance of positional distribution in triacylglycerols (TAG)

Knowledge of the positional distribution of fatty acids is important due to its effect on both the functional properties (Love, 1996; Akoh, 1995) and the metabolism of fats and oils (Redgrave *et al.*, 1988; Mortimer *et al.*, 1988; Christensen *et al.*, 1995). Intestinal absorption of fatty acids has been reported to be dependent upon their arrangement in the triacylglycerol (TAG) molecules. Long chain PUFA located on the *sn*-2 position of the TAG are more readily absorbable (Ikeda *et al.*, 1991; Christensen *et al.*, 1995); this extends to other essential fatty acids. Therefore, both the positional distribution and fatty acid composition affect the nutritional value of TAG. Investigation into absorption of fatty acids in a canine model suggested 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 (Jensen *et al.*, 1994). Filer *et al.* (1969) and Tomarelli *et al.* (1968) made a similar observation in human infants.

Microbial lipases have been shown to have both positional and acyl chain specificities (Brockerhoff, 1970). Knowledge of the fatty acid distribution in TAG molecules may provide useful information when selecting microbial lipases for the production of structured lipids. This analysis is based on the modification of TAG by pancreatic lipase. The *sn*-1,3 positions of the TAG are analysed together, while the *sn*-2 position is analysed separately.

2.7 Oxidation of lipids

The oxidation of edible oils containing high amounts of PUFA is a major concern for the food industry because it causes nutritional, flavour, safety and storage problems. Lipid oxidation involves the reaction of unsaturated fatty acids with atmospheric oxygen. This process is accelerated by light, temperature, trace metals and composition of the oil. Characteristic changes associated with oxidative deterioration of vegetable, seed and animal oils include the development of unpleasant odours and tastes as well as changes in colour, specific gravity, viscosity and solubility (Ke and Ackman, 1976; Enser, 1987). The volatile components produced during oxidation are ketones, aldehydes, esters, alcohols and hydrocarbons, among others; however, it is the low threshold ketones and aldehydes that contribute to off-flavour (Gunstone, 1996; Min, 1998). Several authors (Frankel, 1980, 1982; Kanner *et al.*, 1987; Stansby, 1990; Bradley and Min, 1992; Gunstone, 1985, 1996; Min, 1998; Shahidi, 2001) have reviewed the mechanistic aspects of lipid oxidation and its subsequent effects in foods.

2.7.1 Autoxidation

Autoxidation is a natural process whereby molecular oxygen reacts with unsaturated fatty acids in the environment. Autoxidation occurs by a free radical (chemical species with unpaired electrons) chain reaction that consists of initiation, propagation and termination steps. Initiation of this process begins with the abstraction of a hydrogen atom adjacent to a double bond in the fatty acid (RH), catalysed by light, heat, and/or metal/metal ions as well as visible and ultraviolet irradiation in order to form

a free radical (Reaction 1). However, the detailed mechanism for the formation of a free radical (R^{\bullet}) from RH is not fully understood.

The propagation step of the autoxidation process includes an induction period when hydroperoxide formation is minimal (Labuza, 1971; Hawrysh, 1990). The length of the induction period is dependent upon the temperature, availability of oxygen, and the presence or absence of pro-oxidants or antioxidants. The induction period may be decreased by increasing the temperature, amount of light, presence of heavy-metal ions, peroxides or other sources of free radicals (Stansby, 1990). The hydrogen abstraction from oils is dependent upon the number of double bonds present in the lipid molecule. The hydrogen that is α to the double bond in oleic acid has a bond strength of 77 kcal/mol, whereas the bond strength of hydrogen at the doubly allylic methylene group of linoleic acid is only 52 kcal/mol (Min, 1998). The relative rate of autoxidation of oleate, linoleate and linolenate is 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 (Frankel, 1985; Hsieh and Kinsella, 1989). Location of the methylene interrupted double bonds also appears to affect the rate of oxidation as ω 3 fatty acids autoxidize faster than ω 6 fatty acids (Adachi *et al.*, 1995). PUFA such as AA, EPA, DHA, containing 4,5 and 6 double bonds, respectively, are much more labile to oxidation than linoleic or linolenic acids. Porter *et al.* (1981) reported that arachidonic acid oxidizes 2.9 times faster than linolenic acid. The induction period for autoxidation of ethyl esters of EPA and DHA was 3-4 days at 5°C in the dark, while the induction periods of linoleate and linolenate were 20 and 60 days, respectively. Similarly, the oxygen uptake of EPA and DHA ethyl

Initiation:



Propagation:



Termination:



esters after the induction period was 5.2 and 8.5 times faster than that of ethyl linolenate (Cho *et al.*, 1987). Therefore, oils that contain more PUFA are more susceptible to oxidation and hence suffer from stability problems. For this reason, barring of oxygen from oils, especially marine oils, is a major concern for the food industry. The breakdown products (alcohols, aldehydes, ketones, hydrocarbons, etc.) of primary lipid oxidation products (hydroperoxides) possess low flavour threshold values and contribute to the off-flavour development. These compounds may interact with other components of food and cause other functional and nutritional changes (Ke and Ackman, 1976; Enser, 1987).

Stability of hydroperoxides produced by EPA and DHA was much less in comparison with that of linoleate hydroperoxides (Miyashita *et al.*, 1982). In the case of linoleate oxidation, most of the early products were hydroperoxides, whereas the ratios of hydroperoxides-oxygen to total absorbed-oxygen in ethyl EPA and ethyl DHA were 50-60% (Cho *et al.*, 1987). The rate of oxidation of oils is not only affected by the degree of unsaturation, but also by the chain length and positional distribution of the acids on the glycerol molecule (Neff *et al.*, 1993, Endo *et al.*, 1997). If there are two adjacent (1,2 or 2,3) PUFA on one TAG molecule, the rate of oxidation is greater than when the PUFA are separated by a saturated fatty acid in the 2-position (Endo *et al.*, 1997). The high oxidation rates and instability of their hydroperoxides result in a rapid formation of secondary products, leading to flavour revision. Possible autoxidation of oleate, linoleate and linolenate is shown in Figures 2.8 and 2.9 (Frankel, 1985). Autoxidation of oleate involves hydrogen abstraction from C-8 and C-11 with the formation of two allylic radicals (Figure 2.8a) which react with oxygen to form a mixture of 8-, 9-, 10- and 11-allylic hydroperoxides. Hydrogen abstraction on the double allylic C-11 of the linoleate produces a pentadienyl radical, which reacts at both ends with oxygen to produce a mixture of conjugated 9- and 13-diene hydroperoxides (Figure 2.8b). Hydrogen abstraction of the linolenate occurs on the two active methylenes on C-11 and C-14 and produces two pentadienyl radicals which react with oxygen at the carbon ends to form a mixture of conjugated diene-triene 9-, 12-, 13- and 16-hydroperoxides (Figure 2.9). Studies by Yamauchi *et al.* (1983) and van Rollins and Murphy (1984) have shown that the autoxidation of EPA and DHA produces 8 monohydroperoxides and 10 hydroperoxide isomers, respectively (Figures 2.10 and 2.11).

Figure 2.8 Mechanisms of autoxidation of oleic acid (a) and linoleic acid (b) and formation of possible primary and secondary oxidation products adapted from Frankel, 1985)

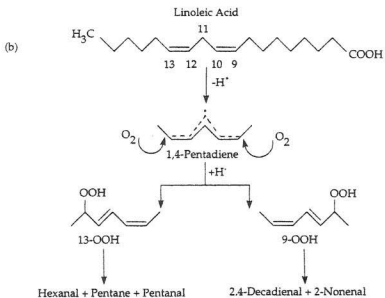
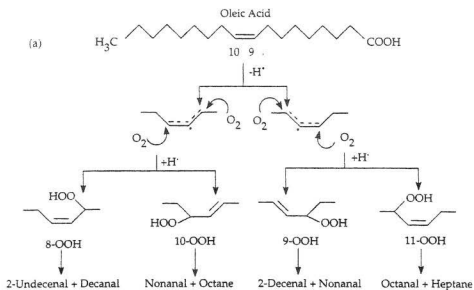
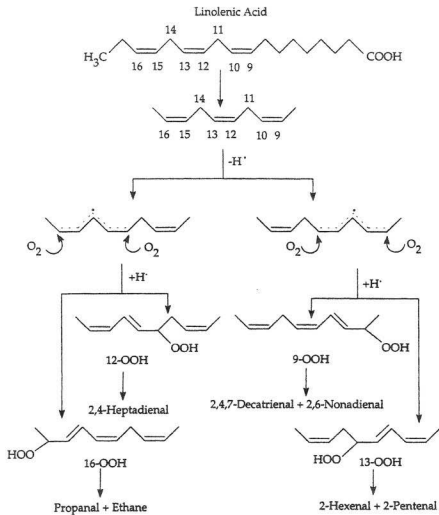


Figure 2.9 Mechanism of autoxidation of α -linolenic acid and formation of possible primary and secondary products (adapted from Frankel, 1985)



2.7.2 Photooxidation

The exposure of fats and oils to light brings about changes in their flavour characteristics. This can be explained by both photolytic autooxidation and photosynthesized oxidation. Photolytic oxidation is the production of free radicals from lipids during exposure to light (Min, 1998). Photosynthesized-oxidation involves the reaction between a double bond and highly reactive singlet oxygen in the presence of light and a suitable sensitizer (Gunstone, 1985; 1996). During photooxidation, singlet oxygen ($^1\text{O}_2$) is generated by the interaction of light and a photosensitizer (Gunstone, 1984) which then reacts with a methylene group adjacent to the double bonds of the unsaturated fatty acids to form hydroperoxides (Chan, 1977; Frankel, 1985). Some photosensitizers are dyes (eosin, erythrosine, rose bengal or methylene blue), pigments (chlorophyll, riboflavin, protoporphyrin IX, hemoglobin, myoglobin), metallic salts (cadmium sulphide, zinc oxide, zinc sulphide), transition metal complexes and polycyclic aromatic hydrocarbons (Min *et al.*, 1989; Davis *et al.*, 1995). These compounds are good photosensitizers because their conjugated double bond systems easily absorb visible light energy (Bradley and Min, 1992).

There are two types of emissions for electrons excited by light absorption—fluorescence and phosphorescence. For fluorescence, a ground state electron absorbs light energy and is boosted to a higher energy level. The sensitizer is referred to as an excited singlet state photosensitizer ($^1\text{Sen}^*$) which has a short life time and returns to ground state by emitting fluorescent light. During phosphorescence, $^1\text{Sen}^*$ is converted to an excited triplet sensitizer ($^3\text{Sen}^*$) via an intersystem crossing (ISC) which decays to

Figure 2.10 Mechanism of autoxidation of eicosapentaenoic acid and formation of possible primary and secondary products (adapted from Fujimoto, 1989)

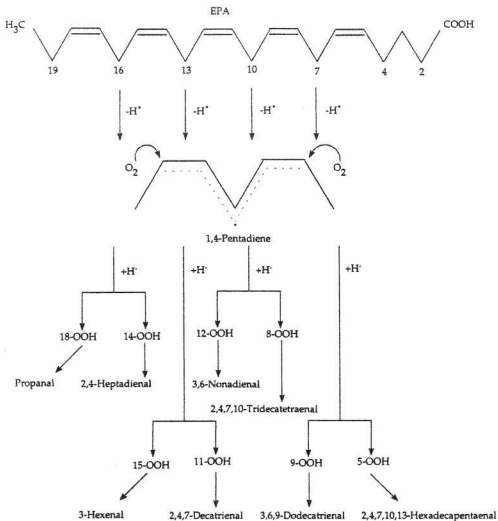
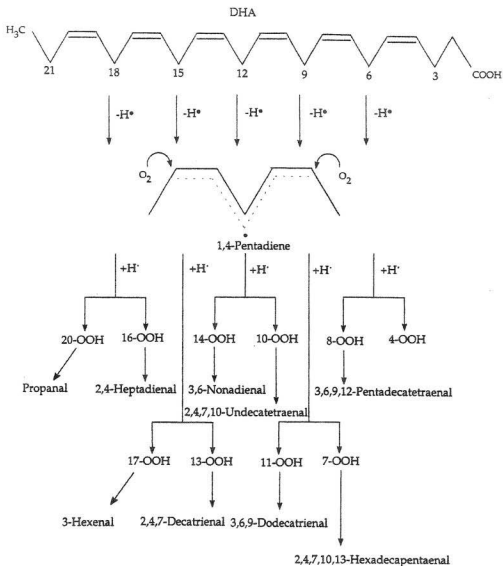


Figure 2.11 Mechanism of autoxidation of docosahexaenoic acid and formation of possible primary and secondary products (adapted from Fujimoto, 1989)

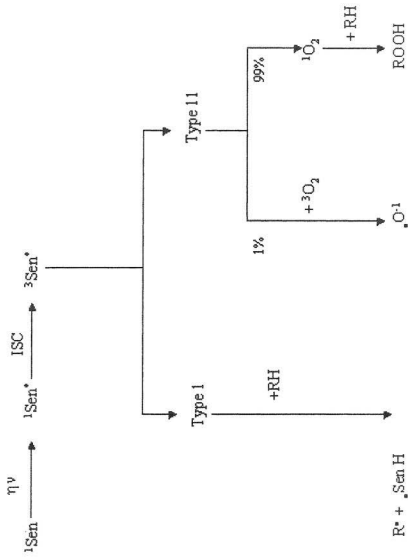


ground state $^1\text{Sen}^*$ slowly by emitting phosphorescence light. During photosensitized oxidation it is the $^3\text{Sen}^*$ that is the reactive intermediate (Min, 1998). The $^3\text{Sen}^*$ takes two major pathways (Types I and II) in order to oxidize unsaturated fatty acids (Figure 2.12).

In the Type I pathway, a hydrogen atom is abstracted from a lipid molecule (RH) and transferred to a $^3\text{Sen}^*$ to produce free radicals. The free radicals then react with triplet oxygen ($^3\text{O}_2$) to form oxidized products, i.e. hydroperoxides (ROOH) and free radicals that can initiate free radical chain reactions of lipid oxidation. This type of reaction is dependent upon the concentration and type of sensitizer and substrate (Davis *et al.*, 1995; Min, 1998). In the Type II pathway, $^3\text{Sen}^*$ reacts with $^3\text{O}_2$ to form singlet oxygen ($^1\text{O}_2$). The singlet oxygen reacts with a RH to form ROOH. There is also a 1% chance of an electron transfer from $^3\text{Sen}^*$ to triplet oxygen to produce a superoxide radical anion ($\text{O}_2^{\bullet-}$) and sensitizer radical cation ($\text{Sen}^{\bullet+}$) (Min, 1998). Electron rich compounds such as olefins, dienes and aromatic compounds favour this pathway. The type II pathway is dependent upon the solubility and concentration of oxygen present in the food. Since oxygen is more soluble in nonpolar solvents and lipids than in water, small amounts of photosensitizers in oil would promote Type II photosensitized oxidation (Bradley and Min, 1989; Davis *et al.*, 1995; Min, 1998). The involvement of singlet oxygen in the photosensitized oxidation is of Type II which occurs rapidly and accounts for most of the photosensitized reactions (Bradley and Min, 1989; Davis *et al.*, 1995; Min, 1998).

There are many differences between type II photooxidation and autooxidation: (1) it involves reaction with singlet oxygen produced from triplet oxygen by light and a sensitizer; (2) it is an ene reaction and not a radical chain reaction; (3) there is no

Figure 2.12 Photosensitized oxidation of lipids (RH) (adapted from Min, 1998)



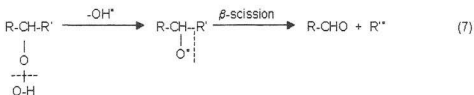
induction period; (4) the reaction is not affected by antioxidants used to inhibit autoxidation, but is inhibited by singlet oxygen quenchers; (5) it causes double bond migration with a change in configuration from *cis* to *trans*; (6) the products of photosensitized oxidation are both conjugated and non-conjugated hydroperoxides, whereas autoxidation only produces conjugated hydroperoxides; (7) the reaction is faster than autoxidation and its rate is determined by the number of double bonds rather than the number of doubly activated allylic bonds (Gunstone, 1996; Min, 1998). Carotenoids have been shown to act as quenchers for either singlet oxygen or triplet sensitized in singlet lipid oxidation (Davis *et al.*, 1995). Tocopherols are also known as free radical scavengers and singlet oxygen quenchers (Min *et al.*, 1989).

2.7.3 Decomposition of hydroperoxides

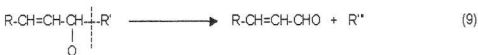
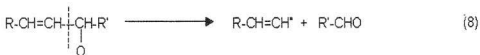
The breakdown of hydroperoxides follows a very complex mechanism that is not completely understood. Depending on the conditions, the decomposition of hydroperoxides can yield a variety of compounds, including aldehydes, ketones, esters, alcohols, acids, hydrocarbons, polymers and volatile components that produce off-flavours and off-odour. Some of these products are unstable and undergo further decomposition to lower molecular weight products (Frankel, 1980; Gardner 1987; Hilder, 1998).

The first step in hydroperoxide decomposition is the cleavage between the oxygen-oxygen bond in R-O-O-H, that is $\text{R-O-O-H} \rightarrow \text{RO}^\bullet + \bullet\text{OH}$ instead of $\text{R-O-O-H} \rightarrow \text{ROO}^\bullet + \text{H}^\bullet$. The reason for this is that the activation energy of the cleavage between the -O-O- bond

is 44 kcal/mol, whereas the activation energy of the cleavage between the oxygen and the hydrogen of R-O-O-H was 90 kcal/mol (Hiatt *et al.*, 1968). Therefore, hydroperoxides are cleaved by homolysis producing an alkoxy radical and a hydroxy radical (Reaction 7):



The alkoxy radical then undergoes homolytic β -scission, which is the most important free radical reaction leading to the breakdown products causing flavour deterioration of lipids. The alkoxy radical can cleave the carbon-carbon bond by β -scission on either side of the oxygen containing carbon atom producing two types of aldehydes, an olefinic and an alkyl radical (Reactions 8 and 9):



The olefinic radical is very reactive and may undergo the following reactions

(Reaction 10-12):



The corresponding alkyl radical may undergo similar reactions to produce alcohols, hydrocarbons or hydroperoxides (Reactions 13-15):



Secondary oxidation products are still unstable and undergo further decomposition such as condensation, rearrangement, dimerization and polymerization. Despite the extensive research on the breakdown of hydroperoxides, there are still many possible pathways of decomposition that lead to the origin of the hydroperoxides being completely lost.

2.7.4 Factors affecting lipid oxidation

The oxidation of fats and oils is a very complex subject that is affected by many factors such as components of the oils and non-lipid components. These factors may

enhance or suppress the rate of oxidation of the oil. One factor is the effect of the number, position, and geometry of the double bonds in the unsaturated fatty acids. The rate of autooxidation of saturated fatty acids is very slow at room temperature, while unsaturated fatty acids tend to oxidize readily. *Cis* acids oxidize more freely than *trans* isomers and conjugated double bonds are more reactive than nonconjugated ones (Nawar, 1985).

It is known that both temperature and oxygen pressure have profound effects on the rate of oxidation of oils. In general, as temperature increases by 10°C the rate of oxidation doubles (Meyer, 1987). The oxidation rate of lipids is independent of oxygen pressure when the oxygen supply is unlimited. However, at low oxygen pressures the rate of oxidation is proportional to the oxygen pressure. Temperature and surface area also affect the partial pressure of oxygen (as the temperature increases, oxygen becomes less soluble) (Nawar, 1985).

Light has an accelerating effect on the decomposition of oils, but this is in part dependent upon the wavelength. Visible light accelerates the decomposition of hydroperoxides formed upon primary oxidation, while the effect of ultraviolet (UV) light is more pronounced (Stansby, 1967). Ultraviolet light promotes the formation of free radicals, thereby initiating autooxidation and creating conjugated systems which absorb UV light strongly at certain wavelengths (Hilder, 1997). This may be due to photosensitized oxidation as discussed earlier.

The transition metals, particularly those possessing two or more valency states with a suitable oxidation-reduction potential between them (i.e. cobalt, copper, iron, and

nickel), are major prooxidants (Gordon, 1990). These metals catalyze the formation of free radicals and should be avoided or removed during processing. In order to avoid pro-oxidant effects the maximum levels recommended in refined oils are 0.01 ppm for copper, 0.1 ppm for iron and 0.1 ppm for nickel (Hilder, 1997). If these metals are present above the recommended levels, they can decrease the induction period and increase the rate of oxidation.

The effect of moisture and its role in oxidation of oils is much less clear. Karel (1980) showed that dried fruits with a water activity of less than 0.1 oxidize very rapidly while a water activity of 0.3-0.7 retards lipid oxidation. This protective effect of water occurs by reducing the catalytic activity of metals, by quenching free radicals and impeding the access of oxygen into foods (Nawar, 1985; Hilder, 1997). At lower water activities, the hydration effect is negligible, leading to higher oxidation rates. At higher levels, the rate of oxidation increases again due to increased mobility of metals, enzymatic attack, formation of free fatty acids, hydrolytic rancidity and leaching of iron (Hilder, 1997).

2.8 Control of lipid oxidation

After examining mechanisms of lipid oxidation in previous sections, it is quite evident that there are various tactics that can be used to control oxidation. Knowledge of the mechanisms that initiate lipid oxidation enables one to develop methodologies to control lipid oxidation. Some methods that may be used to control lipid oxidation are hydrogenation, removal of oxygen, use of appropriate packaging material, removal of

trace metals and the use of antioxidants. The most commonly used strategy to control lipid oxidation is the use of antioxidants, as discussed in the following sections.

2.8.1 Use of antioxidants

By far the most important defence mechanism against lipid oxidation is the presence of antioxidants, which can delay or slow down the rate of oxidation of oxidizable materials. However, antioxidants can not improve the quality of an already oxidized product (Dziezak, 1986). Antioxidants operate by suppressing or reducing free radical formation by promoting termination reactions which increase the induction period (Hilder, 1997). According to the United States Drug Administration Code of Federal Regulations [21 CFR 170.3 (0) (3)], "antioxidants are substances used to preserve food by retarding deterioration, rancidity or discoloration due to oxidation" (Dziezak, 1986). The type and the amount of antioxidants that are permitted in foods are strictly controlled. However, certain antioxidants that are allowed in some countries are not permitted in others. For example, tertiary-butylhydroquinone (TBHQ) is allowed in the USA and its use has only been permitted by the Canada Food inspection Agency since 1999, but it is not permitted in Europe and Japan (Gunstone, 1996). Many factors influence the effectiveness of an antioxidant such as activation energy, rate constant, oxidation-reduction potential, ease of antioxidant loss or destruction and solubility. Ideal food-grade antioxidants should not affect the colour and flavour of the food, and must be effective at low concentrations, survive processing, easy to incorporate, stable, low cost and safe (Coppen, 1983).

2.8.2 Mechanism of action of antioxidants

To control oxidation or at least to minimise it, several types of antioxidants may be utilised. There are two distinct types of antioxidants, primary and secondary. Primary antioxidants interfere with the chain reactions that cause oxidation of the oils. A primary antioxidant reacts either with the original free radical or one forming early in the oxidation process to form an intermediate that is not able to continue the chain process (Labuza, 1971; Stansby, 1990; Gordon, 1990; Gunstone, 1996). These are primarily phenolic compounds that are most common in foods and can donate a hydrogen atom to the lipid molecules. Some examples of primary antioxidants are tocopherols, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) tert-butylhydroquinone (TBHQ) (Nawar, 1985) and plant phenolics (Shahidi and Wanasundara, 1992).

There are two drawbacks associated with primary antioxidants, time of addition and the concentration of antioxidant. The time of addition of antioxidant is critical because if the peroxide levels are too high then the effectiveness of the antioxidant is masked. Also, at high concentrations the phenolic compound may lose its antioxidant activity and act as a prooxidant by reacting with peroxides (Labuza, 1971; Cillard *et al.*, 1980).

Secondary antioxidants operate by controlling the source of free radicals before the propagation step, thereby preventing the chain-initiation process (Gunstone, 1996). These may operate by several mechanisms including chelation of metal catalysts, scavenging of oxygen, decomposition of hydroperoxides to non-radical species,

deactivation of singlet oxygen and absorption of UV radiation (Labuza, 1971; Gordon, 1990; Gunstone, 1996). Only very low concentrations of trace metals are sufficient to reduce the shelf-life of fats. For example, the shelf-life of lard at 98°C is reduced by 50% when the concentration of iron is 0.6 ppm and copper is present at 0.05 ppm (Gunstone, 1996). The activation energy is reduced by trace metals which aid in the initiation of the oxidation process. Citric acid, ethylenediaminetetraacetic acid (EDTA) and phosphoric acid are excellent metal chelators that inhibit oxidation in foods. Ascorbic acid and its derivatives control oxidation in fatty foods. It acts as an antioxidant by several mechanisms; these include action as oxygen scavenger; function as an electron donor; act as a metal chelator; and may also participate in tocopherol regeneration (Nawar, 1985; Erickson, 1998). Carotenoids, as fat-soluble pigments, are also known to inhibit lipid oxidation initiated by xanthine oxidase, but only at low oxygen concentrations. At high oxygen concentrations carotenoids may act as prooxidants. Carotenoids may also function similarly to tocopherols in that they scavenge singlet oxygen and peroxy radicals (Erickson, 1998)

2.8.3 Synthetic Antioxidants

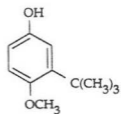
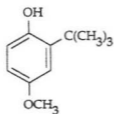
Synthetic antioxidants, mostly phenolic compounds, have been used in foods since the late 1940's (Sherwin, 1990). In the United States of America, the Federal Food, Drug and Cosmetics Act regulates the addition of antioxidants to foods. Some of the accepted synthetic antioxidants in the United States are PG, TBHQ, BHT and BHA (Figure 2.13; Nawar, 1985). United States Food and Drug Administration (US-FDA)

regulations require that antioxidants and their carrier be stated on the ingredient label of products and this should be followed by an explanation of their intended purpose (Dziezak, 1986). In general, the total concentration of antioxidant added to foods, singly or in combination, must not exceed 0.02% by weight of the total fat in the food (Nawar, 1985). Some antioxidants are under review by the FDA due to their possible toxicity and/or mutagenicity (Branen, 1975). BHA was removed from the list of generally accepted as safe (GRAS) antioxidants in the early 1990's due to its possible conversion into toxic substances or carcinogens by increased secretion of microsomal enzymes of the liver and hepatic organs (Wattenburg, 1986). However, after further investigation, BHA was added back to the GRAS list. TBHQ has never been approved in Japan or European countries (Barlow, 1990). Antioxidants are necessary additives to extend the shelf-life of fatty foods, however, it may be necessary to replace synthetic antioxidants with natural alternatives.

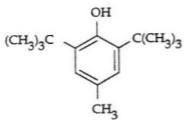
2.8.4 Natural Antioxidants

The public is concerned with the addition of chemical additives into foods, therefore scientists are engaged in search for new antioxidants that occur naturally in foods. Phenolics, found in all parts of plants, are the primary sources of natural antioxidants that can be extracted from foods (Hudson, 1990). Plant phenolics may act as antioxidants by reducing free radicals, chelating metals, and quenching singlet oxygen (Shahidi and Wanasundara, 1992). The activity of natural antioxidants depends on the chemical nature of the active ingredients, the preparation methods and concentration of

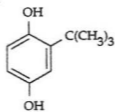
Figure 2.13 Structures of some synthetic antioxidants and α -tocopherol



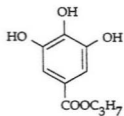
BHA (two isomers)



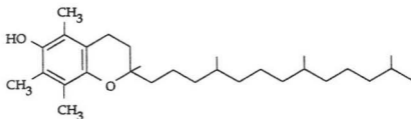
BHT



TBHQ



PG



α -Tocopherol

the active components in the final products. Some sources of natural antioxidants are oils and oilseeds, cereals and legumes, herbs and spices and tea. Some common antioxidative compounds are tocopherols (Figure 2.13), flavonoids, carotenoids, esters of caffeic and ferulic acids, carnosol, capsaicin, and catechins. The most commonly used natural antioxidants are tocopherols. There are four isomers of tocopherol, namely α -, β -, δ - and γ -tocopherols. Alpha-tocopherol has the highest vitamin E activity but shows the lowest antioxidant activity. The order of antioxidant activity is thus $\delta > \gamma > \beta > \alpha$ however, this activity is dependent upon light and temperature. The level of tocopherols added to foods is usually up to 500 ppm. At concentrations above 1000 ppm tocopherols may act as a prooxidant (Gunstone, 1996).

2.9 Measurement of lipid oxidation

There are many physical, chemical and sensory methods available for the measurement of lipid oxidation in foods. However, there is no one standard method for detecting all oxidative changes in all food systems. These methods may be divided into two groups: those that measure primary changes in lipids and those that quantitate secondary oxidation products. Primary changes are measured as loss of unsaturated fatty acids, weight gain, peroxide value and conjugated diene value. Secondary changes are monitored by quantitation of carbonyls (by gas chromatography), malonaldehyde and/or other aldehydes (thiobarbituric acid reactive substances, TBARS value), hydrocarbons (pentane or hexane content), and fluorescence products (1-amino-3-iminopropane compounds). Individual aldehydes, such as hexanal and propanal may also be measured.

The method of choice is usually dependent upon the nature and history of the oxidized sample, the amount of sample available, the type of information needed, time available and test conditions. Some of these methods will be discussed in subsequent sections.

2.9.1 Primary changes

2.9.1.1 Measurement of hydroperoxides

In the oxidation of lipids, hydroperoxides are the first oxidation products formed. The initial rate of hydroperoxide formation exceeds that of its decomposition, but this is reversed at later stages. Therefore, it is possible to determine if the lipid is in the growth or decay portion of the hydroperoxide concentration curve by monitoring the amount of hydroperoxides as a function of time. This information could be used as a guide to assess the quality of a product with respect to its deterioration. The classical method used to determine the amount of hydroperoxides is "peroxide value". This method determines the amount of hydroperoxide by an iodometric method, which is based on the titration of iodine released from potassium iodide by peroxides in a biphasic system with a thiosulphate solution (Reactions 24 and 25):



Drawbacks of this method are absorption of iodine at the unsaturation sites of fatty acids and liberation of iodine from potassium iodide by oxygen present in the solution to be

titrated (Gray, 1978). Although determination of peroxide value is quite common, it is only useful for measuring the initial stages of oxidation.

2.9.1.2 Measurement of conjugated dienes

The conjugated diene test is often used in conjunction with peroxide value in order to determine the amount of primary deteriorative products such as hydroperoxides in a sample. The reason for this is that the reactions causing peroxidation of polyunsaturated fatty acids such as linoleic and linolenic acids are also responsible for production of conjugated diene hydroperoxides (Rossell, 1991). Peroxidation involves initial abstraction of hydrogen from the allylic position followed by double bond migration resulting in the formation of conjugated dienes (Logani, 1980). These conjugated double bonds absorb light at 234 nm (St. Angelo *et al.*, 1975). Farmer and Sutton (1946) indicated that the absorption increase is proportional to the oxygen uptake and formation of peroxides during the early stages of oxidation. Wanasundara *et al.* (1995) reported that conjugated dienes and peroxide values during the oxidation of canola and soybean oils were directly related.

St. Angelo *et al.* (1975) reported that the number of conjugated dienes in peanut butter steadily increased with time. This corresponded to the expected increased oxidation of the product. A limitation of this method is that it is nonspecific and extinction coefficients used for biological systems are only approximate (Logani and Davies, 1990). However, the method of using absorption increases at 234 nm is advantageous because it is simple, fast, does not depend on chemical reactions or colour development and requires a small sample size (St. Angelo *et al.*, 1975).

2.9.2 Secondary oxidation changes and their measurement

Hydroperoxides are intermediary compounds that undergo breakdown into different secondary oxidation products. Hydroperoxides are colourless and odourless products, however, secondary oxidation products cause off-flavour and off-odour. For this reason, secondary oxidation products can be used as indices of lipid oxidation. Secondary oxidation products include carbonyls (ketones and aldehydes), hydrocarbons, carboxylic acids and alcohols, among others. There are many test methods for measuring secondary oxidation products, some of which are described in subsequent sections.

2.9.2.1 2-Thiobarbituric acid reactive substances (TBARS)

The 2-thiobarbituric acid (TBA) test is widely used for monitoring the oxidative state of lipid-containing foods. It provides a convenient method for measuring the content of secondary oxidation products, referred to as TBA reactive substances (TBARS). One of the carbonyl decomposition products of food lipids is malonaldehyde. Malonaldehyde reacts with two TBA molecules and the intensity of the resultant coloured chromogen, is measured spectrophotometrically at 532 nm. Other TBARS may also react with the TBA reagent and the intensity of the chromogen at 532 nm is proportional to the amount of TBARS present in food. Thus, the TBA test serves as a useful procedure for monitoring the extent of rancidity development in foods (Shahidi *et al.*, 1991).

The TBARS number is defined as mg of malonaldehyde (MA) equivalents per kg of sample. It is calculated by multiplying the absorbance of the TBA-MA complex at 532 nm by a constant. This constant is obtained from a standard line using a precursor of

malonaldehyde such as 1,1,3,3-tetramethoxypropane (TMP) and monitoring the absorption intensity of the chromogen at 532 nm (Shahidi, 1991).

The validity of TBA methodology has been questioned as it has never been adequately standardized as an indicator of lipid oxidation (Shahidi and Pegg, 1994). Malonaldehyde may interact with food constituents such as amino acids, proteins, glycogen and other components, so all the malonaldehyde may not be available to react fully with the TBA reagent, thus leading to under-estimation of TBARS values (Gray and Monahan, 1992). Also, the formation and accumulation of malonaldehyde in meat samples depend on the degree of unsaturation of polyunsaturated fatty acid (PUFA), malonaldehyde precursors from non-lipid origin, types of catalysts and conditions and reactivity of malonaldehyde with biological materials (Raharjo and Sofos, 1993). Finally, in biological samples, the TBA reaction lacks specificity for malonaldehyde (Bidder and Sipka, 1989). However, it has been suggested that TBARS provide an excellent means for evaluating relative oxidative status as affected by processing or storage (Gray, 1978; Hoyland and Taylor, 1991).

2.9.2.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is considered to have the potential for monitoring lipid oxidation as it has been successfully used to evaluate oxidative deterioration of fat-containing foods (Shahidi, 1992). It provides a useful means for following oxidation because polyunsaturated fatty acids are converted to hydroperoxides and then further broken down into a complex mixture of secondary products. This is

accompanied by a decrease in the relative number of olefinic and an increase in the number of aliphatic protons of fish oils which appear at δ 5.1 - 5.6 and δ 0.6 - 2.5, respectively, on the NMR spectrum (Saito and Udagawa, 1992). Because of this decrease in the relative number of olefinic protons and increase in aliphatic protons, NMR appears to be useful in studying lipid oxidation in foods. Wanasundara and Shahidi (1993) reported that the relative number of olefinic and diallylmethylenic protons decreased with the storage time of bulk oils, while that of aliphatic protons increased. This was due to the oxidation of polyunsaturated fatty acids and further confirmed the findings of Saito and Nakamura (1990) on oxidative deterioration of fish meal.

In evaluation of oxidative state of food lipids, it is recommended that several indicators that measure both the primary and secondary oxidation be used. This would provide information both about the current state of lipids involved and their history.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Samples

Borage oil was obtained from Bioriginal Food and Science Corp. (Saskatoon, SK). Refined-bleached and deodorized (RBD) harp seal (*Phoca groenlandica*) oil and menhaden oil, devoid of any additives, were obtained from local sources in Newfoundland and Omega Protein (USA) Inc. (Reedville, VA), respectively. Three 1 kg containers of each type of oil were received and stored at -20°C until use.

3.1.2 Chemicals

Lipozyme-IM (*Mucor miehei*; immobilized on a macroporous anion-exchange resin) was provided by Novo Nordisk (Bagsvaerd, Denmark). Lipase PS-30 (*Pseudomonas sp.*) was obtained from Amano Enzyme USA Co., Ltd. (Lombard, IL). Fatty acid methyl esters were purchased from either Supelco (Oakville, ON) or NU-Chek (Elysian, MN) companies. All other chemicals were of American Chemical Society (ACS) grade or better.

3.2 Production of gamma-linolenic acid (GLA) concentrates from borage oil (BO)

The GLA concentrate was prepared from BO, following hydrolysis, by employing urea complexation as explained below.

3.2.1 Preparation of free fatty acids from BO

Preparation of free fatty acids from BO was carried out according to the scheme given in Figure 3.1. BO (25 g, treated with 200 ppm of butylated hydroxytoluene; BHT) was saponified by refluxing for 1 h at the boiling temperature of the mixture ($62 \pm 2^\circ\text{C}$) under a blanket of nitrogen using a mixture of KOH (5.75 g), water (11 mL) and 95% (v/v) aqueous ethanol (66 mL). To the saponified mixture, distilled water (50 mL) was added and the unsaponifiable matter extracted into hexane (2 x 100 mL) and discarded. The aqueous layer containing the saponifiable matter was acidified ($\text{pH}=1.0$) with 3M HCl. The mixture was subsequently transferred to a separatory funnel and the liberated fatty acids were extracted into 50 mL of hexane. The hexane layer containing the free fatty acids was then dried over anhydrous Na_2SO_4 and the solvent removed at 40°C to recover free fatty acids which were then stored under nitrogen at -60°C until used.

3.2.2 Preparation of γ -linolenic acid (GLA) concentrates from borage oil (BO) by urea complexation

The separation of $\omega 6$ fatty acids from the mixture of hydrolyzed fatty acids (Section 3.2.1) of BO 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 to 60°C while stirring until the whole mixture became a clear homogenous solution. The ratio of urea-to-fatty acid was changed by using different amounts of urea.

Figure 3.1 Flowsheet for preparation of free fatty acids from borage oil (BO)

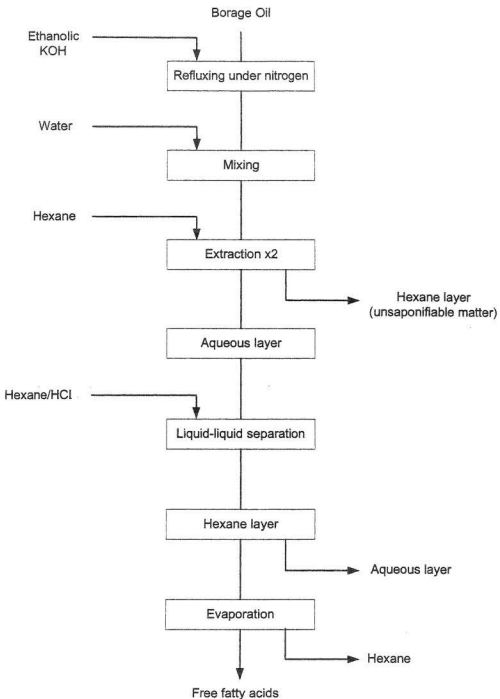
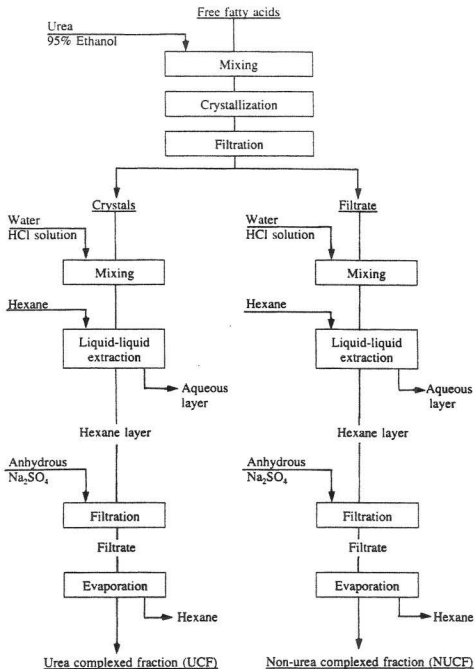


Figure 3.2 Flowsheet for preparation of γ -linolenic acid (GLA) concentrates by urea complexation



Initially, the urea-fatty acid adduct was allowed to crystallize at room temperature, but colder temperatures (-12, -3 and +6 °C) were later maintained over varying time periods (12, 18 and 24 h) for further crystallization. The crystals formed (urea-fatty acid adducts were referred to as urea complexing fraction, UCF) were separated from the liquid (non-urea complexing fraction, NUCF) by filtration under suction using a Buchner funnel lined with a Whatman #4 filter paper. The NUCF (filtrate) was diluted with an equal volume of water and acidified to pH 4-5 with 6 M HCl; an equal volume of hexane was subsequently added, the mixture stirred thoroughly for 1 h, and then transferred to a separatory funnel. The hexane layer containing the liberated fatty acids was separated from the aqueous layer containing urea. The hexane layer was washed with distilled water to remove any remaining urea and then dried over anhydrous Na_2SO_4 and the solvent was removed at 40 °C using a rotary evaporator. Fatty acids from the UCF were recovered after addition of water/6 M HCl and hexane in a similar manner to the NUCF. The two fractions (UCF and NUCF) were weighed separately and the percentage recovery of each was calculated. The fatty acid composition of the two fractions was determined using a gas chromatographic procedure (Section 3.7). Details of the experimental design to determine optimum conditions (urea-fatty acid ratio, crystallization time, and crystallization temperature) for concentration of GLA by urea complexation are described in Section 3.3.

Table 3.1 Independent variables and experimental design levels used for face-centred cube design in the optimization of GLA concentrate production by urea complexation of borage oil

Independent Variables	Symbols	Coded Variable Levels		
		-1	0	1
Reaction time (h)	X_1	12	18	24
Reaction temperature ($^{\circ}\text{C}$)	X_2	-12	-3	+6
Urea-to-fatty acid ratio (w/v)	X_3	2	3	4

Table 3.2 Coded and uncoded variables of face centred cube design (FCCD) in optimization of GLA concentration from borage oil (BO) by urea complexation

Run	Coded variables			Uncoded variables		
	x_1	x_2	x_3	X_1	X_2	X_3
1	-1	-1	-1	12	-12	2
2	-1	-1	+1	12	-12	4
3	-1	+1	-1	12	+6	2
4	-1	+1	+1	12	+6	4
5	+1	-1	-1	24	-12	2
6	+1	-1	+1	24	-12	4
7	+1	+1	-1	24	+6	2
8	+1	+1	+1	24	+6	4
9	-1	0	0	12	-3	3
10	+1	0	0	24	-3	3
11	0	-1	0	18	-12	3
12	0	+1	0	18	+6	3
13	0	0	+1	18	-3	4
14	0	0	-1	18	-3	2
15	0	0	0	18	-3	3
16	0	0	0	18	-3	3
17	0	0	0	18	-3	3

X_1 = Reaction time (h), X_2 = reaction temperature ($^{\circ}\text{C}$) and X_3 = urea-to-fatty acid ratio

3.3 Optimization procedure for production of GLA concentrate via urea complexation of borage oil (BO)

3.3.1 Experimental design and data analysis

A 3-factor, 3-level, face-centred cube design (FCCD) was employed (Gao and Mazza, 1996) to study the response (Y), which is the percent GLA concentrate formed by were the independent variables (Xi). Table 3.1 shows the independent variables studied and their levels used in urea complexation. Duplicate reactions were carried out at all design points except for the centre point (0,0,0) where reactions were carried out in triplicate. All reactions were carried out in a randomized order to minimize the effect of unexplained variability in the observed responses due to extraneous factors. Coded (x) and uncoded (X) variables of the FCCD and treatment combinations used for optimization of GLA concentration by urea complexation are given in Table 3.2.

A generalized second order polynomial model was assumed for predicting individual Y variables. The model proposed for each response was:

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

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 analytical system (SAS, 1990) was used for multivariate regression analysis, analysis of variance (ANOVA), canonical analysis and analysis of ridge maximum of data in the response surface regression (RSREG) procedure. Response surfaces and contour plots were developed using the fitted quadratic polynomial equations obtained from the

RSREG analysis. This was achieved by holding the independent variable with the least effect on the response at a constant value and changing the levels of the other two variables.

3.4 Preparation of structured lipids containing ω 3 and GLA fatty acids by enzymatic acidolysis

3.4.1 Determination of lipase activity

Lipase activity was measured by assaying fatty acids produced from the hydrolysis of triacylglycerols. All experiments were carried out in triplicate in screw-capped test tubes. Triolein was used as a substrate. Triolein was emulsified at a concentration of 50 mM in 5% (w/v) gum arabic for 1 min using a Polytron (800 rpm; PT-3000; Brinkmann, Littau-Switzerland). The assay mixture contained 1 mL substrate-emulsion and the enzyme (10-100 mg). Reactions were carried out for up to 1 h in a shaking water bath at 35°C. Fatty acids were released linearly for more than 1 hour. The released fatty acids were assayed colorimetrically as copper soaps using cupric acetate-pyridine reagent (Lowry and Tinsley, 1976; Kwon and Rhee, 1986). Purity of triolein was verified by thin layer chromatography-flame ionization detection (TLC-FID) and no breakdown products were detected.

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 and stirred for 90 s in a vortex; the absorbance of the upper isooctane layer was measured at 715 nm (Arribère *et al.*, 1994). One unit (U) of

lipase activity was defined as nanomoles of fatty acids (equivalent to oleic acid) produced per minute per gram of enzyme.

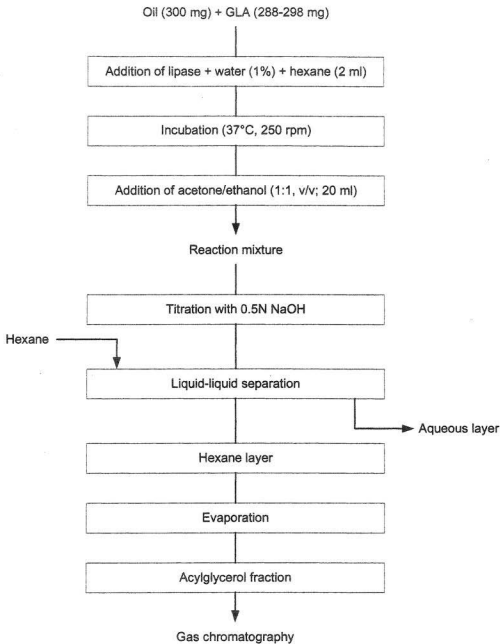
3.4.2 Acidolysis of seal blubber oil (SBO) and menhaden oil (MO) with the GLA concentrate

Acidolysis of SBO and MO (oils were stabilized with 200 ppm of tertiary-butylhydroxyquinone (TBHQ)) by microbial lipase was carried out according to the scheme given in Figure 3.3. In general, 300 mg of seal blubber or menhaden oil were mixed with a GLA concentrate at a mole ratio of 1:1 - 5:1 (GLA:TAG), in a screw-capped test tube. Lipase from *Mucor miehei* or *Pseudomonas sp.* (100-700 enzyme activity units /g oil) and water (1% of the weight of substrates and enzyme) and hexane (2 mL) were subsequently added to the test tube. The mixture was stirred in a Gyrotory water bath shaker (Model G76, New Brunswick Scientific Co. Inc., New Brunswick, NJ) at 200 rpm under a blanket of nitrogen and at different temperatures (30, 40, 50, 60°C). Individual samples were removed and analyzed at different time periods (0 - 24 h).

3.4.3 Separation of acylglycerols and free fatty acids after interesterification

Once the reaction was complete, the enzymes were removed by filtration. Samples were placed in a 250 mL conical flask 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 in order to remove any remaining free fatty acids. The mixture was transferred to a separatory funnel and thoroughly mixed with 25 mL of hexane.

Figure 3.3 Flowsheet for the preparation of structured lipids containing GLA and ω 3 fatty acids by microbial lipase



The lower aqueous layer was separated and discarded. The upper hexane layer containing triacylglycerols was passed through a bed of anhydrous sodium sulphate and subsequently the solvent was removed using a rotary evaporator. The fatty acid composition of the triacylglycerols was analyzed using gas chromatography (Section 3.5).

3.5 Analysis of fatty acid composition of lipids

3.5.1 Preparation of fatty acid methyl esters

The fatty acid composition of lipids was determined after their conversion to corresponding methyl esters. Approximately 15 mg of oil were weighed into a 6 mL Teflon-lined, screw capped conical vial. Transmethylation reagent (2 mL, freshly prepared 3 mL of concentrated sulphuric acid made up to 100 mL with spectral grade methanol and 15 mg 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 (Senanayake and Shahidi, 2000a). After incubation and subsequent cooling, distilled water (1 mL) was added and the mixture, after thorough mixing, extracted 3 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 twice with 1.5 mL of distilled water. In the first water wash, the aqueous layer was removed and in the second water wash, the hexane layer was separated and evaporated under a stream of nitrogen. The dried residue was dissolved in 1 mL of CS₂ and GC analysis was performed, as described below.

3.5.2 Analysis of fatty acid methyl esters (FAMES) by gas chromatography (GC)

Fatty acid methyl esters (FAMES) were determined on a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Mississauga, ON) equipped with a flame-ionization detector and a split/splitless injector. A Supelcowax 10 column (30 m x 0.25 mm ID; 0.25 μ m film thickness; Supelco Canada, Oakville, ON) was used for analyzing FAMES. Chromatographic parameters were set as follows: injector temperature, 250°C; detector temperature, 270°C; oven temperature programming, hold for 10.25 min at 220°C then raise to 240°C at 30°C/min followed by a hold period of 9 min. Total run time was 19.92 min. Helium was used as the carrier gas. HP 3365 Series II Chemstation software (Hewlett Packard, Palo Alto, CA) was used for data handling. The FAMES were tentatively identified by comparison of their retention times with those of authentic reference standard mixtures (GLC-416; Nu-Chek) and quantified using area normalization.

3.6 Distribution of structured lipids

Hydrolysis of modified SBO and MO by pancreatic lipase was carried out according to the method described by Christie (1982). Tris-hydrochloric acid buffer (5 mL; 1.0 M, pH 8.0), 0.5 mL of calcium chloride (2.2%, w/v) and 1.25 mL of sodium taurocholate (0.05%, w/v) were added to 25 mg of oil in a glass test tube. The entire mixture was allowed to equilibrate at 40°C in a water bath for 1 min and subsequently 5.0 mg of porcine pancreatic lipase (EC. 3.1.1.3, Sigma) were added to it. Pancreatic lipase was added to catalyze the hydrolysis of emulsified esters of glycerol and long chain fatty acids. Pancreatic lipase preferentially attacks the primary ester groups leaving a 2-

monoacylglycerol product. In this way, *sn*-1 and *sn*-3 esters were separated from the *sn*-2 ester allowing for a positional distribution.

The mixture was then placed in a Gyrotory water bath shaker (Model G76, New Brunswick Scientific Co. Inc., New Brunswick, NJ) 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 the 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 thin layer chromatographic (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 3.5.2.

3.7 Comparison of the oxidative stability of modified and unmodified SBO and MO

The oxidation of oils proceeds via three steps: absorption of oxygen, formation of peroxides and degradation of peroxides to secondary oxidation products. There are many

factors that affect the oxidation of oils by acting as initiators. These factors include oxygen, light, metals and heat. Two initiators, heat and light, were considered in this study in order to accelerate the oxidation process. The first experiment involved heating the oil to 60°C in the dark and testing its stability after varying time periods. The second experiment was initiated by light. The oil was placed under fluorescent light and tested periodically. The analyses performed were designed to investigate both primary and secondary oxidation products. Primary oxidation products were measured spectrophotometrically at 234 nm; the wavelength at which conjugated dienes (CD) are observed. The thiobarbituric acid reactive substances (TBARS) was used for measuring the secondary oxidation products. As oxidation proceeds, the primary oxidation products are broken down into secondary products of oxidation such as aldehydes and ketones. Finally, nuclear magnetic resonance (NMR) spectroscopy is considered a tool that is capable of measuring both the primary and secondary oxidation products. NMR absorption peaks of olefinic, diallylmethylenic and aliphatic protons are estimated by integration. During oxidation the number of olefinic and diallylmethylenic protons decrease, while the number of aliphatic protons increase.

3.7.1 Assessment of the stability of oils by accelerated oxidation and photooxidation methods

Borage oil (BO), gamma-linolenic acid (GLA), seal blubber oil (SBO), menhaden oil (MO), modified seal blubber oil (MSBO) and modified menhaden oil (MMO) were examined for their oxidative stability. The storage conditions for the auto-oxidation

experiment were carried out under Schaal-oven test conditions at 65°C. It is generally accepted that each day (24 h) of storage of oils under Schaal oven test conditions at 65°C is equivalent to one month of storage at ambient conditions (Evans *et al.*, 1973).

The experiments carried out under Schaal oven test conditions were carried out as follows. Each oil (1 g), in triplicate, was placed in open glass test tubes (10 ml) and stored in a forced-air oven (Thelco, Model 2, Precision Scientific Co., Chicago, IL) at 65°C. To estimate oxidative stability (conjugated dienes value; Section 3.7.2, 2-thiobarbituric acid reactive substances value; Section 3.7.3 and nuclear magnetic resonance; Section 3.7.4), samples were removed periodically at 0, 24, 48, 72 and 120 h from the oven, cooled to room temperature, flushed with nitrogen for 30s, covered with aluminum foil-parafilm and stored at -20°C until analysed (usually with 7 days).

For the photooxidation study, each oil (1 g), in triplicate, was placed in open glass test tubes (10 ml) and stored under intense fluorescent light. The samples were placed in a box (70 cm length x 35 cm width x 25 cm height) equipped with two 40 Watts cool white fluorescent lights which were suspended approximately 10 cm above the surface of the oil containers. The remaining open space was covered with aluminum foil. The fluorescent radiation was at a level of 2650 Lux and the temperature inside the containers was maintained at 25°C. Oil samples were removed from the box after 12, 24, 48 and 72 h for stability tests (analysed as mentioned above).

3.7.2 Conjugated dienes

Conjugated dienes (CD) of oil samples were measured by the IUPAC method (1987). Oil samples (0.01 - 0.04 g) were weighed into 25 mL volumetric flasks, dissolved in isooctane (2,2,4-trimethylpentane) and made up to the mark with the same solvent. The solution was thoroughly mixed and the absorbance was read at 234 nm using a Hewlett Packard 8452A diode array spectrophotometer (Hewlett Packard Co., Mississauga, ON). Pure isooctane was used as a reference.

$$CD = \frac{A}{c \times d}$$

where,

CD = concentration of dienes

c = absorptivity (g/100 mL)

d = light path (1 cm)

A = Absorbance of solution

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

The direct TBARS 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 millilitres of this solution were transferred into a dry test tube to which 5 mL of TBA reagent (200 mg 2-thiobarbituric acid in 100 mL 1-butanol and a few crystals of BHA) were 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., Mississauga, ON). A standard curve was prepared using 1,1,3,3-tetramethoxypropane (TMP) as a malonaldehyde (MA) precursor (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 $C = (0.435A_{532})/w$, $r = 0.999$. Where, C is the concentration of MA, A is the absorbance of the coloured complex at 532 nm and w is the mass of the oil.

3.7.4 Nuclear Magnetic Resonance (NMR) Spectrometric Analysis

NMR spectra were recorded using a General Electric GN-300 NMR spectrometer (General Electric Inc., Fremont, CA) at 300 MHz in CDCl_3 . Tetramethylsilane (TMS) was used as an internal standard. Solutions containing approximately 35 mg oil in 0.7 mL of CDCl_3 were used and the spectra recorded. The number of protons of a given kind relative to the number of methylene protons in the methylene moieties of glycerol residues was calculated from the ratio of the respective peak areas (Wanasundara and Shahidi, 1993).

3.8 Statistical Analysis

All experiments and/or measurements were replicated three times. Mean \pm standard deviations (SD) were reported for each case. SigmaStat (Jandel Scientific, USA) was used to transform the data, then analysis of variance (ANOVA) and Tukeys' studentized test were performed at a level of $p < 0.05$ to evaluate the significant differences among mean values. Simple linear and multiple regression analyses were also performed

using the same software for the response surface regression (RSREG) procedures.

Section 3.3.1 explains data analysis carried out for the optimization study.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Preparation of GLA concentrate from borage oil (BO) by urea complexation

The simplest and most efficient technique for obtaining GLA in the free fatty acid form is urea complexation. Urea forms crystalline inclusion compounds with fatty acids. Urea usually crystallises in a tetragonal structure; however, an X-ray study by Traitler *et al.* (1988) showed that urea inclusion compounds consist of hexagonal crystal forming canals which are capable of including long chain molecules. The formation of these inclusion compounds depends on the degree of unsaturation of the fatty acids; the more unsaturated, the less will be the likelihood of their inclusion into the urea crystals.

Initially, the TAG of the oil are split into their constituent fatty acids by alcoholic KOH; these fatty acids are then mixed with an ethanolic solution of urea for complex formation. The saturated and monounsaturated fatty acids easily complex with urea and crystallise out on cooling, which may subsequently be removed by filtration. The liquid or non-urea complexing fraction (NUCF) is enriched in GLA. Factors such as crystallisation time (h, X_1), crystallisation temperature ($^{\circ}\text{C}$, X_2) and urea-to fatty acid ratio (w/w, X_3) (Table 4.1) were studied collectively in order to optimise the conditions to obtain a maximum concentration of GLA. The initial conditions chosen for the optimization reaction were based on a literature review. Experimental values obtained for the percentage recovery and GLA content in the NUCF and urea complexing fraction (UCF) for the 17 design points are given in Table 4.2.

Table 4.1 Independent variables and experimental design levels used for face-centred cube design

Independent Variables	Symbols	Coded Variable Levels		
		-1	0	1
Reaction time (h)	X_1	12	18	24
Reaction temperature ($^{\circ}\text{C}$)	X_2	-12	-3	+6
Urea-to-fatty acid ratio (w/v)	X_3	2	3	4

Table 4.2 Face-centred cube design arrangement and response^a

Run Number	Independent Variables ^b			Recovery ^c (%)	Response ^d	
	X ₁	X ₂	X ₃		NUCF ^e	UCF ^f
1	-1	-1	-1	82.3 ± 3.8	50.1 ± 4.3	5.73 ± 1.2
2	-1	-1	+1	63.4 ± 1.6	88.0 ± 3.0	2.57 ± 0.4
3	-1	+1	-1	79.3 ± 0.1	46.7 ± 0.9	1.99 ± 1.0
4	-1	+1	+1	74.7 ± 1.3	80.6 ± 1.9	3.18 ± 0.7
5	+1	-1	-1	81.3 ± 7.9	48.8 ± 6.4	1.58 ± 0.1
6	+1	-1	+1	61.2 ± 0.1	80.1 ± 5.0	7.61 ± 0.5
7	+1	+1	-1	81.0 ± 1.3	48.4 ± 1.2	2.01 ± 1.1
8	+1	+1	+1	73.3 ± 0.4	81.9 ± 0.1	4.25 ± 0.8
9	-1	0	0	75.2 ± 1.1	75.8 ± 0.4	3.75 ± 0.6
10	+1	0	0	69.5 ± 0.7	81.1 ± 6.4	4.27 ± 0.2
11	0	0	0	61.8 ± 0.1	83.6 ± 0.2	6.89 ± 1.6
12	0	0	0	74.2 ± 0.9	73.2 ± 2.7	2.86 ± 0.4
13	0	+1	+1	54.3 ± 1.9	86.8 ± 1.1	7.76 ± 2.3
14	0	-1	-1	81.4 ± 0.4	50.0 ± 1.5	2.36 ± 0.7
15	0	0	0	67.8 ± 6.3	85.6 ± 2.9	3.97 ± 0.4
16	0	0	0	65.7 ± 2.9	84.7 ± 1.9	3.51 ± 0.2
17	0	0	0	69.1 ± 0.6	87.9 ± 3.8	3.36 ± 0.1

^a Nonrandomized^b Coded symbols and levels of independent variables refer to Table 1^c % Recovery = % GLA in the experimental run/ % GLA in the original oil x 100^d Average ± standard deviations of duplicate determinations from different experiments^e NUCF, non-urea complexing fraction ^f UCF, urea complexing fraction

GLA was found almost exclusively in the NUCF in some of the treatment conditions, however, a small amount of GLA invariably complexed with urea and was detected in the UCF. Traitler *et al.* (1988) has reported similar results for the urea complexation of blackcurrant seed oil, while Huang *et al.* (1999) have reported similar results for borage seed oil. The highest GLA content (88%) was obtained with a urea-to-fatty acid ratio of 4, a crystallisation time of 12 h and a crystallisation temperature of -12°C. The content of GLA in the original borage oil was 21.6%, but by urea complexation a 4-fold increase in the GLA content was achieved. Urea complexation of BO resulted in an increase in the total PUFA up to 99.1% (Table 4.3). Thus, the saturated, monounsaturated and most of the diunsaturated fatty acids were separated by urea complexation. Enrichment of GLA in the concentrate varied inversely with decreasing urea-to-fatty acid ratio and crystallisation temperature. Therefore, these experimental variables should be carefully controlled in order to achieve an optimum level of GLA in the concentrate with a reasonable recovery.

4.1.1 Optimisation of process conditions to maximise the content of GLA in borage oil concentrates

A face-centred cube design was employed (Table 4.2); the actual levels of the variables used in each experimental run are shown in Table 4.1. The independent variable levels are coded for an experimental design (Table 4.1). The centre point for each individual variable is given a code of zero. The highest and lowest levels of interest for

Table 4.3 Fatty acid composition (%) of borage oil and its gamma-linolenic acid concentrate (GLA)^c.

Fatty acid	Borage oil ^a	GLA Concentrate ^a
16:0	10.3 ± 0.13	ND ^b
18:0	4.49 ± 0.04	ND ^b
18:1ω9	16.6 ± 0.41	ND ^b
18:2ω6	37.1 ± 0.96	7.60 ± 0.04
18:3ω6	21.6 ± 0.10	91.5 ± 3.14
20:1ω9	4.68 ± 0.09	ND ^b
22:1ω9	3.14 ± 0.03	ND ^b
24:1ω9	2.15 ± 0.03	ND ^b

^a Means ± SD (n=3)

^b Not Detected

^c The concentrate was prepared under optimum conditions (3.7 urea-to-fatty acid ratio, - 7°C, 16 h)

Table 4.4 Regression coefficients of the predicted quadratic polynomial model

Coefficient ^a	% GLA ^{cd}
β_0	-116.3664***
Linear	
β_1	3.1109
β_2	-0.8607
β_3	95.9412***
Quadratic	
β_{11}	-0.0724
β_{22}	-0.0329
β_{33}	-12.6817***
Interactions	
β_{12}	0.0287
β_{13}	-0.1442
β_{23}	-0.0244
β_{123}	
R^{2b}	0.9728

^a Coefficients refer to the general model.^b R^2 = coefficient of determination^{c***} Significant at the 1% level.^d GLA = gamma-linolenic acid

each independent variable are coded +1 and -1, respectively. The main advantage of this design is that it enables one to study one or more variables simultaneously in a single experimental design (Montgomery 1991).

4.1.2 Diagnostic checking of fitted model

Table 4.2 shows experimental data obtained for the response (% GLA concentrate) for borage oil. Multiple regression coefficients obtained by employing a least-squares technique to predict a quadratic polynomial model for the concentration of GLA in borage oil are summarised in Table 4.4. Examination of these coefficients with the t-test indicated that the linear and quadratic terms of urea-to-fatty acid ratio were highly significant ($P < 0.01$), but the reaction time and reaction temperatures were not significant ($P > 0.01$) for the concentration of GLA. Therefore, the results suggest that linear and/or quadratic effect of urea-to-fatty acid ratio is the primary determining factor for the concentration of GLA. No statistically significant ($P > 0.05$) interaction existed between any two of the three factors. The coefficient of determination (R^2) for the concentration of GLA was 0.97. This indicated that most of the variations observed for the design points could be explained by the fitted model. The coefficient of independent variables (reaction time, X_1 ; reaction temperature, X_2 ; and urea-to-fatty acid ratio, X_3) determined for the quadratic polynomial model (Table 4.4) were:

$$Y = -116.366 + 3.111X_1 - 0.861X_2 + 95.941X_3 - 0.0724X_1^2 - 0.0330X_2^2 - 12.682X_3^2 + 0.0287X_1X_2 - 0.144X_1X_3 - 0.0244X_2X_3$$

The model predicted for Y was adequate as indicated by error analysis that showed the lack of fit as insignificant ($P>0.05$), therefore, the fitted model was appropriate for the description of response surface.

4.1.3 Response surface plotting and optimisation based on canonical analysis

Variables giving the largest absolute coefficients in the fitted model (Table 4.4) were chosen as the axes (urea-to-fatty acid ratio and reaction temperature) for the response plots. Figure 4.1 shows the response surface of the independent and dependent variables. A contour plot (Fig. 4.2) was generated on the predicted quadratic polynomial model to examine the overall shape of the response surface curves, and it was used to characterise the nature of the stationary points. Canonical analysis is a mathematical approach used to locate the stationary point of the response surface and to determine whether it represents a maximum, minimum or saddle point (Mason *et al.*, 1989). Results of the canonical analysis of the response surface are shown in Table 4.5. The stationary point for the concentration of GLA by urea complexation predicted a maximum of 89.5% at a urea-to-fatty acid ratio of 3.7, crystallisation time of 16 h, and crystallisation temperature of -7°C . The contour plot derived from the results of the analysis showed ellipsoidal contours at a maximum point (Fig. 4.2). The adequacy of the model predicted was examined by performing independent experiments under optimal conditions, which revealed that the predicted values from this model were reasonably close to the observed values (Table 4.5). Therefore, under optimum conditions, the concentration of GLA can be increased

Table 4.5 Predicted observed values for response variable (concentration of GLA) in urea complexation experiment of borage oil

Response Variable	<u>Critical values of independent variables</u>			Stationary Point	Predicted Value	Observed Value ^c
	Time ^a (h)	Temperature ^b (°C)	Urea/fatty acid ratio (w/w)			
GLA (%)	16	-7	3.7	Maximum	89.5	91.5 ± 3.14

^a Crystallisation time

^b Crystallisation temperature

^c Mean ± SD (n=3)

Figure 4.1 Response surface for the effect of urea-to-fatty acid ratio and crystallisation temperature on the concentration of γ -linolenic acid (GLA) from borage oil by urea complexation.

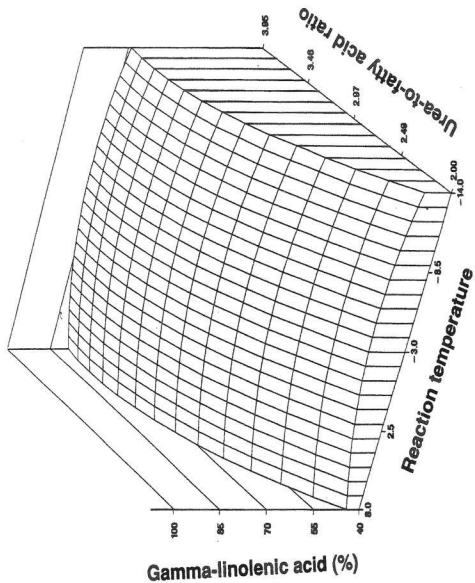
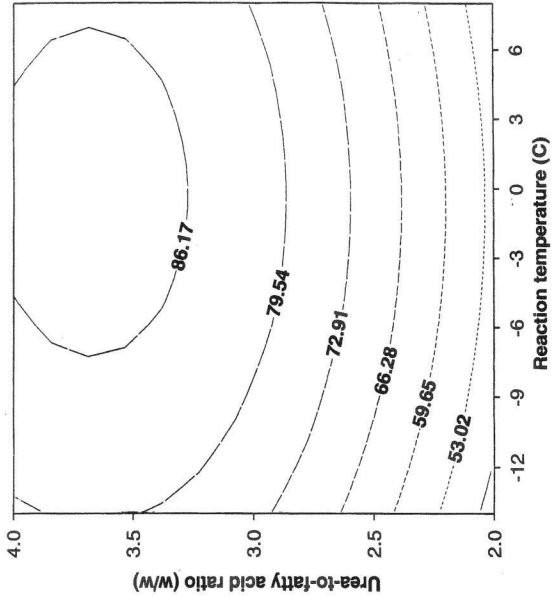


Figure 4.2 Contour plot for the effect of urea-to-fatty acid ratio and crystallisation temperature on the concentration of γ -linolenic acid (GLA) from borage oil by urea complexation.



from 21.6% to 91.5% with a 67% recovery of the weight of the GLA in the original borage oil.

4.2 Preparation of structured lipids by enzyme-assisted acidolysis of menhaden (MO) and seal blubber oil (SBO) with gamma-linolenic acid (GLA)

From a marketing point of view, mono-, di-, and triacylglycerol are often promoted as being more "natural" than other fatty acid derivatives such as free fatty acids and their methyl and ethyl esters (Haraldsson and Hoskuldsson, 1989). Therefore, in this study, a TAG containing GLA and ω 3 PUFA was produced via enzymatic acidolysis.

Until recently, ω 3 PUFA rich and GLA rich oils have been used individually or as physical mixtures in feeding trials (Chapkin *et al.*, 1988; Miller *et al.*, 1990; Fan and Chapkin, 1992). Incorporation of GLA from borage oil into marine oils, by enzyme catalysed reactions, would provide a unique speciality oil which might have an advantage over that of physical mixtures.

4.2.1 Determination of reaction conditions for acidolysis

Some preliminary experiments were performed in order to determine the optimum amount of water, substrate and type of enzyme in the acidolysis reaction. In an enzymatic process, the content of water in the mixture affects the rate of the reaction. There are two reasons for this: first, a trace amount of water is necessary for the functioning of the enzyme and maintenance of its three-dimensional structure (Christie, 1982); second, water thermodynamically favours hydrolysis of the TAG instead of acidolysis. Therefore,

an experiment was performed in order to determine the optimum water content (Table 4.6). It was found that 1% water (based on the weight of substrate and enzyme) provided the ideal environment for the acidolysis reaction. Higher percentages of water brought about a decrease in the incorporation of GLA. This result was in agreement with that of Cerdan *et al.* (1998) who also reported that 1% water was best for production of TAG.

The substrate concentration was also investigated because large volumes of solvent decrease the concentration of reactants. It was found that 2 mL of solvent provided the maximum acidolysis activity (Table 4.7). With a volume of 2 mL hexane in the reaction mixture, GLA incorporation of 23.8 and 26.5% was reached for SBO and MO, respectively. Increasing the solvent volume decreased the GLA incorporation into oils. A possible explanation for the decrease of GLA incorporation with an increase in solvent may be due to a reduction in the relative amount of water present that might affect the most effective three-dimensional configuration of the enzyme and hence its activity.

As a result of an extensive literature review, the lipases from *Mucor miehei* (IM, *sn*-1,3 specific) and *Pseudomonas species* (PS-30, non-specific) were chosen for interesterification of GLA with SBO and MO. Both a nonspecific and *sn*-1,3 specific enzymes were chosen in order to incorporate the GLA fatty acid onto the *sn*-2 position of the TAG molecule. IM and PS-30 have an activity of 13,613 and 11,936 units, respectively. One unit of lipase activity was defined as the nanomoles of fatty acid (oleic acid equivalents) produced per minute per gram of enzyme.

Table 4.6 Effect of water content on acidolysis of seal blubber oil or menhaden oil with gamma-linolenic acid (GLA) using the non-specific lipase, *Pseudomonas sp.* (PS-30).

Water (% weight) (weight of substrate and enzyme)	Degree of GLA incorporation	
	Seal blubber oil	Menhaden oil
0.5	16.5 ± 0.4	21.7 ± 0.4
1	22.0 ± 0.5	24.5 ± 0.5
2	21.6 ± 0.3	18.0 ± 0.2
3	17.9 ± 0.1	16.8 ± 0.3

The reaction conditions were a 3:1 mole ratio (GLA:TAG) of substrates over a period of 24 h at 37°C using an enzyme concentration of 300 units/g of oil.

Table 4.7 Effect of solvent volume on the degree of gamma-linolenic acid (GLA) incorporation upon acidolysis of seal blubber oil or menhaden oil using a non-specific lipase *Pseudomonas sp.* (PS-30)^a

Solvent volume (mL)	Seal blubber oil	Menhaden oil
1	23.5 ± 0.6	26.1 ± 0.3
2	23.8 ± 0.6	26.5 ± 0.8
3	22.1 ± 0.4	20.4 ± 0.2
4	19.9 ± 0.3	17.0 ± 0.5
5	18.9 ± 0.4	16.9 ± 0.3

^aThe reaction was performed with a 3:1 mole ratio (GLA:TAG) of substrates (300 mg oil: 288-298 mg GLA) over a period of 24 h at 37°C with lipase PS-30 (300 units/g of oil)

4.2.2 Effect of individual variables on the degree of incorporation of gamma-linolenic acid (GLA) into seal blubber oil (SBO) and menhaden oil (MO)

Many factors may affect the product yield (% GLA incorporation) of lipase-catalysed interesterification of acylglycerols. These include the temperature of the reaction medium, reaction time, enzyme concentration and the mole ratio of TAG from SBO or MO, to GLA. Therefore, it is necessary to study the influence of each variable independently with both enzymes in order to determine the optimum conditions required for maximum incorporation of GLA into SBO and MO.

4.2.2.1 Effect of enzyme concentration on the incorporation of gamma-linolenic acid (GLA)

Figures 4.3 and 4.4 illustrate the effect of enzyme concentration on the incorporation of GLA into SBO and MO by lipase PS-30 and Lipozyme-IM, respectively. The degree of incorporation increased with increasing enzyme load until it reached 500 units/g of oil. Higher concentrations of enzyme did not increase the degree of incorporation significantly ($p > 0.05$) so there was no advantage to using more enzyme. Therefore, it was concluded that 500 units of enzyme/g of oil was suitable to catalyse the synthesis of structured lipids. In this study, the water content in the reaction mixture was based on the weight of the reactants and was maintained at 1%. A possible explanation for the constant level of reaction rate with an increase in enzyme load may be due to the insufficient amount of water in the reaction mixture, which leads to a decrease in enzyme function because the enzyme may not be able to assume its most effective three-dimensional configuration.

Figure 4.3. The effect of varying enzyme concentration on the incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase PS-30 from *Pseudomonas species*. The reaction mixture contained 300 mg oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 100-700 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C for 24 h. Some standard deviations are within the domain of the symbols

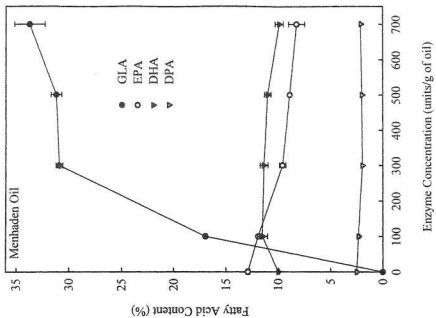
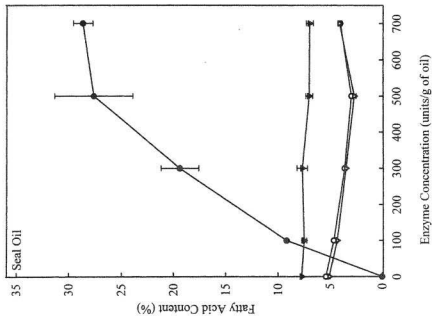
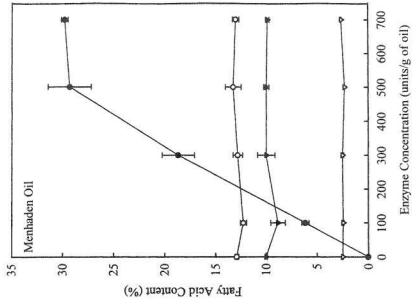
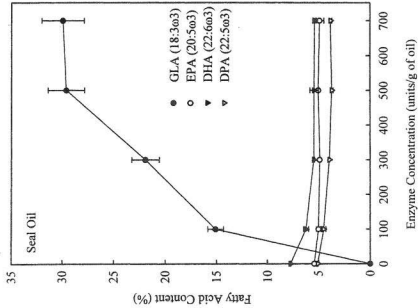


Figure 4.4 The effect of varying enzyme concentration on the incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase Lipozyme-IM from *Mucor miehei*. The reaction mixture contained 300 mg oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 100-700 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C for 24 h. Some standard deviations are within the domain of the symbols.



4.2.2.2 Effect of the mole ratio of gamma-linolenic acid (GLA) to triacylglycerol (TAG) from seal blubber oil (SBO) and menhaden oil (MO) on the incorporation of GLA

Figures 4.5 and 4.6 show the percent incorporation of GLA at various GLA to TAG mole ratios using Lipozyme-IM and PS-30, respectively. The amount of enzyme was kept constant at 500 units/g of oil. For all samples, as the number of moles of GLA was increased, its percent incorporation increased; a higher incorporation was achieved for both oils with PS-30 enzyme (approximately 34%) than Lipozyme-IM (approximately 29%). The steep part of the curve occurred from 1:1 to 3:1 mole ratio of GLA to TAG; from 3:1 to 4:1 mole ratio the incorporation was not statistically significant ($P > 0.05$) for all samples. For the MO samples with both enzymes, the mole ratios 3:1 and 5:1 were statistically different ($P < 0.05$) from one another. However, for economic reasons a mole ratio of 3:1 was chosen as optimum. This result agrees with that of Senanayake and Shahidi (2000) who also found that the stoichiometric ratio of fatty acid to TAG of 3:1 was optimal for production of TAG as there are three acyl groups per molecule of TAG. At higher mole ratios, incorporation of GLA into TAG may be slow because the interchange reaction between the TAG and GLA may compete with the product and slow the reaction (Huang *et al.*, 1994). Mutua and Akoh (1993) reported a decreased EPA incorporation into biosurfactants by IM-20 when the mole ratio of EPA to phospholipid exceeded 2:1. However, no decrease in GLA incorporation was observed at high mole ratios employed in this study. Osterberg *et al.* (1989) postulated that TAG molecules possessing two or more GLA acyl groups are sterically hindered from entering the active

Figure 4.5 The effect of varying the mole ratio (GLA:TAG) on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase PS-30 from *Pseudomonas species*. Mole ratios of GLA to TAG were varied from 1:1 to 5:1. The reaction mixtures contained enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C for 24 h. Some standard deviations are within the domain of the symbols.

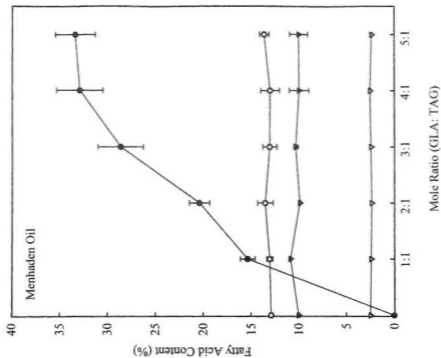
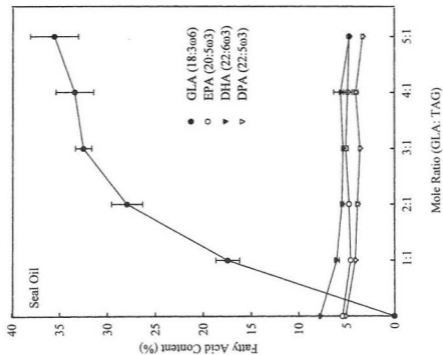
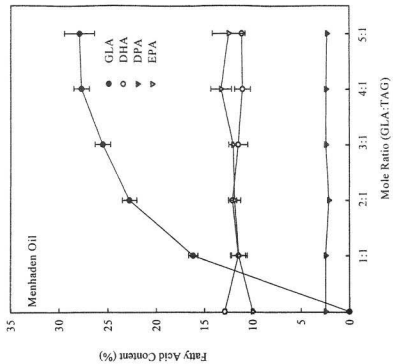
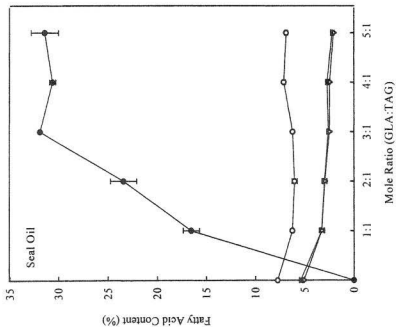


Figure 4.6 The effect of varying the mole ratio (GLA:TAG) on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase Lipozyme-IM from *Mucor miehei*. Mole ratios of GLA to TAG were varied from 1:1 to 5:1. The reaction mixtures contained enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C for 24 h. Some standard deviations are within the domain of the symbols

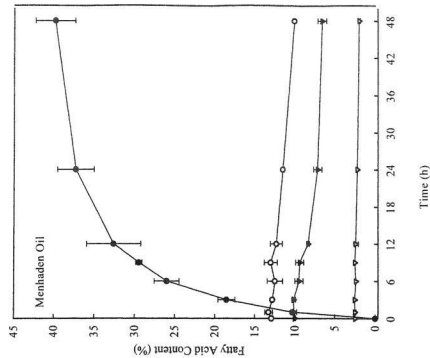
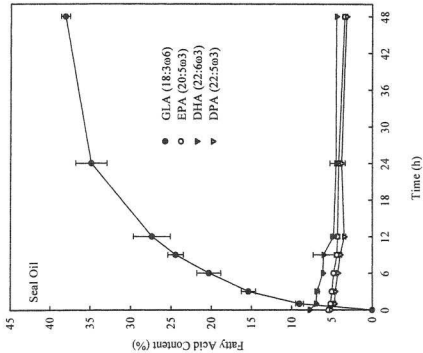


site of lipases to act as an acyl acceptor because of the “stiffness” of the molecules caused by the double bonds at the Δ -6 position. This may be a possible reason for limitation of GLA incorporation into the oils at higher mole ratios.

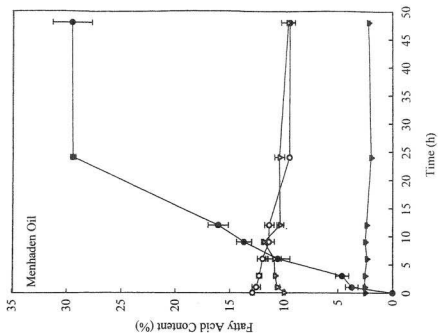
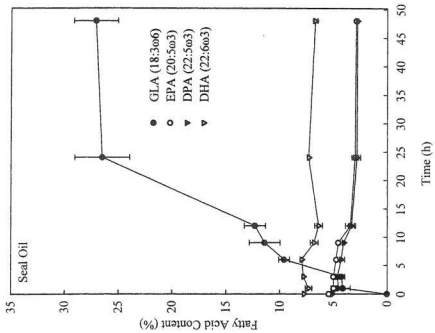
4.2.2.3 Effect of reaction time on the incorporation of gamma-linolenic acid (GLA) into seal blubber oil (SBO) and menhaden oil (MO)

The time course is useful in monitoring the progress of enzymatic reactions. It helps determine the shortest time necessary to obtain good yields and minimise production costs. The time course also allows following product formation and reactant disappearance with time and gives an indication of when to stop the reaction, depending on the desired product. Figures 4.7 and 4.8 show the time course of lipase-catalysed acidolysis of GLA into SBO and MO by lipase PS-30 and Lipozyme-IM, respectively. Incorporation of GLA into SBO and MO increased to 27 and 32%, respectively, for the PS-30 lipase after 12 h, and essentially reached equilibrium at 24 h (35% GLA in SBO and 37% in MO), after which further incorporation was minimal. The increase in the incorporation of GLA by Lipozyme-IM was more gradual than that with PS-30 for both SBO and MO. At 24 h, the percentage of GLA incorporated into SBO and MO levelled off at 26 and 29%, respectively, for the lipase Lipozyme-IM, after which further incorporation was insignificant ($P > 0.05$). This result is in agreement with that of Akoh *et al.* (1996) and Moussata and Akoh (1997) who both reported that 24 h was the optimum reaction time for incorporation of EPA into evening primrose oil and oleic acid into melon seed oil, respectively.

Figure 4.7 The effect of varying the reaction time on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase PS-30 from *Pseudomonas species*. The reaction mixture contained 300 mg oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C. Some standard deviations are within the domain of the symbols.



- Figure 4.8 The effect of varying the reaction time on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase Lipozyme-IM from *Mucor miehei*. The reaction mixture contained 300 mg oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 37°C. Some standard deviations are within the domain of the symbol.



4.2.2.4 Effect of reaction temperature on the incorporation of gamma-linolenic acid (GLA) into seal blubber oil (SBO) and menhaden oil (MO)

The optimum performance temperature of enzymes depends on their source, the nature of the immobilisation or chemical modification, and the pH of the reaction mixture (Dordick, 1989). The temperature range tested was 30 to 60°C. Figures 4.9 and 4.10 illustrate the effect of temperature on incorporation of GLA into MO and SBO by the lipase PS-30 and Lipozyme-IM, respectively. The results show that the maximum incorporation of GLA was achieved at 60°C, similar to that reported by Rahmatullah *et al.* (1994). However, a temperature increase from 40 to 60°C produced a minimal increase in the incorporation of GLA. Therefore, 40°C was chosen as the best temperature because there was no significant difference between 40 to 50°C for any of the reactions. The 60°C temperature was rejected as optimum because at this temperature thermal degradation of PUFA may occur (Akoh and Vista, 1995). Since the acidolysis reaction was carried out in sealed test tubes, the increase in temperature could not have evaporated the water from the reaction mixture thus enhancing the extent of hydrolysis as a side reaction. The increase in temperature did not have a profound effect on GLA incorporation into the oils.

4.2.3 Conditions chosen as optimum for production of structured lipids

From the foregoing results, it is evident that incorporation of GLA into marine oils through lipase catalysed acidolysis is possible. Lipase PS-30 showed a better

Figure 4.9 The effect of varying the temperature on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase PS-30 from *Pseudomonas species*. The reaction mixture contained 300 mg oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 30-60°C for 24 h. Some standard deviations are within the domain of the symbols.

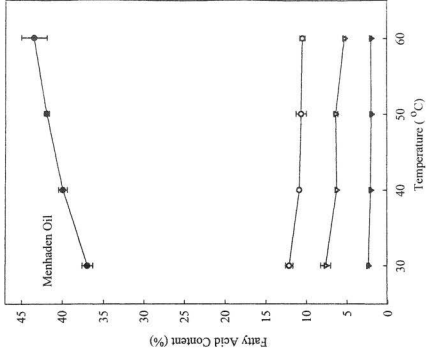
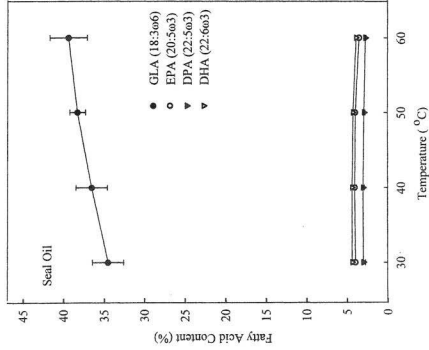
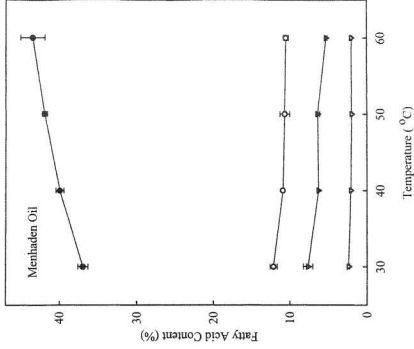
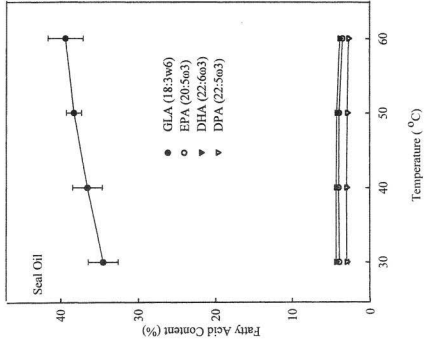


Figure 4.10 The effect of varying the temperature on the percent incorporation of gamma-linolenic acid (GLA) into SBO and MO with lipase Lipozyme-IM from *Mucor miehei*. The reaction mixture contained 300 mg oil, 288-298 mg GLA (3:1 mole ratio of GLA:TAG), enzyme concentration of 500 units/g, 1% water and 2 ml hexane. Reactions were carried out at 30-60°C for 24 h. Some standard deviations are within the domain of the symbols.



acidolysis activity than *Mucor miehei*. The optimum reaction conditions were: enzyme concentration of 500 units/g of oil, a 3:1 mole ratio of GLA to TAG for both SBO and MO, a reaction time of 24 h and a reaction temperature of 40°C. Table 4.8 shows the fatty acid profile of unmodified and lipase-catalysed modified SBO. The fatty acid profile of the modified SBO produced under optimum reaction conditions indicated that GLA, EPA, DPA, and DHA were present at 37.1, 3.80, 2.99 and 4.36%, respectively. The content of the ω 3 PUFA was decreased from 18.2 to 11.2% after the interesterification reaction. The ratio of ω 3 to ω 6 PUFA was 1:3.6. Similarly, the content of GLA, EPA, DPA and DHA in the modified MO was 39.6, 11.0, 2.07 and 6.56%, respectively, and the ω 3 PUFA decreased by 1.3% after the interesterification reaction (Table 4.8). The corresponding mole ratio of ω 3 to ω 6 PUFA was 1:2.2. After modification by *Pseudomonas sp.* lipase, the contents of EPA and DHA in both SBO and MO decreased by 1.6 and 3.4%, respectively. This indicates that DHA was more readily replaced in these oils than EPA.

Since there has been no research conducted on the incorporation of GLA into marine oils, a direct comparison of the results is not possible. Nonetheless, considerable research has been conducted on the addition of ω 3 PUFA into borage, primrose and vegetable oils. Senanayake and Shahidi (1999) modified the fatty acid composition of borage and evening primrose oils by incorporating EPA and DHA, using a non-specific PS-30 lipase from *Pseudomonas sp.* They reported that incorporation of EPA+DHA into borage and evening primrose oils was 35.5 and 33.6%, respectively. At a substrate mole

Table 4.8 Fatty Acid Composition (w/w %) of Refined-Bleached Deodorised SBO and MO Triacylglycerols (TAG) Before and After Enzymatic Interesterification with γ -Linolenic Acid (GLA)^a

Fatty acid (w/w%)	SBO-TAG before modification ^b	SBO-TAG after modification ^b	MO-TAG before modification ^b	MO-TAG after modification ^b
12:0	ND ^c	0.05 ± 0.01	ND ^c	0.06 ± 0.00
14:0	3.36 ± 0.08	2.40 ± 0.04	8.18 ± 0.08	4.55 ± 0.08
14:1 ω 5	1.09 ± 0.04	0.72 ± 0.01	0.36 ± 0.01	0.20 ± 0.00
15:0	0.23 ± 0.00	0.13 ± 0.00	0.70 ± 0.02	0.35 ± 0.01
16:0	5.14 ± 0.03	3.04 ± 0.03	19.8 ± 0.24	8.78 ± 0.06
16:1 ω 7	14.5 ± 0.04	9.67 ± 0.00	11.5 ± 0.13	5.84 ± 0.01
17:0	0.92 ± 0.01	0.56 ± 0.01	1.68 ± 0.02	0.23 ± 0.01
17:1	0.55 ± 0.02	0.25 ± 0.00	1.43 ± 0.03	0.86 ± 0.02
18:0	1.02 ± 0.01	0.56 ± 0.00	3.83 ± 0.02	1.50 ± 0.01
18:1 ω 9	22.6 ± 0.06	14.1 ± 0.01	9.86 ± 0.11	4.24 ± 0.10
18:1 ω 11	4.88 ± 0.02	2.69 ± 0.01	3.71 ± 0.05	1.52 ± 0.01
18:2 ω 6	1.51 ± 0.02	3.80 ± 0.01	1.76 ± 0.09	3.81 ± 0.02
18:3 ω 6	0.59 ± 0.00	37.1 ± 0.16	0.43 ± 0.00	39.6 ± 0.07
18:3 ω 3	ND ^c	0.33 ± 0.00	1.31 ± 0.02	0.66 ± 0.03
20:1 ω 9	17.3 ± 0.02	8.30 ± 0.06	1.62 ± 0.06	0.83 ± 0.10
20:4 ω 6	0.46 ± 0.01	0.32 ± 0.01	0.86 ± 0.02	0.70 ± 0.01
20:5 ω 3	5.40 ± 0.08	3.80 ± 0.01	12.9 ± 0.18	11.0 ± 0.10
22:1 ω 1	2.01 ± 0.04	0.56 ± 0.00	0.12 ± 0.04	0.30 ± 0.01
22:5 ω 3	5.07 ± 0.01	2.99 ± 0.01	2.48 ± 0.03	2.07 ± 0.01
22:6 ω 3	7.73 ± 0.02	4.36 ± 0.06	10.0 ± 0.11	6.56 ± 0.04
Total ω 6	2.56	41.2	3.05	44.1
Total ω 3	18.2	11.5	26.7	20.3
Total PUFA	20.8	52.7	29.7	64.3
Ratio ω 3/ ω 6	7.1 : 1	1 : 3.6	8.7 : 1	1 : 2.2

^a Restructured SBO and MO were prepared under optimum conditions (enzyme concentration of 500 units/g of oil, 24 h, 40°C, 3:1 mole ratio, 2 mL hexane, 1% water) ^b Mean ± SD ^c ND = not detected

ratio of 1:0.5:0.5 (TAG:EPA:DHA) the corresponding $\omega 3/\omega 6$ PUFA ratio was 0.9:1 for borage oil and 0.63:1 for evening primrose oil. Akoh and Sista (1995) and Akoh *et al.* (1996) incorporated EPA into borage and evening primrose oil, respectively, by non-specific immobilised SP435 lipase from *Candida antarctica* as the biocatalyst. The maximum percentage of EPA incorporation was 21.1% with a $\omega 3/\omega 6$ PUFA ratio of 1:2.4 for borage oil and 34.0% with a $\omega 3/\omega 6$ PUFA ratio of 1:1.6 for evening primrose oil.

4.3 Determination of the positional distribution of fatty acids in triacylglycerols of the enzymatically modified seal blubber oil (MSBO) and menhaden oil (MMO)

Fatty acid distributions of the TAG of modified seal blubber oil (MSBO) and modified menhaden oil (MMO) are given in Table 4.9. Results indicate that lipase PS-30 incorporated 25% of GLA, at the *sn*-2 position of TAG of MSBO and MMO. Thus, the middle position of the TAG has been involved in the acidolysis process when using the non-specific PS-30 (*Pseudomonas sp.*) lipase. In MSBO, the *sn*-2 position had EPA and DHA contents of 3.0 and 2.0%, respectively. In MMO, the *sn*-2 position contained 14.3% EPA and 6.1% DHA. Lipase PS-30 was also able to incorporate large quantities of GLA (34% in MSBO and 36% in MMO) at the *sn*-1,3 positions of TAG. The primary positions (*sn*-1,3) of TAG of MSBO had 8.2% EPA and 3.8% DHA. On the other hand, *sn*-1,3 positions of MMO contained 13.8 and 7.3% EPA and DHA, respectively. Thus, structured lipids containing GLA, EPA and DHA in the same TAG molecules were successfully produced and may have potential health benefits. The fact that GLA was

incorporated into all positions on the TAG of SBO and MO is in agreement with a study performed by Chandler (2001) confirming that the *Pseudomonas* genus exhibited activity toward all positions of the TAG molecules.

Brockerhoff *et al.* (1966) studied the fatty acid distribution of fatty fish and marine mammals and found that long chain PUFA tended to be in the *sn*-2 position of fish TAG and in the *sn*-1 and *sn*-3 positions of TAG of marine mammals. Ando *et al.* (1996) determined the positional distribution of fatty acids in TAG of fish oils (bonito, tuna and sardine) and seal oil. They found that fish oils rich in DHA did not follow the general tendency of having the PUFA in the *sn*-2 position, but predominated in the *sn*-3 position of the TAG for both Bonito and tuna oils. Myher *et al.* (1996) found contradictory evidence because they found that DHA was mainly located in the *sn*-2 position of the TAG of tuna oil. Aursand *et al.* (1995) studied the positional distribution of Atlantic salmon, cod liver oil and seal oil by nuclear magnetic resonance (NMR). They found that DHA was preferentially esterified at the *sn*-2 position of the TAG of salmon and cod liver oil, whereas EPA was randomly distributed throughout positions of the TAG of SBO and MO. In SBO, EPA, DPA and DHA were located mainly in the *sn*-1 and *sn*-3 positions, whereas saturated fatty acids usually occupied the *sn*-2 position of the TAG molecules. In MO, DPA and DHA were located mainly in the *sn*-2 position of the TAG, however, EPA was equally distributed amongst the *sn*-2 and *sn*-3 positions and was present only in small amounts in the *sn*-1 position. In addition, Ota *et al.* (1994) found that in flounder liver and flesh lipids there was no preference for the *sn*-2 position, which

Table 4.9 Positional Distribution of Structured Lipids Produced using Lipase PS-30^a

Fatty acid (w/w%)	MSBO-TAG		MMO-TAG	
	<i>sn</i> -1,3 positions	<i>sn</i> -2 position	<i>sn</i> -1,3 positions	<i>sn</i> -2 position
8:0	0.1 ± 0.03	0.2 ± 0.01	0.1 ± 0.01	0.1 ± 0.03
10:0	0.1 ± 0.01	0.2 ± 0.03	0.1 ± 0.01	0.1 ± 0.01
12:0	0.2 ± 0.01	0.1 ± 0.01	0.1 ± 0.04	0.1 ± 0.04
14:0	2.1 ± 0.15	3.0 ± 0.15	2.8 ± 0.31	4.9 ± 0.22
14:1ω5	0.9 ± 0.20	0.7 ± 0.09	0.2 ± 0.05	0.1 ± 0.04
15:0	0.1 ± 0.04	0.1 ± 0.02	0.3 ± 0.02	0.4 ± 0.01
16:0	2.4 ± 0.03	4.6 ± 0.36	5.4 ± 0.29	9.4 ± 0.27
16:1ω7	6.9 ± 0.27	9.5 ± 0.48	3.5 ± 0.61	5.1 ± 0.58
17:0	0.6 ± 0.12	0.7 ± 0.08	0.8 ± 0.11	0.2 ± 0.01
17:1	5.4 ± 0.48	3.8 ± 0.15	0.2 ± 0.03	4.8 ± 0.31
18:0	0.6 ± 0.07	1.0 ± 0.10	0.8 ± 0.04	1.2 ± 0.08
18:1ω9	8.2 ± 0.31	18.8 ± 1.10	2.2 ± 0.13	3.8 ± 0.52
18:1ω11	1.5 ± 0.19	2.4 ± 0.05	0.9 ± 0.02	1.1 ± 0.33
18:2ω6	6.6 ± 0.52	2.9 ± 0.18	2.0 ± 0.04	3.6 ± 0.09
18:3ω6	33.7 ± 1.02	25.1 ± 1.70	35.8 ± 0.83	24.7 ± 0.91
18:3ω3	0.4 ± 0.05	0.4 ± 0.09	0.5 ± 0.10	0.5 ± 0.07
20:0	0.1 ± 0.02	0.2 ± 0.03	0.1 ± 0.03	0.1 ± 0.06
20:1ω9	2.8 ± 0.11	4.5 ± 0.29	0.1 ± 0.01	0.8 ± 0.04
20:2ω6	0.2 ± 0.04	0.2 ± 0.08	0.2 ± 0.03	0.2 ± 0.06
20:3ω6	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.04	0.1 ± 0.03
20:4ω6	0.3 ± 0.01	0.3 ± 0.07	1.1 ± 0.06	1.0 ± 0.05
20:5ω3	8.2 ± 0.34	3.0 ± 0.11	13.8 ± 0.84	14.3 ± 0.61
22:0	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.02
22:1ω11	0.4 ± 0.05	0.4 ± 0.05	0.4 ± 0.05	0.6 ± 0.08
22:4ω6	0.1 ± 0.01	0.1 ± 0.02	0.2 ± 0.03	0.2 ± 0.01
22:5ω3	2.3 ± 0.09	1.3 ± 0.10	2.6 ± 0.18	2.6 ± 0.29
22:6ω3	3.8 ± 0.06	2.0 ± 0.06	7.3 ± 0.01	6.1 ± 0.12

^a Modified SBO and MO (MSBO and MMO) were prepared under optimum conditions (enzyme concentration of 500 units/g of oil, 24 h, 40°C, 3:1 mole ratio, 2 mL hexane, 1% water). The results are mean of triplicate determinations from different experiments.

also contrasts the general tendency for distribution of long chain PUFA of fish oils in this position.

4.4 Chemical and instrumental analysis of modified and unmodified SBO and MO

4.4.1 Conjugated dienes

Figure 4.11 shows the CD content of modified and unmodified seal blubber and menhaden oils under accelerated storage conditions at 60°C. When oxidation was induced at 60°C, the unmodified menhaden oil had a slightly higher ($P < 0.05$) rate of conjugated double bonds formation than its modified counterpart, but the modified and unmodified seal blubber oils were not significantly ($P > 0.05$) different from one another. All samples followed an increasing trend throughout the experiment. As lipid peroxidation proceeded, more primary products such as hydroperoxides and conjugated dienes were formed. The CD in the menhaden oil increased to 26.7% after 5 days of storage under Schaal oven conditions at 60°C. However, the corresponding conjugated value for the modified menhaden oil did not exceed 20.5%. In the case of seal blubber oil, both the modified and unmodified oils reached an approximate CD value of 16%.

Figure 4.12 shows the CD content of modified and unmodified seal blubber and menhaden oils that were stored at room temperature under fluorescent lighting. All oils examined showed an increasing trend for their CD content throughout the experimentation. However, when the oils were subjected to high intensity fluorescent light the MSBO and MMO had a much higher conjugated diene value than their unmodified counterparts. The CD value for MMO after 3 days of storage under fluorescent light increased to 36.9%,

while its unmodified counterpart rose only to 29.1%. Similarly, the CD value of MSBO reached 25.8 as compared to that of 21.7 for the unmodified SBO. The continual increase in CD throughout the experiment was in agreement with the results reported by Senanayake and Shahidi (2002) and Moussata and Akoh (1998) who also found that the amount of CD increased continuously during the entire storage period for structured lipids produced by incorporating PUFA. In addition, Wanasundara and Shahidi (1996) reported a constant increase in CD of SBO and MO over a six day storage period at 65°C. For the oil stored at 60°C, the high content of CD in the enzymatically modified oils may arise from the high proportion of readily oxidisable GLA. Farmer and Sutton (1946) and Jackson (1981) found that the formation of lipid hydroperoxides coincides with that of CD upon oxidation. The higher CD values in the modified oils during photooxidation may be explained when considering the presence of photosensitising pigments such as chlorophylls and carotenoids. Edible oils containing natural pigments such as chlorophylls and pheophytins have been reported to be very susceptible to photooxidation (Davis *et al.*, 1995; Lee *et al.*, 1997). Khan and Shahidi (2000) who reported that stripped borage and evening primrose oils had significantly lower CD values than their non-stripped counterparts.

4.4.2 Thiobarbituric acid reactive substances (TBARS)

TBARS are used to measure secondary products of lipid oxidation and are commonly used to monitor the quality of edible oils. The determination of TBARS is

Figure 4.11 Conjugated diene values of menhaden oil and seal blubber oil-based structured lipids and unmodified oils stored under Schaal oven conditions at 60°C

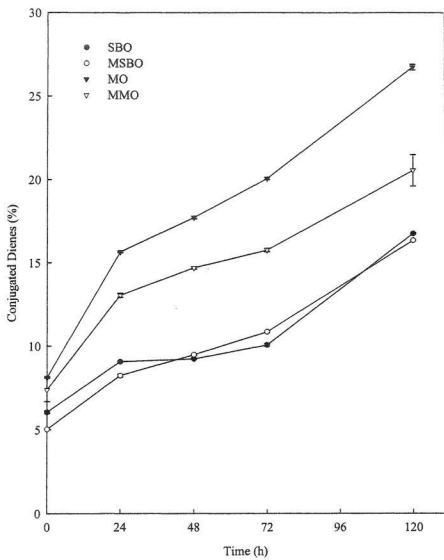
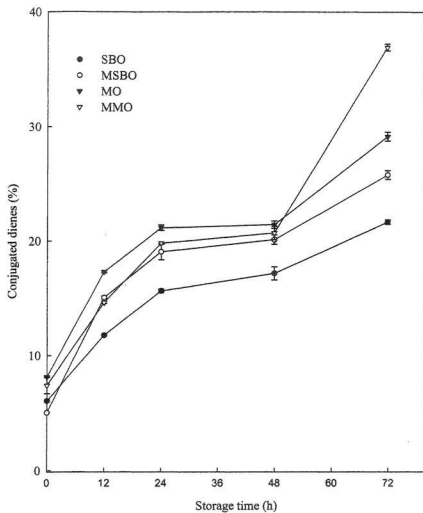


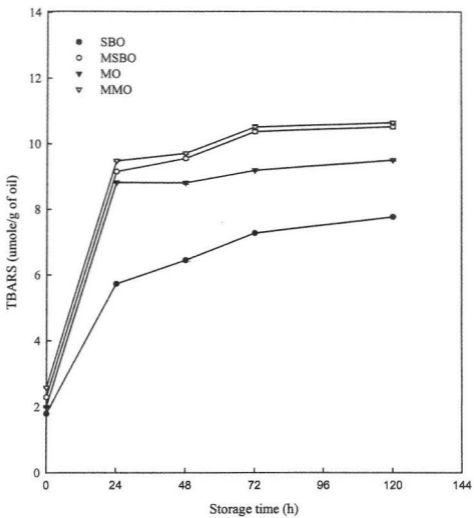
Figure 4.12 Conjugated diene values of menhaden oil and seal blubber oil-based structured lipids and unmodified oils stored under fluorescent lights at room temperature



based on colour intensity of the reaction between TBA and secondary oxidation products of PUFA, including malonaldehyde (MA). TBARS value is expressed as μ moles of MA equivalents per gram of oil. Production of TBARS in modified and unmodified oils stored at 60°C is given in Figure 4.13. The TBARS value progressively increased over the entire storage period. Nevertheless, enzymatically modified oils had a significantly higher ($P < 0.05$) TBARS than those of their unmodified counterparts. The general increase in TBARS occurred because as oxidation proceeded, the lipid hydroperoxides tended to break down to produce secondary oxidation products, as supported by Strange *et al.* (1997) and Akoh and Moussata (2001). These results are in agreement with those of Senanayake and Shahidi (2002) who also found that borage oil esterified with DHA had higher TBARS values than those of the original borage oil during autoxidation at 60°C. Akoh and Moussata (2001) also reported higher TBARS values for structured lipids produced from MO and caprylic acid than the original MO when stored at 60°C in a dark container. The high TBARS levels for modified oils may be due to the loss of natural protective antioxidants in the original oils and/or incorporation of high proportion of GLA, which is highly unsaturated and prone to oxidation. During the acidolysis process some of the minor components such as tocopherols and phospholipids may have been stripped off, thus reducing the oils' stability. In a salmon model system, phospholipids effectively inhibited the formation of TBARS (King *et al.*, 1992).

Figure 4.14 shows the TBARS values for modified and unmodified oils stored under fluorescent lighting. The amount of secondary oxidation products formed increased throughout the storage period. Furthermore, enzymatically modified oils had higher

Figure 4.13 TBARS values of menhaden and seal blubber oil-based structured lipids stored under Schaal oven conditions at 60°C



TBARS values than their unmodified counterparts. The MSBO had significantly higher ($P < 0.05$) TBARS values than unmodified SBO, whereas the difference between MMO and MO was not significant ($P > 0.05$). The higher TBARS values in the modified oils may be explained by their higher degree of unaturation and possible presence of photosensitising chlorophylls. Oils containing natural pigments are very susceptible to photooxidation (Davis *et al.*, 1995 Lee *et al.*, 1997). Aranda and Fregapane (1998) reported that olive oil contained naturally occurring pigments such as chlorophylls and pheophytins as well as carotenoids. Khan (1999) reported the presence of chlorophylls and carotenoids in borage. Since borage oil was the source for GLA it is possible that some pigments were transferred into the modified lipids which then acted as photosensitisers for photooxidation. Thus stabilization of these oils might be difficult. Yasei *et al.* (1996) revealed that phenolic antioxidants such as butylated hydroxytoluene and tertiary butylhydroquinone did not effectively protect fats from oxidation due to involvement of singlet oxygen. However, Kamal-Eldin and Appelqvist (1996) reported that tocopherols have a quenching ability for singlet oxygen. The quenching efficiency for tocopherols on singlet oxygen decreased in the order of α - > β - > γ - > δ -. However, in the late stages of photooxidation, γ - and δ -tocopherols may be more effective than α -tocopherol. Hall and Cuppett (2000) reported that rosemariquinone had a synergistic effect when combined with mixed natural tocopherols during autoxidation. However, during photooxidation, this combination was not synergistic. In addition, Lee *et al.* (1997) showed that ascorbyl palmitate was successful in quenching the chlorophyll-sensitised photooxidation of oils.

4.4.3 Nuclear magnetic resonance (NMR)

In this study, olefinic, aliphatic and diallylmethylenic protons of extracted lipids were identified for each oil. The NMR protons' absorption peaks appeared at δ 5.1-5.6, δ 0.6-2.5 and δ 2.6-2.9, respectively (Saito and Udagawa, 1992). The relative number of protons in each group was calculated based on the integration of the methylene protons (δ 4.0-4.4) of the glycerol portion of the triacylglycerol backbone (4 protons in the 2 methylene groups of the glycerol portion of triacylglycerol moiety (Wanasundara and Shahidi, 1993).

The ^1H NMR exhibited relative changes in total number of aliphatic ($\text{CH}_3 + [\text{CH}_2]_n + \text{CH}_2\text{-CH}_2\text{-CH=}$ + $\text{CH}_2\text{-CH=}$ + $\alpha\text{-CH}_2$; 0.6-2.5), olefinic (-HC=CH- ; 5.1-5.4) and diallylmethylenic ($\text{=C-CH}_2\text{C=}$; 2.6-2.9) protons of the modified and unmodified SBO and MO stored at 60°C. The ratios of aliphatic to olefinic (R_{ao}) protons and aliphatic to diallylmethylenic (R_{ad}) protons are shown in Figures 4.15 and 4.16, respectively. In both the modified and unmodified SBO and MO there was a continuous increase in the R_{ao} and R_{ad} values for all oils throughout the storage period. This is in agreement with the reports of Shahidi *et al.* (1994) and Shahidi and Spurvey (1996) who also reported a steady increase in the R_{ao} and R_{ad} values throughout the storage period in their experiments with SBO and cod liver oil, and with mackerel oil, respectively. The SBO had the highest R_{ao} and R_{ad} when compared to the MO and the modified oils. Shahidi *et al.* (1994) also showed that SBO had higher R_{ao} and R_{ad} values than cod liver oil, which was attributed to its lower number of total olefinic and diallylmethylenic protons. As the GLA was incorporated into the modified oils, their R_{ao} and R_{ad} values were decreased due to the increase in the total olefinic and diallylmethylenic protons.

Figure 4.14 TBARS values of menhaden and seal blubber oil-based structured lipids stored under fluorescent light at room temperature

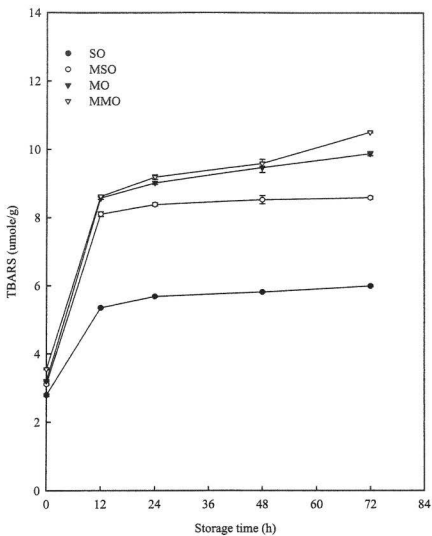


Figure 4.15 Relationship between storage time at 60°C and the ratio of aliphatic to olefinic (Rao) protons of modified and unmodified seal blubber oil (SBO) and menhaden oil (MO)

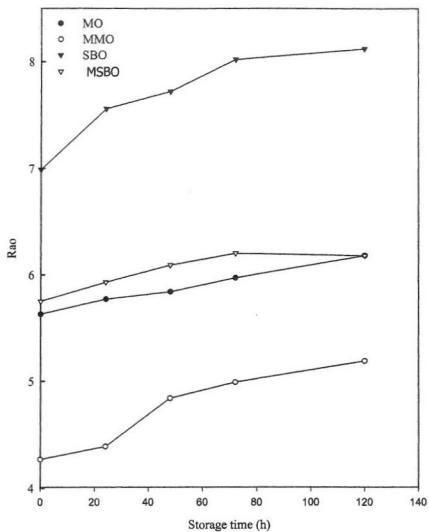
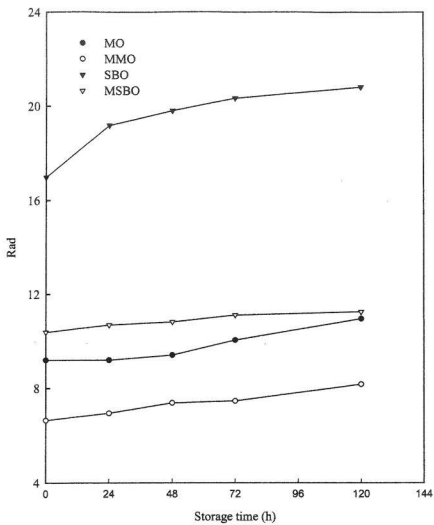


Figure 4.16 Relationship between storage time at 60°C and the ratio of aliphatic to diallylmethylenic (Rad) protons of modified and unmodified seal blubber oil (SBO) and menhaden oil (MO)



Figures 4.17 and 4.18 illustrate the change in the R_{ao} and R_{ad} values for both the modified and unmodified MO and SBO, respectively, stored under fluorescent light. For all oilsexamined, there was a steady increase in the R_{ao} and R_{ad} values throughout the experiment. Again, the SBO had the highest R_{ao} and R_{ad} values of the four oils followed by MO and modified oils. This agrees with the fact that SBO has the lowest number of total olefinic and diallylmethyleneic protons. In Table 4.8 the amount of PUFAs is listed for the modified and unmodified SBO and MO. The number of double bonds increased in the order of SBO < MO < MSBO < MMO. This shows that the amount of unsaturation in the SBO is the lowest of the oils tested, therefore the R_{ao} and R_{ad} values would be the highest. There is a lack of information that study the effect of photooxidation by NMR, therefore results will be compared with those of autooxidation. As stated above, reports by Wanasundara and Shahidi (1993), Shahidi *et al.* (1994) and Shahidi and Spurvey (1996) all show the continued increase in the R_{ao} and R_{ad} values for various types of edible oils upon oxidation. Wanasundara and Shahidi (1993) reported that the relative number of olefinic and diallylmethyleneic protons decreased with the storage of bulk oils, while that of aliphatic protons increased. This was due to the oxidation of polyunsaturated fatty acids and further confirmed the findings of Saito and Nakamura (1990) and Saito (1997) on oxidative deterioration of fish meal. This accounts for the gradual increase of the R_{ao} and R_{ad} values throughout the experiments.

Figure 4.17 Relationship between storage time at 25°C under fluorescent light and the ratio of aliphatic to olefinic (Rao) protons of modified and unmodified seal blubber oil (SBO) and menhaden oil (MO)

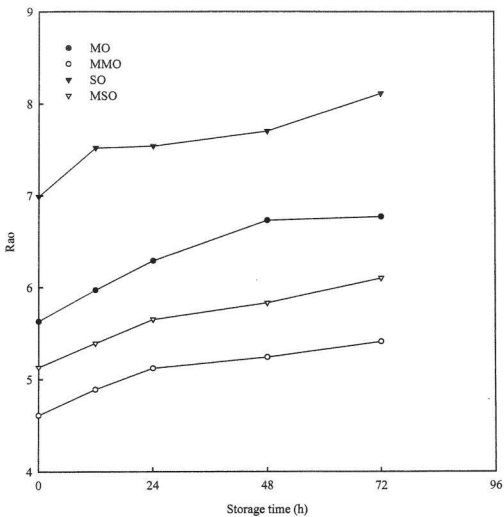
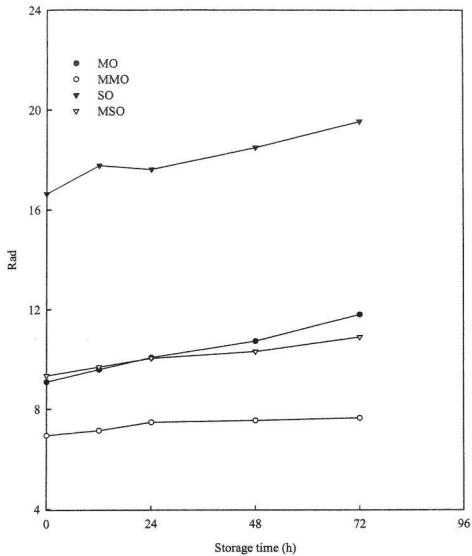


Figure 4.18 Relationship between storage time at 25°C under fluorescent light and the ratio of aliphatic to diallylmethylenic (Rad) protons of modified and unmodified seal blubber oil (SBO) and menhaden oil (MO)



4.4.4 Summary of the oxidation results

Seal blubber and menhaden oils and their modified counterparts containing GLA were continuously degraded throughout the storage period. The most stable oil was SBO due to its lower number of double bonds. The modified oils were the least stable, due to the loss of natural protective antioxidants and/or incorporation of high proportions of GLA, which is highly unsaturated and prone to oxidation. In addition, during the acidolysis process some of the minor components such as phospholipids may have been stripped off, thus reducing the oils' stability.

In the case of photooxidation, the stability of oils decreased in the order of SBO > MSBO > MO > MMO, possibly due to existing differences in their degree of unsaturation and presence photosensitisers. Khan (1999) reported the presence of chlorophylls, carotenoids and tocopherols in borage oil, while Sensidoni *et al.* (1996) identified chlorophylls in borage oil. It has been reported that edible oils containing natural pigments such as chlorophylls and pheophytins are very susceptible to photooxidation (Davis *et al.*, 1995; Lee *et al.*, 1997). Sotiropoulos and Tsimidou (2002) studied the effect of photooxidation on the stability of virgin olive oil. They concluded that photosensitisers such as chlorophyll and pheophytin *a* were lost during exposure to light, while β -carotene and lutein acted as singlet oxygen quenchers and their contents remained almost unchanged throughout the experiment.

SUMMARY AND CONCLUSION

Production of gamma-linolenic acid (GLA) fatty acid concentrate from borage oil by urea complexation was optimized, under laboratory conditions. Process parameters considered were urea-to-fatty acid ratio, reaction temperature and reaction time and the production conditions were optimized using response surface methodology (RSM) with a face-centred cube design (FCCD). The maximum amount of GLA (91.5%) from borage oil was obtained at urea-to-fatty acid ratio of 3.7, reaction temperature of -7°C and reaction time of 16 h.

Enzymatic acidolysis of seal blubber oil (SBO) and menhaden oil (MO) with lipase from *Mucor miehei* and *Pseudomonas sp.* was studied. The reaction parameters considered were the mole ratio of GLA to triacylglycerol (GLA:TAG), reaction temperature, reaction time and enzyme concentration. The lipase *Pseudomonas species* was chosen over *Mucor meihei* to catalyze the interesterification reaction due to higher incorporation of GLA. The best conditions were a mole ratio of GLA to TAG of 3:1, reaction temperature of 40 °C, reaction time of 24 h and enzyme concentration of 500 units/g of oil. Under these conditions, incorporation of GLA was 37.1% for SBO and 39.6% for MO. The resultant oils containing both $\omega 3$ and $\omega 6$ fatty acids are considered important for clinical as well as nutritional purposes.

The positional distribution of the fatty acids in the TAG molecules of the modified seal blubber oil (MSBO) and modified menhaden oil (MMO) were examined by stereospecific analysis. In MSBO, eicosapentaenoic acid (EPA), docosahexaenoic acid

(DHA) and docosapentaenoic acid (DPA) were located mainly in the *sn*-1 and *sn*-3 positions of the TAG molecules. In MMO, EPA, DHA and DPA were equally distributed throughout amongst the *sn*-1, *sn*-2 and *sn*-3 positions of the TAG. GLA was distributed throughout all positions of the TAG of MMO and MSBO. This indicates that the lipase *Pseudomonas species* (PS-30) was able to involve the middle position of the TAG in the acidolysis process. Thus, structured lipids containing EPA, DHA, DPA and GLA were successfully produced and may have potential health benefits. Therefore, the structured lipid produced from the SBO may be better assimilated in the body than the MO structured lipid. However, clinical studies should be carried out to verify this assumption.

The antioxidant activity of both the modified and unmodified oils was assessed under both autoxidative and photooxidative conditions using Schaal oven conditions at 60°C and room temperature under fluorescent lighting, respectively. During autoxidation, the modified oils were the least stable oils due to the high amount of polyunsaturated fatty acids (PUFA) and loss of natural antioxidants during the acidolysis process. In the case of photooxidation, both modified and unmodified SBO oils were more stable than their MO counterparts. An explanation for this may be the presence of photosensitizers such as chlorophylls in MO, which might accelerate photooxidation.

Future work should concentrate on *in-vivo* studies to establish consumption, assimilation and metabolism of fatty acids in the blood and specific tissues. The influence of the fatty acids in modified oils should also be compared with that of physical mixtures of oils in order to examine their comparative health benefits.

REFERENECES

- Fishery Statistics Commodities FAO Year Book (1993), Vol.77, Food and Agriculture Organization of the United Nations. Rome.
- Canadian Scientific Review Committee (1990). Nutrition Recommendations: Minister of National Health and Welfare. Ottawa, ON.
- British Nutrition Foundation (1992). Report of the Task Force on Unsaturated Fatty Acids. Chapman and Hall, London.
- Ackman, R.G. (1982). Fatty acid composition of fish oils. In *Nutritional Evaluation of Long-Chain Fatty Acids in Fish Oils*, ed. S.M. Barlow and M.E. Stansby, Academic Press, New York, NY, pp. 25-88.
- Ackman, R.G. (1988). The year of fish oil. *Chem. Ind.*, 3: 139-145.
- Ackman, R.G. (1990). Canola fatty acids - an ideal mixture for health, nutrition and food use. In *Canola and Rapeseed, Production, Chemistry, Nutrition and Processing Technology*, ed. F. Shahidi, van Nostrand Reinhold, New York, NY, pp. 81-98.
- Ackman, R.G., Sipos, J.C., Hooper, S.N. and Dube, G. (1977). Selection of internal standards for determining quantitative recovery of isoprenoid acids after urea complexing. *J. Am. Oil Chem. Soc.*, 54: 199-201.
- Adachi, S., Ishiguro, T. and Matsuno, R. (1995). Autoxidation kinetics for fatty acids and their esters. *J. Am. Oil Chem. Soc.*, 72: 547-551.
- Adachi, S., Okumura, K., Ota, Y. and Mankura, M. (1993). Acidolysis of sardine oil by lipase to concentrate eicosapentaenoic and docosahexaenoic acids in glycerides. *J. Ferm. and Bioeng.*, 75: 259-264.
- Aitzetmuller, K. and Tsevegsuren, N. (1994). Occurrence of gamma-linolenic acid in *ranunculaceae* seed oils. *J. of Plant Physio.*, 143: 578-580.
- Akoh, C.C. (1995). Structured lipids-enzymatic approach. *INFORM*, 6: 1055-1061.
- Akoh, C.C. (1998). Structured lipids. In *Food Lipids- Chemistry, Nutrition and Biotechnology*, ed. C.C. Akoh and D.B. Min, Dekker, New York, NY, pp. 699-727.
- Akoh, C.C. and Huang, K.H. (1995). Enzymatic synthesis of structured lipids: transesterification of triolein and caprylic acid. *J. Food Lipids*, 2: 219-230.

- Akoh, C.C., Jennings, B.H. and Lillard, D.A. (1995). Enzymatic modification of trilinolein: incorporation of n-3 polyunsaturated fatty acids. *J. Am. Oil Chem. Soc.*, 72: 1317-1321.
- Akoh, C.C., Jennings, B.H. and Lillard, D.A. (1996). Enzymatic modification of evening primrose oil: incorporation of n-3 polyunsaturated fatty acids. *J. Am. Oil Chem. Soc.*, 73: 1059-1062.
- Akoh, C.C. and Moussata, C.O. (2001). Characterization and oxidative stability of enzymatically produced fish and canola oil-based structured lipids. *J. Am. Oil Chem. Soc.*, 78: 25-30.
- Akoh, C.C. and Sista, R.V. (1995). Enzymatic modification of borage oil: incorporation of eicosapentaenoic acid. *J. Food Lipids*, 2: 231-238.
- Akoh, C.C. and Yee, L.N. (1997). Enzymatic synthesis of position-specific low-calorie structured lipids. *J. Am. Oil Chem. Soc.*, 74: 1409-1413.
- Al, M.D.M., Hornstra, G., Schouw, Y.T., Bulstra-Remakers, M.E.E.W. and Huistes, G. (1990). Biochemical EFA status of mothers and their neonates after normal pregnancy. *Early Hum. Dev.*, 24: 239-248.
- Ahmad, J.I. (1998). Omega three fatty acids - the key to longevity. *Food Sci. Technol. Today*, 12: 139-146.
- Anderson, L.A., Dibble, M.V., Turkki, P.R., Mitchell, H.S. and Rynbergen, H.J. (1982). In *Nutrition in Health and Disease*, ed. J.B Lippincott Co., Philadelphia, PN, pp. 35-40.
- Ando, Y., Ota, T., Matsuhiro, Y. and Yazawa, K. (1996). Stereospecific analysis of triacyl-sn-glycerols in docosahexaenoic acid-rich fish oils. *J. Am. Oil Chem. Soc.*, 73: 483-487.
- Angelo, A.J.S., Ory, R.L. and Brown, L.E. (1975). Comparison of methods for determining peroxidation in processed whole peanut products. *J. Am. Oil Chem. Soc.*, 14: 34-35.
- Angelo, A.J.S., Ory, R.L. and Brown, L.E. (1975). Comparison of methods for determining peroxidation in processed whole peanut products. *J. Am. Oil Chem. Soc.*, 14: 34-35.

- Anonymous. (1986). Fish Oil Production and Potential in Atlantic Canada. In *Project Report No. 115*, Fisheries Development Branch, Halifax, NS.
- Anonymous. (1999). Fish meal and oil production estimates 1998. *Ifoma*, 84: 12-16.
- AOCS. (1990). Official Methods and Recommended Practices of the American Oil Chemists' Society, 4th edn. ed. D. Firestone, American Oil Chemists' Society, Champaign, IL.
- Arribere, M.C., Priolo, N.S. and Caffini, N.O. (1994). Characterization of the lipolytic system of germinated sunflower seeds. *Acta Aliment.*, 23: 313-324.
- Aursand, M., Gorgensen, L. and Grasdalen, H. (1995). Positional distribution of omega-3 fatty acids in marine lipid triacylglycerols by high-resolution ^{13}C nuclear magnetic resonance spectroscopy. *J. Am. Oil Chem. Soc.*, 72: 293-297.
- Bang, H.O., Dyerberg, J. and Hjerne, N. (1976). The composition of food consumed by Greenland Eskimos. *Acta Med. Scand.*, 200: 59-73.
- Barber, A.J. (1988). Evening primrose oil: a panacea? *Pharmaceut. J.*, 240: 723-725.
- Barlow, S.M. (1990). Toxicology of antioxidants used as food additives. In *Food Antioxidant*, ed. B.J.F. Hudson, Elsevier Applied Science, London, pp. 253-307.
- Barlow, S.M. and Young, V. (1988). New uses for fish oils. *Food Manufacture*: October, pp. 1-4.
- Barre, D.E., Holub, B.J. and Chapkin, R.S. (1993). The effects of borage oil supplementation on human platelet aggregation, thromboxane B_2 , Prostaglandin E_1 and E_2 formation. *Nutr. Research*, 13: 739-751.
- Bengmark, S. (1998). Ecoimmunonutrition: a challenge for the third millennium. *Nutrition*, 14: 563-572.
- Bhatena, S.J. (1992). Fatty acids and diabetes. In *Fatty Acids in Foods and Their Health Implications*, ed. C.K. Chow, Dekker, New York, NY, pp. 823-855.
- Bidder, T.G. and Sipka, B.M. (1989). A modification of the thiobarbituric acid reaction. *Lipids*, 24: 656-658.
- Bimbo, A.P. (1989). Technology of production and industrial utilization of marine oils. In *Marine Biogenic Lipids, Fats and Oils*, ed. R.G. Ackman, CRC Press Inc., Boca Raton, FL, pp. 401-433.

- Bimbo, A.P. (1990). Processing of fish oils. In *Fish Oils in Nutrition*, ed. M.E. Stansby, Van Nostrand Reinhold, New York, NY, pp. 181-225.
- Bimbo, A.P. (1991). Fish oils: processing beyond crude oil. *Infofish Int.*, 6: 20-25.
- Bimbo, A.P. and Crowther, J.B. (1992). Fish meal and oil: current issues. *J. Am. Oil Chem. Soc.*, 69: 221-227.
- Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, 37: 911-917.
- Bohannon, M.B. and Kleiman, R. (1976). γ -Linolenic acid in acer seed oils. *Lipids*, 11: 157-1159.
- Boissonneault, G.A. and Hayek, M.G. (1992). Dietary fat, immunity and inflammatory disease. In *Fatty Acids in Foods and Their Health Implications*, ed. C.K. Chow, Dekker, New York, NY, pp. 707-734.
- Bordoni, A., Biagi, L.P., Masi, M., Ricci, G., Fanelli, C., Patrizi, A. and Ceccolini, E. (1988). Evening primrose oil Efanol in the treatment of children with atopic eczema. *Drugs Exp. Clin. Res.*, 14: 291-297.
- Bougnoux, P., Chajes, V., Germain, E., Hubert, B., Lhuillery, C., LeFloch, O., Body, G. and Calais, G. (1999). Cytotoxic drug efficacy correlates with adipose tissue docosahexaenoic acid level in locally advanced breast carcinoma. *Lipids*, 34: S109-S110.
- Bradley, D.G. and Min, D.B. (1992). Singlet oxygen oxidation of foods. *CRC Crit. Rev. Food Sci. Nutri.*, 31: 211-236.
- Branden, L.M. and Carroll, K.K. (1986). Dietary polyunsaturated fats in relation to mammary carcinogenesis in rats. *Lipids*, 21: 285-288.
- Branen, A.C. (1975). Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. *J. Am. Oil Chem. Soc.*, 52: 59-63.
- Brenner, R.R. (1974). The oxidative desaturation of unsaturated fatty acids in animals. *Mol. Cell. Biochem.*, 3: 41-52.
- Brenner, R.R. (1982). Nutritional and hormonal factors influencing desaturation of essential fatty acids. *Prog. Lipid Res.*, 20: 41-48.

- Brockerhoff, H. (1970). Substrate specificity of pancreatic lipase: Influence of the structure of fatty acids on the reactivity of esters. *Biochem. Biophys. Acta.*, 212: 92-101.
- Brockerhoff, H., Hoyle, R.J. and Huang, P.C. (1966). Positional distribution of fatty acids in the fats of a polar bear and a seal. *Can. J. Biochem.*, 44: 1519-1525.
- Brown, J.B. and Kolb, D.K. (1955). Application of low temperature crystallization in the separation of the fatty acids and their compounds. *Prog. Chem. Fats Lipids*, 3: 57-94.
- Bruckner, G. (1992). Fatty acids and cardiovascular disease. In *Fatty Acids in Foods and Their Health Implications*, ed. C.K. Chow, Dekker, New York, NY, pp. 735-752.
- Burr, G.O. and Burr, M.M. (1929). A new deficiency disease produced by rigid exclusion of fat from the diet. *J. Biol. Chem.*, 82: 345-367.
- Burr, M.L., Fehily, A.M., Gilbert, J.F., Rogers, S., Holliday, R.M., Sweetnam, P.M., Elwood, P.C. and Deadman, N.M. (1989). Effect of changes in fat, fish and fibre intakes on death and myocardial function: diet and reinfarction trial (DART). *Lancet*, ii: 757-762.
- Carlson, S.E., Rhodes, P.G. and Ferguson, M.G. (1986). Docosahexaenoic acid status of preterm infants at birth and following feeding with human milk of formula. *Am. J. Clin. Nutr.*, 44: 798-802.
- Carr, R.A. (1978). Refining and degumming systems for edible fats and oils. *J. Am. Oil Chem. Soc.*, 55: 765-771.
- Carroll, K.K. (1990). Experimental and epidemiological evidence on marine lipids and carcinogenesis. In *Omega-3 Fatty Acids in Health and Disease*, ed. R.S. Lees and M. Karel, Dekker, New York, NY, pp. 99-114.
- Carter, P.J. (1988). Gamma-linolenic acid as a nutrient. *Food Technol.*, 42: 72-76.
- Cerdan, E.L., Medina, A.R., Gimenez, A.G., Ibanez, M.J. and Grima, E.M. (1998). Synthesis of polyunsaturated fatty acids-enriched triglycerides by lipase-catalyzed esterification. *J. Am. Oil Chem. Soc.*, 10: 1329-1337.
- Chajes, V., Hulten, K., Kappel, A.L.V., Winkvist, A., Kaaks, R., Lenner, G.H.G. and Riboli, E. (1999). Fatty acid composition in serum phospholipids and risk breast cancer: A prospective cohort study in northern sweden. *Lipids*, 34: S113-S114.

- Chan, H.W.S. (1977). Photosensitized oxidation of unsaturated fatty acid methyl esters: the identification of different pathways. *J. Am. Oil Chem. Soc.*, 54: 100-104.
- Chandler, I.C. (2001). Determining the regioselectivity of immobilized lipases in triacylglycerols acidolysis reactions. *J. Am. Oil Chem. Soc.*, 78: 737-742.
- Chang, S.S. (1967). Processing of fish oils. In *Fish Oils*, ed. M.E. Stansby, AVI Publishing Co., West Port, CT, pp. 206-221.
- Chapkin, R.S., Sommers, S.D., Schumaker, L. and Erickson, K.L. (1988). Fatty acid composition of macrophage phospholipids in mice fed fish or borage oil. *Lipids*, 23: 380-383.
- Chatterjee, T. and Bhattacharyya, D.K. (1998). Synthesis of monoterpene esters by alcoholysis reaction with mucor miehei lipase in a solvent-free system. *J. Am. Oil Chem. Soc.*, 75: 651-654.
- Chawla, P. and deMan, J.M. (1990). Measurement of the size distribution of fat crystals using a laser particle counter. *J. Am. Oil Chem. Soc.*, 67: 329-332.
- Chen, H.C. (1994). Response-surface methodology for optimizing citric acid fermentation by *Aspergillus foetidus*. *Process Bioc.*, 29: 399-405.
- Cho, S.Y., Miyashita, K., Miyazawa, T., Fujimoto, K. and Kaneda, T. (1987). Autoxidation of ethyl eicosapentaenoate and docosahexaenoate. *J. Am. Oil Chem. Soc.*, 64: 876-879.
- Christensen, M.S., Hoy, C.E., Becker, C.C. and Redgrave, T.G. (1995). Intestinal absorption and lymphatic transport of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and decanoic acids: dependence on intramolecular triacylglycerol structure. *Am. J. Clin. Nutr.*, 61: 56-61.
- Christie, W.W. (1982). Analysis of complex lipids. In *Lipid Analysis*, 2nd edn., Pergamon Press, New York, NY, pp. 107-134.
- Chung, H.Y., Park, J., Kim, J.H. and Kong, U.Y. (1996). Preparation of sorbitol fatty acid polyesters, potential fat substitutes: optimization of reaction conditions by response surface methodology. *J. Am. Oil Chem. Soc.*, 73: 637-643.
- Cillard, J., Cillard, P. and Cormier, M. (1980). Effect of experimental factors on the prooxidant behaviour of tocopherol. *J. Am. Oil Chem. Soc.*, 57: 255-261.

- Cisowski, W., Zielinska-Stasiek, M., Luczkiewicz, M. and Stolyhwo, A. (1993). Fatty acids and triglycerols of developing evening primrose (*Oenothera biennis*) seeds. *FITOTERAPIA*, LXIV: 155-162.
- Coppen, D.P. (1983). Use of antioxidants. In *Rancidity in Foods*, ed. J.C. Allan and R.J. Hamilton, Applied Science, London, pp. 76-87.
- Crozier, G.L., Fleith, M., T.Traitle and Finot, P.A. (1989). Black currant seed oil feeding and fatty acids in liver lipid classes of guinea pigs. *Lipids*, 24: 460-466.
- Crozier, G.L. and Secretin, M.C. (1996). γ -Linolenic acid in infant formula. In *γ -Linolenic acid: metabolism and its roles in nutrition and medicine*, ed. Y.S. Huang and D.E. Mills, AOCS Press, Champaign, IL, pp. 246-251.
- Cunnane, S.C., Francescutti, V., Brenna, J.T. and Crawford, M.A. (2000). Breast-fed infants achieve a higher rate of brain and whole body docosahexaenoate accumulation than formula-fed infants not consuming dietary docosahexaenoate. *Lipids*, 35: 105-111.
- Davidson, M.H., Burns, J.H., Subbaiah, P.V., Conn, M.E. and Drennan, K.B. (1991). Marine oil capsule therapy for the treatment of hyperlipidemia. *Arch. Intern. Med.*, 151: 1732-1740.
- Davis, L., Smith, G. and Hole, M. (1995). Lipid oxidation in salted-dried fish: II. The effect of photosensitises on the rate of oxidation of a fish oil. *J. Sci. Food Agric.*, 67: 493-499.
- Davis, L., Smith, G. and Hole, M. (1995). Lipid oxidation in salted-dried fish: II. The effect of photosensitivities on the rate of oxidation of a fish oil. *J. Sci. Food Agric.*, 67: 493-499.
- Defeme, J.L. and Leeds, A.R. (1992). The Antihypertensive Effects of Dietary Supplementation With a 6-Desaturation Essential Fatty Acid Concentrate as Compared With Sunflower Seed Oil. *J. Hum. Hypertens.*, 6: 113-119.
- DeMichele, S.J., Karlstad, M.D., Bistran, B.R., Istfan, N., Babayan, V.K. and Blackburn, G.L. (1989). Enteral nutrition with structured lipids: effect on protein metabolism in thermal injury. *Am. J. Clin. Nutr.*, 50: 1295-1300.
- Dijkstra, A.J. and Opstal, M.V. (1989). The total degumming process. *J. Am. Oil Chem. Soc.*, 66: 1002-1009.

- Dordick, J.S. (1989). Enzymatic catalysis in monophasic organic solvents. *Enzyme Microb. Technol.*, 11: 194-198.
- Dudek, S.G. (1993). Principles of Nutrition. In *Lipids*, ed. S.G. Dudek, J.B Lippincott, Philadelphia, PN, pp. 58-65.
- Dyerberg, J. (1986). Linolenate-derived polyunsaturated fatty acids and prevention of atherosclerosis. *J. Nutr. Rev.*, 44: 125-134.
- Dziezak, J.D. (1986). Preservatives antioxidants. *Food Technol.*, 40 (9): 94-102.
- Endo, Y., Hoshizaki, S. and Fujimoto, K. (1997). Oxidation of synthetic triacylglycerols containing eicosapentaenoic and docosahexaenoic acids: effect of oxidation system and triacylglycerol structure. *J. Am. Oil Chem. Soc.*, 74: 1041-1045.
- Engler, M.M., Bellenger-Germain, S.H., Engler, M.B., Narce, M.M. and Poisson, J.P.G. (2000). Dietary docosahexaenoic acid affects stearic acid desaturation in spontaneously hypertensive rats. *Lipids*, 35: 1011-1015.
- Engler, M.M. and Engler, M.B. (1998). The effects of dietary evening primrose, black currant, borage and fungal oils on plasma, hepatic and vascular tissue fatty acid composition in the spontaneously hypertensive rat. *Nutr. Research*, 18: 1533-1544.
- Engler, M.M., Engler, M.B. and Paul, S.M. (1992). Effects of dietary borage oil in gamma-linolenic acid on blood pressure and vascular reactivity. *Nutr. Research*, 12: 519-528.
- Enser, M. (1987). What is lipid oxidation. *Food Sci. Technol. Today*, 1: 151-153.
- Erickson, M.C. (1998). Lipid oxidation of muscle foods. In *Food Lipids*, ed. C.C. Akoh and D.B. Min, Dekker, New York, NY, pp. 297-332.
- Evans, C.D., List, G.R., Moser, H.A. and Cowan, J.C. (1973). Long term storage of soyabean and cottonseed salad oils. *J. Am. Oil Chem. Soc.*, 50: 218-222.
- Fan, Y.Y. and Chapkin, R.S. (1992). Mouse peritoneal macrophage prostaglandin E₁ synthesis is altered by dietary gamma-linolenic acid. *J. Nutr.*, 122: 1600-1606.
- Farmer, E.D. and Sutton, D.A. (1946). Peroxidation in relation to olefinic structure. *Trans. Faraday Soc.*, 42: 228-232.

- Fatima, R. and Qadri, R.B. (1993). Omega-3 polyunsaturated fatty acids (n-3 PUFAs) a nature's bounty to this modern and mechanized world. *J. Chem. Soc. Pak.*, 15: 82-91.
- Filer, L.J., Mattson, F.H. and Formon, S.J. (1969). Triglyceride configuration and fat absorption by the human infants. *J. Nutr.*, 99: 293-298.
- Fineberge, H. and Johnson, A.G. (1967). Industrial use of fish oils. In *Fish Oils - Their Chemistry, Technology, Stability, Nutritional Properties and Uses*, ed. M.E. Stansby, AVI publishing Co., West Port, CT, pp. 222-238.
- Fiocchi, A., Sala, M., Signoroni, P., Banderai, G., Agostoni, C. and Riva, E. (1994). The efficacy and safety of γ -linolenic acid in the treatment of infantile atopic dermatitis. *J. Int. Med. Res.*, 22: 2-32.
- Fischer, S. (1989). Dietary polyunsaturated fatty acids and eicosanoid formation in humans. *Adv. Lipid Res.*, 23: 169-198.
- Folgia, T.A. and Sonnet, P.E. (1995). Fatty selectivity of lipases: gamma-linolenic acid from borage oil. *J. Am. Oil Chem. Soc.*, 72: 417-420.
- Forssell, P., Parovouri, P., Linko, P. and Poutanen, K. (1993). Enzymatic transesterification of rapeseed oil and lauric acid in a continuous reactor. *J. Am. Oil Chem. Soc.*, 70: 1105-1109.
- Foundation, B.N. (1970). Report of the task force on Unsaturated fatty acids. Chapman and Hall, London
- Frankel, E.N. (1980). Lipid oxidation, A review. *Prog. Lipid Res.*, 19: 1-22.
- Frankel, E.N. (1982). Volatile lipid oxidation products, A review. *Prog. Lipid Res.*, 22: 1-33.
- Frankel, E.N. (1985). Chemistry of autoxidation mechanism, product and flavour significance. In *Flavour Chemistry of Fats and Oils*, ed. D.B. Min and T.H. Smouse, AOCS Monograph 15, American Oil Chemists' Society, Champaign, IL, pp. 1-37.
- Fujimoto, K. (1989). Flavour chemistry of fish oils. In *Flavour Chemistry of Lipid Foods*, ed. D.B. Min and T.H. Smouse, American Oil Chemists' Society, Champaign, IL, pp. 190-195.

- Fukada, H. and Morikawa, H. (1987). Enhancement of γ -linolenic acid production by *Mucor ambigua* with nonionic surfactants. *Appl. Microbiol. Biotechnol.*, 27: 15-19.
- Galli, C. and Simopoulos, A.P. (1994). Effects of fatty acids and lipids in health and disease. *World Rev Nutr Diet.*, 76: 1-149.
- Gao, L. and Mazza, G. (1996). Extraction of anthocyanin pigments from purple sunflower hulls. *J. Food Sci.*, 61: 600-603.
- Gardner, H.W. (1987). Reaction of hydroperoxide-products of high molecular weight. In *Autoxidation of Unsaturated Lipids*, ed. H.W.S. Chan, Academic Press, New York, NY, pp. 51-93.
- Gensen, G.L., McGarvey, N., Taraszewski, R., Wixon, S.K., Seidner, D.L., Pai, T., Yeh, Y.Y., Lee, T.W. and DeMichele, S.J. (1994). Lymphatic absorption of enterally-fed structured triacylglycerol versus physical mix in a canine model. *Am. J. Clin. Nutr.*, 60: 518-524.
- Ghebrenesk, K., Min, Y., Crawford, M.A., Nam, J.H., Kim, A., Koo, J.N. and Suzuki, H. (2000). Blood fatty acid composition of pregnant and nonpregnant Korean women: red cells may act as a reservoir of arachidonic acid and docosahexaenoic acid for utilization by the developing fetus. *J. Am. Oil Chem. Soc.*, 35: 567-574.
- Goderis, H.L., Ampe, G., Feyten, M.P., Fouwe, B.L. and Guffens, W.M. (1987). Lipase-catalyzed ester exchange reactions in organic media with controlled humidity. *Biotechnol., Bioeng.*, 30: 258-261.
- Gonzalez, M.J. (1995). Dietary fat, fish oil and tumor growth: a perspective. *J. of Optimal Nutr.*, 4: 5-8.
- Gordon, M.H. (1990). The mechanism of antioxidant action *in vitro*. In *Food Antioxidants*, ed. B.J.F. Hudson, Elsevier Applied Science, London, pp. 1-18.
- Gray, J.I. (1978). Measurement of lipid oxidation, a review. *J. Am. Oil Chem. Soc.*, 55: 539-546.
- Gray, J.I. and Monahan, F.J. (1992). Measurement of lipid oxidation in meat and meat products. *Trends in Food Sci. and Technol.*, 3: 315-319.
- Gunstone, F. (1996). In *Fatty Acid and Lipid Chemistry*, ed. F. Gunstone, Blackie Academic & Professional, London,

- Gunstone, F.D. (1984). Reaction of oxygen and unsaturated fatty acids. *J. Am. Oil Chem. Soc.*, 61: 441-447.
- Gunstone, F.D. (1994). Chemical properties. In *Lipid Handbook 2nd ed.*, ed. F.D. Gunstone, J.L. Harwood and F.D. Padley, Hall and Chapman, London, pp. 594-595.
- Gunstone, F.D. (1999). What else besides commodity of oils and fats. *Lipid*, 101: 124-131.
- Gunstone, F.D. (1999a). High-resolution NMR spectroscopy as an analytical tool. *Lipid Tech.*, 11: 39-41.
- Gurr, M.I. (1997). Lipids and nutrition. In *Lipid Technologies and Applications*, ed. F.D. Gunstone and F.B. Padley, Dekker, New York, pp. 79-112.
- Gurr, M.I. and Harwood, J.L. (1991). In *Lipid Biochemistry*, ed. M.I. Gurr and J.L. Harwood, Chapman & Hall, New York, NY,
- Hall, C.A. and Cuppette, S.L. (2000). Rosmariquinone interactions in autoxidation and light-sensitized oxidation of stripped soyabean oil. *J. Am. Oil Chem. Soc.*, 77: 937-943.
- Hamilton, J., Grainer, R., Salem, N. and Kim, H. (2000). n-3 fatty acid deficiency decreases phosphatidylserine accumulation selectly in neuronal tissues. *J. Am. Oil Chem. Soc.*, 35: 863-869.
- Hansson, L. and Dostalek, M. (1988). Effect of culture conditions on mycelial growth and production of γ -linolenic acid by the fungus *Mortierella ramanniana*. *Appl. Microbiol. Biotechnol.*, 28: 240-246.
- Haraldsson, G.G. (1984). Separation of saturated/unsaturated fatty acids. *J. Am. Chem. Soc.*, 61: 219-222.
- Haraldsson, G.G. (1992). Using biotechnology to modify marine lipids. *INFORM*, 3: 626-629.
- Haraldsson, G.G. and Hoskuldsson, P.A. (1989). The preparation of triglycerides highly enriched with ω -3 polyunsaturated fatty acids via lipase catalyzed interesterification. *Tetrahedron Letters*, 30: 1671-1674.

- Haraldsson, G.G., Hoskuldsson, P.A., Sigurdsson, S.T., Thorsteinsson, F. and Gudbjarnason, S. (1989). The preparation of triglycerides highly enriched with omega-3 polyunsaturated fatty acids via lipase catalyzed interesterification. *Tetrahedron Letters*, 30: 1671-1674.
- Haro-Bailon, A.D. and Rio, M.D. (1998). Isolation of chemically induced mutants in borage (*Borago officinalis L.*). *J. Am. Oil Chem. Soc.*, 75: 281-283.
- Hassam, A.G. (1985). The role of evening primrose oil in nutrition and disease. In *The Role of Fats in Human Nutrition*, ed. F.B. Padley and J. Podmore, Ellis Horwood Ltd., Chichester, England,
- Hasulo, S. (1998). Nothing fishy about this... *Lab Business*, Fall 98: 8-11.
- Hawrysh, Z.J. (1990). Stability of canola oil. In *Canola and Rapeseed, Production, Chemistry, Nutrition and Processing Technology*, ed. F. Shahidi, Van Nostrand Reinhold, New York, NY, pp. 99-122.
- Hiatt, R., Mill, T. and Mayo, F.R. (1968). Homolytic decomposition of hydroperoxides: I. Summary and implication. *J. Org. Chem.*, 33: 1416-1420.
- Hilder, M.H. (1997). Oil storage, transport and handling. In *Lipid Technologies and Applications*, ed. F.D. Gunstone and F.B. Padley, Dekker, New York, NY, pp. 169-198.
- Hills, M.J., Kiewitt, I. and Mukherjee, K.D. (1989). Enzymatic fractionation of evening primrose oil by rape lipase: enrichment of gamma-linolenic acid. *Biotechnol. Letters*, 2: 629-632.
- Hills, M.J., Kiewitt, I. and Mukherjee, K.D. (1990). Enzymatic fractionation of fatty acids: enrichment of γ -linolenic acid and docosahexaenoic acid by selective esterification catalyzed by lipase. *J. Am. Oil Chem. Soc.*, 67: 561-564.
- Holman, R.T. (1998). The slow discovery of the importance of omega-3 essential fatty acids in human health. *Amer. Soc. for Nutr. Sci.*, 128: 427s-423s.
- Holmer, G. (1989). Triglycerides. In *Marine Biogenic Lipids, Fats and Oils, Vol I*, ed. R.G. Ackamn, CRC Press, Boca Raton, FL, pp. 140-174.
- Horrobin, D.F. (1990). Gamma-linolenic acid: an intermediate inessential fatty acid metabolism with potential as an ethical pharmaceutical and as a food. *Rev. Contemp. Pharmacother.*, 1: 1-41.

- Horrobin, D.F. (1992). Clinical applications of n-6 essential fatty acids: Atopic eczema and inflammation, diabetic neuropathy and retinopathy, breast pain and viral infections. In *Essential Fatty Acids and Eicosanoids*, ed. A. Sinclair and R. Gibson, American Oil Chemists Society, Champaign, IL, pp. 367-372.
- Horrobin, D.F. (1994). Unsaturated lipids: a new approach to the treatment of cancer. In *Effects of Fatty Acids and Lipids in Health and Disease*, ed. C. Galli, A.P. Simopoulos and E. Tremoli, Karger, Basel, Switzerland, pp. 77-80.
- Horrobin, D.F. and Manku, M.S. (1990). Clinical biochemistry of essential fatty acids. In *Pathophysiology and Roles in Clinical Medicine*, ed. D.F. Horrobin, Alan R. Liss, New York, pp. 21-53.
- Hoyland, D.V. and Taylor, A.J. (1991). A review of the methodology of the 2-thiobarbituric acid test. *Food Chem.*, 40: 271-291.
- Hsieh, R.J. and Kinsella, J.E. (1989). Oxidation of polyunsaturated fatty acids, mechanism, products and inhibition with emphasis on fish tissues. *Adv. Food Nutr. Res.*, 33: 233-241.
- Huang, F.C., Ju, Y. and Huang, C. (1997). Enrichment of gamma-linolenic acid from borage oil via lipase-catalyzed reactions. *J. Am. Oil Chem. Soc.*, 74: 977-981.
- Huang, F.C., Ju, Y.H. and Chiang, J.C. (1999). γ -Linolenic acid-rich triacylglycerols derived from borage oil via lipase-catalyzed reactions. *J. Am. Oil Chem. Soc.*, 76: 833-837.
- Huang, K., Akoh, C.C. and Erickson, M.C. (1994). Enzymatic modification of melon seed oil: incorporation of eicosapentaenoic acid. *J. Agric. Food Chem.*, 42: 2646-2648.
- Huang, K.H. and Akoh, C.C. (1994a). Lipase-catalyzed incorporation of n-3 polyunsaturated fatty acids into vegetable oils. *J. Am. Oil Chem. Soc.*, 71: 1277-1280.
- Huang, Y.S., Lin, X., Redden, P.R. and Horrobin, D.F. (1995). *In vitro* hydrolysis of natural and synthetic gamma-linolenic acid-containing triacylglycerols back pancreatic lipase. *J. Am. Oil Chem. Soc.*, 72: 625-631.
- Hubbard, V.S. and McKenna, M.C. (1987). Absorption of safflower oil and structured lipid preparations in patients with cystic fibrosis. *Lipids*, 22: 424-430.

- Hudson, B.J. (1984). Evening primrose (*Oenothera spp.*) oil and seed. *J. Am. Oil Chem. Soc.*, 61: 540-545.
- Hudson, B.J.F. (1990). Food antioxidants. Elsevier Applied Science, London.
- Ikedo, I., Tomari, S., Sugano, M. and Nagata, J. (1991). Lymphatic absorption of structured glycerolipids containing medium chain fatty acids and linoleic acid, and their effect on cholesterol absorption in rats. *Lipids*, 26: 369-373.
- Illingworth, D. and Ullmann, D. (1990). Effects of omega-3 fatty acids on risk factors for cardiovascular diseases. In *Omega-3 Fatty Acids in Health and Disease*, ed. R.S. Lees and M. Karel, Marcek Dekker Inc., New York, NY, pp. 39-69.
- Ingold, K.U. (1960). Inhibition of oil oxidation by 2,6-di-t-butyl-4-substituted phenols. *J. Phys. Chem.*, 64: 1636-1642.
- Innis, S.M., Auestad, N. and Siegman, J.S. (1996). Blood docosahexaenoic and arachidonic acid in term gestation infant fed formulas with high docosahexaenoic acid, low eicosapentaenoic acid fish oil. *J. Am. Oil Chem. Soc.*, 31: 617-625.
- IUAPAC. (1987). Standard Methods for the Analysis of Oils and fats and Derivatives, 7th edn. ed. C. Paquot and A. Hautfenne, Blackwell Scientific Publishing Ltd., Oxford.
- Iverson, J.L. and Weik, R.W. (1967). Correlation of fatty acid structure with preferential order of urea complex formation. *J. Assoc. Off. Anal. Chem.*, 50: 1111-1118.
- Jackson, H.W. (1981). Techniques for flavor and odor evaluation of soy oil. *J. Am. Oil Chem. Soc.*, 58: 227-231.
- Jafar, S.S., Hultin, H.O., Bimbo, A.P., Crowther, J.B. and Barlow, S.M. (1994). Stabilization by antioxidants of mayonnaise made from fish oil. *J. Food Lipids*, 1: 295-311.
- Jandacek, R.J., Whiteside, J.A., Holcombe, B.N., Volpenhein, R.A. and Taulbee, J.D. (1987). The rapid hydrolysis and efficient absorption of triglycerides with octanoic acid in the 1 and 3 position and long chain fatty acid in the 2 position. *Am. J. Clin. Nutr.*, 45: 940-945.
- Jantti, J., Nikkari, T., Solakivi, T. and Vapaatalo, H. (1989). Evening primrose oil in rheumatoid arthritis: changes in serum lipids and fatty acids. *Ann Rheum. Diseases*, 48: 124-127.

- Jensen, G.L. and Jensen, R.G. (1992). Specialty lipids for infant nutrition:II. Concerns new developments and future applications. *J. Pediatr. Gastroenterol. Nutr.*, 15: 382-387.
- Jensen, J.L., McGarvey, N., Taraszewski, R., Wixson, S.K., Seiner, D.L., Pai, T., Yeh, Y.Y., Lee, T.W. and DeMichele, S.J. (1994). Lymphatic absorption of externally fed structured triacylglycerols vs. physical mixture in a canine model. *Am. J. Clin. Nutr.*, 60: 518-523.
- Johnson, M.M., Swan, D.D., Surette, M.E., Stagner, J., Chilton, T., Fonteh, A.N. and Chilton, F.H. (1997). Dietary supplementation with gamma-linolenic acid alters fatty acid content and eicosanoid production in healthy humans. *J. Nutr.*, 127: 1435-1444.
- Kalo, P., Huotari, H. and Antila, M. (1990). *Pseudomonas fluorescens* lipase-catalyzed interesterification of butter fat in the absence of a solvent. *Milchwissenschaft*, 45: 281-285.
- Kalo, P., Vaara, K. and Antila, M. (1986). Changes in triglyceride composition and melting properties of butter fat solid fraction/rapeseed oil mixtures induced by lipase catalyzed interesterification. *Fette Seifen Anstrichmittel*, 88: 362-365.
- Kamal-Elden, A. and Appelqvist, L. (1996). The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids*, 31: 671-701.
- Kanner, J., German, J.B. and Kinsella, J.E. (1987). Initiation of lipid peroxidation on biological systems. *CRC Crit. Rev. Food Sci. Nutr.*, 25: 317-364.
- Katan, M.B. (1995). Fish and heart disease. *New England J. Med.*, 332: 1024-1025.
- Ke, P.J. and Ackman, R.G. (1976). Metal-catalysed oxidation in mackerel skin and meat lipids. *J. Am. Oil Chem. Soc.*, 53: 636-640.
- Ke, P.J., Ackman, R.G., Linke, B.A. and Nash, D.M. (1977a). Differential lipid oxidation in various parts of frozen mackerels. *J. Food Technol.*, 12: 37-47.
- Kennedy, J.P. (1991). Structured lipids: fats of the future. *Food Technol.*, (45): 76-79.
- Khan, M.A. (1999). Oxidative stability of stripped and non-stripped borage and evening primrose oils and their oil-in-water emulsions. *MSc thesis, Memorial University of Newfoundland, St. John's, NF., Canada*.

- Khan, M.A. and Shahidi, F. (2000). Oxidative stability of stripped and non-stripped borage and evening primrose oils and their emulsions in water. *J. Am. Oil Chem. Soc.*, 77: 963-968.
- Kies, C. (1989). Evening primrose oil: a source of gamma linolenic acid. *Cereal Food World*, 34: 1016-1020.
- King, I., Childs, M.T., Dorsett, C., Ostrander, J.G. and Morsen, E.R. (1990). Shellfish: proximate composition, minerals, fatty acids and sterols. *J. Am. Diet. Assoc.*, 90: 677-685.
- King, M.F., Boyd, L.C. and Sheldon, B.W. (1992). Antioxidant properties of individual phospholipids in a salmon oil model system. *J. Am. Oil Chem. Soc.*, 69: 545-551.
- Kinsella, J.E. (1986). Food components with potential therapeutic benefits: the n-3 polyunsaturated fatty acids of fish oils. *Food Technol.*, 40: 89-97.
- Knapp, H.R. and Fitzgerald, G.A. (1989). The antihypertensive effects of fish oil. *New England J. Med.*, 320: 1037-1043.
- Kolanowski, W., Swiderski, F. and Berger, S. (1999). Possibilities of fish oil application for food products enrichment with ω -3 PUFA. *Int. J. Food Sci. Nutr.*, 50: 39-49.
- Kreulen, H.P. (1976). Fractionation and winterization of edible fats and oils. *J. Am. Oil Chem. Soc.*, 53: 393-396.
- Kromhout, D., Bosschieter, E.B. and Coulander, C.D.L. (1985). The inverse relation between fish consumption and 20-years mortality from coronary heart diseases. *New England J. Med.*, 312: 1205-1209.
- Kunkel, S.L., Ogawa, H., Ward, P.A. and Zurier, R.B. (1981). Suppression of chronic inflammation by evening primrose. *Prog. Lipid Res.*, 20: 885-888.
- Kwon, D.Y. and Rhee, J.S. (1986). A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *J. Am. Oil Chem. Soc.*, 63: 89-92.
- Labuza, T.P. (1971). Kinetics of lipid oxidation in foods, A review. *CRC Crit. Rev. Food Technol.*, 2: 355-405.
- Lee, A.G., East, J.M. and Froud, R.G. (1986). Are essential fatty acids essential for membrane function. *Prog. Lipid Res.*, 25: 41-46.

- Lee, C.C. and Hoseney, R.C. (1982). Optimization of the fat-emulsifier system and the gum-egg white-water system for a laboratory-scale single stage cake mix. *Cereal Chem.*, 59: 392-396.
- Lee, K. and Akoh, C.C. (1998). Structured lipids: synthesis and applications. *Food Rev. Int.*, 14: 17-34.
- Lee, K. and Akoh, C.C. (1998a). Characterization of enzymatically synthesized structured lipids containing eicosapentaenoic, docosahexaenoic and caprylic acids. *J. Am. Oil Chem. Soc.*, 75: 495- 499.
- Lee, K.H., Jung, M.Y. and Kim, S.Y. (1997). Quenching mechanism and kinetics of ascorbyl palmitate for the reduction of the photosensitized oxidation of oils. *J. Am. Oil Chem. Soc.*, 74: 1053-1057.
- Leifert, W.R., Jahangiri, A. and McMurchie, E.J. (1999). Antiarrhythmic fatty acids and antioxidants in animal and cell studies. *J.Nutr. Biochem.*, 10: 252-267.
- Leu, S., Jhaver, S.N., Karakoltsidis, P.A. and Constantinides, S.M. (1981). Atlantic mackerel: Seasonal variations in proximate composition and distribution of chemical nutrients. *J. Food Sci.*, 46: 1635-1638.
- Li, Y.T. and Regenstein, J.M. (1990). Use of fish oil in mayonnaise. In *Advances in Fisheries Technology and Biotechnology for increased Profitability*, ed. M. Voigt and J.R. Botta, Technomic Publishing Inc., Lancaster, PA, pp. 155-161.
- Lichtenstein, A.H. (1999). Dietary fat: a history. *Nutrition Reviews*, 57: 11-14.
- Lindeberg, S. (1994). *Apparent absence of cerebrocardiovascular disease in Melanesians*. Lund University, Lund, Sweden
- Lindskov, R. and Holmer, G. (1992). Polyunsaturated fatty acids in plasma, red blood cells and mononuclear cell phospholipids of patients with atopic dermatitis. *Allergy*, 47: 517-521.
- Logani, M.K. and Davies, R.E. (1980). Lipid oxidation: biological effects and antioxidants. A review. *Lipids*, 15: 485-495.
- Love, J. (1985). Factors affecting lipid oxidation. In *Flavour Chemistry of Fats and Oils*, ed. D.B. Min and T.H. Smouse, American Oil Chemists' Society, Champaign, IL, pp. 101-108.

- Love, J.A. (1996). In *Bailey's Industrial Oil and Fat Products*, 5th edition, pp. 10-13. New York, NY: Wiley
- Lovern, J.A. (1964). The lipids of marine organisms. *ocenogr. Mar. Biol. Ann. Rev.*, 2: 169-191.
- Lowry, R.R. and Tinsley, I.J. (1976). Rapid colorimetric determination of free fatty acids. *J. Am. Oil Chem. Soc.*, 53: 470-472.
- Macrae, A.R. (1983). Lipase-catalyzed interesterification of oils and fats. *J. Am. Oil Chem. Soc.*, 60: 291-294.
- Mangos, T.J., Jones, K.C. and Foglia, T.A. (1999). Lipase-catalyzed synthesis of structured low-calorie triacylglycerols. *J. Am. Oil Chem. Soc.*, 76: 1127-1132.
- Manku, M.S., Horrobin, D.F., Morse, N.L., Wright, S. and Burton, J.L. (1984). Essential fatty acids in the plasma phospholipids of patients with atopic eczema. *Br. J. Dermatol.*, 110: 643-648.
- Marangoni, A.G. and Rousseau, D. (1995). Engineering triacylglycerols: the role of interesterification. *Trends in Food Sci. and Technol.*, 6: 329-335.
- Markley, K.S. (1964). Techniques of separation. Distillation, salt solubilities methods and low temperature crystallization. In *Fatty Acids*, 2nd edn., Part III, ed. K.S. Markley, Interscience Publishers, New York, NY, pp. 1983-2123.
- Mascioli, E.D., Babayan, V.K., Bistran, B.R. and Blackburn, G.L. (1988). Novel triglycerides for medical purposes. *J. Parent. Enteral nutr.*, 12: 127S-130S.
- Mason, R.L., Gunst, F.R. and Hees, J.L. (1989). Response-surface designs. In *Statistical Design and Analysis of Experiments*, ed. Wiley, New York, NY, pp. 204-233.
- Mason, R.L., Gunst, R.F. and Hess, J.L. (1989). Response-surface designs. In *Statistical Design and Analysis of Experiments*, Wiley, New York, pp. 204-233.
- Mason, R.L., Gunst, R.F. and Hess, J.L. (1989). In *Statistical Design and Analysis of Experiments with Applications to Engineering and Science*, ed. John Wiley, New York, NY,
- McKenna, M.C., Hubbard, V.S. and Pieri, J.G. (1985). Linoleic acid absorption for lipid supplementation in patients with cystic fibrosis with pancreatic insufficiency and in control subjects. *J. Pediatr. Gastroenterol. Nutr.*, 4: 45-50.

- McNeill, G.P., Shimizu, S. and Yamane, T. (1991). High- yield enzymatic glyceride oils. *J. Am. Oil Chem. Soc.*, 68: 1-6.
- McNeill, G.P., Shimizu, S. and Yamane, T. (1991). High-yield glycerolysis of fats and oils. *J. Am. Oil Chem. Soc.*, 68: 1-5.
- McNeill, G.P. and Sonnet, P.E. (1995). Low-calorie triglyceride synthesis by lipase-catalyzed esterification of monoglycerides. *J. Am. Oil Chem. Soc.*, 72: 1301-1307.
- Meland, E., Fugelli, P., Lerum, E. and Sandvik, R. (1989). Effect of fish oil on blood pressure and blood lipids in men with mild to moderate hypertension. *Scand. J. Prim. Health Care*, 7: 131-135.
- Menzel, D.B. and Olcott, H.S. (1964). Positional distribution of fatty acids in fish and other animal lecithins. *Biochem. Biophys. Acta.*, 84: 133-137.
- Meusel, D., Muschter, A., Tuelsner, M. and Paul, W. (1990). Lipase-catalyzed exchange of fatty acids in the manufacture of shortenings. *Lebensmittelindustrie*, 37: 63-66.
- Meyer, L. 1987. *Verfahren zur Herstellung eines hydrolysierten lecithins*. Eur. Patent Appl.No. 0260573
- Miller, C.C., Austin, H., Posorske, L. and Gonzalez, J. (1988). Characteristics of an immobilized for the commercial synthesis of esters. *J. Am. Oil Chem. Soc.*, 65: 927-931.
- Miller, C.C. and Ziboh, V.A. (1988). γ -Linolenic acid-enriched diet alters cutaneous eicosanoids. *Biochem. Biophys. Res. Commun.*, 154: 967-974.
- Miller, C.C., Ziboh, V.A., Wong, T. and Fletcher, M.P. (1990). Dietary supplement with oils rich in n-3 and n-6 fatty acids influence *in vivo* levels of epidermal lipoxygenase products in guinea pigs. *J. Nutr.*, 120: 36-44.
- Miller, S.A. (1995). Biochemistry and nutrition of low-calorie triglycerides. *INFORM*, 6: 461-465.
- Min, D.B. (1998). Lipid oxidation of edible oils. In *Food Lipids- Chemistry, Nutrition and Biotechnology*, ed. C.C. Akoh and D.B. Min, Marcel Dekker Inc., New York, NY, pp. 283-295.
- Min, D.B., Lee, S.H. and Lee, E.C. (1989). Singlet oxygen oxidation of vegetable oils. In *Flavour Chemistry of Fats and Oils*, ed. D.B. Min and T.H. Smouse, American Oil Chemists' Society, Champaign, IL, pp. 57-97.

- Miyashita, K., Fujimoto, K. and Keneda, T. (1982). Formation of dimers during the initial stage of autoxidation in methyl linoleate. *Agric. Biol. Chem.*, 46: 751-755.
- Mok, K.T., Maiz, A., Yamakazi, K., Sobrado, J., Babayan, V.K., Moldawen, L., Bistran, B.R. and Blackburn, G.L. (1984). Structured medium chain and long chain triglyceride emulsions are superior to physical mixtures in sparing body protein in the burned rat. *Metabolism*, 33: 910-916.
- Montgomery, D.C. (1991). Response-surface methods and designs. In *Designs and Analysis of Experiments*, 3rd ed, Wiley, New York, pp. 521-568.
- Morice, I.M. (1967). Seed fats of *Astelia* and *Collospermum*, family Liliaceae. *J. Sci. Food Agric.*, 18: 343-346.
- Mortimer, B.C., Simmonds, W.J., Joll, C.A., Stick, R.V. and Redgrave, T.G. (1988). Regulation of the metabolism of lipid emulsion model lipoproteins by saturated acyl chain at the 2-position of the triacylglycerol. *J. Lipid Res.*, 29: 713-720.
- Moussata, C.O. and Akoh, C.C. (1997). Lipase-catalyzed incorporation of oleic acid into melon seed oil. *J. Am. Oil Chem. Soc.*, 74: 177-179.
- Moussata, C.O. and Akoh, C.C. (1998). Influence of lipase-catalyzed interesterification on the oxidative stability of melon seed oil triacylglycerols. *J. Am. Oil Chem. Soc.*, 75: 1155-1159.
- Mukherjee, K.D. (1990). Lipase-catalyzed reactions for modification of fats and other lipids. *Biocatalysis*, 3: 277-281.
- Mukherjee, K.D. (1995). Fractionation of fatty acids and other lipids via lipase-catalyzed reactions. *Oleag. Corps. Gras Lipides*, 2: 365-370.
- Mukherjee, K.D. and Kiewitt, I. (1991). Enrichment of γ -linolenic acid from fungal oil by lipase-catalyzed reactions. *Appl. Microbiol. Biotechnol.*, 35: 579-584.
- Mutua, L.N. and Akoh, C.C. (1993). Lipase-catalyzed modification of phospholipids: incorporation of n-3 fatty acids into biosurfactant. *J. Am. Oil Chem. Soc.*, 70: 125-128.
- Myher, J.J., Kuksis, A., Park, P.W. and Diersen-Schade, D.A. (1996). Sterospecific analysis of triacylglycerols rich in long-chain polyunsaturated fatty acids. *J. Am. Oil Chem. Soc.*, 31: 207-215.

- Nawar, W.W. (1985). Lipids. In *Food Chemistry*, ed. O.R. Fennema, Marcel Dekker, New York, NY, pp. 139-244.
- Neff, W.E., Mounts, T.L. and Rinsch, W.M. (1997). Oxidative stability as affected by triacylglycerol composition and structure of purified canola oil triacylglycerols from genetically modified normal and high stearic acid and lauric acid canola varieties. *Lebensm.-Wiss.-u.-Technol.*, 30: 793-799.
- Neff, W.E., Mounts, T.L., Rinsch, W.M. and Konishi, H. (1993). Photooxidation of soyabean oils as affected by triacylglycerol composition and structures. *J. Am. Oil Chem. Soc.*, 70: 163-166.
- Neilson, T. (1985). Industrial application possibilities of lipase. *Fat Sci. Technol.*, 87: 15-19.
- Nestel, P.J. (1990). Effects of n-3 fatty acids on lipid metabolism. *Ann. Rev. Nutr.*, 10: 149-167.
- Nettleton, J.A. (1985). In *Seafood Nutrition*, ed. Van Nostrand Reinhold, New York, NY, pp. 55-80.
- Nielson, G.L., Faarvang, K.L., Thomsen, B.S., Teglbjaerg, K.L., Dierberg, J. and Ernst, E. (1992). The effects of dietary supplementation with n-3 polyunsaturated fatty acids in patients with rheumatoid arthritis: a randomized, double blind trial. *Eur. J. Clin. Invest.*, 22: 687-691.
- Nielson, T. (1985). Industrial application possibilities for lipase. *Fette Seifen Anstrichmittel*, 87: 15-19.
- Nordoy, A. (1999). Dietary fatty acids and coronary heart disease. *Lipids*, 34: S19-S22.
- O'Carroll, P. (1995). Oils & Fats - Impact of R&D. *The World of Ingredients*, 5: 32-36.
- Osterberg, E., Blomstrom, A.C. and Holmberg, K. (1989). Lipid catalyzed transesterification of unsaturated lipids in a microemulsion. *J. Am. Oil Chem. Soc.*, 66: 1330-1337.
- Ota, T., Kawabata, Y. and Ando, Y. (1994). Positional distribution of 24:6 (n-3) in triacylglycerols from flathead flounder liver and flesh. *J. Am. Oil Chem. Soc.*, 71: 475-478.

- Parrish, C.C. (1987). Separation of aquatic lipid classes by chromarod thin-layer chromatography with measurement by iatrosan flame ionization detection. *Can. J. Fish. Aquat. Sci.*, 44:722-731.
- Patterson, H.B.W. (1989). Safeguarding quality and yield. In *Handling and Storage of oilseeds, oils, fats and meals*, ed. H.B.W. Patterson, Elsevier Applied Science, New York, NY, pp. 1-86.
- Peat, J.K. (1996). Prevention of asthma. *Eur Respir J*, 9: 1545-1555.
- Piconneaux, A., Grandgirard, A. and Sebedio, J. (1985). Identification of an unusual polyunsaturated fatty acid in liver of rats fed with heated linseed oil. *Comptes Rendus de l'Académie des Sciences. Series 3. Science de la Vie*, 300: 353-358.
- Pigott, G.M. and Tucker, B.W. (1987). Science opens new horizons for marine lipids in human nutrition. *Food Rev. Int.*, 3: 105-138.
- Porter, N.A., Lehman, L.S., Weber, B.A. and Smith, K.J. (1981). Unified mechanisms for polyunsaturated fatty acid autoxidation. Comparison of peroxy radical hydrogen atom abstraction. Beta scission and cyclization. *J. Am. Chem. Soc.*, 103: 6447-6455.
- Quinlan, P. and Moore, S. (1993). Modification of triglycerides by lipases: process technology and its application to the production of nutritionally improved fats. *INFORM*, 4: 580-585.
- Raharjo, S. and Sofos, J.N. (1993). Methodology for measuring malonaldehyde as a product of lipid peroxidation in muscle tissues: A review. *Meat Sci.*, 35: 145-169.
- Rahmatullah, M.S.K., Shukla, V.K.S. and Mukherjee, K.D. (1994a). Enrichment of gamma-linolenic acid from evening primrose oil and borage oil via lipase-catalyzed hydrolysis. *J. Am. Oil Chem. Soc.*, 71: 569-573.
- Rahmatullah, M.S.K., Shukla, V.K.S. and Mukherjee, K.D. (1994b). γ -linolenic acid concentrates from borage and evening primrose oil fatty acids via lipase-catalysed esterification. *J. Am. Oil Chem. Soc.*, 71: 563-567.
- Rangheard, N.S., Langrand, G., Triantaphylides, C. and Baratti, J. (1989). Multicomponent enzymatic reactions in organic media: a simple test for the determination of lipase specificity. *Biochem. Biophys. Acta*, 20: 1004-1010.

- Ratnayake, W.M., B.Olsson, Matthews, D. and Ackman, R.G. (1988). Preparation of omega-3 fatty PUFA concentrates from fish oil via urea complexation. *Fat Sci. Technol.*, 90: 381-386.
- Redgrave, T.G., Kodali, D.R. and Small, D.M. (1988). The effects of triacyl-*sn*-glycerol structure on the metabolism of chylomicron and triacylglycerol-rich emulsions in the rat. *J. Biol. Chem.*, 263: 5118-5123.
- Roberts, J.B. and Stevens, R. (1963). The seed fat of hops (*Humulus lupulus*). *Chem. Ind.*, 1963: 608-609.
- Robinson, G.K., Alston, M.J., Knowles, C.J. and Cheetham, P.S.J. (1994). Investigation into the factors influencing lipid-catalyzed intramolecular microaqueous systems. *Enzyme Microb. Technol.*, 16: 855-892.
- Rossell, B. (1991). How to measure oxidative rancidity in fats and fatty foods. *Lipid Tech.*, Oct-Dec: 122-126.
- Saito, H. (1997). Evaluation method for lipid oxidation by nuclear magnetic resonance. In *Flavor and Lipid Chemistry of Seafoods*, ed. F. Shahidi and K.R. Cadwallader, American Chemical Society, Washington, DC, pp. 219-239.
- Saito, H. and Nakamura, K. (1990). Application of the NMR method to evaluate the oxidative deterioration of crude and stored fish oils. *Agric. Biol. Chem.*, 54: 533-534.
- Saito, H. and Udagawa, M. (1992). Applications of nuclear magnetic resonance to the evaluate the oxidative deterioration of brown fish meal. *J. Sci. Food Agric.*, 58: 135-137.
- Sanders, T.A.B. (1991). Influence of omega-3 fatty acids on blood lipids. *World Rev Nutr Diet.*, 66: 358-366.
- SAS Institute, I. (1990). Statistical Analytical Systems User's Guide. SAS Institute, Inc, Cary, NC
- Schlenk, H. (1961). Crystallization of fatty acids. *J. Am. Oil Chem. Soc.*, 38: 728-736.
- Schnepf, M., Spencer, G. and Carlat, J. (1991). Chemical and sensory characteristics of stored menhaden oil/soyabean oil blends. *J. Am. Oil Chem. Soc.*, 68: 281-284.

- Schuch, R. and Mukherjee, K.D. (1988). Interesterification of lipids by an *sn*-1,3-specific triacylglycerol lipase. In *World Conference on Biotechnology for the Fats and Oils Industry*, ed. V.K.S. Shukla and G. Holmer, American Oil Chemist's Society, Champaign, IL, pp. 328.
- Scott, D. (1989). Speciality enzymes and products for food industry. In *Biocatalysts in Agricultural Biotechnology*, ed. J.R. Whitaker and P.E. Sonnet, ACS Symposium Series 389, Washington, pp. 176-192.
- Senanayake, N. and Shahidi, F. (2002). Oxidative stability of structured lipids produced from borage (*Borago officinalis* L.) and evening primrose (*Oenothera biennis* L.) oils with docosahexaenoic acid. *J. Am. Oil Chem. Soc.*,
- Senanayake, S.P.J.N. and Shahidi, F. (1999a). Enzymatic incorporation of docosahexaenoic acid into borage oil. *J. Am. Oil Chem. Soc.*, 76: 1009-1015.
- Senanayake, S.P.J. and Shahidi, F. (1999b). Enzyme-assisted acidolysis of borage and evening primrose oil: incorporation of omega-3 polyunsaturated fatty acids. *J. Agric. Food Chem.*, 47: 3105-3112.
- Senanayake, S.P.J.N. and Shahidi, F. (2000a). Concentration of docosahexaenoic acid (DHA) from algal oil via urea complexation. *J. Food Lipids*, 7: 51-61.
- Senanayake, S.P.J.N. and Shahidi, F. (2000b). Structured lipids containing long-chain omega-3 polyunsaturated fatty acids. In *Seafood in Health and Nutrition. Transformations in Fisheries and Aquaculture: Global Perspectives*, ed. F. Shahidi, Science Tech Publishing, St. John's, NF, pp. 29-44.
- Sensdoni, A., Bortolussi, G., Orlando, C. and Fantozzi, P. (1996). Borage oil (*Borago officinalis* L.)- an important source of gamma-linolenic acid. 2. Tocopherols and chlorophyll content and sensory analysis of borage oils extracted by different techniques and blended with extra virgin oil. *Industry Alimentary*, 35: 664-669.
- Shahidi, F. and Dunajski, E. (1994). Lipid fatty acids, growth and compositional characteristics of farmed cod (*Gadus morhua*). *J. Food Lipids*, 1: 265-271.
- Shahidi, F. and Pegg, R.B. (1994). Hexanal as an indicator of meat flavour deterioration. *J. Food Lipids*, 1: 177-186.
- Shahidi, F. and Spurvey, S.A. (1996). Oxidative stability of fresh and heat-processed dark and light muscles of mackerel (*Scomber scombrus*). *J. Food Lipids*, (3): 13-25.

- Shahidi, F. and Wanasundara, P.K.J. (1992). Phenolic antioxidants. *CRC Crit. Rev. Food Sci. Nutr.*, 32: 67-103.
- Shahidi, F., Wanasundara, P.K.J. and Hong, C. (1991). Antioxidant activity of phenolic compounds in meat model systems. In *Phenolic Compounds in Food and Their Effects on Health I, Analysis, Occurrence and Chemistry*, ed. C.T. Ho, C.Y. Lee and M.T. Huang, American Chemical Society, Washington, DC, pp. 214-222.
- Shahidi, F., Wanasundara, U.N. and Brunet, N. (1994). Oxidative stability of oil from blubber of harp seal (*Phoca groenlandica*) as assessed by NMR and standard procedures. *Food Research International*, 27: 555-562.
- Sherwin, E.R. (1990). Oxidation and antioxidants in fat and oil processing. *J. Am. Oil Chem. Soc.*, 55: 809-814.
- Shieh, C.J., Akoh, C.C. and Koehler, P.E. (1995). Four factor response surface optimization of the enzymatic modification of triolein to structured lipids using capric acid. *J. Am. Oil Chem. Soc.*, 72: 619-623.
- Shimada, Y., Maruyama, K., Sugihara, A., Morivama, S. and Tominaga, Y. (1997). Purification of docosahexaenoic acid from tuna oil by a two-step enzymatic method: hydrolysis and selective esterification. *J. Am. Oil Chem. Soc.*, 74: 1441-1446.
- Shimada, Y., Sakai, N., Sugihara, A., Fujita, H., Honda, Y. and Tominaga, Y. (1998). Large-scale purification of γ -linolenic acid by selective esterification using *Rhizopus deleamar* lipase. *J. Am. Oil Chem. Soc.*, 75: 1539-1544.
- Shimada, Y., Suenaga, M., Sugihara, A., Nakai, S. and Tominaga, Y. (1999). Continuous production of structured lipid containing γ -linolenic acid and caprylic acids by immobilized *Rhizopus deleamar* lipase. *J. Am. Oil Chem. Soc.*, 76: 189-193.
- Shimada, Y., Sugihara, A., Shibahiraki, M., Fujita, H., Nakano, H., Nagao, T., Terai, T. and Tominaga, Y. (1997). Purification of γ -linolenic acid from borage oil by a two-step enzymatic method. *J. Am. Oil Chem. Soc.*, 74: 1465-1470.
- Shimizu, S., Kawashima, H., Akimoto, K., Shinmen, Y. and Yamada, H. (1989). Microbial conversion of an oil containing alpha-linolenic acid to an oil containing eicosapentaenoic acid. *J. Am. Oil Chem. Soc.*, 66: 342-347.
- Simopoulos, A., Kifer, R. and Martin, R. (1987). In *Health Effects of Dietary Polyunsaturated Fatty Acids in Seafoods*, ed. Academic Press, New York, NY

- Simopoulos, A.P. (1990). Omega-3 fatty acids in growth and development. In *Omega-3 Fatty Acids in Health and Disease*, ed. R.S. Lees and M. Karel, Dekker, New York, NY, pp. 115-156.
- Simopoulos, A.P. (1991). Omega-3 fatty acids in health and disease and in growth and development. *Am. J. Clin. Nutr.*, 54: 439-463.
- Simopoulos, A.P. (1997). Essential fatty acids in health and chronic disease. *Food Rev. Int.*, 13: 623-631.
- Simopoulos, A.P., Kifer, R.R. and Martin, R.E. (1986). In *Health Effects of Polyunsaturated Fatty Acids in Seafoods*, ed. Academic Press, New York, NY,
- Singh, G. and Chandra, R.K. (1988). Biochemical and cellular effects of fish and fish oils. *Prog. Food Nutr. Sci.*, 12: 371-419.
- Singleton, W.S. (1960). Solution properties. In *Fatty Acids, 2nd edn., Part I*, ed. K.S. Markley, Interscience Publishers, New York, NY, pp. 609-678.
- Sobrado, J., Moldawer, L.L., pompeselli, J.J., Mascioli, E.A., Babayan, V.K., Bistran, B.R. and Blackburn, G.L. (1985). Lipid emulsions and reticuloendothelial system function in healthy and burned guinea pigs. *Am. J. Clin. Nutr.*, 42: 855-890.
- Spielman, D., Bracco, V., Traitler, H., Crozier, G., Holman, R., Ward, M. and Cotter, R. (1988). Alternative lipids to usual ω 6 PUFAS: γ -linolenic acid, α -linolenic acid, Stearidonic acid, EPA, etc. *J. Enteral and Parenteral Nutr.*, 12: 1115-1119.
- Sprecher, H. (1981). Biochemistry of essential fatty acids. *Prog. Lipid Res.*, 20: 13-22.
- Spurvey, S. and Shahidi, F. (2000). Production and optimization of gamma-linolenic acid (gla) concentrate from borage oil. *J. Food Lipids*, 7: 163-174.
- Stansby, M.E. (1967). In *Fish Oils- Their Chemistry, Technology, Stability, Nutritional Properties and Uses*, ed. M.E. Stansby, The AVI Publishing Company, Westport, CT
- Stansby, M.E. (1990). Deterioration. In *Fish Oils in nutrition*, ed. M.E. Stansby, AVI Publishers Co Inc., Westport, CT, pp. 120-140.
- Stout, V.F., Nilsson, W.B., Krzynowek, J. and Schlenk, H. (1990). Fractionation of fish oils and their fatty acids. In *Fish Oils in Nutrition*, ed. M.E. Stansby, Van Nostrand Reinhold, New York, NY, pp. 73-119.

- Strange, E.D., Konstance, R.P., Lu, D., Smith, P.W., Onwulata, C.I. and Holsinger, V.H. (1997). Oxidative and functional stability during storage of butter oil encapsulated with sucrose or flour. *J. Food Lipids*, 4: 245-260.
- Strocchi, A. and Bonaga, G. (1975). Correlation between urea inclusion compounds and conformational structure of unsaturated C-18 fatty acid methyl esters. *Chem.Phys. Lipids*, 15: 87-94.
- Takahata, K., Monobe, K., Tada, M. and Weber, P.C. (1998). The benefits and risks of n-3 polyunsaturated fatty acids. *Biosci. Biotechnol. Biochem.*, 62: 2079-2085.
- Tanaka, A., Kawamoto, T., Kawase, M., Nanko, T. and Sonomoto, K. (1988). Immobilized lipase in organic solvents. In *World Conference on Biotechnology for the Fats and Oils Industry*, ed. V.K.S. Shukla and G. Holmer, American Oil Chemist's Society, Champaign, IL, pp. 123.
- Tanaka, T., Ono, E., Ishihara, M. and Yamanaka, S. (1981). Enzymatic acyl exchange of triglycerides in n-hexane. *Agric. Biol. Chem.*, 45: 2387-2389.
- Teo, T.C., DeMichele, S.J., Selleck, J.M., Babayan, V.K., Blackburn, G.L. and Bistran, R.B. (1989). Administration of structured lipid composed of MCT and fish oil reduces net protein catabolism in enterally fed burned rats. *Ann. Surg.*, 210: 100-106.
- Teo, T.C., Selleck, J.M., Wang, J.M.F., Pomposelli, J.J., Babayan, V.K., Blackburn, G.L. and Bistran, R.B. (1991). Long term feeding with structured lipid composed of medium-chain and n-3 fatty acids ameliorates endotoxic shock in guinea pigs. *Metabolism*, 40: 1152-1160.
- Thein, F.C.K., Mencia-Huerta, J. and Lee, T.H. (1993). Dietary fish oil effects on seasonal hay fever and asthma in pollen-sensitive subjects. *Am. Rev. Respir. Dis.*, 147: 1138-1143.
- Thomas, A.J. and Patton, S. (1972). Phospholipids of fish gills. *Lipids*, 7: 76-77.
- Timms, R.E. (1997). Fractation. In *Lipid Technologies and Applications*, ed. F.D. Gunstone and F.B. Padley, Dekker, New York, NY, pp. 199-222.
- Tinker, L.F., Parks, E.J., Behr, S.R., Schneeman, B.O. and Davis, P.A. (1999). (n-3) fatty acid supplementation in moderately hypertriglyceridemic adults changes postprandial lipid and apolipoprotein B responses to a standardized test meal. *Amer. Soc. for Nutr. Sci.*, 1999: 1126-1134.

- Tomarelli, R.M., Meyer, B.J., Weaver, J.R. and Bernhart, F.W. (1968). Effects of positional distribution of the absorption of the fatty acids of human milk and infant formulas.
- Tomobe, Y.I., Morazawa, K., Tsuchida, M., Hibino, H., Nakano, Y. and Tanaka, Y. (2000). Dietary docosahexaenoic acid suppresses inflammation and immunoresponses in contact hypersensitivity reaction in mice. *J. Am. Oil Chem. Soc.*, 35: 61-69.
- Traitler, H., Wille, H.J. and Studer, A. (1988). Fractionation of blackcurrant seed oil. *J. Am. Oil Chem. Soc.*, 65: 755-760.
- Traitler, H., Winter, H., Richli, U. and Ingenbleek, Y. (1984). Characterization of γ -linolenic acid in ribes seed. *Lipids*, 19: 923-928.
- Tsevegsuren, N. and Aitzetmuller, K. (1993). γ -Linolenic acid in Anemone spp. seed lipids. *Lipids*, 28: 841-846.
- Tsevegsuren, N. and Aitzetmuller, K. (1996). γ -Linolenic acid and stearidonic acids in mongolian boraginaceae. *J. Am. Oil Chem. Soc.*, (73): 1681-1684.
- Uauy-Dagach, R. and Valenzuela, A. (1996). Marine oils: the health benefits of n-3 fatty acids. *Nutrition Reviews*, 54: S102-S108.
- Valenzuela, A. and Uauy, R. (1999). Consumption patterns of dietary fats in Chile: n-6 and n-3 fatty acids. *International Journal Food Sciences and Nutrition*, 50: 127-133.
- Van-Rollins, M. and Murphy, R.C. (1984). Autoxidation of docosahexaenoic acid: analysis of the isomers of hydroxy docosahexaenoate. *J. Lipid Res.*, 25: 507-517.
- Wanasundara, U.N. and Shahidi, F. (1993). Application of NMR spectroscopy to assess oxidative stability of canola and soyabean oils. *J. Food Lipids*, 1: 15-24.
- Wanasundara, U.N. and Shahidi, F. (1996a). Stabilization of seal blubber and menhaden oils with green tea catechins. *J. Am. Oil Chem. Soc.*, 73: 1183-1190.
- Wanasundara, U.N. and Shahidi, F. (1997). Positional distribution of fatty acids in triacylglycerols of seal blubber oil. *J. Food Lipids*, 4: 51-64.
- Wanasundara, U.N. and Shahidi, F. (1998). Lipase-assisted concentration of n-3 polyunsaturated fatty acids in acylglycerols from marine oils. *J. Am. Oil Chem. Soc.*, 75: 945-951.

- Wanasundara, U.N. and Shahidi, F. (1999). Concentration of omega-3 polyunsaturated fatty acids in seal blubber oil by urea complexation of reaction conditions. *Food Chem.*, 65: 683-688.
- Wanasundara, U.N., Shahidi, F. and Jablonski, J.R. (1995). Comparison of standard and NMR methodologies for assessment of oxidative stability of canola and soyabean oils. *Food Chem.*, 52: 249-253.
- Wattenburg, L.W. (1986). Protective effects of 2(3)-tert-butyl-4-hydroxy anisole on chemical carcinogenesis. *Food Chem. Toxicol.*, 24: 1099-1102.
- Weber, P.C. and Leaf, A. (1991). Cardiovascular effects of omega-3 fatty acids. *World Rev Nutr Diet.*, 66: 218-232.
- Weihrauch, J.L. and Son, Y.S. (1983). The phospholipid content of food. *J. Am. Oil Chem. Soc.*, 60: 1971-1978.
- Whpkey, A., Simon, J.E. and Janick, J. (1988). *In vivo* and *in vitro* lipid accumulation in *Borago officinalis* L. *J. Am. Oil Chem. Soc.*, 65: 979-984.
- Williams, S.R. and Anderson, S.L. (1993). Lipids. In *Nutrition and Diet Therapy*, ed. S.R. Williams and S.L. Anderson, Mosby-Year Book, St Louis, MO, pp. 65-75.
- Willis, W.M. and Marangoni, A.G. (1998). Enzymatic interesterification. In *Food Lipids-Chemistry, nutrition and Biotechnology*, ed. C.C. Akoh and D.B. Min, Marcel Dekker Inc., New York, NY, pp. 665-698.
- Wisdom, R.A., Dunnill, P. and Lilly, M.D. (1984). Enzymatic interesterification of fats: factors influencing the choice of support for immobilized lipase. *Enzyme Microb. Technol.*, 6: 443-446.
- Wisdom, R.A., Dunnill, P. and Lilly, M.D. (1987). Enzymatic interesterification of fats: laboratory and pilot-scale studies with immobilized lipase from *Rhizopus arrhizus*. *Biotechnol., Bioeng.*, 29: 1081-1085.
- Wolfe, R.B., Kleiman, R. and England, R.E. 1983. *J. Am. Oil Chem. Soc.*, 60: 1858-1862.
- Wright, S. and Burton, L.J. (1982). Oral evening primrose oil improves atopic eczema. *Lancet*, 2: 1120-1123.

- Yadwad, V.B., Ward, O.P. and Noronha, L.C. (1991). Application of lipase to concentrate the docosahexaenoic acid fraction of fish oil. *Biotechnol., Bioeng.*, 38: 956-959.
- Yamane, T., Suzuki, T., Sahashi, Y., Vikersveen, L. and Hoshino, T. (1992). Production of n-3 polyunsaturated fatty acid-enriched fish oil by lipase-catalyzed acidolysis without solvent. *J. Am. Oil Chem. Soc.*, 69: 1104-1107.
- Yamauchi, R., Yamada, T., Kato, K. and Ueno, Y. (1983). Monohydroperoxides formed by autoxidation and photosensitized oxidation of methyl eicosapentaenoate. *Agric. Biol. Chem.*, 47: 2897-2902.
- Yasaei, P.M., Yang, G.C., Warner, C.R., Daniels, D.H. and Ku, Y. (1996). Singlet oxygen oxidation of lipids resulting from photochemical sensitizers in the presence of antioxidants. *J. Am. Oil Chem. Soc.*, 73: 1177-1181.
- Yee, L.E., Akoh, C.C. and Phillips, R.S. (1997). Lipase PS-catalyzed transesterification of citronellyl butyrate and geranyl caproate: effects of reaction parameters. *J. Am. Oil Chem. Soc.*, 74: 255-260.
- Yokochi, T., Usita, M.T., Kamisaka, Y., Nakahara, T. and Suzuki, O. (1990). Increase in the γ -linolenic acid content by solvent winterization of fungal oil extracted from *Mortierella* Genus. *J. Am. Oil Chem. Soc.*, 67: 846-851.
- Yokozeki, K., Yamanaka, S., Takinami, K., Hirose, Y., Tanaka, A., Sonomoto, K. and Fukui, S. (1982). Application of immobilized lipase to regio-specific interesterification of triglyceride in organic solvent. *Eur. J. Appl. Microbiol. Biotechnol.*, 14: 1-5.
- Yu, T.C. and Sinnhuber, R.O. (1967). An improved 2-thiobarbituric acid (TBA) procedure for the measurement of autoxidation in fish oils. *J. Am. Chem. Soc.*, 44: 256-258.
- Zurier, R.B., Rossetti, R.G., Jacobson, E.W., Demarco, D.M., Liu, N.Y., Temming, L.E., White, B.M. and Laposata, T.E. (1996). γ -Linolenic acid treatment of rheumatoid arthritis - a randomized placebo-controlled trial. *Arthri. Rheumat.*, 39: 1808-1817.

PUBLICATIONS

- Spurvey, S., Senanayake, S.P.J.N. and Shahidi, F. (2001) Enzyme-Assisted Acidolysis of Menhaden and Seal Blubber Oils With Gamma Linolenic Acid, Amer. Oil Chem. Soc., 78, 1105-1112
- Spurvey, S and Shahidi, F. (2000) Production and optimization of gamma linolenic acid (GLA) concentrate from borage oil. J. Food Lipids, 7, 163-174.

