MARINE OILS
STABILIZATION, STRUCTURAL CHARACTERIZATION AND OMEGA-3 FATTY ACID CONCENTRATION

UDAYA NAYANAKANTHA WANASUNDARA

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STABILIZATION, STRUCTURAL CHARACTERIZATION AND OMEGA-3 FATTY ACID CONCENTRATION

BY

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THIS WORK IS DEDICATED TO

MY LOVING PARENTS, WIFE & SON

ABSTRACT

Marine oils are obtained from the flesh of fatty fish, liver of lean fish and blubber of marine mammals. Seal blubber, a major product from the seal fishery, is abundantly available in Newfoundland and Labrador, but its characteristics have not been adequately defined. In this work, seal blubber oil (SBO) and cod liver oil (CLO) were extracted and refined under laboratory conditions. During each step of refining, oils were assessed for their oxidative stability by accelerated oxidation under Schaal oven conditions at 65°C over a 6 days period. Progression of oxidation was monitored by employing peroxide value (PV) determinations and 2-thiobarbituric acid reactive substances (TBARS) tests. Oxidative stability of processed oils after alkali-refining, degumming and deodorization steps was lower than that of crude oils, which is due in part to the removal of natural antioxidative compounds during refining. Oxidative stability of refined-bleached and deodorized (RBD) SBO and CLO was compared with that of commercially available menhaden oil (MHO); SBO exhibited a higher oxidative stability than CLO or MHO, perhaps due to its low content of polyunsaturated fatty acids (PUFA).

Different procedures were examined for improving the oxidative stability of SBO and/or MHO. Particularly emphasis was placed on the use of natural antioxidants. Dechlorophyllized green tea extracts (DGTE, 100 to 1000 ppm), individual tea catechins [(-)epicatechin (EC), (-) epigallocatechin (EGC), (-)epigallocatechin gallate (EGCG), (-)epicatechin gallate (ECG)] and commercially-available flavonoids (apigenin, kaempferol, morin, myricetin, naringin, naringenin, quercetin, rutin and taxifolin) at 200

ppm levels were added to both test oils. Oxidative stability of treated oils was determined and compared to those treated with conventional antioxidants such as α-tocopherol at 500 ppm and BHA, BHT and TBHQ at 200 ppm levels. Progression of oxidation was monitored by employing weight gain, PV and TBARS tests. DGTE at 500 and 1000 ppm and individual tea catechins at 200 ppm exhibited better antioxidant activity than α-tocopherol (500 ppm), BHA and BHT (200 ppm) in these oils. Antioxidant activity of individual tea catechins in both oils was in the order of ECG > EGCG > EGC > EC; antioxidant activity of ECG was slightly better than that of TBHQ (200 ppm). Among flavonoids tested, myricetin, morin, quercetin, naringin and naringenin were more effective than α-tocopherol, BHA and BHT in retarding oxidation of both oils. Myricetin was the most effective flavonoid tested. Therefore, DGTE, isolated catechins and some flavonoids could be used as effective natural antioxidants for stabilization of highly unsaturated marine oils.

In another set of studies, microencapsulation of SBO with different starch materials such as β-cyclodextrin, corn-syrup solids and maltodextrins was carried out in order to improve the oxidative stability of SBO. Encapsulated SBO was stored at room temperature for 49 days and its stability was monitored by measuring fatty acid composition, PV and TBARS. β-Cyclodextrin was the most effective encapsulating material used, as the resultant SBO retained 89% of its total PUFA.

Stereospecific analysis was carried out to establish positional distributions of fatty acids in the triacylglycerols (TAG) of SBO and MHO. In SBO, eicosapentaenoic acid

(EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) were located mainly in the *sn*-1 and *sn*-3 positions of the TAG molecules. In MHO, 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. Therefore, EPA, DPA and DHA from SBO might be better assimilated in the body than those from MHO.

In order to prepare ω3 fatty acid concentrates from SBO and/or MHO, three different approaches, namely low temperature crystallization, urea complexation and enzymatic hydrolysis were employed. Low temperature crystallization of free fatty acids of SBO gave a higher amount of total ω3 fatty acids in the non-crystalline fraction (concentrate) as compared to intact TAG. Approximately 58.3 and 66.7% of total ω3 fatty acids were obtained at -60 and -70°C when hexane was used as the solvent; corresponding recoveries were 39.0 and 24.8%.

Urea complexation of saturated and/or monounsaturated fatty acids resulted in concentration of ω3-PUFA in the non-urea complexed fraction, thus allowing facile separation of ω3 fatty acids from marine oils. Process parameters such as urea-to-fatty acids ratio, crystallization temperature and crystallization time were optimized by response surface methodology (RSM) with a central composite rotatable design (CCRD) to obtain maximum amounts of ω3 fatty acids in the SBO concentrate. Total ω3 fatty acids of 88.2% (recovery of 24.5%) were obtained at a urea-to-fatty acid ratio of 4.5, a crystallization time of 24 h, and a crystallization temperature of -10°C.

Preparation of ω3 fatty acid concentrates from SBO and MHO using enzymatic hydrolysis was carried out by first screening microbial lipases from *Aspergillus niger; AN, Candida cylindracea; CC, Chromobacterium viscosum; CV, Geotrichum candidum, GC, Mucor miehei; MM, Pseudomonas spp.; PS, Rhizopus niveus; RN* and *Rhizopus oryzae; RO* for their activities; *CC*-lipase was the most efficient enzyme examined. Optimization of hydrolysis parameters, namely enzyme concentration, reaction time and reaction temperature, gave a maximum of 54.3% total ω3 fatty acids from SBO (recovery of 30%) at an enzyme concentration of 308 U/g oil, a reaction time of 40 h, and a reaction temperature of 37°C. Similarly, a maximum of 54.5% total ω3 fatty acids was obtained from MHO (recovery of 43%) at an enzyme concentration of 340 U/g oil, a reaction time of 45 h, and a reaction temperature of 38°C. Therefore, low temperature crystallization, urea complexation and enzymatic hydrolysis by *CC*-lipase may be used to prepare ω3 fatty acid concentrates from marine oils.

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LIST OF ABBREVIATIONS

AA	- Arachidonic acid
AN	- *Aspergillus niger*
ANOVA	- Analysis of variance
ACS	- American Chemical Society
p-AnV	- p-Anisidine value
AOCS	- American Oil Chemists' Society
AOM	- Active oxygen method
AV	- Acid value
BHA	- Butylated hydroxyanisole
BHT	- Butylated hydroxytoluene
CC	- Column chromatography
CC	- *Candida cylindracea*
CCRD	- Central composite rotatable design
CHD	- Coronary heart diseases
CLO	- Cod liver oil
CNO	- Canola oil
CV	- *Chromobacterium viscosum*
DAG	- Diacylglycerol
DGTE	- Dechlorophyllized green tea extract
DHA	- Docosahexaenoic acid
DPA	- Docosapentaenoic acid
EC	- Epicatechin
ECG	- Epicatechin gallate

EDTA - Ethanoldiaminetetraacetic acid

EGC - Epigallocatechin

EGCG - Epigallocatecnin gallate

EPA - Eicosapentaenoic acid

ESR - Electron spin resonance

FAME - Fatty acid methyl ester

FAO - Food and Agriculture Organization

FDA - Food and Drug Administration

FID - Flame ionization detector

GC - *Geotrichum candidum*

GC - Gas chromatography

GC-MS - Gas chromatography-Mass spectrometry

GLA - γ-Linolenic acid

GLC - Gas liquid chromatography

GLM - General linear model

GRAS - Generally recognized as safe

HDL - High density lipoprotein

HPLC - High pressure liquid chromatography

HUFA - Highly unsaturated fatty acids

IR - Infra-red

ISC - Inter system crossing

IV - Iodine value

IUPAC - International Union of Pure and Applied Chemistry

LDL - Low density lipoprotein

LPC - Lysophosphatidylcholine

MA - Malonaldehyde

MAG - Monoacylglycerol

NFMOA - National Fish Meal and Oil Association

MHO - Menhaden oil

MM - *Mucor miehei*

MUFA - Monounsaturated fatty acids

NL - Neutral lipids

NMR - Nuclear magnetic resonance

NUCF - Non-urea complexed fraction

OSI - Oil stability instrument

PC - Phosphatidylcholine

PE - Phosphatidylethanolamine

PS - Phosphatidylserine

PS - *Pseudomonas spp*

PI - Phosphatidylinositol

PG - Propyl gallate

PL - Phospholipids

PUFA - Polyunsaturated fatty acids

PV - Peroxide value

RD - *Rhizopus delemer*

RB - Refined bleached

RBD - Refined bleached deodorized

RJ - *Rhizopus javanicus*

RN	- *Rhizopus niveus*
RO	- *Rhizopus oryzae*
RSM	- Response surface methodology
SAS	- Statistical analysis system
SBO	- Seal blubber oil
SD	- Standard deviation
SEM	- Scanning electron micrograph
SFE	- Supercritical fluid extraction
SV	- Saponification value
TAC	- Total allowable catch
TAG	- Triacylglycerols
TBARS	- Thiobarbituric acid reactive substances
TBHQ	- tert-Butylhydroxyquinone
TLC	- Thin layer chromatography
TX	- Thromboxene
USDA	- United States Department of Agriculture
UV	- Ultraviolet
UCF	- Urea complexed fraction
VLDL	- Very low density lipoprotein

CHAPTER 1

INTRODUCTION

The importance of polyunsaturated fatty acids (PUFA) in human nutrition and disease prevention was scientifically recognized three decades ago. Epidemiological studies in the early 1970's postulated that the low incidence of coronary heart disease of Greenland Eskimos might be related to their distinctive dietary habit and use of lipids rich in PUFA (Bang *et al.*, 1976). Eskimos were also found to have a reduced prevalence of other chronic and inflammatory diseases such as arthritis, psoriasis, asthma and diabetes. Several sources of information suggest that humans originally consumed a diet with a ratio of ω6 to ω3 fatty acids of about 1:1 whereas today this ratio ranges from 10:1 to 20-25:1 in the affluent western societies. Therefore, the western diets are deficient in ω3 fatty acids compared with the diet on which humans were evolved and their genetic patterns established (Simopoulos, 1991).

The beneficial effects of PUFA have been ascribed to their ability to lower serum triacylglycerol and cholesterol levels and enhance their excretion, to increase membrane fluidity and by conversion to eicosanoids to reduce thrombosis (Kinsella, 1986). The ω3 fatty acids are considered essential for normal growth and development throughout the life cycle and may play an important role in the prevention and treatment of coronary artery disease, hypertension, arthritis, other inflammatory and autoimmune disorders and cancer (Branden and Carroll, 1986; Kinsella, 1986). A significant amount of docosahexaenoic acid (DHA) is found in human milk (Yongmanitchai and Ward, 1989). High levels of DHA are also found in human brain and retina. Therefore, DHA

in breast-milk or infant formula meets the requirements of developing human brain and visual parts (Neuringer *et al.*, 1988). The PUFA composition of cell membranes is largely dependent on their dietary intake. Therefore, consumption of appropriate amounts and proportions of ω6 and ω3 fatty acids need to be considered.

In the USA, it is recommended that the total fat intake should be 30% of the total calories with 10% saturated fatty acids, 10% monounsaturated fatty acids and 10% PUFA, the latter being equally divided between ω6 and ω3 fatty acids. The 1990 Canadian Nutrition Recommendations have included separate values for the two classes of long-chain ω3 PUFA; eicosapentaenoic acid (EPA) and DHA. The amount of ω3 and ω6 fatty acids are given in grams, based on energy expressed as daily requirements, for the various age groups from birth to 75+ years. During pregnancy additional amounts of ω3 and ω6 fatty acids are recommended in amounts that increase from the 1st to the 2nd trimester. There is no increase between the 2nd and 3rd trimester. Additional ω3 and ω6 fatty acids are recommended during lactation.

The long-chain ω3 fatty acids (EPA or DHA) may be obtained mainly from seafoods or derived from dietary α-linolenic acid by chain elongation and desaturation. EPA and DHA are synthesized mainly by both uni- and multicellular marine plants such as 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, particularly those living at low temperatures, probably due to the acids' ability to maintain fluidity in such environments. Therefore, increased consumption of

marine lipids has been suggested in order to increase the intake of ω3 fatty acids in our diet. However, despite health benefits, highly unsaturated fatty acids are very sensitive to oxidative deterioration and thus pose practical problems (Cho *et al.*, 1987).

Degradation of highly unsaturated fatty acids, via a free radical chain mechanism, results in changes in odour and flavour of oils or oil-containing foods (Labuza, 1971). Chemical reactions involved in oxidative processes require low activation energies (4-14 kcal.mol^{-1}) and their rates are not changed significantly by lowering of the storage temperature (Labuza, 1971). Therefore, to overcome this problem, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) are often incorporated into oils or oil-containing foods (Sherwin, 1990). However, there is a decline in the use of synthetic antioxidants perhaps due to consumer preference for natural ingredients (Hudson, 1990).

The choice of natural antioxidants to stabilize marine oils for human consumption is restricted to a few substances, with α-tocopherol (or its synthetic analog) being most frequently used. Although tocopherols are considered as safe natural antioxidants, they do not always provide effective protection against *in vitro* oxidation (Frankel, 1980). Therefore, research on other natural antioxidants has gained momentum as they are considered, rightly or wrongly, to pose no health risk to consumers. Naturally-occurring antioxidative components in foods include flavonoids, phenolic acids, lignan precursors, terpenes, mixed tocopherols, phospholipids,

polyfunctional organic acids and also plant extracts such as those of rosemary and sage (Hudson, 1990). However, activity of natural antioxidants in highly unsaturated marine oils has not received much attention.

Other options available for stabilization of marine oils are hydrogenation, interesterification of PUFA oils with saturated oils/fatty acids or encapsulation. Hydrogenation and interesterification reduce unsaturation and thus negate the beneficial effects of PUFA in the original oils. Encapsulation of oil provides a barrier between oil and atmospheric oxygen/light by coating oil droplets and preventing them from oxidation. Such products may improve the handling of the oils due to their dry form and free-flowing nature (Shahidi and Han, 1993).

It has been suggested that PUFA concentrates devoid of more saturated fatty acids are much better than marine oils themselves since they would keep the daily total lipid intake as low as possible. With the growing public awareness of the nutritional benefits of consuming PUFA concentrates the market for these products is expected to grow in the future. In response to this demand, pharmaceutical industries have used different methods for preparing PUFA concentrates from marine oils. Chromatographic separation, fractional distillation, low temperature crystallization, supercritical fluid extraction and urea complexation are currently practised for preparation of PUFA concentrates from marine oils. Selective enzymatic hydrolysis of saturated fatty acids which would concentrate the ω3 PUFA in the acylglycerol form is a recent biotechnological advance that may be extended to large scale operations once

economical constraints are addressed.

1.1 Objectives of this study

The harp seal (*Phoca groenlandica*) is the principal species of seal found in the waters of Newfoundland and Labrador. Due to an increase in the population of harp seals over the past several years, the government of Canada has increased the total allowable catch (TAC) from 186,000 to 250,000 animals as of 1996. Seal blubber which constitutes about 20% of the carcass weight is a good source of lipid rich in PUFA that could be totally utilized as an edible oil for food formulation and/or pharmaceutical use. The main objective of this study was to examine utilization of seal blubber oil as an edible oil by comparing its characteristics with those of cod liver and menhaden oils. Stability and stabilization of marine oils by naturally-occurring antioxidants as well as by microencapsulation were also examined in order to improve their food utilization. Stereospecific analysis of seal blubber and menhaden oils was carried out to determine the existing differences in positional distribution of PUFA in the triacylglycerol molecules. In addition, three different methods, namely low temperature crystallization (physical), urea complexation (chemical) and enzymatic hydrolysis (biotechnological) were studied for the preparation of ω3 fatty acid concentrates from seal blubber and menhaden oils, and the process parameters were optimized in order to obtain highly concentrated ω3 preparations.

CHAPTER 2

LITERATURE REVIEW

2.1 Marine lipids and their significance

Fish has been a major source of animal protein for man since early civilizations. Lipids obtained from the body and organs of fish and marine mammals may have been used as fuel, food or medicine. Lipids from marine sources have gained significance due to the recognition of the nutritional and health related benefits of polyunsaturated fatty acids in prevention or possible curing of human diseases. The use of marine oils as food ingredients is being expanded. Peru, Chile, the United States, Iceland, Norway, Japan and Denmark are the leading countries in having established marine oil industries. Canada contributes approximately 1.1×10^4 metric tonnes (MT) per year of marine oils as compared with the total annual global production of 2.0×10^5 MT (Fishery Statistics Commodities, FAO Year Book, 1993).

2.1.1 Sources of marine lipids

Lipid is an integral component of living tissues of animals and plants. In most marine organisms lipid is the second largest constituent after protein. In addition to serving as structural components of cells and tissues, lipids function as a source of energy and provide buoyancy to marine animals. Depot fats comprise the largest part of total lipids of marine organisms (Pigott and Tucker, 1987). Fish such as anchovy, capelin, herring, mackerel, menhaden and salmon contain considerable amounts of depot fat in their skeletal muscles and skin while lean fish such as cod, halibut and shark have lipids stored mainly in their livers. The blubber (subcutaneous depot fat layer) of marine

mammals such as seal, whale and walrus is exceptionally rich in lipids.

The content and composition of depot lipids of fish and marine mammals reflect their diet, phylogen, physiological conditions (e.g. age, spawning etc.) and living environment (e.g. geographical location, season of the year, water temperature and availability of food). Table 2.1 provides the average content and fatty acid composition of total lipids of selected fish and marine mammal species and compares them with those of important vegetable oils.

2.1.2 Chemistry and composition of marine lipids

Triacylglycerols (TAG) are the major component of marine oils while phospholipids contribute little to the total amount. Some species of fish also contain wax esters, carotenoids and sterols in addition to glyceryl esters (Singh and Chandra, 1988).

2.1.2.1 Triacylglycerols and fatty acids

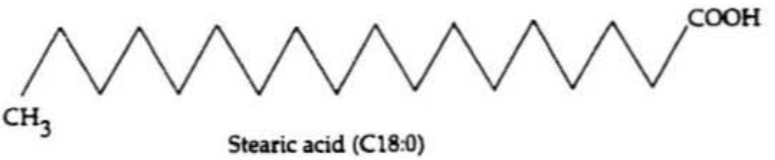
Triacylglycerols (TAG) contain a glycerol molecule esterified with three fatty acids. The fatty acids have an even number of carbon atoms, they are usually unbranched and are saturated or unsaturated (Taylor, 1973). However, branched and odd carbon numbered fatty acids are also found in marine lipids in small quantities (Patterson, 1989). When the carbon atoms in the hydrocarbon chain of a fatty acid hold their full complement of hydrogen they are defined as saturated fatty acids (Figure 2.1). Saturated fatty acids are most stable either in the free state or in the TAG form and pack together more easily in the solid state due to their contour arrangement. This behaviour favours

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

Component	Atlantic herring[1]	Cod liver (wild cod)[2]	Blubber (Gray seal)[3]	Blubber (Harp seal)[4]	Eel[1]	Canola[1]	Soybean[1]
Total lipid content	12-14	55-64	85-95	85-95	20-27	45-48	18-24
14:0	6.8	7.3	3.9	4.4	4.2	0.1	0.1
16:0	14.8	12.7	6.2	7.9	19.6	3.6	10.8
18:0	1.1	2.2	1.1	1.2	3.3	1.4	4.0
20:0	-	-	-	-	0.1	0.4	-
22:0	-	-	-	-	-	0.2	-
14:1	-	-	-	1.1	0.3	-	-
16:1	7.8	9.8	12.7	17.1	12.7	0.3	0.2
18:1[5]	16.6	18.4	16.5	21.8	32.6	55.6	23.8
20:1	0.4	16.1	0.3	13.5	2.9	1.8	0.2
22:1	15.9	9.7	2.60	4.4	-	1.6	-
18:2[5]	0.8	1.1	22.6	1.9	1.4	21.9	53.3
18:3[5]	13.4	-	0.7	0.2	0.3	13.0	7.1
18:4ω3	-	1.1	2.1	0.9	0.6	-	-
20:4ω6	-	-	0.3	0.4	0.6	-	-
20:5ω3	4.5	7.6	7.0	7.9	4.3	-	-
22:5ω3	-	1.4	5.9	5.1	0.7	-	-
22:6ω3	2.9	4.7	14.5	6.9	6.0	-	-

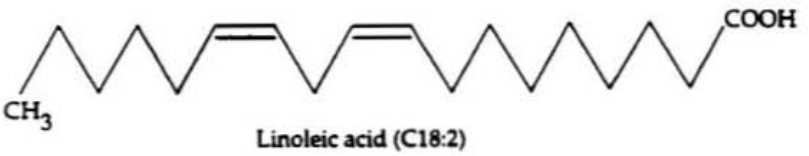
[1]Ackman (1982, 1990), [2]Shahidi and Dunajski (1994), [3]Piggot and Tucker (1987), [4]Jangaard and Ke (1968), [5]All isomers included

Figure 2.1 Chemical structures of saturated (stearic), monounsaturated (oleic), and polyunsaturated (linoleic, linolenic, eicosapentaenoic, docosapentaenoic and docosahexaenoic) fatty acids



Stearic acid (C18:0)

Oleic acid (C18:1)

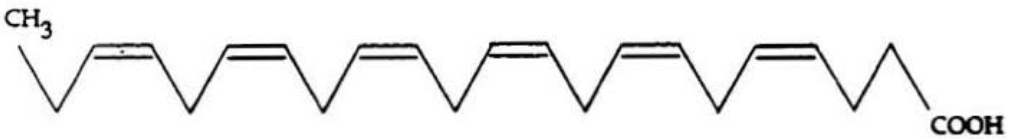


Linoleic acid (C18:2)

Linolenic acid (C18:3)

Eicosapentaenoic acid; EPA (C20:5)

Docosapentaenoic acid; DPA (C22:5)



Docosahexaenoic acid; DHA (C22:6)

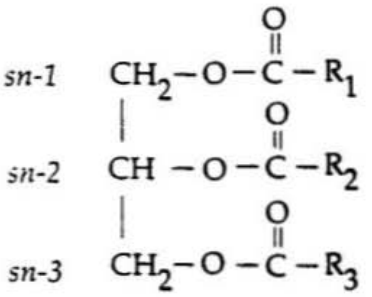
a higher melting point of the fat. As the chain length of fatty acid increases the melting point of the material rises steadily (Patterson, 1989).

Fatty acids with two or more double bonds are referred to as polyunsaturated fatty acids (PUFA) (Figure 2.1). The double bonds in natural unsaturated fatty acids exist in the *cis* (*Z*) form which is associated with softness and liquidity of lipids. The double bonds and the methylene (-CH_2-) group immediately adjoining them (α methylene group) are notably reactive. When the methylene group lies between two double bonds (i.e., diallyl methylene group such as that in linoleic acid, C18:2) the activity is further enhanced (Patterson, 1989).

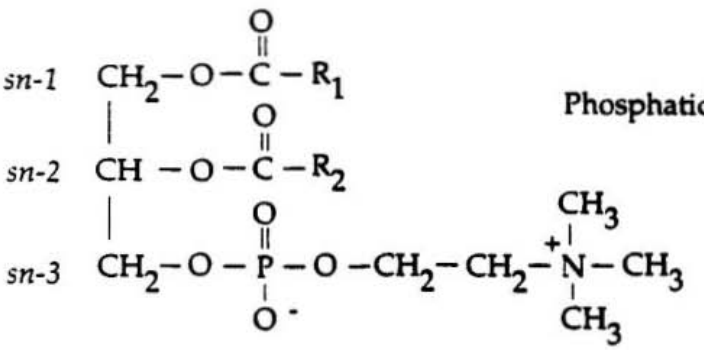
The fatty acids esterified with a glycerol molecule may be identical or different. According to the nomenclature adopted by the International Union of Pure and Applied Chemistry (IUPAC) Commission on Biochemical Nomenclature, the primary ester groups previously considered as α and the secondary ester group as β, are now identified as *sn*-1 or *sn*-3 and *sn*-2, respectively (Figure 2.2). TAG are widespread in nature and serve as the most concentrated form of energy (generally 9 kcal/g lipid) and in animal kingdom occur in tissues or as droplets in cells. In addition to their function as energy storage, TAG may also serve as insulating materials for warm-blooded animals living in aquatic environments. Thus, marine mammals such as seals and whales have enormous layers of lipids under their skin allowing them to survive in the icy cold waters of the Arctic and Antarctic (Hølmer, 1989).

The fatty acid composition of marine lipids varies considerably, especially when compared with vegetable oils (Table 2.1). The level of PUFA in marine oils is high and

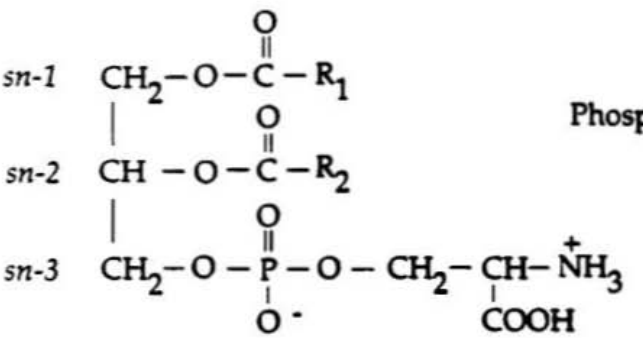
Figure 2.2 Basic structures of triacylglycerol (TAG) and phospholipid molecules



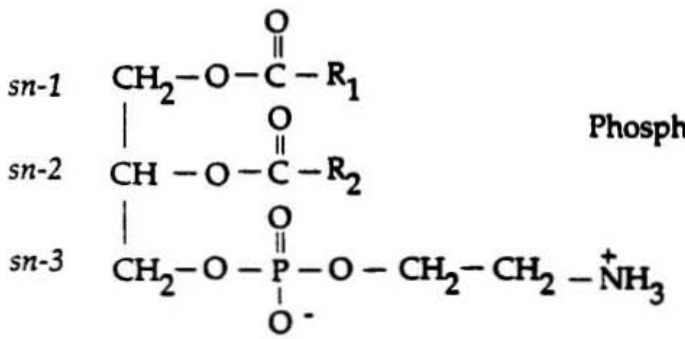
Triacylglycerol (TAG)



Phosphatidyl choline (PC)



Phosphatidyl serine (PS)



Phosphatidyl ethanolamine (PE)

comprised mainly of those with 5 or 6 double bonds. Most of these fatty acids are long-chain with 20 to 22 carbon atoms and have omega-3 (ω3) configurations (i.e. double bond starting on carbon number 3 from methyl end group) (Figure 2.1). Some species of fish contain small amounts of C17 and C19 saturated fatty acids (Piggot and Tucker, 1987). These unique features differentiate lipids of marine species from land animals. The two most common and also important long chain PUFA in marine lipids are eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) which belong to the ω3 family (Figure 2.1). Most PUFA are formed in unicellular phytoplankton and multicellular sea algae and eventually pass through the food web and become incorporated into the body of fish and other higher marine species (Yongmanitchai and Ward, 1989). The high content of ω3 fatty acids in marine lipids is suggested to be a consequence of cold temperature adaptation, because at lower habitat temperatures ω3-PUFA remain liquid (Ackman, 1988) and oppose any tendency to crystallize.

Total lipids of aquatic species may be isolated by common extraction methods such as that of Bligh and Dyer (1959) using chloroform/methanol/water mixtures. Separation of the TAG fraction from total lipids can be achieved using column chromatography (CC) or thin layer chromatography (TLC) on silicic acid by employing non-polar solvents (Christie, 1982). The amount of TAG in the total lipid fraction can be determined by a number of methods including spectrophotometry, densitometry on TLC plates, gas liquid chromatography (GLC) and radioisotopic derivatization techniques (Hølmer, 1989). A method based on a combination of TLC on silica gel-coated quartz rods and detection by flame ionization detector (TLC-FID IATROSCAN®) has been

reviewed by Parrish (1987).

2.1.2.2 Phospholipids

In phospholipids, acyl groups occur in the *sn*-1 and *sn*-2 positions of the glycerol while a polar head group involving a phosphate is present in the *sn*-3 position of the molecule (Figure 2.2). Phospholipids are major constituents of cell membranes and therefore, are regarded as structural lipids in living organisms. The major types of phospholipids in marine species are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl inositol (PI) with minor amounts of sphingomylene, lysophosphatidyl choline (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. Lovern (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 the PUFA of menhaden oil are predominantly located on the *sn*-2 position of the TAG molecule. The *sn*-2 position of PC in menhaden oil contained 29 and 42% of EPA and DHA, respectively, whereas the *sn*-1 position contained only 1.7 and 12.5%, respectively. However, the phospholipid content of fully refined-bleached and deodorized oils is very low (Young, 1982), due to the removal of polar components during the degumming process.

Phospholipids are usually extracted with the total lipids when using the Bligh and

Dyer (1959) extraction procedure. Silicic acid column chromatography with methanol after eluting neutral lipids can be used to recover phospholipids. Two dimensional TLC (Christie, 1982) and TLC-FID IATROSCAN® provide a means for separating individual phospholipids (Parrish, 1987).

2.1.2.3 Fat soluble vitamins

Many species of fish store vitamin A (fatty acid esters of retinol) and D_2 (Ergocalciferol) in their livers. Therefore, fish liver oils are considered as exceptionally rich sources of vitamin A and D (Davidson *et al.*, 1991). Since these vitamins are soluble in lipids they are also accumulated in other lipid-rich tissues owing to their chemical nature. Vitamin E or/and tocopherols are also present in marine lipids. None of these vitamins are synthesized in the fish body, therefore, their concentration in oils is directly related to the dietary habits of the fish. Ackman and Cormier (1967) and Kinsella (1987) have reported that α-tocopherol was the major form of tocopherol present in fish oils. Bao (1988) reported that processed menhaden oil contained 68.9 ppm of α-tocopherol and that no other tocopherol isomers were detected.

2.1.2.4 Wax esters

Wax esters are found in lipids of a number of marine species. These compounds, which presumably serve as an energy reserve, comprise a long chain fatty alcohol (usually hexadecanol and octadeca-9-enol) esterified with a fatty acid, usually of low unsaturation. Wax esters are mainly present in marine invertebrates such as sea anemones and

crustaceans (Bergmann *et al.*, 1956) and can be accumulated in other organisms of the food web. Nevenzel and co-workers (1965) have found that lipids of muscle and liver from gempylid fish contain 91.5 and 3.9% of wax esters, respectively. Wax esters are also present in large amounts in some marine mammals such as dolphins and bottle and sperm whales (Lovern, 1964).

2.1.2.5 Carotenoid pigments

The natural yellow colour and red pigments found in fish skin, muscles and reproductive organs are due to the presence of carotenoids. They occur both in the free form as well as esters, glycosides, sulphates and as carotenoproteins. Carotenoids consisting of eight isoprenoid units in a molecule are called carotenes and their oxidized derivatives are known as xanthophylls. Fish and marine mammals do not synthesize carotenoids *de novo*, and those found in their body are either direct accumulation from food or are partly modified through metabolic cycles (Matsuno and Hirao, 1989). Carotenoids are usually extracted with lipids in extracting solvents owing to their non-polar nature.

2.1.3 Industrial uses of marine lipids

Traditional industrial uses of marine oils were based on their highly unsaturated nature, particularly as drying oils or varnishes. Commercial marine oils are used in a variety of applications, but these are not generally known because of the confidential nature of these novel uses. The major industrial uses of marine oils take advantage of

either the unique type and high degree of unsaturation of the oils to produce elastic durable polymers or the diverse mixture of long chain fatty acids that adds to the lubricity, detergency and plasticity functionalities (Fineberg and Johnson, 1967). The hydrogenated fatty acids and derivatives from marine oils are used in metallic greases, textile soaps, polishes, carbon paper and crayons (Harwood, 1954). In addition, marine oils are also used in variety of products such as cutting oils, glazings, core oils, printing inks, ceramics, linoleum, fermentation substrates, polyethylene foams, ore flotation and plasticizers (Bimbo, 1987; Bimbo and Crowther, 1992). Low quality marine oils are used in leather tanning (Bimbo, 1989).

2.1.4 Food uses of marine lipids

Over one million metric tonnes of marine oils are annually used as food ingredients (Stansby, 1978; Bimbo 1989, 1990). Most of the world's marine oil production is used in Europe, South America and Japan for making salad oils, frying fats, table margarine and low calorie spreads. Marine oils are also used in shortenings that are used in breads, pastries, cakes, cookies, biscuits and synthetic creams where they are mostly in the hydrogenated form. It is also used in the production of food emulsifiers.

The report provided by Barlow and Young (1988) on novel uses of fish oils suggests that the optimum fish oil incorporation into meat products (e.g. salami, frankfurters) is 9 to 16.5% of their total lipids. Incorporation of fish oils in highly flavoured foods is preferred since any off-flavour development from the use of fish oils is masked. Schnepf *et al.* (1991) have reported that overall acceptability of French salad

dressings prepared with menhaden/soybean oil blends (maximum 30% menhaden oil) up to eight weeks of storage at 22°C was similar to those prepared with soybean oil alone. Li and Regenstine (1990) have shown that mayonnaise prepared with 100% menhaden oil can be stored under a blanket of nitrogen for 8 weeks at 30°C without changes in its physical appearance. Jafar *et al.* (1994) have shown that mayonnaise prepared from menhaden oil had a shelf-life of one day without any added antioxidants. However, added citric acid or sodium citrate and propyl gallate in the oil phase and ethylenediamine tetraacetic acid (EDTA) and ascorbic acid in the aqueous phase increased the shelf-life to an average of 49 days at room temperature. In addition to the direct use of marine oils in foods, concentrates of EPA and DHA may be applied as PUFA supplements in a number of dairy products, mayonnaise, margarine and other table spreads, edible vegetable oils, emulsified or non-emulsified shortenings, hard fats, pastry fats, biscuits, icings and bread doughs (Bimbo, 1989).

Most of the marine oils used in food applications are hydrogenated to various degrees. Modern hydrogenation processes of high selectivity make it possible to produce a high proportion of mono- and polyunsaturates without increasing the level of saturated fatty acids, and the degree of hydrogenation can be controlled to produce oils with a wide range of physical and chemical properties in order to meet various application demands (Bimbo, 1989). However, the beneficial health effects of PUFA, especially the ω3 type, is compromised by the hydrogenation process. In 1986, the National Fish Meal and Oil Association (NFMOA) filed a petition with United State (US) Food and Drug Administration (FDA) to affirm that menhaden oil and partially hydrogenated menhaden

oil are "generally recognized as safe (GRAS)" for direct human food ingredient application and this was granted in 1989.

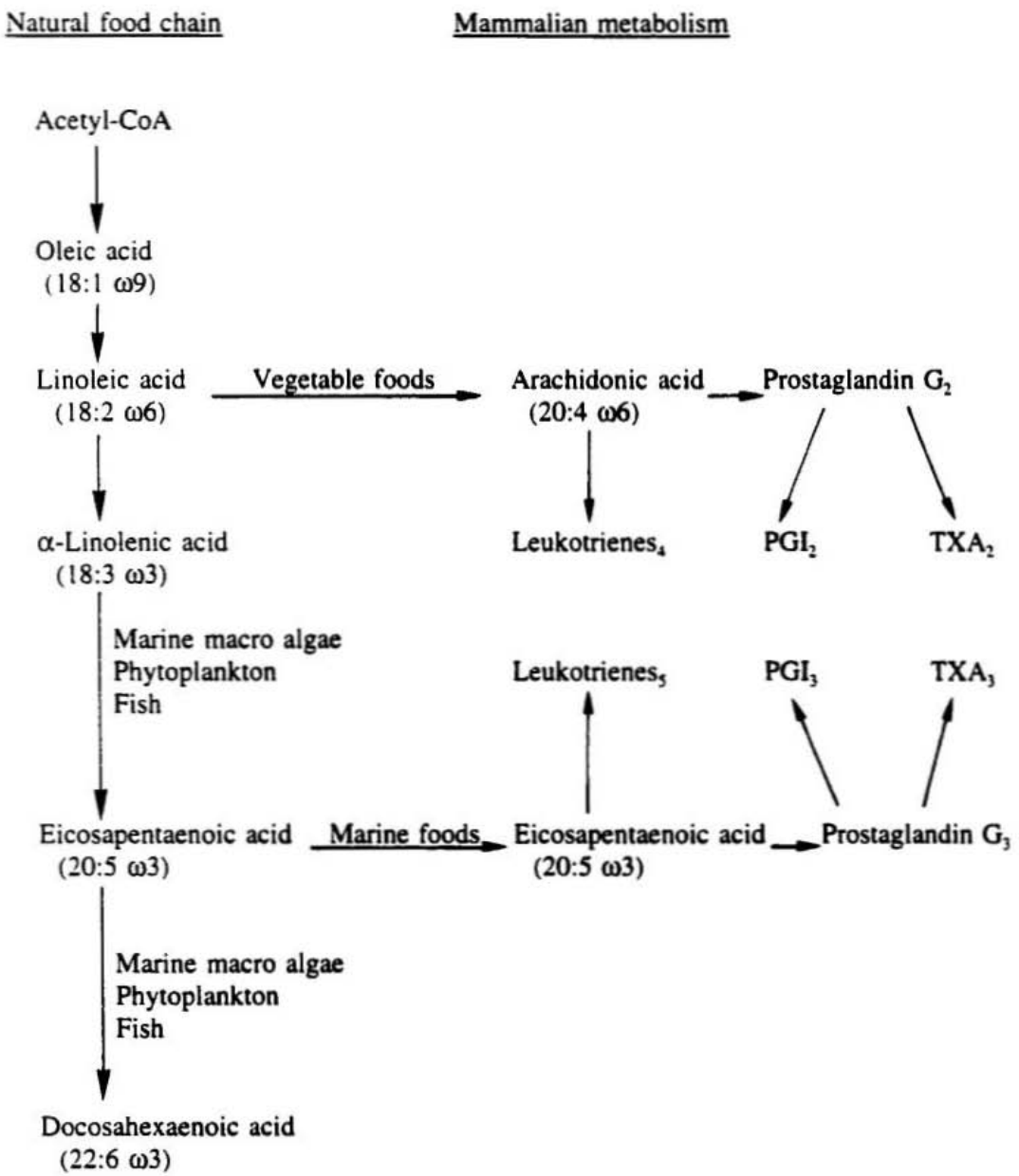
2.1.5 Marine lipids in human nutrition and disease prevention

Recognition of the health benefits associated with consumption of seafoods (ω3 fatty acids) is one of the most promising developments in human nutrition research in the past three decades. Epidemiological studies in the early 1970s postulated that low incidence of coronary heart diseases in Eskimos might be related to their distinctive dietary habit. Eskimos living in Greenland consume a diet rich in seafoods (which seal meat and blubber make an important contribution) compared to their counterparts living in Denmark who consumed limited amounts of seafoods (Bang *et al.*, 1976). Subsequent analyses revealed that Greenland Eskimos had significantly lower concentration of serum cholesterol, triacylglycerols, very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) and higher levels of high-density lipoproteins (HDL) compared to the Dane population who consumed a typical European diet. Furthermore, low death rate from coronary heart diseases (CHD) in Japanese population was attributed to the higher consumption of fish, particularly in Okinawa where the lowest death rate was noted and fish consumption exceeds 200g/day. Burr *et al.* (1989) showed improved survival rate among men who had already experienced a heart attack and subsequently increased their consumption of fatty fish. According to the prospective longitudinal study of middle-age Dutch men, consumption of at least 30g/day of fish reduced the risk of CHD (Kromhout *et al.*, 1985).

Since these early investigations, several mechanisms have been developed to explain beneficial health effects of dietary marine oils. It has been proposed that the decrease in plasma lipids by reduced hepatic synthesis of fatty acids and VLDL could result from consumption of marine oils (Illingworth and Ullmann 1990). Nestel (1990) has suggested that ω3 fatty acids have a direct effect on the heart muscle itself, increase in blood flow, decrease in arrhythmias, improve in arterial compliance, decrease in the size of the infarct and reduction in several chemical and cellular processes that compromise heart function. It is also suggested that marine oils may retard atherogenesis through their effects on platelet function, platelet-endothelial interactions and on the inflammatory response. Most of these effects are mediated, at least in part, by alterations in eicosanoids formation in the human body (Fischer, 1989).

Arachidonic acid (AA) and EPA are precursors of eicosanoids (Branden and Carroll, 1986), such as prostaglandins (PG), thromboxanes (TX) and leukotrienes, all of which are oxygenated derivatives of C20 fatty acids. Figure 2.3 provides a summary of eicosanoid formation from dietary fats. The eicosanoids from these two fatty acids (AA and EPA) are different in structure and function (Fischer, 1989). Eicosanoids have a broad spectrum of biological activity. Physiological effects, both positive and negative, of ω3 fatty acids have been observed in the areas of heart and circulatory, immune response and cancer. The first category includes prevention or treatment of atherosclerosis (Dyerberg, 1986; Mehta *et al.*, 1988), thrombosis (Kinsella, 1986), hypertriglyceridemia (Phillipson *et al.*, 1985) and high blood pressure (Dyerberg, 1986).

Figure 2.3 Metabolic pathways of ω3 and ω6 fatty acids and biosynthesis of eicosanoids (adapted from Branden and Carroll, 1986)


Natural food chain
Mammalian metabolism
Acetyl-CoA
Oleic acid
(18:1 ω9)
Linoleic acid
(18:2 ω6)
Vegetable foods
Arachidonic acid
(20:4 ω6)
Prostaglandin G2
α-Linolenic acid
(18:3 ω3)
Leukotrienes4
PGI2
TXA2
Marine macro algae
Phytoplankton
Fish
Leukotrienes5
PGI3
TXA3
Eicosapentaenoic acid
(20:5 ω3)
Marine foods
Eicosapentaenoic acid
(20:5 ω3)
Prostaglandin G3
Marine macro algae
Phytoplankton
Fish
Docosahexaenoic acid
(22:6 ω3)

The second area relates to the treatment of asthma, arthritis (Singh and Chandra, 1988), migraine headache, psoriasis and nephritis (Kinsella, 1986). The third category involves cancer of breast (Branden and Carroll, 1986), prostate and colon (Singh and Chandra, 1988).

In general, the metabolism of ω3 fatty acids that facilitates prevention and treatment of the diseases and disorders has been addressed by considering changes in the eicosanoids in the circulatory system. Since eicosanoids are ultimately derived from PUFA provided by the diet, it is clear that quantitative and qualitative changes in the supply of dietary PUFA will have a profound effect on the production of eicosanoids. There is an emerging consensus that ω3 fatty acids are essential nutrients. As a structural component of brain, retina, testis and sperm, DHA appears linked to proper tissue function and needs to be supplied in sufficient amounts during tissue development (Neuringer *et al.*, 1988). Recent studies suggest that a relative deficiency of long-chain ω3 PUFA occurs during pregnancy and lactation. Some investigation have shown a progressive deterioration of the mother's DHA status during pregnancy, possibly indicating that the mother's capacity to meet the high fetal requirement for DHA is working at its limit and may even be inadequate (Al *et al.*, 1990). Carlson *et al.* (1986) have shown that premature babies have lower levels of DHA in their tissues than full-term babies. Infants fed formula supplemented with marine oils accumulated as much DHA as full-term breast-fed infants (Carlson *et al.*, 1987). Feeding of infants with formula devoid of ω3 fatty acids resulted in lack of deposition of DHA in their visual and neural tissues. Long-term dietary studies with marine oils enriched with ω3 fatty acids

indicated that enrichment of foods with these fatty acids is practical. Therefore, dietary fat modification is now considered to be an effective tool for changing the lipid composition of cell membranes.

The Canadian Scientific Review Committee of Nutrition Recommendations (1990) have suggested daily requirements for PUFA (ω3 and ω6) based on age and energy needs. Additional amounts of ω3 and ω6 fatty acids are recommended for lactating and pregnant women with an increasing amounts from first to second trimester of pregnancy. British Nutrition Foundation Task Force (1992) on unsaturated fatty acids has recommended that 5% of total daily energy supply should come from ω3 fatty acids.

2.2 Processing of marine oils

Marine oils are produced from different raw materials: small fish, blubber of marine mammals and discards of food fish industry. Also fish which are either not acceptable for direct table consumption or surplus by-catch such as mackerel, sardines, anchovies, herring, capelin, sprat, menhaden and sand eel are used to manufacture oil.

The basic processing steps of manufacturing of marine oils involve cooking or rendering to release the oil, degumming, alkali-refining, bleaching and deodorizing. During processing, impurities such as free fatty acids, mono- and diacylglycerol, phospholipids, sterols, vitamins, hydrocarbons, pigments, proteins and their degradation products, suspended mucilaginous and colloid-like matters and oxidation products of fatty acids are removed from the oil (Bimbo and Crowther, 1991). Processing of marine oils is similar to that of vegetable oils, however, the quality of crude marine oils is less

uniform than crude vegetable oils. High quality crude oils may be obtained by proper handling of raw material such as minimizing damage to fish and proper chilling after landing.

2.2.1 Cooking or rendering

Heat treatment is necessary to denature the protein and to break cell walls so that oil and water can be easily removed from tissues (Bimbo, 1989). The lipid containing raw material is first cooked with water in a continuous cooker at 50-70°C. The denatured protein in stickwater may be then separated from the oil by centrifugation or removing of liquors (oil and water) by applying pressure in a screw-type continuous press in which oil can be separated by centrifugation (Lee, 1963). The separated crude oil is then used for further processing/refining.

2.2.2 Degumming

Degumming is the process by which phospholipids and certain other ill-defined mucilaginous materials are removed from crude oil by treatment with water/steam or with an aqueous solution of boric acid or salt such as sodium chloride at 30 to 50°C. Deposited hydrated phospholipids referred to as gums may be separated from oil by centrifugation (Chang, 1967). It is recommended that air should be excluded in the process in order to prevent oxidation at high temperatures of refining (Carr, 1976). The amount of water used should also be minimized for effective degumming because excess water may promote hydrolysis (Carr, 1978). In some cases, crude oils are pretreated with

phosphoric acids before being degummed (Dijkstra and Van Opstal, 1989; List *et al.*, 1978) which may help in reducing the iron content by converting iron into an easily removable form (Dijkstra and Van Opstal, 1989). Marine oils are usually not degummed before they are refined. The removal of phospholipids and mucilaginous materials are, therefore, accomplished together with the removal of free fatty acids by alkali-refining process itself.

2.2.3 Winterization

Winterization processes involve chilling of the oil at a prescribed rate, allowing the "stearin" or solid portions to crystallize and finally separating the two phases, usually by filtration, while cold (Bimbo, 1989). Winterization is an old practice that evolved from the observation that storage of oils in outdoor tanks during cold weather caused deposition of high-melting TAG at the bottom and clear liquid oil on the top. The clear oil was decanted and used as a light oil. Today marine oils are winterized for a number of reasons: (i) to remove waxes and other non-TAG constituents, (ii) to remove naturally-occurring high-melting TAG and (iii) to remove TAG formed during partial hydrogenation (List and Mounts, 1980).

2.2.4 Alkali-refining

Alkali-refining is practised as a purifying treatment designed to remove free fatty acids, phospholipids and gums, pigments, insoluble matters, etc. from crude oil (Bimbo, 1989). The alkali (normally diluted sodium hydroxide) added to oil reacts with free fatty

acids present and forms soap. Gums absorb alkali and are coagulated by hydration, much of the pigments are degraded, adsorbed on the gums or made water-soluble by the alkali and the insoluble matters are entrained with the other coagulated materials (Bimbo and Crowther, 1991). After adding alkali to crude oil, the mixture is slightly heated to break the emulsion and then the soap stock is removed by centrifugation (Cowan, 1976; Carr, 1978). The refined oil is washed with warm water to remove the last traces of soap (Kwon *et al.*, 1984).

2.2.5 Bleaching

After alkali-refining, the oil is usually bleached. Although the main objective of bleaching is to reduce the amount of coloured compounds and natural pigments, some suspended mucilaginous and colloid-like matters are also removed (Chang, 1967). Any traces of soap, if still present, are also adsorbed by the bleaching materials. Bleaching materials commonly used are natural clay, activated clay and carbon (Cowan, 1976). Activated carbon is normally used at 5-10% in combination with the clay. Natural clay is used if the oil is readily bleachable. However, acid-activated clay is usually used because its bleaching power is greater than that of natural clay (Boki *et al.*, 1989; Morgan *et al.*, 1985; Richardson, 1978).

2.2.6. Hydrogenation

Hydrogenation is direct addition of hydrogen to the double bonds of fatty acids and is the most widely used single reaction in the edible oil industry (Bimbo and

Crowther, 1991). For hydrogenation, hydrogen gas, liquid oil and solid catalyst (nickel) must be brought into intimate contact at a suitably elevated temperature and under an appropriate pressure of hydrogen. Under such conditions, the proportion of unsaturated fatty acids in the oil is reduced by direct addition of hydrogen to their double bonds. Also migration of the position of double bonds and the formation of *trans* isomers in the TAG molecule may take place (Allen, 1978, 1986). Primarily, hydrogenation is a means of converting liquid oils to semi-solid plastic fats suitable for shortening and margarine manufacturing. Recently, hydrogenation is performed on highly unsaturated oils, especially marine oils in order to stabilize them against oxidative deterioration (Bimbo and Crowther, 1991). Hydrogenation also improves the colour of oil by decomposing objectionable colour and taste-active components of the crude oil, particularly those in marine oils (Chang, 1967).

2.2.7 Deodorization

Deodorization is the last major processing step in the refining process of edible oils and used to remove undesirable odourous substances that occur naturally or are generated during processing or storage (Gavin, 1978). Before deodorization, the oil may contain volatile odour and flavour-active components originally present in the crude oil, the "soapy" odour created by alkali-refining, the "earthy" odour generated by bleaching and the typical hydrogenation odour caused by hardening (Chang, 1967). It should be noted that some compounds may impart undesirable odour or flavour to oils at concentrations of 1 to 10 ppm or even lower (Chang, 1967). The deodorization process

is essentially a steam distillation process where the volatile compounds are stripped from the non-volatile oil (Bimbo and Crowther, 1991) and also destroys peroxides in the oil and removes any aldehydes or other volatile products which might have resulted from atmospheric oxidation (Lin *et al.*, 1990). This process also serves to decrease the free fatty acid content and improves colour of the oil. The conventional procedures used for deodorization (i.e. steam stripping at 200-240 °C) are not suitable for marine oils since they may cause oxidation of PUFA (Bimbo and Crowther, 1991). Therefore, modifications of conventional deodorization process have been sought to deodorize marine oils. Dinamarca *et al.* (1990) have developed a pilot-scale procedure for deodorizing fish oil by high vacuum distillation at low temperatures (below 150 °C) and produced a bland oil without destroying the long-chain PUFA.

2.3 Oxidation of marine lipids

Lipid oxidation, leading to rancidity, has been recognized since antiquity as a problem in the storage of fats and oils and lipid-containing foods. Characteristic changes associated with oxidative deterioration of vegetable and animal fats, especially marine oils, includes the development of unpleasant tastes and odours as well as changes in colour, viscosity, specific gravity and solubility (Labuza, 1971; Ke and Ackman, 1976; Enser, 1987). Several authors (Labuza, 1971; Frankel, 1980, 1982; Kanner *et al.*, 1987; Hsieh and Kinsella, 1989; Min *et al.*, 1989; Bradley and Min, 1992) have reviewed the mechanistic aspects of lipid oxidation and its subsequent effects in foods. Since this study is limited to marine oils (unsaturated fatty acids), their oxidation is discussed in

terms of autoxidation, photooxidation and thermal oxidation.

2.3.1 Autoxidation

Autoxidation is a natural process that takes place between molecular oxygen and unsaturated fatty acids in the environment. Autoxidation of unsaturated fatty acids occurs via a free radical (chemical species with an unpaired electron) chain mechanism that consists of basic steps of initiation, propagation and termination. Initiation of this process begin with the abstraction of a hydrogen atom adjacent to the double bond in the fatty acid (RH), catalyzed by light, heat or metal/metal ions in order to form a free radical (Reaction 1).

Initiation:

$$RH \xrightarrow{\text{initiator}} R^{\bullet} + H^{\bullet} \qquad (1)$$

Propagation:

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \qquad (2)$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet} \qquad (3)$$

Termination:

$$R^{\bullet} + R^{\bullet} \rightarrow RR \qquad (4)$$

$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR \qquad (5)$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2 \qquad (6)$$

The resultant alkyl free radical ($R^{\bullet}$) reacts with atmospheric oxygen to form an unstable peroxy alkyl free radical (Reaction 2) which may in turn abstract a hydrogen atom from

another unsaturated fatty acid to form a hydroperoxide (ROOH) and a new alkyl free radical (Reaction 3). The new alkyl free radical initiates further oxidation and contributes to the chain reaction. The chain reaction (or propagation) may be terminated by formation of non-radical products (Reactions 4-6). In the presence of air, termination reaction (6) is most important. Termination reactions (4) and (5) become more important when the oxygen concentration is low and away from the surface of a lipid-containing system (Frankel, 1985).

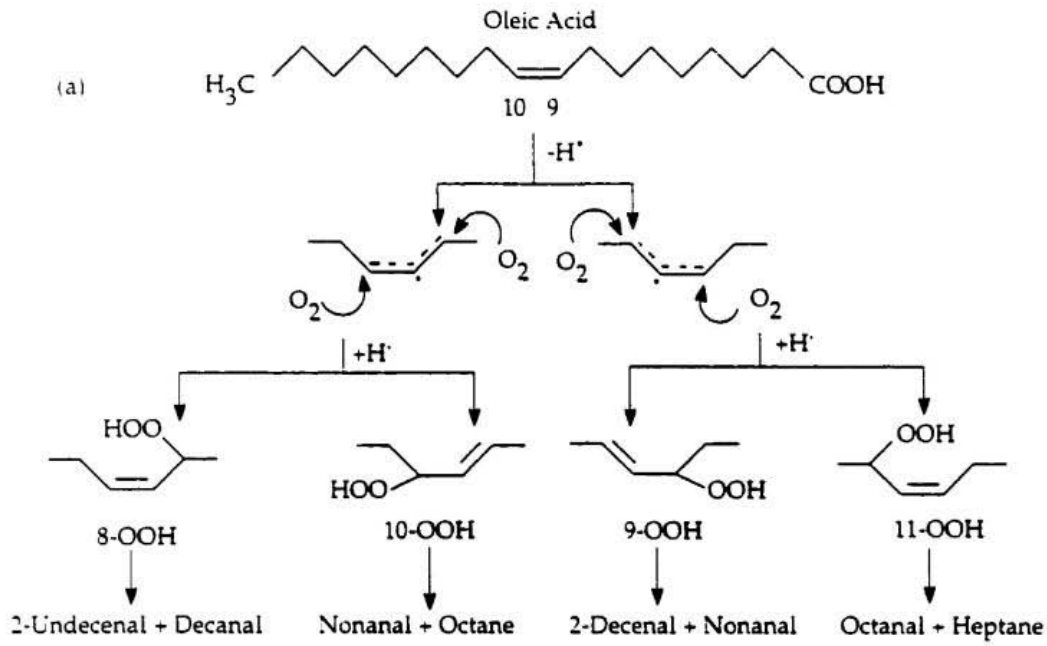
The propagation step of the autoxidation process includes an induction period when hydroperoxide formation is minimal (Labuza, 1971; Hawrysh, 1990). The rate of oxidation of fatty acids increases in relation to their degree of unsaturation. The relative rate of autoxidation of oleate, linoleate and linolenate was reported to be in the order of 1:40-50:100 on the basis of oxygen uptake and in the order of 1:12:25 on the basis of peroxide formation (Hsieh and Kinsella, 1989). Polyunsaturated fatty acids such as arachidonic acid, EPA and DHA, containing 4, 5, and 6 double bonds, respectively, are much more labile to oxidation than linoleic and linolenic acids. Arachidonic acid was reported to be oxidized 2.9 times faster than linoleic acid (Porter *et al.*, 1981). Ethyl esters of EPA and DHA are oxidized rapidly even at 5°C in the dark after an induction period of 3-4 days, whereas the induction periods of linoleate and linolenate were 20 and 60 days, respectively. Similarly, oxygen uptake of EPA and DHA ethyl esters after induction period was 5.2 and 8.5 times faster than that of ethyl linolenate (Cho *et al.*, 1987). Therefore, oils that contain relatively higher amounts of PUFA pose stability problems. Thus, inhibition of oxidation is a major criterion when marine oils are

incorporated into food products. Also the breakdown products (alcohols, aldehydes, ketones, hydrocarbons, etc.) of primary lipid oxidation products (hydroperoxides) contribute to off-flavour development. These compounds may also interact with other components of the food and cause several other functional and nutritional changes (Sherwin, 1978).

The ease of breaking down of hydroperoxides depends on the number of double bonds present in the molecule. Stability of hydroperoxides produced by EPA and DHA was much less in comparison with linoleate hydroperoxides (Miyashita *et al.*, 1982). In the case of linoleate oxidation, most of the oxidation products in the early stages were exclusively hydroperoxides. However, the ratios of hydroperoxide-oxygen to total absorbed-oxygen in ethyl EPA and ethyl DHA were 50-60%, even during the early stages of oxidation (Cho *et al.*, 1987). The high oxidation rates of EPA and DHA together with the instability of their hydroperoxides results in rapid formation of volatile secondary products such as aldehydes and other compounds which may cause flavour reversion of oils.

Possible autoxidation products of oleate, linoleate and linolenate are shown in Figures 2.4 and 2.5 (Frankel, 1985). Autoxidation of oleate involves hydrogen abstraction from carbon-8 and carbon-11 with formation of two allylic radicals (Figure 2.4a) which react with oxygen to form mixture of 8-, 9-, 10- and 11-allylic hydroperoxides. Hydrogen abstraction on the double allylic carbon-11 of linoleate produces a pentadienyl radical, which reacts at both ends with oxygen to produce a mixture of conjugated 9- and 13-diene hydroperoxides (Figure 2.4b). Hydrogen abstraction of linolenate occurs on the two

Figure 2.4 Mechanism of autoxidation of oleic acid (a) and linoleic acid (b) and formation of possible primary and secondary products (adapted from Frankel, 1985)


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

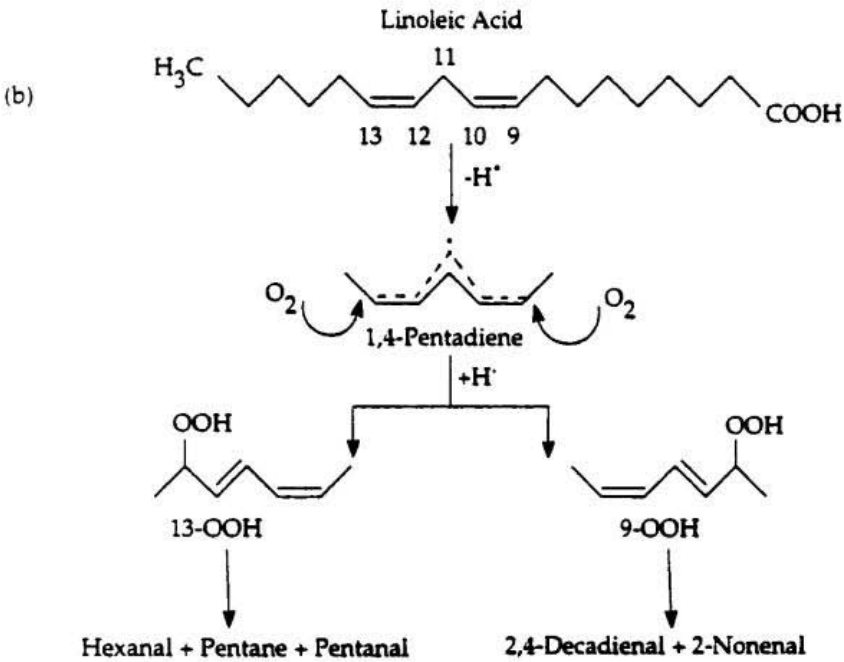
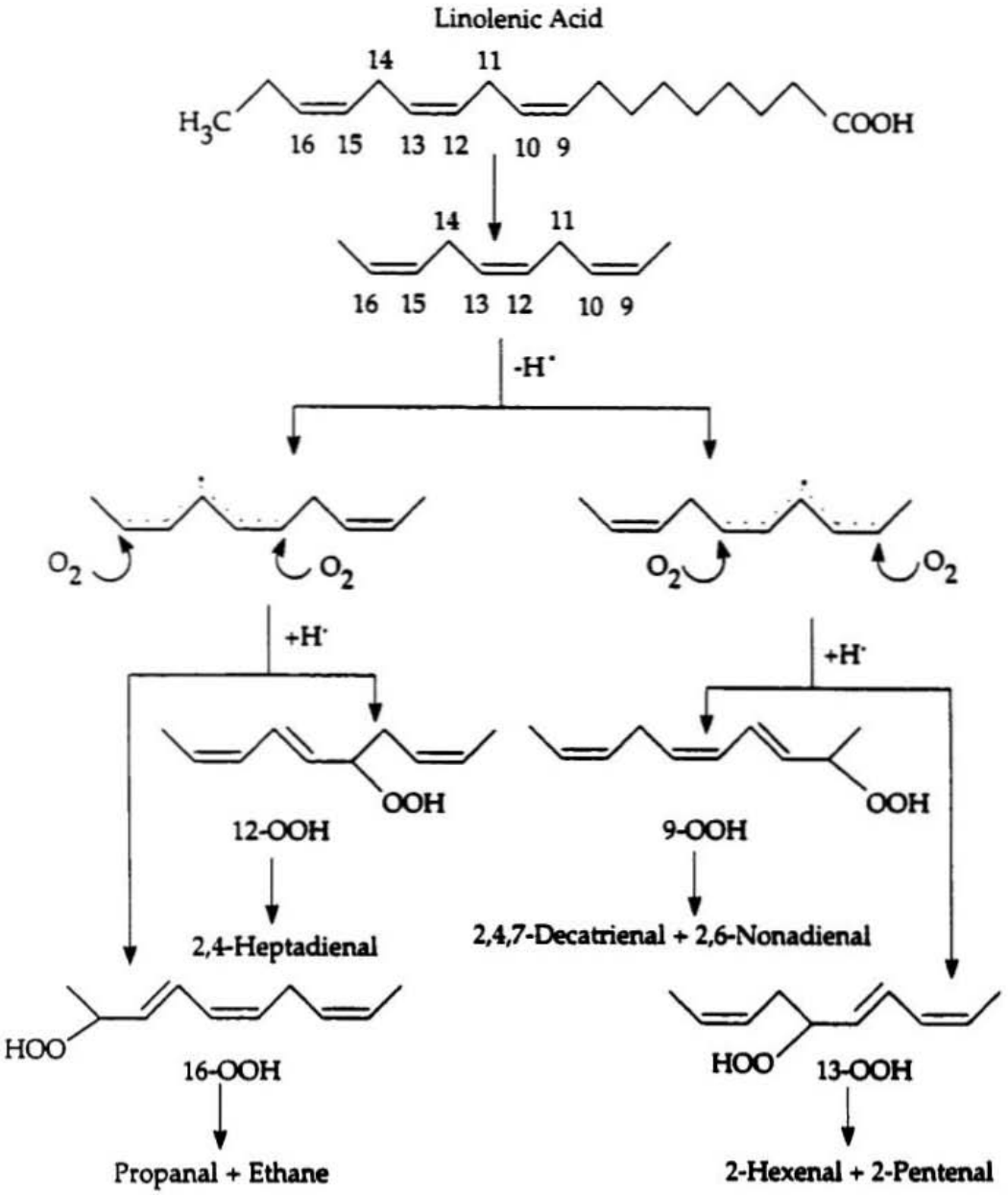

(b)
Linoleic Acid
H_3C
11
COOH
13 12 10 9
-H·
O_2
1,4-Pentadiene
+H·
OOH
13-OOH
9-OOH
Hexanal + Pentane + Pentanal
2,4-Decadienal + 2-Nonenal

Figure 2.5 Mechanism of autoxidation of linolenic acid and formation of possible primary and secondary products (adapted from Frankel, 1985)


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

active methylenes on carbon-11 and carbon-14 and produces two pentadienyl radicals which react with oxygen at the end carbon to form a mixture of conjugated diene-triene 9-, 12-, 13- and 16-hydroperoxides (Figure 2.5). Yamauchi *et al.* (1983) reported that myoglobin-catalyzed autoxidation of EPA produces eight monohydroperoxide isomers (5-, 8-, 9-, 11-, 12-, 14-, 15- and 18-hydroperoxides; Figure 2.6). Van Rollins and Murphy (1984) have found that autoxidation of DHA produces ten hydroperoxide isomers (4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17-, and 20-hydroperoxide; Figure 2.7).

Quantitative studies of isomeric hydroperoxides showed that only linoleate had the product distribution corresponding to that predicted by free radical mechanism (Frankel, 1980). For oleate, the 8- and 11-hydroperoxides were present in slightly but consistently higher amounts than the 9- and 10-hydroperoxides (Table 2.2). The exact reason for this difference has not been established, however it might be due to a greater oxygen attack on carbon-8 and carbon-11 than carbon-9 and carbon-10 or to allylic rearrangement of hydroperoxides. In linolenate, the 9- and 16-hydroperoxides were formed in significantly higher proportions than the 12-, and 13-hydroperoxides which might be attributed to the: (i) pentadiene radicals of linolenate prefer to react with oxygen at the end of carbon-9 and carbon-16 either because autoxidation is regioselective or due to steric effects; (ii) 12- and 13-hydroperoxides are more easily decomposed; and (iii) 12- and 13-hydroperoxides undergo either 1,4-cyclization into six-membered peroxides or 1,3-cyclization into prostaglandin-like endoperoxides (Frankel, 1980). Among the eight hydroperoxides produced by EPA oxidation, the 5- and 18-hydroperoxide isomers occurred in higher yields than the inner 8-, 9-, 11-, 12-, 14- and 15-hydroperoxide isomers (Table 2.2).

Figure 2.6 Mechanism of autoxidation of eicosapentaenoic acid (EPA) and formation of possible primary and secondary products (adapted from Fujimoto, 1989)

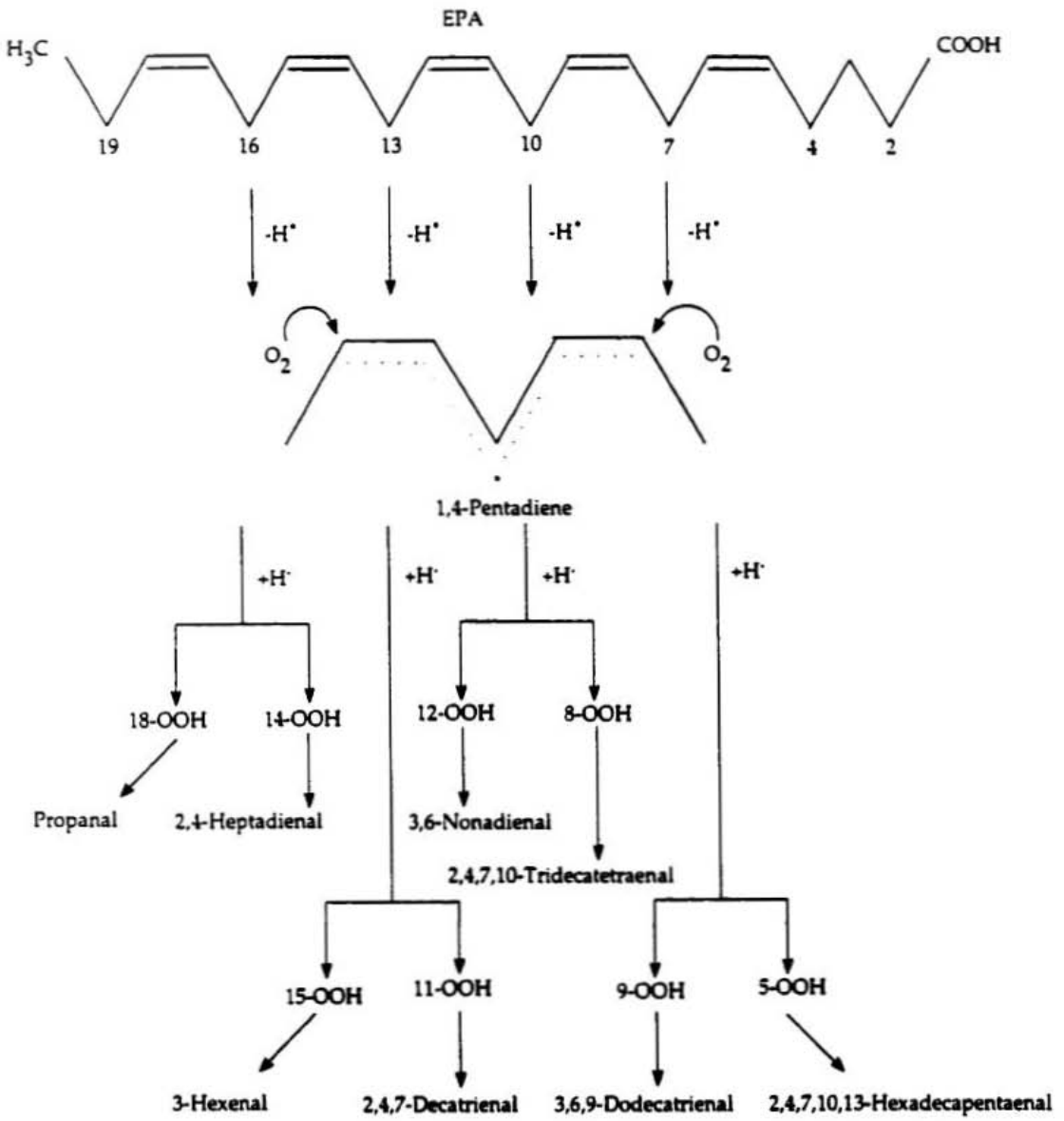

EPA
H_3C
COOH
19
16
13
10
7
4
2
-H•
-H•
-H•
-H•
O_2
O_2
1,4-Pentadiene
+H·
+H·
+H·
+H·
18-OOH
14-OOH
12-OOH
8-OOH
Propanal
2,4-Heptadienal
3,6-Nonadienal
2,4,7,10-Tridecatetraenal
15-OOH
11-OOH
9-OOH
5-OOH
3-Hexenal
2,4,7-Decatrienal
3,6,9-Dodecatrienal
2,4,7,10,13-Hexadecapentaenal

Figure 2.7 Mechanism of autoxidation of docosahexaenoic acid (DHA) and formation of possible primary and secondary products (adapted from Fujimoto, 1989)

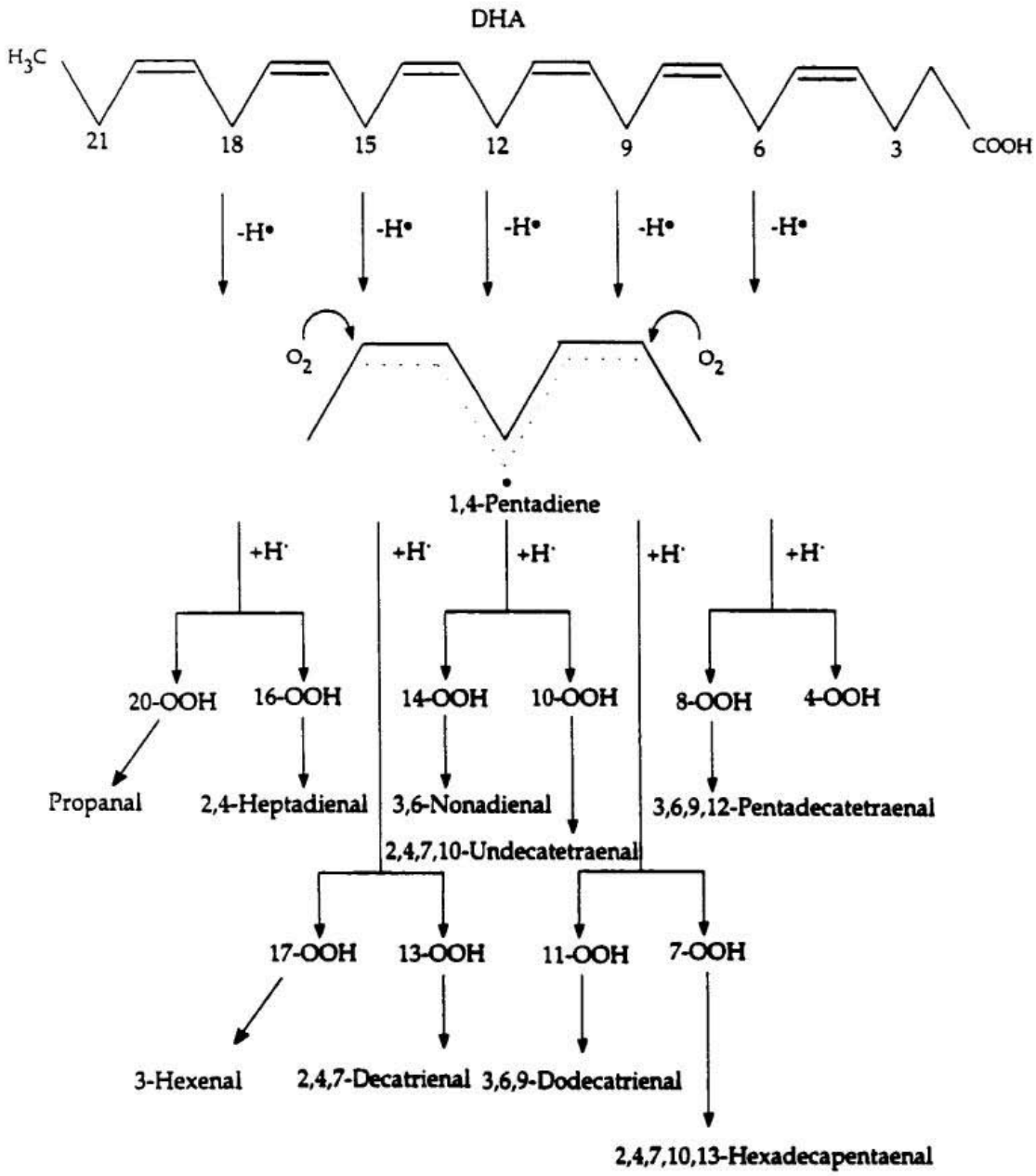

DHA
H3C
21
18
15
12
9
6
3
COOH
-H•
-H•
-H•
-H•
-H•
O2
O2
1,4-Pentadiene
+H·
+H·
+H·
+H·
+H·
20-OOH
16-OOH
14-OOH
10-OOH
8-OOH
4-OOH
Propanal
2,4-Heptadienal
3,6-Nonadienal
3,6,9,12-Pentadecatetraenal
2,4,7,10-Undecatetraenal
17-OOH
13-OOH
11-OOH
7-OOH
3-Hexenal
2,4,7-Decatrienal
3,6,9-Dodecatrienal
2,4,7,10,13-Hexadecapentaenal

Table 2.2 Relative percentage of isomeric hydroperoxides of autoxidized fatty acid methyl esters

Fatty acid	Relative distribution (%)					
Methyl-oleate		8-OOH 26-28	9-OOH 22-25	10-OOH 22-24	11-OOH 26-28	
Methyl-linoleate			9-OOH 48-53	13-OOH 48-53		
Methyl-linolenate		9-OOH 28-35	12-OOH 8-13	13-OOH 10-13	16-OOH 41.52	
Methy-EPA[1]	5-OOH 22	8-, 9-OOH 19	11-OOH 14	12-, 14-OOH 16	15-OOH 7	18-OOH 22

[1]Eicosapentaenoic acid

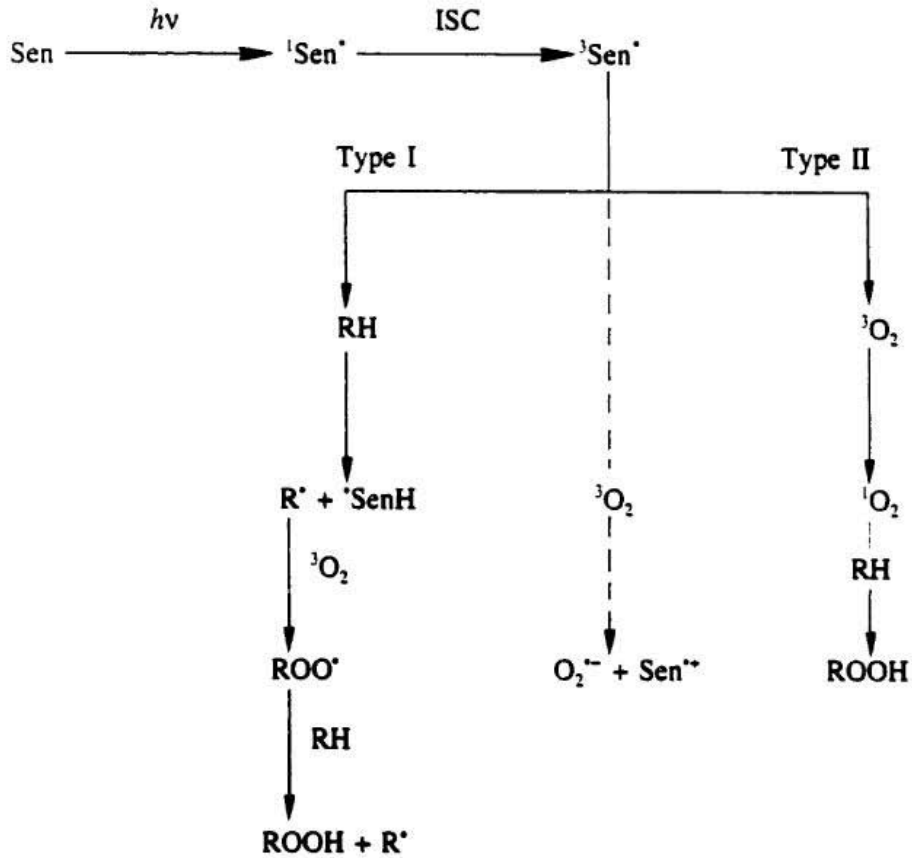
From Frankel (1980), Yamamoto *et al.* (1982) and Yamauchi *et al.* (1985)

2.3.2 Photooxidation

The degradation of fats and oils during exposure to light is an important factor influencing flavour stability. Photooxidation or photosensitized oxidation occurs in the presence of a photosensitizer and visible light, and proceeds faster than autoxidation (Hawrysh, 1990). During photooxidation, singlet oxygen ($^1O_2$) is generated by the interaction of light and a photosensitizer (Gunstone, 1984) which then reacts with the methylene group adjacent to the double bonds of the unsaturated fatty acids to form hydroperoxides (Chan, 1977: Frankel, 1985). Photosensitizers can be dyes (eosin, erythrosine, methylene blue, rose bengal), natural pigments (chlorophyll, protoporphyrin IX, riboflavin, haematoporphyrin, haemoglobin, myoglobin), metallic salts (cadmium sulphide, zinc oxides, zinc sulphide), transition metal complexes and polycyclic aromatic hydrocarbons such as anthracene (Min *et al.*, 1989; Davis *et al.*, 1995).

The ground state photosensitizer (Sen) absorbs visible or near ultraviolet light and becomes the excited singlet state photosensitizer ($^1Sen^*$) which has a short life time. $^1Sen^*$ returns rapidly to the ground state by emitting fluorescent light or converts to the excited triplet state sensitizer ($^3Sen^*$) by intersystem crossing (ISC). $^3Sen^*$ has a much longer life time than $^1Sen^*$ and decays to ground state slowly by emitting phosphorescent light. Efficient sensitizers for the generation of singlet oxygen are long lived $^3Sen^*$ (Min *et al.*, 1989; Bradley and Min, 1992). The triplet excited state sensitizer ($^3Sen^*$) takes two major reaction pathways (Type I and Type II) in order to accomplish photosensitized oxidation of unsaturated oils (Figure 2.8).

Figure 2.8 Photosensitized oxidation of lipids (RH)


hν
ISC
Sen
^{1}Sen*
^{3}Sen*
Type I
Type II
RH
$^3O_2$
R* + *SenH
$^3O_2$
$^1O_2$
$^3O_2$
RH
ROO*
O_2^{*-} + Sen^{+*}
ROOH
RH
ROOH + R*

In the Type I mechanism (sensitizer-substrate), $^3Sen^*$ serves as a photochemically-activated free radical initiator and reacts with substrate (RH) to produce free radicals by hydrogen transfer or electron transfer. The free radicals so formed react with triplet state oxygen ($^3O_2$) to produce the oxidized products that readily break down to form free radicals that can initiate free radical chain reactions. The rate of Type I reaction is dependant on the type and concentration of the sensitizer and substrate (Bradley and Min, 1992; Davis *et al.*, 1995).

In the Type II mechanism (singlet oxygen), $^3Sen^*$ reacts with triplet oxygen ($^3O_2$) to generate singlet oxygen ($^1O_2$). The singlet oxygen so produced reacts with the substrate (RH) to give ROOH. There is also an electron transfer from $^3Sen^*$ to triplet oxygen to produce superoxide radical anion ($O_2^{\bullet-}$) and sensitizer radical cation ($Sen^{\bullet+}$) with a chance of less than 1% (Min *et al.*, 1989). Electron-rich compounds such as simple olefins, dienes and aromatic compounds favour the Type II pathway. The rate of the Type II reaction depends mainly on the solubility and concentration of oxygen present in the food system. Traces of the sensitizer present in oils would tend to promote photosensitized oxidation by the Type II pathway because oxygen is more soluble in lipids and non-polar solvents than in water (Bradley and Min, 1992; Davis *et al.*, 1995). The involvement of singlet oxygen in the photosensitized oxidation is of Type II which occurs rapidly and thus accounts for almost all photosensitized oxidation reactions (Min *et al.*, 1989; Davis *et al.*, 1995).

Photooxidation of edible oils is a major concern in the food industry as they contain natural photosensitizes and are commercially sold under light (Labuza, 1971;

Frankel, 1980: Simic, 1980; Davis *et al.*, 1995)). The products of photosensitized oxidation include both non-conjugated and conjugated diene hydroperoxides, compared to autoxidation which produces only conjugated diene hydroperoxides (Rawls and Van Santen, 1970). Carotenoids have been known to act as quenchers for either singlet oxygen or triplet sensitizer in singlet oxygen lipid oxidation (Davis *et al.*, 1995). Tocopherols are also known to serve as free radical scavengers and singlet oxygen quenchers (Min *et al.*, 1989).

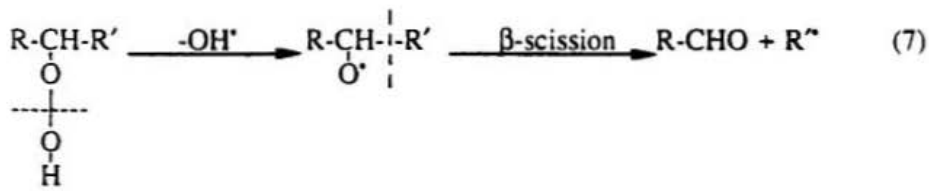
2.3.3 Thermal oxidation

During heating (as in deep fat frying), the oil is subjected to high temperatures (180-190°C) in the presence of air and moisture. Under such conditions, oxidation, hydrolysis and thermal degradation are relatively rapid and no induction period is detected (Hawrysh, 1990). As oxidation, hydrolysis and thermal reactions proceed, functional properties, sensory and nutritional qualities of the frying oils are changed (Stevenson *et al.*, 1984). The breakdown products formed from oil during heating (at frying temperatures) include volatile and non-volatile products. Formation of volatile decomposition products, affects the flavour of both the oil and the fried food (Chang *et al.*, 1978). Formation of non-volatile decomposition products is largely due to thermal oxidation and polymerization of the unsaturated fatty acids of the fried fat. These products cause physical changes such as darkening of colour, increase in viscosity and decrease in the smoke point of the fat as well as inducing chemical changes such as increase in the content of free fatty acids and carbonyl value (Hawrysh, 1990).

2.3.4 Decomposition of hydoperoxides

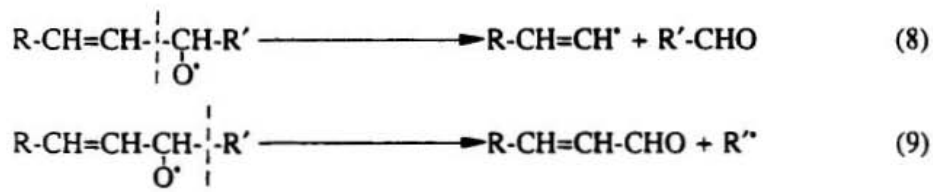
Hydroperoxides of unsaturated fatty acids are thermally unstable and undergo decomposition to generate a wide variety of breakdown products including the volatile compounds causing off-flavour and off-odour. Some of the decomposition products are unstable and will undergo further decomposition to produce lower molecular weight products (Frankel, 1980; Chan and Coxon, 1987; Gardner, 1987).

The first step in hydroperoxide decomposition is scission at the oxygen-oxygen bond of hydroperoxide group, giving rise to an alkoxy radical and a hydroxy radical (Reaction 7):



(7)

The alkoxy radical then undergoes homolytic β-scission, which is the most important free radical reaction leading to breakdown products causing flavour deterioration of lipids. The corresponding alkoxy radical from allylic hydroperoxides may undergo carbon-carbon cleavage on either side of the carbon bearing oxygen to produce two types of aldehydes, an olefinic and an alkyl radical (Reactions 8 and 9):



(8)

(9)

The olefinic radical is very reactive and unstable and may undergo the following reactions (Reactions 10-12):

$$R\text{-}CH{=}CH^{\bullet} + {}^{\bullet}OH \longrightarrow R\text{-}CH{=}CH\text{-}OH \longrightarrow R\text{-}CH_2\text{-}CHO \quad (10)$$

$$R\text{-}CH{=}CH^{\bullet} + {}^{\bullet}H \longrightarrow R\text{-}CH{=}CH_2 \quad (11)$$

$$R\text{-}CH{=}CH^{\bullet} + O_2 + {}^{\bullet}H \longrightarrow R\text{-}CH{=}CH\text{-}OOH \xrightarrow{-OH^{\bullet}} R\text{-}CH_2\text{-}CHO \quad (12)$$

The corresponding alkyl radical would undergo similar reactions to produce alcohol, hydrocarbon or hydroperoxide (Ractions 13-15):

$$R'^{\bullet} + {}^{\bullet}OH \longrightarrow R'\text{-}OH \quad (13)$$

$$R'^{\bullet} + {}^{\bullet}H \longrightarrow R'H \quad (14)$$

$$R'^{\bullet} + O_2 + {}^{\bullet}H \longrightarrow R'\text{-}OOH \quad (15)$$

Secondary products of hydroperoxide decomposition, especially unsaturated aldehydes, alkyl and alkoxyl radicals, are still unstable and undergo further reactions such as condensation, rearrangement and polymerization. Despite the intensive research on the decomposition of hydroperoxides, the multiplicity of the many possible pathways results in a very complex pattern of autoxidation products in such away that the origin of their hydroperoxide is completely obliterated.

2.3.5 Factors affecting lipid oxidation

Food systems contain numerous non-lipid components that affect the oxidation of lipids; while some enhance the rate of lipid oxidation, some tend to suppress it (Love, 1985). The number, position and geometry of the double bonds of unsaturated fatty acids affect the rate of lipid oxidation. The *cis* isomers oxidize more rapidly than the *trans* isomers and conjugated double bonds are more reactive than non-conjugated olefines (Nawar, 1985).

The oxidation rate of lipids is independent of oxygen pressure when the oxygen supply is unlimited (zero order reaction). At low oxygen pressures, however, the rate is approximately proportional to the oxygen pressure (Korycka-Daht and Richardson, 1978; Sherwin, 1978). Temperature and surface area also affect the partial pressure of oxygen (oxygen becomes less soluble at higher temperature) (Nawar, 1985). In general, the rate of oxidation increases with increasing temperature (Erickson and List, 1985). Ultraviolet and near ultraviolet lights have strong accelerating effects on fat and oil oxidation (Sherwin, 1978). This may be due to photosensitized oxidation, as discussed earlier.

Transition metals, particularly those possessing two or more valency states, with a suitable oxidation-reduction potential between them (e.g. Co, Cu, Fe, Mn, Ni), are major prooxidants (Gordon, 1990). At concentrations as low as 0.1 ppm, they can decrease the induction period and increase the rate of oxidation (El-Zeany *et al.*, 1974). Trace amounts of heavy metals are encountered in most edible oils. They may originate from equipment used in processing and storage of oils. Trace metals are also naturally present in all plant and animal tissues and fluids of biological origin, both in the bound and free

forms (Nawar, 1985). The contribution of metals ions to lipid oxidation involves two radical-producing reactions, one (Reaction 16) involves the metal in its lower oxidation state, and the other (Reaction 17) in its higher oxidation state (Hiatt *et al.*, 1968).

$$M^{n+} + ROOH \rightarrow M^{(n+1)+} + OH^- + RO^{\bullet} \quad (16)$$

$$M^{(n+1)+} + ROOH \rightarrow M^{n+} + ROO^{\bullet} + H^+ \quad (17)$$

The relative importance of these reactions varies with the type of metal as well as other factors (e.g. solvent, substrate), but the rate of Reaction (16) is generally much faster than that of Reaction (17). The two reactions can operate as a cycle so that the overall effect of the metal ion would be to produce more radicals.

The enzyme lipoxygenase, present in most plants tissues and fish skin and gills, specifically oxygenates PUFA and PUFA esters containing a *cis, cis*-1,4-pentadiene moiety located between carbons 6 and 10 from the methyl terminus. Off-flavour development in soybean and soybean products is highly dependent on the action of various endogenous lipoxygenases as subsequent decomposition of the resulting hydroperoxides yields rancid flavours (Richardson and Hyslop, 1985). The potential role of fish lipoxygenase in catalysing the oxidation of fish lipids has been reported by several researches. German and Kinsella (1986) suggested that lipoxygenase from skin of fish could accelerate lipid deterioration in underlying muscle tissues. Hsieh and Kinsella (1986) found that lipoxygenase activity in the skin of trout and this might explain why skin lipids of mackerel are more susceptible to oxidation than those of their muscle tissues as reported by Ke *et al.* (1977a and b).

2.4 Control of lipid oxidation

Since oxidation of lipids containing unsaturated fatty acids can proceed via different mechanisms, several strategies are possible to minimize oxidation. Knowledge of the key mechanism(s) for the initiation of lipid oxidation allows adequate devising of methodologies to control lipid oxidation. Several methods that might be employed for control of oxidation of food lipids are described in subsequent sections.

2.4.1 Removal of oxygen

Since oxygen is an essential reactant in lipid oxidation, control of oxygen availability is a critical variable in minimizing oxidation of unsaturated fatty acids. As discussed earlier, the rate of oxidation of unsaturated fatty acids is affected by oxygen pressure when the partial pressure of oxygen in the reaction system is less than 100 mm (Pryor, 1973; Sherwin, 1978). The level of available oxygen may be controlled by vacuum packaging (Lindsay, 1977; Josephson *et al.*, 1985) and by using oxygen scavengers such as glucose oxidase and ascorbic acid oxidase (Hsieh and Kinsella, 1989). These precautions reduce the rate and extent of lipid oxidation, especially when combined with antioxidants and low temperature storage in the dark.

2.4.2 Hydrogenation

A high level of unsaturated fatty acids, particularly PUFA, which also confers liquidity to oils, is responsible for the early development of off-flavours and off-odours. When an oil is hydrogenated, the combination of the hydrogen with double bonds of the

fatty acids raises the melting point of the oil but also improves its flavour and odour stability. Hydrogenation of marine oils has been patented as a method of stabilizing them. Selective hydrogenation of menhaden oil is practised in the United States (Bimbo and Crowther, 1991). This process reduces the PUFA content of the oil and products so obtained are used in the preparation of table margarine, shortenings, etc. (Bimbo 1989). However, hydrogenation reduces the degree of unsaturation of fatty acids and lowers the nutritional value of PUFA-containing foods. Details of this process have been discussed in Section 2.2.6.

2.4.3 Use of antioxidants and synergists

Antioxidants are added to fats and oils to retard oxidation and to reduce development of rancidity. However, antioxidants cannot improve the quality of already oxidized food products (Dziezak 1986). According to the USDA Code of Federal Regulations [21 CFR 170.3 (0) (3)], "antioxidants are substances used to preserve food by retarding deterioration, rancidity or discolouration due to oxidation" (Dziezak, 1986). Synergists are substances that enhance the activity of antioxidants without having their own antioxidant effect (Nawar, 1985). Ideal food-grade antioxidants in addition to being safe, should not affect the colour and flavour of food and must be effective at low concentrations, easy to incorporate, survive after processing and be stable in the finished product (carry-through properties) as well as being available at a low cost (Coppen, 1983).

2.4.3.1 Mechanism of antioxidant activity

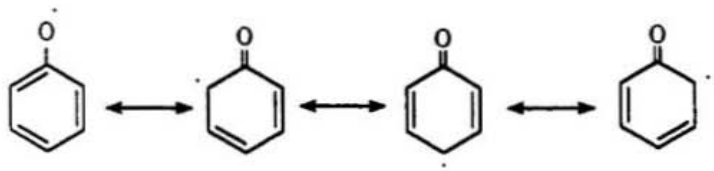
On the basis of lipid oxidation processes, antioxidants can be grouped into two mechanistically distinct classes. One group of antioxidants can inactivate two important radical species, involved in chain propagation steps, alkyl peroxy ($ROO^{\bullet}$) and alkyl ($R^{\bullet}$) radicals, and they can be grouped as chain breakers or primary antioxidants (Heish and Kinsella, 1989; Gordon, 1990). This group includes the most common food antioxidants (phenolic antioxidants; AH) which interfere with lipid oxidation by rapid donation of a hydrogen atom to lipid radicals according to Reactions (18) and (19).

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \qquad (18)$$

$$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet} \qquad (19)$$

Primary antioxidants are able to donate a hydrogen atom to a lipid radical and produce a radical from the antioxidant which is more stable than the lipid radical or is converted to other stable products (Gordon, 1990). Even though phenol itself is inactive as an antioxidant, alkyl substituted phenolic compounds are the most effective antioxidants used in foods (Uri, 1961; Sherwin, 1990). Substitution of alkyl groups in the 2, 4 or 6 position increases the electron density on the hydroxy group by an inductive effect and thus increases their reactivity with lipid radicals. Substitution at the 4th position with an ethyl or t-butyl group rather than a methyl group improves the activity of a phenolic antioxidants, however, longer chain or branched alkyl groups in this position decrease the activity (Ingold, 1960). The strong electron donating effect of a methoxy substituent is an important contributor to the effectiveness of 2-tert-butyl-4-methoxyphenol (BHA) as an antioxidant.

The radical formed from the reaction of a phenol with a lipid radical is stabilized by delocalization of the unpaired electron around the aromatic ring as indicated by the valence bond isomers (Reaction 20).



(20)

The stability of the phenoxy radical (A$^{\bullet}$) reduces the rate of propagation of the autoxidation chain reaction since propagation Reactions such as (21)-(23) are very slow as compared with Reactions (2) and (3).

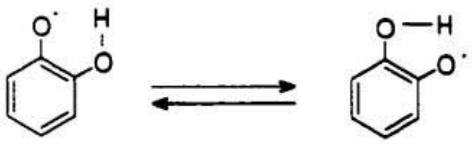
$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet} \qquad (21)$$

$$AOO^{\bullet} + RH \rightarrow AOOH + R^{\bullet} \qquad (22)$$

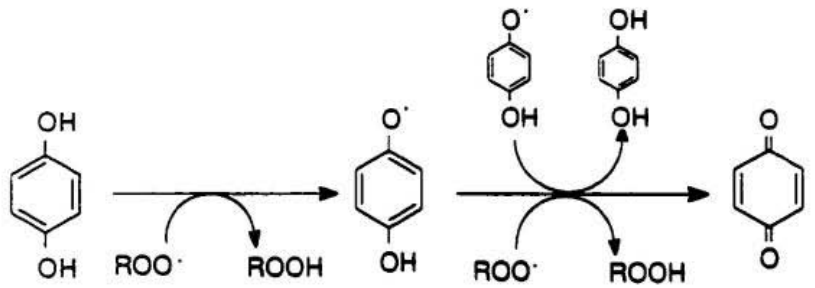
$$A^{\bullet} + RH \rightarrow AH + R^{\bullet} \qquad (23)$$

Stability of the phenoxy radical is further increased by the presence of bulky groups in the 2 and 6 positions as in 2,6-di-tert-butyl-4-methylphenol, BHT (Gordon, 1990). However, the presence of bulky substituent in the 2 and 6 positions also reduces the rate of reaction of the phenol with lipid radicals (Reactions 18 and 19). The presence of second hydroxy group at the 2 or the 4 position of a phenol increases the antioxidant activity. The effectiveness of a 1,2-dihydroxybenzene derivative is increased by the stabilization of a phenoxy radical by an intramolecular hydrogen transfer (Reaction 24). The antioxidant activity of dihydroxybenzene derivatives is partly due to the fact that the semiquinoid radical produced initially can further oxidize to a quinone via reaction with

another lipid radical or may disproportionate to a quinone and a hydroquinone molecule (Reaction 25) (Gordon, 1990).



(24)



(25)

Hydroquinone Quinone

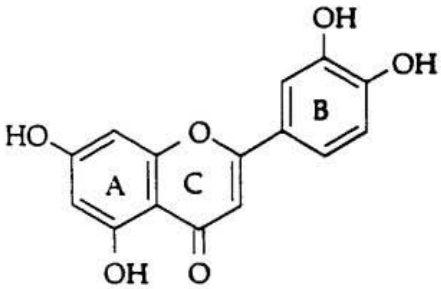
The effect of antioxidant concentration on the rate of autoxidation depends on several factors including antioxidant structure, oxidation conditions and the substrates involved. Often the antioxidant activity of phenolic compounds is lost at high concentrations and they may act as prooxidants due to involvement in initiation reactions (Cillard *et al.*, 1980; Lundberg *et al.*, 1947).

Secondary antioxidants prevent the introduction of chain initiation radicals into the system (Heish and Kinsella, 1989). These may operate by a variety of mechanisms including binding of metal ions, scavenging of oxygen, decomposition of hydroperoxides

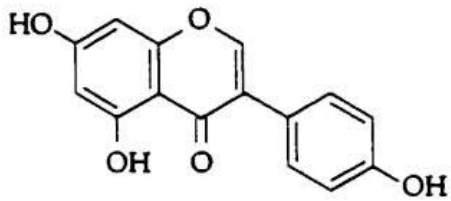
to non-radical species, absorption of UV radiation or deactivation of singlet oxygen. Secondary antioxidants usually show antioxidant activity if a second minor component is present in the system (Gordon, 1990). Citric acid, EDTA and phosphoric acid derivatives (polyphosphates) may extend the shelf life of lipid-containing foods by chelation of metal ions which act as prooxidants. Ascorbic acid, ascorbyl palmitate, erythrobic acid (isoascorbic acid) or sodium erythrobate are also used to stabilize fatty foods. Ascorbic acid is oxidized to dehydroascorbic acid when it functions as an oxygen scavenger, its activity is enhanced in the presence of tocopherols. Ascorbyl palmitate is more effective as an antioxidant because of its increased solubility in the lipid phase (Cort, 1974). It has been shown that enzymes-like superoxide dismutase and catalase can remove formed superoxide radical anion ($O_2^{\cdot-}$) and hydrogen peroxide, respectively, which are important in lipid oxidation of biological system (Kellog and Fridovich, 1975). Similarly β-carotene can inhibit lipid oxidation initiated by xanthine oxidase, perhaps due to its quenching effect of singlet oxygen. Amino acids have also been implicated as having some chelating ability, however, their application in oils is limited due to solubility problems (Labuza, 1971).

Many of the flavonoids and related phenolic compounds show marked antioxidant properties (Mehta and Seshadri, 1959). Structures of these flavonoids and related compounds are given in Figure 2.9. Flavonoids and related compounds are known as primary antioxidants and act as free radical acceptors and chain breakers. Flavonoids are also known to chelate metal ions at the 3-hydroxy-4-keto group and/or the 5-hydroxy-4-keto group (when the A ring is hydroxylated at position 5; Shahidi *et al.*, 1991a, 1993).

Figure 2.9 Structure of flavonoids and related compounds (adapted from Shahidi and Wanasundara, 1992)

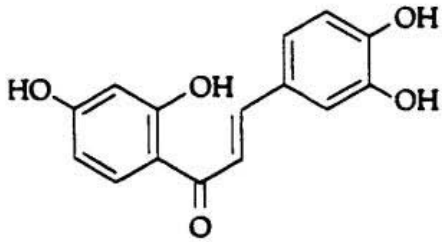


Flavones (luteolin)

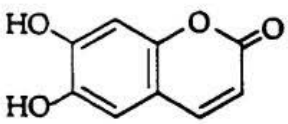


Isoflavones (daidzein)

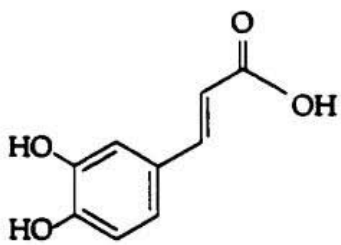
Flavanones (eriodictyol)



Chalcones (butein)



Coumarins (aesculetin)



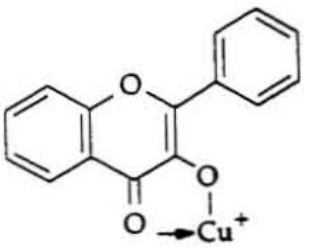
Cinnamic acids (caffeic acid)

An *O*-quinol group at the B ring can also demonstrate metal chelating activity (Pratt and Hudson, 1990). Hudson and Lewis (1983) have demonstrated the ability of flavonoids to form complexes with cupric ion (Figure 2.10), using ultraviolet spectral studies. Such complexation may contribute to the antioxidative action of flavonoids. Chelation of metal ions renders them catalytically inactive.

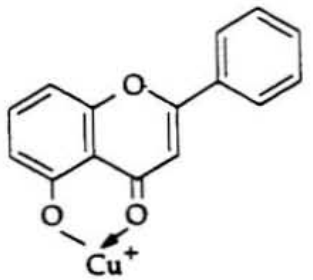
2.4.3.2 Synthetic antioxidants

Use of synthetic antioxidants, mainly phenolic compounds, in foods has been in practice since the late 1940's (Sherwin, 1990). The application of antioxidants to foods is governed by Federal regulations. United States-Food and Drug Administration (US-FDA) regulations require that antioxidants and their carriers be declared on the ingredient labels of products and should be followed by an explanation of their intended purpose (Dziezak, 1986). The commonly used synthetic antioxidants today are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butylhydroquinone (TBHQ) (Figure 2.11). Possible toxicity and/or mutagenicity of synthetic antioxidants has been a subject of study for many years (Branen, 1975). There are reports on the effect of BHA on conversion of ingested materials into toxic substances or carcinogens by the increased secretion of microsomal enzymes of liver and extra-hepatic organs, such as the lungs and gastrointestinal tract mucosa (Wattenberg, 1986). Therefore, at the present time, the FDA has removed BHA from the GRAS list (Nieto *et al.*, 1993). After Ito *et al.* (1985) reported findings showing that BHT to be carcinogenic in rats, BHT is in the process of being carefully scrutinized. TBHQ has never been

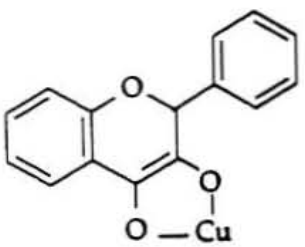
Figure 2.10 Forms of copper complexes with flavones and flavanones (adapted from Hudson and Lewis, 1983)



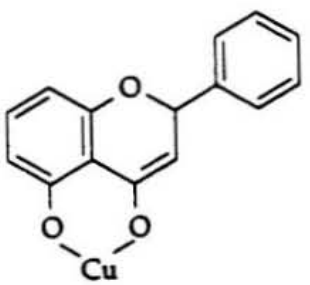
3-Hydroxyflavone



5-Hydroxyflavone

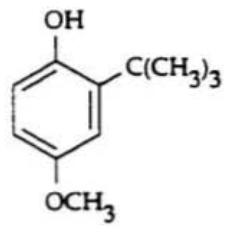


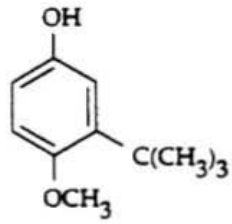
3-Hydroxyflavanone



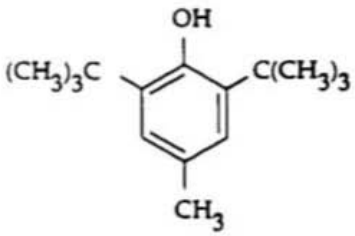
5-Hydroxyflavanone

Figure 2.11 Structures of some synthetic antioxidants and α-tocopherol

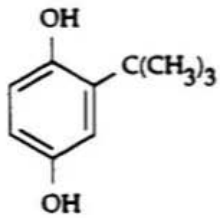




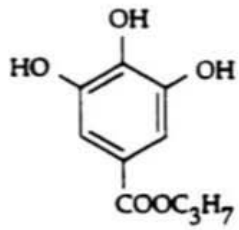
BHA (two isomers)



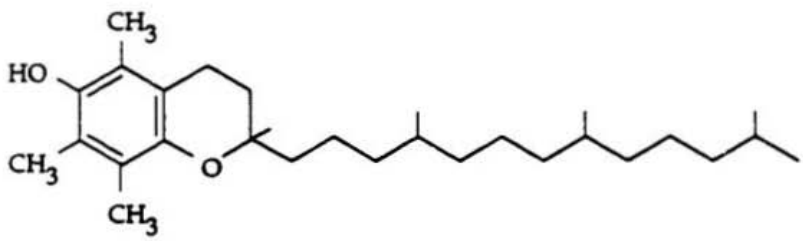
BHT



TBHQ



PG



α-Tocopherol

approved in Japan, Canada and certain European countries (Barlow, 1990). Since antioxidants are unavoidable additives, very soon the food, pharmaceutical and cosmetic industries will have to examine other options in order to replace synthetic antioxidants with natural analogues.

2.4.3.3 Natural antioxidants

Efforts to find natural alternatives to synthetic antioxidants have intensified over the last few years due to concerns about their safety when used in foods (Marshell, 1974). Natural antioxidants can be extracted from foods and non-food sources. Naturally-occurring antioxidative components are primarily phenolics that are present in all parts of plants (Hudson, 1990; Dugan, 1980). Plant phenolics are multifuctional and may act as reducing agents (free radical terminators), metal chelators and singlet oxygen quenchers (Shahidi and Wanasundara, 1992). The activity of natural antioxidants depends on the chemical nature of their active ingredient(s), the preparation methods and concentration of the active component(s) in the final products. The sources of natural antioxidative compounds, their properties and their main active components are briefly discussed below.

2.4.3.4 Sources of natural antioxidants

2.4.3.4.1 Oils and Oilseeds

Antioxidative compounds from oils and oilseeds such as canola, cottonseed, flax, mustard, peanut, sesame and soybean have been investigated in the past. Soybean flour as well as other soybean products are sources of a large variety of antioxidants belonging

to the isoflavone glycoside family and their derivatives, phospholipids, tocopherols, amino acids and peptides (Naim *et al.*, 1973, 1974, 1976; Herrmann, 1976; Hays *et al.*, 1977; Chen *et al.*, 1995). Methanolic extracts of peanut and glandless cottonseed have been shown to possess antioxidant activity in lipid oxidation model systems catalysed by metmyoglobin (Rhee *et al.*, 1979; Whittern *et al.*, 1984). Pratt and Miller (1984) and Duh *et al.* (1992) have identified dihydroquercetin, taxifolin as antioxidants extracted with hot methanol from peanut and quercetin and rutin as major flavonoids from delinted cottonseed. Methanolic and aqueous extracts of chia seeds possessed antioxidant activities as they retarded the bleaching of β-carotene and their activity was due to flavonol glycosides as well as chlorogenic and caffeic acids. The hydrolysed extracts of chia contained caffeic acid and flavonol aglycones namely, kaempferol, quercetin and myricetin (Taga *et al.*, 1984). Ohta *et al.* (1994) have reported that corn bran hemicellulose possessed antioxidant activity and found that sugar esters of ferulic acid were the most active components present. Among phenolic acids found in corn flour and oil, ferulic acid was most abundant (Sosulski *et al.*, 1982). The ethanolic extracts of defatted canola meal exhibited remarkable antioxidant activity in canola oil when compared with BHA and BHT (Wanasundara and Shahidi, 1994b). The compound with the strongest antioxidant activity was identified as 1-O-β-D-glucopyranosyl sinapate (Wanasundara *et al.*, 1994). The antioxidant activity of ethanolic extracts of mustard and flaxseed has also been reported in a β-carotene/linoleate and muscle food systems (Shahidi *et al.*, 1991a, 1994; Amorowicz *et al.*, 1993).

It has been reported that sesame seed oil has superior oxidative stability when

compared to other vegetable oils; this has been attributed to the presence of sesamin, sesamolin and sesamol as well as δ-tocopherol in the seeds (Lyon, 1972; Shahidi *et al.*, 1996). Sesamol which is found in processed sesame oil was as effective as BHT and BHA and better than PG in inhibiting oxidation of lard (Lyon, 1972). Fukuda *et al.* (1985) and Osawa *et al.* (1985) have reported strong antioxidant activity for acetone extracts of sesame seeds in linoleic acid model system and the active compound was identified as bisepoxylignan or sesamolinol.

2.4.3.4.2 Cereals and Legumes

Cereals are among the most common ingredients that may be added to many food products. Oat extracts were among the first antioxidants proposed for stabilization of fats and oils and lipid-containing foods (Chang *et al.*, 1977). Esters of caffeic and ferulic acids appear to be the most important antioxidants found in oats (Duve and White, 1991). The methanolic extracts of rice hulls from long-life and short-life varieties exhibited a superior antioxidant activity when compared with α-tocopherol (Ramarathnam *et al.*, 1986, 1988, 1989). Maillard and Berest (1995) have found that methanolic extract of germinated barley contained three major phenolic acids namely, *trans*-ferulic, *trans*-*p*-coumaric and *cis*-ferulic as main antioxidative components that prevent oxidation of prepared beer. Tsuda *et al.* (1993) have demonstrated a strong antioxidant activity for methanolic extracts of pea bean in a linoleic acid model system as measured by the thiocyanate method. Ethanolic extracts of navy bean hulls added to soybean and sunflower oils showed delayed oxidative deterioration of the oils as reflected in their

peroxide values during the extended storage. The antioxidant activity of this extract was better than BHA, BHT and rosemary (Onyeneho and Hettiarachchy, 1991).

2.4.3.4.3 Herbs and Spices

Chipault *et al.* (1952, 1956) have investigated the antioxidant potency of spices and herbs in various lipid systems. All spices (ginger, clove, garlic, capsicum, tumeric, etc.) and herbs (sage, oregano, rosemary, thyme, etc.) were shown to possess antioxidant activity in all types of lipid systems examined. Spice extracts have attracted much interest in recent years since they could be freely added to lipids. However, many extracts possess a strong odour and a bitter taste and thus are of limited value for incorporation into many food products. Chang *et al.* (1977) were able to prepare odourless and flavourless natural antioxidants from rosemary and sage. The extracts of rosemary leaves contained a phenolic diterpene, namely carnosol (Houlihan *et al.*, 1984, 1985; Nakatani and Inatani, 1984; Schuler 1990; Frankel *et al.*, 1996). Commercial antioxidant extracts from rosemary are available as a fine powder. Depending on their content of active antioxidants, they are recommended for use at concentrations ranging between 200 and 1000 ppm in processed products. Cuvelier *et al.* (1994) isolated six major antioxidative compounds from sage oleoresin and identified them as carnosol, carnosic acid, rosmadial, romanol, epirosmanol and methyl carnosate. Meanwhile, the main antioxidative compound of oregano was identified as a phenolic glycoside (Nakatani and Kikuzaki, 1987; Kikuzaki and Nakatani, 1989). The strong antioxidant activity of Papua mace was related to the presence of 2-allylphenols and a number of lignans

(Nakatani and Ikeda, 1984). Capsicin, a pungent antioxidant component of capsicum, and a ferulic amide of tyramine as well as piperine-related compounds with an open methylenedioxy ring formed in black pepper had a stronger antioxidant activity than tocopherols. These compounds are fat-soluble and are odourless and tasteless (Nakatani *et al.*, 1986). In addition, tetrahydroxy curcumin which is a colourless and heat resistant antioxidative compound was found in turmeric (Osawa *et al.*, 1989). Jitoe *et al.* (1992) and Masuda and Jitoe (1994) have isolated curcuminoids as strong antioxidative components from rhizomes of tropical ginger.

2.4.3.4.4. Natural antioxidants from tea

Green tea leaves contain approximately 36% (dry weight basis) of polyphenols, however, their composition varies with climate, season, variety and maturity of leaves (Lunder, 1989). Catechins are the predominant group of substances of green tea polyphenols and are comprised of (-) epicatechin (EC), (-)epicatechin gallate (ECG), (-)epigallocatechin (EGC), (-)epigallocatechin gallate (EGCG), (+)catechin and (+)gallocatechin (GC) (Huang *et al.*, 1992; Shahidi *et al.*, 1992; Ho *et al.*, 1994; Amarowicz and Shahidi, 1995). The content of different catechins in fresh tea leaves is shown in Table 2.3 (Ho *et al.*, 1994). Among catechins, EGCG in the dominant catechin in green tea leaves. In recent years, catechins have been shown to possess physiological potential with respect to their antimutagenic and antitumorigenic activities (Hara *et al.*, 1989; Conney *et al.*, 1992; Hara, 1994). Epidemiological studies have also suggested that tea polyphenols are effective in cancer prevention (Kim *et al.*, 1994). Furthermore,

Table 2.3 Content of different catechins in fresh green tea leaves (% dry weight)[a]

Component	Content
(-)Epigallocatechin gallate (EGCG)	9 - 13
(-)Epigallocatechin (EGC)	3 - 6
(-)Epicatechin gallate (ECG)	3 - 6
(-)Epicatechin (EC)	1 - 3
(+)Catechin	1 - 2
(+)Gallocatechin	3 - 4

[a]Ho *et al.* (1994)

catechins and other flavonoids have been recognized as efficient antioxidants capable of scavenging oxygen radicals and chelating metal ions (Sorata *et al.*, 1984; Husain *et al.*, 1987; Chen *et al.*, 1990; Shahidi *et al.*, 1991a, 1993; Shahidi and Wanasundara, 1992; Wanasundara and Shahidi, 1994a). Hara (1994) has evaluated the antioxidative potency of crude extracts of green tea and individual catechins in lard by the active oxygen method. Crude tea catechins reduced the formation of peroxides more effectively than α-tocopherol or BHA. The antioxidant potency of individual catechins, evaluated in the same manner, was in the order of EC< ECG < EGC < EGCG. However, there has been no studies on the evaluation of the antioxidative activity of individual tea catechins in marine oils which contain a large proportion of highly unsaturated fatty acids.

2.5 Measurements of lipid oxidation

There are various methods available for measurement of lipid oxidation in foods. Changes in chemical, physical, or sensory properties of the oil during oxidation may be monitored in order to assess the oxidative status of lipids. However, there is no uniform and standard method for detecting all oxidative changes in all food systems. The available methods to monitor lipid oxidation in foods and biological systems may be divided into two groups. The first group measures primary changes and the other group measures secondary changes that occur in oxidizing lipids. Primary changes are generally measured as (i) loss of unsaturated fatty acids, (ii) oxygen uptake by weight gain or other methods (iii) hydroperoxide values and (iv) conjugated diene value. Secondary changes are monitored by quantification of (i) carbonyls (as dinitrophenyl hydrazone or by gas

chromatography), (ii) malonaldehyde and other aldehydes (thiobarbituric reactive substances, TBARS value), (iii) hydrocarbons (ethane or pentane content), and (iv) fluorescence products (1-amino-3-iminopropane compounds). The method of choice depends on a number of factors including the nature and the history of the oxidized sample, the type of information required, the time available and the test conditions. Nonetheless, it is advisable to use a combination of methods to assess both primary and secondary oxidative changes in oils. However, results must be in agreement with sensory perception and acceptability of foods.

2.5.1 Primary changes and their measurement

2.5.1.1 Measurement of changes of reactants

Methods that measure primary changes of lipids may be classified as those that quantify loss of reactants (unsaturated fatty acids), addition of oxygen or formation of primary lipid oxidation products (hydroperoxides). These methods are more suitable to measure low levels of oxidation in uncooked products at low temperatures (Coxon, 1987). Measurement of changes in fatty acid composition is not widely used in assessing lipid oxidation since it requires total lipid extraction and conversion into derivatives suitable for gas chromatographic analysis. On the other hand changes of fatty acid composition can not be used in more saturated oils since this indicator shows only changes in unsaturated fatty acids during oxidation. Therefore, oxidative changes in marine oils and highly unsaturated vegetable oils may only be monitored in this manner. Similarly, monitoring of changes in iodine value due to loss of unsaturation during accelerated

oxidation studies may be considered (Hudson, 1983).

2.5.1.2 Measurement of weight gain

It is generally accepted that the addition of oxygen to lipids and formation of hydroperoxides is reasonably quantitative during initial stages of autoxidation. Therefore, the measurement of induction period from weight gain data is theoretically sound. In this method oil (lipid) samples, are kept at 60-65°C in an oven and the weight change of the samples is recorded at different time intervals. Olcott and Einset (1958) reported that marine oils exhibit a fairly sharp increase in weight at the end of the induction period and are rancid by the time they gain 0.3-0.5% in weight (at 30-60°C). However, surface exposure of the sample to air is an important variable in determining the rate of oxidation. Therefore, use of equal size containers to store samples is very important in carrying out such experiments.

The weight gain method has some disadvantages: (i) the weighing frequency hinders monitoring of fast kinetics (a higher frequency would involve nocturnal weighing), and low or moderate temperatures would result in long analysis times for stable samples, (ii) discontinuous heating of the sample (which must be cooled before weighing) may give rise to irreproducible results, so the heating and cooling intervals must be accurately controlled, (iii) the method involves intensive human participation and (iv) the working conditions (sample size, shape of the container and temperature) influence the results. Nevertheless, this method offers advantages such as low instrumentation cost and unlimited capacity for sample processing.

2.5.1.3 Measurement of hydroperoxides

In the oxidation of fats and oils, the initial rate of formation of hydroperoxides exceeds their rate of decomposition, but this is reversed at later stages. Therefore, monitoring the amount of hydroperoxides as a function of time will indicate whether a lipid is in the growth or decay portion of the hydroperoxide concentration curve. This information can be used as a guide for considering the acceptability of a food product with respect to the extent of its deterioration. By monitoring the incubation period before the appearance of hydroperoxides, one can assess the effectiveness of added antioxidants on the stability of a food lipid.

2.5.1.3.1 Peroxide value

The classical method for quantification of hydroperoxides is the determination of "peroxide value" (PV). The hydroperoxide content, generally referred to as peroxide content, is determined by an iodometric method which is based on the titration of the iodine released from potassium iodide by peroxides in a biphasic system with a thiosulphate solution (Reactions 26 and 27):

$$ROOH + 2H^{+} + 2I^{-} \rightarrow I_2 + ROH + H_2O \qquad (26)$$

$$I_2 + 2S_2O_3^{--} \rightarrow S_4O_6^{--} + 2I^{-} \qquad (27)$$

Potential drawbacks of this method are absorption of iodine at unsaturation sites of fatty acids and liberation of iodine from potassium iodide by oxygen present in the solution to be titrated (Gray, 1978). The official iodometric method Cd 8-53 (AOCS, 1990) for determination of PV is applicable to all normal fats and oils, but is highly

empirical and any variation in procedure may produce different results. The official method also fails to adequately measure low PV because of difficulties encountered in determination of the titration end point. Therefore, the official method has been modified by replacing the titration step with an electrochemical technique in which the liberated iodine is reduced at a platinum electrode maintained at a constant potential to increase the sensitivity. Peroxide values ranging from 0.06 to 20 meq/kg lipid have been determined with this method. During the analysis, it is essential to deaerate all solutions since the presence of oxygen may lead to further formation of peroxides. Although determination of peroxide value is a common, its usefulness is generally limited to the initial stages of lipid oxidation.

2.5.1.4 Measurement of conjugated dienes

Oxidation of PUFA is accompanied by an increase in the ultraviolet absorption of the product. Lipids containing methylene interrupted dienes or polyenes show a shift in their double bond position during oxidation due to isomerization and conjugation formation (Logani and Davies, 1980). The resulting conjugated dienes exhibit intense absorption at 234 nm. Farmer and Sutton (1946) indicated that the absorption increase is proportional to the uptake of oxygen and formation of peroxides in the early stages of oxidation. Wanasundara *et al.* (1995) reported that conjugated dienes and peroxide values during oxidation of canola and soybean oils were directly related. Determination of conjugated dienes is faster than PV determination, is much simpler, does not depend upon any chemical reaction or colour development, and requires a small sample size.

2.5.2 Secondary changes and their measurement

The hydroperoxides of lipids are transitionary intermediates which decompose into various secondary products. Measurement of secondary oxidation products as an index of lipid oxidation is more appropriate since such compounds are generally flavour-active, in contrast to primary oxidation products which are colourless and flavourless. Secondary oxidation products include carbonyls (ketones and aldehydes), hydrocarbons, alcohols and carbonylic acids, among others. There are a number of methods that are used to measure the formation of secondary products of lipid oxidation, is described in subsequent sections.

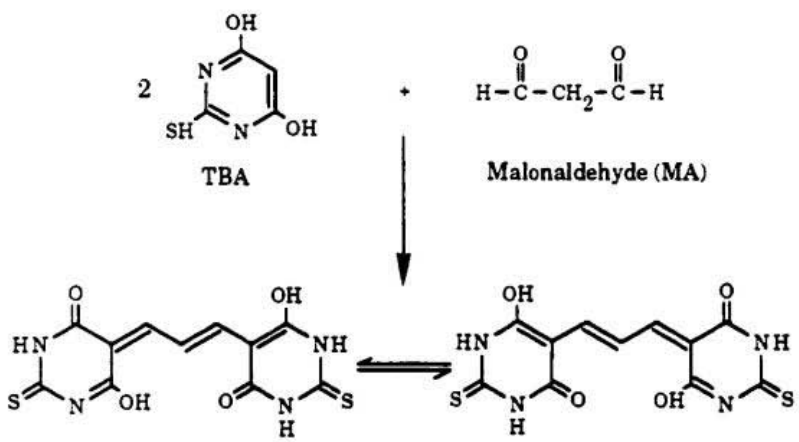
2.5.2.1 p-Anisidine value

p-Anisidine value (p-AnV) is used to measure secondary products of lipid oxidation. This method determines the amount of aldehydes (mainly 2-alkenals and 2,4-alkadienals) in animal fats and vegetable oils. Aldehydes in an oil and the p-anisidine reagent react under acidic conditions (IUPAC, 1987; method number 2.504). List *et al.* (1974) reported a highly significant correlation between p-AnV of salad oils processed from undamaged soybeans and their flavour acceptability scores.

2.5.2.2 The 2-thiobarbituric acid reactive substances (TBARS)

One of the oldest and the most frequently used test for assessing lipid oxidation in foods and other biological systems is the 2-thiobarbituric acid (TBA) test. The extent of lipid oxidation is reported as the TBA value and is expressed as milligrams of

malonaldehyde (MA) equivalents per kilogram of sample (results may also be expressed in μmoles MA equivalents/g sample). Malonaldehyde is a relatively minor product of oxidation PUFA which reacts with the TBA reagent to produce a pink-coloured complex with an absorption maximum at 530-532 nm (Tarladgis *et al.*, 1964). The adduct is formed by condensation of 2 moles of TBA with 1 mole of MA:



TBA-MA adduct

Dahle *et al.* (1962) postulated a mechanism for the formation of MA and indicated that only peroxides which possess unsaturation β or γ to the peroxide group are capable of undergoing cyclization with the ultimate formation of MA. Such peroxides may be produced from fatty acids containing three or more double bonds (Dahle *et al.*, 1962). Other products of lipid oxidation, such as aldehydes, alkenals and alkadienals (e.g. alka-2,4-dienals) may also react with the TBA reagent to form a pink-coloured complex with the same absorption maximum as the MA-TBA complex (Marcuse and Johansson, 1973). Therefore, the term "thiobarbituric acid-reactive substances" (TBARS) is now commonly used in place of TBA value (Ke *et al.*, 1984; Gray and Pearson, 1987).

There are certain limitations when using the TBA test for the evaluation of the oxidative state of foods and biological systems due to chemical complexity of these systems. Dugan (1955) has reported that sucrose and some compounds in woodsmoke react with the TBA reagent to give a red colour which interferes with the TBA test. Baumgartner *et al.* (1975) have found that a mixture of acetaldehyde and sucrose when subjected to the TBA test produced a 532 nm absorbing pigment identical to that produced by MA and TBA. Modifications of the original TBA test have been reported by Marcuse and Johansson (1973), Ke and Woyewoda (1979), Robbles-Martinez *et al.* (1982), Pokorny *et al.* (1985), Shahidi *et al.* (1987, 1991b), Thomas and Fumes (1987) and Schmedes and Hølmer (1989). However, it has been suggested TBARS provide an excellent means for evaluating relative oxidative status of a system as affected by storage or processing variables (Gray, 1978).

2.5.2.3 Measurement of carbonyls

An alternative approach for monitoring the extent of lipid oxidation in fats and oils is to measure the total or individual volatile carbonyl compounds formed from degradation of hydroperoxides. Hexanal, one of the major secondary products formed during the oxidation of linoleic acid in oils (Shahidi and Pegg, 1994; Frankel *et al.*, 1981), and other aldehydes have been used to follow lipid oxidation in meat products. Shahidi *et al.* (1987) reported a linear relationship between hexanal content, sensory scores and TBA numbers of cooked ground pork, while St. Angelo *et al.* (1987) established a similar correlation for cooked beef. These studies suggested that compounds

usually associated with lipid oxidation could be used as marker compounds to follow development of off-flavours in lipid-containing foods.

2.5.3 Recent developments for quantification of lipid oxidation

Lipid oxidation in foods and biological systems has conventionally been studied by monitoring either primary or secondary oxidation products. Over the last twenty years or so advances in pulse radiolysis (Simic, 1980) and electron spin resonance (ESR; Schaich and Borgi, 1980) techniques have facilitated the detection and study of short-lived free radical intermediates. ESR spectroscopy selectively detects species with an unpaired electron, such as free radicals (Brand-Williams *et al.*, 1995). During oxidation of food lipids, changes occur to the type of protons in an oxidizing molecule. These changes may be monitored by employing proton nuclear magnetic resonance (^{1}H NMR) spectroscopy as described by Saito and Udagawa (1992) and Wanasundara and Shahidi (1993). Infrared (IR) spectroscopy has also been used for the measurement of rancidity, and it is of particular value in the recognition of unusual functional groups and in the study of fatty acids with *trans* double bonds (Gray, 1978). Production of hydroperoxides during oxidation of lipids gives rise to a band at about 2.93 μm whereas the disappearance of a band at about 3.20 μm indicates the replacement of a hydrogen on a double bond, or polymerization (Van de Voort *et al.*, 1994).

2.6 Production of ω3 fatty acid concentrates

As an alternative to the substitution of generally used dietary lipids by rich sources of ω3 fatty acids, supplementation has been recommended. Fish oil supplements are currently available as non-prescription drugs in the United States (Herzberg, 1987) and European countries (Ratnayake, 1987), but not in Canada. For clinical applications, capsules containing fish oils, especially fish liver oil (cod), have been used. The dosage of cod liver oil required to achieve the desired biological effects carries the risk of vitamin A and D overdose and subsequent toxic effects as well as an increase in the intake of cholesterol and other saturated fatty acids (Davidson *et al.*, 1991). Therefore, concentrated forms of ω3 fatty acids from marine oils have been developed. Marine oils could be concentrated in the form of natural or modified TAG and as free fatty acids or as their simple alkyl esters. Most of the marine oil products sold over the counter today are in the natural TAG form, but more recently products in the free fatty acids or ethyl ester form have been sold. A number of companies in the world, especially in Japan, the United States and Europe have started producing marine oil ω3 concentrates, in the capsule or microcapsule forms (Table 2.4). Most of the marine oil capsules available in United States contain 18% EPA and 12% DHA; Promega is an exception and contains 28% EPA and 12% DHA.

Several investigations on the utility of marine oil capsules/concentrates in reducing serum VLDL and cholesterol levels have been carried out (Nestel *et al.*, 1984; Singer *et al.*, 1984; Phillipson *et al.*, 1985; Sullivan *et al.*, 1986). It has been concluded that ω3 concentrates devoid of saturated and monounsaturated fatty acids are much better than

Table 2.4 Marine oil concentrates available on the market as capsules and their contents of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (as weight %)

Product name	Manufacturer/Distributor	EPA	DHA
Res-q-1000	Res-q-International	18	12
Super EPA	Pharmacaps	18	12
Promega	Parke-Davis	28	12
Sonergx	Sonergx Nutrional Products	18	12
Your Life	P. Leiner Nutr. Products	18	12
EPA-PLUS	Superon Natural Vitamins	18	12
MaxEPA	Solgar Co., Inc.	18	12
SUPER MaxEPA	Twinlab	18	12
ProEPA	Parke-Davis	18	12

Source: Ratnayake (1987)

marine oils themselves since they keep the daily intake of total lipids as low as possible (Haagsma *et al.*, 1982). Therefore, concentrated forms of ω3 fatty acids are preferred items for pharmaceutical applications as well as enrichment of foods.

Marine oils are abundant in ω3 fatty acids and have traditionally been used as the raw material for preparation of ω3 fatty acid concentrates. Since marine oils are complex mixtures of fatty acids with varying chain lengths and degrees of unsaturation, separation of individual fatty acids is difficult for production of highly concentrated ω3 components. Therefore, commercial production of marine oil concentrates with high percentages of EPA and DHA is now a major challenge for food scientists and biotechnologists engaged in research in this area.

2.6.1 Methods of concentration of ω3 fatty acids

Methods for concentration of ω3 fatty acids include adsorption chromatography, fractional or molecular distillation, enzymatic splitting, low-temperature crystallization, supercritical fluid extraction and urea complexation. All these techniques have their own advantages and drawbacks. The following provides a background to each of these methods.

2.6.1.1 Chromatographic methods

It is possible to separate fatty acids according to their carbon number or degree of unsaturation using appropriate adsorbents (Brown and Kolb, 1955). High performance liquid chromatography (Beebe *et al.*, 1988) and silver resin chromatography (Adlof and

Emiken, 1985) have been used for preparation of ω3 concentrates. Teshima *et al.* (1978) have used a silver nitrate-impregnated silica gel column to separate EPA and DHA from squid-liver oil after forming methyl esters. These authors were able to isolate 85-96% EPA and 95-98% DHA with a yield of 39 and 48%, respectively. Recently, Hayashi and Kishimura (1993) have isolated 63-74% pure DHA from skipjack tuna eye orbital oil by stepwise elution with n-hexane, diethylether/n-hexane and diethylether on a silicic acid column. Adlof and Emiken (1985) were able to enrich the ω3 content of commercial ω3 fatty acid concentrates from 76.5 to 99.8% using isocratic elution from a silver resin column. However, use of organic solvents, loss of resolution of the column on repeated use and difficulties in scaling up the process for commercial production and possible presence of silver residues in the resultant concentrates pose problems (Mishra *et al.*, 1993).

2.6.1.2 Distillation method

Distillation has been used for the partial separation of mixtures of fatty acid esters. This method takes advantage of differences in the boiling point and molecular weight of fatty acids at reduced pressure (Brown and Kolb, 1955). This is a rather old technique and requires high temperatures of approximately 250°C (Berger and McPherson, 1979). Short-path distillation or molecular distillation uses lower temperatures and short heating intervals. However, fractionation of marine oil esters is difficult since separation of these components becomes less effective with increasing molecular weight (Weitkamp, 1955; Breivik, 1992).

Exposure of long-chain ω3 fatty acids to high temperatures during distillation may induce hydrolysis, thermal oxidation, polymerization and isomerisation. Possible degradation products of long-chain PUFA are cyclic fatty acids and high-molecular-weight polymers (Ackman, 1988; Wijesundara *et al.*, 1989). Privett *et al.* (1959) and Privett and Nickell (1963) found marked decomposition of arachidonic acid when it was distilled slowly in a spinning band column. Even when the catalytic effects of metal parts were eliminated to an all-glass apparatus, high temperature and exposure to oxygen caused major losses of ω3 fatty acids (Ackman *et al.*, 1973a). Therefore, design of a method for preparation of ω3 fatty acid concentrates which involves low process temperature and time to minimize thermal damage is desirable.

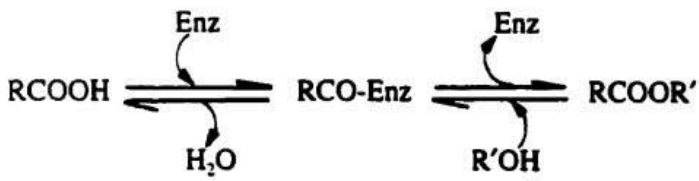
2.6.1.3 Enzymatic hydrolysis

Lipases 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 ester formation is shown in Figure 2.12. The reaction is reversible and under low water activity conditions the enzyme functions "in reverse", that is the synthesis of an ester bond rather than its hydrolysis (Miller *et al.*, 1988).

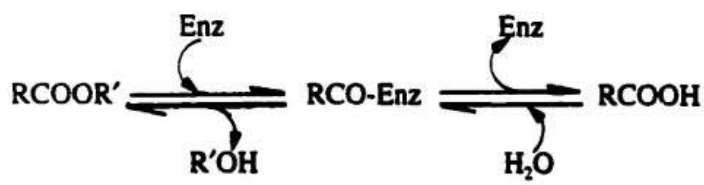
Bottino *et al.* (1967) have reported that PUFA-TAG of marine mammals were resistant to pancreatic lipolysis *in vitro*. This was suggested to be due to an inhibitory effect brought about by proximity of the double bond to the ester linkage or to a steric

Figure 2.12 Enzymatic ester synthesis and hydrolysis (adapted from Miller *et al.*, 1988)

Enzymatic ester synthesis
[R'OH] >> [H_2O]



Enzymatic ester hydrolysis
[H_2O] >> [R'OH]



hinderance resulting from proximity of the terminal methyl groups of some of these fatty acids to the esterified carboxylic groups. Lipases from microbial sources (e.g, *Mucor miehei, Candida cylindracea, Aspergillus niger*) were also found to discriminate against polyunsaturated fatty acids/acyl moieties in both esterification and hydrolysis reactions (Miller *et al.*, 1988; Hoshino *et al.*, 1990; Yadwad *et al.*, 1991). Therefore, it is possible to prepare PUFA-rich acylglycerols by selective hydrolysis of saturated or monounsaturated fatty acids in the TAG of marine oils. Hoshino *et al.* (1990) have reported that selective hydrolysis of cod liver oil and sardine oil using lipases from *Candida cylindracea* and *Aspergillus niger* gave acylglycerols with more than a two-fold increase in the content of their ω3 PUFA compared to that of the original oils. According to Yadwad *et al.* (1991) 1,3-specific lipase of *Rhizopus niveus* under suitable conditions was able to produce a monoacylglycerol concentrate containing 29.17% (w/w) DHA from a cod liver oil with an original content of 9.64% (w/w) DHA. Production of ω3-enriched acylglycerols using a similar approach may be of interest.

2.6.1.4 Low temperature crystallization

Low-temperature crystallization was originally developed to separate TAG, fatty acids, esters and other lipids which are highly soluble in organic solvents at temperatures above 0°C but which become sparingly soluble at temperatures down to -80°C (Brown and Kolb, 1955). The solubility of fats in organic solvents decreases with increasing mean molecular weight and increases with increasing unsaturation (Chawla and deMan, 1990). Singleton (1960) and Stout *et al.* (1990) have determined the solubility of

numerous fatty acids (Figure 2.13) and esters in a variety of solvents and revealed the following rules: when the acids are saturated, long-chains are less soluble than short chains; saturated are less soluble than monoenoic and dienoic acids of equal chain length; *trans* isomers are less soluble than *cis* isomers, and normal acids are less soluble than branched acids. The melting point of fatty acids changes considerably with the type and degree of unsaturation (Table 2.5) and thus separation of mixtures of saturated and unsaturated fatty acids may become possible (Haraldsson, 1984). At low temperatures, long chain saturated fatty acids which have higher melting points crystallize out and PUFA remain in the liquid form.

It has been reported that use of different organic solvents and temperatures affect the concentration of PUFA (Brown and Kolb, 1955; Yokochi *et al.*, 1990). Therefore, proper choice of solvent and temperature is necessary to achieve the optimum yield of the concentration of ω3 fatty acids. The fungal oil extracted from *Mortierella genus* was used for concentration of γ-linolenic acid (GLA) by low temperature crystallisation (Kreulen, 1976; Yokochi *et al.*, 1990). The concentration of GLA using different solvents was achieved 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). Solvent crystallization of fatty acids is an indispensable method for preparing pure fatty acids. This method requires the least amount of equipments and the simplest apparatus (Schlenk, 1961; Markely, 1964). Briefly, the process consists of cooling the oil or fatty acids in a solvent, holding for a specified period of time, and removing the crystallized fraction by filtration.

Figure 2.13 Solubilities of some fatty acids in acetone at different temperatures (adapted from Stout *et al.*, 1990)

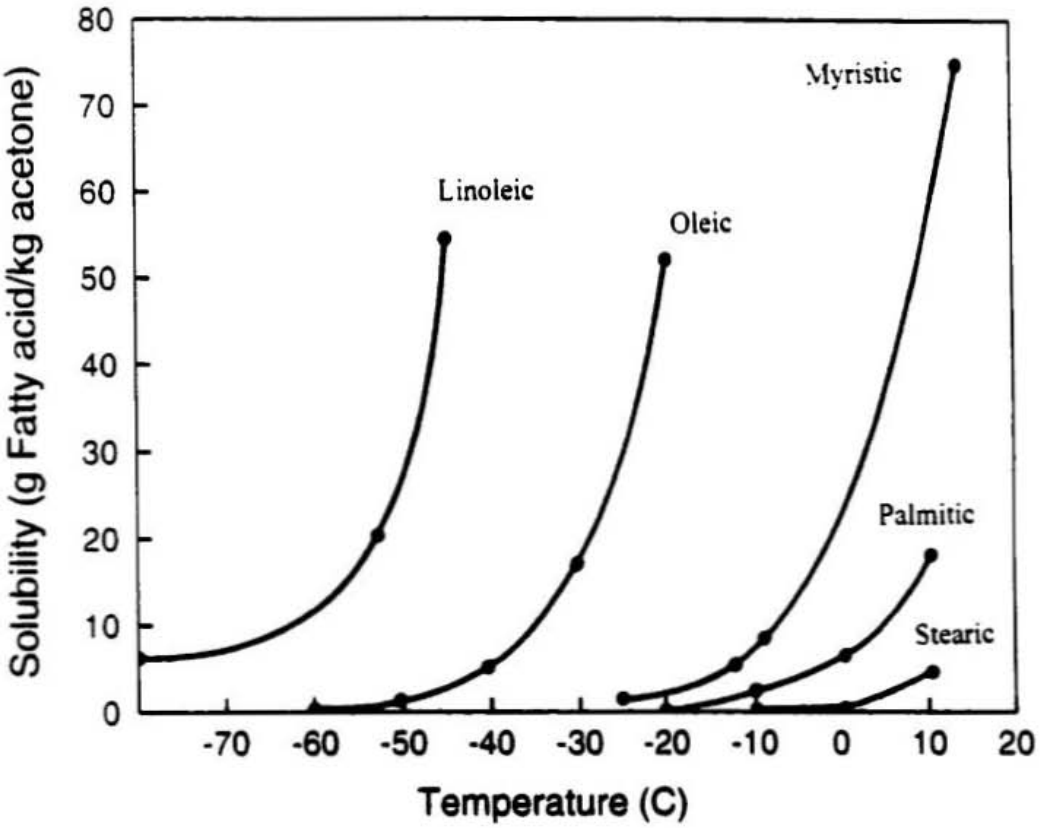

Solubility (g Fatty acid/kg acetone)
80
70
60
50
40
30
20
10
0
-70
-60
-50
-40
-30
-20
-10
0
10
20
Temperature (C)
Linoleic
Oleic
Myristic
Palmitic
Stearic

Table 2.5 Melting points of some fatty acids and cholesterol present in lipids[a]

Systematic name	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			
cis-9-Tetradecenoic acid	Myristoleic acid	14:1ω5	-4.5 to -4
cis-9-Hexadecenoic acid	Palmitoleic acid	16:1ω7	-0.5
cis-9-Octadecenoic acid	Oleic acid	18:1ω9	13.4
trans-9-Octadecenoic acid	Elaidic acid	18:1	43 to 45
cis-13-Docosenoic acid	Erucic acid	22:1ω9	33 to 35
Polyunsaturates			
cis-9,12-Octadecadienoic acid	Linoleic acid	18:2ω6	-6.5
cis-6,9,12-Octadecatrienoic acid	γ-Linolenic acid	18:3ω6	-11
cis-9,12,15-Octadecatrienoic acid	α-Linolenic acid	18:3ω3	-12.8
cis-5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4ω6	-49.5
cis-5,8,11,14,17-Eicosapentaenoic acid	-	20:5ω3	-54 to -53
cis-4,7,10,13,16,19-Docosahexaenoic acid	-	22:6ω3	-44.5 to -44.1
	Cholesterol		148.5

[a]Adapted from Merck Index (1983)

2.6.1.5 Supercritical fluid extraction

Supercritical fluid extraction (SFE) is a relatively new separation process that circumvents some of the problems associated with the use of conventional separation techniques. A number of gases are known to have good selective solvent properties when raised to pressures above their critical values. These pressures are very high, on the order of 1000-2000 psig. For food commodities, CO_2 is the solvent of choice because it has moderate critical temperature and pressure (31.1°C, 1070 psig) and is inert, inexpensive, non-flammable, environmentally acceptable, readily available and safe (Mishra *et al.*, 1993). The separation of PUFA is dependant on the molecular size rather than unsaturation; therefore, a prior concentration step is needed to achieve a higher concentration of PUFA in the final product (Mishra *et al*, 1993). Oils to be used for ω3 concentration by SFE require preparation steps of extraction, hydrolysis and esterification by conventional methods (Eisenbach, 1984; Nilsson *et al.*, 1989). The use of SFE for extraction of oil and concentration of ω3 fatty acids from fish oil and seaweed has been reported (Yamagouchi *et al.*, 1986; Choi *et al.*, 1987; Mishra *et al.*, 1993). SFE has been effectively used to refine fish oils and remove cholesterol, polychlorinated biphenyls (PCB), vitamin E and other components (Rizvi *et al.*, 1986). The disadvantages of this process are the use of extremely high pressures and the high capital cost.

2.6.1.6 Urea complexation

Urea alone crystallizes in a tightly packed tetragonal structure. However, in the presence of long straight-chain molecules it crystallizes in a hexagonal structure with

channels of 0.8-1.2 nm diameter within the hexagonal crystals (Smith, 1952). The formed channels are sufficiently large to accommodate aliphatic chains (Schlenk, 1954). While straight-chain saturated fatty acids with six carbons or more are readily adducted, the presence of double bonds in the carbon chain increases the bulk of the molecule and reduces the likehood of complexation. Monoenes are more readily complexed than dienes which, in turn, are more readily complexed than trienes. Therefore, the stability of fatty acid-urea adducts parallels the geometry of the molecules. Any deviation from a straight chain arrangement weakens the stability of the adduct. Therefore, formation of urea inclusion compounds depends on the degree of unsaturation of the fatty acids (Belitz and Grosch, 1987). During this process, the oil (aclyglycerol) is split into fatty acids using alcoholic KOH or NaOH and unsaponifiable compounds such as sterols, vitamins A and D and xenobiotics (e.g. PCB) and other undesirable components are removed from it. The free fatty acids mixed with an alcoholic solution of urea are then allowed to cool to a particular temperature depending on the degree of concentration desired. The saturated fatty acids, monoenes and, to a lesser extent, dienes are crystallised with urea and non-crystallized fatty acids in the solution can be separated by filtration. Alternatively, this procedure can be carried out using methyl or ethyl esters of fatty acids rather than free fatty acids. There are advantages to each of these options. Fatty acids are soluble to a higher degree in alcohol than their corresponding esters, therefore, the volume of alcohol required is much lower than when the ester form is used. If the ester form is chosen, the process step of re-esterification of the concentrates is eliminated.

Many publications have described the application of urea complexation as both an

analytical and a preparative tool 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 application of this technique to concentrate specific fatty acids: furanoid fatty acids (Gunstone *et al.*, 1978), isoprenoid acids (Ackman *et al.*, 1977), *cis-trans* isomers (Piconneaux *et al.*, 1985), an unusual fatty acid bearing a methyl branch and a double bond on the same carbon atom; i.e. 7-methyl-7-hexadecenoic acid (Ackman *et al.*, 1973b) and preparation of ω3 fatty acid concentrates from cod liver oil (Haagsma *et al.*, 1982).

Urea complexation has the advantage that complexed crystals are extremely stable, and filtration does not necessarily have to be carried out at the very low temperatures at which solvent crystallization of fatty acids would require (Anon, 1986). This method is also favoured by many researchers because complexation depends upon the configuration of the fatty acid moieties due to the presence of multiple double bonds, rather than pure physical properties such as melting point or solubility.

2.7 Process optimization in food product development

Experimental design is a systematic approach that enables several variables to be studied simultaneously, allowing the acquisition of a considerable amount of data from the minimum number of experiments and at the lowest cost. Experimental designs also allow 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 strategies can reduce time, cost, wastage and rework during production.

In the product development, process optimization is done in two stages. At the first stage, information about specific variables that affect the result is gathered in order to identify the variables. In the second stage, the variables are further tested to establish their optimum levels. Experimental designs are used in both stages: screening designs in the first stage and optimization in the second. The general approach to implement design experiments in either stage of product development can be summarised as follow. The first requirement is to define the purpose of study and to identify factors (independent variables) and responses (dependant variables). Then one must develop a model for each response to be evaluated. A model which could be a first, second or third order equation can predict response values for different factor levels after data have been collected and analyzed. For the optimization experiments, second order polynomial models are often used (see Section 3.9). Regression analysis or the Yates algorithm is used for data analysis to fit the model to data and this would enable one to obtain estimates for parameters (Dziezak, 1990).

Response surface methodology (RSM), first introduced by Box and Wilson (1951), is an effective statistical technique for investigation of complex processes and optimization. This method has been successfully adapted in optimization studies in food science (Hill and Hunter, 1966; Lee and Hoseney, 1982; Shieh *et al.*, 1995). The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. It is a faster and less expensive method for gathering research results than classical one-variable-at-a-time or full-factorial experimentation (Hill and Hunter, 1966; Lee and Hoseney, 1982).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Samples

Fresh, blubbers of harp seal (*Phoca groenlandica*; from 9 animals; 1993 hunting season) were obtained from local sources in Newfoundland. Cod (*Godus morhua*) livers were obtained from the experimental offshore cod farm of the Sea Forest Plantation (Bay Bulls, NF). Refined-bleached and deodorized (RBD) menhaden oil (MHO) and canola oil (CNO) devoid of any additives were obtained from Zapata Protein (USA) Inc. (Reedville, VA) and CanAmera (Saskatoon, SK), respectively. Green tea leaves were obtained from Anhui province of China. β-Cyclodextrin was procured from the American Maize-Products Company (Hammond, IN); corn-syrup solids (Maltrin M250) and maltodextrin (Maltrin M500) were acquired from the Grain Processing Corporation (Muscatine, IA).

3.1.2 Chemicals

Synthetic antioxidants namely tert-butylhydroquinone (TBHQ), butylated hydoxyanisole (BHA) and butylated hydoxytoluene (BHT) as well as α-tocopherol and flavonoids, namely apigenin, kaempferol, morin, myricetin, naringenin, naringin, quercetin, rutin and taxifolin were obtained from either Sigma (St. Louis, MO) or Aldrich Chemical Company (Milawaukee, WI).

Eight types of microbial lipases used were provided by different manufacturers as shown in Table 3.1. Fatty acid methyl esters were purchased from either Supelco

Table 3.1 Microbial lipases employed, their suppliers and characteristics

Origin	Manufacturer	Optimum temperature (°C)	Optimum pH	Positional specificity	Activity (U/g)[1]
Aspergillus niger (AN)	Amano Enzyme	30 - 40	5.0 - 7.0	1-, 3- >> 2-	15,500
Mucor miehei (MM)	Novo Nordisk	30 - 45	6.5 - 7.5	1-, 3- >>> 2-	10,000
Rhizopus oryzae (RO)	Amano Enzyme	30 - 45	5.0 - 8.0	1-, 3- >>> 2-	82,000
Rhizopus niveus (RN)	Amano Enzyme	30 - 45	5.0 - 8.0	1-, 3- >>> 2-	10,000
Candida cylindracea (CC)	Amano Enzyme	30 - 50	5.0 - 8.0	Random	22,500
Chromobacterium viscosum (CV)	Asahi Chemicals	-	-	Random	90,000
Geotrichum candidum (GC)	Amano Enzyme	30 - 45	6.0 - 8.0	Random	3000
Pseudomonas spp (PS)	Amano Enzyme	40 - 60	5.0 - 9.0	Random	35,000

[1]Enzyme activity was determined as given in Section 3.8.4.1

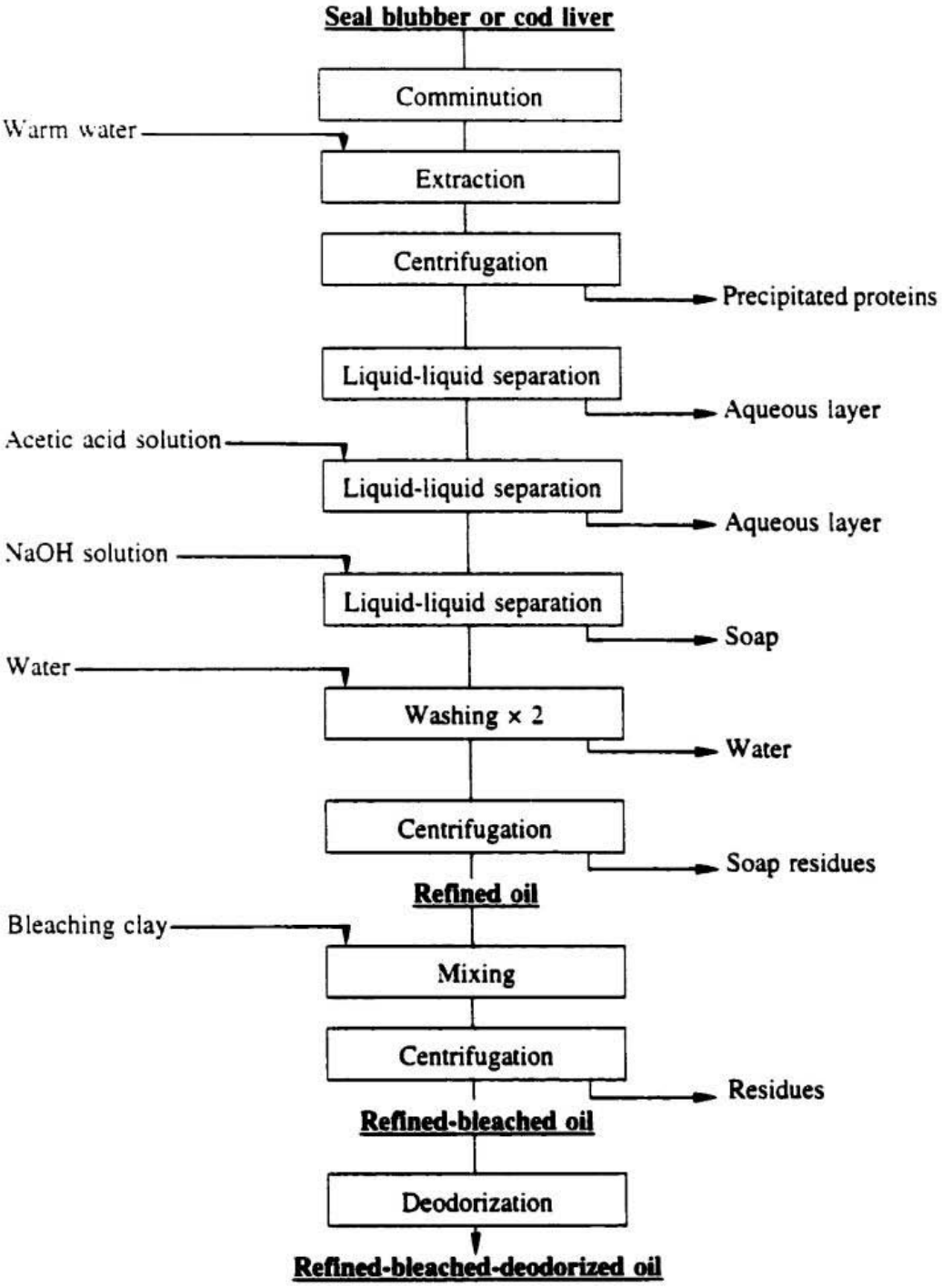
(Oakville, ON) or Nu-Check (Elysian, MN) companies. Standard tocopherols (α-, β-, γ- and δ) were purchased from Hoffman-La Roche Ltd. (Basel, Switzerland). Authentic aldehydes, namely propanal, butanal, pentanal, hexanal, heptanal, etc. were purchased from Sigma. All other chemicals used in this study were of American Chemical Society (ACS) grade or better.

High performance liquid chromatographic (HPLC)-grade chemicals were used for analysis and preparation of reagents as required. Glass-distilled water was used for preparation of reagents. Water was demineralized and its organic matter removed using Ultrapure Banstead Reverse Osmosis system (Banstead, Boston, MA) coupled with organic removal, demineralization and submicron filtration connected to a Nanopore II system for HPLC analyses.

3.2 Extraction and refining of seal blubber oil (SBO) and cod liver oil (CLO)

Seal blubber oil (SBO) and cod liver oil (CLO) were prepared from raw blubber of harp seal and fresh livers of farmed cod, respectively. The extraction and refining of oils were carried out according to the scheme given in Figure 3.1. Small chunks of seal blubber and cod liver were first comminuted using a Commercial Waring blendor (Waring Products, New Hartford, CT) and then heated in water (water/solids, 3:2, v/w) at 65°C for 15 min. Denaturated proteins were removed by centrifugation (at 3100 × g for 10 min) and crude oils were separated from the aqueous phase using a separatory funnel. A 5% (v/v) acetic acid solution was added to the oil (acid/oil, 3:2, v/v) to precipitate residual proteins. After 5 min, the aqueous layer containing residual proteins was

Figure 3.1 Flowsheet for preparation of refined-bleached and deodorized (RBD) seal blubber oil (SBO) and cod liver oil (CLO)


Seal blubber or cod liver
Comminution
Warm water
Extraction
Centrifugation
Precipitated proteins
Liquid-liquid separation
Aqueous layer
Acetic acid solution
Liquid-liquid separation
Aqueous layer
NaOH solution
Liquid-liquid separation
Soap
Water
Washing × 2
Water
Centrifugation
Soap residues
Refined oil
Bleaching clay
Mixing
Centrifugation
Residues
Refined-bleached oil
Deodorization
Refined-bleached-deodorized oil

removed. The oil was then treated with a 0.1 N NaOH (NaOH/oil, 3:2, v/v) solution at room temperature to saponify impurities. Saponified materials were washed two times with water (water/oil, 3:2, v/v) and residual soap was removed by centrifugation. The alkali-refined oil so obtained was then bleached with 4% (w/w) activated clay at 60°C with vigorous stirring for 8 min. The refined-bleached oil, obtained after centrifugation at 3100 × g for 10 min were deodorized using a laboratory scale vacuum steam distillation apparatus. The process parameters used for vacuum steam distillation were : oil, 2300 ± 10 g; temperature, 100 ± 5°C; pressure, 0.01-0.03 mm Hg; duration, 4 h and rate of steam generation, 45-50 g/h. Refined-bleached and deodorized (RBD) oil was then transferred to amber coloured glass bottles, flushed with nitrogen, capped tightly and stored at -60°C until use. Recoveries of RBD-SBO and RBD-CLO from their source materials were 78 and 63%, respectively.

3.3 Assessment of oxidative stability of oils by accelerated oxidation methods

Comparison of the oxidative stability of marine oils (SBO, CLO and MHO) as well as SBO and CLO, obtained at different stages of refining was 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 temperatures (Evans *et al.*, 1973).

The specification of the experiments carried out under Schaal oven test conditions were as follows. Each oil (20 mL) in triplicate, was placed in open glass containers (30 mm diameter and 60 mm height) and stored in a forced-air oven (Thelco, Model 2,

Precision Scientific Co, Chicago, IL) at 65°C. To estimate oxidative stability by chemical methods (peroxide value; Section 3.10.4 and 2-thiobarbituric acid reactive substances value; Section 3.10.6), samples were removed periodically at 0, 24, 48, 84 and 144 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 within 10 days).

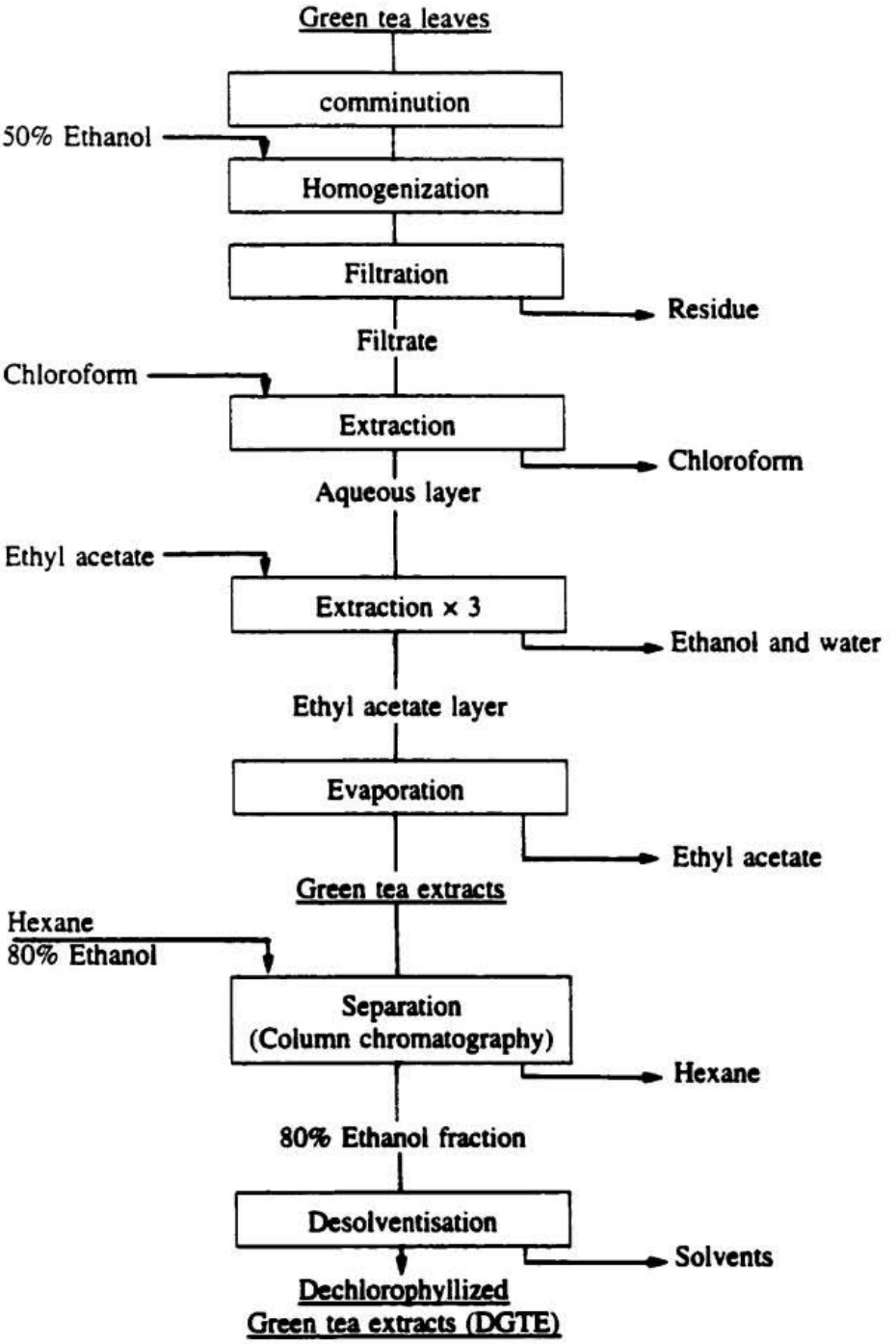
For the weight gain study, 2.0 g of each oil (in triplicate) was placed in a petri dish (60 mm diameter and 15 mm height), traces of water removed overnight in a vacuum oven (NAPCO Model 5831, Napco Scientific Co. Tualatin, OH) containing drierite (Hammond Drierite Co., Xenia, OH) at 35°C. Each sample was reweighed and stored in a forced-air oven at 65°C. The rate of oxidation in terms of weight increase was recorded at 6-h intervals over a 162-h period. The time required for a 0.5% weight increase for each oil was taken as an index of stability (Olcott and Einest, 1958).

3.4 Preparation of green tea extracts and catechins as natural antioxidants

3.4.1 Preparation of crude green tea extracts

The dried green tea leaves were obtained from Anhui Province of China and were then ground using a Moulinex coffee grinder. Preparation of green tea extracts was carried out as given in Figure 3.2. The ground tea leaves (10 g) were homogenized with 50% aqueous ethanol (100 mL) at room temperature using a Polytron homogenizer (PT-3000; Brinkmann, Rexdale, ON). The homogenate was then filtered through a Whatman #1 filter paper. The collected filtrate was mixed with an equal volume of chloroform and allowed to separate. The upper aqueous layer was extracted three times with an equal

Figure 3.2 Flowsheet for preparation of dechlorophyllized green tea extracts (DGTE)


Green tea leaves
comminution
50% Ethanol
Homogenization
Filtration
Residue
Filtrate
Chloroform
Extraction
Chloroform
Aqueous layer
Ethyl acetate
Extraction × 3
Ethanol and water
Ethyl acetate layer
Evaporation
Ethyl acetate
Green tea extracts
Hexane
80% Ethanol
Separation
(Column chromatography)
Hexane
80% Ethanol fraction
Desolventisation
Solvents
Dechlorophyllized
Green tea extracts (DGTE)

volume of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under vacuum at 40°C using a rotary evaporator (BÜCHI RE 111, Büchi Laboratoriums-Technik, Flawil, Switzerland).

3.4.2 Dechlorophyllization of green tea extracts

In order to remove chlorophylls in the crude tea extracts, a column (1.25 cm internal diameter and 20 cm height) packed with TOYO PEARL HW-40 (Bioseparation Specialists, Montgoneyvile, PA) was used. Crude extract (2 g) was dissolved in 5 mL of 80% (v/v) aqueous ethanol and subsequently introduced onto the column. The column was eluted with hexane until all the residual green colour had disappeared. The column was then washed with 80% (v/v) aqueous ethanol to obtained dechlorophyllized green tea extracts (DGTE). Ethanol was removed by evaporation under vacuum at 40°C using a rotary evaporator and the residual water was removed by lyophilization (Labconco 5 freeze dryer, Labconco Co. Kansas City, MO) at -60°C, under 0.01 torr pressure. The yield of DGTE was 15-20% from the original tea leaves. The extracts so obtained were transferred into air-tight glass vials and stored at -20°C until use.

3.4.3 Separation of individual tea catechins from dechlorophyllized green tea extracts (DGTE)

Separation of individual tea catechins from DGTE was carried out by employing both column and thin layer chromatographic procedures. The DGTE was separated into six fractions according to their content of total phenolics (Section 3.10.10) and UV

absorbance (Section 3.10.9) following Sephadex LH-20 column chromatography. The major fractions were further separated by thin layer chromatography (TLC) and semi-preparative high performance liquid chromatography (HPLC).

3.4.3.1 Sephadex LH-20 column chromatography

A glass column (1.5 cm internal diameter and 75 cm height) was packed with Sephadex LH-20 (particle size 25-100 μm, Pharmacia, Uppsala, Sweden). A 0.5 g sample of DGTE was dissolved in 5 mL of ethanol and subsequently introduced onto the column. The same solvent was used for elution and 8 mL fractions were collected using a fraction collector (LKB Bromma 2112 Radirac; Pharmacia).

The column fractions were pooled into six major fractions (I, II, III, IV, V and VI) according to their content of total phenolics (Section 3.10.10) and UV absorbance values (Section 3.10.9). These major fractions were used for TLC and HPLC separation of individual catechins.

3.4.3.2 Thin layer chromatography (TLC)

Different fractions (I to VI; Section 3.4.3.1) separated by Sephadex LH-20 chromatography were loaded onto TLC plates (Silica gel, 60 Å mean pore diameter, 2-25 μm mean particle size, 250 μm thickness, Sigma). Chromatograms were developed in a rectangular glass chamber (22 cm × 22 cm × 10 cm; Fisher Scientific Ltd. Toronto, ON) using chloroform/methanol/water (65:35:10, v/v/v, lower phase) as the mobile phase (Amorowicz *et al.*, 1992). After drying, separated catechin bands were viewed using a

spraying reagent prepared by dissolving 1 g of vanillin in 50 mL of absolute ethanol and 10 mL of concentrated HCl. Catechins are known to give a red colour with this reagent (Price *et al.*, 1978; Price and Spitzer, 1993).

3.4.3.3 High performance liquid chromatography (HPLC)

Fractions (III, IV, V and VI; Section 3.4.3.1) obtained from Sephadex LH-20 column were used for isolation of individual catechins, namely (-)epicatechin; EC, (-)epigallo catechin; EGC, (-)epicatechin gallate; ECG and (-)epigallocatechin gallate; EGCG, using semi-preparative HPLC. A Shimadzu (Kyoto, Japan) HPLC system consisting of two pumps (model LC-6A) with a mixing chamber, SPD-6AV UV-VIS spectrophotometric detector, a system controller (model SCL-6B) and a model CR-501 chromatopac data processor were used. A semi-preparative Hilbar pre-packed column RT (10 × 250 mm) with Lichrosorb RP-18 (7 µm; Merck, Darmstad, Germany) was used with mobile phase; water/acetonitrile/ methanol/acetic acid (79.5:18:2:0.5, v/v/v/v; Saijo, 1982). The flow rate was 3 mL/min and an injector volume of 500 mL was used for the analysis.

Purity of the isolated catechins was confirmed by comparing their retention times with those of pure standards of catechins (Amarowicz and Shahidi, 1995) using a HPLC equipped with an analytical column (CWSL 4.5 × 250 mm) packed with Spherisorb-ODS-2 (10 µm; Chromatography Sciences Company Inc. Montreal, PQ). Injection volume was 20 µl and a flow rate of 0.8 mL/min was used. For both semi-preparative and analytical HPLC, the UV detector was set at 280 nm.

3.5 Stabilization of seal blubber oil (SBO) and menhaden oil (MHO) with antioxidants

A number of antioxidants were used to stabilize the oils. These included dechlorophyllized green tea extracts (DGTE), individual tea catechins (EC, EGC, ECG, and EGCG), α-tocopherol and commercially-available flavonoids, namely apigenin, kaempferol, morin, myricetin, naringenin, naringin, quercetin, rutin and taxifolin, as well as BHA, BHT and TBHQ. Oils used were refined-bleached and deodorized. Antioxidants were used at different levels as shown in Table 3.2. Antioxidants were introduced into the oils by first dissolving them in a minimum amount of absolute ethanol, then adding to the oil and mixing thoroughly for 10 min using an ultrasonic water bath (ULTRASONIK-300, Fisher Scientific Ltd.). Control samples of oil were prepared to contain the same minimum amount of ethanol used to dissolve the antioxidants. Oxidative stability of treated oils was monitored under accelerated Schaal-oven test conditions as described in Section 3.3. Peroxide value (Section 3.10.4) and 2-thiobarbituric acid reactive substances value (Section 3.10.6) were used as chemical indices to determine stability of antioxidant-treated oils.

3.6 Stabilization of seal blubber oil (SBO) by microencapsulation

Microencapsulation of refined-bleached and deodorized (RBD) seal blubber oil (SBO) was employed as a means of extending its oxidative stability. Encapsulating materials used were β-cyclodextrin, corn-syrup solids and maltodextrin. These were dissolved in distilled water (30%, w/w) at 45-50°C in a water bath until clear solutions

Table 3.2 Types and levels of antioxidants used in refined-bleached and deodorized seal blubber oil (SBO) and menhaden oil (MHO)

Antioxidants	Level	
Dechlorophyllized green tea extracts (DGTE)	100 ppm	(DGTE-100 ppm)
	200 ppm	(DGTE-200 ppm)
	500 ppm	(DGTE-500 ppm)
	1000 ppm	(DGTE-1000 ppm)
(-)Epicatechin (EC)	200 ppm	
(-)Epigallocatechin (EGC)	200 ppm	
(-)Epicatechin gallate (ECG)	200 ppm	
(-)Epigallocatechin gallate (EGCG)	200 ppm	
Apigenin	200 ppm	
Kaempferol	200 ppm	
Morin	200 ppm	
Myricetin	200 ppm	
Naringenin	200 ppm	
Naringin	200 ppm	
Quercetin	200 ppm	
Rutin	200 ppm	
Taxifolin	200 ppm	
Butylated hydroxyanisole (BHA)	200 ppm	
Butylated hydroxytoluene (BHT)	200 ppm	
tert-Butyl hydroquinone (TBHQ)	200 ppm	
α-Tocopherol	500 ppm	

were obtained. For β-cyclodextrin, 2 to 3 drops of a 50% (w/v) NaOH were added to the mixture in order to attain complete solubility. RBD-SBO was emulsified in an ice bath with solutions of encapsulating materials (oil/encapsulating material, 3:17, w/w) using a Polytron homogenizer (10 min at 10,000 rpm; PT-3000, Brinkmann). Tween-80 (9% w/v of the oil phase) was added as an emulsifier during homogenization. Prepared emulsions were then spray dried using a mini spray-dryer (BÜCHI 190, Büchi Laboratoriums-Technik) under nitrogen as the spray flow-through gas to minimize contact with oxygen. The inlet and outlet temperatures were 145 and 80°C, respectively.

Unencapsulated or encapsulated SBO samples (10 g) were placed in amber glass bottles (40 mm diameter and 60 mm height) separately and stored under nitrogen at room temperature. To determine oxidative stability of unencapsulated and encapsulated oils, samples were removed periodically (separate sample containers for each day) for performing chemical analyses.

3.6.1 Extraction of oil from encapsulated matrices and determination of their oxidative stability

To extract oil from microcapsules, encapsulated products (10 g) were dissolved in 0.88% (w/v) KCl solution (50 mL) to dissolve the wall materials, and then mixed with a mixture of chloroform (100 mL), methanol (50 mL) and a few crystals of BHT. This sample was homogenized using a Polytron homogenizer for 2.5 min at 12,000 rpm, then transferred to a separatory funnel, the chloroform layer was separated and the solvent was removed at 40°C using a rotary evaporator. Oxidative stability of the resulting oil was

evaluated by monitoring changes in its fatty acid composition (Section 3.10.12), peroxide value (Section 3.10.4) and 2-thiobarbituric acid reactive substances value (Section 3.10.6).

3.6.2 Scanning electron microscopy of encapsulated samples

For scanning electron microscopy (SEM), wall materials, as such or with oil (encapsulated oil), were attached to the aluminium SEM holders with double sided adhesive tape. The samples were then sputter coated with gold under vacuum and observed using a Hitachi S-570 scanning electron microscope operated at 20 kV. The photographs were taken using a Polaroid type 665 positive/negative film.

3.7 Stereospecific analysis of seal blubber oil (SBO) and menhaden oil (MHO)

3.7.1 Removal of constituents other than triacylglycerol in seal blubber oil (SBO) and menhaden oil (MHO)

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

3.7.2 Grignard reaction on purified seal blubber oil (SBO) and menhaden oil (MHO)

Grignard reaction was performed on purified SBO and MHO (Section 3.7.1) according to the method described by Brockerhoff *et al.* (1963) and Brockerhoff (1971) with some modifications. The purified oil (1 g) was dissolved in anhydrous diethyl ether (50 mL) and mixed with methyl magnesium bromide (3.5 mL, 3.0 M CH_3MgBr, Sigma). The Grignard reaction was allowed to proceed with continuous stirring until a clear solution was obtained. To stop the reaction, glacial acetic acid (1.0 mL) was slowly added to the mixture followed by 10% (w/v) boric acid solution (10 mL; to minimize acyl migration). Stirring of the reaction mixture was continued for another 2 to 3 min. The whole mixture was then transferred to a separatory funnel and allowed to separate into two layers. The top ether layer was removed and the lower aqueous layer was washed twice with diethyl ether. The combine ether layers were washed successively with 10 mL of water, 10 mL of 2% (w/v) aqueous sodium bicarbonate and 10 mL of water and then dried over anhydrous sodium sulphate.

3.7.3 Separation of individual lipids after Grignard reaction

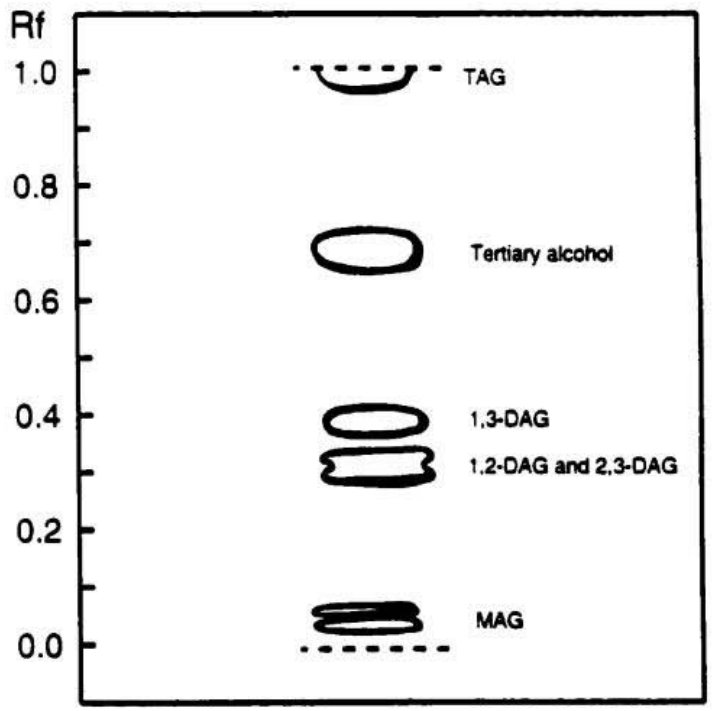
Products of the Grignard reaction from Section 3.7.2 were dissolved in a minimum amount of chloroform and applied to several TLC plates (20 x 20 cm; Silica gel, 60 Å mean pore diameter, 2-25 µm mean particle size, 500 µm thickness, with dichlorofluorescein, Sigma) impregnated with 5% (w/v) boric acid. The plates were developed in two different solvent systems of diethyl ether/petroleum ether (boiling point

30-60°C) (8:92, v/v) and diethyl ether/petroleum ether (40:60, v/v), respectively. After drying, the bands were located by viewing under short (254 nm) and long (356 nm) UV lights (Spectraline, Model ENF-240C, Spectronics Co. Westbury, NY). From the separated bands of triacylglycerol (TAG; R_f = 1.0), tertiary alcohol (R_f = 0.7), 1,2- (1,2-DAG) and 2,3-diaclylglycerols (2,3-DAG; R_f = 0.3), 1,3-diacylglycerol (1,3-DAG; R_f = 0.4) and monoacylglycerol (MAG; R_f = 0.03) (Figure 3.3), 1,2- and 2,3-diacylglycerol bands were scraped and then extracted with diethyl ether. The ether layer was evaporated under nitrogen to obtained 1,2- and 2,3-diacylglycerols. After removing a small sample for fatty acid analysis, the diacylglycerol fractions were used to prepare synthetic phospholipids.

3.7.4 Preparation of synthetic phospholipids from diacylglycerol fraction

The 1,2- and 2,3-diacylglycerols, obtained as described in Section 3.7.3, were dissolved in 1.0 mL of diethyl ether and mixed with 2.5 mL pyridine/diethyl ether/phenyl dichlorophosphate (1:1:0.5, v/v/v). The reaction mixture was then allowed to stand at room temperature for 1 h, after which 5 mL of pyridine, 3.0 mL of diethyl ether and few drops of water were added while cooling in an ice bath. The content of the flask was subsequently mixed with 86 mL of methanol/water/chloroform/ triethylamine (30:25:30:1, v/v/v/v). After standing, the lower chloroform layer containing synthetic phospholipids (1,2-diacyl-3-phosphatide and 2,3-diacyl-1-phophatide) was separated, and the solvent removed at 40°C using a rotary evaporator. The recovered synthetic phospholipids were used for stereospecific hydrolysis by the phospholipase A_2 enzyme.

Figure 3.3 TLC chromatogram of Grignard deacylation products

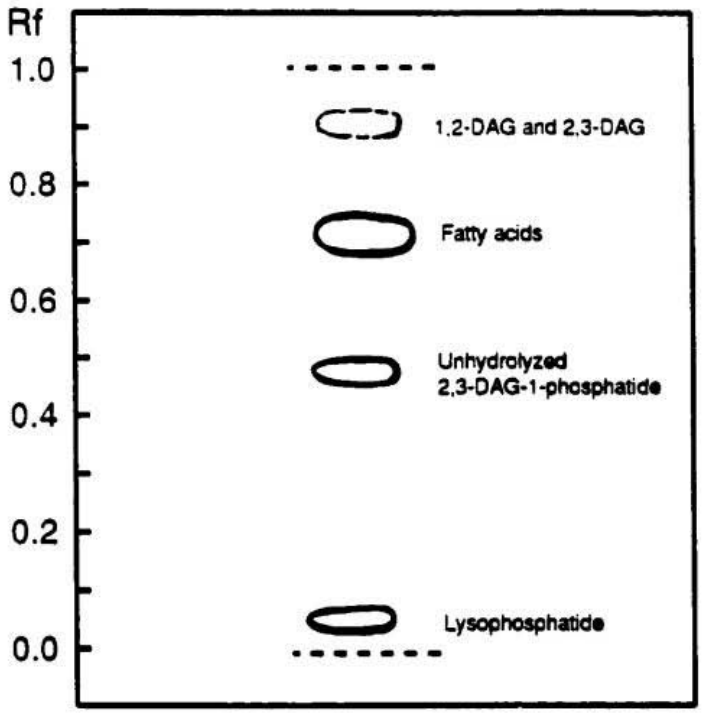

Rf
1.0
0.8
0.6
0.4
0.2
0.0
TAG
Tertiary alcohol
1,3-DAG
1,2-DAG and 2,3-DAG
MAG

3.7.5 Stereospecific hydrolysis of synthetic phospholipids by phospholipase A_2

The synthetic phospholipids (1,2-diacyl-3-phosphatide and 2,3-diacyl-1-phophatide) obtained in Section 3.7.4 were dissolved in 3.0 mL of diethyl ether and transferred to a solution containing 15 mL of 0.1 M triethylammonium bicarbonate (pH 7.5), 100 µl of 0.1 M calcium chloride and 2.0 mg of phospholipase A_2 (EC. 3.1.1.4; Sigma) obtained from snake venom (*Crotalus adamantus*). The mixture was then shaken gently overnight in a Gyrotory water bath shaker (Model G76, New Brunswick Scientific Co. Inc., New Brunswick, NJ) and water in the mixture was evaporated at 40°C using a rotary evaporator. In order to prevent foaming during evaporation, 15 mL of isobutanol was added to the mixture.

The hydrolyzed products were dissolved in 1.0 mL of chloroform/methanol (1:1, v/v) containing one drop of glacial acetic acid. The dissolved hydrolytic products were applied to TLC plates (20 × 20 cm; Silica gel, 60 Å mean pore diameter, 2-25 µm mean particle size, 500 µm thickness, with dichlorofluorescein, Sigma) impregnated with 5% (w/v) boric acid. The plates were developed in diethyl ether/petroleum ether (40:60, v/v), dried in a fume hood and then kept over concentrated aqueous ammonia for 10 min and redeveloped in aqueous ammonia/methanol/diethyl ether (2:15:83, v/v/v). After drying, the bands were located by viewing under short (254 nm) and long (356 nm) UV light (Spectraline, Model ENF-240C, Spectronics Co.). The separated bands; free fatty acids (hydrolysed from *sn*-2 position of 1,2-diacyl-3-phosphatide; $R_f = 0.71$), unhydrolyzed 2,3-diacyl-1-phosphatide ($R_f = 0.49$), lysophosphatide ($R_f = 0.05$) and traces of 1,2- and 2,3-diacylglycerols ($R_f = 0.91$) (Figure 3.4) were scraped out and extracted into chloroform/

Figure 3.4 TLC chromatogram of products of phospholipase A_2 hydrolysis


Rf
1.0
0.8
0.6
0.4
0.2
0.0
1,2-DAG and 2,3-DAG
Fatty acids
Unhydrolyzed
2,3-DAG-1-phosphatide
Lysophosphatide

methanol (1:1, v/v). After removing a small sample for fatty acid analysis, the unhydrolyzed 2,3-diacyl-1-phosphatide fraction was hydrolysed by porcine pancreatic lipase using the procedure described in Section 3.7.6 to obtain 2-monoacyl-1-phosphatide and free fatty acids (hydrolysed from *sn*-3 position). All separated lipid fractions were analyzed for their fatty acid composition by employing the gas chromatographic procedure described in Section 3.10.12.

3.7.6 Hydrolysis of seal blubber oil (SBO) and menhaden oil (MHO) by pancreatic lipase

Hydrolysis of purified oils as well as separated 2,3-diacyl-1-phosphatide by pancreatic lipase was carried out according to the method described by Christie (1982). Tris-hydrochloric buffer (5 mL; 1.0 M, pH 8.0), 0.5 mL of calcium chloride (2.2%, w/v) and 1.25 mL of sodium taurocholate (0.05%, w/v) were added to 25 mg of oil in a glass test tube. The whole mixture was allowed to equilibrate at 40°C in a water bath for 1.0 min and subsequently 5.0 mg of porcine pancreatic lipase (EC. 3:1:1:3; Sigma) were added to it. The mixture was then placed in a Gyrotory water bath shaker (Model G76, New Brunswick Scientific Co. Inc.) at 200 rpm under nitrogen for 8 to 10 min at 40°C. Ethanol (5 mL) was added to stop the enzymatic hydrolysis followed by addition of 5.0 mL of 6.0 M 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 TLC plates (20 × 20 cm; Silica gel, 60 Å mean

pore diameter, 2-25 μm mean particle size, 500 μm thickness, with dichlorofluorescein, Sigma) impregnated with 5% (w/v) boric acid. The plates were developed 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) UV lights (Spectraline, Model ENF-240C, Spectronics Co.). The bands were scraped off and their lipids were 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 3.10.12.

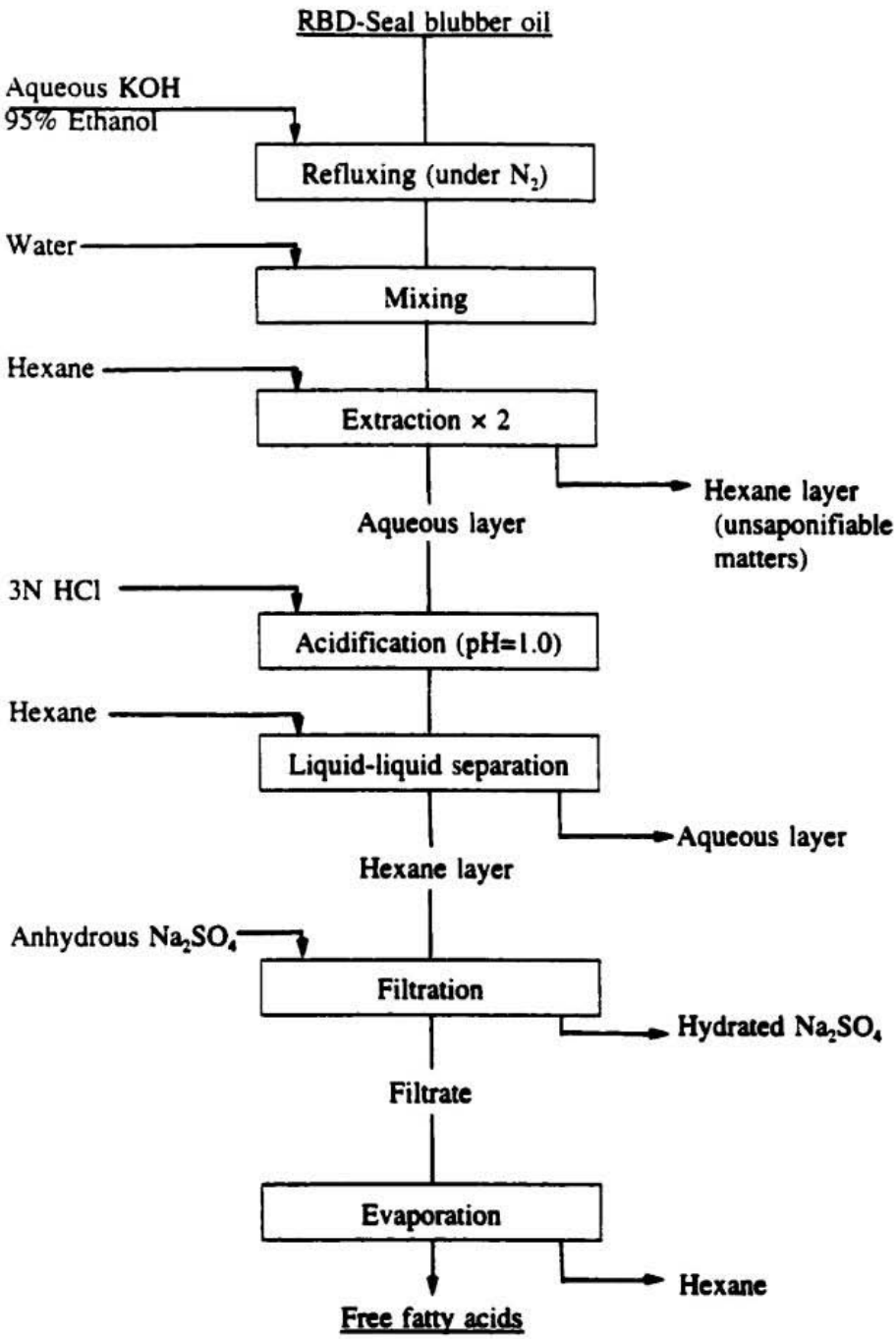
3.8 Preparation of ω3 fatty acid concentrates from seal blubber oil (SBO) and menhaden oil (MHO)

Omega-3 fatty acid concentrates were prepared from SBO and MHO by employing physical, chemical and biotechnological methods. Low temperature crystallization (physical), urea complexation (chemical) and enzymatic hydrolysis (biotechnological) were studied for their efficiency in concentrating total ω3, EPA and DHA.

3.8.1 Preparation of free fatty acids from seal blubber oil (SBO)

Preparation of free fatty acids from RBD-SBO was carried out according to the scheme given in Figure 3.5. Seal blubber oil (25 g, treated with 200 ppm BHT) was saponified by refluxing for 1 h at the boiling temperature of the mixture (62±2 °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 was extracted into hexane (2 × 100 mL) and discarded.

Figure 3.5 Flowsheet for preparation of free fatty acids from refined-bleached and deodorized (RBD) seal blubber oil (SBO)


RBD-Seal blubber oil
Aqueous KOH
95% Ethanol
Refluxing (under N_2)
Water
Mixing
Hexane
Extraction × 2
Hexane layer
(unsaponifiable
matters)
Aqueous layer
3N HCl
Acidification (pH=1.0)
Hexane
Liquid-liquid separation
Aqueous layer
Hexane layer
Anhydrous Na_2SO_4
Filtration
Hydrated Na_2SO_4
Filtrate
Evaporation
Hexane
Free fatty acids

The aqueous layer containing saponifiable matter was acidified (pH = 1.0) with 3 N HCl. The mixture was transferred to a separatory funnel and the liberated fatty acids were extracted into 50 mL of hexane. The hexane layer containing free fatty acids was then dried over anhydrous sodium sulphate and the solvent removed at 40°C to recover free fatty acids which were then stored at -60°C until use.

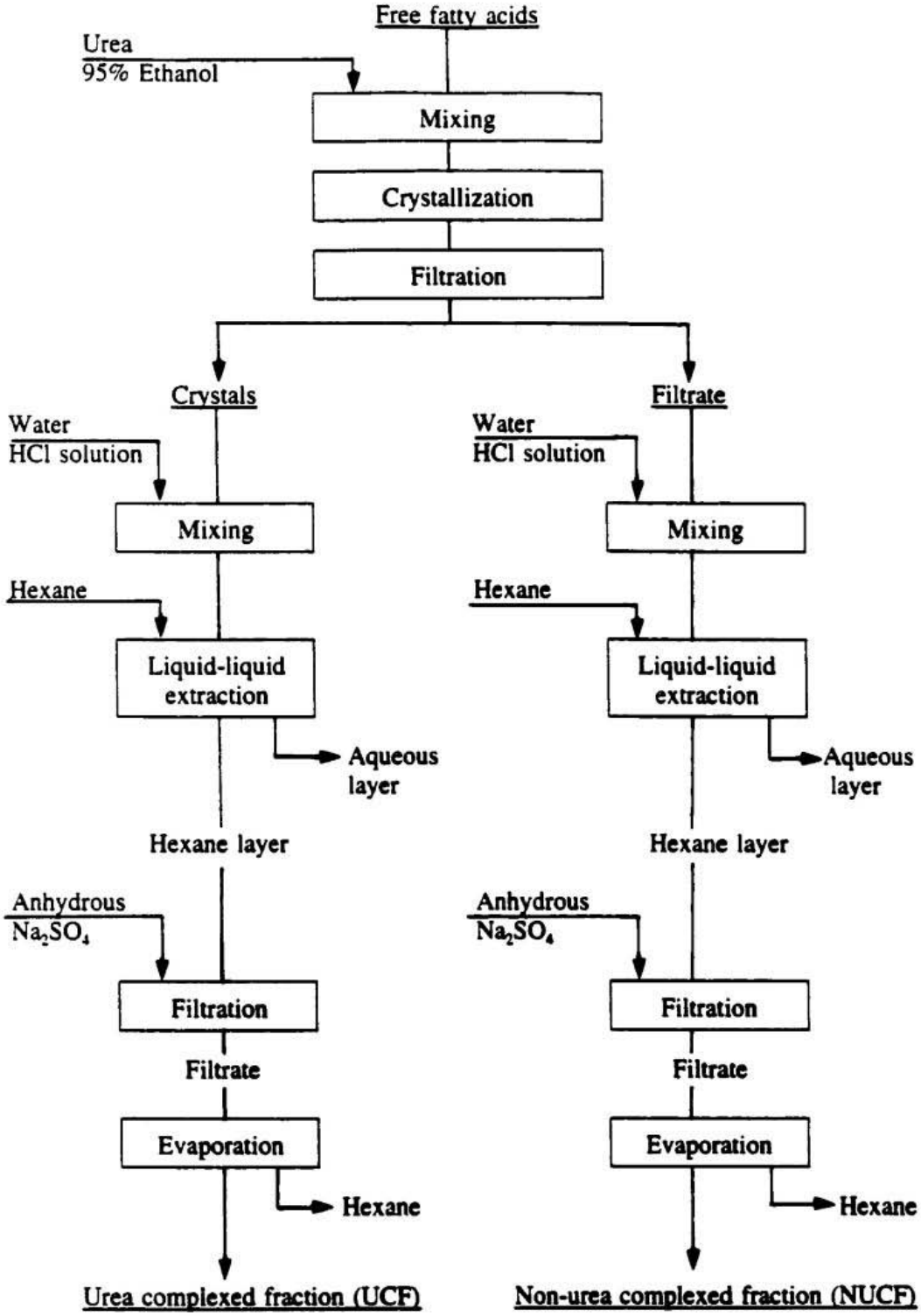
3.8.2 Preparation of ω3 fatty acid concentrates from seal blubber oil (SBO) by low temperature crystallization

The separation of ω3 fatty acids from SBO and its free fatty acid mixture prepared in Section 3.8.1 was carried out by solvent crystallization at freezing and sub-freezing temperatures. Free fatty acids (10 g) were mixed with 40 mL of acetone (melting point -94°C), hexane (melting point -95°C) or isopropanol (melting point -88.5°C) in glass containers (30 mm diameter and 60 mm height). These mixtures were stored at different temperatures (0, -10, -20, -40, -60 and -70°C) for 24-h to induce crystal formation. The crystals formed during low temperature storage were separated from the liquid (non-crystallized fraction) by filtration on a Buchner funnel lined with a thin layer of glass wool. Filtration was carried out at the temperature of crystallization. The non-crystallized fraction containing long-chain polyunsaturated fatty acids was recovered after evaporation of the solvent at 40°C using a rotary evaporator and its fatty acid composition was determined by gas chromatographic means (Section 3.10.12). The percentage recovery of non-crystallized fraction was calculated on a percent weight basis.

3.8.3 Preparation of ω3 fatty acid concentrates from seal blubber oil (SBO) by urea complexation

The separation of ω3 fatty acids from the hydrolyzed fatty acid mixture (Section 3.8.1) of SBO was carried out by urea-fatty acid adduct formation according to the scheme given in Figure 3.6. Free fatty acids (10 g) were mixed with urea (20%, w/v) in 95% aqueous ethanol and heated at 60°C with stirring until the whole mixture turned into a clear homogenous solution. The ratio of urea-to-fatty acids was changed by using different amounts of urea. Initially, the urea-fatty acid adduct was allowed to crystallize at room temperature but colder temperatures (-24, -18, -9, 0 and +6 °C) were maintained later for different periods for further crystallization. The crystals formed (urea-fatty acid adducts were also refers to as the urea complexing fraction; UCF) were separated from the liquid (non-urea complexing fraction, NUCF) by filtration under suction on a Buchner funnel lined with a thin layer of glass wool. The NUCF (filtrate) was diluted with an equal volume of water and acidified to pH 4-5 with 6 N HCl; an equal volume of hexane was subsequently added and the mixture was stirred thoroughly for 1 h, then transferred to a separatory funnel. The hexane layer containing 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 sodium sulphate and the solvent was then removed at 40°C using a rotary evaporator. Fatty acids from UCF were recovered after addition of water/6 N HCl and hexane in a similar manner (Figure 3.6). The two fractions (NUCF and UCF) were weighed separately and percentage recovery of each was calculated. The fatty acid composition of the two fractions was

Figure 3.6 Flowsheet for preparation of ω3 fatty acid concentrates by urea complexation


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

determined using a gas chromatographic procedure (Section 3.10.12). Details of the experimental design to determine optimum conditions (urea-to-fatty acid ratio, crystallization temperature and crystallization time) for concentration of ω3 fatty acid by urea complaxation are described in Section 3.9.

3.8.4 Preparation of ω3 fatty acid concentrates from seal blubber oil (SBO) and menhaden oil (MHO) by enzymatic hydrolysis

3.8.4.1 Determination of enzyme activity of microbial lipases

Lipolytic activity of microbial lipases used was determined using a pH-Stat titration procedure according to the Novo Nordisk's analytical method (AF 95) with some modifications.

Unit definition: One unit of lipolytic activity is defined as the amount of enzyme which liberates 1 μmol of titratable butyric acid from tributyrin per min under the following standard conditions.

Standard conditions:

Temperature	35±1°C
pH	6.0 or 7.0
Emulsifier	gum arabic
Substrate	tributyrin

3.8.4.1.1 Preparation of the emulsifying reagent for enzyme activity determination

Sodium chloride (17.9 g), potassium dihydrogen phosphate (0.41 g), demineralized water (400 mL) and glycerol (540 mL) were mixed vigorously in a glass beaker while

adding gum arabic (6 g), stirring was continued until complete dissolution of gum arabic was achieved. The mixture was then transferred to a 100 mL volumetric flask and the volume made up to the mark with demineralized water.

3.8.4.1.2 Preparation of substrate-emulsion for enzyme activity determination

Tributyrin (15 mL; Sigma), the emulsifying reagent (50 mL) prepared in Section 3.8.4.1.1 and demineralized water (235 mL) were mixed in a 500 mL glass beaker. The mixture was then homogenized using a Polytron (1 min, 12,000 rpm; PT-3000; Brinkmann) to prepare the substrate-emulsion. The substrate-emulsion was freshly prepared every day.

3.8.4.1.3 Preparation of the lipase solutions for enzyme activity determination

The enzymes were dissolved in a 0.1 M potassium phosphate buffer (pH=7.0) to obtain a concentration of approximately 0.5-3.5 U/mL. In the preparation of the enzyme solution of *Aspergillus niger*, a pH of 6.0 and a 0.1 M potassium phosphate buffer were employed.

3.8.4.1.4 The pH-Stat method

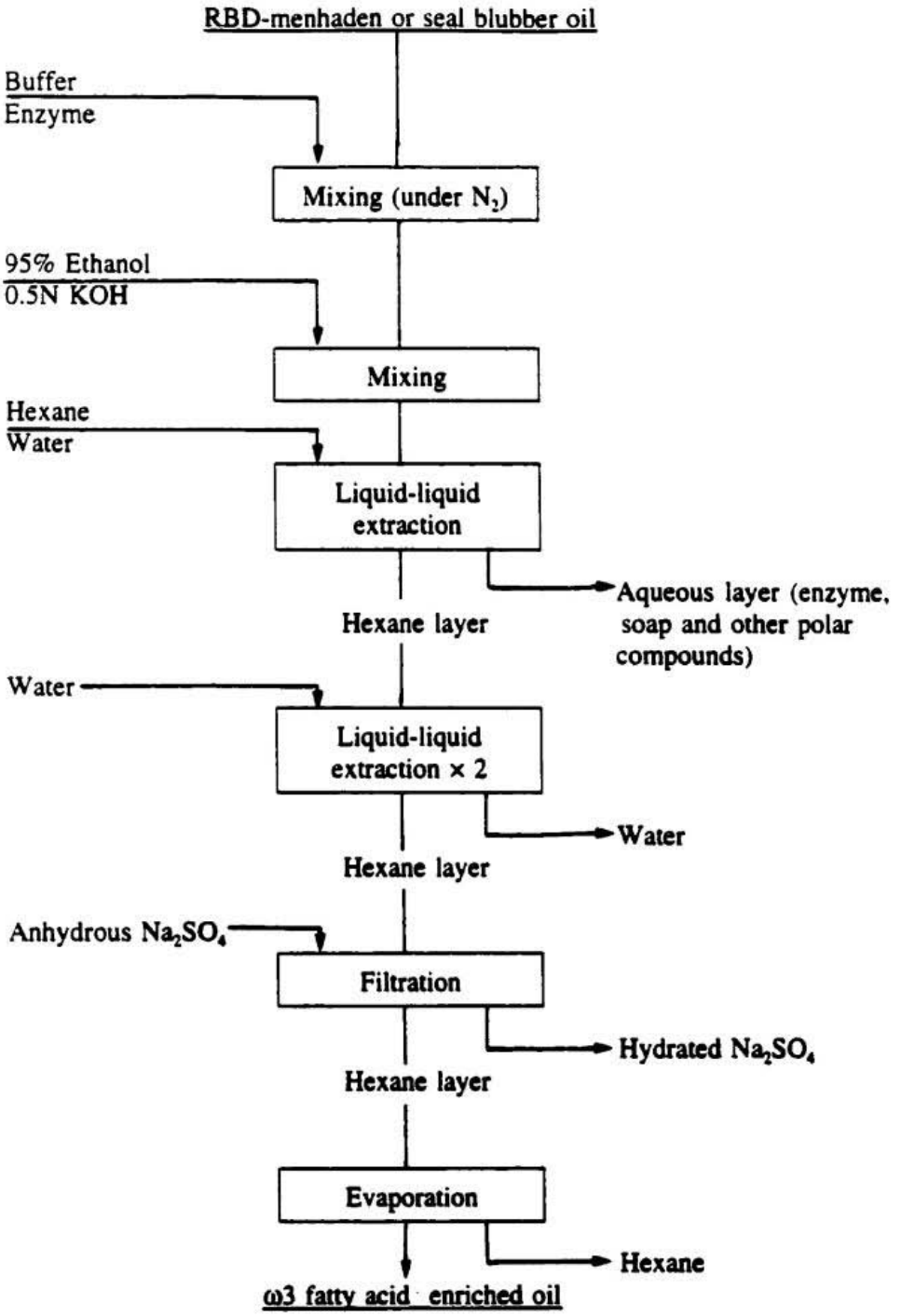
The amount of base needed to maintain a pH of 6.0-7.0 in the emulsion during the lipase-assisted hydrolysis of tributyrin was measured by the pH-stat method. Substrate-emulsion (20 mL, Section 3.8.4.1.2) was transferred into the reaction vessel (glass beaker; 4 cm diameter and 6.5 cm height). The substrate-emulsion was pre-heated for 3-4 min

to 35°C in a water bath. The enzyme solution (1 mL) prepared in Section 3.8.4.1.3 was then added to the substrate-emulsion and the reaction vessel was placed in the titration set-up (Metrohm Auto-Titrator, Model SM Titrino 702, Metrohm Ltd, Herisau, Switzerland). The pH of the mixture was adjusted to 6.8-6.9 with 0.05 N sodium hydroxide in the autoburette and the pH-Stat titration for all enzymes was carried out while stirring the mixture at 200 rpm (magnetic bar size 25 mm long and 7.9 mm diameter) at 35°C. The titration was stopped after 3 min of constant (linear) rate of alkali addition. The chart recorder speed was 1 cm/min and recorder amplitude was 1 cm/50µl. Lipolytic activity was calculated as described in the Appendix (Figure A.1).

3.8.4.2 Hydrolysis of seal blubber oil (SBO) and menhaden oil (MHO) by microbial lipases

Hydrolysis of SBO and MHO (oils were stabilized with 200 ppm of BHT) by microbial lipases and separation of the ω3-enriched fraction was carried out according to the scheme given in Figure 3.7. Oil (4 g) and 6.0 mL of 0.1 M phosphate buffer (pH 6.0 or 7.0) containing 800 units (200 U/g oil) of lipase were placed in a glass container (4 cm diameter and 7 cm height). The container was flushed with nitrogen and sealed with a rubber cap and parafilm. Containers were then placed in a Gyrotory water bath shaker at 35±1°C operating at 200 rpm. Hydrolysed samples were removed periodically (separate sample container for each time) to determine the percentage of hydrolysis. Lipolytic activity was quenched by introducing 2 mL of methanol to the mixture.

Figure 3.7 Flowsheet for preparation of ω3 fatty acid concentrates by enzymatic hydrolysis


RBD-menhaden or seal blubber oil
Buffer
Enzyme
Mixing (under N_2)
95% Ethanol
0.5N KOH
Mixing
Hexane
Water
Liquid-liquid extraction
Aqueous layer (enzyme, soap and other polar compounds)
Hexane layer
Water
Liquid-liquid extraction × 2
Water
Hexane layer
Anhydrous Na_2SO_4
Filtration
Hydrated Na_2SO_4
Hexane layer
Evaporation
Hexane
ω3 fatty acid enriched oil

3.8.4.3 Determination of hydrolysis percentage

Unhydrolysed oil (SBO and MHO), hydrolysed-acylglycerols and free fatty acids were extracted into hexane and used to determine the acid value according to the method described in Section 3.10.1. The hydrolysis percentage of the oils after enzyme treatment was calculated as:

$$\text{Hydrolysis (\%)} = \frac{\text{Acid value}_{(\text{Hydrolysed oil})} - \text{Acid value}_{(\text{Unhydrolysed oil})}}{\text{Saponification value} - \text{Acid value}_{(\text{Unhydrolysed oil})}} \times 100$$

Where, the acid value is expressed as the number of mg of KOH required to neutralize free fatty acids present in 1 g of oil (see Section 3.10.1) and the saponification value is defined as the number of mg of KOH required to saponify 1 g of oil (see Section 3.10.5).

3.8.4.4 Separation of acylglycerols and free fatty acids after enzymatic hydrolysis

After adding the required amount of 0.5 N KOH to neutralize fatty acids released during hydrolysis (the required amount of KOH was determined by the acid value), the mixture was transferred into a separatory funnel and thoroughly mixed with 100 mL of hexane and 50 mL of distilled water (Figure 3.7). The lower aqueous layer was separated and discarded. The upper layer (hexane) containing tri-, di- and monoacylglycerols was washed 2 times with 50 mL distilled water and then passed through a bed of anhydrous sodium sulphate. The acylglycerols were subsequently recovered following hexane removal at 45°C using a rotary evaporator. The fatty acid composition of the acylglycerols was determined according to the method described in Section 3.10.12. All

enzymes listed in Table 3.1 were screened for their efficacy in concentrating ω3 fatty acids of both oils (SBO and MHO). Optimization of hydrolysis parameters (enzyme concentration, reaction time and reaction temperature) to obtain a maximum concentration yield of ω3 fatty acids from oils is described in Section 3.9.

3.9 Optimization procedure for production of ω3 fatty acid concentrates via enzymatic hydrolysis and urea complexation of seal blubber oil (SBO) and menhaden oil (MHO)

3.9.1 Experimental design and data analysis

A three-factor central composite rotatable design (CCRD, Figure 3.8, Box, 1954; Cornell, 1992) was employed to study the responses such as concentration of total ω3 fatty acids, EPA and DHA (Y variables) by urea complexation and enzymatic hydrolysis of SBO and/or MHO. The urea-to-fatty acid ratio (X_1), crystallization time (X_2) and crystallization temperature (X_3) were independent variables studied to optimize Y variables in the urea complexation experiment. In the enzymatic hydrolysis experiment, enzyme concentration (X_4), reaction time (X_5) and reaction temperature (X_6) were independent variables studied. Tables 3.3 and 3.4 show the independent variables studied and their levels used in urea complexation and enzymatic hydrolysis experiments, respectively. Duplicate reactions were carried out at all design points except at the centre point (0,0,0) where five replications were performed to allow the estimation of the "pure error". All experiments were carried out in a randomized order to minimize the effect of unexplained variability in the observed responses due to extraneous factors. Coded (x)

Figure 3.8 Central composite rotatable design for three variables (points are given as coded variable levels)

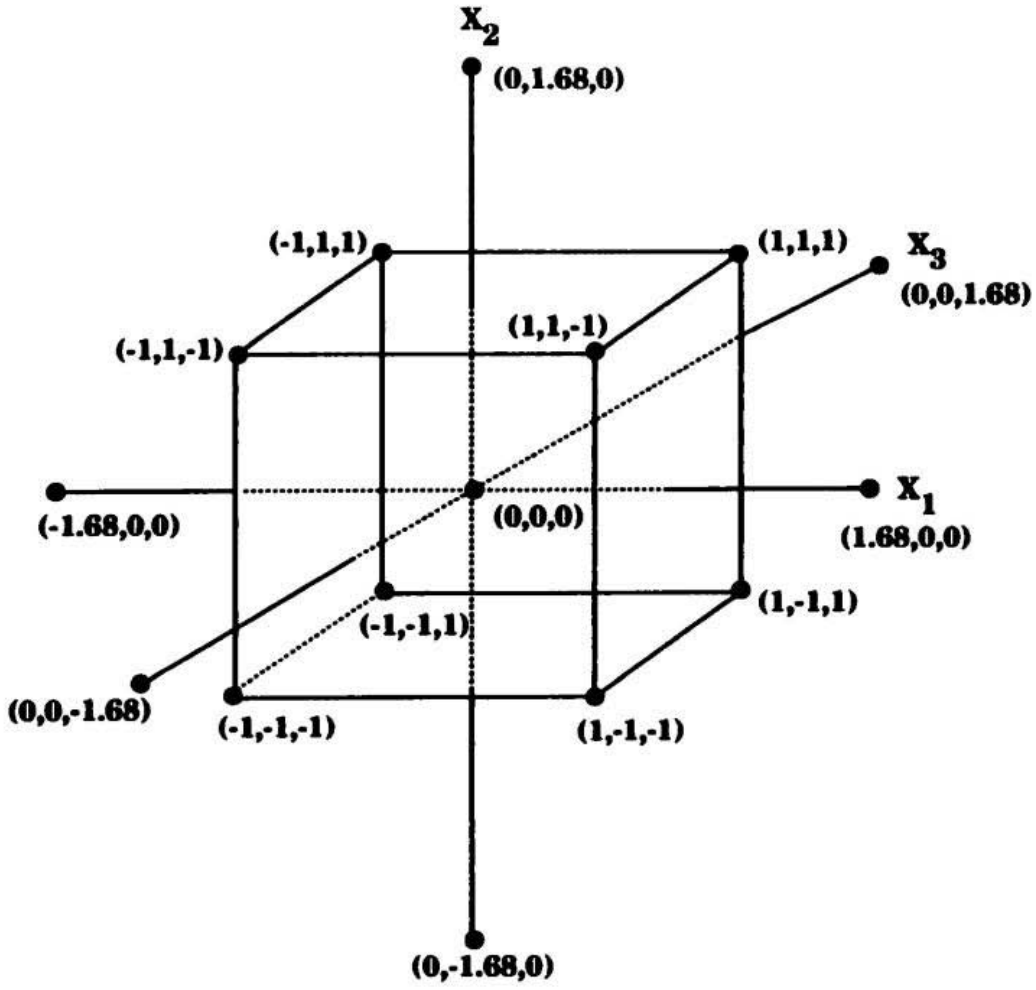

X2
(0,1.68,0)
(-1,1,1)
(1,1,1)
X3
(0,0,1.68)
(-1,1,-1)
(1,1,-1)
X1
(-1.68,0,0)
(0,0,0)
(1.68,0,0)
(1,-1,1)
(-1,-1,1)
(0,0,-1.68)
(-1,-1,-1)
(1,-1,-1)
(0,-1.68,0)

Table 3.3 Independent variables and their levels for central composite rotatable design (CCRD) in optimization of ω3 fatty acid concentrate production by urea complexation of seal blubber oil (SBO)

Independent variables	Symbol	Coded variable levels[a]				
		-1.68 (-α)	-1	0	1	1.68 (α)
Urea-to-fatty acid ratio (w/w)	X_1	1	2	3.5	5	6
Crystallization time (h)	X_2	8	12	18	24	28
Crystallization temperature (°C)	X_3	-24	-18	-9	1	6

[a]Transformation of coded variable (X_i) levels to uncoded variable (x_i) levels could be obtained from: $x_1 = 1.5X_1 + 3.5$, $x_2 = 6X_2 + 18$ and $x_3 = 9.5X_3 - 8.5$

Table 3.4 Independent variables and their levels for central composite rotatable design (CCRD) in optimization of ω3 fatty acid concentrate production by enzymatic hydrolysis of seal blubber oil (SBO) and menhaden oil (MHO)

Independent variables	Symbol	Coded variable levels[a]				
		-1.68 (-α)	-1	0	1	1.68 (α)
Enzyme concentration (U/g oil)	X_4	45	140	280	420	515
Reaction time (h)	X_5	3	12	25	38	47
Reaction temperature (°C)	X_6	26.6	30	35	40	43.4

[a]Transformation of coded variable (X_i) levels to uncoded variable (x_i) levels could be obtained from: $x_4 = 140X_4 + 280$, $x_5 = 13X_5 + 25$ and $x_6 = 5X_6 + 35$

and uncoded variable (X) of CCRD and treatment combinations used for optimization of ω3 concentrates production by urea complexation and enzymatic hydrolysis are given in Tables 3.5 and 3.6, respectively.

A quadratic polynomial regression model was assumed for predicting individual Y variables. The model proposed for each response of **Y** was:

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

where β_o, β_i, β_{ii} and β_{ij} are 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 multiple regression analysis, analysis of variance (ANOVA), canonical analysis and analysis of ridge maximum of data in the RSREG procedure. Response surfaces and contour plots were developed using the fitted quadratic polynomial equations obtained from response surface regression (RSREG) analysis and holding the independent variables with the least effect on the response at a constant value and changing the levels of the other two variables.

3.10 Chemical and instrumental analyses

3.10.1 Determination of the acid value (AV)

The acid value of oil samples was determined according to the Official Method of the American Oil Chemists' Society (AOCS 1990, Method Cd 3a-63). Samples (2-10 g of oil) were weighed into a 250 mL glass Erlenmeyer flask and mixed with 50 mL of

Table 3.5 Coded and uncoded variables of central composite rotatable design (CCRD) in optimization of ω3 production by urea complexation of seal blubber oil (SBO)

Run	Coded variables			Uncoded variables		
	x_1	x_2	x_3	X_1	X_2	X_3
1	-1	-1	-1	2	12	-18
2	1	-1	-1	5	12	-18
3	-1	1	-1	2	24	-18
4	1	1	-1	5	24	-18
5	-1	-1	1	2	12	0
6	1	-1	1	5	12	0
7	-1	1	1	2	24	0
8	1	1	1	5	24	0
9	-1.68	0	0	1	18	-9
10	1.68	0	0	6	18	-9
11	0	- 1.68	0	3.5	8	-9
12	0	1.68	0	3.5	28	-9
13	0	0	-1.68	3.5	18	-24
14	0	0	1.68	3.5	18	+6
15	0	0	0	3.5	18	-9
16	0	0	0	3.5	18	-9
17	0	0	0	3.5	18	-9
18	0	0	0	3.5	18	-9
19	0	0	0	3.5	18	-9

X_1 = Urea-to-fatty acid ratio, X_2 = crystallization time (h) and X_3 = crystallization temperature (°C)

Table 3.6 Coded and uncoded variables of central composite rotatable design (CCRD) in optimization of production of ω3 concentrates by enzymatic hydrolysis of seal blubber oil (SBO) and menhaden oil (MHO)

Run	Coded variables			Uncoded variables		
	x_4	x_5	x_6	X_4	X_5	X_6
1	-1	-1	-1	140	12	30
2	1	-1	-1	420	12	30
3	-1	1	-1	140	38	30
4	1	1	-1	420	38	30
5	-1	-1	1	140	12	40
6	1	-1	1	420	12	40
7	-1	1	1	140	38	40
8	1	1	1	420	38	40
9	-1.68	0	0	45	25	35
10	1.68	0	0	515	25	35
11	0	- 1.68	0	280	3	35
12	0	1.68	0	280	47	35
13	0	0	-1.68	280	25	26.6
14	0	0	1.68	280	25	43.4
15	0	0	0	280	25	35
16	0	0	0	280	25	35
17	0	0	0	280	25	35
18	0	0	0	280	25	35
19	0	0	0	280	25	35

X_4 = Enzyme concentration (U/g oil), X_5 = reaction time (h) and X_6 = reaction temperature (°C)

95% (v/v) aqueous ethanol (neutralized with 0.5 N KOH) and 2 mL of 1% phenolphthalein indicator. The mixture was heated to 70°C while stirring, and titrated against standardized 0.1 N alcoholic potassium hydroxide (KOH) solution to attain a permanent pink colour. A blank titration was conducted each time. The AV was expressed as the amount of KOH, in mg, required to neutralize free fatty acids present in 1 g of oil.

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

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

3.10.2 Determination of cholesterol in oils

The cholesterol content of NUCF of seal blubber oil (SBO) was determined according to Rudel and Morris (1973) using *O*-phthalaldehyde reagent (Sigma). Samples (0.07-0.08 g of oil) were weighed into 15 mL screw capped tubes and mixed thoroughly with 0.3 mL of 33% (w/v) KOH and 3.0 mL of 95% (v/v) aqueous ethanol. Tubes were then heated to 60°C in a water bath for 15 min, cooled, then added 10 mL of hexane and 3 mL of distilled water and mixed thoroughly using a vortex. The whole mixture was allowed to separate into two layers. Aliquots (1 mL) of upper hexane layer were pipetted into clean tubes and the solvent was then evaporated under a stream of nitrogen. Two mL of the 0.05% (w/v) *O*-phthalaldehyde reagent in glacial acetic acid and 1 mL of concentrated sulphuric acid were carefully added to the tubes and then mixed thoroughly.

After 10 min, the absorbance of the solutions was read at 550 nm. A standard curve was prepared using a cholesterol standard (Sigma). Cholesterol content of the oil was expressed as mg/100 g oil and calculated using the equation $C = 45.455A_{550} \times F$, $r = 0.992$. Where, C is the concentration of cholesterol, A is the absorbance of the solution at 550 nm and F is the correction factor (for details see Figure A.2 in the Appendix).

3.10.3 Determination of the iodine value (IV)

The iodine value of oil samples was determined according to the Official Method of the American Oil Chemists' Society (AOCS 1990, Method Cd 1-25). Samples (0.1-0.2 g of oil) were weighed into 250 mL glass-stoppered Erlenmeyer flasks and dissolved in 10 mL of chloroform. After thorough mixing, the flask was wrapped with aluminium foil and 25 mL of Hanus iodine solution were added to it and the mixture was allowed to stand for 30 min in the dark. Afterwards 10 mL of 15% (w/v) potassium iodide (KI) solution and 100 mL of distilled water were added to the sample. The mixture was subsequently titrated against a standardized 0.1 N solution of sodium thiosulphate ($Na_2S_2O_3$) with constant shaking, until the yellow colour of the mixture disappeared, upon which 0.5 mL of starch indicator solution (1%, w/v) was added to the mixture and titration was continued until the blue colour of starch-iodine complex had disappeared. A blank titration was conducted each time. The IV was expressed as the uptake of iodine in grams by 100 g of oil.

$$IV = \frac{(V_{Blank} - V_{Sample}) \times N_{Na_2S_2O_3} \times 12.692}{\text{Mass of sample (g)}}$$

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

3.10.4 Determination of the peroxide value (PV)

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

$$PV = \frac{(V_{Sample} - V_{Blank}) \times N_{Na_2S_2O_3} \times 1000}{\text{Mass of sample (g)}}$$

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

3.10.5 Determination of the saponification value (SV)

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

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

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

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

The direct TBARS value determination of the American Oil Chemists' Society (AOCS 1990, Method Cd 19-90) was employed. Oil (50-200 mg) was accurately weighed into a 25 mL volumetric flask and dissolved in a small volume of 1-butanol and made up to the mark with the same solvent. Five mL of this solution was transferred into a dry test tube to which 5 mL of fresh TBA reagent (200 mg 2-thiobarbituric acid in 100

mL 1-butanol and 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. 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 $C = (0.355A_{532})/w$, $r = 0.995$. Where, C is the concentration of MA, A is the absorbance of the coloured complex at 532 nm and w is the mass of oil (for details see Figure A.3 in the Appendix).

3.10.7 Determination of tocopherols in oils

The content of tocopherol in seal blubber oil (SBO) and cod liver oil (CLO) as affected by the refining process was determined by high performance liquid chromatography (HPLC). Tocopherols in the oils were separated using a Lichrosorb Si-60 (Merck, 3.2 × 20 mm, 5 μ) analytical column by employing diethyl ether/hexane (5:95, v/v) as the mobile phase (Thompson and Hatina, 1979). The system used for the analysis was a Shimadzu HPLC equipped with two LC-6A pumps, SPD-6AV UV-VIS spectrophotometric detector and C-R4A Chromatopac for data handling. A 20 μL sample (1 g oil was dissolved 10 mL of mobile phase) and a flow rate of 1 mL/min were used for the analysis. The tocopherols in the oils were detected by the UV detector set at 295 nm and identified by comparing their retention times with those of known tocopherol standards (Hoffmann-La Roche Ltd.).

3.10.8 Separation of oils into neutral lipids (NL) and polar lipids (PL) fractions

Separation of oils into different lipid classes was carried out according to the method described by Christie (1982). Oil samples (1.75 g) were applied onto a silicic acid column (1.25 cm internal diameter and 20 cm height; mesh 100 silicic acid powder, Mallinckrodt Canada Inc.). Neutral lipid (NL) fraction of each oil was eluted with chloroform (1200 mL) while methanol (1200 mL) was subsequently used to elute polar lipids (PL). Solvents were removed at 40°C using a rotary evaporator. All fractions were weighed and their percentage in the oils calculated.

3.10.9 Measurement of UV absorbance of dechlorophyllized green tea extract (DGTE) fractions following Sephadex LH-20 column chromatography

One tenth of a mL of each sample fraction was mixed with 2 mL of spectral grade ethanol. The absorbance of each sample at 280 nm was read using a Hewlett-Packard 8452A diode array spectrophotometer.

3.10.10 Determination of total phenolics in dechlorophyllized green tea extracts (DGTE) and DGTE fractions following Sephadex LH-20 column chromatography

The total phenolic content of DGTE was determined colorimetrically by the method of Price *et al.* (1978). Tea extracts (20-25 mg) were weighed into a 10 mL volumetric flask and dissolved in absolute ethanol. To 1.0 mL of ethanolic solution of tea extracts, 5.0 mL of 0.5% (w/v) solution of vanillin reagent or 5.0 mL of 4% (v/v) solution of HCl in ethanol was added. The absorbances of the samples and blank were

read at 500 nm after standing 20 min at room temperature. (+)Catechin (Sigma) was used as the standard compound and the content of total phenolics in the extracts was expressed as catechin equivalents, using the equation $C = k(2.2467A_{500})$, $r = 0.9963$. Where, C is the concentration of catechins, A is the absorbance at 500 nm and k is the dilution factor (Figure A.4 in the Appendix). The content of phenolics in the extracts was expressed as mg per 100 g of material.

The concentration of total phenolics in each fraction, separated by Sephadex LH-20 column chromatography, was estimated colourimetrically using the Vanillin-HCl reagent as described by Price *et al.* (1978). To 0.5 mL of the test solution, 0.5 mL of vanillin-HCl reagent were added and then mixed thoroughly and absorbances were read at 500 nm after standing 20 min at room temperature.

3.10.11 The colour of the oil

The effect of processing on the colour of the seal blubber oil (SBO) and cod liver oil (CLO) was monitored using a Lovibond Tintometer (Model E, The Tintometer Ltd., Salisbury, England). The colour of the oils was measured using a 133.5 mm glass cuvette with a depth of 25 mm, as supplied with the instrument and values expressed in terms of the intensity of the yellow and red colours were determined and compared with a series of standard reference lenses.

3.10.12 Analysis of fatty acid composition of lipids

3.10.12.1 Preparation of fatty acid methyl esters (FAMEs)

Fatty acid composition of lipids was determined by their conversion to methyl esters. About 10 mg of each oil was weighed into a 6 mL well-cleaned Teflon-lined, screw capped conical vial. The internal standard (250 ng/100µl chloroform, methyl tricosanoate; C23:0) was added to the vial and the solvent in the oil-internal standard mixture was evaporated under a stream of nitrogen. Transmethylation reagent (2 mL, freshly prepared 6 mL of concentrated sulphuric acid made up to 100 mL with spectral grade methanol and 15 mg of hydroquinone as an antioxidant) was added to the sample vial and mixed by vortexing. The mixture was incubated overnight at 60°C and subsequently cooled (Keough and Kariel (1987). Distilled water (1 mL) was added to the mixture and after thorough mixing, extracted three times with 1.5 mL of pesticide-grade hexane. A few crystals of hydroquinone were added to each vial prior to extraction with hexane. Hexane layers were separated, combined and transferred to a clean tube and then washed two times with 1.5 mL of distilled water. In the first wash, the aqueous layer was removed and in the second wash, the hexane layer was separated and evaporated under a stream of nitrogen. Fatty acid methyl esters were then dissolved in 1 mL of carbon disulphide and used for gas chromatographic analysis (see below).

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

A Hewlett Packard 5890 Series II gas chromatograph (Hewlett Packard, Toronto, ON) equipped with a Supelcowax-10 column (0.25 mm diameter, 30 m length, 0.25 µm

film thickness; Supelco Canada Ltd., Oakville, ON) was used for analyzing FAMEs. The oven temperature was initially 220°C for 10.25 min and then ramped to 240°C at 30°C/min and then held there for 9 min. The injector and detector (flame ionization, FID) temperatures were both at 270°C. UHP helium was used as a carrier gas (15mL/min). HP 3365 Series II ChemStation software (Hewlett Packard, USA) was used for data handling. The FAMEs were tentatively identified by comparison of their retention times with those of authentic standard mixtures (PUFA 1; Supelco Canada Ltd. and GLC-416; Nu-Check). The area under each peak was calculated on a weight percentage basis using methyl tricosanoate (C23:0) as an internal standard.

3.10.13 Analysis of headspace volatiles of oxidized and unoxidized oils

A Perkin-Elmer 8500 gas chromatograph and HS-6 headspace sampler (Perkin-Elmer Corp., Montreal, PQ) were used for analysis of volatiles produced during oxidation of oil samples. The volatiles in the headspace of oxidized oils (obtained from the accelerated oxidation method) were separated using a high polarity Supelcowax-10 fused silica capillary column (0.32 mm internal diameter, 30 m length, 0.10 μm film thickness; Supelco Canada Ltd.). UHP helium was the carrier gas. The inlet column pressure was 17.5 psig and the split ratio was 7:1. The oven temperature was maintained at 40°C for 5 min and then ramped to 200°C at 20°C/min and held there for 5 min. The injector and flame ionization detector (FID) temperatures were held at 280°C.

For headspace analysis, 0.10 g of each oil was transferred to a 5 mL headspace vial (Chromatographic Specialties Inc., Brockville, ON). The vials were capped with

Teflon-lined septa and crimped. The vial was then preheated in the headspace magazine assembly at 40°C for a 45 min equilibration period. Pressurization time of the vial was 6 s, and the volume of the vapour phase drawn was approximately 1.5 mL. The area under each peak was expressed as integrator count units. Volatile compounds were identified by comparison of their retention times with those of authentic compounds. Quantitative determination of dominant volatiles (mainly hexanal and propanal) was accomplished using 2-heptanone as an internal standard (Shahidi and Pegg, 1994).

3.11 Statistical analyses

All experiments in this study were replicated at least three times. Data are reported as mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed and differences in mean values were determined using Tukey's studentized test at $P < 0.01$ or 0.05 and employing ANOVA and TUKEY'S procedures of statistical analysis system (SAS, 1990). Simple linear and multiple regression analyses were also performed using the same software in the general linear model (GLM) and response surface regression (RSREG) procedures, respectively. Section 3.9 explains data analysis carried out for the optimization study. Experimental designs used in the study are described in the appropriate sections.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of processing on the content of major and minor components and oxidative stability of seal blubber oil (SBO) and cod liver oil (CLO)

Triacylglycerols are the predominant constituents of marine oils; however, the presence of minor amounts of other compounds plays an important role on their quality and oxidative stability. Therefore, crude oils are further processed in order to improve their quality by degumming, alkali-refining, bleaching and deodorization. Each processing step has a specific function in removing certain minor compounds which may change the quality of the finished oil. Alkali-refining removes free fatty acids, phopholipids and metals. Subsequent bleaching removes coloured materials, peroxides and residual free fatty acids. Finally deodorization removes volatile compounds, free fatty acids and tocopherols and may decompose carotenoids and peroxides in order to improve flavour quality and stability of the oil.

4.1.1 Changes in neutral and polar lipids, tocopherol, free fatty acids and colour of seal blubber oil (SBO) and cod liver oil (CLO) during processing

Table 4.1 show changes in the contents of neutral and polar lipids, tocopherols, free fatty acids and colour of SBO and CLO during processing. Processing of oils by alkali-refining, bleaching and deodorization removed approximately 39 and 71% of polar lipids in crude SBO and CLO, respectively. The highest amount of polar lipids was removed during alkali-refining of the oils; the amounts were reduced from 1.02 to

Table 4.1 Changes of neutral and polar lipids (%), tocopherol (mg/100g oil), free fatty acid contents (acid value, mg KOH/g oil)) and colour of seal blubber oil (SBO) and cod liver oil (CLO) during processing[a]

Sample	Neutral lipids	Polar lipids	α-Tocopherol	Acid value (%)[b]	Colour (133.5mm cell)
			Seal blubber oil (SBO)		
Crude	98.98 ± 0.05	1.02 ± 0.05	2.8 ± 0.18	2.72 ± 0.10 (1.367%)	Y 0.92 ± 0.02*
Alkali-refined	99.36 ± 0.34	0.64 ± 0.14	3.2 ± 0.11	0.08 ± 0.00 (0.040%)	Y 0.71 ± 0.01
Refined-bleached (RB)	99.27 ± 0.17	0.72 ± 0.07	3.1 ± 0.12	0.03 ± 0.01 (0.015%)	Y 0.41 ± 0.00
Refined-bleached and deodorized (RBD)	99.38 ± 0.16	0.62 ± 0.06	2.4 ± 0.09	0.04 ± 0.01 (0.020%)	Y 0.30 ± 0.00
			Cod liver oil (CLO)		
Crude	98.17 ± 0.11	1.83 ± 0.02	10.8 ± 0.15	1.87 ± 0.15 (0.940%)	Y 1.10 ± 0.05
Alkali-refined	99.44 ± 0.40	0.56 ± 0.04	11.7 ± 0.21	0.07 ± 0.00 (0.030%)	Y 0.95 ± 0.02
Refined-bleached (RB)	99.33 ± 0.10	0.67 ± 0.09	10.0 ± 0.13	0.04 ± 0.00 (0.020%)	Y 0.70 ± 0.01
Refined-bleached and deodorized (RBD)	99.47 ± 0.15	0.53 ± 0.02	7.4 ± 0.11	0.05 ± 0.00 (0.025%)	Y 0.12 ± 0.00

[a]All values are mean of three replicates ± standard deviation
[b]content expressed as percent oleic acid, *Y = Yellow

0.64% in SBO and from 1.83 to 0.56% in CLO. The oils after alkali-refining, bleaching and deodorization had 98.5% (for SBO) and 97.4% (for CLO) of their free fatty acids removed as compared to their crude counterparts. The highest amounts were removed during alkali-refining (from 1.37 to 0.04% in SBO and from 0.94 to 0.03% in CLO). The addition of alkali to crude oils results in the conversion of free fatty acids to soap and the gums which absorb alkali become coagulated by dehydration. Therefore, these can be easily removed with water during the washing process. In industrial specifications, the free fatty acid content of processed marine oils should be less than 0.5% (Bimbo, 1989).

Tocopherol is another important minor component that may act as an antioxidant in marine oils. The tocopherol content of oils depends on the source material (organ, muscles, liver or blubber), PUFA content of the oil, as well as processing and storage conditions. The content of α-tocopherol in CLO was significantly ($P < 0.05$) higher than that of SBO; no other tocopherols were detected in these oils. According to Ackman and Cormier (1967) and Kinsella (1987) α-tocopherol is the major tocopherol present in marine oils. The content of α-tocopherol in both oils decreased during processing, especially at the bleaching and deodorization steps. A significant ($P > 0.05$) decrease (22.6% in SBO and 26.0% in CLO) in the content of α-tocopherol was noted during the deodorization process. Tocopherols are heat-labile (Jung *et al.*, 1989) and may also volatilize at high temperatures (100-200°C) reached during deodorization (Ferrari *et al.*, 1996).

Colour determined using a Lovibond tintometer, showed that the intensity of yellow colour in both oils was decreased during processing; the crude SBO had a slightly lower yellow intensity than crude CLO. The decrease in the intensity of yellow colour was from 0.92 to 0.30 for SBO and from 1.10 to 0.12 for CLO. In marine lipids, carotenoid pigments are responsible for their yellow colour (Stansby, 1978). Bleaching is used to improve the colour of the oil, but other processing methods such as alkali-refining and deodorization may also remove some of the coloured components (Bimbo, 1989). Although colour of marine oils is not included in their industrial specifications, removal of the coloured compounds (bleaching) is generally practised in their processing (Bimbo, 1989).

4.1.2 Oxidative stability of seal blubber oil (SBO) and cod liver oil (CLO) at different processing stages

The effect of processing on the oxidative stability of SBO and CLO was determined by measuring peroxide values (PV) and 2-thiobarbituric acid reactive substances (TBARS). During storage at 65°C, crude oils produced significantly ($P < 0.05$) lower amounts of hydroperoxides than alkali-refined, refined-bleached (RB) and refined-bleached and deodorized (RBD) oils (Figures 4.1a and 4.2a). The highest PV were observed in RB oil followed by alkali-refined oil. However, differences in PV of alkali-refined and RB-SBO were not significant ($P > 0.05$). The TBARS values of both oils at different stages of refining also showed that RB oils produced the highest

Figure 4.1 Effect of processing steps on peroxide (a) and TBARS (b) values of seal blubber oil (SBO) stored under Schaal oven conditions at 65°C (all values are mean of three replicates ± SD)

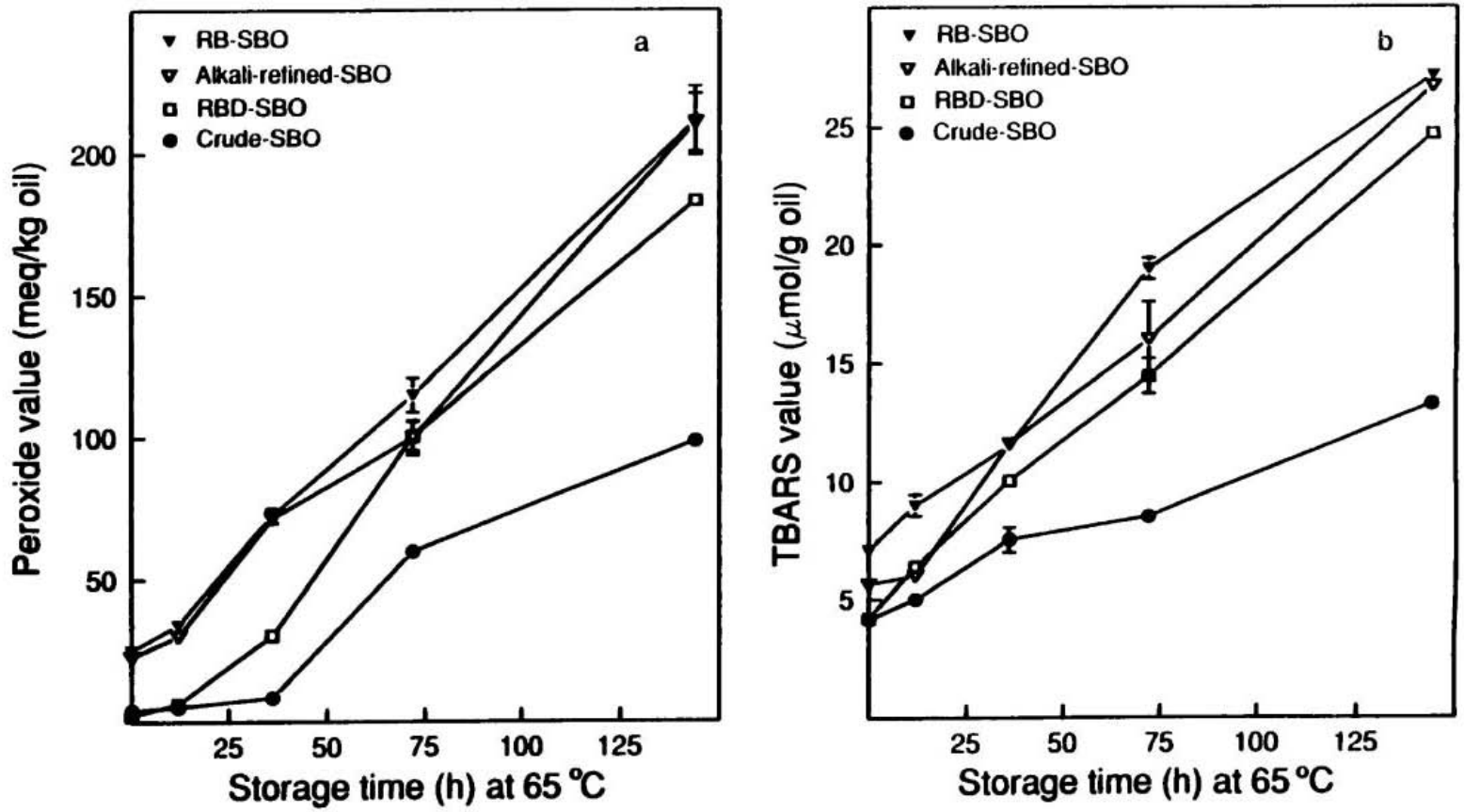
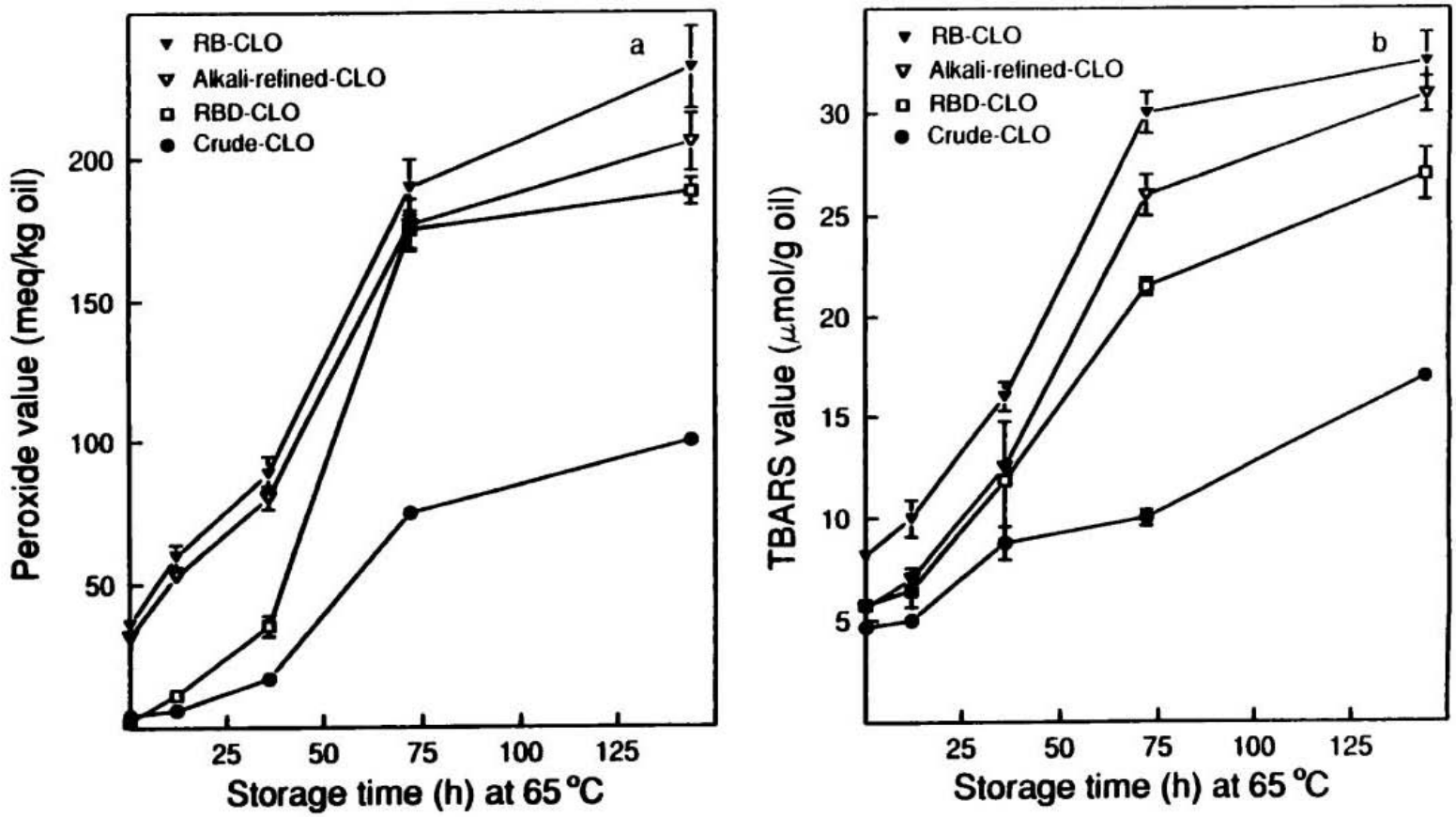

a
RB-SBO
Alkali-refined-SBO
RBD-SBO
Crude-SBO
Peroxide value (meq/kg oil)
200
150
100
50
25
50
75
100
125
Storage time (h) at 65 °C
b
RB-SBO
Alkali-refined-SBO
RBD-SBO
Crude-SBO
TBARS value (μmol/g oil)
25
20
15
10
5
25
50
75
100
125
Storage time (h) at 65 °C

Figure 4.2 Effect of processing steps on peroxide (a) and TBARS (b) values of cod liver oil (CLO) stored under Schaal oven conditions at 65°C (all values are mean of three replicates ± SD)


RB-CLO
Alkali-refined-CLO
RBD-CLO
Crude-CLO
a
Peroxide value (meq/kg oil)
200
150
100
50
25
50
75
100
125
Storage time (h) at 65 °C
RB-CLO
Alkali-refined-CLO
RBD-CLO
Crude-CLO
b
TBARS value (μmol/g oil)
30
25
20
15
10
5
25
50
75
100
125
Storage time (h) at 65 °C

and crude oils produced the lowest amounts of secondary oxidation products during storage (Figures 4.1b and 4.2b).

The high stability of crude oils from both SBO and CLO may be due the presence of antioxidative compounds, particularly α-tocopherol in the oils. During processing the content of α-tocopherol in both oils decreased significantly (Table 4.1). Jung *et al.* (1989) reported that crude soybean oil was most stable, while deodorized, refined and bleached oils were less stable in a decreasing order as determined by molecular oxygen content in their headspace. It has been suggested that decreased oxidative stability of oils during processing is due to the loss of natural antioxidants and synergists such as tocopherols and phospholipids (Ferrari *et al.*, 1996). Deodorization may increase oxidative stability of the oil because prooxidants such as moisture, monoacylglycerols and free fatty acids remaining in the bleached oil are removed during deodorization (Jung *et al.*, 1989).

Furthermore, although tocopherol content of CLO was high, its oxidative stability was lower than that of SBO. The presence of higher amounts of PUFA in CLO as compared to SBO might explain the observed trends.

4.2 Comparison of oxidative stability of refined-bleached and deodorized (RBD) seal blubber oil (SBO), cod liver oil (CLO) and menhaden oil (MHO)

4.2.1 Chemical properties of RBD seal blubber, cod liver and menhaden oils

RBD oils used in this study had iodine values of 145, 159 and 172, g iodine/100

g SBO, CLO and MHO, respectively. Their respective PV were 1.09, 1.99 and 3.05 meq/kg oil and their acid values (free fatty acid contents) were 0.04, 0.05 and 0.07 mg KOH/g oil (Table 4.2). Canola oil (CNO) which was used for comparative purposes had an iodine value, a PV and an acid value of 111 g iodine/100 g, 0.20 meq peroxides/kg and 0.05 mg KOH/g oil, respectively. The slightly higher PV of MHO as compared to SBO and CLO may be due to the formation of more primary oxidation products (hydroperoxides) in this oil which contains more unsaturated fatty acids (i.e. high iodine value) and is more prone to oxidation than other oils. Furthermore, MHO was received when it was two weeks old and this might have also influenced on its PV. The fatty acid composition of oils showed that CLO and MHO had higher amounts of EPA (11.2% in CLO and 13.2% in MHO) and DHA (14.8% in CLO and 10.1% in MHO) as compared to SBO, but the latter had a higher content of DPA (4.66%) which is less abundant in fish oils (1.14% in CLO and 2.40% in MHO). However, the contents of EPA and DHA of CLO used in this study were slightly higher than values reported in the literature (e.g. Haagsma *et al.*, 1982). This might be due to the fact that CLO used in this study was from farmed cod and differences may have originated from the dietary regime of the farmed cod.

Table 4.3 shows the neutral and polar lipids as well as tocopherol contents of these marine oils. The content of polar lipids in all three marine oils was less than 1.0% of the total amount. During the refining (degumming) process, most of the polar lipids were removed. The content of polar lipids in CNO was slightly higher than those

Table 4.2 Iodine, peroxide and acid values and fatty acid composition of refined-bleached and deodorized seal blubber, cod liver, menhaden and canola oils[a]

Parameter	Seal blubber (SBO)	Cod liver (CLO)	Menhaden (MHO)	Canola (CNO)
Iodine value (g iodine/100g oil)	145 ± 0.35	159 ± 4.41	172 ± 3.01	111 ± 1.99
Peroxide value (meq/kg oil)	1.09 ± 0.03	1.99 ± 0.02	3.05 ± 0.20	0.20 ± 0.01
Acid value (mg KOH/g oil)	0.04 ± 0.01	0.05 ± 0.00	0.07 ± 0.01	0.05 ± 0.00
Fatty acid (w/w%)				
14:0	3.73 ± 0.08	3.33 ± 0.01	8.32 ± 0.12	-
14:1ω5	1.09 ± 0.04	0.15 ± 0.00	0.38 ± 0.01	-
15:0	0.23 ± 0.00	0.19 ± 0.00	0.71 ± 0.02	-
16:0	5.98 ± 0.03	11.1 ± 0.11	17.1 ± 0.24	4.20 ± 0.02
16:1ω7	18.0 ± 0.04	7.85 ± 0.02	11.4 ± 0.13	0.26 ± 0.01
17:0	0.92 ± 0.00	0.61 ± 0.01	2.45 ± 0.12	-
17:1	0.55 ± 0.02	0.44 ± 0.00	1.86 ± 0.03	-
18:0	0.88 ± 0.00	3.89 ± 0.01	3.33 ± 0.02	1.91 ± 0.02
18:1ω9	20.8 ± 0.06	16.6 ± 0.17	8.68 ± 0.12	57.6 ± 1.51
18:1ω11	5.22 ± 0.03	4.56 ± 0.01	3.46 ± 0.07	-
18:2ω6	1.51 ± 0.02	0.74 ± 0.01	1.42 ± 0.09	23.4 ± 1.00
18:3ω6	0.19 ± 0.00	0.22 ± 0.00	0.51 ± 0.00	9.10 ± 0.11
18:3ω3	0.40 ± 0.00	0.24 ± 0.01	1.31 ± 0.00	-
18:4ω3	1.00 ± 0.02	0.61 ± 0.00	2.90 ± 0.05	-
20:0	0.11 ± 0.00	0.05 ± 0.00	0.20 ± 0.01	0.81 ± 0.01
20:1ω9	12.2 ± 0.02	10.4 ± 0.41	1.44 ± 0.06	2.00 ± 0.10
20:2ω6	0.16 ± 0.00	0.12 ± 0.00	0.21 ± 0.00	-
20:3ω6	0.09 ± 0.00	-	0.25 ± 0.00	-
20:3ω3	0.05 ± 0.00	-	0.21 ± 0.03	-
20:4ω6	0.46 ± 0.01	0.22 ± 0.00	0.83 ± 0.02	-
20:5ω3	6.41 ± 0.08	11.2 ± 0.01	13.2 ± 0.18	-
22:0	-	-	0.12 ± 0.00	0.34 ± 0.00
22:1ω11	2.01 ± 0.04	9.07 ± 0.03	0.12 ± 0.05	0.38 ± 0.00
22:2	-	-	0.02 ± 0.00	-
22:4ω6	0.11 ± 0.01	-	0.19 ± 0.03	-
22:5ω3	4.66 ± 0.01	1.14 ± 0.00	2.40 ± 0.03	-
22:6ω3	7.58 ± 0.02	14.8 ± 1.01	10.1 ± 0.11	-

[a]Mean ± SD (n = 3)

Table 4.3 Neutral and polar lipids and tocopherol content of refined-bleached and deodorized seal blubber, cod liver, menhaden and canola oils[a]

Sample	Neutral lipids (%)	Polar lipids (%)	Tocopherols (mg/100 g oil)
RBD-seal blubber oil (SBO)	99.38 ± 0.16	0.62 ± 0.06	2.4 ± 0.09[1]
RBD-cod liver oil (CLO)	99.47 ± 0.15	0.53 ± 0.02	7.4 ± 0.11[1]
RBD-menhaden oil (MHO)	99.33 ± 0.13	0.67 ± 0.04	6.9 ± 0.21[1]
RBD-canola oil (CNO)	97.60 ± 0.80	1.02 ± 0.16	63.1 ± 0.82[2]

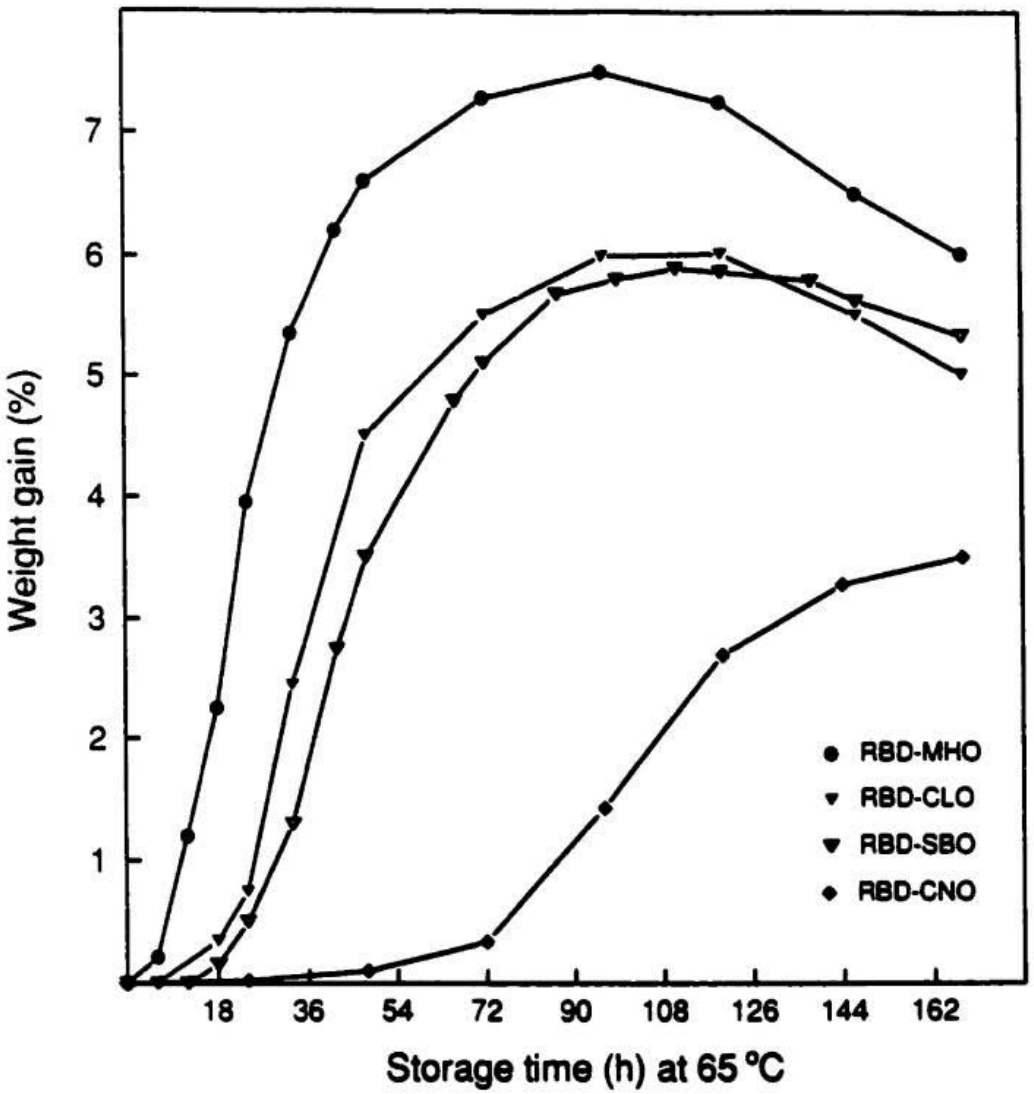
[a]Mean ± SD (n = 3)
[1]α-Tocopherol
[2]α-,γ- and δ-Tocopherols

in marine oils examined, even after the refining process. Both MHO and CLO contained considerably higher amounts of α-tocopherol than SBO.

4.2.2 Oxidative stability of seal blubber, cod liver and menhaden oils measured by various physical and chemical indices

Figure 4.3 summarizes the weight gain data of SBO, CLO and MHO under Schaal oven test conditions over a 162 h storage period; CNO is given for comparative purposes. The time required for a 0.5% weight gain (Olcott and Einset, 1958) of oil samples was taken as the length of the induction period and was 24, 9, 20 and 77 h for SBO, MHO, CLO and CNO, respectively. CNO had a much higher induction period as compared to all marine oils. Canola oil, being a vegetable oil, contains much less polyunsaturates and more naturally-occurring antioxidants such as tocopherols and sterols as well as phospholipid synergists. Among the marine oils tested, SBO had the longest induction period as compared to CLO and MHO. The weight of oils continued to increase rapidly up to 108 h for SBO and up to 90 h for MHO and CLO, after which the rate of weight increase was decreased. However, the rate of weight gain of CNO was not as fast as the marine oils, and no weight loss was detected throughout the course of the experiment. The weight increase of oils is due to the addition of oxygen to lipid molecules leading to the formation of hydroperoxides during the primary stages of autoxidation. Further storage of marine oils, for up to 162 h, resulted in a relatively small loss of weight, perhaps due to the breakdown of heat-labile hydroperoxides and

Figure 4.3 Weight gain data of refined-bleached and deodorized (RBD) seal blubber (SBO), cod liver (CLO), menhaden (MHO) and canola (CNO) oils stored under Schaal oven conditions at 65°C, (deviation from mean for each data point is within ± 1% of the absolute values, n = 3)

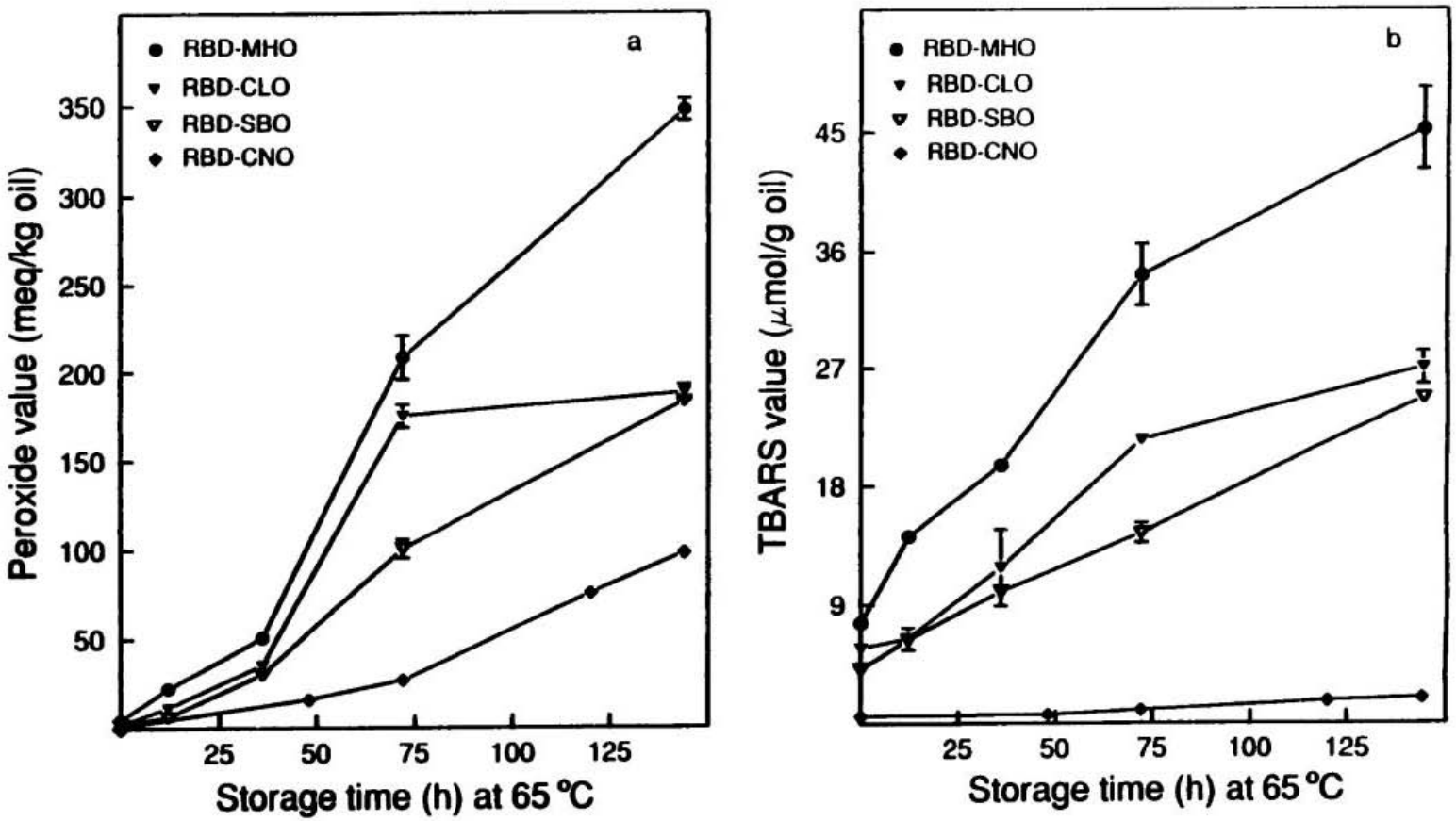

Weight gain (%)
7
6
5
4
3
2
1
18
36
54
72
90
108
126
144
162
Storage time (h) at 65 °C
RBD-MHO
RBD-CLO
RBD-SBO
RBD-CNO

volatilization of some of the secondary oxidation products formed under the experimental conditions. Another significant feature of the weight gain data was that addition of oxygen to marine oils was much faster and higher than that of CNO; among the marine oils, MHO had the highest weight gain during the storage period due to its higher degree of unsaturation.

Privett and Nickell (1956) have reported that addition of oxygen to lipids to form hydroperoxides is reasonably quantitative during the initial stages of autoxidation. According to Olcott and Einset (1958) the weight gain during the course of oxidation serves as a useful indicator for evaluating the oxidative stability of edible oils. The weight gain method is simple, sufficiently reproducible, and may be used to compare oxidation of marine oils (Ke and Ackman, 1976). However, for comparative purposes, it is necessary to report the surface exposure and the quantity of samples used for evaluation (Kwon *et al.*, 1984).

Figure 4.4a shows progression of formation of hydroperoxide during storage of oils under investigation. Marine oils (SBO, CLO and MHO) oxidized rapidly, thus producing more than 50 meq peroxides/kg sample within 52 h of storage. In contrast, CNO oxidized slowly and produced similar amounts of hydroperoxides (50 meq peroxides/kg oil) after 100 h of storage. Among marine oils, MHO and CLO produced a significantly ($P < 0.05$) higher amounts of hydroperoxides as they contained higher proportions of PUFA, especially EPA and DHA as compared to SBO. Thus, SBO is a more stable oil than CLO and MHO.

Figure 4.4 Peroxide (a) and TBARS (b) values of refined-bleached and deodorized (RBD) seal blubber (SBO), cod liver (CLO), menhaden (MHO) and canola (CNO) oils stored under Schaal oven conditions at 65°C (all values are mean of three replicates ± SD)


a
RBD-MHO
RBD-CLO
RBD-SBO
RBD-CNO
Peroxide value (meq/kg oil)
350
300
250
200
150
100
50
25
50
75
100
125
Storage time (h) at 65 °C
b
RBD-MHO
RBD-CLO
RBD-SBO
RBD-CNO
TBARS value (μmol/g oil)
45
36
27
18
9
25
50
75
100
125
Storage time (h) at 65 °C

Since hydroperoxides are the primary products of lipid oxidation, PV provides a clear indication of the oxidative state of marine oils. However, due to the instability of hydroperoxides in the oxidation pathway, measurement of PV provides information about only the initial oxidation potential of the oil.

The 2-thiobarbituric acid reactive substances (TBARS) test, which measures secondary products of lipid oxidation, is frequently used for monitoring stability of edible oils. Figure 4.4b displays changes of the TBARS values of SBO, CLO, MHO and CNO during storage at 65°C. Among the marine oils examined, MHO produced the highest amount of TBARS followed by CLO and SBO. Again the high content of TBARS in MHO may reflect the presence of high proportions of PUFA in the oil, which are highly labile to oxidative deterioration. The content of TBARS in CNO remained significantly ($P < 0.05$) lower than all marine oils tested.

Oxidative stability of marine oils namely SBO, CLO and MHO was also compared with that of CNO by measuring volatile products formed during storage using gas chromatographic (GC) headspace analysis. The main volatile products identified in marine oils were propanal, and to a lesser extent, butanal, hexanal and pentanal (Figure 4.5a). In CNO, hexanal, and to a lesser extent, propanal, pentanal and heptanal were the major volatile products of oxidation (Figure 4.5b). The content of propanal produced by SBO, CLO and MHO was significantly ($P < 0.05$) higher than that produced by CNO (Figure 4.6b). In contrast, the content of hexanal produced by CNO was higher than that from marine oils tested (Figure 4.6a). This difference in the

Figure 4.5 Chromatograms of the headspace volatiles of refined-bleached and deodorized (RBD) seal blubber (SBO), cod liver (CLO), menhaden (MHO) and canola (CNO) oils after 48h storage under Schaal oven conditions at 65°C

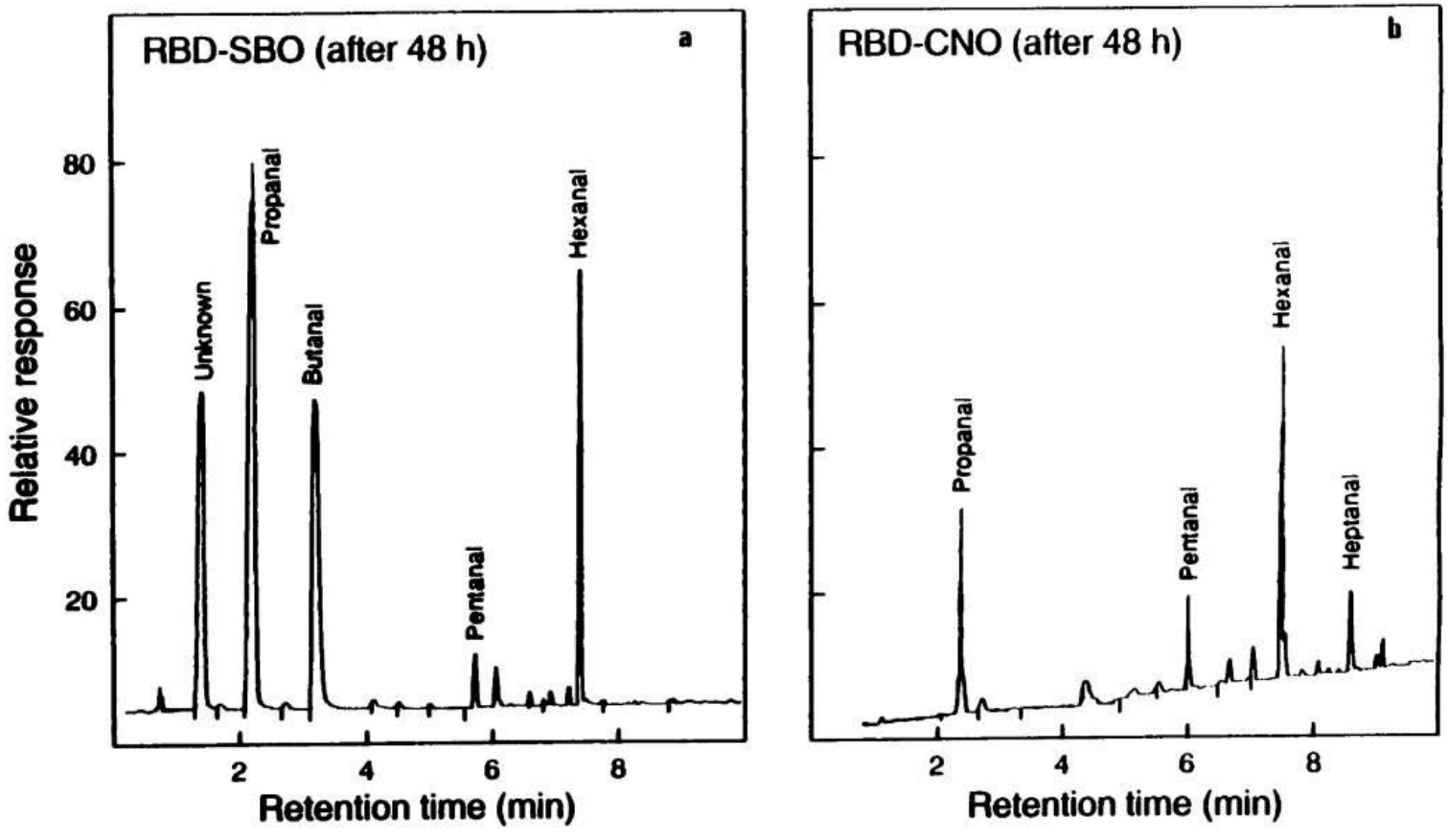
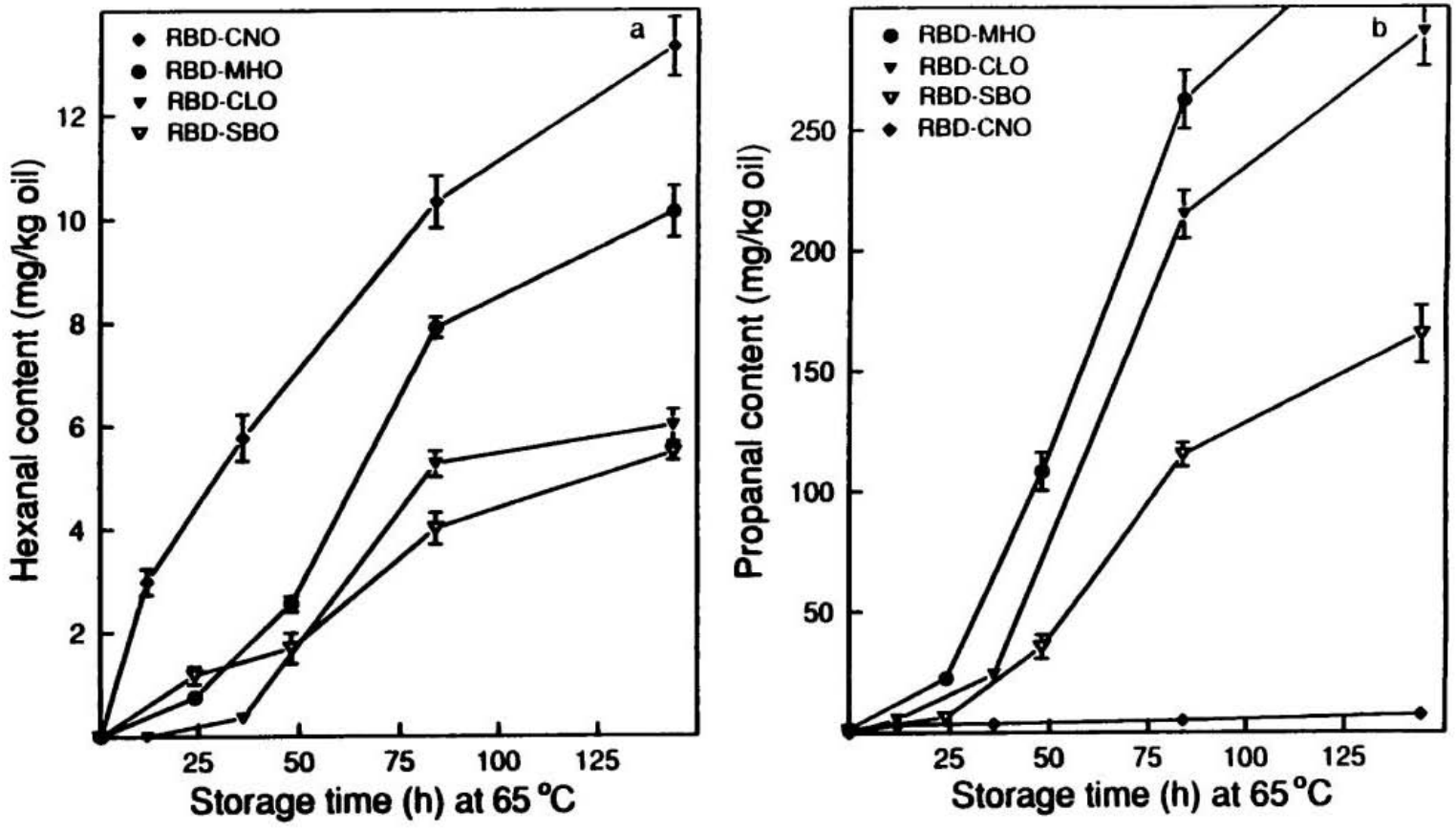

RBD-SBO (after 48 h)
a
Relative response
80
60
40
20
Unknown
Propanal
Butanal
Pentanal
Hexanal
2
4
6
8
Retention time (min)
RBD-CNO (after 48 h)
b
Propanal
Pentanal
Hexanal
Heptanal
2
4
6
8
Retention time (min)

Figure 4.6 Hexanal (a) and propanal (b) contents of refined-bleached and deodorized (RBD) seal blubber (SBO), cod liver (CLO), menhaden (MHO) and canola (CNO) oils stored under Schaal oven conditions at 65°C (all values are mean of three replicates ± SD)


a
RBD-CNO
RBD-MHO
RBD-CLO
RBD-SBO
Hexanal content (mg/kg oil)
2
4
6
8
10
12
25
50
75
100
125
Storage time (h) at 65 °C
b
RBD-MHO
RBD-CLO
RBD-SBO
RBD-CNO
Propanal content (mg/kg oil)
50
100
150
200
250
25
50
75
100
125
Storage time (h) at 65 °C

formation of volatile compounds of marine oils and CNO may be due to high content of ω3 fatty acids present in marine oils which produced more propanal during oxidation than CNO with a higher content of ω6 fatty acids as compared to its content of ω3 fatty acids. During the oxidation process, ω3 fatty acids, especially EPA and DHA, produce more 18- and 20-hydroperoxides than other hydroperoxides which may give propanal upon homolytic cleavage, respectively (Figure 4.7). Similarly, ω6 fatty acids, especially linoleic acid, produce more of the 13-hydroperoxide as their primary product of oxidation and produce hexanal upon homolytic cleavage (Figure 4.8). Therefore, formation of propanal in MHO, CLO and SBO was appreciable after 25, 30 and 50 h, respectively. Under similar oxidation conditions, CNO showed no significant formation of propanal (Figure 4.6b). However, hexanal content in CNO was significantly ($P < 0.05$) higher than that in all marine oils tested (Figure 4.6a). Frankel (1993) made a similar observation for fish oils (menhaden, sardine and bonita) and vegetable oils (soybean and safflower). Frankel *et al.* (1981) and Frankel (1982) have reported that hexanal is one of the major secondary products formed during the oxidation of ω6 fatty acids in edible oils. Shahidi *et al.* (1987) have found a direct relationship between hexanal content and sensory scores of cooked ground pork which contains a high amount of linoleic acid (ω6). Therefore, it is necessary to consider dominant fatty acids of oils before selecting specific aldehyde as an indicator for determining their oxidative stability.

Polyunsaturated fatty acids are among the most easily oxidizable components

Figure 4.7 Autoxidation of docosahexaenoic acid (DHA) and production of propanal

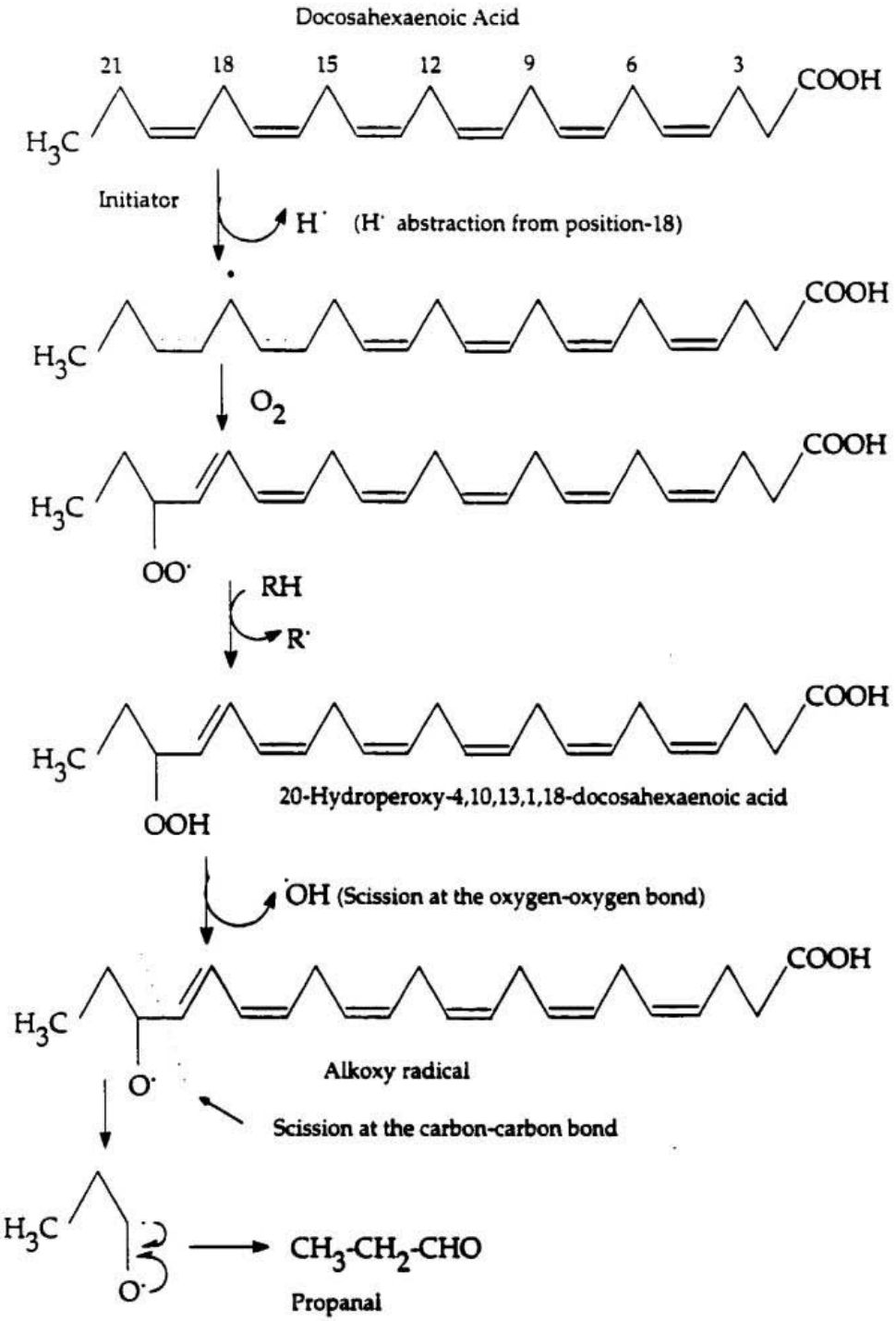
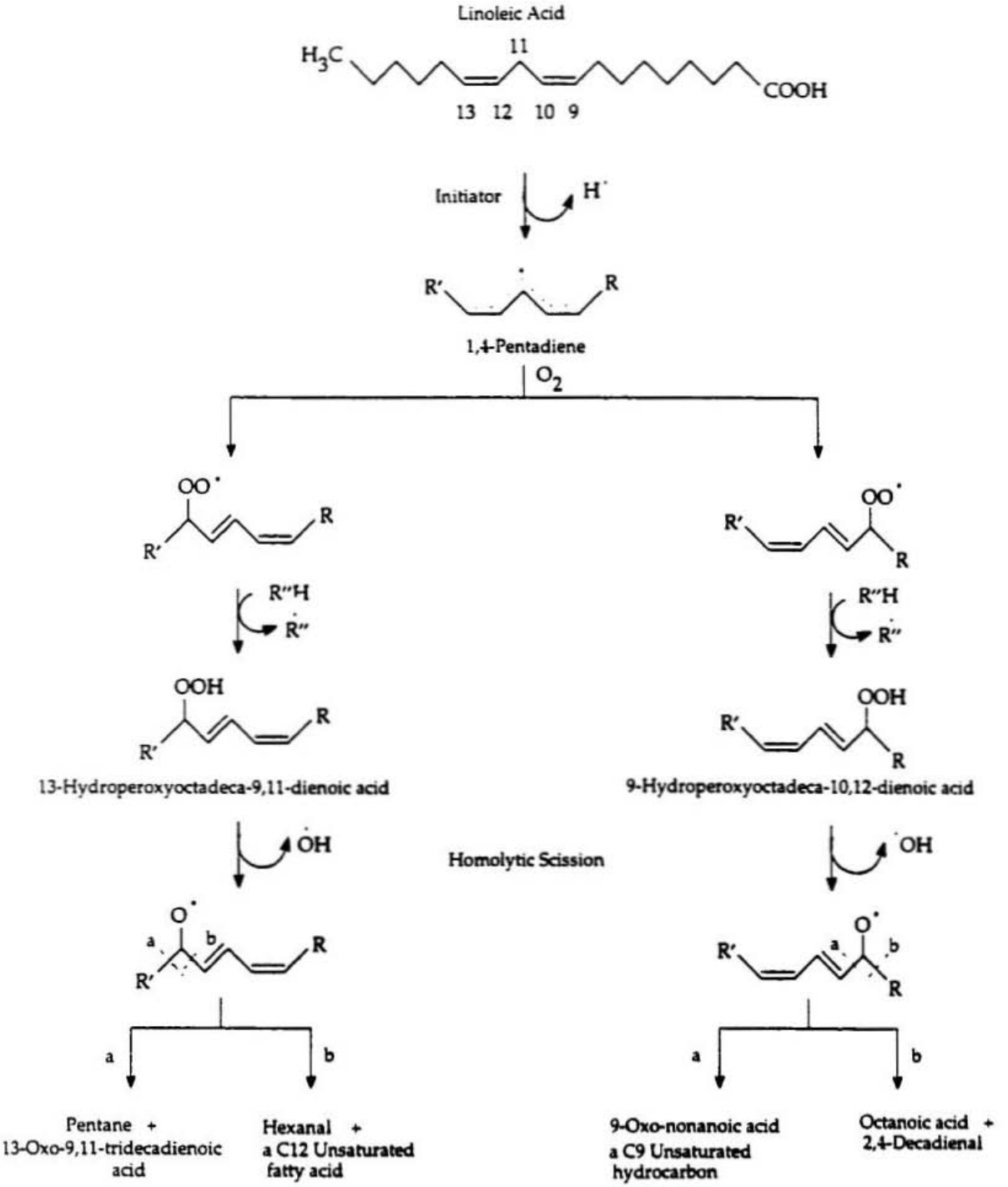

Docosahexaenoic Acid
21
18
15
12
9
6
3
COOH
H_3C
Initiator
H·
(H· abstraction from position-18)
O_2
OO·
RH
R·
OOH
20-Hydroperoxy-4,10,13,1,18-docosahexaenoic acid
·OH (Scission at the oxygen-oxygen bond)
O·
Alkoxy radical
Scission at the carbon-carbon bond
CH_3-CH_2-CHO
Propanal

Figure 4.8 Autoxidation of linoleic acid and production of hexanal


Linoleic Acid
H_3C
11
13 12 10 9
COOH
Initiator
H·
R'
R
1,4-Pentadiene
O_2
OO·
R"H
R"·
OOH
13-Hydroperoxyoctadeca-9,11-dienoic acid
9-Hydroperoxyoctadeca-10,12-dienoic acid
·OH
Homolytic Scission
O·
a
b
Pentane +
13-Oxo-9,11-tridecadienoic acid
Hexanal +
a C12 Unsaturated fatty acid
9-Oxo-nonanoic acid
a C9 Unsaturated hydrocarbon
Octanoic acid +
2,4-Decadienal

of foods and cell membrane lipids; many of the oxidized products of lipids, including peroxides, free radicals and aldehydes are toxic and mutagenic (Pearson *et al.*, 1983). The ease of non-enzymatic oxidation (autoxidation) of unsaturated fatty acids is proportional to the number of allylic methylene groups between double bonds; thus marine oils with a high content of polyunsaturated fatty acids are more prone to oxidation than CNO. Due to the presence of high proportions of PUFA in marine oils, protection of PUFA is essential to counterbalance any harmful effects which may arise as a result of their oxidation in order to take full advantage of their nutritional and health-related benefits related to their ω3 components.

4.3 Stabilization of refined-bleached and deodorized (RBD) seal blubber oil (SBO) and menhaden oil (MHO) with naturally occurring antioxidants

Oxidative deterioration of marine oils is known to have a significant effect on their nutritional value. Because of the presence of PUFA, marine oils are easily oxidized via a free radical chain mechanism. Therefore, in order to arrest free radical-induced oxidation, different antioxidants, both natural and synthetic, are incorporated into oils. In this study, antioxidant activity of dechlorophyllized green tea extracts (DGTE), individual tea catechins and flavonoids was compared with those of commonly used food-grade antioxidants such as α-tocopherol, BHA, BHT and TBHQ in SBO and MHO.

4.3.1 Stability of refined-bleached and deodorized (RBD) seal blubber oil (SBO) and menhaden oil (MHO) as affected by the addition of dechorophyllized green tea extracts (DGTE) and different flavonoids

The effect of DGTE at 100, 200, 500 and 1000 ppm, α-tocopherol at 500 ppm and BHA, BHT and TBHQ at 200 ppm, on weight gain of SBO and MHO during accelerated oxidation is presented in Figure 4.9. The time required to achieve a 0.5% weight increase for samples was 33, 40, 42, 50, 49, 66, 76 and 112 h for SBO containing α-tocopherol-500, DGTE-100, BHA-200, BHT-200, DGTE-200, DGTE-500, DGTE-1000 and TBHQ-200, respectively, as compared to a 24 h period for the control sample. The time for a 0.5% weight gain for treated MHO with the same antioxidants was 15, 20, 19, 22, 35, 42, 49 and 93 h, respectively, as compared to a 9 h period for the control sample. The weight gain data for SBO and MHO treated with different flavonoids as compared to α-tocopherol, BHA, BHT and TBHQ are shown in Figures 4.10 and 4.11, respectively. Flavonoid-treated samples of SBO and MHO showed a delayed induction period when compared to the control samples. The time required to achieve a 0.5% weight increase by SBO and MHO was 36 and 13 h for apigenin, 36 and 14 h for taxifolin, 40 and 19 h for naringin, 42 and 21 for naringenin, 45 and 21 h for rutin, 53 and 31 h for kaempferol, 56 and 36 h for quercetin, 62 and 29 h for morin, and 138 and 72 h for myricetin, respectively. It has been suggested that each storage day (24 h) under Schaal oven test conditions at 65°C is equivalent to one month of storage at ambient temperatures (Evans *et al.*, 1973).

Figure 4.9 Effect of dechlorophyllized green tea extracts (DGTE) and conventional antioxidants on weight gain data of refined-bleached and deodorized seal blubber oil (SBO) and menhaden oil (MHO) stored under Schaal oven conditions at 65°C. (deviation from mean for each data point is within ± 1% of the absolute values, n = 3)

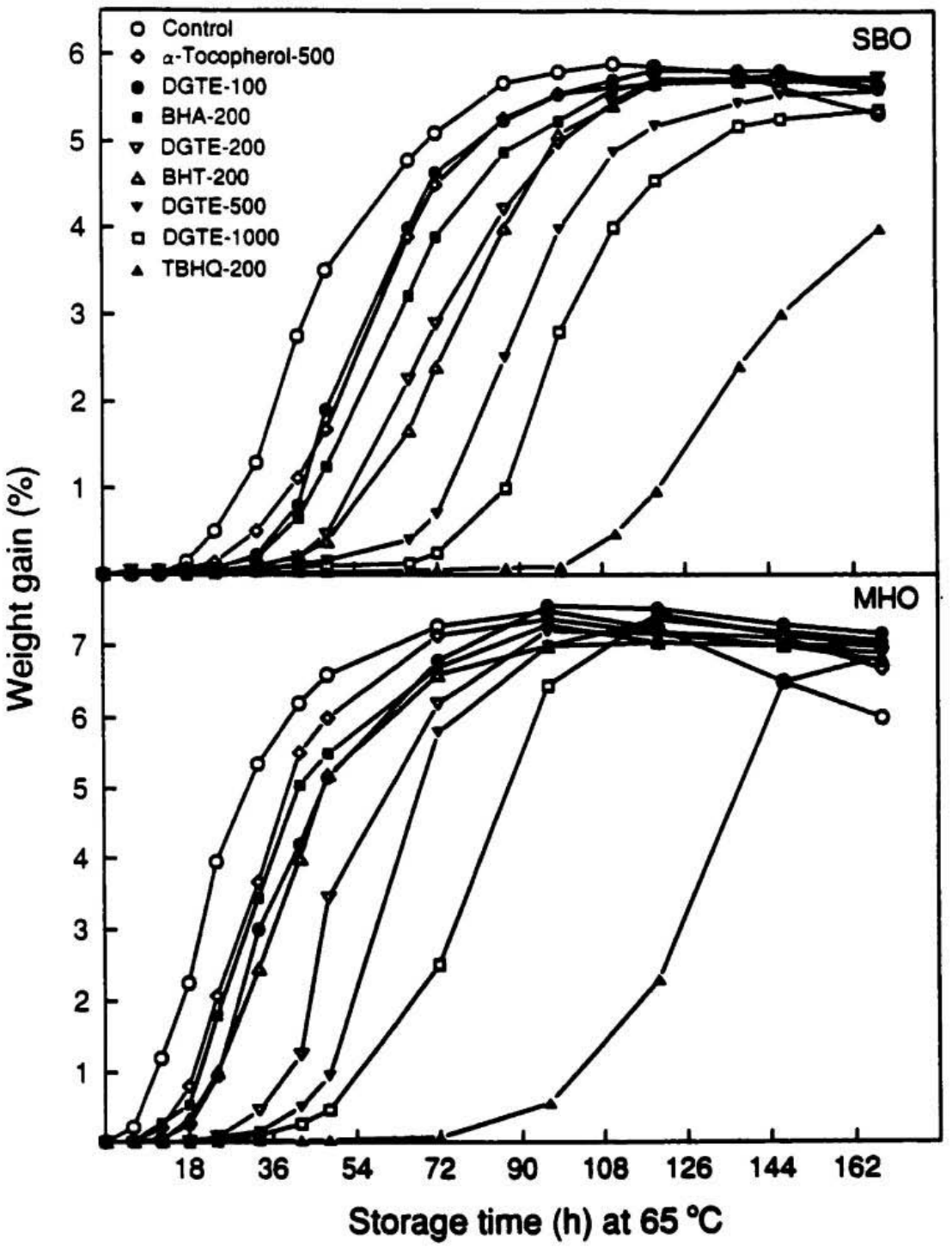

Control
α-Tocopherol-500
DGTE-100
BHA-200
DGTE-200
BHT-200
DGTE-500
DGTE-1000
TBHQ-200
SBO
MHO
Weight gain (%)
Storage time (h) at 65 °C
18
36
54
72
90
108
126
144
162

Figure 4.10 Effect of flavonoids and conventional antioxidants on weight gain data of refined-bleached and deodorized seal blubber oil (SBO) stored under Schaal oven conditions at 65°C, (deviation from mean for each data point is within ± 1% of the absolute values, n = 3)

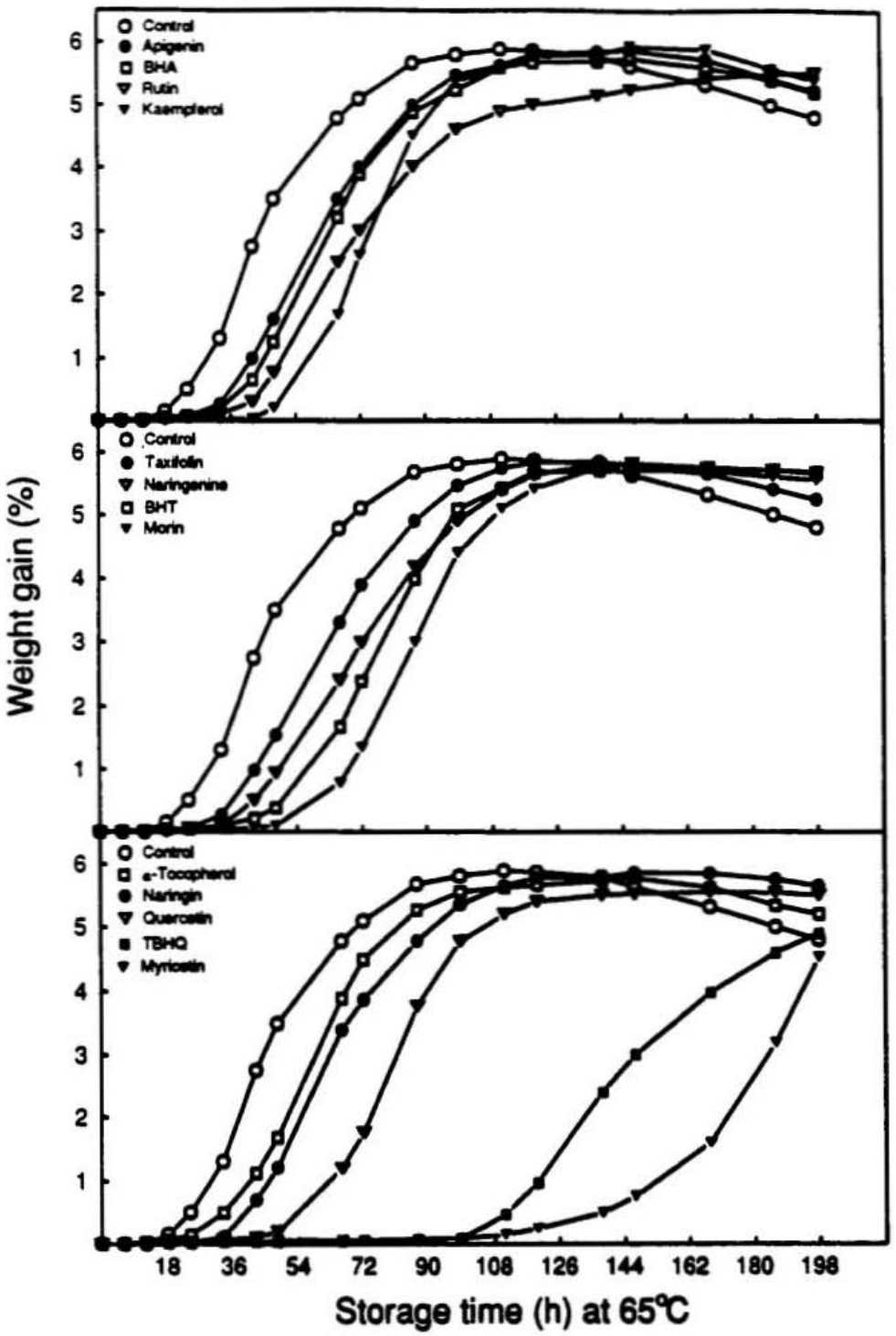
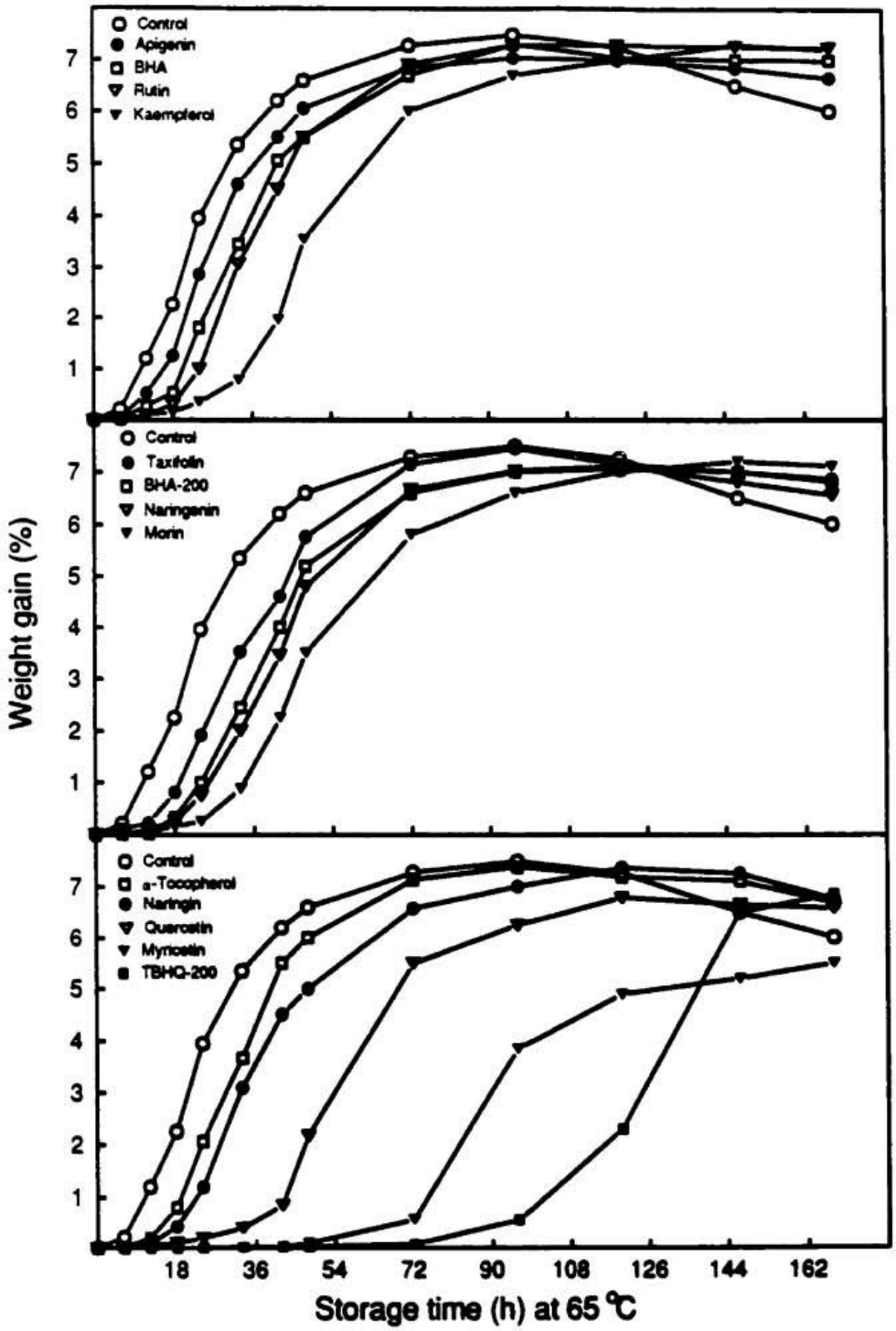

Control
Apigenin
BHA
Rutin
Kaempferol
Control
Taxifolin
Naringenine
BHT
Morin
Control
α-Tocopherol
Naringin
Quercetin
TBHQ
Myricetin
Weight gain (%)
1
2
3
4
5
6
18
36
54
72
90
108
126
144
162
180
198
Storage time (h) at 65℃

Figure 4.11 Effect of flavonoids and conventional antioxidants on weight gain of refined-bleached and deodorized menhaden oil (MHO) stored under Schaal oven conditions at 65°C, (deviation from mean for each data point is within ± 1% of the absolute values, n = 3)


Control
Apigenin
BHA
Rutin
Kaempferol
Control
Taxifolin
BHA-200
Naringenin
Morin
Control
α-Tocopherol
Naringin
Quercetin
Myricetin
TBHQ-200
Weight gain (%)
1
2
3
4
5
6
7
18
36
54
72
90
108
126
144
162
Storage time (h) at 65 ℃

The extension of the induction period of oils treated with DGTE-500, DGTE-1000 and TBHQ was 2.8, 3.2 and 4.7 times that of the control for SBO and 4.7, 5.4 and 10.3 times for MHO, respectively. Furthermore, SBO and MHO containing over 200 ppm of DGTE delayed the induction period more than that of α-tocopherol, BHA and BHT. Extension of the induction period by kaempferol, quercetin, morin and myricetin was 2.2, 2.3, 2.6 and 5.8 times that of the control for SBO and 3.4, 4.0, 3.2 and 8.0 times that of the control for MHO, respectively. Furthermore, while rutin, kaempferol, quercetin, morin and myricetin were more effective than α-tocopherol, BHA and BHT, myricetin was even more effective than TBHQ, the strongest synthetic antioxidant used by the food industry.

Peroxide values (PV) of SBO and MHO samples containing DGTE, flavonoids, α-tocopherol and synthetic antioxidants are presented in Tables 4.4, 4.5, 4.6 and 4.7. Addition of DGTE over 200 ppm to both SBO and MHO significantly ($P < 0.05$) decreased their PV during accelerated oxidation. However, samples treated with α-tocopherol, BHA and BHT showed higher PV as compared to DGTE-treated oils under similar conditions. These data indicate that an increase in the addition level of DGTE paralleled a decrease in the formation of hydroperoxides in both oils. For up to 144 h, the PV of the control SBO sample increased from 2.09 meq/kg (fresh oil) to 183 meq/kg (oxidized oil) and that of MHO increased from 4.53 meq/kg (fresh oil) to 348 meq/kg (oxidized oil). The corresponding values for oils treated with DGTE-500 and DGTE-1000 changed from 2.18 to 115 and from 2.21 to 85.0 meq/kg in SBO and

Table 4.4 Effect of dechlorophyllized green tea extracts (DGTE) and conventional antioxidants on peroxide values (meq/kg oil) of refined-bleached and deodorized seal blubber oil (SBO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	2.09 ± 0.06^{a}	25.2 ± 0.79^{a}	49.3 ± 1.22^{a}	119 ± 4.24^{a}	183 ± 2.34^{a}
DGTE-100	2.13 ± 0.04^{a}	13.2 ± 0.99^{c}	36.2 ± 0.85^{c}	90.0 ± 4.88^{c}	180 ± 7.14^{a}
DGTE-200	2.20 ± 0.14^{a}	11.7 ± 0.12^{cd}	29.2 ± 0.58^{d}	77.5 ± 5.21^{d}	172 ± 1.86^{ab}
DGTE-500	2.18 ± 0.05^{a}	8.92 ± 0.38^{de}	18.9 ± 0.66^{e}	64.6 ± 5.86^{ef}	115 ± 6.19^{e}
DGTE-1000	2.21 ± 0.11^{a}	8.23 ± 0.47^{e}	11.8 ± 1.48^{f}	40.7 ± 0.76^{gh}	85.0 ± 6.60^{d}
α-Tocopherol-500	2.18 ± 0.01^{a}	21.5 ± 2.05^{b}	44.5 ± 0.91^{b}	110 ± 0.80^{ab}	166 ± 1.23^{b}
BHA-200	2.19 ± 0.02^{a}	13.3 ± 1.27^{c}	42.6 ± 0.01^{b}	71.3 ± 1.38^{de}	124 ± 2.68^{c}
BHT-200	2.11 ± 0.06^{a}	13.3 ± 0.18^{c}	31.6 ± 0.42^{d}	58.8 ± 2.18^{f}	94.9 ± 2.11^{d}
TBHQ-200	2.09 ± 0.07^{a}	8.04 ± 0.24^{e}	10.4 ± 0.31^{f}	29.9 ± 0.86^{h}	53.4 ± 1.20^{e}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2]Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

Table 4.5 Effect of dechlorophyllized green tea extracts (DGTE) and conventional antioxidants on peroxide values (meq/kg oil) of refined-bleached and deodorized menhaden oil (MHO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	4.53 ± 0.41^{a}	38.1 ± 0.77^{a}	96.5 ± 1.91^{a}	283 ± 4.58^{a}	348 ± 5.82^{a}
DGTE-100	4.65 ± 0.23^{a}	37.9 ± 0.31^{a}	67.6 ± 2.00^{bc}	257 ± 5.82^{b}	280 ± 1.39^{b}
DGTE-200	4.45 ± 0.45^{a}	28.6 ± 0.24^{c}	53.9 ± 0.48^{c}	151 ± 1.32^{d}	166 ± 4.17^{e}
DGTE-500	4.80 ± 0.70^{a}	16.9 ± 1.31^{de}	41.1 ± 0.29^{f}	109 ± 1.67^{fg}	160 ± 1.80^{ed}
DGTE-1000	4.71 ± 0.01^{a}	16.0 ± 1.02^{de}	37.8 ± 0.98^{f}	89.7 ± 2.16^{g}	148 ± 7.70^{d}
α-Tocopherol-500	4.10 ± 0.12^{a}	37.3 ± 0.42^{a}	66.3 ± 0.50^{c}	227 ± 5.59^{c}	280 ± 6.10^{b}
BHA-200	4.27 ± 0.06^{a}	36.1 ± 1.44^{ab}	71.3 ± 0.94^{b}	135 ± 4.90^{de}	163 ± 3.60^{ed}
BHT-200	4.43 ± 0.01^{a}	32.9 ± 1.63^{b}	63.3 ± 1.18^{cd}	120 ± 0.82^{ef}	137 ± 2.70^{d}
TBHQ-200	4.22 ± 0.02^{a}	15.1 ± 1.30^{e}	15.1 ± 0.14^{g}	42.8 ± 1.57^{h}	101 ± 1.02^{e}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2]Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

Table 4.6 Effect of flavonoids and conventional antioxidants on peroxide values (meq/kg oil) of refined-bleached and deodorized seal blubber oil (SBO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	2.09 ± 0.06^{a}	25.2 ± 0.79^{a}	49.3 ± 1.22^{a}	119 ± 4.24^{a}	183 ± 2.34^{a}
Flavones:					
Apigenin	2.13 ± 0.09^{a}	12.9 ± 0.79^{bc}	36.3 ± 1.12^{c}	110 ± 1.05^{b}	169 ± 3.03^{ab}
Flavonols:					
Kaempferol	2.15 ± 0.01^{a}	13.6 ± 2.12^{b}	16.7 ± 0.60^{f}	37.6 ± 3.57^{ghi}	100 ± 1.36^{ef}
Morin	2.09 ± 0.01^{a}	12.1 ± 0.01^{bcd}	20.6 ± 0.73^{f}	43.6 ± 2.17^{fg}	109 ± 1.15^{e}
Myricetin	2.10 ± 0.00^{a}	8.23 ± 0.06^{d}	9.81 ± 0.71^{g}	34.5 ± 0.91^{hi}	64.1 ± 5.64^{g}
Quercetin	2.10 ± 0.01^{a}	9.56 ± 0.14^{cd}	12.0 ± 2.71^{g}	47.1 ± 2.42^{f}	92.6 ± 4.95^{f}
Rutin	2.12 ± 0.01^{a}	11.6 ± 0.69^{bcd}	25.4 ± 1.45^{e}	43.1 ± 0.08^{fgh}	129 ± 8.35^{cd}
Flavanones:					
Naringenin	2.14 ± 0.01^{a}	10.1 ± 0.11^{bcd}	18.4 ± 0.37^{f}	61.3 ± 1.65^{e}	136 ± 3.61^{cd}
Naringin	2.12 ± 0.03^{a}	11.6 ± 0.60^{bcd}	19.2 ± 1.44^{f}	72.3 ± 3.10^{d}	140 ± 1.83^{c}
Flavononols:					
Taxifolin	2.08 ± 0.01^{a}	22.1 ± 1.34^{a}	42.9 ± 1.00^{b}	81.1 ± 1.28^{c}	156 ± 2.58^{b}
α-Tocopherol-500	2.18 ± 0.01^{a}	21.5 ± 2.05^{a}	44.5 ± 0.91^{b}	110 ± 0.80^{b}	166 ± 1.23^{b}
BHA-200	2.19 ± 0.02^{a}	13.3 ± 1.27^{bc}	42.6 ± 0.01^{b}	71.3 ± 1.38^{d}	124 ± 2.68^{d}
BHT-200	2.11 ± 0.06^{a}	13.3 ± 0.18^{bc}	31.6 ± 0.42^{d}	58.8 ± 2.18^{e}	94.9 ± 2.11^{f}
TBHQ-200	2.09 ± 0.07^{a}	8.04 ± 0.24^{d}	10.4 ± 0.31^{g}	29.9 ± 0.86^{i}	53.4 ± 1.20^{g}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2]Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

Table 4.7 Effect of flavonoids and conventional antioxidants on peroxide values (meq/kg oil) of refined-bleached and deodorized menhaden oil (MHO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	4.53 ± 0.41^{a}	38.1 ± 0.77^{a}	96.5 ± 1.91^{a}	283 ± 4.58^{a}	348 ± 5.82^{a}
Flavones:					
Apigenin	4.21 ± 0.03^{a}	35.1 ± 0.50^{ab}	72.9 ± 0.99^{ab}	217 ± 1.36^{b}	274 ± 5.44^{c}
Flavonols:					
Kaempferol	4.17 ± 0.06^{a}	23.5 ± 0.63^{de}	54.3 ± 1.68^{gh}	111 ± 6.60^{d}	140 ± 1.68^{g}
Morin	4.58 ± 0.51^{a}	23.3 ± 0.13^{de}	36.4 ± 1.73^{i}	111 ± 3.75^{d}	138 ± 3.74^{g}
Myricetin	4.02 ± 0.01^{a}	10.4 ± 0.33^{g}	13.3 ± 0.41^{k}	42.3 ± 0.41^{e}	42.4 ± 2.23^{h}
Quercetin	4.57 ± 0.01^{a}	22.7 ± 0.74^{e}	51.7 ± 2.16^{h}	114 ± 4.47^{d}	142 ± 7.17^{fg}
Rutin	4.46 ± 0.63^{a}	23.2 ± 0.24^{de}	43.0 ± 1.19^{i}	115 ± 5.78^{d}	182 ± 7.25^{e}
Flavanones:					
Naringenin	4.36 ± 0.15^{a}	26.1 ± 0.01^{cd}	58.0 ± 1.33^{fg}	129 ± 4.01^{cd}	230 ± 7.14^{d}
Naringin	4.38 ± 0.28^{a}	28.2 ± 0.22^{c}	59.8 ± 2.32^{ef}	224 ± 5.04^{b}	229 ± 1.50^{d}
Flavononols:					
Taxifolin	4.46 ± 0.02^{a}	33.3 ± 1.12^{b}	57.5 ± 1.03^{fg}	206 ± 14.0^{b}	306 ± 9.01^{b}
α-Tocopherol-500	4.10 ± 0.12^{a}	37.3 ± 0.42^{a}	66.3 ± 0.50^{cd}	227 ± 5.59^{b}	280 ± 6.10^{c}
BHA-200	4.27 ± 0.06^{a}	36.1 ± 1.44^{ab}	71.3 ± 0.94^{bc}	135 ± 4.90^{c}	163 ± 3.60^{f}
BHT-200	4.43 ± 0.01^{a}	32.9 ± 1.63^{b}	63.3 ± 1.18^{de}	120 ± 2.82^{cd}	137 ± 2.70^{g}
TBHQ-200	4.22 ± 0.02^{a}	15.1 ± 1.30^{f}	15.1 ± 0.14^{k}	42.8 ± 1.57^{e}	101 ± 1.02^{h}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2]Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

from 4.80 to 160 and from 4.71 to 148 meq/kg in MHO, respectively. DGTE was quite effective at 1000 ppm and gave a much lower PV than the control, α-tocopherol, BHA, BHT and other DGTE levels in both oils. However, among these treatments, TBHQ remained the most effective and gave the lowest PV in both SBO and MHO.

Addition of flavonoids to SBO and MHO reduced their PV compared to those of the control samples (Tables 4.6 and 4.7). Among flavonoids used, flavonols were most effective in lowering PV in both oils followed by flavanones, flavones and flavononols. All flavonols (kaempferol, morin, myricetin, quercetin and rutin) tested were able to reduce PV by approximately 50% during the storage of SBO. Myricetin was the only flavonoid that was effective in reducing PV of MHO by 50%. Flavanones such as naringenin and naringin were also effective in reducing PV. Addition of naringenin to SBO inhibited formation of hydroperoxides by 59.9, 62.6, 48.4 and 25.0% after 24, 48, 84 and 144 h of storage, respectively, and its inhibitory effect in MHO was 31.4, 40.0, 54.3 and 33.7%, respectively. Among the flavonoids tested, the best inhibition of hydroperoxide formation was conferred by myricetin in both oils; the values were 67.4, 80.0, 71.0 and 65.0% for SBO and 72.8, 86.2, 85.0 and 87.8% for MHO after 24, 48, 84 and 144 h of storage, respectively.

Addition of DGTE, flavonoids, α-tocopherol and other synthetic antioxidants to both SBO and MHO had a significant ($P < 0.05$) effect in lowering the formation of TBARS as compared to the control samples (Tables 4.8, 4.9, 4.10 and 4.11). Among these additives, TBHQ was most effective in retarding TBARS formation at 200 ppm.

Table 4.8 Effect of dechlorophyllized green tea extracts (DGTE) and conventional antioxidants on 2-thiobarbituric acid reactive substances (TBARS) values (μmol/g oil) of refined-bleached and deodorized seal blubber oil (SBO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	4.19 ± 0.01^{a}	8.78 ± 0.23^{a}	12.3 ± 0.37^{a}	19.4 ± 0.54^{a}	24.7 ± 0.20^{a}
DGTE-100	4.96 ± 0.91^{a}	6.60 ± 0.09^{bc}	8.24 ± 0.12^{b}	15.3 ± 0.05^{b}	21.6 ± 0.53^{bc}
DGTE-200	4.45 ± 0.28^{a}	4.87 ± 0.10^{e}	8.24 ± 0.60^{b}	12.5 ± 0.17^{cd}	20.1 ± 0.12^{cd}
DGTE-500	4.37 ± 0.25^{a}	4.82 ± 0.15^{e}	6.39 ± 0.20^{c}	10.2 ± 0.28^{de}	18.2 ± 0.54^{de}
DGTE-1000	4.19 ± 0.02^{a}	4.46 ± 0.04^{de}	6.14 ± 0.13^{c}	9.82 ± 0.29^{e}	14.9 ± 0.87^{f}
α-Tocopherol-500	4.32 ± 0.14^{a}	7.67 ± 0.47^{ab}	11.8 ± 0.06^{a}	13.8 ± 0.64^{bc}	22.9 ± 0.20^{ab}
BHA-200	4.55 ± 0.34^{a}	6.89 ± 0.27^{bc}	8.97 ± 0.50^{b}	14.8 ± 0.11^{bc}	20.0 ± 0.32^{cd}
BHT-200	4.59 ± 0.46^{a}	6.35 ± 0.39^{cd}	7.80 ± 0.11^{b}	10.6 ± 0.27^{de}	16.8 ± 0.97^{ef}
TBHQ-200	4.54 ± 0.62^{a}	4.53 ± 0.06^{e}	5.50 ± 0.42^{c}	7.07 ± 0.14^{f}	10.5 ± 0.28^{g}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2]Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

Table 4.9 Effect of dechlorophyllized green tea extracts (DGTE) and conventional antioxidants on 2-thiobarbituric acid reactive substances (TBARS) values (μmol/g oil) of refined-bleached and deodorized menhaden oil (MHO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	7.65 ± 0.49[a]	17.8 ± 0.36[a]	25.8 ± 1.11[a]	42.3 ± 0.41[a]	45.2 ± 3.03[a]
DGTE-100	7.63 ± 0.33[a]	12.6 ± 0.58[cd]	21.8 ± 1.15[bc]	30.9 ± 0.78[b]	31.9 ± 0.48[cd]
DGTE-200	7.61 ± 0.26[a]	12.4 ± 0.35[cd]	19.9 ± 0.17[c]	26.1 ± 2.48[c]	27.2 ± 0.48[ef]
DGTE-500	7.42 ± 0.31[a]	11.8 ± 0.68[de]	17.2 ± 1.59[d]	20.0 ± 0.66[d]	24.5 ± 1.16[ef]
DGTE-1000	7.18 ± 0.14[a]	11.5 ± 0.45[de]	16.1 ± 1.14[d]	18.3 ± 0.33[d]	23.3 ± 0.33[gh]
α-Tocopherol-500	6.95 ± 0.06[a]	15.0 ± 0.18[b]	25.5 ± 0.21[a]	33.4 ± 0.78[b]	36.7 ± 2.21[b]
BHA-200	7.06 ± 0.01[a]	14.4 ± 0.36[b]	23.0 ± 0.45[b]	25.4 ± 0.54[c]	31.1 ± 1.61[cd]
BHT-200	7.43 ± 0.02[a]	12.5 ± 0.59[cd]	21.7 ± 0.12[bc]	24.2 ± 0.57[c]	28.4 ± 0.80[de]
TBHQ-200	7.61 ± 0.14[a]	10.5 ± 0.42[e]	13.1 ± 0.13[e]	17.3 ± 1.57[d]	20.5 ± 0.48[h]

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2]Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

Table 4.10 Effect of flavonoids and conventional antioxidants on 2-thiobarbituric acid reactive substances (TBARS) values (μmol/g oil) of refined-bleached and deodorized seal blubber oil (SBO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	4.19 ± 0.01^{a}	8.78 ± 0.23^{a}	12.3 ± 0.37^{a}	19.4 ± 0.54^{a}	24.7 ± 0.20^{a}
Flavones:					
Apigenin	4.38 ± 0.16^{a}	5.72 ± 0.56^{def}	9.52 ± 0.23^{bc}	14.9 ± 0.12^{bc}	22.5 ± 0.16^{b}
Flavonols:					
Kaempferol	4.50 ± 0.03^{a}	5.03 ± 0.04^{efg}	8.48 ± 0.08^{cde}	14.6 ± 0.49^{bcd}	19.2 ± 0.04^{cd}
Morin	4.43 ± 0.08^{a}	6.10 ± 0.02^{cde}	7.44 ± 0.54^{e}	11.4 ± 0.22^{efg}	17.5 ± 0.47^{de}
Myricetin	4.26 ± 0.05^{a}	4.53 ± 0.35^{fg}	5.59 ± 0.31^{f}	9.66 ± 0.32^{g}	11.3 ± 0.27^{f}
Quercetin	4.39 ± 0.23^{a}	5.36 ± 0.11^{efg}	7.36 ± 0.01^{e}	12.8 ± 0.79^{cde}	18.8 ± 0.28^{cd}
Rutin	4.30 ± 0.08^{a}	5.23 ± 0.13^{efg}	7.51 ± 0.27^{e}	12.5 ± 0.97^{def}	16.4 ± 0.04^{e}
Flavanones:					
Naringenin	4.27 ± 0.07^{a}	5.63 ± 0.19^{def}	9.49 ± 0.13^{bc}	14.0 ± 0.11^{bcd}	17.8 ± 0.44^{de}
Naringin	4.43 ± 0.02^{a}	6.11 ± 0.75^{cde}	8.54 ± 0.35^{cde}	14.6 ± 0.22^{bcd}	19.7 ± 0.53^{c}
Flavononols:					
Taxifolin	4.37 ± 0.10^{a}	7.15 ± 0.33^{bc}	9.92 ± 0.16^{b}	15.6 ± 0.45^{b}	21.9 ± 1.05^{b}
α-Tocopherol-500	4.32 ± 0.14^{a}	7.67 ± 0.47^{ab}	11.8 ± 0.06^{a}	13.8 ± 0.64^{cd}	22.9 ± 0.20^{ab}
BHA-200	4.55 ± 0.34^{a}	6.89 ± 0.27^{bcd}	8.97 ± 0.50^{bcd}	14.8 ± 0.11^{bc}	20.0 ± 0.32^{c}
BHT-200	4.59 ± 0.46^{a}	6.35 ± 0.39^{bcde}	7.80 ± 0.11^{de}	10.6 ± 0.27^{fg}	16.8 ± 0.97^{e}
TBHQ-200	4.54 ± 0.62^{a}	4.53 ± 0.06^{g}	5.50 ± 0.42^{f}	7.07 ± 0.14^{h}	10.5 ± 0.28^{f}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2]Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

Table 4.11 Effect of flavonoids and conventional antioxidants on 2-thiobarbituric acid reactive substances (TBARS) values (μmol/g oil) of refined-bleached and deodorized menhaden oil (MHO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	7.65 ± 0.49^{a}	17.8 ± 0.36^{a}	25.8 ± 1.11^{a}	42.3 ± 0.41^{a}	45.2 ± 3.03^{a}
Flavones:					
Apigenin	6.99 ± 0.02^{a}	13.3 ± 0.02^{bc}	23.8 ± 0.22^{abc}	37.1 ± 2.00^{ab}	35.9 ± 0.68^{bcd}
Flavonols:					
Kaempferol	6.99 ± 0.01^{a}	10.7 ± 0.07^{cde}	18.1 ± 0.05^{de}	28.6 ± 2.95^{def}	34.7 ± 1.60^{bcdef}
Morin	7.29 ± 0.35^{a}	10.6 ± 0.21^{cde}	18.7 ± 1.10^{de}	23.9 ± 1.00^{fg}	29.5 ± 0.18^{defg}
Myricetin	6.99 ± 0.01^{a}	10.0 ± 0.01^{e}	11.9 ± 0.19^{g}	21.0 ± 0.29^{gh}	23.4 ± 1.71^{gh}
Quercetin	7.29 ± 0.35^{a}	10.7 ± 0.03^{cde}	16.1 ± 0.98^{ef}	24.6 ± 0.67^{fg}	28.2 ± 0.23^{fg}
Rutin	7.11 ± 0.05^{a}	11.0 ± 0.02^{cde}	16.3 ± 1.69^{ef}	26.0 ± 0.60^{efg}	32.5 ± 1.00^{bcdef}
Flavanones:					
Naringenin	7.08 ± 0.12^{a}	12.3 ± 1.72^{bcde}	19.9 ± 1.49^{cde}	30.7 ± 1.80^{cde}	35.4 ± 0.21^{bcde}
Naringin	7.03 ± 0.02^{a}	12.9 ± 0.86^{bcd}	20.8 ± 2.14^{cd}	36.8 ± 2.25^{b}	37.0 ± 3.87^{bc}
Flavononols:					
Taxifolin	7.10 ± 0.07^{a}	13.9 ± 1.45^{b}	18.4 ± 0.12^{cd}	34.3 ± 0.99^{bc}	38.9 ± 3.77^{ab}
α-Tocopherol-500	6.95 ± 0.06^{a}	15.0 ± 0.18^{b}	25.5 ± 1.21^{ab}	33.4 ± 0.78^{bcd}	36.7 ± 2.21^{bc}
BHA-200	7.06 ± 0.01^{a}	14.4 ± 0.36^{b}	23.0 ± 0.45^{abc}	25.4 ± 0.54^{efg}	31.1 ± 1.61^{cdef}
BHT-200	7.43 ± 0.02^{a}	12.5 ± 0.59^{bcde}	21.7 ± 0.12^{bcd}	24.2 ± 0.57^{fg}	28.4 ± 0.80^{efg}
TBHQ-200	7.61 ± 0.14^{a}	10.5 ± 0.42^{de}	13.1 ± 0.13^{fg}	17.3 ± 1.57^{h}	20.5 ± 0.48^{h}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2]Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

The ability of TBHQ to lower TBARS values of stored marine oils has been reported for whale oil (Chihine and MacNell, 1974), mackerel (Ke *et al.*, 1977b) and capelin oil (Kaitaranta, 1992). The effect of DGTE (at > 200 ppm) was equivalent or slightly better than that of BHA and BHT in reducing TBARS in both SBO and MHO. At levels of 500 and 1000 ppm, DGTE was able to lower TBARS values more effectively than α-tocopherol, BHA and BHT even after 144 h storage at 65°C. Percentage inhibition of TBARS formation of DGTE-1000-treated samples was 49, 50, 49 and 40 for SBO and 36, 38, 57 and 49 for MHO after 24, 48, 84 and 144 h of storage, respectively; inhibition values for BHA and BHT-treated samples were 21 and 28, 27 and 37, 23 and 46, and 19 and 32 for SBO and 19 and 30, 11 and 16, 40 and 43, and 31 and 37, for MHO, respectively. In general, flavonols and flavanones were more active than flavones and flavononols in inhibiting TBARS formation in marine oils. Among the flavonoids tested, morin, myricetin and quercetin were able to inhibit TBARS formation by >30% for up to 84 h of storage in both oils (Tables 4.10 and 4.11). All flavonoids tested exhibited better antioxidant activity than α-tocopherol for up to 84 h, however, the antioxidant activity of apigenin and taxifolin was lower than that of α-tocopherol in both oils after 84 h of storage. Myricetin was the most effective flavonoid and its effect in reducing TBARS values was similar to that of TBHQ throughout the storage period of both oils. The overall order of potency of flavonoids on inhibition of TBARS formation was:

Myricetin > quercetin > morin > rutin > kaempferol > naringenin > naringin > apigenin > taxifolin.

TBARS measure the secondary lipid oxidation products, mainly aldehydes (or carbonyls) that contribute to off-flavours in oxidized oils. Results of this study indicate that DGTE and some of the flavonoids tested have a marked effect on the inhibition of formation of TBARS in both SBO and MHO. The effect of DGTE (at > 200 ppm) in suppressing TBARS formation was better than α-tocopherol (500 ppm), BHA (200 ppm) and BHT (200 ppm), but less than that of TBHQ (200 ppm). Effectiveness of myricetin was similar to that of TBHQ.

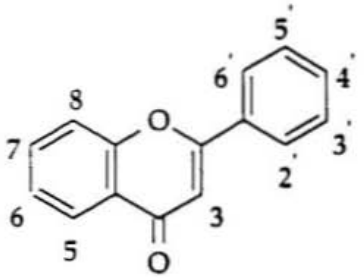
The antioxidant activity of DGTE is evidenced by weight gain, peroxide and TBARS values for both SBO and MHO. Effectiveness of DGTE at 500 and 1000 ppm levels was superior to that of α-tocopherol at 500 ppm and BHA and BHT at 200 ppm, but less than that of TBHQ at 200 ppm. These findings lend further support to previous literature reports that tocopherols, BHA and BHT are not as effective as TBHQ in stabilizing marine oils (Ke *et al.*, 1983; Kaitaranta, 1992; Nieto *et al.*, 1993). Apart from being a stronger antioxidant, DGTE did not impart any visible colour or perceivable odour change to the treated SBO or MHO. Thus, use of dechlorophyllized green tea extracts as a natural source of antioxidant for highly unsaturated marine oils is possible.

Previous literature is replete with results on studies that used extracts from natural sources with proven antioxidant activity, especially in vegetable oils and other

lipid model systems. These extracts have been reported to be more effective in many instances than commonly used synthetic antioxidants. Methanolic extracts of oregano, dittany, thyme, marjoram, spearmint, lavender and basil have shown strong antioxidative effect in lard (Economou *et al.*, 1991). Chevolleau *et al.* (1992) have shown strong antioxidant activity for methanolic extracts obtained from sixteen Mediterranean plant leaves in a β-carotene-linoleate model system. Most of these plant antioxidative compounds are of phenolic nature. However, only a few reports are available on antioxidant activity of green tea extracts in lipid systems. Hara (1994) has evaluated the antioxidative potency of crude extracts of green tea in lard and found that the antioxidant activity of the extract is better than than of α-tocopherol and BHA. It is well known that green tea leaves have a high content of catechins. Dechlorophyllized green tea extracts used in this study had 63.1 ± 1.68% total phenolics, expressed as (+)catechin equivalents. Therefore, the presence of high amounts of phenolic catechins in the extract may contribute to its high antioxidant activity when compared to α-tocopherol, BHA and BHT.

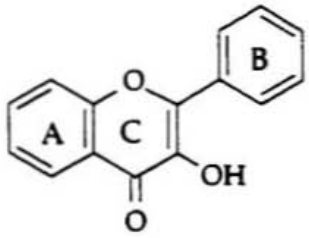
Among the flavonoids tested in this study, the flavonols namely, myricetin, morin and quercetin (Figure 4.12) had the strongest antioxidant activity and effectively stabilized both SBO and MHO. The flavonones such as naringenin and naringin (Figure 4.12) were also effective, but were less potent than those of flavonols. Flavonoids may act as primary antioxidants by donating a hydrogen atom to the peroxy radicals derived from oxidizing fatty acids (Torel *et al.*, 1986) and may also function

Figure 4.12 Chemical structures of some flavonoids and resonance stabilization of their free radicals



Flavones

Apigenin: 5,7,4', tri-OH-flavone



Flavonols

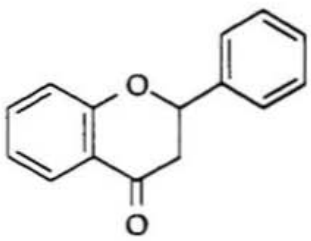
Kaempferol: 3,5,7,4', tetra-OH-flavonol

Quercetin: 3,5,7,3',4', penta-OH-flavonol

Myricetin: 3,5,7,3',4',5', hexa-OH-flavonol

Morin: 3,5,7,2',4', penta-OH-flavonol

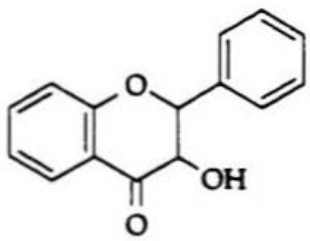
Rutin: 3-O-rutinose,5,7,3',4', tetra-OH-flavonol



Flavanones

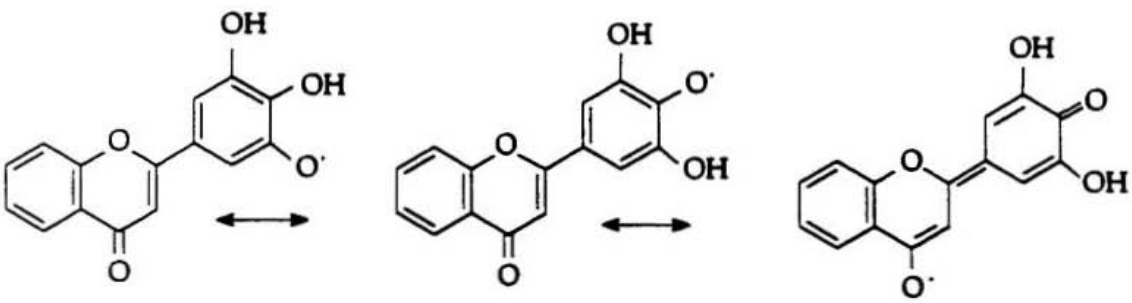
Naringinin: 5,7,4', tri-OH-flavanone

Naringin: 7-rhamnoglucose,5,4',di-OH-flavanone



Flavanonols

Taxifolin: 3,5,7,3',4', penta-OH-flavanonol



as free radical acceptors or chain breakers as well as possibly serving as metal chelators (Hudson and Lewis, 1983). Larson (1988) has reported that quercetin and some flavonoids may act as potent quenchers of singlet oxygen.

The antioxidative activity of flavonoids is generally governed by their chemical structures (Shahidi *et al.*, 1991a). All flavonoids possessing a 3′, 4′-dihydroxy (B-ring) configuration have antioxidant activity. Myricetin with an additional hydroxy group at the 5′ position showed a better antioxidant activity than its corresponding flavonols devoid of a 5′-hydroxy group, i.e., quercetin (Figure 4.12). Naringenin and narigin with a single hydroxy group on the B-ring possessed only slight antioxidative activity. Therefore, hydroxylation of the B-ring is a major consideration for antioxidant activity of flavonoids. Rutin with an etherified sugar moiety at carbon 3 of the C-ring showed a lower antioxidant activity than its deglycosilated counterpart, namely quercetin. Therefore, glycosyl substitution of flavonoids reduces their antioxidant activity, because of their inability to donate a hydrogen atom to lipid free radicals. The presence of multiple hydroxy groups on the B-ring provides an advantage since their corresponding phenoxy radicals are stabilized by delocalization of the unpaired electrons around the aromatic rings as indicated by the valence isomers (Figure 4.12). Among the flavonoids tested, myricetin with the largest number of hydroxyl groups was the most effective flavonoid in inhibiting oxidation of both oils. This gains further support for the findings of Das and Pereira (1990) for palm oil, Ramanathan and Das (1992) for ground fish and Wanasundara and Shahidi (1994a) for canola oil. Our consumption of

food flavonoids amounts to 1 g/day (Kühnau, 1976) and these have been considered as important food components since antiquity (Pokorny, 1991). In addition, food-derived flavonoids such as quercetin, kaempferol and myricetin have been shown to have anticarcinogenic and antimutagenic effects as evaluated in both *in vivo* and *in vitro* studies (Kato *et al.*, 1983; Huang *et al.*, 1983; Mukhtar *et al.*, 1988; Francis *et al.*, 1989; Deschner *et al.*, 1991).

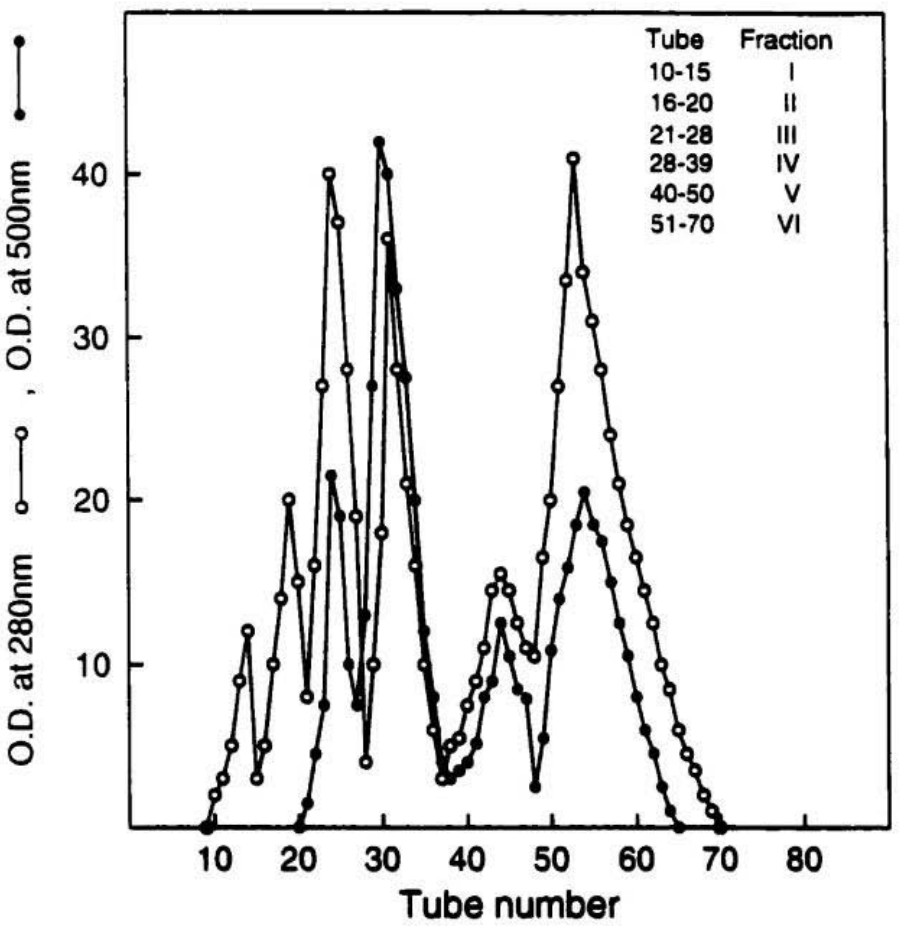
4.3.2 Isolation of individual catechins from dechlorophyllized green tea extracts (DGTE)

Since DGTE exhibited a strong antioxidative effect in SBO and MHO, isolation of individual tea catechins, namely (-)epicatechin (EC), (-)epigallocatechin (EGC), (-) epigallocatechin gallate (EGCG) and (-)epicatechin gallate (ECG) from DGTE was successfully carried out. Individual catechins were then evaluated for their antioxidative activity in SBO and MHO.

4.3.2.1 Fractionation of dechlorophyllized green tea extracts (DGTE)

Fractionation of DGTE was carried out according to their molecular weight and polarity using Sephadex LH-20 column chromatography. Sephadex LH-20 is regarded as an efficient medium for separation of plant phenolics (Amarowicz *et al.*, 1992). The UV absorbance (at 280 nm) of different fractions of DGTE and the content of their phenolic compounds are presented in Figure 4.13. Peaks six and four were clearly

Figure 4.13 Eluates following Sephadex LH-20 column chromatography: UV absorbance of phenolics prior to colour development (280 nm) and after colour development (500 nm)


Tube Fraction
10-15 I
16-20 II
21-28 III
28-39 IV
40-50 V
51-70 VI
O.D. at 280nm —o— , O.D. at 500nm —•—
10
20
30
40
10 20 30 40 50 60 70 80
Tube number

defined according to the absorbance values at 280 nm and their phenolic contents, respectively. Based on these data, fractions of DGTE following Sephadex LH-20 column chromatography were pooled into six major fractions (I, II, III, IV, V and VI) (Figure 4.13). Figure 4.14 shows the amount of each major fraction recovered following column chromatography of DGTE. The percentage recovery was 3.0, 5.4, 15.0, 19.0, 5.8 and 37.0 for fractions I, II, III, IV, V and VI, respectively. In order to characterize compounds present in these fractions, thin layer chromatography was carried out. Compound characterized by vanillin-HCl spray showed the presence of catechins in fractions II, III, IV, V and VI (Figure 4.15) by displaying a red colour upon spraying (Price *et al.*, 1978; Price and Spitzer, 1993). The red band from fraction II was weak. In addition, two green and two yellow bands were visualized in this fraction. Two less polar green compounds were also observed near the solvent front for fractions I and II. Strong red bands of catechins were noted in fractions III, IV, V and VI and these exhibited different R_f values. Therefore, fractions III, IV, V and VI were used to isolate individual tea catechins by a semi-preparative high performance liquid chromatography.

4.3.2.2 Isolation of individual tea catechins by semi-preparative high performance liquid chromatography (HPLC)

The semi-preparative HPLC chromatograms of fractions III, IV, V and VI, shown in Figure 4.16, exhibited one main peak in each fraction. Retention times of

Figure 4.14 Scheme for separation of individual catechins: (-)epicatechin (EC), (-) epigallocatechin (EGC), (-)epicatechin gallate (ECG) and (-) epigallocatechin gallate (EGCG)

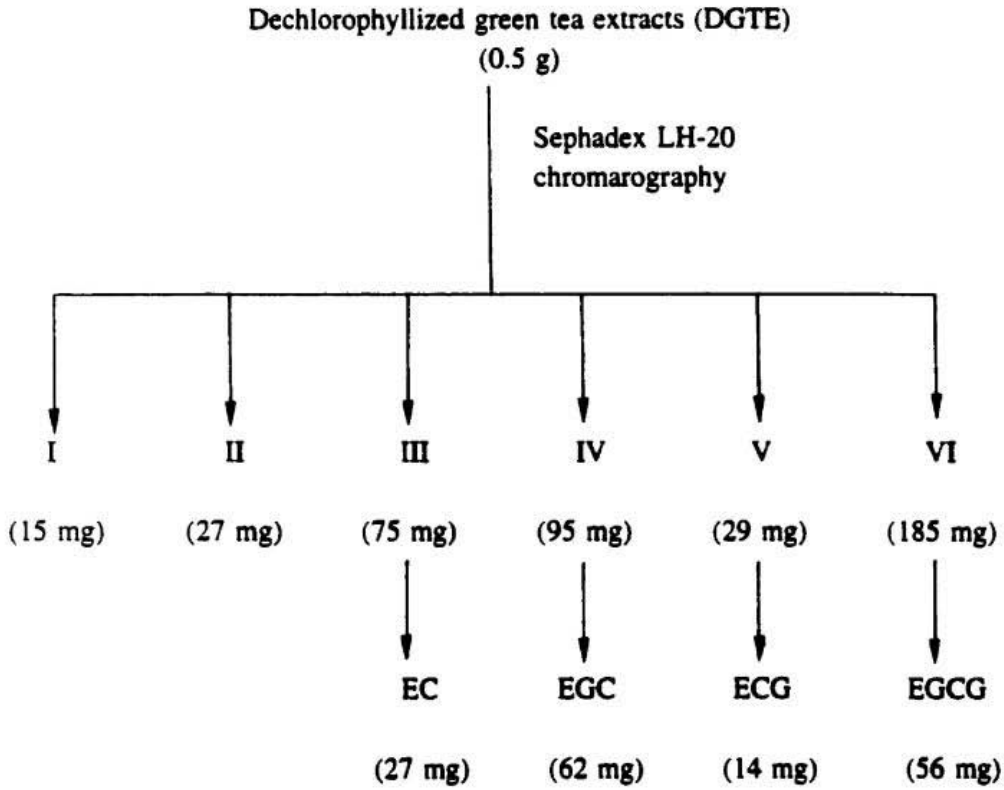

Dechlorophyllized green tea extracts (DGTE)
(0.5 g)
Sephadex LH-20
chromarography
I
II
III
IV
V
VI
(15 mg)
(27 mg)
(75 mg)
(95 mg)
(29 mg)
(185 mg)
EC
EGC
ECG
EGCG
(27 mg)
(62 mg)
(14 mg)
(56 mg)

Figure 4.15 TLC chromatogram of fractions of DGTE separated on a Sephadex LH-20 column

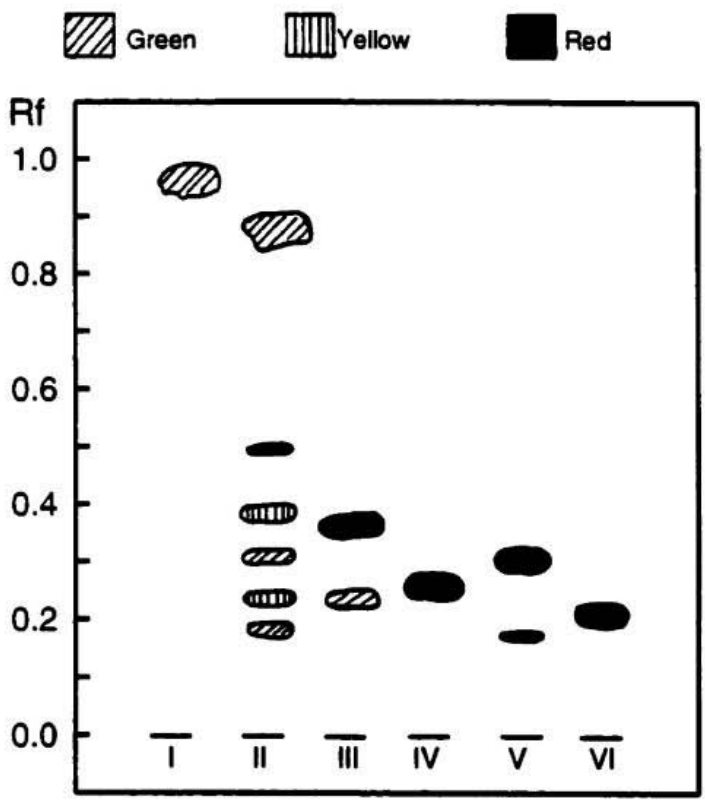
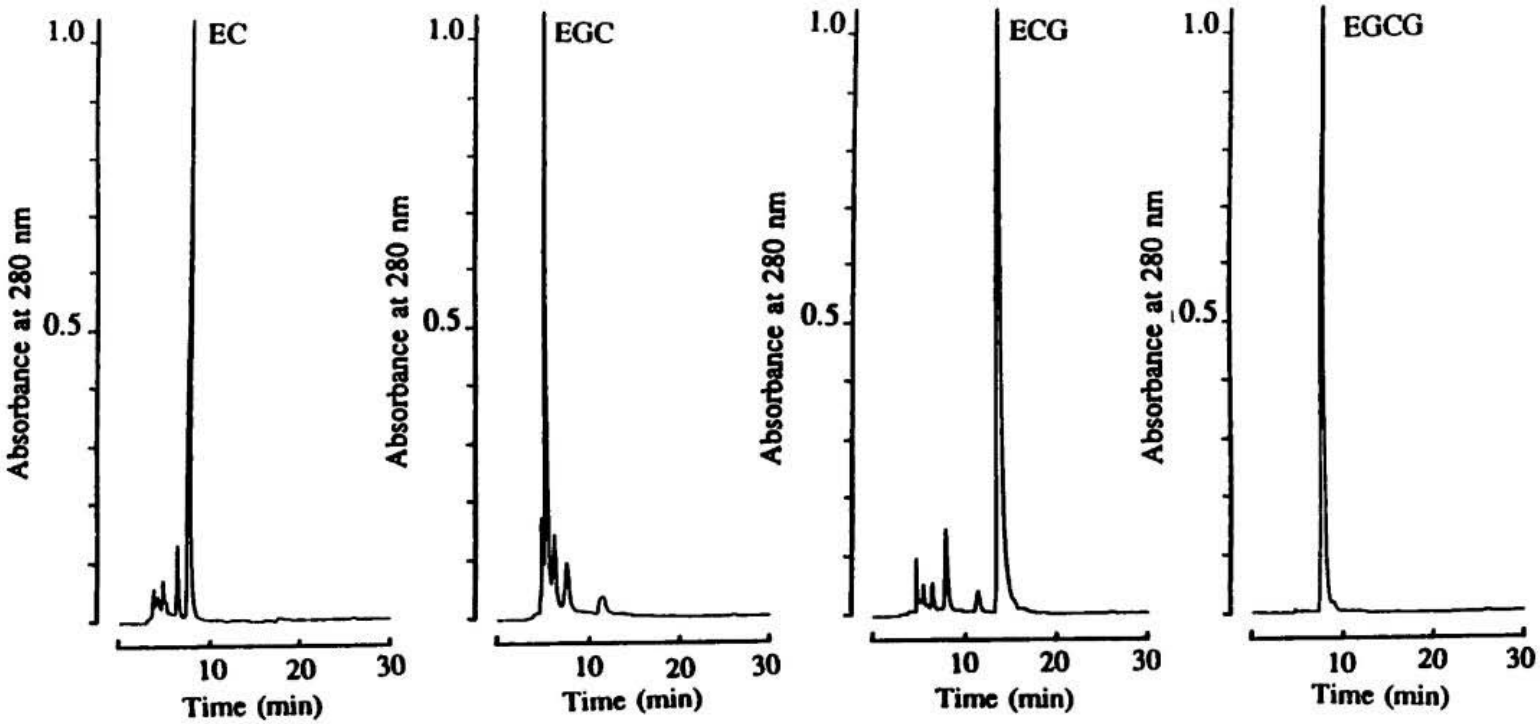

Green
Yellow
Red
Rf
1.0
0.8
0.6
0.4
0.2
0.0
I
II
III
IV
V
VI

Figure 4.16 Semi-preparative HPLC chromatograms of fractions DGTE separated on a Sephadex LH-20 column


EC
EGC
ECG
EGCG
1.0
0.5
Absorbance at 280 nm
10
20
30
Time (min)

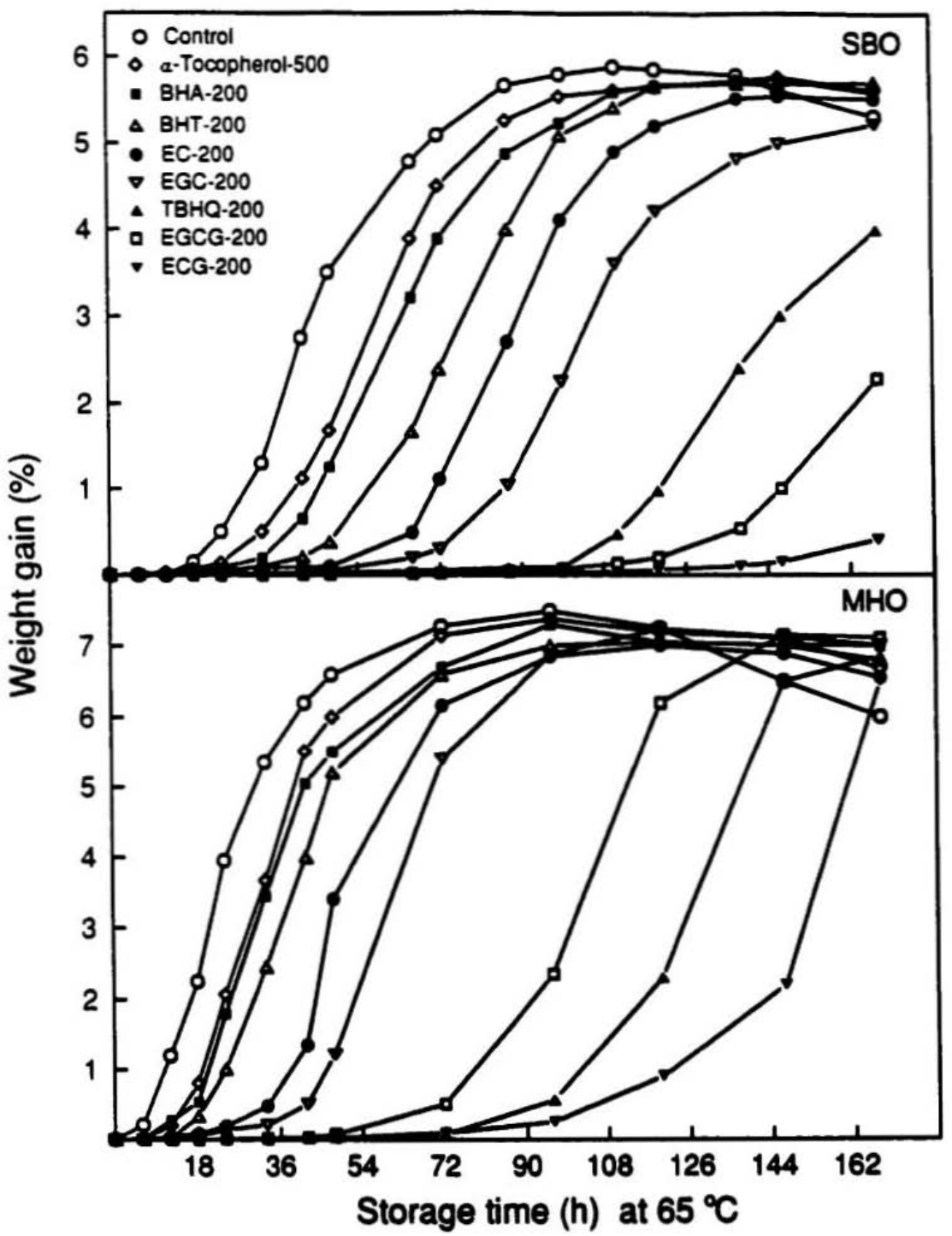
these peaks were 9.10 (fraction III), 8.21 (fraction IV), 14.9 (fraction V) and 9.82 min (fraction VI). These peaks were identified as (-)epicatechin (EC), (-)epigallocatechin (EGC), (-)epicatechin gallate (ECG) and (-)epigallocatechin gallate (EGCG), respectively, by comparison with reference standards. The content of individual catechins isolated from each fraction is shown in Figure 4.14. The amounts were: EC, 36%; EGC, 65%; ECG, 48%; and EGCG, 30% from fractions III, IV, V and VI, respectively.

4.3.3 Stability of refined-bleached and deodorized (RBD) seal blubber oil (SBO) and menhaden oil (MHO) as affected by the addition of individual tea catechins

The antioxidant activity of individual catechins isolated was compared with those of α-tocopherol, BHA, BHT and TBHQ in RBD-SBO and RBD-MHO. Similar to previous studies, testings were carried out under Schaal oven test conditions at 65°C.

Weight gain data of treated oils, stored for a 162 h period, are presented in Figures 4.17. The time required to achieve a 0.5% weight increase for samples was 33, 42, 49, 70, 76, 112, 137 and 170 h for SBO oil containing α-tocopherol, BHA, BHT, EC, EGC, TBHQ, EGCG and ECG, respectively. The corresponding values for MHO treated with the same antioxidants were 15, 19, 22, 35, 42, 93, 72 and 112 h, respectively. The extension of the induction period of oils treated with tea catechins was much longer than those treated with α-tocopherol, BHA or BHT. Furthermore, EGCG and ECG extended the induction period of SBO by 5.7 and 7.1 times that of the

Figure 4.17 Effect of tea catechins and conventional antioxidants on weight gain of refined-bleached and deodorized seal blubber oil (SBO) and menhaden oil (MHO) stored under Schaal oven conditions at 65°C, (weight increase variations are within ± 1%)


Control
α-Tocopherol-500
BHA-200
BHT-200
EC-200
EGC-200
TBHQ-200
EGCG-200
ECG-200
SBO
MHO
Weight gain (%)
Storage time (h) at 65 ℃
18
36
54
72
90
108
126
144
162

control and 8.0 and 12.4 times for MHO, respectively.

The PV of SBO and MHO treated with tea catechins decreased (at least by 60% even after 144 h storage) significantly ($P < 0.05$) during their storage at 65°C (Tables 4.12 and 4.13). Among tea catechins tested, ECG served as the best antioxidant in lowering hydroperoxide formation in SBO, even to a greater extent than TBHQ. However, in MHO, ECG was more powerful than TBHQ, as evidenced by PV, only during the first 48 h of oil storage, after which TBHQ was most effective. The potency of ECG was 5.6 and 6.8 times that of α-tocopherol and 5.4 and 7.4 times that of BHA in SBO and MHO after 48 h of storage, respectively.

Addition of tea catechins to both SBO and MHO showed a significant ($P < 0.05$) effect in lowering the formation of TBARS in comparison with α-tocopherol, BHA and BHT (Tables 4.14 and 4.15). The order of potency of these catechins in inhibiting the formation of TBARS in both oils was in the decreasing order of ECG > EGCG >EGC > EC. Among tea catechins tested, ECG and EGCG exhibited >45% inhibition of TBARS formation in both oils. ECG showed a better antioxidant activity than TBHQ during the first 48 h of storage, after which TBHQ gave lower TBARS values. However, TBHQ is not yet licensed for food use in Canada and in the European countries.

Tea catechins exhibited excellent antioxidant properties in both SBO and MHO as evidenced by weight-gain, peroxide and TBARS values. The order of potency of the catechins in marine oils was ECG > EGCG > EGC > EC. Similar to other

Table 4.12 Effect of tea catechins and conventional antioxidants on peroxide values (meq/kg oil) of refined-bleached and deodorized seal blubber oil (SBO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	2.09 ± 0.06^{a}	25.2 ± 0.79^{a}	49.3 ± 1.22^{a}	119 ± 4.24^{a}	183 ± 2.34^{a}
EC-200	2.03 ± 0.03^{a}	6.70 ± 0.13^{de}	15.8 ± 0.80^{d}	45.9 ± 3.21^{e}	79.3 ± 3.91^{e}
EGC-200	2.19 ± 0.15^{a}	6.20 ± 0.21^{de}	9.96 ± 0.31^{ef}	41.3 ± 0.97^{e}	59.9 ± 1.07^{f}
EGCG-200	2.16 ± 0.01^{a}	5.64 ± 0.60^{de}	9.49 ± 0.20^{ef}	37.9 ± 0.94^{ef}	51.3 ± 1.70^{fg}
ECG-200	2.08 ± 0.09^{a}	4.62 ± 0.09^{e}	7.90 ± 0.08^{f}	27.6 ± 0.78^{g}	47.0 ± 2.59^{g}
α-Tocopherol-500	2.18 ± 0.01^{a}	21.5 ± 2.05^{b}	44.5 ± 0.91^{b}	110 ± 0.80^{b}	166 ± 1.23^{b}
BHA-200	2.19 ± 0.02^{a}	13.3 ± 1.27^{c}	42.6 ± 0.01^{b}	71.3 ± 1.38^{c}	124 ± 2.68^{c}
BHT-200	2.11 ± 0.06^{a}	13.3 ± 0.18^{c}	31.6 ± 0.42^{c}	58.8 ± 2.18^{d}	94.9 ± 2.11^{d}
TBHQ-200	2.09 ± 0.07^{a}	8.04 ± 0.24^{d}	10.4 ± 0.31^{e}	29.9 ± 0.86^{fg}	53.4 ± 1.20^{fg}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2](-)Epicatechin (EC), (-)epigallocatechin (EGC), (-)epigallocatechin gallate (EGCG), (-)epicatechin gallate (ECG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

Table 4.13 Effect of tea catechins and conventional antioxidants on peroxide values (meq/kg oil) of refined-bleached and deodorized menhaden oil (MHO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	4.53 ± 0.41^{a}	38.1 ± 0.77^{a}	96.5 ± 1.91^{a}	283 ± 4.58^{a}	348 ± 5.82^{a}
EC-200	4.05 ± 0.44^{a}	14.2 ± 1.10^{e}	47.6 ± 3.03^{d}	97.4 ± 2.26^{e}	133 ± 3.54^{d}
EGC-200	4.14 ± 0.20^{a}	12.1 ± 1.42^{cd}	31.7 ± 0.95^{e}	92.0 ± 2.05^{e}	133 ± 3.57^{d}
EGCG-200	4.10 ± 0.16^{a}	9.23 ± 0.32^{de}	14.8 ± 0.97^{f}	81.1 ± 1.60^{f}	113 ± 3.77^{e}
ECG-200	4.09 ± 0.13^{a}	7.62 ± 0.52^{e}	9.69 ± 0.26^{g}	75.9 ± 1.31^{f}	101 ± 2.10^{f}
α-Tocopherol-500	4.10 ± 0.12^{a}	37.3 ± 0.42^{a}	66.3 ± 0.50^{bc}	227 ± 5.59^{b}	280 ± 6.10^{b}
BHA-200	4.27 ± 0.06^{a}	36.1 ± 1.44^{ab}	71.3 ± 0.94^{ab}	135 ± 4.90^{c}	163 ± 3.60^{c}
BHT-200	4.43 ± 0.01^{a}	32.9 ± 1.63^{b}	63.3 ± 1.18^{c}	120 ± 0.82^{d}	137 ± 2.70^{d}
TBHQ-200	4.22 ± 0.02^{a}	15.1 ± 1.30^{e}	15.1 ± 0.14^{f}	42.8 ± 1.57^{g}	101 ± 1.02^{f}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2](-)Epicatechin (EC), (-)epigallocatechin (EGC), (-)epigallocatechin gallate (EGCG), (-)epicatechin gallate (ECG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

Table 4.14 Effect of tea catechins and conventional antioxidants on 2-thiobarbituric acid reactive substances (TBARS) values (μmol/g oil) of refined-bleached and deodorized seal blubber oil (SBO) stored at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	4.19 ± 0.01^{a}	8.78 ± 0.23^{a}	12.3 ± 0.37^{a}	19.4 ± 0.54^{a}	24.7 ± 0.20^{a}
EC-200	4.31 ± 0.05^{a}	4.76 ± 0.25^{ef}	7.77 ± 0.25^{c}	12.0 ± 1.25^{bc}	15.6 ± 0.30^{d}
EGC-200	4.30 ± 0.11^{a}	5.70 ± 0.02^{de}	7.61 ± 0.64^{c}	10.6 ± 0.89^{c}	14.0 ± 0.16^{e}
EGCG-200	4.07 ± 0.07^{a}	4.53 ± 0.04^{f}	5.87 ± 0.09^{d}	10.1 ± 0.07^{cd}	12.1 ± 0.08^{f}
ECG-200	4.05 ± 0.05^{a}	4.27 ± 0.22^{f}	5.24 ± 0.17^{d}	7.31 ± 0.02^{d}	9.10 ± 0.32^{g}
α-Tocopherol-500	4.32 ± 0.14^{a}	7.67 ± 0.47^{b}	11.8 ± 0.06^{a}	13.8 ± 0.64^{b}	22.9 ± 0.20^{b}
BHA-200	4.55 ± 0.34^{a}	6.89 ± 0.27^{bc}	8.97 ± 0.50^{b}	14.8 ± 0.11^{b}	20.0 ± 0.32^{c}
BHT-200	4.59 ± 0.46^{a}	6.35 ± 0.39^{cd}	7.80 ± 0.11^{c}	10.6 ± 0.27^{c}	16.8 ± 0.97^{d}
TBHQ-200	4.54 ± 0.62^{a}	4.53 ± 0.06^{f}	5.50 ± 0.42^{d}	7.07 ± 0.14^{d}	10.5 ± 0.28^{g}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2](-)Epicatechin (EC), (-)epigallocatechin (EGC), (-)epigallocatechin gallate (EGCG), (-)epicatechin gallate (ECG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

Table 4.15 Effect of tea catechins and conventional antioxidants on 2-thiobarbituric acid reactive substances (TBARS) values (μmol/g oil) of refined-bleached and deodorized menhaden oil (MHO) stored under Schaal oven conditions at 65°C[1]

Treatment[2]	Storage time, h				
	0	24	48	84	144
Control	7.65 ± 0.49^{a}	17.8 ± 0.36^{a}	25.8 ± 1.11^{a}	42.3 ± 0.41^{a}	45.2 ± 3.03^{a}
EC-200	7.58 ± 0.01^{a}	11.5 ± 0.43^{cd}	14.2 ± 0.71^{c}	25.4 ± 1.49^{cd}	24.8 ± 0.99^{de}
EGC-200	6.93 ± 0.08^{a}	10.5 ± 0.50^{de}	13.4 ± 0.61^{cd}	21.6 ± 0.37^{d}	23.4 ± 1.57^{e}
EGCG-200	6.83 ± 0.07^{a}	9.69 ± 0.05^{e}	11.9 ± 0.18^{d}	21.9 ± 1.22^{cd}	22.6 ± 0.71^{e}
ECG-200	7.00 ± 0.28^{a}	9.45 ± 0.64^{e}	11.5 ± 0.69^{d}	17.7 ± 0.70^{e}	22.2 ± 0.45^{e}
α-Tocopherol-500	6.95 ± 0.06^{a}	15.0 ± 0.18^{b}	25.5 ± 0.21^{a}	33.4 ± 0.78^{b}	36.7 ± 2.21^{b}
BHA-200	7.06 ± 0.01^{a}	14.4 ± 0.36^{b}	23.0 ± 0.45^{b}	25.4 ± 0.54^{c}	31.1 ± 1.61^{c}
BHT-200	7.43 ± 0.02^{a}	12.5 ± 0.59^{c}	21.7 ± 0.12^{b}	24.2 ± 0.57^{cd}	28.4 ± 0.80^{cd}
TBHQ-200	7.61 ± 0.14^{a}	10.5 ± 0.42^{de}	13.1 ± 0.13^{cd}	17.3 ± 1.57^{e}	20.5 ± 0.48^{e}

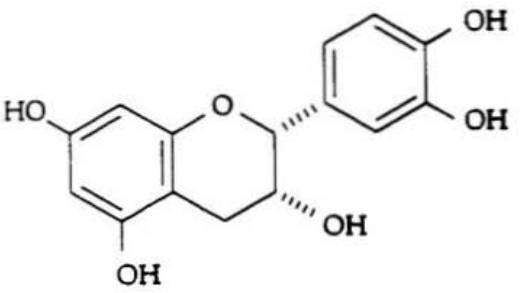
[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)
[2](-)Epicatechin (EC), (-)epigallocatechin (EGC), (-)epigallocatechin gallate (EGCG), (-)epicatechin gallate (ECG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ)

flavonoids, catechins possessing multiple hydroxyl groups in their structures (Figure 4.18) exhibited good antioxidant activity and extended the induction period of both SBO and MHO to a larger extent than BHA and BHT which have only one hydroxyl group in their chemical structures. When in the *ortho* configuration, multiple hydroxyl groups may participate in chelation of metal ions, in addition to their free-radical scavenging effect. Torel *et al.* (1986) have demonstrated that catechins suppress lipid oxidation in biological tissues and subcelluler fractions. Chen and Ho (1995) have tested the inhibitory effect of tea catechins for production of superoxide and have found their order of potency as EGCG > ECG > EGC > EC. The existing differences between the present results and those of Chen and Ho (1995) may be due to the influence of molecular weight of these catechins on their activity; all catechins were used at 200 ppm regardless of their molecular weight. The present findings demonstrated that tea catechins may be used as natural antioxidants for stabilization of highly unsaturated marine oils.

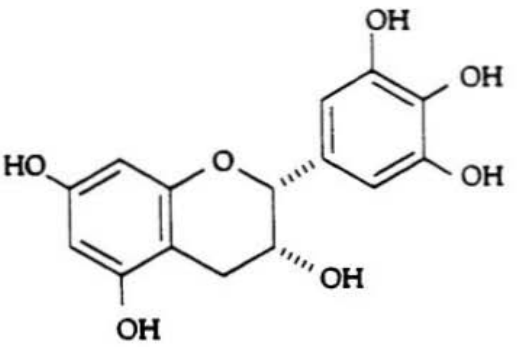
4.4 Stabilization of refined-bleached and deodorized (RBD) seal blubber oil (SBO) by microencapsulation

Oxidation of PUFA can proceed via different mechanisms, therefore devising complementary strategies to minimize its occurrence is mandatory. Because oxygen is an essential reactant in the process, control of oxygen availability is a critical variable in minimizing oxidation of PUFA. Microencapsulation of PUFA-rich oils would confer

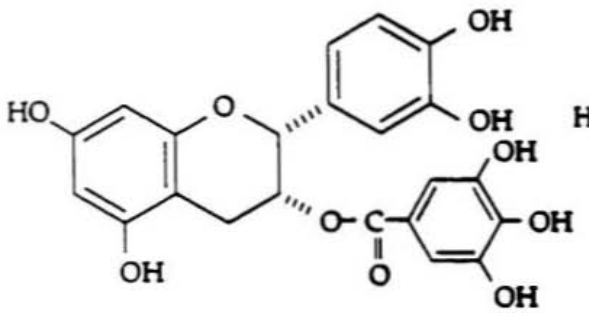
Figure 4.18 Chemical structures of green tea catechins



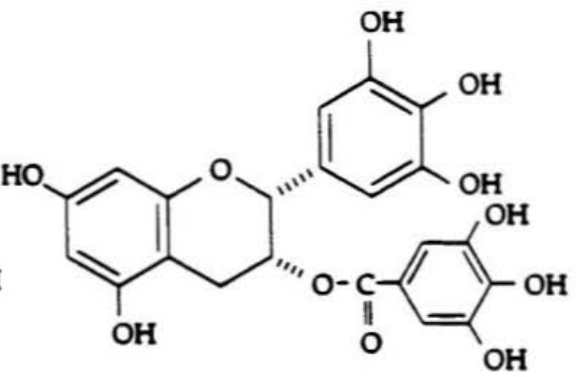
(-)Epicatechin (EC)



(-)Epigallocatechin (EGC)



(-)Epicatechin gallate (ECG)



(-)Epigallocatechin gallate (EGCG)

better stability to the product by preventing contact of the oil with oxygen and light. Microencapsulation is a technique by which oil is entrapped into a wall matrix and thus protected from oxygen, moisture and light.

Changes in fatty acid composition of stored SBO, both in the encapsulated and unencapsulated forms, are shown in Table 4.16. The total PUFA content of unencapsulated SBO decreased from 22.6% (on day-0) to 11.5% (on day-49), but PUFA of SBO encapsulated in β-cyclodextrin or corn-syrup solids decreased to 20.2 and 17.8%, respectively. The total omega-3 fatty acids in β-cyclodextrin encapsulated SBO remained nearly unchanged even after 21 days of storage while those for unencapsulated SBO changed from 20.1 to 10.6%.

Table 4.17 illustrates the progression of peroxide formation in unencapsulated and encapsulated SBO stored at room temperature. Encapsulated samples showed low PV throughout the storage period. Among the encapsulating materials tested, β-cyclodextrin served best in controlling the formation of peroxides followed by corn-syrup solids. The PV of the control samples (unencapsulated oil) increased from 2.08 to 29.8 meq/kg oil over a 49-day storage period. The corresponding values for encapsulated oils in β-cyclodextrin and corn-syrup solid matrices were smaller, changing from 3.01 to 10.2 and from 4.01 to 20.0 meq/kg oil, respectively. It is of interest to note that the initial PV of encapsulated samples were higher than those of the control sample, however, encapsulated samples were able to maintain lower PV throughout the storage period than the control samples. The higher initial PV of

Table 4.16 Effect of microencapsulation on changes in polyunsaturated fatty acids of refined-bleached and deodorized seal blubber oil (SBO) stored at room temperature[1]

Fatty acid (w/w %)	RBD-Seal blubber oil			β-cyclodextrin		Corn-syrup solids		Maltodextrin	
	Day-0	Day-21	Day-49	Day-21	Day-49	Day-21	Day-49	Day-21	Day-49
Total Polyunsaturated	22.6 ±0.02	13.1 ±0.25	11.5 ±0.35	21.3 ±0.45	20.2 ±0.85	19.0 ±0.75	17.8 ±0.85	15.0 ±0.95	11.9 ±0.80
EPA (20:5 ω3)	6.41 ±0.08	3.33 ±0.01	3.02 ±0.04	6.11 ±0.06	6.02 ±0.15	5.86 ±0.15	5.42 ±0.34	3.35 ±0.25	3.00 ±0.25
DPA (22:5 ω3)	4.66 ±0.01	2.14 ±0.02	2.09 ±0.02	4.24 ±0.03	4.22 ±0.25	3.68 ±0.23	3.49 ±0.35	2.95 ±0.25	2.15 ±0.15
DHA (22:6 ω3)	7.58 ±0.02	3.75 ±0.02	3.01 ±0.15	7.02 ±0.04	6.19 ±0.23	6.13 ±0.25	5.48 ±0.15	3.86 ±0.25	3.19 ±0.15
Total ω3	20.1 ±0.02	10.6 ±0.25	9.22 ±0.27	19.9 ±0.11	18.8 ±0.64	16.7 ±0.75	14.5 ±0.81	10.7 ±0.80	9.25 ±0.55
Total ω6	2.53 ±0.01	2.45 ±0.05	2.26 ±0.04	2.37 ±0.01	2.34 ±0.15	2.33 ±0.05	2.32 ±0.01	2.26 ±0.11	2.00 ±0.15
ω3/ω6	7.94	4.33	4.10	8.40	8.03	7.15	6.26	4.74	4.62

[1]Mean ± SD (n = 3)

Table 4.17 Effect of microencapsulation on peroxide values (meq/kg oil) of refined-bleached and deodorized seal blubber oil (SBO) stored at room temperature[1]

Treatment	Storage time, days					
	0	7	14	21	35	49
Control	2.08 ± 0.12^{a}	6.33 ± 0.51^{a}	13.0 ± 2.10^{a}	17.0 ± 2.65^{a}	31.9 ± 2.98^{a}	29.8 ± 3.66^{a}
β-cyclodextrin	3.01 ± 0.01^{b}	3.30 ± 0.10^{d}	4.21 ± 0.40^{d}	7.32 ± 0.39^{c}	9.22 ± 0.41^{c}	10.2 ± 1.21^{c}
Maltodextrin	4.41 ± 0.31^{c}	5.12 ± 0.31^{b}	9.22 ± 0.81^{b}	14.1 ± 2.43^{ab}	18.9 ± 1.32^{b}	27.4 ± 2.35^{a}
Corn-syrup solids	4.01 ± 0.21^{c}	4.55 ± 0.51^{c}	6.41 ± 0.94^{c}	12.0 ± 1.22^{b}	15.1 ± 0.92^{b}	20.0 ± 0.36^{b}

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)

encapsulated samples may be due to the formation of oxidation products during high temperature conditions of spray drying.

Changes in the content of TBARS of encapsulated SBO as compared with the unencapsulated oil are shown in Table 4.18. The β-cyclodextrin matrix was able to reduce the formation of TBARS of SBO by 74%, even after 49 days of storage, thereby indicating the effectiveness of β-cyclodextrin as an encapsulating agent. The corn-syrup solid matrix was also effective in lowering TBARS production after 49 days of storage of SBO, thus making it as a reasonably effective encapsulating agent for use in marine oils but maltodextrin appears to only serve as a carrier rather than an effective encapsulating material for SBO. Gejl-Hansen and Fink (1977) reported that linoleic acid encapsulated in a maltodextrin coating was resistant to oxidative deterioration even though maltodextrin is not known as a good encapsulating agent. Rice bran oil embedded in granules containing corn-syrup solids and pork polypeptone which was vacuum-dried, underwent little oxidation on exposure to air at a high temperature (Ono and Aoyama, 1979). Taguchi *et al.* (1992) reported that the oxidative stability of sardine oil embedded in spray-dried egg white powders and use of the product as a source of ω3-PUFA for fortification of cookies. The same researchers have reported that use of encapsulated sardine oil in fortified cookies did not affect their sensory quality. In addition, spray-dried egg white powder inclusive of sardine oil was stable during prolonged storage (6 months) at room temperature.

Table 4.18 Effect of microencapsulation on 2-thiobarbituric reactive substances (TBARS) values (μmol/g oil) of refined-bleached and deodorized seal blubber oil (SBO) stored at room temperature[1]

Treatment	Storage time, days					
	0	7	14	21	35	49
Control	4.14 ± 0.02[a]	8.77 ± 0.30[a]	9.71 ± 0.50[a]	13.4 ± 0.41[a]	20.7 ± 1.21[a]	28.9 ± 3.12[a]
β-cyclodextrin	4.79 ± 0.10[a]	4.96 ± 0.41[c]	5.46 ± 0.41[c]	5.94 ± 0.23[d]	6.57 ± 0.71[c]	7.51 ± 0.25[d]
Maltodextrin	5.21 ± 0.20[a]	6.66 ± 0.10[b]	7.10 ± 0.20[b]	9.96 ± 0.30[b]	15.9 ± 0.98[b]	21.9 ± 1.92[b]
Corn-syrup solids	5.10 ± 0.02[a]	5.02 ± 0.09[c]	6.01 ± 0.50[c]	7.51 ± 0.20[c]	18.0 ± 3.00[b]	17.8 ± 2.00[c]

[1]Values in the same column bearing different superscripts are significantly ($P < 0.05$) different, Mean ± SD (n = 3)

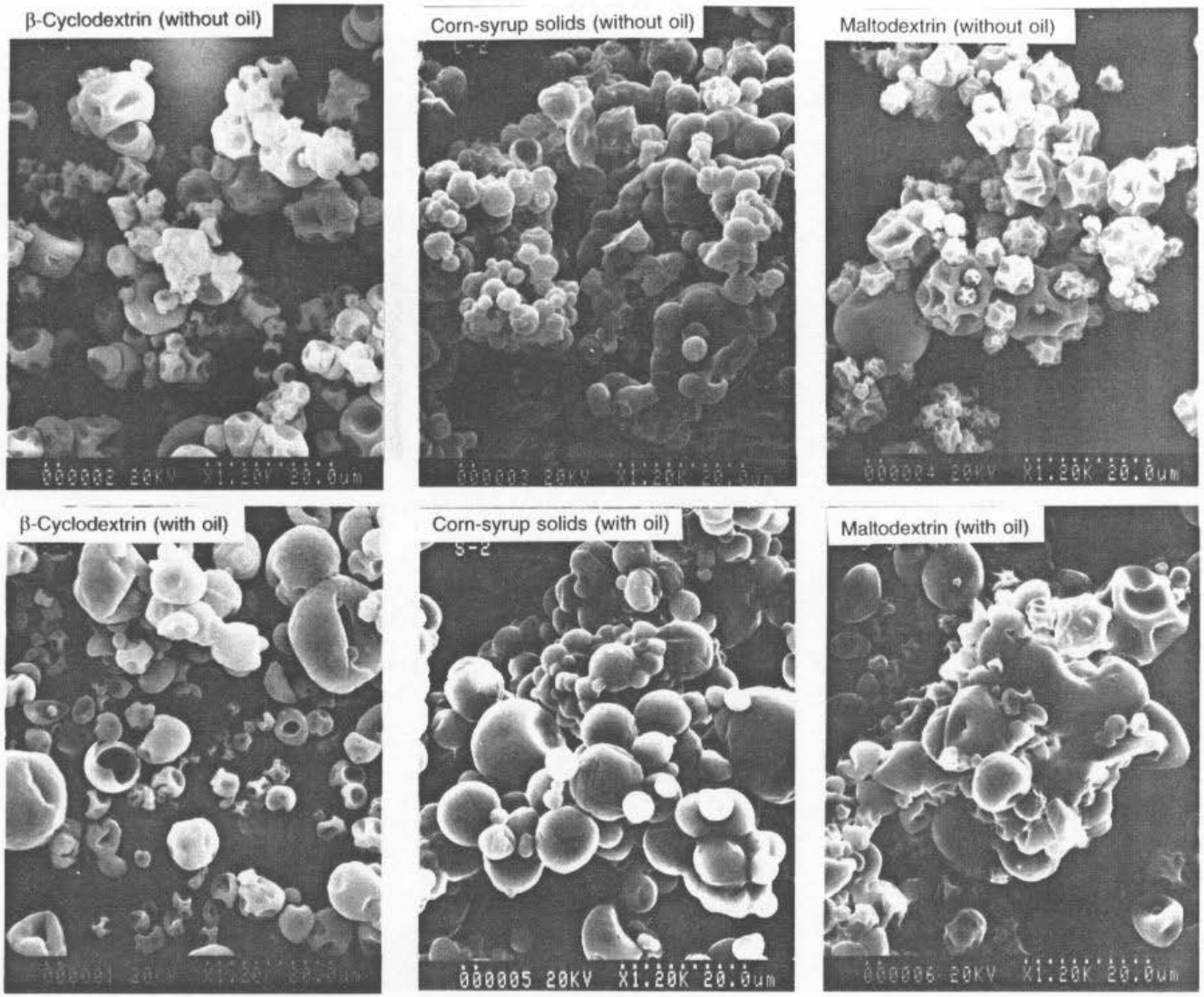
A close scrutiny of scanning electron micrographs (SEM) of spray-dried encapsulating materials, both in the absence and presence of SBO, indicated that some creases or dents were evident on the surface of encapsulating materials devoid of SBO (Figure 4.19). However, starch particles containing SBO were globular in shape with smooth surfaces and were variable in size. The β-cyclodextrin and, to a lesser extent, corn-syrup solid particles containing SBO were more globular in shape than corresponding maltodextrin particles, a further indication that maltodextrin was ineffective in forming microencapsules of SBO. Iwami *et al.* (1988) reported similar changes of encapsulating materials upon entrapment of fatty acids where gliadin particles containing fatty acids exhibited spherical shapes with smooth surfaces. Some creases or dents were seen on gliadin surfaces devoid of fatty acids.

Results of this study clearly demonstrates that microencapsulation offers an effective means for prevention of oxidative deterioration of SBO and preservation of the integrity of nutritionally important fatty acids of marine oils. β-cyclodextrin was the most effective encapsulating material examined. The products so obtained may be easily incorporated into food formulations in order to enrich with ω3 fatty acids.

4.5 Determination of positional distributions of fatty acids in triacylglycerols of seal blubber oil (SBO) and menhaden oil (MHO)

Intestinal absorption of fatty acids has been reported to be dependent on their arrangement in the triacylglycerol (TAG) molecules. Investigation of the absorption

Figure 4.19 Scanning electron micrographs of spray-dried wall materials with and without seal blubber oil (SBO)

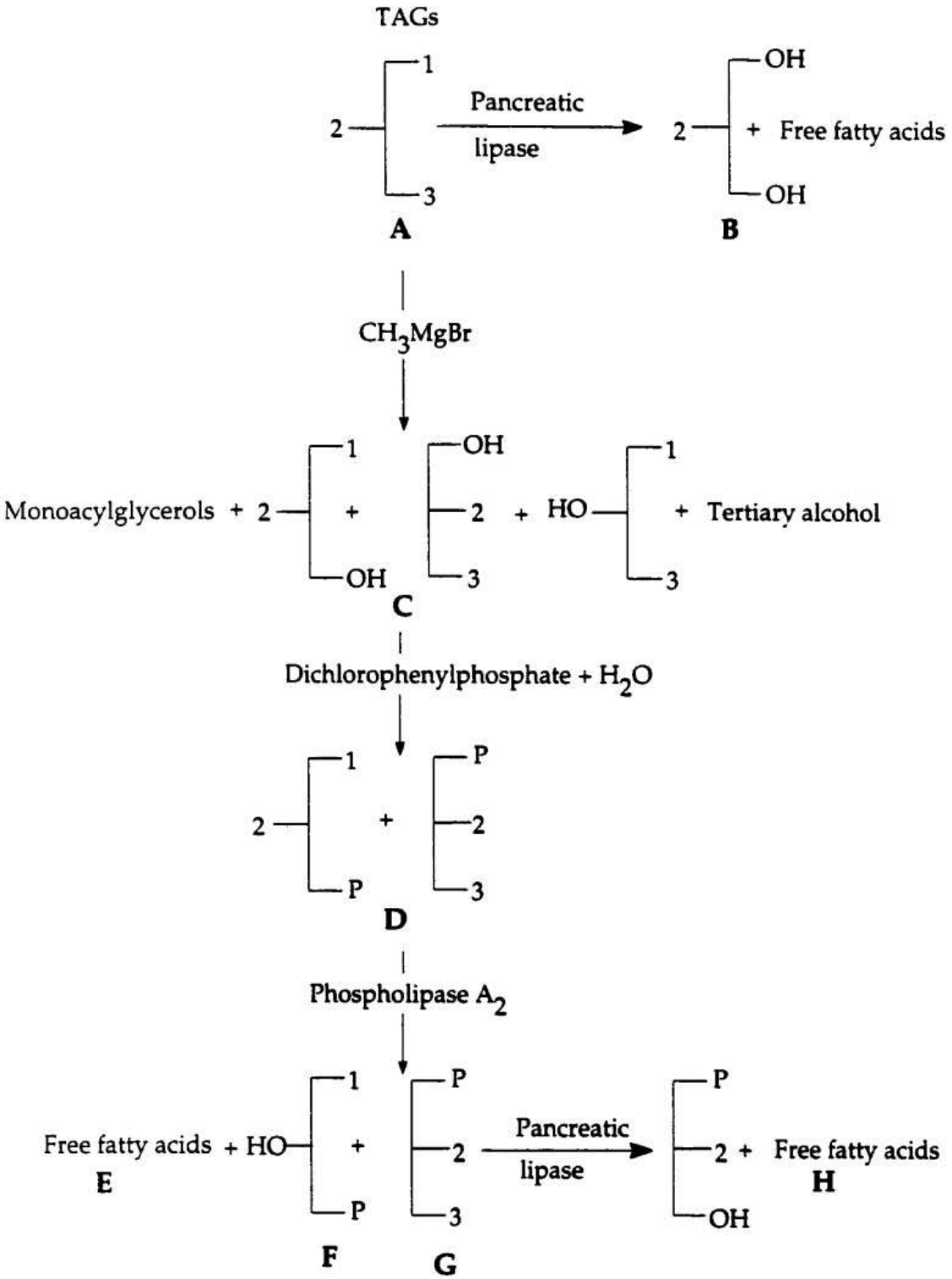

β-Cyclodextrin (without oil)
Corn-syrup solids (without oil)
Maltodextrin (without oil)
β-Cyclodextrin (with oil)
Corn-syrup solids (with oil)
Maltodextrin (with oil)
000005 20KV X1.20K 20.0um

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. On the other hand, different microbial lipases have been used to prepare ω3 concentrates from marine lipids via a hydrolysis process. Some microbial lipases have both positional and acyl-chain specificities (Brockerhoff, 1970). Therefore, knowledge of the fatty acid distribution in TAG molecules may provide useful information when selecting appropriate microbial lipases for hydrolysis of specific fatty acids in order to obtain ω3-enrichment. Stereospecific analysis allows determination of the distribution of fatty acids over the three positions of TAG molecules (Brockerhoff, 1971). The analysis is based on the modification of TAG by Grignard degradation followed by synthesis of phophatides and subsequent hydrolysis of fatty acids by stereospecific phospholipase A_2.

4.5.1 Stereospecific analysis of triacylglycerols of seal blubber oil (SBO) and menhaden oil (MHO)

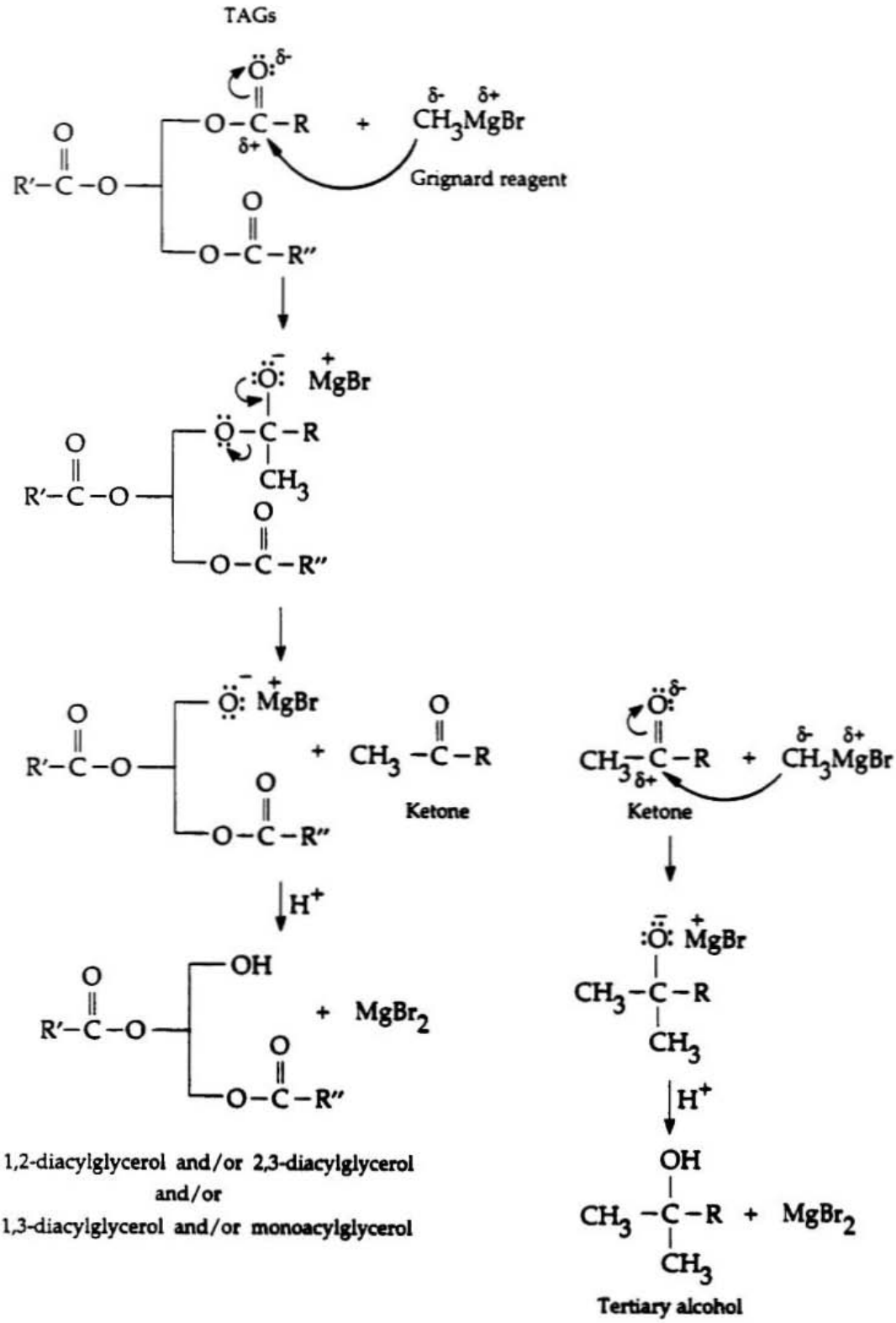
Stereospecific analyses of SBO and MHO and the intermediates involved are shown in Figure 4.20. In the first step, TAG of both SBO and MHO were hydrolysed by porcine pancreatic lipase in order to split fatty acids at the *sn*-1 and *sn*-3 positions, yielding 2-monoacylglycerols, which accurately provides the fatty acid composition of the *sn*-2 position of TAG. In the second step, TAG of both SBO and MHO were

Figure 4.20 Procedure for the stereospecific analysis of triacylglycerol (TAG) of seal blubber (SBO) and menhaden (MHO) oils


TAGs
1
2
3
Pancreatic
lipase
OH
2
+ Free fatty acids
OH
A
B
CH_3MgBr
Monoacylglycerols + 2
1
+
OH
OH
2
3
+ HO
1
3
+ Tertiary alcohol
C
Dichlorophenylphosphate + H_2O
1
2
P
+
P
2
3
D
Phospholipase A_2
Free fatty acids + HO
E
1
+
P
P
2
3
F
G
Pancreatic
lipase
P
2 +
OH
Free fatty acids
H

modified by Grignard degradation using CH_3MgBr. Laakso and Christie (1990) and Itobashi *et al.* (1990) used Grignard reaction to obtain partially deacylated triacylglycerols, i.e., diacylglycerols. In this reaction, the electrons in the C=O bond (sigma and pi) of the carbonyl groups in TAG are drawn towards the electronegative oxygen atom (Figure 4.21). The carbon atom of the carbonyl group carries a partial positive charge and can be attacked by the nucleophilic carbon of the Grignard reagent. The products of the reaction are a ketone and a magnesium salt of acylglycerol. In the presence of an aqueous acid, the magnesium salt of acylglycerols yields acylglycerols and an inorganic magnesium bromide (Figure 4.21). The resulting ketone reacts further with the Grignard reagent to yield a tertiary alcohol and an inorganic magnesium bromide. The products of the Grignard degradation of TAG may be separated on TLC plates (Figure 3.3); these consisted of monoacylglycerols (R_f = 0.03), 1,2- and 2,3-diacylglycerols (R_f = 0.30), 1,3-diacylglycerol (R_f = 0.40) and a tertiary alcohol (R_f = 0.70). Among these bands, the band with an R_f value of 0.30 (1,2- and 2,3-diacylglycerols) was isolated and used to prepare synthetic racemic phosphatides by reacting them with dichlorophenylphosphate (Figure 4.20). This reaction produced 1,2-diacylglycero-3-phosphatide (L-isomer) and 2,3-diacylglycero-1-phosphatide (D-isomer). These phophatides were hydrolysed by stereospecific phospholipase A_2 enzyme extracted from snake venom. This enzyme reacts only with 1,2-diacylglycero-3-phosphatide (L-isomer; naturally present) and releases free fatty acids from the *sn*-2 position (Verheij and Dijkstra, 1994). The products of phospholipase A_2 hydrolysis

Figure 4.21 Chemical reactions involved during Grignard degradation of triacylglycerol (TAG) of seal blubber (SBO) and menhaden (MHO) oils


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

(free fatty acids from *sn*-2 position and L-lysophophatide) and the unchanged 2,3-diacylglycero-1-phosphatide (D-isomer; un-natural) were separated on a TLC plate (Figure 3.4). The separated bands were identified as being free fatty acids from the *sn*-2 position of 1,2-diacyl-3-phosphatide (R_f = 0.71), unchanged 2,3-diacylglycero-1-phosphatide (R_f = 0.49) and L-lysophosphatide (R_f = 0.05). The band with an R_f value of 0.05 (L-lysophosphatide) was isolated, extracted into chloroform/methanol (1:1, v/v) and then used for fatty acid analysis. These allowed identification of the fatty acid composition at the *sn*-1 position of TAG of SBO and MHO.

In order to determine the fatty acid composition at the *sn*-3 position of TAG, a band with an R_f value of 0.49 (unchanged 2,3-diacylglycero-1-phosphatide) was isolated and extracted into chloroform/methanol (1:1, v/v). The latter compound was subjected to porcine pancreatic lipase hydrolysis (Figure 4.20), the products of which were 2-monoacylglycero-1-phosphatide and free fatty acids released from the *sn*-3 position of TAG of SBO and MHO.

4.5.2 Positional distribution of fatty acids in the triacylglycerols of seal blubber oil (SBO) and menhaden Oil (MHO)

Fatty acid distribution at three different positions of the TAG of SBO and MHO are given in Table 4.19. In SBO, saturated fatty acids such as 14:0, 15:0, 16:0 and 17:0 were preferentially located at the *sn*-2 position, followed by the *sn*-1 and *sn*-3 positions. However, the saturated fatty acids in MHO were randomly distributed over

Table 4.19 Fatty acid distribution in different positions of triacylglycerols of seal blubber and menhaden oils

Fatty acids (w/w %)	Seal blubber oil (SBO)			Menhaden oil (MHO)		
	sn-1[a]	*sn*-2[b]	*sn*-3[c]	*sn*-1[a]	*sn*-2[b]	*sn*-3[c]
14:0	2.23±0.12	8.08±0.01	0.12±0.00	12.1±0.10	10.5±0.09	5.23±0.01
14:1ω9	0.23±0.00	2.35±0.03	0.22±0.00	0.30±0.01	0.60±0.00	0.16±0.00
15:0	0.10±0.00	1.25±0.01	0.41±0.01	1.02±0.01	0.31±0.00	0.80±0.01
16:0	3.12±0.13	12.4±0.15	2.99±0.04	24.1±0.55	19.9±0.13	6.91±0.01
16:1ω7	8.22±0.16	35.3±0.80	12.3±0.12	18.1±0.32	10.6±0.18	9.20±0.10
17:0	0.79±0.01	1.80±0.01	0.80±0.01	4.39±0.12	3.65±0.03	1.01±0.00
17:1	0.20±0.00	1.30±0.00	0.12±0.00	2.11±0.09	2.23±0.13	1.23±0.03
18:0	1.01±0.01	1.64±0.06	0.71±0.02	1.21±0.02	2.06±0.00	4.12±0.01
18:1*	38.3±1.00	22.6±1.00	18.0±0.17	13.1±0.21	7.47±0.01	13.6±0.09
18:2ω6	0.32±0.00	1.24±0.09	2.01±0.08	0.81±0.00	0.49±0.00	3.11±0.03
18:3ω3	0.96±0.01	0.45±0.01	1.21±0.01	1.12±0.01	0.41±0.01	0.99±0.03
18:4ω3	1.82±0.16	0.45±0.02	0.33±0.09	4.51±0.02	0.34±0.03	4.94±0.01
20:0	0.10±0.00	0.43±0.00	0.09±0.00	0.21±0.00	0.11±0.00	1.21±0.01
20:1ω9	13.0±0.10	4.33±0.13	17.2±0.11	0.71±0.00	0.50±0.01	2.20±0.03
20:2ω6	0.21±0.00	0.15±0.00	0.20±0.00	0.40±0.00	0.14±0.00	0.10±0.00
20:3*	0.19±0.00	0.13±0.00	0.22±0.01	0.59±0.00	0.42±0.00	0.61±0.00
20:4ω6	0.21±0.02	0.14±0.00	0.91±0.01	1.11±0.01	1.13±0.02	0.27±0.01
20:5ω3	8.36±0.12	1.60±0.09	11.2±0.04	3.12±0.03	17.5±0.19	16.3±0.18
22:0	-	-	-	0.21±0.02	0.08±0.00	0.21±0.02
22:1ω11	3.00±0.04	0.13±0.01	3.25±0.03	0.13±0.00	0.12±0.00	0.09±0.00
22:2	-	-	-	0.20±0.00	0.15±0.00	0.18±0.00
22:4ω6	0.01±0.00	0.05±0.00	0.22±0.00	0.20±0.01	0.31±0.02	0.04±0.00
22:5ω3	3.99±0.05	0.79±0.06	8.21±0.08	1.21±0.01	3.11±0.02	2.31±0.05
22:6ω3	10.5±0.10	2.27±0.12	17.9±0.11	4.11±0.03	17.2±0.19	6.12±0.02

[a]L-lysophosphatide (F[1]), [b]2-Monoacylglycerol (B[1]), [c]3-Fatty acids (H[1]), [1]Refer to Figure 4.20 *Include all isomers

the three positions. SBO contained very high amounts (over 59%) of monounsaturated fatty acids, in which 18:1 was preferentially located at the *sn*-1 position, whereas 16:1 and 20:1 were abundant at the *sn*-2 and *sn*-3 positions, respectively. In MHO, monounsaturated fatty acids were randomly distributed. SBO and MHO were also different in the dominancy and distribution of long-chain ω3 fatty acids in their TAG molecules. In SBO, EPA (20:5ω3), DPA (22:5ω3) and DHA (22:6ω3) occurred mainly at the *sn*-1 and *sn*-3 positions of TAG and their quantities were EPA, 8.36 and 11.2%; DPA, 3.99 and 8.21%; and DHA, 10.5 and 17.9%, respectively. In MHO, DPA and DHA occurred mainly at the *sn*-2 position of TAG at 3.11 and 17.2%, respectively. However, EPA was equally distributed at the *sn*-2 (17.5%) and *sn*-3 (16.3%) positions and was present only in small amounts (3.12%) at the *sn*-1 position.

Brockerhoff *et al.* (1966) have pointed out the general tendency of long-chain PUFA to be preferentially esterified at the *sn*-2 position of fish oil TAG, whereas in marine mammal lipids, these fatty acids are mainly found at the *sn*-1 and *sn*-3 positions. Recently, Aursand *et al.* (1995) determined the positional distribution of ω3 fatty acids in cod liver and Atlantic salmon oils (fish) and seal blubber oil (marine mammal) by high-resolution ^{13}C nuclear magnetic resonance spectroscopy and found that in cod liver and salmon oils DHA was concentrated at the *sn*-2 position of TAG, whereas EPA was nearly randomly distributed in all three positions. In seal oil, DHA was primarily present at the *sn*-1 and *sn*-3 positions. Similar observations were made by Brockerhoff *et al.* (1963; 1966) for fish (herring and mackerel) and marine mammal

depot lipids (seal blubber oil). However, the major drawback of their method is that their results for the *sn*-3 position is subject to cumulative error because fatty acid distribution in this position was not determined directly. Ando *et al.* (1992) have determined the positional distribution of fatty acids in TAG of fish oils (capelin, herring, menhaden, sardine and saury) by high-performance liquid chromatography using a chiral stationary phase and found that in these oils DHA was present mainly at the *sn*-2 position of TAG. However, Ota *et al.* (1994) found that in flounder liver and flesh lipids there was no preference for the *sn*-2 position in contrast to the general tendency for distribution of long-chain PUFA of fish oils in this position.

4.6 Preparation of ω3 concentrates from seal blubber oil (SBO) via low-temperature crystallization

The melting point of a fatty acid changes considerably with its degree of unsaturation and chain length. When the temperature of a fatty acid mixture is lowered, the fatty acids with higher melting points, such as saturated and long-chain monounsaturated fatty acids start to crystallize and long-chain PUFA remain in the liquid form. Therefore, fractional crystallization may provide a simple way to concentrate PUFA of marine oils. In order to prepare ω3 fatty acid concentrates from SBO, low temperature fractional crystallization was carried out. In this study SBO in the triacylglycerol (TAG) form as well as free fatty acid form were subjected to solvent crystallization at different temperatures.

Figure 4.22 shows the enrichment of total ω3 fatty acids, EPA and DHA following low temperature crystallization of SBO in TAG form dissolved in hexane or acetone. Total ω3 fatty acids, EPA and DHA contents in the non-crystalline fraction (the concentrate) were increased with the lowering of crystallization temperature in both solvents. At each temperature acetone gave the highest concentration of total ω3 fatty acids, EPA and DHA. When acetone was used as a solvent, total ω3 fatty acid contents increased up to 36.4, 43.8 and 47.9% at -40, -60 and -70°C crystallization temperatures, respectively, whereas hexane at the same temperatures gave 26.3, 30.5 and 35.1% total ω3 fatty acids, respectively. However, percentage recovery of the non-crystallized fraction of the TAG of SBO in acetone was lower than that in hexane in all crystallization temperatures (Table 4.20). At -60 and -70°C hexane gave 16.3 and 14.4% of concentrate recovery, respectively. Corresponding values in acetone were 5.8 and 4.1%, respectively. At -70°C, levels of EPA and DHA in the concentrate increased 1.8 and 2.1 fold in hexane and 2.4 and 2.5 fold in acetone, respectively.

Low temperature crystallization of SBO in the free fatty acid form at -60 and -70°C in hexane resulted in total ω3 fatty acids up to 58.3 and 66.7%, respectively, with concentrate recovery of 39.0 and 24.8% (Figure 4.23 and Table 4.20). However, the content of total ω3 fatty acids in acetone increased up to 56.7 and 46.8% and recovery of the concentrate was 15.9 and 12.9%, respectively. Enrichment of total ω3 fatty acid, EPA and DHA as well as recovery of the concentrate was considerably higher in the free fatty acid form as compared to the TAG form of SBO. The use of free fatty acids,

Figure 4.22 Enrichment of total ω3 fatty acids, EPA and DHA upon low temperature crystallization of seal blubber oil in triacylglycerol (TAG) form (all values are mean of three replicates ± SD)

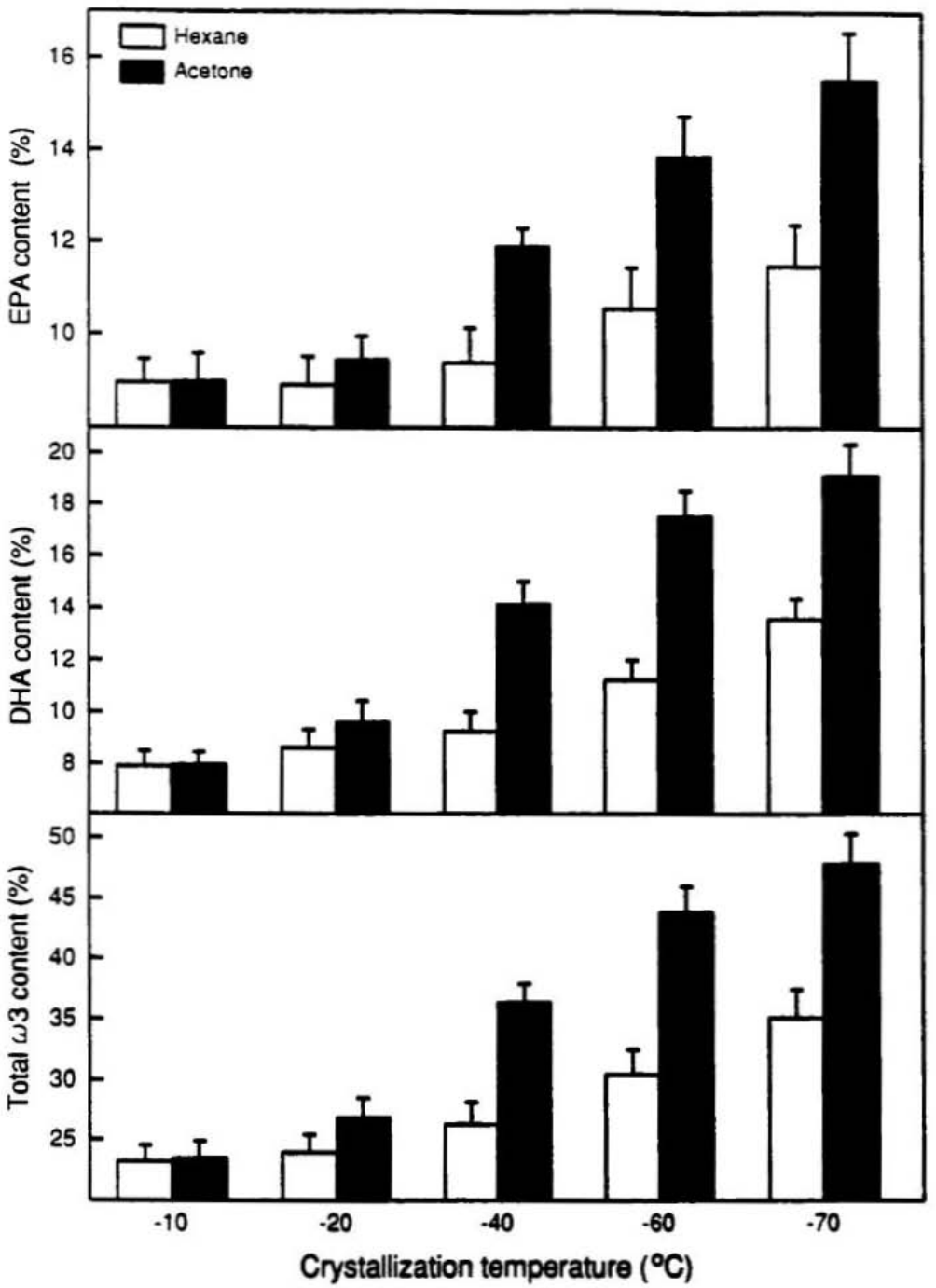

Hexane
Acetone
EPA content (%)
16
14
12
10
DHA content (%)
20
18
16
14
12
10
8
Total ω3 content (%)
50
45
40
35
30
25
-10
-20
-40
-60
-70
Crystallization temperature (°C)

Figure 4.23 Enrichment of total ω3 fatty acids, EPA and DHA upon low temperature crystallization of seal blubber oil in free fatty acid form (all values are mean of three replicates ± SD)

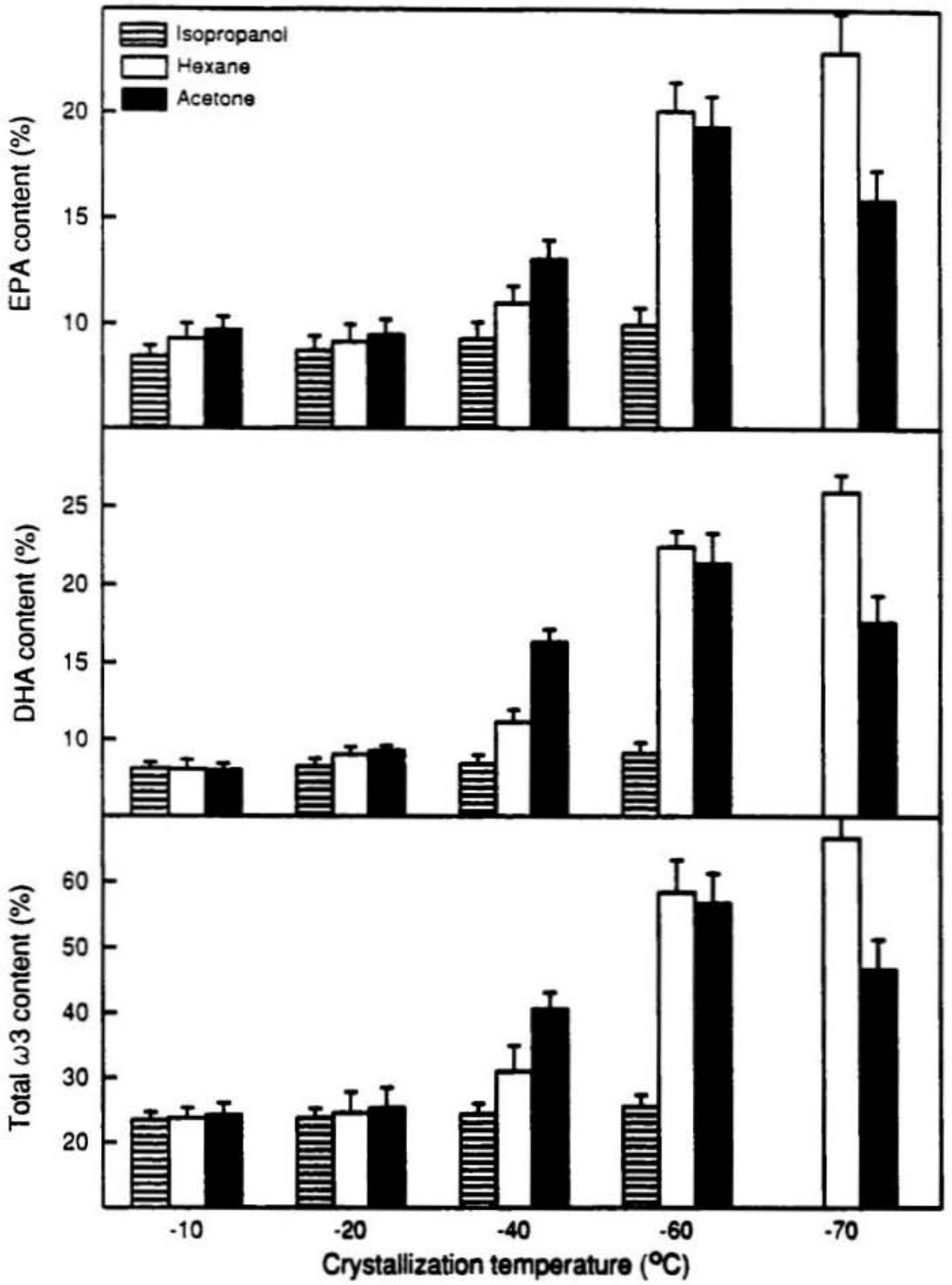

Isopropanol
Hexane
Acetone
EPA content (%)
DHA content (%)
Total ω3 content (%)
-10
-20
-40
-60
-70
Crystallization temperature (°C)

Table 4.20 Percentage recovery of non-crystallized fraction of triacylglycerols (TAG) or free fatty acids of seal blubber oil (SBO) upon low temperature crystallization[1]

Storage temperature (°C)	Triacylglycerols (TAG)		Free fatty acids		
	Acetone	Hexane	Isopropanol	Acetone	Hexane
-10	67.9 ± 0.5	88.0 ± 2.1	86.6 ± 2.3	73.9 ± 3.4	85.8 ± 3.0
-20	35.8 ± 1.8	83.2 ± 1.2	82.6 ± 1.5	57.9 ± 2.5	64.4 ± 2.3
-40	11.6 ± 0.5	50.9 ± 2.1	37.4 ± 1.9	30.5 ± 2.9	54.2 ± 3.1
-60	5.8 ± 0.2	16.3 ± 0.3	9.9 ± 0.8	15.9 ± 1.1	39.0 ± 1.6
-70	4.1 ± 0.1	14.4 ± 0.2	-	12.9 ± 0.9	24.8 ± 1.1

[1]Mean ± SD (n = 3)

which accentuate differences in physical properties, improve the enrichment of total ω3 fatty acids in the concentrate. The other advantage of using free fatty acids for low temperature crystallization is their greater solubility in different solvents. SBO in the TAG form was not soluble in isopropanol, therefore, limiting the choice to acetone and hexane for solvent crystallization. Free fatty acids obtained from SBO were soluble in isopropanol down to a temperature of -60°C, however, enrichment of total ω3 fatty acids, EPA and DHA in isopropanol was lower than in hexane and acetone.

Solvent crystallization of SBO at low temperatures increased the total ω3 fatty acid content of the non-crystallized fraction for both free fatty acids and TAG. The highest enrichment and recovery was obtained when hexane was used as a solvent and the oil was in the free fatty acids form. Kinsella (1990) has reported that low temperature crystallization of menhaden oil (free fatty acid form) at -18 and -35°C from acetone increased the total ω3 fatty acids to 26 and 30%, with a recovery of 87 and 70%, respectively. Low temperature crystallization of skipjack eye orbital oil in hexane at -60, -70 and -80 °C enriched the content of DHA and EPA to 39.3 and 8.82%, 42.4 and 9.54%, and 46.4 and 11.1%, respectively (Jeong, 1993).

4.7 Preparation of ω3 concentrates from seal blubber oil (SBO) via urea complexation

The simplest and most efficient technique for obtaining ω3-PUFA concentrates in the form of free fatty acids is urea complexation. This is a well established

technique for elimination of saturated and monounsaturated fatty acids. Initially the TAG of oil is split into their constituent fatty acids by alkaline hydrolysis using alcoholic KOH or NaOH and these free 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 crystallize out on cooling and may subsequently be removed by filtration. The liquid or non-urea complexed fraction (NUCF) is enriched with ω3-PUFA. In this study, urea complexation of SBO was carried out to concentrate ω3 fatty acids of the oil. Factors (variables) such as urea-to-fatty acid ratio (w/w, X_1), crystallization time (h, X_2) and crystallization temperature (°C, X_3) were studied collectively in order to optimize the conditions to obtain a maximum concentration of ω3-PUFA.

Experimental values obtained for responses; PUFA, total ω3 fatty acids, EPA, DPA and DHA in NUCF of SBO as well as percentage recovery of NUCF for nineteen design points are given in Table 4.21. Table 4.22 shows values of the above mentioned responses for urea complexed fraction (UCF) of SBO. Among the major ω3-PUFA, DHA was found almost exclusively in the NUCF of some treatment conditions. Although a major proportion of EPA was recovered in the NUCF, a small proportion invariably complexed with urea and was detected in UCF. The amount of EPA in the UCF was considerable (6.69-6.77%) with a urea-to-fatty acid ratio of 3.5, crystallization time of 18 h and a crystallization temperature of -9°C. Therefore, these results showed that EPA has more tendency to form urea adducts than DHA. Haagsma *et al.* (1982)

Table 4.21 Central composite design arrangement and responses for non-urea complexed fraction of seal blubber oil (SBO)

Run	Variable levels			Responses, Y (non-urea complexed fraction, NUCF)					
	Urea/FAs[a] (X_1)	Time[b] (X_2)	Temperature[c] (X_3)	Yield[d] (%)	PUFA (%)	EPA (%)	DPA (%)	DHA (%)	Total ω3 (%)
1	2	12	-18	40.6	69.2	22.1	12.7	24.4	63.4
2	5	12	-18	22.9	87.3	18.1	2.72	56.8	86.7
3	2	24	-18	41.3	68.8	21.9	12.6	24.4	62.9
4	5	24	-18	20.0	89.5	17.7	2.10	59.5	88.4
5	2	12	0	45.6	57.5	18.3	10.6	19.9	52.3
6	5	12	0	20.9	87.1	21.6	5.18	50.7	84.6
7	2	24	0	45.2	59.6	18.9	11.0	20.7	54.2
8	5	24	0	20.3	87.4	20.6	4.90	52.3	85.1
9	1	18	-9	52.8	33.7	10.4	6.46	11.2	30.1
10	6	18	-9	20.0	87.8	20.3	3.59	54.2	85.9
11	3.5	8	-9	19.6	86.7	11.4	0.53	70.3	86.4
12	3.5	28	-9	20.8	87.7	11.6	0.64	67.3	87.3
13	3.5	18	-24	18.2	80.8	11.0	1.36	57.1	80.8
14	3.5	18	6	34.6	80.8	24.9	2.07	43.2	77.2
15	3.5	18	-9	19.3	88.4	10.9	2.35	66.8	88.3
16	3.5	18	-9	20.4	85.3	10.9	3.31	60.8	88.5
17	3.5	18	-9	20.7	85.9	7.82	3.11	65.9	84.1
18	3.5	18	-9	19.3	87.6	7.68	2.20	66.4	85.9
19	3.5	18	-9	20.0	87.7	7.61	2.96	65.9	86.0

[a]Urea-to-fatty acid ratio (w/w), [b]crystallization time (h), [c]crystallization temperature (°C), [d]percentage recovery of NUCF

Table 4.22 Central composite design arrangement and responses for urea complexed fraction of seal blubber oil (SBO)

Run	Variable levels			Responses, Y (urea complexed fraction, UCF)					
	Urea/FAs[a] (X_1)	Time[b] (X_2)	Temperature[c] (X_3)	PUFA (%)	MUFA (%)	EPA (%)	DPA (%)	DHA (%)	Total ω3 (%)
1	2	12	-18	4.95	73.6	1.23	1.21	0.90	3.66
2	5	12	-18	19.0	63.3	6.03	4.17	5.00	16.2
3	2	24	-18	5.33	73.5	1.36	1.32	0.99	4.00
4	5	24	-18	19.3	63.0	6.28	4.43	4.74	16.4
5	2	12	0	6.87	70.4	1.86	1.36	1.97	5.59
6	5	12	0	16.3	65.3	5.59	3.79	3.54	13.7
7	2	24	0	5.55	71.3	1.79	1.28	0.60	4.15
8	5	24	0	15.1	68.5	5.61	3.89	0.13	10.4
9	1	18	-9	7.04	64.7	2.68	1.68	0.78	5.66
10	6	18	-9	17.2	64.6	5.85	3.92	3.90	14.5
11	3.5	8	-9	20.2	62.4	6.86	4.36	5.52	17.7
12	3.5	28	-9	19.5	62.7	6.57	3.94	5.07	16.6
13	3.5	18	-24	14.5	69.8	6.50	4.27	0.27	12.1
14	3.5	18	6	11.7	69.3	4.46	3.93	0.35	9.31
15	3.5	18	-9	19.3	62.8	6.77	3.92	5.27	17.0
16	3.5	18	-9	19.1	62.7	6.69	3.73	5.31	16.7
17	3.5	18	-9	19.2	62.5	6.71	3.80	5.29	16.8
18	3.5	18	-9	18.2	63.8	6.72	3.92	4.19	15.7
19	3.5	18	-9	18.2	63.7	6.77	3.97	4.23	15.8

[a]Urea-to-fatty acid ratio (w/w), [b]crystallization time (h), [c]crystallization temperature (°C)

and Ratnayake *et al.* (1988) have reported similar results for urea complexation experiments carried out for cod liver and menhaden oils, respectively. The highest DPA content (12.6%) was achieved with a urea-to-fatty acid ratio of 2, a crystallization time of 12 h and a crystallization temperature of -18°C (Table 4.21). The content of DPA in the original SBO was 4.66%, but by urea complexation a 3-fold increase in the DPA content may be achieved. Urea complexation of SBO resulted in an increase in the total PUFA content up to 89.5% in the NUCF. It is difficult to remove all saturated fatty acids to obtain 100% PUFA in the concentrate. Ratnayake *et al.* (1988) have also reported that complete removal of saturated fatty acids by urea complexation may be impossible since some of the saturated fatty acids do not complex with urea during crystallization. Long chain monounsaturated fatty acids (MUFA), especially those of the C20 and C22, form complexes with urea more readily than those of shorter chain saturated fatty acids (C14 and C16) thus the amount of MUFA in UCF was increased up to 73% in some treatment conditions. Enrichment of total ω3 fatty acids in the concentrate and overall recovery varied inversely with increasing urea-to-fatty acid ratio as well as decreasing crystallization temperatures. Therefore, these experimental variables should be carefully controlled in order to achieve a maximum content of total ω3 fatty acids in the concentrate with a reasonable recovery.

The amount of cholesterol in the NUCF of SBO under different treatment conditions are given in Table 4.23. Although it was assumed that the content of cholesterol in the NUCF may increase due to its large molecular size, the use of

Table 4.23 Cholesterol content of non-urea complexed fraction (NUCF) of seal blubber oil at different treatment conditions[a]

Treatment			Cholesterol content (mg/100 g oil)
Urea-to-fatty acid ratio (w/w)	Time[b] (h)	Temperature[c] (°C)	
2	12	-18	72.1 ± 6.0
5	12	-18	71.6 ± 4.0
2	24	-18	70.3 ± 5.2
5	24	-18	68.4 ± 3.9
2	12	0	129 ± 4.5
5	12	0	109 ± 2.1
2	24	0	119 ± 8.7
5	24	0	116 ± 4.5
1	18	-9	90.1 ± 3.6
6	18	-9	90.4 ± 7.2
3.5	8	-9	95.9 ± 3.2
3.5	28	-9	88.2 ± 3.1
3.5	18	-24	60.3 ± 4.2
3.5	18	6	128 ± 8.0
3.5	18	-9	95.2 ± 4.5
RBD-seal blubber oil			106 ± 22.0

[a]Mean ± SD (n = 3)

[b]Crystallization time (h), [c]Crystallization temperature (°C)

crystallization temperatures below 0°C, lowered the content of cholesterol. The melting point of cholesterol is 148.5°C, so at low temperatures it may solidify with urea (without complexing) and separate along with the UCF. Original SBO contained 106 mg cholesterol/100 g oil. When crystallization temperatures were set at -9, -18 and -24°C, the amount of cholesterol in NUCF was reduced down to 88-95, 68-72 and 60 mg/100 g oil, respectively.

4.7.1 Optimization of process conditions to maximize contents of total ω3 fatty acids, EPA and DHA of seal blubber oil (SBO) concentrate via urea complexation

Optimization of process conditions such as urea-to-fatty acid ratio (X_1), crystallization time (X_2) and crystallization temperature (X_3) to maximize contents of total ω3 fatty acids, EPA and DHA in the prepared concentrate was carried out. The response surface methodology (RSM) was employed for this purpose.

4.7.1.1 Diagnostic checking of fitted models

Multiple regression coefficients obtained by employing a least squares technique to predict quadratic polynomial models for contents of total ω3 fatty acids (Y_1), EPA (Y_2) and DHA (Y_3) are summarized in Table 4.24. Examination of these coefficients with the t-test indicated that linear and quadratic terms of urea-to-fatty acid ratio and crystallization temperature were highly significant ($P < 0.01$) but crystallization time was not for total ω3 fatty acids content in the concentrate. For the content of EPA in

Table 4.24 Regression coefficients of predicted quadratic polynomial model for response variables (total ω3, EPA and DHA contents) in urea complexation experiment of seal blubber oil (SBO)

Variables[1]	Coefficients (β)		
	Total ω3, % (Y_1)	EPA, % (Y_2)	DHA, % (Y_3)
Intercept	-18.98212	38.05204**	-61.545841**
Linear			
X_1	44.17949***	-7.015524	52.360500***
X_2	0.267651	-1.509582	0.886283
X_3	-1.450864***	0.649557	-1.914253
Quadratic			
X_{11}	-4.721517***	1.315469**	-6.248160***
X_{22}	-0.005895	0.044117	-0.029410
X_{33}	-0.037687***	0.050363***	-0.095827***
Interaction			
X_{12}	0.009264	-0.024361	0.048181
X_{13}	0.134343	0.121981	-0.046639
X_{23}	0.002613	0.000218	-0.000734
X_{123}	-	-	-
R^2	0.99	0.71	0.93

[1]See Table 4.21 for description of abbreviations

**$P < 0.05$

***$P < 0.01$

the concentrate, none of the linear terms of independent variables was significant ($P > 0.05$) but quadratic terms of urea-to-fatty acid ratio and crystallization temperature were significant at $P < 0.05$ and $P < 0.01$, respectively. The coefficients obtained for DHA content of the concentrate showed that linear term of urea-to-fatty acid ratio was highly significant ($P < 0.01$), whereas crystallization time and temperature were not. However, quadratic terms of urea-to-fatty acid ratio and crystallization temperature were significant at $P < 0.01$. Therefore, these results suggest that linear and/or quadratic effect of urea-to-fatty acid ratio and crystallization temperature are the primary determining factors for the amounts of total ω3 fatty acids, EPA and DHA in the prepared concentrate of SBO. No statistically significant interactions existed between any two of the three factors. The contribution of linear and quadratic terms to the models was 0.66 and 0.32 for the total ω3, 0.09 and 0.58 for the EPA and 0.47 and 0.46 for the DHA, respectively. The coefficients of independent variables (urea-to-fatty acid ratio: X_1, crystallization time; X_2 and crystallization temperature; X_3) determined for the quadratic polynomial models (Table 4.24) for total ω3 fatty acids (Y_1), EPA (Y_2) and DHA (Y_3) of the prepared concentrate are given below:

$$Y_1 = -18.982 + 44.179X_1 + 0.268X_2 - 1.451X_3 - 4.72152X_1^2 - 0.00589X_2^2 - 0.03769X_3^2 + 0.00926X_1X_2 + 0.13434X_1X_3 + 0.00261X_2X_3$$

$$Y_2 = 38.052 - 7.016X_1 - 1.511X_2 + 0.65X_3 + 1.31547X_1^2 + 0.04412X_2^2 + 0.05036X_3^2 - 0.02436X_1X_2 + 0.12198X_1X_3 + 0.00022X_2X_3$$

$$Y_3 = -61.546 + 52.361X_1 + 0.886X_2 - 1.914X_3 - 6.24816X_1^2 - 0.02941X_2^2 - 0.09583X_3^2 + 0.04818X_1X_2 - 0.04664X_1X_3 - 0.00073X_2X_3$$

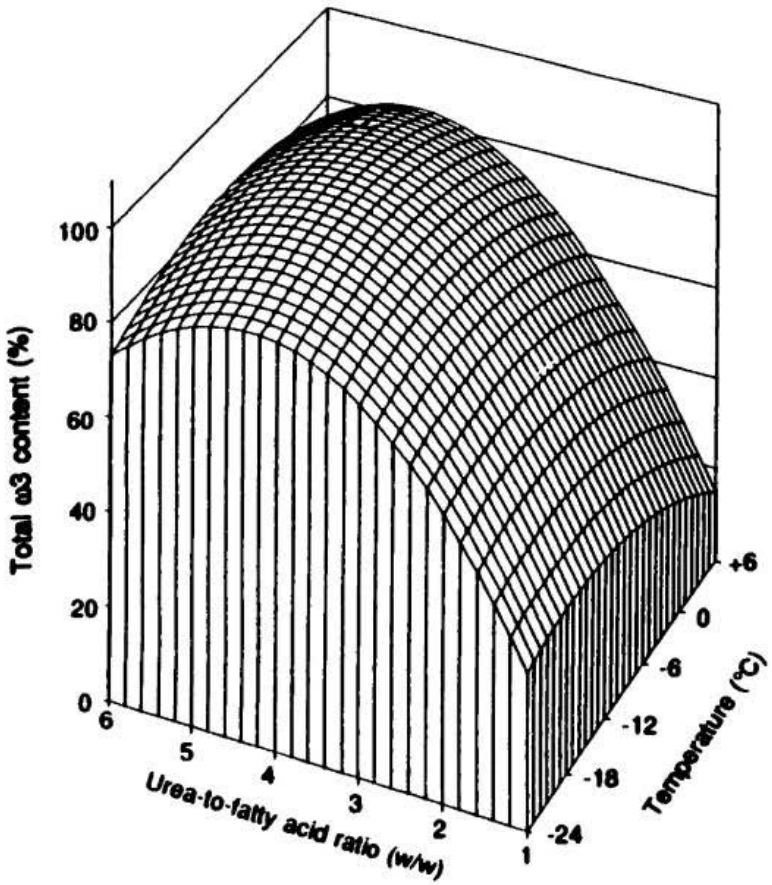
The models predicted for Y_1, Y_2 and Y_3 were adequate as indicated by error analysis that showed non-significant ($P > 0.05$) lack-of-fit. The regression models for data on total ω3 fatty acids and DHA were highly significant ($P < 0.01$) with satisfactory coefficient of determinations (R^2) 0.99 and 0.93, respectively. However, the coefficient of determination for data on EPA was 0.71. The models indicated that urea-to-fatty acid ratio (X_1) was a significant variable with the most linear effect on both total ω3 fatty acids and DHA contents in the concentrate as it had the largest linear coefficients of 44.179 and 52.361, respectively (Table 4.24).

4.7.1.2 Response surface plotting and optimization based on canonical analysis

Variables giving linear and quadratic terms with the largest absolute coefficients in the fitted models (Table 4.24) were chosen as the axes (urea-to-fatty acids ratio and crystallization temperature) for the response surface plots. The relationship between independent and dependent variables are shown in the three-dimensional representation of the response surfaces generated for the models developed for the contents of total ω3 fatty acids, EPA and DHA in the concentrates are given in Figures 4.24, 4.25 and 4.26, respectively.

Canonical analysis was performed on the predicted quadratic polynomial models to examine the overall shape of the response surface curves and used to characterize the nature of the stationary points. Canonical analysis is a mathematical approach used to locate the stationary point of the response surface and to determine whether it

Figure 4.24 Response surface and contour plot for the effect of urea-to-fatty acid ratio and crystallization temperature on total ω3 fatty acid content of the prepared concentrate of seal blubber oil by the urea complexation method


Total ω3 content (%)
100
80
60
40
20
0
6
5
4
3
2
1
Urea-to-fatty acid ratio (w/w)
+6
0
-6
-12
-18
-24
Temperature (°C)

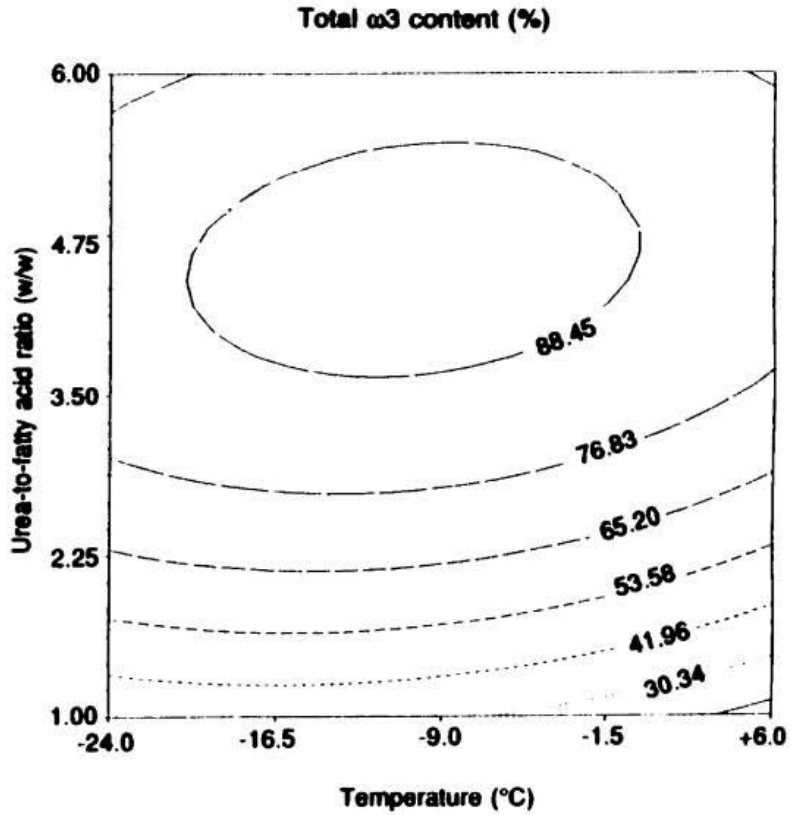
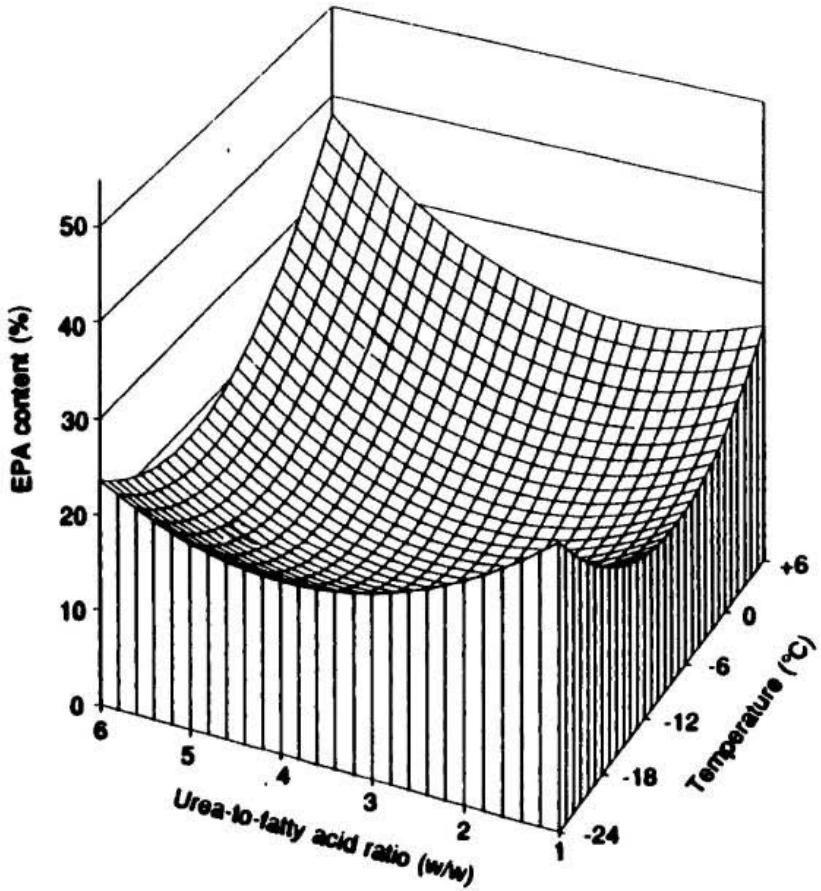

Total ω3 content (%)
6.00
4.75
3.50
2.25
1.00
Urea-to-fatty acid ratio (w/w)
88.45
76.83
65.20
53.58
41.96
30.34
-24.0
-16.5
-9.0
-1.5
+6.0
Temperature (°C)

Figure 4.25 Response surface and contour plot for the effect of urea-to-fatty acid ratio and crystallization temperature on EPA content of the prepared concentrate of seal blubber oil by the urea complexation method


EPA content (%)
50
40
30
20
10
0
6
5
4
3
2
1
Urea-to-fatty acid ratio (w/w)
-24
-18
-12
-6
0
+6
Temperature (°C)

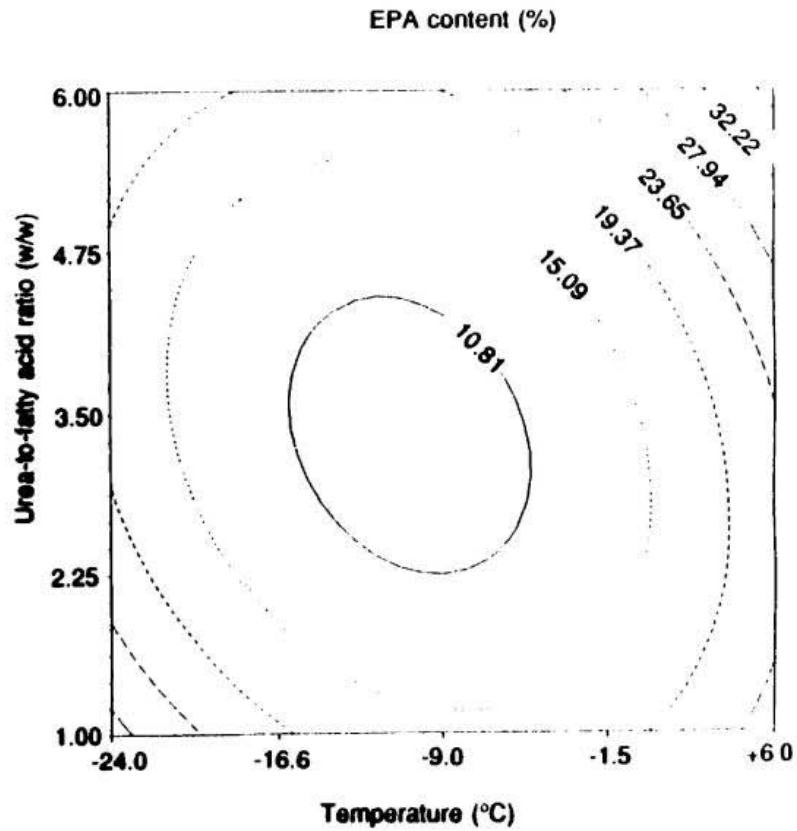
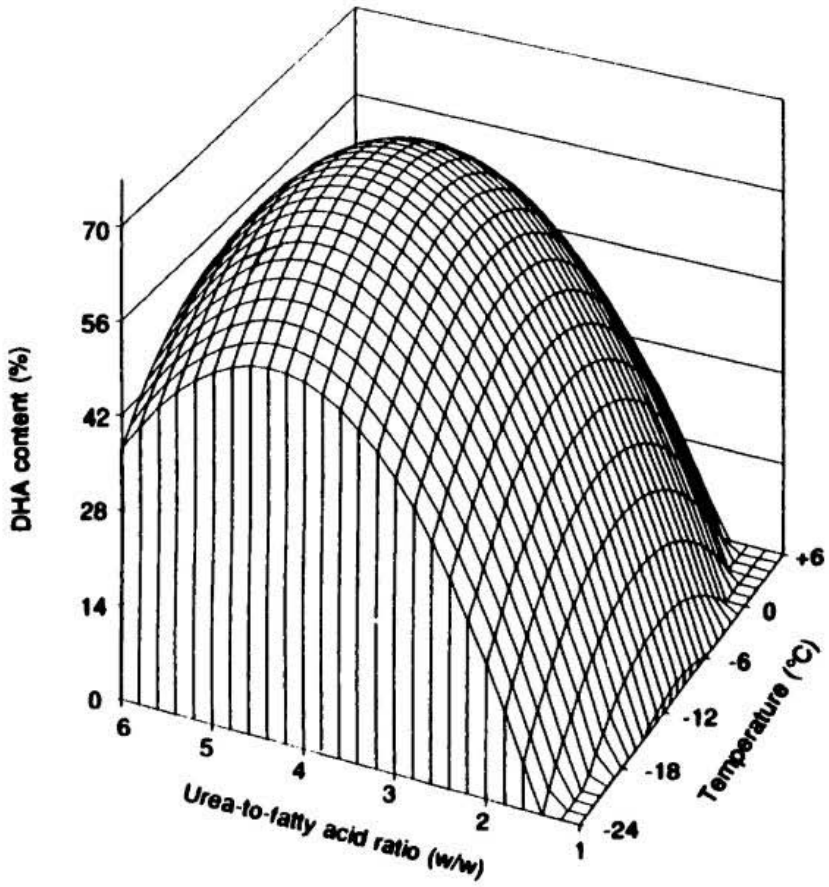
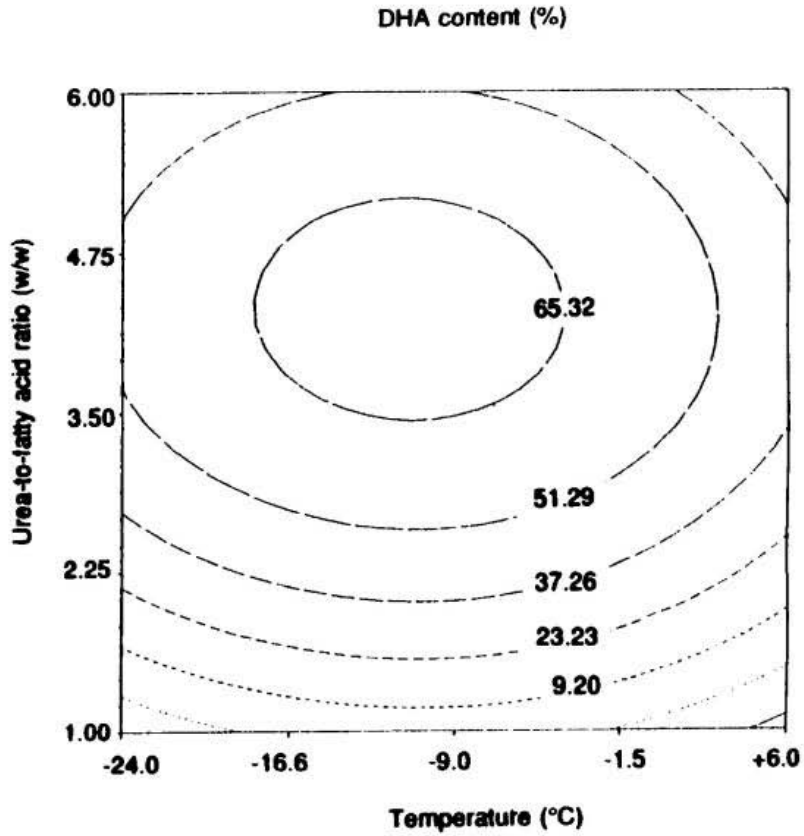

EPA content (%)
6.00
4.75
3.50
2.25
1.00
Urea-to-fatty acid ratio (w/w)
32.22
27.94
23.65
19.37
15.09
10.81
-24.0
-16.6
-9.0
-1.5
+6 0
Temperature (°C)

Figure 4.26 Response surface and contour plot for the effect of urea-to-fatty acid ratio and crystallization temperature on DHA content of the prepared concentrate of seal blubber oil by the urea complexation method


DHA content (%)
70
56
42
28
14
0
6
5
4
3
2
1
Urea-to-fatty acid ratio (w/w)
+6
0
-6
-12
-18
-24
Temperature (°C)


DHA content (%)
6.00
4.75
3.50
2.25
1.00
Urea-to-fatty acid ratio (w/w)
65.32
51.29
37.26
23.23
9.20
-24.0
-16.6
-9.0
-1.5
+6.0
Temperature (°C)

represents a maximum, minimum or saddle point (Mason *et al.*, 1989). Results of canonical analysis of the response surfaces are given in Table 4.25. The stationary point for total ω3 fatty acids content of the prepared concentrates by urea complexation predicted as a maximum of 92.3% at urea-to-fatty acid ratio, crystallization time and temperature of 4.5, 24 h and -10°C, respectively. The contour plot derived from the results of canonical analysis showed ellipsoidal contours at the maximum point (Figure 4.24).

In a contour plot, curves of equal response values are drawn on a plane whose coordinates represent the levels of the independent variables (factors). Each contour represents a specific value for the height of the surface, above the plane defined for combination of the levels of the factors. Therefore, different surface height values enables one to focus attention on the levels of the factors at which changes in the surface height occur. The contour plots show the combination of levels of urea-to-fatty acid ratio and crystallization temperature that can afford the same amount of total ω3 fatty acids in the concentrate.

At the maximum level of total ω3 fatty acids of the concentrate, DHA comprised a major proportion of it; its content was predicted to increase up to 68.2% (calculated from the predicted equation for DHA [Y_3]) (Table 4.25 and Figure 4.26), but the content of EPA in the concentrate was predicted to decrease with the increasing DHA content (Table 4.25 and Figure 4.25). Therefore, in order to obtain a high content of EPA in the concentrate, process variables should be changed but the DHA content

Table 4.25 Predicted and observed values for response variables (total ω3, EPA and DHA contents) in urea complexation experiment of seal blubber oil (SBO)

Response variables	Critical values of independent variables			Stationary point	Predicted value	Observed value[1]
	Urea-to-fatty acid ratio (w/w)	Crystallization time (h)	Crystallization temperature (°C)			
Total ω3 fatty acids (%)	4.5	24	-10	Maximum	92.3	88.2 ± 3.44
EPA (%)	3.3	18	-10	Minimum	9.36	-
DHA (%)	4.3	19	-11	Maximum	70.1	67.6 ± 2.54

[1]Mean ± SD (n = 3)

may be reduced accordingly. The contour plot for EPA content shows an increase with increasing urea-to-fatty acid ratio and crystallization temperature (Figure 4.24). At the same time, the content of DHA in the concentrate should also be considered using the contour plot approach. Therefore, if a high DHA and a low EPA content in the concentrate was desired, the urea-to-fatty acid ratio of 4.3, crystallization time of 19 h and crystallization temperature of -11°C may be suitable. However, if a higher content of EPA was needed in the concentrate, the levels of these three variables has to be changed and it would result in a lower content of DHA.

The adequacy of the models predicted was examined by performing independent experiments at the optimal conditions for both the total ω3 fatty acids and the DHA contents. Verification results revealed that the predicted values from these models were reasonably close to the observed values (Table 4.25). Therefore, using urea-to-fatty acid ratio of 4.5, crystallization temperature of -10°C and crystallization time of 24 h the amount of total ω3 fatty acids can be increased up to 88.2 ± 3.44% with a recovery (yield) of 24.5% of the weight of the original SBO by urea complexation.

The results of one-factor-at-a-time experiments do not reflect actual changes in the environment as they ignore interactions between factors which are present simultaneously. The response surface methodology can describe concomitant effects more fully and helps in more accurate optimization of factors that affect the process.

The central composite rotatable design (CCRD) is the preferred class of

experimental design for fitting polynomial models to analyze response surfaces of multi-factor combinations. The design is considered rotatable because the variance of the predicted response, Y, at the point X is a function only of the distance of the point from the design centre irrespective of the direction. This implies that the variance contour of predicted response are concentric circles. Also rotatable design has the property that the variance of predicted response does not change when the design is rotated around the centre point (Montgomery, 1984). CCRD with response surface methodology is a very effective tool for reducing the number of experiment combinations required without compromising the validity of results in studies where a large number of independent variables are included.

4.8 Preparation of ω3 concentrates from seal blubber oil (SBO) and menhaden oil (MHO) via enzymatic hydrolysis

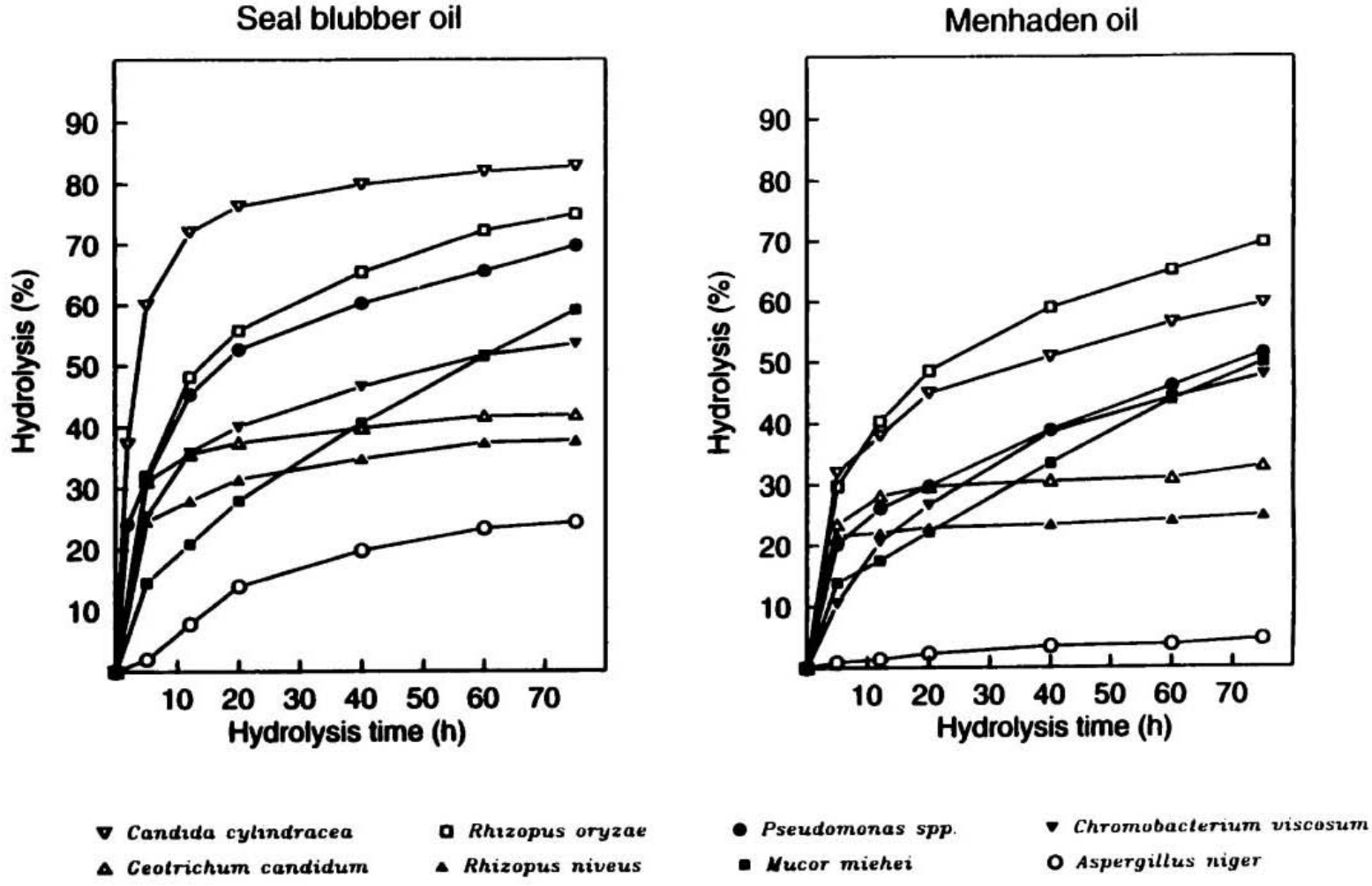
The acylglycerol form of PUFA is considered to be nutritionally more favourable than methyl or ethyl esters of fatty acids due to the impaired intestinal absorption of alkyl esters of ω3 fatty acids observed in laboratory animals (Hamazaki *et al.*, 1982; El Boustani *et al.*, 1987; Lawson and Hughes, 1988). It has also been shown that methyl and ethyl esters of unsaturated fatty acids hydrolyse at a slower rate than their corresponding TAG (Yang *et al.*, 1989). From a marketing point of view, mono-, di- and triacylglycerols are often promoted as being more "natural" than other fatty acid derivatives such as free fatty acids and their methyl or ethyl esters

(Haraldsson and Hoskuldsson, 1989). Therefore, in this study, preparation of ω3-PUFA enriched acylglycerols from SBO and MHO was attempted via enzymatic hydrolysis. Several microbial lipases (see Table 3.1) were used to screen suitable enzyme(s) in order to enrich ω3-PUFA in both oils.

4.8.1 Screening of microbial lipase(s) to enrich ω3 fatty acids of seal blubber oil (SBO) and menhaden oil (MHO)

Hydrolysis percentages of both oils as a function of time are shown in Figure 4.27. All microbial lipases tested were able to hydrolyse fatty acids in both oils, but at different rates. Among the lipases tested *CC*-lipase gave the highest degree of hydrolysis of SBO followed by *RO*-lipase, however, in MHO, *RO*-lipase gave the highest degree of hydrolysis. Other lipases studied gave lower degrees of hydrolysis than *CC* and *RO* in both oils. At a given hydrolysis time all lipases had considerably higher degrees of hydrolysis in SBO than in MHO. This difference may be due to the presence of higher amounts of PUFA, especially EPA and DHA in MHO than SBO which exhibit resistance to enzymatic hydrolysis. The degree of hydrolysis of SBO after 9 h of action of *CC*-lipase was 70% and the same degree of hydrolysis was achieved after 60 h of hydrolysis using *RO*-lipase. However, none of the lipases used were able to afford 70% hydrolysis in MHO over a 75 h hydrolysis period.

Figure 4.27 Time courses of seal blubber oil (SBO) and menhaden oil (MHO) hydrolysis by different microbial lipases (deviation from mean for each data point is within ± 2% of the absolute values, n = 3)


Seal blubber oil
Menhaden oil
Hydrolysis (%)
Hydrolysis time (h)
10
20
30
40
50
60
70
80
90
▽ Candida cylindracea
□ Rhizopus oryzae
● Pseudomonas spp.
▼ Chromobacterium viscosum
△ Geotrichum candidum
▲ Rhizopus niveus
■ Mucor miehei
○ Aspergillus niger

Figures 4.28 and 4.29 show the changes in the content of total ω3 fatty acids, EPA and DHA in the non-hydrolyzed fraction (acylglycerols) of both oils upon enzymatic (lipase) hydrolysis. Among the lipases tested *CC*-lipase significantly ($P < 0.05$) increased the total ω3 fatty acids, EPA and DHA contents of SBO as the hydrolysis reaction progressed. However, at 80% hydrolysis, the same lipase resulted in a decrease in the content of EPA. After 12 h of hydrolysis, *CC*-lipase gave a two-fold increase of total ω3 fatty acids in SBO. But in MHO this lipase was able to increase the content of total ω3 fatty acids only by 11% (from 30% in original oil to 41% after hydrolysis) during the same hydrolysis period. In MHO the highest contents of total ω3 fatty acids and DHA were obtained by *RO*-lipase-assisted hydrolysis. But using this lipase the EPA content of both oils was decreased during the course of the reaction (Figures 4.28b and 4.29b). This may be due to the fact that *RO*-lipase selectively hydrolyses EPA in the oils by exhibiting acyl-chain specificity. Tanaka *et al.* (1992) have also reported that EPA content of tuna oil was decreased upon hydrolysis by lipase from *Rhizopus* spp; *R. delemer* (*RD*) and *R. javanicus* (*RJ*). Kotting and Eble (1994) have reported that lipases from *Rhizopus* spp are 1,3-positional specific. In SBO the EPA content was decreased from 6.4 to 4.3% during a 75 h *RO*-lipase-assisted hydrolysis. However, upon hydrolysis of MHO by *RO*-lipase, EPA content was increased up to 9 h of hydrolysis, after which it began to decline. During 75 h of hydrolysis the content of EPA in MHO decreased from 13.2 to 12.5%.

Figure 4.28 Changes of total ω3 fatty acids (a), EPA (b) and DHA (c) contents during hydrolysis of seal blubber oil (SBO) by different microbial lipases, (deviation from mean for each data point is within ± 2.5% of the absolute values, n = 3)

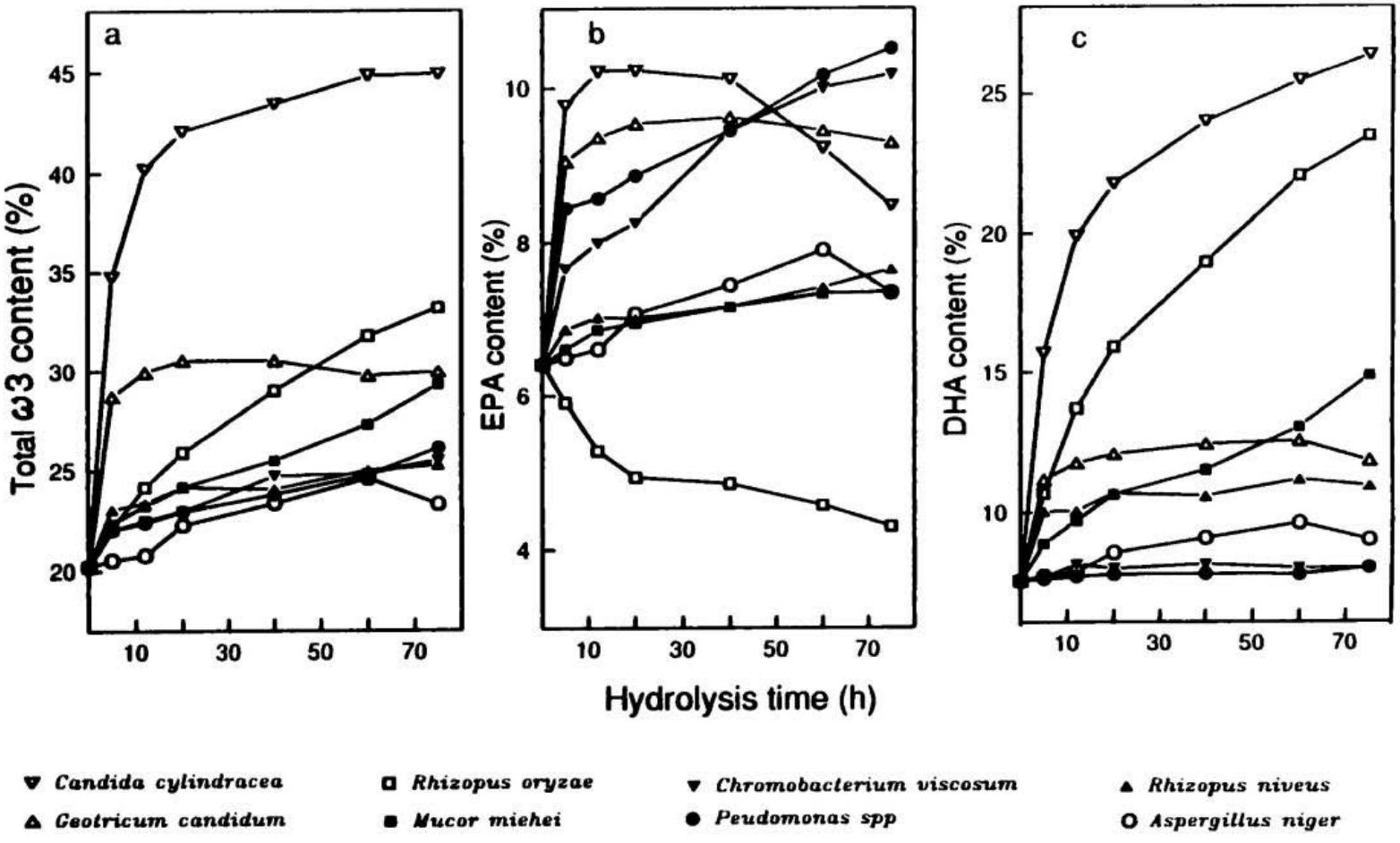
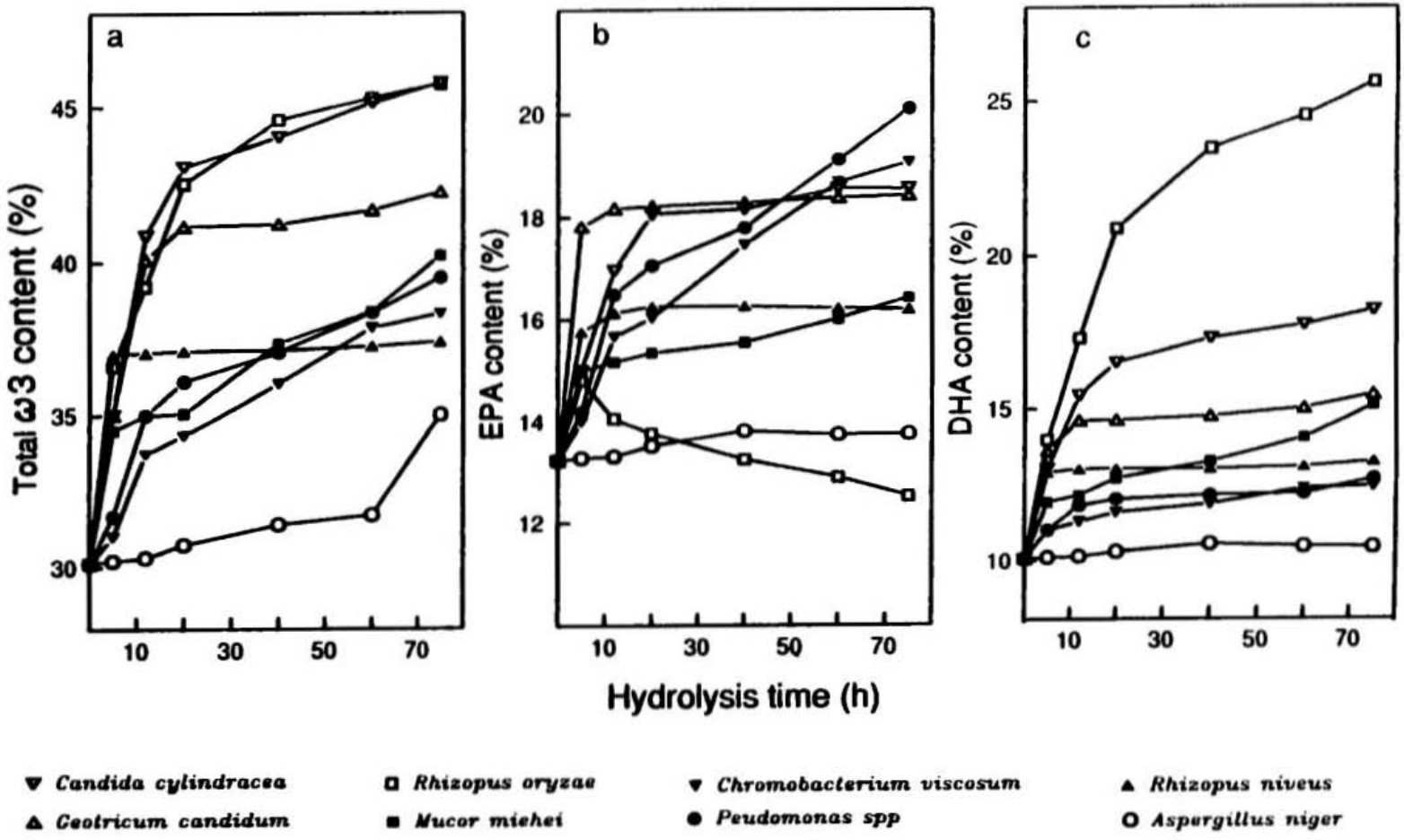

a
b
c
Total ω3 content (%)
EPA content (%)
DHA content (%)
Hydrolysis time (h)
Candida cylindracea
Geotricum candidum
Rhizopus oryzae
Mucor miehei
Chromobacterium viscosum
Peudomonas spp
Rhizopus niveus
Aspergillus niger

Figure 4.29 Changes of total ω3 fatty acids (a), EPA (b) and DHA (c) contents during hydrolysis of menhaden oil (MHO) by different microbial lipases. (deviation from mean for each data point is within ± 2.5% of the absolute values, n = 3)


a
b
c
Total ω3 content (%)
EPA content (%)
DHA content (%)
Hydrolysis time (h)
45
40
35
30
20
18
16
14
12
25
20
15
10
10
30
50
70
▼ Candida cylindracea
△ Geotricum candidum
□ Rhizopus oryzae
■ Mucor miehei
▼ Chromobacterium viscosum
● Peudomonas spp
▲ Rhizopus niveus
○ Aspergillus niger

Therefore, the rate of hydrolysis of EPA from SBO was much higher than that from MHO. This may be due to the cumulative effect of both 1,3-positional and acyl-chain specificity of *RO*-lipase. In Section 4.5, it was demonstrated that EPA was located mainly in *sn*-1 and *sn*-3 positions of the TAG in SBO, however, EPA in MHO was equally distributed over the *sn*-2 and *sn*-3 positions and was present only in small amounts in the *sn*-1 position (Table 4.19). Therefore, the higher hydrolysis rate of EPA from SBO may reflect the abundance of this fatty acid in the *sn*-1 and *sn*-3 positions which is highly vulnerable to 1,3-specific *RO*-lipase hydrolysis.

In MHO, the total ω3 fatty acid content increased from 30% (original oil) to 44.6, 44.1 and 41.7% after a 40 h hydrolysis by *RO*-, *CC*- and *GC*-lipases, respectively. The corresponding increase in DHA content in this oil was from 10.1% to 23.5, 17.3 and 14.8%, respectively. In SBO the maximum increase of total ω3 fatty acids, from 20.2 to 45.0%, was reached when *CC*-lipase was used under similar experimental conditions. Other lipases were less effective in increasing the content of ω3 fatty acids in the oil and gave a maximum of 33.2, 30.6, 29.3, 26.1, 25.5, 25.3 and 24.6% using *RO*-, *GC*-, *MM*-, *PS*-, *CV*-, *RN*- and *AN*-lipases, respectively, during the course of hydrolysis. The lipase from *Pseudomonas spp.* showed a slightly higher degree of hydrolysis for both oils than lipases from *CV*, *GC*, *RN*, *MM* and *AN*. Although enrichment of total ω3 fatty acids and DHA in the oils was small, the content of EPA was increased from 6.4 to 10.5% in SBO and from 13.2 to 20.1% in MHO.

Among the lipases tested in this experiment, *AN*-lipase gave the lowest total ω3 fatty acids in the non-hydrolysed fraction of both oils. Even though this lipase is 1,3-specific, it was not able to hydrolyse much of the saturated fatty acids present in the *sn*-1 position of MHO (about 43.3% of saturated fatty acids are present in the *sn*-1 position of MHO, Table 4.19). Similarly, 1,3-specific *MM*-lipase was not able to hydrolyse much of the saturated fatty acids in both marine oils. Therefore, no correlation was found between positional specificity and ease of hydrolysis of fatty acids for both oils in this study. This implies that the concept of positional specificity of lipases alone cannot be used to explain the observed hydrolysis differences of both oils. Hoshino *et al.* (1990) have shown that the course of hydrolysis of marine oils by lipases is decided by cumulative effects of various factors such as differences in substrate specificities, including fatty acid and positional specificity of lipase, differences in the rate of the reverse reaction which occurs during hydrolysis, differences in fatty acid composition of the oil and reactivity of each lipase towards partial acylglycerols (mono- and diacylglycerols).

Among the lipases tested, *CC*-lipase seems to be the most active one in increasing the contents of total ω3 fatty acids, EPA and DHA in the non-hydrolysed fraction of both SBO and MHO. *RO*-lipase was also effective in enriching the total ω3 fatty acids and DHA in both oils but this lipase selectively hydrolysed EPA from the TAG of both oils, therefore, the contents of EPA in the final products were lowered. Use of enzymes to produce ω3 fatty acid concentrates has an advantage over traditional

methods (chromatographic separation, molecular distillation, etc.) of concentration since such methods involve extremes of pH and high temperatures which may partially destroy the natural all-*cis* ω3-PUFA by oxidation and by *cis-trans* isomerization or double bond migration. Therefore, the mild conditions (temperature less than 50°C, pH between 6-8 and less chemicals) used in enzymatic hydrolysis provide a promising alternative that could also save energy and increase product selectivity. In addition, the enzymatic hydrolysis method produces ω3 fatty acids in the acylglycerol form and this is considered to be nutritionally favourable.

Bottino *et al.* (1967) have illustrated the mechanism of resistivity of lipases towards the long-chain ω3-PUFA in marine oils. The presence of carbon-carbon *cis* double-bonds in the fatty acids results in bending of the chains. Therefore, the terminal methyl group of the fatty acid lies close to the ester bond which may cause a steric hinderance effect on lipases. The high bending effect of EPA and DHA due to the presence of 5 and 6 double-bonds, respectively, enhanced the steric hinderance effect, therefore, lipases cannot reach the ester-linkage of these fatty acids and glycerol. However, saturated or monounsaturated fatty acids do not present any barriers to lipase and they could be easily hydrolysed. Therefore, fatty acid selectivity of a lipase for EPA and DHA has allowed separation of these fatty acids from the remaining fatty acids in marine oils, which is very important in the production of ω3 fatty acid concentrates. Also, lipases have been frequently used to discriminate between EPA and DHA in concentrates containing both these fatty acids; this enables the preparation of

EPA- as well as DHA-enriched concentrates.

Research in recent years has received much attention for using microbial lipases to produce ω3 fatty acid concentrates either by hydrolysis or transesterification reactions of marine oils. Tanaka *et al.* (1992) have used six types of microbial lipases (*AN*, *CC*, *CV*, *RD*, *RJ* and *PS*) to hydrolyse tuna oil and found that *CC*-lipase was the most effective one in increasing the DHA content in the concentrates. It increased the DHA content in the non-hydrolysed fraction to three times that in the original tuna oil, however, other lipases did not increase DHA content in the oil. Hoshino *et al.* (1990) have also used several lipases for selective hydrolysis of cod liver and sardine oils. The best hydrolysis results were obtained for the non-regiospecific *CC*- and 1,3-specific *AN*-lipases, but none of the lipases was able to raise the EPA content of the acylglycerols considerably. However, over 50% of total ω3 fatty acids were produced when these two lipases were employed. Shimada *et al.* (1994) have reported that hydrolysis of tuna oil by *GC*-lipase has increased the content of both EPA and DHA from 32.1 to 57.5%. Osada *et al.* (1992) have employed *CV*- and *CC*-lipases for direct esterification of glycerol with individual free fatty acids, including EPA and DHA. The *CV*-lipase was superior to *CC*-lipase and 89-95% incorporation levels were obtained. With the latter lipase, 71-75% incorporation was obtained for all fatty acids, except DHA which reached 63% incorporation. More recently, Akoh *et al.* (1996) were able to incorporate EPA into evening primrose oil (EPO) by its esterification with *Candida*

antarctica-lipase. They were able to incorporate up to 43% EPA in EPO with this lipase.

4.8.2 Optimization of enzymatic hydrolysis of seal blubber oil (SBO) and menhaden oil (MHO) for preparation of ω3 fatty acids concentrates

According to the screening study of lipases for their efficiency at concentrating ω3 fatty acids by selective hydrolysis of SBO and MHO, *Candida cylindracea* lipase (*CC*-lipase) exhibited the highest efficiency at the concentration of 200 U lipase/g oil and the reaction temperature of 35°C (Section 4.8.1). However, there are many other factors that affect the product yield (concentration of ω3 fatty acids) of lipase-catalysed hydrolysis of TAG, which include, pH and temperature of the reaction medium, reaction time, substrate and enzyme concentrations, etc. (Yamane, 1987). Therefore, it is necessary to study these factors collectively to find the optimum reaction conditions to obtain the maximum ω3-PUFA from SBO and MHO by the most effective enzyme; *Candida cylindracea*. For this study, reaction parameters such as enzyme concentration (X_4), reaction time (X_5) and reaction temperature (X_6) were selected for optimization. Enzyme concentration and reaction time are major factors that affect the cost of preparation of ω3 fatty acids concentrates via lipase hydrolysis. The *CC*-lipase possesses a wide range of optimum pH activity (5.0 - 8.0), therefore, in accordance with the literature (Hishino *et al.*, 1990), a pH of 7.0 was used in the present studies. Furthermore, temperature of the reaction medium and also reaction time can be considered important as they influence the oxidative status of the prepared

ω3 fatty acids concentrates.

4.8.2.1 Diagnostic checking of fitted models

Tables 4.26 and 4.27 provide the values obtained for contents of PUFA, total ω3 fatty acids, EPA, DPA and DHA and percentage recovery (yield) of the non-hydrolysed fractions of SBO and MHO, respectively. For all these responses (PUFA, total ω3 fatty acids, EPA, DHA and yield) higher values were obtained for MHO than SBO, except DPA which was higher in SBO than MHO. The contents of PUFA, total ω3 fatty acids, EPA and DHA were higher in the original MHO than SBO, while the content of DPA was higher in the original SBO than MHO (Table 4.2). Therefore, during enzymatic hydrolysis a similar pattern of abundance of these fatty acids was observed in the non-hydrolysed fraction of SBO and MHO, since these fatty acids are resistant to lipase hydrolysis and are retained in the intact acylglycerol form.

The data obtained for total ω3 fatty acids, EPA and DHA from the nineteen experiments points in both SBO and MHO were used for statistical analysis to optimize the process variables; enzyme concentration, reaction time and reaction temperature. Multiple regression coefficients obtained using the least squares method to predict quadratic polynomial models for total ω3 fatty acids (Y_1), EPA (Y_2) and DHA (Y_3) for both SBO and MHO are summarized in Tables 4.28 and 4.29, respectively. Examination of these coefficients with the t-test indicated that in SBO, linear and quadratic terms for test variables, enzyme concentration, reaction temperature were

Table 4.26 Central composite design arrangement and responses for enzymatic hydrolysis experiment of seal blubber oil (SBO)

Run	Variable levels			Responses (Y)					
	Enzyme conc.[a] (X_4)	Time[b] (X_5)	Temperature[c] (X_6)	Yield[d] (%)	PUFA (%)	EPA (%)	DPA (%)	DHA (%)	Total ω3 (%)
1	140	12	30	35.8	43.3	13.9	7.62	17.6	41.1
2	420	12	30	29.2	45.7	13.2	7.80	20.5	43.3
3	140	38	30	25.4	46.2	13.4	8.10	20.5	43.9
4	420	38	30	22.2	49.3	12.8	8.01	24.2	46.8
5	140	12	40	40.8	41.4	14.1	7.92	17.4	41.4
6	420	12	40	29.1	49.3	14.9	8.12	22.1	47.0
7	140	38	40	21.8	51.5	13.9	8.30	25.3	49.3
8	420	38	40	23.6	51.2	12.2	8.36	26.6	48.9
9	45	25	35	41.1	41.2	13.9	7.80	15.4	39.0
10	515	25	35	25.6	50.9	15.0	6.88	25.0	48.5
11	280	3	35	35.0	45.0	14.0	7.63	19.4	42.9
12	280	47	35	23.5	56.2	15.5	8.20	26.3	53.9
13	280	25	26.6	26.1	44.5	12.0	8.30	19.9	42.1
14	280	25	43.4	23.1	47.8	14.1	8.31	21.3	45.5
15	280	25	35	25.6	53.2	17.1	7.13	24.3	50.9
16	280	25	35	25.9	53.2	16.3	7.43	24.3	50.8
17	280	25	35	27.1	53.8	17.1	7.74	24.8	51.5
18	280	25	35	29.4	54.0	16.3	7.38	26.2	52.7
19	280	25	35	25.9	54.6	16.5	7.47	26.4	52.2

[a]Enzyme concentration (U/g oil), [b]reaction time (h), [c]reaction temperature (°C), [d]percentage recovery of non-hydrolysed fraction of SBO

Table 4.27 Central composite design arrangement and responses for enzymatic hydrolysis experiment of menhaden oil (MHO)

Run	Variable levels			Responses (Y)					
	Enzyme conc.[a] (X_4)	Time[b] (X_5)	Temperature[c] (X_6)	Yield[d] (%)	PUFA (%)	EPA (%)	DPA (%)	DHA (%)	Total ω3 (%)
1	140	12	30	47.5	48.8	17.2	3.89	17.5	45.3
2	420	12	30	44.0	48.2	17.9	3.79	18.7	44.7
3	140	38	30	52.9	50.5	18.5	4.30	19.2	46.7
4	420	38	30	36.2	54.3	19.8	4.37	21.5	50.4
5	140	12	40	54.2	49.7	18.3	4.03	17.4	46.1
6	420	12	40	43.6	52.1	19.6	3.95	19.6	48.5
7	140	38	40	52.7	54.3	18.5	3.96	21.9	50.7
8	420	38	40	40.1	56.5	19.9	4.46	22.9	52.7
9	45	25	35	65.7	42.0	17.0	3.59	14.6	38.6
10	515	25	35	49.3	53.0	20.2	3.92	21.2	49.5
11	280	3	35	64.4	45.7	16.9	3.36	19.8	45.7
12	280	47	35	45.7	55.8	20.2	4.61	24.4	55.8
13	280	25	26.6	37.8	51.1	16.7	4.58	20.3	47.5
14	280	25	43.4	47.0	55.5	20.2	4.19	22.0	51.8
15	280	25	35	50.9	57.2	20.3	3.86	24.8	53.7
16	280	25	35	49.2	57.0	20.0	3.78	25.7	52.9
17	280	25	35	46.8	55.9	20.9	3.81	24.6	52.4
18	280	25	35	48.0	58.0	21.3	3.94	25.8	54.4
19	280	25	35	49.1	58.1	20.2	3.96	25.1	54.5

[a]Enzyme concentration (U/g oil), [b]reaction time (h), [c]reaction temperature (°C), [d]percentage recovery of non-hydrolysed fraction of MHO

Table 4.28 Regression coefficients of predicted quadratic polynomial model for response variables (total ω3, EPA and DHA contents) in enzymatic hydrolysis experiment of seal blubber oil (SBO)

Variables[1]	Coefficients (β)		
	Total ω3, % (Y_1)	EPA, % (Y_2)	DHA, % (Y_3)
Intercept	-110.5355***	-48.60494***	-61.24276**
Linear			
X_4	0.101038**	0.006971	0.067948**
X_5	0.375423	0.185442	0.031836
X_6	7.691455***	3.410418***	3.970611***
Quadratic			
X_{44}	-0.000139***	-0.000037***	-0.000082***
X_{55}	-0.006311**	-0.003578	-0.003950
X_{66}	-0.108737***	-0.048938***	-0.058109***
Interaction			
X_{45}	-0.000371	-0.000018	-0.000179
X_{46}	-0.000000	0.000436	-0.000079
X_{56}	0.006808	0.000038	0.011077
X_{456}	-	-	-
R^2	0.94	0.91	0.93

[1]See Table 4.26 for description of abbreviations

**$P < 0.05$

***$P < 0.01$

Table 4.29 Regression coefficients of predicted quadratic polynomial model for response variables (total ω3, EPA and DHA contents) in enzymatic hydrolysis experiment of menhaden oil (MHO)

Variables[1]	Coefficients (β)		
	Total ω3, % (Y_1)	EPA, % (Y_2)	DHA, % (Y_3)
Intercept	-37.56674	-28.04923**	-67.05821***
Linear			
X_4	0.091352**	0.018149	0.086240***
X_5	0.257621	0.415054**	0.242539
X_6	3.779578**	2.127598***	4.135811***
Quadratic			
X_{44}	-0.000168***	-0.000032***	-0.000134***
X_{55}	-0.005393	-0.003720***	-0.006709***
X_{66}	-0.052472**	-0.027114***	-0.059370***
Interaction			
X_{45}	0.000277	0.000039	-0.000014
X_{46}	0.000275	0.000114	-0.000044
X_{56}	0.003462	-0.005389	0.005981
X_{456}	-	-	-
R^2	0.92	0.89	0.97

[1]See Table 4.27 for description of abbreviations

**$P < 0.05$

***$P < 0.01$

significant ($P < 0.01$ or 0.05) for total ω3 content (Table 4.28). The linear term for reaction time for total ω3 content was not significant ($P > 0.05$) but quadratic term was significant ($P < 0.05$). For the content of EPA in SBO, only the linear term of reaction temperature and the quadratic terms of enzyme concentration and reaction temperature were highly significant ($P < 0.01$). The coefficient obtained for DHA content in SBO showed that linear and quadratic terms of enzyme concentration and reaction temperature were significant ($P < 0.01$ or 0.05), whereas reaction time was not. In MHO, linear terms of enzyme concentration and reaction temperature were significant ($P < 0.01$ or 0.05) for both total ω3 fatty acids and DHA contents but for EPA content reaction time and reaction temperature were significant at $P < 0.05$ and $P < 0.01$, respectively. All quadratic terms were highly significant ($P < 0.01$) for EPA and DHA contents of MHO, whereas for total ω3 fatty acids content only enzyme concentration and reaction temperature were significant at $P < 0.01$ and $P < 0.05$, respectively. No statistically significant interactions existed between any two of the three factors observed. Therefore, these results suggest that linear and/or quadratic effect of enzyme concentration, reaction time and reaction temperature are the primary determining factors for the amounts of total ω3 fatty acids, EPA and DHA in the prepared concentrates of both SBO and MHO by *CC*-lipase hydrolysis. The contribution of linear and quadratic terms to the models of SBO concentrate was 0.44 and 0.49 for total ω3 fatty acids, 0.12 and 0.77 for EPA and 0.63 and 0.26 for DHA, respectively. The contribution of these two terms to the models of MHO concentrate was 0.45 and 0.46

for total ω3 fatty acids, 0.51 and 0.35 for EPA and 0.32 and 0.64 for DHA, respectively. The coefficients of independent variables, enzyme concentration (X_4), reaction time (X_5) and reaction temperature (X_6) determined for quadratic polynomial models for total ω3 fatty acids (Y_1), EPA (Y_2) and DHA (Y_3) of prepared concentrate of SBO (Table 4.28) were:

$$Y_1 = -110.536 + 0.101X_4 + 0.375X_5 + 7.691X_6 - 0.00014X_4^2 - 0.00631X_5^2 - 0.10873X_6^2 - 0.00037X_4X_5 + 0.00681X_5X_6$$

$$Y_2 = -48.605 + 0.007X_4 + 0.185X_5 + 3.410X_6 - 0.00004X_4^2 - 0.00358X_5^2 - 0.04894X_6^2 - 0.00002X_4X_5 + 0.00044X_4X_6 + 0.00004X_5X_6$$

$$Y_3 = -61.243 + 0.068X_4 + 0.032X_5 + 3.971X_6 - 0.00082X_{42} - 0.00395X_5^2 - 0.05811X_6^2 - 0.00018X_4X_5 - 0.00008X_4X_6 + 0.01108X_5X_6$$

The quadratic polynomial models for Y_1, Y_2 and Y_3 for the prepared concentrate of MHO (Table 4.29) were:

$$Y_1 = -37.567 + 0.091X_4 + 0.258X_5 + 3.779X_6 - 0.00017X_4^2 - 0.00539X_5^2 - 0.05247X_6^2 + 0.00028X_4X_5 + 0.00028X_4X_6 + 0.00346X_5X_6$$

$$Y_2 = -28.049 + 0.018X_4 + 0.415X_5 + 2.128X_6 - 0.00003X_4^2 - 0.00372X_5^2 - 0.02711X_6^2 + 0.00004X_4X_5 + 0.00011X_4X_6 - 0.00539X_5X_6$$

$$Y_3 = -67.058 + 0.086X_4 + 0.243X_5 + 4.136X_6 - 0.00013X_4^2 - 0.00671X_5^2 - 0.05937X_6^2 - 0.00001X_4X_5 - 0.00004X_4X_6 + 0.00598X_5X_6$$

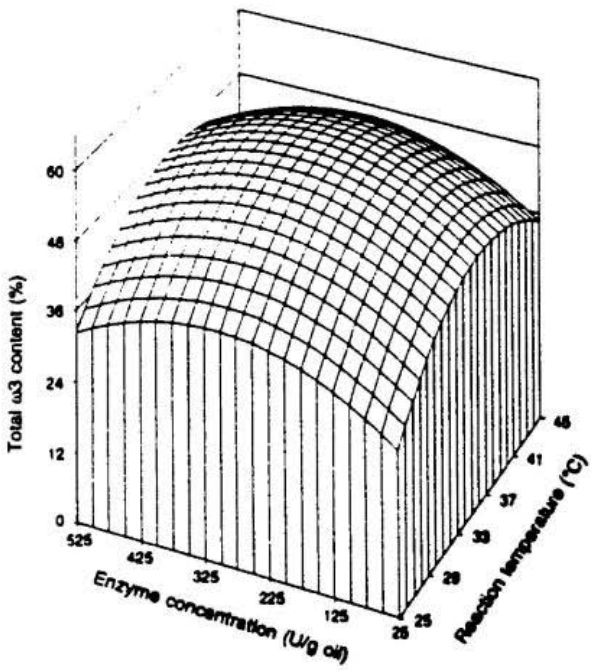
These models predicted for Y_1, Y_2 and Y_3 for both oils were adequate as indicated by error analysis that showed non-significant ($P > 0.05$) lack-of-fit. The regression models for total ω3 fatty acids, EPA and DHA were highly significant ($P < 0.01$) with satisfactory coefficients of determination (R^2) of 0.94, 0.91 and 0.93 for SBO (Table 4.28) and 0.92, 0.89 and 0.97 for MHO (Table 4.29), respectively.

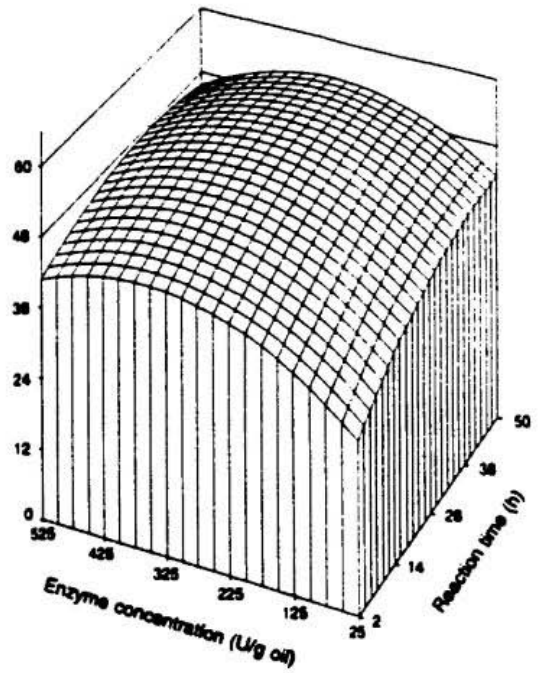
4.8.2.2 Response surface plotting and optimization based on canonical analysis

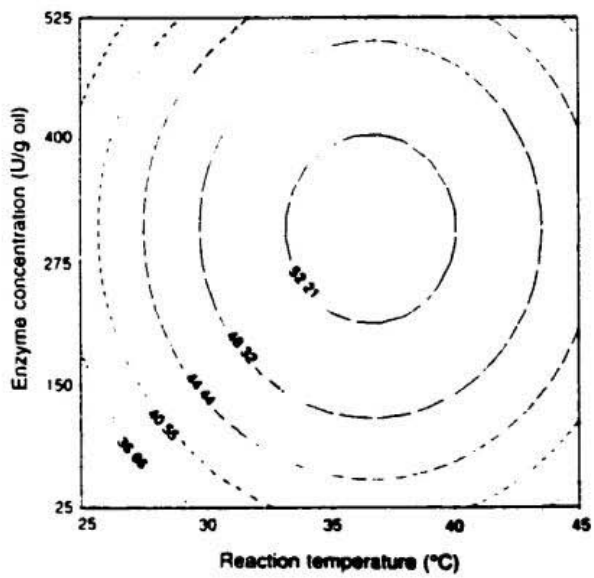
The relationship between independent and dependent variables are shown in the three-dimensional representation of the response surfaces generated for the models developed for the contents of total ω3 fatty acids, EPA and DHA in SBO concentrate (Figures 4.30, 4.31 and 4.32, respectively). Canonical analysis was performed on the predicted quadratic polynomial models to examine the overall shape of the response surface curves and used to characterize the nature of the stationary points. This showed that all three responses; total ω3 fatty acids, EPA and DHA contents in SBO had maximum stationary points (Table 4.30). The maximum of 53.5% for the total ω3 fatty acids was predicted at an enzyme concentration, reaction time and reaction temperature of 308 U/g oil, 40 h and 37°C, respectively. The EPA content of the SBO concentrate was predicted to increase from 6.41% (original oil) to 16.5% (concentrate) at an enzyme concentration of 297 U/g oil, reaction time of 26 h and reaction temperature of 36°C. The DHA content was maximized to 28.1% at an enzyme concentration of 342 U/g oil, reaction time of 51 h and reaction temperature 39°C. The maximum points for total ω3 fatty acids, EPA and DHA contents are clearly indicated graphically in the contour plots (Figures 4.30, 4.31 and 4.32, respectively) and also located in the experimental region.

Response surfaces for total ω3 fatty acids, EPA and DHA contents of MHO concentrate are given in Figures 4.33, 4.34 and 4.35, respectively. Canonical analysis of MHO data on these responses also showed maximum stationary points similar to

Figure 4.30 Response surface and contour plots for the effect of enzyme concentration, reaction time and reaction temperature on total ω3 fatty acid content of the prepared concentrate of seal blubber oil (SBO) by enzymatic hydrolysis


Total ω3 content (%)
60
48
36
24
12
0
525
425
325
225
125
25
Enzyme concentration (U/g oil)
25
29
33
37
41
45
Reaction temperature (°C)


60
48
36
24
12
0
525
425
325
225
125
25
Enzyme concentration (U/g oil)
2
14
26
38
50
Reaction time (h)


Enzyme concentration (U/g oil)
525
400
275
150
25
25
30
35
40
45
Reaction temperature (°C)
53.21
48.32
44.44
40.55
36.66

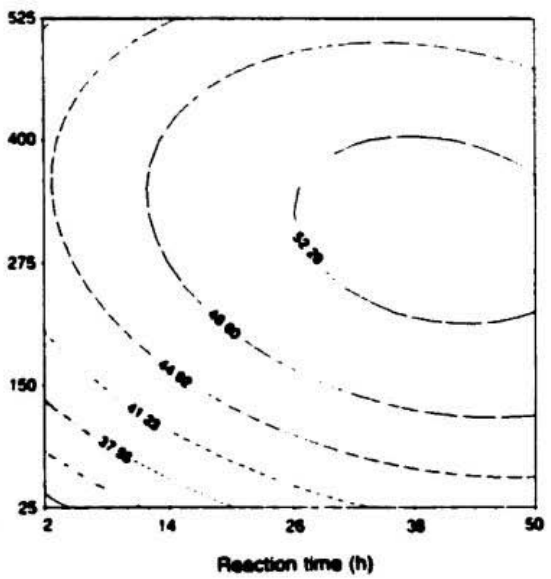

525
400
275
150
25
2
14
26
38
50
Reaction time (h)
52.28
48.60
44.92
41.23
37.55

Figure 4.31 Response surface and contour plots for the effect of enzyme concentration, reaction time and reaction temperature on EPA content of the prepared concentrate of seal blubber oil (SBO) by enzymatic hydrolysis

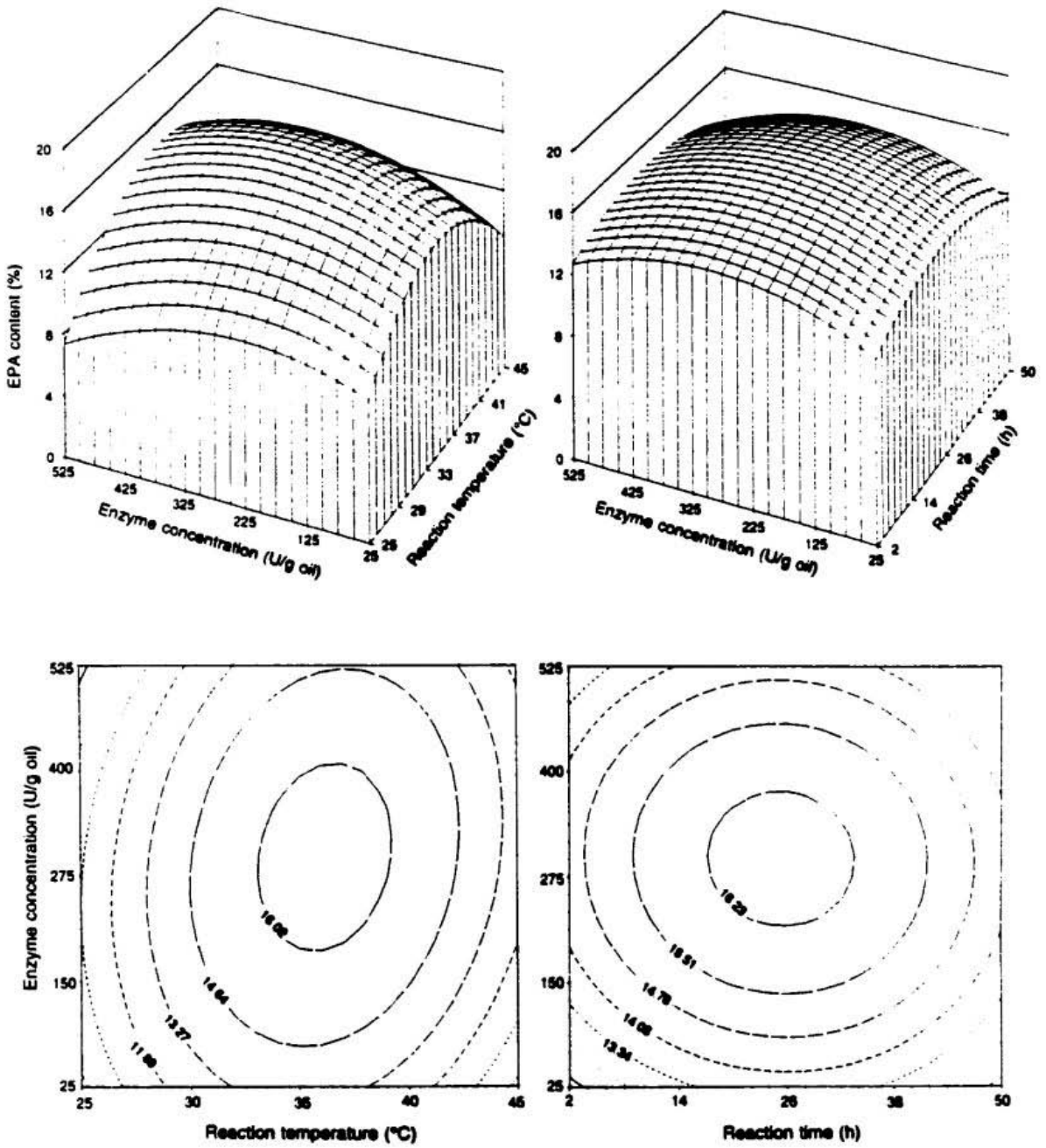

EPA content (%)
20
16
12
8
4
0
525
425
325
225
125
25
Enzyme concentration (U/g oil)
25
29
33
37
41
45
Reaction temperature (°C)
2
14
26
38
50
Reaction time (h)
Enzyme concentration (U/g oil)
400
275
150
16.08
14.64
13.27
11.89
Reaction temperature (°C)
30
35
40
16.23
15.51
14.79
14.08
13.36
Reaction time (h)

Figure 4.32 Response surface and contour plots for the effect of enzyme concentration, reaction time and reaction temperature on DHA content of the prepared concentrate of seal blubber oil (SBO) by enzymatic hydrolysis

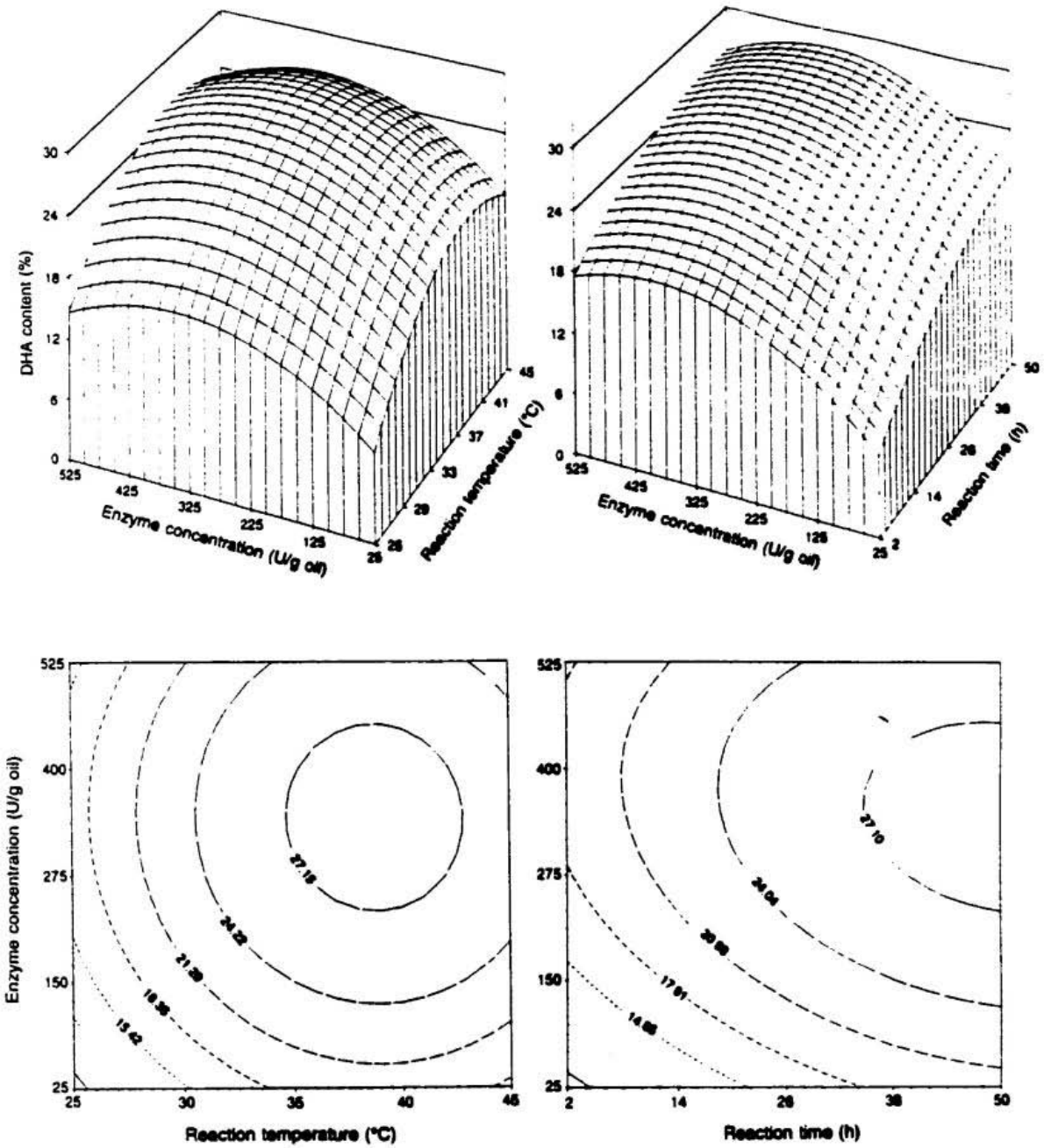
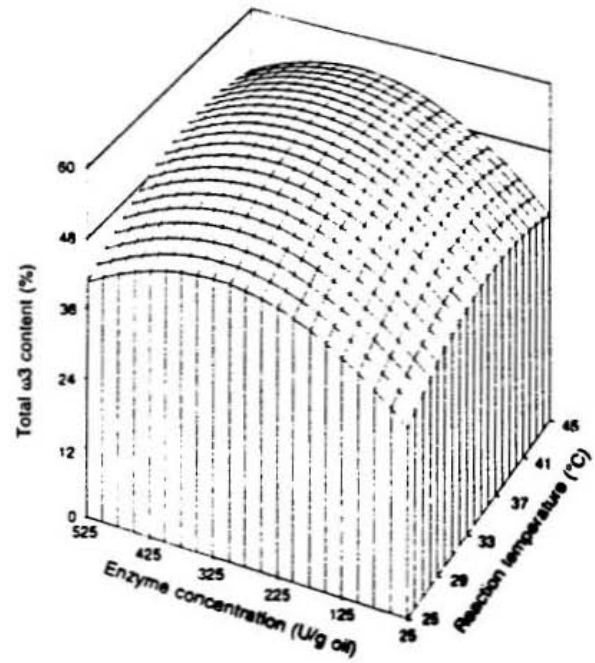

DHA content (%)
30
24
18
12
6
0
525
425
325
225
125
25
Enzyme concentration (U/g oil)
26
29
33
37
41
45
Reaction temperature (°C)
2
14
26
38
50
Reaction time (h)
Enzyme concentration (U/g oil)
400
275
150
27.19
24.22
21.26
18.30
15.42
30
35
40
Reaction temperature (°C)
27.10
24.04
20.99
17.91
14.85
Reaction time (h)

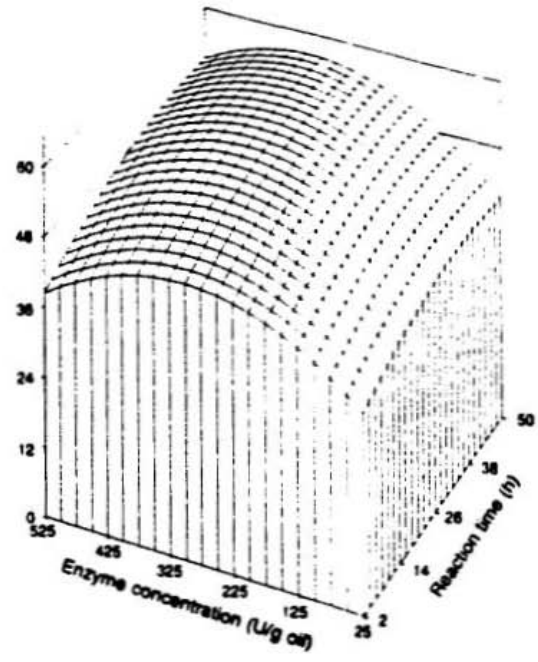
Table 4.30 Predicted and observed values for response variables (total ω3, EPA and DHA contents) in enzymatic hydrolysis experiment of seal blubber oil (SBO)

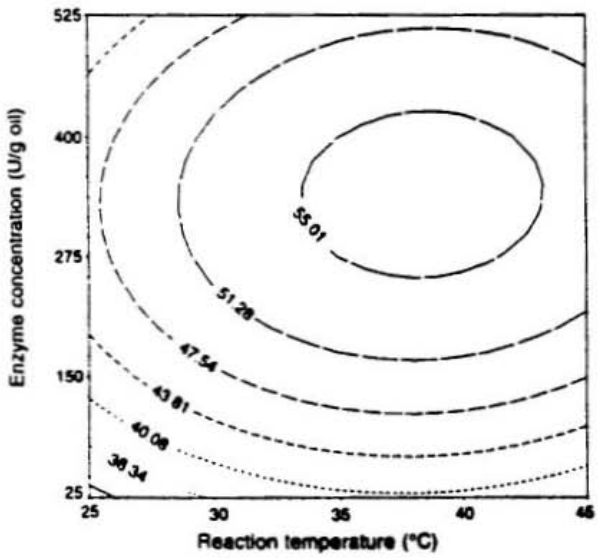
Response variables	Critical values of independent variables			Stationary point	Predicted value	Observed value[1]
	Enzyme concentration (U/g oil)	Reaction time (h)	Reaction temperature (°C)			
Total ω3 fatty acids (%)	308	40	37	Maximum	53.5	54.3 ± 3.22
EPA (%)	297	26	36	Maximum	16.5	19.4 ± 2.51
DHA (%)	342	51	39	Maximum	28.1	27.6 ± 2.11

[1]Mean ± SD (n = 3)

Figure 4.33 Response surface and contour plots for the effect of enzyme concentration, reaction time and reaction temperature on total ω3 fatty acid content of the prepared concentrate of menhaden oil (MHO) by enzymatic hydrolysis


Total ω3 content (%)
60
48
36
24
12
0
525
425
325
225
125
25
Enzyme concentration (U/g oil)
25
29
33
37
41
45
Reaction temperature (°C)


60
48
36
24
12
0
525
425
325
225
125
25
Enzyme concentration (U/g oil)
2
14
26
38
50
Reaction time (h)


Enzyme concentration (U/g oil)
525
400
275
150
25
55.01
51.28
47.54
43.81
40.08
36.34
25
30
35
40
45
Reaction temperature (°C)

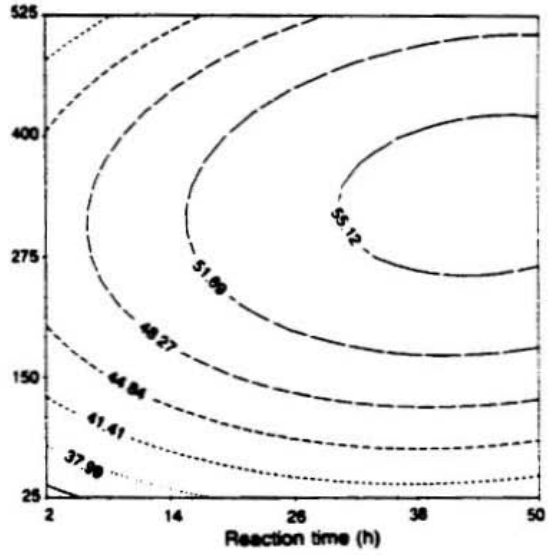
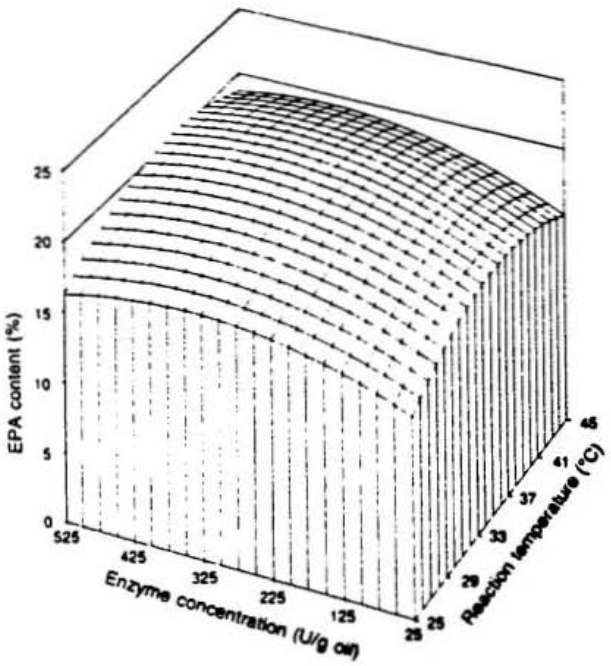
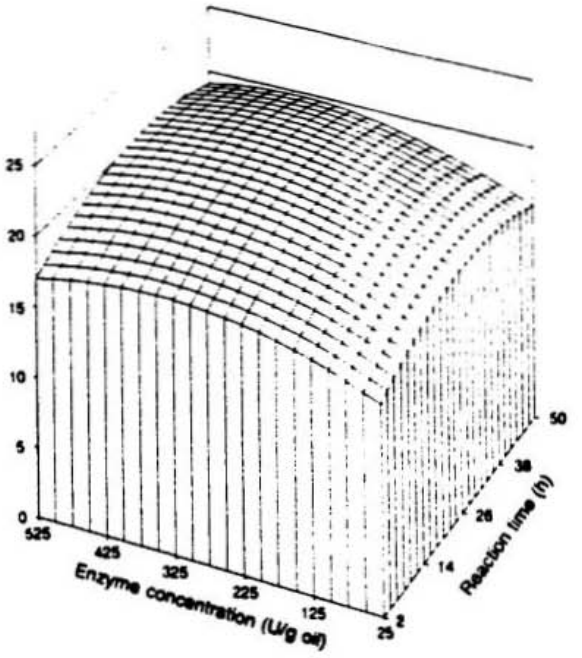
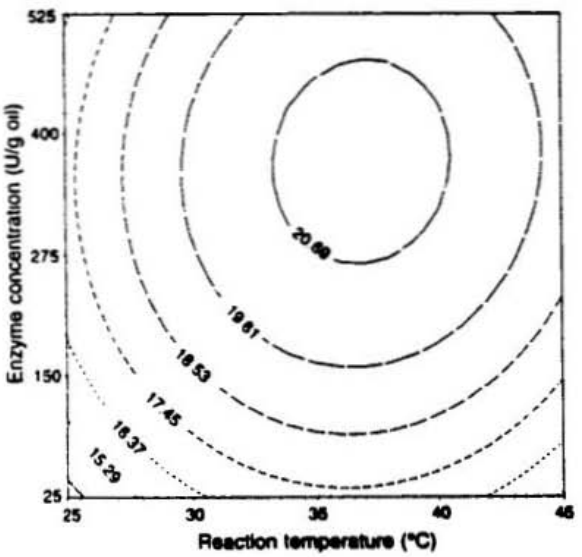

525
400
275
150
25
55.12
51.69
48.27
44.84
41.41
37.98
2
14
26
38
50
Reaction time (h)

Figure 4.34 Response surface and contour plots for the effect of enzyme concentration, reaction time and reaction temperature on EPA content of the prepared concentrate of menhaden oil (MHO) by enzymatic hydrolysis


EPA content (%)
25
20
15
10
5
0
525
425
325
225
125
25
Enzyme concentration (U/g oil)
25
29
33
37
41
45
Reaction temperature (°C)


25
20
15
10
5
0
525
425
325
225
125
25
Enzyme concentration (U/g oil)
2
14
26
38
50
Reaction time (h)


Enzyme concentration (U/g oil)
525
400
275
150
25
20.69
19.61
18.53
17.45
16.37
15.29
25
30
35
40
45
Reaction temperature (°C)

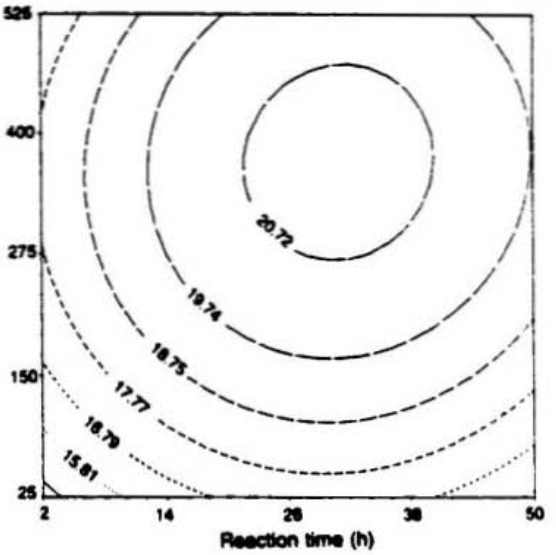
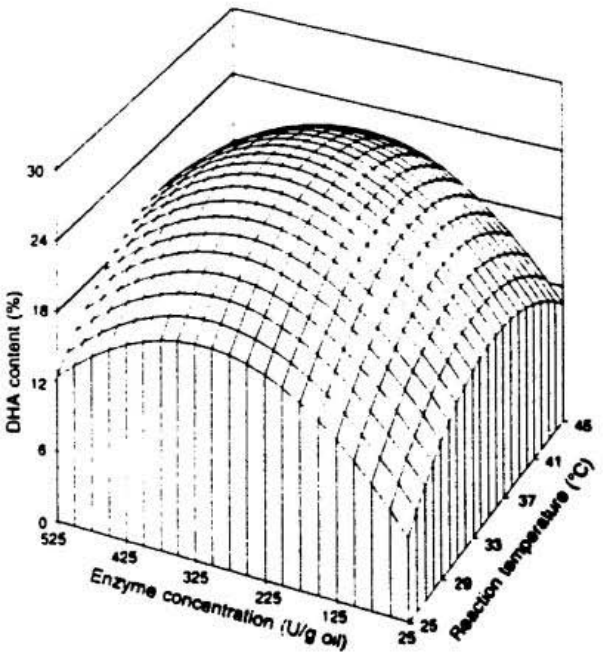
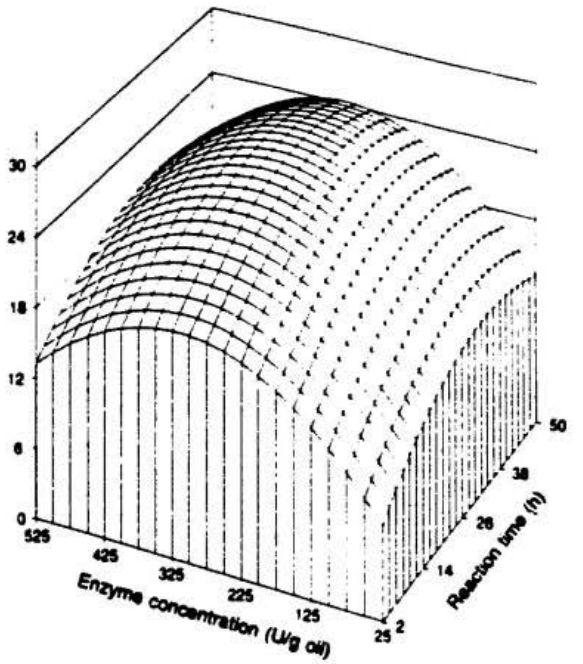
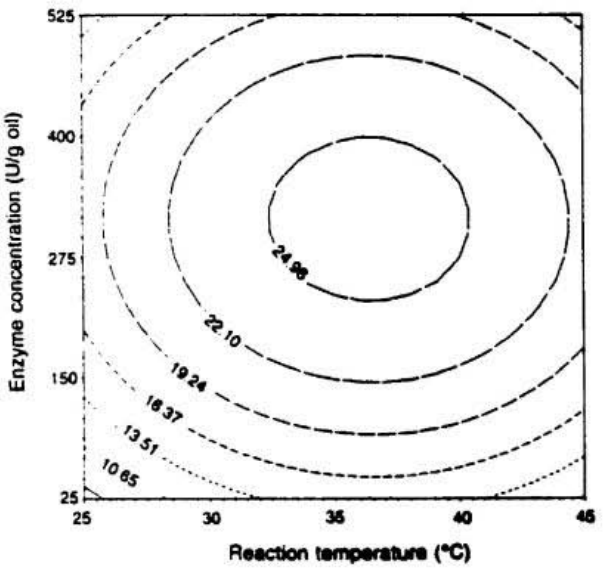
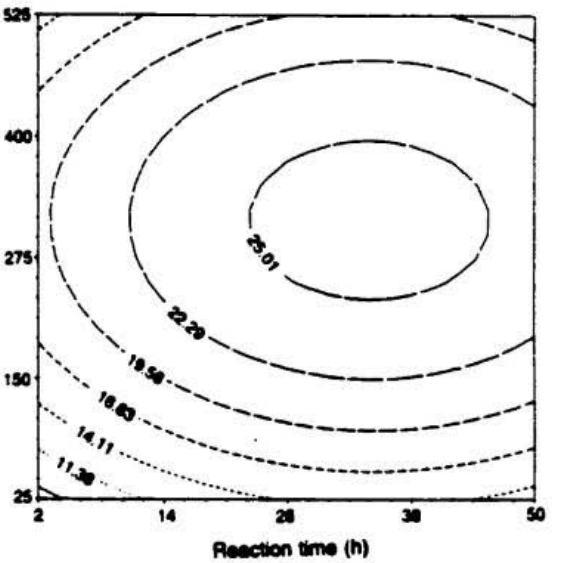

525
400
275
150
25
20.72
19.74
18.75
17.77
16.79
15.81
2
14
26
38
50
Reaction time (h)

Figure 4.35 Response surface and contour plots for the effect of enzyme concentration, reaction time and reaction temperature on DHA content of the prepared concentrate of menhaden oil (MHO) by enzymatic hydrolysis


DHA content (%)
30
24
18
12
6
0
525
425
325
225
125
25
Enzyme concentration (U/g oil)
25
29
33
37
41
45
Reaction temperature (°C)


30
24
18
12
6
0
525
425
325
225
125
25
Enzyme concentration (U/g oil)
2
14
26
38
50
Reaction time (h)


Enzyme concentration (U/g oil)
525
400
275
150
25
24.98
22.10
19.24
16.37
13.51
10.65
25
30
35
40
45
Reaction temperature (°C)


525
400
275
150
25
23.01
22.29
19.58
16.83
14.11
11.39
2
14
26
38
50
Reaction time (h)

SBO (Table 4.31). A maximum of 56.3% total ω3 fatty acids was predicted in MHO at an enzyme concentration of 340 U/g oil, reaction time of 45 h and reaction temperature of 38°C. Maximum values of 21.1 and 25.9% EPA and DHA in the MHO concentrate were predicted at enzyme concentrations of 370 and 314 U/g oil, reaction times of 31 and 34 h and reaction temperatures of 37 and 36°C, respectively. Graphical representations of data on contour plots (Figure 4.33, 4.34 and 4.35) showed that these maxima are located in the experimental region.

Results of independent experiments carried out to examine the adequacy of the predicted values by the models for both oils showed very close values for all three responses (Tables 4.30 and 4.31). These verification results revealed that the predicted values from models were reasonable and reproducible. Therefore, hydrolysis of SBO and MHO by *Candida cylindracea*-lipase can increase the content of total ω3 fatty acids up to 54.3±3.22 and 54.5±2.33%, with recovery (yield) of 30 and 43% of the weight of original oils, respectively.

Table 4.31 Predicted and observed values for response variables (total ω3, EPA and DHA contents) in enzymatic hydrolysis experiment of menhaden oil (MHO)

Response variables	Critical values of independent variables			Stationary point	Predicted value	Observed value[1]
	Enzyme concentration (U/g oil)	Reaction time (h)	Reaction temperature (°C)			
Total ω3 fatty acids (%)	340	45	38	Maximum	56.3	54.5 ± 2.33
EPA (%)	370	31	37	Maximum	21.1	18.1 ± 2.76
DHA (%)	314	34	36	Maximum	25.9	26.1 ± 3.44

[1]Mean ± SD (n = 3)

SUMMARY AND CONCLUSIONS

Refining, bleaching and deodorization of seal blubber oil (SBO) and cod liver oil (CLO) was effective in the removal of coloured compounds, free fatty acids and polar lipids; however, the resultant oils were less stable to oxidative deterioration. Possibly, this might arise from the removal of naturally occurring antioxidants and synergists from the oils. Among the marine oils tested, refined-bleached and deodorized (RBD) SBO had a higher oxidative stability than CLO and menhaden oil (MHO).

Dechlorophyllized green tea extracts (DGTE) as well as individual tea catechins, namely (-)epicatechin (EC), (-)epigallocatechin (EGC), (-)epicatechin gallate (ECG) and (-)epigallocatechin gallate (EGCG), exhibited strong antioxidant properties when added to SBO and MHO. DGTE at addition levels of 500 and 1000 ppm as well as isolated tea catechins at 200 ppm exhibited antioxidant efficacy superior to that of α-tocopherol (500 ppm), BHA and BHT (200 ppm) treated oils. The potency of catechins in retarding oxidation of both oils was in the decreasing order of ECG > EGCE > EGC > EC; ECG was somewhat more effective than TBHQ (200 ppm). Therefore, DGTE and isolated tea catechins could be used as effective natural antioxidants for the stabilization of highly unsaturated marine oils.

The antioxidant activity of a number of flavonoids was also investigated in this study. Results indicated that myricetin, morin, quercetin (all flavonols) were the most effective flavonoids in stabilizing SBO and MHO. Furthermore, naringin and naringenin (flavonones) were found to be effective, but to a lesser extent than the flavonols tested.

Microencapsulation of SBO using starch based wall materials improved the oxidative stability of the oil and preserved the integrity of nutritionally important polyunsaturated fatty acids. β-Cyclodextrin was the most effective encapsulating material examined in this study.

The positional distribution of fatty acids in the triacylglycerol (TAG) molecules of SBO and MHO was determined by employing stereospecific analysis; eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) were located mainly in the *sn*-1 and *sn*-3 positions of the TAG molecules of SBO. In MHO, DPA and DHA were found mainly in the *sn*-2 position of the TAG, however, EPA was equally distributed in the *sn*-2 and *sn*-3 positions and was present only in small amounts in the *sn*-1 position. Therefore, EPA, DPA and DHA in SBO might be assimilated in the body more effectively than those in MHO (fish oil). However, clinical studies should be carried out to verify this assumption.

Concentration of ω3 fatty acids from SBO and MHO was achieved by low temperature crystallization, urea complexation and enzymatic hydrolysis. Low temperature crystallization of SBO, in the free fatty acid form, produced considerably higher amounts of total ω3 fatty acids in the non-crystalline fraction (the concentrate) of the oil as compared to that in the TAG form. The content of total ω3 fatty acids of SBO was 58.3 and 66.7% at -60 and -70°C, respectively, when hexane was used as a solvent. The corresponding recoveries were 39.0 and 24.8%.

Production of ω3 fatty acid concentrates from SBO by urea complexation was optimized, under laboratory conditions, for process parameters such as urea-to-fatty acid

ratio, crystallization time and temperature using response surface methodology (RSM) with a central composite rotatable design (CCRD). The maximum amount of total ω3 fatty acids (88.2%) from SBO was obtained at a urea-to-fatty acid ratio of 4.5, a crystallization time of 24 h, and a crystallization temperature of -10°C (recovery of 24.5%).

Enzymatic hydrolysis of SBO and MHO by lipases from *Aspergillus niger; AN, Candida cylindracea; CC, Chromobacterium viscosum; CV, Geotrichum candidum; GC, Mucor miehei; MM, Pseudomonas spp.; SP, Rhizopus niveus; RN* and *Rhizopus oryzae; RO* was carried out. The highest concentration of total ω3 fatty acids was obtained when *CC*-lipase was used for hydrolysis. Optimization of reaction parameters namely, enzyme concentration (*CC*-lipase), reaction time and temperature gave a maximum of 54.3% total ω3 fatty acids from SBO in the acylglycerol form (recovery of 30%) at an enzyme concentration of 308 U/g oil, a reaction time of 40 h and a reaction temperature of 37°C. Similarly, the maximum of 54.5% total ω3 fatty acids from MHO was produced (recovery of 43%) at an enzyme concentration of 340 U/g oil, a reaction time of 45 h and a reaction temperature of 38°C.

Therefore, low temperature crystallization, urea complexation and enzymatic hydrolysis by *CC*-lipase can be used to concentrate ω3 fatty acids from SBO and/or MHO. Under optimum conditions, approximately 88 and 54% of total ω3 fatty acids could be obtained by urea complexation and *CC*-lipase hydrolysis, respectively. However, low temperature crystallization produced a maximum of 58 and 67% of total ω3 fatty acids at -60 and -70°C temperatures, respectively.

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APPENDIX 1

Examples of standard lines (curves) used for different determinations as described in Materials and Methods

"One unit of lipolytic activity is defined as the amount of enzyme which liberates one μmol of titratable butyric acid from tributyrin per min"

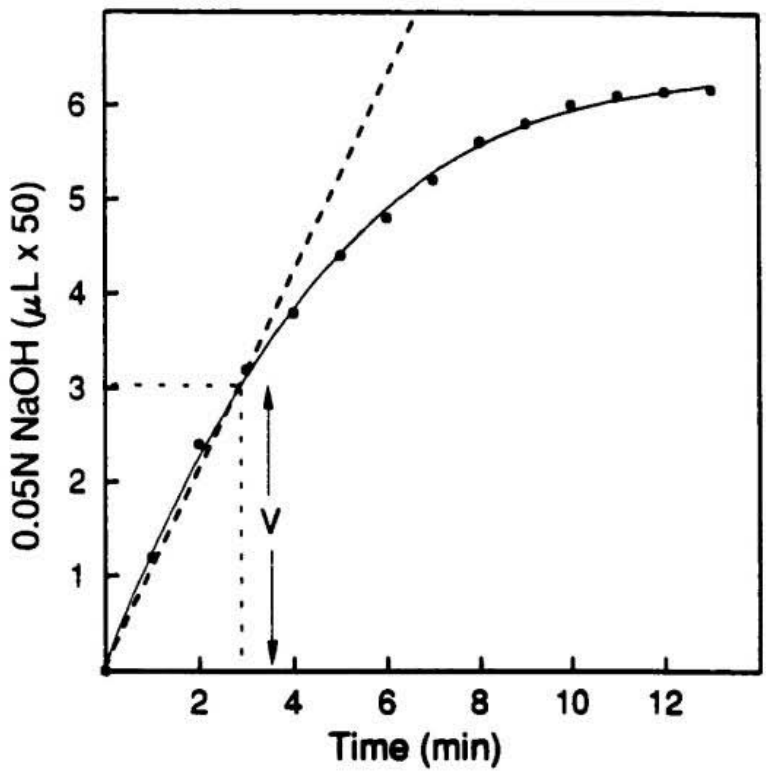

Figure A.1 Titration curve used for lipolytic activity determination by pH-Stat method

Titration rate at first 3 min of the curve was measured by drawing a straight line where it corresponds best to the titration curve.

$$\text{Lipolytic activity (U/g)} = \frac{V}{3} \times \frac{1}{20} \times k$$

Where: V = Volume (μL) of 0.05N NaOH consumed during first 3 min of titration

$\frac{1}{20}$ = Conversion of μL 0.05N NaOH to μmol NaOH

k = Dilution factor of enzyme (g/mL)

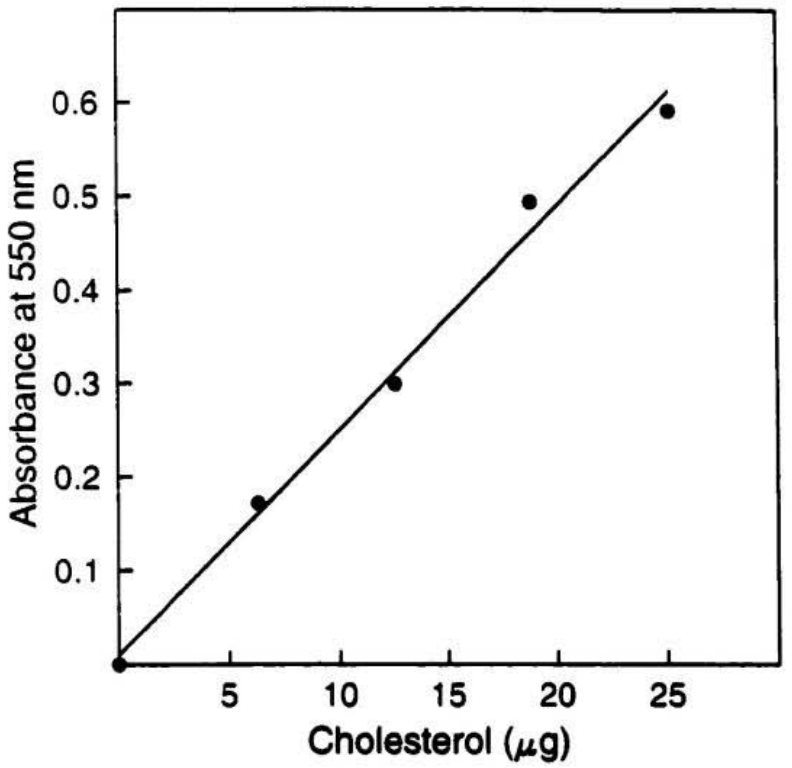

Figure A.2 Standard line of cholesterol concentration dependence on absorbance at 500 nm

Regression coefficient (r) = 0.992
Equation of the line (Y=aX+b) where,

Y = absorbance at 550 nm (A_{550})
X = concentration of cholesterol in 1 mL hexane, μg (C)
a = 0.022
b = 0.0

$A_{550} = 0.022 \times C$

Therefore, $C = 45.455A_{550}$

Hence, cholesterol content (mg/100 g oil) = $45.455A_{500} \times F$

Where, F = Total volume of hexane used for extraction of w grams of oil × 100/weight of oil (g) × 1000

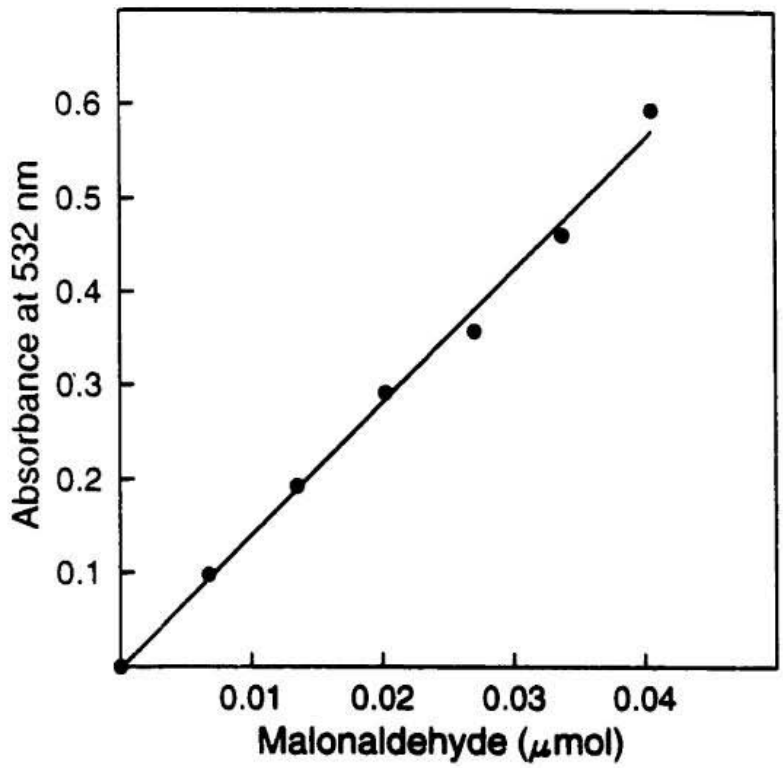

Figure A.3 Standard line of concentration dependence of TBARS as reflected in the absorbance of the TBA-malonaldehyde complex

Regression coefficient (r) = 0.995
Equation of the line (Y=aX+b) where,

Y = absorbance at 532 nm (A_{532})
X = concentration of malonaldehyde (MA) in 5 mL solution, μmole (C)
a = 14.116
b = 0.0

$A_{550} = 14.116 \times C$

Therefore, $C = 0.071A_{532}$

Since the w grams of oil dissolved in 25 mL solution, the MA concentration is:

C (μmole of MA/g oil) = $(0.355A_{532})/w$

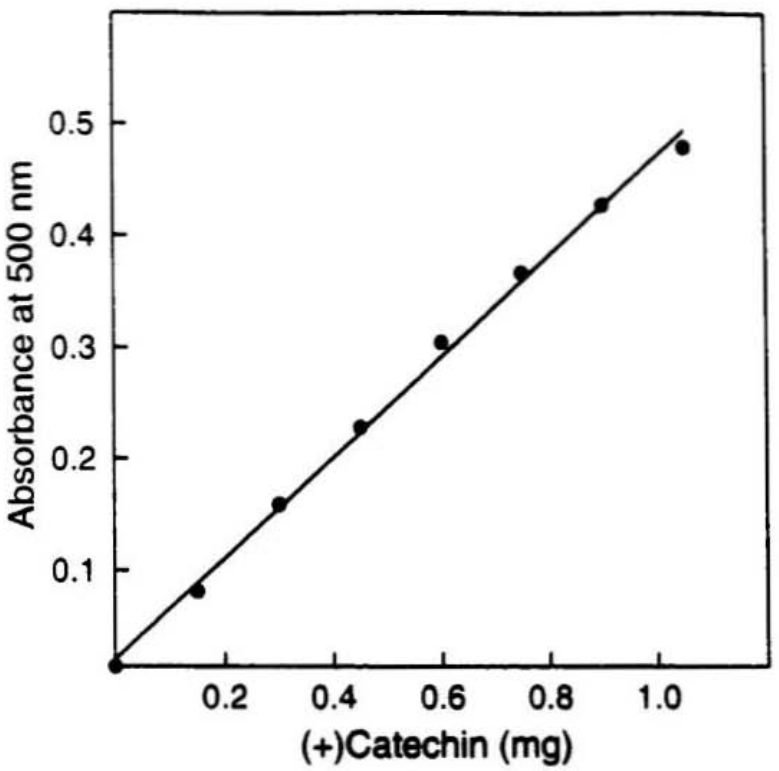

Figure A.4 Standard line of concentration dependence of total phenolics as reflected in the absorbance of the (+)catechin-vanillin complex

Regression coefficient (r) = 0.996
Equation of the line (Y=aX+b) where,

Y = absorbance at 500 nm (A_{500})
X = concentration of (+)catechin in solution, mg (C)
a = 0.445
b = 0.0

$A_{500} = 0.445 \times C$

Therefore, $C = 2.247A_{500}$

Since tea extracts have to be diluted, then:

$C\ (mg) = k(2.247A_{500})$

Where k = dilution factor

APPENDIX 2

Details of data analysis obtained by SAS programme for optimization of production of ω3 fatty acid concentrates via enzymatic hydrolysis of seal blubber oil (SBO)

Y = Total Omega-3 fatty acids (%)
X1 = Enzyme concentration (U/g oil)
X2 = Reaction time (h)
X3 = Reaction temperature (C)

Coding Coefficients for the Independent Variables

Factor	Subtracted off	Divided by
X1	280.000000	235.000000
X2	25.000000	22.000000
X3	35.000000	8.400000

Response Surface for Variable Y: Total Omega-3 fatty acids

Response Mean	46.944737
Root MSE	1.571872
R-Square	0.9390

Regression	Degrees of Freedom	Type I Sum of Squares	R-Square	F-Ratio	Prob > F
Linear	3	159.169431	0.4367	21.474	0.0002
Quadratic	3	177.903946	0.4880	24.001	0.0001
Crossproduct	3	5.211450	0.0143	0.703	0.5737
Total Regress	9	342.284827	0.9390	15.393	0.0002

Residual	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F
Lack of Fit	5	19.571167	3.914233	5.873	0.0555
Pure Error	4	2.665880	0.666470		
Total Error	9	22.237047	2.470783		

Parameter	Degrees of Freedom	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEPT	1	-110.535527	23.984020	-4.609	0.0013
X1	1	0.101038	0.031439	3.214	0.0106
X2	1	0.375423	0.336826	1.115	0.2939
X3	1	7.691455	1.235711	6.224	0.0002
X1*X1	1	-0.000139	0.000021779	-6.400	0.0001
X2*X1	1	-0.000371	0.000305	-1.215	0.2554
X2*X2	1	-0.006311	0.002492	-2.532	0.0321
X3*X1	1	-1.01794E-18	0.000794	-13E-16	1.0000
X3*X2	1	0.006808	0.008550	0.796	0.4464
X3*X3	1	-0.108737	0.017051	-6.377	0.0001

Parameter	Parameter Estimate from Coded Data
INTERCEPT	51.628071
X1	3.222819
X2	4.274259
X3	2.100689
X1*X1	-7.697202
X2*X1	-1.917445
X2*X2	-3.054641
X3*X1	-2.00942E-15
X3*X2	1.258062
X3*X3	-7.672458

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F
X1	4	155.100633	38.775158	15.693	0.0004
X2	4	108.626727	27.156682	10.991	0.0016
X3	4	123.380266	30.845066	12.484	0.0010

Canonical Analysis of Response Surface
(based on coded data)

Factor	Critical Value Coded	Uncoded
X1	0.121990	308.667679
X2	0.701378	40.430322
X3	0.194401	36.632968

Predicted value at stationary point 53.527771

Canonical Analysis of Response Surface
(based on coded data)

Eigenvalues	Eigenvectors X1	X2	X3
-2.786486	-0.190102	0.973728	0.125360
-7.679467	0.515959	-0.009544	0.856560
-7.958348	0.835253	0.227514	-0.500590

Stationary point is a maximum.

Estimated Ridge of Maximum Response for Variable Y:

Coded Radius	Estimated Response	Standard Error	Uncoded Factor Values X1	X2	X3
0.0	51.628071	0.702195	280.000000	25.000000	35.000000
0.1	52.149429	0.699159	291.566811	26.750428	35.296601
0.2	52.571936	0.691247	299.900292	28.698273	35.567869
0.3	52.907221	0.682154	305.328975	30.781321	35.811184
0.4	53.164427	0.678311	308.442712	32.942821	36.028892
0.5	53.350099	0.688745	309.828442	35.144027	36.225449
0.6	53.468722	0.723773	309.953843	37.362410	36.405347
0.7	53.523341	0.792308	309.158596	39.585976	36.572323
0.8	53.516044	0.899328	307.681773	41.808696	36.729260
0.9	53.448294	1.045543	305.691259	44.027732	36.878326
1.0	53.321135	1.229045	303.306256	46.241906	37.021146

BIO DATA

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1982-1986	Undergraduate student, Faculty of Agriculture, University of Peradeniya, Kandy, Sri Lanka
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