# STUDIES ON THE PREPARATION OF STRUCTURED LIPIDS FROM RICE BRAN OIL

A thesis submitted to the

**UNIVERSITY OF MYSORE**

in fulfillment of the requirements of the degree of

**DOCTOR OF PHILOSOPHY**

in

**BIOCHEMISTRY**

By

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**April, 2008**

## DECLARATION

I declare that the thesis entitled '**STUDIES ON THE PREPARATION OF STRUCTURED LIPIDS FROM RICE BRAN OIL'** submitted to the **UNVERSITY OF MYSORE** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOCHEMISTRY** is the result of the work carried out by me under the guidance of **Dr. K. SAMBAIAH**, Scientist F, Department of Biochemistry and Nutrition, Central Food Technological Research Institute (CFTRI), Mysore, during the period of April, 2003 – April, 2008. I further declare that the results presented in the thesis have not been submitted for the award of any other degree or fellowship.

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Place: Mysore

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## CERTIFICATE

This is to certify that the thesis entitled **'STUDIES ON THE PREPARATION OF STRUCTURED LIPIDS FROM RICE BRAN OIL'** submitted by **RAJNI CHOPRA** for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOCHEMISTRY** to the **UNVERSITY OF MYSORE** is the result of the research work carried out by her in the department of Biochemistry and Nutrition under my guidance during the period of April, 2003- April, 2008.

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## ACKNOWLEDGEMENTS

I wish to express my deep sense of gratitude and indebtedness to my research mentor, Dr. K. Sambaiah, Scientist F, Department of Biochemistry and Nutrition for his excellent guidance, incessant encouragement and support throughout this research investigation.

I am grateful to Dr. V. Prakash, Director, CFTRI, for giving me an opportunity to work in this premier institute and providing the required facilities.

My whole-hearted and sincere thanks to Dr. P.V. Salimath, Head, Biochemistry & Nutrition and Dr. S. G. Bhat, former Head, Biochemistry & Nutrition for their encouragement and inspiring support during this work.

I thank Dr. S. Yella Reddy, Scientist, Department of Lipid Science and Traditional Foods and Dr. N.K. Rastogi, Scientist, Department of Food Engineering for their help and valuable suggestions to complete my research work.

I thank Dr. B. R. Lokesh, Head, Department of Lipid Science & Traditional Foods for his valuable suggestions guidance during the research period.

I am grateful to the Head and staff of Central Instruments Facilities and Services, Animal house staff, Library (Fostis), Administration, Stores and Purchase Sections and IFTTC Hostel for providing the necessary facilities and services throughout this research investigation and my stay in the institute.

I am thankful to Dr. Rekha Melhotra, Associate Professor and Head of Department of Food Science and Nutrition, CSKHPKV, Palampur for her sincere encouragement which made me to pursue my research career.

I am thankful to my loving and dear most friends Dr. Chethana S, Amit Baranwal, Dr. Venkatesh KS, Chithra MC, Dr. Kumaresan N, Reena MB, Krithika, Kavitha BC and Sowmya SK for their constant encouragement, love and emotional support.

I do bear in my mind the help of seniors, Dr. Ramaprasad TR and Mr. Vidhyashankar S during my stay in the institute.

I convey my sincere thanks to Dr. Ganapathi Patil, Dr. Jessie Suneetha, Ravindra Babu, Chethn Nayak, Raghavendera, Dr. Mohan Dhale, Veer Bala, Dr. Shrilatha, Dr. Ajila CM, Deepa Prashant, Anitha Gopal, Smitha J, Shobha, Shyamla, Srikanta, Supriya, Madhu MS, RLR Reddy and Vivek for their help and friendly atmosphere to me for successful completion of my work. Listing would be lengthy, however I thank many more friends and colleagues in the department and institute for their kind help and co-operation which made my stay here a memorable one.

I am deeply indebted to my beloved parents, sisters, and brothers in-law for their enlivening support, understanding and inspiration.

I am grateful to my brother Mr. Bhupender Rana and other cousins for their affection, continued support and encouragement through out my career.

I acknowledge the financial assistance provided by University Grant Commission (UGC), New Delhi, in the form of Junior and Senior Research fellowships.

**(RAJNI CHOPRA)**

To

My Beloved Daddy and mummy

# Contents

| Abbreviations | Expansions |
|---|---|
| α | Alpha |
| β | Beta |
| µ | Micro |
| ALA | α- linolenic acid |
| CHD | Coronary heart disease |
| CLO | Cod liver oil |
| DTNB | Dithiobis nitrobenzoic acid |
| DPA | Docosapentaenoic acid |
| DHA | Docosahexaenoic acid |
| $^{0}C$ | Degree Celsius |
| ECN | Equivalent carbon number |
| EPA | Eicosapentaenoic acid |
| EDTA | Ethylene diamine tetra acetic acid |
| FA | Fatty acid (s) |
| FFA | Free fatty acid (s) |
| g | Gram (s) |
| GC | Gas chromatography |
| GNO | Groundnut oil |
| h | Hour (s) |
| HPLC | High performance liquid chromatography |
| HDL | High density lipoprotein (s) |
| L | Litre (s) |
| LA | Linoleic acid |
| LC PUFA | Long chain polyunsaturated fatty acid (s) |
| LDL | Low density lipoprotein (s) |
| LSO | Linseed oil |
| mL | Milliliter (s) |
| mm | Millimeter (s) |
| min | Minute (s) |
| mM | Millimolar |
| mU | Milliunits |
| mg | Milligram (s) |
| M | Molar |

| | |
|---|---|
| MDA | Malondialdehyde |
| MUFA | Monounsaturated fatty acid (s) |
| µg | Microgram |
| µM | Micromolar |
| µL | Microlitre |
| nm | Nanometre |
| N | Normal |
| OD | Optical density |
| PUFA | Polyunsaturated fatty acid (s) |
| PG | Prostaglandin |
| PPP | Platelet poor plasma |
| PRP | Platelet rich plasma |
| RBO | Rice bran oil |
| RSM | Response surface methodology |
| SA | stearic acid |
| SL | Structured lipids |
| SFA | Saturated fatty acid (s) |
| sec | Second (s) |
| SOD | Superoxide dismutase |
| TCN | Total carbon number |
| TAG | Triacylglycerol |
| TLC | Thin layer chromatography |
| TX | Thrmoboxane |
| U | Units |
| UV | Ultra violet |
| V | Volume |
| v/v | Volume/Volume |
| VLDL | Very low density lipoprotein (s) |
| w/w | Weight/weight |

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# Synopsis

***Synopsis of the thesis submitted for the award of Ph.D. degree (Biochemistry) of the University of Mysore.***

**Title of the thesis: 'Studies on the preparation of structured lipids from rice bran oil'**

**Candidate : Rajni Chopra**

## Introduction

Rice bran oil (RBO) is the byproduct of rice milling and it is consumed widely in Asia. It is produced from the bran and polish of rice, which are by-products of the rice milling industry. India is the second largest producer of rice in the world and has high potential to produce RBO. It is used for both edible and industrial applications. RBO is unconventional oil with its fatty acid composition very close to that of groundnut oil and has high content of unsaponifiable matter. There has been much interest in RBO in recent years because of its nutraceuticals like tocopherols, tocotrienols, and γ-oryzanol, which have been found to have several health beneficial effects. Structured lipids are triacylglycerols (TAGs) that have been modified to incorporate new fatty acids or have been restructured to change the position of fatty acids to produce novel/new TAGs. Structured lipids can be produced to alter the physical characteristics, or improve the nutritional quality of fats and oils. RBO contains approximately 38 % oleic acid, 34 % linoleic acid and 18.6 % palmitic acid and it lacks n-3 PUFA. The physical properties of these fatty acids do not confer any special properties for its use in food applications. It is interesting to note that stearic acid content of RBO is very low. Though stearic acid is a saturated fatty acid, studies have been shown non-atherogenic nature of stearic acid. Saturated fatty acids are known to impart various physico-chemical and thermal stability properties to oils and fats. Cocoa butter which is rich in stearic acid is currently the fat of choice in the confectionery industry. Because of its demand for confectionery and

desirable physical and nutritional quality, cocoa butter substitutes and cocoa butter equivalents have recently become more common. It is of interest to see of whether structured lipid with RBO containing n-3 fatty acids such as $\alpha$ - linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are having several health benefits, can be synthesized. Therefore, the objective of this study was to modify the RBO to produce structured lipids containing stearic acid and n-3 fatty acids so that utilization of RBO can be increased and value added products can be prepared from RBO.

**Objectives and plan of work**

The major objectives of the present investigation are to prepare structured lipids from RBO rich in stearic acid, $\alpha$- linolenic acid and EPA+DHA by enzyme catalyzed reaction using immobilized lipase from *Rhizomucor miehei*. The structured lipids enriched with stearic acid and n-3 PUFA were evaluated for their physico-chemical and hypocholesterolemic and anti-aggregation effects respectively and accordingly the following work plan has been envisaged.

1. Standardization of different reaction conditions like incubation time, temperature, enzyme concentration and substrates molar ratio on the incorporation of stearic acid into RBO. Purification of reaction product and studies on physico-chemical studies.
2. Optimization different reaction conditions like incubation time, temperature, enzyme concentration and substrates molar ratio using response surface methodology on the incorporation of n-3 PUFA into RBO. Synthesis of large scale structured lipids rich in n-3 PUFA and their purification.

3. Evaluation of structured lipids rich in n-3 PUFA for their hypocholesterolemic effects and hepatic antioxidant enzymes and platelet aggregation in rats fed n-3 PUFA rich structured lipids.

**Chapter I:**

**Introduction**

This chapter deals with the general introduction of fats and oils, lipid classification, different types of fatty acids and their physiological effects, biological significance and health benefits of n-6 and n-3 fatty acids, metabolic pathway of n-6 and n-3 fatty acid synthesis. This chapter also includes the effects of dietary lipids on health, health benefits of n-3 PUFA on cardiovascular system, enzymes regulating calcium ion channels, in the vascular retina, haemostatic system, endothelial function and nervous system. A description on the edible oils used in India, dietary recommendation of different fatty acids and need for the balance of fatty acids in the diet is given. Uses and importance of structured lipids, importance of immobilized enzymes in modification of fats and oils are described in this chapter.

**Chapter II:**

**Materials and methods**

In this chapter brief protocols of all procedures carried out throughout the investigation have been given with appropriate references. Described here are standardization of different reaction conditions for the synthesis of structured lipids enriched in stearic acid, purification of newly synthesized TAG by thin layer chromatography, quantitation of triglycerides, analysis of fatty acids by gas chromatography and fatty acids at *sn-2* position of triglyceride and differential scanning calorimetric studies of structured lipids rich in stearic acid. Analytical methods for minor constituents like $\gamma$- oryzanol, tocopherols and tocotrienols of native and modified RBO were also given. This chapter describes the preparation of free fatty acids by the

hydrolysis of linseed and cod liver oil and preparation n-3 fatty acid concentrate from linseed oil by urea inclusion method. Optimization of reaction conditions like incubation time, temperature, enzyme concentration and substrates molar ratio by using response surface methodology for the acidolysis reaction were also described. Scale up of the enzyme catalyzed acidolysis and large scale preparation of structured lipids rich in n- 3 PUFA and preparation of blended oil from RBO with linseed oil and cod liver oil and nutritional evaluation of these structured lipids were enumerated.

Chapter III:

**Synthesis of structured lipids with RBO enriched in stearic acid**

This chapter begins with the brief introduction of RBO, structured lipids, importance of structured lipids for the improvement of physical properties of fats and oils and importance and effect of stearic acid on cholesterol level. Enzymatic acidolysis reactions were carried out in 50 mL stoppered conical flasks under inert atmosphere. Different reaction conditions like incubation time, temperature, substrates molar ratio and enzyme concentration on the incorporation of stearic acid into RBO were studied. The effect of reaction time on the incorporation of stearic acid into RBO was studied from 0 to 48 h keeping other parameters constant. The effect of reaction temperature on the incorporation of SA into RBO was studied from 25 to 60 $^{0}$C keeping other parameters constant. The effect of molar ratio of substrates on incorporation of SA into RBO was studied from 1:1 to 1:10. The effect of enzyme concentration on incorporation of SA into RBO was studied from 1 to 10% of the weights of both the substrates. Stearic acid was successfully incorporated (48.5 %) into RBO under optimal conditions. After incorporation of stearic acid into RBO, fatty acid profile and *sn-2* positional analysis was carried out in modified and original RBO using pancreatic lipase catalyzed reaction.

Analysis of oryzanol, tocopherols and tocotrienols were carried out in native and modified RBO. The type of the triacylglycerols in modified and native rice bran oil were analyzed by HPLC. After incorporation of stearic acid into RBO, physical properties of native and modified RBO were studied by differential scanning calorimeter and physical properties of modified RBO were compared with vanaspati and cocoa butter. The chapter was concluded by discussion of the results obtained.

Chapter IV:

**Enrichment of RBO with n-3 PUFA by enzymatic acidolysis: optimization of parameters by RSM**

This chapter begins with the brief introduction about the importance of n-3 PUFA in human health and need of balance between n-6 and n-3 PUFA. The importance of response surface methodology (RSM) for optimizing different reaction conditions for incorporation of n-3 PUFA into the RBO was elaborated. Fatty acid concentrate from linseed oil (LSO) rich in $\alpha$- linolenic acid (ALA) and free fatty acids from cod liver oil (CLO) were prepared and further used for acidolysis reaction to enrich RBO with ALA and EPA+DHA respectively. Two structured lipids (SL) were obtained with RBO, one was rich in ALA and other rich in EPA+DHA. RSM was used to optimize the reaction conditions for lipase-catalyzed incorporation of n-3 PUFA into RBO. A CCRD with four variables was used to study the response pattern. Purification of the reaction product was done with TLC and column chromatography. Pancreatic lipase hydrolysis was used to determine the fatty acids at *sn-2* position in triacylglycerol. The optimum incorporation of ALA (n-3 PUFA) into RBO was about 49 % when incubation time and temperature were 11.5 h and 43.75$^0$C respectively and substrates molar ratio and enzyme concentration (%) was maintained at 7.75. The optimum incorporation of EPA+DHA into RBO was about 10 % at 37 h and 32.5 $^0$C incubation time and temperature respectively,

where as enzyme concentration (%) and substrates molar ratio was 7.75. The fatty acid composition of substrates and reaction products were analyzed by gas chromatography. Large scale synthesis of these SL rich in PUFA was done. The chapter ends with the discussion.

Chapter V:

**Effects of SL and blends from RBO enriched with n-3 PUFA on liver and serum lipids in rats**

This chapter focused on the effect of SL with RBO rich in n-3 PUFA on the lipid profile in rats. This chapter begins with a brief mention on the role of dietary fatty acids in health and disease prevention in humans and cardiovascular protective effects of n-3 PUFA. This chapter deals with the hypocholesterolemic effect of RBO rich in n-3 PUFA. Different experimental diets were prepared by including SL with ALA and EPA+DHA and blended oils (RBO and LSO and RBO and CLO) having similar fatty acid composition was fed to rats. Growth and food intake were monitored and food efficiency (FER) was calculated. The serum lipid parameters like total cholesterol, triglycerides, phospholipids, low density and high density cholesterol were estimated. Liver lipid parameters like total cholesterol, triglycerides and phospholipids were estimated. Fatty acid analysis was done for serum, liver, heart, eye, brain and adipose tissues. The chapter was concluded with the discussion.

Chapter VI:

**Antioxidant activity and platelet aggregation in rats fed SL and blends from RBO enriched with n-3 PUFA**

This chapter begins with a brief introduction about the importance of n-3 PUFA in prevention of inflammatory diseases, including cancer and coronary heart diseases. Effect of RBO rich in n-3 PUFA on platelet aggregation of rats was studied. Antioxidant enzymes like catalase, superoxide dismutase, glutathione reductase and

glutathione transferase activity in liver homogenate and serum of rats fed structured lipids enriched with n-3 fatty acid was studied. Lipid peroxides in liver and serum were done. Total $Na^+$, $K^+$ ATPase activity in RBC membrane of rats fed SL rich in PUFA was also studied. Structured lipids enriched with n-3 fatty acid were studied for their inhibitory effects on platelet aggregation of rats. The chapter concludes with discussion.

The summary and conclusions of the present findings were given and the thesis concludes with the bibliography arranged alphabetically in sequential order.

# CHAPTER - I

# INTRODUCTION

Dietary lipids provide desirable organoleptic, physical, nutritional and biological functions to foods. On the other hand too much fat can be deleterious. Eating large amounts of high-fat foods adds excess calories, which can lead to weight gain and obesity. Obesity is a risk factor for several diseases, including diabetes, heart disease, cancer, gallstones and osteo-arthritis etc. Too much of certain types of fats - such as saturated fat or *trans* fat - can increase blood cholesterol level and risk of coronary artery disease.

Fats supplies 9 Cal/g compared to 4 Cal/g by protein or carbohydrates. Food lipids provide essential fatty acids such as linoleic and linolenic acids. In addition, dietary fat carries fat-soluble vitamins - A, D, E and K and carotenoids from food into body. Fat also acts as a 'food solvent' for phytochemicals in food products and also enhance the bioavailability of phytochemicals (Spector and York, 1985). It regulates cholesterol metabolism. Fat besides being an energy source, it is a nutrient used in the formation of cell membrane, as well as in several hormone-like compounds called eicosanoids. These compounds help to regulate blood pressure, heart rate, blood vessel constriction, blood clotting and the nervous system. Fat also helps maintain healthy hair and skin, protects vital organs, keeps body insulated, and provides a sense of fullness.

Many foods inherently contain fat. These include meat, dairy products, poultry, fish, nuts and vegetable oils. The edible products of fats and oils are often referred to as food lipids. Dietary fat (vegetable or animal) consists of mainly triacylglycerol (TAG) and other minor lipid components like phospholipids, free fatty acids, phytosterols, glycolipids, and sulpholipids. TAG makes the majority of the lipids in edible fats and oils. They are composed of glycerol esters of fatty acids. They contain essential fatty acids which are precursor substrates for prostaglandins and regulate many physiological processes. Fatty acids are oxidized to provide energy or stored in adipose tissue as a reserve source of energy. TAG composed of saturated fatty acids (SFA) (eg. myristic,

palmitic and stearic) have high melting point and are generally solid at ambient temperatures, whereas TAG consisting of unsaturated fatty acids (oleic, linoleic, and linolenic) are usually liquid at room temperature.

Cholesterol is required for life and occurs in the plasma membranes of many mammalian cells for normal cellular function, and is either synthesized in the endoplasmic reticulum, or derived from the diet. Cholesterol is carried in the blood by the lipoproteins and taken up by the cells via LDL receptor-mediated endocytosis in clathrin-coated pits, and then hydrolysed in lysosomes. Cholesterol is primarily synthesized from acetyl CoA through the HMG-CoA reductase pathway in many cells and tissues. About 20 – 25% of total daily production (~1 g/day) occurs in the liver and other tissues including intestine, adrenal glands and reproductive organs. Cholesterol comprises about 0.2 % of normal body weight. Most of it is in the brain and nervous system, where its functions have been probed beyond suggesting that its major activity is as an insulator. Almost about one third of body's cholesterol is in muscle, where it is a structural component. Cholesterol also serves as substrates for vitamin $D_3$, bile acids, adrenocortico hormones and sex hormones. Thus it is one of the important biological substances.



**LIPID CLASSIFICATION**

Lipids are classified as simple or complex.

**Simple lipids** - esters of fatty acids with various alcohols.

a) Fats- esters of fatty acids with glycerol. A fat in liquid state is known as oil.
b) Waxes- esters of fatty acids with higher alcohols than glycerol.

**Complex lipids** - esters of fatty acids containing groups in addition to an alcohol and a fatty acid.

a) Phospholipids - lipids containing, in addition to fatty acids and an alcohol, a phosphoric acid residue, nitrogen - containing base and other substituents. Eg, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositiol, phosphatidyl serine and shingomyelins.

b) Glycolipids - lipids containing a fatty acid, and carbohydrate but no phosphoric acid.

c) Other complex lipids - lipids such as sulpholipids and aminolipids. Lipoproteins may also be placed in this category.

**Precursor and derived lipids** – substances derived from the above groups by hydrolysis. These include fatty acids, glycerol, steroids, alcohols in addition to glycerol and sterols, fatty aldehydes, and the proteins of lipoproteins.

In general terms the lipids can be hydrolyzed into two different components, usually an alcohol and an acid. Complex lipids include glycerophospholipids (phospholipids), glyceroglycolipids (glycolipids), and shingolipids. These structures yield three or more different compounds on hydrolysis. Fatty acids are the main components of neutral lipids. Fatty acids occur mainly as esters in fats and oils but do not occur generally as unesterified form as free fatty acids, a transport form found in the plasma. Fatty acids that occur in natural fats are usually straight chain derivatives and contain an even number of carbon atoms because they are synthesized from two carbon units. The chain may be saturated (containing no double bonds) or unsaturated (containing one or more double bonds). Fatty acids vary in their chain length and in number, position, and in configuration of double bonds which influence their chemical and biological properties.

**Functions of food lipids**

The various components of food lipids perform many desirable organoleptic, physical, nutritional and biological functions that must be considered in making broad

recommendations regarding dietary lipids. Lipids are very important components in determining food selection, and the properties they account for their general desirability in foods (Table 1.1).

**Table 1.1: Various functions of food lipids**

| Functions | |
|---|---|
| **Food quality** | Colour – carotenoids |
| | Texture, structure – cocoa butter |
| | Flavor, aroma – carbonyl compounds |
| | Lubricity – mouthfeel |
| | Satiety |
| **Nutritional** | Source of energy via β oxidation |
| | Carriers of fat – soluble vitamins |
| | Source of essential fatty acids |
| | Physical functions – micelle formation/bile: facilitate absorption of fat soluble vitamins |
| **Biological** | Vitamin A, D, E and K – numerous effects |
| | Cholesterol – precursor of vitamin $D_3$, corticosteroids, bile acids |
| | Linoleic acid – component of skin acylglucoceramides |
| | Inositol phospholipids – receptor signaling, signal transduction |
| | Arachidonic acid (AA) – eicosanoids and lipoxins |
| | Docosahexaenoic acid – specific membrane functions |

Source: Kinsella JE (1988) Food lipids and fatty acids: Importance in food quality, nutrition and health. Food Tech 42; 124-144.

Edible fats and oils are excellent cooking media. They can be heated at higher temperature for rapid cooking and simultaneously they impart surface texture and flavour to fried foods. Texture and consistency are important characteristics of edible fats that are determined by the relative concentrations of saturated and unsaturated fatty acids in the component TAG. They have important functions in the application of edible oils. In this regard, most of the fats used in contemporary vegetable oils, shortenings and margarines have relatively high levels of unsaturated fatty acids for a specific consistency.

**Nutritional and biological effects of food lipids**

Dietary lipids are hydrolyzed by pancreatic lipase and the free fatty acids and monoacylglycerol are absorbed in the upper segment of the small intestine. These are mostly resynthesized into TAG in the mucosal epithelial layer and assembled into chylomicrons, which enter the blood stream via the lymphatic system. These chylomicrons are metabolized in liver and tissues. Following uptake by the liver, both exogenous and endogenous fatty acids and cholesterol are incorporated into VLDLs and LDLs, secreted into blood and transported to peripheral tissues where, via lipoprotein lipase, they provide fatty acids for the various tissues (Grundy, 1986; McNamara, 1987). Once dietary lipids are absorbed they perform many diverse metabolic, structural and regulatory functions.

**Different types of fatty acids and their physiological effects**

Fatty acids are components of TAG, phospholipids, and cholesterol esters and also have structural, energetic and metabolic functions (Table 1.2). Fatty acids are distinguished from each other on the basis of their chemical structure. All have chain like structure with an acid or carboxyl group (HO-C=O) at one end and a methyl group ($CH_3$) at the other. The rest of the molecule consists of a hydrocarbon ($CH_2$) chain varying in length from 2 to 20 or more carbons.

**Table 1.2: Functions and effects of the various dietary fatty acids**

| Fatty acids | Functions/effect |
|---|---|
| **Short chain and medium chain** | Rapid source of calories and energy |
| **Saturated** | |
| Lauric (12:0) | |
| Myristic (14:0) | Hyperlipidemic-hypercholesterolemic, prothrombic |
| Palmitic (16:0) | |
| Stearic (18:0) | Neutral or hypolipidemic, precursor of oleic acid |
| **Monounsaturated** | |
| Oleic (18:1 n-9) | Hypolipidemic, hypocholesterolemic, precursor of eicosatrienoic acid in essential fatty acids insufficiency |
| Elaidic (18:1 *trans*) | Analogous to 18:0 |
| Erucic (22:1 n-9) | Impaired FA oxidation in heart of rat |
| **n-6 PUFA** | |
| Linoleic (18:2 n-6) | Essential FA , component of acylglucoceramides, precursor of arachidonic acid (AA), hypolipidemic compared to saturated FA, increase membrane fluidity |
| $\gamma$- Linolenic acid (18:3 n-6) | Precursor of eicosatrienoic acid and AA |
| $\gamma$- Homolinolenic acid (20:3 n-6) | Precursor of $PGE_1$ series of eicosanoids |
| Arachidonic acid (20:4 n-6) | Membrane fluidity, precursor of eicosanoids |
| **n-3 PUFA** | |
| $\alpha$- Linolenic acid (ALA) (18:3 n-3) | Hypolipidemic, membrane fluidity, precursor of EPA and DHA (essential FAs), reduces eicosanoids synthesis |
| Eicosapentaenoic acid (20:5 n-3) | Hypolipidemic, reduces AA synthesis and eicosanoids, precursor of $PGI_3$, $TXA_3$, precursor of $TXB_5$ |
| Docosahexaenoic acid (22:6 n-3) | Hypolipidemic, essential for vision, neural membranes, reduces AA synthesis, reduces eicosanoids in some cells |

Source: Kinsella JE (1988) Food lipids and fatty acids: Importance in food quality, nutrition and health. Food Tech 42; 124-144.

The most common fatty acids in foods have an even number of carbon atoms ranging from 12-22 carbons, though shorter, longer and odd-numbered fatty acids exist. Numerous clinical and experimetal investigations have shown that saturated fat increase circulating concentrations of serum cholesterol and TAG and their replacement by polyunsaturated or monounsaturated fatty acids shows hypocholesterolemic effects. Individual fatty acid or specific dietary fat appears to affect cholesterol metabolism as a result of changes in absorption, synthesis, distribution and excretion of cholesterol. However, the concept about the nature of dietary fatty acids influencing cholesterol metabolism has undergone constant evolution and the field continues to develop.

**Saturated fatty acids**

Dietary saturated fatty acids are major source of energy, and their intake should be closely adjusted to match the energy expenditure because of their capacity to increase plasma cholesterol by impairing LDL cholesterol uptake by liver receptor mechanisms (Medding *et al*, 1987; Spady and Dietschy, 1988). Lauric, myristic and palmitic acids are hyperlipidemic (Keys *et al* 1957), whereas stearic acid may have neligible impact, perhaps because it can be converted to oleic acid (Hegsted *et al* 1965; Bonanome and Grundy, 1988). Saturated fatty acids including stearic acid increase the tendency of platelets to aggregate and hence increase the risk of thrombosis. Temme *et al* (1996) reported that lauric acid raised total cholesterol concentrations more than palmitic acid, which was partly due to an increase in HDL cholesterol. The effects of myristic acid have been examined in several studies with mixed solid foods. Zock *et al* (1994) concluded that myristic acid increased plasma total cholesterol concentration relative to palmitic and oleic acids which was due to an increase in both LDL and HDL cholesterol concentrations. However, TAG concentration was not affected. Several types of dietary fatty acids affect the metabolism of lipoproteins (Harris, 1989). SFAs especially

myristic and palmitic acids are hyperlipidemic and hypercholesterolemic (McNamara, 1987; Hegsted *et al*, 1965). Excessive dietary SFAs may alter membrane composition and lipoprotein metabolism and may influence the metabolism of unsaturated fatty acids and the pattern of acylation of FA into tissue phospholipids pools. Dietary SFAs are also conducive to platelet aggregation (Hornstra, 1974) and have been associated with arrhythmia. Galli *et al*, (1981) reported that higher amounts of arachidonic acid accumulated in the platelets from rabbits consuming butter compared with those fed corn oil. This indicates that the FAs of butter facilitate arachidonic acid synthesis and its deposition in the platelets.

Stearic acid, a long chain 18 carbon dietary saturated fatty acid (SFA) known to have unique beneficial effects because, unlike other long chain SFAs, it has a neutral or lowering effect on plasma cholesterol (Bonanome and Grundy, 1988). However, to determine whether stearic acid is a healthy alternative to SFA and *trans* fatty acids, which increase cholesterol, it is relevant to study its effects on other important risk factors for coronary heart disease. It has been repeatedly suggested that long chain fatty acids are thrombogenic originates from studies performed in the 1960s, when it was shown that stearic acid, added as sodium salt to the blood *in vitro*, accelerated thrombus formation (Connor *et al*, 1963). Effect of stearic acid on LDL cholesterol is more comparable to those of oleic acid than to those of cholesterol raising saturated fatty acids or *trans* monounsaturated fatty acids. Stearic acid may slightly decrease HDL when compared with other saturated fatty acids but may have more favorable effect on the total and HDL cholesterol ratio (Mensink, 2005).

**Monounsaturated Fatty acids**

These are long chain fatty acids (LCFAs) with a single double bond. The predominant monounsaturated fatty acids (MUFA) are palmitoleic acid, (C 16:1, n-7),

oleic acid (C 18:1, n-9), vaccenic acid (C 18:1, n-7), cetoleic acid (20:1) and erucic acid (22:1, n-9). Vegetable oils contain very little 16:1 except palmolein, whereas olive and rapeseed oils are rich in oleic (18:1) and erucic acids (22:1) respectively. Among the MUFA's, oleic acid is identified as hypocholesterolemic fatty acid compared to other MUFAs. Oleic acid occurs in nature in two confirmations.

**Cis – oleic acid (n-9)** - This has been considered as a neutral fatty acid. The fact that the body synthesizes a large quantity of 18:1 suggests that it has a variety of biological functions. Oleic acid does not raise serum LDL cholesterol concentration. This is because it is a favoured substrate for acyl CoA: cholesterol acyl transferase (ACAT), which esterifies cholesterol. Hence excess of 18:1 in the liver is readily esterified. The resulting decrease in the amount of unesterified cholesterol in the liver cell may release the suppressive effect on LDL receptor synthesis (Grundy, 1991). Diets rich in oleic acid have no effect on HDL. (Mensink and Katan, 1989). Oleic acid can inhibit $\Delta$6 desaturase activity, but high intake levels are required to reduce arachidonic acid synthesis. This is also influenced by linoleic acid concentration (Holman, 1964). Lokesh *et al* (1988) have observed that the arachidonic acid content of tissues was lower in mice consuming diets containing olive oil compared with lard. This may indicate some inhibition of $\Delta$6 desaturase by the high level of oleic acid (68 %) in olive oil. Compared with SAFs, hypolipidemic and long term antithrombotic effect of oleic acid may relate to its rapid oxidation, its lack of negative effect on hepatic LDL receptors, its inhibition of $\Delta$6 desaturase, and its associated reduction of arachidonic acid synthesis.

***Trans* oleic acid**- This is produced by hydrogenation of polyunsaturated fatty acids (PUFA). It is known to raise LDL cholesterol concentration when compared with *cis* 18:1 and it also has mild HDL lowering action (Mensink and Katan, 1990). *Trans* MUFAs are more rigid and oils high in them tend to be solid at ambient temperatures. *Trans* MUFA,

like SFAs are a poor substrate for ACAT. Both may inhibit cholesterol esterification in the liver. The resulting increase in unesterified cholesterol concentration could suppress LDL receptor activity, which in turn increase serum LDL cholesterol concentration (Grundy, 1994; Sambaiah and Lokesh, 1999).

**Erucic acid (n-9)**

Erucic acid is a long chain MUFA found in plants, particularly in rapeseeds. This elongation product of oleic acid is an uncharacteristically long chain unsaturated fatty acid for plants. It is known to impair fatty acid oxidation in heart tissue of rats (Christopherson and Bremer, 1972). Erucic acid has been reduced in rapeseeds used for edible oil production by plant breeding. In animals, dietary erucic acid can be retro converted to from oleic acid via β-oxidation.

**Mead acid (n-9)**

Mead acid is hallmark of essential fatty acid deficiency and has the distinction of being the only major MUFA produced de novo by animals. In the absence of dietary n-6 and n-3 fatty acids, the Δ6 desaturase converts oleic acid to 18:2 (n-9), which is further elongated and desaturated (Δ5) to form mead acid (Cook, 1991). It has been speculated that mead acid compensates for the loss of n-3 and n-6 PUFA by increasing the unsaturation of animal cell membranes.

**Polyunsaturated fatty acids**

The unsaturated fatty acids with more than one double bond are classified as polyunsaturated fatty acids (PUFA). The important dietary PUFAs belong to two separate families, n-6 and n-3 (Figure 1.1).

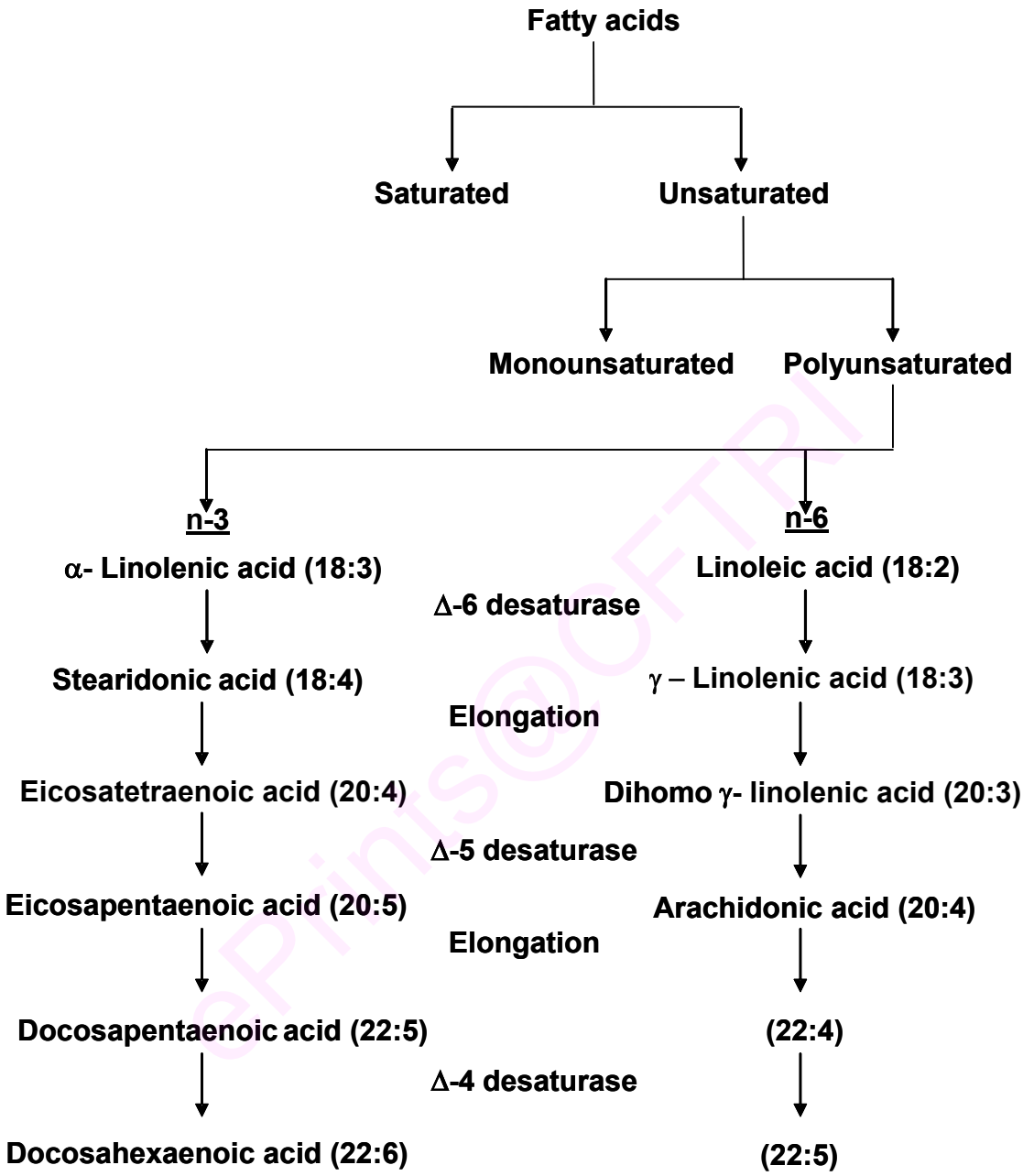


**Figure 1.1: Metabolic pathways of n-6 and n-3 fatty acids**

Source: Siddiqui RA, Harveya KA and Zalogac GP (2007) Modulation of enzymatic activities by n-3 polyunsaturated fatty acids to support cardiovascular health. J Nutr Biochem DOI: 10.1016/j.jnutbio.2007.07.001. (article in press)

**Essential fatty acids**

**n-6 PUFA**

Linoleic acid (LA) (18:2) and arachidonic acid (20:4) are essential fatty acids (EFA) as they are not synthesized by mammals (Burr and Burr, 1929). Hence, they have to be supplied through the diet. About 1-2% of dietary calories from linoleic acid are necessary to cure the symptoms of essential fatty acid deficiency, characterized by scaly dermatitis, excessive water loss through the skin, impaired growth and reproduction and delayed wound healing (Johnston, 1988). Linoleic acid is found in seeds of most plants in large amounts except coconut, palm and cocoa where it is present in very small amounts. In the body, 18:2 is metabolized to 20:4, which is precursor for biosynthesis of eicosanoids. In case of EFA deficiency, 18:1 is desaturated and elongated to eicosatrienoic acid (20:3, n-9) to compensate for arachidonic acid (Holman, 1960). Several studies have shown that 18:2 is less efficient in lowering LDL cholesterol level as compared to 18:1 (Mattson and Grundy, 1985). Mammals possess a series of desaturases and elongases for metabolism of stearic, linoleic and ALA to long chain mono and PUFAs. Four desaturases *viz*; the $\Delta$9, $\Delta$6, $\Delta$5 and $\Delta$4 of the rat liver have been characretized (Brenner and Peluffo, 1966; Brenner *et al*, 1981).

These fatty acids compete with each other especially at the rate limiting $\Delta$6 desaturase step. Dietary linoleic acid at low intake levels and in the absence of other unsaturated fatty acids is efficiently desaturated and elongated to arachidonic acid in the liver (Brenner and Peluffo, 1966; Brenner *et al*, 1982). Other unsaturated fatty acids, *trans* isomers of linoleic acid, particularly ALA, which are also substrates for $\Delta$6 desaturase, are effective competitive inhibitors for this enzyme.

The conversion of linoleic acid to arachidonic acid, especially at low levels of dietary intake, is inhibited by dietary ALA (Mohrhauer and Holman, 1963). This study indicated that as linoleic acid was increased, the level of ALA required to reduce tissue

arachidonic acid was increased. Hence dietary ALA is required to include in the diet to reduce tissue AA levels. Dietary ALA at 0.1 % of calories was sufficient to inhibit the elongation of linoleic acid by 50 % (Holman, 1964). Linoleic acid lowers total cholesterol concentrations. Kris-Etherton *et al* (1993) reported a greater total cholesterol lowering effect of linoleic acid compared with oleic acid whereas McDonald *et al* (1989) reported similar effects of linoleic acid and oleic acid on plasma total and lipoprotein cholesterol concentrations.

**Gamma linolenic acid (18:3 n-6) -** Gamma linolenic acid (GLA) is produced in animals and de novo by plants by the $\Delta$12 desaturation of LA. GLA is obtained from vegetable oils, such as evening primrose (*Oenothera biennis*) oil, blackcurrant seed oil, borage oil and hemp seed oil, and from spirulina, a cyanobacterium. Each contains varying amounts of the fatty acid, with borage oil usually being the most heavily concentrated form. Minute amount can be found in animal tissue (Padley *et al*, 1994). In animals dietary LA is desturated by the $\Delta$6 desaturase to produce GLA as an intermediate in the production of arachidonic acid (AA). Interestingly, dietary GLA is not substantially converted to arachidonic acid. However, a lack of GLA can occur when there is a reduction of the efficiency of the $\Delta$6 desaturase conversion (for instance, as people grow older or when there are specific dietary deficiencies) or in disease states where there is excessive consumption of GLA metabolites. From GLA, the body forms dihomo-gamma-linolenic acid (DGLA). This is one of the body's three sources of eicosanoids (along with AA and eicosapentaenoeic acid (EPA.) DGLA is the precursor of the prostaglandin (PG) PGH1, which in turn forms prostaglandin E1 and the thromboxane $TXA_1$. PGE1 has a role in regulation of immune system function. $TXA_1$ modulates the pro-inflammatory properties of the thromboxane $TXA_2$. Unlike AA and EPA, DGLA cannot yield

leukotrienes. However it can inhibit the formation of pro-inflammatory leukotrienes from AA.

**Arachidonic acid-** In chemical structure, arachidonic acid is a carboxylic acid with a 20-carbon chain with four cis double bonds and the first double bond is located at the sixth carbon from the omega end. Arachidonic acid is a polyunsaturated fatty acid that is present in phospholipids (especially phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol) of membranes of the body's cells, and is abundant in the brain. It is also involved in cellular signaling as a second messenger. Arachidonic acid is freed from phospholipid molecule by the enzyme phospholipase $A_2$. Arachidonic acid is a precursor in the production of eicosanoids: the enzymes cyclooxygenase and peroxidase lead to prostaglandin $H_2$, which in turn is used to produce the prostaglandins (prostacyclin) and thromboxanes and the enzyme 5-lipoxygenase leads to 5-HPETE, which in turn is used to produce the leukotrienes.

**n-3 Fatty acids**

n−3 Fatty acids (commonly spelled as omega-3 fatty acids) are a family of polyunsaturated fatty acids which have in common a carbon-carbon double bond at the n−3 position. Nutritionally important essential n−3 fatty acids are: $\alpha$- linolenic acid (ALA), EPA and DHA. The n-3 PUFAs in dietary fish oils markedly depress both the $\Delta$6 and $\Delta$9 desaturase enzymes, particularly when linoleic acid levels in diets are limiting. The PUFA of ALA family especially (DHA 22:6 n-3), its major end product in animal tissue, may be required for neural, visual and possibly reproductive functions. Dietary ALA is desaturated and elongated by the same enzymes involved in linoleic acid metabolism and there is mutual competition between these two PUFA families (Brenner and Peluffo, 1966). The human body cannot synthesize n−3 fatty acids de novo, but it can form 20-

and 22-carbon unsaturated n−3 fatty acids from the eighteen-carbon n−3 fatty acid (ALA) (Figure 1.1). These conversions occur competitively with n−6 fatty acids, which are essential and closely related chemical analogues that are derived from linoleic acid. Both the n−3 ALA and n−6 linoleic acid are essential nutrients which must be obtained from food. Synthesis of the longer n−3 fatty acids from ALA within the body is competitively slowed by the n−6 analogues. Thus accumulation of long-chain n−3 fatty acids in tissues is more effective when they are obtained directly from food or when competing amounts of n−6 analogs do not greatly exceed the amounts of n−3. By focusing mainly on the effects of fatty acids on blood cholesterol levels, attention remained with n-6 fatty acids for about 25 years. Even though in 1956, Sinclair and others were aware that Inuit (formerly called Eskimos) and some Norwegians were consuming high fat without any reported incidences of atherosclerosis (Sinclair, 1953).

It had been known since the 1940s that Greenland Eskimos did not develop heart disease in spite of their high fat consumption (Kromann and Green 1980). Close examination of their traditional diet revealed not only higher fat and cholesterol content than the typical European diets, but also substantial amounts of n-3 fatty acids (Bang *et al*, 1980). n-3 Fatty acids were virtually absent from many European and North American diets. The suggestion by Bang, Dyerberg and colleagues that n-3 fatty acids were linked to freedom from heart disease ushered in a new era of n-3 fatty acid research (Dyerberg and Bang, 1978; Dyerberg *et al*, 1978). We now regard n-3 fatty acids as fundamental molecules in the structure and activity of the membranes of all cells (Ahmed *et al*, 2002).

**Eicosanoids**

The eicosanoids (prostanoids and leukotrienes) are important signaling agents which affect cell behaviour and cell-to-cell interactions and they are produced from different essential fatty acids (Figure 1.2).

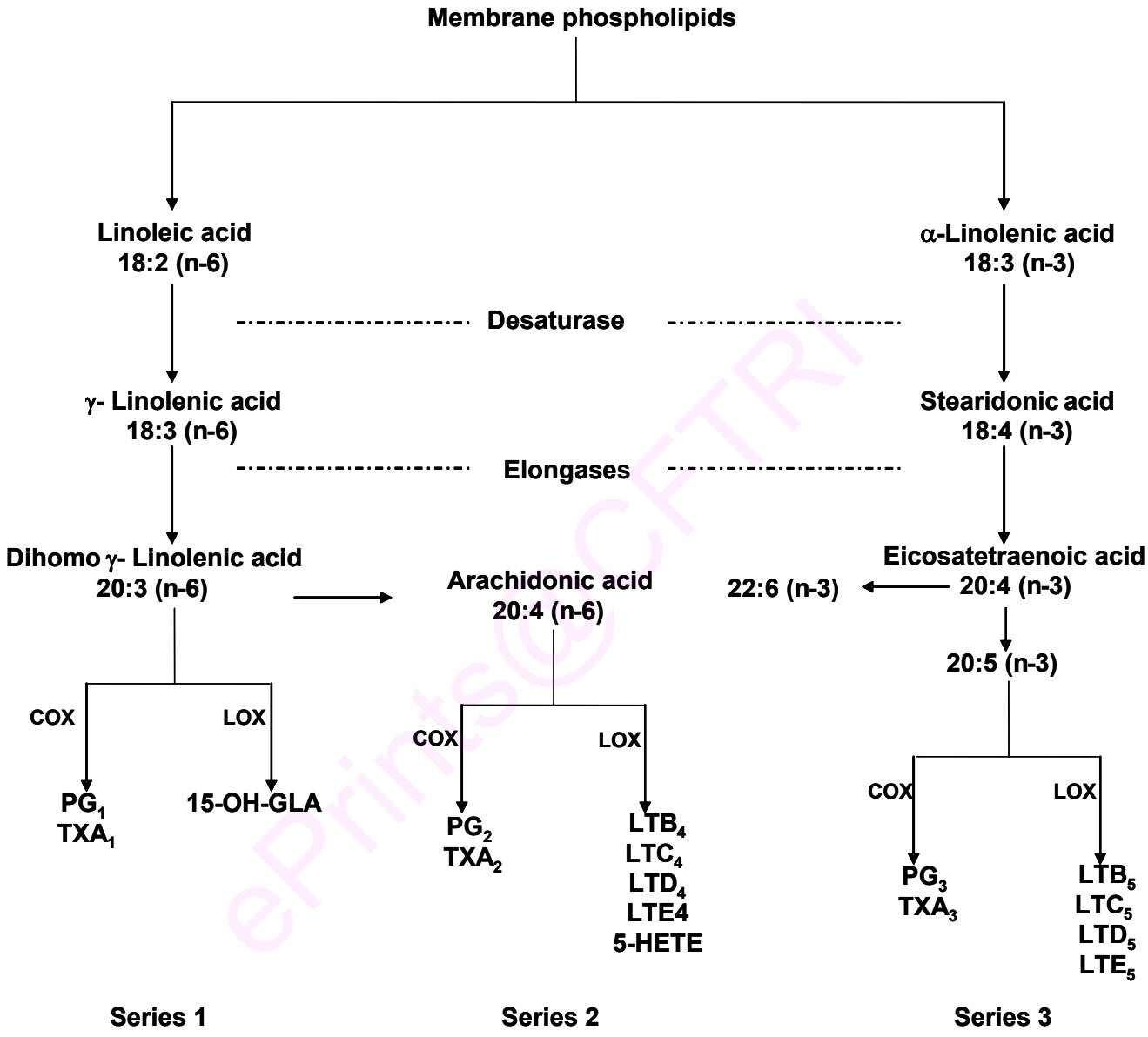


**Figure 1.2: Production of different series of eicosanoids from n-6 and n-3 PUFA**

COX- cyclo-oxygenase; LOX- Lipoxygensase; PG- Prostaglandin; TX- Thromoboxane; LT- Leukotrienes; HETE; Hydroxy eicosatetraenoic acid

They modulate secretory, smooth muscle (contraction or relaxation) and cascade-type reactions (Table 1.3) which are essential to normal health. A deficiency of these compounds results in progressive impairment of functions, while excessive or imbalanced production may result in a number of phathophysiological states, for example: inflammation, immunosupression, arthritis and thrombosis (Marcus, 1984). Eicosanoids are particularly involved in cardiovascular, renal and pulmonary functions and are especially involved in the protective role of blood cells such as platelets, monocytes, macrophages and neutrophils (Goodwin and Webb, 1980). Hence they are actively involved in platelet aggregation, inflammation, anti-infection and immuno protective functions (Goodwin and Webb, 1980). The balanced production of these eicosanoids (many of which acts antagonistically) modulates short term local response to injury or infection that are required for normal health. If chronically produced in excess they may result in pathophysiological states characterized by edema, erythema (pain, swelling and redness) as may occur in arthritis, inflammation, angina pectoris, thrombosis (excess thromboxane), inflammation, asthma and psoriasis (excess localized production of leukotrienes by subepidermal leukocytes (Goodwin and Webb, 1980). The pervasive effects of eicosanoids on vascular functions, atherogenesis and thromboembolic events, the dietary fatty acids gain importance in regulation of eicosanoids production *in vivo*. The production of proaggregatory, vasoconstructive thromboxane ($TXA_2$) by platelets must be counter balanced by continued production of the vasodilatory antiaggregatory prostacyclin ($PGI_2$) by the vascular endothelium to maintain vessel tone and blood circulation. An excess of $TXA_2$ or impaired synthesis of $PGI_2$ is condusive to platelet aggregation, which may form a thrombin in a stenotic artery and cause infraction. This together with cardiac arrhythmia is the most common event causing lethal heart attack (Davies and Thomas, 1984).

**Table 1.3: Some biological functions of prostanoids and leukotrienes**

| Organ system | Effects | Species involved [a] |
|---|---|---|
| **Prostanoids** | | |
| Blood vessels | Vasodilation | $PGI_2 > PGI_3 > PGE_1$ |
| | Vasoconstriction | $TXA_2$ |
| Platelets | Ahesion, aggregation | $TXA_2$ |
| | Antiaggregatory | $PGI_2 > PGI_3 > PGE_1$ |
| Lung | Bronchiole constriction | $PGF_2$, $TXA_2$, $PGD_2$ |
| | Bronchiole dilation | $PGE_2$, $PGI_2$ |
| Kidney | Glomerular filtration rate | $PGE_2$, $PGI_2$, $TXA_2$ |
| | Renin secretion | $PGI_2$, $PGF_2$ |
| Stomach | Acid secretion | $PGE_2$, $PGE_1$ |
| Small intestine | Peristalsis | $PGE_2$, $PGF_2$ |
| Pancreas | Amylase secretion | $PGE_2$, $PGI_2$ |
| | Insulin secretion | $PGE_2$ |
| Hypophysis | Secretion of growth hormone adrenocorticotrophic hormone | $PGE_2$ |
| Tissue | Pain | $PGE_2$ |
| | Cytoprotection | $PGI_2$, dimethyl PGE |
| **Leukotrienes** | | |
| Bronchioles | Constriction | $LTC_4$, $LTD_4$ |
| Illeum | Constriction | $LTC_4$, $LTD_4$ |
| Vascular | Constriction | $LTC_4$, $LTD_4$ |
| | Permeability | $LTC_4$, $LTD_4$ |
| Pancreas | Insulin secretion | $LTB_4$, HETE |
| Neutrophils | Adhesion | $LTB_4$ |
| Monocytes | Chemotaxis/-kinesis | $LTB_4$, HETE |

[a] PG – prostaglandin; $PGI_2$ – prostacyclin, TXA – thromboxane, LT – leukotriene, HETE – hydroxyeicosatetraenoic acid

Source: Kinsella JE (1988) Food lipids and fatty acids: Importance in food quality, nutrition and health. Food Tech 42; 124-144.

## Health effects of n-3 fatty acids

### Lipid levels (lipoproteins)

Dietary n-3 PUFA can modify plasma lipids and lipoprotein metabolism (Kris-Etherton *et al*, 1988). In healthy subjects, increased consumption of n-3 fatty acids is associated with lowering of serum triacylglycerol (TAG)) and very low-density lipoproteins and when used at high doses they would decrease both serum cholesterol and apolipiprotein B concentrations (Sanders *et al*, 1989; Guoping *et al*, 1999). The hypolipidemic effect of n-3 PUFA may be mediated by several mechanisms. n-3 PUFA can reduce TAG synthesis and chylomicron secretion from intestinal cells (Harris, 1989) and suppresses hepatic fatty acid synthesis and TAG production, thereby limiting VLDL secretion. Dietary fish oil decreases the VLDL TAG pool by suppressing TAG synthesis and possibly enhancing the clearance of VLDL TAGs (Nossen *et al*, 1986).

Dietary n-3 PUFA may promote lipoprotein metabolism by altering the activity of certain lipolytic and transfer enzymes functioning in the plasma. Aviram (1986) reported that n-3 PUFA facilitate the transfer of fatty acids from VLDLs to HDLs. Fish oil increased plasma lecithin:cholesterol acyltransferase (LCAT) concentration by 45 % in rats (David *et al*, 1987). The decreased LCAT activity would reduce the cholesterol ester (CE) content and the size of LDL particles. n-3 PUFA consumption from fish oil reduces CEs and size of LDLs in monkeys (Parks *et al*, 1989). Acyl-coenzyme A- cholesterol acyl tranferase (ACAT) regulates the intracellular catabolism of CE. The activity ACAT was higher whereas HMG- CoA reductase activity was lower in intestine and liver of rabbits fed n-3 PUFA from menhaden oil (Field *et al*, 1987).

### Cardiovascular system

n-3 PUFA, mainly those contained in fish oils, are candidates for inclusion in secondary prevention programmes for coronary heart disease based on the results of

recent randomized trials in humans. Marine n-3 PUFA retard coronary atherosclerosis and appear to prevent fatal arrhythmias; and they decrease mortality subsequent to myocardial infarction (Angerer and Clemens, 2000). n-3 PUFAs have many beneficial effects on the cardiovascular system via their effects on several cellular processes. n-3 PUFAs improve the plasma lipid profile. TAG lowering effect was shown in normal and hypertriglyceridemic patients when n-3 PUFA were given (7 g or less of n-3 PUFA /day) for two weeks. Recent studies also concluded that higher levels of n-3 PUFAs for longer duration have beneficial effects on plasma lipid profile and n-3 PUFAs also have antiatherogenic actions (McKenney and Sica, 2007). Various studies concluded that the n-3 PUFA effect on new plaque development appeared to be due to antithrombotic as well as antiatherosclerotic properties of n-3 PUFA (Willis *et al,* 1986).

**Enzymes regulating calcium ion channels**

An increase in cellular $Ca^{2+}$ is associated with enhanced cell contraction, vasoconstriction and cell proliferation and, thus, may be involved in the development of cardiovascular diseases (Locher *et al*, 1991). Intracellular free calcium ions ($Ca^{2+}$) serve as a cofactor for several enzymatic processes required for cellular growth. Among various biochemical processes, the increase in intracellular $Ca^{2+}$ plays an important role in the development of arrhythmia (Niggli, 2007) and cardiac hypertrophy (Berridge, 2006). Under normal physiological conditions, intracellular levels of $Ca^{2+}$ are tightly controlled by a number of key enzymes, including voltage dependent channels, $Na^{+}/Ca^{2+}$ exchanger, receptor mediated calcium channels and the ryanodine receptor (RyR) and $Ca^{2+}$ ATPase pump. Any abnormalities in any of these key regulators contribute to abnormal $Ca^{2+}$ handling and, thus, lead to cardiac dysfunction, including arrhythmia generation, hypertrophy and myocardial stunning. The primary action of cardiac glycosides is to inhibit this enzyme, which is also known as the sodium pump. With

inhibition of this enzyme, sodium ions accumulate in the cell and intracellular concentrations of potassium decrease. Increased intracellular sodium activity favors the accumulation of calcium ions in the cell via the $Na^+/Ca^{2+}$ antiport system. Ion channels, and other cellular processes, including various active phospholipases, receptor-bound enzymes and protein kinases, also play an important role in regulating intracellular calcium and other cellular processes. The activities of these enzyme systems are also modulated by n-3 PUFAs to beneficially affect the cardiovascular system. Phospholipase $A_2$ ($PLA_2$) is physiologically important enzyme whose activity is also modulated by n-3 PUFAs. $PLA_2$ catalyzes the hydrolysis of fatty acids from the sn-2 position of membrane phospholipids, resulting in the production of pro-inflammatory AA-derived eicosanoids. Most of the effects of $PLA_2$ believed to be involved in inflammation (Oestvang and Johansen, 2006), atherogenesis (Reiss and Edelman, 2006) and cardiac functions (Giles, 1990) are mediated through eicosanoid generation (Gerritsen, 1996). The omega-3 index has been validated as a surrogate for myocardial omega-3 FA composition in the human and as such reflects the omega-3 status of the most critical organ (Harris, 2007).

**Vascular retina**

Docosahexaenoic acid, a major dietary n-3 LCPUFA, is also a major structural lipid of retinal photoreceptor outer segment membrane. Biophysical and biochemical properties of DHA may affect photoreceptor membrane function by altering permeability, fluidity, thickness, and lipid phase properties. Tissue DHA status affects retinal cell signaling mechanisms involved in phototransduction. DHA may operate in signaling cascades to enhance activation of membrane-bound retinal proteins and may also be involved in rhodopsin regeneration. Tissue DHA insufficiency is associated with alterations in retinal function. Visual processing deficits have been ameliorated with DHA supplementation in some cases (SanGiovanni and Chew, 2005).

**Haemostatic system**

Blood flow is affected by the ability of various blood cells to adapt their shape to the nature of the passage way to squeeze through narrow passages and around obstacles. Red blood cells also need to adjust their shape in order to pass through capillary channels that are often to the size of the red cell. The process of thrombosis involves the formation of a blood clot at the site of tissue or vascular injury (Furie and Furie, 2005). This process is essential to repair cuts and wounds and maintain blood vessel integrity but potentially fatal if a large clot forms in, or is delivered to blood vessels of heart, lung and brain. Platelet aggregation requires fibrinogen from the circulation and is stimulated by thromboxane $A_2$ ($TXA_2$), an extremely potent prostaglandin synthesized by the platelets itself (Leung and Nachman, 1982). $TXA_2$ also promotes vasoconstriction, a narrowing of blood vessels, and promotes thrombosis (Kinsella *et al*, 1990). Multiple effects of n−3 PUFAs on function of thrombocytes, coagulation including lowering of thrombocytes and increase of fibrinolytic activity have been documented in experimental studies (Harper and Jacobson, 2001).

**Endothelial function**

The antiatherosclerotic effects of n-3 PUFAs appear to be mediated through their anti-inflammatory effects on platelets and endothelial cells. Platelets, through their interaction with the vascular endothelium, play a critical role in atherogenesis (Ross 1993). Mori *et al*. (1997) observed that human consumption of n-3-PUFAs (3–4 g/day) for 3 weeks reduced platelet aggregation induced by collagen and platelet-activating factor (PAF) regardless of whether n-3 PUFAs were ingested as daily fish meals or fish oil capsules. The ineffectiveness of DHA implies that modulation of platelet aggregation by n-3 PUFAs may be mediated through the eicosanoid pathway rather than being a direct effect of fatty acids on platelets. Several studies have now been published on

effects of n-3 PUFA on endothelial function. When EPA and DHA are ingested together, they have favorable effects on vasodilation in coronary arteries, brachial arteries and the skin microcirculation (Stanley, 2007).

**Nervous system**

Essential PUFA can affect brain functions beyond the critical period of perinatal development. Alterations of n-6 and n-3 status have been associated with psychiatric pathologies in children and adults. Decreases in the blood levels of n-3 and/or n-6 PUFA have been observed in patients with depression (Adams *et al*, 1996; Maes *et al*, 1996; De Vriese *et al*, 2003). The levels of α-LNA, EPA, DPA and DHA in erythrocytes were correlated positively with their respective dietary intakes, which suggest that their lower levels found in depressed patients could be a consequence of a dietary deficiency (Edwards *et al*, 1998). Since the protecting effect of DHA seems to be associated with well-installed food habits, it is possible that the mechanisms implicated concern the concentration of this fatty acid in brain tissue membranes. Docosahexaenoic acid a possible prophylactic means for preventing the learning deficiencies of Alzheimer's disease. (Hashimoto *et al*, 2002).

**Recommended dietary intake of essential fatty acids**

The American Heart Association recommends less than 30 % of total calories from fat. Adequate intakes have been set for LA and ALA. The adequate intake of LA is 17 and 12 g/d for men and women aged 19 to 50 years respectively. The adequate intake of ALA is 1.6 and 1.1 g/d for men and women aged 19 t0 70 years, respectively. The NIH working groups proposed adequate intakes of 2 to 3 % of total calories for LA, 1 % of total calories for ALA, and 0.3 % of total calories for EPA and DHA. The working groups further recommended intakes of EPA and DHA of 650 mg/d and a minimum of 300 mg DHA/d during pregnancy and lactation (Simopolous, 1999b). Health Canada

suggests a minimum of 3 % of energy from n-6 fatty acids and 0.5 % from n-3 fatty acids or 1 % for infants who do not receive a preformed source of EPA and DHA (Scientific Review Committee, 1990). The United Kingdom recommends that 1 % energy be from ALA and 0.5 % from EPA and DHA combined (COMA, 1994). Some experts suggest that vegetarian (and others receiving no direct EPA and DHA) at least double the recommended intakes for ALA. This would suggest an intake of ALA in the range of 1 to 2 %.

The US Food and Drug Administration (FDA, 1997) has formally stated that consumption of upto 3 g/ d of marine-based n-3 fatty acids is generally regarded as safe (GRAS). The FDA (2002) has also approved a health claim for DHA and EPA in supplement form. Governmental regulatory bodies have issued statements concerning the potential for hemorrhagic risk with intake of 43 g/d n-3 LCPUFAs (Kris-Etherton *et al*., 2002). The anti-thrombotic and anti-haemostatic effects of n-3 LCPUFAs operate within physiologic limits at intakes between 1.0 and 3.0 g/d (Dyerberg and Bang, 1979; Levine *et al*., 1989) and at these levels hemorrhagic risk is not considered a major issue. The scientific statement issued by the American Heart Association (AHA) on n-3 fatty acids and cardiovascular disease reviews safety of n-3 fatty acids and fish (Kris-Etherton *et al*, 2002).

**Importance of n-6 to n-3 ratio**

Both n-6 and n-3 PUFA are required for eicosanoids synthesis. The plasma membrane bound enzyme cyclo-oxygenase is a key enzyme in the synthesis of prostaglandins from PUFA. When the substrate for this enzyme is AA, then the prostagldins of 2 series are synthesized but when the substrate is EPA then prostaglndins of the series 3 are synthesized (Figure 1.2). Prostaglandins play a role in promoting both aggregation and inflammation, and in general prostagladins of the

2 - series are more potent than of 3 - series (Table 1.3). If the ratio of AA to EPA in the plasma membrane responds in turn to the dietary n-6:n-3 PUFA ratio then the magnitude of the aggregatory and inflammatory and hence health could be influenced by the type of PUFA consumed. (Stanley *et al*, 2008). While linoleic acid and ALA exert physiological effects in their own right, the essentiality of these fatty acids and the relevance of the ratio of n-6/n-3 PUFAs to cardiovascular health lie in their competitive conversion to longer chain PUFAs via a series of stepwise desaturations and elongations as mentioned earlier. Both fatty acids compete for the activity of a rate-limiting $\Delta$6-desaturase and, although this enzyme shows greater substrate specificity for ALA, the over abundance of dietary linoleic acid gives it a quantitative advantage that limits the conversion of ALA to EPA *in vivo*. Even so, the relationship between the dietary intake of ALA and change in membrane EPA is positive and linear over intakes of ALA between 2 and 10 g but strongly influenced by factors such as age, sex, and the n-6 PUFA (linoleic acid) content of the background diet (Burdge and Calder, 2005). The ratio of n-6/n-3 PUFAs therefore has utility in providing information on the physiological effects of linoleic acid and ALA, and the metabolic conversion of the latter to EPA. The ratio of n-6/n-3 PUFAs does have a number of inherent problems that were concisely reviewed by Harris (2007). Firstly, the ratio makes no distinction between ALA and the metabolically more active EPA/DHA. Secondly, all ratios suffer from the fact that the components can change in different directions or not at all to produce a higher or lower ratio. In terms of n-6 and n-3 PUFAs, this has produced considerable variation in the physiological response to a decrease in the ratio that has confounded the interpretation of intervention studies. Finally, there is an underlying premise that the ratio balances bad and good elements of n-6/n-3 PUFAs, primarily because eicosanoids derived from the principal membrane n-6 PUFA, arachidonic acid, are metabolically more potent in promoting inflammation, platelet aggregation, and immune and vascular reactivity than those

derived from LC n-3 PUFAs (Calder, 2003). It follows from this idea that high or increased intakes of dietary linoleic acid as the dietary precursor of arachidonic acid should increase membrane arachidonic acid and exert adverse effects on CVD when there is epidemiological evidence to suggest that the opposite is true, and that both linoleic acid and ALA exert favourable effects on CVD risk (Asherio *et al*, 1996). Patients with CVD have not only low tissue arachidonic acid status, but also there is no relationship between dietary linoleic acid and tissue membrane arachidonic acid in humans (Harris, 2007; Harris *et al*, 2007). This evidence conflicts with widely held view, and recent cross-cultural evidence (Hibbeln *et al*, 2006) that dietary n-6 PUFAs, chiefly linoleic acid, exerts adverse effects on cardiovascular health, mediated through tissue arachidonic acid. Two recent studies that have attempted to resolve the controversy surrounding the dietary n-6/n-3 ratio include a large, randomly controlled intervention, the quantification of the optimal n-6/n-3 ratio in the UK Diet (OPTILIP) study (Griffin *et al*, 2006; Sanders *et al*, 2006).

Several sources of information suggest that man evolved on a diet with a ratio of fatty acids of ~ 1. Whereas today, this ratio is approximately 10:1 to 25:1 indicating that diets are deficient in n-3 fatty acids compared with diet on which human evolved and their genetic patterns were established (Simopolous 1999a). Studies on the evolutionary aspects of diet indicate that major changes have taken place in our diet, particularly in the type and amount of essential fatty acids (EFA). Present dietary pattern indicates an increase in the total fat consumption compared to the dietary pattern of the society that lived in the era of hunter, gather and agriculture. The consumption of *trans* fatty acids has also increased in the past 50 years (Simopolous, 1991). Intake of n-3 fatty acids is much lower today because of the decrease in fish consumption and industrial production of animal feeds rich in grains containing n-6 fatty acids, leading to the production of meat rich in n-6 and poor in n-3 fatty acids (Cunnane *et al*, 1999). Both n-6 and n-3 fatty acids

are not inter convertible in human body and are important components of practically all cell membranes. n-3 Fatty acids are known to play important role in prevention and treatment of coronary artery diseases, hypertension, diabetes, arthritis, other inflammatory and autoimmune disorders and cancer (Simopolous *et al*, 1986).

**Different edible oils**

There are several kinds of vegetable oils consumed in India and also there are regional preferences in the choice of edible oil (Achaya, 1987). India is one of the largest producers of oilseeds in the world. India accounts for 8.8% of world oilseed production. With oilseed production of about 22 mn tons and oil production of around 7 mn tons, India is the world's fourth largest edible oil economy. It is the world's largest producer of castor seed, the second largest producer of groundnut and the third largest producer of rapeseed. (http://indiaimage.nic.in/pmcouncils/reports/industry/tsld045.htm) The oilseeds area and output is concentrated in central and southern parts of India, mainly in Madhya Pradesh, Gujarat, Rajasthan, Andhra Pradesh and Karnataka. A variety of edible vegetable oils and animal fats are used as dietary fat. The use of particular fat or oil is dictated more by traditional food habits and cost rather than considerations of nutritional qualities. However in recent times, the nutritional quality of oils and fats have gained importance due to their involvement in the diseases of the circulatory and nervous systems. Vegetable oils, in general, differ in their fatty acid composition (Table 1.4). Most of them contain high levels of oleic and linoleic acids along with major SFA, palmitic acid. The fatty acid profile of edible oils play an important role in their stability and nutritional value. No oil by nature provides all fatty acids in optimum amounts as desired by nutritionists. The major oilseeds cultivated in India are groundnut, mustard/rapeseed, sesame, safflower, linseed, niger seed, castorseed, soybean, coconut and sunflower. In non-conventional oils, rice bran oil and cotton seed oil are the most important.

Groundnut, soybean and mustard together contribute about 85 percent of the country's oilseeds production. Groundnut oil is preferred in Western and Southern states except Kerla where coconut oil is used. Sesame oil is also popular in South India. The Northern and Eastern states prefer mustard oil. In Southern Maharashtra and Northern Karnataka safflower oil is preferred.

**Table 1.4: Fatty acid composition of some commonly used vegetable oils**

| Fatty acids | Rice bran oil | Coco-nut oil | Palm oil | Olive oil | Ground-nut oil | Sun-flower oil | Soy-bean oil | Lin-seed oil | Rape-seed oil |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | (%) | | | | |
| 8:0 | - | 8.0 | - | - | - | - | - | - | - |
| 10:0 | - | 6.4 | - | - | - | - | - | - | - |
| 12:0 | - | 48.5 | 0.3 | - | - | - | - | - | - |
| 14:0 | - | 17.6 | 1.1 | - | - | - | - | - | - |
| 16:0 | 21.5 | 8.4 | 45.1 | 13.7 | 11.6 | 11.0 | 11.0 | 4.8 | 2.8 |
| 18:0 | 2.2 | 2.5 | 4.7 | 2.5 | 3.1 | 4.7 | 4.0 | 4.7 | 1.3 |
| 18:1(n-9) | 43.5 | 6.5 | 38.8 | 71.1 | 46.5 | 18.6 | 23.4 | 17.9 | 23.8 |
| 18:2(n-6) | 32.0 | 1.5 | 9.4 | 10.0 | 31.4 | 68.2 | 53.3 | 15.9 | 14.6 |
| 18:3(n-3) | 0.2 | - | - | 0.6 | - | 0.5 | 7.8 | 54.4 | 7.3 |
| 20:0 | - | - | - | 0.9 | 1.5 | 0.4 | 0.3 | - | 0.7 |
| 20:1 | - | - | - | - | 1.4 | - | - | - | 12.1 |
| 20:2 | - | - | - | - | - | - | - | - | 0.6 |
| 22:0 | - | - | - | - | 3.0 | - | - | - | 0.4 |
| 22:1 | - | - | - | - | - | - | - | - | 34.8 |
| 24:0 | - | - | - | - | 1.0 | - | - | - | 1.0 |

Source: Chow CK (1992) Fatty acids in food and their health implications, Marcel Dekker, Inc. New York.

Indian diets are usually cereal-pulse based, and the vegetable oil used as cooking medium, the major source of visible fat (Vinodhini *et al*, 1993). Visible fat (easily extractable) is that is extractable from oilseeds (vegetable oil) or from milk (butter and ghee). Apart from visible fat, every food ingredient contains some amount of invisible fat (difficult to extract). The composition of fatty acids in different fats varies widely and listed in Table 1.5.

**Table 1.5: Approximate fatty acid composition of visible fats**

| Oil | SFA | MUFA | LA | ALA | LA/ALA |
|---|---|---|---|---|---|
| | | | (g/100g) | | |
| Coconut oil | 89 | 7 | 2 | <0.5 | 4 |
| Palm kernel | 82 | 15 | 2 | <0.5 | 4 |
| Ghee | 65 | 32 | 2 | 0.5 | 4 |
| Red palm (raw) | 50 | 40 | 9 | <0.5 | 18 |
| Palm | 45 | 44 | 10 | <0.5 | 20 |
| Olive | 13 | 76 | 10 | <0.5 | 20 |
| Groundnut | 24 | 50 | 25 | <0.5 | 20 |
| Sesame | 15 | 42 | 42 | 1 | 42 |
| Rice bran | 22 | 41 | 35 | 1.5 | 23 |
| Cotton seed | 21 | 25 | 52 | 1 | 52 |
| Corn | 12 | 32 | 55 | 1 | 55 |
| Sunflower | 13 | 27 | 60 | <0.5 | 120 |
| Safflower | 13 | 17 | 70 | <0.5 | 140 |
| Soybean | 15 | 27 | 53 | 5 | 11 |

SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, LA: Linoleic acid, ALA: $\alpha$- Linolenic acid.

Source: Ghafoorunissa (1994) Dietary lipids and heart disease-the Indian context. The National Med J India **7**; 270-275.

The percentage of LA in safflower, sunflower, soybean, corn and cottonseed is more than 50 % of the total fatty acids, while sesame, rice bran and groundnut oil contains 20 – 40 % of LA. Palm oil contains about 10 % LA. Coconut and palm kernel oil contain a high proportion of SFAs of short and medium chain length. Soybean oil contains appreciable amounts of ALA. Ghee is rich source of saturated fatty acids and about 65 % of these are short and medium chain length. Vanaspati contain long chain saturated fatty acids and *trans* fatty acids account about 55 %.

Another type of fat consumed is invisible fat derived from various food items consumed. Every food contains fat as an integral component of the cell (plant or animal). It is seen that cereals and millets that are bulk items in our diets contribute substantially to fat intake. A daily consumption of about 300-500 g of cereals and millets alone provides about 8-12 g invisible fat. The invisible fat of plant foods are good sources of oleic, LA and ALA (Table1.6). While in most of these LA predominates whereas, legumes and green leafy vegetables contain significant proportion of ALA (Ghafoorunissa and Jyotsna, 1993).

**Minor constituents of oils**

Vegetable oils and fat mainly consists of TAG but they also contains some minor constituents as its unsaponifiable fraction. These minor constituents have various health benefits. Butter and ghee contains vitamins A and D. All the vegetable oils contain various tocols (tocopherols and tocotrienols). Tocols are antioxidants and they protect the cells from free radical damage. The total tocol content of palm oil is 1g/100g, of which tocopherols and tocotrienols constitute 30 and 70% respectively. The tocotrienols are hypocholesterolemic due to their inhibitory effect on endogenous cholesterol synthesis and also have antioxidant, antithrombotic and anticarcinogenic properties (Hood and Sidhu, 1992).

**Table 1.6: Invisible fat and fatty acids in plant foods**

| Food | Invisible fat | SFA | MUFA | LA | ALA | LA/ALA |
|---|---|---|---|---|---|---|
| | (g/100g) | | | | | |
| **Cereals** | | | | | | |
| Wheat | 2.9 | 0.5 | 0.3 | 1.1 | 0.17 | 6 |
| Maize | 4.8 | 0.8 | 1.1 | 2.2 | 0.05 | 47 |
| Jowar | 3.3 | 0.6 | 1.0 | 1.5 | 0.05 | 30 |
| Ragi | 1.5 | 0.3 | 0.7 | 0.3 | 0.05 | 6 |
| Bajra | 5.5 | 1.2 | 1.2 | 2.2 | 0.13 | 17 |
| **Legumes** | | | | | | |
| Black gram | 1.7 | 0.3 | 0.2 | 0.1 | 0.7 | 0.2 |
| Rajmah | 2.2 | 0.4 | 0.2 | 0.5 | 0.7 | 0.7 |
| Green gram | 1.7 | 0.5 | 0.05 | 0.6 | 0.2 | 3 |
| Red gram | 2.2 | 0.5 | 0.1 | 1.0 | 0.1 | 8 |
| Lentil | 2.0 | 0.3 | 0.4 | 0.8 | 0.16 | 5 |
| Bengal gram | 6.0 | 0.7 | 1.7 | 1.2 | 2.7 | 1.8 |
| Peas | 2.1 | 0.3 | 0.4 | 0.8 | 0.15 | 5 |
| Soybean | 20 | 2.8 | 5.4 | 10.4 | 1.4 | 7 |
| **Vegetables** | | | | | | |
| Green leafy | 0.4 | 0.09 | 0.025 | 0.04 | 0.15 | 0.3 |
| Others | 0.2 | 0.05 | 0.016 | 0.06 | 0.03 | 2 |
| **Nuts** | | | | | | |
| Coconut | 40 | 36 | 3.2 | 0.6 | - | - |
| Groundnut | 40 | 8.8 | 21 | 10 | 0.2 | 50 |
| Sesame | 40 | 6.0 | 18 | 16 | 0.4 | 40 |
| Mustard | 40 | 2.0 | 5 | 5 | 3.4 | 1.4 |
| Almond | 56 | 25 | 19 | 8 | 0.2 | 40 |

SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, LA: Linoleic acid, ALA: $\alpha$-Linolenic acid

Source: Ghafoorunissa (1996) Fat in Indian diets and their nutritional and health implications. Lipids **31**; 2785-2915.

Rice bran oil contains several minor constituents that exhibit antioxidant properties (Rong *et al*, 1997). These include gamma-oryzanol, tocotrienols, tocopherols, and squalene. The unsaponifiable fraction of sesame oil contain active minor constituents which are responsible for the various health benefits. Sesame oil is rich in vitamin E and minor constituents like sesamin and sesamolin, protect the oil from oxidative rancidity and have health benefits (Hirose *et al*, 1991).

**Linseed oil**

The fatty seed oils of flax (*Linum usitatissimum*) provide a fat profile that makes them useable for nutritional purpose, as well as for medicinal and technical applications. The fatty acid composition of this oil is well known, and its unusual high content of PUFA provides positive nutritional and health effects, as shown in animal and human studies (Tolkachev and Zhuchenko, 2000). Flax also known by its synonym linseed, is cultivated in Europe since the first century. Linseed oil is obtained from raw flax seeds by cold pressing, solvent extraction, or extraction by supercritical $CO_2$. The high content of ALA (50%), which is essential in the development of the brain and retinal tissues of infants. PUFA content of LSO is about 70% and it leads to auto-oxidation and therefore limits the possible use of linseed oil for frying and cooking, as unintentional degradation products may induce contingent unpleasant off-flavours. The high content of essential fatty acids is responsible for numerous health benefits including cholesterol-lowering and anti-cancerogenic effects. The oil has been used in several traditional medicinal purposes, e.g. internally in the treatment of irritations caused by inflammatory agents especially in the gastrointestinal tract (Tolkachev and Zhuchenko, 2000), and some anti-malarial properties of the oil were proposed by Levander and Ager (1995). Linseed oil diet supplementation may favourably change the tissue $\alpha$ - linolenic acid/linoleic acid ratio (Simopoulos, 2002; Kuriki *et al*, 2003).

**Fish oil**

Fish oil is rich in long chain n-3 PUFA such as EPA and DHA. However modern society tends to include very little fish in their diet and increasing fish consumption would involve major dietary changes. More than half (up to 65 %) of the population do not eat fish. Vegetarians in particular do not prefer fish in their diet. Thus there is need for alternative source of n- 3 fatty acids. Therefore alternative ways to increase consumption of n-3 fatty acids have to be explored and assessed (Mantzioris *et al*, 2000).

**Rice bran oil**

Rice bran is a by-product of the rice milling industry. Typically rice bran accounts for 7-8 % of raw rice and contains about 15 % of oil. India produces 7-9 lakh tons of RBO (http://www.mcxindia.com/products_ricebranref_oil.html). RBO contains relatively high proportion of oleic acid (40 %) followed by linoleic and palmitic acids and it may also contain detectable amount of ALA ranging from 1-3 % (Sugano and Tsuji, 1997). The most characteristic component of RBO is γ- oryzanol, the ferulate esters of triterpene alcohols (Figure 1.3). The content of γ- oryzanol differs with the source of RBO and ranges from 115 to 780 ppm, depending on the degree and possibly the method of processing (Rogers *et al*, 1993). Its fundamental molecular structure is the ferulic acid aromatic phenolic nucleus esterified to cyclopentanperhydrophenanthrene (Seetharamaiah and Prabhakar, 1986). RBO contains a little variable quantity of tocotrienols, especially β and γ-tocotrienols. RBO have been shown to possess hypolipidemic activities (Rukmini and Raghuram 1991, Rogers *et al*. 1993) and hypocholesterolemic due to the presence of tocotrienols and oryzanol content (Seetharamaiah and Chandrasekhara, 1989). Through the use of selective refining and fractionation techniques, rice bran oil and concentrates may be produced to optimize composition for specific applications.

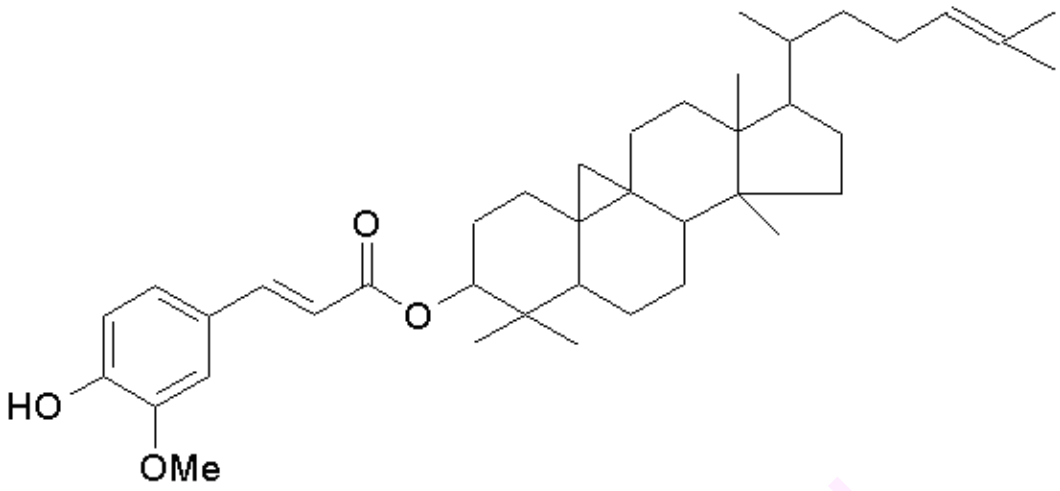


**Figure 1.3: Structure of γ- Oryzanol**

**Need for modification of oils and fats**

Various edible vegetable oils and animal fats are available for human consumption. Most of them contain high oleic and/ or linoleic acid along with palmitic acid as major SFA. The fatty acid profile of edible oils play an important role in their nutritional value and stability. No oil by nature provides all fatty acids in optimum amounts as desired by the nutritionists. In general commonly consumed vegetable oils are rich in MUFA and n-6 PUFA but these oils are poor source of n-3 PUFA. As mentioned earlier there is need to balance the proportion of various fatty acids in the diet. No oil is perfect in respect to its fatty acid composition, therefore here comes the need for modification of oils with desired FAs to obtain oil which fulfills the nutritional requirements. So there is a need for the balancing the various fatty acids in commonly consumed vegetable oils.

**Methods used for modification of TAGs**

Oils and fats play a very important role in human diet, however these days attempts are being made to reduce oils and fats from food products for various health reasons. These concerns are mainly due to cholesterol and its link to diseases like cardiovascular disease and obesity. Fatty acids in TAG can be altered or modified to

achieve specific end uses which impart specific physical, chemical and nutritional properties. In order to widen their range of use, oils and fats can be modified to a certain extent. For example, to obtain lipid with specific melting point and solid fat content or a fat with specific nutritional quality, it is often necessary to modify them in a manner which can not be achieved by blending of different oils.

Hydrogenation, fractionation and interesterification are the processes available to food manufacturers to tailor the physical and chemical properties of food lipids. At present roughly one third of all edible fats and oils in the world are hydrogenated, whereas about 10% are either fractionated or interesterified.

Hydrogenation process constitutes the addition of hydrogen atoms to the double bonds of a molecule through the use of a catalyst. Most hydrogenations involve the direct addition of diatomic hydrogen ($H_2$) but some involve the alternative sources of hydrogen, not $H_2$. These processes are called transfer hydrogenations. The classical example of a hydrogenation is the addition of hydrogen to unsaturated bonds between carbon atoms, converting alkenes to alkanes. Numerous important applications were found in the petrochemical, pharmaceutical and food industries. Health concerns associated with the hydrogenation of unsaturated fats to produce saturated fats and *trans* fats is an important aspect of current consumer awareness.

Fractionation is a separation process in which a certain quantity of a mixture (solid, liquid, solute or suspension) is divided up in a large number of smaller quantities (fractions) in which the composition changes according to a gradient. Fractions are collected based on differences in a specific property of the individual components. A common trait in fractionation is the need to find an optimum between the amount of fractions collected and the desired purity of each fraction. Fractionation makes it possible to isolate more than two components in a mixture in a single run. This property sets it apart from other separation techniques. Fractionation is widely employed in many

branches of science and technology. Fractionation of components also takes place in column chromatography by a difference in affinity between stationary phase and the mobile phase. In fractional crystallization and fractional freezing, chemical substances are fractionated based on difference in solubility at a given temperature. In cell fractionation, cell components are separated by difference in mass. Fractionation is also used for culinary purposes, as palm oil and palm kernel oils are fractionated to produce oils of different viscosities that may be used for different purposes (Trailter and Dieffenbacher, 1985).

Blending of natural oils is a viable option for improving the nutritional quality of oil. Two or more oils can be blended to obtain oil with the desired fatty acid composition. But, in case of blending, TAG retains its original structure because there is no change in their triglyceride structure during blending. So when two oils with different melting points are mixed there is a problem of phase separation and also they retain their original metabolic rates.

**Interesterification**

Another option for modification of oils involves the rearrangement of fatty acids within the TAG by interesterification reactions (Figure 1.4). The term interesterification usually refers to those reactions in which a compound composed of fatty acids esterified to a glycerol backbone reacts with fatty acids (acidolysis), alcohol (alcoholysis) or esters (transesterfication) as acyl donors. The objectives of these modification strategies is to create new TAG species from natural fats with desirable physical, chemical and functional properties (Laning, 1985). Interesterification can be achieved by either chemical or enzymatic approaches.

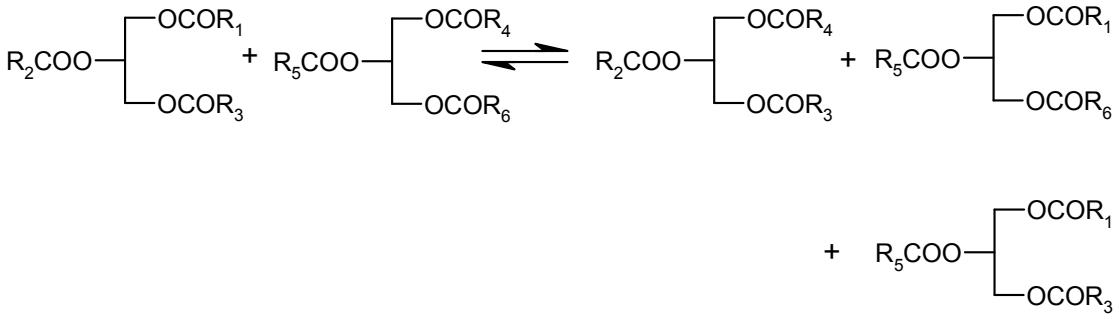


**Figure 1.4: Interesterification reaction where two different TAG molecules exchange their fatty acids**

**Chemical interesterification**

Chemical interesterification with a catalyst like sodium methoxide results in TAG with random distribution of fatty acids within the glycerol molecule. Chemical interesterification using sodium methoxide is a less expensive method for the production of structured lipids and is easy to scale up. However, such reaction lacks specificity and offer little or no control over the positional distribution of fatty acids in the final product (Lee and Akoh, 1998). This randomization distributes the fatty acids equally in all three positions of the TAG and produce a number of unwanted products like *trans* fatty acids which are difficult to remove and have deleterious effects on health (Aro *et al*, 1997). Chemical interesterification has been used in the food industry since 1940s to improve the spreadability and bakeing properties of lard (Weiss *et al*, 1961). In the 1970s there was a renewed interest in this process, particularly as a replacement for the hydrogenation in the manufacture of zero - *trans* margarines.

**Enzymatic interesterification**

Enzymatic interesterification is another method, which is used to produce fats with desirable physical and functional properties for food applications. This method uses lipase enzyme to modify vegetable oils for various purposes such as improving nutritional quality, preparation of low calorie fats and also as an alternative to hydrogenation of vegetable oils to improve their physico-chemical properties (Xu *et al*, 2000a). Depending upon the type of substrate, chemical or enzymatic reactions can be used for synthesis of modified lipids, including direct esterification (reaction of fatty acid and glycerol), reactions between two TAG molecules (transesterification), acidolysis (transfer of acyl group between an acid and ester) (Figure 1.5), and alcoholysis (exchange of alkoxy group between an alcohol and ester) (Osborn and Akoh, 2002).

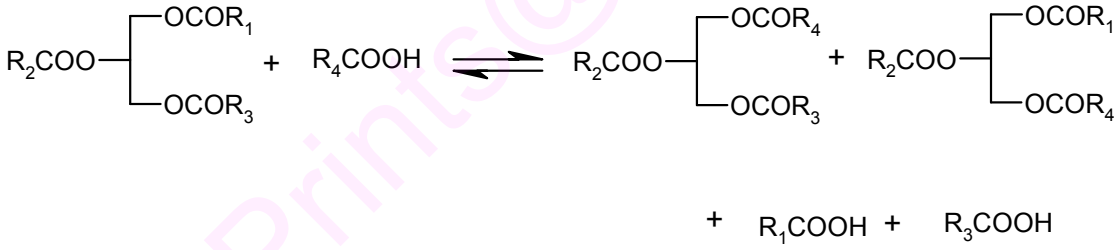


**Figure 1.5: Acidolysis reaction where TAG molecule and free fatty acid exchange fatty acids**.

**Lipase specificity**

The main advantage of enzymatic interesterification from chemical interesterification is their specificity. The fatty acid specificity of lipases has been exploited to produce structured lipids for medical foods and to enrich lipids with specific fatty acids to improve the nutritional properties of fats and oils. There are three main

types of lipase specificity: positional, substrate, and stereo. Positional and fatty acid specificity are usually determined by partial hydrolysis of synthetic TAGs and separation by thin-layer chromatography with subsequent extraction and analysis of the products. Other methods include conversion of fatty acids produced during hydrolysis to methyl esters for gas chromatographic analysis (Sonnet and Gazzillo, 1991). It is well known that lipases catalyze hydrolysis, esterification and transesterification (Yamane, 1987). Generally, efficient high-level processing of oils and fats can be achieved by use of one of these reactions and maximal advantage taken of the enzyme characteristics. The major advantages of using enzymes are their ability to catalyze reactions with high selectivity and specificity. Lipases show positional and substrate specificity. Based on the former, lipases can be subdivided into five different classes (Champ *et al*, 1998).

The first group comprises lipases, which hydrolyze fatty acids from the TAG independent of their type or position on the TAG molecule. They have no regiospecificity. Use of these lipases for interesterification reaction results in the random distribution of fatty acids in TAG molecule. Example for such enzyme include lipases produced by *Candida cylindracea, Corynebacterium acnes, Staphylococcus aureus* etc*.* The second group includes 1,3-specific lipases, which catalyze reactions at sn-1 and sn-3 positions of the TAG molecule. Example for such enzyme include lipase from *Rhizomucor miehei.* The third group covers lipases with different rates of hydrolysis for MAG, DAG and TAG. Some of these enzymes have been found in tissues of rats and humans (Jensen *et al*, 1983). The fourth group of lipases catalyzes the exchange of specific types of fatty acids. An example for this is the exracellular lipase from the fungus *Geotrichum candidum,* which preferentially releases unsaturated *cis*- n-9 fatty acid groups (Macrea, 1985). The fifth group contains special enzymes that show a faster hydrolysis rate for the fatty acids placed on the sn-1 (sn-3) position than the sn-3 (sn-1) position. This is commonly referred to as stereospecifity. Examples include lipoprotein

lipase from milk, adipose tissue and post heparin plasma, which preferentially cleave the ester bond in *sn-1* position and human and rat lingual lipase which react preferentially with the fatty acids at position sn-3 (Jensen *et al*, 1983).

High absorptive structured lipids can be produced using an immobilized 1, 3-positional specific lipase (Shieh *et al*, 1995; Shimada *et al*, 1996a). For the construction of these reaction systems and for prediction of the reaction kinetics, the fatty acid specificity of the respective lipase must be evaluated accurately. The fatty acid specificity of lipases have been investigated mainly by measurement of rates of hydrolysis of simple TAG or methyl (ethyl) esters or by analysis of the composition of fatty acids liberated from natural oil used as a substrate. The former method is not precise because of the differences between the physical states of the substrates, liquid and solid at the reaction temperature. While the latter method is free of this problem, the substrate specificities of 1, 3-positional specific lipases cannot be determined accurately because fatty acids are not homogeneously distributed in triglyceride molecules in natural oils. It was recently suggested that the fatty acid specificities of a lipase in hydrolysis, esterification and transesterification are slightly different (Shimada *et al*, 1996b). However, the fatty acid specificity in esterification and transesterification could not be precisely investigated because the hydrolysis occurred even in a reaction mixture containing only a trace amount of water.

The selectivity and specificity of lipases are highly dependent on the reaction conditions used (eg. water activity, temperature, pH, and immobilization). TAG, the preferred substrate for lipases are insoluble in water. They self-associate to form monomolecular films, micelles or emulsions in aqueous medium. Because of this self-association, lipolysis takes place at the lipid-water interface (Villeneuve and Foglia, 1997). Enzyme catalyzed reactions can occur at low temperatures and non-solvent

systems. Lipase specificity towards different fatty acids of different acylglycerol moieties have also been studied and used for enrichment of fatty acids.

**Lipases in lipid modification**

There are a variety of specific lipases with regiospecificity and stereospecificity, which could be used for the production of specific structured lipids. Although wide spread in nature, lipases have become available only recently in large quantities for industrial purposes. Some of the possible applications of lipases have been established in the last 15-20 years with the realization that lipases could be used in microaqueous organic systems at high temperatures of up to 70 $^{0}$C (Zaks and Klibanov, 1984). The interesterification reactions with sn - 1,3 specific lipase offers high catalytic efficiency, specifity and selectivity. It provides a useful way to improve the nutritional properties of lipids by incorporation of a required acyl group into a specific position of the TAG. The chemical methods do not provide this specificity (Akoh, 1995).

Microbial lipases have attracted great attention, as they are thermostable, functions without co-lipase requirement and posses different specificites (Eigtved, 1992). Lipase from *Carica papaya* latex is an example of the lipase from plant source used for interesterification. Gandhi and Mukherjee (2001) have synthesized structured lipids using papaya (*Carica papaya*) latex lipase. Of the microbial lipases, Lipozyme IM 60 from *Rhizomucor miehei* and SP435 from *Candida antarctica* have been most commonly used for interesterification reactions. Lipozyme IM 60 is immobilized on to an anion exchange resin with well worked specificity. It has been used extensively in the various studies related to interesterification and it is evident from different studies that lipases have varying substrate specificities.

## Structured lipids

Structured lipids (SL) are defined as TAGs which are modified chemically or enzymatically to change the fatty acid (FA) composition and/or positional distribution in the glycerol backbone. SLs are referred to particular molecular species of TAGs with defined molecular structure i.e. specific FA residues in specific positions. Molecular structure of TAGs influences their metabolic rate in organisms i.e. digestion and absorption as well as their physical characteristics e.g. melting point. Consequently, when designing SLs with particular chemical structure, it is possible to control the behavior of TAGs, thereby improving the nutritional and pharmaceutical properties of TAGs. Based on this perspective much attention has been directed to the syntheses of SLs. The synthesis of SLs of particular structure require specific modifications at the desired positions in the glycerol backbone. Although chemical interesterification catalyzed by metal alkoxides is simple and inexpensive, it is not capable of modifying specific positions due to the random nature of the reactions. In contrast, the reactions catalyzed by sn-1,3-specific lipases are more promising for positionally specific modification of lipids.

## Importance of structured lipids in health

The relationship between stereo-specific fatty acid location and lipid nutrition suggests that the process of interesterification, or acidolysis, could be used to improve the nutrition profile of certain TAGs. Manufacturers of speciality food ingredients for infant formulas and enteral supplements can design fats with saturated fatty acids at the sn-2 position to provide increased caloric intake. Structured lipids can be synthesized to target specific metabolic effects or to improve physical characteristics of fats. SL made from fish oil and MCTs was compared with conventional LCTs and found to decrease tumor protein synthesis, reduce tumor growth in Yoshida sarcoma-bearing rats,

decrease body weight, and improve nitrogen maintenance (Ling *et al,* 1991). In addition, the effects of fish/MCT on tumor growth was synergistic with tumor necrosis factor (TNF). A similar study by Mendez *et al*, (1992) compared the effects of a structured lipid (made from fish oil and MCFAs) with a physical mix of fish oil and MCTs and found that the SL resulted in improved nitrogen balance in animals, probably because of the modified absorption rates of SL. Gollaher *et al* (1992) reported that the protein-sparing action associated with SL administration was not seen when the structured lipids provide 50% of protein calories and suggested that the protein-sparing action of SLs may be dependent on the ratio of MCTs to LCTs used to synthesize the SL.

**Aim and scope of the present work**

RBO contains approximately 38 % oleic acid, 34 % linoleic acid and 18.6 % palmitic acid. The physical properties of these fatty acids do not confer any special properties for its use in food applications. Incorporation of stearic acid into RBO can offer alternative use of RBO as solid fat free from *trans* fatty acids and can be used in confectionary and sweet preparations. *Trans* fatty acids have been reported to raise levels of low-density lipoproteins (LDL) and lower the levels of high-density lipoproteins (HDL) in humans (Sun *et al*, 2007). Fatty acids play a unique and important role in human nutrition. They are the major source of energy and also structural components of biological membranes. Dietary fatty acids have been correlated with metabolic and physiologic alterations associated with several complications including coronary heart disease, diabetes, cancer, obesity and hypertension. The major changes that took place with respect to dietary fatty acid consumption may be attributed to agricultural revolution leading to large scale production of edible oils rich in n-6 fatty acids. Dietary survey conducted in India has indicated the need to improve the n-3 fatty acid intake.

One of the aims of this investigation was to incorporate stearic acid into RBO to obtain solid fat from it free from *trans* fatty acids. The second objective of this study was to incorporate n-3 PUFA into RBO which can provide adequate amounts of essential fatty acids to a population who do not get them from other vegetarian sources of oil. The results obtained indicated that it is possible to incorporate desirable amount of n-3 PUFA into RBO by enzymatic acidolysis reaction.

It is necessary to include n-3 PUFA into the dietary fat so that it provides balanced fatty acids. RBO contains negligible amount of n-3 PUFA, so there is need to incorporate n-3 PUFA into RBO. In order to improve the use of RBO, one way is to prepare value added products from RBO, which will have better appeal and usage. In this context lipase catalyzed acidolysis using immobilized lipase from *Rhizomucor miehei* was adapted to modify the fatty acids in RBO. Enzymatic acidolysis reactions were conducted for the incorporation of specific fatty acids and this process is controlled by variables like incubation time, temperature, enzyme concentration and substrates molar ratio. These were optimized individually for the synthesis of three types of structured lipids, enriched in 18:0, 18:3 and 20:5 + 22:6 (long chain PUFA). Linseed oil and cod liver oil are not consumed regularly as edible oils. However they can be exploited as n-3 fatty acids source for their incorporation into RBO. Response surface methodology, a statistical predictive model was used for studying the effects of multiple variables on the incorporation of n-3 PUFA into RBO. Under optimized conditions, the synthesis of structured lipids was scaled up from 100 mg to 100 g levels. The physical properties of the RBO rich in stearic acid were evaluated. The triglyceride structure, distribution of fatty acids, melting profile and solid fat content of the structured lipids were studied. These physical studies would help in understanding changes in physical properties of modified RBO.

Nutritional evaluation of SL from RBO rich in n-3 fatty acids were carried out in experimental animals. The bioavailability, tissue distribution and differences in the distribution of ALA and long chain metabolites (EPA+DHA) were monitored. The beneficial effects of SL from RBO rich in n-3 fatty acids in reducing risk factors for atherogenesis and thrombogenesis were also studied.

# CHAPTER - II

# MATERIALS AND METHODS

**Materials**

Alpha cellulose, thiobarbituric acid, ascorbic acid, adenosine diphosphate, Trizma base, boron trifluride in methanol, dipalmitoylphosphatidylcholine, triolein, triglyceride purifier, sodium metaperiodate, HMG CoA, 3$\alpha$-Hydroxysteroid dehydrogenase, NAD, NADPH, 5,5'-dithiobis (2-nitrobenzoic acid), collagen, heparin, xanthine oxidase, glutathione (reduced and oxidized), glutathione reductase, pancreatic lipase, hydrogen peroxide, 1- choloro 2, 4-dinitrobenzene, dithiothreitol, bovine serum albumin, glycocholic acid, taurocholic acid, taurodeoxycholic acid and thiobarbituric acid were purchased from M/s Sigma Chemical Co., St. Louis, MO, USA. Ferrous sulphate, ferric chloride, ammonium thiocyanate, acetylacetone, HPLC grade solvents like hexane, isopropanol, methanol, butanol and acetonitrile were purchased from M/s Qualigens, Mumbai, India. Stearic acid, silica gel-G, silica gel (100-200 mesh), aluminium oxide, cytochrome C, xanthine, EDTA, hexane, methanol, diethyl ether, chloroform, petroleum ether and benzene were purchased from M/s Sisco Research laboratories, Mumbai, India. Phosphotungstic acid and cadmium acetate were purchased from M/s Sd. Fine Chemicals, Mumbai, India. Carbazole was purchased from M/s Aldrich Chemicals, Milwaukee, USA. DL-methionone and tocopherol acetate were purchased from M/s Hi Media lab, Mumbai, India. Casein was procured from M/s Nimesh Corporation, Mumbai, India. Fatty acid standards were obtained from M/s Nu Chek Prep, Elysian, MN, USA. Immobilzed 1,3 specific lipase Lipozyme IM 60 was a gift from M/s Novo Nordisk Bioindustrial Inc., Danbury, CT, USA. Groundnut oil (GNO), refined rice bran oil (RBO) and cod liver oil (CLO) were obtained from a local super market, Mysore, India. Linseed oil was purchased from local market of Palampur, Himachal Pradesh, India. All solvents used were of analytical grade and distilled prior to use.

## Methods

### Synthesis of structured lipids from RBO enriched in stearic acid

All enzymatic acidolysis reactions were carried out between TAGs of RBO and free fatty acids as described by Jennings and Akoh (2000). Enzymatic interesterification reactions were carried out in 50 mL stoppered conical flasks under inert atmosphere. The reaction mixture consisted of rice bran oil (RBO) and stearic acid (18:0) taken at molar ratio of 1:6. Substrates were dissolved in 4 mL hexane. Immobilized lipase IM 60 was used at a concentration of 10 wt % of the total substrates. Incubations were carried out in an orbital-shaking incubator at a rotary speed of 150 rpm for 6 h and the temperature was $37^0$C.

For general modification of rice bran oil, 100 mg of RBO was mixed with stearic acid (31.8 mg - 318.4 mg) at a mole ratios ranging from 1:1 to 1:10. The reactions were carried out under the specific conditions specified above for different incubation times ranging from 0 to 48 h at temperatures of 25 to 60 ºC. The enzyme level was also varied from 0 to 10 wt % of reactants and the mixture was incubated in an orbital shaking (150 rpm) water bath. All reactions were performed in triplicate and mean values were reported.

### Purification of reaction products

The reaction mixture after enzymatic acidolysis consisted of TAGs, free fatty acids, mono and diglycerides. The immobilized lipase was removed by decanting and passing the reaction mixture through an anhydrous sodium sulphate column. The TAGs were purified and analyzed using thin layer chromatography.

### Thin layer chromatography (TLC)

TLC was used to separate the TAGs from the reaction mixture (Akoh *et al*, 1996) when reaction was carried out at laboratory scale and also to check the purity of

products obtained by column chromatography. A 40 µL of aliquot of the reaction product was applied on a silica gel G coated TLC plate and developed with petroleum ether: diethyl ether: acetic acid (80:20:1, v/v/v). The lipid bands were visualized under ultraviolet light after spraying with 0.2% 2, 7-dichlorofluorescein in methanol. The bands corresponding to TAG were scraped from the silica gel plate and extracted with chloroform: methanol (2:1; v/v) and used for fatty acid analysis.

**Quantitation of triacylglycerol (TAGs)**

Following interesterification reaction, 100 µL of the reaction mixture was spotted on preparatory TLC plates (500 µ thickness coat of silica gel-G). The bands corresponding to TAG standards were separated on TLC as described earlier and eluted with chloroform: methanol (2:1 v/v) and quantitated by glycerol estimation according to method of Fletcher (1968) using triolein as reference standard (30-300 µg). The lipid extract in chloroform was evaporated under a stream of nitrogen and then dissolved in 4 mL of isopropanol, saponified with 0.6 mL of 5 % potassium hydroxide in isopropanol: water (40:60, v/v) at $60^0$C for 15 min. After cooling, 1 mL of sodium metaperiodate prepared from the stock solution of 0.025 M in 1N acetic acid (sodium metaperiodate (12 mL) and 20 mL of isopropanol and made up to 100 mL with 1N acetic acid) was added, mixed and 0.5 mL of acetylacetone was added, stoppered and incubated at $50^0$C for 30 min. After cooling to room temperature, the color intensity was read at 405 nm in Shimadzu 160A spectrophotometer. TAG content was calculated by using triolein as a standard.

**Gas chromatography**

The fatty acid composition of substrates and reaction products was analyzed by gas chromatography. The samples were saponified using methanolic potassium hydroxide (0.5 M) and fatty acid methyl esters were prepared using boron trifluoride in

methanol (Morrison and Smith, 1964). The fatty acid methyl esters were extracted with hexane, dried over anhydrous sodium sulfate, and analyzed by gas chromatography. The fatty acids in the form of methyl esters were analyzed by gas chromatography (Shimadzu 14 B fitted with FID) using fused silica capillary column 25 m x 0.25 mm (Konik Tech, Barcelona, Spain). The injector and detector temperatures were 230 and 240ºC, respectively. The column temperature was held isothermally at 220ºC and nitrogen was used as a carrier gas at a flow rate of 1 mL/min. Individual fatty acids were identified by comparing with retention time of standards.

**TAG analysis by HPLC**

**TAG molecular species of native and modified RBO were monitored according to the method of Swe *et al,* (1994). Individual TAG molecules were separated on a reversed-phase high performance liquid chromatograph (Shimadzu Corp., Tokyo, Japan), equipped with a refractive index detector (RID-10A). A Phenomenex RP-C18 column (250 mm×4.5 mm) of 5µm particle size with acetone/acetonitrile (65:35% v/v) as the mobile phase at a flow rate of 1.0mL/min was used. The samples were solubilised in acetone.**

The elution sequence of TAGs were predicted based on their theoretical carbon number (TCN). Fatty acid composition of RBO showed that more than 90 % of the oil composed of three fatty acids- palmitic (P), oleic (O) and linoleic (L) and fatty acid composition of RBO rich in stearic acid showed that more than 90 % of the modified fat was composed of four fatty acids- palmitic (P), stearic (S), oleic (O) and linoleic (L) acids. Therefore, the TCN of all combinations of P, O and L were calculated according to the equation:

TCN= ECN - (0.7) L – (0.6) O,

ECN=CN-2n.

where

ECN - is the equivalent carbon number,

CN - is the number of carbons in the three fatty acids of the TAG,

n - is the number of double bonds,

L - is the number of linoleic acids and

O - is the number of oleic acids (El-Hamdy and Perkins, 1981).

**TAG molecules were identified by correlating the TCN and retention time of TAG standards. Four standards LLL, OOO, SSS and PPP were used as retention time references to correlate the TCN values with the TAG retention times (Bland *et al*, 1991). To positively characterize the composition of TAG molecule separated by HPLC, the individual fractions were collected. Each of the fatty acid in TAG molecule was esterified to fatty acid methyl esters (FAME) and analyzed by GC. The fatty acid methyl esters were identified based on comparison of retention time with FAME standards.**

**Analysis of fatty acids at *sn-2* position of TAG**

The nature of fatty acids in the *sn-2* position of TAG was determined according to the method of Luddy et al (1964). About 1 mg of TAG separated from modified and native RBO was mixed with 1 mL of 1 mol/L tris- HCl buffer (pH 7.6), 0.25 mL of sodium cholate (0.05 %), 0.1 mL of $CaCl_2$ (2.2 %) and 1 mg of pancreatic lipase was added to the reaction mixture. The mixture was incubated for 3 min at $37^0$C. At the end of incubation, the contents were extracted with 3 mL of diethyl ether, dried over anhydrous sodium sulfate and subjected to TLC with solvent system hexane: diethyl ether: acetic acid (50: 50: 1, v/v/v). The band corresponding to monoacylglycerol was scraped, eluted with chloroform: methanol (2: 1; v/v), methylated and analyzed by GC as described earlier.

**Differential scanning calorimetry**

A Mettler differential scanning calorimeter (DSC-30, Zurich, Switzerland) was used to obtain melting and crystallization characteristics of the samples. The heat flow of the instrument was calibrated using indium. The PT- 100 sensor was calibrated using indium, zinc and lead. To ensure homogeneity and to destroy all crystal nuclei, the samples were heated to 60ºC. About 15 mg of molten sample was accurately weighed into a standard aluminium crucible and the cover crimped in place. An empty aluminium crucible with pierced lid was used as a reference. For melting characteristics, the samples were stabilized according to Paquot and Hautfenne (1987) which includes keeping the samples at 0ºC for 90 min, at 26$^{o}$C for 40 h followed by 0$^{o}$C for 90 min prior to introduction into DSC cell. Although, this tempering method applies to fats with extended stabilization like cocoa butter, it ensures complete transformation to the stable form. Thermograms were recorded by heating at 2ºC / min from –10ºC to 70ºC. The peak temperatures, heat of fusion and percent liquids at various temperatures were recorded directly using TC - 10A data processor and STARe program. The solid fat content (SFC) was calculated from percent liquid. The melting profiles were drawn by plotting SFC against temperature. For studying crystallization behaviour, the samples were kept at 70ºC for 5 min, and then cooled to -10ºC at 5ºC / min. The onset of crystallization, enthalpy and peak temperature were recorded.

**Oryzanol analysis**

Oryzanol content of native RBO and interesterified RBO were determined spectrophotometrically by using molar extinction coefficient of oryzanol $3.58X10^{2}M^{-1}cm^{-1}$. Briefly, 10 mg of each native RBO and modified RBO was taken in 10 mL of hexane and

from this 1 mL was taken and absorbance was measured at 314 nm using in Shimadzu 160A spectrophotometer (De and Bhattacharya, 2000).

**Tocopherol and tocotrienol analysis**

Tocopherols and tocotrienols were analyzed in the RBO before and after enzymatic modification. Modified and native RBO (100 mg each) was dissolved in 2 mL of acetone and 20 µL was injected into high performance liquid chromatography (HPLC) system (Shimadzu LC-10A) fitted with 30 cm in length C-18 column and UV detector was used. The mobile phase consisted of acetonitrile: methanol (60:40) with the flow rate of 1 mL/min (Tan and Krzuskiewicz, 1989).

**Preparation of free fatty acids and fatty acid concentrate from linseed oil (LSO)**

Free fatty acids (FFA) were prepared from LSO as a source for ALA for synthesis of SL rich in ALA (α- linolenic acid) according to the method of Wanasundara and Shahidi (1999). In brief, 100 g of LSO was hydrolyzed with 200 mL of NaOH solution containing 48 g of NaOH and 500 mg of disodium salt of EDTA dissolved in 160 mL of water, followed by addition of 160 mL of ethanol. Refluxing was done for 1 h at 65°C, followed by the addition of conc. HCl to obtain pH 1.0. FFAs were extracted with hexane thrice from the above solution and hexane layers were pooled and anhydrous sodium sulfate was added to remove the water content. Hexane was evaporated completely by using flash evaporator and fatty acids were further used to isolate α- linolenic acid (ALA). ALA was separated from the hydrolyzed fatty acid mixture of LSO by urea-fatty acids adduct formation. Fatty acids obtained after removing hexane mixed in the solution containing 800 mL methanol + 300 g urea. Solution was heated till it becomes clear and allowed to crystallize overnight, and then the solution was filtered. The filtrate obtained was kept at $4^0$C for 3 h, and then filtered again. To each 300 mL filtrate, 100 mL hexane and 50 mL con. HCl was added and solution was stirred for 1 h, then the hexane layer

was separated from the solution and to the lower layer 150 mL water and 100 mL hexane was added and mixed well. Hexane layer was removed and both hexane extracts were combined and the solvent was evaporated to obtain free fatty acid (ALA). Originally in LSO about 54 % of ALA was present whereas the percentage of α- linolenic acid was increased to 88 % after preparing fatty acid concentrate and saturated fatty acids were completely removed during this process. The FFAs were flushed with nitrogen and stored at -20$^0$C till further use.

**Preparation of free fatty acids from cod liver oil (CLO)**

Free fatty acids were prepared from CLO for use as a source of EPA+DHA for the synthesis of SL rich in EPA+DHA. In brief, 100 g of CLO was hydrolyzed with 200 mL of NaOH solution containing 48 g of NaOH and 500 mg of disodium salt of EDTA dissolved in 160 mL of water, followed by 160 mL of ethanol. Refluxing was done for 1 h at 65°C, followed by the addition of conc. HCl to obtain pH 1.0. FFAs were extracted with hexane thrice from the above solution and hexane layers were pooled and anhydrous sodium sulfate was added to remove the water content. Hexane was evaporated completely by using flash evaporator to obtain FFA concentrate from CLO and flushed with nitrogen and stored at -20 $^0$C till further use.

**Synthesis of structured lipids (SL) from RBO enriched in α- linolenic acid (ALA)**

Synthesis of SL from RBO rich in ALA was carried out as described below. The enzymatic acidolysis reaction mixture consisted of 4 mL of hexane, α- linolenic acid obtained from LSO by urea complexation and RBO was incubated at certain temperature, time, enzyme concentration and substrates ratio as per the experimental design. The molecular weights of RBO and α- linolenic acid were 893.5 and 278.5 respectively. The molar ratio of 1: 1 meant RBO and LSO concentrate were 893.5 and 278.5 mg, respectively. The reaction mixture was incubated in a stirred water bath (150

rpm). All the reactions were performed in triplicate and mean values for different parameters were reported.

**RSM experimental design for synthesis of SL from RBO rich in ALA**

In our earlier experiments we have synthesized SLs from RBO containing stearic acid which is a saturated fatty acid and has neutral effect on serum cholesterol level. However, when similar conditions were followed for the incorporation of n-3 PUFA into RBO, the extent of incorporation differed considerably as compared to that of stearic acid. This underscores the need for optimizing reaction conditions for each fatty acid to obtain desired level of incorporation into SL. This necessitates to conduct large number of experiments with each fatty acid. However, this can be circumvented by using response surface methodology (RSM) to optimize reaction conditions with a minimum number of experiments to obtain statistically acceptable results. RSM was used to optimize the reaction conditions for lipase-catalyzed incorporation of n-3 PUFA into rice bran oil. In RSM, both mathematical and statistical techniques were used together in the modeling and analysis of situations in which a response is affected by several variables, alone or in combination. RSM also enables the behavior of different parameters to be predicted under a given set of conditions and provide sufficient information for statistically acceptable results with a reduced number of experiments. Therefore, by choosing the appropriate experimental design, time, cost, wastage and rework during production can be reduced (Can and Ozcelik, 2005). A central composite rotatable design (CCRD) was adopted (Cochran and Cox, 1957) with four variables and used to study the response pattern and to determine the optimum combination of variables. The variables optimized were: reaction time (1 - 15 h or 4 - 48 h), temperature (25 – 50 ºC or 25-55 ºC), enzyme concentration (1- 10 % of total weight of substrates) and substrates molar ratio (1- 10 mol/ mol, RBO/ $\alpha$- linolenic acid) each at 5 levels, -2.0; -1.0; 0; 1.0 and 2.0 (Table 2.1). The CCRD shown in table 2.1 was arranged to allow for fitting an

appropriate regression model using multiple regression program. The CCRD combines the vertices of the hypercubes whose co-ordinates are given by a 2*n* factorial design to provide for the estimation of curvature of the model (Joglekar and May, 1987). Treatment schedule for four factor CCRD for optimization of reaction conditions for the synthesis of SL rich in ALA is given in table 2.2. Seven replicates (treatment 25- 31) at the center of the design were used to allow for estimation of a pure error sum of squares. Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors.

**Table 2.1: Variables and their levels for CCRD for optimization of reaction conditions for the synthesis of SL rich in ALA by RSM**

| Variables | Symbols | | Levels | | | | |
|---|---|---|---|---|---|---|---|
| | Coded | Uncoded | -2.0 | -1.0 | 0 | 1.0 | 2.0 |
| Time of reaction (h) | $X_1$ | $x_1$ | 1 | 4.5 | 8 | 11.5 | 15 |
| Temperature of reaction (°C) | $X_2$ | $x_2$ | 25 | 31.25 | 37.5 | 43.75 | 50 |
| Enzyme concentration (w/w) | $X_3$ | $x_3$ | 1 | 3.25 | 5.5 | 7.75 | 10 |
| Substrate molar ratio | $X_4$ | $x_4$ | 1 | 3.25 | 5.5 | 7.75 | 10 |

where $X_1 = (x_1 - 8)/3.5$; $X_2 = (x_2 - 37.5)/6.25$; $X_3 = (x_3 - 5.5)/2.25$; $X_4 = (x_4 - 5.5)/2.25$

Statistical analysis

A second order polynomial equation was used to fit the experimental data given in table 2.2. The model proposed for the response (*Y*) was

$$Y = a_o + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4$$

where *Y* is the predicted response for percentage incorporation,

$a_o$ is the value of the fitted response at the center point of the design and

$a_i$, $a_{ii}$, and $a_{ij}$ are the linear, quadratic, and cross product terms respectively.

**Table 2.2: Treatment schedule for 4-factor CCRD for optimization of reaction conditions for the synthesis of SL rich in ALA by RSM**

| Exp. No. | Time of reaction (h) $X_1$ | | Temperature of reaction (°C) $X_2$ | | Enzyme concentration (w/w) $X_3$ | | Substrate molar ratio $X_4$ | |
|---|---|---|---|---|---|---|---|---|
| | *Actual* | *Coded* | *Actual* | *Coded* | *Actual* | *Coded* | *Actual* | *Coded* |
| 1 | 4.50 | (-1) | 31.25 | (-1) | 3.25 | (-1) | 3.25 | (-1) |
| 2 | 11.50 | (1) | 31.25 | (-1) | 3.25 | (-1) | 3.25 | (-1) |
| 3 | 4.50 | (-1) | 43.75 | (1) | 3.25 | (-1) | 3.25 | (-1) |
| 4 | 11.50 | (1) | 43.75 | (1) | 3.25 | (-1) | 3.25 | (-1) |
| 5 | 4.50 | (-1) | 31.25 | (-1) | 7.75 | (1) | 3.25 | (-1) |
| 6 | 11.50 | (1) | 31.25 | (-1) | 7.75 | (1) | 3.25 | (-1) |
| 7 | 4.50 | (-1) | 43.75 | (1) | 7.75 | (1) | 3.25 | (-1) |
| 8 | 11.50 | (1) | 43.75 | (1) | 7.75 | (1) | 3.25 | (-1) |
| 9 | 4.50 | (-1) | 31.25 | (-1) | 3.25 | (-1) | 7.75 | (1) |
| 10 | 11.50 | (1) | 31.25 | (-1) | 3.25 | (-1) | 7.75 | (1) |
| 11 | 4.50 | (-1) | 43.75 | (1) | 3.25 | (-1) | 7.75 | (1) |
| 12 | 11.50 | (1) | 43.75 | (1) | 3.25 | (-1) | 7.75 | (1) |
| 13 | 4.50 | (-1) | 31.25 | (-1) | 7.75 | (1) | 7.75 | (1) |
| 14 | 11.50 | (1) | 31.25 | (-1) | 7.75 | (1) | 7.75 | (1) |
| 15 | 4.50 | (-1) | 43.75 | (1) | 7.75 | (1) | 7.75 | (1) |
| 16 | 11.50 | (1) | 43.75 | (1) | 7.75 | (1) | 7.75 | (1) |
| 17 | 1.00 | (-2) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 18 | 15.00 | (2) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 19 | 8.00 | (0) | 25.00 | (-2) | 5.50 | (0) | 5.50 | (0) |
| 20 | 8.00 | (0) | 50.00 | (2) | 5.50 | (0) | 5.50 | (0) |
| 21 | 8.00 | (0) | 37.50 | (0) | 1.00 | (-2) | 5.50 | (0) |
| 22 | 8.00 | (0) | 37.50 | (0) | 10.00 | (2) | 5.50 | (0) |
| 23 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 1.00 | (-2) |
| 24 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 10.00 | (2) |
| 25 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 26 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 27 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 28 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 29 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 30 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 31 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |

Optimization of fitted polynomial was performed by a non-linear mathematical optimization procedure of the Quattro Pro software package (Quattro Pro, Version 4.0, Borland International Inc., USA) (Triveni et al, 2001). Responses were monitored and results compared with model predictions. The fitted polynomial equation was expressed as surface plots in order to visualize the relationship between the response and experimental levels of each factor.

**Synthesis of structured lipids from RBO enriched with EPA+DHA obtained from CLO**

The enzymatic acidolysis reaction mixture consisting of 4 mL of hexane, FFAs obtained from CLO and RBO was incubated at certain temperature, time, enzyme concentration and substrate ratio as per the experimental design. The molecular weights of RBO and CLO FFAs were taken as 893.5 and 319.0 respectively. The molar ratio of 1: 1 meant RBO and cod liver oil FFAs were 893.5 and 319.0 mg, respectively. The reaction mixture was incubated in a shaking water bath (150 rpm). All the reactions were performed in triplicate and mean values for different parameters were reported.

**RSM experimental design for structured lipids from RBO rich in EPA+DHA**

A CCRD (Cochran and Cox, 1957) with four variables was used to study the response pattern and to determine the optimum combination of variables. The variables optimized were reaction time (4 – 48 h), temperature (25 – 55 ºC), enzyme concentration (1 - 10 % of total weight substrates) and substrates molar ratio (1 - 10 mol/ mol RBO/ cod liver oil free fatty acids) each at 5 levels, -2.0; -1.0; 0; 1.0 and 2.0 (Table 2.3). The CCRD shown in table 2.3 was arranged to allow for fitting an appropriate regression model using multiple regression program. The CCRD combines the vertices of the hypercubes whose co-ordinates are given by a 2*n* factorial design to provide for the

**Table 2.3: Variables and their levels for CCRD for optimization of reaction conditions for the synthesis of SL rich in EPA+DHA by RSM**

| Variables | *Symbols* | | *Levels* | | | | |
|---|---|---|---|---|---|---|---|
| | Coded | Uncoded | -2.0 | -1.0 | 0 | 1.0 | 2.0 |
| Time of reaction (h) | $X_1$ | $x_1$ | 4 | 15 | 26 | 37 | 48 |
| Temperature of reaction (°C) | $X_2$ | $x_2$ | 25 | 32.5 | 40 | 47.5 | 55 |
| Enzyme concentration (w/w) | $X_3$ | $x_3$ | 1 | 3.25 | 5.5 | 7.75 | 10 |
| Substrate ratio | $X_4$ | $x_4$ | 1 | 3.25 | 5.5 | 7.75 | 10 |

$X_1 = (x_1 - 26)/11$; $X_2 = (x_2 - 40)/7.5$; $X_3 = (x_3 - 5.5)/2.25$ and $X_4 = (x_4 - 5.5)/2.25$

estimation of curvature of the model (Joglekar and May, 1987). Treatment schedule for four factor CCRD for optimization of reaction conditions for the synthesis of SL rich in EPA+DHA is given in table 2.4. Seven replicates (treatment 25- 31) at the center of the design were used to allow for estimation of a pure error sum of squares. Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors.

Statistical analysis

A second order polynomial equation was used to fit the experimental data given in table 2.4. The model proposed for the response ($Y$) was

$$Y = a_o + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4$$

where $Y$ is the predicted response for percentage incorporation,

$a_o$ is the value of the fitted response at the center point of the design and

$a_i$, $a_{ii}$, $a_{ij}$ the linear, quadratic, and cross product terms, respectively.

of fitted polynomial was performed by a non-linear mathematical optimization procedure of the Quattro Pro software package (Quattro Pro, Version 4.0, Borland International Inc., USA) (Triveni et al.,2001). Responses were monitored and results compared with model predictions. The fitted polynomial equation was expressed as surface plots in order to visualize the relationship between the response and experimental levels of each factor.

**Scale up of interesterification reactions**

**Structured lipids enriched in α- linolenic acid**

Large scale synthesis of structured lipids was carried out in stirred batch reactions. Reactions were carried out in 1 L stoppered conical flasks using optimum conditions derived for incorporation of ALA into RBO by response surface methodology. RBO and α- linolenic acid were taken in 1:5.5 molar ratio. Each batch consisted of 100 g RBO and 170.5 g α- linolenic acid and 100 mL hexane with 2.7 g of immobilized lipase Lipozyme IM 60 (1 % of total substrates weight) taken in a 1 L stoppered conical flask. Incubation was carried out at 37.5 $^0$C for 8 h with constant agitation.

**Structured lipids enriched in long chain n-3 PUFA from cod liver oil**

Reactions were carried out using optimum conditions derived for incorporation of EPA+DHA by response surface methodology. RBO and free fatty acids from CLO were taken in 1:7.75 molar ratio. Each batch consisted of 100 g RBO and 277 g of free fatty acids from cod liver oil, 100 mL hexane and 29 g of immobilized lipase Lipozyme IM 60 (7.75 % of total substrates weight) taken in 1L stoppered conical flask. Incubation was carried out at 32.5 $^0$C for 37 h with constant agitation.

**Table 2.4: Treatment schedule for 4-factor CCRD for optimization of reaction conditions for the synthesis of SL rich in EPA+DHA by RSM**

| Exp. No. | Time of reaction (h) $X_1$ | | Temperature of reaction (°C) $X_2$ | | Enzyme Concentration (v/v) $X_3$ | | Substrate molar ratio $X_4$ | |
|---|---|---|---|---|---|---|---|---|
| | *Actual* | *Coded* | *Actual* | *Coded* | *Actual* | *Coded* | *Actual* | *Coded* |
| 1 | 15 | (-1) | 32.5 | (-1) | 3.25 | (-1) | 3.25 | (-1) |
| 2 | 37 | (1) | 32.5 | (-1) | 3.25 | (-1) | 3.25 | (-1) |
| 3 | 15 | (-1) | 47.5 | (1) | 3.25 | (-1) | 3.25 | (-1) |
| 4 | 37 | (1) | 47.5 | (1) | 3.25 | (-1) | 3.25 | (-1) |
| 5 | 15 | (-1) | 32.5 | (-1) | 7.75 | (1) | 3.25 | (-1) |
| 6 | 37 | (1) | 32.5 | (-1) | 7.75 | (1) | 3.25 | (-1) |
| 7 | 15 | (-1) | 47.5 | (1) | 7.75 | (1) | 3.25 | (-1) |
| 8 | 37 | (1) | 47.5 | (1) | 7.75 | (1) | 3.25 | (-1) |
| 9 | 15 | (-1) | 32.5 | (-1) | 3.25 | (-1) | 7.75 | (1) |
| 10 | 37 | (1) | 32.5 | (-1) | 3.25 | (-1) | 7.75 | (1) |
| 11 | 15 | (-1) | 47.5 | (1) | 3.25 | (-1) | 7.75 | (1) |
| 12 | 37 | (1) | 47.5 | (1) | 3.25 | (-1) | 7.75 | (1) |
| 13 | 15 | (-1) | 32.5 | (-1) | 7.75 | (1) | 7.75 | (1) |
| 14 | 37 | (1) | 32.5 | (-1) | 7.75 | (1) | 7.75 | (1) |
| 15 | 15 | (-1) | 47.5 | (1) | 7.75 | (1) | 7.75 | (1) |
| 16 | 37 | (1) | 47.5 | (1) | 7.75 | (1) | 7.75 | (1) |
| 17 | 4 | (-2) | 40 | (0) | 5.50 | (0) | 5.50 | (0) |
| 18 | 48 | (2) | 40 | (0) | 5.50 | (0) | 5.50 | (0) |
| 19 | 26 | (0) | 25 | (-2) | 5.50 | (0) | 5.50 | (0) |
| 20 | 26 | (0) | 55 | (2) | 5.50 | (0) | 5.50 | (0) |
| 21 | 26 | (0) | 40 | (0) | 1.00 | (-2) | 5.50 | (0) |
| 22 | 26 | (0) | 40 | (0) | 10.00 | (2) | 5.50 | (0) |
| 23 | 26 | (0) | 40 | (0) | 5.50 | (0) | 1.00 | (-2) |
| 24 | 26 | (0) | 40 | (0) | 5.50 | (0) | 10.00 | (2) |
| 25 | 26 | (0) | 40 | (0) | 5.50 | (0) | 5.50 | (0) |
| 26 | 26 | (0) | 40 | (0) | 5.50 | (0) | 5.50 | (0) |
| 27 | 26 | (0) | 40 | (0) | 5.50 | (0) | 5.50 | (0) |
| 28 | 26 | (0) | 40 | (0) | 5.50 | (0) | 5.50 | (0) |
| 29 | 26 | (0) | 40 | (0) | 5.50 | (0) | 5.50 | (0) |
| 30 | 26 | (0) | 40 | (0) | 5.50 | (0) | 5.50 | (0) |
| 31 | 26 | (0) | 40 | (0) | 5.50 | (0) | 5.50 | (0) |

**Column chromatography**

At the end of the incubation, the enzyme was removed from the reaction mixture by decanting. The reaction mixture after scale-up incubation was purified by employing column chromatography as described by Lee and Akoh (1996). The reactants were taken in a round-bottom flask and hexane was removed with a Buchi rotary evaporator (Postfach, Switzerland). A mixture of 30 g each of alumina and silica gel (100 - 200 mesh size) was activated at 150$^0$C for 4 h and cooled in a dessicator. A slurry of this was made in hexane and packed in 55 cm x 3.5 cm glass column. The sample (25 g) was loaded on to the column and eluted with 350 mL of hexane: diethyl ether (95:5, v/v). Fractions containing pure TAG as determined by TLC were pooled and the solvent was removed in a vacuum rotary evaporator. TAGs and fatty acids estimations in the purified product were carried out as described earlier.

**Preparation of blended oils**

Two types of blended oils were prepared by physically mixing RBO: LSO and RBO: CLO. The ratios of blending oils were chosen in such a way that their ALA and EPA + DHA composition was similar to that of the SLs prepared respectively. In one type blended oil RBO: LSO was taken in 3:2 (w/w) ratio and in another type RBO and CLO was taken in 1:1 (w/w) proportion in a round bottom flask and stirred overnight under an inert atmosphere.

**Nutritional studies**

**Experimental animals**

Male Wistar rats 36.0 ± 3.0 g were grouped by random distribution into six dietary groups (n=6 per group). They were placed in individual stainless steel cages with 12 h light: dark cycle with temperature 25 ± 2$^0$C in an approved animal house facility at Central Food Technological Research Institute, Mysore, India. All the animal

experimental protocols were approved by Animal Ethical Committee of the Institute. Animals were given fresh diet daily and left over diets were weighed and discarded. Gain in body weight of animals was monitored weekly.

**Diet composition**

Rats were fed AIN-76 (American Institute of Nutrition) purified diets (Anonymous 1977). The basal composition of AIN- 76 purified diets is given in table 2.5. Fresh diets were given to rats daily for a period of 60 days. Experimental diets differed from control diets only in fat source wherein the fat is either: rice bran oil, blended oils or structured lipids.

**Isolation of serum**

At the end of experimental feeding period of 60 days, rats were fasted overnight and sacrificed under ether anesthesia. Blood was drawn by cardiac puncture and serum was separated. Serum was separated by centrifugation at 1100 X g in a table top Remi 8 C cebtrifuge for 20 min and serum was stored at -80 $^{0}$C till analyzed. Liver, heart, brain, eye, and adipose tissue were removed and rinsed with ice-cold saline, blotted, weighed and stored at –80ºC until analyzed. All the analyses were completed within 15 days of sacrifice.

**Total lipid extraction**

**Serum** – Serum (0.8 mL) was taken in a stoppered tube and 2 mL of methanol was added and shaken well for 30 sec. One mL of chloroform was added and shaken well for 30 sec. Chloroform (1 mL) and water (1 mL) were added and shaken well for another 30 sec (Bligh and Dyer, 1959). The extract was filtered using a Whatman paper No. 1 filter paper. The filtrate was allowed to settle for phase separation. The aqueous methanolic upper layer was removed by aspiration and the lower chloroform layer with total lipid was used for further analysis after drying on anhydrous sodium sulphate.

**Table 2.5: Composition of AIN-76 purified diet**

| Ingredients | g/kg |
|---|---|
| Sucrose | 600 |
| Casein | 200 |
| Fat | 100 |
| Cellulose | 50 |
| AIN 76 vitamin mix[1] | 10 |
| AIN 76 mineral mix[2] | 35 |
| Choline chloride | 2 |
| Methionine | 3 |

**Source: Anonymous (1977). Report on the American institute of nutrition, Ad-hoc committee on standards for nutritional studies. J Nutr** 107**; 1340–1348.**

[1]100 g Vitamin mix contained 60 mg of thiamine hydrochloride, 60 mg riboflavin, 70 mg of pyrodixine hydrochloride, 300 mg of nicotinic acid, 160 mg D-calcium pantothenate, 20 mg folic acid, 2 mg D-biotin, 0.1 mg cynocobalamine, 40,000/IU vitamin A (retiny acetate), 5,000/IU vitamin E (tocopherol acetate), 0.25 mg cholecalciferol, 0.5 mg menadione and made to 100 g with sucrose.

[2]100 g Mineral mix contained 50 g calcium phosphate, 7.4 g sodium chloride, 22 g potassium citrate monohydrate, 5.2 g potassium sulphate, 2.4 g magnesium oxide, 0.35 g manganese carbonate, 0.6 g ferric citrate, 0.001 g sodium selinite, 0.16 g zinc carbonate, 0.03 g cupric carbonate (55% Cu), 0.001 g potassium iodate and 0.0231 g potassium chromate and made to 100 g with sucrose.

**Liver** – Liver lipids were extracted by the method of Folch et al (1957). Tissue (0.5 g) was homogenized with 1 mL of 0.74% potassium chloride in a Potter Elvehjem homogenizer. To the homogenate, 10 mL of chloroform: methanol (2:1 v/v) was added and homogenized for 2 min. The mixture was left overnight and filtered through a Whatman No.1 filter paper. One mL of potassium chloride was added and mixed well by vortexing. The solution was allowed to stand at room temperature. The upper aqueous layer was removed carefully and the lower phase was washed thrice with 2 mL of chloroform: methanol: water, (3: 48: 47, v/v/v) mixture and each time the upper layer was discarded. Finally the lower phase was made into a single phase by the addition of few drops of methanol and this chloroform layer was used for lipid analysis.

**Total cholesterol estimation**

Cholesterol was estimated by the method of Searcy and Bergquist (1960). An aliquot from the chloroform extract was dried under a stream of nitrogen followed by the addition of 1.5 mL of ferric chloride - acetic acid reagent. The reagent was prepared by diluting the stock containing 504 mg/mL anhydrous $FeCl_3$ in 10 mL of glacial acetic acid to 1: 100 dilution with glacial acetic acid. After mixing thoroughly, it was left at room temperature for 15 min. Then, 1 mL of conc. sulphuric acid was added, mixed immediately on vortex (Remi) and left at room temperature in dark for 45 min. The color intensity of the clear solution was measured in spectrophotometer (Shimadzu 160A model) at 540 nm. The cholesterol content in the biological samples was estimated from the standard curve generated with AnalaR cholesterol (30-150 μg).

**HDL cholesterol estimation**

To 0.5 mL of serum, 25 μL heparin (5,000 units/ mL) was added, followed by the addition of 25 μL of 2M manganese chloride (3.969 g/ 10 mL). The solution was vortexed and kept $4^0$C over night (Warnick and Albers, 1978). This was then centrifuged at

1100 X g for 20 min. HDL cholesterol was measured as described earlier after extracting the supernatant with 3 mL of acetone: alcohol (1:1, v/v).

**LDL+ VLDL cholesterol estimation**

The precipitate obtained from serum after adding heparin and manganese chloride was used for LDL + VLDL cholesterol estimation. The precipitate was dissolved in 0.5 mL of saline and cholesterol was extracted in 3 mL acetone: alcohol (1:1, v/v) and cholesterol was estimated as described earlier.

**Phospholipids estimation**

Phospholipids were analyzed by ferrous ammonium thiocyanate method (Stewart, 1980) using dipalmitoylphosphatidyl choline (10 to 100 μg) as reference standard. The lipid extract in chloroform was evaporated under a stream of nitrogen and the residue was dissolved in 2 mL of choloform. Two mL of ferrous ammonium thiocyanate (1.62 g $FeCl_3$ and 3.24 g ammonium thiocyanate in 100 mL water) was added and vortexed for 1 min. After the phase separation, absorbance of chloroform phase was measured at 488 nm in Shimadzu 160A spectrophotometer.

**Triacylglycerol estimation**

The lipid extract in chloroform was evaporated under a stream of nitrogen and then dissolved in 4 mL of isopropanol. Two grams of triglyceride purifier was added, mixed well and centrifuged. Supernatant was transferred to another test tube, and TAGs were estimated by the method of Fletcher (1968) as described earlier.

**Estimation of lipid peroxides in serum**

Serum lipid peroxides were estimated by the method of Yagi (1984). Serum (200 μL) was added to 4 mL of N/12 sulphuric acid and shaken gently, followed by the addition of 0.5 mL of 10 % of phosphotungstic acid and mixed for 5 min at room

temperature and centrifuged at 1600 X g for 10 min. After discarding the supernatant, the sediment was mixed with 2 mL N/12 sulphuric acid and 0.3 mL of 10 % phosphotungstic acid. The tubes were centrifuged again at 1600 X g for 10 min and the sediment was suspended in 4 mL distilled water and 1 mL TBA reagent (mixture of equal volumes of 0.6 % thiobarbituric acid aqueous solution of glacial acetic acid) containing 20 nmoles butylated hydroxyanisole was added. The reaction mixture was heated at $95^0$C for 60 min. The thiobarbituric acid complex was extracted in 5 mL butanol. The color intensity was read at 535 nm in Shimadzu 160 A spectrophotometer. Malondialdehyde (MDA) formed was measured at 535 nm and quantitated using an extinction coefficient of 1.56 x $10^{-5}$ $M^{-1}cm^{-1}$. The lipid peroxides were expressed as nmoles of malondialdehyde (MDA) formed / mg protein. A basal level of MDA was measured in the homogenate in which cofactors were not added.

**Estimation of lipid peroxides in liver homogenate**

**TBARS method**

Liver (0.5 g) was homogenized in 5 mL of 0.15 M potassium chloride in a teflon homogenizer. The homogenate was filtered through cheesecloth and used for assay. Liver homogenate (4 mg protein) in 0.15 M potassium chloride, 0.025 M Tris hydrochloride buffer pH 7.5, 2 mM adenosine diphosphate and 10 μM ferrous sulphate were incubated at 37 $^0$C for 5 min. The reaction was initiated by adding 0.1 mM ascorbic acid and incubated at 37 $^0$C fro 30 min (Buege and Aust, 1978). The final volume of the reaction mixture was 1 mL. The reaction was terminated using 2 mL of thiobarbituric acid (0.037 % thiobarbituric acid, 15% trichloroacetic acid in 0.2 N hydrochloric acid) containing 10 μM butylated hydroxyanisole. Samples were heated for 15 min in boiling water bath. Malondialdehyde (MDA) formed was measured at 535 nm and quantitated using an extinction coefficient of 1.56 x $10^{-5}$ $M^{-1}cm^{-1}$. The lipid peroxides were expressed

as nmoles of malondialdehyde (MDA) formed / mg protein. A basal level of MDA was measured in the homogenate in which cofactors were not added.

**Antioxidant enzymes in liver and serum**

Liver was homogenized in appropriate buffer (1 g/ 10 mL) using glass homogenizer. It was filtered through cheesecloth and centrifuged at 600 X g for 15 min. The homogenate was used for the analysis of antioxidant enzymes. Superoxide dismutase activity was measured by the inhibition of cytochrome C reduction mediated via superoxide anions generated by xanthine-xanthine oxidase and monitored at 550 nm (Kirby and Fridovich, 1982). One unit of superoxide dismutase was defined as the amount required to inhibit the reduction of cytochrome C by 50%. Catalase activity was assayed according to the method of Aebi (1984) by following the decomposition of hydrogen peroxide at 240 nm. Glutathione peroxidase activity was determined by NADPH oxidation in a coupled reaction system consisting oxidized glutathione (Tappel, 1978). Glutathione transferase activity was measured with 1-chloro 2, 4-dinitrobenzene (CDNB) as the substrate. The enzyme activity is expressed as μ moles of CDNB-GSH conjugate formed per minute per mg protein (Jensson et al, 1985).

**Preparation of platelet rich plasma**

Blood was collected by cardiac puncture from anesthetized rats with heparin (50 IU/mL, v/v, heparin: blood, 1:9) as anticoagulant, and centrifuged at 900 X g for 20 min to separate platelets from erythrocytes and leucocytes (Niranjan and Krishnakantha, 2000). The platelet rich plasma (PRP) obtained was then utilized as a source of platelets to study platelet aggregation. The platelet count was adjusted to 500,000-cells/ μL. After obtaining platelet rich plasma, the remaining plasma was again centrifuged for 20 min at 100 X g and platelet poor plasma was collected.

**Platelet aggregation**

Platelet aggregation measurements were performed with in two hours of blood collection. Aggregation experiments were done with PRP (using platelet poor plasma as blank). The percent aggregation was monitored using 10 µL of ADP (20 µM) or 10 µL of collagen (5 mg/10 ml 0.1 N acetic acid) in a Chronolog dual channel aggregometer, (Denmark). Platelet suspension (450 µL) was stirred at and aggregation followed for at least 5 min (Niranjan and Krishanakantha, 2000).

**Fatty acid composition of platelets**

Platelets were isolated and washed as described by Brunauer and Huestis (1993) with slight modification. Platelet rich plasma was obtained as described above. The platelets were separated from plasma by centrifuging for 10 min at 1100 X g. The cells were washed twice in tyrodes buffer pH 7.4 (NaCl 137 mM, KCl 2.7 mM, $NaHCO_3$ 12 mM, EDTA 1 mM, $NaH_2PO_4$ 0.4 mM, $MgCl_2$ 1 mM, glucose 5.6 mM). The final platelet pellet was suspended in the tyrodes buffer and lipid was extracted as described earlier (Bligh and Dyer, 1959). Fatty acid analysis of fat extracted from platelet was done as described earlier.

**Malondialdehyde (MDA) assay in agonist challenged platelets**

To 450 µL of platelet rich plasma suspension which had been challenged by the agonist, 0.1 mL of 1 % butylated hydroxyltoluene (BHT) in ethanol was added, 0.5 mL of TBA (0.12 M in 0.26 M *tris* HCl, pH 7.4) and 0.2 mL of 100 % TCA was added. The samples were then heated at 37 $^0$C for 30 min to develop colour. Absorbance was read at 533 nm using a spectrophotometer. The amount of MDA was calculated using an extinction coefficient of 1.56 x $10^{-5}$ $M^{-1}cm^{-1}$.

**Isolation of erythrocytes and erythrocyte ghosts**

Erythrocyte ghosts were prepared according to method of Fairbanks et al (1971). After removing plasma from freshly drawn heparinised blood, blood cells were washed with 0.9 % saline and centrifuged in a clinical centrifuge at 1100 X g for 10 min. This process was repeated till the supernatant became colorless. The cells (0.5 mL) were lysed by forcing them through 19.5 mL of phosphate buffer in a centrifuge cup. The contents were centrifuged at 7300 X g for 20 min to sediment erythrocyte ghosts. Ghosts membranes were washed with phosphate buffer three to four times till they were almost white or pale pink. Finally, the pellet was suspended in tris buffer. Isolation of erythrocyte and erythrocyte ghosts was done at $4^0$C. The samples were stored at $-80^0$C and used for $Na^+/K^+$ and $Ca^{2+}/ Mg^{2+}$ ATPase activity and fatty acid analysis.

**Measurement of $Na^+/K^+$ ATPase activities**

$Na^+$, $K^+$ ATPase activity was measured by spectrophotometric determination of inorganic phosphate released from ATP with or without ouabain, which is a specific enzyme inhibitor, by the method according to Savitha and Panneerselvam (2006). In brief, the enzyme $Na^+$, $K^+$ ATPase activity was measured in a final volume of 1 mL of total mixture containing 770 μL tris HCl buffer (0.2 M, pH 7.4), 50 μL ouabain (1 mM), 30 μL of membranes, 50 μL NaCl (100 mM), 50 μL KCl (20 mM), 50 μL $MgCl_2$ (5mM) and 50 μL ATP (5 mM). The mixture was incubated for 15 min at $37^0$C. The reaction was stopped by adding 500 μL TCA (5 %) and then reaction mixture was centrifuged for 10 min at 1100 X g. Thirty minutes after addition of 1 mL reagent (containing 1 % (w/v) ammonium heptamolybdate, 40 mg/mL $FeSO_4$ and 1.15 N $H_2SO_4$) to each tube and the released phosphate was measured using a spectrophotometer at 690 nm. The reaction rate was proportional to the amount of protein present. The total ATPase activity was measured with $Na/K^+$and $Mg^{2+}$ present in the reaction mixtures. The $Mg^{2+}$ ATPase was

measured in the presence of 1 mM ouabain, a specific inhibitor of $Na/K^+$ ATPase. Thus, delineation of the $(Na/K^+)$-activated component of ATPase was obtained by the difference between total ATPase and $Mg^{2+}$ ATPase.

**Measurement of $Ca^{2+}$ ATPase activity**

The reaction medium contained 0.2 M tris HCl buffer pH 7.4, EGTA 0.5 mM, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, 4 mM ATP and 30 μL membranes. The reaction was carried out at $37^0$C for 15 min and then stopped by addition of 5 % trichloroacetic acid. Thirty minutes after addition of 1 mL reagent containing 1 % (w/v) ammonium heptamolybdate, 40 mg/mL $FeSO_4$ and 1.15N $H_2SO_4$ to each tube and the released phosphate was measured using a spectrophotometer at 690 nm. The total ATPase activity was measured with $Ca^{2+}$ and $Mg^{2+}$ present in the reaction mixture, while $Mg^{2+}$ ATPase was measured in the presence of 0.5 mM EGTA. $Ca^{2+}$ ATPase activity was obtained by subtracting $Mg^{2+}$ ATPase activity from total ATPase activity (Savitha and Panneerselvam, 2006).

**Protein estimation**

Protein in the serum, liver and erythrocyte membranes was estimated by the method of Lowry et al (1951) using bovine serum albumin as reference standard.

**Statistical analysis**

Data was expressed as Mean ± SEM. Analysis of variance was employed in determining the difference between different groups (Fisher, 1970).

# CHAPTER III

# SYNTHESIS OF STRUCTURED LIPIDS WITH RBO ENRICHED IN STEARIC ACID

## Introduction

Rice bran oil (RBO) is a product of rice milling industry, and is consumed widely in Asia. It is gaining popularity as edible oil in recent past. RBO is a rich source of tocopherols, tocotrienols and γ-oryzanol (Shin et al, 1997). γ-Oryzanol has been reported to possess the hypocholesterolemic and antioxidant activity (Sugano and Tsuji, 1997; Seetharamaiah and Chandrasekhara, 1989; Minhajuddin et al, 2005). Structured lipids (SL) are gaining worldwide recognition as an important avenue for improving nutritional, therapeutic and industrial value of oils and fats. SL are triacylglycerols (TAGs) modified to alter the fatty acid composition or their location in the glycerol backbone by chemical or enzymatic means. The concept of SL for nutritional and medical use was first introduced by Babayan (1987). Improvements or changes in physical and/ or chemical characteristics of a TAG can also be achieved when SL are synthesized. Interesterification is used to produce fats with desirable physical and functional properties for food applications. Interest in interesterification from nutritional and functional standpoints is on the rise because of the possibility to produce *trans* - free margarines, cocoa butter substitutes, and reduced calorie fats; to improve functional and physical properties of fats; and to improve the nutritional quality of fats and oils (Osborn and Akoh, 2002). The use of immobilized lipase catalyzed modification of fats and oils is gaining importance because of their specificity and their mild reaction conditions. Lipase catalyzed interesterification reaction provides a tool to improve the properties of lipids by replacing undesirable fatty acids from triacylglycerols or incorporating desirable ones into specific positions. Enzymatic interesterification using lipases have been employed to modify vegetable oils for various purposes such as improving nutritional properties (Shimada et al, 1999; Xu et al, 2000), preparation of low calorie fats and also as an alternative to hydrogenation of vegetable oils to improve their physico-chemical

properties (Seriburi and Akoh, 1998; Ghosh and Bhattacharya, 1997; Lai et al, 1998). Controlled incorporation of fatty acids at desirable levels into TAG is required to get structured lipids with specific properties. To achieve this, reaction conditions have to be optimized with each fatty acid because it has been observed that lipase catalyzed reactions are specific to each system (Mu et al,1998).

RBO contains approximately 38 % oleic acid, 34 % linoleic acid and 18.6 % palmitic acid (Rukmini and Raghuram, 1991). The physical properties of these fatty acids do not confer any special properties for its use in food applications. It is interesting to note that stearic acid content of RBO is very low. Stearic acid (SA), a unique long-chain SFA has emerged as a candidate to use as a substitute for *trans* fatty acids in food manufacturing and it has become the conventional replacement for *trans* fatty acids. It has the requisite physical attributes that a solid fat imparts and seems to have little effect on important risk factors for cardiovascular disease (CVD) (Penny et al, 2005). Stearic acid is neutral as far as raising serum cholesterol levels in humans is concerned (Grande et al, 1970). Stearic acid as tristearin is well absorbed as found by Baer et al, (2003). Recent studies conducted, to compare the effects of stearic, oleic, and linoleic acids on the serum lipoprotein profile of healthy subjects showed no differential effects of stearic acid, oleic acid, or linoleic acid on lipoproteins particle size and concentrations (Thijssen and Mensink, 2005). Furthermore, detailed studies concluded that stearic acid exerted similar effects on plasma lipoprotein profile as oleic acid (Bonanome and Grundy, 1998). The studies of Mensink (2005) have concluded that stearic acid may decrease the ratio of total to HDL cholesterol slightly when compared with palmitic or myristic acid and also showed that the effects of stearic acid are more favorable than those of *trans* monounsaturated fatty acids. Very recently it was reported that stearic acid rich triacylglycerol in both unrandomized and randomized forms does not adversely affect lipid risk factors for cardiovascular disease (Berry et al, 2007). As a value addition

to RBO and also to encourage its utilization as dietary oil, an attempt has been made to prepare SA rich RBO.

Plastic fats are defined as having a broad melting range, with soft consistency at normal ambient temperature, yet withstanding higher working temperature that are normally employed in bakeries. These fats are normally used in bakeries and also as vanaspati. Vanaspati is a hydrogenated fat often used for culinary purposes and traditional sweets preparation in India and many Southeast Asian countries. These are normally prepared by hydrogenation and there is a great demand for use in frying, shortenings, confectionery, bakeries and margarine industries. However, these hydrogenated fats contain large amounts of *trans* fatty acids, which have been reported to be risk factors involved in cardiovascular diseases. *Trans* fatty acids have been reported to raise levels of low-density lipoproteins (LDL) and lower the levels of high-density lipoproteins (HDL) in humans (Sun et al, 2007). In view of such deleterious effects of hydrogenated fats, the demand for specialty plastic fats with zero *trans* fatty acids by retaining natural fatty acids is increasing throughout. The chemical interesterification process is one of the alternative processes for hydrogenation to prepare *trans* fatty acid-free fats. It was reported that blends formulated by mixing fully hydrogenated soybean oil with the commonly used nine vegetable oils in a ratio of 1:1 (wt/wt) were interesterified and resulted in speciality fats with no *trans* fatty acids (Zeitoum et al, 1993). Bakery shortenings were prepared from tallow and sunflower oil blends in various ratios by interesterification (Rodriguez et al, 2001). The *trans* fatty acid-free bakery shortenings were prepared from vegetable fats and oils by fractionation and blending (Reddy and Jayarani, 2001; Mayamol et al, 2004). One of the main objective of the present study was to prepare a solid fat from RBO with stearic acid which has non-hypercholesterolemic effects. The other objective of the study was to optimize the reaction conditions like the effects of substrates molar ratio, incubation time and

temperature, enzyme concentration on the incorporation of stearic acid into RBO and to study the physicochemical properties of the resultant modified fat.

## Experimental

### Acidolysis reaction between RBO and stearic acid

RBO is used as edible oil and has various health benefits due to its minor constituents like oryzanol, tocopherols and tocotrienols. In the present study attempts were made to incorporate stearic acid which is a saturated fatty acid, so that RBO can be used as alternate source for vanaspati which contains *trans* fatty acids. Acidolysis reaction was carried out between triacylglycerols of RBO and stearic acid as described earlier in chapter II. Enzymatic acidolysis reactions were carried out in 50 mL stoppered conical flasks under inert atmosphere. Different reaction conditions like incubation time, temperature, substrates molar ratio and enzyme concentration on the incorporation of stearic acid into RBO were studied.

### Effect of reaction time

The effect of reaction time on the incorporation of stearic acid into RBO was studied from 0 to 48 h keeping other parameters constant. Reaction temperature, substrates molar ratio, and enzyme concentration were kept at 37 $^{0}$C, 1:6 and 10 % respectively.

### Effect of reaction temperature

The effect of reaction temperature on the incorporation of SA into RBO was studied from 25 to 60 $^{0}$C keeping other parameters constant. Reaction time, substrates molar ratio and enzyme concentration were kept at 6 h, 1:6 and 10 % respectively.

### Effect of substrates molar ratio

The effect of molar ratio of substrates on the incorporation of SA into RBO was studied from 1:1 to 1:10. Molar concentration was calculated based on molecular

weights of RBO and stearic acid. While studying substrates molar ratio, other parameters like incubation time, temperature and enzyme concentration were kept at 6 h, 37 $^{0}$C and 10 % respectively.

**Effect of enzyme concentration**

The effect of enzyme concentration on the incorporation of SA into RBO was studied from 1 to 10% of the weights of both the substrates. While studying enzyme concentration other parameters like incubation time, temperature and substrates molar ratio were 6 h, 37 $^{0}$C and 1:6 molar ratio respectively.

**s*n-2* Positional distribution of fatty acids of RBO and modified RBO**

After incorporation of stearic acid into RBO, *sn-2* positional analysis was done in modified and original RBO using pancreatic lipase catalyzed reaction as described earlier.

**Analysis of oryzanol, tocopherols and tocotrienols**

RBO known to possess antioxidant properties due to the presence of oryzanol, tocopherols and tocotrienols. After incorporation of stearic acid into RBO, these constituents were analyzed in native and modified RBO as described earlier.

**Triglyceride molecular species of modified RBO**

**Triglyceride molecular species of native and modified RBO were monitored according to the method of Swe and Cheman (1994) as described earlier.**

**Differential scanning calorimetric studies of modified RBO**

After incorporation of stearic acid into RBO, physical properties of native and modified RBO were studied by differential scanning calorimeter as described earlier. Vanaspati and cocoa butter were studied to compare with modified RBO.

## Results

### Fatty acid composition of RBO and modified RBO

The fatty acid profile of native and modified RBO with stearic acid catalyzed by immobilized lipase IM 60 from *Rhizomucor miehei* is shown in table 3.1. The fatty acid composition of modified RBO showed that stearic acid was successfully incorporated into RBO. After lipase catalyzed acidolysis reaction, the stearic acid content of RBO was increased to 48.71 % of total fatty acids. There was a 59 % decrease in palmitic acid content after incorporation of stearic acid into RBO. Similarly both oleic and linoleic acids were also decreased by 45 % and 41 % respectively after incorporation of stearic acid into RBO.

**Table 3.1: Fatty acid composition of native and modified RBO**

| Fatty Acids | Native RBO | Modified RBO |
|---|---|---|
| | (%) | |
| 16:0 | 21.53 ± 0.02 | 08.89 ± 0.01 |
| 18:0 | 02.34 ± 0.01 | 48.71 ± 0.02 |
| 18:1 | 43.66 ± 0.04 | 23.95 ± 0.02 |
| 18:2 | 32.10 ± 0.03 | 18.24 ± 0.01 |

Values are mean ± SEM of four separate experiments in duplicate carried out at different times

**Time course of reaction**

Figure 3.1 show that the time course of lipase assisted acidolysis of RBO with stearic acid using immobilized lipase (IM 60) from *Rhizomucor miehei*. Incorporation of stearic acid into RBO increased rapidly with progress of time and reached maximum level of 48 - 50% by 6 h of incubation at 37 $^{0}$C. Prolonging the incubation time did not significantly improve the incorporation. Based on the incorporation level, time and temperature, further experiments were carried out, keeping the reaction time for 6 h.

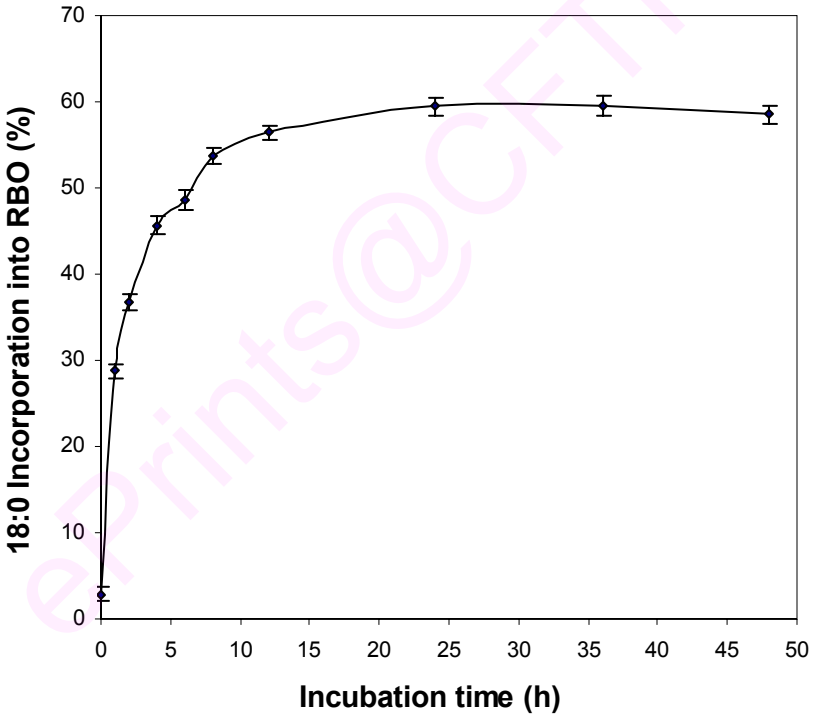


**Figure 3.1: The effect of incubation time on the incorporation of stearic acid into RBO**

Values are mean ± SEM of three separate experiments carried out in duplicate

**Substrates mole ratio**

The effect of molar ratio of substrates on the incorporation of SA into RBO is given in figure 3.2. It is observed that the incorporation was almost doubled when TAG and stearic acid concentration was increased from 1:1 to 1:3. Maximum incorporation of SA into the RBO (54.7 %) was obtained at RBO and SA molar ratio of 1:10. But further increase in substrates molar ratio caused only marginal increase. There is no economic advantage of using excess of stearic acid which is expensive.



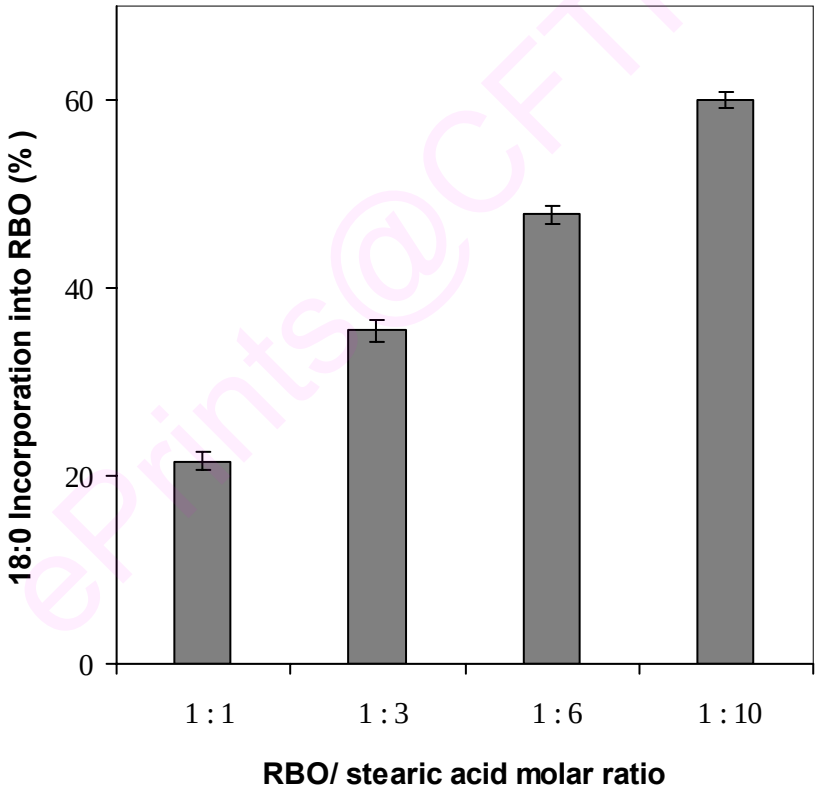


**Figure 3.2: The effect of substrates molar ratio on the incorporation of stearic acid into RBO**

Values are mean ± SEM of three separate experiments carried out in duplicate

**Reaction temperature**

This reaction parameter was investigated to determine the optimum temperature for immobilized lipase IM 60 from *Rhizomucor miehei* for incorporation of SA into the RBO and is shown in figure 3.3. Stearic acid incorporation showed a steady increase from 39 % at 25 °C to 48.5 % at 37 $^{0}$C. A further increase in temperature to 50 and 60 °C did not increase the stearic acid incorporation significantly.

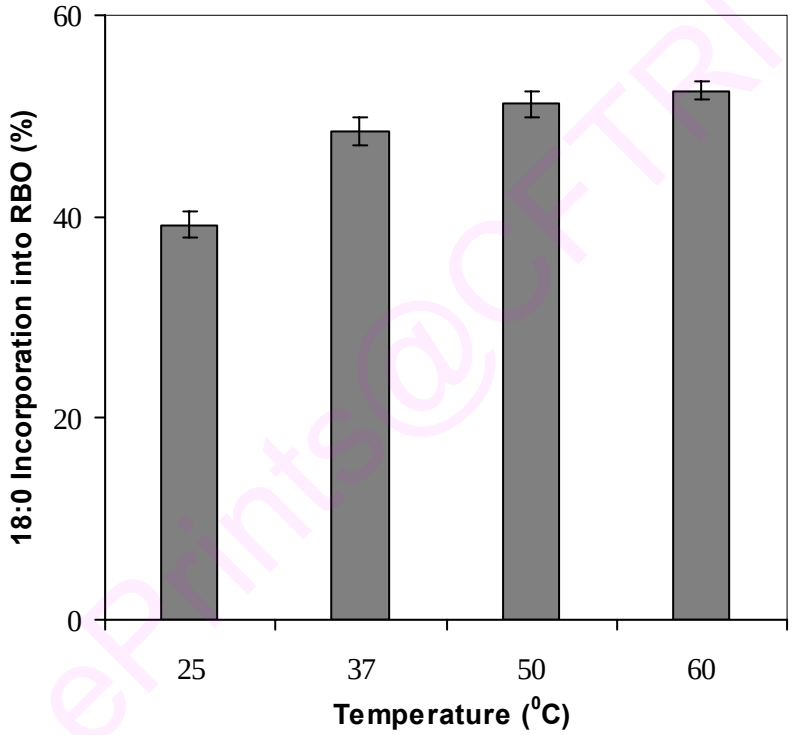


**Figure 3.3: The effect of incubation temperature on the incorporation of stearic acid into RBO**

Values are mean ± SEM of three separate experiments carried out in duplicate

**Enzyme load**

The effect of enzyme load (%) on the incorporation of SA into RBO is shown in figure 3.4. As the enzyme load increased from 1 to 10 %, the incorporation of SA increased gradually reaching maximum (48.5 %) at 10 % level of total substrates weight.

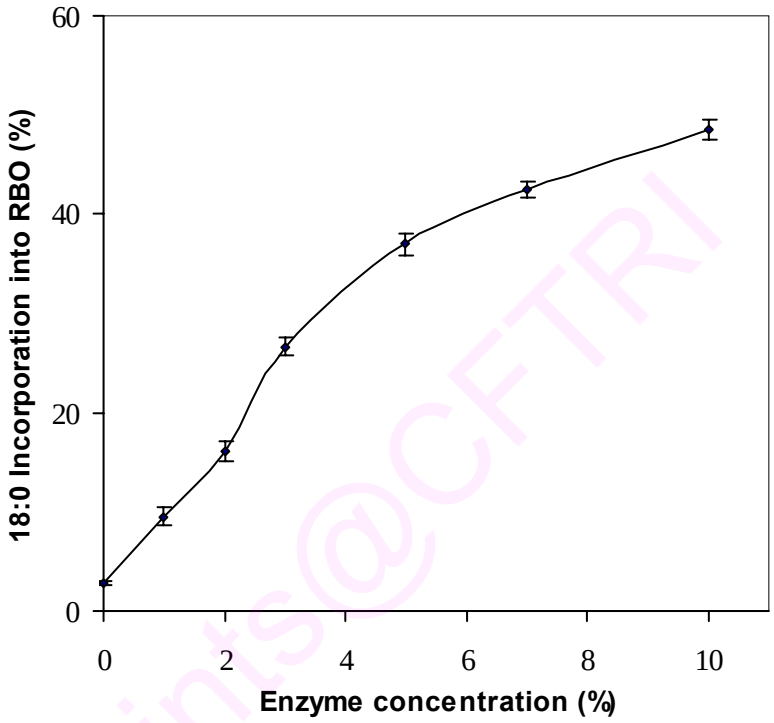


**Figure 3.4: The effect of enzyme load on the incorporation of stearic acid into RBO**

Values are mean ±SEM of three separate experiments carried out in duplicate

**Positional distribution of fatty acids in native and modified RBO**

Pancreatic lipase catalyzed reaction was used to analyze positional distribution of the native and modified RBO fatty acids and is shown in table 3.2. Even though a 1, 3 specific lipase was used, but there was a 17 % stearic acid incorporation at *sn-2* position of modified RBO.

**Table 3.2: Fatty acid composition of native and modified RBO at *sn-2* position**

| Fatty acids | RBO | Modified RBO |
|---|---|---|
| | (%) | |
| 16:0 | nd | Nd |
| 18:0 | nd | 17.0 |
| 18:1 | 52.7 | 51.6 |
| 18:2 | 47.2 | 31.5 |

Values are average of triplicate analysis

**Tocopherol and tocotrienol analysis**

The tocopherols and tocotrienols present in RBO have been shown to possess antioxidant activity against lipid oxidation (Xu et al, 2001). Table 3.3 shows the tocopherol and tocotrienol contents of RBO before and after modification. Tocopherols detected were α-, γ -, and δ- isomers. Total tocopherol content in native RBO was 60.13 µg / g and α- tocopherol accounts for 61.2 % and the rest was accounted by γ -, and δ- tocopherols. There was a 37.6 % loss of total tocopherols after acidolysis. In the modified RBO, α- tocopherol content was less affected as compared to γ- and δ- isomers. Tocotrienols content in RBO and modified RBO was more than tocopherols and after acidolysis they were less affected. Total tocotrienol content of native and modified RBO was 777.62 and 668.27 µg / g respectively. There was a 25 % loss in case of α- tocotrienol, whereas in case of γ- and δ- tocotrienol, the loss was not significant. β- Tocopherol and β- tocotrienol were not detected.

**Table 3.3: HPLC analysis of minor components (tocopherols, tocotrienols and oryzanol) present in native and modified RBO**

| Minor Components | Native RBO (μg/g) | Modified RBO (μg/g) |
|---|---|---|
| **Tocopherols** | | |
| α | 37.00 ± 0.02 | 33.66 ± 0.01 |
| γ | 11.58 ± 0.06 | 1.93 ± 0.01 |
| δ | 11.55 ± 0.03 | 1.71 ± 0.01 |
| Total | 60.13 ± 0.06 | 37.30 ± 0.01 |
| **Tocotrienols** | | |
| α | 71.18 ± 0.07 | 53.17 ± 0.05 |
| γ | 694. 26 ± 0.03 | 603.71 ± 0.02 |
| δ | 12.18 ± 0.01 | 11.39 ± 0.02 |
| Total | 777. 62 ± 0.04 | 668.27 ± 0.03 |
| **Oryzanol (%)** | 1.02 ± 0.03 | 0.66 ± 0.02 |

Values are mean ± SEM of four separate experiments in duplicate

**Oryzanol analysis**

Oryzanol content of RBO and modified RBO is given in table 3.3. There was a 35 % loss in oryzanol content after acidolysis and purification of modified RBO. The loss in the oryzanol content may be due to the isolation procedure employed after acidolysis reaction.

**HPLC profile of triacylglycerol molecular species of native and modified RBO**

The molecular species of triacylglycerol (TAG) of native and modified RBO are shown in figures 3.5 and 3.6, respectively. Incorporation of SA into RBO resulted in the formation of some new TAG species and reduction in some TAG species.

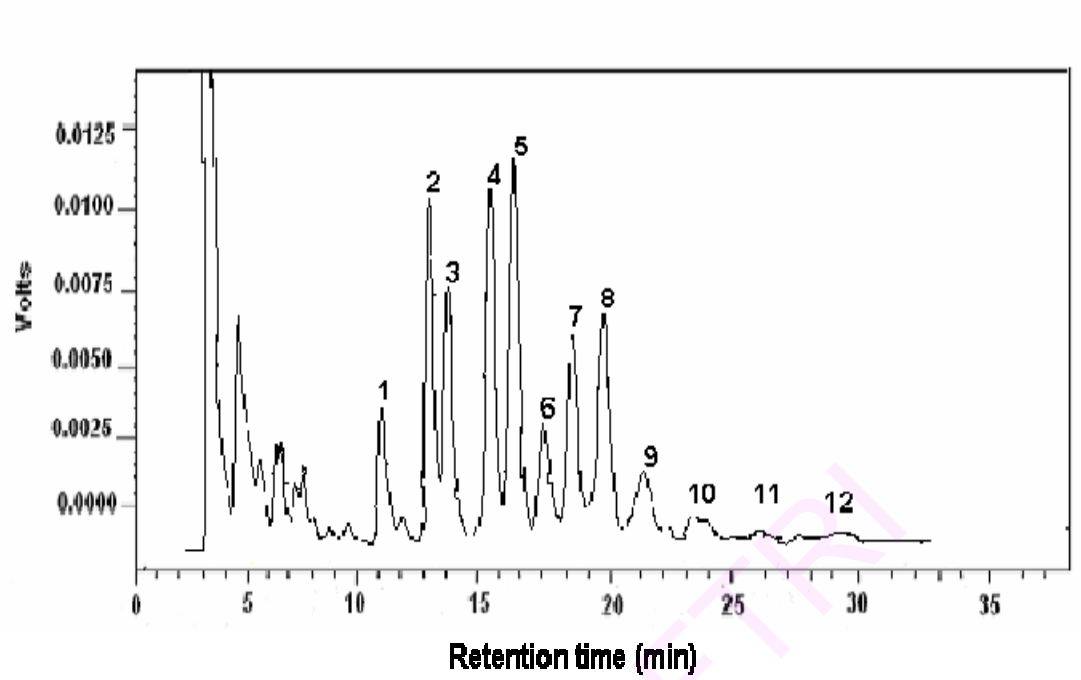


**Figure 3.5: HPLC profile of TAG molecular species of native RBO**

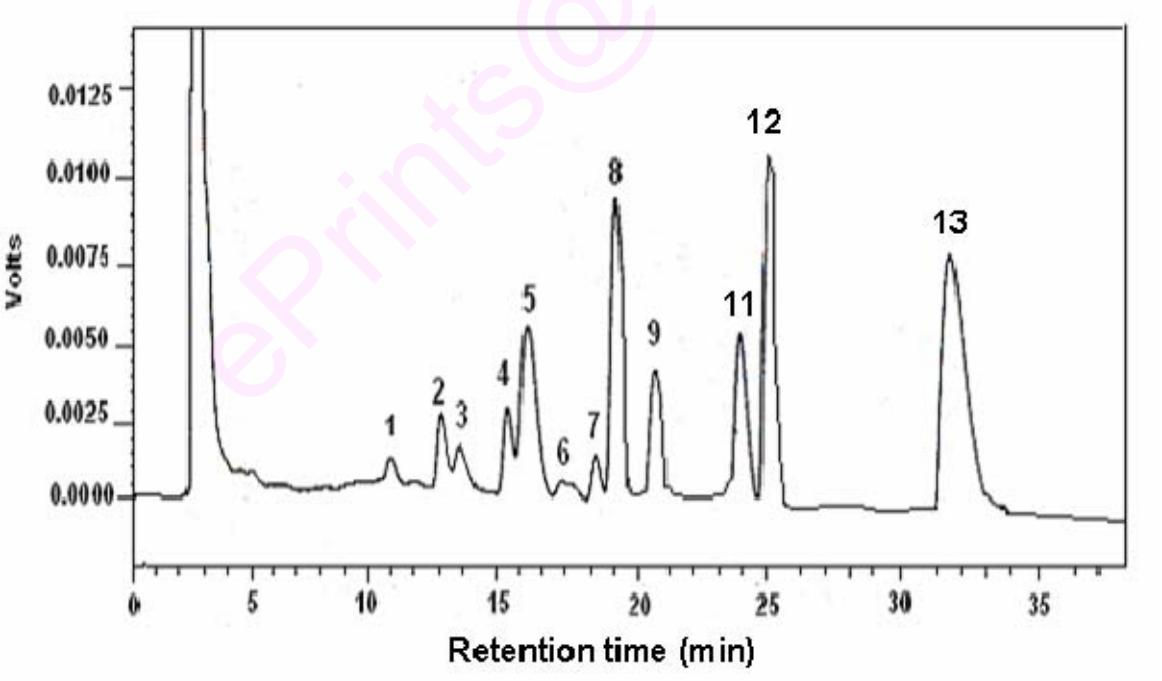


**Figure 3.6: HPLC profile of TAG molecular species of modified RBO**

Peaks *viz*., 1, 2, 3 and 4 of native RBO were reduced significantly in modified RBO. New TAG molecular species (peaks *viz*., 9, 10, 11, and 12) were formed in modified RBO and these were the major peaks. Percent composition of TAG of native and modified RBO is given in table 3.4, which shows that the acidolysis of RBO and SA with lipase resulted in the formation of new TAG species.

***Table 3.4: HPLC separation of TAG species of native and modified RBO***

| Peak No. | Native RBO (%) | Modified RBO (%) | Retention Time (min) | Triglyceride molecular species |
|---|---|---|---|---|
| 1 | 5.5 | 0.5 | 10.0 | LLL |
| 2 | 12.8 | 2.3 | 12.3 | LLO |
| 3 | 11.2 | 0.9 | 13.5 | PLL |
| 4 | 15.2 | 2.9 | 15.3 | LOO |
| 5 | 18.1 | 8.7 | 16.2 | PLO |
| 6 | 5.9 | 0.4 | 17.8 | PLP |
| 7 | 10.2 | 1.5 | 19.3 | OOO |
| 8 | 12.5 | 14.9 | 20.3 | POO |
| 9 | 4.9 | 8.6 | 22.2 | POP |
| 10 | 0.9 | - | 23.5 | PPP |
| 11 | 1.1 | 8.0 | 25.1 | OOS |
| 12 | 0.7 | 29.7 | 27.6 | POS |
| 13 | 0.5 | 21.6 | 33.0 | SOS |

Values are mean of three separate determinations

**DSC analysis**

The thermal behaviour of edible fats is commonly characterized by two physical events *viz.,* melting and crystallization.

**Melting profile of RBO rich in stearic acid, vanaspati and cocoa butter**

Structured lipids with stearic acid levels of 38 and 48 % were synthesized and their melting behaviour was compared with solid fats like vanaspati and cocoa butter. Samples were subjected to cooling scan from 80 to -10 $^0$C at the rate of 10 $^0$C/min. The melting profile of modified RBO, vanaspati and cocoa butter is shown in figure 3.7. The thermal profiles of modified RBO at 30 $^0$C showed that there was an increase in the peak are with the increase in stearic acid level into RBO at its melting temperature whereas melting peaks of vanaspati and cocoa butter were higher than the modified RBO. The enthalpy of endothermic peaks which represented the energy released also showed that with increase in stearic acid into RBO there was a decrease in high melting peak whereas there was not much change in the low melting peak (Table 3.5). In cocoa butter and vanaspati the enthalpy was higher than the modified RBO. Enthalpy of lower endothermic peak was same for modified RBO's and cocoa butter. But for vanaspati it was higher as compared to the other samples. The melting temperature and enthalpy of higher melting peak of the sample with higher 18:0 incorporation was higher than the sample with lower 18:0 (Table 3.5 and Figure 3.8).

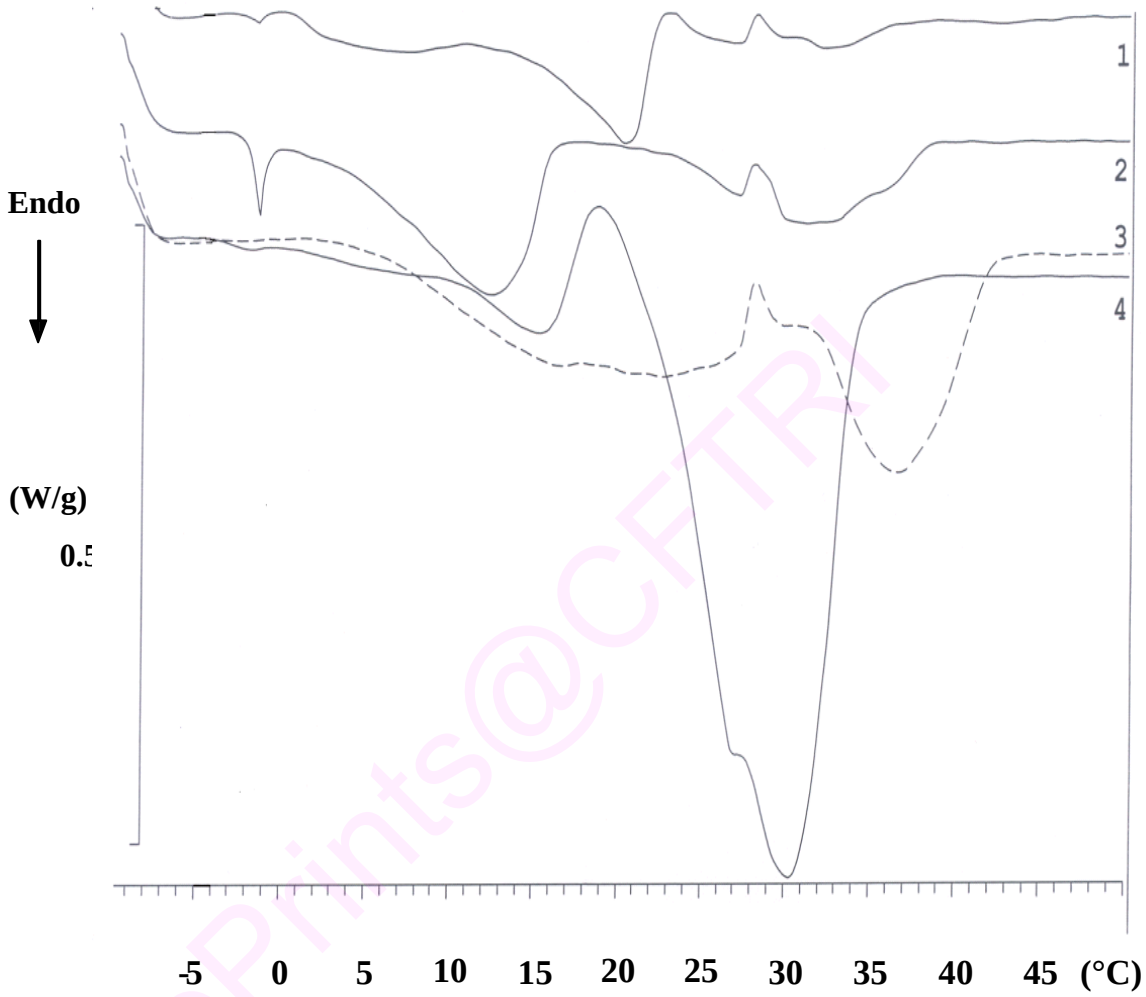


**Figure 3.7: DSC thermograms of modified rice bran oil in comparison with cocoa butter and vanaspati**

1- RBO with 38% stearic acid

2- RBO with 48% stearic acid

3- Vanaspati

4- Cocoa butter

**Table 3.5: Peak temperature and enthalpy of modified RBO, cocoa butter and vanaspati during melting detected by DSC**

| Fat samples | High melting peak | | | | Low melting peak | |
|---|---|---|---|---|---|---|
| | Peak 1 (ºC) | Enthalpy (J/g]) | Peak 2 (ºC) | Enthalpy (J/g) | Peak (ºC) | Enthalpy (J/g) |
| RBO (48% SA) | 32.60 ± 0.12 | 10.70 ± 0.03 | 27.00 ± 0.06 | 1.90 ± 0.03 | 12.50 ± 0.3 | 28.10 ± 0.20 |
| RBO (38% SA) | 32.00 ± 0.20 | 4.20 ± 0.10 | 26.90 ± 0.20 | 2.10 ± 0.10 | 20.20 ± 0.02 | 26.90 ± 0.09 |
| Cocoa butter | 30.26 ± 0.20 | 127.33 ± 0.18 | - | - | 15.30 ± 0.6 | 28.14 ± 0.21 |
| Vanaspati | 36.70 ± 0.18 | 36.90 ± 0.09 | - | - | 22.53 ± 0.2 | 41.72 ± 0.20 |

Values are mean ± SEM of four separate determinations

SA- Stearic acid

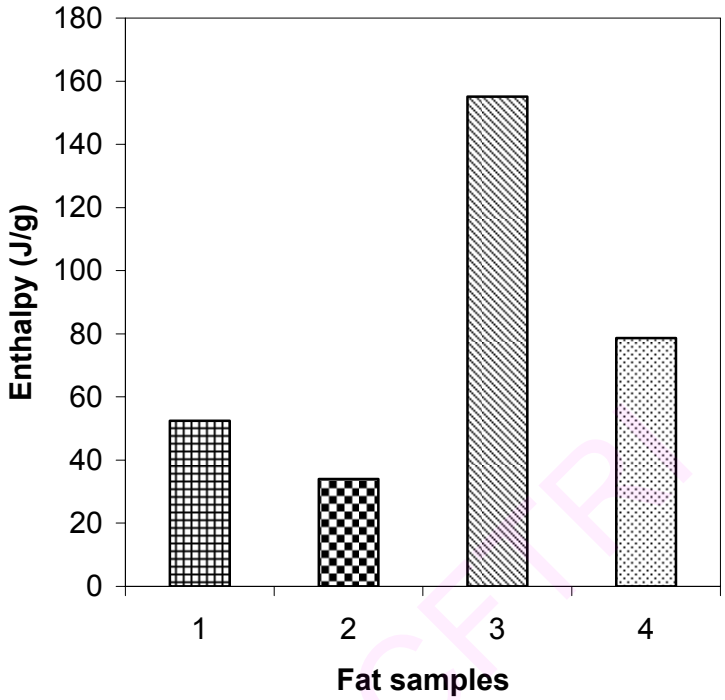


**Figure: 3.8: Enthalpy for melting modified RBO, vanaspati and cocoa butter**

1- RBO with 48% stearic acid
2- RBO with 38% stearic acid
3- Vanaspati
4- Cocoa butter

**Crystallization behaviour of modified RBO, cocoa butter and vanaspati**

RBO with different levels of stearic acid, vanaspati and cocoa butter were subjected to a cooling scan from 80 to -10 $^0$C at the rate of 10 $^0$C/min. There were two endothermic peaks for stearic acid rich RBO but for cocoa butter and vanaspati only one peak was observed. Crystallization studies confirm the melting properties as they showed two distinct peaks indicating heterogeneity of triacylglycerols in modified RBO. The crystallization peak at higher temperature is smaller as it is corresponding to higher melting triglycerides, which observed as small during melting (Table 3.6). With the increase in stearic acid level in RBO, there was an increase in enthalpy for crystallization (Figure 3.9).

**Table 3.6: Crystallization behaviour of modified RBO, cocoa butter and vanaspati**

| Fat samples | Peak 1 | | | Peak 2 | | |
|---|---|---|---|---|---|---|
| | Onset (ºC) | Peak (ºC) | Enthalpy (J/g) | Onset (ºC) | Peak (ºC) | Enthalpy (J/g) |
| RBO (48% SA) | 14.14 ± 0 .10 | 11.46 ± 0.03 | 4.26 ± 0.05 | 4.67 ± 0.03 | 0.25 ± 0.07 | 35.68 ± 0.15 |
| RBO (38% SA) | 12.80 ± 0.24 | 10.38 ± 0.041 | 1.60 ± 0.12 | 6.37 ± 0.01 | 1.60 ± 0.02 | 25.84 ± 0.09 |
| Cocoa butter | - | - | - | 13.99 ± 0.8 | 10.27 ± 0.01 | 75.71 ± 0.18 |
| Vanaspati | - | - | - | 20.34 ± 0.06 | 15.77 ± 0.4 | 71.13 ± 0.13 |

Values are mean ± SEM of four separate determinations

SA- Stearic acid

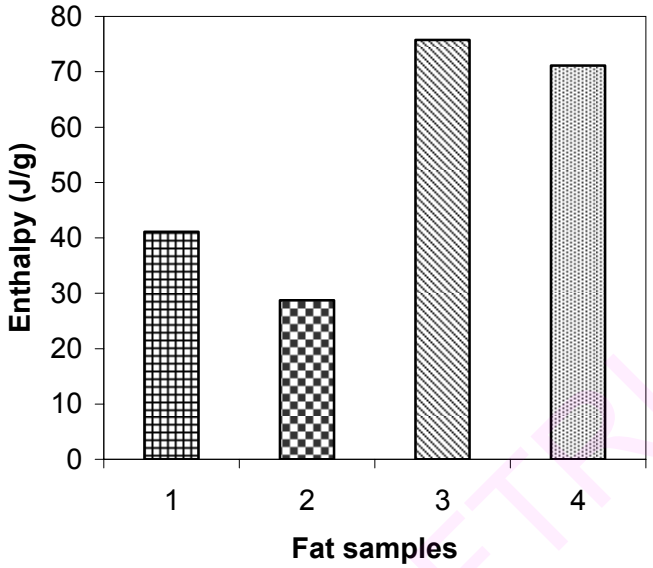


**Figure: 3.9: Enthalpy for cooling modified RBO, vanaspati and cocoa butter**

1- RBO with 48% stearic acid
2- RBO with 38% stearic acid
3- Vanaspati
4- Cocoa butter

**Solid fat content and melting characteristics of RBO rich in stearic acid in comparison with vanaspati and cocoa butter**

The solid fat content (SFC) of modified RBO, cocoa butter and vanaspati subjected to DSC analysis was determined at different temperatures (Figure 3.10). Incorporation of 18:0 into RBO is clearly seen from melting profile and SFC data. RBO is liquid at normal ambient temperature, whereas modified RBO showed solids at all temperatures up to 40 $^{0}$C. Among modified RBO, the sample with higher 18:0 incorporation showed higher SFC compared to those of RBO with lower 18:0 incorporation. Cocoa butter had sharp melting peak and melted completely at 37 $^{0}$C whereas vanaspati showed higher melting point. Cocoa butter had sharp melting peak whereas modified RBO and vanaspati showed broad melting peak.

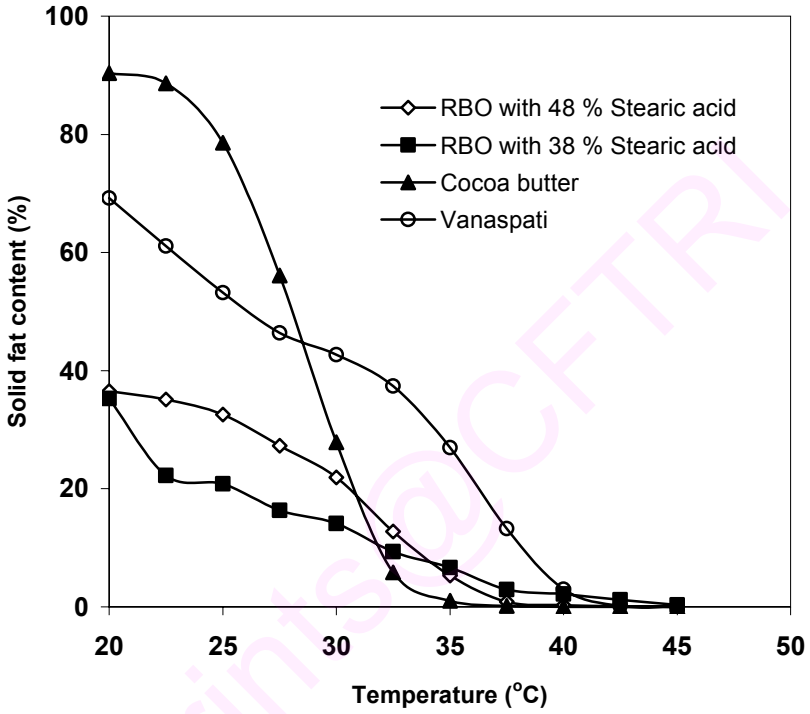


**Figure 3.10: Melting profiles of modified rice bran oil in comparison with cocoa butter and vanaspati**

## Discussion

The present study was carried out to prepare a solid fat, incorporating SA into RBO by acidolysis reaction using immobilized lipase IM 60 from *Rhizomucor miehei* under optimized conditions. SA content of RBO was increased from 2.34 to 48.71 % with a simultaneous decrease in palmitic, oleic and linoleic acids. Enzymatic interesterification has been used as an efficient tool for the modification of the fatty acid composition of fats and oils. Last few years have seen a surge in various approaches for the modification of lipids employing lipase-catalyzed interesterification. This method has interested researchers as well as commercial agencies because of the specificity of lipases, milder reaction conditions, and the fact that it can be used for the modification of a wide range of oils and fats. Most of the studies have concentrated on arriving at optimized conditions for the alteration of the fatty acid composition of oils to modify the physical and nutritional properties. Several factors have prompted the increasing interest in structured lipids. One reason is that longer chain fatty acids are poorly absorbed from the digestive tract compared to unsaturated and short chain fatty acids. Another reason is that pancreatic lipase hydrolyses fatty acids esterified at sn-1,3 positions of dietary TAG. After pancreatic hydrolysis 2 - monoacylglycerol is readily absorbed. Saturated fat like vanaspati is used for many foods preparation as well as in bakery. Vanaspati is produced by hydrogenation of vegetable oil and *trans* fatty acids are produced during this process, which are known for their atherogenic properties. So there is a need for alternate sources to replace such fat and provide a healthy saturated fat. During acidolysis process there is replacement of fatty acids in the TAG molecule which offers the possibility of changing the physical characteristics, specially the crystallization properties of fat and oils. The present study showed that a healthier saturated fat with nutraceuticals like oryzanol, tocopherols and tocotrienols from RBO can be obtained.

The present study showed that the reaction conditions such as incubation time, temperature, substrates and enzyme concentrations play a role in controlling the rate of incorporation of 18:0 into RBO TAG by the lipase catalyzed acidolysis reaction. The incorporation of stearic acid in the structured lipids increased in a time dependent manner with maximum incorporation being observed by 6 h at 37 $^{0}$C. Jennings and Akoh (2000) successfully incorporated capric acid (up to 27 %) into RBO in 72 h using immobilized lipase from *Rhizomucor miehei.* Jennings and Akoh (2001) reported 41.2 % of capric acid incorporation into fish oil. The reaction was also dependent on the ratio of 6: 1 of stearic acid: RBO where optimum incorporation was observed. In many interesterification reactions involving TAG and fatty acids, an optimum incorporation was noticed upon increase in substrates (Fajardo et al, 2003). Similar observations were made by (Reena et al, 2001). Other studies have reported similar observations on the fatty acid incorporation into TAG and has significant effect on the enzymatic interesterification using lipozyme IM *Rhizomucor miehei* of butter fat with rapeseed oil (Xu et al, 1998b; Ronne, 2005; Chang et al, 1990). Whereas, a study has reported that incorporation of DHA into high-laurate canola oil was 42.5% at 24 h (Hamam and Shahidi, 2006).

The reaction rate increased linearly with an increase in enzyme concentration reaching a maximum at 10%. Similar observations were made that with IM 60, (Akoh and Mousata, 1998) SP435 (Akoh et al, 1996) or lipase from *Pseudomonas* species (Moussata and Akoh 1997). These studied showed that there was an increase in the incorporation rates with the increase in the substrates concentration. Studies have shown that increased incorporation of capric acid into rapeseed oil with increasing enzyme load have been reported (Xu et al, 1998b). The cost of substrate, enzyme and energy input must be taken into account in working out the economies of producing the desired product. The present study showed that incubation time, enzyme concentration,

substrates mole ratio and temperature increased the incorporation of SA. These results were consistent with our earlier studies involving enzymatic interesterification of coconut oil with stearic acid (Reena et al, 2001).

γ-Oryzanol from rice bran oil has been reported to possess the property of lowering blood cholesterol (Seetharamaiah and Chandrasekhara 1989; Nicolosi et al, 1993) and antioxidant functionality (Xu and Godber , 1999). There was some loss of oryzanol during the synthesis or isolation of modified RBO. Similar losses were also occurred in tocopherol and tocotrienol levels of modified RBO. This loss in the oryzanol content may be due to the purification procedure employed after acidolysis reaction. The loss in tocopherol and tocotrienol contents may be due to the acidolysis and purification process. In case of γ- and δ- tocotrienol, the loss was not significant. β- Tocopherol and β- tocotrienol were not detected. Similar losses have been reported in another study while incorporating capric acid in RBO (Jennings and Akoh, 2000).

The present study showed that partial replacement of fatty acids present in RBO with stearic acid in the structured lipids changed their TAG composition. However the fatty acid composition of structured lipids in *sn-2* was almost similar to that of RBO. Incorporation of stearic acid at *sn-1,3* position of TAGs brought about a change in their structure. The presence of 18:0 at the s*n-2* position, even though a 1, 3 specific lipase was used, demonstrates some acyl migration. Others have reported that in addition to reaction time, water content, lipase load, temperature, acyl donor type, and lipase type also influence acyl migration (Xu et al, 1998a). Of total fatty acids at 2- position, C 18: 0 represents only 17 % and remaining 83 % of the modified RBO is of long chain fatty acids along with C 18:1 and C 18:2. Fatty acids esterified at the 2-position are easily absorbed, regardless of the type of fatty acid esterified in that position (Haumann, 1997).

HPLC analysis of RBO and structured lipids showed difference in elution profile of peaks representing TAG species. The changes in TAG structure influence the thermal

properties of modified RBO. The RBO rich in stearic acid had higher solid fat content, higher melting point and higher enthalpy. The solid fat content is responsible for many important characteristics of fats like physical appearance, organoleptic properties and spreadabiltiy. This profile also influences the melting properties of specific fat indicating the behaviour of a fat at different temperatures. The plasticity or physical consistency of an edible fat product depends on the amount of solids present. The variation of SFC with temperature and the sharpness of melting range, together with the other factors such as crystal morphology determines the range within which a fat could be considered plastic (Reena et al, 2001; Seriburi and Akoh, 1998). Desirable spreadability occurs within a range of roughly 15- 35 % solids, the so-called plastic range of fats. A SFC value at 10 $^0$C of less than 32 % is essential for good spreadability while the SFC values between 4 and 10 $^0$C determine the spreadability of the product at refrigeration temperatures. In addition, the SFC at 25 $^0$C influences plasticity while that between 33 and 38 $^0$C influences mouth feel (DeMan, 1992). The overall fatty acid composition and their position in TAG molecule plays important role in determining the melting behaviour of fat (Rousseau, 1996). An important factor which determines the physical state of a fat is the polymorphic form of TAG. TAG occurs in a variety of crystal forms (polymorphism) (Birker and Padley, 1987). Due to different packing modes available to the fatty acid residues in TAG, they exhibit complex crystal polymorphism (Bushfield and Prochog, 1990). TAGs of oil and fat may occur in three main polymorphic forms, namely, $\alpha$, $\beta$ and $\beta$' in the order of increasing stability (De Man, 1992). Fats with $\beta$' form are preferred for plastic shortenings as the crystals tend to be small, uniform and impart smooth texture to the product, whereas the $\beta$ form gives sandiness or graininess to the products. A fully hydrogenated $\beta$' hard fat which is usually made from palm oil is incorporated in order to extend the plastic range and to stabilize the $\beta$'-polymorphic form of the fat crystals

(Sabine and Claude, 2003). De Man et al. (1992) studied a variety of hard fats and found that palm hard fat is the most stable β' form. Melting endotherms in the DSC scan represents polymorphic forms. These morphologies directly influence the important properties of that fat such as melting point, solid fat content, plasticity and brittleness (Bushfield and Prochog, 1990). Modified RBO had lower melting point with more assymetrical distribution of fatty acids as compared to cocoa butter and vanaspati. Mixture of TAGs containing different fatty acids have lower melting point (Hagenamm, 1988). Modified RBO's showed broad melting range like vanaspati, though showed lower SFC at all temperatures especially at ambient temperature. However they are entirely different in melting behaviour compared to cocoa butter. These results revealed that modified RBO could be used as plastic fat for culinary purpose, in traditional confectionery and bakery in place of hydrogenated fats and these are nutritionally superior as they do not contain any *trans* fatty acids. In addition to this, a process was developed for shortening fat without *trans* fatty acids which is non-atherogenic and rich in bioactive phytochemicals. The product should also be capable of replacing the vanaspati widely used as shortening in India.

The current finding suggests that RBO may retain many of its health benefits after acidolysis of RBO and stearic acid. Stearic acid can be successfully incorporated into RBO and the modified product can be a good substitute for hydrogenated fats because the solid fat content of modified RBO was increased after acidolysis reaction.

# CHAPTER - IV

# ENRICHMENT OF RBO WITH n-3 PUFA BY ENZYMATIC ACIDOLYSIS: OPTIMIZATION OF PARAMETERS BY RSM

Introduction

Structured lipids are an important avenue for improving nutritional, therapeutic and industrial value of oils and fats. These are modified triacylglycerols with altered fatty acid composition or location in the glycerol backbone. Improvements or changes in physical and/or chemical characteristics of triacylglycerols are also achieved when the structured lipids are synthesized. Chemical interesterification using sodium methoxide is a less expensive method for the production of structured lipids, which is easy to scale up. However, such reaction lacks specificity and offer little or no control over the positional distribution of fatty acids in the final product (Lee and Akoh, 1998). Enzymatic interesterification is another method, which is used to produce fats with desirable physical and functional properties for food applications. This method uses lipase enzyme to modify vegetable oils for various purposes such as improving nutritional quality, preparation of low calorie fats and also as an alternative to hydrogenation of vegetable oils to improve their physico-chemical properties (Xu et al, 2000). Depending upon the type of substrates, chemical or enzymatic reactions can be used for synthesis of modified lipids, including direct esterification (reaction of fatty acid and glycerol), reactions between two TAG molecules (transesterfication), acidolysis (transfer of acyl group between an acid and ester), and alcoholysis (exchange of alkoxy group between an alcohol and ester) (Osborn and Akoh, 2002). Enzymatic acidolysis reactions have been employed by many investigators for the modification of fatty acid composition of vegetable oils to synthesize structured lipids (Hamam et al, 2005b; Xu et al, 2000; Reena et al, 2001). A number of studies have been focused on obtaining TAG having a combination of medium chain fatty acids and PUFA (Lee and Akoh, 1996).

Rice bran is a by-product of rice milling industry. Rice bran oil (RBO) obtained from rice bran is a rich source of tocopherols, tocotrienols and γ- oryzanol (Shin et al, 1997). γ- Oryzanol has been reported to possess the hypocholesterolemic and

antioxidant activity (Sugano and Tsuji, 1997; Seetharamaiah and Chandrasekhara, 1989). Studies have demonstrated that the beneficial effect of natural antioxidants present in RBO, when RBO was used with other edible oils in a 50: 50 blend. (Samia and Karl, 2007). RBO contains approximately 38% oleic acid, 34% linoleic acid and 18.6 % palmitic acid (Rukmini and Raghuram, 1991), however, it contains negligible amount of α- linolenic acid (18:3; n-3).

Clinical and epidemiological studies have shown the cardiovascular protective effects of fish oil and n-3 PUFA due to EPA and DHA. Dietary supplementation of these fatty acids has provided evidence of their cardiovascular protective effects (Siddiqui *et al*, 2007). The present Western and Asian diet is very high in n-6 fatty acids (n-6 to n-3 fatty acids ratio is 20-30:1) (Ghafoorunissa, 1996) because of extentsive use of oils rich in n-6 fatty acids. The ratio of n-6 to n-3 fatty acids is often used to assess the balance between essential fatty acids in the diet. The WHO/ FAO suggests a ratio of 5:1- 10:1 (Bang and Dyerberg, 1972), whereas on the basis of proposed adequate intakes the NIH suggests a ratio of 2:3- 3:1 (Bang and Dyerberg, 1986). The acceration of higher-chain n-3 fatty acids in tissue phospholipids may be dependent on the particular n-3 fatty acid that is provided in the diet (Simopolous et al, 1999). Studies on fatty acid intake among Indians showed that linoleic acid requirements are fully met owing to their high levels in cereals, millets and vegetable oils, but α- linolenic acid intake is low and therefore the n-6/ n- 3 ratio is high. Only a part of α- linolenic acid requirement in human body may be met from the intake of cereals, millets and vegetable oils. As it is not synthesized in our body, it is necessary to increase dietary intake of n-3 fatty acid. Additional data on fatty acid profile of plasma and platelet phospholipids in apparently normal Indian subjects suggested the need to improve the n-3 fatty acid nutritional status in the country (WHO and FAO, 1995). As mentioned earlier one of the major objective of this study is to prepare value added products from RBO so that utilization of RBO is increased. In this

respect preparation of structured lipids with RBO enriched with n-3 PUFA is attempted and so that this new product can provide adequate amounts of n-3 PUFA through an edible oil.

Several factors (variables) influence lipase catalyzed interesterification and these include incubation time, temperature, substrates molar ratio and enzyme concentration. Each of these factors can be individually altered by holding the other parameters constant for optimizing acidolysis reactions. In general, optimization of a process could be achieved by either traditional or statistical methods; the former having limitation towards complete optimization. The traditional one-factor-at-a-time approach to process optimization is time consuming and does not take into account the interaction of the parameters. However, the actual response of the process results from the interactive influence of the various variables. Response surface methodology (RSM) uses mathematical and statistical designs for optimizing reaction conditions. It is used for modeling and analysis of response variables, which are influenced by multiple independent factors (reaction conditions), the interactions between them, and optimizes them in terms of the response variables. Based on the effect of factors on the response variables in the range designed, a response surface that contains the optimal point(s) for the reaction can be obtained. RSM was used to optimize the reaction conditions for lipase-catalyzed incorporation of n-3 fatty acids into rice bran oil. In RSM, both mathematical and statistical techniques were used together in the modeling and analysis of situations in which a response is affected by several variables, alone or in combination. RSM also enables the behavior of different parameters to be predicted under a given set of conditions and provide sufficient information for statistically acceptable results with a reduced number of experiments; therefore, by choosing the appropriate experimental design, time, cost, wastage and rework during production can be reduced.

Such an approach was followed in the classical single factor experimental designs (Shimada et al, 1996a; 2000; Mu et al, 1998). In the present study, a similar approach was attempted for the synthesis of SL from RBO rich in 18:3 fatty acid. However when the experiments were performed in order to enrich RBO with n-3 PUFA under the conditions optimized for stearic acid, variable incorporation rate was observed. This underscored the need for optimizing reaction conditions for incorporation of n-3 PUFA into RBO. However this involves a large number of experiments. This can overcome by using alternate systems. A suitable statistically design can be used for arriving at optimum conditions for reactions by which, the effect of multiple variables can be checked simultaneously with a minimum number of experiments (Huang and Akoh, 1996; Xu et al, 1999; 2000). Response surface methodology is one such design, which can be used for optimizing enzymatic reactions with minimum number of experiments.

Several investigators have used RSM to optimize reaction conditions for enzymatic reaction in organic solvents. Huang and Akoh (1996) optimized enzymatic interesterification reactions using RSM for the incorporation of pure ethyl caprylate into soybean oil and sunflower oil. In this model, incubation time, molar ratio of ethyl caprylate to total TAG and long chain TAG were assumed to be the most important factors affecting reaction. Similarly RSM was used to elucidate acyl migration occurring during enzymatic interesterification between rapeseed oil and capric acid in a solvent free media (Xu et al, 1998b). Five factors, viz. water content, reaction temperature, enzyme load, reaction time and substrates ratio were varied at three levels. RSM was also used to modify menhaden oil to incorporate caprylic acid while conserving the PUFA in the TAG. In this case, parameters such as residence time, substrates molar ratio and reaction temperature were optimized using RSM. All parameters had a positive influence on the incorporation of caprylic acid but residence time and substrates molar ratio had a negative effect on the retention of PUFA (Xu et al, 2000).

In the present study, RSM was used to optimize the conditions for incorporation of n-3 PUFA into RBO by enzymatic acidolysis to the extent such that the ratios of saturated fatty acids to polyunsaturated acids, monounsaturated fatty acids to polyunsaturated fatty acids as well as n-6 to n-3 fatty acids are maintained to most nutritional advantage.

The objectives of the present study are:

(a) to enrich rice bran oil with $\alpha$-linolenic acid (ALA) (n-3 PUFA) obtained from linseed oil (LSO) by using immobilized lipase from *Rhizomucor miehei*, and study the effect of different factors such as incubation time, temperature, enzyme concentration and substrates ratio on the incorporation of ALA, ratios of saturated fatty acids to polyunsaturated fatty acids (SFA/PUFA), monounsaturated fatty acids to polyunsaturated fatty acids (MUFA/PUFA) and n-6 to n-3 fatty acids and to optimize the reaction conditions using RSM.

(b) to enrich RBO with EPA+DHA (long chain n-3 PUFA) obtained from cod liver oil (CLO) by using immobilized lipase from *Rhizomucor miehei*, and study the effect of different factors such as incubation time, temperature, enzyme concentration and substrates ratio on the incorporation of EPA+DHA into RBO using RSM.

**Experimental**

**Preparation of free fatty acid concentrate from LSO and free fatty acids (FFA) from CLO**

FFAs were prepared from LSO and CLO as described in chapter II. Fatty acid concentrate from LSO and CLO rich in ALA and EPA+DHA respectively were used for acidolysis reaction to enrich RBO with ALA and EPA+DHA respectively.

**Acidolysis reaction between RBO and FFA obtained from LSO and CLO**

The enzymatic acidolysis reaction mixture consisting of 4 mL of hexane, ALA obtained from linseed oil by urea complexation and RBO was incubated for a certain

temperature, time, enzyme concentration and substrates ratio as per the experimental design. The molecular weights of RBO and α- linolenic acid were taken as 893.5 and 278.5, respectively. The molar ratio of 1: 1 meant RBO and LSO concentrate were 893.5 and 278.5 mg respectively and reactions were carried out as described earlier. Similarly, the reactions were carried out with RBO and CLO FFAs and their molecular weights taken were 893.5 and 319.0 respectively. The molar ratio of 1: 1 meant rice bran oil and cod liver oil FFAs were 893.5 and 319.0 mg, respectively. All incubations and purification of the products were carried out as described in chapter II.

**Experimental design for RSM**

RSM was used to optimize the reaction conditions for lipase-catalyzed incorporation of n-3 PUFA into RBO. A CCRD with four variables was used to study the response pattern and to determine the optimum combination of variables as described earlier in chapter II.

**Hydrolysis of RBO by pancreatic lipase**

Pancreatic lipase hydrolysis was used to determine the fatty acids at *sn-2* position in triacyleglycerol as described in chapter II.

**Fatty acid composition by gas chromatography**

The fatty acid composition of substrates and reaction products were analyzed as described earlier by gas chromatography.

## Results

### (a) Synthesis of SL from RBO enriched with ALA

The TAG of native RBO contains mainly oleic acid (43%) and linoleic acid (33%) as major fatty acids followed by palmitic acid (21%) and stearic acid (2%). Fatty acid composition of the FFA obtained by the hydrolysis of LSO is given in table 4.1. The FFA obtained from LSO contained 88 % of ALA (n-3 PUFA). When RBO TAGs were subjected to acidolysis with fatty acids from LSO in the presence of lipase, the resulting structured lipids were enriched with n-3 PUFA (ALA).

**Table 4.1: Fatty acid composition of LSO and its fatty acid concentrate**

| Fatty Acids | Linseed oil | Fatty acid concentrate |
|---|---|---|
| | (%) | |
| 16:0 | 6.5 | - |
| 18:0 | 4.4 | - |
| 18:1 | 22.6 | 2.0 |
| 18:2 | 10.0 | 10.0 |
| 18:3 | 54.6 | 88.0 |

Values are average of triplicate determination



### Optimization of reaction conditions for SL with RBO rich in ALA

Acidolysis reactions were carried out between RBO and FFA obtained from LSO. Incubation time, temperature, substrates molar ratio (TAG: FFA) and enzyme concentration were selected as the factors affecting the synthesis of SL rich in ALA. The levels of 4 experimental variables were used to determine the incorporation of ALA into RBO is given in table 4.2. A total of 31 experiments were conducted under different reaction conditions as given in table 4.3. The fatty acid composition of modified RBO with ALA in all the 31 experiments are given in table 4.4. It also gives the ratios of SFA/PUFA, MUFA/PUFA and n-6/n-3.

***Table 4.2: Experimental variables and their levels for CCRD used in the incorporation of ALA into RBO***

| *Variables* | Symbols | | Levels | | | | |
|---|---|---|---|---|---|---|---|
| | Coded | Uncoded | -2.0 | -1.0 | 0 | 1.0 | 2.0 |
| Time of reaction (h) | $X_1$ | $x_1$ | 1 | 4.5 | 8 | 11.5 | 15 |
| Temperature of reaction (°C) | $X_2$ | $x_2$ | 25 | 31.25 | 37.5 | 43.75 | 50 |
| Enzyme concentration (%) | $X_3$ | $x_3$ | 1 | 3.25 | 5.5 | 7.75 | 10 |
| Substrates molar ratio | $X_4$ | $x_4$ | 1 | 3.25 | 5.5 | 7.75 | 10 |

where $X_1 = (x_1 - 8)/3.5$; $X_2 = (x_2 - 37.5)/6.25$; $X_3 = (x_3 - 5.5)/2.25$; $X_4 = (x_4 - 5.5)/2.25$

**Table 4.3: Treatment schedule for 4-factor CCRD and the response for incorporation of ALA into RBO**

| Exp. No. | Time of reaction (h) $X_1$ | | Temperature of reaction (°C) $X_2$ | | Enzyme concentration (%) $X_3$ | | Substrates molar ratio $X_4$ | |
|---|---|---|---|---|---|---|---|---|
| | *Actual* | *Coded* | *Actual* | *Coded* | *Actual* | *Coded* | *Actual* | *Coded* |
| 1 | 4.50 | (-1) | 31.25 | (-1) | 3.25 | (-1) | 3.25 | (-1) |
| 2 | 11.50 | (1) | 31.25 | (-1) | 3.25 | (-1) | 3.25 | (-1) |
| 3 | 4.50 | (-1) | 43.75 | (1) | 3.25 | (-1) | 3.25 | (-1) |
| 4 | 11.50 | (1) | 43.75 | (1) | 3.25 | (-1) | 3.25 | (-1) |
| 5 | 4.50 | (-1) | 31.25 | (-1) | 7.75 | (1) | 3.25 | (-1) |
| 6 | 11.50 | (1) | 31.25 | (-1) | 7.75 | (1) | 3.25 | (-1) |
| 7 | 4.50 | (-1) | 43.75 | (1) | 7.75 | (1) | 3.25 | (-1) |
| 8 | 11.50 | (1) | 43.75 | (1) | 7.75 | (1) | 3.25 | (-1) |
| 9 | 4.50 | (-1) | 31.25 | (-1) | 3.25 | (-1) | 7.75 | (1) |
| 10 | 11.50 | (1) | 31.25 | (-1) | 3.25 | (-1) | 7.75 | (1) |
| 11 | 4.50 | (-1) | 43.75 | (1) | 3.25 | (-1) | 7.75 | (1) |
| 12 | 11.50 | (1) | 43.75 | (1) | 3.25 | (-1) | 7.75 | (1) |
| 13 | 4.50 | (-1) | 31.25 | (-1) | 7.75 | (1) | 7.75 | (1) |
| 14 | 11.50 | (1) | 31.25 | (-1) | 7.75 | (1) | 7.75 | (1) |
| 15 | 4.50 | (-1) | 43.75 | (1) | 7.75 | (1) | 7.75 | (1) |
| 16 | 11.50 | (1) | 43.75 | (1) | 7.75 | (1) | 7.75 | (1) |
| 17 | 1.00 | (-2) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 18 | 15.00 | (2) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 19 | 8.00 | (0) | 25.00 | (-2) | 5.50 | (0) | 5.50 | (0) |
| 20 | 8.00 | (0) | 50.00 | (2) | 5.50 | (0) | 5.50 | (0) |
| 21 | 8.00 | (0) | 37.50 | (0) | 1.00 | (-2) | 5.50 | (0) |
| 22 | 8.00 | (0) | 37.50 | (0) | 10.00 | (2) | 5.50 | (0) |
| 23 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 1.00 | (-2) |
| 24 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 10.00 | (2) |
| 25 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 26 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 27 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 28 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 29 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 30 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |
| 31 | 8.00 | (0) | 37.50 | (0) | 5.50 | (0) | 5.50 | (0) |

**Table 4.4: Composition of various fatty acids and their ratios after incorporation of ALA into RBO**

| Exp. No. | Saturated fatty acids (SFA) | | Monoun-saturated fatty acids (MUFA) | Polyunsaturated fatty acids (PUFA) | | SFA/ PUFA | MUFA/ PUFA | n-6/ n-3 |
|---|---|---|---|---|---|---|---|---|
| | 16:0 | 18:0 | 18:1 | 18:2 (n-6) | 18:3 (n-3) | | | |
| (1) | (2) | (3) | (4) | (5) | (6) | (2+3)/ (5+6) | (4)/(5+6) | (5)/(6) |
| 1 | 17.6 | 2.1 | 30.6 | 26.2 | 23.8 | 0.39 | 0.61 | 1.10 |
| 2 | 15.5 | 1.7 | 30.2 | 25.7 | 26.9 | 0.33 | 0.57 | 0.96 |
| 3 | 12.4 | 2.4 | 34.1 | 25.2 | 25.9 | 0.29 | 0.67 | 0.97 |
| 4 | 12.1 | 1.9 | 30.0 | 25.7 | 30.5 | 0.25 | 0.53 | 0.84 |
| 5 | 14.5 | 1.9 | 25.7 | 22.6 | 36.0 | 0.28 | 0.44 | 0.63 |
| 6 | 12.5 | 1.3 | 22.0 | 22.1 | 42.1 | 0.21 | 0.34 | 0.52 |
| 7 | 9.9 | 1.3 | 23.7 | 22.4 | 42.7 | 0.17 | 0.36 | 0.52 |
| 8 | 10.0 | 1.4 | 21.6 | 21.8 | 45.3 | 0.17 | 0.32 | 0.48 |
| 9 | 14.6 | 2.2 | 28.8 | 23.6 | 30.8 | 0.31 | 0.53 | 0.77 |
| 10 | 14.5 | 2.2 | 29.9 | 24.6 | 29.1 | 0.31 | 0.56 | 0.85 |
| 11 | 14.7 | 1.5 | 28.4 | 23.8 | 31.3 | 0.29 | 0.52 | 0.76 |
| 12 | 11.2 | 1.7 | 27.0 | 24.5 | 35.7 | 0.21 | 0.45 | 0.69 |
| 13 | 12.7 | 1.6 | 24.0 | 23.3 | 38.4 | 0.23 | 0.39 | 0.61 |
| 14 | 11.2 | 1.8 | 24.4 | 23.3 | 39.2 | 0.21 | 0.39 | 0.59 |
| 15 | 11.5 | 1.4 | 21.9 | 22.1 | 43.2 | 0.20 | 0.34 | 0.51 |
| 16 | 8.9 | 1.3 | 18.8 | 21.8 | 49.2 | 0.14 | 0.26 | 0.44 |
| 17 | 15.0 | 2.1 | 29.7 | 22.6 | 30.9 | 0.32 | 0.56 | 0.73 |
| 18 | 11.0 | 2.6 | 24.5 | 22.7 | 39.2 | 0.22 | 0.40 | 0.58 |
| 19 | 15.5 | 2.8 | 30.1 | 22.7 | 28.9 | 0.35 | 0.58 | 0.79 |
| 20 | 12.6 | 1.7 | 27.2 | 23.4 | 35.9 | 0.24 | 0.46 | 0.65 |
| 21 | 16.5 | 2.8 | 35.8 | 24.7 | 19.8 | 0.47 | 0.85 | 1.25 |
| 22 | 10.4 | 2.1 | 21.2 | 21.1 | 45.2 | 0.19 | 0.32 | 0.47 |
| 23 | 15.1 | 2.0 | 30.6 | 21.9 | 30.7 | 0.33 | 0.58 | 0.71 |
| 24 | 13.1 | 2.0 | 25.1 | 21.5 | 38.3 | 0.25 | 0.42 | 0.56 |
| 25 | 13.0 | 2.0 | 27.1 | 22.9 | 35.1 | 0.26 | 0.47 | 0.65 |
| 26 | 12.4 | 2.2 | 26.3 | 23.0 | 35.6 | 0.25 | 0.45 | 0.65 |
| 27 | 13.7 | 2.0 | 27.3 | 21.8 | 35.1 | 0.28 | 0.48 | 0.62 |
| 28 | 13.6 | 2.0 | 27.5 | 22.1 | 35.0 | 0.27 | 0.48 | 0.63 |
| 29 | 14.6 | 1.9 | 29.1 | 21.0 | 33.9 | 0.30 | 0.53 | 0.62 |
| 30 | 14.1 | 2.1 | 28.4 | 22.1 | 34.9 | 0.28 | 0.50 | 0.63 |
| 31 | 13.2 | 1.9 | 27.8 | 22.1 | 35.1 | 0.26 | 0.49 | 0.63 |

**Diagnostic checking of models**

Four responses, namely, percentage incorporation ($Y_1$), ratio of saturated fatty acid to polyunsaturated fatty acid (SFA/PUFA, $Y_2$), ratio of monounsaturated fatty acids to polyunsaturated fatty acids (MUFA/PUFA, $Y_3$) and ratio of n-6 to n-3 fatty acids ($Y_4$), which describe the quality characteristics of the structured lipid, were measured. The coefficients for the actual functional relations for predicting responses ($Y_i$) are presented in table 4.5. The insignificant terms were omitted based on Student's t-ratio (Giovanni, 1983). The responses under different combinations as defined in the design (Tables 4.3) were analyzed using the analysis of variance (ANOVA) appropriate to the experimental design. The ANOVA for the data obtained using CCRD is presented in table 4.6.

**Response surface plotting**

The effect of reaction time, temperature, enzyme concentration and substrates ratio on percentage incorporation of n-3 fatty acid, ratios of saturated fatty acids to polyunsaturated fatty acids (SFA/PUFA), monounsaturated fatty acids to polyunsaturated fatty acids (MUFA/PUFA) and n-6 to n-3 fatty acids reported by the coefficients of second order polynomials were given in table 4.5. Based on the observation of the data as well as the individual responses optimized, the time and temperature of reaction were kept at 4.5 h (coded value -1.0) and 37.5 $^0$C (coded value 0) respectively. A few response surfaces based on these coefficients are shown in figure 4.1 to 4.4, while two variables were kept at the pre-selected levels (time and temperature of the reaction are kept constant at 4.5 h and 37.5 $^o$C, respectively) and varying the other two within the experimental range and their affects on n-3 fatty acid (ALA) incorporation, on SFA/PUFA ratio, on MUFA/PUFA and on n-6 to n-3 fatty acids ratio was studied. In general, exploration of the response surfaces indicated a complex interaction between the variables.

**Table 4.5: Estimated coefficients for the fitted second order polynomial representing the relationship between the different responses and process variables for incorporation of ALA into RBO**

| | Incorporation of ALA into RBO (%) | SFA/PUFA | MUFA/PUFA | n-6/n-3 |
|---|---|---|---|---|
| | ($Y_1$) | ($Y_2$) | ($Y_3$) | ($Y_4$) |
| $a_0$ | 34.957[a] | 0.272[a] | 0.485[a] | 0.633[a] |
| $a_1$ | 1.771[a] | -0.022[b] | -0.031[b] | -0.033[a] |
| $a_2$ | 2.146[a] | -0.032[a] | -0.026[b] | -0.044[a] |
| $a_3$ | 6.421[a] | -0.056[a] | -0.111[a] | -0.174[a] |
| $a_4$ | 1.621[a] | -0.014[c] | -0.031[b] | -0.047[a] |
| $a_{11}$ | 0.372[ns] | -0.008[ns] | -0.013[ns] | 0.003[ns] |
| $a_{22}$ | -0.290[ns] | -0.001[ns] | -0.002[ns] | 0.019[b] |
| $a_{33}$ | -0.340[ns] | 0.007[ns] | 0.014[ns] | 0.054[c] |
| $a_{44}$ | 0.235[ns] | -0.004[ns] | -0.007[ns] | -0.001[ns] |
| $A_{12}$ | 0.581[ns] | -0.001[ns] | -0.013[ns] | -0.008[ns] |
| $A_{13}$ | 0.319[ns] | 0.003[ns] | 0.000[ns] | 0.003[c] |
| $A_{14}$ | -0.431[ns] | 0.001[ns] | 0.013[ns] | 0.022[ns] |
| $A_{23}$ | 0.744[c] | 0.003[ns] | -0.010[ns] | 0.001[b] |
| $A_{24}$ | 0.394[ns] | 0.008[ns] | -0.014[ns] | -0.001[ns] |
| $A_{34}$ | -0.994[c] | 0.005[ns] | 0.016[ns] | 0.051[a] |

[a] Significant at 0.1%, [b] Significant at 1.0%, [c] Significant at 5.0%

[ns] Not significant at 5.0 % level

**Table 4.6: Analysis of variance for the fitted second order polynomial model and lack of fit for different parameters as per CCRD**

| Source of variation | Df | Sum of squares | | | |
|---|---|---|---|---|---|
| | | % incorporation of ALA into RBO | SFA/PUFA | MUFA/PUFA | n-6/n-3 |
| **Regression** | | | | | |
| First order terms | 4 | 1238.27[a] | 0.12 [a] | 0.36 [a] | 0.86 [a] |
| Second order terms | 10 | 49.74 [a] | 0.01 [a] | 0.03 [a] | 0.14 [a] |
| **Total** | 14 | 1288.01 | 0.12 | 0.38 | 1.00 |
| **Residual** | | | | | |
| **Lack of fit** | 10 | 43.03 [ns] | 0.02 [ns] | 0.04 [ns] | $2.06 \times 10^{-2}$ [a] |
| **Pure error** | 6 | 1.60 | 0.00 | 0.00 | $0.89 \times 10^{-3}$ |
| Total error | 16 | 44.63 | 0.02 | 0.04 | $2.15 \times 10^{-2}$ |
| **Grand Total** | 30 | 1332.63 | 0.14 | 0.42 | 1.02 |
| Coefficient of determination ($R^2$) | | 0.97 | 0.86 | 0.91 | 0.98 |

[a] Significant at 5% level, [ns] Not significant

**Effect of enzyme concentration and substrates ratio on incorporation of ALA into RBO**

The percentage incorporation of ALA into RBO was found to be a function of the linear effect of enzyme concentration. The linear effect ($p \leq 0.001$) was positive, which resulted in an increase in percentage incorporation of n-3 fatty acid with an increase in the levels of enzyme concentration (Figure 4.1). The substrates ratio has a prominent

effect on percentage incorporation of ALA and its linear effect was also positive ($p \leq 0.001$), which results in an increase in percentage incorporation of ALA with the substrates ratio for all the levels of enzyme concentration. It is interesting to note that the interaction between the enzyme concentration and substrates ratio was also significant (Figure 4.1). For all the values of enzyme concentration (1.0 - 10.0 %, coded values from -2.0 to +2.0), the percentage incorporation of ALA increased with increase in substrates ratio. At lowest level of substrates ratio (1, coded value -2.0), the percentage incorporation of ALA increased with an increase in enzyme concentration, whereas at the highest level of substrates ratio (10, coded value +2.0) the percentage incorporation of ALA was almost constant with an increase in substrates ratio (Figure 4.1). The maximum percentage incorporation of ALA (49.2%) was obtained for the 7.75 % enzyme concentration and 7.75 molar substrates ratio.

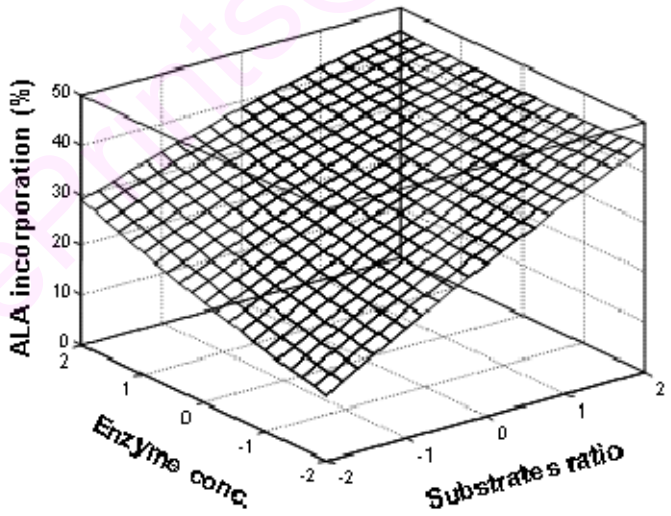


**Figure 4.1: Surface plot showing the effect of enzyme concentration and substrates molar ratio on n-3 PUFA (ALA) incorporation into RBO**

**Effect of enzyme concentration and substrates molar ratio on ratio of saturated fatty acid to polyunsaturated fatty acid (SFA/PUFA)**

The linear effect of enzyme concentration was negatively related ($p \leq 0.001$) with SFA/PUFA ratio and the quadratic effect was not significant, which resulted in decrease in SFA/PUFA ratio with increase in values of enzyme concentration (Figure 4.2). The substrates ratio has a marked effect on SFA/PUFA ratio; its linear effect was negative ($p \leq 0.05$), which resulted in decrease in SFA/PUFA ratio with an increase in the substrates ratio for all the levels of enzyme concentration (1.0 to 10.0%, coded value from -2.0 to +2.0. The interaction term of enzyme concentration and substrates ratio was found to be insignificant (Table 4.6). The decrease in SFA/PUFA ratio was higher at lower levels of substrates ratio (1, coded level -2.0) as compared to higher levels of substrates ratio (10, coded level +2.0). The highest SFA/PUFA ratio (0.46) was observed for enzyme concentration of 1.0% (coded value -2.0) and substrates ratio of 1.0 (coded value -2.0) (Figure 4.2.).

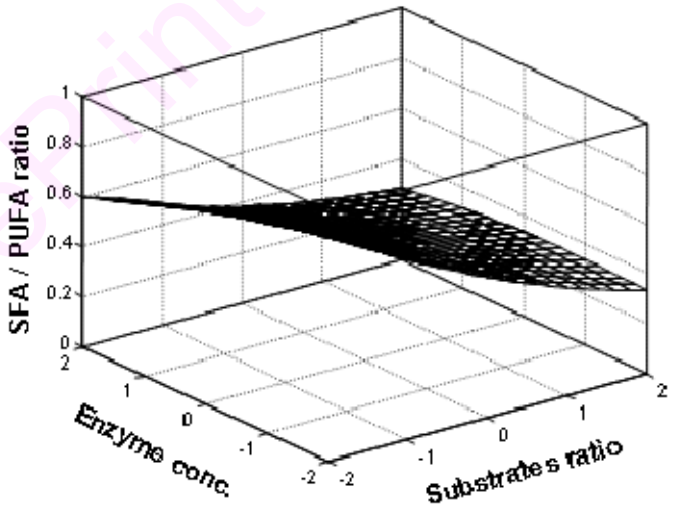


**Figure 4.2: Surface plot showing the effect of enzyme concentration and substrates molar ratio on SFA/PUFA ratio of modified RBO with ALA**

**Effect of enzyme concentration and substrates ratio on ratio of monounsaturated fatty acids to polyunsaturated fatty acids (MUFA/PUFA)**

For all the levels of enzyme concentration (1.0 to 10.0%, coded value from -2.0 to +2.0), the MUFA/PUFA ratio decreased considerably with increase in substrates ratio (Figure 4.3). This effect is due to presence of negative linear term ($p \leq 0.01$) of substrates ratio (Table 4.5). Similarly, for all the levels of substrates ratio (1.0 to 10.0, coded value from -2.0 to +2.0), the MUFA/PUFA ratio decreased with an increase in enzyme concentration, due to the presence of significant linear term of enzyme concentration ($p \leq 0.001$, Table 4.6). Enzyme concentration of 1.0% (coded value -2.0) and substrates ratio of 1.0 (coded value -2.0) resulted in highest MUFA/PUFA ratio (0.96, Figure 4. 3).

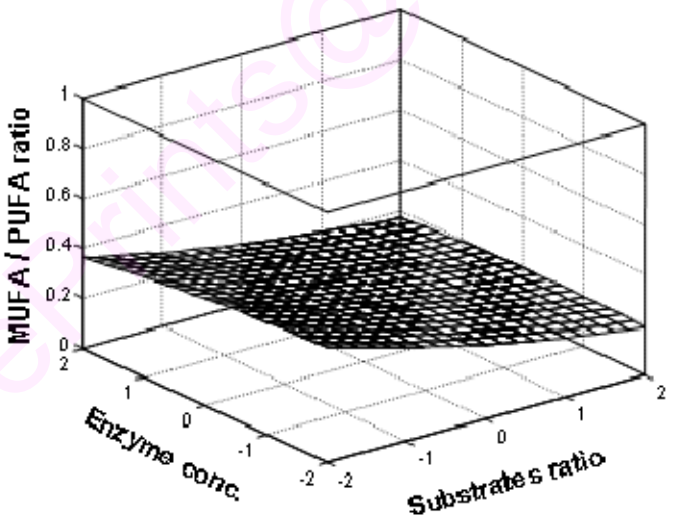


**Figure 4.3: Surface plot showing the effect of enzyme concentration and substrates molar ratio on MUFA/PUFA ratio of modified RBO with ALA**

**Effect of enzyme concentration and substrates molar ratio on the ratio of n-6 to n-3 fatty acids**

The n-6 to n-3 ratio was also found to be dependent on the substrates ratio as its negative linear was significant ($p \leq 0.001$) and its quadratic effect was insignificant (Table 4.6, Figure 4.4). For all the levels of enzyme concentration (1.0-10.0%, coded value -2.0 to +2.0), the n-6 to n-3 ratio increased with an increase in the substrates ratio. The n-6 to n-3 ratio was also found to be a function of the linear and quadratic effects of enzyme concentration. The linear effect ($p \leq 0.001$) is negative, whereas the quadratic effect ($p \leq 0.05$) is positive (Table 4.5). Moreover, the positive interaction parameter between substrates ratio and enzyme concentration was significant ($p \leq 0.001$), which results in the decrease in n-6 to n-3 ratio for lower substrates ratio (1, coded level -2.0), whereas at higher substrates ratio (10, coded level +2.0) the n-6 to n-3 ratio increased with an increase in enzyme concentration (Figure 4.4). The highest value of n-6 to n-3 ratio (0.65) was obtained for lowest enzyme concentration (1.0%, coded value +1.68) and lowest substrates ratio (1.0, coded value -2.0).

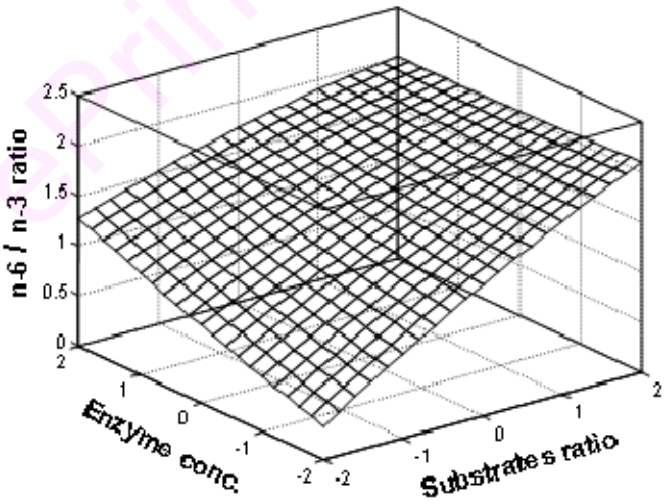


**Figure 4.4: Surface plot showing the effect of enzyme concentration and substrates molar ratio on n-6/n-3 fatty acids ratio of modified RBO with ALA**

**Optimization**

In order to deduce workable optimum conditions, the graphical optimization technique was adopted by fixing two variables, that is, initial reaction time and temperature 4.5 h (coded value -1.0) and 37.5 $^{0}$C (coded value 0) respectively as pre-determined optimum conditions. This drastically reduces the amount of time required for investigation of multifactor and the multi-response systems. It also provides comprehensive and informative insight of the system which leads to process optimization rapidly. The specifications necessary for each response were first set and these also served as constraints for optimization (Cochran and Cox, 1957). An acceptable compromise was made following the criteria for the percentage incorporation of ALA ≤ 19.8%, ratio of saturated fatty acid to polyunsaturated fatty acid (SFA/PUFA) ≥ 0.42, ratio of monounsaturated fatty acid to polyunsaturated fatty acid (MUFA/PUFA) ≥ 0.8, ratio of n-6 to n-3 ≥ 0.65. The contour plots for the response were generated as shown in figure 4.5 to 4.8. The contour plots were superimposed and the regions that best satisfies all the constraints were selected as the optimum conditions. Superimposed contour plots for each response are shown in figures 4.5 to 4.8. Incorporation of ALA was increased with increase in enzyme concentration at lower substrates ratio whereas at higher substrates ratio the incorporation was not increased much with the increase in enzyme concentration (Figure 4.5). At lower substrates ratio, the ratio of SFA/PUFA was decreased with an increase in enzyme concentration whereas at higher substrates ratio there was no change in SFA/PUFA ratio with the increase in enzyme concentration (Figure 4.6). At lowest substrates ratio there was increase in MUFA/PUFA ratio with an increase in enzyme concentration but at higher substrates ratio there no change observed in MUFA/PUFA ratio with increase in enzyme concentration (Figure 4.7). Ratio of n-6/n-3 was increased with the increase in enzyme concentration at lower substrates ratio but at higher substrates ratio there was not much increase observed in the n-6/n-3 ratio.

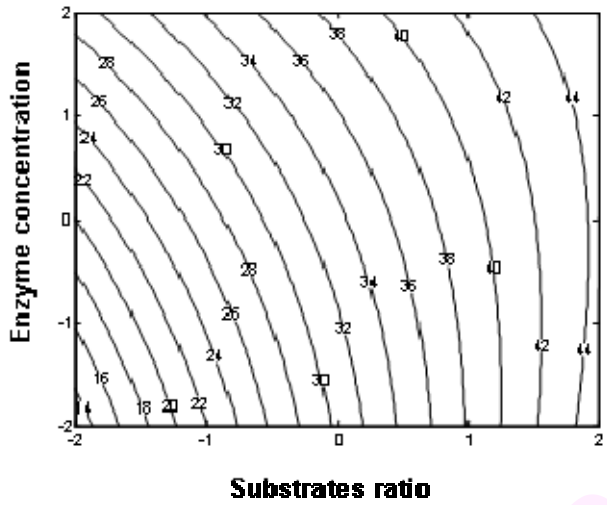


**Figure 4.5: Contour plots showing the effect of enzyme concentration and substrates ratio on incorporation of ALA into RBO**

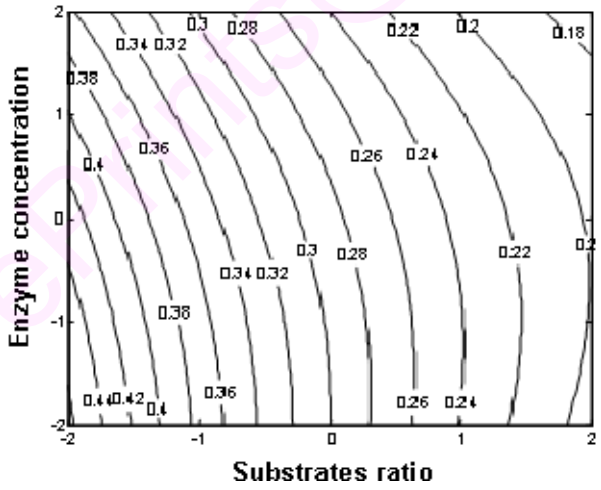


**Figure 4.6: Contour plots showing the effect of enzyme concentration and substrates ratio on ratio of SFA/PUFA of modified RBO**

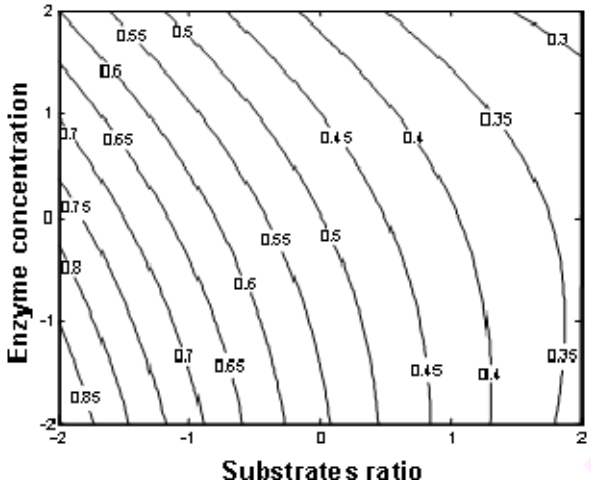


**Figure 4.7: Contour plots showing the effect of enzyme concentration and substrates ratio on ratio of MUFA/ PUFA of modified RBO**

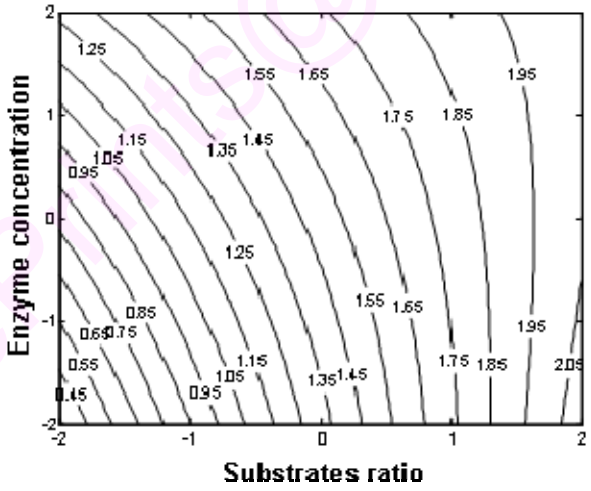


**Figure 4.8: Contour plots showing the effect of enzyme concentration and substrates ratio on ratio of n-6/n-3 of modified RBO**

A combination of optimum working conditions (A, B, C and D) can be selected from the shaded area and presented in figure 4.9. The overlapped area could be recommended as practical optimum zone for enzyme concentration (1.0 to 2.0%, coded level -2.0 to -1.60) and substrates ratio (1.0 to 1.9%, coded level -2.0 to -1.56) (Figure 4.9).

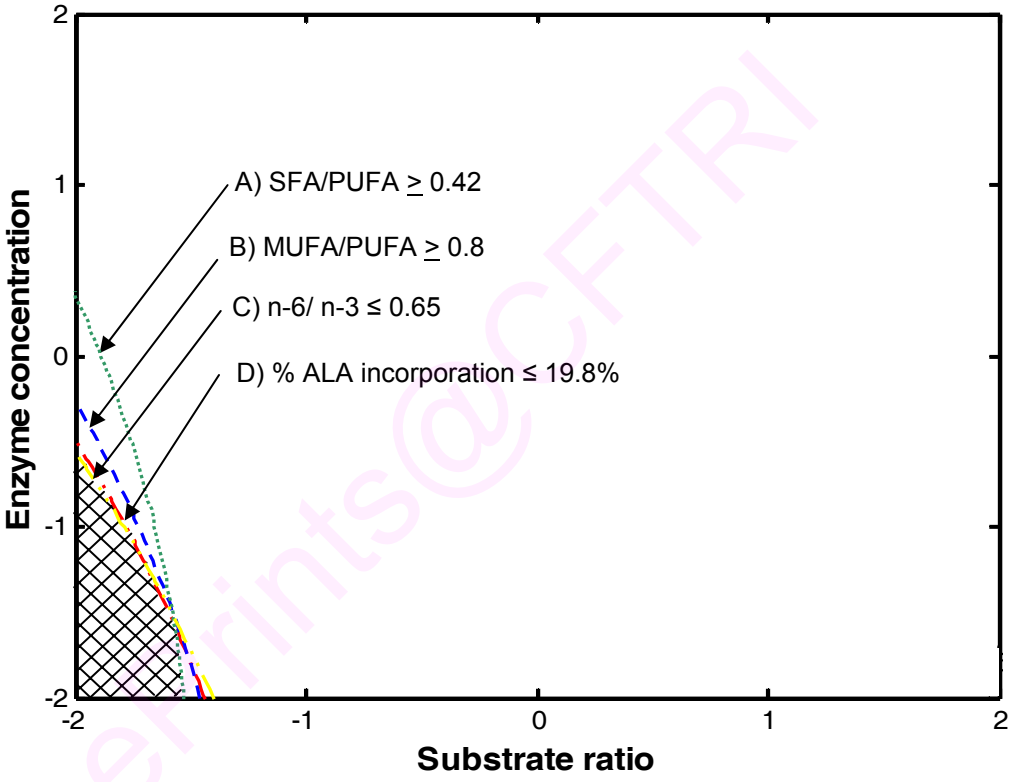




**Figure 4.9: Superimposed contour plots showing the shaded overlapping area for**

**(A) Ratio of saturated fatty acid to poly unsaturated fatty acid (SFA/PUFA) ≥ 0.42.**

**(B) Ratio of mono unsaturated fatty acid to poly unsaturated fatty acid (MUFA/PUFA) ≥ 0.8,**

**(C) Ratio of n-6/n-3 ≤ 0.65,**

**(D) Incorporation of ALA into RBO ≤19.8%.**

**Verification of results**

The suitability of the model equation for predicting the optimum response values were tested using the recommended optimum conditions. This set of conditions was determined to be optimum by a RSM optimization approach, which was also used to validate experimentally and predict the value of the responses using model equations. The experimental value was found to be in agreement with the predicted ones (Table 4.7).

**Table 4.7: Feasible and optimum conditions and predicted and experimental values of response at optimum conditions**

| Optimum conditions | Condition A | | Condition B | | Condition C | | Condition D | |
|---|---|---|---|---|---|---|---|---|
| | Coded levels | Actual levels | Coded levels | Actual levels | Coded levels | Actual levels | Coded levels | Actual levels |
| **Variables** | | | | | | | | |
| Time (h) | -1.0 | 4.5 | -1.0 | 4.5 | -1.0 | 4.5 | -1.0 | 4.5 |
| Temperature ($^0$C) | 0 | 37.5 | 0 | 37.5 | 0 | 37.5 | 0 | 37.5 |
| Substrates molar ratio | -2.00 | 1.00 | -1.60 | 1.90 | -1.53 | 2.06 | -2.00 | 1.00 |
| Enzyme conc. (%) | -0.53 | 4.30 | -1.56 | 2.00 | -2.00 | 1.00 | -2.00 | 1.00 |
| **Responses** | Pred. value | Exp. value[a] | Pred. value | Exp. value[a] | Pred. value | Exp. value[a] | Pred. value | Exp. value[a] |
| % incorporation of ALA | 17.91 | 17.50 | 17.83 | 17.60 | 17.22 | 17.15 | 12.85 | 13.00 |
| SFA/PUFA | 0.44 | 0.43 | 0.42 | 0.40 | 0.42 | 0.39 | 0.46 | 0.47 |
| MUFA/PUFA | 0.82 | 0.83 | 0.81 | 0.80 | 0.81 | 0.83 | 0.90 | 0.88 |
| Ratio of n-6 to n-3 | 1.33 | 1.32 | 1.32 | 1.30 | 1.35 | 1.38 | 1.57 | 1.60 |

[a] Mean value of five determinations, Conditions A, B, C and D have been indicated in figure 4.9

**Positional analyses of fatty acids of native and modified RBO with ALA**

After hydrolysis of native and modified RBO using pancreatic lipase, the fatty acid profile of purified monoacylglycerol indicated that about 15 % of 18:3 fatty acids is ALA (18:3) at *sn-2* position of modified RBO TAG (Table 4.8).

**Table 4.8: *Sn-2* fatty acid composition of native and modified RBO with ALA**

| Fatty acids | RBO | SL with LSO |
|---|---|---|
| | (%) | |
| 16:0 | nd | nd |
| 18:1 | 52.7 | 47.6 |
| 18:2 | 47.2 | 36.5 |
| 18:3 | nd | 15.0 |

Values are average of triplicate analysis

**(b) Synthesis of SL from RBO enriched with EPA+DHA from CLO**

Enzymatic acidolysis in hexane was employed to modify the fatty acid composition of RBO. Fatty acid analysis of unmodified RBO showed absence of long chain n-3 PUFA. When rice bran oil TAG were subjected to acidolysis with fatty acids from cod liver oil in presence of lipase, the resulting structured lipids were enriched in long chain n-3 PUFA (EPA +DHA). Different reaction conditions like time, temperature, enzyme concentration and substrates ratio were standardized using RSM to obtain the optimum incorporation of long chain n-3 PUFA. The values of 4 variables employed for RSM experiments are given in table 4.9. A total of 31 experiments were conducted to test as indicated in the treatment schedule and their predicted and observed EPA+DHA incorporation into RBO (Table 4.10). The optimum incorporation of long chain n-3 PUFA into RBO was about 10 %.

*Table 4.9: Experimental variables and their levels for CCRD used for the incorporation of EPA+DHA into RBO*

| *Variables* | Symbols | | Levels | | | | |
|---|---|---|---|---|---|---|---|
| | Coded | Uncoded | -2.0 | -1.0 | 0 | 1.0 | 2.0 |
| Time of reaction (h) | $X_1$ | $x_1$ | 4 | 15 | 26 | 37 | 48 |
| Temperature of reaction (°C) | $X_2$ | $x_2$ | 25 | 32.5 | 40 | 47.5 | 55 |
| Enzyme Concentration (%) | $X_3$ | $x_3$ | 1 | 3.25 | 5.5 | 7.75 | 10 |
| Substrates molar ratio | $X_4$ | $x_4$ | 1 | 3.25 | 5.5 | 7.75 | 10 |

where $X_1 = (x_1 - 26)/11$; $X_2 = (x_2 - 40)/7.5$; $X_3 = (x_3 - 5.5)/2.25$ and $X_4 = (x_4 - 5.5)/2.25$

**Table 4.10: Treatment schedule for 2-factor CCRD and response of different treatments and EPA+ DHA incorporation into RBO**

| Exp. No. | Time of reaction (h) | Temp. of reaction (°C) | Enzyme conc. (w/w) | Substrates ratio | Observed EPA+DHA incorporation (%) | Predicted EPA+DHA incorporation (%) |
|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | | |
| 1 | 15 | 32.5 | 3.25 | 3.25 | 2.40 | 2.71 |
| 2 | 37 | 32.5 | 3.25 | 3.25 | 1.90 | 2.08 |
| 3 | 15 | 47.5 | 3.25 | 3.25 | 3.30 | 3.96 |
| 4 | 37 | 47.5 | 3.25 | 3.25 | 3.80 | 4.73 |
| 5 | 15 | 32.5 | 7.75 | 3.25 | 3.60 | 3.87 |
| 6 | 37 | 32.5 | 7.75 | 3.25 | 3.70 | 4.22 |
| 7 | 15 | 47.5 | 7.75 | 3.25 | 2.20 | 2.23 |
| 8 | 37 | 47.5 | 7.75 | 3.25 | 4.30 | 3.99 |
| 9 | 15 | 32.5 | 3.25 | 7.75 | 4.00 | 5.08 |
| 10 | 37 | 32.5 | 3.25 | 7.75 | 4.20 | 5.07 |
| 11 | 15 | 47.5 | 3.25 | 7.75 | 2.80 | 3.18 |
| 12 | 37 | 47.5 | 3.25 | 7.75 | 4.10 | 4.59 |
| 13 | 15 | 32.5 | 7.75 | 7.75 | 9.20 | 9.17 |
| 14 | 37 | 32.5 | 7.75 | 7.75 | 10.05 | 10.16 |
| 15 | 15 | 47.5 | 7.75 | 7.75 | 3.80 | 4.39 |
| 16 | 37 | 47.5 | 7.75 | 7.75 | 6.20 | 6.79 |
| 17 | 4 | 40 | 5.5 | 5.5 | 5.00 | 4.19 |
| 18 | 48 | 40 | 5.5 | 5.5 | 6.80 | 5.94 |
| 19 | 26 | 25 | 5.5 | 5.5 | 6.10 | 5.28 |
| 20 | 26 | 55 | 5.5 | 5.5 | 4.00 | 3.15 |
| 21 | 26 | 40 | 1 | 5.5 | 6.10 | 4.49 |
| 22 | 26 | 40 | 10 | 5.5 | 7.90 | 7.84 |
| 23 | 26 | 40 | 5.5 | 1 | 1.50 | 1.04 |
| 24 | 26 | 40 | 5.5 | 10 | 7.40 | 6.19 |
| 25 | 26 | 40 | 5.5 | 5.5 | 6.15 | 6.76 |
| 26 | 26 | 40 | 5.5 | 5.5 | 6.55 | 6.76 |
| 27 | 26 | 40 | 5.5 | 5.5 | 6.55 | 6.76 |
| 28 | 26 | 40 | 5.5 | 5.5 | 7.30 | 6.76 |
| 29 | 26 | 40 | 5.5 | 5.5 | 7.25 | 6.76 |
| 30 | 26 | 40 | 5.5 | 5.5 | 6.60 | 6.76 |
| 31 | 26 | 40 | 5.5 | 5.5 | 6.95 | 6.76 |

**Diagnostic checking of the models**

Incubation temperature, time, substrates molar ratio and enzyme concentration were selected as the factors affecting the reaction. All four parameters were found to influence the incorporation of EPA+DHA into TAG. Fatty acid composition of RBO was changed with the different levels of incorporation of long chain n-3 PUFA (PUFA+DHA) into TAG (Table 4.10). Based on experimental results, the regression coefficient of the response surface model as given by equation were calculated. The coefficients for the actual functional relatives for predicting percentage incorporation of EPA+DHA are presented in table 4.11. The insignificant terms were omitted based on Student's t-ratio (Khuri and Cornell, 1987). The response under different combinations as defined in the design (described in materials and marthods) was analyzed using the analysis of variance (ANOVA) appropriate to the experimental design. The ANOVA for the data obtained using CCRD is presented in table 4.12.

**Response surface plotting**

The effect of reaction time, temperature, enzyme concentration and substrates molar ratio on percentage incorporation of EPA+DHA into RBO is reported by the coefficients of second order polynomials (Table 4.11). The response surfaces based on these coefficients are shown in figures 4.10 to 4.15 with three variables kept at optimum level and varying the other two within the experimental range. In general, exploration of the response surfaces indicated a complex interaction between the variables.

**Effect of various parameters like time, temperature, enzyme concentration and substrates molar ratio on incorporation of EPA+DHA into RBO**

Effect of time with the change in enzyme concentration, substrates molar ratio and temperature on the incorporation of EPA+DHA into RBO is given in figures 4.10 to 4.12. It was observed that there is increase in the incorporation of EPA+DHA with increase in enzyme concentration and substrates ratio (Figures 4.10 and 4.11).

**Table 4.11: Estimated coefficients for the fitted second order polynomial representing the relationship between the response and process variables.**

| | Estimated coefficients | Standard error | t-value |
|---|---|---|---|
| $A_0$ | 6.764 | 0.341 | 19.841 |
| $A_1$ | 0.440 [c] | 0.184 | 2.387 |
| $A_2$ | -0.531 [b] | 0.184 | -2.885 |
| $A_3$ | 0.840 [a] | 0.184 | 4.560 |
| $A_4$ | 1.290 [a] | 0.184 | 7.004 |
| $A_{11}$ | -0.425 | 0.169 | -2.519 |
| $A_{22}$ | -0.637 [b] | 0.169 | -3.779 |
| $A_{33}$ | -0.150 [ns] | 0.169 | -0.889 |
| $A_{44}$ | -0.787 [a] | 0.169 | -4.668 |
| $A_{12}$ | 0.353 [ns] | 0.225 | 1.566 |
| $A_{13}$ | 0.247 [ns] | 0.225 | 1.095 |
| $A_{14}$ | 0.159 [ns] | 0.225 | 0.707 |
| $A_{23}$ | -0.722 [b] | 0.225 | -3.201 |
| $A_{24}$ | -0.784 [b] | 0.225 | -3.478 |
| $A_{34}$ | 0.734 [b] | 0.225 | 3.257 |

[a] Significant at 0.1%, [b] Significant at 1.0%, [c] Significant at 5.0%

[ns] Not significant even at 5% level

**Table 4.12: Analysis of variance for the fitted second order polynomial model and lack of fit for incorporation of EPA+DHA as per CCRD**

| Source of variation | Df | % Incorporation | | |
|---|---|---|---|---|
| | | SS | MSS | F value |
| **Regression** | | | | |
| First order terms | 4 | 68.24 | 17.06 | 97.16 |
| Second order terms | 10 | 59.21 | 5.92 | 33.72 |
| **Total** | 14 | 127.45 | | |
| **Residual** | | | | |
| **Lack of fit** | 10 | 11.96 | 1.20 | 6.81 |
| **Pure error** | 6 | 1.05 | 0.18 | |
| Total error | 16 | 13.02 | | |
| **Grand total** | 30 | 140.47 | | |
| Coefficient of determination ($R^2$) = | | 0.907 | | |

SS = Sum of squares; MSS = Mean sum of squares

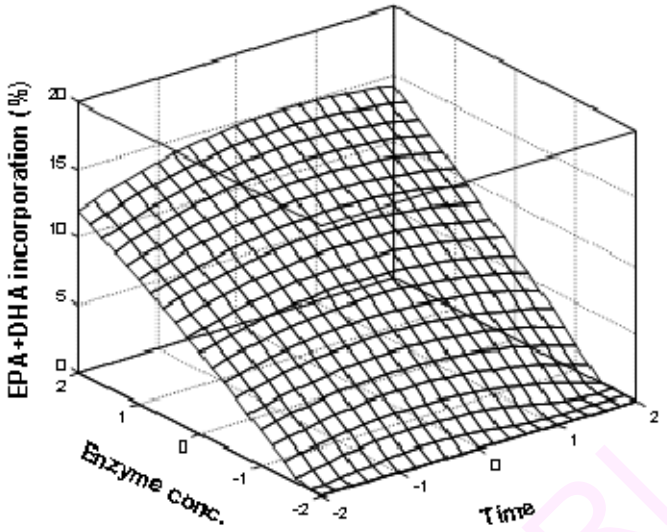


**Figure 4.10: Effect of enzyme concentration and time on EPA+DHA incorporation into RBO**

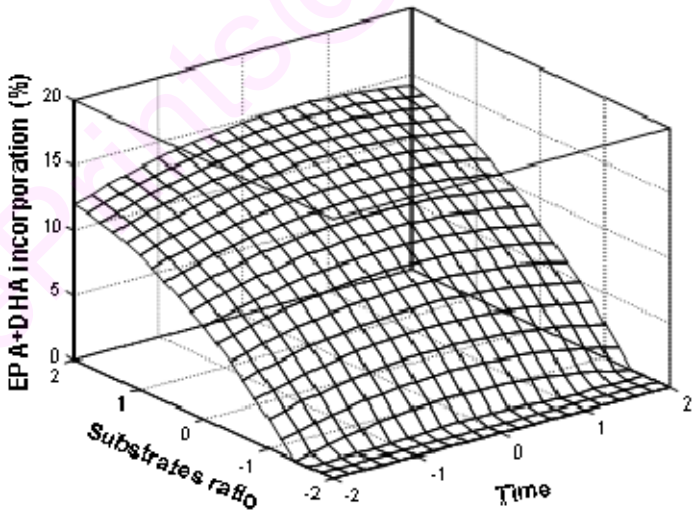


**Figure 4.11: Effect of substrates ratio and time on EPA+DHA incorporation into RBO**

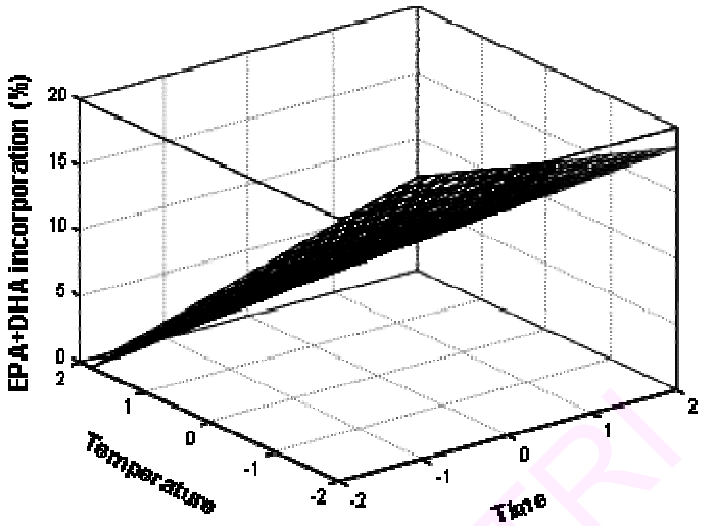


**Figure 4.12: Effect of temperature and time on EPA+DHA incorporation into RBO**

It was observed that temperature has lesser role to play in the incorporation of EPA+DHA with increase in reaction time (Figure 4.12). The increase in temperature from 25 to 55$^{o}$ C decreased the incorporation of EPA+DHA from 6.1 to 4 % (Table 4.10 and Figure 4.13). Figure 4.13 and 4.15 showed that enzyme concentration has increased the incorporation of EPA+DHA from 6.5 to 7.3 % when enzyme concentration was increased from 1 to 10 % respectively. Substrates molar ratio has important role in the incorporation for n-3 long chain PUFA. Percent incorporation was increased from 1.9 to 7.6 % when the substrates molar ratio was increased from 1 to 10 (Figures 4.14 and 4.15).

**Verification of results**

The suitability of the model equation for predicting the optimum response values were tested using the recommended optimum conditions. This set of conditions was determined to be optimum by a RSM optimization approach, which was also used to

validate experimentally and predict the value of the responses using model equations. Experimental value was found to be in agreement with the predicted ones (Table 4.13).

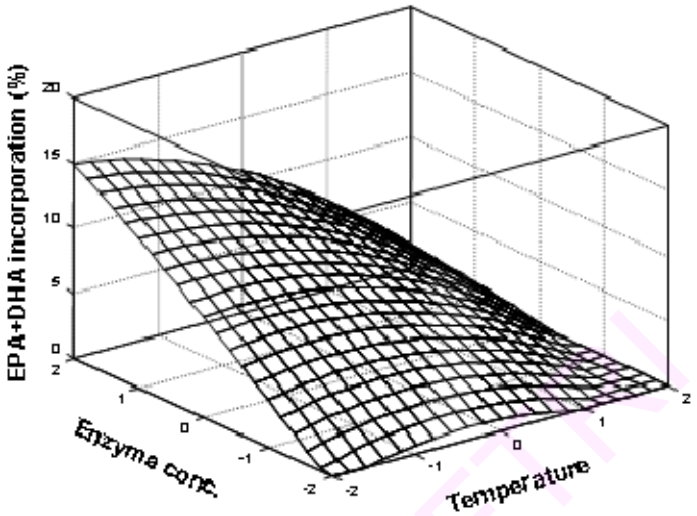


**Figure 4.13: Effect of enzyme concentration and temperature on EPA+DHA incorporation into RBO**

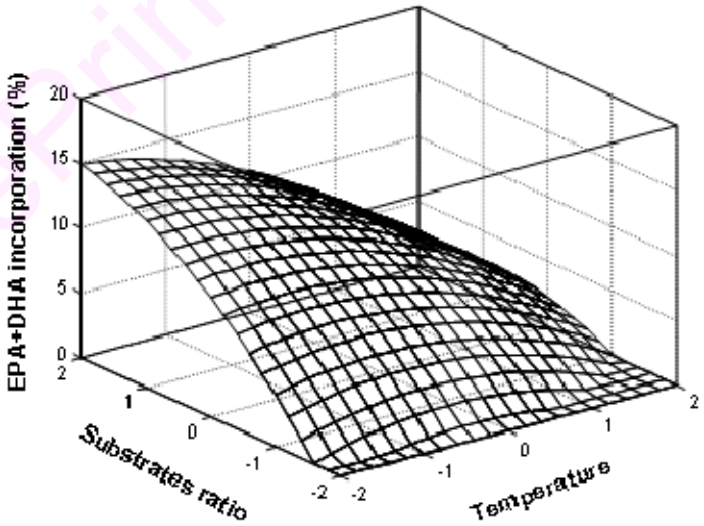


**Figure 4.14: Effect of substrates ratio and temperature on EPA+DHA incorporation into RBO**

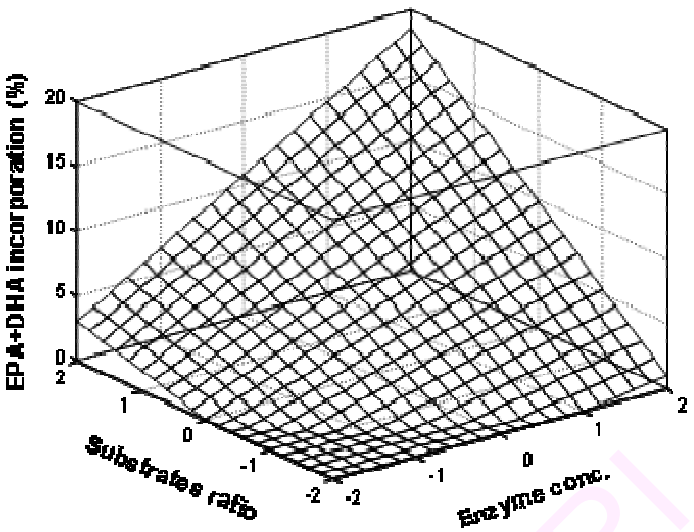


**Figure 4.15: Effect of substrates ratio and enzyme concentration on EPA+DHA incorporation into RBO**

**Table 4.13: Feasible optimum conditions and predicted and experimental value of response at optimum conditions**

| Optimum conditions | **Condition for maximum incorporation of EPA+DHA** | |
|---|---|---|
| | **Coded level** | **Actual level** |
| **Variables** | | |
| Time of reaction (h) ($X_1$) | 0.64 | 33.06 |
| Temperature of reaction (°C) ($X_2$) | -2.00 | 25.00 |
| Enzyme Concentration (w/w) ($X_3$) | 2.00 | 10.00 |
| Substrates ratio ($X_4$) | 2.00 | 10.00 |
| **Responses** | | |
| Predicted | 14.92% | |
| Observed | 14.49% | |

**Fatty acids at *sn-2* position**

After hydrolysis of native and modified RBO using pancreatic lipase, the fatty acid profile of purified monoacylglycerol indicated the presence of EPA+DHA (8.1 %) at *sn-2* position of modified RBO TAG (Table 4.14).

**Table 4.14: Fatty acid composition at *sn-2* position of native and modified RBO with EPA+DHA**

| Fatty acids | RBO | SL with CLO |
|---|---|---|
| | (%) | |
| 16:0 | nd | nd |
| 18:1 | 52.7 | 49.5 |
| 18:2 | 47.2 | 42.7 |
| 18:3 | nd | nd |
| EPA+DHA | nd | 8.1 |

Values are average of triplicate analysis

**Large scale synthesis of structured lipids from RBO rich in ALA**

Large scale synthesis of structured lipids was carried out as shown in figure 4.16. The level of ALA incorporation into RBO was ~ 20 %, which was similar to that obtained under similar conditions at the laboratory scale reactions. The fatty acid composition of the final product is given in table 4.15. Recovery of structured lipids obtained after purification was 86 % for the structured lipids with ALA. These structured lipids were evaluated for their nutritional properties.

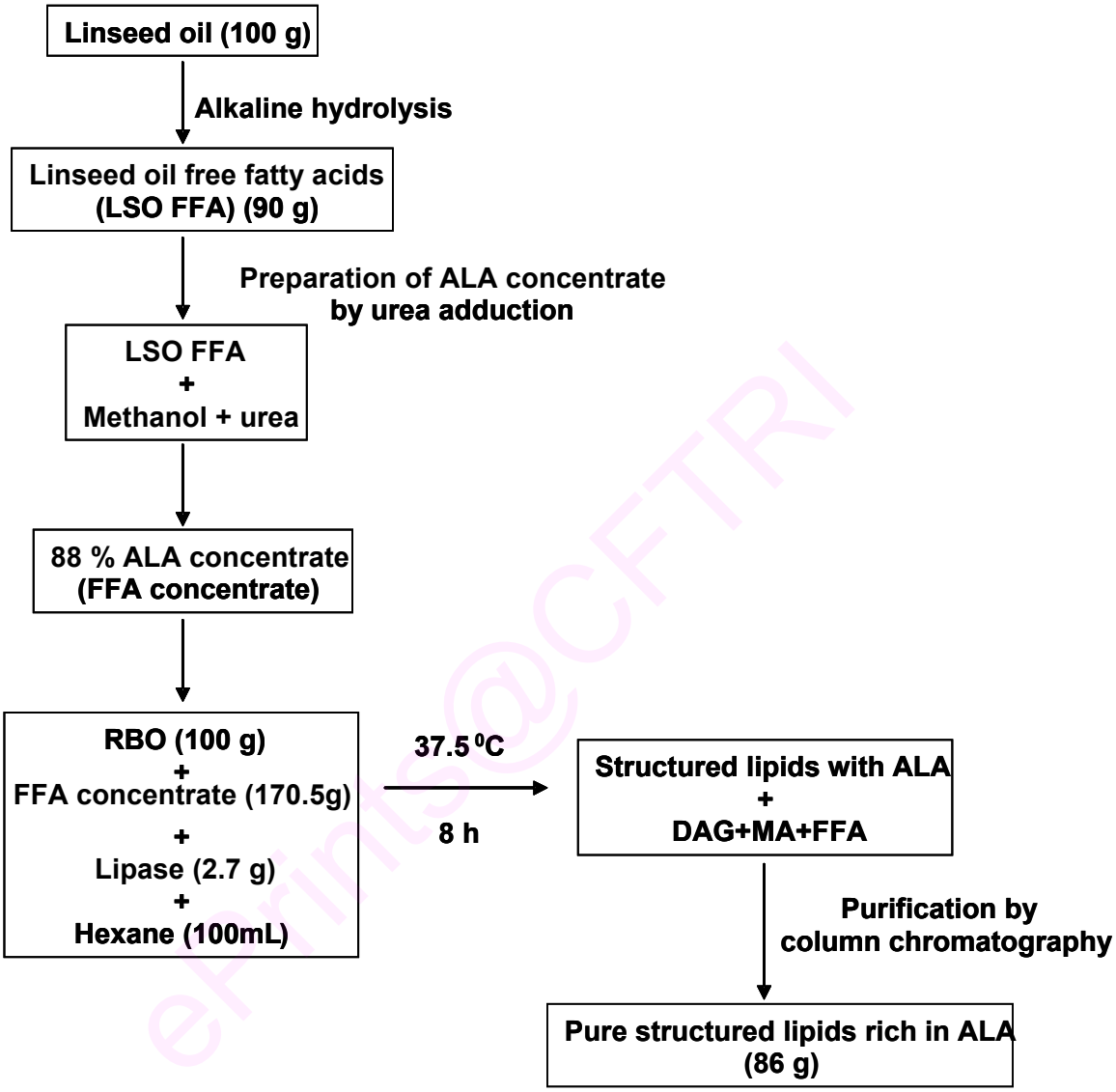


**Figure 4.16: Schematic representation of the scale up process for the production of structured lipids from RBO rich in ALA**

**Table 4.15: Fatty acid composition of native RBO and structured lipids of RBO rich in n-3 PUFA synthesized at optimal conditions at 100 g level**

| Fatty acids | RBO | Structured lipids from RBO enriched with | |
|---|---|---|---|
| | | ALA | EPA+DHA |
| | | (%) | |
| 16:0 | 20.8 | 16.5 | 14.5 |
| 16:1 | - | - | 2.9 |
| 18:0 | 2.2 | 2.1 | 4.2 |
| 18:1 (n-9) | 42.8 | 35.4 | 29.2 |
| 18:2 (n-6) | 33.2 | 24.7 | 25.9 |
| 18:3 (n-3) | - | 19.8 | - |
| 20:1 | - | - | 5.3 |
| 20:4 (n-6) | - | - | 3.2 |
| 20:5 (n-3) | - | - | 5.8 |
| 22:6 (n-3) | | - | 4.3 |

Values are average of triplicate analysis

**Large scale synthesis of structured lipids from RBO rich in EPA+DHA**

Large scale synthesis of structured lipids was carried out as shown in figure 4.17. The level of EPA+DHA incorporation into RBO was ~ 10 %, which was similar to that obtained under similar conditions at the laboratory scale reactions. The fatty acid composition of the final product is given in table 4.15. Recovery of structured lipids obtained after purification was 82 % for the structured lipids with EPA+DHA. These structured lipids were evaluated for their nutritional properties.

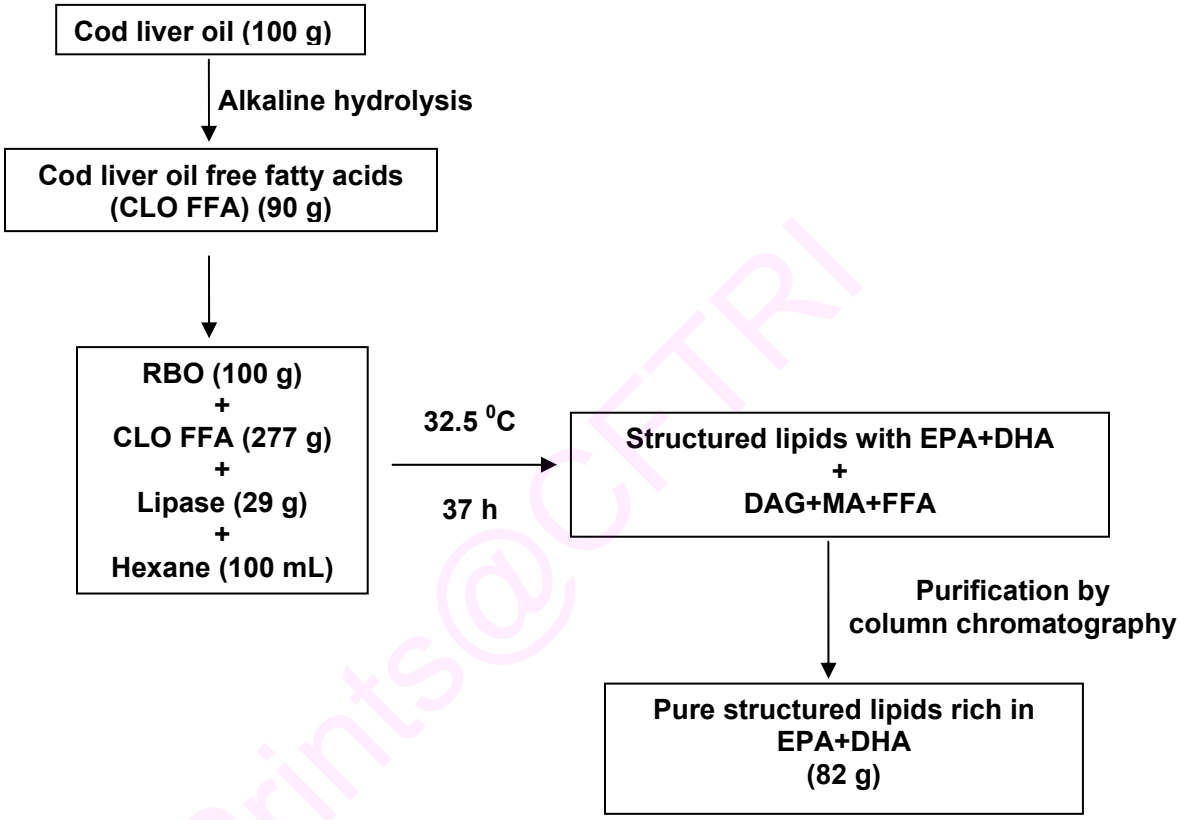


**Figure 4.17: Schematic representation of the scale up process for the production of structured lipids from RBO rich in EPA+DHA**

## Discussion

Enzymatic acidolysis reactions were used to modify the fatty acid composition of RBO. SL rich in n-3 PUFA were synthesized from RBO. Reaction conditions were optimized for incorporating n-3 PUFA into RBO using response surface methodology.

In case of structured lipids from RBO with ALA, incorporation of ALA into RBO was found to be affected by temperature, enzyme concentration, time and substrates molar ratio. However, enzyme concentration showed the most significant effect with high incorporation being shown at high substrates ratio even at low incubation temperature, and time. However, there was little increase in the incorporation of ALA into RBO was observed at very high value of incubation temperature and very long incubation time as compared to minimum incubation time and temperature. This could be attributed to n-3 PUFA being easily prone to lipid peroxidation. Another study reported that there was decrease in the n-3 FA incorporation with the increase in incubation time and temperature (Pedersen and Holmer, 1995; Xu *et al*, 1998a). At higher temperatures and longer periods of time they might have undergone oxidation, causing inhibition and decrease in activity of lipase.

Experimental results illustrate that RSM was found to be an efficient method to obtain optimized parameters for quality characteristics of structured lipids. Based on the set constraints, the optimum conditions have been established. Exploration of the response surfaces indicated a complex interaction between the variables such as time and temperature of reaction, enzyme concentration and substrates ratio. The optimum conditions such as reaction time (4.5 h) and reaction temperature (37.5 $^{o}$C), and substrates ratio (ranging from 1.0 to 1.9) and enzyme concentration (varying from 1.0 to 2.0 %) fulfill the conditions such as percentage incorporation of n-3 PUFA ≤ 19.8%, ratio of SFA/PUFA ≥ 0.42, ratio of MUFA/PUFA ≥ 0.8, ratio of n-6/n-3 (18:2 to 18:3) ≤ 0.65. The response surface methodology provides insight into the interaction and identifies the

optimum combination of variables (within a specified range) for the quality characteristics of structured lipids with the help of a relatively small number of experiments, thus reducing the time and cost of the study. It is evident from the data presented that the first and second (quadratic and cross product) order terms were found to be significant. However, the lack of fit was not significant for the percentage incorporation, ratio of SFA/PUFA, ratio of MUFA/PUFA, but it was significant for the ratio of n-6 to n-3 fatty acids. The lack of fit measures the failure of the model to represent data in experimental domain at points, which are not included in the regression. The high values of coefficient of determination ($R^2$) also suggest that the model is a good fit. The $R^2$ is proportion of variability in response values explained or accounted for by the model (Khuri and Cornell, 1987; Myers, 1971).

Incorporation of long chain n-3 PUFA (EPA+DHA) from cod liver oil into RBO is dependent on reaction time, temperature, enzyme concentration, and substrates molar ratio. Observed results obtained from different conditions were same as predicted results, which show that RSM model was well fitted. Adequacy of model was examined by comparing experimental data with the predicted values and then by performing extra independent experiments at optimal conditions. The value was obtained by substituting the predicted variables with experimental conditions. Verification of results revealed that experimental results and predicted values were close. Lower temperature and higher substrates molar ratio are found to be favorable for maximum incorporation of EPA+DHA. In a study by Chee *et al,* (2007) reported that reaction temperature, reaction time and molar substrates ratio strongly affect medium chain triglyceride synthesis. However, enzyme load and molecular sieve concentration did not have a significant influence on medium chain triglyceride yield. Adlercreutz et al, (2002) have shown that increasing the fatty acid concentration can increase the yield of the esterification reaction.

The response surface methodology provides an insight into the interaction and identifies the optimum combination of variables (within the specific range) for maximum incorporation of n-3 PUFA with the help of a relatively small number of experiments. The four variable and four level experimental design but only 31 combinations were sufficient to predict the optimum conditions using the RSM technique, thus reducing the time and cost of study. It is evident from the data presented that the first and second (quadratic and cross product) order terms were found to be significant and lack of fit was not significant. The lack of fit measures the failure of the model to represent data in experimental domain at points which are not included in the regression. The high values of coefficient of determination ($R^2$) also suggest that the model is a good fit. The $R^2$ is proportion of variability in response values explained or accounted for by the model (Myers, 1971; Montgomery, 1984). Even after using *sn-1, 3* position specific enzyme there was incorporation of EPA and DHA fatty acids at *sn-2* position. However, in the present case the incorporation of ALA and EPA+DHA at *sn-2* position in modified RBO was 15 and 8.0% respectively, which may be attributed to acyl migration (Xu et al, 1998b; 2000; Hamam *et al*, 2005a).

The higher level of ALA incorporated as compared to EPA+DHA might be attributed to higher levels of ALA concentrate (88 %) obtained from LSO used as acyl donor as compared to moderate level of 21 % of EPA+DHA in CLO in these experiments. To overcome these discrepancies, RBO TAG and FFA from LSO were taken at a proportion 1:1 while RBO and CLO free fatty acids were taken at a proportion of 1:7.75. Though the incubations with n-3 PUFA were also carried out for longer periods of time and at higher temperature, only lesser incorporation of long chain PUFA was observed in structured lipids. This could be attributed to the fatty acid specificity of the lipase or steric hindrance associated with the longer chain length and more number of double bonds in the long chain PUFA (EPA+DHA) (Pedersen and Holmer, 1995).

It was observed in the synthesis of SL from RBO rich in ALA as well as SL rich in EPA+DHA that the substrates ratio exerted a very significant influence on the incorporation of fatty acids. At any incubation time or temperature, when the substrates ratio was high, the rate of incorporation was also high. Similarly other studies reported that the incorporation was shown to increase with increase in ratio of 6 or 8 (Akoh *et al*, 1996; Mu *et al*, 1998).

The synthesis of structured lipids from RBO rich in ALA and EPA+DHA were scaled up to 100 g levels using batch reactors and the recovery of purified TAG was in the range of 82-86 %. The level of incorporation of the ALA or EPA+DHA matched with those obtained at the laboratory scale reactions. Optimization studies for the synthesis of structured lipids from RBO rich in ALA and EPA+DHA showed that different reaction variables could influence the rate of incorporation of different fatty acids into RBO TAG. These variables could interact with each other and influence the synthesis of each structured lipids differently. Thus the present study has shown that it is possible to adopt statistical methods such as RSM to synthesize structured lipids from RBO enriched in n-3 PUFA at desired levels. The structured lipids of RBO developed in the present study still retained its MUFA and at the same time contained the beneficial effects of n-3 PUFA.

# CHAPTER - V

# EFFECTS OF SL AND BLENDS FROM RBO ENRICHED WITH n-3 PUFA ON LIVER AND SERUM LIPIDS IN RATS

## INTRODUCTION

Over the past decades, intake of n-6 fatty acids has increased because of excess use of vegetable oils in our diets (Simopoulos, 1999a). Fatty acid composition of the diet plays a key role in health and disease prevention in humans (Simopoulos, 2001). Studies have shown that diet and dietary lipids have an important role to play in the prevention of risk of cardiovascular diseases (Schultz and Hoffmann, 2006; Katan *et al*, 1995). Clinical and epidemiological studies have shown the cardiovascular protective effects of n-3 PUFA (Morris, 1994; Sacks *et al*, 1994). Based on surveys conducted on Indian dietary patterns, Ghafoorunissa (1990; 1996) has estimated that Indians derive 12.6 g of linoleic acid (LA) per day, equivalent to 4.8 energy % (en %) from visible and invisible fat. The requirement of LA as an essential FA is 3 en %, hence Indian diets provide adequate amounts of LA. However the intake of n-3 PUFA by Indians is in the range of 0.3 g in the rural population and 0.6 g in the urban population, equivalent to 0.2 – 0.3 en % (Ghafoorunissa, 1996; Achaya, 1995). Dietary recommendations have been made for n-3 PUFA, including ALA, EPA, and DHA to achieve nutrient adequacy in order to prevent and treat cardiovascular diseases. These recommendations are based on a large body of evidence from epidemiologic and controlled clinical studies. The n-3 PUFA recommendation to achieve nutritional adequacy is defined as the amount necessary to prevent deficiency symptoms is 0.6 –1.2 % of energy for ALA and up to 10% of this can be provided by EPA or DHA. To achieve the recommended ALA intakes, food sources including flaxseed and flaxseed oil, walnuts and walnut oil, and canola oil are recommended. The evidence base supports a dietary recommendation of approximately 500 mg/d of EPA and DHA for cardiovascular disease risk reduction (Domingo, 2007). Most of these recommendations are based on strong evidence from a variety of scientific approaches linking dietary deficiencies of long chain n-3 PUFA with the risk of cardiovascular events, notably sudden death (William, 2007). Even though India has no

specific recommendations for the intake of n-3 PUFA, several studies conducted by the National Institute of Nutrition, an organization of the Indian Council of Medical Research, have suggested that an intake of 0.75 en % from ALA and 0.2 en % from EPA and DHA would have a positive impact on the prevention of coronary heart disease in India (Ghafoorunissa, 1996; 1998; Ghafoorunissa *et al*, 2002; Indu and Ghafoorunissa, 1992). Most of the anti-atherogenic effects have been demonstrated with fish oil derived n-3 PUFA namely EPA and DHA. Lately the attention has also been given to delineation of the cardioprotective potential of plant based n-3 PUFA, such as ALA (Jeffery *et al*, 1996; Harper and Jacobson, 2001). n-3 PUFA are considered to be anti-inflammatory, whereas the n-6 PUFA are considered to be pro-inflammatory (Lauren *et al*, 2005). Rice bran is a by-product of rice milling industry and RBO obtained from it is a rich source of tocopherols, tocotrienols and γ- oryzanol (Shin *et al*, 1997). γ- Oryzanol has been reported to possess the hypocholesterolemic and antioxidant activity (Seetharamaiah and Chandrasekhara, 1989; Sugano and Tsuji, 1997; Wilson *et al*, 2007). RBO contains approximately 38 % oleic acid (18:1, n-9), 34 % linoleic acid (18:2, n-6) and 18.6 % palmitic acid (16:0) (Rukmini and Raghuram, 1991). However, RBO does not contain any long chain n-3 PUFA, hence n-6 to n-3 ratio is high in RBO. The ratio of n-6 to n-3 fatty acids is often used to assess the balance between essential fatty acids in the diet. The WHO/ FAO suggests a ratio of 5:1 - 10:1 (WHO and FAO, 1995), whereas NIH suggests a ratio of 2:3 - 3:1 on the basis of proposed adequate intakes (Simopolous, 1999b). The acceration of higher-chain n-3 PUFA in tissue phospholipids may be dependent on the particular n-3 PUFA that is provided in the diet (Barelo- Coblijin *et al*, 2005). Oily fish can provide sufficient n-3 PUFA such as EPA and DHA (Cughey *et al*, 1996). However many Indians refrain from eating fish, as it is not a vegetarian food. Linseed (*Linum usitatissimum*), which is rich in ALA, is an economically important oilseed crop used for edible purposes in the central and northeastern regions of India.

The beneficial properties of LSO and CLO have been enumerated (Cunnane *et al*, 1993; Jenkins *et al*, 1999; Owen *et al*, 2004). LSO could be exploited as an alternate source of n-3 PUFA. However LSO has very limited use in diet because it is oxidized rapidly due to high level of unsaturated fatty acids. Among all unconventional edible oils, RBO seems to have great promise for use in SL and blends because of its beneficial nutritional properties as well as rich antioxidant content which may confer oxidative stability to PUFA rich oil. The interest in the production of SL containing specific fatty acids have been increasing continuously because the nutritional value of TAG depends both on fatty acid composition as well as the positional distribution of acyl groups within the TAG molecule. Therefore, SL provides an opportunity for using specific end uses by improving physical and nutritional properties. Earlier investigations have revealed that SL which are enriched in n-3 PUFA may exhibit significant beneficial effects when compared to physical mixtures of oils having similar fatty acid composition. The SL may behave differently from physical mixtures by exhibiting differences in their digestion, absorption and transport (Tso *et al*, 1995). The TAG of blended oils retains their original absorption rates, whereas SL in which the TAG are restructured have different absorption rates (Jensen *et al*, 1994). Enhanced lymphatic absorption of MCFA in rats was observed with a structured TAG containing MCFA (C10:0 and C8:0) in the sn-2 position and linoleic acid in the 1- and 3- positions compared with the physical mixture of these fats (Ikeda *et al*, 1991). Lee and Akoh (1999) reported that SL with n- 3 PUFA like EPA and DHA when fed to mice lowered TAG and low density lipoprotein (LDL) cholesterol levels more efficiently compared to soybean oil n- 3 PUFA. Though several groups have studied SL with respect to absorption and transport, very few studies have examined their lipidemic effects. Present study was aimed to study the effects of structured lipids with RBO containing n-3 PUFA (ALA and EPA+DHA) on their influence on lipid parameters related to cardiovascular diseases in experimental animals.

## EXPERIMENTAL

In the present study hypocholesterolemic effect of modified RBO rich in n-3 PUFA was studied. Thirty six male Wistar rats (28 days old) were grouped into six groups with six animals/ group. Fat was incorporated at 10 % level in the diet. GNO was chosen for the control diet because it is the most commonly used edible oil in southern India. Experimental diets differed from control diet only in fat source, where it is either RBO, structured lipids from RBO rich in ALA or EPA+DHA and blended oils with RBO and CLO or LSO. Structured lipids with RBO rich in EPA+DHA from CLO and structured lipids with RBO rich in ALA from LSO were prepared in large quantities as described earlier in chapter IV have been used in the present studies. Similarly blended oils with RBO and CLO or RBO and LSO containing similar fatty acid composition as that of their respective structured lipids were prepared and used.

### Studies on lipid profile of serum

During the study, growth and food intake were monitored and food efficiency (FER) was calculated as the ratio of weight gained by the animal to food consumed. After the experimental period, the animals were sacrificed under ether anesthesia. Blood, liver, heart, eye, brain and adipose tissue were collected and serum was separated from blood as described previously. The serum lipid parameters were estimated as described.

### Studies on lipid profile of liver

Liver was collected after sacrificing the animals and lipid was extracted from liver as described earlier. Liver lipid parameters were estimated as described previously.

### Fatty acid analysis

Tissues like heart, kidney, eye, brain and adipose tissues were collected after sacrificing animals. Fatty acid analysis was done for serum, liver, heart, eye, brain and adipose tissues as described previously.

## RESULTS

### Fatty acid composition of dietary lipids

The fatty acid composition of control and experimental dietary lipids is shown in table 5.1. Analysis of the fatty acid composition of diets revealed that GNO and RBO contained 58 and 43.2 % oleic and 28.4 and 34.8 % linoleic acids respectively. GNO and RBO did not contain any n-3 PUFA. Dietary lipids containing SL with LSO showed 20 % ALA and that with CLO had 5.8 and 4.3 % EPA and DHA respectively. Blends of RBO with LSO and CLO had ALA, EPA and DHA at the same level as their respective SL. Fatty acid composition of RBO with SL and blends showed decrease in palmitic, oleic and linoleic acid contents with incorporation of n-3 PUFA into RBO. The n-6/n-3 ratio in the SLs with CLO and LSO was 1.7 and 1.2 respectively where as in case of blends n-6/n-3 ratios were 2.6 and 1.5 respectively. The linoleic acid content was maintained between 16-30 % in all experimental diets and the control diets containing GNO and RBO were devoid of n-3 PUFA.

Analysis of the fatty acid composition at sn-2 position of different dietary lipids is given in table 5.2. RBO and GNO contained oleic acid and linoleic acid at sn-2 position whereas SL with LSO contained 15 % ALA and SL with CLO contained 8.1 % EPA + DHA. On the other hand, blend with CLO contained 21 % of EPA + DHA and blend with LSO contained 18 % ALA.

### Effect of dietary lipids on growth parameters

The influence of various dietary fats with different fatty acids on food intake, gain in weight and food efficiency ratio (FER) is given in table 5.3. The fat content of all diets was kept constant at 10 %. The amounts of diet consumed by animals in different groups were comparable except in SL with LSO and blend with CLO showed a significant increase in food intake compared to RBO control group. There were no

significant changes in body weight gain and FER except in blend with CLO where it was significantly higher compared to the other groups.

**Table 5.1: Fatty acid composition of diets**

| Fatty acids | GNO | RBO | SL with CLO | SL with LSO | Blend with CLO | Blend with LSO |
|---|---|---|---|---|---|---|
| | (%) | | | | | |
| 14:0 | nd | nd | 4.9 | nd | 2.6 | nd |
| 16:0 | 11.1 | 20.1 | 17.2 | 14.3 | 17.9 | 14.4 |
| 16:1 | nd | nd | 8.2 | nd | 4.4 | nd |
| 18:0 | 2.5 | 1.4 | 3.4 | 2.4 | 2.7 | 2.3 |
| 18:1 | 58.0 | 43.2 | 38.6 | 37.9 | 39.1 | 37.1 |
| 18:2 (n-6) | 28.4 | 34.8 | 16.5 | 24.2 | 25.0 | 29.7 |
| 18:3 (n-3) | nd | nd | nd | 20.1 | nd | 19.4 |
| 20:4 (n-6) | nd | nd | 0.8 | nd | 0.7 | nd |
| 20:5 (n-3) | nd | nd | 5.8 | nd | 5.9 | nd |
| 22:6 (n-3) | nd | nd | 4.3 | nd | 4.1 | nd |
| SFA | 13.6 | 21.5 | 25.5 | 16.7 | 23.2 | 16.7 |
| PUFA | 28.4 | 34.8 | 27.4 | 44.3 | 35.7 | 49.1 |
| SFA/PUFA | 0.4 | 0.6 | 1.0 | 0.3 | 0.6 | 0.3 |
| MUFA/PUFA | 2.0 | 1.2 | 1.7 | 0.8 | 1.2 | 0.7 |
| n-6/n-3 | 28.4 | 34.8 | 1.7 | 1.2 | 2.6 | 1.5 |

Values are average of triplicate analysis

GNO – Groundnut oil; RBO – Rice bran oil; SL with CLO – Structured lipids with RBO rich in EPA and DHA from cod liver oil; SL with LSO – Structured lipids with RBO rich in ALA from linseed oil; Blend with CLO – Blend of RBO with cod liver oil; Blend with LSO – Blend of RBO with linseed oil

nd – not detected

**Table 5.2: Fatty acid composition of dietary lipids at *sn-2* position**

| Fatty acids | GNO | RBO | SL with CLO | SL with LSO | Blend with CLO | Blend with LSO |
|---|---|---|---|---|---|---|
| | (%) | | | | | |
| 16:0 | nd | nd | nd | nd | nd | 9.0 |
| 18:1 | 68.0 | 52.7 | 49.5 | 47.6 | 46.0 | 39.0 |
| 18:2 (n-6) | 30.0 | 47.2 | 42.7 | 36.5 | 32.0 | 33.0 |
| 18:3 (n-3) | nd | nd | nd | 15.0 | nd | 18.0 |
| 20:5+22:6 (n-3) | nd | nd | 8.1 | nd | 21.0 | nd |

Values are average of triplicate analysis

Dietary group legends are as described in table 5.1.

nd – not detected

**Table 5.3: Effect of feeding structured lipids with RBO rich in n-3 PUFA on food intake and gain in weight in rats**

| Groups | Food intake (g/day/rat) | Body wt gained (g) | Food efficiency ratio |
|---|---|---|---|
| GNO | $11.4 \pm 0.31^{ab}$ | $200.6 \pm 8.2^{a}$ | $0.36 \pm 0.01^{a}$ |
| RBO | $10.5 \pm 0.44^{a}$ | $195.8 \pm 11.3^{a}$ | $0.37 \pm 0.01^{ab}$ |
| SL with CLO | $11.5 \pm 0.37^{ab}$ | $208.2 \pm 6.6^{a}$ | $0.39 \pm 0.0^{bc}$ |
| SL with LSO | $12.13 \pm 0.24^{b}$ | $211.2 \pm 4.2^{a}$ | $0.38 \pm 0.02^{ab}$ |
| Blend with CLO | $11.9 \pm 0.49^{b}$ | $217.0 \pm 7.3^{a}$ | $0.41 \pm 0.01^{c}$ |
| Blend with LSO | $11.5 \pm 0.52^{ab}$ | $212.7 \pm 5.8^{a}$ | $0.37 \pm 0.01^{ab}$ |

Values are mean ± SEM of 6 rats.

Values not sharing a common superscript within the column are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.

The effect of feeding various dietary lipids on different organ weights is shown in table 5.4. Weights of heart, brain and spleen of rats fed different dietary fats were comparable. Similarly there were no changes in liver weight in any of the groups.

**Table 5.4: Effect of feeding structured lipids with RBO rich in n-3 PUFA on organ weight in rats**

| Groups | Liver | Heart | Brain | Spleen |
|---|---|---|---|---|
| | (g/ 100 g body wt) | | | |
| GNO | 3.23 ± 0.01$^{a}$ | 0.31 ± 0.0$^{ab}$ | 0.68 ± 0.03$^{ab}$ | 0.22 ± 0.0$^{a}$ |
| RBO | 3.54 ± 0.01$^{a}$ | 0.34 ± 0.01$^{bc}$ | 0.70 ± 0.03$^{b}$ | 0.29 ± 0.03$^{b}$ |
| SL with CLO | 3.55 ± .08$^{a}$ | 0.30 ± 0.02$^{ab}$ | 0.66 ± 0.02$^{ab}$ | 0.20 ± 0.01$^{a}$ |
| SL with LSO | 3.22 ± 0.01$^{a}$ | 0.28 ± 0.03$^{a}$ | 0.70 ± 0.01$^{b}$ | 0.24 ± 0.02$^{a}$ |
| Blend with CLO | 3.56 ± 0.01$^{a}$ | 0.32 ± 0.01$^{b}$ | 0.62 ± 0.01$^{a}$ | 0.24 ± 0.02$^{a}$ |
| Blend with LSO | 3.44 ± 0.03$^{a}$ | 0.37 ± 0.03$^{c}$ | 0.64 ± 0.01$^{ab}$ | 0.31 ± 0.02$^{b}$ |

Values are mean ± SEM of 6 rats.

Values not sharing a common superscript within the column are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.

**Effect of feeding n-3 PUFA on serum lipid profile**

The serum lipid profile of rats in response to different dietary lipids is given in table 5.5. Rats fed rice bran oil showed decrease in serum total cholesterol by 10 % when compared to groundnut oil. Structured lipids with CLO and LSO significantly decreased total serum cholesterol by 18.6 and 21.6 % respectively compared to RBO. In case of blends also the total cholesterol level was decreased significantly as compared to RBO. This reduction occurred both in HDL and LDL fractions. The serum triglycerides

level of rats fed SLs with CLO and LSO decreased by 14 and 16.6 % respectively and in blended oils the decrease in triglycerides level was 21.8 and 16.5 % respectively compared to RBO. Serum phospholipids concentration was increased in rats fed blended oil with CLO but the increase was not statistically significant when compared to RBO and GNO. Whereas incase of other groups there was a significant decrease in phospholids level of rats fed n-3 PUFA as compared to RBO.

**Table 5.5: Effect of feeding structured lipids with RBO rich in n-3 PUFA on serum lipid profile in rats**

| Groups | Total chol. | HDL-C | LDL+ VLDL-C | PL | TG |
|---|---|---|---|---|---|
| | (mg/ dL) | | | | |
| GNO | 93.5 ± 2.9$^{c}$ | 40.6 ± 4.3$^{d}$ | 53.0 ± 2.7$^{c}$ | 135.0 ± 7.3$^{b}$ | 139.6 ± 4.8$^{c}$ |
| RBO | 88.8± 3.8$^{b}$ | 40.7 ± 2.9$^{c}$ | 48.1 ± 1.9$^{b}$ | 128.8 ± 6.2$^{b}$ | 126.9 ± 4.1$^{d}$ |
| SL with CLO | 72.2 ± 4.5$^{a}$ | 30.2 ± 3.3$^{a}$ | 42.0 ± 3.4$^{a}$ | 106.2 ± 5.7$^{a}$ | 108.8 ± 3.7$^{b}$ |
| SL with LSO | 69.6 ± 5.8$^{a}$ | 29.0 ± 5.5$^{a}$ | 40.5 ± 2.2$^{a}$ | 110.4 ± 7.7$^{a}$ | 105.8 ± 2.3$^{ab}$ |
| Blend with CLO | 72.0 ± 4.5$^{a}$ | 33.9 ± 2.1$^{b}$ | 38.2 ± 3.8$^{a}$ | 139.6 ± 4.9$^{b}$ | 99.2 ± 3.6$^{a}$ |
| Blend with LSO | 74.0 ± 5.9$^{a}$ | 33.2 ± 3.6$^{b}$ | 40.8 ±2.2$^{a}$ | 111.7 ± 4.1$^{a}$ | 105.9 ± 4.0$^{ab}$ |

Values are mean ± SEM of 6 rats.

Values not sharing a common superscript within the column are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.

Chol. – Cholesterol; HDL-C – HDL cholesterol; LDL+VLDL-C – LDL+VLDL cholesterol; PL –- phospholipids; TG – triglycerides

**Serum fatty acid profile**

Significant changes were observed in n-3 fatty acids level in serum of rats fed SLs and blends with CLO and LSO (Table 5.6). The ALA content was 5.4 and 4.5 % in SLs and blend with LSO respectively whereas this fatty acid was not detected in other groups. A significant increase in EPA + DHA levels and a concomitant decrease in arachidonic acid were observed in rats fed SLs and blends with CLO and LSO. ALA, EPA and DHA were not detected in serum of rats fed RBO and GNO groups. As a result of feeding of SLs and blends with CLO and LSO significant increase in the levels of all n-3 PUFA occurred whereas SLs rich in n-3 PUFA feeding resulted in significant reduction in arachidonic, oleic and linoleic acids. Arachidonic acid level in serum of rats fed n-3 PUFA was significantly decreased as compared to RBO and GNO. Rats fed SL with EPA+DHA showed 64 % decrease in arachidonic acid content as compared to both GNO and RBO.

**Liver lipid profile**

Liver is an important organ for lipid metabolism and sensitive to changes in the dietary lipids. Liver lipid profile of rats fed n-3 PUFA is given in table 5.7. The total cholesterol content in the liver of rats fed SLs with CLO and LSO was significantly decreased by 14 and 11 % respectively compared to RBO group. In case of rats fed blends with CLO and LSO, the decrease in total cholesterol was 17 and 26 % respectively. The decrease in triglyceride levels of rats fed SLs with CLO and LSO was 21 and 10 % respectively whereas in case of blends the decrease was 11 and 19 % respectively as compared to RBO. Phospholipids level of rats fed SLs with CLO was increased by 21 % as compared to RBO but in case SL with LSO the increase was not statistically significant.

**Table 5.6: Effect of feeding structured lipids with RBO rich in n-3 PUFA on serum fatty acids in rats**

| Fatty acids | GNO | RBO | SL with CLO | SL with LSO | Blend with CLO | Blend with LSO |
|---|---|---|---|---|---|---|
| | (%) | | | | | |
| 16:0 | 22.2 ± 0.2$^{ab}$ | 22.2 ± 0.4$^{ab}$ | 22.9 ± 0.3$^{ab}$ | 21.5 ± 0.6$^{a}$ | 22.4 ± 0.4$^{ab}$ | 23.8 ± 0.9$^{b}$ |
| 16:1 | 2.5 ± 0.04$^{a}$ | 2.4 ± 0.3$^{a}$ | 3.7 ± 0.4$^{b}$ | 2.5 ± 0.2$^{a}$ | 2.4 ± 0.1$^{a}$ | 2.0 ± 0.1$^{a}$ |
| 18:0 | 8.4 ± 0.4$^{a}$ | 9.1 ± 0.2$^{ab}$ | 8.4 ± 0.4$^{a}$ | 9.5 ± 0.3$^{ab}$ | 8.8 ± 0.07$^{b}$ | 9.6 ± 0.5$^{b}$ |
| 18:1 | 32.5 ± 0.3$^{c}$ | 33.2 ± 0.2$^{c}$ | 27.2 ± 0.7$^{b}$ | 27.9 ± 0.4$^{b}$ | 27.0 ± 0.5$^{b}$ | 28.7 ± 1.1$^{a}$ |
| 18:2 (n-6) | 19.1 ± 0.2$^{b}$ | 17.7 ± 0.3$^{b}$ | 15.8 ± 0.5$^{a}$ | 19.2 ± 0.4$^{c}$ | 18.1 ± 0.5$^{ab}$ | 18.2 ± 0.5$^{ab}$ |
| 18:3 (n-3) | nd | nd | nd | 5.4 ± 0.3$^{c}$ | nd | 4.5 ± 0.3$^{b}$ |
| 20:4 (n-6) | 9.4 ± 0.4$^{c}$ | 9.6 ± 0.4$^{c}$ | 3.4 ± 0.1$^{a}$ | 4.7 ± 0.1$^{b}$ | 4.3 ± 0.2$^{ab}$ | 5.0 ± 0.5$^{b}$ |
| 20:5 (n-3) | nd | nd | 6.0 ± 0.5$^{c}$ | 3.2 ± 0.04$^{a}$ | 5.8 ± 0.04$^{b}$ | 3.2 ± 0.05$^{a}$ |
| 22:6 (n-3) | nd | nd | 2.8 ± 0.4$^{c}$ | 2.7 ± 0.04$^{bc}$ | 2.6 ± 0.3$^{b}$ | 2.4 ± 0.1$^{a}$ |

Values are mean ± SEM of 6 rats.

Values not sharing a common superscript within the row are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.

nd – not detected

**Table 5.7: Effect of feeding structured lipids with RBO rich in n-3 PUFA on liver lipid profile in rats**

| Groups | Total cholesterol | Phospholipids | Triglycerides |
|---|---|---|---|
| | (mg/g) | | |
| GNO | 13.4 ± 1.3$^{d}$ | 23.9 ± 1.5$^{a}$ | 16.5 ± 1.6$^{c}$ |
| RBO | 11.5 ± 1.3$^{c}$ | 27.0 ± 1.7$^{b}$ | 15.0 ± 1.5$^{b}$ |
| SL with CLO | 9.9 ± 1.6$^{b}$ | 32.6 ± 2.8$^{c}$ | 11.8 ± 2.6$^{a}$ |
| SL with LSO | 10.2 ± 1.2$^{bc}$ | 28.9 ± 2.0$^{b}$ | 13.5 ± 2.5$^{ab}$ |
| Blend with CLO | 9.8 ± 1.6$^{ab}$ | 32.3 ± 1.7$^{c}$ | 13.3 ± 1.4$^{ab}$ |
| Blend with LSO | 8.5 ± 1.5$^{a}$ | 33.4 ± 3.6$^{c}$ | 12.2 ± 1.6$^{a}$ |

Values are mean ± SEM of 6 rats.

Values not sharing a common superscript within the column are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.

**Liver fatty acid profile**

Table 5.8 shows the fatty acid composition of the liver lipids of rats fed SLs and blended oils with CLO and LSO. Feeding SLs lipids and blends with CLO and LSO resulted in significant reduction in arachidonic acid. The reduction in arachidonic acid level of rats fed SLs with CLO and LSO was 45 and 42 % respectively as compared to RBO. In case of rats fed blends with CLO and LSO, the decrease in arachidonic acid was 41 and 56 % respectively as compared to rats fed RBO and GNO. ALA was detected only in rats fed with SL or blends with LSO. There was significant decrease in the linoleic acid level of rats fed n-3 PUFA as compared to RBO. On the other hand feeding LSO either in SL or blended oils resulted in accumulation of ALA, EPA and DHA

and reduction in the level of arachidonic acid, oleic and linoleic acid compared to RBO fed group. The oleic acid in rats fed SL with CLO and LSO were decreased by 50 and 38 % respectively compared to RBO group whereas in case of rats fed blend with CLO and LSO the decrease was 38 and 24 % respectively compared to RBO fed group.

**Table 5.8: Effect of feeding structured lipids with RBO rich in n-3 PUFA on liver fatty acid profile in rats**

| Fatty acids | GNO | RBO | SL with CLO | SL with LSO | Blend with CLO | Blend with LSO |
|---|---|---|---|---|---|---|
| | | | (%) | | | |
| 16:0 | 19.7 ± 0.4$^{a}$ | 21.5 ± 0.3$^{ab}$ | 24.4 ± 0.2$^{c}$ | 21.8 ± 1.3$^{b}$ | 22.8 ± 0.8$^{bc}$ | 22.3 ± 0.7$^{b}$ |
| 16:1 | 3.2 ± 0.1$^{b}$ | 3.3 ± 0.4$^{b}$ | 2.5 ± 0.2$^{ab}$ | 2.7 ± 0.2$^{ab}$ | 2.0 ± 0.4$^{a}$ | 1.7 ± 0.2$^{a}$ |
| 18:0 | 10.1 ± 0.5$^{a}$ | 9.3 ± 0.4$^{a}$ | 17.6 ± 0.2$^{c}$ | 14.0 ± 1.1$^{b}$ | 14.6 ± 0.6$^{b}$ | 14.9 ± 0.7$^{b}$ |
| 18:1 | 39.0 ± 0.5$^{e}$ | 35.7 ± 1.0$^{d}$ | 17.7 ± 2.6$^{a}$ | 22.0 ± 0.6$^{b}$ | 22.1 ± 0.7$^{b}$ | 27 ± 0.8$^{c}$ |
| 18:2 (n-6) | 14.0 ± 0.4$^{b}$ | 16.6 ± 0.2$^{c}$ | 10.6 ± 0.7$^{a}$ | 13.4 ± 1.1$^{b}$ | 15.5 ± 0.2$^{c}$ | 15.5 ± 0.2$^{c}$ |
| 18:3 (n-3) | nd | nd | nd | 2.1 ± 0.6$^{a}$ | nd | 1.9 ± 0.6$^{a}$ |
| 20:4 (n-6) | 18.4 ± 0.1$^{d}$ | 16.3 ± 0.4$^{c}$ | 9.0 ± 0.02$^{b}$ | 9.4 ± 0.1$^{b}$ | 9.6 ± 0.3$^{b}$ | 7.2 ± 0.3$^{a}$ |
| 20:5 (n-3) | nd | nd | 2.4 ± 0.06$^{c}$ | 1.4 ± 0.04 $^{b}$ | 2.6 ± 0.08 $^{d}$ | 1.2 ± 0.01 $^{a}$ |
| 22:6 (n-3) | nd | nd | 10.3 ± 0.1$^{d}$ | 4.9 ± 0.06$^{a}$ | 8.4 ± 0.3$^{c}$ | 5.4 ± 0.1$^{b}$ |

Values are mean ± SEM of 6 rats.

Values not sharing a common superscript within the row are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.

nd – not detected

**Fatty acid composition of heart, brain, eye and adipose tissues**

The dietary n-3 PUFA content influenced the FA composition of heart (Table 5.9), brain (Table 5.10), eye (Table 5.11) and adipose tissue (Table 5.12). Fatty acid composition of the heart was significantly influenced in rats fed n-3 PUFA containing SLs and blended oils. Rats fed SL and blend with CLO resulted in the significant accumulation of DHA and EPA in the heart tissue of rats. The level of DHA was significantly increased in the heart tissues of rats given SL and blends with CLO. There was a 5.3 fold increase in the DHA level of rats fed SL with CLO compared to RBO, whereas rats fed SL and blend with LSO the increase in DHA was only 2.9 fold compared to RBO. Rats fed SL and blends with CLO significantly decrease the levels of oleic and linoleic and arachidonic acids. The decrease in arachidonic acid level in rats fed SL with CLO and LSO was 28 and 12 % respectively and in case of blends with CLO and LSO the decrease was 21 and 13 % respectively as compared to RBO. Similarly in case of rats fed SL and blends with LSO showed significant amount of ALA in the heart tissue as compared to RBO and GNO fed rats.

In brain small amounts (2.4 and 1.6 %) of ALA was detected in rats fed structured lipids and blend with LSO respectively, whereas this fatty acid was not detected in other dietary groups. The basal level of DHA in RBO and GNO was 4.4 and 4.7 % respectively of the total FAs, whereas it was increased to 10.4 and 8.1 % in SL with CLO and LSO respectively. Similarly it was also increased in the case of blends up to 9.4 and 7.4 % of the total FAs respectively as compared to RBO and GNO. The arachidonic acid was simultaneously decreased significantly along with increase in n-3 PUFA. Fatty acid composition of eye of rats fed n-3 PUFA showed that the arachidonic acid content simultaneously decreased significantly along with increase in n-3 PUFA. DHA content was increased significantly in all groups of experimental animals compared

**Table 5.9: Effect of feeding structured lipids with RBO rich in n-3 PUFA on heart fatty acid profile in rats**

| Fatty acids | GNO | RBO | SL with CLO | SL with LSO | Blend with CLO | Blend with LSO |
|---|---|---|---|---|---|---|
| | | | (%) | | | |
| 16:0 | 15.9 ± 0.2$^{c}$ | 14.7 ± 0.1$^{b}$ | 13.1 ± 0.1$^{a}$ | 17.5 ± 0.2$^{d}$ | 13.0 ± 0.1$^{a}$ | 18.5 ± 0.4$^{e}$ |
| 16:1 | 2.1 ± 0.02$^{c}$ | 1.9 ± 0.02$^{c}$ | 3.6 ± 0.1$^{d}$ | 1.4 ± 0.02$^{b}$ | 3.9 ± 0.02$^{e}$ | 1.1 ± 0.02$^{a}$ |
| 18:0 | 27.9 ± 0.2$^{b}$ | 27.2 ± 0.2$^{b}$ | 25.6 ± 0.4$^{a}$ | 31.0 ± 0.3$^{c}$ | 26.1 ± 0.1$^{a}$ | 32.3 ± 0.4$^{d}$ |
| 18:1 | 21.7 ± 0.2$^{d}$ | 20.6 ± 0.3$^{c}$ | 18.1 ± 0.02$^{b}$ | 16.8 ± 0.1$^{a}$ | 18.7 ± 0.2$^{b}$ | 18.2 ± 0.3$^{b}$ |
| 18:2 (n-6) | 14.8 ± 0.1$^{c}$ | 15.7 ± 0.3$^{d}$ | 12.2 ± 0.1$^{b}$ | 11.4 ± 0.1$^{b}$ | 13.3 ± 0.3$^{b}$ | 10.2 ± 0.1$^{a}$ |
| 18:3 (n-3) | nd | nd | nd | 3.1 ± 0.02$^{a}$ | nd | 3.1 ± 0.02$^{a}$ |
| 20:4 (n-6) | 17.0 ± 0.2$^{d}$ | 17.0 ± 0.3$^{d}$ | 12.3 ± 0.2$^{a}$ | 15.0 ± 0.1$^{c}$ | 13.4 ± 0.2$^{b}$ | 14.7 ± 0.3$^{c}$ |
| 20:5 (n-3) | nd | nd | 2.5 ± 0.02$^{d}$ | 1.4 ± 0.02$^{a}$ | 2.3 ± 0.02$^{c}$ | 2.1 ± 0.02$^{b}$ |
| 22:6 (n-3) | 2.5 ± 0.02$^{a}$ | 2.3 ± 0.02$^{a}$ | 14.5 ± 0.1$^{d}$ | 8.8 ± 0.1$^{b}$ | 12.4 ± 0.2$^{c}$ | 9.3 ± 0.3$^{b}$ |

Values are mean ± SEM of 6 rats.

Values not sharing a common superscript within the row are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.
nd – not detected

**Table 5.10: Effect of feeding structured lipids with RBO rich in n-3 PUFA on brain fatty acid profile in rats**

| Fatty acids | GNO | RBO | SL with CLO | SL with LSO | Blend with CLO | Blend with LSO |
|---|---|---|---|---|---|---|
| | (%) | | | | | |
| 16:0 | 21.5 ± 0.7$^{a}$ | 23.3 ± 0.3$^{b}$ | 24.6 ± 0.2$^{b}$ | 23.8 ± 0.1$^{b}$ | 24.5 ± 0.4$^{b}$ | 23.5 ± 0.3$^{b}$ |
| 16:1 | 3.8 ± 0.2$^{a}$ | 4.3 ± 0.1$^{ab}$ | 4.7 ± 0.4$^{b}$ | 4.3 ± 0.4$^{ab}$ | 4.2 ± 0.1$^{ab}$ | 3.8 ± 0.2$^{a}$ |
| 18:0 | 11.4 ± 0.1$^{a}$ | 11.2 ± 0.1$^{a}$ | 11.9 ± 0.1$^{ab}$ | 11.9 ± 0.6$^{ab}$ | 12.8 ± 0.2$^{bc}$ | 13.1 ± 0.6$^{c}$ |
| 18:1 | 37.2 ± 0.2$^{d}$ | 31.7 ± 0.7$^{c}$ | 29.7 ± 0.1$^{b}$ | 30.0 ± 0.7$^{b}$ | 31.8 ± 0.6$^{c}$ | 27.2 ± 0.4$^{a}$ |
| 18:2 (n-6) | 13.1 ± 0.2$^{d}$ | 10.8 ± 0.1$^{c}$ | 8.8 ± 0.3$^{a}$ | 10.3 ± 0.2$^{bc}$ | 9.5 ± 0.3$^{ab}$ | 9.4 ± 0.2$^{a}$ |
| 18:3 (n-3) | nd | nd | nd | 2.4 ± 0.1$^{b}$ | nd | 1.6 ± 0.02$^{a}$ |
| 20:4 (n-6) | 7.6 ± 0.1$^{d}$ | 7.2 ± 0.3$^{d}$ | 5.0 ± 0.1$^{b}$ | 4.5 ± 0.1$^{ab}$ | 4.1 ± 0.2$^{a}$ | 5.3 ± 0.1$^{c}$ |
| 20:5 (n-3) | nd | nd | 0.6 ± .02$^{ab}$ | 0.8 ± .02$^{b}$ | 0.8 ± 0.08$^{b}$ | 0.6 ± 0.05$^{ab}$ |
| 22:6 (n-3) | 4.7 ± 0.2$^{a}$ | 4.4 ± 0.2$^{a}$ | 10.4 ± 0.1$^{e}$ | 8.1 ± 0.1$^{c}$ | 9.4 ± 0.2$^{d}$ | 7.4 ± 0.02$^{b}$ |

Values are mean ± SEM of 6 rats.

Values not sharing a common superscript within the row are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.
nd – not detected

**Table 5.11: Effect of feeding structured lipids with RBO rich in n-3 PUFA on eye fatty acid profile in rats**

| Fatty acids | GNO | RBO | SL with CLO | SL with LSO | Blend with CLO | Blend with LSO |
|---|---|---|---|---|---|---|
| | (%) | | | | | |
| 16:0 | 21.5 ± 0.7$^{a}$ | 22.9 ± 0.1$^{b}$ | 24.6 ± 0.4$^{c}$ | 23.8 ± 0.1$^{bc}$ | 24.6 ± 0.4$^{c}$ | 23.5 ± 0.3$^{bc}$ |
| 16:1 | 3.8 ± 0.1$^{a}$ | 4.5 ± 0.02$^{ab}$ | 4.6 ± 0.4$^{b}$ | 4.3 ± 0.4$^{ab}$ | 4.2 ± 0.1$^{ab}$ | 3.7 ± 0.2$^{a}$ |
| 18:0 | 11.3 ± 0.1$^{a}$ | 11.4 ± 0.1$^{a}$ | 11.9 ± 0.2$^{ab}$ | 11.9 ± 0.6$^{ab}$ | 12.8 ± 0.2$^{b}$ | 13.1 ± 0.6$^{b}$ |
| 18:1 | 35.4 ± 0.0.9$^{c}$ | 31.9 ± 0.7$^{b}$ | 31.0 ± 0.7$^{bc}$ | 29.0 ± 1.0$^{ab}$ | 29.3 ± 0.2$^{ab}$ | 27.2 ± 0.4$^{a}$ |
| 18:2 (n-6) | 13.2 ± 0.2$^{d}$ | 10.7 ± 0.1$^{c}$ | 8.8 ± 0.3$^{a}$ | 10.3 ± 0.2$^{bc}$ | 9.5 ± 0.3$^{ab}$ | 9.3 ± 0.2$^{a}$ |
| 18:3 (n-3) | nd | nd | nd | 2.4 ± 0.1$^{b}$ | nd | 1.7 ± 0.2$^{a}$ |
| 20:4 (n-6) | 7.4 ± 0.02$^{d}$ | 7.3 ± 0.2$^{d}$ | 5.0 ± 0.1$^{bc}$ | 4.5 ± 0.1$^{ab}$ | 4.2 ± 0.2$^{a}$ | 5.3 ± 0.1$^{c}$ |
| 20:5 (n-3) | nd | nd | 0.6 ± 0.02$^{a}$ | 0.8 ± 0.2$^{b}$ | 0.8 ± 0.02$^{b}$ | 0.6 ± 0.02$^{a}$ |
| 22:6 (n-3) | 4.9 ± 0.2$^{a}$ | 4.7 ± 0.2$^{a}$ | 10.4 ± 0.1$^{c}$ | 7.1 ± 0.5$^{b}$ | 10.1 ± 0.3$^{c}$ | 7.5 ± 0.1$^{b}$ |

Values are mean ± SEM of 6 rats.

Values not sharing a common superscript within the row are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.
nd – not detected

**Table 5.12: Effect of feeding structured lipids with RBO rich in n-3 PUFA on adipose tissue fatty acid profile in rats**

| Fatty acids | GNO | RBO | SL with CLO | SL with LSO | Blend with CLO | Blend with LSO |
|---|---|---|---|---|---|---|
| | (%) | | | | | |
| 14:0 | 3.4 ± 0.2$^{a}$ | 3.4 ± 0.2$^{a}$ | 5.9 ± 0.3$^{d}$ | 4.8 ± 0.1$^{b}$ | 5.3 ± 0.2$^{c}$ | 5.0 ± 0.1$^{b}$ |
| 16:0 | 17.9 ± 0.1$^{a}$ | 22.3 ± 0.4$^{b}$ | 28.6 ± 0.8$^{d}$ | 25.2 ± 0.5$^{c}$ | 25.0 ± 0.2$^{c}$ | 20.9 ± 0.9$^{b}$ |
| 16:1 | 8.2 ± 0.1$^{ab}$ | 8.7 ± 0.1$^{bc}$ | 9.2 ± 0.3$^{c}$ | 8.0 ± 0.1$^{a}$ | 8.9 ± 0.02$^{c}$ | 7.9 ± 0.2$^{a}$ |
| 18:0 | 2.8 ± 0.2$^{ab}$ | 2.4 ± 0.2$^{a}$ | 3.6 ± 0.1$^{d}$ | 2.3 ± 0.1$^{a}$ | 3.5 ± 0.3$^{cd}$ | 3.1 ± 0.02$^{bc}$ |
| 18:1 | 59.6 ±0.4$^{d}$ | 43.4 ± 0.4$^{c}$ | 39.1 ± 0.7$^{a}$ | 39.2 ± 0.5$^{a}$ | 41.4 ± 0.7$^{b}$ | 42.2 ± 0.8$^{b}$ |
| 18:2 (n-6) | 19.7 ± 0.3$^{d}$ | 20.5 ± 0.3$^{d}$ | 10.8 ± 0.4$^{a}$ | 14.8 ± 0.8$^{b}$ | 16.7 ± 0.3$^{c}$ | 17.7 ± 0.4$^{c}$ |
| 18:3 (n-3) | nd | nd | nd | 10.5 ± 0.6$^{a}$ | nd | 10.3 ± 0.2$^{a}$ |
| 20:4 (n-6) | 0.2 ± 0.01$^{a}$ | 0.26± 0.02$^{a}$ | 0.2 ± 0.02$^{a}$ | 0.19 ± 0.01$^{a}$ | 0.3 ± 0.02$^{b}$ | 0.3 ± 0.02$^{b}$ |
| 20:5 (n-3) | nd | nd | 1.0 ± 0.02$^{a}$ | nd | 1.0 ± 0.02$^{a}$ | nd |
| 22:6 (n-3) | nd | nd | 2.7 ± 0.02$^{b}$ | nd | 1.4 ± 0.1$^{a}$ | nd |

Values are mean ± SEM of 6 rats.

Values not sharing a common superscript within the row are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.
nd – not detected

to RBO. The increase in DHA level in rats fed SL with EPA+DHA and blends with CLO were 2.2 and 2.1 folds respectively. Simultaneously there was a decrease in arachidonic acid level of rats fed n-3 PUFA as compared to RBO and GNO. Whereas only small amounts of EPA was detected in these animals. ALA was detected only in LSO fed animals. There was significant reduction in arachidonic acid.

In the adipose tissue of rats fed structured lipids and blend with LSO there was significant accumulation of ALA (10.5 and 10.3 % of ALA respectively of total fatty acids), whereas it was not detected in rats of other dietary groups (Table 5.12). EPA and DHA were found only in small amounts in rats fed SL and blends with CLO. However, feeding SL and blend with CLO resulted in a significant accumulation of EPA and DHA in adipose tissue. The EPA level in rats fed SL with CLO was 1 % in both the groups, whereas DHA level in adipose tissue of rats fed with SL with ALA and blends with LSO was 2.7 and 1.4 % respectively.

**Discussion**

The present investigation was aimed to enrich rice bran oil with ALA (18:3) from linseed oil and EPA + DHA from cod liver oil and study the effect of these modified lipids on changes in the serum and hepatic lipid profile of rats when fed as the sole source of dietary fat. The effect of SLs synthesized was also compared with that of blends of RBO with CLO and LSO with similar levels of n-3 PUFA. Even though 1, 3 positional specific lipase from *Rhizomucor miehei* (Macrae, 1983) was used as a catalyst, some amount of 18:3 (15 %) and EPA+DHA (8.1 %) were incorporated into the SLs synthesized with LSO and CLO respectively. This indicated that most of the ALA and EPA+DHA in the modified lipids were incorporated in the 1 and 3 positions of the triacylglycerol molecule of RBO. The small increase in the ALA and EPA+DHA at the sn-2 position of SLs could be attributed to acyl migration (Xu *et al*, 1998b; 2000). In case of blended oils, though fatty acid composition was similar to that of the SL, they differed in their fatty acid content at the sn-2 position. Numerous studies have shown that SL have unique metabolic pathways and exhibit many superior metabolic benefits in comparison to that of the blended oils. Using a canine model, Jensen *et al*. (1994) measured the absorption of MCFA when given as SL or when administered in the form of a physical blend of lipids with similar fatty acid composition. These studies indicated that the absorption and transport of fatty acids in SL are regulated and differs from that of blended oils with similar fatty acid composition. Even though many studies have clearly shown that there are significant differences in the mode of absorption of fatty acids based on acylglycerol structures, very few studies have addressed the lipidemic effects of modified lipids. The SLs containing n-3 PUFA showed a significant hypolipidemic effect than that of the blended oils when fed as a sole source of fat in the diet. Similarly hypolipidemic effect of SL was also observed in liver. Feeding SL and blends with LSO increased the level of ALA in serum, liver and other tissues lipids. This also resulted in an incremental increase

in the accumulation of EPA+DHA in the serum, liver and other tissues. This indicated that conversion of ALA to long chain PUFA is taking place in rats. Increase in EPA and DHA was higher in rats fed fats containing these fatty acids in preformed state. Both blended oils and SL increased the 18:3 and EPA + DHA levels in the liver and other tissues. One reason for this may be the higher levels of EPA and DHA in the diets containing modified lipids. Another reason could be due to slow conversion of ALA to long chain n-3 PUFA (EPA and DHA). Preformed EPA+DHA was taken up by serum and liver much more efficiently than that obtained from ALA. The level of arachidonic acid in heart tissue was decreased when n-3 PUFA was included in the diet. Earlier studies have shown that feeding diet containing 18:3 can increase the level of ALA as well as the level of its long chain metabolites to varying degrees (Gerster, 1998; Mantzioris *et al*, 1994; Bazinet *et al*, 2003; Ramaprasad *et al*, 2004; Barcelo-Coblijin *et al*, 2005; MacDonald-Wicks and Garg 2004). Rats fed SLs and blends with n-3 PUFA increased the amount of n-3 PUFA in the liver and heart tissue is in agreement with findings of Medeiros *et al* (2007).

The brain is unique organ characterized with the presence of high concentration of DHA, which influences its structure and functional properties (Brenner, 1984). The high level of DHA in brain of a number of mammalian species irrespective of their size led to early speculation that this fatty acid is playing a crucial role in the nervous system. Many studies using ALA deficient diets have shown reduction in level of DHA in brain and loss of many cognitive functions (Ahmed *et al,* 2002). Studies have reported that supplementation of arachidonic acid and DHA can improve the cognitive dysfunction due to organic brain damages or aging (Kotani *et al*, 2006). Feeding SL with EPA+DHA and blends with CLO showed 57 and 45 % increase in brain respectively compared to RBO and GNO fed rats. We have also observed that rats fed preformed DHA in the form of SL and blend with CLO showed a higher levels of DHA in brain as compared to those fed

SL and blend with LSO. This finding is in agreement with earlier report showing that preformed DHA is incorporated efficiently into brain tissue (Ramaprasad *et al*, 2004). Earlier studies have shown little but significant increase in the 18:3 and EPA levels in brain tissue when diet was high in n-3 FAs (Kao *et al*, 2006).

Adipose tissue normally does not contain long chain n-6 and n-3 fatty acids. However accumulation of ALA and EPA+DHA in the adipose tissue increased in response to ALA and EPA+DHA content in the diet. Studies indicated that feeding a diet high in 18:3 to guinea pigs increased the EPA and DHA levels in liver, heart, and adipose tissue compared with a diet low in 18:3 (Zhong and Sinclair, 2002; Wistuba *et al* , 2007). The investigation by Okuno *et al* (1997) in rats showed that feeding a diet containing perilla oil (60.3 % ALA) resulted in accumulation of only 0.9 % and 0.4 % EPA and DHA in adipose tissue respectively. However, in our study EPA and DHA were not detected in adipose tissue when rats fed with SL and blend containing ALA, whereas in the present study the ALA was fed only at 20 % level which could not be enough for the detectable limit of EPA and DHA. That shows the accumulation of EPA and DHA were observed when the rats were fed with higher concentration of ALA in the diet. Previous studies reported that 18:3 enriched diets resulted in the accumulation of 18:3, but not EPA and DHA (Lin and Connors, 1990; Ramaprasad *et al*, 2004). These studies may indicate that high intake of n-3 PUFA, 18:3 can accumulate in adipose tissue but its elongation to EPA and DHA is restricted. However these fatty acids can accumulate in adipose tissue when given in preformed state. This along with our study indicate that adipose tissue has the ability to take up ALA but not be able to convert it to long chain n-3 PUFA. On the other hand adipose tissue readily picks up EPA and DHA if it is provided as preformed long chain n-3 PUFA. Therefore, adipose tissue may not have active desaturase and elongase to convert essential fatty acids to its longer chain metabolites. Eye is an important organ where large amount of DHA is required for the vision.

Docosahexaenoic acid, a major dietary n-3 LCPUFA, is also a major structural lipid of retinal photoreceptor outer segment membranes. Tissue DHA status affects retinal cell signaling mechanisms involved in phototransduction. DHA may operate in signaling cascades to enhance activation of membrane-bound retinal proteins and may also be involved in rhodopsin regeneration. Tissue DHA insufficiency is associated with alterations in retinal function. Visual processing deficits have been ameliorated with DHA supplementation in some cases (SanGiovanni and Chew, 2005). In our study we found that feeding n-3 PUFA resulted in the accumulation of n-3 PUFA into eye of rats.

Structured lipids rich in n-3 PUFA had significant effects on serum and liver lipids. ALA and EPA+DHA containing diets lowered serum total cholesterol and triglyceride levels in rats fed SL and blends with LSO and CLO. Diet containing 10 % EPA+DHA reduced the serum and lipid level as effectively as 20 % of ALA could reduce compared to RBO. So from present study it was noticed that higher concentrations of ALA is required to exhibit comparable effect with EPA+DHA in the diets. American Heart Association recommends for those individuals without heart disease to eat a variety of fish at least twice a weak and include oils and foods rich in ALA (flaxseed, canola, and soybean oils; flaxseed and walnuts) (Kris-Etherton *et al*, 2002).

The SLs used in the present study provides about 2.1 g of ALA and 1.1 g of EPA+DHA when 10.5 g of SL lipids rich in ALA (at 20 % level) or 11 g of SL rich in EPA+DHA (at 10 % level) respectively. Consumption at these levels will meet the daily recommended allowances for these n-3 PUFA.

In conclusion, the present study indicated that 18:3 and EPA + DHA were efficiently absorbed from SLs as well as blended oils. The food intake and growth of animals also showed that the SL was as efficient as blended oil in supporting the growth of the animals without having any adverse effects. Rice bran oil enriched with n-3 PUFA has significantly lowered the serum and liver lipids which are main risk factors for

cardiovascular diseases. These results also confirm the accumulation of preformed n-3 PUFA and conversion of ALA to EPA and DHA in various tissues. Therefore, RBO can be enriched with $\alpha$- linolenic acid and EPA+DHA by lipase catalyzed interesterification reactions and these modified lipids exhibited hypocholesterolemic and hypolipidemic effects in rats when compared to unmodified RBO.

# CHAPTER - VI

# ANTIOXIDANT ACTIVITY AND PLATELET AGGREGATION IN RATS FED SL AND BLENDS FROM RBO ENRICHED WITH n-3 PUFA

## Introduction

Dietary habits varies with respect to regional differences, hence fat consumption pattern also differs. Epidemiological evidence from Greenland Eskimos and Japanese fishing villages suggests that eating fish and marine animals can prevent coronary heart disease. Dietary studies from various laboratories have similarly indicated that regular fish oil intake affects several humoral and cellular factors involved in atherogenesis and may prevent atherosclerosis, arrhythmia, thrombosis, cardiac hypertrophy and sudden cardiac death (Siddiqui *et al*, 2007). Studies have shown that intake of n-3 polyunsaturated fatty acids (PUFA) by Indians are lower than the desired level (Achaya, 1995; Simopoulos, 1999a). Population consuming a western-style n-6 diet are at a significantly greater risk of developing inflammatory diseases, including cancer and coronary heart disease, than those population living on diets rich in fish oils containing n-3 PUFAs (Berg *et al*, 1994; Shekella *et al;* 1985 and Norell *et al*, 1986). The beneficial effects of fish oils are mostly attributed to their n-3 PUFA content, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, another n-3 PUFA, α-linolenic acid (ALA), found in green leafy vegetables, flaxseed, rapeseed and walnuts can be desaturated and elongated in the human body to EPA, docosapentaenoic acid (DPA) and then to DHA (Siddiqui *et al*, 2007). Both epidemiological (Albert *et al*, 1998) and prospective randomized clinical trials (Leaf *et al*, 2005) have reported a decrease in morbidity and mortality from heart disease in patients with diets supplemented with n-3 PUFAs. Studies have also shown that n-3 PUFA enriched milk formulation significantly increased n-3 PUFA level in the liver, heart, brain and adipose tissues of rats and also beneficially modulated the serum lipid levels by exhibiting hypolipidemic effects (Ramaprasad *et al*, 2004). However, PUFAs can also cause deterioration of the antioxidant status due to their liability to get oxidized (Porter *et al*. 1995). A high intake of PUFA has been demonstrated to increase the formation of

lipid radicals and to deplete endogenous antioxidants (Porter *et al*. 1995). Thus, a potential destabilization of antioxidant status remains a concern, particularly when plant oils containing rich in n-3 PUFA were consumed (Harper and Jacobson 2001). Dietary fat affects the fatty acid composition, lipid peroxidation and antioxidant defense systems of the body (Pulla Reddy and Lokesh, 1994). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) defend the host against the damaging effects of free radical species. $Na^+/K^+$ ATPase is an integral membrane protein which catalyzes the ATP dependent transport of $Na^+$ and $K^+$ ions across the cell membranes. It has been reported that dietary PUFAs modulate the activity of this enzyme (Murphy, 1990). The enzyme is more active in the membrane having a fluid lipid environment with PUFA (Xiao *et al*, 2006). Other mechanisms may also be involved in PUFA cardiovascular protective effects such as the lowering of platelet aggregation (Nieuwenhuys and Hornstra, 1998). The n-3 PUFA inhibit vasoconstrictor thromboxane $A_2$ ($TXA_2$) biosynthesis, but blood pressure values are not correlated with plasma $TXA_2$ concentration in rats (Chen *et al*, 1996). However, decreased 20:4 (n-6) levels in platelet lipids might decrease $TXA_2$ synthesis and platelet sensitivity, thus resulting in lower cardiovascular risks. Platelets, through their interaction with the vascular endothelium, play a critical role in atherogenesis (Ross, 1993).

In the present study we have examined the effect of feeding structured lipids with RBO containing n-3 PUFA on lipid peroxidation, hepatic antioxidant enzymes, erythrocyte membrane bound enzymes like $Na^+/K^+$ and $Ca^{2+}/ Mg^{2+}$ ATPase and platelet aggregation in rats.

## Experimental

### Studies on antioxidant status and platelet aggregation

In the present study the influence of SL from RBO enriched with n-3 PUFA on antioxidant status and platelet aggregation was studied. Thirty six male Wistar rats (28 days old) were grouped into six groups with six animals/ group. Experimental diets differed from control diets only in the type of fat. RBO and groundnut oil were the two control diets given and in other 4 groups fat was either SL rich in ALA or EPA+DHA and blends of RBO with CLO or LSO. The composition of diets was as described earlier in chapter V.

### Studies on lipid peroxides in serum, liver homogenate and platelets

Lipid peroxides in serum, liver homogenate and platelets were estimated as described earlier. Lipid peroxides were estimated in 450 µL of pallet suspension which had been challenged by the agonist during platelet aggregation study.

### Studies on $Na^+/K^+$ ATPase activity in erythrocyte membrane

Erythrocyte ghosts were prepared and $Na^+/K^+$ and $Ca^{2+}/Mg^{2+}$ ATPase activities were measured by spectrophotometric method as described earlier.

Studies on platelet aggregation in rats fed structured lipids enriched with n-3 PUFA

**Platelet aggregation measurements were performed within two hours of blood collection. Aggregation experiments were done with platelet rich plasma (PRP) as described earlier. Fatty acid analysis of platelets was also done as described earlier.**

## Results

**Lipid peroxides in liver homogenate**

Lipid peroxides in liver homogenates of rats fed n-3 PUFA is given in figure 6.1. Lipid peroxides of rats fed RBO and GNO were comparable. Lipid peroxides level in liver of rats fed SL rich in EPA+DHA and ALA were increased by 3.4 and 2.9 folds respectively as compared to RBO fed group. In case of blends with CLO and LSO lipid peroxides were also increased by 3.5 and 2.5 folds respectively compared to RBO.

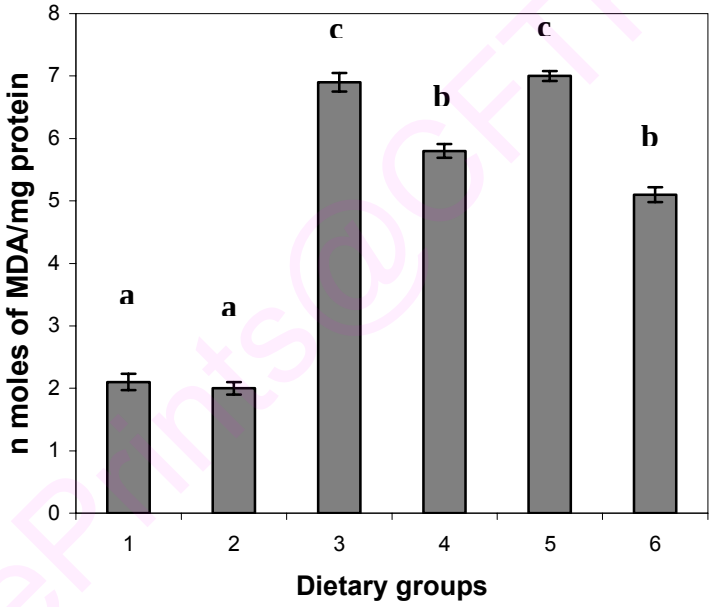


**Figure 6.1: Influence of RBO enriched with n-3 PUFA on liver lipid peroxides in rats**

Values are mean ± SD of 6 rats.

Values not sharing the common superscript are significantly different at $p < 0.05$.

Dietary groups – 1 - GNO, 2- RBO, 3- SL with CLO, 4- SL with LSO, 5- Blend with CLO, and 6- Blend with LSO.

Dietary group legends are as described in table 5.1.

**Lipid peroxides in serum**

Lipid peroxides in serum of rats fed n-3 PUFA is given in figure 6.2. Lipid peroxides of rats fed RBO and GNO were comparable. Lipid peroxide levels in serum of rats fed SL rich in EPA+DHA and ALA was increased by 3.2 and 3.1 folds respectively compared to RBO. In case of blends with CLO and LSO the lipid peroxides were similarly increased by 3.8 and 3.7 folds respectively when compared to RBO.

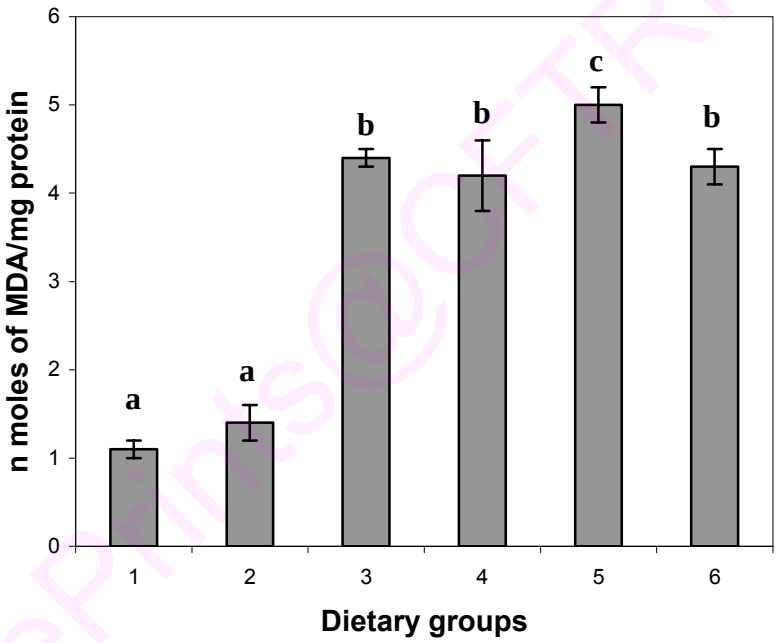


**Figure 6.2: Influence of RBO enriched with n-3 PUFA on serum lipid peroxides in rats**

Values are mean ± SD of 6 rats.

Values not sharing the common superscript are significantly different at $p < 0.05$.

Dietary group legends are as described in figure 6.1 and table 5.1.

**Lipid peroxides in platelets**

Lipid peroxides in platelets of rats fed n-3 PUFA is given in figure 6.3. Lipid peroxides of rats fed RBO and GNO were comparable. On the other hand lipid peroxides level in platelets of rats fed SL rich in EPA+DHA and ALA were increased by 3.1 and 2.5 folds respectively compared to RBO. In case of blends with CLO and ALA the lipid peroxidation was increased by 3 and 2.6 folds respectively as compared to RBO.

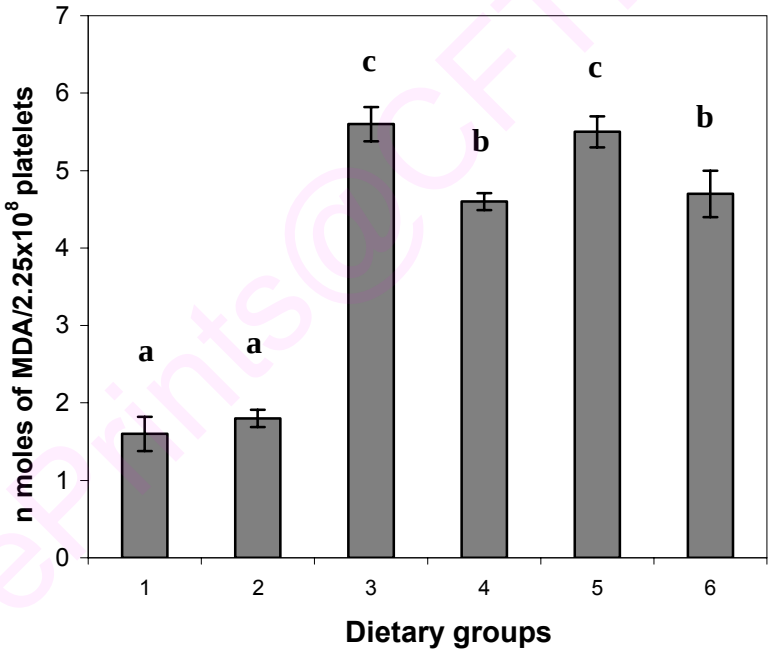


**Figure 6.3: Influence of RBO enriched with n-3 PUFA on platelets lipid peroxides in rats**

Values are mean ± SD of 6 rats.

Values not sharing the common superscript are significantly different at $p < 0.05$.

Dietary group legends are as described in figure 6.1 and table 5.1.

**Fatty acid profile of platelets of rats fed structured lipids enriched with n-3 PUFA**

Dietary fat significantly altered the fatty acid profile of platelets in rats (Table 6.1). In platelets of rats fed SL and blends from LSO, the ALA level was found to be 7.3% and 6.1% respectively. EPA level in rats fed SL and blends from LSO was found to be 2.5 and 1.8% of total fatty acids respectively and this fatty acid was not detected in any other groups. However, DHA was not detected in platelets of rats fed SL and blends with LSO. The platelets of rats fed SL and blends with CLO found to contain both EPA and DHA at 4.3 and 3.8% and 2.4 and 2.2% of total fatty acids respectively. In platelet lipids, arachidonic acid level in rats fed SL with CLO and LSO was decreased by 35.7 and 52.3% respectively compared to RBO group. Linoleic acid content was not altered except in rats fed SL with CLO where it was decreased significantly compared to RBO and GNO groups. Similarly oleic acid was also decreased to an extent of 40% when SL with EPA+DHA was fed to rats. Similarly there was 44.8% decrease in oleic acid content when rats were fed with SL lipids from LSO.

**Antioxidant enzymes activity in liver of rats fed structured lipids from RBO enriched with n-3 PUFA**

The effect of n-3 PUFA enriched SL and blended oils on antioxidant enzyme activity in liver is given in table 6.2. Compared to control groups of rats, SOD activity increased only in SL and blend with LSO group significantly. However, the catalase activity increased by 32 and 21% respectively in rats fed SL and blends with CLO and 27 and 24 % in rats fed SL and blend with LSO respectively. The glutathione reductase activity was increased significantly in all the groups when n-3 PUFA were fed to rats. Similarly, glutathione transferase activity was also increased to an extent of 40-42% in n-3 fatty acid fed groups compared to RBO.

**Table 6.1: Fatty acid composition of platelets of rats fed structured lipids enriched with n- 3 PUFA**

| Fatty acids (%) | GNO | RBO | SL with CLO | SL with LSO | Blend with CLO | **Blend with LSO** |
|---|---|---|---|---|---|---|
| 16:0 | 32.6 ± 0.21$^{b}$ | 32.5 ± 0.35$^{b}$ | 34.9 ± 0.80$^{c}$ | 28.8 ± 0.51$^{a}$ | 32.3 ± 0.31$^{b}$ | 29.8 ± 0.18$^{a}$ |
| 16:1 | 3.0 ± 0.05$^{a}$ | 3.3 ± 0.11$^{a}$ | 3.9 ± 0.24$^{b}$ | 4.0 ± 0.08$^{b}$ | 3.8 ± 0.12$^{b}$ | 4.2 ± 0.08$^{b}$ |
| 18:0 | 19.9 ± 0.23$^{a}$ | 18.8 ± 0.26$^{a}$ | 25.6 ± 0.86$^{ab}$ | 25.5 ± 0.40$^{a}$ | 27.1 ± 0.28$^{cd}$ | 28.2 ± 0.58$^{d}$ |
| 18:1 | 39.1 ± 0.16$^{d}$ | 37.7 ± 0.43$^{c}$ | 18.5 ± 0.62$^{a}$ | 20.8 ± 0.65$^{b}$ | 17.8 ± 0.18$^{a}$ | 17.8 ± 0.30$^{a}$ |
| 18:2 | 5.0 ± 0.08$^{bc}$ | 5.0 ± 0.05$^{bc}$ | 5.5 ± 0.29$^{c}$ | 4.3 ± 0.24$^{a}$ | 4.9 ± 0.08$^{bc}$ | 4.8 ± 0.18$^{ab}$ |
| **18:3** | nd | nd | nd | 7.3 ± 0.28$^{b}$ | nd | 6.1 ± 0.06$^{a}$ |
| **20:4** | 5.0 ± 0.11$^{d}$ | 4.2 ± 0.12$^{c}$ | 2.7 ± 0.18$^{b}$ | 2.0 ± 0.03$^{a}$ | 2.6 ± 0.24$^{b}$ | 2.0 ± 0.12$^{a}$ |
| **20:5** | nd | nd | 4.3 ± 0.01$^{d}$ | 2.5 ± 0.01$^{b}$ | 3.8 ± 0.17$^{c}$ | 1.8 ± 0.01$^{a}$ |
| **22:6** | nd | nd | 2.4 ± 0.24$^{b}$ | nd | 2.2 ± 0.14$^{a}$ | nd |

Values are mean ± SD of 6 rats.

Values not sharing a common superscript within the row are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.

**Table 6.2: Effect of structured lipids from RBO rich in n-3 PUFA on liver antioxidant enzymes activity in rats**

| Groups | Catalase | Glutathione transferase | Glutathione reductase | Superoxide dismutase |
|---|---|---|---|---|
| | (μmoles/min/mg protein) | | | (U/min/mg protein) |
| GNO | 83.4±1.83[a] | 36.1±2.41[a] | 44.8±2.74[a] | 3.5±0.12[a] |
| RBO | 87.5±1.46[a] | 42.5± 1.32[b] | 63.7±2.68[b] | 3.6±0.13[a] |
| SL with CLO | 115.6±2.28[c] | 60.1± 0.93[c] | 66.9±1.39[b] | 3.8±0.13[a] |
| SL with LSO | 110.8±4.90[bc] | 58.0±2.19[c] | 86.2±2.86[c] | 4.3±0.08[b] |
| Blend with CLO | 105.9±3.18[b] | 59.7±2.87[c] | 90.1±3.75[c] | 3.9±0.10[a] |
| Blend with LSO | 108.3±1.31[bc] | 55.8± 2.20[c] | 86.9±3.00[c] | 3.9±0.14[a] |

Values are mean ± SD of 6 rats.

Values not sharing a common superscript within the column are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.

**Antioxidant enzymes activity in serum of rats fed structured lipids from RBO enriched with n-3 PUFA**

The effect of n-3 PUFA enriched SL and blended oils on antioxidant enzyme activity in serum is given in table 6.3. Compared to control groups of rats there was significant increase in SOD activity in SL and blend with CLO and LSO. However, the catalase activity increased by 51.3 and 51.9 % respectively in rats fed SL and blends with CLO and 35.8 and 51.4 % in rats fed SL with LSO respectively. The glutathione reductase activity was increased significantly in all the groups when n-3 PUFA were fed to rats. Similarly, glutathione transferase activity was also increased to an extent of 40 50 % in n-3 fatty acid fed groups compared to RBO.

**Table 6.3: Effect of structured lipids from RBO rich in n-3 PUFA on serum antioxidant enzymes activity in rats**

| Groups | Catalase | Glutathione transferase | Glutathione reductase | Superoxide dismutase |
|---|---|---|---|---|
| | (μmoles/min/mg protein) | | | (U/min/mg protein) |
| GNO | 38.84±0.80$^{a}$ | 27.98±0.74$^{a}$ | 22.24±0.87$^{a}$ | 2.99±0.10$^{a}$ |
| RBO | 37.7±0.88$^{a}$ | 28.30±0.92$^{a}$ | 21.67±0.52$^{a}$ | 2.67±0.16$^{a}$ |
| SL with CLO | 56.68±3.07$^{c}$ | 39.78±1.46$^{c}$ | 32.34±1.30$^{c}$ | 3.65±0.29$^{c}$ |
| SL with LSO | 51.20± 2.59$^{bc}$ | 41.48±2.74$^{b}$ | 31.14±1.00$^{c}$ | 3.77±0.18$^{c}$ |
| Blend with CLO | 57.31±3.26$^{c}$ | 42.78±1.85$^{c}$ | 33.43±2.32$^{c}$ | 3.49±0.12$^{bc}$ |
| Blend with LSO | 57.19±2.10$^{b}$ | 43.34±1.67$^{b}$ | 33.68±1.15$^{b}$ | 4.42±0.14$^{d}$ |

Values are mean ± SD of 6 rats.

Values not sharing a common superscript within the column are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.

$Na^{+}/K^{+}$, $Ca^{2+}$and $Mg^{2+}$ ATPase activities

**$Na^{+}/K^{+}$ ATPase is an integral membrane protein which catalyzes the ATP dependent transport of $Na^{+}$ and $K^{+}$ ion across the cell membrane. Dietary fat modulate the activity of these enzymes. $Na^{+}/K^{+}$ ATPase and $Mg^{2+}$ ATPase activities are given in table 6.4. Total $Na^{+}$, $K^{+}$ ATPase activity in RBC membrane of rats fed SL and blends with CLO increased by 3.5 fold as compared to RBO group. Similarly about 3 fold increase in $Na^{+}$, $K^{+}$ ATPase activity was observed in case of rats fed SL and blends with LSO compared to RBO and GNO. $Mg^{2+}$ ATPase activity in rats fed SL with LSO was increased about 1.5 times as compared to RBO. Similar significant increase was also observed in case of other dietary groups as compared to RBO. $Ca^{2+}$ ATPase activity was also significantly increased in rats fed SL or blends with CLO or LSO.**

**Table 6.4: $Na^+/K^+$ and $Ca^{2+}/Mg^{2+}$ ATPase activity of erythrocyte membrane from rats fed structured lipids enriched with n-3 PUFA**

| Groups | $Na^+/K^+$ ATPase activity | $Mg^{2+}$ ATPase activity | $Ca^{2+}$ ATPase activity |
|---|---|---|---|
| | (n moles pi released / h/mg of protein) | | |
| GNO | 250.7±4.9[a] | 445.5±9.5[a] | 324.7±4.9[a] |
| RBO | 245.9±3.8[a] | 448.4±7.5[a] | 335.6±4.1[a] |
| SL with CLO | 853.0±5.9[d] | 792.2±13.0[d] | 547.2±9.6[c] |
| SL with LSO | 785.2±7.8[c] | 771.8±11.3[c] | 557.1±8.4[c] |
| Blend with CLO | 862.4±9.4[d] | 756.7±12.9[b] | 548.9±7.4[c] |
| Blend with LSO | 760.4±10.5[b] | 752.0±12.2[b] | 480.5±6.6[b] |

Values are mean ± SD of 6 rats.

Values not sharing a common superscript within the column are statistically significant at $p < 0.05$

$Na^+/K^+$, $Mg^{2+}$ and $Ca^{2+}$ ATPase activities were measured as the difference between inorganic phosphate released in the presence of ouabain and EGTA respectively in RBC.

Dietary group legends are as described in table 5.1.

Platelet aggregation in rats fed structured lipids enriched with n-3 PUFA

**Platelet aggregation is an important physiological event, which has implications in thrombosis. Structured lipids enriched with n-3 PUFA fed rats showed a significant decrease in percent aggregation and rate of aggregation of platelets. The percent of ADP induced platelet aggregation was decreased in rats fed SL and blends with CLO by 19.3 and 13 % respectively, whereas, the collagen induced aggregation was decreased by 23 and 9 % respectively compared to rats fed RBO (Table 6.5 and Figure 6.4 and 6.5). Both the SL with LSO and CLO have shown significant decrease in platelet aggregation as compared to blends with LSO and CLO. In rats fed SL and blends with CLO, there was a slight decrease in ADP induced platelet aggregation but it was statistically significant. The rate of**

**ADP induced platelet aggregation in rats fed n-3 fatty acids containing SL was lowered by 40–45 % as compared to RBO. On the other hand the rate of collagen induced platelet aggregation was found to be lower by 40 % in rats fed n-3 fatty acid rich SL.**

**Table 6.5: Platelet aggregation in rats fed structured lipids enriched with n-3 PUFA**

| Groups | ADP induced | | Collagen induced | |
|---|---|---|---|---|
| | Percent aggregation | Rate of aggregation | Percent aggregation | Rate of aggregation |
| GNO | 55.4±3.18$^{d}$ | 5.9±0.59$^{d}$ | 42.0±3.32$^{d}$ | 5.1±0.53$^{d}$ |
| RBO | 50.3±2.91$^{c}$ | 5.0±0.79$^{c}$ | 38.8±2.41$^{c}$ | 4.2±0.64$^{c}$ |
| SL with CLO | 42.2±2.81$^{ab}$ | 3.5±0.67$^{ab}$ | 29.8±1.52$^{a}$ | 2.6±0.75$^{a}$ |
| SL with LSO | 41.3±4.25$^{a}$ | 3.4±0.52$^{a}$ | 31.5±3.35$^{a}$ | 2.4±0.83$^{a}$ |
| Blend with CLO | 45.5±6.25$^{b}$ | 3.9±0.62$^{b}$ | 35.3±4.31$^{b}$ | 3.1±0.63$^{b}$ |
| Blend with LSO | 43.5±2.26$^{b}$ | 3.7±0.42$^{b}$ | 34.1±2.39$^{b}$ | 3.3±0.93$^{b}$ |

Values are mean ± SD of 6 rats.

Values not sharing a common superscript within the column are statistically significant at $p < 0.05$

Dietary group legends are as described in table 5.1.

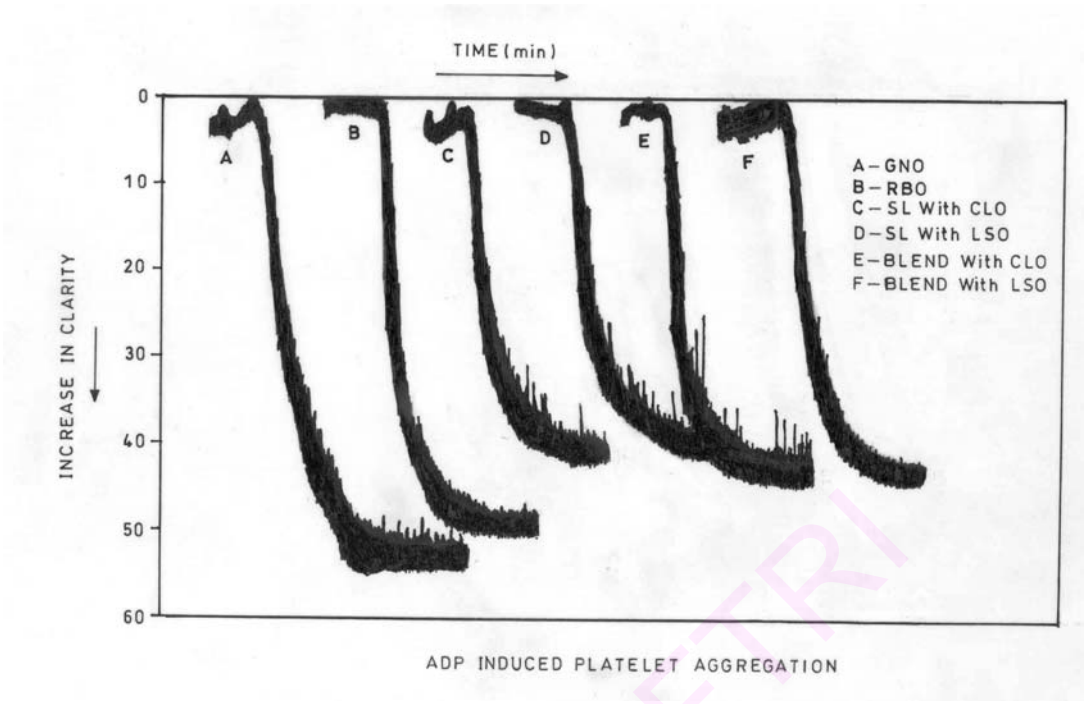


**Figure 6.4: ADP induced platelet aggregation in rats fed RBO rich in n-3 PUFA**

Dietary group legends are as described in table 5.1

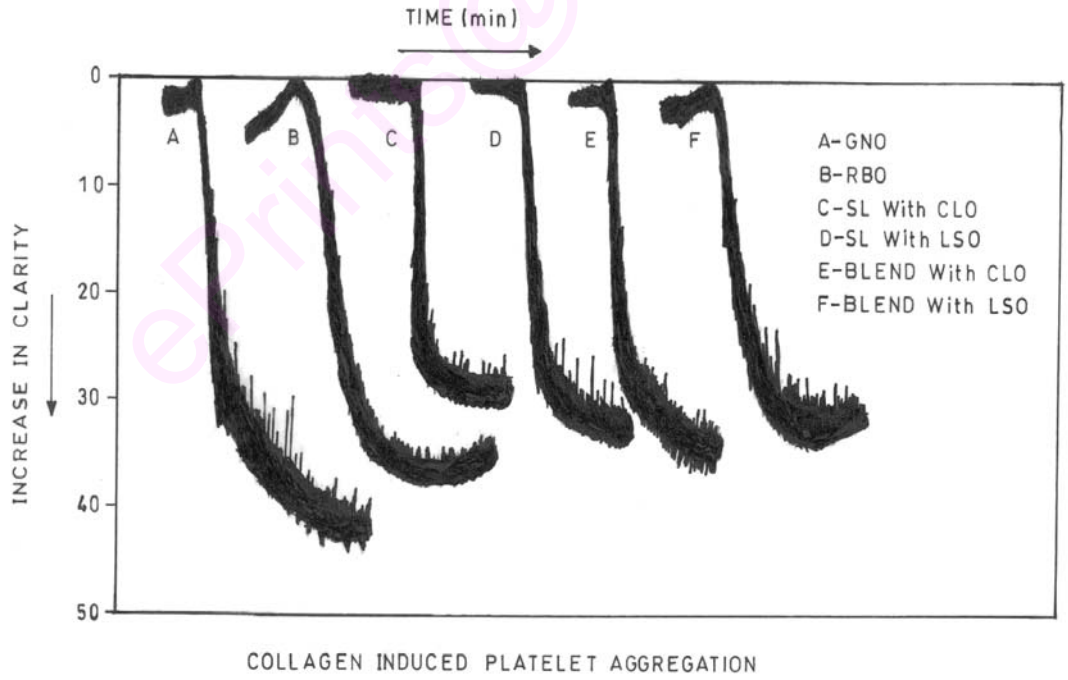


**Figure 6.5: Collagen induced platelet aggregation in rats fed RBO rich in n-3 PUFA**

Dietary group legends are as described in table 5.1

**Discussion**

Population consuming fish or non vegetarian diet can obtain long chain n-3 PUFA. Since plants cannot convert ALA to long chain n-3 PUFA, vegetarians relying on plant based diets must totally depend on endogenous synthesis of long chain n-3 PUFA from ALA by desaturation and elongation (Brenna, 2002). It has been shown that intake of n-3 PUFA by Indians' are lower than the desired level (Achaya, 1995). SL is one of the rout through which n-3 PUFA can be supplied to the needy people. Therefore, to meet the n-3 PUFA requirement of the population consuming vegetarian diet, rice bran oil was enriched with ALA from linseed oil in one case and in another, long chain n-3 PUFA from fish oil were incorporated into RBO. These SLs when fed to rats enhanced the n-3 PUFA levels in serum, liver and other tissues (chapter V). The preformed EPA+DHA from SL and blends with CLO were more effective in enhancing the levels of long chain n- 3 PUFA in serum and various other tissues of rats compared to that derived from ALA in LSO formulation.

The long chain n-3 PUFA are susceptible for lipid peroxidation. It is known that susceptibility of fatty acids to lipid peroxidation increase in proportion to its degree of unsaturation but the inherent antioxidant enzyme systems protect cells from damage caused by peroxidation (Huang and Fwu 1993; L'Abbe *et al*, 1991). Hence lipid peroxidation and antioxidant enzyme activities were monitored after feeding n-3 PUFA enriched SLs and blends in experimental rats. We have observed a significant increase in the lipid peroxides in the liver homogenate of rats fed with n-3 PUFA containing SLs and blends. Lipid peroxidation is known to be initiated by an attack on unsaturated fatty acids by reactive oxygen species. The radical scavenging enzyme, superoxide dismutase (SOD) is known to generate hydrogen peroxide from superoxide anions. The hydrogen peroxide in turn leads highly reactive hydroxyl radicals by Fenton or Haberweis reactions. Hence SOD plays a vital role in the generation of reactive oxygen

species. In our study the activity of SOD was comparable in control and experimental animals except in SL with LSO group and hence may not be enhancing the oxygen radical cascade that feeds into lipid peroxidation in n-3 fatty acid fed animals. They were also shown to act as proliferators for peroxisomes and mitochondria (Reddy and Lalwani, 1983). The crucial enzyme of β - oxidation, fatty acyl CoA oxidase produces hydrogen peroxide increasing oxidative stress (Froyland *et al*, 1998). This needs to be counteracted. In this context catalase plays a crucial role. In the present study, we observed an increased activity of catalase following feeding of n-3 PUFA enriched formulations. The increased activity of catalase may indicate an effective means of removing $H_2O_2$ that may be generated in the cells. If the increased catalase activity is not sufficient to decompose $H_2O_2$, it may lead to increased cellular peroxides and also may influence the glutathione peroxidase activity (Bulur *et al*, 1986). Earlier reports have also shown that n-3 fatty acids can increase the mRNA expression of catalase enzyme and strengthens the antioxidant status (Aguilera, 2003). However, this may not be sufficient to protect highly unsaturated EPA+DHA which underscore the need for external antioxidants when highly unsaturated lipids are fed (Pulla Reddy and Lokesh, 1994). A significant increase in glutathione transferase and glutathione reductase activity were observed in the rats fed experimental diets. Glutathione transferase can act as selenium independent glutathione peroxidase that can reduce a variety of organic hydroperoxides but not hydrogenperoxide and may partly compensate for the lower glutathione peroxidase activity. Hydroperoxides influence the cyclooxygenase activity, which produces thromboxane, a potent platelet-aggregating factor (Piche and Mahadevappa, 1990). Hydorperoxides formation depends on the antioxidant status of the tissues. The present study indicated that antioxidant status was balanced by an increase in catalase, glutathione tranferase and glutathione reductase activity following the feeding of structured lipids rich in n-3 PUFA. Structured lipids in n-3 PUFA also

lowered the arachidonic acid levels in platelets, which is a substrate for thromboxane production. The $PGI_3$ produced by n-3 PUFA is potent anti-aggregator of platelets and hence lower the platelets aggregation. Platelet aggregation is initiated by specific extracellular signals and the platelet plasma membranes play a major role in signal transduction. Plasma membrane associated proteins are involved in changes of platelet morphology of lipid trans-billayer asymmetry (Heemskerk *et al*, 1989). Various studies have reported inhibition in platelet aggregation by feeding n-3 PUFA rich diets to rats (Ramaprasad *et al*, 2005). Both the precursor ALA and preformed EPA+DHA exhibited similar effect on platelet aggregation.

It has been well established that dietary lipids influence the membrane lipid compositions (Spector and York, 1985). Membrane lipids regulate the activities of membrane bound enzymes (Murphy, 1990). The different n-3 PUFA enriched supplementations used either as a precursor or as a source of very long chain PUFA have different effects on $Na^+/K^+$ ATPase and $Mg^{2+}$ ATPase activities in erythrocyte membranes. It has been shown that a deficiency in n-3 PUFA is associated with a variation in $Na^+/K^+$ ATPase activities (Gerbi *et al*, 1999a; 1999b). In the present investigation, we have studied the effect n-3 PUFA on two ATPase activities, $Na^+/K^+$ ATPase and $Mg^{2+}$ ATPase which are membrane bound proteins. It was observed that n-3 PUFA in the diet increased the $Na^+/K^+$ ATPase and $Mg^{2+}$ ATPase activities. Djemli-Shipkolye *et* al (2002) reported that supplementation of n-3 PUFA in diabetic rats increased the $Na^+/K^+$ ATPase and $Mg^{2+}$ ATPase activities of RBC membranes. n-3 PUFA enriched supplementations not only cause modifications in the membrane environment of the enzyme but seem also to modify the pool of enzymes present in the membrane.

The results of our study indicated that it is possible to enrich RBO with n-3 PUFA and to supplement adequate amount of these essential fatty acids in diet. This

also significantly increased the n-3 PUFA levels platelets. Though, lipid peroxidation was increased to certain extent following n-3 fatty acid formulation feeding, the increase in the activity of some enzymes of antioxidant defense system may partly counter the effect of lipid peroxidation. n-3 PUFA enriched RBO increased the $Na^{+}/K^{+}$ and $Ca^{2+}/Mg^{2+}$ ATPase activities and also lowered platelet aggregation in a beneficial manner and there by reduce the risk factors for thrombosis.

# SUMMARY AND CONCLUSIONS

1. Structured lipids were synthesized from RBO enriched with stearic acid (18:0) by lipase catalyzed acidolysis reaction in organic solvent.
2. Incorporation of fatty acids into RBO was influenced by factors like incubation time, temperature, enzyme concentration and substrates molar ratio.
3. Enzymatic acidolysis was used to increase the level of 18:0 from 2 to 48 % and new triglyceride species were observed in the modified RBO.
4. RBO with 48 % stearic acid retained most of its nutraceuticals like oryzanol, tocopherols and tocotrienols.
5. DSC studies confirmed that RBO is liquid at normal ambient temperature, whereas modified RBO showed solids at all temperatures up to 40 $^{0}$C. Among modified RBO the sample with higher 18:0 incorporation showed higher SFC compared to those of RBO with lower 18:0 incorporation.
6. Crystallization studies confirm the melting properties as they showed two distinct peaks indicating heterogeneity of triglycerides in modified RBO.
7. Dietary fat used across the world is not balanced in respect of their fatty acid composition.
8. Various studies indicate that there is an increase in the consumption of dietary n-6 fatty acids resulting in the increase of n-6 to n-3 ratio.
9. Based on the recommendations of the recent dietary guidelines, the intake n-3 PUFA is not adequate in Indian population.
10. Supplementation of n-3 PUFA through edible oils is the best option to provide n-3 PUFA to population.
11. However, the direct use of linseed oil or cod liver oil as single oil or blend has certain limitations with respect to acceptability and sensory quality, hence structured lipids rich in n-3 PUFA from RBO can serve as a vegetarian source of n-3 PUFA with higher acceptability.

12. ALA from linseed oil could be a source of n-3 PUFA for vegetarian population.
13. Structured lipids rich in n-3 PUFA were synthesized from RBO by lipase catalyzed acidolysis reaction in organic solvent and reactions were scaled up from 100 mg to 100 g levels with comparable degree of modification.
14. Response surface methodology was used optimize reaction variables and predict conditions for desired levels of incorporation of n-3 PUFA from linseed oil and cod liver oil into rice bran oil.
15. Structured lipids from RBO enriched with ALA and EPA+DHA were fed to experimental animals for the period of 60 days.
16. RBO rich in n-3 PUFA lowered the serum and liver lipids that have an important role in cardiovascular disease.
17. Feeding structured lipids and blends rich in ALA from RBO as a source of dietary fat increased ALA and its long chain metabolites EPA and DHA levels in serum, liver, brain and heart tissue.
18. However, feeding structured lipids and blends from RBO rich in preformed EPA+DHA increased EAP and DHA levels compared to that obtained after feeding structured lipids and blends rich in ALA in serum, liver, brain, heart and adipose tissue.
19. ALA from linseed oil was elongated and desaturated to long chain n-3 PUFA in different tissues to different levels. The n-3 PUFA showed beneficial effects by lowering serum and liver lipid levels.
20. The lipid peroxide levels in serum, liver homogenate and also in platelets were increased in rats fed structured lipids and blends rich in n-3 PUFA as compared to rats fed RBO and GNO.

21. However, the activities of antioxidant enzymes like catalase, glutathione reductase and glutathione transferase were increased as compared to RBO and GNO.
22. The activity of membrane bound enzymes like $Na^{+}/k^{+}$ and $Ca^{2+}/Mg^{2+}$ ATPase were increased in rats fed structured lipids blends rich in n-3 PUFA.
23. The platelet aggregation induced by ADP and collagen was decreased in rats fed structured lipids and blends rich in n-3 PUFA compared to those fed fat devoid of n-3 PUFA.
24. These studies indicated that RBO be used as a alternate source for solid fat and can offer additional health benefits if it is enriched with n-3 PUFA either from linseed oil or cod liver oil.
25. It is possible to enrich RBO with n-3 PUFA from vegetable oil (linseed oil) or cod liver oil either through structured lipids or blending these oils with RBO. This also beneficially altered serum and tissue lipids having a bearing on cardiovascular function.

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# LIST OF PUBLICATIONS, PATENT, AND POSTER (SYMPOSIA) PRESENTATIONS

## PUBLICATIONS

1. **Rajni Chopra,** Sunki Reddy Yella Reddy, Kari Sambaiah. **2008**. Structured lipids from rice bran oil and stearic acid using immobilized lipase from *Rhizomucor miehei*. **Eur. J. Lipid Sci. Technol. 110**:32-39.

2. **Rajni Chopra** and Sambaiah, K. **2008**. Incorporation of long chain n-3 PUFA into rice bran oil by enzymatic acidolysis. **J. Food Sci. Technol. (accepted).**

3. **Rajni Chopra,** Rastogi, N.K., and Sambaiah, K. Enrichment of rice bran oil by enzymatic acidolysis with α- linolenic acid obtained from linseed oil: optimization by response surface methodology. **J. Am. Oil Chemist's Soc**. (communicated).

4. **Rajni Chopra** and Sambaiah, K. Effects of structured lipids from rice bran oil enriched with long chain n-3 PUFA on serum and liver lipid of rats. **Lipids** (communicated and under revision).

5. **Rajni Chopra** and Sambaiah, K. Effect of dietary long chain n-3 PUFA fed rats on their antioxidant status and platelet aggregation. **Prostaglandins, Leukotrienes and Essential Fatty Acids** (communicated).

## PATENT

**Rajni Chopra**, S Y Reddy, B R Lokesh and Kari Sambaiah. A process for preparation of non-hypercholesterolemic stearic acid rich solid fat from rice bran oil. Indian Patent No. NF/ 67- 2006.

## POSTERS PRESENTED IN NATIONAL/ INTERNATIONAL SYMPOSIA

1. **Rajni Chopra** and Sambaiah K. “Synthesis of structured lipids enriched with stearic acid from rice bran oil” presented at 16th Indian Convention of Food Scientist and Technologists (ICFOST) held during 9-12, Dec. 2004.

2. **Rajni Chopra** and Sambaiah K. “Identification of triglyceride molecular species of structured lipids synthesized from rice bran oil rich in stearic acid” presented at 17th Indian Convention of Food Scientist and Technologists (ICFOST) held during 9-10, Dec. 2005.

3. **Rajni Chopra**, Rastogi N. K. and Sambaiah K. “Structured lipids from Rice bran oil by enzymatic acidolysis” presented at 18th Indian Convention of Food Scientist and Technologists (ICFOST) held during 15-16, Nov. 2006.

4. **Rajni Chopra**, Rastogi N. K. and Sambaiah K. “Enrichment of Rice Bran Oil by Enzymatic acidolysis with linolenic acid obtained from linseed oil” presented at 75th Society of Biological Chemists- India (SBC-I) held during 8- 11, Dec. 2006.