DEVELOPMENT AND NUTRITIONAL EVALUATION OF MILK BASED PRODUCTS ENRICHED WITH OMEGA-3 FATTY ACIDS

A Thesis submitted to the

UNIVERSITY OF MYSORE

For the award of the degree of

DOCTOR OF PHILOSOPHY

In

BIOCHEMISTRY

By

T.R. RAMAPRASAD, M.Sc.,

DEPARTMENT OF LIPID SCIENCE & TRADITIONAL FOODS

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE

MYSORE- 570 020, INDIA

APRIL- 2005

Date

Dr. B.R. Lokesh
Head,
Department of Lipid Science & Traditional Foods

CERTIFICATE

This is to certify that the thesis entitled " DEVELOMENT AND NUTRITIONAL EVALUATION OF MILK BASED PRODUCTS ENRICHED WITH OMEGA-3 FATTY ACIDS" submitted by Mr. T.R. RAMAPRASAD is the result of research work carried out by him in the Department of Lipid Science & Traditional Foods, CFTRI, Mysore under my guidance during the period of 2002-2005.

B.R. LOKESH
Guide

DECLARATION

I hereby declare that the thesis entitled "DEVELOPMENT AND NUTRITIONAL EVALUATION OF MILK BASED PRODUCTS ENRICHED WITH OMEGA-3 FATTY ACIDS" submitted to the University of Mysore for the award of the degree of DOCTOR OF PHILOSOPHY in BIOCHEMISTRY is the result of research work carried out by me under the guidance of Dr. B. R. Lokesh, Head, Department of Lipid Science & Traditional Foods, Central Food Technological Research Institute, Mysore during the period 2000-2005.

I further declare that these results have not been submitted for any other degree or fellowship.

Place: Mysore

Date: **T.R. RAMAPRASAD**

ACKNOWLEDGEMENTS

I am sincerely grateful to

My mentor and guide, Dr. B.R. Lokesh, Head, Department of Lipid Science & Traditional Foods, C.F.T.R.I. Mysore for his invaluable guidance, keen interest and for enabling me to grow with the freedom of thought and expression throughout my stay in his laboratory.

The Director, CFTRI, for giving me an opportunity to work for my PhD programme at CFTRI, Mysore.

Dr. S.G. Bhat, Head Department of Biochemistry & Nutrition for his encouragement.

Dr. K. Sambaiah, for his helpful suggestions, constructive criticism and constant support throughout my stay at CFTRI.

Dr. V. Baskaran, Dr. T.P. Krishnakantha, Dr. S. Divakar and Dr. K. Srinivasan for their useful suggestions on various aspects pertaining to my research.

My Friends at CFTRI, Mr. Vidyashankar, Miss. Anitha, Miss. Reena, Miss. Rajni, Miss. Shrilatha, Miss. Jessy Sunitha, Mr. Suresh, Mr. Manjunath Mr. Murali Hegde, Mr. Raju, Mr. Lakshminarayan, Mr. Madhukumar, Mr. Chandrashekar, Mrs. Hemalatha, Miss. Madhavi and Miss. Chithra for the little drops of kindness that made a mighty impact.

Dr. P. Saibaba and other staff of the Animal house for helping me in Conducting the dietary studies.

Mr. Jayaprakash, Mr. Venkateshmurthy, Mr. Shivakumar and Mr. Mahesh, for their help in pilot plant.

The staff of the Central Instrumentation Facility and Services for helping me in conducting analytical part of my research.

The CSIR, New Delhi for the award of Senior Research Fellowship from 2002-2005.

My parents, sisters, brother-in-law and prabhanjana for their continued support and encouragement throughout my career.

T.R. RAMAPRASAD

.

Dedicated to

My Parents, who encouraged me

My Guide, who enabled me and

My friend, who supported me

CONTENTS

Page No.

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBRIVIATIONS

Chapter I INTRODUCTION

General Introduction

Aim and Scope of the Present Investigation

Chapter II MATERIALS AND METHODS

Chapter III RELATIVE EFFECT OF LINOLENIC ACID AND EICOSAPENTAENOIC PLUS DOCOSAHEXAENOIC ACID ON SERUM AND TISSUE LIPIDS

Introduction

Results

Discussion

Chapter IV SUPPLEMENTATION AND DELIVERY OF OMEGA-3 FATTY ACIDS THROUGH SPRAY-DRIED MILK

Introduction

Results

Discussion

Chapter V INFLUENCE OF SPRAY-DRIED MILK SUPPLEMENTED WITH OMEGA-3 FATTY ACIDS ON CHOLESTEROL BIO SYNTHESIS AND BILIARY SECRETION OF LIPIDS

Introduction

Results

Discussion

Chapter VI INFLUENCE OF SPRAY-DRIED MILK SUPPLEMENTED WITH OMEGA-3 FATTY ACIDS ON ANTIOXIDANT ENZYME ACTIVITIES, PLATELET AGGREGATION AND SERUM PROSTAGLANDINS

Introduction

Results

Discussion

Chapter VII STORAGE RELATED STUDIES ON SPRAY-DRIED MILK SUPPLEMENTED WITH OMEGA-3 FATTY ACIDS

Introduction

Results

Discussion

Chapter VIII GENERAL DISCUSSION AND SUMMARY

REFERENCES

LIST OF TABLES

Page No.

CHAPTER I

1.1 Approximate fatty acid composition of visible fats

1.2 Invisible fat and fatty acids in plant foods

1.3 Fat, fatty acid and cholesterol content of animal foods

1.4 Quality of total fat in Indian diet

CHAPTER II

2.1 Composition of basal AIN-76 purified diet

CHAPTER III

3.1 Fatty acid composition of diets

3.2 Growth and organ weights of rats fed omega-3 fatty acids at incremental levels

3.3 Serum fatty acid profile of rats fed omega-3 fatty Acids at incremental levels

3.4 Serum lipid profile of rats fed omega-3 fatty acids at incremental levels

3.5 Liver tissue fatty acid profile of rats fed omega-3 fatty acids at incremental levels

3.6 Liver lipid profile of rats fed omega-3 fatty acids at incremental levels

3.7 Heart tissue fatty acid profile of rats fed omega-3 fatty acids at incremental levels

3.8 Brain tissue fatty acid profile of rats fed omega-3 fatty acids at incremental levels

3.9 Adipose tissue fatty acid profile of rats fed omega-3 fatty acids at incremental levels

3.10 Calculated levels of EPA+DHA found in different tissues by feeding 100mg of LNA or EPA+DHA in the diet

CHAPTER IV

4.1 Fatty acid composition of diet

4.2 Effect of feeding spray-dried milk supplemented with omega-3 fatty acid on growth parameters

4.3 Effect of feeding spray-dried milk supplemented with omega-3 fatty acid on organs weight

4.4 Serum fatty acid profile of rats fed spray-dried milk supplemented with omega-3 fatty acids

4.5 Serum lipid profile of rats fed spray-dried milk with omega-3 fatty acids

4.6 Liver fatty acid profile of rats fed spray-dried milk supplemented with omega-3 fatty acids

4.7 Liver lipid profile of rats fed spray-dried milk supplemented with omega-3 fatty acids

4.8 Heart tissue fatty acid profile of rats fed spray-dried milk supplemented with omega-3 fatty acids

4.9 Brain tissue fatty acid profile of rats fed spray-dried milk supplemented with omega-3 fatty acids

4.10 Adipose tissue fatty acid profile of rats fed spray-dried milk supplemented with omega-3 fatty acids

CHAPTER V

5.1 Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on HMG Co A reductase activity liver microsomal lipids

5.2 Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on fatty acid composition of rat liver microsomes

5.3 Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on bile secretion and composition

5.4 Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on fatty acid composition of bile

CHAPTER VI

6.1 Fatty acid composition of liver of rats fed spray-dried Milk supplemented with omega-3 fatty

6.2 Fatty acid composition of platelets of rats fed spray-dried Milk supplemented with omega-3 fatty acids

6.3 Platelet aggregation in rats fed spray-dried milk supplemented with omega-3 fatty acids

CHAPTER VII

7.1 Proximate composition of spray-dried milk supplemented with omega-3fatty acids

7.2 Effect of storage temperature and period on peroxide value of spray-dried milk supplemented with omega-3 fatty acids

7.3 Effect of storage temperature and period on fatty acid composition of spray-dried milk supplemented with GNO

7.4 Effect of storage temperature and period on fatty acid composition of spray-dried milk supplemented with LSO

7.5 Effect of storage temperature and period on fatty acid composition of spray-dried milk supplemented with FO

7.5 Sensory property of spray-dried milk supplemented with omega-3 fatty acids

7.6 Physical properties of spray-dried milk supplemented with omega-3 fatty acids

LIST OF FIGURES

Page No.

CHAPTER I

1.1 Hypothetical scheme of the relative percentages of fat and different fatty acid families in human nutrition

1.2 Biosynthesis of polyunsaturated fatty acids

CHAPTER II

2.1 Spray-drying of GNO, LSO or FO supplemented milk

CHAPTER III

3.1 Serum omega-3 fatty acids level of rats fed incremental amount of LNA

3.2 Serum omega-3 fatty acids level of rats fed incremental amount of EPA plus DHA

3.3 Liver omega-3 fatty acids level of rats fed incremental amount of LNA

3.4 Liver omega-3 fatty acids level of rats fed incremental amount of EPA plus DHA

3.5 Heart tissue omega-3 fatty acids level of rats fed incremental amount of LNA

3.6 Heart tissue omega-3 fatty acids level of rats fed incremental amount of EPA plus DHA

3.7 Brain tissue omega-3 fatty acids level of rats fed incremental amount of LNA

3.8 Brain tissue omega-3 fatty acids level of rats fed incremental amount of EPA plus DHA

3.11 Adipose tissue omega-3 fatty acids level of rats fed incremental amount of LNA

3.12 Adipose tissue omega-3 fatty acids profile of rats fed incremental amount of EPA plus DHA

CHAPTER VI

6.1 Lipid peroxides level in liver homogenate of rats fed Spray-dried milk supplemented with omega-3 fatty acid

6.2 SOD activity in liver homogenate of rats fed spray-dried milk supplemented with omega-3 fatty acids

6.3 Catalase activity in liver homogenate of rats fed spray-dried milk supplemented with omega-3 fatty acids

6.4 Glutathione peroxidase activity in liver homogenate of rats fed spray-dried milk supplemented with omega-3 fatty acids

6.5 Glutathione transferase activity in liver homogenate of rats fed spray-dried milk supplemented with omega-3 fatty acids

6.6 Lipid peroxides level in platelets of rats fed spray-dried milk supplemented with omega-3 fatty acids

6.7 ADP and collagen induced platelet aggregation in rats fed milk supplemented with omega-3 fatty acids

6.8 Serum TXB_2 level in rats fed spray-dried milk supplemented with omega-3 fatty acids

6.9 Serum 6-keto $PGF_{1\alpha}$ level in rats fed spray-dried milk supplemented with omega-3 fatty acids

LIST OF SYMBOLS AND ABBRIVIATIONS

α	Alpha
β	Beta
^{0}C	Degree Celsius
CHD	Coronary heart disease
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FO	Fish oil
Fig.	Figure
g	Gram
GC	Gas chromatography
GNO	Groundnut oil
HDL	High density lipoprotein
HMG CoA	Hydroxy methyl gularyl Coenzyme A
Hrs	Hours
LDL	Low density lipoprotein
LNA	Linolenic acid
LA	Linoleic acid
LSO	Linseed oil
ND	Not detected
µg	Micro gram
µL	Micro litre

MDA	Malondialdehyde
min	Minutes
mL	Milliliter
mg	Milligram
ng	Nanogram
nm	Nanometer
OD	Optical density
PG	Prostaglandin
PUFA	Polyunsaturated fatty acids
SD	Standard deviation
SFA	Saturated fatty acids
SOD	Superoxide dismutase
TG	Triglyceride
TX	Thromboxane
v/v	Volume by volume
VLDL	Very low density lipoprotein

CHAPTER I

Introduction

Several sources of information suggest that man evolved on a diet with a ratio of omega-6 to omega-3 fatty acid of approximately one. Whereas today, this ratio is approximately 10:1 to 25:1 indicating that diets are deficient in omega-3 fatty acids compared with the 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-gatherer and agriculture (Fig 1.1). The consumption of trans fatty acids has also been increased in the past 50 years.

Intake of omega-3 fatty acids is much lower today because of the decrease in fish consumption and the industrial production of animal feeds rich in grains containing omega-6 fatty acids, leading to production of meat rich in omega-6 and poor in omega-3 fatty acids (Crawford 1968). The same is true for cultured fish (Van Vliet and Katan 1990) and eggs (Simopolous and Salem 1989). Even cultivated vegetables contain lower amounts of omega-3 fatty acids than do plants in the wild (Simopolous et al 1999).

Omega-6 and omega-3 fatty acids are not inter convertible in the human body and are important components of practically all cell membranes. Today we know that omega-3 fatty acids are essential for normal growth and development. They play an important role in the prevention and treatment of coronary artery diseases, hypertension, diabetes, arthritis, other inflammatory and autoimmune disorders and cancer (Simopolous et al 1986; Galli and Simopolous 1989; Salem et al 1996).

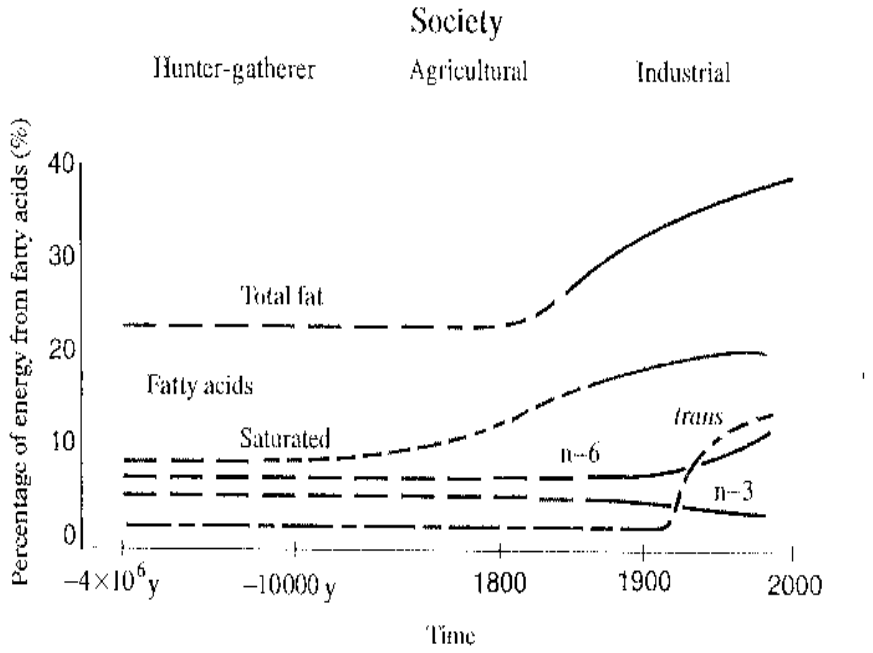


Fig 1.1. Hypothetical scheme of the relative percentages of fat and different fatty acid families in human nutrition (Simopolous 1991)

Dietary fats and oils

Most discussions of fats and health focus on the deleterious effects of these essential nutrients. In such cases, we are really discussing the possibly harmful effects of excess fat and oils.

Dietary fat (vegetable or animal) consists of mainly triglycerides and other minor lipids like phospholipids, free fatty acids, phytosterols, glycolipids and sulpholipids. Triglycerides make up for the majority of the lipids in edible fats and oils. They are composed of glycerol esters of fatty acids. They contain essential fatty acids that are metabolized eventually to provide eicosanoids, substances that possess hormonelike activity and thus may regulate body function.

Natural fats and oils vary in their nutritional and physical properties depending on the type of fatty acids attached to the glycerol backbone. Fats (lipids) supply energy, (fat supplies 9 cal/g

compared to 4 cal/g for protein or carbohydrates), support structural aspects of the body, and provides substances that regulate physiological processes. Fat also contributes to the palatability and flavor of food, hence the enjoyment of eating. Adipose tissue, which is the repository of most of our body fats, serves as an energy reservoir as a heat conservator, and as a shock absorber. Fat is also the transport vehicle for vitamins A, D, E, and K.

Cholesterol, which has absorbed the brunt of the antifat attack, is a compound that is required for life. It is not essential in the sense of essential fatty acids, since the body can synthesize it, but it is a crucially important component of our biological economy. Cholesterol comprises about 0.2% of normal body weight. Most of it (about 33%) is in the brain and nervous system, where its function has been probed beyond suggesting that its major activity is as an insulator. Almost another one-third of body's cholesterol is in muscle, where it is a structural component. Cholesterol is the parent substance for vitamin D_2, bile acids, adrenocortico hormones, and sex hormones. Thus, it is one of the important biological substances.

Lipid Classifications

Classification of lipid structures is made based on physical properties at room temperature (oils are liquid and fats are solid), their polarity (polar, non polar and neutral lipids), their essentiality for humans (essential and nonessential fatty acids) or their structure (simple or complex). Neutral lipids include fatty acids, alcohols, glycerides, and sterols, while polar lipids include glycerophospholipids and glyceroglycolipids. The separation into polarity classes is rather arbitrary, as unlike long chain fatty acids some short chain fatty acids are polar. Based on structure, lipids can be classified as derived, simple, or complex. The *derived lipids*

include fatty acids and alcohols, which are building blocks for the simple and complex lipids. *Simple lipids*, composed of fatty acids and alcohol components, include acylglycerols, ether acylglycerols, sterols and their esters and wax esters. In general terms simple lipids can be hydrolyzed to two different components, usually alcohol and an acid. Complex lipids include glycerophospholipids (phospholipids), glyceroglycolipids (glycolipids), and sphingolipids. These structures yield three or more different compounds on hydrolysis.

Fatty acids and their types

Fatty acids are components of phospholipids, triglycerides, and cholesterol esters and also have structural, energetic and metabolic functions. Fatty acids are distinguished from each other on the basis of their chemical structure. All have a chainlike 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. 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.

Fatty acids serve at least three vital functions: A few, such as linoleic acid (LA), α linolenic acid (LNA) are essential nutrients; others, particularly the short-chain fatty acids, provide energy; long-chain fatty acids are structural components of all cell membranes. Fatty acids seldom exists themselves but are chemically incorporated into larger molecules such as triglycerides and phospholipids.

The main distinguishing features of fatty acids are as follows.

Chain length

Short-chain fatty acids have fewer than 8 carbon atoms; medium-chain fatty acids have from 8 to 12 carbon atoms, whereas those with more than 12 carbons are considered long chain (National Research Council 1989). Short-chain fatty acids are water soluble and absorbed directly from the intestine in to the blood stream. They are usually metabolized for immediate energy needs and are not abundant in most foods.

The long-chain fatty acids with 14 or more carbons have received considerable attention from nutrition and health aspects. These are stored in membranes as components of phospholipids and in adipose tissue as triglycerides. In addition to providing energy, long-chain polyunsaturated fatty acids are constituents of cell membranes where they are available as precursors for several metabolic pathways. They also have pronounced effect on protein function (Murphy 1990).

Saturation

Besides chain length, fatty acids are distinguished by the number of hydrogen atoms they carry. The saturated fatty acids contain the maximum number of hydrogen atoms they can hold. A fatty acid becomes unsaturated when a pair of hydrogen atoms is removed, thereby creating a double bond between the adjacent carbon atoms where the hydrogens is removed. Specific enzymes carry out desaturation process but only at certain carbon sites. Thus the location of double bonds in a fatty acid is tightly controlled.

The increasing number of double bonds in a fatty acid increases its unsaturation. Fatty acids with one double bond are

called monounsaturated fatty acids (MUFA). Those with two or more double bonds are polyunsaturated fatty acids (PUFA).

Cis and _Trans_ isomers

A third distinguishing characteristic of fatty acids is the orientation of the molecule at the site of a double bond. When both sections of the molecule lie on the same side of the double bond, it results in a *cis* configuration. This creates a bend or kink in the fatty acids chain, making the molecule more flexible. Most naturally occurring fatty acids in plants and seafoods have the *cis* configuration.

In the *trans* configuration, each section on either side of the double bond faces in the opposite direction. The *trans* configuration yields a straighter, more rigid molecule. Trans fatty acids are common in bacteria. They are also produced by ruminants and hence find their way into dairy products. *Trans* fatty acids are also formed when unsaturated fatty acids are commercially hydrogenated, as in the manufacture of margarine (Small 1986).

The synthesis and abundance of PUFAs

Despite the considerable variation that is possible in PUFA structure, there are only about 15 unsaturated fatty acids of nutritional importance to humans. These consist primarily of monounsaturated and methylene-interrupted PUFAs in the *cis* configuration. Virtually all the fatty acids consumed in normal diets are members of the omega-3, omega-6, omega-7, or omega-9 families of fatty acids. Non-methylene-interrupted and trans-configured fatty acids have been historically consumed in very small quantities, but the advent of modern food production may have enriched these fatty acids in the food supply. It is believed that the same enzymes catalyze the equivalent steps in omega-7, omega-9, omega-6 and omega-3 fatty acids pathways (Brenner

1977) and there is a competition among the substrates for the same enzyme system (Fig 1.2).

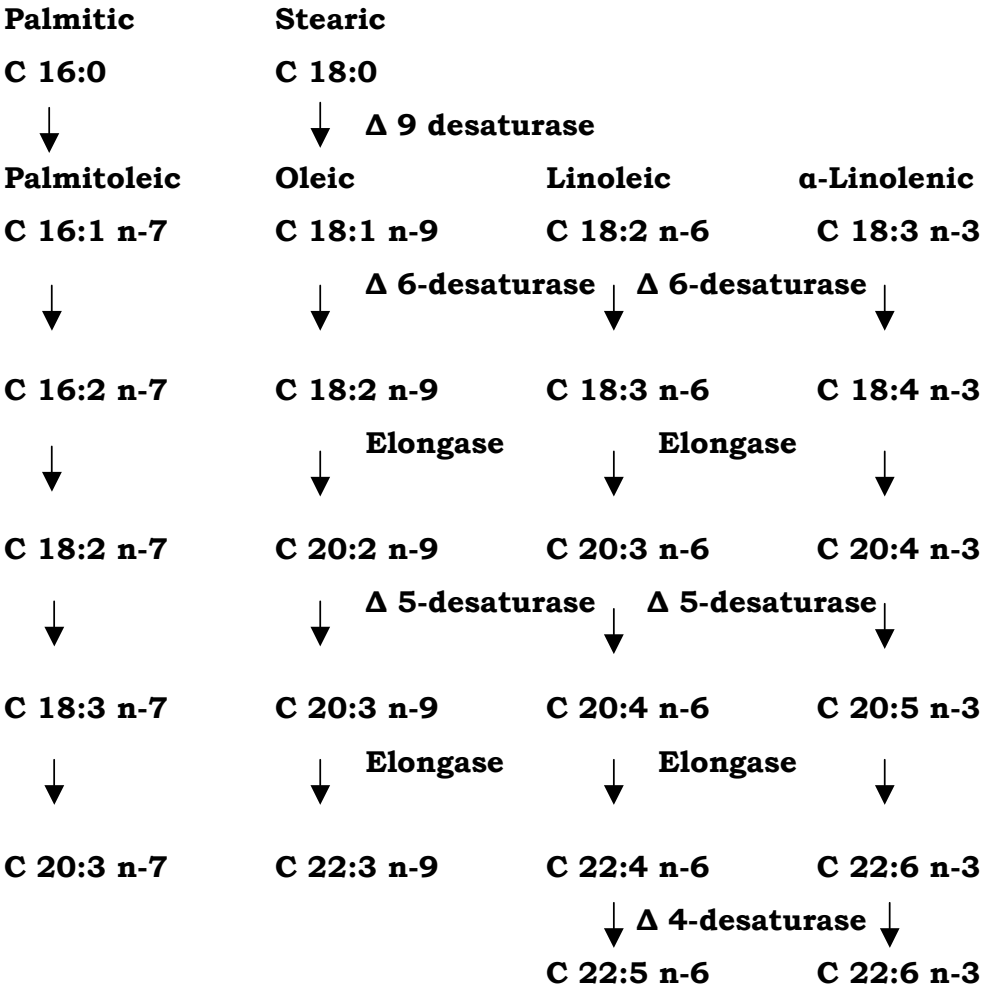


Fig 1.2 Biosynthesis of polyunsaturated fatty acids

The critical enzyme in these reactions is **Δ** 6-desaturase for which the greatest affinity appears to be conferred by the number of double bonds in C18 substrate. The primary unsaturated fatty acids in human nutrition are reviewed below.

A. Omega-7 fatty acids

Palmitoleic acid (16:1 n-7)

Palmitoleic acid is a minor component of both animal and vegetable lipids. Fish oil is particularly enriched in Palmitoleic acid, while some seed oil also represents a significant source of the fatty acids. Palmitoleic acid is produced de novo by both plants and animals by the delta-9 desaturation of palmitic acid.

***Cis*- vaccenic acid**

Cis-vaccenic acid is a major product of bacterial fatty acid synthesis, but is also present in some seed oils. *Cis*-vaccenic acid biosynthesis likely involves the elongation of palmitoleic acid. In animals, cis-vaccenic acid appears to be concentrated in the mitochondrial lipid cardiolipin (Wolff et al 1985).

B. Omega-9 fatty acids

Oleic acid (18:1 n-9)

Oleic acid is a delta 9 desaturase product of stearic acid and is produced de novo in plants, animals, bacteria and algae. Oleic acid is the most common unsaturated fatty acid and is the precursor for the production of most other polyunsaturated fatty acids. Plants produce both omega-3 and omega-6 PUFAs from oleic acid, and animals can elongate and desaturate oleic acid into a variety of omega-9 fatty acids. Olive oil a particularly rich dietary source, but most foods, especially nuts and butter, are rich in oleic acid.

Erucic acid (22:1 n-9)

Erucic acid is a long chain monounsaturated fatty acid found in plants, particularly in rapeseeds. This elongation product of oleic acid is an uncharacteristically long chain unsaturated fatty acids for plants. Erucic acid has been reduced in rapeseeds used for food oil production by plant breeding. In animals, dietary

erucic acid can be retro converted to form oleic acid via β-oxidation.

Mead acid (20:3 n-9)

Mead acid is hallmark of essential fatty acid deficiency and has the distinction of being the only major PUFA produced de novo by animals. In the absence dietary omega-6 and omega-3 fatty acids, the delta-6 desaturase converts oleic acid to 18:2 (n-9), which is further elongated and desaturated (delta-5) to form mead acid (Cook 1991). It has been speculated that mead acid compensates for the loss of omega-3 and omega-6 PUFA by increasing the unsaturation of animal cell membranes.

Other omega-9 fatty acids

The family of omega-9 fatty acids is derived exclusively from the production of oleic acid but can be converted by elongation, desaturation, β-oxidation, and so on. Other rare but naturally occurring omega-9 fatty acid include 17:2, 18:2, 20:2, 20:3, 22:3, 22:4, and 20:1 (Gunstone 1994).

C. Omega-6 fatty acids

Linoleic acid (18:2 n-6)

Linoleic acid (LA) is a primary product of plant PUFA synthesis. LA is produced de novo by plants, and in particular is enriched in seed oils. While nature produces LA at level fairly equitable with LNA, modern agriculture has greatly enriched LA in the food supply. While animals are incapable of producing LA, livestock are fed diets particularly rich in these fatty acids, and thus humans acquire a large portion of their LA from meats and also through seed oil. LA serves as a precursor for the production of the essential fatty acid arachidonic acid, as well as other omega-6 acyl species.

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. Natural sources include evening primrose oil, borage oil and black currant oil, while minute amounts can be found in animal tissue (Padley et al 1994). In animals, dietary LA is desaturated by the delta-12 desaturase to produce GLA an intermediate in the production of arachidonic acid. Interestingly, dietary gamma linolenic acid is not substantially converted to arachidonic acid. There has been a great deal of recent interest in dietary gamma linolenic acid for its antagonistic action of arachidonic acid metabolism. In part this is due to the kinetics of the conversion of gamma linolenic acid to arachidonic acid; but a more significant role probably involves eicosanoids produced directly from the immediate conversion product of GLA, dihomo-gamma linolenic acid.

Dihomo-gamma linolenic acid (20: 3 n-6)

The elongation product of LA, dihomo-gamma linolenic acid (DGLA) is a minor component of animal phospholipids. DGLA serves as the precursor for the formation of essential fatty acid arachidonic acid as well as for the prostaglandin E1 series. Dietary DGLA does not appear to be rapidly converted to arachidonic acid, and because of the prostglandins of E1 series have anti-inflammatory properties, DGLA has received attention as a potential therapeutic agents.

Arachidonic acid (20:4 n-6)

Exceedingly rare as a plant lipid, arachidonic acid is primarily the product of desaturation and elongation of LA in animals. Arachidonic acid is also produced in marine algae. Dietary LA is converted in to arachidonic acid in animals by the concerted activity of delta-6 desaturase, microsomal elongase and delta-5 desaturase. Arachidonic acid referred to as an essential

fatty acid as the precursor for the production of eicosanoids. It appears to be present in all tissues and is particularly enriched in ether linked phospholipid membrane pools.

Docosatrienoic acid (22:4 n-6)

Docosatrienoic acid is the direct elongation product of arachidonic acid and is present in minimal amounts in animal tissues. It has been reported that docosatrienoic acid is a substrate for peroxisomal retro conversion resulting in the formation of arachidonic acid (Hagve and Christophersen 1986).

Other omega-6 fatty acids

The family of omega-6 fatty acids is derived exclusively from the production LA, but can be converted by elongation, desaturation, β-oxidation, and so on. Other rare but naturally occurring omega-6 fatty acids include 15:2, 16:2, 20:2, 22:2, 22:3, 24:2, 25:2, 26:2, and 30:4 (Gunstone 1994).

D. Omega-3 fatty acids

α- linolenic acid (18:3 n-3)

α- linolenic acid is produced de novo by the delta 12 and delta 15 desaturation of oleic acid in plants. Along with LA, LNA constitutes one of the two primary PUFA products of plant fatty acid biosynthesis. It is primarily present in the leaves of plants but is also a component of seed oils. LNA serves as the metabolic precursor for the production of omega-3 fatty acids in animals. The success of the agricultural seed oils has caused a significant shift in the natural balance of LA and LNA, and over the last 100 years the average dietary consumption of LNA has declined significantly (Crawford et al 1989).

Eicosapentaenoic acid (20:5 n-3)

Eicosapentaenoic acid (EPA) is produced de novo by marine algae and in animals by the desaturation or elongation of LNA. EPA is a primary fatty acid of fish oil (approximately 20-25% by

weight), although it is not produced de novo by fish. It has also been reported that significant EPA production can occur in animals by means of the β-oxidation chain shortening of docosahexaenoic acid (Hagve and Christophersen 1986). EPA has been investigated extensively for its action as a competitive inhibitor of arachidonic acid metabolism. While eicosanoids can be produced form EPA, they appear to have either no activity or an activity that opposes arachidonic acid derived eicosanoids (Lowry and Thompson 1994).

Docosapentaenoic acid (22:5 n-3)

Docosapentaenoic acid is the elongation product of EPA and is present in most marine lipids. It has been reported to be actively converted to DHA via a three-step process involving a unique delta-6 desaturation (Voss et al 1991).

Docosahexaenoic acid (22:6 n-3)

Docosahexaenoic acid (DHA) is produced de novo by marine algae and is primary component of fish oil (approximately 8-20% by weight). The production of DHA in animals from LNA occurs via the desaturation and elongation of α- linolenic acid to 24:5 (n-3). This very long unsaturated fatty acid is desaturated by a delta-6 desaturase (possibly a unique delta-6 desaturase enzyme), and the resulting fatty acid undergoes one cycle of β-oxidation to form DHA (Voss et al 1991; Moore et al 1995). Animals appear to have a requirement for DHA for neural function and rely on its production from omega-3 precursors by elongation or desaturation cycles or through the ingestion of intact acid (Crawford 1993). The exact role played by DHA in animal physiology is not understood. The great care with which the fatty acid is preserved in certain tissues implies that it may be an essential component of certain cells. Brain and retinal tissue are particularly enriched with DHA (Ahmad et al 2002).

Omega-3 fatty acids and health

Early history of omega-3 fatty acids

With the sustained publicity given to omega-3 fatty acid in seafood and fish oils during the 1980s, it is startling to think that less than 25 years, few people were aware of these substances. However, early fatty acid analysis had uncovered them and there were isolated reports in the medical literature of their effectiveness in lowering blood lipids (Burr 1942; Bronte-Stewart et al 1956; Keys, et al 1957; Ahrens et al. 1959). Why did they remain obscure until the 1970s?

By focusing mainly on the effects of fatty acids on blood cholesterol levels, attention remained with omega-6 fatty acids for about 25 years. Dissenting voices remained in the wilderness. Even though, in 1956, Sinclair and others were aware that Inuit (formerly called Eskimos) and some Norwegians were consuming high fat diets without any reported incidences of atherosclerosis, the observation went largely unheeded (Ehrstrom 1951; Sinclair 1953). It was not until this anomaly was vigorously pursued by Danish scientist that the importance of the Inuit diet was more widely recognized. Thus, it is popular to mark the investigation of the Inuit diet as the turning point in the fame of omega-3 fatty acids.

It had been known since the 1940s that Greenland Inuit did not develop heart disease in spite of their high fat consumption (Ehrstrom 1951; 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 omega-3 fatty acids (Bang, Dyerberg, and Sinclair 1980). Omega-3 fatty acids were virtually absent from many European and North American diets. The suggestion by Bang, Dyerberg, and colleagues that omega-3 fatty acids were linked to

freedom from heart disease ushered in a new era of omega-3 fatty acid research (Dyerberg and Bang 1978; Dyerberg et al 1978). We now regard omega-3 fatty acids as fundamental molecules in the structure and activity of the membranes of all cells (Ahmed et al 2002).

Epidemiological evidence for the health effects of omega-3 fatty acids.

Observations among Inuit

Epidemiologists study the relationships in human populations between people who do and do not develop diseases (or who have certain characteristics) and examine the circumstances they have common. The virtual absence of heart disease or myocardial infarction among Greenland Inuit was a key epidemiological observation leading to the focus on omega-3 fatty acids. The early literature describing the health and diet patterns among Arctic Inuit documents the unusually low frequency of diseases common in the more "civilized" temperate latitudes (Urquhart 1935).

Observations among Japanese

The Japanese consume more fatty fish and shellfish than Americans. The Japanese have among the world's lowest rates of death from heart disease. The presence of omega-3 fatty acids was interpreted as having a beneficial effect on tissue composition, compared to other factors associated with the reduced risk of heart disease and stroke such as lower blood pressure (Yamori et al. 1985). However, Japanese dietary habit has been changing over the years in the direction of Western eating patterns (Land et al. 1990).

Observations among Swedish

In a 14-year study of nearly 11,000 Swedish men and Women, the consumption of a "high" level of fish was associated

with a substantially lower risk of coronary heart disease mortality and myocardial infarction (Norell et al. 1986). The relative risk of dying from coronary heart disease decreased by 15% in those consuming the fish, compared with those who never or seldom ate fish. The relative risk of having a heart attack decreased by 30% in the same group.

Experimental evidences for the health effects of omega-3 fatty acids

The knowledge on the role of the various fatty acids in growth and development and in health and diseases (Simopolous 1991; Lagarde et al 1999), particularly cardiovascular diseases (De Lorgeril et al 1994; Renaud 1995; De Lorgeril and Salen 2000; Frank et al 2003) are increased in recent times. In terms of potential impact on health, the omega-3 fatty acids are viewed as one of the most important findings in the history of modern nutritional sciences.

Effects on lipids

In healthy subjects, increased consumption of omega-3 fatty acids is associated with lowering of serum triglycerides and very low-density lipoprotein and when used at high doses would decrease both serum cholesterol and apolipoprotein B concentrations (Sanders et al 1989; Kritchevsky et al 1991; Guoping et al 1999). Fish oils have been effective in normal subjects and in patients with common phenotypes of hyperlipidemia in which VLDL concentrations are raised. Omega-3 fatty acids also modify the type of hypertriglyceridemia that is normally inducible by carbohydrates (Harris et al 1988). Omega-3 fatty acids reduce the synthesis of chylomicrons by the intestine and increase their removal from circulation (Bergeron et al 1997). A tendency for the postprandial lipoprotein concentrations to fall was noticed (Weintraub et al 1988). In various dyslipidemias,

moderate supplementation of fish oil would decrease serum triglyceride concentrations with some increase in high-density lipoprotein cholesterol (Prichard 1995). Cholesterol concentration in plasma is decreased by omega-3 fatty acids in patients with type V hyperlipidemia who do not tolerate any other type of dietary fat (Schmidt et al 1993). The effect of long chain omega-3 fatty acids on plasma LDL and HDL, as against the decrease in very low-density lipoproteins and triacylglycerol concentrations, is the result of factors such as the smaller very low-density lipoprotein particle produced, which is more likely to be converted to LDL (Huff and Telford 1989), by the direct effect on the synthesis of LDL by the liver and by lowering the saturated fat intake.

Low-density lipoprotein concentrations showed a tendency to increase in patients with diabetes mellitus when supplemented with fish oil (Schectman et al 1989). In a long term study, Shinozaki et al (1996) observed that when EPA (1800 mg/day) was given for periods ranging from 6 to 24 months the serum levels of lipoprotein (a), total cholesterol, triglycerides and low density lipoprotein lowered significantly. Interestingly, EPA and DHA affect triglyceride levels in different ways: EPA lowers triglyceride levels, whereas, DHA appears to have little or no effect (Bonaa et al 1992). The lowering effect of EPA and lack of effect of DHA on triglycerides levels have been confirmed in rat studies (willumsen et al 1993).

Effects on haemostatic system

Blood flow is affected by the ability of various blood cells to adapt their shape to the nature of the passageway 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 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 1992). 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 activation also affects red blood cell deformability and omega-3 fatty acids are known to affect platelet activity (Harker et al 1993). Thus, omega-3 fatty acids may influence the interaction of red blood cells and platelets, their interaction with the vascular epithelium, and the physiological activity of them selves.

Platelet aggregation requires fibrinogen from the circulation and is stimulated by thromboxane A_2, an extremely potent prostaglandin synthesized by the platelet itself (Leung and Nachman 1986). Thromboxane A_2 also promotes vaso constriction, a narrowing of blood vessel, and promote thrombosis (Kinsella et al 1990). In healthy people minute quantities of thromboxane A_2 are produced, whereas, in people with atherosclerosis, thromboxane A_2 is greatly increased (Fitzgerald et al 1983). Platelets aggregate in response to other stimuli such as collagen, adenosine di-phosphate (ADP), thrombin, serotonin, epinephrine, and bradykinin (Leaf 1990).

Platelet clumping tendencies are opposed by the metabolic products of endothelial cells particularly prostacyclin (PGI_2) that diminishes platelet aggregation and stimulates vasodilation (Kinsella et al 1990). The relative balance between thromboxane A_2 and prostacyclin production has been suggested as a primary means of controlling platelet aggregation (Hornstra 1989).

Omega-3 fatty acids, when given orally, are rapidly incorporated into platelets and compete with arachidonic acid (AA) for the 2-acyl position of membrane phospholipid and as substrate for the cyclo-oxygenase and lipoxygenase enzyme complex. As a result, when stimulated, the platelets will produce less

prothrombotic eicosanoids, such as thromboxane A_3 (TXA_3) (Conquer et al 1999). Similar effects were found after an increase intake of fish or fish oil supplements (Kristensen et al 1989; Ferretti et al 1998).

Mean platelet volume (MPV) is a marker of platelet activation (Willans et al 1995). It has been reported that MPV is increased in patients at high risk for acute myocardial infarction (Endler et al 2002). Large platelets show a higher degree of adhesion and aggregation than small ones (Schoene 1997). The presence of greater number of large platelets increases the risk of thrombosis. Studies have shown that omega-3 fatty acids decrease the MPV in humans (Yongsoon and Harris 2002). Further documentation of the anti-thrombogenic effects of omega-3 fatty acid consumption is provided from the comprehensive study of Harker et al (1993). In this study on baboons fed omega-3 fatty acids and subjected to mechanical vascular injury, the consumption of omega-3 fatty acids eliminated both vascular thrombus and vascular lesion formation. Animals also had increased bleeding time, decreased platelet aggregation, and reduced production of tissue factor, the substance required for the initiation blood clotting. Other clinical manifestations of reduced thrombus formation were also observed in animals receiving vascular graft.

The important signal generated in platelets during activation is the increase in intra cellular ionized calcium and this increase is associated with variety of platelet functions such as shape change, aggregation and secretion (Lebreton et al 1976). Studies had showed that omega-3 fatty acids delay the release if intra cellular ionized calcium and may lower the risk for thrombosis (Podczasy et al 1995). The effect of LNA from linseed oil was compared with EPA plus DHA from fish oil in healthy human subjects. Both the oils decreased the platelet aggregation and thromboxane

production. Thus the 18-carbon omega-3 fatty acid is as effective as fish oil in modulating haemostatic factors (Freese and Mutanen 1997).

Effect on endothelial proliferation and function

Under experimental conditions, EPA and DHA have been shown to reduce adhesion and migration of monocytes and influence processes involving leukocyte-endothelial cell interactions such as atherosclerosis and inflammation that involve increased endothelial expression of leukocyte adhesion molecules or endothelial activation (Prichand et al 1995). Studies have shown that consumption of DHA reduced endothelial expression of vascular cell adhesion molecule 1 (VCAM-1), E selectin, intercellular adhesion molecule (ICAM-1), IL-6 and IL-8 (De Caterina et al 2000). In addition, DHA also reduces the adhesion of human monocytes and monocytic U937 cells to cytokine stimulated endothelial cells and was found to be related to a reduction in VCAM-1 mRNA levels. In a similar study on patients with coronary heart disease, it was demonstrated that when omega-3 fatty acids were given, both tissue plasminogen activator antigen and soluble thrombomodulin decreased, whereas the group which received corn oil as placebo, soluble E-selectin and soluble VCAM- 1 increased. Thus, both these studies suggest that omega-3 fatty acid supplementation decreases haemostatic markers of atherosclerosis, show anti-inflammatory properties and inhibit endothelial activation.

It is known that vascular smooth muscle cell proliferation plays an important role in the pathogenesis of atherosclerosis and restenosis. In a recent study, Pakala et al (1999) showed that EPA and DHA could block smooth muscle cell proliferation induced by serotonin at the sites of vascular injury. Omega-3 fatty acids enhance production of endothelial derived vascular relaxing factor,

which is reduced in atherosclerotic vessels (Israel and Gorlin 1992). Improved endothelial function is evident from the fact that vasodilatation in response to acetylcholine intra-arterially can be restored in coronary arteries of patients who had received heart transplants and took fish oil supplements for 3 weeks, whereas vasoconstriction still occurred in control subjects (Fleischhauer 1993).

The loss of endothelium-derived nitric oxide (NO) may have a key function in early atherosclerosis through the enhancement of platelet aggregation, monocyte adherence and chemotaxis, and loss of vasorelaxation (Napoli and Ignarro 2001). Studies have suggested that both EPA and DHA produce vasodilatation by enhancing the production of NO from the endothelial and non-endothelial-dependent vasodilatation by blocking Ca^{2+} entry into the vascular smooth muscle cells (Tagava et al 1999). Furthermore, both EPA and DHA can also improve systemic large artery endothelial function in subjects with hypercholesterolemia (Goodfellow et al 2000). This may also explain the anti-hypertensive action of EPA and DHA (Engler 1999) since, both NO and calcium is known to have a major role in the pathogenesis of essential hypertension in humans (Kumar et al 1993).

Effect on nervous system

The high levels of brain DHA across mammalian species led to early speculations that DHA was playing crucial role in the nervous system. The human brain is one of the largest “consumer” of DHA. A normal adult brain contains more than 20 g of DHA. Many studies using a diet deficient in omega-3 fatty acids have shown reductions in the level of DHA in brain of different animal species. Low DHA levels have been linked to low brain serotonin levels, which again are connected to an increase tendency to depression, suicide and violence (Barbar, 1997). Associated

changes in brain function (auditory, olfactory, learning, memory, appetite events, neuron size, nerve growth factor levels) have been reported (Greiner et al 1999; Umezawa et al 1999; Ahmad et al 2002, Ahmad et al 2002a). DHA may reduce the development of unipolar depression (Hibbeln et al 1995). The occurrence of depression correlates well with the deficiency of essential omega-3 fatty acids (Adams et al 1996). Maternal depletion of DHA is one factor in post-partum depression in developing fetal nervous system (Hibbeln et al 1995). An adequate intake of omega-3 fatty acids is particularly important during pregnancy and lactation. DHA makes up to 15 to 20% of the cerebral cortex and 30 to 60% of the retina and hence it is absolutely necessary for the normal development of the fetus.

Low levels of DHA are also associated with senile dementia (Alzheimer disease) and schizophrenia. The prevalence of Alzheimer disease correlates positively with high fat and high total calorie consumption and negatively with fish consumption (Grant 1997). A high intake of fish has been linked to a significant decrease in age-related memory loss and cognitive function impairment (Kalmijn 1997)

Various mechanisms have been suggested to account for these physiological changes in the brain (Lauritzen et al. 2001). Briefly, DHA plays a crucial role in membrane disorder (membrane fluidity) that can influence the function of membrane receptors (Feller et al. 2002), regulation of membrane bound enzymes (Na/K-dependent ATPase) (Bowen and Clandinin 2002), dopaminergic and serotoninergic neurotransmission (Zimmer et al. 2000), signal transduction via effects on inositol phosphates, DAG kinase, and protein kinase (Vaidyanathan et al 1994). Studies have also shown the omega-3 fatty acids effect on gene expression (De Urquiza 2000), regulation of phosphatidyl serine levels (Garcia et al 1998),

protection of neural cells from apoptotic death (Akbar 2002) and regulation of nerve growth factor (Ikemoto 1997). When no sources of omega-3 fatty acids are provided in the diet, compensation is provided by the synthesis of docosapentaenoic acid (22:5, n-6) and docosatetraenoic acid (22:4, n-6) from omega-6 fatty acids. However, these fatty acids are present only at very low levels in tissues.

Cytokines and omega-3 PUFA

Recent studies suggested that inflammatory mediators such as tumor necrosis factor α (TNF α) and interleukins (ILs) seem to be involved in CHD. TNF α is released early in the course of acute myocardial infarction (Cain et al 1999), and can decrease myocardial contractility in a dose dependent fashion (Li et al 1999). Myocardial injury and dysfunction induced by TNF α can be reduced by treatment with specific monoclonal antibodies against TNFα (Li et al 1999). Omega-3 fatty acid, EPA and DHA, have been shown to inhibit the production of IL-1, IL-2 and TNF α both in vitro and in vivo (Purasiri et al 1994). This explains why omega-3 fatty acids have beneficial effects on myocardial infarction and CHD. Further, omega-3 fatty acids regulates superoxide anion generation and enhance the production of NO (Das 1994), a vasodilator and platelet anti-aggregator. Even NO has anti-inflammatory actions under certain circumstances (Guidot et al 1996). There is also evidence that inflammatory condition such as ulcerative colitis, Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus, septicemia and septic shock are associated with excess production of TNF α.

Omega-3 fatty acids and lipid peroxidation

Although still not fully understood, lipid peroxidation is thought to be one important mechanism involved in the pathogenesis of inflammation, cancer and atherosclerosis

(Halliwell et al 1993; Dargel 1992). An effective antioxidant defense mechanism is, important to maintain health of animals and man (Machlin and Bendich 1987). The inherent antioxidant defense is composed of antioxidants, such as vitamin E and vitamin C, and antioxidant enzymes. The role of the antioxidant defense system which includes superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione transferase is well characterized and constitutes a major protection for cells against acute oxygen and xenobiotic toxicity (Lieber 1997). The enzymes act biologically to eliminate cellular free radicals, to keep reactive oxygen species at low concentrations and to catalyze the destruction of hydrogen peroxide. Superoxide dismutase removes superoxide radicals (O_2^-) by converting them to triplet oxygen ($^3O_2$). Cytosol is a major source of CuZn-SOD while mitochondria generally contain Mn-SOD.

$$O^-_2 + 2H^+ \rightarrow H_2O_2 + {}^3O_2$$

Catalase then converts the hydrogen peroxide to water.

$$H_2O_2 \rightarrow 2H_2O + {}^3O_2$$

Glutathione peroxidase oxidizes glutathione in the presence of hydroperoxide. The sulfhydril group on one glutathione will react with sulfhydril group of another glutathione to produce oxidized glutathione with a disulphide bond.

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$$

The types of fat in the diet determine the relative composition of biomembrane, and polyunsaturated fatty acids are indeed substrates for free radical reaction leading to lipid peroxidation (Mehta et al 1994). Experimental evidence indicates that free radical-mediated lipid peroxidation can induce endothelial cell injury or dysfunction (Hennig and Chow 1988).

The peroxidizability of lipids depends on the unsaturation index of the fatty acids. The unsaturation index and

peroxidizability index of omega-3 fatty acids are higher than those of omega-6 fatty acids and omega-9 unsaturated fatty acids (Fritsche and Johnston 1988). Many studies have shown that intake of a diet high in omega-3 fatty acids results in high omega-3 fatty acid content in membrane lipids and enhances lipid peroxidation in organs, blood and urine of experimental animals and humans (Kaasgaard et al 1992; Allard et al 1997). Lipid peroxidation is an important event initiated by reactive oxygen species and oxidative stress causing cell death (Droge 2002). Oxidative stress is a term used to denote the imbalance between the concentrations of reactive oxygen and nitrogen species and the defense mechanisms of the body. Indeed, the use of oxygen in the oxidative metabolism of fuel results in reactive oxygen species production (Esposito et al 1999).

Recommended dietary intake of essential fatty acids

Recently, the National Academies released the dietary reference intakes report for energy and macronutrients (Institute of the Medicine of National Academies 2002). Adequate intakes have been set for LA and LNA. The adequate intake for LA is 17 and 12 g/d for men and women aged 19 to 50 years respectively. The adequate intake for LNA is 1.6 and 1.1 g/d for men and women aged 19 to 70 years, respectively. In 1999, the National Institutes of Health (NIH) sponsored an international workshop on the essentiality and recommended dietary intakes for omega-6 and omega-3 fatty acids. The NIH working group proposed adequate intakes of 2 to 3% of total calories for LA, 1% of total calorie for LNA, 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 1999). Health Canada suggests a minimum of 3% of energy from omega-6 fatty acids and 0.5% from omega-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% of energy be from LNA and 0.5% from EPA and DHA combined (COMA 1994). Some experts suggest that vegetarians (and others receiving no direct sources of EPA and DHA) at least double the recommended intakes for LNA. This would suggest an intake of LNA in the range of 1 to 2%.

The ratio of omega-6 to omega-3 fatty acids is often used to assess the balance between essential fatty acids in the diet. For vegetarians and others who consume little, if any EPA and DHA, the omega-6 to omega-3 ratio is of greater relevance than for individuals who consume significant amount of EPA and DHA daily. A number of recommendations have been made on the basis of ratio of omega-6 to omega-3 fatty acids. The WHO/ FAO suggests a ratio of 5:1-10:1 (WHO and FAO 1995), On the basis of proposed adequate intakes; the NIH suggests a ratio of 2:1-3:1 (Simopolous et al 1999). One study found that a ratio of 4:1 allows for adequate conversion to DHA in healthy vegetarians. This can be achieved by increasing the LNA in the diet for the production of EPA and DHA.

Fat intake in India

The role of nutritional factors in chronic degenerative diseases has received wide attention in developed countries. In India, with increasing urbanization, changing work pattern and affluence, there is a steep rise in obesity, diabetes, hypertension and heart attacks (Chadha et al 1990; Gopalan 1994). Studies in Indian immigrants in various countries have shown that Indians are more susceptible to these health risks as compared to respective local populations (Ehas et al 1991). Of several dietary variables, the role of lipids has been extensively investigated.

Indian diets are usually cereal-pulse based, and vegetable oil used as cooking fat, is the major source of visible fat (Vinodini et al 1993). Visible fats are those that are extracted from oilseeds (vegetable oils) or from milk (butter and ghee). Apart from visible fat, every food ingredient provides some amount of invisible fat.

Visible fat

India has several kinds of vegetable oils and there are regional preferences in the choice of oil (Achaya 1995). Groundnut oil (Peanut oil) is preferred in western and southern states except Kerala where coconut oil is used. Sesame oil has been popular in South India. The Northern and Eastern states prefer mustard oil. In Southern Maharastra and Northern Karnataka safflower oil is preferred. In recent years, sunflower and rice bran oils have been introduced on a large scale. The cost and advertisements claiming the cholesterol lowering potential of oils seem to have influenced and modified the traditional and regional choice of oils. While imported palmolein was used over the last decade, because of the palm cultivation that has been launched in India, indigenous palm oil is likely to be important edible oil in the near future.

Vanaspati is produced in India by the hydrogenation of vegetable oil. Both commercial ghee as well as that made at home from milk contributes to its high consumption in middle income and affluent groups.

The visible fats are largely triglycerides. The common fatty acids are palmitic, oleic and LA. The composition of fatty acids in different fats varies widely (Table 1.1). The percentage of LA in safflower, sunflower, soybean, corn and cottonseed is more than 50% of the total fatty acids, while sesame, rice bran, groundnut and mustard oils contains 20 – 40% of LA. Palm oil contains about 10% LA. Coconut and palm kernel oil contain a high proportion of SFA of short and medium chain length. Rapeseed, mustard and

soybean oil contain appreciable amounts of LNA. Rapeseed, and mustard oil contain large quantities of erucic acid. Ghee is a rich source of saturated fatty acid; about 60% of these are of short chain and medium chain length. Vanaspati contains long chain-saturated fatty acid and about 55% trans fatty acids.

Table 1.1 Approximate fatty acid compositions of visible fats (g/100g)

Oil	SFA	MUFA	LA	LNA	LA/LNA
Coconut	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
Cottonseed	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, LNA: Linolenic acid. (Ghafoorunissa 1994)

Invisible fat

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 fats of plant foods are good sources of oleic, LA and LNA (Table 1.2). While in most of

these LA predominates (Ghafoorunissa and Jyotsna 1993), legumes and green leafy vegetables contain high proportions of LNA. Animals foods like meats (muscle and organ) and milk and its products predominantly contain saturated fats (Table 1.2). Lean meat being a structural fat has PUFA as well and is the major source of preformed arachidonic acid (which is otherwise formed in the human body form LA). The overall fatty acid composition of animal foods can vary with animal feed as well as the composition and proportion of depot fat within and around the muscle and organ meats (British Nutrition Foundation 1992).

Cholesterol in foods

Cholesterol is obtained only from animal foods and is not found in plants. The highest cholesterol containing foods are organ meats (brain, liver, kidney) and eggs (Table 1.3). However, eggs provide more cholesterol in our diets than any other food item. Milk and its products, mutton, beef, pork, chicken and seafoods are other sources of cholesterol. While all the above animal foods have large amounts of both cholesterol and saturated fatty acids, egg also contains high levels of LA.

Table 1.2 Invisible fat and fatty acids in plant foods in g/100g of food

Food	Fat	SFA	MUFA	LA	LNA	LA/LNA
Cereals	1.7	0.4	0.4	0.5	0.01	41
Wheat	2.9	0.5	0.3	101	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	32
Ragi	1.5	0.3	0.7	0.3	0.05	5
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	3.5	1.4
Almond	56	25	19	8	0.2	40

SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids, LA: Linoleic acid, LNA: Linolenic acid. (Ghafoorunissa 1996).

Table 1.3 Fat, fatty acid composition and cholesterol content of animal foods

Food	Fat (g/100g)	SFA (g/100g)	Cholesterol (mg/100g)	Others
Butter	80	50	250	
Ghee	100	65	300	
Milk (cow)	4	2	14	Short
Milk (buffalo)	8	4	16	& medium
Milk (Skimmed)	0.1	-	2	fatty acids
Milk (Condensed)	10	6	40	
Cream	13	8	40	
Cheese	25	15	100	
Egg (Whole)	11	4	400	
Egg yolk	30	9	1120	Linoleic
acid				
Chicken with out skin	4	1	60	
Chicken with skin	18	6	100	
Beef	16	8	70	
Mutton	13	7	65	LC n-6 FA
Pork	35	13	90	
Organ meats				
Brain	6	2	2000	n-6& n-3
FA				
Heart	5	2	150	
Kidney	2	1	370	
Liver	9	3	300	LC n-6 FA
Fresh water and seafoods				
Prawns/Shrimps	2	0.3	150	
Fish (lean)	1.5	0.4	45	
Fish (Fatty)	6	2.5	45	n-3 FA

(Holland et al 1993; Gopalan et al 1989)

Quality of fat in the diet with one visible fat

The quantity and source of invisible fat and type of oil determine the intake of different fatty acids in the total diet. Assuming that 50 g of one oil is used as the cooking oil, the probable fatty acid in the total diet is calculated and compared with the suggested upper limits of fatty acid. The table 1.4 shows that most of the oils (if they were to be used as a single source of visible fat) do not provide required levels of PUFA/SFA as well as LA/LNA (omega-6/omega-3) ratios. These estimates are similar to the earlier calculations made from the national survey data on dietary intakes (Ghafoorunissa 1989). Oils such as safflower and sunflower furnish high PUFA/SFA ratios. The use of mustard oil increases the intake of long chain MUFA. The use of coconut oil, vanaspati or ghee furnishes low PUFA/SFA ratios and do not provide adequate EFA. The use of hydrogenated oils increase the intake of trans fatty acids, which are known to have negative effects on health.

Several health authorities have recommended increase in the consumption of omega-3 fatty acids (Scientific Review Committee 1990; British Nutrition Foundation 1992). In India, apex bodies for nutrition and health like Indian Council of Medical Research and National Nutrition Monitoring Bureau also have recommended for the improvement in the omega-3 fatty acids intake by Indian population (Ghafoorunissa 1996; Ghafoorunissa 1998).

Table 1.4 Quality of total fat in Indian diet

Oil	PUFA/SFA	LA/LNA	Minor constituents
Safflower	High	Very high	-
Sunflower	High	Very high	-
Sesame	Fair	High	Sesamol, sesamin
Rice bran	Ideal	Fair	oryzanol
Groundnut	Ideal	High	-
Palmolein	Low	Ideal	Tocotrienols
Mustard	Ideal	Low	-
Soybean	High	Ideal	-
Coconut	Low	Low	-
Vanaspati	Low	Low	-
Ghee	Low	Low	Vitamin A & D

(Ghafoorunissa 1994)

Fish oil is uniquely rich in EPA and DHA. However modern societies tend to include very little fish in their diet and increasing fish consumption would involve major dietary changes. Large percentage (up to 65) of the population do not eat fish. Vegetarians in particular, do not prefer fish in their diet. Thus there is a need for alternative sources of omega-3 fatty acids, such as functional foods, whose unique fatty acid composition could fortify staple foods there by promoting optimal levels of omega-3 fatty acids intake (Helen 1999). Therefore alternative ways to increase consumption of omega-3 fatty acids have to be explored and assessed (Mantzioris et al 2000).

Linseed (*Linum usitatissimum*), which is rich in LNA, is an economically important oil seed crop used for edible purposes in the central and northeastern regions of India. The beneficial properties of linseed oil (LSO) have been studied in detail by earlier researchers (Cunnane et al 1993; Jenkins et al 1999). Linseed consumption in various forms as a food ingredient and for its medicinal properties dates from 5000 BC since its cultivation

(Oomah 2001). Linseed oil may be exploited as an alternate source of omega-3 fatty acids to population who do not consume fish.

Strategies to improve the omega-3 fatty acids status in diet

Efforts to combat deficiencies have centered on supplemental nutrient administration and addition of selected nutrient to the diet in the form of a food fortification (Caballero 2003). Over the past several decades, and as the association between diet and chronic disease became apparent, supplementation and fortification were also targeted at healthy individuals, with the aim of reducing their risk of diseases such as cardiovascular disease, diabetes, and cancer with advancement in age and change of life style. Attempts have been made to enrich eggs with omega-3 fatty acids by feeding flax seeds and fishmeal to poultry (Oomah 2001). Similarly cattle feeds containing flax seed and fishmeal are used to enrich milk with omega-3 fatty acids. In addition, several food products like bread, cookies and muffins are incorporated with omega-3 fatty acids (Gambus et al 2004). Milk has been shown to be an efficient vehicle for various nutrients. Milk fat is highly dispersed in micelles and hence can entrap nutrients including oils and fats (Baro et al 2003).

Milk: Composition, dietary value and spray drying

Milk is a unique fluid that has evolved to sustain life for the newborn mammal. Man is the only species to use the milk of other mammals as a food for adults and, in a modified form. It is the taste of milk, which is first experienced by human infants. Among the various constituents, milk fats are particularly useful for infants and children to meet their energy requirements. Various properties and nutritional benefits of milk have already been described in ancient books of India like Charakasanhita and Astanghirdyam. Charakasanhita has described the various qualities of ghee, the anhydrous milk fat in terms of providing

tenderness to the body, good voice and healthy eyes for the whole life.

Composition of milk fat

Milk fat is composed of mainly triglycerides (96-99%) with a small amount of diglycerides (0.3-1.0%), phospholipids (0.1-0.3%), cholesterol (0.2-4.0%), free fatty acids (0.1-0.4%) and trace of monoglycerides and others. The major fatty acids in milk fat are palmitic acid (24-28%), oleic acid (23-28%), myristic acid (13-14%) and stearic acid (11-12%). The content of short chain fatty acids (up to C-8) in milk fat is relatively high, which is unique characteristic of milk, since other fats and oils contain these fatty acids only in trace amounts. Moreover, it is a good source of fat-soluble vitamins like vitamins A, D, E, and K. The average total vitamin A and vitamin D content in bovine milk is 2000 IU and 20 to 60 IU per 100 g, respectively. Whereas, the vitamin E content is about 3.5 mg per 100 g and that of vitamin K range from 3.6 to 8.9 µg/ lit. Milk fat is low in essential fatty acids (Kansal 1995).

Dietary value

Milk fat is most easily digestible. The degree of digestibility of milk fat is 99%, while that of natural palm oil is 91%. The good digestibility of milk fat is mainly attributed to the state of dispersion of milk fat globule and its fatty acid composition. Milk fat globules can be absorbed without preceding enzymatic hydrolysis and as a result puts relatively little strain on the digestive system (Kansal 1995). Moreover, in milk, the triglycerides contain short chain fatty acids such as butyric and caproic acid on the outer positions which are hydrolyzed more rapidly than triglycerides with long chain fatty acids by lipase. In addition, a fungicidal and bactericidal effect of short chain and medium chain fatty acids against certain acid-resistant bacteria and moulds has been reported (Gurr 1981). Further the short chain fatty acids in

milk fat promote the growth of beneficial micro flora like *Lactobacillus bifidus* in the intestine.

Milk fat contains a number of components, which have the potential to inhibit the process of carcinogenessis. Among these components, sphingomyelin and other phospholipids, which accounts for 0.2 to 1.0% of the total lipids in milk, are having tumor suppressing activity. In addition to its structural functions in membranes, sphingomyelin, through its biologically active metabolites, i.e., ceramide and sphingosine, plays an important role in transmembrane signal transduction and cell regulation (Hannun and Bell 1993; Hannun and Linardic 1993). Bioactive peptides have been identified within the amino acid sequences of milk proteins (Clare and Swaisgood 2000). These peptides directly influence numerous biological processes evoking behavioral, gastrointestinal, hormonal, immunological, neurological, and nutritional responses.

Apart from contributing a wide range of nutrients, milk also supplies a high proportion of certain important nutrients. Calcium and riboflavin are good examples. The bioavailability of these nutrients from milk is generally high when compared with those present in vegetable foods. Milk may also enhance the bioavailability of nutrients in other foods.

Spray drying of milk

Spray drying is the most frequently used method in the food industry, because the process is flexible and efficient, provides good quality powder and is inexpensive (Ashady 1993).

Spray drying is a convective drying technique that uses hot air to transfer heat and remove the water evaporated. It is a short time process in the range of few seconds and if processing conditions are optimized and stabilizers are added it can be

suitable even for heat sensitive enzymes (Yamamoto and Sano 1995).

The principal step in the process of spray drying is the formation of droplets by atomization. A large surface area is created to accomplish maximum water and heat flux and to mix the fine spray with drying air. The initial droplet size (or the specific surface area) has an influence on the drying process and therefore determines many of the final powder properties (particle size, particle density, moisture content etc) of food products (Vanmil et al 1988).

Production of milk in India has increased over years as a result of operation flood. India has overtaken the USA in recent years as the world's largest producer of milk (News Review 2000). Of all the food products, milk is probably the one that is dried in largest quantities. In a country like India, where milk production is surplus, processing milk in to powder may be a suitable option for preservation. Conversion of milk to powder has become a manufacturing process of equal importance to the dairy industry as the traditional processes like manufacturing of butter and cheese.

Milk was used as a vehicle for supplementation with omga-3 fatty acids. It was then spray-dried in to fine powder for preservation and storage. The spray-dried milk can be stored over a period of time under controlled conditions and used as a beverage by reconstituting with water and consumed as and when required. And hence milk was utilized as a vehicle to supplement and deliver omega-3 fatty acids to populations who do not get adequate amount through their regular diet.

Aim and scope of present work

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. Essential fatty acids, LA and LNA are important for normal growth and functioning of body. Dietary fatty acids have been well correlated with metabolic and physiologic alterations associated with several complications including coronary heart diseases, diabetes, cancer, obesity and hypertension. The major changes that took place with respect to dietary fatty acid consumption may be attributed to agricultural revolutions leading to large-scale production of edible oils rich in omega-6 fatty acids. However, historical background of our fatty acid consumption pattern indicates omega-6 to omega-3 ratio of 1:1. But the present dietary omega-6 to omega-3 ratio ranges from 20:1 to 30:1. Indicating an increase of 20-30 fold in the intake of omega-6 fatty acids. Dietary survey conducted in India has indicated the need to improve the omega-3 fatty acid intake.

Linseed oil and marine source like fish are not consumed regularly in India. However, they can be exploited as omega-3 fatty acid source if it can be stabilized and delivered through a medium that is consumed on a regular basis.

Milk is an important dairy products extensively consumed by people of different age groups. Milk contains several factors that are considered to have health beneficial effects. Bovine milk however lacks omega-3 fatty acids. Milk, an every day consumed beverage can be an ideal vehicle to deliver omega-3 fatty acids.

In the present work, it was aimed to incorporate omega-3 fatty acids rich oils like LSO and FO into milk and present it in a form that can be consumed.

Nutritional evaluations of omega-3 fatty acid supplemented spray-dried milk formulation were carried out on experimental

animals. The bioavailability, tissue distribution and differences in the distribution of LNA and long chain metabolites (EPA+DHA) were monitored. The beneficial effects of omega-3 fatty acid supplemented milk formulation in reducing risk factors for atherogenic and thrombogenic events are also studied.

The aim of this investigation was to asses whether spray-dried milk powder containing omega-3 fatty acids can supply adequate amounts of these essential fatty acids to a population who do not get them through their normal diet. The results obtained indicated that it is feasible to deliver omega-3 fatty acids from linseed oil and fish oil through spray-dried milk powder prepared under controlled conditions.

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, 3α hydroxysteroid dehydrogenase, NAD, NADPH, 5,5'-dithiobis(2-nitrobenzoic acid), collagen, heparin, xanthine oxidase, glutathione (reduced and oxidized), glutathione reductase, hydrogen peroxide, flavin adenine dinucleotide, 1 chloro 2,4-dinitrobenzene, dithiothreitol, bovine serum albumin, uronic acid, glycocholic acid, taurocholic acid, taurodeoxycholic acid and glucuronic acid were purchased from Sigma Chemical Co., St. Louis, USA. 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) and thromboxane B_2 (TXB_2) were bought from Cayman Chemical Co., Ann Arbor, MI, U.S.A. 1,1,3,3 tetramethoxy propane was purchased from Fluka (Switzerland). Ferrous sulphate, ferric chloride, ammonium thiocyanate were purchased from Qualigens, Mumbai, India. Cytochrome C, calcium gluconate, xanthine and EDTA were purchased from Sisco Research Laboratory, Mumbai, India. Maltodextrin was purchased from Laxmi Starch Co, (Hyderabad, India). Phosphotungstic acid and cadmium acetate were purchased from Sd. Fine Chemicals, Mumbai, India. Acetylacetone was purchased from veb laborchemie, Apolda, Germany. Urethane and carbazole were purchased from Aldrich Chemicals, Milwaukee USA. HPLC grade hexane, isopropanol, methanol, butanol, acetonitrile, benzene were obtained from E. Merck, Mumbai, India. Choline chloride, DL-methionine, tocopherol acetate were purchased from Hi Media Lab, Mumbai, India. Casein was purchased from Nimesh Corporation (Mumbai, India). Fatty acid standards were obtained from Nu Chek Prep, Elysian, MN, USA. Groundnut oil (GNO), linseed oil (LSO) and fish oil (FO) were purchased from local market. All solvents used were

of analytical grade and were distilled prior to use. Bovine milk was obtained from a state owned dairy.

METHODS

Diet

Spray Drying of omega-3 PUFA Supplemented Milk Formulation

Three batches (70L milk / batch) of spray dried milk formulations were prepared under identical conditions on pilot scale (Fig 2.1) as described by Nagendra et al. (1995) after adding vitamins and minerals to the formulation (Baskaran et al. 1994). LSO or FO was added as omega-3 PUFA source. Groundnut oil (GNO) supplemented spray-dried formulation, which is devoid of omega-3 PUFA served as control.

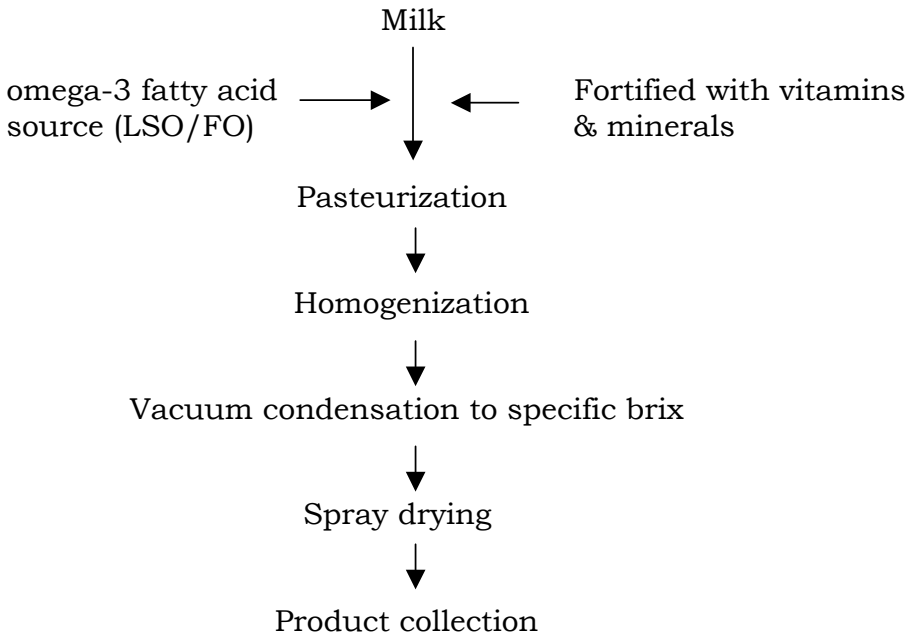


Fig 2.1 Spray-drying of GNO, LSO or FO supplemented milk formulation

After the addition of ingredients, milk was pasteurized at 70^0C, homogenized at 200kg / cm^2, concentrated in a vacuum evaporator to specific brix and spray-dried in the Anhydro drier (Made in Denmark). The spray drying temperature was 180-185^0C and the out let temperature was 80-85^0C. The dried product was collected in a humidity-controlled chamber and packed in tin cans of 100g capacity after flushing with nitrogen. The product was prepared in semi-automatic processing (batch processing) at the Central Food Technological Research Institute pilot plant facility. The product (20kg yield / batch) enriched with GNO/LSO or FO contained 16% fat.

Storage Studies of spray dried milk formulation

The spray-dried formulations were stored in tin cans in duplicates at 4, 27 and 37^0C. Samples were drawn after 1,2,3,4,5 and 6 months for the analysis of physical (solubility index, colour), chemical (moisture, total carbohydrate, crude protein, crude fat, fatty acids, peroxide value, total ash, energy content) characteristics, organoleptic (flavor, mouth feel, overall acceptability) and bacteriological qualities.

Moisture, crude protein, crude fat, total carbohydrate, total ash and energy content of dried milk formulations were determined according to the method of AOAC (2000). Solubility index of the dried formulations were determined according to Bureau of Indian Standards (1981), which measures the volume of sediment obtained by centrifugation of 50mL of reconstituted product, using 20g of product per 100mL of distilled water at 27^0C.

The colour of the product was measured using colour-measuring system (V-2100 spectrophotometer, Shimadzu, Japan) and the percent whiteness determined in this system was taken as an index of browning (Nagendra *et al.* 1995).

The rats were grouped by random distribution and fed either spray-dried milk based diet or AIN-76 (American Institute of Nutrition) purified diets (Anon. 1977). The basal composition of the AIN-76 purified diets is given in table 2.1. Fresh diets (20g/day) were given to rats daily for a period of 60 days.

Table 2.1 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

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

[2]100g mineral mix contained 50g calcium phosphate, 7.4g sodium chloride, 22g potassium citrate monohydrate, 5.2g potassium sulphate, 2.4g magnesium oxide, 0.35g manganese carbonate, 0.6g ferric citrate, 0.001g sodium selinite 0.16g zinc carbonate, 0.03g cupric carbonate (55%Cu), 0.001g potassium iodate and 0.0213g potassium chromate and made to 100g with sucrose.

Experimental animals

Male Wistar rats [OUTB-Wistar, IND-cft (2c)] (*Rattus norvegicus*) weighing 50 ± 3.0g obtained from Central Food Technological Research Institute, Mysore animal house were used.

They were placed in individual cages in an approved animal house facility with 12h light and dark cycles with temperature 25 ± 2ºC and fed fresh diets daily. The animals had free access to food and water throughout the study. The food intake and growth of the animals were monitored at regular intervals.

Isolation of serum

After feeding for 60 days, rats were fasted overnight and sacrificed under ether anesthesia. Blood was drawn by cardiac puncture and kept at 4ºC for 2h. Serum was separated by centrifugation at 2500 rpm (1100g) in a table top Remi 8 C centrifuge for 20 min and serum was stored at -20ºC till analyzed.

Liver and other tissues were removed and rinsed in ice-cold phosphate buffered saline (pH 7.4). The tissues were then blotted, weighed and stored at -20ºC until analyzed. All the analyses were completed within 15 days of sacrifice.

Total lipid extraction

Total lipids were extracted from serum and tissues by the method of Folch et al (1957).

Serum – 0.8ml of serum was taken in a stoppered tube and 2ml of methanol was added and shaken well for 30 seconds. 1ml of chloroform was added and shaken well for 30 seconds. 1ml of chloroform and 1ml of water was added and shaken well for 30 seconds. The extract was filtered using Whatman no 1 filter paper. Filtrate was allowed to settle for phase separation. 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.

Liver - 1g of tissue was homogenized with 1.0ml of 0.74% potassium chloride in a Potter Elvehjem homogenizer. To the extract, 20ml 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. 3ml of 0.74% 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 then lower phase was washed with 3ml of 0.74% potassium chloride and then twice with 3ml of chloroform: methanol: water (3:48:47 v/v) mixture. The chloroform layer was used for lipid analysis.

Total cholesterol estimation

Cholesterol was estimated by the method of Rudel and Morris (1976). An aliquot from the chloroform extract was dried under a stream of nitrogen followed by the addition of 1.5ml of ferric chloride - acetic acid reagent. The reagent was prepared by diluting the stock containing 504mg/ml anhydrous $FeCl_3$ in 10ml of glacial acetic acid to 1:100 dilution with glacial acetic acid. After mixing thoroughly, it was left at room temperature for 15 min. 1ml of concentrated sulphuric acid was added, mixed immediately on a vortex mixer (Remi) and left at room temperature in the dark for 45 min. The color intensity of the clear solution was measured in the spectrophotometer (Shimadzu 160A model) at 540 nm. Cholesterol levels in biological samples were estimated from the standard curve generated with AnalaR cholesterol (30-150µg).

HDL cholesterol estimation

To 0.5ml of serum, 25µl of heparin (5,000 units / ml) was added, followed by the addition of 25µl of 2M manganese chloride (3.969g/ 10ml). The solution was vortexed and kept at 4ºC over night (Warnick and Albers 1978). This was then centrifuged at 3000 rpm for 20 min. HDL cholesterol was measured as described earlier after extracting the supernatant with 3ml of acetone: alcohol (1:1 v/v).

LDL+ VLDL cholesterol estimation

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

Phospholipid estimation

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

Triglyceride estimation

Triglycerides were estimated by the method of Fletcher (1968) using triolein as reference standard (30-300µg). The lipid extract in chloroform was evaporated under a stream of nitrogen and then dissolved in 4ml of isopropanol. Two grams of triglyceride purifier was added, mixed well and centrifuged. Supernatant was transferred to another test tube, saponified with 0.6ml of 5% potassium hydroxide in isopropanol: water (40:60 v/v) at 60°C for 15 min. After cooling, 1 ml of sodium metaperiodate prepared from the stock solution of 0.025M in 1N acetic acid (sodium metaperiodate (12ml) and 20ml of isopropanol and made up to 100ml with 1N acetic acid) was added, mixed and 0.5ml of acetyl acetone was added, stoppered and incubated at 50°C for 30 min. After cooling to room temperature, the color intensity was read at 405 nm in Shimadzu 160 A spectrophotometer.

Peroxide value estimation

Peroxide value was estimated by the method described by Stine et al (1954). To 25mg lipid sample, 4.8mL of chloroform: methanol (3:5 v/v) was added, followed by 25µL of ferrous chloride (0.5%). It was mixed well on a Vortex mixer and colour intensity of the solution was measured at 505 nm. A standard curve of ferric iron (1-20µg) was prepared. Peroxide value of the sample was estimated using the formula

$$\text{meq of } O_2 \text{ / kg fat} = \frac{\text{Net weight of } Fe^{3+} \text{ (µg)}}{\text{Weight of sample taken x } 55.84}$$

Estimation of lipid peroxidation in liver homogenates

TBARS method

1g liver was homogenized in 10ml of 0.15M potassium chloride, in a teflon homogenizer. The homogenate was filtered through cheesecloth and used for assay. Liver homogenates (4mg protein) in 0.15M potassium chloride, 0.025M *Tris* hydrochloride buffer pH 7.5, 2mM adenosine diphosphate, and 10µM ferrous sulphate were incubated at 37°C for 5min. The reaction was initiated by adding 0.1 mM ascorbic acid and incubated at 37°C for 30 min (Berg and Aust 1989). The final volume of the reaction mixture was 1ml. The reaction was terminated using 2ml of thiobarbituric acid (0.375% thiobarbituric acid, 15% trichloroacetic acid in 0.2N hydrochloric acid) containing 10µM butylated hydroxy anisole. Samples were heated for 15 min in a boiling water bath. Malondialdehyde (MDA) formed was measured at 535 nm and quantitated using an extinction coefficient of 1.56 x 10^{-5} $^{-1}$ cm^{-1}. The lipid peroxides were expressed as nmoles of malondialdehyde (MDA) formed/mg protein. Basal levels of MDA was measured in the homogenate in which cofactors were not added (Berg and Aust 1989).

Fatty acid analysis

Fatty acids were analyzed as methyl esters prepared using boron trifluoride in methanol as described by Morrison and Smith (1963) and analyzed by gas chromatography (Shimadzu 14B, fitted with FID) using fused silica capillary column 25m x 0.25mm (Parma bond FFAP-DF-0.25: Machery-Nagel Gm BH co. Duren, Germany). The operating conditions were: initial column temperature 160^0C, injector temperature 210^0C and detector temperature 250^0C; column temperature was programmed to rise at 6^0C per min to the final temperature of 240^0C. Nitrogen gas was used as the carrier. Individual fatty acids were identified by comparing with retention times of standards (NU-Check prep. Inc., Elysian Minnesota, USA) and were quantitated by online chromatopack CR-6A integrator.

Analysis of bile acids

Rats were anaesthetized by injecting urethane (1.2g/kg body weight) by intraperitoneal injection. Laparatomy was performed and common bile duct was cannulated and bile was collected for 3 Hrs with polyethylene tubing [(PE-10) Thomas Scientific Co., New Jersey, USA]. After measuring the volume, it was kept frozen until used.

Biliary lipids were extracted by the method of Bligh and Dyer (1959). Briefly to the 0.8mL of bile 2mL methanol were added, vortexed and kept for 30 min. Then 1mL chloroform and 1mL water were added, vortexed and kept for 15 min, centrifuged and upper methanolic layer was taken for bile acid and uronic analysis and lower chloroform layer for lipid estimation. Total bile acids in methanolic layer were estimated by using 3α-hydroxy steroid dehydrogenase as described by Turley and Dietschy (1978). Individual bile acids in the methanolic layer were separated by TLC using chloroform: methanol: acetic acid: water (65:24:15:9

v/v) and visualized by spraying 10% phosphomolybdic acid in ethanol. Individual bile acids were quantified by densitometry using Camag TLC scanner (model No III, Camag, Germany) as described by Sambaiah et al (1986).

Estimation of uronic acid

Uronic acid in methanol layer of bile extract was estimated by the method of Dische (1947).

An aliquot of methanolic layer of bile (extracted by the Bligh and Dyer method) was made up to 0.5mL with water followed by the addition of 3mL of concentrated sulphuric acid and kept in a boiling water bath for 20 min and then cooled. Alcoholic carbazole (0.1%) 0.1 mL was added and kept in dark for 2 Hrs. and absorbance was measured at 530 nm against a blank sample. Uronic acid was calculated taking glucuronic acid as reference standard.

Assay of HMG CoA reductase activity

Preparation of rat liver microsomes

Rats were sacrificed by stunning between 9.00 to 10.00 p.m. The liver was taken out, kept in ice-cold isotonic saline, blotted and weighed. One gram liver was homogenized with 4mL of 0.1M triethanolamine hydrochloride containing 0.02M EDTA and 2mM DTT pH 7.4, and centrifuged at 1,00,000 g in Beckman LS-50B ultracentrifuge using type 65 rotor for 1hr. The microsomal pellet was washed with 1mL of triethanolamine hydrochloride buffer and homogenized with 2mL of same buffer and used immediately for enzyme activity. The microsomes were also used for estimation of cholesterol, phospholipid and fatty acid analysis.

Enzyme assay

HMG CoA reductase activity was assayed by the method of Hulcher and Oleson (1973) by measuring the monothiols with 5,5' dithiobis (2-nitrobenzoic acid). To the 0.4mL HMG CoA (6.486 mg

/ 6mL), 0.2 mL of dithiothreitol (DTT) (3.086mg/ 2mL) and NADPH 0.2 mL (50mg / 6mL) prepared in 0.1M triethanolamine buffer containing 0.02M EDTA and 2mM DTT pH 7.4 was added to microsomes (≈200µg protein). The mixture was incubated at 37^0C for 30 min. Twenty µL of sodium arsenite (0.01M) was added and after 1 min, the reaction was terminated by the addition of 0.01mL of 2M citrate buffer pH 3.5 containing 3% sodium tungstate to precipitate microsomal proteins. The mixture was again incubated at 37^0C for 10 min. The solution was transferred to plastic tubes and centrifuged at 10,000 rpm for 15 min to remove proteins.

Five minutes before assaying the enzyme, 0.8mL of the supernatant was mixed with 0.2mL of *tris* buffer pH 10.6 and 0.1mL of 2M tris buffer pH 8.0 and 50µL of 5 5'-Dithiobis nitrobenzoic acid (DTNB) (3mM) was added, mixed thoroughly and absorbance was measured for 5 min at 412 nm (Shimadzu 160A spectrophotometer). The absorbance due to monothiols was determined by extrapolating the linear portion of the curve after the addition of DTNB. The difference in absorbance between the complete reaction and that of all the components except NADPH represented the activity due to HMG CoA reductase.

Antioxidant enzymes in liver

Liver was homogenized in appropriate buffer (1g/10mL) using glass homogenizer. It was filtered through cheesecloth and centrifuged at 600 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 (Flohe and Otting 1984). 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 (Hoilg 1974).

Analysis of serum prostglandins

6-keto prostaglandin $F_{1\alpha}$ (6 keto PGF $_{\alpha 1}$) and thromboxane B_2 (TXB_2) were extracted from the serum as described by Lokesh et al (1986). Serum (1.0mL) was mixed with 100μL of 3.0% formic acid to adjust the pH to 3.0. The prostaglandins were extracted in ethyl acetate (2mL added 3 times). The combined ethyl acetate extracts were pooled and evaporated under the stream of nitrogen and loaded on to a Sep Pak C-18 column (Waters, Millipore Corp, Milford MA,. USA). The column was washed with 10mL water, followed by 10mL hexane and finally prostaglandins were eluted in 10mL of ethyl acetate. The prostaglandins were concentrated and loaded on Supelcosil C-18 high performance liquid chromatography column (Supelco, Bellefonte, PA, USA). The prostaglandins were eluted with potassium dihydrogen phosphate (K_2HPO_4) (9g / L): acetonitrile (67.5; 32.5 v/v) at a flow rate of 0.5mL / min and monitored at 200 nm. The prostaglandins were identified and quantified by comparing with authentic standards from Cayman Chemicals, Ann Arbor MI, USA.

Platelet aggregation

Blood was collected in heparinised tubes and platelet rich plasma (PRP) was separated from erythrocytes and leucocytes. Washed platelets were prepared as described by Brunauer (1993) and suspended in tyrodes buffer. Platelets were incubated for 15 min at 37^0C prior to use. The platelet count was adjusted to

400,000-cells/µl. Platelet aggregation measurements were performed using a Chrono-log dual channel aggregometer (Denmark). 450µl of platelet suspension was stirred at 1000 rpm at 37^0C and 10µl of ADP (25µM) or 15µl of collagen (5mg / 10ml 0.1N acetic acid) was added and aggregation followed for at least 5 min. Platelet aggregation was quantitated as described earlier (Niranjan and Krishnakantha 2000).

Protein estimation

Protein was estimated by the method of Lowry et al (1951) using bovine serum albumin as reference standard.

Statistical analysis

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

CHAPTER III

Relative effect of α-linolenic acid and eicosapentaenoic plus docosahexaenoic acid on serum and tissue lipids in rats.

Introduction

On the basis of estimates from studies in Paleolithic nutrition and modern day hunter-gatherer population, it was concluded that human beings evolved consuming a diet that was much lower in saturated fatty acids than is today's diet (Simopolous 2000). Furthermore, earlier diets contained roughly equal amounts of omega-6 and omega-3 fatty acids. The present Western and Asian diet is very high in omega-6 fatty acids (omega-6 to omega-3 fatty acids is 20-30:1) (Kris-Etherton et al 2000; Ghafoorunissa 1996) because of the extensive use of oils rich in omega-6 fatty acids (Reports of the National Cholesterol Education Program 1988). Omega-3 polyunsaturated fatty acids comprises the parent LNA comes mainly from vegetable oil sources like linseed oil, soybean oil, canola oil and walnut oil, and their long chain more unsaturated derivatives like EPA and DHA coming mainly from marine sources like fish.

The health beneficial properties of omega-3 PUFA have been extensively studied by various researchers (Norday et al 2001; Hoffman et al 1993; Lorgeril 2001). The principal omega-3 PUFA consumed by vegetarian populations is LNA. The dietary intake of EPA and DHA in these populations is negligible. The intake of EPA and DHA is restricted to people who consume fish. However many people may not relish fish for various reasons and has to depend on the dietary LNA for obtaining EPA and DHA. Therefore LNA has to play a major role in vegetarian populations for the maintenance of the EPA and DHA supply to the body. Studies have suggested that increased intake of LNA may have beneficial effects in maintaining health and in the control of chronic diseases (Simopolous 1991). Studies on the dose effect of dietary LNA performed by earlier investigators have given, very little information about the ability of the body to absorb, store and

conversion of LNA to long chain omega-3 PUFA (Leyton et al 1987; Mohrhauer and Holman 1963).

The bioavailability of an individual fatty acid is influenced by the presence of other fatty acids (Pudelkewicz et al 1968; Bazinet et al 2003). For many years, studies have focused on apparent differences in the deposition of LA in tissue lipids compared with LNA (Crawford et al 1976; Horobin et al 1984). The LNA did not appear to accumulate to the same extent as that of LA even under circumstances where equal amounts of LA and LNA are fed (Li et al 1999). There are several explanation offered for this observation. These include LNA conversion to EPA and DHA (Sinclair 1975; Salem et al 1996) and preferred β- oxidation of LNA over LA (Ide et al 1996). It is also observed that the extent of β- oxidation of LNA depends on the level of LA intake. LNA oxidation increases with increasing level of LA in the diet (Pan and Storlein 1993). Humans show differences in LA and LNA accumulation (Li et al 1999). Thus the conversion of LNA to EPA and DHA may be an important determinant for maintaining adequate amounts of LNA, EPA and DHA in cells for optimal tissue function. Hence levels of the long chain omega-3 PUFA in serum and tissue are dependent on intake of either their precursors, or preformed product. If an increased intake of LNA is to be promoted as an alternative to long chain omega-3 PUFA, it is necessary to consider to what extent LNA is absorbed, stored and converted in to long chain metabolites. Many researchers have extensively reviewed the essentiality of omega-3 fatty acids and emphasized on dose response effects of dietary omega-3 fatty acids on its incorporation in tissues (Lauritzen and Hansen 2003; Stanley 2004).

In the present investigation, the effect of different levels of LNA or EPA + DHA on the incorporation of omega-3 PUFA into serum and tissues were studied. The implications of this uptake of

omega-3 fatty acids on the risk factors that have a bearing on cardiovascular functions were also studied.

Results

Fatty acid composition of diet

The fatty acid composition of control and experimental diets are given in table 3.1. LSO was added to increase the LNA content of the diet to provide approximately 2.5, 5.0, 10.0 and 25.0% LNA. The LA/LNA ratios in the LSO containing diets were found to be 9.11, 4.52, 1.96 and 0.69. Similarly addition of FO increased the EPA and DHA level of the diet. The diet with FO provided approximately 1.0, 2.5 and 5.0% EPA+DHA. The LA/EPA+DHA ratios in the diet were found to be 16.4, 7.62 and 4.18. The LA content was maintained between 18-24% in all the experimental diets. The control diet containing GNO was devoid of omega-3 fatty acids.

Effect of feeding diets containing LSO or FO on growth parameters

The amounts of diet consumed in different groups were comparable (Table 3.2). There was no significant change in the food efficiency ratio and body weight gained by rats fed diet containing different amounts of LSO or FO. The weight of liver was marginally high in rats fed FO at higher level (at 2.5 and 5.0% level of EPA+DHA) compared to other groups. No significant change was observed in heart weight among rats fed various diets.

Table 3.1 Fatty acid composition (%) of diets

Fatty acids	Control diet	LNA in the diet				EPA + DHA in diet		
		2.5%	5.0%	10.0%	25.0%	1.0%	2.5%	5.0%
14:0	ND	ND	ND	ND	ND	1.5	1.8	2.9
16:0	14.47	13.62	13.16	12.6	10.9	12.5	12.74	14.2
16:1 n-7	ND	ND	ND	ND	ND	2.60	2.64	3.0
18:0	2.56	2.77	3.30	3.50	4.5	2.3	3.5	3.83
18:1 n-9	56.87	56.15	54.50	52.05	40.0	56.5	53.65	46.2
18:2 n-6	26.09	24.44	23.54	21.04	18.0	21.9	20.9	21.2
18:3 n- 3	ND	2.68	5.20	10.71	26.0	ND	ND	ND
20:4 n-6	ND	ND	ND	ND	ND	0.9	2.00	3.16
20:5 n- 3	ND	ND	ND	ND	ND	0.61	1.26	2.43
22:6 n- 3	ND	ND	ND	ND	ND	0.72	1.48	2.64
Saturated (S)	17.03	16.39	16.36	15.6	15.8	16.3	18.04	20.93
PUFA (P)	26.09	27.12	28.02	30.75	44.4	24.13	25.64	29.43
P/S	1.53	1.65	1.71	1.97	2.81	1.48	1.42	1.40
n 6 / n- 3	-	9.11	4.52	1.96	0.69	16.46	7.62	4.18

Values are mean of triplicate samples. ND: not detected

Table 3.2 Growth and organ weights of rats fed omega-3 fatty acids at incremental levels

Parameters	GNO	LNA 2.5%	LNA 5.0%	LNA 10.0%	LNA 25%	EPA+DHA 1.0%	EPA+DHA 2.5%	EPA+DHA 5.0%
Food intake (g / d / rat)	11.7 ± 1.0[a]	11.0 ± 1.1[a]	10.9 ± 0.8[a]	11.3 ± 0.9[a]	11.6 ± 1.4[a]	10.8 ± 0.9[a]	10.9 ± 0.6[a]	11.1 ± 1.1[a]
Body weight gain (g)	240 ± 15[a]	251 ± 13[a]	249 ± 26[a]	250 ± 19[a]	254 ± 18[a]	236 ± 18[a]	247 ± 11[a]	238 ± 20[a]
FER	0.34 ± 0.02[a]	0.33 ± 0.01[a]	0.35 ± 0.01[a]	0.32 ± 0.03[a]	0.31 ± 0.04[a]	0.33 ± 0.3 [a]	0.32 ± 0.01[a]	0.33 ± 0.02[a]
Liver *	3.3 ± 0.1[a]	3.2 ± 0.2 [a]	3.3 ± 0.1[a]	3.3 ± 0.2[a]	3.4 ± 0.1[a]	3.4 ± 0.2[a]	3.6 ± 0.3[ab]	3.9 ± 0.3[b]
Heart *	0.34 ± 0.04[a]	0.31± 0.02[a]	0.30 ± 0.03[a]	0.33 ± 0.01[a]	0.32 ± 0.04[a]	0.35 ± 0.05[a]	0.36 ± 0.03[a]	0.36 ± 0.02[a]

Values are mean ± SD of 6 rats. *(g/100g body weight). FER: Food efficiency ratio. Values not sharing a common superscript within a row are statistically significant ($p<0.001$).

Serum lipid profile of rats fed diets containing different level of LNA or EPA+DHA.

The rats fed LNA containing diets was found to contain 1.0, 1.9, 3.3 and 5.9% of LNA in serum lipids when the diet contained 2.5, 5.0, 10.0 and 25.0% LNA respectively (Table 3.3 and Fig 3.1). The EPA and DHA content in these animals were found to be 1, 1.7, 2.9 and 5.1% respectively. The corresponding ratios of EPA+DHA/LNA in serum lipids were 1.0, 0.89, 0.88 and 0.66 respectively indicating an effective conversion of LNA to long chain omega-3 PUFA in rat serum. The EPA+DHA levels in the serum of rats fed diet containing 1.0, 2.5 and 5.0% of EPA+DHA daily were found to be 3.4, 6.1 and 9.7% of total fatty acids indicating that preformed long chain omega-3 PUFA was absorbed efficiently and reflected on the higher levels of these fatty acids in the serum (Fig 3.2). The omega-3 fatty acids were not detected in control rats fed GNO. A dose dependent decrease in the serum total cholesterol level in both LSO and FO fed rats was observed (Table 3.4). At 2.5, 5.0, 10.0 and 25.0% LNA level in the diet the total cholesterol in serum was decreased by 7, 14, 17 and 26% respectively compared to control. Where as, at 1.0, 2.5 and 5.0% levels of EPA+DHA in the diet, the total cholesterol was decreased by 10, 19 and 25% respectively. Similarly, serum triacylglycerol in rats fed diet with 2.5, 5.0, 10.0 and 25.0% LNA level was decreased by 6, 11, 19 and 28% respectively compared to control animals. In rats fed diet with EPA+DHA at 1.0, 2.5 and 5.0% levels decreased triacylglycerol by 12, 20 and 27% respectively. These studies indicated that both LNA as well as

long chain omega-3 PUFA in the diet can effectively decrease serum cholesterol and triacylglycerol levels in rats.

Table 3.3 Serum fatty acid profile (%) of rats fed omega-3 fatty acids at incremental levels

Fatty Acids	GNO	LNA 2.5%	LNA 5.0%	LNA 10.0%	LNA 25%	EPA+DHA 1.0%	EPA+DHA 2.5%	EPA+DHA 5.0%
16:0	21.9 ± 2.0^{a}	24.9 ± 1.9^{a}	24.9 ± 2.0^{a}	25.9 ± 2.3^{a}	24.9 ± 2.3^{a}	24.6 ± 1.5^{a}	24.1± 1.8^{a}	24.9 ± 3.0^{a}
16:1	0.6 ± 0.3^{a}	1.0 ± 0.2^{a}	0.9 ± 0.2^{a}	1.0 ± 0.3^{a}	0.9 ± 0.3^{a}	2.0 ± 0.4^{b}	2.6 ± 0.3^{b}	3.4 ± 1.0^{c}
18:0	7.2 ± 1.3^{a}	8.6 ± 1.6^{a}	8.9 ± 0.9^{a}	10.4 ± 2.3^{a}	9.9 ± 3.0^{a}	9.4 ± 0.8^{a}	10.1± 2.0^{a}	9.6 ± 2.3^{a}
18:1	41.8 ± 1.3^{a}	38.9± 2.9^{ab}	37.8±2.3^{ab}	36.2 ± 3.0^{b}	35.3 ± 2.7^{b}	39.1± 3.6^{ab}	38.9± 4.8^{ab}	36.7 ± 5.3^{b}
18:2	22.3 ± 2.5^{b}	19.6±4.4^{ab}	18.9± 2.3^{ab}	16.5 ± 3.9^{b}	14.3± 4.0^{ac}	15.6± 2.3^{bc}	13.6± 2.3^{bc}	11.2 ± 2.9^{c}
18:3	ND	1.0 ± 0.08^{a}	1.9 ± 0.08^{b}	3.3 ± 0.1^{c}	5.9 ± 0.4^{d}	ND	ND	ND
20:4	5.9 ± 0.3^{a}	4.8 ± 0.3^{b}	4.4 ± 0.4^{b}	3.6 ± 0.3^{c}	3.1 ± 0.2^{c}	5.4 ± 0.4^{a}	4.2 ± 1.0^{b}	3.1 ± 0.8^{c}
20:5	ND	0.7 ± 0.2^{a}	1.2 ± 0.3^{b}	1.9 ± 0.3^{c}	3.2 ± 0.9^{d}	1.6 ± 0.3^{bc}	3.1 ± 0.6^{d}	4.8 ± 1.0^{e}
22:6	ND	0.3 ± 0.1^{a}	0.5 ± 0.2^{a}	1.0 ± 0.1^{b}	1.9 ± 0.3^{c}	1.8 ± 0.2^{c}	3.0 ± 0.4^{d}	4.9 ± 0.4^{e}

Values are mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant ($p<0.001$).

Fig3.1 Serum omega-3 fatty acids level of rats fed incremental amount of LNA

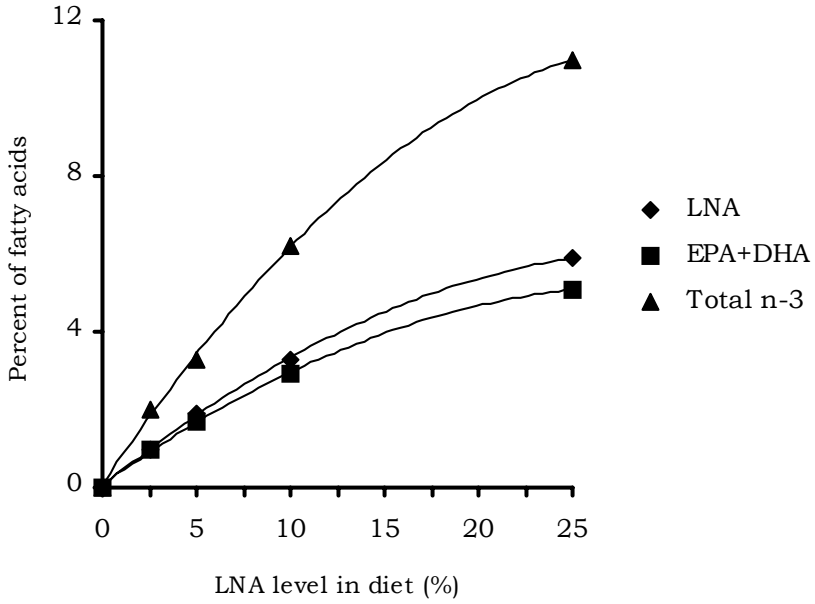


Fig 3.2 Serum omega-3 fatty acids level of rats fed incremental amount of EPA+DHA

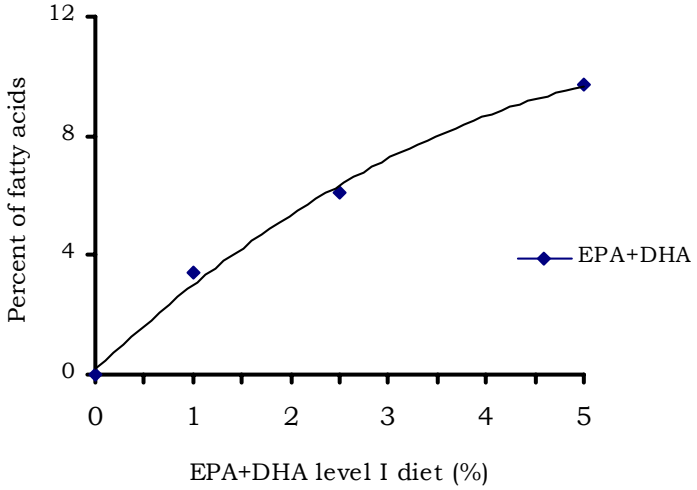


Table 3.4 Serum lipid profile (mg/dL) of rats fed omega-3 fatty acids at incremental levels

Parameters	GNO	LNA 2.5%	LNA 5.0%	LNA 10.0%	LNA 25%	EPA+DHA 1.0%	EPA+DHA 2.5%	EPA+DHA 5.0%
Total cholesterol	93.4 ± 3.8^{a}	86.9 ± 2.6^{b}	80.0 ± 3.1^{cd}	77.2±3.2^{def}	69.2 ± 3.8 ^{e}	83.7± 2.9^{bd}	76.0 ± 4.5^{d}	70.4± 4.7$^{e\ f}$
HDL Cholesterol	48.0 ± 3.0^{a}	44.4 ± 1.9^{a}	38.5 ± 1.5^{bc}	36.0± 2.6$^{b\ c}$	34.6 ± 2.5 ^{c}	41.5 ± 2.2^{b}	36.6 ± 0.9^{c}	35.4 ± 1.9^{c}
VLDL+LDL Cholesterol	45.4 ± 1.9^{a}	42.4 ± 1.2^{a}	41.0 ± 2.7^{ab}	40.0± 3.5^{ab}	34.6 ± 5.5 ^{b}	42.2 ± 3.8^{a}	39.4 ± 4.0^{a}	37.0± 4.1^{ab}
Phospholipids	114.4 ± 0.2^{a}	106.7± 8.5^{a}	104.6 ± 8.4^{a}	108.9± 5.6^{a}	107.9 ± 7.5 ^{a}	114.2± 7.9^{a}	120.6± 7.1^{a}	110.4± 5.5^{a}
Triglycerides	147.5 ± 6.7^{a}	138.9± 3.8^{a}	130.8 ± 6.3 ^{b}	119.6 ± 8.8^{c}	106.7± 10.4^{c}	129.6 ± 4.9 ^{b}	117.3± 8.4^{c}	107.7± 9.7^{c}

Values are mean ± SD of 6 rats. Values not sharing a common superscript within a row are statistically significant ($p<0.001$).

Liver lipid profile of rats fed diets containing different levels LSO or FO.

Liver is an important organ for lipid metabolism and sensitive to changes in the dietary lipids. The fatty acid profile in liver lipids of rats fed diet with different amounts of LNA or EPA+DHA are given in table 3.5. The LNA level of liver lipids in rats fed diet containing 2.5, 5.0, 10.0 and 25.0% LNA was found to be 0.8, 1.9, 3.2 and 4.4% respectively (Fig 3.3). The EPA+DHA level in these rats were found to increase in a dose dependent manner with an increase in the LNA level in the diet. The EPA level in the liver of rats fed diet with LNA content of 2.5, 5.0, 10.0 and 25.0% was found to be 0.7, 1.2, 3.5 and 3.9% of total fatty acids respectively and the DHA level in the liver was found to be 0.4, 0.9, 1.6 and 2.7% of total fatty acids respectively. Control rats fed GNO neither contained LNA nor EPA+DHA. These studies indicated that dietary LNA was taken up and further metabolized to long chain omega-3 fatty acids. Similarly, the EPA+DHA significantly increased the EPA+DHA level in the liver lipids (Fig 3.4). The EPA level in the liver of rats fed diet containing 1.0, 2.5 and 5.0% EPA+DHA were found to be 2.3, 6.2 and 9.5% respectively. The DHA level was found be 2.6, 5.7 and 8.7% respectively. These studies indicated that LNA or EPA+DHA from the diet is effectively taken up and stored in the liver.

Table 3.5 Liver tissue fatty acid profile (%) of rats fed omega-3 fatty acids at incremental levels

Fatty acids	GNO	LNA 2.5%	LNA 5.0%	LNA 10.0%	LNA 25%	EPA+DHA 1.0%	EPA+DHA 2.5%	EPA+DHA 5.0%
16:0	19.3 ± 2.1^{a}	17.7 ± 1.2^{a}	17.4± 2.3^{a}	16.4 ± 2.0^{a}	17.5± 3.0^{a}	16.9 ± 2.3^{a}	18.1 ± 3.0^{a}	17.9 ± 2.9^{a}
16:1	1.1 ± 0.2^{a}	1.0 ± 0.3^{a}	0.8 ± 0.3 ^{a}	0.9 ± 0.1^{a}	1.2 ± 0.1^{a}	1.9 ± 0.1^{b}	2.8 ± 0.4^{c}	4.8 ± 0.7^{d}
18:0	13.0 ± 1.2^{a}	15.3± 1.7^{bc}	20.0 ± 1.1^{b}	21.1 ± 2.3^{b}	23.1 ± 3.1^{b}	18.4 ± 2.3^{bc}	16.8 ± 3.0^{a}	15.9 ± 2.1^{a}
18:1	38.3 ± 3.6^{a}	37.0 ± 4.2^{a}	34.1 ± 3.6^{a}	32.8 ± 4.0^{ab}	28.9 ± 3.0^{b}	34.6 ± 2.9^{ab}	30.4 ± 3.9^{b}	28.6 ± 3.4^{b}
18:2	14.3 ± 1.9^{a}	13.9±1.3^{ab}	12.7± 2.0^{ab}	11.5 ± 1.1^{bc}	9.9 ± 1.3^{d}	11.4 ± 1.9^{bc}	9.6 ± 1.0^{c}	7.1 ± 0.8^{d}
18:3	ND	0.8 ± 0.2^{a}	1.9 ± 0.3^{b}	3.2 ± 0.6^{c}	4.4 ± 0.7^{d}	ND	ND	ND
20:4	13.5± 1.3^{ac}	13.0±1.8^{a}	10.9 ± 1.0^{b}	8.8 ± 0.9^{c}	7.6 ± 0.9^{cd}	11.5 ± 0.9^{be}	9.6 ± 0.8^{c}	6.9 ± 1.0^{d}
20:5	ND	0.7 ± 0.04^{a}	1.2 ± 0.9^{b}	3.5 ± 0.8^{cd}	3.9 ± 0.4^{c}	2.3 ± 0.8^{d}	6.2 ± 0.9^{e}	9.5 ± 1.0^{f}
22:6	ND	0.4 ± 0.08^{a}	0.9 ± 0.09^{b}	1.6 ± 0.2^{c}	2.7 ± 0.7^{d}	2.6 ± 1.0^{d}	5.7 ± 0.7^{e}	8.7 ± 1.1^{f}

Values are mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant ($p<0.001$).

Fig 3.3 Liver tissue omega-3 fatty acids level of rats fed incremental amount of LNA

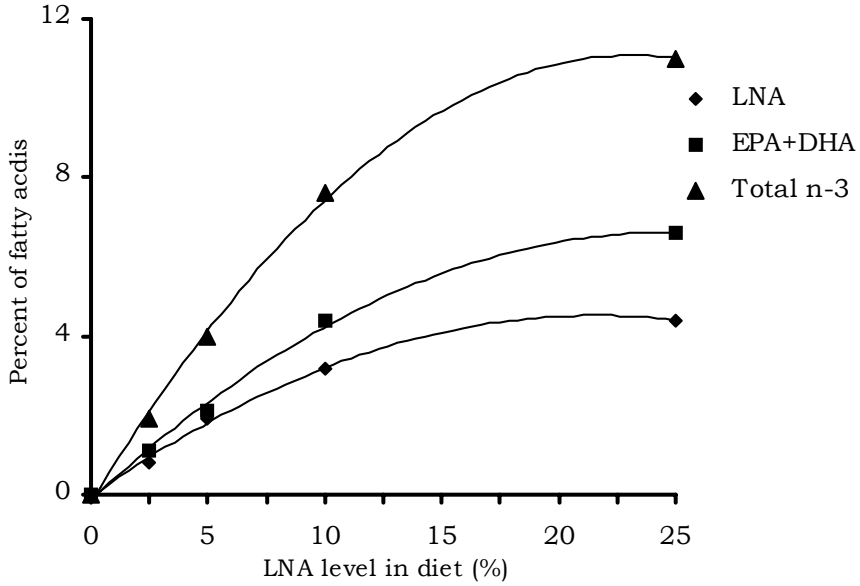
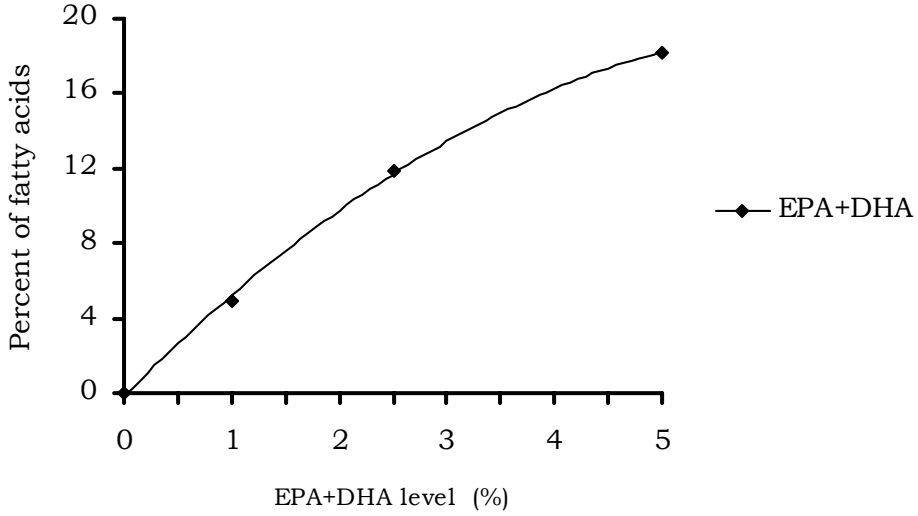


Fig 3.4 Liver tissue omega-3 fatty acids level of rats fed incremental amount of EPA+DHA



The total cholesterol and triacylglycerol level decreased in a dose dependent manner when rats were fed diet containing incremental amounts of LNA or EPA+DHA (Table 3.6). In rats fed diets containing LSO to provide LNA at 2.5, 5.0, 10.0 and 25.0% levels, the total cholesterol level was decreased by 9, 13, 21, and 27% respectively and triacylglycerol level was lowered by 8, 15, 20 and 28% respectively compared to control group. In rats fed diets containing FO with EPA+DHA levels of 1.0, 2.5 and 5.0 % lowered total cholesterol level by 7, 11 and 16% respectively and triacylglycerol levels by 10, 17 and 24% respectively compared to control group. The phospholipids level was not altered in any of the groups.

Table 3.6 Liver lipid profile (mg/g tissue) of rats fed omega-3 fatty acids at incremental levels

Parameters	GNO	LNA 2.5%	LNA 5.0%	LNA 10.0%	LNA 25%	EPA+DHA 1.0%	EPA+DHA 2.5%	EPA+DHA 5.0%
Total cholesterol	10.9 ± 0.4^{a}	9.9 ± 0.2^{b}	9.4 ± 0.1^{cf}	8.6 ± 0.1^{d}	8.0 ± 0.2^{e}	10.1 ± 0.1^{b}	9.7 ± 0.1^{bf}	9.2 ± 0.1^{cf}
Triglycerides	16.9 ± 0.9^{a}	15.5 ± 0.5^{ab}	14.4 ± 0.6^{bc}	13.5 ± 0.6^{cd}	12.1 ± 0.5^{d}	15.3 ± 0.5^{b}	14.0 ± 0.5^{cd}	12.7 ± 0.9^{d}
Phospholipids	25.2 ± 1.4^{a}	26.0 ± 2.9^{a}	28.2 ± 2.3^{a}	28.2 ± 3.0^{a}	28.7 ± 2.0^{a}	25.1 ± 2.3^{a}	25.1 ± 1.3^{a}	25.3 ± 1.3^{a}

Values are mean ± SD of 6 rats. Values not sharing a common superscript within a row are statistically significant ($p<0.001$).

Heart, brain and adipose tissue fatty acid profile of rats fed diet with different levels of LSO or FO.

The fatty acid profile of heart (Table 3.7), brain (Table 3.8) and adipose (Table 3.9) tissues were significantly altered when different levels of LNA or EPA+DHA were fed to rats. EPA was not detected in the heart of control rats as well as in rats fed 2.5% LNA in the diet, but small amounts of which were found when LNA was fed at levels greater than 5.0% in the diet. The initial level of DHA in the heart tissue of control rats was found to be 3.9% of total fatty acids and was raised to 4.2, 4.8 and 6.0% respectively when LNA levels were maintained at 5.0, 10.0 and 25.0% in the diet (Fig 3.5). However, feeding FO at incremental levels significantly increased the DHA level in heart. From the initial level of 3.9% of total fatty acids, the DHA level was raised to 8.2, 11.2 and 15.1% in rats fed diet with 1.0, 2.5 and 5.0% EPA+DHA (Fig 3.6).

Feeding incremental level of LNA to rats resulted in an increase in the levels of DHA in brain tissue. The initial level of DHA in brain tissue of control rats was found to be 8.3% and was raised to 9.4, 10.4, 11.2 and 12.4% respectively when LNA levels were maintained at 2.5, 5.0, 10.0 and 25.0% in the diet (Fig 3.7). LNA was detected in the brain tissue of rats fed diet-containing LNA at 5.0, 10.0 and 25.0%. Feeding diet-containing FO resulted in an increase in the DHA level from 8.3 in control to 11.4, 13.1 and 16.9% in rats when EPA+DHA levels in the diet was maintained at 1.0, 2.5 and 5.0% levels (Fig 3.8).

The level of LNA in the adipose tissue increased when incremental amounts of LNA were given in the diet. The LNA in adipose tissue was found to be 1.9, 3.9, 6.9 and 12.1% of total fatty acids respectively when the LNA content in the diet was kept at 2.5, 5.0, 10.0 and 25.0%

(Fig 3.9). EPA and DHA were not detected in adipose tissue of rats fed diet with different levels of LNA, however, feeding FO resulted in a significant incorporation of EPA and DHA in adipose tissue. The EPA level was found to be 0.8, 2.6 and 5.2% of total fatty acids respectively when a diet containing 1.0, 2.5 and 5.0 % was fed to rats (Fig 3.10). The corresponding DHA levels observed in the adipose tissue of these rats were 0.9, 2.8 and 5.2% respectively.

Table 3.7 Heart tissue fatty acid profile (%) of rats fed omega-3 fatty acids at incremental levels

Fatty acids	GNO	LNA 2.5%	LNA 5.0%	LNA 10.0%	LNA 25%	EPA+DHA 1.0%	EPA+DHA 2.5%	EPA+DHA 5.0%
16:0	14.6 ± 2.1^{a}	13.6 ± 2.3^{a}	14.9 ± 1.9^{a}	15.1 ± 2.0^{a}	17.1 ± 3.3^{a}	14.0 ± 3.1^{a}	14.9 ± 2.3^{a}	14.1± 2.0^{a}
16:1	1.5 ± 0.7^{ab}	1.3 ± .6^{ab}	0.9 ± 0.2^{a}	1.2 ± 0.4^{ab}	1.3 ± 0.3^{ab}	1.8 ± 0.7^{ab}	2.1 ± 0.9^{b}	2.9 ± 1.0^{b}
18:0	28.0± 2.3^{a}	28.6 ± 3.1^{a}	27.9 ± 2.7^{a}	28.0 ± 3.8^{a}	29.1 ± 3.4^{a}	25.6 ± 3.6^{a}	25.9 ± 1.9^{a}	25.2 ± 2.3^{a}
18:1	20.1 ± 2.1^{a}	19.8 ± 3.0^{a}	19.3 ± 2.0^{a}	18.5 ± 3.0^{a}	17.9 ± 2.8^{a}	19.4 ± 3.6^{a}	18.9 ± 1.3^{a}	18.2 ± 2.0^{a}
18:2	14.7± 1.9^{a}	14.8 ± 1.2^{a}	14.2 ± 2.0^{a}	13.3± 3.1^{ab}	10.9 ± 2.4^{b}	13.0± 2.6^{ab}	10.7 ± 1.9^{b}	9.3 ± 2.0^{b}
18:3	ND	0.6 ± 0.2^{a}	1.4 ± 0.3^{b}	2.3 ± 0.7^{b}	3.3 ± 0.6^{c}	ND	ND	ND
20:4	17.0 ± 3.0^{a}	17.1 ± 2.8^{a}	16.7 ± 3.1^{a}	15.8 ± 3.3^{a}	13.0 ± 2.7^{a}	16.1 ± 2.3^{a}	13.9 ± 3.1^{a}	12.0 ± 1.9^{a}
20:5	ND	ND	0.3 ± 0.08^{a}	0.6 ± 0.2^{b}	1.3 ± 0.6^{c}	1.5 ± 0.2^{c}	2.1 ± 0.4^{c}	3.1 ± 0.5^{d}
22:6	3.9 ± 0.6 ^{a}	3.8 ± 1.1^{a}	4.2 ± 1.3^{ab}	4.8 ± 1.0$^{a\ b}$	6.0 ± 1.6^{b}	8.2 ± 1.3^{c}	11.2 ± 1.8^{d}	15.1 ± 2.1^{e}

Values are mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant ($p<0.001$).

Table 3.8 Brain tissue fatty acid profile (%) of rats fed omega-3 fatty acids at incremental levels

Fatty acids	GNO	LNA 2.5%	LNA 5.0%	LNA 10.0%	LNA 25%	EPA+DHA 1.0%	EPA+DHA 2.5%	EPA+DHA 5.0%
16:0	20.9 ± 2.1^{a}	21.2 ± 2.0^{a}	20.4 ± 3.1^{a}	19.3 ± 0.9^{a}	19.0 ± 1.3^{a}	22.1 ± 1.9^{a}	22.1 ± 2.7^{a}	21.1 ± 3.0^{a}
16:1	0.3 ± 0.05^{a}	$0.2 \pm .004^{a}$	0.2 ± 0.03^{a}	0.3 ± 0.05^{a}	0.2 ± 0.06^{a}	0.4 ± 0.06^{b}	0.7 ± 0.03^{c}	0.9 ± 0.07^{d}
18:0	17.1 ± 1.6^{a}	17.0 ± 2.3^{a}	18.1 ± 2.0^{ab}	20.0 ± 1.9^{ab}	22.0 ± 3.0^{b}	15.2 ± 2.3^{ac}	15.0 ± 2.9^{ac}	13.6 ± 3.0^{c}
18:1	37.2 ± 4.1^{a}	35.8 ± 1.9^{a}	34.3 ± 2.6^{a}	33.1 ± 2.6^{a}	29.1 ± 3.6^{a}	35.2 ± 2.3^{a}	33.8 ± 4.0^{a}	32.0 ± 2.6^{a}
18:2	3.4 ± 1.0^{a}	3.5 ± 1.1^{a}	3.2 ± 0.6^{a}	2.6 ± 1.0^{ab}	2.1 ± 0.8^{bc}	3.0 ± 0.9^{ac}	2.8 ± 1.0^{ac}	2.6 ± 0.6^{ac}
18:3	ND	ND	0.6 ± 0.1^{a}	1.0 ± 0.2^{b}	2.1 ± 0.4^{c}	ND	ND	ND
20:4	12.3 ± 2.1^{a}	12.4 ± 1.9^{a}	12.0 ± 0.9^{a}	11.2 ± 2.0^{ab}	10.8 ± 1.2^{ab}	11.9 ± 1.6^{ab}	10.3 ± 2.0^{ab}	9.6 ± 1.0^{b}
20:5	0.4 ± 0.05^{a}	0.3 ± 0.1^{a}	0.6 ± 0.06^{b}	1.1 ± 0.1^{c}	1.9 ± 0.2^{d}	1.0 ± 0.05^{c}	1.9 ± 0.06^{d}	2.3 ± 0.2^{e}
22:6	8.3 ± 1.0^{a}	9.4 ± 0.9^{ab}	10.4 ± 1.3^{abc}	11.2 ± 1.6^{bc}	12.4 ± 1.5^{c}	11.4 ± 2.0^{bc}	13.1 ± 1.2^{c}	16.9 ± 1.6^{d}

[a] Values are mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant ($p<0.001$).

Table 3.9 Adipose tissue fatty acid profile (%) of rats fed omega-3 fatty acids at incremental levels

Fatty acids	GNO	LNA 2.5%	LNA 5.0%	LNA 10.0%	LNA 25%	EPA+DHA 1.0%	EPA+DHA 2.5%	EPA+DHA 5.0%
14:0	2.6 ± 0.06[a]	1.4 ± 0.09[b]	1.3 ± 0.07[b]	1.4 ± 0.1[b]	1.0 ± 0.1[c]	1.9 ± 0.2[d]	1.5 ± 0.4[b d]	1.6 ± 0.09[e]
16:0	17.0 ± 3.6[a]	15.6 ± 1.0[a]	16.5 ± 1.1[a]	15.0 ± 0.9[a]	17.2 ± 1.0[a]	17.9 ± 1.9[a]	16.4 ± 2.1[a]	17.4 ± 1.3[a]
16:1	3.6 ± 1.0[a]	3.7 ± 0.4[a]	4.0 ± 0.6[a]	4.2 ± 1.0[a]	3.7 ± 0.3[a]	4.4 ± 0.8[a]	5.4 ± 1.2[a b]	6.5 ± 0.8[b]
18:0	1.3 ± 0.08[a]	2.0 ± 0.2[b]	1.3 ± 0.1[a]	0.8 ± 0.1[c]	0.5 ± 0.06[d]	1.3 ± 0.2[a]	1.1 ± 0.03[a]	0.9 ± 0.04[b]
18:1	55.0 ± 3.6[a]	54.5 ± 1.9[a]	53.3 ± 1.2[a]	53.2 ± 1.3[a]	49.5 ± 1.0[b]	53.9 ± 2.4[a]	52.4± 3.9[a c]	49.0 ± 1.8[bc]
18:2	19.7 ± 2.6[a]	20.0 ± 1.1[a]	18.2± 2.0[ab]	17.5 ± 0.9[b]	15.5 ± 0.5[c]	17.9 ± 3.4[b]	16.6± 2.9[bc]	13.9 ± 2.9[d]
18:3	ND	1.9 ± 0.1[a]	3.9 ± 0.3[b]	6.9 ± 1.0[c]	12.1 ± 0.3[d]	ND	ND	ND
20:4	0.9 ± 0.02[a]	0.8 ± 0.04[b]	0.9 ± 0.06[a]	0.7 ± 0.02[c]	0.4 ± 0.01[d]	0.7 ± 0.06[c]	0.6 ± 0.05 [c]	0.5 ± 0.01[d]
20:5	ND	ND	ND	ND	ND	0.8 ± 0.01[b]	2.6 ± 0.2 [c]	4.9 ± 0.4[d]
22:6	ND	ND	ND	ND	ND	0.9 ± 0.02[a]	2.8 ± 0.6 [b]	5.2 ± 0.9[c]

[a] Values are mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant ($p<0.001$).

Fig 3.5 Heart tissue omega-3 fatty acids level of rats fed incremental amount of LNA

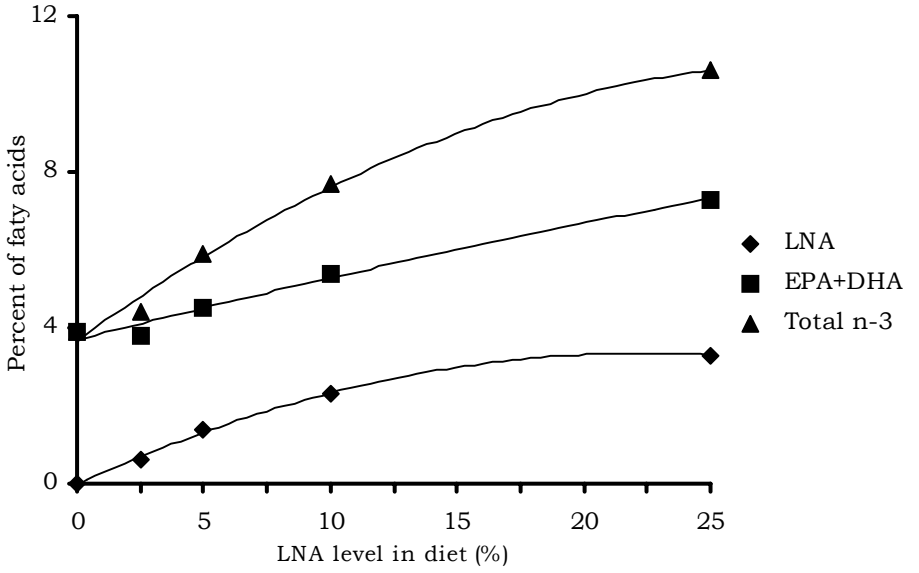


Fig 3.6 Heart tisue omega-3 fatty acids level of rats fed incremental amount of EPA+DHA

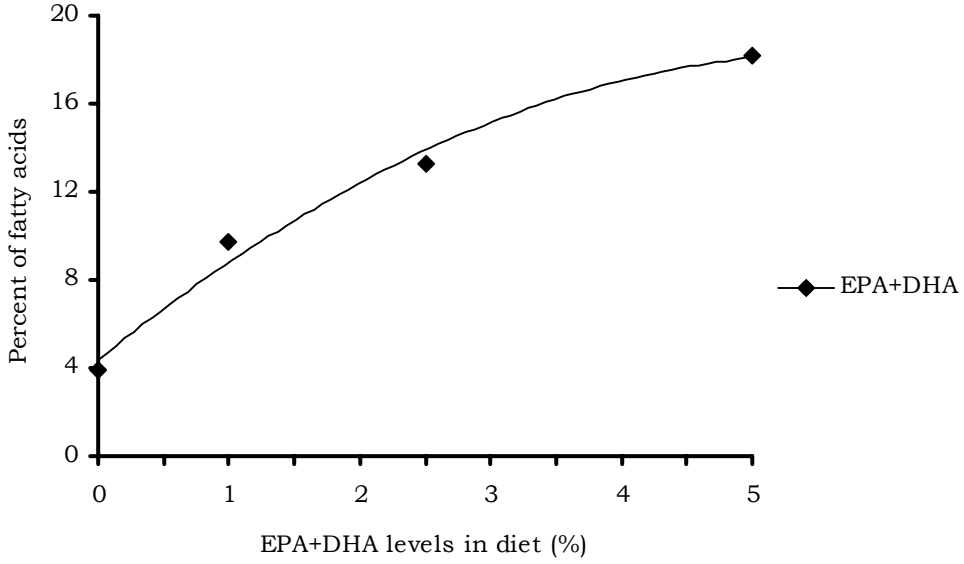


Fig 3.7 Brain tissue omega-3 fatty acids level of rats fed incremental amount of LNA

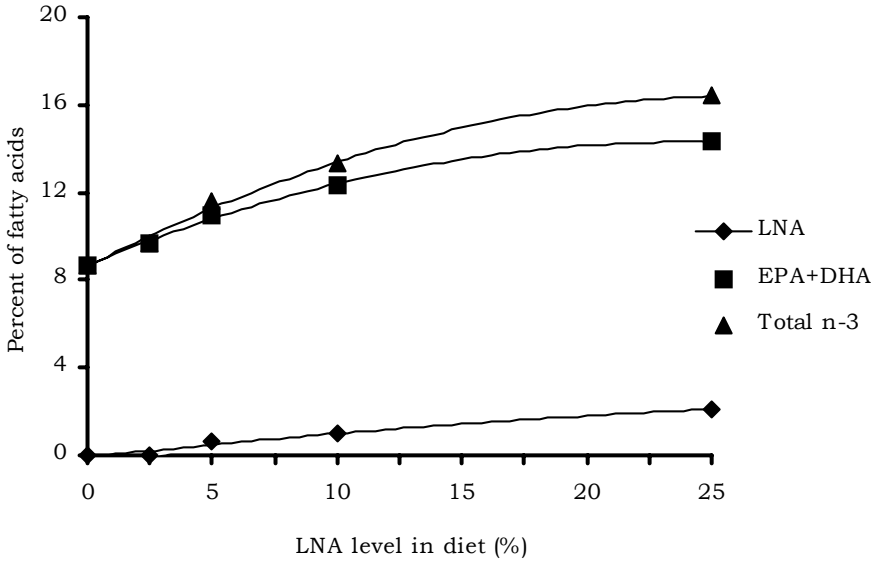


Fig 3.8 Brain tissue omega-3 fatty acids level of rats fed incremental amount of EPA+DHA

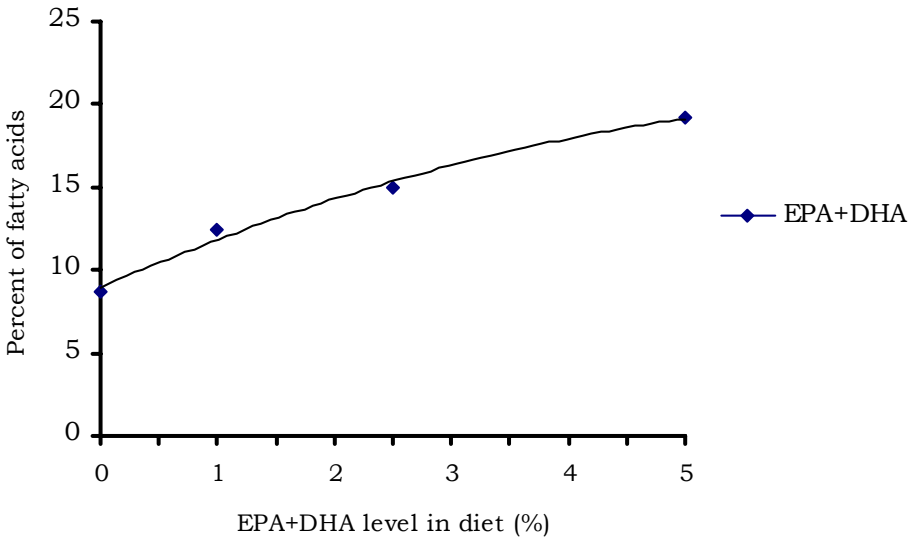


Fig 3.9 Adipose tissue omega-3 fatty acids level of rats fed incremental amount of LNA

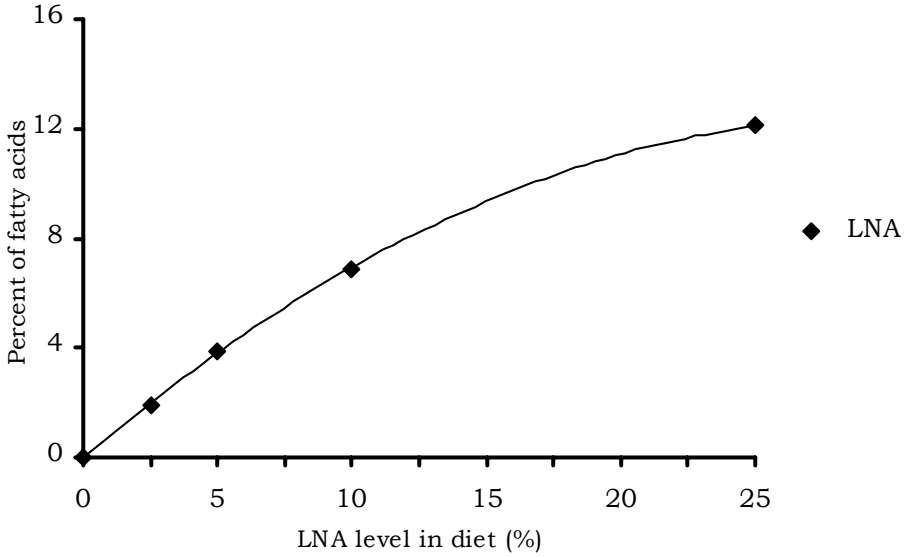
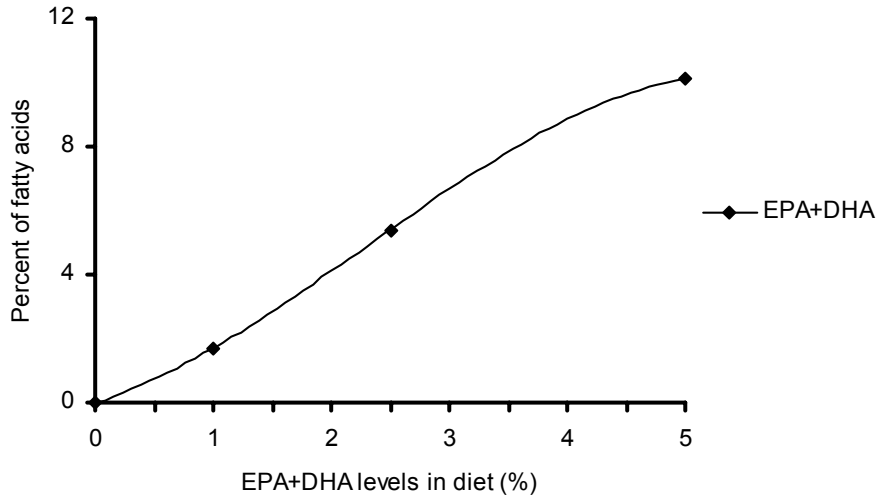


Fig 3.10 Adipose tissue omega-3 fatty acids level of rats fed incremental amount of EPA+DHA



Discussion

The primary objective of this investigation is to evaluate the uptake of LNA from the vegetable oil source and its conversion to long chain omega-3 PUFA. This is then compared with the uptake of preformed long chain omega-3 PUFA obtained from FO. These uptakes were monitored at comparable levels of LA in the diet. α-linolenic acid was fed at four levels of approximately 2.5, 5.0, 10.0 and 25.0% of total fatty acids in dietary lipids. The long chain omega-3 PUFA, EPA+DHA were fed at approximately 1, 2.5 and 5.0% levels in the diet. The LA levels in the diets were maintained at 18-24%.

As the LNA levels were increased incrementally in the diet, there was a corresponding increase in the levels of LNA in serum and liver lipids. This also resulted an incremental increase in the accumulation of EPA+DHA in the serum and liver. This indicated that the conversion of LNA to long chain omega-3 PUFA is taking place in rats and this conversion rate is dependent on the level of LNA in the diet. However, preformed EPA+DHA was taken up by serum and liver much more efficiently than that obtained from LNA. Thus at 2.5 and 5.0% LNA in the diet, EPA and DHA was observed to an extent of 1 and 1.7% in serum and 1.1 and 2.1% in liver lipids. Whereas, at similar levels of EPA+DHA in the diet the accumulation of these long chain omega-3 PUFA was found to be 6 and 9.7% in serum and 11.9 and 18.2% in liver lipids respectively. This indicated that an effective uptake and retention of preformed long chain omega-3 PUFA in serum and liver (Burdge and wooten 2002; Burdge et al 2002).

The ratio of omega-6 to omega-3 PUFA is often considered to be crucial for the conversion of LNA to long chain omega-3 PUFA as omega-6 PUFA (LA, 18:2) can effectively compete with delta-6

desaturase which acts on both omega-6 and omega-3 PUFA (Mathews et al 2000; Cunnane et al 1999; Cho et al 1999). It is also shown that in the presence of LA, the LNA is efficiently oxidized and hence may not be available as substrate for synthesis of long chain omega-3 PUFA (Emken et al 1994). In our studies the ratio of omega-6 to omega-3 fatty acids were varied from 9.1 to 0.69. In spite of such large range of ratios used, LNA was converted to EPA and DHA at all the ratios used. The extent of LNA accumulation was also dependent on the dietary levels of this fatty acid fed to rats. The ratio of LNA to EPA+DHA in serum and liver at all points were comparable. This indicated that LA did not influence delta-6 desaturase responsible for converting LNA to long chain omega-3 PUFA. Further, the LA accumulation and its conversion to arachidonic acid was also found to be dependent on the levels of LA available in the diet. LNA even though varied from 2.5 to 25% in the diet, did not influence accumulation of LA and the conversion to arachidonic acid again indicating that neither LA nor the LNA interfered with each other metabolism to long chain PUFA. Since LNA accumulation and its long chain metabolites remained constant even though the ratio of LA/LNA was varied from 9.11 to 0.69 which again indicated that LA may not be driving LNA to oxidation pathway as has been suggested earlier (Burdge and wooten 2002; Burdge et al 2002). However, when preformed EPA+DHA was used in the diet there was a progressive accumulation of these fatty acids in serum and liver which took place at the expense of LA and arachidonic acid. This may indicate that omega-6 PUFA is effectively displaced by long chain omega-3 PUFA in serum and liver.

However, the change in omega-6 and omega-3 PUFA levels in different tissues in response to dietary lipids was not uniform (Cho et al

1999). The DHA content in heart did not change appreciably up to 10% LNA in the diet even though there was a progressive increase in LNA content of heart. Even at 25% LNA level in the diet there was only 54% increase in DHA content above that found in control animals. However, DHA contents increased dramatically by 110, 187 and 287% when EPA+DHA was included in the diet at 1, 2.5 and 5% respectively. The EPA levels were also significantly increased in FO fed animals as compared to those given higher levels of LNA in the diet. However, the extent of EPA uptake compared to DHA was significantly lower. Studies have shown that the level of incorporation of DHA exceeds that of EPA even if DHA is fed at lower levels (Croset et al 1989). In our study, as observed by earlier investigators, the uptake of DHA mainly occurred at the expense of arachidonic acid (Swanson et al 1988). Our results, supports the general view that heart tissue embraces the omega-3 fatty acids particularly DHA. These studies indicated that preferential uptake of long chain omega-3 PUFA by heart if it is provided as preformed fatty acids.

Brain is another organ, which is highly enriched in DHA. The high levels of DHA in brain of a number of mammalian species irrespective of their size led to early speculations that this fatty acid is playing a crucial role in the nervous system. Many studies using LNA deficient diets have shown reductions in the level of DHA in brain and loss of many cognitive functions (Greiner et al 1999; Ahmed et al 2002). Feeding incremental amounts of LNA in the diets progressively increased DHA levels by 13-49% when LNA levels were increased from 2.5 to 25% in the diet. Similarly, in rats fed 1-5% EPA+DHA in the diet increased DHA levels in brain by 13-49% when LNA levels were increased from 2.5 to 25% in the diet. Similarly in rats fed 1-5%

EPA+DHA in the diet increased DHA levels in brain by 37-104% over that found in control animals. A small but significant increase in EPA levels were also observed in rats fed LNA or EPA+DHA containing diets. Our findings support earlier reports showing better incorporation of dietary DHA to brain tissue rather than that metabolized from precursor LNA (Abedin et al 1999).

Adipose tissue normally does not contain long chain omega-6 or omega-3 PUFA. However, LNA accumulation in the adipose tissue increased linearly in response to increasing LNA content in the diet. Feeding incremental amounts of LNA from 2.5 to 25% in the diet resulted in accumulation of LNA from 1.9 to 12.1% in the adipose tissue. This is of particular interest, since adipose tissue represents one of the important storage tissue for LNA that can be made available to the body when needed (Fu and Sinclair 2000). We however, did not detect any long chain omega-3 PUFA in adipose tissue. Our findings are in agreement with the results reported by Lin and Connors (1990) and also Kajwara et al (1997). Their studies indicated that when rats were given perilla oil which contained LNA to an extent of 60% of total fatty acid, resulted in accumulation of LNA to an extent of 12% in adipose tissue, but less than 1% EPA and DHA was detected. This along with our studies indicated that adipose tissue has the ability to take up LNA but may not be able to convert it to long chain omega-3 PUFA. On the other hand adipose tissue readily picks up EPA+DHA if it is provided as preformed long chain omega-3 PUFA. Therefore, adipose tissue may not have active desaturase and elongase to convert essential fatty acids to its longer chain metabolites.

Both LNA and EPA+DHA containing diets lowered serum cholesterol and triglyceride levels in a dose dependent manner.

However, the ability of EPA+DHA containing diets to lower serum and liver lipid levels at lower levels is significantly higher than that found with LNA containing diet. Based on the levels of these fatty acids in the diet and the extent of lipid lowering observed, it was noticed that 4 to 5 times higher concentrations of LNA is required to exhibit comparable effects with EPA+DHA in the diet.

In the present investigation it was observed that different tissues responded differently to the dietary FA with respect to the type and extent of accumulation of omega-3 PUFA. Based on these results, the amount of dietary omega-3 PUFA required to increase EPA+DHA levels in different tissues was calculated (Table 10). All the values were normalized to 100mg of LNA or EPA+DHA intake in the diet. It was calculated from the data of experiments that consumption of 100mg of LNA will result in the accumulation of EPA+DHA at 2.04%, 0.7%, 1.91% and 1.64% of total FA respectively in liver, heart, brain and serum. Similarly consumption of 100mg of preformed EPA+DHA in the diet resulted in 25.4%, 23.8%, 15.9% and 14.9% of total FA as EPA+DHA in liver, heart, brain and serum respectively (Table 10). This indicated that efficacy of preformed EPA+DHA to increase the long chain omega-3 PUFA in tissues is much higher than that could be achieved by giving precursor LNA in the diet. This raises the point about the efficacy and essentiality of long chain omega-3 PUFA that is currently debated by experts in the field (Lauritzen and Hansen 2003; Sinclair et al 2003). Though adipose tissue accumulated LNA, it failed to elongate it to long chain omega-3 PUFA. However, preformed EPA+DHA were effectively taken up by adipose tissue.

Table. 3.10 Calculated levels of EPA+DHA found in different tissues by feeding 100 mg of LNA or EPA+DHA in the diet.

Tissues	Amount of EPA+DHA (% of total FAs) found after feeding a diet containing		
	LNA (A)	EPA+DHA (B)	* Efficacy (B/A)
Liver	2.04 ± 0.45	25.42 ± 4.31	12.5
Heart	0.71 ± 0.05	23.79 ± 9.02	33.5
Brain	1.91 ± 0.57	15.92 ± 5.01	8.3
Adipose	ND	11.21 ± 1.25	-
Serum	1.6 ± 0.4	14.89 ± 4.48	9.1

ND: Not detected. * Efficacy was caluculated by taking the ratio of long chain omega-3 PUFA found in tissues by feeding equivalent amounts of LNA or EPA+DHA in the diet.

In conclusion, present study indicates that LNA could be a source of omega-3 PUFA for vegetarian sections of population. This could be desaturated and elongated to long chain omega-3 PUFA and also has beneficial effects in lowering serum lipid levels. LA in diet does not seem to influence the metabolism of LNA. However, higher levels of LNA in the diet are required for achieving comparable effects with that of preformed EPA+DHA in the diet. All the tissues tested took up higher amounts of EPA+DHA even when they were provided at lower levels in the diet.

CHAPTER IV

Supplementation and delivery of omega-3 fatty acids through spray-dried milk.

Introduction

The long chain omega-3 fatty acids are abundant in marine sources like fish. Mammalian tissues can elongate and desaturate the precursor LNA to EPA and DHA. Our study indicated incremental amount of LNA from LSO effectively increased the LNA level in serum and tissues in a dose dependent manner. Further, the level of EPA+DHA in serum and tissues was also found to be increased following feeding of LNA. However, it was observed that preformed EPA+DHA from FO increased the EPA+DHA level in serum and tissues much more efficiently than the precursor LNA. Thus, LSO and FO can be exploited as vegetable and marine sources of omega-3 fatty acids respectively to populations, who do not get adequate level of omega-3 fatty acids in their diet.

Based on the survey conducted on dietary patterns it was estimated that Indians derive 12.6g LA per day equivalent to 4.8 en% from visible and invisible fats (Ghafoorunissa 1990; 1996). The requirement for LA as an essential fatty acid is 3 en% (Ghafoorunissa 1990). Hence Indian diets provide adequate amounts of LA. However, the intake of omega-3 fatty acids by Indians is in the range of 0.3g in rural and 0.6g in the urban population equivalent to 0.2-0.3 en% (Ghafoorunissa 1996; Achaya 1995). A number of scientific and regulatory agencies such as committee on Medical Aspects of Food Policy, Department of Health, British Nutrition Foundation, Scientific Committee for Food, Food and Agricultural Organization/World Health Organization have recommended that omega-3 PUFA should go up in the region of 0.7-1.0 en%. The recommendations have been summarized in detail by Roche (1999), Stanley (2004) and Sanderson et al (2002). Recently experts have also debated and agreed upon the essentiality of omega-3 PUFA in the diet. (Sinclair 2003; Lauritzen

and Hansen 2003). Even though there is no specific recommendations for intake of omega-3 PUFA in India, several studies conducted by National Institute of Nutrition, an organization of Indian Council of Medical Research, have recommended that an intake of 0.75 en% from LNA and 0.2 en% from EPA and DHA will have positive impact on the prevention of Coronary Heart Disease. (Ghafoorunissa 1996; 1998; Indu and Ghafoorunissa 1992). Mustard oil, which contains about 10% of LNA, is used in some parts of India. However, it also contains about 50% of erucic acid, (22:1 cis -13) which is a concern for many individuals, as it is shown that erucic acid at levels greater than 7% in the oil of the diet cause myocardial lipidosis and fibrosis as has been observed in experimental animals (Beare-Rogers 1977). These observations in the dietary habit and also the biochemical indices like plasma and platelet phospholipids fatty acid profile of normal Indian subjects with regard to levels of omega-3 fatty acids reinforce the need to improve their omega-3 fatty acids intake (Ghafoorunissa 1996). Oily fish can provide sufficient amount of omega-3 fatty acids like EPA and DHA. However, many people in India refrain from taking fish, as it is a non-vegetarian food. Linseed (*Linum usitatissimum*), rich in LNA is an economically important oil seed crop used for edible purpose by central and northeastern region of India. The beneficial properties of LSO (Caughey et al 1996; Cunnane et al 1993; Jenkins 1999) and FO (Schmidt 1997) are well discussed by earlier workers. LSO can therefore be exploited as an alternate source of omega-3 fatty acids, if it can be delivered in appropriate form. But LSO has very limited utility value due to its rapid polymerization if not processed properly.

The direct use of these omega-3 fatty acid rich oils as a single oil or as blends have certain limitations with respect to sensory

qualities, acceptability and reachability. Hence an alternate way of delivering dietary omega-3 PUFA to the Indian Population could be through enriching a commonly consumed food products such as milk. Milk is a beverage that is consumed by all sections of populations. Milk also contains additional components, which also may have significant health benefits. Milk fat is a good source of conjugated linoleic acid (Aneja and Murthy 1991) with anti atherogenic properties (Lee et al 1994). Milk contains short chain and medium chain fatty acids, which can be more readily absorbed, digested and oxidized by carnitine independent pathways without hindering the formation of chylomicrons (Basal and Kansal 1996). Studies have emphasized the important traditional role of milk as a supplier of nutrients, because of its versatility and high nutrient density. Bovine milk is widely used in India by people of different age groups. However, in spite of its high nutritional value, it is devoid of fatty acids of omega-3 family like LNA, EPA and DHA. Liquid milk can be converted in to powder form by spray-drying techniques and reconstituted with water for consumption as a beverage. Thus, milk can be used as a medium to deliver omega-3 fatty acids.

The present experiment was designed to study the feasibility of using milk in spray-dried form for delivering omega-3 fatty acids from LSO or FO. Their implications on some of the risk factors, which have a bearing on cardiovascular function, were also studied.

Results

Fatty acid composition of spray-dried milk formulation.

Analysis of fatty acid composition of diets revealed that LSO containing spray-dried milk formulation had 20% LNA and those with FO contained 3 and 2% EPA and DHA, respectively. These fatty acids were not detected in the spray-dried milk powder containing GNO (Table 4.1).

Table 4.1 Fatty acid composition (%) of diet

Fatty acids	GNO	LSO	FO
12: 0	1.2	1.9	1.6
14: 0	6.9	7.9	11.0
16: 0	27.7	25.8	35.0
16: 1	1.0	1.0	4.8
18: 0	9.2	11.9	10.6
18: 1	41.3	24.2	25.0
18: 2	9.7	6.8	3.8
18: 3	ND	20.3	ND
20: 0	1.9	ND	ND
20: 4	ND	ND	3.1
20: 5	ND	ND	2.9
22: 0	0.8	ND	ND
22: 6	ND	ND	2.1
PUFA (P)	9.7	27.1	11.9
SFA (S)	47.7	47.5	58.2
P/S	0.20	0.57	0.20

Values are Mean of triplicate samples, ND: Not detected

Effect of feeding spray-dried milk supplemented with omega-3 fatty acid on growth parameters.

The fat level in the diet was kept constant at 16% in all the groups. The amounts of diet consumed in different groups were comparable (Table 4.2). There was no significant change in the food efficiency ratio and body weight gained by rats fed spray-dried milk based diet containing GNO, LSO and FO. The weights of heart, spleen and brain of rats given GNO or LSO or FO based diets were comparable (Table 4.3). The liver weight of rats given FO based diet was higher than those observed in rats given GNO or LSO diets. The hematological parameters in different groups were comparable [hemoglobin (g/dL); 14.66 ± 1.37, red blood cells x 10^6 /μL; 9.82 ± 0.41, total count; 6687 ± 442 and packed cell volume (%); 37.41 ± 1.25] (combined mean ± SD of all groups). These studies indicated that supplementation of milk with omega-3 PUFA rich oils (LSO or FO) had no adverse effect on the growth of animals.

Table 4.2 Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on growth parameters

Parameters	GNO	LSO	FO
Food intake (g / day / rat)	9.9 ± 0.8[a]	10.2 ± 0.6[a]	10.3 ± 1.0[a]
Body wt gained (g)	191 ± 25[a]	194 ± 30[a]	188 ± 19[a]
Food efficiency ratio	0.32 ± 0.04[a]	0.31 ± 0.03[a]	0.31 ± 0.04[a]

Values are Mean ± SD of 6 rats. Values not sharing a common superscript within a row are statistically significant $p < 0.001$

Table 4.3 Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on organs weight

Organs (g/100 g body wt)	GNO	LSO	FO
Liver	3.5 ± 0.2[a]	3.5 ± 0.3[a]	4.1 ± 0.2[b]
Heart	0.32 ± 0.02[a]	0.34 ± 0.03[a]	0.33 ± 0.02[a]
Spleen	0.26 ± 0.08[a]	0.23 ± 0.03[b]	0.24 ± 0.02[b]
Brain	0.67 ± 0.11[a]	0.66 ± 0.08[a]	0.67 ± 0.07[a]

Values are Mean ± SD of 6 rats. Values not sharing a common superscript within a row are statistically significant $p < 0.001$

Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on serum lipids.

Significant changes were observed in omega-3 fatty acid level in rats given milk formulation with LSO or FO (Table 4.4). LNA level in rats given LSO containing formulation was 6.8%. There was also a significant increase in EPA and DHA levels with a concomitant decrease in arachidonic acid levels in rats given LSO containing formulation. LNA was not detected in the serum of rats fed GNO containing formulation. Omega-3 fatty acids fed rats had 12-18% lower levels of oleic acid in serum. Feeding FO supplemented formulation incorporated the long chain omega-3 PUFA like EPA and DHA in serum to a level of 2.3 and 2.6% respectively. These fatty acids were not observed in rats given GNO containing formulation.

Table 4.4 Serum fatty acid profiles (%) of rats fed spray-dried milk supplemented with omega-3 fatty acids

Fatty acids	GNO	LSO	FO
16:0	30.4 ± 3.0^{ab}	29.1 ± 1.6^{a}	34.3 ± 2.0^{b}
16:1	1.5 ± 0.3 ^{a}	2.1 ± 0.5 $^{a\,b}$	3.3 ± 0.7 ^{b}
18:0	11.9 ± 1.5 ^{a}	14.5 ±2.3 ^{ab}	15.5 ±2.0 ^{b}
18:1	41.0 ± 3.9 ^{a}	33.3 ± 2.1 ^{b}	35.8 ± 2.3 ^{ab}
18:2	8.7 ± 2.3 ^{a}	8.3 ± 1.9 ^{a}	3.1 ± 2.3 ^{b}
18:3	ND	6.8 ± 0.8 ^{a}	ND
20:4	6.3 ± 1.3 ^{a}	2.4 ± 1.0 ^{b}	2.6 ± 1.5 ^{b}
20:5	ND	2.0 ± 0.6 ^{a}	2.3 ± 0.1 ^{a}
22:6	ND	0.9 ± 0.1 ^{a}	2.6 ± 0.5 ^{b}

Values are Mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant $p < 0.001$.

Rats given spray dried milk powder containing LSO or FO had 30 and 35% lower level of serum total cholesterol respectively compared to those given GNO containing formulations. Serum triacylglycerol level was decreased by 26 and 29% in LSO and FO formulation fed group respectively compared to rats fed GNO containing formulation (Table 4.5).

Table 4.5 Serum lipid profile of rats fed spray-dried milk supplemented with omega-3 fatty acids

Parameters (mg/ dL)	GNO	LSO	FO
Total cholesterol	90.2 ± 3.2 ^{a}	62.9 ± 5.7 ^{b}	58.2 ± 6.3 ^{b}
HDL cholesterol	37.6 ± 4.3 ^{a}	23.4 ± 3.2 ^{b}	23.4 ± 3.8 ^{b}
LDL+VLDL cholesterol	52.5 ± 4.0 ^{a}	39.5 ± 4.8 ^{b}	34.7 ± 4.8 ^{b}
Phospholipids	147.3 ± 10.7 ^{a}	119.1 ± 9.5 ^{b}	122.6 ± 10.8 ^{b}
Triacylglycerol	136.4 ± 6.4 ^{a}	99.9 ± 12.9 ^{b}	96.4 ± 11.8 ^{b}

Values are Mean ± SD of 6 rats. Values not sharing a common superscript within a row are statistically significant $p < 0.001$

Liver lipid profile of rats fed spray-dried milk supplemented with omega-3 fatty acids.

Liver is an important organ for lipid metabolism. The change in the dietary fat significantly altered the liver fatty acid profile (Table 4.6). The oleic acid level decreased to an extent of 38-41% in the liver of rats fed omega-3 fatty acids. Significant amounts of LNA and long chain omega-3 fatty acids were accumulated in the liver of rats given LSO based diets. The total omega-3 PUFA levels in LSO fed rats were 17.5%. Similarly, FO based diets significantly enriched EPA (6%) and DHA (9.7%) levels in the liver lipids. omega-3 fatty acid enrichment in liver decreased the levels of linoleic acid and arachidonic acid.

Table 4.6 Liver tissue fatty acid profile (%) of rats fed spray-dried milk supplemented with omega-3 fatty acids

Fatty acids	GNO	LSO	FO
16:0	22.7 ± 2.3 [a]	21.1 ± 1.9 [a]	28.2 ± 3.4 [b]
16:1	1.3 ± 0.2 [a]	1.5 ± 0.3 [a]	2.9 ± 0.2 [b]
18:0	11.9 ± 2.1 [a]	17.5 ± 3.4 [b]	17.3 ± 1.6 [b]
18:1	41.2 ± 3.3 [a]	25.3 ± 3.9 [b]	24.0 ± 2.5 [b]
18:2	11.3 ± 1.8 [a]	9.9 ± 2.0 [a]	5.7 ± 1.8 [b]
18:3	ND	7.6 ± 0.8 [a]	ND
20:4	11.4 ± 1.1 [a]	6.9 ± 0.4 [b]	5.9 ± 0.6 [b]
20:5	ND	6.5 ± 1.0 [a]	6.0 ± 1.1 [a]
22:6	ND	3.4 ± 0.6 [a]	9.7 ± 0.9 [b]

Values are Mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant $p < 0.001$.

The total cholesterol level in liver was decreased in rats given LSO and FO containing formulation by 30 and 18%, respectively (Table 4.7). The triacylglycerol level was decreased by 18 and 11% respectively in LSO and FO containing formulation. No significant

changes were observed in the phospholipids level in the liver tissue of animals fed different fat sources.

Table 4.7 Liver lipid profiles of rats fed spray-dried milk supplemented with omega-3 fatty acids

Parameters (mg/g tissue)	GNO	LSO	FO
Total cholesterol	10.7 ± 0.5 [a]	7.5 ± 1.2 [b]	8.8 ± 0.6 [b]
Triacylglycerol	15.1 ± 0.8 [a]	12.3 ± 1.0 [b]	13.5 ± 0.7 [b]
Phospholipids	28.2 ± 2.9 [a]	30.7 ± 7.0 [a]	29.7 ± 2.9 [a]

Values are Mean ± SD of 6 rats. Values not sharing a common superscript within a row are statistically significant $p < 0.001$

Fatty acid composition of heart, brain and adipose tissue of rats fed spray-dried milk supplemented with omega-3 fatty acids.

The dietary omega-3 fatty acids influenced the fatty acid composition of heart (Table 4.8), brain (Table 4.9) and adipose tissue (Table 4.10).

In heart, the LNA levels in the rats fed LSO formulation was found to be 4.9% of total fatty acids, whereas, LNA was not detected in other dietary groups. The basal level of DHA was significantly enhanced in the heart tissue of rats given FO containing diet. There was a 3.5 fold increase in the accumulation of DHA in the heart tissue of animals fed FO formulation compared to GNO containing formulation. The arachidonic acid level in the heart tissue decreased followed by feeding omega-3 fatty acids to the animals.

In brain, rats fed LSO formulations contained LNA to the extent of 2.9% of total fatty acids, whereas, LNA was not detected in other dietary groups. The basal level of DHA in GNO containing formulation fed rats was 9.6% of total fatty acids and found to be increased to 12.7 and 14.4% of total fatty acids respectively in LSO and FO containing formulation fed rats.

Animals fed on LSO and FO formulations had 25 and 45% lower amount of LA in adipose tissue compared to those fed on GNO containing formulation. Adipose tissue of LSO formulation fed rats had 15% of total fatty acid as LNA, where as, LNA was not detected in rats fed on GNO and FO containing formulations. EPA and DHA were found only in animals fed on FO containing formulation and was found to be 1.3 and 1.4% of total fatty acids respectively.

Table 4.8 Heart tissue fatty acid profile (%) of rats fed spray dried milk supplemented with omega-3 fatty acids

Fatty acids	GNO	LSO	FO
16:0	14.3 ± 2.3 [a]	13.5 ± 3.1 [a]	14.8 ± 1.9 [a]
16:1	1.0 ± 0.2 [a]	1.0 ± 0.3 [a]	1.2 ± 0.1 [a]
18:0	17.4 ± 2.5 [a]	23.0 ± 3.1 [b]	22.9 ± 3.5 [b]
18:1	29.7 ± 3.9 [a]	22.2 ± 3.8 [b]	19.4 ± 2.6 [b]
18:2	17.1 ± 2.1 [a]	17.4 ± 3.1 [a]	14.4 ± 3.2 [a]
18:3	ND	4.9 ± 0.4 [a]	ND
20:4	13.6 ± 3.1 [a]	11.2 ± 1.9 [a]	11.2 ± 1.6 [a]
20:5	ND	1.0 ± 0.1 [a]	2.9 ± 0.5 [b]
22:6	4.6 ± 0.4 [a]	5.7 ± 1.3 [b]	15.8 ± 2.1 [c]

Values are Mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant $p < 0.001$.

Table 4.9 Brain tissue fatty acid profile (%) of rats fed spray-dried milk supplemented with omega-3 fatty acids

Fatty acids	GNO	LSO	FO
16:0	24.3 ± 2.9 [a]	25.6 ± 3.6 [a]	23.0 ± 3.0 [a]
16:1	0.4 ± 0.1 [a]	0.5 ± 0.1 [a]	0.4 ± 0.2 [a]
18:0	20.9 ± 3.6 [a]	20.1 ± 4.3 [a]	19.0 ± 3.8 [a]
18:1	29.9 ± 3.6 [a]	25.0 ± 4.2 [a]	29.1 ± 2.8 [a]
18:2	1.8 ± 0.9 [a]	1.6 ± 0.5 [a]	1.4 ± 0.1 [a]
18:3	ND	2.9 ± 0.4 [a]	ND
20:4	11.6 ± 2.8 [a]	9.2 ± 1.6 [a]	8.1 ± 1.2 [a]
20:5	0.6 ± 0.2 [a]	2.1 ± 1.2 [b]	3.0 ± 0.9 [b]
22:6	9.6 ± 0.2 [a]	12.7 ± 0.8 [b]	14.4 ± 0.3 [c]

Values are Mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant $p < 0.001$.

Table 4.10 Adipose tissue fatty acid profile (%) of rats fed spray-dried milk supplemented with omega-3 fatty acids

Fatty acids	GNO	LSO	FO
14:0	6.7 ± 0.6 [a]	7.5 ± 1.0 [a]	10.6 ± 1.0 [b]
16:0	26.6 ± 4.1 [a]	28.2 ± 4.6 [a]	33.1 ± 3.3 [a]
16:1	2.4 ± 0.6 [a]	4.4 ± 1.1 [b]	8.1 ± 1.2 [c]
18:0	4.2 ± 0.3 [a]	4.0 ± 0.6 [a]	3.4 ± 0.3 [a]
18:1	48.9 ± 4.9 [a]	39.1 ± 3.8 [b]	35.3 ± 4.0 [b]
18:2	10.7 ± 2.1 [a]	8.0 ± 0.9 [a]	5.8 ± 1.1 [b]
18:3	ND	15.1 ± 1.2 [a]	ND
20:4	0.5 ± 0.04 [a]	0.2 ± 0.04 [b]	0.4 ± 0.06 [a]
20:5	ND	ND	1.3 ± 0.2 [a]
22:6	ND	ND	1.4 ± 0.3 [a]

Values are Mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant $p < 0.001$.

Discussion

The present investigation was aimed at providing a formulation containing omega-3 fatty acids that can be consumed by those who do not get adequate amount of omega-3 fatty acids from their diet. To cater to such population, milk based instant beverage formulation was developed which can provide omega-3 fatty acids. Few approaches have been made earlier to enrich milk with omega-3 fatty acids by feeding the dairy cows with fish based products (Kitessa et al 2004). However, the metabolic state of animals, season and hormonal variations may interfere with the level of omega-3 fatty acids in the milk. Fresh milk cannot be stored for a long period of time. We developed two milk-based spray-dried formulations that are shelf stable and containing omega-3 fatty acids from vegetable source like LSO or marine source like FO. The beverage provided 640mg of LNA or 160mg EPA+DHA per serving when 20g of formulation dissolved in 100ml water was consumed. This provided 0.75 or 0.2 en% from omega-3 fatty acids provided by LSO and FO formulations respectively.

Rats were fed with these milk based formulations to study the delivery of omega-3 fatty acids to various tissues and its impact on some of the risk factors having a bearing on cardiovascular diseases. The rats fed spray-dried formulation containing LSO showed incorporation of LNA and its metabolites EPA and DHA in serum and tissue. Earlier studies have shown that feeding LNA containing diets can increase the level of this fatty acid along with an increase in the level of long chain metabolites to varying degrees (Gerster 1998; Mantzioris et al 1994; Bazinet et al 2003).

Liver is major site of lipid metabolism and sensitive to alteration in the dietary fatty acids. Feeding LSO containing

formulation significantly increased the LNA, EPA and DHA level in liver tissue. Zhong and Sinclair observed that feeding diet high in LNA to Guinea pigs increases the EPA and DHA level in liver compared to those fed on low LNA diet (Zhong and Sinclair 2000).

In heart tissue, the DHA level increased to an extent of 24% following feeding of spray-dried milk supplemented with LSO compared to control. However, feeding preformed long-chain omega-3 fatty acids significantly increased the level of DHA in heart tissue by 245% compared to control. This indicated an effective uptake of omega-3 fatty acids delivered through milk-based formulation (Zhong and Sinclair 2000).

Brain is a unique organ characterized by the presence of high concentration of DHA, which influences its structural and functional properties (Brenner 1984). Feeding rats with formulations containing omega-3 fatty acids from LSO or FO further increased the DHA level in brain compared to control. It was also observed that rats fed preformed DHA in the form of FO formulation had higher DHA level in brain compared to those fed on LSO formulation. This is in agreement with earlier reports, where it was shown that, preformed DHA is efficiently incorporated in to brain tissue (Abedin et al 1999; Greiner et al 1996). The LSO enriched diets significantly increased the levels of LNA as well as EPA in addition to DHA in brain. Earlier studies have also reported a small but significant increase in LNA and EPA levels in brain tissue when diets high in omega-3 fatty acids were administered (Zhong and Sinclair 2000. Anderson 1994; Philbrick 1987). These studies may indicate differential mechanisms in different tissues for the accumulation of LNA and its conversion to long chain omega-3 PUFA.

However, in adipose tissue, EPA and DHA were not detected following the feeding of LSO containing formulation. Similarly in a study conducted by Lin and Connors (1990) it was observed that LNA enriched diet resulted in the accumulation of LNA but not EPA and DHA in the adipose tissue. The investigations by Kajwara et al (1997) on rats showed that, feeding diet containing perilla oil with 60.3% LNA resulted in the accumulation of EPA and DHA only to an extent of 0.9 and 0.4% respectively, whereas, LNA was deposited to an extent of 12.3% of total fatty acids of adipose tissue. These studies may indicate that though adipose tissue could accumulate LNA at high intake levels but its elongation to EPA and DHA is restricted. However, adipose tissue could accumulate these fatty acids when preformed long chain omega-3 PUFA are given in the diet.

The omega-3 fatty acids obtained through spray-dried milk had significant effects on serum lipids. The serum total cholesterol, VLDL + LDL cholesterol and triacylglycerol were lowered significantly in rats fed formulations containing omega-3 fatty acids compared to control. Similar to serum lipids, the liver lipids were also decreased by feeding omega-3 fatty acids containing formulation. Present investigation also showed that FO formulation containing 5% omega-3 PUFA as EPA and DHA was as effective as LSO formulation containing 20% LNA in modulating serum lipids in beneficial manner. It is generally believed that bio efficacy of long chain omega-3 PUFA is much greater than that of omega-3 PUFA of shorter chain length. Our results are in agreement with this perception.

In conclusion, the present study indicated that spray dried milk formulation enriched in LSO or FO can be used for providing the omega-3 fatty acids. These formulations not only deliver omega-3 fatty acids but also beneficially modulate the serum lipid levels in

reducing risk factors for cardiovascular diseases. The incorporation of omega-3 PUFA and its effect on serum and liver lipids after feeding spray-dried milk powder containing omega-3 fatty acids was comparable to that observed when linseed oil or fish oil were fed as a part of AIN-76 diets (Chapter III).

This indicated that the bioavailability of omega-3 fatty acids from spray-dried milk formulation is not hindered by its processing with milk. The milk associated omega-3 fatty acids is readily acceptable and hence such a formulation will play an important role in providing omega-3 PUFA to the individuals who do not get adequate amounts of these essential fatty acids through their regular diet.

CHAPTER V

Mechanism of hypocholesterolemic effect of spray-dried milk supplemented with omega-3 fatty acids.

Introduction

The synthesis and utilization of cholesterol must be tightly regulated in order to prevent its over accumulation and abnormal deposition within the body. The fatty acid profile of diet plays an important role in determining the serum and tissue cholesterol levels. Dietary saturated fatty acids increases the serum cholesterol concentrations and high concentration of cholesterol in serum is one of the primary factors in the development of atherosclerosis (Martin et al 1986). Studies have predicted that for each 1% rise in serum cholesterol, the risk for coronary heart disease is increased by ≈ 2% (Lipid Research Clinic Program 1984).

The liver plays a central role in the maintenance of cholesterol homeostasis in the body. This mainly involves the biosynthetic pathway regulated by HMG CoA reductase (Mevolanate; NADP oxidoreductase) (Dietschy et al 1993). Reductions in the cholesterol levels can be achieved by drug therapies comprising statins (Shepard et al 1995; Scandinavian Simvastatin Survival Study 1994). Statins inhibit the HMG CoA reductase activity and cholesterol biosynthesis. Cholesterol levels can also be reduced by enhancing its catabolic pathway via bile secretion or by interfering with entero-hepatic circulation.

Bile acids are the by-product of cholesterol and the pathway is regulated by cholesterol 7-α hydroxylase (Botham 1986). Bile acids play an important role in the maintenance of cholesterol homeostasis. Several factors including diet are known to influence the bile metabolism. Dietary fat has been correlated to influence the bile constituents. Diets high in unsaturated fatty acids compared with saturated fatty acids increased the secretion of biliary cholesterol significantly. (Mott et al 1992).

In our study we showed that, feeding rats with spray-dried milk supplemented with omega-3 fatty acids from LSO or FO, efficiently incorporated omega-3 fatty acids in serum and tissues (Ramaprasad et al 2004). The omega-3 fatty acid incorporation was accompanied with a significant reduction in the serum and liver cholesterol levels, which indicated the hypocholesterolemic effect of spray-dried milk containing omega-3 fatty acids from LSO or FO. However, the cholesterol lowering effect of EPA+DHA from FO was higher at lower concentrations compared to LNA in the diet. Bio-efficacy of LNA in terms of its incorporation to tissues and subsequent conversion to EPA and DHA may be an important factor that may modulate the cholesterol homeostasis.

The present investigation was therefore undertaken to understand the efficacy of spray-dried milk containing omega-3 fatty acids from vegetable or marine source on cholesterol metabolism to understand the mechanism by which these formulation causes a reduction in cholesterol levels.

Results

HMG CoA Reductase activity

HMG CoA reductase is a rate-limiting enzyme in the cholesterol biosynthesis. Feeding milk formulation containing omega-3 fatty acids significantly lowered the activity of HMG CoA reductase in rat liver microsomes (Table 5.1). Milk formulation containing LSO lowered the activity of HMG CoA reductase by 17% and FO containing formulation lowered it by 22% compared to control group. The cholesterol level in rat liver microsomes was lowered by 16 and 20% respectively in LSO and FO containing formulation compared to control group. However, no significant change was observed in phospholipids level in rats fed omega-3 fatty acids containing formulations compared to control rats.

The fatty acid composition of liver microsomes indicated that feeding LSO supplemented formulation raised the LNA level from zero in control group to 4.1% in LSO fed group (Table 5.2). The EPA and DHA in this group were found to be 2.7 and 0.9% respectively. Feeding FO significantly incorporated EPA and DHA in microsomes and it was found to be 4.1 and 3.6% respectively. Incorporation of omega-3 fatty acids occurred mainly at the expense of linoleic and arachidonic acid.

Table 5.1 Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on HMG CoA reductase activity, cholesterol and phospholipids in rat liver microsomes

Parameters	GNO	LSO	FO
HMG CoA reductase activity (n moles of Co A formed / min / mg protein)	0.94 ± 0.04 [a]	0.78 ± 0.08 [b]	0.73 ± 0.10 [b]
Cholesterol (µg / mg protein)	44.78 ± 3.4 [a]	37.5 ± 2.9 [b]	35.6 ± 2.0 [b]
Phospholipids (µg / mg protein)	341.3 ± 10.1 [a]	319 ± 15.1 [a]	328.7 ± 17.3 [a]

Values are Mean ± SD of 6 rats. Values not sharing a common superscript within a row are statistically significant ($p<0.001$)

Table 5.2 Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on fatty acid composition rat liver microsomes.

Fatty acids (%)	GNO	LSO	FO
16:0	21.82 ± 2.9 [a]	26.91 ± 1.63 [ab]	28.39 ± 1.78 [b]
16:1	1.27 ± 0.18 [a]	1.20 ± 0.09 [a]	1.32 ± 0.14 [a]
18:0	25.07 ± 2.61 [a]	27.40 ± 2.32 [a]	28.4 ± 2.81 [a]
18:1	20.58 ± 2.00 [a]	21.82 ± 3.53 [a]	21.29 ± 2.13 [a]
18:2	13.46 ± 0.32 [a]	8.72 ± 1.2 [b]	6.88 ± 0.35 [c]
18:3	ND	4.15 ± 0.76 [a]	ND
20:4	17.43 ± 3.26 [a]	6.13 ± 0.15 [b]	5.12 ± 0.88 [b]
20:5	ND	2.72 ± 0.53 [a]	4.09 ± 0.24 [b]
22:6	ND	0.90 ± 0.13 [a]	3.65 ± 0.91 [b]

Values are Mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant ($p<0.001$)

Bile secretion and composition

Bile is one of the important route through which cholesterol is excreted. Feeding spray-dried supplemented with LSO or FO increased the volume of bile flow compared to control rats (Table 5.3). The increase in the bile flow was found to be 23 and 19% respectively in LSO and FO fed group compared to control. The secretion of bile solids increased to an extent of 62% in both LSO and FO supplemented formulation. The increase in the total bile solids was mainly due to increase in the secretion of cholesterol, phospholipids, bile acids and uronic acid. The cholesterol level was increased to an extent of 49-55% in rats fed LSO and FO containing formulation compared to control group. Whereas, phospholipids level was found to be increased to an extent of 140-146% in LSO and FO containing formulation.

The secretion of bile acids was found to be increased significantly in omega-3 fatty acid containing formulation fed group. The major bile acids analyzed includes taurocholic acid increased to an extent of 157-167%, taurodeoxycholic acid increased to an extent of 194-202% and glycocholic acid increased to an extent of 52-64% in LSO and FO containing formulations compared to control group. However, protein level in bile did not alter when omega-3 fatty acid containing formulations were fed.

Table 5.3 Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on bile secretion and composition

Parameters	GNO	LSO	FO
Bile flow (mL/h)	0.43 ± 0.010 [a]	0.53 ± 0.051 [b]	0.51 ± 0.01 [b]
Bile solids (g%)	3.04 ± 0.28 [a]	4.92 ± 0.28 [b]	4.97 ± 0.39 [b]
Cholesterol (µ mol / h)	0.177 ± 0.01 [a]	0.264 ± 0.03 [b]	0.274 ± 0.04 [b]
Uronic acids (µ mol / h)	3.58 ± 0.52 [a]	6.85 ± 0.86 [b]	6.74 ± 0.96 [b]
Total bile acids (µ mol / h)	6.01 ± 0.89 [a]	14.06 ± 1.01 [b]	14.51 ± 0.68 [b]
Taurocholic acid (µ mol / h)	3.00 ± 0.41 [a]	7.71 ± 0.69 [b]	8.01 ± 0.7 [b]
Taurodeoxy cholic acid (µ mol / h)	1.38 ± 0.11 [a]	4.06 ± 0.23 [b]	4.17 ± 0.41 [b]
Glycocholic acid (µ mol / h)	0.793 ± 0.11 [a]	1.21 ± 0.14 [b]	1.30 ± 0.20 [b]
Phospholipids (µ mol / h)	0.861 ± 0.069 [a]	2.07 ± 0.47 [b]	2.12 ± 0.32 [b]
Proteins (mg / h)	3.47 ± 0.49 [a]	3.88 ± 0.36 [a]	3.79 ± 0.41 [a]
Lithogenic index	0.73 ± 0.02 [a]	0.30 ± 0.10 [b]	0.35 ± 0.12 [b]

Values are Mean ± SD of 6 rats. Values not sharing a common superscript within a row are statistically significant ($p<0.001$)

Feeding spray-dried milk supplemented omega-3 fatty acids to rats significantly altered bile fatty acids composition (Table 5.4). The LNA level in bile of control rats was found to be zero and the level were raised to 4.6% in rats fed LSO containing formulation. The level of EPA and DHA in bile of rats fed LSO containing formulation was found to be 1.69 and 0.61% respectively. The EPA and DHA level in bile of FO containing formulation fed rats were found to be 2.3 and 2% respectively. The enrichment of omega-3 fatty acids in bile lipids occurred at the expense of linoleic acid arachidonic acid.

Table 5.4 Effect of feeding spray-dried milk supplemented with omega-3 fatty acids on fatty acid composition (%) of bile

Fatty acids	GNO	LSO	FO
16:0	31.08 ± 2.96 ^{a}	35.44 ± 2.98 ^{ab}	37.59 ± 3.01 ^{b}
16:1	1.08 ± 0.34 ^{a}	1.21 ± 0.46 ^{a}	2.98 ± 0.78 ^{b}
18:0	10.11 ± 2.07 ^{a}	14.0 ± 2.11 ^{ab}	14.91 ± 2.00 ^{b}
18:1	20.3 ± 3.01 ^{a}	15.81 ± 2.33 ^{a}	16.96 ± 2.19 ^{a}
18:2	18.79 ± 2.96 ^{a}	13.00 ± 2.91 ^{a}	11.43 ± 1.41 ^{ab}
18:3	ND	4.66 ± 1.13 ^{a}	ND
20:4	18.53 ± 2.93 ^{a}	13.23 ± 2.31 ^{b}	11.78 ± 1.64 ^{b}
20:5	ND	1.69 ± 0.39 ^{a}	2.3 ± 0.23 ^{b}
22:6	ND	0.61 ± 0.12 ^{a}	1.99 ± 0.37 ^{b}
P/S	0.91	0.67	0.52

Values are Mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant ($p<0.001$)

Discussion

The primary objective of the present investigation was to understand the mechanism of hypocholesterolemic effect exhibited by omega-3 fatty acid containing formulations. Similar levels of hypolipidemic effects were observed when the milk powder contained approximately 20% of LNA from LSO or 5% of EPA+DHA from FO.

The biosynthetic pathway for cholesterol is regulated by the activity of HMG CoA reductase (Dietschy et al 1993). The activity of this enzyme in liver microsomes reduced by 17-20% when omega-3 fatty acid enriched formulations were given to rats. Parallel decreases in cholesterol content of liver microsomes were also observed. However, the phospholipid levels were not significantly altered by omega-3 fatty acid containing formulations. These studies indicated that omega-3 PUFA may affect cholesterol levels by reducing the activity key regulator enzyme of cholesterol biosynthetic pathway. Recently, Le Jossic-Corcos et al (2005) have shown that feeding tuna fish oil reduced cholesterol levels in rodents by down regulating hepatic cholesterol synthesis through inhibition of HMG CoA reductase and farnesyl diphosphate synthase by impairing sterol regulatory binding protein. Similarly, Castillo et al (1999) observed lower activity of HMG CoA reductase in chicken fed menhaden oil. Our results regarding the inhibitory effect of omega-3 PUFA from milk formulations containing LSO or FO on HMG CoA reductase is in agreement with these earlier reports.

The hepato biliary pathway is a major route for the removal of cholesterol from the body either after converting it into bile acids or in the form of free cholesterol. Hence the biliary lipid secretion is an important factor for cholesterol balance in the body. We observed a significant increase in the volume of the bile in rats fed spray-dried

milk powder containing omega-3 fatty acids from linseed oil or fish oil. This resulted in an increase in the secretion of all the bile constituents including cholesterol, bile acids and phospholipids.

However, when the increase in the bile volume was normalized to the volume of bile in the control animals, we still observed 23% increase in all the bile constituents in linseed oil fed animals and a corresponding 20% increase in fish oil fed animals. This indicated that increase in the excretion of bile constituents in omega-3 PUFA fed animals is not merely due to an increase in the volume of bile secreted by animals but an actual increase in bile constituents. An increase in the bile acid secretion may be due to increase in the activity of cholesterol 7 α-hydroxylase (Bothom 1986). Recently, Berard et al (2004) have shown that dietary fish oil increased the D-site binding protein, liver X receptor and also cholesterol 7 α-hydroxylase resulting in an increase in bile acid and cholesterol excretion in mice. Cholesterol secretion in bile also appears to be tightly coupled to phospholipids and bile acid secretion (Robins et al 1991). Bile acids may provide primary stimulus for bile flow and facilitate the secretion of lipids in to bile (Booker et 1990; Tierney et al 1993; Knox et al 1991). Biliary secretion of phospholipids also appears to be dependent on the bile acids. During bile formation, bile acids stimulate hepato-cellular secretion of phospholipids from hepatocytes (Cohen et al 1990). Robins et al (1991) have shown an increased biliary secretion of cholesterol relative to phospholipids with more hydrophilic molecular species of phosphatidylcholine in the bile. The increase in bile secretion however, could not be attributed to higher P/S ratio since rats given LSO or FO formulation had lower P/S ratio compared to control animals given GNO. The P/S ratio of diets containing GNO and FO were comparable, but the

biliary secretions were higher in FO fed animals. Studies have reported that biliary secretion of cholesterol is significantly enhanced in fish oil fed animals compared to those given sunflower oil diets even though P/S ratios of sunflower oil diet is higher than that of fish oil diet (Balasubramaniam et al 1985; herzberg and Rogerson 1988). Thus interdependent mechanisms might have resulted in the increased excretion of bile constituents in rats given omega-3 fatty acids containing formulations.

However, the increased excretion of various bile constituents above a threshold point is also a cause for concern since cholesterol in the bile may precipitate beyond saturation point leading to cholesterol gallstones. Cholesterol is kept in soluble form by balancing the ratio with phospholipids and bile acids in the bile (Carey 1978). Lithogenic index in rats given omega-3 fatty acids from linseed oil or fish oil were 52-59% lower than that observed in animals given groundnut oil which was devoid of omega-3 fatty acids. This indicated that in spite of an increased excretion of cholesterol in bile of rats given omega-3 PUFA enriched diets; the Lithogenic index was kept low because of parallel excretion of bile acids and phospholipids in bile. This is in agreement with the observations made by Bravo et al (1998). There is evidence in literature to indicate that population consuming fish oil have low prevalence of cholesterol gall stone disease (Tierney et al 1993). In fact, the earlier studies have shown that fish oil consumption reduces the incidence of cholesterol gall stone formation in prairie dogs (Magnuson et al 1995). Fish oil also beneficially affects biliary cholesterol nucleation time in obese women (Sanchez et al 2001). Recently Abei et al (2000) demonstrated that fish oil inhibits mucus glycoprotein secretion in gall bladder

epithelia cells, which is a critical factor in the pathogenesis of gallstones.

The fatty acid composition of phospholipids also exhibits significant influence on cholesterol saturation index in monkeys and humans. By linear path analysis Berr et al (1992) have found a positive correlation between the relative amount of cholesterol in human gall bladder bile and that of arachidonic acid in biliary lipids. They also noticed a negative relationship with linoleic acid. The availability of arachidonic acid is an important factor for regulating the rate of prostaglandin synthesis. Omega-3 fatty acids were shown to decrease the incidence of cholesterol crystal formation by lowering prostaglandin synthesis in cholesterol-fed Prairie dogs (Booker et al 1990). We have also demonstrated that feeding spray-dried milk powder containing omega-3 fatty acids replaced arachidonic acid by omega-3 fatty acid in serum and tissue lipids (Ramaprasad et al 2004).

The hydrophobicity of bile acids also plays an important role in the gall stone formation. It was suggested that glycine conjugated bile acids are more hydrophobic which are lithogenic in nature, while taurine conjugates have opposite effects (Trautwein et al 1993; Heuman et al 1989; Armstrong and Carey 1982). Upon feeding omega-3 fatty acid enriched formulation, the taurine conjugated bile acids were increased by 150-200% while the glycine conjugated bile acids increased to an extent of only 50%, thus a favorable ratio of glycine to taurine was maintained in the bile acids which may have a beneficial influence on cholesterol saturation index. It is also known that rat bile acids are conjugated almost exclusively with taurine (Haslewood and Wooton 1950; Jacobson and Smith Jr. 1968). Earlier studies have also shown that hepatic taurine synthesis indirectly

increases cholesterol 7 α-hydroxylase activities there by increasing the synthesis of bile acids (Hajri 1998).

In conclusion, the present study has shown that spray-dried milk powder containing omega-3 fatty acids from linseed oil or fish oil increases bile flow with increased excretion of biliary cholesterol and bile acids. However, the Lithogenic index was lower than that observed in control animals which were given a diet devoid of omega-3 fatty acids. Omega-3 PUFA enriched formulations also inhibited HMG CoA reductase activity. Thus a combined action on cholesterol biosynthesis and increased secretion of cholesterol and its metabolites through bile might have resulted in lowering of serum cholesterol observed in the rats given spray-dried milk formulation containing omega-3 fatty acids. Thus the present investigation addressed the mechanism by which omega-3 fatty acids enriched formulations exhibited hypocholesterolemic effects.

CHAPTER VI

Antioxidant enzyme activities, platelet aggregation and serum prostaglandins in rats fed spray-dried milk supplemented with omega-3 fatty acids

Introduction

The omega-3 fatty acid from spray-dried milk significantly enhanced the omega-3 fatty acids level in serum and tissues. This was followed by a significant reduction in the cholesterol level in serum and liver tissue. However, omega-3 fatty acid is susceptible for oxidation because of high degree of unsaturation (Mehta et al 1994).

The unsaturated fatty acids may also influence the activity of enzymes involved in the antioxidant defense system. It has been established that as the intake of unsaturated fatty acids is increased, the antioxidant capacity of body is challenged. This may cause imbalance in the antioxidant defense and results in oxidative stress.

The susceptibility of a cell to oxidative damage is affected by the efficiency of the antioxidant defense enzymes such as catalase, glutathione peroxidase, glutathione transferase and superoxide dismutase (Leibovitz et al 1990; Rice et al 1995). The antioxidant enzymes, superoxide dismutase, glutathione peroxidase and catalase work with in the cells to remove superoxides and peroxides before they react with metal ions to form more reactive free radical (Gonzales et al 1984). The product of lipid peroxidation may have deleterious effects leading to tissue damage (Morel and Chisolm 1989).

It is well established that lipid peroxidation is implicated in the pathological process and may cause platelet dysfunction (Selvemini and Botting 1993; Nageswari et al 1999). The circulating platelets and their interaction with vascular endothelium play a significant role in the formation of coronary thrombosis that causes acute artery disease (Svaneborg et al 2002; Hennig and Chow 1988). Unlike cellular proteins, which are predominantly genetically determined, cell membrane fatty acids are largely influenced by dietary fat intake

(Simopolous 1991). Any alterations in the dietary lipids provide a non-pharmacologic means of altering eicosanoids synthesis and may inturn influence the aggregability of platelets. The eicosanoids derived from omega-6 fatty acids, primarily arachidonic acid give rise to 2 series of prostaglandins and thromboxanes, where as the omega-3 family of fatty acid give rise to the 3 series of prostaglandins (Needleman et al 1979). The prostanoids of central concern for platelet function are thromboxane A_2 that has pro-aggregatory activity and prostacyclin I_2 that has antiplatelet-aggregatory activity (Mullane and Pinto 1987; Marcus 1984).

The present investigation was undertaken to see whether consumption of omega-3 fatty acid enriched milk formulation modulate the antioxidant enzyme system, thrombotic risk as indicated by serum prostaglandins level and platelet response.

Results

Fatty acid profile of Liver of rats fed spray-dried milk supplemented with omega-3 fatty acid

Liver is an important site for lipid metabolism. Liver is also the source for lipoproteins and antioxidant enzymes. Feeding omega-3 fatty acid supplemented formulation significantly altered the fatty acid profile of liver in rats (Table 6.1). The LNA, EPA and DHA level in liver of LSO formulation fed rats were found to be 7.6, 6.5 and 3.4% of total fatty acids respectively. Feeding FO supplemented formulation significantly deposited EPA and DHA in liver and it was found to be 6 and 9.7% respectively. The oleic acid level decreased to an extent of 40% when omega-3 fatty acids containing formulation were fed to rats. The arachidonic acid level was found to be decreased to an extent of 40-48% in liver of rats fed omega-3 fatty acids supplemented formulation compared to control.

Table 6.1 Fatty acid composition (%) of liver of rats fed spray-dried milk supplemented with omega-3 fatty acid

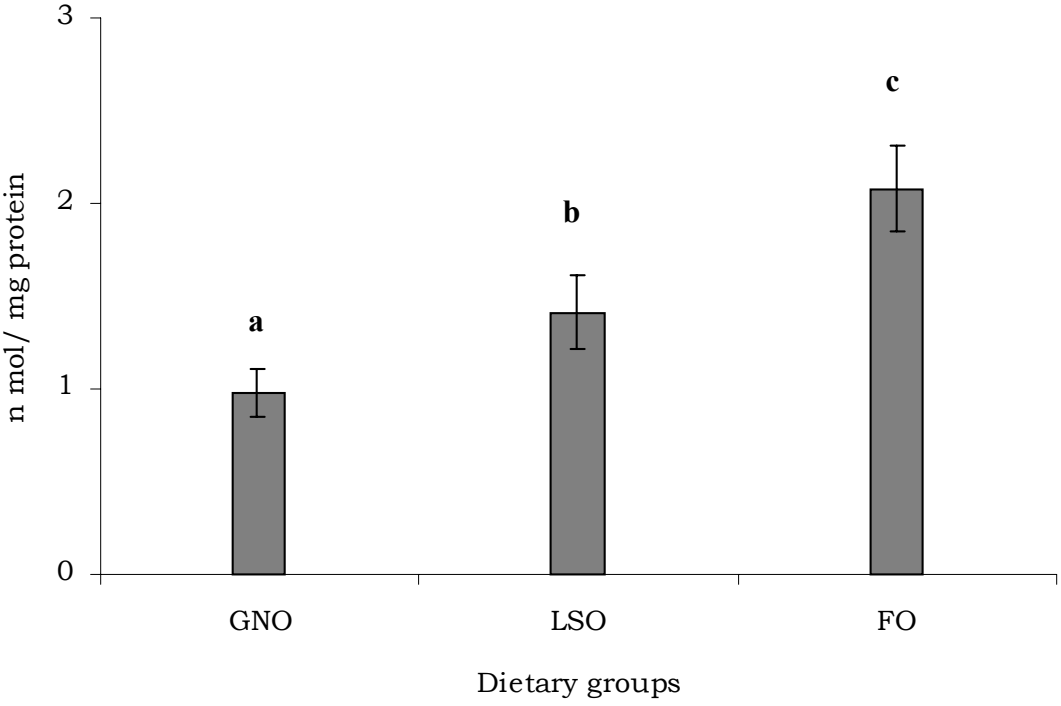
Fatty acids	GNO	LSO	FO
16:0	22.7 ± 2.3 [a]	21.1 ± 1.9 [a]	28.2 ± 3.4 [b]
16: 1	1.3 ± 0.2 [a]	1.5 ± 0.3 [a]	2.9 ± 0.2 [b]
18: 0	11.9 ± 2.1 [a]	17.5 ± 3.4 [b]	17.3 ± 1.6 [b]
18: 1	41.2 ± 3.3 [a]	25.3 ± 3.9 [b]	24.0 ± 2.5 [b]
18: 2	11.3 ± 1.8 [a]	9.9 ± 2.0 [a]	5.7 ± 1.8 [b]
18: 3	ND	7.6 ± 0.8 [a]	ND
20: 4	11.4 ± 1.1 [a]	6.9 ± 0.4 [b]	5.9 ± 0.6 [b]
20: 5	ND	6.5 ± 1.0 [a]	6.0 ± 1.1 [a]
22: 6	ND	3.4 ± 0.6 [a]	9.7 ± 0.9 [b]
PUFA/SFA	0.65 ± 0.07 [a]	0.88 ± 0.08 [b]	0.60 ± 0.10 [a]

Values are Mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant $p < 0.001$.

Lipid peroxides level in liver homogenate of rats fed spray-dried milk supplemented with omega-3 fatty acids

Alteration in dietary fat influences the membrane fatty acids composition which inturn affect the lipid peroxides level in the tissues. Lipid peroxide level in liver homogenate of rats fed LSO and FO enriched formulation was found to be higher by 44 and 112% respectively compared to control group (Fig 6.1).

Fig 6.1 Lipid peroxides level in liver homogenate of rats fed spray-dried milk supplemented with omega-3 fatty acids

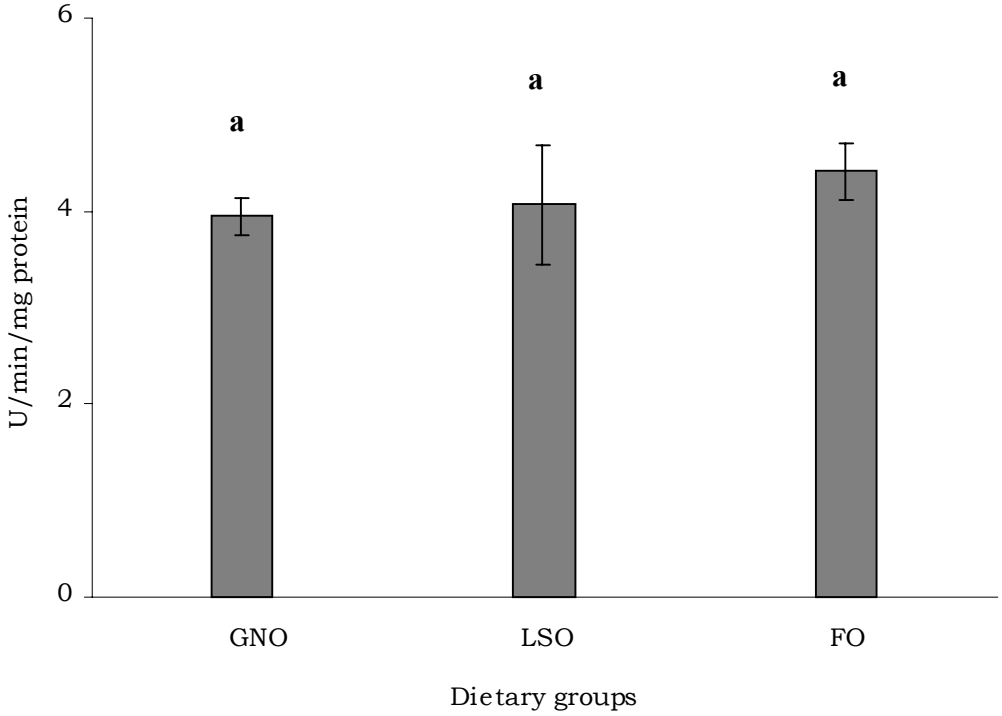


Values are Mean ± SD of 6 rats. Values not sharing the common superscripts are significantly different $p < 0.001$

Antioxidant enzyme activities in liver homogenate of rats fed omega-3 fatty acid enriched formulation

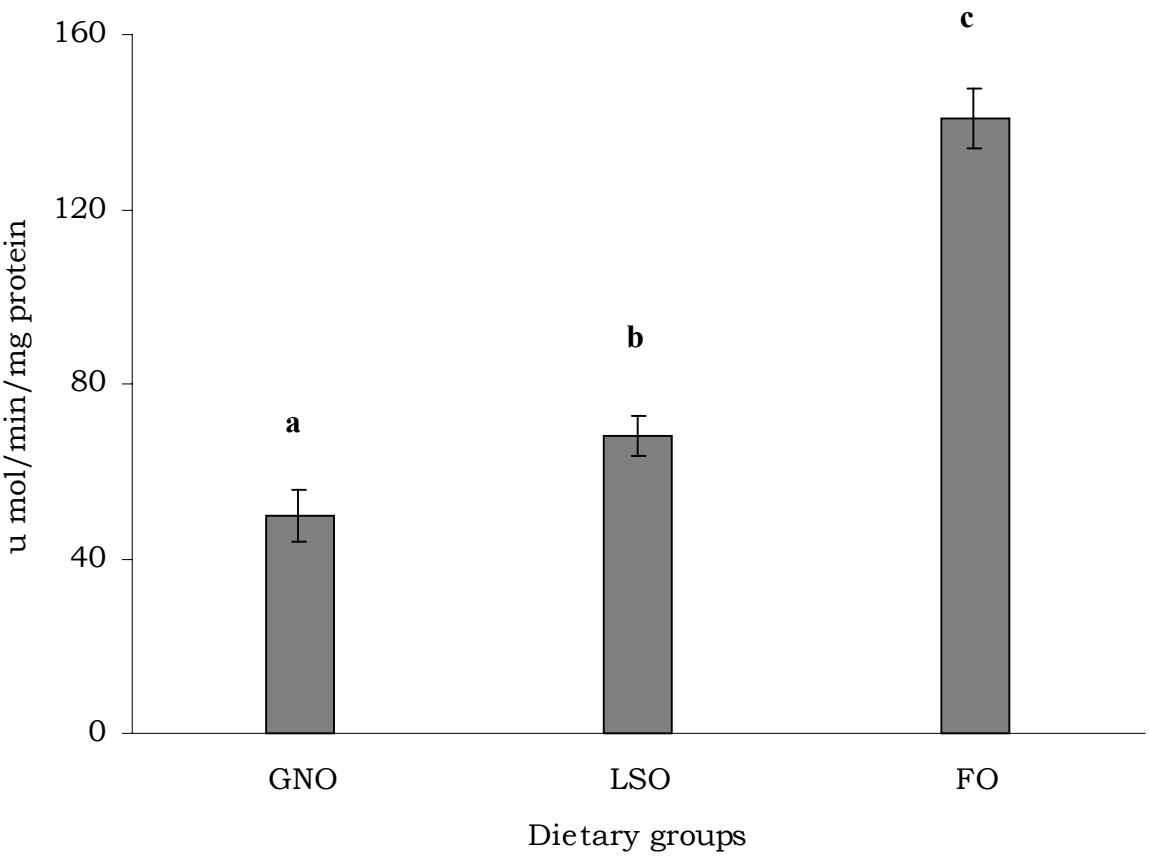
Feeding highly unsaturated fatty acids like omega-3 fatty acids increased the omega-3 fatty acids level in tissue resulting an increase in the susceptibility of tissue to lipid peroxidation. Increase in the lipid peroxidation affects the tissue antioxidant defense. The effect of omega-3 PUFA supplemented formulation on antioxidant enzyme activity in liver is given in Fig 6.2-6.5. Compared to control group of rats, SOD activity increased only marginally in omega-3 fatty acid formulation fed rats, which was not statistically significant. Whereas, the catalase activity increased by 37 and 183% respectively in LSO and FO enriched formulation fed rats. The glutathione peroxidase activity decreased to an extent of 25-36% in omega-3 fatty acid containing formulation fed rats. However, Glutathione transferase activity increased to an extent of 34-39% in omega-3 fatty acid formulation fed group compared to control.

Fig. 6.2 SOD activity in liver of rats fed spray-dried milk supplemented with omega-3 fatty acid



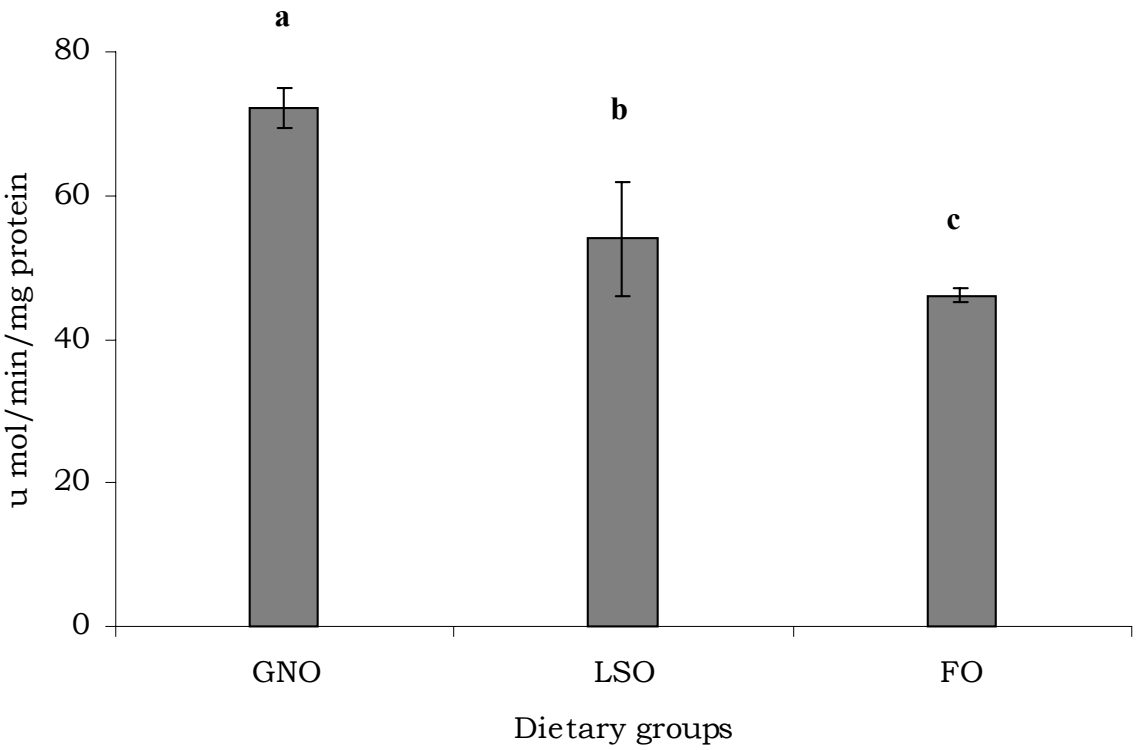
Values are Mean ± SD of 6 rats. Values not sharing the common superscripts are significantly different $p < 0.001$

Fig. 6.3 Catalase activity in liver of rats fed spray-dried milk supplemented with omega-3 fatty acid



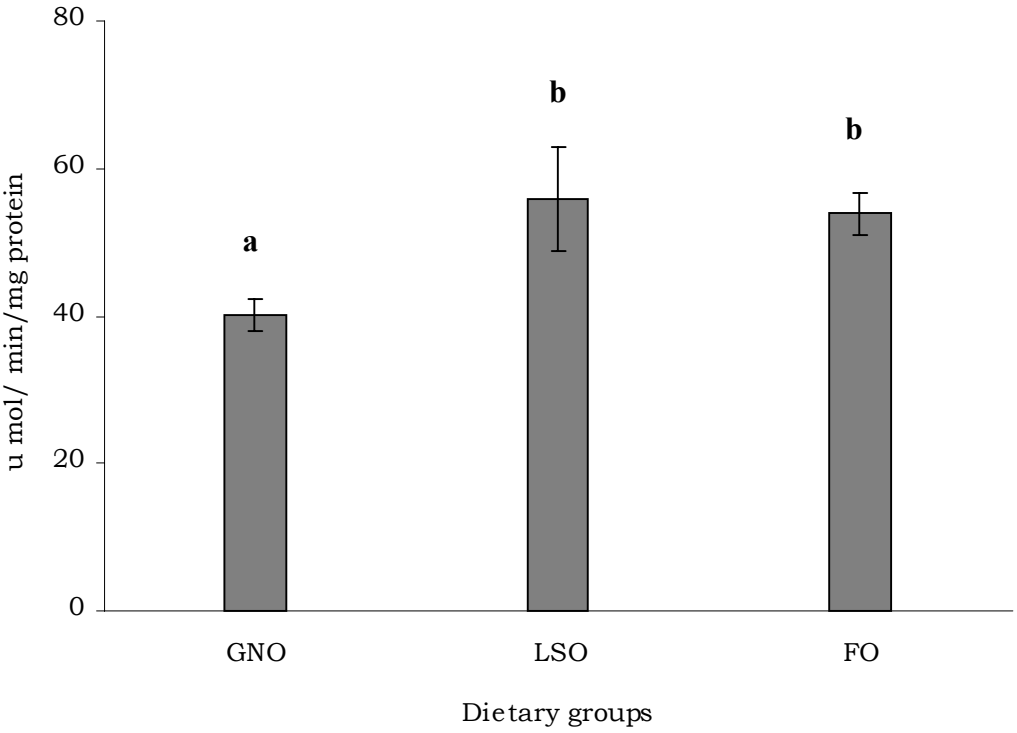
Values are Mean ± SD of 6 rats. Values not sharing the common superscripts are significantly different $p < 0.001$

Fig. 6.4 Glutathione peroxidase activity in liver of rats fed spray-dried milk supplemented with omega-3 fatty acid



Values are Mean ± SD of 6 rats. Values not sharing the common superscripts are significantly different $p < 0.001$

Fig. 6.5 Glutathione transferase activity in liver of rats fed spray-dried milk supplemented with omega-3 fatty acid



Values are Mean ± SD of 6 rats. Values not sharing the common superscripts are significantly different $p < 0.001$

Fatty acid composition of platelets of rats fed spray-dried milk supplemented with omega-3 fatty acid

In addition to liver, the platelet fatty acid composition was also altered in rats fed spray-dried milk formulation containing omega-3 fatty acids (Table 6.2). In rats fed LSO containing formulation, the LNA level in platelets was found to be 7.3% and EPA level was found to be 3% of total fatty acids. However, DHA was not detected in platelets of rats fed LSO supplemented formulation. The EPA and DHA level in platelets of rats fed FO supplemented formulation was

found to be 3.6 and 1.8% of total fatty acids respectively. The oleic acid decreased to an extent of 39-43% in both the experimental group compared to control. Whereas, the arachidonic acid level was decreased to an extent of 39-44% in experimental group compared to control.

Table 6.2 Fatty acid composition (%) of platelets of rats fed spray-dried milk supplemented with omega-3 fatty acid

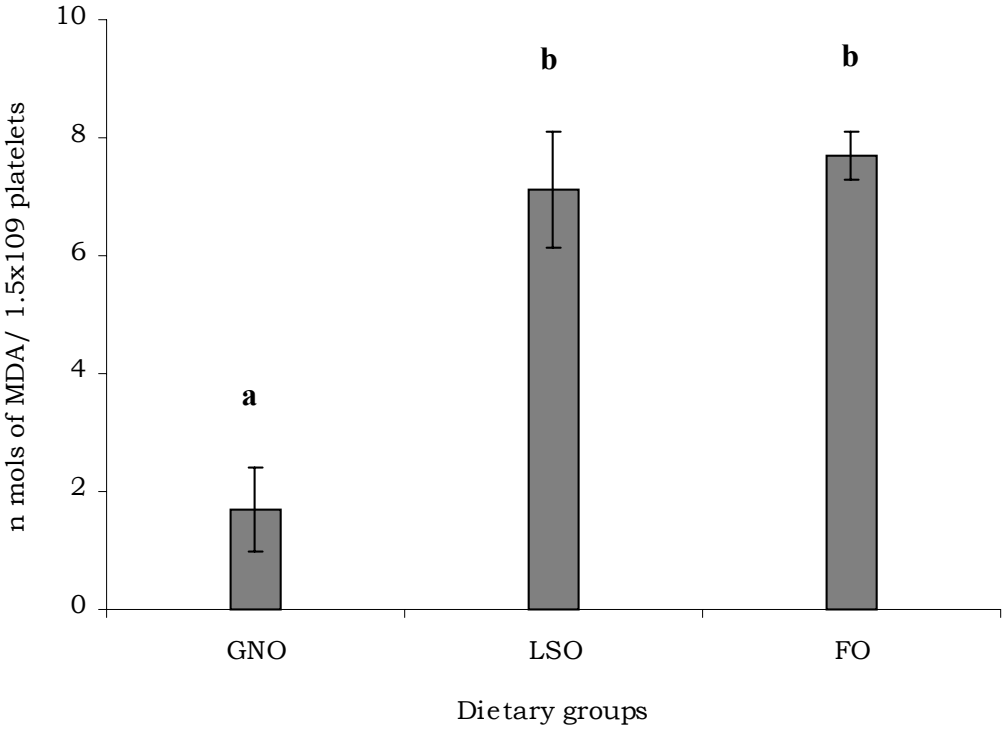
Fatty acids	GNO	LSO	FO
16:0	33.1 ± 3.8 [a]	31.1 ± 4.6 [a]	34.3 ± 3.8 [a]
16:1	3.2 ± 1.1 [a]	3.4 ± 1.0 [a]	3.9 ± 0.9 [a]
18:0	18.3 ± 2.6 [a]	25.0 ± 2.9 [b]	26.4 ± 2.7 [b]
18:1	35.3 ± 4.0 [a]	21.5 ± 3.4 [b]	20.0 ± 3.1 [b]
18:2	5.4 ± 0.2 [a]	5.3 ± 0.6 [a]	5.7 ± 1.3 [a]
18:3	ND	7.3 ± 1.7 [a]	ND
20:4	4.1 ± 0.6 [a]	2.3 ± 0.5 [b]	2.5 ± 0.8 [b]
20:5	ND	3.0 ± 0.9 [a]	3.6 ± 0.4 [a]
22:6	ND	ND	1.8 ± 0.7 [a]
PUFA/SFA	0.18 ± 0.05 [a]	0.31 ± 0.08 [b]	0.22 ± 0.05 [a]

Values are Mean ± SD of 6 rats. ND: Not detected. Values not sharing a common superscript within a row are statistically significant $p < 0.001$

Lipid peroxides level in platelets of rats fed spray-dried milk supplemented with omega-3 fatty acids

The change in the dietary lipids influenced the fatty acid composition and lipid peroxidation in platelets. The lipid peroxides level in platelets of rats fed LSO and FO supplemented formulation was found to be higher by 320 and 355% respectively compared to control.

Fig 6.6 Lipid peroxides level in platelets of rats fed spray-dried milk supplemented with omega-3 fatty acids



Values are Mean ± SD of 6 rats. Values not sharing the common superscripts are significantly different p< 0.001

Platelets aggregation in rats fed omega-3 fatty acid supplemented formulation

Platelet aggregation is an important physiological event, which has implication in thrombosis. The percent of ADP induced platelet aggregation was decreased in the rats fed LSO and FO containing formulation by 17 and 20% respectively, whereas, the collagen induced aggregation was decreased by 17 and 24% in LSO and FO containing formulation respectively compared to control rats (Fig 6.7 & Table 6.3). The rate of ADP induced platelet aggregation in rats fed omega-3 fatty acids containing formulation was lowered by 46-52%.

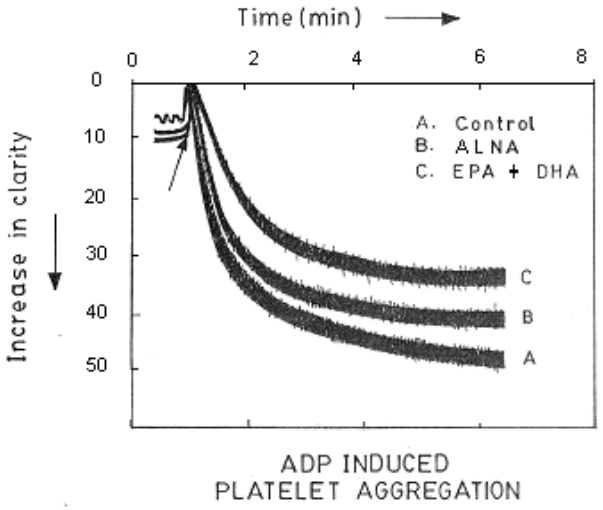
The rate of collagen induced platelet aggregation was found to be lower by 40% in rats fed omega-3 fatty acid supplemented formulation.

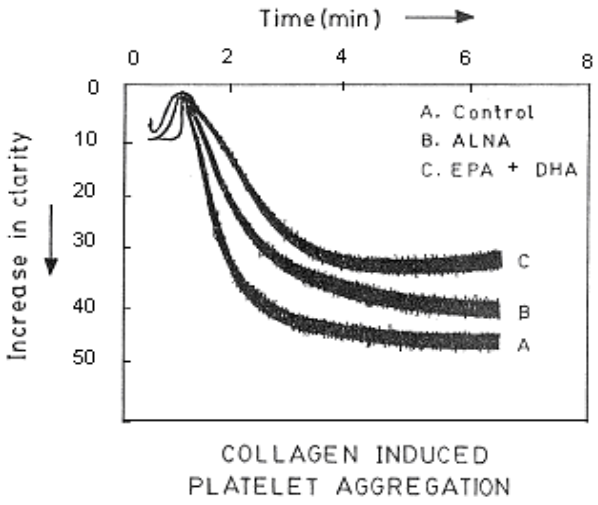
Table 6.3 Platelets aggregation in rats fed spray-dried milk supplemented with omega-3 fatty acid

	GNO	LSO	FO
	ADP induced		
Percent aggregation	50.6 ± 6.1 [a]	42.2 ± 2.0 [b]	40.5 ± 2.5 [b]
Rate of aggregation	5.48 ± 0.74 [b]	2.60 ± 1.3 [b]	2.96 ± 1.2 [b]
	Collagen induced		
Percent aggregation	41.5 ± 0.5 [a]	34.5 ± 3.5 [b]	31.5 ± 4.9 [b]
Rate of aggregation	4.11 ± 0.8 [a]	2.46 ± 0.6 [b]	2.46 ± 0.7 [b]

Values are Mean ± SD of 6 rats. Values not sharing a common superscript within a row are statistically significant $p < 0.001$

Fig 6.7 ADP and collagen induced platelet aggregation in rats fed spray-dried milk supplemented with omega-3 fatty acid

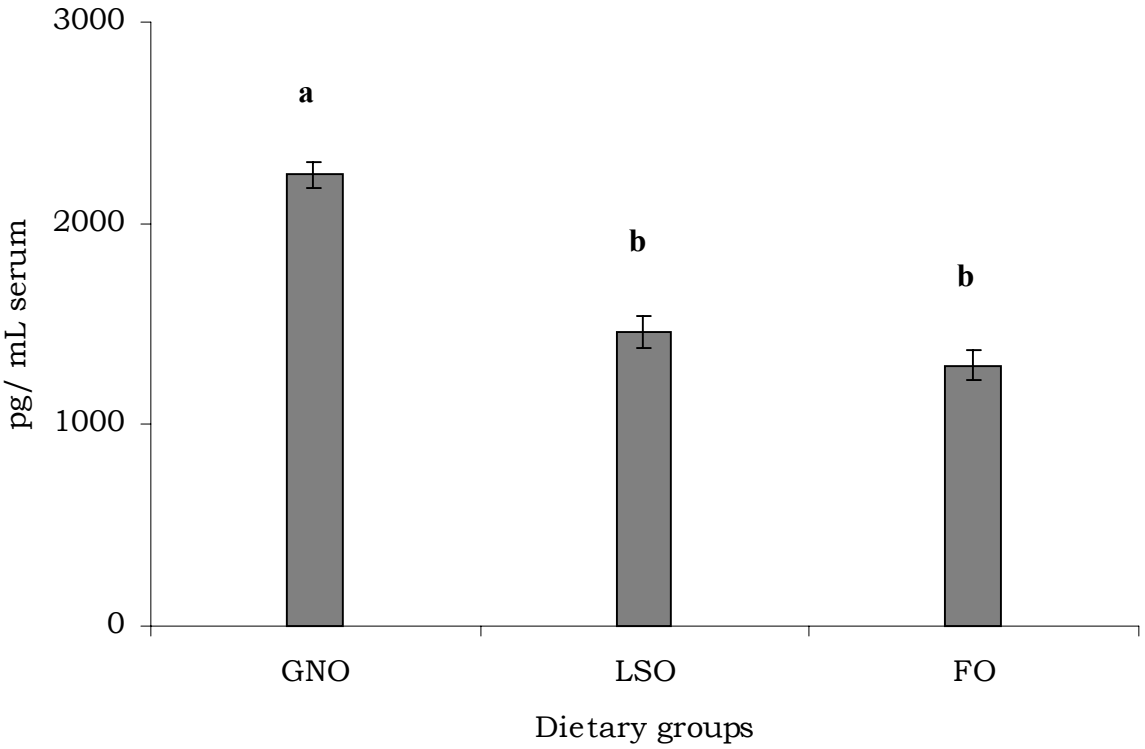




Serum prostaglandin level in rats fed spray-dried milk supplemented with omega-3 fatty acid

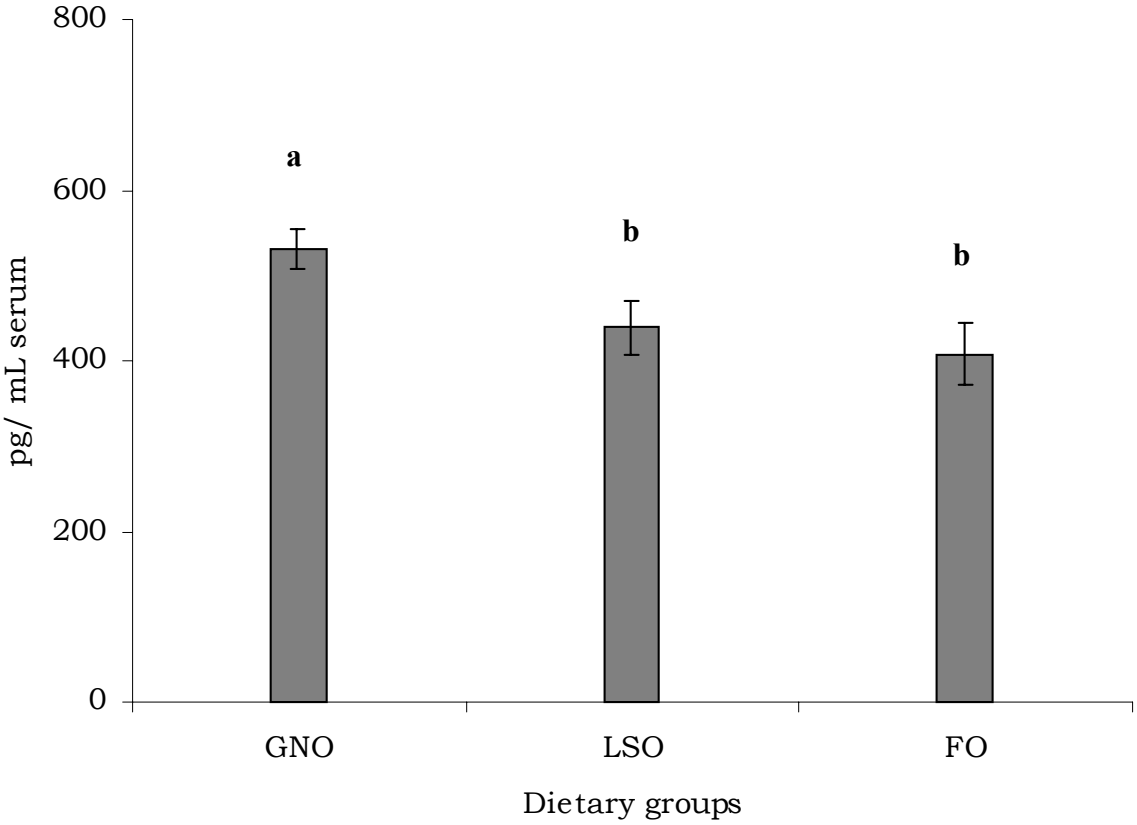
Platelet aggregation is significantly influenced by prostaglandins. Serum prostaglandin levels were significantly decreased in rats fed omega-3 fatty acid enriched formulation (Fig 6.8 & 6.9). Compared to control group of rats, the pro-aggregatory TXB_2 level was decreased by 35 and 42% respectively in LSO and FO enriched formulation fed rats. The anti-aggregatory 6-keto-PGF1α was decreased only by 17 and 23% in LSO and FO formulation fed rats.

Fig. 6.8 Serum TXB_2 level in rats fed spray-dried milk supplemented with omega-3 fatty acid



Values are Mean ± SD of 6 rats. Values not sharing the common superscripts are significantly different $p < 0.001$

Fig. 6.8 Serum 6-keto-PGF1α level in rats fed spray-dried milk supplemented with omega-3 fatty acid



Values are Mean ± SD of 6 rats. Values not sharing the common superscripts are significantly different $p < 0.001$

Discussion

It is known that the susceptibility of fatty acids to lipid peroxidation increase in proportion to degree of unsaturation and hence when incorporated into the cells may likely affect its function. Inherent antioxidant defense system protects cells against oxidative stress. A decrease in the activity of these enzymes may predispose cells to free radical damage (Huang and Fwu 1993; L' Abbe et al 1991). Hence lipid peroxidation and antioxidant enzyme activities were monitored after feeding omega-3 PUFA enriched formulations. We observed a significant increase in the level of omega-3 PUFA and lipid peroxides in the liver homogenate of rats fed spray-dried milk supplemented with omega-3 PUFA.

Lipid peroxidation is initiated by an attack on unsaturated fatty acids by reactive oxygen species. The radical scavenging enzyme, superoxide dismutase is known to generate hydrogen peroxide from superoxide anions. The hydrogen peroxide in turn leads to highly reactive hydroxyl radicals by Fenton or Haberwise reactions. Hence SOD plays a vital role in the generation of reactive oxygen species. In the present study, the activity of SOD was comparable in control and experimental animals and hence may not be enhancing the oxygen radical cascade that feeds in to lipid peroxidation in omega-3 fatty acid fed animals.

However, dietary PUFA, particularly the long chain omega-3 PUFA is known to elevate the peroxisomal β-oxidation (Chen et al 1993). They are also shown to act as proliferators for peroxisomes and mitochondria (Reddy and Lalwani 1983). The key enzyme of β-oxidation, fatty acyl CoA oxidase produces hydrogen peroxide increasing oxidative stress (Froyland et al 1998). This needs to be counter acted. In this context catalase plays a crucial role. In the

present study, we observed an increased activity of catalase following feeding of omega-3 fatty acid enriched formulation. The increased activity of catalase may indicate an effective means of removing H_2O_2 that may be generated in the cells. Though H_2O_2 is a weak oxidant and not very effective in the aqueous environment of cells, it may cross the biological membrane and participate in the iron catalyzed Fenton reaction generating reactive hydroxyl radicals under physiological conditions (Chen et al 1993). 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 1986). We found a decrease in the activity of glutathione peroxidase in rats fed with omega-3 PUFA containing formulation. The decrease in the activity of glutathione peroxidase may lower the cellular capacity to deal with H_2O_2 and possibly lead to an increase in catalase activity as an adaptation process (Schull 1991). Earlier reports have also shown that omega-3 fatty acids can increase the mRNA expression of catalase and strengthens the antioxidant status (Venkatraman et al 1994; Aguilera et al 2003). However, this may not be sufficient to fully protect highly unsaturated EPA+DHA, which underscore the need for external antioxidants when highly unsaturated lipids are fed (Pullareddy and Lokesh 1994).

Although glutathione peroxidase activity was decreased in the rats fed experimental diets, a significant increase in glutathione transferase activity was observed. Glutathione transferase can act as selenium independent glutathione peroxidase that can reduce a variety of organic hydro peroxides and may partly compensate for the lower glutathione peroxidase activity.

Hydroperoxides influence cyclooxygenase activity, which produces thromboxane, a potent platelet-aggregating factor (Piche and Mahadevappa 1990; Land and Byrnes 1982). Hydroperoxides formation depends on the antioxidant status of the tissues. The present study indicated that antioxidant status was balanced by an increase in catalase and glutathione transferase activity following the feeding of omega-3 PUFA supplemented formulations. Omega-3 fatty acids containing spray-dried milk formulation also lowered the arachidonic acid levels in platelets, which is a substrate for thromboxane production. This reflected in 35-42% lowering of serum thromboxane (measured as TXB_2) levels in omega-3 PUFA fed animals. The anti-aggregatory prostacyclin (measured as 6-keto-PGF1α) was lowered only by 17-23%. The PGI_3, produced by omega-3 fatty acids, is a potent anti-aggregator of platelets similar to PGI_2, and hence tilting the balance towards lower platelet aggregation state. Both the precursor LNA and preformed EPA+DHA exhibited similar effect on platelet aggregation.

The results of our study indicated that spray-dried milk containing omega-3 PUFA significantly increased the omega-3 PUFA stores in the tissue as well as platelets. Though, lipid peroxidation was increased to certain extent following omega-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. Omega-3 PUFA enriched formulation also beneficially modulated serum prostaglandins and lowered platelet aggregation in beneficial manner and there by reducing the risk factors for thrombosis.

CHAPTER VII

Storage related studies of spray-dried milk supplemented with omega-3 fatty acids

Introduction

Quality and storage stability of omega-3 PUFA supplemented spray-dried formulation is vital for its acceptance by consumers as the added fat may contribute to product appearance, mouth feel, flavor and palatability. It also carries, enhances and releases the flavors of other ingredients in foods (Haumann 1998). Milk contains macro- and micronutrients that undergo various interactions during processing and storage leading to chemical changes (Baskaran *et al.* 1994; Mc Cluskey *et al.* 1997). The PUFA level in milk is low, but the addition of PUFA rich oils can increase the PUFA content and its vulnerability to oxidation (Christie 1995; Mc Cluskey *et al.* 1997).

The oxidation of fat is a natural process that takes place by the interaction between molecular oxygen and unsaturated fatty acids. The high reactivity of the carbon double bonds in unsaturated fatty acids makes these substances primary targets for free radical attack (Halliwell and Chirico 1993). The oxidation of unsaturated fatty acids is major concern in food industry (Angelo 1996; Vercelotti et al 1992).

Processing and storage treatments can lead to oxidation of lipids and yield lipid oxidation products (Mc Cluskey *et al.* 1997). The level of lipid peroxidation products formed varies with storage conditions (Baldwin and Ackland 1991). Further, the raw materials and their various constituents also play a role in storage quality of foods. Initial quality of milk with respect to microbial load and the extent of deterioration prior to processing can have adverse effect on the stored products (Ispen and Hansen 1988). Milk foods containing high levels of protein, unsaturated fats and reduced sugars tend to undergo browning reactions resulting in rancidity of products (Rolls and Porter 1973). Sensory quality of the products decreases mainly due to off-flavor caused by oxidation of milk fat (Badings and Neeter,

1980). Storage conditions may also alter the quality of the product due to the formation of insoluble Maillard reaction products (Van Mil and Jans 1991).

Since, spray-dried formulations are stored for considerable periods, it is essential to evaluate the quality changes that may occur during storage period. The main objective of the present investigation is to study storage related modification and its influence on sensory qualities that inturn affect the acceptability of milk based omega-3 PUFA formulation.

Results

Freshly prepared spray-dried milk samples enriched in omega-3 PUFA was stored in tin cans as described in methods. These samples were evaluated for chemical properties, bacteriological quality, sensory and physical properties.

Chemical Properties

The initial moisture content of LSO and FO formulations were 3.0 and 2.6%, respectively, and remained practically unaltered throughout the storage period of 6 months under all the temperature conditions (Table 7.1). The crude protein content of control (17.2%), LSO (18.2%) and FO (17.5%) milk formulations remained more or less constant throughout storage period. Similarly, the total fat level in fresh samples were found to be 16.5% in control, 16.2% in LSO and 16.4% in FO formulations and storage conditions did not significantly alter the fat level of the formulations. No significant difference was observed in total carbohydrates and energy value of the milk formulations supplemented with LSO or FO compared with control throughout the study period at all the temperatures.

The initial peroxide value of GNO, LSO and FO milk formulations were 7.88, 9.62 and 11.98 meq of O_2 / kg fat (Table 7.2). The increase in the peroxide value compared to fresh sample was found to be minimal at 4^0 C in all the formulations. At 27^0 C of storage the peroxide value increased by 86% (GNO), 102% (LSO) and 73% (FO) after 6 months compared to fresh sample. At 37^0 C, the increase in peroxide value was found to be 122% (GNO), 174% (LSO) and 141% (FO) compared to fresh sample at the end of 6 months period.

Table 7.1 Proximate composition of spray-dried milk supplemented with omega-3 fatty acids

Temp °C	Time (Months)	Moisture (%)			Total ash (%)			Crude protein (%)			Crude Fat (%)			Total CHO (%) by difference			Energy (K Cal)/100g		
		GNO	LSO	FO	GNO	LSO	FO	GNO	LSO	FO	GNO	LSO	FO	GNO	LSO	FO	GNO	LSO	FO
4	**0**	3.3	3.0	2.6	4.7	4.9	4.9	17.2	18.2	17.5	16.5	16.4	16.9	58.3	56.3	58.1	450	449	454
	1	3.2	3.2	2.8	4.5	4.8	4.5	17.5	18.0	17.0	16.5	16.2	16.5	58.3	57.4	59.2	452	450	453
	2	3.3	3.0	2.6	4.5	4.6	4.5	17.2	18.0	17.0	16.2	16.3	16.5	59.2	57.5	59.4	451	450	454
	3	3.2	3.0	2.5	4.2	4.6	4.4	16.8	17.5	16.5	16.2	16.2	16.2	59.6	58.4	60.4	448	449	453
	4	3.4	3.0	2.8	4.2	4.6	4.4	16.8	17.8	16.5	16.4	16.0	16.0	58.5	58.7	59.9	449	452	450
	5	3.4	3.4	2.5	4.0	3.3	3.8	16.2	16.4	15.8	16.5	16.1	16.2	64.9	58.3	59.4	464	438	438
	6	3.6	3.4	2.5	4.0	3.5	3.5	16.5	16.0	15.2	16.3	16.2	16.2	64.9	58.9	61.2	461	435	437
27	**1**	3.3	3.2	3.4	4.2	4.8	4.5	16.8	18.0	17.5	16.2	16.2	16.4	59.5	58.0	58.2	451	448	450
	2	3.3	3.2	3.4	4.0	4.8	4.5	17.0	17.5	17.5	16.4	16.0	16.4	59.3	57.7	58.0	453	445	450
	3	3.2	3.4	3.6	4.0	5.0	4.5	17.0	17.8	17.5	16.4	16.3	16.2	59.4	58.0	57.8	453	449	447
	4	3.2	3.4	3.4	4.2	4.4	4.4	16.8	17.8	17.0	16.2	16.0	16.2	59.6	57.9	57.8	451	447	445
	5	3.0	3.2	3.2	4.5	4.5	4.0	16.2	16.2	16.4	16.1	16.2	16.0	61.6	60.6	60.4	450	447	451
	6	3.0	3.0	3.0	4.5	4.0	4.0	16.5	16.0	16.0	16.0	16.0	16.0	62.5	62.7	60.5	451	450	450
37	**1**	3.0	3.0	3.2	4.2	4.0	4.2	16.5	17.0	16.5	16.2	16.5	16.2	60.7	60.2	60.9	450	446	446
	2	3.2	3.2	3.2	4.0	4.2	4.2	16.0	17.2	16.5	16.1	16.3	16.0	60.0	60.0	60.9	444	446	445
	3	3.0	3.0	3.1	4.0	4.2	4.0	16.0	17.0	16.0	16.0	16.1	16.0	60.0	59.3	60.3	446	440	431
	4	3.0	3.0	3.0	4.2	4.0	4.0	16.0	16.6	16.0	16.1	16.0	16.2	59.8	64.2	60.4	442	418	431
	5	2.8	3.0	3.0	4.0	3.8	3.8	15.0	15.0	14.8	15.9	16.2	16.1	63.8	64.2	94.6	442	443	440
	6	2.6	2.8	3.0	3.8	3.8	3.6	14.4	14.2	14.2	15.9	16.0	16.0	64.9	65.2	66.2	443	444	439

Values are mean of triplicate samples.

Table 7.2 Effect of storage temperature and period on peroxide value of spray-dried milk supplemented with omega-3 fatty acids

	Temp. °C	Storage period (months)						
		0	1	2	3	4	5	6
GNO	4	7.88 ± 0.67 a	9.56 ± 0.25 b	9.99 ± 0.06 b	10.87 ± 0.28 bc	11.54 ± 0.21 cd	11.65 ± 0.28 cd	12.34 ± 0.25 d
	27	7.88 ± 0.67 a	10.74 ± 0.33 b	11.44 ± 0.43 b	12.37 ± 0.17 bc	13.03 ± 0.11 c	13.94 ± 0.05 cd	14.68 ± 0.40 d
	37	7.88 ± 0.67 a	11.60 ± 0.10 b	12.80 ± 0.46 c	13.94 ± 0.49 d	15.80 ± 0.61 e	17.04 ± 0.70 f	17.54 ± 0.11 f
LSO	4	9.62 ± 0.37 a	11.93 ± 0.20 b	12.64 ± 0.31 b	13.53 ± 0.11 c	14.86 ± 0.26 d	16.79 ± 0.33 e	17.07 ± 0.16 e
	27	9.62 ± 0.37 a	13.71 ± 0.26 b	14.71 ± 0.46 b	15.67 ± 0.20 c	17.04 ± 0.32 d	17.98 ± 1.01 d	19.47 ± 0.21 e
	37	9.62 ± 0.37 a	16.57 ± 0.04 b	17.76 ± 0.59 c	19.76 ± 0.48 d	23.66 ± 0.64 e	25.15 ± 0.57 f	26.44 ± 0.13 g
FO	4	11.98 ± 0.15 a	13.34 ± 0.36 b	14.02 ± 0.20 b	15.60 ± 0.14 c	16.26 ± 0.29 c	16.82 ± 0.19 cd	17.36 ± 0.25 d
	27	11.98 ± 0.15 a	14.57± 0.41 b	15.52 ± 0.29 b	16.35 ± 0.16 c	17.24 ± 0.34 d	19.21 ± 0.42 e	20.73 ± 0.23 f
	37	11.98 ± 0.15 a	18.53± 0.20 b	20.44 ± 0.46 c	21.95 ± 0.55 d	24.56 ± 0.21 e	28.03 ± 0.23 f	28.92 ± 0.40 f

Values are Mean ± SD of 4 samples. Values not sharing a common superscript with in a row are statistically significant $p < 0.001$

Effect of storage temperature and period on fatty acid composition of spray-dried milk supplemented with omega-3 fatty acids

The effect of storage on fatty acid composition of milk formulations supplemented with GNO, LSO and FO is given in table 7.3, 7.4 and 7.5 respectively. Omega-3 PUFA was absent in GNO formulation. The level of LNA in fresh LSO formulation was 20.4% and EPA & DHA in fresh FO formulation was 4.5%. The LNA levels in LSO formulations after 6 months of storage was 18.4, 18.0, and 16.2% respectively, at 4, 27 and 37^0C. In the case of FO formulation, the EPA+DHA levels after 6 months of storage was 3.9, 3.6 and 2.9% respectively at 4, 27 and 37^0 C. These studies indicated that a minimum loss of omega-3 PUFA after 6 months of storage even when stored at higher temperatures.

Bacteriological Quality

The formulations prepared were devoid of coliforms. The counts of mesophilic aerobes were in the range of 6 - 16 X 10^3 CFU/g in products stored at 4^0 C. Those stored at 27^0 C had counts of mesophilic aerobes in the range 5 – 34 X 10^3 CFU/g. The mesophilic aerobes in products stored at 37^0 C were in the range of 11 – 38 X10^3 CFU/g.

Table 7.3 Effect of storage temperature and period on fatty acid composition (%) of spray-dried milk supplemented with GNO

Temp. (°C)	Period (Months)	Fatty acids								
		12:0	14:0	16:0	16:1	18:0	18:1	18:2	20:0	22:0
4	0	1.1	6.6	27.4	1.0	8.9	40.4	9.3	1.8	0.8
	1	1.2	6.4	26.1	1.3	9.2	40.0	9.4	1.5	0.9
	2	1.4	7.6	27.1	1.1	10.1	39.3	9.1	1.5	0.8
	3	1.0	7.8	27.4	1.4	10.9	40.0	9.2	1.4	0.9
	4	1.1	7.3	28.0	1.0	11.0	41.8	8.9	1.4	0.6
	5	0.9	8.0	27.9	1.2	11.1	41.8	8.4	1.3	0.7
	6	0.8	8.1	28.1	1.0	11.3	42.0	7.9	0.9	ND
27	1	1.1	6.3	28.1	1.1	9.1	41.0	9.2	1.4	0.8
	2	0.9	6.2	27.4	1.0	9.8	42.4	9.0	1.4	0.8
	3	1.0	7.0	28.4	0.9	10.0	43.4	8.6	0.9	0.6
	4	0.9	6.9	28.8	0.8	10.4	43.6	8.1	1.0	0.6
	5	0.8	7.2	29.0	0.9	10.8	44.1	7.9	1.0	0.6
	6	0.6	7.6	30.0	0.7	10.0	45.1	7.1	0.7	ND
37	1	1.0	6.8	27.4	0.8	9.4	40.0	8.8	1.3	0.3
	2	1.3	7.3	29.1	1.0	9.1	41.1	9.1	0.9	0.4
	3	1.0	7.2	30.1	0.6	8.9	42.1	8.8	1.0	0.9
	4	0.7	7.9	29.4	0.3	9.4	41.4	8.0	0.4	0.4
	5	0.4	8.3	28.2	0.3	10.0	43.0	7.9	0.6	ND
	6	0.4	9.0	29.4	0.3	10.4	44.7	6.9	0.8	ND

Values are mean of duplicate samples. ND: Not detected

Table 7.4 Effect of storage temperature and period on fatty acid composition (%) of spray-dried milk supplemented with LSO

Temp ^{0}C	Period (Months)	Fatty acids							
		12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
	0	1.9	7.3	25.2	1.0	10.7	24.2	6.5	20.4
	1	1.8	7.8	24.9	0.9	10.8	24.8	6.4	20.0
	2	1.9	8.0	25.0	0.9	11.1	25.1	6.2	19.8
4	3	1.4	8.0	24.8	0.9	11.1	25.2	6.0	19.7
	4	1.5	8.1	25.0	1.0	11.7	26.0	5.9	19.0
	5	1.4	7.8	25.1	1.2	11.5	26.3	5.8	18.8
	6	1.4	7.7	26.0	1.0	11.8	27.0	5.4	18.4
	1	1.4	7.3	25.9	0.9	10.0	24.4	6.4	19.4
	2	1.3	7.6	25.3	0.8	10.1	24.8	6.0	19.3
	3	1.2	7.8	25.8	0.9	10.2	24.4	5.9	19.0
27	4	1.1	8.0	26.0	0.9	10.3	25.0	5.8	18.8
	5	1.3	7.9	26.1	0.9	10.9	25.3	5.7	18.4
	6	1.1	8.0	26.2	0.8	10.8	25.8	5.4	18.0
	1	1.4	7.3	25.9	0.8	10.3	24.8	6.2	19.0
	2	1.3	7.4	26.0	0.9	10.5	25.0	6.0	18.8
37	3	1.7	7.3	26.3	0.8	10.8	25.7	5.9	18.1
	4	1.6	7.6	26.0	0.8	11.2	26.1	5.6	17.4
	5	1.4	8.0	26.8	0.8	11.8	26.8	5.3	17.1
	6	1.5	8.0	27.0	0.9	12.1	27.9	5.0	16.2

Values are mean of duplicate samples.

Table 7.5 Effect of storage temperature and period on fatty acid composition (%) of spray dried milk supplemented with FO

Temp (°C)	Periods (Months)	Fatty acids										
		12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	22:6
4	0	1.6	10.8	35.4	4.6	9.9	24.2	2.6	1.1	2.9	2.4	2.1
	1	1.5	11.0	34.7	4.4	9.8	25.1	2.6	1.0	2.9	2.4	2.0
	2	1.7	10.7	35.1	4.4	10.1	25.8	2.5	1.0	2.8	2.3	1.9
	3	1.4	11.0	35.7	4.5	10.4	26.2	2.6	0.9	2.7	2.4	2.0
	4	1.6	10.9	35.9	4.3	10.6	27.0	2.6	0.9	2.6	2.3	1.8
	5	1.6	11.2	36.0	4.2	10.8	26.9	2.5	0.8	2.4	2.2	1.8
	6	1.4	11.3	36.2	4.3	11.0	27.4	2.5	0.7	2.4	2.2	1.7
27	1	1.6	10.9	35.1	4.3	9.6	26.1	2.5	0.9	2.9	2.4	2.0
	2	1.6	11.2	36.1	4.1	10.0	27.0	2.5	0.9	2.8	2.3	1.9
	3	1.7	11.4	36.9	4.2	10.1	27.7	2.4	0.8	2.7	2.3	1.9
	4	1.4	11.7	37.1	4.1	10.7	27.4	2.3	0.8	2.6	2.3	1.7
	5	1.4	11.7	38.0	4.0	10.8	27.1	2.4	0.8	2.6	2.2	1.7
	6	1.4	11.8	36.4	3.9	10.8	26.8	2.3	0.7	2.5	2.0	1.6
37	1	1.6	10.9	32.8	4.4	10.0	24.8	2.4	1.1	2.8	2.4	1.9
	2	1.4	11.1	33.4	4.2	10.2	24.9	2.3	1.0	2.7	2.3	1.7
	3	1.5	11.2	34.1	4.0	10.8	25.1	2.4	0.9	2.6	2.2	1.7
	4	1.7	11.3	34.8	3.8	11.3	25.5	2.2	0.9	2.5	2.1	1.5
	5	1.8	11.8	35.0	3.8	11.4	26.1	2.0	0.7	2.3	1.9	1.4
	6	1.7	12.0	35.0	3.8	11.6	26.3	2.1	0.7	2.0	1.6	1.3

Values are mean of duplicate samples.

Sensory Properties

Sensory properties play an important role in the commercial viability of a product. Sensory properties of spray-dried milk supplemented with omega-3 fatty acids from LSO or FO were given in table 7.6. No differences were observed in the flavor, mouth feel and overall acceptability of control and formulations with LSO and FO at 4 and 27^0 C of storage. There was, however, a decrease in the sensory qualities of the FO formulations after 5 months when stored at 37^0 C. Both LSO and FO formulations were acceptable, with scores >5, up to 5 months of storage at 4, 27 and 37^0 C.

Physical properties

Similar to sensory qualities, physical properties also affects the commercial value of the product. The mean values for solubility index of LSO, FO and GNO milk formulations ranged between 0.1-0.2 and remained unaltered during the entire storage period (Table 7.7). The percent whiteness also did not alter significantly during the storage period at different temperatures in the control samples, but a significant decrease in the whiteness of the product was observed in LSO and FO containing formulations after 5 months of storage. A maximum decrease of whiteness to an extent of 21.4 and 26.5% were observed in LSO and FO formulations respectively, stored at 37^0 C beyond 5 months, as compared with fresh samples.

Table 7.6 Sensory properties of spray-dried milk supplemented with omega-3 fatty acids

Temp (^{0}C)	Duration (Months)	Flavor			Mouth feel			Overall acceptability		
		GNO	LSO	FO	GNO	LSO	FO	GNO	LSO	FO
4	0	8.8	8.0	7.4	9.0	7.3	6.8	8.0	8.0	7.5
	1	8.8	8.0	7.2	9.0	7.3	6.8	8.0	7.5	7.0
	2	7.8	7.8	7.0	8.5	7.0	6.5	8.0	7.5	7.0
	3	7.6	7.8	6.5	8.0	7.0	6.0	7.8	7.5	6.2
	4	7.3	7.7	6.3	8.0	6.5	6.0	7.5	6.8	6.0
	5	7.0	6.8	6.0	7.0	6.5	5.8	7.0	6.4	6.5
	6	7.0	6.5	6.0	7.0	6.3	5.8	7.0	6.4	5.5
27	1	8.8	8.0	7.0	8.4	7.0	6.8	8.0	8.0	6.3
	2	8.8	8.0	7.0	8.0	7.0	6.8	8.0	7.4	6.0
	3	7.8	7.5	6.5	7.8	6.5	6.5	7.8	7.4	6.0
	4	7.8	7.5	6.5	7.5	6.3	6.5	7.2	6.8	5.8
	5	7.5	6.8	6.0	7.5	5.7	6.0	6.7	6.5	5.3
	6	7.0	6.8	5.8	6.6	5.4	5.4	6.7	6.0	5.0
37	1	8.0	8.0	7.0	8.0	7.5	6.3	8.0	7.2	6.8
	2	8.0	7.8	6.5	7.8	7.4	6.0	7.0	7.0	6.5
	3	7.0	7.5	6.0	7.5	6.8	6.0	7.0	6.6	6.2
	4	7.0	7.5	6.0	7.0	6.8	5.4	6.3	6.2	5.2
	5	6.0	6.5	5.0	6.4	6.0	4.9	5.4	6.0	4.0
	6	6.0	5.0	5.0	6.0	5.5	4.5	5.2	5.0	4.0

Values are mean of triplicate samples

Table 7.7 Physical properties of spray-dried milk supplemented with omega-3 fatty acids

Temp (^{0}C)	Duration (Months)	Solubility Index (%)			Whiteness (%)		
		GNO	LSO	FO	GNO	LSO	FO
4	0	0.2	0.1	0.2	84.9	84.1	83.2
	1	0.2	0.1	0.2	80.3	82.2	82.0
	2	0.2	0.1	0.2	80.3	80.0	80.0
	3	0.2	0.2	0.2	80.0	80.0	78.0
	4	0.2	0.2	0.2	80.0	78.0	78.0
	5	0.1	0.2	0.2	78.0	75.0	76.8
	6	0.2	0.2	0.2	78.0	75.0	76.8
27	1	0.1	0.1	0.2	80.2	80.0	80.0
	2	0.1	0.1	0.2	80.0	80.2	78.2
	3	0.2	0.2	0.2	78.0	78.0	74.8
	4	0.2	0.2	0.2	78.0	78.0	72.4
	5	0.2	0.2	0.2	76.5	72.0	70.0
	6	0.2	0.2	0.2	76.0	70.0	64.5
37	1	0.1	0.2	0.2	80.0	80.0	78.0
	2	0.1	0.2	0.2	78.2	78.0	76.0
	3	0.2	0.2	0.2	76.0	75.0	74.0
	4	0.2	0.2	0.2	75.0	72.0	73.4
	5	0.2	0.2	0.2	72.0	70.0	67.5
	6	0.2	0.2	0.2	72.0	66.0	60.8

Values are mean of triplicate samples

Discussion

The primary objective of the investigation was to provide a shelf stable formulation containing omega-3 fatty acids in a form, which is widely consumed in India. Milk is a wholesome beverage consumed by every age group in the country. However, bovine milk lacks omega-3 fatty acids. Hence an attempt was made to enrich the milk with an omega-3 fatty acid source.

The acceptance of food product depends to a major extent on their quality. Milk powder, widely used as a raw material for various food products is manufactured through many processes such as separation of milk fat, heat treatment, concentration and spray drying. Hence the quality of milk-based product is greatly affected not only by the quality of raw milk but also the changes that occur during processing (Shiratsuchi et al 1994). The milk enriched in omega-3 fatty acids was spray-dried under controlled conditions. Spray drying is a well-known technology in the food industry and is the most commonly used micro encapsulation technique (Rosenberg et al 1990). The spray-dried milk supplemented with omega-3 fatty acids from LSO or FO were stored in tin cans and flushed with nitrogen.

The shelf life of spray dried omega-3 fatty acid enriched formulation were monitored for a period of 6 months by following the sensory quality, physical properties, chemical properties and microbial counts. Sensory evaluation of these products indicated that omega-3 fatty acids enriched formulation was well accepted by panelists. Studies have shown that shelf life of FO containing foods can be extended up to 12-24 months under controlled conditions (Young et al 1993; Rabiskowa et al 1994; Heinzelmann et al 2000).

Spray-dried formulations supplemented with either LSO or FO stored at 27 and 37^0 C for five months had similar solubility index. The proximate composition in terms of moisture, total ash, total fat,

total carbohydrates and energy content were similar to fresh samples when stored at different temperatures over a period of 5 months. However, formulations stored at 37^0 C beyond 5 months showed 16 - 21% reduction in the crude protein. The reduction in the crude protein may be due to Maillard reaction (Mauron 1983; Gothwal and Bhavadasan 1992). Maillard browning is a common phenomenon in milk formulations stored at higher temperatures. Which may account for a moderate reduction in the whiteness of milk formulations observed when stored for a period of more than 5 months. The formulations prepared were enriched in PUFA's. Polyunsaturated fatty acids are susceptible for lipid peroxidation and may generate off flavors (Kolanowsky et al 1999; Keogh, et al 2001). There was no significant change in the PUFA content of samples for 5 months storage periods. This may indicate that milk can be an ideal vehicle to deliver omega-3 fatty acids as milk prevents generation of off flavors. There are studies reporting the inhibitory effect of milk on lipid oxidation (Taylor and Richardson 1980). Milk protein has been reported to inhibit oxidation of lipids promoted by iron and lipoxidase (Colbert and Decker 1991). Several hydrophilic components of milk protein such as casein, α lactalbumin and milk enzymes were suggested to be potent inhibitors of lipid oxidation (Chen and Nawar 1991; Toyosaki et al 1987). Some studies have suggested that processing temperature may lead to denaturation of milk proteins, especially β-lactoglobulin and thus to exposure of sulphydril groups which can act as reducing agents resulting in a decrease of oxidation rate (Walstra and Jenness 1984). The overall acceptability, whiteness of the sample and proximate composition remained similar up to 5 months of storage as compared to fresh samples. The microbial evaluation indicated that product is free from coliforms and mesophilic organisms are within the safe limits (APHA 2001).

The present study indicated that spray-dried milk containing omega-3 fatty acids from LSO or FO has good stability and storing under controlled conditions could extend the shelf life of the formulations for a considerable period of time, which will help in its commercialization.

References

Abedin, L., Lien, E.L., Vingrys, A.J. and Sinclair, A.J. (1999) *Lipids*. 34, 475-482.

Abei, M., Shimizu, M., Yashushi, M., Shoda, Y. and Tanaka, N. (2000) *Gastroenterology*. 118, A714 (abs).

Achaya, K.T. (1995) *J. Sci.and Indust Res*. 54, 91-97.

Adam, O., Wolfram, G. and Zollner, N. (1986) *J. Lipid Res*. 27, 421-426.

Adams, P.B., Lawson, S., Sanigorski, A. and Sinclair, A.J. (1996) *Lipids*. 31, S157-S161.

Aebi ,H. (1984) *Meth Enzymol*. 105, 121-125.

Aguilera, C.M., Mesa, M.D., Ramirez, T.M.C., Quiles, J.L. and Gil, A. (2003) *Clin Nutr*. 22, 379-382.

Ahmad, A. Moriguchi, T. and Salem. N. Jr. (2002) *Pediatr. Neurol*. 26, 210-218.

Ahmad, A., Muthy, M., Greiner, R.S., Moriguchi, T. and Salem, N., Jr. (2002a) *Nutr. Neurosci*. 5, 103-113.

Ahrens, Jr., E.H., Insull, Jr., W., Hirsch, J., Stoffel, W., Peterson, M.L. and Farquhar, J.W. (1959) *Lancet*. 1, 115-119.

Akbar, M. and Kim, H.Y. (2002) *J. Neurochem.* 82, 655-665.

Albert, C.M. (1998) *J. Am. Med Assoc*. 279, 65-66.

Albert. C.M. (2002) *N. Engl. J. Med*. 346, 1113-1118.

Allard, J.P., Kurian, R., Aghdassi, E., Muggli, R. and Royall, D. (1997) *Lipids*. 32, 535-541.

American Public Health Association. (2001) Compendium methods for the microbiological examination of foods 4th edn, 485 p.

Anderson, G.J. (1994) *J. Lipid Res*. 35, 105-111.

Anderson, J.L. (1986) *Aust, New Zeal. J. Med*. 16, 409-415.

Aneja, R.P., and Murthy, T.N. (1991) *Nature*. 350, 280.Baldwin, A.J. and Ackland, J.D. (1991) *Neth. Milk Dairy J*, 45, 169-181.

Anon (1977) *J. Nutr*. 107, 1340-1348.

AOAC. (2000) Official methods of analysis of the association of official analytical chemists, 17th ed, Arlington, USA. Vols 1 and 2.

Appel, L.J. (1993) *Archives Int. Med.* 153, 1429-1438.

Armstrong, M.J. and Carey, M.C. (1982) *J. Lipid Res*. 23, 70-80.

Ashady, R. (1993) J. Microencaps. 10, 413-435.

Aslan, A. and Triadafilipoulous, G. (1992) *Am. J. Gastroenterology*. 87, 432-437.

Balendiran, G.K. Schnutgen, F. Scapin, G. Borchers, T. Xhong, N. Lim, K., Godbout, R. Spener, F. and Sacchettini, J.C. (2000) *J. Biol Chem.* 275, 27045-27054.

Badings, H.T. and Neeter, R. (1980) *Neth. Milk Dairy J*, 34, 9-30.

Bang, H.O., Dyerberg, J. and Sinclair, H.M. (1980) *Am. J. Clin. Nutr*. 33, 2657-2661.

Bansal, P. and Kansal, V.K. (1996) *Ind. Dairyman*. 48, 25-31.

Barbar, L.S. (1997) 32, 248-249.

Baro, L., Fonolla, J., Pena, J.L., Martinez-Ferez, A., Lucena, A., Jimenez, J., Boza, J.J. and Lopez-huertas, E., (2003) *Clin. Nutr.* 22, 175-182.

Baskaran, V., Mahadevamma, Nagendra, R., Daniel, V.A. and Venkat rao, S. (1994) *Ind. J Dairy Sci*, 47, 790-795.

Bazinet, R.P., Douglas, H. and Cunnane, S.C. (2003) *Lipids*. 38, 187-189.

Bazinet, R.P., McMillan, E.G. and Cunnane, S.C. (2003) *Lipids.* 38, 1045-1049.

Beare – Rogers, J.A. (1977) Docosaenoic Acid in Dietary Fats. In: Holman RT (ed). Progress in Chemistry of Fats and Other Lipids, 15, 29-56.

Bemelmans, W.J.E (2002) *Br. J. Nutr*. 88, 573-579.

Berard, A.M., Duman, M.F. and Darman, M. (2004) *FEBS Letters*. 559, 125-128.

Berg, J.A. and Aust. S.D. (1989) *Methods in enzymology*. 52, 302-310.

Bergeron, N. and Havel, R.J. (1997) *Curr. Opin. Lipidol*. 8, 43-52.

Berr, F., Holl, J.H., Jungst, D., Fischer, S., Richter, W.O., Seifferth, B. and Paumgartner, G.D. (1992) *Hepatology*. 16, 960-967.

Bezard, J., Blond, A. Bernard, A. and Clouet, P. (1994) *Reprod. Nutr. Dev.* 34, 539-568.

Bierenbaum, M.L., Reichstein, R. and Watkins, T.R. (1983) *J. Am. Coll. Nutr.* 12, 501-504.

BIS. (1981) Hand book of food analysis. Dairy products Bureau of Indian Standards SP: 18 (partXI), p. 129.

Bligh, E.G. and Dyer, W.J. (1959) *Can. J. Biochem. Physiol.* 37, 911-917.

Boguslawasky, W. and Wrobel, J. (1974) *Nature*. 247, 210-211.

Bonna, K.H., Bjerve, K.S. and Nordoy, A. (1992). *Am. J. Clin. Nutr.* 55, 1126-1234.

Booker, M.L., Scott, T.E. and La Morte, W.W. (1990) *Lipids.*25, 27-32.

Bothom, K.M. (1986) Introduction to cholesterol 7-α hydroxylase: role as rate limiting enzyme in bile acid synthesis, in: R. Fears. J.R. Sabine (Eds.) cholesterol 7-α hydroxylase, CRC Press, Boca Raton, F.L, USA, pp. 21-40.

Bourre, J.M. Francois, M., Youyou, A., Dumout, O., Piciotti, M., Pascal, G. and Durand, G. (1989) *J. Nutr.* 119, 1880-1892.

Bourre, J.M. Youyou, A., Durand, G. and Pascal G. (1987) *Lipids.* 22, 535-538.

Bowen, R.A.R. and Clandinin, M.T. (2002) *J. Neurochem.* 83, 764-774.

Bravo, E., Cantafora, A., DeLuca, V., Tripodi, M., Avella, M. and Botham, K.M. (1998) *Atherosclerosis*. 139, 253-263.

Bravo, E., Ortu, G., Cantafora, A., Lambert, M.S., Avella, M., Mayes, P.A. and Botham, K.M. (1995) *Biochim. Biophys. Acta.* 1258, 328-336.

Brenna, J.T. (2002) *Curr. Opin. Clin. Nutr. Metab. Care*. 5, 127-132.

Brenner, R.R. (1971) *Lipids*. 6, 567-575.

Brenner, R.R. (1977) *Adv. Exp. Med. Biol.* 83, 85-101.

Brenner, R.R. (1984) *Prog. Lipid Res*. 23, 69-96.

British Nutrition Foundation. (1992) Unsaturated fatty acids. Nutrition and physiological significance. The report of the British Nutrition Foundation's task force.

Bronte, S. B., Antonis, A., Eales, L. and Brock, J.F. (1956) *Lancet*. 521-527.

Broughton, K.S. (1997) *Am. J. Clin. Nutr*. 65, 1011-1017.

Brunauer, L.S. and Huestis, W.H. (1993) *Biochem Biophys Acta* . 1152, 109-118.

Bulur, H., Gokkusa, C. and Uysal, M. (1986) *Nutr Rep Int*. 33, 247-251.

Burdge, G.C. and Wootton, S.A. (2002) *Br. J. Nutr*. 88, 411-420.

Burdge, G.C., Jones, A.E. and Wootton, S.A. (2002) *Br. J. Nut*. 88, 355-363.

Burr, G.O. (1942) *Fed. Proc.* 1, 224-233.

Cabellero, B. (2003) *European J. Clin. Nutr.* 57, S76-S78.

Cain, B.S., Harken, A.H., Meldrum, D.R. (1999) *J. Mol Cell Cardiol.* 31, 931-947.

Calder, P.C. (2001) *Nutr. Res.* 21: 309-341.

Carey, M.C. (1978) J. Lipid Res. 19, 945-955.

Castillo, M., Hortal, J.H., Gil-Villarino, A., Luque, P., Iglesias, J. and Peregrin, E.G. (1999) *J. Nutr. Biochem.* 10, 198-204.

Caughey, G.E., Mantzioris, E., Gibson, R.A., Cleland, L.G. and James, M.J. (1996) *Am. J. Clin. Nutr.* 63, 116-122.

Cave, W.T. (1996) *Nutrition.* 12, S39-S42.

Chadha, S.L., Radhakrishnan, S., Ramachandran, K., Kaul, U. and Gopinath, N. (1990) *Ind. J. Med. Res.* 92, 424-430.

COMA (1994) Cardiovascular review group. Report on health and social subjects: nutritional aspects of cardiovascular disease. London: Department of health. 46.

Chautan, M., Chanussot, F., Portugal, H., Pauli, A.M. and Lafont, H. (1990) *Biochim. Biophys. Acta.* 1046, 40-45.

Chen, L.C., Boissonnneault, G., Hayek, M.G. and Chow, C.K. (1993) *Lipids.* 28, 657-662.

Chen, Z.J., Ratnayake, W.M.N. and Cunnane, S.C. (1994) *J. Am. Oil. Chem. Soc.* 71, 629-632.

Chen, Z.Y. and Nawar, W.W. (1991) *J. Food Sci.* 56, 398-401.

Cho, H.P., Nakamura, M.T. and Clarke, S.D. (1999) *J. Biol Chem.* 274, 471-477.

Christie, W.W. (1995) Composition and structure of milk lipids. Advanced Dairy Chemistry and lipids. Vol 2 1-36. P.F.Fox,ed Chapman & Hall London, UK.

Christinsen, J.H. (1996) *Br. Med J.* 312, 677-678.

Clare, D.A. and Swaisgood, H.E. (2000) *J Dairy Sci*. 1187-1195.

Cohen, D.E., Angelico, M. and Carey, M.C. (1990) *J. Lipid Res*. 31, 55-70.

Colbert, L.B. and Decker, E.A. (1991) *J. Food Sci.* 56, 1248-1250.

Connor, W.E., Neuringer, M. and Reisbick, S. (1992) *Nutr Rev*. 50, 21-29.

Conquer, J.A., Cheryk, L.A., Chart, E., Gentry, P.A. and Holub, B.J. (1999) *Thromb Res*. 96, 239-250.

Cook, H.W. Fatty acid desaturation and chain elongation in eukaryotes. In: Biochemistry of Lipids, Lipoproteins and Membranes (D.E. Vance and J. Vance, eds.). Elsevier Science Publishers, Amsterdam, 1991, pp. 141-169.

Craig, W.J. (1999) *Am. J. Clin. Nutr*. 70, 491S-499S.

Crawford, M.A. (1968) *Lancet* 1, 1329-1333.

Crawford, M.A. (1993) *Am. J. Clin. Nutr*. 57, 703S-709S.

Crawford, M.A. Doyle, W. Drury, P. Ghebremeskel K. Harbige, L. Leyton, J. and Williams, G. The food chain for n-6 and omega 3

fatty acids with special reference to animal products. In: Dietary omega 3 and n-6 fatty acids: Biological effects and nutritional essentiality (Galli, C. and Simopolous, A.P. eds.). Plenum press, New York, 1989, pp. 5-20.

Crawford, M.A., Casperd, N.M. and Sinclair, A.J. (1976) *Comp. Biochem. Physiol.* 54B, 395-401.

Croset, M., Black, J.M. Swanson, J.E. and Kinsella, J.E. (1989) *Lipids*. 24, 278-285.

Cunnane, S.C. (1999) *Am. J. Clin. Nutr*. 69, 395-402.

Cunnane, S.C., Ganguly, S., Menaard, C., Liede, A.C., Hamadeh, M.J., Chen, Z.Y. and Woolever, T. (1993) *Br. J. Nutr*. 69, 443-453.

Cunnane, S.C., Menard, C.R., Likhodii, S.S., Brenna, J.T. and Crawford, M.A. (1999) *Prost. Leuko. Essen. Fatty Acids*. 60, 387-392.

Dargel, S. (1992) *Exp Toxicol Pathol*. 44, 169-181.

Das, U.N. (1994) *Prostglandins Leukot Essent Fatty acids*. 51, 207-213.

Daviglus, M.L. (1997 *New Eng. J. Med*. 336, 1046-1053.

De Caterina, R., Liao, J.K. and Libby, P. (2000) *Am. J. Clin. Nutr*. 71, 213S-223S.

De Lorgeril, M., and Salem, P. (2000) Modified Cretan Mediterranean diet in the prevention of coronary heart disease and cancer, in *Mediterranean diets* (Simopolous, A.P., and Visioli, F., eds.) World Review of Nutrition and Dietetics. Vol. 87, S. Karger, Basel.

De Lorgeril, M., Renaud, S., Mamelle, N., Salen, P., Matin J.L, Monjaud, I., Guidollet, J., Touboul, P. and Delaye, J. (1994) *Lancet*. 343, 1454-1459.

Department of health, Report on health and social subjects No 46 Nutritional aspects of cardiovascular disease: Report of the cardiovascular review group, committee on medical aspects of food policy, London: HMSO, 1994.

De Urquiza, A.M., Liu, S., Sjoberg, M., Zetterstrom, R.H., Griffiths, W., Sjovall, J. and Perlmann, T. (2000) *Science.* 290, 2140-2144.

Diamandis, E.P., Novokmet, R., Mehling, C.C., Perera, T., Griffin, L.C. and Oomah, B.D. (2001) *J. Sci. Food and Agri*. 81, 889-894.

Dietschy, J.M., Turley, S.D. and Spady, D.K. (1993) *J. Lipid Res,* 34, 1637-1659.

Dische, Z (1947) *J. Biol. Chem*. 167, 189-198.

Droge, W. (2002) *Physiol. Rev.* 82: 47-51.

Dry, J. and Vincent, D. (1991) *Inter. Archi. Aller. Appli. Immunol*. 95,156-157.

Dyer, A.R., Stamler, J. and Shekelle, R.B. (1992) *Ann Epidemiol*. 15, 1-7.

Dyerberg, J. and Bang, H.O. (1978) *Lancet* I. 152.

Dyerberg, J., Band, H.O., Stoffersen, E., Moncada, S. and Vane, J.R. (1978) *Lancet ii*, 117-119

Eaton, S.B.and Konner, M. (1985) *New Engl J Med*. 312, 283-289.

Ehas, E.A., Yusuf, S. and Mehta, J.L. (1991) *Am. J. cardiol.* 70, 945-949.

Ehrstrom, M.C. (1951) *Acta. Med. Scand.* 140, 416-422.

Emken, E.A., Adlot, R.O. and Gulley, R.M. (1994) *Biochim. Biophys. Acta.* 1213, 277-288.

Endler, G., Klimenseh, A., Sunder-Plassmann, H., Schillinger, M., Exner, M., Mannhalter, S., Jordonova, N., Christ, G., Thalhammer, R., Huber, K. and Sunder-Plassmann, R. (2002) *Br. J. Haematol.* 117, 339-404.

Engler, M.B., Ma, Y.H. and Engler, M.M. (1999) *Am. J. hypertens* 12, 1225-1235.

Enslen, M., Milon, H. and Malnoe, A. (1991) *Lipids.* 26, 203-208.

Esposito, L.A., Melov, S., Panov, A. Cottrell, B. and Wallace, B. (1999) *Proc. Natl. Acad. Sci. USA.* 96, 4820-4823.

Ezaki, O., Takahashi, M., Shingematsu, T., Shimamura, K., Kimura, J., Ezaki, H. and Gotoh, T. (1999) *J. Nutr. Sci. Vitaminol.* 45, 759-762.

Feller, S.E., Gawrisch, K. and MacKerell, A.D. (2002) *J. Am. Chem. Soc.* 124, 318-326.

Ferretti, A. Nelson, G.J. Schmidt, P.S. Bartoloni, G., Kelly, D.S. and Flanagan, V.P. (1998) *J. Nutr. Biochem.* 6, 82-89.

Fisher, R.A. (1970) Statistical Methods for Research Workers, 14th Ed. Oliver and Boya, Edinburg, London.

FitzGerald, G.A., Goodnight, S.H. and Connor, W.E. (1993) *Circulation.* 87, 1017-1029.

FitzGerald, G.A., Pedersen, A. and Patrono, C. (1983) *Circulation* 67, 1174-1179.

Flaten, H. (1990) *Am. J. Clin. Nutr*. 52, 300-306.

Fleischhauer, F.J., Yan W-D. and Fischell, T.A. (1993) *J. Am Coll Cardiol.* 21, 982-989.

Fletcher, M.J. (1968) *Clin. Chim. Acta*. 22, 303-307.

Flohe, L. and Otting, F. (1984) *Meth Enzymol*. 105, 93-104.

Folch, J., Lee, M. and Sloane-Stanley, G.H. (1957) *J. Biol. Chem.* 226, 497-509.

Frank, T., Jennifer, M.C.G. Parveen, Y., Killipan, R., Jennifer, W., Cliff. P.S., Patrick, J.C., Phillip. C.C. and Robert, F.G. (2003) *The Lancet*. 361, 477-485.

Freese, R. and Mutanen, M. (1997) *Am. J. Clin. Nutr.* 66, 591-598.

Fritsche, K.I. and Johnston, P.V. (1988) *J. Nutr*. 118, 425-426.

Froyland, L., Madsen, L., Vaagenes, H., Totland, G.K., Auwerx, J,. Kryvi, H., Staels, B. and Berg, R.K. (1998) *J. Lipid Res*. 39, 583-593.

Fu, Z. and Sinclair, A.J. (2000) *Lipids*. 35, 395-400.

Furie, B. and Furie, B.C. (1992) *New Eng.J.Med*. 326, 800-806.

Galli, C. and Simopolous, A.P. (1989) Dietary omega-3 and omega-6 fatty acids. Biological effects and nutritional essentiality. Plenum press, New York, NY.

Gambus, H., Mikulec, A., Gambus, F. and Pisulewski, P. (2004) *Pol. J. Food Nutr. Sci.* 1, 21-27.

Garcia, M.C., Ward, G., Ma, Y.C. Salem, N. Jr. and Kim, H.Y. (1998) *J. Neurochem.* 70, 24-30.

Gerster, H. (1998) *Internat. J. Vit. Nutr.* Res. 68, 159-173.

Ghafoorunissa (1990) *Lipids.* 25, 763-766.

Ghafoorunissa (1994) *The Natl. Med. J. Ind.* 7, 270-276.

Ghafoorunissa (1996) *Lipids.* 31, S287-S291.

Ghafoorunissa (1998) *Ind. J. Med. Res.* 108, 191-202.

Ghafoorunissa and Jyotsna, P. (1993) *Food Chem.* 47,121-124.

Ghafoorunissa. (1989) *Bull Nutr Foundation India.* 10, 1-5.

Ghfoorunissa, Vani, A., Laxmi, R., and Sesikeran, B. (2002) *Lipids.* 37, 1077 – 1086.

GISSI-Prevenzione Investigators. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E in 11,324 patients with myocardial infarction: results of the GISSI-Prevenzione trial. Lancet 354: 447-455.

Givens, D.I. (2000) *Nutr. Abstr. Rev. Series B* 70, 4-59.

Gogos, C.A. (1998) *Cancer.* 82, 395-402.

Gonzales, R., Auclair, C. and Voisin, E. (1984) *Cancer Res*. 44, 4137-4139.

Goodfellow, J., Bellamy, M.F., Ramsey, M.W., Jones, C.J. and Lewis, M.J. (2000) *J. Am. Coll. Cardio*. 35, 265-270.

Gopalan, C. (1994) *Bull Nutr Foundation India*. 15, 1-4.

Gopalan, C., Ramasastri, B.V., Balasubramanian, S.C. Revised and updated by Narasinga Rao, B.S., Deosthaie, Y.G., Pant, K.C. Nutrient composition of Indian foods. New Delhi: Indian Council of Medical Research, 1989.

Gopinath, N. (1992) *Ind. J. Med Res*. 92, 424-430.

Gothwal, P.P. and Bhavadasan, M.K. (1992) *Ind. J. Dairy Sci*. 45, 146-151.

Grant, W.B. (1997) *Alz Dis Rec*. 2, 42-55.

Greiner, R.C.S., Zhag. Q., Goodman, K.J., Giussani, D.A., Nathanielsz, P.W. and Brenna J.T. (1996) *J. Lipid Res*. 37, 2675-2686.

Greiner, R.S., Moriguchi, T., Hutton, A., Slotnick, B.M. and Salem jr, N. (1999) 34, S239-S243.

Grundy, S.M. and Denkey, M.A. (1990) *J. Lipid Res*. 31, 1149-1172.

Guidot, D.M., Hybertson,B.M. Kitlowski, R.P. and Repine, J.E. (1996) *Am J Physiol*. 271, L225-L229.

Gunstone, F.D. Fatty acid structure. In: The lipid hand book (F.D. Gunstone, J.L. Harwood, and F.B. Padley eds.). Chapman and Hall, London, 1994, pp. 1-19.

Guoping, L., Windsor, S.L. and Harris, W.S. (1999) *J. Nutr. Biochem.* 10, 151-158.

Gurr, M.I. (1981) *J. Dairy Res*. 48, 519-523.

Hagve, T.A. and Christophersen B.O. (1986) *Biochim. Biophys. Acta.* 875, 165-169.

Hajri, T., Pronczuk, A. and Hayes, K.C. (1998) *J. Nutr. Biochem.* 9, 249-257.

Hallaq, H., Smith, T.W. and Leaf, A. (1992) *Proc. Natl. Acad. Sci. USA.* 89, 1760-1764.

Halliwell, B. and Chirico, S. (1993) *Am. J. Clin. Nutr*. 57, 715S-725S.

Hannun, Y.A. and Linardic, C.M. (1993) *Biochim. Biophys. Acta.* 1154, 223-236.

Hannun, Y.A. and Bell, R.M. (1993) *Adv. Lipid Res*. 25, 27-41.

Harker, L.A., Kelly, A.B. Hanson, S.R. Krupski, W., Bass, A., Osterud, B., FitzGerald, G.A., Goodnight, S.H. and Cpnnor, W.E. (1993) *Circulation*. 87, 1017-1029.

Harker, L.A., Kelly, A.B., Hanson, S.R., Krupski, W., Bass, A., Osterud, B., Kristensen, S.D., Scmidt, E.B., and Dyerberg, J. (1989) *J. Intern Med.* 225, S141-S150.

Harris, W. S. (1997) *Am. J. Clin. Nutr*. 65S, 1611S-1616S.

Harris, W.S. (1989) *J. Lipid Res*. 30, 785-807.

Harris, W.S. (1997) *Am. J. Clin. Nutr*. 65, 1645S-1654S.

Harris, W.S., Dujovne, C.A., Zucker, M. and Johnson, B. (1988) *Ann. Intern. Med*. 109, 465-470.

Hasler, C.M., Kundrat, S. and Wool, D. (2000) *Current Atherosclerosis Reports*. 2, 467-475.

Haslewood, G.A.W. and Wooton, V. (1950). *Biochem. J*. 47, 584-597.

Haumann, B.F. (1998) *Inform*. 9, 366-382.

Healy, D.A., Wallace, F.A., Miles, E.A., Calder, P.C. and Newsholme, P. (2000) *Lipids*. 35, 763-768.

Heinzelmann, K., Franke, K., Vaesco, J. and Marquez-Ruiz, G. (2000) *Eur. Food Res. Technol*. 211, 234-239.

Helen, M.R. (1999) 58, 397-401.

Hennig, B. and Chow, K.L. (1988) *Free Rad. Biol. Med*. 4, 99-104.

Herzberg, G.R. and Rogerson, M. (1988) *J. Nutr*. 118, 1061-1067.

Heuman, D.M., Hylemon, P.B. and Vlahcevic, Z.R. (1989). J. *Lipid Res*. 30, 1161-1171.

Hibbeln, J.R., and Salem, N. Jr. (1995) *Am. J. Clin. Nutr*. 62, 1-9.

Hodge, L. (1996) *Med. J. Aus*. 164, 137-140.

Hoffman, D.P., Birch, E.E., Birch, D.G. and Uauy, R.D. (1993) *Am. J. Clin. Nutr*. 57, 807S-812S.

Hoilg, W.H., Pabst, M.J. and Jakoly, W.B. (1974) *J Biol Chem*. 249, 7130-7139.

Holland, B., Welch, A.A., Unwin, I.D. Buss, D.H. Paul, A.A. Southgate, D.A.T. Mc Cance and Widdowson's. (1993) The Composition of Foods, London: Royal Society of Chemistry and Ministry of Agriculture. Fisheries and Food.

Holman, R.T., Johnson, S.B. and Hatchi, T.F. (1982) *Am. J. Clin. Nutr*. 35, 617-623.

Hornstra, G. (1989) *J. Intern. Med*. 225, 53-60.

Horrobin, D.F., Huang, Y.S., Cunnane, S.C. and Manku, M.S. (1984) *Lipids*. 19, 806-811.

Howard, A.N. and Marks, J. (1977) *Lancet II.* 255-256.

Huang, C.J. and Fwu, M.L. (1993) *J Nutr*, 123, 803-810.

Huff, M.W. and Telford, D.E. (1989) *Arteriosclerosis.* 9: 58-66.

Huff, M.W., Telford, D.E., Edmonds, B.W., McDonald, C.G. and Evans, A.J. (1993) *Biochim. Biophys. Acta*. 1210, 113-122.

Hulbert, A.J., Rana, T. and Couture, P. (2002) *Physiol. Biochem. Mol. Biol*. 132: 515-527.

Hulcher, F.H. and Oleson W.H. (1973) J. *Lipid Res*. 14, 625-631.

Ide, T., Murata, M. and Sugano, M. (1996) J. Lipid Res. 37, 448-463.

Ikemoto, A., Nitta, A., Furukawa,S., Ohishi, M., Nakamura, A., Fujii, Y. and Okuyama, H. (2000) *Neurosci. Lett*. 285, 99-102.

Ikemoto, A., Kobayashi, T., Watanabe, S. and Okuyama, H. (1997) *Neurochem. Res*. 22, 671-678.

Illingworth, D.R., Harris W.S. and Connor, W.E. (1984) *Atherosclerosis*. 4, 270-275.

Indu, M. and Ghafoorunissa. (1992) *Nutr. Res.* 12, 569-582.

Institute of Medicine of the National Academies. Dietary References intakes: energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein and amino acids. Washington, DC: National Academies Press, 2002.

Ispen, R. and Hansen, P.S. (1988) Factors affecting the storage stability of whole milk powder. Berentn Statens Forsogsmejeri, No, 274, 35 p.

Israel, D.H. and Gorlin R. (1992) *J. Am. Coll. Cardiol*. 19, 174-185.

Jacobson, J.G. and Smith, L.H., Jr. (1968) *Physiol. Rev*. 48, 424-511.

Jeffrey, B.G. Mitchell, D.C. Hibbeln, J.R. Gibson, R.A. Chedester, A.L. and Salem, N. jr. (2002) *Lipids* 37, 839-848.

Jenkins, D.G., Kendall, C.W., Vidgen, E., Agarwal, S., Rao, A.V., Rosenberg, R.S., Diamandis, E.P., Novokmet, R., Mehling, C.C., Perera, T., Griffin, L.C. and Cunnane, S.C. (1999) *Am. J. Clin. Nutr*. 69, 395-402.

Kaasgaard, S.G. Holmer, G., Hoy, C.E. Beherens, W.A. and Beare-Rogers, J.L. (1992) *Lipids*. 27, 47-52.

Kajwara, M.O.K., Imai, S., Koboyashi, T., Honma, N., Maki, T., Suruga, K., Goda, T., Takase, S., Muto, Y. and Moriwaki, H. (1997) *J. Nutr.* 127, 1752-1757.

Kalmijn, S. (1997) *Am. J. Epidemiol.* 145, 33-41.

Kansal, V.K. (1995) *Indian Dairyman.* 47, 20-27.

Kelly, D.S., Branch, L.B., Love, J.E., Taylor, P.C., Rivera, Y.M. and Lacono, J.M. (1991) *Am. J. Clin. Nutr.* 53, 40-46.

Keogh, M.K., O' Kennedy, B.T. and Kelly, P.M. (2001) *J. Food Sci.* 66, 217-224.

Keys, A., Anderson, J. T. and Grande, F. (1957) *Lancet* 1, 66-68.

Kinsella, J.E. Lokesh, B. and Stone, R.A. (1990) *Am. J. Clin. Nutr.* 52, 1-28.

Kitessa, S.M., Gulati, S.K., Simos, G.C., Ashes, J.R., Scott, T.W., Fleck, E. and Wynn, P.C. (2004) *Br. J. Nutr.* 91, 271-277.

Knox, R., Stein, I., Levinson, D., Tso, P. and Mansbach, C.M. (1991) *Biochim Biophys Acta.* 1083, 65-70.

Kolanowsky, W., Swiderski, F. and Berger, S. (1999) *Int. J. Food Sci. Nutr.* 50, 39-49.

Kremer, J.M. (1995) *Arthritis and Rheumatology.* 38, 1107-1114.

Kremer, J.M. (2000) *Am. J. Clin. Nutr.* 71S, 349S-351S.

Kris-Etherton, P.M., Taylor, D.S., Yu-Poth, S., Huth, P., Moriarty, K., Fishell, V., Hargrove, R.L., Zhoo, G. and Etherton, T.D. (2000) *Am. J. Clin. Nutr.* 71, 179S-188S.

Kristchevsky, D., Tepper, S.A. and Klurfeld, D.M. (1991) *J. Nutr. Biochem*. 1, 133-134.

Kristensen, S.D., Scmidt, E.B. and Dyerberg, J. (1989) *J. Intern. Med*. 225, 141-150.

Kromann, N. and Green, A. (1980) *Acta. Med. Scand*. 208, 401-406.

Kumar, K.V. and Das, U.N. (1993) *Free Rad Res Commun*. 19, 59-66.

Kurowska, E.M., Dresser, G.K., Deutsch, L., Vachon, D. and Khalil, W. (2003) *Prostglan. Leukotri. Essen. Fatty acids*. 68, 207-212.

L'Abbe, M.R., Trick, K.D. and Beare-Rogers, J.L. (1991) *J Nutr.* 121, 1331-1340.

Lagarde, M. Spector, A.A, Galli, C., Hamazaki, T., and Knapp, H.R. (1999) *Lipids*. 34, S341-S350

Land, W.E.M. and Byrnes, M.J. (1982) *Prog Lipid Res*. 20, 287-290.

Land, W.E.M., Hamazaki, T., Yamazaki, K., Okuyama, H., Sakai, K., Goto, Y. and Hubbard, V.S. (1990) *Am. J. Clin. Nutr*. 51, 991-993.

Lapidus, L., Bengtsson, C. and Lindquist, O. (1985) *Acta. Med. Scand*. 217, 418-489.

Lauritzen, L. and Hansen, H.S. (2003) *Lipids*. 38, 889-891.

Lauritzen, L., Hansen, H.S., Jorgensen, M.H. and Michaelson, K.F. (2001) *Prog. Lipid Res*. 40, 1-94.

Lawrence, R. and Sorrel, T. (1993) *The Lancet*. 342, 465-469.

Le Jossic, C.C., Gonthier, C. Z. I., Logette, E.S.I. and Bournot, P. (2005) *Biochem. J*. 385, 787-794.

Leaf, A. (1990) *Circulation*. 82, 624-628.

Le Breton, G.C., Dinerstein, R.S., Roth, L.S. and Feinberg, H. (1976) *Biochem. Biophys. Res. Commun*. 71, 362-370.

Lee, K.N., Kritchevsky, D. and Pariza, M.W. (1994) *Atheroscerosis*. 108, 19-25.

Leibovitz, B., Hu, M.L. and Tappel, A.L. (1990) *J Nutr*. 120, 97-104.

Lemaitre. R.N. (2002) *Am. J. Clin. Nutr*. 76: 319-325.

Leung, L. and Nachman, R. (1986) *Ann. Rev. Med*. 37, 179-186.

Leyton, J., Drury, P. and Crawford, M. (1987) *Br. J. Nutr*. 57, 383-393.

Leyton, J., Drury, P. and Crawford, M. (1987) *Br. J. Nutr*. 57, 383-393.

Li, D. Sinclair, A.J. Wilson, A., Nakkote, S., Kelly, F., Abedin, L., Mann, N.J. and Turner, A.J. (1999) *Am. J. Clin. Nutr*. 69, 872-882.

Li, D., Zhao, L. and Liu, M. (1999) Am. Heart J. 137, 1145-1152.

Lieber, C.S. (1997) *Adv Pharmacol*. 38, 601-628.

Lin, D.S., and Connors, W.E. (1990) *Am. J. Clin. Nutr*. 51, 535-539.

Lipid Research Clinic Program. (1984) *JAMA*. 251, 365-374.

Lokesh, B.R., Hseih, H.L. and Kinsella, J.E. (1986) *Ann Nutr Metab*. 30, 357-364.

Lorgeril, M. Renand, J. Mamelli, ., Salen, P., Martin, J., Monjaud, I., Guidollet, J. Touboul P. and Delaye. J.N. (1994) *Lancet* 343, 1454-1459.

Lorgeril, M.D., Salen, P., Laporte, F. and Leiris, J.D. (2001) *Eu. Heart. J*. 3 (Suppl) D26-D32.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J Biol Chem* 193, 265-275.

Lowry, S.F. and Thompson, W.A. (1994) *New Horiz*. 2, 164-174.

Luo, J. (1998) *Diabetes Care*. 21, 717-724.

Machlin, Z.J. and Bendich, A. (1987) *FASEB J.* 1, 441-445.

Magnuson, T.H., Lillemo, K.D., High, R.C. and Pitt, H.A. (1995) *Surgery*. 118, 517-523.

Mantzioris, E., Cleland, L.G., Gibson, M.J., Neumann, M.A., Demasi, M. and James, M.J. (2000) *Am. J. Clin. Nutr*. 72, 42-48.

Mantzioris, E., James, M., Gibson, R. and Cleland, L.G. (1994) *Am. J. Clin. Nutr*. 69, 872-882.

Mantzioris, E., James, M.J., Gibson, R.A. and Cleland L.G. (1994) *Am. J. Clin. Nutr*. 59, 1304-1309.

Marcus, A.J. (1984) *J Lipid Res*. 25, 1511-1518.

Marshal, R.T. (1993) Standard methods for the examination of dairy products 16th ed. American Public Health Association, Washington D.C.

Martin, M.J., Hulley, S.B., Browner, W.S., Kuller, L.H. and Wentworth, D. (1986) *Lancet* 2, 933-936.

Masden, S., in "Functional Foods. The consumer, the products and the evidence', (Eds Sadler, M.J. Saltmarsh, M.), Cambridge: The Royal Society of Chemistry, 1998, 159-163.

Matthews, K.R. Homer, D.B., Thies, F. and Calder, P.C. (2000) *Br. J. Nutr*. 83, 637-643.

Mauron, J. (1983) Interaction between food constituents during processing. Proc. 6th Int. Congr Food Sci Technol. Dublin. 5, 301-321.

McCluskey, C., Connolly, J.F., Devery, R., O'Brien, B., Kelly, J., Harrington, D. and Stanton, C. (1997) *J Food Sci*. 62, 331-337.

McLennan, P.L., Abeywardena, M.Y. and Charnock, J.S. (1988) *Am. Heart J*. 116, 709-717.

McLennan, P.L., Abeywardena, M.Y. and Charnock, J.S. (1990) *Am. J. Clin. Nutr*. 51, 53-58.

Mehta, R.S. Gunnet, C.A., Harris, S.R. Brunce, O.R. and Hartle, D.K. (1994) *Clin Exp Pharm Physiol*. 21: 881-885.

Mest, H.J., Beitz, J., Heinroth, I., Block, H.U. and Forster, W. (1983) *Klin. Wochenschr*. 61, 187-191.

Miller, D.M. and Aust, S.D. (1989) *Arch. Biochem Biophys*. 271, 113-119.

Mohrhauer, H. and Holman, R.T. (1963) *J. Lipid Res*. 58, 151-159.

Moore, S.A., Hurt, E., Yoder, E., Sprecher, H. and Spector, A. (1995) *J. Lipid Res*. 36, 2433-2437.

Morel, D.W. and Chisolm, G.M. (1989). *J. Lipid Res*. 30, 1827-1834.

Morrison, M.R. and Smith, M. (1963). *J Lipid Res*. 5, 600-608.

Mott, G.E., Jackson, E.M. and Mc Mahan, L.A. (1992) *Am. J. Clin Nutr*. 56: 511-516.

Mullane, K.M. and Pinto, A. (1987) *Fed Proc*. 46, 2422-2433.

Murphy, M.G. (1990) *J. Nutr. Biochem*. 1, 68 – 79.

Nagendra, R., Mahadevamma., Baskaran, V. and Venkat Rao, S. (1995) J. Food. Proc. Preser. 19, 303-315.

Nageswari, K., Banerjee, R. and Menon V.P. (1999) *J. Nutr. Biochem*. 10, 338-344.

Nakashima, Y., Yuasa, S., Hukamizu, Y., Okuyama, H., Ohhara, T., Kameyamma T. and Nabeshima T. (1993) *J. Lipid Res*. 34, 239-247.

Nanua, J.N. and Mc Gregor, U. (2000) *J Dairy Sci*. 83, 2426-2431.

Napoli, C. and Ignarro, L.J. (2001) *Nitric oxide*. 5, 88-97.

National Research council. (1989) Diet and health implications for reducing chronic disease risk. Washington, DC: National Academy press.

Navder, K.P., Bethfryer, E. and Fryer, H.C. (1990) *J. Nutr. Biochem*. 1, 640-646.

Needleman, P., Raz, A. and Minkes, M.S. (1979) *Proc. Natl Acad. Sci.* 76, 944-948.

Nelson, G.J., Ackman, R.G. (1988) *Lipids*. 23, 1005-1014.

Neuringer, M., Anderson, G.J. and Connor, W.E. (1988). *Ann. Rev. Nutr*. 8: 517-541.

Neuringer, M., Connor, W.E., Lin, D.S. Berstad, L. and Luck, S. J. (1986) *Proc. Natl. Acad. Sci. USA*. 83, 4021-4025.

Neuringer, M., Connor, W.E., Lin, D.S., Barstad, L. and Luck, S.J. (1986). *Proc. Natl. Acad. Sci. USA*. 83: 285-294.

News Review. (2000) *Lipid Technology*. 98.

Niranjan, T.G. and Krishnakantha, T.P. (2000) *Nutr Res*. 20, 1125-1138.

Norday, A., Marchili, R., Arnesen, H. and Videbau, J. (2001) *Lipids*. 36, S121-S129.

Norell, S.E., Ahlbom, A., Feychting, M. and Pedersen, N.L. (1986) *Br. Med. J*. 293, 426-429.

Oomah, B.D. (2001) *J. Sci. Food and Agri.* 81, 889-894.

P.M. Kris-Etherton, D.S. Taylor, S. Yu-Poth, P. Huth, K. Moriarty, V. Fishell, R.L. Hargrove, G. Zhoo, T.D. and Etherton, (2000) *Am. J. Clin. Nut*r. 71, 179S-188S.

Padley, F.B. Gunstone, F.D., and Harwood, J.L. Occurrence of and characteristics of oils and fats. In: The lipid hand book (Gunstone, F.D., Harwood, J.L. and Padley, F.B. eds.). Chapman and Hall, London, 1994, pp. 47-223.

Pakala, R., Pakala, R., Sheng, W.L. and Benedict, C.R. (1999) *Arterioscer Thromb Vasc Biol*. 19, 2316-2322.

Pan, D.A. and Storlien, L.H. (1993) *J. Nutr*. 123, 512-519.

Philbrick, D.J., Mahadevappa V.G., Ackman, R.G., and Holub, B.J. (1987) *J. Nutr*. 117, 1663-1670.

Piche, L.A. and Mahadevappa, V.G. (1990) *J Nutr Biochem*. 1, 206-212.

Podczasy, J.J., Church, J.P. and Schoene, N.W. (1995) *J. Nutr. Biochem*. 6, 327-333.

Prichard, B.N.C., Smith C.C.T., Ling K.L.E. and Betteridge, D.J. (1995) *Br. Med J*. 310, 819-810.

Pudelkewicz, C., Seufert, J. and Holman, R.T. (1968) *J. Nutr*. 94, 138-146.

Pullareddy, A.C.H. and Lokesh, B.R. (1994) *J Nutr. Biochem*. 5, 181-188.

Purasiri, P., Murray, A. and Richardson, S. (1994) *Clin Sci*. 87, 711-717.

Rabiskowa, M., Song, J., Opawale, F.O. and Burgess, D.J. (1994) *J. Pharm. Pharmacol*. 46, 631-645.

Ramaprasad, T.R., Baskaran, V. Sambaiah, K. and Lokesh B.R. (2004) *Lipids*, 39, 627-632.

Ramaprasad, T.R. Mohan, B.H., Sambaiah, K. Jamuna, P. and Lokesh, B.R. (2003) *J. Food Lipids.* 10, 237-249.

Ratnayake, W. M. N., Behrens, W.A., Fisher, P.W.F., L' Abbe, M.R., Mongeau, R. and Beare-Rogers, J.L. (1992) *J. Nutr. Biochem*. 3, 232-240.

Reddy, J.K. and Lalwani, N.D. (1983) *Crit. Rev. Toxicol*. 12, 1-58.

Renaud, S., de Lorgeril, M., Delaye, J., Guidollet, J., Jacquardd, F., Mamelle, N., Maartin, J.L., Monjaud, L., Salen, P., and Touboul, P., (1995) *Am. J. Clin. Nutr.* 61, 1360S-1367S.

Reports of the National Cholesterol Education Program. (1988) *Arch. Intern. Med.* 148, 36-69.

Rice –Evans, C.A., Miller, N.J., Bowell, P.G., Bramley, P.M. and Pridham, J.B. (1995) *Free Rad Res*. 22, 375-383.

Rissanen. T. (2000) Circulation 102: 2677-2679.

Roach, P.D., Kambouris, A.M., Trimble, R.P., Topping, D.L. and Nestel, P.J. (1987) *FEBS Lett*. 222, 159-162.

Robins, S.J., Fasulo, J. M. Robins, V.F. and Patton, G.M. (1991) *J. Lipid Res.* 32, 985-992.

Roche, H.M. (1999) *Proc. Nutr. Soc.* 58, 397 - 401.

Rodas, D. Gillilard, S.E. and Maxwell, C.V. (1996) J. Dairy Science. 79, 2121-2128.

Rolls, B.A. and Porter, J.W.G. (1973) *Proc Nutr Soc*. 32, 9-15.

Rosenberg, M., Kopelman, I.J. and Talman, Y. (1990) *J. Agric. Food Chem*. 38, 1288-1294.

Rudel, L.L. and Morris, M.D. (1976) *J. Lipid Res.* 14, 364-366.

Rudel, L.L. Johnson, F.L., Sawyer, J.K., Wilson, M.S. and Parkd J. (1995) *Am. J. Clin Nutr*. 62, 463S-470S

Salem, N. Simopoulos, A.P., Galli, C., Lagarde, M. and Knapp H.R. ed 1996. *Lipids* 31, S1-S326.

Salem, V., Wegner, B., Mena, P. and Uauy, R. (1996) *Proc. Natl. Acad. Sci*. 93, 49-54.

Salvemini, D. and Botting, R. (1993) *Trends in Pharmacol Sci.* 14, 36-42.

Sambaiah, K. Ganesh Bhat, B and Chandrashekara, N (1986) *J. Chromatography* 380, 235-237

Sambaiah, K., and Srinivasan, K., (1991) *J. Food Sci. Technol*. 28, 35-38.

Sanchez, N.M., Gonzalez,V., Aguayo, P., Sanchez, J.M., Tanimoto, M.A., Elizondo, J. and Uribe, M. (2001) *J. Nutr*. 131, 2300-2303.

Sanders, T.A.B., Hinds, A. and Pereira, C.C. (1989) *J. Intern Med.* 225, S99-S104.

Sanderson, P., Finnegan, Y.E., Williams, C.M., Calder, P.C., Burdge, G.C., Wootton, S.A., Griffin, B.A., Millward, D.J., Pegge, N.C., and Beare – Rogers, J.A. (1977) Docosaenoic Acid in Dietary Fats. In: Holman RT (ed). Progress in Chemistry of Fats and Other Lipids, 15, 29-56.

Sanderson, P., Finnegan, Y.E., Williams, C.M., Calder, P.C., Burdge, G.C., Wootton, S.A., Griffin, B.A., Millward, D.J., Pegge, N.C. and Bemelmans, W.J.E (2002) *Br. J. Nutr*. 88, 573-579.

Scandinavian simvastatin survival study group. Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian simvastatin survival study (4S). Lancet. 344: 1383-1389.

Schectman, G., Kaul, S. and Kissebah, A.H. (1989) *Arteriosclerosis*. 9, 345-354.

Schmidt, E.B. (1997) *Dan. Med. Bull*. 44, 1-22.

Schmidt, E.B., Kristensen, E.D., De Caterina, R., Illingworth, D.R. (1993) *Atherosclerosis*. 103, 107-121.

Schoene, N.W. (1997) *Am. J. Clin. Nutr*. 65, 1665S-1668S.

Schull, S., Hemtz, N.H., Periasamy, M., Manohar, M., Janssen, Y.M.W., Marsh, I.P. and Mossman, B.T. (1991) *J Biol Chem.* 266, 24398-24403.

Schwartz, J. (2000) *Am. J. Clin. Nutr*. 71, 393S-396S.

Scientific Review Committee, Nutrition Recommendations Ottawa: Ministry of National Health and welfare. 1990 (H 49 –42/1990E).

Scientific Review Committee. Nutrition recommendations. Ottawa: Canadian Government Publishing Centre, supply and services Canada 1990.

Selvemini, D. and Botting, R. (1993) *Trends Pharmacol. Sci.* 14, 36-42.

Shapiro, D.J. and Rodwell, V.W. (1971) *J. Biol. Chem.* 246, 3210-3216.

Shepard, J., Cobbe, S.M. and Ford, I. (1995) *N. Engl J Med.* 333, 1301-1307.

Shinozaki, K., Kambayashi, J. and Kawasaki, T. (1996) *J. Atherosclerosis Thromb.* 2, 107-109.

Shiratsuchi, H., Shimoda, M., Imayoshi, K., Noda, K. and Osajima, Y. (1994) 42, 1323-1327.

Siguel, E.N. and Lerman, R.H. (1996) *Metabolism.* 45, 12-23.

Simon, J.A., Fong, J. Bernert, J.T. and Browher, W.S. (1995). 26:778-782.

Simonsen, N. (1998) *Am. J. Epidemiol.* 147, 342-352.

Simopolous, A. P. (2000) *Poultry Sci.* 79, 961-970.

Simopolous, A.P. (1991) *Am. J. Clin. Nutr.* 54, 438-463.

Simopolous, A.P. and Salem, N. (1989) *New. Eng. J. Med.* 321, 833-836.

Simopolous, A.P., Kiffer, R.R. and Martin, R.E. ed.1986 Health effects of polyunsaturated fatty acids in seafoods. Academic press, Orlando, FL.

Simopolous, A.P., Leaf, A. and Salem, N. (1999) Workshop on the essentiality of recommended dietary intakes for omega 6 and omega 3 fatty acids. Bethesda, MD: National Institutes of Health.

Simopoulos, A.P. (1999) *Am. J. Clin. Nutr*. 70, S560-S569.

Simopoulos, A.P. Genetic variation and evolutionary aspects of diet. In: Papas A, editor. Antioxidants in nutrition and health: Boca Raton: CRC Press; 1999a. p. 65-88.

Simopoulos, A.P. Nestel, P.J., editors. Genetic variation and dietary response. World Rev Nutr Diet, vol. 80. Basel:Karger; 1997.

Sinclair, A.J. (1975) *Lipids*. 10, 175-184.

Sinclair, A.J. (2003) *Lipids*. 38, 1113-1114.

Sinclair, A.J., Nodia, M. and Attar, B. (2003) *Lipids*. 38, 1113-1114.

Sinclair, H.M. (1953) *Proc. Nutr. Soc*. 12, 69-82.

Singh, R.B. (1997 *Cardiovasc. Drugs. Ther*. 11, 485-491.

Singh, R.B. and Niaz, M.A. (1994) *J. Assoc Phys Ind*. 42, 545-548.

Siscivick, D.S. (1995) J. *Am. Med. Assoc*. 274, 1363-1367.

Small, D.M. (1986) Hand book of Lipid Research, vol 4, The physical chemistry of Lipids. New York: Plenum Press.

Smith, M.J., Vekade, H.J., Havinga, R., Vonk, R.J., Scherpof, G.O., In'tveld, G. and Kuipers, F. (1994) *J. Lipid Res*. 35, 301-310.

Spady, D.K. and Dietschy, J.M. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4526-4530.

St. Angelo, A.J. (1996) *Crit. Rev. Food Sci. Nutr*. 36,175-224.

Stanley, J. (2004) *Lipid Tech.*16, 61-63.

Stewart, J.C.M. (1980) *Anal. Biochem*. 104, 10-14.

Stine, C.M., Harland, H.A., Coulter, S.T. and Jenness, R. 1954. *J. Dairy Sci*. 37, 202-209.

Svaneborg, N., Kristinsen, S.D., Hansen, L.M., Bullow, I., Husted, S. and Schmidt, E.B. (2002) *Thromb Res*. 105, 311-316.

Swanson, J.E. Black, J.M. and Kinsella, J.E. (1988) *Br. J. Nutr.* 59, 535-545.

Tagawa, H., Shmokawa, H. and Tagawa, T. (1999) *J. Cardiovasc Pharmacol*. 33, 633-640.

Tappel, A.L. (1978) *Meth Enzymol*. 52, 506-513.

Taylor, M.J. and Richardson, T. (1980) *J. Dairy Sci*. 63, 1783-1795.

The British Nutrition Foundation. Unsaturated fatty acids-nutritional and physiological significance: The report of the British Nutrition Foundation Task Force: London: Chapman & Hall, 1992.

Thies, F., Miles, E.A., Nebe-von-Caron, G., Powell, J.R. Hunt, J. Newsholme, E.A. and Calder, P.C. (2001) *Lipids*. 36, 1183-1193.

Tierney, S., Ahrendt, S.A., Fox, K., Talbot, M.L., Booker, H.A., Pitt, H.A., La Morte, W.W. and Lillemoe, K.D. (1993) *Gastroenterology.* 104, A380-A384.

Toyosaki, T., Yamamoto, A. and Mineshita, T. (1987) *J. Food Sci.* 52, 88-90.

Trautwein, E., Siddiqui, A. and Hayes, K.C. (1993). *Metabolism.* 42, 1532-1540.

Trozzi, G., Barzanti, V., Biagi, P.L. Lodi, R., Maranesi, M. and Turchetto, E. (1986). *Prog. Lipid Res*. 25, 610-623.

Turley, S.D. and Dietschy, J.M. (1978) *J. Lipid Res*. 19, 924-928.

Uauy, R. and Valenzuela, A. (2000) *Nutrition*. 16, 680-684.

Umezawa, M., Kogishi, K., Tojo, H., Yoshimura, S., Seriu, N., Ohta, A., Takeda, T. and Hosokawa, M. (1999) *J. Nutr*. 129, 431-437.

Urquhart, J.A. (1935) *Can. Med. Assoc. J*. 33, 193-196.

Vaidyanathan, V.V., Rao, K.V.R. and Sastry, P.S. (1994) *Neurosci. Lett.* 179, 171-174.

Van Vliet, T. and Katan, M.B. (1990) *Am. J. Clin. Nutr*. 51, 1-2.

Vanmil, P.J.J.M. and Jans, J.A. (1991) *Neth. Milk Dairy J.* 45, 145-167.

Vanmil, P.J.J.M., Hols, G., and Klok, H.J. Spray drying of concentrated milk: relation between initial droplet size and final particle size. Process Technology Proceedings, 5. Pre concentration

and Drying of food Materials. Elsevier, New York 1988, pp. 193 – 202.

Venkatraman, J.T., Chandrasekar, B., Kim, J.D. and Fernandes, G. (1994) *Lipids.* 29, 561-568.

Ventura, M.A., Woollett, L.A. and Spady, D.K. (1989) *J. Clin. Invest.* 84, 528-537.

Vercelotti, J.R., St. Angelo, A.J. and Spanier, A.M. (1992) Lipid oxidation in foods: An overview, in Lipid oxidation in foods (St. Angelo, A.J. ed.), pp. 1-11, American chemical society symposium series 500, American chemical society, Washington, DC.

Vinodhini, R. Rao, N.P., Sastry,J.G., and Kashinath, K. (1993) Nutrition trends in India. Hyderabad: National Institute of Nutrition.

Von Schacky, C. (1999) *Ann. Intern. Med.* 130, 554-562.

Voss, A. Reinhart, M Sankarappa, S. and Sprecher, H. (1991) *J. Biol. Chem.* 266, 1995.

Walstra, P. and Jenness, R. (1984) *Dairy Chemistry and Physics*. Wiley & Sons, New York.

Warnick, G.R. and Albers, J.J. (1978) *J. Lipid Res.* 19, 65-76.

Weintraub, M.S., Zechner, R., Brown A., Eisen berg S. and Breslow J.L. (1988) *J. Clin Invest.*82, 1884-1893.

WHO and FAO joint consultation: fats and oils in human nutrition. (1995) *Nutr Rev* .53, 202-205.

Willans, D.J., Mill, S.C. and Ranney, E.K. (1995) *Appl. Thrombosis Hemostasis*. 1, 188-201.

Willumsen, N., Skorve, J., Hexeberg, S., Rustan, A.C. and Berge, R.K. (1993) *Lipids*. 28, 683-690.

Wolff, R.L., Combe, N.A. and Entressangles. (1985) *Lipids* 12, 908-912.

Wolfe, M.S., Sawter, J.K., Morgan, T.M., Bulock, B.C. and Rudel, L.L. (1994) *Arterioscl. Thromb* 4, 587-597.

Wong, S.H., Nestel, P.J., Trimble, R.P., Storer, G.B., Illman, R.J. and Topping, D.L. (1984) *Biochim. Biophys. Acta*. 729, 103-109.

Yamamoto and Sano, (1995) *Drying Technology*, 13, 29-41.

Yamori, Y., Nara Y, Y., Iritani, N., Workman, R.J., and Inagami, T. (1985) *J. Nutr. Sci. Vitaminol*. 31, 417-422.

Yang, Y.J. (1999) *Clin. Biochem*. 32: 405-409.

Yamazaki, R.K., Shen, T. and Schade, G.B. (1987) *Biochim. Biophys. Acta.* 920, 62-67.

Yongsoon, P. and Harris, W. (2002) *Lipids*. 37, 941-946.

Young, S.L., Sarda, X. and Rosenberg, M. (1993) *J. Dairy Sci*. 76, 2878-2885.

Zhong, F. and Sinclair, A.J. (2000) *Lipids*. 35, 395-400.

Zimmer,L., Delion-Vancassel,S., Durand, G., Guilloteau, D., Bodard, S., Besnard, J.C. and Chalon, S. (2000) *J. Lipid Res*. 41, 32-40.