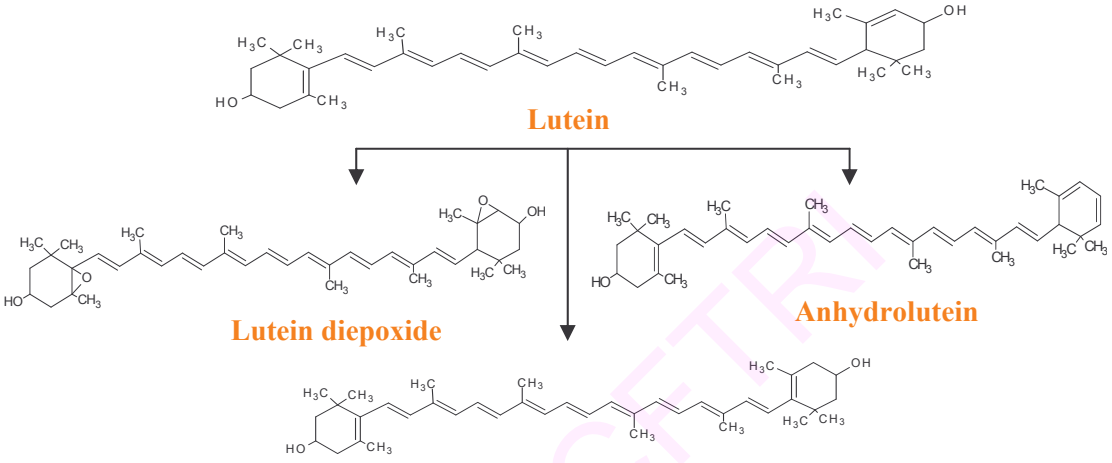
EFFECT OF DIETARY COMPONENTS ON THE BIOAVAILABILITY AND BIOACTIVITY OF XANTHOPHYLLS IN RATS



Thesis submitted to the

UNIVERSITY OF MYSORE

For the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

By

LAKSHMINARAYANA R. *M.Sc., M.Phil.*

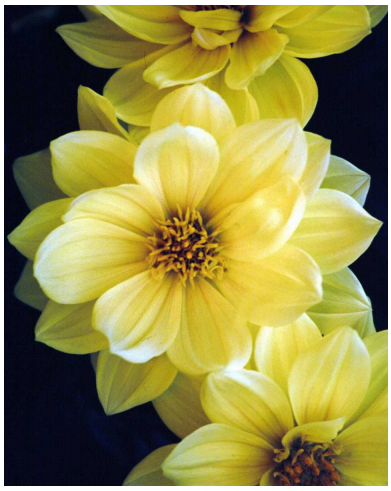
DEPARTMENT OF BIOCHEMISTRY AND NUTRITION
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE

MYSORE - 570 020, INDIA

MARCH - 2008

Dedicated to

My Parents and Teachers



Mr. R.Lakshminarayana
Senior Research Fellow
Department of Biochemistry and Nutrition
CFTRI, Mysore-570 020

DECLARATION

I hereby declare that the thesis entitled "**Effect of dietary components on the bioavailability and bioactivity of xanthophylls in rats**" submitted to the University of Mysore for the award of the degree of **Doctor of Philosophy** in Biochemistry is the results of research work carried out by me under the guidance of Dr. V. Baskaran, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore during the period 2003-2007.

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

Place: Mysore (Lakshminarayana R)
Date:

Date: 10-03-2008

Dr. V. Baskaran, M.Sc, M.Ed, M.Phil, Ph.D.
Scientist,
Department of Biochemistry and Nutrition
CFTRI, Mysore-570 020

CERTIFICATE

This is to certify that the thesis entitled "**Effect of dietary components on the bioavailability and bioactivity of xanthophylls in rats**" submitted by Mr. R.Lakshminarayana for the award of the Degree of Doctor of Philosophy in Biochemistry, to the University of Mysore is the result of research work carried out by him in the Department of Biochemistry and Nutrition under my guidance during the period 2003-2007.

(Dr. V. Baskaran)
Guide

ACKNOWLEDGEMENTS

I am sincerely grateful to my mentor and guide, Dr. V. Baskaran, Scientist, Department of Biochemistry & Nutrition, CFTRI, 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.

I wish to thank Padmashree Dr. V. Prakash, Director, CFTRI, Mysore, for giving me an opportunity to do research for my Ph. D. degree at CFTRI, Mysore.

I profusely thank Dr. P.V. Salimath, Head, Department of Biochemistry & Nutrition for his encouragement and Dr. S.G. Bhat, former Head, Dept. of Biochemistry & Nutrition for his timely suggestions and advice.

I extend my heartfelt gratitude to Dr. T.P. Krishnakantha, Scientist, for his helpful suggestions, constructive criticism and constant support throughout my research at CFTRI.

My thanks are due to Dr. K.S. Jagannatha Rao, Dr. S. Divakar, Mr. K. Rathina Raj, Mr. N. Bhaskar, Dr. G. Muralikrishna Dr. S. Bhattacharya, Dr. B.R. Lokesh, Mrs. Revathy Baskaran, Dr. Shylaja M. Dharmesh, Mr. M.N. Kesava Prakash, Dr. P. Saibaba, Dr. M.C. Varadaraj and Mr. Vijayakumar for their help and useful suggestions on various aspects pertaining to my research.

I take this opportunity to thank all the scientific staff of the Department of Biochemistry & Nutrition for their support during my stay in the department.

My sincere thanks are due to Dr. T. C. Shivashankarmurthy, Department of Botany, University of Mysore for his helping on various aspects.

My sincere thanks are due to my colleagues Mr. M. Raju, Miss. R.K. Sangeetha, Mrs. G. Aruna and Miss. B.S. Mamatha for their help and friends Mr. Chandrashekar, Dr. T. Ramaprasad, Mr. D. Suresh, Mr. Manjunath, Mr. U.V. Sathish, Mr. K. Sreenath, Mr. Vidyashankar, Mr. M.S. Madhukumar, Mr. Srikanta, Mr. Puneeth, Mrs. Veerbala Gupta, Mr. Ramesh, Mr. Vasudev Raju, Mr. Thyagaraju, Mr. B.T. Manu, Mr. Ani, Mr. Raghunath Reddy, Mr. Pramod, Mr. Revanappa, Mr. Rangarao, Mr. Varakumar and other friends for the little drops of kindness that made a mighty impact.

My thanks are also due to Mr. Swaroopkumar, Mr. S. Deepak and Mr. Arun for helping me during my studies in various aspects.

My sincere thanks are due to the staff of the Animal house facility for helping me in conducting animal experiments.

My thanks are due to Miss. M. Asha, Mr. P. Mukund, Mr. Shivaswamy, Mr. K. Vachtravelu, Mr. Anbalagan and other staff of the Central Instrumentation Facility and Services for their timely help in conducting analytical part of my research.

My sincere thanks are due to Dr. A. Nagao, Head, Lipid Laboratory, National Food Research Institute, Tsukuba, Japan for his help in providing standard carotenoids for my research and Prof. Kazuo Miyashita, Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Japan for his valuable suggestions on the subject during his visit to our laboratory.

I thank Council of Scientific and Industrial Research, New Delhi, Government of India for the award of Junior & Senior Research Fellowship from 2003-2007.

Finally, I express my heartfelt thanks to my parents, brother-in-law Dr. A. Somashekar murthy, jayanth and other members of my family for their continued support and encouragement throughout my career.

Lakshminarayana, R.

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

AMD	Age related macular degeneration
α	Alpha
APCI	Atmospheric pressure chemical ionization
AUC	Area under the curve
BC	β-carotene
β	Beta
Cal	Calories
°C	Degree Celsius
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ε	Epsilon
Fig.	Figure
g	Gram
GC	Gas chromatography
GNO	Groundnut oil
GLV	Green leafy vegetable
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography Mass-spectrometry
h	Hour(s)
kg	Kilogram
L	Lutein
L + Z	Lutein + zeaxanthin
L	Liter
LA	Linoleic acid
LDL	Low density lipoprotein
LPC	Lysophosphatidylcholine
μg	Microgram
μL	Microliter
μM	Micromolar
μmol	Micromol
MDA	Malondialdehyde
min	Minutes
mL	Milliliter
mg	Milligram
mM	Millimolar
MUFA	Monounsaturated fatty acids
ng	Nanogram
nm	Nanometer
nmol	Nanomol
ND	Not detected
OA	Oleic acid
OCC	Open column chromatography
OD	Optical density
OO	Olive oil
PC	Phosphatidylcholine
pmol	Picomol
PUFA	Polyunsaturated fatty acids
SD	Standard deviation
SFA	Saturated fatty acids
SFO	Sunflower oil
TGs	Triglycerides
v/v	Volume by volume
Z	Zeaxanthin

LIST OF TABLES

LIST OF FIGURES

Chapter 4

Chapter 5

Chapter 6

Synopsis

Leafy greens, fruit and vegetables are commonly consumed and are accessible throughout the year in India and other parts of the world. India is having rich sources of horticultural crops like fruits, vegetables and is one of the largest producers of these crops in the world. Most of the horticultural crops play an important role in human nutrition and prevention of several diseases since they possess health promoting bioactive phytomolecules like carotenoids and polyphenols. The health benefits of carotenoids are due to their provitamin A and antioxidant properties. Epidemiological studies have demonstrated that a diet rich in lutein and other carotenoids is closely linked with lower incidence of clinical/degenerative disorders such age related macular degeneration (AMD), cataract and certain cancers. Even though plant foods containing carotenoids have been used as major dietary sources of lutein in western countries and in India, AMD/cataract is one of the major health problems in aged peoples in these countries. This is mainly due to inadequate intake of macular pigments rich greens, improper processing techniques adopted to process them, poor intestinal absorption of dietary carotenoids, change in lifestyle and socioeconomic conditions.

In spite these evidence has been shown for lutein and other carotenoids as essential phytomolecules, helps vision and as an antioxidants. The mechanism of intestinal uptake of carotenoids under different conditions, like influence of dietary factors on intestinal uptake, conversion into active components, biodegradation/metabolism, structural futures of metabolites, antioxidant properties and role of various lipids. To increase our understanding on these aspects and also to know the potential health benefits of lutein, we need greater insight into their bioavailability and metabolism.

In specific, the bioavailability of major macular pigments lutein (L) and zeaxanthin (Z) under lutein deficiency status is yet to be studied well. Feeding purified or leafy greens as a source of lutein is demonstrated to modulate the AMD and its associated disorders. Dietary factors like, amount and nature of fat and its fatty acid composition, dietary fiber and nutritional status of an individual are also reported to play a major role in the bioavailability of lutein and zeaxanthin. For an efficient utilization of lutein and

zeaxanthin, maximization of their absorption and its accumulation is important. The efficacy of absorption and utilization of lutein derived from vegetable sources and inclusion of dietary modulators can be an important consideration in dietary approaches to overcome eye related problems (AMD and cataract). The lutein absorption *in vivo* involves sequential steps starting from its release from food matrix to incorporation with lipoproteins. These processes are dependent mostly on the physicochemical properties of the food matrix. Various exogenous and endogenous factors are reported to interfere with the bioavailability of lutein from foods. Of which, the amount and type or mixture of carotenoids present in the food matrix influences the lutein and zeaxanthin bioavailability. Interactions between carotenoids present in the food matrix may also affect the absorption of ingested lutein.

Absorption of carotenoids *in vivo* involves several steps: release of carotenoids from the food matrix, dispersion in lipid emulsion particles, solubilization into mixed bile salt micelles and movement across the unstirred water layer adjacent to the microvilli, uptake by the cells of intestinal mucosa and incorporation into lymphatic lipoproteins. The processes up to solubilization in mixed micelles are dependant mostly on the physicochemical properties of food and carotenoids and on the micelles formation from bile and lipid hydrolysates. A series of events, including competitive interaction among carotenoids can promote or inhibit the intestinal absorption and bioavailability of carotenoids. The bioavailability of carotenoids depends on the type and amount of lipids or fatty acids, the food matrix in which they are present etc. But the role of these components on the intestinal uptake and carotenoids/ lutein metabolism *in vivo* is lacking from detailed studies. Hence, it is proposed to investigate the possible role of dietary components on the bioavailability and bioactivity of lutein and zeaxanthin.

The objective of the present study was to determine the effect of dietary components (phospholipids, fatty acids and mixed carotenoids) on the bioavailability of lutein in rats under lutein sufficient and deficient conditions. It also aims to study the role of dietary components on the status of certain biochemical parameters such as lipid profiles, fatty acid profiles and antioxidant enzymes in selected target tissues. The results of these studies constitute the subject matter for this thesis in seven chapters.

Chapter 1. General introduction

This chapter provides an overview of the functions of lutein and zeaxanthin in physiological processes such as vision, and antioxidant functions. The sources of lutein/zeaxanthin are dark green leafy vegetables (GLVs), fruits and vegetables. The major aspects highlighted in this section include brief information on importance of lutein for eye health, causes and preventive measures. A brief overview of mechanisms involved in the lutein metabolism is discussed. The strategy used for managing AMD and bioavailability of carotenoids is discussed with particular emphasis on the factors affecting the bioavailability. Various dietary factors responsible for the bioavailability of lutein are also discussed. The intake of GLVs with suitable fat source in targeted population, epidemiological and experimental evidences for the beneficial effects of leafy greens are also highlighted. Dietary approach to control the AMD and current strategies available to increase the bioavailability of lutein from natural sources using suitable fat source are discussed in this section. The role of dietary factors like specific phospholipids and fatty acids on intestinal uptake of carotenoids and its conversion into lutein metabolites are highlighted.

It is clear from the literature that L is an important bioactive component, their importance in visual function and understanding the mechanism of its metabolism against photo or other oxidative stress and elucidation of structural features of metabolites are topics for the future research. The dietary components and their influences on L bioavailability and its disposition at ultra level by using appropriate methods are yet to be developed. Further, to date, there have been few studies that examined the effect of L esterification, but this subject is receiving increasing attention. The enzymes involved in the hydrolysis of L esters are not known, and the suitability of esters as substrate for common lipolytic enzymes needs attention.

Chapter 2. Materials and Methods

This chapter provides the details of the materials used and methods employed in the current investigation. Selection of GLVs, methods used for the isolation, purification and quantification of carotenoids from GLVs are described. Preparation of mixed micelles containing lutein and the methods used for studying physicochemical properties (pH, viscosity, particle size and structure) of mixed micelles containing lutein either with

phospholipids or fatty acids are covered. Image processing technique is employed to study the intensity of lutein incorporated into the mixed micelles. The rationale for selecting phospholipids and fatty acids as dietary modulators of lutein bioavailability is due to its fat-soluble nature. The methods used for studying the bioavailability of lutein from mixed micelles containing dietary modulators (phospholipids and fatty acids) in rats are also highlighted. Induction of AMD in rats and re-feeding lutein sufficient diet (lutein rich GLV supplemented diet with vegetable oils as fat source) to determine the possible role of these dietary factors in enhancing the bioavailability and bioconversion of absorbed lutein. Protocols for the determination of lutein and its degraded products under *in vitro* and *in vivo* are determined by high performance liquid chromatography (HPLC), and liquid chromatography mass spectrophotometer were detailed. Estimation of lipid parameters including, total cholesterol, triglycerides, phospholipids and fatty acids in plasma and tissues are described. Methodologies used for the studies on the activity of antioxidant enzymes are described. Experimental data were analyzed for their significant difference against control using appropriate statistical methods.

Chapter 3. Isolation, purification and quantification of xanthophylls (lutein and zeaxanthin) from selected leafy greens, fruits and vegetables by Column chromatography, HPLC and LC-MS techniques

This chapter begins with the brief literature survey on the dietary intake of GLVs, fruits and vegetables as lutein and zeaxanthin source to manage AMD. This chapter mainly aims at determining the carotenoids (L and Z) in locally available, commonly consumed GLVs, fruits and vegetables. The GLVs used in this study have beneficial effects against various health disorders. Some of the GLVs are seasonal, available only during rainy season and are not so familiar to the community. Other GLVs used were common, easily available in the market since cultivated throughout the year. Methodology for the extraction, purification and quantification of carotenoids is described in brief. A simple and reliable newer method for the extraction and purification by open column chromatography and HPLC and LC-MS analysis of L and Z from GLVs are described. Individual carotenoids were purified by open column chromatographic technique. The acetone extracts of GLV was purified by employing an activated alumina column and eluted with various solvent proportions. The purity of an individual eluant was checked by

HPLC and LC-MS techniques and confirmed against respective reference standards. The retention time of L and Z peak was compared with their reference standard's retention time and their characteristic spectrum. Among GLVs studied, the level of lutein and zeaxanthin were ranged from 15-183 mg/100 g dry wt and 0.1-5 mg/100 g dry wt and they were higher than other xanthophylls.

The results revealed that rare GLVs found to contain higher level of lutein compared with those of commonly consumed leafy greens. Among GLVs analyzed, *Commelina benghalensis* L.*,* contained higher levels of L and Z (183 mg/100g dry wt.), hence, used as lutein source for feeding studies. Among vegetables analyzed, *C. annuum* var. Grossa*, L. acutanguta* and *C. annuum* were found to contain comparatively higher level of L+Z, ranging from 430 to 1243 μg/100g edible portions than other vegetables. Among fruits analyzed, *M. indica, C. papaya* and *C. aurantifolia* contained higher level of L and Z ranging from 104.81 to 120.86 μg/100g edible portions. *M. indica* is found to be a richest source while *A. scomusus* is poorest source of L and Z. Interestingly, orange and pineapple contains higher level of Z than L. In conclusion, this study shows that the leafy vegetables are found to contain significantly higher levels of xanthophylls than β-carotene. Interestingly, very rarely consumed GLVs are found to contain higher levels of L and Z. The data generated on the composition of carotenoids in the present study could be helpful to suggest the consumers for better source of L and Z as a part of daily meal to overcome health disorders like AMD and cataract. Further, under utilized GLVs having higher levels of L and Z can be exploited for nutritional purpose.

Chapter 4. Effect of dietary components (phospholipids, fatty acids and mixed carotenoids on physico-chemical properties of mixed micelles and liposome containing lutein

This chapter commences with a brief literature on the role of dietary factors on the mixed micelles formation and the factors interfere with the transfer of carotenoids from the food matrix to the mixed micelles. The present investigation was carried out to evaluate the degree of *in vitro* incorporation of lutein into mixed micelles, which is an essential early step in the absorption process and to assess the role of specific phospholipids and fatty acids on the formation of micelles and the incorporation of lutein into mixed micelles *in vitro*. The micelles were designed to simulate as those appears

during fat digestion in the lumen of the human small intestine with respect to bile salts, lipids, pH and temperature. The composition of the mixed micelles was 2.5 mM monooleoyl glycerol, 7.5 mM oleic acid and 12 mM sodium taurocholate. The concentration of lutein used was 200 μM with 3 mM phospholipids (phosphatidylcholine and lysophosphatidylcholine) or 7.5 mM fatty acids (oleic and linoleic acid). The role of dietary factors (phospholipids, fatty acids and mixed carotenoids) on various physicochemical properties like pH, viscosity, particle size and structure of the mixed micelles were determined to study the extent to which the lutein is incorporated into the micelles. The use of image processing technique gave better results on the intensity of lutein incorporated into the mixed micelles. The factors that determine the incorporation of lutein into the micelles was depend on type of phospholipids, fatty acid, carotenoid and pH of the micellar solution. In the present study, an effort has been made to correlate the physicochemical properties of mixed micelles with the intestinal uptake of lutein from the mixed micelles containing either phospholipids or fatty acids or mixed carotenoids in rats.

Results revealed that inclusion of specific phospholipids or fatty acids to the mixed micelles resulted in altered micellar size and the intensity of L within the micelles. LysoPC and oleic acid found to positively influence the properties of micelles whereas, addition β-carotene along with lutein in mixed micelles affect the property of micelles.

Chapter 5. Effect of dietary components (phospholipids, fatty acids and mixed carotenoids) on lutein bioavailability and certain biochemical parameters in plasma and tissues

This chapter begins with a brief literature survey on lutein bioavailability and the factors associated with it. The main objective of this chapter was to determine the role of phospholipids (phosphotidylcholine and lysophosphatidylcholine) and fatty acids (oleic and linoleic acid) on intestinal lutein uptake. To achieve the objective, two sets of experiments were conducted viz., gavage and dietary studies using dietary factors (phospholipids, fatty acids and mixed carotenoids). Further, in gavage studies, single and repeated dose experiments were employed to determine the time course plasma response of the absorbed lutein and its accumulation in plasma, liver and eyes of rats received lutein sufficient diet. In another study, the rats were fed with an equimolar dose

of lutein and β-carotene in mixed micelles to determine the interaction of carotenoids while uptake by the intestinal cells. The results of the above studies revealed that the plasma response of lutein and zeaxanthin was improved after a single and repeated dose of micellar lutein containing phospholipids and fatty acids compared with controls.

Similarly, in another study rats were fed on a diet deficient with lutein to induce its deficiency and followed they have received GLV supplemented diet (lutein source) with different vegetable oils (fatty acid source) and soy lecithin (phospholipid source) to determine the role of specific fatty acid rich vegetable oils and phospholipid on the intestinal uptake of dietary L and Z. The results demonstrate that the source and type of lipid is critical to achieve an enhanced bioavailability of dietary L *in vivo*. The relative bioavailability of micellar or dietary L could be enhanced by feeding L along with specific fat (phospholipids or fatty acids) sources used. This is an interesting observation that compared L bioavailability among single or repeated (gavage study) or dietary studies using phospholipids, fatty acids and vegetable oils as L carrier lipids. Various biochemical parameters covered in this study were also revealed that dietary factors did not affect the normal status. The comparative observation showing the positive role for OA or OO either they fed to rats by gavages or through diet. These lipid sources were selected after screening various dietary lipids with respect to L bioavailability.

The influence of those dietary factors used in this study was found to influence L bioavailability more significantly in animal deficient with L compared to those of normal ones. Further, the proposed mechanism by which dietary fat influences the L bioavailability is well correlated with the structural properties of mixed micelles, viz. size, surface area and lutein intensity. Fatty acid composition of fat is important to achieve a higher bioefficacy of dietary L.

Chapter 6. Possible degradation/biotransformation of lutein in vitro and in vivo: Isolation and structural elucidation of lutein metabolites by HPLC and LC-MS (APCI)

This chapter commences with a brief literature survey on determination of carotenoid metabolites in general, in human blood and eye tissue and also briefs concerning factors involved in their degradation. Metabolites of lutein are highly

concentrated in the human macula and are known to exhibit protection against age related macular degeneration. The aim of this investigation was to characterize the oxidation products of lutein obtained through photo-oxidation (*in vitro*) and to compare them with biologically transformed dietary lutein in intestine, plasma, liver and eyes of rats. *In vivo* studies involved feeding rats with diet devoid of lutein for 2 weeks to induce deficiency. Rats were divided into two equal groups (n=6/group) and received either micellar lutein by gavages for 10 days or diet supplemented with fenugreek leaves as lutein source for 4 weeks. Lutein metabolites/oxidation products obtained from *in vivo* and *in vitro* studies were characterized by HPLC and LC-MS (APCI) techniques to elucidate their structure. The characteristic fragmented ions resulting from photo-oxidation of lutein were identified as 523 (M^++H^+-3CH_3), 476 (M^++H^+-6CH_3) and 551 (M^++H^+-H_2O). In the eyes, the fragmented molecules resulting from lutein were 13-*Z* lutein, 13'-*Z* lutein, 13-*Z* zeaxanthin, *all-E* zeaxanthin 9-*Z* lutein, 9'-*Z* lutein and 3'-oxolutein. Epoxy-carotenoids were identified in liver and plasma while anhydrolutein was identified in intestine as well. This study emphasizes the essentiality of dietary lutein to maintain its status in retina.

The present study shows that lutein is degraded in the system by photochemical or oxidative reactions and the metabolites/oxidized products formed could be excreted from the biological samples. Further, it is not clear that lutein oxidation products from intestine and blood are transported to and accumulated in retina or whether photo-induced metabolic oxidation of lutein may be responsible for their presence in the circulation. Hence, in depth studies are warranted. The presence of 3-hydroxy-β,ε-caroten-one in eye samples but not *in vitro* samples indicates an evidence for a possible *in vivo* metabolic oxidation of lutein in the eyes. Degradation of lutein in the body indicates that lutein is involved in various photochemical reactions (in eyes) and as an antioxidant in various oxidative reactions (other tissues). Based on the results we have proposed metabolic pathways for conversion of dietary lutein to its oxidation products. This study emphasizes the essentiality of maintaining dietary lutein status especially in retina to function as an antioxidant since it is easily degraded. Further research is needed to identify lutein metabolites in detail in biological samples in order to evaluate their possible biological significance with reference to AMD.

Chapter 7. General discussion and summary

In this chapter, the findings of the present investigation are discussed. Results of investigations of similar nature reported in literature were also discussed highlighting the important findings of the present investigation. The major findings of the investigation are briefly summarized.

At the end of the thesis, a collective list of references, which forms the basis for interpretation of the data obtained in comparison with earlier and contemporary published results, has been given.

Chapter 1

General introduction

Carotenoids are organic pigments that are naturally occurring in plants and some other photosynthetic organisms like algae, some types of fungus and bacteria. Carotenoids belong to the category of tetraterpenoid (contain 40 carbon atoms). Structurally they are in the form of a polyene chain, which is sometimes terminated by rings **(Figure1.1)**. There are over 600 known carotenoids; categorized into two major classes, xanthophylls and carotenes. Carotenoids containing oxygen, such as lutein (L) and zeaxanthin (Z) are known as xanthophylls. They are yellow pigments from the carotenoid group and their molecular structure is based on carotene skeleton, contrary to the carotenes, some hydrogen atoms are substituted by hydroxyl groups and /or by oxygen atoms. Xanthophyll has a chemical formula of $C_{40}H_{56}O_2$. The unoxygenated carotenoids such as α-carotene, β-carotene and lycopene are known as carotenes. Carotenes typically contain only carbon and hydrogen atoms. Carotene has a chemical formula of $C_{40}H_{56.}$ In plants, main function of these carotenoids is the optimization of the photosynthetic process. Xanthophylls being accumulated in the thylakoid membranes of the chloroplast function as accessory pigments help in harvesting light for photosynthesis. Among xanthophylls, L is a major pigment involved in the light harvesting complexes, with its absorption maxima of 445, 421 and 475 nm (**Figure 1.2**). L structure is perfectly designed for its role in harvesting and transferring light energy to the chloroplasts for generation of chemical energy to support the plant cell. The other important function of carotenoids is protecting plants from oxidative damage. By capturing excess energy from excited chlorophyll molecules, they prevent the formation of highly reactive and damaging singlet oxygen (Krinsky et al. 1987). Recently, a similar photo protective function of L and Z is proposed in human tissue. The possible biological functions for L include antioxidant activity and filtering of damaging UV light in the retina (Jughans et al. 2001). In general, out of 600 types of carotenoids, only about 20 have been detected in human plasma and tissues. Chemical structures of few important carotenoids are shown in **Figure 1.3.** Among these, β-carotene and L are well known major carotenoids found in human tissues. L and its metabolites have been detected in

lens and retina (Bernstein et al. 2001). They form the 'macular pigments' in the central region of the retina referred as *macula lutea* and *fovea centralis*.

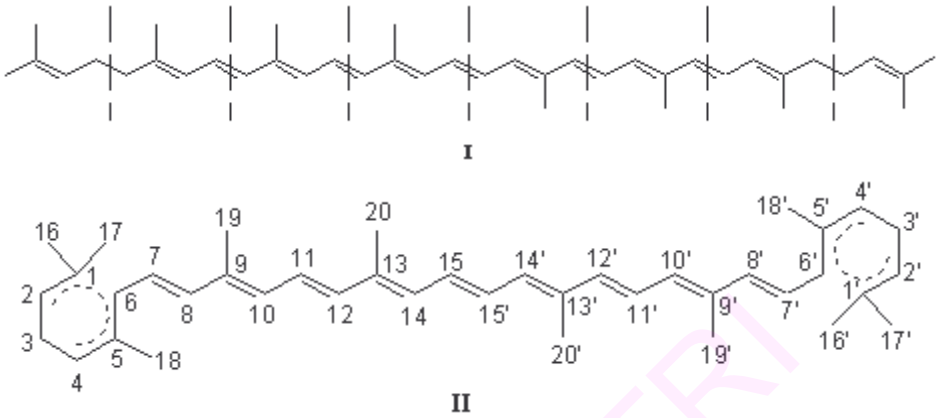


Figure 1.1. Acyclic and cyclic structure of polyene chain showing a long central chain of conjugated double bonds (I). Specific names of carotenoids are based on the stem name carotene, which corresponds to the structure and numbering (II)

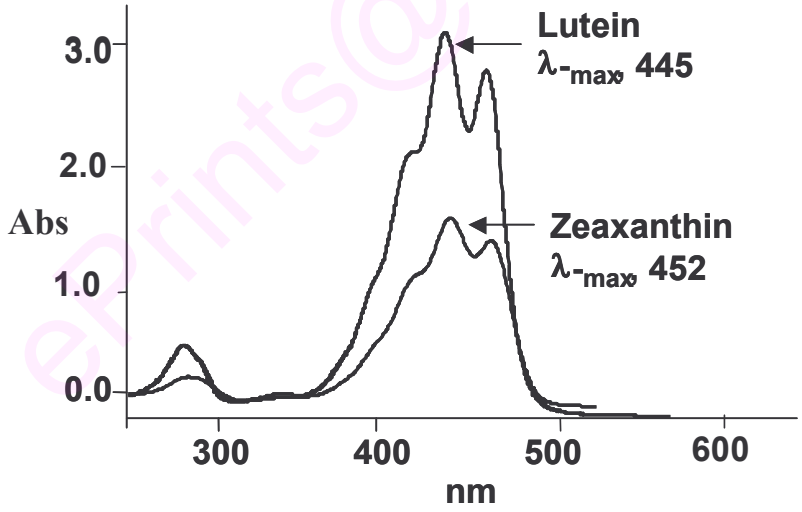


Figure 1.2. Characteristic UV-Vis spectrum of lutein and zeaxanthin.

Since higher animals cannot synthesize L and Z, they depend on dietary source like green leafy vegetables (spinach, kale, collard greens, broccoli etc), orange-yellow fruits and vegetables (corn, peaches, etc.) (DeFreitas, 2000, Lakshminarayana et al. 2005; Raju et al. 2007). L and Z **(Figure 1.3, IV and V)** have recently received the

attention of researchers in various disciplines due to their biological activity. Like β-carotene, L is an important dietary carotenoid and a vital macular pigment (Bone et al. 1993). Clinical and epidemiological studies have indicated that increased dietary intake of L was positively correlated with improved macular pigments density in the retina (Berendscot et al. 2000; Bone et al. 2003), which is inversely correlated with the pathogenesis of age-related macular degeneration (AMD), a major leading cause of blindness in people over 55 years of age (Bone et al. 2001; Beatty et al. 2001). In addition, they play an important role in maintaining ocular health (Schalch et al. 1999). There are evidences to support the protective effect by L against certain cancers and heart disease (Zhang et al. 1991; Kritchevsky et al. 1999; Chew et al. 2003). Before understanding the biological functions of L and Z with regards to human health, intestinal absorption, role of dietary factors on absorption and their bioavailability is need to be studied in detail. This review outlines the chemistry, bioavailability and bioactivity of L and its degradation/metabolism *in vitro* and *in vivo*.

Chemistry of macular pigments

Generally, carotenoids are long chain molecules of 40 carbon atoms with series of centrally located conjugated double bonds (Figure 1.1 and 1.3). Double bonds contribute to the carotenoids colour and ability to quench free radicals. L and Z are dihydroxy carotenoids with the ionone ring system being substituted at both 3 and 3' carbon (Goodwin 1984; Krinsky et al. 1987). There are two classes of ionone rings. In β-ionone ring, the double bond is found between the C5 and C6 carbons. The carbon bearing two hydroxyl groups share an identical '*R*' stereo chemical configuration derived from Z, 3*R,* 3'*R*-zeaxanthin that is found in most of the plants. The other stereo isomers of Z, 3*S,* 3'*S*-zeaxanthin and 3*R,* 3'*S*-*meso*-zeaxanthin have been identified in animal tissues including humans, where significant amount of 3*R,* 3'*S*-*meso*-zeaxanthin is concentrated in the retina (Schmidt et al. 2005). It is reported that these isomers are formed by the result of biochemical transformations and are not of dietary origin (Bernstein et al. 2001). L has both β-ionone and ε-β-ionone rings **(Figure 1.4)**. The presence of the hydroxyl groups at both 3 and 3' carbons suggests a close similarity in physical properties between L and Z.

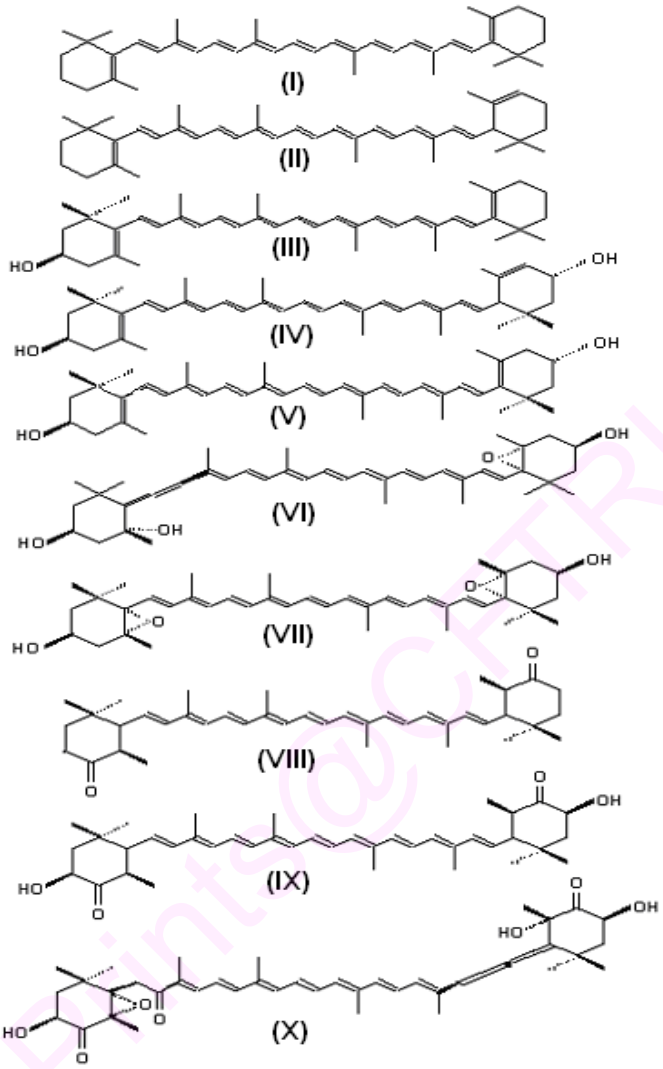


Figure 1.3. Chemical structures of few important carotenoids: β-carotene (β,β-carotene) (I*),* α-carotene (β,ε-carotene) (II), β-cryptoxanthin (β,β -caroten-3-ol) (III), lutein (β,ε -carotene-3, 3'-diol) (IV), zeaxanthin (β,β-carotene-3,3'-diol) (V), neoxanthin (5', 6'-epoxy-6,7-didehydro-5,6,S',6'-tetrahydro-β, β-carotene-3,5,3'-triol) (VI), violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol) (VII), canthaxanthin (β,β-carotene-4,4'-dione) (VIII), astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) (IX), and fucoxanthin (5,6-epoxy-3,3',5'-trihydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro-β,β-caroten-8-one 3'-acetate) (X).

The β-ionone ring has a C4-C5 double bond and an allelic 3'-hydroxyl group. Bone et al. (1993, 2001) reported the first chromatographic features of macular pigments using a HPLC technique and demonstrated the presence of L, Z and *meso*-Z in the *macula* **(Figure 1.4)**. Khachik et al. (1997, 2002) and Bhosale et al. (2005 and 2007) identified various oxidation products and geometric isomers of L such as *3R, 3'S*; *meso*-zeaxanthin, 3*R,* 3*'S,* 6*R*-lutein (3'-epilutein) 3-hydroxy-β-ε-carotene-3-one (3'-oxolutein) and 3-methoxy zeaxanthin in human serum and eyes.

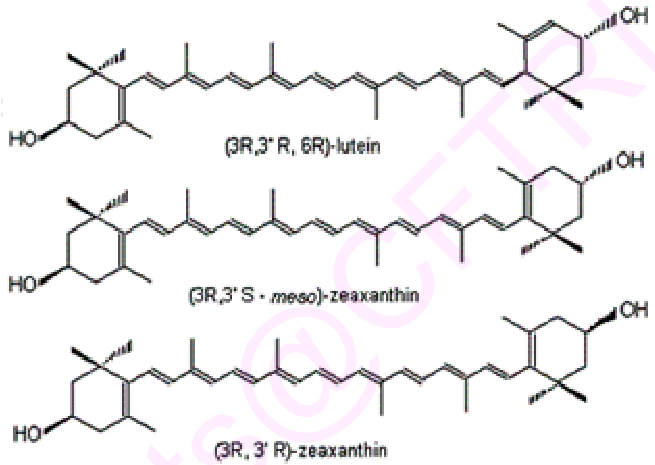


Figure 1.4. Molecular structure of macular pigments.

Health benefits of lutein and zeaxanthin

Age-related macular degeneration. The *macula lutea* or yellow spot in the *retina* is reported to be responsible for central vision and visual function in the human eyes, and have a dual function in tissues, acts as powerful antioxidant and filters high-energy blue light (West et al. 1999; Taylor et al. 1992; Snodderly et al. 1995; Jughans et al. 2001). AMD is a degenerative disorder involving the retinal pigment epithelium, choiocapilaries and retina, which primarily, but not exclusively affect the macular region. Symptoms of AMD include metamorphosia, impaired light adaptation and decreased central vision. AMD is a degradation of the central portion of the *retina* including the macula, and is the principle cause of blindness (vision loss) among aged people. AMD can be classified into two categories: Early (Dry AMD) and Late (Wet AMD). The former is characterized by accumulation of soft drusen caused by phototoxic damage and de-pigmentation of

the retinal epithelium and the later is characterized by neovascularization of the macula and *retina*, and accumulation of scar tissue (Beatty et al. 2000). The macular area and *fovea* becomes compressed due to degeneration of the pigment epithelium behind the *retina*, which leads to formation of drusen and leakage of fluid behind *fovea* **(Figure 1.5).** At this stage, the cones of *fovea* die causing central vision loss, (Algave and Seregard, 2002).

The most influencing factors for AMD are age and sunlight exposure. Cigarette smoking and poor nutritional status are also added to the list of risk factors (Cai et al. 2000; Christen, 1999). Studies demonstrated that high intake of polyunsaturated fatty acids may increase the degree of unsaturation in the macular structures and enhance the susceptibility to oxidative stress. Cho et al. (2001) has shown the association between dietary fat and risk of AMD in the two large prospective cohorts of women and men. AMD at an advanced stage often leads to irreversible blindness for which currently no effective treatment is available (Fine et al. 2000). Epidemiological study shows a direct relationship between L intake and risk of AMD (Seddon et al. 1994). Curren-Clentinlano et al. (2001) have shown a relationship between L intake and macular pigment density (MPD) in a group of 278 healthy volunteers, and also studies have supported the use of L supplements with increased MPD (Bernstein et al. 2002). Bone et al. (2001) showed the first evidence of L and Z concentration verses macular degeneration risk in humans.

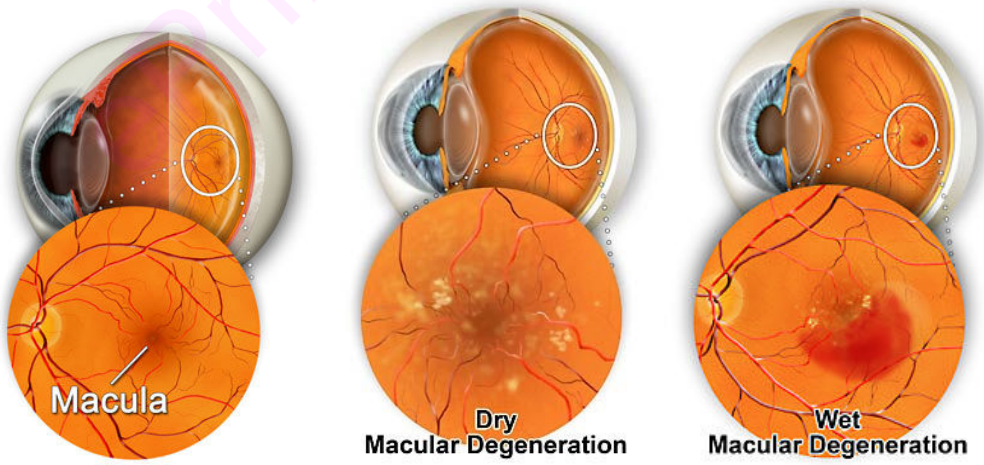


Figure 1.5. Anatomical view of healthy and AMD eyes (**Source:** http:// www. the retina source.com-armd.html)

Bone et al. (1988) quantified the macular L and Z by high performance chromatography in 87 human donors' eyes ranging in age from 3 to 95 years and reported no correlation between age and pigment concentration. However, it has been reported that several factors influence the MPD including smoking, iris colour, lens density and gender (Hammond et al.1996 and 1997). An increased oxidant load and reduced antioxidant defenses have been linked to cigarette smoking (Mezzetti et al. 1995). The inverse relationship between the macula (L and Z density) and oxidative stress may represent a common factor for the pathogenesis such as cataract and AMD (Jacques et al. 1991; Leske et al. 2002; Yeum et al. 1995). Chaine et al. (1998) reported that an individual, who accumulates large quantities of L and Z in the crystalline lens and the *retina*, is less likely to develop cataract and AMD.

Cataract. Cataract is an opacity that develops in the crystalline lens of the eye or in its envelope. In the early stage of age related cataract, the power of the crystalline lens may be increased, causing myopia (near sightedness) and the gradual yellowing and opacification of the lens may reduce the perception of blue colours. Cataract typically progress slowly to cause vision loss and leads to blindness if left untreated. Cataract develops from a variety of reasons, including long-term UV and radiation exposure, diabetes and age. Genetic factors are often causing congenital cataracts. There are various types of cataracts e.g., nuclear, cortical, mature and hyper mature. Cataract is the leading cause of blindness in the world. The most effective and common treatment is to surgically remove the cloudy lens. Several lipophilic components other than vitamin-E, L and Z are well known antioxidants in the human eye (Bernstein et al. 2001; Gale et al. 2001; Shih et al. 2003). Although, they are found within the lens, the dominant antioxidant remains to be vitamin-E. Whereas, L and Z are at lower levels in the lens and are more dominate antioxidants in the *retina* (Augustim et al. 1994). The concentration of vitamins and carotenoids in cataract lenses is higher including the cortex and epithelial cells (Fecondo and Augustyen, 1983). Bates and Cowen (1988) analyzed the concentration of vitamin-E and several carotenoids including β-cryptoxanthin, α-carotene and β-carotene in eyes of guinea pigs. Studies showed that the levels of these carotenoids in lenses were 3 fold higher in the newer epithelial tissues than in the older inner cortex. The epithelial cortex layer comprises 50 % percent

of the tissue, yet it has been found to contain 74% of the total lens L and Z, supporting the hypothesis that these pigments are protective against the oxidative damage causing cataract formation (Yeum et al. 1999). Numerous other observational studies have revealed that increased consumption of foods high in L and Z is associated with a decreased risk for cataracts in both men and women. These studies provide a strong evidence for the protective role for L and Z against development of cataracts (Brown et al. 1999; Tavani et al. 1996). Patients receiving 15 mg L three times weekly were compared with patients receiving 100 mg α-tocopherol for the same period. In patients receiving L, statistically significant improvements in visual acuity and increased serum concentration of L were observed, compared to patients who received α-tocopherol and either no L or the control (Olmedialla et al. 2003).

Bioactivity of lutein

There are several reports showing that L or Z is involved in the photo-protection, radical quenching and immunomodulation reactions in animals and human. Carotenoids may interact with free radicals in three main ways, namely electron transfer (Eq. [A]), hydrogen abstraction (Eq. [B]) and addition of a radical species (Eq. [C])

ROO• 1 CAR3 ROO2 1 CAR•1 [A]

ROO• 1 CAR3 ROOH 1 CAR• [B]

ROO• 1 CAR3 ~ROO 2 CAR! • [C]

In the human body, reactive oxygen species (ROS) include $^1O_2$, $OH^{\bullet}$, $^{\bullet}O^-_2$, and H_2O_2. The mechanisms and rate of scavenging of free radicals by carotenoids in solution is strongly dependent upon the nature of the ROS itself (Britton, 1995).

Among the radicals, carotenoids most efficiently react with peroxyl radicals formed under oxidative conditions *in vivo.* They are generated in the process of lipid per-oxidation, and scavenging of this species interrupts the reaction sequence, which finally leads to damage in lipophilic compartments. Due to their lipophilicity and specific property to scavenge peroxyl radicals, carotenoids are thought to play an important role in the protection of cellular membranes and lipoproteins against oxidative damage (Sies and Stahl, 1995). Martin et al. (1999) have demonstrated that at low partial pressures

many carotenoids are dramatically antioxidant, interrupting substrate oxidation by peroxyl radicals. Deactivation of peroxyl radicals likely depends on the formation of radical adducts forming a resonance stabilized carbon-centered radical.

Light exposure leads to the formation of reactive oxygen species, which damage the biomolecules and affect the integrity and stability of sub-cellular structures, cells and tissues (Stahl and Sies, 2001; Krutmann, 2000). Photo-oxidative processes play an important role in the pathobiochemistry of several diseases of light-exposed tissues, the eye and the skin. The efficacy of carotenoids to filter blue light was investigated in unilamellar liposome (Junghans et al. 2001). In this model, L and Z showed a better filtering efficacy than β-carotene or lycopene. It was suggested that the more prominent efficacy of L and Z is due to differences in the location of the incorporated molecules within the liposome membrane. Literature also offers conflicting evidence about a protective effect conferred by carotenoids, in particular, against UVA-induced cellular alterations.

L is a potent antioxidant and is found to enhance immune function, suppress mammary tumor growth and enhance lymphocyte proliferation (Zhang et al. 1991; Chew et al. 1996; Hadden et al. 1999). L has also been found to protect skin from damages caused by ultraviolet light, and to prevent cardiovascular hardening caused by ageing, coronary heart disease and cancer (Michaud et al. 2000; Slattery et al. 2000). L is one among the 10 phytochemicals recommended by the FDA as GRAS (generally regarded as safe) nutritional supplements (Kruger et al. 2002; http://www.cfsan.fda. gov/ rdb/opa-g110. html). β-Carotene has been found to increase the incidence of lung cancer at high dosages, especially among smokers (Mayne et al. 1996). However, the effects of pure L at higher dosages are not clear. Maccarone et al. (2005) recently reported that Z showed a pro-apoptotic effect in neuroblastoma cells, although these cells are rather resistant to apoptosis, while it is capable of preventing apoptosis in healthy cells.

Bioavailability of lutein

Bioavailability is defined as “the fraction of an ingested nutrient that is available for utilization in normal physiological functions or for storage”. Published information on the bioavailability of L and Z is based on the measurement of their levels in serum or plasma. Dietary matrix or components interfere with the rate of each of the absorption

steps that will affect the overall bioavailability of the ingested carotenoids **(Figure 1.6)**. Carotenoids are released from the food matrix by heat, mechanical and enzymatic treatments during food processing, and in the mouth, by mastication and the action of enzymes in the saliva. The released carotenoids incorporate into the lipid phase, which is emulsified into small lipid droplets in the stomach. From the lipid droplets, carotenoids are transferred to mixed micelles formed by the action of bile salts, biliary phospholipids, dietary lipids, and their hydrolysis products. However, the less lipophilic xanthophylls can also be solubilized directly in mixed micelles. The mixed micelles migrate to the brush border, where carotenoids are absorbed by the intestinal cells, packed into chylomicrons and secreted to the lymphatic system. The uptake of carotenoids from the intestinal lumen takes place by simple diffusion down a concentration gradient through the brush border membrane into the cytoplasm of the enterocytes. However, some reports have suggested the existence of carotenoid transport mediated by scavenger receptor binding protein (SR-BI). The hairpin-like conformation of SR-BI external domain forms a hydrophobic channel that may facilitate the uptake of carotenoids by the enterocytes, without energy expenditure (Yonekura and Nagao, 2007).

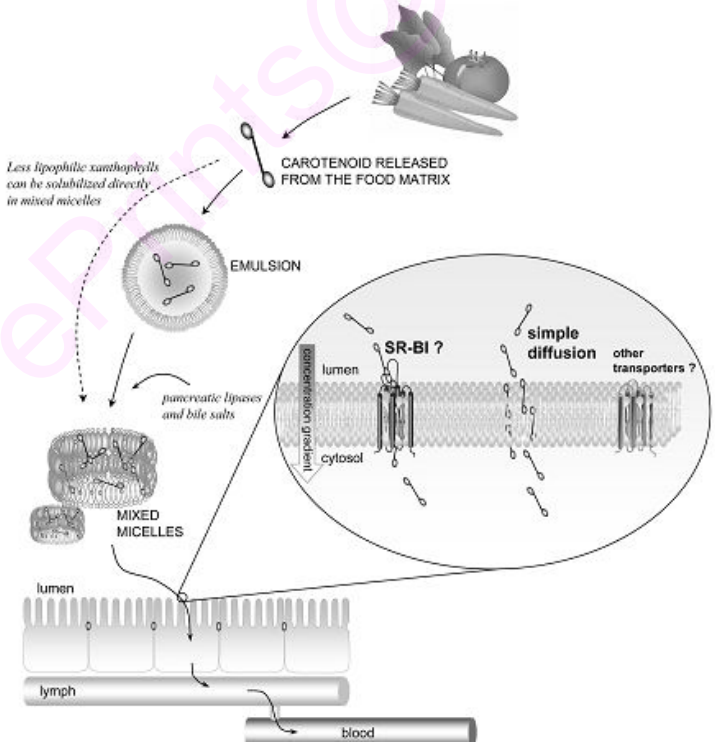


Figure 1.6. Scheme of dietary carotenoid absorption.

Although health benefits of L and Z are documented well, data on their bioavailability and role of dietary factors on their intestinal uptake is limited in the literature. Studies with single dietary doses of L or Z indicate that the L reaches peak concentration in chylomicron fraction at approximately two hours and peak in serum at about 16 hours post ingestion (O 'Neill et al. 1998; Kostic et al. 1995). Absorption of L from purified crystalline supplements is almost twice that from spinach or other vegetable sources (Castenmiller et al. 1999). Non-dietary factors such as age, body composition, gender, malabsorption of fat, alcohol consumption, smoking and liver or kidney diseases also affects the bioavailability (Albanes et al. 1997; Alberg et al. 2002; Brady et al. 1996; Williams et al. 1998).

Mechanism of lutein uptake. Intestinal absorption of dietary lipids including fat-soluble, nonpolar vitamins is a multi step process involving the solubilization of dietary lipids and their hydrolytic products in mixed bile salt micelles, diffusion of micelles through an aqueous phase of the intestinal lumen to the brush border membrane (BBM), and finally passive diffusion across the membrane into the cytoplasm of enterocytes (Tso, 1994; Furr and clark, 1997) **(Figure 1.6)**. In contrast to this diffusion model, kinetic evidence obtained with model systems such as BBM vesicles and Caco-2 cells showed that the uptake into the BBM of cholesterol and other hydrophobic dietary lipids is protein-mediated (Thurnhofer et al. 1990; Compassi et al. 1997). A protein facilitated lipid uptake mechanism is also consistent with the observation of a pronounced inter-individual variability in cholesterol absorption efficiency in both animals and humans that have been attributed to genetic factors at the enterocytes level (Wang et al. 2001). It is noteworthy that there is also inter individual variability in the response to dietary β-carotene (Borek et al.1998), and the absorption is enhanced if the diet contains more fat (Fur and clark, 1997). The cellular uptake and efflux of carotenoids, like cholesterol, likely involve more than one transporter. At present, the identity of the membrane transport protein(s) mediating the uptake of dietary lipids, thought of immediate interest, is still a matter of debate.

During et al. (2005) have shown that the carotenoids transport was decreased with increased polarity of carotenoids. Similarly, Reboul et al. (2005) examined L transport processes using Caco-2, TC-7 monolayer as a model for human intestinal

epithelium. Purified L was mixed with phospholipids, lysophospholipid, cholesterol, mono-olein, oleic acid and taurocholate to obtain L-rich mixed micelles that mimicked those found under physiological conditions. Co-incubation with β-carotene, but not lycopene, decreased the L absorption rate (20%) significantly. Overall, the available literature indicates that L absorption is, at least partly, protein-mediated.

Lutein binding protein and its intestinal uptake mechanisms. The detailed mechanisms for selective uptake, optimum concentration and stabilization of macular carotenoids are not yet fully understood. In the human blood, high-density lipoprotein (HDL) is the major carrier of L and Z. In the mammalian eye it has been reported that retinal tubulin binds with L and Z, possibly as a site for passive deposition in the tissue (Bernstein et al. 1997). Xanthophyll-binding proteins were partially purified and isolated from the human macula and *retina* and shown that L and Z bind specifically to those proteins (Yemelyanov et al. 2001). The concept that adipose tissue and *retina* may compete for dietary L has been suggested; and also hypothesized that if adipose and liver tissues compete with the *retina* for dietary L, its storage in the eye will be reduced (Johnson et al. 2002). The above concepts demonstrate that several basic aspects still remain to be addressed, such as the absolute absorption efficiencies of different carotenoids, the nature of luminal and intracellular factors and mechanism of mediated uptake of L. However, complete information about the specific factors (dietary or non-dietary) affecting or influencing the absorption, transport and metabolism of L is still not available.

Absorption and distribution of lutein and zeaxanthin. The process of L absorption requires movement of the digested food components into the mucosal cells of the intestinal wall. Uptake occurs when the L or its metabolites diffuse through the mucosal cells into the portal or lymphatic system **(Figure 1.6)**. Generally, the intestinal absorption of carotenoids involves five major steps: (1) release from the food matrix, (2) solubilization into mixed lipid micelles in the lumen, (3) cellular uptake by intestinal mucosal cells, (4) incorporation into chylomicrons, and (5) secretion into the lymph (Erdman et al. 1993; van Vliet, 1996; Fur and Clark, 1997; Yeum and Russell, 2002). In the golgi of the enterocytes, carotenoids are incorporated into chylomicrons according to

their polarity. It is hypothesized that L and Z are surface oriented (Deming and Erdman, 1999; Briton, 1995). The chylomicrons are eventually set free into the bloodstream, where they lose triglycerides content and shrink in size by the action of lipoprotein lipase. Eventually, non-triglycerides component of the chylomicron remnant, including surface polar carotenoids are taken up by the extra hepatic tissues or other blood lipoproteins. The remaining L and Z in the chylomicron remnant reach the liver cells **(Figure 1.6).** L and Z remain in the liver or enter into the blood stream back with the help of very low-density lipoproteins (VLDL), are then transferred by either low-density lipoproteins (LDL) or high-density lipoproteins (HDL) to target sites. L binds equally with LDL and HDLs in human blood, in contrast to the hydrocarbon carotenoids, which are preferentially found in LDL fractions (Erdman et al. 1993; Parker, 1996). HDL is the primary transporter of L and Z. Recently, Wang et al. (2007) have reported that the L and Z are transported primarily by lipoprotein in normal and AMD patients. They showed the transport of these carotenoids was not significantly different between the AMD and control groups and further reported that HDL was the major transporter, but other lipoproteins like VLDL and LDL also transport lesser quantities of L and Z.

The approximate concentration of L in human tissues is: serum, 0.1-1.23 μM; liver, 0.1-3.0 μM; kidney, 0.03-2.1 μM and lung, 0.1-2.3 μM (Krinsky et al. 2003; Kaplan et al. 1997). In the human *retina*, the concentration of L and Z reaches its highest levels; ranging between 0.1 and 1 mM (Landrum et al. 1999). Tissues differentially take up carotenoids. Macula of the eye was reported to contain higher levels of L and Z than other tissues.

Although L has received much attention recently due to its antioxidant activity, available information on its pharmacokinetic properties is limited. Further, characterization of its pharmacokinetic properties is warranted to increase our understanding on the clinical importance of L. Itagaki et al. (2006) investigated the pharmacokinetics and disposition of L through pre-cutaneous and pulmonary routes. They suggested that the antioxidant activity of L is dependent on its tissue concentration. Although the concentration of L is lower in the intestine than in the liver, spleen and lung, L has a protective effect on ischemia-reperfusion injury in the small intestine. Since the spleen acts as a reservoir of erythrocytes, results of intra venous injection study have shown the preferential distribution of L in the erythrocytes rather than plasma. Although

L has high lipid solubility, its distribution in the brain and fat tissue was reported to be lower.

Effect of dietary and non-dietary factors on lutein and zeaxanthin bioavailability. Factors that affect carotenoid absorption are shown in **Figure 1.7** (Van het Hof et al. 2000). As mentioned earlier, L is an important molecule since humans do not synthesize it. L and Z concentration in human plasma and tissue depends on their dietary intake and bioavailability. It has been assumed that the highly lipophilic micronutrients such as fat-soluble vitamins, carotenoids, phytosterols and other phytochemicals have the same metabolic process in the human's upper gastrointestinal tract and they follow the same pathway as lipids (Tyssandier et al. 2003). In general, of the ingested dietary carotenoids, only a fraction is available for normal physiological functions. Bioavailability of carotenoids in general, is influenced by various dietary factors namely fat, food matrix, dietary fiber, interaction of hydrocarbon carotenoids and other micronutrients. The mechanism of intestinal absorption of carotenoids from emulsion lipid droplets to mixed micelles is governed by the carotenoid type, their hydrophobicity, pH of emulsion and bile lipid concentration (Borel et al. 1996; Tyssandier et al. 2001).

Garrett et al. (1999) studied the bioavailability of dietary β-carotene, L, lycopene and their aqueous fraction of digesta and determined the quantity of carotenoids transferred from the food to micellar fractions. They found that micellerization of L was higher than that of β-carotene and lycopene. Although, both L and β-carotene share a common lipophilic characteristic, their structural variation affects the process of absorption (Borel et al. 1996; Gartner et al. 1996). In the three main stages of carotenoid absorption (release from the food matrix, incorporation into mixed micelles and intestinal uptake by mucosa), the higher polarity of L seems to have advantage in the mass transfer processes. The main reason is that polar carotenoids are distributed at the surface of emulsions, whereas carotenes are located in the hydrophobic core. Thus, the former can transfer directly from emulsions to mixed micelles. Van het Hof et al. (1999) and Erdman et al. (1999) reported that the bioavailability of β-carotene and L vary substantially among vegetables and found the relative bioavailability of L from vegetables is higher than that of β-carotene. Riso et al. (2003) found that the ingestion of spinach or broccoli increased the levels of L in serum. Van den Berg and Vliet (1998)

observed that L negatively affected the β-carotene absorption when given simultaneously, but lycopene had no effect. The bioavailability of L from food sources was reported to be different and it is dependent on its nature (whole food or supplement), state of the food (raw, cooked or processed), and extent of destruction of the cellular matrix via mastication and digestive enzymes. Food processing is the most important facilitator of carotenoid bioavailability. Food processing (e.g. cooking in the presence of oil) has been reported to affect the availability of carotenoids for absorption. Delia et al. (2004) reported that different cooking processes lowered the L content dramatically, which decreased its bioavailability. L absorption from a green vegetable varies with different degrees of processing. The absorption of L was 37.5% lower from chopped spinach than from whole-leaf spinach. Increased L absorption correlated to slower gastrointestinal transit for the whole-leaf meal (Faulks et al. 2004). Enzymatic disruption of the spinach cell wall structure enhanced the plasma β-carotene level but the level of L was not affected *in vitro* (Castenmiller et al. 1999). Mechanical homogenization or heat treatment resulted in higher plasma response for L from chopped versus whole-leaf spinach (van het Hof et al. 1999). Heat treatment of carotenoid-containing foods converts some of the dominant *all-trans* carotenoids to a *cis*-form. Although *cis*-isomers are apparently better incorporated into the mixed micelles than the *all-trans* form, there is no evidence of higher absorption of *cis*-isomers in laboratory mammals (Levin et al. 1994). Apart from dietary lipids, other dietary factors such as vitamins, dietary fiber and extent of food processing are also reported to affect the L absorption. Tanumihardjo et al. (2005) reported that L is absorbed faster with simultaneous supplementation of vitamin C than E.

Dietary fat and lutein bioavailability. As mentioned elsewhere, a possible reason for normal absorption of carotenoids with 'very low-fat' diets is the presence of endogenous lipid and cholesterol from biliary sources (Furr and Clark, 1997). Among various dietary factors, fat is an important factor facilitate the solubilization and transfer of L from food matrix to tissues. In particular, fatty acids influence the formation of L rich mixed micelles and also influence its physiochemical properties. The fatty acid composition of emulsion droplets and their structure may influence the lipolysis and enhance the bioaccessibility of L at enterocyte level (Tyssandier et al. 2001).

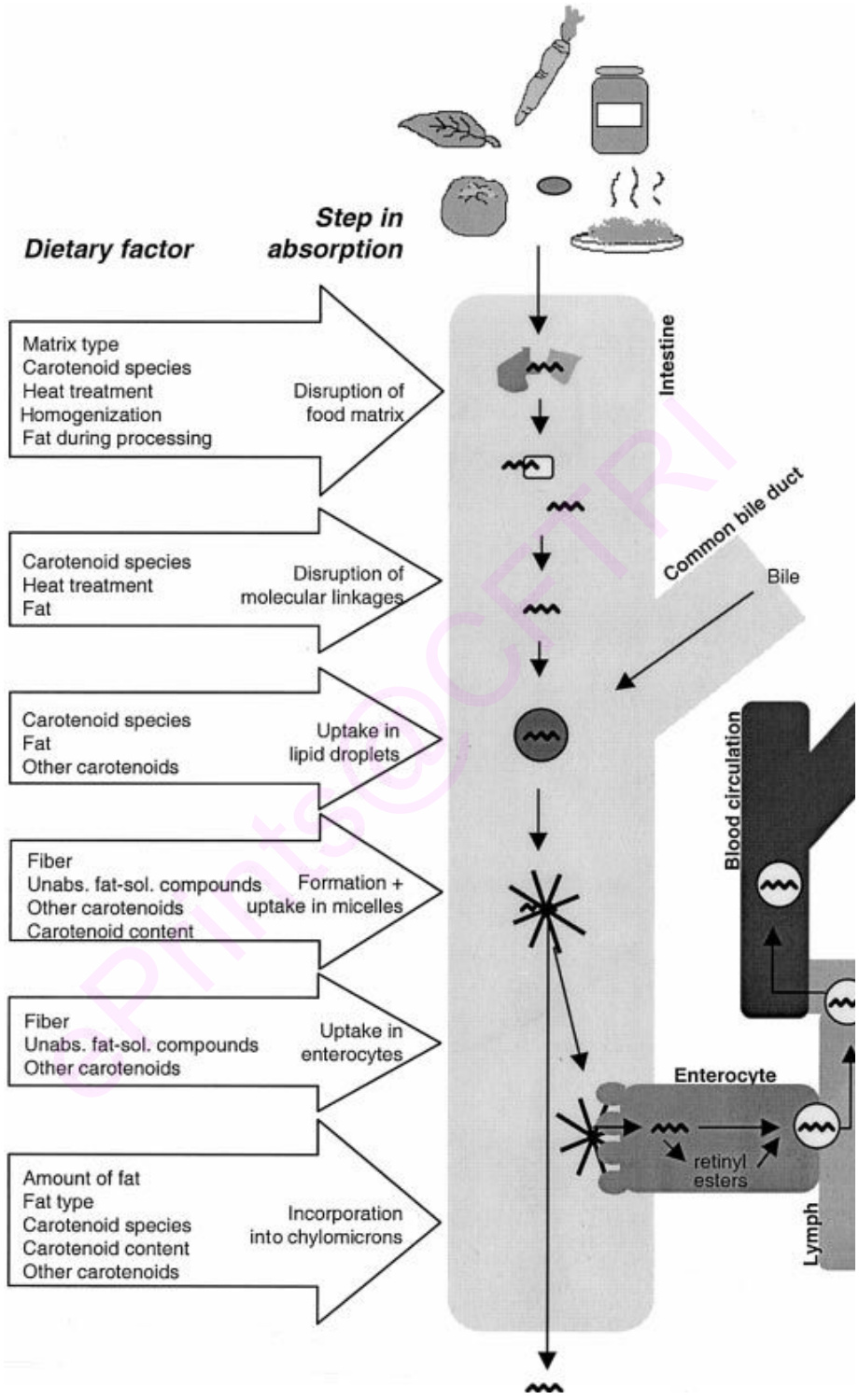


Figure 1.7. Steps of carotenoid absorption and dietary factors that affect carotenoid absorption (Van het Hof et al. 2000).

Sugawara et al. (2001), Raju et al. (2006) and Lakshminarayana et al. (2006 a and b) have reported that specific phospholipids and fatty acids in the mixed micelles positively influence the intestinal absorption of L in human intestinal Caco-2 cells and rats, respectively. The β-carotene response in the triacylglycerol-rich lipoprotein fraction, containing the chylomicrons and chylomicron remnants, was found to be lower after a meal containing sunflower oil as compared to the same dose given with beef tallow (Hu et al. 2000).

Dietary L and Z are esterified with fatty acids either before or after absorption. Esterification decreases their polarity in comparison with that of the corresponding free compounds **(Figure 1.8)**. Thus, the distribution of xanthophyll esters in lipid emulsions normally concentrated in the core, not at the surface, which impairs their diffusion into micelles or binding with lipoproteins, increasing the requirements for bile salts and intestinal enzymes to digest the triacylglycerol bulk (Hollander et al. 1978). Moreover, as no carotene esters have been detected in plasma and peripheral tissues, they must be hydrolyzed, implying a new stage before absorption. Although, Khachik et al. (1992) reported that the xanthophyll esters would be hydrolyzed in the gut before absorption. Experimental data on the absorption of esters in humans were reported by Wingerath et al. (1995). Herbst et al. (1997) and Wingerath et al. (1995) found higher level of esterified L in plasma. Thus, they assumed that the esters were equally absorbed like free L and suggested that hydrolysis of esters is essential. This could be rectified with an adequate intake of dietary fat that has been established as a factor affects the intestinal absorption of carotenoids.

Fat provides an appropriate lipophilic environment and stimulates bile secretions from the gallbladder. The type of fatty acid seems to exert some effect on carotene absorption (Borel et al. 1998). Roodenberg et al. (2000) studied in detail on the role of fat in carotene absorption. The authors compared the absorption of L esters with a low fat (3 g) and high-fat (36 g) spreads. The response was significantly higher in case of high-fat spread, with a 207% increase in L levels than the low fat spread, but the increase with respect to the control subjects was lower (88%). A subsequent study directly compared the absorption of free L with that of esterified L, supplying 12 to 23 g of dietary fat. Contrarily, Bowen et al. (2002) have shown that bioavailability of the L ester supplement was higher (62%) than that of free L. A recent study by Breithaupt et

al. (2002) reported xanthophyll esters as substrates for typical lipases from human pancreas. Chitchumroonchokchai and Failla (2005) showed that dietary Z esters are hydrolyzed to free Z in the small intestine by carboxyl ester lipase and intestinal epithelial cells preferentially take up the free Z. After uptake, free and esterified Z is relatively stable. The above studies suggest that dietary Z esters are hydrolyzed during intestinal phase of digestion and absorption. This selective absorption could be established in some pathway of enzymatic hydrolysis or based on physicochemical properties of the interface of mixed micelles.

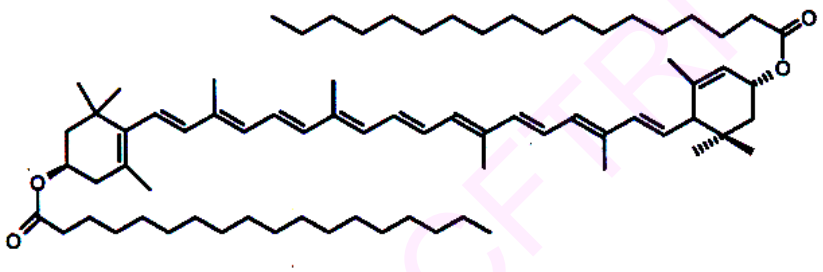


Figure 1.8. Molecular structure of lutein diesters.

Interaction of carotenoids. The interaction between carotenoids is likely to occur at various stages of the absorption process i.e., during micellar incorporation, transport to different organs or within tissues after absorption. Competition between carotenoids for micellar incorporation in the gastro-intestinal tract has been suggested as a possible explanation especially at higher dose. Van den Berg (1999) and van Vliet (1998) studied the interaction between β-carotene and L or lycopene in men. The comparative data showed that between the carotenoid and retinyl palmitate response in triglycerol rich lipoprotein fraction of men given a single dose of 15 mg of β-carotene with that of 15 mg β-carotene plus 15 mg lycopene or 15 mg L. Data on the intestinal absorption of carotenoids other than β-carotene are limited. It seems that more polar carotenoids especially L and Z are absorbed better than carotenes. To support the hypothesis, it was reported that the plasma response for L was twice as high as it was for β-carotene when single doses of those carotenoids were given in oil (Kostic et al. 1995). Both the L and Z were increased in chylomicron compared to that of β-carotene after ingestion of a carotenoid mixture (Gartner et al. 1996). In addition, the relative bioavailability of L from

vegetables was reported to be five times higher than that of β-carotene (Van het Hof et al. 1999), but in the same study the plasma response of L was substantially lower than that of β-carotene after simultaneous ingestion of pure L and β-carotene dissolved in oil.

Dietary fiber and carotenoids bioavailability. Dietary fiber may interfere with the absorption of carotenoids by interfering with micelle formation and its viscosity, volume of intestinal contents and depth of the unstirred water layer on the microvillus surface (Riedl et al. 1999). Pasquier et al. (1996) have reported that soluble dietary fibers could alter the process of intra-gastric lipid emulsification and possibly subsequent triacylglycerol lipolysis. It has been reported that the concentration of viscous fibers increased the size of the emulsified droplets. The droplet size and its surface area were strongly correlated with the medium viscosity. Correlation between the enzymatic release of carotenoids and content of lignin, non-starch polysaccharides was reported by Serrano et al. (2005). Rock et al. (1999) reported that dietary pectin had a negative effect on plasma β-carotene response after a single dose of purified β-carotene administered with a meal. Viscous polysaccharides, such as pectin reported to delay gastric emptying and interfere with micelle formation. Gastric emptying is delayed in human subjects by the addition of 15 g pectin to a meal (Di-Lorenzo et al. 1988).

Assessment of lutein metabolism by isotopes

Recent approaches using stable isotopes, coupled with mass spectral analysis of the carotenoid and its newly synthesized metabolites isolated from the postprandial triglyceride-rich lipoprotein plasma fraction are the most promising methods in terms of accurate measurement of carotenoid absorption (Novotny et al. 1995; Tang et al. 2000; Lin et al. 2000; Yao, et al.2000). Although such methods have a great promise in assessing carotenoid bioavailability and bioefficacy from different food sources in humans (Van Lieshout et al. 2003), they do not provide mechanistic information about the carotenoid absorption process itself.

Recent reports demonstrate that a normal adult woman fed with ^{14}C-lutein (125 nmol, 36 nCi 14C), the ^{14}C first appeared in plasma 1 h after dosing and reached its highest level. L had an elimination half-life ($t_{1/2}$) of approximately 10 d. After feeding, about 45% and 10% of the ^{14}C was eliminated in feces and urine respectively in the first

2 d after dosing (De moura et al. 2005). Lienau et al. (2003) determined relative bioavailability of L from food in humans by stable isotope method. Subjects were administered a single dose of deuterium-labeled carotenoids from intrinsically labeled spinach or collard green. The serum level of labeled L after various time points enabled to calculate its enrichment. Area under the curve analyses of four different subjects yielded serum L responses of 128, 145, 149, and 262 μg-day/mg dietary L, following an acute dose of spinach containing 15.4, 18.8, 18.8 and 9.8 mg labeled L, respectively. These techniques show the efficacy of L bioavailability from different foods of diverse carotenoid composition and/or following various food preparation procedures.

Proposed future line of research

Since, L is a very important bioactive component, their importance in visual function and understanding the mechanism of its metabolism against photo- or other oxidative stress has to be studied in detail. Further, its protective mechanisms to other health benefits like cardiovascular diseases and cancer has to be confirmed with *in vivo* experiments. The dietary components and their influences on L bioavailability and its disposition at ultra level by using appropriate methods are yet to be developed. Biological functions of L metabolites and their structural characterization are yet to be confirmed. Further, to date, there have been few studies that examined the effect of L esterification, but this subject is received increasing attention. The enzymes involved in the hydrolysis of L esters are not known, and the suitability of esters as substrate for common lipolytic enzymes is under investigation.

Aims and scope of the study

Fruits and vegetables are rich in bioactive molecules such as carotenoids, vitamins, minerals etc. Carotenoids are thought to contribute to the inverse relationship between fruit and vegetable consumption and the risk of coronary heart disease, vitamin-A deficiency, cataract, age-related macular degeneration (AMD) and some types of cancer. Among carotenoids, xanthophylls - lutein and zeaxanthin are documented as frontline defense molecules against pathological disorders of the eyes namely cataract and AMD. AMD is a progressive disease of the eye occurring in people over 55 years old due to the aging process. Evidence estimates that by 2050, 20% of people of 55 years and above are likely to suffer from cataract and AMD, which necessitate implementation of effective treatment measures. There are two types of AMD, “wet" and "dry" and they differ in several important respects, including their treatment options. Photodynamic therapy uses different kinds of light-activated drugs to treat wet AMD while laser surgery used to treat dry AMD. These treatments are not affordable for rural population due to expensiveness. The former one can have serious side effects. Hence, we still need to do much more research into the cause and progression of AMD, as well as develop better treatments methods. As the aging population grows, the need will become more desperate. The alternative and inexpensive treatment for AMD is dietary modification/supplementation with natural antioxidants (carotenoids) that help minimize exogenous (UV radiation and pro-oxidants) and endogenous (lipid peroxidation and auto-oxidation) free radical damage and maintain antioxidant – prooxidant ratio in the macula.

Increased consumption of carotenoid rich agri/horticultural produce reported to reverse AMD. Lutein and zeaxanthin that primarily found in leafy greens is among the best risk reducing carotenoids for AMD. These molecules can inhibit light irradiation induced oxidative reactions in the eyes. The most plausible mechanism for the health effects of lutein and zeaxanthin is due to their antioxidant and anticarcinogenic properties. As mentioned elsewhere, because of the increasing size of the elderly population in the world, status of AMD continues to grow. However, there have not been many studies looking at the epidemiology of AMD in India. Randomized controlled trials looking at the utility of antioxidant supplements in the Indian sub-continent is highly

warranted. In such a scenario, efforts for elimination of blindness have pledged support for strategies to reduce the burden of AMD/cataract blindness by the Vision 2020. In India, treatments like cataract operation and dietary supplementation of lutein rich foods helped to reduce the prevalence of blindness among people aged above 50 years from 9.8% to 5%. This figure could be brought down further if dietary approach is implemented seriously. Because of the health benefits associated with lutein and due to its poor bioavailability, dietary factors influencing/affecting its bioavailability, metabolism and biological functions *in vivo* are of current interest.

The absorption of dietary carotenoids involve several steps starting with the mechanical and enzymatic disruption of the food matrix, release of the carotenoids, followed by their incorporation into lipid droplets of the gastric emulsions. The carotenoids are then transferred from the lipid droplets to mixed micelles produced by the action of bile salts, biliary phospholipids, dietary lipids, and their hydrolysis products. After the solubilization in mixed micelles, carotenoids are absorbed by the intestinal cells, packed into chylomicrons and secreted to the lymphatic system. The processes up to solubilization in mixed micelles are dependant mostly on the physico-chemical properties of food and carotenoids and on the micelles formation from bile and lipid hydrolysates. Each step of the carotenoid absorption may be influenced by multiple factors, thus making difficult the task of assessing the effects of each factor on the overall carotenoid bioavailability. Dietary components like lipids and their metabolic products, fatty acids are reported to positively influence the intestinal uptake of polar carotenoids. But the role of these dietary factors on the physico-chemical properties of micelles formed in the intestine, activity of enzymes and lipid profiles, which regulates and transports the absorbed carotenoids and on the formation of metabolic/oxidative components of lutein and their physiological functions are lacking from detailed study.

Hence, it is proposed to investigate the possible role of dietary components (phospholipids and fatty acids) on the bioavailability and bioactivity of polar carotenoid lutein in rats. The present study attempts to explore dietary approach to enhance bioavailability of macular pigment - lutein, nature of its biotransformation (metabolism/oxidation) and bioactivity (biological functions/antioxidant property) with reference to slowing the lutein deficiency in rats. In brief, from the proposed study the following aspects shall be achieved:

- Protocols will be developed for finding suitable dietary modulators to enhance lutein bioavailability in the eyes of rats induced with macular pigment deficiency.
- Possible biodegradation of lutein and characterization of its metabolites will be stabilized.

The results of present investigation will help disseminate the research information to common people about the importance on lutein rich agri/horticultural produce, suitable dietary modulators that can enhance the bioavailability of dietary lutein, possible metabolism of lutein *in vivo* and their health benefits with reference to lutein deficiency.

Chapter 2

Materials and methods

Chemicals

Standard *all-trans*-β-carotene (98%), α-carotene, lutein (99%), DL-α-tocopherol and chlorophylls a and b, monooleoylglycerol, sodium taurocholate, linoleic acid, eicosapentaenoic acid, oleic acid (~99%), β-apo-8'-carotenal, egg-yolk phosphatidylcholine (99%) and lysophosphatidylcholine (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Neoxanthin (95%), violaxanthin (98%) and zeaxanthin (98%) were gifted by Dr. A. Nagao (National Food Research Institute, Tsukuba, Japan). Boron trifluoride in methanol, heparin, thiobarbituric acid, t-butyl hydroperoxide tetramethoxy propane, xanthine oxidase, glutathione (reduced and oxidized), trichloroacetic acid, 5,5'-dithiobis(2-nitrobenzoic acid), glutathione reductase, hydrogen peroxide, NADPH, cytochrome C, xanthine, EDTA, ammonium acetate, acetic acid, ferric chloride, acetyl acetone and HPLC grade acetonitrile, hexane, methanol, ethyl acetate and dichloromethane were purchased from Sisco Research Laboratories (Mumbai, India). Neutral alumina (particle size: 70-230 mesh) was purchased from HiMedia Chemical Laboratories (Mumbai, India). Fatty acid standards were obtained from Nu Chek Prep. (Elysian, MN, USA). Other chemicals and solvents were of reagent grade purchased from E-Merck Co, Ltd. (Mumbai, India).

Materials

Casein, cellulose, sucrose, methionine, food grade vitamins, minerals and soy-lecithin were purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India). Choline chloride was obtained from Loba Chemie Pvt. Ltd. (Mumbai, India). Refined olive, sunflower, and groundnut oils were obtained from a local super market.

Samples

Green leafy vegetables (GLVs) were obtained fresh either from local horticultural farms (familiar, n=16) or collected from agricultural fields (less familiar, n=19). Freshly harvested green leafy vegetables used in this study were collected on the day of analysis during morning (24-26 ^{o}C) and immediately brought to the laboratory in an

icebox. To obtain a homogeneous sample, a known amount (50 g) of each leafy vegetable was washed with deionized water, drained and ground for 5 minutes in a blender and processed immediately. Fruits (n=8) and vegetables (n=10) were purchased from local market. Each vegetable and fruit was collected from three different markets in the city and was used for the analysis of carotenoids to avoid duplication of sample collection and to obtain data on carotenoids in samples from different locations. The botanical, family and common names with health benefits of GLVs, fruits and vegetables used in this study are listed in **Tables 2.1 and 2.2.** The leafy portions were used for the extraction of carotenoids. Photograph of some of the GLVs used in the present study is shown in **Figure 2.1**

Methods

GLVs, fruits and vegetables were screened for their L and other carotenoids composition by HPLC. Carotenoids were purified by column chromatographic technique. Animal experiments were performed to study the bioavailability of purified and dietary L by employing gavages and dietary studies using L sufficient and its deficient rats. Influence of various dietary factors (phospholipids and fatty acids) on the intestinal absorption and metabolism of L was investigated by employing *in vitro* and *in vivo* techniques. Further, influence of dietary factors on the role of L on certain biochemical parameters, lipid peroxidation and antioxidant molecules in plasma and tissues were investigated by employing standard biochemical procedures. Detailed methodologies employed in this study are given below.

Extraction of carotenoids

Carotenoids were extracted from GLVs, fruits and vegetables according to the procedure described by Lakshminarayana et al. (2005). Fresh GLVs (50 g each) or vegetables (50 g each) or fruits (100 g each) were ground well separately in a blender along with sodium sulphate (5 g) and 2 mM of methanolic α-tocopherol (100 μL/g GLVs). Total carotenoids were extracted from the ground leafy vegetables separately using ice-cold acetone and the extraction was repeated until the samples became colourless (total volume, 400 mL).

Figure 2.1. Green leafy vegetables screened for carotenoid composition.

The crude extract (50 mL) was mixed and shaken well with 100 mL of hexane and the upper hexane layer was separated using a separating funnel. The extraction was repeated thrice with hexane (total volume: 250 mL). The pooled hexane extract was dried using anhydrous sodium sulphate (20 g), filtered through Whatmann No.1 filter paper. The filtrate was evaporated to dryness in a rotary evaporator (Buchi, Switzerland) at 30-32^{o} C and re-dissolved in a known volume of hexane. An aliquot (100 μL) of extract was dried under a stream of nitrogen and the residue was redissolved in 1 mL of acetonitrile: methanol: dichloromethane (60:20:20 v/v/v). Samples were analyzed by HPLC to characterize and quantify carotenoids. Extraction of total carotenoids from vegetables and fruits was carried out according to the procedure as described above for GLVs. Handling, homogenization and extraction procedures were carried out on ice or at 4^{o}C, under dim yellow light to minimize photoisomerization and oxidation of carotenoids.

Saponification

In order to quantify xanthophyll carotenoids of our interest (L and Z), crude acetone extract of each sample was subjected to saponification procedure as per Khachik et al. (1986). In brief, one fourth of methanolic KOH (30%) was added to the acetone extract and incubated at room temperature (27^{o}C) for 3 hrs under dark. After incubation, carotenoids were extracted thrice with 50 mL of hexane or until lower phase becomes colour less. To remove the potassium salt soap from the samples, they were washed with deionized water at least three to five times. The hexane extracts of samples were pooled, mixed and then evaporated to dryness by rotary evaporator (Buchi, Switzerland). The residue was re-dissolved separately in a known volume of hexane and applied on to an activated neutral alumina column for purification of L and Z. The purified L was used for *in vitro* and *in vivo* studies. In case of fruits and vegetables all the samples were kept for saponification as per the procedure described above to remove the esters and then analyzed by HPLC.

Column chromatography

L/Z were separated by open column chromatography (OCC, 20 cm x 1.5 cm) on neutral alumina (particle size: 70-230 mesh, SRL, Mumbai) using specific solvent

systems as shown in **Figure 2.2,** by modifying the procedures previously described by Rodriguez-Amaya (1999) and Kimura and Rodriguez-Amaya (2002).

β-Carotene was eluted with hexane, L and Z fraction with methanol: dichloromethane (1:1 v/v), and the fraction rich in violaxanthin and neoxanthin were eluted with ethyl acetate: hexane (5:5 v/v) and ethyl acetate: hexane (1:9 v/v), respectively. The purity of individual eluant was checked by HPLC against respective reference standards. The peak identity, their respective spectra, absorption maxima (λ_{max}) and concentrations of L and other carotenoids were confirmed by HPLC and LC-MS.

HPLC analysis of carotenoids form GLVs, fruits and vegetables

Carotenoids (neoxanthin, violaxanthin, L, Z, β-carotene and α-carotene) were separated on a SGE C-18 (ODS) column, 25 cm x 4.6 mm id, 5 μm, 120A0 (SGE Co., India) according to the procedure described by Lakshminarayana et al (2005). Acetonitrile: methanol: dichloromethane (60:20:20, v/v/v) containing 0.1% ammonium acetate was used as a mobile phase for the separation of carotenoids. The volume of sample injected for HPLC analysis was 20 μL. An isocratic condition was maintained at a flow rate of 1 mL/min. All the carotenoids were monitored at 450 nm with UV-visible detector (Shimadzu, Japan). The peak identities and λ_{max} of carotenoids were confirmed by their retention time and characteristic spectra of standard chromatograms, recorded with a Shimadzu model series equipped with SPD-10AVP detector. They were quantified from their peak areas in relation to the respective reference standards. In case of quantification, the hydrocarbon carotenoids (β-carotene and α-carotene) and xanthophylls other than L and Z are not included here since the objective of the study was to quantify only the L and Z levels in the selected agri/horticultural produce.

Table 2.1. Green leafy vegetables screened for carotenoid composition.

Botanical name	Family	Common name	Medical Application*
Allmania nodiflora (L.) R.Br.	Amaranthaceae	Celosia	Used in snake bite
Alternanthera pungens Kunth	Amaranthaceae	Khaki weed	Diuretic
Alternanthera sessilis (L.) Dc.	Amaranthaceae	Joy weed	Hepatoprotective
Amaranthus gangeticus L.	Amaranthaceae	Amaranth	Antioxidant
Amaranthus tristis L.	Amaranthaceae	Arai keerai	Diuretic
Amaranthus viridis L.	Amaranthaceae	Slender amaranth	Antioxidant
Basella alba L.	Bassellaceae	Indian spinach	Antimutagenic[1]
Basella rubra L.	Bassellaceae	Red spinach	Laxative, Astringent
Beta vulgaris L.	Chenopodiaceae	Beat greens	Laxative & diuretic
Boerhavia diffusa L.	Nyctaginaceae	Hog weed	Anti diabetic[2]
Brassica oleracea var.botrytis L.	Brassicaceae	Broccoli	Anticancer [3]
Chenopodium album L.	Chenopodiaceae	Lamb's Quarters	Anti helminthic
Commelina benghalensis L.	Commelinaceae	Jio	Antileprocy
Coriandrum sativum L.	Apiaceae	Coriander leaves	Anti bacterial[4]
Cucurbita maxima Duch.	Cucurbitaceae	Winter squash	Anti helminthic
Daucus carota L.	Apiaceae	Carrot greens	Purgative
Gynandropsis pentaphylla L.	Capparidaceae	Spider wisp	Anti helminthic

…Contd.

Hibiscus cannabinus L.	Malvaceae	Kenaf	Aphrodisiac
Hydrocotyle asiatica L.	Apiaceae	Indian pennywort	Anti ulcer[5]
Lactuca sativa L.	Asteraceae	Indian lettuce	Anti oxidant
Mentha spicata L.	Lamiaceae	Spear mint	Hysteria
Moringa oleifera Lam.	Moringaceae	Drumstick	Diuretic, Antiulcer
Murraya koenigii L.	Rutaceae	Curry leaf tree	Renal pain
Peucedanum sowa Roxb.	Apiaceae	Indian dill	Carminative, Diuretic
Phyllanthus niruri L.	Euphorbiaceae	Chanca piedre	Jaundice
Piper betle L.	Piperaceae	Betel leaf	Wound healing
Portulaca oleracea L.	Portulacaceae	Purslane	Anti scorbutic
Raphanus sativus L.	Brassicaceae	Radish	Antihelminthic, Antibacterial
Rumex acetosella L.	Polygonaceae	Sheep sorrel	Renal problems
Sesbania grandiflora (L.) Poir.	Fabaceae	Agathi	Antitumor, Laxative
Solanum nigrum L.	Solanaceae	Black night shade	Cirrhosis of liver
Spinacia oleracea L.	Chenopodiaceae	Spinach	Carminative, Febrifuge
Talinum cuniefolium Willd.	Portulacaceae	Ceylon spinach	Aphrodisiac
Trianthema portulacastrum L.	Aizoaceae	Desert horse purslane	Anti-rheumatic, Diuretic
Tribulus terrestris L.	Zygophyllaceae	Puncture vine	Stomachic
Trigonella foenum-graecum L.	Fabaceae	Fenugreek	Laxative, Hypoglycemic

*Useful plants of India (CSIR, 1986), otherwise mentioned. [1]Yen et al. (2001); [2]Pari and Satheesh (2004); [3]Reddy et al. (1999); [4]Kubo et al. (2004); [5]Cheng et al. (2004).

Table 2.2. Vegetables and fruits screened for carotenoid composition

Sl.No	Botanical name	Family	Common name	Medical Application** (Nutritional value)
	Vegetables			
1	*Coccinia cordifolia* L. (Mill)	Cucurbitaceae	Koval	Diabetes
2	*Capsicum annuum var.* Grossa L.	Solanaceae	Giant chilli	Antioxidants
3	*Phaseolus coccineus* L.	Papilionaceae	Beans	Vitamin c
4	*Momordica charantia* L.	Cucurbitaceae	Bitter guard	Malaria and diabetic
5	*Cymopsis tetragonoloba* L.	Papilionaceae	Cluster beans	Colon cancer
6	*Luffa acutanguta* (L.) Roxb.	Cucurbitaceae	Ridge guard	-
7	*Abelmoschus esculentus* (l.) Moench.	Malvaceae	Lady's finger	Diuretic
8	*Cucurbita maxima* Duch.	Cucurbitaceae	Pumpkin	Night blindness
9	*Pisum sativum* L.	Papilionaceae	Peas	Culinary use
10	*Capsicum annuum* L.	Solanaceae	Green chilli	Antioxidants
	Fruits			
1	*Malus sylvestris* L. (Mill)	Rutaceae	Apple	Platelet aggregation
2	*Vitis vinifera* L.	Rhamnales	Grape	Kidney and liver diseases
3	*Psidium guajava* L.	Myrtaceae	Guava	Laxative
4	*Citrus auranatium* L.	Rutaceae	Orange	Scurvey, antioxidant
5	*Mangifera indica* L.	Anacardaceae	Mango	Antioxidants
6	*Citrus aurantifolia* L. (Swingle)	Rutaceae	Lime	Carminative
7	*Carica papaya* L.	Caricaceae	Papaya	Laxative, Digestive, Diuretic
8	*Ananus comosus* L. (Merr.)	Bromeliaceae	Pineapple	Anthelmintic, Diaphoretic

**Encyclopedia: Fruits, Vegetables and Nutrition

Liquid chromatography-Mass spectrometry (LC-MS) for L and Z isolated from GLVs

LC-MS for L and Z was performed according to the procedure of Breithaupt et al. (2002). LC-MS was performed on a Waters 2996 modular HPLC system [auto sampler, gradient pump, thermo regulator, and diode array detector (DAD)] coupled to a Q-TOF Ultima (UK) mass spectrometer. The atmospheric pressure chemical ionization (APCI) source was heated at 130 °C, and the probe was kept at 500 °C. The corona voltage was optimized to 5 kV, the HV lens to 0.5 kV, and the cone voltage to 30 V. Nitrogen was used as a sheath and the flow rate of drying gas at 100 and 300 L/h, respectively.

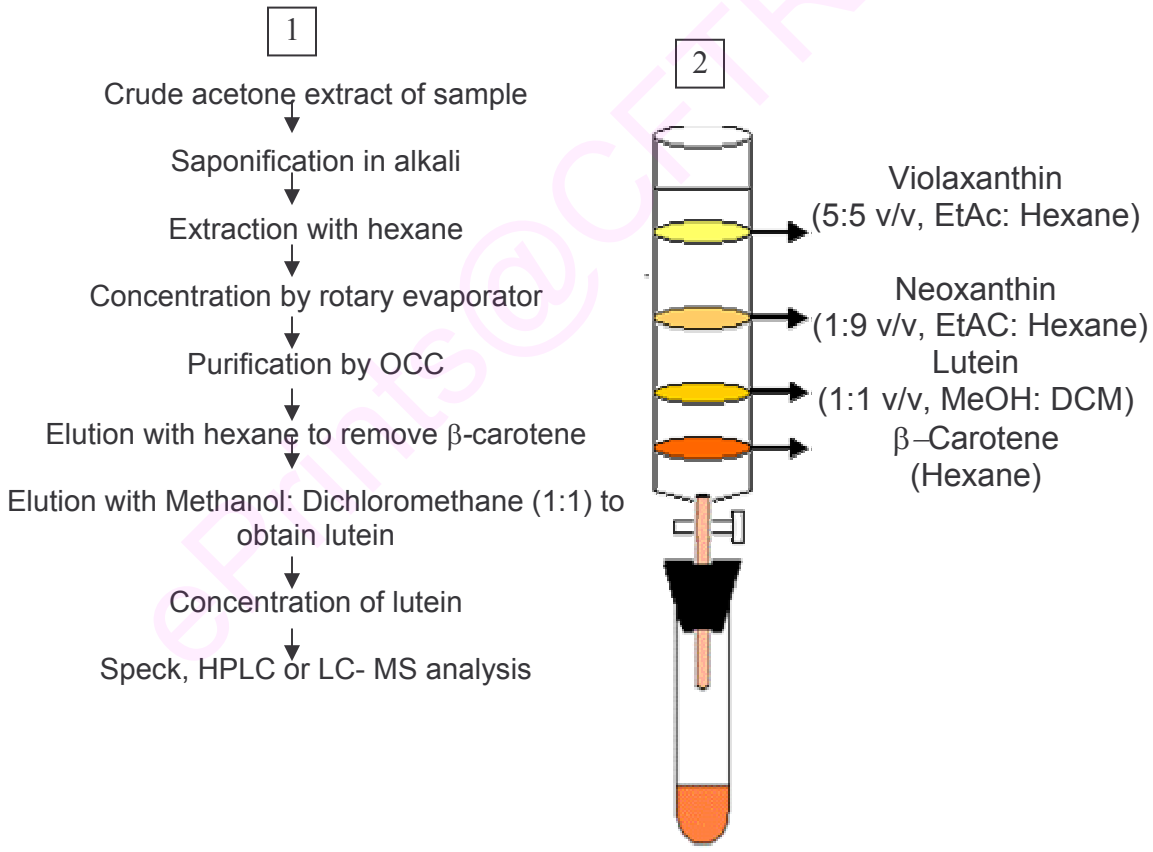


Figure 2.2. Flow-chat showing the step-wise solvent extraction, saponification and purification of carotenoids (1) and schematic representation of open column chromatographic technique adopted for purification of L/Z from GLVs (2).

The spectrometer was calibrated in the positive ion mode (scan range *m*/*z* 80-1200), and the resulting (M + H) + signals as well as the $(M + NH_4)^+$ ions were evaluated. Mass spectra of L and Z were acquired with an *m*/*z* 200-1200 scan range, and the UV absorption was recorded at 450 nm by using a DAD. The MS identities of L and Z in samples were confirmed using respective reference standards. Data were processed with Mass Lynx 3.2 software. For HPLC separation of L and Z, the conditions were as mentioned elsewhere except the mobile phase with no added 0.1% ammonium acetate.

Animals and diet

All animal experiments were conducted after due clearance from the Animal Ethics Committee, Central Food Technological Research Institute, Mysore. Weanling male albino rats of Wistar strain [OUTB- Wistar, IND-cft (2c)] weighing 42 ± 2 g were housed in individual steel cages at room temperature (28 ± 2^{o}C) with 12 hour dark: light cycles in the Institute's animal house facility. Rats received fresh diet daily and were given free access to water, *ad lib*. In case of single and repeated dose studies, rats were fed with a fresh pellet diet (Amrut feeds, Sangli. India). The L level in the casein and pellet diet was determined by HPLC and it was found to be very low (<0.012 μg/kg diet).

Lutein sufficient and deficient diet

For dietary feeding trials, diet with sufficient level of L was prepared by adding *Trigonella foenum-graecum* (**Figure 2.3**) powdered leaves (4.21 g/kg diet). The amount of L and Z together was 2.73-mg/kg diet. The leaves were washed with deionized water, dried under shade, and powdered in a mixer (Mitaso, India). All the ingredients of the diet were similar to the deficient diet except L. Four different types of diets with added L source were prepared. The diet contained 10% fat derived from either olive oil (oleic acid source) or sunflower oil (linoleic acid source) or groundnut oil (control). A separate diet with L source was prepared with soy lecithin (phosphatidylcholine source). L and Z content in the acetone extract of the leaf powder was analyzed by HPLC (Lakshminarayana et al. 2005). Rat diet was prepared according to AIN-76 diet (1976). The fat and protein content of diets were kept isocaloric. The composition of diet with L or devoid of it is given in **Table 2.3**.

Table 2.3. Composition of L sufficient and its deficient diet used for feeding experiment in rats.

Components	Lutein sufficient	Lutein deficient
	g/Kg diet	
Casein	200	200
DL-Methionine	3	3
Sucrose	595.79	600
Cellulose fiber	50	50
Fat	100	100
Vitamin mix	10	10
Mineral mix	35	35
Choline bitartarate	2	2
Leaf powder*	4.21	0

*Dry fenugreek leaf powder was used as a source of L (2.69 mg/Kg diet) and zeaxanthin (0.04 mg/Kg diet).

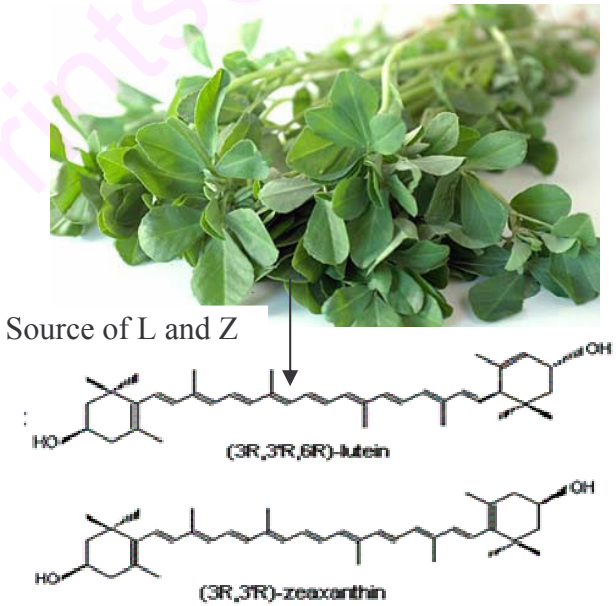


Figure 2.3. The green leafy vegetable (*T. foenum-graecum)* used for animal feeding studies as a source of L and Z.

Gavage studies

Two sets of experiments were conducted. The first set with a single dose and the second set with repeated dose. During the acclimatizing period, rats were fed daily with fresh pellet diet and had free access to tap water. The leftover diets were weighed and discarded. After 7 days of acclimatization, rats were deprived of food for 12-13h before administering L solubilized in mixed micelles (single dose study), otherwise received diet throughout the experimental period (repeated dose study). Diet samples were processed for the analysis of L before feeding to ascertain its level.

Preparation of mixed micelles

Mixed micelles in phosphate buffered saline (200 μL) containing monooleoyl glycerol (2.5 mM), oleic acid (7.5 mM), sodium taurocholate (12 mM), L or β-carotene (200 μM) or L and β-carotene (200 μM) either with 3 mM phospholipids (phosphatidylcholine or lyso-phosphatidylcholine) or with 7.5 mM fatty acids (oleic acid or linoleic acid) were prepared separately. Appropriate amount of these chemicals were dissolved in methanol or dichloromethane and mixed to reach the final concentration (Baskaran et al. 2003). The solvent was evaporated to dryness using nitrogen and the mixture was suspended in phosphate buffered saline (pH, 7.0) with vigorous mixing using a vortex mixer to obtain an optically clear solution. The micelle composition chosen was based on the composition of the clear layer obtained by ultracentrifugation of the duodenal content of healthy adult human subjects given a triglyceride-rich meal (Hernell et al.1990). It would hypothetically produce a mixture of mixed micelles and small unilamellar vesicles (Staggers et al.1990). The vesicles can regroup spontaneously into the mixed micelles as the ratio of lipid to cholic acid decreases during absorption. Thus, the optically clear solution obtained by this procedure was used as the mixed micelles in the present study. The concentration of L in the mixed micelles was determined by HPLC before being fed to the rats.

Intubations and sample collection

Single dose study

For single dose study, three sets of experiments were preformed. For the first set, group of rats (n=30/group) were intubated single dose of L solubilized in mixed micelles containing either with phosphatidylcholine (PC) or lyso-phosphatidylcholine (LPC) or no phospholipids (NoPL). Each group was further divided into five sub-groups (n=6/sub group) to measure L bioavailability after 1, 2, 3, 6 and 9h of intubation. For the second set, group of rats (n=30/group) were intubated single dose of L solubilized in mixed micelles containing either oleic (OA) or linoleic acid (LA). Each group was further divided into five sub-groups (n=6/sub group) to measure L bioavailability after 1, 2, 3, 6 and 9h of intubation. For the third set of experiment, group of rats (n = 30/group) were intubated single dose of L and β-carotene solubilized in mixed micelles containing oleic acid. A separate group of animals (n=6) not fed with mixed micelles was considered as zero-time control. The mixed micelles (0.2 mL/rat) were administered to the rat by direct intubations to the stomach. The amount of L fed was calculated to be 0.445 mg/kg body weight. In a preliminary study, level of L in plasma was undetectable when one tenth of the L was intubated. The volume of intubations had no adverse effect on rats. Rats in the zero time control (n=6) and in each treatment group (n=6/time point) at 1, 2, 3, 6 and 9h after intubations were anesthetized with diethyl ether and sacrificed by exsanguinations. Blood was collected directly from heart into heparinised test tubes, centrifuged (Remi Instruments Ltd, Bombay) at 1000 x g for 15 minutes at 4^{o} C to obtain plasma. The liver was excised and washed with ice-cold isotonic saline, stored at –70^{o}C until analyzed.

Repeated dose study

For the repeated dose experiments, rats were randomly divided into six groups (n=6/group) and housed individually in metabolic cages for 7 days with proper feed and water supply *ad libido* as described earlier. They were fed with L solubilized in mixed micelles containing no phospholipids NoPL (control) or PC or lysoPC or oleic acid or linoleic acid or mixed carotenoids (L + β-carotene). The mixed micelles (0.2 mL/rat) were fed once a day in the morning at 9 A.M for 10 days by direct intubations to the stomach. In addition, food and water was given once in a day. Urine was collected for 6 days in amber bottles containing 2 mL of toluene (to prevent bacterial growth) and 0.2

mL of 2 mM α-tocopherol (antioxidant) for L analysis. Fecal matter was collected separately from the individual rats for 6 days from day 2 to 8 and used for L analysis. Urine volume was determined by a graduated measuring cylinder with 1 mL graduation and corrected for the added toluene and α-tocopherol. Rats were weighed initially and at the termination of the experiments. Control and experimental rats were sacrificed ten days after intubations, to collect blood, liver and eyes, which were processed for L analysis. For analysis of the L in eyes, both right and left eyes from each animal were removed separately and used immediately or stored at –70^{o} C until analysis.

Dietary studies

Two sets of experiments were conducted using adult rats (250 $\pm$ 6.8g). The first set with rats fed on diet with sufficient L and the second set with rats fed on diet devoid of L respectively.

Studies with rats fed on L sufficient diet

Group of rats (n=5/group) were fed with diet (**Table 2.3**) containing fenugreek leaf powder (4.21 g/Kg diet, source of L, 2.73 mg/Kg, here forth referred as L sufficient group) either with 10% olive oil (oleic acid source, OO group) or sunflower oil (linoleic acid source, SFO group) or groundnut oil (control, GNO group) for 4 weeks. A separate group of rats (n =5) was fed on purified soy lecithin (10 g) along with GNO (90 g/kg diet) (PL group). During the experimental run, rats had free access to food and water. The daily food intake and weekly gain-in-body weight were monitored. At the termination of each experiment, rats were anesthetized with diethyl ether and sacrificed. Blood, liver and eyes (both left and right eyes) were sampled immediately and processed for L analysis or stored at –70^{o}C until analyzed.

Studies with rats fed on L - depleted diet

To induce L deficiency, group of rats (n=80) were fed an L- depleted diet (Table 2.3) for 2 weeks (here forth referred as L-deficient group). Initially (0 day, base line) and after 2 weeks of feeding a L-depleted diet, blood was drawn directly from the orbital plexus of randomly selected rats (n=10), plasma was separated by centrifugation and analyzed for L and Z to ensure its base line value and the depletion status

(<0.1pmol/mL). After induction of L deficiency and to study the time-course response of L on post feeding, rats were divided into 4 equal groups (n=20/group). Each group was further divided into 4 sub-groups (n=5/sub group) and the group 1 fed on L supplemented diet (fenugreek leaf powder, 4.21 g/kg diet as L and Z source, 2.73 mg/kg diet) with olive oil (oleic acid source, OOD group) while group 2 and 3 fed on L supplemented diet with sunflower oil (linoleic acid source, SFOD group) or groundnut oil (control, GNOD), respectively. The group 4 was fed on L supplemented diet with lecithin (PL group). Rats in each dietary group at 2, 4, 8 and 16 days after post feeding were sacrificed; blood, liver and eyes (both left and right eyes) were sampled and processed immediately for L and Z analysis by HPLC and LC-MS according to the procedures mentioned elsewhere.

Extraction of L in plasma and tissue homogenate

L and its metabolites were extracted from plasma, liver and eye samples according to the procedure of Baskaran et al. (2003) and Lakshminarayana et al. (2006). To the plasma (0.8 mL), 3 mL of dichloromethane: methanol (2:1, v/v) containing α-tocopherol (2mM) was added and mixed for 1 minute using a vortex mixer. To the mixture, hexane (1.5 mL) was added, mixed well and centrifuged at 1000 g for 5 min, and the resulting upper hexane/DCM phase was collected. The extraction procedure was repeated thrice with 1ml of DCM and 1.5 mL of hexane. The extracts were pooled, evaporated to dryness using nitrogen, redissolved in 100 μL mobile phase (acetonitrile: methanol: dichloromethane (60:20:20, v/v/v) for HPLC analysis.

Liver, eye and feed samples were homogenized (Potter-Elvehjem homogenizer, Remi Instruments Ltd. Mumbai, India) separately with 9 parts ice-cold isotonic saline. The homogenate (0.8 mL) was used for L and its metabolites extraction according to the procedure described above for the plasma. In the case of liver samples, extracts were saponified separately with 2 mL of 10 M KOH at 60 ^{o}C for 45 min. The reaction mixture was vortexed every 15 minutes during saponification with an addition of 2 mL of ice-cold deionized water before L extraction as described above.

An aliquot of either fresh or frozen (-70^{o}C) urine (1 mL) and fecal (1 g) samples from each rat were used for L analysis. Urine (10 mL) samples were filtered through Whatman No.1 filter paper and the fecal matter (2 g) was homogenized with ice-cold acetone, diluted suitably and used for L extraction. The extraction procedure adopted

was the same as described for plasma and tissues. These operations were done in dim yellow light at 4 ^{o}C to minimize isomerization and oxidation of carotenoids by light.

HPLC analysis

L and Z levels in the extracts of plasma, liver, eyes, urine, fecal and feed samples were quantified by HPLC (Baskaran et al, 2003; Lakshminarayana et al. 2006). The HPLC system was consisting of an LC-10AD pump, an SPD-10A UV-vis absorbance detector (Shimadzu, Kyoto, Japan) and personal computer equipped with Ezchrome chromatography data system software (scientific Inc., Pleasanton, CA). L and its metabolites were separated on a TSK gel ODS-80Ts column (Tosoh), 4.6 X 150mm) attached to a precolumn (2 X 20mm) of pelliguard LC-18 (Supelco Inc, Bellafonte, PA). Acetonitrile: methanol: dichloromethane (20:20:20, v/v/v) containing 0.1% ammonium acetate was used as a mobile phase. An isocratic analysis was performed at a flow rate of 1 mL/min at 450 nm with UV-vis absorbance detector (Shinadzy, Japan). The components were quantified from their peak area by use of respective standard curves. The peak identity of the L and Z was further confirmed by their characteristic UV-vis spectra recorded with a model 1100HPLC system equipped with a photodiode array detector (Hewlett-Packard, Palo Alto, CA). In the case of plasma and eye samples, L and Z levels are presented together as L + Z, due to their unsatisfactory resolution.

Liquid chromatography-Mass spectrometry (LC-MS)

LC-MS for L and its metabolites was performed according to the procedure of Lakshminarayana et al. (2007). LC-MS was preformed on a Waters 2996 modular HPLC system (auto-sampler, gradient pump, thermo-regulator and diode array detector (DAD)), coupled to a Q-TOF Ultima (UK) mass spectrometer. The atmospheric pressure chemical ionization (APCI) source was heated at 130^{o} C and the probe was kept at 500^{o} C. The corona (5 kV), HV lens (0.5 kV) and cone (30 V) voltages were optimized. Nitrogen was used as a sheath and drying gas at 100 and 300 L/h, respectively. The spectrometer was calibrated in the positive ion mode and $(M + H)^{+}$ signals and as well as the $(M + NH_4)^{+}$ ions were recorded. Mass spectra of L and its metabolites were acquired with an *m/z* 0-1000 scan range, and the UV absorption was recorded at 450 nm by using a DAD. The MS identities of L and its metabolites in samples were confirmed using

respective reference standards (Kim et al. 2001). Data were processed with Mass Lynx 3.2 software.

Assay for activity of Antioxidant enzymes

Liver samples of rats fed with fenugreek leaves diet incorporated were homogenized in phosphate buffer (pH 7.0) (1 g/10 mL) using glass homogenizer (Remi Instruments Ltd, Mumbai). The homogenate was centrifuged at 600 g for 15 minutes at 40 ^{o}C. The supernatant was used for measuring antioxidant enzymes activity. 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 inhibiting the reduction of cytochrome C by 50%. Briefly, 20 to 50 µL of 1:10 diluted liver homogenate or 20-50 µL of plasma samples were added to the solution containing 50 µM xanthine and 20 µM cytochrome C in 50 mM phosphate buffer (0.1 mM EDTA), reaction was initiated by adding 20 µL of xanthine oxidase (0.2 U/mL in phosphate buffer, pH 7.8), measured at 550 nm for minutes.

Glutathione peroxidase activity was measured by the method of Folhe and gunzler, (1985). The reaction mixture consisted of 500 µL of 0.1 M phosphate buffer (pH, 7.0), 100 µL (50 µg) of protein of enzyme sample, 100 µL of glutathione reductase (0.24U) and 100 µL of 10 mM of Glutathione (GSH). The mixture is pre incubated for 10 minutes at 37 ^{o}C. There after 100 µL of NADPH solution is added and the hydroperoxide, independent consumption of NADPH is monitored for about 3 minutes. Initiate the reaction by adding 100 µL of pre warmed t-butyl hydroperoxide solution and record decrease in absorbance at 340 nm for about 5 minutes. Replacing the enzyme sample by buffer correspondingly assesses the non-enzymatic reaction rate. The activity is expressed as nmol of NADPH oxidized/min/mg protein ($\sum$ 340-6.22 mM-1/cm).

Glutathione level was measured by the method of Owens et al. (1965). 10% of (w/v) liver homogenate or 500 µL of plasma in 5% (w/v) trichloroacetic acid was centrifuged at 2000 rpm for 10 minutes at room temperature. 0.2 mL of deprotenized supernatant was added to 4.75 mL of 0.1 M solution sodium phosphate buffer (pH, 8.0) to which 0.05 mL of 5,5'-dithiobis (2-nitrobenzoic acid) (10 mM in sodium phosphate buffer 0.1 M, pH, 7.0) was added. After 5 minutes, absorbance was read at 412 nm.

Similarly, 200 µL plasma samples were considered. Glutathione was oxidized by 5,5'-dithiobis(2-nitrobenzoic acid) to give reduced glutathione (GSSG) with stoichimetric formation of 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB formation is followed 412 nm.

Protein analysis

Protein content in plasma and tissue samples was estimated by Lowry's method using bovine serum albumin as reference standard (Lowry et al. 1951).

Determination of lipid peroxides

Plasma and tissues levels of lipid peroxides in rats fed L sufficient were measured by TBARS assay following the method of Ohkawa et al. (1979). Briefly, 200 µL of plasma or tissue homogenate (≅ 0.5–1 mg protein) was added to test tubes containing 1.5 mL acetic acid (pH 3.5), 200 µL SDS (0.8%) and 1.5 mL of thiobarbituric acid (0.8%). The reaction mixture was vortexed and the tubes were kept in a boiling water bath for 60 minutes at 90 ^{o}C. The pink colour developed was extracted with 5 mL n-butanol by centrifugation at 2,500 rpm for 15 minutes. The colour was read using spectrophotometer at 532 nm. Malondialdehyde (MDA) formed was measured at 532 nm and quantitated using a standard graph. The results were expressed in terms of nmol MDA/mg protein or gram tissue.

Analysis of lipids profiles

Total lipids were extracted from plasma and tissues by the method of Folch et al. (1957). Triacylglycerides in plasma, liver and eye samples were estimated by the method of Fletcher (1968). Cholesterol levels were quantitated by the method of Searcy and Bergquist (1960). Phospholipids were measured by the method of Stewart (1980) using dipalmitoylphosphatidylcholine as reference standard. 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 25 cm x 0.25 mm (Parma bond FFAP-DF-0.25: Machery-Nagel Gm BH co. Duren, Germany). The operating conditions were: initial column temperature 160 ^{o}C, injector temperature 210 ^{o}C and detector temperature 250 ^{o}C. Column temperature was programmed to rise at 6 ^{o}C/min to the final temperature of

240 ^{o}C. Nitrogen gas was used as the carrier. Individual fatty acid was identified by comparing with retention times of respective fatty acid standards.

Effect of dietary factors on physicochemical properties of mixed micelles

Determination of micellar size

The size, surface area and L intensity of OA and LA micelles were measured using particle size analyzer and image-processing techniques (Tyssandier et al. 2001; Gonzalez and Woods, 2005). The various steps involved in these experimental procedures are given below.

Particle size by particle analyzer

A computerized inspection system (particle size analyzer, CIS-100 M/s Galai production, Israel) capable of measuring micellar size in the range of 0.2-100 μm was employed to find out the size of mixed micelles. A glass cuvette filled with mixed micelles (3 mL) was placed in the laser path and stirred magnetically. The system software automatically generates the particle size distribution in a graphical form with percent cumulative particle size on the 'X' axis and particle size in microns on the 'Y' axis. The mean size of mixed micelles was calculated from the graph.

Measurement of structure, surface area and lutein intensity of mixed micelles using image-processing technique

Image acquisition

Phase contrast microscopy (Olympus BX40 F4, Olympus Optical. Co. Ltd, Japan) was used to acquire the images of micelles containing L (McClements.1999). Mixed micelles (10-20 μL) were placed on a glass slide (Thomas-slides of 1.35 mm, Poly Optic GmbH, Germany), covered with cover glass (0.2 mm), viewed under the microscope (magnification, 1000 x) and photographed using a camera (Olympus SLR-35 mm, Optical Co. Ltd, Japan). These microphotographs were used for image processing using software (Lab VIEW 7.0) (Gonzalez and Woods, 2005).

Determination of micellar surface area

The micelles image is a true colour red (R), green (G), blue (B) image and defined as two-dimensional function f (x, y), where x and y are spatial coordinates. Amplitude at any pair of coordinates (x, y) was considered as intensity or gray level of the image at the point of focus. The digital micellar image is composed of elements, referred as pixels or image elements.

The RGB micellar image defines red, green and blue colour components for each individual pixel and stores it as an m-by-n-by-3 data array. The colour of each pixel of the micellar image was determined by the combination of intensities of those colours stored in each colour plane at the particular pixel's location. The graphics file format of the micelles stores the RGB image as a 24-bit image, where the red, green and blue components are eight bits for each format. The steps involved in digital image processing of micelles are indicated below.

Image pre-processing

The intensity distribution of L in micelles is represented by histogram equalization that maps each gray level into another gray level by a predetermined transformation so that the output gray level is distributed uniformly. This module was used to enhance the low contrast micellar image, which occurs due to non-linearity or small dynamic range of imaging sensor.

Image segmentation

The images of mixed micelles were partitioned into their constituent parts or objects and L was identified individually by using the segmentation procedure.

Representation and description

The images of mixed micelles were converted into a suitable form for further processing by extracting image attributes. The extracted image attributes were described and quantified by differentiating the micelle image objects in terms of pixels.

Recognition and interpretation

The micelle images were recognized through labeling individual objects based on their descriptors. The recognized individual objects of the micelle image were assigned values through the process of interpretation.

Knowledge base

The Knowledge database was created and standardized using a spreadsheet by detailing the regions of the mixed micelles image to locate L for intensity comparison. The areas of micelles containing L were quantified separately based on the inner and outer core and were stored in the spreadsheet for further retrieval and analysis. The distribution of pixel intensities represented by L in a micelle image was described by using histogram equalization technique.

Evaluation of L intensity in micelles

L intensity within a micelle was calculated by converting RGB values into HSI (Hue Saturation Intensity), since the HSI system is more representative to colour perception by humans than RGB. In HSI mode, Hue represents the type of colour; Saturation represents the relative purity or amount of gray in a colour and Intensity represents the brightness. For further analysis of data, colour model transformation technique was employed which quantifies the intensity of L in mixed micelles. Intensity of colour is a measure of brightness and is the average of the colour value *'I'*. The *'I'* was calculated using the equation:

$$I = (R+G+B)/3$$

The saturation (S) or colour purity (lack of whiteness) was measured from the equation:

$$S = 1\min (R, G, B)/I$$

A saturation value of 0 indicates equal values of R, G and B, and corresponds to a gray value. A saturation value of 1 indicates one or two colour values of zero and corresponds to a pure colour. 'Hue' is proportional to the average wavelength of the colour. It gives the gradation of L colour as an angle '*H'* by the equation:

$$H = \cos^{-1} \{[(R - G) + (R-B)] /2 [(R-G)^2 + (R-B)(G-B)]^{1/2}\}.$$

In this polar coordinate system, red corresponds to 0^o, green to 120^o, and blue to 240^o.

The distribution of the pixel intensities represented by L in the micellar image was described by using histogram equalization technique. The histogram is the function, in which 'H' is defined on the grayscale range [0…. k…. 255] such that the number of pixels equal to the gray-level value, 'k' is a histogram count, which was calculated from the equation:

$$H(k) = n_{k,}$$

Where, 'k' is the gray-level value, '$n_{k'}$ is the number of pixels in an image with a gray-level value equal to 'k', and Σn_k from k = 0 to 255 (the total number of pixels in the micellar image).

Determination of pH and viscosity of mixed micelles

The effect of dietary factors like phospholipids and fatty acids on pH of the mixed micelles was determined at different temperatures using a digital pH meter (Cyberscan, Model No. 2500).

The viscosity and rheological behavior of the phospholipids, fatty acids and mixed carotenoids on micellar solution containing L was determined using digital viscometer. L solubilized in mixed micellar solution (25 mL) containing phospholipids (PC and lysoPC), fatty acids (OA and LA) and mixed carotenoids (β-carotene and L) was used for the analysis. The measurements were carried out using rheometer with co-axial system (Model No. RT10, Haake GmbH, Karlreche, Germany, software 2.20). Rheological measurements of samples were performed at 37 ^{o}C and determined at 20 S^{-1}. All samples were measured three times and presented as mean ± SD.

In vitro and *in vivo* oxidation/ metabolism of L

Photo-oxidation products of L in vitro model

Liposome was prepared using phosphotidylcholine (PC, 5 μmol) and L (50 nmol) in 2 mL of Tris-HCl buffer (pH 7.4) according to the procedure of Kim et al. (2001). L was dissolved in dichloromethane (1 mL) was mixed with PC dissolved in chloroform (1 mL) and the mixture was evaporated to dryness under a stream of nitrogen, the residue was re-dissolved in Tris-HCl buffer (pH 7.4) with vigorous mixing for 2 minutes and sonication for 90 seconds. Photo-oxidation of L was preformed as previously described by Jughans et al. (1998) and Dachtler et al. (2001) with slight modification. Liposomes in quartz test

tubes were exposed to direct sunlight (10 AM to 12 PM) for different time intervals (0, 20, 40, 60, 80 and 100 minutes). After set interval of exposure, further oxidation of L was arrested by adding 100 µL of BHT (0.1%) in ethanol and processed immediately for HPLC and LC-MS analysis. Liposomes kept in dark at room temperature were considered as a control sample. Photo-oxidized products of L were extracted and analyzed by HPLC and LC-MS in an atmospheric pressure chemical ionization (APCI) mode, as described elsewhere (Lakshminarayana et al. 2007).

Oxidative products / metabolites of L rat model

Animals and diet

Animal experiments were performed after due clearance from institutional animal ethics committee. Animal studies were conducted at the Institute's animal house facility. Two groups of male albino rats (OUTB-Wistar, IND-CFT 2c) weighing 250 ± 5 g (n=6/group) were housed individually in steel cages at room temperature (28 ± 2 ^{o}C) with a 12 h light/dark cycle. Rats were acclimatized for 7 days on a fresh pellet diet (Amrut Feeds, Sangli, India) and had free access to water. Both the groups received semi-synthetic diet devoid of L for 2 weeks. Thereafter group 1 received a dose of micellar L (for gavage study) daily for 10 days and group 2 received diet supplemented with fenugreek leaf powder as a source of L (for dietary study) for 4 weeks. The concentration of L in the diet was worked out to be 0.0214 mg/day/kg body weight and was comparable to the human daily intake (Richer et al. 2004). Fenugreek leaf powder and synthetic diets were analyzed for L content by HPLC and LC-MS before being fed to animals.

Gavages study

Group 1 was administered a dose of L (200 μM) solubilized in phospholipid (PL) mixed micelles by intubation. L in PL mixed micelles was prepared in phosphate buffered saline (0.5 mL) containing PC (3 mM), monooleoyl glycerol (2.5 mM), oleic acid (7.5 mM), and sodium taurocholate (12 mM). These chemicals were dissolved in either methanol or DCM and solvent was evaporated to dryness under nitrogen followed by re-suspension of the residue in 0.5 mL phosphate buffered saline (pH 7.0) with vigorous mixing using a vortex mixer to obtain an optically clear solution (Sugawara et al.

2002;Baskaran et al. 2003). The volume (0.5 mL) of intubations had no adverse effect on rats. The micelle composition chosen was based on the composition of the clear layer obtained by ultra centrifugation of the duodenal content of healthy adult human subjects received a triglyceride-rich meal (Hernell et al. 1990). Rats were weighed before and after intubations. At termination of set period, rats were sacrificed with diethyl ether to collect blood, liver and eyes, which were analyzed for L and its metabolites. All samples were stored at –70 °C until further analysis. In case of eyes, both right and left eyes from each animal were removed separately and used immediately or stored (Lakshminarayana et al. 2006).

Dietary study

Fenugreek leaf powder at 4.21g / Kg diet afforded 2.69 mg of L and 0.04 mg of Z. Isocaloric diet was prepared according to AIN-76 (1976) and composition of diet is given in **Table 2.3**. Diet contained 10% fat from olive oil. During the 4-week feeding trial, rats had free access to food and water. The daily food intake and weekly gain-in-body weight were monitored. At the termination of experiment, rats were anesthetized with diethyl ether and sacrificed to collect blood, liver, intestine and eyes. All the samples were analyzed for L and its metabolites immediately or stored at –70 ^{o}C until analysis. L and its oxidized products were extracted and analyzed by HPLC and LC-MS in an atmospheric pressure chemical ionization (APCI) mode, as described elsewhere.

Statistical analysis

Data was expressed as mean ± SD. Analysis of variance was employed to determine the significant difference between groups (Fisher, 1970). To quantify the postprandial L in plasma and tissues over 9h (single dose) and 16d (dietary study), the area under the curve (AUC) was calculated by trapezoidal approximation. Data were tested for homogeneity of variances by the Bartlett test. When homogenous variances were confirmed, the data were tested by ANOVA and significant differences in means among groups and at different time intervals (single dose study and deficient dietary study) and between the groups were evaluated by Tukey's test. Differences in means were considered significant at a level of $p<0.05$.

Chapter 3

Isolation, purification and quantification of xanthophylls (lutein and zeaxanthin) from selected leafy greens, fruits and vegetables by column chromatography, HPLC and LC-MS techniques

Introduction

Green Leafy vegetables (GLVs), fruits and vegetables are consumed as part of the daily meal and are available throughout the year in India and other parts of the world. They are rich source of not only major nutrients like protein, lipid and carbohydrates but also rich with micronutrients (vitamins and minerals) and bioactive molecules like carotenoids and polyphenols those triumph several health benefits (Moller et al. 2000) (**Table 2.1, Chapter 2)**. The major carotenoids found in agri/ and horticultural produce are shown in **Figure 3.1**. The health benefits of carotenoids are due to their provitamin A and antioxidant properties (Zanutto et al. 2003). Epidemiological studies have demonstrated that consumption of carotenoid-rich fruits and vegetables is associated with lower incidence of cancer (Bowman and Mobarhan, 1995), cardiovascular disease (Krichevsky, 1999), age-related macular degeneration (AMD) and cataract formation (Landrum and Bone, 2001).

Among the carotenoids, the two major xanthophyll carotenoids found in the human eyes are lutein (β,ε-carotene-3,3'-diol) (L) and its isomer zeaxanthin (β,β-carotene-3,3'-diol) (Z), and are often referred as macular pigments. Their concentration in the *macula* is five fold higher than the peripheral *retina* (Handelman, 1998). They protect the eyes from phototoxic damage (Krinsky, 2003). It has been suggested that there is an association between intake of L-rich plant foods and its density in the *macula* of human subjects (Landrum, 1997). Studies have shown that consumption of GLVs is related, in specific, with lower risk of cataract and AMD (Jacques and Chylack, 1991; Mares-Perman et al. 2001). They also suggested a relationship between the intake of L-rich plant foods and macular pigment density in animal and human subjects. Jacques et al. (1991) reported a correlation between intake of carotenoids and the prevention of

cataract. Case Control Study Group for Eye Diseases of the United States also reported that high dietary intake of GLVs rich in L and Z was linked with 43% lower risk for AMD (Mares-Perman et al. 2001). Scientific data on the level of L and Z in various agri/horticultural produce in India is not available. Hence, it was an objective of this study to screen various GLVs, fruits and vegetables of common and wild varieties for carotenoids composition. The data may help the common people to choose a right dietary source to serve the purpose. Studies have reported the carotenoids composition of plant materials from various geographical regions (Khachik et al. 1986; Granado et al. 1992; West and Poortvliet, 1993; Hart and Scott, 1995; O'Neill et al. 2001). In specific, Khachik et al. (1986) and Hart and Scott, (1995) have studied the quantitative and qualitative aspects of carotenoids in fruits and vegetables. Except for few studies reported on pro-vitamin A carotenoids (Nambiar and Seshadri, 1998; Rajyalakshmi et al. 2001; Singh et al. 2001), comprehensive data on the levels of L and Z in Indian vegetables and fruits were limited. Hence, generating data on those pigments in wild and agri/horticulture produce may be of use from ethno-pharmacological point of view (Grover et al. 2002; Grover and Vats, 2001). Data on L and Z content of GLVs, fruits and vegetables will aid in assessing the dietary intake of these pigments and their relationship with AMD. The data may also provide scientific information directly to the consumers and public health workers to assess their dietary intake. This brief literature survey shows that selection of L rich supplement is vital to safe guard the eyes from AMD/cataract.

Attempts at generating accurate qualitative and quantitative data on carotenoids in greens have resulted in the development of analytical techniques for their separation, identification and quantification (Khachik and Beecher, 1987; Edelenbos et al. 2001). However, newer scientific analytical techniques for the isolation and HPLC separation of polar and non-polar carotenoids from dietary sources are the need of the day. Many reports on the extraction and separation of various classes of carotenoids by HPLC technique are available (Khachik and Beecher, 1987; Kimura and Rodriguez-Amaya, 2003). However, the separation and quantification of major carotenoids from green leafy vegetables has not received as much attention as it deserves. Hence, development of a rapid column chromatographic technique and HPLC methods, which can purify and separate carotenoids within a reasonable time, is warranted, in order to assess their

levels in greens. Chemical structure and systematic names of major carotenoids found in vegetables and fruits are shown in **Figure 3.1** (Rodriguez-Amaya, 1999).

Among the available literature on the HPLC analysis of carotenoids, the work of Kimura and Rodriguez-Amaya (1999), Weller and Breithaupt (2003) and Hart and Scott (1995), who isolated carotenoids from various GLVs and biological samples are noteworthy. Similarly, Khachik et al. (1999) have reported predominant carotenoids and carotenoid fatty acid esters in extracts from various biological samples. The present study was aimed to determine the composition of carotenoids in various GLVs, vegetables and fruits by HPLC. Further, open column chromatographic technique (OCC) was developed to purify individual carotenoids in order to use them for *in vitro* and *in vivo* studies, to assess their bioavailability (Lakshminarayana et al. 2006 and 2007). These HPLC and OCC techniques could be employed to separate both polar (neoxanthin, violaxanthin, L, Z and β-cryptoxanthin) and non-polar (β-carotene and α-carotene) carotenoids. Further, this study provides scientific data on superior food sources of L and Z. The botanical, common and local names of leafy vegetables, vegetables and fruits used in this study are given in **Table 2.1 and 2.2** (Chapter 2)**.** Materials and methods relevant to this chapter are given in **Chapter 2**.

Results

GLVs, vegetables and fruits used in the present study contain two classes of pigments. In the order of chromatographic elution on a C_{18} column, they are xanthophylls (oxygenated carotenoids), chlorophylls and hydrocarbon carotenoids. All the pigments were separated within 22 minutes **(Figure 3.2-3.5)**. The detectable xanthophylls in GLVs, vegetables and fruits comprising carotenoids in the order of elution as neoxanthin (peak 1), violaxanthin (peak 2), L (peak 3) and Z (peak 4), then the chlorophylls (peak 5 and 6), Unidentified (peak 7), α-carotene (8) and β-carotene (peak 9) **(Figure 3.2-3.5)**. Carotenoids were eluted under isocratic condition and confirmed by their retention time and absorption spectra of respective reference standards **(Figure 3.6)**. They were quantified from their peak areas in relation to respective reference standards. It is seen from the results that the major differences in carotenoid composition among leafy vegetables appear to be their levels. For example, the chromatogram from the extract of *C. benghalensis* (**Figure 3.7D**) shows the presence of both hydrocarbon and

xanthophylls carotenoids; however, their relative concentrations was found to be different compared with other leafy vegetables studied **(Table 3.1- 3.2)**.

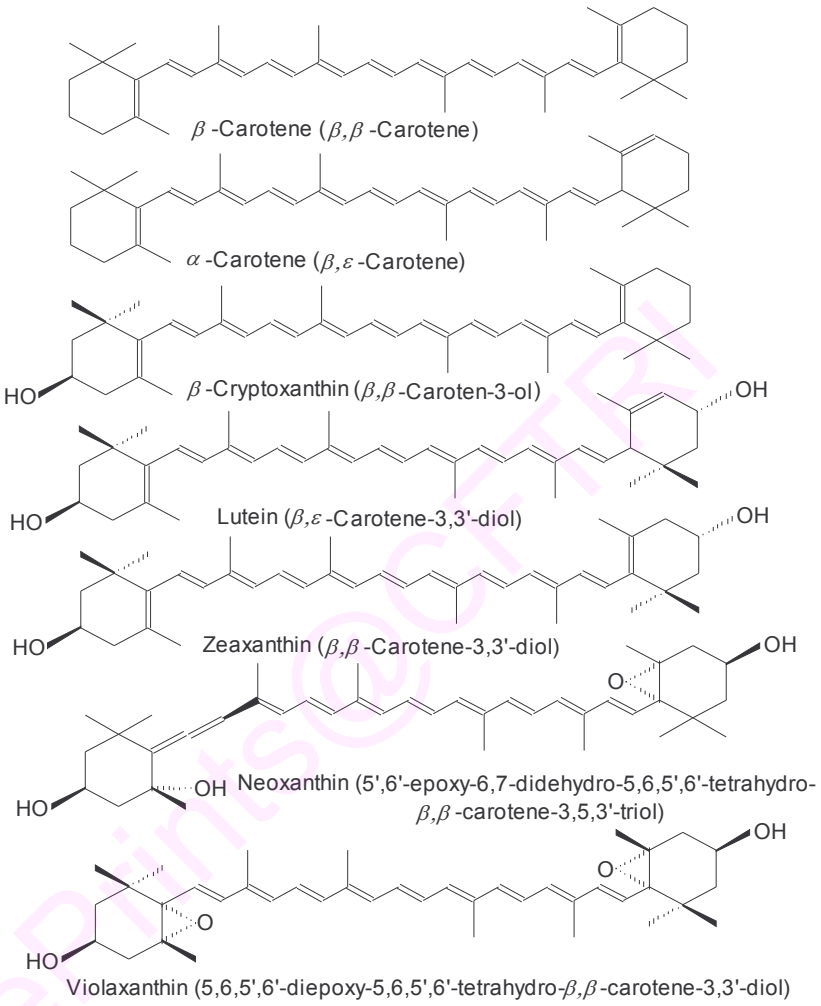


Figure 3.1. Chemical structures of major carotenoids present in leafy greens, vegetables and fruits (Rodriguez-Amaya, 1999).

Results on the levels of L and Z in GLVs, fruits and vegetables are given in **Table 3.1–3.3.** In case of GLVs, they were categorized into familiar and less familiar, based on their consumption and availability. Typical HPLC profile and absorbance spectra of L and Z in *C. benghalensis*, for example, are shown in **Figure 3.7.** The LC-MS

spectra of L and Z, separated from *C. benghalensis* extract further confirmed their identity **(Figure 3.8A).**

The chemical and structural characteristics of L and Z shows that they are composed of two different ionone rings, one with the β-configuration and the other with ε-configuration. The mass spectral (MS) signals are characterized by loss of the water (L) or no loss of water (Z) moiety from the respective quasi molecular ions, which is found in each MS, representing the L backbone ($[M + H - H_2O]^+$; *m/z* 551). Further, the MS of L and Z eluted from the samples in this study were comparable with the MS of the respective reference standards **(Figure 3.8B)**.

Although the HPLC profile of L and Z among GLVs appears analogous, their concentrations are found to be different (**Table 3.1 and 3.2**). For example, less familiar GLVs such as *C. benghalensis* (183.40), *A. pungens* (72.53), *P. niruri* (77.6), *B. rubra* (67.94), *T. cuniefolium* (91.01) and *S. nigrum* (84.38) contained comparatively higher level of L + Z (mg/100g dry weight) than the other leafy vegetables. Among familiar GLVs, *B. alba* (115.58), *C. sativum* (99.27), *P. sowa* (92.24), *A. viridis* (90.59), *L. sativa* (87.12) and *S. oleracea* (77.58) contained higher levels of L + Z (mg/100g dry weight). Results revealed that less familiar GLVs possess equally or even higher level of L + Z compared to those of familiar ones.

The purity of individual carotenoids purified on neutral alumina column ranged between 92 ± 3% (neoxanthin), 94 ± 2% (violaxanthin), 97 ± 2% (L and Z) and 90 ± 3% (β-carotene). β-cryptoxanthin and α-carotene were not amenable for quantitation by the present technique, since they were below the detectable limit (0.1 pmol). The HPLC profiles of L and Z in vegetables analyzed in this study were almost identical, except for differences in the concentration of individual pigments except green and red *C.annuum* (chilli) contained higher level of Z than L **(Table 3.3).** Typical HPLC profiles of carotenoids in vegetables are shown in **Figure 3.4.** It is seen from the results that, in general, L level were higher in all the vegetables studied compared to other carotenoids reported elsewhere. Further, the results show that among vegetables analyzed *C.annuum, L.acutanguta* and *C.annuum* contained comparatively higher level of L + Z, ranging from 430 to 1243 μg/100g edible portions than other vegetables. Among vegetables, *C.annuum* (green chili) contained maximum level (1243 μg/100g edible portion) while *M.charantia* (bitter guard) contained low level (18.04 μg/100gedible

portion) of carotenoids. The level of L+Z in green beans and green peas was estimated to be 173.27 and 195.56 μg/100g edible portions. Z level in green peas was below the detectable limit (0.1 pmol).

The HPLC and quantitative profiles of the L and Z in fruits analyzed in this study are given in **Figure 3.5 and Table 3.3**. Among the fruits analysed, *M.indica, C.papaya* and *C. aurantifolia* contained higher level of L+Z ranging from 104.81 to 120.86 μg/100g edible portions. *M.indica* was found to be the richest source and *A.scomusus* the poorest source of macular pigments among the fruits analyzed. Interestingly, orange and pineapple contain higher levels of Z than L, whereas, other fruits contained higher level of L than Z. HPLC results showed that under the conditions used, the greens, fruits and vegetable extracts contained several unknown, unidentified components eluted between 14 and 16 minutes. On the basis of their retention times and spectral characteristics, these are most likely to be esters of xanthophylls, exhibiting identical UV-vis spectra, but are less polar and elute differently.

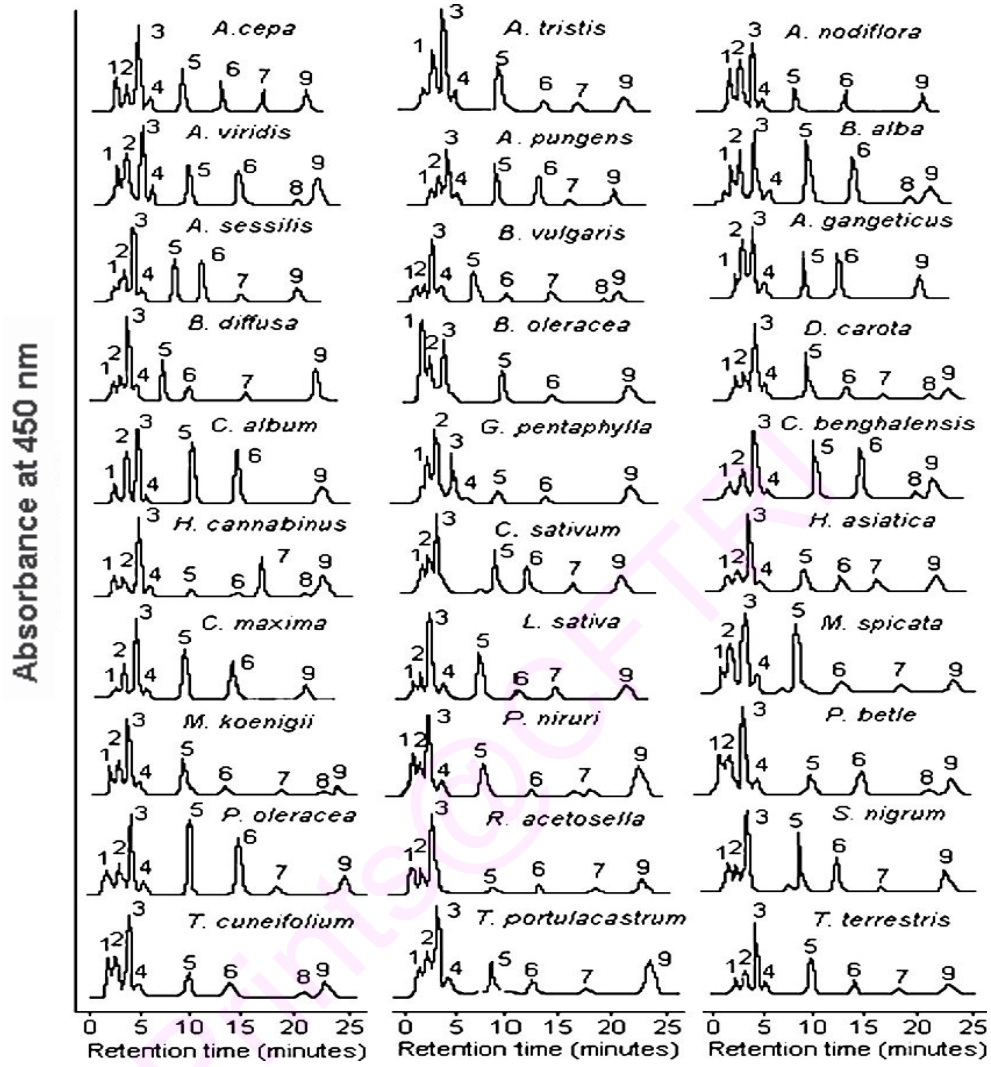


Figure 3.2. HPLC profile of carotenoids in leafy vegetables. Peaks: 1, neoxanthin; 2, violaxanthin; 3, L; 4, Z; 5, chlorophyll b; 6, chlorophyll a; 7, unidentified; 8, α-carotene and 9, β-carotene. HPLC conditions: column - SGE C-18 (ODS), 25 cm x 4.6 mm id, 5 μm, 120A0; mobile phase - acetonitrile/methanol/dichloromethane (60:20:20, v/v/v) containing 0.1% ammonium acetate; volume of sample injected - 20 μL; condition – isocratic; flow rate - 1 mL/min. All the carotenoids were monitored at 450 nm with UV-visible detector. The peak identities and λ_{max} of carotenoids were confirmed by their retention time and characteristic spectra of standard chromatograms, recorded with a SPD-10AVP detector.

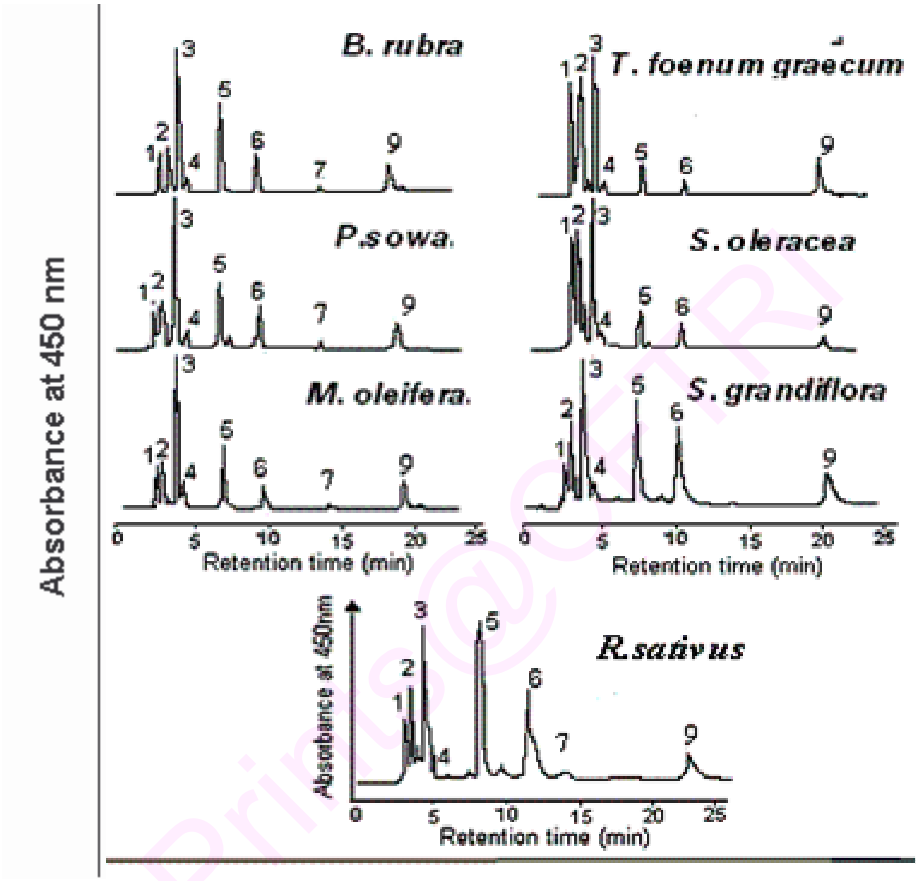


Figure 3.3. HPLC profile of carotenoids in leafy vegetables. Peaks: 1, neoxanthin; 2, violaxanthin; 3, L; 4, Z; 5, chlorophyll b; 6, chlorophyll a; 7, unidentified; 8, α-carotene and 9, β-carotene. HPLC conditions: As given in Figure 3.2.

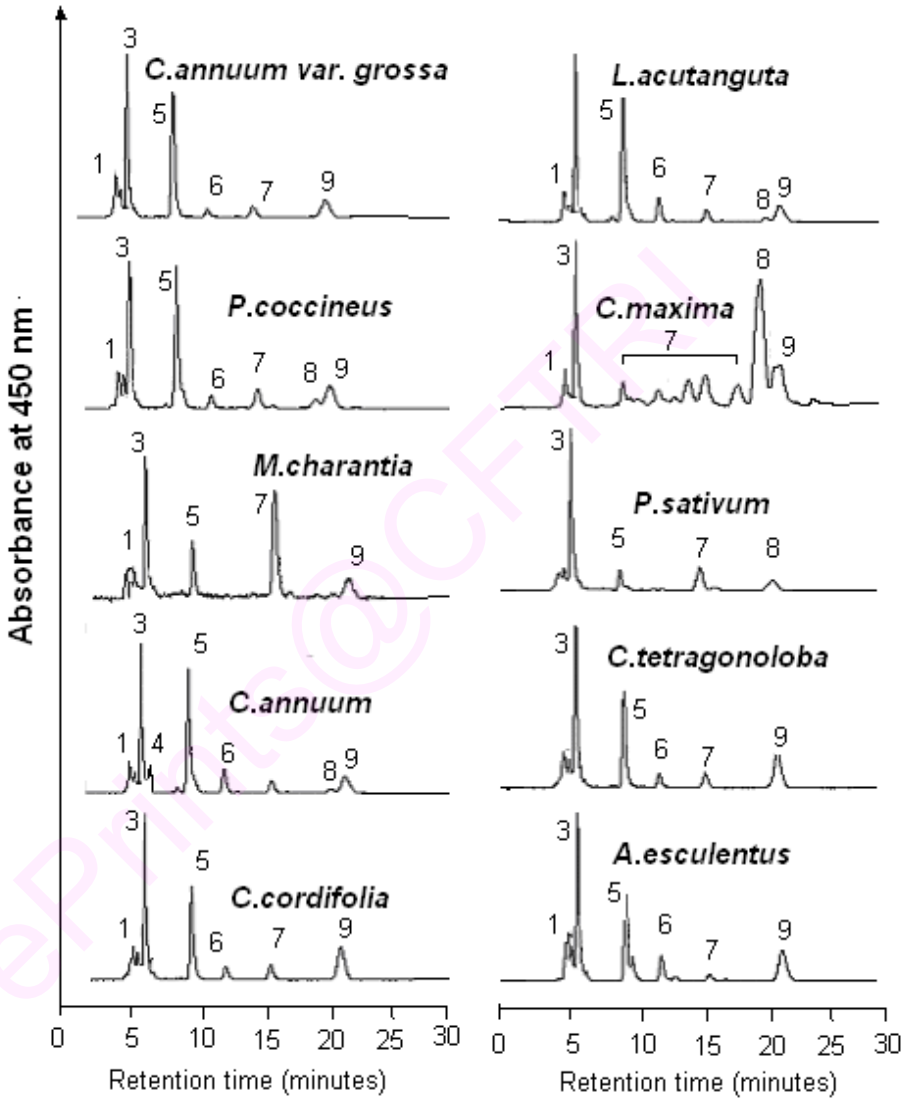


Figure 3.4. HPLC profile of carotenoids in vegetables. Peaks: 1, neoxanthin; 2, violaxanthin; 3, L; 4, Z; 5, chlorophyll b; 6, chlorophyll a; 7, unidentified; 8, α-carotene and 9, β-carotene. HPLC conditions: As given in Figure 3.2

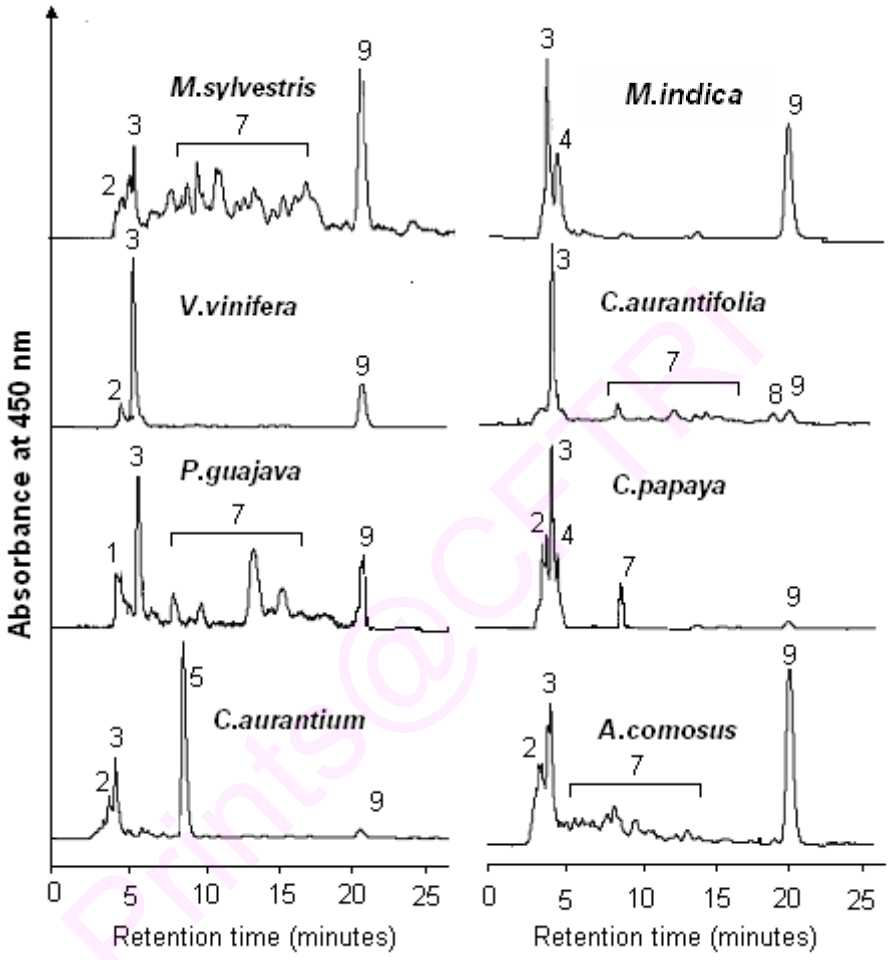


Figure 3.5. HPLC profile of carotenoids in fruits. Peaks: 1, neoxanthin; 2, violaxanthin; 3, L; 4, Z; 5, chlorophyll b; 6, chlorophyll a; 7, unidentified; 8, (-carotene and 9, (-carotene. HPLC conditions: As given in Figure 3.2

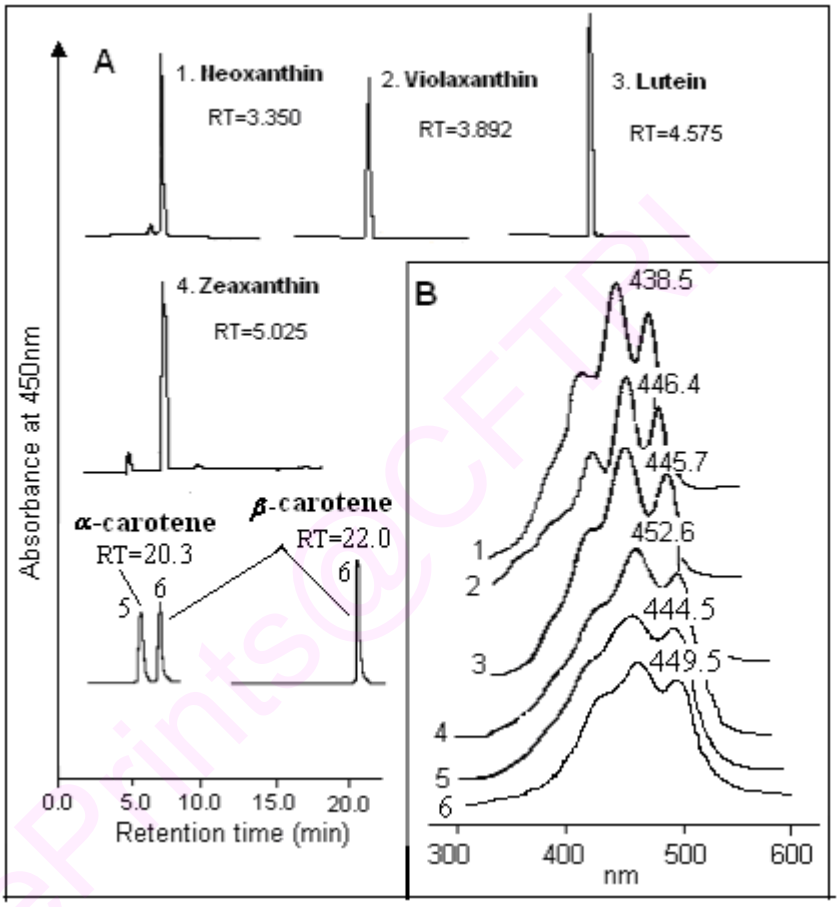


Figure 3.6. HPLC profile of standard carotenoids (A) and their absorption spectra (B) of carotenoids purified from leafy vegetables studied: (1) neoxanthin, (2) violaxanthin, (3) lutein, (4) zeaxanthin, (5) (-carotene and (6) (-carotene. HPLC conditions: As given in Figure 3.2

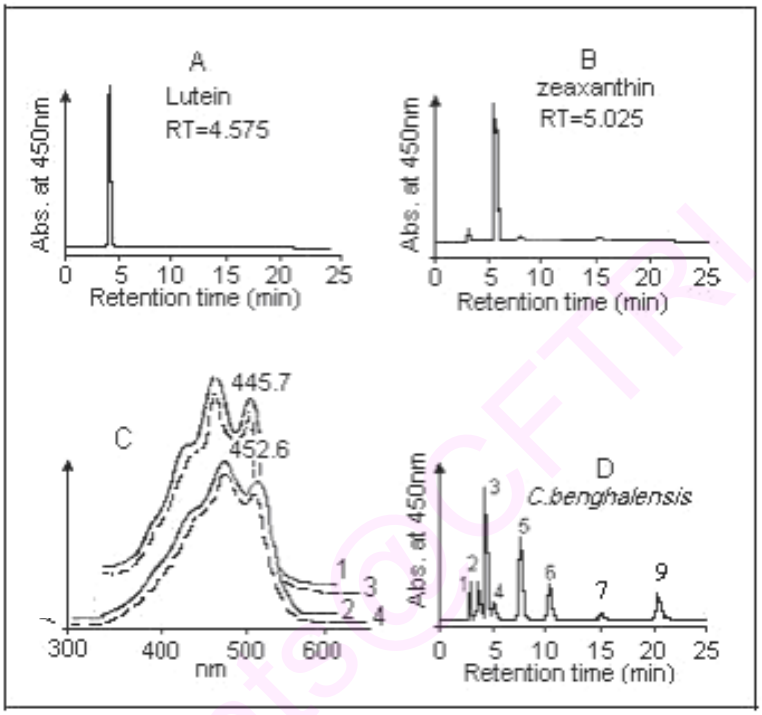


Figure 3.7. HPLC profiles of standard lutein (A), zeaxanthin (B) and their absorption spectra (1 and 2) along with spectra of lutein (3) and zeaxanthin (4) eluted from *C. benghalensis* (C) and its typical chromatogram of carotenoids (D). Peaks: 1, neoxanthin; 2, violaxanthin; 3, L; 4, Z; 5, chlorophyll b; 6, chlorophyll a; 7, unidentified; 8, α-carotene and 9, β-carotene. HPLC conditions: As given in Figure 3.2

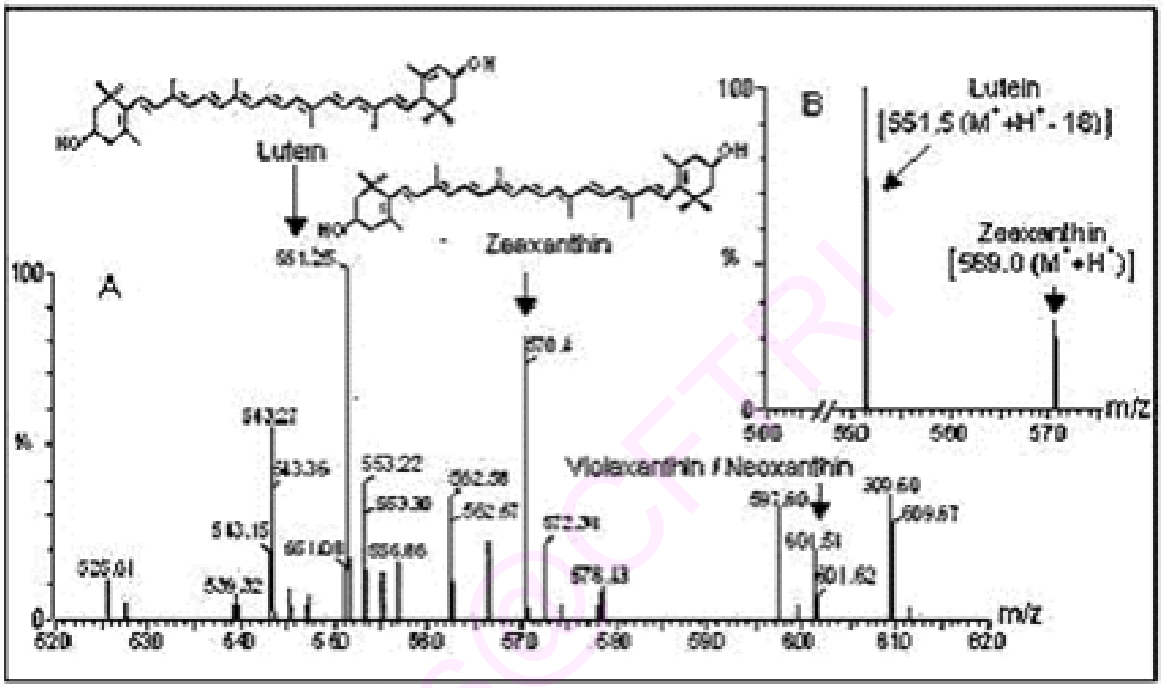


Figure 3.8. Mass spectrum of carotenoids separated from *C. benghalensis* extract **(A)** and standard lutein and zeaxanthin (**B**). LC-MS conditions: Waters 2996 modular HPLC system coupled with mass spectrometer. The APCI source was heated at 130 ^{o}C, and probe was kept at 500 ^{o}C. The corona voltage was optimized to 5 kV, the HV lens to 0.5 kV, and cone voltage to 30 V. Nitrogen was used as sheath and drying gas at 100 and 300 L/h, spectrometer was calibrated in the positive ion mode (scan range *m/z* 80-1200). Mass spectra of lutein and zeaxanthin were acquired with an *m/z* 200-1200 scan range.

Table 3.1. Lutein and zeaxanthin levels in less familiar leafy greens[a].

Botanical name	L*	Z**	L+Z***
Allmania nodiflora (L.) R.Br.	19.71	0.12	19.71
Alternanthera pungens Kunth	71.86	0.67	72.53
Alternanthera sessilis (L.) DC.	62.20	0.50	62.70
Basella rubra L.	67.94	2.25	70.19
Boerhavia diffusa L.	26.83	0.19	27.02
Chenopodium album L.	19.51	0.52	20.03
Commelina benghalensis L.	181.30	2.10	83.40
Daucus carota L.	40.17	0.59	40.76
Gynandropsis pentaphylla L.	42.65	1.28	43.93
Hibiscus cannabinus L.	33.97	0.17	34.14
Hydrocotyle asiatica L.	15.93	ND	15.93
Phyllanthus niruri L.	77.55	1.63	79.18
Piper betle L.	36.43	0.47	36.90
Raphanus sativus L.	22.30	0.75	23.05
Sesbania grandiflora (L.) Poir.	16.90	0.59	17.49
Solanum nigrum L.	84.38	ND	84.38
Talinum cuniefolium Willd.	89.79	1.22	91.01
Trianthema portulacastrum L.	41.51	0.44	41.95
Tribulus terrestris L.	56.39	0.01	56.40

[a]Data presented as mg/100g dry weight (Values are mean of three analysis).

*L= Lutein, **Z=Zeaxanthin, and ***L+Z = lutein + zeaxanthin

ND = Not detected (detection limit = 1pmol)

Table 3.2. Lutein and zeaxanthin levels in familiar leafy greens[a].

Botanical name	L*	Z**	L+Z***
Amaranthus gangeticus L.	20.26	0.21	20.47
Amaranthus tristis L.	25.41	0.19	25.60
Amaranthus viridis L.	90.43	0.16	90.59
Basella alba L.	113.82	1.76	115. 58
Beta vulgaris L.	26.76	0.14	26.90
Brassica oleracea var. botrytis L.	54.5	ND	54.50
Coriandrum sativum L.	99.27	ND	99.27
Lactuva sativa L.	87.12	ND	87.12
Mentha spicata L.	17.74	0.28	18.00
Moringa oleifera Lam.	50.40	4.13	54.53
Murraya koenigii L.	26.96	0.16	27.12
Peucedanum sowa Roxb.	92.99	2.25	95.24
Portulaca oleracea L.	50.84	0.99	51.78
Rumex acetocella L.	53.00	ND	53.00
Spinacia oleracea L.	77.58	1.51	79.09
Trigonella foenum graecum L.	59.60	0.95	60.55

[a]Data presented as mg/100g dry weight (Values are mean of three analysis).

*L= Lutein, **Z=Zeaxanthin, and ***L+Z = lutein + zeaxanthin

ND = Not detected (detection limit = 1pmol)

Table 3.3. Lutein and zeaxanthin content in selected vegetables and fruits[a] .

Botanical name	L*	Z**	L + Z***
Vegetables			
Coccinia cordifolia L. (Mill)	299.27	5.01	304.28
Capsicum annuum var. Grossa L.	425.16	5.33	430.49
Phaseolus coccineus L.	171.37	2.00	173.37
Momordica charantia L.	18.04	ND	18.04
Cymopsis tetragonoloba L.	105.06	ND	105.06
Luffa acutanguta (L.) Roxb.	857.27	13.59	870.8
Abelmoschus esculentus (l.) Moench.	347.70	8.20	355.9
Cucurbita maxima Duch.	153.44	ND	153.44
Pisum sativum L.	195.56	ND	195.56
Capsicum annuum L.	537.40	705.75	1243.15
Fruits			
Malus sylvestris (L.) Mill.	18.91	0.52	19.43
Vitis vinifera L.	11.88	ND	11.88
Psidium guajava L.	66.88	ND	66.88
Citrus auranatium L.	10.09	33.66	43.75
Mangifera indica L.	120.86	ND	120.86
Citrus aurantifolia (L.) Swingle.	104.81	ND	104.81
Carica papaya L.	94.72	20.32	115.04
Ananus comosus L.	1.63	2.36	3.99

[a]Data presented as mg/100g dry weight (Values are mean of three analysis).

*L= Lutein, **Z=Zeaxanthin, and ***L + Z = lutein + zeaxanthin

ND = Not detected (detection limit = 1 pmol)

Discussion

The method adapted for the determination of carotenoids in the GLVs, vegetables and fruits studies is simple, accurate and reliable. Kimura and Rodriguez-Amaya (2003) isolated these carotenoids from lettuce by HPLC under gradient condition, which required 50 minutes to separate them. They were separated within 22 minutes **(Figure 3.2-3.5)** under isocratic condition followed in this study. The purity of column-purified individual carotenoids on neutral alumina was 92% for neoxanthin, 94% for violaxanthin, 97% for L and Z, and 90% for β-carotene. β-Cryptoxanthin was not amenable for quantitation since they were below the detectable limit (0.1 pmol). The values were almost in consistent with the results of Kimura and Rodriguez-Amaya (2002), who purified carotenoids from leafy vegetable using open column chromatography on magnesium oxide: hyflosupercel (1:1). They have reported the purity as 91-97% for neoxanthin, 95-98% for violaxanthin, 92-96% for L, and 90-97% for β-carotene. The absorption maxima (λ_{max}) for xanthophylls and the hydrocarbon carotenoid isolated from leafy vegetables (**Table 3.4**) in the present study were found to be comparable with the reported values (Chen and Chen, 1992, Emenhiser et al. 1995). They have reported λ_{max} for L, 446 nm, β-carotene, 448 nm, violaxanthin 440 nm, neoxanthin 436 nm and for Z 452 nm.

Table 3.4. Reported and observed UV-visible absorption maxima (λ_{max}) for carotenoids isolated from leafy vegetables.

Carotenoids	λ_{max}[a]	λ_{max}[b, c]
Neoxanthin	438.5	436
Violaxanthin	446.4	440
β-Carotene	449.5	448
Lutein	445.7	446
Zeaxanthin	452.6	452[d]

[a]λ_{max} observed in the present study; [b]λ_{max} reported; [c]Chen and Chen (1992) [d]Emenhiser et al. (1995).

Further, L and Z were characterized by using LC-MS (APCI). Breithaupt et al. (2002) and Wingerath et al. (2006) also reported a similar pattern of mass spectra for L and its isomers using MALDI-MS and APCI-MS. The MS of L and Z eluted from the samples in this study were comparable with the MS of the respective reference standards **(Figure 3.8B)**.

The relative concentrations were found to be different compared with other leafy vegetables studied **(Table 3.1- 3.2)**. These differences may be related to species variations and various environmental factors. Chen and Chen (1992) have reported that factors like species, part of the plant, degree of maturity at harvest, cultivation and post harvest handling practices influence the carotenoids levels. Results revealed that less familiar GLVs possess equally or even higher level of L + Z compared to those of familiar ones. The differences in L + Z among greens studied may be attributed to species variations (Ismail and Fun, 2003). Khachik et al. (1986), Hart and Scott (1995) and Tee and Lim (1991) reported β-carotene level in *Spinacia oleracea, Spinacia pumila,* and *Amaranthus viridis* as 3.39, 3.17 and 6.71 mg/100g edible portions respectively. These are different from the values obtained in this study for the same species, which may be due to regional and cultivar differences. Whereas, in this study, the results on the β-carotene in those species was found to be slightly higher (data not shown). The difference in the level of L + Z between samples of cultivated and wild GLVs might be, further attributed to impact of morden agri/horticultural practices (Heinonen, 1990; Mercadante and Rodriguez-Amaya, 1991).

Neoxanthin, which is the most common allene isolated from the green leaves, has also been associated with violaxanthin and L. Earlier studies have reported a transformation in the end group of one of these carotenoids may perhaps be responsible for the formation of the allenic end group (Bonnett et al. 1969). The differences (%) between neoxanthin, violaxanthin and L among leafy vegetables studied may be attributed to the biosynthetic transformations (Britton, 1998; Burns et al. 2003). This study facilitated that among greens screened for carotenoid composition in general and L and Z in specific reveal that *C. benghalensis* and *T. foenum-graecum* could be recommended for supplementation of food as they were found to contain higher levels of L and Z among the GLVs analyzed. Hence, *T. foenum-graecum* was chosen as L source for animal feeding experiments. Further, less familiar GLVs those contained higher level

of L could be exploited as good source of L + Z to derive maximum health benefits for which awareness programs on the importance of those GLVs is needed at present.

The level of total xanthophylls separated in leafy vegetables covered in the present study is about 5-10 times greater than that of the hydrocarbon carotenes. Values for xanthophylls reported here for spinach is about 4-6 times greater than those reported by Khachik et al. (1986). Wills and Rangga (1996) also reported higher levels of xanthophylls in leafy vegetables, *A. tuberosum, A. tricolor* and *B. chinensis* than those of hydrocarbon carotenoids. Further, in general, the hydroxylation of the α-carotene is known to be responsible for the formation of 3-hydroxy cyclic carotenoids and epoxy carotenoids. The absence of α-carotene in the leafy vegetables studied here may therefore be related to the complete conversion of these compounds to L. This may be the reason for the higher content of L (ranged between 16-92 mg/100 g dry wt.) in all the leafy vegetables studied (**Table 3.1 and 3.2**). The level of L+Z in green beans and green peas was estimated to be 173.4 and 195 μg/100g edible portion and Z level in green peas was below the detectable limit (0.1 pmol) (**Table 3.3**). Humpheries and Khachik (2003) reported that L+Z levels in green beans and peas were significantly higher (418.35 and 717.51 μg/100g edible portions, respectivly) than the values obtained in the present study. These differences may be due to different cultivation practices and geographical variation.

HPLC results showed that under the conditions used, the greens, fruits and vegetable extracts contained several unknown, unidentified components eluted between 14 and 16 minutes. On the basis of their retention times and spectral characteristics, these are most likely to be xanthophyll esters, exhibiting identical UV-vis spectra, but are less polar and elute differently. Burns et al. (2003) also reported similar results under isocratic elution in mango and suggested that they could be xanthophylls based on their spectral characteristics.

In conclusion, this study gives L and Z composition of various GLVs, vegetables and fruits. Since low consumption of GLVs in the diet is one of the major factors, which leads to AMD and cataract, an attempt was made to identify and analyze the various underutilized GLVs for their L and Z content from selected regions of southern Karnataka. This is the first report showing HPLC and LC-MS data on L and Z of locally available GLVs. The procedure adapted here for the extraction and analysis of

carotenoids is relatively simple, reliable and accurate for the determination of carotenoids and vitamin A activity of the plant materials. This study shows that the leafy vegetables analyzed are found to contain significantly higher levels of xanthophylls. Interestingly, very rarely consumed GLVs are found to contain higher levels of L+Z. The data generated on the composition of carotenoids in the present study could be helpful to suggest the consumers for better source of L and Z as a part of daily meal to overcome health disorders like AMD and cataract. Further, underutilized GLVs having higher levels of L and Z can be exploited for nutritional purpose.

Chapter 4

Effect of dietary components (phospholipids, fatty acids and mixed carotenoids) on physicochemical properties of mixed micelles and liposome containing lutein

Introduction

Epidemiologic and animal studies have demonstrated that carotenoid-rich diets are associated with a number of health benefits (Clinton, 1998). The potential health benefits of carotenoids, such as their ability to act as antioxidants, immuno-enhancers and inhibitors of premalignant lesions have stimulated investigators' interest (Castenmiller and West, 1998). One of the most biologically plausible roles of carotenoids is the potential effect of dietary L and Z for protection of the *macula* from degeneration (Snodderly, 1995). These possible health benefits seem to relate to the unique geometry of carotenoids. Due to the large vitamin A–deficient population throughout the world, most carotenoid research has focused on hydrocarbon carotenes, particularly the provitamin A carotenes. In recent years, the focus has shifted to other carotenoids, particularly the oxycarotenoids (Castenmiller and West, 1998). Xanthophylls of current interest include L and Z. L and Z have been the primary focus because they are found specifically sequestered in the *macula* of the eye. In addition, there is evidence to suggest that L and Z may also play a role in the prevention of cancers (Yang et al. 1996) and cataract. The focus in this chapter was to study the effect of certain dietary factors on the physicochemical properties of mixed micelles containing L. In humans, the process of nutrient absorption requires movement of the digested food components into the mucosal cells of the intestinal wall **(Figure 1.6 in Chapter 1)**. Uptake occurs when the L or its metabolites enter the intestinal mucosal cells. Absorption is achieved with the movement of the L or its bioactive metabolite through the mucosal cells into the portal or lymphatic system. L bioavailability can be defined as the proportion of the ingested L that is made available (i.e., delivered to the bloodstream) for its intended mode of action. Four major events must take place for optimal absorption of L (Deming and Erdman, 1999). First, L must be released from their food matrix. The second step of absorption is the transfer of L to lipid micelles in the small intestine. This

requires the presence of dietary fat in the small intestine, which stimulates the gallbladder to release bile acids (i.e., emulsifiers). Bile acids are synthesized by the liver and are composed of both polar and non-polar ends, which allow binding of both lipophilic and hydrophilic molecules. Released L then must be assimilated into the mixed lipid micelles in the lumen of the small intestine, most likely orienting them at the micelle surface. Polar compounds make up the exterior of the micelle, acting as a carrier for the L to travel through the hydrophilic chyme in the intestine to the intestinal mucosal cell surface. The third step is uptake by intestinal mucosal cells. It is thought that L passively diffuses through the cell membrane and is released into the enterocyte (Mansbach et al.1975). The intestinal absorption of carotenoids depends upon the fat source in which they are dispersed (Borel et al.1996). The final step in absorption is the transport of the L or its metabolic product to the lymph system. In the golgi of the enterocyte, L is incorporated into chylomicrons. The chylomicrons are eventually delivered to the blood stream and through the action of lipoprotein lipase; chylomicrons lose triglyceride content and shrink in size. It is postulated (Deming and Erdman, 1999) that non-triglyceride components of the chylomicron, including surface molecules such as L may be taken up by extra hepatic tissues or transferred to other blood lipoproteins. Eventually, the chylomicron remnant, including the remaining L is taken up by the liver. L can then remain in the liver or be transported to the bloodstream by VLDL. They are then transferred to LDL and HDL with maturation of the lipoproteins. Tissues differentially take up L specifically accumulating in the *macula* region of the eye. Among carotenoids, L and Z are the major pigments found in the central *retina* of the human eyes and referred as macular pigments (Bone et al.1988). Clinical studies have shown the importance of L and Z in managing the age related macular degeneration (AMD). L and Z levels are reported to be lower in patients with AMD (Beatty et al.2001), which is due to poor absorption of those pigments from foods (Tyssandier et al. 2003).

Some major factors limiting the availability of L include physical disposition in food sources (food matrix), structure of the xanthophyll molecule and interaction of L with other nutrients (mainly dietary fat) and physical properties of micelles formed in the intestine (Castenmiller and West, 1998). Food processing methods such as grinding, fermentation and/or mild heating usually improves bioavailability, most likely as a result of weakening the cell wall of plant tissues, dissociating the protein-oxycarotenoid

complexes and/or dissolving the crystalline carotenoid complexes (Deming and Erdman, 1999). Factors such as general malnutrition or intestinal parasites have been found to substantially reduce the efficiency of carotenoid absorption (Olson, 1999).

Dietary fat is one of the factors that aid L and Z absorption by influencing the release of bile and formation of mixed micelles in the intestine (Borel et al.1996; Tyssandier et al. 2001). Studies have shown that the plasma β-carotene and L response was enhanced after a meal with sufficient fat (Tso, 1994; Jayarajan et al 1980). Earlier *in vitro* and *in vivo* studies have shown that micellar and dietary specific phospholipids and fatty acids markedly improved the plasma, liver and eye level of β-carotene and L in rodents and intestinal Caco-2 cells (Sugawara et al. 2001; Lakshminarayana et al. 2006; Raju et al. 2006). Cho et al. (2001) investigated the association between the fat intake and risk of AMD among human subjects. The above studies demonstrated that the matrix in which carotenoids are in the form of complexes and the physical properties (size and structure) of the complex are considered as the vital factors determining their bioavailability. Emulsification is a characteristic feature of fat (Armand et al.1994). Thus, lipid emulsification and/or micelle formation and their physical properties like size, structure and viscosity which occurs in the stomach and intestine, must be considered as a fundamental step in carotenoid absorption (Armand et al.1996; Tyssandier et al. 2001). Lindenstruth and Muller (2004) investigated the dietary parameters, which influences the droplet size of water/oil emulsions. Emulsification is a characteristic feature of fat (Borel et al. 1994). Properties like micelle size, structure and their organization in the fat are dependent on the nature of lipid in which the carotenoid is solubilized (Baskaran et al. 2003, Lakshminarayana et al. 2006 and Raju et al. 2006). *In vitro* studies have raised a notion that the extent of lipid emulsification/micelle formation affects the intestinal absorption of carotenoids (Carey, 1983). Nevertheless, little information is available on the physicochemical properties of mixed micelles used for enteral feeding. The aim of this study was to correlate the physicochemical properties (micellar size and L intensity in the hydrophobic area) of L solubilized in mixed micelles containing specific phospholipids (phosphatidylcholine, PC; lyso-phosphatidylcholine, LPC), fatty acids (oleic acid, OA and linoleic acid, LA) and mixed carotenoids (L and β-carotene) with L bioavailability.

Results

Effect of phospholipids and fatty acids on physicochemical properties of mixed micelles containing lutein

Micelles size (particle size)

The particle size of the mixed micelles containing L was determined using the particle size analyzer, based on static light scattering, which measures the angular dependence of the scattered light (Farinato and Rowell, 1983; Mikula, 1992). The particle size of mixed micelles containing L with phospholipids or fatty acid or mixed carotenoids is given in **Table 4.1 and Figure 4.1-4.3.** The cumulative percent distribution of various sized micelles in experimental micelles was compared with control micelles (**Figure 4.1-4.3).** Results show that the size of the micelles containing L with PC (13.5 μm) and LPC (9.6 μm) was higher than the control micelles with NoPL (5.2 μm). Further, the data demonstrated that the particle size of the PC micelles was significantly (p>0.05) higher by 28.8% and 45.8% than those of NoPL and LPC micelles. The particle size of micelles containing LPC was higher by 45.8% compared with NoPL group. Addition of β-carotene to the micelles containing PC resulted in 27.4% increase in their particle size demonstrating that addition of β-carotene with L affects the size of L micelles. The higher micelles size may delay or slow the permeation of them through intestinal mucosa. This could be the reason for the lower plasma (12.72 nM/h) or (12.0 nM/h) and liver (31.75 pmol/g/h) or (50.71 pmol/g/h) L levels (AUC) observed in rats fed with either PC or mixed carotenoids, respectively (results given in 5th Chapter).

Results also showed that, particle size of the L solubilized in PC was significantly (p>0.05) higher (26.2%) than mixed micelles containing LPC. Similarly, in case of mixed carotenoid (BC + L) solubilized in PC, the particle size was significantly higher (p>0.05) by 23.1% than the PC containing single carotenoid (lutein).

Results show that the size of the OA micelles containing L was higher by 17.6% than that of NoFA and 32% lower than LA micelles. Further, addition of β-carotene to the OA micelles resulted 45% increase in the micelles size compared to that of OA micelles with no added β-carotene (**Table 4.1**).

Table 4.1. Particle size of the mixed micelles* containing lutein with phospholipids or fatty acids.

Dietary components	Micellar components	Particle size (μm)
	M + L+ NoPL (C)	5.2 ± 0.8^{a}
Phospholipids	M + L + PC	13.5 ± 1.5^{b}
	M + L + LPC	9.6 ± 0.6^{c}
	PC + L	18.3 ± 3.1^{d}
	M (NoFA) ** + L (C)	$4.2 \pm 1.2^{a,e}$
Fatty acids	M (OA) + L	$5.1 \pm 0.8^{a,f}$
	M (LA) + L	7.5 ± 0.9^{g}
	M + BC + L + PC	18.6 ± 3.2^{h}
Mixed carotenoids	PC + L + BC	23.8 ± 2.8^{i}
	M (OA) + L+ BC	9.2 ± 0.6^{j}

*Values are mean ± SD (*n*=3). Values not sharing a common superscript within a **column** are significantly different at $p<0.05$. M- micellar solution containing monoolein, bile salt with oleic (OA) or linoleic acid (LA); L - lutein; BC- β-carotene; PC- phosphatidylcholine; LPC- lysophosphatidylcholine; NoPL- no phospholipids and NoFA- no fatty acid. **Micellar solution without fatty acids (OA and LA) and phospholipids (PC and LPC).

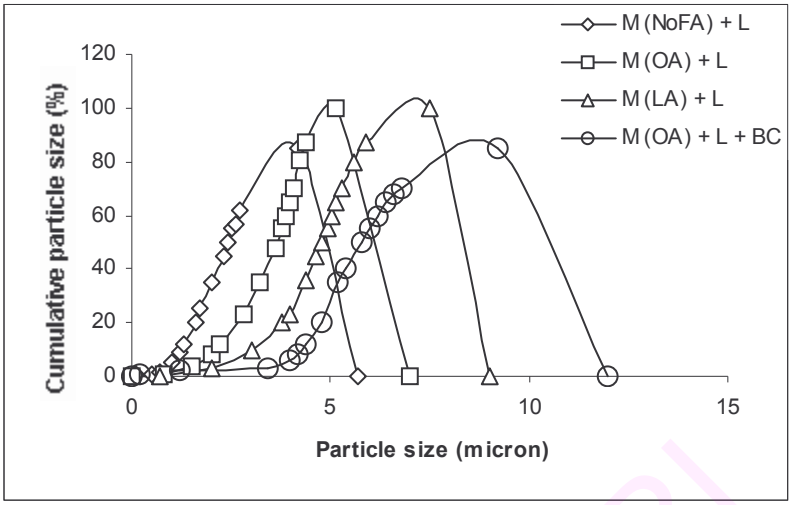


Figure 4.1. Particle size distribution among micellar solutions containing lutein with fatty acids. Micellar solution containing lutein with no fatty acid (◇), oleic acid (□), linoleic acid (△) and mixed carotenoids (O).

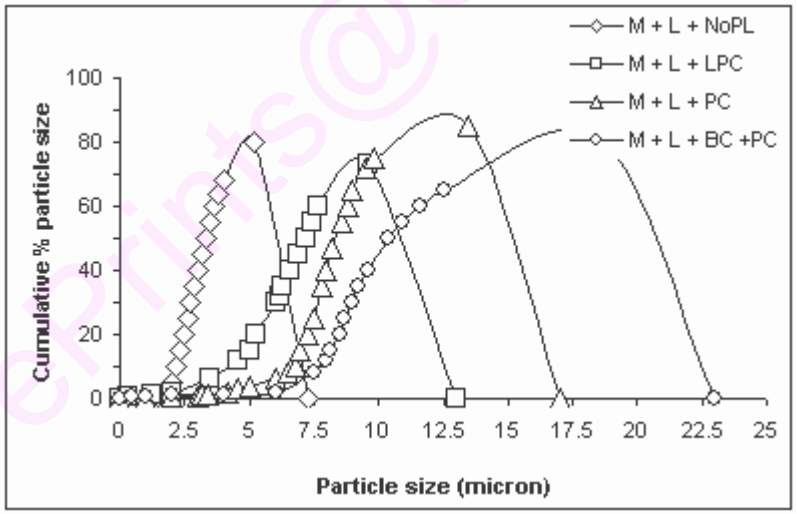


Figure 4.2. Particle size distribution among lutein solubilized mixed micelles containing phospholipids. Micellar solution containing lutein with no phospholipids (◇), lyso phosphatidylcholine (□), phosphatidylcholine (△) and mixed carotenoids (O).

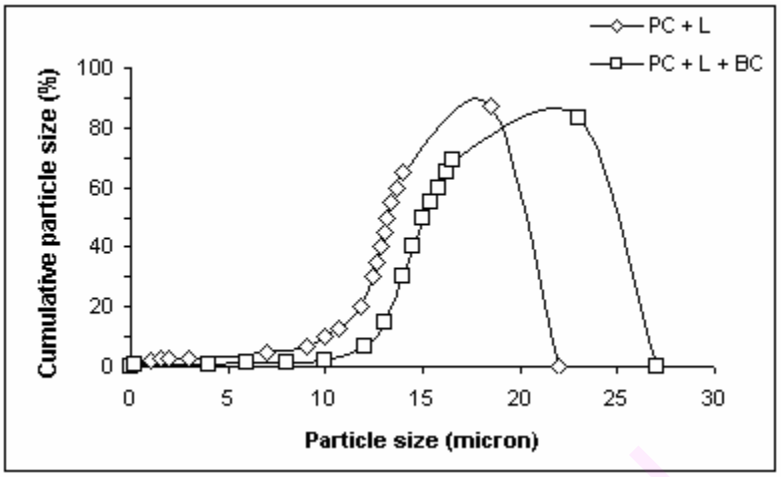


Figure 4.3. Particle size distribution among carotenoids solubilized in phospholipids (phosphatidylcholine). Lutein solubilized in phosphatidylcholine (◇) and mixed carotenoids (BC + L) solubilised in PC (□).

Micelles pH

A physiological variation in pH was postulated to affect L transfer from emulsion to the micelles. In this study, the pH of the micellar solution was measured at 37 ^{o}C using a digital pH meter equipped with temperature probe. Results show that the pH of control mixed micelles containing L was non-significantly altered by the addition of either phospholipids or fatty acids **(Table 4.2).** This slight variation in the pH of micellar solution might have significant effect on the L incorporation into the micelles. The results further demonstrate that pH of the micellar solution is one of the decisive factors like, size of the micelles which determines the intestinal uptake of L. In general, results show that there was no significant change in the pH of the phospholipids and fatty acids containing micellar solution. Addition of PC or LPC resulted in non-significant variation in the pH of respective micellar solution compared with NoPL micelles and the change calculated to be 0.2%. Whereas, micellar solution containing LA exhibited an increased pH by 2.34% and 0.44% compared with OA and NoFA micelles. The pH of the micellar solution containing mixed carotenoids (β-carotene and lutein) with PC had a lower pH by 0.14% compared with micelles containing L alone. But no change was observed in the pH between PC and LPC micellar solutions. The pH of PC mixed with mixed carotenoids (β-carotene and L) was found to have a higher value by 3.27% than micelles with L alone.

Similarly, in case of fatty acid micelles, OA micelles with mixed carotenoids had a lower pH by 0.44% than micelles with L alone.

Table 4.2. pH of the mixed micelles* containing lutein with phospholipids and fatty acids.

Dietary components	Micellar components	pH (37 ^{o}C)
Phospholipids	M + L+ NoPL (C)	6.78 ± 0.03
	M + L + PC	6.77 ± 0.06
	M + L + LPC	6.79 ± 0.07
	PC + L	6.80 ± 0.03
Fatty acids	M (NoFA) ** + L (C)	6.65 ± 0.06
	M (OA) + L	6.78 ± 0.03
	M (LA) + L	6.81 ± 0.02
Mixed carotenoids	M + BC + L + PC	6.76 ± 0.05
	PC + L + BC	7.03 ± 0.03
	M (OA) + L+ BC	6.75 ± 0.08

*Values are mean ± SD (n=3). M- micellar solution containing monoolein, bile salt with oleic (OA) or linoleic acid (LA); L- lutein; BC- β-carotene; PC-phosphatidylcholine; LPC-lysophosphatidylcholine; NoPL- no phospholipid and NoFA- no fatty acid. **Micellar solution without fatty acids (OA and LA) and phospholipids (PC and LPC).

Viscosity of micelles

The viscosity of the micellar solution containing lutein either with phospholipids, or fatty acids or mixed carotenoids (β-carotene + L) was determined using a rheometer containing co-axial or concentric cylindrical system. Shear rate (s^{-1}) was varied from 0.13 - 100 sec^{-1} at the temperature of 37 ± 0.5 ^{o}C. Shear stress (Pa) and apparent viscosity (m Pa) was measured taking 25 data points. The shear rate of 20 s^{-1} was used in this study. The viscosity of various micellar solutions containing **L** either with phospholipids or fatty acids or mixed carotenoids are shown in **Figures 4.4 and 4.5.** Results showed that the viscosity of control micellar solution was non-significantly increased after addition of either phospholipids or fatty acids. Addition of PC and LPC to the control micellar solution resulted in increased viscosity and it was 10.8% and 13.5% higher

compared to that of NoPL micelles. The viscosity of the micellar solution containing LPC was higher by 3.0% compared with PC micelles. Similarly, the viscosity of PC micelles was higher by 5.5% than micelles with PC and mixed carotenoids, whereas, the viscosity of micellar solution containing NoFA was lower by 2% and 11.3% compared with those of OA and LA micelles. It is seen from the results that inclusion of LA increased the viscosity of the micellar solution by 9.5% than OA micelles. Addition of mixed carotenoids to OA micelles resulted in a decrease in viscosity by 2% compared with OA micelles with L alone **(Table 4.3)**. Addition of mixed carotenoids (lutein and β-carotene) to the micellar solution containing PC or OA resulted in decreased viscosity and the effect was higher by 17.7% and 2% respectively compared with lutein alone with PC and OA group. The PC solutions containing mixed carotenoids decreased in viscosity by 5.17% compared with PC with lutein alone (**Table 4.3).**

Table 4.3. Viscosity of the mixed micelles* containing lutein with phospholipids and fatty acids.

Dietary components	Micellar components	Viscosity (m Pa s)
Phospholipids	M (OA)+ L+ NoPL (C)	47.8 ± 0.5^{a}
	M + L + PC	53.6 ± 0.8^{b}
	M + L + LPC	55.3 ± 5.6^{c}
	PC + L	$52.2 \pm 1.2^{b,d}$
Fatty acids	M (NoFA*) + L (C)	$49.5 \pm 4.5^{a,e}$
	M (OA) + L	$50.5 \pm 0.6^{e,f}$
	M (LA) + L	$55.8 \pm 4.8^{c,g}$
Mixed carotenoids	M (OA) + BC + L + PC	44.1 ± 3.5^{h}
	PC + L + BC	$49.3 \pm 1.3^{e,i}$
	M (OA) + L+ BC	$49.5 \pm 2.7^{e,j}$

*Values are mean ± SD (*n*=3). Values not sharing a common superscript within a row are significantly different at $p<0.05$. M – mixed micelles containing monoolein, bile salt with oleic (OA) or linoleic acid (LA); L- lutein; BC- β-carotene; PC- phosphatidylcholine;

LPC- lysophosphatidylcholine; NoPL-no phospholipids and NoFA-no fatty acid. *Micellar solution without fatty acids (OA and LA) and phospholipids (PC and LPC).

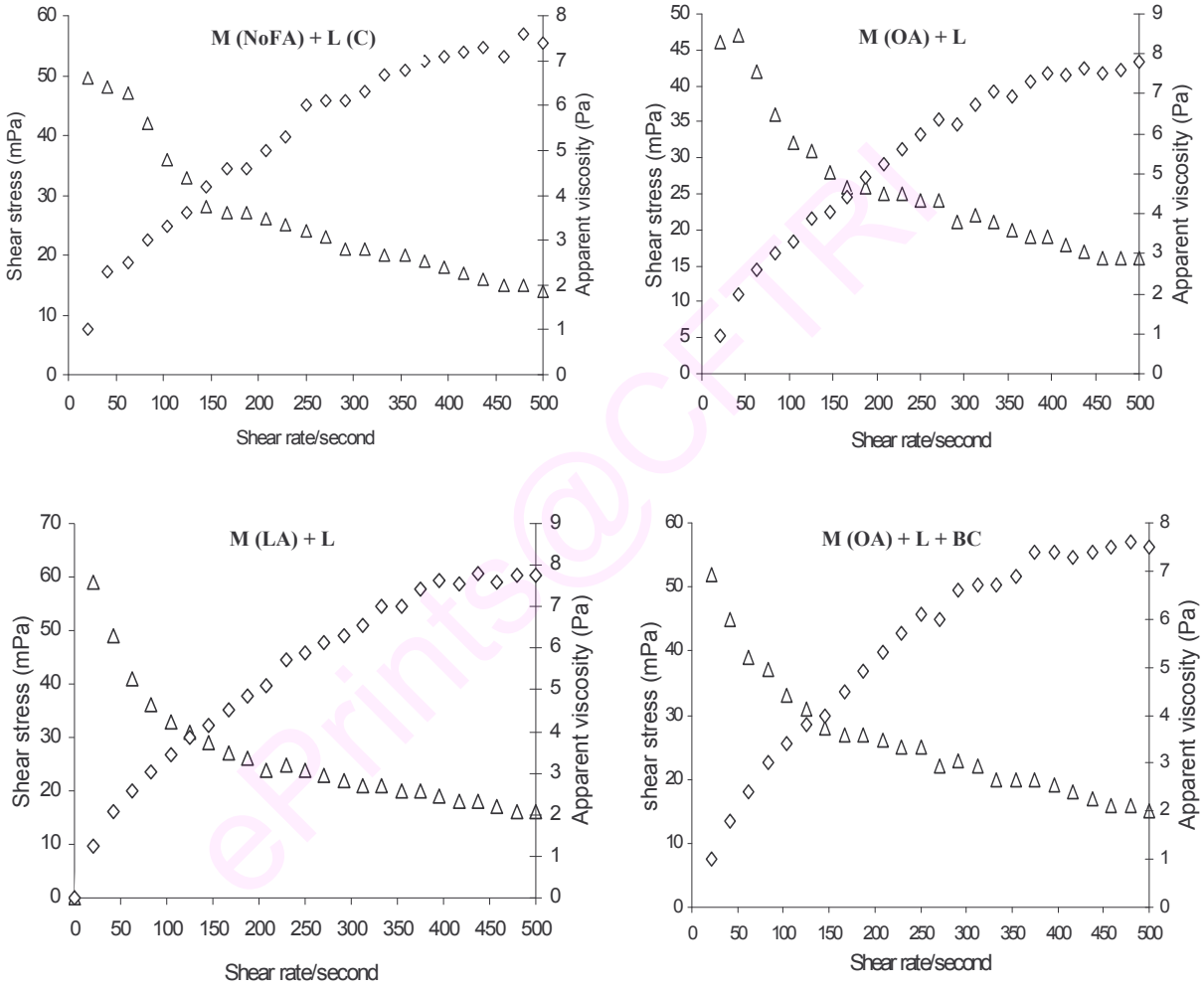


Figure 4.4. Viscosity of the micellar solutions containing lutein with phospholipids and fatty acids. M- micellar solution containing monoolein, bile salt with oleic (OA) or linoleic acid (LA); L- lutein; BC- β-carotene; NoFA-no fatty acid.

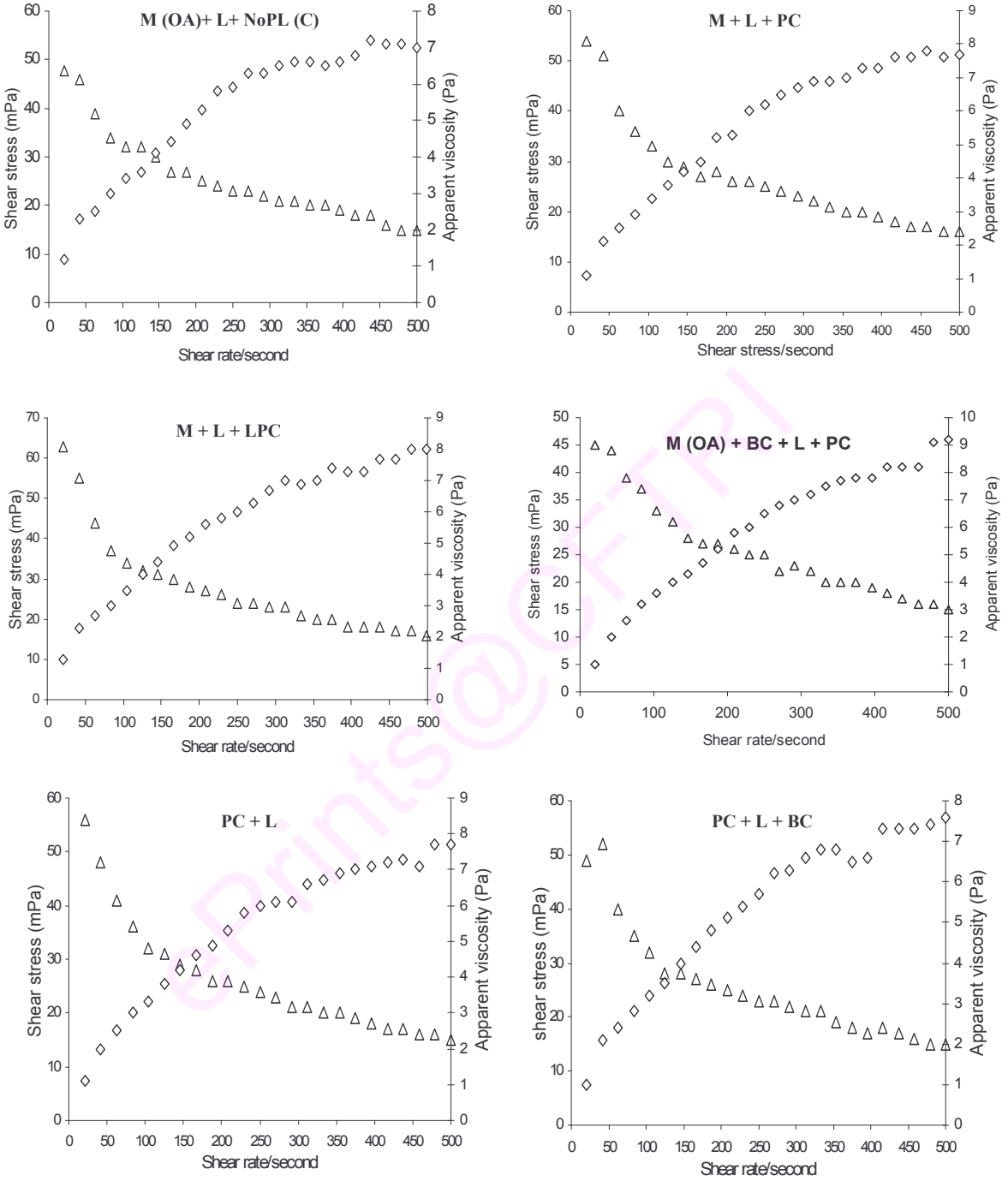


Figure 4.5. Viscosity of the micellar solutions containing lutein with phospholipids and fatty acids. M-micellar solution containing monoolein, bile salt with oleic (OA); L-lutein; BC-β-carotene; PC-phosphatidylcholine; LPC-lysophosphatidylcholine; NoPL-no phospholipid.

Micelles structure, size and L intensity within micelles

The structures of the mixed micelles was examined under phase contrast microscope and photographed at a magnification of 1000X using a camera equipped with phase contrast microscope. The photographs of phospholipids or fatty acids or mixed carotenoids micelles containing lutein are shown in **Figure 4.6.** The phase contrast microscopic structures of individual micelles containing L appeared to be globular in structure with central hydrophobic and outer hydrophilic phases. The LPC and OA micelles were found to contain uniform distribution of L in hydrophobic region compared with other micelles. In case of micelles with mixed carotenoids (β-carotene and L), the L deposition was mostly found at the outer core (next to central hydrophobic core), with less intensity compared with the β-carotene as determined by image processing technique (**Table 4.4**). The peripheral distribution of L is a characteristic feature of L due to its hydrophilic nature. To find out lutein intensity within the micelles, several batches (n=5) of mixed micelles were prepared under similar conditions and examined. The aim of this study was to find out the difference between phospholipids, fatty acids and mixed carotenoids on the L intensity within micelles. The images of the micelles were acquired with magnification at 1000X. To achieve reproducible data, the image acquisition was done very carefully using identical adjustments with respect to aperture stop and brightness. **Figure 4.7** illustrates the microscopic image of a typical micelle used to visualize the outer (hydrophilic region) core where L is distributed and central hydrophobic core. It is seen that the L intensity in the hydrophobic core of OA and LPC micelles is uniformly distributed compared with LA and PC micelles**.** The micelles inner (hydrophobic) and outer (hydrophilic) area was determined by image processing technique and represented as pixels. The RGB (pixels of red, green and blue) values of the micelles were considered for determining L intensity within micelles. The pixel intensity at the hydrophobic core of micelles represents the L intensity and its image is described using histogram equalization technique. The LPC micelles contained higher pixels in the inner core than the PC micelles. Adding β-carotene to PC micelles resulted in comparatively higher level of pixels in the outer core, which may be due to the orientation of L towards outer core compared with inner core (**Table 4.4**).

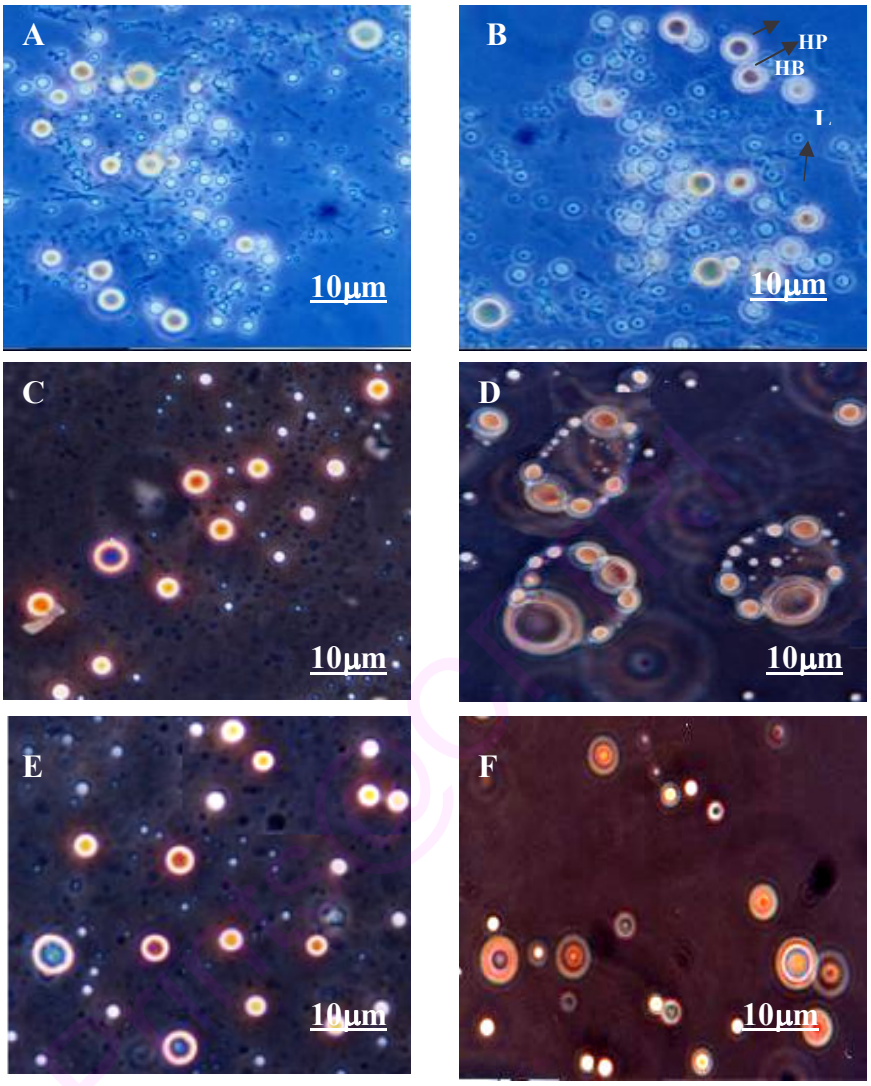


Figure 4.6. Phase contrast microscopic images (1000X) of mixed micelles containing L with phospholipids and fatty acids. (A) M + OA + L, (B) M + LA + L, (C) M + PC + L, (D) M + lysoPC + L and E) PC + L and F) PC + L + BC used to measure the lutein intensity. Where M-micelles*; L-lutein; BC-β-carotene; OA-oleic acid; LA-linoleic acid; PC-phosphatidylcholine and LPC- lysophosphatidylcholine.

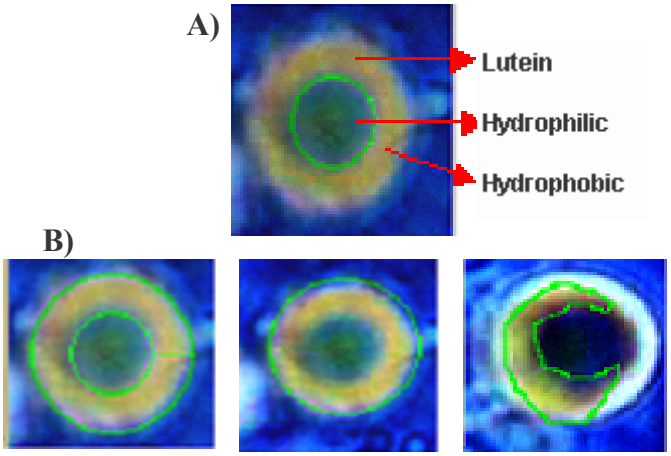


Figure 4.7. Structure of a typical mixed micelle (1000X) showing hydrophilic and hydrophobic regions **(A)** and mode of selection for measurement of L intensity and surface area of the hydrophilic core **(B).**

The relative lutein intensity within PC and LPC micelles were significantly (p<0.05) higher by 16.1% and 41.1% compared with NoPL micelles. Similarly, L intensity within the OA and LA micelles was slightly higher than NoFA micelles. L intensity was 44.7% higher in the case of PC micelles with L alone compared to that of micelles with mixed carotenoids. Similarly, intensity of the mixed carotenoids solubilized in PC was higher by 27.7% than single carotenoid (lutein) containing PC. In case fatty acid micelles, addition of β-carotene to the lutein solubilized in OA micelles was significantly (p<0.05) higher by 34.5% intensity than single carotenoid (lutein) solubilized in OA micelles (**Table 4.4).**

Table 4.4. Surface area and lutein intensity within micelles* containing phospholipids, fatty acids and mixed carotenoids as assessed by image processing technique.

Dietary factor	Micellar components	Area in pixels		Mean lutein intensity (I)**
		Inner core	Outer core	
Phospholipids	M + L+ NoPL (C)	373.2 ± 41.2	2250.2 ± 231.7	189.2 ± 8.9[a]
	M + L + PC	452.1 ± 21.5	3070.2 ±107.2	225.6 ± 9.2[a,b]
	M + L + LPC	652.4 ± 32.8	2700.4 ± 300.2	321.4 ± 8.7[c]
	PC + L	754.5 ± 65.3	4474.1 ± 145.7	287.7 ± 14.5[d]
Fatty acids	M (NoFA*) + L (C)	352.0 ± 25.2	2087.3 ± 178.2	210.5 ± 26.5[a,e]
	M (OA) + L	371.0 ± 10.4	2200.0 ± 427.1	209.8 ± 18.6[a, f]
	M (LA) + L	587.3 ± 14.1	1732.6 ± 464.2	190.5 ± 31.0 [a,g]
Mixed carotenoids	M + BC + L + PC	853.2 ± 123.4	5241.5 ± 354.1	407.2 ± 52.2 [h]
	PC + L + BC	865.2 ± 25.4	5130.7 ± 201.4	397.4 ± 15.2 [h,i]
	M (OA) + L+ BC	482.2 ± 47.2	2900.9 ± 174.5	320.5 ± 12.4 [c,j]

*Values are mean ± SD of 6 randomly selected micelles. The values not sharing common superscripts within a row are significantly different at $p<0.05$. M- micellar solution containing monoolein, bile salt with oleic (OA) or linoleic acid (LA); L-lutein; BC-β-carotene; PC- phosphatidylcholine; LPC-lysophosphatidylcholine; NoPL-no phospholipid and NoFA-no fatty acid. *Micellar solution without fatty acids (OA and LA) and phospholipids (PC and LPC). **I = (R+G+B)/3.

Discussion

Our studies (Chapter 5) have shown that phospholipids and fatty acids used in this study significantly enhanced the bioavailability of micellar and dietary L in rats (Lakshminaryana et al. 2006 and 2007; Raju et al. 2006). This study was conducted to investigate the role of dietary phospholipids, fatty acids and mixed carotenoid on the physicochemical properties (particle size, structure, pH and viscosity) of mixed micelles in which L was solubilised. To understand the possible role of these dietary components on the incorporation of L into the micelles, micelles were prepared and examined under phase contrast microscope. The microscopic images of mixed micelles were used for determining the L intensity within the micelles by image processing technique. Armand et al. (1999) reported that the size of micelles depends on its chemical composition, which in turn determines the rate of its diffusion through intestinal mucosa. Van het Hof et al. (2000) and Mekki et al. (2002) have reported that the emulsion droplet size is one of the factors that influence the intestinal uptake of carotenoids. According to Carey and Small (1970), mixed micellar solutions contain particles of sub-micron size (3-10 nm in diameter). The size of micelles used in the present study ranged from 5-23 μm. The relatively larger size of PC and LA micelles, as observed in the present study might be due to the presence of molecules other than bile salts, fatty acids and phospholipids used to prepare the micelles in which the L was solubilized. The size of micelles containing mixed carotenoids was found to be higher than the micelles with L alone. This may be due to the orientation of L (polar) on the outer hydrophilic region of the mixed micelles apart from the centrally located β-carotene. Addition of lecithin in the micelles increased the micellar size, which positively correlated with their decreased rate of diffusion through the intestinal epithelial cells (Litchfield et al. 1980). Reynier et al. (1985) showed that cholesterol uptake from mixed micelles containing lecithin was lower than lysolecithin containing micelles, which are similar in size. Sugawara et al. (2001) also reported that the apical uptake of carotenoids by Caco-2 cells was greater from micelles containing lysophosphatidylcholine as an alternative to phosphatidylcholine. Nature of fatty acids in micelles is also reported to affect the intestinal L and β-carotene uptake (Lakshminarayana et al. 2007; Raju et al. 2006). It has been suggested that the micelles containing LA or PUFA are larger in size which diffuse more slowly through the enterocytes thereby decreasing the rate of β-carotene absorption in human and rat

models than micelles containing saturated fatty acids (Raju et al. 2006; Kimura et al.1994).The present results suggests that the incorporation of L into the mixed micelles is mostly dependant on the chemical composition of micelles. Inclusion of specific phospholipids or fatty acids to the mixed micelles resulted in altered micellar size and the intensity of L within the micelles.

The structure (shape and size) of a mixed micelle depends both on the architecture of each constituent molecule within it and the solution conditions such as temperature, pH, presence of impurities, etc. Generally, the structure of micelles was reported to be spherical, cylindrical, ellipsoidal or disc-like in shape (Israelachvili et al. 1976). In the present study, the shape of mixed micelles with combination of dietary factors tested was found to be globular in shape as determined by phase contrast microscopy (**Figure 4.6**). The only difference observed among the micelles was the intensity of L incorporated into the mixed micelles, which was determined by image processing technique (**Table 4.4**). Transmission electron microscopic structure of bile salt-lecithin micelles showed disk like shape, which was constructed, based on a molecular model (Mazer et al. 1980). This model was proposed by Small (1967) in which the mixed micelle consists a disk like portion of lecithin bilayer surrounded on its perimeter of bile salt molecules, oriented with their hydrophilic surfaces in contact with the aqueous phase. In the present study, it was observed that the shape of the micelles appeared globular under phase contrast (light) microscopy as they are in solution. Meijer et al. (2001) pointed out that light microscopy and image processing can be used for the analysis of oil-in water emulsions. These principles can be adapted to determine the size and distribution of the oil droplets of multiple emulsions (Scherze et al. 2005).

The transfer of carotenoids into lipid droplets depends on the pH and viscosity of the intestinal content and the composition of the micelles. The pH probably also play a role on lipase activity and solubility of micelles (Tyssandier et al. 2001). In the present study, pH of the OA micelles was kept constant. Inclusion of dietary factors (phospholipids and fatty acids) to the OA micelles resulted in non-significant alterations in the micellar pH. Tyssandier et al. (2001) investigated transfer of carotenoids *in vitro* from lipid droplet emulsions to micelles when incubated with lipase, co-lipase and bile salts to simulate the environment of the small intestinal lumen. They have reported that the transfer of β-carotene and L were maximum at pH 6-7, with bile salt concentrations

of 2-8 mM. In the present study, no significant difference was noticed in the pH of micelles at a constant temperature except the PC micelles containing mixed carotenoids. Moreover, the efficiency of micellarization of L was significantly higher than that of β-carotene and lycopene as reported by Tyssandier et al. (2001), suggesting that transfer of micelles across the intestinal mucosa *in vitro* was inversely proportional to hydrophobicity of the carotenoids. Thus, the association between carotenoid species and the efficiency of micellarization *in vitro* was similar to that observed in human duodenum aspirates (Tyssandier et al. 2002). The investigators also reported that the presence of mixed carotenoids in the lipid droplet decreased the transfer of individual carotenoid to the micelle. This observation suggests that there may be interaction between carotenoids during the process of micelle formation and absorption *in vivo* (Gartner et al.1996). Although, L and β-carotene share a common lipophilic characteristic, their structural variation may affect the processes of absorption (Borel et al. 1996).

The viscosity of the micellar solution is reported to affect the solubilization of carotenoids which inturn interferes with their bioavailability. The water-soluble fibers like pectin, guar and alginate were reported to decrease the absorption of β-carotene, lycopene and lutein (Rock and Swendseid, 1992; Riedl et al. 1999). Possible mechanisms responsible for the fiber-mediated decrease in carotenoid bioavailability include decreased micellarization due to binding of bile acids and phospholipids, inhibition of lipase activity, increased viscosity and volume of luminal contents and increased rate of transit of enterocytes along the villus (Riedl et al. 1999). In the present study, the viscosity of different micelles was found to be altered by the inclusion of dietary fatty acids and phospholipids which could be one of the reasons for an altered L absorption in rats **(Chapter 5).**

From the results, it is clear that phospholipids and fatty acids used in this study interfere with the physicochemical properties of mixed micelles. Addition of either LA or PC to the mixed micelles resulted in increased size, and slightly altered the pH and viscosity of micelles. The intensity within the micelles was also found to be different among the micelles type with lower level of L in the LA and PC micelles than OA and LPC micelles. These effects may be due to the orientation of L in micelles containing phospholipids and fatty acids.

Chapter 5

Effect of dietary components (phospholipids, fatty acids and mixed carotenoids) on lutein bioavailability and certain biochemical parameters in plasma and tissues

Introduction

Dietary ingestion is the only source for lutein (L), as the human body cannot synthesize it. L is ingested along with other carotenoids from food. In general, of the dietary carotenoids ingested, only a fraction is available for normal physiological functions since, they are poorly absorbed. Bioavailability of L is influenced by many exo- and endogenous factors, and the extent to which it is influenced may differ for each carotene (Furr and Clark, 1997). Most of the studies available in the literature investigated the absorption of carotenoids; *in vitro* or *in vivo* have focused mainly on β-carotene (Brown et al. 1989; Johnson and Russell, 1992) for the reason it is cleaved into retinol. Riso et al. (2003) and Lienau et al. (2003) have specifically examined the uptake and transfer thereafter of L by the intestine into plasma but other studies have examined the absorption and/or interaction of carotenoids (Bierer et al. 1995; Gartner et al. 1996). However, knowledge on the specific dietary factors affecting or influencing the intestinal absorption, carrier mediated transportation and metabolism of carotenoids is scanty in the literature. Studies conducted with L supplements fed to human subjects indicated that food matrix is an important factor that interferes with L bioavailability (Kostic et al. 1995; Handelman et al.1999). Herdren et al. (2002) and Scheveigert et al. (2000) reported that intestinal absorption of carotenoids depends on the concentration and origin of dietary fat consumed along with them. For example, rats fed a low fat diet did not absorb β-carotene efficiently, whereas they do so when the diet contained 10% fat (Jayarajan et al. 1980). Baskaran et al. (2003) and Sugawara et al. (2001) reported that specific phospholipids in mixed micelles influence the intestinal uptake of β-carotene and L in mice and human intestinal Caco-2 cells, respectively. Recent nutritional studies have demonstrated that L supplementation was significantly related with the progression of age-related macular degeneration (AMD). Patients with AMD are reported to have

lower plasma L level, due to poor intestinal absorption from foods (Bone et al. 1988; Beatty et al. 2000). The above literature survey clearly reveals that dietary components/factors are prerequisite and associated with L absorption, and are of current interest.

Dietary fat is one of the components known to improve the absorption of L (Dimitrov et al.1988). Dietary fat reported to influence the release of bile from hepatocytes and the formation of emulsification and mixed micelles in the intestine (Tso, 1994; Roodenberg et al. 2000). Studies also showed that the plasma β-carotene and L response improved notably after a meal with sufficient fat, but reduced when dietary fat is absent or too low (Jayarajan et al.1980; Van het Hof et al. 2000). Raju et al. (2005) reported that micellar lysophosphatidylcholine greatly enhanced the plasma level of β-carotene in rats whereas the intestinal absorption of lipid soluble components like cholesterol, fatty acids and α-tocopherol were relatively suppressed by the inclusion of phosphatidylcholine either with diet or mixed micelles in rats (Rampone and Long, 1977; Thomson and Cleland, 1981).

Dietary studies also compared the influence of vegetable oils on carotenoids bioavailability in animal and human subjects. Hu et al. (2000) studied the effect of dietary sunflower oil and beef tallow on the level of β-carotene in plasma triacylglycerides-rich lipoproteins in women subjects. Clark et al. (2000) investigated the influence of corn and olive oil on lycopene and astaxanthin bioavailability in rats. Borel et al. (1998) studied the effect of medium and long chain triglycerides on chylomicron β-carotene level and plasma vitamin A response in men. Hollander and Ruble (1978) compared the effect of PUFA and MUFA on the micellar β-carotene uptake in rat. Despite the importance of dietary lipids as carrier for hydroxy carotenoids, for example, L, little is known about their influence on L bioavailability. Hence, it is appropriate to explore the possibilities of finding suitable dietary components, which can help in improving L bioavailability through dietary means so as to improve visual function and to conquer the problem of AMD.

The aim of this study was to evaluate the influence of dietary lipids such as, phospholipids [phosphatidylcholine (PC) and lysophsophatidylcholine (lysoPC)], fatty acids [oleic acid (OA) and linoleic acid (LA)], vegetable oils [sunflower oil (SFO) and olive oil (OO)] and mixed carotenoids (β-carotene and L) on the L bioavailability in L sufficient and deficient rats.

Results

Gavage studies (Single dose)

Influence of phospholipids on L bioavailability. HPLC profiles of L in plasma and liver samples of rats after a dose of micellar L are shown in **Figure 5.1**. The postprandial L level in plasma and liver was calculated from the HPLC chromatogram. L levels in the plasma after administration of a dose of phospholipids micelles containing L to rats are shown in **Figure 5.2A.** The results indicated that NoPL (control) and PC groups contained maximal levels of L (3.45 and 2.85 pmol/mL) at 1h after gavage, whereas in case of LPC group, it was 4.38 pmol/mL at 2h after gavage. The average level of L at 1 to 6h after gavage was significantly different (p<0.05), while it was not different at 6 to 9h among the three groups by two-way ANOVA. The average level (AUC) of L in the plasma of PC group (1.61 pmol/mL) during 9 h after gavage was significantly (p<0.05) lower (24.84 and 75.16%) than those in the NoPL and LPC groups (2.01 and 2.82 pmol/mL) respectively. Consistent with the average plasma L levels, the AUC values of L in PC group (12.72 pmol/mL/h) was lower (p<0.05) than those in the NoPL (15.85 pmol/mL/h) and LPC (22.27 pmol/mL/h) groups **(Table 5.1).** Plasma L was not detectable before micellar intubations at 0 h (base line value).

The concentration of L in the liver after micellar L administration by gavage is shown in **Figure 5.2B.** In all the groups, the L levels reached maximum at 3 h after its administration. The maximum L levels in the NoPL, PC and LPC groups were worked out to be 8.23, 7.17 and 15.68 pmol/g, respectively, with significant difference between NoPL and other two groups. Interestingly, the L level of the LPC group remained higher up to 9 h after post dose, unlike in the other two groups, those decreased significantly (p<0.05) 6 h after administration of micellar L. The average value of L after 9 h gavages (AUC) in the NoPL, PC and LPC groups were 6.97, 6.04 and 10.37 pmol/g, respectively, and were significantly different between the groups (p<0.05) by two-way ANOVA. Consistent with the mean values, the AUC values of L in PC group (50.71 pmol/g/h) was lower by 13.3% and 41.7% than NoPL (58.52 pmol/g/h) and LPC (87.08 pmol/g/h) groups, respectively and found to be in the order of LPC>NoPL>PC **(Table 5.1A and Figure 5.3).**

Influence of phospholipids on plasma triacylglycerides. The plasma triglycerides (TG) level (corrected with zero time control) after a single dose of micellar L containing either PC or LPC or NoPL are shown in **Figure 5.2C**. The level of plasma TG of LPC (48.2 ± 5.2 mg/dL) group was significantly (p<0.05) higher than PC (39.6 ± 8.2 mg/dL) and NoPL (36.9 ± 7.4 mg/dL) groups. Further, after 2 h of micellar L administration the TG level marginally decreased from the maximum level (**Figure 5.2C**). The higher level of TG in LPC than PC group indicates the involvement of TG for the transport of newly absorbed and higher level of L.

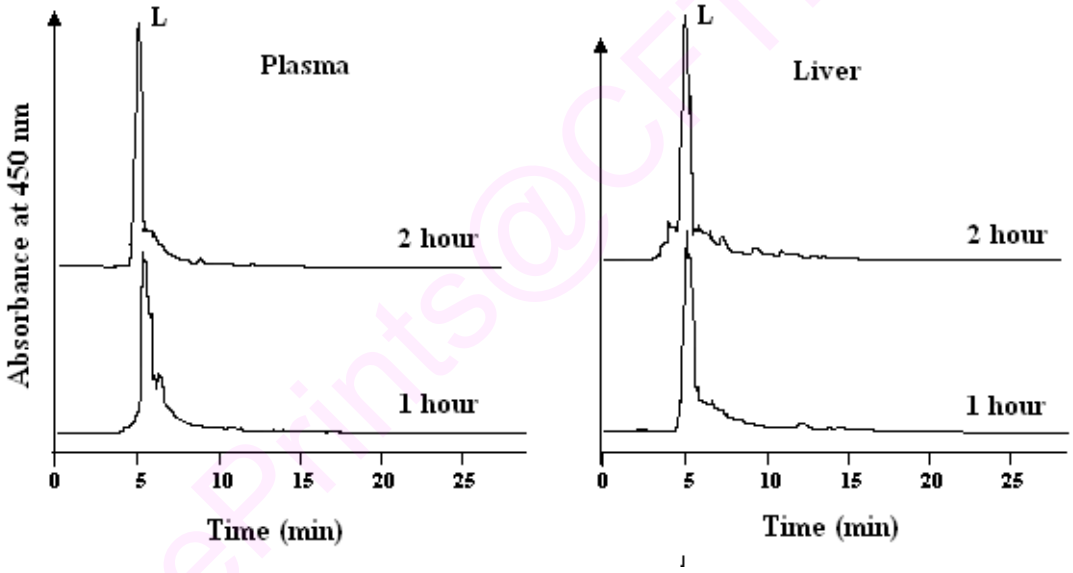


Figure 5.1. HPLC elution of lutein in plasma and liver of rats after single dose admistration of L solubilized mixed micelles with phospholipids.

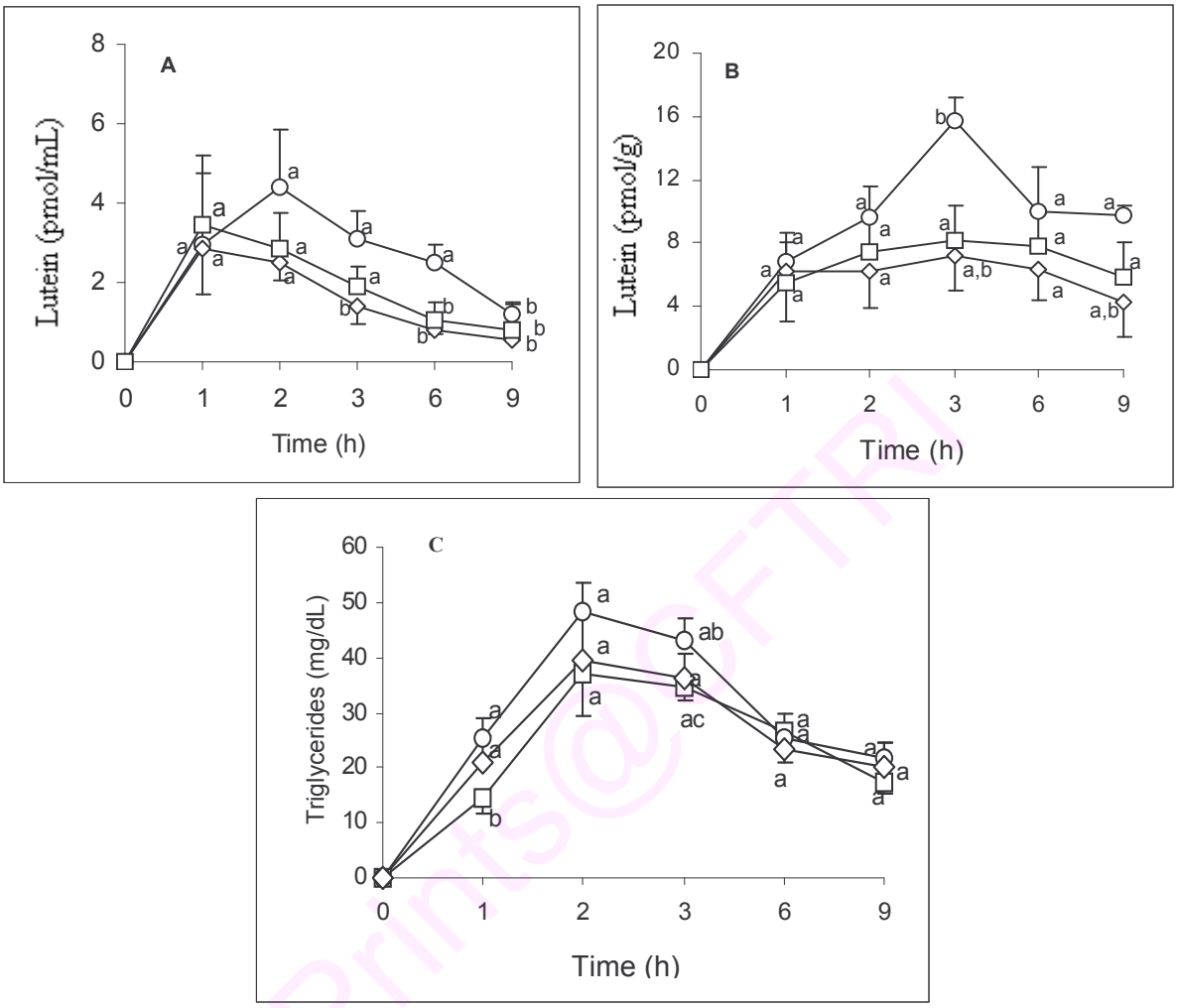


Figure 5.2. Postprandial response of lutein levels in Plasma (A), liver (B) and plasma levels of triglycerides (C) in rats after the administration of a single dose lutein solubilized in mixed micelles* with phospholipids.

*Mixed micelles were composed of 2.5 mM monooleylglycerol, 7.5 mM oleic acid, 12 mM sodium taurocholate and 200 μM L with 3 mM PC (◇), lysoPC (O) or no phospholipids (□). Rats were fed micelles (0.2 mL) and then sacrificed after various time intervals. L in the plasma and liver were analyzed by HPLC. Data represent the mean ± SD (n=6). The values at each time point not sharing a common letter are significantly different (p<0.05) between groups as determined by two-way ANOVA and Tukey's test after log transformation. L at 0 h was not detected (ND).

Table 5.1. Area under the curve (AUC) for lutein levels in the plasma and liver of rats* over 9h after admistration of lutein solubilized in mixed micelles containing different dietary lipids and mixed carotenoids **.

Groups	Plasma (pmol/mL/h)	Liver (pmol/g/h)
	A. Phospholipids	
NoPL	15.85 ± 1.23[a]	58.52 ± 4.31[a]
PC	12.72 ± 2.05[b]	50.71 ± 6.87[b]
LPC	22.27 ± 1.80[c]	87.08 ± 3.98[c]
	B. Fatty acids	
OA	15.9 ± 1.35[a]	55.0 ± 2.86[a]
LA	13.5 ± 0.75[a]	38.3 ± 2.65[b]
	C. Mixed carotenoids	
OA (L)	15.9 ± 1.35[a]	55.0 ± 2.86[a]
OA (L +BC)	12 0 ± 1.25[b]	31.75±7.52[b]

*Values are Mean ± SD (n = 6). Values not sharing a common superscript within a column are statistically significant (p<0.05). **NoPL, group fed mixed micelles containing no phospholipids: PC, group fed mixed micelles containing phosphatidylcholine: LPC, group fed mixed micelles containing lysophosphatidylcholine: OA, group fed mixed micelles containing oleic acid: LA, group fed mixed micelles containing linoleic acid: OA (L) group fed mixed micelles containing oleic acid and L: OA (L + BC) group fed mixed micelles containing oleic acid, L and β-carotene. L was not detected in plasma of rats before intubations.

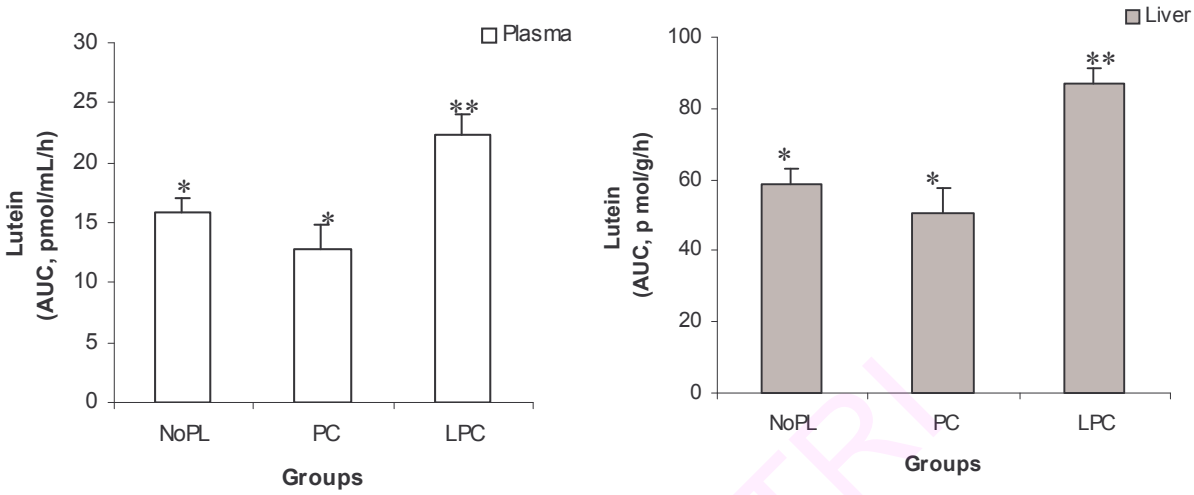


Figure 5.3. Area under the curve for lutein levels in the plasma and liver of rats* over 9 h after administration of lutein solubilized in phospholipids mixed micelles.

*Values are Mean ± SD of 6 rats. Values not sharing common superscripts within similar bars are significantly different $p<0.05$.

Influence of fatty acids on L bioavailability. HPLC profiles of L in plasma and liver samples after a single dose of micellar L containing either OA or LA are shown in **Figure 5.4.** Concentration of L in plasma and liver was calculated from the respective HPLC chromatogram. The postprandial plasma and liver L response 9 h after gavage of micellar L is shown in **Figure 5.5A and 5.5B**. L was not detected at 0 h (base line value), but after gavage, its level reached a maximum of 3.5 and 2.9 pmol/mL ($p<0.05$) in plasma at 2 h and 8.2 pmol/g, 6.4 pmol/g in liver at 3 h of OA and LA groups, respectively. Later the plasma L level decreased from 3 to 9 h ($p>0.05$), whereas its level in liver remained higher in OA group than LA group, in which the difference between the groups was significant (38-45%) after 6 h. The mean AUC values of L in plasma and liver after 9 h gavage of OA and LA micelles were recorded as 15.9 pmol/mL/h, 38.3 pmol/g/h and 13.5 pmol/mL/h, 55.0 pmol/g/h, respectively. From the results it is seen that the AUC value of OA group was higher ($p<0.05$) by 17.8% (plasma) and 44.3% (liver) than LA group (**Table 5.1B and Figure 5.6)**.

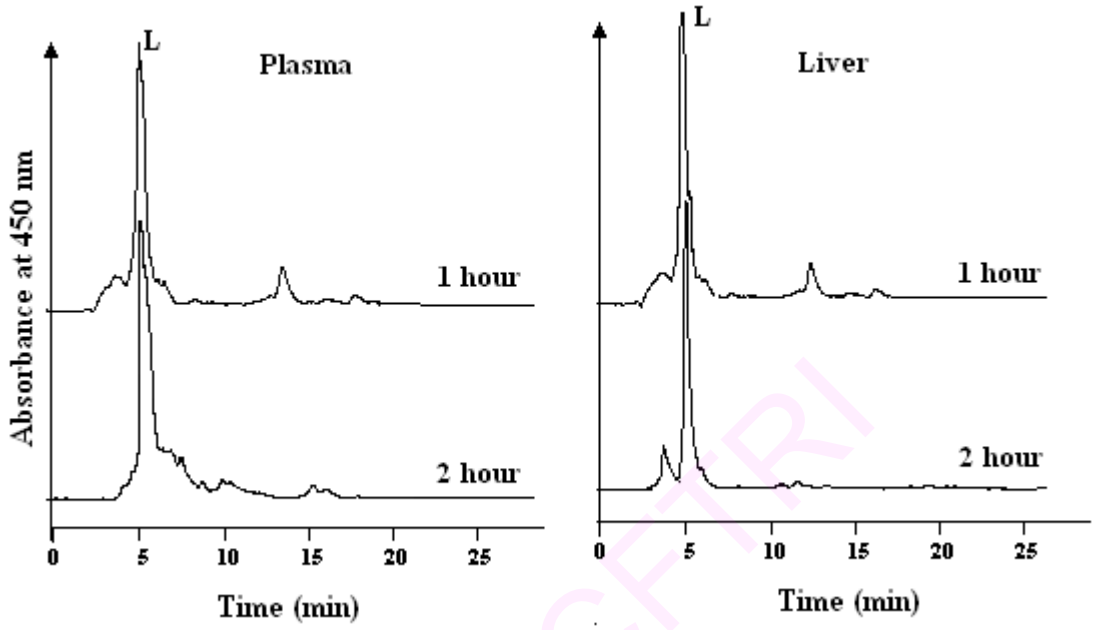


Figure 5.4. HPLC elution of lutein in plasma and liver of rats after a single dose of lutein solubilized in mixed micelles containing oleic acid.

Influence of fatty acids on plasma TG. The plasma TG level (corrected with zero hour control) after a single dose of micellar L containing either OA or LA are shown in **Figure 5.5C.** From the results, it is evident that the level of TG in plasma of OA group was significantly (p<0.05) higher than LA group. Akin to plasma L, the mean TG level peaked at 2 h in OA (69.0 ± 13.2 mg/dL) and LA (49.1 ± 6.2 mg/dL) groups, and then marginally decreased (p>0.05) from the maximum level. The TG level was higher by 40.5% in OA than LA group indicating their requirement for the transport of newly absorbed L (**Figure 5.5C)**.

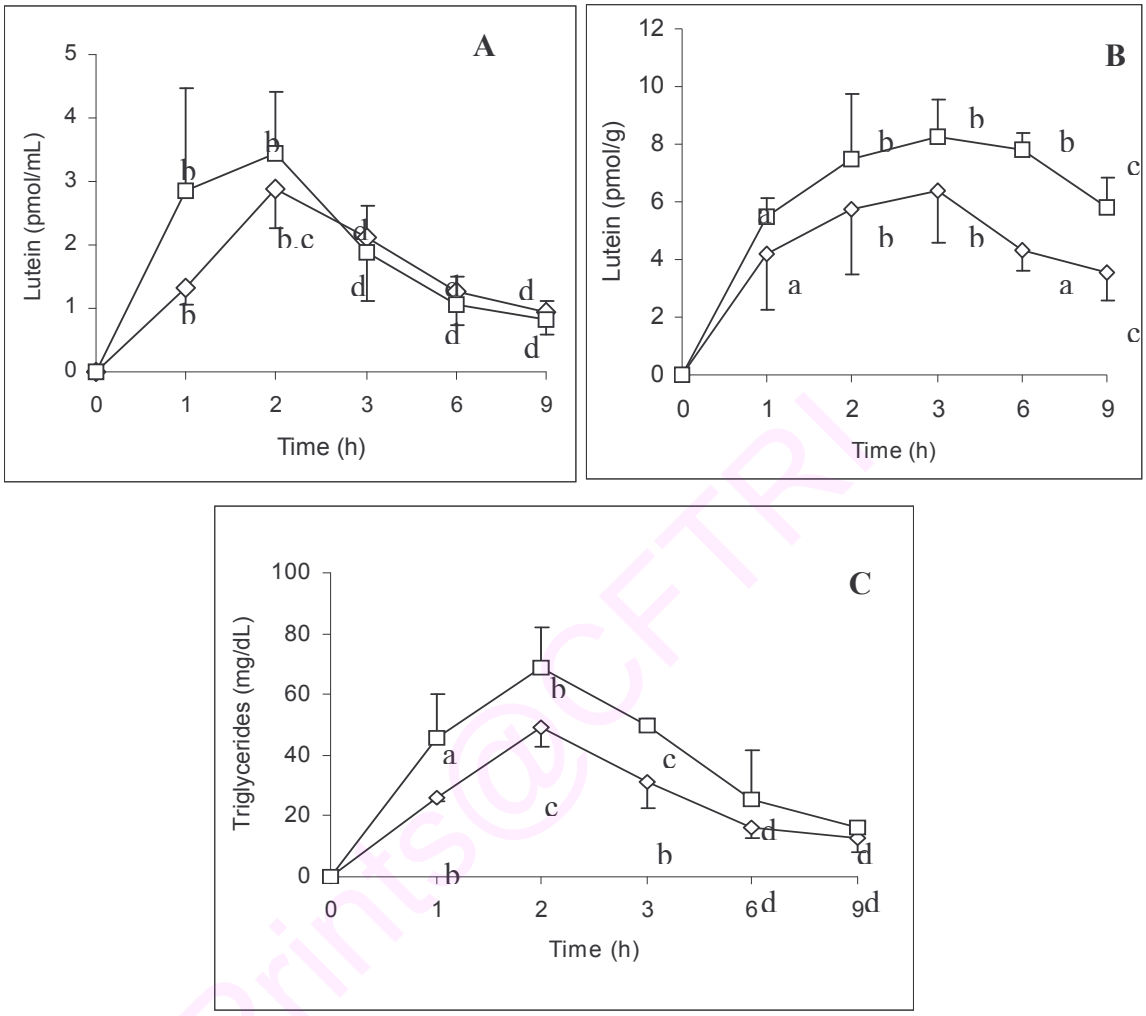


Figure 5.5. Postprandial response of lutein levels in plasma (A), liver (B) and plasma levels of triglycerides (C) in rats after the admistration of a single dose lutein solubilized in fatty acids mixed micelles *.

*Micelles were composed of 2.5 mM monooleoyl glycerol, 12 mM sodium taurocholate, and 200 μM L with 7.5 mM oleic acid (OA, □) or linoleic acid (LA,◇). Data represents the mean±SD (n=6). Values at each time point not sharing common letters are significantly different (p<0.05) between groups determined by ANOVA with Tukey's test after log transformation. No lutein was detected in the plasma at 0 h.

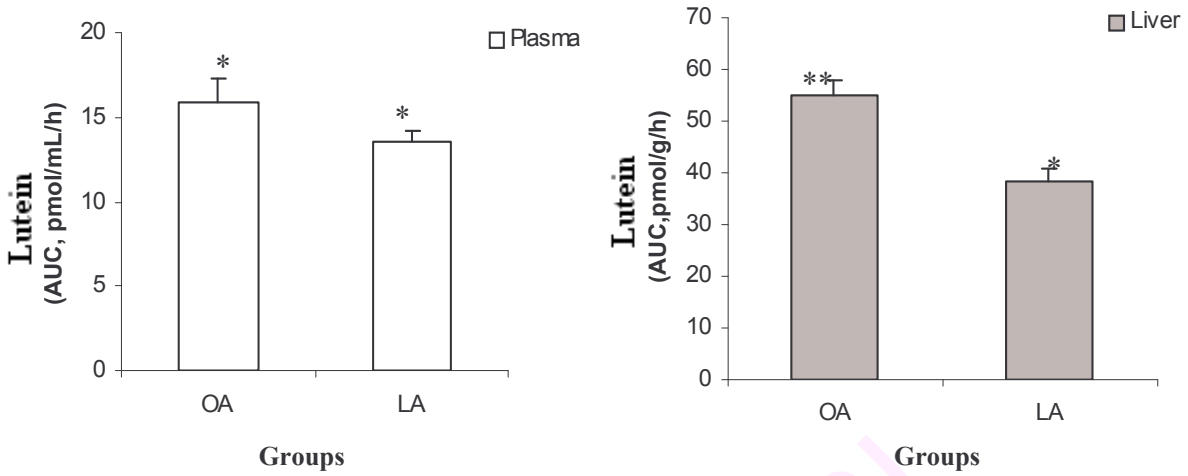


Figure 5.6. Area under the curve for lutein levels in the plasma and liver of rats* over 9 h after administration of lutein solubilized in fatty acids mixed micelles.

*Values are mean ± SD (n = 6). Bars not sharing a common superscript are statistically significant (p<0.05).

Effect of β-carotene on L bioavailability. HPLC profiles of L and β-carotene in plasma and liver samples after a dose of mixed carotenoids (L + β-carotene) are shown in **Figure 5.7**. L level in plasma and liver was calculated from the HPLC chromatogram. The plasma L levels after administration of micellar L alone or L along with equimolar concentration of β-carotene solubilized in OA micelles are shown in **Figure 5.8A**. Results indicated that the maximal level of plasma L was 3.45 and 2.92 pmol/mL, at 2 h after gavage. Further, an average level of L from 1 to 6 h after gavage was significantly different (p<0.05), while it was not different from 6 to 9 h between the groups by two-way ANOVA. Consistent with the average plasma L levels, the AUC value of L group (15.9 pmol/mL/h) was lower (24.5%) than that of L + β-carotene group (12.0 pmol/mL/h) indicating the interference of β-carotene with L absorption (**Table 5.1C and Figure 5.9**).

The liver L levels after gavage of micellar L and L + β-carotene are shown in **Figure 5.8B**. The levels at absorption phase reached maximum at 3 h in L (8.2 ± 1.8 pmol/g) and L + β-carotene (4.32 ± 1.8pmol/g) group, which was found to be significantly different between the groups. In case of L group, the L level remain higher, unlike in the other group, which decreased significantly (p<0.05) from 6 h after gavage. The AUC

values of L after 9 h gavage in the L and L + β-carotene groups were 55.0 and 31.75 pmol/g/h, respectively and were significantly (p<0.05) different (42.2%) between the groups by one-way ANOVA.

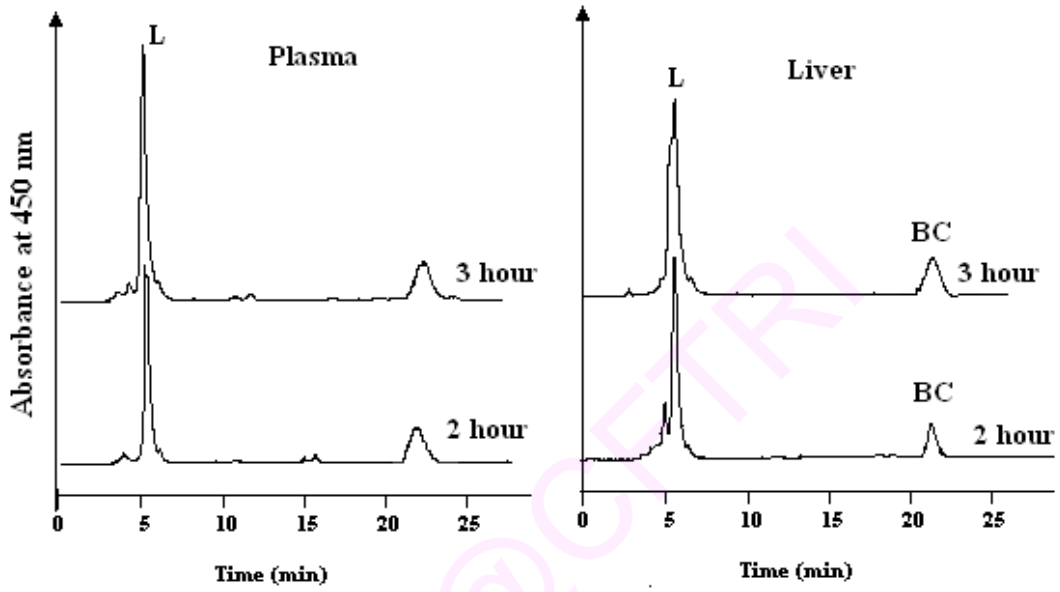


Figure 5.7. HPLC elution of lutein in plasma and liver of rats after a single dose of lutein and β-carotene solubilized in OA mixed micelles.

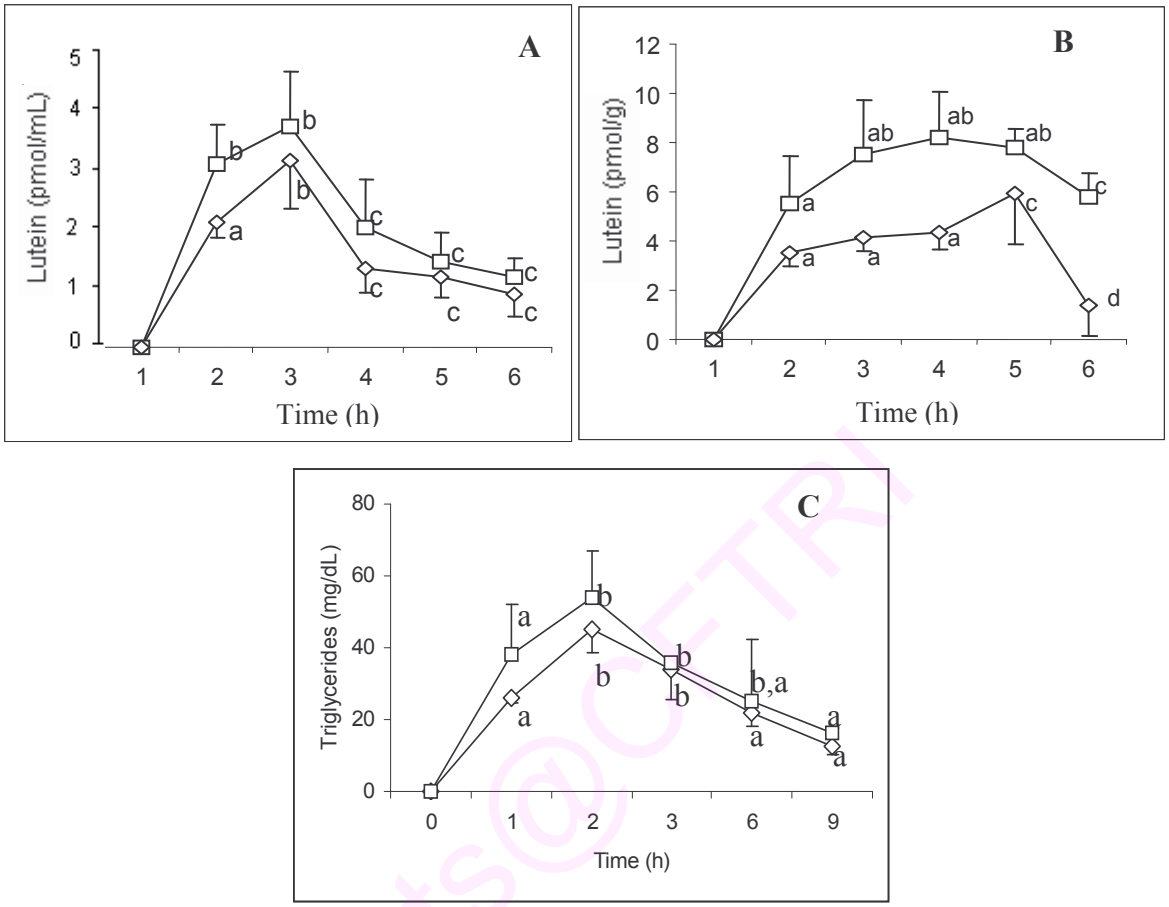


Figure 5.8. Postprandial response of lutein levels in plasma (A), liver (B) and plasma levels of triglycerides (C) in rats after the admistration of a single dose of OA mixed micelles* containing either lutein (□) or mixed carotenoids (◇).

*Micelles were composed of 2.5 mM monooleoyl glycerol, 12 mM sodium taurocholate, 7.5 mM oleic acid and with 200 μM lutein (□) or lutein + β-carotene (◇). Data represents the mean ± SD (n=6). Values at each time point not sharing a common letters are significantly different (p<0.05) between groups determined by ANOVA with Tukey's test after log transformation. No L was detected in the plasma at 0 h.

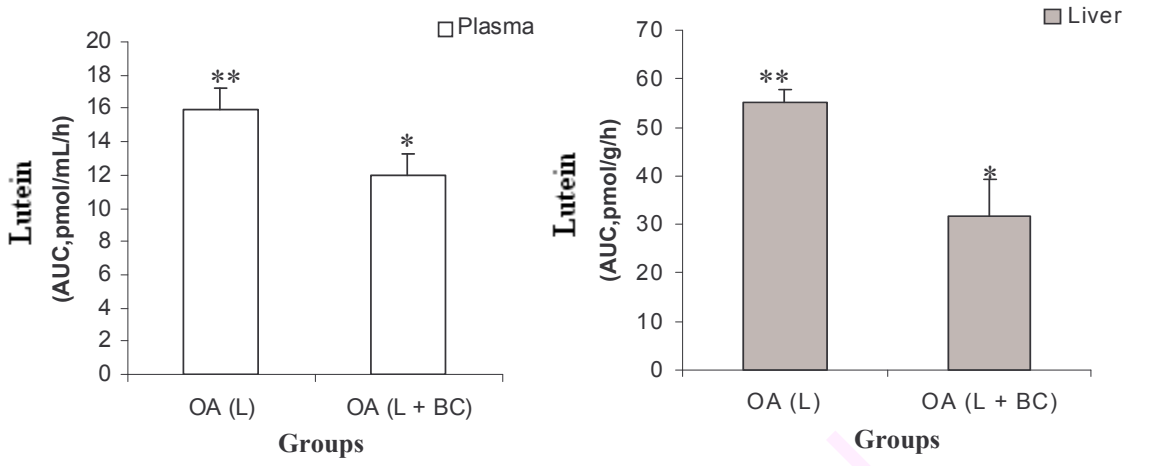


Figure 5.9. AUC for lutein levels in the plasma and liver of rat over 9 h after administration of lutein solubilized in mixed micelles with mixed carotenoids.

Values are mean ± SD (n=6). Bars not sharing a common superscript are statistically significant (p<0.05).

Gavage studies (Repeated dose)

Influence of phospholipids on L bioavailability

Rats were weighed initially and at the end of 10 days repeated (R) intubations (daily) of micellar L either with PC or LPC or NoPL, whereas, food intake was measured daily. Average food intake of the NoPLR, PCR and LPCR groups were 8 ± 0.63, 8.5 ± 0.5 and 8.89 ± 0.45 g/day, respectively. At the beginning of the experiment, the mean body weights of the rats were 42.5 ± 2.5, 42.8 ± 1.6 and 42.4 ± 1.8 g respectively. At the end of the 10th day**,** the mean body weights of these groups were recorded as 53.5 ± 2.5, 53.5 ± 1.6 and 53.3 ± 1.8 g, respectively suggesting the values are not significantly different (p<0.05) among the groups. HPLC data on the level of L in the diet showed that the diet has only L (>1.25 μg/g diet) as the major carotenoid.

The HPLC profiles of L in plasma, liver, eyes, urine and feces and its concentration in NoPLR (control) PCR and LPCR groups after 10 days intubations of micellar L are shown in **Figure 5.10 and Table 5.2.** Results show that the plasma L values for the PCR and LPCR groups were higher (p<0.05) by 18.8% and 31.6% than that of NoPLR group while, values for LPCR group was 40.5% higher (p<0.05)

compared with PCR group. As in the case of plasma L response, the liver L concentration in PCR and LPCR groups was 56% and 68.5% higher (p<0.05) compared with NoPLR group while, in LPCR group it was 18.2% higher than PCR group. Concentration of L (L + Z) in the eye samples of NoPLR, PCR and LPCR groups after repeated dose of micellar L was recorded as 43.99, 66.16 and 76.91 pmol/g, respectively. The data explain that the concentration of L in eye samples of PCR and LPCR groups were significantly (p<0.05) higher by 33.5 and 42.8% compared with NoPLR group. Further, there was an elevated level (21.7%) of L in eyes samples of LPCR than the PCR group (**Figure 5.11**). The plasma and liver content of L in the control samples (0 and 10th day) was below the detectable limit (1 pmol).

Influence of phospholipids on plasma TG

The plasma TG response of rats after repeated dose of micellar L is shown in **Table 5.3.** In contrast to the single dose response (TG level was higher in LPC group), after repeated dose of micellar L, the TG response of PCR group was significantly (p<0.05) higher by 28.6 % than LPCR group.

Influence of phospholipids on the excretion of L. The concentration of L excreted through urine in the case of NoPLR, PCR and LPCR groups were recorded as 11.18 ± 4.46, 15.91 ± 5.9 and 7.82 ± 3.14 pmol/mL/day, respectively. The data shows that the level of L excreted through urine in the case of PCR group was significantly (p<0.05) higher by 30% and 50.8% than those of NoPLR and LPCR groups. Likewise, the level of L excreted in the feces of the NoPLR, PCR and LPCR groups were 1.27 ± 0.23, 1.87 ± 0.96 and 1.10 ± 0.43 nmol/g/day, respectively. The data shows that, as in the case of L in urine, the concentration of L in the feces of PC group were higher by 41.1 and 32% (p<0.05) compared with LPCR and NoPLR groups (**Figure 5.10, Figure 5.11 and Table 5.2)**.

Table 5.2. Lutein and zeaxanthin levels in plasma, liver, eyes, urine and feces of rats after 10 days admistration of either lutein or lutein and β-carotene solubilized in either phospholipids or fatty acid mixed micelles*.

Group	Plasma (nM)	Liver (pmol/g)	Eyes** (pmol/g)	Urine (nM/day)	Feces (nmol/g/day)
			Phospholipids		
NoPLR	4.68 ± 1.5^{a}	5.75 ± 4.5^{a}	43.99 ± 14.0^{a}	11.18 ± 3.5^{a}	1.27 ± 0.2^{a}
PCR	5.77 ± 0.3^{b}	13.07 ± 1.9^{b}	66.16 ± 11.8^{b}	15.91 ± 5.9^{a}	1.87 ± 0.4^{b}
LPCR	6.85 ± 2.0^{c}	18.28 ± 2.7^{b}	76.91 ± 18.3^{c}	7.82 ± 3.1^{b}	1.10 ± 0.5^{a}
			Fatty acids		
OAR	5.85 ± 0.8^{a}	9.15 ± 1.8^{a}	49.68 ± 9.2^{a}	10.26 ± 2.9^{a}	1.25 ± 0.3^{a}
LAR	3.51 ± 0.2^{b}	7.25 ± 2.5^{b}	37.00 ± 11.8^{b}	8.34 ± 1.5^{b}	1.37 ± 0.2^{a}
			Mixed carotenoids		
OAR (L)	5.85 ± 0.9^{a}	9.15 ± 1.8^{a}	49.68 ± 9.2^{a}	10.26 ± 2.9^{a}	1.25 ± 0.3^{a}
OAR	3.42 ± 0.2^{b}	5.92 ± 1.5^{b}	32.0 ± 2.2^{b}	6.53 ± 1.8^{b}	2.32 ± 0.3^{b}

*NoPLR, group fed repeated dose of mixed micelles containing no phospholipids: PCR, group fed repeated dose of mixed micelles containing phosphatidylcholine: LPCR, group fed repeated dose of mixed micelles containing lysophosphatidylcholine: OA, group fed repeated dose of mixed micelles containing oleic acid: LA, group fed repeated dose of mixed micelles containing linoleic acid: OA (L) group fed repeated dose of mixed micelles containing oleic acid and L: OA (L + BC) group fed repeated dose of mixed micelles containing oleic acid, L and β-carotene. Data represent the mean ± SD (n= 6). The values not sharing a common letter are significantly different (p<0.05) between groups as determined by one-way ANOVA. L in the control group before feeding micelles was not detected. **Since L and Z could not be clearly resolved in plasma, tissues and excreted products, results are presented as L + Z.

Table 5.3. Triglycerides level in plasma of rats* after 10 days admistration of either lutein or lutein and β-carotene solubilized in either phospholipids or fatty acid mixed micelles.

Groups	TG (mg/dL)
NoPLR	250.3 ± 25.2^{a}
PCR	$257.3 \pm 27.4^{a, b}$
LPCR	$183.6 \pm 43.2^{c, e}$
OAR	$261.2 \pm 34.1^{a, d}$
LAR	$180.6 \pm 62.2^{c, e}$
OAR (L + BC)	$189.3 \pm 12.0^{c, f}$

*Data represent the mean ± SD (n = 6). The values not sharing a common letter are significantly different ($p<0.05$) between groups as determined by one-way ANOVA. Mixed micelles composition is as given in **Table 5.2.**

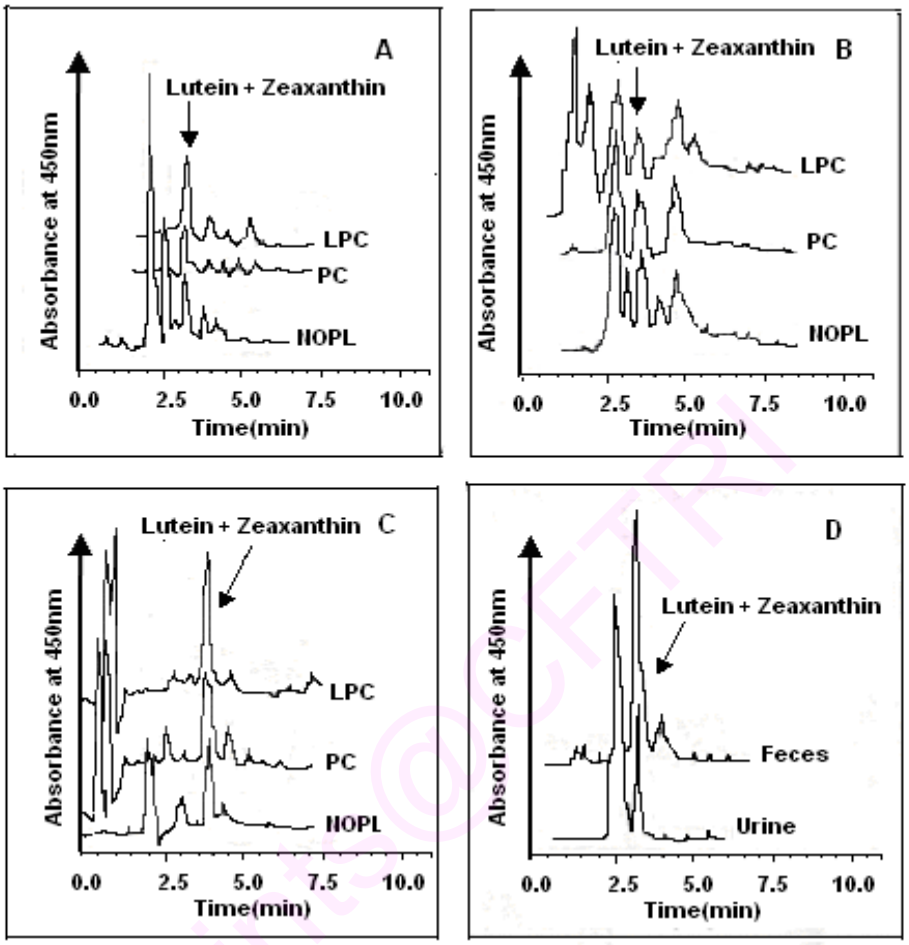


Figure 5.10. HPLC elution of profiles of lutein and zeaxanthin in plasma (A), liver (B), eyes(C) and excretory products (D) of rats after 10 days repeated administration of lutein solubilized in phospholipids mixed micelles.

*Mixed micelles were composed of 2.5 mM monooleoylglycerol, 7.5 mM oleic acid, 12 mM sodiumtaurocholate and 200 μM L with 3 mM phosphatidylcholine (PC), lysophosphatidylcholine (LPC) or no phospholipids (NoPL). Rats (n=6) were fed a dose of micelles (0.2 mL) once in a day for 10 days and then sacrificed. L in the plasma, liver, urine and feces were analyzed by HPLC. HPLC conditions: column- TSK gel ODS-80Ts, precolumn-LC18, mobile phase-Acetonitrile: Methanol: Dichlorormethane (60:20:20, v/v/v) containing 0.1% ammonium acetate, Condition- isocratic, flow rate-1 mL/min.

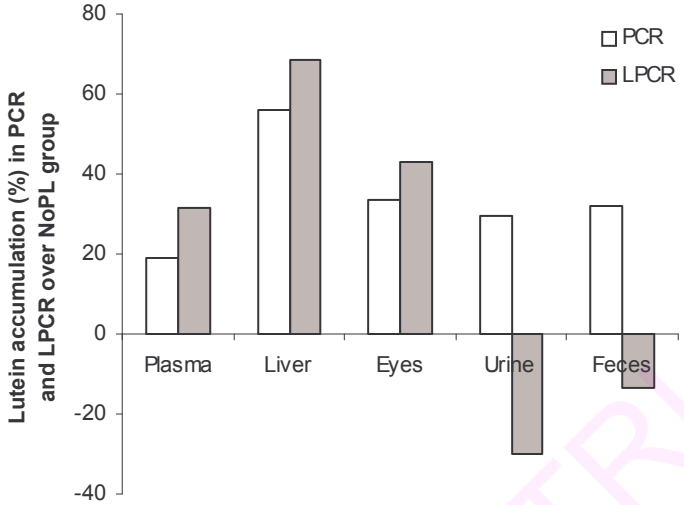


Figure 5.11. Percentage lutein accumulated in plasma, liver, eyes and excreted through urine and feces of PCR (group fed micelles containing phosphatidylcholine) and LPCR (group fed micelles containing lysophosphatidylcholine) over NoPL (group fed micelles containing no phospholipids) during repeated administration of lutein for 10 days.

Influence of fatty acids on L bioavailability.

As in the case of repeated dose of phospholipids micelles with L, rats were weighed initially and at the end of 10 days repeated (R) intubations (daily a dose) of micellar L either with OA or LA, whereas, food intake was measured daily. Results revealed that no difference was found between OAR and LAR groups either in food intake or gain in body weight. HPLC profile of L and its concentration in plasma, liver, eyes, urine and feces of OAR and LAR groups after 10 days intubations of micellar L are given in **Figure 5.12 and Table 5.2**. Results show that the plasma concentration of L for the OAR group was higher by 40% ($p<0.05$) than LAR group. As in the case of plasma L response, the liver L concentration in OAR group was 20.7% higher ($p<0.05$) than LAR group. In eyes, L levels in OAR and LAR groups were 49.7 $\pm$ 9.2 and 37.0 $\pm$ 11.8 pmol/g, respectively and its level in OAR was significantly ($p<0.05$) higher (25.5%) than LAR group (**Figure 5.13A**).

Influence of fatty acids on plasma TG

The plasma TG response of rats after a repeated dose of OA or LA micellar with L is shown in **Table 5.2**. As in the case of single dose response by OA group, significantly (p<0.05) higher (44.8%) level of plasma TG was noticed in OAR than LAR group (**Table 5.3)**.

Influence of fatty acids on L in excretory products

The L level in urine and feces of the OAR and LAR groups after repeated dose of L for 10 days are given in the **Table 5.2.** The concentration of L excreted through urine was recorded as 10.3 ± 2.9 and 8.3 ± 1.5 nmol/L/d and in feces it was 1.3 ± 0.3 and 1.4 ± 0.2 nmol/g/day in OAR and LAR groups, respectively. The level of L excreted in urine was moderately (p<0.05) higher (18.7%) in OAR group and its level in feces was slightly (p>0.05) higher (8.7%) in LAR group (**Figure 5.13B**).

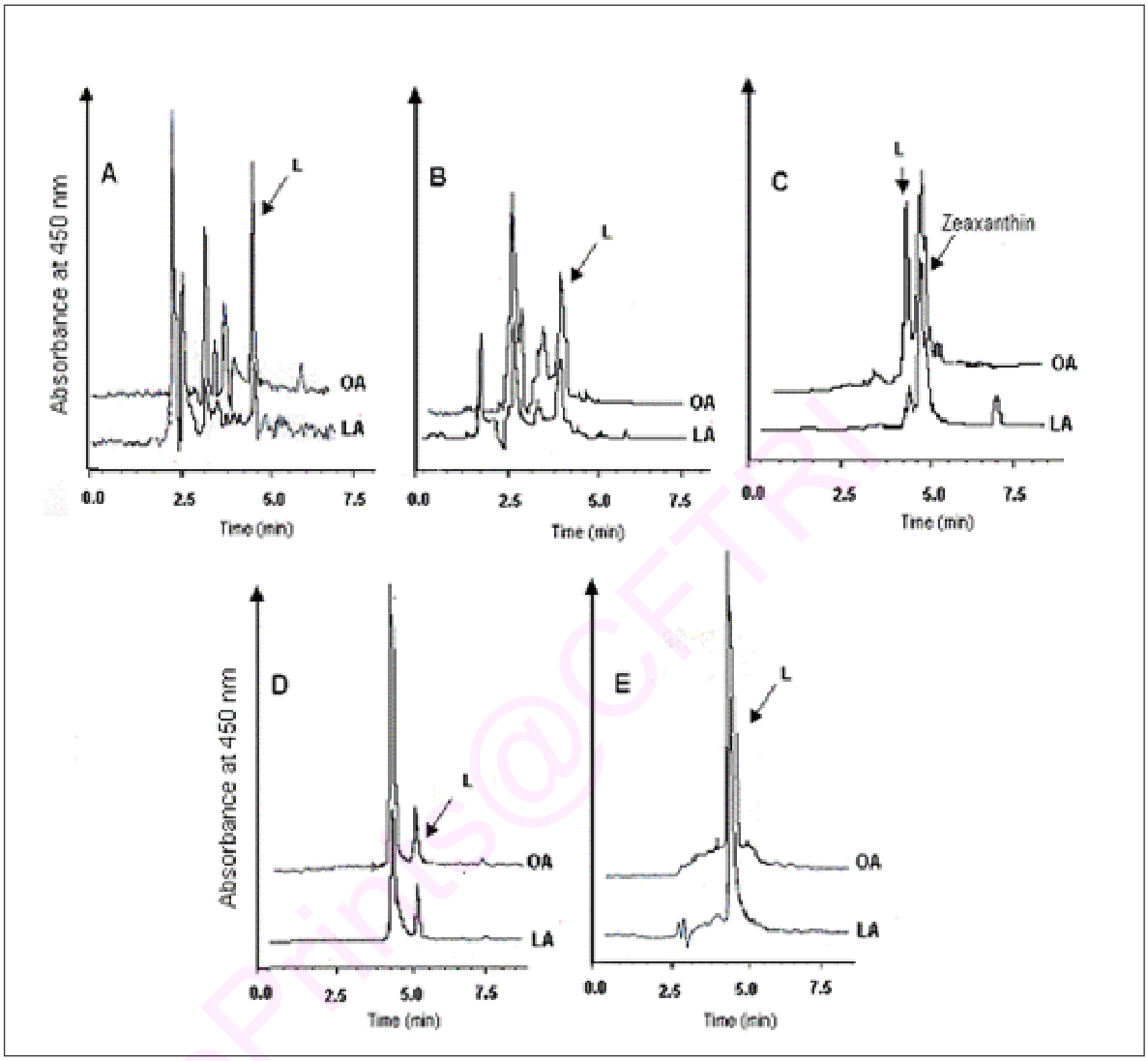


Figure 5.12. HPLC profile of lutein and zeaxanthin in plasma (A), liver (B), eyes (C), urine (D) and feces (E) of rats after repeated dose of lutein solubilized in OA and LA mixed micelles* for 10 days.

*Mixed micelles were composed of 2.5 mM monooleoylglycerol, 12 mM sodiumtaurocholate and 200 μM L and either with 7.5 mM oleic acid (OA) or linoleic acid (LA). Rats (n=6) were fed a dose of micellar L (0.2 mL) once in a day for 10 days and then sacrificed. L in the plasma, liver, urine and feces were analyzed by HPLC. HPLC conditions: column-TSK gel ODS-80Ts, precolumn- LC18, mobile phase-Acetonitrile: Methanol: Dichloromethane (60:20:20, v/v/v) containing 0.1% ammonium acetate, Condition- isocratic, flow rate-1 mL/min.

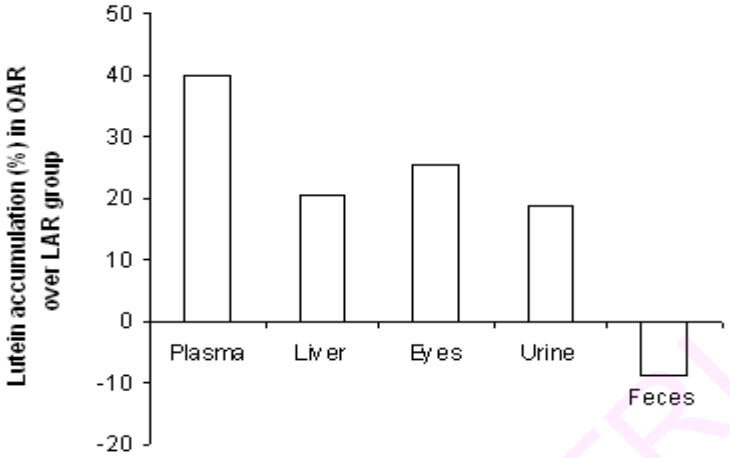


Figure 5.13. Accumulation (%) of lutein in plasma, liver and eyes and excreted through urine and feces of repeated dose of oleic acid over linoleic acid group after daily administration of lutein for 10 days.

Influence of mixed carotenoids on L bioavailability

HPLC profile of L and its concentration in plasma, liver, eyes, urine and feces of OA (L) and OA (L+ BC) groups after 10 days intubations of micellar L are given in **Figure 5.14 and Table 5.2**. Data shows that feeding micellar L along with β-carotene significantly (p<0.05) suppressed the L level in plasma and tissues when compared with that of rats fed with micellar L with no added β-carotene. The plasma L concentration for the L fed group was higher by 41.5% than L + β-carotene group. As in the case of plasma L response, the liver L concentration in L group was 35.3% higher (p<0.05) than L + β-carotene group. In eyes, L levels in L and L + β-carotene groups were 49.7 ± 9.2 and 32.0 ± 2.23 pmol/g, respectively and its level in L group was significantly (p<0.05) higher (34.3%) than L + β-carotene group. As seen after single dose response, the level of plasma TG in L (OA + L) group was significantly (p<0.05) higher by 35.6 % than L + β-carotene group. The amount of L excreted through urine was 10.26 ± 2.9 and 6.53 ±1.8 nmol/L/d and in feces it was 1.25 ± 0.3 and 2.32 ± 0.3 nmol/g/day in L and L+ β-carotene groups, respectively. The level of L excreted in urine was significantly (p<0.05) higher by

36.3% and its level in feces was more significantly higher (46.1%) in L + β-carotene group than L group (**Figure 5.14**).

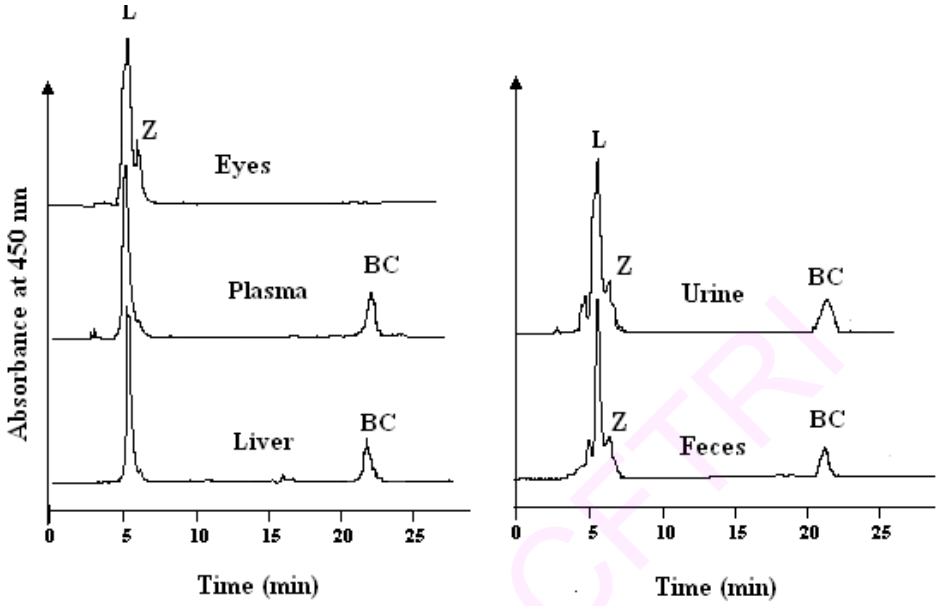


Figure 5.14. HPLC profile of lutein and zeaxanthin in plasma, liver, eyes, urine and feces of rats after repeated dose of either lutein or lutein and β-carotene solubilized in OA mixed micelles* for 10 days.

*Mixed micelles were composed of 2.5 mM monooleoylglycerol, 12 mM sodiumtaurocholate and 7.5 mM oleic acid (OA) and either with 200 μM L or 200 μM of L + β-carotene (L+ BC). Rats (n=6) were fed a dose of micellar L (0.2 mL) once in a day for 10 days and then sacrificed. L and BC in the plasma, liver, urine and feces were analyzed by HPLC. HPLC conditions: column-TSK gel ODS-80Ts, precolumn-LC18, mobile phase-Acetonitrile: Methanol: Dichlorormethane (60:20:20,v/v/v) containing 0.1% ammonium acetate, Condition-isocratic, flow rate-1 mL/min.

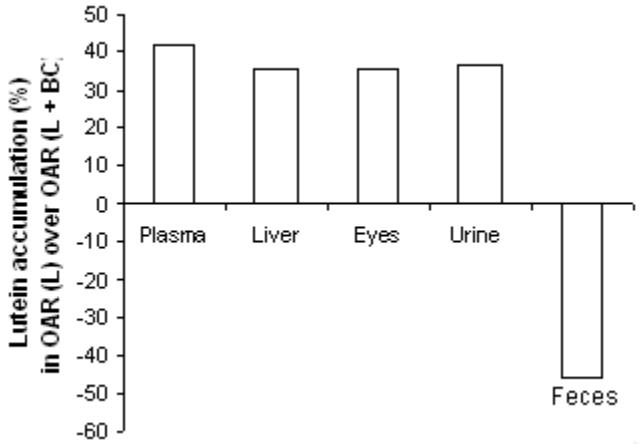


Figure 5.15. Accumulation (%) of lutein in plasma, liver and eyes after and excreted through urine and feces during repeated dose of oleic acid micelles with lutein or lutein and β-carotene to rats for 10 days.

Influence of vegetable oils and lecithin on L bioavailability in rats fed with L sufficient diet.

The purity of lecithin, vegetable oils and their fatty acid composition was confirmed by GC analysis prior to use them for dietary studies. Group of rats (n=5/group) were fed with diet **(Table 2.3)** containing fenugreek leaf powder (4.21 g/Kg diet, source of L, 2.69 mg/Kg, hence forth referred as L sufficient group) either with 10% olive oil (oleic acid source, OO group) or sunflower oil (linoleic acid source, SFO group) or groundnut oil (control, GNO group) for 4 weeks. Results on the growth parameters showed significant difference ($p<0.05$) in gain-in-body weight of the OO group and it was higher by 32.2% compared to that of GNO group and lower by 20.5% than SFO group. The higher gain in body weight of OO group may be attributed to marginally higher food efficiency ratio (0.29 and 0.24), suggesting better palatability of OO diet compared with those of SFO and GNO diets. Further, feeding diet containing powdered fenugreek leaves, as L source along with vegetable oils used in this study had no adverse effect on the food intake and growth of animals.

Semi-synthetic diet was prepared according to AIN-76 and their composition is given in **Table 2.3.** The fatty acid composition of vegetable oils used in this study shows that oleic acid (18:1) is a major fatty acid (77.8%) in olive oil and, linoleic acid (18:2) is a major (57.5%) fatty acid in sunflower oil, whereas, lecithin contained oleic acid as the

major fatty acid (45.4%), followed by 21.1% of linoleic acid. The fatty acid composition of experimental diets added with vegetable oils and lecithin is given in **Table 5.5 and 5.4.** Vegetable oils used in this study were chosen based on their fatty acid composition. To study their influence on tissue L response, the data was compared with the results obtained from gavage studies with specific fatty acids. The HPLC profile of carotenoids in powdered fenugreek leaf and the diet supplemented with fenugreek leaf powder are shown in **Figure 5.16A.** The data shows that fenugreek leaf powder and feed extracts contained L as a major and Z as a minor carotenoid and they were further confirmed by their respective mass spectra **(Figure 5.16D).**

Results on the growth parameters of animals on feeding experimental diets showed significant difference (p<0.05) in gain-in-body weight between OO (39.8%) and SFO (51.7%) groups compared to GNO group, which may be attributed to marginally higher food efficiency, suggesting better palatability of those diets with long chain fatty acids. In the case of lecithin, (PL) fed group, the food efficiency ratio was slightly different compared with control group. Feeding powdered fenugreek leaves, as L source along with vegetable oils and lecithin used in this study had no adverse effect on the food intake and growth of animals (**Table 5.6)**.

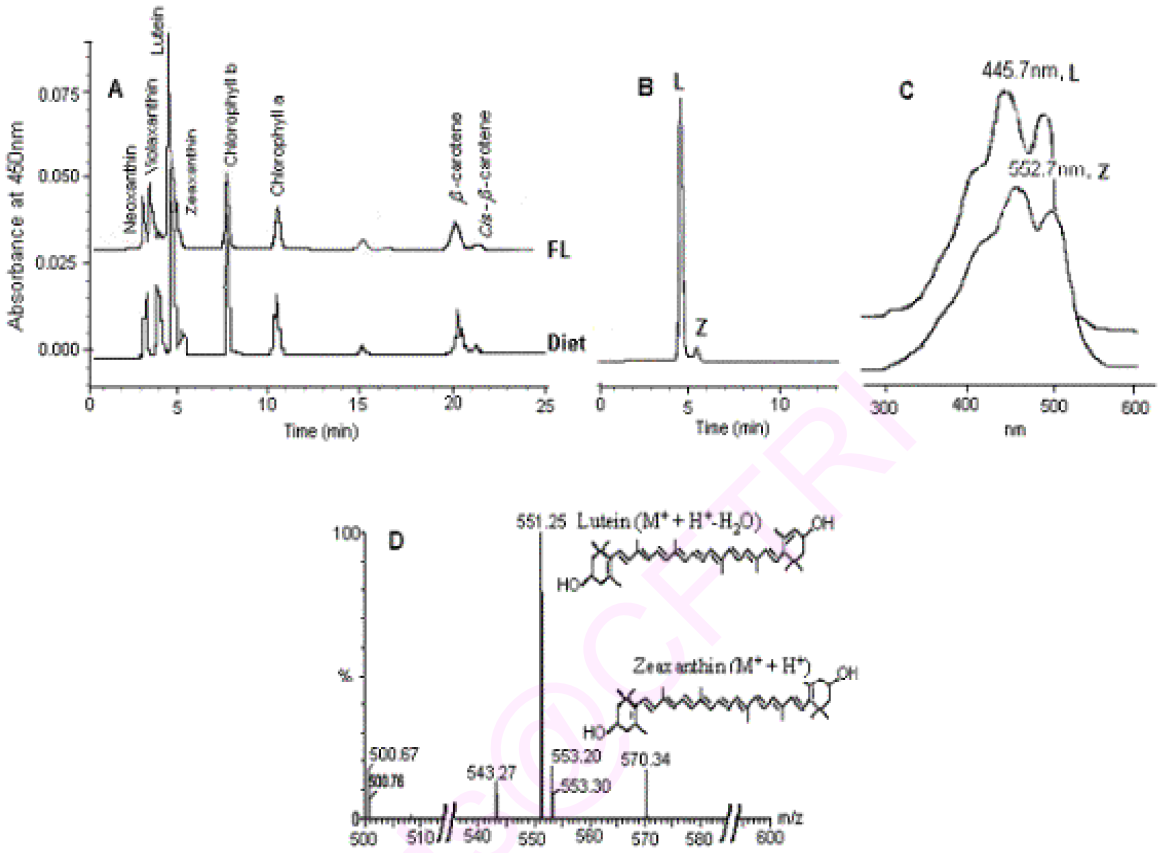


Figure 5.16. HPLC* profile of carotenoids isolated from fenugreek leaf extract (FL) and semi purified diet supplemented with FL (A), standard lutein and zeaxanthin (B), absorption spectra of lutein and zeaxanthin (C) and LC-MS for lutein and zeaxanthin isolated from FL (D).

*HPLC conditions: Column-Tsk-gel ODS-80Ts, Precolumn-LC-18, mobile phase-Acetonitrile: Methanol: Dichloromethane (60:20:20,v/v/v), condition-isocratic, flow rate-1 mL/min. LC-MS conditions: Mode of ionization-APCI, APCI temperature-130^{0}C, APCI probe temperature-500^{o}C, corona voltage-5kV, HV lens voltage-0.5kV, cone voltage-30kV, drying gas-nitrogen at 100 and 300L/h and spectrometer calibration-positive ion mode with scan range of *m/z* 80-1200.

Table 5.4. Fatty acid composition of vegetable oils and lecithin* used for preparing experimental and control diets.

Fatty acid (%)	GNO	OO	SFO	Lecithin
16:0	13.09 ± 2.0 [a]	11.76 ± 1.6 [a]	6.44 ± 1.2 [b]	10.7 ± 2.5 [a]
16.1	ND	0.34 ± 0.0 [a]	1.52 ± 0.6 [b]	4.65 ± 0.1 [c]
18:0	1.77 ± 0.3 [a]	2.31 ± 0.5 [b]	5.14 ± 1.5 [c]	12.2 ± 0.2 [d]
18:1	46.07 ± 7.5 [a]	75.77 ± 6.8 [b]	29.41 ± 4.3 [c]	45.4 ±1.3 [a, d]
18:2 (n-6)	33.71 ± 3.7 [a]	9.84 ± 1.2 [b]	57.46 ± 5.2 [c]	21.1 ± 2.5 [d]
18:3 (n-3)	1.46 ± 0.4 [a]	ND	ND	5.1 ± 0.4 [b]
20:0	ND	ND	ND	1.2 ± 0.1
20:4 (n-6)	0.9 ± 0.1	ND	ND	ND
20:5 (n-3)	ND	ND	ND	ND
22:0	2.96 ± 0.2	ND	ND	ND
22:6 (n-3)	ND	ND	ND	ND

*Values are mean ± SD (n=3). ND: Not detected. Values not sharing a common superscript within a row are statistically significant (p<0.05). Abbreviations: GNO-Ground nut oil; OO- Olive oil; SFO-Sunflower oil.

Table 5.5. Fatty acid composition of semi-synthetic diet* supplemented with vegetable oils and lecithin.

Fatty acid (%)	GNO + GLV	OO + GLV	SFO + GLV	PL + GLV
12:0	ND	ND	ND	ND
14:0	ND	ND	ND	2.1 ± 0.3
16:0	1.30 ± 0.1 [a]	4.44 ± 0.30 [b]	0.35 ± 0.0 [c]	16.1 ± 0.2 [d]
16:1	0.34 ± 0 [a]	ND	0.85 ± 0.3 [a]	5.5 ± 0.5 [b]
18:0	14.99 ± 2.1 [a]	5.0 ± 1.2 [b]	0.35 ± 0.0 [c]	8.7 ± 0.3 [d]
18:1	52.94 ± 5.6 [a]	74.27 ± 7.8 [b]	14.63 ± 2.0 [c]	38.2 ± 2.0 [d]
18:2 (n-6)	19.52 ± 1.3 [a]	1.24 ± 0.1 [b]	64.89 ± 4.7 [c]	19.5 ± 3.2 [a, d]
18:3 (n-3)	6.04 ± 2.1 [a]	7.24 ± 0.7 [a]	11.61 ± 1.3 [b]	5.9 ± 0.2 [a, c]
20:0	0.95 ± 0.3 [a]	2.10 ± 0.2 [b]	5.87 ± 0.8 [c]	3.7 ± 0.4 [d]
20:4 (n-6)	0.40 ± 0.1 [a]	2.65 ± 0.2 [b]	0.18 ± 0.0 [b]	ND
20:5 (n-3)	ND	0.66 ± 0.0	0.33 ± 0.0	ND
22:0	3.37 ± 1.0 [a]	1.37 ± 0.0 [b]	0.74 ± 0.2 [c]	0.34 ± 0.0 [d]
22:6 (n-3)	ND	1.03 ± 0.1	0.29 ± 0.0	ND

*Values are mean ± SD (n=3). ND: Not detected. Values not sharing a common superscript within a row are statistically significant (p<0.05). Abbreviations: GNO + GLV, Diet supplemented with groundnut oil (Control) and green leafy vegetables (L source); OO + L, Diet supplemented with olive oil and green leafy vegetables; SFO + L, Diet supplemented with sunflower oil and green leafy vegetables; PL + L, Diet supplemented with lecithin and green leafy vegetables.

Table 5.6. Gain in body weight and food efficiency ratio of experimental rats* during 4 weeks dietary feeding of lutein along with either vegetable oils (A) or lecithin (B).

Groups (A)	Initial B.wt (g)	Final B.wt (g)	Gain in B.wt (g)	Food intake (g/d)	FER*
GNO + GLV	296.3 ± 4.5	335.6 ± 30.2	39.3 ± 29.5	16.2 ± 1.5	0.41
OO + GLV	328.0 ± 32.3	386.0 ± 41.3	58.0 ± 13.0 [b]	17.2 ± 1.3	0.29
SFO + GLV	315.0 ± 2.0	388.0 ± 5.2	73.0 ± 5.2 [a]	17.8 ± 0.8	0.24

Groups (B)	Initial B.wt (g)	Final B.wt (g)	Gain in B.wt (g)	Food intake (g/d)	FER*
GNO + GLV	296.3 ± 4.5	335.6 ± 30.2	39.3 ± 29.5	16.2 ± 1.5	0.41
PL + GLV	326.0 ± 30.3	366.0 ± 34.3	40.6 ± 10.0	17.5 ± 1.3	0.43

*Values are mean ± SD (n=5). Values not sharing a common letter within a row are significantly different (p<0.05) between groups as determined by repeated measures ANOVA. Diet composition is as given in **Table 2.3**. * Food efficiency ratio.

Abbreviations: GNO + GLV, Diet supplemented with groundnut oil (Control) and green leafy vegetables (L source); OO + L, Diet supplemented with olive oil and green leafy vegetables; SFO + L, Diet supplemented with sunflower oil and green leafy vegetables; PL+ GLVs, Diet supplemented with lecithin and green leafy vegetables.

Lutein response in plasma and tissues

Results on the L level in plasma (nmol/L), liver and eye (pmol/g) samples at zero week (base line value) were 4.7 ± 0.4, 94.5 ± 17.4 and 32.7 ± 3.2, respectively. After 4 weeks feeding of dietary L, its concentration in plasma increased significantly (p<0.05) in all the groups compared with base line value. On comparison, the results revealed that the L level in OO and SFO groups were higher by 43.5 and 13.1% than that of GNO group. While, the values for OO group was 35% (p<0.05) higher compared with SFO group. Similarly, the L level in the eyes of OO and SFO groups were higher (p<0.05) by 37 and 22.7% than GNO group **(Table 5.7)**. Whereas, values for OO group was 18.5% higher compared with SFO group. In contrast, there was no significant (p>0.05) difference (10-20%) in the liver L level between OO and SFO groups compared with GNO group. In the case of PL fed group, the plasma, liver and eye levels of L on its

dietary intake was higher by 7.4, 20.4 and 31.3% compared to control group **(Figure 5.17 and Table 5.7)**.

TG response in Plasma

The plasma TG level of OO and SFO groups were significantly (p<0.05) higher by 26.0 and 22.4% compared with GNO group. Akin to gavages studies, the plasma TG level in the PL group was higher by 15.8% compared with control group **(Figure 5.17 and Table 5.7)**.

Table 5.7. Lutein and triglycerides levels in rats after 4 weeks dietary feeding of lutein either with vegetable oils (A) or lecithin (B) *.

Groups **(A)**	Plasma (nmol/L)	Liver (pmol/g)	Eyes (pmol/g)*	Plasma TG (mg/dL)
GNO + GLV	28.5 ± 6.2 [a]	145.9 ±18.5 [a]	88.4 ± 7.5 [a]	139.0 ± 10.4 [a]
OO + GLV	50.5 ± 3.0 [b]	161.0 ± 4.0 [a]	140.5 ± 9.3 [b]	189.0 ± 20.2 [b]
SFO + GLV	32.8 ± 4.5 [ac]	177.5 ± 11.4 [b]	114.4 ± 13.5 [c]	179.2 ± 12.1 [b]

Groups **(B)**	Plasma (nmol/L)	Liver (pmol/g)	Eyes (pmol/g)*	Plasma TG (mg/dL)
GNO + GLV	28.2 ± 4.2 [a]	145.9 ± 12.5 [a]	88.4 ± 7.5 [a]	139.0 ± 10.4 [a]
PL+ GLV	35.6 ± 2.4 [b]	183.3 ± 10.2 [b]	128.8 ± 12.8 [b]	165.2 ± 16.6 [b]

*Values are mean ± SD (n=5), Values not sharing a common letter within a row are significantly different (p<0.05) between groups as determined by repeated measures ANOVA. *L and Z are expressed together due to their low resolution. Abbreviations: GNO + GLVs, Diet supplemented with groundnut oil (control) and green leafy vegetables (L source); OO + L, Diet supplemented with olive oil and green leafy vegetables; SFO + L, Diet supplemented with sunflower oil and green leafy vegetables; PL+ GLVs, Diet supplemented with lecithin and green leafy vegetables.

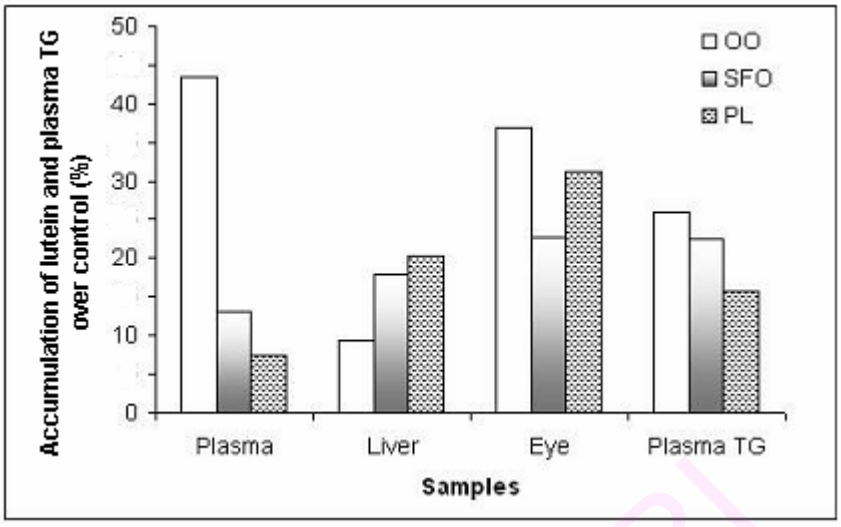


Figure 5.17. Lutein accumulation (%) in plasma, liver, eyes and triglycerides in plasma of olive oil (OO), sunflower oil (SFO) and lecithin (PL) groups compared to groundnut oil (control) group after 4 weeks feeding of lutein sufficient diet. Fenugreek leaf powder was used as lutein source.

Activity of antioxidant enzymes in plasma, liver and eye samples

The influence of vegetable oils and lecithin on the oxidation reaction and/or L bioactivity was assessed by of certain antioxidant enzyme/molecules in different tissues are given in **Table 5.8**. From the data it is seen that the activity of SOD in plasma of SFO group was higher by 17.4%; whereas, in the case of OO group it was lower by 38% when compared with control group. The comparative data on the activity of SOD in the plasma of OO group reveals that the activity was significantly (p<0.05) lower by 40% than SFO group. In case of lecithin fed group, the activity of SOD in plasma was moderately higher (9.3%) than the control group **(Table 5.8A).**

As in the case of plasma, the activity of SOD in liver homogenate of SFO group was slightly higher by 9.2% compared to that of control group; whereas, it was slightly (p.0.05) lower (2%in) in OO group than SFO group. In case of lecithin fed group, the activity of SOD in liver homogenate was lower (p.0.05) by 8.8% than the control group **(Table 5.8B).** The activity of SOD in eye samples of SFO and OO group was slightly higher (p.0.05) by 5.5 and 7.2% than the control group, whereas the activity was lower (2%) than SFO group. In case of eye samples of lecithin fed group, there was no significant difference compared to that of control group **(Table 5.8C)**.

Glutathione peroxidase in plasma of SFO and OO group increased by 8.1% and decreased by 32% than control group, respectively, whereas, the enzyme activity was significantly (p<0.05) lower (30.6%) in OO group than SFO group. In case of lecithin group, the activity of glutathione peroxidase was significantly (p<0.05) higher by 21.4% when compared to that of control group (**Table 5.8A).** Similarly, in case of liver and eye samples the activity of glutathione peroxidase in SFO group was higher by 15.4 and 37.4% and it was lower by 19.1 and 5.7% in OO than the control group. Comparison of results between SFO and OO revealed that the activity of enzyme in liver and eyes of OO group was significantly (p<0.05) lower by 28.9 and 37.4% than the SFO group. The activity of enzyme in the liver and eyes of lecithin fed group was 4.2 and 11.3% higher than that of control group **(Table 5.8B** and **Table 5.8C).**

Results of glutathione levels in plasma of SFO and OO group increased significantly (p<0.05) by 26.6 and 20% than control group, in which the glutathione level was slightly lower in OO group (8.36%) than SFO group. In case of lecithin group, glutathione level was found to be higher (13.6%) than the control group **(Table 5.8A).** Similarly, in case of liver and eye samples of SFO and OO groups, the level of glutathione was found be 17.19, 24.7% and 24.4, 5.0% higher than control group. Further, the data demonstrates that the glutathione level in liver and eyes of OO group was lower by 8.7 and 24.7% (p<0.05) than SFO group. In case of liver and eyes of lecithin fed group, the glutathione levels were slightly higher by 10.1 and 3.7% than control, respectively **(Table 5.8B and 5.8C)**

Lipid peroxides level in plasma, liver and eye samples

Feeding SFO diet (unsaturated fatty acids) with L to rats resulted higher level of linoleic acid in liver tissue. The influence of dietary fatty acids or L on the lipid peroxidation in different tissues is given in **Table 5.8**. Lipid peroxides (Malondialdehyde, MDA) level in the plasma of SFO and OO groups was found to be higher by 39.1 and 9% compared to control group. The MDA level in OO group was significantly (p<0.05) lower by 33% than SFO group. In case of rats fed on dietary L with lecithin, MDA level was significantly (p<0.05) higher by 29% when compared to control group (**Table 5.8A**). MDA level in the liver and eyes homogenates of SFO and OO groups were found to be higher by 40, 23.2% and 15.4, 8.2% respectively compared to control group; whereas,

its level in OO group was significantly (p<0.05) lower by 28 and 16.3% than SFO group. In case of rats fed on dietary L with lecithin, the level of MDA in liver and eyes was significantly (p<0.05) higher by 47.2 and 10.1%, when compared to control (**Table 5.8B and Table 5.8C).**

Table 5.8. Activity of antioxidant enzymes/molecule in plasma, liver and eyes of rats* fed on diet containing powdered fenugreek leaves as lutein source either with vegetables oils or lecithin.

A) Plasma

Groups	GNO (C)	OO	SFO	PL
Superoxide dismutase (U/min/mg protein)	2.9 ± 0.2[a]	2.1 ± 0.2[a]	3.5 ± 0.4[b]	3.2 ± 0.7[a]
Glutathione peroxidase (µmol/min/mg protein)	21.3 ± 1.2[a]	16.1 ± 2.9[b]	23.2 ± 5.5[c]	27.1 ± 1.9[a]
Glutathione (µmol/mL)	22.8 ± 4.3[a]	28.5 ± 6.3 [b]	31.1 ± 7.6[b]	26.4 ± 3.6[b]
MDA (nmol/mg protein)	9.2 ± 2.0[a]	10.1 ± 1.4[a]	15.1 ± 1.2[b]	13.1 ± 2.8 [c]

B) Liver

Groups	GNO (C)	OO	SFO	PL
Superoxide dismutase (U/min/mg protein)	4.9 ± 0.7[a]	4.5 ± 0.62 [a]	5.4 ± 0.8[a]	5.2 ± 1.0[a]
Glutathione peroxidase (µmol/min/mg protein)	31.8 ± 1.2[a]	26.7 ± 4.2[b]	37.6 ± 3.5[c]	33.2 ± 2.2[a]
Glutathione (µmol/mL)	52.0 ± 2.3[a]	68.8 ± 5.2 [b]	62.8 ± 5.7[b]	57.9 ± 3.1[b]
MDA (nmol/mg protein)	15.3 ± 3.2[a]	18.1 ± 2.1[a]	25.1 ± 1.8 [b]	29.1 ± 3.2[b]

C) Eyes

Groups	GNO (C)	OO	SFO	PL
Superoxide dismutase (U/min/ mg protein)	5.1 ± 0.4[a]	5.5 ± 0.4[a]	5.4 ± 0.8[a]	5.2 ± 1.0[a]
Glutathione peroxidase (µmol/min/ mg protein)	38.3 ± 1.2[a]	36.1 ± 4.2[b]	57.7 ± 8.1[c]	43.2 ± 2.2[a]
Glutathione (µmol/mL)	62.0 ± 6.4[a]	78.3 ± 7.1[b]	82.4 ± 9.6[b]	64.4 ± 6.2[b]
MDA (nmol/mg protein)	42.3 ± 4.2[a]	46.1 ± 2.3[a]	55.1 ± 1.7[b]	47.1 ± 2.2[c]

*Values are mean ± SD of 5 samples. Values not sharing a common superscript within a row are statistically significant (p<0.05). Abbreviations: GNO, Diet supplemented with groundnut oil (control) and green leafy vegetables (L source); OO, Diet supplemented with olive oil and green leafy vegetables; SFO, Diet supplemented with sunflower oil and green leafy vegetables; PL, Diet supplemented with lecithin and green leafy vegetables.

Influence of vegetable oils on plasma and tissues lipid profile

Results show that the triacylglycerides level in plasma was higher (p<0.05) in OO group by 21.16 and 35.9% than SFO and GNO groups; whereas, the plasma cholesterol levels in OO and SFO groups lower (p<0.05) by 29.1% and 55.0% than GNO group, respectively. Similarly, phospholipids levels in OO and SFO groups increased by 14.17 and 9.22% than GNO group (**Table 5.9A**). Percent accumulation of lipid profile in plasma of experimental groups over control group is shown in **Figure 5.18.** As in the case of plasma lipid profile, changes were also observed in fatty acid profile in plasma of rats fed on OO or SFO diets compared with that of GNO diet group **(Table 5.5).** In particular, the oleic acid level was found to be 54% in OO group and the linoleic acid level was 21.5% in the SFO group. However, in case of GNO group, the proportion of oleic and linoleic acid levels were 42.6 and 17.9% respectively.

Liver is an important organ for lipid metabolism. The triacylglycerides level in OO group was recorded as 22.5 and 12.7% higher than those of SFO and GNO groups, respectively. In the case of cholesterol, its level was decreased by 8.7% in OO group and 16.2% in SFO group when compared with the GNO group. Feeding dietary L along with OO, SFO and GNO did not alter the phospholipids levels in the liver (**Table 5.9B).** Whereas, fatty acid profile of the liver samples showed that the OA is a major fatty acid (36%) in OO group and LA level increased (29.6%) considerably in SFO group (**Figure 5.18)**.

From the **Table 5.9C**, it is seen that the triacylglycerides level in the eye samples was lower in OO (8.5%) and slightly higher (7.3%) in SFO groups compared with GNO group, respectively. The cholesterol level in eye samples was higher by 14.7% and 9.6% in OO and SFO than GNO group, respectively. No significant changes were observed in the level of phospholipids in all the three groups (**Table 5.9C).** Percent accumulation of lipid profiles in liver of OO and SFO groups over control is shown in **Figure 5.18.**

In case of lecithin fed group, the triglycerides level in plasma of experimental group was lower by 10% and higher by 6.7 and 14.7% in liver and eyes than control group. The cholesterol levels in plasma, liver and eyes of lecithin group were 6.8,1.5 and 21.1% higher than control group respectively, while the phospholipids levels were significantly (p<0.05) higher by 26.3% in plasma, 27% in liver and 21.1% in eyes of lecithin group than control correspondingly.

Dietary vegetable oils used in this study were found to greatly influence the fatty acid composition of plasma, liver and eyes and the data is given in **Table 5.10- 5.13**. Rats fed on SFO and OO diet had 25 and 45% lower level of linoleic acid in plasma compared to those fed on GNO diet. Plasma of rats fed on SFO diet had 15% of total fatty acid as LA, whereas, LA was not detected in rats fed on GNO diet. OA and LA were present only in rats fed on OO diet and they were 1.3 and 1.4% of total fatty acids. In case of liver, rats fed with SFO diet contained LA to the extent of 2.9% of total fatty acids; the basal level of LA and OA in GNO group was 9.6% of total fatty acids and it was increased to 12.7 and 14.4% of total fatty acids, respectively in SFO and OO groups on feeding diet supplemented with respective vegetable oils. In eyes, rats fed with SFO diet contained 4.9% of LA of total fatty acids, whereas, it was not detected in other dietary groups. Further, the basal level of LA was significantly enhanced in the eye tissue of rats fed on SFO diet.

Table 5.9. Lipid profiles of plasma (A), liver (B) and eye (C) tissue of rats* after four weeks feeding diet contained powdered fenugreek leaves along with vegetable oils or lecithin.

A). Plasma

Parameters (mg/L)	GLV + GNO	GLV + OO	GLV + SFO	GLV + PL
Triacylglycerol	139.0 ± 10.4 [a]	189.0 ± 11.2 [b]	149.2 ± 15.1 [c]	125.6 ± 12.2 [d]
Phospholipids	138.7± 9.2 [a]	161.6 ± 15.0 [b]	152.8 ± 12.2 [b]	188.3 ± 21.5 [c]
Cholesterol	219.2 ± 19.9 [a]	155.2 ± 20.8 [b]	140.7± 16.7 [b]	235.4 ± 23.8 [a]

B). Liver

Parameters (mg/g)	GLV + GNO	GLV + OO	GLV + SFO	GLV + PL
Triacylglycerol	120.0 ± 10.4 [a]	137.6 ± 7.0 [b]	106.6 ± 8.5 [c]	128.9 ± 12.5 [a, b]
Phospholipids	160.6 ± 15.7 [a]	180.0 ± 8.0 [b]	159.6 ± 7.9 [a, c]	220.0 ± 10.35 [d]
Cholesterol	231.7 ± 21.8 [a]	212.5 ± 15.5 [b]	194.0 ± 15.8 [c]	235.4 ± 43.5 [a, d]

C). Eye

Parameters (mg/g)	GLV + GNO	GLV + OO	GLV + SFO	GLV + PL
Triacylglycerol	102.5 ± 13.8 [a]	93.8 ± 12.5 [a]	110.6 ± 9.0 [b]	120.3 ± 3.3 [c]
Phospholipids	66.8 ± 4.7 [a]	67.0 ± 9.3 [a]	62.3 ± 4.3 [a]	86.4 ± 2.8 [b]
Cholesterol	112.0 ± 13.6 [a]	131.3 ± 4.4 [b]	118.6 ± 18.6 [a]	142.5 ± 3.6 [c]

*Values are mean ± SD (n=5), Values not sharing a common superscript within a row are statistically significant (p<0.05). Abbreviations: GNO + GLVs, Diet supplemented with groundnut oil (control) and green leafy vegetables (L source); OO + L, Diet supplemented with olive oil and green leafy vegetables; SFO + L, Diet supplemented with sunflower oil and green leafy vegetables; Lecithin + GLVs, Diet supplemented with lecithin and green leafy vegetables

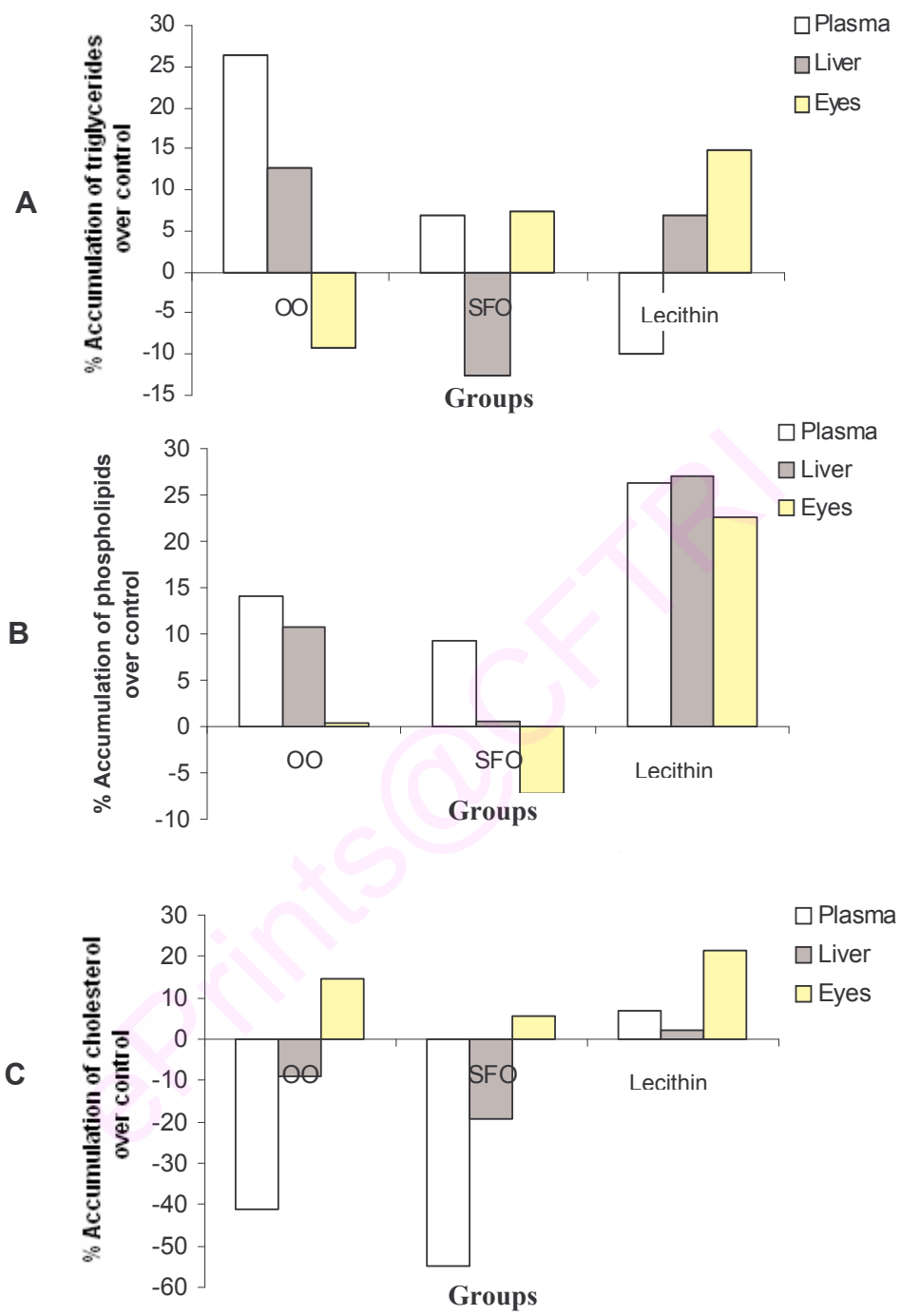


Figure 5.18. Accumulation (%) of triglycerides (A), phospholipids (B) and cholesterol (C) over control in plasma, liver and eyes of rats after 4 weeks dietary feeding of lutein either with vegetable oils or lecithin.

Table 5.10. Fatty acid profile (%) of tissues in control (base line values) rats* used for dietary feeding experiments.

Fatty acid	Plasma	Liver	Eyes
12:0	ND	ND	ND
14:0	ND	ND	ND
16:0	33.8 ±2.5 [a]	26.1 ± 6.5 [b]	24.0 ± 7.9 [b]
16:1	4.7 ± 0.2 [a]	3.0 ± 1.2 [b]	6.3 ± 1.0 [c]
18:0	7.3 ± 0.1 [a]	9.6 ± 2.5 [b]	11.8 ± 0.6 [b]
18:1	16.3 ± 2.7 [a]	18.4 ± 2.1 [a]	16.3 ± 6.6 [a]
18:2 (n-6)	23.9 ± 0.5 [a]	19.2 ± 1.1 [b]	18.8 ± 2.3 [b]
18:3 (n-3)	2.2 ± 0.9 [a]	4.1 ± 1.0 [b]	9.2 ± 2.2 [c]
20:0	ND	ND	ND
20:4 (n-6)	7.1 ± 3.2 [a]	16.5 ± 1.3 [b]	5.6 ± 5.6 [a]
20:5 (n-3)	4.2 ± 0.6 [a]	2.8 ± 0.2 [b]	7.6 ± 6.8 [c]
22:0	ND	ND	ND
22:6 (n-3)	ND	ND	ND

*Values are mean ± SD (n=5). Values not sharing a common superscript within a row are statistically significant (p<0.05).

Table 5.11. Plasma fatty acid profile (%) of rats* after 4 weeks supplementation of diet containing fenugreek leaves with vegetable oils or lecithin.

Fatty acid	GNO + GLV	OO + GLV	SFO + GLV	PL+ GLV
12:0	ND	ND	ND	ND
14:0	ND	ND	ND	3.4 ± 0.2
16:0	21.4 ± 2.2 [a]	19.2 ± 5.2 [a]	23.9 ± 3.7 [b]	18.4 ± 2.5 [a]
16:1	4.7 ± 1.1 [a]	4.3 ±1.6 [a]	3.9 ± 1.1 [a]	10.2 ± 0.5 [b]
18:0	8.1 ± 1.8 [a]	9.8 ± 3.8 [a]	8.4 ± 1.8 [a]	8.7 ± 1.3 [a]
18:1	42.6 ± 2.1 [a]	54.1 ± 3.8 [b]	21.5 ± 1.4 [c]	36.2 ± 5.0 [d]
18:2 (n-6)	17.9 ± 0.6 [a]	8.8 ± 2.0 [b]	32.7 ± 1.5 [c]	14.5 ± 0.9 [d]
18:3 (n-3)	ND	ND	ND	ND
20:0	ND	ND	ND	6.5 ± 0.8
20:4 (n-6)	4.6 ± 0.7 [a]	3.8 ±0.4 [a]	8.7 ± 0.9 [b]	ND
20:5 (n-3)	ND	ND	ND	ND
22:0	ND	ND	ND	1.34 ± 0.0
22:6 (n-3)	ND	ND	ND	ND

*Values are Mean ± SD (n=5). Values not sharing a common superscript within a row are statistically significant (p<0.05). Abbreviations: GNO + GLVs, Diet supplemented with groundnut oil (control) and green leafy vegetables (L source); OO + L, Diet supplemented with olive oil and green leafy vegetables; SFO + L, Diet supplemented with sunflower oil and green leafy vegetables; PL+ GLV, Diet supplemented with lecithin and green leafy vegetables.

Table 5.12. Liver fatty acid profile (%) of rats* after 4 weeks supplementation of diet containing fenugreek leaves with vegetable oils or lecithin.

Fatty acid	GNO + GLV	OO + GLV	SFO + GLV	PL + GLV
12:0	ND	ND	ND	ND
14:0	ND	ND	ND	1.2 ± 0.2
16:0	28.4 ± 1.4 [a]	22.5 ± 1.4 [b]	20.3 ± 2.8 [b]	23.4 ± 2.1 [b]
16:1	5.2 ± 1.1 [a]	8.3 ± 1.9 [b]	7.0 ±1.8 [b]	12.2 ± 0.5 [c]
18:0	7.4 ± 1.8 [a]	7.1 ± 2.7 [a]	7.8 ± 1.1 [a]	10.2 ± 1.3 [b]
18:1	17.8 ± 2.5 [a]	28.4 ± 7.6 [b]	29.6 ± 3.5 [b]	31.3 ± 4.0 [b]
18:2 (n-6)	18.3 ± 4.2 [a]	17.3 ± 3.2 [a]	23.3 ± 2.8 [b]	14.1 ± 0.8 [c]
18:3 (n-3)	12.6 ± 2.0 [a]	10.2 ± 2.7 [a]	5.2 ± 1.0 [b]	2.3 ± 0.4 [c]
20:0	ND	ND	ND	4.4 ± 0.3
20:4 (n-6)	9.6 ± 1.6	ND	ND	ND
20:5 (n-3)	ND	5.7 ± .0.8 [a]	6.1 ± 0.0 [a]	ND
22:0	ND	ND	ND	0.85 ± 0.0
22:6 (n-3)	ND	ND	ND	ND

*Values are Mean ± SD of 5 rats. Values not sharing a common superscript within a row are statistically significant (p<0.05). Abbreviations: GNO + GLV, Diet supplemented with groundnut oil (control) and green leafy vegetables (L source); OO + L, Diet supplemented with olive oil and green leafy vegetables; SFO + L, Diet supplemented with sunflower oil and green leafy vegetables; PL + GLV, Diet supplemented with lecithin and green leafy vegetables.

Table 5.13. Eyes fatty acid profile (%) of rats* after 4 weeks supplementation of diet containing fenugreek leaves with vegetable oils or lecithin.

Fatty acid	GNO + GLV	OO + GLV	SFO + GLV	PL + GLV
12:0	ND	ND	ND	ND
14:0	ND	ND	ND	ND
16:0	33.2 ± 2.9 [a]	35.4 ± 3.1 [a]	28.2 ± 2.4 [b]	30.0 ± 5.1 [a, b]
16:1	5.8 ± 0.2 [a]	4.2 ± 0.8 [b]	7.1 ± 0.7 [c]	6.3 ± 1.2 [c]
18:0	23.4 ± 3.8 [a]	12.7 ± 1.0 [b]	13.7 ± 4.2 [b]	14.1 ± 1.0 [b]
18:1	26.3 ± 2.7 [a]	36.1± 8.2 [b]	21.4 ± 2.5 [c]	28.3 ± 4.0 [a]
18:2 (n-6)	4.6 ± 0.9 [a]	6.5 ± 0.5 [b]	14.2 ± 2.9 [c]	4.1 ± 1.0 [a]
18:3 (n-3)	2.4 ± 0.4 [a]	ND	3.8 ± 0.2 [b]	2.1 ± 0.6 [a]
20:0	ND	ND	ND	1.4 ± 0.1 [d]
20:4 (n-6)	2.2 ± 0.3 [a]	2.7 ± 0.5 [a]	3.2 ± 0.2 [a]	ND
20:5 (n-3)	1.7 ± 0.4 [a]	0.6 ± 0.2 [b]	2.1 ± 0.9 [a]	ND
22:0	ND	ND	ND	0.8 ± 0.0
22:6 (n-3)	ND	1.6 ± 0.2 [a]	6.2 ± 0.4 [b]	2.3 ± 0.1 [c]

*Values are mean ± SD of 5 rats. Values not sharing a common superscript within a row are statistically significant (p<0.05). Abbreviations: GNO + GLVs, Diet supplemented with groundnut oil (control) and green leafy vegetables (L source); OO + L, Diet supplemented with olive oil and green leafy vegetables; SFO + L, Diet supplemented with sunflower oil and green leafy vegetables; PL+ GLV, Diet supplemented with lecithin and green leafy vegetables.

Influence of vegetable oils on L bioavailability in rats fed with L depleted diet

To induce L deficiency, rats were fed L-depleted diet (**Table 2.3**) for 2 weeks (hence forth referred as L-deficient group). Initially (0 day, base line) and after 2 weeks of feeding L-depleted diet, plasma L and Z levels were analyzed, to ensure their base line value and the depletion status (<0.1 pmol/mL). After induction of L deficiency and to study the time-course response of L on post feeding, rats were divided into 4 equal groups. Each group was further divided into 4 equal sub-groups. Group 1 was fed on L supplemented diet (*T. foenum-graecum* leaves, 4.21 g/kg diet as L and Z source, 2.69 +

0.73 mg/kg diet) with olive oil (oleic acid source, OOD group), while group 2 and 3 were fed on L supplemented diet with sunflower oil (linoleic acid source, SFOD group) or groundnut oil (control, GNOD), respectively. The group 4 was fed on L supplemented diet with lecithin (phospholipid group). Rats in each dietary group at 2, 4, 8 and 16 days after post feeding were sacrificed; blood, liver and eyes (both left and right eyes) were sampled and processed for L and Z analysis by HPLC and LC-MS according to the procedures mentioned elsewhere.

In this study, powdered fenugreek leaves was used as L source along with vegetable oils OO and SFO, to study their influence on the efficacy of dietary L. In addition, influence of dietary soy lecithin (PC) was also studied in order to compare the data with single dose results.

Results on the growth parameters revealed that no difference ($p>0.05$) was found with regard to food intake and gain in body weight among the dietary groups. The postprandial plasma and eye responses of L + Z during the 16 day dietary feeding of diet (fenugreek leaf powder) either with OO or SFO or GNO (control) to L deficient rats are shown in **Figure 5.19**. The base line data (before feeding diet devoid of L) on L and Z levels in plasma and eyes of rats was found to be 4.7 pmol/mL and 32.7 pmol/g, respectively. L + Z were detected in plasma after two weeks feeding of diet devoid of L, whereas, their level in eye was 19.0 ± 2 pmol/g. But after feeding diet with fenugreek leaf powder as L source for 16 days, the plasma L + Z level (AUC) of OOD group was significantly ($p<0.05$) higher by 37.6 and 40.9% than those of SFOD and GNOD groups. Similarly, in eyes, their level was higher by 22.7 and 30.8% in OOD group compared to SFOD and GNOD groups. In case of phospholipids fed group, the plasma and eye L level (AUC) was higher by 43.7 and 19.3% compared to control group (**Figure 5.20)**. Further, the TG response in OOD was significantly higher than SFOD (42.7%) and controls (70.8%) groups. The TG response of lecithin fed group was also higher by 33.7% than control group.

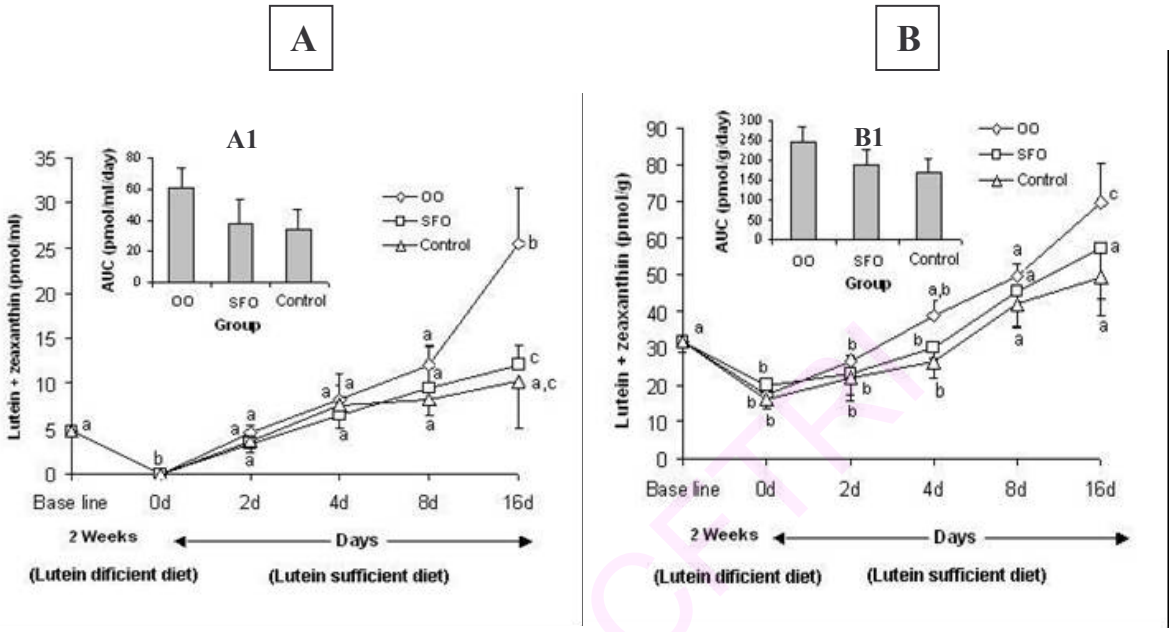


Figure 5.19. Lutein and zeaxathin levels in plasma (A) and eyes (B) of rats* fed lutein sufficient diet either with olive oil (OO) or sunflower oil (SFO) or groundnut oil (control) after induction of lutein deficiency.

Area under the curve for L + Z in plasma (A1) and eyes (B1) of rat over 16 days after supplementation of L sufficient diet. *Data represent the mean ± SD (n=5). The values at each time point not sharing a common letter are significantly different (P<0.05) between groups as determined by repeated measures ANOVA and Tukey's test.

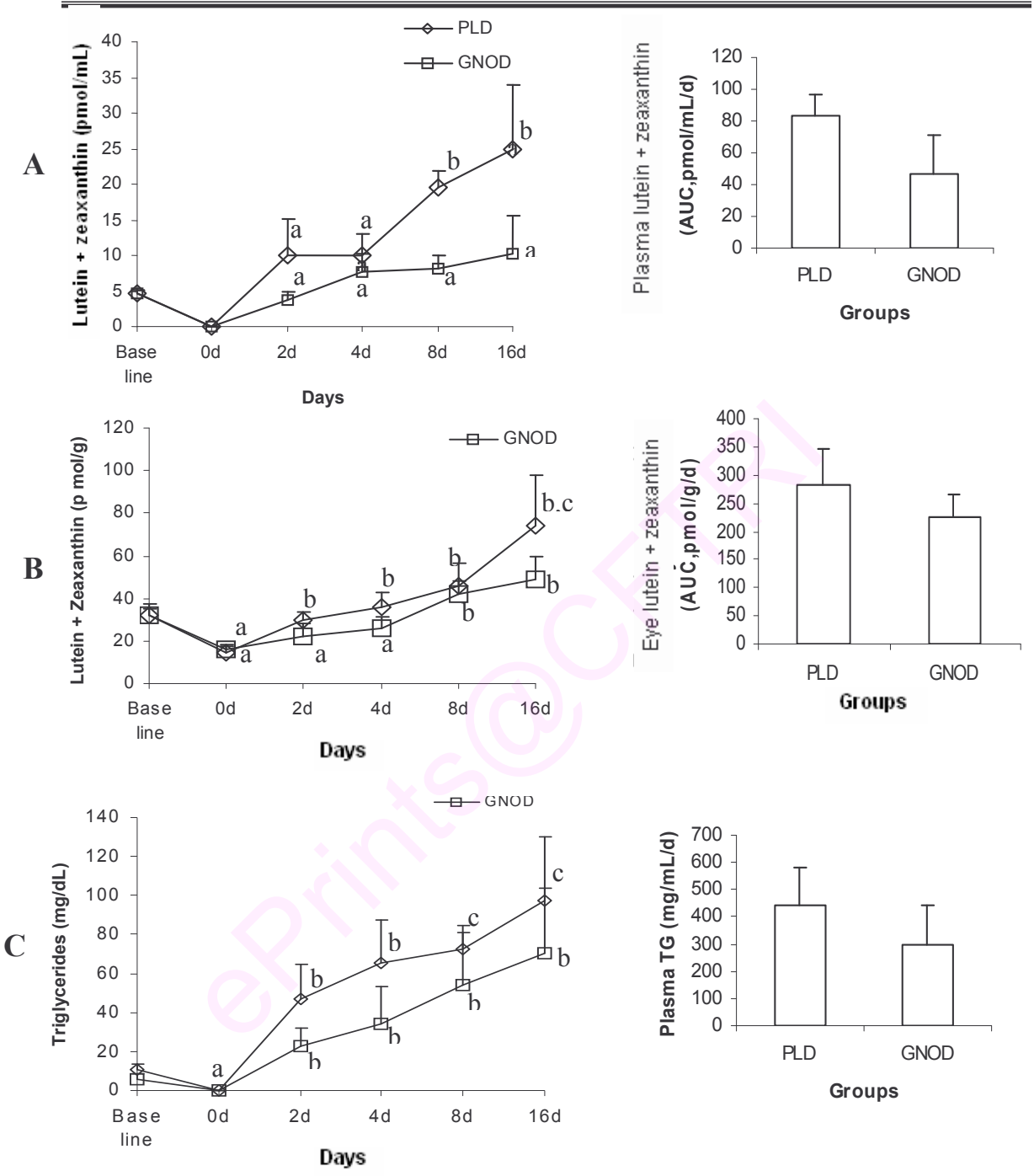


Figure 5.20. Postprandial response of lutein in plasma (A), eye (B) and plasma triglycerides response (C) after dietary feeding of lutein either with lecithin or ground nut oil (control) to lutein deficient rats*. *Data represent the mean ± SD (n=5). The values at each day point not sharing a common letter are significantly different (P<0.05) between groups as determined by repeated measures ANOVA and Tukey's test.

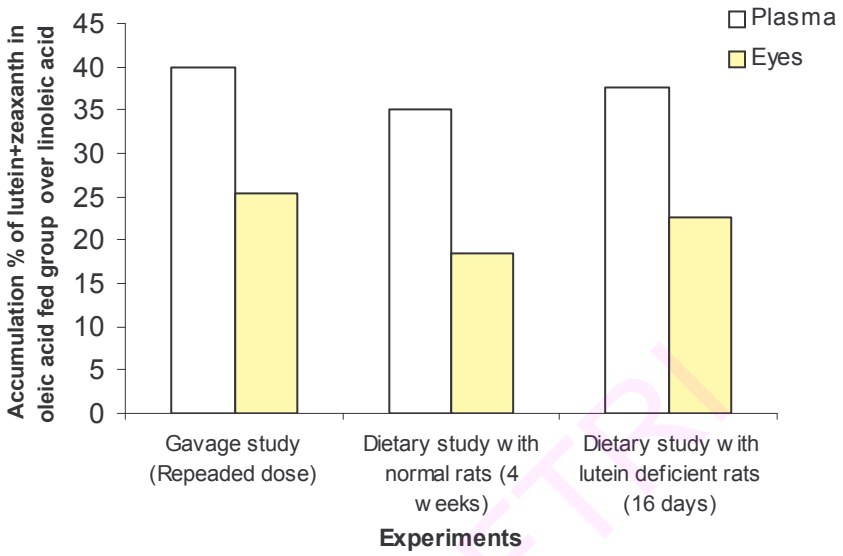


Figure 5.21. Percent accumulation of lutein and zeaxanthin in oleic acid or OO fed group over linoleic acid or SFO group in plasma and eyes of repeated dose or dietary (normal and deficient rats) lutein.

Discussion

The intestinal absorption and plasma response of dietary carotenoids is governed by various exogenous (food matrix) and endogenous (mixed micelles, permeation etc.) factors (Hollander and Ruble, 1978). One such dietary factor appears to be the nature and level of fat present in the food matrix. Previous studies from our lab (Baskaran et al. 2003; Raju et al. 2006) showed that micellar lysoPC increases the plasma level of β-carotene and L in mice and rats, suggesting that phospholipids may modulate the intestinal absorption of carotenoids. The present study demonstrates the effect of dietary phospholipids, fatty acids and mixed carotenoids on the intestinal L uptake either from mixed micelles or diet and its response in plasma, liver and eyes of rats fed on L sufficient or its deficient diets. To achieve complete solubilization and required concentration of L, it was solubilized in respective mixed micelles or vegetable oils and rats were then intubated or fed. The postprandial appearance of L in plasma was considered as a measure of intestinal absorption after single and repeated dose of micellar or dietary L.

In the case of single dose study, the results show that lysoPC micelles significantly enhanced the plasma L concentration, compared with PC and NoPL micelles. It is interesting to emphasize that the peak plasma concentration of L after a single dose of micellar L reached maximum after 1 h in PC and NoPL and 2 h in LPC groups. The concentration was significantly higher in the case of LPC group than other two groups. Further, to our knowledge, this is the first report to demonstrate a positive role for lysoPC in L absorption in rats. Earlier, Sugawara et al. (2001), Raju et al. (2005) and Nishimukai and Hara (2004) proposed the influence of phospholipids on β-carotene and lycopene absorption *in vitro* and *in vivo* using Caco-2 cells, mice and rat models.

This study indicates that mixed micelle containing lysoPC, when intubated to rats profoundly influenced the intestinal uptake and plasma response of L compared to PC. However, the influence of lysoPC on the intestinal uptake of L in rats was poor compared with mice (Baskaran et al. 2003). The present results are in agreement with the observations on carotene absorption and their response in rat tissues (Shapiro et al. 1984). The exact mechanism by which micellar lysoPC influences the intestinal L uptake is still remains unclear. Evidence from *in vitro* studies by Sugawara et al. (2001) and Noh and Koo (2003) suggests that the inhibitory effects of PC or sphingomyelin - structurally

similar to PC on β-carotene and α-tocopherol absorption may be mostly mediated within the intestinal cells. Phosphatidylcholine is hydrolyzed more slowly and incompletely in the intestinal lumen (Noh and Koo, 2003; Duan, 1998). Thus, the slow and incomplete hydrolysis of PC in the upper segment of the intestine, where much of the lipid hydrolysis occurs, may allow for interaction between intact PC and other lipids in the intestinal environment, affect the rate of hydrolysis, micellar solubilization and transfer of lipids from mixed micelles to the enterocytes. Evidence from an *in vitro* study (Homan and Hamlehle, 1998) show that the presence of intact PC in mixed micelles slows the transfer of more hydrophobic components such as cholesterol and α-tocopherol from the micellar matrix. Although no direct evidence is available for such interactions between PC and other lipids or lipophilic components in micelles, studies with lipid vesicles and membrane system indicate that PC interacts more tightly with fat-soluble components (Slotte, 1999). This may be a reason for significantly higher level of L excreted through urine and feces by the rats fed with PC micelles compared to lysoPC.

Though PC lowers plasma and tissue level of L after single dose of micellar L compared with NoPL group, results of the repeated dose study show that L levels in the plasma, liver and eyes of PC group were significantly elevated compared with NoPL group. It is possible that repeated intake of PC leads to stimulation of phospholipases which hydrolyses PC to lysoPC resulting in increased L absorption in the PC group when compared to the single dose study (Baskaran, et al. 2003; Sugawara et al. 2001). The level of L in the LPC group was significantly higher than the PC and NoPL groups in single and repeated dose studies. Thus, the results clearly suggest that PC in the mixed micelles suppresses the plasma L level and its accumulation in liver and retina of rats compared with lysoPC. It is not sure whether the mixed micelles fed directly to the stomach by gavages in the present study reached the intestine or were reconstituted as micelles in the intestinal tract after disintegrating in the stomach. Nonetheless, the present results were basically in consistent with *in vitro* results of Sugawara et al. (2001), in which they have reported that the carotenoids solubilized in mixed micelles were directly incubated with cultured human intestinal Caco-2 cells. The decline in the plasma and liver L levels after reaching maximal (single dose study), reflects its transport to other tissues like liver, eyes and adipose tissues.

The results of the present and the previous *in vivo* and *in vitro* studies using mice and human intestinal Caco-2 cell models (Baskaran et al. 2003 and Sugawara et al. 2001), suggest that the hydrolysis of phospholipids in the intestinal tract by phospholipase-A_2 is vital for the efficient uptake of carotenoids by the enterocytes, although PC plays a significant role in the solubilization of carotenoids in lipid emulsions (Borel et al.1996). The mechanism underlying these effects is not yet fully understood.

Physical properties like low viscosity and larger size of the PC micelles compared with those of lysoPC micelles, as found in this study, may also be other reasons for the poor bioavailability of L by the intestinal cells. Further, PC with two long chain acyl moieties is more hydrophobic than lysoPC, which has one acyl moiety and a free hydroxyl group. Therefore, PC has a greater affinity for hydrophobic carotenoid molecules than lysoPC (Sujak et al. 2000). The uptake of PC itself by the intestinal cells is known to be much lower than that of lysoPC (Homan and Hamlehle, 1998; Reynier et al. 1985). Thus, PC can strongly retain the hydrophobic carotenoids in the mixed micelles so that the absorption by the intestinal cells is suppressed. The cells of the jejunum can take up lysoPC across the unstirred water layer, whereas bile acids are taken up in the ileum. Further, lysoPC might strongly associate with L and facilitate its diffusion across the water layer from mixed micelles to the brush border membrane of intestinal cells. Moreover, the lysoPC taken up into the intestinal cells is quickly converted to PC and triglycerides, which then stimulate the synthesis of triglycerides and the secretion of chylomicron (Field et al. 1994).

The effect of phospholipids on L uptake in rats found in the present study was in agreement with those reported on the uptake of cholesterol and α-tocopherol (Koo and Noh, 2001; Noh and Koo, 2003). The intestinal uptake of such highly hydrophobic substances like L may partly follow a similar mechanism. These properties of phospholipids would make it possible to modify the bioavailability of hydrophobic substances with diet and supplements rich in phospholipids. Since carotenoids were fed to rats after direct solubilization in mixed micelles in the present study, it is unsure whether phospholipids present in foods can influence similarly, the bioavailability of dietary carotenoids. However, the amount of PC or lysoPC fed to rats in the present study was ca.19 mmol/kg body weight, which was comparable to the daily intake of dietary PC (0.91-1.85mmol) in the Western diets (Akesson, 1982). Moreover, dietary

supplementation with egg PC or sphingomyelin was reported to suppress cholesterol absorption in the human intestine (Kasaniemi and Grundy, 1986) and in rats (Noh and Koo, 2003). Consequently, dietary phospholipids have high potential and play an important and crucial role in enhancing the bioavailability of carotenoids not only in L sufficient animals but also under condition like L deficiency (Beatty et al. 2000) as the dietary lecithin was found to support the above hypothesis.

In case of rats fed with different fatty acid micelles, the results revealed that the influence of oleic acid on the plasma L + Z level was significantly higher than that of linoleic acid. This study compared the postprandial plasma and eye response to L in rats fed with OA and LA (gavage study) or L deficient and sufficient diets with OO or SFO. Maximum postprandial plasma L was observed with the OA and LA micelles after 2-3 h (single dose) and comparable returns to base line within 9 h. The results reveal that the influence of micellar OA on the plasma L level was 2-3 folds higher than that of LA. Clark et al. (2000) reported that olive oil significantly influenced the absorption of lycopene and astaxanthin compared with corn oil in lymph cannulated rats suggesting that the type of lipid ingested matters on carotenoids uptake, whereas, Ahuja et al. (2006) did not find any difference between the effect of olive and sunflower oil on lycopene absorption in human subjects. The differences in plasma triglycerides reflected those of the fatty acid or phospholipids ingested, indicating that the ingested micellar fatty acid or phospholipids readily alters the plasma TG as a result of the efficient processing of fat digestion, absorption and intestinal secretion. Tso (1994), Hollander and Ruble (1978) and Raju et al. (2006) found that the rate of β-carotene disappearance from micellar perfusates in rats was higher when oleic acid was added to perfusates or mixed micelles than linoleic acid which stated that the rate of transport depends upon the hydrophobicity of fatty acids.

Van het Hof et al. (2000) and Mekki et al. (2002) reported that the size of emulsion droplet is one of the factors that affect the bioaccessibility of lipids and lipid soluble nutrients. In this study, plasma response of newly absorbed L was significantly higher in OA and OAR groups than LA and LAR groups. The process of emulsification occurring in the stomach and mixed micelles thereafter in the intestine generates different sizes of droplet with diameters ranging between 10-100 μm and 4-20 μm in the aqueous medium; it is likely that the ingested LA or SFO forms larger sized micelles (17

μm) compared with OA or OO (9 μm) as reported by Armand et al. (1996). Fave et al. (2004) also reported that physico-chemical properties of lipids such as droplet size and their organization and the structure of the droplet surface, greatly influence the emulsification, enzymatic hydrolysis, solubilization and transport functions involved in dietary fatty acid bioavailability. They also suggested that lipid-droplet size directly affects the lipid water-interface area and influences the fatty acid bioavailability. LA or SFO may not be a suitable substrate for gut lipase, which acts at the aqueous interface of lipid droplet. The OA or OO used in this study are expected to provide TG more conducive to physicochemical domain. A significantly larger size (7.52 μm) of the LA micelles could be one of the reasons for the lower level of plasma L (AUC=13.46) compared with smaller size (5.13 μm) of OA micelles (AUC=15.85). Apart from the micellar size, the intensity of L within the mixed micelles correlated positively with the plasma L level after a single dose of micellar L (chapter 4). After L is transferred to mixed micelles, it was found that the micelles containing LA or PUFA were larger in size with lesser L, diffusion was slower through enterocytes thereby decreasing the rate of β-carotene absorption in human and rat models, than micelles containing saturated fatty acids (Raju et al. 2006; Van het Hof et al. 2000).

An elevated L level in the eyes of rats after repeated dose of L solubilized in OA micelles (49.7 ± 9.2 pmol/g) than LA micelles (37.0 ± 11.8 pmol/g) suggests that, OA not only influences the intestinal accessibility but also sways the transport of newly absorbed L to the target tissue. An elevated level of plasma TG further supports the above hypothesis being a carrier molecule for L. Lower level of plasma L after 4 h and 10 d of intubation and its higher level in eyes further supports the basis for clearance of absorbed L via TG **(Tables 5.1 and 5.2 and Figure 5.2 and 5.5).** A similar trend was reported for β-carotene in rats by Raju et al. (2006). Van Greevenbroek et al. (1996) and Tso (1994) also reported that olive and sunflower oil did influence difference in postprandial plasma and eye L response suggesting a higher retention of oleic acid than linoleic acid in the digestive tract and blood. Whereas, Hussain et al. (2000) and De Bruin et al. (1993) reported higher level of plasma TG chylomicron response in men after single and repeated ingestion of olive oil rich meal, as compared with safflower oil rich meal. These data along with the results of this study suggest that, the oleic acid rich

olive oil could be used to influence the intestinal accessibility and other steps involved in the metabolism of L *in vivo*.

Although the same amount of fats were fed to the rats, the LA diet elucidated significantly lower plasma and eye L response (40 and 52% for 0-16 d AUC) than the OO diet, but the levels were 18 and 21% higher ($p<0.05$) compared with control diet. This observation agrees with the data obtained by others using dairy fats or unesterified saturated fat or butter (Mekki et al. 2002; Thoisterup et al. 2001). In contrast, studies showed no difference after providing saturated fat compared with unsaturated fat (Juhel et al.1999). Differences in the amount and type of fa**t,** composition of test meals and experimental model used could partly explain such discrepancies. Several mechanisms could be involved in the reduced postprandial response elicited by LA or SFO as observed in this study when considering the physicochemical state of SFO and its fatty acid composition (Mekki et al. 2002).

Bell et al. (1997) suggested that the re-secretion of fatty acids from the enterocytes into the circulation is likely to be another vital step involved for the differences between dietary fats. Further, Bracco (1994) suggested that the bioavailability of fatty acids can be improved or altered depending on the structure of the triglycerides. The lipid sources used in this study (OO and SFO) also differ in their triglycerides (TG) and fatty acid composition **(Table 5.5).** OO is rich in oleic acid whereas, SFO is rich in linoleic acid. The structure of TG fatty acids has an impact on the activity of lipase because it releases short and medium chain fatty acids earlier than long chain fatty acids located at the sn-1 and sn-3 positions on the glycerol backbone (Jandacek et al.1987). Also, the difference in the structure of the TG in vegetable oils may affect lipid droplet size and activity of lipases (Armand et al. 1992 and Borel et al.1994). Thus, the selection of vegetable oil or source of lipid or fatty acids used in bioavailability studies, as L carrier, is very important. The structure of the TG in the vegetable oil could influence lipid digestion by affecting the lipid droplet organization and fatty acids absorption as observed in this study. Hamilton et al (1996) and Innis et al (1997) have reported that TG located in the lipid droplet surface varies depending on the unsaturation and the long-chain fatty acids are poorly absorbed from the intestinal lumen when they are esterified at positions sn-1 and sn-3 of the glycerol backbone due to their strong tendency to form insoluble soaps. Thus, this study suggest that intestinal re-

secretion of triglyceride rich lipoproteins-long chain fatty acids in the circulation may differ in the following decreasing order: oleic> linoleic acid or olive >sunflower oil.

Short- and medium-chain fatty acids are readily transported from the enterocytes membrane, as complexes with albumin via the portal route (Jensen et al.1994). In contrast, Borel et al. (1998) and Odeberg et al. (2003) reported that incorporation of β-carotene and astaxanthin into chylomicrons in human subjects was lower with emulsion containing medium-chain than long-chain triglycerides and suggested that medium-chain triglycerides are mainly transported by portal blood and not favoring lymphatic transport of lipoproteins (Caliph et al.2000). Given that 15 and 78% short- and medium-chain fatty acids in olive oil, one might expect a reduction in their level in the plasma postprandially. The replacement of butter (rich in medium-chain FA) by long-chain oleic and stearic acids increased postprandial chylomicron response in human subjects (Thiosterup et al.1998). In fact, the overall reduction in plasma TG observed in the present study is higher after single, repeated dose and dietary feeding implicating the above mechanism.

Apart from the nature and chemical composition of fats, the level of triglycerides and fatty acid profile of plasma, liver and eyes of rats were determined after feeding diet supplemented with L along with phospholipids and fatty acids, as they were carriers for the absorbed L to the target tissues. In another study, rats were induced with L deficiency by feeding them a diet devoid of L. On confirmation of L deficiency, rats were fed on diet supplemented with green leafy vegetable (*Trigonella foenum-graecum* L.) rich in L along with soy lecithin and vegetable oils (as fat source) to determine their effects on bioavailability of dietary L. The data was compared with the results of gavages studies. The results obtained from dietary studies are found to be in line with the results of gavages studies and rats fed with L sufficient diet indicating that selection of specific lipid or fatty acid is very essential to positively manipulate the bioavailability of L not only in normal but also animals deficient with L. The results of this study may have direct impact on human kind in combating/slowing the progression of AMD.

Although, specific fatty acids or vegetable lipids play an important role on enhancing the bioavailability of L, in addition, they are found to be involved in the regulation of oxidative mechanism. The present results demonstrate that either monounsaturated fatty acid or its rich olive oil is found to protect tissues from peroxidation attack compare to that of PUFA - rich sunflower oil (Mataix et al.1998).

The effects of oleic acid rich diet (olive oil) on liver lipid composition, antioxidant enzyme activities, were compared with those of SFO and control rats. Compared with the control diet fed groups, plasma and hepatic total triacylglycerides concentrations were higher in the rats fed on OO diet and it was decreased in those of SFO fed rats. Further, changes in the liver fatty acid composition found in this study may be due to unsaturated lipids in SFO, in turn they enhance the efficiency of the antioxidant system (Ruiz- Gutierrez et al.1999; Venkataraman et al. 1998). Further, the accumulation of tissue fatty acid depends on the source of dietary lipids intake. In this study, rats fed on diet with OO significantly increased the oleic acid (18:1) level in the tissues, whereas SFO produced an elevated level of linoleic acid (18:2). The data demonstrates an inverse correlation between dietary lipids mediated oxidative stress and antioxidant enzyme activities. Since carotenoids have been known as good antioxidants, especially L and Z are involved in the protective action against oxidative reaction. Hence, bioavailability of L from GLVs may positively involve in the protection of oxidative damage to the tissues (Krinsky, 2002). This has been supported by studies using *in vitro* retinal cell culture models showing that treatment with antioxidants, including L and Z, substantially decreased oxidative stress-induced lipid peroxidation and apoptosis (Cai et al. 2000; Winkler et al.1999; Wrona et al. 2001). The present data suggest that high levels of dietary unsaturated fat should be avoided if oxidative stress is a critical issue in nutrition-related diseases (Slim et al. 1996).

In conclusion, the choice of carrier lipid is critical to achieve an enhanced bioavailability of dietary L *in vivo*. The relative bioavailability of L could be improved by its incorporation into OA or OO combination either by single, repeated and dietary doses. This is an interesting observation that compared enhanced L bioavailability from micelles containing different fatty acids or vegetable oils (source of fatty acid) in L sufficient and its deficient rat model. The proposed mechanism by which dietary OA or OO influences the L bioavailability is well correlated with physico-chemical properties of mixed micelles, viz. size, surface area and L intensity. Fatty acid composition of fat is another important factor to be considered to influence the bioefficacy of L more effectively in macular pigments deficient rats. Further, L supplementation with physiological emulsions or micelles or diet containing OA or OO may improve the plasma and eye L status (**Figure 5.21**) which in turn help in overcoming macular

pigments deficiency related disorders. These new observations could be of interest in enteral nutrition of AMD subjects. Further, the present findings demonstrate for the first time, the application of image processing technique to measure the size of micelles, L content therein and to correlate these properties with postprandial plasma and eyes levels of L.

Chapter 6

Possible degradation/biotransformation of lutein *in vitro* and *in vivo*: isolation and structural elucidation of lutein metabolites by HPLC and LC-MS (APCI) techniques

Introduction

Age related macular degeneration (AMD) is a leading cause of vision loss in the elderly for which treatment options are limited (Krinsky et al. 2003). Increased consumption of dark green leafy vegetables (GLVs) rich in lutein (L) and zeaxanthin (Z) is reported to delay progression of AMD (Bone et al. 2003). The major dietary carotenoids present in human plasma are lycopene, β-carotene, α-carotene, β-cryptoxanthin, γ-carotene, phytofluene, phytoene, L and Z. Among these L, Z and meso-Z are reported to be major pigments in human *retina* (Bone et al. 1993) and are the best antioxidants for reversing AMD (Klein et al. 1995). L and its metabolites may guard the photoreceptors in *retina* from exposure to harmful short wave length blue light and protect the *macula* from photo-oxidation (Schalch and Dayhaw-Barker, 1999). Hence, these pigments are referred as the macular pigments (MP). Studies have indicated that the consumption of GLVs containing L (~6 mg/d) lowers the risk of AMD by 43%. Earlier reports (Bone et al. 1993; Lakshminarayana et al. 2006; Khachik et al. 2002) indicate the intestinal absorption and possible biotransformation of L and Z *in vivo*.

In general, carotenoid metabolites are reported to be involved in the chemoprevention of cancer (Khachik et al.1995a; King et al. 1997) and increase the gap junction communication (Hanush et al. 1995). The MP has been postulated to improve acuity through the amelioration of the effects of chronic aberration (Reading and Weale, 1974) to preserve (Hammond et al. 1998) and protect (Snodderly, 1995; Beatty et al. 1999) the central *retina*. In natural systems, L and Z are found in different chemical environments (Bone et al. 1993). These xanthophylls (L and Z) and *meso*-zeaxanthin (*meso*-Z) are isolated from *macula* and characterized using HPLC technique (Bone et al. 2001). Apart from the dietary carotenoids, 3'-epilutein, 3' dehydrolutein, (3R, 3'S) *meso*-Z, 3'-oxolutein and 3-methoxy zeaxanthin were also reported in human ocular tissues and serum (Bone et al. 1993; Khachik et al.2002; Khachik et al.1997; Bernstein et

al.2001; Bhosale and Bernstein, 2001; Bhosale et al.2007). 3'-Oxolutein was reported as a major oxidative derivative formed by direct oxidation of L and Z. The presence of the direct oxidation product of L in human *retina* suggests that L and Z may perform as antioxidants to protect the *macula* (Krinsky et al. 2003). However, the pathways of biotransformation and biological functions of L metabolites (oxidative breakdown products) and their isomers have not been fully understood. Khachick et al. (1999, 2002) for the first time reported oxidation products of L and Z in human and non-primate models. Earlier reports suggest that carotenoids with increased number of oxo- and hydroxyl- functional groups show an enhanced antioxidant capacity (Hurst et al. 2004; Siems et al.1999). Carotenoids could be oxidized to form cleavage products by various agents, which include oxidizing agents, azo compounds, free radical reactions, etc., (Stratton and Liebler, 1997; Scheidegger et al.1998; Sommerburg et al. 2003). Conversions of β-carotene and other provitamin A carotenoids into *retina*l and retinoic acid are well documented in rats and humans (Ganguly and Sastry, 1985). However, not much scientific information is available on the oxidative breakdown/metabolism of non-provitamin A carotenoids (Astorg et al.1994; Gradelet et al.1996a and 1996b).

Several biochemical studies have demonstrated that L and Z can be bleached rapidly *in vitro* by photolysis (Mortensen and Skibsted, 1997). They have reported that the bleaching rate and the loss in absorption (λ_{max}) as a function of exposure time are similar for L and Z in the presence of radicals. L is highly reactive with free radicals and other reactive oxygen species. It is hypothesized that oxidized L products may be involved in protecting eyes from the ultraviolet rays and may act as better antioxidants than the parent compound itself. Epidemiological studies suggest that complete characterization of carotenoids and their metabolites in *retina* may help in understanding their functional properties (Khachik et al. 1996). Hence, it is vital to assess the formation of L oxidation products and its cleavage/fragmentation pattern before understanding their biological function. Although considerable attention has been directed towards elucidating the functions of L and Z in the *retina*, there is no detailed study on the identification and characterization of their full spectrum in human tissues. Therefore, a sensitive and specific analytical method and studies are needed to characterize the oxidative metabolites of L with regard to their antioxidant properties to rationalize their

bioactivity. Against this background, the present work was undertaken to elucidate possible biotransformation of L *in vitro* (photo-oxidation) as well as *in vivo (*rat model).

Results

This study was aimed at optimizing the analytical conditions for enumeration of L and its metabolites using HPLC and LC-MS (APCI) from *in vitro* (liposome) and *in vivo* (eyes, liver, plasma and intestine) samples. The peaks of L and its fragments in the scanned range between 0 to 1000 *m/z*, were designated as A, B, C, D, E, F, G, H, I and J **(Table 6.1)**. The APCI detector parameters were adjusted suitably to give ion signal intensity for identification of peaks. The solvents in mobile phase such as acetonitrile (60%), methanol (20%) and DCM (20%) appeared to have no effect on formation of positive ions. An integrated chromatographic peak area was measured by PDA at 450 nm, HPLC-MS was very sensitive, permitting detection limits at 10-pmoles with a signal-to-noise criteria of 10. Under the conditions mentioned, a prominent peak at *m/z* 551.5 was detected for L in spite of its molecular weight 568.9 indicating the elimination of water molecule from the protonated 569 amu $(M+1)^+$ ion, which is characteristic of hydroxy- carotenoids like L. This peak is more intense in L fed intestinal sample than *in vitro* samples. As reported by Dachtler et al. (2001), the hydroxyl- group at C 3' position is allylic in L and the loss of water molecule from the protonated parent ion gives a more stable allylic radical cation **(Figure 6.1)**. For convenience, in-chain geometrical isomers of carotenoids, the terms *all-E* and *Z* that refer to *all-trans* and *cis*-isomers of carotenoids respectively are used in this text instead of the old nomenclature.

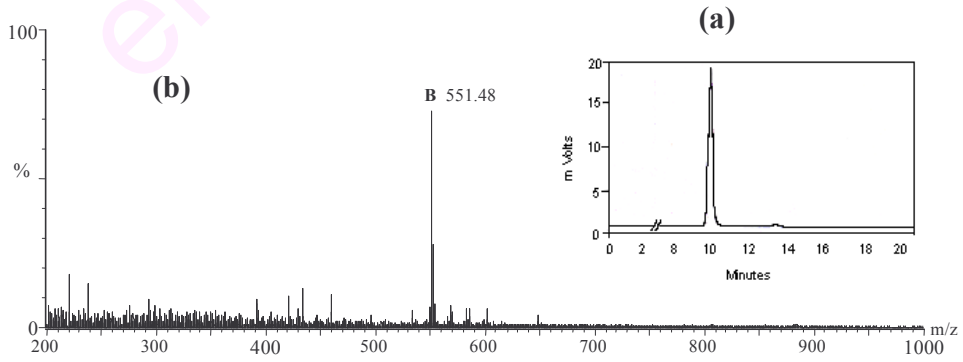


Figure 6.1. A typical HPLC profile of standard lutein (a) and its mass spectrum (b). HPLC and LC-MS conditions adopted were outlined under materials and method section.

Photo-oxidation products of L *in vitro*

In the present study, photo-induced degradation of L was investigated *in vitro*. The typical HPLC profiles of L and its oxidized products are shown in **Figure 6.2a**. During the course of light exposure, optical density of sample was measured. The results on the optical density of L extract after sunlight exposure showed L concentration depletion with increased duration of sunlight exposure indicating bleaching of L, which was not found in control samples kept at dark. An initial (0 min exposure) concentration of L (20 nmol/2 mL) measured at 450 nm was decreased to less than 1 nmol/2 mL after 100 min exposure indicating that L was involved in a photochemical reaction (**Figure 6.2b**) which resulted in slack in its chemical structure. The characteristic UV visible spectra lend further support to the hypothesis that L is degraded to various isomers and photolytic products (**Figure 6.2c and 6.2d**). The characteristic fragmented ions (C, D, E and F) formed from L in the samples exposed to sunlight are C corresponding to an exact breakage of the parent molecule M^++H^+-$2H_2O$ into two fragments, D (M^++H^+-$3CH_3$), E (M^++H^+-$6CH_3$) and F (M^++H^+-W) (**Figure 6.3a** and **Table 6.1**). It is an established fact that carotenoid pigments are made up of a system of conjugated double bonds, which makes them vulnerable to photo-oxidation leading to the cleavage/oxidation and molecular reactions involving the double bonds (Bonnie and Choo, 1999). A similar process might have occurred in the present study, when L was exposed to direct sunlight. A study on bleaching mechanism of carotenoids from red pepper due to light exposure was reported by Jung and Lee (2006). They have demonstrated that carotenoids in red pepper were degraded when exposed to light. The present *in vitro* study and the results of Jung and Lee (2006) demonstrate that pigments are either in bound or pure form and may degrade due to oxidation. Lu et al. (2001) demonstrated a light induced degradation of xanthophyll pigments in leaves which further supports that carotenoids within the xanthophyll cycle undergo epoxidations and de-epoxidations to different extents in response to light exposure.

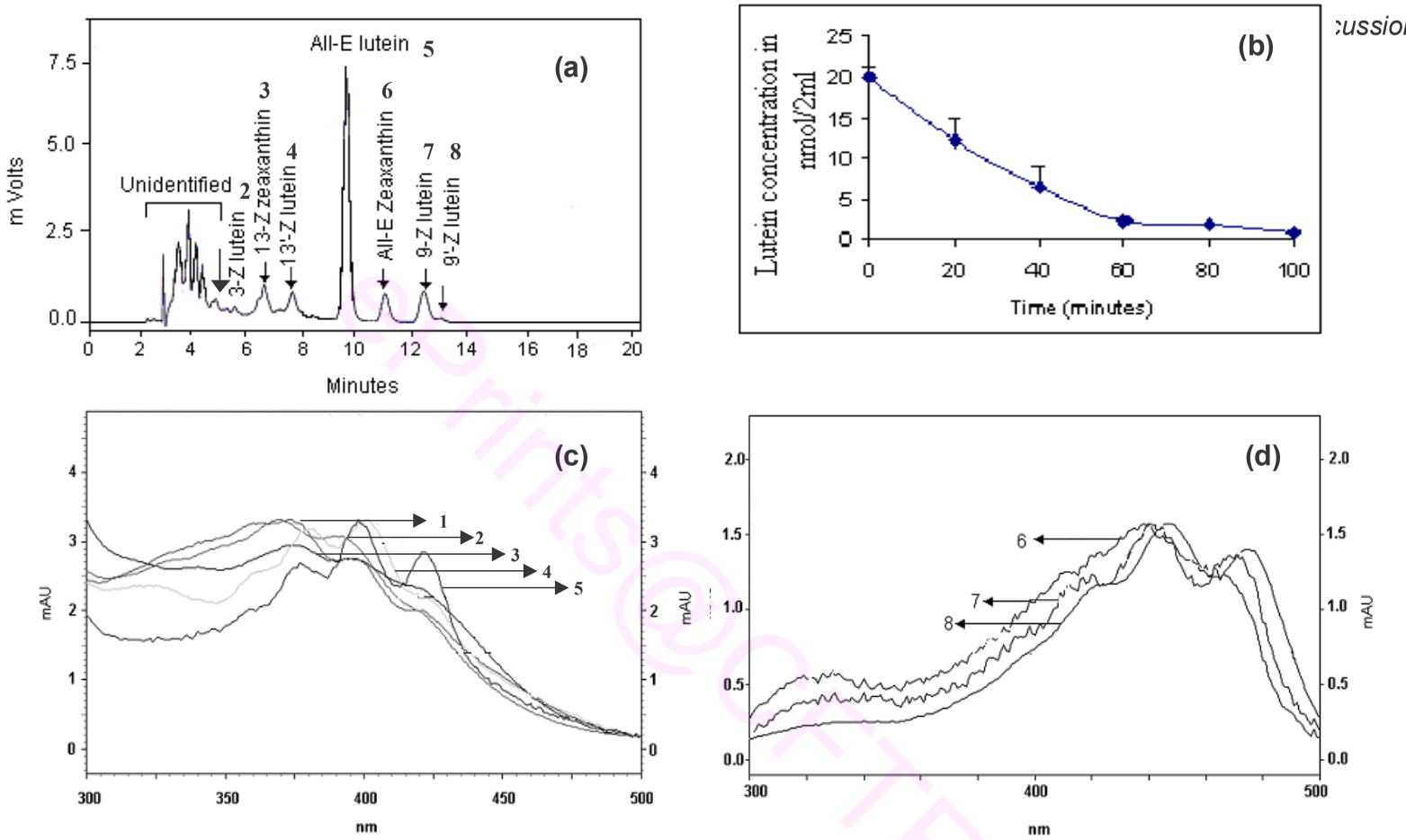


Figure 6.2. HPLC elution profile of photo-oxidized products of lutein resulted after liposome containing lutein exposed to direct sunlight for 100 minutes (A), optical density of lutein at 450 nm *vs* duration of sunlight exposure showing the lutein degradation (B) overlaid spectrums of lutein and its oxidized compounds (C, D). HPLC and LC-MS conditions were outlined in materials and methods section. In Figures. C and D, spectra 1 to 8 refer to components 1 to 8 in A.

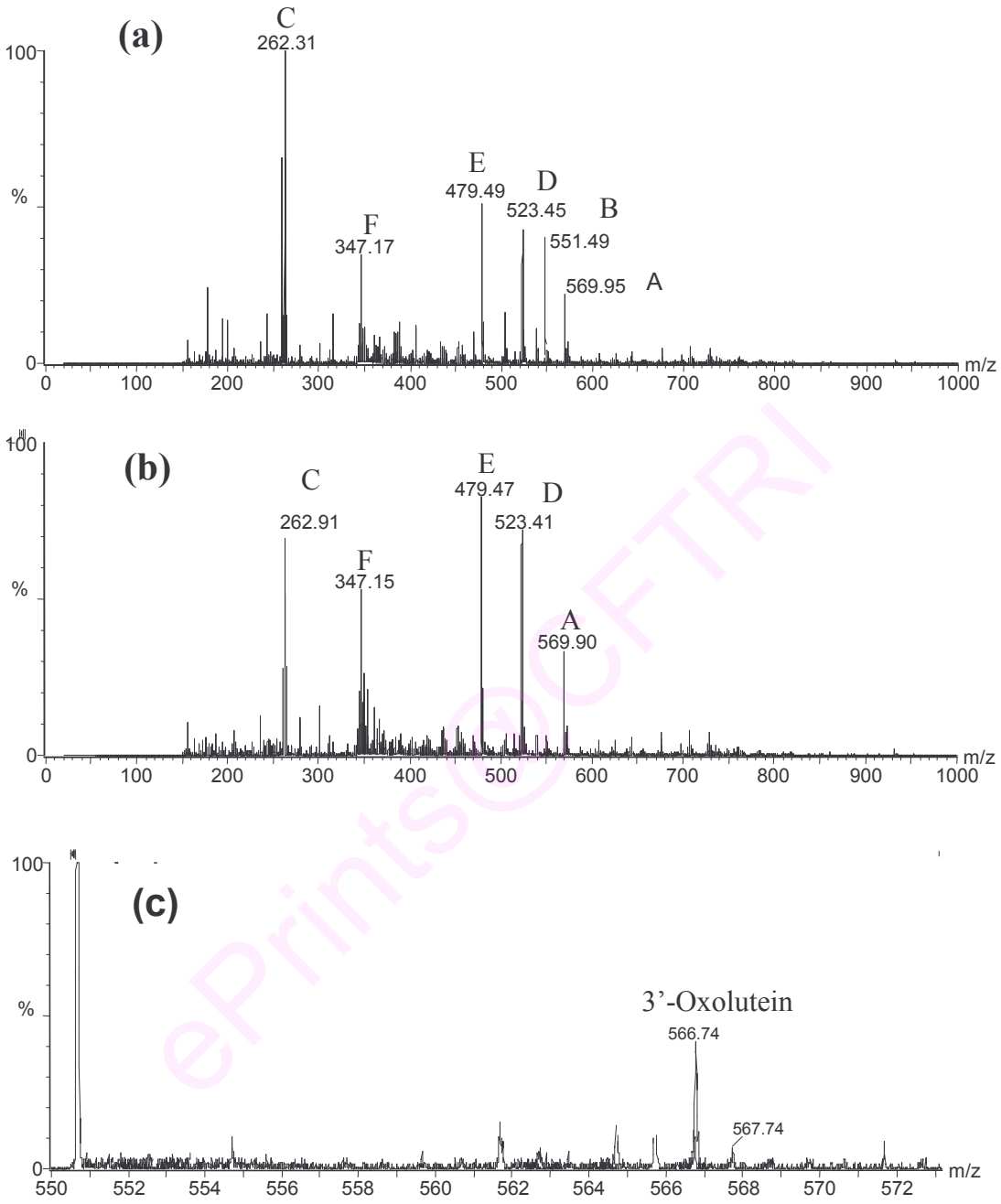
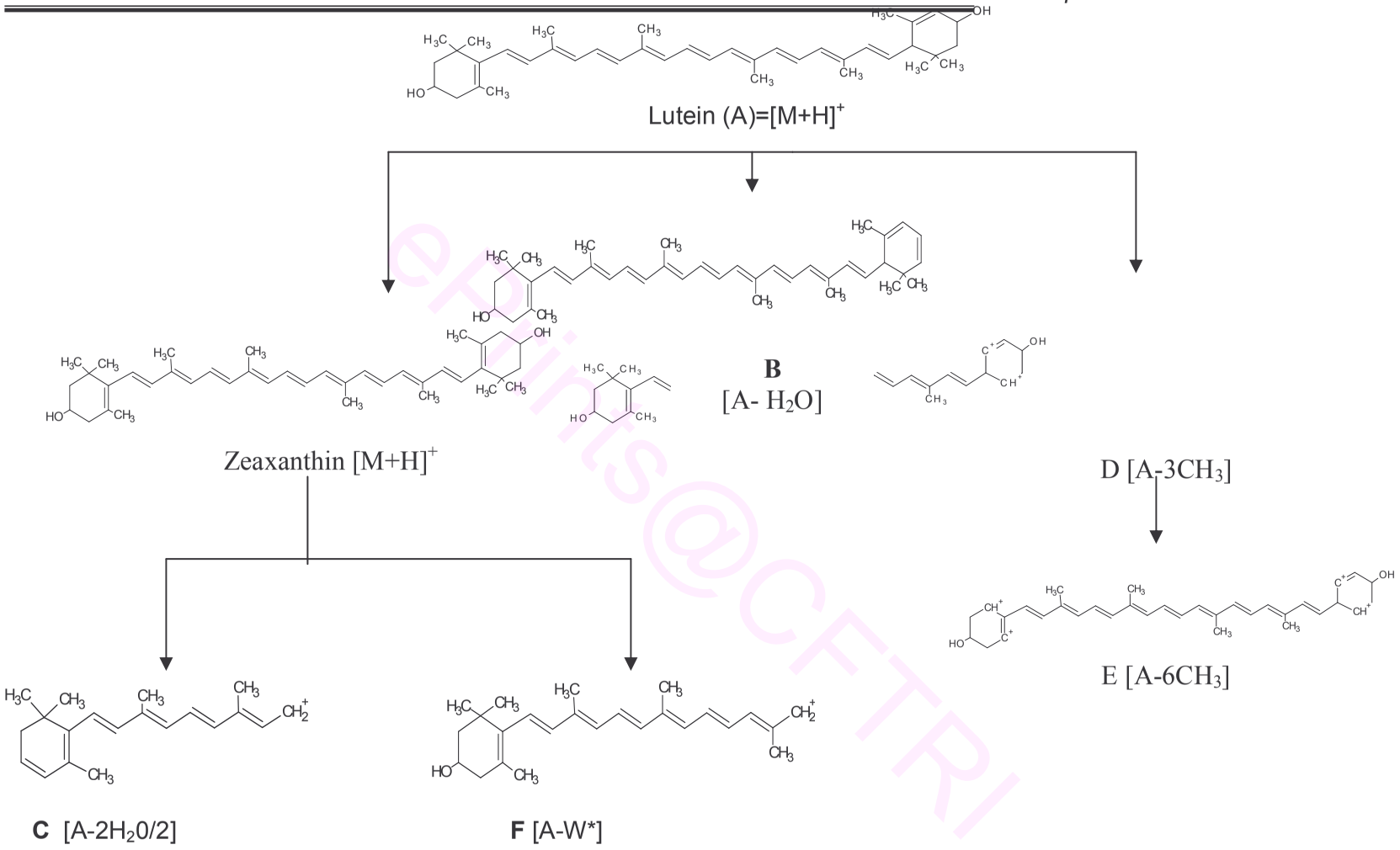


Figure 6.3. APCI-MS profiles of lutein and its sunlight induced oxidized products (a) and lutein and its metabolites isolated from pooled rat eyes (b) and (c). Refer materials and methods section for LC-MS conditions. Refer Table 6.1 for respective mass spectra of A to Z (shown within the bracket).



*Structure given in Table 2.

Figure 6. 4. Proposed pathway of lutein fragmentation *in vitro* by photolysis (Scheme 1)

Lutein and its metabolites *in vivo*

Biotransformation of L in eyes. Rats were divided into two groups, Group 1 (Gavage study) and Group 2 (Dietary study). The objective of the later experiment was to confirm the degradation of L when fed along with dietary matrix. In the case of former experiment, L was intubated to animals in the form of mixed micelles. The results obtained in both the experiments were found to be almost similar indicating that the degradation of L appears to take place at the intestinal level or after absorption. Since the concentration of L metabolites was found to be below the detectable limit in a single pair of eyes, pooled eyes (n=6 x 2) were used for extraction. The characteristic fragmented ions obtained in eye samples were A, C, D, E and F (**Table 6.1 and Figure 6.3b**). Some of the L isomers (13-*Z* lutein, 13'-*Z* lutein, 13-*Z* zeaxanthin, *all-E* zeaxanthin 9-*Z* lutein, and 9'-*Z* lutein) were well separated and identified by HPLC (**Figure 6.5d**) in eye samples. Similar to the earlier reports (Khachik et al. 1997; Bhosale and Bernstein, 2005) 3'-oxolutein was identified in the rat eye samples, but it is detected in very low abundance at *m/z* base peak of 567.5. The retention times for 3'-oxolutein, L and Z were 8.5, 9.8 and 10.9 min by HPLC **(Figure 6.3c and Figure 6.5d)**. According to the metabolic transformation, L metabolites are listed in **Table 6.1** and may be inter-converted through a series of oxidation-reduction and double isomerization reactions as proposed by Khachick et al. (1997). Oxidation of L to 3`-oxolutein may be due to the activation of the hydroxyl- group at C-3' by the neighboring allylic double bond. However, in (3*R*, 3'*R*)-Z, because of non-allylic nature of the OH groups at C-3 and C-3', it may not be directly oxidized or may therefore undergo double bond isomerization to yield epilutein before oxidation (Khachik et al. 1997). The presence of *meso*-Z in the eye samples and the stereochemistry of macular carotenoids in human have been well stabilized by Bone et al. (1997).

Biotransformation of L in liver. In case of liver, the characteristic fragmented ions obtained from L were B, G, H, I and J (**Figure 6.7c and Table 6.1**). The identified base peak G [L-oxidized] corresponds to L diepoxide (5,6, 4', 5'-diepoxy-5, 6; 4', 5'-tetrahydro-β,β-carotene 3,3'-diol), and J (G-X) to 5,6 epoxy-3 hydroxy-12'-β,ε-carotene-12'al, and these metabolites may be formed due to L oxidation in the liver itself. Other

characteristic peaks H (B-Y) corresponding to (2*E*, 4E)-3-methyl-5- (2,6,6-trimethylcyclohexa-2, 4-dien-1-yl) penta-2, 4-dien-1-ylium and I (B-Z) to 2,6,6-trimethylcyclohex-2-ene-1, 4-bis (ylium) could have been formed from fragmentation of anhydrolutein (B). Z, the isomer of L was also identified in liver (**Figure 6.5c and Figure 6.7c**). In HPLC, a metabolite eluted at 4.8 min and was identified as L diepoxide, confirmed by UV visible spectrum and its characteristic m/z peak. L and its diepoxides were identified in liver and plasma samples of both the rat groups fed with L either by gavages or dietary supplementation. These diepoxides are not detected in eye samples indicating that they have not been mobilized from eyes to liver. Hence, it is assumed that diepoxides might be formed in liver itself or mobilized from intestine via plasma.

Biotransformation of L in plasma. In plasma, the fragments resulting from L were identified as B, G, H, I, and J (**Figure 6.7b and Table 6.1**). The L oxidative metabolites pattern in plasma was almost similar to that in liver. The HPLC profile of plasma extract showed a well-separated major peak, which was identified as L along with its diepoxide and Z (**Figure 6.5b**). Only few reports are available on epoxy-carotenoids in plasma (Lakshminarayana et al. 2006; Barua and Olson, 1998; Asai et al. 2004). The present results are in agreement with the observations of Barua and Olson (2001) who also reported epoxy carotenoids and Z *in vitro* and *in vivo.* Whereas, Bone et al. (1992) did not detect *meso*-Z in human plasma after dietary intake of L; they hence assumed it was derived from chemical processes within the eye.

Biotransformation of L in intestine. In case of intestine, anhydro-L was the only compound found (**Figure 6.7a**). The HPLC profile of intestinal homogenate showed anhydro-L, with a retention time of 15.3 min **(Figure 6. 5a).** The MS peaks obtained were B, H and I **(Table 6.1)**.

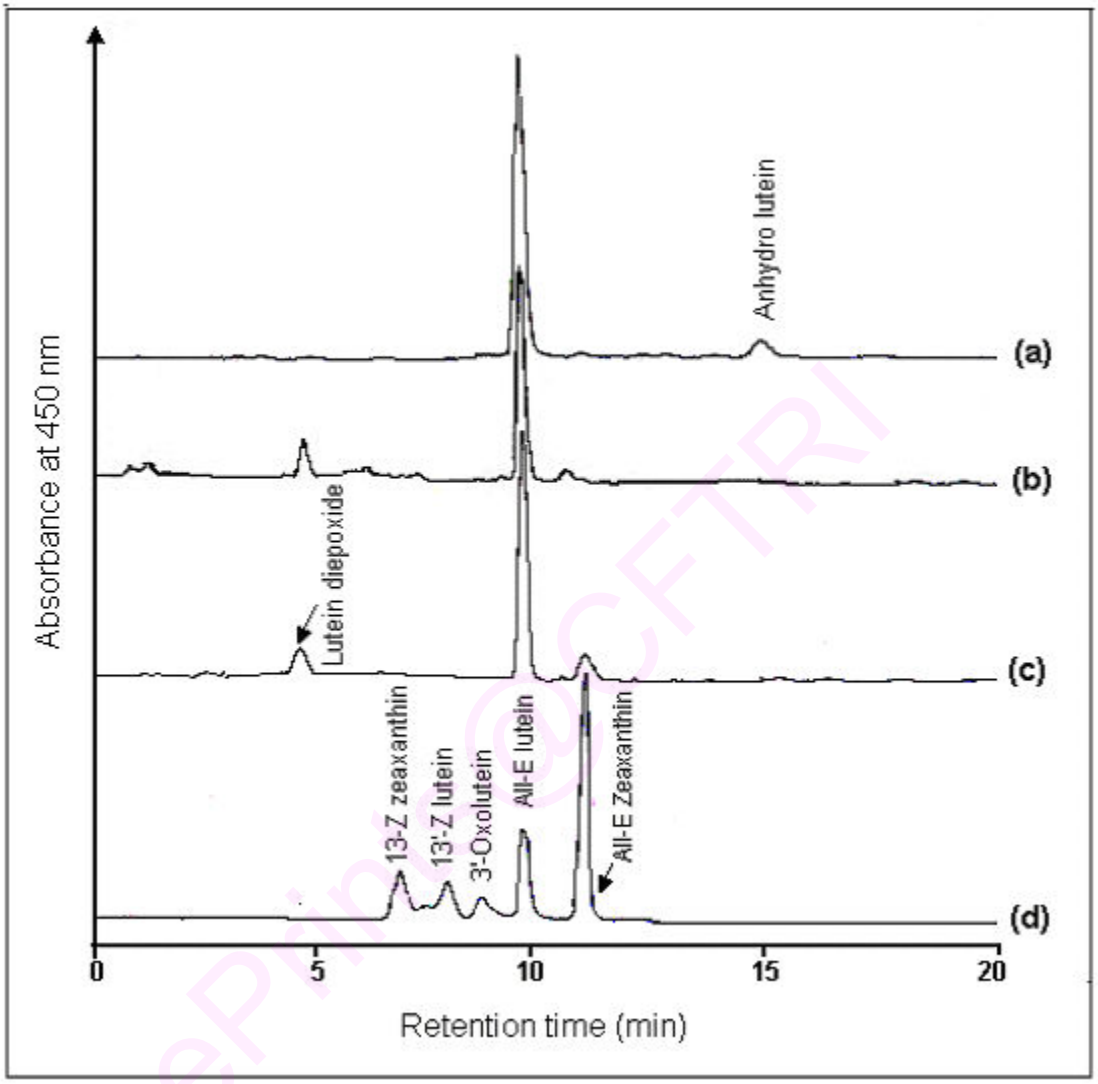
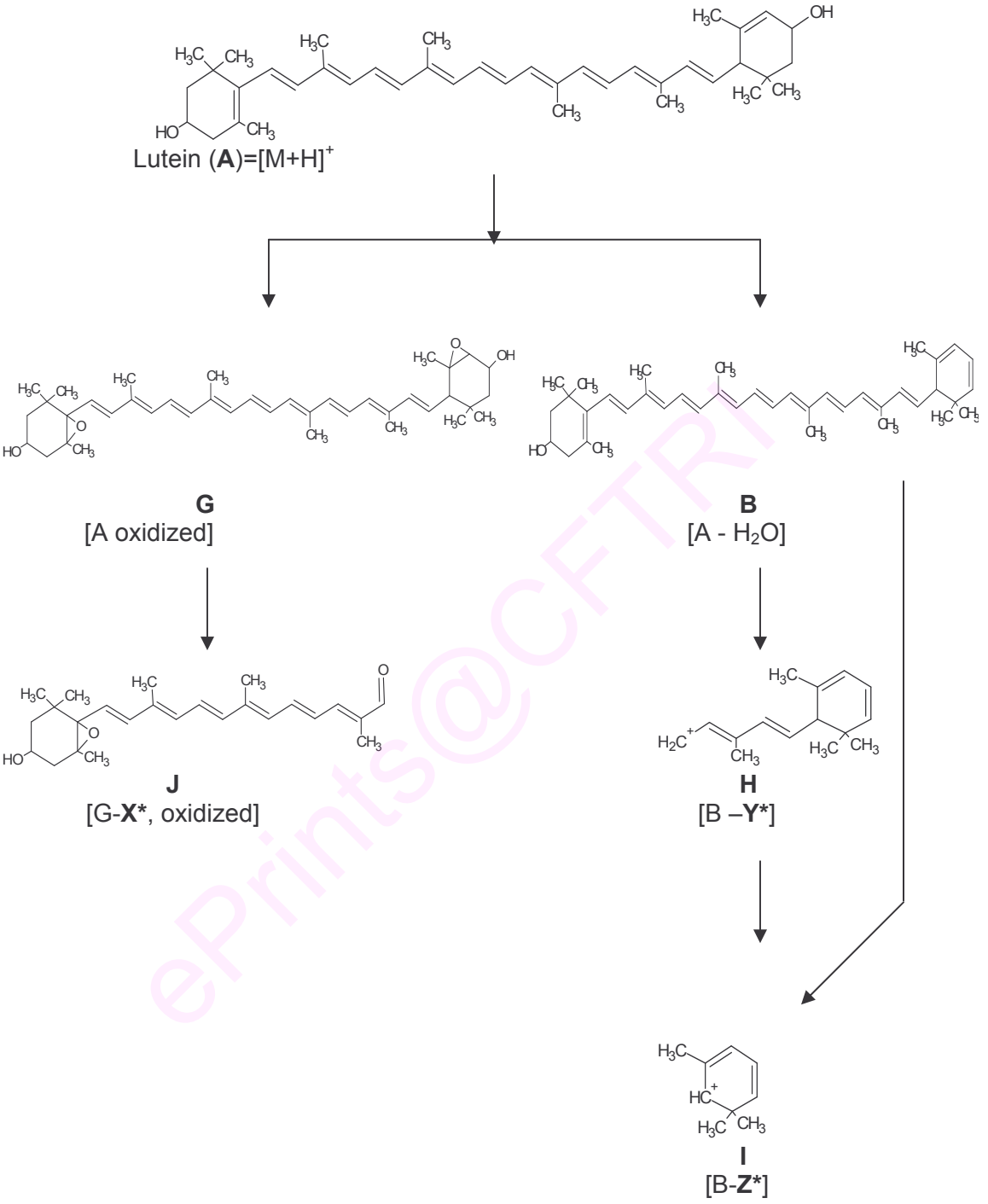


Figure 6.5. HPLC profiles of lutein and its metabolized/oxidized products eluted from intestine (a), plasma (b), liver (c) and eyes (d) after feeding lutein to rats.



* Structure given in **Table 6.1**

Figure 6.6. Proposed pathway of lutein biotransformation by oxidation/metabolism *in vivo* (Scheme 2)

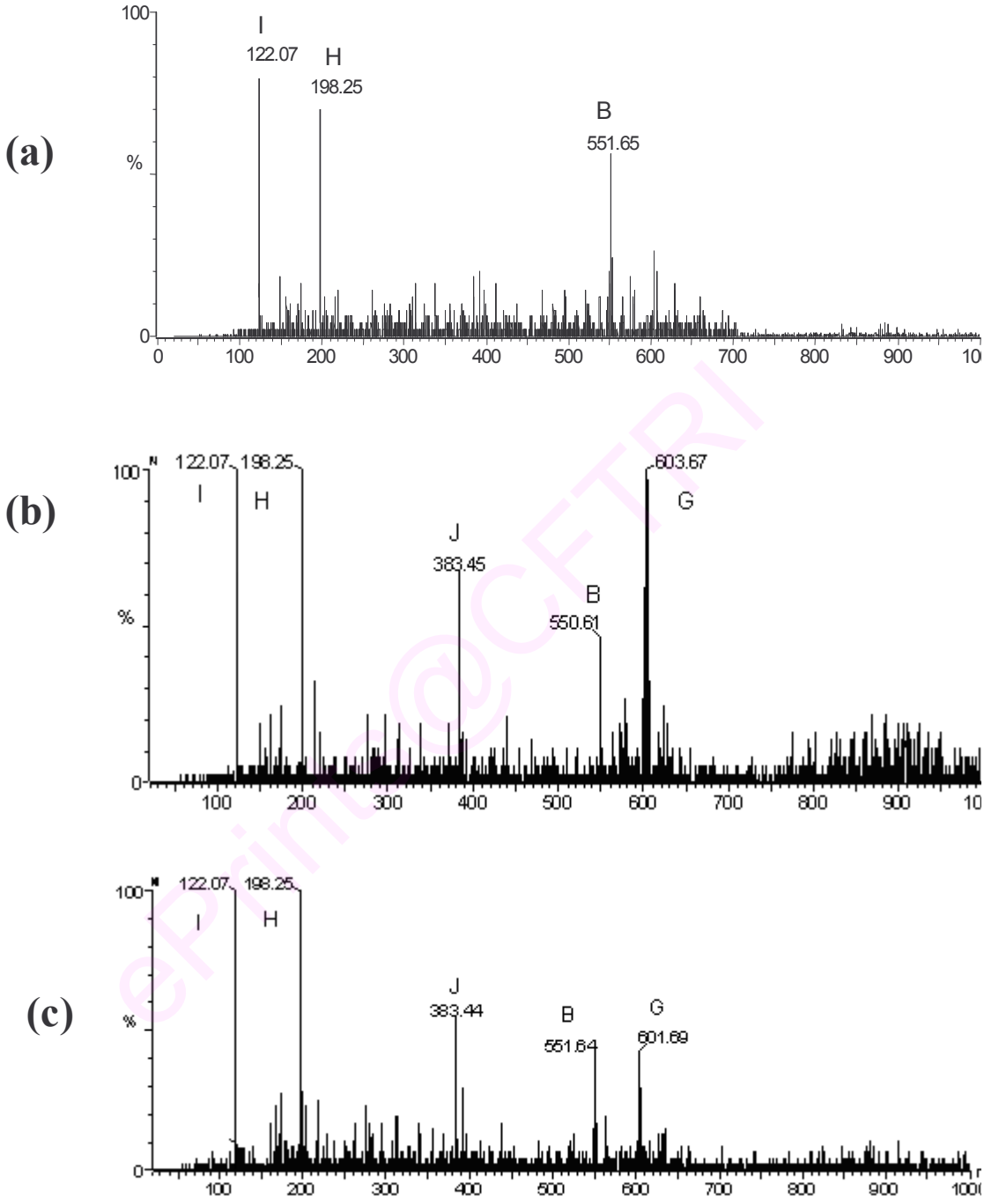
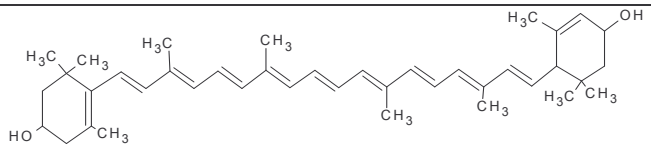
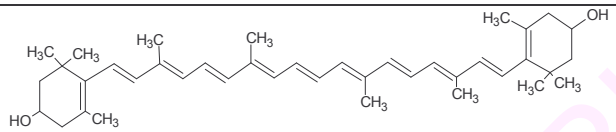
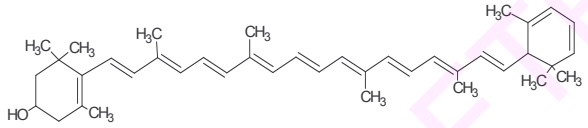
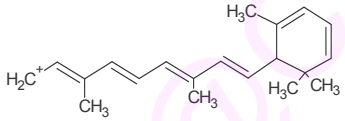
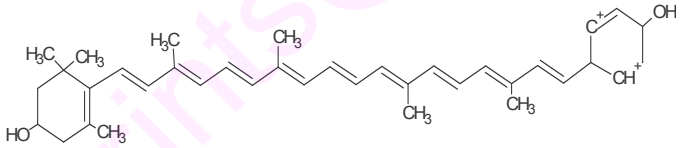
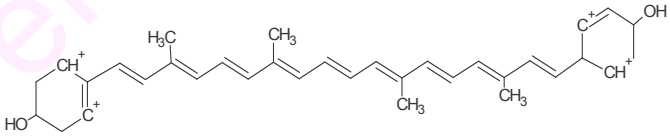
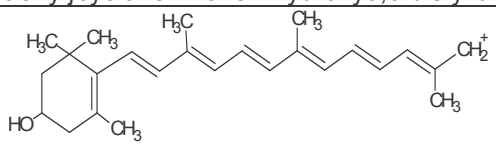


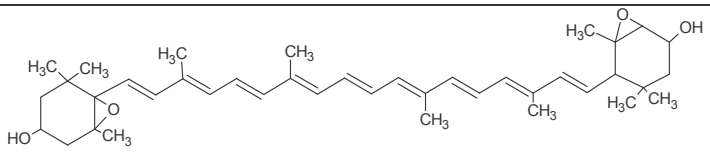
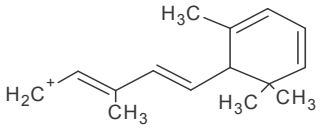
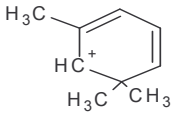
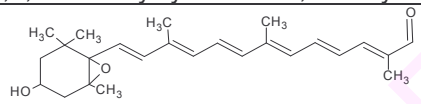
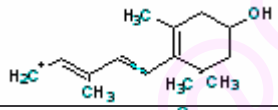
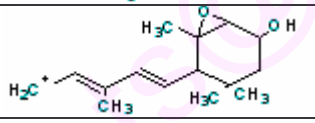
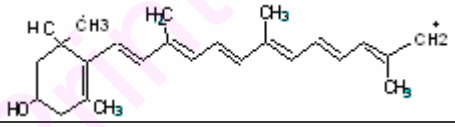
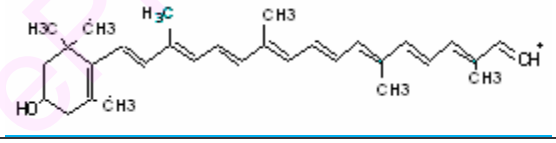
Figure 6.7. APCI-MS profiles of lutein and its oxidized/metabolized products isolated from rat intestine (a), plasma (b) and liver (c). Refer materials and methods for APCI-MS conditions. Refer Table 6.1 for alphabets given against mass spectra

Table 6.1. Fragmented ions derived from lutein *in vitro* and *in vivo,* elucidated by $APCI^+$ ion mode showing their chemical structure, molecular mass and molecular formula.

Alphabet	Structure of the compound	MM*	MF**
A	 Lutein	568.9	$C_{40}H_{56}O_2$
	 Zeaxanthin		
B	 Anhydrolutein	551.5	$C_{40}H_{56}O$
C	 3,7-dimethyl-9-(2,6,6-trimethylcyclohexa-2,4-dien-1-yl)nona-2,4,6,8-tetraen-1-ylium	267.4	$C_{20}H_{27}$
D	 4{18-[4-hydroxy 2,6,6 trimethyl cyclohex-2-en1-yl]-3,7,12,16-tetramethyl-1, 3,5,7,9,11,13,15,17-octa-decanonaenyl} cyclohex-2-ene1-hydroxy3, 5 bisylium	523.7	$C_{37}H_{47}O_2$
E	 4{18-[4-hydroxy cyclohex-2-ene 2,6 bis(ylium)-1-yl]-3,7,12,16-tetramethyl-1,3,5,7,9,11,13,15,17-octa decanonaenyl}cyclohex2-ene1-hydroxy3,5 bis ylium.	476.6	$C_{34}H_{36}O_2$
F	 3-hydroxy-12'-β,ε-carotene-12'-ylium	351.5	$C_{25}H_{35}O$

*MM, App. Molecular mass; **MF, Molecular Formula.

…Contd.

G	 Lutein diepoxide	601.8	$C_{40}H_{56}O_4$
H	 (2*E*,4*E*)-3-methyl-5-(2,6,6-trimethylcyclohexa-2,4-dien-1-yl)penta-2,4-dien-1-ylium	201.3	$C_{15}H_{21}$
I	 2,6,6-trimethylcyclohexa-2,4-dienylium	121.2	C_9H_{13}
J	 5,6-Epoxy-3-hydroxy-12'-β,ε-carotene-12'-al	382. 5	$C_{25}H_{34}O_3$
W		220	$C_{15}H_{23}O$
X		236	$C_{15}H_{24}O_2$
Y		352	$C_{25}H_{35}O$
Z		430	$C_{31}H_{41}O$

Discussion

The present study demonstrates that L is photolysed *in vitro* and metabolized/oxidized in rat tissue to several components. The analysis of mixed micelles and diet used in this study for intubation (gavages) and feeding (dietary) revealed that there were no oxidation products of L commonly found in biological samples. This demonstrates that these oxidation products appeared only after feeding of L. Further, these metabolites/oxidation products were not detected in control samples incubated at dark (*in vitro*) or animals fed with no L *(in vivo*). The available literature provides an indication that L and Z may play an active role in modulating the course of AMD. Yet, critical evidence of the beneficial effect of L and Z or their metabolites/oxidation products has not been elucidated in detail (Mares-Perlman et al.1995). Among the carotenoids identified in intestine, plasma, liver and eyes of rats after feeding L, only (*all-trans*, 3*R*, 3`*R*, 6`*R*)-L and (*all-trans*, 3*R*, 3`*R*)-Z are of the dietary origin. Samples were handled carefully, with all precautions to avoid the possibility of oxidation of L and Z. Further, we have previously found that Z in rat plasma is in the form of dietary (3*R*, 3'*R*)-Z and *meso*-Z (meager concentration) (Lakshminarayana et al. 2007). Therefore, the fact that, Bone et al. (1993) have reported that (3'*R*, 6'*R*)-L, (3*R*, 3'*R*)-Z and (3*R*, 3'*R*)-*meso*-Z are present in the human *macula* and postulated that the *meso*-Z may be formed from double bond isomerization of dietary L. They also demonstrated that under non-physiological conditions, a base catalyzed conversion of L to Z yields only *meso*- Z. The only evidence available in support of transformation is the mere presence of 3-hydroxy-L, epi-L and *meso*-Z in the eye samples.

As suggested by Khachik et al. (2002), the transport and the metabolic inter-conversion between L and Z in the eyes are most probably induced by sunlight as evidenced *in vitro*. Hartmann et al. (2004) reported that dosing Z to human volunteers resulted in considerable accumulation of *all-E*-3-dehyro-L in plasma and postulated that since L concentration remains unaffected by Z dosing, the increase in *all-E*-3-dehydro-L might have derived from Z. Another study (Thurman et al. 2005) demonstrated that long-term intake of L resulted in an accumulation of 3`dehydro-L in human plasma. Based on the results given in **Table 6.2,** we assume that formation of L oxidation products under *in vitro* is possibly due to photolysis of L which splits the molecule into two fragments as seen in peak C and F, and the removal of methyl groups from D and E **(Figure 6.3)**.

Similar fragmented ions were also identified in eye samples, which indicate the occurrence of photochemical reaction in eyes possibly because of exposure to light.

Table 6.2. Comparison of the L fragmented ions identified *in vitro* and *in vivo* experiments.

Samples	Identified fragmented ions									
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
In vitro conditions (photo-oxidation)	+	+	+	+	+	+	-	-	-	-
Eyes	+	-	+	+	+	+	-	-	-	-
Liver	+	+	-	-	-	-	+	+	+	+
Plasma	+	+	-	-	-	-	+	+	+	+
Intestine	-	+	-	-	-	-	-	+	+	-

+ Represents the presence of molecule

- Represents the absence of molecule

Refer Table 6.1 for abbreviations A to J used in this Table.

From the above data, a probable scheme for the formation of photolysis products from L is proposed (**Figure 6.4**). The HPLC profile of the L exposed to sunlight (*in vitro)* shows some of the isomers of L **(Figure 6.2a)** that are already reported earlier in human ocular tissues, which further supports the possible pathway of photochemical reaction in the eye. These results are significant, as earlier reports have suggested that light damages *retina* by generation of free radicals (Khachik et al. 2002).

L may be converted into several metabolites *in vivo*. In liver and plasma, oxidized molecules like G and J may be formed due to oxidative reactions, which indicate that L is involved in the chain breaking peroxyl- radical or quenching of the singlet oxygen (Stratton and Liebler, 1997; Yamauchi et al. 1998). The formation of oxidized product G indicates the formation of L epoxide, which is very similar to

neoxanthin, and may have more antioxidative capacity. It has been reported earlier that increase in the number of hydroxyl-groups in the carotenoid molecule amplifies the antioxidant capacity. Apart from these two compounds, B, H and I were also formed in the plasma, liver and intestine. H and I may have been formed due to fragmentation of anhydrolutein. Based on these results, a probable scheme of formation of the above-described molecules is proposed **(Figure 6.6) (Scheme 2)**.

The present study shows that L is degraded in the system by photochemical or oxidative reactions and the metabolites/oxidized products formed could be excreted from the biological samples (Bausch et al. 1999). Further, it is not clear that L oxidation products from intestine and blood are transported to and accumulated in *retina* via circulatory system or whether photo-induced metabolic oxidation of L may be responsible for their presence as suggested by Khachick et al. (2002). Hence, in depth studies are warranted in this regard. The presence of 3-hydroxy-β,ε-caroten-one in eye samples but not *in vitro* samples indicates an evidence for a possible *in vivo* metabolic oxidation of L in the eyes. Degradation of L in the body indicates that L is involved in various photochemical reactions (in eyes) and as an antioxidant in various oxidative reactions (other tissues). Based on the results we have proposed metabolic pathways for conversion of dietary L to its oxidation products. This study emphasizes the essentiality of maintaining dietary L status especially in *retina* to function as an antioxidant since it is easily degraded. Further research is needed to identify L metabolites in detail in biological samples in order to evaluate their possible biological significance with reference to AMD.

Chapter 7

General discussion and Summary

Lutein (L) and Z (Z) are hypothesized to play a similar role in humans as in plants: function as potent antioxidants and effective screeners of high-energy blue light (Krinsky, 2002). Known mostly for its importance for eye health, consumption and serum/plasma levels of L have been shown to be inversely related to the risk for ocular diseases, including age-related macular degeneration (AMD) (Mares-Perlman et al. 2001; Seddon et al. 1994), and cataracts (Brown et al. 1999; Gale et al. 2001; Lyle et al. 1999a). National Health and Nutritional Examination Survey reported that diets high in fruits and vegetables were inversely associated with the risk of AMD (Goldberg et al. 1988; Sommerburg et al. 1998). As is the case with AMD, a number of epidemiological studies have also reported that L intake and/or serum/plasma levels are inversely associated with the risk of cataract (Brown et al. 1999; Chasan-Taber et al. 1999; Lyle et al. 1999a; Jacques et al., 2001; Moeller et al., 2000).

Findings from placebo-controlled intervention trials demonstrated that ingestion of L rich foods or supplements results in increased macular pigment optical density (Johnson et al., 2000; Landrum, 2000), and suggested help in improving visual function in patients suffering from AMD and other ocular diseases (Dagnelie et al. 2000; Olmedilla et al. 2003; Richer et al. 2002). The benefit of L in humans does not stop with eye health. Recent studies suggest that, L and Z may help in maintaining the function of heart by reducing the risk of atherosclerosis (Dwyer et al. 2001; Mares-Perlman et al. 2002). Presence of L in skin (Peng et al. 1995; Wingerath et al. 1998) and its consumption resulting in reduction of UV-induced damage (Chen et al., 2002; Faulhaber, 2001) suggest it may be important for health of the skin as well. Despite knowing the health importance of L, the most recent data show that human intake level have declined, likely due to a decrease in the consumption of greens and vegetables (National Institute of Medicine, 2001; O'Neill et al. 2001). L can be found extensively in the human diet, most abundantly in dark leafy green vegetables (U.S. Department of Agriculture, 1998). Foods with yellow color, such as corn and egg yolks also serve as good dietary sources of L (Sommerburg et al. 1998). Given evidence supporting that biological role of

L and Z in the eye and their prevalence in the human diet, researchers have attempted to correlate their intake with reduced risk for eye disease.

Reported epidemiological studies provide a basis for the hypothesis that dietary carotenoids may have protective effect against AMD and cataract. Combined with the fact that L and Z are the only carotenoids found in the *macula* and comprise the macular pigment, the protective function and their level in the eye is directly related to intake of leafy greens and vegetables. Since data on the composition of L and Z varies significantly in agri/horticultural produce of different geographical origin and lack of data on the carotenoid composition of leafy greens and fruits and vegetables of Indian origin, in this study, data was generated mainly on the L and Z levels in those produce. For better comparison the produce was categorized in to familiar and less familiar (Lakshminarayana et al. 2007; Raju et al. 2007). Results show that the leafy vegetables examined contain significantly higher levels of xanthophylls than hydrocarbon carotenoids like β-carotene. Interestingly, very rarely consumed (less familiar) GLVs are found to contain higher levels of L and Z. The data generated on the composition of carotenoids in the present study could be helpful to suggest the consumers for selection of better source of L and Z rich GLVs as a part of daily meal to combat health disorders like AMD and cataract. Further, under utilized GLVs having shown higher levels of L and Z, they can be exploited for nutritional purpose.

The intestinal absorption and plasma response of dietary carotenoids is governed by various exogenous (food matrix) and endogenous (mixed micelles, permeation etc.) factors (Hollander and Ruble, 1978). One such dietary factor appears to be the nature and level of fat present in the food matrix. Previous studies from our laboratory (Baskaran et al. 2003; Raju et al. 2006) showed that micellar lysoPC increases the plasma level of β-carotene and L in mice and β-carotene in rat suggesting that phospholipids may modulate the intestinal absorption of carotenoids. The present study demonstrates the effect of dietary phospholipids, fatty acids and mixed carotenoids on the intestinal L uptake of either micellar or dietary L and its response in plasma, liver and eye of rats fed on L sufficient or its deficient diets. Further, the plasma response of L on feeding was correlated with the physicochemical characteristics of mixed micelles/micro emulsions. To achieve complete solubilization and required concentration of L, it was solubilized in respective mixed micelles, and rats were

intubated. The postprandial appearance of L in plasma was considered as a measure of intestinal absorption after single and repeated dose of micellar L.

The L absorption *in vivo* involves release from food matrix, dispersion in emulsion, solubilization into mixed micelles and permeation to intestinal mucosal cells and incorporation into lymphatic lipoproteins (Dimitrov et al. 1988; Olson, 1994; Furr and Clark, 1997). Thus, the carotenoids must be solubilized in mixed micelles before cellular uptake. These processes are dependent mostly on the physicochemical properties of the food matrix (Jalal et al. 1998; Tyssandier et al.2001).

The results of gavage studies show that lysoPC micelles significantly enhanced the plasma L concentration, compared with PC and NoPL micelles. This study indicates that mixed micelle containing lysoPC when intubated to rats profoundly influenced the intestinal uptake and plasma response of L compared to PC. However, the influence of lysoPC on the intestinal uptake of L in rats was depressed compared with mice (Baskaran et al. 2003). The present results are in agreement with the observations of carotene absorption and their response in rat tissues (Shapiro et al. 1984). The exact mechanism by which micellar lysoPC influences the intestinal L uptake still remains unclear. Evidence from *in vitro* studies by Sugawara et al. (2001) and Noh and Koo (2003) suggest that the inhibitory effects of PC or sphingomyelin - structurally similar to PC on β-carotene and α-tocopherol absorption may be mostly mediated within the intestinal cells. PC is hydrolyzed more slowly and incompletely in the intestinal lumen (Noh and Koo, 2003; Duan, 1998). Thus, the slow and incomplete hydrolysis of PC in the upper segment of the intestine, where much of the lipid hydrolysis occurs, may allow for interaction between intact PC and other lipids in the intestinal environment, influencing the rate of hydrolysis, micellar solubilization and transfer of lipids from mixed micelles to the enterocytes. Although no direct evidence is available for such interactions between PC and other lipids or lipophilic components in micelles, studies with lipid vesicles and membrane system indicate that PC interacts more tightly with fat-soluble components (Slotte, 1999). This may be a reason for significantly higher level of L excreted through urine and feces by the rats fed with PC micelles compared to lysoPC.

Carotenoids may interact with each other during the absorption, metabolism, and transport processes. Majority of the studies have focused on interaction of L with β-carotene. Several studies have suggested that single dose and long-term carotene

supplementation may inhibit L absorption (Albanes et al. 1997; Kostic et al. 1995). Tyssandier et al. (2002) reported that the addition of a second carotenoid to a meal diminishes the chylomicron response of the carotenoid already present in the diet. The present results also suggest that β-carotene suppress the bioavailability of L in rats.

The variability in β-carotene and L bioavailability from different studies, may reside on the amount of fat used in the simulated digestions *in vitro* and in the meals used on the intervention trials. The amount of fat in the diet is known to improve carotenoid bioavailability. With a limited oil phase, the micelle formation of the highly lipophilic carotenes is inhibited, while the relatively polar xanthophylls seem to travel more freely from the food matrix to the lipid and micellar phases of the digesta. In human, the dietary fat may also promote carotenoid absorption by stimulating bile secretion, raising the luminal concentration of bile salts that act as surfactants in the formation of mixed micelles. Nevertheless, addition of even a small amount of fat to the diet improves the absorption of carotenoids from vegetables (Jayarajan et al. 1980; Jalal et al. 1998; Roodenburg et al. 2000; Van het Hof et al. 2000). The type of fat in the meal ingested with β-carotene reported to influence the degree of absorption; beef tallow resulted in a greater absorption when compared with sunflower oil (Hu et al. 2000) and long-chain triglycerides were better than medium-chain triglycerides, which primarily are absorbed (Borel et al. 1998b).

The present results have shown that phospholipids and fatty acids used in this study significantly enhanced the bioavailability of micellar and dietary L in rats (Lakshminaryana et al. 2006 and 2007; Raju et al. 2006). Armand et al. (1999) reported that the size of micelles depends on its chemical composition, which in turn determines the rate of its diffusion through intestinal mucosa. Van het Hof et al. (2000) and Mekki et al. (2002) have reported that the emulsion droplet size is one of the factors that influence the intestinal uptake of carotenoids. The size of micelles used in the present study ranged from 5-23 μm. The relatively larger size of PC and LA micelles, as observed in this study might be due to the presence of molecules other than bile salts, fatty acids and phospholipids used to prepare the micelles in which the L was solubilized. The size of micelles containing β-carotene + L was found to be higher than the micelles with L alone. This may be due to the orientation of L (polar) on the outer hydrophilic region of

the mixed micelles apart from the centrally located β-carotene. Addition of lecithin in the micelles increased the micellar size, which positively correlated with its decreased rate of diffusion through the intestinal epithelial cells (Litchfield et al. 1980). Sugawara et al. (2001) also reported that the apical uptake of carotenoids by Caco-2 cells was greater from micelles containing lysophosphatidylcholine as an alternative of phosphatidylcholine. It has been suggested that the micelles containing LA or PUFA are larger in size which diffuse more slowly through the enterocytes thereby decreasing the rate of β-carotene absorption in human and rat models than micelles containing saturated fatty acids (Raju et al. 2006; Kimura et al.1994).

The transfer of carotenoids into lipid droplets also depends on the pH and viscosity of the intestinal content and the composition of the micelles. The pH probably plays a role on lipase activity and solubility of micelles (Tyssandier et al. 2001). In the present study, pH of the OA micelles was kept constant. Inclusion of phospholipids or fatty acids to the OA micelles resulted in a non-significant alteration in the micellar pH. Tyssandier et al. (2001) have reported that the transfer of micellar β-carotene and L was higher at pH 6-7, with bile salt concentrations of 2-8 mM. In the present study, no significant difference was noticed in the pH of micelles at a constant temperature except for PC micelles contained L + β-carotene. Moreover, the efficiency of micellarization of L was significantly higher than that of β-carotene and lycopene as reported by Tyssandier et al. (2001), suggesting that transfer of micelles across the intestinal mucosa *in vitro* was inversely proportional to hydrophobicity of the carotenoids. Thus, the association between carotenoid species and the efficiency of micellarization *in vitro* was similar to that observed in human duodenum aspirates (Tyssandier et al. 2002). Although, L and β-carotene share a common lipophilic characteristic, their structural variation may affect the processes of their intestinal absorption (Borel et al. 1996).

The results of the present study with fatty acids in rats demonstrate that the plasma response of newly absorbed L was significantly higher in OA group compared with LA group (Lakshminarayana et al. 2007). A significantly larger particle size of the mixed micelles containing linoleic acid could be one of the reasons for the lower level of plasma L compared with smaller particle size of micelles containing oleic acid. The present results and the previous *in vitro* and *in vivo* studies using rat and Caco-2 cells

(Rahman, 2000; Jackson, 2002); suggest that the lipolysis or hydrolysis of triglycerides and release of fatty acids in the intestinal tract by lipase is vital for the efficient uptake or esterification of carotenoids. Fatty acids play a significant role in the solubilization of carotenoids in lipid micelles and their efficient uptake by the enterocytes (Borel et al. 1996).

The bioavailability of pure L is not representative of the L in foods, as several factors such as the species and amount of carotenoids, matrix in which it is incorporated modifiers, amount and type of fat and nutrient status of the individual may influence the availability greatly. In this study, we used the L rich green leafy vegetable as L source with vegetable oils (as fat source) *viz*., groundnut, sunflower (linoleic acid source), olive (oleic acid source) and soy PC (phospholipids source) to evaluate their effect on the bioefficacy of L. The results show, improved L status in L deficient rats fed GLV with olive and soybean oil than other oils used in this study. This is in agreement with Clark et al. (2000) who found more efficient absorption of carotenoids by rats when the lycopene and astaxanthin were administered in olive oil than in corn oil. The positive role of olive oil may be due to its increased synthesis of triacylglycerol rich chylomicron, which helps in better incorporation of L and thereby reflects its level in plasma. Although, the exact mechanism of olive oil on the intestinal uptake of L is not clear, the possible role may be on secretion of bile, which in turn facilitates the formation of micelles and incorporation of L to mixed micelles and its intestinal uptake.

Phospholipids, especially PC, are present in the diet either as a natural component of the food matrix or as an emulsifier/ stabilizer in processed foods (Artz, 1990; Zeisel, 2003). Soy PC influences increased absorption of dietary L and accumulation in the present study. Feeding rats with diet supplemented with soybean lecithin may stimulate bile formation and secretion rate of bile acids, phospholipids and cholesterol (LeBlanc et al. 1998). Additionally, the bile constitutes a large physiological pool of phospholipids (mainly PC) (Tso, 1991). Although both dietary and biliary PC are important for the emulsification of dietary lipids in the digestive tract, the presence of PC in mixed micelles inhibits the absorption of carotenoids by human intestinal Caco-2 cells (Sugawara et al. 2001; Yonekura, 2006) and mice (Baskaran et al. 2003), most likely by shifting the carotenoid partition into the micellar phase. However, during the normal digestive process, most of the dietary and biliary PC are hydrolyzed by phospholipases,

producing lysophosphatidylcholine (LPC) which restore or even enhance β-carotene and L absorption by Caco-2 cells and experimental animals (Sugawara et al. 2001; Baskaran et al. 2003; Lakshminarayana et al. 2006).

Results on the biochemical parameters after single and repeated dose of micellar L with either phospholipids or fatty acids revealed significant changes in the plasma lipid profile. The plasma triglycerides, phospholipids, cholesterol and fatty acids level were increased significantly in the rats fed with micellar L either with phospholipids or fatty acids.

Results on the metabolism/oxidation of L demonstrate that L is photolysed *in vitro* and metabolized/oxidized in rat tissue to several components. The analysis of mixed micelles and diet used in this study for intubation (gavages) and feeding (dietary) revealed that there were no oxidation products of L commonly found in either micelles or in diet. This demonstrates that those oxidation products seen in the biological samples may be due to metabolite formed after feeding of L. Further, the metabolites/oxidation products were not detected in control samples. The available literature provides an indication that L and Z may play an active role in modulating the course of AMD. Yet, critical evidence for the beneficial effect of L and Z or their metabolites/oxidation products has not been elucidated in detail (Mares-Perlman et al, 1995). Among the carotenoids identified in the intestine, plasma, liver and eyes of rats after feeding L, only (*all-E*, 3*R*, 3'*R*, 6'*R*)-L and (*all-E*, 3*R*, 3'*R*)- Z are of the dietary origin. Samples were handled carefully, with all precautions to avoid the possibility of oxidation of L and Z.

Previously, we have reported (3R, 3'R)- Z and *meso*- Z (meager concentration) in rat plasma after intubations of L (Lakshminarayana et al, 2007). Therefore, the fact that, Bone et al (Bone et al, 1993) have reported that (3'*R*, 6'*R*)-L, (3*R*, 3'*R*)- Z and (3*R*, 3'*R*)-*meso*- Z are present in the human *macula* and postulated that the *meso*- Z may be formed from double bond isomerization of dietary L. They also demonstrated that under non-physiological conditions, a base catalyzed conversion of L to Z yields only *meso*- Z. The only evidence available in support of transformation of L *in vivo* is the mere presence of 3-hydroxy L, epi L and *meso* Z in the eye samples. As suggested by Khachik et al (Khachik et al 2002), the transport and the metabolic inter-conversion between L and Z in the eyes are most probably induced by sunlight as evidenced *in vitro* in the present study. Hartmann et al (2004) reported that dosing Z to human volunteers

resulted in considerable accumulation of *all-E*-3-dehyro L in plasma and postulated that since L concentration remains unaffected by Z dosing, the increase in *all-E*-3-dehydro L might have derived from Z. Another study (Thurman et al 2005) demonstrated that long-term intake of L resulted in an accumulation of 3`dehydro-L in human plasma.

Based on the results **(Chapter 6)** obtained in this study, we speculate that formation of L oxidation products under *in vitro* is possibly due to photolysis of L which splits the molecule into two fragments as seen in peak C and F, and the removal of methyl groups from D and E **(Figure 6.4)**. Similar fragmented ions were also identified in eye samples, which indicate the occurrence of photochemical reaction in eyes possibly because of exposure to light. From the above data, a probable scheme for the formation of photolysis products from L is proposed **(Figure 6.4)**. The HPLC profile of the L exposed to sunlight (*in vitro)* shows some of the isomers of L that are already reported earlier in human ocular tissues, which further supports the possible pathway of photochemical reaction in the eye. These results are significant, as earlier reports have suggested that light damages *retina* by generation of free radicals.

L may be converted into several metabolites *in vivo* (Chapter 6). In liver and plasma, oxidized molecules like G and J **(Figure 6.6)** may be formed due to oxidative reactions, which indicate that L is involved in the chain breaking peroxyl radical or quenching of the singlet oxygen (Yamauchi et al, 1998). The formation of oxidized product G indicates the formation of L epoxide, which is very similar to neoxanthin, and may have more antioxidative capacity. It has been reported earlier that increase in the number of hydroxyl groups in the carotenoid molecule amplifies the antioxidant capacity. Apart from these two compounds, B, H and I were also formed in the plasma, liver and intestine. H and I may have been formed due to fragmentation of anhydro L. Based on these results, a probable scheme of formation of the above-described molecules is proposed **(Figure 6.6)**.

In conclusion, the present study shows that the less familiar green leafy vegetables are good sources of L. The data generated on the composition of carotenoids in the present study could be helpful to suggest the consumers, particularly the rural community, for better source of L and Z as a part of daily meal to overcome health disorders like AMD and cataract. Further, under utilized GLVs having shown higher levels of L and Z can be exploited for nutritional purpose.

Physiochemical properties of the mixed micelles containing L with phospholipids and fatty acids were found to be limiting factors for L bioavailability. This gives an idea about the process of intestinal L uptake and its conversion. Phospholipids, fatty acids and mixed carotenoids used in this study were found to affect the L bioavailability after a single, repeated dose and dietary feeding to L sufficient and its deficient rats. Feeding micellar L with oleic acid, lysoPC and olive oil to L deficient rats improved its bioavailability faster than L sufficient rats.

The assessment of carotenoid bioavailability has long been hampered by the limited knowledge on their absorption mechanisms as well as by the limitations in experimental approaches involving laboratory animals and humans. Recently, with the use of *in vitro* cell culture systems and molecular biology techniques, the mechanisms of carotenoid absorption at cellular level have started to be unveiled. Such systems have also enabled the investigation of factors involved in each step of carotenoid absorption, identifying the steps influenced by each factor. Detailed *in vitro* approaches modeling the gastrointestinal environment have characterized the emulsification and micellarzation steps occurring prior to carotenoids uptake by the intestinal cells. These steps may be largely affected by the food matrix and other dietary components, being the main determinants of carotenoid bioavailability from foodstuffs. Although the investigations on the intestinal absorption of carotenoids other than L are still limited, they would be useful to elucidate the differential absorption of individual carotenoids.

Although, the conversions of β-carotene and other provitamin A carotenoids into retinal and retinoic acid are well documented in rat and humans (Goodman and Huang, 1965; Olson and Hayaishi, 1965) little information is available on the metabolism of non-provitamin A carotenoids like L. Earlier, L, Z and *meso*-Z (*meso*-Z) are isolated and characterized in *macula* using HPLC (Bone et al. 2001). Apart from the dietary carotenoids, 3'-epi L, 3' dehydro L, (3R, 3'S) *meso*-Z, and 3'–oxo L and 3-methoxy Z were also reported in human ocular tissues and serum (Khachik et al. 2002; Bhosale et al. 2007). 3'-Oxo L was reported as a major oxidative derivative formed by direct oxidation of L and Z. The presence of the direct oxidation product of L found in this study suggests that L may act as an antioxidant to guard the *macula* from the oxidative damage. Reports also suggest that carotenoids with increased number of oxo-and

hydroxyl-functional groups show an enhanced antioxidant capacity (Hurst et al. 2004; Siems et al.1999).

Several biochemical studies have demonstrated that L can be bleached rapidly *in vitro* by photolysis (Mortensen and Skibsted, 1997). It is hypothesized that oxidized L products may be involved in protecting eyes from the ultraviolet rays and may act as superior antioxidants. Epidemiological studies suggest that complete characterization of carotenoids and their metabolites in *retina* may help in understanding their functional properties (Khachik et al. 1996). Hence, it is vital to assess the formation of L oxidation products and its cleavage/fragmentation pattern before making conclusions about their biological function. The present study shows that L is degraded in the system by photochemical or oxidative reactions and the metabolites/oxidized products formed could be excreted from the biological samples. Further, it is not clear that L oxidation products from intestine and blood are transported to and accumulated in *retina* via circulatory system or whether photo-induced metabolic oxidation of L may be responsible for their presence as suggested by Khachick et al. (2002). The presence of 3-hydroxy-β,ε-carotene-one in eye samples but not *in vitro* samples indicates an evidence for a possible *in vivo* metabolic oxidation of L in the eyes. Degradation of L in the body indicates that L is involved in various photochemical reactions (in eyes) and as an antioxidant in various oxidative reactions (other tissues). Based on the results obtained, we have proposed a scheme of possible degradation and formation of some novel metabolites/ oxidized product of L both *in vitro* and *in vivo*.

Summary

1. This study revealed the L and Z concentrations of selected familiar and less familiar GLVs, fruits and vegetables. This is the first report showing HPLC and LC-MS data on L and Z levels in locally available GLVs, in particular, less familiar GLVs.
2. The procedure adapted in this study for the extraction, purification by open column chromatography and HPLC analysis of L and Z is relatively simple, reliable and accurate for the determination of the L and Z compared with those of available procedures in the literature.

3. Interestingly, results revealed that most of the less familiar GLVs are found to confirm richest sources of L. Hence, those GLVs could be exploited as good sources of L to combat degenerative disorders like AMD and cataract.
4. The nutritional significance of the present findings is clear since the GLVs screened in this study are found to contain L as major pigment hence considered an important source of macular pigments. Some of these GLVs are regularly consumed by a majority of the rural communities in southern part of India. The generated data on the L and Z content in GLVs could be very helpful to create nutritional awareness with regard to L and Z security among various communities.
5. This study shows, in general, that the leafy vegetables analyzed are found to register significantly higher levels of xanthophylls than β-carotene. Further, under utilized GLVs having shown higher levels of L and Z can be exploited for nutritional purpose.
6. Effect of specific phospholipids and fatty acids on particle size, structure, pH and viscosity of the mixed micelles was determined *in vitro*. Results revealed that these physicochemical properties play role in the solubilization of L in the mixed micelles and its bioavailability *in vivo*.
7. Inclusion of linoleic acid or sunflower oil or phosphatidylcholine to mixed micelles is found to enhance particle size, pH and viscosity of micelles with lower L retention compared to that of oleic acid, olive oil and lysoPC micelles. The difference may be due the level of unsaturation in the lipids used. Similarly, addition of phosphatidylcholine to the micelles resulted in lower intensity of L within the micelles than lysophosphatidylcholine. The possible reason for the above result may be due to the orientation of L in micelles containing phospholipids and fatty acids.
8. Application of image processing technique to analyze L incorporation/intensity within the micelles found to be a newer method, which provided a direct sign on the amount of L incorporated in the micelles. The techniques used in this study for determining various physicochemical properties of mixed micelles will offer an insight to understand the configuration of micelles and their characteristic features.

9. Phospholipids and fatty acids used in this study did improve or facilitate an enhanced bioavailability of dietary L both in L sufficient and as well in L deficient rats. The influence of those dietary substances was superior in L deficient rats than those of control rats.
10. The mechanism of interaction of β-carotene on L bioavailability in rats fed L + β-carotene dispersed in mixed micelles or dietary lecithin was discussed. The results revealed that β-carotene interferes with the L absorption.
11. The studies on bioavailability of L clearly demonstrated that the extent of intestinal L uptake and its accessibility *in vivo* were found to be mostly dependent on the physicochemical properties of mixed micelles intubated or formed at the intestinal level.
12. Feeding rats with L rich fenugreek leaves diet along with phospholipids or fatty acids used in this study show promising results on the alleviation of L deficiency and this strengthens the efficacy of food-based strategy as preventive measure in humans against AMD and cataract.
13. The plasma and tissues lipid profiles in rats fed on L along with lipids also illustrate a positive response with reference to fatty acid profile, lipid peroxidation and certain antioxidant enzymes/molecules.
14. Further, feeding rats with vegetable oils - sunflower oil and olive oil found to exhibit similar results as found in the case of single and repeated gavages studies with specific fatty acids showing olive oil (source of oleic acid) significantly enhance the absorption and transportation of dietary L to target tissues.
15. Studies *in vitro* and *in vivo* revealed that absorbed L is quickly metabolized /degraded in to various oxidation products in intestine, plasma, liver and eye tissues, for example, 13-*Z* L, 13'-*Z* L, 13-*Z* Z, *all-E* Z 9-*Z* L, and 9'-*Z* L. The proposed metabolic/degradation pathway clearly demonstrates various structural features of L metabolites. From this study we could characterize 13 L degraded/oxidized compounds by LC-MS.
16. However, in (3*R*, 3'*R*) - Z, because of non-allelic nature of the hydroxyl groups at C-3 and C-3', it may not be directly oxidized or undergo double bond isomerization to yield epiL before oxidation in the eyes. The presence of *meso*-Z

in the eye samples and the stereochemistry of macular carotenoids in human have been well correlated with reported literature.

17. The identified base peak, L-oxidized in liver corresponds to L diepoxide (5,6, 4', 5'-diepoxy-5, 6; 4', 5'-tetrahydro-β,β-carotene 3,3'-diol), and 5,6-Epoxy-3-hydroxy-12'-β,ε-carotene-12'-al to 5,6 epoxy-3 hydroxy-12'-β,ε-carotene-12'al, and these metabolites may be formed due to L oxidation in the liver itself. Other characteristic peaks (2*E*, 4*E*)-3-methyl-5- (2,6,6-trimethylcyclohexa-2, 4-dien-1-yl) penta-2, 4-dien-1-ylium and 2,6,6-trimethylcyclohex-2-ene-1, 4-bis (ylium) respectively could have been formed from fragmentation of anhydro L.
18. The L oxidative metabolites pattern in plasma was almost similar to that in liver.
19. In case of intestine, anhydro- L was the only compound found indicating that most of the oxidation or degradation of L taking place either in the eye or liver.
20. Further, the proposed mechanism by which dietary fat influences the L bioavailability is well correlated with structural properties of mixed micelles, *viz*. size, surface area and L intensity. Fatty acid composition of fat is important to achieve a higher bioefficacy of dietary L. L supplementation with physiological emulsions or micelles or diet containing oleic acid or olive oil and phospholipids or lyso-lecithin may improve the plasma and eye L status which in turn help in overcoming macular pigments deficiency disorders. These new observations could be of interest in enteral nutrition of AMD subjects.

References

1. Ahuja KD, Pittaway JK, Ball MJ: Effects of olive oil and tomato lycopene combination on serum lycopene, lipid profile, and lipid oxidation. Nutrition 22: 259-265, 2006
2. Akesson B: Content of phospholipids in human diets studied by the duplicate-portion technique. Br J Nutr 47: 223-229, 1982
3. Albanes D, Virtamo J, Talo PR, Rautalahti M, Pietinen P, Heinonen OP: Effects of supplemental β-carotene, cigarette smoking and alcohol consumption on serum carotenoids in the α-tocopherol, β-carotene cancer prevention study. Am J Clin Nutr 66: 366-372, 1997
4. Alberg A: The influence of cigarette smoking on circulating concentrations of antioxidant micronutrients. Toxicology 180:121-137, 2002
5. Algave PV, Seregard S: Age related maculopathy pathogenetic features and new treatment modalities. Acta Opthamol Scand 80: 136-143, 2002
6. Armand M, Borel P, Dubois C, Senft M, Peryot J, Saducci J, Lanfont H, Lairon, D: Characterization of emulsions and lipolysis of dietary lipids in human stomach. Am J Physiol 266: G372-381, 1994
7. Armand M, Borel P, Pasquier B, Dubois C, Senft M, Andre M, Peryot J, Saducci J, Lairon D: Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. Am J Physiol 271: G172-G183, 1996
8. Armand M, Borel P, Pasquier B, Dubois C, Senft, M, Andre M, Peryot J, Saducci J, Lairon D: Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. Am J Physiol; 271: G172-G183, 1996
9. Armand M, Borel P, Ytheir P, Dutot G, Melin C, Senft, M, Lafont H, Lairon D: Effects of droplet size, triglycerol composition, and calcium on the hydrolysis of complex emulsions by pancreatic lipase: an *in vitro* study. J Nut Biochem 3: 333-341,1992
10. Armand M, Pasquier B, Andre M, Borel P, Dubois C, Senft, M, Peryot, J, Saducci

J, Portugal H, Jaussan V, Lairon D: Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract. Am J Clin Nutr 70: 1096-1106, 1999

11. Artz WE: Emulsifiers In: Branen AL, Davidson PM, Salminen S. (eds.), Food Additives, Marcel Decker, New York, pp. 347-394, 1990
12. Asai A, Terasaki M, Nagao A: An epoxide furanoid rearrangement of spinach neoxanthin occurs in the gastrointestinal tract of mice and *in vitro*: formation and cytostatic activity of neochrome stereoisomers. J Nutr 134: 2237-2243, 2004
13. Astorg P, Gradelet S, Leclerc J, Canivenc MC and Siess HM: Effects of β-carotene and canthaxanthin on liver xenobiotic-metabolizing enzymes in the rat. Food Chem Toxicol 32: 735-742, 1994
14. Augustin AJ, Boker SHH, Blumenroder, Lutz J, Spitznas M: Free radical scavenging and antioxidant activity of haloperidol and oxypurinol in experimental lens-induced uveitis. Invest Opthamol Vis Sci 35: 3897-3904, 1994
15. Barua AB, Olson JA: Reversed-phase gradient high-performance liquid chromatographic procedure for simultaneous analysis of very polar to nonpolar retinoids, carotenoids and tocopherols in animal and plant samples. J Chromatography B 707: 69-79, 1998
16. Barua AB, Olson JA: Xanthophylls epoxides, unlike β-carotene monoepoxides are not detectibly absorbed by humans. J Nutr 131: 3212-3215, 2001
17. Baskaran V, Sugawara T, Nagao A: Phospholipids affect the intestinal absorption of carotenoids in mice. Lipids 38: 705-711, 2003
18. Bates CJ, Cowen TD: Effects of age and dietary vitamin C on the contents of ascorbic acid and acid-soluble thiol in lens and aqueous humour of guinea pigs. Exp Eye Res 46: 937-945,1988
19. Bauernfreind JC, Adams C, Marusich WL: Carotenes and other vitamin A precursors in animal feed. In carotenoids as colorants and vitamin A precursors, (Eds, J.C Bauernfeind) Academic press, New York, pp 563-743, 1981
20. Bausch J, Licchite H, Oesterhelt G, Kister A: Isolation and identification of a

major urinary canathaxanthin metabolite in rats. Int J Vitaminol Nutr Res 69: 268-272, 1999

21. Beatty S, Boulton M, Henson D, Koh HH, Murray IJ: Macular pigment and age related macular degeneration. Br J Ophthalmol 83: 667-877, 1999
22. Beatty S, Koh H, Henson DB, Boulton ME: The role of oxidative stress in the pathogenesis of age-related macular degeneration. Surv Ophthalmol 45: 115-134, 2000
23. Beatty S, Murray IJ, Henson DB, Carden D, Koh H, Boulton ME: Macular pigment and risk for age related macular degeneration in subjects from a Northern European population. Invest Ophthalmol Vis Sci 42: 439-446, 2000
24. Beatty S, Murray IJ, Henson DB, Carden D, Koh H, Boulton ME: Macular pigment and risk for age related macular degeneration in subjects from a Northern European population. Invest Opthalmol Vis Sci 42: 439–446, 2001
25. Bell SJ, Bradley D, Forse RA, Bistrian BR. The new dietary fats in health and disease. J Am Diet Assoc 97: 280-286, 1997
26. Bennekum AV, Werder M, Thuahnai ST, Han C, Duong P, Williams DL: Wettstein P, Schulthess, Phillips MC, Hauser H: Class-B scavenger-mediated intestinal absorption of dietary β-carotene and cholesterol. Biochemistry 44: 4517-4525, 2005
27. Berendscot TT, Goldbohm RA, Klopping WA, Van de Kraats J, van Norren D: Influence of lutein supplementation on macular pigment assessed with two objective techniques. Invest Opthalmol Vis Sci 41: 3322-3326, 2000
28. Bernstein PS, Balashov NA, Tsong ED, Rando RR: Retinal tubulin binds macular carotenoids. Invest Ophthalmol Vis Sci 38: 167-175, 1997
29. Bernstein PS, Khachik F, Carvalho LS, Muir GJ, Zhao DY, Katz NB: Identification and quantitation of carotenoids and their metabolites in the tissues of the human eye. Exp Eye Res 72: 215-223, 2001
30. Bernstein PS: New insights into the role of the macular carotenoids in age related macular degeneration. Resonance Raman studies. Pure Appl Chem 74: 1419-1425, 2002
31. Bhosale P, Bernstein PS: Quantitative measurement of 3'-oxolutein from human retina by normal-phase high-performance liquid chromatography coupled to

atmospheric pressure chemical ionization mass spectrometry. Anal Biochem 345: 296-301, 2005

32. Bhosale P, Zhao da Y, Serban B, Bernstein PS: Identification of 3-methoxy zeaxanthin as a novel age-related carotenoid metabolite in the human macula. Invest Ophthalmol Vis Sci 48: 1435-1440, 2007
33. Bierer TL, Merchen NR, Erdman W: Comparative absorption and lipoprotein transport of five common carotenoids in the preruminant calves. Am Inst Nutr125: 1569-1577, 1995
34. Bone RA, Landrum JT, Cains A: Optical density spectra of the macular pigments in vitro and in vivo. Vision Res 32: 105-110, 1992
35. Bone RA, Landrum JT, Fernandez L, Tarsis SL: Analysis of macular pigment by HPLC: retinal distribution and age study. Invest Ophthalmol Vis Res 29: 843-849, 1988
36. Bone RA, Landrum JT, Frieds LM, Gomez CM, Kilburn MD, Menendez E, Vidal I, Wang W: Distribution of lutein and zeaxanthin stereoisomers in the human retina. Exp Eye Res 64: 211-218, 1997
37. Bone RA, Landrum JT, Guerra LH, Ruiz CA: Lutein and zeaxanthin dietary supplements raise macular pigment density and serum concentrations of these carotenoids in humans. J Nutr 133: 992-998, 2003
38. Bone RA, Landrum JT, Hime GW, Cains A, Zamor J: Stereochemistry of the human macular carotenoids. Invest Ophthalmol Vis Sci 34: 2033-2040,1993
39. Bone RA, Landrun JT, Mayne ST, Gomez CM, Tibor SE, Twaroska EE: Macular pigment in donor eyes with and without AMD: a case-control study. Invest Opthalmol Vis Sci 42: 235-240, 2001
40. Bonnett R, Mallams AK, McCormick A, Spark AA, Tee TL, Weedon BCL: Carotenoids and related compounds. Part XX. Structure and reactions of fucoxanthin. J Chem Soc C: 429-454, 1969
41. Bonnie TYP, Choo YM: Oxidation and thermal degradation of carotenoids. J Oil Palm Res 11: 62-78,1999
42. Boode K, Walstra P, Degrootmostert, AEA: Partial coalescence in oil in water emulsions, 1. Influence of the properties of the fat. Colloids and Surfaces A 81: 139-143, 1993
43. Borek P, Grolier P, Mekki N, Boiric Y, Rochette Y, Le Roy B, Alexaner gouabou

MC, Lairon D, Azais-Braesco V: Low and high responders to pharmacological doses of β-carotene: proportion in the population, mechanisms involved and consequences on β-carotene metabolism. J Lipid Res 39: 2250-2200, 1998

44. Borel P, Armand M, Ytheir P, Dutot G, Melin C, Senft M, Lafont H, Lairon D. Hydrolysis of emulsions with different triglycerides and droplet sizes by gastric lipase in vitro. Effect on pancreatic lipase activity. J Nut Biochem 5: 124-133, 1994
45. Borel P, Grolier P, Armand M, Partier A, Lafont H, Lairon D, Azais-Braesco V: Carotenoids in biological emulsions: Solubility, Surface-to-core distribution and release from lipid droplets. J Lipid Res 37: 250-261, 1996
46. Borel P, Tyssandier V, Mekki N, Grolier P, Rochette Y, Alexandre-Gouabau M C, Azais-Braesco: Chylomicron β-carotene and retinyl palmitate responses are dramatically diminished when men ingest β-carotene with medium-chain rather than long-chain triglycerides. J Nutr 128: 1361-1367, 1998
47. Borel P, Tyssandier V, Mekki N, Grolier P, Rochette Y, Alexandre-Gouabau MC, Lairon D, Azais-Braesco V : Chylomicron β-carotene and retinyl palmitate responses are dramatically diminished when men ingest β-carotene with medium chain rather than long chain triglycerides. J Nutr 128: 1361-1367, 1998b
48. Bowen PE, Herbst-Espinosa SM, Hussain EA, Stacewicz-Sapuntzakis M: Esterification does not impair lutein bioavailability in humans. J Nutr 132: 3668-3673, 2002
49. Bowman PE, Mobarhan S: Evidence from cancer intervention and biomarker studies and the development of biochemical markers. Am J Clin Nutr 62: 1403S-1409S, 1995
50. Bracco U. Effect of triglyceride structure on fat absorption. Am J Clin Nutr 60: 1002S-1009S, 1994
51. Brady WE, Mares-Perlman JA, Bowen P, Stacewicz-Sapuntzakis M: Human serum concentrations are related to physiologic and life style factors. J Nutr 126: 129-137,1996
52. Breithaupt D, Bamedi A, Wirt U: Carotenol fatty esters: easy substrates for

digestive enzymes? Comp Biochem Physiol (Part B) 132: 721-728, 2002

53. Breithaupt DE, Wirt U, Bamedi M: Differentiation between lutein monoesters and detection of lutein diesters from marigold flowers (Tageters erecta L.) and several fruits by liquid chromatography–mass spectrometry. J Agric Food Chem 50: 66-70, 2002

54. Britton G, Liaaen-Jensen S, Pfander H (eds). Overview of carotenoid biosynthesis. In Carotenoids: Biosynthesis and metabolism. Birkhauser Verlag, Basel, 1998

55. Britton G. Structure and properties of carotenoids in relation to function. FASEB J 9: 1551-1558, 1995

56. Brown ED, Micozzi MS, Craft NE, Bieri JG, Beecher G, Edwards BK, Rose A, Taylor PR, Smith JC: Plasma carotenoids in normal men after a single dose ingestion of vegetables or purified β-carotene. Am J Clin Nutr 49: 1258-1265, 1989

57. Brown L, Rimm EB, Seddon JM, Giovannucci EL, Chasan-Taber L, Spiegelman D, Willett WC, Hankinson SE: A prospective study of carotenoid intake and risk of cataract extraction in US men. Am J Clin Nutr 70: 517-524, 1999

58. Burns J, Fraser PD, Bramley PM: Identification and quantification of carotenoids, tocopherol and chlorophylls in commonly consumed fruits and vegetables. Phytochemistry 62: 939-947, 2003

59. Bush RA, Reme CE, Malnoe A: Light damage in the rat retina: the effect of dietary deprivation of n-3 fatty acids on acute structural alterations. Exp Eye Res 53: 741-752, 1991

60. Cai J, Nelson KC, Wu M, Steinberg P, Jr. Jones DP: Oxidative damage and protection of the retinal pigment epithelium. Prog Ret Eye Res 19: 205-221, 2000

61. Caliph SM, Charman WN, Porter CJ: Effect of short-, medium-, and long chain fatty acid-based vehicles on the absolute oral bioavailability and intestinal lymphatic transport of halofantrine and assessment of mass balance in lymph-cannulated and non-cannulated rats. J Pharm Sci 89: 1073-1084, 2000

62. Carey MC, Small DM: The characteristics of mixed micellar solutions with particular reference to bile. Am J Med 49: 590-608, 1970

63. Carey MC: Lipid digestion and absorption. Ann Rev Physiol 45: 651-677,1983

64. Castenmiller JJM, West CE, Linsssen JPH, van het Hof KH, Voragen AGJ: The food matrix of spinach is a limiting factor in determining the bioavailability of β-carotene and to a lesser extent of lutein in humans. J Nutr 129: 349-355, 1999

65. Castenmiller JJM, West CE: Bioavailability and bioconversion of carotenoids. Ann Rev Nutr 18: 19-38, 1998

66. Chaine G, Hullo A, Sahel J, soubrane, Espinase-berrod M, Schutz D, Bourguinon C, Harpey C, Brault Y, Coste M, Moccatti D, Bourgeois H: Case -control study of the risk factors for age-related macular degeneration. France-DMLA study group. Br J Ophalmol 82: 996-1002, 1998

67. Chasan-Taber L, Willett WC, Seddon JM, Stampfer MJ, Rosner B, Colditz GA, Speizer FE, Hankinson SE: A prospective study of carotenoids and Vitamin A intakes and risk of cataract extraction in US women. Am J Clin Nutr 70: 509-516, 1999

68. Chen BH, Chen YY: Determination of carotenoids and chlorophylls in water convolvulus (Ipomea aquatica) by liquid chromatography. Food Chem 45: 129-134,1992

69. Chen J, Wu A, Pathak MA, Rius-Diaz F, Mihm CM, Goukassian D, Gonzalez S: Dietary lutein and zeaxanthin partially prevent UVB-induced skin carcinogenesis in the SKh-1 hairless mouse model (Abstract). In: Society for Investigative Dermatology. Los Angeles, CA. 2002

70. Cheng CL, Guo JS, Luk J, Koo MWL: The healing effects of Centella asiatica extract and asiaticoside on acetic acid induced gastric ulcers in rats. Life Sci 74: 2237-2249, 2004

71. Chew BP, Brown CM, Park JS, Mixtor PF: Dietary lutein inhibits mouse mammary tumor growth by regulating angiogenesis and apoptosis. Anticancer Res 23: 3333-3339, 2003

72. Chew BP, Wong MW, Wong TS: Effects of lutein from marigold extract on immunity and growth of mammary tumors in mice. Anticancer Res 16: 3689-3694, 1996

73. Chitchumroonchokchai Ch, Failla M: Hydrolysis of zeaxanthin esters by carboxyl ester lipase during digestion facilitates micellarization and uptake of

xanthophylls by Caco-2 human intestinal cells. J Nutr 136: 588-594, 2006

74. Cho E, Humg S,Willett WC, Spiegel man D, Rimmm EB, Seddon JM, Colditz GA, Hankinson SE: Prospective study of dietary fat and the risk of age-related macular degeneration. Am J Clin Nutr 73: 209-218, 2001

75. Christen WG: Antioxidant vitamins and age related eye disease. Proc Assoc Am Phys 111: 16-21, 1999

76. Clark RM, Yao L, She L, Furr HC: A comparison of lycopene and astaxanthin absorption from corn oil and olive oil emulsions. Lipids 37: 803-806, 2000

77. Clinton SK: Lycopene: chemistry, biology and implications for human health and disease. Nutr Rev 56: 535-511,1998

78. Compassi S, Werder M, Weber FE, Boffelli D, Hauser H, Schulthess G: Comparison of cholesterol and sitosterol uptake in different brush border membrane models. Biochemistry 36: 6643-6652,1997

79. Congming L, Qingtao Lu, Jianhua Zhang, Qide Zhang, Tingyun Kuang: Xanthophyll cycle light energy dissipation and photosystem II down-regulation in senescent leaves of wheat plants grown in the field. Australian Journal of Plant Physiology 28: 1023-1030, 2001

80. Curren-Clentinlano J, Hammond BR, Ciulla TA, Cooper DA, Pratt LM, Danis RB: Relation between dietary intake, serum concentrations and retinal concentrations of lutein and zeaxanthin in adults in a Midwest population. Am J Clin Nutr 74: 796-802, 2001

81. Dachtler M, Glaser T, Kohler K, Albert K: Combined HPLC-MS and HPLC-NMR online coupling for the separation and determination of lutein and zeaxanthin stereoisomers in spinach and in retina. Anal Chem 73: 667-674, 2001

82. Dagnelie G, Zorge IS, McDonald TM: Lutein improves visual function in some patients with retinal degeneration: a pilot study via the Internet. Optometry 71: 147-164, 2000

83. De Bruin TWA, Brouwer CB, van Linde-Sibensius Trip M, Jansen H, Erkelens D W: Different metabolism of olive oil and soybean oil: a possible mechanism of the high-density lipoprotein conserving effect of olive oil. Am J Clin Nutr 58: 447-483, 1993

84. De La Paz MA, Anderson RE: Region and age-dependent variation in

susceptibility of the human retina to lipid per oxidation. Invest Opthalmol Vis Sci 33: 3497-3499, 1992

85. De Moura FF, Ho CC, Getachew, Hickenbottom SI, Clifford AJ: Kinetics of 14C distribution after tracer dose of 14C-lutein in an adult woman. Lipids 40: 1069-73, 2005

86. DeFreitas Z: Prevalence of Lutein Vs Lutein Eaters in Human Diets USDA-NNCC Carotenoid Database for U.S.Foods, 1998a. Kemin Foods, L.C., Des Moines, IA. 2000

87. Delia J, Molnar P, Osz, E, Toth G, Zsita F: Epimerization of lutein to 3'-Epilutein in processed foods. Biorg Med Chem Lett 14: 925-928, 2004

88. Deming DM, Erdman JW: Mammalian carotenoid absorption and metabolism. Pure Appl Chem 71: 2213-2223, 1999

89. Di Lorenzo C, Williams CM, Hajnal F, Valenzuela JE: Pectin delays gastric emptying and increases satiety in obese subjects. Gastroenterology 95: 1211-1215,1988

90. Dimitrov NV, Meyer C, Ulrey DE, Chenoweth W, Michelakis A, Malone W, Boone C, Fink G: Bioavailability of β-carotene in humans. Am J Clin Nutr 48: 298-304, 1988

91. Duan RD: Sphingomyelin hydrolysis in the gut and clinical implications in colorectal tumorigenesis and other gastrointestinal diseases. Scand J Gastroenterol 33: 673-683, 1998

92. During A, Hussain MM, Morel DW, Harrison EH: Carotenoid uptake and secretion by Caco-2 cells: β-carotene isomer selectivity and carotenoid interactions. J Lipid Res 43: 1086-1095, 2002

93. During A, Dawson HD, Horrison EH: Carotenoid transport is decreased and expression of the lipid transporters SR-BI, NPC1L1and ABCA1 is down regulated in Caco-2 cells treated with estimable. J Nutr 135: 2305-2312, 2005

94. Dwyer JH, Navab M, Dwyer KM, Hassan K, Sun P, Shircore A, Hama-Levy S, Hough G, Wang X, Drake T, Merx CN, Fogelman AM: Oxygenated carotenoid lutein and progression of early atherosclerosis: The Los Angeles Atherosclerosis Study. Circulation 103: 2922-2927, 2001

95. Edelenbos M, Christensen LP, Grevsen K: HPLC Determination of chlorophyll

and carotenoid pigments in processed green pea cultivars (*Pisum sativum* L.). J Agric Food Chem 49: 4768-4774, 2001

96. Emenhiser C, Sander L, Schwartz S: Capability of a polymeric C_{30} stationary phase to resolve cis-trans carotenoid isomers in reversed-phase liquid chromatography. J Chromatography A 707: 205-216, 1995
97. Encyclopedia: Fruits and Vegetables.
98. Erdman JW, Bierer TL, Gugger ET: Absorption and transport of carotenoids. Ann NY Acad Sci 691: 76-85, 1993
99. Erdman JW: Variable bioavailability of carotenoids from vegetables. Am J Clin Nutr 70: 179-180, 1999
100. Farinato RS and Rowell RL: Optical properties of emulsions, in Encyclopedia of Emulsion Technology, Vol. 1, Becher P, Marcel Dekker, New York. Chap.8. 1983
101. Faulhaber D: Lutein inhibits UVB radiation-induced tissue swelling and suppression of the induction of contact hypersensitivity (CHS) in the mouse. In: Proceedings of the Investigative Dermatology Conference Washington, DC; J Invest Dermatol 2001
102. Faulks RM, Hart D, Brett GM, Dainty JR, Southon S: Kinetics of gastro-intestinal transit and carotenoid absorption and disposal in ileostomy volunteers fed spinach meals. Eur J Nutr 43: 15-22, 2004
103. Fave G, Coste TC, Armnad M: Physicochemical properties of lipids: New strategies to mange fatty acid bioavailability. Cell Mol Biol 50: 815-831, 2004
104. Fecondo JV, Augustyen RC: Superoxide dismutase, Catalase and glutathione peroxides in the human cataractous lens. Exp Eye Res 36: 15-23,1983
105. Field FJ, Born E, Murthy S, Mathur SN: Lysophosphatidylcholine increases the secretion of cholesterol ester-poor triacylglycerol-rich lipoproteins by Caco-2 cells. Biochem J 304: 35-42, 1994
106. Fisher RA. Statistical methods for research workers, 14th ed. Oliver and Boya, Edinburgh, London, 1970
107. Fine SL, Berger JW, Maguire MG, Ho AC: Age related macular degeneration. J Med 342: 483-492, 2000
108. Fletcher MJ: A Colorimetric Method for Estimating Serum Triacylglycerol, Clin

Chem Acta 22: 303-307,1968

109. Flohe L, Otting F: Superoxide dismutase assays. Methods in Enzymol 105: 93-104, 1984

110. Folch J, Lee M, Sloane SGH: A simple method for isolation and purification of total lipids from animal tissue. J Biol Chem 226: 497-509, 1957

111. Flohe L, and Gunzler W. Assays of glutathione peroxidase.Methods in Enzymol 105: 114-121,1984

112. Furr HC, Clark RM: Intestinal absorption and tissue distribution of carotenoids. J Nutr Biochem 8: 364-377, 1997

113. Gale CR, Hall NFI, Phillips DI, Martyn CN: Plasma antioxidant vitamins and arytenoids and age-related cataract. Ophthalmology108: 1992-1998, 2001

114. Ganguly J, Sastry PS: Mechanism of conversion of β-carotene into vitamin-A central cleavage versus random cleavage. World Rev Nutr Diet 45: 198-220, 1985

115. Garret DA, Failla ML, Sarama RJ, Craft N: Accumulation and retention of micellar β-carotene and lutein by Caco-2 human intestinal cells. J Nutr Biochem 10: 573-581, 1999a

116. Garret DA, Failla ML, Sarama RJ: Development of an in vitro digestion method to assess carotenoid bioavailability from meals. J Agri Food Chem 47: 4301-4309, 1999b

117. Gartner C, Stahl W, Sies H: Preferential increase in chylomicron levels of the xanthophylls lutein and zeaxanthin compared to β-carotene in the human. Int J Vitaminol Nutr Res 66: 119-125, 1996

118. Goldberg J, Flowerdew G, Smith E, Brody JA, Tso MO: Factors associated with age-related macular degeneration. An analysis of data from the first National Health and Nutrition Examination Survey. Am J Epidemiol 128: 700-710, 1988

119. Gonzalez RC, Woods RE. Digital Image Processing. Second Edition, Pearson Education, and Singapore pvt. Ltd., 2005

120. Goodman DS, Huang HS: Biosynthesis of vitamin A with rat intestinal enzymes. Science 149: 879-880,1965

121. Goodwin TW: The Biochemistry of the carotenoids. London: Chapman and Hall.

Pp 377, 1984

122. Gradelet S, Astorg P, Leclerc J, Chevalier J, Vernevaut MF, Sies MH: Effects of canthaxanthin, astaxanthin, lycopene and lutein on liver xenobiotic-metabolizing enzymes in the rat. Xenobiotica 26: 49-63, 1996a

123. Gradelet S, Leclerc J, Siess MH, Astorg P: '[3-Apo-8'-Carotenal, But Not if-Carotene, is a Strong Inducer of Liver Cytochromes P450 1 A1 and1A2 in the Rat'. Xenobiotica 26: 909-919,1996b

124. Granado F, Olmedilla B, Blanco I, Rojas E, Hidalgo: Carotenoid composition in raw and cooked Spanish vegetables. J Agric Food Chem 40: 2135-2140, 1992

125. Grover JK, Vats V: Shifting paradigm “from conventional to alternate medicine”, an introduction on traditional Indian medicine. Asia-Pacific Biotech News 5. 28-32, 2001

126. Grover JK, Yadav S, Vats V: Medicinal plants of India with antidiabetic potential. J Ethnopharm 81: 81-100, 2002

127. Hadden WL, Watkins RH, Levy LW, Regalado E, Rivadeneira DM, van Breemen RB, Schwartz SJ: Carotenoids composition of marigold (Tagetes erecta) flower extract used as nutritional supplement. J Agric Food Chem 47: 4189-4194,1999

128. Hamilton JA, Vural JM, Carpentier YA, Deckelbaum RJ: Incorporation of medium chain triglycerols into phospholipid bilayers: effects of long chain triglycerols, cholesterol and cholesteryl esters. J Lipid Res 37: 773-782, 1996

129. Hammond BR, Johnson EJ, Russell RM, Krinsky NI, Yeum KJ, Edwards RB, Snodderly DM: Dietary modification of human macular pigment density. Invest Ophthalmol Vis Sci 38: 1795 -1801, 1997a

130. Hammond BR, Wooten BR, Snodderly DM: Preservation of visual sensitivity of older subjects: association with macular pigment density. Invest Ophthalmol Vis Sci 39: 397-406,1998

131. Hammond BR, Fuld K, Snodderly DM: Iris color and macular pigment optical density. Exp Eye Res 62, 293-297,1996

132. Handelman GJ, Dratz EA, Reay CC, Van Kujik JG: Carotenoids in the human macula and whole retina. Invest. Ophthalmol Vis Sci 29: 850-855,1998

133. Handelman GJ, Nightingale ZD, Liechtenstein AH, Schaefer EJ, Blumberg JB: Lutein and zeaxanthin concentration in plasma after dietary supplementation with egg yolk. Am J Clin Nutr 70: 247-251, 1999

134. Hanush M, Stahl W, Schulz WA, Sies H: Induction of gap junctional communication by 4-oxoretinoic acid generated from its precursor canathaxanthin. Arch Bichem Biophys 317: 423-428,1995

135. Hart DJ, Scott KJ. Development and evaluation of an HPLC method for the analysis of carotenoids in food, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. Food Chem 54: 101-111, 1995

136. Heartman D, Thurmann PA, Spitzer V, Schalck W, Birke M, Cohn W: Plasma kinetics of zeaxanthin and 3`-dehydro-lutein after multiple oral doses of synthetic zeaxanthin. Am J Clin Nutr 79: 410-417, 2004

137. Heinonen MI: Carotenoids and provitamin A activity of carrot cultivars. J Agric Food Chem 38: 609-612, 1990

138. Herbst S,Bowen PE, Hussain E, Stacewicz-Sapuntzakis M, Damayanti B, Burns J: Evaluation of the bioavailability of lutein (L) and lutein diesters (LD) in humans. FASEB J 11: 444-447, 1997

139. Herden E, Diaz V, Svanberg U: Estimation of carotenoids accessibility from carrots determined by an *in vitro* digestion method. Eur J Clin Nutr 56: 425-430, 2002

140. Hernell O, Staggers JE, Carey MC: Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. Biochemistry 29: 2041-2056, 1990

141. Hoilg WH, Pabst MJ, Jakoly WB: Glutathione-S- transferase. The first enzymatic step in mercapturic acid formation. J Biol Chem 249: 7130-7139, 1974

142. Hollander D, Ruble PE: β-Carotene intestinal absorption: bile, fatty acids, pH and flow rate effects on transport. Am J Physiol 235: E686-E691, 1978

143. Homan R, Hamlehle KL: Phospholipase A_2 relieves phosphatidylcholine inhibition of micellar cholesterol absorption and transport by human intestinal cell line Caco-2. J Lipid Res.39: 1197-1209, 1998

144. Hu X, Jandacek RJ, White WS: Intestinal absorption of β-carotene ingested with

a meal rich in sunflower oil or beef tallow: postprandial appearance in triacylglycerol-rich lipoproteins in women. Am J Clin Nutr 71: 1170-1180, 2000

145. Humpheries JM, Khachik F: Distribution of lutein, zeaxanthin and related geometrical isomers in fruits, vegetables, wheat, and pasta product. J Agri Food Chem 51: 1322-1327,2003

146. Hurst JS, Contreras JE, Siems WG, Van Kuijk FJ: Oxidation of carotenoids by heat and tobacco smoke. Biofactors. 20: 23-25, 2004

147. Hussain MH. A proposed model for the assembly of chylomicrons. Atherosclerosis 148: 1-15, 2000

148. Innis SM, Dyer R: dietary triglycerols with palmitic acid (16:0) in the 2 position increase 16:0 in the position of plasma and chylomicron triglycerols, but reduce phospholipid arachidonic and docosahexaenoic acids, and alter cholesteryl ester metabolism in formula- fed piglets. J Nutr 127: 1311-1319, 1997

149. Ismail A, Fun CS: Determination of vitamin C, β-Carotene and riboflavin contents in five green vegetables organically and conventionally grown. Mal J Nutr 9: 31-39, 2003

150. Israelachvili JN, Mitchell DJ, Ninham BW: Theory of self-assembly of hydrocarbon amphiphiles into micelles and bilayers. J Chem Soc Far Trans 2 72: 1525-1568, 1976

151. Itagaki S, Ogura W, Sato Y, Noda T, Hirano T: Characterization of the disposition of lutein after i.v administration to rats. Biol Pharmcol Bull 29: 2123-2125, 2006

152. Jackson KG, Robertson MD, Fielding BA, Frayn KN, Williams CM: Olive oil increases the number of triacylglycerol-rich chylomicron particles compared with other oils: an effect retained when a second standard meal is fed. Am J Clin Nutr 76: 942-949, 2002

153. Jacques PF, Chylack Jr LT, Hankinson SE, Khu PM, Rogers G, Friend J, Tung W, Wolfe JK, Padhye N, Willen WC, Taylor A: Long-term nutrient intake and early age-related nuclear lens opacities. Arch Ophthalmol 119: 1009–1019, 2001

154. Jacques PF, Chylack LT: Epidemiological evidence of a role for the antioxidant

vitamins and carotenoids in cataract prevention. Am J Clin Nutr 53: 352S-355S, 1991

155. Jalal F, Nesheim MC, Agus Z, Sanjur D, Habicht JP: Serum retinol concentrations in children are affected by food sources of β-carotene, fat intake and anthelmintic drug treatment. Am J Clin Nutr 68: 623-629, 1998

156. Jalal F: Effects of deworming, Dietary fat and carotenoid rich diets on vitamin A status of preschool children infected with Ascaris lumbricoides in West Sumatra province, Indonesia. Ph.D. dissertation. Cornell University, Ithaca, NY, 1991

157. Jandacek RJ, Whiteside JA, Holocombe BN, Volpenhein RA, Taulbee JD: The rapid hydrolysis and efficient absorption triglycerides with octanoic acid in the 1 and 3 positions and long chin fatty acid in the 2 positions. Am J Clin Nutr 45: 940-945, 1987

158. Jayarajan P, Reddy V, Mohanram M: Effect of dietary fat on absorption of β-carotene from green leafy vegetables in children. Ind J Med Res 71: 53-56, 1980

159. Jensen MM, Christensen MS, Hoy CE; Intestinal absorption of octanoic, decanoic and linoleic acids: effect of triglyceride structure. Ann Nutr Metabol 38: 104-116, 1994

160. Johnson EJ, Hammond BR, Yeum KJ, Qin J, Wang XD; Castaneda C, Snodderly DM, Rusell RM: Relation among serum and tissue concentrations of lutein and zeaxanthin and macular pigment density. Am J Clin Nutr 71: 1555–1562, 2000

161. Johnson EJ, Russel RM: Distribution of orally administered β-carotene among lipoprotein in healthy men. Am J Clin Nutr 56: 128-135, 1992

162. Johnson EJ: The role of carotenoids in human health. Nutr Clin Care 5: 56-65, 2002

163. Jughans A, Siems H, Stahl W: Carotenoid-containing unilamellar liposomes loaded with Glutathione: a Model to Study Hydrophobic-Hydrophilic Antioxidant Interaction. Free Radic Res 33: 801-808, 1998

164. Jughans A, Sies H, Stahl W: Macular pigments lutein and zeaxanthin as blue filters studied in liposomes. Arch Biochem Biophys 391: 160-164, 2001

165. Juhel C, Senft M, Pafumi Y, Lairon D: Acute digestion of different dietary fatty acid species modulates postprandial lipid responses in New Zealand white rabbits. J Nutr Biochem 10: 458-466, 1999

166. Jung JW, Lee SK: Photo bleaching, light-induced discoloration in red peppers. J Sci Food Agric 86: 2296-2301, 2006

167. Kaplan LA, Lau JM, Stein EA: Carotenoid composition, concentrations, and relationships in various human organs. Clin Physiol Biochem 8: 1-10, 1990

168. Kasaniemi YA, Grundy SM: Effects of dietary polyenylphosphatidylcholine on metabolism of cholesterol and triglycerides in hypertriglyceridemic patients. Am J Clin Nutr 43: 98-107, 1986

169. Khachik F, Beecher GR, Jr Smith JC: Lutein, lycopene and their oxidative metabolites in chemoprevention of cancer. J Cell Biochem 22: 236S-246S, 1995a

170. Khachik F, Beecher GR, Whittaker NF: Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extract of several green vegetables by liquid chromatography. J Agric Food Chem 34: 603-616, 1986

171. Khachik F, Beecher GR: Application of a C-45-β-carotene as an internal standard for the quantification of carotenoids in yellow /orange vegetables by liquid chromatography. J Agric Food Chem 35: 732-738, 1987

172. Khachik F, Bernstein PS, Garland DL: Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. Invest Ophthalmol Vis Sci 38:1802–1811,1997

173. Khachik F, Bertram JS, Huang MT, Fahey JW, Talalay P: Dietary carotenoids and their metabolites as potentially useful chemoprevantive agents against cancer. In: Packer I., Hiramatsu M, Yoshikawa T, Eds. Antioxidant food supplements in human health. Tokyo: Academic Press: 203-229, 1999

174. Khachik F, de Moura FF, Chew EY, Douglass LW, Ferris IFL, Kim J, Thompson DJS: The effect of lutein and zeaxanthin supplementation on metabolites of these carotenoids in the serum of persons aged 60 or older. Invest Ophthalmol Vis Sci 47: 5234-5242, 1996

175. Khachik F, de Moura FF, Zbao DU, Aebiscber CP, Bernstein PS: Transformation of selected carotenoids in plasma, liver, and ocular tissues of humans and

in nonprimate animal models. Invest Ophthalmol Vis Sci 43: 3383-3392, 2002

180. Khachik F, Goli MB, Beecher GR, Holden J, Lusby WR, Tenorio MD, Barrera MR: The effect of food preparation on qualitative and quantitative distribution of major carotenoid constituents of tomatoes and several green vegetables. J Agric Food Chem 40: 390-398, 1992

181. Khachik F, Steck A, Pfander, H: Isolation and structural elucidation of (13Z, 13'Z, 3R, 3'R, 6'R) -lutein from marigold flowers, kale and human plasma. J Agric Food Chem 47: 455-461,1999

182. Kiefer C, Sumser E, Wernet MF, von Lintig J: A class B-Scavenger receptor mediates the cellular uptake of carotenoids in Drosophila. Proc Natl Acad Sci 99: 10581-10586, 2002

183. Kim SJ, Nara E, Kobayashi H, TeraoJ, Nagao A: Formation of cleavage products by auto oxidation of lycopene. Lipids 36: 191-199, 2001

184. Kimura M, Rodriguez-Amaya DB: A scheme for obtaining standards and HPLC quantification of leafy vegetable carotenoids. Food Chem 78. 389-398, 2002

185. Kimura M, Rodriguez-Amaya DB: Carotenoid composition of hydroponic leafy vegetables. J Agric Food Chem 51: 2603-2607, 2003

186. Kimura M, Rodriguez-Amaya DB: Sources of errors in the quantitative analysis of food carotenoids by HPLC. Arch Latinoam Nutr 49: 58S-66S, 1999

187. Kimura M, Shizuki M, Miyoshi K, Sakai T, Hidaka H, Takamura H, Matoba T: Relationship between the molecular structures and emulsification properties of edible oils. Biosci Biotech Biochem 58: 1258-1261,1994

188. King T J, Khachik E, Bortkiewicz H, Fukishima LH, Morioka S, Bertram JS: Metabolites of dietary carotenoids as potential cancer preventive agents. Pure Applied Chem 69, 2135-2140, 1997

189. Klein R, Rowlad ML, Hartis MI: Racial /ethnic differences in age related maculopathy-Third National health and Nutrition Examination. Surv Ophthalmol 102: 371-381, 1995

190. Koo SI, Noh SK: Phosphatidylcholine inhibits and lysophosphatidylcholine enhances the lymphatic absorption of α-tocopherol in adult rats. J Nutr 131: 717-722, 2001

191. Kostic D, White WS, Olson JA: Intestinal absorption, serum clearance and interactions between lutein and β-carotene when administered to human adults in separate or combined oral doses. Am J Clin Nutr 62: 604-610, 1995

192. Krichevsky SB: β-carotene, carotenoids and the prevention of coronary heart disease. J Nutr 129: 5-8, 1999

193. Krinsky NI, Mathews-Roth MM, Taylor RF. Carotenoids: Chemistry and Biology. Plenum press, New York and London, 1987

194. Krinsky NI: Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. Ann Rev Nutr 23: 171-201, 2003

195. Krinsky NI: Possible biologic mechanisms for a protective role of xanthophylls. J Nutr 132: 540S–542S, 2002

196. Kruger et al. 2002, http://www.cfsan.fda.gov/_rdb/opa-g110.html

197. Krutmann J: Ultraviolet a radiation-induced biological effects in human skin: relevance for photoaging and photodermatosis. J Dermatol Sci 23: S22–S26, 2000

198. Kubo I, Fijita K, Kubo A, Nehei K, Gura T: Antibacterial activity of coriander volatile compounds against Salmonella choleraesuis. J Agric Food Chem 52: 3329-3332, 2004

199. Lakshminarayana R, Raju M, Krishnakantha TP, Baskaran V: Determination of major carotenoids in few Indian leafy vegetables by High-Performance Liquid Chromatography. J Agric Food Chem 53: 2838-2842, 2005

200. Lakshminarayana R, Raju M, Krishnakantha TP, Baskaran V: Enhanced bioavailability of lutein by lyso-phosphatidylcholine in mixed micelles, Mol Cell Biochem 281: 103-110, 2006a

201. Lakshminarayana R, Raju M, Krishnakantha TP, Baskaran V: Lutein and zeaxanthin in leafy greens and their bioavailability: Olive oil influences the absorption of dietary lutein and its accumulation in adult rats. J Agric Food Chem 55: 6395-6400, 2007

202. Lakshminarayana R, Vasanthi G, Keshava Prakash MN, Raju M; Krishnakantha TP, Baskaran V: Manipulation of lutein and zeaxanthin levels in plasma and eyes of aged rats deficient in macular pigments through dietary means.

Symposium on Building Leadership Skills in food and Nutrition Essential for National development, CFTRI- Mysore, 23-25th June 2006b

203. Landrum JT, Bone RA, Joa H, Kilbum MD, Moore LL, Spargue KE: A one-year study of the macular pigment: The effect of 140 days of a lutein supplement. Exp Eye Res 65: 57-62, 1997

204. Landrum JT, Bone RA, Joa H, Kilburn MD, Moore LL, Sprague KE: A one-year study of the macular pigment: the effect of 140 days of a lutein supplement. Exp. Eye Res 65: 57-62, 1997a

205. Landrum JT, Bone RA, Moore LL, Gomez CM: Analysis of zeaxanthin distribution within individual human retinas. Methods Enzymol 299: 457-467.1999

206. Landrum JT, Bone RA: Lutein, zeaxanthin, and the macula pigment. Arch Biochem Biophys 385: 28-40, 2001

207. Landrum JT: Serum and macular pigment response to 2.4 mg dosage of lutein (Abstract). Assoc Res Vision Ophthalmol 41: S60, 2000

208. LeBlanc MJ, Gavino V, Perea A, Yousef IM, Levy E, Tuchweber B: The role of dietary choline in the beneficial effects of lecithin on the secretion of biliary lipids in rats. Biochem Biophys Acta 1393: 223-234, 1998

209. Leske MS, Wu SY, Nemesure B, Hennis A: Risk factors for incident nuclear opacities. Ophthalmology. 109: 1303-1308, 2002

210. Levin MS: Intestinal absorption and metabolism of vitamin-A. In Physiology of the gastrointestinal tract (Johnson LR), Ravan press, NY. Vol 2: 1957-1978, 1994

211. Lienau A, Glaser T, Tang G, Dolnikowski GG, Grusak MA, Albert K: Bioavailability of lutein in humans from intrinsically labeled vegetables determined by LC-APCI-MS. J Nutr Biochem 11: 663-670, 2003

212. Lim BP, Nagao A, Terao J, Tanaka K, Suzuki T, Takama K: Antioxidant activity of xanthophylls on peroxyl radical-mediated phospholipid peroxidation. Biochim Biophys Acta 1126**:** 178-184,1992

213. Lin Y, Dueker SR, Burri BJ, Neidlinger TR, Clifford AJ: Variability of the conversion of β-carotene to vitamin A in women by using a double-tracer study design. Am J Clin Nutr 71: 1545-1554, 2000

214. Lindenstruth K, Muller BW: W/O/W multiple emulsions with diclofenac sodium. Eur J Pharm Biopharm 58: 621-627, 2004

215. Litchfield C, Tyszkiewicz J, Dato V: Triacontatrienoic acid, principal fatty acid of the marine sponge Chondrilla nucula. Lipids 15: 200-202, 1980

216. Lowry OH, Rosebrough NJ Farr AL Randall RJ: Protein estimation with Folin phenol reagent. J Biol Chem 193: 265-275, 1951

217. Lu QY, Hung JC, Heber D, Go VL, Reuter VE, Cordon-Cardo C, Scher HI, Marshall JR, Zhang ZF: Inverse associations between plasma lycopene and other carotenoids and prostate cancer. Cancer Epidemiol Biomark Prev 10: 749-56, 2001

218. Lyle BJ, Mares-Perlman JA, Klein BE, Klein IL, Greger JL: Antioxidant intake and risk of incident age-related nuclear cataracts in the Beaver Dam Eye Study. Am J Epidemiol 149: 801-809,1999a

219. Maccarone MM, Bari V, Gasperi B, Demmig-Adams: The photoreceptor zeaxanthin induces cell death in neuroblastoma cells. Anticancer Res 25: 3871-3876, 2005

220. Mansbach CM, Cohen RS, Leff PB: Isolation and properties of the mixed micelles present in intestinal content during fat digestion in man J Clin Invest 56: 781-791, 1975

221. Mares-Perlman JA, Brady WE, Klein R, et al: Serum antioxidants and age related macular degeneration in a population based case control study. Arch Ophthalmol 113: 1518-1523, 1995

222. Mares-Perlman JA, Millen AE, Ficek TL, Hankinson SE: The body of evidence to support a protective role for lutein and zeaxanthin in delaying chronic disease. J Nutr132: 518S-524S, 2002

223. Mares-Perlman JA, Fisher A, Klein R, Palta M, Block G, Millen AE, Wright JD: Lutein and zeaxanthin in the diet and serum and their relation to age-related maculopathy in the third national health and nutrition examination Survey. Am J Epidem 153: 424-432, 2001

224. Martin HD, Ruck C, Schmidt M, Sell S, Beutner S, Mayer B, Walsh R: Chemistry of carotenoid oxidation and free radical reactions. Pure Appl Chem 71: 2253-2262, 1999

225. Mataix J, Quiles JL, Huertas JR, Battino M, Manas M: Tissue specific interactions of exercise, dietary fatty acids and vitamin-E in lipid peroxidation. Free Radic Biol Med 24: 511-521, 1998

226. Mayne ST, Handelman GJ, Beecher G: β-carotene and lung cancer promotion in heavy smokers a plausible relationship? J Natl Cancer Institute 88:1513-1515, 1996

227. Mazer NA, Benedek GB and Carey MC: Quasielastic light-scattering studies of aqueous biliary lipid systems. Mixed micelle formation in bile salt-lecithin solutions. Biochemistry 19: 601-615, 1980

228. McCarty C, Taylor HR: Light and risk for age-related diseases. In: Taylor, A, Editor, Nutritional and environmental influences on the eye, CRC Press, Boca Taton, p.135, 1999

229. Meijer N, Abbes H and Hansen WG. 2001. Particle size distribution and dispersion of oil-in-water emulsions: An application of light microscopy. www.iscpubs.com/articles/al/a0104mei.pdf

230. Mekki N, Charbonnier M, Borel P, Leonardi J, Juhel C, Portugal H, Lairon D: Butter differs from olive oil and sunflower oil in its effects on postprandial lipemia and triglycerol-rich lipoproteins after single mixed meals in healthy young men. J Nutr 132: 3642-3649, 2002

231. Mercadante AZ, Rodriguez-Amaya DB: Carotenoid composition of a leafy vegetable in relation to some agricultural variables. J Agric Food Chem 39: 1094-1097,1991

232. Mezzetti A, Lapenna D, Pierdomenico SD, Calafiore AM, Costantini F, Riario-sforza G, Imbastaro T, Neri M, Cuccurullo F: Vitamins E, C and lipid peroxidation in plasma and arterial tissue of smokers and non smokers. Atherosclerosis 112: 91-99, 1995

233. Michaud DS, Feskanich D, Rimm EB, Colditz GA, Speizer FE, Willett WC, Giovannucci E: Intake of specific carotenoids and risk of lung cancer in 2 prospective US cohorts. Am J Clin Nutr 72: 990-997, 2000

234. Mikula RJ. Emulsion characterization, in Emulsions: Fundamentals and applications in petroleum industry, Schramm LL. Ed., American Chemical Society, Washington, DC, Chap.3, 1992

235. Moller SM, Jacques PF, Blumberg JB: The potential role of dietary xanthophylls in cataract and age-related macular degeneration. J Am Coll Nutr 19: 522S-527S, 2000

236. Morrison MR, Smith M: Preparation of Fatty Acids Methyl Esters and Dimethyl Acetyls from Lipids with Boron Fluoride Methanol. J Lipid Res 5: 600-608, 1963

237. Mortensen A, Skibsted LH: Free radical transient in photo bleaching of xanthophylls and carotenes. Free Radic Res 26: 549-563, 1997

238. Nambiar VS, Seshadri SA: Study on β-carotene content of some green leafy vegetables of Western India by high performance liquid chromatography. J Food Sci Tech 35: 365-367, 1998

239. National Institute of Medicine, DRI's for Vit A, Vit As, B, Cr, Cu, I, Fe, Mn, Mo, Ni, Si, V, and Zn. National Academy Press, National Institute of Medicine, Washington, DC. 2001

240. Nishimukai M, Hara H: Enteral administration of soybean phosphatidylcholine enhances the lymphatic absorption of lycopene, but reduces that of α-tocopherol in rats. J Nutr 134: 1862-1866, 2004

241. Noh SK, Koo SI: Egg sphingomyelin lowers the lymphatic absorption of cholesterol and α-tocopherol in rats. J Nutr 133: 3571-3576, 2003

242. Novotny JA, Ducker SR, Zech LA, Clifford AJ: Compartmental analysis of the dynamics of β-carotene metabolism in an adult volunteer. J Lipid Res 36:1825-1838, 1995

243. O'Neill ME, Thurnham DI: Intestinal absorption of β-carotene, lycopene and lutein in men and women following a standard meal: response curves in the triglycerol-rich lipoprotein fraction. Br J Nutr 79: 149-159, 1998

244. Odeberg JM, Lingnell A, Pettersson A, Hoglund P: Oral bioavailability of the antioxidant astaxanthin in humans is enhanced by incorporation of lipid based formulations. Eur J Pharm Sci 19: 299-304, 2003

245. Ohkawa H, Ohishi N, Yagi H: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95: 351-358, 1979

246. Olmedialla B, Granado F, Blanco I, Vaquero M: Lutein but not a α-tocopherol supplementation improves visual function in patients with age-related cataracts: A 2-Y double blind, placebo-controlled study. Nutrition 19: 21-25, 2003

247. Olson JA, Hayaishi O: The enzymatic cleavage of β-carotene into vitamin A by soluble enzymes of rat liver and intestine. Proc Natl Acad Sci USA 54: 1364-1370, 1965

248. Olson JA: Carotenoids In: Modern Nutrition in Health and Disease (Shils ME, Olson JA, Shike M, Ross AC. Eds.), 9th ed. pp. 525-541. Williams and Wilkins, Philadelphia, PA. 1999

249. Olson JA: Serum levels of vitamin A and carotenoids as reflectors of nutritional status. J Natl Cancer Inst 73: 1439-1444, 1984

250. O'Neill ME, Carroll Y, Corridan B, Olmedilla B, Granado F, Blanco I, Van den Berg H, Hininger I, Rousell AM, Chopra M, Southon S, Thurnham DI: A European carotenoid database to assess carotenoid intakes and its use in a five-country comparative study. Br J Nutr 85: 499-507, 2001

251. Owens CWI, Belcher RV: A colorimetric micro-method for the determination of glutathione. Biochem J: 94, 705-711, 1965

252. Pari L, Satheesh MA: Antidiabetic activity of *Boerhaavia diffusa* L.: effect on hepatic key enzymes in experimental diabetes. J Ethnopharmacol 91: 109-113, 2004

253. Parker RS: Absorption, metabolism and transport of carotenoids. FASEB J 10: 542-551,1996

255. Pasquier B, Armand M, Castelain C, Guillon F, Borel P, Lafont H, Lairon D: Emulsification and lipolysis of triglycerols are altered by viscous soluble dietary fibres in acidic gastric medium in vitro. Am J Physiol 271: G172-183, 1996

256. Peng Y-M, Peng Y-S, Lin Y, Moon T, Roe DJ, Ritenbaugh C: Concentrations and plasma-tissue-diet relationships of carotenoids, retinoids and tocopherols in humans. Nutr Cancer 23: 233-246, 1995

257. Rahman MH, Avella MA, Botham KM: The fatty acid composition of chylomicrons influences the rate of their lipolysis in vivo. Nutr Metab Cardiovasc Dis 10: 121-125, 2000

258. Raju M, Lakshminarayana R, Krishnakantha TP, Baskaran V: Influence of phospholipids on β-carotene absorption and conversion into vitamin A in rats. J Nutr Sci Vitaminol 51: 216-222, 2005

259. Raju M, Lakshminarayana R, Krishnakantha TP, Baskaran V: Micellar oleic and

eicosapentaenoic acid but not linoleic acid influences the β-carotene absorption and its cleavage into retinol in rats. Mol Cell Biochem 288: 7-15, 2006

260. Raju M, Varakumar S, Lakshminarayana R, Krishnakantha TP, Baskaran V: Carotenoid composition and vitamin A activity of medicinally important green leafy vegetables. Food Chem101: 1621-1628, 2007

261. Rajyalakshmi P, Venkatalaxmi K, Venkatalakshmamma K, Jyothsna Y, Balachandramanidevi K, Suneetha V: Total carotenoid and β-carotene contents of forest green leafy vegetables consumed by tribals of South India. Plant Food for Hum Nutr 56: 225-238, 2001

262. Rampone AJ, Long LR: The effect of phosphatidylcholine and lysophosphatidylcholine on the absorption and mucosal metabolism of oleic acid and cholesterol in vitro. Biochem Biophys Acta 486: 500-510, 1977

263. Reading VM, Weale RA: Macular pigments and chronic aberrations. J Ops Soc Am 64: 231-234,1974

264. Reboul E, Aboul L, Mikail C, Ghiringhelli O, Andre M, Portugal H, Jourdhevil-Rahmani D, Amiot MJ, Lairon D, Borel P: Lutein transport by Caco-2, TC-7 cells occurs partly by a facilitated process involving the scavenger receptor class B type 1 (SR-B1). Biochem J 387: 455-461, 2005

265. Reboul E, Klein A, Bietrix F, Gleize B, Malezet-Desmoulins C, Schneidu M, Marigolte, Lagrost L, Collet X, Borel P: Scavenger receptor class B type 1 (SR-B1) is involved in vitamin-E transport across the enterocytes. J Biol Chem 281: 4739-4745, 2006

266. Reddy BS, Kawamori T, Lubet R, Steele V, Kelloff G, Rao CV: Chemopreventive effect of S-methylmethne thiosulfonate and sulindac administered together during the promotion/progression stages of colon carcinogenesis. Carcinogenesis 20: 1645-1648, 1999

267. Report of the American Institute of Nutrition Ad. Hoc committee on standards for nutritional studies. J Nutr 170: 1340-1348, 1977

268. Reynier MO, Lafont H, Crotte C, Sauve P, Gerolami A: Intestinal cholesterol uptake: Comparison between mixed micelles containing lecithin or lysolecithin. Lipids 20: 145-150, 1985

269. Rich GT, Faulks RM, Wickham MS, Fillery-Travis A: Solubilization of carotenoids from carrot juice and spinach in lipid phase: II. Modeling the duodenal environment. Lipids 38: 947-956, 2003

270. Richer S, Stiles W, Statkute L, Pei KY, Frankowski J, Nyland J, Pulido J, Rudy D: The lutein antioxidant supplementation trial (LAST). In: Proceedings of the Association for Research in Vision and Ophthalmology. Ft. Lauderdale, FL, pp, B539, 2002

271. Richer SW, Stilies L, Statkute J, Pulido J et al. Double–masked, placebo-controlled, randomized trial of lutein and antioxidant supplementation in the intervention of atrophic age related macular degeneration: the veterans LAST study (Lutein antioxidant Supplementation Trial), Optometry 75: 216-230, 2004

272. Riedl J, Linseisen J, Hoffmann J, Wolfram G: Some dietary fibres reduce the absorption of carotenoids in woman. J Nutr 129: 2170-2176, 1999

273. Riso P, Brusanolino A, Ciappellano S, Porrini M: Comparison of lutein bioavailability from vegetables and supplement. Int J Vitaminol Nutr Res 73: 201-205, 2003

274. Rock CL, Swendseid ME: Plasma β-carotene response in humans after meals supplemented with dietary pectin. Am J Clin Nutr 55: 96-99, 1992

275. Rock CL, Thornquist MD, Kristal AR, Patterson RE, Cooper DA, Neuhouser ML, Newmarle-Sztainer D, Cheskin LJ: Demographic, dietary and life style factors differentially explain variability in serum carotenoids and fat soluble vitamins: base line results from the sentinel site of the Olestra post marketing surveillance study. J Nutr 129: 855-864,1999

276. Rodriguez-Amaya DB: A guide to carotenoids analysis in foods. ILSI Press, Washington, 1999

277. Roodenberg AJC, Leenen R, Van het hof KH, Westsrate JA, Tijburg LBM: Amount of fat in the diet affects bioavailability of lutein esters but not of β-carotene, and vitamin-E in humans. Am J Clin Nutr 71: 1187-1193, 2000

278. Ruiz-Gutierrez V: Perez-Espinosa A: Vazquez CM: Santa-Maria C: Effect of dietary fats (fish, olive and high-oleic acid sunflower oils) on lipid composition and antioxidant enzymes in rat liver. Br J Nutr 82: 233-41,1999

279. Schalch W, Dayhaw–Barker P, Barker FM: The carotenoids of the human retina. In: Taylor A, ed. Nutritional and Environmental Influences on the Eyes. Washington DC: CRC; 215-250,1999

280. Scheidegger R, Pande AK, Bounds LP, Koppenol WH: The reaction of peroxynitrite with zeaxanthin; Nitric oxide: Biology and Chemistry Vol. 2, No. 1, pp. 8-16,1998

281. Scherze I, Knofel R, Muschiolik G: Automated image analysis as a control tool for multiple emulsions. Food Hydrocoll 19: 617-624, 2005

282. Schmidt-Erfurth U: Nutrition and retina. Dev Ophthalmol 38: 120-147, 2005

283. Schweigert FJ, Bok V: Vitamin A in blood plasma and urine of dogs is affected by the dietary level of vitamin A. Int J Nutr Res 70: 84-91, 2000

284. Searcy RL, Bergquist LM: A new color reaction for the quantification of serum cholesterol. Clin Chem Acta 5: 192-196, 1960

285. Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC, Farber MD, Gragoudas ES, Haller J, Miller DT, Yannuzzi LA, Willett W: Dietary carotenoids, vitamins A, C, E and advanced age-related macular degeneration: a multicenter study. J Am Med Assoc 242: 1413-1420, 1994

286. Serrano J, Goni I, Saura-Calixto F: Determination of β-carotene and lutein available from green leafy vegetables by an in vitro digestion and colonic fermentation method. J Agri Food Chem 53: 2936-2940, 2005

287. Shapiro SS, Mott DJ, and Machlin LJ: Kinetic properties of β-carotene uptake and depletion in rat tissue. J Nutr 114: 1924-1933, 1984

288. Shih S, Weng YM, Chen S, Huang SL, Huang CH, Chen W: FT-Raman spectroscopic investigation of lens protein of tilapia treated with dietary vitamin E. Arch Biochem Biophys 420: 79-86, 2003

289. Siems WG, Sommerburg O, van Hujik, FJGM: Lycopene and β-carotene decompose more rapidly than lutein and zeaxanthin open exposure to various pro-oxidants in vitro. Biofactorts 10:105-108, 1999

290. Sies H, Stahl W: Vitamins E and C, β-carotene, and other carotenoids as antioxidants. Am J Clin Nutr 62: 1315S-1321S, 1995

291. Singh G, Kawatra A, Sehgal S: Nutritional composition of selected green leafy vegetables, herbs, and carrots. Plant Food for Hum Nutr 56: 359-364, 2001

292. Slattery ML, Benson J, Curtin K, Ma KN, Schaeffer D, Potter JD: Carotenoids and colon cancer. Am J Clin Nutr 71: 575-582, 2000

293. Slim M, Toborek M, Watkins BA, Boissonneault GA, Hennig B: Susceptibility to hepatic oxidative stress in rabbits fed different animal and plant fats. J Am Coll Nutr 15: 289-294, 1996

294. Slotte JP: Sphingomyelin-cholesterol interactions in biological and model membranes. Chem Phys Lipids 102: 13-27, 1999

295. Small DM: Physicochemical studies of cholesterol gallstone formation. Gastroenterology 52: 607-610, 1967

296. Snodderly DM: Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. Am J Clin Nutr 62: 1448S-1461S, 1995

297. Sommerburg O, Arnold J, Leichsenring M, Salerno C, Crifo C, Hoffmann GF, Debatin KM, Siems WG: β-Carotene cleavage products after oxidation mediated by hypochlorous acid: a model for neutrophil-derived degradation. Free Radic Biol Med 35:1480-1484, 2003

298. Sommerburg O, Keunen JEE, Bird AC, van Kuijk FJGM: Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eye. Br J Ophthalmol 82: 907-910,1998

299. Staggers JE, Hornell O, Stafford RJ, Carey MC: Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 1. Phase behavior and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings. Biochemistry 29: 2028-2040, 1990

300. Stahl W, Junghans A, de Boer B, Driomina ES, Briviba K, Sies H: Carotenoid mixtures protect multilamellar liposomes against oxidative damage: synergistic effects of lycopene and lutein. FEBS Lett 427: 305-308,1998

301. Stahl W, Sies H: Protection against solar radiation- protective properties of antioxidants. In: Giacomoni PU. (Ed.), Sun Protection in Man. Elsevier Science, BV, 561–572, 2001

302. Stewart JCM: Colorimetric Estimation of Phospholipids with Ammonium Ferrothiocyanate. Anal Biochem 104: 10-14, 1980

303. Stratton SP, Liebler DC: Determination of singlet oxygen-specific versus radical-

mediated lipid peroxidation in photosensitized oxidation of lipid bilayers: effect of β-carotene and α-tocopherol. Biochemistry 36: 12911-12920, 1997

304. Sugawara T, Baskaran V, Tsuzuki W, Nagao A: Brown algae fucoxanthin is hydrolyzed to fucoxanthinol during absorption by Caco-2 human intestinal cells and mice. J Nutr 132: 946-95, 2002

305. Sugawara T, Kushiro M, Zhang H, Nara E, Ono H, Nagao A: Lysophosphatidylcholine enhances carotenoid uptake from mixed micelles by Caco-2 human intestinal cells. J Nutr 131: 2921-2927, 2001

306. Sujak A, Okulski W, Gruszecki WI: Organization of xanthophyll pigments lutein and zeaxanthin in lipid membrane formed with dipalmitoylphosphatidylcholine. Biochem Biophys Acta 1509: 255-563, 2000

307. Tang G, Qin I, Doinikowski GG, Russell RM: Vitamin A equivalence of β-carotene in women as determined by a stable isotope reference method. Eur J Nutr 39: 7-11, 2000

308. Tang G, Serfaty-Lacrosniere C, Camilo ME, Russell RM: Gastric acidity influences the blood response to a β-carotene dose in humans. Am J Clin Nutr 64: 622-626, 1996

309. Tanumihardjo SA, Li J, Dosti MP: Lutein absorption is facilitated with co supplementation of ascorbic acid in young adults. Am Diet Assoc 105: 114-118, 2005

310. Tappel AL: Glutathione peroxidase and hydroperoxides. Methods Enzymol 52: 506-513, 1978

311. Tavani A, Negri E, La Vechia C: Food and nutrient intake and risk of cataract. Ann Epidemiol 6: 41-46, 1996

312. Taylor A, Jacques PF, Dorey CK: Oxidation and aging Impact on vision. Toxicol Ind Health 9: 349-371, 1993

313. Tee ES, Lim CL: Carotenoid composition and content of Malaysian vegetables and fruits by the AOAC and HPLC methods. Food Chem 41: 309-339,1991

314. Thoisterup T, Sandstorm B, Bysted A, Holmer G: Effect of 6 dietary fatty acids on the post parandial lipid profile, plasma fatty acids, lipoprotein lipase and cholesterol ester transfer activities in healthy young men. Am J Clin Nutr

73: 198-208, 2001

315. Thoisterup T, Sandstorm B, Hermansen JE, Holmer G: Effect of modified dietary fat on postprandial and fasting plasma lipids and lipoproteins in healthy young men. Lipids 33: 11-21, 1998

316. Thomson ABR, Cleland L: Intestinal cholesterol uptake from phospholipid vesicles and from simple and mixed micelles. Lipids 16: 881-887, 1981

317. Thumann PA, Schalck W, Aebischer JC, Tenter U, Cohn W: Plasma kinetics of lutein, zeaxanthin and 3`dehydro-lutein after multiple oral doses of a lutein supplement. Am J Clin Nutr 82: 88-97,2005

318. Thurnhofer H, Hauser H: Uptake of cholesterol by small intestinal brush border membrane is protein mediated. Biochemistry 29: 2142-2148,1990

319. Tso P: Intestinal lipid absorption. In physiology of the gastro intestinal tract, 3rd, ed.; Johnson LR, Alpas DH, Christensen J, Jacobson ED, Walsh JH, Eds. Raven press: New York, vol.2, 1867-1908, 1994

320. Tyssandier V, Lyan B, Borel P: Main factors governing the transfer of carotenoids from emulsion lipid droplets to micelles. Biophy Biochem Acta 1533: 285-292, 2001

321. Tyssandier V, Reboul E, Dumas JF, Demange CB, Armand M, Marcand J, Sallas M, Borel P: Processing of vegetable born carotenoids in the human stomach and duodenum. Am J Physiol Gastro Liver Physiol 284: 913-923, 2003

322. Unlu NZ, Bohn T, Clinton SK, Schwartz SJ: Carotenoid from salad and salsa by humans is enhanced by the addition of avocado or avocado oil. J Nutr 135: 431-436, 2005

323. U.S. Department of Agriculture,USDA-NCC. 1998. Carotenoid database for US foods.www.nal.usda.gov/fnic/foodcomp/Data/car98/car98.html.

325. Useful plants of India (CSIR, 1986)

326. Van den Berg H, van Vilet T: Effect of simultaneous, single oral doses of β-carotene with lutein or lycopene on the β-carotene and retinyl esters responses in the triglycerol- rich lipoprotein fraction in men. Am J Clin Nutr 68: 82-89, 1998

327. Van den Berg H: Carotenoid interactions. Nutr Rev 57: 1-10, 1999

328. Van den Berg TJ, Ijspeert JK, de Waard PW: Dependence of intraocular stray light on pigmentation and light transmission through the ocular wall. Vision Res 31: 1361-1367, 1991

329. Van Greevenbroek MMJ, Van meer GD, Erkelens DW, De Bruin TWA. Effects of saturated, mono-and polyunsaturated fatty acids on the secretion of apoB containing lipoproteins by caco2 cells. Atherosclerosis 121: 139-150, 1996

330. Van Het Hof KH, Brouwer IA, West CE, Haddeman E, Steegers-Theunissen RP, van Dusseldorp MV, Weststrate JA, Eskes TKAB, Hautvast JGAJ: Bioavailability of lutein from vegetables is 5 times than that of β-carotene. Am J Clin Nutr 70: 261-268,1999

331. Van het Hof KH, West CE, Weststrate JA, Hautvast JGA: Dietary factors that affect the bioavailability of carotenoids. J Nutr 130: 503-506, 2000

332. Van Kuijik FJ, Buck P: Fatty acid composition of the human macula and peripheral retina. Invest Opthalmol Vis Sci 33: 3493-3496,1992

333. Van Lieshout M, West CE, Van Breemen RB: Isotopic tracer technique for studying the bioavailability and bioefficacy of dietary carotenoids particularly β-carotene in humans; A review. Am J Clin Nutr 77: 12-28, 2003

334. Van Vliet T, Schreurs WHP, Van den Berg H: Intestinal β-carotene absorption and cleavage in men: response of β-carotene and retinyl esters in the triglyceride-rich lipoprotein fraction after a single oral dose of β-carotene. Am J Clin Nutr 62: 110-116, 1995

335. Van Vliet T, Van Vlissingen MF, Van Schaik F, Van den Berg H: β-Carotene absorption and cleavage in rats is affected by the vitamin A concentration of the diet. J Nutr 126: 499-508,1996

336. Van Vliet: Absorption of β-carotene and other carotenoids in humans and animal models. Eur J Clin Nutr 50: 32-37, 1996

337. Venkatraman JJ, Angkeow P, Satsangi N, Fernandes G: Effects of dietary n-6 and n-3 lipids on antioxidant defense system in livers of exercised rats. J Am Coll Nutr 17: 586-594,1998

338. Wang DO: regulation of intestinal cholesterol absortption. Ann Rev physiol 69: 221-248, 2007

339. Wang OM, Tsao R, Zhang S, Dong Z, Yang R, Gong J, Pei Y: Antioxidant activity, mutagenicity/anti-mutagenicity, and clastogenicity/anti-clastogenicity of lutein from marigold. Food and Chem Toxicol 44: 1522-1529, 2006

340. Wang W, Connor SL, Johnson EJ, Klein ML, Hughes S, Connor WE: Effect of dietary lutein and zeaxanthin on plasma carotenoids and their transport in lipoproteins in age-related macular degenaration. Am J Clin Nutr 85: 762-769, 2007

341. Wang XD, Tang GW, Fox JG, Krinsky NI, Russell RM: Enzymatic conversion of β-carotene into β-apocarotenals and retinoids by human, monkey, ferret and rat tissues. Arch Biochem Biophys 285: 8-16, 1991

342. Warnick GR, Albers JJ: A comprehensive evaluation of the heparin–manganese chloride precipitation procedure for estimating HDL–cholesterol. J. Lipid Res 19: 65-76, 1978

343. Weller P, Breithaupt D: Identification and quantification of zeaxanthin esters in plants using liquid chromatography-mass spectrometry. J Agric Food Chem 51: 7044-7049, 2003

344. West CE, Poortvliet EJ: The carotenoid content of foods with special reference to developing countries. Washington DC: US Agency for International Development, 1993

345. West SK: Smoking and the risk of eye diseases. In: Taylor A, Editor, Nutritional and environmental influences on the eye, CRC Press, Boca Raton. Pp.151, 1999

346. Westergaard H, Dietschy JM: The mechanism whereby bile acid micelles increase the rate of fatty acid and cholesterol uptake into the intestinal mucosal cell. J Clin Invest 58: 97-108, 1976

347. Williams AW, Boileau TW, Erdman JW: Factors influencing the uptake and absorption of carotenoids. Proc Soc Exp Biol Med 218:106-108,1998

348. Wills RBH, Rangga A: Determination of carotenoids in Chinese vegetables. Food Chem 56: 451-455, 1996

349. Wingerath T, Sies H, Stahl W: Xanthophyll esters in human skin. Arch Biochem Biophys 355: 271-274, 1998

350. Wingerath T, Stahl W, Sies H: β-Cryptoxanthin selectively increased in human chylomicrons upon ingestion of tangerine concentrate rich in β-cryptoxanthin esters. Arch Biochem Biophys 324: 385-390,1995

351. Winkler BS, Boulton ME, Gottsch JD, Sternberg P: Oxidative damage and age-related macular degeneration. Mol Vis 5: 32, 1999

352. Wrona M, Rozanowska M, Czub-Pelech B, Sarna T: Antioxidant action of zeaxanthin in protection of human RPE cells against oxidative damage. Assoc Res Vis Ophthalmol 42: S576. 2001.

353. Yamauchi R, Tauchihashi K, Kato K: Oxidation products of β-carotene during the peroxidation of methyl linoleate in the bulk phase. Biosci Biotechnol Biochem 62: 1301-1306, 1998

354. Yang Y, Huang CY, Peng SS, Li J: Carotenoid analysis of several dark-green leafy vegetables associated with a lower risk of cancers. Biomed Environ Sci 9: 386-392, 1996

355. Yao L, Liang Y, Trahanosky WS, Serfass RE, White WS: Use of a ^{13}C tracer to quantify the plasma appearance of a physiological dose of lutein in humans. Lipids 35: 339-348, 2000

356. Yemelyanov AY, Katz NB, Bernstein PS: Ligand-binding characterization of xanthophyll carotenoids to solubilized membrane proteins derived from human retina. Exp Eye Res 72: 381-392, 2001

357. Yen GC, Chen HY, Peng HH: Evaluation of cytotoxicity, mutagenicity and antimutagenicity of emerging edible plants. Food Chem Toxicol 39: 1045-1053, 2001

358. Yeum KJ, Russell RM: Carotenoid bioavailability and bioconversion. Ann Rev Nutr 22: 483-504, 2002

359. Yeum KJ, Shang F, Schalch W, Russell RM, Taylor A: Fat-soluble nutrient concentrations in different layers of human cataractous lens. Cur Eye Res 19: 502-505,1999

360. Yeum KJ, Taylor A, Tang G, Russell RM: Measurement of carotenoids, retinoids, and tocopherol in human lenses. Invest. Ophthalmol Vis Sci 36: 2756-2761, 1995

361. Yonekura L, Nagao A: Intestinal absorption of dietary carotenoids.1Mol Nutr Food Res 51: 107-115, 2007

362. Yonekura L, Tsuzuki W, Nagao A: Acyl moieties modulate the effects of phospholipids on β-carotene uptake by Caco-2 cells. Lipids 41: 629-636, 2006

363. Zanutto ME, Jordao AA, Vannucchi H: Improvement in vitamin status with consumption of dark green vegetables: a bioavailability study in rabbits. Nutr Res 23: 271-278, 2003

364. Zeisel SH, Mar MH, Howe JC, Holden JM: Concentrations of choline-containing compounds and betaine in common foods. J Nutr 133: 1302-1307, 2003

365. Zhang LX, Coney RV, Bertram JS: Carotenoids enhance gap junction communication and inhibit lipid peroxidation in C3H/10T/2 cells: relationship to their cancer chemo preventive action. Carcinogenesis 12: 109-114, 1991

Appendix

Publications:

1. **Lakshminarayana R**, Raju M, Krishnakantha TP, Baskaran V**:** Determination of major carotenoids in a few Indian leafy vegetables by high performance liquid chromatography. J Agric Food Chem 53: 2838-2842, **2005**

2. **Lakshminarayana R**, Raju M, Krishnakantha TP, Baskaran V: Enhanced bioavailability of lutein by lysophosphatidylcholine in mixed micelles. Mol Cell Biochem 281: 103-110, **2006**

3. **Lakshminarayana R**, Raju M, Krishnakantha TP, Baskaran V: Lutein and zeaxanthin in leafy greens and their bioavailability: Olive oil influences the absorption of dietary lutein and its accumulation in adult rats. J Agric Food Chem 55: 6395-6400, **2007**

Paper presentations in symposia:

1. **R. Lakshminarayana**, M. Raju, K. Rathinaraj and V. Baskaran. Isolation and purification of carotenoids from few leafy vegetables. **Presented at IFCON-2003**.

2. **R. Lakshminarayana**, T. P. Krishnakantha and V. Baskaran. Phospholipids and fatty acids in mixed micelles modulate the intestinal absorption of lutein and its accumulation in macula of rats. **National symposium SBC (I), 21-24th November 2004. G.B. Pant Agricultural University, Uttaranchal.**

3. **R. Lakshminarayana** and V. Baskaran. Evaluation of macular pigments in green leafy vegetables and dietary approach in modulating their levels in eyes of adult rats. **International Conference on Ethnopharmacology and Alternative Medicine will be held at Amala nagar, Trissur on 20-22nd January 2006.**

4. **R. Lakshminarayana**, G.vasanthi, M.N.Keshava Prakash, T.P.Krishnakantha and V.Baskaran. Manipulation of lutein and zeaxanthin levels in plasma and eyes of aged rats deficient in macular pigments through dietary means. **International Symposium on Food and Nutrition, 23-25th June 2006, CFTRI- Mysore**.

5. **R.Lakshminarayana**, T.P. Krishnakantha and V.Baskaran. Identification and structural elucidation of photo and auto-oxidized products of lutein in vitro**. ICFOST, 16-17th Nov-2006, Hyderabad.**