BIOAVAILABILITY OF β-CAROTENE AS INFLUENCED BY FOOD PROCESSING AND PRESENCE OF FACTORS SUCH AS SPICES

THESIS

submitted to the
UNIVERSITY OF MYSORE

for the award of the degree of

DOCTOR OF PHILOSOPHY
in
BIOCHEMISTRY

by

SUPRIYA VEDA

Under the supervision
of
DR. K. SRINIVASAN

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION,
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE,
MYSORE - 570020, INDIA

MAY 2009

Supriya Veda
Senior Research Fellow
Biochemistry and Nutrition Department
Central Food Technological Research Institute,
Mysore-570020, INDIA.

DECLARATION

I hereby declare that this thesis entitled "**BIOAVAILABILITY OF β-CAROTENE AS INFLUENCED BY FOOD PROCESSING AND PRESENCE OF FACTORS SUCH AS SPICES**" submitted to the University of Mysore for the award of degree of **DOCTOR OF PHILOSOPHY in BIOCHEMISTRY** is the result of the research work carried out by me in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, India under the guidance of **Dr. K. Srinivasan**, during the period November 2004 – May 2009.

I further declare that the research work embodied in this thesis has not been submitted previously for the award of any other degree / diploma.

(SUPRIYA VEDA)

Place: Mysore
Date: May 2009

Dr. K. Srinivasan,
Senior Scientist,
Department of Biochemistry and Nutrition.

CERTIFICATE

This is to certify that the thesis entitled "**Bioavailability of β-carotene as influenced by food processing and presence of factors such as spices**" submitted by **Supriya Veda** to the University of Mysore for the award of the degree of '**Doctor of Philosophy' in Biochemistry**, is the result of the research work carried by her in the Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, under my guidance during the period of November 2004 – May 2009

(K Srinivasan)
(Guide)

Place: Mysore
Date: May 2009

ACKNOWLEDGEMENT

I express my deep sense of gratitude and heartfelt thanks to my guide **Dr.K.Srinivasan**, Senior Scientist, Department of Biochemistry & Nutrition for his multi-dimensional support as a supervisor for suggesting the research problem, a guide for his inspiring guidance, a scientist for introducing me in to the field of biochemistry, a researcher for inculcating in me the research culture with respect to proper planning, execution of the research tasks, compilation of the research data and their rational interpretation, and finally, lucid presentation of the scientific data both orally and in this written form.

I am highly indebted to Dr. V.Prakash, Director, CFTRI for providing me an opportunity and the necessary infrastructure to pursue this doctoral programme in this institution.

I am thankful to Dr. S.G.Bhat, former Head and Dr. P.V.Salimath, Head, Department of Biochemistry & Nutrition for their support during this research tenure.

I deem it as a proud privilege to have received gratuitous help and guidance throughout this doctoral programme from Dr. Kalpana Platel, Scientist, Department of Biochemistry & Nutrition. I am extremely thankful to her for the constant interest and encouragement shown on me.

I am profoundly indebted to the staff of Central Instrumentation Facility and Services, especially Mr. Mukund, Mr. Kantha, Mr. Vatcharavelu, Ms. Asha, Mr. Rangaih, Mr. Umapathi, and Mr. Shivaswamy, and to Mrs. Rita Das and other staff of Animal House facility for providing me invaluable technical assistance / necessary facilities in my research work.

I sincerely thank Dr.M.C.Vardaraj (Head, Human Resource Department), Dr. G.S.Joseph (Scientist, HRD), Dr.B.R.Lokesh (Head, Lipid Science and Traditional Foods), Dr.S.G.Prapulla (Scientist, Fermentation Technology and Bioengineering) for their excellent support during the investigation.

I wish to convey heartful thanks to all my fellow labmates: Dr. H.Manjunatha, Dr. D.Suresh, Dr. S.Hemalatha, Dr. S.Vidyashankar, Mr.R.L.R.Reddy, Mrs.Usha Prakash, Ms.Smita Gautam, Ms.Bhoomika Tripathi, Ms. Shubhra Pande, Mr.B.S.Suresha, Ms. M.C.Shubha, Ms.B.C.Kavita, Ms. Akshaya Kamath, Ms. S.H.Bhavyashree, Ms.Chetana for providing an excellent ambience at the work place and also for their cooperation in my research activities. I have also taken help on many occasions from other colleages of my department and also from colleagues in other departments. I am grateful to each one of them. My warmest thanks to Meesha, Shobhana, Neetika, Rajani, Divya, Ajit, Ayapan, Kumaresan, and Desai for their immense help.

I owe a deep sense of respectful gratitude to all my family members especially to my parents, parents-in law, husband, brother, and grand parents for their constant encouragement, moral support and blessings. Gracefully, all these dearest people have put up with my continuous absence from home in pursuit of my goal.

I owe a lot to the **University Grants Commission**, New Delhi for awarding me Junior and Senior Research Fellowships, which enabled this scientific pursuit and also sustained my research tenure.

Supriya Veda

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
° C	Degree Celsius
µg	Micro gram
µl	Micro litre
µmol	Micro mole
ANOVA	Analysis of variance
AOAC	Analytical official analysis control
AUC	Area under the curve
BBM	Brush border membrane
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
g	Gram
g/l	Gram per litre
GLV	Green leafy vegetable
h	Hour
HCl	Hydrochloric acid.
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
I.U.	International units
ICMR	Indian Council of Medical Research
kDa	kilo Dalton
LD_{50}	Lethal Dose for 50% mortality.
LDL	Low- density lipoprotein
M	Molar concentration
mg	Milli gram
mg/ml	Milli gram per litre

min	Minute
ml	Milli litre
mmol/L	Millimole per litre
mol	Moles
mol/L	Moles per litre
NAD	β-Nicotinamide adenine dinucleotide
NADH	β-Nicotinamide adenine dinucleotide (reduced form)
m	Nanometer
nmol	Nano mole
NRC	National Research Council
p.o.	Post operative
p.s.i	Pounds per square inch
PBS	Potassium phosphate saline
PC	Phosphatidylcholine
PDA	Photo diode array
pmol	Pico mole
RBC	Red blood corpuscles
RDA	Recommended dietary allowance
rpm	Revolution per minute
sec	Second
SEM	Standard error of the mean
Tris	Tris (hydroxyl methyl) amino methane
TRL	Triglyceride–rich- lipoprotein
uM	Micro molar
USP	United States Pharmacopeia
v/v	Volume by volume
viz.	Namely
VLDL	Very low density lipoprotein

w/v	Weight by volume
w/w	Weight by weight
WBC	White blood corpuscles
x g	Times gravity

ABSTRACT

Micronutrient deficiency is a major public health problem in the developing countries, India accounting for nearly half of the world's prevalence. Among the micronutrient deficiencies, deficiency of vitamin A is recognized as a serious public health problem leading to blindness. Animal foods such as eggs, milk and liver are good sources of preformed vitamin A. A majority of the population in India is dependent on plant foods, which provide carotenes, especially β-carotene, to meet their requirement of vitamin A. β-carotene is abundantly found in green leafy and yellow-orange vegetable. Several factors such as diet composition and methods employed for food processing affect the bioavailability of β-carotene from foods.

Vitamin A malnutrition being widely prevalent, understanding the bioavailability of dietary β-carotene from plant foods, and its subsequent conversion to vitamin A is of utmost importance. Spices such as black pepper alter the ultra structure and permeability characteristics of the intestine, thus modifying the process of absorption. In vertebrates, provitamin A carotenoids are converted to retinol by the enzyme β-carotene-15, 15'-dioxygenase and retinal reductase. Since the enzymes are located in the intestinal cells which are directly exposed to various food components, actions of dietary components such as spices on the enzyme activity might affect the bioavailability of β-carotene derived from plant foods, and its bioconversion to vitamin A. It was therefore relevant to examine if such a spice also influence the β-carotene cleavage enzyme present in the intestinal enterocytes.

Such information may lead to optimization of dietary approaches to increase the bioavailability of dietary β-carotene. Knowledge of the bioavailability of β-carotene from dietary sources is also important in order to rationalize the RDA for the same. In addition to the provitamin-A activity, β-carotene and other carotenoids are of much value as antioxidants. In this context, information on the bioavailability of β-carotene from plant foods assumes greater importance. *In vitro* methods which essentially provide inaccessibility value of β-carotene from foods offer quick and cost effective alternative to the more expensive and cumbersome *in vivo* procedures. Thus in present investigation an *in vitro* method was employed in screening a large number of foods and also for evaluating the influence of heat processing, presence of food acidulants and presence of antioxidant spices on the bioaccessibility of β-carotene.

In view of the probable influence of a few specific spices on the ultra structure and permeability characteristics of intestines, animal studies were also carried out to assess the influence of specific dietary spices such as black pepper (or piperine), red pepper (or capsaicin) and ginger on the absorption of β-carotene and its bioconversion to vitamin A.

Thus the present investigation has evidenced that strategies such as heat processing as encountered in domestic cooking, especially pressure-cooking, open-pan-boiling and stir-frying, inclusion of food acidulants – lime and amchur, inclusion of antioxidant spices – turmeric and onion, inclusion of milk along with fruit pulp, and consumption of spices, such as black pepper, red pepper and ginger would be useful in deriving β-carotene in higher amounts from its potential plant sources.

Synopsis of the thesis entitled

BIOAVAILABILITY OF β-CAROTENE AS INFLUENCED BY FOOD PROCESSING AND PRESENCE OF FACTORS SUCH AS SPICES

Submitted to the University of Mysore

For the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

by

SUPRIYA VEDA

Department of Biochemistry and Nutrition

Central Food Technological Research Institute

Mysore – 570020, India

May 2009

SYNOPSIS OF Ph.D. THESIS

Title: BIOAVAILABILITY OF β-CAROTENE AS INFLUENCED BY FOOD PROCESSING AND PRESENCE OF FACTORS SUCH AS SPICES

Candidate: SUPRIYA VEDA **No. Ex / 9.2 / PhD / SV / 05-06**

Micronutrient deficiency is a major public health problem in the developing countries, India accounting for nearly half of the world's prevalence. Among the micronutrient deficiencies, deficiency of vitamin A is recognized as a serious public health problem leading to blindness. It has been estimated that globally, 2.8 million preschool children are at risk of blindness. Deficiency of vitamin A is wide spread in India leading to the blindness of about 60 thousand children below the age of five years every year. Animal foods such as eggs, milk and liver are good sources of preformed vitamin A. A majority of the population in India is dependent on plant foods, which provide carotenes, especially β-carotene, to meet their requirement of vitamin A. β-carotene is abundantly found in green leafy and yellow-orange vegetable. Several factors such as diet composition and methods employed for food processing affect the bioavailability of β-carotene from foods. Dietary factors such as fat, fiber and protein are documented to influence β-carotene bioavailability. Earlier investigations have evidenced that inclusion of phospholipids and certain specific fatty acids in the diet significantly improve the vitamin A status of experimental animals. Studies have shown that the absorption of carotenoids from uncooked food is low and mild cooking enhances the absorbability of β-carotene. However, heat treatment especially in presence of light and oxygen causes isomerization of carotene as well as its oxidative destruction thus decreasing its biological activity.

In vertebrates, provitamin A carotenoids are converted to retinal by the enzyme β-carotene- 15,15'-dioxygenase, the activity of which is expressed specifically in intestinal epithelium and in liver. The intestinal enzyme determines whether provitamin A carotenoids are converted to vitamin A or circulated in the body as intact carotenoids.

Thus, the bioconversion of β-carotene to retinal is dependent on the regulation of the activity of this enzyme. Since the cleavage enzyme is located in the intestinal cells which are directly exposed to various food components, actions of dietary components such as spices on the enzyme activity might affect the bioavailability of β-carotene derived from plant foods, and its bioconversion to vitamin A.

Vitamin A malnutrition being widely prevalent, understanding the bioavailability of dietary β-carotene from plant foods, and its subsequent conversion to vitamin A is of utmost importance. Such information may lead to optimization of dietary approaches to increase the bioavailability of dietary β-carotene. Knowledge of the bioavailability of β-carotene from dietary sources is also important in order to rationalize the RDA for the same. In addition to the provitamin-A activity, β-carotene and other carotenoids are of much value as antioxidants. In this context, information on the bioavailability of β-carotene from plant foods assumes greater importance. *In vitro* methods which essentially provide bioaccessibility value of β-carotene from foods offer quick and cost effective alternative to the more expensive and cumbersome in vivo procedures. They can therefore be employed in screening a large number of foods and also for evaluating the influence of various factors on the bioavailability of β-carotene.

Considerable amount of β-carotene are lost from vegetables during pressure- cooking or open- pan- boiling. Presence of food acidulants affects the retention of β-carotene from some vegetables. The antioxidant spices – turmeric or onion generally improve the retention of β-carotene. A combination of food acidulant and antioxidant spice improved the retention from specific green leafy vegetables synergistically. Heat processing methods, presence of acidulants and presence of antioxidant spices may similarly influence the bioavailability of β-carotene from plant foods, which remains to be verified. Spices such as black pepper alter the ultrastructure and permeability characteristics of the intestine, thus modifying the process of absorption. Several spices have also been evidenced to enhance the activity of terminal digestive enzymes of the small intestine. Piperine has been evidenced to produce a proliferation of endoplasmic reticulum of the enterocytes of the intestine, which is associated with an increased absorptive surface and

microvilli length. It would therefore be relevant to examine if such a spice would also influence the β-carotene cleavage enzyme present in the intestinal enterocytes.

This research programme involved screening of selected green leafy and yellow-orange vegetables for the bioaccessibility of β-carotene as influenced by factors such as heat processing, presence of food acidulants, antioxidant spices or their combination. The programme also included a study of the varietal differences in the content and bioaccessibility of β-carotene from mango and papaya fruits.

In view of the probable influence of a few specific spices on the ultrastructure and permeability characteristics of intestines, animal studies were carried out to assess the influence of specific dietary spices such as black pepper (or piperine) on the intestinal permeability characteristics and absorption of β-carotene. Animal Studies were also extended to examine the effect of these dietary spices on the bioconversion of β-carotene to retinol in these animals which involved measurement of retinol in tissues following an oral dose of β-carotene and the activity of enzymes involved in carotenoid cleavage and subsequent reduction.

The thesis is presented in five chapters as follows:

Chapter-I :

GENERAL INTRODUCTION

Chapter-I presents general introduction which encompasses an outlay of the following: preamble: Vitamin A deficiency, particularly, the Indian scenario, Chemistry and classification of carotenoids, Sources of carotenoids, Requirements of β-carotene, Biological functions of carotenoids, Digestion and absorption of carotenoids, Metabolism of carotenoids, Bioavailability of carotenoids, The methods for determining bioavailability of carotenoids, both *in vivo* and *in vitro*. This general introduction culminates in the scope of the present investigation and its objectives.

Chapter-II:

COMPARISON OF *IN VITRO* METHODS FOR THE DETERMINATION OF BIOACCESSIBILITY OF β-CAROTENE IN VEGETABLES

This chapter discusses the optimization of an *in vitro* procedure for the determination of bioaccessibility of β-carotene from vegetables. The suitability of procedural alternatives for the determination of bioaccessibility of β–carotene in this *in vitro* method which involved simulated gastro-intestinal digestion followed by the separation of aqueous micellar fraction containing the bioaccessible β–carotene was examined. In this study, membrane filtration and equilibrium dialysis were examined to separate the micellar fraction as an alternative to ultracentrifugation. Values of β-carotene bioaccessibility from vegetables obtained with the membrane filtration method were similar to those obtained by the ultracentrifugation method, and hence it was inferred that membrane filtration to separate the aqueous micellar fraction containing the bioaccessible β-carotene is satisfactory. The procedure employed for further studies in this investigation, involved initial simulated gastrointestinal digestion of the food material, followed by ultracentrifugation to separate the micellar fraction containing the bioaccessible β-carotene and its quantitation.

Chapter-III:

BIOACCESSIBILITY OF β-CAROTENE FROM YELLOW-ORANGE AND GREEN LEAFY VEGETABLES AS INFLUENCED BY HEAT PROCESSING AND PRESENCE OF FOOD ADJUNCTS

This chapter consists of two sections, namely:

A] Bioaccessibility of β-carotene from yellow-orange and green leafy vegetables as influenced by heat processing

B] Influence of food acidulants and antioxidant spices on the bioaccessibility of β-carotene from vegetables

A majority of the population in India is dependent on plant foods, which provide carotenes, especially β-carotene, to meet their requirement of vitamin A. Several factors such as methods employed for food processing and diet composition and presence of

various food adjuncts affect the bioaccessibility of β-carotene from foods. Section-A presents data on the bioaccessibility of β-carotene from different yellow-orange vegetables (carrot and pumpkin) and green leafy vegetables (fenugreek, amaranth, spinach, drumstick, coriander and mint) commonly consumed in India, and influence of heat processing on the same. Heat treatment of these vegetables by pressure-cooking, open-pan-boiling and stir-frying generally had a beneficial influence on the bioaccessibility of β-carotene from these vegetables.

In the second section (Section-B) of this chapter, data on the influence of food adjuncts such as acidulants and antioxidant spices on the bioaccessibility of β-carotene from representative green leafy vegetables and yellow-orange vegetables are discussed. Four common food acidulants- amchur, lime, tamarind and kokum, and two antioxidant spices– turmeric and onion were examined for their influence on bioaccessibility of β-carotene. Amchur and lime generally enhanced the bioaccessibility of β-carotene from these vegetables in many instances. Such an improved bioaccessibility was evident in both raw and heat-processed vegetables. The effect of lime juice was generally more pronounced than that of amchur. Turmeric significantly enhanced the bioaccessibility of β-carotene from all the vegetables tested, especially when heat-processed. Onion enhanced the bioaccessibility of β-carotene from pressure-cooked carrot and amaranth leaf and from open-pan-boiled pumpkin and fenugreek leaf. Lime juice and the antioxidant spices turmeric and onion minimized the loss of β-carotene during heat processing of the vegetables. In the case of antioxidant spices, improved bioaccessibility of β-carotene from heat-processed vegetables is attributable to their role in minimizing the loss of this provitamin. Lime juice which enhanced the bioaccessibility of this provitamin from both raw and heat-processed vegetables probably exerted this effect by some other mechanism in addition to minimizing the loss of β-carotene. Thus, the presence of food acidulants- lime juice / amchur and antioxidant spices – turmeric / onion prove to be advantageous in the context of deriving maximum β-carotene from the vegetable sources. This study also indicated that a combination of food acidulant and an antioxidant spice which individually produced a higher bioaccessibility of β-carotene, did

not have any significant additive or synergistic effect with regard to enhancing the bioaccessibility of β-carotene.

Chapter-IV:

VARIETAL DIFFERENCES IN THE BIOACCESSIBILITY OF β-CAROTENE FROM MANGO (*Mangifera indica*) AND PAPAYA (*Carica papaya*) FRUITS.

Mango and papaya, which are rich sources of β-carotene, are widely consumed in India. This chapter documents β-carotene content and its bioaccessibility determined in six locally available varieties of mango *viz*; *Badami*, *Raspuri*, *Mallika*, *Malgoa*, *Totapuri* and *Neelam*, and two varieties of papaya namely *Honey Dew* and *Surya*. Varietal differences were evident in both β-carotene content and its bioaccessibility in the case of mango. β-Carotene content (mg/100 g) in ripe mango ranged from 0.55 ± 0.03 in the *Malgoa* variety to 3.21 ± 0.25 in the *Badami* variety. Similarly, among the *Honey Dew* and the *Surya* varieties of papaya, β-carotene content (mg/100 g) was 0.70 ± 0.10 and 0.74 ± 0.12, respectively. Bioaccessibility of β-carotene ranged from 24.5% in the *Badami* to 39.1% in the *Raspuri* varieties of mango. Considering both the percent bioaccessibility and the inherent β-carotene content, the amount of bioaccessible β-carotene was highest in the *Mallika* variety (0.89 mg/100 g), followed by *Badami* (0.79 mg/100 g). Since mango and papaya are also consumed as a blend with milk, influence of the presence of milk on the bioaccessibility of β-carotene from these fruits was also examined. Addition of milk generally brought about a significant increase in the bioaccessibility of β–carotene from mango, the increase ranging from 12 to 56%. Bioaccessibility of β–carotene from the two varieties of papaya examined was similar (31.4-34.3%). Addition of milk increased this bioaccessibility by 19 and 38 % in these two varieties. Considering the β–carotene content of mango and papaya, the latter has to be consumed roughly 3 times that of mango, to derive the same amount of β–carotene. Thus, this study has indicated that varietal differences exist in the content and bioaccessibility of β–carotene in mango, and that the addition of milk is advantageous in deriving this provitamin A from the fruit pulp of mango and papaya.

Chapter-V :

ANIMAL STUDIES ON THE INFLUENCE OF DIETARY SPICES ON ABSORPTION AND BIOCONVERSION OF β-CAROTENE TO VITAMIN A.

This chapter consists of two sections, namely:

A] Influence of dietary spices – black pepper, red pepper and ginger on the uptake of β-carotene by rat intestines.

B] Influence of dietary spices on the *in vivo* absorption of ingested β-carotene and its bioconversion to vitamin A in experimental rats.

Spices are very commonly used in Indian culinary. Specific spices may alter the ultra-structure and permeability characteristics of intestines. Piperine, the major alkaloid present in black pepper is known to increase bioavailability of drugs and other phytochemicals, which may be attributed to increased absorption, resulting from alteration in membrane lipid dynamics and change in the conformation of enzymes in the intestine. Whether such dietary spices which have potential to alter the ultrastructure and permeability of intestinal brush border beneficially influence the absorption of β-carotene and its subsequent conversion to vitamin A are discussed in this chapter.

Section-A deals with the uptake of β-carotene by the intestinal segments isolated from rats fed black pepper, red pepper, ginger, piperine and capsaicin. Higher absorption of β-carotene in the intestines was evidenced in all the spice-fed animals. Dietary piperine and ginger increased the uptake of β-carotene by 147% and 98% respectively. While black pepper and red pepper fed animals showed an increase in absorption by 59 and 27 %, dietary capsaicin increased the same by 50%. Thus, pungent spices alter permeation characteristics presumably by increasing absorptive surface, and thereby enhance intestinal absorption of β-carotene, which could form a strategy to reduce vitamin-A deficiency.

Animal studies were also conducted to evaluate the influence of these dietary spices on the *in vivo* absorption of orally administered β-carotene and the efficacy of its conversion to vitamin A, results of which are presented in Section-B of this chapter. Young male Wistar rats were maintained on specific spice containing diets for 8 weeks.

These rats were administered a single oral dose of β-carotene. After 4 h *p.o.* administration, concentration of β-carotene and retinol in serum, liver and intestine were determined. There was a significant increase in β-carotene concentration in the serum, liver and intestine of piperine and ginger fed rats as compared to control. This suggests that dietary piperine and ginger improve intestinal absorption of β-carotene leading to an increased β-carotene concentration in circulation and in tissues. However, the concentration of retinol was not significantly changed in these spice fed groups as compared to control, suggesting that bioconversion of β-carotene to vitamin A was not similarly influenced.

This was further verified in a separate animal study, wherein the activities of the two enzymes specifically involved in the bioconversion of β-carotene to vitamin A were measured in the intestine and liver of spice fed animals. Activity of intestinal β-carotene-15,15'-dioxygenase was rather lowered in capsaicin and ginger fed animals, while it was comparable to control in piperine treatment. β-Carotene- 15,15'-dioxygenase activity was also lower in the liver of capsaicin fed animals. Activity of retinal reductase either in liver or intestine was not influenced by dietary spices.

The spice active compounds – piperine, capsaicin, and gingerone were also examined for their *in vitro* influence on the activity of β-carotene cleavage enzyme and retinal reductase, results of which are also presented in Section-B of this chapter. Rat intestinal and liver homogenate was used as the enzyme source. Capsaicin significantly decreased the activity of liver β-carotene- 15,15'- dioxygenase when included in the assay medium at 1 x 10^{-6}M, 1 x 10^{-5}M, 1 x 10^{-4}M level, while piperine and gingerone inhibited this activity only at 1 x 10^{-4}M level in the assay medium. Intestinal β-carotene- 15,15'-dioxygenase activity was significantly decreased by capsaicin and gingerone present at 1 x 10^{-5} M and 1 x 10^{-4}M, while piperine showed the inhibitory effect at 1 x 10^{-4}M in the assay medium. The activity of hepatic and intestinal retinal reductase on the other hand, was increased by the presence of either of the three spice compounds at 1 x 10^{-4}M level in the assay system. In the absence of a simultaneous promotion of the bioconversion of β-carotene, the benefit of increased blood and tissue levels of β-carotene brought about

by dietary spices suggests an effective enhancement of antioxidant protection by this provitamin.

General Summary is given at the end of these chapters which highlights the salient observations of this investigation. On the whole, the salient observations of this Ph.D. programme points to food based strategies to maximize the bioavailability of β-carotene from the conventional food sources. The thesis culminates in Bibliography pertaining to the five individual chapters and given in alphabetical order.

Supriya Veda
(Candidate)

Dr. K. Srinivasan
(Guide)

Date: th May 2009

CHAPTER – I

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Vitamin A deficiency is a major public health problem that affects more than 190 million children and as many as nineteen million pregnant women in more than 100 countries across the globe (WHO, 2009). The incidence of vitamin A deficiency in women and children is particularly high in South and Southeast Asia and Sub-Saharan Africa. Deficiency of Vitamin A is widespread in developing countries including India, influencing the growth of young children severely (West, 2002; WHO, 2009). Vitamin A deficiency causes growth retardation, xerophthalmia, often leading to blindness, anemia, reduced resistance to infection, and increased severity of infectious diseases which may result in loss of life. More than 2.5 lakh children become blind as a result of vitamin A deficiency each year. Vitamin A deficiency in young children is a consequence of low concentrations in breast milk, inadequate intake after weaning, and depletion of stored vitamin A due to chronic illness (Miller *et al*., 2002). UNICEF and WHO opine that improving the vitamin A status of young children with marginal deficiency may reduce mortality by about 23%. In addition to supplementation programmes, dietary approaches that promote bioavailability of the precursor of vitamin A are also needed to ensure adequate intakes of this vitamin.

Because humans lack the ability to synthesize vitamin A, they are dependent on dietary intake to provide adequate levels of this vitamin. Dietary sources of vitamin A include preformed vitamin A primarily in the form of retinyl esters in animal products and the provitamin A carotenoids in plant foods. Vitamin A fortified foods (e.g., milk) and pharmaceutical supplements containing provitamin A and preformed vitamin A are readily available in affluent countries; thus, the provitamin A carotenoids in fruits and vegetables generally account for less than one-third of the total retinol intake in nutritionally diverse diets consumed in such countries (Rodriquez-Amaya, 1997). In contrast, a majority of the population in developing countries lack access to animal products and pharmaceutical supplements; thus in developing countries, where vitamin A deficiency is widely prevalent, most dietary vitamin A is obtained from plant sources in

the form of provitamin A carotenoids. Among carotenoid pigments, which are widely distributed in plant tissues, β-carotene possesses the highest vitamin A potential. (Jayarajan *et al*., 1978; Ribaya-Mercado *et al*., 2000).

Micronutrient deficiencies, especially of iron, vitamin A, iodine and zinc, are most widely prevalent in India, accounting for nearly half of the world's prevalence (WHO, 2003). Vitamin A deficiency itself leads to the blindness of about 60 thousand children below the age of five years every year. Animal foods such as eggs, milk, and liver are good sources of preformed vitamin A. A majority of population in India, however, is dependent on plant foods, which provide provitamin A carotenes, especially β-carotene, to meet their requirement of vitamin A. β-Carotene is abundantly found in green leafy vegetables and yellow- orange vegetables and fruits (Gopalan *et al*., 2004).

1. Chemistry and classification of carotenoids

Among the most common and most important natural pigments are the carotenoids. Carotenoids are a class of natural fat-soluble pigments found principally in plants, algae, and photosynthetic bacteria, where they play a critical role in the photosynthetic process. These are responsible for many of the red, orange, and yellow hues of plant leaves, fruits, and flowers, as well as the colors of some birds, insects, fish, and crustaceans. They also occur in some non-photosynthetic bacteria, yeasts, and molds, where they may carry out a protective function against damage by light and oxygen. Some familiar examples of carotenoid coloration are the orange of carrots and citrus fruits, the red of peppers and tomatoes, and the pink of flamingoes and salmon (Pfander, 1992). Although animals appear to be incapable of synthesizing carotenoids, many animals derived carotenoids from their diet. Within animals, besides being a source for vitamin A activity, carotenoids provide bright coloration, and serve as antioxidants (Ong and Tee, 1992; Britton *et al*., 1995).

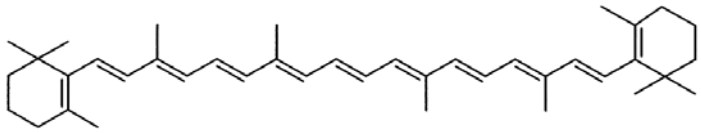
The name carotene was given to the yellow pigment of carrot from which it was first isolated in 1832. β-Carotene, the principal carotenoid in carrots, is a familiar carotene, while lutein, the major yellow pigment of marigold petals, is a common xanthophyll.

Carotenoids are defined by their chemical structure. According to the accepted definition Karrer and Jucker (1950), carotenoids are yellow to red pigments of aliphatic or alicyclic structure composed of isoprene units linked so that the two methyl groups nearest to the centre of the molecule are in positions 1- 6 and all other lateral methyl groups are in positions 1-5; the series of conjugated double bonds constitutes the chromophoric system of carotenoids. Some 600 different carotenoids are known to occur naturally (Ong and Tee, 1992), and new carotenoids continue to be identified (Mercadante, 1999). All carotenoids are derived from a basic C-40 isoprenoid structure. The basic structure of carotenoids is illustrated by the structure of β-carotene, a symmetrical hydrocarbon with 40 carbon atoms, 11 double bonds and 2 rings (Fig.A). Carotenoids generally contain a conjugated polyene structure which is efficient in absorbing light, and are the major yellow and red pigments in many fruits and vegetables. The carotenes – hydrocarbon carotenoids (e.g. α- and β -carotene and lycopene) contain only carbon and hydrogen, while the term 'xanthophylls' refers to compounds which contain hydroxyl groups (lutein, zeaxanthin, β-cryptoxanthin) or keto groups (canthaxanthin) or both (astaxanthin) i.e., each contain at least one oxygen group (alcohol or carbonyl). The hydrocarbon carotenoids are known as carotenes, while oxygenated derivatives of these hydrocarbons are known as xanthophylls.

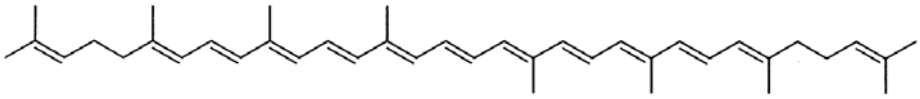
Carotenoids are also classified as provitamin A or non-provitamin A compounds. The former serve as dietary sources of vitamin A because they can be enzymatically cleaved to yield either one (e.g., β-cryptoxanthin and α-carotene) or two (β-carotene) molecules of retinal. β-Carotene is the most potent source of vitamin A in the diet. Configurational isomers, such as α- and β- carotene, may be used differently by the body. Most carotenoids in plants exist in the all-*trans* configuration, although *cis*- isomer may be formed during food processing, especially heat-treatment (Rodriguez-Amaya, 1997). *Cis* isomers of both dietary carotenoids and their retinoid metabolites are found in tissues (Ross, 1999). The polyene carotenoids are found largely in the all-*trans* configuration but they also have *cis*-geometrical isomers, which may have different roles in metabolism (Furr and Clark, 1997).

Carotenes

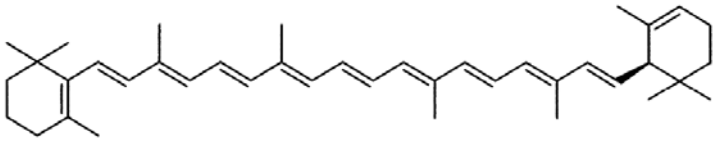
β-Carotene



Lycopene

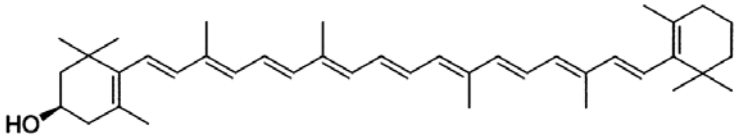


α-Carotene

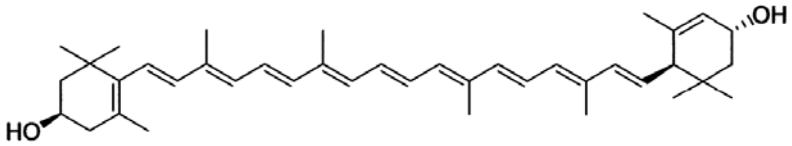


Xanthophylls

β-Cryptoxanthin



Lutein



Zeaxanthin

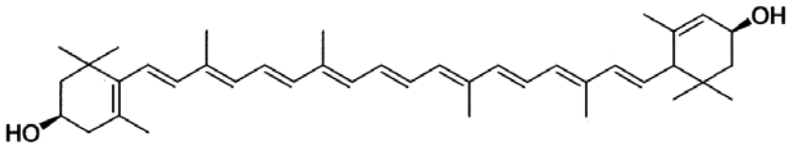


Fig.A. Structures of major carotenoids

The structure of a carotenoid ultimately determines what potential biological function(s) that pigment may have. The distinctive pattern of alternating single and double bonds in the polyene backbone of carotenoids is what allows them to absorb excess energy from other molecules, while the nature of the specific end groups on carotenoids may influence their polarity. The former may account for the antioxidant properties of biological carotenoids, while the latter may explain the differences in the ways that individual carotenoids interact with biological membranes (Britton, 1995).

Carotenoids termed as pro-vitamins have certain physical and chemical properties. They are fat–soluble and also readily soluble in organic solvents such as chloroform, benzene, carbon disulfide and petroleum ether. They are sensitive to oxidation, auto oxidation and light. They are stable to heat in oxygen-free environment. They have characteristic absorption spectra, and the λ_{max} shifts with use of different solvents (Freed, 1966). The stability of carotenoids in foods varies depending on the matrix. Oxidation of carotenoids takes place both in presence and absence of enzymes. These pigments may auto oxidize by reacting with atmospheric oxygen. Oxidation of carotenoids depends on light, heat and presence of pro-and antioxidants. They don't undergo hydrolysis very easily; the hydrocarbons are tightly bound and hence do not break. Carotenoids change their colour by undergoing isomerization from *trans* to *cis* configuration. The orange-red colour is changed to lemon yellow on isomerization. The change is facilitated in the presence of acid and temperature. Prolonged cooking and blanching also causes thermal destruction of carotenoids (Manay and Shadaksharaswamy, 2002).

2. Sources of carotenoids

β-Carotene is obtained from a number of fruits and vegetables. Rich source of β-carotene are green leafy vegetables, such as agathi (*Sisbania grandiflora*), spinach (*Spinacia oleracea*), amaranth (*Amaranthus gangeticus*), coriander (*Coriandrum sativum*), drumstick leaves (*Moringa oleifera*), curry leaves (*Murriya koenigii*), mint (*Mentha spicata*), radish leaves (*Raphanus sativus*) etc. Ripe yellow fruits such as mango

(*Mangifera indica*), papaya (*Carica papaya*) and tomato (*Lycopersicum esculentum*) are also rich in carotene. Among other vegetables, carrot (*Daucus carota*) and pumpkin (*Cucurbita maxima*) are good sources. Generally, greener the leafy vegetable, higher the content of carotene and thus the outer green leaves of cabbage have more carotene than inner white leaves. Crude Red palm oil (RPO) is also a very good source of β-carotene, besides being a source of edible oil. One g of RPO contains 800 μg of β-carotene. However, in the refining process of crude red palm oil, particularly during bleaching process, β-carotene is lost.

β-Carotene requirement varies with age and physiological condition. The daily requirement of β-carotene for the Indian population varies from 1200 μg for infants to 2400 μg for adults. Lactating women require an additional 1400 μg of β-carotene per day (Gopalan *et al*., 2004).

3. Biological functions of carotenoids

3.1. *Provitamin A activity of carotenoids*

Provitamin A activity is the most important biological activity of carotenoids in human beings. Vitamin A, which has many vital systemic functions in humans, can be formed within the body from certain carotenoids, notably β-carotene (Britton *et al*., 1995). Other provitamin A carotenoids include α-carotene and cryptoxanthin. For vitamin A activity, a carotenoid must have at least one unsubstituted β-ionone ring with an attached polyene side chain of at least eleven carbon atoms. Consistent with these important structural requirements, the following carotenoids have been known to possess provitamin A activity, β-carotene, α-carotene, γ-carotene, and cryptoxanthin. Vitamin A activity of β-carotene, expressed as μg retinol equivalents (RE) is calculated as

RE = μg β-carotene ÷ 6

The other three carotenoids mentioned, above possess only one unsubstituted β-ionone ring and are expected to have about 50% of the biological activity of β-carotene.

The formula used for these three provitamin A carotenoids is therefore

RE = μg carotenoid ÷ 12 (National Academy of Sciences, 1980).

Recently, equivalencies are raised to 12 μg β–carotene and 24 μg of other provitamin A carotenoids to 1 μg RE (Thurnham, 2007).

An arbitrary provitamin A value assigned by United States Pharmacopeia (USP) for β-carotene is 0.60 μg of β-carotene = 1 USP unit of vitamin A (Freed, 1966). It is the usual practice to express vitamin A value of food stuffs in terms of International Units of vitamin A. The carotene content of plant foods is usually converted to vitamin A value assuming that 0.6 μg of carotene is equivalent to 1 I.U. of vitamin A. However, in view of a lack of definite information about the conversion of carotene to vitamin A in the body, the values for carotene are given now as μg per 100 g of the foodstuff. The values for vitamin A are also given as μg retinol per 100 g assuming that one international unit of vitamin A is equivalent to 0.3 μg of retinol (Gopalan *et al*., 2004).

3.2. *Antioxidant potential of carotenoids*

β-Carotene, apart from being an important precursor of vitamin A, also offers several other vital benefits for mankind. It is an unusual type of lipid antioxidant found in nature. It imparts several of its health benefits through its antioxidant property (Young and Lowe, 2001). Carotenoids also play an important role in human health by acting as natural antioxidants, protecting cells and tissues from the damaging effects of free radicals and singlet oxygen. Lycopene, the hydrocarbon carotenoid that gives tomatoes their red color, is particularly effective at quenching the destructive singlet oxygen (Di Mascio *et al*., 1989). The xanthophylls lutein and zeaxanthin are believed to function as protective antioxidants in the macular region of the human retina (Snodderly, 1995). Astaxanthin is another naturally occurring xanthophyll with potent antioxidant properties (Di Mascio *et al*., 1991). The consequence of such antioxidative potential of carotenoids includes health benefits such as enhancement of immune system function (Bendich, 1989), protection from sun-burn (Mathews-Roth, 1990) and inhibition of the development of certain types of cancer (Nishino, 1998).

The hydrocarbon carotenoids such as β-carotene are known to reduce the risk of cancer and heart disease, whereas xanthophylls such as lutein and zeaxanthin may be more important for age related macular degeneration and cataract formation. The most plausible mechanism for these health effects of carotenoids is their antioxidant property, although function in the control of gene expression has also been studied (Furr and Clark, 1997). In plants, they serve as antioxidants to protect the highly reactive photo systems and also act as accessory photo pigments (Krinsky, 1993).

The antioxidant activity of carotenoids is a direct consequence of the chemistry of their long polyene chain: a highly reactive, electron rich-system of conjugated double bonds susceptible to attack by electrophilic reagents and forming stabilized radicals. Burton and Ingold (1984) described the mechanism by which β-carotene acts as a chain-breaking antioxidant. The addition of a peroxyl radical ($ROO^{\cdot}$**)** to a suitable double bond of the carotenoid (CAR) could be the first step in the scavenging of a peroxyl radical (reaction-1). The resulting carbon-centered radical ($ROO\text{-}CAR^{\cdot}$) reacts rapidly and reversibly with oxygen to form a new chain carrying peroxyl radical ($ROO\text{-}CAR\text{-}OO^{\cdot}$) (reaction-2). The carbon-centered radical is resonance stabilized to such an extent, that when the oxygen pressure is lowered the equilibrium of reaction-2 shifts sufficiently to the left, to effectively lower the concentration of peroxyl radicals and hence reduce the extent of autooxidation in the system (Britton, 1995a). Furthermore, the carotene radical adduct can also undergo termination by reaction with another peroxyl radical(reaction-3).

$$CAR + ROO^{\cdot} \longrightarrow ROO\text{-}CAR^{\cdot} \qquad (1)$$

$$ROO\text{-}CAR^{\cdot} + O_2 \longleftrightarrow ROO\text{-}CAR\text{-}OO^{\cdot} \qquad (2)$$

$$ROO\text{-}CAR^{\cdot} + ROO^{\cdot} \longrightarrow \text{inactive products} \qquad (3)$$

Reaction- 3 is thought to take place at low oxygen pressure (such as < 150 torr) so that peroxyl radicals would be eventually consumed to allow carotenoids to act as a chain-breaking antioxidants (Kiokias and Gordon, 2004).

The antioxidative property of β-carotene makes it an important nutrient in prevention of several life threatening diseases like cardio-vascular disease, and several types of cancer. As an important precursor it has a major role to play in normal vision. It brings about immunomodulation by stimulating immune response. The important health protective effects of β-carotene are given in (Fig.B).

3.3. *β-Carotene and cardiovascular diseases*

β-Carotene, as a powerful singlet oxygen quencher, scavenges free radicals and thus protects against cardio-vascular diseases (CVD) (Burton and Ingold, 1984). Several studies have shown that high intake of β-carotene is associated with reduced risk of heart attack or stroke. An intervention study (Heinekens and Eberlein, 1985) involving 22,000 male physicians aged 40-84 years, who received aspirin (325 mg) and / or β-carotene (50 mg), showed significant reduction (50%) in cardio-vascular events in the β-carotene supplemented group. Short term intake of β-carotene (300 mg/day) caused significant increase in serum HDL in healthy adults; this protective effect of β-carotene supplementation was significant after 2 years of treatment (Ringer *et al*., 1991). Ghaziano *et al*. (1991) showed that β-carotene significantly reduced the risk of cardio-vascular events in patients who had a history of stable angina pectoris.

3.4. *β-Carotene and vision*

Dietary intake of β-carotene is well established to protect against cataract. One molecule of dietary β-carotene yields two molecules of vitamin A in the human system, which is essential for normal vision of an individual (Bamji *et al*., 1996). The protein present in the eye lens can undergo oxidative damage under pro-oxidant conditions as encountered in diabetes mellitus or aging, which may lead to cataract (Taylor, 1992). Low carotene in the diet is a risk factor for cataract (Schlipalius, 1997). Significant reduction was seen in nuclear cataract of adults aged 65-74 years who were supplemented with multiple vitamins and minerals along with 15 mg β-carotene/day for 6 years (Sperduto *et al*., 1993). Senile cataract was shown to be delayed by the intake of dietary β-carotene (Jacques *et al.*, 1988; Jacques and Chylack, 1991).

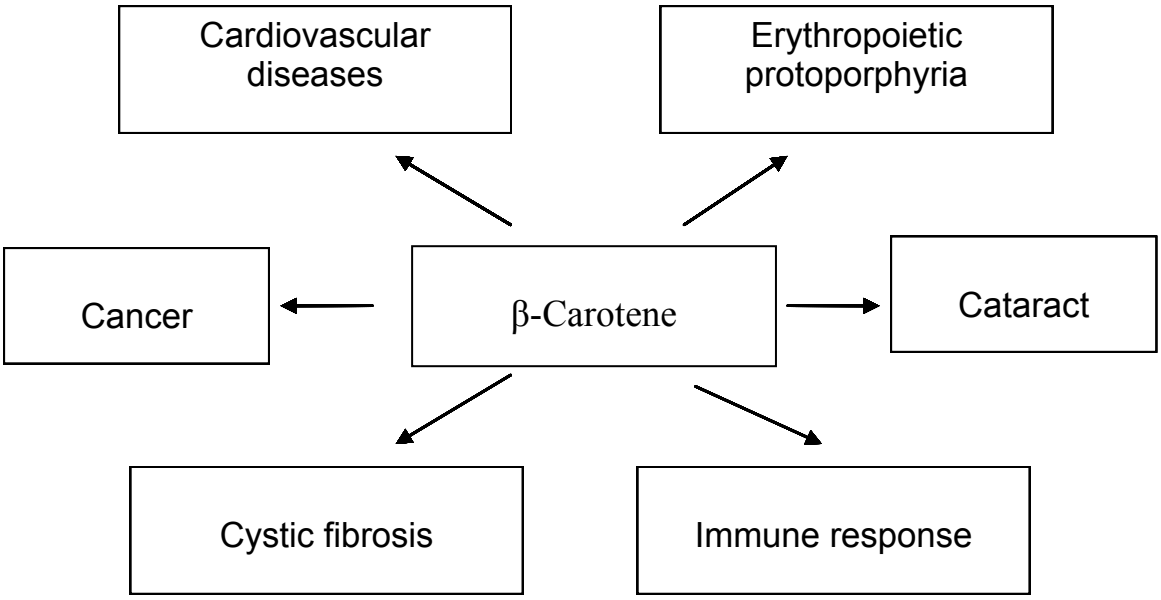


Fig.B. Health protective influences of β-carotene

3.5. *β-Carotene and cancer*

β-Carotene is an anticancer agent by virtue of its radical scavenging antioxidant action (Burton and Ingold, 1984). Several epidemiological studies demonstrated a lower incidence of cancer in people who consumed higher amounts of fruits and vegetables (Schlipalius, 1997). β-Carotene can prevent various types of cancer especially of lung, mouth, larynx, pharynx, oesophagus, stomach, and breast, colon / rectum, prostrate and skin.

Mayne *et al*. (1994) reported that intake of yellow and green leafy vegetables significantly protects against lung cancer in non-smoking men and women. Several studies have also shown that β-carotene does not protect smokers from lung cancer (Naves and Moreno, 1998). Ziegler *et al*. (1996) found that dietary β-carotene is strongly associated with reduced risk of lung cancer.

Several studies suggest that β-carotene plays a protective role in oral, pharynx, and larynx cancer. Observational studies strongly suggest that fruits and vegetables, especially those rich in β-carotene, have inhibitory effect on mouth and throat cancers (Mayne, 1996). Dietary intervention trials in humans using carotene in the prevention of oral leukoplakia (pre-cancerous changes) showed that β-carotene is one of the agents responsible for protective effects against mouth and throat cancers.

Blot *et al*. (1993) conducted a study on 30,000 people, men and women, aged 40-69. Combinations of nutrients like retinol + zinc, riboflavin + niacin, ascorbic acid + molybendum, β–carotene + selenium + α-tocopherol were given to test their efficacy against stomach cancer. After an intervention period of 9 years, the highest reduction in cancer rate was seen in individuals who received β-carotene + selenium + α-tocopherol, indicating that β-carotene has a beneficial role against stomach cancer. Several studies show an inverse relationship between colorectal cancer and fruit and vegetable intake. A study conducted by National Cancer institute on 2000 men and women for a 4-year follow-up period showed that carotenes have a role in the prevention of colorectal cancer.

Several dietary antioxidants such as vitamin C, vitamin E and β-carotene are protective against development of breast cancer (Mckeown, 1999). A study on 678 women with breast cancer indicated the association of the disease with higher intake of saturated fats and lower intake of β-carotene (Jain *et al*., 1994), indicating the protective role of β-carotene against breast cancer.

Studies on carotenoids and prostrate cancer have shown mixed results. One study by Giovannucci *et al*. (1995) showed that intake of fresh tomato and tomato-based products such as tomato sauce was associated with lower risk for prostrate cancer. Limited data available on the relationship between β-carotene and skin cancer, has suggested that supplementation with 50 mg of β-carotene per day for 5 years did not reduce the occurrence of skin cancer in persons with non–melanoma skin cancer (Greenberg *et al.*,1990).

3.6. *β-Carotene and erythropoietic protoporphyria*

β-Carotene has been successfully used to treat erythropoietic protoporphyria (EPP) for over 25 years. EPP is a common genetic disease which is thought to be inherited as an autosomal dominant trait with partial penetrance, but might possibly be an autosomal recessive disease. An intake of 180 mg of β-carotene/day is recommended for patients with EPP; such higher doses of β-carotene are not associated with any serious side effects in these patients (Burri, 1997).

3.7. *β-Carotene and cystic fibrosis*

Several studies have shown a decreased concentration of β-carotene in cystic fibrosis, a genetic disease that produces lung infection and inflammation in children (Bui, 1988; Portal *et al*., 1995). Oxidative damage appears to be high in cystic fibrosis (Portal *et.al*., 1995; Lepage *et al*., 1996). A study conducted by Lepage *et al*. (1996) showed that an increase in β-carotene concentration with continuous ingestion of 13.3 mg β-carotene /day for 2 months decreases lipid peroxidation, suggesting that β-carotene has a beneficial role in cystic fibrosis by decreasing lipid peroxidation through its antioxidant property.

3.8. *β-Carotene and immune response*

β-Carotene can- (a) protect phagocytic cells from auto oxidative damage, (b) enhance T- and B- lymphocyte proliferative responses, (c) stimulate effectors T-cell functions, (d) enhance macrophages, cytotoxic T-cells, and tumoricidal capacity of natural killer cells, and (e) increase the production of certain interleukins. Since vitamin A is a relatively poor antioxidant and cannot quench singlet oxygen, β-carotene may have more importance as an antioxidant than simply serving as a precursor of vitamin A (Bendich, 1989) (Table-A). β-Carotene stimulates human immune response, which includes cytokine release, increase natural killer cells and activated lymphocytes (Prabhala *et al*., 1991). Alexander *et al*. (1985) found an increase in T-cells, helper T-cells and cytotoxic T-cells in individuals who were given 180 μg of β-carotene/day. An *in vitro* study showed that β-carotene can increase helper T-cells and the percentage of natural killer cells (Prabhala *et al*., 1991). Increase in natural killer cells is a part of immune system that fights against infection, especially of viruses, and combats tumors, thus offering protection against cancer (Schlipalius, 1997). Rhodes and Stokes (1982) showed an increased production of interferon by T- lymphocytes with increased intake of carotenes. Carotenes can stimulate certain lymphocytes, which assist in fighting infections including HIV (Schlipalius, 1997).

4. Bioavailability of carotenoids

Bioaccessibility is the amount of β-carotene that is released from the food matrix and available for absorption. Bioavailability is defined as the fraction of carotenoid that is absorbed and available for utilization in normal physiological functions or for storage. Bioconversion is the proportion of absorbed carotene that is converted to retinol. Thus, a bioconversion rate of 100% means that all of the absorbed β-carotene is converted to retinal and then reduced to retinol. Bio-efficacy combines absorption and bioconversion and has been defined as the efficiency with which ingested dietary provitamin A carotenoids are absorbed and converted to active retinol. A bio-efficacy of 100% means that 1 μmol of dietary β-carotene results in 2 μmol of retinol (van Lieshout *et al*., 2001; Tanumihardjo, 2002) (Fig.C).

Table-A. Effect of carotenoids on immune function

Carotenoid	Effect
β-carotene	Prevented stress- and radiation- induced thymus involution and lymphopenia
β-carotene	Increased graft vs. host response
β-carotene + Bixin	Enhanced regression of virus- induced tumors
β-carotene	Increased helper T- lymphocytes (human)
β-carotene + Canthaxanthin	Enhanced T and B cell proliferation
β-carotene + Canthaxanthin + Astaxanthin	Increased cytotoxic macrophage and T cell activities in tumor models
β-carotene + Canthaxanthin	Maintained macrophage receptors for antigen
β-carotene + α-carotene	Increased natural killer cell lysis of tumor cells

(Source: Bendich, 1989)

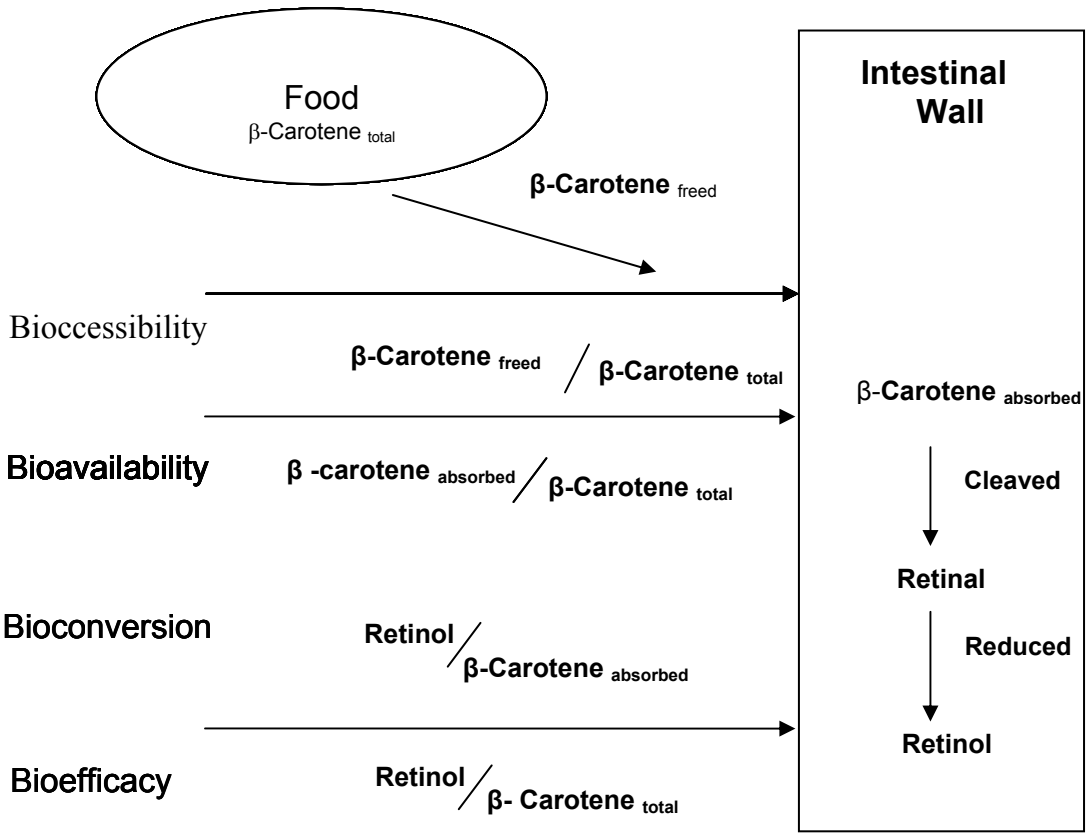


Fig.C. Interrelationship between bioaccessibility, bioavailability, bioconversion and bioefficacy (Tanumihardjo, 2002).

4.1. *Factors affecting the bioavailability of carotenoids*

In 1996, De Pee and West coined the term SLAMENGHI as a mnemonic to represent potential factors that may affect carotenoid bioavailability (De Pee and West, 1996). Although not all factors are well studied, the following have been identified as having significant effects on the bioavailability of carotenoids (Castenmiller and West, 1998).

- Species of carotenoids: *cis / trans* configuration.
- Linkage of alkyl groups: Esterification
- Amount in the meal
- Matrix properties of the plant
- Effectors of absorption and bioconversion
  - -Enhancement: protein, lecithin, fat
  - -Impairment: certain drugs, fibre, alcohol
- Nutrient status: Especially Vitamin A, zinc, protein
- Genetic factors
- Host related factors
- Interaction between factors

The bioavailability of carotenoids from food sources is quite variable, because the release from the food matrix, lipid micelle formation, uptake of carotenoids by intestinal mucosal cells, and transport of carotenoids and their metabolic products are all affected by a complex set of factors, as detailed below (Yeum and Russell, 2002):

Chemical speciation or carotenoid type: Absorption of dietary provitamin A carotenoids is influenced by numerous factors in addition to the amount ingested. The physiochemical properties of the carotenoid are of interest. The bioavailability of hydrocarbon carotenoids such as β-carotene is relatively lower than that of oxygenated carotenoids such as lutein and zeaxanthin. Owing to their more polar nature, oxygenated carotenoids can more easily be incorporated into the outer portions of lipid micelles within the gastrointestinal tract and therefore can more easily be taken up by enterocyte

membranes and eventually chylomicrons, hence increasing their bioavailability. This is supported by the work of van Het-Hof *et al*. (1999), who showed that the absorbability of lutein from vegetables was five times higher than that of β-carotene. Likewise lutein was absorbed more efficiently than β-carotene when the carotenoids were administered in oil to human subjects (Kostic *et al*., 1995; Castenmiller *et al*., 1999; van Het-Hof *et al*., 1999).

Food matrix: In nature, carotenoids in a wide variety of plants, animals and microorganism are found complexed with protein. Therefore release from the food matrix is an important initial step in the absorption process. It has been suggested that carotenoproteins have an inhibitory effect upon carotenoid digestion and absorption (Dietz *et al*., 1988; Bryant *et al*., 1992). Several investigators have found that pure β-carotene dissolved in oil or aqueous dispersion is efficiently absorbed (>50%), whereas carotenoids in uncooked vegetables such as β-carotene in the carrot (Rodriguez and Irwin, 1972) or lycopene in tomato juice (Stahl and Sies, 1992) are poorly absorbed (<3%). The bioavailability of β-carotene has also been reported to be influenced by the food matrix with absorption from carrots being higher than that from broccoli and spinach (Micozzi *et al*., 1992; Castenmiller *et al*., 1999).

Food processing: Moderate cooking, mashing and juicing increase carotenoid bioavailability (van Het-Hof *et al*., 1998; Edward *et al*., 2002; Livny *et al*., 2003). Such processes destroy the plant matrix, thereby increasing surface area and interactions of hydrolytic enzymes and emulsifiers with food particles during the gastric and small intestinal phases of digestion. The effect of food processing on carotenoid bioavailability can be illustrated by comparing the blood responses after eating a raw food compared with food that has been heat treated and / or mechanically homogenized to disrupt the food matrix (Britton, 1995). Castenmiller and colleagues (1999) examined serum carotenoid responses after continuous consumption (3 weeks) of various spinach products such as whole leaf spinach with intact food matrix, minced spinach with the matrix partially distrupted, enzymatically liquefied spinach in which the matrix was further disrupted and liquefied spinach to which dietary fibre was added. The bioavailability of

β-carotene from whole leaf, minced, enzymatically liquefied spinach and liquefied spinach plus added dietary fibre was 5.1, 6.4, 9.5 and 9.3%. The enzymatic disruption of the matrix enhanced the bioavailability of β-carotene.

In a 4-week cross–over feeding study (Rock *et al*., 1998) the plasma response of β-carotene was three times greater after feeding spinach and carrots that were pureed and thermally processed than when these vegetables were consumed raw. Dietz and colleagues (1988) reported an increase in extractability of carotenoids in carrots by steaming. However, more prolonged exposure to high temperatures (boiling) reduced the bioavailability of carotenoids by increasing the oxidative destruction and production of more isomeric forms that are less absorbable.

Isomeric forms: Several different geometric isomers of β-carotene (all-*trans*, 9-cis, 13-*cis* and 15-*cis* isomeric forms) exist in food and human tissues. The major β- carotene isomer in the circulation of humans is all-*trans*- β-carotene, with a small amount of 13-*cis* and 9-*cis*-β-carotene. However, circulating levels of the *cis* isomers of β-carotene are not responsive to increased consumption of these *cis* isomers. Data examining the serum response to a single large oral dose of either all-*trans*-β-carotene or 9-*cis*-β-carotene in men indicated that the all–*trans* isomer attains a far greater postprandial concentration than the *cis* form (Gaziano *et al*., 1995). In an attempt to determine if these serum response differences were due to differences in tissue uptake, the concentration of β-carotene isomers in human serum, breast milk and buccal mucosal cells after continuous oral doses of β-carotene isomers (all-*trans* and 9-*cis*) were examined in healthy lactating women (Johnson *et al*., 1997). The changes of all-*trans* and 9-*cis*-β-carotene in milk and buccal mucosal cells followed a pattern similar to that for serum, suggesting that the differences in the serum response of all-*trans*-β-carotene versus 9-*cis*-β-carotene reflect selective intestinal absorption of the all-*trans*-β-carotene or conversion of 9-*cis*-β-carotene to the all-*trans* form. In fact, the work of You *et al*. (1996) using stable isotopes indicates that substantial proportions of oral doses of 9-*cis*-β-carotene can undergo isomerization to all-*trans*-β-carotene between ingestion and appearance in plasma.

Dietary factors: a) *Fat:* Dietary fat increases carotenoid bioavailability by providing an ambience for hydrophobic compounds released from the food matrix, stimulating the secretion of bile salts and pancreatic lipases required for micelle formation, and inducing chylomicron synthesis (Borel, 2003). Dimitrov and colleagues (1988) showed that dietary fat increases the plasma response to β-carotene supplements, although the amount of fat required for optimal absorption of vegetable carotenoids may be quite small. Approximately 5 -10 g fat in a meal is required for efficient absorption of carotenoids (Reddy and Mohanram, 1980). The type of fat may also affect carotenoid absorption. For example, absorption of carotenoids by rats was more efficient when the carotenoids were administered in olive oil than in corn oil (Clark *et al*., 2000). Similarly, the presence of unsaturated fatty acids, particularly oleate, in micelles stimulated β-carotene absorption from perfused rat intestine (Hollander and Ruble, 1978). Hu *et al*. (2000) reported that the efficiency of carotene absorption by human subjects increased when the meal was rich in sunflower oil compared with beef tallow. Also, dietary triacylglycerol with long chain rather than medium chain fatty acids enhanced the absorption of β-carotene and retinyl palmitate (Borel *et al*., 1998b). Inhibitors of lipid absorption such as olestra (Cooper *et al*., 1997; Weststrate and van Het-Hof, 1995) and phytosterols (Richelle *et al*., 2004) decrease carotenoid bioavailability primarily by decreasing micellarization. The potential of phospholipids to affect carotenoid bioavailability is supported by the observation that lysophosphtidylcholine increases carotenoid absorption in mice (Baskaran *et al*., 2003).

b) *Fibre:* The water soluble fibre: pectin, guar gum and alginate decrease the absorption of carotene, lycopene and lutein (Riedl *et al*., 1999). Rock and Swendseid (1992) examined the effect of dietary fibre (12 g citrus pectin) on serum β-carotene response. The increase in plasma β-carotene concentration after ingestion of β-carotene in a capsule (25 mg) was significantly reduced by pectin (42% reduction). Possible mechanisms responsible for the fibre–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).

c) Interactions between carotenoids: Carotenoids in the same food or meal may influence the absorption of one another. Kostic *et al.* (1995) examined serum responses as area under the curve (AUC, i.e. area under the serum response curve) after single doses of β-carotene and lutein, both alone and after an equimolar mixture (each being 0.5 μmol/kg body weight). In combination, β-carotene significantly reduced the serum responses for lutein to 53-61% of control values, suggesting the interaction between these two carotenoids. The reduced absorption of lutein by β-carotene was supported by O'Neill and Thurnham (1998) who examined intestinal absorption of β-carotene, lutein and lycopene using the response curves in the triglyceride- rich- lipoprotein fraction after a single oral dose of 40 mg of β-carotene taken with either 31.2 mg of lutein or 38 mg of lycopene. The estimated absorptions (determined by AUC) were similar for β-carotene and lycopene but were significantly lower for lutein.

In another study, lutein impaired β-carotene absorption in human subjects, but did not affect the secretion of retinyl esters in chylomicrons (van den Berg and van Vliet, 1998); in contrast, β-carotene absorption was not affected by lycopene in these subjects. Tyssandier *et al*. (2003) reported that the absorption of β-carotene, lutein and lycopene from a single vegetable was greater when the food was administered alone than when it was co-administered with either a second carotenoid rich vegetable or the purified carotenoid from the second vegetable. Possible sites for pre-absorptive interactions between carotenoids include their competition for incorporation into micelles in the lumen, uptake from the micelle by intestinal cells, competitive binding to β-carotene 15,15'-monooxygenase (BCO1) and incorporation into chylomicrons (van den Berg, 1999).

β-Carotene also appears to reduce the absorption of canthaxanthin (Paetau *et al*., 1997). Combined doses of β-carotene and canthaxanthin (25 mg each) resulted in plasma canthaxanthin responses, which are significantly lower than canthaxanthin alone; on the

other hand, canthaxanthin did not inhibit the appearance of β-carotene in plasma. Yeum *et al*. (1996) showed high serum concentrations of α-carotene and cryptoxanthin, but low serum responses for lutein relative to the dietary intake, which indicates selective absorption of carotenoids. Carotenoids can interact with each other at any point during the absorption, metabolism and transport process in the intestinal mucosa. Carotenoids may also inhibit or enhance the activity of carotenoid cleavage enzymes. In circulation there may be an exchange of carotenoids among plasma lipoproteins, which could be affected by the type and amount of carotenoid present. There may also be inhibition or enhancement of tissue uptake and / or release of one carotenoid by another.

Physiological and pathophysiological factors: *a) Gut health:* The absorption of dietary carotenoids and their bioactive products is also modulated by phenotypic characteristics of the host that affect processes associated with digestive and absorptive events. These include the composition and activity of luminal fluids and the morphological and functional integrity of the absorptive epithelium. For example, the plasma response to a single dose of β-carotene was significantly lower in subjects administered omeprazole to increase gastric pH to the neutral range compared with the same subjects when gastric pH was acidic (Tang *et al*., 1996). In addition, cholestasis, pancreatic insufficiency, biliary cirrhosis, cystic fibrosis, and other syndromes responsible for fat malabsorption decrease carotenoid bioavailability and can induce vitamin A deficiency, especially in children (Olson, 1999; 1999a).

Intestinal parasites can impair carotenoid absorption or utilization. Metabolism of carotenoids by parasites residing in the intestinal lumen, parasite associated changes in the numbers and maturation of absorptive cells along the villi, and cytokine mediated decreases in lipid absorption associated with parasite infection may all contribute to a decline in carotenoid absorption. Absorption and utilization of β-carotene were enhanced after deworming children infected with ascaris (Jalal, 1998). In contrast, plasma retinol concentration in helminth-infected preschool children in Ghana fed a stew with dark Green cassava and kapok supplemented with fat and carotene were not further elevated by administration of anti-helminthics (Takyi, 1999).

b) Nutritional status: Nutritional status can affect the bioavailability of provitamin A carotenoids. The plasma vitamin A response curve following the administration of carotene to protein deficient rats was decreased compared with rats fed a protein sufficient diet (Parvin and Sivakumar, 2000). This suppression was due to decline in the activity of BCO1. Because of the central role of retinoic acid in cellular differentiation, vitamin A deficiency compromises with the integrity of epithelial barriers. Mild vitamin A deficiency reduced the number of duodenal goblet cells per villus and luminal mucous, and decreased cellular division in the crypts of intestinal villi (McCullough *et al*., 1999). Gastrointestinal integrity, assessed by the dual-sugar gastrointestinal permeability test, was markedly improved when vitamin A-deficient children in Gambia and India ingested β-carotene-rich mango and received vitamin A supplementation, respectively (Thurnham *et al.*, 2000). A decreased uptake of micellarized β-carotene by brush border membrane vesicles isolated from vitamin A deficient Mongolian gerbils and rats was observed as compared to those from animals fed vitamin A adequate diets (Moore *et al*., 1996; Boileau *et al*., 2000).

c) Genotype: Recent studies using tracer isotope techniques have confirmed earlier observations of a marked variability in the absorption of β-carotene by human subjects (Lin *et al*., 2000; Hickenbottom *et al*., 2002). Moreover, just plasma β-carotene and vitamin A were not predictive for the absorption or conversion of β-carotene. These differences in absorption efficiency originally resulted in the classification of individuals as 'responders', 'low responders', and 'non- responders'. Explanations for the observed variation among healthy subjects tested under well controlled conditions have included differences in the rate of cleavage of β-carotene to retinal, the efficiency of incorporation of the carotenoid into chylomicrons, and the rate and extent of clearance from circulation (Borel, 2003). Lin *et al*. (2000) also suggested that differences in the ability to transfer the carotenoid from a complex matrix to the absorptive cell may be the basis for the reported variability, because all individuals were 'responders' when administered high doses of β-carotene in oil (Borel *et al*., 1998a). Genetic factors are also likely to affect the efficiency of carotenoid absorption and conversion. Polymorphism in genes whose

products are required for the various reactions affecting the transfer of carotenoids from food matrix to micelles during digestion, assembly and secretion of chylomicrons, and the kinetics of post-absorptive delivery of carotenoids and retinoids to tissues may all contribute to the observed variations in the absorption and conversion efficiency of provitamin A carotenoids in individuals. However, a lack of knowledge about the characteristics and regulation of carotenoid transport and metabolism precludes consideration of specific polymorphisms at this time. Also, variability might exist within an individual over a period of time and lifestyle factors may also affect carotenoid absorption.

d) Aging: The greatest change in gastrointestinal physiology affecting nutrient bioavailability that has been identified with advancing age is atrophic gastritis, which occurs in a large percentage (20%) of the elderly population and results in reduced stomach acidity. Atrophic gastritis appears to affect the bioavailability of carotenoids, the absorption of which is pH dependent (Russell, 2001), because the pH in the proximal intestinal lumen can affect the surface charges of both the micellar particles and the luminal cell membrane, with less diffusion resistance at a lower pH. Tang and colleagues (1996) reported that a decrease in gastric acidity decreases the blood response to β-carotene, thereby implicating a negative effect of atrophic gastritis on β-carotene absorption.

e) Alcohol consumption: Subjects with alcoholic liver diseases have low concentrations of plasma β-carotene. Yet, among heavy drinkers who have normal liver enzyme levels, β-carotene concentrations are positively correlated with amount of alcohol consumed (Ahmed *et al*., 1994). Similarly, baboons who received 50% of energy as alcohol had higher levels of β-carotene in response to eating a carrot daily than did controls who received no alcohol (Leo *et al*., 1992). These observations seem counter-intuitive because alcohol is a pro-oxidant. However, the explanation appears to be that alcohol inhibits the conversion of β-carotene to vitamin A, as evidenced by delayed clearance of β-carotene and relative low concentrations of plasma vitamin A, even though

β-carotene concentrations in plasma and liver were elevated. Information is scarce concerning the impact of alcohol at moderate intake levels on circulating carotenoid concentrations. However, data from an intervention study suggest that the positive association of plasma carotenoid concentrations with alcohol consumption is not limited to those who are heavy consumers of alcohol. In another study, in women who consumed alcohol (equivalent of two drinks per day for 3 months), concentrations of circulating α- and β-carotene increased by 19 and 13% respectively; in contrast, concentrations of lutein / zeaxanthin were lowered by 17% (Forman *et al*., 1995).

5. Digestion and absorption of carotenoids

Carotenoids are lipid soluble and follow the same absorptive pathways as other dietary lipids (Onstad and Zeive, 1972). Carotenoids do not undergo any digestive hydrolysis; nevertheless, they have to be released from the associated proteins in the food matrix (Erdman *et al*., 1993). Therefore, release of carotenoids from the food matrix and dissolutions in the lipid phase are critical steps in the absorption process. The amount of carotenoids incorporated into micelles depends on the polarity of the carotenoid and on the micellar fatty acid composition and saturation. Poor *et al*. (1993) observed that physically altering food by cooking, blending, and finely chopping improves the release of some carotenoids such as α- and β-carotene from the food matrix. Caray and Hernell (1992) found that once the food is ingested, its mechanical breakdown continues as it is chewed, swallowed, and mixed in the stomach. Gastric hydrolysis of dietary lipids and proteins results in partial release of carotenoids and lipids from the food matrix. Once they are released, however, the lipophilic carotenoids would dissolve in the oily phase of lipid droplets.

With mixing, the lipid droplets in the gastric contents become emulsified particles. Shearing forces from normal digestive tract motility bring about the formation of a fine lipid emulsion as the contents of the stomach pass into the duodenum. Tso (1994) observed that emulsion has a triacylglycerol core surrounded by a monomolecular layer of partially digested proteins, polysaccharides and lipids especially phospholipids and

partially ionized fatty acids. The solubility and location of the polar carotenoids (xanthophylls) and the non polar carotenoids (carotene) in emulsion differ.

Absorption of carotenoids involves several steps from the breakdown of the food matrix and release of carotenoids into the lumen of the gastrointestinal tract to their incorporation into lymphatic lipoproteins. These include mechanical and chemical disruption of the food matrix, dispersion in lipid emulsion particles, solubilization into mixed bile salt micelles, movement across the unstirred water layer adjacent to the micro villi, uptake by the enterocytes and incorporation into lymphatic lipoproteins namely chylomicrons. Carotenoids are absorbed by the mucosa of the small intestine (mainly in duodenum) via passive diffusion to become packaged in to triacylglycerol-rich-chylomicrons (Parker, 1996). Carotenes are believed to be incorporated almost exclusively in the triacylglycerol core of the emulsion, whereas the more polar xanthophylls distribute preferentially at the emulsion surface (Borel *et al*., 1996). The significance of location in an emulsion is that the surface components can spontaneously translocate from the lipid droplets to mixed micelles, whereas components associated with the emulsion core require digestion of triacylglycerol before transfer.

The product of lipid digestion and minor dietary lipids including the carotenoids transfer from the emulsion particle to mixed bile salt micelles. The solubility of carotenoids in mixed micelles is limited and varies with intra- luminal concentration of the carotenoid. A major difference between absorption of other dietary lipids and carotenoids is that the carotenoids seem to have an absolute requirement for bile salt micelles (El-Gorab and Underwood, 1973) whereas fatty acids, the major product of lipid digestion, can be absorbed in the absence of micelles (Carey and Hernell, 1992).

Canfield *et al*. (1990) studied the incorporation of β-carotene into mixed micelles designed to resemble those seen in the lumen of the small intestine. The incorporation of β-carotene into the micelles varied from approx. 4 - 13% with the percent incorporated decreasing with increasing initial concentration of carotenoid. Whereas the solubility of carotenoids differs in emulsions, the polar and nonpolar carotenoids have similar

solubility in bile salt micelles. Depending on their polarity, carotenoids may solubilize independently into different regions of the bile salt micelle.

El-Gorab and Underwood (1973) studied the solubility of β-carotene (nonpolar) and retinol (polar) into solutions containing bile salt micelles. β-Carotene was solubilized in the hydrophobic core of the micelle, whereas retinol was incorporated into the surface. Rather than competing for solubilization into bile salt micelles, retinol expanded the micelle and enhanced β-carotene solubilization into the internal core of the micelle. The steps involved in carotenoid transfer from mixed micelles to the enterocytes are not completely understood. Sugawara *et al*. (2001) observed that an important step for the absorption of carotenoids and other lipophilic compounds is the cleavage of phospholipids by phospholipase A2 (PLA2). Baskaran *et al*. (2003) found that although the cleavage of phospholipids is not a prerequisite for the formation of mixed micelles, the presence of phosphatidylcholine (PC) in the mixed micelles inhibits carotenoid absorption in rats. Sugawara *et al*. (2001) reported that the action of PLA2 can restore the absorption of lipophilic compounds that were inhibited by PC indicating the importance of PLA2 in the absorption of carotenoids, along with other lipophilic compounds such as cholesterol, vitamin A and vitamin E.

Hollander and Ruble (1978) observed that the uptake of carotenoids by the enterocytes has been thought to occur by simple diffusion, similar to many other dietary lipids. A simple diffusion mechanism was indicated by linear responses to increasing carotenoid concentrations in perfused rat intestines and in rat small intestinal cells. Additionally, only a small inhibition of ^{14}C-labeled carotenoid uptake was observed when those cells were incubated at 48˚C (compared to their incubation at 37˚C), or when an excess of unlabeled carotenoid was added to the medium (Scita *et al*., 1992). According to the simple diffusion mechanism, the micelles migrate through the unstirred water layer to the brush border membrane, the carotenoid then leaves the micellar structure and diffuses through the membrane into the cytoplasm of enterocytes. Cell membranes are basically formed by lipid bilayers, thus in the absence of a specific transporter, lipophilic substances can diffuse more easily through the membrane than the hydrophilic ones.

Gastric secretions and their effect on the pH of the upper small intestine may influence carotenoid absorption. In humans, the acid environment of the stomach facilitates the absorption of β-carotene; however if intestinal pH becomes too low (pH<4.5) the solubilization of carotenoids into bile salt micelles markedly decreases, thus decreasing carotenoid absorption. It seems that β-carotene is absorbed best under slightly acidic conditions.

The mixed micelles are thought to penetrate the unstirred water layer, a series of water lamellae adjacent to the microvillus surface. The unstirred water layer and the microvillus membrane form two barriers through which the carotenoids must pass. The bile salt micelle serves as a reservoir for carotenoids and other lipids, which then move across the unstirred water layer as monomers down a concentration gradient to the brush border membrane (Westergaard and Dietschy, 1976).

The products of lipid digestion provide an intact hydrophobic domain where minor dietary lipids, such as fat soluble vitamins and carotenoids are dissolved and flow from the mixed bile salt micelles to the micro villi membrane. The concept of an uninterrupted hydrophobic domain dispenses with the problem of the unstirred water layer and would have significant implications for carotenoid absorption (Kuksis, 1986). Whereas the mechanism of carotenoid transfer from the bile salt micelle through the micro - villi membrane is unclear, there is general agreement that rate of transfer is dependent on intra micellar concentration of carotenoids and that the carotenoids are taken up intact by the mucosal cells.

There are evidences that bile salts have a more extensive role in carotenoid absorption than just solubilization. In a rat everted gut sac study, El-Gorab *et al*. (1975) reported that β-carotene was absorbed from micellar solutions made with bile salt mixtures, whereas when β-carotene was solubilized in micellar solutions made with non-ionic detergents (Tween-20), there was only a limited uptake by the intestines. In a similar study with slices of intestinal tissues by Olson (1964), β-carotene dispersed in a micellar solution

with a non-ionic detergent was not absorbed and converted to retinyl ester by the intestine unless bile or conjugated bile salts were present. The authors of both these studies suggest that bile salts not only serve to solubilize carotenoids in the small intestine, but may also be required for interaction with and transport through the brush-border membrane.

Provitamin A carotenoids such as β-carotene, α-carotene and cryptoxanthin, are partly converted to vitamin A, primarily retinyl esters, and incorporated into chylomicrons which are secreted into lymph for delivery to the blood stream, where they are rapidly degraded by lipoprotein lipase. The resulting chylomicron remnants containing carotenoids are rapidly taken up by the liver (Parker, 1996).

Carotenoids are secreted into blood from the liver in association with very low density lipoproteins (VLDL), while in the fasting state most plasma carotenoids are associated with low density lipoproteins (LDL) and high density lipoproteins (HDL). Chylomicron carotenoid levels peak early (4-8 h) after ingestion of carotenoids owing to intestinal excretion, whereas LDL carotenoid levels in the circulation peak at 24-48 h, and HDL levels peak at 16-48 h (Cornwell *et al*., 1962). Erdman *et al*. (1993) reported that in the fasting state, up to 75% of hydrocarbon carotenoids such as β-carotene and lycopene are found in LDL, and the remaining carotenoids are associated with HDL and to a lesser degree, with VLDL. The more polar carotenoids such as lutein and zeaxanthin are evenly distributed between LDL and HDL fractions in fasting blood. Lipophilic carotenoids are mainly located in the core of lipoprotein, which may not allow their transfer between lipoproteins at an appreciable rate, whereas the more polar carotenoids, which are mainly present on the surface of lipoproteins, are likely to undergo rapid surface transfer, resulting in an equilibration between LDL and HDL.

Non-provitamin A carotenoids (e.g., lutein, zeaxanthin, lycopene) are also absorbed intact, although oxidative cleavage of these could occur to some extent before absorption from the intestinal lumen. Carotenoid concentrations vary substantially from tissue to

tissue varying in their LDL receptors; tissues such as liver and adipose that have large number of LDL receptors probably accumulate carotenoids passively. However, the variable concentrations and forms of carotenoids in different tissues suggest that other factors play a role in uptake of carotenoids by tissues. For example, the macular pigments of the eye are primarily lutein and zeaxanthin, suggesting the presence of a binding protein (Bone *et al*., 1993; 1997; 2000).

Recent studies reported the existence of receptor-mediated transport of β-carotene and lutein in the apical membrane of enterocytes, with strong indications for the involvement of the scavenger receptor class-B type-I (SR-BI) in this transport (Reboul *et al*., 2005; van Bennekum *et al*., 2005; During *et al*., 2005) (Fig.D). Rigotii *et al*. (2003) and Yancey *et al*. (2003) found that SR-BI, a member of the ATP-binding cassette (ABC) transporter super-family. The first evidence that SR-BI was important for carotenoid transport was observed in Drosophila, whose gene encoding a SR-BI-homologous protein was essential for the cellular uptake of carotenoids in this species. Kiefer *et al*. (2002) reported that the absorption of dietary β-carotene and α-tocopherol by mice and the uptake and transport of β-carotene and lutein by Caco-2 cells seem to be at least partly mediated by SR-BI. In contrast to most of the protein-mediated transport, the carotenoid absorption via SR-BI seems to occur without energy expenditure. Yancey *et al.* (2003) observed that the hairpin-like conformation of SR-BI external domain forms a hydrophobic channel that may facilitate a bidirectional flux of lipophilic substances, and similar to the simple diffusion mechanism, the net flux via SR-BI will depend on the direction of the concentration gradient. Thus, efficient solubilization of carotenoids into mixed micelles and their release to the aqueous phase would favour carotenoid uptake by both simple diffusion and SR-BI mediated transport. At apical concentrations of carotenoids above the level that would be reached by a nutritional dose, the uptake of carotenoids by Caco-2 cells does not respond linearly to the initial concentration at the apical side (During *et al*., 2002).

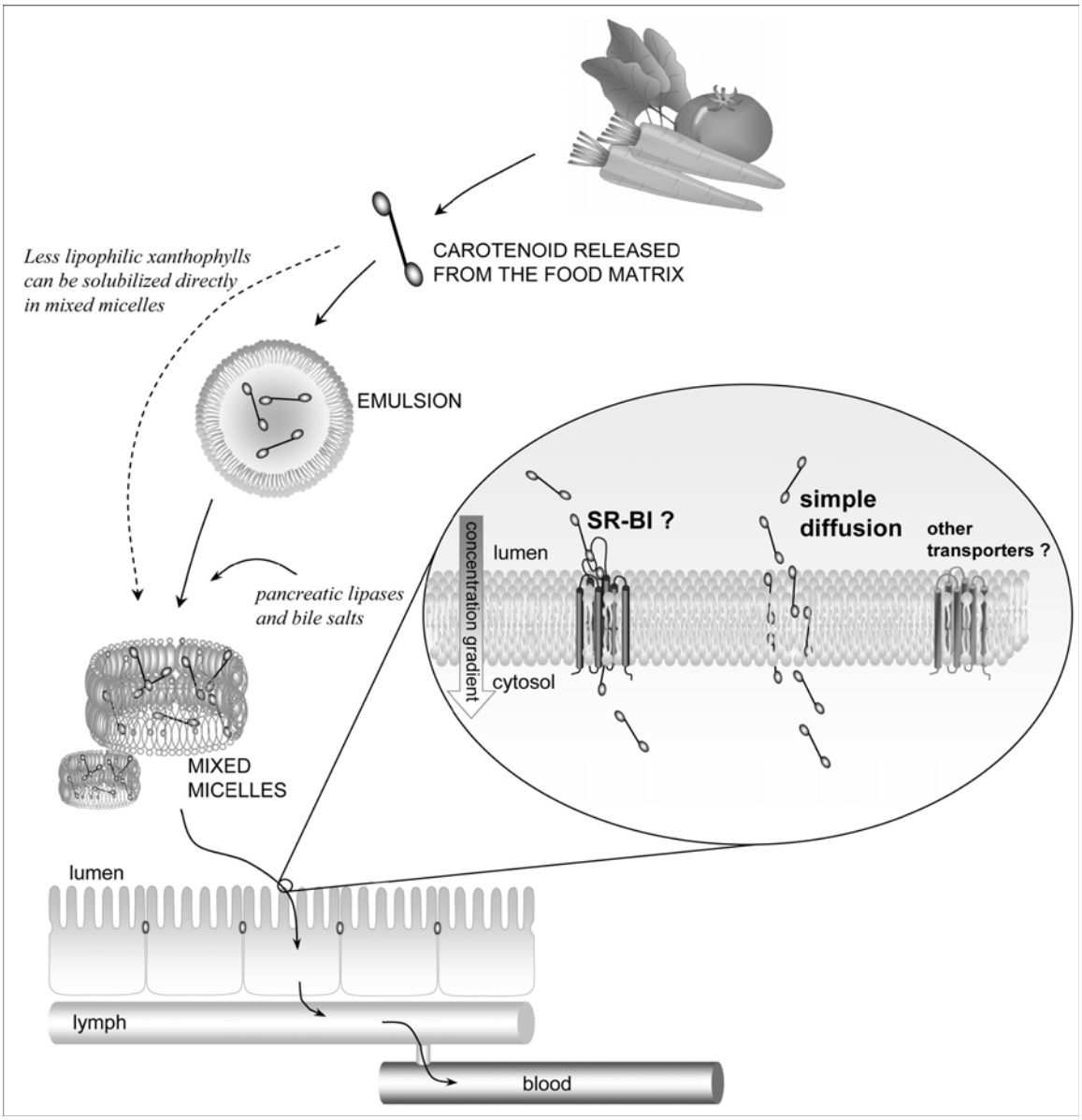


Fig.D. Scheme of dietary carotenoid absorption (Nagao and Yonekura, 2007)

6. Metabolism of carotenoids

Absorbed β-carotene and presumably the other provitamin A carotenoids undergo oxidative cleavage in intestine as well as in other organs such as liver. Based on the identification of several oxidative products of carotenoids in *in vitro* studies involving tissue homogenates / postmitochondrial fractions from animals or humans, and by identifying the carotenoid cleaving enzymes at the molecular level, various mechanisms for breakdown of carotenoids have been suggested (Fig.E).

The central cleavage mechanism splits β-carotene at the central double bond (15,15') by a specific enzyme β-carotene-15,15'-dioxygenase yielding retinal in intestinal cells and liver cytosol. The cleavage product retinal can be reversibly reduced to retinol (vitamin A) or irreversibly oxidized to retinoic acid (Olson and Lakshmanan, 1990).

Several *in vitro* and an *in vivo* studies have shown almost exclusive central cleavage in the intestines of guinea pig, pig, rat and hamster. Therefore, central enzymatic cleavage of β-carotene is an essential step in the formation of vitamin A in vertebrates. An additional random cleavage process for carotenoids was first proposed by Glover and Redfearn (1954), who observed cleavage at several double bonds in the polyene chain of β-carotene in addition to the central 15,15'-double bonds to produce β-apo-8'- carotenals, which can be subsequently converted to retinal. Random cleavage of β-carotene was supported by the identification of β-apo- carotenoids in the intestine of chickens after β -carotene supplementation. Furthermore, Wang *et al*. (1991) demonstrated the formation of β-apo-carotenals from *in vitro* incubations of β-carotene with the post nuclear fraction of intestinal tissues from humans, monkeys, ferrets and rats, as well as from *in vivo* studies using ferrets. Kiefer and colleagues (2001) identified an enzyme that exclusively catalyzes the asymmetric oxidative cleavage of β-carotene at the 9',10'-double bond of β-carotene, resulting in the formation of β-apo-10'-carotenal and β-ionone. Dmitrovskii and colleagues (1997) also reported an enzyme, which is different from β-carotene- 15,15'-dioxygenase, involved in the enzymatic oxidation of β–apo-8'-carotenal to β-apo-14'-carotenal.

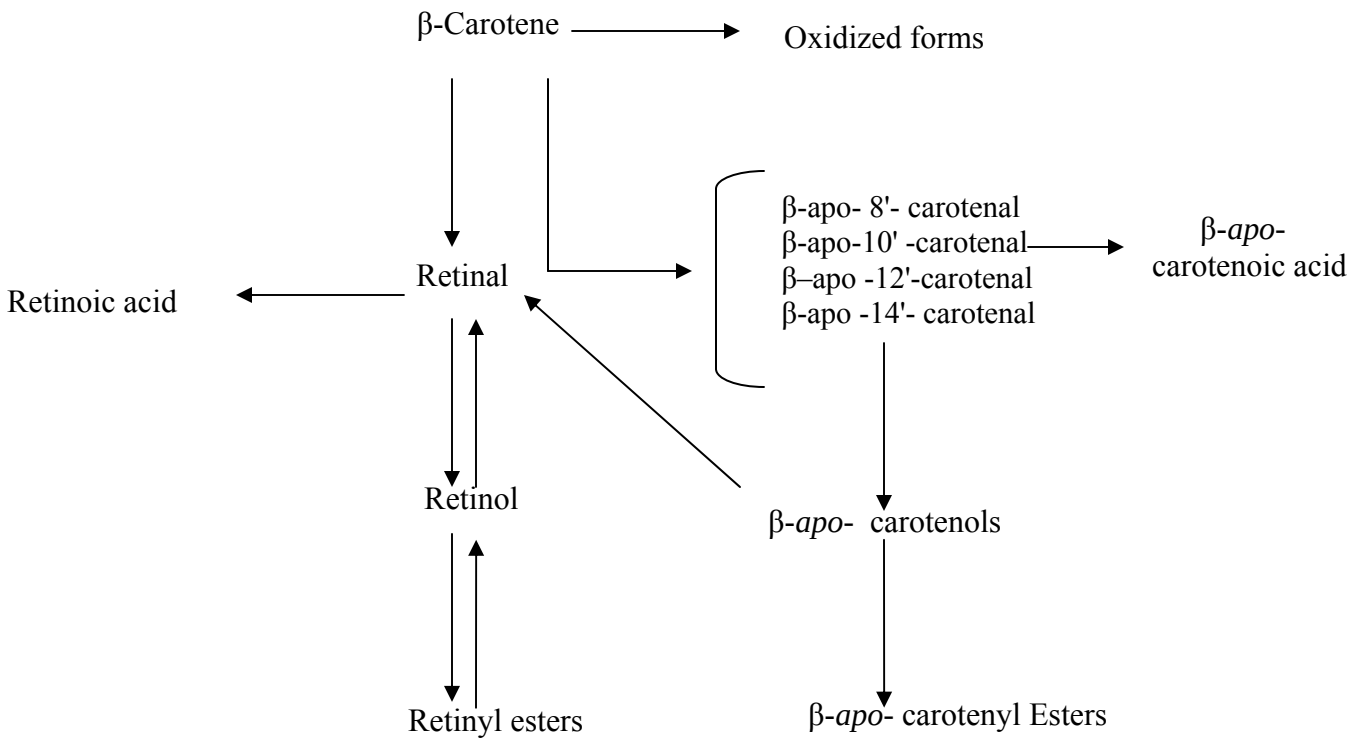


Fig.E. Possible mechanism of transformation of β-carotene in mammals (Rock, 1997)

Carotenoids also can be broken down by free radicals produced by enzymes such as lipoxygenase. Gessler and colleagues (2001) reported that free radical oxidation of arachidonic acid with lipoxygenase inhibited the central cleavage of β-carotene. Moreover, a lipoxygenase inhibitor and antioxidants promoted conversion of β-carotene to retinal. Yeum and colleagues (2000) demonstrated that both central and random cleavage of β-carotene can take place in the post–mitochondrial fraction of rat intestine, depending on the presence or absence of the antioxidant α-tocopherol. In their work on the presence of α-tocopherol central cleavage mainly occurred (i.e., β-carotene was converted to retinal), whereas in the absence of α-tocopherol both random cleavage and central cleavage took place (i.e. both retinal and β-apo-carotenals were produced). Gomboeva and colleague (1998) reported the activity of β-carotene- 15,15'-dioxygenase to be decreased in the presence of oxidant but protected by antioxidants, thus providing more evidence for an important role of antioxidants in promoting central cleavage of β -carotene.

Thus, it appears that β-apo-carotenals with different carbon chain lengths can be produced by enzymatic reactions, cooxidation by lipoxygenase, autooxidation or direct reaction with free radicals. Considering that the stoichiometry of retinal production per mole of β-carotene is 1.72 - 2.00 mol and that the total amount of the β-apo-carotenoid is <5% of the retinoids formed in the intestine from β-carotene, it is certain that the enzymatic central cleavage of β-carotene plays a major role in β-carotene breakdown under normal conditions when an adequate supply of antioxidants is available. However, under conditions of oxidative stress or when high β-carotene concentrations are present, both enzymes-related and radical induced random cleavage can play a role in β-carotene breakdown.

7. Methods to determine bioavailability of carotenoids

In vivo and *in vitro* approaches are used to determine and predict, respectively, the relative bioavailability of provitamin A carotenoids from complex food matrices. These methods include:

In vivo methods:

i) Balance techniques: (a) Metabolic mass balance, (b) Ileostomy mass balance, (c) Gastrointestinal lavage

ii) Plasma response techniques: (a) Changes in carotenoid concentration in plasma, (b) Appearance-disappearance of carotenoids in plasma triglyceride-rich-fraction after dosing, (c) Isotopic methods

iii) Sampling from gastrointestinal lumen after ingestion

iv) Intestinal perfusion techniques

In vitro methods:

i) Simulation of gastric and small intestinal phases of digestion

ii) Uptake by isolated intestinal segments

iii) Uptake and metabolism by Caco-2 human intestinal cell line

iv) Coupled in vitro digestion / Caco-2 cell model

(Failla and Chitchumroonchokchai, 2005)

7.1. *Bioavailability studies involving human models*

Carefully controlled investigations using human subjects are necessary for accurate determination of the relative bioavailability and conversion of provitamin A carotenoids. Balance studies and plasma response curves have been used to estimate relative bioavailability, whereas functional improvement in vitamin A status (e.g., restoration of night vision) has been used to assess bioefficacy of intervention programs in vitamin A deficient populations (Christian *et al.*, 2000; Congdon and West, 2002). Sampling of gastrointestinal contents during the digestive process provides insights into the stability of carotenoids and their transfer from matrix to oil droplets and micelles (Tyssandier *et al.*, 2002).

(a) *Balance studies*: Metabolic balance studies represent a traditional method for estimating the absorption and excretion of compounds that are not metabolized in the gastrointestinal tract. (i) Comparison of carotenoid consumption with its fecal excretion (i.e., balance) has been used for the estimation of absorption of carotenoids, particularly

from foods. Because elimination in feces represents the major excretory route for ingested carotenoids. It is assumed that absorption can be estimated by carefully monitoring intake and fecal output. A primary advantage of this approach is that it is noninvasive (Bowen *et al.,* 1993; van Lieshout *et al.*, 2003). This balance method has major limitations: It does not account for carotenoid degradation in the upper (chemical oxidation) or lower (microbial degradation or alteration) regions of the gastrointestinal tract or the excretion of endogenously secreted carotenoids. Thus the oral–fecal balance studies have yielded considerable variation in estimates of carotenoid `absorption, even with seemingly similar carotenoid sources or preparations. (ii) Bowen *et al.* (1993) modified the method by using gastrointestinal lavage (washout) after allowing a defined period for digestion and absorption. The advantage of this approach is that it controls the residence time of non absorbed carotenoids in the lower gut, thus limiting degradation by microflora. However, the duration of the allowed absorption period in this approach is arbitrary and it may alter gastrointestinal physiology. (iii) Livny *et al.* (2003) and Faulks *et al.* (1997) have used intestinal effluent from subjects with ileostomy to estimate carotenoid absorption.

The sample collections in these above models contain ingested carotenoids that were not transferred from the food matrix to absorptive cells, as well as carotenoids that were absorbed and subsequently returned to the lumen of the gastrointestinal tract with bile and pancreatic secretions, retro-transported across the apical surface of the mucosal epithelium, and retained within cells sloughed from intestinal and colonic villi. However, sample collection and extraction in these methods are labor intensive.

(b) *Plasma response techniques*: Bioavailability has also been estimated by monitoring changes in plasma concentration of carotenoids after feeding purified compounds or enriched test foods for a period of days or weeks. Plasma or serum carotenoid responses (concentration vs. time curves) have been widely used to measure carotenoid bioavailability, because this method provides an estimate of relative bioavailability using simple procedures. In this method known amounts of carotenoids

are ingested and changes in serum concentration of carotenoids are measured at various time intervals following ingestion. Comparisons of relative bioavailability can be made between different carotenoids, formulations (e.g., purified vs. food), food preparations (e.g., processed vs. unprocessed food) or individuals. Serum response curves are carried out using either single or multiple doses. A rise in serum concentration followed by a fall is generally measured. However, in chronic dose trials, serum carotenoid concentrations reach a constant elevated plateau level of various magnitudes.

Serum response curves to determine bioavailability is limited by several factors. (i) Serum response to a single oral dose of carotenoid is highly variable. (ii) Concentration of carotenoid in serum represents a balance between intestinal absorption, breakdown, tissue uptake, and release from body stores. (iii) This approach lacks sensitivity due to a relatively high level of endogenous carotenoids in plasma, fails to account for the cleavage of provitamin A carotenoids, and assumes that different carotenoids have similar rates of plasma clearance (van den Berg *et al*., 2000; Parker *et al.*, 1999).

For these reasons, relatively large doses, usually exceeding the typical daily intake by at least fivefold, are needed for a significant increase in carotenoid concentrations over baseline levels. However, it is probable that large doses overwhelm transport and metabolic processes, or at least alter rate constants of metabolism or transport, thereby making interpretation of results difficult.

Carotenoid concentrations in triglyceride-rich- lipoprotein (TRL) fractions (mixtures of chylomicron and VLDL) have also been used to estimate intra as well as inter-individual variability in β-carotene absorption and intestinal conversion to retinyl esters (van Vliet *et al.,* 1995). Advantages of this method over the serum response curve method are that (a) the method accounts for intestinal conversion to retinyl esters; (b) it improves the distinguishability of newly absorbed carotenoids from endogenous pools; (c) it allows for the use of smaller doses.

However, this method is not able to separate the liver derived VLDL from the intestine derived chylomicrons. The limitation of this approach is that food matrices that are slowly digested result in slow rates of carotenoid absorption and thus yields little or no rise of carotenoids in the TRL fraction. Like serum response curves, TRL response curves are highly variable. This may be due to variability in carotenoid absorption as well as in the kinetics of chylomicron secretion and clearance (van den Berg and van Vliet, 1998).

Administration of physiologic doses of purified carotenoids or plant foods that are intrinsically labeled with stable isotopes (^{2}H and ^{13}C) facilitates the study of *in vivo* absorption and metabolism of carotenoids. The development of stable isotope labeled carotenoids has made it possible to (i) distinguish between dosed and endogenous carotenoids, (ii) assess the extent of intestinal conversion of vitamin A, (iii) estimate absolute absorption and post absorptive metabolism for subsequent empirical or compartmental modeling and (iv) use of doses that are low enough to avoid influencing endogenous pools (Novotny *et al.*, 1995).

A stable isotope method which includes the isolation and quantification of all-*trans* octadeuterated β-carotene and tetradeuterated retinol derived from the former following an oral dose using reverse phase high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) with electron ionization (Dueker *et al.*, 1994). Oral doses of ^{13}C- labeled β-carotene as low as 0.5 mg have been used in another a stable isotope tracer method in a human study, using a high precision gas isotope ratio mass spectrometer (Parker *et al*., 1993). The use of accelerator mass spectrometry for examining the absorption and excretion of trace amounts of ^{14}C-β-carotene in human subjects has also been explored (Dueker *et al.*, 2002; Lemke *et al.*, 2003; Burri and Clifford, 2004). This powerful approach is yielding important new insights in to the absorption and whole body metabolism of dietary carotenoids. However, owing to extensive fraction purification required, these methods are labour intensive and expensive. Thus, instrumentation and labour intensiveness of stable isotope technology

preclude its consideration as a tool for screening the bioavailability of provitamin A carotenoids.

(c) *Aspirates from stomach and small intestine:* The collection and analysis of aspirates from stomach and small intestine of human subjects provides investigators with the ability to investigate the stability and partitioning of dietary compounds within the gastrointestinal lumen during digestion (Armand *et al*., 1996; Borel *et al.*, 2001). This method has been effectively used to examine the gastrointestinal processing of β-carotene, lutein, and lycopene from pureed vegetables but does not lend itself to the initial screening of multiple types of foods prepared in diverse manners.

7.2. *Bioavailability studies involving animal models*

The primary advantages of animal models for investigating nutritional problems relevant to humans include the ability to induce dietary deficiencies and excesses, administer radioisotopes, collect tissues of interest, and induce acute and chronic diseases (Erdman, 1999). The central issue concerns the selection of an animal that absorbs and metabolizes carotenoids in a manner comparable to human subjects have critically reviewed this subject. Carotenoid absorption, metabolism, and function have been investigated to varying degrees in mouse, rat, gerbil, ferret, pre-ruminant calf, non-human primates, birds and amphibians. Because mice and rats efficiently convert ingested provitamin A carotenoids to retinol in the intestine, they do not absorb intact carotenoids unless supra-physiologic doses are administered. Thus, these rodents are not particularly relevant to the human situation regarding the bioavailability of provitamin A carotenoids from foods. The rat however, is a preferred model for investigating the patho-physiological consequences of an inadequate supply of this essential nutrient because vitamin A deficiency can be induced. Pre-ruminant calves, ferrets, and gerbils, like humans, absorb a portion of dietary provitamin A carotenoid intact and also produce retinyl esters in enterocytes. It is important to recognize that these species differ from humans in some characteristics of vitamin A metabolism. Gerbils store high levels of vitamin A in the liver, making it difficult to induce vitamin A deficiency, and ferrets have

markedly higher amounts of retinyl esters in their plasma compared with human subjects. These differences do not however, diminish the overall usefulness of the two animal species for investigating many problems related to the bioavailability of provitamin A carotenoids (Lee *et al*., 1999).

The inherent limitations in experimental design, data interpretation, cost of instrumentation, and labor intensiveness of these *in vivo* methods involving human subjects limit their utility for screening the bioavailability of carotenoids for large numbers of cultivars that may be prepared for ingestion in many different ways.

7.3. *Studies using in vitro methods*

Simulated digestive processes, isolated intestinal cells and segments, and brush-border and basolateral membrane vesicles represent models for studying specific nature and the mechanism associated with digestion and absorption. These models have been used to investigate the effects of chemical speciation, food matrix and processing, and dietary components on the digestive stability, accessibility, and intestinal transport and metabolism of carotenoids from foods and supplements.

Simulated gastric and small intestinal digestion has been widely used to investigate the digestion of proteins (Lindberg *et al.*,1998), starch (Englyst *et al.*, 1999), lipids (Fouad *et al.*,1991), polyphenols (Gil-Izquierdo *et al.,* 2002), transgenic plant DNA (Netherwood *et al.*, 2004), and recombinant proteins (Richards *et al.*, 2003) in complex food matrices. This method has also been used to examine carotenoid stability and partitioning during the digestion of foods, meals, and supplements (Garrett *et al*., 1999a; Garrett *et al.*, 2000; Ferruzzi *et al*., 2001; Chitchumroonchokchai *et al*., 2004). Comparison of the carotenoid profile before and after simulated digestion provides information about the stability of the carotenoids during the gastric and small intestinal phases of digestion. The effects of processing, dietary components, and luminal conditions on transfer of carotenoids from the food matrix to oil droplets (gastric digestion) and from oil droplets to micelles (small intestinal digestion) can also be studied employing this simple method.

A multi-compartmental, computer-controlled system that accurately reproduces various physiological factors during gastric and small intestinal digestion has been developed to offset some of the limitations of the commonly used static methods (Minekus *et al.*, 1995). This system utilizes inputs on the effects of meal composition on gastric pH, transit rates, and mean concentrations of bile and digestive enzymes to program the computer operated system to accurately mimic the luminal environment after the ingestion of a specific food or meal. Also, the complex design of the system precludes the efficient screening of numerous test foods. Differences between the intact organism and simple biochemical and cellular models dictate the need for caution when using results from *in vitro* studies to conceptualize the more complex environment *in vivo*. Nevertheless, *in vitro* approaches are useful tools for defining key questions that merit more rigorous investigation *in vivo*, and also yield insights into the mechanisms underlying observations in humans and animals (Minekus *et al*., 2001).

Human Caco-2 cells are being increasingly used as a model system for determining the absorption of biomolecules. Caco-2 is a cell line originating from human colonic carcinoma that exhibits some morphological and functional characteristics similar to those of differentiated epithelial cells that line the intestinal mucosa (Sambruy *et al.*, 2001). These cells spontaneously differentiate to an enterocyte-like phenotype when monolayers reach confluency and are maintained using conventional culture conditions (Pinto *et al.*, 1983; Hidalgo *et al.*, 1989, 1999). Garrett *et al*., (1999b, 2000) developed a two component coupled digestion / Caco-2 human intestinal cell system to examine cellular uptake of micellarized carotenoids and other lipophiles from foods, supplements, and meals following simulated gastrointestinal digestion.

Ultimately, studies on human subjects are required to determine the efficiencies of the absorption and conversion of provitamin A carotenoids from foods prepared in various ways and ingested in traditional meals. However, the difficulty and expenditure of conducting well-controlled human studies precludes systematic investigation of the effects of plant genotype, meal preparation method, and host factors on provitamin A bioavailability.

Thus, the *in vitro* digestion system appears to provide a useful alternative to animal and human models for rapidly screening carotenoid bioavailability from foods and meals. The ability to carefully control the *in vitro* environment should be useful for systematically investigating the impact of various processing methods and dietary factors on the bioavailability of carotenoids from specific foods.

In vitro biochemical and cellular methods represent cost-effective surrogates for an initial screening of relative bioavailability of provitamin A carotenoids. Support for this hypothesis is provided by direct comparison of the observations from *in vivo* and *in vitro* studies. Human studies have consistently shown that bioavailability is increased when the plant matrix is destroyed by cooking and by other forms of processing, and when carotenoids solubilized in oil are ingested instead of natural foods. These factors also increase the micellarization of carotenoids during simulated digestion. In addition, impaired secretion of digestive enzymes, bicarbonate, and bile in response to a meal can decrease absorption of fat-soluble compounds, just as reduced concentrations of pancreatin and bile extract decrease micellarization of carotenoids during simulation of the small intestinal phase of digestion. Collectively, these results indicate that the relative bioavailability of provitamin A carotenoids from staple food crops can be probed by monitoring the extent of micellarization of provitamin A carotenoids during simulated digestion.

8. Scope of present investigation

Micronutrient deficiency is a major public health problem in the developing countries, India accounting for nearly half of the world's prevalence. Among the micronutrient deficiencies, deficiency of vitamin A is recognized as a serious public health problem leading to blindness (WHO, 2009). It has been estimated that globally, 2.8 million preschool children are at risk of blindness (WHO, 1998). Deficiency of vitamin A is wide spread in India leading to the blindness of about 60 thousand children below the age of five years every year. Xerophthalmia afflicts 4.4 million preschool aged children across the globe. Forty percent of all preschool-aged children with xerophthalmia (1.8 million) in the developing world live in India, a number that accounts for 88% of all cases in South and Southeast Asia (West, 2002). Animal foods such as eggs, milk and liver are good sources of preformed vitamin A. A majority of the population in India is dependent on plant foods, which provide carotenes, especially β-carotene, to meet their requirement of vitamin A. β-carotene is abundantly found in green leafy and yellow-orange vegetable (Gopalan *et al*., 2004). Several factors such as diet composition and methods employed for food processing affect the bioavailability of β-carotene from foods. Dietary factors such as fat, fiber and protein are documented to influence β-carotene bioavailability (Yeum and Russell, 2002). Studies have shown that the absorption of carotenoids from uncooked food is low and mild cooking enhances the absorption of β-carotene (Rodriguez and Irwin, 1972). However, heat treatment especially in presence of light and oxygen causes isomerization of carotene as well as its oxidative destruction thus decreasing its biological activity (Ogulensi and Lee, 1979).

In vertebrates, provitamin A carotenoids are converted to retinal by the enzyme β-carotene-15,15'-dioxygenase, the activity of which is expressed specifically in intestinal epithelium and in liver. The intestinal enzyme determines whether provitamin A carotenoids are converted to vitamin A or circulated in the body as intact carotenoids. Thus, the bioconversion of β-carotene to retinal is dependent on the regulation of the activity of this enzyme. Since the cleavage enzyme is located in the intestinal cells which are directly exposed to various food components, actions of dietary components such as

spices on the enzyme activity might affect the bioavailability of β-carotene derived from plant foods, and its bioconversion to vitamin A (Nagao, 2004).

Vitamin A malnutrition being widely prevalent, understanding the bioavailability of dietary β-carotene from plant foods, and its subsequent conversion to vitamin A is of utmost importance. Such information may lead to optimization of dietary approaches to increase the bioavailability of dietary β-carotene. Knowledge of the bioavailability of β-carotene from dietary sources is also important in order to rationalize the RDA for the same. In addition to the provitamin-A activity, β-carotene and other carotenoids are of much value as antioxidants. In this context, information on the bioavailability of β-carotene from plant foods assumes greater importance. *In vitro* methods which essentially provide inaccessibility value of β-carotene from foods offer quick and cost effective alternative to the more expensive and cumbersome *in vivo* procedures. They can therefore be employed in screening a large number of foods and also for evaluating the influence of various factors on the bioavailability of β-carotene.

Considerable amounts of β-carotene are lost from vegetable during pressure cooking or open pan boiling (Gayathri *et al*., 2004). Presence of food acidulants affects the retention of β-carotene from some vegetables. The antioxidant spices – turmeric or onion generally improve the retention of β-carotene. A combination of food acidulant and antioxidant spice improved the retention from specific green leafy vegetables synergistically. Heat processing methods, presence of acidulants and presence of antioxidant spices may similarly influence the bioavailability of β-carotene from plant foods, which remains to be verified. Spices such as black pepper alter the ultra structure and permeability characteristics of the intestine, thus modifying the process of absorption. Several spices have also been evidenced to enhance the activity of terminal digestive enzymes of the small intestine. Piperine has been evidenced to produce a proliferation of endoplasmic reticulum of the enterocytes of the intestine, which is associated with an increased absorptive surface and microvilli length. It would therefore be relevant to examine if such a spice would also influence the β-carotene cleavage enzyme present in the intestinal enterocytes.

Objectives of this investigation

The present investigation envisaged screening of green leafy and yellow-orange vegetables, and fruits for the bioaccessibility of β-carotene as influenced by factors such as heat processing, presence of food acidulants and presence of antioxidant spices. In view of the probable influence of a few specific spices on the ultra structure and permeability characteristics of intestines, animal studies were also carried out to assess the influence of specific dietary spices such as black pepper (or piperine) on the absorption of β-carotene. Animal Studies were extended to examine the effect, if any, of these food components (spices) on the activity of the enzymes involved in the bioconversion of β-carotene to vitamin A. Thus, the objectives of this investigation were:

1) Screening of selected green leafy and yellow-orange vegetables for the bioaccessibility of β-carotene. Influence of heat processing encountered during cooking on the same.

2) Studies on the influence of dietary factors such as presence of food acidulants and antioxidant spices and their combinations on the bioaccessibility of β-carotene from selected green-leafy and yellow-orange vegetables.

3) Studies on the varietal differences in the content and bioaccessibility of β-carotene from mango and papaya fruits.

4) Studies on the influence of specific dietary spices – black pepper (or piperine), red pepper (or capsaicin) and ginger on the absorption of β-carotene in experimental animals.

5) Studies on the effect of the above dietary spices on the concentration of vitamin A in tissues following an oral dose of β-carotene, and the activity of the carotenoid conversion enzymes as a measure of bioconversion of β-carotene in experimental animals.

CHAPTER – II

COMPARISON OF *IN VITRO* METHODS FOR THE DETERMINATION OF BIOACCESSIBILITY OF β-CAROTENE IN VEGETABLES

Comparison of *in vitro* methods for the determination of bioaccessibility of β-carotene in vegetables

INTRODUCTION

Bioavailability of a nutrient has been defined as the fraction of the ingested amount available for utilization in normal physiological functions and storage. The concept of the term 'bioavailability of a particular nutrient' comprises different steps, and as such the amount of this food component that is released from the food matrix is commonly referred to as 'bioaccessibility' (digestibility). In other words, the amount of an ingested nutrient that is available for absorption in the gut after digestion is referred to as bioaccessibility. For some components, this constitutes the maximum amount available for absorption (Hedren *et al.*, 2002).

There are various factors that may affect the bioavailability of nutrients such as carotenoids from its food sources including the food matrix, particle size of the food, presence of fat, etc. (Erdman *et al*., 1993). Fat soluble components must be incorporated into mixed micelles before absorption. Thus, the efficiency of micellarization (quantities transferred in to the aqueous micellar fraction) is used as an estimate of relative bioavailability of carotenoids (Failla and Chitchumroonchokchai, 2005).

Epidemiological studies have shown that the consumption of carotenoid rich foods is associated with a reduced risk of developing several chronic diseases, especially cardiovascular diseases (Kohlmeier and Hastings, 1995; Kritchevsky, 1999) and cancer (Van Poppel and Goldbohm, 1995; Ziegler, 1996). Carotenoids are recognized for their antioxidant properties (Canfield *et al*.,1992; Sies *et al.,*1992) besides acting as precursor to vitamin A. Vitamin A malnutrition being widely prevalent, understanding the bioaccessibility of dietary β-carotene from plant foods is of utmost importance. Such information may lead to optimization of dietary approaches to increase the bioaccessibility of dietary β-carotene. Knowledge of the bioaccessibility of micronutrients including β-carotene from dietary sources is also important in order to rationalize the RDA for vitamin A.

In vitro models have been developed as simple, inexpensive and reproducible tools to study digestibility, micellarization, intestinal transport and metabolism and to predict the bioavailability of food components such as carotenoids (Granado-Lorencio *et al*., 2007; Chitchumroonchokchai *et al*., 2004). The *in vitro* digestion system appears to provide a useful alternative to animal and human models for rapidly screening carotenoid bioavailability from foods and meals. The *in vitro* system should be useful for systematically investigating the impact of various processing methods and dietary factors on the bioavailability of carotenoids from specific foods. The *in vitro* approach would also be useful for estimating the bioavailability of provitamin A from different cultivars with the aim of selecting cultivars that merit further examination in animal models. *In vitro* methods to determine the bioaccessibility of β-carotene from foods appear to provide a cost effective alternative to the more expensive and cumbersome *in vivo* procedures.

An *in vitro* digestion method to estimate carotenoid bioaccessibility from meals similar to the one employed for determination of iron bioaccessibility was developed by Garrett *et al*. (1999). This method essentially involves simulated gastrointestinal digestion followed by ultracentrifugation to separate the micellar aqueous fraction containing the bioaccessible fraction of β-carotene and determination of the same. In the present investigation, alternative methods were explored to separate the micellar fraction containing the bioaccessible β-carotene after simulated gastrointestinal digestion. These alternative methods could be simpler, faster, economical and suitable for routine screening of plant foods for β-carotene bioaccessibility. These methods were employed to determine the bioaccessibility of β-carotene from two representative green leafy and yellow-orange vegetables.

MATERIALS AND METHODS

Materials

Fresh carrot (*Daucus carota*), pumpkin (*Cucurbita maxima*), amaranth (*Amaranthus gangeticus*) leaves and fenugreek (*Trigonella foenum-graecum*) leaves were locally

procured. All chemicals used were of analytical grade. Solvents were distilled before use. Porcine pancreatic pepsin, and pancreatin and bile extract (porcine) were procured from Sigma Chemicals Co., St. Louis, USA. Double-distilled water was employed through out the entire study. All glassware used was acid washed.

Bioaccessibility of β-carotene in vitro

The bioaccessibility of β-carotene *in vitro* was determined by the method of Garrett *et al.* (1999). Briefly, the method involved subjecting the sample to simulated gastric digestion at pH 2.0 in the presence of pepsin at 37ºC, followed by simulated intestinal digestion in the presence of pancreatin-bile extract mixture, pH 7.5 at 37ºC for 2 h. For this purpose, the test vegetable (10 g) was homogenized and mixed with approx. 90 ml water in a 250 ml Erlenmeyer flask. After adjusting the pH to 2.0 using 6M HCl, 3 ml of freshly prepared pepsin solution [16 g pepsin (from porcine stomach) in 100 ml 0.1M HCl] was added to the sample and the volume was made up to 100 ml. The samples were incubated at 37ºC for 2 h in an incubator shaker (110 strokes/min).

After this gastric digestion, an aliquot (20 ml which represents 2 g of vegetable sample) of the gastric digesta was taken out and transferred to a 100 ml Erlenmeyer flask. The pH of this gastric digesta was adjusted to 5.3 with 0.9M sodium bicarbonate. Five millilitres of freshly prepared pancreatin - bile extract mixture (4 g porcine pancreatin and 25 g porcine bile extract in 1000 ml of 0.1M $NaHCO_3$) was added to the sample and the pH was further raised to 7.5 using 1N sodium hydroxide. The samples were incubated at 37ºC in the incubator shaker for 2 h (110 strokes/min). At the end of simulated intestinal digestion, the aqueous micellar fraction, which contained the bioaccessible β-carotene, was separated from the digesta by ultracentrifugation at 70,000 x g for 120 min in a Beckman L7-65 ultracentrifuge (Beckman Instruments , Palo Alto, CA, USA), using polycarbonate centrifuge bottles (10.4 ml capacity) for spinning the samples in a Type 65 rotor. The aqueous micellar fraction was collected from the centrifuge tubes using an 18-guage needle attached to a 10 ml syringe. (Method-1) (Fig.1).

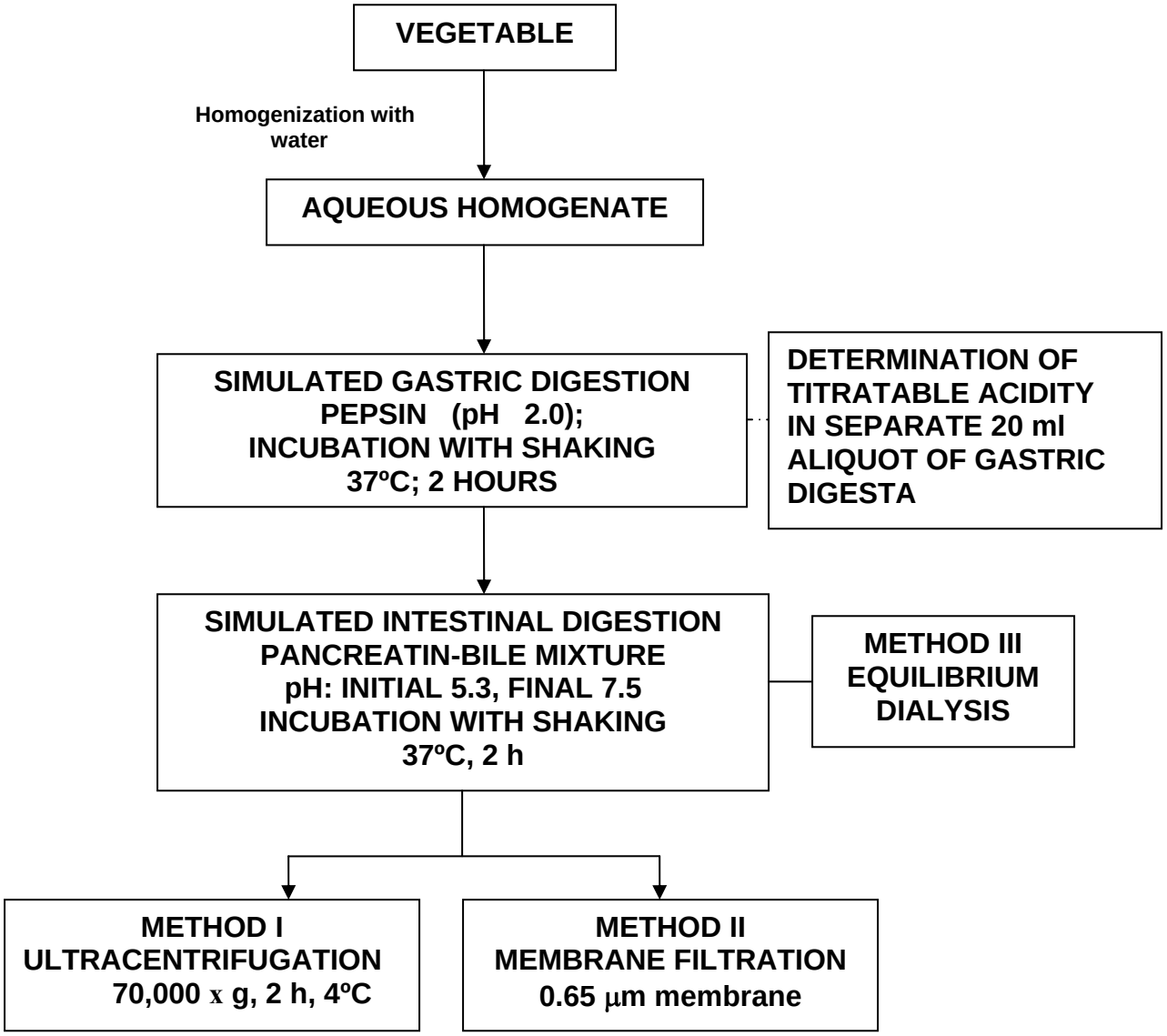


Fig.1. Flow Chart of *in vitro* procedures for the determination of bioaccessibility of β-carotene

One alternative method to separate the micellar fraction examined for its suitability in this investigation was: filtration of the digesta through a Millipore membrane (0.65 μm size; 25 mm diameter) after a preliminary centrifugation at 5000 x g for 20 min using polyallomer tubes (40 ml capacity) in a SS-34 rotor and Sorvall RC-5B super speed refrigerated centrifuge, instead of ultracentrifugation (Method-2) (Fig.1).

The second alternative method to separate micellar fraction examined for its suitability in this investigation was: equilibrium dialysis by insertion of a dialysis bag containing sodium bicarbonate equimolar to the titratable acidity, and mixed micelles to facilitate the movement of the lipid-soluble β-carotene into the dialysis bag, during simulated intestinal digestion (Method-3) (Fig.1). At the end of gastric digestion as described above, titratable acidity was determined in an aliquot of the gastric digesta. An aliquot of gastric digesta (20 ml) was brought to room temperature and 5 ml of pancreatin-bile extract mixture was added. The pH was adjusted to 7.5 with 0.2M sodium hydroxide placed in a burette. After 30 min, the pH was checked and readjusted to 7.5, if necessary. Titratable acidity was defined as the amount of 0.2M sodium hydroxide required to obtain pH of 7.5.

During simulated intestinal digestion, segments of dialysis tubing (Molecular mass cut off: 10 kDa) containing 25 ml sodium bicarbonate solution, being equivalent in moles to the NaOH needed to neutralize the gastric digest (titratable acidity) were placed in Erlenmeyer flasks containing the gastric digest and incubated at 37ºC with shaking for 30 min or longer until the pH of the digest reached 5.0. Five ml of the pancreatin-bile extract mixture was then added and incubation was continued for 2 h or longer until the pH of the digest reached 7.0. Mixed micelles were prepared using a mixture of phosphatidyl-choline and deoxycholic acid in the molar ratio 1:2 as described by Began *et al*. (1999). Solutions of phosphatidyl-choline and deoxycholic acid were made in chloroform-methanol (2:1 v/v); after mixing the two solutions, the solvent was evaporated in a flash evaporator and dried under a stream of nitrogen. The resulting thin film was solubilized in 50 mM Tris-HCl buffer, pH 7.4 by sonication for 5 min in a bath type sonicator.

The concentration of mixed micelles (prepared as described above) taken in the dialysis bag along with sodium bicarbonate (equivalent of titratable acidity) was 100 μM in terms of phosphatidyl-choline. This amount was optimized in a preliminary trial, by measuring the amount of β-carotene from raw amaranth dialyzed as a function of increasing concentrations of the mixed micelles in the dialysis bag (Fig.2). The amount of β-carotene that was dialyzed from the digesta of amaranth, which was insignificant in the absence of any mixed micelles, increased with the inclusion of the same in the dialysis bag. Dialyzability of β-carotene increased linearly with increasing micellar concentration in the dialysis bag up to 100 μM (in terms of phosphatidyl choline). Further increase in micellar concentration did not proportionately improve β-carotene dialyzability. On the other hand, increase in micellar concentration beyond 100 μM also resulted in an undesirable turbidity of the dialyzate. Thus, micellar concentration in the dialysis bag was limited to 100 μM in terms of phosphatidyl choline in all determinations involving Method-3.

Analysis of β-carotene by Spectrophotometric method

Total as well as bioaccessible β-carotene were determined by extraction of the pro-vitamin from the samples with acetone followed by petroleum ether (60 - 80ºC), and fractionated on neutral alumina column using 3% acetone in petroleum ether. The color intensity of β-carotene eluent was measured at 450 nm in a Shimadzu UV/Visible spectrophotometer, and compared with that of β-carotene standard (Ranganna, 1977). In Methods 1, 2 and 3, β-carotene was quantitated from both the micellar fraction as well the residue, and the bioaccessibility of β-carotene expressed as a percentage of this recovered amount.

During the entire procedure, namely simulated gastrointestinal digestion, ultra-centrifugation / membrane filtration, extraction of β-carotene and column chromatography, adequate precautions were taken to minimize the exposure of samples to light and air and thus prevent oxidative destruction of β-carotene. Air was replaced by nitrogen before stoppering the flask at all stages of incubation and storage. The

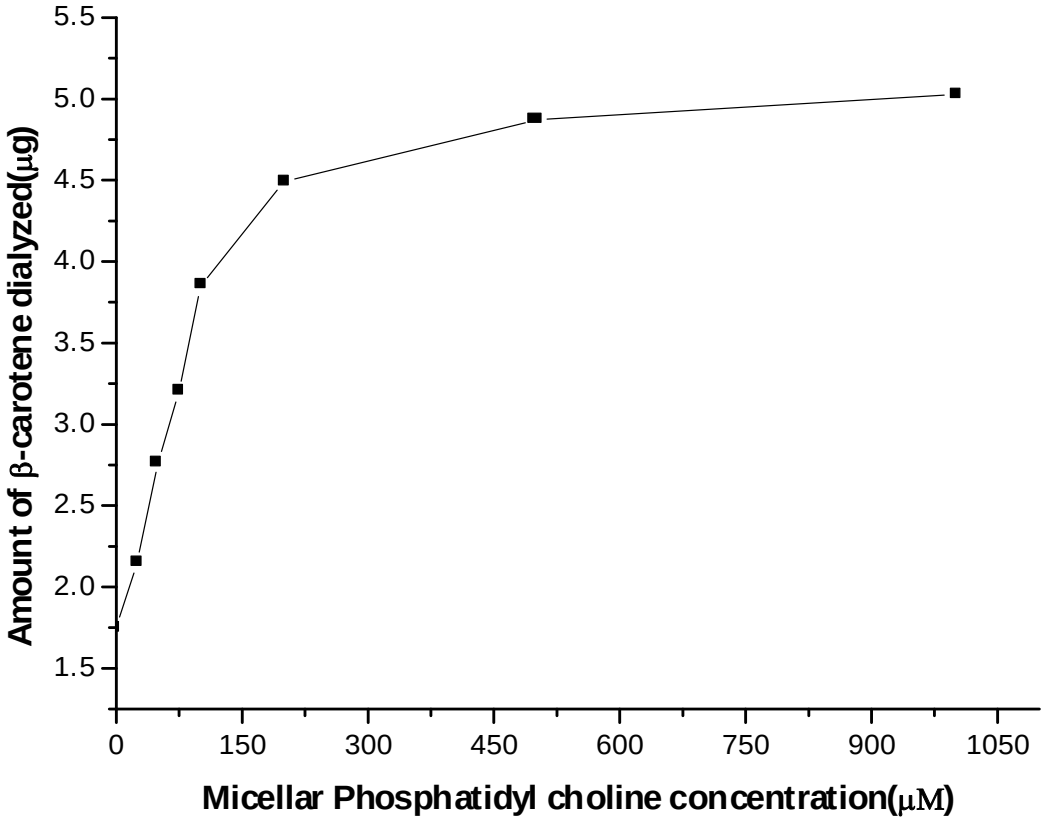


Fig.2. Dialyzability of β-carotene from the digesta as a function of micellar phosphotidyl choline concentration in the dialysis bag

experiments were carried out under yellow lighting and all the glassware was covered with black cloth to prevent penetration of light.

Vegetable (2 g) sample was pressure-cooked with 10 ml of distilled water at 15 p.s.i. for 10 min. Another sample of the vegetable was subjected to stir-frying by frying 2 g of chopped vegetable in a shallow pan in the presence of 185 mg of refined groundnut oil for 10 min at 100°C. These samples were homogenized before being subjected to simulated gastrointestinal digestion.

Statistical analysis

All determinations were made in pentuplicates and the average values are reported. Data were analyzed statistically according to Snedecor and Cochran (1976).

RESULTS AND DISCUSSION

Optimization of extraction of β-carotene from aqueous samples

Conventionally, β-carotene from fresh plant tissues is extracted directly by blending with the organic solvent system. Since we need to analyze β-carotene essentially in aqueous samples in our investigation, trials were done to optimize and ensure maximum extractability of β-carotene from the aqueous blends of plant tissues, and compared with β-carotene extracted from fresh plant tissue.

In a preliminary experiment, extraction of β-carotene from the aqueous samples was tried using different solvent systems, to optimize maximum extraction of β-carotene. For this purpose, amaranth leaves homogenized to thin slurry with distilled water was extracted with different solvent systems as listed in Table-1. Results of this trial showed that maximum extraction of β-carotene (96%) could be achieved using acetone and petroleum ether. Acetone and petroleum ether were used for extraction sequentially [Initially the aqueous blend was mixed with acetone (1:1 v/v), subsequently this homogenous mixture was extracted with petroleum ether (2:1 v/v)]. An equally high

Table-1. Extraction of β-carotene from the aqueous samples of amaranth blends using different solvents

Solvent employed	Amount of β-carotene extracted from aqueous phase (mg/100 g)	% Recovered
Acetone + Petroleum ether	8.070	96.4
Acetone + ethanol+ Petroleum ether	8.075	96.4
Petroleum ether alone	2.969	35.4
Ethanol + petroleum ether	7.559	90.3
Ethanol + acetone + hexane	7.680	91.7

Values are average of pentuplicate samples.

Total β-carotene in fresh amaranth leaves = 8.369 mg/100 g

recovery of β-carotene was also envisaged with the solvent system - ethanol: acetone (1:1 v/v), (instead of acetone alone), and subsequent extraction with petroleum ether.

Saponification is an additional step during the extraction of carotenoids, in order to achieve removal of undesirable chlorophylls (because of green color that interferes with spectrophotometric determination of carotenoids) and also removal of unwanted lipids (Riso and Porrini, 1997; Granado-Lorencio *et al*., 2001). Thus, in these optimization trials, β-carotene extraction from aqueous blends was also tried with and without saponification during the extraction. The saponification which involved treatment with methanolic KOH for 3 h in the dark as described by Kimura *et al.* (1990) gave an almost comparable recovery of β-carotene in both amaranth leaves and carrot (Table-2). Saponification for extended duration of 16 h in the dark as employed by Chen *et al.* (1995) gave a slightly lower recovery of β-carotene from these plant materials.

Thus, in all our subsequent experiments in this investigation, the solvent system consisting of acetone-ethanol (1:1 v/v) and petroleum ether was used for the extraction of β-carotene from aqueous samples (Hedren, *et al*., 2002). Additionally, saponification for 3 h was employed in the case of samples from green leafy vegetables in order to achieve removal of chlorophylls.

Bioaccessibility of β-carotene from food matrix during different phases of simulated gastrointestinal digestion

In a separate study, we also examined the contribution of different phases of *in vitro* digestion to the transfer of β-carotene from food matrix from a representative vegetable, viz., carrot, into the aqueous micellar fraction. The transfer of β-carotene from the food matrix was only partial in the case of samples which did not go through simulated gastro-intestinal digestion, or in samples which went through only gastric phase of digestion (Table-3). The transfer of β-carotene was maximal in the case of carrot sample that went through both gastric and intestinal phase of digestion; and it was close to maximum in the

Table-2. Suitability of saponification during extraction of β-carotene from liquidized carrot and amaranth leaves

Vegetable	Extraction procedure	Amount of β- carotene extracted from aqueous phase (mg/100 g)	Percent Recovered
Carrot	Without saponification	6.958	96.9
	With saponification	6.819	94.9
Amaranth	Without saponification	7.920	95.8
	With saponification	7.740	93.6

Values are average of pentuplicate samples.

Total β-carotene in fresh carrot = 7.179 mg/100 g

Total β-carotene in fresh amaranth = 8.270 mg/100 g

Table-3. Contribution of different phases of *in vitro* digestion to the transfer of β-carotene from food matrix to the aqueous micellar fraction

Samples	β-carotene content in filtrate (μg/100 g)	Percent Bioaccessibility
Carrot sample without digestion	27.5	0.35
Carrot sample + gastric phase	159	2.04
Carrot sample + intestinal phase initiation	1334	17.1
Carrot sample + gastric phase + pancreatin	277.5	3.6
Carrot sample + complete digestion (both gastric and intestinal phase)	1495	19.2
Carrot sample without digestion	27.5	0.35
Carrot sample + gastric phase	159	2.04

Total amount of β-carotene in carrot = 7785 μg/100 g

sample that contained pancreatin plus bile salts (added to initiate intestinal phase of digestion), even in the absence of gastric phase of digestion.

β- Carotene content of test vegetables

Table-4 presents the total β-carotene content of the four test vegetables, carrot, pumpkin, amaranth and fenugreek leaves. Fenugreek had the highest concentration of β-carotene (9.15 mg/100 g), followed by amaranth (8.17 mg/100 g), carrot (8.14 mg/100 g) and pumpkin (1.90 mg/100 g). These values are comparable to those reported by the Indian Council of Medical Research (Gopalan *et al.*, 2004), where the β-carotene content of fenugreek leaves, amaranth, carrot and pumpkin are reported as 9.10, 8.34, 6.46 and 1.17 mg/100 g, respectively.

Comparison of three methods to determine bioaccessible β-carotene

Fig.3 presents a comparison of the bioaccessibility of β-carotene from the four test vegetables as determined by the three methods described above. Bioaccessible β-carotene was calculated as the percentage of the total amount recovered in the residue and micellar aqueous fractions, at the end of simulated gastrointestinal digestion. Some losses in the recovery of β-carotene during prolonged processing procedures are to be expected, as this provitamin is highly susceptible to destruction by exposure to light and oxygen. Among the alternatives tried for Method-1, Method-2 which involved membrane filtration to separate the micellar fraction, produced values for bioaccessible β-carotene that were comparable with those of Method-1 in all the test vegetables examined. On the other hand, Method-3, where the bioaccessible fraction of β-carotene was separated by equilibrium dialysis, gave values that were several folds lower than those obtained by Methods -1 and -2 (Table-4).

The percent bioaccessible β-carotene from raw carrot was 20.3 as determined by Method-1, while it was 19.5 by Method-2 and 3.58 by Method-3. The bioaccessiblity of β-carotene from raw pumpkin was 16.3, 19.0 and 2.1% as determined by Methods -1, -2 and -3, respectively. Among the green leafy vegetables tested, the bioaccessibility of

Table-4. Estimation of bioaccessibility of β-carotene from fresh vegetables employing alternative *in vitro* methods

Vegetable	β-Carotene content (mg/100 g)	Percent bioaccessible β-carotene		
		Method-1	Method-2	Method-3
Carrot	8.14 ± 0.14	20.3 ± 0.58	19.5 ± 0.03	3.58 ± 0.10*
Pumpkin	1.90 ± 0.27	16.3 ± 0.84	18.0 ± 0.17	2.10 ± 0.12*
Amaranth	8.17 ± 0.54	10.6 ± 0.58	11.0 ± 0.49	3.10 ± 0.09*
Fenugreek leaves	9.15 ± 0.05	6.70 ± 0.23	7.10 ± 0.29	1.20 ± 0.06*

Method-1: Ultracentrifugation to separate micellar fraction

Method-2: Membrane filtration to separate micellar fraction

Method-3: Dialysis bag to separate micellar fraction

Values are mean ± SEM of pentuplicate determinations;

*Statistically different ($P < 0.05$) compared to the Method-1.

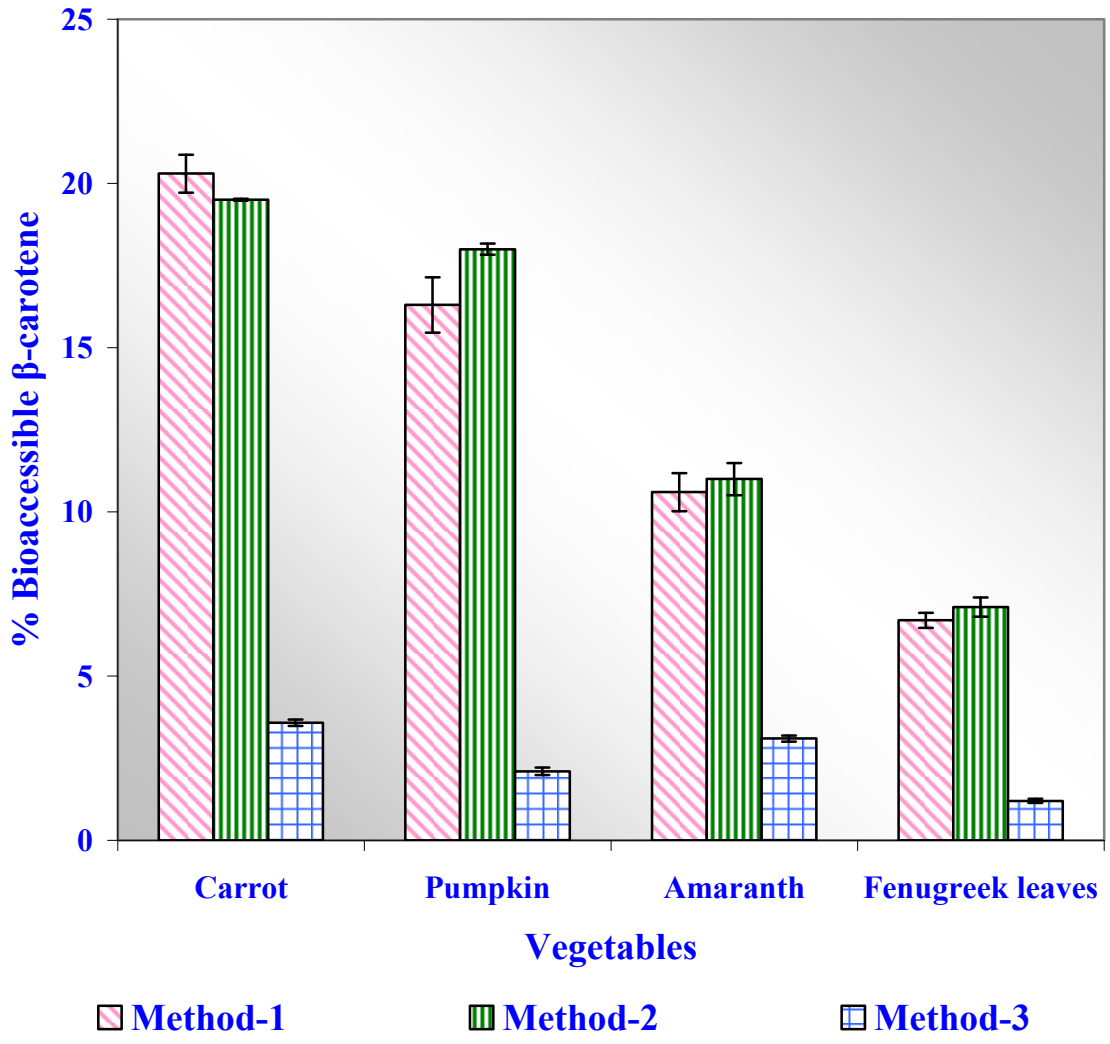


Fig.3. Bioaccessibility of β-carotene as determined by three variations of the *in vitro* procedure

Method 1: Ultracentrifugation to separate micellar fraction;
Method 2: Membrane filtration to separate micellar fraction;
Method 3: Equilibrium dialysis

β-carotene from raw amaranth as determined by Method-1 was 10.6%, while it was 11.0 and 3.10% by Methods -2 and -3, respectively. The percent bioaccessible β-carotene from raw fenugreek leaves was 6.7, 7.1 and 1.2 as obtained by Methods -1, -2, and -3, respectively (Table-4).

Similarly, the three alternatives in the *in vitro* method for the determination of bioaccessibility of β-carotene were compared in the case of heat processed vegetables (Table-5). The percent bioaccessible β-carotene from pressure- cooked carrot was 24.2 as determined by Method-1, while it was 21.0 by Method-2 and 4.27 by Method-3. The bioaccessiblity of β-carotene from stir- fried carrot was 32.9% as determined by Method-1, while it was 28.0% by Method-2 and 4.49% by Method-3. The percent bioaccessible β-carotene from pressure-cooked pumpkin was 15.3, 19.0 and 4.05% as determined by Methods -1, -2 and -3, respectively. The percent bioaccessible β-carotene from stir-fried pumpkin was 24.9 as determined by Method-1, while it was 20.9 by Method-2 and 4.13 by Method-3. (Table-5). Thus, Method-1 and Method-2 gave closer values of bioaccessible β-carotene for these heats processed yellow-orange vegetables, but not Method-3.

The percent bioaccessible β-carotene from pressure-cooked amaranth was 15.7 as determined by Method-1, while it was 16.5 by Method-2 and 3.95 by Method-3. The bioaccessiblity of β-carotene from stir-fried amaranth was 30.6% as determined by Method-1, while it was 23.5% by Method-2 and 5.45% by Method-3. The percent bioaccessible β-carotene from pressure-cooked fenugreek leaves was 13.4 as determined by Method-1, while it was 15.2 by Method-2 and 2.38 by Method-3. The percent bioaccessible β-carotene from stir-fried fenugreek leaves was 24.3, 21.5 and 3.28 as determined by Methods -1, -2 and -3, respectively (Table-5). Thus, Method-1 and Method-2 gave closer values of bioaccessible β-carotene for these heat processed green leafy vegetables, but not Method-3.

Thus, membrane filtration to separate the bioaccessible fraction of β-carotene after simulated gastrointestinal digestion, has provided values for bioaccessible β-carotene

Table-5. Estimation of bioaccessibility of β-carotene from heat processed vegetables employing alternative *in vitro* methods

Vegetable		Percent bioaccessible β-carotene		
		Method-1	Method-2	Method-3
Carrot	Pressure-cooked	24.2 ± 0.38	21.0 ± 0.35	4.27 ± 0.08*
	Stir-fried	32.9 ± 0.09	28.0 ± 0.40	4.49 ± 0.10 *
Pumpkin	Pressure-cooked	15.3 ± 0.64	19.0 ± 0.09	4.05 ± 0.07*
	Stir-fried	24.9 ± 1.18	20.9 ± 0.64	4.13 ± 0.12*
Amaranth	Pressure-cooked	15.7 ± 0.32	16.5 ± 0.87	3.95 ± 0.10*
	Stir-fried	30.6 ± 1.20	23.5 ± 0.06	5.45 ± 0.13*
Fenugreek leaves	Pressure-cooked	13.4 ± 0.06	15.2 ± 0.26	2.38 ± 0.09*
	Stir-fried	24.3 ± 1.04	21.5 ± 0.06	3.28 ± 0.06 *

Method-1: Ultracentrifugation to separate micellar fraction

Method-2: Membrane filtration to separate micellar fraction

Method-3: Dialysis bag to separate micellar fraction

Values are mean ± SEM of pentuplicate determinations.

*Statistically different ($P < 0.05$) compared to the Method-1.

similar of those obtained by ultracentrifugation. On the other hand, the values obtained by Method-3 which involved equilibrium dialysis were lower than those obtained by the other two methods. In this method, the migration of the bioaccessible fraction of β-carotene, a lipid soluble provitamin, from the gastric digesta into the dialysis tube may have been restricted by the fact that the tubing contains an aqueous solution of sodium bicarbonate. Although the amount of β-carotene that was dialyzed from the digesta linearly increased with the inclusion of mixed micelles up to 100 μM (in terms of phosphatidyl choline) in the dialysis bag, addition of mixed micelles at the level used here to the tubing did not result in β-carotene dialyzability comparable to the micellar β-carotene found in the other two methods. Membrane filtration after a preliminary low speed centrifugation to separate the bioaccessible fraction of β-carotene may be considered as a suitable alternative to ultracentrifugation. This alternative method may be less expensive, and can probably be used when the availability of an ultracentrifuge is a limitation. On the other hand, simulated gastrointestinal digestion involving equilibrium dialysis, an *in vitro* method employed in the determination of mineral bioaccessibility, is not suitable for the determination of the bioaccessibility of the lipid-soluble β-carotene. The use of mixed micelles in this method at an appropriate higher concentration would also prove expensive.

SUMMARY

An *in vitro* method currently in use for the determination of β-carotene bioaccessibility involves simulated gastrointestinal digestion followed by ultracentrifugation to separate the micellar fraction containing bioaccessible β-carotene and its quantitation. The suitability of procedural alternatives for the determination of bioaccessibility of β–carotene in this *in vitro* method was examined. In this study, membrane filtration and equilibrium dialysis were examined to separate the micellar fraction as an alternative to ultracentrifugation. Values of β-carotene bioaccessibility from vegetables obtained with the membrane-filtration method were similar to those obtained by the ultracentrifugation method, and hence it was inferred that membrane filtration to separate the aqueous micellar fraction containing the bioaccessible β-carotene is satisfactory, and can be employed in the absence of an ultracentrifuge. The simulated gastrointestinal digestion method of Miller *et al.* (1981) involving equilibrium dialysis did not produce satisfactory results.

CHAPTER - III

BIOACCESSIBILITY OF β-CAROTENE FROM YELLOW-ORANGE AND GREEN LEAFY VEGETABLES AS INFLUENCED BY HEAT PROCESSING AND PRESENCE OF FOOD ADJUNCTS

Section-A

Bioaccessibility of β-carotene from yellow-orange and green leafy vegetables as influenced by heat processing

INTRODUCTION

A majority of the population in India is dependent on plant foods, which provide carotenes, especially β-carotene to meet their requirement of vitamin A. β-Carotene is abundantly found in green leafy and yellow-orange vegetables (Gopalan *et al*., 2004). In addition to the carotenoid content of food, bioavailability of the same is influenced by a number of factors such as the food matrix, food processing – including cooking, and the presence of other constituents such as fibre, fat, protein, etc. (Garrett *et al.,* 2000; Hedren *et al.,* 2002; O'Connel *et al.,* 2007). Earlier investigations have evidenced that inclusion of phospholipids and certain specific fatty acids in the diet significantly improves the vitamin A status of experimental animals (Suruga *et al*., 1995; Baskaran *et al*., 2003).

Cooking and application of heat is a necessary step in making the food palatable. Cooking also causes some desirable changes in food, viz., alteration in colour, flavor and texture, thus making it more appealing and also improved digestibility of food components (Sareen, 1999). Besides this, destruction of pathogenic microorganisms and inactivation of undesirable enzymes are the other two important preservative effects of cooking. Certain undesirable changes associated with cooking are reduction in nutrient content. Loss of nutrients can also be attributed to enzymatic destruction or oxidation of chemicals in the food. Knowledge on the effect of food processing on vitamin content of foods is very important since many vitamins like thiamine, riboflavin, and ascorbic acid are sensitive to heat and their loss is very rapid. β-Carotene, the major precursor of vitamin A is unstable to heat in presence of light (Barnell and Hollingsworth, 1956).

Carotenoid micellarisation can vary from one carotenoid to another in a given food as well as for the same carotenoid in different foods, and the extent of micellarisation can

range from 5 to 100% depending on the carotenoid and the food tested (Granado-Lorencio *et al*., 2007). Absorption of carotenoids from uncooked food is reported to be low and mild cooking enhances the absorbability of β-carotene (Rock *et al.*, 1998). β-Carotene is unstable to heat in presence of light (Barnell and Hollingsworth, 1956). Heat treatment especially in presence of light and oxygen causes isomerization of carotene as well as its oxidative destruction, thus decreasing its biological activity (Ogulensi and Lee, 1979). Heating causes isomerization of β-carotene (Weckel *et al.,* 1962), partially converting the *trans*-isomer to the *cis*-form. Increase in the proportion of *cis*-isomer decreases vitamin A value of β-carotene (Ogulensi and lee, 1979). Prolonged heating causes complete destruction of carotenoids, causing molecular rearrangement, thus loss of nutritive value (Ratantunga *et al*., 1978).

Knowledge on the effects of food processing such as domestic cooking, with respect to losses of this provitamin and changes in the bioaccessibility of the same is critical for evolving food based strategies for deriving adequate / optimal amounts of β-carotene. While much information is available on the losses of carotenoid content of foods during heat treatment, there is less information on the bioaccessibility of β-carotene from plant foods processed by domestic cooking procedures. In this context, pressure-cooking, stir frying and open-pan-boiling – the heat treatments encountered in domestic kitchen practices, were evaluated for their influence on the bioaccessibility of β-carotene. These heat treatment procedures are expected to release the provitamin A carotenoids from the food matrix, thus facilitating their absorption. At the same time, these heat treatment procedures cause some amount of destruction of the provitamin compound and isomerization of all- *trans* isomeric form to *cis*-form; both destruction and isomerization result in lesser bioavailability of β-carotene from foods. Representative green leafy and yellow-orange vegetables were examined in this context by subjecting them to the above domestic cooking procedures.

MATERIALS AND METHODS

Materials

Fresh carrot (*Daucus carota*), pumpkin (*Cucurbita maxima*), amaranth (*Amaranthus gangeticus*) leaves, fenugreek (*Trigonella foenum-graecum*) leaves, Spinach leaves (*Spinacia oleracea*), Drumstick leaves (*Moringa oleifera*), Coriander leaves (*Coriandrum sativum*), and Mint leaves (*Mentha spicata*) were locally procured. All chemicals used were of analytical grade. Solvents were distilled before use. Porcine pancreatic pepsin, pancreatin and bile extract (porcine) were procured from Sigma Chemicals Co., St. Louis, U.S.A. Double-distilled water was employed through out the entire study. All glassware used was acid washed.

Determination of bioaccessibility of β-carotene in vitro

The bioaccessibility of β-carotene *in vitro* was determined by the method of Garrett *et al.* (1999). Briefly, the method involved subjecting the sample to simulated gastric digestion at pH 2.0 in the presence of pepsin at 37ºC (16 g in 100 ml 0.1M HCl), followed by simulated intestinal digestion in the presence of pancreatin-bile extract mixture (4 g porcine pancreatin and 25g porcine bile extract in 1000 ml of 0.1M $NaHCO_3$), pH 7.5 at 37ºC for 2 h. At the end of simulated intestinal digestion, the micellar fraction, which contains the bioacessible β-carotene, was separated by ultracentrifugation at 70,000 x g for 120 min using a Beckman L7-65 ultracentrifuge (As in Chapter-II). β-apo-8'-carotenal was added as an internal standard to these samples

Extraction and Analysis of β-carotene

β-Carotene was extracted from the samples according to the method of Hedren *et al.* (2002) with slight modification. β -Carotene was extracted from the samples initially with a mixture of acetone : ethanol (1:1) containing 0.1% (w/v) butylated hydroxytoluene and subsequently with petroleum ether. The process was repeated several times to ensure complete extraction of β-carotene. In the case of GLVs, the extract was saponified (in an additional step to remove chlorophyll) with 30% methanolic potassium hydroxide at

room temperature for 3 h. Following saponification, the alkali was removed completely by repeated washing, and the solvent was evaporated to dryness in a rotary evaporator. The residue was redissolved in petroleum ether and stored in the cold pending analysis. Prior to analysis, the petroleum ether was evaporated under nitrogen and the residue was dissolved in the mobile phase used for determination by HPLC.

Quantitation of β-carotene was carried out by HPLC, using a Shimadzu HPLC LC-10AVP system consisting of a photo diode array detector (SPD-M 20A), system controller (SCL-10AVP), solvent delivery system / pump (LC-10AT VP) and manual sample injector. Chromatographic separation was accomplished using SGE 250 x 4.6 mm ODS C_{18} 5-µm column (SS Excil, Australia). β-Carotene containing sample was injected on to the reverse phase column and eluted with an isocratic mobile phase which consisted of a mixture of 65% (v/v) acetonitrile, 15 % (v/v) methylene chloride and 20% (v/v) methanol containing 1.3 mmol/L ammonium acetate at a flow rate of 1ml/min (Martin *et al.*, 1996). β-Carotene (all-*trans* form) was monitored at a wavelength of 450 nm. The peak identities and λ_{max} were confirmed by their retention time and characteristic spectra of standard chromatograms. Quantitation of β-carotene was made from peak area, which was based on a calibration curve generated from standard β-carotene. Assay performance and reproducibility were confirmed using β-apo-8'-carotenal as an internal standard. HPLC profile of Standard β-carotene, carotenoid extracts of fresh and heat processed vegetable samples (both total and bioaccessible) are given in Fig.4 -7.

During the entire procedure, namely simulated gastrointestinal digestion, ultra-centrifugation extraction of β-carotene and during chromatography, adequate precautions were taken to minimize the exposure of samples to light and air and thus prevent oxidative destruction of β-carotene. Air was replaced by nitrogen before stoppering the flask at all stages of incubation and storage. The experiments were carried out under yellow lighting and all the glassware was covered with black cloth to prevent penetration of light.

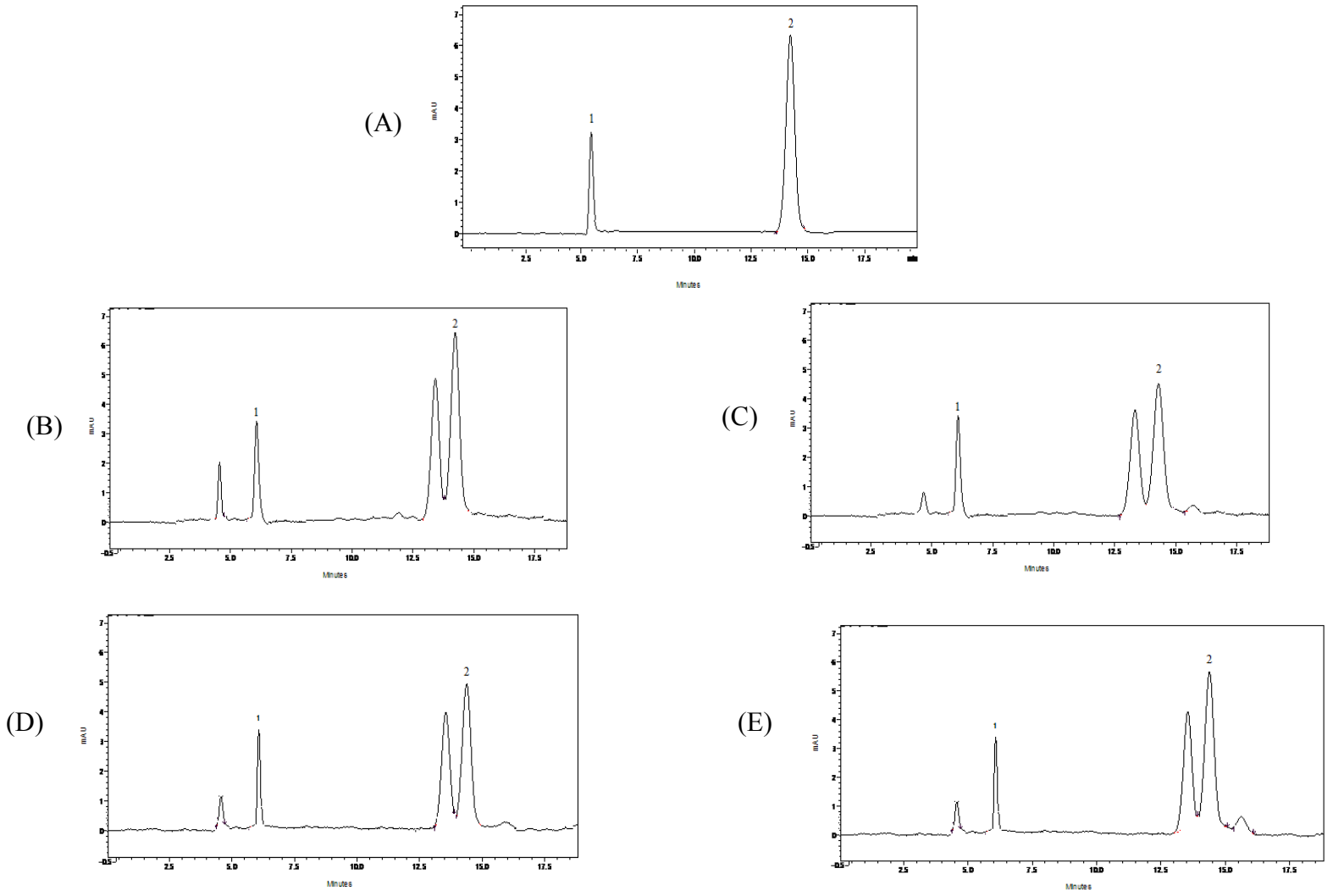


Fig.4. HPLC profile of carotenoid extracts of fresh and heat-processed carrot

A) Standard β-carotene; (B) Fresh carrot - Total; (C) Pressure-cooked carrot - Total; D) Open-pan-boiled carrot - Total; (E) Stir-fried carrot - Total

Peak #1: β-apo-8'-carotenal (Internal standard); Peak #2: β-carotene

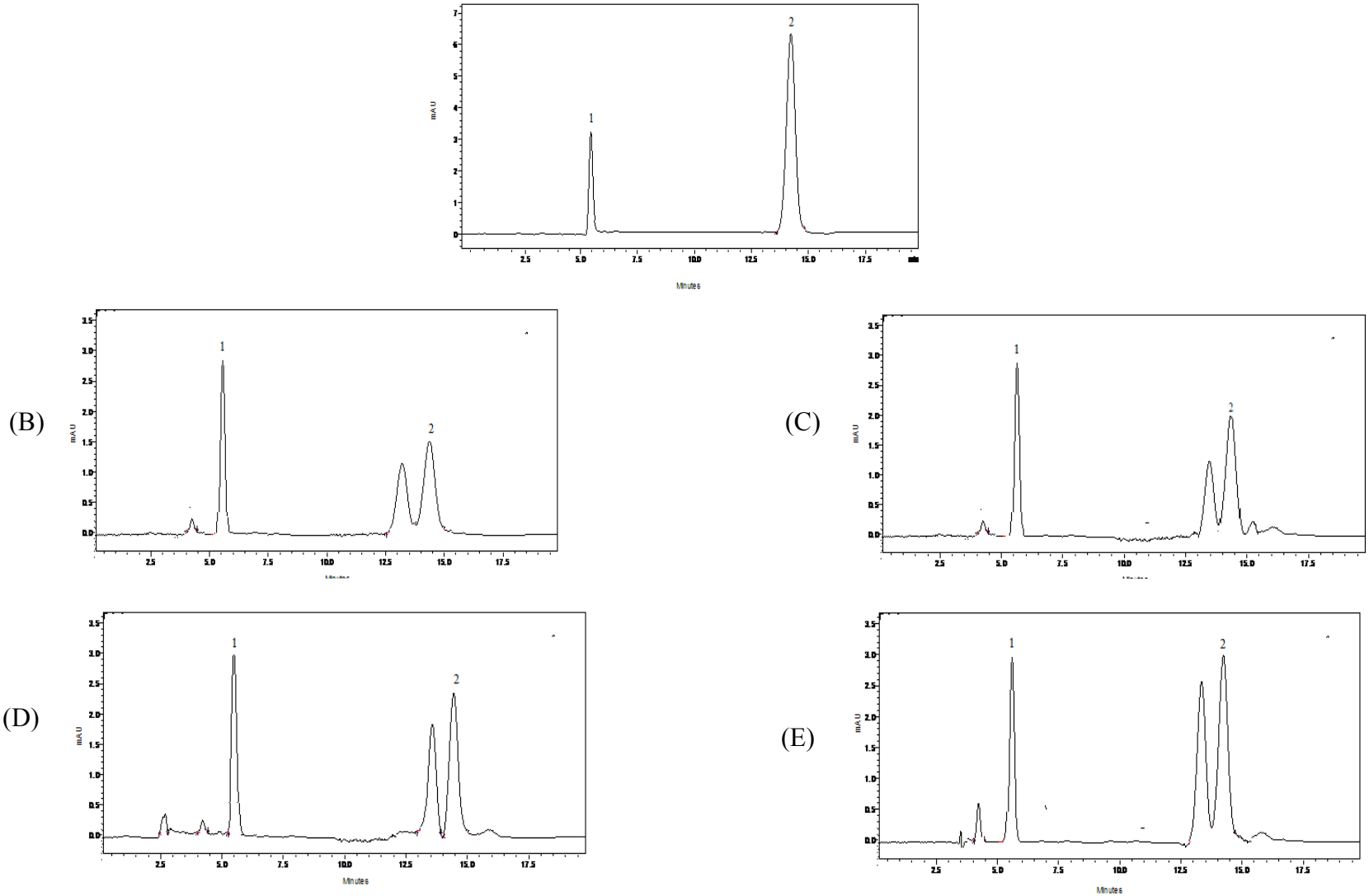
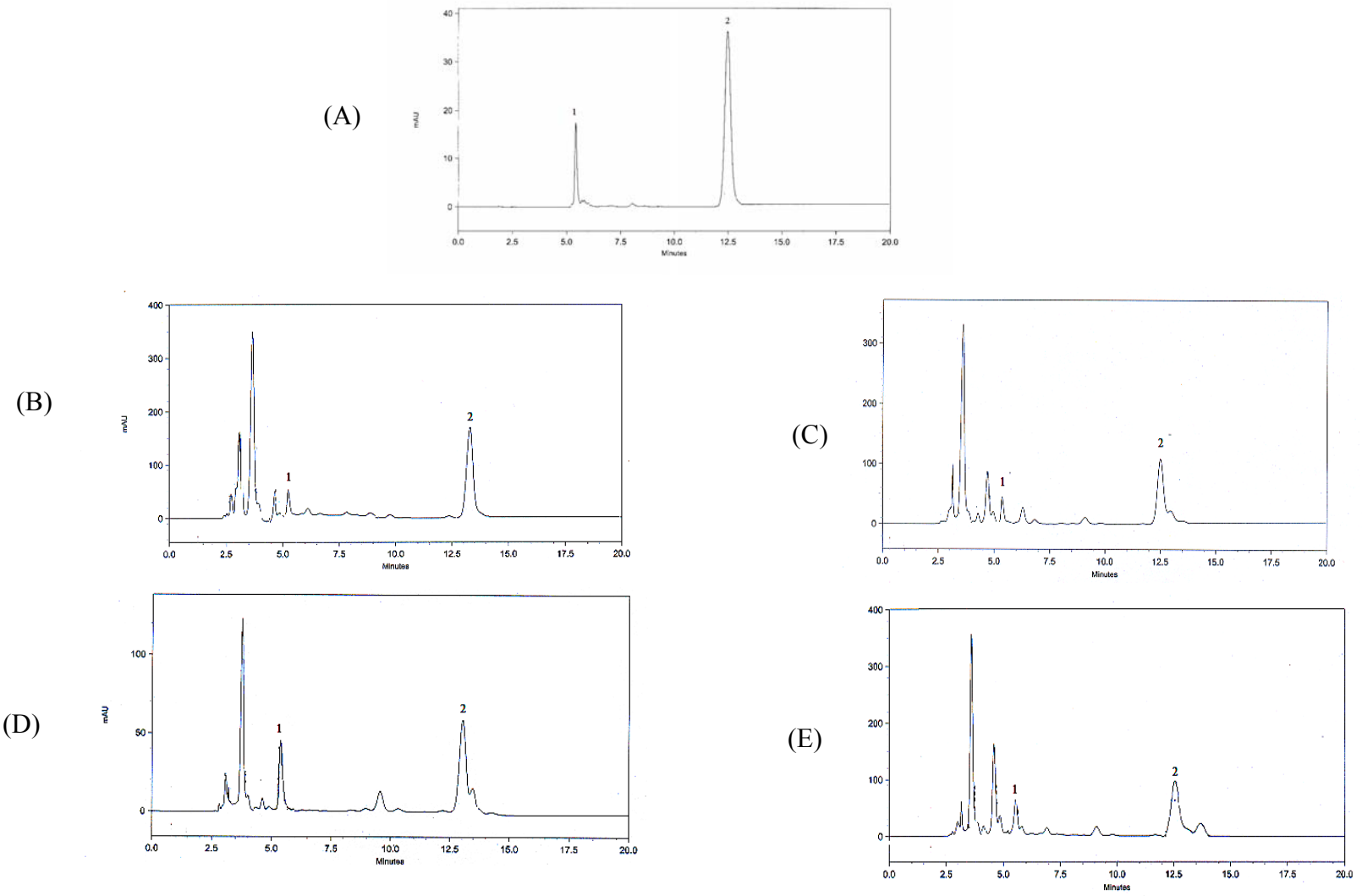


Fig.5. HPLC profile of carotenoid extracts of fresh and heat-processed carrot

A) Standard β-carotene; (B) Fresh carrot - Bioaccessible; (C) Pressure-cooked carrot - Bioaccessible; D) Open-pan-boiled carrot - Bioaccessible; (E) Stir-fried carrot - Bioaccessible

Peak #1: β-apo-8'-carotenal (Internal standard); Peak #2: β-carotene



. **Fig.6.** HPLC profile of carotenoid extracts of fresh and heat-processed amaranth
(A) Standard β-carotene; (B) Fresh amaranth – Total; (C) Pressure-cooked amaranth – Total; (D) Open-pan-boiled amaranth - Total (E) Stir-fried amaranth – Total
Peak #1: β--apo-8'-carotenal (Internal standard); Peak #2: β-carotene

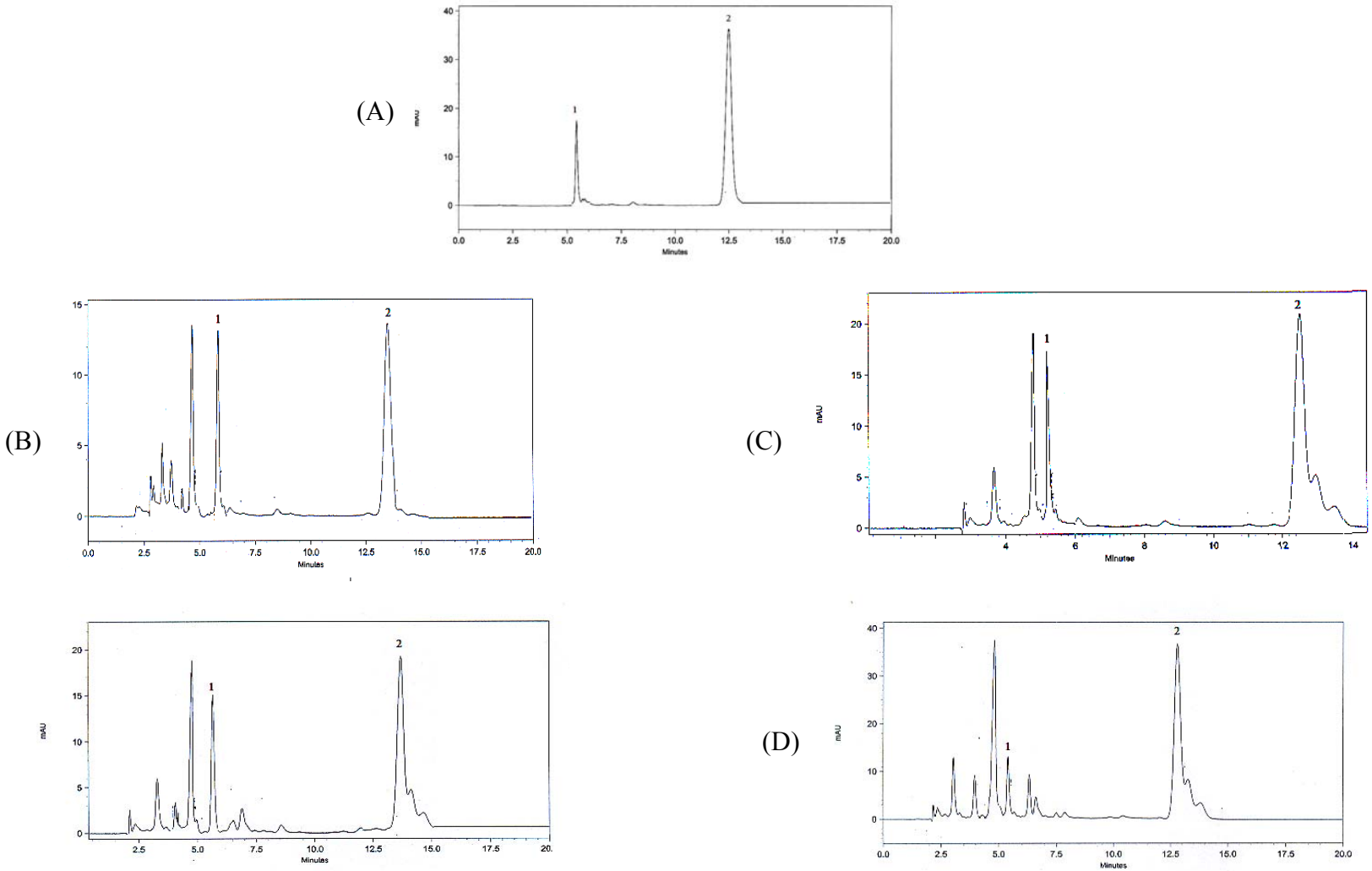


Fig.7. HPLC profile of carotenoid extracts of fresh and heat-processed amaranth
(A) Standard β-carotene; (B) Fresh amaranth - Bioaccessible; (C) Pressure-cooked amaranth - Bioaccessible; (D) Open-pan-boiled amaranth - Bioaccessible (E) Stir-fried amaranth – Bioaccessible
Peak #1: β--apo-8'-carotenal (Internal standard); Peak #2: β-carotene

Heat processing of food samples

Three methods of heat processing, namely, pressure-cooking, stir-frying and open-pan-boiling were employed in the study. For pressure-cooking, 2 g of the vegetable sample was cooked with 10 ml of distilled water at 15 p.s.i. for 10 min. In the case of stir frying 2 g of chopped vegetable samples were fried in a shallow pan in the presence of 185 mg of refined groundnut oil for 10 min at 100°C. In the case of open- pan- boiling, 2 g food materials were boiled in a open vessel in presence of 15 ml water for 10 min. (Water content was maintained throughout by appropriate intermittent additions).The heat-processed samples were homogenized before being subjected to simulated gastro-intestinal digestion.

Statistical analysis

All determinations were made in pentuplicates and the average values are reported. Data were analyzed statistically according to Snedecor and Cochran (1976).

RESULTS

Bioaccessibility of β-carotene from raw and heat processed yellow-orange vegetables

Table-6 and Fig.8 presents data on the bioaccessibility of β-carotene from the two fleshy yellow-orange vegetables subjected to heat processing by pressure-cooking, stir-frying, open-pan-boiling. Percent bioaccessibility of β-carotene in the two raw vegetables were 16.8 in carrot and 15.4 in pumpkin. All the three types of heat treatments had in general, a significant enhancing influence on the bioaccessibility of β-carotene from both the vegetables examined. Among the three cooking methods employed, stir-frying showed the maximum increase in percent bioaccessibility in both the yellow- orange vegetables, and the increases were 81 and 67% in the case of carrot and pumpkin, respectively. Open-pan-boiling produced higher increase in the % bioaccessibility as compared to pressure-cooking in case of these yellow-orange-vegetables. The bioaccessibility of this provitamin as a result of open pan boiling was increased by 36 and 23% in carrot and pumpkin respectively. Pressure-cooking produced 21% increase in the

Table-6. Effect of pressure-cooking, open-pan-boiling and stir-frying on the bioaccessibility of β-carotene from yellow-orange vegetables

Vegetables	Fresh		Pressure-cooked		Open-pan- boiled		Stir-fried	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Carrot	7504.0 ± 80.4	1260.7 ± 42.6	5134.5 ± 75.3	1532.7 ± 38.4	6740.3 ± 58.6	1716 ± 17.0	5926.4 ± 106.6	2280 ± 92.9
Pumpkin	1794.8 ± 5.03	276.0 ± 6.60	790.1 ± 16.1	294.1 ± 8.10	1205.3 ± 58.1	339.6 ± 7.80	817.6 ± 14.5	461.2 ± 18.5

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

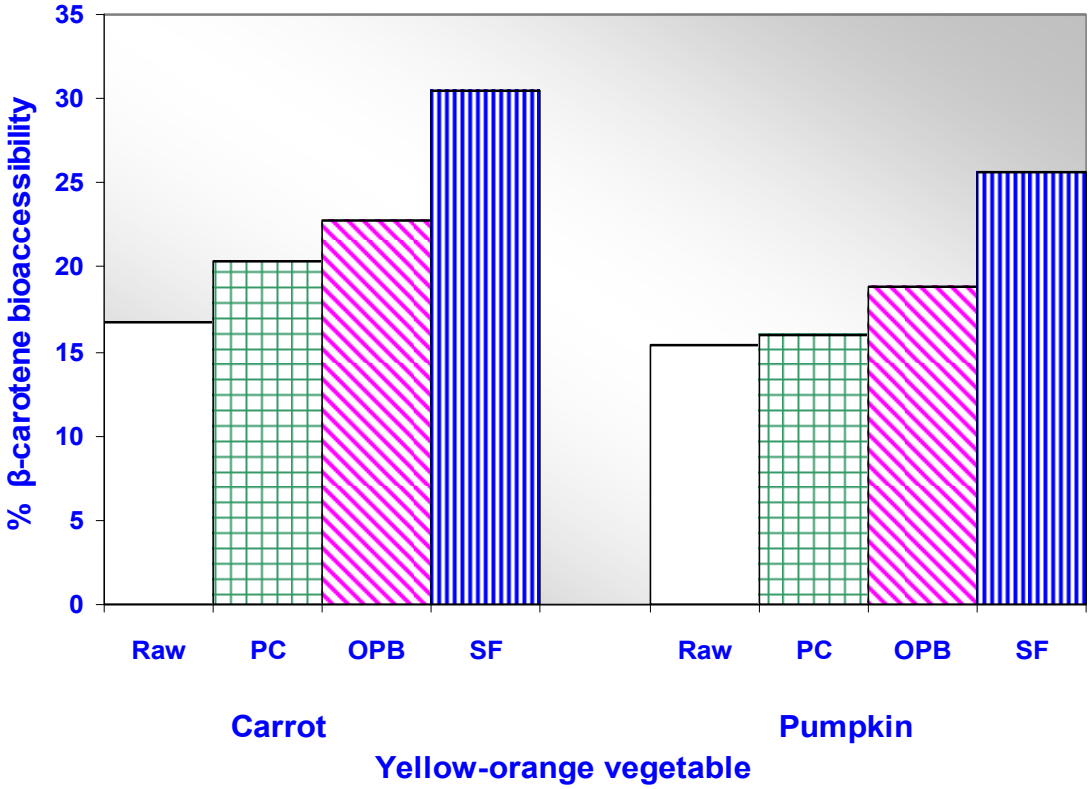


Fig.8. Bioaccessibility of β-carotene from raw and heat-processed yellow-orange vegetables

PC: Pressure-cooked; OPB: Open-pan boiled: SF: Stir-fried

% bioaccessibility of β-carotene in the case of carrot, while it did not have any significant influence on the same from pumpkin (Fig.8).

Bioaccessibility of β-carotene from raw and heat processed green leafy vegetables

Table-7 and Fig.9 presents data on the bioaccessibility of β-carotene from amaranth and fenugreek leaves subjected to heat processing by pressure-cooking, stir-frying, open-pan-boiling. Percent bioaccessibility of β-carotene in these two raw vegetables were 9.5 in amaranth and 7.5 in fenugreek leaves. All the three types of heat treatments significantly enhanced the bioaccessibility of β-carotene from both the green leafy vegetables examined. Stir-frying the vegetable in the presence of a small quantity of oil (9% w/w) brought about an enormous increase in the bioaccessibility of β-carotene from both the green leafy vegetables examined, the extent of increase being 192 and 158% in fenugreek leaves and amaranth, respectively. The extent of increase in the percent bioaccessibility of this provitamin as a result of pressure-cooking was 66 and 52% in fenugreek leaves and amaranth, respectively, whereas the increase in β-carotene bioaccessibility was 25 to 27% in the case of open- pan- boiling.

Table-8 and Fig.10 presents data on the bioaccessibility of β-carotene from few other green leafy vegetables subjected to heat processing by pressure-cooking and stir-frying. Since in the case of amaranth and fenugreek leaves, the increase in percent bioaccessibility produced by open-pan-boiling was least among the three heat treatment procedures tested, we have limited the evaluation of the influence of heat processing to two types viz. pressure-cooking and stir-frying for the other four green leafy vegetables (Table-8). Stir-frying these green leafy vegetables – spinach, drumstick leaves, coriander leaves, and mint leaves in the presence of a small quantity of oil (9% w/w) brought about an enormous increase in the bioaccessibility of β-carotene from these vegetables, the extent of increase being 149% (coriander leaves),136% (spinach), 128% (drumstick leaves), and 105% (mint leaves). Among these green leafy vegetables studied the extent of increase in the percent bioaccessibility of this provitamin as a result of pressure-cooking was highest in coriander leaves (84.2%) followed by spinach (52.9%), mint leaves (35.1%), and drumstick leaves (33.5%).

Table-7. Effect of pressure-cooking, open-pan- boiling and stir- frying on the bioaccessibility of β-carotene from green leafy vegetables

Vegetables	Fresh		Pressure-cooked		Open-pan-boiled		Stir-fried	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Amaranth	8046.3 ± 47.4	762.0 ± 21.9	5786.2 ± 29.0	1156.3 ± 36.1	4002.4 ± 56.4	956.3 ± 12.3	4810.3 ±72.5	1965.7 ± 0.5
Fenugreek leaves	9371.3 ± 204.3	704.7 ± 41.3	6145.4 ± 116.8	1169.5 ± 75.2	5037.6 ± 83.9	896.4 ± 10.9	6428.3 ± 106.9	2057.3 ± 95.9

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

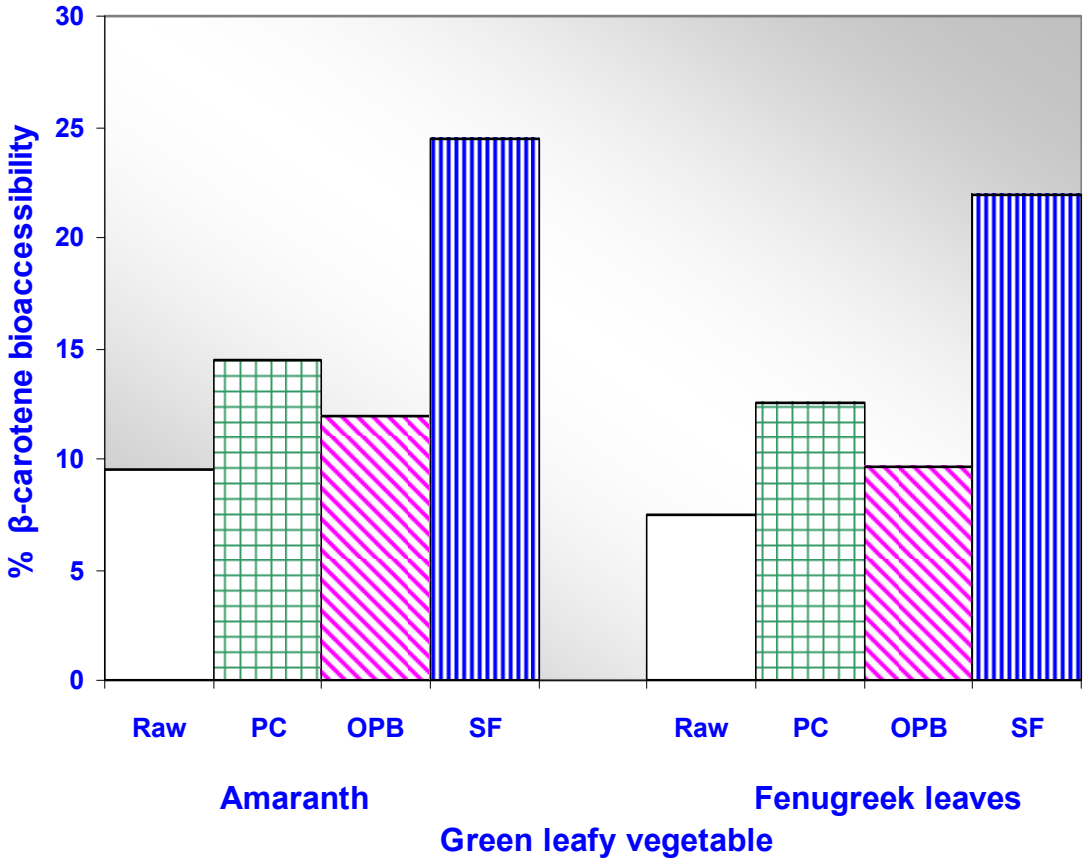


Fig.9. Bioaccessibility of β-carotene from raw and heat-processed green leafy vegetables

PC: Pressure-cooked; OPB: Open-pan boiled: SF: Stir-fried

Table-8. Effect of pressure- cooking and open-pan- boiling on the bioaccessibility of β-carotene from green leafy vegetables

Vegetable	Fresh		Pressure-cooked		Open-pan- boiled	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Spinach	4797.6 ± 174.1	580.2 ± 50.8	3037.2 ± 58.4	887.3 ± 43.4	3436.4 ± 38.9	1366.5 ± 64.0
Drumstick leaves	15735.4 ± 250.7	1811.3 ± 90.5	10411.2 ± 418.0	2418.5 ± 98.7	9157.2 ± 346.7	4129.4 ± 69.4
Corriandor leaves	4864.3 ± 196.3	683.2 ± 21.8	3722.2 ± 59.3	1256.3 ± 39.4	2971.6 ± 68.2	1699.5 ± 73.9
Mint leaves	5334.3 ± 53.9	606.7 ± 6.10	4828.4 ± 39.5	820.0 ± 9.80	4571.6 ± 23.5	1245.5 ± 38.1

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

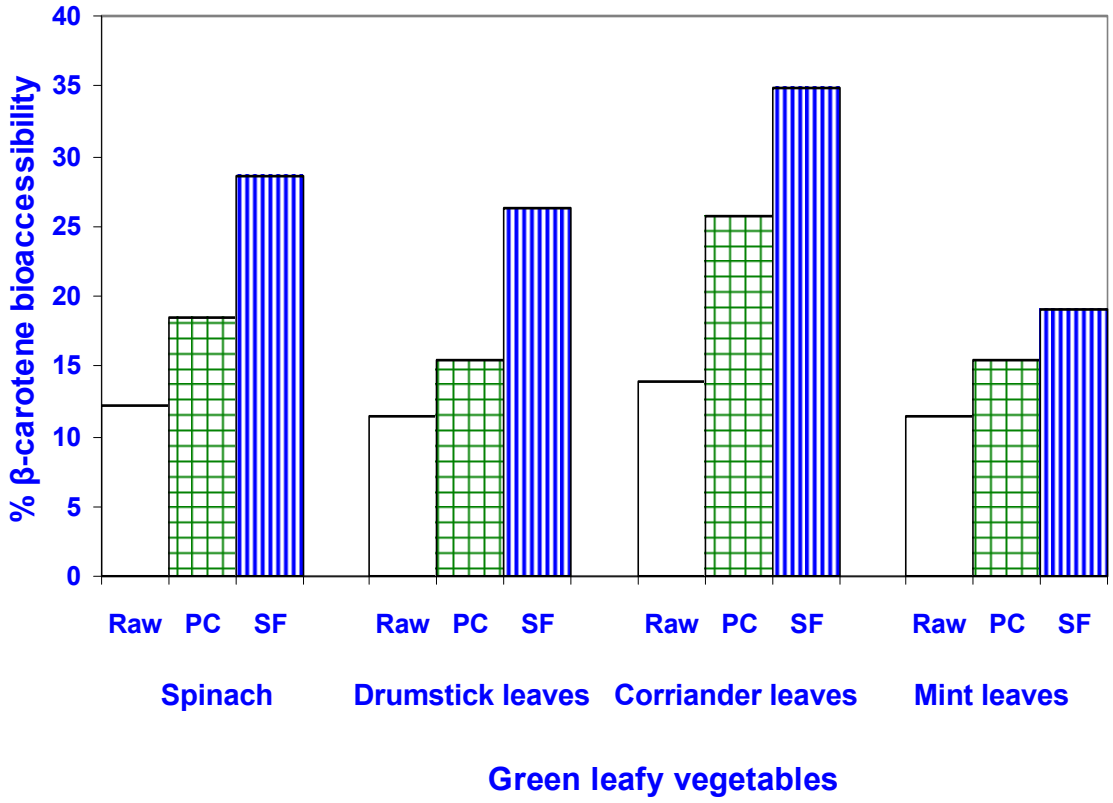


Fig.10. Bioaccessibility of β-carotene from raw and heat- processed green leafy vegetables

PC: Pressure-cooked; OPB: Open-pan- boiled: SF: Stir-fried

DISCUSSION

In view of the widespread prevalence of vitamin A deficiency, it is important to understand the extent of bioaccessibility of its precursor, β-carotene from plant foods which are the main sources of this pro-vitamin especially in India. In this investigation we have examined the influence of three types of heat processing, viz., pressure-cooking, stir-frying, and open-pan-boiling generally employed in Indian culinary practices. While *in vivo* methods to determine the same are tedious and time consuming, *in vitro* methods offer the advantages of being simpler, economical and less time-consuming. Such methods can be employed for the routine screening of several diverse plant foods for bioaccessibility of β-carotene.

The present investigation has evidenced that domestic cooking procedures that involve heat treatment either by pressure-cooking, stir-frying or open-pan-boiling considerably enhance the bioaccessibility of β-carotene from both fleshy yellow-orange vegetables as well as green leafy vegetables. Heat processing is believed to improve the bioaccessibility of β-carotene by loosening the food matrix and thus facilitating its absorption. Food processing, according to the type and intensity, influences food matrix and hence the bioavailability of carotenoids (van Het-Hof *et al.*, 2000).

Stir-frying of these vegetables produced highest increase in the bioaccessibility of β-carotene from all the vegetables tested. The oil *per se* used in stir-frying did not contribute to the β-carotene content of the sample. It is well known that presence of fat improves the bioavailability of β-carotene, which is lipophilic (Yeum and Russell, 2002). Similar increases in the bioaccessibility of β-carotene from carrot and amaranth as a result of heat processing in the presence of oil have been reported by other workers. An *in vivo* study conducted by Huang *et al*. (2000) reported bioavailability of 33% from carrot stir-fried in presence of 15% oil. The *in vitro* accessibility of β-carotene from carrot, cooked in the presence of 2% oil was found to be 39% (Hedren *et al*., 2002a). Thus, stir-frying could offer a good strategy to derive maximum amounts of bioavailable

β-carotene from dietary sources. While the bioaccessibility of β-carotene was higher from raw fleshy vegetables, heat processing brought about a higher increase in the same from the leafy vegetables. This difference could be due to the differences in the alteration of the matrices of these two varieties of vegetables as a result of heat processing.

Serrano *et al*. (2005) determined β-carotene available from green leafy vegetables by an *in vitro* digestion method and reported a β-carotene bioaccessibility of 22-67% from the food matrix by the action of digestive enzymes. Hornero-M and Minguez-M, (2007) who investigated the effects of cooking on the release and micellarization of carotenes from carrots observed that although cooking reduced the β-carotene content of carrot, a positive effect on the micellarization of carotenes and therefore on their bioaccessibility was found.

Hedren *et al*. (2002a, 2002) reported values of 21 and 18% respectively for the bioaccessiblity of β-carotene from carrot and amaranth leaves. The values obtained in the present study are lower (17 and 10%, respectively) compared to these reported values. The values reported for other green leafy vegetables, i.e., cooked sweet potato leaves and cooked pumpkin leaves were 9 and 10% respectively (Hedren *et al*., 2002). The value obtained in our study for the flesh portion of the pumpkin (16.3%) is thus higher than that reported for pumpkin leaves.

While the current investigation has documented the beneficial enhancing effect of cooking procedures on the bioaccessibility of β-carotene from the potential vegetable sources of the same, it should also be noted that heat treatment of plant foods generally results in decrease in the content of this provitamin carotenoid. Several researchers have studied the effect of cooking on the total content of carotenoids in vegetables. Losses of β-carotene during cooking of green leafy vegetables have been reported in the range of 24-50% (Jayarajan *et al*., 1980), 51-83% (Dikshit *et al*., 1988), and 18-61% (Padmavati *et al*., 1992). Our present study is in agreement with studies that have reported losses of β-carotene from vegetables due to cooking. We recorded 10-56% losses of β-carotene from vegetables subjected to pressure-cooking, 10-50% losses when vegetables were

open-pan-boiled and 21-39% losses when vegetables were subjected to stir-frying. The differences in the amount of β-carotene retained among different vegetables cooked by the same method is possibly due to differences in the degree of susceptibility of these vegetables to heat treatment as a result of differences in their matrices.

Carotene losses in some selected vegetables cooked by different methods have been reported by Rao and Reddy (1979). Loss of carotene in amaranth, spinach and drumstick leaves during stir frying has been reported to be as much as 58-70% (Rao and Reddy, 1979). Loss of carotene during stir-frying, open- boiling, and pressure-cooking was 55, 50, and 14% respectively for cabbage and 55.5, 7.4, and 2.6% respectively for carrot. Padmavati *et al*. (1992) have reported that prolonged cooking and cooking methods preceded by grinding and chopping resulted in progressive loss of β-carotene. Loss of β-carotene was twice in deep fried vegetables when compared to shallow fried vegetables.

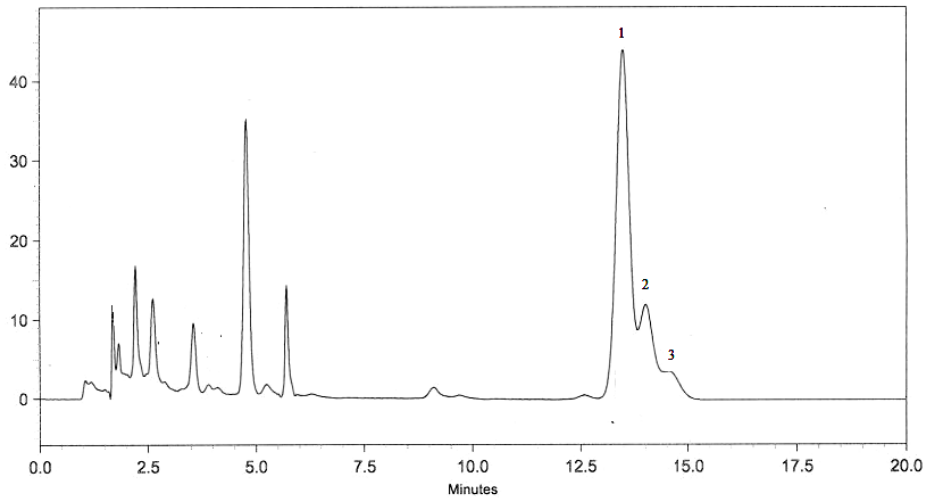
The carotene losses from green leafy vegetables have been reported to be more in traditional method of cooking (open-boiling in excess water) and least in pressure–cooking (Sood and Bhat, 1974). Higher losses in traditional method have been attributed to: (i) enzyme oxidation during time lag between preparation and cooking, (ii) longer time involved in cooking, and (iii) practice of cooking in the open which exposes the food material to atmospheric oxidation.

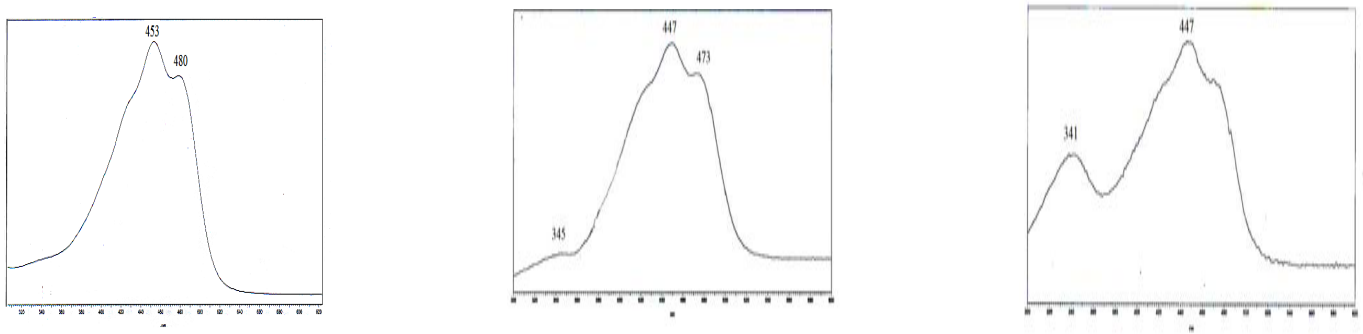
Gayathri *et al*. (2004) studied the extent of retention of β-carotene in carrot, pumpkin, amaranth and drumstick leaves during their cooking by pressure-cooking and open-pan boiling. Among the two heat processing methods employed in the study, pressure cooking reduced the β-carotene content of the two fleshy vegetables to a greater extent than did boiling in water for a similar period. On the other hand, higher losses of β-carotene occurred during open-pan-boiling of leafy vegetables than during pressure cooking. Higher losses of β-carotene during open-pan-boiling of amaranth and drumstick leaves as compared to pressure-cooking could be attributed to higher oxidative destruction in the open system occurring in the case of leafy vegetables.

Heat processing has also been found to induce the formation of *cis* isomers from all *trans*- β-carotene and the quantity formed is related to the severity and length of heat treatment (Chandler and Schwartz, 1988). In our study, we observed that heat processing of green leafy vegetables resulted in the formation of 9-*cis* and 15-*cis*- β-carotene to an extent of 8-10% relative to all- *trans*-β-carotene; while these *cis* isomers were present only in trace amounts in the raw vegetable (Fig.11). Around 16% formation of 9-*cis*-β-carotene relative to all-*trans*-β-carotene in vegetables cooked for 15-30 min has been reported (Mulokozi *et al*., 2004). The lesser formation of *cis i*somers in our study could be due to the shorter duration of cooking employed by us, viz.,10 min.

The carotenoids were identified by the UV-visible absorption spectra, specifically the wavelength of maximum absorption. The *cis*-isomers were tentatively identified by their λ_{max} which was slightly lower than those of the all-*trans*-carotenoids and also by the presence of the '*cis*' peak at about 142 nm below the longest-wavelength absorption maximum of the all-*trans* form. The location of the *cis*-double bond was indicated by the percentage of ratio of peak heights A_B/A_{II}, where A_B is the height of the *cis* peak and A_{II} is the height of the middle main absorption peak (Britton, 1995). This ratio is the indicator of the intensity of the '*cis'* peak, which is greater when the *cis*-double bond is closer to the centre of the molecule. The *cis*-isomers were first perceived by the visible absorption spectra having λ_{max} 2-6 nm lower than those of the all-*trans* carotenoids and the appearances of a '*cis'* peak. The % A_B/A_{ii} of 16 and 57 were thus identified as 9-*cis*-β-carotene and 15-*cis*-β-carotene, respectively (Mercadante *et al.*, 1999).

In the case of aqueous micellar fraction obtained after *in vitro* digestion of the vegetables (which represented the bioaccessible β-carotene), low or even undetectable amounts of 9-or 15-*cis*-β-carotene were present (Fig.11). This is consistent with the fact that all-*trans*-β-carotene is the more bioavailable form of this provitamin (Erdman *et al*., 1998). Thus, our studies which have mainly focused on the bioaccessible amounts of β-carotene, have limited the quantitation to all-*trans*-β-carotene alone in the aqueous micellar fraction.





A B C

Fig.11. HPLC profile of *trans* and *cis* isomers of β-carotene isolated from heat-processed amaranth (A) all-*trans*-β-carotene; (B) 9-*cis*-β-carotene; (C) (15-*cis*- β-carotene.

Garrett *et al*. (2000) have recently demonstrated that the *in vitro* digestion model, which was earlier used for determining the bioaccessibility of β-carotene in highly processed infant foods, is also suitable for minimally processed foods such as stir-fried vegetables (which involved stir-frying in vegetable oil at 177^{o}C for 4 min). This *in vitro* digestion system serves as a simple model for screening the relative bioavailability of carotenoids in various plant foods. This quick procedure may particularly be useful for providing insights about dietary strategies for improving vitamin A status in populations that heavily rely on plant foods as the primary source of this vitamin.

Thus, heat processing of yellow-orange and green leafy vegetables by pressure-cooking, stir-frying, and open-pan-boiling was found to significantly improve the bioaccessibility of β-carotene. The higher bioaccessibility was particularly prominent when the vegetables were stir-fried in the presence of oil. This study suggests that the use of suitably heat-processed vegetable sources of β-carotene could form a dietary strategy to derive this micronutrient maximally.

SUMMARY

Effect of heat treatment involved in domestic cooking procedures on the bioaccessibility of β-carotene from yellow-orange as well as green leafy vegetables was evaluated. Heat treatment of these vegetables by pressure cooking, stir-frying, and open-pan boiling had a beneficial influence on the bioaccessibility of β-carotene from these vegetables. The extent of increase in the percent bioaccessibility of β-carotene as a result of pressure-cooking was 21 - 84%. Stir-frying in presence of a small quantity of oil brought about an enormous increase in the bioaccessibility of β-carotene from these vegetables, the extent of increase being 67 - 192%. Open-pan-boiling of vegetables increased the bioaccessibility of β-carotene in the range 23 – 36%. This observation suggests that the use of suitably heat-processed vegetable sources of β-carotene could form a dietary strategy to derive this micronutrient maximally.

SECTION-B

Influence of food acidulants and antioxidant spices on the bioaccessibility of β-carotene from vegetables

INTRODUCTION

Deficiency of vitamin A is a serious public health problem leading to blindness. A majority of the population in India is dependent on plant foods, which provide carotenoids, especially β-carotene; to meet their requirement of vitamin A. β-Carotene is abundantly found in green leafy and yellow-orange vegetables (Gopalan *et al*., 2004). Studies have shown that absorption of carotenoids from uncooked foods is low, and that mild cooking enhances the same (Rock *et al.* 1998). However, heat treatment especially in the presence of light and oxygen causes isomerization of carotenes as well as its oxidative destruction, thus decreasing its biological activity (Sweeney and Marsh, 1971). Apart from food processing methods, several factors such as diet composition could affect the bioaccessibility of β-carotene from foods (Rodriguez and Irwin, 1972).

An earlier study in our laboratory revealed that inclusion of food acidulants - citric acid and tamarind, and antioxidant spices - turmeric and onion during heat processing of vegetables generally improved the retention of β-carotene in the same (Gayathri *et al*., 2004). In view of the fact that a majority of the Indian population is dependent on plant foods to meet their requirement of vitamin A, it is desirable to evolve dietary strategies to improve the bioavailability of β-carotene from these sources. Organic acids such as citric, ascorbic, malic acid, etc. are known to promote the bioavailability of iron from plant foods (Gillooly *et al*., 1983). Similar enhancement of bioaccessibility of zinc from cereals and pulses by these food acidulants / organic acids has also been recently evidenced by us (Hemalatha *et al*., 2005). It is possible that these common food ingredients may have a beneficial promoting effect on the bioaccessibility of β-carotene from its vegetable

sources through modification of food matrix under conditions of low pH, and also contributing to higher retention of this provitamin during food processing.

The food acidulants commonly used in Indian cuisine include amchur (dry raw mango [*Mangifera indica*] powder), lime (*Citrus)* juice, tamarind (*Tamarindus indica*) and kokum (*Garcinia indica*). Turmeric (*Curcuma longa*) and onion (*Allium sepa*) are the spices that are well known to possess antioxidant property, and are also commonly used in Indian households (Srinivasan, 2005) (Fig.12,13). These antioxidant spices, when present in foods could contribute to minimizing the oxidative destruction of β-carotene during heat processing of foods. Information on the effect of food acidulants and antioxidant spices on the bioaccessibility of β-carotene from vegetables is, at present, lacking.

Knowledge on the effects of other food ingredients such as acidulants and antioxidants, with respect to retention of this provitamin and consequent changes in the bioaccessibility of the same is necessary for evolving food based strategies for deriving adequate / optimal amounts of β-carotene. The present investigation was therefore undertaken to examine the effect of these food ingredients on the bioaccessibility of β-carotene from select green leafy and yellow-orange vegetables. In this context, the commonly used food acidulants – lime (Citrus), amchur (*Mangifera indica*), tamarind (*Tamarindus indica*) and kokum (*Garcinia indica*), and antioxidant spices – turmeric (*Curcuma longa*) and onion (*Allium cepa*) were evaluated for their influence on the bioaccessibility of β-carotene from plant foods – both yellow-orange as well as green leafy vegetables.

MATERIALS AND METHODS

Materials

Fresh carrot (*Daucus carota*), pumpkin (C*ucurbita maxima*), amaranth (*Amaranthus gangeticus*) leaves and fenugreek (*Trigonella foenum-graecum*) leaves were procured from local vendors, cleaned and the edible portions used for the study. The other

ingredients employed, namely, amchur (*Mangifera indica*), lime (Citrus), tamarind (*Tamarindus indica*), kokum (*Garcinia indica*), turmeric (*Curcuma longa*) and onion (*Allium cepa*) powder were locally procured. All chemicals used were of analytical grade. Solvents were distilled before use. Standard β-carotene, porcine pancreatic pepsin, and pancreatin and bile extract (porcine) were procured from Sigma Chemicals Co., St. Louis, USA. Double-distilled water was employed throughout the entire study. All glassware used was acid washed.

Food sample preparation

Carrot and pumpkin were cut to a uniform size, while the edible portion of the two leafy vegetables was finely chopped. The test vegetables (10 g portions) were used for the study in the following combinations: (1) Test vegetable alone; (2) Test vegetable + acidulant (amchur / lime / tamarind / kokum); test vegetable + antioxidant spice (turmeric powder / onion powder). The acidulants were added at amounts that reduced the pH of the food by 1 unit, the levels added being 3, 7, 2.5 and 3% for amchur, lime juice, tamarind and kokum, respectively. Turmeric and onion powder were included at 1% level. The above combinations were also subjected to heat processing by two methods, namely, pressure-cooking and open-pan-boiling.

In a separate experiment, to evaluate the influence of a combination of a food acidulant and an antioxidant spice on the bioaccessibility of β-carotene, carrot and amaranth were chosen. These test vegetables (10 g) were used for the study in the following combinations: (1) Test vegetable alone; (2) Test vegetable + acidulant (7% lime / 2.5% tamarind); Test vegetable + 1% turmeric powder, and Test vegetable + 7% lime / 2.5% tamarind + 1% turmeric powder. The above combinations were also subjected to heat processing by two methods, namely, pressure-cooking and open-pan-boiling.

Determination of bioaccessibility of β-carotene in vitro

The bioaccessibility of β-carotene *in vitro* was determined by the method of Garrett *et al* (1999) with suitable modification (Veda *et al*., 2006). Briefly, the method involved subjecting the sample to simulated gastric digestion at pH 2.0 in the presence of pepsin at 37°C (16 g in 100 ml 0.1M HCl), followed by simulated intestinal digestion in the presence of pancreatin-bile extract mixture (4 g porcine pancreatin and 25 g bile extract (porcine) in 1000 ml of 0.1M $NaHCO_3$), pH 7.5 at 37°C for 2 h. At the end of simulated intestinal digestion, the aqueous micellar fraction was separated by ultracentrifugation at 70,000 x g for 120 min using a Beckman L7-65 ultracentrifuge. β-Carotene present in the aqueous micellar fraction represents the portion that is bioaccessible (As in Chapter-II).

Analysis of β-carotene

β-Carotene was extracted from the samples initially with a mixture of acetone : ethanol (1:1) and subsequently with petroleum ether (Hedren *et al*., 2002). The process was repeated several times to ensure complete extraction of β-carotene. In the case of GLVs, the extract was saponified (in an additional step to remove chlorophyll) with 30% methanolic potassium hydroxide at room temperature for 3 h. Following saponification, the alkali was removed completely by repeated washing, and the solvent was evaporated to dryness in a rotary evaporator. The residue was redissolved in petroleum ether and stored in the cold pending analysis. Prior to analysis, the petroleum ether was evaporated under nitrogen and the residue was dissolved in the mobile phase used for HPLC determination.

Determination of β-carotene was carried out by reverse-phase HPLC (Shimadzu LC 10 AVP), equipped with a PDA detector. β-Carotene was separated on a C_{18} column (S.S.Excil, Australia). The mobile phase consisted of a mixture of 65% (v/v) acetonitrile, 15% (v/v) methylene chloride and 20% (v/v) methanol containing 1.3 mmol/l ammonium acetate. β-Carotene (all-*trans*-isomer) was monitored at a wavelength of 450

nm. The peak identities and λ_{max} were confirmed by their retention time and characteristic spectra of standard chromatograms.

During the steps of simulated gastrointestinal digestion, ultracentrifugation and extraction of β-carotene, precautions were taken to minimize the exposure of samples to light and air and thus prevent oxidative destruction of β-carotene. Air was replaced by nitrogen before stoppering the flask at all stages of incubation and storage. The experiments were carried out under yellow lighting and all the glassware was covered with black cloth to prevent exposure to light.

Statistical analysis

All determinations were made in pentuplicates and the average values are reported. Data were analyzed statistically according to Snedecor and Cochran (1976).

RESULTS

Effect of food acidulants on the bioaccessibility of β-carotene from vegetables

The effect of food acidulants- amchur, lime, tamarind and kokum on the bioaccessibility of β-carotene from the test vegetables is presented in Tables 9-12. Table-9 presents the influence of food acidulants on the bioaccessibility of β–carotene from pumpkin. Inclusion of amchur significantly increased the bioaccessibility of β-carotene from raw as well as heat-treated pumpkin. The increase brought about in percent bioaccessible fraction was from 15.8 to 21.0 in the case of raw vegetable, from 17.1 to 24.0 in the case of pressure-cooked vegetable, and from 19.4 to 21.4 in the case of open-pan-boiled vegetable (Fig.14). Thus, the extent of increase in bioaccessibility of β-carotene from pumpkin caused by amchur amounted to 32.6, 40.0 and 10.0% from raw, pressure-cooked and open-pan-boiled pumpkin, respectively. A similar significant increase in the percent bioaccessible fraction of β-carotene from raw as well as heat-treated pumpkin was also evidenced with the addition of lime juice, the extent of increase in the bioaccessibility of β-carotene being 102% (an increase from 15.8 to 31.8%)

32.2% (an increase from 17.1 to 22.6%) and 16.4% (an increase from 19.4 to 22.6%) in raw, pressure-cooked and open-pan-boiled pumpkin, respectively. Unlike amchur and lime juice, tamarind and kokum generally did not influence the bioaccessibility of β-carotene from pumpkin. On the other hand, these significantly decreased the same from open-pan-boiled pumpkin (14.7 and 24.5%, respectively). Tamarind also negatively influenced bioaccessibility of β-carotene, from raw pumpkin (23.6%).

The effect of acidulants on the bioaccessibility of β–carotene from carrot is presented in Table-10. Lime juice brought about an increase in percent bioaccessibility of β-carotene from raw carrot, the extent of increase being 14.2% (from 15.5 to 17.7%) (Fig.15). Amchur, on the other hand, had no effect on the bioaccessibility of β-carotene from either raw or heat-processed carrot. As in the case of pumpkin, tamarind negatively influenced the bioaccessibility of β-carotene from open-pan-boiled carrot (36.6% decrease), while kokum decreased the same from pressure-cooked carrot by 11.5% (Fig.15).

Table-11 presents the influence of acidulants on β–carotene bioacessibility from fenugreek leaf. Amchur brought about a significant increase in the bioaccessibility of β–carotene from pressure-cooked fenugreek leaf, the percent increase being 22.2% (increase from 14.4 to 17.6%) (Fig.16). The bioaccessibility of β–carotene from fenugreek leaf was also increased by the presence of lime juice, the percent increase being 12.4, 14.6 and 28 in the raw (increase from 7.8 to 8.75%), pressure-cooked (from 14.4 to 16.5%) and open-pan boiled (from 9.1 to 11.6%) leafy vegetable, respectively (Fig.16). Tamarind and kokum did not have any effect on the bioaccessibility of β–carotene from either raw or heat-processed fenugreek leaf.

The effect of acidulants on the bioaccessibility of β-carotene from amaranth leaf is presented in Table-12. Amchur brought about a significant increase in the bioaccessibility of β-carotene from pressure-cooked amaranth leaf (19.6% increase) (Fig.17).

In the case of lime juice, the positive effect was limited to open-pan-boiled amaranth leaf, where the percent β-carotene bioaccessibility was 14.7, compared to 11.8 in the absence of the food acidulant. Tamarind, on the other hand, brought about a decrease in the bioaccessibility of β-carotene from raw amaranth leaf (23.8% decrease), while kokum decreased the same from pressure-cooked amaranth leaf (13.1% decrease) (Fig.17).

Effect of antioxidant spices on the bioaccessibility of β-carotene from vegetables:

The effect of turmeric and onion on the bioaccessibility of β-carotene from pumpkin and carrot is shown in Table-13 and 14. In the case of pumpkin, the beneficial influence of turmeric was evident in both open-pan-boiled as well as pressure-cooked pumpkin, the effect being much more in the case of the former (Table-13). The percent increase in β-carotene bioaccessibility was 54.2 and 23.4, for open-pan-boiled and pressure-cooked pumpkin, respectively (Fig.18). As in the case of turmeric, onion did not have any effect on the bioaccessibility of β-carotene from raw pumpkin. Onion enhanced the bioaccessibility of β-carotene from open-pan-boiled pumpkin (13.9%) (Fig.18).

While turmeric did not have any effect on the bioaccessibility of β-carotene from the raw vegetables when included at 1% level, it had an enhancing influence on the same from pressure-cooked carrot (24.6% increase) (Fig.19). Onion brought about a 27.8% increase in β-carotene bioaccessibility from raw carrot. Onion enhanced the bioaccessibility of β-carotene from pressure-cooked carrot (24.5% increase) (Fig.19).

The effect of the antioxidant spices turmeric and onion on the bioaccessibility of β–carotene from the two green leafy vegetables – amaranth and fenugreek leaves is presented in Tables 15 and 16. As in the case of carrot and pumpkin, turmeric significantly increased the bioaccessibility of β-carotene from the heat-processed green leafy vegetables, this effect being more prominent in the case of open-pan-boiled GLV. The extent of increase in percent bioaccessibility of β-carotene was 68.7 and 36.3 from open-pan-boiled amaranth and fenugreek leaf, while it was 24.2 and 15.3 from pressure-cooked amaranth and fenugreek leaf (Fig.20 and 21). Onion enhanced the

bioaccessibility of β-carotene from pressure-cooked amaranth leaf (22%) (Fig.20), and open-pan-boiled fenugreek leaf (26.6%) (Fig.21).

Effect of a combination of acidulant and turmeric on the bioaccessibility of β-carotene from vegetables:

The effect of a combination of a food acidulant - lime / tamarind and turmeric on the bioaccessibility of β-carotene from carrot and amaranth is presented in Tables 17 and 18. While lime and turmeric individually enhanced the bioaccessibility of β-carotene from raw carrot, a combination of lime and turmeric did not produce any higher effect (compared to the individual effects) on the same, (Table-17 and Fig.22). Similarly, the combination of lime and turmeric did not produce an effect higher than that of turmeric alone in the case of pressure-cooked carrot or than that of lime in the case of open-pan-boiled carrot (Table-17 and Fig.22).

In the case of the combination of tamarind and turmeric, the content of bioaccessible β-carotene was lower than the sample having turmeric alone in raw, pressure-cooked or open-pan-boiled carrot (Table-17 and Fig.22). The positive effect of turmeric on the bioaccessibility of β-carotene was completely countered in the case of pressure-cooked carrot by the presence of tamarind.

The content of bioaccessible β-carotene in open-pan-boiled carrot in presence of tamarind and turmeric was significantly decreased to a value lesser than that of open-pan- boiled carrot alone. In other words, the combination had a negative influence on the bioaccessibility of β-carotene (Table-17 and Fig.22).

Whereas lime had a positive influence on the content of bioaccessible β-carotene in the case of open-pan-boiled amaranth, and turmeric had a similar influence on the same in both pressure-cooked and open-pan-boiled amaranth, a combination of lime and turmeric did not have any additive effect with regard to bioaccessibility of β-carotene in raw / pressure-cooked / open-pan-boiled amaranth (Table-18 and Fig.23).

The presence of tamarind along with turmeric totally countered the positive effect of turmeric on the bioaccessibility of β-carotene from pressure-cooked and open-pan-boiled amaranth. In case of raw amaranth the values of bioaccessible β-carotene in presence of combination of tamarind and turmeric was lower than that of amaranth alone and was comparable to that of amaranth + tamarind (Table-18 and Fig.23).

Thus, there was no additive or synergestic effect on the bioaccessibility of β-carotene when lime and turmeric were used in combination, while these two individually generally enhanced the same. Tamarind which by itself did not have a beneficial influence on the bioaccessibility of β-carotene from vegetables, even countered the positive influences of turmeric in heat processed vegetables (Table-18 and Fig.23).

In order to verify if the higher bioaccessibility of β-carotene observed in the presence of exogenous food acidulants / antioxidant spices was a result of minimized loss of this provitamin during heat treatment, concentrations of β–carotene recovered in the two types of heat processing in the presence and absence of the tested food acidulants and antioxidant spices were computed and are presented in Tables 19-22. Incidentally, higher retention of β-carotene was observed in all the four vegetable samples subjected to either pressure-cooking or open-pan-boiling in the presence of lime juice (Table-19-22). Such a prevention of loss of β-carotene by heat processing was restricted to fenugreek leaves in the case of amchur (Table-21). Tamarind and kokum also had a sparing action on the loss of β-carotene from the test vegetables in a few instances. The antioxidant spice turmeric had a remarkable protective effect on the loss of β-carotene during heat processing of the test vegetables, the effect being more prominent in the case of yellow-orange vegetables (Tables 19-20). Onion too offered a similar protection during the heat processing of the four test vegetables (Tables 19-22), the effect being more prominent in the case of carrot (Table-20).

DISCUSSION

The present investigation has documented the beneficial bioaccessibility enhancing effect of two common food acidulants- amchur and lime juice in both raw and cooked vegetables. The beneficial effect of antioxidant spices was observed only during heat processing of these vegetables. We have earlier observed that acidulants (citric acid and tamarind) and antioxidant spices (turmeric and onion) prevented the loss of β-carotene during heat processing of vegetables (Gayathri *et al*., 2004). In the present study, the acidulant lime juice, and the antioxidant spices turmeric and onion improved the retention of β-carotene in vegetables during heat-processing.

Among the four food acidulants examined, lime juice and amchur enhanced the bioaccessibility of β-carotene from the test vegetables (both yellow-orange and green leafy), while tamarind and kokum did not have a similar effect. The enhancing effect of lime juice on the bioaccessibility of β-carotene seems to be higher than that of amchur. These findings on the effect of food acidulants on the bioaccessibility of β-carotene are in agreement with our earlier observation on their effect on mineral bioaccessibility, where citric acid and amchur generally enhanced the bioaccessibility of zinc and iron, while tamarind and kokum did not have a similar effect (Hemalatha *et al*., 2005). Whether the lack of an enhancing effect of tamarind and kokum on β-carotene bioaccessibility in spite of a decrease in the pH similar to amchur or lime and their negative effect in a few instances could be attributable to the high tannin content present in these two acidulants needs to be ascertained. These two acidulants contain significant amounts of tannin, as observed by us earlier (Hemalatha *et al*., 2005). Thus, food acidulants appeared to have an influence on β-carotene bioaccessibility from vegetables very similar to their influence on bioaccessibility of iron and zinc from staple grains. The food acidulants amchur and lime probably exerted a favourable influence on β-carotene bioaccessiblity through loosening of the matrix, thereby rendering β-carotene more bioaccessible.

In a previous study, it was indicated that bioaccessibility of β-carotene from six specific mango varieties roughly corresponded with the organic acid content of these fruits. *i.e*. the variety with highest organic content also showed the highest β-carotene bioaccessiblity, and *vice versa* (Veda *et al*., 2007). While organic acids are not the only modifiers of β-carotene bioaccessibility; and that other factors, especially fibre and carotenoids other than β-carotene (which have not been determined here) may also influence the same. While it is known that acids promote the isomerization of all-*trans*-β-carotene to *cis*-isomers which are less absorbed than the all-*trans*-isomer, it is to be noted that the food acidulants exogenously added here did not make the system drastically acidic (lowered the pH by just one unit) so as to cause significant extent of isomerization of β-carotene.

Among the two antioxidant spices, the effect of turmeric appeared to be higher in the case of vegetables subjected to open-pan-boiling. These antioxidant spices have contributed to higher retention of β-carotene (minimizing the loss due to heat / exposure to air) and hence its bioaccessibility. This is consistent with the observation that the effect of these spices on the bioaccessibility of β-carotene was not apparent in the case of raw vegetables. In the case of antioxidant spices, the improved bioaccessibility of β-carotene from heat-processed vegetables is attributable to their role in minimizing the loss of this provitamin during heat treatment. The food acidulant–lime juice which enhanced the bioaccessibility of this provitamin from both raw and heat-processed vegetables probably exerted this effect by some other mechanism in addition to minimizing the loss of β-carotene.

Heat processing of vegetables by pressure-cooking or open-pan-boiling generally improved the bioaccessibility of β-carotene, which is consistent with our earlier observation with pressure-cooked or stir-fried vegetables (Chapter–IIIA). In the present study, the bioaccessibility enhancing effect of pressure-cooking was greater than that of open- pan- boiling in the case of the two green leafy vegetables, while both the methods of heat treatment had a similar effect on the yellow-orange vegetables . The

bioaccessibility enhancing effect of either the food acidulants or antioxidant spices discussed above is over and above that brought about by heat processing of the vegetables alone.

The *in vitro* method employed here for the estimation of β-carotene availability is based on simulation of gastro-intestinal digestion and estimation of the proportion of this nutrient convertible to an absorbable form in the digestive tract, by measuring the fraction that gets into the aqueous micellar portion. This method has been well standardized and internationally accepted. The bioaccessibility of β-carotene gives a fair estimate of its availability for absorption *in vivo* (bioavailability). Such *in vitro* methods are rapid, simple and inexpensive. The *in vitro* method that measures bioaccessibility provides relative rather than absolute estimates of β-carotene absorbability since they are not subjected to the physiological factors that can affect bioavailability.

Reports on the effect of acidulants and antioxidant spices on the bioaccessibility of β-carotene from vegetable sources are lacking, and, to our knowledge, this is the first report of its kind. These food ingredients, especially antioxidant spices, have the double advantage of minimizing loss of β-carotene during heat processing, as well as of enhancing the bioaccessibility of thus retained provitamin from vegetables. The amounts of food acidulants and antioxidant spices observed to bring about such a beneficial influence on β-carotene bioaccessibility are those that are normally encountered in traditional food practices in India. It is a common practice to add turmeric powder before heat processing of dishes containing vegetables. Onion is also used in a majority of such dishes. The amount of onion commonly used as a vegetable in many dishes along with green leafy and yellow-orange vegetables probably surpasses the amount evidenced here to produce a beneficial effect on bioaccessibility of β-carotene; therefore, the magnitude of this effect may actually be even higher. Thus, probably a majority of our population is already practicing a food-based strategy to maximize β-carotene bioavailability.

Population in the northern parts of India commonly uses amchur as the food acidulant in preference to tamarind or kokum, which are commonly used in southern parts of India. Lime juice is used in dishes throughout the subcontinent. Thus, the liberal presence of food acidulants such as lime juice and amchur and antioxidant spices- turmeric and onion prove to be advantageous in the context of deriving maximum β-carotene from the vegetable sources.

On addition of any acidulant or antioxidant spices to the vegetables, we have observed an increase in the retention of β-carotene indicating that such additions are beneficial from the point of view of β-carotene retention. However, among the four acidulants examined, lime juice and amchur enhanced the bioaccessibility of β-carotene from the test vegetables, whereas tamarind and kokum did not have the same benefit. Among the two antioxidant spices, turmeric significantly enhanced the bioaccessibility of β-carotene from all the vegetables tested. Thus, a combination of lime, an acidulant and turmeric, each of which having positive influence on the bioaccessibilty of β-carotene, was also examined for their influence on the bioaccessibility of β-carotene. This study indicated that a combination of food acidulant and an antioxidant spice which individually produced a higher bioaccessibility of β-carotene, did not have any additive or synergistic effect with regard to enhancing the bioaccessibility of β-carotene from either yellow-orange-fleshy vegetables or green leafy vegetables. Tamarind which by itself did not have a beneficial influence on the bioaccessibility of β-carotene from vegetables, countered the positive influence of turmeric in heat processed vegetables when included along with turmeric.

When tamarind was used in combination with turmeric, the bioaccessibility of β-carotene was lower than when turmeric alone was added. This could be due to the fact that rather than antioxidant property which helps in the retention of β-carotene, the inhibitory role of tannin during micellar formation could be more dominant. Cholesterol micelle formation is well known to boost lipid molecule absorption, and tannins (astringent polyphenols) have been reported to inhibit lipid uptake in the intestine by inhibiting cholesterol micelle formation in *in vivo* situation (Osada *et al.*, 1997).

Summary

Four common food acidulants- amchur, lime, tamarind and kokum, and two antioxidant spices-turmeric and onion were examined for their influence on the bioaccessibility of β-carotene from two fleshy and two leafy vegetables. Amchur and lime generally enhanced the bioaccessibility of β-carotene from these test vegetables in many instances. Such an improved bioaccessibility was evident in both raw and heat-processed vegetables. The effect of lime juice was generally more pronounced than that of amchur. Turmeric significantly enhanced the bioaccessibility of β-carotene from all the vegetables tested, especially when heat-processed. Onion enhanced the bioaccessibility of β-carotene from pressure-cooked carrot and amaranth leaf and from open-pan-boiled pumpkin and fenugreek leaf. Lime juice and the antioxidant spices turmeric and onion minimized the loss of β-carotene during heat processing of the vegetables. In the case of antioxidant spices, improved bioaccessibility of β-carotene from heat-processed vegetables is attributable to their role in minimizing the loss of this provitamin. Lime juice which enhanced the bioaccessibility of this provitamin from both raw and heat-processed vegetables probably exerted this effect by some other mechanism in addition to minimizing the loss of β-carotene. Thus, the presence of food acidulants-lime juice / amchur and antioxidant spices – turmeric / onion prove to be advantageous in the context of deriving maximum β-carotene from the vegetable sources. The bioaccessibility enhancing effects of lime and turmeric were not additive when these two enhancers were used in combination.

Table-19. Effect of food acidulants and antioxidant spices on the retention of β-carotene in heat-processed pumpkin

	Raw	Pressure-cooked	Open-pan- boiled
Pumpkin	100.0 ± 1.9	43.6 ± 1.7	73.7 ± 1.9
Pumpkin + Amchur	99.3 ± 1.3	45.8 ± 2.2	66.9 ± 3.6
Pumpkin + Lime	98.8 ± 1.1	57.3 ± 2.9	76.3 ± 3.2
Pumpkin + Tamarind	99.4 ± 0.4	66.2 ± 3.4	62.1 ± 2.1
Pumpkin + Kokum	98.5 ± 1.0	51.9 ± 3.2	60.8 ± 2.9
Pumpkin + turmeric	101.0 ± 1.6	61 2 ± 1.7	88.0 ± 2.6
Pumpkin + onion	101.0 ± 1.8	45.2 ± 1.1	76.1 ± 1.2

Values (given as per cent) are average of pentuplicates

Values are given as percent relative to β-carotene content of raw vegetables taken as 100%

Table-20. Effect of food acidulants and antioxidant spices on the retention of β-carotene in heat-processed- carrot.

	Raw	Pressure-cooked	Open-pan- boiled
Carrot	100.0 ± 1.4	66.8 ± 0.8	86.8 ± 1.3
Carrot + Amchur	98.8 ± 0.9	65.1 ± 0.8	80.2 ± 1.7
Carrot + Lime	98.7 ± 2.8	74.7 ± 4.1	92.1 ± 1.1
Carrot + Tamarind	90.6 ± 1.5	73.7 ± 0.9	82.8 ± 1.7
Carrot + Kokum	91.0 ± 1.5	77.7 ± 1.5	82.1 ± 0.9
Carrot + turmeric	102.0 ± 0.9	81.4 ± 3.8	97.7 ± 0.9
Carrot + onion	95.6 ± 0.9	72.2 ± 2.3	92.3 ± 0.9

Values (given as per cent) are average of pentuplicates

Values are given as percent relative to β-carotene content of raw vegetables taken as 100%

Table-21. Effect of food acidulants and antioxidant spices on the retention of β-carotene in heat-processed fenugreek leaves.

	Raw	Pressure-cooked	Open-pan- boiled
Fenugreek	100.0 ± 0.8	68.3 ± 2.4	56.6 ± 0.7
Fenugreek + Amchur	98.8 ± 0.6	71.6 ± 1.4	60.2 ± 3.2
Fenugreek + Lime	99.9 ± 0.7	77.1 ± 0.5	58.3 ± 0.4
Fenugreek + Tamarind	96.8 ± 1.2	69.6 ± 1.6	60.9 ± 1.1
Fenugreek + Kokum	98.1 ± 0.7	67.5 ± 1.2	52.2 ± 0.6
Fenugreek + turmeric	102.0 ± 1.0	75.5 ± 0.6	67.1 ± 0.4
Fenugreek + onion	101.0 ± 2.1	77.2 ± 0.5	63.6 ± 1.0

Values (given as per cent) are average of pentuplicates

Values are given as percent relative to β-carotene content of raw vegetables alone taken as 100%

Table-22. Effect of food acidulants and antioxidant spices on the retention of β-carotene in heat-processed amaranth leaves.

	Raw	Pressure-cooked	Open-pan-boiled
Amaranth	100.0 ± 0.8	69.4 ± 0.7	50.0 ± 0.4
Amaranth + Amchur	98.6 ± 0.6	70.2 ± 1.9	46.2 ± 2.6
Amaranth + Lime	98.6 ± 0.7	76.5 ± 0.7	70.2 ± 2.1
Amaranth + Tamarind	98.1 ± 0.4	67.5 ± 0.7	67.9 ± 0.8
Amaranth + Kokum	96.1 ± 0.7	70.3 ± 1.1	44.0 ± 3.2
Amaranth + turmeric	98.6 ± 0.7	73.2 ± 0.7	53.1 ± 2.0
Amaranth + onion	99.2 ± 0.8	73.9 ± 1.8	59.3 ± 2.6

Values (given as per cent) are average of pentuplicates

Values are given as percent relative to β-carotene content of raw vegetables alone taken as 100%

Lime

Amchur

Tamarind

Kokum

Fig.12. Food acidulants:

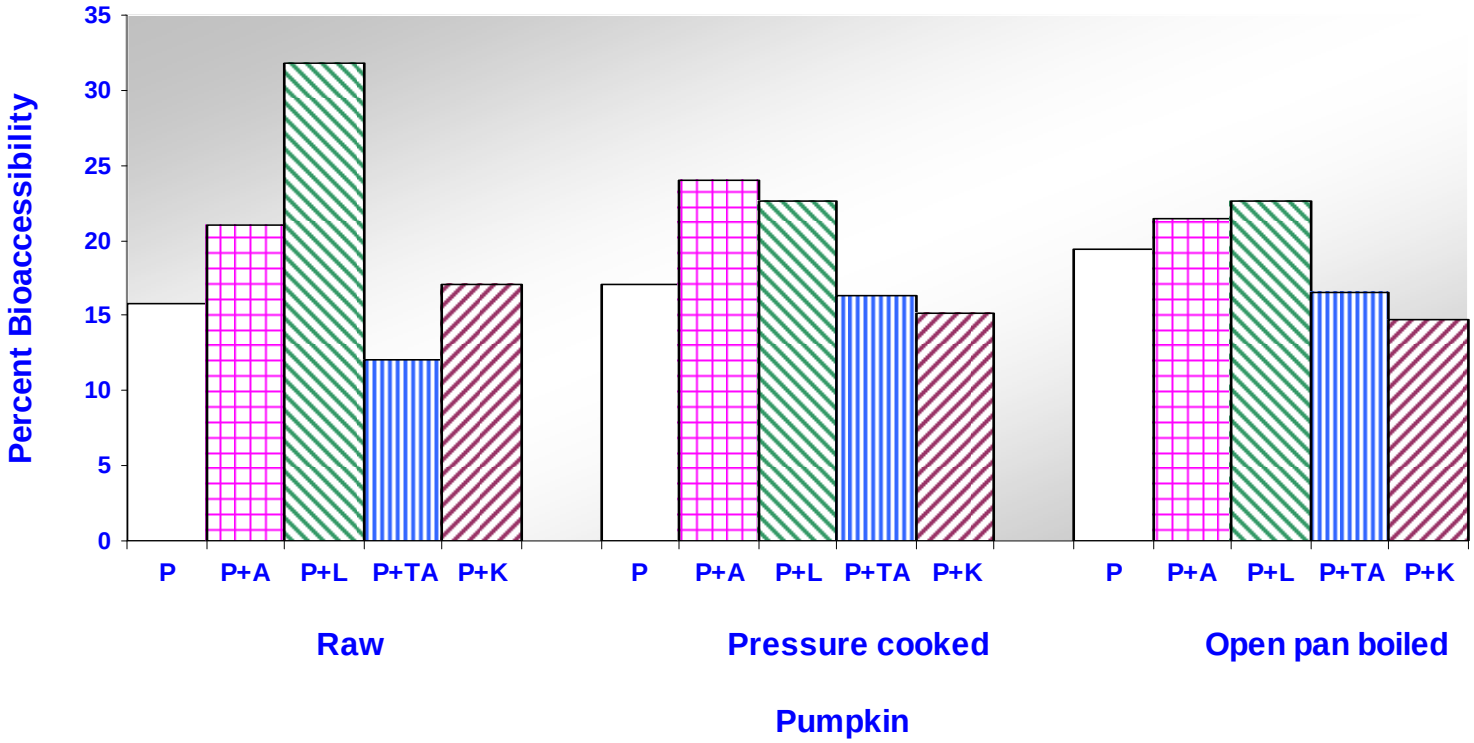
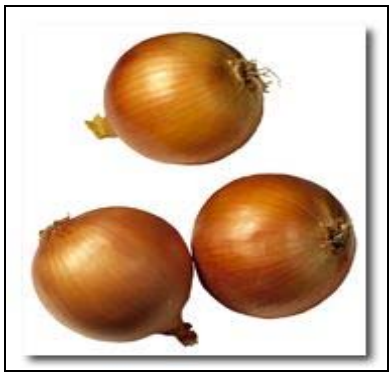


Fig.14. Effect of food acidulants on the percent bioaccessibility of β-carotene from pumpkin.

P = Pumpkin; P+A = Pumpkin + Amchur; P+L = Pumpkin + Lime; P+T = Pumpkin + Tamarind; P+K = Pumpkin + Kokum

Turmeric



Onion

Fig.13. Antioxidant spices:

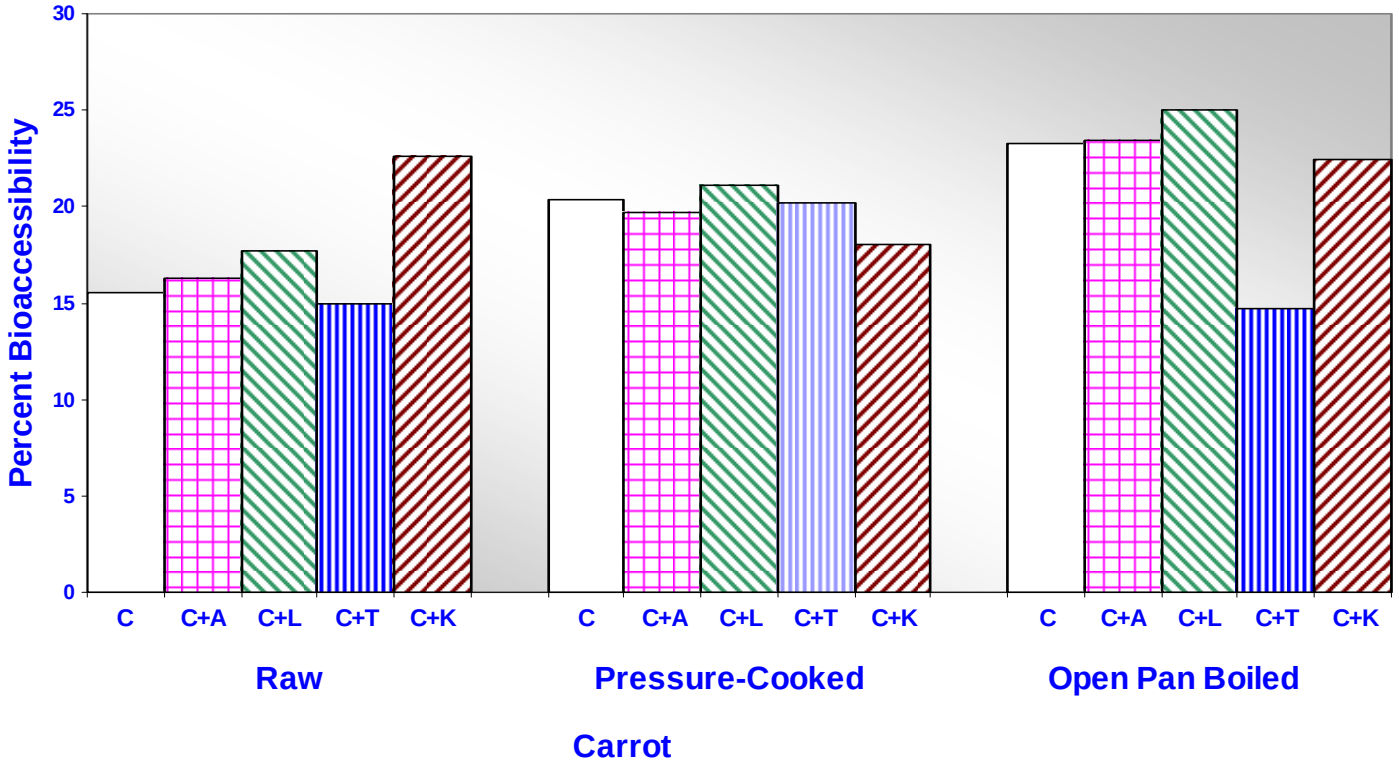


Fig.15. Effect of food acidulants on the percent bioaccessibility of β-carotene from carrot.

C = Carrot; C+A = Carrot + Amchur; C+L = Carrot +Lime; C+T = Carrot + Tamarind; C+K = carrot. Carrot + Kokum

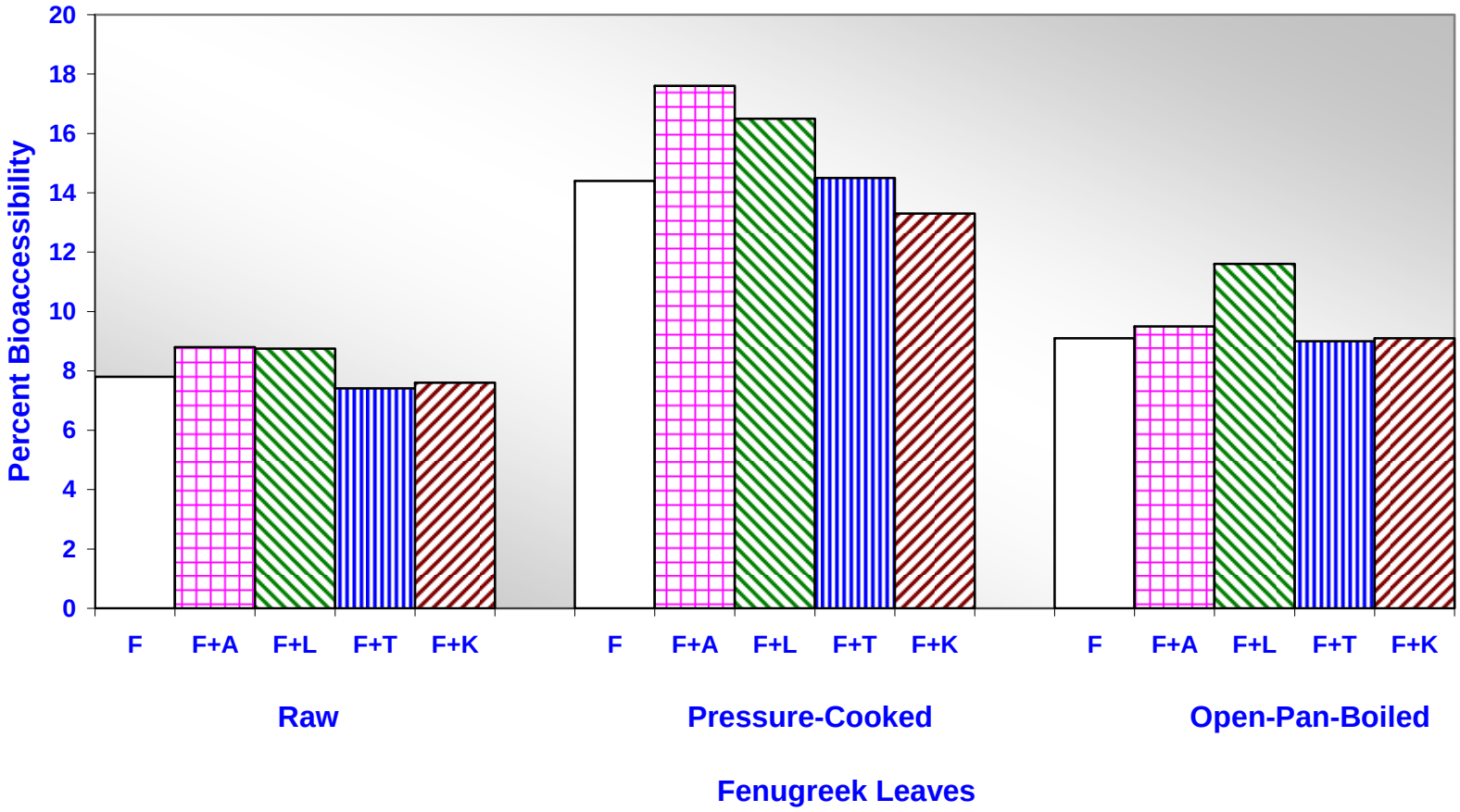


Fig.16. Effect of food acidulants on the percent bioaccessibility of β-carotene from fenugreek leaves.

F = Fenugreek leaves; F+A = Fenugreek leaves +Amchur; F+L = Fenugreek leaves + Lime; F+T = Fenugreek leaves + Tamarind; F+K = Fenugreek leaves + Kokum

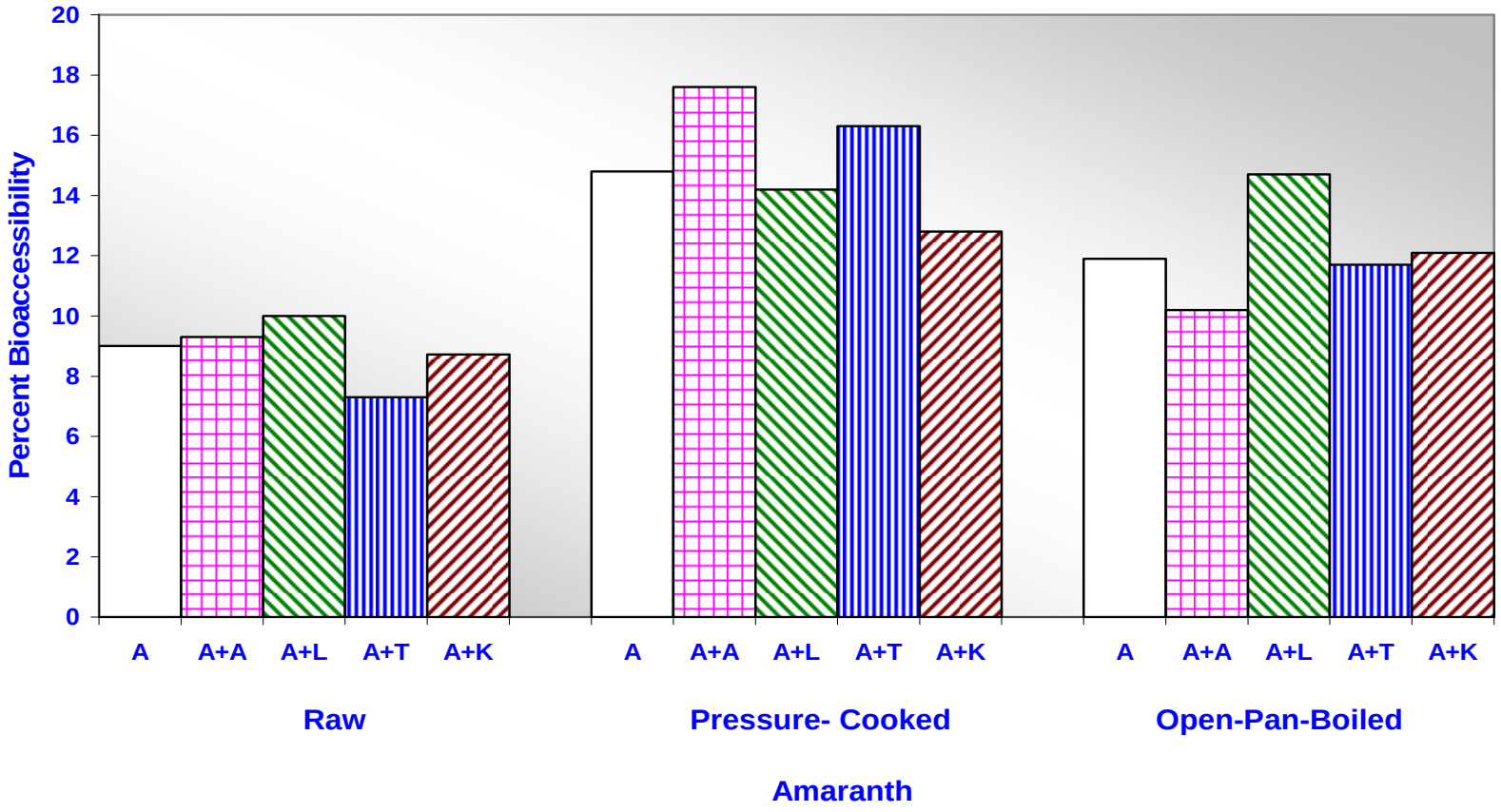


Fig.17. Effect of food acidulants on the percent bioaccessibility of β-carotene from amaranth.

A = Amaranth; A+A = Amaranth +Amchur; A+L = Amaranth + Lime; A+T = Amaranth +Tamarind; A+K = Amaranth + Kokum

Table-11. Effect of food acidulants on the bioaccessibility of β-carotene from fenugreek leaves

Food Acidulant	Raw		Pressure-cooked		Open-pan- boiled	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Fenugreek leaves	9024.5 ± 67.6	703.0 ± 3.8	6165.1 ± 217.0	1296.8 ± 21.7	5108.6 ± 61.8	819.7 ± 50.0
Fenugreek leaves + Amchur	8922.6 ± 56.2	791.2 ± 2.1	6458.3 ± 129.0	1585.0 ± 92.2*	5433.5 ± 290.0	859.6 ± 27.4
Fenugreek leaves + Lime	9022.7 ± 64.3	790.1 ± 5.8*	6965.0 ± 43.6	1485.7 ± 11.4*	5260.5 ± 39.0	1050.0 ± 43.6*
Fenugreek leaves + Tamarind	8740.0 ± 107.5	669.4 ± 35.0	6282.0 ± 143.8	1316.4 ± 30.4	5496.5 ± 95.0	800.0 ± 7.1
Fenugreek leaves + Kokum	8846.8 ± 61.5	683.4 ± 13.4	6092.0 ± 105.7	1190.0 ± 57.0	4715.3 ± 55.0	817.5 ± 2.1

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

*Significant increase compared to the value in vegetable alone ($p<0.05$)

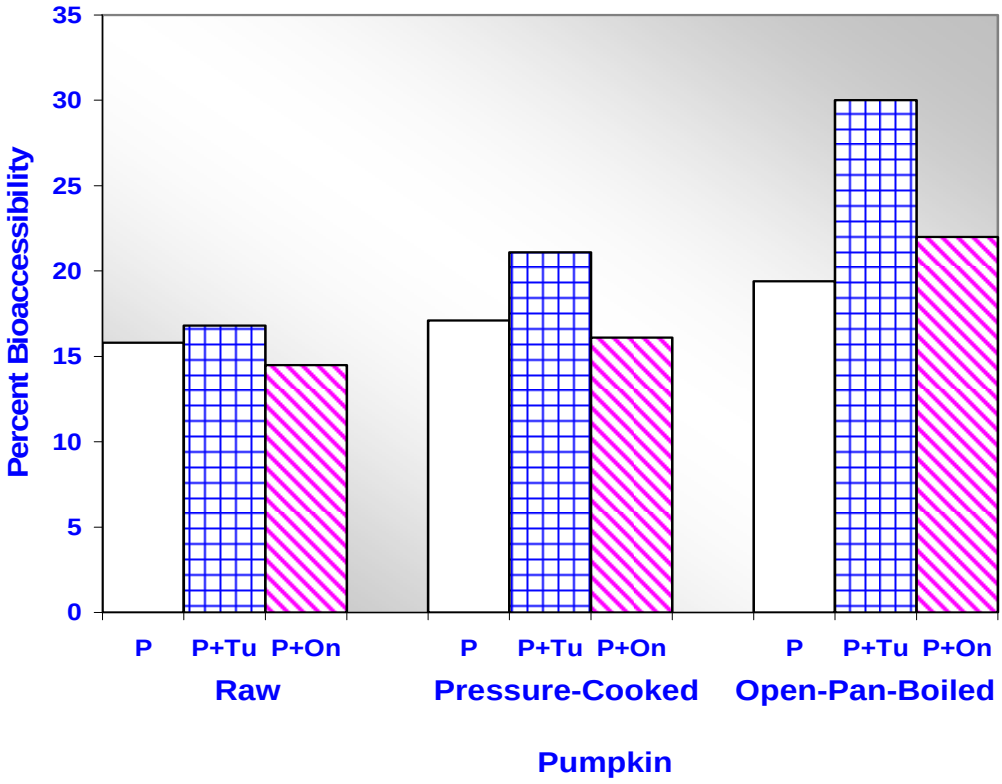


Fig.18. Effect of antioxidant spices on the percent bioaccessibility of β-carotene from pumpkin.

P = Pumpkin; P+Tu = Pumpkin + Turmeric; P+On = Pumpkin + Onion

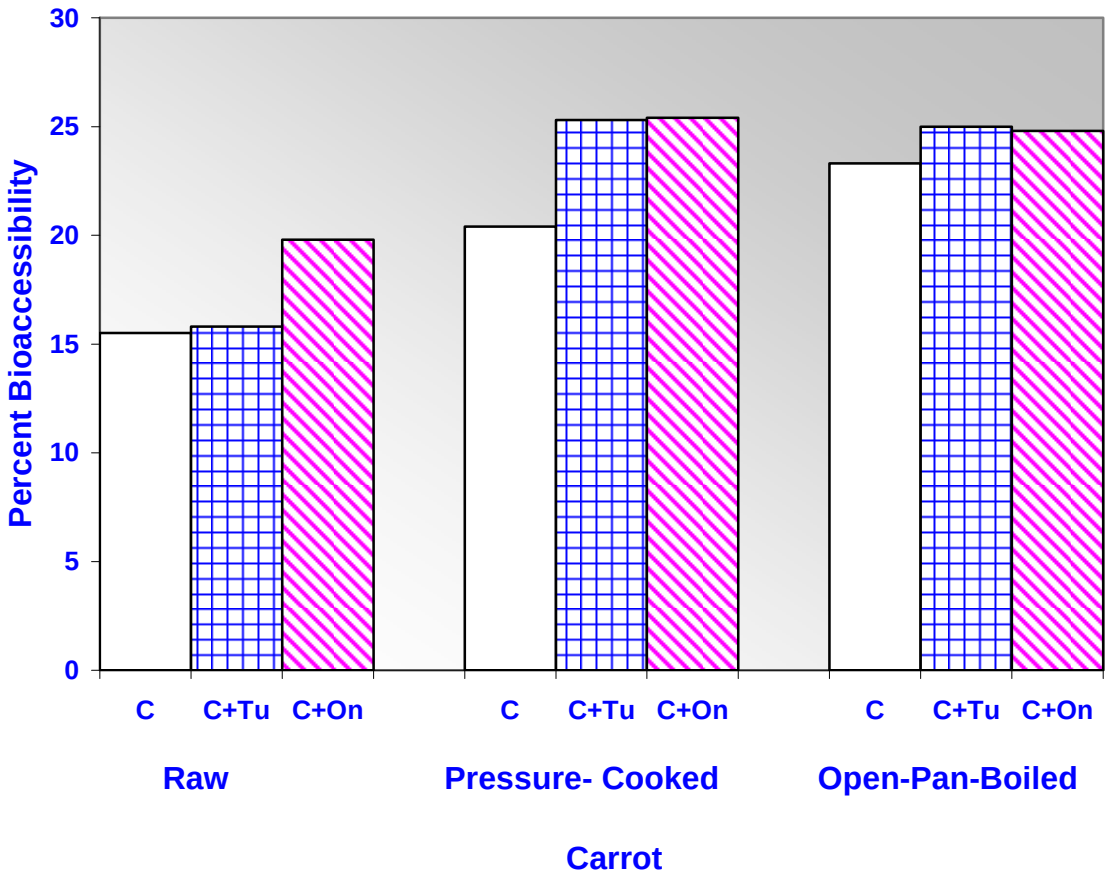


Fig.19. Effect of antioxidant spices on the percent bioaccessibility of β-carotene from carrot.

C = Carrot; C+Tu = Carrot + Turmeric; C+On = Carrot + Onion

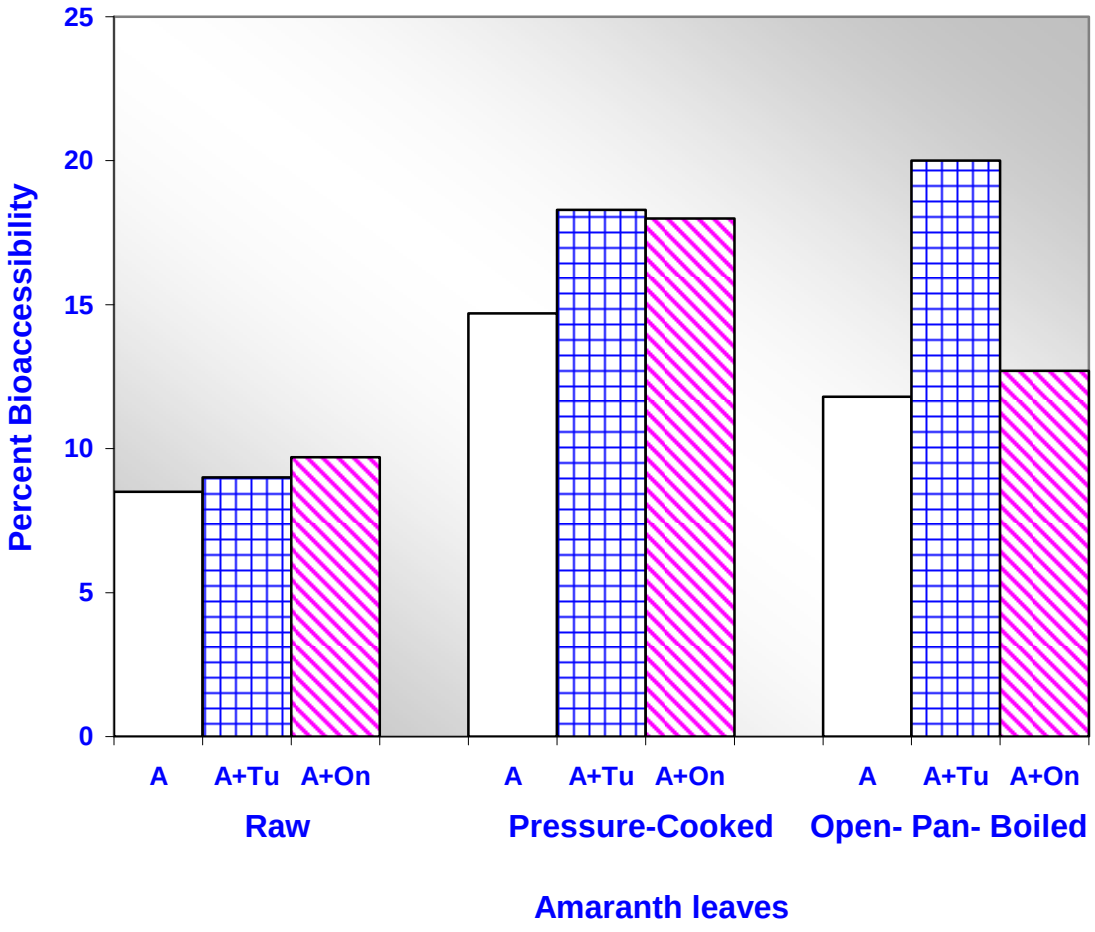


Fig.20. Effect of antioxidant spices on the percent bioaccessibility of β-carotene from amaranth.

A = Amaranth; A+Tu = Amaranth + Turmeric; A+On = Amaranth + Onion

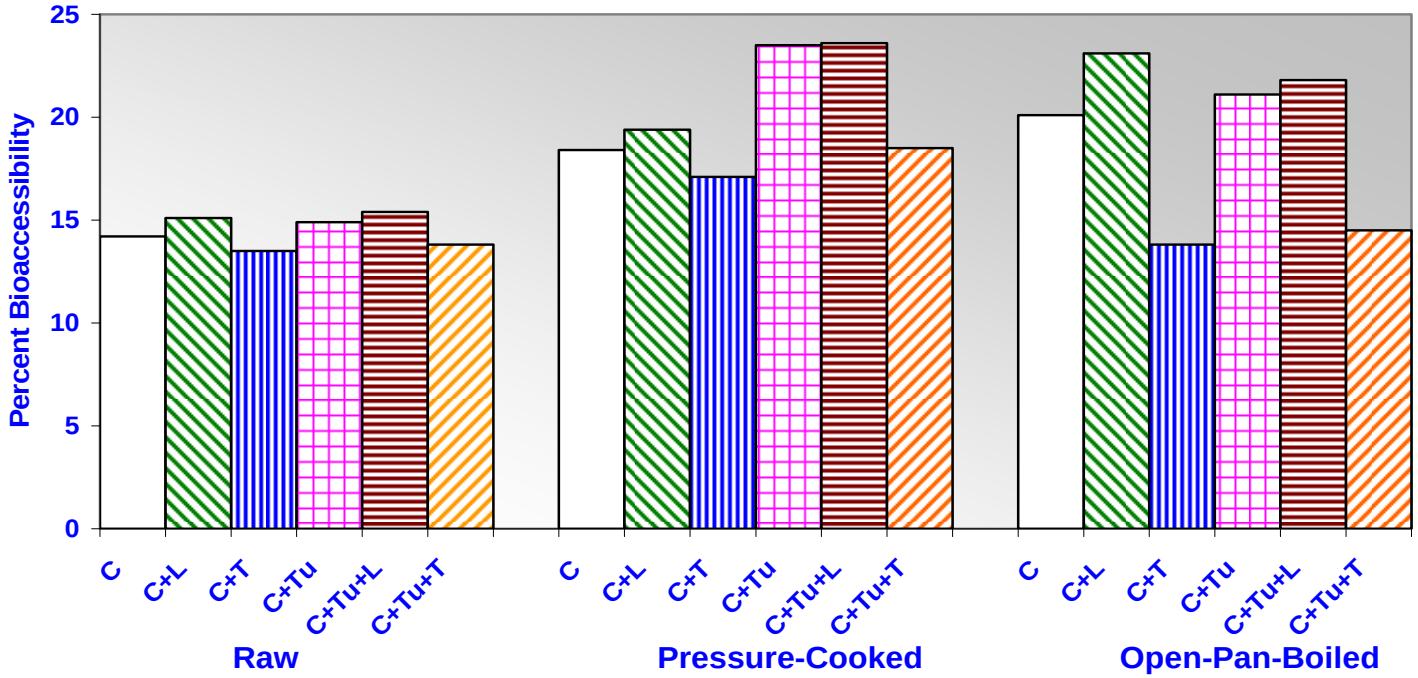


Fig.22. Effect of combination of a food acidulant and turmeric on the bioaccessibility of β-carotene from carrot

C = Carrot; C+L = Carrot +Lime; C+T = Carrot + Tamarind; C+Tu = Carrot +Turmeric
C+Tu+L = Carrot + Turmeric + L; C+Tu + T= Carrot + Turmeric + Tamarind

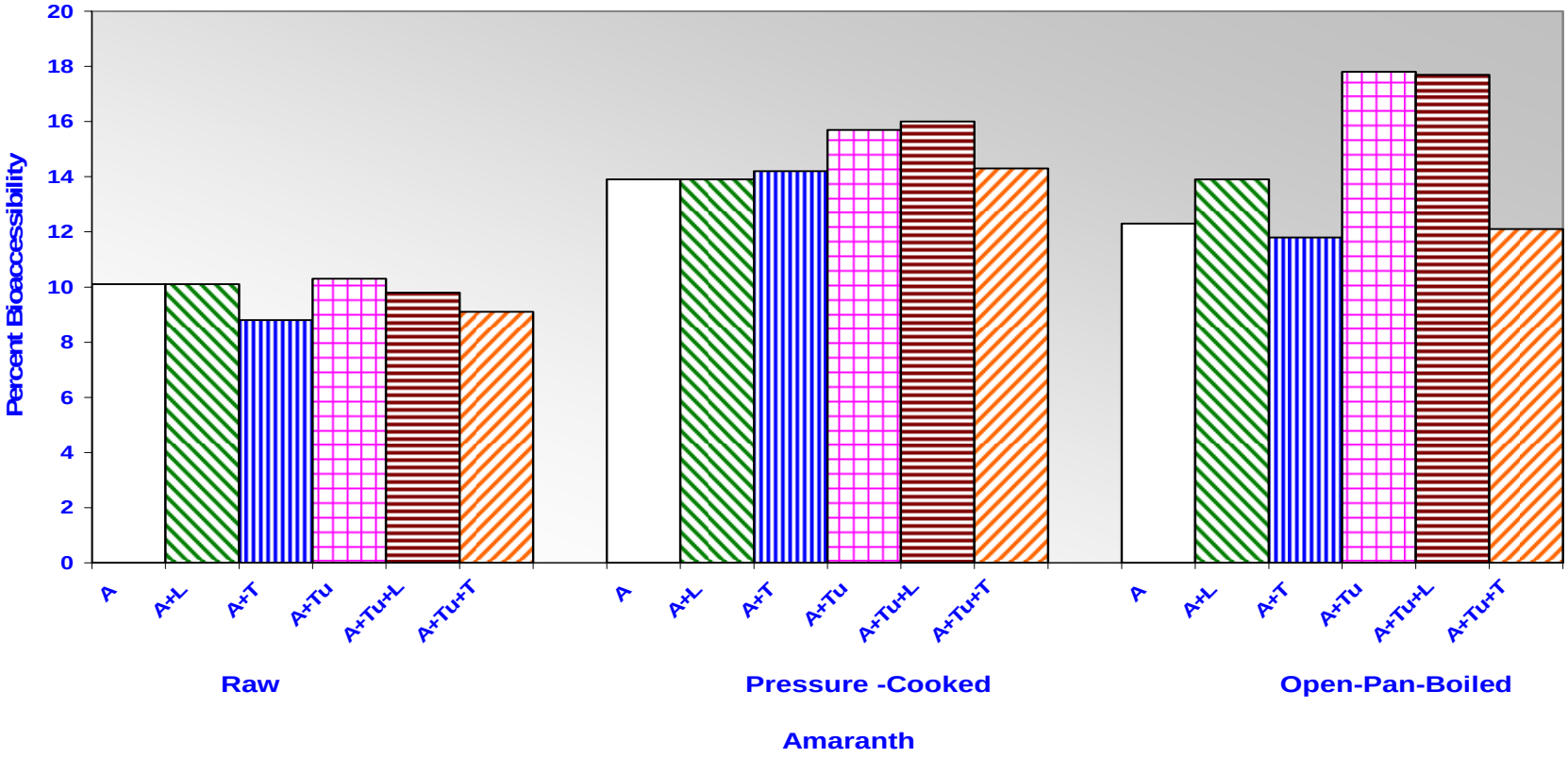


Fig.23. Effect of combination of a food acidulant and turmeric on the bioaccessibility of β-carotene from amaranth

A = Amaranth; A+L = Amaranth +Lime; A+T = Amaranth +Tamarind; A+Tu = Amaranth + Turmeric; A+Tu+L= Amaranth + Turmeric + Lime; A+Tu+T= Amaranth + Turmeric + Tamarind

Table-12. Effect of food acidulants on the bioaccessibility of β-carotene from amaranth

Food Acidulant	**Raw**		**Pressure-cooked**		**Open-pan- boiled**	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Amaranth	7930.8 ± 65.5	716.2 ± 16.6	5504.0 ± 59.0	1170.0 ± 38.7	3965.1 ± 30.0	942.4 ± 30.0
Amaranth + Amchur	7836.4 ± 47.1	740.6 ± 28.6	5566.2 ± 146.7	1399.4 ± 46.1*	3667.8 ± 203.7	813.4 ± 62.4
Amaranth + Lime	7825.0 ± 54.0	768.6 ± 29.0	6067.3 ± 56.0	1124.7 ± 74.2	5569.2 ± 170.0	1173.1 ± 54.0*
Amaranth + Tamarind	7781.5 ± 28.1	578.4 ± 25.2**	5360.3 ± 58.0	1295.8 ± 51.4	5388.1 ± 60.5	930.7 ± 25.5
Amaranth + Kokum	7625.6 ± 58.3	691.6 ± 31.1	5577.0 ± 85.4	1016.1 ± 30.1**	3490.0 ± 251.2	963.3 ± 60.2

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

*Significant increase compared to the value in vegetable alone ($p<0.05$);

**Significant decrease compared to the value in vegetable alone ($p<0.05$)

Table-13. Effect of antioxidant spices turmeric and onion on the bioaccessibility of β-carotene from pumpkin

	Raw		Pressure-cooked		Open- pan- boiled	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Pumpkin	1707.8 ± 13.4	269.0 ± 5.2	745.1 ± 30.0	292.1 ± 5.7	1258.8 ± 33.2	331.6 ± 14.8
Pumpkin + Turmeric	1725.3 ± 26.6	286.9 ± 7.7	1044.7 ± 29.4	360.5 ± 19.4*	1503.2 ± 44.0	511.3 ± 20.0*
Pumpkin + Onion	1718.7 ± 19.8	250.0 ± 12.0	772.8 ± 28.4	277.0 ± 6.5	1300.0 ± 3.20	377.8 ± 13.4*

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

*Significant increase compared to the value in vegetable alone ($p<0.05$)

Table-14. Effect of antioxidant spices turmeric and onion on the bioaccessibility of β-carotene from carrot

	Raw		Pressure-cooked		Open- pan-boiled	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Carrot	7594.3 ± 81.7	1179.0 ± 30.0	5075.8 ± 60.0	1546.6 ± 35.5	6599.1 ± 95.7	1770.3 ± 66.1
Carrot + Turmeric	7737.0 ± 68.6	1198.7 ± 31.5	6183.5 ± 518.9	1927.1 ± 39.0*	7425.2 ± 64.3	1766.7 ± 59.0
Carrot + Onion	7259.0 ± 67.6	1507.1 ± 117.4*	5488.3 ± 172.4	1926.3 ± 42.2*	7014.8 ± 66.7	1886.2 ± 102.2

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

*Significant increase compared to the value in vegetable alone ($p<0.05$)

Table-15. Effect of antioxidant spices turmeric and onion on the bioaccessibility of β-carotene from amaranth

	Raw		Pressure-cooked		Open- pan- boiled	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Amaranth	7930.8 ± 65.5	678.0 ± 39.5	5504.0 ± 59.0	1170.0 ± 38.7	3894.7 ± 73.5	942.4 ± 30.0
Amaranth + Turmeric	7823.0 ± 53.8	713.3 ± 12.4	5807.6 ± 58.2	1453.0 ± 27.4*	4213.5 ± 156.2	1590.3 ± 76.6*
Amaranth + Onion	7868.0 ± 66.6	771.3 ± 27.8	5859.0 ± 145.0	1427.7 ± 36.5*	4704.7 ± 205.0	1007.3 ± 27.0

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

*Significant increase compared to the value in vegetable alone ($p<0.05$)

Table-16. Effect of antioxidant spices turmeric and onion on the bioaccessibility of β-carotene from fenugreek leaves

	Raw		Pressure-cooked		Open- pan- boiled	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Fenugreek leaves	9024.5 ± 67.6	703.0 ± 3.80	6412.8 ± 277.5	1296.8 ± 21.7	5075.3 ± 69.0	819.7 ± 50.0
Fenugreek leaves + Turmeric	9083.7 ± 89.0	727.2 ± 12.0	6815.7 ± 52.7	1494.8 ± 53.5*	6052.6 ± 36.6	1117.5 ± 54.5*
Fenugreek leaves + Onion	9145.5 ± 186.7	718.0 ± 31.3	6968.0 ± 43.3	1368.8 ± 30.4	5743.4 ± 89.3	1038.2 ± 67.5*

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

*Significant increase compared to the value in vegetable alone ($p<0.05$)

Table-17. Effect of combination of a food acidulant and turmeric on the bioaccessibility of β-carotene from carrot

	Raw		Pressure-cooked		Open- pan- boiled	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Carrot	7698.4 ± 83.0	1090.6 ± 22.0	5252.5 ± 63.1	1420.2 ± 32.6	6436.4 ± 93.3	1549.2 ± 57.8
Carrot + Lime	7661.9 ± 212.0	1164.4 ± 45.7	5829.4 ± 321.3	1491.8 ± 42.2	6723.5 ± 76.9	1781.5 ± 39.0*
Carrot + Tamarind	7598.7 ± 114.5	1040.7 ± 36.4	5307.8 ± 65.2	1315.9 ± 19.4	5854.6 ± 120.4	1064.9 ± 41.0**
Carrot + Turmeric	7782.9 ± 69.0	1150.2 ± 30.2	6432.4 ± 39.7	1810.8 ± 36.6*	7159.2 ± 62.0	1627.2 ± 24.3*
Carrot + Turmeric + Lime	7719.1 ± 223.0	1185.1 ± 47.0	6376.2 ± 351.0	1820.2 ± 52.0*	7071.1 ± 81.0	1679.3 ± 37.0*
Carrot + Turmeric + Tamarind	7557.3 ± 112.3	1064.9 ± 37.2	5564.5 ± 68.4	1425.9 ± 21.0	6013.0 ± 123.6	1115.3 ± 43.0**

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

*Significant increase compared to the value in vegetable alone ($p<0.05$);

**Significant decrease compared to the value in vegetable alone ($p<0.05$)

Table-18. Effect of combination of a food acidulant and turmeric on the bioaccessibility of β-carotene from amaranth

	Raw		Pressure-cooked		Open- pan- boiled	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Amaranth	7943.5 ± 65.6	800.1 ± 19.0	5916.8 ± 63.4	1109.9 ± 36.7	3854.1 ± 29.2	976.9 ± 31.1
Amaranth + Lime	7840.6 ± 54.1	802.8 ± 30.2	6639.0 ± 61.3	1110.3 ± 73.2	5117.6 ± 156.2	1107.6 ± 51.0*
Amaranth + Tamarind	7828.9 ± 28.3	702.4 ± 30.6	5662.6 ± 61.3	1126.3 ± 44.7	3860.2 ± 43.3	940.1 ± 25.7
Amaranth + Turmeric	7947.9 ± 54.8	816.0 ± 15.0	6426.8 ± 64.4	1249.6 ± 23.6*	4263.2 ± 158.0	1411.4 ± 67.9*
Amaranth + Turmeric + Lime	7940.5 ± 54.8	782.3 ± 30.0	6851.2 ± 63.2	1273.1 ± 44.0*	5132.5 ± 156.6	1404.2 ± 64.6*
Amaranth + Turmeric + Tamarind	7898.7 ± 28.5	716.0 ± 31.2	6299.2 ± 68.2	1134.0 ± 45.0	4235.6 ± 47.2	962.0 ± 26.3

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

*Significant increase compared to the value in vegetable alone ($p<0.05$);

Table-10. Effect of food acidulants on the bioaccessibility of β-carotene from carrot

Food Acidulant	Raw		Pressure-cooked		Open-pan- boiled	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Carrot	7594.3 ± 81.7	1179.0 ± 30.0	5075.8 ± 60.0	1546.6 ± 35.5	6599.1 ± 95.7	1770.0 ± 66.1
Carrot + Amchur	7503.2 ± 67.0	1239.0 ± 48.3	4947.7 ± 57.2	1498.7 ± 41.3	6091.0 ± 127.4	1774.0 ± 73.7
Carrot + Lime	7500.1 ± 207.6	1341.7 ± 52.7*	5677.8 ± 313.0	1602.3 ± 45.3	6995.0 ± 80.1	1901.0 ± 41.6
Carrot + Tamarind	6887.1 ± 103.8	1128.8 ± 39.5	5600.3 ± 68.8	1535.3 ± 22.6	6288.6 ± 129.3	1120.9 ± 43.2**
Carrot + Kokum	6914.8 ± 116.1	1250.1 ± 29.0	5375.0 ± 112.4	1368.1 ± 51.9**	6263.4 ± 70.6	1702.3 ± 29.8

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

*Significant increase compared to the value in vegetable alone ($p<0.05$);

**Significant decrease compared to the value in vegetable alone ($p<0.05$)

Table-9. Effect of food acidulants on the bioaccessibility of β-carotene from pumpkin

Food Acidulant	**Raw**		**Pressure-cooked**		**Open-pan- boiled**	
	Total	Bioaccessible	Total	Bioaccessible	Total	Bioaccessible
Pumpkin	1707.8 ± 33.4	269.0 ± 5.2	745.1 ± 30.0	292.1 ± 9.7	1258.8 ± 33.2	331.6 ± 14.8
Pumpkin + Amchur	1696.4 ± 21.8	356.8 ± 22.0*	836.2 ± 36.7	409. 0 ± 12.3*	1143.3 ± 61.0	364.6 ± 7.5*
Pumpkin + Lime	1687.5 ± 19.1	543.8 ± 43.7*	979.1 ± 49.3	386.2 ± 17.1*	1302.0 ± 54.4	386.1 ± 20.1*
Pumpkin + Tamarind	1697.6 ± 7.6	205.4 ± 5.0**	1131.1 ± 57.4	279.3 ± 19.7	1060.1 ± 36.4	282.8 ± 12.2**
Pumpkin + Kokum	1681.3 ± 17.2	292.5 ± 3.1	903.5 ± 55.0	260.0 ± 10.2	1037.7 ± 49.2	250.5 ± 28.0**

Values of β-carotene are expressed as μg/100 g fresh weight and are given as mean ± SEM of pentuplicates.

*Significant increase compared to the value in vegetable alone ($p<0.05$);

**Significant decrease compared to the value in vegetable alone ($p<0.05$)

CHAPTER – IV

VARIETAL DIFFERENCES IN THE BIOACCESSIBILITY OF β-CAROTENE FROM MANGO (*Mangifera indica*) AND PAPAYA (*Carica papaya*) FRUITS

Varietal differences in the bioaccessibility of β-carotene from mango (*Mangifera indica*) and papaya (*Carica papaya*) fruits

INTRODUCTION

Fruits such as mango (*Mangifera indica*) and papaya (*Carica papaya*) which are rich sources of β-carotene are abundantly grown and consumed in India (Fig.24 and 25). While mango is a seasonal fruit, papaya is found throughout the year, often grown in kitchen gardens. Several popular varieties of mango are available and consumed during the season. There are hundreds of varieties of mango fruits grown in India, a few of which are of commercial importance. The most well-known varieties of mango in India are '*Langra'*, '*Alphonso*', '*Dashehari*', '*Banganpalli*', '*Malgoa',* '*Neelam'*, '*Kesar*', '*Mallika*', '*Raspuri*', '*Totapuri*', '*Amrapali*', *etc*. Many regionally grown wild varieties of papaya are consumed in India. '*Honey Dew'* popularly known as '*Madhubindu*' is extensively cultivated for commercial use. The variety bears greenish-yellow oblong-shaped big sized fruits with orange thick flesh and good flavour. A hybrid variety '*Surya*' has also been in popular use, whose medium and uniformly sized (1-2 kg) seedless fruits with deep orange flesh have excellent flavour.

The typical yellow-orange color of ripened mango fruits is due to the presence of carotenoids (Vázquez-Caicedo *et al*., 2005) with all-*trans*-*β*-carotene being the most abundant (Mercadante *et al*., 1997). The relatively high content of all-*trans*-β-carotene suggests the possibility of mango contributing to the health of human population consuming this fruit, since β-carotene is believed to prevent certain types of human cancer (Pradeep and Kuttan, 2003) and the oxidation of LDL (Krinsky and Jhonson, 2005), a process implicated in the development of atherosclerosis.

Numerous factors affect the bioavailability of carotenoids from foods, which include food matrix, type and intensity of food matrix processing, carotenoid speciation, and some components such as the amount of fat and fibre present in the meal. These factors have been described in the mnemonic "SLAMENGHI" (West and Castenmiller, 1998).

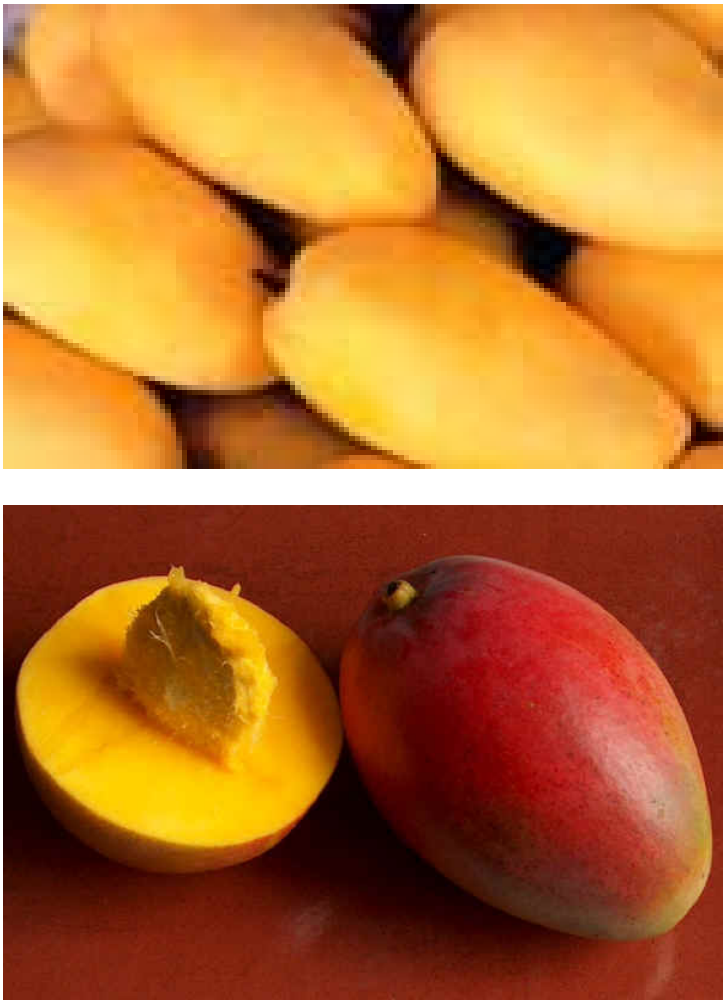


Fig.24. Mango fruits (*Mangifera indica*)

Fig.25. Papaya fruits (*Carica papaya*)

Food matrix refers to the combined effects of all factors from a food that simultaneously promotes or reduces the bioavailability of endogenous carotenoids. The matrix certainly contributes to the variable bioavailability of carotenoids in different foods and meals (Tyssandier *et al*., 2002). The carotenoids in dark green leafy vegetables (DGLV) are entrapped as complexes with proteins in chloroplasts within the cell structures. Such entrapment is probably responsible for the poor bioavailability of carotenoids from DGLV. The cell wall structure of fruits is relatively weaker than that in leaves, and carotenoids in fruits are present in oil droplets in chromoplasts, hence may be more easily extracted during digestion (West and Castenmiller, 1998; Thurnham, 2007).

The different matrices in a fruit at different stages of ripening are likely to affect carotenoid bioavailability. Marked qualitative and quantitative changes in carotenoids, organic acids, lipids, phenolics, volatile compounds, and nonstructural and structural carbohydrates have been reported during the short duration of the ripening process of mango fruit (Kudachikar *et al.,* 2001; Vázquez-Caicedo *et al.*, 2004). Effects of such compositional changes might modify the bioavailability of carotenoids from mango at different stages of ripening.

A few studies have reported differences in the β-carotene content of different cultivars of mango (Mercadante and Rodriguez-Amaya, 1998; Pott *et al*., 2003; Vazquez-Caicedo *et al*., 2005). Climatic conditions have been found to influence the β-carotene content of fruits (Mercadante and Rodriguez-Amaya, 1998). Information on the varietal differences, if any, in the concentration of this provitamin in mango and papaya fruits native to India is lacking. It would also be interesting to see if varietal differences exist in the bioaccessibility of β-carotene from these fruits in view of the choice of varieties available for consumption. The present investigation was therefore undertaken to examine the β-carotene content as well as its bioaccessibility in six popular varieties of mango, and two varieties of papaya found in the local market. Mango and papaya are also consumed along with milk in the form of milk shake. Hence, the influence of milk, if any, on the bioaccessibility of β-carotene from mango and papaya was also examined in this investigation.

MATERIALS AND METHODS

Materials

Six popular cultivars of mango, namely, *Badami*, *Raspuri*, *Mallika*, *Malgoa*, *Totapuri* and *Neelam*, and two cultivars of papaya, *Honey Dew* (conventional) and *Surya* (hybrid, seedless) variety were procured from the local market. These fruits were of a ripeness ideal for consumption, and were uniform for all the varieties. Fresh edible pulp of the fruits was used for the study. Each variety of mango, the annual fruit available during summer, was procured from five different vendors, and mangoes from each vendor were analyzed on different days, in duplicate. The whole pulp of the fruits was homogenized and used for analysis. In the case of papaya which is available more or less throughout the year, the fruits were collected from five different vendors, each at three different seasons.

All chemicals used were of analytical grade. Solvents were distilled before use. Standard β-carotene, porcine pancreatic pepsin, and pancreatin and bile extract (porcine) were procured from Sigma Chemicals Co., St. Louis, USA. Double-distilled water was employed throughout the entire study. All glassware used was acid washed.

Determination of bioaccessibility of β-carotene in vitro

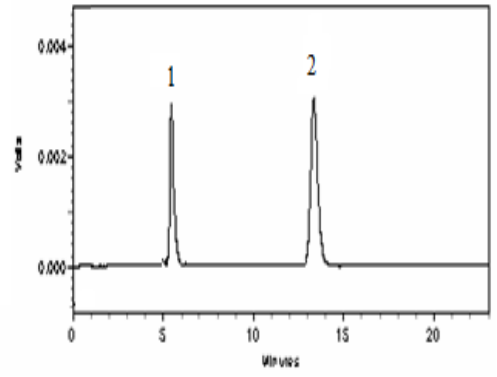
The bioaccessibility of β-carotene *in vitro* was determined by the method of Garrett *et al.* (Garrett *et al*., 1999). Briefly, the method involved subjecting the sample (10 g of fresh fruit pulp) to simulated gastric digestion at pH 2.0 in the presence of pepsin at 37^{o}C (16 g in 100 ml 0.1M HCl), followed by simulated intestinal digestion in the presence of pancreatin-bile extract mixture (4 g porcine pancreatin) and 25 g bile extract (porcine) in 1000 ml of 0.1M $NaHCO_3$), pH 7.5 at 37^{o}C for 2 h. At the end of simulated intestinal digestion, the micellar fraction was separated by ultracentrifugation at 70,000 x g for 120 min using a Beckman L7-65 ultracentrifuge. The β-carotene present in the aqueous micellar fraction represents the portion that is bioaccessible (As in Chapter-II).

Analysis of β-carotene

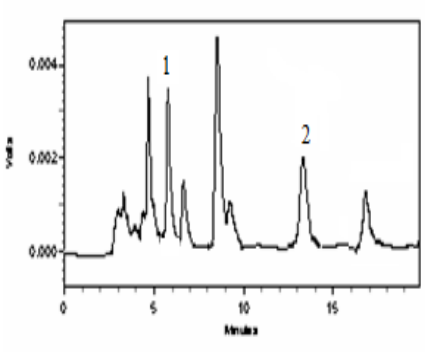
β-Carotene was extracted from the fruit pulp initially with a mixture of acetone : ethanol (1:1) and subsequently with petroleum ether. The process was repeated several times to ensure complete extraction of β-carotene. The extract was saponified with 30% methanolic potassium hydroxide at room temperature for 3 h. Following saponification, the alkali was removed completely by repeated washing, and the solvent was evaporated to dryness in a rotary evaporator. The residue was redissolved in petroleum ether and stored in the cold pending analysis. Prior to analysis, the petroleum ether was evaporated under nitrogen and the residue was dissolved in the mobile phase. β-apo-8'-carotenal was used as an internal standard.

Determination of β-carotene was carried out by reverse-phase HPLC (Shimadzu LC 10 AVP), equipped with a PDA detector. β-Carotene was separated on a SGE 250 x 4.6 mm C_{18} 5 μm column (S.S.Excil, Australia). The mobile phase consisted of a mixture of 65% (v/v) acetonitrile, 15% (v/v) methylene chloride and 20% (v/v) methanol containing 1.3 mmol/l ammonium acetate. An isocratic analysis was performed at a flow rate of 1ml/min. β-Carotene was monitored at a wavelength of 450 nm. The peak identities and λ_{max} were confirmed by their retention time and characteristic spectra of standard chromatograms. Quantitation of β-Carotene was made from peak area ratio, which was based on a calibration curve generated from standard β-carotene (Fig.26).

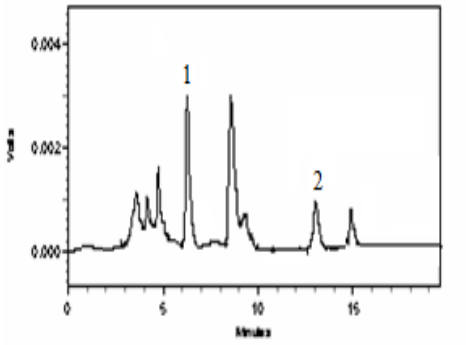
During the steps of simulated gastrointestinal digestion, ultracentrifugation and extraction of β-carotene, precautions were taken to minimize the exposure of samples to light and air and thus prevent oxidative destruction of β-carotene. Air was replaced by nitrogen before stoppering the flask at all stages of incubation and storage. The experiments were carried out under yellow lighting and all the glassware was covered with black cloth to prevent exposure to light.



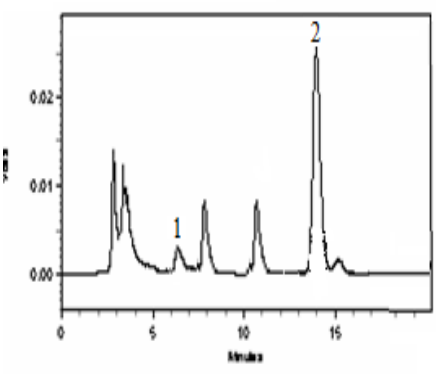
Standard



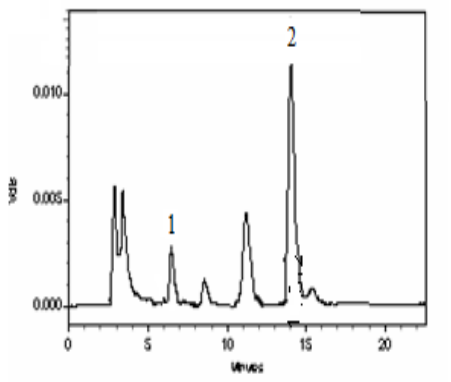
Fresh papaya
Total β-carotene



Fresh papaya
Bioaccessible β-carotene



Fresh mango
Total β-carotene



Fresh mango
Biooaccessible β-carotene

Fig.26. HPLC profile of carotenoid extract of mango (‘*Raspuri’* variety) and papaya (‘*Honeydew*’ variety) fruits.
Peak #1: β-apo-8’-carotenal (Internal standard); Peak #2: β-carotene

Determination of total organic acids in fruit pulp

Fresh fruit pulp was blended in a Sorval Omni mixer provided with stainless steel blade assembly. The pulp blend (2.5 g) was boiled for 1 h and the volume was made up to 50 ml with distilled water. Total organic acid content of the fruit pulp was determined by titrating the boiled aqueous pulp (10 ml) against 0.005N NaOH, previously standardized using standard oxalic acid (AOAC, 1960). Percent organic acid was calculated as:

% Total organic acid =

$$\frac{\text{Titration value x Normality of alkali x 50 x Equivalent weight of acid x 100}}{\text{10 x 2.5 x 1000}}$$

Preparation of mango and papaya milk shake

To examine the effect of the presence of milk, 10 g of the fresh fruit pulp was blended with 50 ml of milk and 10 g of sugar, and the bioaccessibility of β-carotene from this blend was determined as above. For this purpose, commercially available pasteurized cow's milk was boiled in the laboratory, as usually practiced in Indian households.

Statistical analysis

All determinations were made in five experiments using fruits bought from five different vendors, and the average values are reported. Statistical analysis of data was done employing Analysis of Variance (ANOVA), and the differences between means were determined by Duncan's Multiple Range test and were considered significant when $p < 0.05$ (Duncan, 1955).

RESULTS

β-Carotene content and bioaccessibility from different varieties of mango

Table-23 presents the β-carotene content and its bioaccessibility from six different

Table-23. Varietal differences in the content and bioaccessibility of β-carotene from mango (*Mangifera indica*)

Mango variety	Total β-carotene (mg/100 g)	Bioaccessible β-carotene (mg/100 g)	Percent Bioaccessibility
Badami	3.21 ± 0.25^{a}	0.79 ± 0.03^{a}	24.5
Raspuri	1.83 ± 0.08^{b}	0.71 ± 0.03^{a}	39.1
Malgoa	0.55 ± 0.03^{c}	0.18 ± 0.01^{b}	32.5
Mallika	2.77 ± 0.09^{a}	0.89 ± 0.04^{c}	31.9
Totapuri	1.27 ± 0.10^{d}	0.48 ± 0.03^{d}	38.1
Neelam	1.45 ± 0.23^{d}	0.45 ± 0.03^{d}	30.8

Values are Mean ± SEM of five independent determinations, each being in duplicate.

Within the same column values with different superscripts are significantly different ($p<0.05$).

varieties of mango. The β-carotene content (mg/100 g fresh pulp) varied widely among the different varieties of mango, the highest being present in *Badami* (3.21), followed by *Mallika* (2.77), *Raspuri (*1.83), *Neelam* (1.45), *Totapuri* (1.27) and *Malgoa* (0.55). Thus, there is a 6-fold difference in β-carotene between the variety with the highest (*Badami*) and that with lowest content (*Malgoa*). Incidentally the ripe fruits of *Malgoa* variety are pale yellow in colour, whereas the hue of the *Badami* variety is the most intense.

Varietal differences were also evident in the bioaccessiblity of β-carotene from mango, which were however confined to a range 24.5 - 39%, unlike the wide variation seen in their β-carotene content (Table-23). The bioaccessibility values of these mango varieties were also independent of the inherent content of this provitamin. The percent bioaccessible β-carotene was highest in the *Raspuri* variety (39.0) and lowest in the *Badami* variety (24.5), while it was 38, 32.5, 32, and 31 in *Totapuri*, *Malgoa*, *Mallika*, and *Neelam* variety respectively. Considering both the total content and percent bioaccessibilty, the *Mallika* variety provides the highest amount of β-carotene (0.89 mg/ 100 g), followed by the *Badami* (0.79 mg/100 g) and *Raspuri* (0.71 mg/100 g) varieties for the same amount of edible pulp consumed.

Total organic acid content in the edible pulp portions of these mango varieties is indicated in Fig.27. Total organic acid content ranged from 0.13% in *Mallika* to 0.50% in *Raspuri* variety. The organic acid profile of the test varieties of mango indicated that bioaccessibility of β-carotene from specific mango varieties roughly corresponded with the organic acid content of the fruits of *Raspuri, Totapuri, Malgoa*, *and Badami* variety.

β-*Carotene content and bioaccessibility from two varieties of papaya*

β-Carotene content of two tested cultivars of papaya fruits and its percent bioaccessibility are presented in Table-24. There was no significant difference in the content of β-carotene between the two varieties of papaya examined in this study, namely *Honey dew* and *Surya*. While the β-carotene content of the *Surya* (seedless) variety was 0.73 mg/100 g, the same was 0.70 mg/100 g in the Honey *Dew* (conventional) variety.

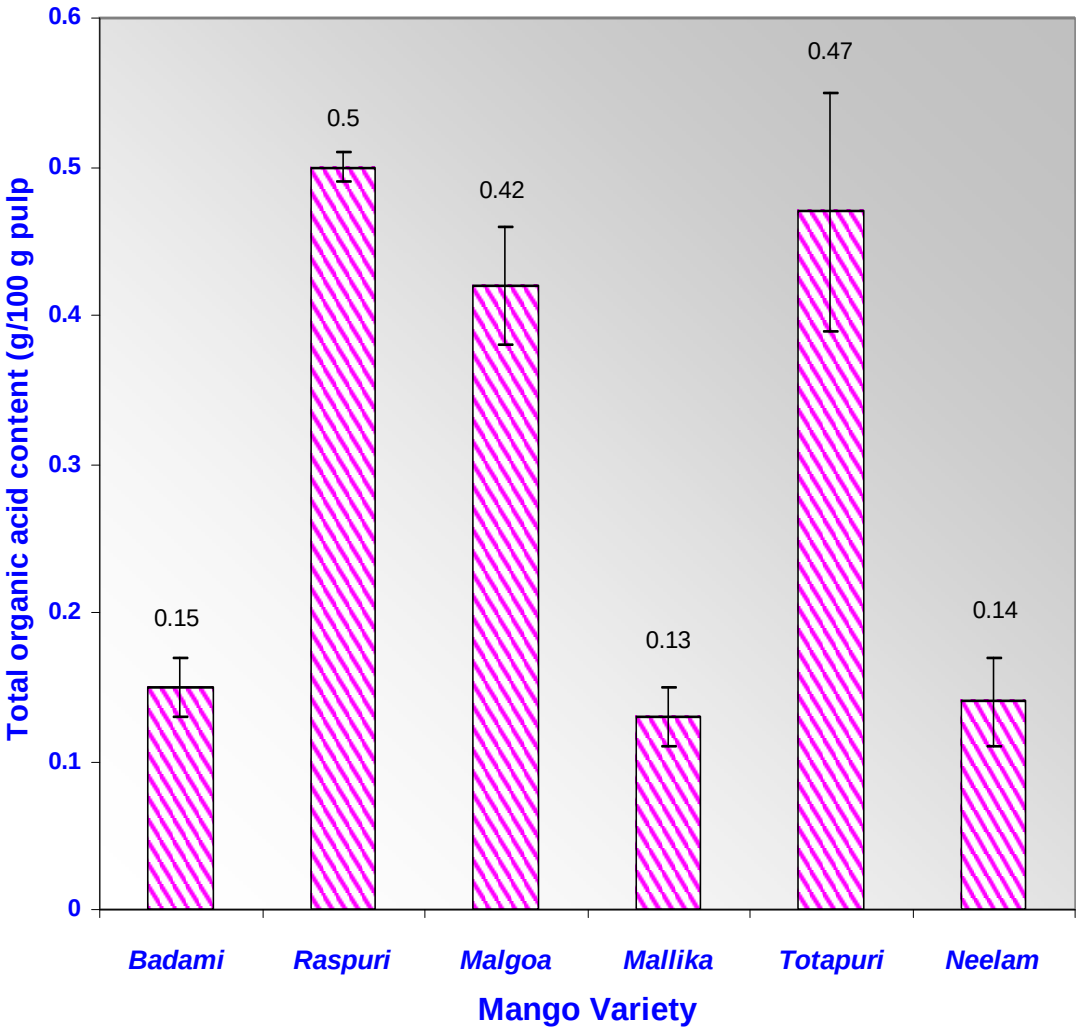


Fig.27. Organic acid content in mango varieties

Table-24. Varietal difference in the content and bioaccessibility of β-carotene from papaya (*Carica papaya*)

Papaya variety	Total β-carotene (mg/100 g)	Bioaccessible β-carotene (mg/100 g)	Percent Bioaccessibility
Honey dew	0.70 ± 0.10^{a}	0.24 ± 0.02^{a}	34.3
Surya	0.73 ± 0.12^{a}	0.23 ± 0.04^{a}	31.4

Values are Mean ± SEM of five independent determinations, each being in duplicate.

Within the same column values with different superscripts are significantly different ($p<0.05$).

Similarly, no varietal differences were evident in the percent bioaccessibility of β-carotene from papaya, the same being 34.3 and 31.4 from the *Honey Dew* and *Surya* variety, respectively. The total organic acid content of the two tested varieties of papaya was 0.14% (*Honey dew*) and 0.20% (*Surya*) (Fig.28). Unlike in the case of mango, the organic acid content of papaya did not seem to influence the bioaccessibility of β-carotene.

Effect of the presence of milk on the bioaccessibility of* β*-carotene from mango and papaya.

Table-25 presents the influence of milk on the bioaccessibility of β-carotene from the six different varieties of mango fruits. Addition of milk to the fruit pulp generally enhanced the bioaccessibility of β-carotene from all the varieties of mango (Fig.29). The percent increase in the bioaccessibility of β-carotene in the presence of milk compared to fruit pulp alone ranged from 12 (*Raspuri) to* 56 (*Badami).* The enhancement in the bioaccessibility of β-carotene brought about by the presence of milk was minimal in *Raspuri* and *Malgoa* varieties (12 and 13.6% respectively). Considering the absolute amount of bioaccessible β-carotene, the blend of milk and the *Badami* variety of mango provides the highest amount (1.2 mg/100 g), followed by those of *Mallika* (1.1 mg/100 g), *Raspuri* (0.8 mg/100 g), *Neelam* (0.64 mg/100 g), *Totapuri* (0.61 mg/100 g) and *Malgoa* (0.20 mg/100 g).

Presence of milk had a similar beneficial influence on the bioaccessibility of β-carotene from both the varieties of papaya examined (Table-26). Milk brought about a 38% increase in the bioaccessibility of β-carotene from the *Surya* variety of papaya, while the same was 18% from the *Honey Dew* variety (Fig.30). Thus, varietal differences existed in papaya only with respect to the influence of exogenous milk on the bioaccessibility of β-carotene. The exogenous milk added to the fruit pulp in this study did not contribute any β-carotene by itself.

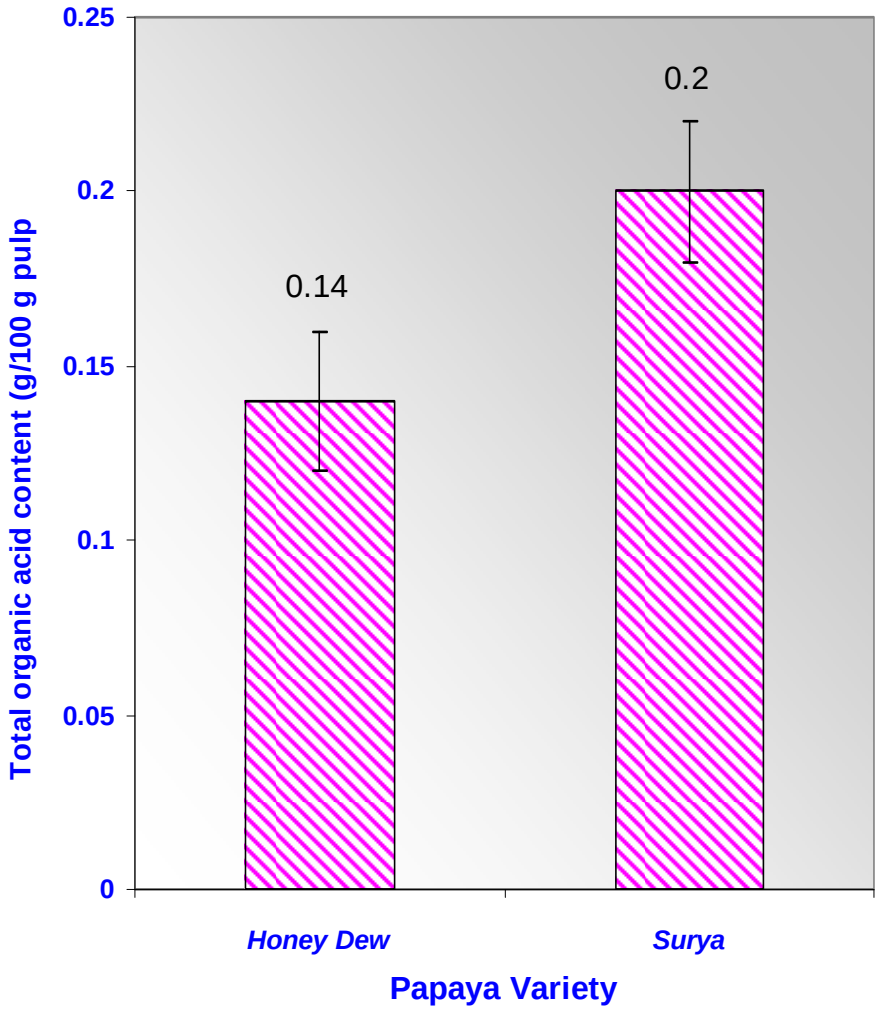


Fig.28. Organic acid content in papaya varieties

Table-25. Varietal difference in the bioaccessibility of β-carotene from mango (*Mangifera indica)* in the presence of milk

Mango variety	Total β-carotene (mg/100 g)	Bioaccessible β-carotene (mg/100 g)	
		Without milk	With milk
Badami	3.21 ± 0.25[a]	0.79 ±0.03[a]	1.23 ± 0.04[a]
Raspuri	1.83 ± 0.08[b]	0.71 ± 0.03[a]	0.80 ± 0.05[b]
Malgoa	0.55 ± 0.03[c]	0.18 ± 0.01[b]	0.20 ± 0.04[c]
Mallika	2.77 ± 0.09[a]	0.89 ± 0.04[c]	1.14 ± 0.03[a]
Totapuri	1.27 ± 0.10[d]	0.48 ± 0.03[d]	0.61 ± 0.05[d]
Neelam	1.45 ± 0.23[d]	0.45 ± 0.03[d]	0.64 ± 0.03[d]

Values are Mean ± SEM of five independent determinations, each being in duplicate.

Within the same column values with different superscripts are significantly different ($p<0.05$).

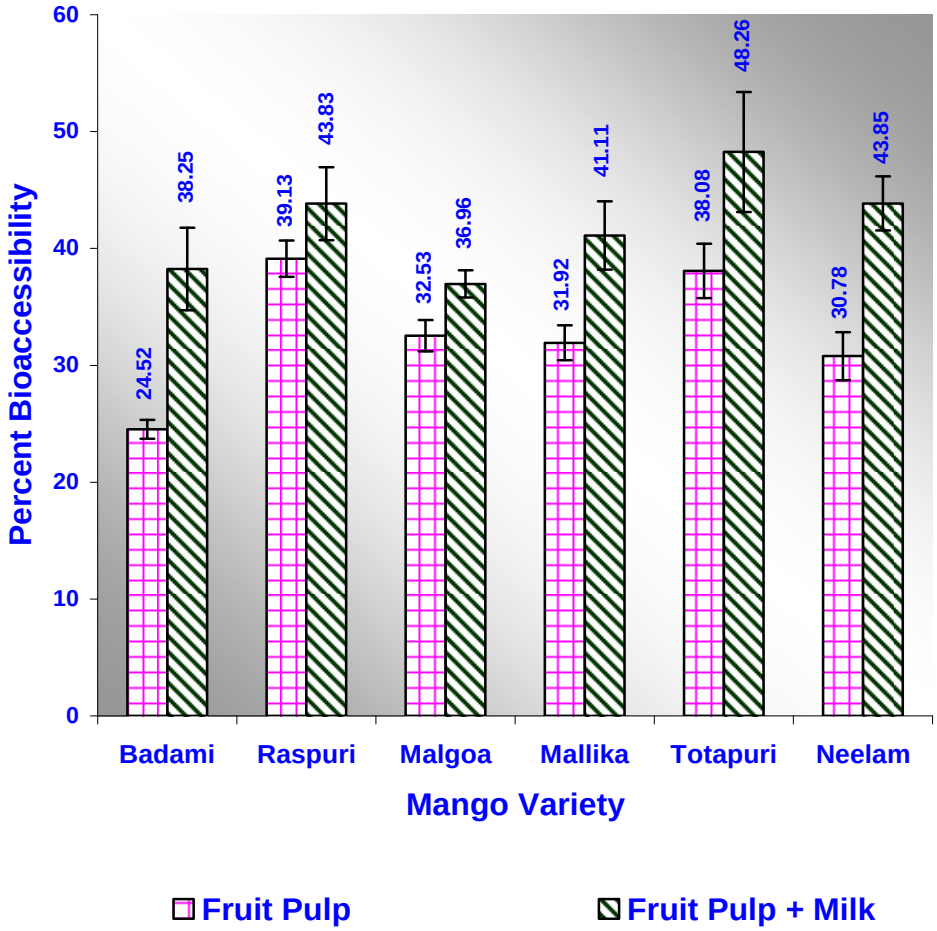


Fig.29. Influence of the presence of milk on the bioaccessibility of β-carotene from mango fruits.

Table-26. Varietal difference in the content and bioaccessibility of β-carotene from papaya (*Carica papaya)* in presence of milk

Papaya variety	Total β-carotene (mg/100 g)	Bioaccessible β-carotene (mg/100 g)	
		Without milk	With milk
*Honey de*w	0.70 ± 0.10[a]	0.24 ± 0.02[a]	0.28 ± 0.04[a]
Surya	0.73 ± 0.12[a]	0.23 ± 0.04[a]	0.32 ± 0.02[a]

Values are Mean ± SEM of five independent determinations, each being in duplicate.

Within the same column values with different superscripts are significantly different ($p<0.05$).

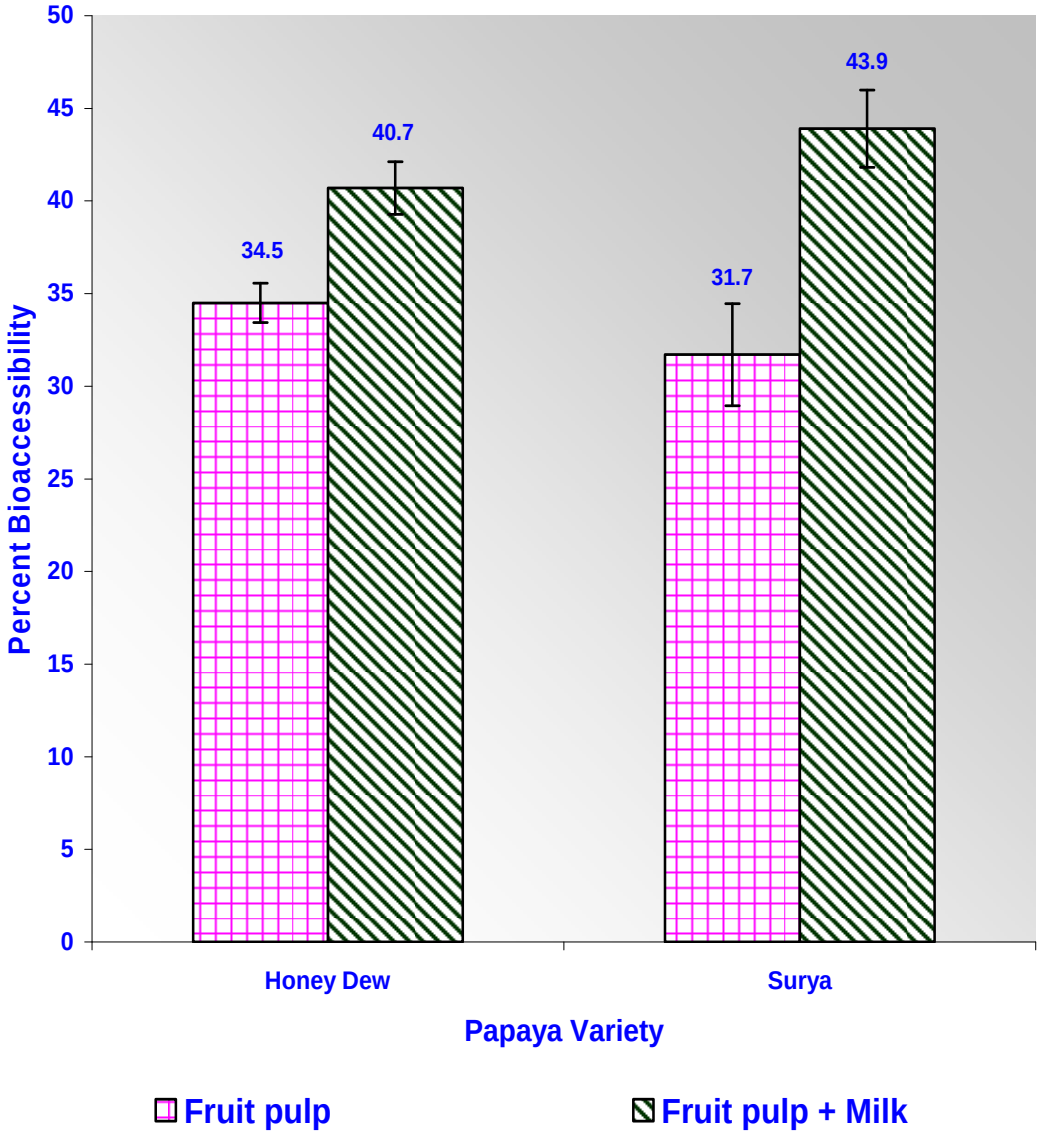


Fig.30. Influence of the presence of milk on the bioaccessibility of β-carotene from papaya fruits.

DISCUSSION

The β-carotene content of a mango cultivar (variety not specified) as reported by the National Institute of Nutrition (Gopalan *et al.*, 2004) is 1.99 mg/100 g. This value is close to that of the *Raspuri* variety of mango (1.83 mg/100 g) determined by us in the present study. Varietal differences in the concentration of β-carotene in mango grown in Brazil and Thailand have been reported by other workers (Mercadante and Rodriguez-Amaya, 1998; Pott *et al.*, 2003a; Vazquez-Caicedo *et al.*, 2005). Mercadante and Rodriguez-Amaya (1998) found that the *Keitt* variety of mango contained higher amounts of *all-trans* β-carotene (0.67 mg/100 g) compared to the *Tommy Atkins* variety (0.58 mg/100 g). The *Keitt* vartiety of mango brought from Bahia state of Brazil, however, had a significantly higher content of *all-trans*-β-carotene (1.5 mg/100 g). These authors observed that climatic conditions in which the fruits are grown too have an influence on the carotenoids content, with fruits being grown in hot regions having a generally higher carotenoid concentration. Pott *et al.* (2003a) reported differences in the carotenoid content of three cultivars of mangoes. The *all-trans*- β-carotene content in the *Kaew* variety was 11.6 mg/100 g, while the same was 4.60 and 3.70 mg/100 g in the *Kent* and *Tommy Atkins* varieties, respectively. Recently, Vazquez-Caicedo and co-workers (2005) reported differences in the *all-trans*- β-carotene and its *cis* isomers in nine different cultivars of mango grown in Thailand. Thus, the β-carotene content of the Indian cultivars of mango studied here was higher than that of *Keitt* and *Tommy Atkins* varieties grown in Brazil.

Other than the above reports on the total β-carotene content of mango, information on the bioavailability of this provitamin from mango fruits is lacking. Our current study is probably the very first attempt to understand the bioaccessibility of β-carotene from this abundant source and also the existent varietal differences in the same.

This study which has essentially envisaged varietal differences in the content and bioaccessibility values of β-carotene in mango has indicated that the latter is independent

of the former. Considering both the total content and percent bioaccessibilty, the *Mallika* variety provides the highest amount of β-carotene, followed by the *Badami* and *Raspuri* varieties for the same amount of edible pulp consumed. Thus, among the average sized fruit of any of the varieties of mango (around 200 g edible pulp), the above three varieties would provide 1.4 - 1.8 mg of β-carotene which corresponds to 60-75% of the RDA (2.4 mg) of this provitamin for Indians (ICMR, 2000). The other three varieties (*Totapuri*, *Neelam* and *Malgoa*) provide less than 1 mg of β-carotene per fruit of similar size.

Although mango fruits are consumed even in raw stages, ripe mangoes are the choices for deriving the maximum amounts of provitamin A. Carotenoid content increases with ripening of the mango fruit, with all-*trans*-*β*-carotene being the predominant carotenoid at all stages of ripening (Ornelas-Paz *et al*, 2008). Accumulation of carotenoids correlates with the ripening stage, with a change in mango flesh color from white to yellow-orange which has also been suggested as a reliable maturity index (Vázquez-Caicedo *et al,* 2005; Ueda *et al*, 2000). Besides providing higher amounts of provitamin A compared to the raw fruit, ripe fruits are also associated with higher bioaccessibility of the same. Softening of the pulp during ripening might increase the accessibility of carotenoids by facilitating the mechanical and enzymatic disruption of the pulp during digestion with release of carotenoids to oil droplets and enhance the potential for absorption (Ornelas-Paz *et al*, 2008). Thus ripening likely has a similar effect as homogenization and thermal processing that disrupt cell walls to provide digestive enzymes with access to macromolecules to facilitate transfer of carotenoids to micelles and enhance the potential for absorption (van Het- Hof, 2000; Hedren, 2002).

Determination of the total organic acids present in the edible portions of these mango varieties indicated that bioaccessibility of β-carotene from specific mango varieties roughly corresponded with the organic acid content of the fruits of *Raspuri, Totapuri, Malgoa and Badami* variety. i.e. the variety with highest organic content also showed the highest β-carotene bioaccessiblity, and *vice versa.* Such a correspondence was however not seen in *Mallika* and *Neelam* varieties. This could be due to the fact that organic acids are not the only modifiers of β-carotene bioaccessibility. Other factors, especially fiber

and carotenoids other than β-carotene (which have not been determined here) may also influence the same.

Besides organic acids, other factors may influence bioaccessibility of β-carotene in mango fruits. The amount of pectin present in mango pulp may also influence the extent of bioaccessibility of β-carotene, and the varietal differences in the same may be attributable in part to the variable pectin content in mango cultivars. Several investigators have reported that ingestion of pectin and other soluble fibre decreases carotenoid bioavailability in human subjects (Riedl *et al.,* 1999). The negative effects of mango pectin can be attributed to its ability to alter the process of mixed micelle formation.

We have earlier reported the beneficial influence of food acidulants on the retention of β-carotene in vegetables (Gayathri *et al*., 2004). Inclusion of tamarind and citric acid (0.1 and 0.01%, respectively) along with green leafy vegetables during heat processing brought about a significant improvement in the retention of β-carotene. Results of the present study showed that higher the organic acids inherent in the fruit, higher were the bioaccessibility of β-carotene. Thus, organic acids either exogenous or inherent help in the retention of β-carotene in food matrix during heat processing as well as improve the bioaccessibility of the same.

There was no significant difference in the content of β-carotene between the two varieties of papaya examined in this study, and so also, no varietal differences were evident in the percent bioaccessibility of β-carotene from these two papaya cultivars. Unlike in the case of mango, the organic acid content of papaya did not seem to influence the bioaccessibility of β-carotene. Despite a higher amount of organic acid in the *Surya* variety compared to the *Honey Dew* variety, the percent bioaccessibility of β-carotene from both the varieties was similar. The absence of a direct relationship between organic acid content and β-carotene bioaccessibility in these two varieties of papaya suggests that factors other than mere organic acids could also be responsible for the observed trend.

Absence of any difference in the β-carotene content of five cultivars of papaya grown in Brazil, values ranging from 0.23 to 0.37 mg/100 g of the ripe fruit pulp has been

reported (Wilberg and Rodriguez-Amaya, 1995). Setiawan *et al.* (2001) reported a β-carotene content of 0.44 mg/100 g fresh edible portion of papaya. The concentration of β-carotene of papaya in our study (0.69 and 0.73 mg/100 g for the *Surya* and *Honey Dew* varieties, respectively) is higher than that reported by these two studies. These values agree with those reported by The National Institute of Nutrition, India (0.88 mg/ 100 g) for papaya grown in India (Gopalan *et al*., 2004). This indicates that as in the case of mangoes, geographical location probably influences the β-carotene content of papaya. In the absence of any information on the bioavailability of β-carotene from papaya, ours is the first observation on the same. Although the percent bioaccessiblity of β-carotene is similar in both mango and papaya, considering the total content of this provitamin, papaya has to be consumed nearly three times the amount of mango to derive the same amount of β-carotene. This is feasible in view of the relative abundance and low cost of papaya in this country, where this fruit is especially affordable by the lower economic segments of population, who are at risk of vitamin A deficiency.

The β-carotene content of any of the varieties of mango and papaya examined in this investigation is much less compared to the amounts present in the commonly consumed green leafy vegetables (GLV) (4.8 to 15.7 mg/100 g) and carrot (8.1 mg/100 g) (Veda *et al*., 2006). Although the percent bioaccessibility of β-carotene from the fruit pulp of mango and papaya is generally higher than that from pressure-cooked GLV (12.5-25.8%) and carrot (20.4), considering the total amount of this provitamin latent in them, bioaccessible β-carotene per 100 g pulp of mango and papaya turns out to be lesser than that from the same amount of pressure-cooked GLV (0.9 - 2.4 mg/100 g), or carrot (1.5 mg/100 g) [Previous Chapter]. However, considering the amounts of GLV or carrot consumed in a day's menu and probably the relatively higher amount of either mango or papaya generally consumed, the latter would still be better providers of bioaccessible β-carotene.

Addition of milk to the fruit pulp generally enhanced the bioaccessibility of β-carotene from all the varieties of mango as well as the two varieties of papaya. The percent

increase in the bioaccessibility of β-carotene in the presence of milk compared to fruit pulp alone was as high as 56 in *Badami* variety of mango. Considering the absolute amount of bioaccessible β-carotene, the blend of milk and the *Badami* variety of mango provides the highest amount of bioaccessible β-carotene (1.2 mg/100 g), followed by the blend of milk with fruit pulp of *Mallika* (1.1 mg/100 g).

Presence of milk had a similar beneficial influence on the bioaccessibility of β-carotene from both the varieties of papaya examined, viz., 40% increase in the *Surya* variety and 18% increase in *Honey Dew* variety. Thus, varietal differences existed in papaya only with respect to influence of exogenous milk on the bioaccessibility of β-carotene.

The enhancing effect of milk on the bioaccessibility of β-carotene from both mango and papaya could probably be attributed to protein as well as fat present in it. Small amounts of fat are essential for the optimal absorption of carotenoids, which are fat-soluble (Thurnham, 2007). The presence of protein in the small intestine has been found to aid stabilization of fat emulsions and enhance micelle formation (West and Castenmiller, 1998). Addition of fermented milk to a green leafy vegetable local to Tanzania is reported to significantly enhance the bioaccessibility of *all-trans*- β-carotene (Mulokozi *et al*, 2004). This, as well as our observation of the enhancing effect of milk on the bioaccessibility of β-carotene from mango and papaya suggests that fat and protein are effective even *in vitro*, where they probably aid the incorporation of β-carotene into the micellar fraction.

Similar promotive influence of fat and protein through chicken enhanced the bioaccessibility of carotenoids from mango in an *in vitro* study. The authors suggested that the fat increases the micellarization of β-carotene, while proteins contribute to the stabilization of fat emulsification (Ornelas-Paz *et al*., 2008).

The accumulation of vitamin A in rat liver fed with a diet containing mango suggests that all-*trans*-β-carotene in this fruit is readily bioavailable (Yuyama *et.al*.,1991).

However, supplementation with retinyl palmitate and mango was insufficient to correct vitamin A deficiency in Senegalese children (Carlier, 1992). An increase in the plasma β-carotene content of children supplemented with dried mango with and without fat, for a period of 4 months has been reported (Drammeh *et al*., 2002). There was also an improvement in the plasma retinol concentration in children receiving fat in addition to dried mango suggesting that fat plays an important role in the absorption of β-carotene and its conversion to vitamin A.

Thus, the present investigation which examined the bioaccessibility of β-carotene from six varieties of mango and two of papaya, has shown that varietal differences exist in both the content of β-carotene and its bioaccessibility in the case of mango. The bioaccessibility of β-carotene from mango roughly corresponded with the organic acid content of the fruits. Considering the total content and percent bioaccessibility, the *Mallika* variety of mango provides the highest amount of β-carotene, followed by the *Badami* variety for the same amount of pulp. There were no significant varietal differences in the β-carotene content or its bioaccessibility from papaya. Consumption of mango and papaya in the form of milk shake seems to be an ideal approach to improve the bioaccessibility of β-carotene. Thus, if consumed alone, the *Mallika* variety of mango provides more β-carotene, while the amount from *Badami* variety can be maximally derived if consumed as milk shake. Although the percent bioaccessiblity of β-carotene is similar in both mango and papaya, considering the total content of this provitamin, papaya has to be consumed nearly 3 times the amount of mango to derive the same amount of β-carotene. The present study suggests that differences may also exist in those varieties of mango and papaya not examined here, with respect to both the content and bioaccessibility of β-carotene.

Summary

Mango and papaya, which are rich sources of β-carotene, are widely consumed in India. In this study, β-carotene content and its bioaccessibility were determined in six locally available varieties of mango *viz*; *Badami*, *Raspuri*, *Mallika*, *Malgoa*, *Totapuri* and *Neelam*, and two varieties of papaya namely *Honey Dew* and *Surya*. Varietal differences were evident in both β-carotene content and its bioaccessibility in the case of mango. β-Carotene content (mg/100 g) in ripe mango ranged from 0.55 ± 0.03 in the *Malgoa* variety to 3.21 ± 0.25 in the *Badami* variety. Similarly, among the *Honey Dew* and the *Surya* varieties of papaya, β-carotene content (mg/100 g) was 0.70 ± 0.10 and 0.73 ± 0.12, respectively. Bioaccessibility of β-carotene ranged from 24.5% in the *Badami* to 39.1 % in the *Raspuri* varieties of mango. Considering both the percent bioaccessibility and the inherent β-carotene content, the amount of bioaccessible β-carotene was highest in the *Mallika* variety (0.89 mg/100 g), followed by *Badami* (0.79 mg/100 g). Since mango and papaya are also consumed as a blend with milk, influence of the presence of milk on the bioaccessibility of β-carotene from these fruits was also examined. Addition of milk generally brought about a significant increase in the bioaccessibility of β-carotene from mango, the increase ranging from 12 to 56%. Bioaccessibility of β-carotene from the two varieties of papaya examined was similar (31.4-34.3%). Addition of milk increased this bioaccessibility by 18 and 38% in these two varieties. Considering the β-carotene content of mango and papaya, the latter has to be consumed roughly 3 times that of mango, to derive the same amount of β-carotene. Thus, this study has indicated that varietal differences exist in the content and bioaccessibility of β-carotene in mango, and that the addition of milk is advantageous in deriving this provitamin A from the fruit pulp of mango and papaya.

CHAPTER – V

ANIMAL STUDIES ON THE INFLUENCE OF DIETARY SPICES ON THE ABSORPTION AND BIOCONVERSION OF β-CAROTENE TO VITAMIN A

A] Influence of dietary spices – black pepper, red pepper and ginger on the uptake of β-carotene by rat intestines

INTRODUCTION

Deficiency of vitamin A is a serious public health problem leading to blindness among children in India (WHO, 2009). While animal foods (egg, milk, liver) are good sources of preformed vitamin A, majority of Indian population is however dependent on plant foods, which provide carotenes, especially β-carotene. Several factors such as diet composition (fat, fiber, protein) and methods employed for food processing affect the bioaccessibility of β-carotene from foods (Rodriguez and Irwin 1972). Studies have shown that absorption of carotenoids from uncooked foods is low, and that mild cooking enhances the same (Poor *et.al* 1993; Our study reported in Chapter III-A).

Presence of dietary factors such as food acidulants and antioxidant spice ingredients influences retention and bioaccessibility of β-carotene. An earlier study in our laboratory revealed that inclusion of food acidulants (tamarind and citric acid) and antioxidant spices (turmeric and onion) during heat processing of vegetables generally improved the retention of β-carotene in the same (Gayathri *et al*., 2004; Our study reported in Chapter III-B). In view of the fact that a majority of the Indian population is dependent on plant foods to meet their requirement of vitamin A, it is desirable to evolve dietary strategies to improve the bioavailability of β-carotene from these sources. Food acidulants- amchur and lime beneficially enhanced the bioaccessibility of β-carotene from green leafy and yellow-orange vegetables (Veda *et al*., 2008). This improved bioaccessibility was evident in both raw and heat-processed vegetables. Presence of the spice turmeric significantly enhanced the bioaccessibility of β-carotene from these vegetables, especially when heat-processed, while the presence of onion also enhanced the bioaccessibility of β-carotene from pressure-cooked carrot and amaranth leaf and from open-pan-boiled pumpkin and fenugreek leaf (Veda *et al*., 2008).

Spices are very commonly used in Indian culinary. Specific spices may alter the ultra-structure and permeability characteristics of intestines. Piperine, the major alkaloid present in black pepper is known to increase bioavailability of drugs and other phytochemicals, which may be attributed to increased absorption, resulting from alteration in membrane lipid dynamics and change in the conformation of enzymes in the intestine (Srinivasan 2007, 2009). The lipophilic spice compounds–capsaicin (red pepper), and gingerol and gingerone (phytochemicals of ginger) share a considerable amount of structural homology with piperine**.** Whether such dietary spices that have the potential to alter the ultra structure and permeability of intestinal brush border beneficially influence the absorption of β-carotene needs to be evidenced in animal studies.

Spices are a group of esoteric food adjuncts that have been in use for thousands of years to enhance the sensory attributes of foods. The quantity and variety of spices consumed in tropical countries is particularly extensive. These spice ingredients impart characteristic flavors, aroma and attractive color to foods (Srinivasan, 2008). Apart from these sensory qualities, a host of beneficial physiological influences are also attributed to spices. Among these, ability to stimulate digestion, beneficial influence on lipid metabolism, efficacy as antidiabetics, antioxidant property, anti inflammatory potential and cancer preventive potential have been extensively documented (Srinivasan, 2005).

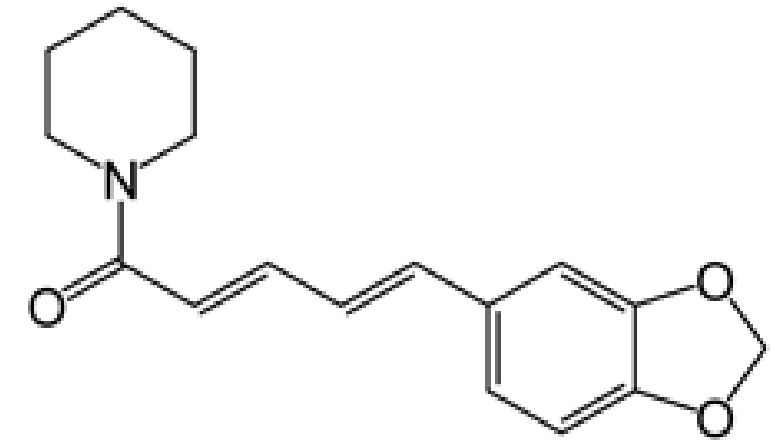
Evaluation of the effect of specific dietary spices on the absorption of β-carotene in particular by the everted sacs of intestinal segments isolated from experimental rats is the objective of this study. Dietary black pepper, red pepper, ginger and their active principles have been examined in particular in this investigation for any influence on β-carotene absorption by virtue of alteration in the ultra structure and fluidity of intestinal brush border (Fig-31, 32). Such basic information on the bioavailability of β-carotene are necessary to optimize dietary approaches to improve the same, and it also helps in rationalizing the RDA for vitamin A (β-carotene).

Black pepper

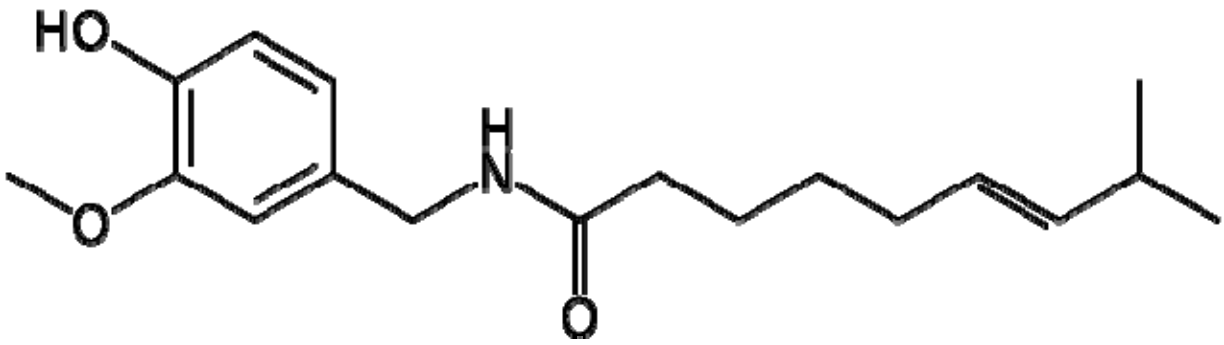
Ginger

Red pepper

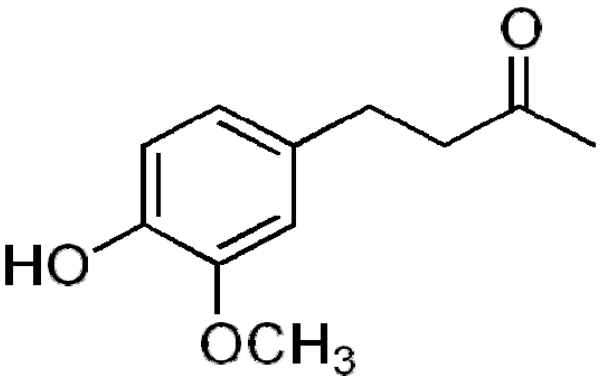
Fig.31. Dietary spices evaluated in this study



Piperine



Capsaicin



Gingerone

Fig.32. Structure of spice compounds evaluated in this study

MATERIALS AND METHODS

Materials

Fresh carrot (*Daucus carota*) was procured from local market, cleaned and used as source of β-carotene in this study. All chemicals used were of analytical grade and the solvents were distilled before use. The spice bioactive compounds- piperine and capsaicin were procured from M/s Fluka Chemie, Buchs, Switzerland. Standard β-carotene, porcine pancreatic pepsin, and pancreatin and bile extract (porcine) were procured from Sigma Chemicals Co., St. Louis, MO, USA. Double-distilled water was employed throughout the entire study. All glassware used was acid washed.

Animal treatment

Animal experiments were carried out taking appropriate measures to minimize pain or discomfort in accordance with the guidelines laid down regarding the care and use of animals for experimental procedures and with due approval from the Institutional Animal Ethics Committee. Young male Wistar rats (8 per group) weighing 80-85 g obtained from the Experimental Animal Production Facility of this Institute were maintained on specific semi-synthetic diets for 8 weeks. The basal diet comprised of (%): casein, 21; cane sugar, 10; corn starch, 54; refined peanut oil, 10; Bernhart-Tommarelli modified Salt mixture, 4 and NRC vitamin mixture, 1. Spices- black pepper (0.5%), red pepper (3.0%), ginger (0.05%), spice bioactive compounds piperine (0.02%) and capsaicin (0.01%) were included in this basal diet to give various experimental diets. The animals were housed in individual stainless steel cages and had free access to food and water. The diet consumption and the gain in body weight during the experimental regimen in all spice groups were comparable to controls.

Food source of β-carotene

Carrot was finely chopped and mashed. It was subjected to simulated gastric digestion at pH 2.0 in the presence of pepsin at 37°C (16 g in 100 ml 0.1M HCl), followed by

simulated intestinal digestion in the presence of pancreatin-bile extract mixture (4 g porcine pancreatin and 25 g bile extract in 1000 ml of 0.1M $NaHCO_3$), pH 7.5 at 37°C for 2 h (As described in Chapter II). The resultant carrot digesta was used as a source of β-carotene in the study of its intestinal uptake.

In vitro intestinal absorption studies

The rats were stunned and after laporatomy, the small intestine was quickly excised. After thoroughly washing both inside and outside with 0.9% saline, it was everted and cut in to segments of uniformly 10 cm length. Uptake of β-carotene *in vitro* by these segments of intestines isolated from spice pre-treated animals was evaluated.

Each intestinal everted sac was filled with Krebs-Ringer phosphate buffer containing 10 mM glucose. Absorption of β-carotene was examined by incubating aerobically, the everted rat intestinal segments in the same Krebs-Ringer phosphate buffer - 10 mM glucose medium (10 ml) placed in 25 ml conical flask containing a known amount of β-carotene source (carrot digesta containing 24 µg β-carotene) that was subjected to simulated gastro-intestinal digestion employing pepsin, pancreatin and bile salts according to a standardized procedure described above. The flasks were aerated with 95% oxygen and 5% carbon dioxide mixture and incubated at 37°C in a Julabo shaking water bath for 3 h (110 strokes/min). At the end of incubation, the sacs were removed, the mucosal surface was washed and serosal contents were collected (Suresh and Srinivasan, 2007). The mucosal medium, serosal fluid and the intestinal tissue were extracted for β-carotene employing an appropriate procedure (described below) and the extracts were analyzed for β-carotene by an appropriate HPLC procedure (described below). Amount of β-carotene absorbed was computed by the values of β-carotene present in the serosal and mucosal side and the intestinal epithelial tissue.

Analysis of β-carotene

β-Carotene in the aqueous, serosal and mucosal medium samples after incubation was extracted initially with a mixture of acetone : ethanol (1:1 v/v) and subsequently with

petroleum ether (Hedren *et al*., 2002). The process was repeated several times to ensure complete extraction of β-carotene. The extracts were pooled and the solvent was evaporated to dryness in a rotary evaporator. The residue was redissolved in petroleum ether and stored in the cold pending analysis. Prior to analysis, petroleum ether was evaporated under nitrogen and the residue was re-dissolved in the mobile phase used for HPLC determination. β-Carotene in the intestinal tissue samples was extracted according to the method of Mercado *et al*. (1989). The intestinal tissue was homogenized in 10 ml chloroform: methanol (2:1 v/v) in a tissue homogenizer fitted with a Teflon pestle. The homogenate was mixed with 2 ml 0.9% saline and then vortexed. The blend was allowed to settle and separate in to two layers and centrifuged at 2500 x g for 10 min. The bottom chloroform layer was separated and evaporated to dryness under a stream of nitrogen gas. The residue was re-dissolved in the mobile phase used for HPLC determination of β-carotene.

Determination of β-carotene was carried out by reverse-phase HPLC (Shimadzu LC 10 AVP), equipped with a PDA detector. β-Carotene was separated on a C_{18} column (S.S.Excil, Australia). The mobile phase consisted of a mixture (v/v) of 65% acetonitrile, 15% methylene chloride and 20% methanol containing 1.3 mmol/l ammonium acetate. β-Carotene was monitored at a wavelength of 450 nm. The peak identities and λ_{max} were confirmed by their retention time and characteristic spectra of standard chromatograms.

During the steps of incubation, and extraction of β-carotene, precautions were taken to minimize the exposure of samples to light and air and thus prevent oxidative destruction of β-carotene. Air was replaced by nitrogen before stoppering the flask at all stages of incubation and storage. All operations were carried out under yellow lighting and the glassware were covered with black cloth to prevent exposure to light.

Statistical analysis

All determinations were made in pentuplicates and the average values are reported. Data were analyzed statistically according to Snedecor and Cochran (1976).

RESULTS AND DISCUSSION

The amount of carrot digesta to be included in the incubation medium as a source of β-carotene was optimized in a trial study using identical length of intestinal segments and varying the concentration of the source of β-carotene. An amount of digesta equivalent to 0.08 g dry carrot present in the incubation medium provided maximum intestinal uptake of β-carotene under the experimental conditions of duration of incubation and the length of rat intestinal segment (Fig.33). The dietary levels of piperine and capsaicin used in this study roughly correspond to the dietary level of their respective parent spices- black pepper and red pepper, respectively used in this animal study.

The uptake of β-carotene from carrot homogenate by everted intestinal segments from rats fed various spices is presented in Table-27. A significantly increased absorption of β-carotene in the intestinal segments from the spice fed animals was generally evidenced. Among the test spices, dietary piperine produced the highest increase in β-carotene absorption, and was 147% of the control value. Whereas dietary ginger increased the intestinal uptake of β-carotene by 98%, dietary black pepper, capsaicin and red pepper brought about an increase of 59, 50 and 27%, respectively, in the intestinal uptake of this provitamin.

Thus, both black pepper and its bioactive constituent- piperine are evidenced here to promote β-carotene absorption in the intestine (Fig.34). The other pungent spice – red pepper and its pungent constituent-capsaicin were also effective in promoting intestinal β-carotene absorption, but to a comparatively lesser degree. Ginger, the third pungent spice was observed here to significantly enhance β-carotene uptake by the intestines, much more than either black pepper or red pepper (Fig.34).

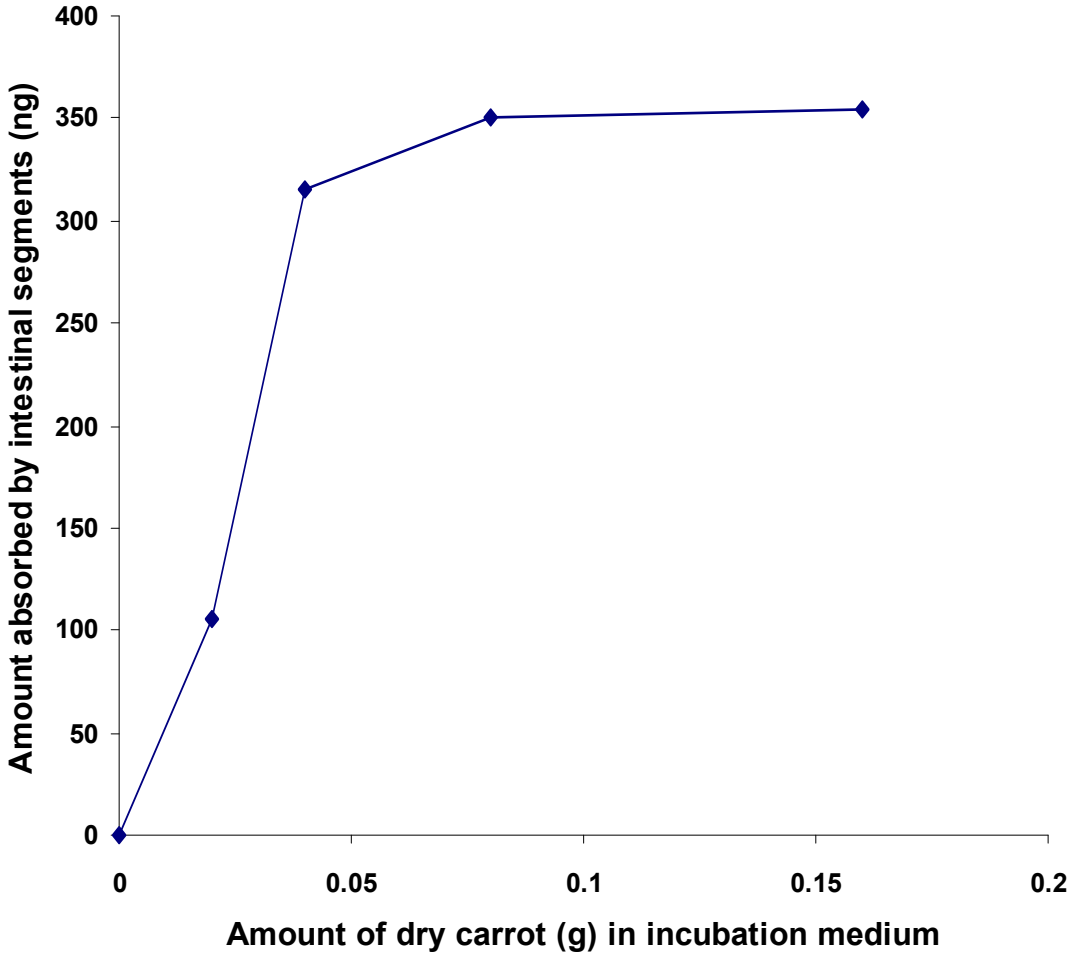


Fig.33. Standardization of uptake of β-carotene by everted intestinal segments from the carrot digesta.

Table-27. Uptake of β-carotene from carrot homogenate by everted intestinal segments from rats fed spices

Rat group	Recovery of β-carotene after 3 h of incubation (μg)			
	Mucosal fluid	Serosal fluid	Intestinal Epithelium	Percent Absorption
Control	22.09 ± 0.52	0.166 ± 0.022	0.256 ± 0.023	1.87
Black pepper	21.30 ± 0.51	0.261 ± 0.032	0.392 ± 0.046	2.97*
Red pepper	22.51 ± 0.47	0.169 ± 0.018	0.379 ± 0.052	2.37*
Ginger	22.03 ± 0.40	0.384 ± 0.043	0.465 ± 0.060	3.71*
Piperine	21.27 ± 0.36	0.332 ± 0.047	0.695 ± 0.097	4.61*
Capsaicin	22.36 ± 0.52	0.332 ± 0.036	0.315 ± 0.032	2.81*

Values are mean ± SEM of eight independent determinations.
* Denotes significantly higher compared to Control group ($p<0.05$).

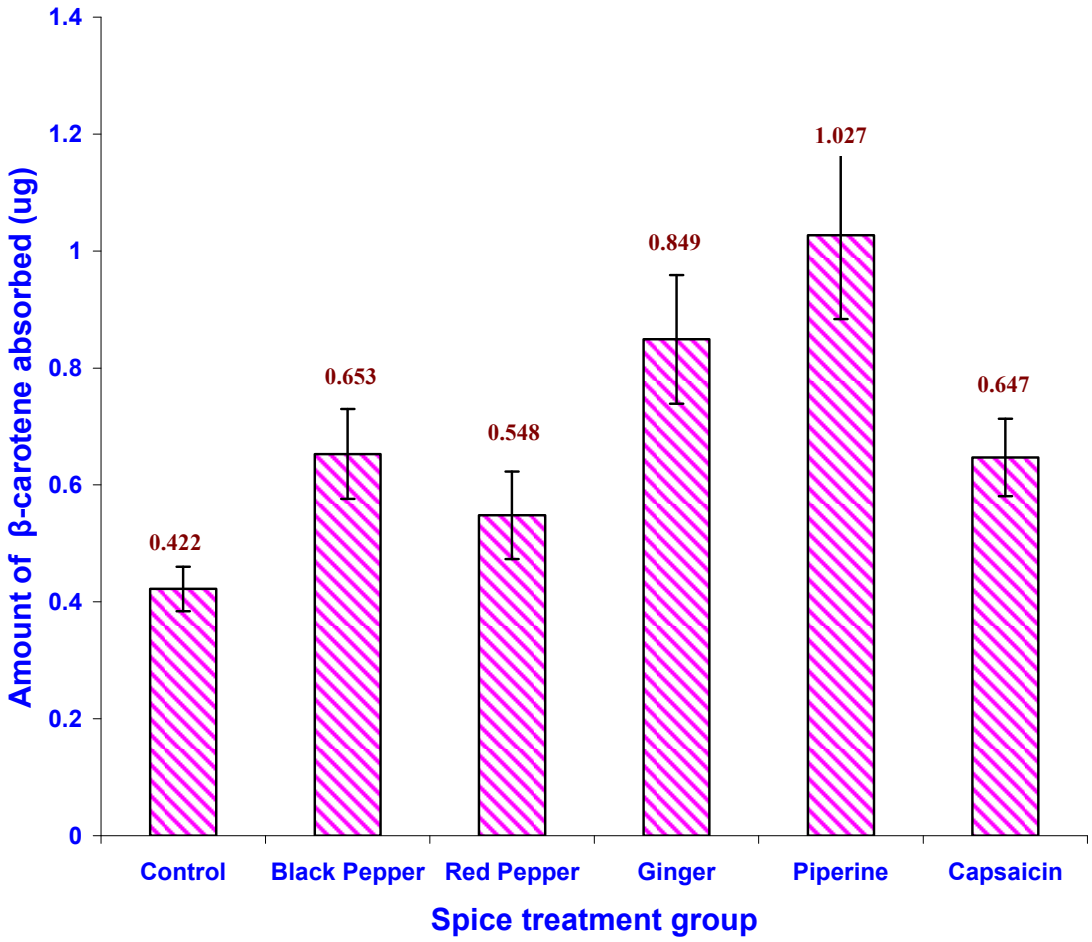


Fig.34. *In vitro* absorption of β-carotene by intestinal segments of spice fed rats.

Values are mean ± SEM of eight independent determinations.
* Significantly higher compared to Control group.

Piperine, the bioactive constituent of the commonly used spice- black pepper is now established as a bioavailability enhancer of various structurally and therapeutically diverse drugs and other substances (Srinivasan, 2007). Potential of piperine or its parent spice – black pepper to increase the bioavailability of drugs in humans is of great pharmacological value. Atal *et al.* who evaluated the scientific basis of the use of long pepper (*Piper longum*), black pepper and ginger in a large number of prescriptions in the indigenous *Ayurvedic* system of medicine have inferred that these constituents increase the bioavailability of drugs either by promoting rapid absorption from the gastrointestinal tract, or by protecting the drug from being metabolized in its first passage through the liver after being absorbed, or by a combination of these two mechanisms (Atal *et al*.,1981).

In vitro studies on the effect of piperine on the absorptive function of the intestine using freshly isolated epithelial cells of rat jejunum have showed that piperine (25-100 μM) significantly stimulates γ-glutamyl transpeptidase (γ-GT) activity and the uptake of amino acids (Johri *et al*., 1992a). The kinetic behaviour of intestinal γ-GT is altered in the presence of piperine, suggesting that piperine may interact with the lipid environment to produce effects leading to increased permeability of the intestinal cells. It is hypothesized that piperine's bioavailability-enhancing property may be attributed to increased absorption, which may be due to alteration in membrane lipid dynamics and change in the conformation of enzymes in the intestine (Khajuria *et al*., 2002). Results of membrane fluidity studies using an apolar fluorescent probe, pyrene (which measures the fluid properties of hydrocarbon core), showed an increase in intestinal brush border membrane fluidity. Piperine also stimulated leucine amino peptidase and glycyl-glycine dipeptidase activity, due to the alteration in enzyme kinetics. This suggests that piperine could modulate the membrane dynamics due to its apolar nature by interacting with surrounding lipids and hydrophobic portions in the protein vicinity, which may decrease the tendency of membrane lipids to act as steric constrains to enzyme proteins and thus modify enzyme conformation. Ultra structural studies with piperine showed an increase in microvilli length with a prominent increase in free ribosomes and ribosomes on the

endoplasmic reticulum in enterocytes, suggesting that synthesis or turnover of cytoskeletal components or membrane proteins may be involved in the observed effect (Khajuria *et al*., 2002). Thus, it is suggested that piperine may induce alterations in membrane dynamics and permeation characteristics, along with induction of the synthesis of proteins associated with cytoskeletal function, resulting in an increase in the small intestine absorptive surface, thus assisting efficient permeation through the epithelial barrier.

Dietary spices– black pepper, red pepper, ginger and spice bioactive compounds – piperine and capsaicin have been recently evaluated in experimental rats for their influence on the membrane fluidity in intestinal brush border membrane (BBM), activity of intestinal enzymes whose activity is dependent on the interaction with the lipid micro environment of membrane and ultra structural alterations in the intestinal epithelium (Usha Prakash and Srinivasan, 2009). Results of membrane fluidity studies using an apolar fluorescent probe, diphenyl hexatriene (which measures the fluid properties of hydrocarbon core), showed an increase in BBM fluidity in spice fed animals. Dietary spices were shown to stimulate the activities of glycyl-glycine dipeptidase, leucine amino peptidase and γ-glutamyl transpeptidase in jejunal mucosa. Increased activities of these intestinal enzymes suggest that the test pungent spice compounds could modulate the membrane dynamics due to their apolar nature by interacting with surrounding lipids and hydrophobic portions in the protein vicinity, which may decrease the tendency of membrane lipids to act as steric constrains to enzyme proteins and thus modify enzyme conformation. Scanning electron microscopic observation of the intestinal villi from spice/spice principles fed animals revealed alteration in the ultra stucture, especially an increase in microvilli length and perimeter which would mean a beneficial increase in the absorptive surface of the small intestine, providing for an increased bioavailability of micronutrients.

The present study suggests that besides piperine and its parent spice- black pepper, other pungent spices such as ginger and red pepper and its pungent constituent-

capsaicin also have the potential to beneficially influence the intestinal permeability characteristics of the small intestine. It is speculated that pungent spices- black pepper, red pepper and ginger or their active principles could induce alteration in membrane dynamics and permeation characteristics along with induction in the synthesis of proteins associated with cytoskeleton function resulting in an increase in the small intestine absorptive surface, thus assisting efficient permeation through the epithelial barrier. Such an influence of dietary pungent spices needs further in-depth investigation, with regard to *in vivo* absorption of micronutrients. Thus, dietary pungent spices could enhance the absorption of β-carotene and help reduce this micronutrient deficiency. Such promising basic information is likely to help evolve diet based strategies to combat vitamin A deficiency diseases.

Thus, the present study on the uptake of β-carotene by the intestinal segments isolated from rats fed black pepper, red pepper, ginger, piperine and capsaicin indicated higher absorption of β-carotene in the intestines of these spice-fed animals. This effect was highest in the case of dietary piperine followed by ginger and capsaicin. Thus, pungent spices alter permeation characteristics presumably by increasing absorptive surface, and thereby enhance intestinal absorption of β-carotene, which could form a strategy to reduce vitamin-A deficiency.

Summary

In view of the wide-spread deficiency of vitamin-A in population dependent on plant foods, it is desirable to improve the bioavailability of β-carotene. Specific dietary spices may alter the ultra structure and permeability characteristics of intestines. Few common spices were studied here for their possible promoting influence on intestinal absorption of β-carotene by examining the uptake of β-carotene by the intestines from rats fed black pepper, red pepper, ginger, piperine and capsaicin. Higher absorption of β-carotene in the intestines was evidenced in all the spice-fed animals. Dietary piperine and ginger increased the uptake of β-carotene by 147% and 98% respectively. While black pepper and red pepper fed animals showed an increase in absorption by 59 and 27%, dietary capsaicin increased the same by 50%.

CHAPTER – V

Animal studies on the influence of dietary spices on the absorption and bioconversion of β-carotene to vitamin A

B] Influence of dietary spices on the *in vivo* absorption of ingested β-carotene and its bioconversion to vitamin A in experimental rats

INTRODUCTION

β-Carotene is a major source of vitamin A from plant based diets for a large segment of world's population, especially for those in developing countries. As an important precursor of vitamin A, it has a major role to play in normal vision. Besides this, β-carotene also plays an important role in human health by acting as a natural antioxidant, protecting cells and tissues from the damaging effects of free radicals and singlet oxygen. Since vitamin A is a relatively poor antioxidant and cannot quench singlet oxygen, β-carotene may have more importance as an antioxidant than simply serving as a precursor of vitamin A (Bendich, 1989). β-Carotene imparts several of its health benefits through its antioxidant property (Young and Lowe, 2001), which makes it an important nutrient in the prevention of life threatening diseases, such as cardiovascular disease and several types of cancer. The important protective effects of β-carotene are in cardiovascular diseases, erythropoietic protoporphyria, cancer, cataract, immune response, and cystic fibrosis. It brings about immunomodulation by stimulating immune response. β-Carotene can protect phagocytic cells from autooxidative damage, enhance T- and B- lymphocyte proliferative responses, stimulate effectors of T-cell functions, enhance macrophages, cytotoxic T-cells, enhances tumoricidal capacity of natural killer cells, and increase the production of certain interleukins.

Spices, the natural food additives that contribute immensely to the taste and flavor of our foods have been in use for thousands of years. Spices have been recognized to possess several medicinal properties and have been effectively used in the indigenous systems of medicine in India and other countries (Nadkarni and Nadkarni, 1976). Apart from the traditional use, a host of beneficial physiological effects have been brought to the fore by extensive animal studies and numerous human trials during the past three

decades (Srinivasan, 2005; 2005a). Among these are their ability to stimulate digestion (Platel and Srinivasan, 2004), beneficial influence on lipid metabolism (Srinivasan *et al*., 2004), antilithogenic influence (Srinivasan *et al*., 2004), anti-diabetic effect (Srinivasan, 2005b), antioxidant property (Srinivasan, 2009a), anti-inflammatory potential (Srinivasan, 2005), and cancer preventive potential (Srinivasan, 2009a).

Piperine – the bioactive compound of black pepper, has been endowed with an ability to enhance the bioavailability of a number of therapeutic drugs as well as phyto-nutrients (Srinivasan, 2007). It is also evident that bioavailability of these compounds by piperine is partly attributable to an increased absorption, facilitated by alterations in the intestinal epithelial membrane lipid dynamics and permeation characteristics. It would be most relevant to understand whether these compounds also enhance the bioavailability of β-carotene by facilitating its intestinal absorption. Higher uptake of β-carotene *ex vivo* by the intestinal segments was evidenced in animals fed black pepper / piperine, ginger, red pepper / capsaicin with dietary piperine increasing the uptake of β-carotene by as much as 2.5 - fold (Previous section of this chapter). Thus, there is an indication that the three pungent spices tested here, might alter the permeation characteristics presumably by increasing the absorptive surface, and thereby enhance the intestinal absorption of β-carotene. It would be most relevant to evidence the potential of these specific dietary spices to beneficially alter the absorbability of β-carotene in *in vivo* system thus contributing to its increased bioavailability.

In vertebrates, provitamin A carotenoids are converted to retinal by the enzyme β-carotene-15,15'-dioxygenase, and further to retinol by retinal reductase, the activity of which is expressed specifically in intestinal epithelium and in liver. The intestinal enzyme determines whether provitamin A carotenoids are converted to vitamin A or circulated in the body as intact carotenoids. Thus, the bioconversion of β-carotene to retinal is dependent on the regulation of the activity of these enzymes. Since the cleavage enzyme is located in the intestinal cells which are directly exposed to various food components, actions of dietary components such as spices on the enzyme activity might

affect the bioavailability of β-carotene derived from plant foods, and its bioconversion to vitamin A. Thus, the possible modulation of the efficacy of conversion of β-carotene to vitamin A by dietary factors such as spices also merits investigation.

The present study was conducted to evaluate the potential of dietary piperine, capsaicin and ginger on the absorption of the orally administered fat soluble nutrient, β-carotene. This animal study also evaluated the influence of these dietary spices on the efficacy of its conversion to vitamin A. The study was further extended to know the activities of two enzymes specifically involved in the bioconversion of β-carotene to vitamin A in the intestine and liver of spice fed animals. In this study, the spice active principles– piperine, capsaicin, and gingerone were also examined for their *in vitro* influence on the activities of β-carotene-15,15'-dioxygenase and other enzymes involved in the intestinal conversion of β-carotene.

MATERIALS AND METHODS

All-*trans*-retinol, all-*trans*-retinal, all-*trans*- retinyl acetate, β-apo-8-carotenal, β-carotene, phosphatidylcholine, nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide reduced form (NADH), glutathione reduced, bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, USA. Capsaicin (N-vanillyl-6-nonanamide), sodium taurocholate and dithiothreitol were obtained from M/s Fluka Chemie, Buchs, Switzerland. Piperine (1-Piperoyl piperidine) and gingerone were from M/s Aldrich Chemical Co., Milwaukee, USA. Ginger was locally procured. All other organic solvents used were of analytical reagent or HPLC grade.

Animal experiments were carried out taking appropriate measures to minimize pain or discomfort in accordance with standard guidelines laid down for the care and use of animals for experimental procedures and with due approval from the Institute's Animal Ethics Committee. Adult male Wistar rats (8 per group) weighing 80-85 g were maintained on specific semi synthetic diets for 8 weeks. The basal diet comprised of (%)

casein, 21; cane sugar, 10; corn starch, 54; refined peanutoil, 10; Bernhardt-Tommarelli modified Salt mixture, 4 and NRC vitamin mixture. Spices – black pepper (0.5%), red pepper (3.0%), ginger (0.05%), spice bioactive compounds – piperine (0.02%) and capsaicin (0.01%) were included in this basal diet to give various experimental diets. The animals were housed in individual stainless steel cages and had free access to food and water. The diet consumption and the gain in body weight during the experimental regimen in all spice groups were comparable to controls. After 8 weeks of feeding, the overnight fasted rats were given a single oral dose of β-carotene (5.6 µmol or 3 mg) in 1ml of refined peanut oil. Rats from each group were sacrificed at zero time to obtain baseline values. After 4 h *p.o.* administration, the animals were sacrificed (Fig.28). Blood collected from the heart was allowed to clot, and serum was obtained by centrifugation at 1200 x g for 5 min. Small intestine and liver were removed and weighed. Serum and tissues were kept frozen at -20ºC.

In a separate animal experiment, groups of male Wistar rats weighing 80-85 g (n=6 per group) were maintained on specific semi synthetic diets containing spices – black pepper (0.5%), red pepper (3.0%), ginger (0.05%), spice bioactive compounds – piperine (0.02%) and capsaicin (0.01%) for 8 weeks. At the end of the feeding trial, these animals were fasted overnight and sacrificed under ether anaesthesia. Liver and small intestine were quickly excised, washed with ice cold saline, and processed for enzyme activity determinations.

Extraction of β-Carotene and retinoids

β-Carotene and retinol in serum were extracted under yellow light at 4^{o}C by a slight modification of the procedure given by Barua *et al* (1998). Serum was mixed with ethanol (1 ml), dilute acetic acid (3.3 mol/l, 0.1 ml), ethyl acetate (1 ml) and hexane (1 ml). The mixture was vortexed (30 sec) and then centrifuged (1200 x g) for 1 min. The supernatant was removed, and the pellet was extracted with hexane (1 ml). The pooled extracts were vortexed with water (0.5 ml) and then centrifuged at 1200 x g for 1 min. The organic extract was evaporated to dryness under a stream of nitrogen. The residue was dissolved in the mobile phase and injected in to the HPLC column. The recovery of

internal standard retinyl acetate was 90-95%. The efficiency of β-apo-8'-carotenal was similar to retinyl acetate under the same conditions.

Liver and intestine samples were weighed (approx. 1 g) and homogenized in 5 ml of chloroform ($CHCl_3$) and methanol (CH_3OH) (2:1, v/v) using a Potter–Elvehjem homogenizer. Another 5 ml of $CHCl_3$ and CH_3OH was used to rinse the polytron glass homogenizer and the rinse was combined with the homogenate. After adding 2 ml of 0.9% saline, the mixture was vortexed for 2 min and then centrifuged at 2500 rpm at 4°C for 10 min. The $CHCl_3$ layer was evaporated to dryness under nitrogen. The residue was redissolved in 5 ml of mobile phase and a 20 µl aliquot was injected in to the HPLC column.

Determination of β-carotene and retinoids

Determinations of β-carotene and retinol were carried out by reverse phase HPLC using a Shimadzu system (Model LC 10AVP) equipped with a PDA detector. β-Carotene and retinol were separated on a C_{18} column (S.S Excil). The mobile phase consisted of a mixture of 65% (v/v) acetonitrile, 15% (v/v) methylene chloride, and 20% (v/v) methanol containing 1.3 mmol/l ammonium acetate. An isocratic analysis was performed at a flow rate of 1ml/min. β-Carotene was monitored at a wavelength of 450 nm and retinol at a wavelength of 325 nm with a PDA detector. They were quantified from their peak areas by comparing with the standard curves of their reference compounds (Fig.35 -37). The peak identities and λ_{max} values were confirmed by their retention time and characteristic spectra of standard chromatograms.

Activity of β-Carotene 15,15'-dioxygenase (EC 1.13.11.21)

The assay procedure was a modification of the method described by van Vleit *et al.* (1996). The small intestine of rats were flushed with ice cold phosphate buffer saline (PBS) containing 16.5 mmol/l sodium taurocholate to remove mucus and blotted gently with absorbent paper. The duodenum and jejunum portion of intestines (approx. the first 60 cm) was cut lengthwise; the mucosa was scrapped off with a glass slide. The mucosal scrapping was homogenized in a Potter–Elvehjem homogenizer using 5 ml of ice cold

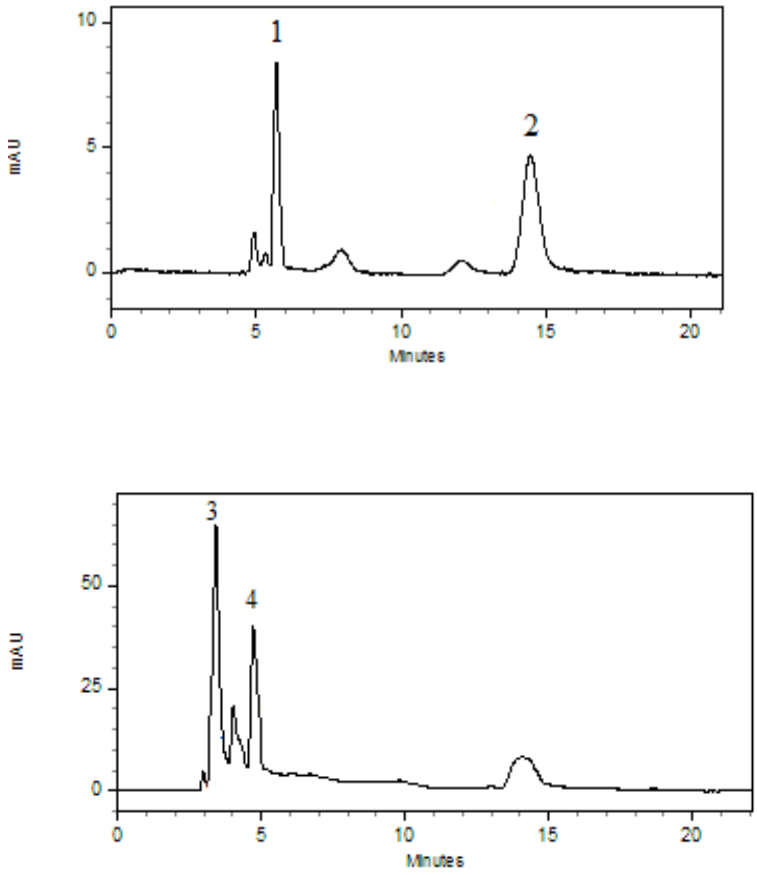


Fig.35. HPLC profile of carotenoids and retinoids present in serum of rats at 4 h after single dose administration of β-carotene (A) β-carotene (detected at 450 nm) and (B) retinol (detected at 325nm)
Peak #1: β-apo-8'-carotenal (Internal standard); Peak #2: β-carotene; Peak #3: Retinal; Peak # 4: Retinyl acetate (Internal standard)

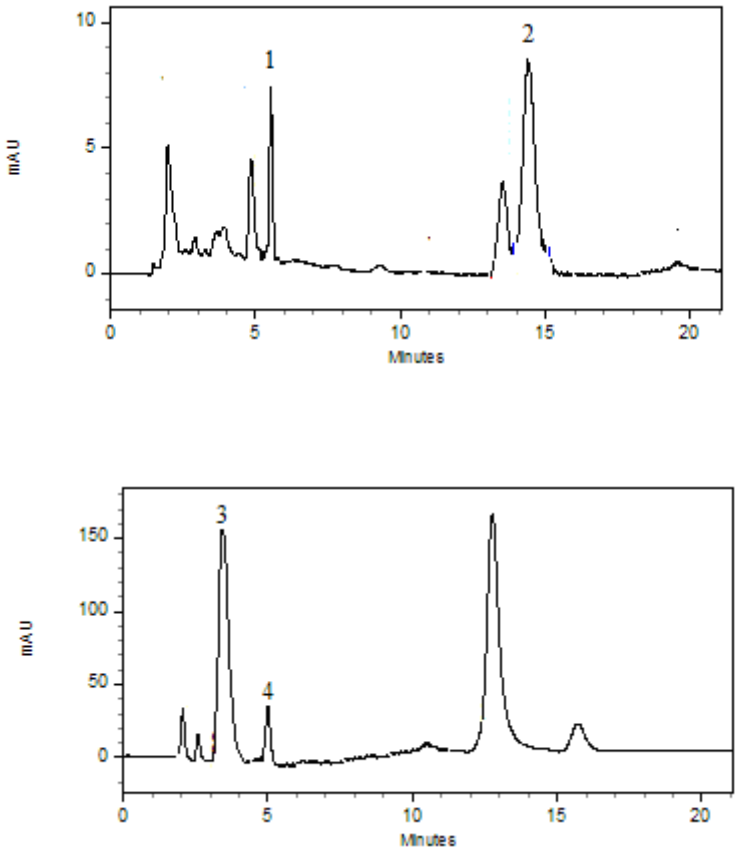


Fig.36. HPLC profile of carotenoids and retinoids present in intestine of rats at 4 h after single dose administration of β-carotene (A) β-carotene (detected at 450 nm) and (B) retinol (detected at 325nm)
Peak #1: β-apo-8'-carotenal (Internal standard); Peak #2: β-carotene; Peak #3: Retinal; Peak # 4: Retinyl acetate (Internal standard)

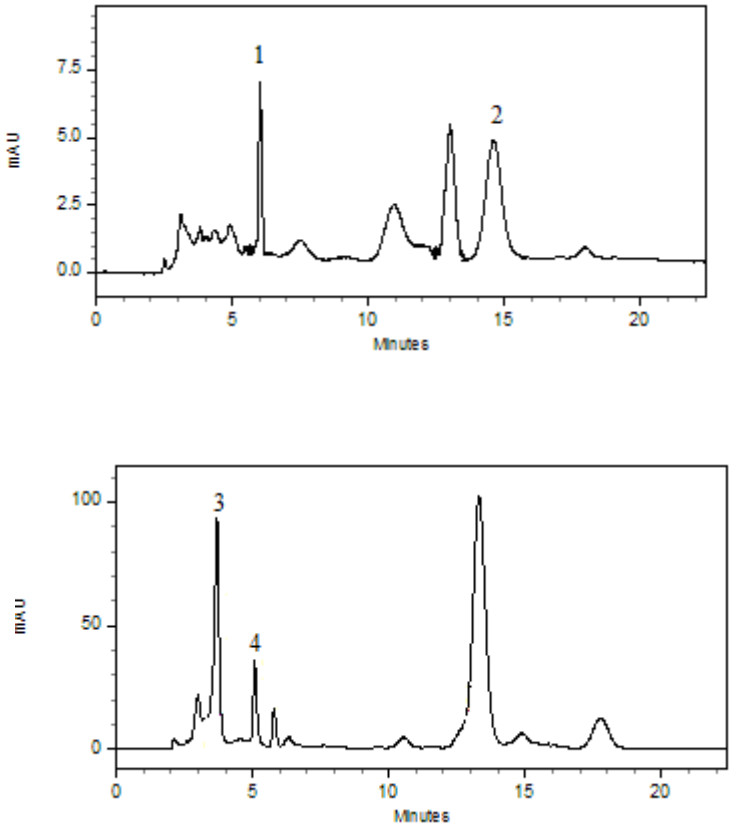


Fig. 37. HPLC profile of carotenoids and retinoids present in liver of rats at 4 h after single dose administration of β-carotene (A) β-carotene (detected at 450 nm) and (B) retinol (detected at 325nm)
Peak #1: β-apo-8'-carotenal (Internal standard); Peak #2: β-carotene; Peak #3: Retinal; Peak # 4: Retinyl acetate (Internal standard)

potassium phosphate buffer (in mmol/l:100.0 potassium phosphate, 4.0 magnesium chloride, and 1.0 dithiothreitol (Sigma Chemical Co., St. Louis, MO, USA), pH 7.7. The homogenate was centrifuged at 9000 x g at 4^{o}C for 30 min and the supernatant collected. Protein was measured by procedure of Lowry *et al.* (1951) using bovine serum albumin as reference. Liver lobe weighing approx.1 g was homogenized with 9 volumes of 100 mmol/l potassium phosphate buffer, pH 7.4, with 50 mmol/l potassium chloride in a glass homogenizer for 2 min to get 10% (w/v) liver homogenate.

The β-carotene-15,15'-dioxygenase enzyme activity was assayed in an incubation mixture (2.0 ml) that consisted of (mmol/L): potassium phosphate buffer, 100.0 (pH 7.7); NAD, 30.0; $MgCl_2$, 2.0; reduced glutathione, 5.0; sodium dodecyl sulfate, 1.7; sodium taurocholate, 6.0; egg phosphatidyl choline, 0.2 g/l; and 25 µl α-tocopherol solution in ethanol (0.125 g/l) and 3 µg of β-carotene (added as solution in 20 µl acetone). The reaction was started by the addition of 3.5 mg of intestinal supernatant protein. The mixture was incubated in a shaking water bath at 37^{o}C in the dark for 1 h. The reaction was stopped by the addition of 2 ml of ethanol. Parallel controls were run without added β-carotene, and also without added enzyme preparation. Under the conditions used, retinal was the only product which could be detected.

At the end of enzyme reaction, the assay mixture was mixed with an equal volume of ethanol containing 1% pyrogallol and extracted twice with an equal volume of hexane. After a brief centrifugation, the upper phase was transferred into a vial, dried under nitrogen and resuspended in mobile phase. Retinal formed in the reaction was determined by reverse phase HPLC in a Shimadzu system (Model LC 10AVP) equipped with a photo diode array detector. Retinal was separated on a C_{18} 5 µm reverse phase column, 250 x 4.6 mm (S.S Excil). The mobile phase consisted of a mixture of 65% (v/v) acetonitrile, 15% (v/v) methylene chloride, and 20% (v/v) methanol containing 1.3 mmol/l ammonium acetate. Unutilized β-carotene was also quantitated by monitoring at a wavelength of 450 nm, while the product retinal was monitored at a wavelength of 380

nm. The peak identities and λ_{max} values were confirmed by their retention time and characteristic spectra of standard chromatograms.

Activity of Retinal reductase (EC: 1.1.1.1)

The assay procedure was a slight modification of the method described by Sundaresan *et al.* (1977). Intestinal mucosal scrapings were homogenized for 2 min in a polytron glass homogenizer in 10 ml of 250 mmol/l sucrose solution containing 25 mmol/l KCl. The homogenate was centrifuged at 14,000 x g for 15 min in a Sorvall (RC-5B) super speed refrigerated centrifuge, and the supernatant fraction at 100,000 x g for 60 min in an ultracentrifuge (Beckman L2-65B) at 4^{o}C. The supernatant was used for the assay of retinal reductase. The assay was performed with 500 nmol all-*trans*-retinal (Sigma Chemical Co., St Louis, MO, USA) in 2 ml of potassium phosphate buffer [100.0 mmol/l potassium phosphate, 30.0 mg Tween-40, 0.5 mmol/l NADH, 1.0 mmol/l reduced glutathione (pH 7.2)] in a shaking water bath at 37^{o}C in dark for 1 h. The reaction was stopped by adding 5 ml of ethanol. Controls with identical reaction mixtures, but with added ethanol at zero time itself were also included. After the addition of ethanol, samples were extracted twice with an equal volume of hexane. The upper phase was dried under nitrogen and resuspended in mobile phase. Retinal reductase activity was expressed as nmol of retinol formed/h/mg of protein. The mobile phase consisting of 100% methanol and 0.5 % ammonium acetate pumped at a flow rate of 1 ml/min.

Effect of spice compounds on activities of β-carotene- 15,15'-dioxygenase and retinal reductase in vitro

In a separate animal experiment, a group of male Wistar rats weighing 150-160 g were maintained on basal semi synthetic diet (described above) for 1 week for acclimatization. These animals were fasted overnight and sacrificed under ether anaesthesia. Liver and small intestine were quickly excised, washed with ice cold saline, and processed for enzyme activity determinations in the presence of test spice compounds added to the assay mixture. Stock solutions of spice compounds- capsaicin, piperine and gingerone

were prepared in acetone (mg/ml). Suitable aliquots of these stock solutions were included in the assay systems of β-carotene- 15,15'-dioxygenase and retinal reductase to give a final concentration of 1 x 10^{-6}M, 1 x 10^{-5}M, and 1 x 10^{-4}M. Activities of β-carotene-15,15'-dioxygenase and retinal reductase were determined as outlined previously. All assays were conducted in quadruplicates

Statistical analysis

Results are expressed as mean ± SEM and comparisons between groups were made by means of an unpaired Student's t-test (Snedecor and Cochran, 1976). Differences were considered significant when $p < 0.05$.

RESULTS

Influence of dietary spices on intestinal absorption of orally administered β-carotene

In an initial study with control rats, it was observed that β-carotene concentration reaches maximum concentration in circulation around 4 h following its oral administration (Fig.38). Hence, all subsequent experiments involving oral administration of β-carotene were terminated at 4 h.

An animal study was conducted to evaluate the influence of dietary spices- piperine, capsaicin and ginger on the *in vivo* absorption of orally administered β-carotene and also its conversion to vitamin A. Young male Wistar rats were maintained on specific spice containing diets for 8 weeks. These rats were administered a single oral dose of β-carotene at the end of this feeding trial. After 4 h *p.o.* administration, concentration of β-carotene and retinol in serum, liver and intestine were determined. There was a significant increase in β-carotene concentration in the serum, liver and intestine of piperine and ginger fed rats as compared to control (Table-28). β-Carotene concentration in the serum was 70 and 51% higher in these animals respectively, as compared to control animals (Fig.39). Similarly, intestinal β-carotene was 69 and 55% higher in dietary piperine and ginger treatment, respectively (Fig.40). Hepatic β-carotene was 118% higher in dietary piperine group after 4 h *p.o.* administration of this provitamin (Fig.41).

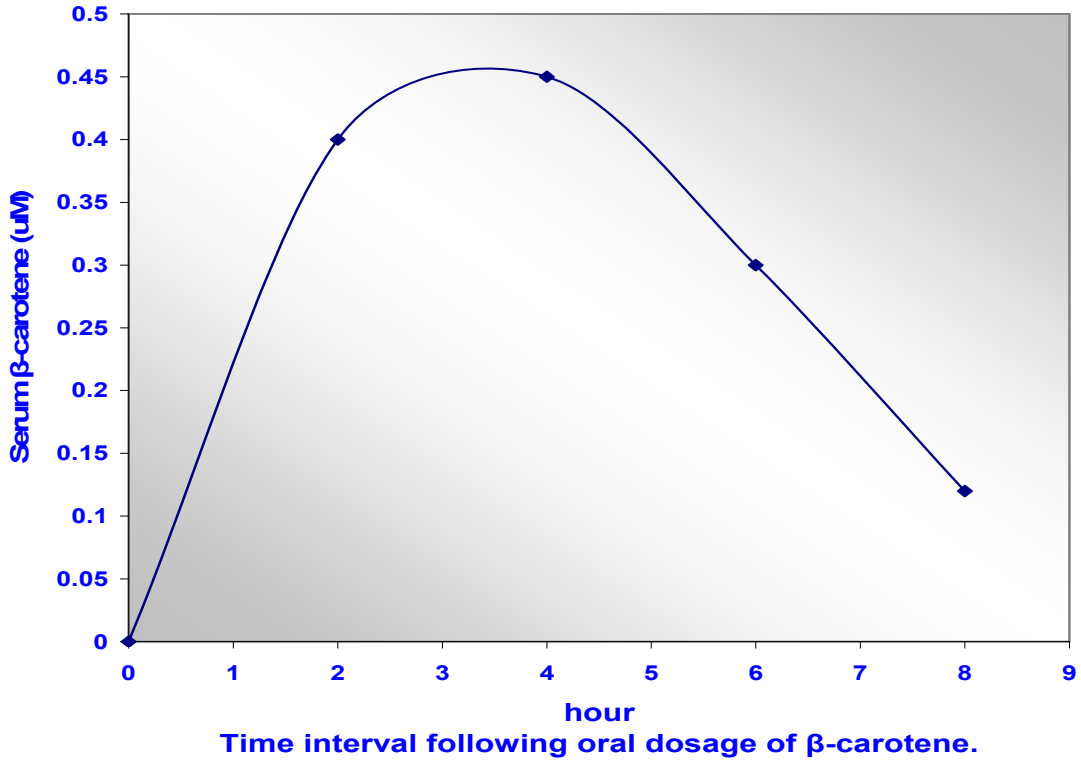


Fig.38. Serum β-carotene concentration as a function of time following its oral administration

Table-28. β-Carotene concentration in the tissues of spice fed animals 4 h after single oral administration.

Diet group	Serum (μM)	Intestine (nmole/g)	Liver (nmole/g)
Control	0.494 ± 0.012	4.15 ± 0.25	0.382 ± 0.06
Piperine	0.840 ± 0.045*	7.02 ± 0.43*	0.832 ± 0.03*
Capsaicin	0.517 ± 0.012	4.01 ± 0.13	0.549 ± 0.05*
Ginger	0.746 ± 0.060*	6.45 ± 0.23*	0.450 ± 0.019*

Values are mean ± SEM of 6 animals per group.

* Significantly different compared to Control group ($P < 0.05$)

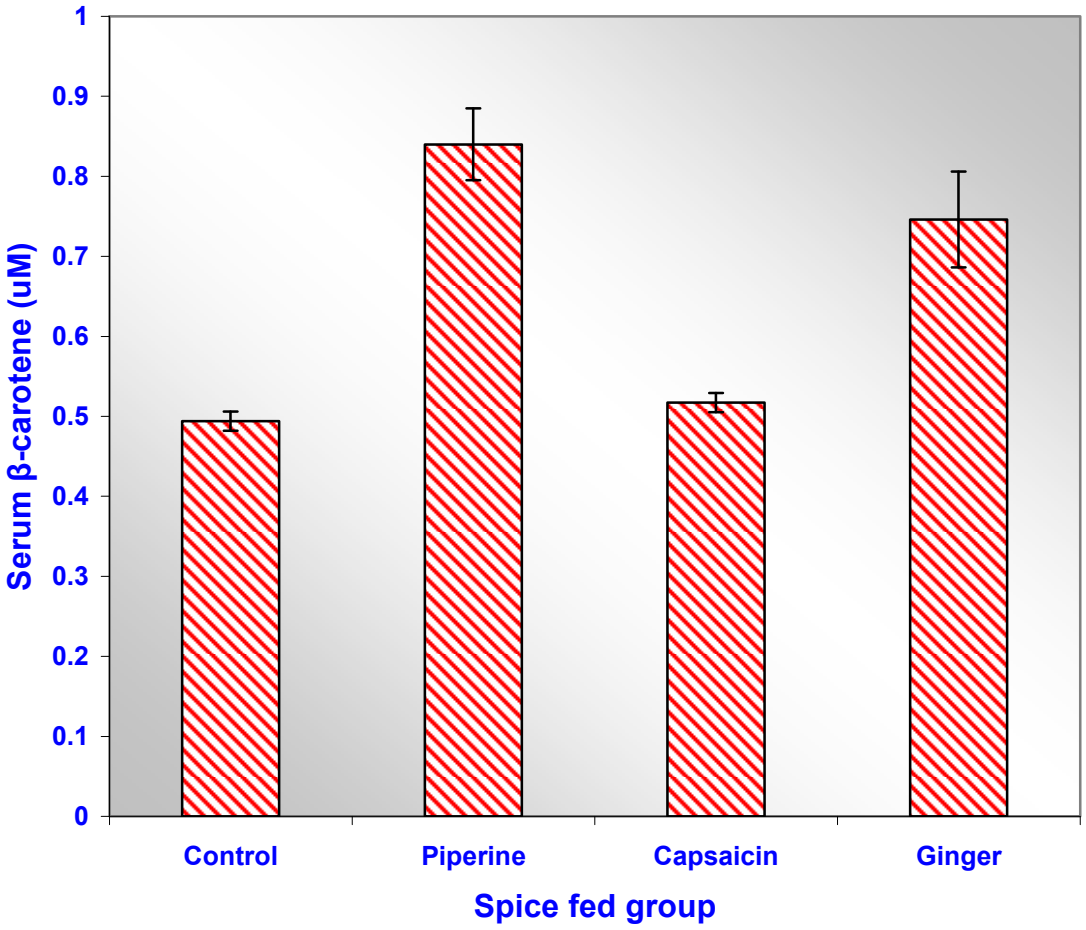


Fig.39. β-Carotene concentration in the serum of spice fed animals 4 h after single oral dosage

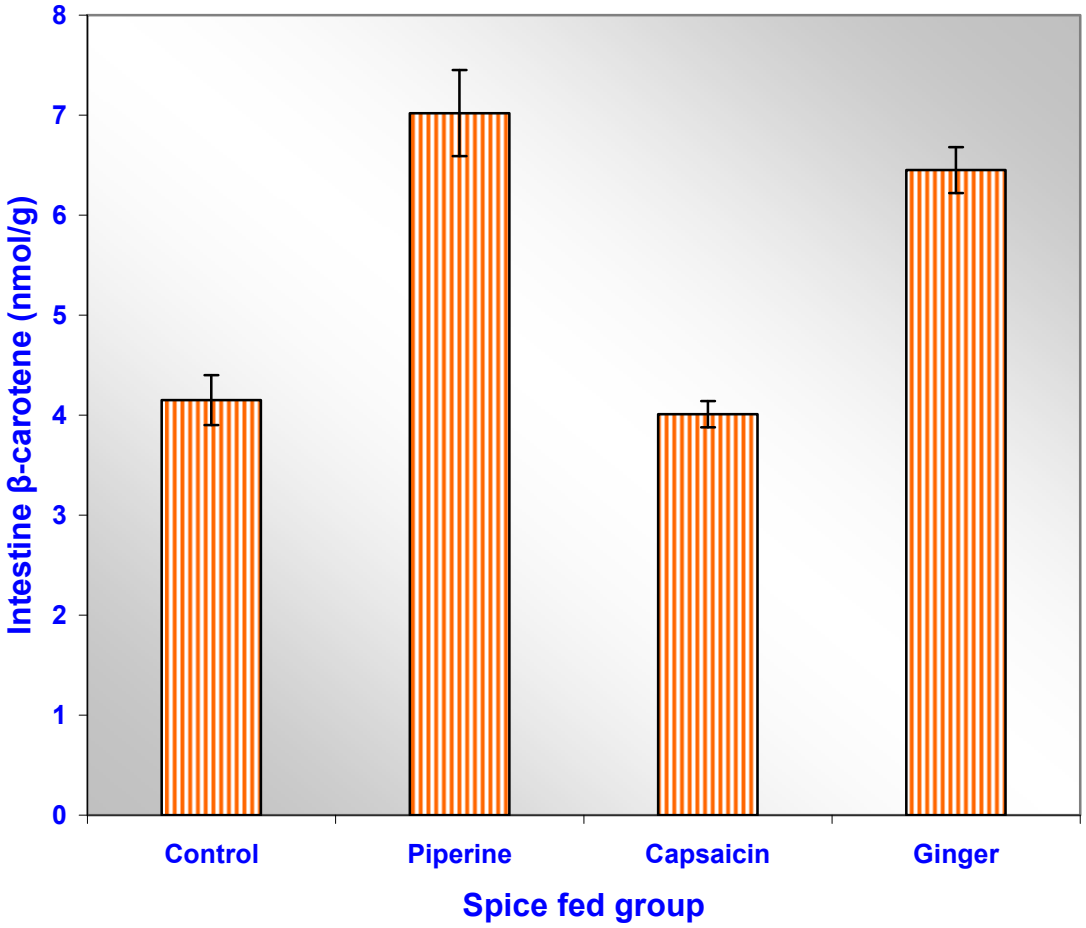


Fig.40. β-Carotene concentration in the intestine of spice fed animals 4 h after single oral dosage

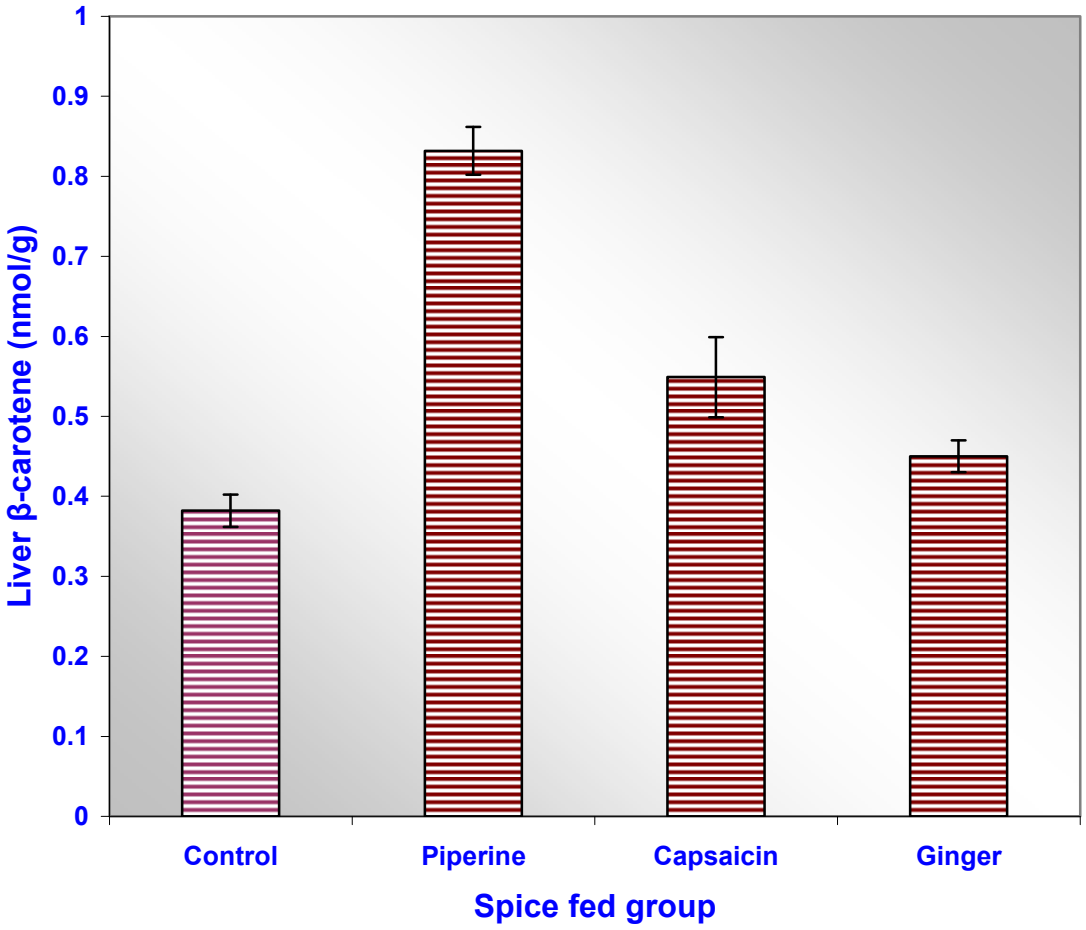


Fig.41. β-Carotene concentration in the liver of spice fed animals 4 h after single oral dosage

β-Carotene concentration was also higher in the liver of capsaicin fed group (by 44%) and in the liver of ginger fed group (by 18%). The results on tissue β-carotene concentrations following its oral administration suggested that dietary piperine and ginger improve intestinal absorption of β-carotene leading to an increased β-carotene concentration in circulation and in tissues.

Retinol concentration in the tissues of spice fed animals 4 h after oral dosage of β-carotene is presented in Table-29. The concentration of retinol was not significantly changed in these spice fed groups as compared to control, suggesting that bioconversion of β-carotene to vitamin A was not significantly influenced by these dietary spices (Fig.42 -44).

Influence of dietary spices on the activities of intestinal and hepatic enzymes involved in the bioconversion of β-carotene

The present study conducted to evaluate the potential of dietary piperine, capsaicin and ginger to influence the absorption of fat soluble nutrient, β-carotene and the efficacy of its conversion to vitamin A, was further extended to know if the activities of two enzymes specifically involved in the bioconversion of β-carotene to vitamin A in the intestine and liver are influenced by these test spices. In young male rats which were maintained on specific spice containing diets for 8 weeks, the activities of two enzymes specifically involved in the bioconversion of β-carotene to vitamin A were measured in the intestine and liver of spice fed animals (Table-30 and Table-31).

Activity of intestinal β-carotene-15, 15'-dioxygenase was comparable to control in piperine treatment (Fig.45). On the other hand, the enzyme activity was significantly lowered in capsaicin and ginger fed animals (Table-30). The extent of decrease in the enzyme activity was 20 and 19% in capsaicin and ginger groups, respectively. β-Carotene 15,15'-dioxygenase activity was also lower in the liver of capsaicin fed animals, the extent of decrease being 19% (Table-30; Fig.46). Activity of retinal reductase either in intestine or liver was not influenced by the test dietary spices (Table-31; Fig.47 and Fig.48).

Table-29. Retinol concentration in the tissues of spice fed animals 4 h after single oral dosage of β-carotene.

Diet group	Serum (μM)	Intestine (nmole/g)	Liver (nmole/g)
Control	1.36 ± 0.10	4.02 ± 0.29	3.09 ± 0.46
Piperine	1.32 ± 0.02	4.95 ± 0.61	4.03 ± 0.75
Capsaicin	1.15 ± 0.07	4.65 ± 0.89	3.14 ± 0.41
Ginger	1.27 ± 0.03	3.69 ± 0.38	2.84 ± 0.21

Values are mean ± SEM of 6 animals per group.

***** Significantly different compared to Control group ($P < 0.05$)

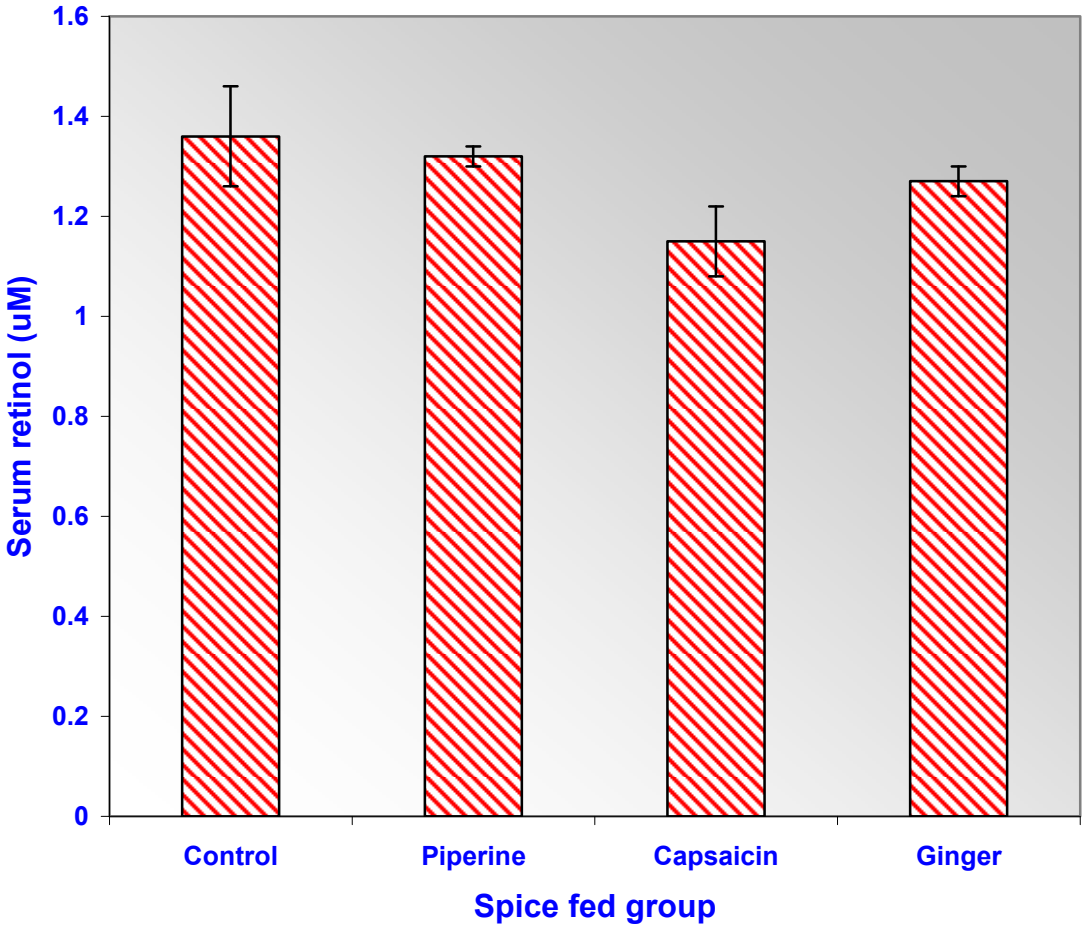


Fig.42. Retinol concentration in the serum of spice fed animals 4 h after single oral dosage of β-carotene.

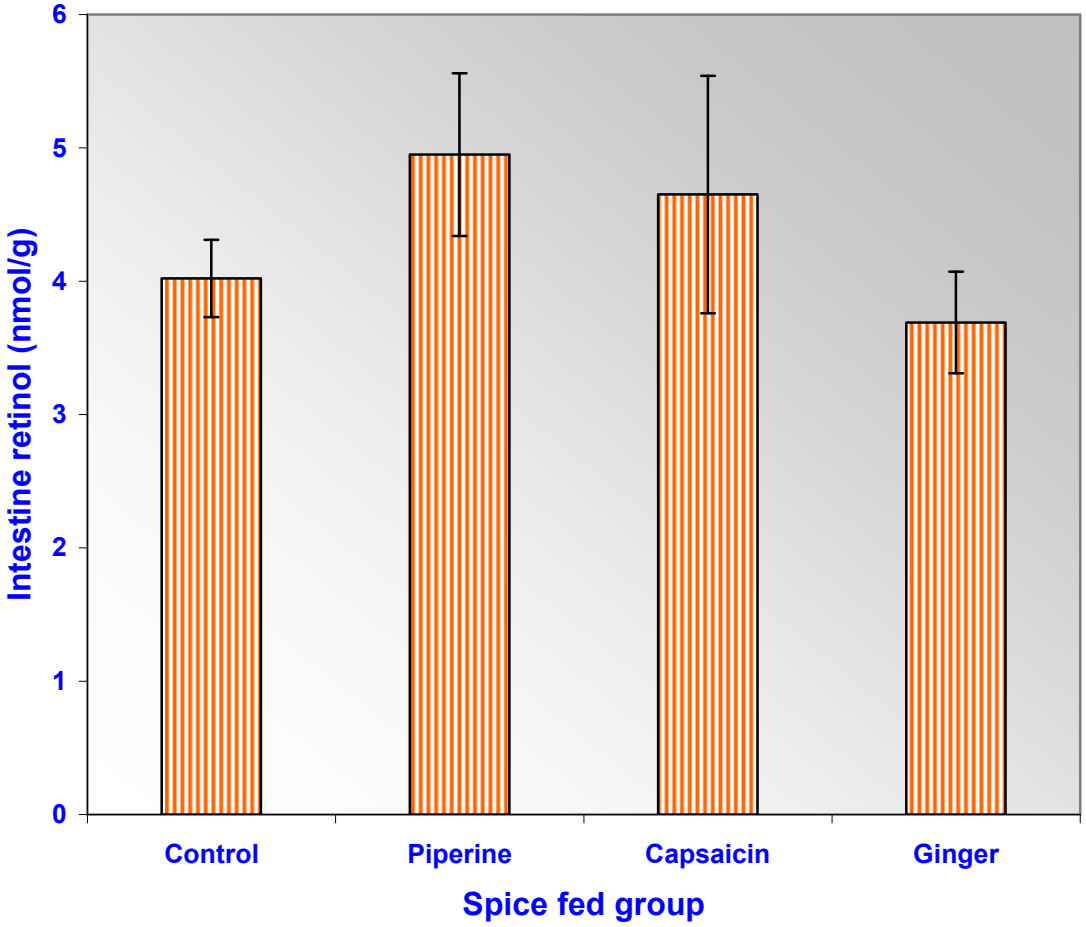


Fig.43. Retinol concentration in the intestine of spice fed animals 4 h after single oral dosage of β-carotene

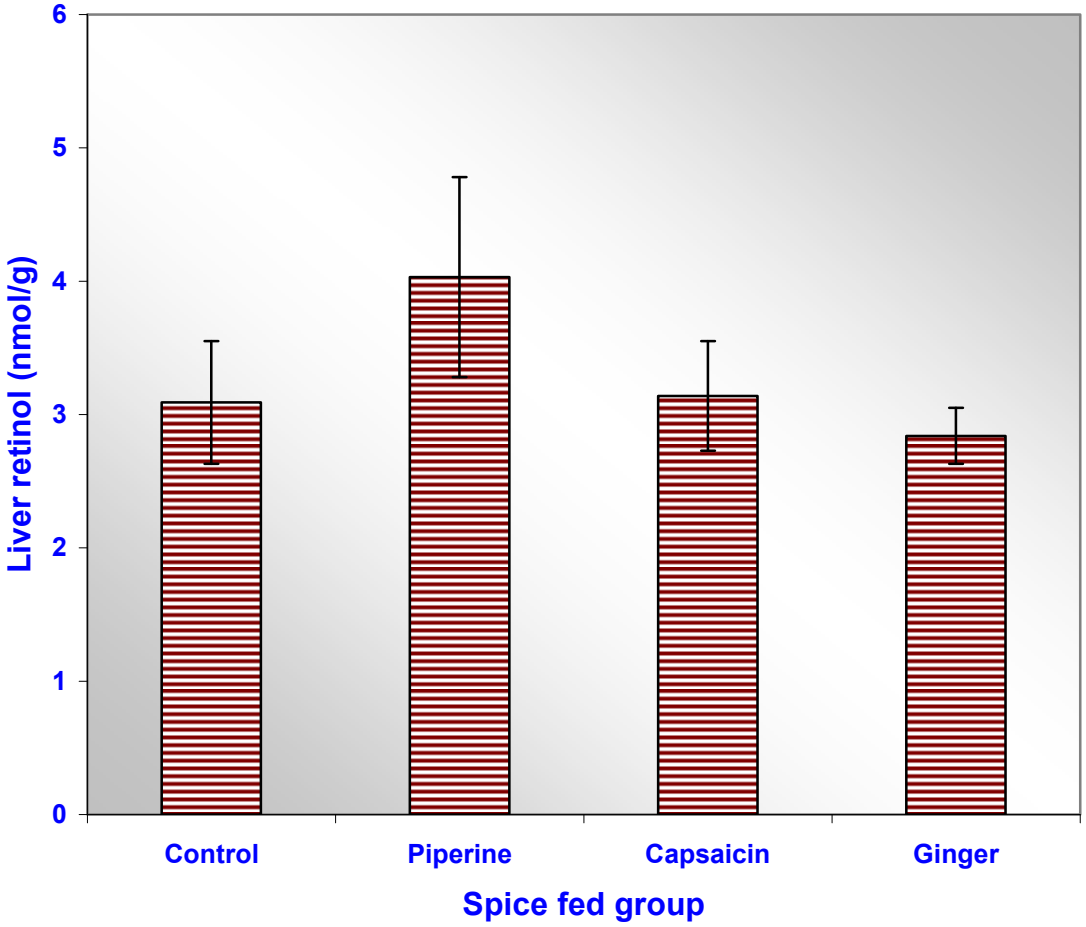


Fig.44. Retinol concentration in the liver of spice fed animals 4 h after single oral dosage of β-carotene.

Table-30. Activity of intestinal and liver β- carotene- 15, 15'-dioxygenase in spice fed rats.

Diet group	Intestine	Liver
Control	75.8 ± 4.68	50.3 ± 4.19
Piperine	74.8 ± 1.95	52.2 ± 1.37
Capsaicin	60.7 ± 2.93*	40.6 ± 0.16*
Ginger	61.1 ± 8.79*	52.5 ± 2.09

Values (expressed as pmole retinal formed/h/mg protein) are mean ± SEM of 6 animals per group

Table-31. Activity of intestinal and liver retinal reductase in spice fed animals

Diet group	Intestine	Liver
Control	5.89 ± 0.34	19.37 ± 1.14
Piperine	6.55 ± 0.38	19.20 ± 1.12
Capsaicin	6.75 ± 0.49	18.40 ± 0.71
Ginger	7.05 ± 0.84	19.08 ± 0.90

Values (expressed as pmole retinal formed/h/mg protein) are mean ± SEM of 6 animals per group

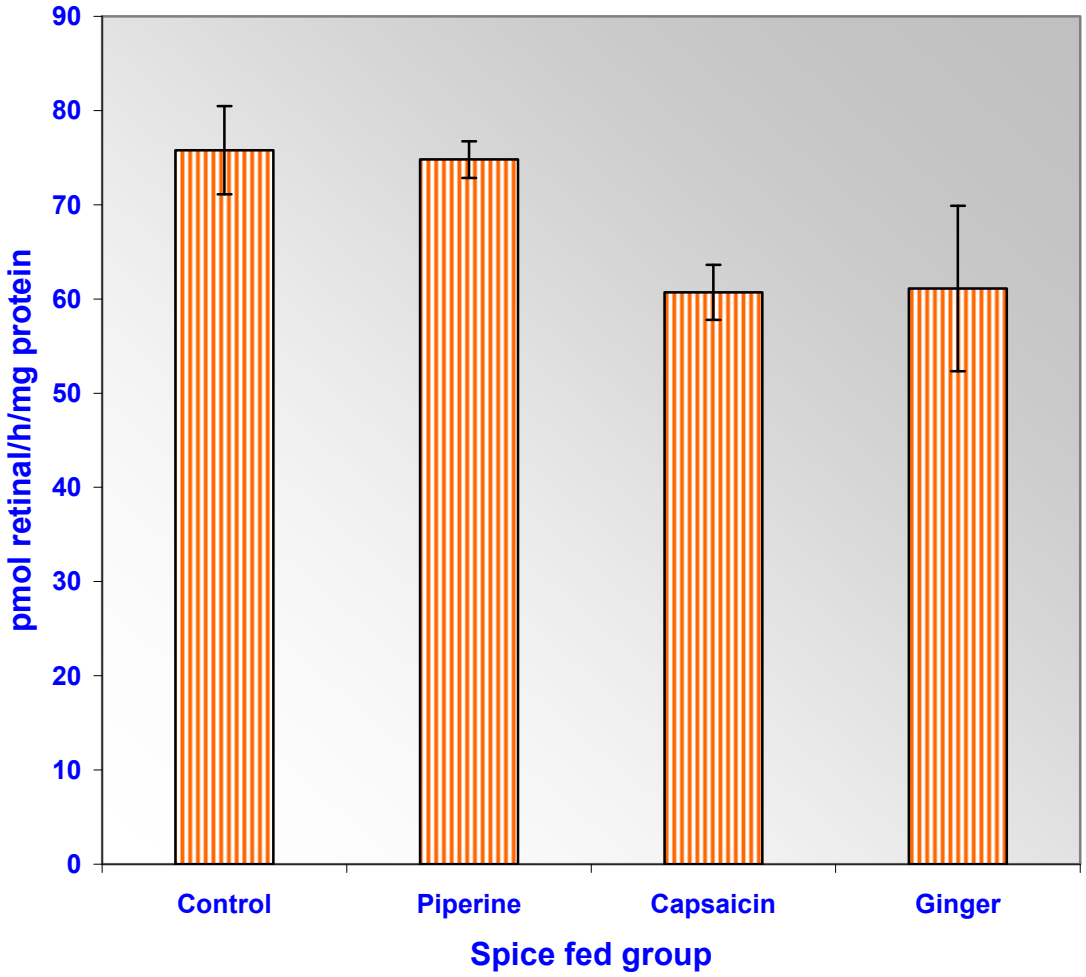


Fig.45. Intestinal β-carotene 15,15'-dioxygenase activity in spice fed animals

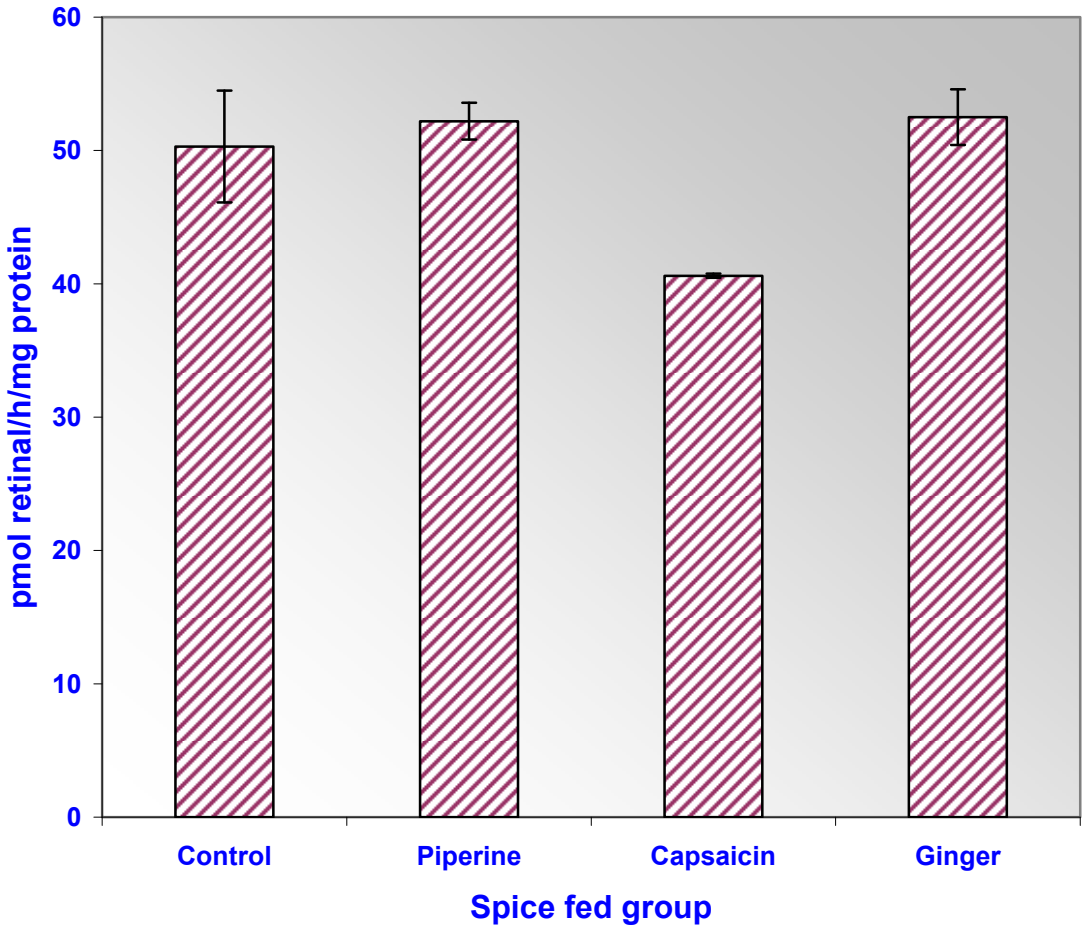


Fig.46. Liver β-carotene 15,15'-dioxygenase activity in spice fed animals

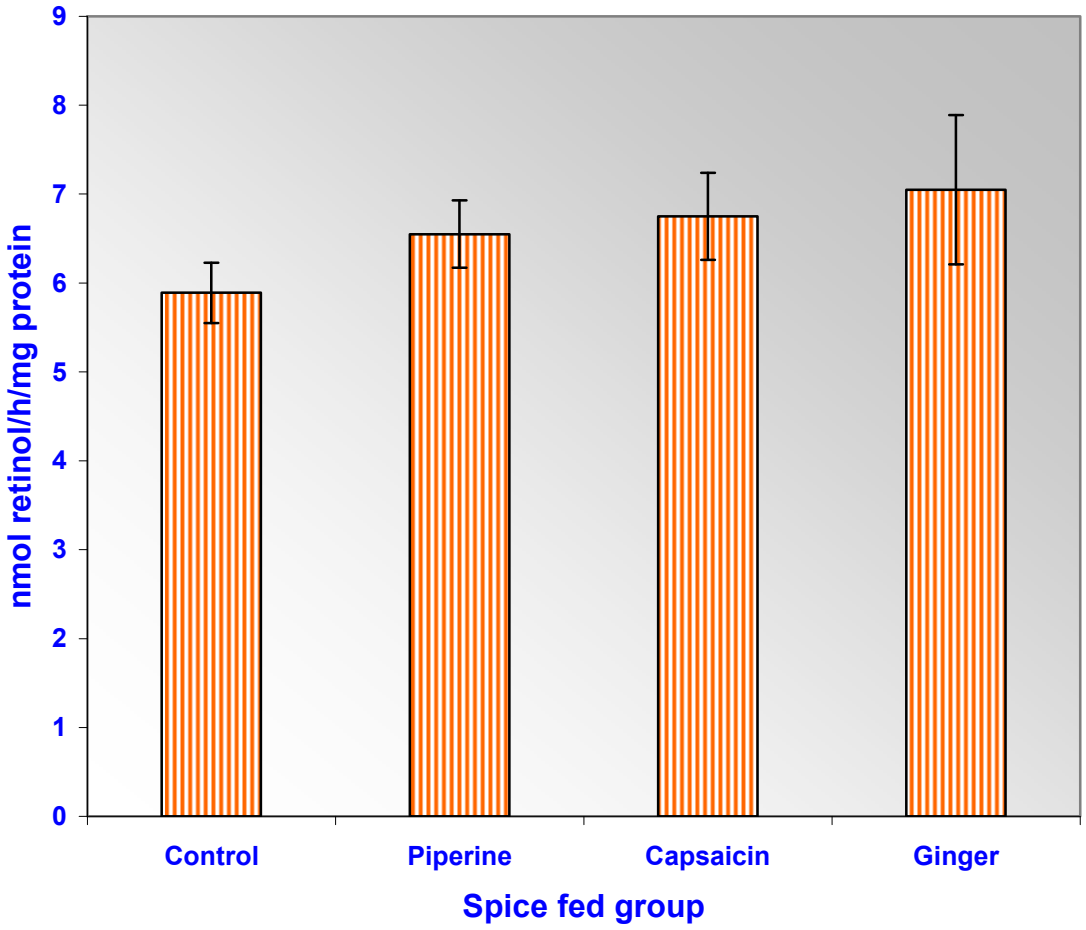


Fig.47. Intestinal retinal reductase activity in spice fed animals

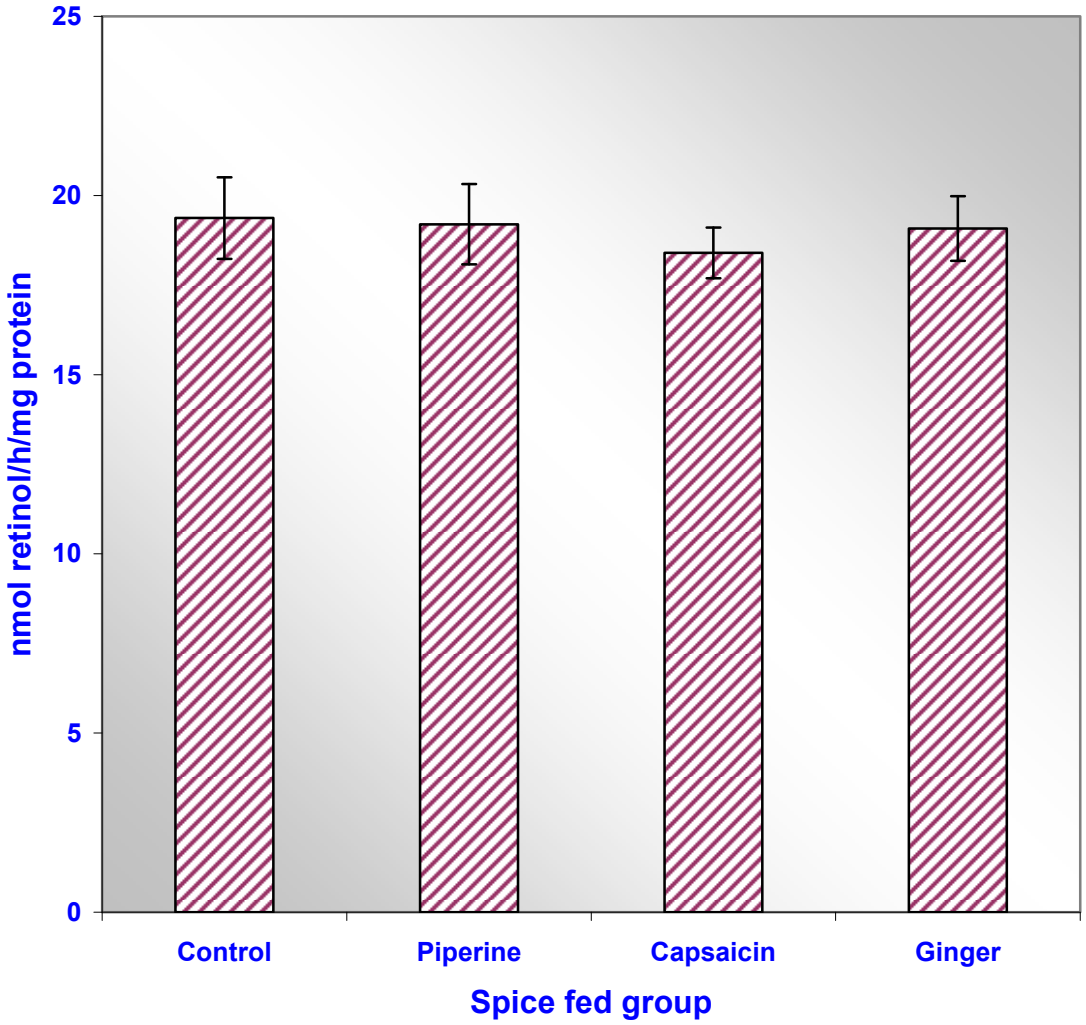


Fig.48. Liver retinal reductase activity in spice fed animals

Effect of spice compounds on the activities of rat intestinal and hepatic β-carotene-15,15'-dioxygenase and retinal reductase in vitro

Since dietary test spices did not have a positive influence on the activities of either hepatic or intestinal β-carotene cleavage enzyme and retinal reductase, it was imperative that these spices did not enhance the bioconversion of β-carotene to vitamin A under the experimental conditions. On the other hand, dietary capsaicin even lowered the activities of the cleavage enzyme involved in the bioconversion of β-carotene in the intestine and liver, while dietary ginger lowered the activity of the cleavage enzyme in the intestines. Hence, in this study, the spice active principles– piperine, capsaicin, and gingerone were also examined for their *in vitro* influence on the activities of the β-carotene cleavage enzyme 15,15'- dioxygenase as well as retinal reductase involved in the bio-conversion of β-carotene. Rat intestinal and liver homogenate was used as the enzyme source. The enzyme activities were determined in presence of spice compounds in the assay system at three different concentrations, viz., 1 x 10^{-6}M, 1 x 10^{-5} M, 1 x 10^{-4} M.

The *in vitro* influence of spice compounds on the activities of intestinal and liver β-carotene- 15,15'-dioxygenase is presented in Table-32. Capsaicin significantly decreased the activity of liver β-carotene- 15,15'-dioxygenase when included in the assay medium at 1 x 10^{-6}M, 1 x 10^{-5} M, 1 x 10^{-4} M level, while piperine and gingerone inhibited this activity only at 1 x 10^{-4} M level in the assay medium (Table-32). The decrease in hepatic β-carotene-15,15'-dioxygenase activity by capsaicin was roughly proportional to its concentration in the assay medium.

Intestinal β-carotene-15,15'-dioxygenase activity was significantly decreased by capsaicin present at 1 x 10^{-5} M and 1 x 10^{-4} M. Similarly, gingerone showed the inhibitory effect at 1 x 10^{-5} M and 1 x 10^{-4} M in the assay medium, while piperine produced this effect only at a concentration of 1 x 10^{-4} M in the assay medium. The decrease in intestinal β-carotene 15, 15'-dioxygenase activity by capsaicin or gingerone was roughly proportional to their concentration in the assay medium.

Table-32. *In vitro* effect of spice compounds on the activities of intestinal and liver β-carotene- 15,15'-dioxygenase

Spice compound	Concentration in the assay medium	Liver	Intestine
None		68.24 ± 1.83	77.14 ± 2.02
Piperine	1 x 10^{-6}M	65.00 ± 1.01	75.11 ± 3.10
	1 x 10^{-5}M	60.07 ± 2.74	67.89 ± 4.10
	1 x 10^{-4}M	41.60 ± 0.27*	49.13 ± 0.40*
Capsaicin	1 x 10^{-6}M	53.74 ± 0.73*	69.52 ± 2.60
	1 x 10^{-5}M	49.88 ± 0.92*	45.47 ± 2.80*
	1 x 10^{-4}M	25.93 ± 0.29*	37.99 ± 0.41*
Gingerone	1 x 10^{-6}M	62.59 ± 1.47	73.11 ± 2.11
	1 x 10^{-5}M	61.05 ± 2.55	63.72 ± 2.60*
	1 x 10^{-4}M	39.67 ± 1.10*	46.89 ± 2.50*

Values (expressed as pmole retinal formed /h/mg protein) are mean ± SEM of 5 values in each group

* Significant decrease compared to Control group ($P < 0.05$).

In vitro effect of spice compounds on the activities of liver and intestinal retinal reductase is presented in Table-33. Piperine when present in the assay medium at 1 x 10^{-5} M and 1 x 10^{-4} M level significantly increased the activity of hepatic retinal reductase, the increase being as much as 2.5-fold at the latter concentration. Similar increase in the activity of the hepatic enzyme was seen with capsaicin and gingerone only at a concentration of 1 x 10^{-4} M in the assay medium. Whereas, intestinal retinal reductase activity was significantly increased by all the three tested concentrations of piperine, such an effect was seen only at 1 x 10^{-4} M level in the assay medium in the case of either capsaicin or gingerone. The increase in intestinal enzyme activity was more than 3-fold in the higher two concentrations of piperine.

DISCUSSION

The present animal study suggests that the intestinal absorption of β-carotene is higher in animals fed the with test spices (black pepper, red pepper and ginger) as indicated by the concentration of β-carotene in serum, liver and intestinal tissue following the oral intake of β-carotene. On the other hand, these dietary spices have no similar beneficial influence on the bioconversion of the absorbed β-carotene to vitamin A either in intestine or liver, as indicated by the tissue concentrations of retinol as well as the activities of hepatic and intestinal enzymes involved in the cleavage of β-carotene and further reduction during its bioconversion to vitamin A. Results of the *in vitro* study corroborates with the *in vivo* observation with regard to the influence of dietary spices on the bioconversion of β-carotene to vitamin A.

The *in vitro* influence of the tested spice compounds on the activities of β-carotene-15,15'-dioxygenase involved in the bioconversion of β-carotene to vitamin A was in fact negative. Lack of positive influence of the test spices on the bioconversion of β-carotene to vitamin A could be attributed to the fact that the animals were in nutritionally well fed state, and that there was no deficiency in the vitamin A status. As such, the higher β-carotene absorption did not result in a parallel increase in its bioconversion, in the absence of any need for that. The possibility of the test spices having a positive influence

Table-33. *In vitro* effect of spice compounds on the activities of intestinal and liver retinal reductase.

Spice compound	Concentration in the assay medium	Liver	Intestine
None	-	16.29 ± 0.35	8.20 ± 0.22
Piperine	1 x 10^{-6}M	16.67 ± 0.12	10.42 ± 0.45*
	1 x 10^{-5}M	27.25 ± 1.91*	27.90 ± 1.40*
	1 x 10^{-4}M	41.51 ± 2.20*	28.20 ± 1.55*
Capsaicin	1 x 10^{-6}M	15.22 ± 0.48	7.94 ± 0.30
	1 x 10^{-5}M	17.61 ± 0.29	8.37 ± 0.50
	1 x 10^{-4}M	20.9 ± 1.07*	13.76 ± 0.40*
Gingerone	1 x 10^{-6}M	17.72 ± 0.57	9.16 ± 0.29
	1 x 10^{-5}M	17.16 ± 0.44	8.31 ± 0.23
	1 x 10^{-4}M	23.16 ± 0.42*	16.14 ± 0.61*

Values (expressed as pmole retinal formed /h/mg protein) are mean ± SEM of 5 values in each group

* Significant decrease compared to Control group ($P < 0.05$).

on the same needs to be examined in vitamin A deficiency state. Even in the absence of any positive influence of the dietary spices - piperine, capsaicin, and ginger, the appreciable increase in the intestinal absorption of orally administered β-carotene is a highly desirable effect, since apart from its role as provitamin, β-carotene can act as a very good antioxidant, and hence higher absorption of the same contributes to an improved antioxidant status in the animal.

Piperine, the active principle of black and long pepper has been established as a bioavailability enhancer of various structurally and therapeutically diverse drugs and other phytochemicals (Zutshi *et al*., 1985; Bano *et al.*, 1987; 1991). Piperine increases the serum response of β-carotene by non-specific mechanisms which operate directly on the gastrointestinal tract and the liver. This mechanism may involve increased micelle formation (Annamalai and Manavalan, 1990), epithelial cell wall modification due to lipophilic nature of piperine (Johri *et al.*, 1992) or an increase in the bioenergetic processes of the gastrointestinal epithelium due to the thermogenic properties of piperine (Reanmongkol *et al.,* 1988).

We have earlier evidenced that a few common spices have a promoting influence on intestinal absorption of β-carotene wherein the uptake of β-carotene by the intestines isolated from rats fed with black pepper, red pepper, ginger, piperine and capsaicin were higher compared to control (Previous Section). Dietary piperine and ginger increased the uptake of β-carotene by 147% and 98% respectively. While black pepper and red pepper fed animals showed an increase in absorption by 59 and 27%, dietary capsaicin increased the same by 50%. It was inferred that these pungent spices altered permeation characteristics presumably by increasing absorptive surface, and thereby enhance intestinal absorption of β-carotene. Our present *in vivo* animal study which evidenced significantly increased absorption of orally administered β-carotene in all the spice fed groups is consistent with the previous *ex vivo* intestinal uptake study.

Badmaev *et al.* (1999) have evaluated the effectiveness of an extract from the fruit of black pepper, consisting of a minimum of 98.0% pure alkaloid piperine, for its ability to

improve serum response of β-carotene during oral supplementation using a double-blind, crossover study design. In this double blind cross over study on humans, the subjects ingested a daily β-carotene dose (15 mg) either with 5 mg of piperine or placebo during each of two 14-day supplementation period. The results indicated that significantly greater increases ($P<0.01$) in serum β-carotene occurred during supplementation with β-carotene plus piperine (49.8 ± 9.6 μg/dl vs. 30.9 ± 5.4 μg/dl) compared to β-carotene plus *placebo*. Supplementation with β-carotene plus piperine for 14 days produced a 60% greater increase in area under the serum β-carotene curve (AUC) than was observed during supplementation with β-carotene plus placebo. The authors suggested that the serum response during oral β-carotene supplementation was improved through the non-specific thermogenic property of piperine which is an active thermonutrient. In this study involving 12 healthy male volunteers, non-smokers, abstaining from alcohol, not taking nutritional supplements or prescription drugs during the period of cross-over study, β-carotene was compared in volunteers who received the formula with and without Bioperine® for a period of 14 days. A formula having a trademark Bioperine® which consists of 98% piperine has been found to act as enhancer of nutrient absorption.

Khajuria *et al.* (1998) have pointed out that piperine may act as an apolar molecule and form apolar complex with drugs and solutes. It may modulate membrane dynamics due to its easy partitioning thus helping in efficient permeability across the barriers. Khajuria *et al.* (2002) also pointed out that piperine may be inducing alterations in membrane dynamics and permeation characteristics, along with induction in the synthesis of proteins associated with cytoskeletal function, resulting in an increase in the small intestine absorptive surface, thus assisting efficient permeation through the epithelial barrier.

Piperine is reported to have stimulated the activities of intestinal leucine aminopeptidase and glycine-glycine dipeptidase, located on the external surface of brush border membrane (BBM), and whose activity is dependent on the interaction with the lipid micro environment of the membrane (Ugolev *et al*., 1977; McDonald and Barrette,1986). It is suggested that piperine could modulate the membrane dynamics due

to its apolar nature by interacting with surrounding lipids and hydrophobic portions in the protein vicinity, which may decrease the tendency of membrane lipid to act as stearic constrains to enzyme proteins and thus modify enzyme conformation. Ultra structure studies with piperine showed an increase in micro villi length with a prominent increase in free ribosomes and ribosomes on the endoplasmic reticulum in enterocytes suggesting that synthesis or turnover of cytoskeletal components or membrane proteins may be involved in the observed effect. In a recent study from our laboratory, dietary spices – black pepper / piperine, red pepper / capsaicin and ginger were evidenced to induce alteration in membrane dynamics and permeation characteristics, associated with an induction in the increased microvilli length and perimeter, resulting in increased absorptive surface of the small intestine (Usha Prakash and Srinivasan, 2009).

The probable mechanism of piperine's effect on the bioavailability of β-carotene is its effect on gastrointestinal events that lead to increased absorption of this nutrient and most probably other nutrients as well. Our current animal study has not only reiterated the bioavailability enhancing effect of piperine with respect to the micronutrient β-carotene, but also has documented that two other pungent spices-red pepper (*Capsicum annuum*) or its pungent constituent, capsaicin and ginger (*Zingiber officinale*) also have the potential of significantly enhancing the intestinal absorption of the micronutrient β-carotene.

Earlier reports however, have indicated that capsaicin does not have the ability to affect serum response of nutrients such as glucose and aminoacids (Monsereenusorn and Glinsukon, 1978; Sambaiah *et al.*, 1984). Our present observation of enhanced absorption of β-carotene in capsaicin fed animals is in disagreement with these two earlier reports; this could be attributed to differences in the system studied, nature of the nutrient (hydrophilic or lipophilic), etc.

Carotenoids have been linked with protective roles against diseases associated with aging, including cancer, cardiovascular disease, cataracts, and age-related- macular degeneration. Because of the potential importance of carotenoids as protective factors

against chronic diseases, more attention to food-related practices associated with carotenoid intake by population is warranted. Our current animal study has evidenced that dietary spices like black pepper or its pugent principle, piperine, red pepper or its pungent constituent, capsaicin, and ginger can effectively enhance the absorption of β-carotene. Although the dosage of these spices employed in this study are much higher than the usually encountered levels in our diet, such higher doses have been proved to be safe and without any deleterious effects.

Acute, subacute and chronic toxicity studies of piperine in laboratory animals indicate that piperine used in a broad range of doses, does not cause any abnormality in the general growth pattern, body to organ weight ratio, clinical symptomatology and blood chemistry (Johri and Zutshi, 1992). The dose of piperine considered as bioenhancing absorption of nutrients is considered to be 4 μg - 150 μg of piperine/kg body weight. This concentration is many thousand times less than the LD_{50} dose of piperine established in mice and rats. The LD_{50} data indicate a relatively high therapeutic index for piperine, which means high degree of safety in nutritional use of piperine.

Bile salts have a prominent role in carotenoid absorption through solubilization of the dietary carotenoid, and facilitating their transport in the form of micelles from intestinal lumen to the brush border. In a rat everted gut sac study, El-Gorab *et al*. (1975) have reported that β-carotene was well absorbed from micellar solutions made with bile salt mixtures, but not from micellar solutions made with non-ionic detergents. In a similar study with slices of intestinal tissues, β-carotene dispersed in a micellar solution made with a non-ionic detergent was not absorbed and converted to retinyl ester unless bile or conjugated bile salts were present (Olson, 1964). Thus, both these studies suggest that bile salts not only serve to solubilize carotenoids in the small intestine, but may also be required for their transport through the brush-border- membrane. Incidentally, dietary capsaicin / red pepper and ginger, among several other spices are documented to cause higher production and secretion of bile with significantly enhanced titres of bile salts (Bhat *et al*., 1984, 1985; Sambaiah and Srinivasan, 1991; Platel and Srinivasan, 2001).

Thus, the capacity of these dietary spices to enhance secretion of bile salts which participate in the absorption of lipophilic compounds including β-carotene could also be responsible for the observed higher absorption of orally administered β-carotene in present animal study.

The bioenhancing dose of piperine as used in human trials is a maximum of approximately 15 mg/person/day, or no more than 20 mg/day in divided doses, which corresponds to from several thousands to up to 40,000 times less than the LD_{50} dose of piperine, as established in various experiments on rodents. Human consumption of black pepper in Indian population constitutes 0.02% of the diet, which corresponds to 2 mg of powdered pepper/kg/day (Bhat and Chandrasekhara, 1986). Based on this assumption, black pepper, and its components oleoresin containing 40% piperine, and pure piperine, was fed to rats at doses calculated as 5 to 20 times the average daily intake for humans. This particular diet with pepper and its components did not affect food intake, growth pattern of fed animals, the organ weights and also produced no clinical symptoms. Comparison of the blood chemistry tests results of the treated and untreated animals showed no alterations in RBC, WBC, the differential count, levels of hemoglobin, serum proteins, and other metabolites, and levels of serum aminotransferases and phosphatases.

Hiwale *et al.* (2002) found that co-administration of piperine significantly enhanced the bioavailability of β-lactam antibiotics, amoxicillin trihydrate and cefotaxime in rats. The increased bioavailability is attributed to the effect of piperine on microsomal drug metabolizing enzyme system.

In vertebrates, provitamin A carotenoids are converted to retinal by β-carotene-15,15'-dioxygenase. The enzyme activity is expressed specifically in intestinal epithelium and in liver. The intestinal enzyme not only plays an important role in providing animals with vitamin A, but also determines whether provitamin A carotenoids are converted to vitamin A or circulated in the body as intact carotenoids. High fat diet has been found to enhance the β-carotene dioxygenase activity together with the cellular retinol binding protein type II level in rat intestines (Nagao, 2004).

Nagao *et al*. (2000) have reported the effect of antioxidants and dietary flavanoids on the activity of β-carotene dioxygenase *in vitro* using pig intestinal homogenate as the enzyme source. Flavonols having a catechol structure in the B-ring and 2,6-di-*tert*-butyl-4-methylphenol inhibited the dioxygenase activity of pig intestinal homogenates. BHT, (2,6-di–*tert*–butyl-4-methylphenol), a synthetic antioxidant, strongly inhibited the activity at the level of 10^{-6} M (a mixed type of inhibition), whereas butylated hydroxyanisole (BHA), nor-dihydroguaiaretic acid, n-propyl gallate and curcumin were moderately inhibitory. Flavanoids such as luteolin, quercetin, rhamnetin and phloretin remarkably inhibited the dioxygenase activity noncompetitively, whereas flavanones, isoflavanones, catechins and anthocyanins were less inhibitory. Canthaxanthin and zeaxanthin inhibited carotenoid dioxygenase activity competitively *in vivo* as well as *in vitro* and α-carotene, β-cryptoxanthin and lutein, but not lycopene inhibited carotenoid dioxygenase enzyme activity *in vitro*. Ershov *et al*. (1994) found that lycopene, lutein and asthaxanthin competitively inhibited carotenoid dioxygenase activity and that antioxidants such as BHT, BHA and ascorbic acid inhibited the activity. Thus, the conversion of β-carotene to vitamin A can be modulated by inhibitory effects of various dietary components on carotenoid dioxygenase activity.

Flavonols with a catechol structure in the B-ring also inhibited the conversion of β-carotene to retinol in Caco-2 human intestinal cells (Nagao *et al*., 2000). Thus, the bioavailability of dietary provitamin A carotenoids might be modulated by other food components ingested. Regulation of the dioxygenase activity and its relation to the retinoid metabolism as well as to lipid metabolism deserve further study in the context of understanding the beneficial effects of carotenoids on human health.

The significantly lowered activity of the β-carotene cleavage enzyme in the liver and intestines as a result of dietary capsaicin and ginger in our *in vivo* study, was also corroborated by the inhibitory effects of the spice compounds on this enzyme in *in vitro* study. Although this appears to be a point for concern with regard to the bioconversion of β-carotene to vitamin A, it is to be noted that the observed *in vivo* effect is only in the vitamin A adequate status; and in spite of a decrease in the activity of β-carotene

cleavage enzyme, retinol status in the tissues was comparable to the untreated controls. Secondly, the concentrations of the spice compounds that produced the inhibitory effect on β-carotene cleavage enzyme in the *in vitro* system would generally never be encountered in the tissues when the animals are fed spices through diet. Even in the laboratory rats administered with these spice compounds – capsaicin or piperine as a single high oral dose (Suresh and Srinivasan, 2009), the tissue levels of intact administered spice compounds were far below the concentrations that are evidenced in our study to produce drastic reduction in the enzyme activity.

Although the activity of retinal reductase was increased *in vitro* by the three spice compounds in this study, the same were comparable to control animals in the *in vivo* situation. Higher activity of this enzyme was not discerned in spice fed animals probably due to not encountering such higher levels of spice compounds in the tissues (10^{-6} to 10^{-4} M) which would have brought about the enhancing effect. Further, since β-carotene cleavage enzyme, which is the rate limiting enzyme in the bioconversion of β-carotene rather than retinal reductase, was not similarly influenced, the positive influence on the latter enzyme alone was of no consequence.

The doses of the three test spices that enhanced the bioavailability of β-carotene would most likely not interfere with the metabolism of a majority of drugs as previously discussed. In fact, the doses of test spices or their bioactive compounds as used in this study do not affect the metabolic pathways of this nutrient in the body, as measured by the blood levels of retinol which remained unchanged throughout the experiment. Retinol, or vitamin A, is a product of metabolic conversion of β-carotene, and its blood levels would be affected by enzymatic inhibition / stimulation with piperine. In the context of toxic effects of an overdose of vitamin A, our observation that piperine does not elevate the conversion of β-carotene to vitamin A under conditions of adequate vitamin A status is an important finding. The benefit of increased blood levels of β-carotene without the risk of vitamin A toxicity translates in to the safe and effective enhancement of antioxidant protection provided by β-carotene.

Summary

Animal studies were conducted to evaluate the influence of dietary spices- piperine, capsaicin and ginger on the *in vivo* absorption of orally administered β-carotene and the efficacy of its conversion to vitamin A. Young rats were maintained on these spice containing diets for 8 weeks. These rats were administered a single oral dose of β-carotene. After 4 h *p.o.* administration, concentration of β-carotene and retinol in serum, liver and intestine were determined. There was a significant increase in β-carotene concentration in the serum, liver and intestine of piperine and ginger fed rats as compared to control. This suggests that dietary piperine and ginger improve intestinal absorption of β-carotene leading to an increased β-carotene concentration in circulation and in tissues. However, the concentration of retinol was not significantly changed in these spice fed groups as compared to control, suggesting that bioconversion of β-carotene to vitamin A was not similarly influenced. This was further verified in a separate animal study, wherein the activities of two enzymes specifically involved in the bioconversion of β-carotene to vitamin A were measured in the intestine and liver of spice fed animals. Activity of intestinal β-carotene- 15,15'-dioxygenase was rather lowered in capsaicin and ginger fed animals, while it was comparable to control in piperine treatment. β-Carotene 15,15'-dioxygenase activity was also lower in the liver of capsaicin fed animals. Activity of retinal reductase either in liver or intestine was not influenced by dietary spices. *In vitro* influence of the tested spice compounds on the activities of the enzyme involved in bioconversion of β-carotene to retinal was also negative, thus corroborating with the *in vivo* observation with regard to the influence of dietary spices on the bioconversion of β-carotene to vitamin A. In the absence of a simultaneous promotion of the bioconversion of β-carotene, the benefit of increased blood and tissue levels of β-carotene brought about by dietary spices without the risk of vitamin A toxicity ensures an effective enhancement of antioxidant protection.

GENERAL SUMMARY

GENERAL SUMMARY

Vitamin A malnutrition being widely prevalent, understanding the bioavailability of dietary β-carotene from plant foods, and its subsequent conversion to vitamin A is of utmost importance. Such information may lead to optimization of dietary approaches to increase the bioavailability of dietary β-carotene. In the present investigation, several green leafy and yellow-orange vegetables were screened for the bioaccessibility of β-carotene as influenced by factors such as heat processing encountered in domestic cooking, presence of food acidulants, and presence of antioxidant spices.

- The suitability of procedural alternatives for the determination of bioaccessibility of β–carotene in an *in vitro* method was examined. Membrane filtration and equilibrium dialysis were examined to separate the micellar fraction that contains bioabsorbable β–carotene as an alternative to ultracentrifugation.

- Values of β-carotene bioaccessibility from vegetables obtained with the membrane filtration method were similar to those obtained by the ultracentrifugation method, and hence it was inferred that membrane filtration to separate the aqueous micellar fraction containing the bioaccessible β-carotene is satisfactory, and can be employed in the absence of an ultracentrifuge.

- Effect of heat treatment involved in domestic cooking procedures on the bioaccessibility of β-carotene from yellow-orange as well as green leafy vegetables was evaluated. Heat treatment of these vegetables by pressure-cooking, stir-frying, and open-pan- boiling had a beneficial influence on the bioaccessibility of β-carotene from these vegetables.

- The extent of increase in the percent bioaccessibility of β-carotene as a result of pressure-cooking was 21 - 84%.

- Open-pan-boiling of vegetables increased the bioaccessibility of β-carotene in the range 23 – 36%.

- Stir-frying in presence of a small quantity of oil brought about an enormous increase in the bioaccessibility of β-carotene from these vegetables, the extent of increase being 67 - 192%.

- This observation suggests that the use of suitably heat-processed vegetable sources of β-carotene could form a dietary strategy to derive this micronutrient maximally.

- Four common food acidulants – amchur, lime, tamarind and kokum, and two antioxidant spices–turmeric and onion were examined for their influence on the bioaccessibility of β-carotene from two fleshy and two leafy vegetables.

- Food acidulants – Amchur and lime generally enhanced the bioaccessibility of β-carotene from these test vegetables in many instances. Such an improved bioaccessibility was evident in both raw and heat-processed vegetables. The effect of lime juice was generally more pronounced than that of amchur. Lime juice which enhanced the bioaccessibility of this provitamin from both raw and heat-processed vegetables probably exerted this effect by some other mechanism in addition to minimizing the loss of β-carotene.

- The antioxidant spice – turmeric significantly enhanced the bioaccessibility of β-carotene from all the vegetables tested, especially when heat-processed. Onion enhanced the bioaccessibility of β-carotene from pressure-cooked carrot and amaranth leaf and from open-pan- boiled pumpkin and fenugreek leaf. Turmeric and onion minimized the loss of β-carotene during heat processing of the vegetables; and this could be responsible for the observed improved bioaccessibility of β-carotene from heat-processed vegetables in presence of these antioxidant spices.

- The bioaccessibility enhancing effects of lime and turmeric were not additive when these two enhancers were used in combination.

- Thus, the presence of food acidulants- lime juice/ amchur and antioxidant spices – turmeric/ onion prove to be advantageous in the context of deriving maximum β-carotene from the vegetable sources.

- In this investigation several varieties of mango and papaya fruits were examined for their β-carotene content as well its bioaccessibility. The varieties of mango studied were: *Badami*, *Raspuri*, *Mallika*, *Malgoa*, *Totapuri* and *Neelam*, and two varieties of papaya were: *Honey Dew* and *Surya*.

- Varietal differences were evident in both β-carotene content and its bioaccessibility in the case of mango. β-Carotene content (mg/100 g) in ripe mango ranged from 0.55 ± 0.03 in the *Malgoa* variety to 3.21 ± 0.25 in the *Badami* variety.

- Bioaccessibility of β-carotene ranged from 24.5 % in the *Badami* to 39.1 % in the *Raspuri* varieties of mango. Considering both the percent bioaccessibility and the inherent β-carotene content, the amount of bioaccessible β-carotene was highest in the *Mallika* variety (0.89 mg/100 g), followed by *Badami* (0.79 mg/100 g).

- Similarly, among the *Honey Dew* and the *Surya* varieties of papaya, β-carotene content (mg/100 g) was 0.70 ± 0.10 and 0.73 ± 0.12, respectively. Bioaccessibility of β–carotene from the two varieties of papaya examined was similar (31.4-34.3%).

- Since mango and papaya are also consumed as a blend with milk, influence of the presence of milk on the bioaccessibility of β-carotene from these fruits was also examined. Addition of milk generally brought about a significant increase in the bioaccessibility of β–carotene from mango, the increase ranging from 12 to 56%. Addition of milk increased this bioaccessibility by 18 and 38% in these two varieties.

- Thus, this study has indicated that varietal differences exist in the content and bioaccessibility of β–carotene in mango, and that the addition of milk is advantageous in deriving this provitamin A from the fruit pulp of mango and papaya.

- Specific dietary spices may alter the ultra structure and permeability characteristics of intestines. Few common spices were studied here for their possible promoting influence on intestinal absorption of β-carotene by examining the uptake of β-carotene by the intestines from rats fed black pepper, red pepper, ginger, piperine and capsaicin.

- Higher *in vitro* absorption of β-carotene in the intestines was evidenced in all the spice-fed animals. Dietary piperine and ginger increased the uptake of β-carotene by 147% and 98% respectively. While black pepper and red pepper fed animals showed an increase in absorption by 59 and 27%, dietary capsaicin increased the same by 50%.

- Animal studies were conducted to evaluate the influence of dietary spices- piperine, capsaicin and ginger on the *in vivo* absorption of orally administered β-carotene and the efficacy of its conversion to vitamin A.

- To rats maintained on these spice containing diets for 8 weeks, a single oral dose of β-carotene was administered. After 4 h *p.o.* administration, concentration of β-carotene and retinol in serum, liver and intestine were determined.

- There was a significant increase in β-carotene concentration in the serum, liver and intestine of piperine and ginger fed rats as compared to control, suggesting that dietary piperine and ginger improve intestinal absorption of β-carotene leading to an increased β-carotene concentration in circulation and in tissues.

- The concentration of retinol was not significantly changed in these spice fed groups as compared to control, suggesting that bioconversion of β-carotene to vitamin A was not similarly influenced.

- The activities of two enzymes specifically involved in the bioconversion of β-carotene to vitamin A were measured in the intestine and liver of spice fed animals.

- Activity of intestinal β-carotene- 15,15'-dioxygenase was rather lowered in capsaicin and ginger fed animals, while it was comparable to control in piperine treatment.

- β-Carotene- 15,15'-dioxygenase activity was also lower in the liver of capsaicin fed animals. Activity of retinal reductase either in liver or intestine was not influenced by dietary spices.

- *In vitro* influence of the tested spice compounds on the activities of the enzyme involved in bioconversion of β-carotene to retinal was also negative, thus corroborating with the *in vivo* observation with regard to the influence of dietary spices on the bioconversion of β-carotene to vitamin A.

- Thus, the present animal study has evidenced the beneficial influence of dietary spices in enhancing the absorption of orally consumed β-carotene, which in turn offers health benefits through antioxidant protection, besides being a provitamin.

- On the whole, the salient observations of this investigation lead to evolving optimal food based strategies to maximize the bioavailability of β-carotene from the conventional food sources.

- Strategies such as heat processing as encountered in domestic cooking, especially pressure cooking, open-pan-boiling and stir-frying, inclusion of food acidulants – lime and amchur, inclusion of antioxidant spices – turmeric and onion, inclusion of milk along with fruit pulp, and consumption of spices, such as black pepper, red pepper and ginger would be useful in deriving β-carotene in higher amounts from its potential plant sources.

BIBLIOGRAPHY

BIBLIOGRAPHY

Chapter –I

Ahmed, S.M.; Leo, A.; Lieber, C.S. Interactions between alcohol and β-carotene in patients with alcoholic liver disease. *Am.J.Clin.Nutr.* **1994**, *10*, 430-436.

Alexander, M.; Newmark, H.; Miller, R.G. Oral β-carotene can increase the number of OKT4 + cells in human blood. *Immunology Lett.* **1985**, *9*, 221-224.

Armand, M.; Borel, P.; Pasquier, B.; *et al*. Physicochemical characteristics of emulsions during fat digestion in human stomach and duodenum. *Am.J.Physiol.* **1996**, *271*, G172–G183.

Bamji, M.S.; Rao, P.N.; Reddy,V. Fat soluble vitamins. In: *Text book of human Nutrition*, Oxford and IBH publishing Pvt Ltd. **1996**, pp.64-75.

Baskaran, V.; Sugawara, T.; Nagao, A. Phospholipids affect intestinal absorption of carotenoids in mice. *Lipids.* **2003**, *38*, 705–711.

Bendich, A . Carotenoids and the immune response. *J.Nutr.* **1989**, *119*, 112-115.

Blot, W.J.; Taylor, Li J.Y.; Guo, P.R.; *et al.* Nutrition intervention trials in linxian, China supplementation with specific vitamin /mineral combinations ,cancer evidence and disease specific mortality in general population. *J.Natl.Cancer.Inst.* **1993,** *85*, 1483-1491.

Boileau, A.C. ; Lee, C.M. ; Erdman, J.W.Jr. Vitamin A deficiency reduces uptake of β-carotene by brush border membrane vesicles but does not alter intestinal retinyl ester hydrolase activity in the rat. *J.Nutr.Biochem*. **2000**, *11*, 436-442.

Bone, R.A.; Lantrum, J.T.; Hime G.W.;Cains,A, Zamor,J. Stereochemistry of the human macular carotenoids. *Invest.Ophthamol.Vis.Sci.***1993**, *34,2033*-2040.

Bone, R.A.; Lantrum, J.T.; Friedes, L.M.; Gomez, C.M.; Kilburn, M.D. Distribution of lutein and zeaxanthin stereoisomers in the human retina. *Exp.Eye Res*. **1997**, *64*, 211-218.

Bone, R.A.; Lantrum, J.T.; Dixon, Z.; Chen, Y; Llererne,C.M. Lutein and zeaxanthin in the eyes serum and diet of human subjects. *Exp.Eye Res.* **2000,** *71*, 239-245.

Borel, P.; Grolier, P.; Armand, M.; *et al.* Carotenoids in biological emulsions: Solubility, surface-to-core distribution, and release from lipid droplets. *J.Lipid Res.* **1996**, *37,* 250–261.

Borel, P.; Grolier, P.; Mekki, N.; *et al.* 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.* **1998a**, *30*, 2250–2260.

Borel, P.; Tyssandier, V.; Mekki, N.; *et al.* 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.* **1998b**, *128*, 1361–1367.

Borel, P.; Pasquier, B.;Armand, M.; *et al.* Processing of vitamin A and E in the human gastrointestinal tract. *Am.J.Physiol*. **2001**, *280*, G95–G98.

Borel, P. Factors affecting intestinal absorption of highly lipophilic food micro constituents (fat-soluble vitamins, carotenoids and phytosterols). *Clin.Chem.lab.Med.* **2003**, *41,* 979–994.

Bowen, P.E.; Mobarhan, S. Smith, J.C. Jr. Carotenoid absorption in humans. *Meth. Enzymol.* **1993**, *214*, 3–17.

Britton, G.; Liaaen-Jensen, S.; Pfander, H. Carotenoids today and challenges for the future. In: Britton, G., Liaaen-Jensen, S., Pfander H. (Eds), *Carotenoids*, Vol. 1A, Basel, Birkhäuser, Switzerland. **1995**.

Britton, G. Structure and properties of carotenoids in relation to function. *FASEB.J.* 1**995a,** *9,* 1551-1558.

Bryant, J.D.; McCord, J.D.; Unlu, L.K.; Erdman, J.W. The isolation and partial characterization of α- and β- carotene carotene-protein (s) from carrot (*Daucus carota)* root chromoplasts. *J.Agric.Food.Chem.* **1992,** *40,* 545-549.

Bui, M.H. Vitamin A, provitamin A and fibrosis patients. *Int.J.Vitam.Nutr.Res.* **1988,** *58*, 354-355.

Burri, B.J. β-carotene in human health: A review of current research. *Nutr.Res.* **1997**, *17*, 547-580.

Burri, B.J.; Clifford, A.J. Carotenoid and retinoid metabolism: insights from isotope studies. *Arch.Biochem.Biophys*. **2004**, *430*, 110–119.

Burton, G.W.; Ingold, K.U. β-carotene an unusual type of lipid antioxidant. *Science.* **1984,** *224,* 569-573.

Canfield,L.M.; Fritz, T.A.; Tarara, T.E. Incorporation of β-carotene into mixed micelles. *Meth.Enzymol*. **1990,** *189*, 418-422.

Carey, M.C.; Hernell, O. Digestion and absorption of fat. *Semin.Gastrointest.Dis.* **1992**, *3*, 189-208.

Castenmiller, J.J.; West, C.E. Bioavailability and Bioconversion of carotenoids. *Annu.Rev.Nutr.***1998**, *18,* 19-38.

Castenmiller, J.J.; West, C.E.; Linssen, J.P.; van Het hof, K.H.; Voragen, A.G. 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.* **1999**, *129*, 349-355.

Chitchumroonchokchai, C.; Schwartz, S.J.; Failla, M.L. Assessment of lutein bioavailability from meals and supplement using simulated digestion and Caco-2 human intestinal cells. *J.Nutr.* **2004**, *134*, 2280–2286.

Christian, P.; West, K.P. Jr.; Khatry, S.K. *et.al*. Night blindness during pregnancy and subsequent mortality among women in Nepal: effects of vitamin A and β-carotene supplementation. *Am J Epidemiol.* **2000**, *152*, 542–547.

Clark, R.C.; Yao, L.; She, L.; Furr, H.C. A comparison of lycopene and astaxanthin absorption from corn oil and olive oil emulsions. *Lipids.* **2000**, *35,* 803–806.

Congdon, N.G.; West, K.P. Jr. Physiologic indicators of vitamin A status. *J.Nutr.* **2002**, *132, 2889S*–2894S.

Cooper DA, Webb DR, Peters JC. Evaluation of the potential for olestra to affect the availability of dietary phytochemicals*. J.Nutr.*1**997,** *127*, 1669S-1709S.

Cornwell, D.G.; Kruger, F.A.; Robinson, H.B. Studies on the absorption of β-carotene and the distribution of total carotenoid in human serum lipoproteins after oral administration. *J.Lipid Res.***1962**, *3*, 65-70.

De Pee, S.; West, C.E. Dietary carotenoids and their role in combating vitamin A deficiency: a review of the literature. *Eur.J.Clin.Nutr*. **1996,** *50,* S38-S53.

Dietz, J.M.; Sri Kantha,S.; Erdman, J.W. Jr. Reversed phase HPLC analysis of α-and β -carotene from selected raw and cooked vegetables*. Plant Foods Hum.Nut*r.**1988**, *38,* 333-341.

Di- Mascio, P., Kaiser, S., Sies, H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch. Biochem. Biophys*.**1989**, *274*, 532-538.

Di- Mascio, P., Murphy, M. E.; Sies. M. E. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. *Am.J.Clin.Nutr*. **1991**, *53*, 194S-200S.

Dimitrov, N.V.; Meyer, C.; Ultrey, D.E.; Chenoweth, W.; Michelakis, A. Bioavailability of β-carotene in humans. *Am.J.Clin.Nutr.* **1988**, *48*, 298-304.

Dmitrovskii, A.A.; Gessler, N.N.; Gomboeva, S.B.; Ershov, Yu.V.; Bykhovsky,V. Enzymatic oxidation of β-apo-8'-carotenal to β-apo-14'-carotenal by an enzyme different from β-carotene 15-15'-dioxygenase. *Biochemistry*, **1997**, *62*, 787-792.

Dueker, S.R.; Johnes, A.D.; Smith, G.M.; Clifford, A.J. Stable isotope methods for the study of β-carotene–d8 metabolism in humans utilizing tandem mass spectrometry and high performance liquid chromatography. *Anal.Chem*. **1994***, 66,*4177-4185.

Dueker, S.L.; Lin, Y.; Buchholz, P.D.; *et al.* Long-term kinetic study of β-carotene, using accelerator mass spectrometry in an adult volunteer. *J.Lipid.Res.* **2002,** *41*, 1790– 800.

During, A., Hussain, M. M., Morel, D. W., Harrison, E.H. Carotene uptake and secretion by Caco-2 cells : β-carotene isomer selectivity and carotenoid interactions. *J.Lipid Res.* **2002***, 43*, 1086-1095.

During, A., Dawson, H. D., Harrison, E. H. Enrichment of tomato paste with 6% tomato peel increases lycopene and β-carotene bioavailability in men. *J.Nutr.* **2005**, *135*, 2305–2312.

Edwards, A.J.; Nguyen, C.H.; You, C.; Swanson, J.E.; Emenhiser, C.; Parker, R.S. α- and β-carotene from a commercial puree are more bioavailable to humans than from boiled-mashed carrots, as determined using an extrinsic stable isotope reference method. *J.Nutr*. **2002,** *132*, 159–167.

El-Gorab, M.; Underwood,B.A. Solubilization of β-carotene and retinol into aqueous solutions of mixed micelles. *Biochim.Biophys.Acta*. **1973**, *306*, 58-66.

El-Gorab, M.I.; Underwood, B.A.; Loerch, J.D. The roles of bile salts in the uptake of β-carotene and retinol by rat everted sacs. *Biochim.Biophy.Acta.* **1975,** *401, 265*–277.

Englyst, K.N.; Englyst, H.N.; Hudson, G.J.; Cole, T.J.; Cummings, J.H. Rapid available glucose in foods: an *In vitro* measurement that reflects the glycemic response. *Am.J.Clin.Nutr.* **1999**, *69,* 448–454.

Erdman, J.W.; Bierer, T.L.; Gugger, E.T. Absorption and transport of carotenoids. *Ann. N.Y.Acad.Sci*. **1993**, *691*, 76-85.

Erdman, J.W.Jr. Review of animal models in carotenoid research. *J.Nutr*. **1999**, *129*, 2271–2277.

Failla, M.L.; Chitchumroonchokchai, C. *In vitro* models as tools for screening the relative bioavailabilities of provitamin A carotenoids in foods. *Harvest Plus Technical Monograph*, **2005,** *3*, p.12.

Faulks, R.M.; Hart, D.J.; Wilson, P.D.G.; Scott, J.K.; Southon, S. Absorption of all-*trans* and 9-*cis*-β-carotene in human ileostomy volunteers. *Clin.Sci.* **1997**, 93, 582–591.

Ferruzzi, M.G.; Failla, M.L.; Schwartz, S.J. Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an *In vitro* digestion and Caco-2 human cell model. *J.Agric.Food Chem.* **2001**, *49*, 2082-2089.

Forman, M.R.; Beecher, G.R.; Lanza, E.; *et al.* Effect of alcohol consumption on plasma carotenoid concentrations in premenopausal women: a controlled dietary study. *Am.J. Clin.Nutr.* **1995**, *62*, 131-135.

Fouad, F.M.; Farrel, P.G.; Marshall, W.D.; van de Voort, F.R. *In vitro* model for lipase-catalyzed lipophile release from fats. *J.Agric.Food Chem.* **1991**, *39*, 150–153

Freed M. Carotene. In: *Methods of vitamin assay*, 3rd Edn, Interscience Publishers, NewYork**. 1966**, pp.97-121

Furr, H.C.; Clark, R.M. Intestinal absorption and tissue distribution of carotenoids. *J.Nutr.Biochem.* **1997,** *8*, 364-377.

Gaziano, J.M.; Johnson, E.J.; Russell, R.M.; Manson, J.E.; Stampfer, M.J. Discrimination in absorption or transport of -carotene isomers after oral supplementation with either all-*trans* or 9- *cis* β–carotene. *Am.J.Clin.Nutr*.**1995,** *61*, 1248-1252.

Gessler, N.N.; Gomboeva, S.B; Shumaev, K.B.; Bykhovskii, V.Y.; lankin, V.Z. Free radical lipid peroxidation inhibits enzymatic conversion of β-carotene into vitamin A. *Bull.Exp.Biol.Med.* **2001**, *131,* 451- 453.

Garrett, D.A.; Failla, M.L.; Sarama, R.J. Development of an *in vitro* digestion model for estimating the bioavailability of carotenoids from meals. *J.Agric.Food.Chem.* **1999a**, *47*, 4301–4309.

Garrett, D.A. ; Failla, M.L. ; Sarama, R.J. ; Craft, N.E. Accumulation and retention of β-carotene and lutein by Caco-2 human intestinal cells. *J.Nutr.Biochem*. **1999b**, *10*, 573–581.

Garrett, D.A .; Failla, M.L. ; Sarama, R.J. Estimation of carotenoid bioavailability from fresh stir-fried vegetables using an *in vitro* digestion / Caco-2 cell culture model. *J.Nutr.Biochem*. **2000**, *11,* 574–580.

Gayathri, G.N.; Platel, K.; Prakash, J.; Srinivasan, K. Influence of antioxidant spices on the retention of β- carotene in vegetables during domestic cooking processes. *Food Chem.* **2004**, *84*, 35-43.

Ghaziano, J.M.; Manson, J.E.; Ridker, P.M.; Buring, J.E.; Hennekens, C.H. β-Carotene therapy for chronic stable angina. *Circulation*, **1991.** *82,* Abstract No. 0796.

Gil-Izquierdo, A.; Zafrilla, P.; Tomas-Barberan, F.A. An *in vitro* method to stimulate phenolic compound release from the food matrix in the gastrointestinal tract. *Eur.Food Res.Technol.* **2002,** *214*, 155–159.

Giovannucci, E.; Ascherio, A.; Rimm, E.B.; Stampfer,M.J.; Colditz, G.A.; Willett, W.C. Intake of carotenoids and retinal in relation to risk of prostrate cancer. *J.Natl.Cancer Inst.* **1995**, *87*, 1767-1776.

Glover, J.; Redfearn, E.R. The mechanism of the transformation of β-carotene to vitamin A in *vivo. Biochem.J.* **1954,** *58,* XV-XVI.

Gomboeva, S.B.; Gessler, N.N.; Shumaev, K.B.; Khomich,T.I.; Moiseenok, A.G.; Bykhovskii, V.Y. Some natural and synthetic antioxidants as stabilizers of β-carotene to Vitamin A. *Biochemistry.* **1998**, *63*, 185-190.

Gopalan, G.; Ramasastri, B.V.; Balasubramanian, S.C. In: *Nutritive value of Indian Foods.* Indian Council of Medical Research, New Delhi, **2004**.

Greenberg, E.R.; Baron, J.A.; Stukel, T.A.; *et al*. A clinical trial group of β-carotene to prevent basal cell and squamous cell cancers of the skin. *N.Engl.J.Med.* **1990,** *323*, 789-795.

Heinekens, C.H.; Eberlein,K. A randomized trial of aspirin and β–carotene. Toxicology **1985**, *36*, 91-100.

Hickenbottom, S.J.; Follett, J.R.; Lin, Y.; Dueker, S.R.; Burri, B.J.; Neidlinger, T.R.; Clifford A. Variability in conversion of β-carotene to vitamin A in men as measured by using a double-tracer study design. *Am.J.Clin.Nutr.* **2002,** *75*, 900–907.

Hidalgo, I.J.; Raub, T.J.; Borchardt, R.T. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **1989**, *96,* 736–749.

Hidalgo, I.J.; Raub, T.J.; Borchardt, R.T. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial carotenoids in human subjects. *Proc.Nutr.Soc.* **1999**, *58*, 155–162.

Hollander, D.; Ruble, P.E. β-carotene absorption: bile, fatty acid, pH and flow rate effects on transport. *Am.J.Physiol.* **1978**, *235,* E686–E691.

Hu, X.; Jandacek, R.J.; White, W.S. Intestinal absorption of β-carotene ingested with a meal rich in sunflower oil or beef tallow: postprandial appearance in triglycerol-rich lipoproteins in women. *Am.J.Clin. Nutr.* **2000**, *71*, 1170–1180.

Jacques, P.F.; Hartz, S.C.; Chylark, L.T.; McGrandy, R.B.; Sadowski, J.A. Nutritional status in persons with and without senile cataract , blood vitamin and mineral level. *Am.J.Clin.Nutr*. **1988***, 48,* 152-158.

Jacques, P.F.; Chylack, L.T.Jr. Epidemiologic evidence of a role for the antioxidant vitamin carotenoids in cataract prevention. *Am.J.Clin.Nutr.* **1991**, *23,* 110-113.

Jain, M.; Miller, A.B.; To, T. Premorbid diet and prognosis of women with breast cancer. *J.Natnl.Cancer.Inst*. **1994**, *86*, 1390-1397.

Jalal, F.; Nesheim, M.C.; Agus, Z.; Sanjur, D.; Hibachi, J.P. Serum retinol concentrations are affected by food sources of β-carotene, fat intake, and antihelminthic drug treatment. *Am.J.Clin.Nutr* **1998**, *68*, 623–629.

Jayarajan, P.; Reddy, V.; Mohanram, M. Effect of dietary fat on absorption of β-carotene from green leafy vegetables in children. *Indian J.Med.Res.* **1978**, *71,* 53-56.

Johnson,E.J.; Qin,J.; krinsky,N.I.; Russell,R.M. β-carotene isomers in humans serum, breast milk and buccal mucosa cells after continuous oral doses of all–*trans* and 9-*cis* β-carotene. *J.Nutr.* **1997**, *127,* 1993-1999.

Karrer,P.; Jucker,E. *Carotenoids.* Elsevier Publishing Co. New-York. USA .**1950.**

Kiefer, C.; Hessel, S.; Laederer M.O. Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. *J.Biol.Chem.* **2001,** *276*, 14110-14116.

Kiefer, C., Sumser, E., Wernet, M. F., Von Lintig, J. A class B receptormadiates the cellular uptake of carotenoids in Drosophilla. *Proc.Natl.Acad.Sci*. USA. **2002**, *99*, 10581 –10586.

Kiokias,S.; Gordon,M.H. Antioxidant properties of carotenoids in vitro and in vivo. *Food Rev. Internat.* **2004,** *20*, 99-121**.**

Kostic, D.; White, W.S.; Olson, J.A. Intestinal absorption, serum clearance, and interactions between lutein and β-carotene when administered to human adults in separated or combined oral doses. *Am.J.Clin.Nutr.* **1995,** *62,* 604–610.

Krinsky, N.I. Actions of carotenoids in biological systems. *Ann.Rev.Nutr*. **1993**, *13*, 561.

Kuksis, A. *Absorption of fat soluble vitamins*. In: Fat absorption, Vol 11, CRC press, Boca Raton, USA, **1986**, pp. 65-86.

Lee, C.M.; Boileau, A.C.; Boileau, T.W.; Williams, A.W.; Swanson K.S.; Heintz, K.A.; Erdman, J.W. Jr. Review of animal models in carotenoid research. *J Nutr*. **1999,** *129*, 2271-2277.

Lemke, S.L.; Ducker, S.R.; Follett, J.R.; *et al.* Absorption and retinol equivalence of β-carotene in humans is influenced by dietary vitamin A intake. *J.Lipid Res.* **2003**, *44*, 1591–1600.

Leo, M.A.; Kim, N.; Lowe, I.; Liber, C.S. Interaction of ethanol with β-carotene: Delayed blood clearance and enhanced hepototoxicity; *Hepatology.* **1992,** *15,* 883-891.

Lepage, G.; Chamagne, J.; Ranco, N.; *et al.* Supplementation with carotenoids corrects increased lipid peroxidation in children with cystic fibrosis. *Am.J.Clin.Nutr*. **1996**, *64,* 87-93.

Lin, Y., Dueker, S.R.; Burri, B.J.; Neidlinger, T.R.; Clifford, A.J. Variability of the conversion of β-carotene to vitamin A in women measured by using a double-tracer study design. *Am.J. Clin.Nutr.* **2000,** *71*, 1545–1554.

Lindberg, T.; Engberg, S.; Sjoberg, L.B.; Lonnerdal, B. *In vitro* digestion of proteins in human milk fortifiers and in preterm formula. *J.Pediatric.Gastroenterol.Nutr* **1998**, *27*, 30–36.

Livny, O., Reifen, R., Levy, I., *et al*., β-carotene bioavailability from differently processed carrot meals in human- ileostomy volunteers. *Eur.J.Nutr*. **2003**, *42*, 338–345.

Manay, S.N.; Shadaksharaswamy, M. Pigments and colors. In: *Food facts and principles,* New Age Internationals (P) Ltd., New Delhi. **2002,** pp.101-121.

Mathews-Roth, M.M. Plasma concentration of carotenoids after large doses of β-carotene. *Am.J.Clin.Nutr.* **1990,** *52*, 500-501.

Mayne, S.T.; Janerich, D. T.; Greenwald, P.; *et al.* Dietary β-carotene and lung cancer risk in U.S nonsmokers. *J.Natl.Cancer.Inst*. **1994,** *86*, 33-38.

Mayne, S.T. β-carotene, carotenoids and disease prevention in humans. *FASEB J.* **1996**, *10*, 690-701.

McCullough, F.S.W.; Northrop-Clewes, C.A.; Thurnham, D.I. The effect of vitamin A on epithelial integrity. *Proc.Nutr.Soc*. **1999,** *58,* 289–293.

Mckeown, I. Antioxidants and breast cancer. *Nutr.Rev.* **1999**, *57*, 321-323.

Mercadante, A. New carotenoids: recent progress. *Abstracts of the 12th International Carotenoid Symposium*, Cairns, Australia, **1999.**

Micozzi, M.S.; Brown, E.D.; Edwards, B.K.; *et al.* Plasma carotenoid response to chronic intake of selected foods and β-carotene supplements in men. *Am.J.Clin.Nutr.* **1992,** *55*, 1120–1125.

Miller, M.; Humphrey, J.; Johnson, E.; Marinda, E.; Brookmeyer, R.; Katz, J. Why do children become vitamin A deficient? *J.Nutr*. **2002**, *132*, 2867S–2880S.

Minekus M, Marteau P, Havenaar R, Huis Veld, J.H.J. A multi-compartmental dynamic computer-controlled model simulating the stomach and small intestine. *Alternatives. Lab. Animals*. **1995,** *23*, 197–209.

Minekus, M.; Lelieveld, J.; van den Berg, H.A. A dynamic model of the stomach and small intestine to study the bio-accessibility of carotenoid from vegetables and the effect of processing. In: *Abstract Book of the Bioavailability Congress,* Interlaken, Switzerland, **2001.**

Moore, A.C.; Gugger, E.T.; Erdman, J.W. Jr. Brush border membrane vesicles from rats and gerbils can be used to study uptake of all-*trans* and 9-*cis* β-carotene. *J .Nutr.* **1996**, *126*, 2904–2912.

Nagao, A. Oxidative conversion of carotenoids to retinoids and other products. *J.Nutr*. **2004**, *134*, 237S-240S.

Nagao, A.; Yonekura, L. Intestinal absorption of dietary carotenoids. *Mol.Nutr.food Res*. **2007**, *51*, 107-115.

National Academy of Sciences. Fat soluble vitamins: Vitamin A. In: *Recommended Dietary allowances*, (9th edn), National Academy of Sciences, Washington D.C., **1980**, pp 55-60.

Naves,V.M.M.; Moreno,S.F. β-carotene and cancer chemoprevention from epidemiological association into cellular mechanism of action. *Nutr. Res.* **1998**, *18,* 1807-1842.

Netherwood, T.; Martin-Orue, S.M.; O'Donnell, A.G.; *et al.* Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. *Nature Biotechnol.* **2004**, *22*, 204–209.

Nishino, H. Cancer prevention by carotenoids. *Mutat.Res.* **1998**, *402*, 159-163.

Novotny, J.A.; Dueker, S.R.; Zech,L.A.; Clifford, A.J. Compartmental analysis of the dynamics of β-carotene metabolism in an adult volunteer. *J.Lipid.Res.* **1995**, *36*, 1825-1838.

Ogulensi, A.T.; Lee, C.Y. Effect of thermal processing on the stereoisomerization of major carotenoids and vitamin A value of carrots. *Food.Chem*. **1979**, *4*, 311-318

Olson, J.A. The effect of bile and bile salts on the uptake and cleavage of β-carotene in to retinol ester (vitamin A ester) by intestinal slices*. J.Lipid.Res.* **1964**, *5, 402*-408.

Olson, J.A.; lakshmanan, M.R. Carotenoid conversions. *Meth.Enzymol.* **1990**, *189*, 425-432

Olson, J.A. Bioavailability of carotenoids. *Arch.Latinoamericanos de Nutr.* **1999**, *49*, 21S-25S.

Olson J.A. Carotenoids. In: Shils M.E.; Olson J.A.; Shike M.; Ross C.A. (Eds).*Modern Nutrition in Health and Disease*, 9th Edn. Lippincott Willams & Wilkins, NewYork, **1999a,** 525–541.

Ong, A.S.H.; Tee, E.S. Natural sources of carotenoids from plants and oils. *Meth. Enzymol.* **1992**, *213*, 142-167.

O'Neill, M.E.; Thurnham, D.I. Intestinal absorption of β-carotene, lycopene and lutein in men and women following a standard meal: response curves in the triacylglycerol-rich lipoprotein fraction. *Br.J.Nutr*. **1998**, *79*, 149-159.

Onstad, G.R.; Zeive,L. Carotenoid absorption .A screening test for steatorrhea *JAMA*. **1972,** *221*, 677-679.

Paetau, I.; Chen, H.; Goh, N.M.; White,W.S. Interactions in the postprandial appearances of β-carotene and canthaxanthinin plasma triacylglycerol rich lipoproteins in humans. *Am.J.Clin.Nutr.* **1997,** *66*, 1133-1143.

Parker,R.S.; Swanson,J.E.; Marmor,B.; Goodman,K.J.; Spielman,A.B. A study of β-carotene metabolism in humans using ^{13}C-β -carotene and high precision isotope ratio mass spectrometry. *Ann.NY.Acad.Sci.***1993**, *691*, 86-95.

Parker, R.S. Absorption, metabolism, and transport of carotenoids. *FASEB. J.* **1996,** *10*, 542–551.

Parker, R.S., Swanson, J.E.; You, C.; Edwards, J.; Huang, T. Bioavailability of carotenoids in human subjects. *Proc.Nutr.Soc.* **1999**, *58*, 155–162.

Parvin, S.G.; Sivakumar, B. Nutritional status affects intestinal carotene cleavage activity and carotene conversion to vitamin A in rats. *J.Nutr*. **2000**, *130*, 573–577.

Pfander, H. Carotenoids: an overview. *Meth.Enzymol.* **1992**, *213*, 3-13.

Pinto, M.; Robine-Leon, S.; Appay, M.; *et al.* Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol.Cell.* **1983**, *47*, 323–330.

Poor, C.L.; Bierer,T.L.; Merchen, N.R.; Fahey,G.C.; Erdman, J.W. The accumulation of α- and β- carotene in serum and tissues of pre-ruminant calves fed raw and steamed carrot slurries. *J.Nutr.* **1993,** *123,* 1296-1304.

Portal, B.C.; Richard, M.J.; Foure, H.S.; Hadjian, A.J.; Favier, A.E. Altered antioxidant status and increased lipid peroxidation in children with cystic fibrosis. *Am.J.Clin.Nutr.* **1995,** *61,* 843-847.

Prabhala, H.R.; Garewal, S.H.; Meylskena, L.F.; Dwatson, R.R. Immunomodulation in humans caused by β -carotene and vitamin A. *Nutr.Res.* **1991**, *10*, 1473-1486.

Reboul, E. ; Borel, P.; Mikail, C.; *et al*., Carotenoid transport is decreased and expression of the lipid transporters SR-B1,NPC1L1 and ABCA1 is downregulated in Caco-2 cells treated with Ezetimibe. *J. Nutr*. **2005**, *135*, 790–794.

Reddy, J.P.; Mohanram, M. Effect of dietary fat on absorption of β-carotene from green leafy vegetables in children. *Indian.J.Med.Res.* **1980,** *72*, 53–56.

Rhodes, J.; Stokes R. Interferon induced changes in monocyte membrane. *Immunology.* **1982**. *45,* 531-536.

Ribaya–Mercado, J.D.; Solon, F.S.; Solon, M.A. Bioconversion of plant carotenoids to vitamin A in Filipino school-aged children varies inversely with vitamin A status. *Am.J. Clin.Nutr.* **2000**, *72*, 455-465.

Richards, H.A., Han, C.T.; Hopkins, R.G., Failla, M.L.; Ward, W.W., Stewart C.N .Jr. Safety assessment of recombinant green fluorescent protein orally administered to weaned rats. *J.Nutr.* **2003**, *133*, 1909–1912

.

Richelle, M.; Enslen, M.; Hager, C.; *et al.* Both free and esterified plant sterols reduce cholesterol absorption and the bioavailability of β-carotene and α-tocopherol in normocholesterolemic humans. *Am.J.Clin.Nutr*. **2004,** *80*, 171–177.

Riedl, J.; Linseisen, J.; Hoffmann, J.; Wolfram, G. Some dietary fibers reduce the absorption of carotenoids in women. *J. Nutr*. **1999,** *129,* 2170–2176.

Rigotti, A., Miettinen, H. E., Krieger, M. The role of the high –density lipoprotein receptor SRB1 in the lipid metabolism of endocrine and other tissues. *Endocr.Rev*. **2003,** *24*, 357–387.

Ringer, T.V.; Deloof, M.J.; Winterwad, G.E.; Francom, S.F.; Gaylor, S.K.; Ryan, J.A.; Sander, M.E.; Huges, G.S. β-carotene effect on serum lipoproteins and immunologic indices in humans. *Am.J.Clin.Nutr.* **1991**, *53*, 688-694.

Rock, C.L.; Swendseid, M.E. Plasma β-carotene response in human after meals supplemented with dietary pectin. *Am.J.Clin.Nutr*. **1992**, *55*, 96–99.

Rock, C.L. Carotenoids: Biology and treatment. *Pharamacol.Ther.***1997**, *75,185*-197.

Rock, C.L.; Lovalvo, J.L.; Emenhiser, C.; Ruffin,M.T.; Flatt,S.W.; Schwartz, S.J. Bioavailability of β-carotene is lower in raw than in processed carrots and spinach in women. *J. Nutr.* **1998**, *128*, 913-916

Rodriguez, M.S. Irwin, M.I. A conspectus of research on vitamin A requirement of man. *J.Nutr*. **1972**, *102,* 909-968.

Rodriguez-Amaya, D.B. Carotenoids and food preparation: the retention of provitamin A carotenoids in prepared, processed, and stored foods. Washington DC - OMNI project. **1997,** pp.93

Ross, C.A. Vitamin A and retinoids. In: Shies, M.E., Olson, J.A., Shike, M., Ross, C.A. (Eds). *Modern Nutrition in Health and Disease*, 9th Edn., Lippincott Williams & Wilkins, New York, **1999**, pp,305-327.

Russell, R.M. Factors in aging that affect the bioavailability of nutrients. *J.Nutr.* **2001**, *131*, 1359S-1361S.

Sambruy Y, Ferruzza S, Ranaldi G, De Angelis I. Intestinal cell culture models. *Cell.Biol. Toxicol.* **2001,** *17*, 301–317.

Schlipalius E.L. Action mechanism of carotenoids in the human body. *Lipid Technol*. **1997**, *9*, 39-43.

Scita, G.; Aponte, G.W.; Wolf, G. Uptake and cleavage of β-carotene by cultures of rat small intestinal cells and human lung fibroblasts. *J.Nutr.Biochem.* **1992**, *3*, 118-123.

Snodderly, D.M. Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *Am.J.Clin.Nutr.* **1995**, *62*(suppl), 1448S-1461S.

Sperduto, R.D.; Milton, Hu.T.S.; *et al.* The Linxian cataract studies two nutrition intervention studies. *Arch. Opthamol.* **1993**, *111*, 1246-1253.

Stahl, W.; Sies, H. Uptake of lycopene and its geometrical isomers is greater from heat processed than from unprocessed tomato juice in humans. *J.Nutr.* **1992**, *122*, 2161-2166.

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.* **2001**, *131*, 2921–2927.

Takyi, E.E. Children's consumption of dark green, leafy vegetables with added fat enhances serum retinol. *J.Nutr*. **1999**, *129*, 1549-1554.

Tang,G.; Serfaty-Lacrosniere,C.; Camilo, M.E.; Russell, R.M. Gastric acidity influences the blood response to a β- carotene dose in humans. *Am.J.Clin.Nutr*. **1996,** *64*, 622-626.

Tanumihardjo, S.A. Factors influencing the Conversion of Carotenoids to Retinol: Bioavailability to Bioconversion to Bioefficacy. *Int.J.vitam.Nutr.Res.* **2002**, *72*, 40-45.

Taylor A. Role of nutrients in delaying cataract.Ann.N.Y.Acad.Sci.1992, 669,111-123.

Thurnham, D.I.; Northrop-Clewes, C.A.; McCullough, F.S.; Das, B.S.; Lunn, P.G. Innate immunity, gut integrity, and vitamin A in Gambian and Indian infants. *J Infect Dis.* **2000**, *182*, S23–S28.

Thurnham, D.I. Bioequivalence of β-carotene and retinol. *J. Sci.Food Agric*. **2007**, *87*, 13-39.

Tso, P. Intestinal lipid absorption .In: *Physiology of the. Gastrointestinal Tract*, Johnson, L.R. (Ed), Raven Press, New York, USA **1994,** pp.1867-1907

Tyssandier, V.; Cardinault, N.; Caris-Veyrat, C.; *et al.* Vegetable-borne lutein, lycopene, and β-carotene compete for incorporation into chylomicrons, with no adverse effect on the medium-term (3-wk) plasma status of carotenoids in human. *Am.J.Clin.Nutr*. **2002**, *75,* 526–534.

Tyssandier, V.; Reboul, E.; Dumas, J.; *et al.* Processing of vegetable-borne carotenoid in human stomach and duodenum. *Am.J.Physiol.* **2003**, *284*, G913–G922.

van Bennekum, A.,Werder, M., Thuahnai, S.T., *et al.* Class β-scavenger receptor – Mediated intestinal absorption of dietary β-carotene and cholesterol. Biochemistry. **2005**, *44*, 4517–4525.

van den Berg, H.; van Vliet, T. Effect of simultaneous, single oral doses of β-carotene with lutein or lycopene on the β-carotene and retinyl ester responses in the triglyceride-rich fraction of men. *Am.J.Clin.Nutr.***1998**, *68,* 82–89.

van den Berg, H. Carotenoid interactions. *Nutr. Rev.* **1999**, *57*, 1–10.

van den Berg, H.; Faulks, R.; Fernando-Granado, H.; *et al.* The potential for the improvement of carotenoid levels in foods and the likely systemic effects. *J.Sci.Food Agric.* **2000**, *80*, 880–912.

van Het-Hof, K.; Gartner, C.; West, C.E.; Tijberg,L.B.M. Potential of vegetable processing to increase the delivery of carotenoids to man. *Int.J.Vit.Nutr.Res.* **1998**, *68*, 366–370.

van Het-Hof, K.; Brouwer, I.A.; West, C.E.; Haddeman, E.; Steegers-Theunissen,R.P.; van Dusseldorp, M.; Weststrate, J.A.; Hautvast, J.G.A.J. Bioavailability of lutein from vegetables is 5 times higher than that of β-carotene. *Am.J.Clin.Nutr.* **1999**, *70*, 261–268.

van Lieshout, M.; West, C.E.; Permaeshi, M.D.; *et al.* Bioefficacy of β-carotene dissolved in oil studied in children in Indonesia. *Am.J.Clin.Nutr*. **2001**, *73*, 949-958.

van Lieshout, M.; West, C.E.; van de Bovenkamp, P.; *et al.* Extraction of carotenoids from feces, enabling the bioavailability of β-carotene to be studied in Indonesian children. *J. Agric.Food.Chem.* **2003**, *51,* 5123–5130.

van Lieshout, M.; West, C.E.; van Breemen, R.B. Isotopic tracer techniques for studying the bioavailability and bioefficacy of dietary carotenoids, particularly β-carotene, in humans : a review. *Am.J.Clin.Nutr.* **2003a**, *77*, 12–28.

van Vliet, T.; Schreurs, W.H.P.; 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.* **1995**, *62*, 110–116.

Wang, X.D.; Tang, G.W.; Krinsky, J.G.; Russell, R.M. Enzymatic conversion of β-carotene into β-apo-carotenals and retinoids by human, monkey, ferret and rat tissues. *Arch.Biochem.Biophys*. **1991**, *285,* 8–16.

West, K.P. Jr. Extent of vitamin A deficiency among preschool children and women of reproductive age. *J.Nutr.* **2002**, *132*, 2857S–2866S.

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

Weststrate, J.A.; van Het-Hof, K.H. Sucrose polyester and plasma carotenoids concentrations in healthy subjects. *Am.J.Clin.Nutr*.**1995**, *62*, 591–597.

World Health Organization. Studies Rebut Concept that body stores vitamin A making substance. WHO: Geneva, Switzerland **1998**, pp.1-2.

World Health Organization Report. *Nutrition Micronutrient Deficiencies: combating vitamin A deficiencies*. Geneva: www.who.int/nut. **2003**.

World health organization Global prevalence of vitamin A deficiency in populations at risk1995-2005.WHO Global database on vitamin A deficiency, Geneva: www.who.int/nut **2009.**

Yancey, P. G., Bortnick, A. E., Kellner-Weibel, G., *et al*. Importance of different pathways of cellular cholesterol efflux. *Arterioscler.Thromb. Vasc. Biol*. **2003**, *23*, 712–719.

Yeum, K.J.; Booth, S.L.; Sadowski, J.A.; Liu, C.; Tang, G. Human plasma carotenoid response to the ingestion of controlled diets high in fruits and vegetables. *Am.J.Clin.Nutr.* **1996,** *64*, 594-602.

Yeum, K.J.; dos-Anjos-Ferreira, A.l.; Smith, D.; Krinsky, N.I.; Russell, R.M. The effect of α-tocopherol on the oxidative cleavage of β–carotene. *Free Radic.Biol.Med.* **2000**, *29*, 105-114.

Yeum,K.I.; Russell, R.M. Carotenoid bioavailability and bioconversion. *Annu.Rev.Nutr.* **2002,** *22,* 483-504.

You, C.S.; Parker, R.S.; Goodman, K.J.; Swanson, J.E.; Corso, T.N. Evidence of *cis–trans* isomerization of 9-*cis* β-carotene during absorption in humans. *Am.J.Clin.Nutr* **1996**, *64*,177-183.

Young, J.A., Lowe, M.G. Anti oxidant and pro oxidant properties of carotenoids. *Arch. Biochem.Biophys.* **2001***, 385,* 20-27.

Ziegler, R.G.; Colavito, E.A.; Hartage, P.Mc.; *et al.* The importance of α-carotene, β-carotene and other phytochemicals in the etiology of lung cancer. *J.Natnl.Cancer Inst.* **1996**, *88*, 612-615.

Chapter – II

Began, G.; Sudharshan, E.; Udhayashankar, K.; Rao, A.G.A. Interaction of curcumin with phosphatidyl choline. A spectrofluorometric study. *J.Agric.Food Chem*. **1999**, *47*, 4992-4997.

Canfield, L.M.; Forage, J.W.; Valenzuela J.G. Carotenoids as cellular antioxidants. *Proc.Soc.Exp.Biol.Med.* **1992**, *200*, 260-265.

Chen, B.H.; Peng, H.Y.; Chen, H.E. Changes of carotenoids ,color,and vitamin A contents during processing of carrot juices. *J.Agri.Food.Chem*. **1995**, *43*, 1912-1918.

Chitchumroonchokchai, C.; Schwartz, S.J.; Failla, M.L. Assessment of lutein bioavailability from meals and supplement using simulated digestion and Caco-2 human intestinal cells. *J.Nutr.* **2004**, *134*, 2280–2286.

Erdman, J.W.; Bierer, T.L.; Gugger, E.T. Absorption and transport of carotenoids. *Ann. N.Y.Acad.Sci*. **1993**, *691*, 76-85.

Failla, M.L.; Chitchumroonchokchai, C. *In vitro* models as tools for screening the relative bioavailabilities of provitamin A carotenoids in foods. *Harvest Plus Technical Monograph*., **2005**, *3*, 12-32.

Garrett, D.A.; Failla, M.L.; Sarama, R.J. Development of an in vitro digestion method to assess carotenoid bioavailability from meals. *J.Agric.Food Chem*. **1999**, *47*, 4301-4309.

Gopalan, G.; Ramasastri, B.V.; Balasubramanian, S.C. *Nutritive value of Indian Foods*. .Indian Council of Medical Research, New Delhi, **2004.**

Granado-Lorencio, F.; Olmedilla, B.; Gil-Martinez, E.; Blanco, I. A fast, reliable and low cost saponification protocol for analysis of carotenoids in vegetables. *J.Food Comp. Anal.* **2001**, *14,* 479- 489.

Granado-Lorencio,F.; Olmedilla-Alonso,B.; Herrero-Barbudo,; Blanco-Navarro,I.; Perez-Sacristan,B.; Blazquez-Garcia, S. *In vitro* bioaccessibility of Carotenoids and tocopherols from fruits and vegetables*. Food.Chem.* **2007**, *102,* 641-648.

Hedren, E.; Mulkozi, G.; Svanberg, U. *In vitro* accessibility of carotenes from green leafy vegetables cooked with sunflower oil or red palm oil. *Int.J.Food Sci.Nutr.* **2002**, *53*, 445-453.

Kimura, M.; Rodriguez-Amaya, D.B.; Godoy, H.T. Assessment of the saponification step in the quantitative determination of carotenoids and provitamins A. *Food Chem.* **1990**, *35,* 187-195.

Kohlmeier, l.; Hastings, S.B. Epidemiological evidence of a role of carotenoids in cardiovascular disease preventation. *Am.J.Clin.Nutr.* **1995**, *62S*, 370S-1376S

Kritchevsky, S.B. β-carotene, carotenoids and the prevention of coronary heart disease. *J.Nutr.* **1999**, *129*, 5-8.

.

Miller, D.D.; Schricker, B.R., Rasmussen, R.R., Vancanpen, D.R. An *in vitro* method for the estimation of iron availability from meals. *Am.J.Clin.Nutr*. **1981,** *34*, 2248-2256.

Ranganna, S. Plant pigments. In: *Manual of Analysis of Fruits and Vegetable Products*. Tata McGraw-Hill Co., New Delhi, **1977**, pp.73-77.

Riso, P.; Porrini, M. Determination of carotenoids in vegetable foods and plasma. *Int.J. Vit.Nutr.Res.* **1997**, *67*, 47-54.

Sies, H.; Stahl,W.; Sunquist, A.R. Antioxidant function of vitamins. Vitamin E and C, β-carotene, and other carotenoids. *Ann.N.Y.Acad.Sci.* **1992**, *669*, 7-20.

Snedecor, G.W.; Cochran, W.G. *Statistical Methods*. Sixth Edition. Iowa State Univ. Press, Ames, **1976**, p.298.

Van Poppel,G.; Goldbohm,R.A. Epidemiological evidence for β-carotene and cancer prevention..*Am.J.Clin.Nutr*. **1995**, *62*, 1493-1503.

Ziegler, R. Importance of α-carotene, β-carotene and other phytochemicals in the etiology of lung cancer. *J.Natl.Cancer.Inst*. **1996**, *88*, 612-615.

Chapter -III

Barnell, H.R.; Hollingsworth, D.F. The impact of food technology on the nutrition of man and animals. *Proc.Nutr.Soc.* **1956**, *15,* 142-147.

Baskaran, V.; Sugawara, T.; Nagao, A. Phospholipids affect the intestinal absorption of carotenoids in mice. *Lipids*. **2003**, *38*, 705-711.

Britton, G. UV/VIS spectroscopy. In: Britton G, Liaaen-Jensen S, Pfender H. (Eds) *Carotenoids*, Vol..1B, Basel, Birkhauser, Switzerland, **1995**, pp.13–62.

Chandler, L.A.; Schwartz, S.J. Isomerization and losses of *trans* β - carotene in sweet potato as affected by processing treatments. *J.Agric.Food Chem.* **1988**, *36*,129-133.

Dikshit, S.N.; Udipi, S.A.; Rao, A.; Manohar, V. Separation of carotenoids and estimation of beta–carotene content of selected Indian food and food preparations by HPLC. *J.Food Sci.Tech.* **1988**, *2*, 39-41.

Erdman, J.W. Jr, Thatcher, A.J.; Hofmann, N.E.; *et al.* All-*trans* β-carotene is absorbed preferentially to 9-*cis*- β-carotene, but the latter accumulated in the tissues of domestic ferrets *(Mustela putorius puro). J.Nutr.* **1998**, *128*, 2009–2013.

Garrett, D.A.; Failla, M.L.; Sarama, R.J. Development of an in vitro digestion method to assess carotenoid bioavailability from meals. *J.Agric.Food Chem*. **1999**, *47*, 4301-4309.

Garrett, D.A.; Failla, M.L.; Sarama, R.J. Estimation of carotenoid bioavailability from fresh stir-fried vegetables using an *in vitro* digestion / Caco-2 cell culture model. *J.Nutr.Biochem.* **2000**, *11*, 574-580.

Gayathri, G.N.; Platel, K.; Prakash, J.; Srinivasan, K. Influence of antioxidant spices on the retention of β- carotene in vegetables during domestic cooking processes. *Food Chem.* **2004**, *84*, 35-43.

Gillooly, M.; Bothwell, T.H.; Torrance, J.D.; MacPhail, A.P.; Derman, D.P.; Bezwoda, W.R.; Mills, W.; Charlton, R.W.; Mayet, F. The effect of organic acids, phytates, and polyphenols on the absorption of iron in vegetables. *Br.J.Nutr.* **1983**, *49*, 331-342.

Gopalan, G.; Ramasastri, B.V.; Balasubramanian, S.C. *Nutritive value of Indian Foods*. Indian Council of Medical Research, New Delhi, **2004**.

Granado-Lorencio,F.; Olmedilla-Alonso,B.; Herrero-Barbudo,; Blanco-Navarro,I.; Perez-Sacristan, B.; Blazquez-Garcia, S. *In vitro* bioaccessibility of Carotenoids and tocopherols from fruits and vegetables. *Food Chem.* **2007**, *102***,** 641-648.

Hedren, E.; Mulkozi, G.; Svanberg, U. *In vitro* accessibility of carotenes from green leafy vegetables cooked with sunflower oil or red palm oil. *Int.J.Food Sci.Nutr.* **2002**, *53*, 445-453.

Hedren, E.; Diaz, V.; Svanberg, U. Estimation of carotenoid accessibility from carrot determined by in vitro digestion method. *Eur.J. Clin. Nutr*. **2002a,** *56,* 425-430.

Hemalatha, S; Platel, K.; Srinivasan, K. Influence of food acidulants on bioacessibility of zinc and iron from selected food grains. *Mol.Nutr.Food Res.* **2005,** *49*, 950-956.

Hornero, M.; Minguez, M. Bioaccessibility of carotenes from carrots: effect of cooking and addition of oil. *Innov.Food Sci.Emerg.Tech.* **2007**, *8*, 407–412

Huang, C.; Tang, Y.; Chen, Y.; Chen, M.; Chu, C.; Hseu, C. The bioavailability of β-carotene in stir or deep fried vegetables in men determined by measuring the serum response to a single injection. *J.Nutr.* **2000,** *130*, 534-540.

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

Martin, K.R.; Failla, M.L; Smith, J.C.; Jr. β-carotene and lutein protect Hep G2 human liver cells against oxidant–induced damage. *J.Nutr.* **1996,** *126***,** 2098-2106**.**

Mercadante, A.Z.; Steck, A., Pfander, H. Carotenoids from guava (*Psidium guajava L*); isolation and structure elucidation. *J.Agri.Food Chem*. **1999**, *47*,145-151.

Mulokozi, G.; Hedren, E.; Svanberg, U. In vitro accessibility and intake from cooked green leafy vegetables and their estimated contribution to vitamin A requirement. *Plant Foods Hum. Nutr.* **2004**, *59*, 1-9.

O'Connell, O.; Ryan, L.; O'Brien, N. Effect of fibre on the bioaccessibility of carotenoids from a combination of vegetables. Abstract of original communitation. *Proc.Nutr.Soc.* **2007,** *66*,113A.

Ogulensi, A.T.; Lee, C.Y. Effect of thermal processing on the stereoisomerization of major carotenoids and vitamin A value of carrots. *Food.Chem*. **1979**, *4*, 311-318

Osada, K.; Ogino, Y.; Nakamura-Kanda, T.; Yanagida, T. Effect of dietary apple polyphenol metabolic disorder of lipid in rats given oxidized cholesterol. *Jap.Con. Biochem.Lipids*. **1997,** *39*, 317

Padmavati, K.; Udipi, S.A.; Rao; M. Effect of different cooking methods on β –carotene content of vegetables. *J.Food Sci.Technol.* **1992**, *29, 137*-140

Rao, S.; Reddy, M.V. Effect of different cooking methods on carotene content of selected foods; *Proc.First Indian Conven.Food Scientists and Technologists,* **1979.** *2*, 4-19.

Ratantunga, M.; Setty, R.G.; Saroja, S.; Swamy, N.A.M. Effect of processing on vitamins and minerals in fruits and vegetables. *Indian Food Packer.* **1978**, *6*, 26-61

Rock, C.L.; Lovalvo, J.L.; Emenhiser, C.; Ruffin,M.T.; Flatt,S.W.; Schwartz, S.J. Bioavailability of β-carotene is lower in raw than in processed carrots and spinach in women. *J. Nutr.* **1998**, *128*, 913-916

Rodriguez, M.S.; Irwin, M.I. A prospectus of research in vitamin requirement of man. *J.Nutr.* **1972**, *23*, 105-108.

Sareen, S. *Heat processing, in food Preservation*; 1st Edn; Sarup and Sons, New Delhi, **1999,** pp.386-431.

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.Agric.Food Chem*. **2005**, *53*, 2936-2940.

Snedecor, G.W.; Cochran, W.G. *Statistical Methods*. Sixth Edition. Iowa State Univ. Press, Ames, **1976**, p.298.

Sood, R.; Bhat, C.M. Changes in ascorbic acid and carotene content of green leafy vegetables on cooking. *J.Food.Sci.Technol.* **1974**, *11*, 131-133.

Srinivasan, K. Role of spices beyond food flavouring: Nutraceuticals with multiple health effects. *Food Rev.Int.* **2005**, *21*, 167-188.

Suruga, K.; Suzuki, R.; Goda, T.; Takase,S. Unsaturated fatty acids regulates gene expression of cellular retinol binding protein Type II in rat jejunum. *J.Nutr*. **1995**, *125*, 2039-2044.

Sweeney, J.P.; Marsh, A.C. Effect of processing on provitamin A in vegetables. *J.Am.Diet.Assoc.***1971,** *59***,** 238.

van Het-Hof, K.H.; De Boer, B.C.J.; Tijburg, L.B.M.; Lucius, B.R.H.M.; Zijp, I.; West, C.E.; Hautvast, J.G.A.J.; Weststrate, J.A. Carotenoid bioavailability in humans from tomatoes processed in different ways determined from the carotenoid response in the triglyceride-rich lipoprotein fraction of plasma after a single consumption and in the plasma after four days of consumption. *J.Nutr.* **2000***, 130*, 1189–1196.

Veda, S.; Kamath, A.; Platel, K.; Begum, K.; Srinivasan, K. Determination of bioaccessible β-carotene from green leafy and yellow-orange vegetables by in vitro procedures. *Mol.Nutr.Food Res.* **2006**, *50*, 1047-1052.

Veda, S.; Platel, K.; Srinivasan, K. Varietal differences in the bioaccessibility of β-carotene from mango and papaya fruits. *J.Agric.Food Chem*. **2007**, *55*, 7931-7935.

Weckel, K.G.; Santos, B.; Hernan- Laferriare, L. Gabetman, W.H. Carotene components of frozen and processed carrots. *Food.Tech*. **1962**, *16*, 91-94

Yeum, K.J.; Russell, R.M. Carotenoid bioavailability and bioconversion. *Ann.Rev.Nutr.* **2002**, *22*, 483-504.

Chapter –IV

AOAC Official Methods of Analysis. 9th Edition, 1960, pp.217-272

Carlier, C.; Etchepare, M.; Ceccon, J.F.; Mourey, M.S.; Amédée-Manesme O. Efficacy of massive oral doses of retinyl palmitate and mango (*Mangifera indica.*.) consumption to correct an existing vitamin A deficiency in Senegalese children. *Br.J.Nutr.* **1992**, *68*, 529–540.

Drammeh, B.S.; Marquis, G.S.; Funkhouser, E.; Bates, C.; Eto, I.; Stephensen, C.B. Randomized, 4- month mango and fat supplementation trial improved vitamin A status among young Gambian children. *J.Nutr.* **2002**, *132*, 3693 – 3699.

Duncan, B.B. Multiple ranges and multiple F test. *Biometrics*. **1955**, *11*, 1-5.

Garrett, D.A.; Failla, M.L.; Sarama, R.J. Development of an in vitro digestion method to assess carotenoid bioavailability from meals. *J.Agric.Food Chem*. **1999**, *47*, 4301-4309.

Gayathri, G.N.; Platel, K.; Prakash, J.; Srinivasan, K. Influence of antioxidant spices on the retention of β-carotene in vegetables during domestic cooking processes. *Food Chem.* **2004,** *84*, 35 – 43.

Gopalan, G.; Ramasastri, B.V.; Balasubramanian, S.C. *Nutritive value of Indian Foods*. Indian Council of Medical Research, New Delhi. **2004.**

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

Indian Council of Medical Research. Nutrient Requirements and Recommended Dietary Allowances for Indians. ICMR, New Delhi, **2000**.

Krinsky, N. I.; Jhonson, E. J. Carotenoid actions and their relation to health and disease. *Mol.Aspects Med.* **2005**, *26*, 459–516.

Kudachikar, V. B.; Kulkarni, S. G.; Keshava Prakash, M. N.; Vasantha, M. S.; Aravinda Prasad, B.; Ramana, K. V. R. Physicochemical changes during maturity of mango (Mangifera indica L.) variety 'Neelum'. *J.Food Sci.Technol*. **2001**, *38*, 540–542.

Mercadante, A. Z.; Rodríguez-Amaya, D. B.; Britton, G. HPLC and mass spectrometric analysis of carotenoids from mango. *J.Agric.Food Chem*. **1997**, *45*, 120–123.

Mercadante, A. Z.; Rodríguez-Amaya, D. B. Effects of ripening, cultivar differences, and processing on the carotenoid composition of mango. *J.Agric.Food Chem*. **1998**, *46*, 128-130.

Mulokozi, G.; Hedren, E.; Svanberg, U. *In vitro* accessibility and intake of β-carotene from cooked green leafy vegetables and their estimated contribution to vitamin A requirements. *Plant Foods Hum.Nutr*. **2004**, *59*, 1-9.

Ornelas-Paz, J.De.J.; Failla, M.L.; Yahia, E.M.; Gardea-Bejar, A. Impact of the stage of ripening and dietary fat on in vitro bioaccessibility of β-carotene in (Atalufo mango). *J.Agri.Food Chem.***2008,** *56*,1511-1516

Pott, I.; Breithaupt, D. E.; Carle, R. Detection of unusual carotenoid esters in fresh mango (Mangifera indica L. Cv. Kent). *Phytochemistry.* **2003**, *64*, 825–829.

Pott, I.; Marx, M.; Neidhart, S.; Muhlbauer, W.; Carle, R.; Neidhart,S. Quantitative determination of β-carotene stereoisomers in fresh dried, and solar dried mangoes (*Mangifera indica).J.Agri.Food Chem.***2003a**, 51, 4527-4531.

Pradeep, C. R.; Kuttan, G. Effect of β-carotene on the inhibition of lung metastasis in mice. P*hytomedicine.* **2003**, *10*, 159-164.

Riedl, J.; Linseisen, J.; Hoffmann, J.; Wolfram, G. Some dietary fibers reduce the absorption of carotenoids in women. *J.Nutr*. **1999**, *129*, 2170–2176.

Setiawan, B.; Sulaeman, A.; Giraud, D.W.; Driskell, J.A. Carotenoid content of selected Indonesian fruits. *J.Food Comp.Anal*. **2001**, *14*, 169-176.

Thurnham, D.I. Bioequivalence of β-carotene and retinol. *J. Sci.Food Agric*. **2007**, *87*, 13-39.

Tyssandier, V.; Cardinault, N.; Caris-Veyrat, C.; Amiot, M.J.; Grolier, P.; Bouteloup, C.; Azais-Braesco, V.; Borel, P. Vegetable borne lutein, lycopene, and β-carotene compete for incorporation into chylomicrons, with no adverse effect on the medium-term(3 week) plasma status of carotenoids in humans. *Am.J.Clin.Nutr*. **2002,** *75*, 526–534.

Ueda, M.; Sasaki, K.; Utsunomiya, N.; Inaba, K.; Shimabayashi,Y. Changes in physical and chemical properties during maturation of mango fruit (*Mangifera indica* L. "Irwin") cultured in a plastic greenhouse. *Food Sci.Technol.Res*. **2000,** *6*, 299–305.

van Het-Hof, K.H.; de Boer, B.C.J.; Tijburg, L.B.M.; Lucius, B.R.H.M.; Zijp, I.; West, C.E.; Hautvast, J.G.A.J.; Weststrate, J.A. Carotenoid bioavailability in humans from tomatoes processed in different ways determined from the carotenoid response in the triglyceride-rich lipoprotein fraction of plasma after a single consumption and in the plasma after four days of consumption. *J.Nutr*. **2000**, *130*, 1189–1196.

Vázquez-Caicedo, A.L.; Neidhart, S.; Carle, R. Postharvest Vázquez-Caicedo, A.L.; Neidhart, S.; Carle, R. Postharvest ripening behavior of nine Thai mango cultivars and their suitability for industrial applications. *Acta Hortic*. **2004,** *645*, 617–625.

Vazquez-Caicedo, A.L.; Sruamsiri, P.; Carle, R.; Neidhart, S. Accumulation of all-trans-β-carotene and its 9-cis and 13-cis isomers during post-harvest ripening of nine Thai mango cultivars. *J.Agric.Food Chem.* **2005**, *53,* 4827-4835.

West, C. E.; Castenmiller, J. J. M. Quantification of the"SLAMENGHI" factor for carotenoid bioavailability and bioconversion. *Int.J.Vitam.Nutr.Res*. **1998,** *68*, 371–377.

Wilberg, B.C.; Rodriguez-Amaya, D. HPLC quantitation of major carotenoids of fresh and processed guava, mango and papaya. *Lebenson.Wiss..Technol*. **1995**, *28,* 474-480.

Yuyama, L.K.O.; Fávaro, R.M.D.; Yuyama, K.; Vannucchi, H. Bioavailability of vitamin A from peach palm (*Bactris gaspiaes*) and from mango (*Mangifera indica* L.) in rats. *Nutr.Res*. **1991,** *11*, 1167–1175.

Chapter - V

Annamalai A.R.; Manavalan, R. Effect of 'Trikatu' and its individual components and piperine on gastrointestinal tracts: Trikatu – a bioavailable enhancer. *Indian Drugs.* **1990**, *27*, 595-604.

Atal, C.K.; Zutshi, U.; Rao, P.G. Scientific evidence on the role of Ayurvedic herbals on bioavailability of drugs. *J. Ethnopharmacol*. **1981**, *4*, 229-232.

Badmaev V.; Majeed M.; Norkus E.P. Piperine, an alkaloid derived from black pepper increases serum response of β-carotene during 14-days of oral β-carotene supplementation. *Nutr.Res*. **1999,** *19*, 381-388.

Bano, G.; Amla, V.; Raina, R.K.; Zutshi, U.; Chopra, C.l. The effect of piperine on pharmacokinetics of phenytoin in healthy volunteers. *Planta.Med.* **1987**, *53*, 568-570.

Bano, G.; Raina, R.K.; Zutshi,U.; Bedi,K.L.; Johri,R.K.;Sharma,S.C. The effect of piperine on bioavailability and pharmacokinetics of propranolol and thephylline in healthy volunteers. *Eur.J.Clin.Pharmacol***. 1991,** *41*, 615-618.

Barua A.B.; Furr H.C. Properties of retinoids. Structure, handling, and preparation. *Mol. Biotechno*l. **1998,** *10*, 167-182. Review.

Bendich, A . Carotenoids and the immune response. J.Nutr. 1989, 119, 112-115.

Bhat, B.G.; Srinivasan, M.R.; Chandrasekhara, N. Influence of curcumin and capsaicin on the composition and secretion of bile in rats. *J.Food Sci.Technol*. **1984**, *21*, 225-227.

Bhat, B.G.; Sambaiah, K.; Chandrasekhara, N. The effect of feeding fenugreek and ginger on bile composition in the albino rat. *Nutr.Rep.Internat*. **1985**, *32*, 1145-1151.

Bhat B.G.; Chandrasekhara N. Lack of adverse effect of piperine, pepper or pepper oleoresin on weanling rats. *J.Food Safety*.1**986**, *7*, 215-223.

El-Gorab, M.I.; Underwood, B.A.; Loerch, J.D. The roles of bile salts in the uptake of β-carotene and retinol by rat everted sacs. *Biochim.Biophy.Acta.* **1975,** *401*, 265–277.

Ershov Y.V.; Bykhovsky VY.; Dmitrovskii A.A. Stabilization and competitive inhibition of β- carotene 15,15'-dioxygenase by carotenoids. *Biochem.Mol.Biol.Int*. **1994**, *34*, 755-763.

Gayathri, G.N.; Platel, K.; Prakash, J.; Srinivasan, K. Influence of antioxidant spices on the retention of β-carotene in vegetables during domestic cooking processes. *Food Chem.* **2004**, *84*, 35-43.

Hedren, E.; Mulkozi, G.; Svanberg, U. *In vitro* accessibility of carotenes from green leafy vegetables cooked with sunflower oil or red palm oil. *Int.J.Food Sci.Nutr.* **2002**, *53,* 445-453.

Hiwale, A.R; Dhuley, J.N.; Naik, S.R. Effect of co-administration of piperine on pharmacokinetics of β-lactam antibiotics in rats. *Indian J.Exp.Biol*. **2002**, *40*, 277-281.

Johri R.K.; Zutshi U. An Ayurvedic formulation 'Trikatu' and its constituents. *J.Ethno-pharmacol*. **1992**, *37*, 85-91

Johri, R.K.; Thusu, N.; Khajuria, A.; Zutshi, U. Piperine-mediated changes in the permeability of rat intestinal epithelial cells: Status of γ-glutamyl transpeptidase activity, uptake of amino acids and lipid peroxidation. *Biochem. Pharmacol*. **1992a,** *43,* 1401-1407.

Khajuria, A.; Zutshi, U.; Bedi, K.L. Permeability characteristics of piperine on oral absorption - an active alkaloid from peppers & a bioavailability enhancer, *Indian J. Exp. Biol.* **1998,** *36*, 46-50.

Khajuria, A.; Thusu, N.; Zutshi, U. Piperine modulates permeability characteristics of intestine by inducing alterations in membrane dynamics: influence on brush border membrane fluidity, ultra structure & enzyme kinetics, *Phytomedicin.* **2002,** *9*, 224-231.

Lowry O.H.; Rosebrough N.J; Farr A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J.Biol.Chem.* **1951,** *193*, 265-275.

McDonald, J.K.; Barrette, A.J. *Leucyl aminopeptidase*. . In. Mammalian Proteinases. A glossary and bibiliography. McDonald J .K.; Barrette A.J. (Eds), Academic Press, London, **1986,** pp.23-100.

Mercado, R.J.D.; Holmgren, S.C.; Fox, J.G.; Russel, R.M. Dietary β-carotene absorption and metabolism in ferrets and rats. *J. Nutr*. **1989**, *119*, 665-669.

Monsereenusorn Y., Glinsukon, T. Inhibitory effect of capsaicin on intestinal glucose absorption *in vitro*. *Food Cosmet.Toxicol*. **1978**, *16*, 469-473.

Nadkarni, K.M.; Nadkarni, A.K. *Indian Materia Medica*, Popular Prakashan Pvt. Ltd, Mumbai, India, **1976.**

Nagao, A.; Maeda, M.; Lim, B.P.; Kobayashi, H.; Terao, J. Inhibition of β-carotene 15, 15'-dioxygenase activity by dietary flavonoids. *J.Nutr.Biochem*. **2000**, *11*, 348-355.

Nagao, A. Oxidative conversion of carotenoids to retinoids and other products. *J.Nutr*. **2004**, *134*, 237S-240S.

Olson, J.A. The effect of bile and bile salts on the uptake and cleavage of β-carotene in to retinol ester (vitamin A ester) by intestinal slices. *J.Lipid Res.* **1964**, *5,* 402-408.

Platel, K.; Srinivasan, K. Stimulatory influence of select spices on bile secretion in rats. Nutr.Res. **2001**, 20, 1493-1503.

Platel, K.; Srinivasan K. Digestive stimulant action of spices: A myth or reality? *Indian J.Med.Res*. **2004**, *119*, 167-179.

Poor, C.L.; Bierer,T.L.; Merchen, N.R.; Fahey,G.C.; Erdman, J.W. The accumulation of α- and β- carotene in serum and tissues of pre-ruminant calves fed raw and steamed carrot slurries. *J.Nutr.* **1993,** *123,* 1296-1304.

Reanmongkol, W.; Janthasoot, W.; Wattanatorn, W.; Dhumma-Upakorn, P.; Chudapongse, P. Effects of piperine on bioenergetic functions of isolated rat liver mitochondria, *Biochem.Pharmacol*., **1988,** *37*, 753-757.

Rodriguez, M.S.; Irwin, M.I. A prospectus of research in vitamin requirement of man. *J.Nutr*. **1972**, *23*, 105-108.

Sambaiah, K.; Srinivasan M.R.; Satyanarayana, M.N. ; Chandrasekhara N. Influence of capsaicin on the absorption of amino acids and fat in rats. *J.Food Sci.Technol*. **1984,** *21*, 155-157.

Sambaiah, K.; Srinivasan, K. Secretion and composition of bile in rats fed diets containing spices. *J.Food Sci.Technol*. **1991**, *28*, 35-38.

Snedecor, G.W.; Cochran, W.G., *Statistical Methods*. Sixth Edition. Iowa State Univ. Press, Ames, USA, **1976,** p.298.

Srinivasan, K.; Sambaiah, K.;Chandrasekhara N. Spices as beneficial hypolipidemic food adjuncts: A Review. *Food Rev.Int*. **2004**, *20*, 187-220.

Srinivasan K. Spices as influencers of body metabolism: An overview of three decades of research. *Food Res.Int.* **2005**, *38*, 77-86.

Srinivasan K. Role of spices beyond food flavouring: Nutraceuticals with multiple health effects. *Food Rev.Int*. **2005a,** *21,* 167-188.

Srinivasan K. Plant foods in the management of diabetes mellitus: Spices as potential antidiabetic agents. *Int.J.Food Sci.Nutr*. **2005b,** *56*, 399-414.

Srinivasan K. Black pepper and its pungent principle - piperine: A review of diverse physiological effects. *Crit.Rev.Food Sci.Nutr*., **2007,** *47*, 735-748.

Srinivasan K. Reason to season: Spices as functional food adjuncts with multiple health effects. *Indian Food Industry.* **2008**, *27* (5), 36-47.

Srinivasan, K. Black Pepper (*Piper Nigrum*) and its bioactive compound piperine. *In*: *Molecular Targets and Therapeutic uses of Spices: Modern Uses for Ancient Medicine*. B.B.Aggarwal, and A.B.Kunnumakkara (Eds), World Scientific Publishing Co. Inc., Ames, USA; Chapter-3, **2009**, In Press

Srinivasan K. Antioxidant potential of spices and their active constituents. *Crit.Rev.Food Sci.Nutr*. **2009a**, *49*, In Press.

Sundaresan, P.R.; Cope, F.O.; Smith, J.C. Jr. Influence of zinc deficiency on retinal reductase and oxidase activities in rat liver and testes. *J.Nutr*. **1977,** *107*, 2189-2197.

Suresh. D.; Srinivasan K. Studies on the in vitro absorption of spice principles: curcumin , capsaicin and piperine in rat intestines. *Food.Chem.Toxicol*. **2007**, *45,* 1437-1442.

Suresh.D.; Srinivasan K. Tissue distribution and elimination of the spice bioactive compounds following oral intake in rats. *Indian J.Med.Re*s. **2009**, *124*, In press.

Ugolev, A.M.; Timofeera, N.M.;Smirnova, L.F.; *et.al*. Membrane and intracellular hydrolysis of peptide after initiation role and relationship with transport .In *Ciba Foundation Symp Peptide transport and hydrolysis*.**1977**, *50*, 221-243.

Usha Prakash N.S.; Srinivasan K. Beneficial influence of dietary spices on the ultrastructure and fluidity of intestinal brush border in experimental rats. **2009**, *J.Gastroenterol*., In Press.

van Vliet T.; van Vlissingen, M.F.; 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*. **1996,** *126*, 499-508.

Veda, S.; Platel, K.; and Srinivasan, K. Influence of food acidulants and antioxidant spices on the bioaccessibility of β-carotene from selected vegetables. *J. Agric. Food Chem.* **2008,** *56*, 8714-8719.

World health organization Global prevalence of vitamin A deficiency in populations at risk1995-2005. WHO Global database on vitamin A deficiency, Geneva, **2009.**

Young, A.J.; Lowe, G.M. Antioxidant and pro-oxidant properties of carotenoids. *Arch.Biochem.Biophys*. **2001**, *385*, 20-27.

Zutshi, R.K.; Singh, R.; Zutshi, U.; Johri, R.K.; Atal, C.K. Influence of piperine on Rifampicin blood levels in patients of pulmonary tuberculosis. *JAPI*. **1985**, *53*, 568-569.