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Green Extraction methods and Environmental applications of Carotenoids-A Review

Aarti Singh[a], Sayeed Ahmad[b] and Anees Ahmad*[a]

[a]Department of Chemistry, Aligarh Muslim University, Aligarh, UP, India,

[b]Department of Pharmacognosy and phytochemistry, Jamia Hamdard, New Delhi

Abstract

This review covers and discusses various aspects of carotenoids including their chemistry, classification, biosynthesis, extraction methods (conventional and non-conventional), analytical techniques and biological roles in living beings. Carotenoids play a very crucial role in human health through foods, cosmetics, neutraceuticals and pharmaceuticals. Carotenoids cannot be synthesized by humans so they are usually consumed with natural sources like fruits and vegetables. Among carotenoids, lycopene acts as best antioxidant. Carotenoids have been categorised into two classes as xanthophylls and carotenes, which have substantial roles in the prevention of chronic diseases. Various extraction methods have been employed for extraction of carotenoids: solvent extraction, soxhlet extraction, centrifugation and non-conventional methods of extraction such as ultrasound-assisted, microwave-assisted, enzymatic and the innovative technique Supercritical Carbondioxide (SC-CO_2) extraction. The green and environmentally friendly technique for extraction of carotenoids is SC-CO_2 extraction which extracts pure compound in high yield without the use of harmful organic solvents, it operates at lower temperature so it is useful for extraction of thermolabile compounds. This technique uses SC-CO_2 as green solvent and other solvents as modifiers which are Generally recognized as safe (GRAS) solvents. Green technology is the need of present time in order to keep environment healthy, pollution free and sustainable for

coming generation. Present review includes several analytical techniques used to identify and quantify carotenoids are: Thin layer chromatography (TLC), High performance thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC), Liquid chromatography-mass spectrometry (LC-MS), Nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FTIR), Ultra performance liquid chromatography - tandem mass spectrometer (UPLC-MS), UV-Vis (Ultraviolet-Visible) spectrophotometry; out of these, NMR and FTIR have been explored the least for carotenoid analysis.

Keywords: *Supercritical Carbondioxide Extraction, GRAS, Carotenoids, Green technique*

Corresponding author: as87822@gmail.com (Anees Ahmad)(+91-9536322688)*

Introduction

Carotenoids are known to be organic pigments present in the chromoplasts and chloroplasts of the plants and various algae, fungi and certain bacteria in which photosynthesis process occur. In certain cases, carotenoids are also present in non-photosynthetic yeasts, moulds and bacteria, where their function is to protect from the damage caused by oxygen and light.[1] All these organisms produce carotenoids from fats and other fundamental organic metabolic building blocks. Quality of fruits and vegetables are defined well by carotenoids.[2] Colours of fruits, vegetables, flowers, leaves and also in case of certain insects, fish, birds and crustaceans are due to carotenoids, for example colours in peppers, tomatoes, which are red and pink colour of salmon and flamingos.[3] Yellow-orange colour of fruits and vegetables is due to α-carotene and β-carotene, orange fruits contain α-cryptoxanthin, tomato and its products contain lycopene which has been found to be maximum in its peel. Dark green vegetables are found to have lutein and zeaxanthin. During maturity of leaf when chlorophyll degrades, carotenoids are responsible for the colour of the leaf.[4] More than 600 different carotenoids from natural sources have been isolated and characterized and novel carotenoids are still to be isolated and identified.[5] The structures of 563 have been elucidated till now, 14 carotenoids have been found in the serum of humans and it has been elucidated that human body can metabolize and absorb 50 of them. α-carotene, β-carotene, lutein and lycopene constitute 90% of total carotenoids of the human diet.[6] Important carotenoids with their natural sources along with their functions are given in **Table 1**. Human serum and milk have been found to contain 34 carotenoids.[7] Animals consume carotenoids in their diet as they are unable to produce them biologically.[8] Carotenoids (fruits and vegetables) play a crucial role in reducing risk of various diseases in humans, which includes cancer, cardiovascular diseases, eye-related diseases (cataract, macular degeneration, photo-induced skin disorders and other unrelieved diseases and they have been served as best sources of vitamin A and as

antioxidants, and also in immunoenhancement.[2,4,9] Among all carotenoids, lycopene possesses highest antioxidant property.[10] Adipose tissue accumulates maximum carotenoids.[11] There is a need to identify and isolate novel carotenoids from diverse natural sources to understand their human health benefits.

Structure and chemistry of carotenoids

Carotenoids are lipid-soluble tetraterpenoids (having 8 isoprene molecules) containing 40 carbon atoms in their structure, having conjugated double bonds.[12] Naturally carotenoids occur in *trans*-form in fruits and vegetables, but on their processing it is converted to *cis*-isomer form thereby increasing its percentage from 10 to 39%.[13] Majority of carotenoids has a central carbon chain with alternate single and double bonds along with dissimilar cyclic and acyclic end groups (rings). With the increment in the number of double bonds, electrons involved in the conjugation gets more space to move about, and changes states at lower energy. Naturally occurring carotenoids are *trans*-form but they get isomerised to *cis*-form due to conjugation on processing or environmental conditions (heat or light).[14] Time and strength of heat determine the extent of isomerisation of carotenoids. Unesterified carotenoids are present in green leaves, while ripened fruits contain esterified carotenoids along with fatty acids. Carotenoids have very important light absorbing property because of which they give colour to various fruits and vegetables as a result of conjugation of double bonds. Lycopene gives red colour to several fruits and vegetables, contain 11 conjugated and 2 unconjugated double bonds.[15] Stability of various forms of lycopene: 5-*cis*>all-*trans*>9-*cis*>13-*cis*>15-*cis*>7-*cis*>11-*cis*. Its antioxidant activity follows the order: 5-*cis*>9-*cis*>7-*cis*>13-*cis*>11-*cis*>all-*trans* isomers.[16] Carotenoids were given trivial names and are known by their common names from which they have been isolated, but further nomenclature (semi systematic) of carotenoids have been devised on the basis of their structure, as given in the **Table 2.**

Carotenoids classification and their bio-regulation

Classification of Carotenoids

There are over 600 known carotenoids. They have been divided into two classes, xanthophylls (which contain oxygen) and carotenes (which are purely hydrocarbons, and contain no oxygen). Carotenoids are lipid soluble C40 tetraterpenoids. Most of the carotenoids are derived from a 40-carbon polyene chain, which could be considered as a backbone of the molecule and this chain is terminated by cyclic end-groups. **Fig. 1** shows classification of naturally occurring carotenoids. The classification of carotenoids according to the structure is as follows:

Xanthophyll: These are oxygenated carotenoids that are synthesized within the plastids and chromatographically separated from other carotenes as they differ in their polarity. Among all other xanthophylls (e.g. lutein, zeaxanthin, neoxanthin, violaxanthin, and α- cryptoxanthin), β-cryptoxanthin is the only xanthophyll that is known to possess pro-vitamin A activity for mammals. Xanthophylls do not require light for synthesis, so they are present in all young leaves as well as in etiolated leaves and have an important function as accessory pigments (capturing certain wavelength of sunlight not absorbed by chlorophyll).

Carotenes: These are purely hydrocarbons and oxygen free carotenes (α-carotene, β-carotene and lycopene). Carotene is an orange photosynthetic pigment important for photosynthesis. Carotenes contribute to photosynthesis by transmitting the light energy they absorb from chlorophyll. They also protect plant tissues by absorbing the energy from singlet oxygen, an excited form of the oxygen molecule (O_2) which is formed during photosynthesis.

Carotenoid biosynthesis and regulation in plants

Carotenoids Biosynthesis

In the plastids, where carotenoids biosynthesis takes place, Isopentenyl pyrophosphate (IPP) is synthesized through the plastid-specific DOXP (1-deoxyxylulose 5-phosphate) pathway. Four desaturation reactions take place. Two reactions are catalyzed by the membrane associated phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) which results in the formation of the pink lycopene from the colourless phytoene. The cyclization of lycopene represents a branch point in the pathway, and two products can be formed depending on the position of the double bond on the cyclohexane ring. On one side, lycopene β-cyclase, for which there are two forms in tomato, one specific to green tissues (LCY-B) and the other to chromoplasts (CYC-B), first produces γ-carotene containing one β-ring which is subsequently converted to β-carotene by the same enzyme. On the other side, lycopene ε-cyclase (LCY-E) produces δ- carotene. The formation of α- carotene, the precursor for lutein, involves formation of a β-ring on δ-carotene by lycopene β-cyclase. The α- and β-carotenes are the precursors for the xanthophylls, which are oxygenated carotenoids generated by β- and ε-ring specific hydroxylases. β-carotene is converted to zeaxanthin by the carotenoid β-ring hydroxylases (HYD- B), encoding a non-heme diiron enzyme for which there are two genes in Arabidopsis. The hydroxylation of the ε- ring is carried out by the carotenoid ε-ring hydroxylase (HYD-E), a cytochromeP450 enzyme, CYP97C1, encoded by the Arabidopsis LUT1 locus. In addition to displaying activity towards the ε-ring, LUT1 can also hydroxylate the β-ring. Hydroxylation of the β-ring of α- carotene is also mediated by a P450 enzyme.[18] Lutein is the main carotenoid present in the petals of marigold, and the broad range of colors that characterize marigold flowers is due to different levels of this xanthophyll. The formation of ketocarotenoids, such as astaxanthin, requires the addition of keto groups in each β-ring of zeaxanthin.[19] Scheme for biosynthesis of carotenoids has been shown in **Fig. 2.**

Regulation of Carotenoid Biosynthesis

Carotenoid accumulation occurs in most plant tissues, including green shoots, flowers, fruits, seeds and roots.[20] Animals and human beings are incapable of carotenoid biosynthesis, but they can modify some of them when absorbed from plant food, for example β-carotene, which can be converted to retinol (vitamin A).[21] There are several regulatory pathways involved for biosynthesis of carotenoids and these are Isopentyl pyrophosphate (IPP), phytoene biosynthesis, lycopene biosynthesis and its cyclization. In IPP pathway, carotenoids biosynthesis is being operated by regulating DXS gene.[22] In plant, yield of carotenoid content is regulated in phytoene biosynthesis pathways through expressing different phytoene synthase gene (PSY1, PSY2 and PSY3),[23] but in case of bacteria, bacterial phytoene synthase gene is responsible.[24] Expression of PSY, leads to the condensation of two C-20 geranylgeranyl diphosphate (GGPP) molecules to form phytoene whereas lycopene biosynthesis is catalyzed by phytoenedesaturase (PDS) gene,[25] but epigenetic regulation reveals that another gene, SDG8 is involved for lycopene biosynthesis,[26] The carotenoids biosynthesis pathway take branches after the formation of lycopene and these are being regulated by different responsible enzyme.[27] Carotenoids accumulation is regulated by some genetic variation and these are over expression of PSY gene[28] and silencing of €-CYC.[27] Similarly, degradation of carotenoids is regulated by several gene family and these are Cleavage dioxygenase (CCD) and 9-cis-epoxycarotenoids dioxygenases (NCED).[29]

Accumulation, Storage and insights from biofortification

The storage of carotenoids requires a lipophilic environment, usually within the membranes of plastid organelles, which behave as a sink for carotenoid accumulation. The colourless pluripotent progenitor proplastid differentiates into specialised plastids that can store chlorophylls and carotenoids.[30] Carotenoids are usually synthesised de novo in differentiated plastids of roots, flowers, fruits and seeds, accumulating mostly in chloroplasts (green

photosynthetic plastids) and chromoplasts (coloured plastids), but also in amyloplasts (starch-storing plastids), leucoplasts (colourless plastids), etioplasts (dark-grown precursors of the chloroplast) and elaioplasts (lipid-storing plastids). The regulation of carotenoid targeting, storage and sequestration within various plastid types is a process to modulate a sink for carotenoid accumulation.[25] For example, the *high-pigment 1* tomato mutant, hp1, displays an increased pigmentation because of increased chromoplast compartment size.[31] A naturally occurring mutation in the *Brassica oleracea* orange-curd (or) gene changes a normally white cauliflower curd into an orange, which accumulates high levels of β-carotene.[32] The introduction of foreign exogenous carotenoid biosynthetic genes into crops is another means to modulate the sink for carotenoid storage. Carotenoid accumulation has been achieved in oil seeds of canola (*Brassica napus*) through the overexpression of PSY, which resulted in 43-50-fold increase in total seed carotenoid content.[33] 'Golden rice 2' is a high β-carotene accumulating line overexpressing PSY from maize as well as bacterial CrtI (from *Erwinia uredovora*) and accumulates 23-fold more carotenoids than was the case for the initial 'golden rice', which overexpressed PSY from daffodil.[24] Level of carotenoid accumulation depends on sequestration and availability of storage compartment. Carotenoid accumulation is accompanied by changes in the anatomical structure of plastids, resulting in enhanced ability to store carotenoids.[28] In chloroplasts, most carotenoids accumulate in the form of chlorophyll-carotenoid-protein complexes in the thylakoid membranes associated with light-harvesting antenna but in chromoplasts, significant amounts of carotenoids may be stored in membranes, oil bodies or other crystalline structures within the stroma.[20] Seed carotenoids are compartmentalized to elaioplasts (lipid–storing plastids), which use specialized lipoprotein-sequestering structures to store large quantities of carotenoids.[20]

Catabolism and degradation

Degradation of carotenoids in foods is complex in nature and knowledge about it is fragmentary. Various factors such as nature and composition of foods, processing treatments, packaging and storage conditions, activity of lipooxygenase and other enzymes, and coupled oxidation with lipids are considered to play a vital role for degradation of carotenoids. The polyene chain is the cause of instability of carotenoids including their susceptibility to oxidation and geometric isomerization. Heat, light and acids promote isomerization of *trans*-carotenoids to the *cis*-form. Oxidation depends on the available oxygen, the carotenoids involved, and their physical conditions. It is generally accepted that the initial stage of oxidation involves epoxidation and formation of apocarotenals.[34] Subsequent fragmentation results in a series of low molecular weight compounds similar to those obtained in fatty acid oxidation, which contribute to the desirable flavor of wine and tea but can be responsible for the off flavor of dehydrated carrots and sweet potato flakes. Carotenoid degradation by enzymatic oxidative cleavage produces an array of terpenoid products collectively known as apocarotenoids and these include abscisic acid, strigolactones, and other volatile and non-volatile compounds. The possible scheme for carotenoid degradation is shown in **Fig. 3.**

Carotenoids are relatively stable compounds that accumulate in diverse types of tissues. Recently, it was demonstrated by $^{14}CO_2$ uptake experiments that carotenoid turnover appears to be much greater than expected.[35] In mature leaves, the active degradation of carotenoids by CCD (carotenoid cleavage dioxygenases) and NCED (9-*cis*-epoxycarotenoid dioxygenase) enzymatic turnover. Members of these gene families are involved in the biosynthesis of the phytohormone ABA (NCED2, 3, 5, 6, 9), which controls abiotic stress signalling pathways and strigolactone (CCD1, 4, 7, 8), which controls shoot growth and root-mycorrhizal symbiosis. The active degradation of the xanthophylls by CCD activity can reduce lutein content in strawberries as well as changes in the pigmentation in chrysanthemums from white

to yellow.[36] In maturing Arabidopsis seeds a loss of function of CCD1 activity leads to higher carotenoid levels and may have a role in synthesis of apocarotenoid flavour and aroma volatiles.[37] Similarly, in tomato (*Lycopersicon esculentum*) LeCCD1 activity contributes to the formation of the flavour volatiles β-ionone, pseudoionone and geranylacetone.[38] The Crocus, zeaxanthin 7,8 (70,80)-cleavage dioxygenase (CsZCD) and 9,10 (90,100)- cleavage dioxygenase (CsCCD) initiate the biogenesis of carotenoid derivatives such as crocetin glycosides, picrocrocin and safranal (saffron).

Bioavailability from the diet and tissue distribution

Bioavailability is defined as the fraction of an ingested nutrient that becomes available to the body for utilisation in physiological functions or for storage.[39] There are several factors that influence the bioavailability of carotenoids; species of carotenoid, molecular linkage, amount consumed in a meal, matrix in which the carotenoid is incorporated, effectors of absorption and bioconversion, nutrient status of the host, genetic factors, host-related factors and interactions. Since carotenoids are lipid- soluble they are taken up from the intestine far better from a fatty diet.[40] The uptake of β-carotene from vegetables is low compared to purified β-carotene added to a simple matrix. *Cis*-isomers of carotenoids appear to be more bioavailable than the all-*trans* forms, perhaps because they are more soluble in bile acid micelles and so preferentially incorporated into chylomicrons. It has been suggested that individual carotenoids antagonise absorption of each other, e.g. canthaxanthin inhibits lycopene uptake[41] and it is likely that uptake by intestinal cells is a facilitated process. Once ingested, carotenoids appear in plasma, initially in the VLDL and chylomicron fractions and later in LDL and HDL. The highest levels are found in LDL. Studies of β-carotene uptake and plasma clearance, using human ileostomy volunteers showed that absorbed β-carotene is rapidly cleared from the plasma to an unobservable pool at a rate similar to that of chylomicron triacylglycerols. Lycopene is found in most human tissues, but is not deposited

uniformly. These differences suggest that there are specific mechanisms for the preferential deposition of lycopene, particularly in the adrenals and testes.[42]

Recommended dietary intake of carotenoids in humans

Intake of β-carotene (15-60 mg/day), lutein (6-20 mg/day) and zeaxanthin (2-5 mg/day) are recommended in age-related macular degeneration, and lutein intake of 15 mg/week in humans suffering from cataract. Intake of 60 mg/day of lycopene is needed in case of hypercholesterolemia.[43] Intake of lycopene varies from region to region, i.e. 3.7 to 16.2 mg, 25.2 mg, 1.3 mg, 1.1 mg, 0.7 mg in USA, Canada, Germany, UK, and Finland, respectively.[44] Recommended levels for lycopene intake have been reported to be 5-7 mg for healthy human body[45] but, in case of a diseased person, increased amount of lycopene is required which ranges from 35-75 mg.[46]

Methods of extraction for carotenoids

Various Extraction techniques have been worked upon in order to extract bioactive and natural compounds from different parts (such as leaves, roots, fruits, peels, seed, bark etc.) of various plants. A range of conventional techniques which includes Hydrodistillation, organic solvent extraction and steam distillation.[47] Conventional Low pressure solvent extraction (LPSE) techniques depend on the selection of the solvents along with other conditions like temperature, stirring, solubility etc. Carotenoids are extremely sensitive to heat and light so they can easily undergo thermal degradation and photodegradation.[48a] Due to their high molecular weight they cannot be extracted by traditional techniques. LPSE techniques have generally been used conventionally for the extraction of carotenoids from various fruits and vegetables.

LPSE offers various disadvantages associated firstly with thermal degradation of bioactive compounds due to high temperature during vaporisation stage; secondly due to utilization of more quantity of organic solvents which are hazardous to environment and human health, so

a majority of organic solvents have been restricted for the extraction of bioactive compounds; thirdly it consumes more energy, time and processing labor cost.[47] US Environmental Protection Agency has given a list of 189 hazardous air pollutants in which n-hexane occupies number 1 position, [49] which reduces its usage levels from 5 mg/kg of product to 1 mg/kg.

Low pressure extraction (LPSE) (conventional methods)

LPSE depends upon the solubility of the extractable compound in particular solvents. Various LPSE techniques have been used for the extraction in research labs such as centrifugation, agitation, soxhlet, etc. Selection of the extraction methods is based on extraction conditions like temperature, pressure and shaking and solvent.[50] LPSE techniques are commonly used in fragrance and flavour industries as they are simple to execute. Several LPSE techniques used for carotenoids extraction are given below.

Agitation, Homogenization and Shaking

This is the traditional method used by extraction industries for the extraction of natural compounds. Agitation involves combination of an agitator to the container containing the raw material. In homogenization process, the solvent and the raw material are mixed and then leaving them in contact with each other while in case of shaking, agitation or perturbation is carried out in container containing the solvent and raw material. Liquid solvents makes the dispersion of the particles which enhances the rate of extraction of the components avoiding saturation in the immediate closeness of the surface of the solid to be extracted.[51] In extraction of the carotenoids, high temperature is avoided because degradation will occur, as in case of β-carotene.[48a] Thermal degradation of carotenoids results in the production of volatile and large non-volatile compounds such as beta-apo-13-carotenone, beta-apo-15-carotenal, beta-carote-5,6-epoxide, beta-carotene-5,8-epoxide, mutachrome, aurochrome etc.[48b,c] Agitation, homogenization and shaking process are used for extraction of a variety of

carotenoids, a lot of work have been done in past years on extraction using these conventional techniques, detailed process and the carotenoids extracted has been given in the **Table 3**.

Solvent Extraction

Other name for solvent extraction is liquid-liquid extraction in which extraction is based on the solubility of the compound to be extracted and involves two immiscible solvents like organic solvents and water. Carotenoids in certain solvents such as chloroform undergo photodegradation which produces carotenoid radical, carotenoid radical cation or free radical adduct.[48d] Carotenoids have been extracted by solvent extraction from the freeze-dried samples of pumpkin (0.1 g) using 2 ml of ethanol (95 ml/100 ml) which was vortexted for a duration of 1 min and repeatedly extracted with equal volume of hexane to get maximum yield[52,54]. Extraction of lycopene and other carotenoids from fermented cell mass of *Blakeslea trispora* NRRL 2895 and 2896 was done by using petroleum ether and acetone (1:1, v/v) , temperature (30±2 ^{o}C). The upper organic layer contains lycopene and other lipotropic carotenoids.[53] Solvent extraction have been used to extract total carotenoids extraction from rapeseed and the conditions of extraction viz. temperature, time and solvent to solid ratio was optimized.[55] Protocol for the extraction of carotenoids by liquid-liquid extraction has been shown in the **Fig. 4.**

Soxhlet Extraction

Soxhlet is the usual extraction technique which transcends the performance of other traditional techniques except for the extraction of thermolabile compounds which undergo thermal degradation due to high temperature [48b,c,56]. In this technique, thimble contains raw material which is joined to a flask containing the solvent for extraction. The extraction is carried out by vapours of the solvent. Soxhlet has widely been used for the extraction of carotenoids. Extraction of carotenoids from pink shrimp (*P. brasisiliensis and P. paulensis*) residue has been carried out by soxhlet using following solvents: n-hexane, ethanol, acetone,

isopropanol and isopropanol: hexane (50:50, v/v), all these solvents have been permitted as GRAS solvents and are generally used in food and flavour industry, astaxanthin yield was maximum in hexane: isopropanol followed by acetone, isopropanol, ethanol and hexane.[57] Carotenoids have been extracted from freeze-dried carrots using 50 mL hexane and was refluxed for 4h, 1832 μg β-carotene/g dry carrot was extracted by soxhlet extraction.[58] β-carotene was also extracted by soxhlet extraction from freeze-dried skin powder samples of Aloe vera using 100 ml petroleum ether for a duration of 8 hours.[59]

Centrifugation Extraction

Very rare extractions of natural compounds have been done using centrifugation technique. It is carried out by mixing of raw material and solvent in a container and is allowed for centrifugation which proceeds by filtration. Total carotenoids have been extracted by means of centrifugation from frozen apical tips of *Gracilaria tenuistipitata*.[60] Centrifugation process of extraction was used for carotenoids extraction from *Haematococcus pluvialis* and *Dunaliella salina*.[61]

Non-Conventional or Green Extraction Methods

These are green extraction methods as either these are free from hazardous organic solvents, or require reduced quantity of solvents and hence target compounds are free from hazardous solvents. These extraction methods are employed under controlled temperature or without the involvement of heat hence advantageous for extraction of thermolabile compounds preventing them from degradation. Short period of time is required for extraction of carotenoids. Carotenoids extracted by green extraction methods are obtained in high purity and yield with reduction in quantity or no solvents with negligible wastage (as dry solid waste remains after extraction can be reused for increasing fertility of soil and animal feeding as well. Ultrasound-Assisted Extraction (UAE), Microwave-Assisted Extraction (MAE), Pressurised Liquid Extraction, Pulsed Electric Field (PEF) Assisted Extraction, Enzymatic

Extraction, Supercritical CO_2 Extraction are environmentally friendly or green extraction techniques for carotenoids.

UAE

In UAE the sound waves of frequencies higher than 20 kHz which causes rupture of the cell wall and diffusion of the solvent inside the cell results in extraction of bioactive compounds.[50, 62] β-carotenes and other carotenoids have been extracted from carrot peels by means of UAE via intermittent radiation which enhances the yield of carotenoids without thermal degradation.[63, 64]

MAE

MAE is a novel and inexpensive technique which involves combination of microwave and conventional solvent technique in which microwaves are accounted for heating the solvents for the extraction of the carotenoids in short period of time, with reduction in solvent quantity.[65] β-carotene and other carotenoids have been extracted via MAE in which hexane was chosen as a solvent due to its lower dielectric constant so that microwave energy is absorbed at a lower level.[66] Lycopene was extracted quickly from tomato peels through MAE as the carotenoids are highly sensitive to heat and light so short duration of its extraction is advantageous for their extraction.[67] A variety of 30 solvent mixtures have been used to extract carotenoids from powder of paprika (*Capsicum annuum*) by means of MAE.[68a]

PLE & PEF-Assisted Extraction

Carotenoids have been extracted via PLE in last few years. The technique is somewhat similar to soxhlet extraction. But in PLE high pressure is applied which keeps the solvent below its boiling point (prevents degradation of thermolabile compounds) and allows diffusion of solvent in the sample and hence increases the solubility of the desired bioactive compounds in the solvent with reduced quantity of solvent. But nowadays mostly water is used as the solvent and so it keeps the environment free from hazardous organic solvents.[71a]

Carotenoids having antioxidant activity have been extracted from *Haematococcus pluvialis* microalgae via PLE.[69] Extractions of carotenoids and chlorophyll from *Chlorella vulgaris* were carried out by PLE.[70] GRAS solvent ethanol was used to extract β-carotene from carrot by PLE.[71b] PEF-assisted extraction technique has been used for the extraction of carotenoids using olive oil (as a substituent for organic solvent) from the carrot pulp which enhances the yield of total carotenoids and is also a green and sustainable technique.[71c]

Enzymatic extraction

Enzyme aided extraction has also been used for the extraction of carotenoids. Cellulase and pectinase enzymes have been used for the extraction of lycopene from tomatoes. The yield of lycopene is 132 μg/g (198%) and 108 μg/g (224%) in cellulose and pectinase treated samples, respectively.[72] Various proteolytic enzymes gave enhanced yield of carotenoids from shrimp head waste.[73] Pectinases and cellulases were obtained from fermented portions of *Penicillium oxalicum* and *Trichoderma reesei,* respectively.[74] Carotenoids have been extracted from orange peel, sweet potato and carrot by varying concentrations of cellulase and pectinase.[75] Combinations of enzyme and edible oil have also been used to extract carotenoids from orange flavedo.[76] Carotenoids extraction from marigold flower have been done via Enzyme-mediated solvent extraction.[77] In certain cases enzymatic cell disruption have been carried out by enzymatic complex from a combination of β-(1, 3-1, 4)-glucanase, xylanase, cellulase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, amylase and protease for the extraction of total carotenoids and effect of temperature and enzymatic complex concentration have been evaluated.[78] Enzymatic extraction have been implemented for the extraction of lycopene from tomato tissues,[79] tomato paste,[80] carotenoids from marigold flower[77,81] and chillies.[82] Enzyme-aided extraction gives better yield in comparison to other traditional methods of extraction and also no involvement of heat so thermolabile compounds can be extracted without degradation. [75,77]

SC-CO_2 Extraction

SFE is an advanced and green technique for the extraction of carotenoids. This technique uses GRAS solvents or in some cases no organic solvents for the extraction of bioactive compounds. It reduces time of extraction and is most important technique for thermolabile compounds such as carotenoids.[83a] In most of the extractions via SFE uses CO_2 as supercritical fluid (solvent) which is inexpensive, chemically inert, non-inflammable, easily available in high purity and recyclable. [83a,108] Due to lack of polarity as in case of CO_2, modifiers are used for increasing the extractability of the polar bioactive compounds.[83b] Addition of modifiers enhances the yield of the carotenoids which has been reflected in various extractions as in the extraction of lycopene from tomato where lycopene yield was enhanced by addition of vegetable oil as modifier.[84] Presence of modifier also increases the antioxidant activity of the extract[85] and effect of modifier also depends upon the bioactive compound to be extracted.[86] SFE is a best alternative for LPSE and other conventional techniques. On increasing the pressure, density of the solvent increases and on increasing the temperature vapour pressure of the solute will increase, both the conditions of temperature and pressure will increase solubility but suggested temperature for the carotenoid extraction is 40 ^oC in order to avoid thermal degradation[87] whereas other researchers have used temperature up to 70 ^oC for the extraction of the carotenoids which have shown better yields.[88] A lot of work have been done in this field. SC-CO_2 has been used to carry out extraction of lycopene and β-carotene from tomato paste waste where parameters were temperature (35, 45, 55 and 65 ^oC), pressure (200, 250 and 300 bar) addition of co solvent (5, 10 and 15% ethanol), time (1, 2 and 3 h) and flow rate of CO_2 (2, 4 and 8 kg/h).[83] *Trans* lycopene have been extracted from Portuguese tomato industrial waste in which 93% *trans* lycopene was obtained at (60 ^oC, 300 bar), flow rate of the solvent was 0.59 g/min and particle size was 0.36 mm.[89a,89b] Extraction of carotene from pressed palm oil has been

carried out by SC-CO_2 with following conditions: pressure (250 and 300 bar), temperature (45 and 55 ^oC) and flow rate being 1.2-2.0 g/min.[90] SC-CO_2 has been used to extract carotene from freeze-dried powder of the marine microalgae *Nannochloropsis gaditana* with following parameters: pressures ranging from 100, 200, 300, 400 and 500 bar with temperatures of 40, 50 and 60 ^oC.[91] Carotenoids from freeze-dried pulp of pitanga fruits (*Eugenia uniflora* L.) have been extracted via SC-CO_2, at temperatures of 40 and 60 ^oC with a range of pressures, 100, 150, 200, 250, 300, 350 and 400 bar. Total carotenoid content in extract was found to be 5474 µg/g with 66% lycopene and 32% rubixanthin.[92] Xanthophylls have also been extracted via SC-CO_2 from *Capsicum annuum*.[68b] A range of temperature from 40-70 ^oC and pressure of 250-350 bar have been used for carotenoids extraction via SFE (**Table 4**). SFE have widely been used for extraction of a variety of carotenoids in pure form with marvellous yields in short duration of time without their degradation and is an environmentally friendly green technique.

Saponification

After the extraction of carotenoids, next step prior to their analysis is the saponification to make separation, quantification and qualification of carotenoids simpler, which involves removal of undesirable lipids, fatty acids, chlorophylls and carotenoid esters are hydrolyzed. Carotenoids are mostly esterified by fatty acids in fruits and vegetables.[151] Extent of esterification depends upon the number of hydroxyl groups present in the structure of different xanthophylls. β-cryptoxanthin have one hydroxyl group so it can get to monoester while others like lutein and zeaxanthin having two hydroxyl groups are esterified to diester by fatty acids, so it becomes complicated and difficult to identify peaks in HPLC.[152] Saponification may lead to degradation or transformation of structures.[153,154] Studies have shown that recoveries for β-carotene and lutein were found to be higher after saponification as compared to those without saponification.[153] In case of table olives, β-carotene recovered

was found to be less after saponification[155,156] whereas in case of coriander there was loss of β-carotene by 20-30% and other carotenes by 50% as a result of saponification.[154] Saponification involves alkali treatment,[157] epoxycarotenoids are sensitive to alkaline treatments so saponification is not preferred if sample contains these pigments.[158] It has been reported that better yield was obtained when saponification was carried out with 0.02 M KOH in methanol as compared with higher concentrations.[159] Lutein yield was increased to near about twice when 2.5% KOH was used.[160] Time for which saponification has been carried out and the concentration of the alkaline solutions decides the yield of the carotenoids, for low fat content milder conditions are required and for higher fat content stronger conditions are required.[157] Saponification should be avoided in lower-lipid containing samples as in case of leafy vegetables, tomato, and carrot.[161] In case when gradient elution is used as in lipid corn, saponification is not required.[162] Saponification procedure is needed to detect presence of carotenal ester which may lead to difficulty during analysis of carotenoids. Saponification conditions have been mentioned in **Table 5**. as reported in literature.

Methods for the Analysis of Carotenoids

Several analytical techniques have been used by the researchers for the analysis of extracted carotenoids from various natural sources. Carotenoids have been analysed by using TLC, HPTLC, HPLC, NMR, FTIR**,** UPLC-MS and UV-Vis spectrophotometry.

TLC

TLC has been used since years to identify bioactive compounds in extraction labs. Carotenoids have been separated by TLC using 50% acetone in heptane (v/v) as mobile phase.[177] Carotenoids (astaxanthin, lutein, and β-carotene with R_f value being 0.20, 0.17 and 0.97 respectively) from fancy carp (Cyprinus carpio) serum have been identified through TLC using petroleum ether-diethyl ether-acetone (75:15:10, v/v/v) as mobile phase.[178] TLC

have been implemented to separate carotenoids in tissues of white storks (*Ciconia ciconia*) using petroleum ether:acetone:diethylamine, 10:4:1 (eluant A) hexane:acetone, 3:1 (eluant B) and benzene: ethyl acetate 1:1 (eluant C).[179,180] Solvent system methanol: acetone, 1:1, v/v has been used to separate dietary carotenoids in food supplements by TLC.[181] New methods have been devised to carry out analysis of carotenoids separated by TLC.

HPTLC

It is a chromatographic technique generally used for qualitative and quantitative analysis of the components in the extract obtained from natural sources.[182] Lutein, lycopene and β-carotene were evaluated by HPTLC in which plates were developed with methanol-dichloromethane (1:1, v/v) with 0.5% triethylamine (TEA), solvent system methanol-acetone (1:1, v/v) which was used with or without 0.5% TEA, 0.1% 2,6-di-tert-butyl-4-methylphenol (BHT) or 0.1% f 2-tert-butylhydroquinone (TBHQ).[181] This analytical technique has been used for identification and quantification of lycopene extracted from *Calendula officinalis*.[183]

HPLC

In reversed phase chromatography monomeric octyl (C_8) and octadecyl (C_{18}) are most commonly used stationary phases for its packing. Geometric isomers (*cis-trans*) are not resolved effectively using C_8 and C_{18} columns. These provide good resolution with molecules having short chains. C_{30} columns are used to isolate long chain molecules effectively, and they also give excellent separation for *cis-trans* isomers. C_{30} column gives profiles with longer chromatographic duration in comparison to traditional C_{18} columns.[184] C_{30} columns are used for less polar samples.[185] Carotenoids were first detected in oil of red orange using silica micro-HPLC normal-phase column and a reverse-phase monolithic C_{18} column in two dimensions.[186] Hence HPLC with reversed-phase C_8, C_{18} or C_{30} columns is the ideal for isolating carotenoids in samples extracted from various natural sources. HPLC has been

widely used for the quantitative and qualitative analysis of a range of carotenoids, which has been compiled in the **Table 6**.

NMR

Carotenoids have also been detected by using NMR, in which structure of each carotenoid is identified by splitting pattern of ^{1}H NMR and ^{13}C NMR. The spectra is found to be in the olefinic region which is due to conjugation of double bonds (5.8-7 ppm), as this is the region for identification of carotenoids via NMR. This method gives a complete practical analysis of carotenoids.[203] Carotenoids in tomato juice has been analysed by NMR, it gives chemical shift data according to the structure.[204]

FTIR

Elucidation of carotenoids structures by FTIR spectroscopy has not yet been explored much.[205] FTIR gives information about the nature of functional groups present in the molecule. In lycopene 3450 cm^{-1}, 2924 cm^{-1}, 2854 cm^{-1}, 1643 cm^{-1} and 1510 cm^{-1} were attributed to OH, CH_2 asymmetrical, CH_2 symmetrical, C=C of olefin and C=C, respectively.[206] Crocetin C=O (1664 cm^{-1}), C-O (1243 cm^{-1}), O-H (3400 cm^{-1}), C=C (1540 cm^{-1}), C-C (1166 cm^{-1}); for β-carotene C=C (1517 cm^{-1}), C-C(1160 cm^{-1}), C=O (1687), O-H (3372 cm^{-1}).[203,207] Further analysis of carotenoids through FTIR can be investigated to get better results.

UPLC-MS

Carotenoids have been separated by UPLC using gradient system with the mobile phases: ACN :MeOH (7:3, v/v) as solvent A and H_2O (100%).[208] Hydroxycarotenoids (such as β-cryptoxanthin, all-*trans*-zeaxanthin, all-*trans*-lutein) and β-carotene in nectarine and pumpkin have been identified via UPLC technique.[209] UPLC separation technique have been found to be efficient for separation of epoxycarotenoids (increased peak efficiently) over HPLC methods.[210] Polar carotenoids and β-carotene have been found to be retained for a longer

duration on HSST3 column as compared to BEH C_{18} column.[211] Carotenoids have generally been determined qualitatively and quantitatively through LC in combination with UV-Vis instruments, so later the carotenoids have been detected by using Mass Spectrometry (MS). It was found to be impractical to elucidate the molecular structures of various unidentified carotenoids in the analyte by means of UV and PDA , spectral interferences occurring in UV-Vis can be overpowered by using MS,[212] hence molecular structure of carotenoids are elucidated by using molecular mass and fragmentation patterns obtained via MS. Carotenoids having similar molecular mass can be differentiated on the basis of different fragmentation patterns.[213] Structurally-correlated molecules and their epoxidized forms can be differentiated by using HPLC/MS-MS.[214] Several methods for ionization of carotenoids have been used which includes: electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI), atmospheric pressure chemical ionization(APCI), atmospheric pressure photoionization (APPI) and atmospheric solids analysis probe (ASAP).[215] Samples can be analysed directly without preparation of sample or chromatographic separation via ASAP, Raman spectroscopy and MALDI-TOF-MS as well.[216] Hence carotenoids have been efficiently determined by MALDI-TOF-MS.[217] Carotenoid analysis by using FAB-MS have been carried out very rarely.[218] Rearrangement and degradation of the structures of carotenoids can be reduced by FAB ionization. Carotenoid analysis is mostly done by using API which is coupled to MS and has substituted FAB ionization method.[219] Carotenoids in spinach leaves have been detected by ASAP.[220] LC coupled with ESI and APCI have been found to be the most used and APCI has been found to be an excellent ionization technique for non-polar and lipophilic pigments (carotenoids), whereas for more polar compounds ESI technique for ionization is mostly used.[221] MS and NMR are important for determination of carotenoid structures, which are also used in combination with other separation techniques, namely; as Liquid chromatography-nuclear

magnetic resonance (LC-NMR), Gas chromatography-mass spectrometry (GC-MS), Liquid chromatography-mass spectrometry (LC-MS), and Capillary electrophoresis–mass spectrometry (CE-MS).[222] Many studies have been done on the analysis of carotenoids and are still under way due to high demand and importance of carotenoids in food industry, pharmaceuticals and also as neutraceuticals for human beings.

UV-Vis spectrophotometry

UV-Vis spectroscopy refers to absorption or reflectance spectroscopy in the UV-spectral region. It uses light in visible, near-UV and near-IR region as well. Molecules undergo electronic transitions in electromagnetic spectrum. This technique is vice-versa of fluorescence spectroscopy. Molecules containing π-electrons gets excited to higher energy levels by absorbing energy from UV- light, those which gets excited easily, absorbs light with longer wavelength. β-carotene and lycopene absorb light and have λ_{max} values of 470 and 502 nm respectively.[223] UV-spectrophotometry has been implemented in many studies to detect carotenoids in the extracted samples. In one study, for the detection of β-carotene, UV spectrum was scanned in the range from 200-800 at λ_{max} = 461 nm.[224] In UV-spectra of all-*trans*, 7-*cis* and 15-*cis* forms of β-carotene have found to give λ_{max} at 456 nm and 484 nm.[225]

Extraction and Estimation of Carotenoid in human plasma

The most common solvent employed for plasma extraction of carotenoids is n-hexane[226,227], or a mixture of n-hexane and other solvents (e.g., hexane/ether[228], hexane/ethanol/acetone/toluene (HEAT)[229,230], hexane/dichloromethane[231] and hexane/chloroform[232]). The suitability of n-hexane for carotenoid extraction was confirmed in a previous review article that high- lighted nine studies between 1990 and 2002 in which n-hexane was used as the main extraction solvent. For carotenoid extraction in serum, n-hexane was the most commonly applied solvent.[233-236] Hsu et al.[215] used a mixture of hexane and ethyl acetate, while heptane was the solvent selected by Connolly et al. and Matsubara et al.

in their studies.[237,238] Tetrahydrofuran (THF) was used for carotenoid extraction by Ferreiro-Vera et al.[239] Most studies with human samples have used butylhydroxytoluene (BHT) to prevent carotenoids from oxidation and echinone as internal standard. Ethanol was also used to precipitate proteins in serum and plasma, since these proteins may cause clogging of the standards in the HPLC columns after a few injections and interfere with chromatographic separation. A combination of protein precipitation by organic solvents, salts or acids, followed by centrifugation, is the most widely applied technique for protein removal from blood-derived materials.[240] Most of the studies follow these steps; Deproteination, extraction and vortex, centrifugation and concentration and re-dissolution. **Table 7** gives a brief summary for extraction and analysis of carotenoids in human plasma.

Biological activities of carotenoids in humans

Carotenes, xanthophylls and their derivatives have been found to play an important role in all living beings (**Fig. 5**).

Provitamin A activity

At present, pro vitamin A carotenoids (α-carotene, β-carotene and β-cryptoxanthin) play a vital role in humans to provide Vitamin A source for the growth, development and proper functioning of immune system and vision. Carotenoids having β-ring as end groups like β-cryptoxanthin, β-carotene and zeaxanthin shows pro vitamin A activity. It was being reported years ago that the intake of these carotenoids in diet were found to be cleaved by an intestinal 15-15'-dioxygenase to give retinal, a key molecule involved in vision.[244] In recent years mammalian genes were cloned. It was found that mammalian β-carotene 15-15'-dioxygenase (beta-CD) is a key enzyme for the development and metabolism that controls the formation of vitamin A from plant-derived precursors.[245] Several biochemical studies were done.[246] Two carotenoid cleavage enzymes have been detected, firstly the central cleavage enzyme 15,15'-carotenoid monooxygenase (CMO I) and secondly eccentric cleavage enzyme carotenoid

monooxygenase-II(CMO II).[245-248] CMO I causes the cleavage of the provitamin A carotenoids such as α-carotene, β-carotene and β-cryptoxanthin to vitamin A, and CMO II which cleaves β-carotene at the 9,10 (or 9',10') causes chain shortening of β-apocarotenals and oxidation of retinal to retinoic acid.[249] The recombinant human enzyme, named BCO, is produced highly in the liver and the digestive system, converts provitamin A carotenoids obtained from plasma into retinal in the vicinity.[248] β-ring carotenoids are inequitably cleaved to give a variety of apocarotenoids by cleaving double bond at 9', 10' position in order to form β-ionone and β-apo-10'-carotenal which acts as precursor of retinoic acid. Lycopene can also be cleaved by 9', 10'-dioxygenase.[250] The recommended amounts of the provitamin A carotenoids must be taken in diet by humans in order to avoid vitamin A deficiency. Researchers play a very important role in order to develop such food supplements from natural sources efficiently.

Role in the prevention of diseases

Diet including more intake of carotenoids results in reduced threat of various chronic diseases such as cancer and coronary heart diseases.[251] In developing countries a major portion of the population lacks in proper diet (including fruits and vegetable) and hence suffering from various diseases. Carotenoids such as lycopene, lutein, β-carotene and zeaxanthin have been studied for their roles in humans health as antioxidants, immune-enhancement, decreased risk of cataract and prevention of cancer.[252,253] Carotenoids having antioxidant activities reduces the risk of several chronic diseases.[254] In certain cases, as in case of smokers, high doses of carotenoids leads to harmful effects due to their pro-oxidant properties.[255] Role of β-carotene on moderative and heavy oxidative stress is shown in **Fig. 6**. Carotenoid loses its antioxidant capacity by increase in pO_2 (50-100 mmHg) and function as a prooxidant at pO_2 varying from 100-760 mmHg.[256] Apoptosis have been induced in T-lymphocyte cell lines by carotenoids [257] and genome stability can be protected.[258] Majority of the diseases can be

prevented by including recommended amount of individual carotenoids (fruits and vegetables) in the diet. Studies have shown that hypertensive patients have lower level of lycopene in plasma.[259] Lower levels of lycopene have been found in semen in men with antibody-mediated infertility.[260] In an study 8 mg/day of lycopene was consumed by infertile men for a period of 12 months showed a significant increase in levels of lycopene in serum along with enhancement in sperm motility, sperm motility index, sperm morphology and efficient sperm concentration. 36% successful pregnancies were reported by lycopene treatment. Further studies are in progress for the valuable function of lycopene related to infertility in male. Researchers have reviewed and presented the role of lycopene in neurodegenerative diseases including Alzheimers's disease[261], Parkinson's disease and vascular dementia patients.[262] Austrian Stroke Prevention study have shown that lower levels of lycopene in serum increases the probability of microangiopathy.[236] Lycopene was also known to prevent Amyotrophic Lateral Sclerosis (ALS) syndrome in humans.[264] Lycopene also have preventive function for emphysema. Role of lycopene needs to be investigated further for various other human diseases including ocular and dermatological disorders, diabetes, rheumatoid arthritis, periodontal diseases and inflammatory disorders.[265]

Carotenoids and eye health

Two xanthophylls present in eye lens (retina, plasma and other tissues) are lutein and zeaxanthin.[266] Zeaxanthin is concentrated in the central macula and lutein being dispersed all over the retina.[267,268] Macular pigment density and lens density are inversely related to each other, indicating that the macular pigments may serve as the indicator for xanthophylls in the lens.[269] The blue light filtering efficiency of the carotenoids in liposomes have been reported to be maximum in lutein followed by zeaxanthin , β-carotene and lycopene.[270] Oxidation products of lutein and zeaxanthin have been identified in human retina[271,272], lens[272] and other ocular tissues[272] which suggests antioxidant function of xanthophylls present in human

eye. Lutein and zeaxanthin have been found to be present in the outer segment membrane of human rod where the concentration of long-chain polyunsaturated fatty acids and receptiveness to oxidation is maximum.[273] Lutein and zeaxanthin plays a vital role to protect photoreceptor cells from light generated oxygen radicals and thus they are crucial in prevention of advanced macular degeneration (AMD).[272,274] Deficiency of vitamin A causes disease xerophthalmia, intake of provitamin A carotenoids prevents similar ocular issues.[275] WHO in 2010 has given the main reasons for visual impairment as: cataracts (33%), diabetic retinopathy (1%), uncorrected refractive errors (43%), glaucoma (2%), age-related macular degeneration (AMD) (1%), and around 18% comes under unidentified type.[276] AMD and Cataract are mainly caused due to aging, oxidative stress (access of oxygen radical species), adverse environmental factors, exposure to ultraviolet light (380 nm) and blue light (400-500 nm), high polyunsaturated fatty acids and smoking.[277] Supplements of lutein for a duration of 140 days amplified lutein level in serum.[278] The addition of lycopene to cell cultures in vitro studies on human lens epithelial cells have shown to prevent vacuolization in HLEC.[279] It has been found that increased supplements of β-carotene in combination with diet having high proportion of lutein and zeaxanthin lowers the concentration of lutein and zeaxanthin in retina, plasma and tissue.[280] At present, clinical trial on the Age-related Eye Disease Study 2 (AREDS2) is under progress to assess the effects of lutein and zeaxanthin supplements on the development of advanced AMD . The Risk of age-related macular degeneration (AMD) can be reduced by including carotenoids containing high amount of lutein and zeaxanthin in the diet. There is a scope for researchers to develop high xanthophyll supplements (lutein and zeaxanthin) to overcome eye related diseases among poor population which lacks in proper diet.

Cardiovascular diseases (CVD)

Protective efficiency of carotenoids for CVD has been studied and reported.[281] Lycopene play a vital role for the protection from CVD. Higher levels of lycopene in adipose tissue prevent myocardial infarction in non-smokers.[282] Hypercholesterolemic patients consuming 15 mg/day of lycopene have shown decreased systolic and diastolic blood pressure.[283] Intake of processed tomato products has found to decrease lipoprotein susceptibility to oxidative damage.[284] Lower level of lycopene in serum increases the probability of atherosclerotic vascular actions in aged men.[285] β-carotene have little or no role to play in the reduction of mortality rates by CVD whereas it increases the risk of lung cancer.[286] Studies have shown that lycopene prevents the oxidation of native LDL (low-density lipoprotein) along with inhibition of cholesterol formation.[287,288] Hence, carotenoid containing fruits and vegetables should be included in diet to avoid cardiovascular diseases.

Osteoporosis

Oxidative stress caused by Reactive Oxygen Species (ROS) and antioxidant carotenoids leads to pathogenesis of osteoporosis.[289] Oxidative stress plays a vital role in both osteoclasts[290] and osteoblasts.[291] Lycopene have shown stimulatory effect on the multiplication of the cells, demarcation indicator alkaline phosphatise of osteoblasts ,[292] along with its inhibitory effects on osteoclast formation and resorption.[293,294] Clinical studies have been conducted which have shown that lycopene in serum is directly related to reduced risk of osteoporosis in postmenopausal women.[295] Reduced plasma antioxidants were reported in women suffering from osteoporosis.[296] There have been found a correlation between decrease in bone density with increase in oxidative stress biomarker 8-iso-prostaglandin F alpha (8-iso-PGFα).[297]

Role of carotenoids in Cancer

Various studies have been done extensively on the function of lycopene in the prevention of prostate cancer. Intake of tomatoes (lycopene) is inversely related to the reduced probability

of prostate cancer.[298] Around 72 studies were conducted in 1999 which show that increase in the consumption of lycopene and its high concentration in serum have reduced risk of various cancers (breast, liver, cervical, prostrate, ovarian and other organ parts).[299-301] Studies have been carried out to understand the role of antioxidant and oxidative stress in patients suffering from prostate cancer.[302] Various studies on animals have shown that consumption of lycopene in the diet decreases the expansion of spontaneous and transplanted tumours.[303] In vivo and in vitro studies have shown that capsanthin, capsanthin diester and capsorubin diester extracted from *Capsicum annuum* found to acquire anti-tumour activity.[304] Fucoxanthin plays a vital role as anticarcenogenic, as shown in the **Fig. 7**. Cancer is the foremost cause for the mortality in the world. Many studies have to be done with lycopene on various cancers that will be advantageous to human health.

Lung cancer

In 1960s and 1970s studies were conducted and were found that the diet containing fruits and vegetables reduces the risk of lung and other cancers.[305] It was hypothesised that β-carotene may be responsible for this but later it was reported that β-carotene have no role to play in the prevention of lung cancer,[306] rather it was found that β-carotene acted as prooxidant not as antioxidant in case of extensive male smokers.[307] In a study over 27,000 Finnish men smokers it was found that threat of lung cancer decreases with the consumption of lycopene, β-cryptoxanthin, lutein, zeaxanthin and total carotenoids.[308] One more study on over 58,000 Dutch males was conducted and was found that consumption of β-cryptoxanthin, lutein and zeaxanthin in diet reduces the possibility of lung cancer.[309] Smoking has been found to be the main cause for the lung cancer. Fruits and vegetables having variety of carotenoids must be included in diet to reduce risk of lung cancer.[310,311]

Breast cancer

Various studies were conducted and it was found that increase in the consumption of β-carotene and lycopene in the diet lowers the possibility of breast cancer.[312-314]

Prostate cancer

Among various cancers, studies on prostate cancer have been done extensively. Diets having high levels of lycopene along with lipid lowers down the possibility of prostate cancer.[298] Lycopene levels in serum and prostrate have found to be enhanced by intake of tomato sauce.[315] Consumption of tomatoes and its products increases lycopene levels in plasma hence decreases the threat of prostate cancer[316] but it was not confirmed that lycopene was alone responsible for this activity because tomato also contain other carotenoids or phytochemicals which may be responsible for this effect.[317] Prostate cancer patients are advised to include tomatoes in their diet because it lowers down the leukocyte oxidative DNA damage and oxidative damage of prostrate tissues.[318] In vitro studies have shown that lycopene decreases the multiplication of prostrate LNCaP cancer cells.[319] Studies were conducted over 58,000 Dutch males and were found that consumption of lycopene in diet has no role to play in the reduction of possibility of prostate cancer.[320] There is a considerable need for the studies on relation of lycopene enriched diet with the prevention of prostate cancer.[321]

Colorectal cancer

Intake of lutein in diet also reduces the risk of colon cancer in humans.[322] β-carotene consumption is inversely related to the reduced risk of colorectal cancer.[323] Other carotenoids such as β-cryptoxanthin, lycopene and zeaxanthin were found to have no role to play in this regard. Recently a study have been carried out which is contradictory with earlier ones showing that consumption of carotenoids have no relation with colorectal cancer.[324]

Ovarian cancer

Consumption of diet with increased amounts of lutein along with zeaxanthin decreases the probability of ovarian cancer by about 40%.[325,326] Uptake of lutein have no effect on the bladder cancer.[327]

Role of carotenoids in photosensitivity skin disorders

The roles of carotenoids in the treatment of erythema have been studied.[328] Keratinocytes present in the epidermis absorbs Ultraviolet-B (UV-B) radiations (280-315 nm) which leads to erythema (sunburn).[329] Studies have been carried out and contradictory results are obtained. Garmyn et al. have shown that β-carotene do not play any role in protection from erythema,[330] whereas Gollnick et al. have found that β-carotene suppresses the progression of erythema.[331] It was investigated that β-carotene along with vitamin E is more efficient because of their antioxidant properties.[332] In a study it has been found that intake of tomato paste decreases erythema considerably.[333] In vitro studies have shown that UV-induced lipid peroxidation of human skin fibroblast cells have been decreased by adding up of carotenoids such as β-carotene, lycopene and lutein[334] where as phytoene and phytofluene have protective role in photo-induced skin disorders.[335] Photocarcinogenesis (skin cancer) is caused due to the interaction of UV radiation with nucleic acids. Absorption of Ultraviolet-A (UV-A) radiation (315-400 nm) induces the formation of vitamin D in humans, but it may lead to photoageing by production of ROS.[336] Carotenoids have positive effect on photoageing (dryness, wrinkling, hyperpigmentation, telangiectasia and skin inelasticity).[337] Carotenoids are found to be shielding agents in skin photo-induced disorders because they are exceptional quenchers of singlet molecular oxygen and scavenges ROS very well. Protoporphyria, an endogenous photosensitizers are found to be effectively scavenged in its excited triplet states by β-carotene and canthaxanthin.[338] β-carotene and canthaxanthin have been found to be useful for curing polymorphous light eruption.[339] Studies have found to show positive effect

of consumption of lutein on basal cell carcinoma.[340] Many studies have yet not confirmed that whether the carotenoids intake is related to the decrease in the risk of non-melanoma photocarcinogenesis.[341] Further research needs to be done to validate the beneficial role of carotenoids in the photo-induced disorders.

Role of carotenoids as antioxidants

Carotenoids are very crucial for plants and humans as they protect from photooxidative damage and are excellent antioxidants quenching singlet molecular oxygen and peroxyl radicals.[342] During aerobic metabolism and pathological processes reactive species of oxygen and nitrogen are produced which damage DNA, lipids or proteins.[343] Peroxy radicals and singlet oxygen molecules produced electronically by excited sensitizer molecules, these sensitizer molecules are deactivated by carotenoids effectively.[344] Carotenoids scavenges singlet oxygen molecule by physical and chemical scavenging.[342] Scavenging by β-carotene and other carotenoids depend on degree of conjugation.[345] Peroxyl radicals are quenched by carotenoids particularly at low oxygen tension and prevent lipid peroxidation.[346] β-carotene along with vitamins E, C scavenges reactive nitrogen species through cooperative synergistic effects as compared to antioxidant only.[347] In biological membranes, β-carotene and α-tocopherol together work as radical-scavenging antioxidants.[348] Zeaxanthin and α-tocopherol are found to have cooperative effect against photosensitized lipid peroxidation.[349] They show cooperative effect among hydrophilic ascorbic acid (vitamin C), hydrophobic α-tocopherol (vitamin E) and β-carotene (provitamin A) which protects cell from reactive nitrogen species (RNS).[347] Carotenoids have been found to be excellent quenching agents for singlet oxygen molecule and scavenges reactive oxygen species in cellular lipid bilayers.[350] Xanthophylls, α-carotene, β-carotene, cryptoxanthin and zeaxanthin, present in serum and tissues of human have been found to be efficient singlet oxygen quenchers.[351]

Carotenoids in age-related macular degeneration

Carotenoids play a crucial role in the protection against AMD, which has been found to be a main reason for irreparable blindness in people over age of 65 years.[352] There is a yellow spot called as macula lutea present in the centre of the retina which maintains sharp central vision. Macula has only two carotenoids: lutein and zeaxanthin which are responsible for the colour.[353] Lutein and zeaxanthin present in retina are inter-convertible biochemically in the macula.[354, 355] Xanthophyll-binding proteins are involved in intake, transfer and metabolism of xanthophylls present in retina. Zeaxanthin-binding protein present in human macula is an isoform of glutathione S-transferase (GSTP 1).[356] Xanthophyll pigments present in retina have protective nature. Those who are suffering from AMD found to have lower levels of lutein and zeaxanthin in retina.[357] Atrophic AMD patients are advised to intake lutein along with other nutrients.[358] Occular tissues are protected from photooxidation degradation firstly by filtering blue light and secondly by quenching singlet oxygen molecule and scavenging ROS.[352]

Role of carotenoids in immunology

Carotenoids play a crucial role in enhancing the immune system of humans as in case of kids having lack of vitamin A in their diet suffers from various contagious diseases due to weak immune response. Studies have shown an increase in natural killer cell property activity by regular consumption of β-carotene for long duration in men of 65 to 86 years of age.[359] One pathway have been given in which immune cells operates more effectively due to β-carotene.[360] Enhancement in the lymphocyte response to mitogens was observed due to carotenoids.[361] But it has yet not been confirmed whether the carotenoids are specifically involved in strengthening immunity, hence there is a need to explore it further.

Interactions of carotenoids with foods

Alcohol

Clinical trials show that intake of high levels of β-carotene leads to increased probability of lung cancer which is further enhanced in the high alcohol consumers.[362] Those consuming alcohol regularly suppress the transformation of β-carotene into retinol.[363]

Olestra

Olestra is a fat substitute sucrose polyester that does not adds calories, fats and cholesterol to the food products, it is also known by another name as Olean. Studies have shown that intake of 18 g olestra per day for a period of 3 weeks found to decrease levels of carotenoid in serum by 27%, [364] while those consuming 2g/day olestra found to have reduced carotenoid levels in serum by 15%.[365] In one study it was found that 9.7% carotenoids levels in serum was reduced on consumption of about 4.4 g olestra weekly.[366]

Foods Containing Plant Sterol-or Stanol

Consumption of plant sterol-containing spreads, reduces the concentration of α-carotene, β-carotene and lycopene in plasma by 10-20% .[367,368] It was found that those consuming plant sterol-or stanol-containing margarines to intake additional supplement of carotenoids rich in fruits and vegetables regularly, inhibits the reduction of carotenoids levels in plasma.[369]

Carotenoid-Carotenoid Interactions

Plasma lutein level decreases when 12 mg or 30 mg β-carotene was taken for a period of 6 weeks regulary.[370] Several investigations have been done on interaction between lutein and β-carotene. A study to understand the effect of β-carotene on lutein, a combination of pure crystalline lutein and β-carotene were incorporated, which showed a decrease in the serum area under the curve value for lutein due to β-carotene.[371] Studies conducted have shown that absorption of lutein and β-carotene are correlated to each other.[372] Further studies must be done in order to explore the interaction between various other carotenoids.

Undesirable effects of carotenoids

Lycopenodermia

Consumption of diet containing high levels of lycopene (tomatoes) causes deep orange patches on the skin. Intensity of colour in case of lycopene is high so it leads to lycopenodermia at lower levels in comparison to other carotenoids.[373]

Carotenodermia

It results when levels of carotene in plasma increases which leads to yellow patches on the skin. Consumption of over 30 mg/day of β-carotene for a long duration causes this condition.[374] Carotenodermia can be cured by reducing or completely cutting down the intake of carotene in the diet.

Other harmful effects

High intake of carotene causes higher risk of prostate cancer, leukopenia, allergic reactions, reproductive disorder, and retinopathy.[374-376] The recommended amounts of carotenoids must be taken in diet for its beneficial effects and avoiding harmful effects.

Conclusion:

Carotenoids being photosensitive are liable to various reactions such as oxidation and isomerisation on exposure to heat, light, acids and oxygen. Hence great care must be taken in order to reduce errors during extraction, qualitative and quantitative analysis of a variety of naturally occurring carotenoids.

Among all extraction methods employed for carotenoids till now, Supercritical Carbon-dioxide extraction have been found to be the best for extraction of carotenoids under optimized conditions leading to best yield, high purity and an environmental friendly technique as well. Saponification process needs to be employed prior to analysis of the extracts to remove undesired components such as lipids which interfere during the analysis of carotenoids. UPLC-MS have been found to be of great importance in the analysis of a variety

of carotenoids. There is a lot of scope for the extraction and analysis of novel carotenoids from unexplored natural sources and their valuable roles in the humans and animals which is of high need in developing countries where major portion of population are lacking proper diet.

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FIGURE CAPTIONS:

Fig. 1: Classification of naturally occurring carotenoids on the basis of their chemical nature

Fig. 2: Mechanism based biosynthesis of carotenoids involving epoxidation and de-epoxidation of xanthophylls along with cellular enzymes

Fig. 3: Schematic diagram of carotenoid degradation via oxidation and their break down into lower molecular compounds

Fig. 4: Schematic diagram to show the liquid-liquid extraction of carotenoids

Fig. 5: Roles of various carotenes, xanthophylls and their derivatives in nutrition, human health, behaviour, reproduction and survival, hormones and signalling molecules as well as in photosynthesis and photoprotection

Fig. 6: Effect of β-Carotene on oxidative stress indicating β-Carotene as antioxidant in non-smokers and as pro-oxidant in smokers

Fig. 7: Schematic diagram showing Anti-carcinogenic effect of fucoxanthin via apoptosis, cell cycle arrest and metastasis suppression

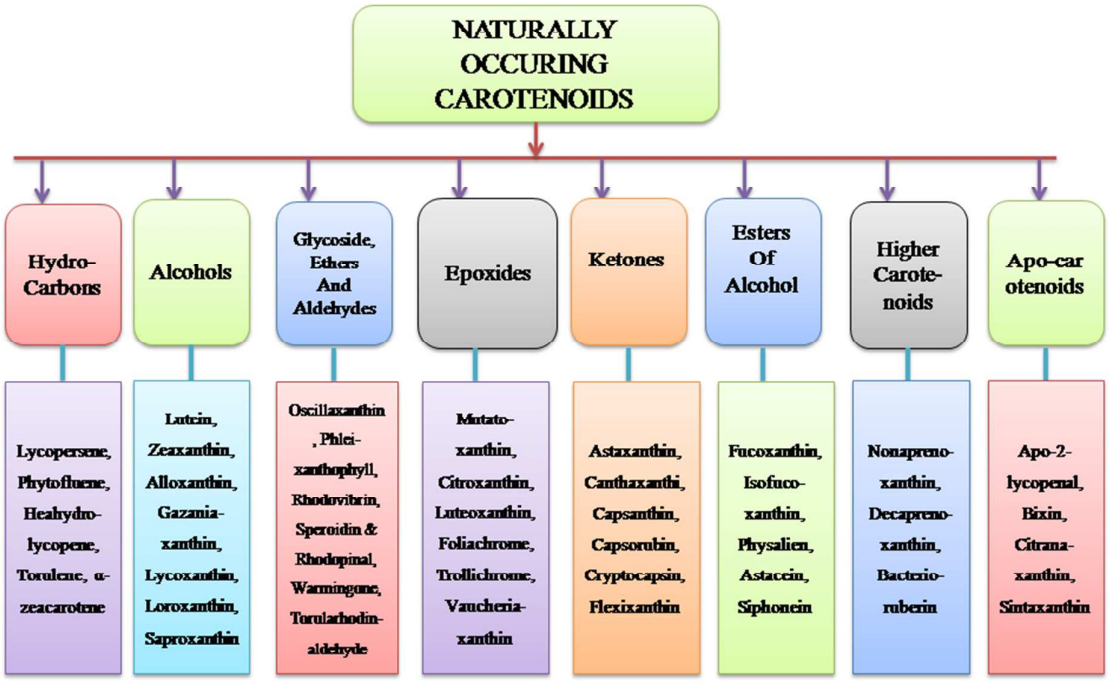


Fig. 1

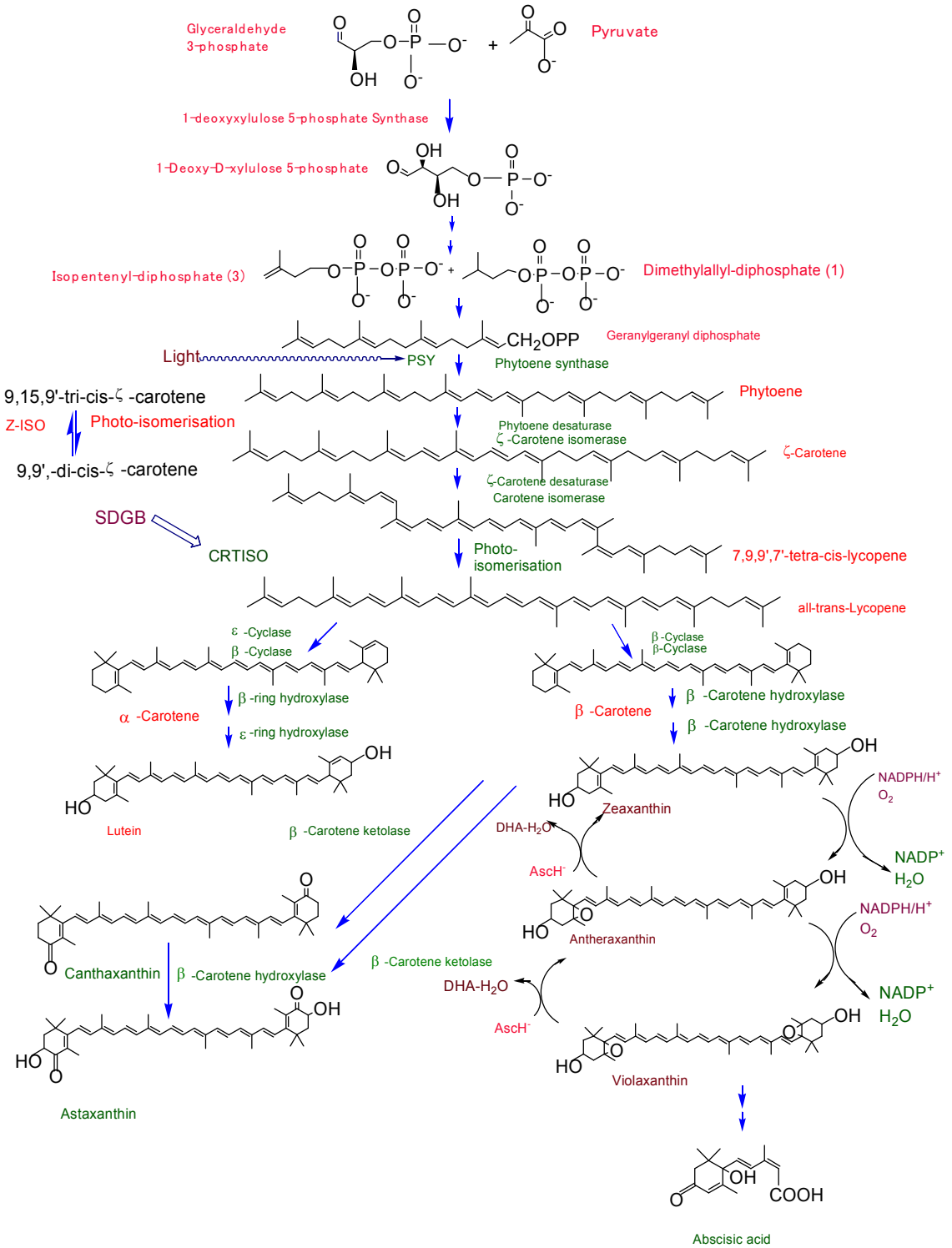


Fig. 2

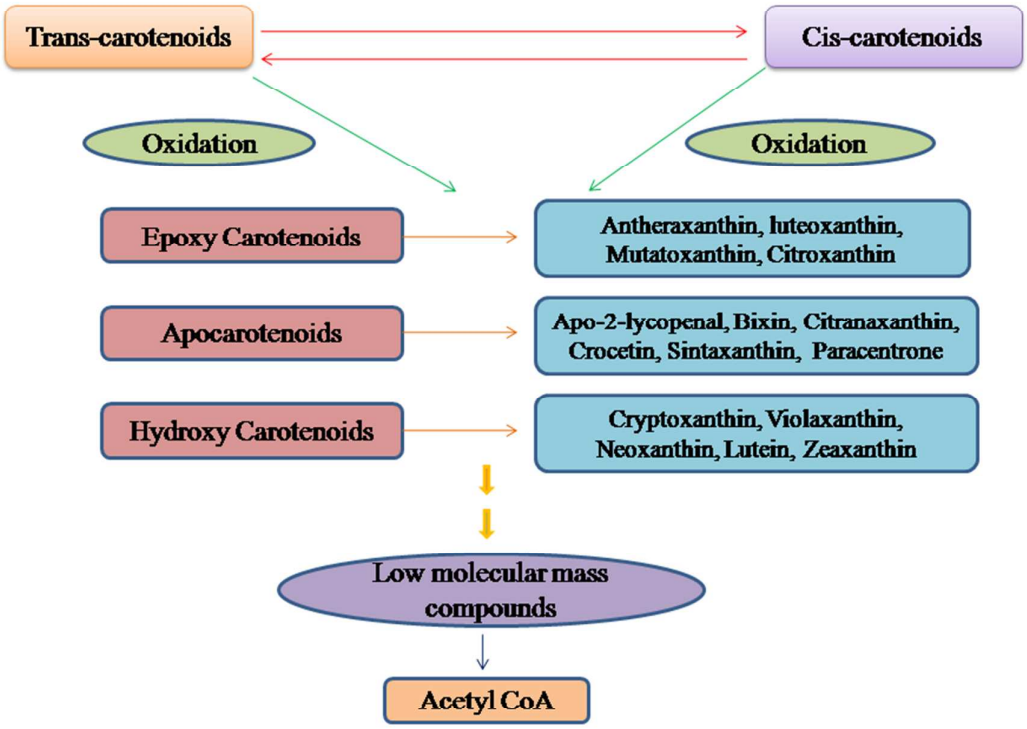


Fig. 3

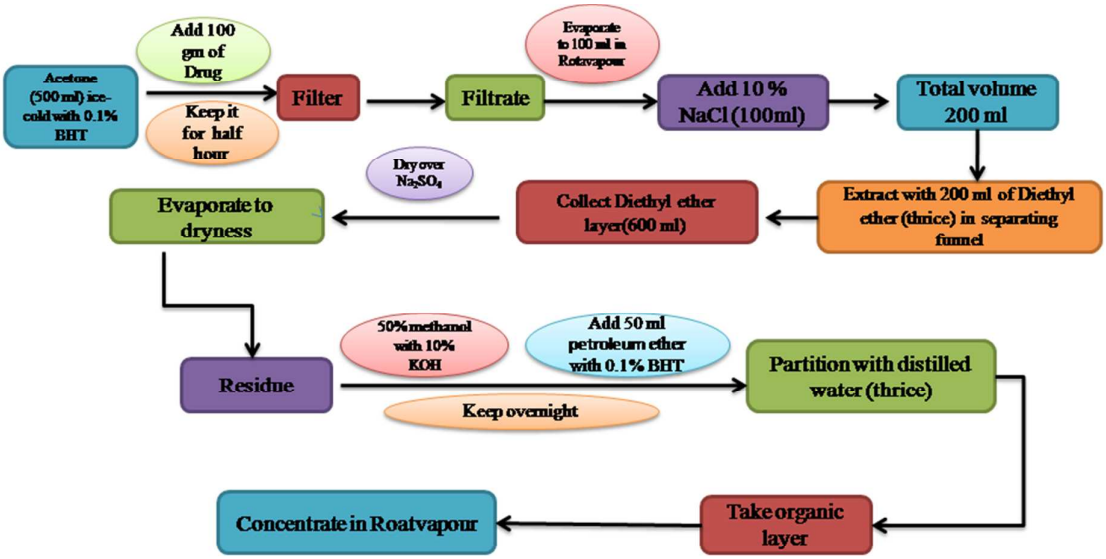


Fig. 4

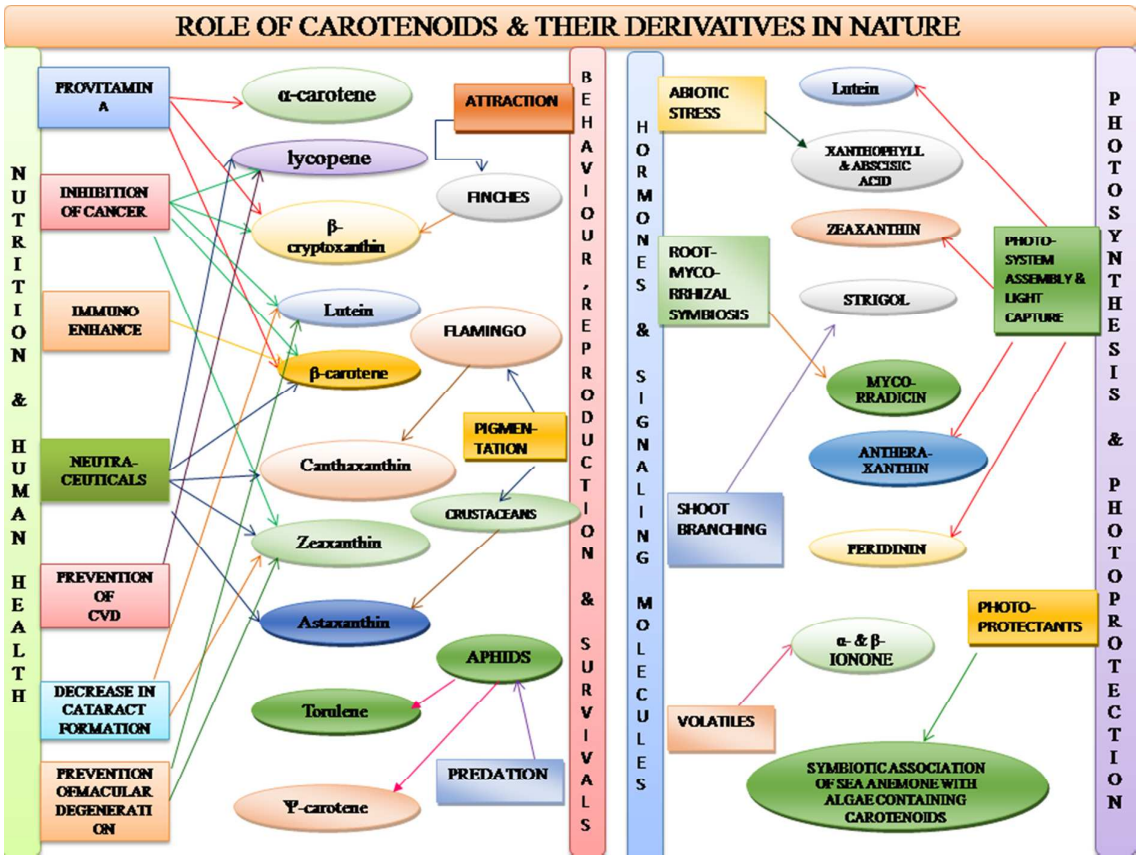


Fig. 5

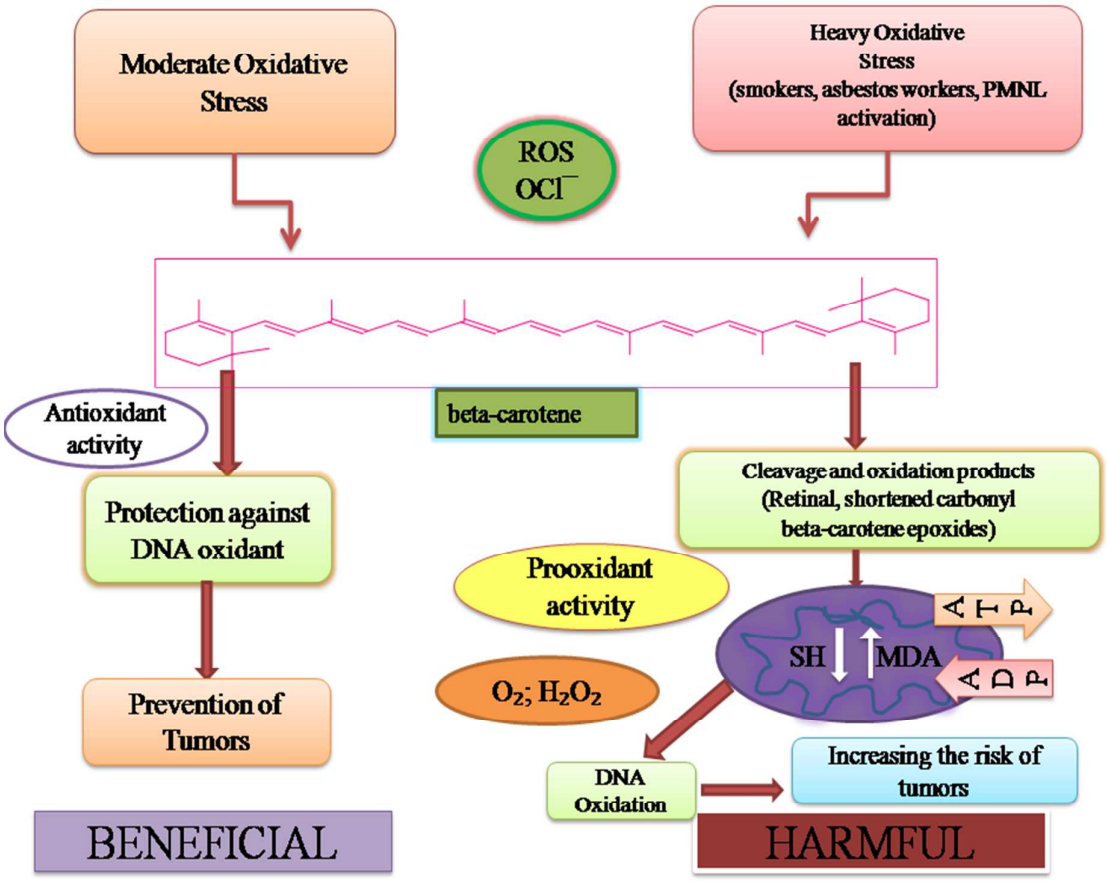


Fig .6

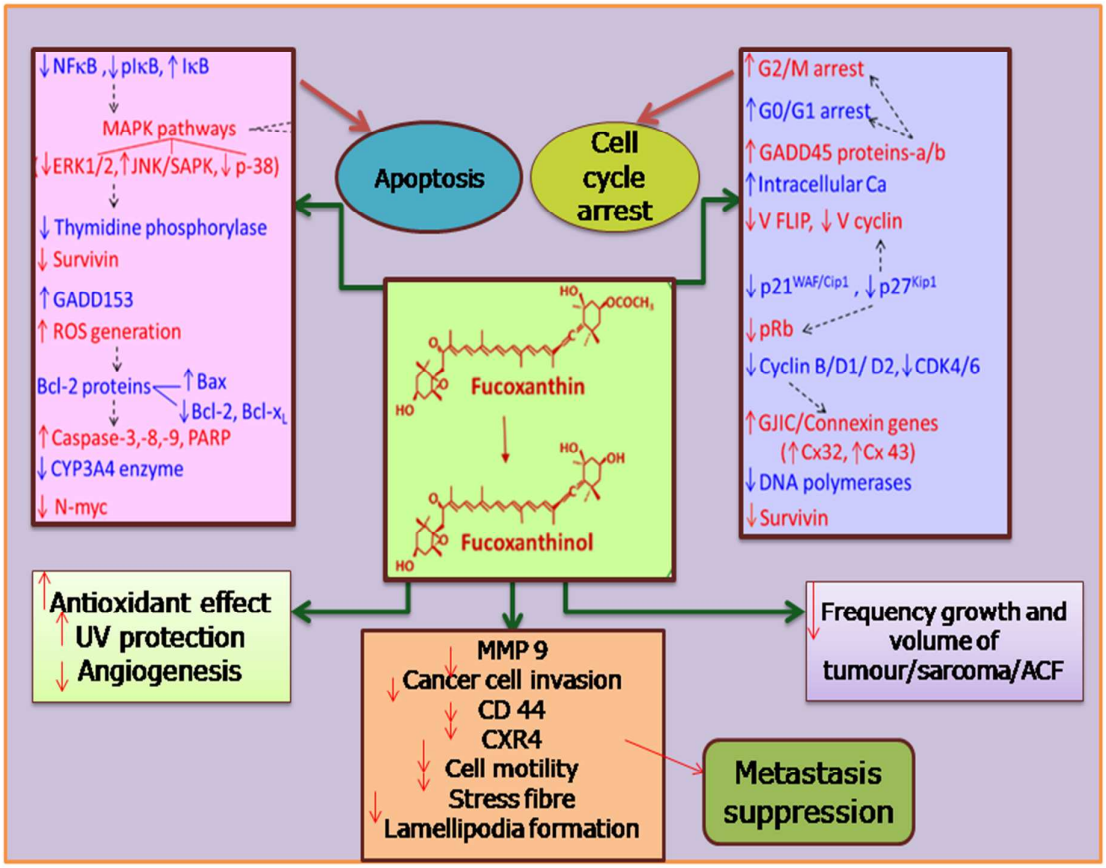


Fig. 7

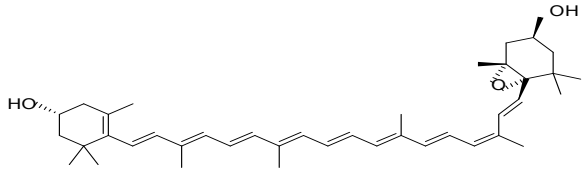
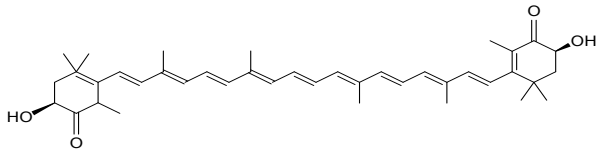
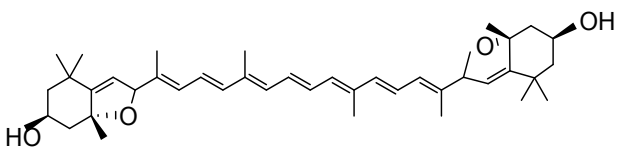
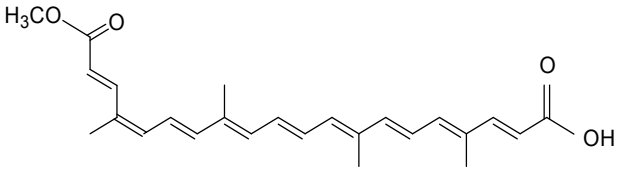
Table 1. Source with their content, functions, and marketed formulations of different carotenoids

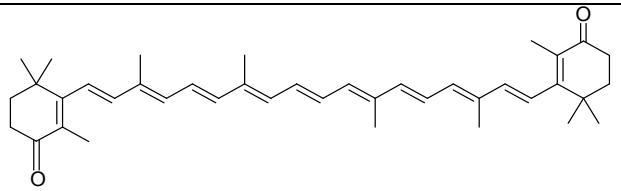
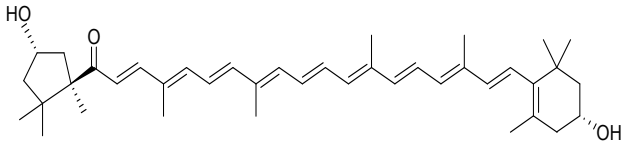
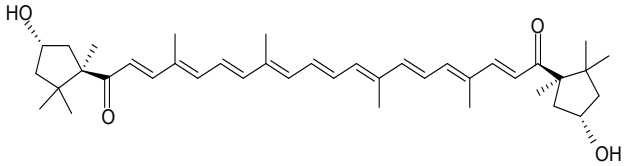
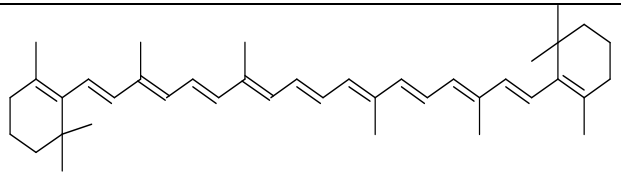
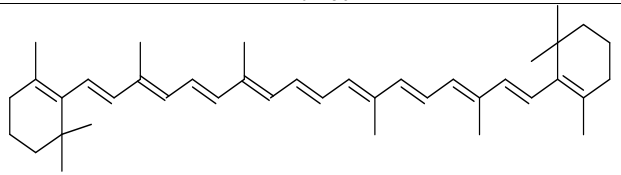
Carotenoids	Dietary Sources-µg per 100 grams	Function/effects[references]	Market formulation available
α-Carotene	Pumpkin canned-28215,Carrots-23851,vegetables mixed (corn, lima beans,peas,green beans,carrots)-14247,peas-7121,squash,cooked-5650,peppers,sweet,red-4414,peppers,hotchilli,red-4247,pumpkin,raw-3962,pumpkin,raw-3962squash,raw-3707,pumpkin,cooked-3480,balsam-pear,raw-2176,collards,raw-1587,fiddlehead ferns,raw-1535,grape leaves,raw-1352,spices,chilli powder-1331,plantains,raw-718,tomatoes,green,raw-678,nopales,raw-600,chard swiss,raw-474,kumquats,raw-437,coriander leaves,raw-313,pepper,banana,raw-289	Provitamin A activity; Antioxidant,lowers risk of lung cancer/ Immune-enhancement; stimulate cell to cell communication; Decreases risk of some cancers.[17, 245, 250]	Alpha GPC Capsules, Mcobacin,
β-Carotene	Green leafy vegetables and orange and yellow fruits and vegetables (carrots, apricots, Spinach, sweet potatoes, pumpkin, pepper, kale, cantaloupe)/Spices (Paprika, Cayenne, Chili)-26162; Sun Dried Chili Peppers-14844; Sweet Potato Chips-14205; Sweet Potato(Baked)-11509; Carrot Juice-9303; Kale-9226; Kale (Frozen, Cooked)- 8823; Sweet potato-8509; Carrots (Cooked)- 8332, Carrots-8285, Canned Pumpkin-6940, Mustard greens-6300, Dark Green Leafy Vegetables (Spinach, Cooked)-6288, Mixed Vegetables (Canned)- 5670, Spinach-5626, Lettuce-5226, Parsley-5054, Dried Herbs (Marjoram, Sage, Coriander)- 4806, Butternut Squash (Cooked)- 4570, Garden Cress-4150,Cilantro (Coriander)-3930, Collard greens-3842 , Swiss chard-3647, Basil-3142, Pumpkin -3100, Cime di Rapa (Broccoli Raab, Cooked)-2720, Chives-2612, Thyme -2264,Dried Apricots-2163, Cantaloupes-2020, Watercress-1914	Provitamin A activity; Antioxidant/ Immune-enhancement; Decreases risk of some cancers and some cardiovascular events; high-dose supplementation may increase the risk of lung cancer among smokers.[247, 282, 285, 305]	Dietfold,Leroy Capsules,Lycovia,Lycoza,Pevit, Bel-3,UK-TOP,Avencare, Alpene,Nidze-LP,Fokus,Attovita plus,Alfabeta tablets,Lycozin,Ybeta-S,Mycopene, Nurorose-Red,B-Fact,Betavit-AFR,Multi-carotene,Lycolide-Red,Spirulina capsules
Lycopene	Tomatoes-2937 , water melon, apricot, peaches; Sun dried	Antioxidant/ Decreases risk	Lycomits, SAC, Telvit-18, ARIA-L,

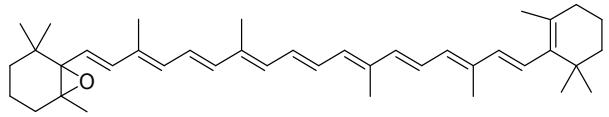
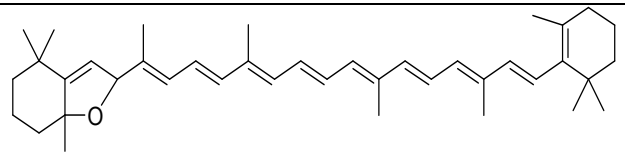
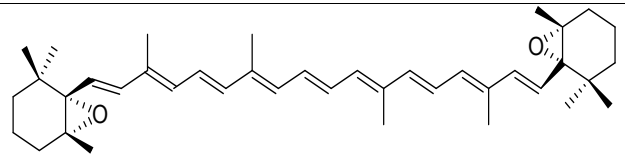
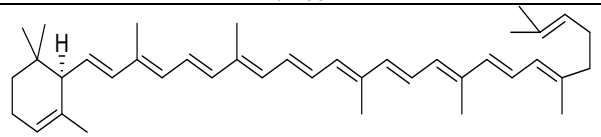
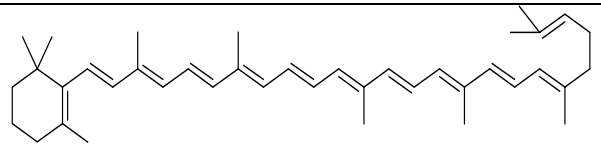
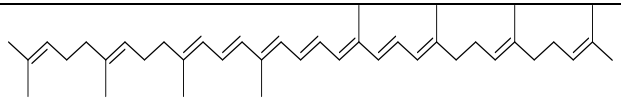
	tomatoes-45902, Tomato puree-21754, Rose hips-6800, Guava-5204, Watermelon-4532, Tomatoes cooked-3041, Papaya-1828, Grapefruit-1135, Sweet red peppers-484, Dried herbs and spices (basil)- 393	of some cancers and some cardiovascular events, dibetes, and osteoporosis.[17, 290]	Algen, Lyrisel, Litecap soft gelatine capsules, L-TO, Lycobury, Lycofun, Lycoza, Lyco, Becogold, Lycosure-SG, Tricopen-Forte, Lycotone-X_X, Lyene-G, Avit, Lyomuch, Litovit,
β-Cryptoxanthin	peppers,sweet,red,cooked-16876,pumpkin,raw-16500,squash,cooked-15583,squash,raw-15429,pumpkin,cooked-14497,squash,frozen-5488,peppers,hot chilli,red-4714,peppers,sweet,red,frozen-3800,peppers,sweet,red,raw-3161,coriander leaves,raw-1756,carrots,canned-1252,carrots,frozen,cooked-1076,dandelion greens,raw-538,collards raw-538,cucumber,with peel,raw-347,cucumber,peeled,raw-300,corn,sweet,yellow,raw-267,peppers,hot chilli, green,raw-250,peppers,jalapeno,raw-227,chrysanthemum,garland,raw-200	Provitamin A activity; Anti-oxidant/ Anti-inflammatory effects; Inhibits risks of some cancer and cardiovascular events; Immune enhancement. [301, 283]	
Lutein/Zeaxanthin	Dark green leafy vegetables (spinach, kale), red peppers, maize, tomatoes, corn, and egg yolks; kale,raw-39551,dandelion greens,raw-13609,spices,paprika-13158,turnips greens,raw-12824,cress,garden,raw-12499,spinach,raw-12197,chard,swiss,raw-10999,chicory greens,raw-10301,mustard greens,raw-9899,collards,raw-8832,radicchio,raw-8832,watercress,raw-5767,basil,fresh-5649,parsley,raw-5561,coriander leaf,dried-5529,parsley,dried-5529,peppers,sweet,green,freeze-dried-5198,celery flakes,dried-5076,chrysanthemum,garland,raw-3834,lambsquarters,raw-3616,arugula,raw-3555,spinach,dry-3487,peas,green,raw-2477,lettuce,cos or romaine,raw-2312,taro leaves,raw-1932,leeks,raw-1900,grape leaves,raw-1747,lettuce,green leaf,raw-1730	Anti-photosensitizing agent and photosynthetic pigment; Acts as antioxidants and blue light filters,Antimutagenic & anticarcenogenic/ Decrease age-related macular degeneration, cataract, and risk of cardiovascular disease and certain cancers.[17, 329, 352]	FloraGLO®-lutein, ,Lycoark,Pevit,Avencare,Alpene, Nidze-LP,Lycozin,Mycopene, Lycolide-Red; OPTISHARP® (zeaxanthin), Swanson-ultra,Nordic Naturals Ultimate DHA Eye + Lutein & zeaxanthin,Pure encapsulations Lutein/Zeaxanthin, TruNature Vision Complex with Lutein & zeaxanthin,Best Naturals Lutein, Vitacost Lutein
Astaxanthin	Green algae, salmon, trout, crustacean;salmonids-5,plankton-60,krill-120,arctic shrimp(P borealis)-1200,phaffia yeast-10000,Haematococcus pluvialis-40000	Antioxidant; Coloration/ Prevention certain cancers, cataract, diabetes, and	Lycotone-X_X,Zenith Nutrition Astaxanthin, Healthy-Origins Astaxanthin,Nutrex BioAstin

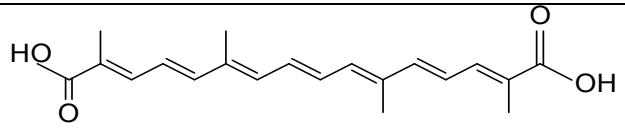
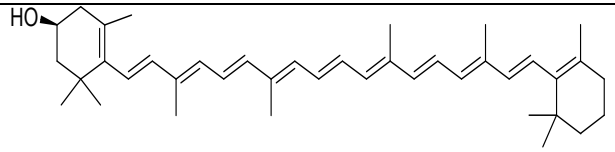
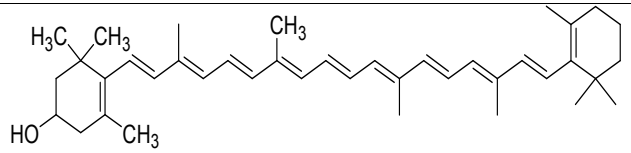
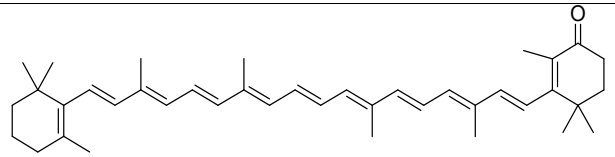
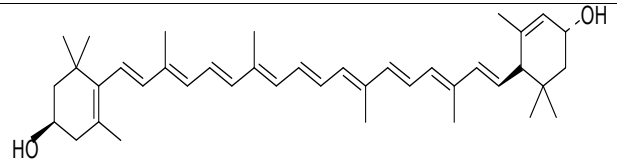
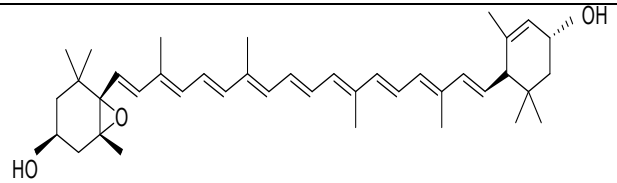
		inflammatory neurodegenerative and risk of cardiovascular disease and certain cancers.[285,342]	Hawaiian Astaxanthin, Neptune Krill Oil, Nutrex Hawaii BioAstin Natural Astaxanthin,, Best Naturals-Astaxanthin, Now Foods Astaxanthin, Nutrigold Astaxanthin gold, MasoN natural Triple Strength Krill oil with Astaxanthin, Bluebonnet Astaxanthin,
Canthaxanthin	Salmon, crustacean, green algae, bacteria, fish (carp, golden mullet, seabream & trush wrasse)	Antioxidant; Coloration/ Immune enhancement; Decreases risk of some cancers.[17, 300, 301]	Bronze EZee,Orobronze
Fucoxanthin	Brown algae, heterokonts	Antioxidants/ Anti-cancer, anti-allergic, anti-obese, anti-inflammatory, and anti-osteoporotic activities.[292, 295, 299]	Vitabase-Fucoxanthin Plus,Souce Naturals-FucoxanTHIN-90 capsules, Fucogreen,Fucothin,Diet 360,Fucomax,Natural Balance-Slimcare, Natural care-Slimcare,Fucoslim,,Fucoxanthin Patch-500Fucoxanthin Solaray,Absonutrix Fucoxanthin Slim Patch, Vitaplus Fucoslim,Fucoxanthin Plus, Fucoxanthin-Slim,RawTrim Fucoxanthin

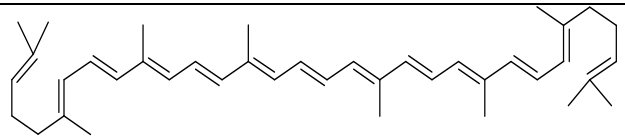
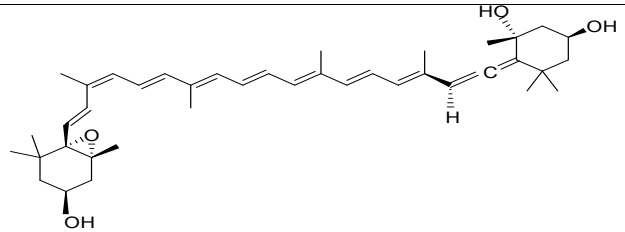
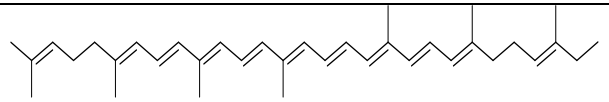
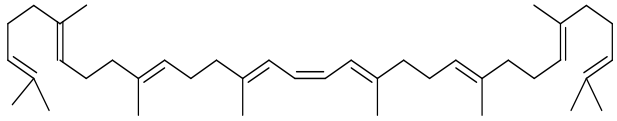
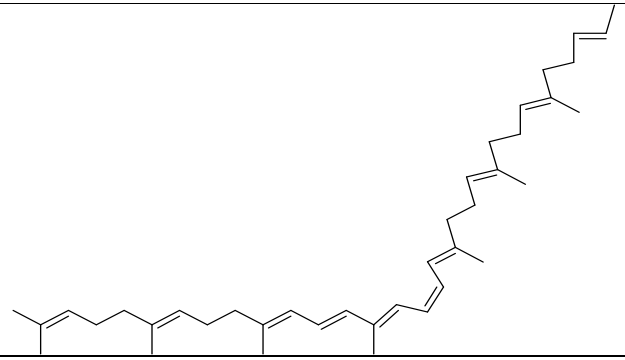
Table 2. Physico-chemical properties of different carotenoids

Trivial Name/Semisystematic Name	Structure/Chemical formula	Mass fragmentation	
		Precursor ion $[M+H]^+$ (m/z)	**Product ion (m/z)**
Antheraxanthin/5,6-epoxy-5,6-dihydro-β,β-caroteno-3,3'-diol M.P.: 197 ^{0}C M.W.: 584.87 λ_{max} (nm): 447	 $C_{40}H_{56}O_3$	585	567, 549, 493, 475.
Astaxanthin/3,3'-dihydroxy-β,β-caroteno-4,4'-dione M.P.: 216 ^{0}C M.W.: 596.84 λ_{max} (nm): 478	 $C_{40}H_{52}O_4$	596	580, 564, 504,490
Auroxanthin/5,8,5',8'-diepoxy-5,8,5',8'-tetrahydro-β,β-carotene-3,3'-diol M.P.:203 ^{0}C M.W.:600.87 λ_{max} (nm): 384, 400, 425	 $C_{40}H_{56}O_4$	600	582, 520, 419, 379, 352
Bixin/Methyl hydrogen 9'-*cis*-6,6'-diapocarotene-6,6'-dioate M.P.: 217°C M.W.:394.5 λ_{max} (nm): (chloroform) 502, 471 for *cis*-form, (chloroform): 507, 476 for *trans*-form O. R.: -20°C	 $C_{25}H_{30}O_4$		

Canthaxanthin/ β,β-carotene-4,4'-dione M.P.:212 ^{0}C M.W.:564.8 λ_{max} (nm): 455.0 to 465.0 (Acetone)	 $C_{40}H_{52}O_2$	551.6	535, 471, 444
Capsanthin/3,3'-dihydroxy-β,κ-carotene-6'-one M.P.:176 ^{0}C M.W.:584.87 λ_{max} (nm): 455 to 465 (Acetone)	 $C_{40}H_{56}O_3$		
Capsorubin/3,3'-dihydroxy-κ,κ-carotene-6,6'-dione M.P.:201 ^{0}C M.W.:600.87 λ_{max} (nm): 460,489,523	 $C_{40}H_{56}O_4$		
α- Carotene/ β,ε- carotene M.P.: 187.5 ^{0}C M.W.:536.8 λ_{max} (nm):444 O. R.: 18643 + 385^0	 $C_{40}H_{56}$	536	480 , 444 , 430, 388, 378, 374
β- Carotene/ β,β –carotene M.P.: 176-184 ^{0}C M.W.:536.8 λ_{max} (nm): 454	 $C_{40}H_{56}$	537	444, 430, 119, 109

β-Carotene-5,6-epoxide/5,6-epoxy-5,6-dihydro-β,β-carotene M.W.:552.8 λ_{max} (nm):	 $C_{40}H_{56}O$		
β – Carotene-5,8-epoxide (mutatochrome)/ 5,8-epoxy-5,8-dihydro-β,β-carotene M.W.:552.87	 $C_{40}H_{56}O$		
β-Carotene-5,6,5',6'-diepoxide/5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene M.P.: M.W.:568.42	 $C_{40}H_{56}O_2$		
δ-Carotene/ ε, ψ-carotene M.W.:536.87 λ_{max} (nm): 421,456,489	 $C_{40}H_{56}$	536.4	444.4, 177.4, 137.4
Υ-Carotene/ β, ψ-carotene M.P.:160-162 ^{0}C M.W.: 536.87 λ_{max} (nm): 435,461,490 O. R.: 276	 $C_{40}H_{56}$	536	467, 444, 375
ζ –Carotene/7,8,7',8'-tetrahydro-ψ,ψ-carotene M.W.:540.9 λ_{max} (nm): 377, 400, 424	 $C_{40}H_{60}$		

Crocetin/8,8'-diapocarotene-8,8'-dioic acid M.P.:285 ^{0}C M.W.:328.4 O. R.: 308	 $C_{20}H_{24}O_4$		
α –Cryptoxanthin/ β, ε-carotene-3'-ol M.W.:552.87 O. R.: 274	 $C_{40}H_{56}O$		
β –Cryptoxanthin/ β, β –carotene-3-ol M.P.: 169^{O}C M.W.:552.8 λ_{max} (nm):452	 $C_{40}H_{56}O$	553	535, 497, 461
Echineone/ β, β –carotene-3-ol M.W.:550.86 λ_{max} (nm): 457	 $C_{40}H_{54}O$	550	535, 558 , 471, 444 , 392, 347, 133, 91, 69
Lutein/ β, ε-carotene-3,3'-diol M.W.:568.87 λ_{max} (nm):445	 $C_{40}H_{56}O_2$	569	551, 533, 416, 376
Lutein-5,6-epoxide (taraxanthin)/ 5,6-epoxy-5,6-dihydro-β, ε-carotene-3,3'-diol M.P.:190^{o}C M.W.: 584 λ_{max} (nm): 472, 443, 420	 $C_{40}H_{56}O_3$		

Lycopene/ Ψ, ψ-carotene M.P.: 172-173 ^{0}C M.W.:536.8 λ_{max} (nm):470	 $C_{40}H_{56}$	536.7	467.4, 444.7, 69
Neoxanthin/5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro-β,β-carotene-3,5,3'-triol M.W.:600.8 λ_{max} (nm):438	 $C_{40}H_{56}O_4$	600	584, 582, 566, 564
Neurosporene/7,8-dihydro-ψ,ψ-carotene M.W.:538.8 λ_{max} (nm): 419, 442, 470	 $C_{40}H_{58}$		
Phytoene/7,8,11,12,7',8',11',12'-octahydro-ψ,ψ-carotene M.P.:620^{O}C M.W.:544.9 λ_{max} (nm):286	 $C_{40}H_{64}$	545	450, 339, 81
Phytofluene/7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene M.W.:542.9 λ_{max} (nm):348		543	406, 338

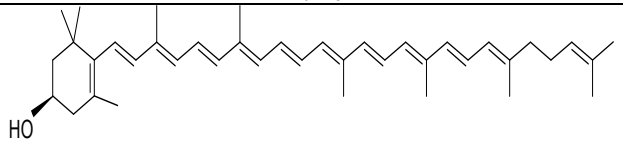
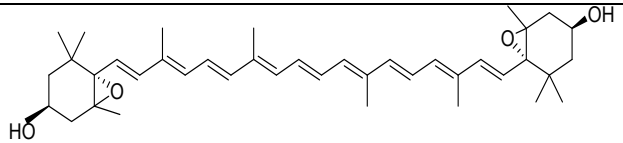
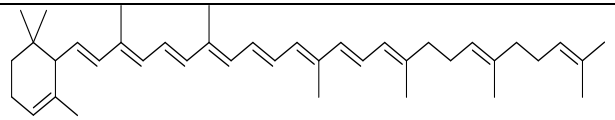
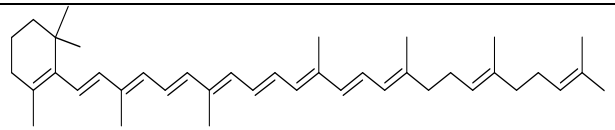
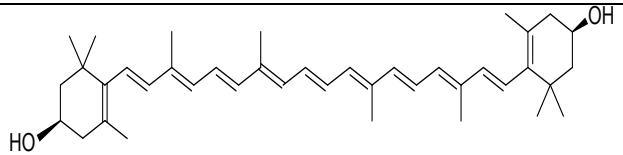
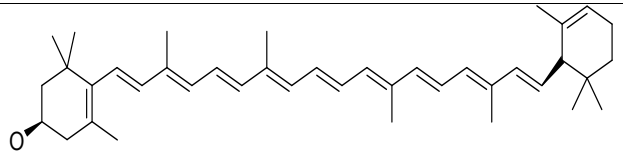
	$C_{40}H_{62}$		
Rubixanthin/ β, ψ-carotene-3-ol M.W.:552.8 λ_{max} (nm): 509, 474, 439 (chloroform)	 $C_{40}H_{56}O$		
Violaxanthin/5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro-β,β-carotene-3,3'-diol M.W.:600.8 λ_{max} (nm):443	 $C_{40}H_{56}O_4$	601	583, 565, 547, 509
α –Zeacarotene/7',8'-dihydro-ε,ψ-carotene M.W.:538.8	 $C_{40}H_{58}$		
β –Zeacarotene/7',8'-dihydro-β,ψ-carotene M.W.:538.89	 $C_{40}H_{58}$	538	446, 401
Zeaxanthin/ β, β-carotene-3,3'-diol M.P.: 215.5 ^{0}C M.W.:568.88 λ_{max} (nm):452	 $C_{40}H_{56}O_2$	568	550, 532, 489, 458
Zeinoxanthin/ β, ε-carotene-3-ol M.W.:552.87 λ_{max} (nm): 433,459, 488	 $C_{40}H_{56}O$		

Table 3. Conventional techniques and conditions for carotenoid extraction

S. No.	Extraction Methods Used	Solvent used	Carotenoids extracted	Fruits & Part	References
	Homogenization	acetone: ethanol(1:1, v/v), 5% aqueous solution of pyrogallol	β-carotene, *cis*-β-carotene, α-carotene, Antheraxanthin, Lutein, Zeaxanthin, Violaxanthin, Neoxanthin, Lycopene	Apricot, Avocado, Blackberry, Banana, Broccoli, Bilberry, Carrot, Grape, Kale, Kiwi fruit, Lemon, Leuttuce, Mirabelle, Nectarine, Papaya, Parsley Leaf, Peach, Plum, Raspberry, Red Paprika, Strawberry, Tomato, Spinach	93
		Diethyl ether and Acetone	β-carotene, β-cryptoxanthin, Lutein, Violaxanthin	Acerola fruits	94
		Tetrahydrofuran: methanol (1:1, v/v), petroleum ether	Lutein, α-carotene, β -carotene, *cis*-β-carotene	Beans, Broccoli, Brussels, Cabbage, Carrot, Cucumber, Greens, Leeks, Lettuce, Marrow, Pepper, Sweet corn, Tomato,	95
		Ethanol: Pentane: Sodium Chloride: Water (10:16:2:5 v/v/v/v)	β-carotene, α-carotene	Broccoli, Carrot, Collard, Corn, Squash, Turnip	96

	Immersion	Hexane	Total Carotene	Apricot, Cntaloupe, Grape Fruits, Peach	97
		Tetrahydrofuran	Neoxanthin, Violaxanthin, Lutein 5,5-epoxide, *Trans*-lutein, *Cis*-lutein, Total lutein, α-carotene, β-carotene	Beans, Broccoli,	98
	Agitation	Petroleum ether: Methanol (1:1 v/v)	β-carotene	Apricot	99
		Acetone: Ethanol: Hexane (1:1:2 v/v/v)	Lycopene, Carotenoids, β-carotene, Lutein	Guava, Red Navel, Tomato	100-102
		Hexane: Ethyl acetate (85:15 v/v)	β-carotene	Olive oil	93
		Dicholoromethane: Acetone: Methanol (2:1:1 v/v/v)	β-carotene, Carotenoids	Paprika	103
		Tetrahydrofuran	β-carotene, Lutein	Spinach	104
		Hexane	Lycopene, β-carotene	Tomato	105
		Water: 1-butanol	α-carotene, β-carotene, Lutein, Lycopene	Wheat	106
	Soxhlet	Hexane	β-carotene, Carotenoids, Lutein	Buriti, Marigold, Palm oil, Paprika, Tomato	107- 111
		Diethyl Ether	α-carotene β-carotene	Cloudberry	112

		Acetone, Hexane	β-carotene, , β-cryptoxanthin, Zeaxanthin,, Capsanthin, *Trans*-lycopene, *Cis*-lycopene	Paprika, Tomato	113, 114
		Chloroform	Lycopene	Tomato	115
		Dichloromethane	Lycopene, β-carotene	Tomato	116
		Ethanol, Hexane	Polyoxy xanthophylls, Lutein , α-cryptoxanthin,β cryptoxanthin, Lycoxanthin, *Cis*-lycoxanthin, Lycopene, Neolycopene, Y-carotene, β-carotene	Tomato Skins and Seed	117, 118
	Ultrasound	Ethyl acetate	Bixin, β-carotene	Annatto, Carrot	119, 120
		Chloroform: acetone	β-carotene, Lutein	Stinging nettle	121
		Chloroform	Lycopene	Tomato skins	122, 123
	Shaking	1,2-dichloroethane: Acetone	β-carotene	Cardamom	124
		Methanol: Tetrahydrofuran (1:1 v/v)	Lutein, Zeaxanthin, β-cryptoxanthin	Corn, Wheat	125
		Chloroform: Methanol	α-carotene, β-carotene, Lycopene, Lutein, , Zeaxanthin, β-cryptoxanthin	Japanese persimmon	48a
	Centrifugation	Zinc sulphate + monohydrate potassium	*Cis*-violaxanthin, β-carotene,	Orange	109

		ferrocyanide	Lutein		
		Ethanol: Water: Butylated Hydroxytoluene (95:5:0.05 v/v/v)	β-carotene, Cryptoxanthin, α-carotene, Lutein, Lycopene	Pumpkin	126
		Ethanol: Hexane (1:1)	Lutein, α-carotene , β-carotene Lutein esters,Lycopene	Pumpkin, Tomato	127, 128
		Tetrahydrofuran: Methanol (1:1 v/v)	Lutein, Zeaxanthin, β-cryptoxanthin, *Trans* - β-carotene	Spinach, Tomato, Tangerine	129

Table 4. Supercritical Fluid Extraction (SFE) conditions used in carotenoid extraction

Carotene	Source	SFE Condition			References
		Temperature (K)	Pressure (bar)	Time (min)	
β-carotene	Apricot	313-350	304-507	90-150	99, 130, 131
	Broccoli	313	342	30	96
	Buriti	313-328	200-300	95-210	107
	Cardamom	298-328	80-300		124
	Carrot	310-330	150-250	60-300	132
	Collard greens	313	342	30	96
	Crude Palm Oil	333	140	60	133-135
	Mustard greens, Squash, Turnip greens, Zucchini	313	342	30	96
	Rosa canina	343	300		136
α-carotene, β-carotene	Carrot	303-342	404-606		96, 137, 138
	Cloudberry	313-333	90-300		112
	Corn,Vegetables	313	342	30	96
Carotenoids	Capsicum	313-333	140-300	360	139
	Crude palm oil	313-353	140-350		140, 141

	Lotus	301-344	159-441	150	142
	Sea Buckthorm	305-340	128-472	9-116	143
	Walnut	323-343	200-400	182-470	144
	Paprika	333-353	300-500		145
Lycopene	Gauava	328	300	180	100, 146
	Papaya, Pomelo red, Rosehip fruits	363	400	15	141, 146
	Tomato	313-373	200-500	330	105, 115, 123, 128, 146-149
	Watermelom	333-363	207-414	35	150

Table 5. Saponification conditions employed in carotenoid extraction

Sample	Analyte	Saponification conditions	References
Juiceof red Navel Orange (Cara cara)	Neoxanthin (a,b), neochrome, violaxanthin, luteoxanthin, antheraxanthin, mutatoxanthin, lutein, isolutein, zeaxanthin, α- and β-cryptoxanthin, phytoene, phytofluene, α-, β- and Y-carotene, lycopene	10% methanolic KOH sol. (Overnight, room T, darkness)	163
Ultrafrozen orange juice	Lutein, zeaxanthin, lutein 5,6-epoxide, antheraxanthin, β-cryptoxanthin	10% methanolic KOH sol. (1 h, room T, darkness)	164
Fatty foods (fat-cured crude sausage ‘‘Sobrassada’’)	Capsorubin, violaxanthin, capsanthin, anteraxanthin, lutein+zeaxanthin, cantaxanthin, β-cryptoxanthin, β-carotene	10% methanolic KOH sol. containg 0.01% BHA (5 min, 50 ^{0}C)	165
Marigold (Tagetes erecta) flower extract	All-*trans*-*cis* isomers of zeaxanthin, all-*trans*-*cis* isomers of lutein, lutein	15% methanolic KOH sol. (1 h, darkness)	166

	esters		
Standard Reference Material 2383 (Baby Food Composite)	Lutein, zeaxanthin, β-cryptoxanthin, lycopene, *trans-cis* α- and β-carotene, retinol, retinylpalmitate, δ-, Y- and α-tocopherol	40% methanolic KOH sol. (30 min, room T)	167
Raw and cooked Spanish vegetables (lettuce, artichokes, Brussel sprouts, green beans, asparagus (green), beet, green peppers, spinach, tomato, red peppers, carrots, red cabbage, cucumber, squash, potato, onion, cabbage, cauliflower)	Lutein, zeaxanthin, lycopene, bcryptoxanthin, α-, β- and Y-carotene	Saturated methanolic KOH sol. (Under nitrogen atmosphere, 30 min, darkness)	168
Edible wild vegetable Stinging Nettle (Urtica dioica L.)	Lutein, lutein isomers, β-carotene, β-carotene isomers, neoxanthin, violaxanthin, lycopene	Methanolic KOH sol. (room T)	169
Virgin olive oil	α-tocopherol and β-carotene	76% ethanolic KOH sol. (Under nitrogen atmosphere, 30 min, 70 ^{0}C)	170

Corn	Lutein, zeaxanthin, and β-cryptoxanthin	80% ethanolic KOH sol. (In a water bath at boiling point, 10 min)	171
Fresh and processed vegetables (broccoli, carrots and green beans)	*Trans*-β-carotene	100% ethanolic KOH sol. (30 min, 70 ^{0}C)	172
Sweetpotato (Ipomoea batatas, L.)	α-carotene, β-carotene	10% ethanol:water (50:50, v/v) for 1 h, 80 ^{0}C	173
Milk samples	β-carotene	60% aqueous KOH sol. containing pyrogallol as antioxidant (30 min, 30 ^{0}C)	174
Fortified foods (fortified breakfast cereal, peanut butter and margarine)	All-rac-alpha-tocopheryl acetate, retinyl palmitate, β-carotene	60% aqueous KOH sol. containing pyrogallol as antioxidant (under nitrogen atmosphere, 30 min, 70 0C)	175
Kale (Brassica oleracea var. Acephala cv. Vates)	Lutein, β-carotene, retinol, phylloquinone	80% aqueous KOH sol. (15 min, 70 ^{0}C)	176

Table 6. HPLC analysis of carotenoids

S.No.	Analyte & Wave-length	Mobile phase	Column/ Detection	References
	β-carotene & 451 nm	Methanol 65%, THF 27% and water 6%	C_{18} Column	59
		65% methanol, 27% acetonitrile, 4% methylenediclorure and 4% hexane.	C_{18} RS 5 µm column	131
		Methanol with 10% (v/v) acetonitrile	C_{18} Column	187
		Acetonitrile (ACN), dichloromethane (DCM) and methanol (MeOH) 5:4:1 v/v/v, containing 0.1% BHT.	Luna C18 coupled to a Jupiter C18 column	188
		Methanol, water and t-BME	ODS-C_{18} Tracer Extrasil (5 µm)/ PDA	189
		Acetonitril, water and ethyl acetate		190
		Acetonitrile, propranolol, methanol and water	5C18-MS Waters column	191
		Acetonitrile, methanol, dichloromethane	ODS2 Column; STR-ODS-II Column	192, 193

		Methanol/methyl tert-butyl ether (MTBE)/water (81:15:4, v/v/v; A), and methanol/ MTBE/ water (4:92:4, v/v/v; B).	C_{30} column	127, 194
	Neoxanthin, β- cryptoxanthin, lutein, α-, β-carotene, violaxanthin, violeoxanthin, Lutein and several *cis* isomers	Methanol, propranolol and dichloro methane	Silica C_{30} (5 µm)/ PDA	195, 196
		Methanol, t-BME	C_{18} Nucleosil (5 µm)/ LC-MS	197
	Lycopene, β-cryptoxanthin, α-, β-carotene, lutein, zeaxanthin isomers	Acetonitrile, dichloromethane and t-BME; Acetonitrile: dichloromethane (65:35 v/v)	C_{18} Spherisorb (5 µm)/ PDA; C_{18} octadecyl silane	53, 198
	α-, β-Carotene, lutein, lycopene, β-cryptoxanthin, phytoene, phytoene	Acetonitrile, methanol and propanol	C_{18} Spherical (5 µm)/ PDA	199
	Lycopene, β-Carotene	Methanol, THF and water	C_{18} Symmetry (5 µm)/ PDA	83b
	Lutein, α-, β-carotene, β-cryptoxanthin, zeaxanthin	Acetonitrile, dichloromethane, methanol	Silica C_{18} Nucleosil C_{18} Techsphere ODS Spherisorb ODS Spherisorb C_8 (3 µm, 5 µm)/ UV-MS	200
	Valenciaxanthin, neochrome, α-, β-cryptoxanthin, lutein, antheraxanthin, trolichrom, neoxanthin, auroxanthin, leutoxanthin, phytofluene	t-BME, Methanol and water	Silica C_{30} Spheres (3 µm)/ PDA	201
	Astaxanthin, zeaxanthin, canthaxanthin, echineone, lycopene, β-carotene	Methanol, t-BME	Silica C_{30} (3 µm)/ UV-MS	202

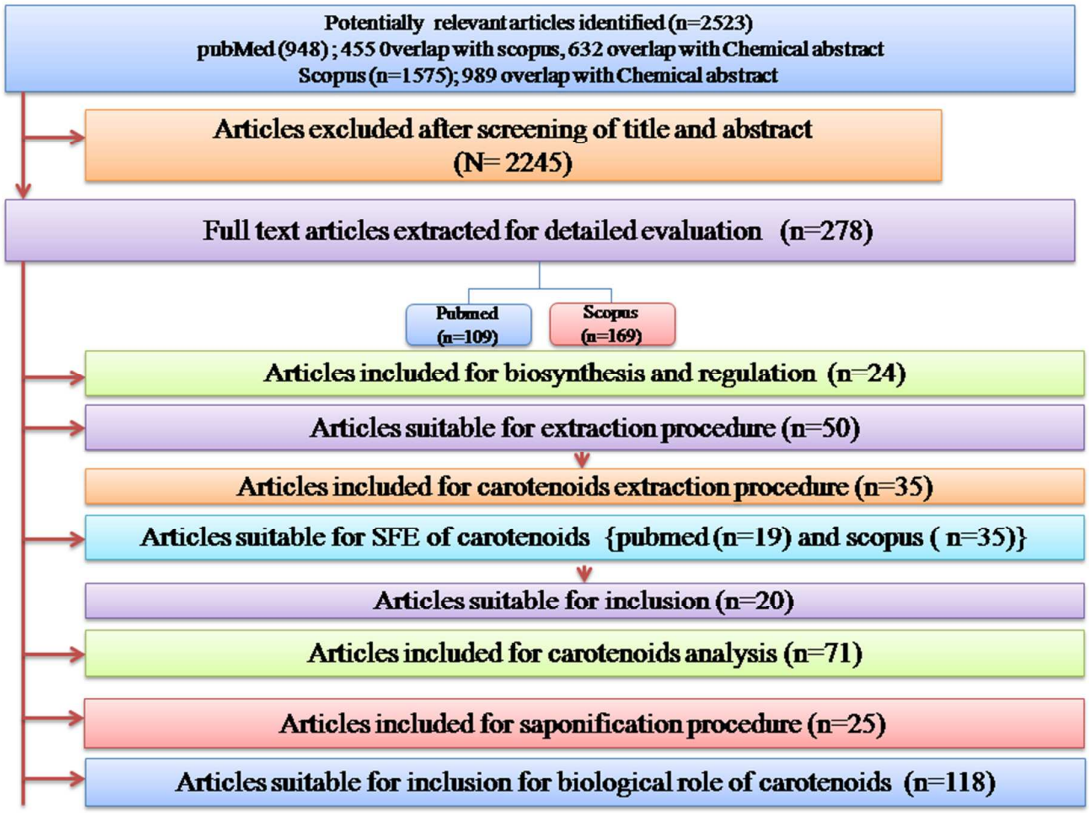
Table 7. Brief summary of carotenoid analysis in human samples

Sample	Analyte	Extraction protocol	Detection system	References
Plasma	Lutein Zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, Lycopene, Phytoene, Phytofluene, 36 different carotenoid isomers	Centrifuged blood treated with ethanol (0.1% BHT) and heated with hexane, ethanol, acetone and toluene; Sometimes saline treated blood extracted with ethyl acetate.	LC-PDA, C30 column, Mobile Phase: methanol:MTBE:H_2O:2% Aqueous ammonium acetate solution, 88:5:5:2, v/v/v/v	229
			LC-PDA Si60 (250mm? 4 mm, 5 lm) column Mobile phase: n-hexane and acetone (19% by vol)	232
			LC-PDA C18 column Mobile phase: (A) 0.0125% ammonium acetate in MeOH, (B) 100% CHCl3 and (C) CH3CN with 0.1% triethylamine Gradient elution	227
			UPLC-PDA-APCI(-)/MS C30 (150mm? 4.96 mm, 5 lm) column Mobile phase: (A) MeOH/0.1% aqueous formic acid solution (80:20) (B) MTBE /MeOH/0.1% aq. formic acid solution (78:20:2)	230
Serum	Lutein Zeaxanthin, β-	200 µL plasma extracted twice with 1mL of hexane containing 0.01% BHT. Dried under N2; reconstituted	LC-PDA-APCI (+)/MS C30 column Mobile phase: (A) MeOH/ACN/water (84:14:4, v/ v/v) and (B) DCM Gradient	212

	cryptoxanthin, α-carotene, β-carotene, Lycopene, Phytoene, Phytofluene	in mobile phase	elution	
			SFC/MS/MS ODS (250mm? 4.6 mm, 5 lm) column Mobile phase: Carbon dioxide (99.99% grade). Modifier: MeOH with 0.1% (w/v) ammonium formate	238
			LC-UV-Vis C30 (4.6mm? 25 mm, 5 lm) column, guard C30 column Mobile phase: (A) MeOH: ACN (40:60, v/v) (B) MeOH: ACN (25:75, v/v) and (C) MTBE Gradient elution	235
			LC-PDA ODS 2 C18 (3mm? 250 mm, 3 lm) column connected with a ODS 2 guard column Mobile phase: ACN: methylene chloride: methyl alcohol (7:2:1,v/v/v) Isocratic elution	233
			LC-PAD RP C18 Mobile phase: MeOH/THF/H2O(94:5:1)	241
			Reversed phase HPLC C30 (250 ? 4.6 mm, 5 lm) column in line with a C18 pre-column Mobile phase: (A) MeOH/water (90:10, v/v, with 0.4 g/l ammonium acetate in H2O)	226

			(B) MeOH/ MTBE/water (8:90:2 v/v/v, with 0.1g/l ammonium acetate in H2O).	
			LC-APCI (+)/MS RP C30 (150mm × 4.6 mm, 3 lm) Mobile phase: (A) MeOH/tert-butyl methyl ether/ water (83:15:2, v/v/v) and (B) MeOH/MTBE/water (8:90:2, v/v/v) Gradient elution	242
			HPLC-APCI(+)/MS C30 (4.6 × 250 mm, 5 lm) carotenoid column Mobile phase: (A) MeOH/MTBE/water (8:90:2) containing 2.6 mmol/L of ammonium acetate.	243
Red blood cells	Lutein Zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, Lycopene, Phytoene, Phytofluene	2,5 mL + 2,5 water + 5 ml pyrogallol (0–400 mmol/L in ethanol) + 1 mL aqueous potassium hydroxide (0–7 mmol/L) and 40 lL of echinone (1 lmol/L in ethanol); sonicated for 5 min; vortexed for 2 min, incubated at various temp. (20–70) for different time periods (0–24 h). Mixed; extracted with 1.25 mL of 0.1 mol/L sodium dodecyl sulfate aqueous solution and 15 ml of hexane/dichloromethane (5:1, v/v) containing 1.2 mmol/L BHT; sonicated, vortexed, centrifuged at 1000g for 10 min. Supernatants collected extraction repeated. Supernatants evaporated under	LC-PDA-APCI(+) /MS C30 (250mm× 4,6mm I.D, 5 lm) column Mobile phase: (A) MeOH/MTBE/water (83:15:2, v/ v/v) containing 3,9 mmol/L ammonium acetate (B) MeOH/MTBE/water (8:90:2, v/v/v) containing 2,6 mmol/L ammonium acetate	231

		N2; reconstituted in 3mL of hexane/acetone (2:1, v/v) and eluted with 7mL of hexane/acetone (2:1, v/v); eluent evaporated and residue dissolved in 100 lLofMeOH/MTBE (2:3, v/v) 100		



Graphical Abstract (Overview of data included)