CHAPTER-3

Extraction, isolation and purification of Lutein, Zeaxanthin, Meso-Zeaxanthin and β-Cryptoxanthin

Lutein is a non-provitamin-A xanthophylls and is one of the major Carotenoids present in serum of most populations (Olmedilla et al.1997). It is predominantly transported in HDL and shows a specific distribution pattern in human tissues (Olmedilla et al.1997a). Vegetables, fruits and egg yolks are the most important food sources of Lutein. Fruits and vegetables differ widely in their xanthophylls composition. Lutein and Zeaxanthin are often found together in foods and human tissues. These are present in the green leafy vegetables and also in yellow and red colored fruits. Generally, compared to Zeaxanthin, Lutein is present in greater amount in the diet and human blood and tissues (Khachik et al.1992). Lutein from diet can be either in free form or bound in ester form. Lutein shows a high antioxidant activity (Chopra and Thurnham, 1993), and in vivo oxidative metabolites have been described in human subjects after Lutein supplementation (Khachik et al.1995), indicating biological activity as a potential cancer-prevention agent (King et al. 1997). Data from epidemiological studies indicate a protective effect of dietary Carotenoids against a variety of chronic diseases (Mayne ST, 1996) and, more specifically, Lutein and Zeaxanthin have been associated with a lower risk of eye disease in the elderly (Bunce, 1994). Marigold flowers are richest sources of Lutein esters and considered to be the best possible natural l source for obtaining Lutein esters. The marigold oleoresin is typically produced by solvent extraction of dehydrated marigold meal in milled form prepared by drying of fresh or fermented flowers. The xanthophylls esters are found 15-30% in the oleoresin depending on the quality of meal, extraction condition and cultivation practices.

The current practices of marigold oleoresin production may be summarized as follows: Fresh marigold flowers containing high level of moisture is used. Anaerobic fermentation of marigold flower is carried out in the designated silos for extended period of time, dewatering by pressing the fermented flower, followed by drying using a rotary or flash dryers to reduce the moisture content to about 10% and
pulverising the dehydrated material to obtain marigold meal with xanthophylls content of around 1.5-2.5% by weight.

The traditional process for lutein consists of solvent extraction of marigold flower to get marigold oleoresin and saponification, crystallization which gives Lutein (More-Pale et al. 2007). A method of isolating, purifying and re-crystallizing Lutein from marigold oleoresin (Khachik, 1995), this method is a laborious involving a multiple solvents for extraction and purification. Another study describes a process of saponification of marigold oleoresin using alcohol, water and alkali at a temperature of 45-50°C (Shao et al. 2007), even though the high yield the process was uneconomical because of multiple process steps and high amount of alkali used.

Zeaxanthin is one of the most common Carotenoid found in fruit, vegetables and flowers and is important in the Xanthophyll cycle. Animals get Zeaxanthin from natural source. Zeaxanthin is one of the two primary xanthophyll Carotenoids present within the retina of the eye. The eye is selective and preferentially dietary Zeaxanthin accumulates in the very center of the macula, the most critical area of central vision, whereas in the peripheral retina, Lutein predominates. Zeaxanthin containing supplements are used to treat many eye disorders and no side effects reported. Lutein and Zeaxanthin are isomers and have identical chemical formulas. The difference between Lutein and Zeaxanthin are in the location of the double bond in one of the end rings. The macula mainly contains the (RR) and meso-Zeaxanthin (RS-) forms and trace amounts of SS-Zeaxanthin. The macular pigment constitute of Lutein and Zeaxanthin concentrated in the fovea of the eye. (Landrum et al. 2001). In humans, Zeaxanthin accumulates in the central area of the macula (Landrum et al. 1999). Thinning of the macular pigment, known as age-related macular degeneration, is associated with irretrievable loss of central vision. (Beatty et al. 1999; Landrum et al. 2001). The small red berry known wolfberry, and is rich in Zeaxanthin. The fruit contain Zeaxanthin dipalmitate around 90% (Lam and But, 1999, Zhou et al 1999, Weller and Breithaupt, 2003).

Dietary sources of Zeaxanthin are green, yellow/orange fruits and vegetables such as corn, oranges, papaya and squash. Capsicum annum is another most common spice widely used which is a good source of Zeaxanthin. Wolfberry (Lycium
barbarum, fructus lycii or Gou Qi Zi) plant has small red berries has been shown to have high constant of Zeaxanthin which are commonly used in Chinese food. The dried fruit of wolfberry is used as a therapeutic agent for eye diseases by Chinese herbalists. In a randomised, single-blind cross-over study, volunteers were administered R,R-Zeaxanthin (5 mg) from Wolfberry (Lycium barbarum) suspended in yoghurt together with a balanced breakfast. The chiral APCI-MS was used to confirm the appearance of RR-Zeaxanthin in pooled plasma samples (Breithaupt et al. 2004).

The macular pigment of the eye is composed of three xanthophyll Carotenoids, namely (R,R)-Lutein, (R,R)-Zeaxanthin and (R,S)-Zeaxanthin along with minor Carotenoids like o xo-Lutein, epi-Lutein and ε,ε-carotene 3,3′-dione (Landrum et al.2001). The highest concentration of the xanthophyll pigments are found in the macula lutea region of the retina, including the fovea. The concentration of the xanthophylls increases progressively towards the center of the macula and in the fovea. Compared to other human tissues, the concentration of these pigments are much higher in macula and fovea (Landrum et al. 1992). The fovea is a relatively small area within the macula, in which the cone photoreceptors reach their maximal concentration. About 50% of the total amounts of the xanthophylls is concentrated in the macula where Zeaxanthin dominates over Lutein by a ratio of 2:1 (Handelman et al.1992; Billsten et al.2003). At the center of the retinal fovea, Zeaxanthin is a 50:50 mixture of RR-Zeaxanthin and RS-Zeaxanthin along with small quantity of SS-Zeaxanthin (Landrum et al. 2001). Since the fovea is important for proper visual function, disease and damage to this area is known to result in blindness. Age-related macular degeneration (AMD) is generally diagnosed by pathological changes in the retina, retinal pigment epithelium (RPE) and the choroid. The study of xanthophylls and XBP by femto-second transient absorption spectroscopy showed better stability for RS-Zeaxanthin enriched XBP compared to RR-Zeaxanthin while the photophysical properties of the xanthophylls RR-Zeaxanthin and RS-Zeaxanthin are generally identical. It is likely that the RS-Zeaxanthin is better accommodated with XBP wherein the protein protects the xanthophylls Carotenoids from degradation by free radicals. The above mentioned complex may have better antioxidant property than the free form of xanthophylls, facilitating improved protection of ocular tissues form oxidative damage (Billsten et al.2003). The reduced vision function due AMD
and cataract can be controlled by consuming vegetables, fruits. The supplements containing Lutein, Zeaxanthin and meso-Zeaxanthin also helps to control the progression of AMD and cataract. The meso-Zeaxanthin present in eye is considered a metabolic product originating from Lutein in the human body, the dietary supplementation containing meso-Zeaxanthin is now considered to improve the MPD. (Landrum et al. 2001). The shrimps, fish, turtle, etc are the dietary source of meso-Zeaxanthin thereby the vegetarian population is deprived of meso-Zeaxanthin. Khachik et al. have reported metabolic pathways of formation meso-Zeaxanthin from dietary Lutein and its presence in human plasma.

Among the Carotenoids detected in mammalian plasma and tissues, β-cryptoxanthin is one of the major Carotenoids along with Lutein, Zeaxanthin, beta-carotene and lycopene (Bieri at al.1985). β-Cryptoxanthin is a provitamin -A, is playing an important role in the diet, finally converting in the human body into an active form of vitamin A (retinol), which is a important nutrient for vision, immune function, skin and bone health. β-Cryptoxanthin has about one half the vitamin A activity of the major vitamin precursor, β-carotene. In addition, β-cryptoxanthin acts as an antioxidant in the body. The bioavailability of the Carotenoids from paprika oleoresin has shown the presence of β-cryptoxanthin and β-carotene in higher amounts in chylomicrons compared to Zeaxanthin among the volunteers (Perez-Galvez et al. 2003, Burri et al.2011). Beta-cryptoxanthin in substantial amounts were detected both in human chylomicrons and in serum (Wingerath et al.1995).

β-cryptoxanthin is present in certain specific foods such as capsicum species, citrus fruits, mango, papaya, pumpkin. The β-cryptoxanthin is present in paprika in ester form and mandarin fruits. High correlation of plasma β-cryptoxanthin have been reported in many tropical countries due to the consumption of papaya fruit (Irwig et al. 2002). In the common Spanish diet ,citrus fruits accounted for 68% of the total β-cryptoxanthin (Garcia – Closas et al, 2004). The red chillies, tangerine and oranges are containing highest amount of β-cryptoxanthin esters (Breithaupt and Bamedi, 2001). Later, Breithaupt et al., in a randomized, single-blind crossover study using a single dose of esterified or non-esterified β-cryptoxanthin in equal amounts found no difference in the resulting plasma response among 12 volunteers suggesting a comparable bioavailability. (Breithaupt et al.2003, Takayanagi and Mukai, 2009).
Vitamin A is metabolically derived from oxidative cleavage of provitamin A Carotenoids (\(\alpha\)-carotene, \(\beta\),\(\beta\)-carotene, and \(\beta\)-cryptoxanthin) by 15,15\(\epsilon\)-dioxygenases. Humans are unable to synthesize vitamin A. (Lintig et al.2000, Wyss Wt al.2000). Due to its hydroxyl group, \(\beta\)-cryptoxanthin can be present as a mono acyl ester in plants. The main \(\beta\)-cryptoxanthin esters occurring in plants are \(\alpha\)-cryptoxanthin laurate, myristate, and palmitate (Khachik et al.1988, Wingerath et al. 1995), purified by preparative HPLC methods, and used as reference material for the quantification and identification of the respective Carotenoid esters.

The chemically induced rat colon and mouse lung tumorigenesis inhibit by \(\beta\)-cryptoxanthin prepeared from Citrus unshiu (Tanaka et al.2002). In vitro and in vivo studies in aged female rats, \(\beta\)-cryptoxanthin has showed an anabolic effect on bone components (Uchiyamma et al. 2004), In human lung cancer, it acts as a chemopreventive agent. (Yuan et al. 2003). It belongs to the major xanthophylls found in the human brain. In the frontal cortex had higher concentrations of antioxidants than other parts which are generally susceptible to Alzheimer’s disease (Craft et al.2004).

Dietmar et al in 2001 reported a method for the isolation of \(\beta\)-Cryptoxanthin from fresh papayas, by extraction with light petroleum ether/diethyl ether. The extracts were combined, dried over anhydrous sodium sulphate and filtered. The solvent was evaporated under reduced pressure, and the residue was dissolved in diethyl ether. Methanolic potassium hydroxide was used for the r saponification of \(\beta\)-cryptoxanthin esters. The solution was washed several times with distilled water to remove excess alkali and dried over anhydrous sodium sulphate, filtered, and evaporated to dryness. The residue obtained was dissolved in \(n\)-hexane and subjected to open column chromatography on silica gel. Light petroleum ether and acetone were used as mobile phases. The first band, obtained by elution with light petroleum ether, consists mainly of \(\alpha\),\(\alpha\)-carotene second band obtained by elution with mixture of light petroleum ether/acetone, 9:1 v/v, consist a single component which was analysed by HPLC. The Beta-cryptoxanthin was identified by comparison of the HPLC retention time and absorption spectra (Locher et al. 1971, Isler et al. 1957, Khachik et al. 2006).
The main objective of the study was to extract and isolate ophthalmic Carotenoids, Lutein (L), Zeaxanthin (Z), Meso-Zeaxanthin (MZ) and β-cryptoxanthin (BCX) from natural source. The present study describes

(1) A process for obtaining the xanthophylls crystals with high Lutein content: Oleoresin containing Xanthophyll esters (obtained from the extraction and concentration of Dried Marigold flower with hexane) is saponified using an aliphatic alcohol and alkali and the resulting reaction solution is subjected to purification by neutralizing the alkali and addition of acetone, heating, filtering the slurry and the residue obtained after washing with water followed by purification with alcohol to remove the solvent soluble impurities, thereby resulting in Lutein crystals with high levels of trans-Lutein.

(2) A process for the preparation of Zeaxanthin from red bell pepper extract, through saponification, extraction with ethyl aceate and purification with alcohol

(3) A process for the preparation meso-Zeaxanthin crystals of high purity from Lutein through thermo alkali treatment, which has no toxic effects, less solvent residues and is safe for human consumption.

(4) A process for the preparation of β-cryptoxanthin from paprika oleoresin through extraction, isolation and chromatographic separation by using selective solvent to get high purity product.
Material and methods:

Electronic micro balance 0.001mg (Mettler), Spectrophotometer-Dual beam, (PerkinElmer), HPLC System- Agilent 1200 series, isocratic& Gradient with UV/VIS and PDA detector, Column – SILICA , YMC-Carotnoid and ChiralPack AD. All solvents and chemicals used for the extraction and analysis were of analytical grade and the solvents used for HPLC were of HPLC grade purchased from Merck, Qualigens, Mumbai. The analysis of the chromatographic data was carried out on a Chem Station software (Agilent)

- Red Bell Pepper oleoresin obtained from OmniActive Health Technologies Ltd, Mumbai.
- Paprika oleoresin sourced from Kancor Ingredients Ltd, Cochin.
- β-cryptoxanthin standard purchased Carotenature, Switzerland.
- Lutein, Zeaxanthin and meso-Zeaxanthin standards obtained from Omni Active Health Technologies Ltd, Mumbai.

Isolation of Lutein from Marigold extract:

The marigold oleoresin was mixed with ethyl alcohol in which alkali (Potassium Hydroxide) dissolved and forming alcoholic alkali solution . The ratios of the three constituents of the saponification reaction mixture were about 1 :3:0.25 part oleoresin, s alcohol and KOH respectively by w/v/w. 6 gm of KOH was dissolved in 75 ml of ethyl alcohol in a 250 ml round bottom flask, into which 25 gm of marigold oleoresin was added. The flask was shaken well and kept in a water bath at 70°C. for a period of 3 hours to ensure complete saponification of esters. The saponification of the oleoresin results in formation of free Xanthophylls along with alkali salts of fatty acids. The progress of the saponification reaction was monitored by HPLC analysis to determine the completion of saponification which is indicated by the complete disappearance of the Lutein ester peak. The analysis was performed by taking an aliquot (1 ml) of the saponification reaction mixture in to a 100 ml amber coloured standard flask and added 30 ml extractant (hexane: acetone: toluene: absolute alcohol 10:7:7:6 v/v). Added 30 ml of hexane, shaken for 1 minute and was then diluted with 10% sodium sulphate solution and kept in the dark for 30 minutes. The upper phase was analysed by HPLC. Silica column 254mm x 4.5mm, 5 µm. Hexane /Ethyl acetate 75:25 was used as a mobile phase for the separation. An isocratic condition was
maintained at flow rate 2 ml /min. The volume of sample injected was 20 µl. Detection wave length was set at 474 nm.

The alcohol was removed from the reaction mixture by vacuum distillation. The resultant saponified product was then homogenized with water at room temperature for 30 minutes resulting in a brownish yellow oily liquid containing free form of xanthophylls, fatty acid soaps and other impurities. The solution obtained was then subjected to neutralize with the addition of 25% acetic acid solution followed by the addition of acetone. The temperature of the solution was increased to 80-85°C and the mixture was stirred continuously for a period of 15 minutes. The resultant mixture was filtered off (Buckner funnel) and the filtrate was discarded. The Xanthophylls separate out as crude crystals and the impurities were dissolved in the acetone/ water which were removed by filtration. This retained crude crystals of Lutein washed with distil water until the filtrate was almost colourless and the pH was neutral. The resulting Lutein obtained showed Xanthophyll content of around 50 to 60% purity by spectrophotometric analysis. Final purification was accomplished by crystallization in ethyl alcohol by dissolving 60% pure crystals in ethyl alcohol. The crystals were then filtered off and dried in vacuum at 50-60°C. The purity of this product was greater than 85% (analyzed by spectrophotometer) and Lutein content was over 90% (AUC % by HPLC method).
Fig 3.1 Scheme for conversion of Lutein and Zeaxanthin esters to free form
Isolation of Zeaxanthin from Red Pepper

The Red pepper oleoresin obtained was stored under controlled conditions and Xanthophylls content was analysed by spectrophotometry. The composition of the Carotenoids were analysed by HPLC before isolation trial. The xanthophylls Carotenoids were separated (Lutein, Zeaxanthin, β-carotene, β-cryptoxanthin and capsanthin) on a Silica column 254 mm x 4.5 mm, 5 µm. Hexane/Ethyl acetate 75:25 was used as a mobile phase for the separation. An isocratic condition was maintained at flow rate 2 ml/min. The volume of sample injected was 20 µl. Detection 450 nm. Sample was saponified with alcoholic alkali prior to the injection.
Red bell pepper oleoresin (45g) containing 6.70% total xanthophylls content and colour value 87000 cu (ASTA), Zeaxanthin comprised 57% and the remaining were trans-capsanthin, β-carotene and β-cryptoxanthin (by HPLC), was weighed on a analytical balance and transferred into a 500 ml round bottom flask. 100 ml of ethyl alcohol was added and warm until a homogenous solution was obtained. 50 ml of aqueous solution of (40%) potassium hydroxide were added to the flask. The flask was placed on a hot water bath and refluxed on low temperature (75°C) with stirring for a period of 3 hrs. The degree of hydrolysis was monitored by HPLC during the saponification stage. The solution was taken off from the water bath and ethyl alcohol was distilled off from the reaction mixture under vacuum. The solids obtained was stirred with water (200 ml) at room temperature to get a homogenous mixture. The mixture was then taken into a separatory funnel and extracted with equal volume of ethyl acetate (250 ml) 3 times by this way all the Carotenoids were recovered to ethyl acetate from the reaction mixture. Ethyl acetate layer was collected and washed with distilled water for removing the alkali. The washing of the ethyl acetate layer was repeated two more times at which point all the alkali was shown to have been removed and the pH of the aqueous wash was at pH 7. The ethyl acetate layer was evaporated in rotary evaporator (Buchi) under reduced pressure to get saponified product. The saponified product containing Zeaxanthin was dissolved in ethyl alcohol to commence crystallization. The mixture was stirred at room temperature for about 30 minutes and the solids were separated out by filtration. After removal of the filtrate, the Zeaxanthin crystals were dried under high vacuum for about 24 hrs at room temperature. This gave 0.97 gm Zeaxanthin (2.16%) which was shown by UV-Visible–HPLC to 91.70% Xanthophyll content and HPLC composition of the product gave Zeaxanthin content 75.10%.
**Preparation meso-Zeaxanthin from Lutein**

Lutein crystals prepared according to the procedure mentioned above, was refluxed with 1-proponolic alkali at higher temperature for isomeric conversion of Lutein to meso-Zeaxanthin at elevated temperature.

The Lutein crystal (20g) containing 79.52% total xanthophylls (measured by spectrophotometric method) and Lutein and RR-Zeaxanthin were 90.23% 5 and 8.344 % AUC respectively by HPLC was transferred into a three necked RB flask (250 ml capacity) followed by the addition of 1-propanol (40g) and 20g potassium hydroxide. To one neck of RB flask a water condenser was fixed passing water and through the other neck nitrogen gas was bubbled and through the central neck a stirrer was fixed for uniform mixing. The flask was fixed in an oil bath maintaining the temperature of the oil between 100-110°C. The reaction of xanthophylls ester saponification and of Lutein isomerisation was allowed for 12-18 hrs after which the resulting red coloured product (80 g) was removed and subjected to three stage
process, (i) water was added (100 ml) to get a homogenous solution (ii) pH of the homogenous solution was adjusted to neutral (pH7) with 50% acetic acid solution (iii) heated the solution to 85-90°C. The heated mixture was filtered through a Buchner funnel under vacuum and the filtrate was discarded. The crude crystals were washed with water three times at which point all the excess alkali was shown to have been removed and the pH of the aqueous phase was neutral. The crude meso-Zeaxanthin was dissolved in ethyl alcohol to commence crystallization. The mixture was stirred at room temperature for about 30 minutes and the solids were separated by vacuum filtration (Buchner). The wet meso-Zeaxanthin crystals were dried under high vacuum to obtain meso-Zeaxanthin crystal. The total xanthophylls content was 76.52% by spectrophotometry analysis (452 nm E1% 2348).

The stereo isomeric compositions of the resultant product were quantified by Chiral - HPLC using a Photo diode array (PDA) detector (452 nm) and solvent gradient consisting of n-hexane (A) and iso-propanol (B). The column used was a Chiral Pak AD, packed with silica gel (10 µm), coated with amylose tris-(3,5-dimethylphenylcarbamate) as a selector. The reference standards like meso-Zeaxanthin, Lutein and Zeaxanthin were sourced from Omni (Mumbai). The following gradient was used (min. % A 0/94.5; 40/94.5; 50/85; 55/50; 90/50; 91/94.5; 120/94.5) and flow rate was 0.7 ml/min (Breithaupt et al. 2004).

Further reconfirmation of the stereo isomeric composition was done by analysis of their dibenzoate derivatives, using the following procedures described by Ruttiman, Schiedt and Vecci (1983). The Zeaxanthin collected after drying was dissolved in anhydrous pyridine / benzene (1:1 v/v). To this was added S)-(1)-(1-Naphthyl) ethyl isocyanate, and the reaction allowed to proceed at room temperature for 2 days. The bicarbonate derivatives thus produced were analysed by HPLC, column Sumichiral OA-2000, solvent n-hexane : CHCl₃ (48:8), flow rate of 2 ml/min, Detection was at 452nm.

NMR analysis were performed on a Varian INOVA 500 MHz NMR spectrophotometer. H-NMR spectra were recorded in the stop-flow mode with 1000-2000 transients per H1-NMR spectrum. COSY spectra were acquired using 48-160 repetitions for 128 increments and 2K data points with a spectral width of 9000 hz. All spectra were measured in CDCl₃ at 25°C.
Fig 3.4 Scheme for Lutein to meso-Zeaxanthin

Fig 3.5. Thermal isomerization of Lutein, isolation and purification of meso-Zeaxanthin from Lutein.
Preparation of β-cryptoxanthin from Paprika oleoresin.

Quantification of the samples was performed by UV-VIS spectral analysis using published specific absorption coefficients. HPLC analysis was performed on a Agilent HPLC 1200 series with PDA detector with an attenuation of 0.5 AUFS. YMC-Carotenoid, 254 x 4.5 mm column was used for the chromatographic separation. Mobile phase methanol / chloroform (80:20 v/v) at flow rate 1.5 ml/minute. The peak areas were quantified with Agilent Chem Station data processor. Wave length was 450 nm.

Paprika oleoresin (1g) taken into 100 ml standard flask, dissolved in 30 ml of HEAT (hexane : Ethyl alcohol : acetone : hexane) and 5 ml of methanolic KOH (40%) was added, refluxed 30 minutes at 80°C, 30 ml hexane was added and diluted to the mark with 10% sodium sulphate solution and allowed the layers to separate. The aqueous layer was discarded, the organic layer collected and washed with distilled water to neutral pH. The organic phase was filtered through a solid bed of anhydrous sodium sulphate and evaporated off on a water bath with the aid of nitrogen gas. The residue was immediately dissolved in 10 ml of HPLC grade methanol. After passing through 0.2 mm filter 20 µl chromatographed using the condition described above. Quantitation of the sample carried out by comparing with reference standard. Retention time and area of standard and sample used for identification and comparison. HPLC has been the most effective and accurate method for the separation, identification and quantification of Carotenoids (Maoka et al. 2001a; Vesper and Nitz, 1997). As paprika contains Carotenoids esterified with fatty acids, as well as free Carotenoids, fatty acids can be removed by saponification leaving free pigments in the sample. HPLC analysis of saponified extract separates free pigments, giving details about the pigments present in the sample.

β-cryptoxanthin standard (10 mg) was taken into 100 ml volumetric flask, 30 ml of THF (Tetra Hydro Furan) added and sonicated for 5 minutes, and diluted to volume with ethyl acetate. 10ml of the solution diluted to 50 ml with methanol, filtered through 0.2 mm filter and 20 µl of this standard solution was injected into HPLC.

50mg of β-Cryptoxanthin obtained was ground in a mortar. From the ground sample 0.05 g. of β-Cryptoxanthin was taken into a 100 ml volumetric flask and 30
ml THF was added and diluted to volume with Ethyl acetate. 5 ml of the above solution was diluted to 50 ml with methanol and 20 micro litre of this solution was used for HPLC analysis.

Paprika oleoresin (50 g) containing 6.50% total Xanthophylls and a colour value of 1,05,457 units, the HPLC profile of the oleoresin being β-carotene -15.73%; β-cryptoxanthin-9.07%; Zeaxanthin -10.54% and trans capsanthin-31.38%, was weighed on a analytical balance and transferred into a 250 ml round bottom flask. 50 ml of ethanol and 12.5g of potassium hydroxide pellet were added. The reaction mixture was heated to a temperature of 80°C with stirring. The reaction process was maintained for 4-5 hours at 80-85°C with gentle agitation to ensure complete saponification of esters. The progress of the reaction was monitored by HPLC analysis. The reaction mixture was allowed to cool, and then ethanol was distilled out from the mixture under vacuum and brownish red saponified mass collected. 100 ml of water was added to the solid mass mixture and kept for agitation until a homogenous solution was obtained. The solution was then taken into a separatory funnel and extracted three times with an equal volume of ethyl acetate. The Ethyl Acetate layer was pooled and washed with water at which point alkali had been removed. The ethyl acetate layer was concentrated in a Buchi Rotary evaporator under vacuum. The residue was then dissolved in hexane and stirred at room temperature for about 30 minutes. The washing of the residue was repeated three times, and the pooled hexane extract was evaporated to dryness under vacuum by rotary evaporator (Buchi). The HPLC profile of the hexane concentrate showed β-cryptoxanthin around 10%.

β-carotene, β-Cryptoxanthin, Zeaxanthin and trans-capsanthin were separated by column chromatography. Glass column was used for the experiment. Silica was used as the stationary phase (100-200 mesh). The concentrate obtained in the previous step was re-dissolved in minimum volume of hexane and loaded on the column. β-carotene was eluted with hexane, fraction rich in β-cryptoxanthin was eluted with Hexane/Ethyl acetate and finally Zeaxanthin and capsanthin were eluted with acetone. All fractions were concentrated separately and analysed by spectrophotometry and HPLC. The product obtained after the concentration of hexane/ethyl acetate fraction which is rich in beta-cryptoxanthin (Fraction 2) was
dissolved in ethanol (1:2w/v) and stirred for 1 hr and chilled for 8hrs at 10°C. The slurry from the above crystallization, the β-cryptoxanthin was separated by filtration and dried under vacuum to get β-cryptoxanthin crystals. The crystal thus obtained (0.22 g) with more than 40% total Xanthophylls content. The HPLC profile of the crystal showed β-cryptoxanthin 98.3% AUC.
Results and Discussion:

Table 3.1. Experimental data of Lutein process

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<th>No</th>
<th>Sample wt (gm)</th>
<th>% xanthophylls</th>
<th>KOH (gm)</th>
<th>Alcohol (ml)</th>
<th>Reaction time (hrs)</th>
<th>pH of the solution</th>
<th>Acetone (ml)</th>
<th>Dried crystal (gm)</th>
<th>Xanthophylls %</th>
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Fig 3.6 HPLC analysis of Marigold Oleoresin

HPLC analysis of Marigold Oleoresin chromatographed on Silica column using Hexane/Ethyl acetate (75:25) mobile phase at flow rate 2ml/min. Injection volume was 20 µl. Absorbance monitored at 474 nm with a UV-VIS detector. Peak No. 5 – Lutein, 6 – Zeaxanthin, 7, 8, 9 cis & epoxides.

Fig 3.7 HPLC analysis of Lutein crystal

HPLC analysis of Lutein crystal. Chromatographed on Silica column using Hexane/Ethyl acetate (75:25) mobile phase at flow rate 2ml/min. Injection volume was 20 µl. Absorbance monitored at 474 nm with a UV-VIS detector. Peak no. 4 - Lutein, 5 - Zeaxanthin.
Fig 3.8 CD spectra of Lutein

CD spectra of Lutein in Et2O at room temperature with a JASCO J-720 WI spectrophotometer.

Fig 3.9 -¹H-NMR of Lutein in CDCL3
$^1$H-NMR of Lutein in CDCL3 (500 MHz) analysed in Varian Inova 500 MHz NMR,
Fig 3.10 HPLC analysis of red pepper oleoresin

HPLC analysis of red pepper oleoresin after saponification. chromatographed on Silica column using Hexane/Ethyl acetate (75:25) mobile phase at flow rate 2ml/min.Detection at 450 nm. RT -19.9 RR-Zeaxanthin.

Fig 3.11 HPLC analysis of red pepper isolate

HPLC analysis of red pepper isolate. chromatographed on Silica column using Hexane/Ethyl acetate (75:25) mobile phase at flow rate 2ml/min.Detection at 450 nm RT-20.00 RR Zeaxanthin.
Table 3.2. Experimental data of Zeaxanthin isolation from Red pepper

<table>
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<tr>
<th>Sl No</th>
<th>Oleo quantity (g)</th>
<th>40% alc.KoH (ml)</th>
<th>Saponification time Hrs</th>
<th>Saponification temperature °C</th>
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<td>90</td>
<td>3</td>
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<td>2.16</td>
<td>91.70</td>
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<tr>
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<td>95</td>
<td>3</td>
<td>80-85</td>
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<td>1.91</td>
<td>88.59</td>
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Table 3.3. Preparation of meso-Zeaxanthin from Lutein - experimental data.

<table>
<thead>
<tr>
<th>Lutein gm</th>
<th>Solvent</th>
<th>Solvent ml</th>
<th>Base gm</th>
<th>Reaction time hrs</th>
<th>Reaction temperature ◦C</th>
<th>Chiral analysis Lut/RR/RS-zea</th>
</tr>
</thead>
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<tr>
<td>20</td>
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<td>30</td>
<td>26</td>
<td>10</td>
<td>102</td>
<td>81.50/8.3/5.5</td>
</tr>
<tr>
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<td>30</td>
<td>26</td>
<td>15</td>
<td>102</td>
<td>74.49/8.35/9.30</td>
</tr>
<tr>
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<td>26</td>
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<td>66.65/8.56/17.70</td>
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<tr>
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<td>20</td>
<td>5</td>
<td>105</td>
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<td>20</td>
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<td>105</td>
<td>8.13/7.92/83.04</td>
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Fig 3.1-Chiral HPLC analysis of Lutein

Chiral HPLC analysis of Lutein. Chromatographed on ChiralPak AD column using n-hexane / iso-propanol mobile phase, gradient elution at flow rate 0.7ml/min. PDA detection at 452 nm. Peak 1-RR-Zeaxanthin, Peak 2-Lutein.

Fig 3.13-Chiral HPLC analysis of RR-Zeaxanthin

Chiral HPLC analysis of RR-Zeaxanthin. Chromatographed on ChiralPak AD column using n-hexane / iso-propanol mobile phase, gradient elution at flow rate 0.7ml/min. PDA detection at 452 nm.
Chiral HPLC analysis of Meso-Zeaxanthin crystals. Chromatographed on ChiralPak AD column using n-hexane / iso-propanol mobile phase, gradient elution at flow rate 0.7ml/min. PDA detection at 452 nm. Peak 1-RS –Zeaxanthin (meso-Zeaxanthin), Peak 2-RR-Zeaxanthin, Peak 3-Lutein.
Chiral HPLC composition of Zeaxanthin dibenzoate. Chromatographed on SumiChiral OA column, mobile phase n-hexane : chloroform (48:8), flow rate 2ml/min and detection at 452nm. meso-Zeaxanthin : RR-Zeaxanthin (86.6:13.4).
Normal phase HPLC of mos-Zeaxanthin product chromatographed on Silica column, n-hexane: acetone (9:1) mobile phase. Lutein : Zeaxanthin isomers, 8.08:92.06
Fig 3.17. NMR of Zeaxanthin

$^1$H-NMR NMR of Zeaxanthin

NMR of Zeaxanthin measurements was performed on Varian Inova 500 MHz NMR.

Fig 3.18-NMR of Zeaxanthin dibenzoate

$^1$H-NMR NMR of Zeaxanthin dibenzoate

NMR of Zeaxanthin dibenzoate measurement was performed on Varian Inova 500 MHz NMR.
Fig 3.19 $^1$H-NMR of Zeaxanthin benzoate in CDCL$_3$

$^1$H-NMR of Zeaxanthin benzoate in CDCL$_3$ (500 MHz)

$^1$H-NMR of Zeaxanthin benzoate in CDCL$_3$ measurements were performed on a Varian Inova 500 MHz NMR.
Fig 3.2 $^1$H-NMR of Zeaxanthin benzoate in CDCl$_3$

$^1$H-NMR of Zeaxanthin benzoate in CDCl$_3$ measurements were performed on a Varian Inova 500 MHz NMR.
Fig. 3.21 COSY NMR of Zeaxanthin

COSY NMR of Zeaxanthin measurements were performed on a Varian Inova 500 MHz NMR.
Fig 3.22-1H NMR of Zeaxanthin in CDCl3

1H NMR of Zeaxanthin in CDCl3 measurements were performed on a Varian Inova 500 MHz NMR.
Fig. 3.23 Analysis of β-cryptoxanthin

Analysis of β-cryptoxanthin standard chromatographed on YMC-Carotenoid column, mobile phase methanol / chloroform (80 :20 v/v) at flow rate 1.5 ml /minute and detection at 450 nm.

Fig 3.24 HPLC analysis of Paprika oleoresin

HPLC analysis of Paprika oleoresin chromatographed on YMC-Carotenoid column, mobile phase methanol / chloroform (80:20 v/v) at flow rate 1.5 ml /minute and detection at 450 nm.
Fig 3.25 HPLC analysis of β-cryptoxanthin isolated from paprika

HPLC analysis of β-cryptoxanthin isolated from paprika chromatographed on YMC-Carotenoid column, mobile phase methanol / chloroform (80 :20 v/v) at flow rate 1.5 ml /minute and detection at 450 nm.

Fig 3.26 HPTLC data of Lutein, Zeaxanthin and meso-Zeaxanthin

HPTLC data of Lutein, Zeaxanthin and meso-Zeaxanthin. scanning was performed on a CAMAG TLC. Mobile phase – Dichlromethane : Ethyl acetate (8:2)v/v. silica gel thickness : aluminium backed silica plates (Merck F254), chamber saturation time: 15 mins, temperature: RT (28-32C), length of chromatogram run :8 mm, absorbance mode:254 nm
HPTLC data of Lutein, Zeaxanthin and meso-Zeaxanthin scanning was performed on a CAMAG TLC. Mobile phase—Dichloromethane:Ethyl acetate (8:2)v/v.

Graph -Stability study data of Lutein 0-90 days. Stored at RT with nitrogen and -20 with nitrogen. 5gm each packed in a poly propylene packet, nitrogen was purged before sealing the packet, each product 14 x 5 gm packet.
Fig 3.29 Graph- stability study data of Zeaxanthin 0-90 days.

Graph- stability study data of Zeaxanthin 0-90 days. Stored at RT with nitrogen and -20 with nitrogen. 5gm each packed in a poly propylene packet, nitrogen was purged before sealing the packet, each product 14 x 5 gm packet.

Fig 3.30 Graph-Stability study data of meso-Zeaxanthin 0-90 days.

Graph-. Stability study data of meso-Zeaxanthin 0-90 days. Stored at RT with nitrogen and -20 with nitrogen. 5gm each packed in a poly propylene packet, nitrogen was purged before sealing the packet, each product 14 x 5 gm packet.
Fig 3.31 Graph- stability study data of beta-Cryptoxanthin 0-90 days.

Graph- stability study data of beta-Cryptoxanthin 0-90 days. Stored at RT with nitrogen and -20 with nitrogen. 5gm each packed in a poly propylene packet, nitrogen was purged before sealing the packet, each product 14 x 5 gm packet.
The process of isolation of Lutein from Marigold Oleoresin was done as explained in isolation of Lutein from Marigold extract and Fig.3.2 of process of flow diagram of isolation of Lutein. As explained, Lutein esters were saponified with alcohol and potassium hydroxide, followed by the distillation of alcohol and addition of water /acetone mixture, pH correction and filtration. The obtained semi purified crystals were further purified and dried in vacuum to get product. The method provides the preparation of high purity Lutein crystals with high yield.

Further it was analyzed by UV spectrophotometer and HPLC. The obtained final product contains 70-80% Xanthophylls, which contain more than 90% trans-Lutein and 4 to 5% of Zeaxanthishin (Fig 3.7 and 3.8). The Lutein isolated by this method, has high purity and contains no toxic compounds or hazardous solvent residues and hence suitable for human consumption.

To confirm the structure of free Lutein after purification, the sample was analysed by H-NMR. The H-NMR result in Fig.3.10 showed the similar peak pattern of Khachik work (Khachik et al.1995). From the data it can be concluded that the purified Lutein through the current process contains only free Lutein (Fig 3.9 & 3.10).

The process of isolation of Zeaxanthishin from red pepper extract was done as explained in isolation of Zeaxanthishin form red pepper extract and Fig.3.3 of process of flow diagram of isolation of Zeaxanthishin. Fig 3.11 and 3.12 are HPLC analysis of red pepper oleoresin and isolate chromatographed on Silica column using Hexane/Ethyl acetate (75:25) mobile phase at flow rate 2ml/min. Detection at 450 nm. The HPLC analysis showed Zeaxanthishin comprised 57% and the remaining were trans-capsanthin, β-carotene and β-cryptoxanthishin. The pigment content in paprika fruits is depended on the verity, degree of ripeness, time of harvest, growing and storage conditions. (Deli et al.1996 ; Molnar et al.2001b).

Table 3.2 gives experimental data for the preparation Zeaxanthishin from paprika oleoresin. It can be seen that the xanthophylls content of the isolate showed more than 85% and HPLC composition showed more than 70%. The method employed for the preparation of Zeaxanthishin, saponification and extraction with ethyl acetate, followed by the crystaillation technique helped us to selective elimination of unwanted material such as waxes, sterols and other associated Carotenoids present in the starting material. Figs 3.11 and 3.12 shows the HPLC analysis of red pepper oleoresin and
Zeaxanthin isolate. The acute oral toxicity study of Zeaxanthin isolated from paprika on animals studied are described further in chapter 4.

Table 3.3 gives experimental data of Lutein to meso-Zeaxanthin by thermo alkali treatment, analysed by chiral column chromatography (Breithaupt et al., 2004). It can be seen that the chiral composition of the product showed more than 83% meso-zeaxanthin in one product. Fig 3.12, 3.13 and 3.14 shows chiral chromatogram of Lutein crystal, RR-Zeaxanthin and thermo alkali treated Lutein crystal respectively. It is observed that the Lutein crystal sample not showing a new peak is indicative of the absence of R,S-Zeaxanthin. However, the thermally treated reaction condition showing a clear separation of R,R-Zeaxanthin and R,S-Zeaxanthin. Further, the Lutein peak gets reduced indicating that the R,S-Zeaxanthin is derived from Lutein. Fig 3.17 shows the normal phase HPLC of the thermo alkali treated Lutein, which contains 92% Zeaxanthin isomers. Fig 3.16 the benzoate derivative of thermo alkali treated Lutein, the composition of the stereoisomer’s showing 86.60% R,S-Zeaxanthin and 13.40% R,S-Zeaxanthin. Fig3.18, 3.19, 3.20, 3.21 and 3.22 are the structural elucidation of the meso-Zeaxanthin by NMR.

The isolation and chromatographic separation methodologies employed provides high purity β-cryptoxanthin from paprika extract. Yamaguchi proposed that β-cryptoxanthin may be a preventive remedy for bone diseases such as osteoporosis (Yamaguchi et al.2004). In humans β-cryptoxanthin acts as a chemo preventive agent for lung cancer. (Yuan et al.2003). Craft et al. found that the major xanthophylls found in the human brain belongs β-cryptoxanthin.. Takuji et al. found that special fractions of Satsuma mandarins (Citrus unshiu) containing β-cryptoxanthin inhibit chemically induced rat colon and mouse lung tumorigenesis (Tanaka et al.2002). Fig 3.25 shows HPLC chromatogram of saponified paprika extract. This demonstrates that the β-cryptoxanthin peak is detected after saponification. Several small and big peaks eluted before and after the β-cryptoxanthin peak were identified as β-carotene, Zeaxanthin, Lutein, capsanthin and capsorubin etc. The investigation into the colouring matter of paprika were pioneered by Zechmeister and Cholnory (1937), who identified red colour component as capsanthin and capsorubin and yellow component as beta carotene, Zeaxanthin and cryptoxanthin (Cholnoky et al.1937). The percentual proportion of the cryopxanthin was 3-5%.
The study data shows that it provides a β-cryptoxanthin concentrate, which contains more than 40% by weight total Xanthophylls out of which 98% by weight being β-cryptoxanthin by HPLC analysis which comprises mixing the paprika oleoresin with an aliphatic alcohol, saponifying the Xanthophylls esters present in the oleoresin with an alkali by applying heat to elevate the temperature. The alcohol was distilled off and water was added to the reaction mixture followed by the addition of ethyl acetate. The ethyl acetate layer was collected and concentrated under vacuum. The residue obtained washed with hexane, concentrated the hexane layer and subjected to column chromatography separation.

The β-cryptoxanthin collected from the Ethyl Acetate/ Hexane fraction was concentrated and further washed with alcohol to get the crystals of high purity. The HPLC analysis of the crystal showed more than 98.30% AUC by HPLC analysis which is clearly demonstrates that other associated caroteneoids present in the paprika get eliminated during the process stage their by resulting in greater purity.(Fig .3.26). Cholnoky et al (1958) established a column chromatography method using a saponified extract of paprika following chromatography on a calcium carbonate and calcium hydroxide column. The fractions were separated on the column eluted from the column with methanol. Egger and Voigt (1965) reported that polyamide is suitable for as solvent system for the separation of Carotenoids. Montag (1962) tried to separate the Carotenoids by stepwise development in an activated Kieselgel G layer, using chloroform; acetic acid (65:2) in the first step and benzene in the second step. Vinkler and Kiszel –Richter (1970) studied the chromatographic method to determine the pigment content in the paprika.

The Thin –Layer Chromatography of isolated samples (Fig 3.27 & 3.28) were carried out using CAMAG IV (CAMAG,Switzerland). The samples were spotted on percolated silica gel with 0.25 mm thickens (MerckF254)using sample applicator. Mobile phase –Dichlromethane : Ethyl acetate (8:2)v/v, chamber saturation time : 15 mins, temperature : RT (28-32C), length of chromatogram run : 8 mm, absorbance mode : 254 nm.
Reference:

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