Research Article

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STUDIES ON CHIRAL ISOMERS COMPOSITION OF THERMO ALKALI TREATED MARIGOLD EXTRACT

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ABSTRACT:

Lutein and zeaxanthin are the xanthophylls carotenoid pigments contributing the characteristic yellow-reddish colour to fruits, vegetables, corn and egg yolk. These carotenoids may be present in free and as well in ester form. Marigold flower petals and its extracts are rich sources of (3R,3'R,6'R)-β ε-carotene-3,3'- diol (R,R-lutein) and (3R,3'R) -ββ-carotene-3,3'-diol (RR-zeaxanthin) . Lutein and zeaxanthin show both optical (chiral) and geometrical (trans,E and cis,Z) isomers. The chiral isomers of zeaxanthin comprise of R, R-, R, S- (meso-) and S,S-zeaxanthin . Lutein and plant extracts containing lutein treated with alkali and at high temperatures undergo reaction process wherein lutein is converted into R,S-zeaxanthin(meso-) by allylic isomerisation while R,R-zeaxanthin remains unaltered. The present study describes the reaction conditions, the identification and quantification of R,R- and R,S-zeaxanthin (meso-) and lutein in the reaction products, using Chiral Chromatography .

KEYWORDS:

Marigold flower petals, Lutein and zeaxanthin, Chiral Isomers Composition

INTRODUCTION

The characteristic yellow-red colour of marigold flower petals and its extracts is due to the xanthophylls carotenoids comprising of lutein and its stereo isomer zeaxanthin. Lutein and zeaxanthin are found particularly leaves, fruits and vegetables and help in light harvesting process in photosynthesis. Lutein and zeaxanthin are present in human body, blood, adipose tissue, skin and brain and higher amounts accumulating in macular pigment in macular region of retina and in other parts of eye in trace amounts. Mammalian species do not synthesise carotenoids and therefore both lutein and zeaxanthin have to be obtained from dietary sources such as fruit, vegetables and egg yolks.

In addition to lutein (L) and zeaxanthin (Z) found in macular pigment one more isomeric form of zeaxanthin ,R,S-zeaxanthin (MZ)in substantial quantities is found within the retina.The biological functional role of these carotenoids is its ability to filter light from phototoxic damage to the tissues and the eye and fight against free radicals in the retina. Dietary supplementation of lutein extracts containing Lutein,Zeaxanthin and meso-Zeaxanthin is reported to increase macular pigment density and helps in visual function by reducing the damaging effect causing cataract and Age Related Macular Degeneration (ARMD) . (Bone RA,Landrum JT, Cao Y , Nutr Metab ,2007).

In continuation of our studies on lutein extracts we investigated the chiral isomeric composition of (1) saponified extract of marigold isolate (2) saponified extract of marigold isolate subjected to higher temperature and different period using 1-propanolic alkali free of water. The resulting products of the above were analysed by normal phase HPLC and chiral methods. The chromatographic data showed lutein isomerisation resulting to R, S-zeaxanthin which can be identified only chiral HPLC.

There have been a number of studies attempting to convert lutein into zeaxanthin. In 1971-72 Buchecker et al. assigned R-chirality to lutein based on PMR analysis and attempts to isomerise lutein to R, R-zeaxanthin failed (Chimia, 25, 192, 1971; ibid, 26, 134, 1972). Andrewes et al. (1974) in the Journal Acta Chem. Scand., B28, 139 (1974) reported the stereo chemical aspects of isomerisation reaction of (3R, 3'R, 6R)-lutein (optically active) which resulted in (3R, 3'S)-zeaxanthin which was trans-isomeric and optically inactive based on CD spectral studies. The above process resulted in low yield of 10 to 15% optically inactive (3R, 3'S, meso)-zeaxanthin and used benzene and DMSO. Rodriguez described a method of isomerising lutein to yield a mixture of zeaxanthin epimers by employing non aqueous media and heating a mixture of alkali and propylene glycol. (U.S.Pat. No. 5,973,211, 1999). Torres- Corodona reported the conversion of lutein containing substrate either in free form or in ester form into zeaxanthin using aqueous alkali solution under pressure reaction as well as atmospheric condition resulting in a product with higher pigmenting activity. The patent reports 15-24% conversion of lutein into zeaxanthin as analysed in the resultant mixture (US patent No.5, 523, 494, 1996). US Patent No. 5,780,693 July 1998 Bernhard et al , developed a process for converting lutein and its esters in the presence of different organic solvents and finally obtained sufficiently increased amount of zeaxanthin in the resultant product . In all the above studies the use of normal phase column, although showed increased zeaxanthin peak, the conformation of R,S-zeaxanthin is possible only by CD spectral and chiral HPLC.

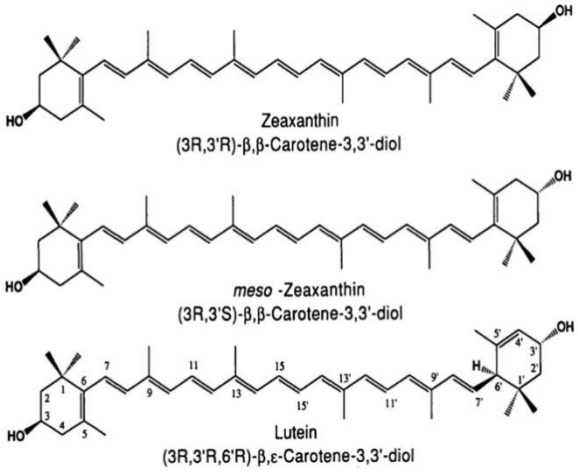

Fig.1. Chemical structure of Lutein ,Zeaxanthin and meso-Zeaxanthin.

MATERIALS AND METHODS

Chemicals: Ethyl alcohol, acetone, hexane, ethyl acetate, 1-proponol, n-hexane, 2-propanol, methanol, Toluene, Sodium sulphate were purchased form Merck; potassium hydroxide was purchased from a local dealer. All solvents were checked for their purity by GC Analysis. High purity water was prepared with a Millipore water purification system.

Instrumentation;

The analysis of the lutein cake were carried out by normal phase HPLC analysis .The Agilent Technology 1200 HPLC system equipped with with DAD, gradient pump, a degasser, column thermostat and auto Sampler . The separation was carried out in a Phenomenex silica analytical column 250 x 4.6 mm id; particle size 5 um with a Silica guard column was used for normal phase analysis .The mobile phase consisting of hexane and ethyl acetate (75/25). All Solvents were filtered through a syringe filter prior to the analysis. The flow rate was 2 mil /min for total run time 45 min. the Detector was set at 474 nm and Chemstation

software was used for data processing. The injection volume was 20 uL.

For the identification of R, S-Zeaxanthin **,** R,R-zeaxanthin and R,R-lutein in the composition of the present study, a chiral HPLC column was used and employing chiral liquid chromatography (LC). The xanthophylls present in the composition were quantified by HPLC Agilent Model 1260, using a Photo diode array detector (450 nm) and solvent gradient consisting of n-hexane 100 % (A) and iso-propanol and 1-propanol (1:1) (B). The column used was a ChiralPak AD-H 250mm x 4.6 mm ,5u, coated with amylase tris-(3,5-dimethylphenylcarbamate) as a selector. The reference standards R,S-zeaxanthin ,lutein and R,R -zeaxanthin were sourced from OmniActive Health Technologies Ltd,Mumbai.

The following gradient (flow rate 0.5 ml/min.) was used (min. 0-50 A90/ B10 ; min 50-60 A60/B40 ; min 60-70 A90/B10).column temperature 25 degree.C. Sample was prepared 5 g of the reaction mass dissolved in 100 ml of HEAT solution (Hexane /Ethyl alcohol/ Acetone / Toluene, 10:6:7:7) and 1 ml of the solution evaporated by using vacuum pump and reconstitute with 10 mil mobile phase . Filter the solution using 0.22 u nylon membrane filter paper and inject into the HPLC.

Experiment;

Marigold meal (dehydrated marigold flower) was obtained from OmniKan Earth Science dehydration plant in Hassan, Karnataka, India. The meal was extracted with hexane solvent in the laboratory to get marigold oleoresin. The experiments were carried out at various scale 500 gm to 1000 gm. The extracts were determined by weight after hexane evaporation in a Buchi evaporator at 55-60 degree.C under vacuum. The totoal xanthophylls were determined by specrophtometrically at 474 nm by AOAC method. The xanthophylls concentration ranged from 17-18% in the extracts.

The lutein crystals were separated from the oleoresin by following the method mentioned in US patent 6,743,953, the extract was saponified with alcoholic alkali solution (40%) at controlled temperature, and the progress of the reaction was monitored by HPLC. Form the resultant mixture the alcohol was removed by vacuum distillation. Water was added to the reaction mixture and the resultant reaction mixture was extracted with ethyl acetate solvent (4 times). The combined ethyl acetate layer was washed with equal volume of water. The ethyl acetate layer was separated and filtered through anhydrous sodium sulphate. The ethyl acetate layer was evaporated to get saponified oleoresin. The lutein crystals were separated from the saponified oleoresin by mixing saponified oleoresin with mixture of hexane/acetone (80/20), filtered and resultant residue was again washed with ethyl alcohol. The filtered wet cake was dried under vacuum at 55-60 degree's. The xanthophylls concentration of the crystal was 80.20% w/w, estimated by AOAC method) and the relative percentage of lutein and RR-zeaxanthin were estimated by normal phase HPLC. (92.13% trans-lutein and 8.25 % R, R- zeaxanthin).

Table 1: Data of Lutein and Zeaxanthin composition (%) in marigold flower and its products.

Sl No	Lutein products	Lutein %	Zeaxanthin %	L/Z Ratio
1	Marigold flower	88-90	4-4.5	1:20-22
2	Marigold Extract	76-78	4-4.5	1:17-19
3	Marigold Isolate (ester)	90-91	7-8	1:11-12
4	Marigold Isolate (saponified)	91-92	8-8.5	1:11-12

For preparation of R,S-zeaxanthin(meso-) ,the lutein crystal obtained was refluxed with alkali and 1-propanol at elevated temperature and the resultant mixture was analysed at various intervals and estimated the trans-lutein and isomeric composition of zeaxanthin by chiral HPLC analysis. It can be seen that from the given data the meso-zeaxanthin level increased to 83.04 % at ratio of lutein : 1 propanol : pot hydroxide at 1:1:1 ratio. The reaction time was 20 hrs at 105^0C.

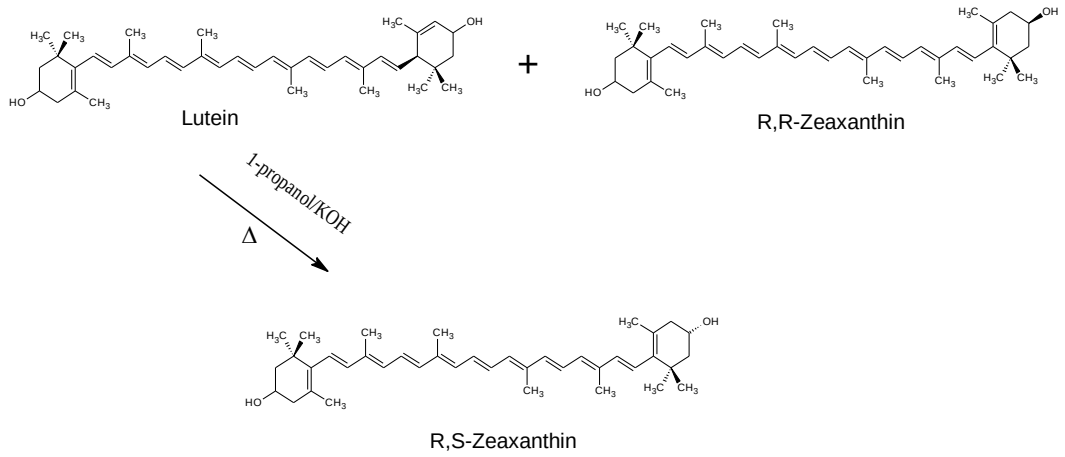

Fig.2 .Scheme of reaction of Lutein isomerisation to R,S-zeaxanthin.

Table 2: Data of reaction condition of Lutein conversion to R,S-zeaxanthin and chiral isomers composition (using normal and chiral HPLC)

	Relative Initial Product Composition					**Relative Composition**	**Final Product**
Experiment	**Initial L /Z**	**Solvent**	**Solvent /Base Ratio**	**Reaction Time hrs**	**Reaction temperature**	**Final L/Z**	**Chiral analysis % L,RR and RS Zea**
1	91.2/8.3	1-Propanol	1:1.5	10	10	81/13.8	81.50 ,8.3,5.5
2	91.2/8.3	1-Propanol	1:1.5	15	15	74/17	74.49,8.35,9.30
3	92.13/8.1	1-Propanol	1:1.5	25	25	66/26	66.65 ,8.56,17.70
4	92.13/8.1	1-Propanol	1:1	5	5	60/31	60.62,8.42,23.48
5	92.13/8.1	1-Propanol	1:1	10	10	29/63	29.02,8.38,55.61
6	92.13/8.1	1-Propanol	2:1	15	15	23/72	23.49,8.62,64.10
7	92.13/8.1	1-Propanol	2:1	20	20	8.13/90	8.13,7.92,83.04

Table 3.Comparative data of experiments of Lutein conversion to Zeaxanthin carried out by earlier research groups.

	Relative Initial Product Composition					**Relative Final Product Composition**		
Experiment	Initial L /Z	Solvent	Solvent / Alkali Ratio	Reaction Time hrs	Reaction temperature	Final L/Z	Chiral analysis % L,RR and RS Zea	Remarks
	93.1 /6.6	Hexane	4:1 (7M KOH)	15	64 C	15.8/ 83.1	__	Starting material
	93.1 /6.6	Heptane	4:1 (7MKOH)	1.5	94 C	18.1 / 80.0	---	
	93.1 /6.6	Petroleum Ether	3:1 (7MKOH)	1.5	84 C	16 /84.0	---	Marigold oleoresin
	93.1 /6.6	Hexane	2:1	4.5	66 C	21.4 /77.1	---	23.9 g/kg lutein
Bernhard et al, US Patent No. 5,780,693 ,1998.	91.3 / 6.6	DMSO	1:1 (10.7 M KOH)	23	82 C	24 /68	__	FloraGlo Lutein Xanthophyll content 739 g/kg
	91.3/6.6	DMSO	1:1 (10.7 M KOH)	22	83 C	28.8 / 68.2	---	
	91.3 / 6.6	DMSO	2:1(14.7MKOH)	1	107 C	2.9 /27.2	----	
	91.3/ 6.6	DMSO	2:1 (14.3 MKOH)	1	98-99	24.5/ 74.1	----	
	90.9 /5.3	DMSO	2:1 (10.7 M KOH)	21	80 C	40.2 / 54.5	----	Xantopina Plus 351 g/ kg
Torres – Cardona et al, US Patent No. 5, 523, 494, 1996.	85.5 / 4.5	water	1:1 (KOH)	48	80 C	68.9 / 16.1	----	97 gm / kg xanthophylls content.
	85.5/ 4.5	water	1:1 (NaOH)	30	90 C	66.2/ 18.7	----	
	85.5/ 4.5	water	1:1(Na2CO3)	3	105 C	60.8 / 24.0	----	
	85.5/ 4.5	water	1:1(KOH)	36	95 C	68.3/ 15.8	-----	
Rodriguez, US patents No .5, 973,211, 1999.	4.80 zeaxanthin	PG	1: 0.5 (KOH).25 (NaOH)	5	100-106 C	59.80 Zeaxanthin	----	

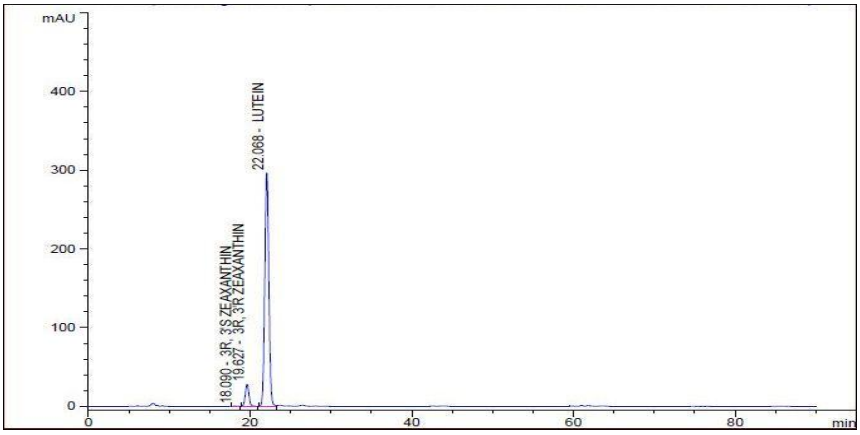

Fig 3 .Chiral HPLC chromatogram of saponified marigold isolate, Lutein crystals

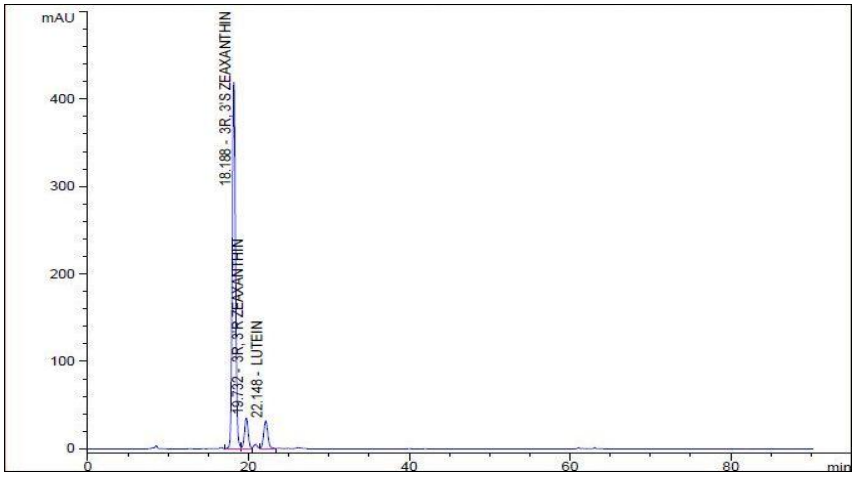

Fig 4: Chriral HPLC chromatogram of thermally treated saponified marigold isolate.

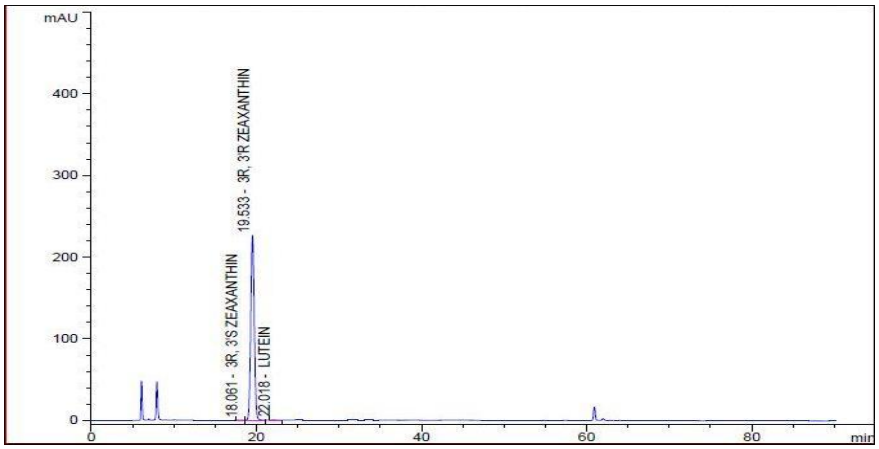

Fig 5: Chiral HPLC chromatogram of RR-zeaxanthin.

RESULTS AND DISCUSSION

Table 1 gives data on normal phase chromatographic separation of fresh marigold flower, marigold extract, marigold ester concentrate and marigold saponified isolate, lutein crystals. Tabale-2 gives data on normal phase chromatographic separation and also chiral composition data of Lutein ,Zeaxanthin and meso-Zeaxanthin of lutein crystals and thermal treated lutein crystals. Table 3 shows comparative study data of experimental condition of lutein conversion to zeaxanthin reported by earlier research groups. Fig 1,2 and3 show chiral chromatogram of saponified marigold isolate, lutein crystal , thermo alkali treated saponified marigold isolate , lutein cryatals and R,R-zeaxanthin standard.

The data of table 1 shows a clear separation of lutein and R,R-zeaxanthin in all the samples , marigold fresh flower petals , marigold extract , marigold ester concentrate and saponified marigold isolate. Table 2 show data on chromatographic separation of Lutein and Zeaxanthin in lutein crystals obtained by normal phase and also chiral HPLC column . It can be observed lutein crystal sample not showing a new peak is indicative of the absence of R,S-zeaxanthin . However, the thermally treated reaction condition show (1) normal phase column showing enhanced zeaxanthin peak and (2) chiral column showing a clear separation of R,R-zeaxanthin and R,S-zeaxanthin . Further , the lutein peaks gets reduced indicating that the R,S-zeaxanthin is derived from lutein. Table 3 shows a summary of the thermally treated reaction condition, change of concentration of reagents and pressure, carried out by earlier research groups. This table also includes data obtained from their laboratory.

It may be observed that for the effective conversion of lutein to zeaxnthin depends on the factors such as solvents,ratio of alkali,reaction time and temperature.Also the identification in n-HPLC only shows the product composition of lutein and zeaxanthins whereas Chiral analysis gives the optically active stereo isomeric compositions of the zeaxanthin isomers. Chiral analysis is a valuable tool to obtain reliable results of chiral isomeric composition of zeaxanthin.

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