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Original Research Article

Simple and rapid extraction method for determination of carotenoids in the edible parts of *Vitis vinifera*, *Vaccinum* sect. *cyanococcus*, *Ipomoea batatas* and *Capsicum annum*

ABSTRACT

Fruits and vegetables are rich source of carotenoids. The aim of this study was to find out the rapid and simple extraction method for carotenoids in grapes, blueberries, sweet potato and green chilli using HPLC analysis. Four different extraction methods; MeOH:DCM [methanol: dichloromethane], MeOH:DCM exhausted, MeOH simple and hexane exhausted were evaluated for the determination of carotenoids. Among them, MeOH: DCM has yielded higher in all carotenoids concentrations than the other three methods. Using the MeOH: DCM method, lutein was found predominantly in green chilli (12.8 μg g^{-1}) followed by blueberries, sweet potato and grapes. Consequently, β-carotene was rich in sweet potato (69.2 μg g^{-1}). Intake of 100g sweet potato can provide 96.1 % RDA of vitamin A for 9-13 year males and females and 75% RDA for pregnant women. The result of this study could be useful in future pharmacological and nutraceutical research.

Key words: Lutein; β-carotene; violaxanthin; zeaxanthin; HPLC

1. INTRODUCTION

Fruits and vegetables are some of the excellent sources of minerals and carotenoids. Among the carotenoids, only lutein and zeaxanthin are accumulated in macula of the retina and these are collectively referred to as macular pigment [1]. Consumption of carotenoids rich in fruits and vegetables help to protect against Age related macular degeneration (ARMD) [2],

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cataracts and cardiovascular diseases [3]. Deficiency of Vitamin A is a major health problem in children and adults from India. However, β-carotene act as precursor for Vitamin A and helps protect night blindness, xerophthalmia, corneal ulceration and vision disability [4].

Grapevine (*Vitis vinifera* L.), blueberries (*Vaccinium* sect. *Cyanococcus* Rydb.) and sweet potato (*Ipomoea batatas* L.) are rich in phytochemicals including flavonoids. Green chilli (*Capsicum annum* L.) is an immature fruit, contains vitamin A, and C, quercetin, luteolin and capsaicinoids [5]. Isolation and determination of carotenoids through high performance liquid chromatography (HPLC) equipped with diode array detector (DAD) is a sensitive, reliable and accurate method. The aim of this study was to quantify and discover nutritionally important carotenoids concentration in edible parts of grapes, blueberries, sweet potato and green chilli. The results of the study will be useful for identification of new sources of bioactive carotenoids from vegetables, fruits and tubers.

2. MATERIAL AND METHODS

2.1. General experimental procedures

Two fruits (grapes and blueberries) and one vegetable (green chilli) as well as one tuber (sweet potato) were collected during the year 2017, from local supermarket and vegetable market in Kumbakonam, Tamil Nadu, India. The samples was identified and authenticated from Department of Agricultural Botany, PRIST University, School of Agriculture, (Thanjavur, Tamil Nadu, India). The details of botanical name, common name, family and edible part tested in each species are presented (Supplementary Table S2; online only). All the samples were cleaned before they were used and analyzed in triplicate for carotenoids concentration through HPLC. All of the sample extraction and purification procedures were carried out under dim yellow light conditions at room temperature of 20°C to protect the carotenoids from degradation through the

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process of photo-oxidation [6]. The edible portion of each fresh fruits and vegetables (50g) was ground well (particle size ~50 µm) separately in a blender.

2.2. Extraction methods

2.2.1. MeOH: DCM method

This method is little modified method of our earlier study of evaluation of carotenoids in dried seed samples of pea and chickpea [7]. MeOH and DCM extraction solvent (v/v; 1:1) were initially premixed with an antioxidant 0.1% butylated hydroxyl toluene (BHT) and added at the rate of 5 ml for 1 g of sample tissue in the 15 ml Pyrex tubes. Samples were vortexed gently followed by shaking at 200 rpm for 1 h and then 750 µl extract was treated with 750 µl of diluent 100% acetonitrile to remove proteins and some lipids and centrifuged at 10,000g for 5 min. Subsequently, the supernatant was filtered with 0.2 µm membrane filter, placed in 2 ml amber glass vials and analyzed through HPLC.

2.2.2. MeOH:DCM exhausted method

This method is unpublished, and it's similar to the method of [8] but it has fewer modifications. The MeOH and DCM extraction solvent (v/v; 1:1) were initially premixed with an antioxidant 0.1% BHT and added at the rate of 10 ml in 5 g of sample tissue in the 15 ml Pyrex tubes, then vortexed gently. Later, soaked samples were kept at 4°C for 16 h followed by shaking at 150 rpm for 1 h and vortex again and let rest for 10 min. The supernatant off settled sample was decanted, pour the extract and place in to 25 ml beaker then purged with nitrogen gas until complete dry. The dry sample was dissolved with 1 ml of extraction solvent. Subsequently, the content was shaked it gently followed by 1ml of sample was centrifuged using 2 ml

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eppendorf tube at 11,000 rpm for 5 min. Supernatant was filtered with 0.2 µm membrane filter, filtrate was analyzed by HPLC.

2.2.3. MeOH simple method

This is a little modified method of [9], 100% methanol solvent was initially premixed with an antioxidant 0.1% BHT and added at the rate of 10 ml in 5 g of sample tissue in the 15 ml Pyrex tubes. Samples were vortexed followed and then kept at 4 °C for 16 h, later shaked at 200 rpm for 1 h and vortexed again and let rest for 5 min. Pipet as 1.5 ml of extraction solvent and centrifuged at 11,000 rpm for 5 min. Subsequently, the supernatant was filtered with 0.2 µm membrane filter and filtrate used for HPLC analysis.

2.2.4. Hexane exhausted method

It is a modified method of [10]. In this method, 100% hexane was initially premixed with 0.1% BHT and it was added at the rate of 10 ml in 5 g of sample tissue and vortexed gently. The sample was kept at 4 °C for 16 h, followed by shaking 150 rpm for 1 h. Then the sample was smoothly vortexed and let rest for 10 min. The supernatant of extract was poured and placed into 25 ml beaker and purged with nitrogen gas until complete dry and later the dry sample was dissolved with 1 ml of extraction solvent, and then centrifuged at 10,000g for 5 min. Subsequently, the supernatant was filtered with 0.2 µm membrane filter and filtrate was analyzed through HPLC.

2.3. Reagents and calibration of standards

All chemicals and organic solvents used in the study were of HPLC grade (Sigma-Aldrich, Mumbai, India) unless otherwise noted. Reference compounds of authenticated carotenoid standards of violaxanthin, lutein, zeaxanthin, and β-carotene (Sigma-Aldrich) were

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used to construct linear standard curves by injecting in the range of 4-80 µg [11]. Carotenoids standards were isolated and purified in our lab with individual purity not less than 98% (HPLC assay, UV/Vis detection). The reference chemical compounds were weighed to 0.1 mg and all the reference stock solutions were stored at -80°C. Diluted working solutions were prepared freshly for each HPLC analysis.

2.4. Separation of carotenoids

Chromatography was performed using the HPLC system (Agilent 1100 serial) equipped with diode array detector (DAD). Carotenoids separation was done on YMC Carotenoid C30 carotenoid column (3 µm, 4.6 × 250 mm), preceded by a C30 guard column were used at 24°C. The extracts were eluted with 40 min isocratic elution (58:22:20, CH_3CN:CH_3OH:CH_2Cl_2) at the flow rate of 0.8 ml/min, to separate the compounds in the extracts, and injection volume was 10 µl/sample [11]. All individual carotenoids peaks were detected at 450 nm [6]. All carotenoids identified by UV-Vis were compared with their retention time with the authentic standards [12].

2.5. Statistical analysis

Results of each carotenoid concentration were converted to µg g^{-1} fresh weight. Total carotenoid concentrations were calculated as the sum of the mean values of four individual carotenoids. Mean comparison for individual and total carotenoids across the four species was done using Duncan's Multiple Range Test (DMRT) at the 0.05 significance level using statistical software SAS 9.4 version for windows [13].

3. RESULTS AND DISCUSSION

3.1. Range of linearity and accuracy

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The standards of carotenoids (violaxanthin, lutein, zeaxanthin and β-carotene) and their molecular structure, molar mass (g/mol) and purity percentage were presented (Supplementary Table 1; Figure S1, online only). The linearity was investigated for authenticated standards of four carotenoids through blotting the peaks against the injected volume that resulted in good correlation of linearity. Retention time (RT), linear regression (LR) equation and correlation coefficient (CC) determined from the standards are summarized (Table 1). The precision of analytical method was examined through at least triplicate the analysis of each sample. The accuracy of the extraction method was assessed by determination of recovery of all the carotenoids of violaxanthin, lutein, zeaxanthin and β-carotene with mean value of 99.5, 99.9, 99.0, and 99.5 % being attained, respectively. The intra-day and inter - day relative standard deviation (RSD) for standard concentrations were 0.60 - 2.20% and 1.12 - 3.10% respectively, validating that a high reproducibility was achieved through using this method. All the carotenoids standard peaks were detected at 450 nm [6].

3.2. Identification of carotenoids

Two fruits, one tuber and a vegetable species were extracted by four different modified method of MeOH: DCM [7], MeOH: DCM exhausted [8], MeOH simple [9] and Hexane exhausted [10], was used for identification of and carotenoids. Among the extraction methods, MeOH: DCM recorded higher carotenoids concentration than other three methods for all four species (Table 2). Of the carotenoids, β-carotene was the prime component, followed by lutein, violaxanthin and zeaxanthin.

3.3. Determination of carotenoids concentration

Mean concentration of carotenoids was significantly differed within four species (Table 2). Lutein was previously reported as the major source of carotenoids in several vegetables

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including green chilli [8], wheat [6], pea and chickpea [11]. In the present study, it was confirmed that lutein was the major component in green chilli (12.8 μg g^{-1}), blueberries (2.3μg g^{-1}) and grapes (0.5 μg g^{-1}). However, β-carotene concentration was predominant in sweet potato. Of the four species, zeaxanthin was present in green chilli and other species, the concentration of which was found to below deductable limit (0.5 ng). Violaxanthin was ranged from 0.1 μg g^{-1} to 3.5 μg g^{-1} (Table 2).

Among the four species studied, β-carotene concentration was greater in sweet potato (69.2 μg g^{-1}), followed by green chilli (3.3 μg g^{-1}), blueberries (0.5 μg g^{-1}) and grapes (0.2 μg g^{-1}). The present study revealed that β-carotene concentrations of sweet potato was within the range of 14 sweet potato cultivars (53.2 to 84.3 μg g^{-1}) [14] and 43 fold richer than in endosperm of golden rice (1.6 μg g^{-1}) [15] and 10 fold higher β-carotene in sweet potato [16]. Hence, this study suggests that MeOH: DCM method could be reliable, simple and rapid determination of estimation β-carotene and carotenoids in fruits, vegetables and tubers. Additionally, the present study recommends that the consumption of sweet potato could address the prevention of vitamin A malnutrition deficiency in people from India, Africa and other developing countries. A typical chromatogram of the carotenoids profile of sweet potato and green chilli are presented (Figure 1).

The recent epidemiological studies have shown that consumption of high carotenoid containing foods are associated with reduction of oxidative stress and helps protect cardiovascular diseases [17], ARMD and cataracts [18]. Total carotenoid was calculated as the sum of four individual carotenoids and was ranged from 0.7 μg g^{-1} to 70.1 μg g^{-1}. Total carotenoids concentration was greatest in sweet potato (70.1 μg g^{-1}), followed by green chilli (20.2 μg g^{-1}), blueberries (2. 9μg g^{-1}) and grapes (0.7 μg g^{-1}) (Table 2).

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Comparison of grapes, blueberries, sweet potato and green chilli β-carotene with percent recommended dietary allowance (% RDA)

Percent recommended dietary allowance (% RDA) for vitamin A was calculated based on the daily value (DV) of retinol activity equivalents (RAE) μg/day from 100g serving of each species. The RAE was calculated by 12μg dietary β-carotene converted to 1μg retinol (REA ratio 12:1) is presented in Table 2. The United States (U.S) advised % RDA's required as RAE 300 μg/day and 400 μg/day for 1 to 3 years and 4 to 8 years children respectively. The results of the present study showed that daily consumption of 100 g of sweet potato would be enough to meet more than 100% RDA among 1 to 8 year old children. Consumption of 100g sweet potato can provide 96.1 % RDA of vitamin A for 9 to 13years males and females and 75% RDA for >19 year aged pregnant women (Table 3).

4. Conclusion

Among the four extraction methods, MeOH:DCM simple had 2 fold increase in total carotenoids than MeOH:DCM exhausted, and 12 fold greater total carotenoids than MeOH simple and hexane exhausted methods. Additionally, MeOH:DCM simple method is very easy, less time consuming for extraction and rapid detection of carotenoids in fruits, vegetables and tubers. Among the four species evaluated, lutein concentration was greatest in green chilli and blueberries. Sweet potato was rich in β-carotene concentration, which is greater than those reported in rice, wheat, cassava and potato. Therefore, this study suggests the consumption of sweet potato could be a good strategy to address the problem of vitamin A and age related macular degeneration (ARMD) deficiencies among peoples in developing countries.

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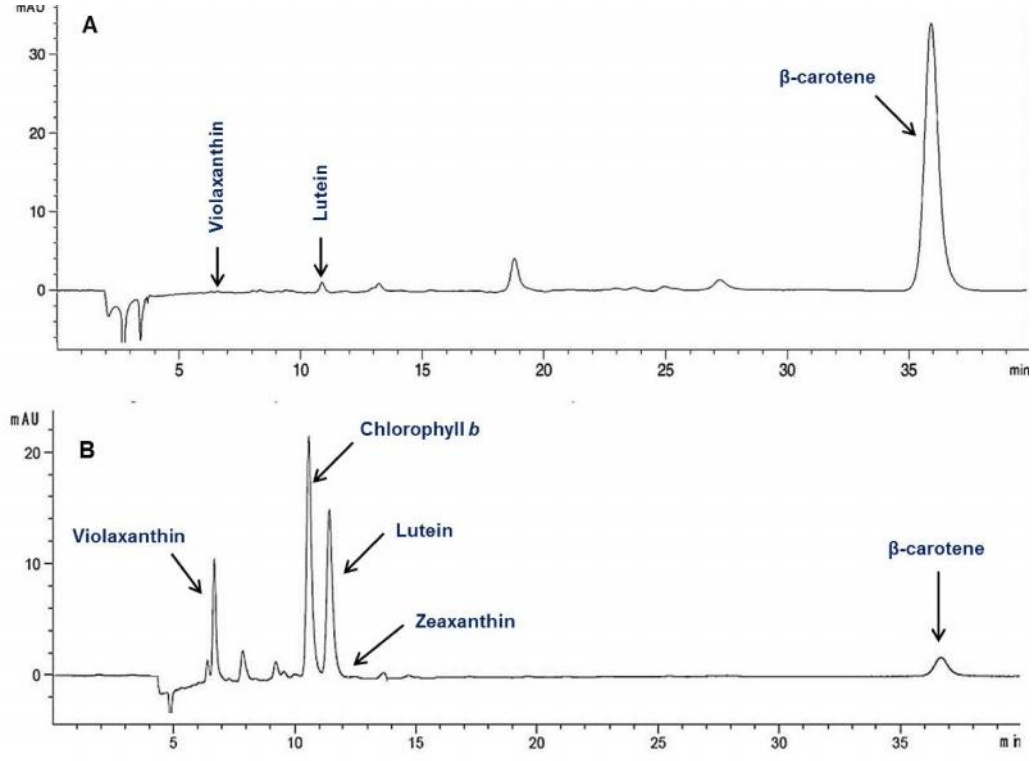

Figure. 1. Typical chromatogram of nutritionally important carotenoid profile of sweet potato (A), and green chilli (B)

Table 1. Summary of calibration data of individual carotenoids

Sl. No.	Compounds	RT (minutes) §	Lambda max†	Linear regression equation	R^2	Recovery (%)‡	Intra-day RSD (%)*	Inter-day RSD (%)*
Carotenoids								
1.	Violaxanthin	6.20	450	$y = 14.731x - 7.5619$	0.997	99.5	2.12	3.10
2.	Lutein	11.70	450	$y = 12.294x + 18.121$	0.998	99.9	0.60	1.12
3.	Zeaxanthin	12.45	450	$y = 12.504x - 12.211$	0.998	99.0	2.20	3.10
4.	β-carotene	34.00	450	$y = 10.202x + 34.441$	0.999	99.5	1.56	2.11

§Retention time
† Absorbance spectrum wavelength (nanometer)
‡Average recovery (n=3)
*Relative standard deviation (%)
R^2- Regression coefficient

Table 2. Determination of carotenoids concentration using four different extraction methods

Sl. No.	Species	Extraction method	Type	Mean carotenoid concentration (µg g^{-1} FW)§				
				Violaxanthin	Lutein	Zeaxanthin	β.carotene	Total carotenoids
1	Grapes	MeOH:DCM	Fruit	ND	0.5^{c}	ND	0.2^{c}	0.7^{d}
2	Blueberries	MeOH:DCM	Fruit	0.1^{b}	2.3^{b}	ND	0.5^{c}	2.9^{c}
3	Sweet potato	MeOH:DCM	Tuber	0.1^{b}	0.8^{c}	ND	69.2^{a}	70.1^{a}
4	Green chilli	MeOH:DCM	Vegetable	3.5^{a}	12.8^{a}	0.6^{a}	3.3^{b}	20.2^{b}
1	Grapes	MeOH:DCM Exhausted	Fruit	ND	ND	0.1^{a}	0.1^{c}	0.2^{d}
2	Blueberries	MeOH:DCM Exhausted	Fruit	ND	1.5^{b}	ND	0.3^{c}	1.8^{c}
3	Sweet potato	MeOH:DCM Exhausted	Tuber	ND	0.5^{c}	ND	39.9^{a}	40.4^{a}
4	Green chilli	MeOH:DCM Exhausted	Vegetable	0.8^{a}	5.3^{a}	0.1^{a}	0.8^{b}	7.0^{b}
1	Grapes	MeOH Simple	Fruit	ND	0.1^{b}	ND	ND	0.1^{b}
2	Blueberries	MeOH Simple	Fruit	ND	ND	ND	ND	ND
3	Sweet potato	MeOH Simple	Tuber	ND	ND	ND	ND	ND
4	Green chilli	MeOH Simple	Vegetable	1.1^{a}	6.5^{a}	0.1^{a}	0.2^{a}	7.9^{a}
1	Grapes	Hexane Exhausted	Fruit	ND	ND	ND	ND	ND
2	Blueberries	Hexane Exhausted	Fruit	ND	ND	ND	ND	ND
3	Sweet potato	Hexane Exhausted	Tuber	ND	ND	ND	9.5^{a}	9.5^{a}
4	Green chilli	Hexane Exhausted	Vegetable	ND	0.1^{a}	ND	0.3^{b}	0.4^{b}

§Within a column, means followed by different letters differed significantly according to Duncan's Multiple Range Test (DMRT) $P< 0.05$.
MeOH: DCM (methanol: dichloromethane), MeOH (methanol), FW; Fresh weight.

Table 3. Percentage of RDA on Vitamin A from 100g serving

Sl. No	Species	β-carotene concentration (µg/100g FW)	RAE (µg/day)‡	RDA % for vitamin A from 100g serving§											
				Children		Males			Females			Pregnancy		Lactation	
				1-3yrs	4-8yrs	9-13yrs	14-18yrs	>19yrs	9-13yrs	14-18yrs	>19yrs	<19yrs	>19yrs	<19yrs	>19yrs
1	Grapes	20	1.7	0.6	0.4	0.3	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.1	0.1
2	Blueberries	50	4.2	1.4	1.0	0.7	0.5	0.5	0.7	0.6	0.6	0.6	0.5	0.3	0.3
3	Sweet potato	6920	576.7	192.2	144.2	96.1	64.1	64.1	96.1	82.4	82.4	76.9	74.9	48.1	44.4
4	Green chilli	330	27.5	9.2	6.9	4.6	3.1	3.1	4.6	3.9	3.9	3.7	3.6	2.3	2.1

§Recommended dietary allowance (RDA) for vitamin A was calculated based on daily value (DV) of retinol activity equivalents (RAE) µg/day from 100 g serving of each species. The United States (U.S), RDAs required RAE 300 µg/day and 400 µg/day for 1-3 years and 4-8 years children respectively; 600 µg/day for 9-13 years males and females; 900 µg/day for 14-18 years and >19 years males; 700 µg/day for 14-18 years and >19 years females; 750 µg/day and 770 µg/day for <19 years and >19 years pregnant women, respectively; 1200 µg/day and 1300 µg/day for <19 years and >19 years lactating mother, respectively.
‡RAE was calculated by 12 µg dietary β-carotene converted to 1 µg retinol (REA ratio 12:1).

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Table S1. Molar mass, molecular formula and purity of individual carotenoids

Sl. No	Compound	Molar mass (g/mol)	Molecular formula	Purity (%)
	Carotenoids			
1.	Violaxanthin	600.85	$C_{40}H_{56}O_4$	>97.0
2.	Lutein	568.87	$C_{40}H_{56}O_2$	>98.0
3.	Zeaxanthin	568.88	$C_{40}H_{56}O_2$	>98.0
4.	β-carotene	536.88	$C_{40}H_{56}$	>97.0

Table S2. Details of fruits, tuber and vegetable used in this study

Sl. No.	**Common Name**	**Botanical Name**	**Family**	**Local name (Tamil)**	**Edible part tested**
	Fruit				
1.	Grapes (black)	*Vitis vinifera* L.	Vitaceae	*Kapputhiratchai*	Fruit
2.	Blueberries	*Vaccinium* sect. *Cyanococcus* Rydb.	Ericaceae	*Avurinelli*	Fruit
	Tuber				
3.	Sweet potato	*Ipomoea batatas* L.	Convolvulaceae	*Sarkaraivalli kizhangu*	Tuber
	Vegetable				
4.	Green chilli	*Capsicum annum* L.	Solanaceae	*Pachamilakai*	Immature fruit

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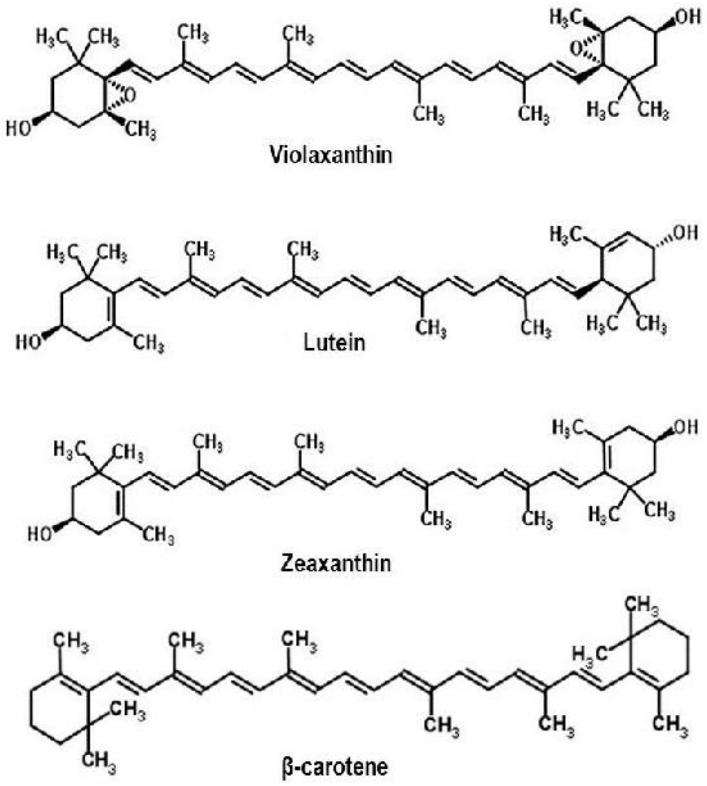

Figure. S1. Molecular structure of the nutritionally important carotenoids identified in grapes, blueberries, sweet potato and green chilli.