"Extraction, Purification and Comparison of Carotenoids from Some Vegetables, Fresh Leafy Vegetables and Fruits"

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ABSTRACTS

Carotenoids comprise a large group of natural pigments widely distributed in the plant and animal kingdoms. They are yellow-orange in colour, insoluble in water but soluble in organic solvents. They are present as pigments in many vegetables and fruits and are associated with chlorophyll in higher plants, playing important roles during photosynthesis by passing on the light energy they absorb to chlorophyll; they also protect the chlorophyll from the excess light and oxidation. Carotenoids are of two types: - carotenes and xanthophylls. The most widespread and important carotene is β-carotene which is found abundantly in some plants. The carotenoids are widespread naturally occurring antioxidants; their importance is related to their functions. They act as provitamin A and food colorants and are being extensitively studied for their potential role in reducing the risk for cancer and other chronic diseases. This study was conducted estimate the total amount of carotenes and its counterparts in vegetables such as carrot, spinach and beetroot. The main objective was to estimate and purify the amount of carotenoids using solvent extraction followed by calorimetry and TLC method. In addition to that a comparison of the estimated amounts of caroteness with the other samples was made in this study. The solvent extraction was done using hexane: acetone (1:1) along with ethanol, 10% NaCl solution was also prepared in the laboratory which was used in the extraction. A calorimeter was used to observe the absorbance at 630nm. Silica gel and calcium sulphate hemihydrates were used in the ratio 4:1 along with double the amount of water to prepare the thin layer chromatography (TLC) plates. The mobile phase consisted of a mixture of hexane and acetone in the ratio of 3:2. The experimental yielded appreciable results and the samples were graded in the order of their respective carotene content. The most notable amount of carotenoid was obtained from carrots.

Key words: - Carotenoids, Chlorophyll, TLC etc.

INTRODUCTION

A diet rich in vegetables is recommended along with fruits and whole grains. An epidemiological study found that a diet of this composition has a negative association with risk of chronic diseases. Carotenoids in vegetables and fruits are of the significant importance,

besides other vitamins, minerals, flavanoids and phytochemicals, which have been reported to contribute to health. Carotenoids are the natural compounds with lipophilic properties. About 500 different carotenoids have been identified, among them β-carotene is the most important. It can act as an antioxidant under low pressure of O_2. β-carotene usually functions in association with vitamins C and E. Lycopene, a fat soluble pigment is a carotenoid. It is responsible for colour of certain fruits and vegetables (eg; tomato). Lycopene possess antioxidant property. Lutein and xeaxanthin are also carotenoid pigments that impart yellow or green colour to fruits and vegetables. These pigments can also serve as antioxidants. A precursor to Vitamin A, β-carotene is commonly found in many vegetables. β-carotene is also converted to Vitamin-A in the intestinal wall and stored in the liver. This makes β-carotene an important natural product of organic chemistry. To mitigate the harmful/damaging effects of free radicals, the aerobic cells have developed antioxidant defense mechanisms. A biological antioxidant may be defined as a substance (present in low concentrations compared to an oxidizable substrate) that significantly delays or inhibits oxidation of a substrate. Antioxidants may be considered as scavengers of free radicals. Free radicals have been implicated in the causation and progress of several diseases such as cardiovascular diseases, cancer, inflammatory diseases such as Rheumatoid arthritis, Respiratory diseases, Diabetes etc. The production of free radicals and their neutralization by antioxidants is a normal bodily process.

Carotenoids, the tetraterpenoid (C_{40}) compounds, are ubiquitous in plants. These terpenoids existing as hydrocarbons (carotenes) or oxygenated derivatives, are accessory pigments in photosynthetic systems and give characteristic colour to plant parts, particularly flowers and fruits. Carotenes occurring in different chemical forms have characteristic features and functions. Their levels are altered during physiological and pathological conditions. Lycopene is responsible for the red colour of tomato and the fleshy part of water melon. It is a carotene having the formula $C_{40}H_{56}$. Though it has no nutritional value, its contribution to the colour of tomato has a great role in consumer acceptability.

The carotenoids in the sample can be extracted in acetone and then taken up in petroleum ether. Lycopene has absorption maxima at 473nm and 503nm. One mole of lycopene when dissolved in one litre light petroleum (40-60ºC) and measured in spectrophotometer at 503nm in 1cm light path gives absorbance of 17.2×10^4. Therefore, a concentration of 3.1206µg lycopene/mL gives unit absorbance. (Ranganna, 1976). The populations of underdeveloped and developing countries, such as Brazil, commonly suffer undernourishment and so-called heidden hunger, which can cause diseases from both caloric/proteic and micronutrients deficiencies. Vitamin A deficiency constitutes a public health problem and affects mainly children and women. Interest in raw materials of vegetal origin that contain high levels of carotenoids and provitamin A activity, has increased substantially in recent years. Some cultivars of pumpkin (Cucurbita) staining intense yellow to orange have revealed high levels of carotenoids, mainly β-carotene and α- carotene. (Arima,1988 and Azevedo *et al*, 2007)

Oxidative stress is an important contributor to the risk of chronic diseases. Dietary guidelines recommend increased consumption of fruits and vegetables to combat the incidence of human diseases such as cancer, cardiovascular diseases, osteoporosis and diabetes. Fruits and vegetables are good sources of antioxidant phytochemicals that mitigate the damaging effect of oxidative stress. Carotene is an orange photosynthetic pigment important for photosynthesis. Carotenes are all coloured to the human eye. Carotenes are valuable preventive medicines, too. Research shows that people, who eat a lot foods rich in beta-carotene, the carotenoid with the greatest vitamin A value, are less likely to develop lung cancer. They are metabolized by hydroxylation, epoxidation, isomerization, oxidation-reduction and degradation. In addition to being potent antioxidants some carotenoids also contribute to dietary vitamin A. They are all synthesized by higher plants, algae and bacteria and are widely distributed in animals, which acquire them via their diet. In the plant carotenoids act as photosynthetic accessory pigments and also play a protective function as scavengers of oxygen radicals released from chloroplasts during photosynthesis, thus protecting cellular constituents such as DNA from free radical damage. Carotenoids generally cannot be manufactured by species in the animal kingdom so animals obtain carotenoids in their diets, and may employ them in various ways in metabolism. They are split into two classes, xanthophylls (which contain oxygen) and carotenes (which are purely hydrocarbons, and contain no oxygen). All carotenoids are tetraterpenoids, meaning that they are produced from 8 isoprene molecules and contain 40 carbon atoms. Carotenoids in general absorb blue light. They serve two key roles in plants and algae: they absorb light energy for use in photosynthesis, and they protect chlorophyll from photodamage. In humans, three carotenoids (beta-carotene, alpha-carotene, and beta-cryptoxanthin) have vitamin A activity (meaning they are converted to retinal), and these and other carotenoids can also act as antioxidants. In the eye, certain other carotenoids (lutein, astaxanthin and zeaxanthin) apparently act directly to absorb damaging blue and near-ultraviolet light, in order to protect the macula of the retina, the part of the eye with the sharpest vision. Food coloring carotenoids include beta-carotene, paprika, lycopene, lutein, carrot oil and saffron. Each contains different types and ratios of carotenoids.

Xanthophylls (originally phylloxanthins) are yellow pigments that form one of two major divisions of the carotenoid group. The name is from Greek *xanthos* (yellow) and *phyllon* (leaf), due to their formation of the yellow band seen in early chromatography of leaf pigments. Their molecular structure is similar to carotenes, which form the other major carotenoid group division, but xanthophylls contain oxygen atoms, while *carotenes* are purely hydrocarbons with no oxygen. Xanthophylls contain their oxygen either as hydroxyl groups and/or as pairs of hydrogen atoms that are substituted by oxygen atoms acting as a bridge (epoxide). Like other carotenoids, xanthophylls are found in highest quality in the leaves of most green plants, where they act to modulate light energy and perhaps serve as a non-photochemical quenching agent to deal with triplet chlorophyll (an excited form of chlorophyll), which is overproduced at high light levels in photosynthesis.

A process for the extraction of carotenoid dyes from pre dried natural starting materials is described using compressed gases such as propane and/or butane in which organic entraining agents can be added in order to facilitate and complete the extraction process. With the aid of this process highly concentrated carotenoid dyes are obtained in high yield. The methods of TLC and UV were used usually for the separation and identification of carotenoid pigments. The essential role of β-carotene as dietary source of vitamin A has been known for many years (Britton, 1995). Among the provitamin A carotenoids in food namely beta-carotene, alpha-carotene, gamma-carotene and beta-cryptoxanthin, beta-carotene is one that is most efficiently converted to retinol (Olson et al; 2000). Vitamin A is essential for a variety of biological processes, amny of which are related to growth cellular differentiation and interactions of cells with each other or with extracellular matrix. Its deficiency, even in its relatively early stage, results in impairments in linear growth, cartilage and bone development and epithelial cell differentiation and function (Roberts and Sporn, 1984)

Deficiency of vitamin A in the diets represents one of the key challenges affecting the developing world. However, beta-carotene is the most available/ important source of pro-vitamin A in the diets of most people living in developing countries. It reportedly provides about 66% of vitamin A in their diets. In West Africa, much carotenoid is obtained from red palm oil, which is widely used in cooking. However, it has been reported that these available sources of pro-vitamin A are very often neglected by children and pregnant women who are more vulnerable or at risk of vitamin A deficiency. Also amount of dietary intakes of beta-carotene of Nigerians is not known. This study therefore aimed at determining the beta-carotene contents of some selected commonly consumed food items. The determination of carotene contents of the extracts was done using the UV visible spectrophotometer.

The quenching of singlet oxygen is the major antioxidative activity of carotene. Due to their system of conjugated double bonds carotenoids are extremely reactive and consequently unstable. Precaution steps are taken during the isolation and analysis include the protection from light, avoiding the exposure to oxygen, use of antioxidants (eg; BHT, pyrogallol, vitamin E), operation at reduced temperatures and the need for completing the analysis in the shortest possible time. For foods with a high fat content the saponification is being employed prior to (or after) extraction in order to hydrolyze the carotenoid esters and remove fatty material. Although this optional step facilitates subsequent separation, identification and quantification of carotenes, it prolongs the time of analysis and might also lead to a degradation of carotenoids. Hence the saponification in the analytical procedure should be omitted whenever possible. Carotenoids are used as colorants (eg; carotene (E160a), annatto dye-stuffs norbixin and bixin (E160b), lycopene, lutein, capsanthin, etc) in foodstuffs such as beverages, cookies, cereals, margarines, butter, cheese, etc. Natural carotenoid pigments (carrot extract, palm oil, saffron, annatto or paprika) or synthetic carotenoids are used to impart yellow colour to margarines. Antioxidant fortified margarine increases the antioxidant status in humans.

Quantification of carotenoid colorants in foodstuffs is therefore very important from nutritional, epidemiological and food quality points of view.

OBJECTIVE OF THE STUDY

- ✓ Collection of samples such as vegetables, leafy vegetables and fruits
- ✓ Grinding the samples using mortar and pestle.
- ✓ Filtering the crude extracts using Whatman filtepaper No:1
- ✓ Extracting the pigments using a Separating funnel.
- ✓ To estimate the amount of carotenoids using solvent extraction followed by calorimetry.
- ✓ Purification of the extracted carotenoid by TLC method
- ✓ To compare the estimated amounts with the other samples present in the study.

MATERIALS AND METHODS

This study was conducted estimate the total amount of carotenes and its counterparts in vegetables, leafy vegetables and fruits. A method to extract total carotenoids from fresh plant materials and to separate most of the components from each other is given below. The total carotenoids were extracted and partitioned in organic solvents on the basis of their solubility. The separation of individual components was effected by chromatography. The procedure is as follows:-

Sample Collection

The fresh vegatables, leafy vegetables and fruits were used for this study. The vegetables and fruits used were brought fresh from the market and preserved in the refrigerator. They are shown in the figure1, 2 and 3.

Fig 1: Vegetables taken for the study

Fig 2: Leafy vegetables taken for the study

Fig 3: Fruits taken for the study

TLC Plates Preparation

Simple TLC plates were prepared using microscopic slides. A mixture of silica gel (finely powdered) and calcium sulphate hemihydrates were mixed together and double the amount of water was added so as to form a paste. This paste was evenly distributed over the surface of the slide and kept aside to dry. Prior to usage, slides were activated by baking in the oven at 120ºC for 30-40 minutes.

Solvent extraction of carotenoids

The fruits and vegetables were cut separately and 2g of each sample was weighed and kept separately. The same extraction procedure was followed for all vegetable and fruit samples. 2g of sample was placed in a mortar and crushed with a pestle. A mixture of hexane and acetone in the ratio of 1:1 was added into the mortar and the sample was crushed. About 5 ml of acetone was added slowly at regular intervals. The solvents were collected separately and the process was repeated with the sample again for double extraction. The solvents containing carotenoids were filtered through a filter paper and then transferred into a separating funnel. 50ml of distilled water was added along with 5oml of 10% NaCl solution. The mixture was shaken vigorously and kept aside for the layers to separate. The upper layer contained carotenoids and it was collected separately after the removal of the water and NaCl solution. The extract was collect in tubes. Using a calorimeter, the absorbance of carotenoid was noted at 630nm. The amount of carotenoid present in 100g of each food sample was calculated.

Purification process using Thin Layer Chromatographic method

A thin line was drawn on the activated TLC plate about 1.5cm above the bottom. A spot of the extract was placed on the line and allowed to dry. This was followed by a repeated addition of the extract on the same spot. The developing chamber was a beaker containing a mixture of petroleum ether (hexane) and acetone in the ratio of 3:2. The TLC plate was placed inside the developing chamber and the top was covered. The solvents were allowed to rise on the plate till it reached 1.5cm close to the top. It was then taken out and the Rf was calculated.

RESULTS AND DISCUSSION

The results of the study entitled "**Extraction, Purification and Comparison of Carotenoids from Some Vegetables, Fresh Leafy Vegetables and Fruits"** undertaken at Postgraduate Department of Bioscience, SSV College, Ernakulam is as follows:-

The project work mainly included the following aspects:-

Sample collection

The samples were collected from a market near to SSV College, Valayanchirangara, Ernakulam and preserved in the refrigerator. The following procedures were done:-

Fig 4: Grinding the sample using mortar and pestle

Fig 5: Extraction of pigments from carrot

Fig 6: Filtering the extracts

Fig 7: Collection of extracted pigments

The extraction of carotenoid from the vegetable and fruit samples using solvent extraction method in a separating funnel is shown in the above figures 5, 6 and 7. The different samples were collected in test tubes for further analysis. The procedure for this experiment proved to be quite interesting although a few changes were required in certain aspects. The extraction procedure using solvents proved to be highly efficient. Calorimetric reading of the extracted samples was taken at 630nm. This was in good agreement with that reported in the literatures of previously reported many articles and journals by giving a value in already reported ranges. This confirmed that the extraction procedure was valid and the extract contained carotenoid.

Spotting of the extracted pigments in TLC plates

Purification using thin layer chromatography was not very easier and effective for certain samples. The TLC showed the orange band at lower Rf value than the yellow band. The TLC plates on which a spot of the extract was made is shown in the figure 8. The TLC plates was placed and kept in developing chamber to separate into different bands and developed TLC plate with the orange spot are shown in figure 9 and 10.

Fig 8: Spotting of the extracts

Fig 9: TLC plates inside the developing chamber

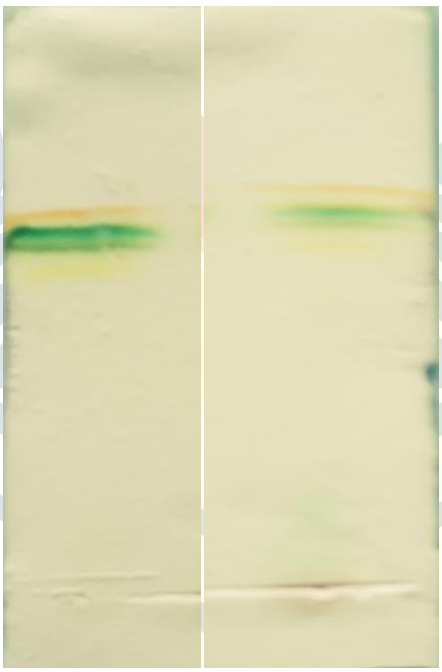

Fig 10: TLC plates after separation

The absorbance value of the extract which was noted and it was used to calculate the carotenoid content in each sample. The result is tabulated below in table 1. It can inferred that per 100g of the sample of vegetables and fruits amaranthus, drumstick, orange, carrot etc showed a high carotenoid content compared to others.

Serial Number	Items	Scientific Name	Carotenoid Content (mg/100g of the sample)
Vegetables			
1	Beet root	***Beta vulgaris***	3.5
2	Tomato	***Solanum lycopersicum***	12.7
3	Carrot	***Daucus carota***	15.9
4	Pumpkin	***Cucurbita maxima***	4.8
Leaf Vegetables			
1	Amaranthus (green)	***Amaranthus acanthochiton***	20.1
2	Spinach	***Spinacia oleracea***	17.3
3	Drum stick	***Moringa oleifera***	27.1
4	Amaranthus (red)	***Amaranthus cruentus***	25.2
Fruits			
1	Orange	***Citrus sinensis***	28.9
2	Papaya	***Carica papaya***	16.4
3	Apricot	***Prunus armeniaca***	16.5
4	Mango	***Mangifera indica***	5.5 in green mango 50 in ripe mango

Table 1: Total Amount of Carotenoid present in each sample – a comparison

The extract was purified using Thin Layer Chromatography method and the retardation factor was calculated as shown in Table 2.

Serial Number	Items	Scientific Name	Rf factor	
			Carotene	Xanthophyll
Vegetables				
1	Beet root	***Beta vulgaris***	0.84	0.42
2	Tomato	***Solanum lycopersicum***	0.92	0.38
3	Carrot	***Daucus carota***	0.95	0.31
4	Pumpkin	***Cucurbita maxima***	0.86	0.34
Leaf Vegetables				
1	Amaranthus (green)	***Amaranthus acanthochiton***	0.88	0.38
2	Spinach	***Spinacia oleracea***	0.77	0.44
3	Drum stick	***Moringa oleifera***	0.81	0.52

4	Amaranthus (red)	***Amaranthus cruentus***	0.94	0.36
Fruits				
1	Orange	***Citrus sinensis***	0.90	0.53
2	Papaya	***Carica papaya***	0.92	0.49
3	Apricot	***Prunus armeniaca***	0.89	0.32
4	Mango	***Mangifera indica***	0.83	0.48

Table 2: The retardation factor of different samples

Results of Thin Layer Chromatography

The results obtained from the present study were either same or marginally different for all the vegetables when compared to a few similar studies that were undertaken based on carotenoids. The present study states the absorbance change in accordance to the kind of solvents used, pigment concentration, experimental results and calculation. However, the results should be similar to the following typical value: Carotene - 0.95, Xanthophylls – 0.35. Using the works published in many journals and research articles comparisons were made between the results of both the studies and they were found to be in accordance with one another.

CONCLUSION

The term carotenoid refers to a family of more than 600 different plant pigments, which are responsible for many colors (red, orange and yellow etc) of plant leaves, fruits and flowers, as well as the colors of some birds, insects, fish and crustaceans. Carotenoids have polyisoprenoid structures, are generally found in plants, algae, photosynthetic bacteria, non-photosynthetic bacteria, yeasts and molds. One of the important physiological functions of carotenoids in human nutrition is to act as pro-vitamin A (Vitamin A precursors). Pro-vitamin A carotenoids support the maintenance of healthy epithelial cell differentiation, normal reproductive performance, and visual functions. Additionally, non pro-vitamin A carotenoids (eg: lutein, astaxanthin, zeaxanthin and lycopene) also play an important role in human health as biological antioxidants, protecting cells and tissues from the oxidative damaging effects of free radicals and singlet oxygen. Many studies show strong correlations between carotenoids intake and a reduced risk of some diseases, such as cancer, atherogenesis, bone calcification, eye degeneration, immune function and neuronal damage. Among the carotenoids β-carotene is popular to consumers. β-carotene belongs to the carotene class, which is one of the most abundant found in the diet and is used as food colorants. The present study was conducted to estimate the total amount of carotenes and its counterpart, xanthophylls in some vegetables, leafy vegetables and fruits. The experiment yielded appreciable results and the samples were graded in the order of their respective carotene content. Based on the results, it can be noted

that the amaranthus, drumstick, orange, carrot contained the highest concentration of carotenoids followed by others in the respective order as given in the table, Pumpkin and beetroot was reported to have the least amount of carotenoids of the six vegetables that were chosen for the study.

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