Chapter 2

Characterization of Carotenoids
SUMMARY

The content of carotenoids in vegetables depends on their growth/maturity stage, concentration of carotenoid isomers, and food processing methods. This chapter focuses on the growth characteristics and the accumulation of carotenoids in foliage of ten commercially important varieties of coriander (*Coriandrum sativum* L.) and partial characterization of carotenoids, and their stability after processing. For this, coriander varieties were grown at identical greenhouse conditions and major carotenoids were analysed by HPLC-MS in seeds and foliage at different growth stages of coriander plants. In all varieties, total carotenoids and β-carotene contents were higher in foliage at mature stage than at seedling stage. Under our experimental set of conditions, the variety GS4 Multicut was found to produce highest biomass 6.18 g/plant, total carotenoids (217.50 mg/100 g DW) β-carotene (73.64 mg/100 g DW) and lutein (130.26 mg/100 g DW) at pre-flowering stage. Lutein was the predominant carotenoid among xanthophylls. Violaxanthin and neoxanthin were the other carotenoids present in very low concentrations. Lycopene and zeaxanthin - the intermediary compounds were not detectable, probably because of their complete utilization for the synthesis of downstream carotenoids such as β-carotene and lutein. The antioxidant activity of the total carotenoid fraction obtained after acetone extraction and saponification showed a strong antioxidant activity, rendered strong protection to DNA against oxidation by Fenton’s reagent. For the stability of carotenoids in coriander foliage, the application of microwave drying showed much highest retention of both carotenoids and chlorophylls, better intactness of β-carotene (as analysed by HPLC-MS) as well as enhanced extractability of pigments after processing.

Publication

2.1. Introduction

Vitamin A deficiency is one of the most important health concerns in India. An estimated 250,000 to 500,000 vitamin A-deficient children become blind every year globally, of which nearly 50% die within 12 months of losing their sight (Micronutrient Initiative, 2009). The frequency is very high in preschool children belonging to low income groups. Alleviation of vitamin A deficiency is possible by enriching diet with carotenoid-rich green leafy vegetables and other fresh food sources. The green leafy vegetables (GLVs) are less expensive and are rich in pro-vitamin A carotenoids such as β-carotene, α-carotene etc. The foliage of coriander is a very good source provitamin A carotenoids (β-carotene 12 mg/100 g FW, Girenko 1982), Vitamin B12 (6 mg/100 g FW, Prakash 1990), polyphenols, phytochemicals such as vitamin C (160 mg/100 g FW) and essential oils that are popular for rendering health benefits (Silva et al. 2011; Samojlik et al. 2010; Darughe et al. 2012). Coriander, like many spices, contains antioxidants, which can delay or prevent the spoilage of food seasoned with this spice and provide similar protections upon its ingestion (Darughe et al. 2012). India is the highest producer, consumer and exporter of coriander in the world (see previous Chapters). Most of the research studies in coriander have been focused on its seeds (Dhanapakiam et al. 2007; Srinivasan 2011) and very little attention is paid to analyse the nutritional constituents in leaves (Aruna and Baskaran 2010), which are more frequently consumed all over the world than seeds. Among foliar vegetables, coriander foliage is one of the richest sources of carotenoids (see Table G1.5 under the section “review of literature”).

Carotenoids are biosynthesized by photosynthetic organisms as well as non-photosynthetic bacteria and fungi. Carotenoids are present in photosynthetic tissues in which they perform the dual role of being accessory light-harvesting pigments at wavelengths where chlorophylls cannot absorb light, and thus carotenoids protect chlorophylls from photo oxidation (Bartley and Scolnik 1995). Dark GLVs and other vegetables are therefore not only the rich sources of carotenoids but also display a distinct qualitative pattern. Among the different carotenoids in GLVs, lutein is the predominant carotenoid, followed by β-carotene, violaxanthin and neoxanthin (Lakshminarayana et al. 2005; Raju et al. 2007).
Plant carotenoids are dominated by C40 isoprenoids with polyene chains containing 15 conjugated double bonds, which can be divided into two groups: hydrocarbons (carotenes) and their oxidation derivatives (xanthophylls). These compounds are not only responsible for yellow, orange and red colors of foods (Meléndez-Martínez et al. 2007), but also function as the precursors of vitamin A (Sánchez-Moreno et al. 2003) in mammals. The carotenoids are efficient singlet oxygen quenchers and function as chain-breaking antioxidants, protecting cells and other body components from the attack by free radicals. Moreover, carotenoids play multiple roles in the prevention of diseases, imparting better health by way of providing protection against oxidative stress under conditions of cardiovascular diseases, cancers and age-related macular degeneration (Bartley and Scolnik 1995). The predominant carotenoids playing a role in macular pigment synthesis are lutein and zeaxanthin, as has been demonstrated that an increased dietary intake of lutein or zeaxanthin increases the macular carotenoid levels (Landrum and Bone 2001).

Carotenoid pigments, particularly the β-carotene, present in GLVs are precursors for vitamin A in mammals, the deficiency of vitamin A is prevalent in many tropical and under-developed temperate countries. Although many leafy vegetables, carrots and yellow fruits are rich sources of β-carotene, such food items are either not liked by many for routine consumption or inaccessible to poor people of developing countries, for various reasons. Certain GLVs grown and traditionally used routinely in some of these countries can form a good source to alleviate vitamin A deficiency, provided – a) more organized research and product development are considered for their versatile applications and b) there is a further enhancement in the concentration of carotenoids by judicious eco-friendly manner. Chemical structures of some major carotenoids present in GLVs are shown in Figure 2.1.
Coriander is one such GLV having very versatile applications, liked mostly by all, can be routinely consumed, could be an ingredient in various processed products, and is a rich source of β-carotene. Both seeds and leaves of coriander contain diverse primary
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and secondary metabolites, some of which have medicinal properties. Coriander has been used as a folk medicine for the relief of anxiety and insomnia in Iran. Volatile components in essential oil, from both seeds and leaves, have antimicrobial activity, and inhibition of lipid peroxidation is also reported (Tanabe et al. 2002). Wangensteen et al. (2004) reported that, phenolic compounds from coriander leaves, seeds, may be involved in the inhibition of lipid peroxidation and the inclusion of both seeds and leaves of coriander in the cuisine will increase the content of natural antioxidants, and thus probably prevent oxidative damage of food. Although, a few preliminary studies have been conducted on the estimation of total carotenoids and β-carotene content (Aruna and Baskaran 2010) no detailed study on carotenoids in different varieties of C. sativum has been done so far. Therefore, it is very important to obtain more detailed information about the carotenoids, their stability after processing and the levels of antioxidant activities in different cultivars of coriander.

Several factors make the carotenoid analysis difficult in coriander, which are the following:

- Existence of a large number of carotenoids.
- Existence of large variations in growth patterns in different coriander varieties
- Both qualitative and quantitative variations of carotenoids among different organs of the same plant.
- Matrix variations
- Carotenoids are prone to isomerization and oxidation during analysis as well as during storage.

The objective of this research was to identify the carotenoids in the foliage of coriander and compare the differences between the overall composition and contents of carotenoids in the foliage of different coriander cultivars using HPLC-MS with a C18 reverse phase column. Besides, the effect of different stage of maturation on the contents of carotenoids in the leaves and in immature fruit was also investigated, since there was no record on the levels of carotenoids in such lipid-rich coriander seeds. Similarly, there has been no report on the bio-efficacy of carotenoids from coriander, particularly in terms of DNA protection. It was also necessary to check the stability of
carotenoids after processing to obtain dehydrated product. In this context certain cost-effective modern drying methods such as microwave drying was not so far applied for coriander foliage. Therefore, this chapter has been focused on such studies to explore the usefulness of coriander both as a source of carotenoids as well as a natural food source of antioxidants in fresh and processed form.

2.2. Materials and methods

2.2.1. Collection of plant material

Certified seeds of eleven newly improved and commercially cultivated varieties of *C. sativum* were obtained from local seed markets from various parts of India. The germination and growth patterns were monitored under controlled identical conditions in green house, using a mixture of soil containing equal proportions of red soil, garden compost and sand. The growth pattern of the plants and biomass were periodically recorded by using 3 sets of plants each set having 10 randomly picked plants after removing the adherent soil and surface blotting. Each variety was grown in three different plots simultaneously to rule out changed environmental effects (if any) and 10 plants from each plot were randomly selected each time for biomass determination at different phases of growth. Total carotenoids and β-carotene contents at different phases of growth in ten varieties were also determined.

2.2.2. Extraction of carotenoids

All extractions and handling of sample was done under dark condition (by wrapping containers with black paper and under subdued light) to prevent photo-oxidation of the sample. A known quantity of different samples, such as fresh and dried seeds and foliage, was ground using mortar and pestle. Total carotenoids were extracted in to ice-cold acetone, where the extraction was repeated thrice until the residue appeared colorless. The excess water in the extract was removed by partitioning against petroleum ether and measured for carotenoids.

2.2.3. Saponification of carotenoids

The petroleum ether extract was subjected to saponification with methanolic KOH (10% w/v). Saponification is an effective method for removing chlorophylls as
well as lipids from the plant materials that contain a mixture of fatty acids, because such compounds make the chromatographic separation difficult and complicated. The crude extract was washed with water for removing alkali, after which the extract was concentrated at 40 °C in vacuo using rotavapour. The concentrated sample (5 ml) was passed through silica (110 Å) column (15 cm long and 1 cm dia), eluted with varying concentration of acetone in petroleum ether and the eluent was used for quantification of carotenoids. Whenever, necessary, the extract was concentrated under nitrogen gas and stored in freezer (-20 °C) for not more than one week and used for quantification and characterization.

2.2.4. Spectrophotometric determination of total carotenoids

The concentration of total carotenoids in the extract was estimated by spectrophotometric reading by using the following equation (Rodriguez-Amaya et al. 2004).

\[
\text{Total carotenoids (µg/g)} = \frac{A \times \text{volume (ml)} \times 10^4}{A_{1\%}^{1\text{cm}} \times \text{sample weight (g)}}
\]

Where \( A = \) absorbance; \( \text{volume} = \) total volume of extract; \( A_{1\%}^{1\text{cm}} = \) absorption coefficient of \( \beta \)-carotene in PE (2592).

2.2.5. Spectrophotometric determination of total carotenoids, chlorophyll a and chlorophyll b

For the simultaneous analysis of both carotenoids and chlorophylls, total carotenoids content in the extract was estimated by spectrophotometric reading by using the following equation (Lichtenthaler 1987).

\[
\text{Chlorophyll a, Ca} = 11.24 \ A_{\text{661.5}} - 2.04 \ A_{\text{645}}, \\
\text{Chlorophyll b, Cb} = 20.13 \ A_{\text{645}} - 4.19 A_{\text{661.5}}, \\
\text{Total carotenoids, Cx + c} = (1000 \ A_{\text{470}}) - [(1.9Ca) + (63.14Cb)] \ V \times DF/214
\]

Where \( V = \) Total volume of the extract, \( DF = \) Dilution factor
2.2.6. HPLC analysis of carotenoids from coriander foliage and seeds

The sample was re-dissolved in 1 mL of mobile phase (acetonitrile: methanol: ethyl acetate in the proportion of 80:10:10 v/v) and injected into the HPLC system (LC-10A; Shimadzu, Kyoto, Japan), equipped with a Shimadzu Photo Diode Array detector (PDA) for analysis of major carotenoids. Carotenoids were quantified by integrating peak areas in the HPLC chromatograms. Each sample was analyzed in duplicate. If analysis of duplicate samples were different by more than 10%, it was repeated in duplicate. Carotenoid analysis was performed two to six times. All the carotenoids were separated on a Phenomenex Gemini C18 reverse phase stainless steel column (250×4.6 mm) in isocratic mode with a flow rate of 1 mL min⁻¹. Chromatogram was acquired at 450 nm. Peak identities were done by their retention time and respective spectra recorded with the PDA detector. Quantification of β-carotene was performed by analysing their peak area in relation to the concentration of the standard β-carotene concentration. The standard curves were constructed by the injection in triplicate of standard solutions at five different concentrations.

2.2.7. Mass spectral analysis of carotenoids

This was done using a Phenomenex Gemini 5 µm C18 110 A reverse phase column, 250 x 4.6 mm i.d. Conditions of resolving total carotenoids using APCI +ve mode were Corona (µA): 1.7, Cone: 100 V, Source Temp: 120 °C, APCI Probe Temp: 500 °C, Cone Gas Flow: 100 L/hr, Desolvation gas flow: 250 L/hr.

The variety of coriander which exhibited highest biomass accumulation in shortest time as well as highest total carotenoids and β-carotene contents was selected for further studies.

2.3. Antioxidant activity of carotenoids

2.3.1. DPPH scavenging Activity

Free radical scavenging activity was carried out by reaction with stable free radical DPPH’ (Blois 1958). Briefly, an aliquot of extract in methanol mixed with 0.5 mL of 0.15 mM DPPH solution in methanol. The solution was mixed well and allowed to stand for 30 min at room temperature. UV absorbance was read at 517 nm. Gallic
acid was used as positive control. The concentration required to scavenge 50% DPPH free radicals (IC\textsubscript{50}) was calculated.

DPPH\textsuperscript{•} scavenging activity was calculated using the equation:

DPPH\textsuperscript{•} scavenging effect (%) = \left\{\frac{(A_0 - A_1)}{A_0}\right\} \times 100

where ‘A\textsubscript{0}’ is the absorbance of the control and ‘A\textsubscript{1}’ is the absorbance of the sample.

### 2.3.2. Hydroxyl radical induced DNA damage

Hydroxyl radical induced DNA damage was evaluated by the competition between deoxyribose and carotenoid fraction for hydroxyl radical generated by Fenton’s reagents (Fe\textsuperscript{3+}-ascorbate-EDTA-H\textsubscript{2}O\textsubscript{2} system) according to (Cao et al. 2008) with slight modification. The reaction mixture (1 mL) contained calf thymus DNA (8 mg/mL), Fe\textsuperscript{3+} chloride (10 mM), EDTA (10 mM), and H\textsubscript{2}O\textsubscript{2} (2 mM), without and with the test extract of varying concentrations in sodium phosphate buffer (pH 7.4). Ascorbic acid (10 mM) was added to trigger reaction, which reduces Fe\textsuperscript{3+} to Fe\textsuperscript{2+}, and the reaction mixture was kept at 37 °C for 30 minutes. Fenton’s assay reagents were prepared just prior to use. To 1 mL of the above mixture, TBA in 25 mM NaOH (1 mL, 0.5%) and TCA (1 mL, 10% w/v aqueous solution) were added. The mixture was incubated in a boiling water bath at 80 °C for 90 min. After centrifugation at 3000 rpm for 10 min, pink supernatant chromogen produced was spectrophotometrically measured at 532 nm. Hydroxyl radical scavenging activity was calculated by the following equation:

\% hydroxyl radical scavenging activity = (1 – As/Ac) \times 100

Where ‘As’ is the absorbance of the sample and ‘Ac’ is the absorbance of the control.

### 2.3.3. DNA degradation assay by gel electrophoresis

The ability of coriander carotenoid fraction to protect DNA, from the attack by hydroxyl radicals, was studied according to (Lee et al. 2002) with minor modifications. The reaction mixture contained 5 \(\mu\)g of calf thymus DNA, Fenton’s reagent (H\textsubscript{2}O\textsubscript{2} 30 mM, Ascorbic acid 50 \(\mu\)M, FeCl\textsubscript{3} 80 \(\mu\)M) in TE buffer, and extracts in DMSO in a final volume of 20 \(\mu\)L. The mixture was incubated for 30 min at 37 °C and DNA was analyzed by loading on to 1% agarose gel, electrophoresed followed by ethidium
bromide staining and documented by the Hero Lab Documentation unit (Herolab 442K, E.A.S.Y., Germany).

2.4. Stability of pigments during processing

To standardize the drying conditions for best retention of pigments, first the moisture content in fresh coriander leaves of var. GS4 Multicut was determined by drying foliage (wrapped in blotter sheets) in hot air oven at 70 °C overnight (Therdthai & Zhou, 2009). In case of drying in hot-air oven, 5 to 10 g fresh samples were wrapped in single layered blotters, placed in a perforated steel tray and incubated in an oven at 45 ±2 °C until a constant weight was noted, which generally occurred within 24 h. For microwave (MW) drying, the samples were similarly wrapped and drying was done at five power levels (850 W, 600 W, 450 W, 300 W & 180 W) for five different periods (30 s, 60 s, 90 s, 120 s and 150 s). Dry weight, chlorophyll a, chlorophyll b and total carotenoids contents were estimated after acetone extraction followed by spectrophotometric reading (Lichtenthaler, 1987), as explained earlier. Mean gravimetric values of 3 sets of samples, each having 4–5 shoots were recorded. The quantity of pigments in samples after MW drying were compared with those of fresh sample and those obtained after oven drying. The MW-dried samples were also subjected to HPLC analysis where, sets of three similar leaves of fresh and after MW-drying were used, and total extract was quantitatively analyzed.

2.5. Results

2.5.1. Growth pattern of different coriander cultivars

The data on growth patterns of different cultivars of coriander are presented in an earlier section (Chapter 1, Table 1.2). Briefly, seed germination in all cultivars started within 4–10 days of sowing. GS4 Multicut reached maximum biomass after 35 days, whereas Amar and Surabhi showed lowest quantity of biomass. In mature stage, along with GS4 Multicut, commander also attained highest biomass yield. Except the cultivar Amar, all other cultivars showed minor variation in total biomass. For floral leaf induction most of the cultivars took 90–96 days, in the case of GS4 Multicut, GC Gold 99 and Kalmi Gutchedar, flowering stage started much earlier (57–68).
2.5.2. Effect of saponification on carotenoids

GLVs are rich in lutein, which is a polar xanthophyll. Xanthophylls are carotenoids characterized by the presence of hydroxyl groups and may form esters with fatty acids. Alkaline saponification was suggested in the analytical procedures for xanthophylls, which is done usually by using aqueous-ethanolic or methanolic solutions of potassium hydroxide (Oliver and Palou 2000), although in this study, saponification of the carotenoids was done overnight at room temperature in petroleum ether, with equal volume of 10% methanolic KOH, which is known to retain β-carotene and completely hydrolyze the carotenol esters. However, even with this saponification, xanthophylls such as lutein and violaxanthin degraded significantly, along with chlorophylls. Other studies reported the loss of carotenoids during saponification by degradation (Deli et al. 1992; Ittah et al. 1993). Provitamin A carotenoids (α-carotene, β-carotene, γ-carotene, β-cryptoxanthin) may resist saponification (Kimura et al. 1990), although losses of lutein, violaxanthin, and other dihydroxy, trihydroxy and epoxycarotenoids can occur during saponification and subsequent washing steps (Khachik et al. 1986). In the analysis of vegetables, tomato and carrot which are low lipid materials and essentially free of carotenol esters, saponification may not be necessary. Since coriander leaves of mature stage contain oil, saponification was essential during extraction. The loss of carotenoids from fresh coriander leaves and microwave dried (MWD*) leaves during saponification has been shown in Figure 2.2. Saponification resulted in substantial loss of carotenoids, accounting for more than half of the carotenoids lost during this step.
2.5.3. Qualitative composition of carotenoids present in coriander leaves

Mercadante and Rodriguez-Amaya (1990) reported highest carotenoid content in leaves of coriander and parsley than in other vegetables. The vast number of carotenoids and their instability to heat, light, and air makes the extraction, isolation, identification and quantification challenging. Because of the various biological functioning of carotenoids, there is a great need for the detection and identification of carotenoids from food. The most sensitive method for the analysis of carotenoids is the HPLC with photodiode array detector or UV-visible detector. The trace level could be detected by mass spectrometry with APCI positive mode. APCI become the widely accepted mode for carotenoid analysis because of the linearity compared with other modes. All samples were overlaid with nitrogen prior to storage at –20 °C until carotenoid analysis in order to avoid degradation of β-carotene due to presence of oxygen. All analyses were completed within 7 days after extraction.

2.5.4. HPLC analysis of carotenoid content of leaves and young seeds in different cultivars

Since carotenoids are thermally unstable, analysis is usually carried out by HPLC with reverse phase column instead of gas chromatography. Therefore, only the most sensitive analytical method for the analysis of such delicate compounds is the HPLC
fitted with the photodiode array or UV-Visible detector. At the trace level, carotenoid identification may be confirmed by combining data such as HPLC retention times, photodiode-array absorbance spectroscopy, mass spectrometry (van Breemen et al. 1995). Carotenoids separated well within 30 min in HPLC by using C18 column. Major carotenoids found in coriander were β-carotene, lutein, violaxanthin and neoxanthin. Lutein is a macular pigment not synthesized by humans and should obtain the same through diet. Carotenoid concentration in vegetables depends on their growth/maturity stage, levels of carotenoid isomers, and food processing methods.

Table 2.1. Lutein content (mg/100 g DW) in the foliage of different coriander cultivars analysed by HPLC

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Cultivar</th>
<th>Lutein content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GS4 Multicut</td>
<td>132.27</td>
</tr>
<tr>
<td>2</td>
<td>GC Gold 99</td>
<td>130.95</td>
</tr>
<tr>
<td>3</td>
<td>Kalmi Gutchedar</td>
<td>108.07</td>
</tr>
<tr>
<td>4</td>
<td>Surabhi</td>
<td>108.44</td>
</tr>
<tr>
<td>5</td>
<td>Super 5</td>
<td>113.56</td>
</tr>
<tr>
<td>6</td>
<td>Mahak</td>
<td>87.93</td>
</tr>
<tr>
<td>7</td>
<td>Evergreen</td>
<td>108.07</td>
</tr>
<tr>
<td>8</td>
<td>Commander</td>
<td>106.78</td>
</tr>
<tr>
<td>9</td>
<td>Nutan</td>
<td>108.65</td>
</tr>
<tr>
<td>10</td>
<td>Nutan</td>
<td>102.65</td>
</tr>
<tr>
<td>11</td>
<td>Mysore local</td>
<td>98.35</td>
</tr>
</tbody>
</table>

Data presented in Table 2.1 regarding the concentration of carotenoids shows that lutein is the predominant carotenoid among xanthophylls, violaxanthin and neoxanthin were the other carotenoids present in this sample, but their concentrations were small. Lycopene and zeaxanthin were not detected. GS4 Multicut was found to contain highest total carotenoids (217.50 mg/100 g DW) and β-carotene content (73.64 mg/100 g DW) at mature stage, whereas, Kalmi Gutchedar seeds showed highest carotenoids among all (Table 2.2). The content of β-carotene was found to increase with foliage maturation in all varieties. The cv. GS4 Multicut was also rich in lutein with cv. Evergreen containing lowest lutein content.
Table 2.2. Total carotenoids and β-carotene concentrations (mg/100 g DW) in leaves and seeds of commercially important coriander varieties

<table>
<thead>
<tr>
<th>Coriander variety</th>
<th>In leaves at young plant stage</th>
<th>In leaves at mature plant stage</th>
<th>In fresh seeds</th>
<th>In dry seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total carotenoids</td>
<td>β-carotene</td>
<td>Total carotenoids</td>
<td>β-carotene</td>
</tr>
<tr>
<td>GS4 Multicut</td>
<td>169.15</td>
<td>45.58</td>
<td>217.50</td>
<td>73.64</td>
</tr>
<tr>
<td>GC Gold 99</td>
<td>167.06</td>
<td>40.36</td>
<td>212.94</td>
<td>70.43</td>
</tr>
<tr>
<td>Kalmi Gutchedar</td>
<td>158.19</td>
<td>38.55</td>
<td>213.38</td>
<td>68.44</td>
</tr>
<tr>
<td>Surabhi</td>
<td>157.62</td>
<td>35.51</td>
<td>205.26</td>
<td>57.78</td>
</tr>
<tr>
<td>Super 5</td>
<td>160.44</td>
<td>37.40</td>
<td>210.40</td>
<td>69.72</td>
</tr>
<tr>
<td>Mahak</td>
<td>152.79</td>
<td>22.55</td>
<td>197.73</td>
<td>39.04</td>
</tr>
<tr>
<td>Evergreen</td>
<td>159.57</td>
<td>30.53</td>
<td>207.79</td>
<td>68.01</td>
</tr>
<tr>
<td>Commander</td>
<td>159.11</td>
<td>27.14</td>
<td>203.55</td>
<td>58.57</td>
</tr>
<tr>
<td>Nutan</td>
<td>162.55</td>
<td>40.75</td>
<td>215.53</td>
<td>65.54</td>
</tr>
<tr>
<td>Amar</td>
<td>165.09</td>
<td>47.51</td>
<td>216.87</td>
<td>68.69</td>
</tr>
<tr>
<td>Mysore local</td>
<td>142.54</td>
<td>26.98</td>
<td>186.21</td>
<td>63.08</td>
</tr>
</tbody>
</table>

Values for β-carotene are after saponification.

Figure 2.3. HPLC chromatogram of trans- β-carotene standard

In the present study, main carotenoids identified were lutein (β,ε-carotene-3,3’-diol), β-carotene (β,β-carotene), violaxanthin (5,6,5’,6’-diepoyxy-5,6,5’,6’-tetrahydro-β,β-carotene-3,3’-diol), and neoxanthin (5’,6’-epoxy-6,7-didehydro-5,6,5’,6’-tetrahydro-β,β-carotene-3,5,3’-triol).
Lutein: The absorption spectrum, with $\lambda_{\text{max}}$ at 424, 448, and 476 nm in the mobile phase and less fine structure ($\% \text{III/II} = 60$), was typical of a carotenoid with 10 conjugated double bonds: 9 in the polyene chain and 1 in the $\beta$-ring. The chromatographic behavior was that of a dihydroxy carotenoid.

$\beta$-carotene: The $\lambda_{\text{max}}$ at 428 (shoulder), 455, and 480 nm in the mobile phase with low spectral fine structure ($\% \text{III/II} = 25$) was compatible with a chromophore of 11 conjugated double bonds, 2 of which were situated in the $\beta$-rings. The chromatographic behaviour was that of a dicyclic carotene. Structurally Vitamin A is one-half of the $\beta$-carotene molecule, consequently $\beta$-carotene is the most potent and wide spread provitamin A (Rodriguez-Amaya 1999). Chromatogram of standard $\beta$-carotene as in Figure 2.3.

Violaxanthin: The $\lambda_{\text{max}}$ at 417, 442, and 471 nm in the mobile phase and the high spectral fine structure ($\% \text{III/II} = 96$) of the visible spectrum were characteristic of a carotenoid with nine conjugated bonds in the polyene chain. It behaved chromatographically as a dihydroxy carotenoid with other less polar substituents.

Neoxanthin: The $\lambda_{\text{max}}$ at 415, 438, and 467 nm in the mobile phase and the defined spectral fine structure ($\% \text{III/II} = 88$) were consistent with a carotenoid having eight conjugated double bonds and an allenic group in the polyene chain. Chromatographically, it behaved as a trihydroxy carotenoid. HPLC profiles of above mentioned major ($\beta$-carotene and lutein) and minor (neoxanthin and violaxanthin) carotenoids presented in the Figure 2.4.
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2.5.5. Mass spectral analysis of carotenoids

HPLC-MS analysis is important for the analysis of carotenoids from natural sources, since these compounds are present in low quantity and are always associated with cellular membranes and matrices. Commonly used mode for carotenoid analysis in MS is ESI and APCI. ESI forms molecular ion with low fragmentation, whereas APCI produces molecular ion with fragment (deprotonated or protonated) especially for xanthophylls.

Figure 2.4. HPLC chromatogram of carotenoids after saponification
2.6. Antioxidant activity of carotenoids extract

2.6.1. DPPH scavenging activity

The IC₅₀ value of DPPH radical scavenging activity of coriander carotenoid fraction was 303.36 ± 6.8 µg/mL. An earlier study stated that lipophilic extracts of coriander leaves were inactive in this assay as the reaction medium was more hydrophilic (Wangensteen et al. 2004). However, this study reported a higher DPPH IC₅₀ value of 389 ± 5 µg/mL for the coriander ethanolic extract, depicting a much lesser efficacy than in the present study.

2.6.2. DNA Degradation assay by Spectrophotometric method

Coriander carotenoids extract was found to show higher potential for scavenging hydroxyl radicals and protecting DNA from degradation (IC₅₀, 14.29 ± 1.68 µg/mL) than the standard gallic acid (IC₅₀ 357.21 ± 4.29 µg/mL). Among the free radicals, hydroxyl radicals are the most deleterious ones, since they directly react with DNA. While each carotenoid has potential benefits, combination of all carotenoids is probably more effective than single compound. β-carotene, lutein and other carotenoids exhibit antioxidant functions by quenching hydroxyl and other free radicals (Sies and Stahl
1995). Antioxidant activity of ether extract of coriander analyzed by β-carotene/linoleic acid model was reported by (Guerra 2005), where they found that individual carotenoids are inferior to the whole carotenoid functionalities.

2.6.3. DNA degradation assay by gel electrophoresis

DNA protective effect of the coriander carotenoids against hydroxyl radicals generated by the Fenton’s reaction was further confirmed by the gel electrophoresis pattern of the DNA. Coriander carotenoid fraction was found to inhibit the degradation induced by Fenton’s reagent more effectively than the standard gallic acid (Figure 2.6).

![Figure 2.6. Gel showing the protective activity of coriander carotenoid fraction on hydroxyl radical induced DNA damage](image)

1. DNA+Standard (Gallic acid 50 µg in DMSO)
2. DNA+Standard (Gallic acid 100 µg in DMSO)
3. DNA treated with Fenton’s reagent – the pro-oxidant
4. DNA+Sample (50 µg in DMSO)
5. DNA+Sample (100 µg in DMSO)
6. DNA in Buffer

2.7. Stability of pigments during microwave drying

HPLC analysis of total carotenoids in microwave-dried foliage indicated that trans-β-carotene was found to be more stable when compared to other carotenoids, but partial degradation of lutein and other carotenoids were observed. The bioavailability of trans form is higher than that of cis-isomer. HPLC profile of carotenoids and
quantification of trans-β-carotene before and after microwave drying showed no trans to cis isomerization of β-carotene.

**Figure 2.7. Effect of microwave drying on levels of total carotenoids and chlorophylls**

Provitamin A carotenoids are sensitive to heat, light and prolonged processing. Therefore, storage of raw as well as processed materials need to be addressed carefully, for which there is a lack of information regarding the retention of provitamin A carotenoids from traditional processing methods. The data presented in **Figure 2.7** show that microwave drying is an efficient method for the processing of coriander leaves because, there was no decrease in the carotenoids especially β-carotene, lutein and other biologically active carotenoids as shown in the **Figure 2.9**. Moreover, a slight increase in total carotenoids was observed, which may be due to the improved extractability of carotenoids after drying or due to the reduced enzymatic activity, that prevents auto-catalytic degradation of important bio-molecules. No significant difference in the content of trans-β carotene content was observed when analyzed before and after microwave drying.
Figure 2.8. Loss of moisture (gravimetric %) from coriander leaves during microwave drying at different power levels.
Other minor carotenoids, neoxanthin and violaxanthin were also found to be stable after processing indicating that microwave processing is an efficient method for retaining the stability of carotenoids in GLVs.

2.8. Discussion

2.8.1. Growth patterns of different cultivars of coriander

For the export market as well as for domestic use there is a great demand for coriander material which has the capacity to yield high biomass with good nutritional quality. Shanu et al. (2013) reported the factors responsible for increasing productivity and quality of coriander which plays important role for obtaining improved growth, yield and quality of the crop. Different coriander varieties screened in this study showed marked differences in growth pattern and biomass yield (Table 1.2.), which are known to occur because of inherent genetic variations. Both fertilizer application and irrigation are expected to result in higher yields of coriander (Tomar et al. 1994) than in the present green-house study. The effects of cutting management and different planting
dates on the growth and yield of coriander crop have been examined in an earlier study (Baboo and Rana 1995). While most of the varieties of coriander showed a short germination period of 4–10 days, there were variable initial growth phase of 20–25 days, followed by a lag phase characterized by slow growth and variable periods of profuse growth phase leading to flowering stage. Three varieties, GS4 Multicut, GC Gold 99 and Kalmi Gutchedar required shorter time to produce higher amount of foliage than the rest, with Variety GS4 Multicut being the best, attaining the initial lag phase between 20–25 days after germination with an average biomass of 0.90 g/plant. It reached the mature stage between 37th and 42nd days of growth with an average biomass of 6.18 g/plant. Dhanasekar et al. (2000) determined the growth pattern of 12 coriander genotypes, where significant increase in leaf and stem height was recorded from 25 to 35 DAS, irrespective of the season. Kofidis et al. (2008) evaluated the effects of the two gibberellin inhibitors on certain growth and anatomical characteristics of coriander. These studies indicate that several environmental factors as well as hormone applications have direct effects on the biomass and quality of the crop.

2.8.2. Quantitation of different carotenoids in coriander leaves in young and mature stage by HPLC-MS

The concentration of total carotenoids contents was higher in foliage than in seeds in all the varieties (Table 2.1). When the concentration of β-carotene before and after saponification was compared (Figure 2.2), from 15 to 20% of β-carotene and 50% of other carotenes were found to be lost during the process of saponification. Other studies have also recorded that prolonging the saponification process significantly reduced the recovery of β-carotenes (Hart and Scott 1995; Inbaraj et al. 2008). Conversely, shortening saponification time, or avoiding saponification wherever possible has been suggested for reducing such pigment losses (Rodriguez-Amaya et al. 2004). Among the factors affecting carotene content, varietal differences due to genetic variability is a prominent factor (Sass-Kiss et al. 2005). Differences among cultivars are well documented. For instance, the red fleshed papayas Solo, Formosa and Tailandia produced in the Brazilian state differed in the lycopene content with the Formosa papayas twice when compared to other two cultivars (Kimura et al. 1990). The process
of ripening enhanced carotenoid accumulation in some fruit and vegetables such as mango (Mercadante and Rodriguez-Amaya 1998) and pepper (Rahman and Buckle 1980; Howard et al. 1994; Minguez-Mosquera and Hornero-Mendez 1994). β-carotene content of sweet potato cultivars, varied from 10 to 26,600 µg/100 g (Takahata et al. 1993; Hagenimana et al. 1998). In squashes and pumpkins, some cultivars were found to contain α-and β-carotene as principal carotenoids, whereas lutein was found to predominate in others (Arima and Rodriguez-Amaya 1988). Wide variations in carotenoids can occur within the same variety at varying growth conditions (Hart and Scott 1995). However, the present study conducted at identical growth conditions indicate that the differences were purely due to varietal differences resulting from inherent genetic make-up, which remain significantly different from each other. In different stages of plant growth, greater differences would be expected. In mature stage, greater carotenoid content than the immature stage as observed in Curcurbita moschata cultivar Menina Verde, in which β-carotene content increased dramatically during maturation (Arima and Rodriguez-Amaya 1988). In mature leaves of lettuce (12 mg/g) and endive (14 mg/g), the content β-carotene content was about three times higher than in that of young leaves (3.5 and 4.2 mg/g, respectively) (Mercadante and Rodriguez-Amaya 1998). Carotenogenesis may be inhibited by the presence of certain agrochemicals. Kale leaves at the same stage of maturity, produced in neighboring farms were compared. All the components of carotenoids were significantly higher in samples collected from an organic farm than in those taken from a conventional farm that used herbicide (Mercadante and Rodriguez-Amaya 1991). This indicated that carotenoid biosynthesis in the leaves is inhibited by one or many chemicals used in the latter farm.

In coriander leaves, the content of lutein (Table 2.1) was higher than that of β-carotene as in the case of other GLVs (Aruna and Baskaran 2010). Similar studies conducted by Singh et al. (2001) also revealed that coriander leaves possess higher concentration of β-carotene compared to spinach and Amaranthus – the vastly recommended green vegetables as sources of vitamin A, and 3-folds more than that in carrots. Perry et al. (2009) reported various carotenoids present in coriander leaves such
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as β-carotene (5.5 mg/100 g) and lutein (7.7 mg /100 g). This data was compiled after a tentative identification of carotenoids because of the lack of mass spectral analysis.

In plant foods, β-carotene is mostly found as all-trans isomers and lesser as cis-isomers, with the relative abundances in the following order: all-trans> 9-cis> 13-cis> 15-cis. Cis-provitamins A have long been associated with lower vitamin A activity than their trans-isomers. Apart from this, all-trans form of carotene has higher bioavailability than its cis counterpart (Gaziano et al. 1995; Sies and Stahl 1995; Ben-Amotz and Levy 1996), while β-carotene and β-apo-12’-carotenal have the highest bioconversion rate at 100% and 120% respectively (Tee and Lim 1991; Castenmiller and West 1998).

2.8.3. Antioxidant activity of carotenoids

In addition to their biochemical activities as provitamin A carotenoids, these molecules also have physiological activities that are attributed to their strong antioxidant property, specifically the ability to quench singlet oxygen and interact with free radicals (Palozza et al. 1992). Fruits and vegetables rich in vitamin C, vitamin E (tocopherols) and carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin and lycopene) have been suggested as a natural source of antioxidants. Antioxidant functions are associated with the reduction in DNA damage, diminished lipid peroxidation, improved immune functions, which are believed to prevent or postpone the occurrence of chronic diseases (Sies and Stahl 1995; Rao and Rao 2007). Apart from this, α-carotene, β-carotene and β-cryptoxanthin are considered as provitamin A carotenoids (Raju et al. 2007). Moreover, it is generally accepted that the consumption of vegetables can play an important role in maintaining health and reducing the risk of chronic diseases (Kalt 2005; Marinova and Ribarova 2007). For these reasons, vegetables as well as fruits are widely recommended as healthy foods (De Azevedo and Rodriguez-Amaya 2005). The functionality of carotenoids against diseases has been attributed to their strong antioxidant activity, specifically due to their ability to quench singlet oxygen and interact with free radicals (Palozza and Krinsky 1992). However, one cannot ignore the other physiological roles played by carotenoids such as the modulation of carcinogen metabolism, enhancement of cell differentiation,
regulation of cell growth, inhibition of cell proliferation, stimulation of cell-to-cell gap junctional communication, retinoid-dependent signalling and filtering of blue light into eye ball (EL-Qudah 2009). Previous studies have demonstrated that both leaves and seeds of coriander contain antioxidants, but the leaves were found to have stronger effects than that of seeds (Wangensteen et al. 2004), attributed to the high content of carotenoids in leaves. In view of the wide prevalence of vitamin A deficiencies among nutritionally deficient populations, the foliage of coriander holds great promise due to their high content of carotenoids, particularly the β-carotene – an immediate precursor of vitamin A.

2.8.4. Stability of pigments during drying

Food processing, particularly the heat treatment, may lead to the degradation of thermo-labile nutrients such as carotenoids, biologically active compounds and substances important to food quality such as food colorants. Since carotenoids are highly unsaturated, they are prone to degradation resulting in the loss of colour and biological activity. Lessin et al. (1997) reported the prevalence of 9-cis isomer as the highest in processed GLVs. There are quite a large number of literatures available on the processing of fruits and vegetables. Difficulty in interpreting the results is because of the following reasons,

- Processing conditions are partially described.
- Different samples are processed differently, so comparison is difficult
- Different parameters (time and temperature) are used for the same processing method.
- Most of the earlier studies focused on total carotenoids, not individual ones.
- Moisture contents before and after processing were not considered.

In the present work, all the above-mentioned points were taken care of while processing and sample analysis.

Processing of plant materials by thermal treatments generally results in the loss of biologically active compounds. Oven drying at 70 °C overnight was found to remove the moisture from the foliage, yielding a material with uniform weight for the next 12 hours. At this level, the moisture content was found to be 87%. Analysis of drying of
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Foliation of different crops have shown that sun drying and shade drying resulted in maximum loss of β-carotene, chlorophyll and ascorbic acid (Ndawula et al. 2004). However, it is well known that brief pre-treatment such as blanching enhances pigments by freeing them from bound forms and also arresting the endogenous enzymes that may act on the foliage material. For processing of vegetables, blanching has been a well-accepted method followed routinely for many commodities. Despite some advantages, this method also leads to nutrient loss, predominantly vitamins, and loss of pigments and aroma. Negi and Roy (2000) analysed the effect of different blanching and drying conditions on β-carotene, ascorbic acid and chlorophyll retention of some leafy vegetables grown in India.

In the current study, the microwave drying at different power levels for different periods indicated that drying at 850W for 90s was found ideal for drying coriander foliage to match with the moisture content equal to that of oven-dried (Figure 2.7). The oven drying of coriander foliage at a low temperature (45 °C) resulted in substantial loss of both chlorophylls (65%) and carotenoids (35%). Further experiments with various power levels and drying time indicated that microwave drying, similar to blanching, increased the extractable total chlorophylls, carotenes and β-carotene levels, at all power levels up to 90s (Figure 2.8). A similar study in pumpkin also showed an increase in β-carotene content (Azizah et al. 2009) and comparable effects were observed during heat processing of fruits (Jatunov et al. 2010). Another study conducted on the effects of cooking and processing on fruits and vegetables revealed about 8–10% destruction of total carotenoids (Mazzeo et al. 2011) and α- and β-carotene as a result of heat treatment (Khachik and Beecher 1987), with significant variations in response to treatment conditions. Thus, there is a need to screen and evaluate the specific drying requirements to preserve the bio-active pigments in different plant materials. Also our comparative analysis of fresh and microwave samples showed that the carotenoid yield upon microwave drying increased to an extent of 35 to 49% (data not shown), probably due to higher extractability and liberation of carotenoid molecules that are bound to membranes and other pigments such as chlorophyll. Such Increases have also been observed during steaming (Mazzeo et al. 2011). During saponification, nearly 20% was lost in fresh samples whereas only about 10% was lost in microwave
dried samples, indicating that more loss occurs in fresh samples probably due to carotenoid bondage with chlorophylls, the latter are removed during saponification. However, for obtaining β-carotene, saponification is an essential step accounting for a loss of only 15 to 20%.

The period of exposure to microwave treatment was found to affect the total chlorophylls and carotenoids contents. As the exposure time increased, carotenoids content decreased, particularly when exposed to lower power levels (180W) for longer periods such as 90 seconds and 120 seconds. However, chlorophyll a decreased (from 600W to 180W power level) with the increase in treatment time (from 60 seconds to 150 seconds) probably because of the lower thermo-stability of chlorophyll a compared with that of chlorophyll b. HPLC analysis of total carotenoids in microwave-dried foliage indicated that trans-β-carotene was found to be more stable when compared to other carotenoids, but partial degradation of lutein and other carotenoids were observed. HPLC profile of carotenoids and quantification of trans-β-carotene before and after microwave drying showed no trans to cis isomerization of β-carotene. Since the bioavailability of trans form is higher than that of cis-isomer, the present study suggests microwave treatment as an efficient cost-effective method for drying of trans-β-carotene-rich leafy materials. Studies in human subjects have revealed the presence of higher levels of all-trans β-carotene in plasma and serum than cis-β carotene (Deming et al. 2002) and hence the dried coriander foliage obtained after microwave drying could be easily made into powder to serve as an ingredient in various culinary processes and as a taste enhancement ingredient in snack foods such as potato chips and other crispy delights, which is expected to partially, if not fully, alleviate vitamin-A deficiency.

2.9. Conclusion

The present comprehensive study on total carotenoids and β-carotene contents in commercially important coriander cultivars reveal that there exist large variations in growth patterns and the pattern of accumulation of carotenoids in both foliage and seeds. By evaluating the changes in growth phases, it has been established that foliage biomass is highest at the stage before the onset of flowering in all varieties of coriander.
This stage also coincides with high β-carotene content and other carotenoids. Coriander carotenoids were found to be significantly effective in scavenging the highly reactive hydroxyl radicals and also helpful in protecting DNA from oxidative damage. Microwave drying was found to have no effect on cis/trans-isomerization of β-carotene with little loss in nutrient content, allowing biologically active trans-β-carotene rich dried-foliage for direct use in various processed food products. The data also suggests that more detailed analyses of carotenogenic biosynthetic pathway is needed for further improvement in the cultivation and processing conditions to make this crop an important routine source of provitamin-A with additional benefit of offering anti-oxidative effects.

Chapter highlights

- Carotenoids in different coriander cultivars were characterised and the best cultivar with high content of carotenoids was found to be GS4 Multicut, and hence selected for further study.

- Microwave processing parameters were optimized for the better stability of major carotenoids in coriander leaves.