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Berberine and lycopene profiling during the ontogeny of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms fruit

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Tinospora cordifolia **(Menispermaceae) fruits were studied for pigment profile, carbohydrate content, weight and water content during ontogeny. Carotenoid pigment lycopene appeared in yellow fruits and attained maximum level in matured (red) fruits whereas chlorophyll *a* and *b* disappeared after intermediate (yellow) stage. In addition, isoquinoline alkaloid berberine was more in early (green) stage than intermediate and matured stages. Carbohydrate content increased 1.3-fold on maturation, whereas weight and water content did not change significantly.**

Keywords: Berberine, fruit ontogenesis, lycopene, *Tinospora cordifolia*.

Tinospora cordifolia (Willd.) Miers ex Hook. F. & Thoms (*Tc*) (Menispermaceae) is highly exploited for pharmaceutical purposes in Ayurvedic and Homeopathic systems of medicine[1,2]. The climbing shrub is widely distributed throughout India and neighbouring countries, like Bangladesh, Pakistan and Sri Lanka, and South East Asian countries such as Malaysia, Indonesia and Thailand[3]. It is reported to bear distinct male and female flowers[3]. However, its red fruits, a forest produce, have not yet been studied. Chlorophylls, carotenoids and flavonoids, including anthocyanins, and betalains are pigments involved in leaf and fruit colouration in plants. The pigment content changes during ontogeny to adapt to the environmental conditions, and various stresses and damages[4–6]. Fruit ontogenesis is completed in two phases: fruit induction to 'maturation' which is marked by changes in carpel followed by maximum organ expansion, followed by 'ripening' during which structure and chemical composition of the organ undergo striking modifications[7]. During the maturation process, Fleancu[8] observed a negative correlation between fruit diameter and photosynthesis rate, respiration rate, fruit chlorophyll *a* or *b* content, and a positive correlation between diameter and fruit carotenoid content. Thus, quantity and ratio of pigments determine many important physiological characteristics[9,10].

Lycopene is a red carotenoid pigment synthesized exclusively by plants and microorganisms. Its functions include absorption of light during photosynthesis to protect plants from photosensitization. Sometimes the green chlorophyll pigments mask the red colour of lycopene in fresh fruits. However, as the fruit matures, chloroplasts are transformed to chromoplasts resulting in the loss of chlorophylls, increase in carotenoids, tissue softening, and alterations in the metabolism of organic acids and monosaccharide[11]. As carotenoids increase, chlorophylls disappear during ontogeny, thereby accumulating carotenoids such as lycopene in matured fruits (pineapple, orange, lemon, grapefruit, strawberry, tomato, paprika and rose hip) and many flowers (*Eschscholtzia* and *Narcissus*)[12].

Berberine (natural colour 18), a benzyl tetra isoquinoline alkaloid, is pharmaceutically important and has been used in traditional Chinese and North American medicine. It has been reported in nine botanical families, including Menispermaceae. Therapeutic potential of ber-

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berine includes antileukaemia, antihepatoma[13], cardioprotective[14], anticancer[15] and activation of AMP-activated protein kinase resulting in beneficial metabolic effects in diabetic and insulin-resistant states[16]. Thus, there is great demand for berberine from pharmaceutical companies. In view of this, it is imperative to find alternative sources of berberine alongside other bioactive compounds in the same source plant. Alkaloids tend to decrease during the ontogeny of fruits[17]. Therefore, a study of the evolution of fruit size, weight, colour in correlation with components such as soluble solids, and polyphenolics can contribute in the establishment of the nutraceutical value at a particular stage of maturity[18].

Recently, we have reported pigment identification, nutritional component characterization, and antioxidant potential of pigment-rich extracts of *Tc* fruits[19]. We had studied lycopene and berberine profiles along with physical and chemical changes during the ontogeny of fruits of *Tc*.

The *Tc* fruits were collected from the Central Food Technological Research Institute, Mysore campus during January–April 2008. The plant was authenticated by depositing herbarium sheets at the Herbarium Collection Centre (SKU – accession no. 11199), Sri Krishnadevaraya University, Anantapur, India. Three ontogenial stages of the fruit – green (immature), yellow (intermediate), deep-red (ripened) – were used for this study. HPLC-grade hexane, methanol, *tert*-methyl butyl ether and acetone were obtained from the Sisco Research Laboratory (Mumbai). β-Carotene, lycopene and berberine HCl were obtained from the Sigma–Aldrich Co. (St Louis, MO, USA). Anthrone reagent and other chemicals were obtained from the Ranbaxy Fine Chemicals Limited (New Delhi, India). All other chemicals used were of analytical grade. For HPLC analysis extra pure water (18.2 MΩ) was used. Physical dimensions of *Tc* ripened fruit were measured as soon as the fruits were collected.

Colour of the fruits and extracts was measured by Hunter's *Lab* method[20] in the visible range (380–700 nm) using barium sulphate as a standard. The *L*, *a* and *b* values were determined using the colour-measuring instrument, Labscan XE (M/s Hunter Associates Laboratory Inc., VA, USA). *L* indicates lightness, whereas *a* (+ to –) and *b* (+ to –) indicate the change in hue from 'red' to 'green', and 'yellow' to 'blue' respectively. The values were recorded using illuminant 'C', 2° observer angle and 5 mm slit width. Chroma was calculated as $(a^2 + b^2)^{0.5}$, whereas hue angle was calculated as arctan(b/a).

For pigment extraction, fresh fruits of *Tc* at three different stages were crushed, after separating the seeds manually, using mortar and pestle in the presence of neutralized sand. For complete extraction, the fruit homogenate without seed was soaked in the respective extraction solvents (viz. hexane, methanol and acetone) and kept for 3 h for extraction on a rotary shaker. The extract was filtered under vacuum passing through 0.22 μm membrane filters. The extract was then dried under reduced pressure and suitably diluted with the respective solvents. A double-beam spectrophotometer (Model UV-160 A, Shimadzu Corporation, Kyoto, Japan) was used to read the absorbance of the extract in the range 200–800 nm. Chlorophyll *a*, *b*, total chlorophyll, total carotenoids[21] and lycopene content[22] were calculated.

$$\text{Chl } a \text{ (μg/ml)} = 11.93 \times \text{OD (664 nm)} - 1.93 \times \text{OD (647 nm)}.$$

$$\text{Chl } b \text{ (μg/ml)} = 20.36 \times \text{OD (647 nm)} - 5.50 \times \text{OD (664 nm)}.$$

$$\text{Lycopene (mg lycopene/100 g fruit)} = 31.50 \text{ (for 100 ml of extraction volume)} \times \text{OD (503 nm)/ sample fresh weight (g)}.$$

The extract was chromatographed following the procedure of Sander *et al.*[23] using Shimadzu LC 10A (Shimadzu Corp., Kyoto, Japan) equipped with CLASS-VP integrator software for data-processing, UV–VIS detector and fitted with YMC 30 column (YMC Europe GmbH, Germany) of 250 × 4.6 mm i.d. and 5 μm pore size. The absorbance was recorded at 450 nm at ambient temperature.

Berberine was analysed in methanol extract of *Tc* fruits using a spectrophotometer at 266 nm and the calibration curve prepared using standard berberine HCl was used for quantification of berberine in the fruit extract. For HPLC determination of berberine in methanol extract, a chromatographic study was carried out under the following conditions: RP C_{18} column (Sunfire, USA) of 250 × 4.6 mm i.d. and absorbance was recorded at 266 nm in a UV–VIS detector as reported by Srinivasan *et al.*[24]. Total carbohydrate content of deseeded *Tc* fruits was analysed using phenol sulphuric acid assay[25]. Weight of *Tc* fruits in different stages was measured using an analytical balance. The fruits were dried at 50°C overnight and the reduction in weight was calculated[26] as water content according to AOCS official method Da 2a-48.

Statistical significance was analysed using two-way ANOVA test in Microsoft Excel program of Windows 2007 software. *P* value of 0.05 was considered statistically significant.

Physical dimensions of *Tc* ripened fruits are given in Table 1. Circumference of the whole fruit was 2.9 cm, whereas seeds had a circumference of 1.8 cm. This was in agreement with an earlier report[19]. The weight of 100 fruits was approximately 43 g, whereas 100 seeds weighed around 9.7 g. The whole fruit ranged from 2.4 to 3.4 cm in circumference, whereas the circumference of the seeds ranged from 1.6 to 2.1 cm. The seed weight constituted approximately 22.5% of the whole fruit weight in all the stages.

The fruits are red in colour when ripened, whereas they are green and yellow during immature and intermediate

Table 1. Morphological description of ripened *Tinospora cordifolia* fruit

Circumference of whole fruit (cm)	Circumference of seed (cm)	Shape and colour of pericarp	Hundred fruits weight (g)	Hundred seeds weight (g)
2.9 ± 0.5	1.8 ± 0.2	Round and red	42–44	9.2–10.2

Values are mean ± SD of ten fruits.

Figure 1. *Tinospora cordifolia* fruit bunch containing green, yellow and red fruits.

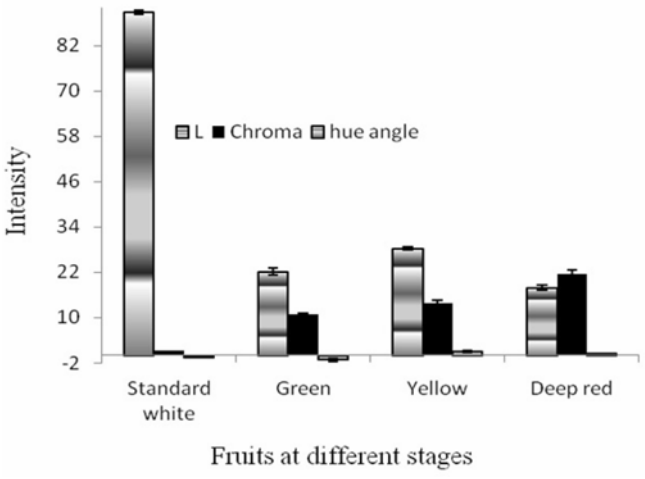

Figure 2. Colour intensity (Hunter's *Lab*) of *T. cordifolia* fruits in different stages of ontogenesis. Values are mean ± SD of four observations.

stages respectively (Figure 1). Colour of *Tc* fruits from different ontogenial stages is given in Figure 2. Chroma value increased from green to red through yellow, whereas lightness and hue angle were more during the yellow (intermediate) stage of the fruit. Accumulation of carotenoids in the advanced stage of fruit ontogeny has been reported[11]. Lycopene, the red carotenoid, was found to be accumulated (Table 2) in the red stage of *Tc* fruit development, which resulted in decreased lightness and negative hue angle, and increase in chroma value. Figure 3 shows the different colours of *Tc* ripened fruit extracts using different extraction solvents. Lightness of methanol extract was more than five-fold, chroma was eight-fold, and hue angle was more than three-fold compared to the hexane extract. This may be because the methanol extract is rich in berberine (yellow colour), whereas the hexane extract is rich in carotenoids, predominantly lycopene (red colour; Table 2). Pigment profile of the fruits of *Tc* in three stages of ontogenesis is given in Table 2. There was a slight increase in chlorophyll *a*, whereas chlorophyll *b* level reduced from the green to yellow stage. However, total chlorophyll content of the green and yellow fruits was almost the same, which indicates that the photosynthesis rate in green fruits is similar to that of yellow fruits. Red fruits did not have any of the chlorophylls. Chlorophyll levels have been shown to decrease on ripening, which in turn indicates that the rate of photosynthesis decreases on maturation[7,27] possibly due to breakdown of the chloroplast while chromoplast develops, which synthesizes coloured pigments such as carotenoids[28].

Lycopene, as confirmed by HPLC (Figure 4 *a*), appeared in yellow fruits (3.1 mg/100 g fruit) and reached maximum level at the deep red (58.1 mg/100 g fruit) stage, as shown in Table 2. Total yield of hexane extract was 0.69% fresh weight of red berries[19]. The lycopene level, 58.1 mg/100 g ripened fruit, is by far high compared to that of tomato. This observation was in close agreement with our previous report[19]. The average lycopene content of tomatoes was reported to be 3–5 mg/100 g raw material; however 15 mg/100 g and 0.5 mg/100 g was observed in few deep red, and yellow varieties respectively[29]. Many reports have shown a decrease in chlorophyll and increase in carotenoids during the maturation of fruits[8,11]. In addition, it was reported that tomato fruits grown under controlled environment (greenhouse) either in summer or winter had lesser lycopene level than fruits produced with abundance of sunlight in summer; also green or storage-ripened fruits were modest in lycopene content than vine-ripened fruits[30]. This may be due to high light intensity available during summer. A recent observation suggests the role of light in fruit ripening[31], and opportunities for modification of fruit quality and nutrient content. Whereas ethylene has been reported[32] to regulate carotenoid accumulation and carotenogenic gene expression, however, in tomato, accumulation of lycopene is regulated by fruit-localized phytochromes,

Table 2. Pigment and berberine profile of *T. cordifolia* fruit during ontogeny

Sample	Green	Yellow	Deep red
Chl *a* (acetone)	9.98 ± 0.1	6.38 ± 0.1	ND
Chl *b* (acetone)	5.8 ± 0.2	9.93 ± 0.2	ND
Total Chl	15.78 ± 1	16.31 ± 1.2	ND
Lycopene (hexane)	ND	3.15 ± 0.4	58.1 ± 0.4
Berberine (methanol)	0.13 ± 0.01	0.11 ± 0.009	0.1 ± 0.01

Chl, Chlorophyll; ND, Not detected. Results are expressed in mg/100 g fresh weight of deseeded fruits. Values are mean ± SD; $n = 3$.

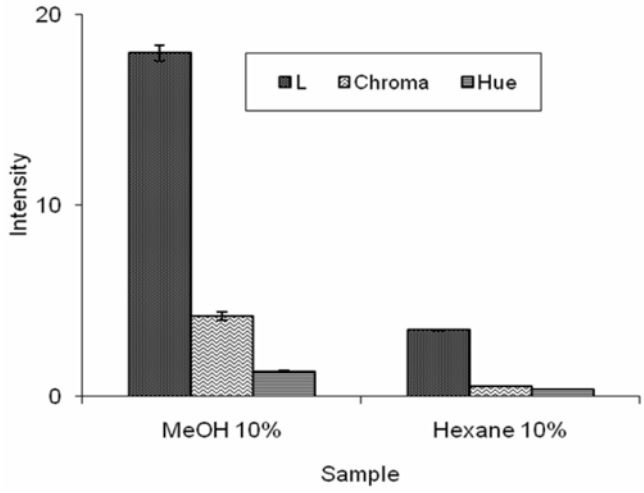

Figure 3. Colour intensity (Hunter's *Lab*) of *T. cordifolia* ripened fruit extracts. The quantity of pigment corresponds to 10 mg in 100 ml extraction volume of methanol (based on berberine content) and hexane (based on lycopene content) on fresh weight. Values are mean ± SD; $n = 3$.

independent of ethylene production[33]. The fruits analysed in the present study were collected from wild climbers during the dry season, which is also the fruiting season of *Tc*. The average precipitation (cm), and maximum and minimum temperatures (°C) during fruit collection were: January – 0.17 cm, 28°C, 16°C; February – 0.36 cm, 31°C, 18°C; March – 0.63 cm, 33°C, 20°C, and April – 0.48 cm, 34°C, 22°C (data provided by India Meteorological Department, Bangalore station). It was not studied in detail whether high accumulation of lycopene in *Tc* fruits in study was due to geographical factors such as light intensity, rainfall, etc.

Among the solvents used for the extraction of carotenoids, hexane gave the maximum extraction, which was confirmed by the spectrophotometer readings. The hexane extract yield was 0.6% fresh weight[19]. HPLC analysis provided further confirmation by comparing with commercial standards (Figure 4 *a*). Among the carotenoids analysed in *Tc* fruit extract, β-carotene and β-cryptoxanthin were identified as minor carotenoids along with some unidentified carotenoid isomers (Figure 4 *a*). β-Carotene was about 2.9% of lycopene content but β-cryptoxanthin was not quantified. It was found that the carotenoids were mainly localized in the skin of the fruit.

Content of berberine ($C_{10}H_{18}NO_4$), a yellow isoquinoline alkaloid (m.p. 145°C)[34] in the methanol extract was found to be 0.13, 0.11 and 0.1 g/100 g fresh weight in red, yellow and green deseeded fruits respectively (Table 2 and Figure 4 *b*). Total methanolic extract yield was 6.4% fresh weight in case of red berries[19]. Berberine was confirmed by employing standard berberine HCl using HPLC (Figure 4 *b*). This alkaloid has been reported from other parts of the plant[1]. Berberine has been observed to have anti-neoplastic activity in Ehrlich ascites carcinoma mouse model[15]. The concentration of berberine was found to be more in flesh (pulp), whereas skin had only minor berberine content. Gulfraz *et al.*[35] reported berberine content in root and fruit of *Berberis lyceum royle*, and root and leaf of *Justicia adhatoda* L. It was 4.5% and 2.9% respectively, in the former and the latter root had 0.3% of dry weight, and berberine was absent in the leaves. Alkaloid (like brachycerine) concentration decreases on ripening of the fruits[17]. Berberine content decreased slightly during *Tc* fruit ontogeny (Table 2 and Figure 4 *c*). In general, most such alkaloids synthesized by plants, play several roles due to antimicrobial, feeding deterrent or allelopathic properties. It has been observed that inflorescences had the highest alkaloid content, whereas in mature fruit pulp much lower amounts were present[17].

Total carbohydrate content increased significantly (*P* value was 0.005) on ripening of *Tc* fruits (Figure 5 *a*). Although total carbohydrate content in green (3.35%) and yellow (3.34%) fruits did not have significant difference, the deep red or ripened (4.6%) fruits had significantly (*P* value was 0.005) higher (1.3 times) carbohydrate level. During ripening many biochemical changes occur, such as increase in sugar content reported in sapota[36] and cherry fruits[37]. Carbohydrate content increases in ripened fruits may be because of reduction in metabolic activities, which provides carbohydrate during seed formation, responsible for growth and development[36,37]. At fruit-fall stage, the sugar concentration declines, level of organic acids increases, and short-chain fatty acids appear, indicating that sugars are being degraded[11,37].

There was no significant (*P* value was more than 0.05) increase in either the fruit weight (Figure 5 *b*) or moisture content (Figure 5 *c*) on ripening. However, it was observed that *Tc* fruits become more juicy (oozing out gummy substance) and soft on ripening (data not presented). It

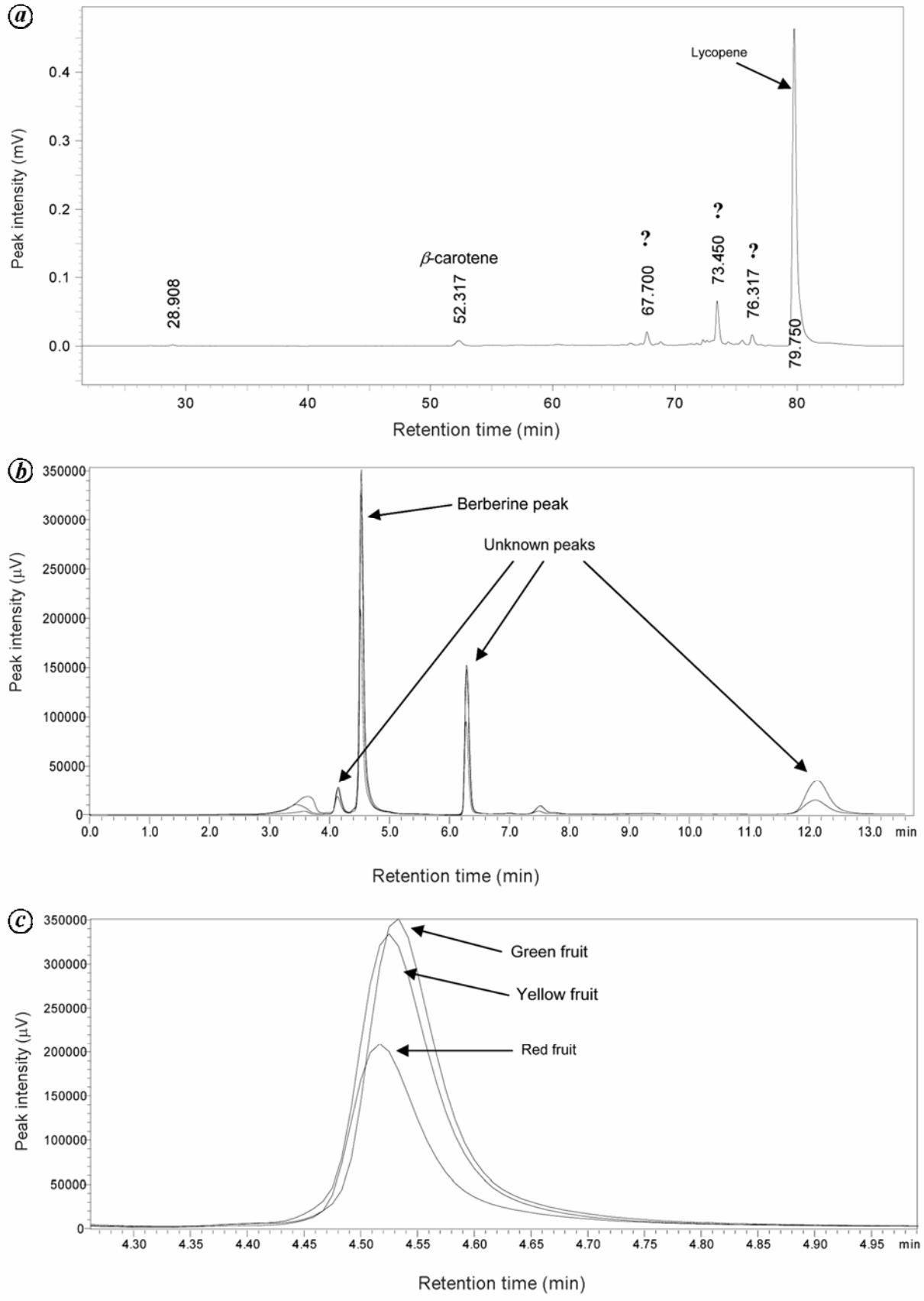

Figure 4. Representative HPLC profile of carotenoids in hexane extract (***a***), berberine profile in methanol extract (***b***), and zoomed berberine peaks (***c***) of *T. cordifolia* fruits during different stages.

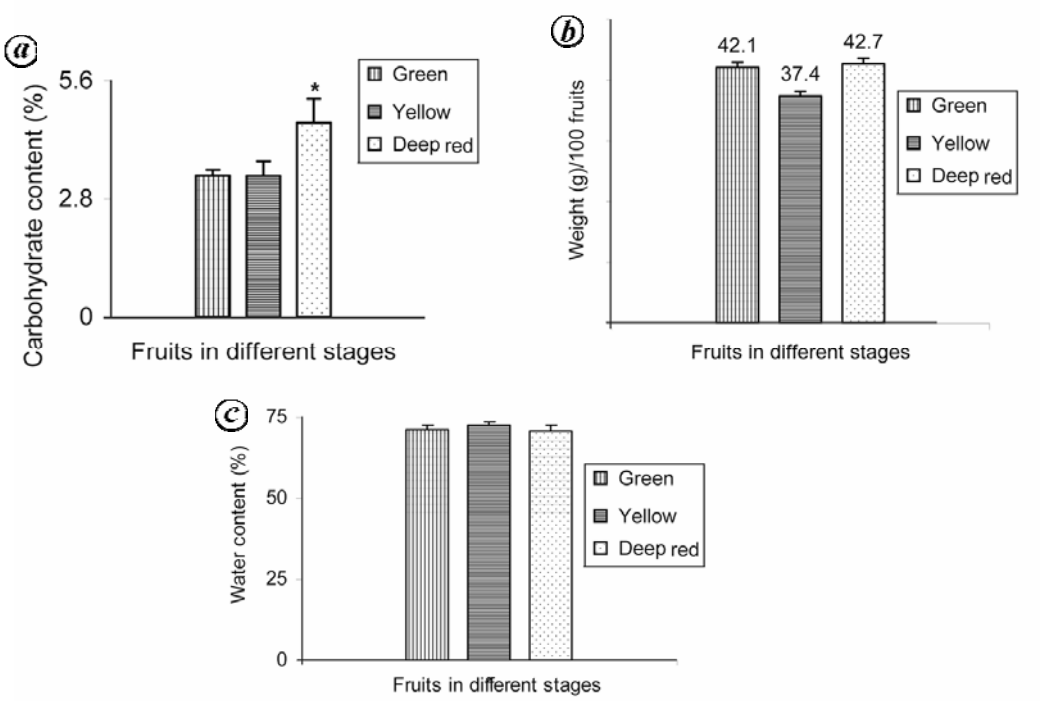

Figure 5. Total carbohydrate content (***a***), and changes in fresh weight (***b***) and water content (***c***) of *T. cordifolia* fruits during ontogeny stages. Values are mean ± SD. *P* value was more than 0.05.

was reported that water content on maturation increased in fruits[36,37]. The increase in water content in fruits during maturation is closely associated with cell metabolism and growth[38,39]. However, we did not observe any significant change in water content during ontogeny of *Tc* fruits (Figure 5 *c*). On ripening the water activity increases in fruits as reported by Ishida *et al.*[36], which makes the fruits juicy. They reported that on ripening the diffusion coefficient of water in the pericarp increases, thereby resulting in increased mobility. It was reported[36] that soluble compounds like sugars increased on ripening; however the sugar level decreased after ripening resulting in loss of sweetness and increased softness.

Similarly, there is a lot of ecological significance associated with changing pigment content during the ontogeny of fruits. According to Looney and Patterson[40], chlorophyllase activity increases sharply at the onset of the climacteric, rises to a peak which coincides with the climacteric peak, and then falls to near zero in the post-climacteric period. Consequent to this, transition of chloroplast to chromoplast takes place. The green to red colour transition of ripening fruits is largely due to this. The accumulation of specific carotenoids in chromoplasts of fruits is developmentally regulated[28]. During tomato fruit ripening, the expression of phytoene synthase and phytoene desaturase was found to increase, whereas both lycopene β-cyclase and lycopene ε-cyclase disappeared, leading to massive lycopene accumulation[41]. The change in colour helps in attracting birds, which eat the fruits and disseminate the seeds. This is one of the natural ways of seed dispersal, and development of variety, thereby helping in maintaining ecological balance.

From these findings, we conclude that *T. cordifolia* fruit can be a source of bioactive food colourants such as lycopene and berberine, and ripened fruits which may contain other nutraceuticals as well, will be best suited for such commercial exploitation. An agronomic strategy may be needed for sustainable plant yield of *Tc*. The parameters studied in this work could not be correlated, but the work is expected to create interest among researchers and the industry.

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