University of Thi-Qar Journal of agricultural research

Thi-Qar Journal of agricultural research

ISSN Onlin:2708 -9347, ISSN Print: 2708 -9339 Volume 15, Issue 1 (2026)

https://iasj.rdd.edu.iq/journals/journal/issue/20226 https://doi.org/10.54174/utjagr.v13ii 71.

Detection of Carotenoids concentrations and their associated genes in sweet pepper plant (Capsicum carotenoids L.)

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1. Abstract

Background: The color of fruit and the concentration of carotenoids are significant traits for both consumers and breeders among the diverse cultivars of the genus Capsicum. The study aimed to measure the carotenoid concentration and their related genes in sweet pepper plant. **Methods:** Eight Imported sweet pepper fruits were collected from local Iraqi markets in Baghdad Governorate used for extraction of carotenoids by High- performance Liquid chromatography (HPLC). B-carotene related genes(*Ccs* and *Lcyb*) were measured by Polymerase chain reaction(PCR).**Results:** beta-carotene concentrations in the studied sweet pepper sample recorded was 160.98 µg/ml. The gel electrophoresis for 2% agarose indicate presence of Ccs gene in 2(25%) studied pepper samples with 450 bp. The gel electrophoresis for 2% agarose indicates presence of *Lcyb* gene in 6 (75%) studied pepper samples with 320 bp. **Conclusion:** HPLC analysis revealed correct identification and quantification of β-carotene in sweet pepper samples. The chromatographic peak from the extract matched the retention time of the standard β-carotene solution, indicating the presence of this pigment in the samples. Quantitative investigation showed β-carotene as a significant natural source of carotenoids with nutritional and antioxidant benefits. The discovered genes may contribute to β-carotene biosynthesis and accumulation in sweet pepper fruits, confirming genetic heterogeneity in carotenoid production among examined samples

Keywords: Capsicum carotenoids, Carotenoids, Ccs gene, Lcyb gene, HPLC.

I. Introduction

Pepper, or capsicum, is a major crop grown all over the world that is prized for both its nutritional worth and economic significance (Tripodi et al., 2019). Because of their strong antioxidant activity and distinctive chromophore, carotenoids play a number of functions in plants, including free radical scavengers, auxiliary photosynthetic pigments, and precursors of phytohormones including strigolactones and abscisic acid. Carotenoids must be obtained through diet because humans are

ISSN Onlin:2708 -9347, ISSN Print: 2708 -9339 Volume 15, Issue 1 (2026)

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unable to synthesis them from scratch. Epidemiological and intervention studies have demonstrated that consumption of fruit and vegetables high in carotenoids can delay the onset of chronic disease states, including several types of cancer, age-related macular degeneration, and cardiovascular illnesses (Jaiswal et al., 2021). The concentration of several carotenoids, which greatly enhance the fruit's nutritional worth in addition to its aesthetic appeal, is what gives pepper fruits their vivid colors (Karim et al., 2021). Phytochemicals as anthocyanins, vitamins, phenolic acids, flavonoids, carotenoids, and capsaicinoids are abundant in peppers (Zia-Ul-Haq,2021). Numerous studies have shown the advantages of peppers' bioactive substances both in vivo and in vitro. Antioxidant, anti-inflammatory, and antibacterial properties, a lower incidence of type 2 diabetes and obesity, protection against hypercholesterolemia, and a lower incidence of atherosclerotic cardiovascular illnesses are just a few of the nutritional and physiological benefits these compounds offer. (wang et al., 2023; Filyushin et al., 2020; Roy et al., 2026).

For instance, β-carotene is a precursor of vitamin A, essential for cell growth, immune function and vision (Sharma et al., 2024). In addition, a carotenoid that is unique to red peppers, capsanthin, has been shown to have promising anti-inflammatory and anti-cancer properties. The carotenoids found in Capsicum species are also used in the production of functional foods and nutraceuticals for human health, and they contribute to the nutritional value of peppers (Akter,2025).

The *Ccs* gene is 1497 bp in length of coding region consisting of single exon . it reported that a large deletion located in the upstream region of the *Ccs* gene was responsible for the non- red fruit color (Tian et al., 2017).

Lycopene-β-cyclase (LCYB) is essential to this pathway because it catalyzes the final stage of β-carotene production. In pepper, overexpression of LCYB increases resistance to abiotic stressors (such as dehydration and salt(Yu et al., 2026). Furthermore, it has been demonstrated that higher levels of β-carotene, violaxanthin, lutein, and zeaxanthin in plants enhance their resistance to abiotic stressors such high light, UV radiation, and salt stress by scavenging ROS(Li et al., 2024).The study aimed to measure the carotenoid concentration and their related genes in sweet pepper plant.

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II. Methods

Plant sample collection

Eight Imported sweet pepper fruits were collected from local Iraqi markets in Baghdad Governorate and classified based on fruit shape due to the absence of stems and leaves.

Carotenoid extraction from bell pepper

Carotenoids extraction was performed according to the method of Howard et al. (2000) with modest changes. Briefly, 100 g of pepper fruits were frozen in liquid nitrogen and crushed to a fine powder. The pulverized material was combined with 10 mL of cold acetone in order to efficiently extract carotenoids and to prevent pigment degradation. The homogenate was passed through four layers of cheesecloth, and the residue was re-extracted with fresh cold acetone; this washing step was repeated three times to guarantee optimum pigment recovery. The combined filtrate was centrifuged at 4000 rpm for 10 minutes and the supernatant was collected and filtered through Whatman filter paper (0.45 µm) to obtain a clean extract suitable for HPLC analysis. The filtrate was concentrated using a Büchi rotary evaporator (Germany) to remove acetone and the dry residue was reconstituted in 1 mL of mobile phase. Then 20 µL of the finished extract was put into the HPLC apparatus. The mobile phase consisted of Solvent A (acetonitrile:water) and Solvent B (ethyl acetate) and the carotenoids were separated depending on their retention durations. Identification and quantification of β-carotene were done by comparing the peak area and retention duration of the samples with the standard β-carotene solution (25 µg/mL) and its concentration was determined using the equation, (sample peak area/standard peak area) x standard concentration × dilution factor.

Agarose gel preparation

A 1.5% agarose gel was prepared for the RAPD-PCR experiments and for the beta-carotene gene detection experiments by dissolving 0.6 g and 0.8 g of it, respectively, in 40 mL of triborate buffer (TBE). The mixture was heated to 60°C using a hot plate until melted and poured into the designated transfer tank.

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Extraction of Bell pepper DNA

The whole DNA were extracted according to the study of Kang and Yang(2004).Samples were placed in a thermocycler for DNA amplification, and the program was set to obtain the reaction conditions, as below:

Steps	Temperature and cycles		Time(min)
Denature template	94°		5
Initial denaturation	94°	35 cycles	1
Annealing	55°		1
Extension	72°		2
Final Extension	72°		5
Final incubation	4		optional

In their 2% agarose gel compartments, bell pepper samples were loaded with DNA amplification results from the aforesaid primers. The 1500-base-pair genetic molecular marker was loaded per the leaflet's directions. Each sample received 3 microliters of bromophenol blue loading dye and was electrophoresed from negative (black) to positive (red) electrodes at 75 V for two hours. The amplified DNA fragments were examined and imaged using a gel documentation system with a dedicated camera after 15 minutes of EtBr staining the agarose gel.

PCR master mix

The Go Taq® Green Master Mix (2X) for Promega /USA were used in amplification of studied genes according to instruction of manufacturer

Primers used in the study

The primers used in current study shown in table(1) of genes that were associated with B. carotene. All primers were dissolved using nuclease free water according to manufacturer instructions.

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Table(1): Primers used in the study

Gene	Nucleotide sequences
Ccs	F: 5′- CCTTTTCCATCTCCTTTACTTTCCATT- 3′
	R: 5′- AAGGCTCTCTATTGCTAGATTGCCCAG- 3′
Lcyb	F: 5′- GCACCTTGTTGGGAAAATATGGATACGC- 3′
	R: 5′- GATCCCAGATAAGTCGAATTCATTC- 3′

Statistical analysis

The experiments were carried out in a Completely Randomized Design (CRD) with three replications for each treatment. The data obtained were analyzed by analysis of variance (ANOVA) and mean comparisons were made by Duncan's multiple range test in MSTAT-C software. The differences were regarded as significant with a value of $P<0.05$.

III. Results

estimation of B-carotene in Pepper

The concentration of beta-carotene was determined quantitatively and qualitatively in sweet pepper samples using high-performance liquid chromatography (HPLC). The separation process resulted in the plotting of the standard compound, which appeared at a retention time of 3.439 minutes. The retention time that appeared on the chromatogram of each sample was comparable to the retention time of the standard solution, indicating the presence of the pigment in these extracts. Table(2) shows the beta-carotene concentrations in the studied sweet pepper sample. The concentration recorded was 160.98 µg/ml.

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Table(2): concentration of B-carotene in sweet Pepper

	Dilution	Retention time	Peak aera	β. Carotene(µg/ml)
Capsicum carotenoids L	**10**	**3.200**	**13999**	**160.98**

- **Standard retention time;3.439, peak area; 25953, Standard conc.;24 µg/ml.**

Detection of B-carotene genes

Detection of Ccs gene in pepper sample

The gel electrophoresis for 2% agarose indicate presence of Ccs gene in 2(25%) studied pepper samples with 450 bp as shown in figure(1):

Figure(1): agarose gel electrophoresis of Ccs gene in sweet pepper using primers with 2% agarose and 75 V for 2 hours

Detection of *Lcyb* gene in pepper sample

The gel electrophoresis for 2% agarose indicate presence of *Lcyb* gene in 6 (75%) studied pepper samples with 320 bp as shown in figure(2).

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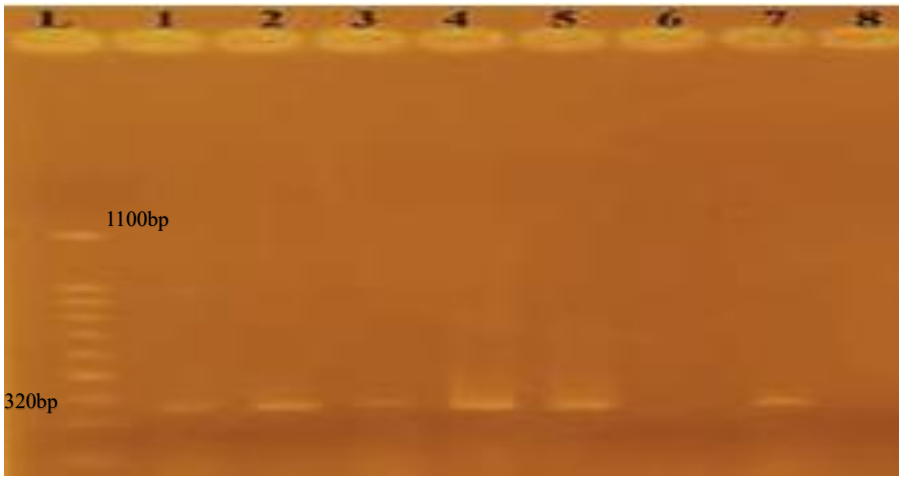

Figure(2): agarose gel electrophoresis of Lcyb gene in sweet pepper using primers with 2% agarose and 75 V for 2 hours

Discussion

The present study was conducted to confirm the presence of β-carotene in sweet pepper fruits by HPLC analysis, in which the retention time of the chromatographic peak of the fruits was found to be similar to the standard β-carotene solution. The level of β-carotene detected was 160.98 µg/mL, suggesting that sweet pepper fruits can be regarded as one of the most important natural sources of carotenoids. The main pigments found in most plants' fruits and flowers are called carotenoids. The kinds and concentrations of carotenoids in various colored fruit varieties change as pepper fruits develop. The color of pepper fruits is influenced by the makeup and amount of carotenoids(Solovchenko et al., 2019).

In the current study, the molecular analysis showed the successful amplification of the gene Ccs and Lcyb in pepper samples. Twenty-five percent of the samples examined contained the Ccs gene, whose size of the amplification product was about 450 bp, and 75% of the samples contained the Lcyb gene exhibiting an amplification product size of about 320 bp. It is suggested that Lcyb might play an important role in β-carotene biosynthesis and accumulation in the pepper fruits analysed in this study because it was detected more frequently than Ccs. The Lcyb gene encodes lycopene β-cyclase which catalyzes lycopene to β-carotene in the carotenoid biosynthetic pathway. Thus, the presence of this gene in most samples may account for the high level of β-carotene detected from the HPLC results.

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Recent studies indicate that the regulation of the carotenoid pathway in bell pepper fruits is dependent on a complex regulatory network, including structural genes, transcription factors and epigenetic regulation, and not only the presence of genes such as LCYB and Ccs (Weiguo,2025). Recent genomic investigations have identified the LCYB enzyme as a crucial regulatory point in channeling pathway flux towards β-carotene synthesis, resulting to higher accumulation in yellow and orange fruits (Na et al., 2025).

According to earlier research, mutations in the carotenoid production pathway genes Psy or Ccs are responsible for producing non-red pepper fruit colors like orange and yellow. Catalyzing the conversion of geranylgeranyl pyrophosphate to phytoene, the Psy gene encodes the enzyme (Tian,2014). At the very end of the process, the capsanthin-capsorubin synthase enzyme is encoded by the Ccs gene. This enzyme converts the intermediates antheraxanthin and violaxanthin into the ultimate product, capsanthin. Mutations in the Ccs gene mostly generated non-red fruit color in the major cultivated pepper species, C. annuum L(Kilcrease et al., 2015).

When Popovsky and Paran (2000) used polymerase chain reaction (PCR) to amplify the whole Ccs gene, they found no evidence of amplification in the yellow fruit cultivar . Using red and orange fruit cultivars, Lang et al. (2004) conducted Southern hybridization for the Ccs gene. They found that the downstream coding region was conserved, but the upstream coding and 5' flanking portions of the Ccs gene were absent in the orange fruit cultivar. These findings point to a loss of at least 1 kb in the Ccs gene of peppers that don't have a red fruit color, which would affect both the promoter and the region immediately upstream of the coding region.

Pepper plants contain two copies of the Lcyb gene. Both Lcyb1 and Lcyb2 are involved in the β-carotene and β-ε-carotene branches, respectively. In papaya (Carica papaya), a red-fleshed variety that stores lycopene in its flesh is produced by a single-base mutation in the Lcyb coding area that alters enzyme activity (Liu et al., 2020).

In yellow-fruited peppers, a deletion of 211 and 220 base pairs was found at the 3' end of the Ccs gene, according to many studies (Feng et al., 2024; Filyushin et al., 2025). According to del Rocío Gómez-García and Ochoa-Alejo (2013), an orange-fruited annual pepper called 'Fogo' had a frameshift mutation in its Ccs coding region that led to the premature cessation of transcription. The pepper's fruit mostly contained β-carotene and zeaxanthin, but it did not contain capsanthin.

Recent transcriptome studies also showed that the variance in fruit color in peppers was directly correlated with changes in gene expression levels during ripening stages. Transcription factors,

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https://iasj.rdd.edu.iq/journals/journal/issue/20226 *https://doi.org/10.54174/utjagr.v13ii 71.*

regulate carotenoid pathway genes, which affect the activity of the structural genes in the metabolic pathway (Wie et al., 2025). This leads to a diversion of carbon flux to β-carotene accumulation instead of the final red carotenoids. However, employing genomic analysis have demonstrated a decrease or mutation of the Ccs gene results in a reduction in capsatin synthesis and change in fruit color to yellow or orange owing to the accumulation of intermediate carotenoids. This is in line with the actual observations of a decreased presence of this gene in certain of the samples (Wang et al., 2023).

Moreover, recent studies have demonstrated that carotenoids are not only responsible for the color of fruits, but also play an important role in strengthening plant tolerance to oxidative stress. β-carotene is a powerful antioxidant, which protects cell membranes from salt and drought damage (Babaei et al., 2022). This can be explained by the association between high amounts of β-carotene and higher activity of genes involved in the carotenoid pathway. Moreover, recent epigenetic studies have indicated that DNA methylation modification in the promoter region can affect the expression of carotenoid genes without any mutations in the gene sequence itself, which explains the difference in the intensity of gene signals between samples (Kim et al., 2012).

Conclusion

HPLC analysis revealed correct identification and quantification of β-carotene in sweet pepper samples. The chromatographic peak from the extract matched the retention time of the standard β-carotene solution, indicating the presence of this pigment in the samples. Quantitative investigation showed β-carotene concentration of 160.98 µg/mL, showing sweet pepper as a significant natural source of carotenoids with nutritional and antioxidant benefits. The pepper samples had key carotenoid production genes found by PCR and agarose gel electrophoresis. The Ccs gene was found in 2 (25%) samples with a 450-bp amplification product, while the Lcyb gene was found in 6 (75%) samples with a 320-bp fragment. The discovered genes may contribute to β-carotene biosynthesis and accumulation in sweet pepper fruits, confirming genetic heterogeneity in carotenoid production among examined samples.

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