International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(6): 486-489

Received: 19-09-2017
Accepted: 20-10-2017

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HPTLC quantification of β-carotene in papaya (*Carica papaya* L.) fruit treated with 1-methylcyclopropene (1-MCP)

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Abstract
During ripening, papaya fruit accumulate massive amounts of carotenoid, thereby changing the skin color to yellow. The rapid metabolic process in the fruit leads to skin freckles that depreciate fruit appearance. Loss of greenness, or yellowing, in most products is inhibited by 1-methylcyclopropene (1-MCP). Thus, β-carotene contents were evaluated using HPTLC in papaya fruit samples treated with and without 1-MCP stored under ambient and cold storage conditions. In general, the results reveal that 1-MCP delayed the increase in the accumulation of beta-carotene.

Keywords: Carotenoid, climacteric, ripening, shelf life, HPTLC

1. Introduction
Physiologically, papaya is a climacteric fruit and during ripening it shows a climacteric rise in respiration and ethylene production which in turn triggers other metabolic processes that hasten ripening. Hence, papaya fruit accumulate massive amounts of carotenoid, thereby changing the skin color to yellow, mainly all-trans-lycopene, all-trans-β-cryptoxanthin and all-trans-β-carotene (Barreto *et al.,* 2011) [1]. β-carotene is the compound responsible for orange color in papaya (*Carica papaya* L.). The rapid metabolic process in the fruit leads to skin freckles that depreciate fruit appearance and lower its commercial value. The ripening process can be slowed down by inhibiting perception and action of the ripening hormone, ethylene.
Loss of greenness, or yellowing, in most products is inhibited by 1-methylcyclopropene (1-MCP). For many products, especially leafy vegetables and certain fruit such as apple, maintenance of green color is desirable in the marketplace as yellowness is regarded as a sign of senescence. However, for many fruit loss of chlorophyll and development, or unmasking, of colored pigments is an essential aspect of ripening (Kays, 1997) [5].
Carotenoid biosynthesis in fruit can be inhibited by 1-MCP, depending on the carotenoid compound and whether pulp ripening in the species is regulated by endogenous ethylene in a climacteric or non-climacteric process (Marty *et al.,* 2005; Kita *et al.,* 2007; Barreto *et al.,* 2011; Deaquiz *et al.,* 2014) [8, 6, 1, 2]. 1-MCP inhibited anthocyanin increases in strawberry fruit (Jiang *et al.,* 2001), but loss of chlorophylls and development of colors (anthocyanins, lycopene) eventually reached desirable levels of those of untreated fruit. 1-MCP applied before ripening also inhibited the expression of PSY, ZDS, LCYB and BCH genes involved in carotenoid synthesis in nectarine (Ziliotto *et al.,* 2008) [11]. In contrary, findings of Marty *et al*. (2005) [8] who used 1-MCP treatments to investigate ethylene regulation of carotenoid accumulation and carotenogenic gene expression in orange and white apricot cultivars, reported that 1-MCP inhibited ethylene production of apricot cultivars, while effects on pigment accumulations were small.
Thus, the objective was to evaluate β-carotene contents in papaya fruits samples treated with and without 1-MCP stored under ambient and cold storage conditions.
Carotenoid analysis in food products may be carried out by different methods: HPLC or colour evaluation. Although spectrophotometry can be used to rapidly assess the β-carotene content of papaya, a highly versatile, sensitive and selective method such as High Performance Thin Layer Chromaography (HPTLC) is needed for reliable analysis of food samples. HPTLC can be used as high sample throughput and low-cost technique for the analysis of food samples. The major advantage of HPTLC is in reducing analysis time and cost per analysis (Rathee *et al.,* 2010) [9]

Materials and methods

Estimation of β-carotene content in 1-MCP treated and control fruit under cold and ambient storage conditions using HPTLC

1-MCP application

Papaya (TNAU var. CO.8) fruit harvested at color break stage were selected for uniformity, devoid of injury or diseases and separated into two batches for administration of the 1-MCP treatments. One batch was treated with 1-MCP at 900 ppb, while the other batch was left untreated. The fruit were pre-treated with 100 ppm ethylene to trigger the ethylene receptor in order to avoid rubbery texture as reported in previous studies (Manenoi *et al.,* 2007) [7]. 1-MCP (obtained from Agrofresh Inc., Mumbai) at 900 ppb concentration was applied to papaya fruit by vapourization in a closed container for a duration of 14 hours @ 14 °C, RH 90-95 per cent.

After 1-MCP vaporization, half of the fruit from each treatment box were loaded into crates and kept at ambient temperature of 27±2 °C and rest of the fruit were kept at cold storage 14 °C. All boxes with 1-MCP-treated and untreated fruit were divided at random into three equal replications. Thus, the treated and untreated fruits were allowed to undergo normal ripening at the respective storage conditions.

β-carotene content was estimated in 1-MCP treated and control papaya fruit stored under ambient and cold storage conditions at 4th and 7th day of storage. At day 0, the fruits stored under ambient and cold storage conditions had no significant color changes. Since, the shelf life of ambient stored fruit could be extended only up to 7days, to study the significant difference between fruit at different storage conditions; the analysis was carried out on day 4 and day 7. Nevertheless, the fruit treated with 1-MCP stored under cold storage conditions had a better shelf life compared to 1-MCP treated fruit stored under ambient storage.

Extraction of carotenoids and analysis in HPTLC

Carotenoids were extracted under dim light conditions to minimize light-induced changes. Carotenoid substances extraction in papaya fruit peel and pulp was carried out according to procedures described by Wilberg and Rodriguez-Amaya (1995) [10]. Papaya fruit slurry was weighed and quantitatively transferred to test tube with about 20 mL of ethanol : hexane (1:1) solvent mixture, and homogenized in a blender. Homogenate was filtered in a vacuum filter system with the help of 0.45 micra pore diameter PTFE membranes for aqueous and organic solvents. This solution was used as test solution for HPTLC analysis. 10µL of test solutions and five different concentrations of 1µL, 2µL, 3µL, 4µL and 5µL of standard solutions (1mg in 1mL Chloroform) were loaded as 5mm band length in 10 x 10 Silica gel 60F$_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. β-carotene (≥95% (HPLC), crystalline) standard was purchased from Sigma-Aldrich. All chemicals used for HPLC were of analytical grades and organic solvents used were of HPLC grade. HPLC-grade water was purchased from Merck. The column temperature was maintained at 24°C.

Spot development

The samples loaded plate was kept in TLC twin trough developing chamber after saturated with Solvent vapor with respective mobile phase and the plate was developed up to 90mm. The mobile phase consisted of 1:1 (v/v) methanol and tert-methyl butyl ether (TBME). Yellow, Yellowish orange coloured zone at visible mode were present in the tracks, it was observed from the chromatogram.

Photo-documentation

The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at Visible light, UV 254nm and UV 366nm.

Scanning

The plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at 454nm. The Peak table, Peak display and Peak densitogram were noted. The software used was winCATS 1.3.4 version.

Results and Discussion

Identification of carotenoids and preparation of a β-carotene calibration curve in HPTLC

Carotenoids absorb light in the visible-UV range. Thus, the identification of carotenoids was achieved by the comparison of retention factor relative to the β-carotene standard (Sigma). The β-carotene standard was quantified and a calibration curve prepared as detailed below.

Standard	Rf	Concentration	Peak Height	Peak Area
S1	0.93	1µg	177.16	2549.67
S2	0.92	2µg	661.88	13039.53
S3	0.92	3µg	696.47	15360.10
S4	0.92	4µg	742.26	18527.29
S5	0.92	5µg	758.38	20402.63

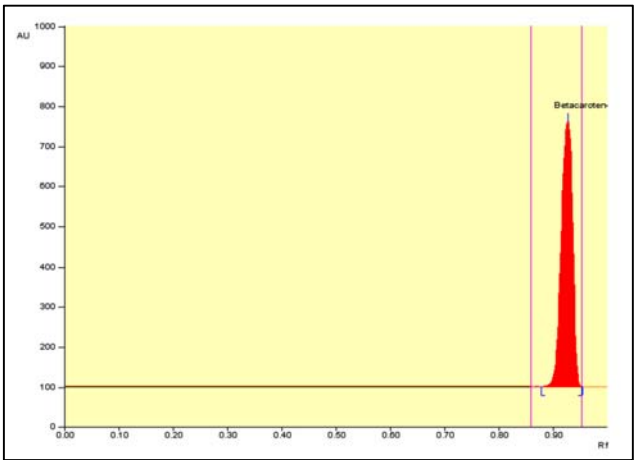
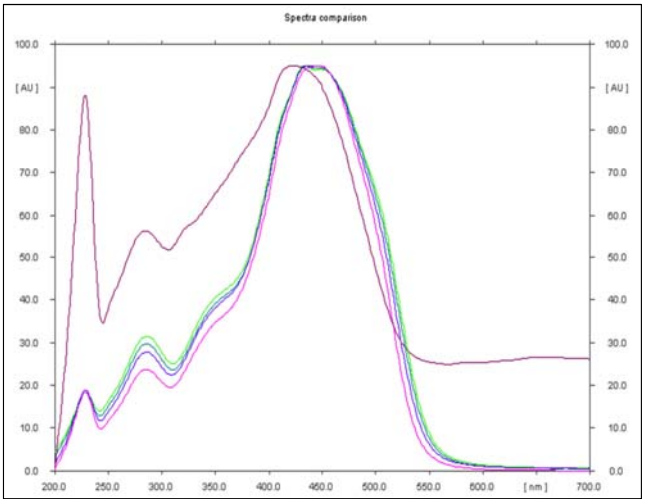
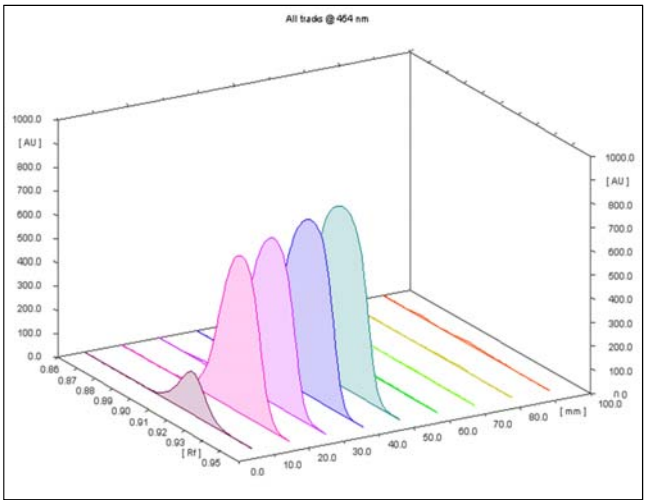


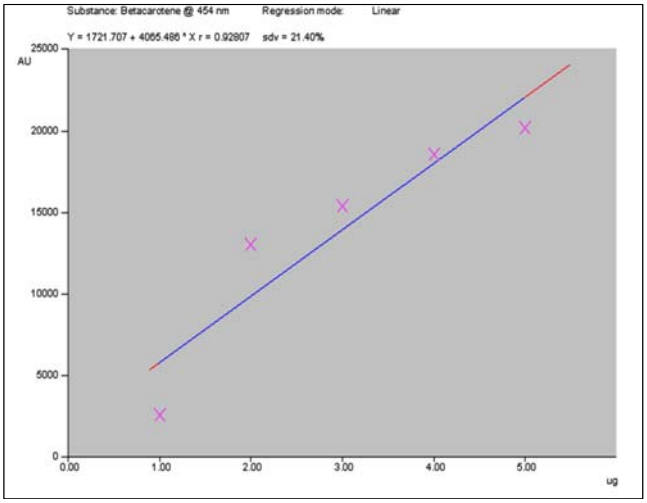
Fig 1: Chromatogram of standard, AU – area under curve, Rf 0.92



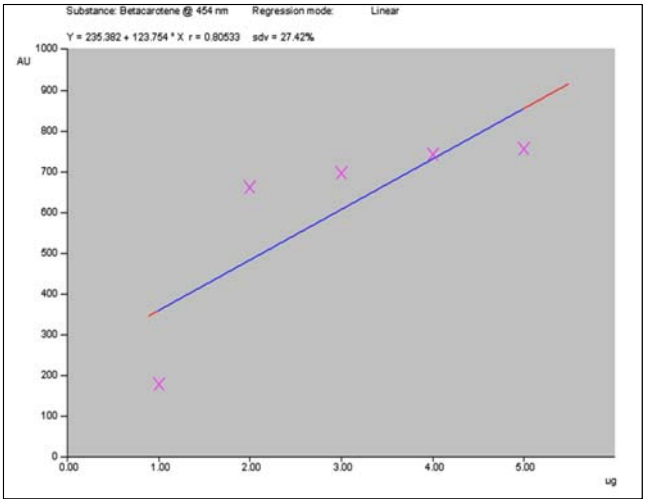
a. Spectrum of all standard tracks



b. 3D display of all tracks



c. Linearity graph on the basis of height



d. Linearity graph on the basis of area

Fig 2: HPTLC densitometric scan at 454 nm of β-carotene standard

Quantification of β-carotene in the samples

The peak was automatically identified and quantified by comparing its retention time of the sample with the standard retention time. β-carotene content estimated on day 4 and day 7 in treated and untreated samples (peel and pulp) are presented below.

Day 4		Ambient storage (27±2 °C)		Cold storage (27±2 °C)	
Sample Name	**Rf**	**Concentration (mg/g) in peel**	**Concentration (mg/g) in pulp**	**Concentration (mg/g) in peel**	**Concentration (mg/g) in pulp**
Control	0.92	0.69±0.008	0.94±0.009	0.56±0.003	0.83±0.001
1-MCP treated	0.92	0.57±0.004	0.80±0.002	0.45±0.009	0.72±0.009
Values represent Mean ± SE					

Day 7		Ambient storage (27±2 °C)		Cold storage (27±2 °C)	
Sample Name	**Rf**	**Concentration (mg/g) in peel**	**Concentration (mg/g) in pulp**	**Concentration (mg/g) in peel**	**Concentration (mg/g) in pulp**
Control	0.92	0.58±0.011	0.72±0.009	0.79±0.009	1.12±0.023
1-MCP treated	0.92	0.63±0.010	0.87±0.013	0.67±0.014	0.85±0.016
Values represent Mean ± SE					

In general, the β-carotene content was higher in fruit pulp than the peel. On day 4, the β-carotene contents in control fruit was higher than the 1-MCP treated fruit under both the storage conditions. This marked that; 1-MCP delayed the increase in the accumulation of beta-carotene. The accumulation of β-carotene in fruits under control treatment increased steadily under cold storage, reaching maximum level of 1.12 mg g^{-1} at the end of 7 days of storage. However, under ambient storage, control fruits showed a decrease in the β-carotene levels at the end of 7th day due to the development of skin freckles and diseases.

On the contrary, β-carotene content in papaya fruit subjected to 900ppb of 1-MCP treatment, progressively increased from day 4 to 7, reaching maximum levels of 0.63 and 0.87 under

ambient storage and 0.67 and 0.85 under cold storage, respectively in fruit peel and pulp. At the end of the storage period, 1-MCP treated fruits retained relatively higher beta-carotene levels compared to untreated control under ambient storage and delayed ripening (indicated by slower color change) under cold storage for enhanced shelf life. The results were in agreement with the findings of Fabi *et al*. (2007) [3] who reported that when papaya fruits, which are climacteric, were exposed to 1-MCP, carotenoid accumulation was reduced compared to untreated control fruit. Although ethylene treatment of papaya fruit induced ripening resulting in the anticipated appearance of some ripening-acquired characteristics, such as pulp softening, the final amounts of lycopene, beta-carotene and beta-cryptoxanthin were similar to 1-MCP treated fruits (Fabi *et al.,* 2007) [3].

Conclusion

The results of this study show that 1-MCP can be used to effectively delay ripening changes in 'TNAU CO.8.' papaya fruit, thereby prolonging the shelf life and extending the marketing period. Therefore, successful 1-MCP use requires a delay, but not irreversible inhibition, of the processes involved in pigment metabolism.

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